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VALORISATION DE DÉCHETS AGRO-INDUSTRIELS PAR BIOPRODUCTION FONGIQUE D'UN IMPORTANT PRODUIT DE PLATE FORME (L'ACIDE CITRIQUE) AVEC EXTRACTION SIMULTANÉE DE CHITOSANE

Par

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RÉSUMÉ

Au regard de la demande toujours croissante en acide citrique (AC), due à l'émergence de nombreuses applications avancées, il existe un besoin urgent de chercher des substrats peu coûteux et novateurs pour la production faisable d'AC. Dans ce contexte, divers déchets agro-industriels, comme les déchets solides de jus de pomme (en anglais : AP), les déchets solides de micro-brasseries, les déchets d'agrumes (CW), la tourbe de sphaigne (SPM), les boues d'ultrafiltration de jus de pomme (APS), les eaux usées des industries d'amidon (SIW), les boues municipales secondaires (MSS) et le lactosérum (LS) ont été utilisés pour leur potentiel de production d'AC par fermentation à l'état solide (SSF) et par fermentation à l'état liquide (SmF), par *Aspergillus niger* NRRL 567 et *A. niger* NRRL 2001, respectivement.

Pendant la sélection des déchets agro-industriels, l'utilisation des AP a abouti à la production de 66.0 ± 2.0 g/kg de substrat sec (ds) d'AC et pour les APS, de 9.0 ± 0.3 g/l et se sont avérés être des substrats solides et liquides potentiels par la croissance de *A. niger* NRRL 567. Les substrats sélectionnés, AP et APS ont été utilisés pour de nouvelles études portant sur l'optimisation des paramètres tels que : le niveau d'humidité initial (IML), les inducteurs par SSF, les matières solides en suspension (SS) et la concentration d'inducteurs par SmF. Pour se faire, la méthodologie de surface de réponse (RSM) a été utilisée. L'application de la MRS a mené à une bio-production plus élevée de l'état solide d'AC de 342 g/kg de substrat sec dans des conditions optimales de 75% (v/p) de IML accompagnée de 3% (v/p) de MeOH après une période de fermentation de 144 h et une taille de particule allant de 1.7 à 2.0 mm, respectivement. De même, par SmF, les AP ont donné une bio-production plus élevée d'AC de 44.9 g/kg ds, avec des paramètres optimaux de 25 g/l de SS accompagnées de 3% (v/v) de MeOH après une période de fermentation de 144 h. Les travaux d'optimisation par RSM ont révélé le potentiel des AP et APS comme substrats novateurs pour la bio-production économique d'AC.

La production d'AC a été augmentée dans le laboratoire à l'échelle des fermenteurs. La SSF dans le fermenteur conduit à la production plus élevée d'AC de 294 ± 14 g/kg ds avec 3% (v/p) de MeOH, avec une agitation intermittente de 1 h toutes les 12 h à 2 trs/min, 1 vvm de taux d'aération, 120 h de temps d'incubation dans des conditions d'extraction optimales: temps d'extraction de 20 min, taux d'agitation de 200 trs/min et volume d'extraction de 15 ml. De même, la production à l'état liquide d'AC dans le laboratoire à l'échelle des fermenteurs a mené

à une bio-production d'AC de 40.3 ± 2.0 g/l de APS avec 3% (v/p) de MeOH, respectivement, après 132 h de fermentation par *A. niger* NRRL 567. Les études des profils rhéologiques pour les APS des bouillons contenant le champignon filamenteux *A. niger* NRRL 567, pendant la fermentation d'AC, ont été menées à l'échelle du fermenteur de 7.5 L. Le contrôle sans inducteurs a démontré un comportement pseudo-plastique non-newtonien en raison d'une plus grande croissance filamenteuse. Cependant, dans le traitement complété avec le méthanol, la forme de boulette a été observée pendant la fermentation des bouillons non-newtoniens et moins visqueux. Le modèle de loi de puissance a été reproduit avec une grande précision (77.8-88.9%) tout au long de la fermentation sans inducteurs. Cependant, la loi de puissance a été observée de manière modérée (65.3-75.9%) au début de fermentation jusqu'à 48 h, puis suivi plus tard d'un bon indice de confiance (75.9-88.2%) jusqu'à 144 h de fermentation contrôlée. Des études rhéologiques devront être menées afin d'accroître la bio-production d'AC.

Les déchets du mycélium fongique résultant de la fermentation par SSF et SmF ont été simultanément utilisés pour l'extraction d'un important coproduit, le chitosane (CTS). L'extrait de CTS était plus élevé dans le contrôle sans inducteurs de, respectivement, 6.40% et 5.13% de biomasse séchée, avec le mycélium fongique résultant de la fermentation par SSF et SmF. Le degré de dé-acétylation du CTS variait entre 78% et 86% pour la biomasse fongique obtenue par SSF et SmF avec des inducteurs différents. La viscosité de CTS fongique s'est trouvée être augmentée de 1.02 $Pa.s^{-1}$ à 1.18 $Pa.s^{-1}$, ce qui est considérablement plus bas que celle du coproduit commercial CTS se trouvant dans la carapace du crabe. Ces résultats indiquaient la possibilité d'une co-extraction de CTS de qualité supérieure de la biomasse des déchets fongiques résultant de diverses industries biotechnologiques fongiques incluant l'AC.

La production combinée d'AC et de CTS accompagnée de déchets différents (déchets de mycélium fongique résultant de la fermentation, la biomasse fermentée accompagnée d'AC et la biomasse fongique, la biomasse activée après l'extraction de CTS a été utilisé pour la biosorption de métaux toxiques (As, Cr et Cu) provenant de solutions aqueuses issues de la décontamination de déchets de bois traité au CCA (arséniate de cuivre chromaté). L'effet des différents paramètres, tels que la concentration de biosorbant, la concentration métallique et le temps de contact, a été examiné. Parmi les isothermes d'adsorption testés, l'isotherme de Langmuir a donné la meilleure estimation de la valeur des coefficients de détermination (R^2) de trois métaux différents (la biomasse par SSF) s'étendant de 0.89 à 0.97; de 0.96 à 0.99 et de 0.76 à 0.95 pour As, Cr et Cu, respectivement. De même, l'adsorption significative de métaux (>60% dans le lixiviat 2) provenant des lixiviats de déchets de bois traité au CCA, a été réalisé

avec des biomatériels (BMs) différents. Ainsi, cette étude démontre que ces déchets de BMs produits pendant la production d'AC et l'extraction de CTS, pourrait être utilisés pour adsorber les métaux lourds présents dans les effluents contaminés et purifier ainsi ce type de rejet liquide.

Un autre aspect étudié est la synthèse de ZnO-CTS basé sur des nanoparticules (NPs). Différents stabilisateurs ont été utilisés pour la synthèse de NPs par séchage de nano-vaporisation et par méthode de précipitation. Le ZnO-CTS basé sur NPs a montré une activité significative antimicrobienne et inhibitrice de biofilm contre des souches bactériennes pathogènes opportunistes, *Micrococcus luteus* et *Staphylococcus aureus*.

L'utilisation des déchets agro-industriels avec une production minimale de déchets et l'application de déchets de biomatériaux à des fins sanitaires constituent les objectifs essentiels de ce projet de recherche. Les études ont montré que des milieux coûteux ne sont pas nécessaires lors de l'ajout de déchets agro-industriels de faible coût pour la production d'AC. Ainsi, les conditions optimales pourraient être appliquées pour accroître la production d'AC afin de satisfaire la demande toujours croissante en raison des applications variées. De plus, l'extraction simultanée du coproduit CTS se consolidera davantage et rendra la biosynthèse d'AC plus économique. Le projet a également plusieurs buts : (1) l'utilisation économique de déchets agro-industriels de faible coût ; (2) la séquestration environnementale des déchets de carbone menant à la réduction du changement climatique; (3) la production minimale de déchets et leur utilisation durable dans des applications de biotraitement environnemental; (4) la synthèse de NPs de ZnO-CTS possédant des propriétés antimicrobiennes et inhibitrices de biofilm contre des souches bactériennes pathogènes leur donnant un rôle prometteur en biomédecine.

ABSTRACT

In view of the ever increasing demand of citric acid (CA) due to many advanced applications coming to light, there is an urgent need to look for inexpensive and novel substrates for feasible production of CA. In this context, various agro-industrial wastes, such as apple pomace (AP), brewery spent grain (BSG) CW (citrus waste), sphagnum peat moss (SPM) and apple pomace ultrafiltration sludge (APS), starch industry water (SIW), municipal secondary sludge (MSS) and lactoserum (LS) were utilized for their potential for CA production through solid-state fermentation (SSF) and submerged fermentation (SmF), respectively, by *Aspergillus niger* NRRL 567 and *A. niger* NRRL 2001. During the screening of agro-industrial wastes, AP resulted in CA production of 66.0±1.9 g/kg of dry substrate (ds) and APS 9.0±0.3 g/l respectively and proved to be the potential solid and liquid substrate by *A. niger* NRRL 567. The screened substrates, AP and APS were used for further studies for optimization of parameters [initial moisture level (IML) and inducers in SSF and suspended solids (SS) and inducers concentration in SmF] through response surface methodology (RSM). The application of RSM leads to higher solid-state CA bioproduction of 342.4 g/kg ds with optimum conditions of 75% (v/w) IML and along with 3% (v/w) MeOH after fermentation period of 144 h and particle size of 1.7-2.0 mm, respectively. Similarly, in SmF, APS rendered higher CA bioproduction of 44.9 g/100g ds, respectively through optimum parameters of 25 g/l SS along with 3% (v/v) MeOH after 144 h fermentation period. RSM optimization results indicated the potential of AP and APS as novel substrate for economical CA bioproduction.

CA production was scaled-up in the lab scale fermenters. SSF in fermenter resulted in the higher CA production of (294.19±13.9 g/kg ds with 3% (v/v) MeOH, intermittent agitation of 1 h after every 12 h at 2 rpm and 1 vvm of aeration rate and 120 h incubation time and with optimum extraction conditions: extraction time of 20 min, agitation rate of 200 rpm and extractant volume of 15 ml by RSM. Similarly, submerged CA production in lab scale fermenter leads to CA bioproduction of 40.34±1.98 g/l APS with 3% (v/v) MeOH, respectively, after 132 h of fermentation by *A. niger* NRRL 567. Rheological profile studies for APS broth with filamentous fungus, *Aspergillus niger* NRRL-567 during CA fermentation are reported for the biomass developed in a 7.5 l bench scale fermenter. The control demonstrated non-Newtonian pseudoplastic behavior due to more filamentous growth. However, in treatment supplemented

with methanol, pellet form was observed during fermentation with less viscous non-Newtonian broths. The power law model was followed with good confidence of fit (77.8–88.9%) throughout the fermentation in treatment with MeOH as an inducer. However, power law was followed with moderate fit of confidence (65.3–75.9%) at the beginning of fermentation until 48 h and later followed a good confidence of fit (75.9–88.2%) until 144 h of fermentation in control. Rheological studies will further aid in achieving higher CA bioproduction.

The waste fungal mycelium resulting from SSF and SmF was concomitantly used for extraction of important co-product, chitosan (CTS). Extractable CTS was found to be higher in control with 6.40% and 5.13% of dried biomass, respectively with the fungal mycelium resulting from the SSF and SmF. Degree of deacetylation of the CTS was ranged from 78-86 % for fungal biomass obtained from SSF and SmF with different inducers. The viscosity of fungal CTS was found to be ranging from 1.02-1.18 $Pa.s^{-1}$ which is considerably lower than the commercial crab shell CTS. These results indicated the possibility of co-extraction of superior quality CTS from waste fungal biomass resulting from various fungal-based biotechnological industries including CA. In-house produced CA and CTS along with different waste streams (waste fungal mycelium resulting from fermentation, the fermented biomass along with CA and fungal biomass, and activated biomass after CTS extraction) was used for the biosorption of toxic metals (As, Cr and Cu) from aqueous solutions and waste CCA (chromated copper arsenate) woods leachates. The effect of different parameters such as biosorbate concentration, metal concentration and contact time was investigated. The fitness of biosorption data for Freundlich and Langmuir adsorption models was investigated by employing batch adsorption technique. Among the adsorption isotherm tested, Langmuir isotherm gave the best fit with correlation coefficients (R^2) value of three different metals (SSF biomass) ranging from 0.89-0.97; 0.96-0.99 and 0.76-0.95 for As, Cr and Cu, respectively. Similarly, the significant removal of metals (> 60% in leachate 2) from waste CCA wood leachate was achieved with the different BMs. Therefore, this study demonstrated that waste BMs resulting during CA production and CTS extraction which serves as an environmental pollutants could be used to adsorb heavy metals from contaminated waste waters and achieve cleanliness thereby abating environmental nuisance caused by the these wastes. Another application aspect of this project is the facile synthesis of ZnO-CTS-based NPs. Different stabilizers were used for the synthesis of NPS through Nano-spray drying and precipitation methods. ZnO-CTS based NPS displayed significant antimicrobial and biofilm inhibiting activity against pathogenic bacterial strains, *Micrococcus luteus* and *Staphylococcus aureus*.

The agro-industrial waste utilization with minimal waste production and the applications of the waste biomaterials for environmental cleanliness formulate the vital objectives of this research project. The studies showed that expensive media is not required for supplementation of negative cost agro-industrial wastes for CA production. Therefore, the optimized conditions could be applied for enhanced CA bioproduction to meet the ever increasing demand due to wide ranging applications. Moreover, the simultaneous extraction of co-product, CTS will further strengthen and makes the CA biosynthesis as more economical. The project serves multifold purpose of: (1) economical use of negative cost agro-industrial wastes and; (2) environmental sequestration of waste carbon leading to mitigation of climate change; (3) minimal waste production and their sustainable utilization for environmental clean-up applications and; (4) facile synthesis of ZnO-CTS-based NPs which possesses antimicrobial and biofilm inhibiting properties against pathogenic bacterial strains implicating their promising role in biomedicine.

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LISTE DES ABRÉVIATIONS

AAIM- acid-and alkali insoluble material

AIM- alkali-insoluble material

ANOVA- analysis of variance

AP- apple pomace

APS- apple pomace ultrafiltration sludge

A. niger- Aspergillus niger

ARS- agricultural research services

ATP- adenosine triphosphate

BOD biological oxidation demand

BSG brewery spent grain

BSL Brewery spent liquor

CA citric acid

CCA chromated copper and arsenate

CCD central composite design

CTS chitosan

CFUs colony forming units

COD chemical oxidation demand

Ct total carbon

CW citrus waste

DP degree of polymerization

DTPA diethylene triaminepentaacetic acid

EDTA ethylene diaminetetraacetic acid

EtOH ethanol

gds gram dry substrate

GHGs greenhouse gases

GRAS generally recognized as safe

HMF hydroxyl-methyl furfural

ICA isocitric acid

ICP-AES inductively coupled plasma atomic emission spectroscopy

IML initial moisture level

ISPR *in-situ* product recovery

LS lactoserum

MeOH methanol

MOs microorganisms

MSS municipal secondary sludge

Mw molecular weight

NPs nanoparticles

NRRL National regional research laboratory

Nt total nitrogen

PAW pine apple waste

PDA potato dextrose agar

PEG polyethylene glycol

POC poly(1, 8-octanediol-co-citric acid)

rpm revolutions per minute

RSM response surface methodology

SD standard deviation

SF surface fermentation

SIS starch industry water sludge

SIW starch industry water

SMB	simulated moving bed
SmF	submerged fermentation
SPM	sphagnum peat moss
SS	suspended solids
SSF	solid-state fermentation
TS	total solids
VAPs	value-added products
VSS	volatile suspended solids

SYNTHÈSE

PARTIE 1. BIO-PRODUCTION D'ACIDE CITRIQUE

1. REVUE DE LITTÉRATURE

1.1 INTRODUCTION

L'acide citrique (AC, $C_6H_8O_7$; Fig 1.1 A), un intermédiaire du cycle de Krebs, appelé également cycle des acides tricarboxyliques, est l'un des produits chimiques les plus importants en raison de sa large utilisation en alimentation (70%), pharmaceutique (12%), et dans d'autres secteurs (18%) (Ates et al., 2002). La production mondiale d'AC a augmenté de 1.7 million de tonnes en 2007 selon les estimations de Business Communications Co. (BCC, http://www.bccresearch.com). En raison de ses nombreuses applications, le volume de production de l'AC par fermentation ne cesse d'augmenter avec une élévation du taux annuel de production de 5% (Finogenova et al., 2005). Cependant, les conditions défavorables du marché ont réduit le prix de l'AC entre 1.0 et 1.3 $/kilogramme. La valeur de ce produit chimique au niveau du marché dépassa les 2 milliards de dollars en 2009 (http://www.foodnavigator-usa.com).

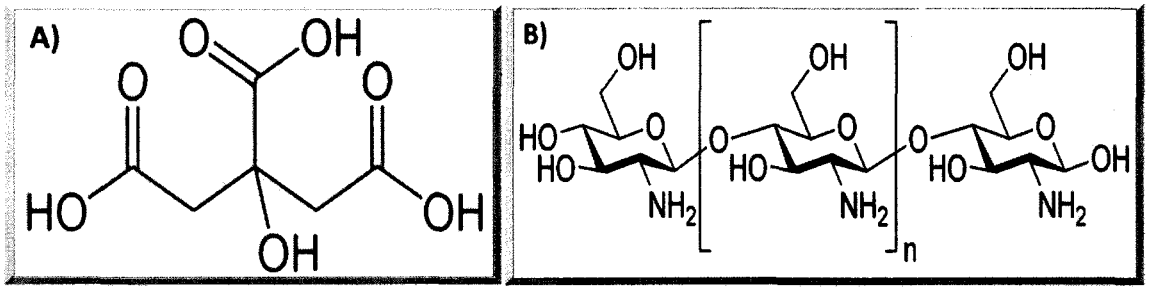


Figure 1. 1 Structure de: A) acide citrique; B) chitosane

L'AC est mondialement reconnu comme GRAS (*"generally recognized as safe"*), par la commission mixte FAO/OMS d'experts en additifs alimentaires (Carlos et al., 2006). L'AC et ses sels, principalement le sodium et le potassium, sont utilisés dans de nombreuses applications industrielles comme agent chélateur, solution tampon, correcteur de pH et comme agent de dérivation. On utilise cet acide dans les détergents à lessive, le shampoing, les produits cosmétiques, les produits chimiques de nettoyage, et également pour améliorer la récupération des huiles (Soccol et al., 2006). Les solutions aqueuses d'AC sont d'excellentes solutions tampons lorsqu'elles sont partiellement neutralisées. L'AC est un acide faible comportant trois

groupes carboxyliques. Par conséquent, il possède trois pKa à 20°C $pK_1 = 3.15$, $pK_2 = 4.77$, et $pK_3 = 6.39$, résultant d'une action tampon à pH variant entre 2.5–6.5. Des études révèlent l'utilisation potentielle d'AC au niveau des biopolymères, des médicaments, dans la culture de variétés de cellules et dans de nombreuses autres applications biomédicales prometteuses, mais aussi écologiques par l'utilisation durable d'AC qui permet d'enlever efficacement les résidus de soudure (*soldering flox*) faites par les militaires (Robin et al., 1995; Ashkan et al., 2010; Guillermo et al., 2010).

La majorité de l'AC est produit par voie biologique, principalement par fermentation submergée (SmF) de l'amidon/saccharose (en utilisant la mélasse comme ingrédient), exclusivement à l'aide des champignons filamenteux, *Aspergillus niger* (Jianlong 2000; Lesniak et al., 2002; Schuster et al., 2002) qui possèdent un grand pouvoir de production d'AC à pH faible sans la sécrétion de sous-produits toxiques. L'AC est considéré comme un métabolite du métabolisme énergétique dont la concentration pourrait augmenter à des concentrations intéressants dans certaines conditions du métabolisme non équilibré. Durant ces dernières années, divers résidus et sous-produits agricoles ont été étudiés pour leur potentiel d'utilisation comme substrat pour a production d'AC par fermentation en milieu solide (SSF), tels que la mélasse, les résidus de pomme, le son de blé, les écailles de café et la bagasse de manioc, qui sont généralement compostés ou jetés dans des décharges causant de graves problèmes environnementaux (Singhania et al., 2009; Kuforiji et al., 2010). Les industries subissent d'énormes pertes à cause de ces déchets qui doivent être éliminés de façon sécuritaire. La bio-production de produits à valeur ajoutée (VAPs), telles que l'AC à partir de substrats provenant des déchets agro-industriels présente énormément d'avantages au niveau de la gestion des déchets et au niveau des coûte des substrats qui sont faibles. De même, l'extraction de chitosane (CTS) simultanée durant la production d'AC à partir des déchets par les mycéliums fongiques va permettre de renforcer la production économique d'AC.

Le chitosane est un autre polymère tout aussi important présent dans de nombreuses applications. Le CTS est un polymère de polysaccharide (Fig 1.1B), polycationique, chélatant et qui présente des propriétés de formation de films en raison de la présence de groupements fonctionnels hydroxyles et amines. Le CTS présente également un ensemble de propriétés biologiques, tels que l'activité antimicrobienne, une résistance aux maladies au niveau des plantes et diverses propriétés stimulantes ou inhibitrices à l'égard de certaines lignées cellulaires humaines. Grâce à ces propriétés, le CTS est largement utilisé dans divers domaines allant de la médecine, les nanosciences, l'alimentation, le génie chimique, les produits

pharmaceutiques et l'agriculture. Le CTS est un ingrédient actif qui sert à la formation de différentes nanoparticules potentielles (NPs) servant dans de nombreux domaines. La forte demande en chitosane est due au fait qu'il soit non-toxique, biodégradable, biocompatible et respectueux de l'environnement.

Traditionnellement, le CTS dérive naturellement de la chitine, généralement de la chitine des crustacés tels que les crabes, les crevettes. Il est produit par la dé-acétylation chimique à l'aide de traitements agressifs. Toutefois, ces traitements peuvent avoir des effets négatifs sur le CTS, tel qu'une contamination des protéines. Il peut y avoir des niveaux de dé-acétylation incompatibles et donc un changement du poids moléculaire (Mw), qui aboutit à des variations physico-chimiques du CTS. Il existe également d'autres problèmes, tels que les questions qui se posent sur l'environnement et qui sont liées à l'utilisation de produits chimiques toxiques, la limitation en approvisionnement en fruits de mer, la limitation géographique, les variations saisonnières et les coûts élevés. Par conséquent, les approches biologiques pour la synthèse du CTS doivent être explorées. Une paroi cellulaire fongique contient jusqu'à 50% de chitine par rapport aux carapaces des crustacés qui contiennent 14-27% de chitine sur la base de la biomasse sèche (Zamini et al., 2007). Dans cette perspective, la production, la purification et la transformation de la chitine fongique en CTS par dé-acétylation dans des conditions contrôlées offre un grand potentiel pour l'obtention d'un produit homogène.

L'industrie des produits forestiers représente le premier secteur pour l'exportation canadienne. Le bois fait partie intégrante de la vie humaine, il est utilisé pour la fabrication des poteaux des maisons, des meubles et des services publics, entre autres. Le bois a toujours joué un rôle important dans l'économie canadienne. Il s'agit d'une industrie diversifiée, produisant à la fois des produits de base et des produits à valeur ajoutée dans toutes les régions du pays. La contribution de l'industrie forestière au Canada du produit intérieur brut est d'environ 1,9%. La valeur des exportations canadiennes de produits forestiers était de 30.1-33.6 CAN $ milliards en 2007-2008 (Source: Statistics Canada, merchandise trade data, monthly http://canadaforests.nrcan.gc.ca/rpt). L'arséniate de cuivre chromaté (ACC) est le traitement chimique le plus utilisé pour la préservation du bois et pour le protéger contre toutes attaques fongiques et contre les insectes. Cependant, il devient impératif de décontaminer les bois usagés traités à l'ACC avant qu'ils ne soient jetés. Le bois traité doit être éliminé conformément aux règlements locaux, provinciaux et fédéraux. L'AC était utilisé pour la biorémédiation des métaux lourds provenant de sites contaminés (Williams et Cloate, 2010). L'AC peut lixivier efficacement le Cd, Cu, Pb et le Zn des sols contaminés. L'utilisation potentielle d'AC a été

démontrée dans l'élimination du potassium à partir de concentrés de minerais de fer de qualité inférieure (Williams et Cloete, 2010). L'AC contient trois groupes carboxyliques qui tendent à céder des protons (H^+), ces groupements carboxyles sont alors chargés négativement, et deviennent stables en se liant à des cations tels que le potassium.

À l'avenir, l'AC pourrait être utilisé pour la lixiviation chimique des impuretés des minerais naturels afin d'extraire des métaux d'intérêt. Parmi les acides organiques faibles, l'AC produit par l'*A. niger* présente l'avantage d'être respectueux de l'environnement par rapport aux agents chélateurs chimiques et acides forts, tels que l'EDTA (ethylene diaminetetraacetic acid), DTPA (diethylenetriaminepenta acetic acid), HCl, HNO_3 et H_2SO_4. La biomasse fermentée (contenant de l'AC et de la biomasse fongique) et la biomasse activée (après extraction du CTS), offrent également un grand potentiel pour être utilisées pour la biorémédiation des métaux lourds toxiques issus de milieux contaminés, tels que les bois contaminés par l'ACC.

Ainsi, en regardant les demandes en hausse d'AC, il est urgent de trouver des moyens pragmatiques pour augmenter les rendements de production. Ce projet vise à évaluer le potentiel des déchets agro-industriels pour la production réalisable et durable d'AC et pour l'extraction du CTS à partir des déchets par des mycéliums fongiques Les nanoparticules composées de CTS et d'AC combinées à des oxydes métalliques seront fabriquées par un procédé vert, par vaporisation micro-fine et par la méthode de précipitation. L'application de la biomasse fongique des déchets, la biomasse fermentée (contenant de l'AC et un mycélium fongique), et la biomasse active (biomasse résiduelle lors de l'extraction du CTS), sera évaluée pour la biorémédiation des métaux lourds à partir de solutions aqueuses et les lixiviats contaminés en ACC seront traités.

1.2. Produits plate-formes

Parmi les acides carboxyliques, l'AC a été largement utilisé pour la fabrication de divers produits chimiques. La demande grandissante pour les substituts en polymères biodégradables dans divers secteurs attire l'attention sur la nécessité d'une amélioration des procédés de production de l'AC. L'AC est obtenu par la fermentation aérobie du glucose par glycolyse et par la dérivation glyoxylate. La fermentation aérobie conduisant à la formation de l'AC est l'une des voies de conversion microbienne le mieux établies. L'AC est considéré comme l'un des produits chimiques le plus important. Il a été inclus dans la liste des 30 premiers candidats d'intrants de construction qui: [1] présentent de multiples fonctionnalités appropriées pour la conversion plus

que les dérivés ou les familles moléculaires ; [2] peuvent être produits à partir des matières lignocellulosiques et de l'amidon ; [3] étaient en monomères C1-C6 ; [4] ne sont pas des produits aromatiques dérivés de lignine et ; [5] n'étaient pas des super-produits chimiques (NREL, 2004). Etant donné que l'AC est non toxique, comestible et biodégradable, il offrira d'importants avantages environnementaux aux nouveaux produits plate-formes. L'AC à moindre coût pourrait ouvrir des marchés importants des polymères et d'autres composés importants en tant que constituant monomère important.

L'AC est un acide carboxylique multiforme qui a un potentiel énorme dans l'alimentation, le domaine pharmaceutique, les cosmétiques, les détergents et les secteurs agricoles. Récemment, une série d'applications de pointe ont été découvertes, telles que dans l'industrie biomédicale pour la synthèse de biopolymères pour les médicaments, la culture d'une grande variété de lignées cellulaires humaines; la nanotechnologie, pour la bioremédiation des métaux lourds dans le sol et pour la protection du bois à base d'eau (Carlos et al., 2006; Ashkan et al., 2010; Guillermo et al., 2010). Cependant, depuis les dernières années, le marché de l'AC a été sous une pression énorme qui continue de se balancer avec une réduction des prix. Une énergie élevée associée à des coûts de matières premières élevés a mis la production de l'AC dans un marché peu rentable. En même temps, la consommation de l'AC accroît progressivement en raison de demandes vers des applications nouvelles. Différentes techniques pour la surproduction d'AC sont à l'étude depuis les dernières décennies. Par conséquent, il existe un besoin évident d'envisager de nouveaux moyens pratiques pour parvenir à la bio-production industriellement réalisable et écologiquement durable d'AC. L'utilisation de déchets agro-industriels non coûteux et leurs sous-produits par fermentation en milieu solide par des souches microbiennes existantes et génétiquement modifiées est une voie potentielle.

1.3 Micro-organismes

Un grand nombre de microorganismes, tels que les bactéries, les champignons et les levures ont été cultivés dans une variété de substrats pour la bio-production d'AC comme indiqué au Tableau 1 (Crolla et al., 2004; Kuforiji et al., 2010). Le champignon de la pourriture blanche, *A. niger*, constitue le meilleur choix de microorganismes pour la bio-production d'AC. Les souches d'*Aspergillus* sont les MOs les plus adaptées et adéquats pour se développer dans des substrats différents. La régulation fine et le contrôle des flux glycolytique, la sécrétion d'AC par la mitochondrie et le cytosol, les caractéristiques de croissance et de l'adaptabilité d'*A. niger*

dans les divers milieux de culture contribuent à l'accumulation massive d'AC. La réglementation des différentes enzymes métaboliques associés à l'effet de divers facteurs positifs sur le flux glycolytique favorise une production élevée d'AC et une dégradation faible par l'intermédiaire du cycle d'AC (Levente et al., 2003). Malgré le fait que les études classiques de production d'AC impliquent des moules, l'utilisation des bactéries et de la levure s'avère de grande importance (Carlos et al., 2006; Rymowicz et al., 2010). Parmi les levures, *Yarrowia lipolytica* a été largement utilisée pour sa capacité à produire de grandes quantités d'AC. Un inconvénient de la fermentation de la levure réside dans le fait qu'elle produit des quantités importantes d'acide iso-citrique (AIC), un sous-produit indésirable. Les principaux avantages d'*A. niger* parmi de nombreux microorganismes sont sa facilité de manipulation/récolte, sa capacité à fermenter une variété de matières premières, de préférence les résidus de déchets agricoles, et les rendements élevés de production. Dans la littérature, la plupart des études mentionnent l'utilisation de souches d'*A. niger* en tant que microorganismes le plus favorable pour la production d'AC. Cependant, avec les progrès de la biotechnologie, il y a eu développement de nouvelles souches génétiquement modifiées et l'amélioration de souches existantes productrices d'AC par mutagénèse et sélection de souches potentielles de productivité plus élevés d'AC. Cependant, l'utilisation effective de ces nouvelles souches en question dans l'application industrielle et leur stabilité sur une période de temps, s'appuyaient sur les souches classiques. À cet égard, le rôle des substrats est également un facteur essentiel pour améliorer le rendement de production d'AC.

Table 1. 1 Micro-organismes producteurs d'acide citrique

Champignons	*Aspergillus niger, A. awamori, A. clavatus, A. nidulans, A. fonsecaeus, A. luchensis, A. phoenicus, A. wentii, A. saitoi, A. flavus, Absidia* sp., *Acremonium* sp., *Botrytis* sp., *Eupenicillium* sp., *Mucor piriformis, Penicillium citrinum, P. janthinellu, P. luteum, P. restrictum Talaromyces* sp,. *Trichoderma viride, Ustulina vulgaris*
Levure	*Candida tropicalis, C. catenula, C. guilliermondii, C. intermedia, Hansenula, Pichia, Debaromyces, Torula, Torulopsis, Kloekera, Saccharomyces, Zygosaccharomyces, Yarrowia lipolytica*
Bactéries	*Arthrobacter paraffinens, Bacillus licheniformis, Corynebacterium* ssp.

1.4. Substrats

En raison de la hausse des prix, la réduction des ressources non renouvelables, la faible disponibilité des matières premières en raison de la concurrence avec l'approvisionnement alimentaire, et les questions de la pollution environnementale, l'accent a été mis sur la récupération, le recyclage et la valorisation de la biomasse ligno-cellulosique. Cela est particulièrement vital pour l'alimentation et l'industrie agro-alimentaire qui génère d'énormes quantités de résidus de déchets (solides et liquides) pendant le traitement, ce qui peut souvent être mis à profil pour générer des bioproduits à haute valeur ajoutée. Ces déchets posent des risques de pollution de l'environnement et représentent une perte de biomasses et de nutriments. Dans le passé, ces déchets ont souvent été sous-évalués ou utilisés dans des applications de faible valeur, tels que les aliments pour les animaux ou comme engrais. Au cours des dernières années, cependant, en raison de la nécessité croissante de prendre en considération les aspects visant à prévenir la pollution de l'environnement, ainsi que pour des raisons économiques, et la nécessité d'économiser de l'énergie et de nouveaux matériaux, de nouvelles méthodes et politiques de gestion et de traitement des déchets ont été introduits dans la récupération, la bioconversion, et l'utilisation des éléments de valeur de ces déchets. En général, les déchets de transformation des aliments ont un potentiel de recyclage des matières premières ou pour la transformation en produits à haute valeur, ou pour l'utilisation comme denrées alimentaires ou aliments pour animaux/fourrage après le traitement biologique. En particulier, la bioconversion des déchets de transformation des aliments a reçu un intérêt accru. Tout en considérant les différents substrats, il est certainement plus facile de produire l'AC à partir de substrats synthétiques où la source de carbone est simple à dégrader. Cependant, avec la hausse des coûts des matières premières et l'abondance des déchets agro-industriels (en vertu de la population en plein essor) résultant des coûts d'élimination, la perte de carbone précieuse et la libération de gaz à effet de serre (GES), il existe un besoin crucial d'utiliser ces déchets agro-industriels pour la production de l'AC. Toutefois, les déchets agro-industriels sont souvent complexes exigeant des prétraitements pour augmenter la disponibilité des éléments nutritifs; améliorer la capacité rhéologique (diminuant la viscosité et la taille des particules) favorisant le transfert accru d'oxygène et un transfert de matière de nutriments, et d'accroître le rendement de production par l'utilisation efficace du substrat complet.

1.5. Rhéologie

La morphologie du champignon est un paramètre important qui influe sur les caractéristiques physiques du bouillon de fermentation et donc le rendement final du produit. Le comportement rhéologique du champignon est étroitement lié à la morphologie et à la concentration de la biomasse (Jayanta et al., 2001). La rhéologie du bouillon de fermentation détermine les phénomènes de transport dans les bioréacteurs et le rendement de production du produit désiré. Le comportement de l'écoulement non-newtonien est l'aspect fondamental des systèmes de fermentation fongique, en particulier les champignons filamenteux. Le bouillon de fermentation présente des caractéristiques non-newtoniennes distinctes, même si la teneur en matière sèche est présente en faible quantité (Henzler et al., 1987). Par exemple, dans les systèmes de fermentation d'*A. niger,* les formes filamenteuses ou granulées peuvent être distinguées dans le bouillon pendant le procédé de fermentation. Au cours du stade filamenteux, l'enchevêtrement des hyphes du mycélium avec une forte concentration de la biomasse peut conduire à un comportement non-newtonien très visqueux. Cependant, dans la forme granulée, le mycélium développe des agrégats sphériques très stables, constitués d'un réseau ramifié plus dense, partiellement lié par des hyphes et donne généralement des bouillons non-newtoniens moins visqueux.

Pour la fermentation à l'état liquide d'AC, la forme granulée d'*A. niger* est fortement recommandée. Le comportement rhéologique des bouillons de fermentation affecte le mélange du contenu moyen et donc de masse et des processus de transfert de chaleur qui, à son tour, empêche le transfert de l'oxygène vers les cellules, ce qui est souvent l'étape cinétiquement limitante de la fermentation. Une voie possible pour réduire au minimum la limitation du transfert d'oxygène vers les cellules est de stimuler la formation de petites boulettes de cellules sphériques. Le bouillon de fermentation contenant un mycélium déficient en manganèse (Mn^{2+}) a une rhéologie beaucoup plus favorable et un taux de transfert d'oxygène accru due à la formation de granules (Levente et al., 2003). Toutefois, en même temps, le Mn^{2+} est essentiel pour la croissance d'*A. niger* et il a été constaté que l'anabolisme cellulaire d'*A. niger* est altéré lors de carence en Mn^{2+} total (Rohr et al., 1996). La concentration initiale de Mn^{2+} dans le milieu de fermentation doit être optimisée afin d'accroître la bio-production d'AC. Cependant, il est intéressant de noter que le caractère non-newtonien et très visqueux des suspensions mycéliennes conduit à des difficultés dans le mélange, ce qui affecte fortement le transfert à l'interface de l'oxygène. L'utilisation de bioréacteurs agités mécaniquement est nécessaire pour obtenir un bon transfert de matière et de la chaleur et de l'oxygène dans les bouillons de

fermentation non-newtoniens. La rhéologie de bouillon de fermentation résulte de l'effet net de la complexité du substrat, de l'augmentation de la biomasse lors de la croissance, ainsi que de la formation de métabolites différents causant des problèmes de transfert de l'oxygène et une faible productivité d'AC.

1.6. Différentes techniques de fermentation pour la bio-production d'AC

Actuellement, 99% de l'AC total produit dans le monde est obtenu par fermentation (Kuforiji et al., 2010). La moisissure *A. niger* est le producteur préféré et le plus performant d'AC. La bio-production d'AC peut être divisée en trois étapes, comprenant la préparation et l'inoculation du milieu de fermentation, la fermentation, et la récupération/purification de l'AC tel que décrit dans la Figure 1.2. Les méthodes les plus utilisées de bio-production d'AC sont la fermentation à l'état liquide (SmF) et la fermentation à l'état solide (SSF) comme illustré au Tableau 2. Une large gamme de substrats pourrait être utilisée pour la production efficace et réalisable d'AC selon le type de fermentation.

1.6.1. Fermentation à l'état liquide (SmF)

Il est estimé qu'environ 80% de la production mondiale d'AC est obtenue par SmF en utilisant la moisissure *A. niger* cultivée dans un milieu contenant du glucose ou du saccharose (Leangonet al., 2000; Kumar et al., 2003), principalement à partir de sous-produits de l'industrie sucrière. En dehors de cela, diverses autres matières premières, telles que les hydrocarbures, les résidus de déchets agro-industriels et plusieurs autres matériaux riches en féculents ont été utilisés comme source de carbone pour la SmF d'AC (Jianlong et al., 2000; Mourya et al., 2000; Vandenberghe et al., 2000; Ambati et al., 2001; Rivas et al., 2008). Le Tableau 2 illustre l'effet de différents types de fermentation qui utilisent différents substrats sur la production d'AC. En comparaison aux autres types de fermentation, la SmF présente plusieurs avantages, comme une productivité et un rendement élevés, des coûts de main-d'œuvre plus faibles et un risque moindre de contamination. Selon les conditions de fermentation, elle est réalisée généralement en 5 à 12 jours. Bien que la SmF est la méthode la plus largement utilisée pour la production en masse d'AC, il existe toujours un besoin d'explorer une méthode alternative pour la production industrielle viable et durable d'AC afin de faire face à la baisse des prix et de répondre à la demande croissante du marché en AC. La SSF est une approche potentielle pour réaliser un

rendement élevé d'AC en exploitant un grand choix de déchets agro-industriels naturels et renouvelables et leurs sous-produits et qui ne peut pas être réalisable par SmF. En outre, la SSF a repris son envol dans les pays occidentaux suite aux besoins urgents de résoudre les problèmes croissants associés à la gestion des déchets agro-industriels.

1.6.2. Fermentation à l'état solide (SSF)

Au cours des dernières années, la production d'AC par SSF a fait l'objet d'un grand intérêt, elle offre de nombreux avantages pour la production de produits chimiques en vrac (par exemple AC) et des enzymes (Romero-Gomez et al. 2000). En effet, les procédés de fermentation à l'état solide ont de faibles besoins énergétiques, produisent beaucoup moins d'effluents et donc engendrent moins de préoccupations environnementales. Afin de rendre la bio-production d'AC industriellement réalisable par SSF, la recherche de substrats appropriés a été faite surtout sur des résidus agro-industriels. Ceci est principalement dû au fait qu'ils sont facilement disponibles, peu coûteux, riches en glucides et en autres nutriments essentiels, et en raison de leur avantage potentiel de la récolte des champignons filamenteux, qui sont capables de pénétrer dans les parties de ces substrats suite à la pression de turgescence dans la partie supérieure du mycélium (Ramachandran et al., 2004). En outre, la biotransformation des résidus agro-industriels contribue à leurs problèmes d'élimination. L'utilisation des déchets agricoles pour la production d'AC a beaucoup amélioré son efficacité économique. Le choix d'un substrat approprié pour le procédé de SSF dépend de plusieurs facteurs qui sont principalement liés au coût et à la disponibilité de celui-ci. Selon Chundakkadu (2005), la SSF est définie comme étant un procédé de fermentation dans lequel les MOs se développent sur des matériaux solides en absence de liquide libre. Lors de la SSF, l'humidité nécessaire à la croissance microbienne existe dans un état absorbé ou complexé à l'intérieur de la matrice solide. Ce substrat solide est généralement un composé naturel provenant soit de produits agricoles, de sous-produits et résidus agro-industriels, ou d'une matière synthétique (Pandey, 2003). Différents types de fermenteurs ont été utilisés pour la production d'AC par SSF, comme les erlenmeyers, les incubateurs en verre, plateaux, bioréacteurs à tambour horizontal tournant, bioréacteur à colonne garnissage, lit de percolation à simple couche, lit de percolation à multi-couches, entre autres (Papagianni et al., 1999; Pandey et al., 2000; Vandenbergh et al., 1999, 2004).

Table 1. 2 Effet de différentes fermentations utilisant différents substrats alternatifs sur la production d'AC

Type de fermentation	Substrat	Concentration d'AC	Micro-organismes	Température /durée	Remarques	Références
SmF	Rafles de maïs	604 ± 31[d]	*A. niger* NRRL 2001	30°C/72 h	Pré-traitée avec NaOH & enzyme	Hang et al., 2001
	Mélasse noire	31.1 ± 2.0 [e]	*A. niger* GCB 75	30°C/120 h	-	Haq et al., 2001
	Mélasse de betterave	68.7	*Y. lipolytica* A-101	-	-	Lesniak et al., 2002
	Mélasse de canne	114 g/l	*A. niger* GCMC 7	30°C/168 h	Rendement élevé après le pré-traitement avec le ferrocyanure et H_2SO_4	Haq et al., 2004
	n-paraffine	40 g/l[e]	*C. lipolytica* NRRL Y 1095	28±1°C/7 jrs	-	Crolla et al., 2004
	Huile de soja	115[c]	*C. lipolytica* N-5704	-	-	
	Huile de coco	99.6[c]	*C. lipolytica*	28°C/10 jrs	-	Soccol et al., 2006
	Huile de palme	155[c]	*C. lipolytica* N-5704	-	-	William et al.,2007
	Huile d'olive	112 g/l[e]	*C. lipolytica* N-5704	-	-	
	Glycérol brut	119[c]	*Y. lipolytica* NRRL YB-423	10 jrs	-	Levinson et al., 2007
	Huile de colza	66.6 [e]	*Y. lipolytica* N 1	96 h	-	Kamzolova et al., 2007
	Pelures d'orange	53% [a]	*A. niger* CECT- 2090	30°C	4% MeOH	Rivas et al., 2008
	Eaux usées d'huilerie (olives)	28.8 g/l[e]	*Y. lipolytica*	28±1°C	-	Seraphim et al., 2008
	Tourbe de sphaigne + glucose	354 g [d]	*A. niger* NRRL 567	-	Rendement maximal avec 19 g phytate, 49 g d'huile d'olive & 37 g MeOH/kg dw	Suzelle et al., 2009
	Déchets contenant du glycerol (biodiesel l'Industrie)	55.7%	*Y. lipolytica* A-101-1.22	158 h	-	Rymowicz et al., 2010

Type de fermentation	Substrat	Concentration d'AC	Micro-organismes	Température /durée	Remarques	Références
	Algues rouges (*Gelidiella acerosa*) + 10% de saccharose	50 g/l	*A. niger*	30°C/ 10 jrs	-	Ramesh et Kalaiselvum, 2011
SSF	Épis de maïs	259±10 g[d]	*A. niger* NRRL 2001	120 h/ 30°C	70% humidité	Hang et al.,2000
	Marc de pommes	124 g[d]	*A. niger* BC-1	30°C/ 5 jrs	78% humidité ; PS : 0.6-1.18 mm	Shojaosadati et al., 2002
	Déchets d'ananas	51.4[a]	*A. niger* DS-1	30°C/ 8 jrs	70% humidité; 4% MeOH	Kumar et al., 2003
	Déchets de moasmi	50[a]	*A. niger* DS-1	30°C/ 8 jrs	3% MeOH	Kumar et al., 2003
	Déchets d'ananas	46.4%	*A. niger* NRRL 328	6 jrs	54.8% humidité	Kuforiji et al., 2010
	Pelures de bananes	~ 180[d]	*A. niger* MTCC 282	72 h/ 28°C	70% humidité	Karthikeyan et al., 2010
	Marc de pommes	46 g/kg	*A. niger* van. Tieghem MTCC	30°C/ 5 jrs	4% MeOH	Kumar et al., 2010
	Déchets d'ananas + 15% (p/v) saccharose et nitrate d'ammonium (0.25% p/v)	60.6 g/kg	*Aspergillus niger* KS-7	30°C/ 5 jrs	2% MeOH; 65% humidité	Kareem et al., 2010
	Huile de palme grappes de fruits vides + solution minérale	369 g/kg of dry EFB	*A. niger* IBO-103 MNB (IMI396649)	33.1°C/ pH 6.5	2% MeOH; 70% humidité	Bari et al., 2010
	Déchets industriels solides de pommes de terre	32.7 g/kg sec SPW	*A. niger* MAF 3	25°C/ 5 jrs	3% MeOH	Afifi 2011
	Marc de pommes + sulfate d'ammonium	159 g/kg	*A. niger* A	93.9 h	80% humidité	Hoseyini et al., 2011

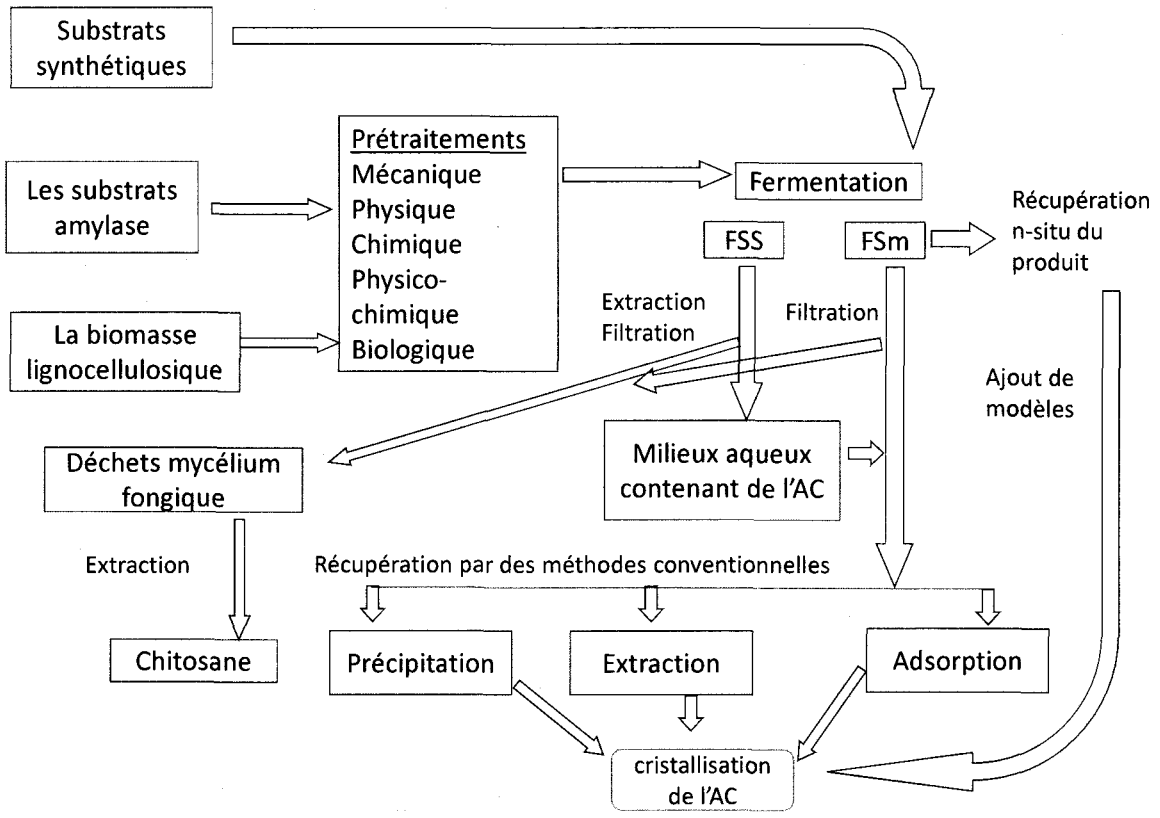


Figure 1. 2 Organigramme illustrant le processus de production d'AC à partit de différents substrats

Au cours de ces dernières années, plusieurs chercheurs ont évalué les deux procédés (SSF et SmF). Ils ont rapporté que le procédé SSF était nettement plus avantageux que le procédé SmF. Les avantages de celui-ci sont illustrés au Tableau 1.3. Bien qu'au cours de ces dernières décennies la SFF a beaucoup été étudiée pour la production durable d'AC et d'autres produits industriels, il reste encore beaucoup d'amélioration à apporter aux niveaux de ce procédé afin d'augmenter les rendements de production de l'AC. Il existe sans doute un fort potentiel dans le procédé de SSF pour développer une technologie de production commerciale d'AC avec une faisabilité économique, mais une plus grande automatisation du processus est nécessaire pour accroître son exploitation industrielle pour la production d'AC, ce qui peut être réalisée avec l'amélioration de la conception des réacteurs. En outre, l'utilisation durable de la biomasse renouvelable et abondante et l'optimisation des différents paramètres de fermentation pourraient conduire à une production techno économiquement viable d'AC.

Table 1. 3 Avantages et inconvénients des différents types de fermentations avec leurs effets sur la production d'AC

Type	Avantages et inconvénients	Production d'AC	Références
SF	**Avantages :** Coûts de fonctionnement, d'installation et d'énergie moindres; microorganismes de surface, très peu **Inconvénients :** Production importante de chaleur, long temps de réaction, besoin de grand espace, sensible à la contamination par *Penicillium*, *Aspergillus* et autres, des levures et des bactéries lactiques	Utilisés dans les industries de petite ou moyenne envergure	Soccol et al., 2003
SmF	**Avantages :** Mécanismes de contrôle sophistiqués, baisse des coûts de main-d'œuvre, une productivité et un rendement accrus **Inconvénients :** Coûts élevés des milieux, sensible à l'inhibition par les métaux traces, en risque de contamination, quantité importante d'effluents à traiter	80% de la production mondiale d'AC	Vanderberghe et al., 1999
SSF	**Avantages** : Technologie simple, rendement plus élevé, grand choix de milieux à faible coût qui ressemble à l'habitat naturel de plusieurs microorganismes, meilleure circulation d'oxygène, moin sensible à l'inhibition par les éléments en traces, moins d'énergie et de coût, faible risque de contamination bactérienne, moins coûteux par la valorisation des déchets **Inconvénients :** Difficultés de mise à l'échelle, contrôle difficile des paramètres opératoires tels que le pH, l'humidité, la température, les nutriments, la détermination rapide de la croissance microbienne, les produits d'impuretés et des coûts plus élevés des produits de récupération	Rendements plus élevés, procédé de production d'AC économiquement efficace et industriellement réalisable en raison de l'utilisation efficace et la valeur ajoutée des déchets	Gowethaman et al., 2001; Durand et al., 2003; Robinson et al., 2003 ; Holker et al., 2004; Susana et al., 2006

1.7. Facteurs influant la production d'AC

Les conditions de biosynthèse affectent la croissance fongique et la production d'AC. De nombreux chercheurs ont montré qu'une forte production d'AC ne peut être obtenue que lorsque la production de biomasse est limitée (Couto et al., 2006). Différents facteurs agissant sur la production d'AC par divers procédés de fermentation peuvent être optimisés afin de maximiser la production microbienne d'AC.

1.7.1. Constituants du milieu

La production d'AC par *A. niger* est influencée par un certain nombre de paramètres au niveau de la culture, en particulier les éléments retrouvés en traces métalliques tel que présenté au Tableau 1.4. Ce microorganisme a besoin de certains éléments en traces métalliques pour sa croissance. Les métaux essentiels comprennent le Zn, Mn, Fe, Cu, entre autres. Certains ions métalliques (Fe^{2+}, Mn^{2+}, Zn^{2+}, Cu^{2+} et autres) inhibent la production d'AC par *A. niger* en SmF, même à très faible concentration. La production d'AC par *A. niger* en SmF est extrêmement sensible aux métaux en traces présents dans la mélasse (Majolli et al., 1999). Par conséquent, la concentration de ces métaux lourds doit être faible afin d'obtenir une croissance optimale d'*A. niger* et une production maximale d'AC (Majolli et al., 1999). La concentration optimale de Fe^{2+} requise pour obtenir une production maximale d'AC dépend de la souche de champignons. Des études ont révélé que la production d'AC par *A. niger* en SmF, en utilisant la mélasse comme substrat a été fortement affecteé par la présence de fer à une concentration aussi faible que 0,2 ppm, mais l'ajout de cuivre à 0,1 à 500 ppm au moment de l'inoculation ou au cours des premières 50 h de fermentation a permis d'atténuer l'effet nocif de fer. D'autres chercheurs ont également démontré l'effet bénéfique du Cu^{2+} sur l'atténuation de l'effet négatif de Fe^{2+} (Haq et al., 2002). Il a été montré que l'ajout de Mn^{2+} à des concentrations aussi faibles que 3 µg/l réduit considérablement le rendement d'AC même dans des conditions optimales de production (Rohr et al., 1996). L'anabolisme cellulaire d'*A. niger* est altéré en absence de manganèse total et/ou par la limitation en azote et en phosphate. L'importance du manganèse a également été démontrée dans de nombreuses autres fonctions cellulaires, notamment dans la synthèse de la paroi cellulaire, la sporulation et la production de métabolites secondaires. Les concentrations des éléments traces métalliques dans les milieux de culture peuvent être contrôlé de deux façons: [1] par la purification du support afin d'éliminer certains ions métalliques, puis par l'ajout de quantités connues d'ions métalliques nécessaires et, [2] par l'ajout d'agents chélateurs aux

métaux afin de diminuer la concentration des ions métalliques libres. Dans cette méthode, le complexe métallique agit comme un tampon métallique dont la dissociation est réversible ce qui permet la libération des ions métalliques nécessaires à la croissance des microorganismes. La présence d'éléments traces métalliques à des concentrations toxiques peut causer un énorme problème au cours de la SmF des substrats bruts qui servent à la production de produits à valeur ajoutée. Cependant, la SSF permet l'obtention d'un rendement élevé d'AC sans aucune inhibition liée à la présence de métaux à forte concentration. Les sels de fer sont essentiels pour la production d'AC. En effet, ils activent la production de l'acétyle coenzyme A qui est importante pour la production de l'AC (Milsom et al., 1985). Pendant ce temps, l'excès de fer va activer la production d'aconitate hydratase (aconitase), l'enzyme qui est responsable de la production de l'isomère d'acide citrique (l'IAC). L'IAC est un sous-produit de la fermentation qui n'est pas souhaité car il réduit le rendement potentiel d'AC. Le type et la concentration d'hydrate de carbone sont également des facteurs importants qui influent sur la concentration finale du produit désiré. Contrairement à l'effet d'autres facteurs, un nombre relativement faibles d'études ont été publiées sur l'effet de la concentration en sucre sur la fermentation par des champignons filamenteux tels qu'*A. niger*. Selon Anwar et al. (2009), la teneur élevée en sucre dans le substrat est considérée comme étant favorable à une production accrue d'AC. Les constituants du milieu jouent un rôle essentiel dans la production globale d'AC. Il est très important de prendre en considération la concentration des différents métaux et des autres constituants présents dans la biomasse selon le type de fermentation et selon les exigences physiologiques du microorganisme, afin d'obtenir des rendements élevés d'AC. Par exemple, les concentrations en métaux lourds toxiques en trace doivent être soigneusement optimisées pour assurer une croissance maximale du microorganisme. La morphologie du champignon est un paramètre important qui influence le rendement et la production globale d'AC et dépend en grande partie des constituants de la culture.

1.7.2. Conditions environnementales

Un certain nombre de paramètres ont été considérés au niveau des processus biotechnologiques (température, humidité, pH, minéraux, type d'inoculum et ajout d'éléments nutritifs, etc.). En outre, divers autres paramètres ont également été jugés critiques au cours de la SSF, tels que la vitesse d'agitation, la taille des particules du substrat et la charge de lit (Shojaosadati et Babaeipour, 2002; Kumar et al., 2003). Les champignons préfèrent un milieu humide pendant leur croissance. Pour la production de la biomasse, un niveau d'humidité optimal doit être maintenu puisque une humidité inférieure a tendance à réduire la diffusion des

éléments nutritifs, la croissance microbienne, la stabilité de l'enzyme et le gonflement du substrat (Chundakkadu, 2005).

Les niveaux d'humidité les plus élevés conduisent à l'agglomération des particules, la limitation du transfert de gaz et la concurrence des bactéries (Gowthaman et al., 2001). En général, les niveaux d'humidité dans les processus SSF varient entre 30 et 85%. Le niveau d'humidité doit être soigneusement optimisé en fonction de la nature du substrat utilisé pour un meilleur rendement d'AC par *A. niger*. La température est notamment la variable la plus importante de toutes les variables physiques pouvant affecter la performance de SSF, car elle affecte la croissance des microorganismes et la production d'enzymes ou de métabolites. L'importance de la température dans le développement d'un processus biologique réside dans le fait qu'elle peut engendrer des effets importants, tels que la dénaturation des protéines, l'inhibition de l'enzyme, l'accélération ou la suppression de la production d'un métabolite en particulier, et surtout la mort des cellules (Pandey et al., 2001). Les champignons peuvent se développer sur une large gamme de températures de 20 à 55°C, et la température optimale pour la croissance pourrait être différente de celle de la formation du produit. Une limitation de la SSF réside dans son incapacité à évacuer la chaleur excédentaire produite par le métabolisme des microorganismes en raison de la faible conductivité thermique du milieu solide. Dans la pratique, la SSF exige une bonne aération qui lui sert comme source d'oxygène, mais qui lui sert surtout pour assurer la dissipation thermique. Afin de réaliser une production optimale d'AC, des conditions environnementales appropriées devraient être fournies pour la croissance efficace des microorganismes. Il devrait y avoir un besoin urgent du développement de la technologie pour le contrôle adéquat des différents paramètres de la SSF pour une production élevée d'AC. Le soucis principal de l'aération est de fournir une quantité appropriée d'O_2 pour la croissance microbienne et pour éliminer le CO_2. L'aération assure également une fonction critique dans la dissipation de la chaleur et de l'humidité (Pandey et al., 2000; Shojaosadati et Babaeipour 2002), régulant ainsi la température du milieu de fermentation, la distribution de la vapeur d'eau (régulation de l'humidité), et les composés volatils produits au cours du métabolisme. Le débit d'aération est donc déterminé par plusieurs facteurs, tels que les besoins de croissance des microorganismes, la production de métabolites gazeux et volatils, l'évolution de la chaleur et il dépend de la porosité du support; les pO_2 et pCO_2 doivent être optimisés pour chaque type de milieu, de microorganismes et de processus (Chundakkadu, 2005). Les équations suivantes décrivent la stœchiométrie pour la production microbienne d'AC.

Table 1. 4 Macro- et micro-nutriments nécessaires à la production d'acide citrique

Principaux nutriments	Type de nutriments	Concentration requise	Effet sur la production d'AC	Références
Macronutriments				
Sucres	Saccharose (préféré), le glucose, le lactose, le maltose, le mannose, le galactose	Concentration initiale: 14-22%	Positif	Hossain et al., 1984; Xu et al., 1989
Azote	Urée, chlorure d'ammonium / sulfate / tartrate, oxalate, sulfate, nitrate, chlorure, nitrate de sodium, les peptones, la levure / extrait de malt, les acides aminés	0.1 à 0.4 g/l	Rendement en AC directement influencé par la source d'azote, des niveaux élevés conduisent à la production de biomasse et la diminution du pH	Xu et al., 1989; Rohr et al., 1996; Chundakkadu, 2005, Soccol et al., 2006
Micronutriments				
Éléments traces	Zn^{2+}	0.3 ppm (faibles teneurs)	Amélioration du rendement d'AC	Pandey et al., 2000
	Mn^{2+}	Faibles teneurs	Réduit la production d'AC dans des conditions autrement optimisés	Rohr et al., 1996
	Fe^{2+}	1.3 ppm	Concentration optimale	Haq et al., 2002
	Cu^{2+}	0.1–500 ppm	Contre-agit sur l'effet délétère du fer dans la fermentation de la mélasse par SMF et l'amélioration du rendement d'AC	Pandey et al., 2000
	Mg^{2+}	0.02- 0.025%	Essentielle à la croissance ainsi qu'à la production d'AC	Soccol et al., 2006
	Phosphore (source préférée et dihydrogénophosphate de potassium)	0.5-5.0 g/l	Les faibles niveaux favorisent la production d'AC	Soccol et al., 2006
Baisse	Méthanol, éthanol, iso-propanol, méthyl acetate, *n*-propanol	1-4% (v/p)	Améliorer le rendement d'AC	Sikander et al., 2005; Nadeem et al., 2010
Alcools				
Autres composes	Na^{+}, Ca^{++}, Ni^{+}, K^{+}, Mo^{++}, B, composés organiques tels que thiamine, biotine, acide folique et stéroïdes	De faibles concentrations	Amélioration de la sporulation	Pandey et al., 2000

Équation 0.1 $C_6H_{12}O_6 + 1.5O_2 \rightarrow C_6H_8O_7 + 2H_2O$

Équation 0.2 $C_6H_{12}O_6 + 6O_2 \rightarrow 6CO_2 + 6H_2O$

Les équations ci-dessus décrivent le rôle important de l'oxygène dans la production de l'AC. Il a été trouvé que l'augmentation de la concentration en oxygène dissous est accompagnée par une augmentation significative de l'accumulation d'AC. La concentration d'AC a été également augmentée par un facteur de 1,4 fois en ajoutant du n-dodécane (5% v/v) en tant que vecteur de l'oxygène dans le milieu de fermentation d'*A. niger* (Jianlong, 2000). Cependant, il est intéressant de noter que l'aération excessive peut produire une contrainte de cisaillement qui a un effet nocif sur la morphologie du champignon filamenteux et peut canaliser le lit garni (Shojaosadati et Babaeipour, 2002).

L'agitation est un autre paramètre important dans la fermentation aérobie car elle assure l'homogénéité de la température et de l'environnement gazeux et fournit une zone interfaciale pour le transfert du gaz au liquide et du liquide au gaz (Trilli et al., 1986). Il est évident d'après les rapports précédents que l'agitation facilite l'élimination des produits métaboliques volatils, empêche la formation d'agglomérats, améliore le transfert de chaleur, protège le support contre la dessiccation locale ou l'humidification excessive, et améliore les conditions de la croissance microbienne dans tout le volume du lit (Mitchell et al., 1998). L'agitation permet une distribution uniforme et plus efficace de la suspension de spores, de l'eau nécessaire pour le contrôle d'humidité *et/ou de toutes* autres solutions nutritives, le cas échéant (Suryanarayan, 2003). Cependant, il faut souligner que l'agitation n'est pas utilisée dans de nombreux procédés de SSF aérobies effectués dans des réacteurs statiques, comme les fermenteurs à plateaux. En revanche, l'agitation est souvent une part essentielle dans les bioréacteurs de SSF périodiquement ou continuellement agités. L'agitation est également connu pour avoir des effets néfastes sur la porosité du substrat en raison de la compression des particules de substrat, la perturbation de l'attachement des champignons sur les solides et l'altération des mycéliums fongiques qui est due à des forces de cisaillement dans les systèmes de la SSF (Lonsane et al., 1992). L'agitation discontinue a été jugée plus appropriée que l'agitation continue car elle permet d'éviter la destruction des mycéliums

et les perturbations de l'attachement des microorganismes sur les particules solides dans certains cas.

Le pH est un autre paramètre important dans le processus de fermentation, et peut varier en réponse aux activités métaboliques. La raison la plus évidente est la sécrétion d'acides organiques qui causent la baisse du pH. D'autre part, l'assimilation des acides organiques, qui peuvent être présents dans certains médias, conduira à une augmentation du pH, et l'hydrolyse de l'urée se traduira par une alcalinisation (Raimbault, 1998). Chaque microorganisme possède une gamme de pH optimale pour sa croissance et pour son activité métabolique. Les champignons filamenteux peuvent croître sur une large gamme de pH allant de 2 à 9, avec une gamme optimale de 3,8 à 6,0. Cette flexibilité typique aux pH des champignons peut être avantageusement exploitée pour prévenir ou minimiser la contamination bactérienne, en particulier en choisissant un pH plus bas. Le pH du milieu de fermentation est important au cours de la production d'AC. Tout d'abord, les spores ont besoin d'un pH > 5 pour germer. Deuxièmement, le pH de la production d'AC doit être faible (pH ≤ 2). Un pH faible réduit le risque de contamination de la fermentation avec d'autres microorganismes et inhibe également la production d'acides organiques indésirables (acide gluconique, acide oxalique), ce qui simplifie la récupération d'AC à partir du bouillon de fermentation (Levente et al., 2003). Le changement du pH peut également se produire en fonction de la source d'azote choisie, ainsi qu'en fonction des caractéristiques de croissance (Gowthaman et al., 2001). L'utilisation de l'urée comme source d'azote plutôt que les sels d'ammonium est un moyen de contrôler le pH. Une tentative a été suggérée pour surmonter le problème de la variabilité du pH au cours du processus SSF. Cependant, cette tentative s'est basée sur la formulation du substrat en utilisant différents composants ayant un pouvoir tampon ou sur l'utilisation d'une formulation de tampon avec des composants qui n'ont aucun effet négatif sur l'activité biologique. Afin d'hyper-produire le produit souhaité, les microorganismes doivent être cultivés dans des conditions sous-optimales pour la formation de la biomasse. La taille et la forme de boulettes de mycéliums jouent un rôle direct dans la biosynthèse d'AC au cours de la SmF. La croissance sous forme de boulettes de diamètre ≤ 1 mm a été associée à des taux de production et des rendements élevés (Magnuson et al., 2004).

1.7.3. Taille des particules

La taille des particules du substrat est un facteur critique car elle est liée à la rhéologie du bouillon de fermentation, qui détermine la capacité du système pour interagir avec la croissance microbienne et le transfert de chaleur et de matière au cours du processus de SSF. En outre, il affecte le rapport surface/volume des particules, ce qui détermine la fraction du substrat, qui est initialement à la disposition des microorganismes et la densité de tassement à l'intérieur de la masse surfacique (Krishna, 1999). La taille du substrat détermine l'espace vide, qui est occupé par de l'air. Puisque le taux de transfert d'oxygène dans l'espace vide affecte la croissance des microorganismes, le substrat doit contenir des particules d'une taille appropriée pour augmenter le transfert de masse (Krishna, 1999). En règle générale, les petites particules de substrat fourniraient une plus grande surface pour la croissance microbienne mais des particules trop petites peuvent entraîner l'agglomération du substrat, ce qui peut interférer avec la respiration/aération microbienne et entraîner une faible croissance. Les particules de petite taille sont également favorables pour le transfert de chaleur et l'échange d'oxygène et de dioxyde de carbone entre l'air et la surface solide. Au même temps, les particules les plus grosses également fournissent une meilleure efficacité de respiration/aération, mais offrent une surface limitée de l'action microbienne (Pandey et al., 2000). Ainsi, une taille moyenne optimale de particules de 0,6 à 2,0 mm est souhaitée pour une production élevée d'AC.

1.8. Effet des stimulateurs

De nombreux chercheurs ont étudié le rôle stimulant du méthanol ou de l'éthanol (EtOH) sur le rendement d'AC (Sikander et Haq, 2005; Rivas et al., 2008; Suzelle et al., 2009; Nadeem et al., 2010). L'augmentation de la production d'AC avec l'ajout d'EtOH peut être attribuée à la lente dégradation d'AC en raison de la réduction de l'activité d'aconitase. L'ajout d'EtOH a également entraîné une légère augmentation de l'activité des autres enzymes du cycle de TCA. Il y a aussi une possibilité que EtOH peut être converti en acétyl-CoA, un substrat métabolique nécessaire à la formation d'AC. Le MeOH n'est pas métabolisé/assimilé par *A. niger*, son rôle exact dans l'amélioration de la production d'AC n'est pas clair. L'ajout de MeOH pourrait augmenter la perméabilité de la cellule au citrate. En outre, l'ajout de MeOH dans le milieu de fermentation a baissé remarquablement la synthèse protéique cellulaire sans affecter l'absorption

d'azote, ce qui provoque une augmentation des acides aminés, des peptides et des protéines de faibles poids moléculaires mis en commun dans le mycélium pendant la phase précoce de la culture (Usami et al., 1961). Par ailleurs, le MeOH a légèrement modifié l'activité de certaines enzymes du cycle TCA favorisant l'accumulation d'AC. L'effet stimulant du méthanol sur le rendement d'AC peut être expliqué en termes de morphologie mycéliene ainsi que la forme et la taille des granules. Le MeOH a un effet direct sur la morphologie mycélienne et elle favorise la formation de granules. Il augmente également la perméabilité de la membrane cellulaire pour provoquer une plus grande excrétion d'AC à partir des cellules de mycélium. Il a été proposé que la morphologie mycélienne des champignons filamenteux, sous la forme de petits granulés ronds (diamètre < 3 mm) pourrait être utilisée pour la production maximale d'AC. Il a été généralement constaté que l'ajout de MeOH, EtOH, d'iso-propanol et d'acétate de méthyle améliore la production d'AC (Pandey et al., 2000).

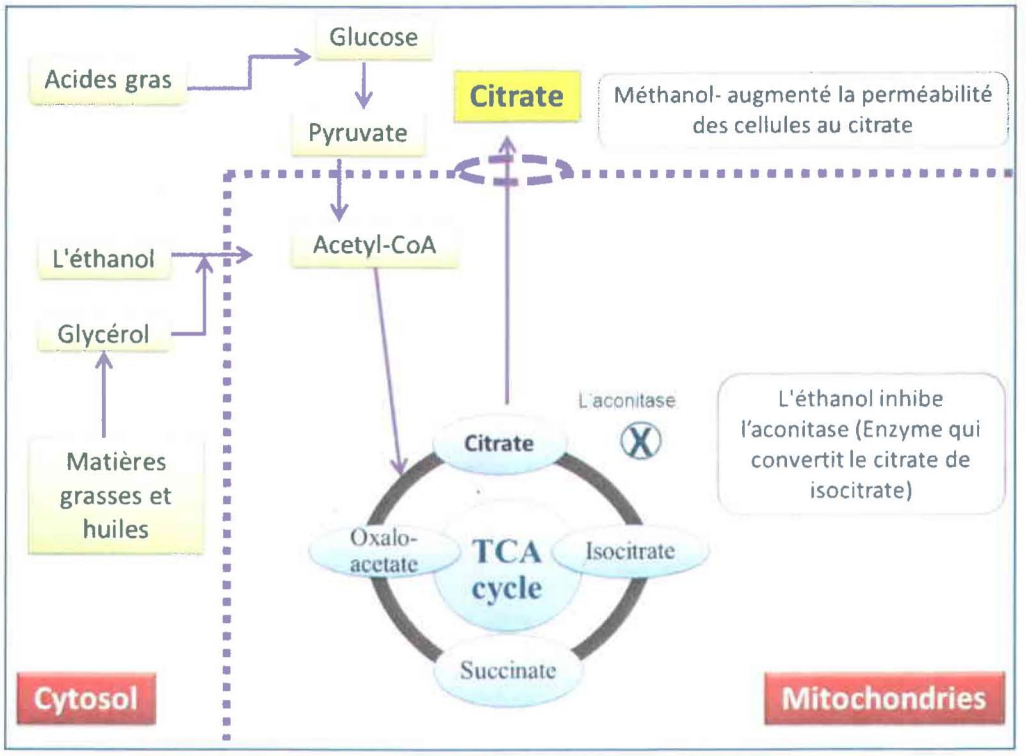


Figure 1. 3 Effet de différents stimulateurs sur la production d'acide citrique

L'ajout d'huile végétale dans le milieu de culture augmente également la formation d'AC (Nehad et al., 2002; Sikander et Haq, 2005). Les graisses et les huiles végétales agissent en tant que sources de carbone et sont dégradées en glycérol et acides gras. Le glycérol pénètre directement dans le cycle de Krebs par la formation d'acétyl-CoA et

les acides gras entrant par la glycolyse (Figure 1.3). Il a été montré qu'il est préférable d'utiliser le glycérol directement au lieu des acides gras afin d'améliorer le rendement d'AC (Papanikolaou et al., 2002, 2003; Finogenova et al., 2005; Papanikolaou et Aggelis, 2009). Divers chercheurs ont démontré que certains additifs, tels que l'alcool polyvinylique, le polyéthylène glycol, la CMCase, jouent le rôle de protecteurs et de stimulateurs (Michaels et al., 1990, 1991, Rugsaseel et al., 1995). Le rôle stimulateur des alcools et des autres inducteurs sur la production d'AC peut être expliqué en termes de morphologie du mycélium de champignon, ainsi que la forme et la taille de granules, ce qui est une condition indispensable pour obtenir une production élevée d'AC. Des rendements élevés d'AC pourraient être obtenus en exploitant les ressources disponibles et en ajoutant les agents stimulateurs au milieu de fermentation. Le mode d'action de certains inducteurs pour améliorer la production d'AC est expliqué en détail à la Figure 1.3.

1.9. Applications de l'acide citrique

La demande d'AC est en hausse, en raison de son caractère *GRAS*. La taille du marché d'AC est en expansion continue en raison de son utilisation très répandue dans les aliments, les produits pharmaceutiques, les produits de consommation liés à la santé et la biomédecine (Figure 1.4 et Tableau 1.5). L'AC présente des effets bactéricides et bactériostatique contre *Listeria monocytogenes*, un pathogène d'origine alimentaire capable de croître à des températures basses (régrégéteurs) et en présence de concentrations salines élevées (Bal `A et al., 1998). *L. monocytogenes* contamine fréquemment les produits de charcuterie, y compris les saucisses de Francfort (Nickelson et al., 1999). Ces dernières années, un intérêt mondial très considérable est porté à l'AC, en raison de sa large gamme de propriétés, telles que la biodégradabilité, la biocompatibilité, la non toxicité et ses propriétés antibactériennes. Il existe des rapports sur l'utilisation prometteuse d'AC comme copolymère dans les nanomatériaux pour être utilisé dans la nano-médecine. Une étude réalisée par Ashkan et al. (2010) a démontré l'utilisation potentielle d'AC dans la synthèse des macromolécules dendritiques linéaires de poly (AC)-bloc-poly (éthylène glycol). Ces copolymères sont utilisés dans les médicaments car ils se dégradent de nouveau en molécules individuelles qui peuvent être métabolisées par le corps. Une autre étude scientifique réalisée par Guillermo et al. (2010) a mis au point un nano-polymère à base d'AC

destiné à être utilisé comme échafaudage biocompatible, qui peut être utilisé pour la culture d'une variété de cellules, comme les cellules endothéliales, les tissus ligamentaires, les cellules musculaires, les cellules osseuses, les cellules cartilagineuses et l'administration de médicaments. De nombreux chercheurs ont étudié des échafaudages fabriqués à partir de polyesters élastiques, en particulier à partir du poly(octanediol citrate) (CEP) ou de poly(glycérol sebacate) (PGS), qui sont jugés compatibles pour la régénération de différents tissus, tels que les vaisseaux sanguins (Yang et al., 2005;. Gao et al., 2006), le cartilage (Kang et al., 2006), le tissu osseux (Qiu et al., 2006) et nerveux (Sundback et al., 2005). Actuellement, il existe de nouvelles applications d'AC qui ont été découvertes. Williams et al. (2010) ont démontré la possibilité d'utiliser l'AC pour l'élimination du potassium du concentré de minerai de fer de qualité inférieure. Le traitement du minerai de fer de qualité inférieure contribue à résoudre le problème de la rareté de cette précieuse ressource non-renouvelable et contribue à atténuer le problème de l'épuisement continu des dépôts de minerais de fer à travers le monde. À l'avenir, l'AC pourrait être utilisé pour la lixiviation chimique des impuretés des minerais naturels pour extraire les métaux d'intérêt. Ainsi, l'AC a un rôle potentiellement très important à jouer dans l'hydrométallurgie qui est de plus en plus axée sur des techniques biologiques écologiques.

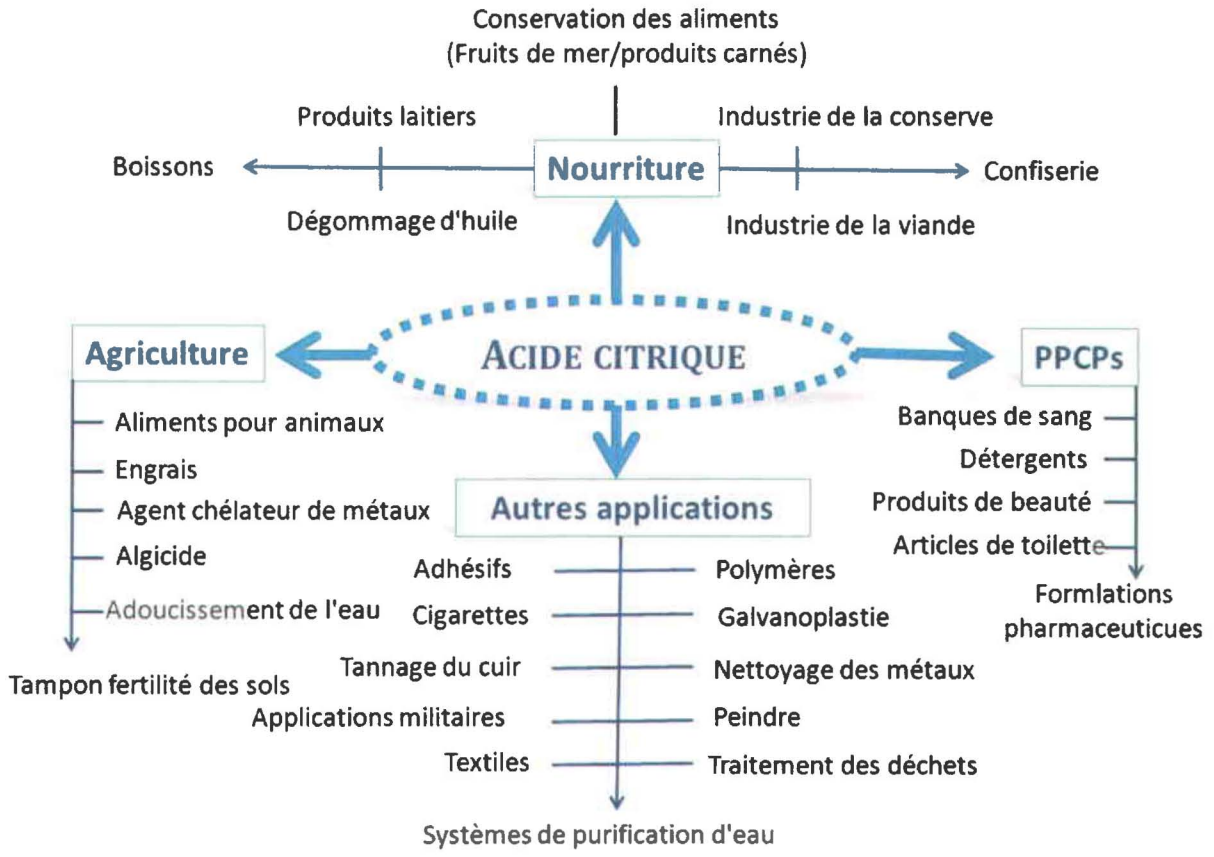


Figure 1. 4 Différentes applications conventionnelles d'AC (PPCPs- Produits pharmaceutiques et de soins personnels)

1.10. Perspectives et défis futurs

La demande croissante d'AC a permis une meilleure exploration des applications spécialisées de celui-ci et donc une production plus efficace d'AC. La fermentation à l'état solide est un moyen d'augmenter la productivité d'AC. En effet, de telles stratégies novatrices pourront réduire le coût de production d'AC. L'utilisation de déchets agro-industriels comme substrat pour la production d'AC contribuera certainement à la réduction des coûts de production associés à des substrats synthétiques coûteux.

L'augmentation de la production d'AC est principalement attribuable à la vaste gamme d'applications de celui-ci dans différents secteurs industriels, tels que les produits chimiques, les produits pharmaceutiques, cosmétiques et alimentaires comme additif alimentaire polyvalent et sûr. Afin de répondre à la demande croissante d'AC, il existe un besoin urgent de développer une technologie de production rentable et

écologiquement durable. La meilleure façon pour atteindre cet objectif est la modification ou le remplacement de la procédure mise en place. L'utilisation d'un procédé de fermentation pour la production d'AC à partir d'*A. niger* a un impact moindre sur l'environnement. Dans ce contexte, la bio-utilisation de déchets agro-industriels et leurs sous-produits par des processus existants, SSF avec des souches génétiquement modifiées ou nouvelles et le raffinement des procédés de récupération actuels d'AC pourrait être une voie prometteuse pour atteindre les plus forts taux de production d'AC.

Table 1. 5 Différentes applications avancées de l'acide citrique

Domaine d'application	Industrie	Utilités	Pureté requise	Références
Biomédicaments	Nano-médicaments	Utilisés comme copolymères dans les nanomatériaux qui peuvent encapsulés des petites molécules biologiquement actives, peuvent être utilisés pour l'auto-guérison auto-polymères, produits de soins, comme des échafaudages et biocompatibles, utilisés pour une variété de culture de cellules, administration de médicaments	Haute	Ashkan et al., 2010;Gillermo et al., 2010
	Ingénierie tissulaire	Utilisé pour fabriquer des élastomères de polyester ayant une utilisation potentielle dans l'ingénierie tissulaire	Haute	Ivan et al., 2009; Yang et al., 2004
Préservation du bois	Matériaux de construction	Protège le bois contre les champignons et les insectes	Modérée	Forest products lab. US Dept. of agriculture.
Traitement du minerai de fer de qualité inférieure	Mines de minerai de fer	Utilisé pour la lixiviation de potassium à partir du concentré de minerai de fer	Modérée	Williams et al., 2010

1.11. Extraction de coproduits, le chitosane

Le chitosane (CTS) est un biopolymère important qui a de nombreuses applications. Le CTS est un biopolymère polysaccharide polycationique, chélatant et a des propriétés filmogènes en raison de la présence de groupes fonctionnels amines et hydroxyles. Le CTS présente également un ensemble de propriétés biologiques, telles que l'activité antimicrobienne, la résistance aux maladies induites chez les plantes et diverses propriétés stimulantes ou inhibitrices à l'égard d'un certain nombre de lignées cellulaires humaines. Grâce à ces propriétés, le CTS est largement utilisé dans divers domaines allant de la médecine, les nano-sciences, le génie chimique, la pharmaceutique, la nutrition et l'agriculture, comme décrit au Tableau 1.6. Le CTS est un ingrédient actif utilisé pour la formation de nanoparticules (NPs) différentes qui trouvent des applications potentielles dans de nombreux domaines, tels que les NPs d'argent ayant des applications émergentes contre la résistance aux antibiotiques. L'augmentation de la demande élevée de CTS est principalement due à des attributs souhaités, tels que leur nature non-toxique, biodégradable, biocompatible, l'absorption des lipides et la nature respectueuse de l'environnement.

Table 1. 6. Différentes applications du chitosane

Champ	Applications
Biomédecine et produits pharmaceutiques	Agent antimicrobien dans les pansements, sutures chirurgicales, formulations de pommades pour les plaies, implants dentaires, de la peau artificielle, ophtalmologie, orthopédie et sport; agent de l'administration médicaments à libération lente, système de transfert de gènes, nanomatériaux, ingénierie tissulaire, vaccination, tissu de régénération de la peau et cicatrisation des plaies, abaisse la pression artérielle, activité antioxydante; abaissement du cholestérol, traitement des maladies parodontales, inhibe la croissance des cellules tumorales, anticoagulant
Nourriture	Immobilisation d'enzymes, conservation des aliments, émulsions alimentaires, films et revêtements comestibles
Agriculture	Agent de lutte biologique, réponses active de défense des plantes, revêtements de protection de semences, feuilles, fruits et légumes, libération contrôlée des produits agrochimiques, engrais, amélioration de la production agricole, activateur de croissance végétale
Environnement	Floculation, adsorption de métaux, des colorants et des composés organiques

1.11.1. Sources traditionnelles de CTS

Traditionnellement, le CTS est dérivé de la chitine naturelle, provenant généralement des carapaces de crustacés, tel que le crabe, et à partir des carapaces de crevettes par *N*-désacétylation ou par l'utilisation de traitements chimiques agressifs. Toutefois, les CTS obtenus par ces traitements souffrent de certaines inconvénients, comme la contamination des protéines, des niveaux incompatibles de dé-acétylation et poids moléculaires qui ont abouti à des variations des caractéristiques physico-chimiques du CTS. Il existe quelques problèmes supplémentaires, tels que les questions d'environnement liés à l'utilisation de produits chimiques toxiques, la limitation en approvisionnement des coquilles de fruits de mer, la limitation géographique, les variations saisonnières et les coûts très élevés. Par conséquent, les approches biologiques pour la synthèse de CTS doivent être explorées.

1.11.2. Sources fongiques de chitosane

Les parois cellulaires des champignons contiennent jusqu'à 50% de chitine par rapport aux carapaces de crustacés qui comprennent entre 14 et 27% sur une base de biomasse sèche. Dans cette perspective, la production de la CTS de parois cellulaires des champignons cultivés sous des conditions contrôlées offre un grand potentiel d'obtention d'un produit très homogène. La présence naturelle de chitine dans de nombreuses souches de champignons pourrait être considérée comme une bonne source de CTS. De plus, ces souches fongiques sont largement utilisées pour la variété de procédés biotechnologiques à l'échelle industrielle. Les déchets de mycélium de différentes souches de champignons issus de différents procédés de fermentation peuvent être utilisés pour la production réalisable de CTS.

Les souches *d'Aspergillus niger* sont largement utilisées pour la production d'AC et d'autres produits pharmaceutiques et biotechnologiques à l'échelle industrielle. Les déchets fongiques mycéliens de l'AC ou de l'industrie antibiotique peuvent être une source peu coûteuse et une alternative de CTS, en plus de la source industrielle traditionnelle des déchets de crustacés. En outre, les CTS fongiques peuvent avoir des propriétés uniques par rapport à ceux issus de crustacés. La fraction insoluble en milieu alcalin de la paroi cellulaire des résidus de la biomasse d'*A. niger* est principalement constituée de chitine, CTS et β-glucanes, avec une fréquence importante de (1, 3)-β-D-glucane. La production mondiale annuelle d'AC est estimée à 1,7 Mt qui se traduiront

par 0,34 Mt de déchets de mycélium d'*A. niger* par an et, par ailleurs, l'industrie continue à augmenter avec un taux de croissance annuel de 5% (Wu et al., 2005). *A. niger* contient environ 15% de chitine (poids à sec), qui peut être séparé et transformé en CTS (Kucera, 2004). Cependant, au cours des dernières années, le marché d'AC a été sous une pression énorme et continue de se balancer vers une réduction des prix. Les coûts élevés d'énergie et de substrats rendent le marché de production d'AC non-rentable. Il y a un besoin évident de développer une technologie intégrée pour utiliser le mycélium gaspillé et illimité résultant de l'industrie de production d'AC qui contribuera également à la stabilisation des prix de l'AC. La production par fermentation de CTS par des champignons de culture sur les déchets a un bon marché et il est une source infinie et économique. Compte tenu des quantités importantes de déchets fongiques accumulés par les industries biotechnologiques et pharmaceutiques, ainsi que le coût impliqué dans la gestion des déchets, l'extraction de produits à valeur ajoutée, tel que le CTS peut fournir une solution rentable pour ces industries.

Généralement, toutes les études ont été concentrées sur la production d'AC à partir des déchets agro-industriels. Toutefois, le mycélium fongique des déchets résultant des industries d'AC est une riche source de CTS qui peut être extrait à partir du mycélium ce qui contribue encore à la stabilisation des marchés d'AC et l'obtention des revenus supplémentaires pour les industries de transformation de fruits. Le développement des procédés de valorisation des déchets résultant de mycélium des industries biotechnologiques peuvent aussi accroître la rentabilité des prochaines industries biotechnologiques en raison de l'utilisation de la biomasse des déchets pour la production de produits à valeur ajoutée. L'approche de la valeur ajoutée génère des revenus supplémentaires pour les industries et aussi réduire le coût du produit biotechnologique primaire. En outre, il permettra de renforcer l'économie et il aidera également à réduire la pollution de l'environnement.

Ainsi, le but du présent projet de recherche est d'explorer une approche pour l'utilisation des ressources avec un minimum de production de déchets. La production d'AC a été réalisée à l'aide de déchets agro-industriels par *A. niger,* suivie d'une extraction de CTS à partir du mycélium fongique des déchets. Divers déchets solides et liquides agro-industriels ont été sélectionnés pour leur potentiel de production supérieur d'AC par SSF et SmF en utilisant *A. niger* NRRL 567 et 2001. D'autres études d'optimisation par la méthodologie de surface de réponse (RSM) ont été réalisées avec les substrats et la

souche d'*Aspergillus*. De façon précise, l'extraction de CTS a été réalisée à l'aide du mycélium fongique des déchets résultant de la bio-production d'AC. Les déchets de mycélium fongique, la biomasse fermentée (contenant d'AC et mycélium) et la biomasse résiduelle activée (après l'extraction de CTS), ont été utilisés pour la bioremédiation des métaux lourds toxiques dans les effluents de décontamination des déchets de bois traités à l'ACC (arséniate de cuivre chromaté).

HYPOTHÈSES, OBJECTIFS ET ORIGINALITÉ

2. HYPOTHÈSES

Afin d'évaluer le potentiel des déchets agro-industriels pour la bioproduction fongique d'AC et pour l'extraction du CTS à partir des déchets grâce aux mycéliums, les hypothèses suivantes ont été formulées.

2.1. Hypothèse 1

Il a été rapporté que les champignons sont capables de croître sur divers déchets agro-industriels en fonction de la complexité des substrats. La plupart du temps, les déchets agro-industriels sont très riches en glucides et en autres nutriments essentiels qui sont présents en quantité suffisante. Cela permettrait l'utilisation de ces substrats bruts sans aucune supplémentation en nutriments pour les champignons. Ceci serait applicable pour la production des composés à valeur ajoutée, tels l'AC.

2.2. Hypothèse 2

Divers facteurs physico-chimiques, tels le niveau initial d'humidité, les matières en suspension, la taille des particules et la concentration des micronutriments, affectent le rendement du produit désiré pendant la SSF et la SmF. De nombreux composés agissent comme des inducteurs favorisant la formation des produits désirés. En outre, pendant la fermentation, les différentes variables présentent des effets interactifs qui ont un impact sur la formation du produit. Des techniques comme la méthodologie de surface de réponse (RSM) pourraient être utilisées pour étudier les effets interactifs entre les variables.

2.3. Hypothèse 3

Les procédés biotechnologiques effectués au niveau d'un flacon à l'échelle laboratoire et d'un fermenteur diffèrent au niveau de la formation du produit en raison des conditions de fonctionnement qui sont différentes. Les processus à grande échelle permetraient d'évaluer l'efficacité du microorganisme.

2.4. Hypothèse 4

Le développement morphologique du champignon au cours de la SmF a un impact important sur la formation du produit final et sur le traitement qui permet d'obtenir la récupération élevée du produit. Les études rhéologiques de fermentation fongique pourraient aider à optimiser les conditions physico-chimiques permettant d'atteindre des taux plus élevés au niveau de la formation du produit avec une grande facilité au niveau du traitement.

2.5. Hypothèse 5

De nombreux champignons contiennent de la chitine dans leurs parois cellulaires. La production d'AC fongique résulte en grandes quantités des déchets des mycéliums représentant une source de CTS riche, abondante et peu coûteuse. La production et l'extraction du CTS à partir de la paroi cellulaire des champignons pourraient être réalisées sous des conditions environnementales ambiantes, par des légers traitements alcalins et acides, à des températures plus basses que celles des sources traditionnelles d'extraction du CTS qui se font par des moyens chimiques à partir des carapaces de crustacés.

2.6. Hypothèse 6

Les nanomatériaux (NMs) gagnent un grand intérêt en raison de leurs fonctionnalités améliorées et de leurs bioactivités. Ils sont issus d'un processus de synthèse en respect avec l'environnement et sont fabriqués à partir des biomatériaux renouvelables.

2.7. Hypothèse 7

La biomasse fongique résultante de l'extraction du CTS est un type de biomasse activée. L'activation de n'importe quel matériau améliore ses propriétés adsorbantes. *La biomasse activée sera très poreuse et aura donc une grande surface disponible pour les réactions d'adsorption ou pour les réactions chimiques.* La biomasse activée fongique peut être utilisée pour la bioremédiation des métaux lourds provenant des lixiviats de déchets de bois contaminés en ACC, des sols et des eaux usées. Les champignons sont activement impliqués dans la bioremédiation des métaux toxiques. La biomasse fongique séchée, la biomasse fermentée (contenant d'AC et des mycéliums), l'AC et le

CTS pourraient être également utilisés pour la bioremédiation des métaux lourds et d'autres composés toxiques provenant des environnements pollués.

3. OBJECTIFS DE RECHERCHE

L'objectif global de cette étude est d'identifier les conditions optimales de production d'AC (le type de substrat, la souche, les paramètres de fermentation et les stimulateurs) pour les souches fongiques *A. niger* NRRL 567 et NRRL 2001 en utilisant deux méthodes de fermentation: (1) la fermentation à l'état solide et; (2) la fermentation submergée.

Les objectifs specifiques mettent l'accent sur l'optimisation de la production d'AC par *A. niger* en utilisant un déchet agro-industriel à faible coût.

3.1. Objectif 1

Etudier l'effet du substrat solide et liquide provenant de déchets agroindustriels sur la bio-production d'AC par *A. niger* NRRL 567 et NRRL 2001.

3.2. Objectif 2

Optimiser des paramètres d'opération du procédé par la méthodologie de surface de réponse:

3.2.1. Le niveau d'humidité initiale et la concentration de l'inducteur servant à la bio-production d'AC en utilisant le substrat à l'état solide pré-sélectionné et la souche d'*Aspergillus* choisie lors des expériences.

3.2.2. Les matières en suspension et la concentration de l'inducteur pour la fermentation submergée d'AC en utilisant le substrat liquide sélectionné et la souche d'*Aspergillus* choisie lors des expériences.

3.3. Objectif 3

Mise à l'échelle d'un procédé de fermentation en substrat solide (AP) utilisant *A. niger* 567 NRRL basée sur la méthodologie de surface de réponse.

3.3.1. Mise à l'échelle d'un procédé de fermentation du substrat à l'état solide (AP) par *A. niger* 567 NRRL pour la production d'AC basée sur la RSM et qui a été réalisé dans un fermenteur à tambour rotatif de 12 L.

3.3.2. Mise à l'échelle d'un procédé de fermentation submergée pour le substrat liquide (APS) basé sur la RSM et produit par *A. niger* 567 NRRL dans un volume de 4,5 L placé dans un fermenteur ayant une capacité de 7,5 L.

3.4. Objectif 4

Études de la rhéologie de l'APS pendant la fermentation submergée qui conduit à la production d'AC au niveau du fermenteur.

3.5. Objectif 5

Développer et valider une méthode d'extraction du CTS à partir des déchets de mycélium fongique issus de la fermentation à l'état solide et submergé pour produire de l'AC basée sur divers principes physico-chimiques.

3.6. Objectif 6

Produire des nanoparticules de CTS à base d'oxydes métalliques par des procédés de nano-pulvérisation et la méthode de précipitation. L'action antimicrobienne et antibiofilm contre les bactéries pathogènes par des NPs d'oxydes metalliques sera evaluée.

3.7. Objectif 7

La traitement des métaux lourds et des déchets de bois contaminés par l'CCA peut être effectué par les déchets de la biomasse fongique, par un substrat fermenté (contenant d'AC et un mycélium fongique) et par la biomasse activée (après l'extraction du CTS), CTS et d'AC.

4. ORIGINALITÉ

D'après les hypothèses qui précèdent, cette étude affirme bien son originalité par différentes raisons:

4.1. Gestion des déchets pour la production des produits à valeur ajoutée, tels que l'AC et le CTS. L'application des déchets de la biomasse activée résultante de l'extraction du chitosane pour la biorestauration des déchets de bois contaminés par l'ACC, on aura donc à la fin zéro déchet.

4.2. L'APS qui est riche en glucides et en autres nutriments essentiels permettant la bio-production fongique d'AC n'a pas été jusqu'à à ce jour utilisé pour la biosynthèse des PVA.

4.3. Bien que le substrat AP a déjà été utilisé pour la production d'AC à des faibles rendements, cette étude a donc permis d'optimiser aux mieux les conditions d'expérimentation afin d'obtenir une plus grande bio-production fongique d'AC.

4.4. Toutes les études ce sont jusqu'ici concentrées sur la production d'AC à partir des déchets agro-industriels. Toutefois, les déchets des mycéliums fongiques résultant des industries qui produisent d'AC représentent une source très riche en CTS qui peut être extraite de façon simultanée du mycélium déchets. Ceci va donc contribuer à la stabilisation des marchés de production d'AC et donc à l'obtention de revenus supplémentaires pour les industries de transformation des fruits, ce qui permettrait de stimuler l'économie.

4.5. Optimisation des paramètres du procédé d'AC grâce à la méthodologie RSM qui permet d'évaluer les interactions entre les différents paramètres du procédé.

4.6. Les études rhéologiques affectent la croissance fongique au niveau du fermenteur et, par conséquent, la production d'AC. Les études rhéologiques au cours de la SmF de l'APS permettent de faciliter le traitement, ce qui conduit à la récupération élevée d'AC, ceci rend donc cette étude intéressante et plus originale.

4.7. L'AC et le CTS présentent de gros potentiels dans le domaine biomédical, dans l'environnement, dans les secteurs pharmaceutiques, alimentaires et agricoles, il est donc important d'explorer ces bioproduits. Le traitement des métaux lourds toxiques des

déchets de bois contaminés en ACC d'effluents, par de la biomasse fongique séchée, la biomasse fermentée (contenant d'AC et de la biomasse fongique), la biomasse résiduelle activée (après l'extraction du CTS), et par d'AC et du CTS représente une alternative viable et en respect avec l'environnement qui permet l'élimination des métaux lourds provenant des sites contaminés.

4.8. Cette recherche aurait donc des répercussions positives sur les industries de transformation des fruits. En effet, l'utilisation de leurs déchets générés à partir de la ligne de traitement qui représentaient auparavant un problème majeur, serait maintenant un atout pour l'achèvement de la boucle de gestion durable de ces déchets.

Dans l'ensemble, l'originalité de la recherche proposée est la suivante:

- *La production simultanée d'acide citrique et de chitosane à partir de déchets agro-industriels par Aspergillus niger.*

5. RÉSUME DES ÉTUDES PRÉSENTÉES DANS LE CORPS DE LA THÈSE

Les résultats obtenus dans cette thèse sont présentés en quatre parties:

5.1. Bio-production fongique d'acide citrique: études de criblage et d'optimisation (5 articles publiés).

La partie 1 traite de la production et de l'optimisation de la production économique d'acide citrique par différents déchets agro-industriels. Les déchets de l'industrie de la transformation de jus de pomme ont été choisis comme substrats potentiels et les paramètres importants ont été optimisés, ce qui a conduit à la production plus élevée d'acide citrique. Les résultats de cette étude ont aidé à effectuer des études à l'échelle fermenteurs.

5.2. Fermentation à l'état solide et submergé de l'AC dans les fermenteurs (2 articles publiés).

La partie 2 traite à l'échelle des fermenteurs de la production d'acide citrique en utilisant les déchets de l'industrie de pomme. Les résultats de cette étude ont indiqué que ces déchets peuvent éventuellement être utilisés pour la production d'acide citrique. En outre, le processus peut être rendu plus économique en utilisant l'approche de bioraffinerie visant à l'utilisation durable et maximum de déchets agro-industriels. Le mycélium fongique des déchets résultant au cours de la production d'acide citrique peut être utilisé pour les produits à valeur ajoutée.

5.3. Extraction du co-produit (chitosane) durant la fermentation à l'état solide et submergé (1 article publié; 1 article communiqué).

La partie 3 a été élaborée sur la base de la valorisation du mycélium fongique des déchets résultant de la production d'acide citrique. L'extraction du chitosane est une alternative lucrative pour l'industrie de l'acide citrique et fournira un avantage économique complémentaire en plus d'être respectueux de l'environnement.

5.4. Applications des biomatériaux issus de différents déchets pendant la fermentation d'AC (1 article publié; 2 articles communiqués).

La partie 4 traite avec des applications des biomatériaux déchets (BM) résultant en production d'acide citrique et extraction de chitosane. Les BMs ont été utilisés comme biosorbants pour le traitement des lixiviats de déchets de bois de l'ACC. Les nanoparticules à la base de ZnO-CTS ont été fabriquées en utilisant la nano pulvérisation et la méthode de précipitation chimique. Les nanoparticules ont montré des propriétés importantes antimicrobiennes et antibiofilms contre des bactéries pathogènes opportunistes.

5.1. Bio-production d'acide citrique fongique: études de criblage et d'optimisation

L'AC a été reconnu comme étant un produit chimique important avec une large gamme d'applications dans divers domaines. Diverses études ont été orientées vers la recherche de substrats économiques pour la bio-production d'AC, comme les déchets agro-industriels, afin de surmonter le coût élevé de l'énergie et des substrats. Des études de littérature sur les progrès récents dans la bio-production d'AC ont été réalisées (Chapitre 2, Partie I et II). Une revue complète de la littérature a démontré la

nécessité de sélectionner un substrat à faible coût et à développer des conditions optimales de fermentation pour la production d'AC.

5.1.1. Criblage de différents déchets agro-industriels solides et liquides pour la bio-production d'acide citrique

5.1.1.1 Utilisation des différents déchets agro-industriels pour la bio-production durable d'acide citrique par *Aspergillus niger* (Chapitre 3, Partie I)

En se basant sur la littérature, des études ont été menées pour sélectionner les substrats solides et liquides ayant un fort potentiel pour la production d'AC par *A. niger* NRRL 567 et NRRL 2001. En utilisant les meilleurs substrats, les effets inducteurs, EtOH et MeOH ont été optimisés et ont augmenté la production d'un facteur de 3 à 4. La production d'AC a été réalisée par la SSF et la SmF avec l'aide de différents déchets agro-industriels, tels que les déchets solides [AP, BSG, CW et SPM] et les déchets liquides [APS-1, APS-2, LS, SMS, SMS (hydrolysés) et SIW]. Parmi tous les supports solides utilisés, AP à 66,0 ± 1,9 g/kg MS s'est avéré être un excellent substrat pour la production d'AC par *A. niger* 567 NRRL à 72 h d'incubation. *A. niger* NRRL 2001 a entraîné une production d'AC légèrement inférieure de 61,0 ± 1,9 g/kg MS avec le même temps d'incubation. APS-1 (marc de pomme ultrafiltration boues-1) a augmenté la production de l'AC de 9,0 ± 0,3 g/l et 8,9 ± 0,3 g/l de substrat par *A. niger* NRRL 567 et NRRL 2001 avec la SmF. Les substrats sélectionnés (AP et APS) ont été combinés avec l'EtOH et le MeOH (3-4%) afin de stimuler les souches d'*A. niger* pour la production d'AC. L'ajout de 3% (v/p) EtOH et 4% (v/p) MeOH aux AP a augmenté les valeurs de production d'AC de façon significative de 127,9 ± 4,3 g/kg et 115,8 ± 3,8 g/kg MS par *A. niger* 567 NRRL par la SSF. Des valeurs plus élevées d'AC de 18,2 ± 0,4 g/l et de 13,9 ± 0,4 g/l de l'APS-1 ont été obtenues après addition de 3% (v/v) EtOH et 4% (v/v) MeOH, respectivement par *A. niger* NRRL 567. Les substrats AP et APS ont été sélectionnés pour des études d'optimisation supplémentaires par RSM.

5.1.1.2 Potentiel biotechnologique des déchets industriels pour la bio-production économique d'acide citrique par *Aspergillus niger* par fermentation submergée (Chapitre 3, Partie II)

Différents déchets liquides [Liqueur de Brasserie (BSL), lactosérum (LS) et les boues d'amidon de l'industrie (SIS)] ont été testées pour la production d'AC par *A. niger* 567 NRRL par la SmF. La fermentation a été réalisée en faisant varier la température (25-35°C), pH (3-5), avec l'addition d'inducteurs, le temps d'incubation et de supplémentation avec des proportions différentes d'APS. Les résultats indiquent qu'avec 3% (v/v) de MeOH, la concentration optimale de 11.34 g/l d'AC a été obtenue à l'aide de BSL à pH 3,5 et à la température de 30 ± 1°C après 120 h d'incubation. L'ajout de MeOH a entraîné une augmentation de la production d'AC de 56%. Avec les mêmes heures d'incubation et dans les mêmes conditions, une concentration plus élevée de 18.34 g/l d'AC a été enregistrée à l'aide d'une addition de BSL avec 40% (v/v) APS avec une concentration de solides en suspension de 30 g/l. La présente étude met en évidence le potentiel de BSL complété par l'APS dans la production d'AC de façon économique.

5.1.2. Optimisation des principeux paramètres de fermentation pour la production d'acide citrique par la méthodologie de surface de réponse

5.1.2.1 Amélioration de la fermentation à l'état solide par la bio-production d'acide citrique à partir des déchets de pomme en utilisant la méthodologie de surface de réponse (chapitre 3, Partie III)

La fermentation en milieu solide a été reconnue comme étant un procédé de fermentation à faible coût. En effet, la production d'AC se fait à partir de déchets agro-industriels. La production d'AC par *A. niger* est fortement influencée par la composition physico-chimique du milieu, tels que le carbone et d'autres micronutriments essentiels, l'humidité et la concentration des inducteurs. Afin d'améliorer la bio-production d'AC, le niveau d'humidité initiale et la concentration des inducteurs ont été optimisées dans des flacons à travers la méthodologie de surface de réponse (RSM) par *A. niger* 567 NRRL cultivé sur AP. Le plan composite central (CCD) a été appliqué pour évaluer les interactions entre les variables. Enfin, les résultats obtenus ont été comparés à ceux obtenus par des études de criblage.

L'humidité et le méthanol a eu un effet positif important sur la production d'AC par *A. niger* (p <0.05). Des valeurs plus élevées d'AC sur AP par *A. niger* (342.41 et 248.42 g/kg sec AP) ont été obtenues avec 75% (v/v) d'humidité par utilisation de deux inducteurs [3% (v/v) de MeOH et 3% (v/v) EtOH] avec un rendement de fermentation 93.90% et 66.42%, respectivement, en fonction de la totalité du carbone utilisé après 144 h de période d'incubation. Avec les mêmes paramètres optimisés, la fermentation classique a été menée. Des concentrations d'AC de 187.96 g/kg avec 3% (v/v) EtOH et 303.34 g/kg sec AP avec 3% (v/v) ont été obtenues avec le MeOH, ce qui représente une efficacité de fermentation de 50.80% et 82.89% en fonction de l'utilisation du carbone après 120 h d'incubation. Les résultats ont montré la pertinence de l'AP pour la production d'AC et l'étude a été appliquée à plus grande échelle dans un bioréacteur à tambour rotatif de 12 litres.

5.1.2.2 L'ultrafiltration des boues de pommes- un nouveau substrat pour la bio-production fongique de l'acide citrique: études d'optimisation (Chapitre 3, Partie IV)

La fermentation submergée de l'AC par *A. niger* est un procédé de fabrication bien établi vu l'importance de ce produit chimique. La production optimale d'AC n'est atteinte que si les paramètres importants du procédé sont minutieusement optimisés. Les matières en suspension (MES) présentes dans le milieu liquide présente une grande influence sur le rendement de la fermentation vu qu'elle est liée à la rhéologie des bouillons de fermentation. En même temps, la concentration des inducteurs, tels que les alcools (EtOH et MeOH) devraient également être optimisés afin de stimuler les champignons pour une meilleure sécrétion d'AC. Ce chapitre traîte de l'optimisation des MES et de la concentration des inducteurs en utilisant APS. Le nouveau substrat APS a été utilisé la première fois dans cette étude pour une meilleure production d'AC à travers la RSM. La fermentation submergée a été réalisée à l'aide de pomme (*Malus domestica*), l'ultrafiltration des boues de pomme permet d'avoir un substrat bon marché pour la bio-production d'AC, en utilisant *A. niger* NRRL567. Les paramètres essentiels, tels que les MES et la concentration en inducteur, ont été optimisés par la RSM pour la production d'AC. Les concentrations optimales d'AC sont de 44.9 g/100 g et 37.9 g/100 g SD et elles ont été obtenues à partir de 25 g/l de solides totaux initiaux et 3% (v/v) MeOH et d'une concentration de 3% (v/v) de EtOH, après la 144 h de fermentation. Les résultats indiquent que la concentration des solides totaux, et de l'inducteur MeOH, ont été efficaces et ont permis des rendements d'AC plus élevés. Ils ont également confirmé la

possibilité d'utiliser les boues de pomme comme substrat pour une production économique d'AC. En effet, les boues APS ont été utilisées à l'échelle laboratoire pour la production d'AC en optimisant les paramètres à l'aide de la RSM.

5.2 Fermentation à l'état solide et submergée dans les fermenteurs d'AC

5.2.1. Bio-production et optimisation des extractions de l'acide citrique à partir d'un substrat solide par Aspergillus niger en utilisant un bioréacteur à tambour rotatif (Chapitre 4, Partie I)

La présente étude consiste en la fermentation à l'état solide de l'AC en utilisant AP. L'étude vise à extraire de façon efficace l'AC de la biomasse solide fermentée. L'étude a été réalisée dans un bioréacteur à tambour rotatif de type 12 L. Les conditions optimales pour l'obtention de concentrations supérieures d'AC (220.6±13.9 g/kg DS) sont les suivantes: 3% (v/v) MeOH, sous agitation intermittente d'une 1 h après chaque 12 h avec 2 tours par minute, avec 1 vvm du taux d'aération et un temps d'incubation de 120 h. L'optimisation des différents paramètres d'extraction, tels que le temps d'extraction (min), l'agitation (tours par minute) et le volume d'extraction (H_2O distillée) (ml/g de substrat) pour une meilleure extraction d'AC à l'aide de la RSM a été réalisée. L'extraction d'AC de 294.19 g/kg MS a été réalisée dans des conditions optimales avec un temps d'extraction de 20 min, une vitesse d'agitation de 200 tours par minute et un volume d'extraction de 15 ml. Les résultats de l'étude indiquent que l'AP peut servir de substrat potentiel pour la production industrielle d'AC.

5.2.2. Études rhéologiques au cours de la fermentation submergée de l'acide citrique par Aspergillus niger en utilisant l'ultrafiltration des boues de pommes (Chapitre 4, Partie II)

La totalité de la bio-production d'AC par *Aspergillus niger* NRRL-567 a été réalisée dans un fermenteur de 7,5 l à l'échelle pilote en utilisant le substrat APS. Des études du profil rhéologiques de l'APS avec les champignons filamenteux ont été réalisées afin de développer la biomasse au cours de la fermentation. La bio-production de 23.67 ± 1.0 g/l d'AC a été obtenue au bout de 120 h avec 40.34 ± 1.98 g/l APS et 3% (v/v) de l'inducteur MeOH après 132 h de fermentation. Les propriétés rhéologiques du bouillon de fermentation y compris les granules ont été analysées. Le contrôle non-Newtonien a

montré un comportement pseudo-plastique en raison de la croissance qui est plus filamenteuse. Toutefois, l'inducteur (MeOH) qui est sous forme de granules a permis un traitement complet du bouillon au cours de la fermentation qui est rendu moins visqueux et non-newtonien. Le modèle a été suivi avec un intervalle de confiance de (77.8 à 88.9%) tout au long de la fermentation en utilisant le MeOH comme inducteur. D'importants changements rhéologiques se sont produits dans le bouillon pendant la phase de production maximale d'AC avec une augmentation de la biomasse sous forme de mycéliums granulés. Les résultats ont montré que la valeur ajoutée de l'APS pour la production d'AC sur une plateforme chimique peut être réalisée à l'échelle industrielle.

5.3. Extraction du coproduit (chitosane) pendant la fermentation

L'extraction du CTS à partir des déchets de mycélium offre un grand potentiel pour le développement durable du procédé de production d'AC et dans la stabilisation du marché de l'AC, tout en générant des revenus supplémentaires pour l'industrie et en atténuant le flux des déchets. Une revue exhaustive de la littérature a été réalisée pour étudier l'extraction du CTS à partir des déchets de la biomasse fongique (chapitre 5, Partie I).

5.3.1. Extraction concomitante du chitosane à partir des déchets du mycélium d'Aspergillus niger au cours de la bio-production d'acide citrique (chapitre 5, Partie II)

Les différentes classes de champignons contiennent des quantités importantes de chitine dans leurs parois cellulaires. Les déchets disponibles en abondance comme les mycéliums fongiques qui résultent de divers procédés biotechnologiques industriels représentent une source prometteuse de CTS par rapport aux sources traditionnelles, tels que les carapaces de crustacés. Dans cette étude, les déchets des mycéliums résultant de la production d'AC à partir des fermentations ont été utilisés pour la production durable et économique du CTS. La perspective de co-extraction du CTS à partir des déchets des mycéliums résultant de la production d'AC est intéressante, car elle peut conduire à une diminution partielle des coûts de production d'AC. En effet, le développement des procédés de valorisation des déchets des mycéliums fongiques d'*A. niger* stimule les industries biotechnologiques. Actuellement, les industries souffrent d'énormes pertes qui sont dues à l'élimination des déchets, ce qui fait souvent augmenter le coût de la production des produits. L'approche des produits à valeur

ajoutée va générer des revenus supplémentaires pour les industries et va aussi réduire le coût du produit principal. En effet, il permettra de renforcer l'économie et aidera également à réduire la pollution de l'environnement. En outre, l'extraction de CTS des déchets du mycélium fongique a été réalisée dans des conditions ambiantes et peut être considérée comme une approche écologique qui donnera l'opportunité de remplacer les méthodes traditionnelles d'extraction du CTS qui présentent beaucoup d'inconvénients.

5.4 Applications des biomatériaux issus des différents déchets pendant la fermentation d'AC

Ces dernières années, l'utilisation de matériaux dérivés de voies biologiques a beaucoup augmenté dans diverses applications, car ils sont non-toxiques et leurs produits de dégradation sont compatibles avec l'environnement. Les préoccupations énergétiques et environnementales sont les principaux facteurs qui accélèrent le développement des procédés écologiques. De plus, ces procédés sont principalement basés sur l'utilisation de la biomasse des déchets abondants et renouvelables qui contribuent à la conservation de nos ressources non renouvelables. Les bioproduits, tels que l'AC et le CTS, ont déjà prouvé leur potentiel à servir comme substrats écologiques pour les polymères issus de ressources non renouvelables, du fait qu'ils peuvent être produits de façon écologique en utilisant des stocks d'alimentation renouvelables. Différentes applications dérivées de la production d'acide citrique par formation de biopolymères ont été discutées dans cet article (chapitre 6, Partie I).

5.4.1. Utilisation des CTS, d'AC et d'autres biomatériaux résultant de différents déchets lors de la production d'AC et du CTS pour la traitement des lixiviats de CCA du bois (chapitre 6, Partie II).

L'arséniate de cuivre chromaté (CCA) a été largement utilisé comme agent de conservation du bois dans les années passées. Les bois traités au CCA posent de graves problèmes environnementaux dus à la lixiviation des métaux toxiques. La présente étude a été réalisée pour évaluer le potentiel des nouveaux biomatériaux issus de la production de l'AC et du CTS pour la biosorption des métaux toxiques qui sont présents dans les solutions aqueuses contenant du Cu, Cr et As des lixiviats de bois traité au CCA.

5.4.2 Synthèse facile du nanoparticules d'base de chitosan à base d'oxyde de Zn (ZnO) par nano-pulvérisation en utilisant différents stabilisants organiques et l'évaluation de leurs activités antimicrobiennes et antibiofilm contre les bactéries pathogènes (chapitre 6, Partie III)

La présente étude explore de nouveaux procédés de préparation d'une nouvelle classe de nanoparticules (NPs) d'base de chitosan (CTS) à base d'oxyde de Zn (ZnO) stabilisées avec divers composés organiques (CA, le glycérol, l'amidon et poudre de lactosérum) grâce à de nouveaux nano-pulverisation et des procédés de précipitation. Les composés organiques solubles dans l'eau servent comme stabilisant et agent dispersant qui empêche les NPs résultants de s'agglomérer, ce qui prolonge leur réactivité et permet le maintien de leur intégrité physique. Les NPs ont été caractérisées par Vis-UV pour des mesures des spectrophotomètres, des tailles grâce à la nano-sizer, zeta-potential et Microscope électronique à balayage. L'activité antimicrobienne et antibiofilm des NPs a été examinée. Les NPs d'base de CTS à base d'oxyde de Zn affichent des propriétés antimicrobiennes et inhibitrices de biofilm contre les souches bactériennes pathogènes opportunistes, *Micrococcus luteus* et *Staphylococcus aureus*, suggérant un rôle prometteur en médecine.

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PARTIE II.

FUNGAL CITRIC ACID BIOPRODUCTION: SCREENING AND OPTIMIZATION STUDIES

RECENT ADVANCES IN CITRIC ACID BIO-PRODUCTION

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RÉSUMÉ

La consommation d'acide citrique s'intensifie progressivement, au regard du taux de croissance annuel élevé dû à l'émergence d'applications de plus en plus avancées. La présente revue examine les différents aspects de la fermentation et effet de divers paramètres environnementaux et traite des différentes manières possibles d'accroître le rendement de l'acide citrique afin de répondre aux demandes croissantes de cet acide organique commercialement important. Différentes techniques d'hyper production d'acide citrique sont constamment à l'étude depuis les dernières décennies et il existe encore un fossé. Par conséquent, il est évident d'envisager de nouveaux moyens pragmatiques de réaliser une bioproduction d'acide citrique industriellement faisable et écologiquement durable. . Une des voies potentielles est d'utiliser des déchets agro-industriels peu coûteux ainsi que leurs sous-produits par fermentation en milieu solide, grâce à des souches existantes et génétiquement modifiées. Cette revue porte également sur la transformation en aval qui prend en compte les approches classiques et avancées; celles-ci nécessitent également une amélioration significative. La méthode *in-situ* de produit de récupération qui conduit à des rendements et à une productivité améliorés, peut encore être optimisée pour une production à grande échelle et la récupération de l'acide citrique.

Mots-clés: acide citrique; *Aspergillus niger*; *Yarrowia lipolytica*; fermentation à l'état liquide; fermentation à l'état solide; déchets agro-industriels; prétraitement; récupération

ABSTRACT

Citric acid consumption is escalating gradually, witnessing high annual growth rate due to more and more advanced applications coming to light. The present review discusses different aspects of fermentation and effects of various environmental parameters and deals with the potential ways to increase the yield of citric acid to meet the ever increasing demands of this commercially important organic acid. Different techniques for the hyper production of citric acid are continuously being studied from the past few decades and still there is a gap, and hence, there is an obvious need to consider new pragmatic ways to achieve industrially feasible and environmentally sustainable bio-production of citric acid. The utilization of inexpensive agro-industrial wastes and their by-products through solid-state fermentation by existing and genetically engineered strains is a potential route. This review also deals with downstream processing considering the classical and advanced approaches, which also need significant improvement. In situ product recovery method which leads to improved yields and productivity can be further optimized for large-scale production and recovery of citric acid.

Keywords: Citric acid; *Aspergillus niger*; *Yarrowia lipolytica*; Submerged fermentation; Solid-state fermentation; Agro-industrial waste; Pretreatment; Recovery

INTRODUCTION

Citric acid (CA), an intermediate of the tricarboxylic acid cycle, is one of the most important commercially valuable products due to its widespread use mainly in food (70%), pharmaceuticals (12%), and others (18%) (Tran et al. 1998; Wang 1998; Ates et al. 2002). The global production of citric acid has increased to 1.7 million tons in 2007, as estimated by Business Communications Co. (http://www.bccresearch.com). Due to its numerous applications, the volume of citric acid production by fermentation is continually increasing at a high annual rate of 5% (Finogenova et al. 2005; Francielo et al. 2008) and also witnessing steadily increasing demand/consumption. The adverse market conditions have decreased the price of CA, which is about $1.0-1.3/kg. Meanwhile, its consumption rate is rising day by day due to its numerous applications; considering the slight increase in price, the market value for this commodity chemical will exceed $2 billion in 2009 (Partos 2005).

It is accepted worldwide as a GRAS (generally recognized as safe), as approved by the Joint FAO/WHO Expert Committee on Food Additives (Rohr et al. 1996; Carlos et al. 2006). CA and its salts (primarily sodium and potassium) are used in many industrial applications: as a chelating agent, buffer, pH adjustment, and derivatization agent. Applications include laundry detergents, shampoos, cosmetics, enhanced oil recovery, and chemical cleaning (Soccol et al. 2006). Aqueous solutions of CA are an excellent buffer when partially neutralized as citric acid is a weak acid and has three carboxylic groups; hence, three pKa's at 20 °C pK1=3.15, pK2=4.77, and pK3=6.39 (Weast 1989), resulting in buffering action in the pH range 2.5–6.5 (Blair et al. 1991).

Nowadays, there is a growing trend to increase CA production due to many advanced applications coming to light. The studies reveal the potential use of CA in biopolymers, for drug delivery, tissue engineering for culturing a variety of cells, and many other promising biomedical applications, besides environmentally sustainable use of CA for the efficient removal of post-soldering flux residues by the military (Robin et al. 1995; Ashkan et al. 2010; Guillermo et al. 2010). Most of the citric acid is manufactured through biological means, mainly through submerged fermentation of starch/sucrose-based media (molasses) exclusively by the filamentous fungus *Aspergillus niger* (Jianlong 2000; Vandenberghe et al. 2000; Lesniak et al. 2002; Schuster et al. 2002) due to its high citric acid productivity at low pH without the secretion of toxic by-products. CA is regarded as a metabolite of energy metabolism whose concentration will rise to

appreciable amounts only under conditions of substantial metabolic imbalances. In recent years, various agricultural waste residues and by-products have been investigated for their potential to be used as a substrate for CA production by solid-state fermentation (SSF). Among them are molasses, fruit pomace waste, wheat bran, coffee husk, and cassava bagasse, among others which otherwise are used in composting or dumped in landfills and cause environmental hazards (Medeiros et al. 2000; Singhania et al. 2009; Kuforiji et al. 2010). The bioproduction of value-added products using agri-waste substrates provides advantages from both the standpoint of waste material management and of lower capital costs for carbon substrates.

Thus, looking at the surge in the demand for CA, there is a critical need to find ways to increase its production yield and develop advanced low loss recovery methods. In this light, this review highlights the different production methods for citric acid, the importance of various environmental parameters, possibility of pretreatment of complex alternative wastes to enhance CA production, and conventional and advanced citric acid recovery techniques.

MICROORGANISMS

A large number of microorganisms such as bacteria, fungi, and yeast have been harvested on a variety of substrates for the bio-production of CA, as given in Table 1 (Crolla and Kennedy 2001; Papagianni 2007; Kuforiji et al. 2010). A white rot fungus, *A. niger*, is exclusively the preferred choice of microorganism for the citric acid bio-production process. These filamentous fungi are the most adapted and suitable microorganisms to grow on various substrates. The fine regulation and control of glycolytic flux, secretion of citric acid from the mitochondria and the cytosol, and the growth characteristics and adaptability of *A. niger* on diverse habitats together contribute toward the massive accumulation of CA. The regulation of different metabolic enzymes coupled with the effect of various positive factors on glycolytic flux favors high CA formation and further low degradation via the citric acid cycle (Levente and Christian 2003). Wehmer (1893) first observed that "Citromyces" (now Penicillium) accumulated CA in a culture medium that contained sugars and inorganic salts. Many other microorganisms have since been found to accumulate citric acid, including many other strains of *A. niger*, as further illustrated in Table 1. Currie (1917) discovered that some strains of *A. niger* grew abundantly in a nutrient medium that had a high concentration of

sugar and mineral salts and an initial pH of 2.5–3.5 and produced large quantities of CA. This discovery laid down the basis for industrial exploitation of this strain for the bio-production of citric acid. Prior to this, *A. niger* was a known producer of oxalic acid, but the low pH suppressed the formation of oxalic and gluconic acids. Currie's finding formed the basis of CA production established by Pfizer in 1923 in the USA.

Despite the fact that the mainstream CA production studies involve molds, the use of bacteria and yeast has been considered as well. Several bacteria, such as *Arthrobacter paraffinens, Bacillus licheniformis,* and *Corynebacterium* ssp. (Carlos et al. 2006), and yeast strains are known to produce large amounts of CA from n-alkanes and carbohydrates (Mattey 1999; Rymowicz et al. 2010; André et al. 2007). Yeasts are also well known to produce CA from various carbon sources. Among these yeasts, *Yarrowia lipolytica* has been widely utilized for its ability to produce large amounts of CA. One drawback of yeast fermentation is that they produce substantial quantities of isocitric acid, an undesired by-product. This problem should be the key motivation to look for different ways to enhance the CA production and at the same time alleviate the problem of co-production of isocitric acid by *Y. lipolytica*. Selection for mutants with very low aconitase activity has been used in attempts to reduce the production of isocitric acid. Genetic alteration of microorganisms has not been explored much for CA production but offers a potentially useful approach for improving yields and fermentation rates.

The main advantages of *A. niger* over many other microorganisms are its ease of handling/harvesting, its ability to ferment a variety of raw materials, preferably agricultural waste residues, and high product yields. In addition to the economically efficient *A. niger* strain, the yeast Y. lipolytica has been gaining attention as a microbial cell factory for CA production. It grows efficiently on *n*-paraffins and fatty acids, besides glucose and sucrose yielding high CA concentrations (Crolla and Kennedy 2004; Papanikolaou 2006; Forster 2007; André et al. 2007). Most of the literature studies exclusively mention the use of *A. niger* strains as the most favorable microorganism for the production of CA. There are other strains too capable of producing CA, albeit at low yields. Nevertheless, with the advances in biotechnology, there has been a development of new genetically engineered strains and improvement in the existing citric acid-producing strains by mutagenesis and the selection of higher potential strains of production of CA. However, the actual use of these novel strains in the industrial application and stability over a period of time is questionable, thus relying on

conventional strains. In this regard, the role of substrates is also a principal factor to enhance the yield of CA.

SUBSTRATES

Most of the industrial-level production of CA involves submerged fermentation using *A. niger* grown on media containing glucose or sucrose (Leangon et al. 2000; Kumar et al. 2003), especially from by-products of the sugar industry. Apart from this, various other raw materials, such as hydrocarbons, agro-industrial waste residues, and several other starchy materials, have been employed as carbon sources for submerged fermentation of CA (Hang and Woodams 1998; Raukas et al. 1998; Jianlong et al. 2000; Mourya and Jauhri 2000; Vandenberghe et al. 2000; Ambati and Ayyanna 2001). Table 2 illustrates the effect of different fermentation types employing various substrates on CA production. Several other synthetic routes using different starting materials have since been published, but chemical methods have so far proved uncompetitive with fermentation as the starting materials are worth more than the final product.

In recent years, the utilization of solid-state fermentation (SSF) has shown some potential as an alternative for the production of CA (Romero-Gomez et al. 2000). In order to make the bio-production of citric acid industrially feasible by SSF, the research for suitable substrates for SSF has mainly focused on agro-industrial residues. This is mainly due to the fact that they are easily available, inexpensive, carbohydrate-rich comprising other vital nutrients, and due to their potential advantage of harvesting filamentous fungi, which are capable of penetrating into the solid portions of these solid substrates aided by the turgor pressure at the top of the mycelium (Ramachandran et al. 2004). In addition, these inexpensive agro-industrial waste residues not only find applications in various bioprocesses but also help solve their disposal problems (Pandey et al. 1999c). The utilization of agro-industrial waste or by-products for CA production has greatly enhanced its economic efficiency. The selection of a suitable substrate for SSF process depends on several factors mainly related with cost and availability.

Agro-industrial residues are generally considered as the best substrates for SSF processes as they supply the needed nutrients for the growth of microbes. The substrates include sugarcane bagasse, fruit pomace, wheat, rice, maize and grain bran, wheat and rice straw, coconut coir pith, newspaper, fruit wastes, tea and coffee wastes,

cassava waste, and distiller grains among others (Krishna and Chandrasekaran 1995, 1996; Hang and Woodams 2000; Vandenberghe 2000; Gowthaman et al. 2001; Pandey et al. 2001; Shojaosadati and Babaeipour 2002; Kumar et al. 2003; Xie and West 2006; Karthikeyan and Sivakumar 2010; Kuforiji et al. 2010). There are various other inexpensive agro-waste residues which can be potentially utilized for the production of CA.

Owing to the high carbohydrate content, other vital nutrients, high-moisture content, and abundant availability, apple pomace can be utilized as an ideal substrate to cultivate different microorganisms for the production of CA (Shojaosadati and Babaeipour 2002). Presently, only a small proportion of apple pomace is utilized as a feed for the ruminants, added to soil as a fertilizer, and the large proportion of this inexpensive biomass goes to the composting sites and landfills, resulting in the release of greenhouse gases and causing environmental nuisance and jeopardizing the health of people.

Glycerol, the important side product of biodiesel production, is another potential substrate for CA bioproduction. Furthermore, glycerol is produced by several other industries, such as fat saponification and alcoholic beverage production units (Makri et al. 2010). The increasing demand and production of biodiesel also leads to the production of abundant quantities of glycerol as a side product (Wen et al. 2009). According to Thompson and He (2006), for each gallon of biodiesel produced, approximately 0.35 kg (0.76 lb) of crude glycerol is also produced. However, in spite of the increasing supply of crude glycerol, only a few reports of the value-added product formation from this substrate have been published. CA had been synthesized from glycerol by Grimoux and Adams (1880). Ever since, there was a major gap in the research owing to the high material cost. Recent studies showed that *Y. lipolytica* cultivated on glycerol produced high quantities of CA (Papanikolaou and Aggelis 2009; Makri et al. 2010; Rymowicz et al. 2010). Taking into account the huge quantities of glycerol produced and its low cost, it can be potentially used in future for industrial CA production by *Y. lipolytica*.

While considering the different substrates, it is certainly much easier to produce CA from synthetic substrates where the carbon source is simple to degrade. However, with the rising raw material costs and abundance of agro-industrial wastes (by virtue of the

increase in population) resulting in disposal costs, loss of precious carbon, and release of greenhouse gases, there is a critical need to utilize these agro-industrial wastes for the production of CA. The lower raw material price of the agro-industrial wastes might alleviate the total production cost of CA and make it an economically viable process. However, agro-industrial wastes are often complex, mandating pretreatment to increase nutrient availability, enhance rheological capability (decreased viscosity and particle size) favoring enhanced oxygen transfer and mass transfer of nutrients, and increase product yield by efficient utilization of the complete substrate.

RHEOLOGY

Morphology of fungus is an important parameter which influences the physical characteristics of the fermentation broth and final yield of the product. The rheological behavior of fungus is closely related to the morphology and biomass concentration (Jayanta et al. 2001). The broth rheology determines the transport phenomena in bioreactors and yield of the desired product (Gehrig et al. 1998). Non-Newtonian flow behavior is the fundamental aspect of fungal fermentation systems, especially the filamentous fungi. The fermentation broth exhibits distinct non- Newtonian characteristics even if the solid content is present in low amount (Henzler and Schafer 1987). For instance, in *A. niger* fermentation systems, the filamentous or pellet forms can be distinguished in broth during the fermentation process. During filamentous stage, the entanglement of mycelial hyphae along with high concentration of biomass may lead to highly viscous non-Newtonian behavior. However, in the pellet form, the mycelium develops very stable spherical aggregates consisting of more dense, branched, and partially intertwined network of hyphae and usually gives less viscous non-Newtonian broths (Berovic and Cimerman 1982).

For the submerged CA fermentation, pellet growth form of *A. niger* is highly recommended (Berovic et al. 1991, 1993). The rheological behavior of fermentation broths affects the mixing of the medium contents and thus the mass and heat transfer processes, which in turn hinder oxygen transfer to the cells, which is often the rate-limiting step in fermentation. One possible way to minimize oxygen mass transfer limitation to the cells is to stimulate the formation of small spherical cell pellets. Small pellets can reduce clump formation and viscosity of the broth during fermentation. The fermentation broth containing Mn^{2+}-deficient mycelium has a much better rheology and

oxygen transfer rate possibly due to pellet formation (Levente and Christian 2003). However, at the same time, manganese is essential for the growth of *A. niger*, and it was found that cellular anabolism of *A. niger* is impaired under total manganese deficiency (Rohr et al. 1996). The prerequisite for the desired morphology of fungus for high CA yield needs to be carefully optimized for the initial concentration of manganese in the fermentation medium. The studies conducted by Rugsaseel et al. (1995) demonstrated that the supplementation of viscous substances, such as agar, carrageenan, carboxy methylcellulose, and polyethylene glycol among others in synthetic medium, markedly showed high productivity of citric acid in semi-solid and surface cultures of *A. niger*, which otherwise does not metabolize these substances. The authors therefore suggested that these viscous substances act as protectants for the mycelium from physiological stresses due to shaking. The studies also demonstrated that with the addition of gelatin in the medium, the resulting mycelia were thick with stable spherical aggregates, consisting of a denser, branched, and partially intertwined network of hyphae with more bulbous cells, which represent the most productive form of the fungus. These morphological characteristics were found to be similar with the other *A. niger* strains grown in high CA medium (Ujcova et al. 1980; Legisa et al. 1981). However, it is worth noting that highly viscous and the non-Newtonian character of mycelial suspensions lead to difficulty in mixing, which strongly affects the interface transfer of oxygen. For high yield of CA in non-Newtonian fermentation broths, the mechanically agitated bioreactors are required for the efficient mass and heat transfer and the proper circulation of oxygen.

The rheology of fermentation broth results from the net effect of complexity of the substrate, increase in biomass during growth, and formation of different metabolites causing mass and oxygen transfer problems, resulting in low CA productivity. In order to better utilize the agro industrial wastes, pretreatment can serve a dual purpose: reduce particle size, which in turn influences the rheology of the medium, and weaken the molecular interactions in biomass, providing better hydrolysis rate, finally resulting in high yields of CA.

PRETREATMENT

All solid substrates have a common feature, which is their basic macromolecular structure being starch, cellulose, hemi-cellulose, lignin, pectin, and other

polysaccharides.Preparation and pretreatment are the necessary steps to convert the raw substrate into a form suitable for use. Starchy feed stocks are easily hydrolyzed by the microorganisms. These materials required mild acidic and enzymatic pretreatments for utilization by the microorganisms, whereas the bio-utilization of lignocellulosic material is restricted by several factors, such as crystallinity of cellulose, available surface area, and lignin content. Several mechanical, thermal, chemical, and biological methods could be employed for the pretreatment, as depicted in Table 3.

Milling is a common type of mechanical pretreatment for cutting the complex biomass, which often imposes significant energy costs (Berlin et al. 2006). Depending upon the complexity of the biomass, extrusion can also be employed for the mechanical pretreatment, which has many advantages over milling. The lignin hydrolysis by extrusion is limited; however, the combination of extrusion with other pretreatments, such as thermal treatment, might result in an increase in the susceptibility of biomass to chemical and biological degradation for efficient utilization of biomass for high CA production (Lee et al. 2007; Seung-Hwan et al. 2009). Hot water treatment and steam explosion are other options of thermal pretreatment which offer the advantage of delignification (Laser et al. 2002; Kim and Holtzapple 2006; Hendriks and Zeeman 2009).

To obtain a much harsher treatment, chemical methods can also be used. Compared with acidic or oxidative reagents, alkali treatment appears to be the most effective method in breaking the ester bonds between lignin, hemicellulose, and cellulose (Gaspar et al. 2007). Other advanced pretreatment options include ammonia and carbon dioxide pretreatment, which, however, can lead to losses of hemicellulose and cellulose (Sun and Cheng 2002; Alizadeh et al. 2005; Kim and Lee 2005). Depending upon the complexity of the substrate and the type of fermentation, the pretreatment process can be carefully optimized for the proper utilization of available carbon for the feasible production of CA. The production of CA from starchy substrates requires the conversion of starch polymer into glucose, which can be accomplished by using enzymes, α-amylase to loosen the structure of the molecule and thus lower the viscosity, and amyloglucosidase for the final formation of glucose, albeit with low treatment rate (Sun and Cheng 2002). Thus, the best pretreatment method choice for CA production depends on the complexity of biomass and type of fermentation adopted as SSF usually

works closer to the niche conditions of the fungus when compared to submerged fermentation (SmF).

DIFFERENT METHODS OF CITRIC ACID BIO-PRODUCTION

At present, 99% of the CA produced in the world is obtained by fermentation (Kuforiji et al. 2010). The filamentous fungus *A. niger* is a preferred and is a potent producer of CA. Bio-production of CA can be divided into three steps, which include preparation and inoculation of the fermentation broth, fermentation, and recovery/purification of the product, as described in Fig. 1. The three widely used methods of CA bio-production are submerged fermentation, surface fermentation, and solid-state fermentation, as already depicted in Table 2. A wide range of substrates could be employed for the efficient and feasible production of CA according to the type of fermentation.

Surface Fermentation

Surface fermentation (SF) refers to the process in which the microorganisms grow on the surface of the fermentation media. In CA production by surface fermentation process, *A. niger* grows as a thick floating mycelial mat over the surface of the media used. Surface fermentation is used in many small- and medium-scale industries as it requires less effort in operation, simple equipment, and lower energy cost. The process is carried out in fermentation chambers where a large number of trays are arranged in shelves. The trays are made of high-purity aluminum, special type steel, or polyethylene; however, steel trays supply better yields of citric acid (Soccol and Vandenberghe 2003). The fermentation chambers are provided with an effective air circulation which passes over the surface in order to control humidity and temperature by evaporative cooling. This air is filtered through a bacteriological filter and the chambers should always be in aseptic conditions and must be conserved principally during the first 2 days when spores germinate. The most frequent contaminations are mainly caused by Penicillia, other Aspergilli, yeasts, and lactic acid bacteria. During fermentation which is completed in 8–12 days (Yokoya 1992), a large amount of heat is generated, so high aeration rates are needed in order to control the temperature and to supply air to the microorganism. After fermentation, the tray contents are separated into crude fermentation broth and mycelial mats which are washed to remove the impregnated CA. CA production by surface

fermentation could further be enhanced by the efficient utilization of various new substrates and improvement in the existing CA-producing microorganisms by various means. In spite of high CA yields obtained through surface fermentation, the process is not much popular in large industrial scale due to some disadvantages, such as large space requirement, being time consuming, contamination risk, generation of large amounts of heat during process and liquid waste, and high production costs. There is an imperative need to design tray bioreactors similar to the ones which are used in SmF processes to alleviate the aforementioned problems. Surface fermentation has the potential to compete with SmF, which has been a promising and well-established method for large-scale production of CA in the last few decades.

Submerged Fermentation

It is estimated that about 80% of world CA production is obtained by submerged fermentation using *A. niger* grown on media containing glucose or sucrose (Vandenberghe et al. 1999; Leangon et al. 2000; Kumar et al. 2003), especially from by-products of the sugar industry. Apart from this, various other agro-industrial wastes and their byproducts have been employed as carbon sources for submerged fermentation of CA (Jianlong et al. 2000; Mourya and Jauhri 2000; Vandenberghe et al. 2000; Ambati and Ayyanna 2001; Rivas et al. 2008). Over other fermentation types, SmF presents several advantages, such as higher productivity and yield, lower labor costs, and lower contamination risk. Submerged fermentation processes can be carried out in batch and fed-batch fermentation. Depending on the fermentation conditions, it is concluded in 5–12 days. Although submerged fermentation is the widely employed method for the bulk production of CA, there still is a need to explore some alternative methods for the industrially feasible and sustainable production of citric acid in order to cope with the decreasing prices and fulfill the increasing market demand of CA. SSF is the potential approach to accomplish the task of achieving high yield of CA by exploiting wide range of natural and renewable agro-industrial wastes and their by-products which may not be feasible with SmF. Moreover, SSF has taken over as an important process in the western countries too with urgent needs to address the escalating problems of agro-industrial wastes.

Solid-State Fermentation

In the past few years, CA production using SSF has been a subject of great interest as it offers numerous advantages for the production of bulk chemicals and enzymes (Roukas 1999; Shojaosadati and Babaeipour 2002). This is partly because solid-state processes have lower energy requirements and produce much less wastewater and thus less environmental concerns. According to Chundakkadu (2005), SSF is defined as a fermentation process in which microorganisms grow on solid materials without the presence of free liquid. In SSF, the moisture necessary for microbial growth exists in an absorbed or complexed state within the solid matrix. This solid material is generally a natural compound consisting of agricultural and agro-industrial by-products and residues, urban residues, or a synthetic material (Pandey 2003). Various kinds of fermenters have been used for CA production in solid-state fermentation, such as Erlenmeyer conical flasks, glass incubators, trays, rotating and horizontal drum bioreactors, packed bed column bioreactor, single-layer packed bed, and multilayer packed bed among others (Papagianni et al. 1999; Vandenberghe et al. 1999, 2004; Pandey et al. 2001).

A closer assessment of these two processes in recent years by various researchers has revealed the enormous economical and practical advantages of SSF over SmF, as illustrated in Table 4. Although SSF fermentations are gaining global attention from the past few decades for the sustainable and feasible production of citric acid and many other industrial products, still there is much room for improvement of these processes in order to get the highest yield of CA. No doubt there is a great scope in SSF process for developing commercial CA production technology with economic feasibility, but greater automation of the process is needed for increasing its industrial exploitation for CA production, which can be achieved with the improvement in reactor designs. Furthermore, the sustainable use of renewable and abundantly available biomass and optimization of different fermentation parameters could lead to the techno-economically feasible production of CA.

DIFFERENT FACTORS GOVERNING CITRIC ACID PRODUCTION

The conditions under which biosynthesis occurs affect the fungal growth and CA production differently. Many researchers reported that a high concentration of CA is

produced only under the conditions of limited biomass production (Grewal and Kalra 1995; Couto and Sanroman 2006). Similarly, Vandenberghe et al. (2000) attained good growth of a fungal culture with high CA production. However, Elzbieta (2008)) also obtained maximum citric acid yield (150.5 g/substrate dry matter) under the following conditions: 0.2 dm3kg−1min−1, mixing (periodical) 1 min once an hour, and bed loading 30% of the bioreactor working volume. Different factors governing CA production by employing various fermentation processes can be optimized carefully for the improved microbial production of CA.

Medium Constituents

CA production by *A. niger* is influenced by a number of culture parameters, especially trace metal ions, as provided in Table 5. This organism requires certain trace metals for growth (Mattey 1999). The metals that must be limiting include Zn, Mn, Fe, Cu, heavy metals, and alkaline metals. Some metal ions (Fe^{2+}, Mn^{2+}, Zn^{2+}, Cu^{2+}, and others) are known to be inhibitory to CA production by *A. niger* in submerged fermentation even at a very low concentration (Kapoor et al. 1987). CA production through submerged fermentation by *A. niger* is extremely sensitive to trace metals present in molasses (Pera and Callieri 1997; Majolli and Aguirre 1999). Therefore, the concentration of these heavy metals should be decreased well below the concentration required for optimal growth as well as maximum CA production (Maria and Wladyslaw 1989; Majolli and Aguirre 1999). The optimum concentration of Fe^{2+} required for maximal CA production has been found to vary with the fungus strain. Studies revealed that CA production by *A. niger* under submerged fermentation conditions using molasses as the substrate was severely affected by the presence of iron at a concentration as low as 0.2 ppm; however, the addition of copper at 0.1–500 ppm at the time of inoculation or during the first 50 h of fermentation was found to counteract the deleterious effect of iron. Researchers have also reported such beneficial effects of Cu^{2+} in counteracting the Fe^{2+} effect (Haq et al. 2002). Additions of Mn^{2+} at concentrations as low as 3 µg/L have been shown to drastically reduce the yield of CA under otherwise optimal conditions (Rohr et al. 1996). Research by Rohr et al. (1996) confirmed the key regulatory role of Mn2+ ions. Cellular anabolism of *A. niger* is impaired under total manganese deficiency and/or nitrogen and phosphate limitation. Manganese has also been shown to be important in many other cell functions, most notably cell wall synthesis, sporulation, and production of secondary metabolites.

The concentrations of these trace metals in culture media may be controlled in two ways. One way is to purify the medium in order to remove certain metal ions and then add known amounts of the required metal ions. The second method is to add metal chelating agents to the medium to decrease the concentration of free metal ions in the required amounts. This method has the advantage of the metal complex acting as a "metal buffer" which reversibly dissociates to release ions as they are utilized by the growing microorganism (Jianlong 1998). The presence of trace metals in toxic concentrations can be a significant problem during the submerged fermentation of crude substrates into useful value-added products. However, solid-state fermentation gives high CA yield without inhibition related to the presence of metals at high concentration. Iron salts are essential for the production of CA as these activate the production of acetyl co-enzyme A, which is important for the production of CA (Milsom and Meers 1985). Meanwhile, excess concentration of iron will activate the production of aconitate hydratase (aconitase), the enzyme responsible for the production of isocitric acid. Isocitric acid is a by-product of the fermentation that is not desired as an isomer of CA; it reduces potential CA yield. Potassium dihydrogen phosphate has been found to be the most suitable phosphorus source, and low levels of phosphorus were found to favor citric acid production. The addition of methyl acetate, copper, and zinc was found to enhance CA production, while magnesium was found to be essential for growth as well as for citric acid production (Sato and Sudo 1999; Pandey et al. 2000).

The type and concentration of carbohydrate is also one of the important factors which determine the final concentration of the desired product. In contrast to the effect of other factors, relatively little has been published on the effect of sugar concentration on the important fermentation parameters with filamentous fungi such as *A. niger*. However, according to Anwar et al. (2009), the high content of sugars in substrate is considered favorable for higher production of CA. Nitrogen sources stimulate fungal conidiation, as already given in Table 5. CA production is directly influenced by the nitrogen source used in the fermentation, and ammonium salts such as urea, ammonium chloride, and ammonium sulfate are preferred (Chundakkadu 2005). An advantage of using ammonium salts is that the pH declines as the salts are consumed; a low pH is a requirement for CA fermentation (Mattey 1999).

Medium constituents play a vital role in the overall production yield of CA. It is of utmost importance to consider the concentration of different trace metals and other constituents

present in the biomass according to the type of fermentation and physiological requirements of microorganism for the high yields of CA. For instance, the toxic heavy metals should be removed and the required trace metal concentration should be carefully optimized for the efficient growth of microorganisms. The morphology of fungus is an important parameter which affects the overall production yield of CA, and it largely depends upon the culture constituents.

Environmental Conditions

A number of parameters have been considered to influence biotechnological processes (temperature, humidity, pH, minerals, type of inoculum, and addition of nutrients among others). Besides, various other parameters are also found to be very critical during solid-state fermentation, such as agitation rate (Shojaosadati and Babaeipour 2002), particle size of the substrate (Roukas 1999; Shojaosadati and Babaeipour 2002; Kumar et al. 2003), and the extent of the bed loading (Lu et al. 1997; Shojaosadati and Babaeipour 2002). Fungi are well known to accommodate/prefer a moist environment for their growth. For the biomass production, an optimum moisture level has to be maintained as lower moisture tends to reduce nutrient diffusion, microbial growth, enzyme stability, and substrate swelling (Chundakkadu 2005). Higher moisture levels lead to particle agglomeration, gas transfer limitation, and competition from bacteria (Gowthaman et al. 2001). In general, the moisture levels in SSF processes vary between 30% and 85%. For filamentous fungi, the moisture of the solid matrix could be as wide as 20–70%. The moisture level should be carefully optimized according to the nature of the substrate used for a better yield of CA by *A. niger*.

The temperature is notably the most important of all the physical variables affecting SSF performance as it adversely affects microorganism growth and production of enzymes or metabolites. The significance of temperature in the development of a biological process lies in the fact that it could determine some important effects, such as protein denaturation, enzyme inhibition, acceleration or suppression on the production of a particular metabolite, and, importantly, cell death (Pandey et al. 2001). As in the case of pH, fungi can grow over a wide range of temperatures of 20–55 °C, and the optimum temperature for growth could be different from that of product formation (Yadav 1988). One limitation of SSF is its inability to remove excess heat generated by metabolism of microorganism due to the low thermal conductivity of the solid medium. In practice, SSF

requires more aeration for heat dissipation than as a source of oxygen (Viesturs et al. 1981). In order to achieve the hyperproduction of CA, proper environmental conditions should be provided for the efficient growth of the microorganisms. There should be an urgent need of the technology development for the proper control of different parameters in SSF for high production of CA.

The main objective of aeration is to supply an appropriate amount of oxygen for microbial growth and to remove carbon dioxide. Aeration also performs a critical function in heat dissipation and moisture transfer (Raimbault 1998; Pandey et al. 2000; Shojaosadati and Babaeipour 2002), thus regulating the temperature of the fermentation medium, distribution of water vapor (regulating humidity), and volatile compounds produced during metabolism. The aeration rate is therefore determined by several factors, such as the growth requirements of the microorganism, the production of gaseous and volatile metabolites, and heat evolution, and it depends on the porosity of the medium; pO_2 and pCO_2 should be optimized for each type of medium, microorganism, and process (Chundakkadu 2005). The following equations describe the stoichiometry for the microbial production of CA (Briffaud and Engasser 1979).

$$C_6H_{12}O_6 + 1.5O_2 \rightarrow C_6H_8O_7 + 2H_2O \quad (1)$$

$$C_6H_{12}O_6 + 6O_2 \rightarrow 6CO_2 + 6H_2O \quad (2)$$

The above equations describe the important role of oxygen in the production of citric acid (Rane and Sims 1994). CA accumulation was found to be increased significantly with an increase in dissolved oxygen concentration. CA concentration was also increased by a factor of 1.4-fold by adding n-dodecane (5%, v/v) as an oxygen vector to the fermentation medium by *A. niger* (Jianlong 2000). However, it is worth noting that excessive aeration can produce shear stress (which has a harmful effect on the morphology of filamentous fungus) and can channel the packed bed (Lu et al. 1997; Shojaosadati and Babaeipour 2002).

Agitation is considered as one of the most important parameters in aerobic fermentations as it ensures homogeneity with respect to temperature and gaseous environment and provides a gas–liquid interfacial area for gas-to-liquid as well as liquid-to-gas transfer (Trilli 1986). It has been evident from the previous reports that agitation

facilitates the removal of volatile metabolic products, prevents the formation of agglomerates, enhances heat exchange, protects the medium against local desiccation or excessive moistening, and improves the conditions for microbial growth within the entire bed volume (Mitchell and Berovic 1998). Agitation enables an even and more effective distribution of the spore suspension, water required for moisture control, and/or of any other nutrient solutions, if necessary (Suryanarayan 2003). However, it must be pointed out that agitation is not used in many aerobic SSF processes carried out in static reactors, such as tray fermenters. In contrast, agitation is usually an essential part of periodically or continuously agitated SSF bioreactors. Agitation is also known to have adverse effects on substrate porosity due to the compacting of the substrate particles, disruption of fungal attachment to the solids, and damage to fungal mycelia due to shear forces in SSF systems (Lonsane et al. 1992). Application of occasional rather than continuous agitation was found to be more appropriate to prevent damage to the mycelia and disruption of mycelial attachment to solids in certain cases.

pH is another important aspect in any fermentation process, and it may vary in response to metabolic activities. The most evident reason is the secretion of organic acids that will cause the pH to drop. On the other hand, the assimilation of organic acids, which may be present in some media, will lead to an increase in pH, and urea hydrolysis will result in alkalinization (Raimbault 1998). Each microorganism possesses a pH range for its growth and activity with an optimum value within the range. Filamentous fungi have reasonable growth over a broad range of pH 2–9, with an optimal range of 3.8–6.0. On the other hand, yeasts have a pH optimum between 4 and 5 and can grow in a large pH range of 2.5–8.5. This typical pH versatility of fungi can be beneficially exploited to prevent or minimize bacterial contamination, especially by choosing a lower pH. pH of the fermentation medium is important at two different times during the CA production. Firstly, the spores require a pH>5 in order to germinate. Secondly, the pH for citric acid production needs to be low (pH≤2). Low pH reduces the risk of contamination of the fermentation with other microorganisms. A low pH also inhibits the production of unwanted organic acids (gluconic acid, oxalic acid), and this makes the recovery of CA from the broth simpler (Levente and Christian 2003). The pH change can also occur according to the nitrogen source selected as well as the growth characteristics (Gowthaman et al. 2001). Use of urea as a nitrogen source rather than ammonium salts is one way of controlling the pH (Lonsane et al. 1992). An attempt to overcome the

problem of pH variability during SSF process, however, is obtained by substrate formulation considering the buffering capacity of the different components employed or by the use of buffer formulation with components that have no detrimental effects on the biological activity.

In order to hyper produce the actual product desired; the microorganisms must be cultivated under suboptimal conditions for biomass formation. Several advantages have been cited in the use of spores rather than vegetative cells for inoculum. According to Larroche (1996), spores can serve as a biocatalyst in bioconversion reactions as they are often able to carry out the same reactions as the corresponding mycelium. Other advantages include convenience, greater flexibility in the coordination of inoculum preparation, prolonged storability for subsequent use, and higher resistance to damage caused by mishandling during transfer (Gowthaman et al. 2001; Krishna and Nokes 2001). However, they do have a few disadvantages, such as longer lag time during growth, different optimal conditions for spore germination, and vegetative growth and larger inoculum size requirement. The fact that the spores are metabolically dormant hence requires that the metabolic activities must be induced and appropriate enzyme systems must be synthesized before the fungus begins to utilize the substrate and grow (Krishna and Nokes 2001). The size and form of mycelial pellets play a direct role in citric acid biosynthesis during submerged fermentation (Saha et al. 1999). Growth in the form of pellets of ≤1 mm in diameter was associated with high production rates and yields (Magnuson and Lasure 2004).

Particle Size

Particle size of the substrate is a critical factor as it is related to rheology of fermentation broth which determines the capacity of the system to interchange with microbial growth and heat and mass transfer during SSF process. Moreover, it affects the surface area-to-volume ratio of the particle, which determines the fraction of the substrate, which is initially available to the microorganism and the packing density within the surface mass (Krishna 1999). The substrate size determines the void space, which is occupied by air. Since the rate of oxygen transfer into the void space affects growth, the substrate should contain particles of suitable size to enhance mass transfer (Krishna 1999). Generally, smaller substrate particles would provide larger surface area for microbial action, but too

small particles may result in substrate agglomeration, which may interfere with microbial respiration/aeration and poor growth. Smaller particle size was also favorable for heat transfer and exchange of oxygen and carbon dioxide between the air and the solid surface. At the same time, larger particles also provided better respiration/aeration efficiency, but provided limited surface for microbial action (Pandey et al. 2000). Thus, an optimal average particle size of 0.6–2.0 mm is desired for high production of CA.

Other Factors

The effect of alcohols on CA production was first investigated by Moyer (1953). He observed that the addition of methanol enhanced the production of CA from commercial glucose and other crude carbohydrate substrates. Many researchers investigated the stimulating role of methanol or ethanol on CA yield (Haq et al. 2003; Sikander and Haq 2005; Rivas et al. 2008; Suzelle et al. 2009; Nadeem et al. 2010). The enhanced citric acid production with ethanol addition can be attributed to the slow degradation of CA due to reduction in aconitase activity. Addition of ethanol also resulted in the slight increase in the activity of other tricarboxylic acid (TCA) cycle enzymes. There is also a possibility that ethanol might be converted to the acetyl-CoA, a metabolic substrate required for CA formation. Methanol is not metabolized/assimilated by *A. niger*; its exact role in enhancing citric acid formation in not much clear. The addition of methanol might increase the permeability of cell to citrate. Usami and Taketomi (1961) studied that the methanol addition to the fermentation medium remarkably depressed the cellular protein synthesis without affecting nitrogen uptake, thus causing an increase in amino acids, peptides, and low molecular-weight protein pooled in the mycelium during the early stage of cultivation. In addition, methanol addition also slightly changed the activity of some TCA cycle enzymes favoring CA accumulation. The stimulatory effect of methanol on CA yield can be explained in terms of mycelia morphology as well as pellet shape and size (Srivasta and Kamal 1979). Methanol has a direct effect on mycelial morphology and it promotes pellet formation. It also increases the cell membrane permeability to provoke more citric acid excretion from the mycelial cells. They proposed mycelial morphology of filamentous fungi in the form of small round pellets (<3-mm diameter) for maximal acid production. It has been generally found that addition of methanol, ethanol, isopropanol, and methyl acetate was found to enhance CA production (Sato and Sudo 1999; Pandey et al. 2000).

Addition of vegetable oil also increases CA formation (Adham 2002; Sikander and Haq 2005). Fats and vegetable oils act as carbon sources and are degraded to glycerol and fatty acids. Glycerol enters directly in the TCA cycle by the formation of acetyl-CoA and fatty acids enters via glycolysis, as given in Fig. 2. Nowadays, worldwide biodiesel production has flooded the market with its byproduct, glycerol, and decreased the value of this valuable carbon source in a few years. Valorization of glycerol is sought in order to utilize this available substrate. Instead of old approaches to supplement the fermentation media with fatty acids to enhance the yield of CA, it is better to use glycerol directly to produce citric acid (Papanikolaou et al. 2002, 2003, 2009; Finogenova et al. 2005). Addition of inhibitors of metabolic pathways such as calcium fluoride, sodium fluoride, sodium arsenate, sodium malonate, sodium azide, potassium fluoride, and iodoacetic acid and mild oxidizing agents such as hydrogen peroxide, napthaquinone, and methyl blue to the fermentation medium was also found to increase citric acid accumulation (Bruchmann 1966; Adham et al. 2008). Most of these metabolic pathway inhibitors are found to enhance citric acid production at low levels. However, at higher concentrations, they completely inhibit fungal growth and resulted in a remarkably low yield of CA.

EFFECT OF SOME INDUCERS

Many researchers have tried to improve the production of citric acid by various additives besides enhancing the effect of lower alcohols and lipids, which are illustrated in Table 6. The studies showed that the addition of 4% (v/v) ram horn hydrolysate enhanced citric acid yield by 52%, reduced residual sugar concentration, and stimulated mycelial growth in the submerged fermentation with *A. niger* (Kurbanoglu and Kurbanoglu 2004). Various researchers demonstrated the effects of additives such as polyvinyl alcohol, polyethylene glycol, CMCase, serum and pluronic F68 as protectants (Kunas and Papourtsakis 1990; Michaels and Papoutsakis 1990, 1991; Rugsaseel et al. 1995). The addition of various inducers to fermentation medium is a common practice these days to enhance citric acid production. The stimulatory role of alcohols and other inducers on citric acid production can be explained in terms of the mycelium morphology of fungus as well as pellet shape and size, which is a vital condition for high CA production. High CA yields could be obtained by exploiting available resources and adding the stimulatory

agents to the fermentation medium. The mode of action of some inducers to enhance CA production is demonstrated in Fig. 2.

CA RECOVERY AND PURIFICATION

Recovery of CA from fermented broth entails classical and advanced methods, as presented in Fig. 3.

Precipitation

One of the conventional CA fermentation broth downstream processing technologies used in industry is precipitation as a calcium salt using calcium carbonate and sulfuric acid (Pazouki and Panda 1998). Despite the heating which incurs energy requirements, the precipitation process is only 90–95% complete, which has to be further explored (Annadurai et al. 1996). Karklin et al. (1984) separated CA from the fermentation broth by adjusting the pH between 6.1 and 7.5. The clarification was performed by treatment with activated carbon and kieselgur and mixed with $CaCl_2$, calcium acetate, or $Ca(OH)_2$ to precipitate CA. Calcium citrate was then separated by filtration under vacuum, washed with hot water, and dried at 90–105 °C. Optimum pH and temperature were 7–7.2 and 80 °C, respectively. This classical multistep, lime–H_2SO_4 precipitation process suffers from the use of large amounts of chemicals, which increases the production cost and generates considerable amount of an environmentally harmful waste (for 1×10^3 kg of CA production, approximately 30 m3 CO_2, 40×103 kg of wastewater, and 2×10^3 kg of gypsum are generated) (Jinglan et al. 2009).

The classical approach of CA production, despite being simple and yet long, has many drawbacks as mentioned before, the principal being the formation of by-product salts generating secondary pollution. At this juncture, there is a need to shorten the method and at the same time enhance CA recovery using some advanced techniques. In order to eliminate the generation of CO2 and gypsum as byproducts, many separation techniques, i.e., solvent extraction (Pazouki and Panda 1998), supercritical CO_2 extraction (Shishikura et al. 1992), electrodialysis (Pinto et al. 2002; Widiasa et al. 2004; Luo et al. 2004), and membrane separation (Friesen et al. 1991) have been studied as possible methods for CA recovery and purification.

Solvent Extraction

Although classical precipitation method is the most used technique in large-scale industrial processes, solvent extraction has also been developed for the separation of CA. Amine extraction has been found to be a promising method of separation of carboxylic or hydroxycarboxylic acid from aqueous solution. CA is readily extracted into a number of organic solvents, such as high-molecular-weight aliphatic amines (Pazouki and Panda 1998). Baniel and Gonen (1990) attained 90% recovery of CA with the solvent extraction method. Wenneresten (1980) studied the possibility of using solvent extraction technique for the recovery of citric acid from aqueous solution. Wenneresten (1980, 1983) found that tertiary amines were effective extractants for CA. The amine extraction method takes advantage of low pH (below the lowest pKa) of fermentation medium where CA is present in its protonated form. Due to high reactivity in this form, it forms a complex with weakly basic tertiary amines. The amine extraction has the advantage of consuming negligible amounts of mineral acids and bases and production of salt by-products over the conventional method. It helps relieve product inhibition and also eliminates the need to evaporate large quantities of water. The eco-friendly solvent extraction method with tertiary amines can be further refined for the technically feasible extraction of CA at pilot scale.

Adsorption

Generally, it is difficult to achieve high recovery and concentrated ratio using conventional separation techniques. The use of ion-exchange resins for organic acids and sugar recovery and purification have been studied by several authors. Juang and Chou (1996) studied the adsorption of CA from aqueous solution on macroporous resins (Amberlite XAD-4 and XAD-16) impregnated with tri-n-octylamine. Gluszcz et al. (2004) measured the adsorption properties of 18 types of different ion exchange resins for CA and lactic acid recovery from aqueous model solutions and found that the weakly basic resins possessed the highest adsorption capacity for the solutes studied. Takatsuji and Yoshida (1997, 1998) also investigated the adsorption mechanisms of three different organic acids, acetic acid, malic acid, and CA, on a commercial weakly basic resin, Diaion WA30 (Mitsubishi Chemical Co.). The adsorption isotherms of the organic acids were highly favorable and the adsorption capacity depended on the pH value of the solution. The fact that the fermentation broth is highly acidic in CA fermentation

processes can be explored for the feasible and environmental-friendly recovery of CA. The simultaneous fermentation and adsorption of CA from fermentation broth with weakly basic resins might also help avoid product inhibition and thus increase the production rate and sustain the cell viability.

The promising method of CA recovery from its fermentation broth using the simulated moving bed (SMB technology) is described in a few UOP (Universal Oil Products, Chicago, Palatine IL, USA) patents. Different types of non-specific commercially available ion-exchange resins have been used as adsorbents, and the eluent is either water–acetone mixture or dilute H_2SO_4 (Kulprathipanja 1990). Jinglan et al. (2009) has proposed an innovative CA refining process based on the SMB technology. A novel tailor-made tertiary PVP resin has been developed and used as an adsorbent (Peng 2002). This specific resin has a high selectivity to CA, while the other components (impurities) present in the fermentation broth are hardly retained. Moreover, the bonding energy between CA and the resin is not as strong as found in the existing commercial resins (Kulprathipanja 1990). Therefore, pure water can be used as an eluent. The SMB technology was developed by UOP in the early 1960s (Broughton and Gerhold 1961) and has emerged as a powerful continuous countercurrent chromatographic technique (Minceva and Rodrigues 2005). Despite the moderate success of these adsorption technologies, the process would be laden with some challenges, such as resin shelf-life, their disposal, and regeneration capacity.

In-situ Product Recovery

Many fermentation processes have low productivity and yield which may be due to product inhibition or hydrolysis of product by further catalytic reactions (Lye 1999). Thus, attempts to decrease this inhibition and degradation by optimizing physiological and technological parameters are fundamental in the development of successful biocatalytic processes. This can be accomplished by keeping the dissolved product concentration low in the fermentation medium. An approach that can accomplish this task is the implementation of an in situ product recovery (ISPR). This technique could be potentially applied in the fermentation process for the improved yield and productivity of organic acids such as CA. In principle, in situ recovery can be achieved using one possible ISPR technique, i.e., template induced crystallization (TIC). With TIC, templates are added to the fermentation solution as a specific surface upon which the solute preferably

crystallizes. The governing principles of TIC are not well understood and template selection criteria are lacking (Schügerl 2000; Alba-Perez 2001; Stark and Von 2003; Von and Vander-Wielen 2003; Schügerl and Hubbuch 2005). Urbanus et al. (2008) showed that the addition of TiO_2 and ZrO_2 templates decreased the induction times of cinnamic acid crystallization. TiO_2 and ZrO_2 therefore appeared effective for template-induced crystallization. Colloidal gold nanoparticles increased induction times and therefore were ineffective templates for TIC. This in situ process integration technique leads to the recovery processes where the product is removed from the fermentation medium as soon as it is formed. By this technique, the number of downstream processing steps as well as the generation of post-recovery waste residues are reduced (Buque-Taboda et al. 2006). This in situ crystallization of CA during fermentation could be explored as potential future technology to make the biosynthesis of CA feasible and economical on the industrial level. Moreover, *A. niger* strains are known to produce in situ nanoparticles (Kaur et al. 2004; Samuel 2005). In fact, this strategy can be used to cultivate in situ nanoparticles which can further act as a template improving the recovery process, adding novelty to the process.

APPLICATIONS OF CITRIC ACID

The demand of CA is escalating steadily due to its GRAS nature. The size of the citric acid market is continuously expanding, primarily because of its widespread use in food, pharmaceuticals, health-related consumer products, and advance biomedicine applications such as in nano biotechnology, tissue engineering, and drug delivery. CA is the most widely used for acidulation, antioxidant, chelation, and preservation of foods, beverages, pharmaceuticals, chemicals, cosmetics, and other products, as depicted in Table 7. The environmentally friendly nature of sodium citrate is a major factor in the use of citrates in the detergent industry. CA exhibits bacteriocidal and bactiostatic effects against *Listeria monocytogenes*, a food-borne pathogen capable of growth at refrigerated temperatures and in high-salt environments (Buchanan and Golden 1998; Bal'A and Marshall 1998). *L. monocytogenes* frequently contaminates post-process ready-to-eat meat products, including frankfurters (Nickelson and Schmidt 1999).

In recent years, citric acid is gaining worldwide attention as an innocuous molecule due to its wide range of properties such as biodegradability, biocompatibility, non-toxicity, being antibacterial, inexpensive, easily available, and highly versatile. There are reports

about the promising use of CA as a copolymer in nanomaterials to be used in nanomedicines. A study conducted by Ashkan et al. (2010) demonstrated the potential use of CA in the synthesis of linear-dendritic macromolecules of poly(citric acid)-block poly(ethylene glycol). These copolymers find use in nanomedicines as they will degrade back into individual molecules that can be metabolized by the body. Guillermo et al. (2010) has developed a nano-CA polymer for use as biocompatible scaffolds, which can be used to culture a variety of cells such as endothelial cells, ligament tissue, muscle cells, bone cells, cartilage cells, and drug delivery. The study conducted by Ivan et al. (2009) has demonstrated the potential use of CA in the synthesis of polyester elastomers. These CA-based elastic polyesters represent a new generation of advanced biocompatible and biodegradable synthetic materials with promising biomedical applications.

Prior to these works, many researchers have reported the scaffolds fabricated from elastic polyesters, in particular from poly(octanediol citrate) (POC) or poly(glycerol sebacate), which are found to be compatible for the regeneration of different tissues such as blood vessels (Yang et al. 2005; Gao et al. 2006), cartilage (Kang et al. 2006a), bone (Qiu et al. 2006), and nervous tissue (Sundback et al. 2005). POC-based scaffolds synthesized by the polyesterification reaction between 1, 8-octanediol and CA and fabricated by solvent-casting/particulate-leaching technique are permissive to *in vitro* chondrocyte proliferation and formation of extracellular matrix within the scaffold porous structure (Kang et al. 2006b). Currently, there are new applications of citric acid coming to light. Williams and Cloete (2010) demonstrated the potential use of CA in the removal of potassium from the lower quality iron ore concentrate. The processing of the lower quality iron ore helps solve the problem of scarcity of this valuable renewable resource and helps alleviate the problem of continuous depletion of iron ore deposits worldwide. In the future, citric acid could be employed for the chemical leaching of impurities from the natural ores to extract metals of interest. Thus, citric acid has a potentially very important role to play in hydrometallurgy, which is more and more focusing on eco-friendly biological techniques.

FUTURE PERSPECTIVES AND CHALLENGES

Increasing demand of citric acid in specialty applications has led to the ways to be explored for its efficient production. Solid-state fermentation is a way to increase the

productivity of citric acid. Appropriately, high citric acid-yielding strains need to be explored, and nowadays, there is a trend to manufacture *in situ* product, if possible. In fact, such innovative strategies can decrease the cost of production and recovery, saving the environment from harmful chemicals being used in conventional citric acid recovery methods. The use of agro-industrial wastes as a substrate for CA production certainly helps in the reduction of production costs associated with costly synthetic substrates. However, techno-economic studies should be carried out to check whether the overall production of CA is economical on the pilot scale, including the downstream processing step. Recovery and purification of end products are the major challenges that have been stimulating researchers to thrive hard to find the solutions. The integrated fermentation and product recovery methods have to be developed for the efficient recovery of products such as organic acids. *In situ* product recovery of citric acid during fermentation or tertiary amine extraction could be one of the possible alternatives, but still a lot has to be done to make this bioprocess industrially feasible. There is also a need of new technology innovations in bioreactor designs which could overcome the problems of scale-up in solid-state fermentation processes and also the online monitoring and control of several parameters.

CONCLUSIONS

The increase in production of citric acid is mainly attributed to the wide range of applications in different industrial sectors, such as chemical, pharmaceuticals, cosmetics, and food industries, as a versatile and safe alimentary additive. In order to meet the increasing demand of citric acid, there is an urgent need of cost-effective and environmentally sustainable production technology. A possible way to achieve this goal is either by the modification or replacement of the established but environmentally unsafe *A. niger* fermentation process by a process with increased citric acid yield and less impact on the environment. In this context, bio-utilization of a variety of agro-industrial wastes and their by-products by solid-state fermentation processes with the existing genetically engineered or new strains and the refinement of the present recovery processes of citric acid could be a promising way to achieve the highest rates of citric acid production. Further advancement in tertiary amine extraction method and in situ product recovery methods can make the overall citric acid production economically feasible.

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ABBREVIATIONS

AFEX- Ammonia fibre explosion; *A.niger- Asperigillus niger*; BCC- business communications co.; CA- citric acid; DP- degree of polymerization; ECM- extra cellular matrix; GRAS- generally recognized as safe; HMF- hydroxyl-methyl furfural; ISPR-in situ product recovery; LHW- liquid hot water; OD- 1,8 octanediol; ODC- ozone depleting chemicals; PEGDM- polyethylene glycol dimethyl ether; PGS- polyglycerol sebacate; POC—poly(1,8 octanediol-co-citric acid); [p(OCS)]- polyoctanediol citratesebacate; RHH- ram horn hydrolysate; SA- sebacic acid; SCO- single-cell oil; SF- surface fermentation; SMB- simulated moving bed; SmF- Submerged fermentation; SSF- Solid-state fermentation; TIC- template induced crystallization; TOA- tri-octylamine

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Table 2.1. 1 Citric acid producing microorganisms

Fungi:	*Aspergillus. niger, A. awamori, A. clavatus, A. nidulans, A. fonsecaeus, A. luchensis, A. phoenicus, A. wentii , A. saitoi, A. flavus, Absidia sp., Acremonium sp., Botrytis sp., Eupenicillium s., Mucor piriformis, Penicillium citrinum, P. janthinellu, P. luteum, P. restrictum Talaromyces sp,. Trichoderma viride, Ustulina vulgaris,*
Yeast:	*Candida tropicalis, C. catenula, C. guilliermondii, C. intermedia,Hansenula, Pichia, Debaromyces, Torula, Torulopsis, Kloekera, Saccharomyces, Zygosaccharomyces, Yarrowia lipolytica*
Bacteria:	*Arthrobacter paraffinens, Bacillus licheniformis, Corynebacterium ssp*

Table 2.1. 2 Effect of different fermentation by using alternate substrates on CA production

Type	Substrate	CA yield	Microorganism	Incubation temperature/ time	Remarks	References
Surface fermentation	Cotton waste	-	*A.niger* ATCC 9142	30°C/7 days	-	Hildergard et al., 1981
	Brewery waste	78.5[a]	*A.niger* ATCC 9142	30°C/14 days	-	Roukas et al., 1986, 87
	Turnip whey (supplemented with molasses)	27-46[a]	*A.niger* NCIM 595	28°C/9-12 days	High CA yield on 9 day	Chanda et al., 1990
	Sweet potato starch hydrolysate	45.9±4.2[e]	*A.niger* IIB-A6	30°C/ 264h	With addition of 200 ppm of $K_4Fe(CN)_6$ into the medium just before inoculation	Anwar et al., 2009
Submerged fermentation	Corn Cobs	603.5 ± 30.9[d]	*A.niger* NRRL 2001	30°C/72 h	Pre-treated with NaOH & Enzyme	Hang et al., 2001
	Black strap molasses	31.1±2 [e]	*A.niger* GCB 75	30°C/120 h	-	Haq et al., 2001
	Beet molasses	68.7	*Y. lipolytica* A-101	-	-	Lesniak et al., 2002
	Cane Molasses	114 g/l	*A.niger* GCMC 7	30°C/168 h	High yield after pre-treatment with potassium ferro-cyanide and H_2SO_4	Haq et al., 2004
	n-paraffin	40g/l[e]	*C.lipolytica* NRRL Y 1095	28±1°C/7 days	-	Crolla et al., 2004
	Soybean oil	115[c]	*C.lipolytica* N-5704	-	-	
	Coconut oil	99.6[c]	*C.lipolytica*	28°C/10 days	-	Soccol et al., 2006
	Palm oil	155[c]	*C.lipolytica* N-5704	-	-	William et al.,2007

Type	Substrate	CA yield	Microorganism	Incubation temperature/ time	Remarks	References
	Olive oil	112g/l[e]	*C.lipolytica* N-5704	-	-	
	Crude glycerol	119[c]	*Y.lipolytica* NRRL YB-423	10 days	-	Levinson et al., 2007
	Rapeseed oil	66.6 [e]	*Y.lipolytica* N 1	96h	-	Kamzolova et al., 2007
	Orange peel	53% [a]	*A. niger* CECT- 2090	30°C	4% MeOH	Rivas et al., 2008
	Olive mill waste water media	28.8g/l[e]	*Y. lipolytica*	28±1°C	-	Seraphim et al., 2008
	SPM + glucose	354g [d]	*A.niger* NRRL 567	-	Maximum yield with 19g phytate, 49g olive oil & 37g MeOH/kg dw	Suzelle et al., 2009
	Glycerol containing waste (Biodiesel Industry)	55.7%	*Y. lipolytica* A-101-1.22	158 h	-	Rymowicz et al., 2010
	Red seaweed (Gelidiella acerosa) + 10% sucrose	50 g/l	*A. niger*	30°C /10 days	-	Ramesh and Kalaiselvum, 2011
Solid-state fermentation	Sugarcane pressmud	79%	*A.niger* CFTRI 30	120 h	2% MeOH	Shankaranand et al., 1993
	Coffee husk	150g[d]	*A.niger* CFTRI 30	72 h	Supplemented with Fe, Cu and Zn	Shankaranand et al., 1994
	Pine apple waste	62.4[a]	*A.foetidus* ACM3996	4 days	70% mois. & 3% MeOH	Tran and Mitchell 1995
	Corn cobs	254g[d]	*A.niger*	72 hrs/30°C	-	Hang et al., 1998
	Pineapple peel	74%[a]	*A.niger* ACM 4992	30°C/4 days	65% mois., 3% (v/w) MeOH, 5ppm Fe^{2+}	Tran et al., 1998
	Carob pod	264g[d]	*A.niger* ATCC 9142	28-30°C/12 days	6% MeOH	Roukas, 1999
	Corn husk	259±10 g[d]	*A.niger* NRRL 2001	120 h/30°C	70 % mois.	Hang et al.,2000
	AP	124g[d]	*A.niger* BC-1	30°C/5 days	Mois. -78% (w/w), PS-0.6-1.18mm)	Shojaosadati et al., 2002
	Pine apple waste	51.4[a]	*A.niger* DS-1	30°C /8 days	High yield at 70% moist. & 4% MeOH	Kumar et al., 2003
	Moasmi waste	50[a]	*A.niger* DS-1	30°C /8 days	3% (v/w) MeOH	Kumar et al., 2003

Type	Substrate	CA yield	Microorganism	Incubation temperature/ time	Remarks	References
	Pine apple waste	46.4%	*A.niger* NRRL 328	6 days	54.8% mois.	Kuforiji et al., 2010
	Banana peels	~ 180[d]	*A.niger* MTCC 282	72h /28°C	70% mois.	Karthikeyan et al., 2010
	AP	46 g/kg	*A.niger* van. Tieghem MTCC	30°C /5 days	MeOH- 4% (v/w)	Kumar et al., 2010
	Pumpkin supplemented with Prescott salt	-2.86 mg/ml for *A. niger* 14/20 -2.7 mg/ml for *A. niger* 79/20.	*A. niger* 14/20 and 79/20 (14/20 and 79/20 are step mutants derived from the strain HB3 which is the step mutant from the wild type strain CA16	30°C /13 days	-	Majumder et al., 2010
	Cane molasses supplemented with Prescott salt	-4.88 mg/ml for *A. niger* 14/20 -4.75 mg/ml for *A. niger* 79/20	*A. niger* 14/20 and 79/20	30°C /13 days	-	Majumder et al., 2010
	Mixed medium (equal amounts of pumpkin and cane molasses + Prescott salt	-14.86 mg/ml for *A. niger* 14/20 -14.44 mg/ml for *A. niger* 79/20	*A. niger* 14/20 and 79/20	30°C /13 days	-	Majumder et al., 2010
	Pineapple waste + 15% (w/v) sucrose and ammonium nitrate (0.25% w/v)	60.61 g/kg	*Aspergillus niger* KS-7	30°C /5 days	MeOH- 2% (v/v) & Mois. 65%	Kareem et al., 2010
	Oil palm empty fruit bunches (EFB) +mineral solution	369.16 g/kg of dry EFB	*A. niger* IBO-103 MNB (IMI396649)	33.1°C/pH 6.5	MeOH- 2% Mois. **70.3% (v/w)**	Bari et al., 2010
	Industrial solid potato	32.68 g/kg	*A. niger* MAF 3	25°C /5 days	MeOH- 3% (v/w)	Afifi 2011

Type	Substrate	CA yield	Microorganism	Incubation temperature/ time	Remarks	References
	waste (SPW)	dry SPW				
	AP + Ammonium sulfate	159.14 g/kg	*A. niger* A	93.9 h	Mois. 80% w/w,	Hoseyini et al., 2011

Abbreviations: AP- apple pomace; MeOH- methanol; Mois. –moisture; PS-particle size

[a]based on sugar consumed; [b]based on total sugar; [c]based on fatty acids; [d]based on per kg dry mass of substrate; [e]based on g/l

Table 2.1. 3 Different pre-treatment technologies for lignocellulosic and other complex biomass for CA production

Pretreatment technology	Mechanism	Formation of inhibitor compounds	Effect on CA production	References
Mechanical				
1) Milling	Cutting the lignocellulosic biomass, causes the increase in specific surface area, reduction of DP and shearing of the biomass	-	Increases total hydrolysis of biomass & thus product yield	Chang et al., 2000; Delgenes et al., 2002
2)Extrusion	Performs many unit operations such as mixing, grinding, pressing, formulating, expansion, drying, sterilization & cooling among others	-	Solubilize various plant cell wall materials and increases CA yield	Jae-Kwan et al., 1998.
Thermal				
1)Heating	Heating of lignocellulosic biomass at 150-180°C, solubilization of hemicelluloses followed by lignin.	Above 180°C, the solubilization of lignin forms phenolic & heterocyclic compounds such as vanillin, vanillin alcohol & furfurals & HMF	Phenolic compounds have toxic effects on bacteria, yeast & others, can recondensate & precipitate on biomass sometimes along with furfurals and HMF and effect CA yield	Negro et al., 2003; Ramos, 2003
2)Hot water	Used to solubilize mainly the hemicelluloses to make the cellulose more accessible at pH4-7	Lower risk of inhibitor formation	Observed 2-5 fold increase in enzymatic hydrolysis and thus CA yield	Mosier et al., 2005; Hendricks et al., 2009
3)Steam/steam explosion	Biomass is treated in large vessels at high temperature, up to 240°C & pressure for few minutes. After the set time the steam is released & biomass is cooled down quickly	Risk of formation of inhibitory compounds such as furfurals, HMF & soluble phenolic compounds	80-100% hemicelluloses hydrolysis, increasing accessibility of cellulose for enzymes	Laser et al., 2002
Chemical				
1)Alkali	Treatment with different conc. of	Furfurals	Causes swelling of the biomass,	Laser et al., 2002;

Pretreatment technology	Mechanism	Formation of inhibitor compounds	Effect on CA production	References
Mechanical				
	alkali, such as NaOH, KOH, NH_4 and lime etc.		making it more accessible for enzymatic reactions	
2)Acid	Hydrolysis of Starchy & lignocellulosics at different acid (H_2SO_4, HCl, HNO_3) conc.	Furfurals, HMF, and other volatile products at higher acid concentrations	Increases accessibility of cellulose fraction	Liu et al., 2003; Ramos, 2003; Taherzadeh et al., 2007
3)Oxidative	Treatment of biomass with oxidizing agent, such as H_2O_2 or peracetic acid in water	High risk of formation of inhibitory compounds in case oxidant is not selective	Increases enzymatic hydrolysis of cellulose	Hon et al., 2001;
4)Ammonia	Biomass treated with ammonia (equal ratio) at 90-120°C for several minutes	No inhibitor formation	Swelling of cellulose, delignification, 6-fold increase in enzymatic hydrolysis	Sun et al., 2002; Alizadeh et al., 2005; Kim et al., 2005
5)CO_2 explosion	Explosive steam pre-treatment with high pressure CO_2	No inhibitor formation	Cellulose conversion >75%	Sun et al., 2002
Biological	-Lignocellulosic biomass treated with cellulases, hemicellulases, ligninases, lignin peroxidases, polyphenoloxidases, laccase & quinine-reducing enzymes	-	Increases overall CA yield	Sun et al., 2002; Jun et al., 2009
	-Starchy feed stocks treated with a-amylases & amyloglucosidases		Increases CA yield many fold	Tengerdy et al., 2003

Abbreviations: CA- citric acid; HMF- hydroxymethyl furfurals

Table 2.1. 4 Advantages and disadvantages of different types of fermentations with their effects on CA production

Type	Advantages	Disadvantages	CA Production	References
SF	Less effort in operation, installation & energy cost	Very few surface micro-organisms, Large amount of heat generation, time consuming and needs large area/space, sensitive to contamination by Penicillia, other Asperigilli, yeasts and lactic acid bacteria	Used in small/ medium scale industries	Soccol et al., 2003
SmF	Have sophisticated control mechanisms, lower labor cost, higher productivity and yield	High cost media, sensitive to trace metal inhibition, Risk of contamination, High amount of post recovery waste water generation	80 % of the world CA production,	Vanderberghe et al., 1999
SSF	Simple technology, Higher yield, Wide range of low cost media which resembles the natural habitat for several micro-organisms, Better oxygen circulation, Less susceptible to trace elements inhibition, lower energy and cost requirements, Low risk of bacterial contamination, Less amount of post recovery waste	Difficulties in scale up, difficult control of process parameters such as pH, moisture, temperature, nutrients etc, rapid determination of microbial growth, Higher impurity products thus higher recovery product costs	Higher CA yields, economically efficient & industrially feasible process due to efficient utilization and value addition of wastes,	Gowethaman et al., 2001;Durand et al., 2003; Robinson et al., 2003 ; Holker et al., 2004; Susana et al., 2006

Table 2.1. 5 Macro and micro nutrients required for CA production

Type of Nutrients	Type	Required concentration	Effect on CA production	References
Macronutrients				
Sugars	Sucrose(most preferred), glucose, lactose, maltose, mannose, galactose	Initial concentration 14-22%	Positive	Gupta et al., 1976; Hossain et al., 1984; Xu et al., 1989
Nitrogen	Urea, ammonium chloride/ sulphate/ tartarate, oxalate, sulfate, nitrate, chloride, sodium nitrate, peptones, yeast/malt extract amino acids	0.1 to 0.4 g/l	CA yield directly influenced by the nitrogen source, high levels leads to biomass production and decrease in pH	Xu et al., 1989; Larroche, 1996; Rohr et al., 1996; Chundakkadu, 2005, Soccol et al., 2006
Micronutrients				
Trace Elements	Zn^{2+}	0.3 ppm (low levels)	Enhanced CA yield	Sato et al., 1999; Pandey et al., 2000
	Mn^{2+}	Low levels	Reduces CA production under otherwise optimized conditions	Rohr et al., 1996
	Fe^{2+}	1.3 ppm	Optimal concentration	Haq et al., 2002
	Cu^{2+}	0.1–500 ppm	Counter-act the deleterious effect of iron in molasses fermentation by SmF and enhanced CA yield	Sato et al., 1999; Pandey et al., 2000
	Mg^{2+}	0.02- 0.025%	Essential for growth as well as CA production	Soccol et al., 2006
	Phosphorous (preferred source is Potassium dihydrogen phosphate)	0.5-5.0g/l	Low levels favour CA production	Soccol et al., 2006
Lower Alcohols	Methanol, ethanol, iso-propanol, methyl acetate, *n*-propanol	1-4% (v/w)	Enhance CA yield	Sikander et al., 2005; Nadeem et al., 2010
Other compounds	Na^{+}, Ca^{++}, Ni^{+}, , K^{+}, Mo^{++}, boron, organic compounds, such as thiamine/ biotin/ folic acid, thiamine/ biotin and steroids	Low concentrations	Enhanced sporulation	Sato et al., 1999; Pandey et al., 2000

Table 2.1. 6 Effect of different inducers on CA yield

Inducer	Substrate	Micro-organism	Inducer concentration	Incubation time	CA yield: Ctrl/ inducer	Reference
Lower alcohols						
MeOH	Galactose	*A.Carbonarius* IMI41873	1% (v/v)	3 days	4%/ 25%/ galactose utilized	Maddox et al., 1986
	Galactose	*A.niger* ATCC 12846	1% (v/v)	3 days	0.3%/31%	Maddox et al., 1986
	Galactose	*A.niger* ATCC 26036	1% (v/v)	3 days	0.4%/44%	Maddox et al., 1986
	Galactose	*A.niger* ATCC 26550	1% (v/v)	3 days	0.1%/27%	Maddox et al., 1986
	Galactose	*A.niger* IMI 83856	1% (v/v)	3 days	1.5%/20%	Maddox et al., 1986
	Galactose	*A.niger* MH 15-15	1% (v/v)		-/21%	Maddox et al., 1986
	Date syrup	*A.niger* ATCC 9142	4% (v/v)		55/90±1.5 g/l	Roukas, et al., 1997
	Pine apple waste	*A.niger* ACM 4992	3% (v/w)	4 days	67%/ kg sugar consumed	Tran et al., 1998
	Cane molasses	*A.niger* GCB-47	1% (v/v)	24 h	(90:02±2:2 g/l)	Haq et al., 2003
	AP	*A.niger* NRRL 567	3-4%	5 days	77-88%	Hang et al., 1986
EtOH	SB	*A.niger* MNNG115	3% (v/w)		30.1g/kg to 200g/kg	Sikander et al., 2005
Sodium Phytate	Sucrose medium	*A.niger* W1-2	1%	6 days	1.1-1.7 fold increase	Jianlong et al., 1997
Phytate	Beet molasses	*A.niger* W1-2	10g/l	-	3.1 fold increase	Jianlong, 1998
Potassium ferrocyanide	Sugarcane pressmud	*A.niger* CFTRI 30	80ppm	96 h	79% to 88%	Shankaranand et al., 1993

Inducer	Substrate	Micro-organism	Inducer concentration	Incubation time	CA yield: Ctrl/ inducer	Reference
Lower alcohols						
Fluoro-acetate	SB	*A.niger* MNNG 115	10^{-2} M (added after 6 h of fermentation)	72 h	30.1g/kg to 183g/kg	Sikander et al., 2005
Groundnut oil	SB	*A.niger* MNNG115	3% (v/w)	48 h	48-104 g/kg	Sikander et al., 2005
Mustard oil	SB	*A.niger* MNNG115	3% (v/w)	48 h	48-85 g/kg	
Oleic oil	SB	*A.niger* MNNG115	3% (v/w)	48 h	48-186 g/kg	
Coconut oil	SB	*A.niger* MNNG115	3% (v/w)	48 h	48 to 224g/kg	
Olive oil	Beet molasses	*A.niger* A-20	4% (v/v)	12 days	20.3-49.8%	Adham, 2002
Sunflower oil	Beet Molasses	*A.niger* A-20	4% (v/v)		39.0%	
Maize oil	Beet molasses	*A.niger* A-20	4% (v/v)		40.3%	
Viscous substances						
Gelatin	Glucose (Shake culture)	*A.niger* Yang no.2	6 mg/ml	9 days	15.4-53.3 mg/ml	Rugsaseel et al., 1995
Agar			5mg/ml	9 days	47.3 mg/ml	
Carrageenan			5mg/ml	9 days	52mg/ml	
CMC			2.5 mg/ml	9 days	54.7mg/ml	
PEG- 6000			2.5 mg/ml	9 days	39.2mg/ml	

Abbreviations: CMC- carboxymethyl cellulose; EtOH- ethanol; MeOH- methanol; PEG- poly ethylene glycol; SB- sugarcane baggase

Table 2.1. 7 Different applications of CA

Field of application	Industry	Uses	Required form	References
Conventional				
1) Food	Beverages (Wines /Juices/Soft drinks/Syrups)	As an acidulant, used in carbonated and non-carbonated beverages for flavoring and buffering, used in dry powder beverages for flavor and pH control.	Moderate	BalÀ et al.,1998
	Confectionary (Jams/jellies /Candies)	Antioxidant, increase effectiveness of anti-microbial preservatives, control sugar inversion, provides tartness and enhance flavor, control the product pH for optimum gelation	Moderate	
	Dairy products	Sodium citrate is used in creamers to stabilize the casein, prevents feathering of the creamers when added to hot beverages, sodium citrate functions as an emulsifying salt to stabilize the water & oil phases of the cheese and improves body and texture,	Moderate	
	Canning industry	Acts as chelating agent, helps preserve the natural color and prevent discoloration in canned mushrooms, kidney beans and canned corn. Acts as an antioxidant synergist and anti-microbial agent that can be applied to the surfaces of cured meat products prior to packaging.	Moderate	Christopher et al., 2003
	Frozen fruits/vegetables	Along with ascorbic acid, inhibits enzymatic and trace metal-catalyzed oxidation reactions which can cause color and flavor deterioration	Moderate	
	Oils	Used in canola oil de gumming in the deodorization and hydrogenation steps in oil processing to chelate trace metals which can catalyze rancidity reactions.	Moderate	
	Sea-food	Prevent discoloration & the development of off-odors and flavors by chelating trace metals	Moderate	
	Meat industry	Sodium citrate is used in slaughter houses to prevent the coagulation or clotting of fresh blood.	Moderate	
	Blood banks and other formulations	Used as an anticoagulant, as effervescent combined with bicarbonates or carbonates in antacids and dentrifices, as a flavoring & stabilizing agent in liquid suspensions and solutions, imparts a desirable tart taste	High	

Field of application	Industry	Uses	Required form	References
		that helps mask medicinal flavors, calcium citrate is used as a dietary		Soccol etal.,
2)Pharma-ceutical		calcium supplement		2006
	Detergents/ Cosmetics/ Toiletries	Added to hair care formulations, cosmetics, detergents to adjust the pH, as buffering agent & chelate metal ions to prevent discoloration & stabilizes the formulation.	Moderate	
	Animal feed	CA increases solubilty, easily digestible chelates of essential metal nutrients, enhance response to antibiotics, enhance flavor, to control gastric pH and improve the efficiency of the feed, used in pet food as flavor enhancer	Moderate	
3)Agriculture		Chelates Fe, Cu, Mg & Zn, is used to correct soil deficiencies, enhance	Moderate	
	Fertilizers	phosphorous availability in plants.		
	Soil fertility	Removal of lead from contaminated soils		
	Buffering/	Used to chelate copper in formulations used to kill algae in reservoirs	Moderate	
	chelating agent – algicide, water softening	and natural waters. CA is mixed in the salt to chelate iron from fouled water softener resins.	Moderate	Dilara et al., 2007
	Textiles	In textile finishing, CA is used to adjust pH, as a buffer & as a chelating	Low /Moderate	
4)Other Industrial applications		agent in dye operations and in durable-press finishes using glyoxal resins.		Soccol et al., 2006
	Cigarettes	Flavoring agent	Moderate	
		Ammoniated CA is used to clean metal oxides from the steam boilers,	Moderate	Robin et al.,
	Metal cleaning	Cleaning of iron and copper oxides, utilized in nuclear reactors to remove mill scale from welding operations.		1995
		Used to retard the setting of titanium dioxide, the most common	Moderate	
	Paint	pigment used in paints and other coatings.		
		Copper electroplating, as a chelating agent to control the deposition	Moderate	
	Electroplating	rate of metals in electroplating		
		CA solutions are used to remove iron, calcium and other cations which	High	
	Water purification systems	foul the cellulose acetate membranes used in reverse-osmosis systems		
	Military	Utilized in removal of post soldering flux residues	Moderate	

Field of application	Industry	Uses	Required form	References
	applications Others	Used in non-toxic, non-corrosive and biodegradable processes such as waste treatment. Leather tanning, printing inks, photographic reagents, concrete, plaster, floor cement, adhesives, polymers, controls paper staining etc.	Low/Moderate	
Advanced applications				
1)Biomedicines	Nanomedicines Tissue engineering	Used as copolymer in nanomaterials which can encapsulates small biologically active molecules, can be used for self-healing polymers & self-care products, & as biocompatible scaffolds, used to culture variety of cells, drug delivery. Used to make polyester elastomers having potential use in tissue engineering	High	Ashkan et al., 2010; Guillermo et al., 2010 Ivan et al., 2009; Yang et al., 2004
2)Wood preservation	Water-based wood preservative	Protects wood against fungi, insects and marine borers, very effective against difficult to treat wood species such as Douglas-fir	Moderate	Forest products lab. US Dept. of agriculture.
3)Processing of lower quality Iron ore	Iron ore mines	Used for the leaching of potassium from the iron ore concentrate	Moderate	Williams et al., 2010

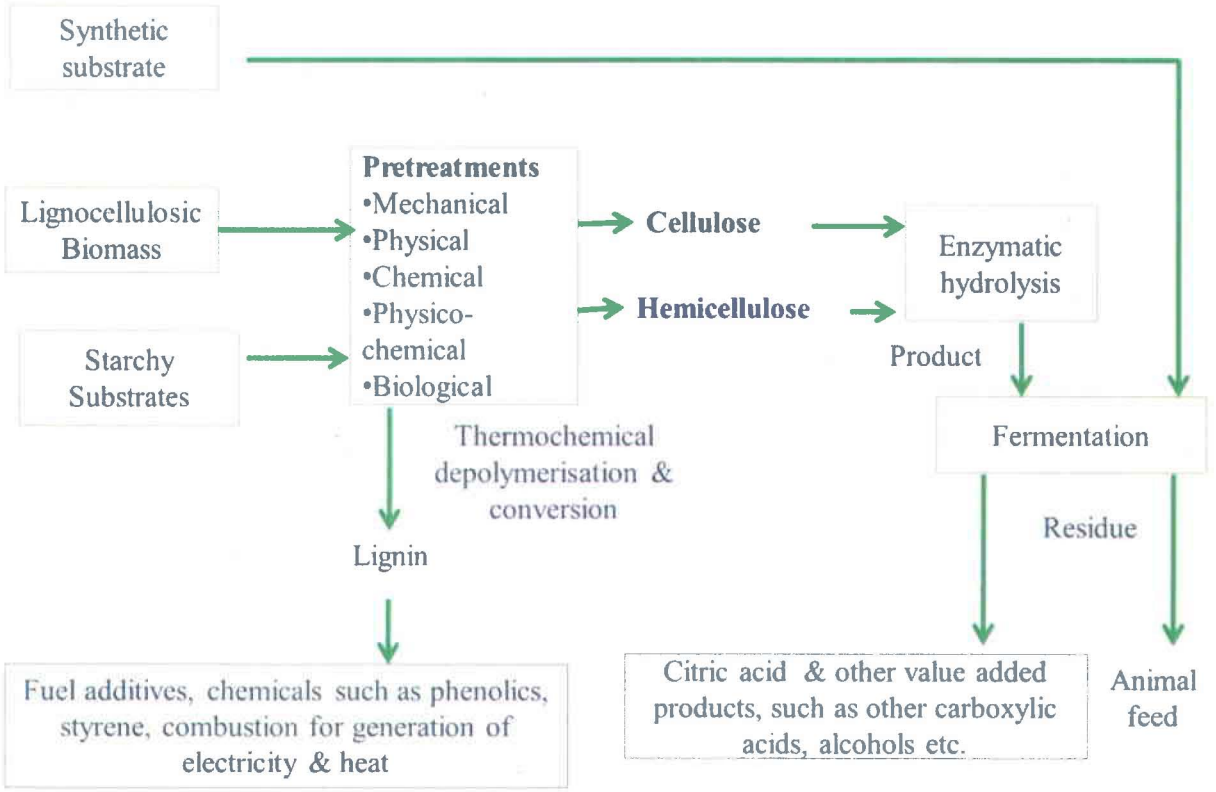


Figure 2.1. 1 Flowchart showing the entire CA production process from different substrates

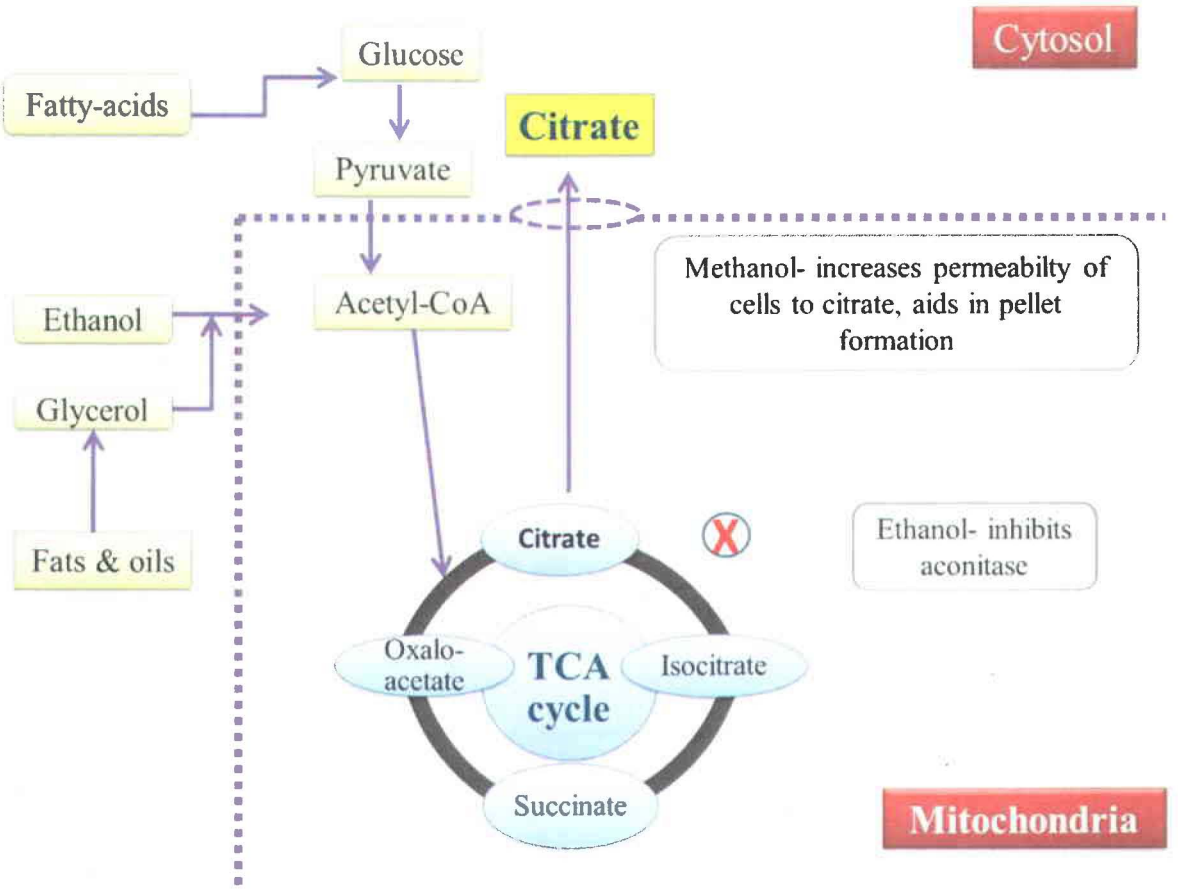


Figure 2.1. 2 Effect of different inducers on CA production

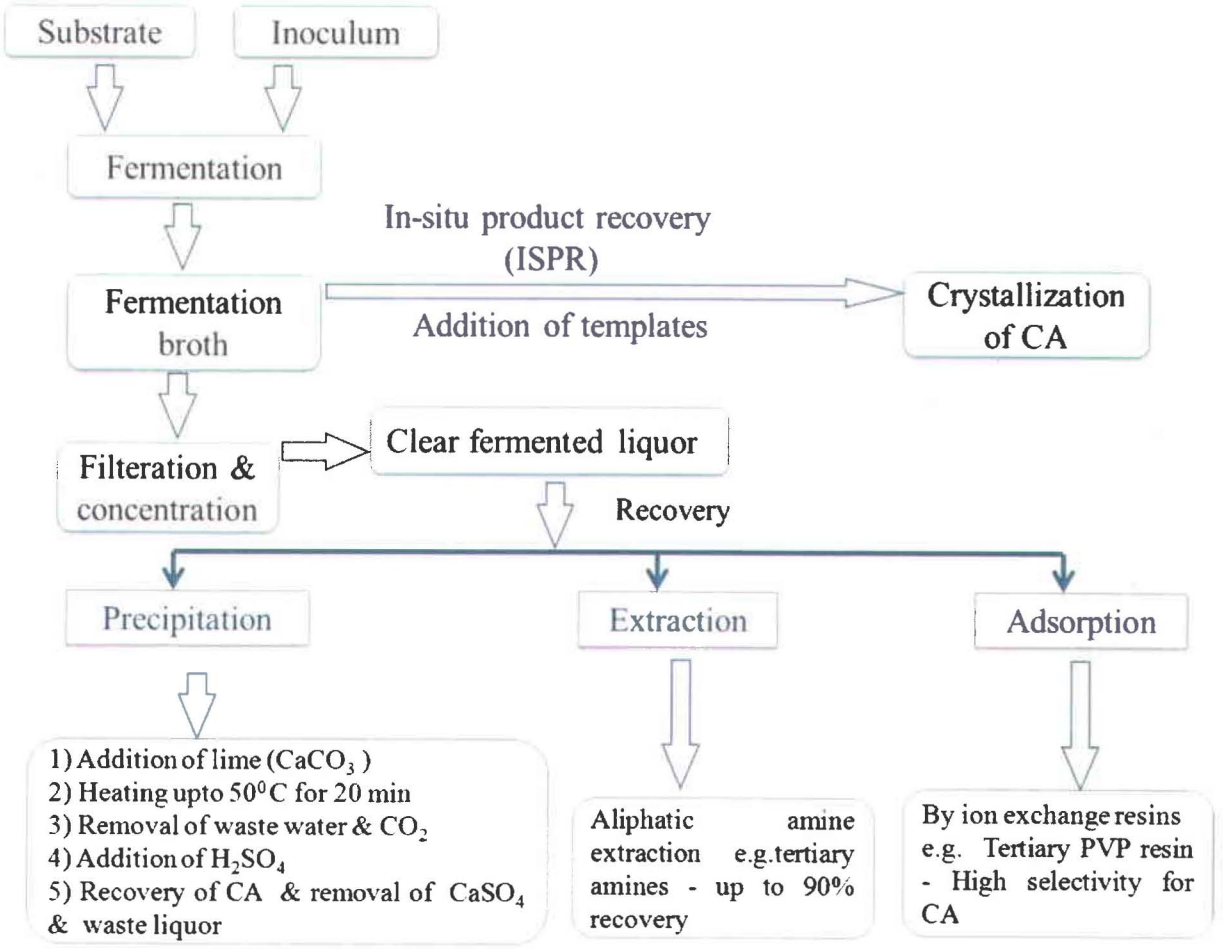


Figure 2.1. 3 Schematic for citric acid recovery by conventional and proposed method

UTILIZATION OF DIFFERENT AGRO-INDUSTRIAL WASTES FOR SUSTAINABLE BIOPRODUCTION OF CITRIC ACID BY ASPERGILLUS NIGER

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RÉSUMÉ

Une revue détaillée de la littérature a démontré la nécessité de sélectionner des substrats à faible coût et de développer des conditions de fermentation optimum pour la production d'AC. La fermentation Koji a été reconnue comme étant un système de fermentation relativement peu coûteux et approprié pour la production d'AC à l'aide des sous-produits agro-industriels. La production d'AC par *A. niger* est fortement influencée par la composition du milieu, notamment du carbone, de l'azote et d'autres micronutriments essentiels. La nature et les constituants des substrats affectent également la production d'acide citrique fongique. Par conséquent, des études ont été menées pour examiner le potentiel des substrats solide et liquide à haut potentiel pour la production d'AC par *A. niger* NRRL 567 et NRRL 2001. En utilisant les meilleurs substrats, les effets des inducteurs EtOH et MeOH (% 3-4), sur la production d'acide citrique ont été optimisés grâce à une stratégie classique, dans le but de se rapprocher de leur valeur optimale pour une production d'AC plus élevée. La production d'AC a été réalisée par FSS et FSm à l'aide de différents déchets agro-industriels, tels que les déchets solides (AP, BSG, CW et SPM), les déchets liquides (APS-1, APS-2, LS, MSS, MSS (hydrolysé) et SIW. Parmi tous les supports solides utilisés, AP à 66,0 ± 1,9 g/kg de substrat sec s'est avéré être un excellent substrat pour la production d'acide citrique par *A. niger* 567 NRRL à 72 h d'incubation. *A. niger* NRRL 2001 a entraîné une concentration légèrement plus faible d'AC de 61,0 ± 1,9 g /kg de substrat sec au moment même de l'incubation. APS-1 (boues d'ultrafiltration de jus de pomme -1) a donné le taux de production d'AC le plus haut : de 9,0 ± 0,3 g/l et 8,9 ± 0,3 g/l de substrat, respectivement, par *A. niger* 567 NRRL 2001 par fermentation à l'état liquide. Les substrats étudiés (AP et APS) ont été complétés avec EtOH et MeOH (3-4%) afin de stimuler les souches d'*A. niger* pour une production plus élevée d'AC. L'ajout de 3% (v/p) d'éthanol et de 4% (v/p) de méthanol aux déchets solides de jus de pomme a donné des valeurs d'acide citrique significativement plus élevées de 127,9 ± 4,3 g / kg et 115,8 ± 3,8 g / kg de substrat sec en utilisant *A. niger* NRRL 567 par fermentation en milieu solide. Des valeurs plus élevées d'AC de 18,2 ± 0,4 g / l et de 13,9 ± 0,4 g / l de l'APS-1 ont été obtenues après l'addition de 3% (v / v) d'éthanol et 4% (v / v) de méthanol, respectivement, par *A. niger* NRRL 567.

ABSTRACT

In view of ever growing demand of citric acid, there is an urgent need to look for inexpensive and novel substrates for feasible production of citric acid. In this context, the present study was carried out to evaluate the potential of different agro-industrial wastes for hyper production of citric acid through solid-state and submerged fermentation by *Aspergillus niger* NRRL 567 and NRRL 2001. It was found that among all the solid substrates utilized, apple pomace with 66.0±1.9 g/kg of dry substrate proved to be an excellent substrate for citric acid production by *A. niger* NRRL 567 at 72 h of incubation. *A. niger* NRRL 2001 resulted in slightly lower citric acid concentration of 61.0±1.9 g/kg of dry substrate at the same incubation time. APS-1 (apple pomace ultrafiltration sludge-1) gave highest citric acid production rate of 9.0±0.3 g/l and 8.9±0.3 g/l of substrate by *A. niger* NRRL 567 and NRRL 2001 by submerged fermentation, respectively. Further study with apple pomace and apple pomace ultrafiltration sludge-1 by *A. niger* NRRL 567 was carried out. Addition of 3% (v/w) ethanol and 4% (v/w) methanol to apple pomace gave significantly higher citric acid values of 127.9±4.3 g/kg and 115.8±3.8 g/kg of dry substrate by *A. niger* NRRL 567 by solid-state fermentation. Higher citric acid values of 18.2±0.4 g/l and 13.9±0.4 g/l of apple pomace ultrafiltration sludge-1 were attained after addition of 3% (v/v) ethanol and 4% (v/v) methanol, respectively by *A. niger* NRRL 567. Apple pomace solid waste and apple pomace ultrafiltration sludge-1 thus proved to be an excellent source for citric acid production, of the different substrates chosen.

Keywords: Agro-industrial waste; *Aspergillus niger*; Citric acid; Solid-state fermentation; Submerged fermentation

1. INTRODUCTION

Citric acid is one of the most versatile and important carboxylic acid intermediate of metabolism in most plants and animals. Due to its innocuous nature, citric acid is extensively used in food preparations, pharmaceuticals and cosmetics. About 70% citric acid is used in food industry and remaining 30% in other industries [1]. Due to their chelating property and powerful sequestering action with various transient metals, such as iron, copper, nickel, cobalt, chromium and magnesium citric acid is used for the phytoremediation of heavy metals from the contaminated soils and sediments [2, 3]. Nowadays, citric acid is also increasingly utilized as a monomer for the manufacture of biodegradable polymers which are widely used in various medical applications [4–7]. Due to its numerous applications and it is generally recognized as safe (GRAS) nature, global production of citric acid has reached 1.7 million tonnes per year as estimated by Business Communications Co. (BCC, http://www.bccresearch.com) and is increasing at annual growth rate of 5% due to the possibility of expanding utilization of citric acid in biomedicine, biopolymer production and various other applications. Considering high consumption rate and slight increase in price, the market value for this commodity chemical is expected to exceed $2 billion in 2009 [8]. Recent prices of citric acid have been around $1–$1.3 per kilo [9]. The ever-increasing demand for citric acid mandates the use of alternative fermentation processes using inexpensive raw materials, such as agro-industrial wastes through solid state fermentation. Large-scale production of citric acid has been carried out solely by the filamentous fungus *Aspergillus niger* in submerged fermentation on beet or cane molasses, sucrose or glucose syrup. In recent years, solid state fermentation process has gained global attention as an alternative to submerged fermentation [9]. The use of agro-industrial wastes in solid state fermentation is economically important and can minimize various environmental problems. A cost reduction in citric acid production can be achieved by using less expensive raw materials. Apple pomace solid waste (AP) and apple pomace ultrafiltration sludge (APS) for example, would be particularly interesting raw materials and low-cost carbon substrate for the production of citrates by *A. niger*. *A. niger* is well established and grows efficiently on different fruit pomace wastes giving higher citric acid yields of 120–264 g/kg dry substrate [1].

In the recent years, apple pomace has been used for the production of organic acids, such as citric acid, protein-enriched feeds, edible mushrooms, ethanol, aroma

compounds, natural antioxidants and various enzymes, among others through solid state fermentation [10, 11]. A single isolated study reported solid-state citric acid production using apple pomace, but the yield was relatively lower (124 g/kg dry AP) [10] and apple pomace ultrafiltration sludge has not been utilized so far for value addition. However, owing to high carbohydrate content present in apple pomace, citric acid yield can be further improved possibly by careful optimization of process parameters. Brewery spent grain has been used for lactic acid production, polyphenol extraction, enzymes production, mushrooms and actinobacteria cultivation [12, 13]. El-Holi and Al-Delaimy [14] and El-Aasar [15] utilized lactoserum for the submerged fermentation of citric acid by *A. niger* achieving low yield. Citrus pulp and peels are also used for the production of citric acid, albeit at low amount of 57.6% [16]. Owing to the high carbohydrates present in the above potential wastes and possibility of getting higher citric acid concentration, present study was taken. As per the published literature so far to the best of our knowledge, there is no study reported on the citric acid bio-production using apple pomace ultrafiltration sludge, brewery spent grain, starch industry water and municipal secondary sludge.

In Canada, waste residues and by-products derived from fruit processing industry, beer production and starch industries are abundant sources of less expensive organic matter, which were utilized in the present study to investigate their suitability for citric acid production. This study comprised solid-state fermentation (SSF) to test the production of citric acid by *A. niger* strains NRRL 567 and NRRL 2001, while using different agro-industrial wastes, such as apple pomace solid waste (AP), citrus waste (CW), brewery spent grain (BSG) and sphagnum peat moss (SPM). The study also investigated submerged citric acid fermentation using liquid wastes, such as APS-1 (apple pomace ultrafiltration sludge-1), APS-2 (apple pomace ultrafiltration sludge-2), lactoserum (LS), municipal secondary sludge (MSS) and starch industry water (SIW).

2. MATERIALS AND METHODS

2.1. Microorganisms

Aspergillus niger (NRRL 567 and NRRL 2001) was procured from Agricultural Research Services (ARS) culture collection, IL, USA. These microbial strains were selected due to their citric acid hyper production capacity. The strains were obtained from ARS and

cultivated as freeze-dried form and were revived onto potato dextrose broth medium (PDB) for 7–8 days at 30±1 ◦C and 200 rpm. The microorganisms were maintained on potato dextrose agar (PDA) plates for 96 h at 30±1 ◦C and stored at 4±1 ◦C in a refrigerator for future use. The cultures were renewed every four weeks.

2.2. Spore suspension

The spores were harvested from the PDA plates by using 0.1% (v/v) Tween-80 solution after incubating for 5–6 days. The harvested spores were initially filtered through glass wool to remove mycelial contamination and recover only the spores. Spores were counted by a haemocytometer to adjust the count approximately to 1×108 spores/ml and stored in test tubes at −20 ◦C for maximum of 4 weeks.

2.3. Solid-state fermentation (SSF)

2.3.1. Substrate procurement and treatment

Different agro-industrial biomass, such as AP (from Lassonde Inc., Rougemont, Montreal, Canada), BSG (from LA BARBERIE, Quebec, Canada),CWand SPM were used as solid substrates to evaluate their suitability for the production of citric acid. SPM was supplemented with nutrient solution per kg dry peat moss (DPM): 250 g glucose, 15.4 g $(NH_4)2SO_4$, 43.9 g KH_2PO_4 and 4.0 g NaCl and maintained to desired moisture content with additional amount of distilled water. The physico-chemical characterization of the solid substrates is given in Table 2.2.1 (APHA, AWWA, WPCF, 2005). The most suitable solid substrate was used for further experimental study for hyper production of citric acid using optimized conditions.

2.3.2. SSF fermentation

The moisture of the substrates was analyzed using a moisture analyzer (HR-83 Halogen, Mettler Toledo, Switzerland). Fifty grams of solid substrate in a 500 ml Erlenmeyer flask were thoroughly mixed and autoclaved at 121±1 ◦C for 45 min. The substrates were inoculated with spore suspension having 1×107 spores/g dry substrate (gds) and initial moisture was adjusted to 75% (v/w). After thorough mixing, Erlenmeyer flasks and their contents were incubated in an environmental chamber at 30±1 ◦C and 75% relative humidity for 6 days. Unless specified otherwise, these fermentation

conditions were maintained throughout the study. All the experiments were conducted in duplicates.

2.4. Submerged fermentation (SmF)

2.4.1. Substrate procurement and treatment

Different agro-industrial wastes, such as APS-1 and APS-2 (from Lassonde Inc., Rougemont, Montreal, Canada), SIW (from ADM Ogive; Candiac, Quebec, Canada), MSS (from Kruger Wayagamack Inc., Trois-Rivieres, Quebec, Canada) and LS (from Saputo Inc., Montreal, Quebec, Canada) were selected as fermentation medium for their potential to be used for citric acid production. The apple pomace ultrafiltration sludge is the liquid waste which is obtained after the ultrafiltration of crude juice. The apples are mashed in the initial step of processing and the solid waste is separated, and the crude juice obtained is again filtered through ultrafiltration devices twice. In LS the initial carbohydrate concentration was adjusted to 200 g/l. In one treatment, MSS was hydrolyzed by thermo-alkaline pre-treatment. The concentrated sludge (35 g/l suspended solids concentration) was adjusted to pH 10.0±0.1 by 10M NaOH solution. Thermo-alkaline pre-treatment was conducted at 100rpm stirrer speed and 140 ◦C at 30 bar pressure for 30 min in a microwave digestion system (Perkin-Elmer, Montreal, Canada). The aim of thermo-alkaline pretreatment was to make the sugars accessible for the growth of fungus. The physico-chemical characterization of the liquid wastes is provided in Table 2.2.2 (APHA, AWWA,WPCF, 2005).

2.4.2. SmF fermentation

One hundred fifty milliliter of liquid substrate was dispensed in a 500 ml Erlenmeyer flask and thoroughly mixed and autoclaved at 121±1 ◦C for 30 min. The substrates were inoculated with spore suspension of 1×107 spores/25 ml. After thorough mixing, Erlenmeyer flasks and their contents were incubated in a shaker at 30±1 ◦C at 200 rpm. Unless specified otherwise, these fermentation conditions were maintained throughout the study. All the experiments were conducted in duplicates.

2.5. Sampling details

For the SSF, 3 g of a wet sample was harvested from each flask after every 24 h in aseptic conditions and dispensed in 15 ml sterilized distilled water. The extraction of citric acid was carried out by incubating the samples in an incubator shaker for 60 min at 180 rpm at 25 ◦C. The supernatant was filtered through glass wool for removal of solid substrate and fungal mycelia. Similarly for SmF, 5ml samples at regular intervals of 24 h were collected under aseptic conditions from each flask and were filtered through glass wool for removal of solid particles and fungal mycelia. About 100 µl sample was taken in Eppendorf tubes from both SSF and SmF for viability of fungus (total spore count) using haemocytometer, the remaining sample was centrifuged (Sorvall RC 5C plus by Equi-Lab Inc. Quebec, Canada) at 9000×g for 20 min and the supernatant was analyzed for citric acid concentration. In SmF, the changes in pH and viscosity were also analyzed initially and at the end of experiment to obtain an understanding of the morphological changes occurring in the medium during fermentation.

2.6. Citric acid analysis

Citric acid concentrations were determined spectrophotometrically by the modified method of Marier and Boulet [17]. Appropriately diluted 1ml samples of SSF and SmF each were taken in a test tube and 1.3 ml pyridine was added. The contents were mixed manually by swirling and 5.7 ml acetic anhydride was added to the test tubes. The contents were again mixed by swirling and immediately placed in a constant-temperature (22 ◦C) water bath and incubated for 30 min. The optimal density was recorded at 420 nm with the blank set on 100% transmission. Citric acid concentration was determined by referring to a standard curve for citric acid concentration. Citric acid concentrations were expressed as grams per kilogram (g/kg) of dry weight for solid substrate fermentation and grams per liter (g/l) for submerged fermentation.

2.7. Statistical analysis

Data means and standard deviation were analyzed with individual Student's t-test to distinguish differences among treatments. Data were statistically analyzed by using the multiple analyses of variance (ANOVA) by STATGRAPHICS Centurion XV, version 15.1.02. The tests were performed at the level of $p < 0.05$ to determine the significance of the difference between the treatments and the strains to produce citric acid.

3. RESULTS AND DISCUSSION

3.1. Screening of solid substrates for SSF

Citric acid production using different solid wastes by *A. niger* NRRL 567 and *A. niger* NRRL 2001 is given in Table 2.2.3 A. Significant amount of citric acid can be produced by growing *A. niger* on solid fermentation substrates, such as AP, CW, and BSG. Higher citric acid production of 65.6±1.9 g/kg and 59.3±1.8 g/kg dry substrate was achieved with AP followed by CW by *A. niger* NRRL 567 in 72 h incubation period. However, the highest values for citric acid concentration of 61±1.9 g/kg and 63.6±2.9 g/kg dry substrate were observed with AP and CW as solid substrate by *A. niger* NRRL 2001, respectively. Similarly, the citric acid production of 14.0±1.2 g/kg, 32±1.8 g/kg and 11.8±0.7 g/kg, 24.6±1.3 g/kg dry substrate were attained when BSG and SPM were utilized as solid substrates with *A. niger* NRRL 567 and NRRL 2001, respectively. SPM can retain 15–20 times its own weight in water and has a low bulk density even when wet, allowing for a high porosity and good air diffusion even under moist conditions [2]. The variation in citric acid production with different substrates was due to the difference in the complexity of substrates used, the microbial strain and the optimum conditions for fermentation.

The viability of fungus during solid-state citric acid fermentation is provided in Fig. 2.2.1A and B. As the principal objective of the study was citric acid production, the viability was analyzed only up to incubation period where maximum citric acid was achieved. In the beginning of fermentation, the initial spore count of *A. niger* NRRL 567 and *A. niger* NRRL 2001 decreased till 24 h in AP and BSG, mainly due to the active growth of fungus in the beginning and then it started increasing due to proper adaptation of fungus. Similarly, in CW and SPM, the spore count of *A. niger* NRRL 567 and *A. niger* NRRL 2001 decreased until 72 h and then started increasing. The viability varied with the cultivation medium. The maximum viability for *A. niger* NRRL 567 was 1.6×108, 4×108, 5.5×106 and 6.5×106 colony forming units/g (cfu/g) and for *A. niger* NRRL 2001 were 5.8×107, 3×108, 7.5×106 and 7.0×106 cfu/g when cultivated on AP, BSG, CW and SPM, respectively. The viability assays correlated well with citric acid production during course of fermentation.

The results of the screening experiment confirmed the possibility of utilization of AP as potential substrate for further experiments (fermentation by *A. niger* NRRL 567) for

optimization of growth and citric acid production parameters. Owing to the high total carbon concentration (128 g/kg) present in apple pomace as seen in (Table 2.2.1), citric acid concentration was expected to increase with the optimized parameters and inducer supplementation.

3.2. Submerged citric acid fermentation

Table 2.2.3 B demonstrates the results of citric acid submerged fermentation using different liquid substrates by *A. niger* NRRL567 and NRRL 2001. Higher citric acid production of 9.0±0.3 g/l of substrate was observed when *A. niger* NRRL 567 was grown on APS-1and higher value of 8.9±0.3 g/l was achieved with *A. niger* NRRL 2001 using APS-1. APS-2 and LS resulted in 8.3±0.2 g/l, 6.5±0.2 g/l and 8.2±0.3 g/l, 6.8±0.3 g/l citric acid by using *A. niger* NRRL 567 and *A. niger* NRRL 2001, respectively. El-Aasar [15], achieved 4.6 g/l, 24.7 g/l and 27.1 g/l citric acid from LS, LS+ crude cane molasses and whey+ crude beet molasses respectively. Similarly, El-Holi and Al-Delaimy [14], achieved 2.4 g/l, 47.5 g/l, 54.2 g/l, 59.7 g/l, and 57.8 g/l with LS, LS+ 15% sucrose, LS+ 15% glucose, LS+ 15% fructose and LS+ 15% galactose respectively. Further, enhanced yield of 92.4 g/l was observed with the addition of 1% (v/v) methanol to LS+ 15% sucrose. The low CA production from LS alone was due to the presence of galactose moiety in the disaccharide lactose. *A. niger* was not able to readily utilize galactose. The nature of sugar source has been found to have marked effect on citric acid production [18,19]. The preferred sugars for *A. niger* are sucrose followed by glucose, fructose and maltose [20]. Due to its relatively low molecular weight, sucrose is readily transported into microbial cells and is hydrolyzed by intracellular enzymes [21]. The presence of either galactose or its metabolic products causes inhibition of citric acid production and reduction in the rate of glucose utilization. Galactose interferes with the glucose repression of key enzyme, 2-oxoglutarate dehydrogenase. Moddax et al. [22] demonstrated that there is a strong relationship between citric acid production and activities of 2-oxoglutarate dehydrogenase and pyruvate dehydrogenase enzymes in cell free extracts.

The viability of fungus during submerged citric acid fermentation is shown in Fig. 2.2.2 A and B. The initial spore count decreased until 72 h due to the growth phase and then increased until 144 h after depletion of the substrates. Drastic increase in spore formation was observed after 96 h due to nutrients depletion. The maximum viability for

A. niger NRRL 567 in APS-1, APS-2, MSS, SIW and LS were 1.7×106, 1.8×106, 1.9×10^6, 2.0×10^6 and 2.1×10^6 cfu/ml, respectively and for *A. niger* NRRL 2001 were 1.8×10^6, 2.1×10^6, 1.9×10^6, 2.2×10^6 and 2.4×10^6 cfu/ml, respectively. The main objective of this study was citric acid production, so the viability of fungus was not measured after 144 h. Surprisingly, SIW and MSS which contained approximately 25.0 g/l and 70.0 g/l total carbon were not able to induce citric acid production by both strains. Lower citric acid production of 0.19±0.04 g/l and 0.42±0.04 g/l was observed using SIW and MSS by *A. niger* NRRL 567. Citric acid concentration of 0.24±0.02 g/l and 0.34±0.02 g/l was attained using SIW and MSS by *A. niger* NRRL 2001. Even *A. niger* NRRL 567 was not able to grow in the hydrolyzed municipal secondary sludge with thermo-alkaline pretreatment and gave only 0.40±0.03 g/l of citric acid. The aim of this pretreatment was to make the sugars accessible for the growth of fungus. Inability of *A. niger* to grow was due to the high concentrations of Fe^{2+}, Cu^{2+} and other metal ions present in SIW, MSS and hydrolyzed MSS as evident from Table 2. These metal ions profoundly inhibit citric acid production by *A. niger* strains in submerged fermentation Study conducted by Mirminachi et al. [23] showed that iron concentration values above 200 g/l resulted in significant reduction in citric acid productivity. In view of the screening results, APS-1 was further selected for the submerged citric acid fermentation by *A. niger* NRRL 567 for optimization of growth and citric acid production parameters.

3.3. Effect of inducers on citric acid concentration

The effect of lower alcohols on citric acid solid-state and submerged fermentation is shown in Fig. 2.2.3 A and B. The stimulatory effect of lower alcohols, such as methanol (MeOH) and ethanol (EtOH) permits their application in the industrial production of citric acid. The increase in citric acid concentration was observed during solid-state and submerged fermentation. Addition of inducers to the solid substrates enhanced the citric acid concentrations. Supplementation with 3% (v/w) EtOH and 4% (v/w) MeOH increased citric acid production gradually during fermentation period which reached its highest values of 127.9±4.3 g/kg and 115.8±3.9 g/kg dry apple pomace by *A. niger* NRRL 567 at 96 h as seen in Fig. 2.2.3 A. The citric acid concentration decreased after 96 h incubation time which might be due to four principal reasons: inhibitory effect of high citric acid accumulation in the medium; decay in enzyme system responsible for citric acid biosynthesis; depletion of available sugar content and decrease in amount of

nitrogen content available in fermentation medium [25,26]. In order to observe the combined effect of the two inducers, MeOH and EtOH, 2% (v/w) was added in one treatment, but the concentration of citric acid achieved was less than as compared to the treatments in which ethanol or methanol was added singly. In fact, literature studies have reported the beneficial effects of the inducers when added singly. Tran et al. [27] obtained higher citric acid concentration with 3% MeOH by solid-state fermentation of pineapple waste. The increased citric acid production from citric pulp by *A. niger* was observed with the addition of 4% MeOH [28]. They observed the drastic increase in citric acid production from 261.2 g/kg to 380.9 g/kg of citric pulp. These results are in concordance with Shojaosadati and Babaeipour [10] who reported citric acid concentration of 124 g/kg dry AP in a multi-layer packed bed solid-state fermenter. Moreover, in this study, nearly 128 g citric acid/kg dry substrate was achieved at flask level; thus further increase in citric acid is expected during scale-up of the process to fermenter level.

Similarly, in SmF, the citric acid concentration was found to be best with the addition of 4% (v/v) MeOH and 3% (v/v) EtOH to APS-1 which was selected from the screening studies based on citric acid production. The inducers were added to the fermentation medium after sterilization. Addition of 3% (v/v) EtOH and 4% (v/v) MeOH to the APS-1 resulted in citric acid concentration of 18.2±0.4 g/l and 13.9±0.4 g/l whichwas1-fold and 0.55-fold higher than the control as evident from Fig. 2.2.3 B. Less citric acid concentration was observed in the treatment having combined MeOH and EtOH, each at 2% (v/v). Rivas et al. [29] observed higher citric acid production with 4% MeOH during submerged fermentation on orange peel autohydrolysate. Ali and Ikram-ul-Haq [30] observed increase in citric acid production on sugarcane bagasse supplemented with MeOH 2% (v/w) and EtOH 3% (v/w). EtOH supplementation of 3% (v/w) resulted in highest citric acid yield in SSF and SmF. Further, increasing the ethanol concentration to 4% (v/w) resulted in decreased citric acid production which might be due to the fact that higher ethanol concentration in the medium disturbed the fungal morphology and metabolism.

3.4. Effect of inducers on fungal morphology

Fig. 2.2.4 A and B shows viability of *A. niger* NRRL 567 cultivated on solid substrates, such as AP and SPM and submerged fermentation of APS-1. The initial spore count

decreased till 48 h due to the growth of spores and then increased till 144 h in all the treatments except AP with 4% MeOH due to the depletion of the substrates during solid state fermentation. Higher colony forming units were observed in AP and SPM controls as compared to other treatments supplemented with inducers. The maximum viabilities for *A. niger* NRRL 567 cultivated on SPM-MeOH 4% and AP-MeOH+ EtOH were 3.5×106 and 3.4×106 cfu/g as compared to AP control and SPM control which were 7.6×107and 5.0×107 cfu/g, respectively. As compared to treatment without inducers, very little sporulation was observed in the treatments with inducers which was mainly due to the fact that addition of alcohols is known to reduce mycelial growth, inhibit sporulation and increase the fermentation efficiency [24]. The initial particle size of substrate also has effect on the viability of fungus. The colony forming units were higher in control during screening and the control used in the solid state fermentation with optimized parameters having particle size 1.7–2.0 mm as shown in Figs. 1A and B and 4A. Table 4A and B represents the change in viscosity and pH during submerged citric acid fermentation. An increase in viscosity during the course of fermentation was observed with different substrates during screening studies. The increase in viscosity of the fermentation medium was due to the mycelium growth and metabolism of fungus. However, the addition of MeOH and EtOH resulted in the decrease in viscosity as compared to control in which viscosity was higher during the course of incubation. The viscosity at 144 h was found to be lower than the control which was mainly due to the pellet formation and reduced mycelial growth due to the effect of alcohols. The morphology of fungus is considered as an important parameter which influences the physical characteristics of the fermentation medium which in turn can have an effect on final yield of citric acid. The rheological behaviour of fungus was closely associated to the morphology and biomass concentration [1]. The broth rheology also determined the transport phenomena in bioreactors and final concentration of citric acid produced. Less viscous non-Newtonian broths are usually indicative of pellet form of fungus in which the mycelium develops very stable spherical aggregates consisting of more dense, branched, and partially intertwined network of hyphae. The pellet form is highly recommended for high yield of citric acid during submerged fermentation [1].

In this context, the present study also reflected the role of broth rheology. Higher citric acid production was achieved with APS-1 which contained lower total solids (115.0±5.0 g/l) than APS-2 (135.0±5.0 g/l). The initial total solids concentration can be further optimized for high citric acid production from apple pomace ultrafiltration sludge. The

stimulating effect of EtOH on citric acid fermentation suggested that ethanol could be used as a carbon source by the fungus *A. niger* and was converted into citric acid via TCA cycle. Ethanol also increased the permeability of the cell membrane and thus higher secretion of citric acid [31, 32]. Similarly, MeOH is well known for depressing the synthesis of cell wall proteins in the early stages of cultivation [33]. MeOH also acts as an inducer for the activity of enzyme citrate synthase [34]. MeOH and EtOH also markedly increased the tolerance levels of trace metals, such as manganese, iron and zinc far above the required levels for inoculum growth. The addition of alcohols thus permitted utilization of crude carbohydrate sources, such as agro-industrial waste residues and by-products for citric acid production [33].

5. PRELIMINARY ECONOMICAL EVALUATION OF CA PRODUCTION

Every year thousands of tons of apple pomace and apple sludge are generated worldwide. AP is mainly used as a feed component, a low value-added application whereas APS did not find any potential application and cause environmental nuisance. The industries incur losses due to treatment of waste and transportation costs for dumping into landfills. In this context, citric acid production from nutrient rich wastes, such as AP and APS is lucrative alternative for apple processing industries. In 2006–2007, the global production of apples was 46.1 million tons with China alone contributing more than 50% (24.5 million tons) [35]. Out of the total apple production 70–75% of the apple is consumed fresh, while 25–30% of the fruit is processed into juice, cider, and frozen and dried processed products. Almost 64% of the total of all processed apple products is apple juice and concentrate throughout the entire world. Global apple juice production was estimated at 1.4 million metric tons during 2004–2005. The total recovery of the juice in industrial production process is about 70–75%, generating 25–30% apple pomace and 5–11% sludge i.e. liquid waste obtained after clarification and sedimentation with bentonite clay as summarized in Fig. 2.2.5 [11, 35]. This sludge contains up to 10% apple juice and bentonite particles which are added during clarification of the juice. There seems to be a direct loss of about 10% juice, which can be recovered easily by adoption of efficient processing techniques. Moreover, this is a time consuming process. In some industries, instead of bentonite clarification,

ultrafiltration process is applied for efficient recovery of juice, which generates around 10% sludge (approximately having 115–135 g/l total solids).

Taking into consideration the annual production of apples during the year 2006–2007, a total of 7.4–8.8 million tons of apples were processed for juice concentrate worldwide. On average, 1.8–2.7 million tons of apple pomace and 0.7–0.9 million tonnes of sludge are generated. The apple pomace being rich in carbohydrates and other vital nutrients and having high moisture content (70–75%) and biodegradable organic load (high BOD and COD values), makes it highly susceptible to microbial attack. The direct disposal of this agro-industrial residue as a waste has negative impact on environment and represents an important loss of biomass. It could be used as a potential substrate for bio-production of citric acid, having a high demand these days. According to our results, higher citric acid concentration of 128 g/kg of dry apple pomace was achieved. Approximately 339.2 thousand metric tons citric acid will be produced which will cost around 441 million dollars (current price of citric acid being $1.3) from apple pomace. Similarly, 18.2 g citric acid/l of sludge was attained. From the total sludge produced during processing of apples, 161 hundred metric tons citric acid can be produced which will contribute to an output value of 20.9 million dollars. Further, these values will increase on scale-up of these studies further enhancing the cost-efficiency. Thus, resource utilization of wastes for citric acid production will have significant social, economic and environmental impacts. Subsequent fermenter studies are in progress in our laboratory which will lead to the detailed calculation of cost-economics of the process.

6. CONCLUSIONS

The results obtained in the present study suggested that both apple pomace solid waste and apple pomace ultrafiltration sludge, the by-products from apple processing industry were promising substrates for citric acid production through submerged and solid state fermentation by *A. niger* NRRL 567. The following conclusions can be drawn:

(1) Citric acid concentration of 66.0±1.9 g/kg and 59.3±1.9 g/kg dry substrate was achieved using apple pomace followed by citrus waste by *A. niger* NRRL 567 and 61±1.9 g/kg and 63.6±2.9 g/kg dry substrate was attained with apple pomace and citrus waste as solid substrate by *A. niger* NRRL 2001 respectively in 72 h incubation period.

(2) Submerged citric acid fermentation resulted in higher citric acid concentration of 9.0 g/l and 8.9 g/l of apple pomace ultrafiltration sludge-1 by *A. niger* NRRL 567 and *A. niger* NRRL 2001, which was higher than other substrates used in this study.

(3) Addition of 3% (v/v) EtOH and 4% (v/v) MeOH to apple pomace during solid-state citric acid production resulted in increased citric acid concentration up to 90% and 60% g/kg dry apple pomace as compared to apple pomace control by *A. niger* NRRL 567. Similarly in submerged fermentation using apple pomace ultrafiltration sludge-1 addition of 3% (v/v) EtOH and 4% (v/v) MeOH resulted in approximately 100% and 55% g/l increase in citric acid production by *A. niger* NRRL 567 as compared to apple pomace ultrafiltration sludge control.

(4) The spore viability results correlated well with citric acid production by solid-state and submerged fermentation. According to preliminary economical validity study, approximately 355.3 thousand metric tons citric acid will be produced from apple pomace and apple pomace ultrafiltration sludge. Further these values will increase on scale up of these studies. Thus, resource utilization of apple industry wastes for citric acid bioproduction will have significant social, economic and environmental impacts.

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LIST OF ABBREVIATIONS

AP	apple pomace
APS-1	apple pomace sludge with 45±5 g/l suspended solids
APS-2	apple pomace sludge with 80 g/l suspended solids
BSG	brewery spent grain
cfu	colony forming units
CW	citrus waste
MeOH	Methanol
MSS	municipal secondary sludge
SIW	starch industry water
SmF	submerged fermentation
SPM	sphagnum peat moss

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Table 2.2. 1 Composition of different solid waste substrates

Constituents	Composition (Dry weight basis)			
	AP[a]	BSG[b,c,d]	CW[e]	SPM[f]
Moisture (% wet basis)	70.5[a]	65.4[a]	77.9[a]	-
Suspended solids (g/l)	202±1.3	-	-	-
Total solids (g/kg)	256±1.3	-	-	-
Total carbon (C_t) (g/kg)	127.9	-	-	53.1
Total nitrogen (N_t) (g/kg)	6.8	-	-	-
Protein (%)	2.9-5.7	15.2-24.0	12.2±0.7	-
Lipids	-	10.6	-	-
Cellulose (W/dry weight)	7.2	13.1-25.4	8.8±0.7	-
Hemicellulose (W/dry weight)	-	28.4-29.96	4.4±0.55	-
Lignin (W/dry weight)	23.5	11.9-27.8	3.7±0.1	-
Total carbohydrates (%)	48.0-62.0	-	-	-
Fibre (%)	4.70-51.10	-	-	-
Ash (%)	0.5-6.1	2.4-5.8	5.9±0.3	2.8
Reducing sugars (%)	10.8-15.0	-	-	-
Glucose	22.7	21.5	4.6±0.1	-
Fructose	23.6	-	9.8±0.2	-
Sucrose	1.8	-	3.9±0.2	-
Xylose	0.1	17.6	1.0±0.1	-
Galactose	-	1.3	-1.4±0.	-
Minerals				-
Phosphorous (%)	0.07-0.076	-	-	0.01
Potassium (%)	0.4-1.0	-	-	0.15
Calcium (%)	0.06-0.1	-	-	-
Magnesium (%)	0.02-0.36	-	-	-
Copper (mg/kg)	1.1	-	-	-
Zinc (mg/kg)	15.0	-	-	-
Manganese (mg/kg)	3.96-9.0	-	-	-
Iron (mg/kg)	31.8-38.3	-	-	-

Citation sources: [b] [12]; [c] [35]; [d] [37]; [e] [36]; [f] [2] ; **Data** with superscript [a] was analyzed during this study

Table 2.2. 2 Composition of different liquid waste substrates (concentration mg/kg unless stated)

Components	APS-1[a]	APS-2[a]	MSS[a]	MSS (Hydrolysed)[b]	SIW[c]	LS
pH±0.1	3.3±0.1[a]	3.4±0.1	5.5±0.1[a]	8.5±0.1	3.4±0.1[a]	5.9±0.1[a]
Total solids (TS) (g/l)	115±5.0	135±5.0	26.5±1.5[a]	53.5±0.1	16±1.1 a	-
Total volatile solids (TVS) (g/l)	38±2.4	48±2.9	18±0.3	28.4±0.1	14.4±0.1a	-
Suspended solids (SS) (g/l)	41.5±2.0	46.84±2.4	19±1.1[a]	44±0.23	2.3±0.11a	-
Volatile suspended solids (VSS) (g/l)	-	-	7.2±0.7[a]	2.3±0.4	2.3±0.4a	-
Total carbon (C_t)	44.3 g/l	51.9 g/l	253.7±3.5[a]	418977±785	31402±5346	-
Total nitrogen (N_t)	2.2 g/l	2.9 g/l	35.9±43.6[a]	39989±2211	37880±4567	-
Total phosphorous (P_t)			6.963±324[a]	15702±1734	8001±467	-
Carbohydrates (g/l)	56.2±1.3	66±1.7	-	-	-	76.5% (Lactose 65% minimum)
Lipids (g/l)	5.1±0.2	5.9±0.3	-	-	-	0.2%
Protein (g/l)	28.8±1.2	33.8±2.0	-	-	-	12.3%
Micronutrients (mg/kg)						
Al	259.4±5.2	304.6±17	4999 ±437[a]	13978±2522	58797±1422	-
Ca	912.2±58	1070.8±0.67	14011 ±511[a]	23697±336	11987±126	6870
Cd	0.02±0.01	0.023±0	3.01 ±0.9[a]	0.3±0.1	-	-
Cr	0.57±0.03	0.670±0.02	27.9 ±1.1[a]	71±23.6	1.0±0.06	-
Cu	13.4±1.2	15.731.6	401 ±157[a]	300±161	345.7 ±159.5	-
Fe	333.1±45	391.1±56	11987 ±603[a]	8061.9±758.6	2188±1318.6	9.2
K	6825.3±30	8012.3±296	998 ±371[a]	23.3±4.2	23578 ±3432	-
Pb	0.3±0.02	0.35±0.03	27 ±4.3[a]	4.1±1.9	2.7 ±1.1	-
S	2200±128	2582.6±157	4369 ±538[a]	5978±332.3	22301 ±61.7	-
Zn	18.1±2.5	21.25±2.3	325 ±197[a]	395.8±100.2	241 ±86.2	-
Na	405.2±3	417±45	1456 ±408[a]	15287±1021	2203.5 ±225	60 g/kg

Citation souces: [b] [38] [c] [39]; Data with superscript [a] was analyzed during this study

Table 2.2. 3 Citric acid production during (A) solid-state and; (B) submerged fermentation of agro-industrial wastes

(A) **Treatment**	**Citric acid production at 72 h (g/kg of dS) by *A.niger* NRRL-567**	**Citric acid production at 72 h (g/kg of ds) by *A.niger* NRRL-2001**
AP	65.6±1.9	61.1±1.9
(BSG)	14.0±1.2	11.8±0.7
CW	59.3±1.8	63.6±2.9
SPM	31.6±1.	24.6±1.3
(B) **Treatment**	**CA production at 96 h (g/L of substrate) by *A.niger* NRRL-567**	**CA production at 96 h (g/L of substrate) by *A.niger* NRRL-2001**
APS-1	9.0±0.3	8.9±0.3
APS-2	8.3±0.2	8.2±0.3
SIW	0.19±0.043	0.2±0.02
LS	6.53±0.2	6.9±0.3
MSS	0.4±0.04	0.3±0.02
MSS (Hydrolyzed)	0.4±0.03	-

All the experiments are performed in duplicate values are mean ± standard deviation.

Abbreviations: AP-apple pomace; BSG-brewery spent grain; CW- citrus waste; SPM-sphagnum peat moss; APS 1- apple pomace ultrafiltration sludge; APS 2- apple pomace ultrafiltration sludge; SIW-starch industry water; LS-lactoserum; MSS- municipal secondary sludge

Table 2.2. 4 Change in different parameters in SmF during (A) screening of different wastes by *A. niger* NRRL-567 and *A. niger* NRRL-2001 (B) *A. niger* NRRL-567 with APS-1

A) Substrate type	pH		Viscosity (cP)	
	Initial(0 h)	Final (144 h)	Initial (0 h)	Final (144 h)
APS-1 *A. niger* NRRL-567	3.3±0.1	3.1±0.1	3.4	32.0
A. niger NRRL-2000	-	3.2±0.1		74.8
APS-2 *A. niger* NRRL-567	3.4±0.1	3.2±0.1	14.4	243.9
A. niger NRRL-2000	-	3.1±0.1		214
SIW *A. niger* NRRL-567	3.4±0.1	7.5±0.1	1.5	71.6
A. niger NRRL-2000	-	5.4±0.1		56.0
LS *A.niger*NRRL-567	5.9±0.1	5.2±0.1	2.8	188.0
A. niger NRRL-2000	-	4.8±0.1		120.0
MSS *A. niger* NRRL-567	5.5±0.1	8.0±0.1	1.4	44.0
A. niger NRRL-2000	-	4.8±0.1		34.0
MSS (Hydrolysed) *A. niger* NRRL 567	3.5±0.1	8.2±0.1	2.8	11.9

B) Substrate type	pH		Viscosity	
	Initial(0 h)	Final (168 h)	Initial (0 h)	Final (168 h)
APS-1 (control)	3.5±0.1	3.2±0.1	3.7	22.0
APS-1 (Met. 3%)	3.5±0.1	2.7±0.1	3.7	4.0
APS-1 (Met. 4%)	3.5±0.1	2.9±0.1	3.7	3.8
APS-1 (Eth. 3%)	3.5±0.1	2.7±0.1	3.7	4.2
APS-1 (Eth. 4%)	3.5±0.1	2.8±0.1	3.7	4.0

A) Substrate type	pH		Viscosity (cP)	
	Initial(0 h)	Final (144 h)	Initial (0 h)	Final (144 h)
APS-1 (Eth. + Met.)	3.5±0.1	3.4±0.1	3.7	3.2

Abbreviations: APS 1- apple pomace ultrafiltration sludge; APS 2- apple pomace ultrafiltration sludge; SIW-starch industry water; LS-lactoserum; MSS- municipal secondary sludge

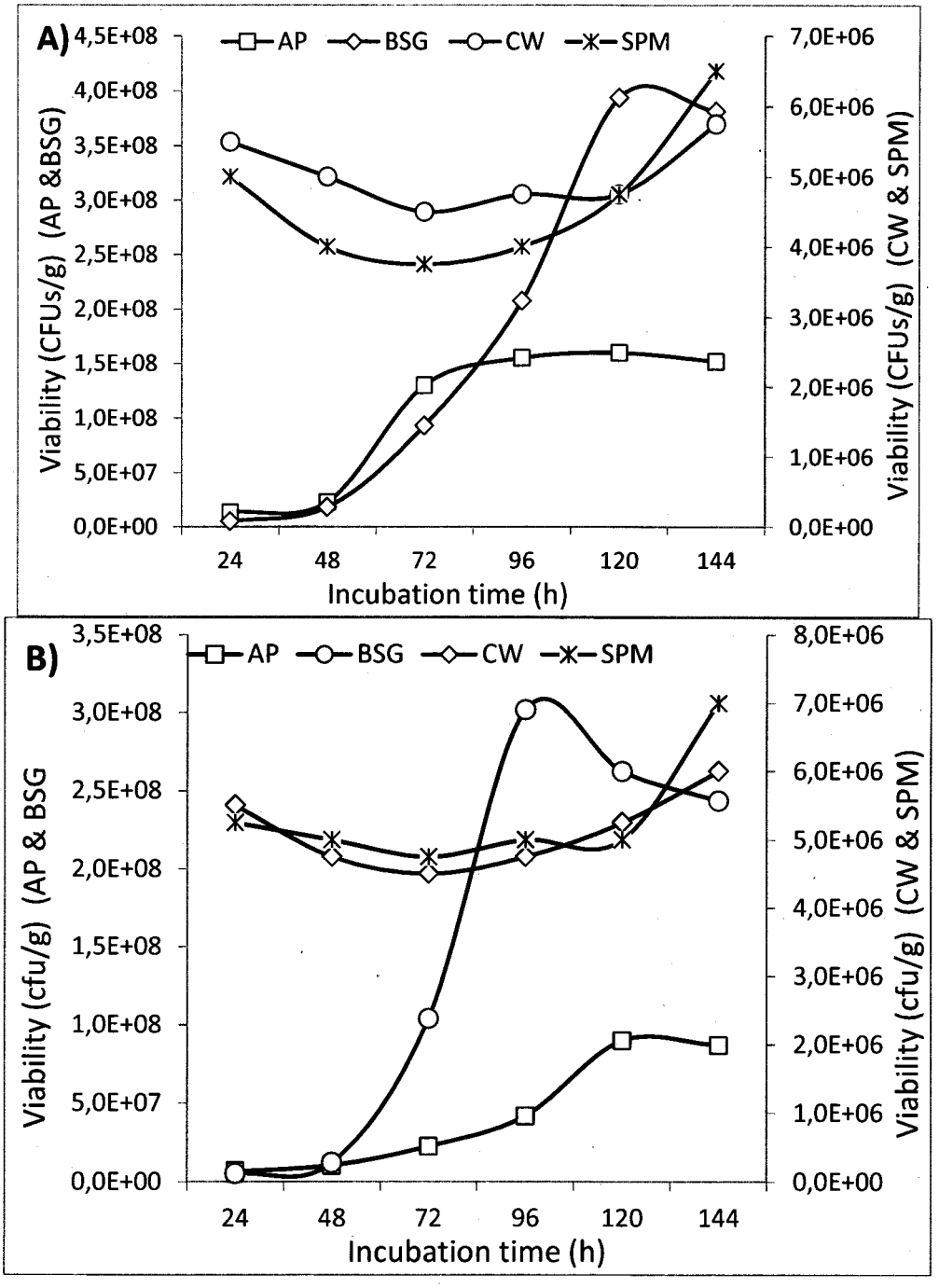


Figure 2.2. 1 A & B Viability of fungus cultivated on wastes (apple pomace, brewery spent grain, citrus waste and sphagnum peat moss) through solid-state fermentation by (A) *Aspergilllus niger* NRRL 567; (B) *Aspergilllus niger* NRRL 2001.

Abbreviations: AP-apple pomace; BSG-brewery spent grain; cfu-colony forming units; CW- citrus waste; SPM-sphagnum peat moss

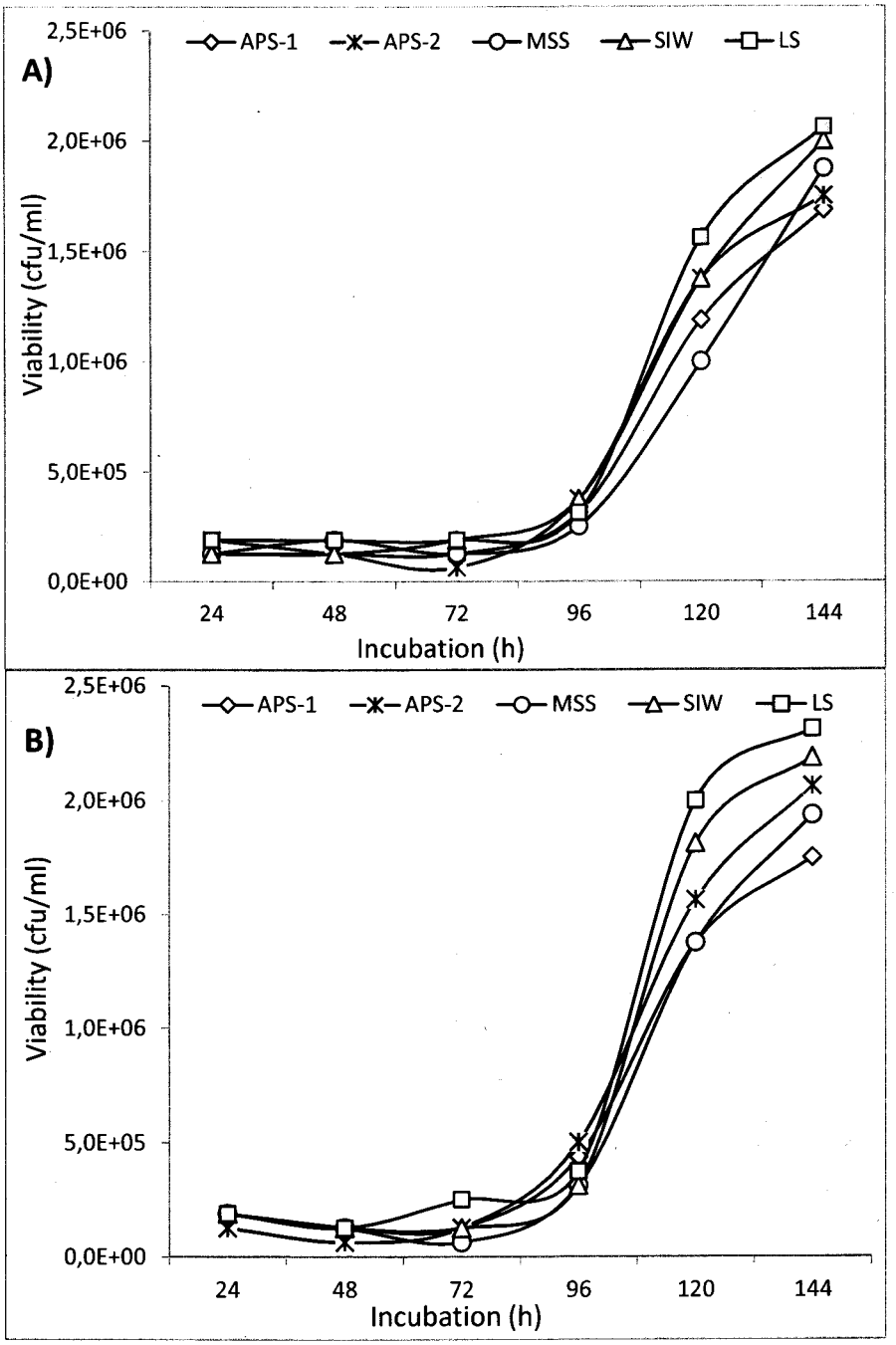


Figure 2.2. 2 A & B. Viability of fungus cultivated on wastes (apple pomace ultrafiltration sludge 1 and 2, municipal secondary sludge, starch industry water and lactoserum)) through submerged fermentation by (A) *Aspergilllus niger* NRRL 567; (B) *Aspergilllus niger* NRRL 2001

Abbreviations: APS 1- apple pomace ultrafiltration sludge; APS 2- apple pomace ultrafiltration sludge; cfu-colony forming units; SIW-starch industry water; LS-lactoserum; MSS- municipal secondary sludge

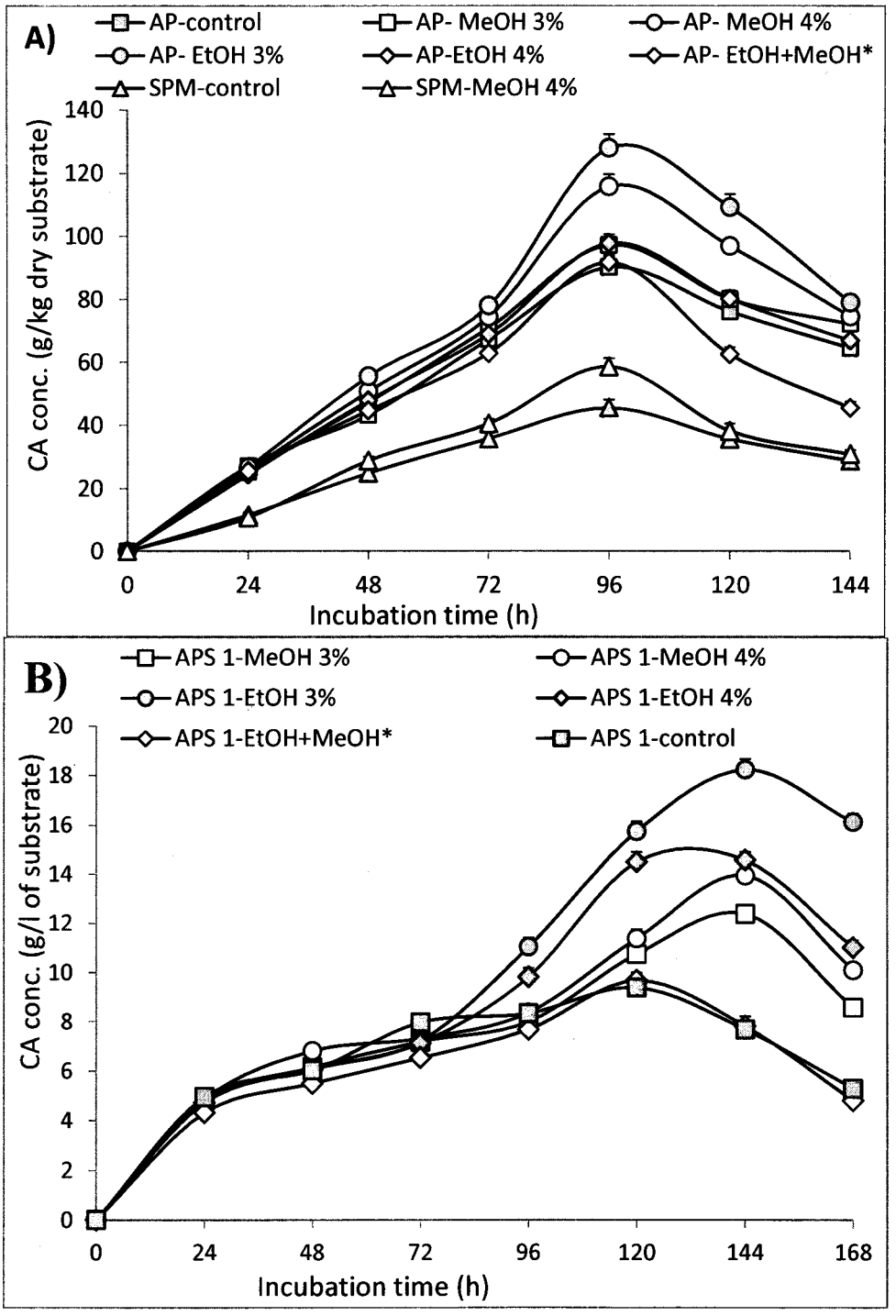


Figure 2.2. 3 (A) Solid-state CA fermentation using AP and; (B) submerged CA fermentation using APS-1 by *Aspergilllus niger* NRRL 567

Abbreviations: AP, apple pomace; APS-1, apple pomace ultrafiltration sludge; CA, citric acid; SPM-sphagnum peat moss

*In treatment with combined effect of inducers, 2% (v/v) EtOH and MeOH was added.

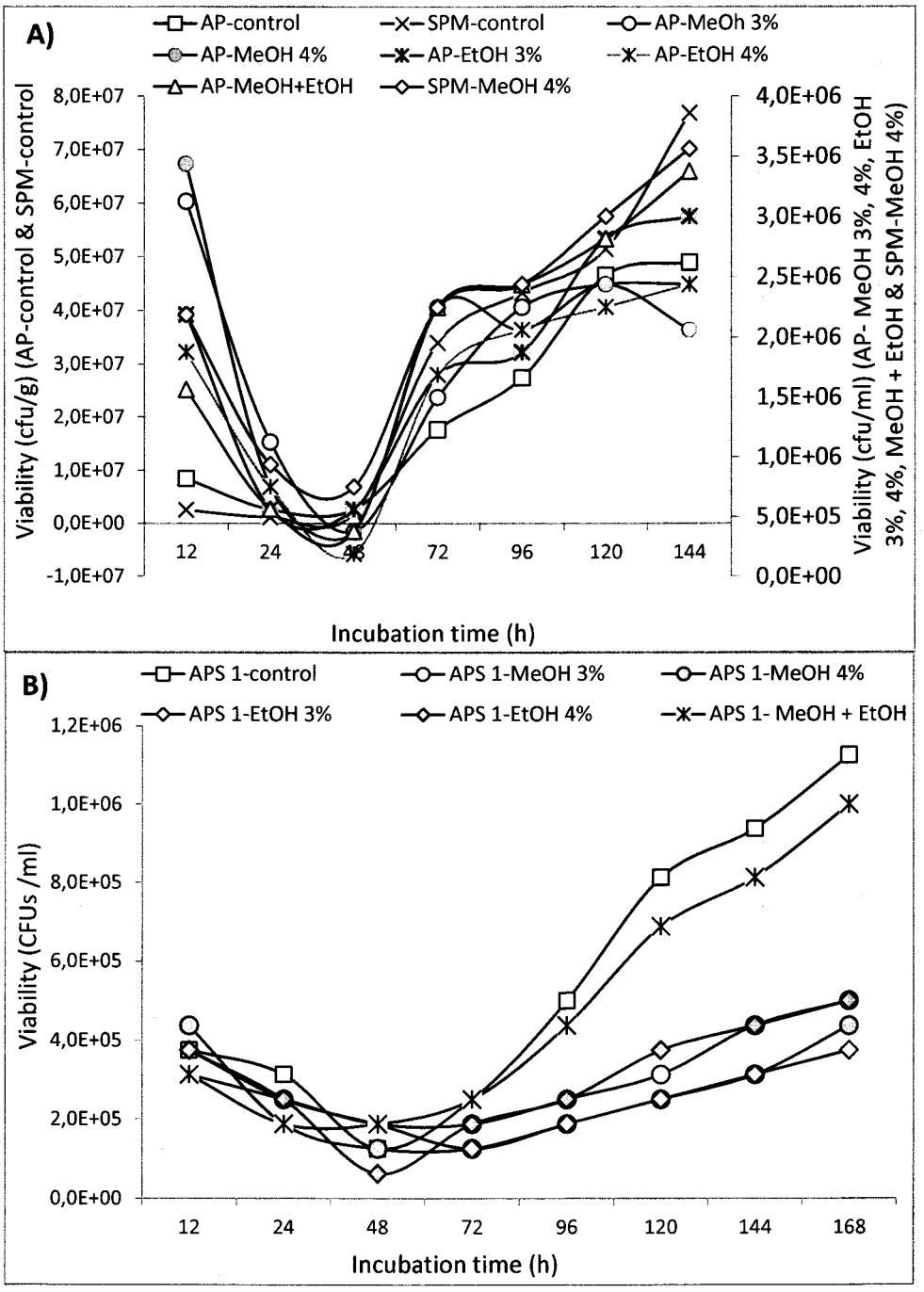


Figure 2.2. 4 Viability of *Aspergilllus niger* NRRL 567 cultivated on (A) apple pomace and sphagnum peat moss through solid-state fermentation (B) apple pomace ultrafiltration sludge 1 through submerged fermentation

*In treatment with combined methanol and ethanol, 2% (v/w) of each was added

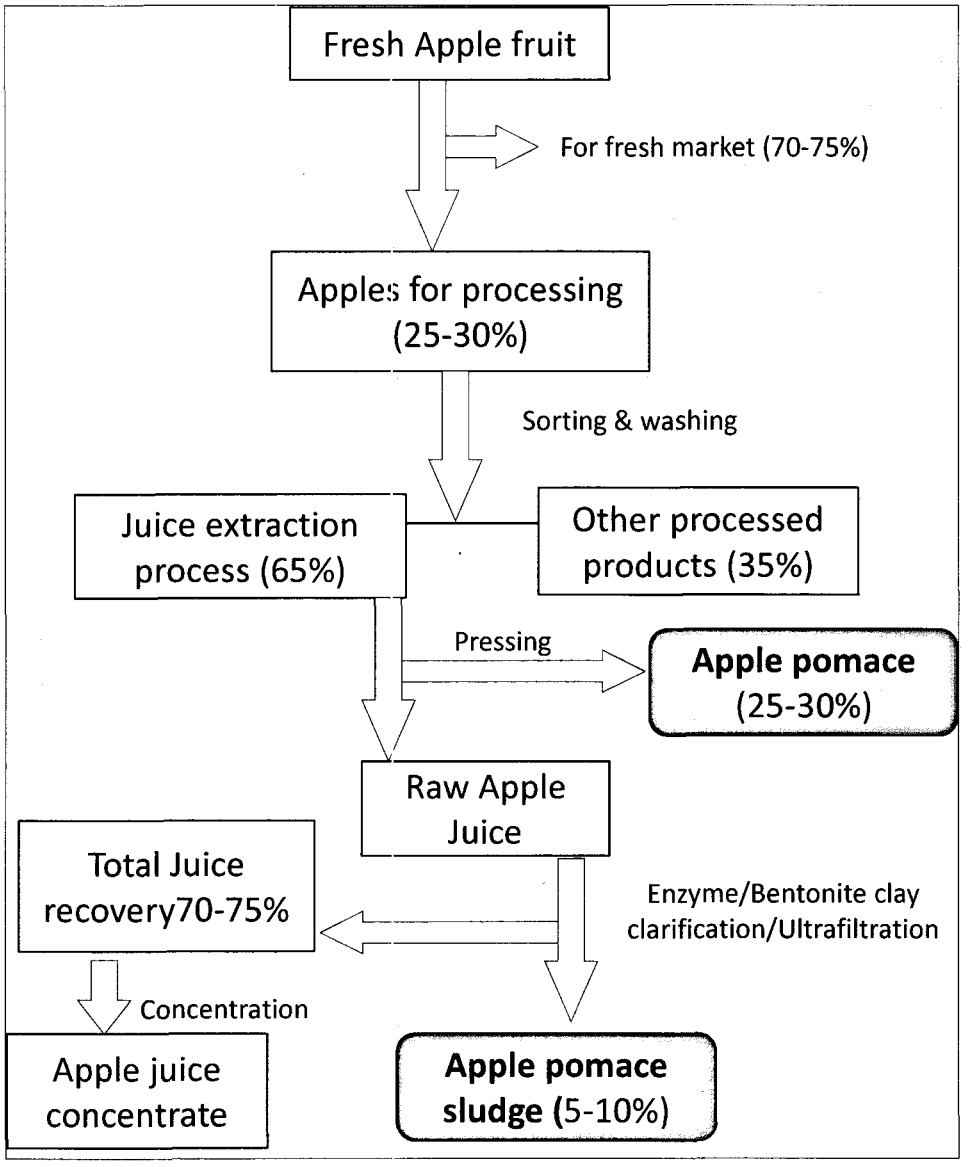


Figure 2.2. 5 Flow chart showing processing of apple and generation of apple pomace and sludge waste.

BIOTECHNOLOGICAL POTENTIAL OF INDUSTRIAL WASTES FOR ECONOMICAL CITRIC ACID BIOPRODUCTION BY ASPERGILLUS NIGER THROUGH SUBMERGED FERMENTATION

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ENHANCED SOLID-STATE CITRIC ACID BIOPRODUCTION USING APPLE POMACE WASTE THROUGH RESPONSE SURFACE METHODOLOGY

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APPLE POMACE ULTRAFILTRATION SLUDGE- A NOVEL SUBSTRATE FOR FUNGAL BIOPRODUCTION OF CITRIC ACID: OPTIMIZATION STUDIES

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RÉSUMÉ

La demande croissante de l'acide citrique (AC) et le besoin urgent de sources alternatives ont servi de force motrice pour les travailleurs dans la recherche de substrats novateurs et économiques. La fermentation à l'état liquide a été réalisée à l'aide de boues d'ultrafiltration de jus de pomme (Malus domestica) utilisées comme substrat pour la bioproduction d'AC, et utilisant *l'Aspergillus niger* NRRL567. Les paramètres essentiels, tels que les matières solides en suspension et la concentration d'inducteur, ont été optimisés par la méthodologie de réponse de surfaces pour une production plus élevée d'AC. Les concentrations optimales d'AC de 44,9 g/100 g et de 37,9 g pour 100 g de substrat sec ont été obtenus avec 25 g / l de solides totaux initiaux et 3% (v / v) de méthanol, et 25% g/l de matières solides totales et 3 % (v/v) d'éthanol, respectivement, après 144 h de fermentation. Les résultats indiquent d'une part, que la concentration totale de solides, avec le méthanol comme inducteur, a été efficace en ce qui concerne le rendement plus élevé d'AC, et d'autre part, la possibilité d'utiliser les boues de jus de pomme comme substrat potentiel pour la production économique d'AC.

Mots-clés: boues d'ultrafiltration de jus de pomme; inducteur, la méthodologie de réponse de surface; fermentation à l'état liquide; solides totaux en suspension

ABSTRACT

Ever-growing demand for citric acid (CA) and urgent need for alternative sources has served as a driving force for workers to search for novel and economical substrates. Submerged fermentation was conducted using apple (*Malus domestica*) pomace ultrafiltration sludge as an inexpensive substrate for CA bioproduction, using *Aspergillus niger* NRRL567. The crucial parameters, such as total suspended solids and inducer concentration, were optimized by response surface methodology for higher CA production. The optimal CA concentrations of 44.9 g/100 g and 37.9 g/100 g dry substrate were obtained with 25 g/l of initial total solids and 3% (v/v) methanol and 25 g/l of total solids and 3% (v/v) ethanol concentration, respectively, after the 144 h of fermentation. Results indicated that total solids concentration, and methanol as an inducer, were effective with respect to higher CA yield and also indicated the possibility of using apple pomace sludge as a potential substrate for economical production of CA.

Keywords: Apple pomace ultrafiltration sludge; inducer; response surface methodology; submerged fermentation; total suspended solids

1. INTRODUCTION

Citric acid (CA) production by *Aspergillus niger* is one of the finest and most commercially utilized examples of higher accumulation of intermediate products during fungus metabolism. CA dominates the category of organic acids, with the global production estimated to be more than 1.6 million tons (Sauer, Porro, Mattanovich, & Branduardi, 2008) and the volume of CA production by fermentation is constantly rising at a high annual rate of 5% (Francielo, Patricia, & Fernanda, 2008), with future escalating trends. CA is an important multi-functional organic acid with a broad range of versatile applications, e.g. in food, pharmaceuticals and cosmetics. Currently, many advanced applications are coming to light, such as: (1) biomedicine, e.g. synthesis of biopolymers for culturing a variety of human cell lines; (2) nanotechnology, such as drug delivery systems and; (3) agriculture, such as bioremediation of heavy metals due to its powerful sequestering action with various transitional metals (Dhillon, Brar, & Verma, 2010b).

The world's existing demand for CA is almost entirely (over 99%) met by fermentative processes, using *A. niger* in submerged or static liquid cultures (Dhillon, Brar, Verma, & Tyagi, 2010a). However, for the past few years, the CA market has been under tremendous pressure and continues to swing with reduction in prices. High energy, coupled with raw material costs, has squeezed CA production into an unprofitable market. The search for inexpensive substrates as an alternative to high cost substrates is vital to reduce the production cost of CA. In recent years, considerable interest has been focused on agro-industrial wastes for CA bioproduction (which serves to solve waste management problem faced by agro-industries). Moreover, industries will also be benefitted by extra revenue from the value addition of agro-industrial wastes. Every year, thousands of tons of apple pomace (AP) and apple pomace sludge (APS) are generated by apple processing industries in Canada. In 2008–2009, out of world's total apple production (69, 603, 640 tonnes), Canada contributed 455, 361 tonnes (more than 25% by Quebec alone) (Bhusan, Kalia, Sharma, Singh, & Ahuja, 2008; Food, 2008; Dhillon, Brar, Verma, & Tyagi, 2011; Statistics Canada 2010; Vendruscolo, Albuquerque, & Streit, 2008). The processing of apples will lead to production of 25–30% AP and 5–10% APS (Dhillon et al., 2011). The apple processing industries incur losses due to treatment of waste and transportation costs for dumping into landfills. In this context, CA

production from nutrient-rich organic wastes, such as AP and APS (total carbon 51.9 g/l, total nitrogen 2.94 g/l, carbohydrates 66.0 ± 1.7 g/l, lipids 5.9 ± 0.32 g/l, protein 33.8 ± 2.0 g/l) is a lucrative alternative for the apple processing industries (Dhillon et al., 2011). Due to the complexity of the metabolic state in fungus for the enhanced accumulation of desired product, there is an obvious need to optimize the important process parameters, depending upon the substrate. Optimization of different parameters, by the traditional 'one-factor-at-a-time' method, requires a considerable amount of time and effort. An alternative potential approach is a statistical approach, such as response surface methodology (RSM), one of the most widely used statistical techniques for bioprocess optimization (Liu & Tzeng, 1998). RSM can be used to evaluate the relationship between a set of controllable experimental factors and outcomes. The interactions among the possible influencing factors can be evaluated with a restricted number of experiments.

The aim of the present study is a higher CA bioproduction using APS as a novel substrate through submerged fermentation by *A. niger* NRRL 567. The effects of two crucial process parameters, namely total solids and inducers (ethanol and methanol), on CA production were optimized, using an experimental design, and analyzed by RSM. The design used in this study is CCD which is a first-order (2N) design amplified by additional centre and axial points to allow estimation of the tuning parameters of a second-order model. Total suspended solids concentration plays an important role in broth rheology and hence affects the final concentration of CA (Dhillon et al., 2011). As in the published literature, so far, to the best of our knowledge, there is no study reporting the: (1) utilization of APS for CA production; (2) coordinate influence of total suspended solids and the two different inducers on CA production by *A. niger* NRRL-567 under submerged culturing conditions.

2. MATERIALS AND METHODS

2.1. Microorganisms and inoculum preparation

A. niger NRRL-567 was procured from the Agricultural Research Services (ARS) culture collection, IL-USA and cultivated as the freeze-dried form. The culture was revived onto potato dextrose broth medium (PDB) for 7–8 days at 30 ± 1 °C and 200 rpm. *A. niger*

spores were produced on potato dextrose agar (PDA) plates for 5– 6 days at 30 ± 1 °C. The spores were harvested from the PDA plates by adding 10 ml of 0.1% w/v Tween-80 solution to each plate. The harvested spores were initially filtered through glass wool to remove mycelial contamination and recover only the spores. Spores were counted microscopically, using a Neubauer chamber to adjust the count to approximately 1×10^8 spores/ml and stored in test tubes at -20±1 °C for a maximum of 4 weeks. The harvested spores were used as an inoculum.

2.2. Substrate procurement and pretreatment

Apple pomace ultrafiltration sludge (Lassonde Inc., Rougemont, Montreal, Canada), was selected as fermentation medium due to its potential for CA production, based on our previous screening studies (Dhillon et al., 2011). APS is the liquid sludge which is obtained after the ultrafiltration of crude juice. The apples are mashed in the initial step of processing and the solid waste is separated, and the crude juice so obtained is filtered twice through the ultrafiltration system which leaves apple pomace sludge, accounting for 5–10% of total processed apples. For experimental purposes, the desired total solids concentrations were made up by adding distilled water. The pH was adjusted to 3.5 ± 0.1 and initial viscosity of all the samples was analyzed.

2.3. Parameter range finding

One important factor that affects the performance of submerged CA fermentation is the total suspended solids concentration of the medium. During screening studies, higher CA production was achieved with APS-1 which had a lower total solids content (115 ± 5 g/l) than had APS-2 (135 ± 5 g/l) (Dhillon et al., 2011). In order to determine the effect of total solids concentration on response (CA), three levels of total solids concentration, namely 30 g/l, 40 g/l and 50 g/l, were made up, using APS sludge having an initial total solid concentration of 135 g/l. Previous studies have shown that the supplementation of lower alcohols, such as ethanol (EtOH) and methanol (MeOH), at concentrations of 1–4% (v/v), resulted in a marked increase of CA formation by *A. niger* on various wastes. The increase was mainly attributed to the increased cell permeability level and decreased end-product inhibition of related enzyme systems (Moddax, Hosain, &

Brooks, 1986). To determine the effects of lower alcohols (EtOH and MeOH) concentrations, separately, on responses (CA productivity), three levels, of 2%, 3% and 4% (v/v), were selected. Other important parameters, such as initial pH (3.5), temperature (30 ± 1 °C), rpm (200/min), and inoculum level (1 × 10^7/25 ml of medium) were selected, based on the literature. The initial pH of the medium is an important parameter, which also affects the performance of submerged CA fermentation by *A. niger*, which grows very well in lower pH for higher CA production (Dhillon et al., 2010a). Temperature also exerts a profound influence on the fungal production of CA. *A. niger* is known to produce a higher yield of CA at temperatures ranging between 25 and 35 °C. Adinarayana and Prabhakar (2003) showed that higher temperatures lead to enzyme denaturation and moisture loss while lower temperatures lead to reduced metabolic activity. Fawole and Odunfa (2003) reported that a temperature of 30 °C was optimum for metabolite production by *A. niger*. In the absence of an ideal temperature during fermentation, the fungal cells showed signs of adverse growth and metabolic production (Ellaiah, Srinivasalu, & Adinarayana, 2003).

2.4. Submerged fermentation (SmF)

One hundred and fifty milliliters of liquid substrate, with pH 3.5 ± 0.1, were dispensed into a 500 ml Erlenmeyer flask and thoroughly mixed and autoclaved at 121 ± 1 °C for 30 min. After sterilization, the desired inducer concentrations were added to the substrates and were inoculated with a spore suspension of 1 × 10^7 spores/25 ml. After thorough mixing, Erlenmeyer flasks and their contents were incubated in a shaker at 30 ± 1 °C at 200 rpm. Unless specified otherwise, these fermentation conditions were maintained throughout the study. All the experiments were conducted in duplicate.

2.5. Experimental design and optimization

In order to identify the significant factors that affect the response (CA production), an effort was made to improve the composition of the medium by comparing different levels of two factors that were found to have more influence on the production of CA by *A. niger* NRRL 567. The impact of two independent quantitative variables, including total

suspended solids (X_1) and ethanol (X_2), in one set of experiments and total solids (X_3) and methanol (X_4), in second set of experiments, were evaluated by a central composite design (CCD) to find the most favorable concentrations of these two factors. In this regard, a set of 13 experiments, including, five centre points (0, 0, 0) and four axial points (a = 1.414) and 4 points corresponding to a matrix of 22, which incorporates 4 experiments, including 3 variables (+1, -1, 0), were carried out. Each variable was studied at two different levels (-1, +1) and centre point (0) which is the midpoint of each factor range. The minimum and maximum range of variables investigated and the full experimental plan with respect to their actual and coded values are listed in Tables 2.5.1 and 2.5.2, respectively. A multiple regression analysis of the data was carried out by STATISTICA 6 of STATSOFT Inc. (Thulsa, US) by surface response methodology and the second-order polynomial equations, that define predicted responses (Y_i) in terms of the independent variables for experiment set 1 (X_1, X_2) and experiment set 2 (X_3, X_4), are shown in Equations. (1) and (2):

$$Y_i = b0_i + b1_i X_1 + b2_i X_2 + B11_i X_2 + b22_i X_2 + b12_i X_1 X_2 \quad [1]$$

$$Y_i = b0_i + b3_i X_3 + b4_i X_4 + B33_i X_4 + b44_i X_4 + b34_i X_3 X_4 \quad [2]$$

where, Y_i = predicted response (CA production), $b0_i$ is intercept term, $b1_i$ and $b2_i$, linear coefficients, $b11_i$ and $b22_i$ squared coefficients and $b12_i$, the interaction coefficient and i refer to the response in Eq. (1) and similarly, for Eq. (2). Combination of factors (such as X_1X_2 and X_3X_4) represents an interaction between the individual factors in the respective term. The response, i.e. CA production, is a function of the level of factors. The response surface graphs specify the effect of variables, individually and in combination, and determine their optimum levels for maximal CA production. The data were fitted into a second-order polynomial function [Eq. (1) and (2)] and a correlation was drawn between experimental data and the predicted values by the model (Fig. 2.5.1 A and B). The quality of the model fit was evaluated by the coefficient R^2 and its statistical significance was determined by an *F*-test. R^2 represents the proportion of variation in the response data that can be explained by the fitted model. High R^2 was considered as evidence for the applicability of the model in the range of variables included. R^2 values range from 0 to 1; values greater than 0.75 indicate high consistencies between the predicted and the

observed values. The R^2 value also provides a measure of the fraction of total variability in the data explained by the regression.

2.6. Sampling details

At regular intervals of 24 h, 5 ml samples were collected, under aseptic conditions, from the fermentation medium from each flask, to be used for analysis. The extracted medium from SmF was filtered through glass wool for removal of solid substrate and fungal mycelia. About 100 µl of sample were taken in Eppendorf tubes from the filtrate for viability (total spore count), using a haemocytometer and the remaining sample was centrifuged (Sorvall RC 5C plus by Equi-Lab Inc., Québec, Canada) at 9000 × g for 20 min; the supernatant was analyzed for CA concentration. The changes in pH and viscosity were also analyzed initially and at the end of the experiment to obtain an understanding of the morphological changes occurring in the medium during fermentation.

2.7. Analytical techniques

Viscosity measurement was carried by using a rotational viscometer Brookefield DV II PRO + (Brookefield Engineering Laboratories, Inc., Stoughton, MA, USA). A LV-1 spindle was used with a sample cup volume of 30 ml. The pH of the substrates was measured with a pH-meter equipped with a glass electrode. The total solids content of APS was estimated by drying a 25 ml sample in an oven at 105 ± 5 °C to constant weight. For experimental purposes, the required total solids concentrations were made up with distilled water. In order to measure the amount of living fungal biomass in solid-state cultivation, viability assay was used as an indicator. Total spores were counted microscopically, using a Neubauer chamber. CA concentrations were determined gravimetrically by the modified method of Marier and Boulet (1958). Details of the estimation procedure have been provided by Dhillon et al. (2011). CA concentrations were expressed as grams per 100 g of dry substrate (g/100 g ds).

3. RESULTS AND DISCUSSION

Response surface methodology was adopted to optimize the total solids and inducer concentrations with respect to the CA yield. Owing to the high carbohydrate content and other vital nutrients, APS was utilized for the bioproduction of CA, using *A. niger*, by submerged fermentation (Dhillon et al., 2011). Central composite design was used to find the optimized values of the important variables on the citric acid production by *A. niger* NRRL 567, by using APS as fermentation medium. The results of CCD experiments consisted of experimental data for studying the effects of two independent variables: set (1) total solids concentration and EtOH and set (2) total solids concentration and MeOH on citric acid production and the results are depicted in Table 2.

3.1. Effects of variables on CA production

CA production exhibited different responses with varied concentrations of two inducers, namely EtOH and MeOH, and total solids concentration. The response at various coded levels of total solids and inducer concentrations is shown in Figs. 2.5.2 and 2.5.3, respectively, for CA production. Figs. 2.5.2 and 2.5.3 present response surface profiles depicting quantitative values for CA production, whereas total solids and inducer concentrations are represented as their coded values. The 3D response surface is the graphic representation of the regression equation for CA production. The effect of pairwise interaction of the parameters is prominently depicted in the three-dimensional graphs. It represents an infinite number of combinations of two test variables.

Statistical analysis was performed with the data having higher CA production at 144 h of fermentation as no significant increase in CA production was observed after 144 h of incubation time. CA production at 144 h varied from 16.8 g/100 g ds (trial 4 having 50 g/l TS and 4% v/v EtOH) to 37.9 g/100 g ds (trial 5 having 25.9 g/l TS and 3% v/v EtOH) for experiment set 1. Similarly, a CA production difference of 24.7 g/100 g ds (trial 4 having 50 g/l TS and 4% v/v MeOH) to 44.9 g/100 g ds (trial 5 having 25.9 g/l TS and 3% v/v MeOH) for the experiment set was observed. Higher CA production was obtained with 25.9% (g/l) initial TS which declined by 56% and 45%, respectively for sets 1 and 2 on increase in the TS concentration to 50 g/l. When MeOH was used as an inducer, a

significantly higher concentration of CA was achieved. It can be concluded that MeOH exerts an enhancing effect on the CA concentration.

The initial total solids concentration of the fermentation medium plays an important role in the submerged CA production by filamentous fungus, *A. niger*. Usually, an increase in viscosity of the fermentation medium was observed during the course of fermentation, mainly, due to the mycelium growth and metabolism of fungus. The morphology of the fungus is considered to be an important parameter which influences the physical characteristics of the fermentation medium which in turn can have an effect on the final yield of CA. The rheological behavior of the fungus was closely associated with the morphology and biomass concentration. The broth rheology also determined the transport phenomena in bioreactors and the final concentration of CA produced. Less viscous non-Newtonian broths are typically indicative of a pellet form of fungus, in which the fungus mycelium develops very stable spherical aggregates consisting of a denser, branched, and partially intertwined network of hyphae. The pellet form is highly recommended for a high yield of CA during submerged fermentation (Dhillon et al., 2010a).

Due to the stimulatory effect of lower alcohols, such as methanol (MeOH) and ethanol (EtOH), they are routinely used as inducers in the microbial production of citric acid. The effect of lower alcohols in increasing CA concentrations appears to be a common practice in strains of *A. niger* (Sikander & Haq, 2005; Rivas, Torrado, Torre, Converti, & Dominguez, 2008; Barrington, Kim, Wang, & Kim, 2009; Nadeem, Syed, Baig, Irfan, & Nadeem, 2010). MeOH was found to have a more pronounced effect on CA production (g/100 g ds) than EtOH by *A. niger*. Methanol is not metabolized by *A. niger*; however, it is recognized for depressing the synthesis of cell wall proteins in the early stages of cultivation (Dhillon et al., 2010a). Moreover, MeOH also acts as an inducer for the activity of citrate synthase, which ultimately leads to a higher accumulation of CA (Barrington & Kim, 2008). However, it should be noted that MeOH, at higher concentrations, also produces negative effects on the growth of fungus. The stimulating effect of EtOH on CA fermentation suggested that EtOH could be used as a carbon source by the fungus, *A. niger*, and it was metabolized into CA via the tricarboxylic acid (TCA) cycle. It is possible that ethanol might be converted to acetyl-CoA, a metabolic

substrate required for CA formation. EtOH also causes reduction in aconitase activity, which can result in massive accumulation of CA. Addition of ethanol also resulted in a slight increase in the activity of other tricarboxylic acid (TCA) cycle enzymes. Furthermore, EtOH is also known to increase the permeability of the cell membrane and thus higher secretion of CA (Navaratnam, Arasaratnam, & Balasubramaniam, 1998; Jianlong, 2000). In submerged fermentations, lower alcohols, such as MeOH and EtOH, also markedly increase the tolerance levels of trace metals, such as manganese, iron and zinc, far above the required levels for fungus growth. Comparison of our present and previous studies indicated the role of lower alcohols on the morphology of *A. niger*. Addition of alcohols resulted in the pellet form, which is considered better for higher CA production (Dhillon et al., 2010a). The results of the second order response surface model fitting, in the form of ANOVA, are given in Table 2.5.3. The data were best fitted into a second-order polynomial function [Eq. (1) and (2)], as it can be inferred from the good agreement of experimental data with those predicted by the model (Fig. 1A and B). In all models, the coefficients of determination (R^2 value) higher than 0.75 (0.86 for set 1 and 0.88 for set 2) indicated that the models fitted well into the experimental results, presenting about 86.0–88.0% variability in the response (Table 2.5.3).

The significance of each coefficient was evaluated with the *p*-values (Ghodke, Ananthanarayan, & Rodrigues, 2009). The smaller the magnitude of *p*, the more significant is the corresponding coefficient. $p<0.05$ indicates that the model terms are significant. The estimated coefficients and the corresponding *p*-value indicate that, among the tested variables used in the present study, X_1 (TS) and X_2 (inducers) have a negative significant effect on CA production in both set 1 and set 2 ($p < 0.05$). It can be inferred that variables can act as limiting factors and variation in their concentration will also alter the product formation rate. The interactions between the two variables (TS and inducers) showed a positively insignificant effect in all models. It can be concluded that the variables alone may have a positive significant effect on the response. The quadratic term of X_2 (EtOH) showed a negative significant effect on CA production by *A. niger* ($p<$ 0.021). However, a positive insignificant effect was observed with the quadratic term X_1 (TS) in set 1. Similarly, in set 2, the quadratic term of X_2 (EtOH) showed a highly negative significant effect ($p < 0.005$) and the quadratic term X1 (TS) showed a positive insignificant effect on CA production by *A. niger*. The interactive effect of total solids and

inducers for citric acid production using APS has not been described earlier in the literature.

3.2. Effect of variables on physicochemical properties of fermentation broth

Table 2.5.4 represents the change in viscosity and pH during submerged CA fermentation. The maximum levels of CA production were observed with a 25 g/l total solids concentration, in both sets of experiments. This could be explained by the fact that, in SmF, initial solids concentration played an important role in broth rheology. Higher total solids concentration resulted in higher initial and final viscosity of the broth, as given in Table 2.5.4. An increase in viscosity during the course of fermentation was observed with different treatments during fermentation. However, results of the present study showed that the addition of MeOH and EtOH resulted in a decrease in viscosity as compared to the control, in which viscosity was higher during the course of incubation. The morphology of the fungus is an important parameter which influences the physical characteristics of the fermentation medium which, in turn, affect the final yield of CA. The rheological behavior of the fungus was closely associated with the morphology and biomass concentration (Dhillon et al., 2010a). The broth rheology affects the transport phenomena in bioreactors and the final concentration of CA produced. Less viscous non-Newtonian broths are usually indicative of the pellet form of the fungus, in which the mycelium develops very stable spherical aggregates consisting of a denser, branched, and partially intertwined network of hyphae. The small round pellets were observed in all treatments with addition of alcohols. The lower alcohols have a direct effect on mycelial morphology and they promote pellet formation instead of entangled hyphae. The mycelial morphology of filamentous fungi, in the form of small round pellets (<3 mm diameter), is also considered best for maximal CA production during submerged fermentation (Dhillon et al., 2010a). In this context, the present study reflected the role of broth rheology in the final CA yield.

3.3. Effect of variables on fungal growth

Fig. 2.5.4 represents the total spore count, which reflects the physiological state of *A. niger* during the course of fermentation. Initially, until 72 h of fermentation, the spore

count decreased, due to the vegetative growth of the fungus. After 72 h, the total number of spores started increasing which might be due to the depletion of nutrients, so the fungus started adapting itself according to the availability of nutrients in the medium. The alcohols are also known to inhibit sporulation and increase the fermentation efficiency (Anwar, Ali, & Sardar, 2009). Thus, the present study established the suppressive role of alcohols with respect to spore formation.

3.4. Determination of optimal conditions and optimal responses

Using the method of experimental factorial design and response surface analysis, the optimal conditions to obtain a higher CA concentration were determined. The validity of the model was proved by fitting different values of the variables into the model equation and by carrying out the experiment at those values of the variables. The best conditions for the citric acid production with APS were: 25 g/l total solids concentration and inducer concentrations at 3% (v/v) for both EtOH and MeOH as provided in Table 2.5.3. These parameters rendered higher CA production in APS (37.9 g/100 g ds with EtOH and 44.9 g/100 g ds with MeOH). No addition of expensive media is required and the use of inexpensive agro-industrial wastes, such as APS, will have important economic and environmental advantages. Therefore, these optimized conditions could be applied for enhanced CA production, which has a great potential in the biomedicine industry, e.g. raw material for polymer synthesis and drug delivery agents. APS could be used as a potential substrate, due to the fact that the increment of cost of raw substrates for CA production raises interest in fermentation processes for production of this compound from renewable resources. Currently, the sludge produced by apple processing industries is viewed as a waste product and disposed of through landfills, land application on farms, or incineration. Due to the fact that the wastes generated by the fruit processing industries cannot be dumped into the environment directly, the industries incur additional losses due to treatment of sludge and transportation costs for dumping into landfills which finally adds to the cost of processed products. The biological oxidation demand (BOD) of the sludge is 72,000 mg/g and the BOD to chemical oxidation demand (COD) ratio is very high (0.6), thus, indicating that APS is a highly biodegradable material. So, the disposal of APS poses formidable environmental challenges. The landfills and land-spreading of sludge are sources of secondary

pollution as they: (1) contaminate the underground water table, due to run off in rainy seasons, (2) increase greenhouse gases concentration in the environment and (3) produce negative effects on human health as they create breeding grounds for many human disease vectors which can cause epidemics. Thus, CA production from APS is a viable alternative to sewage disposal which will help to alleviate the negative impacts of its disposal into the environment.

3.5. Fermentation efficiency and carbon capture

Table 2.5.2 shows the fermentation efficiency of CA production using APS by *A. niger* during parameter optimization of total solids and inducer concentrations. Taking initial total carbon/kg dry solids into account, the fermentation efficiency of citric acid production was calculated. The initial total carbon was found to be 328 g/kg dry weight of APS having a total solids concentration of 135 g/l and total carbon of 44.3 g/l. A higher concentration of citric acid was achieved with 3% (v/w) of MeOH and EtOH, used as inducers. The concentration of 449 g/kg of dry substrate was obtained with 3% (v/w) (30 ml/kg = 23.6 g/kg) MeOH. The amount of carbon (g/kg) present in 449 g of citric acid is 168 g/kg. The efficiency achieved with MeOH was 49.9%. Similarly, a citric acid concentration of 379 g/kg of dry substrate was achieved with 3% (v/v) (30 ml/kg = 23.7 g/kg) EtOH. The amount of carbon (g/kg) present in 379 g citric acid is 142 g/kg. The efficiency achieved with EtOH was 41.7%. Future studies will concentrate on scale up of the process, with optimized parameters for enhanced citric acid bioproduction, by using apple pomace ultrafiltration sludge with *A. niger* NRRL 567.

4. CONCLUSIONS

The present study has shown a promising potential for utilizing apple pomace ultrafiltration sludge as a novel substrate for the production of citric acid by *A. niger*. The statistically based optimization procedure, using response surface methodology, was applied in this study, and proved to be an effective technique in optimizing submerged fermentation conditions. Utilization of apple pomace ultrafiltration sludge served in sequestration of carbon, which is an important element of greenhouse gas emissions.

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Table 2.5. 1 Experimental range of the two variables studied using central composite design (CCD) in terms of actual and coded factors

Set 1		**Coded levels**				
Variables	**Symbol**	**-1.414**	**Low (-1)**	**Mid (0)**	**High (1)**	**+1.414**
TS (g/l)	X_1	25.86	30	40	50	54.14
EtOH (% v/v)	X_2	1.59	2	3	4	4.41
Set 2		**Coded levels**				
Variables	**Symbol**	**-1.414**	**Low (-1)**	**Mid (0)**	**High (1)**	**+1.414**
TS (g/l)	X_3	25.86	30	40	50	54.14
MeOH (% v/v)	X_4	1.59	2	3	4	4.41

Abbreviations: EtOH- ethanol; MeOH- methanol; TS-total solids

Table 2.5. 2 Results of experimental plan by central composite design for apple pomace ultrafiltration sludge (shaded cells represent maximum value)

Trial	X_1/ X_3	X_2/ X_4	CA (g/100 g ds) Set 1	Fermentation efficiency* (%)	CA (g/100 g ds) Set 2	Fermentation efficiency* (%)
1	30.00	2.00	30.74	34.27	39.88	44.77
2	30.00	4.00	23.24	25.29	28.23	31.14
3	50.00	2.00	23.07	25.72	32.94	36.98
4	50.00	4.00	16.83	18.31	24.74	27.29
5	25.86	3.00	37.89	41.73	44.87	49.93
6	54.14	3.00	24.20	26.65	31.77	35.35
7	40.00	1.59	25.93	29.05	31.95	36.0
8	40.00	4.41	21.44	23.22	26.51	29.14
9 C)	40.00	3.00	27.43	30.21	34.80	38.72
10(C)	40.00	3.00	27.39	30.17	34.95	38.89
11(C)	40.00	3.00	26.93	29.66	35.79	39.83
12 (C)	40.00	3.00	27.19	29.95	34.94	38.88
13 (C)	40.00	3.00	26.98	29.71	35.94	39.99

Abbreviations: $X_1$and X_2 represents variables i.e. total solids concentration and inducer (ethanol) concentration for experiment set 1

X_3 and X_4 variables i.e. total solids concentration and inducer (methanol) concentration for experiment set 2

*Fermentation efficiency was calculated based on the total carbon utilized.

Table 2.5. 3 Model coefficients estimated by central composite design and best selected prediction models (where TS is total solids concentration, APS is apple pomace ultrafiltration sludge, EtOH is ethanol and MeOH is methanol)

Set 1	**CA (g/100g ds)**			**Set 2**	**CA (g/100g ds)**		
	Coefficient	***t*-value**	***p*-value**		**Coefficient**	***t*-value**	***p*-value**
Constant	139.78*	25.15*	0.000*	**Constant**	104.81*	33.58*	0.000
Linear				**Linear**			
TS	-8.36*	-4.89*	0.0018*	TS	-7.24*	-4.36*	0.003*
EtOH	-5.02*	-2.94*	0.022*	MeOH	-6.89*	-4.14*	0.004*
Interactions				**Interactions**			
TS × EtOH	0.63	0.26	0.80	TS × MeOH	1.73	0.73	0.48
Quadratic				**Quadratic**			
TS	1.91	1.04	0.33	TS	1.87	1.05	0.32
EtOH	-5.44*	-2.97*	0.020*	MeOH	-7.22*	-4.05*	0.005*
R-sqr	0.86*			**R-sqr**	0.88*		

Reduced Equations for CA production: best selected models:

Citric acid (AP + EtOH) = 139.78 – 8.36 M – 5.02 EtOH + 1.91 M – 5.45 EtOH + 0.63 M × EtOH

Citric acid (AP + MeOH) = 104.81 – 7.24 M – 6.89 MeOH + 1.87 M – 7.22 MeOH + 1.73 M × MeOH

*Significant (p<0.05).

Table 2.5. 4 Changes in different physical-chemical parameters during submerged fermentation with various treatments

Treatment		Initial	Final pH	Final pH	Initial	Final	Final
SS (g/l)	Inducer (% v/v)	pH (±0.1)	(±0.1) (Inducer EtOH)	(±0.1) (Inducer MeOH)	Viscosity (cP)	Viscosity-EtOH (cP)	Viscosity-MeOH (cP)
30.0	2.0	3.5	2.48	2.45	1.46	3.0	2.9
30.0	4.0	3.5	2.92	2.85	1.36	3.40	3.14
50.0	2.0	3.5	2.65	2.65	2.14	25.59	19.4
50.0	4.0	3.5	2.78	2.76	2.68	4.0	5.20
25.86	4.0	3.5	2.66	2.62	1.40	7.0	4.40
54.14	3.0	3.5	2.55	2.54	2.54	26.39	12.20
40.0	1.59	3.5	2.60	2.54	1.72	2.40	13.40
40.0	4.41	3.5	2.70	2.68	2.46	2.80	3.60
40.0	3.0	3.5	2.44	2.44	2.05	8.0	9.80
40.0	3.0	3.5	2.50	2.40	2.10	2.40	8.80

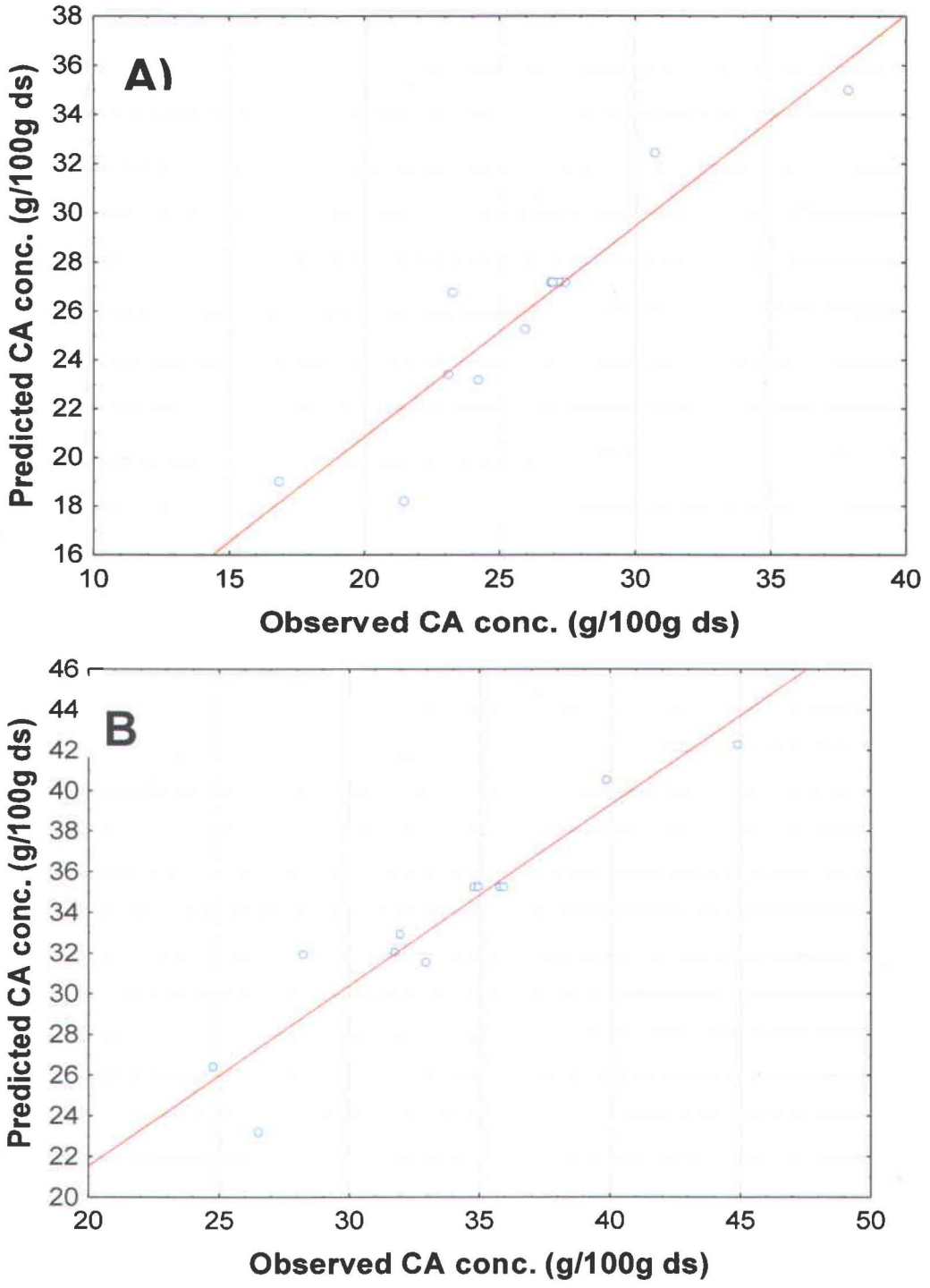


Figure 2.5. 1 Parity plot showing the distribution of experimental vs. predicted values of citric acid production (apple pomace) supplemented with inducer: A) ethanol and; B) methanol

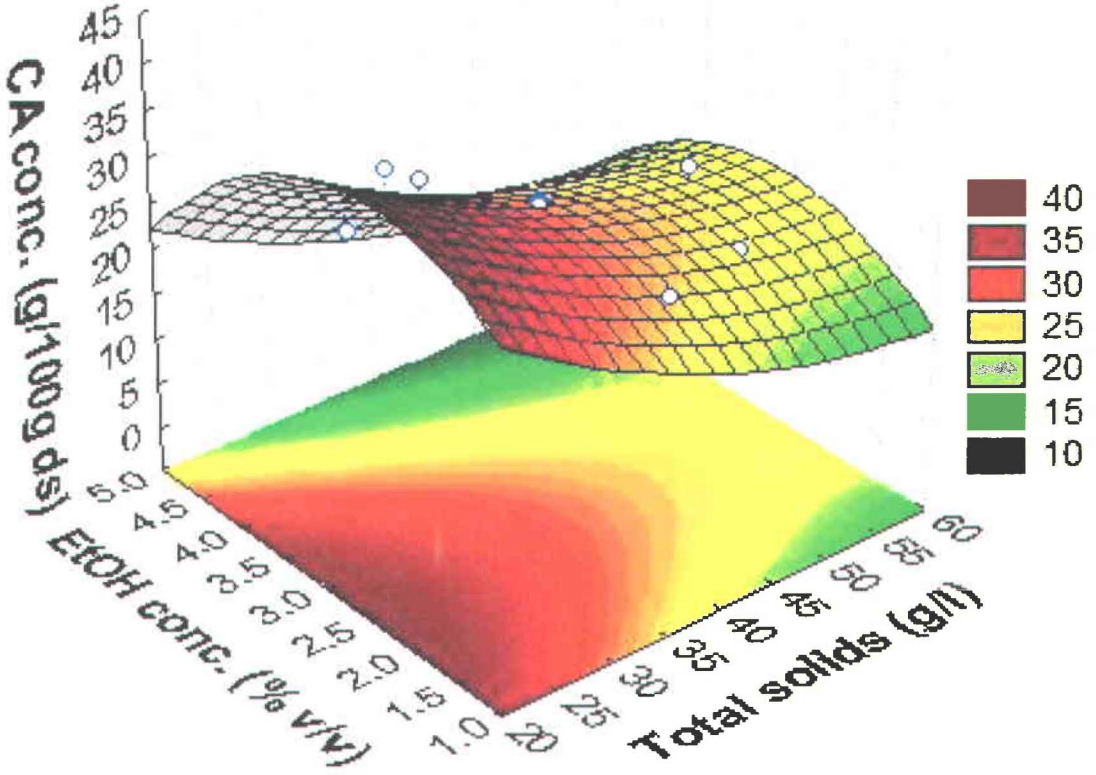


Figure 2.5. 2 Response surface plot of citric acid production using apple pomace ultrafiltration sludge obtained by varying: a) moisture (X_1) and; b) the concentration of inducer i.e. ethanol (X_2).

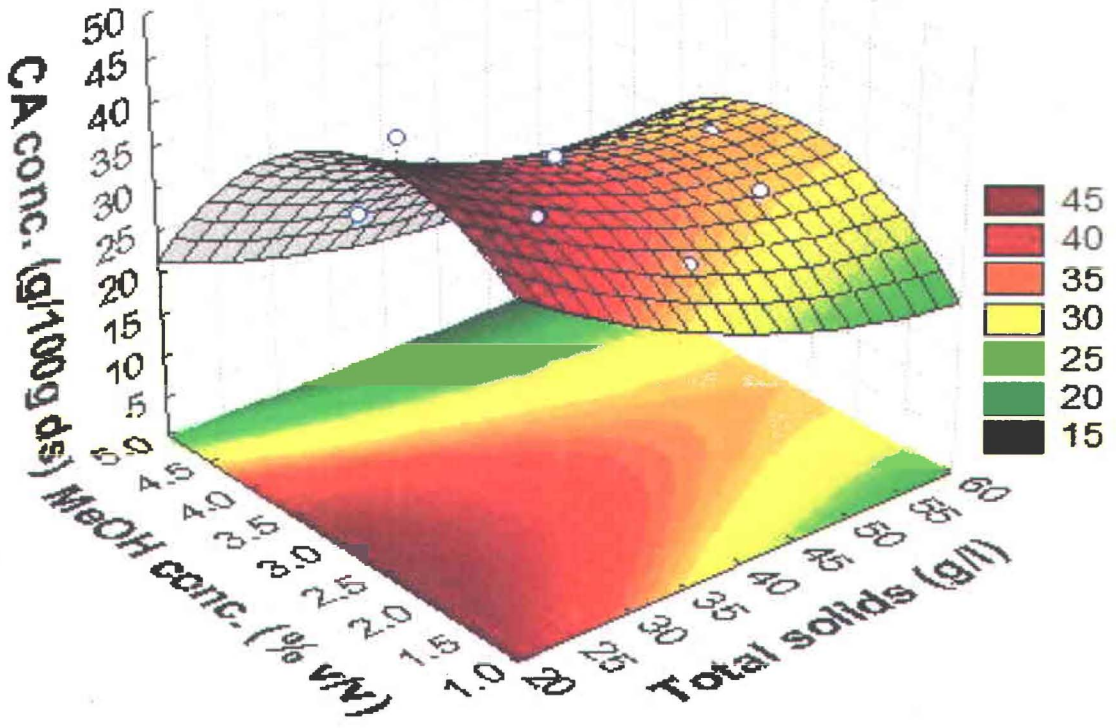


Figure 2.5. 3 Response surface plot of citric acid production using apple pomace ultrafiltration sludge obtained by varying: a) moisture (X_3) and b) the concentration of inducer i.e. methanol (X_4).

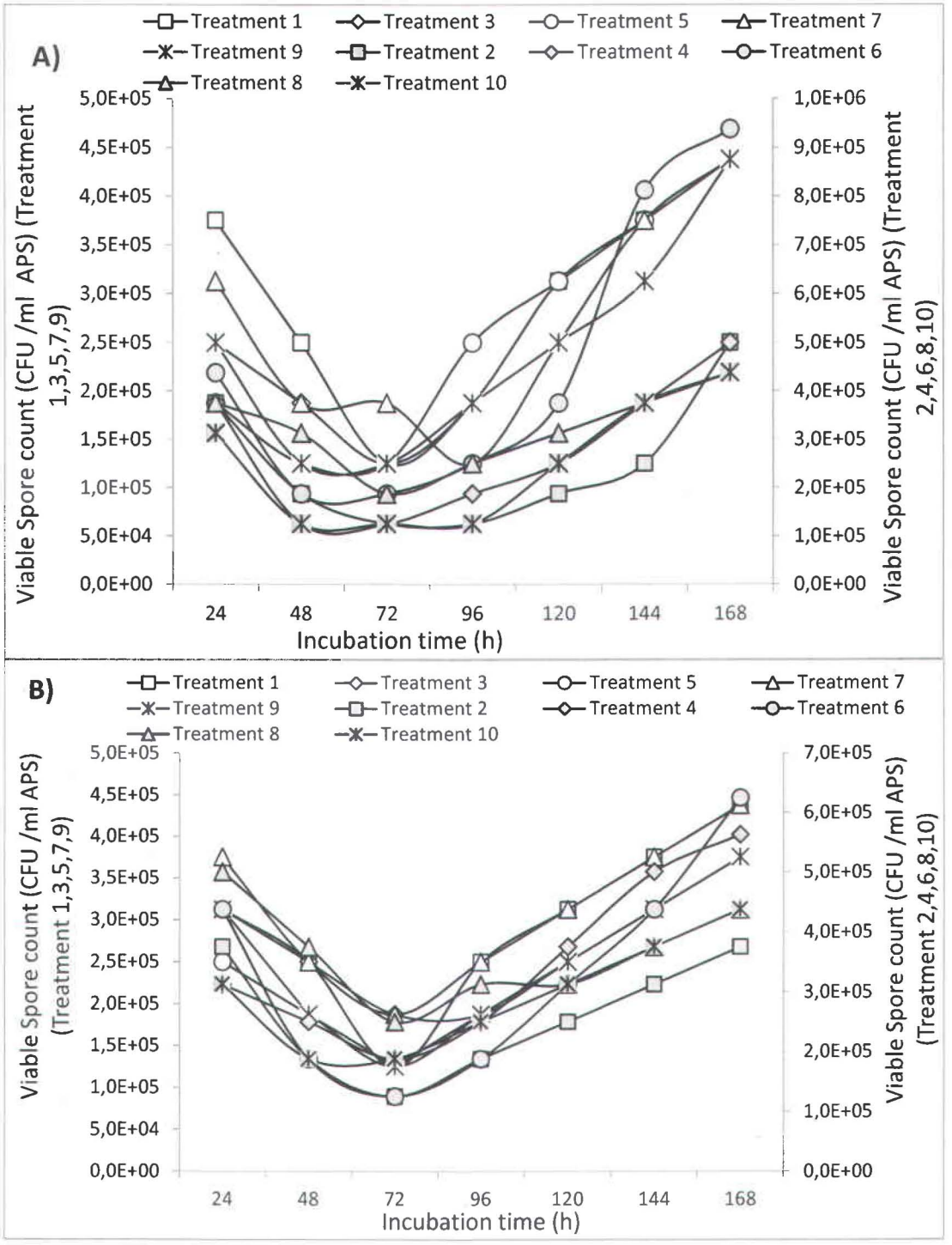


Figure 2.5. 4 Viability of *A. niger* NRRL 567 cultivated on apple pomace ultrafiltration sludge through solid-state fermentation supplemented with inducer: (A) ethanol and (B) methanol. For treatments refer to Table 2.

PARTIE III.

SOLID-STATE AND SUBMERGED CITRIC ACID FERMENTATION IN THE BENCH SCALE FERMENTERS

BIOPRODUCTION AND EXTRACTION OPTIMIZATION OF CITRIC ACID FROM ASPERGILLUS NIGER BY ROTATING DRUM TYPE SOLID-STATE BIOREACTOR

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RÉSUMÉ

La fermentation à l'état solide de l'acide citrique a été réalisée dans un bioréacteur à tambour rotatif de 12 litres L'effet des inducteurs, l'éthanol et le méthanol, a été étudié sur la bioproduction d'acide citrique par *Aspergillus niger* NRRL 567, cultivé sur des déchets solides de jus de pomme utilisés comme substrat solide. Les conditions optimales pour atteindre une bioproduction plus élevée d'acide citrique (220,6 ± 13,9 g / kg de solides secs, DS) ont été de 3% (v / v) méthanol, sous agitation intermittente de 1 h après chaque 12 h à 2 tours par minute et 1 vvm de taux d'aération et 120 h de temps d'incubation. L'optimisation de la réponse de surface s'est avérée efficace pour une extraction plus grande d'acide citrique à partir de substrat solide fermenté. L'extraction la plus élevée de 294,19 g/kg DS d'acide citrique a été réalisée dans des conditions optimales suivantes: temps d'extraction de 20 min, vitesse d'agitation de 200 tours par minute et volume de solvant d'extraction de 15 ml par réponse de surface.

Mots-clés: déchets solides de jus de pomme; *Aspergillus Niger*, l'acide citrique, l'optimisation d'extraction; fermentation en milieu solide

ABSTRACT

Solid-state citric acid fermentation was conducted in a 12-L rotating drum type bioreactor. The effect of inducers, ethanol and methanol were studied on citric acid bioproduction by *Aspergillus niger* NRRL 567 cultivated on apple pomace as a solid-substrate. Optimum conditions achieved for higher citric acid bioproduction (221±14 g/kg dry solids, DS) were 3% (v/v) methanol, intermittent agitation of 1 h after every 12 h at 2 rpm and 1 vvm of aeration rate and 120 h incubation time. The response surface optimization proved effective for higher citric acid extraction from fermented solid-substrate. Higher citric acid extraction of 294 g/kg DS was achieved at optimum conditions: extraction time of 20 min, agitation rate of 200 rpm and extractant volume of 15 ml by response surface methodology.

Keywords: Apple pomace; *Aspergillus niger;* citric acid; extraction optimization; solid-state fermentation

1. INTRODUCTION

Citric acid (CA) is the world's second largest fermentation product synthesized after industrial ethanol fermentation. CA dominates the organic acids category with an estimated bioproduction of more than 1.7 million tons per year and the volume of CA bioproduction is constantly rising at a high annual rate of 5% (Francielo et al., 2008) with future escalating trends. CA is an important multi-functional commodity chemical with a broad range of versatile applications, such as in: (1) biomedicine, e.g. synthesis of biopolymers for culturing a variety of human cell lines; (2) nanotechnology, such as drug delivery systems; and (3) agriculture, such as bioremediation of heavy metals due to its powerful sequestering action with various transitional metals (Dhillon et al., 2011a, 2011b). Considering high consumption rate and slight increase in price, the market value for this multifunctional acid was expected to exceed $2 billion in 2009 (Partos, 2005). Moreover, the demand of CA is increasing at faster pace due to various new applications coming to light which mandates the need for possible ways to increase its production. However, CA industry is currently facing challenges for economical and sustainable process development due to high substrate and energy costs.

The world's existing demand for CA is almost entirely (over 99%) met by fermentation processes, especially by *Aspergillus niger* sub-merged fermentation (~80%) (Francielo et al., 2008). However, in recent years, increasing interest has been shown in utilizing fruit pomace and other agro-industrial wastes by solid-state fermentation (SSF) for sustainable and economical CA process development (Karthikeyan and Sivakumar, 2010; Kuforji et al., 2010; Kumar et al., 2010; Dhillon et al., 2011c, 2011d). SSF is gaining worldwide attention for the production of CA due to the numerous advantages it offers, such as ability of filamentous fungus to grow efficiently and utilization of low cost agro-industrial solid waste as substrates. In fact, there are two trends for the development of the feasible process for CA bioproduction: (1) use the abundant low cost substrates, such as agro-industrial wastes; and (2) efficient extraction of CA from the fermented solid biomass. As evident from the literature, various researchers have attempted to optimize the fer-mentation medium and the process parameters for decreasing the production cost of CA for commercializing the process to industrial scale (Shojaosadati and Babaeipour, 2002; Rivas et al., 2008; Karthikeyan and Sivakumar, 2010; Kuforji et al., 2010; Kumar et al., 2010; Dhillon et al., 2011c, 2011d, 2011e).

Besides, extraction of CA from fermented solid biomass is also an important aspect of SSF. However, there is a dearth of information for CA extraction studies from fermented solid substrate. The present study involves the effect of lower alcohols using apple pomace (AP) to cultivate *A. niger* NRRL 567 for solid-state CA fermentation in rotating drum type bioreactor. The study also aims at efficient extraction of CA from the fermented solid biomass. We have not come across any report or published literature where CA extraction has been optimized using statistical design to improve CA extraction from fermented solid biomass. The present study was therefore carried out to optimize different extraction parameters, such as extraction time (min), agitation (rpm) and extractant volume (distilled H2O) (ml/g substrate) for higher extraction of CA by response surface methodology (RSM).

2. MATERIALS AND METHODS

2.1. Microorganisms and inoculum preparation

The microorganism used in this study was *A. niger* NRRL 567, procured from Agricultural Research Services (ARS) culture collection, IL, USA. This strain was maintained and periodically sub-cultured on potato-dextrose-agar (PDA) medium at 4±1 ◦C. The culture of *A. niger* was grown on PDA Petri plates and incubated for 7–8 days at 30±1 ◦C and 200 rpm. The spores were harvested from the sporulation medium plates by adding 10 ml 0.1% Tween-80 solution to each plate. To recover fungal spores devoid of mycelial contamination, filtration of harvested spores was carried out by using glass wool. Quantitative estimation of spores was performed microscopically using Nauber chamber and stored in test tubes at −20±1 ◦C for maximum of 4 weeks. The spore suspension adjusted to 1×10^7 spores/ml count was used as an inoculum.

2.2. Substrate procurement and pretreatment

Fresh apple pomace (AP) was procured from Lassonde Inc., Rougemont, Montreal, Canada. AP was selected as fermentation substrate for its potential to be used for CA production based on our previous study using flasks and tray bioreactor (Dhillon et al., 2011d). AP used in this study was already supplemented with 1% (w/w) rice husk as a common industrial practice during the extraction of juice for a better hold on the apples during meshing and filtration. AP was completely dried at 50±1 ◦C in a hot air dehydra-

tor till constant weight, grounded and was passed through sieves to get the desired particle size of 1.7–2.0 mm which was used for this study.

2.3. Solid-state fermentation

The fermentation was carried out in a 12-L rotating drum type solid-state bioreactor, Terrafor (Infors HT, Switzerland). Approximately, 3 kg of rehydrated AP (moisture 75%, v/w) was sterilized in autoclave (121±1 ◦C, 15 psi for 30 min) and was transferred into the sterilized bioreactor under aseptic condition. After transferring the substrate into the bioreactor the sterilization was done again to ensure complete decontamination and was supplemented with 3% (v/w) of inducers, ethanol and methanol, respectively. The initial pH (3.5±0.1) was taken as such without any adjustment. The inoculation was done with the spore suspension having 1×10^7 spores/g substrate. For final moisture content of 75% (v/w), the volume of inducers and spore suspension was also taken into account. The fermentation was carried out in a controlled environment at 30±1 ◦C, intermittent agitation rate of 2 rpm (for one hour after every 12 h) and aeration rate of 1 vvm. The fermentation was carried out for 12 days, and the sampling was done periodically after every 24 h. Fermented samples were extracted with distilled water and analyzed for CA and total spore count analysis.

2.4. Citric acid extraction

For CA estimation, the samples were harvested from each flask after every 24 h under aseptic conditions. CA was extracted from a solution prepared with 3 g of a fermented sample macerated with 15 ml of distilled water and shaken for 30 min in an incubator shaker at 200 rpm at 25±1 ◦C. The supernatant was filtered through glass wool for removal of solid substrate and fungal mycelia. About 100 µl sample was taken in Eppendorf tubes for viability (total spore count) assay using haemocytometer and the remaining sample was centrifuged (Sorvall RC 5C plus by Equi-Lab Inc., Québec, Canada) at 9000 × g for 20 min and the supernatant was analyzed for CA concentration.

2.5. Central composite design for CA extraction optimization

In separate experiments, optimization of CA extraction parameters from 120 h MeOH induced fermented AP was carried out using RSM. Application of RSM for the

optimization of parameters having impact on CA extraction will help in achieving higher CA extraction from fermented solid substrates. RSM helps in overcoming the limitations of time consuming conventional optimization method of 'one factor-at-a-time' (at each stage, a single factor is changed while other factors remain constant). Moreover, the statistical optimization method can evaluate the effective factors and help in building models to study interaction and select optimum conditions of variables for a desirable response. In the response surface method, the factors, such as extraction time (min) (X_1), agitation (rpm) (X_2) and extractant volume (ml/g) (X_3), were considered as independent variables and CA extraction (g/kg DS) as dependent variable.

To begin with, the screening experiments were carried out to determine the direction of optimal domain of each process. Once the provisional optimal values were determined, a central composite design (CCD) was used to find the optimal conditions of these three factors (X_1, X_2 and X_3). CCD is a first-order (2N) design augmented by additional center and axial points to allow estimation of the tuning parameters of a second-order model. In order to identify the significant factors that affect the responses, an attempt was made to improve the extraction of CA from fermented solid substrate by comparing different levels of several factors that were found to have more influence on dependent variable, i.e. CA extraction. A study of determination of the provisional optimal values of these independent variables was carried out by using the steepest ascent method and was found to be significant. In this regard, a set of 17 experiments including, 3 center points (0, 0, 0) and six axial points (α=1.68) and 8 points corresponding to a matrix of 23 which incorporates 8 experiments including 3 variables (+1, −1, 0), were carried out. Each variable was studied at two different levels (1, +1) and center point (0) which is the midpoint of each factor range. The levels of each factor along with their codes and values of the experimental design and full factorial plan are listed in Table 1 and Table 2, respectively.

A multiple regression analysis of the data was carried out by STATISTICA 7 of STATSOFT Inc. (Tulsa, U.S.) by RSM. After running the CCD experiments, a second-order polynomial regression equation that defines predicted responses (Y_i) in terms of the independent variables (X_1, X_2 and X_3) was fitted to the data [Eq. (1)]:

$$Y_i = b_{0i} + b_{1i}X_1 + b_{2i}X_2 + b_{3i}X_3 + b_{11i}X_{21} + b_{22i}X_{22} + b_{33i}X_{23} + b_{12i}X_1X_2 + b_{23i}X_2X_3 + b_{13i}X_1X_3$$

[1]

where Y_i is the predicted response, b_{0i} is intercept term, b_{1i}, b_{2i}, b_{3i} linear coefficients, b_{11i}, b_{22i}, b_{33i} squared coefficients and b_{12i}, b_{23i}, b_{13i} are interaction coefficients and i refer to the response. Combination of factors (such as X_1X_2) represents an interaction between the individual factors in the respective term. The response (CA extraction g/kg DS) is a function of the level of factors. The significance of the second-order model as shown in Eq. [1] was evaluated by analysis of variance (ANOVA). The insignificant coefficient was eliminated after the F (Fisher)-test and the final model was obtained. The analysis of variance (ANOVA) and surface plots were generated using data analysis software system version 7.0, and the optimized value of three independent variables for best response was determined using a numerical optimization pack-age of the same software. The various response surface graphs presented are function of level of factors and indicate the effect of variables individually and in combination and determine their optimum levels for CA extraction. The response surface graphs presented the second-order polynomial model which showed the predicted response of two factors at a time, holding the other two factors at fixed zero level and are in fact more helpful in interpreting the main effect and the interactions.

2.6. Analytical techniques

The moisture of the substrates was analyzed using a moisture analyzer (HR-83 Halogen; Mettler Toledo, Switzerland). For pH measurement, 1 g of substrate was mixed in 10 ml of dis-tilled water using magnetic stirrer, and the pH was recorded using pH-meter equipped with glass electrode. CA concentrations were determined gravimetrically by the modified method of Marier and Boulet (1958) as already described in Dhillon et al. (2011e). CA concentrations were expressed as g/kg of dry substrate (DS).

2.7. Statistical analysis

All the experiments were assayed in triplicates, and an average of triplicates was calculated along with the standard deviation (±SD). Database was subjected to an analysis of variance (ANOVA). One-way ANOVA followed by Student's test was used to

determine significant differences among treatment groups for CA production. For all analysis, differences were considered to be significant at $p<0.05$.

3. RESULTS AND DISCUSSION

3.1. Effect of inducers on citric acid production

Citric acid bioproduction was scaled up in a rotating drum type solid-state bioreactor based on our previous studies conducted in flasks and plastic trays using AP as substrate (Dhillon et al., 2011c, 2011d). Citric acid bioproduction trend during SSF of AP by *A. niger* NRRL 567 with intermittent agitation was given in Fig. 3.1.1 A. Significantly higher CA production was achieved with the apple pomace supplemented with methanol (221±14 g/kg DS) followed by ethanol (190±12 g/kg DS) after 120 h of incubation ($p<0.05$). However, CA production in control treatment was significantly lower (~28–38%) as compared to treatments supplemented with inducers ($p<0.05$). The enhancing effect of lower alcohols on CA production by *A. niger* NRRL 567 has been already demonstrated in previous studies (Dhillon et al., 2011c, 2011e, 2011f, 2012a). During initial experiment in bioreactor, the formation of clumps (1–2 cm balls) of AP was observed with continuous agitation of 2 rpm during fermentation. Our results were not in accordance with Mitchell and Berovic (1998) who described that agitation prevents the formation of agglomerates. Moreover, the moisture level was decreased from initial level of 75% (v/w) to 65.4% (v/w) within 7 days of incubation. Hence, the experiments were conducted with intermittent agitation for 1 h (2 rpm) after every 12 h and before sampling in order to get a homogenous sample. The moisture was analyzed after every 12 h and was maintained by spraying sterilized distilled water in the bioreactor.

Citric acid production obtained during intermittent agitation (137±11 g/kg DS) was higher as compared to continuous agitation (103±14 g/kg DS) after 120 h for control treatment. This may be due to the fact that continuous agitation during the exponential phase can break mycelia and cause the alteration of metabolism in *A. niger*. Moreover, the formation of clumps of AP with continuous agitation might cause nutrient concentration gradients and allow nutrients to become limiting at the surface. The nutrients at the interior may become inaccessible to the fungus. Mixing allowed the addition and distribution of water in the system and also enhanced efficient conductive heat transfer via the bioreactor wall. However, mixing during SSF can have detrimental effect on the

growth of the microorganism especially during the exponential phase of growth. Mechanical action leads to change in the development of the morphology of the fungus and the breakage of hyphae (Palma et al., 1996; Cho et al., 2002).

Optimum moisture content during fermentation is important for fungal growth and product formation. The moisture level of the substrate changes during the fermentation process, thus it is a prerequisite to optimize the moisture level while carrying SSF. Enough moisture must be present in the substrate to support the fungal growth and metabolism during solid-state fermentation. Lower moisture level gives a lower degree of substrate swelling and higher water tension and reduces the solubility of nutrients. AP has higher water holding capacity and therefore, allows the level of available water required for fungal growth. The growth of the microorganism is enhanced with the increase in moisture content to a certain limit and generally, decreases beyond that point. Excess moisture level might be a limiting factor to the fungal growth by decreased porosity and oxygen exchange in the SSF by forming the clumps of the solid particles (Dhillon et al., 2012b; Kaur et al., 2012). Higher moisture level also increases formation of aerial hyphae. Therefore, it becomes impervious to maintain optimum moisture content during SSF for enhanced CA bioproduction. Generally, AP has sticky nature which can result in the formation of clumps. However, in this study, AP used was supplemented with rice husk which provides advantage of increasing the porosity of AP, hence improve oxygen exchange and prevent the formation of AP clumps during the fermentation process to some extent (Dhillon et al., 2011a).

The comparison of CA production using various solid substrates and different fermentation techniques is provided in Table 3. Higher product formation in rotating drum type bioreactors can be achieved only when the drum is filled to 30% of its capacity, otherwise agitation is not efficient (Couto and Sanroman, 2006). In the present study, we used 3 kg substrate which is <30% of the bioreactor volume. Shojaosadati and Babaeipour (2002) conducted a study using a packed bed bioreactor for CA production with *A. niger* using AP as a substrate. Under the optimized conditions, the authors achieved CA production of 124 g/kg dried AP with yield of 80% based on total sugar. Similarly, Tran et al. (1998) compared CA bioproduction in flasks, trays and rotating drum bioreactor using pineapple peels by different *A. niger* strains. Higher CA production of 140 g/kg DS with 74% yield/kg sugar consumed was achieved in flasks after 3 days. The optimum conditions observed were: 65% (w/w) initial moisture content, 3% (v/w)

MeOH, 30±1 ◦C, an unadjusted initial pH of 3.4 and particle size 2 mm. However, CA in trays and rotating drum bioreactor peaked at 2 days at less than 80 g/kg dry substrate and rapidly decreased to undetectable concentrations which might be due to utilization of CA by fungus. As evident from Table 3, higher CA production was obtained in the present study using rotating drum type solid-state bioreactor as compared to previous studies using different fermentation methods.

Some studies have indicated that the bed of solid substrate can be continuously mixed without detrimental effects. Nagel et al. (2001) studied continuously mixed SSF with *Aspergillus oryzae* cultivated on wheat grain in a paddle mixer. They concluded that continuous mixing improves process control, because it allows addition and distribution of water during fermentation, reduces gradients in the bed, and improves heat transfer to the bioreactor wall. They also concluded that continuous mixing does not have a negative impact on the fermentation by showing that respiration rates were comparable to those in unmixed cultures. Nagel et al. (2001) also observed that the fungus grew primarily inside the wheat grain during continuously mixed SSF; hardly any mycelium was observed on the grain surface and the amount of aerial mycelium was negligible. In contrast, Han et al. (1999) investigated the effect of intermittent mixing on enzyme production during cultivation of *Rhizopus* on soybeans and concluded that intermittent mixing may negatively affect enzyme production. Compared with the work of Nagel et al. (2001) there are four differences: (1) the morphology of the fungus; (2) the structure and firmness of the solid substrate; (3) the fermenter layout; and (4) the mixing regime. Hence, mixing during SSF is beneficial, but it should be discontinuous rather than continuous. Our results clearly showed that the intermittent agitation during SSF of *A. niger* cultivated on AP significantly increased the CA production ($p<0.05$). At the same time, agitation facilitates the removal of volatile metabolic products, enhances heat exchange, protects the medium against local desiccation or excessive moistening, and improves the conditions for microbial growth. Agitation enables a more effective distribution of the spore suspension, water required for moisture control, or of any other nutrient solutions, if necessary (Suryanarayan, 2003). Another benefit of agitation is a more evenly distributed airflow in the medium. However, higher agitation can break the growing mycelium of filamentous fungus hampering the growth and product formation.

In SSF, the temperature of the fermentation medium tends to increase with the increase in incubation period owing to the metabolic activities and reduced heat removal. The

elevated temperatures in SSF proved to be detrimental to the fungal growth and hence the product formation. The primary objective of aeration is to supply an appropriate amount of oxygen for microbial growth and to remove carbon dioxide. However, aeration also performs a critical function in heat and moisture transfer between the solids and gas phase (Shojaosadati and Babaeipour, 2002). It is essential to note that excessive aeration can produce shear stress which has a detrimental effect on the morphology of filamentous fungus (Shojaosadati and Babaeipour, 2002). The aeration is normally a very important parameter in solid state fermentation of aerobic microorganisms. Aeration of solid state culture plays four functions, namely to maintain aerobic conditions, for the elimination of carbon dioxide, for the regulation of temperature in culture and the regulation of water content (Raimbault, 1998). Aeration during solid state fermentation is easier than in liquid fermentation as on the one hand, there is rapid diffusion of oxygen in the wet film surrounding the particles substrate, and also there is large contact surfaces between the gas phase, the substrate and aerial mycelia. The lack of aeration decreases the heat and mass transfer in SSF (Shojaosadati and Babaeipour, 2002).

3.2. Fungal viability

The results of *A. niger* viability in terms of total spore count (CFUs/g) during the fermentation of AP in bioreactor is presented in Fig. 3.1.1 B. These results showed that the supplementation with inducer, i.e. lower alcohols influenced the viability of fungus. During AP fermentation without inducer, higher viability of *A. niger* (8.8×10^7 CFUs/g) was obtained after 144 h of incubation. How-ever, in the other treatments supplemented with ethanol and methanol, respectively, lower viability of *A. niger* (3.6×10^7 and 2.75×10^7 CFUs/g) was obtained after 144 h of incubation time. The effect of lower alcohols on sporulation and growth has been described in previous studies (Dhillon et al., 2011c, 2011d).

3.3. Effect of extraction parameters on CA extraction optimization

Extraction of products from the solid substrate is an important aspect of SSF. An ideal extraction process could extract the product selectively and efficiently at ambient temperature and agitation with minimal contact time. The recovery of CA from fermented solid substrates depends upon the interactive forces between the acid and substrates.

Higher extraction can be achieved by optimizing important extraction parameters leading to reduction of these interactive forces. The hydrophobic/hydrophilic nature of fungal mycelium, Vander Waals forces and ionic and hydrogen bonds also determine the efficacy of the product recovery. Using the method of experimental factorial design and response surface analysis, the optimal conditions to obtain higher CA extraction (g/kg DS) from fermented AP was determined. The validity of the model was proved by fitting different values of the variables into the model equation. The data were fitted into a second-order polynomial function (Eq. (1)) and a correlation was drawn between experimental data and the predicted values by the model as given in Fig 3.1.2. The statistical significance of the second-order polynomial model was verified by ANOVA. The quality of the model fit was evaluated by the coefficient R^2 which represents the proportion of variation in the response data and it can be explained by the fitted model. High R^2 was considered as an evidence for the applicability of the model in the range of variables included. It should be noted that an R^2 value > 0.75 indicates the aptness of the model. The analysis indicated that the second-order polynomial model resulted in a determination coefficient R^2 value of 0.876, which ensured a satisfactory adjustment of the quadratic model to the experimental data.

Analysis of variance including the ratio of the level mean square (MS) and the residual MS following a Fisher (F) distribution with degrees of freedom (df) are shown in Table 3.1.3. Other main effects have MS higher than the residual MS which showed that most of the variation in the data of CA extraction is accounted by the separate effect of independent variables except for linear effect of agitation (X_2) and quadratic effect of X_1, X_2 and X_3. The overall quadratic model was significant with R^2 value of 0.88 for CA extraction, indicating that 88% of the total variation around the average could be explained by the regression. Statistical analysis indicated that the first-order effects of X_2 variable were statistically significant with a good confidence interval (p<0.002). The second-order interaction between X_1X_2, X_1X_3 and X_2X_3 are significant at a 90–95% confidence level (Table 3.1.3).

The significance of three factors, such as extraction time (min) (X_1), agitation rate (rpm) (X_2) and extractant volume (ml/g) (X_3) and their interaction was judged by its p-value being closer to 0. The p-value should be less than 0.1, 0.05 and 0.01 for a 90%, 95%, and 99% confidence level, respectively for the factors to be termed as significant. Based on the effects of variables using the Pareto chart (Fig. 3.1.3), both agitation and

extractant volume should be in optimal values for effective extraction of CA. It is also clear from the results that both agitation and extractant volume should be in optimal values for effective extraction of CA. The final response function to predict CA extraction after eliminating the non-significant terms at a confidence level of 95% is given in Eq. (2) (Table 3.1.4).

$$\text{CA extraction (Solid substrate)} = 289.2 + 80.1X_2 + 97.5X_{22} - 102.3X_{23} \quad [2]$$

3.4. Model graphs

The response surface graphs shown in Figs. 3.1.4–3.1.6, present the second-order polynomial model which showed the experimental response of two factors varied within their experimental range and holding the third factor at fixed center level and are in fact more helpful in interpreting the main effect and interactions. The response for the interactive factors, extraction time (X_1) and agitation (X_2), when extractant volume was fixed at central point is depicted in Fig. 3.1.4. Higher CA extraction was obtained in the range of 20 min extraction time and 200–240 rpm agitation rate. Further increase in agitation rate can have negative effect on response. The response surface shown in Fig. 3.1.5 was based on the final model in which agitation rate was kept at central value and extraction time and extractant volume was varied within its experimental range. It is clear from the model graph responses that the interaction of these two variables had a significant effect on response. Higher CA extraction was obtained at extraction time of 20 min and extractant volume of 15 ml. Similarly, the response surface graph of CA extraction affected by the agitation and extractant volume at constant extraction time (Fig. 3.1.6) showed that highest CA extraction (~294 g/kg DS) occurred at 15 ml extractant volume and 200 rpm agitation rate. Further increase in value of both the variables had negative effect on the response. Therefore, these optimized conditions could be applied for enhanced CA production using AP. AP could be used as a potential substrate, due to the fact that the increment of cost of raw substrates for CA production raises interest in fermentation processes for production of this compound from renewable resources. No addition of expensive media is required and the use of inexpensive agro-industrial wastes, such as AP, will have important economic and environmental advantages.

4. CONCLUSIONS

Apple pomace supplemented with rice husk for culturing *A. niger* NRRL 567 for citric acid bioproduction and extraction of citric acid from fermented solid substrate through response surface method-ology led to the following conclusions:

(1) Supplementation with methanol and ethanol rendered higher citric acid production of 221±14 and 190±12 g/kg DS after 120 h of incubation (p<0.05) which was 37.7% and 27.6% higher as compared to control.

(2) The second-order response surface model proved adequate to achieve higher citric acid extraction from fermented solid-substrate within three independent variables namely, extraction time (min) (X_1), agitation rate (rpm) (X_2) and extractant volume (ml/g) (X_3).

(3) All the three independent variables showed significant effect on citric acid extraction.

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Table 3.1. 1 Experimental range of the three independent variables studied using central composite design (CCD) in terms of actual and coded factors

		Coded levels				
Variables	**Symbol**	**−1.682**	**Low (−1)**	**Mid (0)**	**High (1)**	**+1. 682**
Extraction time (min)	X_1	11.10	15	20	25	28.94
Agitation rate (rpm)	X_2	110.56	150	200	250	289.44
Extractant vol. (ml/g)	X_3	6.10	15	20	25	23.94

Table 3.1. 2 Results of experimental plan by central composite design (CCD) for apple pomace (shaded cells indicate higher citric acid (CA) extraction)

Trial	X_1	X_2	X_3	CA (g/ kg DS)
1	-1.0	-1.0	-1.0	115.98
2	-1.0	-1.0	1.0	103.74
3	-1.0	1.0	-1.0	165.72
4	-1.0	1.0	1.0	183.61
5	1.0	-1.0	-1.0	129.10
6	1.0	-1.0	1.0	113.44
7	1.0	1.0	-1.0	185.70
8	1.0	1.0	1.0	197.38
9	-1.68	0.0	0.0	247.61
10	1.68	0.0	0.0	252.85
11	0.0	-1.68	0.00	72.45
12	0.0	1.68	0.0	243.98
13	0.0	00.0	-1.68	98.70
14	0.0	0.0	1.68	202.62
15 (C)	0.0	0.0	0.0	294.20
16 (C)	0.0	0.0	0.0	290.31
17 (C)	0.0	0.0	0.0	294.24

Abbreviations: X_1, X_2 and X_3 represents three independent variables i.e. extraction time (min), agitation (rpm) and Extractant volume (ml/g), respectively.

Table 3.1. 3 Analysis of variance (ANOVA) for CA extraction from fermented solid substrate as a function of three independent variables

Model	CA extraction				
	Sum of squares (SS)	Degrees of freedom (df)	Mean square (MS)	*F*-ratio	*p-value*
X_1	301.66	1	301.66	0.19	0.67
X_2	23124.2	1	23124.2	15.01	0.006*
X_3	2444.6	1	2444.6	1.59	0.24
X_1X_2	15.10	1	15.10	0.009	0.92
X_1X_3	11.46	1	11.46	0.007	0.93
X_2X_3	412.0	1	412.0	0.27	0.62
X_1^2	5352.2	1	5352.1	3.47	0.10
X_2^2	31785	1	31785	20.63	0.002*
X_3^2	34939.6	1	34939.6	26.68	0.002*
Residual	1540.7	-	-	-	-
Total	87183	16	-	-	-
R^2 coefficient	0.876				

*Significant (p<0.05)

Table 3.1. 4 Comparison of CA production using various solid substrates using different fermentation techniques

Aspergillus strains	Substrate/ fermentation technique	CA yield (g/kg ds)	Optimum conditions	Incubation time/ temperature	References
A. niger NRRL 2001	Corn husk	259±10 g	70 % moisture	120 h/30 °C	Hang and Woodams (2000)
A. niger BC-1	AP/ multilayer packed bed solid-state bioreactor	124 g	Moisture -78 % (w/w), PS-0.6-1.18 mm)	30 °C/5 days	Shojaosadati and Babaeipour (2002)
A. niger NRRL 567	SPM + glucose/ column bioreactor	123.9 g	Aeration rate of 0.84 vvm, bed depth of 22 cm	32 °C/ 12 days	Barrington et al. (2009)
A. niger van. Tieghem MTCC	AP/ Flasks	46 g	4 % (v/w) MeOH	30 °C /5 days	Kumar et al. (2010)
A. niger MTCC 282	Banana peels/flasks	180 g	70 % moisture	28 °C/72h	Karthikeyan and Sivakumar (2010)
A. niger NRRL 328	Pine apple waste/		54.8 % moisture	144 h	Kuforji et al. (2010)
A. niger ATCC 9142 (NRRL 599)	Orange peels/flasks	193 g	Inoculum of 0.5×10^6 spores/gds, and moisture 70 %	30 °C /84 h	Torrado et al. (2011)
A. niger NRRL 567	AP + rice husk (1 % w/w)/Plastic trays	342.4 g	75 % (v/w) moisture, 3 % v/w) MeOH,	30±1 °C/120 h	Dhillon et al. (2011d)
A. niger NRRL 567	AP + rice husk (1 % w/w)/ Rotating drum type solid-state bioreactor	294.19 g	75 % (v/w) moisture, 3 % v/w) MeOH, intermittent agitation of 1 h after every 12 h at 2 rpm and 1vvm of aeration rate	30±1 °C/120 h	Present study

Abbreviations: AP- apple pomace; DS- dry substrate; MeOH- methanol; PS-particle size

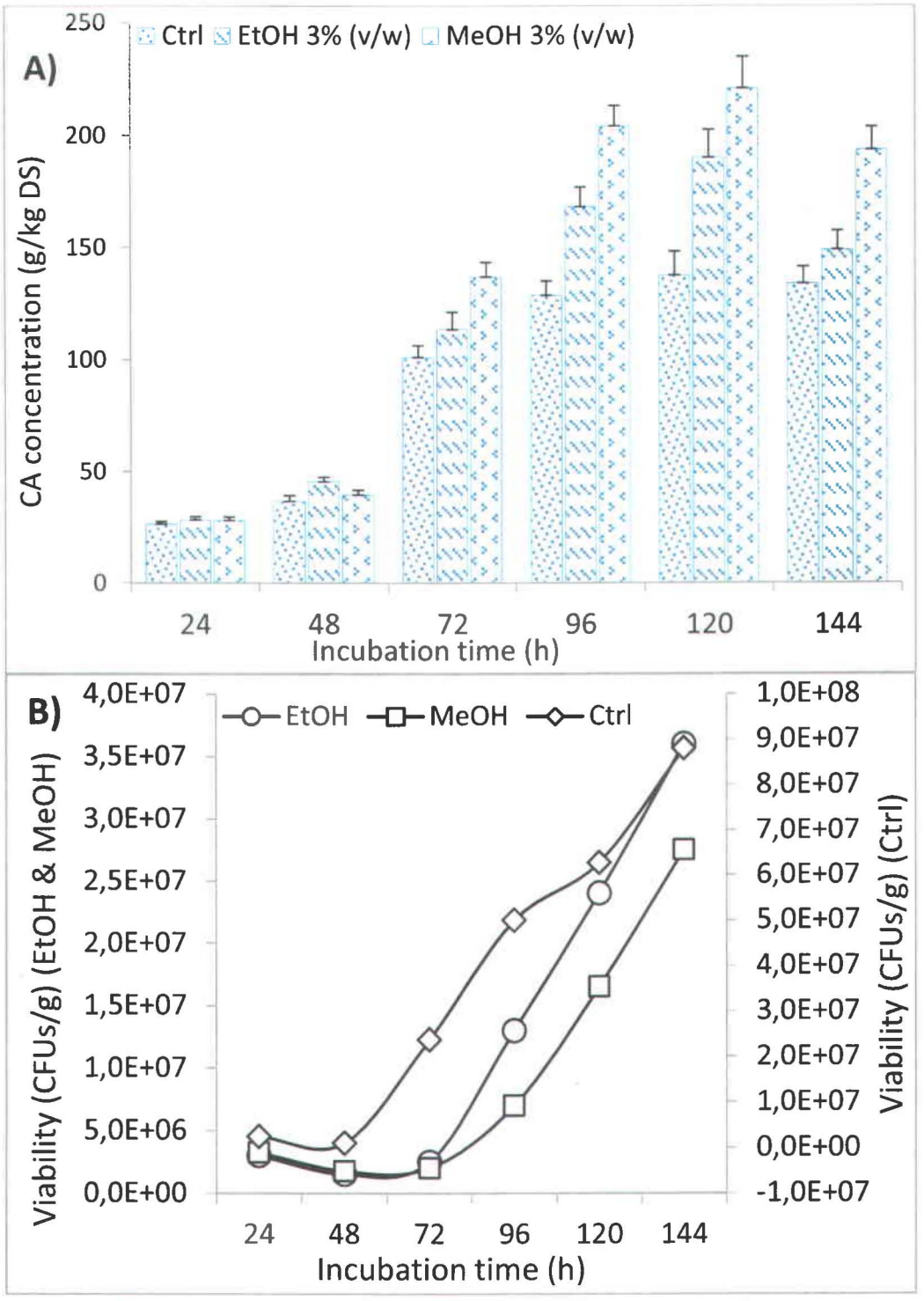


Figure 3.1. 1 (A) Citric acid (CA) production (g/kg dry substrate) and (B) viability trend of *A. niger* NRRL 567 during solid-state fermentation using apple pomace (AP) as a substrate.

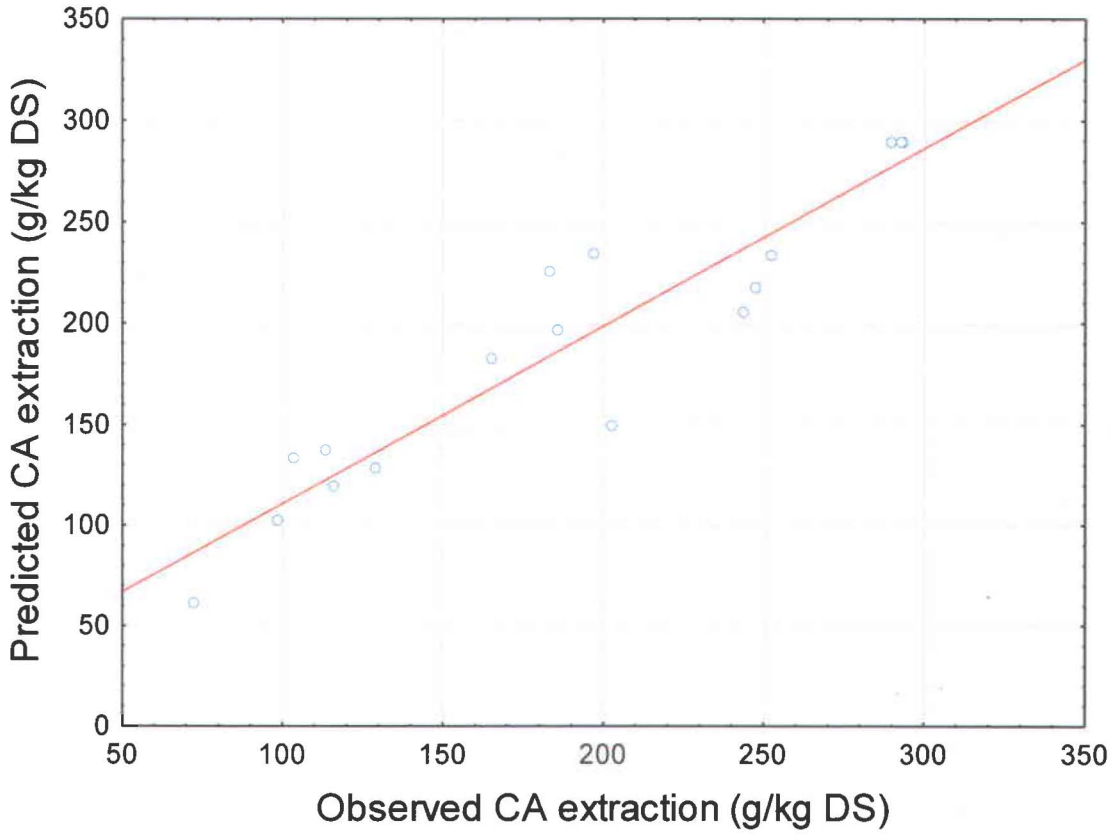


Figure 3.1. 2 Parity plot showing the distribution of experimental data versus predicted value by the model for citric acid (g/kg dry substrate) extraction from fermented apple pomace.

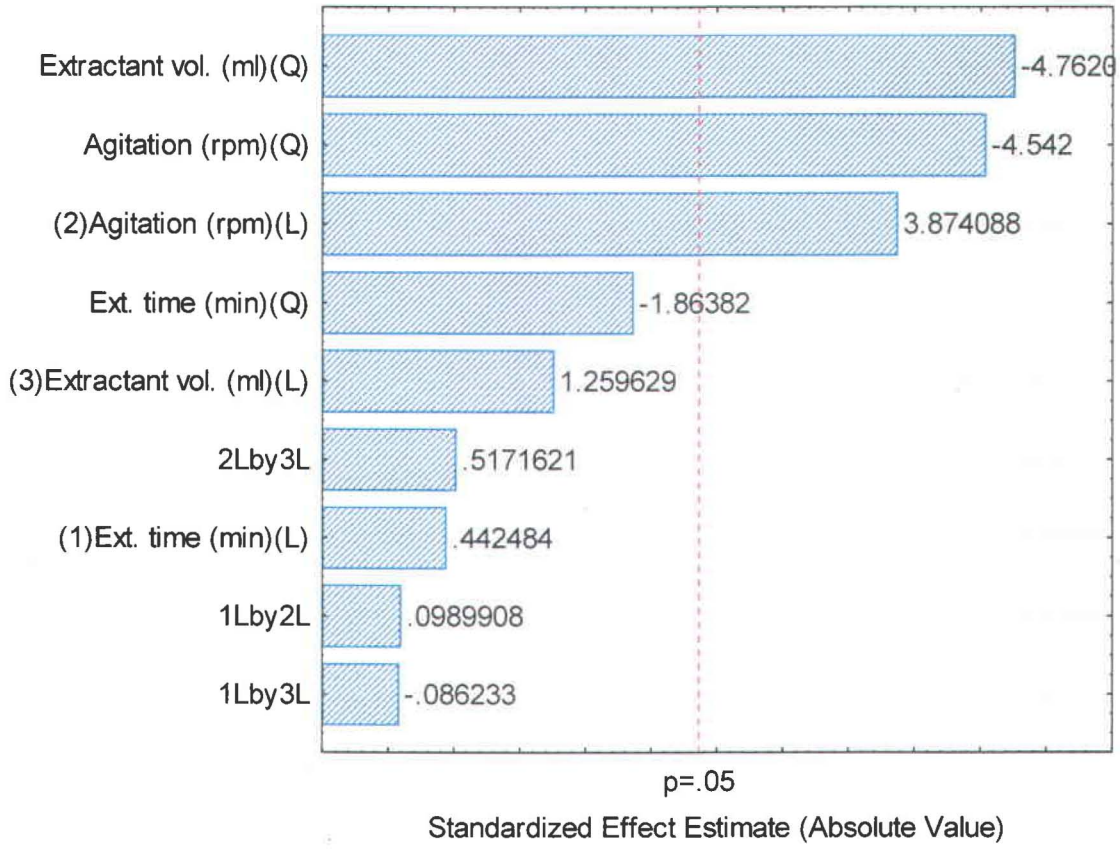


Figure 3.1. 3 Pareto charts showing the effect of variables i.e. extraction time (min) (X_1); agitation rate (rpm) (X_2) and extractant vol. (ml/g) (X_3) on: A) response i.e. CA production and; B) viability of *A. niger* NRRL in 22 factorial design

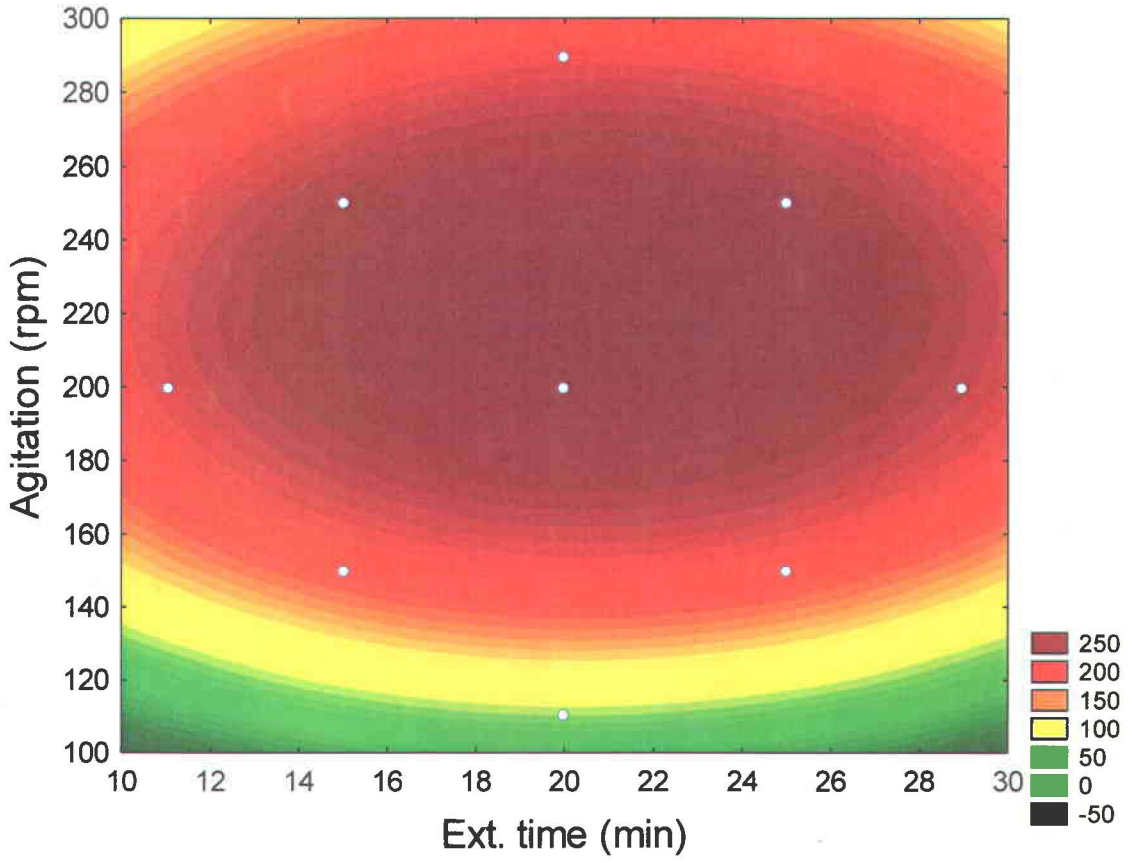


Figure 3.1. 4 Response surface plots of citric acid extraction (g/kg dry substrate) as a function of (1) extraction time (min) and (2) agitation rate (rpm) (extractant volume constant).

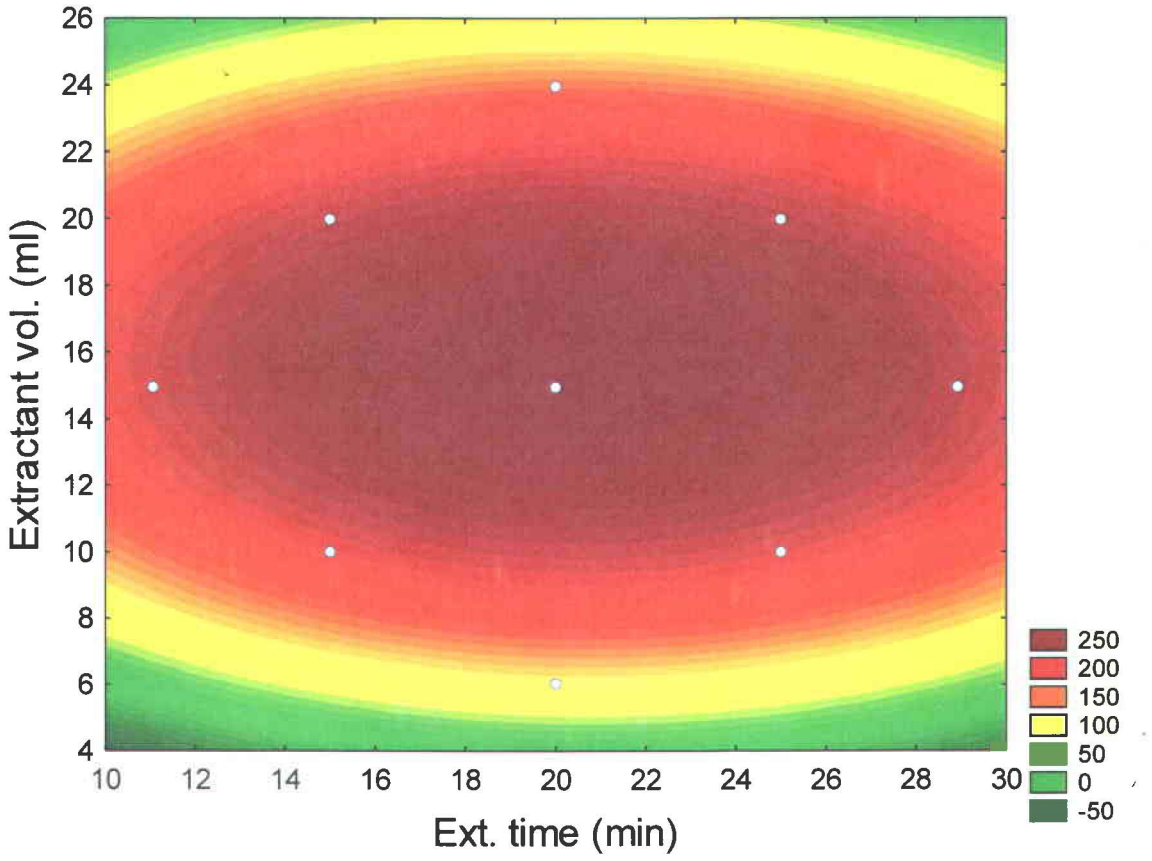


Figure 3.1. 5 Response surface plots of citric acid extraction (g/kg dry substrate) as a function of: (1) extraction time (min) and (2) extractant volume (ml/g) (agitation rate constant).

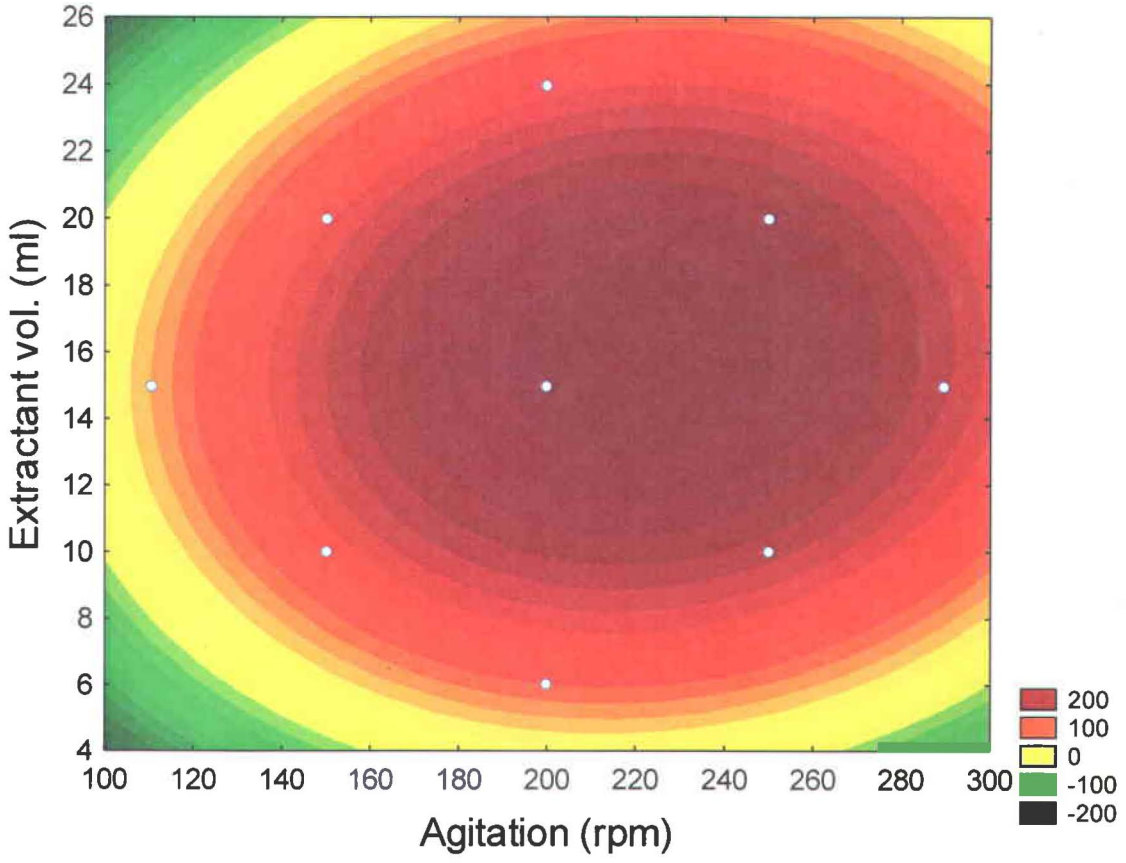


Figure 3.1. 6 Response surface plots of citric acid extraction (g/kg dry substrate) as a function of: (1) agitation rate (rpm) and (2) extractant volume (ml/g) (extraction time constant).

RHEOLOGICAL STUDIES DURING SUBMERGED CITRIC ACID FERMENTATION BY *ASPERGILLUS NIGER* IN STIRRED FERMENTER USING APPLE POMACE ULTRAFILTRATION SLUDGE

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RÉSUMÉ

Des études de profil rhéologiques pour les boues d'ultrafiltration de jus de pomme (BUF) de bouillon de champignons filamenteux, *Aspergillus niger* NRRL-567, au cours de la fermentation de l'acide citrique (AC) ont été réalisées pour la biomasse et développées dans un fermenteur de 7,5 l. Les bioproduction d'AC de 23,7 ± 1 g / l et d'APS de 40,3 ± 2 g / l ont été obtenues durant le contrôle (120 h) et en traitement par un inducteur, MeOH 3% (v / v), respectivement, après 132 h de fermentation par *A. niger* NRRL 567. Les propriétés rhéologiques du bouillon de fermentation y compris celles des boulettes ont été analysées. Le contrôle a montré un comportement pseudo-plastique non-newtonien en raison d'une croissance plus filamenteuse. Toutefois, durant le traitement complété par un inducteur (méthanol), la forme de boulette a été observée pendant la fermentation avec des bouillons moins visqueux et non-newtoniens. Le modèle de loi de puissance a été suivi avec un bon indice de confiance (77,8 à 88,9%) tout au long de la fermentation, durant le traitement avec le MeOH comme inducteur. Cependant, la loi de puissance a été suivie avec un indice de confiance plus modéré (65,3 à 75,9%), au début de la fermentation jusqu'à 48 h, puis, suivi plus tard d'un bon indice de confiance (75.9 à 88.2%) jusqu'à 144 h de fermentation en contrôle. D'importants changements rhéologiques se sont produits dans le bouillon pendant la phase de production maximale d'AC et ont été accompagnés d'une augmentation de la biomasse sous forme de boulettes de mycélium.

Mots-clés: boues d'ultrafiltration de jus de pomme: comportement non-newtonien; rhéologie; fermentation à l'état liquide

ABSTRACT

Rheological profile studies for apple pomace ultrafiltration sludge (APS) broth with filamentous fungus, *Aspergillus niger* NRRL-567 during citric acid (CA) fermentation are reported for the biomass developed in a 7.5 l bench scale fermenter. CA bioproduction of 23.7±1 g/l APS and 40.3±2 g/l APS was obtained in control (120 h) and in treatment with inducer, MeOH 3% (v/v), respectively, after 132 h of fermentation by *A. niger* NRRL 567. The rheological properties of the fermentation broth including pellets were analyzed. The control demonstrated non-Newtonian pseudoplastic behavior due to more filamentous growth. However, in treatment supplemented with inducer (methanol), pellet form was observed during fermentation with less viscous non-Newtonian broths. The power law model was followed with good confidence of fit (77.8–88.9%) throughout the fermentation in treatment with MeOH as an inducer. However, power law was followed with moderate fit of confidence (65.3–75.9%) at the beginning of fermentation until 48 h and later followed a good confidence of fit (75.9–88.2%) until 144 h of fermentation in control. Important rheological changes occurred in the broth during maximal CA production phase along with increase in biomass in the pelleted mycelium form.

Keywords: apple pomace ultrafiltration sludge: *Aspergillus niger*; Non-Newtonian behavior; rheology; submerged fermentation

INTRODUCTION

A large number of industrial fermentations use filamentous microorganisms for value-addition applications, such as organic acids, antibiotics and enzymes production, among others. The rheological properties of fermentation broth employing filamentous microorganisms are of considerable importance as it describes the transport phenomenon in bioreactors. The rheology of broth during fermentation is defined as the suspension of microorganisms and other components resulting from the net effect of conversion and degradation of initial growth medium components to different metabolites, change in morphology of microorganisms and increase in biomass (Brar et al. 2007). Non-Newtonian flow behavior is the fundamental aspect of fungal fermentation systems, especially the filamentous fungi. The fermentation broth exhibits distinct non-Newtonian characteristics even if the solid content is low (Dhillon et al. 2011a). The problem of mass and heat transfer as well as energy consumption is directly related to the rheological behavior of broths which results in decreased process productivity. Furthermore, these problems are increased during scale-up of the process due to complexity of the medium and generally lower agitation rates.

The processing of apples to various products generates several million tonnes of apple wastes [5–10% apple pomace sludge (APS) and 25–30% apple pomace (AP)] (Dhillon et al. 2011b). Every year, thousands of tons of apple industry wastes [AP and APS] are generated by apple processing industries in Canada. In 2008–2009, worldwide apple production exceeded 69,603,640 tonnes (Food and Agriculture Organization (FAO) of the United Nations, http://faostat.fao.org) out of which Canada contributed 455,361 tonnes. AP has been used for the production of citric acid, hydrolytic enzymes and for the extraction of polyphenols in previous studies (Ajila et al. 2011; Dhillon et al. 2011b, c, d, e; Kaur et al. 2012). Currently, APS finds no value-added applications.

In our previous study, we used APS for the first time for CA bioproduction with *Aspergillus niger* NRRL 567 through response surface methodology (Dhillon et al. 2011f). Higher CA production was achieved with suspended solids (SS) concentration of 25 g/l APS. Suspended solids concentration plays an important role in broth rheology and affects the final concentration of CA. Higher SS concentrations resulted in highly viscous broths affecting the mixing of the medium contents and thus the mass and heat transfer processes which in turn hinders the oxygen transfer to the fungal cells. This is

considered as the rate-limiting step of CA fermentation. In view of these limitations, an understanding of the broth flow behavior is necessary to develop design strategies that will help overcome possible limitations in mass and heat transfer in bioreactors, and finally, downstream processing. In this context, this study reported the bench-scale process with previously optimized SS solid (25 g/l APS) concentration for higher CA production. The rheological behavior during submerged CA fermentation by *A. niger* NRRL-567 using APS was studied. So far, to the best of our knowledge, there is no study reported on the rheological studies using APS for the bioproduction of CA, an important chemical commodity.

MATERIALS AND METHODS

Microorganism and Inoculum Preparation

A. niger NRRL-567 was obtained from Agricultural Research Services (ARS) culture collection, IL-USA and cultivated in freeze-dried form. It was revived onto potato dextrose broth medium (PDB) for 7–8 days at 30±1 °C and 200 rpm. The culture conditions and maintenance of fungus have been already described in Dhillon et al. (2011b). The inoculum was prepared by culturing *A. niger* on PDB for 36 h at 30±1 °C and 200 rpm.

Fermentation Medium

Apple pomace ultrafiltration sludge (Lassonde Inc., Rougemont, Montreal, Canada) was used as fermentation medium due to its higher potential for CA production, based on previous screening studies (Dhillon et al. 2011c). APS contains high macro- and micronutrient contents (total carbon 51.9 g/l, total nitrogen 2.94 g/l, carbohydrates 66.0±1.7 g/l, lipids 5.9±0.3 g/l, protein 33.75±2.0 g/l, among others) (Dhillon et al. 2011b). For experimental purposes, the desired total solids concentrations (25 g/l of APS) were made up by adding distilled water. The pH of the medium was adjusted to 3.5±0.1 using a pH meter equipped with glass electrode.

Submerged CA Fermentation

APS having 25 g/l SS concentration was used as a substrate for CA bioproduction. All the experiments were performed in a 7.5 l capacity fermenter with 4.5 l working volume (Labfors, HT Bottmingen, Switzerland). APS was sterilized at 121±1 °C for 30 min. The medium was inoculated with 10% (v/v) inoculum concentration. After sterilization, methanol (MeOH) was added at 3% (v/v) concentration as an inducer for CA production. Temperature and pH of the fermentation medium was controlled at 30±1 °C and 3.5, respectively. To maintain dissolved oxygen concentration above 20% saturation (critical oxygen concentration), the medium was agitated at a speed of 300 rpm, and the air flow rate was automatically controlled using a computer controlled system. Samples were withdrawn from the fermenter at 12-h intervals for the viability assay, citric acid and rheological properties (viscosity) of the fermented broth.

Broth Rheology

Rheological properties of fermented APS were determined by using a rotational viscometer Brookefield DV II PRO+ (Brookfield Engineering Laboratories, Inc., Stoughton, MA, USA) equipped with Rheocalc32 software (for rheological models). Three different spindles, namely, SC-34 (small sample adaptor), L-22 and ULA (ultralow centipoise adapter) were used with a sample cup volume of 20–50 ml (spindle dependent). The gaps between spindle and sample chamber were 1.235 mm for ULA (viscosity range, 1.0–30 mPa s) and 4.830 mm for SC-34 (viscosity range, ≥30 mPa S) to adapt to the analyzed samples of APS. Time-dependent profile was studied at low shear rate (7.34 s−1), and viscosity at each sampling point was measured at 36.71 s−1. The shear rate behavior was determined from 0.1 to 200 s−1. The viscosity and rheological analysis was performed by using Rheocalc V2.6 software, (BEAVIS: Brookfield Engineering Advanced Viscometer Instruction Set). All measurements were performed at 25±1 °C with a measurement time lag of 1 min between consecutive shear rates. Viscosity was referred to as "apparent viscosity", unless stated otherwise. Different rheological models were considered using the viscosity input information as described in "Results and Discussion" section.

Microscopic Analysis and Fungal Viability Assay

The fungal mycelium was stained with aniline blue (0.1 mg/ ml in distilled water) to observe the cell morphology or any cellular damage during fermentation using a Zeiss Axioplan light microscope with a ×10 and × 40 objective. Viability of fungal mycelium was assayed using most probable number method (Thomas 1942). Viability assay was used as an indicator or measure of the growth in terms of amount of living fungal biomass in solid-state fermentation. The fermented APS was incubated in wrist action shaker (Burrell Scientific, PA, USA) for 30 min in order to separate mycelium from pomace particles. After incubation, the sample was filtered through muslin cloth, and the clear supernatant obtained was used for viability assay. In total, five dilutions per sample were used for the viability assay. Each dilution (six spots×10 µl) was aseptically poured on an agar plate at six different spots, and for each sample, two plates were made. The plates were incubated for 24 h, and the growing spots were calculated. While calculating the results, "the first dilution where all the pipetted spots did not grow and the last dilution where at least one pipetted spot showed positive results and all the samples between these spots were taken into consideration". The results in colony-forming units (CFUs) were calculated according to the following formula:

$$MPN = P \div (N_n \times N_k)^{0.5}$$

Where P = the number of positives in all the accounted series; Nn = amount of sample in the negative parallels (grams); N_k = amount of sample in all the accounted series (grams).

Analytical Methods

The total solid content of APS was estimated by drying 25 ml sample in an oven at 105±5 °C to constant weight. The pH of the substrate was measured using a pH meter (EcoMet, Istek, Seoul, South Korea) equipped with glass electrode. For experimental purpose, the required suspended solid concentration was made up with distilled water. CA concentrations were gravimetrically measured by the modified method of Marier and Boulet (1958). Details of CA analysis method have been given in Dhillon et al. (2011c).

Statistical Analysis

CA values presented are an average of three replicates along with the standard deviation (±SD). Database was subjected to an analysis of variance (ANOVA), and multiple range tests among data were carried out using the Statistical Analysis System Software (STATGRAPHICS Centurion, XV trial version 15.1.02 year 2006, Stat Point, Inc., USA), and the results which have $p<0.05$ were considered as significant.

RESULTS AND DISCUSSION

Citric Acid Production Trend

Figure 3.2.1 illustrates CA production and viability of *A. niger* NRRL-567 using APS at different time intervals in a control and inducer treatment fermentation. CA production of 23.7±1 g/l APS and 40.3±2 g/l APS, respectively, was obtained in control (120 h) and treatment with inducer, MeOH 3% (v/v) after 132 h of fermentation period ($p< 0.05$). Similarly, higher viability of 5.89×10^8 CFUs/ml after 108 h and 4.14×10^8 CFUs/ml after 102 h incubation time, respectively, was observed in control and MeOH supplemented treatment.

Supplementation with 3% (v/v) MeOH gradually increased CA production during fermentation which reached its highest values of 40.3±2 g/l APS by *A. niger* NRRL 567 at 132 h as seen in Fig. 1. Addition of MeOH to the fermentation medium enhanced CA concentration by 41.32% as compared with control ($p<0.05$). In our previous study, CA production of 44.9 g and 37.9 g was obtained in flasks using APS (25 g/l SS) through response surface methodology when supplementing with MeOH and EtOH [3% (v/v)] as an inducer (Dhillon et al. 2011c). The physical properties of mycelium suspensions are different from bacterial and yeast cultures. The fermentation broths involving mycelial microorganisms possess two types of characteristic growth patterns: (1) filamentous and (2) pellet forms. These morphological growth forms can have significant effects on fermentation broth rheology and thereby affect bioreactor performance. During filamentous stage, the entanglement of mycelial hyphae along with high concentration of biomass may lead to highly viscous non-Newtonian behavior. Various studies demonstrated that supplementation of lower alcohols, such as MeOH resulted in pellet morphology (Dhillon et al. 2011a, b). The suspension of pellets is usually less viscous as compared with suspensions of filamentous growth. In pellet form, the mycelium usually

adopts stable spherical aggregate form comprising of more or less dense, branched and partially intertwined network of hyphae having diameter of several millimeters and usually gives less viscous non-Newtonian broths. The pellet growth form is highly recommended for enhanced submerged CA fermentation (Papagianni 2004; Dhillon et al. 2011a, b, c). Small pellets can reduce clump formation and viscosity of the broth during fermentation and hence render higher CA production (Papagianni 2004; Dhillon et al. 2011b, c). The effect of lower alcohols on submerged CA fermentation has been well documented (Barrington and Kim 2008; Rivas et al. 2008; Nadeem et al. 2010; Dhillon et al. 2011b, c). MeOH also acts as an inducer for the activity of enzyme citrate synthase (Barrington and Kim 2008). MeOH and EtOH markedly increased the tolerance levels of fungus for trace metals, such as manganese, iron and zinc far above the required levels for inoculum growth. Owing to their stimulatory effect, lower alcohols, such as MeOH and ethanol (EtOH), are widely used in the industrial production of CA. The addition of lower alcohols thus permitted utilization of crude carbohydrate sources, such as agro-industrial waste residues and by-products for higher CA production (Dhillon et al. 2011b, c). CA concentration decreased in control and MeOH after 120 and 132 h of incubation time, respectively, which might be due to four principal reasons: (1) inhibitory effect of high CA accumulation in the medium; (2) decay of enzymes responsible for CA biosynthesis; (3) depletion of available sugar content and; (4) decrease in amount of nitrogen content available in fermentation medium (Arzumanov et al. 2000; Al-Sheri and Mostafa 2006).

Viscosity and DO Profiles During Submerged Fermentation

The viscosity and dissolved oxygen (DO) profiles during submerged CA fermentation by *A. niger* NRRL 567 using APS is provided in Fig. 3.2.2 A, B respectively. The viscosity of the medium increased until 84 and 96 h, respectively, for treatment with MeOH as an inducer and control having higher viscosity during fermentation. High viscosity and non-Newtonian feature of mycelia suspensions often leads to difficulty in mixing which in turn affects oxygen transfer. A highly viscous broth causes the formation of so called "dead zones" usually in the bottom of bioreactor where the broth can stay stagnant for a long period of time due to lower agitation effect. Such stagnant zones also appear in shear thinned non-Newtonian broths, although not much pronounced. These zones can easily decrease the productivity of the microorganism and cause the production of undesirable

metabolites in the bioreactor. Homogenous mixing in such broths demands higher agitation which also results in decreased productivity due to breakage of mycelium (Berovic et al. 1991).

The continuous decrease in DO values till 60 h of fermentation suggests the active growth of fungus during this period (Fig. 3.2.2 B). The DO values were maintained throughout the experiment by adjusting agitation speed. Higher biomass was achieved in control experiment as compared with treatment supplemented with 3% (v/v) MeOH as evident from Fig. 3.2.3 A,B. The broth rheology in fungal fermentations is frequently related to the morphology and yield of mycelial biomass. As evident from the Fig. 3.2.3 A, B, there is an increase in biomass concentration with time, which considerably alters the rheology of the fermentation broth to usually pseudoplastic behavior (Roels et al. 1974). Relatively few studies have been aimed at quantifying the influence of biomass accumulation on broth rheology. In our previous studies, it was demonstrated that addition of lower alcohols, such as MeOH, decreased mycelial growth, resulted in pellet formation, inhibited sporulation and increased the fermentation efficiency (Dhillon et al. 2011b, c). MeOH is well known for depressing the synthesis of cell wall proteins in the early stages of cultivation (Moyer 1953).

Broth Rheology

Figure 3.2.3 A, B shows the visible observation of biomass growth trend during submerged CA fermentations. An increase in viscosity during the course of fermentation was observed in both treatments until 84–96 h of fermentation period. The increase in viscosity of the fermentation medium was due to the mycelium growth and metabolite production by fungus. However, the viscosity was lower in treatment supplemented with MeOH as compared with control during the course of incubation which was mainly due to the pellet formation and reduced mycelial growth caused by MeOH. The morphology of fungus is considered as an important parameter which influences the physical characteristics of the fermentation medium finally affecting CA production. In fact, it is well known that the rheological behavior of fermentation broths during the process is closely correlated to the type of medium and morphological changes of mycelia, so that viscosity could be used as a parameter for process monitoring and regulation (Papagianni 2004). The rheology is equally and highly dependent on physical, chemical and biological characteristics of the fermentation medium. Generally, mycelial growth

increases the viscosity of the medium as compared with pellet growth (Pazouki and Panda 2000). Pelleted cultivation broth may be Newtonian of low viscosity and more economical in the separation of the biomass. However, in mycelial growth, the entanglement of hyphae occurs resulting in highly branched network rendering the broths highly viscous. The much more viscous broth leads to heterogeneous, stagnant, non-mixed zone formations, which make the cultivation challenging and more expensive to operate (Oncu et al. 2007).

Table 3.2.1 showed that APS during submerged CA fermentation followed various rheological models with varying degree of confidence of fits. Table 2 illustrates shear rate versus shear stress and viscosity behavior of control and MeOH supplemented treatments, respectively, at different fermentation times. The shear rate and shear stress values all through the fermentation showed non-linear relationship which is indicator of strong non-Newtonian behavior. The shear stress increased from 0 to 96 h (control) and from 0 to 120 h (MeOH) possibly due to the resistance offered by actively growing fungal cells. However, the shear stress decreased during stationary phase (96/120 to 168 h) possibly due to filament breakage (confirmed by microscopy), sporulation and pellet disruption. The viscosity profile showed varied patterns with respect to time (Table 3). Higher viscosity values were observed for treatments from 72–120 min control and treatment with inducer. The microbial submerged fermentation broths showed different rheology changes from initial Newtonian to pseudoplastic behavior during growth period, in particular during exponential phase (Hwang et al. 2004). The pseudoplastic behavior will have a significant effect on mass and heat transfer in the bioreactor due to higher shear rates near the impeller and low elsewhere. Various studies showed that the complex morphology of filamentous fungi is responsible for highly viscous fermentation broths, characterized by shear rate- dependent viscosities and yield stress (Riley et al. 2000; Papagianni 2004; Gupta et al. 2007) resulting in varied hydrodynamic properties of the bioreactor including mixing and mass transfer performance (Sinha et al. 2001a).

The models used in this study (Table 3.2.1) were most commonly used for rheological analysis of mycelial cultivation broths and are widely used in non-Newtonian transport correlations (Pollard et al. 2002). Both the fermentations (control and with inducer) followed Bingham plastic law throughout the fermentation from 0–168 h. The yield stress (τ_0) increased until 96 and 120 h, respectively, in control and in treatment with MeOH. Bingham model is a simple rheological model that relates shear stress and shear rate

and quantifies yield stress and high-shear viscosity. The Bingham law is mathematically represented by Eq. 1.

Bingham Plastic $$\sqrt{\tau} = \sqrt{\tau_0} + \sqrt{\eta D} \quad (1)$$

The Bingham model includes the yield stress, which must exceeded to certain extent before any flow is induced in fluid. The existence of yield stress is important, because it will determine start-up power requirement in pumping through pipelines and mixers and the existence of dead regions in bioreactors (Olsvik and Kristiansen 1994). Likewise, Casson model was followed with 76.7–88% confidence of fits for control treatment from 24–120 h and with 84–86.5% confidence of fits for treatment with MeOH as inducer from 48–120 h, respectively. The plastic viscosity increased from 24–120 h in both control with higher η value of 5.41 and in treatment with inducer (MeOH) with η value of 3.37. The value of τ0 was found to increase until 96 h in control and 120 h in treatment with MeOH as inducer during fermentation. Casson model quantifies yield stress and high shear viscosity, a model which incorporates features of the power law and the Bingham plastic equations. The mathematical representation of Casson law is given as Eq. 2.

Casson Law $$(1+a)\sqrt{\tau} = 2\sqrt{\tau_0} + (1+a)\sqrt{\eta D} \quad (2)$$

The decrease in the plastic viscosity and yield stress towards the end of fermentation indicated that the fermented broth would require lower yield stress to maintain flow. The shear stress is known to affect the flow in a similar manner as pseudo-plasticity with more pronounced effects. This will help in the pump design for centrifugation facilities where cell debris aggregates are identified as being reversibly de-aggregated by low shear stresses which might exist in the separation zone of the disc space of a disc stack centrifuge (Maybury et al. 2000).

Similarly, NCA–CMA Casson (modified Casson law) followed a good confidence of fit (76.7–88.1%) for control from 24–120 h and 84.5–86.5% fit for MeOH treatment from 48 until 120 h of fermentation. NCA–CMA Casson model has been derived from the standard set forth by the National Confectioners Association (NCA) and the Chocolate Manufacturers Association (CMA). Although based on the original Casson equation, this

implementation has been tailored by the NCA and CMA specifically to applications involving chocolate-type behaviour. The NCA–CMA model is mathematically represented by Eq. 3.

NCA/CMA Casson $\tau = \tau_0 + \eta D$ (3)

Power Law Behavior of Fermented APS

Power law model is a useful rheological model that describes the relationship between viscosity or shear stress and shear rate over the range of shear rates where shear thinning occurs in a non-Newtonian fluid. It quantifies overall viscosity range and degree of deviation from Newtonian behavior and is represented by Eq. 4.

Power Law $\tau = kD^n$ (4)

The power law model was followed with moderate fits of confidence (65.3–75.9%) as observed at the beginning of fermentation until 48 h and later followed a good confidence of fits (75.9–88.2%) until 144 h of fermentation in control. However, in treatment with MeOH as an inducer, good confidence of fit (77.8–88.9%) was followed throughout the fermentation. Power law has been used widely to determine the rheology of several microbial fermentation broths (Hwang et al. 2004). The consistency index (K, measure of the thickness of the fermented broth) increased until 96 and 144 h, respectively, in control and treatment with inducer. The increase in "K" can be attributed to the increase in medium consistency with fungal cell growth and increase in the phase volume (volume of suspended materials/volume of continuous phase) which has been reported to increase K in fermentation broths (Hwang et al. 2004). As the value of flow behavior index "n" approaches towards 1, the shear-thinning properties normally increase so as to reach Newtonian behavior. However, the "n" values [n≤ 0.83 in control (0–120 h) and n≤0.92 (24–96 h)] indicated that APS fermentation exhibited a shear-thinning response towards the end. The viscosity continuously increased until 84–96 min control and inducer treatments, respectively (Table 3.2.2, Fig. 3.2.2). The increase in viscosity can be attributed to the increase in viability (colony-forming units per milliliter) from 96–108 h of fermentation (Fig. 3.2.1). The viscosity was lower in the treatment with MeOH due to lower viability (colony-forming units per milliliter) and predominant pellet-type morphology.

The consistency index, K, has been used as the sole indicator for the viscosity of filamentous cultivations (Olsvik and Kristiansen 1992a, b). The broth rheology parameters, K and n, were found to be influenced by the concentration of the biomass and morphology, such as the average pellet size, and slightly by the fluffiness of the pellet (Rodriguez Porcel et al. 2005). The consistency index K has been also strongly influenced by an increasing size of pellets but was not sensitive to an increase in the concentration of pellets of a fixed size (Rodriguez Porcel et al. 2006). In general, the power law of Ostwald-deWaele model most adequately describes the broth rheological profiles over the whole shear rate range and due to its relative simplicity and easy handling (Pollard et al. 2002; Gupta et al. 2007; Oncu et al. 2007).

Similar to power law, IPC paste model is intended to calculate the shear sensitivity factor and the 10 RPM viscosity value of pastes. IPC paste model followed a good confidence of fit (77.8–89.5%) for treatment with MeOH throughout the fermentation. However, in the control, fair fit was observed until 48 h, and afterwards good confidence of fit (78.7–88.2%) until 120 h fermentation was observed. The IPC paste model law is primarily used in the solder paste industry, thus the name IPC (Institute for Interconnecting and Packaging Electronic Circuits) and is mathematically derived according to Eq. 5.

IPC Paste $\eta = KR^n$ (5)

The broth rheology in fungal fermentations is closely associated to the morphology and yield of mycelial biomass, and CA production (Dhillon et al. 2011a). Broth rheology causes mixing problems and influences transport phenomena, which in turn may lead to maintenance problems inhibiting cell growth and hence lower production of CA. Less viscous non-Newtonian broths are usually indicative of pellet form of fungus in which the mycelium develops very stable spherical aggregates consisting of more dense, branched and partially intertwined network of hyphae. Product formation is closely related to the form of biomass growth, as the product is said to be mainly expressed at the tips of the hyphae. Optimized growth leads to extensive product formation and therefore high productivity. The pellet form is highly recommended for CA production during submerged fermentation (Berovic et al. 1991; Papagianni 2004; Dhillon et al. 2011a). Small pellets are known to reduce formation and viscosity of the broth during fermentation. In our previous studies, the role of broth rheology was clearly reflected. Higher CA production was achieved with APS-1 which contained lower total solids

(115.0±5.0 g/l) than APS-2 (135.0±5.0 g/l). The addition of lower alcohols resulted in the pellet formation due to which the fermentation medium was less viscous as compared with control (Dhillon et al. 2011b, c).

Filamentous fungi, such as *A. niger*, is commercially important for production of organic acids, enzymes and many other biotechnological products. Fungi show two distinct morphologies in submerged fermentation: (1) pellet and (2) free filamentous or dispersed form. These growth forms are determined by a number of factors including genotype, medium pH, medium constituents, types of inocula and other physical conditions (Amanullah et al. 2000; Cho et al. 2002). The two morphological growth forms can have significant effects on fermentation broth rheology and thereby affect bioreactor performance. Among two typical fungal morphologies, i.e. filamentous and pellet form, latter morphology was cited as one of the key factors that directly affected CA fermentation productivity. The pelleted form is considered of larger importance with respect to citric acid (*A. niger*) (Papagianni 2004). In a submerged culture of ascomycetes, Paecilomyces japonica pellets with high compactness proved to be more productive morphological forms compared with free filamentous mycelia fermentation (Sinha et al. 2001b). Mycelium form of filamentous organisms in submerged cultures consists of branched and unbranched hyphae (freely dispersed) and clumps or aggregates. Non-Newtonian rheology resulting from increased viscosity is usually related to particular growth morphology, i.e. filamentous growth. The resulting broth morphology may reduce oxygen transfer rate and enzyme synthesis which in turn affects CA secretion ability of fungus. *Penicillium chrysogenum* is a good example due to the fact that its viscosity is higher when cells grow as filaments instead of pellets (Tucker and Thomas 1993). Thus, the enhancing role of MeOH over control (resulting in more filamentous type fungus growth) having higher viscosity was clearly evident due to the pellet morphology of fungus.

Variation of Sedimentation Velocity in Different Submerged CA Fermentations

Sedimentation velocity profiles of SmF indicated that treatment supplemented with inducer (MeOH) resulting in higher sedimentation velocity (v_{sed}) of 2.386E-10 ms^{-1} as compared with control having lower v_{sed} of 8.695E-11 ms^{-1}. According to Stoke's law, sedimentation velocity of particles in a quiescent liquid is given by Eq. 6

$$V_{sed} = \frac{g}{18} \frac{[(\rho_S - \rho_L)d^2]}{\eta} \quad (6)$$

As sedimentation velocity v_{sed} is influenced by the square of particle size (PRS), an increase in PRS will favor settling as seen from Eq. 6. The v_{sed} was calculated by using Eq. 6, where g = 9.81 (meters per square second), η = viscosity during higher CA fermentation stage (kg m^{-1} s^{-2}); density of particle ρ_S = 1.02 kg/l (experimental value); density of medium ρ_L = 1.000 kg/l (experimental value); and *d*= particle size of pomace during each stage.

Ideally, Stoke's equation is valid only for discrete particles in dilute suspension under laminar flow condition. However, concentration of sludge varying from suspended solids of 10 to 40 g/l (25 g/l SS in present study) could be correlated with Stoke's equation as a conventional estimate for determining sedimentation velocity as established by earlier studies (Rector and Bunker 1995). The calculated v_{sed} was interpreted in order to draw correlation between different treatments for centrifugation of fermented broths. The v_{sed} decreased with SS proportional to PRS (morphology in this case) and viscosity.

A pellet type of morphology is preferred in industrial cultivations due to ease in downstream processing because of the lower viscosity of the broth (Zhou et al. 2000). In these cultivations, the mass transfer of oxygen and nutrients is considerably superior. Moreover, the subsequent separation of the pellets from the cultivation broth is simpler than in mycelia cultivations. Easy agitation and aeration ensure low operating costs, as much lower power input is needed (Oncu et al. 2007). Although the heat and mass transfer within the bioreactor is not limited, concentration gradients within the pellet results in a depletion of nutrients, especially oxygen, in the central regions of the pellets (Hille et al. 2005). The present study highlighted the importance of pellets in downstream processing due to higher vsed in treatment with MeOH displaying pelleted growth.

In the CA production process, centrifugation of the fermented broth is required to remove cell biomass, spores and other particles from fermented broth. Thus, lower centrifugal force may be required to settle larger particles (e.g. pellets) providing valuable data for downstream processing. The pellets will contribute to higher v_{sed} as compared with control treatment having higher suspendibility and lower settleability. For CA fermented broths, higher v_{sed} was desirable for CA extraction which could be attained at lower

centrifugal force/in lesser time, thus lowering centrifugal costs. A laborious and expensive purification procedure leads to an uneconomical overall production process. Fungal morphology influences not only the productivity of the fermentation process, but through its impact on rheology, it also has an influence on mixing and mass transfer within the bioreactor. To ensure high metabolite secretion and at the same time a low viscosity of the cultivation broth, it is necessary to tailor the morphology of filamentous fungi during industrial cultivations. The morphological type and the related physiology strongly depend on environmental conditions in the bioreactor (Znidarsic and Pavko 2001), which can be controlled by regulation of the process parameters.

In conclusion, rheological behavior in submerged fermentation broths largely depends on two parameters: (1) type of medium that governs the inter-particle interactions and; (2) type of microorganism and growth pattern that affects the hydrodynamic regime of medium, for example, fungus, Aspergillus sp. produces either mycelia or pellets during exponential phase, among others. The rheology of the broth has an impact on the purification of the product, as high viscosities complicate the downstream processing, i.e. recovery of the CA.

CONCLUSIONS

The rheological features of *A. niger* grown in submerged culture using APS were studied. There was an important impact of inducer on the mycelial morphology, mycelial growth and CA production in fermentor. Supplementation of 3% (v/v) MeOH resulted in less viscous non-Newtonian broth rendering higher CA production (41.32% higher CA as compared with control). The fermentation of apple pomace ultrafiltration sludge supplemented with methanol followed power law and pseudoplasticity decreased as compared with fermented broth in control, enhancing the flow properties. The power law constants, consistency index increased with a peak at 96 and 144 h for treatment with MeOH and control, respectively, and later decreased, whereas, the flow behavior index also increased continuously till 96 and 120 h for treatment with MeOH and control, respectively, and later decreased. The viscosity of apple pomace ultrafiltration sludge supplemented with methanol decreased with shear rate which could improve broth rheology. Morphology of *A. niger* is a good indicator for identifying the cell activity for higher CA production and ease in downstream processing. The study is beneficial for

further optimization of other *A. niger* species fermentation processes for the large-scale production of CA.

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List of Symbols	
τ	shear stress (10^{-1} kg m^{-1} s^{-2})
τ_0	yield stress (shear stress at 0 rpm of spindle, (10^{-1} kg m^{-1} s^{-2})
D	shear rate (s^{-1})
K	consistency index (10^{-3} kg m^{-1} s^{-n})a
N	flow behaviour index (dimensionless)
H	Plastic viscosity and/or viscosity (10^{-3} kg m^{-1} s^{-1})
R	rotational speed (rpm)
η_{10}	10 rpm viscosity (10^{-3} kg m^{-1} s^{-1})

ABBREVIATIONS

ANOVA Analysis of variance; APS- Apple pomace ultrafiltration sludge; ARS- Agricultural research services; CA- Citric acid; DO- Dissolved oxygen; EtOH- Ethanol; MeOH- Methanol; SD- Standard deviation; SS- Suspended solids

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Table 3.2. 1 Different rheological model fits of fermented apple pomace ultrafiltration sludge (APS) for citric acid production through *A. niger* NRRL 567 at different time intervals

Rheological model	Incubation time (h)							
	0	24	48	72	96	120	144	168
Bingham law								
Plastic viscosity (η, 10^{-3} kg m^{-1} s^{-1})	2.88 [1.63]	1.93 [1.50]	1.09 [1.37]	3.12 [4.68]	4.86 [6.26]	7.12 [8.30]	1.80 [5.02]	1.64 [5.19]
Yield stress (τ_0, 10^{-1} kg m^{-1} s^{-2})	0.03 [0.20]	0.08 [0.22]	0.35 [1.59]	0.33 [3.25]	1.69 [3.70]	2.56 [3.38]	0.08 [3.51]	0.25 [3.22]
Confidence of fit (%)	74.2 [80.3]	84.9 [78.8]	85.6 [81.7]	82.0 [81.5]	85.4 [85.9]	89.8 [88.2]	88.3 [81.6]	80.9 [76.7]
Casson law								
Plastic viscosity (η, 10^{-3} kg m^{-1} s^{-1})	7.31 [4.92]	2.36 [3.88]	2.58 [4.37]	2.70 [4.40]	3.12 [4.60]	3.37 [5.41]	2.16 [5.09]	2.15 [3.08]
Yield stress (τ_0, 10^{-1} kg m^{-1} s^{-2})	0.27 [0.14]	0.09 [0.13]	0.12 [1.23]	0.15 [2.55]	0.33 [4.17]	1.43 [3.26]	0.22 [3.76]	0.13 [3.37]
Confidence of fit (%)	54.5 [74.2]	68.0 [76.7]	86.2 [79.8]	84.5 [82.8]	86.5 [76.8]	85.3 [88.1]	74.8 [73.3]	62.6 [75.6]
NCA/CMA Casson								
Plastic viscosity (η, 10^{-3} kg m^{-1} s^{-1})	7.31 [4.92]	2.36 [3.88]	2.58 [4.37]	2.70 [4.40]	3.14 [4.60]	3.37 [5.41]	2.16 [5.09]	2.15 [3.09]
Yield stress (τ_0, 10^{-1} kg m^{-1} s^{-2})	0.24 [0.13]	0.08 [0.12]	0.09 [1.21]	0.03 [2.53]	0.29 [3.80]	0.81 [2.97]	0.10 [3.43]	0.07 [3.38]
Confidence of fit (%)	52.8 [74.2]	68.0 [76.7]	86.2 [79.8]	84.5 [82.8]	86.5 [76.8]	85.3 [88.1]	74.8 [73.3]	62.8 [75.6]
Power law								
Consistency index (K, 10^{-3} kg m^{-1} s^{-n})	20.68 [23.4]	18.74 [18.9]	69.6 [126.6]	125.7 [248.6]	271.5 [305.1]	147.8 [314.0]	120.6 [421.4]	68.14 [364.1]
Flow behaviour index (n)	0.44 [0.42]	0.53 [0.46]	0.52 [0.18]	0.60 [0.65]	0.92 [0.74]	0.87 [.83]	0.81 [0.68]	0.62 [0.62]
Confidence of fit (%)	77.8 [65.3]	83.7 [71.6]	86.6 [75.9]	85.5 [84.0]	88.9 [78.7]	89.5 [88.2]	88.4 [83.2]	80.2 [76.9]
IPC paste								
Shear sensitivity factor (n_s)	4.01 [5.45]	3.77 [4.90]	7.09 [16.1]	25.15 [29.5]	110.0 [30.7]	77.3 [27.9]	18.78 [30.2]	9.24 [27.8]
10 rpm viscosity (η_{10})	1.64 [1.58]	1.03 [1.54]	0.48 [0.82]	0.40 [0.85]	0.48 [0.92]	0.63 [0.97]	0.25 [1.05]	0.32 [1.03]
Confidence of fit (%)	77.8 [65.3]	83.7 [71.6]	86.6 [75.9]	85.5 [84.0]	78.9 [78.7]	89.5 [88.2]	88.4 [83.2]	80.2 [76.9]

*The value outside the parenthesis corresponds to treatment supplemented with inducer (MeOH) whereas values in the parenthesis corresponds to control

Table 3.2. 2 Rheology data of APS during fermentation showing shear stress profile in treatment supplemented with 3% (v/v) MeOH (outside parenthesis) and control (inside parenthesis) during submerged citric acid production by *A. niger* NRRL 567 in fermenter

(A) Shear rate (s^{-1})	**Shear stress (10^{-1} kg m^{-1} s^{-2})**							
	0 h	24 h	48 h	72 h	96 h	120 h	144 h	168 h
5	0.34 [0.40]	0.24 [0.49]	0.34 [1.79]	0.54 [2.98]	2.24 [3.89]	3.82 [2.65]	0.67 [3.53]	0.17 [3.27]
25	0.47 [0.56]	0.38 [0.65]	0.84 [2.25]	1.51 [3.05]	3.56 [4.17]	4.04 [3.40]	0.59 [3.86]	0.34 [3.10]
50	0.67 [0.83]	0.50 [0.91]	0.87 [2.71]	1.68 [3.18]	3.60 [4.38]	5.54 [3.46]	0.84 [3.80]	0.67 [3.21]
75	0.66 [1.17]	0.50 [1.19]	0.84 [2.35]	1.85 [4.53]	3.36 [4.15]	6.05 [3.53]	1.01 [3.03]	0.67 [3.24]
100	0.84 [1.56]	0.50 [1.50]	1.01 [2.95]	2.20 [4.60]	4.00 [4.32]	6.05 [3.59]	0.94 [3.39]	0.67 [3.16]
125	0.84 [2.37]	0.54 [2.02]	1.07 [3.42]	2.60 [4.58]	3.86 [4.36]	6.18 [3.85]	1.18 [3.77]	0.84 [3.64]
150	0.84 [2.92]	0.59 [2.71]	1.18 [3.74]	2.70 [5.01]	3.90 [4.63]	6.21 [4.26]	1.01 [4.07]	0.84 [3.22]
(B) Shear rate (s^{-1})	**Viscosity (10^{-1} kg m^{-1} s^{-2})**							
5	3.96 [4.80]	14.04 [10.56]	48.35 [24.80]	75.94 [65.07]	45.90 [51.8]	40.23 [71.16]	15.48 [37.71]	6.24 [21.86]
25	2.86 [4.70]	2.50 [9.98]	8.21 [19.96]	13.32 [52.02]	13.44 [40.0]	17.04 [64.21]	2.78 [22.18]	1.87 [15.23]
50	1.58 [3.01]	1.90 [6.73]	2.64 [14.58]	3.84 [22.24]	5.16 [15.06]	6.36 [16.95]	1.93 [6.93]	1.72 [6.21]
75	1.14 [1.57]	1.75 [2.52]	2.30 [6.73]	2.30 [9.65]	4.96 [6.69]	6.08 [10.92]	1.79 [5.28]	1.71 [5.76]
100	1.16 [1.39]	1.51 [2.45]	1.98 [2.75]	2.30 [6.22]	4.93 [5.31]	5.18 [6.75]	1.77 [4.66]	1.50 [3.81]
125	1.14 [1.40]	1.50 [1.82]	1.89 [2.71]	2.13 [5.85]	4.91 [5.23]	5.13 [4.80]	1.38 [4.57]	1.41 [3.57]
150	1.12 [1.39]	1.31 [1.82]	1.75 [2.60]	2.11 [4.99]	4.78 [4.91]	5.11 [3.34]	1.17 [4.37]	1.38 [3.41]
175	1.02 [1.34]	1.30 [1.74]	1.61 [2.61]	1.97 [3.52]	4.61 [4.30]	5.01 [3.21]	1.17 [3.89]	1.32 [3.07]
200	1.01 [1.32]	1.21 [1.68]	1.35 [1.31]	1.91 [2.63]	4.60 [3.00]	4.92 [3.25]	1.11 [3.18]	1.21 [3.20s]

Table 3.2. 3 Rheology data of APS during fermentation showing viscosity Vs. time profile in treatment supplemented with 3% (v/v) MeOH (outside parenthesis) and control (inside parenthesis) during submerged citric acid production by *A. niger* NRRL 567 in fermenter

Time	Viscosity (10^{-3} kg m^{-1} s^{-1})							
(min)	0 h	24 h	48 h	72 h	96 h	120 h	144 h	168 h
1	2.0 [11.74]	15.40 [18.10]	66.99 [50.87]	142.37 [69.45]	81.98 [95.10]	111.98 [99.45]	13.60 [106.62]	7.34 [108.58]
10	1.40 [6.36]	53.39 [14.74]	63.79 [78.01]	151.37 [82.17]	265.94 [108.96]	260.0 [102.17]	14.60 [136.84]	12.47 [108.92]
20	1.40 [4.40]	42.99 [30.08]	72.19 [148.60]	185.36 [215.81]	228.93 [282.43]	205.96 [285.81]	9.20 [114.96]	10.76 [65.29]
30	1.40 [3.67]	42.79 [19.13]	99.18 [147.76]	173.20 [243.64]	229.93 [290.13]	189.96 [278.64]	9.20 [118.12]	9.54 [58.69]
40	1.40 [3.18]	42.99 [23.48]	178.56 [63.58]	196.76 [172.63]	239.92 [251.0]	165.96 [202.63]	4.40 [120.20]	6.11 [50.05]
50	1.40 [2.93]	42.99 [25.65]	143.37 [62.36]	191.16 [158.08]	207.92 [175.89]	143.97 [188.08]	4.0 [50.38]	8.07 [49.88]
60	1.40 [3.18]	34.99 [25.68]	142.57 [53.80]	185.36 [133.28]	205.91 [116.27]	120.20 [173.28]	3.20 [45.51]	7.09 [54.53]

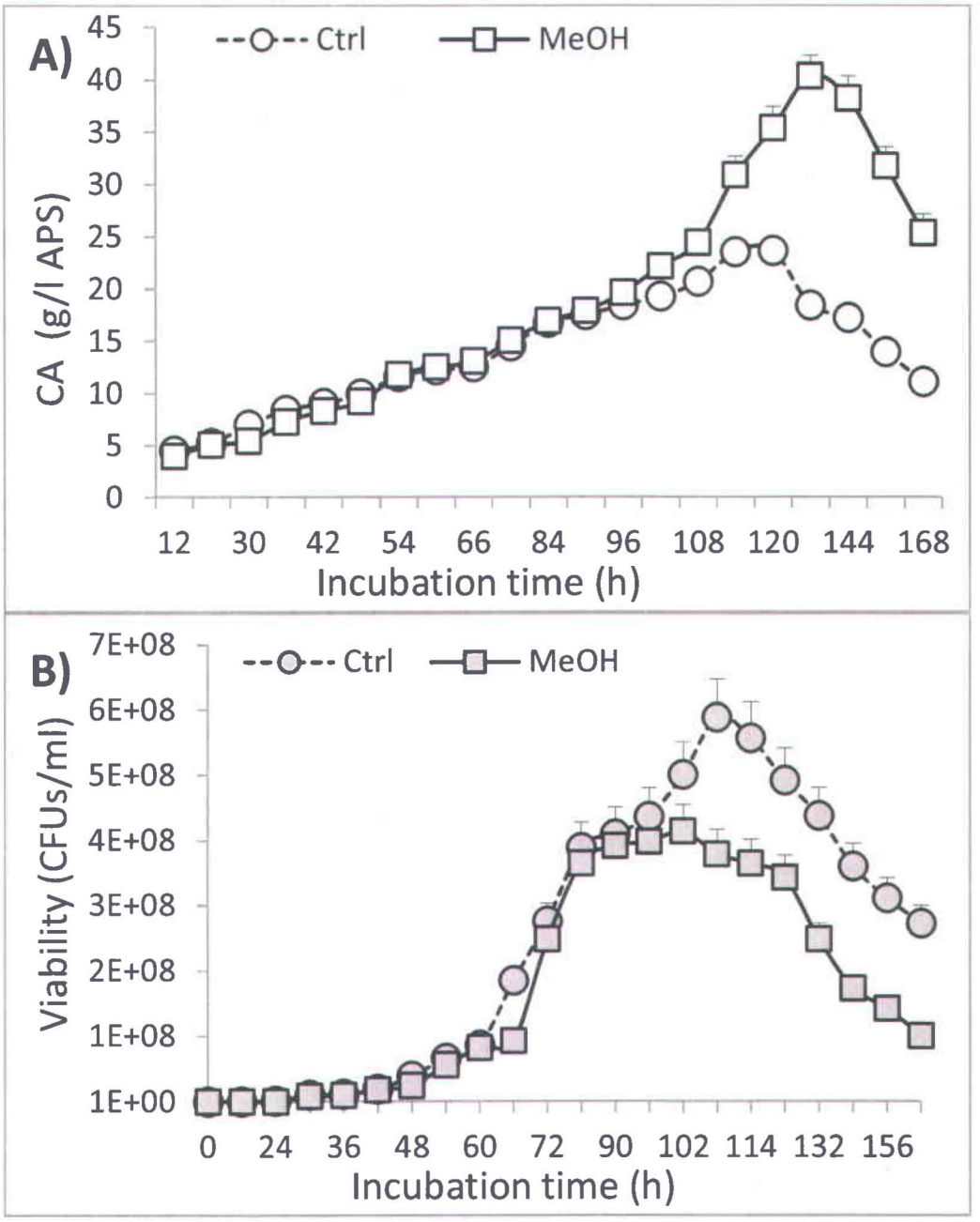


Figure 3.2. 1 Citric acid (CA) and viability (CFUs/ml) during SmF by *Aspergillus niger* NRRL-567 on apple pomace ultrafiltration sludge (A) control and; (B) inducer methanol (MeOH)- 3% (v/v) concentration

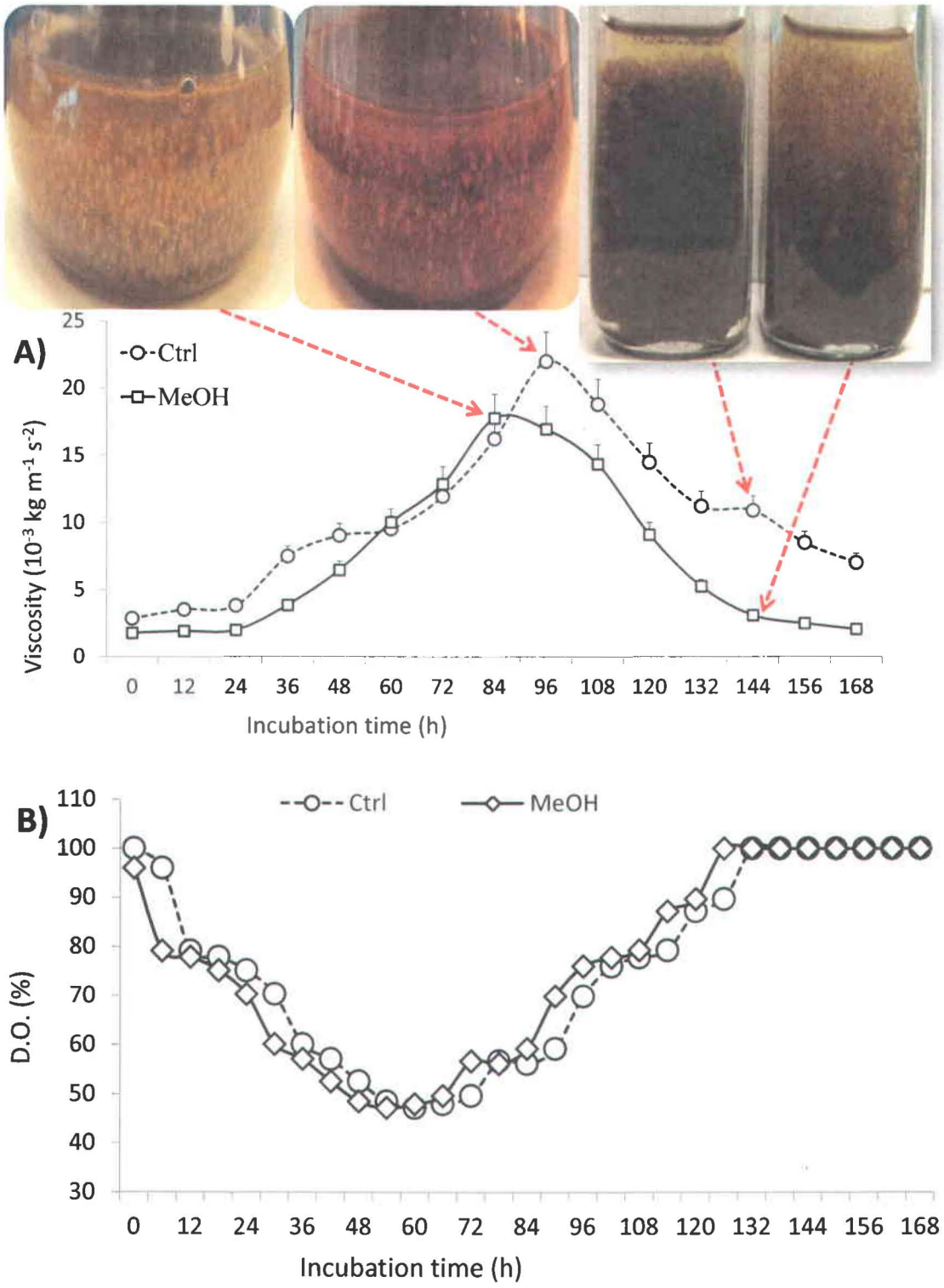


Figure 3.2. 2 Changes in (A) viscosity and; (B) DO (%) profiles during citric acid (CA) fermentation by *Aspergillus niger* NRRL-567 with control apple pomace ultrafiltration sludge and with inducer methanol (MeOH) in a concentration of 3% (v/v). *Arrows indicating the time for different morpholological features.

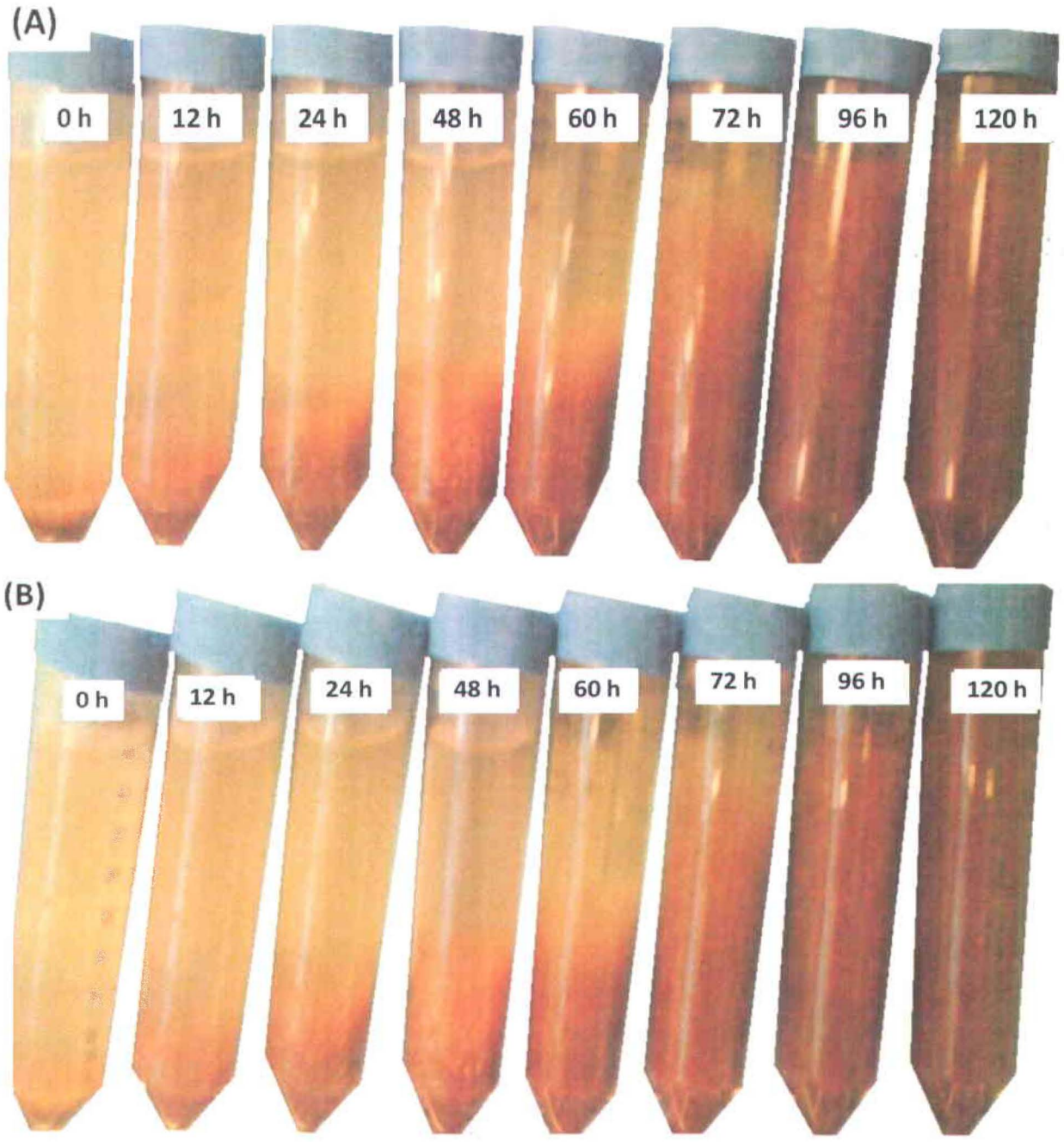


Figure 3.2. 3 Biomass concentration of *A. niger* NRRL 567; (A) control and; (B) treatment supplemented with 3% (v/v) MeOH during submerged citric acid production in fermenter

PARTIE IV.

CO-PRODUCT (CHITOSAN) EXTRACTION FROM WASTE FUNGAL MYCELIUM FROM CA FERMENTATION

GREEN SYNTHESIS APPROACH: EXTRACTION OF CHITOSAN FROM FUNGUS MYCELIUM

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RÉSUME

Le chitosane, un copolymère de glucosamine et N-acétyl glucosamine, est principalement dérivé de la chitine et présent dans les parois cellulaires de certains Crustacés et des autres micro-organismes, tels que les champignons. Le chitosane est en train de devenir un biopolymère important ayant de nombreuses applications dans des différents domaines. À l'échelle commerciale, le chitosane est principalement obtenu à partir des carapaces de Crustacés, mais jamais reporté à partir des sources fongiques. Les méthodes employées pour l'extraction de chitosane sont chargés de nombreux inconvénients. D'autres options de production de chitosane à partir de biomasse fongique existent avec des propriétés physico-chimiques importantes. Les chercheurs du monde entier tentent actuellement à commercialiser la production de chitosane et son extraction à partir de sources fongiques. Le chitosane extrait de sources fongiques a le potentiel de remplacer complètement le chitosane provenant des crustacés. Dans ce contexte, cette étude examine le potentiel de la biomasse fongique produits comme une source peu coûteuse de chitosane, résultant de industries biotechnologiques diverses ou cultivés sur des déchets agricoles et industriels a coûts négatifs. Le chitosane d'origine biologique fongique offre des avantages prometteurs par rapport à celui obtenus à partir de carapaces de Crustacés ayant des différentes caractéristiques physico-chimiques importantes. Les différents aspects de méthodes d'extraction de chitosane fongique et les divers paramètres ayant un effet sur le rendement de chitosane sont discutés en détail. Le présent rapport traite également avec des attributs essentiels de chitosane pour la haute valeur ajoutée pour des applications dans différents domaines.

Mots clés: matières insolubles Alkalines (AIM), matériau alcalin et insoluble dans l'acide (AAIM); chitine; chitosane; degré d'acétylation (DA); champignons; approche synthétique verte

ABSTRACT

Chitosan, copolymer of glucosamine and *N*-acetyl glucosamine is mainly derived from chitin which is present in cell walls of crustaceans and some other microorganisms, such as fungi. Chitosan is emerging as an important biopolymer having broad range of applications in different fields. On commercial scale, chitosan is mainly obtained from the crustacean shells but not from the fungal sources. The methods employed for extraction of chitosan are laden with many disadvantages. Alternative options of producing chitosan from fungal biomass exist, in fact with superior physico-chemical properties. Researchers around the globe are attempting to commercialize chitosan production and extraction from fungal sources. Chitosan extracted from fungal sources have the potential to completely replace crustacean derived chitosan. In this context, the present review discusses the potential of fungal biomass resulting from various biotechnological industries or grown on negative/low cost agricultural and industrial wastes and their by-products as an inexpensive source of chitosan. Biologically derived fungal chitosan offers promising advantages over the chitosan obtained from crustacean shells with respect to different physico-chemical attributes. The different aspects of fungal chitosan extraction methods and various parameters having effect on the yield of chitosan are discussed in detail. This review also deals with essential attributes of chitosan for high value-added applications in different fields.

Key words: Alkali insoluble material (AIM); alkali- and acid- insoluble material (AAIM); chitin; chitosan; degree of acetylation (DA); fungi; green synthesis approach

1. INTRODUCTION

Chitin is a biopolymer composed of [β (1-4) linked *N*-acetyl -2-amino-2-deoxy-D-glucose] units. It is a primary constituent of crustacean shells, insect cuticles and a component of the cell walls of fungi and algae. Chitin is a natural polysaccharide biopolymer produced by a number of other living organisms in the lower plant and animal kingdoms, serving in various functions where reinforcement and strength are sought. The annual production and the steady-state amount in the biosphere are approximately 10^{12}-10^{14} kg (Poulicek et al., 1998) with production rate just next to cellulose, which is being produced by plants. As per literature data on chitin production by arthropods, the total annual chitin production in aquatic environments was estimated to be 2.8×10^{10} kg and 1.3×10^{12} kg chitin/year for for freshwater and marine ecosystems, respectively (Cauchie 2002). The chemical structure of chitin is very close to cellulose except that the hydroxyl group in C-2 of cellulose is replaced by an acetamido group in chitin. This structural similarity between cellulose and chitin can be viewed as serving similar structural and defensive functions.

Chitin serves as the raw material for all commercial production of chitosan and glucosamine with estimated annual production of 2000 and 4000 tons, respectively (Sandford, 2003). Chitin can be enzymatically or chemically deacetylated to chitosan, a more flexible and soluble polymer. However, deacetylation is rarely conducted to full completion; hence the chitosan polymeric chain is generally described as a copolymeric structure comprised of β-(1-4)-2-acetamido-D-glucose (acetylated unit) and β-(1-4)-2-amino-D-glucosamine (deacetylated unit) residues. According to some researchers, chitosan is a polymer having at least 60% of D-glucosamine residues in the chain (Aiba, 1992). However, the degree of deacetylation (DD) of chitosan usually exceeds 80% (Wang et al., 2010). Chitosan also occurs naturally in some fungi (*Mucoraceae*) (Roberts, 1998). Fungal cell walls are composed of polysaccharides and glycoproteins. Polysaccharides, such as chitin and glucan are the structural components, whereas the glycoproteins, namely mannoproteins, galactoproteins, xylomannoproteins and glucuronoproteins form the interstitial components of fungal cell walls (Cabib et al., 1988; Bowman and Free, 2006). The glucan component is mainly composed of β-1,3-glucan, long linear chains of β-1,3-linked glucose. Glucans having alternate linkages, such as β-1,6-glucan, are also found within some fungal cell walls. Chitin is synthesized as chains of β-1,4-linked *N*-acetylglucosamine residues and is typically less abundant than either

the glycoprotein or glucan portions of the cell wall. The composition of the cell wall is subjected to change and may even vary within a single fungal isolate depending upon the conditions and stage of growth. The glycoprotein, glucan and chitin components are extensively cross-linked together to form a complex network, which forms the structural basis of the cell wall (Bowman and Free, 2006).

The development of applications for chitosan in different areas of life has expanded rapidly in recent years due to the following major characteristics: (1) it possesses defined chemical structure; (2) it is polycationic, innocuous, biodegradable and biocompatible with many organs, tissues, and cells; (3) it is physically and biologically active; (4) it can be chemically or enzymatically modified and; (5) it can be processed into several forms, such as flakes, beads, powders, membranes, gels, sponges, cottons, and fibres. It has been used in enzyme immobilization, wastewater treatment, as a food additive and anti-cholesterolemic, for wound healing, and in pharmaceuticals in several drug delivery systems (Jolles and Muzzarelli, 1999; Amorim et al., 2003). Conventionally, on an industrial scale, chitosan is mainly derived from the waste product of crustacean exoskeletons obtained after the industrial processing of seafood, such as shrimp, crabs, squids and lobsters shell by chemical deacetylation by using hot concentrated base solution (30-50% w/v) at high temperatures (<100 °C) for forprolonged time (Roberts, 1992, Aranaz et al., 2009). However, the chitosan obtained by such treatments suffers some inconsistencies, such as protein contamination, inconsistent levels of deacetylation and high molecular weight (MW) which results in variable physico-chemical characteristics (Knorr and Klein, 1986; No et al., 2000). There are some additional problems, such as environmental issues due to the large amount of waste concentrated alkaline solution, seasonal limitation of seafood shell supply and high cost (Muzzarelli et al., 1980; Wu et al., 2005).

In this context, production and purification of chitosan from the cell walls of fungi grown under controlled conditions offers advantage of being environmentally friendly and provides greater potential for consistent product (Table 1). Fungal mycelium can be obtained by simple fermentation regardless of geographical location or season. The extraction of chitosan is achieved by mild alkaline and acidic treatments as compared to chemical extraction method and it can be considered a green approach. Moreover, fungal mycelia have lower levels of inorganic materials compared to crustacean shells, and no demineralization treatment is required during processing (Teng et al., 2001). The

physico-chemical properties and yields of chitosan isolated directly from a fungus can be optimized by controlling fermentation and processing parameters. Furthermore, β-glucan, that can be isolated from the mycelia chitosan-glucan complex, has important applications in biomedicine (Pomeroy et al., 2001). There are other advantages of chitosan derived from fungi as well, such as: (1) free of allergenic shrimp protein and; (2) MW and DD of fungal chitosan can be controlled by varying the fermentation conditions (Arcidiacono & Kaplan 1992, Stevens et al., 1997). The chitosan obtained from crustacean sources possesses high molecular MW (about 1.5×10^6 Da) whereas fungal derived chitosan has medium-low MW of 1-12 $\times$ 10^4 Da. The high MW of chitosan results in a poor solubility at neutral pH values and high viscosity aqueous solutions, limiting its potential uses in the fields of food, health and agriculture (Wibowo et al., 2007). The medium-low MW chitosan derived from fungi can be used as a powder in cholesterol absorption and as a thread or membrane in many medical-technical applications (Ikeda et al., 1993; Nwe and Stevens, 2002a). Additionally, fungal chitosan is thought to have higher bioactivity than chitosan obtained from traditional sources, which is especially important in medical applications (Jaworska and Konieczna, 2001).

Fungal biomass can be produced by solid-state fermentation (SSF) and submerged fermentation (SmF). SmF has specific advantage as this fermentation method provides easier control of fermentation parameters, such as pH, temperature and nutrient concentration in the fermentation medium (Arcidiacono and Kaplan, 1992). However, SSF is known to produce larger quantities of biomass as compared to SmF. Generally, the media reported for chitosan production from fungi contains yeast extract, D-glucose, and peptone. Recently, studies have been focused on the utilization of inexpensive carbon sources, such as biowastes for culturing fungi for chitosan production (Nwe and Stevens, 2002a; Khalaf, 2004; Streit et al., 2009; Kannan et al., 2010; Tai et al., 2010). Fermentative production of chitosan by culturing fungi on inexpensive biowaste is an infinite and economical source. Considering the significant amounts of fungal-based waste materials accumulated from biotechnological and pharmaceutical industries, and the cost involved in managing the wastes, extraction of highly functional value-added products, such as chitosan may provide a lucrative solution to these industries.

In this context, the present review discusses the different fungal sources of chitosan in detail, various factors affecting the yield of extractable chitosan, different extraction protocols and the attributes required for important applications of chitosan in various

fields. To date, to the best to our knowledge, there is no review article that discusses the different fungal sources of chitosan in detail. Thus, this review will ensure a better understanding of various advances in this upcoming field of biopolymers.

2. TRADITIONAL SOURCES OF CHITOSAN

Various protocols have been developed and proposed over the last few decades for preparation of pure chitosan. Several of these protocols form the basis of chemical processes for industrial production of chitosan from crustacean shell waste. The most abundantly available raw materials (69-70%) for chitin production are the shells of crab, shrimp, and prawn (Muzzarelli, 1986; No and Meyers, 1997; Kurita, 2006). However, due to complex organization of chitin with other constituents, harsh treatments are mandatory to remove them from chitinous material to prepare chitin and then chitosan on a commercial scale. Proteins are removed from ground shells by treating them with either sodium hydroxide or by digestion with proteolytic enzymes, such as papain, pepsin, trypsin, and pronase (Sandford, 1989). Minerals, such as calcium carbonate and calcium phosphate are extracted with hydrochloric acid. Pigments, such as melanin and carotenoids are removed with 0.02% potassium permanganate at 60 °C or hydrogen peroxide or sodium hypochlorite. Formation of chitosan from chitin is generally achieved by hydrolysis of its acetamide groups by severe alkaline hydrolysis treatment due to the resistance of such groups imposed by the *trans*-arrangement of the C2-C3 substituents in the sugar ring (Horton and Lineback, 1965). Thermal treatments of chitin under concentrated aqueous alkali (sodium or potassium hydroxide solution [40–50%]) are usually required to obtain partially deacetylated chitin (DA < 30%) regarded as chitosan. Generally, deacetylation is achieved by treatment with concentrated sodium or potassium hydroxide solution (40–50%) at 100 °C (No and Meyers, 1997; No et al., 2000).

Deacetylation of chitin releases amine groups (NH_2) and imparts a cationic characteristic to chitosan. This is particularly remarkable in an acid environment where the majority of polysaccharides are usually neutral or negatively charged. The deacetylation process is performed either at room temperature (homogeneous deacetylation) or at elevated temperature (heterogeneous deacetylation), depending on the nature of the final product desired. However, the latter is preferred for industrial purposes. In some cases, the deacetylation reaction is carried out in the presence of thiophenol as a scavenger of

oxygen or under N_2 atmosphere to prevent chain degradation that invariably occurs due to peeling reaction under strong alkaline conditions (Dung et al., 1994). The chitosan obtained from tradional sources is laden with many disadvantages as described above. In this context, the cultivation of selected fungi can provide an environmentally safe alternative source of chitin and chitosan.

3. CHITOSAN FORMATION IN FUNGI

The mycelium of several fungi, such as Mucor rouxii, Absidia glauca, Aspergillus niger, Gongronella butleri, Pleurotus sajor-caju, Rhizopus oryzae, Lentinus edodes and Trichoderma reesei have been considered as possible sources of chitin and chitosan due to their presence in the cell walls (Crestini et al., 1996; Brown and Thornton, 1998; Hu et al., 1999; Nwe et al., 2002a; 2002b; 2002c; Pochanavanich and Suntornsuk, 2002; Suntornsuk et al., 2002). Generally, the fungi belonging to zygomycetes class has higher amounts of chitin and chitosan in their cell walls when compared to other classes of fungi (Chandy and Sharma, 1990; Rinaudo et al., 1992; Feofilova et al., 1996; Tan et al., 1996; Pochanavanich and Suntornsuk, 2002; Sousa et al., 2003; Synowiecki and Al-Khateeb, 2003; Hu et al., 2004; Nemtsev et al., 2004). There is already evidence that the enzymatic deacetylation takes place in fungi and certain bacteria to form chitosan (Gooday et al., 1991). The formation of chitosan in cell walls of fungi is a result of the complex synergistic action of two enzymes: (1) chitin synthase (EC 2.4.1.16) and; (2) chitin deacetylase (EC 3.5.1.41). In the first step, chitin synthase synthesizes a chain of chitin using the chitin precursor, uridino-di phospho N-acetylglucosamine (UDP-Glc-NAc). In the next step, chitin deacetylase hydrolyzes acetic groups from N-acetylglucosamine (GlcNAc), transforming it into glucosamine (GlcN) and finally forming chitosan (Davis and Bartnicki-Garcia 1984; Bartnicki-Garcia 1989; Kafetzopoulos et al., 1993) as depicted in Fig 1. During chitin biosynthesis, monomers of GlcNAc are coupled in a reaction catalyzed by the membrane-integral enzyme chitin synthase, a member of the family 2 of glycosyltransferases. The polymerization requires UDP and GlcNAc as a substrate and divalent cations as co-factors. Chitin biosynthesis can be divided into three distinct steps: (1) the catalytic domain of chitin synthase facing the cytoplasmic site forms the polymer; (2) the translocation of the nascent polymer across the membrane and its release into the extracellular space and; (3) single polymers spontaneously

assemble to form crystalline microfibrils of varying diameter and length (Merzendorfer, 2006).

The MW of the chitin depends upon the chain length which is influenced by the activity of chitin synthase. Similarly, the degree of acetylation (DA) i.e. the content of GlcNAc groups in the chitosan chain is influenced by the activity of chitin deacetylase. The properties of nascent chitosan, such as DA and MW depend on the activity of these two enzymes which are largely influenced by the presence of various compounds and metal ions in the medium (Table 2). Chitin deacetylase plays an essential role in chitosan biosynthesis in fungi. Deacetylases have been isolated from different types of fungi, such as Aspergillus nidulans, Colletotrichum lindemuthianium, Mucor rouxii and Rhizopus oryzae (Chatterjee et al., 2008). However, the activity of deacetylases is severely inhibited by the insolubility of the substrate i.e. chitin. Researchers have attempted to use amorphous chitin having a high DA as a substrate for the deacetylase enzyme in vitro but failed to isolate acid-soluble chitosan (Martinou et al., 1998). The insolubility of chitin with high DA in aqueous solvents represents the practical barrier for the synthesis of chitosan using deacetylases where in vivo activity is already achieved. Enzymatic deacetylation by the enzyme chitin deacetylase (CDA) can be explored for more consistent product and environment friendly technique.

4. FUNGAL SOURCES OF CHITOSAN

Chitin is widely distributed in Fungi Kingdom, occurring in *Ascomycetes*, *Zygomycetes, Basidiomycetes* and *Phycomycetes*, where it is a component of the cell walls and structural membranes of mycelia, stalks, and spores. Fungi provide the highest amount of chitin in the soil with nearly about 6–12 % of the chitin biomass, which is in the range 500–5000 kg/ha (Shahidi and Abuzaytoun, 2005). The amount of chitin varies from traces up to 40-45% of the organic fraction, the rest being mostly proteins, lipids, glucans and mannans (Roberts, 1992). However, not all fungi contain chitin, and the polymer may be absent in one species that is closely related to another. Variations in the amounts of chitin may depend on physiological changes in natural environment as well as on the fermentation conditions during biotechnological processing of fungal cultures. Chitin is the chief component in primary septa between mother and daughter cells of *Saccahromyces cerevisiae*. Chitin is vital to the integrity of the fungal cell wall and septum, as it provides strength through the hydrogen bonding of multiple chitin chains

arranged into microfibrils (Minke and Blackwell, 1978). Interestingly, chitin, with its importance in the fungal cell wall, accounts for only 1 to 2% of dry cell weight of yeasts (Klis, 1994), although in filamentous fungi, the proportion of chitin can be as great as 40-45% (Bartnicki-Garcia and Lippman, 1969).

Chitosan, the deacetylated derivative of chitin is an important constituent of the cell wall at various times during the life cycle of some fungal species (Christodoulidou et al., 1996). Chitosan is not directly synthesized rather the deacetylase enzyme can act efficiently to convert chitin to chitosan. *N*-deacetylation is a common step in the modification of sugar moieties, which may confer resistance to lysozyme action (Muzzarelli, 1977). Chitin deacetylases are thought to be in close proximity to the regions where chitin traverses the plasma membrane (Araki and Ito, 1975; Kafetzopoulos et al., 1993). As chitin is synthesized, the deacetylase enzyme converts it to chitosan. Chitosan is a polycation that is much more soluble than neutrally charged chitin. Of the few fungal species shown to synthesize chitosan, those belonging to the class *Mucorales* have been shown to generate chitosan during the vegetative growth phase (Orlowski, 1991), whereas *S. cerevisiae* produces chitosan only during the sporulation stage (Christodoulidou et al., 1996). Chitosan occurs as a natural component of cell walls of fungi belonging to various classes of fungi, such as *Zygomycetes* (e.g. *Absidia, Mucor, Rhizopus, Gongronella*) and can be produced by extraction from fungus cell walls (White et al., 1979). Chitosan isolation from various fungal sources and their extraction methods are depicted in Table 3. Recent advances in fermentation technology offers numerous possibilities for production and extraction of chitosan having predefined properties using fungi at industrial scale.

4.1. Zygomycetes

The cell wall of the *Zygomycetes* class is mainly composed of 20-50% chitin and chitosan however the polymers of glucuronic acid are present in lower concentrations. In this class of fungi, glucose is found only in spores (Ruiz-Herrera, 1992). Phosphates which are considered major contaminants also occur in the cell walls of these fungi (Gow et al., 1995). In the cell wall, chitosan may interact with other polysaccharides and phosphates in a complex such that common acidic treatments are not able to break and extract the chitosan from the cell wall completely. In these cases, a considerable amount

of chitosan remains in the alkali- and acid insoluble material (AAIM). In contrast to chitin, chitosan is less abundant in nature and has so far been found only in the cell walls of certain Mucoraleae fungi (*Zygomycetes*) in particular, *Mucor, Absidia* and *Rhizopus* genera (Muzzarelli, 1986; Ruiz-Herrera, 1992; Tan et al., 1996; Synoweicki and Al-Khateeb, 1997; Khor, 2001). Among *Zygomycetes* class, the *Mucorales* are the only one that has both chitin and chitosan in the cell walls. Both chitin and chitosan have been isolated from *Mucor rouxii* (White et al., 1979), *Absidia coerulea* (McGahren et al., 1984) and various other *Mucoraceae* strains (Amorim et al., 2001; 2003). In *Mucor rouxii* chitosan is the principal fibrous polymer of the cell wall in addition to chitin (Amorim et al., 2001). Chitosans isolated from *Mucorales* typically show MW in the range 4×10^5 to 1.2×10^6 Daltons.

4.1.1 Rhizopus

Nadarajah et al. (2001) demonstrated the extraction of chitosan from mycelia of different *Zymomycetes* strains, such as *Rhizopus* sp. KNO1, *Rhizopus* sp KNO2, *Mucor* sp KNO3 and *A. niger* obtained after submerged fermentation on synthetic media. They showed that the highest amount of chitosan can be obtained during the exponential growth phase. *Mucor* sp KNO3 produced the highest yield of 25% chitosan per unit dry mycelia mass. An alternative medium was used by Hang (1990) with *R. oryzae* mycelia using rice and corn as carbon sources, yielding 601 mg/l of extractable chitosan in the rice medium for a 48 h cultivation period. Four different filamentous fungi representing four different species, *A. niger* TISTR 3245, *R. oryzae* TISTR 3189, *Lentinus edodes* and *Pleurotus sajo-caju* and two yeast strains, *Zygosaccharomyces rouxii* TISTR5058 and *Candida albicans* TISTR 5239 were investigated for their potential to produce chitosan on synthetic media. A higher chitosan yield of 138 mg/g dry weight (DW) (14% chitosan) was achieved by the *R. oryzae* followed by 107 mg/g DW by *A. niger* (Pochanavanich & Suntornsuk, 2002).

In an attempt to utilize agricultural residues for economical production of chitosan, Suntornsuk et al. (2002) studied chitosan production from four fungal strains cultivated in solid-state fermentation with soybean and mungbean residues from food processing industries, containing limited nitrogen. The authors found that *R. oryzae* TISTR3189 produced the highest chitosan yield of 4.3 g/kg of substrate on soybean residue compared to chitosan yielded on mungbean residue (1.6 g/kg substrate). Recently,

(Yoshihara, 2003) studied the effect of the rare sugar D-psicose on chitosan production by *R. oryzae* and found that D-psicose supplementation in a medium containing a low amount of D-glucose resulted in 17.6% increase in the fungal chitosan productivity. Production and characterization of chitosan by different isolates, such as *A. niger, P. citrinium, F. oxysporum and R. oryzae* under solid-state fermentation conditions using rice straw supplemented with nutrients was investigated by Khalaf (2004). The maximum chitosan yield 5.63 g/kg of fermented biomass resulted with *R. oryzae* after 12 days of incubation followed by *A. niger*. The chitosan derived from these strains was found to have a deacetylation degree of 73-90% and viscosity of 2.7-6.8 milli Pascal-sec (mPa.s) The chitosan produced by *R. oryzae* was found to exhibit higher antibacterial activity against various pathogenic bacterial strains, such as *B. cereus, P. aeruginosa, Salmonella* sp and *E.coli* compared to chitosan from crab shells.

Chatterjee et al. (2008) studied the effect of some plant growth hormones (PGH), such as gibberellic acid (GA), indole-3-acetic acid (IAA), indole-3-butyric acid (IBA), and kinetin on improvement of growth, chitosan production and physico-chemical properties of chitosan by *Rhizopus oryzae* in deproteinized whey medium. The results indicated that hormones supplemented at different concentrations increase the mycelial growth by 19–32%. However, increase in chitosan content of the mycelia was comparatively small (1.7–14.3%) as compared to the control. Supplementation of GA resulted in maximum increase in growth and chitosan production. Addition of GA in the whey medium at a concentration of 0.1 mg/l increased both mycelial growth and chitosan content by 32% and 14.3%, respectively. The effect of plant growth hormones on different physico-chemical properties was also studied. DD of chitosan isolated from *R. oryzae* grown in presence of hormone was more or less the same in all cases. Polydispersity index (PDI), the measure of the distribution of MWs in a given polymer, was greatly decreased when hormone, especially IAA, GA or kinetin, was added to the whey medium. Average MW was found to be increased in all treatments by about 2-fold from 120 kDa to maximum 271 kDa due to hormone addition.

More recently, Tai et al. (2010) investigated the potential of hemicellulose hydrolysate (HH) of corn straw as substrate for *R. oryzae* growth and chitosan fermentation. The authors concluded that compared to xylose medium, HH resulted in increased mycelial growth and chitosan content (0.6 g/l). Sulphuric acid hydrolysis of corn straw resulted in the formation of different sugars (xylose, glucose, mannose) and inhibitors (formic acid,

acetic acid and furfurals). The authors showed that these microbial inhibitors at various concentrations increased mycelial growth and chitosan production by 24.5-37.8% and 60.1-207.1%, respectively. The authors also speculated that inhibitors, such as formic, acetic acid and furfural acts as natural stimulators of chitin and chitosan. In fact, these inhibitors stimulate *R. oryzae* to synthesize more chitin and chitosan during the fermentation process to better resist the invasion of these inhibitors. The enhanced chitin and chitosan production increases the density and thickness of the cell wall which now becomes resistant to the effect of inhibitors. Generally, depending on the environmental conditions, cells may undergo specific changes or develop a system to alter the composition of the cell wall that is more adapted to the specific environment.

Kleekayai and Suntornsuk (2010) used the potato chip processing waste, including trimmed potato, potato peel and low-quality potato chips as substrates for culturing *Rhizopus oryzae* for chitosan production for 21 days at 30±2° C and 70% moisture content. Results indicated that fermented potato peel resulted in the highest yield of 10.8 g/kg substrate after 5 days of fermentation with optimum conditions having 70% moisture content, pH of 5 and peel size of less than 6 mesh and extraction condition using 1M NaOH at 46 °C for 13 h followed by 2% acetic acid at 95 °C for 8 h. Therefore, potato peel could be applied as a low cost substrate for chitosan production from *R. oryzae. Rhizopus oryzae* is widely used in the food industry and its products are generally recognized as safe. Flood and Kondu, (2003) conducted studies for the safety evaluation of lipase produced from *R. oryzae* and found that lipase D enzyme produced by *R. oryzae* was regarded as safe when used in the processing of dietary fatty acids and glycerides of fatty acids.

4.1.2 Mucor

Mucor rouxii has been an extensively investigated fungus for chitosan production as is evident from the literature showing chitosan yields ranging from 6 to 9% of the dry cell mass (White et al., 1979). Synoweicki and Al-Khateeb (1997) investigated the influence of the growth time of *M. rouxii* on the contents of the chitosan as well as the yield of chitosan during the isolation process. The authors showed that the amount of extractable chitosan reached a maximum yield of 7.3% of dry mycelia after 48 h of incubation and the yield of extractable chitosan was not influenced by a prolonged growth of up to 5 days. Amorim et al. (2003) reported a chitosan yield of 3.3-5% after 2

and 5 days using the *M. rouxii* ATCC 24 905 strain. Amorim et al. (2001) reported rapid chitosan production by Mucoralean strains, *Mucor racemosus* IFM 40781 and *Cunninghamella elagans* URM 46109 within 24 h in submerged fermentation. A chitosan yield of 35.1 mg/g and 20.5 mg/g dry mycelia weight was achieved. After 24 h, a decline in extractable chitosan was observed which was due to physiological changes in the fungal cell wall. pH was noted to be 3.5 after 24 h of cultivation time which was a stimulating factor for the production of chitosan. Rane and Hoover, (1993) also suggested that the pH of the culture medium influenced the yield and the degree of *N*-acetylation of chitosan from *Absidia coerulea* possibly due to the pH optimum of chitin deacetylase, the enzyme responsible for the conversion of chitin to chitosan in fungus cell wall.

4.1.3 Rhizomucor

Belonging to *Zygomycetes*, *Rhizomucor pusillus* CCUG 11292 has been cultivated for the extraction of chitosan by dilute sulphuric acid (Zamani et al., 2007).The fungus was cultivated on the xylose-rich wastewater of an industrial ethanol plant and a chitosan yield of about 45.3% of alkali insoluble material (AIM) was achieved using dilute sulphuric acid treatment.

4.1.4 Absidia

Jaworska and Konieczna (2001) investigated the influence of ferrous ions, manganese ions, cobalt ions, trypsin and chitin, as individual supplements to the nutrient medium, on the *in vivo* activity of chitin synthase and chitin deacetylase to form chitosan in the fungus *Absidia orchidis*. They successfully demonstrated that manganese and ferrous ions gave the most significant results with increased chitosan yields through an increase in biomass production rather than an increase of chitosan content in cell walls. Manganese and ferrous ions were found to exert their effect by lowering the activity of the chitin deacetylase enzyme. However, their influence on the activity of chitin synthase was more complex. Rane and Hoover (1993) studied the effect of media on chitosan production from *Absidia coerulea* and showed that media with greater amounts of glucose and protein, and supplemented with minerals gave higher chitosan yields and lower degrees of *N*-acetylation of chitosan compared to cultures grown in minimal medium, with no added minerals. The authors attempted to optimize the alkaline and

acidic conditions for chitosan extraction and a maximum yield of 10.73% of dry weight mycelium was achieved with alkaline extraction at 121 °C for 30 min and hydrochloric acid extraction at 95 °C for 12 h. Similarly, Arcidiacono and Kaplan (1992) also reported that removal of peptone or yeast extract reduced biomass to around 30 and 40%, respectively. In an attempt to extract high-quality chitosan, Hu et al. (1999) demonstrated that pure chitosan with a high degree of extraction can be produced from *Absidia glauca* grown under submerged culturing conditions.

4.1.5 Cunninghamella

Amorim et al. (2006a) conducted a study aiming to evaluate the influence of culture medium on chitosan production by the mucoralean strain of *Cunninghamella bertholletiae*. Traditionally, the growth medium for mucoralean strains is comprised of yeast extract, peptone, and D-glucose as a carbon source giving a maximum chitosan yield of 55 mg/g of dry mycelia of *C. bertholletiae* in 72 h submerged culture conditions. After a 72 h incubation period, the decline in extractable chitosan was observed in the late exponential growth phase possibly due to physiological changes in the fungal cell wall. In the stationary phase, more chitosan was anchored to the cell wall of the zygomycetes by binding to chitin and other polysaccharides and the extraction became more difficult (McGahren et al., 1984; Amorim et al., 2006a).

There have been some studies reporting chitosan production from non-commercial substrates of the sugarcane processing industry, such as sugar cane juice and molasses supplemented with 0.3% yeast extract. The optimal production of chitosan was found to be 128 mg/g of dry mycelia in batch flasks at 28 °C (Amorim et al., 2006a). The utilization of sugarcane juice did not require high concentrations of sugar cane and gave a good yield of 580 mg/l of medium MW chitosan produced within 48 h. The study also demonstrated that molasses did not demonstrate the potential to be a good carbon source for chitosan production. Sugar cane juice was found to be a potential alternative for chitosan production from *C. bertholletiae*, especially because it does not need a high concentration of sugar and can reach a good yield of chitosan within 48 h of culture with chemical properties that enable it to be used in biological applications. Tan et al. (1996) during screening of different *Zygomycetes* strains reported the potential of *C. echinulata* as a prospective fungus for chitosan production with a yield of 7.14%. Amorim et al. (2006b) isolated a new strain of *Syncephalastrum racemosum* from the

dung of herbivores. The newly isolated was used for chitosan production in media with sugar cane juice and molasses as carbon sources. Higher yields of chitosan (74 mg/g of dry mycelia) were obtained from sugar cane juice with a sucrose concentration of 21 g/l and an increase in sucrose concentration did not improve the chitosan yield. However, molasses was not found to be a good carbon source for chitosan production (Amorim et al., 2006b).

4.1.6 Gongronella butleri

An improved method for the extraction of chitosan was demonstrated by Nwe and Stevens (2002a) using solid-state culturing of *G. butleri* USDB 0201 for 6 days, followed by the enzymatic extraction of chitosan. The chitosan from *G. butleri* was extracted by using 11 M NaOH at 45 °C and then by 0.35 M acetic acid at 95 °C and the resulting extract was clarified using a heat-stable α-amylase enzyme. Chitosan yields of of 4-6% dry mycelia and average molecular weight of 44-54 kDa were obtained. The authors (Nwe and Stevens 2002b) extracted chitosan from the chitosan/glucan complex isolated from the mycelia of the fungus, *G. butleri* USDB 0201 cell wall using amylolytic enzymes. The chitosan/glucan complex was cleaved with a heat-stable α-amylase at 65° C for 3 h which resulted in the removal of the glucan side chain and gave a chitosan yield of 4 g/kg sweet potato with 100 times lower turbidity.

Yokoi et al. (1998) used alternative media, such as barley/buckwheat and sweet potato, from shochu distillery wastewater for the production of chitosan with *G. butleri* IFO 8081, and a maximum chitosan yield of 730 mg/l was achieved using sweet potato medium after a 120 h incubation period. However, Tan et al. (1996) while using the same microorganism but different strain (*G. butleri* USDB 0201) under varied culturing conditions, using a synthetic medium containing glucose, yeast extract, peptone and other vital nutients obtained 470 mg/l chitosan. Nwe et al. (2004) studied the influence of nitrogen source and urea in the media comprising sweet potato in SSF, in order to optimize the chitosan production and they obtained a chitosan yield of. 11% of the dry cell mass.

Recently in another study, Streit et al. (2009) investigated the production of chitosan using liquid cultivation of *G. butleri* CCT4274 using apple pomace as substrate. A chitosan yield of 1.19 g/l culture medium including 40 g/l of reducing sugars and 2.5 g/l

of sodium nitrate was produced after 72 h incubation period which represented around 21% of the biomass content. The authors concluded that the reducing sugars and sodium nitrate had a significant effect on the yield of chitosan. Increasing the concentration of sugars resulted in a reduction of chitosan yield, although an increase in biomass was observed.

4.2. Ascomycetes

Chitin and cellulose are present in the cell walls of *Ascomycetes*, *Ophiostoma ulmi* and *Colletotrichum lindemuthianum*, whereas the ascomycetes, *F. oxysporum* contain only chitin (Cherif et al., 1993). *O. ulmi*, *C. lindemuthianum* and *F.oxysporum,* among others are causal organisms of many diseases in plants. For example, *C. lindemuthianum* is the causal agent of anthracnose disease of common bean (*Phaseolus vulgaris*), one of the most serious diseases in tropical areas. However, a few non-pathogenic strains of *C. lindemuthianum* are also known (Veneault-Fourrey et al., 2005), which can be possibly harvested for the production of chitosan. The natural occurrence of chitin in the related fungal strains could be viewed as a good source of chitosan. Moreover, these fungal strains are widely employed for variety of biotechnological processes on an industrial scale. The mycelium wastes of different fungal strains resulting from various fermentation processes can be utilized for the feasible chitosan extraction.

4.2.1 Aspergillus

Aspergillus niger strains are widely employed for the production of citric acid (CA) and other biotechnological and pharmaceutical products on an industrial scale. Fungal mycelial wastes from the antibiotic or CA industry can be an inexpensive and rich alternative source of chitosan besides the traditional industrial source of shellfish waste materials. The alkali-insoluble cell-wall residue of the *A. niger* biomass consists mainly of chitosan, chitin and β-glucans, with a significant prevalence of (1,3)-β-D-glucan. Chitin is thought to be present as microfibrils physically embedded in the β-glucan matrix. Nadarajah et al. (2001) carried out optimization studies for chitosan production during fungal growth by using different fungal strains, such as *Rhizopus* sp. KNO1, *Rhizopus* sp KNO2, *Mucor* sp KNO3 and *A. niger* in submerged fermentation on synthetic media. The extractable chitosan content in *A. niger* was 11.2% per dry cell weight (DCW) which was the lowest among all the fungal strains. Maghsoodi et al. (2008) investigated the

effect of different nitrogen sources on the amount of chitosan produced by *A. niger* PTCC 5012. The authors utilized naturally occurring waste substrates, such as soya bean, corn seed and canola residue as substrates for culturing *A. niger* and concluded that produces the highest amount of chitosan (17.05±0.95 g/kg DS) was produced using soya bean residues having a moisture content 37% and nitrogen content of 8.4±0.26% after a 12 day incubation period. However, corn seed residues having 1.9±0.4% nitrogen content resulted in much lower chitosan yields. Recently, Maghsoodi et al. (2009) studied the effect of glucose supplementation on chitosan production in the submerged fermentation of *A. niger* BBRC 20004. The addition of 8% glucose in sobouro dextrose broth resulted in the highest yield of chitosan 0.912 g/l as compared to 0.845 g/l without glucose after 12 days of cultivation.

Aspergillus niger strains are widely employed for the bioproduction of citric acid (Dhillon et al., 2011a, 2011b). The annual worldwide production of CA is estimated to be 1.7 million tons which will result in 0.34 million tons of *A. niger* mycelium waste per annum and furthermore the industry continues to increase with an annual growth rate of 5% (Wu et al., 2005; Dhillon et al., 2011b). *Aspergillus niger* strains contain approximately 15% chitin, which can be separated and transformed into chitosan (Kucera, 2004). There is an obvious need to develop some integrative technology to utilize the unlimited waste mycelium resulting from CA industries.

4.2.2. Penicillium

Penicillium chrysogenum is widely used for the large-scale production of antibiotics. As a by-product of the antibiotic industry, a large amount of *P. chrysogenum* mycelia are disposed off by incineration. Only a small percentage is used as an additive for cattle feed and in agriculture as fertilizers. Utilizing this biomass to produce chitosan not only has a commercial advantage, but also an ecological benefit. The extraction of chitosan from mycelia of *P. chrysogenum* using 0.5 M NaOH has been studied (Tan et al., 2002). Recently, Tianqi et al. (2007) developed an integrative extraction method for chitosan, ergosterol and (1-3)-α-D-glucan from *P. chrysogenum* mycelia provided by a pharmaceutical company. A chitosan yield of 5.7% was achieved with an 86% DD. The fungal waste mycelium resulting from the biotechnological and pharmaceutical industries, such as *Penicillium* waste from antibiotic production among others are promising zero cost sources for extraction of fungal chitosan.

4.3. Basidiomycetes

The mycelia, caps and stalks of fruiting bodies of four edible mushrooms, *Lentinus edodes* (shiitake mushroom), *Lycophyllum shimeji, Pleurotussajor- caju,* and *Volvariella volvacea* contain chitin as a minor component (Cheung, 1996). There is only one report on the presence of chitosan in a *Basidiomycete*, *Lentinus edodes* (Shiitake mushroom) (Crestini et al., 1996). Basidiomycete *Rhizoctonia solani* also contains chitin (Cherif et al., 1993). Crestini et al. (1996) studied the production of chitosan from *Lentinus edodes* SC-945 by solid-state and submerged fermentation. A chitosan yield of 120 mg/l under submerged conditions and 6.18 g/kg of fermented medium under solid-state fermentation was obtained. In one of the recent studies, Kannan et al. (2010) successfully utilized three mushrooms belonging to the *Basidiomycetes* family including *Agaricus* sp., *Pleurotus* sp. and *Ganoderma* sp. for the production of chitosan under solid-state fermentation using different compositions of saw dust and rice straw solid substrate. The authors showed that in both alkaline and acidic treatments during the extraction of chitosan the incubation time, temperature and concentration of base and acid significantly affected the production and physico-chemical properties of chitosan, such as DD and MW. As evident from the literature, the higher fungal chitosan yields were achieved mainly during the exponential growth phase which suggested that the chitosan formed by chitin deacetylase prevailed at this stage. There is also a possibility that during the growth phase, chitin is less crystalline and thus more susceptible to chitin deacetylase (Davis and Bartinicki-Garcia, 1984).

Edible mushrooms, such as white button mushroom *Agaricus bisporus*, among others are produced and consumed at large scale. The amount of waste remaining after removing the edible part mainly consists of stalks and mushrooms with irregular dimensions and shapes and accounts for 5-20% of the total production volume. In the US alone, mushroom production results in nearly 50, 000 metric tons of mushroom waste material per year with no suitable commercial application (Wu et al., 2005). Taking into consideration the huge amount of wastes of commercially important edible mushrooms, such as *Agaricus bisporus, Lentinus edodes*, *Pleurotus* species and *Volvariella volvacea,* among others there is a need to look for its potential applications including extraction of high value-added product chitosan, which nowadays finds promising applications in various fields. The research should be focused on non-pathogenic fungal strains for chitosan production and extraction. As evident from the literature, researchers have utilized various phytopathogenic strains for chitosan

extraction, namely *Ascomycetes*, such as *Ophiostoma ulmi*, *Colletotrichum lindemuthianum*, and *F. oxysporum* are phytopathogens responsible for agricultural economic losses. The genus *Fusarium* includes a number of economically important plant pathogenic species and produces mycotoxins in cereal crops that can affect human and animal health, if they enter the food chain. *Fusarium* species are known to produce toxins, named fumonisins and trichothecenes. Some species may cause a range of opportunistic infections in humans (Guarro and Gene, 1995). *Ophiostoma ulmi* is responsible for a fungal disease in elm trees. *Colletotrichum lindemuthianum* is also a plant pathogen, responsible for causing "anthracnose" in a wide range of plants from both temperate and tropical environments, such as common bean (*Phaseolus vulgaris*) (Roca et al., 2003). Similarly, in the genus zygomycetes, some species, such as *Rhizopus* and *Absidia* are also pathogens. *Rhizopus oryzae* is an opportunistic human pathogen and causative agent of zygomycosis (mucormycosis) (Pipet et al., 2007). Some species of *Absidia* also cause zygomycosis, in the form of mycotic spontaneous abortion in cows. They can also causes mucormycosis in humans with low immune systems, typically affecting vital organs, such as lungs, nose, brain, eyes and skin (Morales-Aguirre et al., 2004).

Specific care needs to be taken by the scientific community during research related to the pathogenic strains during chitosan production and extraction. Nevertheless, there are chances of their dispersal to some isolated areas which are still less affected or unaffected. It is worth noting that fungal sources are gaining interest among researchers due to the potentially greener synthetic approaches available compared to traditional crustacean sources of chitosan which generate large quantities of chemical wastes (Freepons, 1991; Roberts, 1992; Tayel et al., 2010). At the same time, future research should also concentrate on the use of safe microorganisms. In this regard, the studies should focus on the fungal waste mycelium resulting from various biotechnological industries and more importantly, non-pathogenic fungal strains.

5. FACTORS AFFECTING YIELD OF CHITOSAN

The yield of chitosan derived from fungal biomass largely depends on several factors, such as the strain of fungi used, cultivation methods, such as submerged shaking culture, bath culture, continuous culture, solid-state culture and surface culture and process parameters, such as pH, temperature, mixing rate, incubation time, particle size

and broth rheology. An increase in the chitosan yield can be obtained either by increasing biomass yield or by an increase in the cell wall content of chitosan (Jaworska & Konieczna, 2001). The SSF method of culturing fungi for biomass production offers advantages over SmF, such as higher chitosan yield (mg/kg mycelia) due to higher amount of mycelia produced. Among the various groups of microorganisms studied, filamentous fungi are the most exploited for their ability to grow on solid substrates (Nwe et al., 2002c; 2004). However, SMF has specific advantages over SSF as it provides easier control of fermentation parameters, such as pH, temperature and nutrient concentration in the fermentation medium (Durand et al., 1996). The initial pH of the SSF medium strongly affects the rate and extent of fungal growth (Barbosa et al., 1997). Most of white rot fungi grow well at slightly acidic pH values between 4 to 5 (Dube, 1990). Nwe and Stewens (2004) showed that the weight average MW (Mw) and polydispersity of the chitosan were lower at pH 3.77 and 4.92 and higher at pH about 5.5 in SSF. Arcidiacono and Kaplan (1992) observation that the biomass of *Mucor rouxii* and MW of chitosan reduced at pH 3 and molecular weight slightly increased at pH 6 in SmF.

As chitosan is a nitrogen containing biopolymer, fungi require an inorganic or organic nitrogen source as nutrient to produce the chitin/chitosan for their cell wall. Fungi use ammonium ions directly as a nitrogen source, while other inorganic forms of nitrogen are first reduced to the redox level of ammonium (Moore-Landecker 1996). The nitrogen source is considered as one of the important factors for the production of chitosan by fungi. Among various nitrogen sources, urea is found to be the best nitrogen source for the production of fungal chitosan by solid substrate (SS) fermentation (Nwe et al., 2002c). Nwe and Stewens (2004) studied the production of chitosan by the fungus *Gongronella butleri* USDB 0201 grown on sweet potato pieces supplied with different amounts of urea at 26 °C for 7 days under sterilized and humidified air supply. The results demonstrated that the initial pH and the amount of urea affect the yield of fungal mycelia and chitosan. The higher yield of mycelia up to 40 g/kg of sweet potato supplied with 7.2 g urea was observed. Further increase in urea resulted in a decrease in the mycelium production and above 15 g urea/kg SS resulted in a poor growth. However there are no significant differences between the total amounts of chitosan, 3.59±0.29 and 4.31±0.65 g, obtained from the 1 kg of solid substrate supplemented with 7.2 or 14.3 g urea, respectively. The authors also demonstrated that in *G. butleri*, the MW of chitosan increased with increasing amounts of urea supplied to the SSF media. The

large difference between number average MW (Mn) and Mw also expressed by the polydispersity (Mw/Mn), indicates that the chitosan polymers are in homogeneous in size and that a fraction of considerable higher MW chitosan is present. Arcidiacono and Kaplan (1992) showed that MW of chitosan could be increased by using more N-source (peptone) in the submerged fermentation medium.

6. EFFECT OF EXTRACTION METHODS ON THE PHYSICO-CHEMICAL CHARACTERISTICS OF CHITOSAN

The commercial scale extraction of chitosan from the traditional sources, such as exoskeleton of crustaceans employ harsh chemical treatments which often lead to inconsistent physico-chemical characteristics of the chitosan produced, such as high MW, low DD and purity of product. Chitosan obtained from deacetylation of crustacean chitin may have a MW over 100 kDa. MW and DA are considered as basic physico-chemical characteristic of the chitosan which govern most of the other important physico-chemical properties of chitosan, such as viscosity, solubility, degree of polymerization and polydispersity index. The extraction method is a vital factor that determines the specific characteristics of the products, such as MW, DD and crystallinity of chitosan. Viscosity of linear molecules is also affected by the MW (Billmeyer, 1971). There are several important factors that contribute to chitosan solubility, such as temperature and time of deacetylation, alkali concentration, prior treatments applied to chitin isolation, ratio of chitin to alkali solution, particle size, etc. A study conducted by Rao et al. (2007) on intrinsic viscosity, FTIR, and powder X-ray diffraction (XRD) showed that the MW and DA are collectively responsible for the solubility in the condition of random deacetylation of acetyl groups, which resulted fromthe intermolecular force. The solution properties of chitosan, thus, depend not only on its average DA but also on the distribution of the acetyl groups along the main chain in addition of the MW (Domard et al., 1983; Muzzarelli, 1983; Kurita et al., 1991; Kubota and Eguchi, 1997; Peniche and Arguelles-Monal, 2001). Apart from the DD, the MW is also an important parameter that controls significantly the solubility and other properties (Chatelet et al., 2001; Zhang and Neau , 2001; Guo et al., 2002; Khan et al., 2002; Prashanth et al., 2002; Lu et al., 2004; Schiffman and Schauer, 2007; Zhou et al., 2008). In this context, the extraction process should be selected based on the properties of chitosan desired. The various routes

applied for the extraction of chitosan from the fungal mycelium are depicted in Fig 2. Although chitin is insoluble in most solvents, chitosan is soluble in most acidic solutions, such as formic, acetic, citric, hydrochloric, lactic and tartaric acids at pH < 6.5 (Peniston and Johnson, 1980; LeHoux and Grondin, 1993). However, it is insoluble in phosphoric and sulfuric acid at room temperature with possible solubility in boiling solutions.

Chitosan is traditionally produced from the cell wall of fungi by a two-step extraction process involving base and acid treatments as depicted in Fig 2. Proteins, lipids, and alkali-soluble carbohydrates are first dissolved in alkaline solution, such as 2–4% NaOH at 90–121 °C for 15–120 min and the remaining cell wall material containing chitosan is obtained as AIM. The AIM is then treated with an aqueous solution of an acid, such as 2–10% acetic acid at 25–95 °C for 1–24 h to dissolve the acetic-acid-soluble material (AcSM) and separated from the remaining material in the cell wall, which is called alkali- and acid-insoluble material (AAIM). AcSM is generally considered to be rich in "fungal chitosan" and it can be precipitated by raising the pH to 9–10 followed by centrifugation and washing with acetone and ethanol (Tan et al., 1996; Synowiecki and Al-Khateeb, 1997; Chatterjee et al., 2005). In one study, Rane and Hoover (1993) investigated chitosan extractions of mycelia from *Absidia coerulea* ATCC 14076 using hot alkaline and acid treatments. Alkaline treatments were carried out at 95 °C and 121 °C using NaOH with varying extraction times. Acid treatments were carried out at 95 °C using hydrochloric, formic and acetic acids with various extraction times. The highest yields of chitosan were obtained with alkaline extraction at I21 °C for 30 min and hydrochloric acid extraction at 95 °C for 12 h. The effects on the DA and viscosity of the chitosan of various treatments were also examined.

The requirement of different physico-chemical characteristics of chitosan is sought in different applications. The extraction protocols which have large impact on the physico-chemical characteristics of the chitosan should be carefully selected depending upon the desired application of chitosan. Moreover, recent advances in fermentation technology have made possible the large-scale controlled production of chitosan by culturing the microorganisms, such as fungi which contain chitosan in their cell walls. Various factors, such as base and acid concentration, temperature, incubation period and particle size among others have been found to affect the physico-chemical properties of chitosan. However, the possibility of manipulating the different physicochemical properties of chitosan through the regulation of factors, such as growth media composition and

processing parameters in the extraction protocols have made fungi an ideal research candidate.

6.1. Alkaline treatment

The concentration of base used in the extraction process significantly affects the DD. Kannan et al. (2010) demonstrated that increasing the concentration of base significantly resulted in increased DD along with increased incubation time and temperature of the chitosan extracted. The concentration of base should be carefully optimized for the production of highly consistent product.

6.2. Acidic treatment

The nature of acid used can affect the final yield of chitosan during extraction. The utilization of formic acid (6% v/v) as the extracting solution was found to have higher yield of chitosan compared to acetic acid followed by hydrochloric acid (Kannan et al., 2010). The authors also observed that during chitosan extraction, various acids produceded different effects and interactions at the same incubation time and concentration. The extraction of chitosan using hydrochloric acid gave a significantly higher DD compared to acetic acid and formic acid. Hydrochloric acid being a strong acid compared to acetic and formic acid caused a greater extent of hydrolysis of the acetyl moieties along with hydrolysis within the network of monomers in the chitosan polymer. Increasing the concentration of different acids also resulted in increased DD. High acid concentration and high temperature produced darker coloured chitosan whereas milder treatments gave lighter coloured chitosan.

6.3. Temperature and incubation period

Higher temperatures and longer incubation periods are necessary to allow for effective interactions between base, such as NaOH and the constituents of the fungal cell wall which in turn will lead to the possibility of extracting higher concentration of chitosan. Moreover, the longer incubation time also provides longer reaction time for base to act on the chitin and chitosan structures to separate them from other cell wall polysaccharides. Kannan et al. (2010) achieved a maximum yield of chitosan at 121 °C after 30 min incubation time. The DD was also found to increase with an increase in

incubation time as well as temperature and concentration of base and acid used during the extraction of chitosan (Kannan et al., 2010).

6.4. Particle size and density of chitin

Particle size and density of chitin affects the DD by affecting the penetration rate of the alkali solution into the amorphous regions and to some extent also the crystalline regions of the polymer required for hydrolysis to take place (Kannan et al., 2010).

7.METHODS FOR DETERMINATION OF PHYSICO-CHEMICAL CHARACTERISTICS OF CHITOSAN

Various methods have been reported to be applied for the determination of various physico-chemical characteristics of the chitosan (Table 4). Chitosan possesses three types of reactive functional groups, an amino/acetamido group as well as both primary and secondary hydroxyl groups at C-2, C-3 and C-6 positions, respectively. The amino contents of chitosan chain are the main factors contributing to differences in their structures and physico-chemical properties. Moreover, their distribution is random, which makes it easy to generate intra- and inter-molecular hydrogen bonds.

Zhang et al. (2010) synthesized many novel chitosan derivatives by chemical modification using the reactive activities of hydroxy- and amino groups. The amino group possesses nucleophilic characteristics, allowing easy formation of imine by reaction with aldehyde or corresponding amide derivatives in acylating reagents. In acidic solution, the amino groups showed alkaline properties and received protons to generate salts, presenting cationic polymer properties. Moreover, the amino functional group has also been associated with properties, such as chelation, flocculation and various biological functions. The characterization of a chitosan sample requires the determination of its average DA. The solubility of the chitosan is affected by the distribution of acetyl groups (random or blockwise) along the chain, and the inter-chain interactions due to H-bonds and the hydrophobic character of the acetyl group. The distribution of acetyl groups has been determined by various techniques, such as IR, elemental analysis, enzymatic reaction, UV, ^{1}H liquid-state NMR and solid-state ^{13}C NMR. Diad and triad frequencies have been calculated for homogeneous and heterogeneous chitosan with different values of DA by Rinaudo (2006).

In chitin, the acetylated units prevail (DA ≥ 90%), whereas chitosan is fully or partially deacetylated derived with a DA of < 30%. The search for quick, user-friendly, economical, and accurate method to determine the DA has been one of the major concerns over many decades. There are several destructive or non-destructive techniques to carry out DA measurements, such as IR, FTIR, nuclear magnetic resonance (NMR) and UV spectroscopies, pyrolysis gas, gel permeation, chromatography (GPC), thermal analysis, acid hydrolysis, dye absorption method and various titration methods (Muzzarelli and Rocchetti, 1985; Varum et al., 1991; Roberts 1992;; Koide, 1998; Brugnerotto et al., 2001; Duarte et al., 2001, 2002; Jiang et al., 2003; Van de Velde and Kiekens, 2004; Guinesi and Cavalheiro, 2006; Kasaai, 2008, 2010; Wu and Zivonovic, 2008). The non-destructive methods, such as FTIR offer the advantage of avoiding manipulations of the polymers, such as hydrolysis, pyrolysis, or derivatization, the consequences of which are not always well known. Especially, FTIR spectroscopy is considered to be a very attractive technique, as it is fast, sensitive, user-friendly, economical, and suitable for both soluble and nonsoluble samples (Duarte et al., 2002). IR-spectrophotometry is also valuable technique for the determination of *N*-acetylation degree of the chitosan based on the ratio between the absorbance of the amide II band at approximately 1655 cm^{-1} and the C-H band at 3450 cm^{-1} (Roberts, 1992). In high value product applications, usually NMR is used as it gives the most precise DA number. Proton NMR spectroscopy is a convenient and accurate method for determining the chemical structure of chitosan and its derivatives (Van de Velde and Kiekens, 2004; Lebouc et al., 2005; Kasaai, 2010). NMR measurements of chitosan compounds are, however, limited to samples that are soluble in the solvent, which limits the analysis of chitosan with DA values lower than 0.3 in aqueous solutions. Low cost and rapid methods, such as titration or dye adsorption can also be used that are rapid and convenient methods yielding comparative results.

Likewise, MW of chitosan also affects its applications in various fields. As polysaccharides in general, chitosans are polydisperse with respect to MW. The MW of chitosan depends on its source and deacetylation conditions, such as incubation time, temperature, and concentration of alkali. Chitosan obtained from deacetylation of crustacean chitin may have a MW over 100 kDa. Therefore, it is crucial to reduce the MW by chemical or enzymatic methods to much lower MW for easy application and high biological activity. The solubility of the samples and dissociation of aggregates often

present in polysaccharide solutions is the main problem encountered during MW calculations. Various systems have been suggested concerning choice of a solvent for chitosan characterization, such as an acid at a given concentration for protonation together with a salt to screen the electrostatic interaction. The limited choice of solvents, and the stability of the solutions formed, is the main problems for MW determination of chitosan. The selection of the solvent also becomes imperative when MW has to be calculated from intrinsic viscosity using the Mark-Houwink relation. The MW of a polymer is related to the intrinsic viscosity [η] by the Mark-Houwink-Sakurada-equation

$$[\eta] = K \times MW^{a} \qquad (3)$$

where ***K*** and the exponent ***a*** both are viscometric constants for a given well-defined polysaccharide solvent system (Tanford, 1961). The values of the parameters "***K***" and "***a***" depends on both the polymer-solvent system and the temperature (Flory, 1953). The exponent "***a***" is a polymer conformation parameter that decreases with increasing molecular compactness. There are many reports concerning MW calculations from intrinsic viscosity using the Mark-Houwink relation as reviewed by Yomata et al. (1993). It is well known that, in aqueous solutions, polyelectrolyte polymers are expanded by the electrostatic repulsion forces between the charged groups and, therefore, their viscosities decreased linearly with the increase of ionic strength. In order to characterize the properties of polymers, such as intrinsic viscosity and MW, it is necessary to choose the appropriate solvent system to eliminate ionic effects.

The viscometric constants for chitosan were determined by various authors as described by Yomata et al. (1993). Roberts and Domszy, 1982 determined the viscometric constants *a* and *Km* in the Mark-Houwink equation for chitosan in 0.1 M acetic acid 0.2 M sodium chloride solution, using the approach of Sharples and Major. The number-average molecular weights (Mn) were determined by absorbance measurements on solutions of the phenylosazone derivatives. The results obtained a = 0.93, $Km = 1.81 \times 10^{-3}$ cm^3 g^1 were found in agreement with values found for other ionic polysaccharides having related β-(1→4)-linked structures. Similarly, the values of *k* and α in the Mark-Houwink equation have been determined for chitosans with different DD values of 69, 84, 91 and 100% respectively, in 0.2 M CH_3COOH/0.1 M CH_3COONa aqueous solution at 30 °C by the light scattering method (Wang et al., 1991). The results indicated that the values of α decreased from 1.12 to 0.81 and the values of *k* increased from 0.104×10^{-3}

to 16.80 × 10^{-3} ml/g, when the DD varied from 69 to 100%. This can be attributed to the reduction of rigidity of the molecular chain and an increase of the electrostatic repulsion force of the ionic groups along the polyelectrolyte chain in chitosan solution, when the DD of chitosan increases gradually.

The authors used different solvent systems, such as: (1) 0.2 M acetic acid/0.1 M NaCl/4 M urea; (2) 0.167 M acetic acid/0.47 M NaCl; (3) 0.1 M acetic acid/0.2 M NaC1 and; (4) 0.5 M acetic acid/0.5 M CH3COONa. However, as evident from these studies, the authors used different solvent systems, preparation methods of the samples, MW ranges, and MW measurements, which make its difficult to compare the results. A sharp decrease (40-60% in one day) in chitosan viscosity dissolved in some organic acid solutions was observed by Kim et al. (2000). However, the viscosity of chitosan solution stored at 4° C was found to be relatively stable (No et al., 1999). Studies reported a decrease in the viscosity of chitosan (1% chitosan in 1% acetic and/or lactic acid solution) with increased storage time and temperature (No et al. 2006). The decrease in viscosity observed over time was related to the partial degradation of chitosan by the organic acid solutions.

MW of chitosan can also be measured by gel permeation chromatography (GPC), light scattering or viscometry (Rinaudo et al., 1993; Terbojevich and Cosani, 1997; Brugnerotto et al., 2001). Due to its simplicity, viscometry is the most commonly used methods although its accuracy is influenced by many factors, such as concentration, temperature, ionic strength, pH and type of acids as well as the MW. High MW chitosans (HMWCs) form solutions with higher viscosities as compared to lower molecular weight chitosans (LMWCs). Chitosan polymer having high viscosity is found to be soluble in acidic conditions and is insoluble at pH values above 6.5 (the pKa of chitosan is ~6.5). Chitosan is only soluble in acidic aqueous solutions and precipitates when neutralized. However, it was recently discovered that chitosan dissolved in solutions containing glycerol phosphate was soluble at near neutral pH and produced a sol-gel transition when heated (Filion et al. 2007). The study described the role of temperature-dependent chitosan intrinsic monomeric dissociation constant pK_0 (T) in context with solubility. The resulting chitosan pK_0 values at 25 °C were in the range from 6.63 to 6.78 for all chitosans and salt contents tested. The temperature dependence of chitosan ionization was found to strongly reduce pK_0(T) by 0.023 units per °C, for example, resulting in a reduction of chitosan pK_0(T) from 7.1 at 5 °C to 6.35 at 37 °C for f_D (the fraction of

glucosamine monomers/deacetylated monomers in chitosan) of 0.72 in 150 mM salt (Filion et al. 2007).

However, chitosan oligomer possesses low viscosity, and is freely soluble at neutral pH. LMWCs and chitooligosaccharides (COS) can be produced by the hydrolysis of chitosan either by chemical or enzymatic methods. The chemical method needs high energy and is difficult to control therefore the enzymatic hydrolysis of chitosan has been gaining interest. Chitosanolytic enzymes are used for depolymerization of chitosan to make LMWCs and COS. However, the applications of chitosanases are hindered by their high cost and lower availability. Various other enzymes, such as cellulases, lipases, lysozyme, papain and pectin lyase have been utilized for hydrolysis of chitosan to chitosan preparations with different molecular masses (Terbojevicb et al., 1996; Shin-Ya et al., 2001; Lin et al., 2002; Liu and Xia, 2006; Xia et al., 2008). During preparation of different MW chitosans, viscosity is used as a parameter to determine the MW. Unlike most polysaccharides, chitosan [positively charged only under acidic conditions (pH<6.5)], LMWCs and COS possesses positive charges, which allows them to bind with high affinity to negatively charged surfaces. This property is responsible for many of the observed biological activities (Kurita, 1998). However, it is worth mentioning that chitosans with different structures show variable biological activities, and not all biological activities are found in single kind of chitosan. For speciality applications in biomedical and pharmaceuticals, special types of bioactive chitosan can be synthesized by chemical modification and enzymatic hydrolysis.

8. ATTRIBUTES REQUIRED FOR CHITOSAN AS HIGH VALUE-ADDED INGREDIENT

For high value-added applications of chitosan in various sectors, the three main features of chitosan are generally considered: (1) MW; (2) DA; and (3) purity (absence of contaminating bacteria). Besides these features, other physicochemical characteristics, such as degree of polymerization (DP), polydisperity index (PI), viscosity, solubility, charge density, and crystallinity of the polymer are also taken into account for several speciality applications.

8.1. Molecular weight/ degree of polymerization (DP) and polydisperity index (PI)

Since chitosan is a polymer formed by repeating units of D-glucosamine sugar, the total length of the molecule is an important attribute of the molecule. As a result, the MW is a key feature for a particular application of chitosan. Several studies have established that the length of chains (MW) affects the degradation rates i.e. biodegradability (Tomihata and Ikada, 1997; Zhang and Neau, 2001; Huang et al., 2004). The understanding and control of the degradation rate of chitin/chitosan-based biomolecules, such as drug carriers and scaffolds is of utmost importance as degradation is essential in many applications related to release of bioactive molecules in the body and functional tissue regeneration applications. In ideal conditions, the rate of scaffold degradation should be equivalent to the rate of new tissue formation or adequate for the controlled release of bioactive molecules. The biocompatibility of the molecule is also affected by its degradation rate since very high rates of degradation will result in the accumulation of the amino sugars in large concentration which will produce an inflammatory response in the body. Studies have revealed that differences in degradation rates were due to variations in the distribution of the acetamide groups in the chitosan molecule (Aiba, 1992; Shigemasa et al., 1994; Aranaz et al., 2009). Chitosan with a low DD provoke acute inflammatory response due to high degradation rates and vice versa.

Similarly, higher DP of chitosan poses limitations in various applications due to its high viscosity and low solubility at neutral pH. In order to overcome this problem, LMWCs and chitooligomers having lower DP can be prepared by hydrolysis of the chitosan chains. These depolymerized products have been found to be more befitting in some specific applications, such as in biomedicine, pharmaceuticals, food and agriculture (Wang et al., 2008). The solubility of the chitosan depends upon the chain length of chitosan and chitosan with shorter chain could be dissolved directly in water without the need for acid treatment. The higher solubility in water is particularly useful for specific applications, such as in cosmetics or in medicine when the pH should be close to 7.0.

Depolymerization of chitosan can be carried out by chemical, physical or enzymatic methods. The chemical processes produce a large amount of short-chain oligomers having low yields, high separation cost and sre not environmental friendly. Several enzymes, such as chitosanase, chitinase, gluconase and some proteases can also

effectively used for enzymatic depolymerization for the production of chitosans having low DP with high water solubility (Qin et al., 2004; Aam et al., 2010; Xia et al., 2010).

The preparation of oligomers can also be carriedout with with the aid of specific enzymes, such as chitinases and chitosonases produced by microorganisms cultured on cheap waste biomass which can provide advantage of being highly reproducible, low cost and environmental friendly (Lee et al., 2006; Su et al., 2006). However, due to the unavailability and cost of specific chitosanases and chitinases, various non-specific enzymes are widely employed for their capability to depolymerize chitosan including lysozyme, lipases, amylases, cellulases and pectinases (Lin et al., 2002; Qin et al., 2004; Wang et al., 2004; Liu and Xia 2006; Lee et al., 2008; Lin et al., 2009; Xia et al., 2008). The efficiency achieved by the non-specific enzymes is often comparable to that obtained by chitosanases. The COS and LMWCs prepared by depolymerization of chitosan/chitin with chitosanases/chitinases and other non-specific enzymes have several potential applications in biomedicine, pharmaceuticals, food and agriculture (Aam et al., 2010; Luis et al., 2010; Xia et al., 2010). LMWCs and COS possesses positive charges which allows them to bind strongly to negative charged surfaces. This specific attribute of hydrolyzed chitosan products is responsible for many biological activities (Kurita, 1998). The DP and chain length of chitosan optimize the balance between stability and unpacking of polyplex containing chitosan and DNA during gene transfer. An increased chitosan chain length could lead to reduction in transgene expression (Hoggard et al., 2003). The authors also showed that chitosan oligomer (24-mer) was excellent for gene carrier.

Similarly, the PI value of chitosan and its derivatives also influences their applications in various domains. In analytical chemistry, chitosan is used as a chiral selector for separating optically active isomers (Budanova et al., 2004). Generally, these applications of chitosan involve the use of chitosan derivatives with a MW of nearly 60 kDa and low PI value. The fermentative hydrolysis used for chitosan hydrolysis usually gives samples with a high degree of polydispersity i.e. more than 3 which makes difficult to make proper correlation between the observed biological effect and the MW. The preparation of low-disperse samples of chitosan by fractionation represents an important limitation. Currently the methods, such as gel-permeation chromatography and fractional precipitation are used for obtaining low dispersed chitosans. However, the fractional precipitation of chitosan carried out using organic solvents or by increasing pH does not

produce the desired product as the process occurs under heterogeneous conditions. Low-disperse chitosan can be obtained in larger scale using successive ultrafiltration on membranes.

Lopatin et al. (2009) described that the chitosan sample can be fractioned by ultrafiltration using a combination of solvents and chitosan hydrolysis products with MW 10–30 kDa and low PI value of 1.56 can be obtained. Recently, Kleekayai and Suntornsuk (2010) studied the production and characterization of chitosan from *Rhizopus oryzae* grown on potato chip processing waste. The results indicated that MWs of fungal chitosan samples range between from 81 to 128 kDa (lower than crab and shrimp chitosans with 1,239 and 206 kDa, respectively), 86-90% DD and viscosity of 3.1–6.1 mPa s. The PI values of fungal chitosan, which show the distribution of the individual molar mass of the chitosan chain length, were ranging from 4.4 to 5.4.

8.2. Degree of acetylation (DA)

Degree of acetylation is one of the most important chemical characteristics that can influence the performance of chitosan in many applications (Baxter et al., 1992; Li et al., 1992). Chitosan is fully or partially N-deacetylated derivative with a DA < 30 %. The structure of chitosan is generally defined by the overall content of D-hexosamine residues and their distribution in the polymer chain. The molar fraction of *N*-acetyl D-glucosamine and D-glucosamine are expressed as a DA or DD, respectively. The DD represents the proportion of monomeric units from which the acetylic groups have been removed, indicating the proportion of free amino groups (reactive after dissolution in weak acid) on the polymer. The presence of free amine groups along the chitosan polymer chain due to a high DD allows this macromolecule to dissolve in diluted aqueous acidic solvents due to the protonation of amine groups. The extent of the DD is affected by various factors, such as pre-treatment, concentration of the base, particle size and density of chitin. The last two factors affect the penetration rate of base into the amorphous regions and to some extent in the crystalline regions of the biopolymer, required for the hydrolysis to occur. Practically, a DD value of 70 to 85% can be achieved in a single alkaline treatment (Roberts, 1998). This parameter is vital since it indicates the cationic charge of the molecule after dissolution in a weak acid. DD is a structural parameter which influences physico-chemical properties of chitosan (Tomihata

and Ikada, 1997). Fungal chitosan with high DD possesses large positive charge density due to free amine groups, making it unique for biological applications.

It is evident from the studies that chitosan has been used as a support material for gene delivery (Saranya et al., 2011).The parameters, such as DD, MW, charge of chitosan and the pH of media are important in determining the transfection efficiency of polyplex containing chitosan and DNA (Ishii et al., 2001; Huang et al., 2005; Lavertu et al., 2006; Wang et al., 2010). MacLaughlin et al. (1998) showed that chitosan with high MW formed the most stable plasmid/chitosan polyplex. The increased DD resulted in higher DNA binding ability, and high transgene expression due to higher charge density along the chain (Kiang et al., 2004; Lavertu et al., 2006; Centelles et al., 2010). Low DD in chitosan induced a rapid dissociation of chitosan/pDNA complex before lysosomal escape whereas high DD containing chitosan formed the highly stable and inefficient complex which did not dissociate even after 24 h (Thibault et al., 2010). Bozkir et al. (2004) showed that encapsulation efficiency of pDNA was directly proportional to DD and inversely proportional to MW of chitosan. DD also influences many biological properties, including biodegradation by lysozyme and wound healing properties (Varum et al., 1997; Cho et al., 1999).

The physico-chemical properties of LMWCs affect its hypocholesterolemic and hypolipidemic activities and *in vitro* binding capacities (Zhou et al., 2006; Liu et al., 2008; Liu et al., 2008a). The hypocholesterolemic activity of LMWCs was higher when its DD was higher (90%) at equal MW and particle size; this might be due to the electrostatic attraction between LMWCs and anionic substances, such as fatty acids and bile acids (Deuchi et al., 1995). The fat-binding capacity of LMWCs was found increase with higher DA and MW, while the cholesterol-binding ability did not show significant variation with changes in DA and MW. The bile-salt binding capacity was greatly affected by MW. The chitosans with higher MW showed the best binding capacity for the bile salts. The hypocholesterolemic effects of LMWCs with different physiochemical properties were further investigated *in vivo*. Rats fed diets containing the lowest-DA chitosan showed significantly lowered plasma triglyceride, total cholesterol and low-density-lipoprotein cholesterol (LDL-C) levels as well as elevated high-density-lipoprotein cholesterol (HDL-C) levels, although not all differences were significant. Moreover, the food-efficiency ratio also decreased with decreasing DA (Liu et al., 2008). LMWCs with higher MW limited the body-weight gain of adult rats significantly, reduced the food-efficiency ratio and

lowered plasma lipids (Li et al., 2007; Liu et al., 2008). These results confirmed the effect of viscosity on hypocholesterolemic activity but also indicated that the viscosity was not the major factor influencing the hypocholesterolemic effects of chitosan in the upper gastrointestinal tract. Above a certain viscosity, the effect was small with increasing MW. The particle size of LMWCs also evidently affected its hypocholesterolemic effect. LMWCs with a fine particle size effectively lowered plasma and liver lipid levels in rats (Sugano et al., 1980). In addition, the powdered form of LMWCs exhibited a greater rate of adsorption of oil than the flake type (Ahmad et al., 2005). The particle size of LMWCs was the main property affecting its hypocholesterolemic effect (Zhang et al., 2010). This is consistent with the report that powdered chitosans exhibited better cholesterol-binding capacity as compared to cellulose, while chitosan in the flake form bound less cholesterol than cellulose (Li et al., 2007).

Chatelet et al. (2001) investigated the role of the DA on some biological properties of chitosan films *in vitro*. The authors found that inspite of the DA, all chitosan films were cytocompatible towards keratinocytes and fibroblasts. They also demonstrated that higher the DA of chitosan, the lower was the cell adhesion on the films. Fibroblasts appear to adhere twice as much as keratinocytes on these materials. The authors observed that keratinocyte proliferation increases with the decrease of DA of chitosan films in contrast to fibroblasts which do not proliferate on chitosan films. Thus, DA influences the cell growth in the same way as cell adhesion. This behaviour can be linked to an extremely high adhesion on this kind of material, which certainly inhibits cell growth. In conclusion, DA plays a key role in cell adhesion and proliferation, but does not affect the cyto-compatibility of chitosan.

As the majority of the biological properties are related to the cationic nature of chitosan, the parameter having the most pronounced effect is the DD. However, MW also plays a predominant role in some cases. Some other factors also need consideration, such as chain conformation and degree of solubility. For instance chitosans having a block arrangement of acetylated and deacetylated units have the tendency to form aggregates in aqueous solutions (Anthonsen et al., 1994). Extensive aggregation result in more intermolecular interactions in the chitosan molecule limiting the number of available sites, particularly the amine groups which in turn affects biological functionality of the polymer. The distribution of acetyl groups also affects the biodegradability of the polymer

as the absence of acetyl groups or their homogeneous distribution results in low rate of enzymatic degradation (Aiba, 1992; Francis et al., 2000).

8.3. Purity

The purity of the product is important for high-value product applications, particularly in the field of biomedicine, pharmaceuticals or cosmetics. The purity is quantified in terms of the remaining ashes, proteins, insoluble fraction as well as in terms of bio-burden i.e. microbes, yeasts and moulds and various endotoxins produced by these microorganisms. Residual proteins in chitosan can cause allergic reactions, such as hypersensitivity (Aranaz et al., 2009) resulting in limiting the use of chitosan in the biomedicine sector. However, pure chitosan has been potentially used in various speciality applications, such as an internal hemostatic dressing, as a drug delivery agent, tissue scaffolding and in numerous other health related products (Baker and Wiesmann, 2008; Baldrick, 2010). As a natural product derived from the shells of marine organisms and other microorganisms, chitosan carries a variety of contaminants, common to biologically derived materials, such as protein and endotoxin, which varies with extraction protocols and thus must be carefully purified. For use in medically related applications, the resultant stimulatory immune response due to the presence of these contaminants should be understood (Peniston and Johnson, 1978; Sannan et al., 2003).

Recently, various chitosan-containing medical devices, such as the hemostatic celox for the treatment of bleeding, and bandages, such as HemCon or QuikClot for the control of bleeding are currently flooded in markets in Europe and US (Baldrick, 2010). These products are manufactured according to the Good Manufacturing Practice (GMP) guidelines. The companies utilize processes that eliminate contaminants, such as proteins, bacterial endotoxins and toxic metals. In addition, there are many studies reported on the preparation of high purity chitosan for medical grade through getting rid of the protein, lipid, metals and endotoxin (Lin et al., 2003; Percot et al., 2003; Van De Velde and Kiekens, 2004; Hung et al., 2006; Cooper et al., 2007; Shafaei et al., 2007; Abdou et al., 2008; Trombotto et al., 2008; Nguyen et al., 2009a, 2009b). For various applications, such as waste water treatment where lower purity chitosan is used, purity is a factor of concern as the remaining ashes or proteins tend to block active sites of chitosan, especially the amine grouping. Due to the blockage of these sites, the sites will

not be available to bind; hence, a larger quantity of chitosan is required for effective treatment.

8.4. Viscosity

Viscosity is an important parameter in the determination of chitosan MW and hence determining its industrial applications. Higher MW chitosan often render highly viscous solutions, which may not be desirable for industrial applications. In this regard, lower viscosity chitosans are prefered. The solution viscosity of chitosan depends on its molecular size, cationic character, and concentration as well as the pH and ionic strength of the solvent (Rinaudo 2006).

8.5. Solubility

Chitin and chitosan degrade before melting, which are typical for polysaccharides with extensive hydrogen bonding. This makes it essential to dissolve them in an appropriate solvent system to impart functionality. For each solvent system, polymer concentration, pH, counter ion concentration, and temperature effects on the solution viscosity must be known. When the DA of chitin reaches about 50%, it becomes soluble in aqueous acidic media and is called chitosan. Chitosan contains free amino groups on C-2 of GlcN unit and hence, is insoluble in water. However, under acidic pH conditions, amino groups can undergo protonation thus, making it soluble in water. For this reason, solubility of chitosan depends upon the distribution of free amino and *N*-acetyl groups (Sannan et al., 1976). Generally, 1–3% aqueous acetic acid solutions are used to solubilize chitosan (Rinaudo et al., 1999). The macromolecule chains are stretched caused by electrostatic repulsion of the NH^{+3}groups. The stretched chains will tend to be coiled with addition of salt because of the charge screening effect of added salt. The extent of solubility depends on the concentration and type of acid, whereas the solubility decreases with increasing concentration of acid and aqueous solutions of some acids, such as phosphoric, sulfuric, and citric acids are not good solvents (Gross et al., 1983).

8.6. Charge density

The charge density (the degree of protonation of amino groups) of chitosan is determined by the chemical composition, MW, and external variables, such as pH and

ionic strength. Dissociation constants (*pKa*) for chitosan range from 6.2 to 7, depending on the type of chitosan and conditions of measurement (Domard, 1987; Rinaudo and Domard, 1989; Sorlier et al., 2001; Strand et al., 2001). Generally, the solubility decreases with an increase in MW (Rathke and Hodson, 1994; Hudson and Jenkins, 2001). Researchers have made attempts to enhance chitosan's solubility in organic solvents (Nishimura et al., 1991; Kurita et al., 1994; Holappa et al., 2004). Various studies have been conducted to enhance the solubility of chitosan in water as most biological applications require the material to be processible and functional at neutral pH. Hence, obtaining a water soluble derivative of chitosan is an important step towards the further application as a biofunctional material (Jia et al., 2001; Kim and Choi, 2002; Badawy, 2010).

8.7. Crystallinity

Since chitosan is a heterogeneous polymer consisting of GlcN and GlcNAc units, its properties depend on the structure and composition. Ogawa and Yui (1993) studied the crystalline structure of different chitin/chitosan samples prepared by two different procedures: (1) the partial deacetylation of chitin, and (2) the partial reacetylation of a fully deacetylated chitin (pure chitosan). The results indicated that the partially reacetylated material was less crystalline than pure chitosan and also forms less anhydrous crystals. Generally, the step of dissolving the polymer results in a decrease in the crystallinity of the material. At the same time, it also depends on the secondary treatment, such as reprecipitation, drying, and freeze-drying. In addition, the origin may affect the residual crystallinity of chitosan, which in turn controls the accessibility to internal sorption sites and the diffusion properties (rehydration and solute transport) and also deacetylation procedure may affect the solid state structure of chitosan (Rout et al., 1993; Harish Prashanth et al., 2002; Jaworska et al., 2003).

9. CONCLUSIONS AND FUTURE PROSPECTIVE

Recently, the production of chitin and chitosan using fungi has received much attention due to the need for cheap and environmental friendly alternative sources. The abundantly available fungal mycelium wastes resulting from various industrial biotechnological processes represents promising source of chitosan as compared to traditional sources, such as crustacean shells. The various classes of fungi containing

significant amounts of chitin in their cell walls can be cultured using low cost agro-industrial wastes through fermentation processes for sustainable and economical production of chitosan. The extraction of chitosan from waste fungal mycelium can be considered as green synthesis approach and has the possibility to replace traditional methods which are laden with plethora of disadvantages. The chitosan can be obtained from fungal mycelium at ambient extraction conditions as compared to crustacean shells which are very recalcitrant and requires harsh condition for chitin extraction and its deacetylation to chitosan. Morevoer, the extraction of chitosan from crustacean shells results in large quantities of acid and alkali rich waste waters. The treatment of the waste water before its disposal in environment results in higher production costs. However, the chitosan obtained from fungal sources possesses superior physico-chemical characteristics. It can be tailored with predefined properties for various speciality applications, such as in biomedicines by careful manipulation of growth medium, fermentation process and extraction parameters. Furthermore, β-glucan can be isolated as a valuable side product from the mycelia chitosan-glucan complex. The waste biomass obtained during chitosan extraction from fungal biomass can be used as animal feed or as a biosorbent for adsorption of various pollutants from waste waters, such as heavy metals dyes, pesticides among others. This biomaterial will be highly activated during the extraction process which comprises alkali and acidic treatments and thus can replace high cost activated carbon as a biosorbent.

Moreover, recently the studies of chitin and chitosan conversion to chitooligomers have drawn attention. These oligomers are water soluble and have immense potential in biomedicine, pharmaceuticals, food and agriculture sectors. Traditionally, the chitin/chitosan derived oligomers are prepared through chemical processes which produce a large amount of short- chain oligomers having low yield, high separation cost and are not environmental friendly. Alternatively, these can also be prepared with the aid of chitinases and chitosonases produced by microorganisms using cheap waste biomass which can provide advantage of being highly reproducible, low cost and environmental friendly. The development of processes for utilization of fungal mycelium wastes resulting from biotechnological industries can also boost the upcoming fungal-based biotechnological industries. Currently, the industries incur losses for the safe disposal of wastes which finally adds to the production cost of the products. The value-addition approach will generate extra revenues for industries and also curtail the cost of

primary biotechnological product. Moreover, it will strengthen the economy and it will also help alleviate environmental pollution.

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DECLARATION OF INTEREST

The authors report no declarations of interest.

ABBREVIATIONS

ABA- abscisic acid ; AcSM- **A**cetic-acid-soluble material**;** AIM- alkali insoluble material; AAIM- alkali-and acid-insoluble material; ATCC- American type culture collection; BABA- β-aminobutyric acid ; CRL- *Candida rugosa* lipase; COS- chitooligosaccharides; CA- citric acid; CDA- chitin deacetylase; CS- chitosan sponge; DA- degree of acetylation; DD- degree of deacetylation; DP- degree of polymerization; AgNPs- gold nanoparticles; GlcN- glucosamine; HH- hemicellulose hydrolysate; HMWCs- High molecular weight chitosans; Kc- Kendal Cops®; LMWCs- lower molecular weight chitosans; MIC- minimum inhibitory concentration; MBC- minimum bactericidal concentration; MW- molecular weight; GlcNAc- *N*-acetylglucosamine; NOCC- *N,O*-carboxymethylchitosan (NOCC); NPs- nanoparticles; NMR- nuclear magnetic resonance; Mn- number average molecular weight; OCs- organochlorine compounds; OCPs- organochlorine pesticides; O/W- oil-in-water; PAMPs/AMPs- pathogen/microbe associated molecular patterns; PMCP- Partially myristoyl chitosan pyrrolidone carboxylic acid salt; PRRs- pattern recognition receptors; PtNPs- Platinum NPs; PANI- polyaniline; PI- polydisperity index; SAR- systemic acquired resistance; SPME- solid-phase microextraction; SmF- submerged fermentation; SS- solid-substrate; SSF-solid-state fermentation; TEM- transmission electron microscopy; TNV- tobacco necrosis virus; TP- total phenol; UDP-Glc-NAc- uridino-di phospho *N*-acetylglucosamine; Mw- weight average molecular weight

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Table 4.1. 1 Pros and cons of chitosan from different sources

Source	Pros and cons	References
Crustacean shells	**Pros** Well established method for Industrial production of chitosan **Cons** Seasonal and limited supply, high cost and laborious process and not environmental friendly Large quantities of chemicals, such as alkali and acids, higher temperatures and long processing time are required for extraction. Generally alkali concentration of 30-50% w/v and temperature > 100°C is required. Demineralization treatment is required to remove calcium carbonate which accounts for 30-50% of crustacean shells. Possesses high molecular weight and protein contamination which limits its applications in biomedicines	Aranaz et al., 2009
Fungi	**Pros** Medium-low molecular weight which is suitable for many biomedical applications Higher degree of deacetylation can be achieved Free of allergenic shrimp protein The molecular weight and degree of deacetylation of fungal chitosan can be controlled by varying the fermentation conditions Supply of fungal biomass is infinite, largely from the biotechnological and pharmaceutical industries Cheap biowastes can be used as economic substrates fro culturing fungi **Cons** Processes not scaled up to industrial level	Pochanavanich & Suntornsuk, 2002; Streit et al., 2009; Kannan et al., 2010

Table 4.1. 2 Various compounds and ions known to influence the activity of chitin synthase and chitin deacetylase

Enzyme	Metal ion/ compound	Microorganism used	References
Chitin synthase	Trypsin	*Neurospora crassa* *Saccharomyces cerevisiae* *Benjaminiella poitrassi* *Candida albicans*	Leal-Morales et al. (1994) Cabib et al. (1996) Deshpande et al. (1997) Gooday et al. (1997)
	Mn^{2+}	*Saccharomyces cerevisiae* *Candida albicans*	Cabib et al. (1996) Gooday et al. (1997)
	Co^{2+}	*Saccharomyces cerevisiae*	Cabib et al. (1996)
	Mg^{2+}	*Candida albicans* *Saccharomyces cerevisiae*	Gooday and Schofield (1995); Cabib et al. (1996)
Chitin deacetylase	Co^{2+}	*Colletotrichum lindemuthianum*	Tokuyasu et al. (1996) Kołodziejska et al. (1999)
	Fe^{2+}	*Absidia orchidis*	Jaworska & Konieczna, 2001
	Mn^{2+}	*Absidia orchidis*	Jaworska and Konieczna, 2001
		Mucor rouxi	Kołodziejska et al. (1999)
	Zn^{2+}	*Colletotrichum lindemuthianum* *Mucor rouxi*	Tokuyasu et al. (1996) Kołodziejska et al. (1999)
	Ca^{2+}	*Mucor rouxi*	Kołodziejska et al. (1999)

Table 4.1. 3 Different fungal sources for chitosan extraction

Microorganism	Type of culture medium/fermentation type	Chitosan yield/dry weight mycelium	Extraction conditions	Degree of deacetylation /viscosity /Mol. weight	Remarks	References
Absidia coerulea ATCC 14076	Synthetic medium	10.73±0.41 %	121 °C/30 min- 1 N NaOH 95 °C/12 h– 2 % HCl	90±2.5 % 2.69±0.19	-	Rane & Hoover, 1993
Aspergillus flavus	PDB medium	57 mg/g	121 °C/20 min- 1 N NaOH 95 °C (1:30 w/v)/8 h- 2% (v/v) acetic acid	-	-	George et al., 2011
Absidia Glauca	Synthetic medium/SmF	594.7±10.2 mg/L^{-1} g AIM	121 °C/15 min- 1 M NaOH 121 °C/15 min – 1M Hcl	75.6±0.9 %	-	Hu et al., 1999
Aspergillus niger BBRC 20004	Synthetic medium/SmF	0.912 g/L^{-1}	121 °C/20 min-1N NaOH 95 °C/ 6 h- 2 % (v/v) acetic acid	-	-	Maghsoodi et al., 2009
Aspergillus niger PTCC 5012	Soya bean, canola and corn seed residues/SSF	17.053±0.95 g/kg ds soya bean 12.73±1.22 g/kg canola reisude	121 °C/20 min-1N NaOH 95 °C/ 6 h- 2 % (v/v) acetic acid	-	Corn seed resulted in very low yield due to agglomeration of substrate which hinders oxygen diffusion within the substrate	Vida, et al., 2008
Basiodiomycetes (*Agaricus* Sp, *Pleurotus* Sp and *Ganoderma* Sp)	Different ratios of saw dust and paddy straw	0.976 mg	121 °C/25 min-1M NaOH	82 %	Increase in alkali concentration, Incubation time and temperature resulted in	Kannan et al., 2010

Microorganism	Type of culture medium/fermentation type	Chitosan yield/dry weight mycelium	Extraction conditions	Degree of deacetylation /viscosity /Mol. weight	Remarks	References
					increased chitosan production	
Cladosporium cladosporioides	Potato Dextrose Broth medium	25.2 mg/g	121 °C/20 min- 1 N NaOH 95 °C (1:30 w/v)/8 h- 2% (v/v) acetic acid	-	-	George et al., 2011
Cunninghamella bertholletiae IFM 46.114	YPD medium &sugarcane juice /SmF	55mg/g of dry mycelia (YPD) 128 mg/g dry mycelia (sugarcane juice)	NaOH/ acetic acid	88.20 % DD (YPD) 89.70 % DD (sugarcane juice)	-	Amorim et al., 2006a
Gongronella butleri USDB 0201	Sweet potato/Solid substrate fermentation	4-6 g/100g mycelia	11M NaOH-45 °C/13 h 0.35 M acetic acid-95 °C/5 h followed by clarification with α-amylase	Mol. Weight 44-54 kDa	-	Nwe & Stevens (2002a)
Gongronella butleri USDB 0201	Sweet potato/SSF	4 g/kg sweet potato		90.9 % DD/61 kDa mol. wt.	Chitosan solution with out any turbity was achieved after clarification with amylolytic enzymes	Nwe & Stevens (2002b)
Gongronella butleri CCT4274	Apple pomace/SmF	1.19 g/L representing 21 % of biomass content	2 % NaOH /200rpm//90 °C/ 2h 10 % acetic acid /150 rpm/60 °C/6h	-	Increasing of RS above 40g/L resulting in more biomass, but with less chitosan content	Streit et al., 2009

Microorganism	Type of culture medium/fermentation type	Chitosan yield/dry weight mycelium	Extraction conditions	Degree of deacetylation /viscosity /Mol. weight	Remarks	References
Lentinus edodes SC-495	Wheat straw (SSF)	6.18 g/kg fermented biomass (SSF) 120 mg/L SmF	1 M NaOH -120 °C/1.5 h/ 95 °C/14 h/ 2% acetic acid	Nearly 88-90 % DD		Crestini et al., 1996
Mucor sp KNO3	Synthetic/SmF	27 % of dried biomass	Method of white et al., 1979	-	Highest amount of chitosan obtained at late exponential phase	Nadarajah et al., 2004
Mucor racemosus IFM 40781	Synthetic (YPD medium) /SmF	35.1 mg/g dry mycelia weight	1M NaOH in 95% ethanol/100 °C/90 min 1M HCl- 95 °C/5h	51 % DD	Rapid production at 24 h incubaton time	Amorim et al., 2001
Mucor rouxii ATCC 24 905	Synthetic (YPD medium) /SmF	3.3-5.0 % /dry mycelia	Extraction by method of Synoweicki & Al-Khateeb, 1997	-	-	Amorim et al., 2003
Mucor rouxii		6-9 % /dry mycelia				Synoweicki & Al-Khateeb, 1997
Penicillium chrysogenum	Waste mycelia provided by pharmaceutical industry	5.7 %	95 °C/1.5 h/0.1 M NaOH/ 45 °C/1.5 h/1M acetic acid	86 % DD	Ergosterol and (1-3)-α-D glucan can also be extracted simultaneously	Tianqi et al. (2007
Phoma sp.	Potato Dextrose Broth medium	31.1 mg/g	121 °C/20 min- 1 N NaOH 95 °C (1:30 w/v)/8 h- 2% (v/v) acetic acid	-	-	George et al., 2011
Rhizomucor pusillus CCUG 11292	Xylose-rich wastewater from ethanol plant	45.7 %/ AIM	90 °C/2 h/0.5 M NaOH	-	-	Zamani et al., 2007

Microorganism	Type of culture medium/fermentation type	Chitosan yield/dry weight mycelium	Extraction conditions	Degree of deacetylation /viscosity /Mol. weight	Remarks	References
			95 °C-121°C/20 min-12 h/0.5-2 % H_2SO_4			
Rhizopus arrhizus UCP 402	Corn steep liquor and honey	29.3 mg/g	121 °C/15 min-1M NaOH 100 °C/ 15 min – 2 % (v/v) acetic acid	86 % DD	-	Cardoso et al., 2012
Rhizopus oryzae ME-F12 (mutant of *R.oryzae* ATCC 20344)	Hemicelluloses hydrolysate of corn straw obtained after H2SO4 hydrolysis/SmF	Nearly 0.58 g/L^{-1}	121 °C/15 min-1M NaOH 95 °C/ 24 h – 2 % (v/v) acetic acid	89-90 % DD	Inhibitors present in the hydrolysate induced fungal growth	Tai et al., 2010
Rhizopus oryzae	Rice straw+ nutrients/SSF for 12 days	5.63 g/kg of fermented medium	121 °C/20 min-1M NaOH 95 °C/ 8 h – 2 % (v/v) acetic acid	73-90 % 2.7-6.8 cP	Exhibited higher antibacterial as compared to crab shell chitosan activity	Khalaf, 2004
Rhizopus oryzae TISTR 3189	Potato dextrose broth (PDB) for fungi and mushrooms	138 mg/g DW (14 %)	121 °C/15 min-1M NaOH 95 °C/ 8 h – 2 % (v/v) acetic acid	83.8 – 90 % /3.1-6.2cP /2.7 × 10^4 - 1.9 × 10^5 Da	-	Pochanavanich and Suntornsuk, 2002
Aspergillus niger TISTR 3245		107 mg/g DW (11 %)				
Lentinus edodes		33 mg/g DW (3.3 %)				
Pleurotus sajo-caju		12 mg/g (1.2 %)				
Zygosaccharomyces rouxii TISTR5058	Yeast malt extract broth (YMB) for yeast	36 mg/g (3.6 %)				
Candida albicans TISTR 5239		44 mg/g (4.4%)				

Microorganism	Type of culture medium/fermentation type	Chitosan yield/dry weight mycelium	Extraction conditions	Degree of deacetylation /viscosity /Mol. weight	Remarks	References
Rhizopus oryzae TISTR 3189 *Aspergillus niger TISTR 3245* *Zygosaccharomyces rouxii* TISTR5058 *Candida albicans* TISTR 5239	Soyabean and moongbean residues/SSF	Higher yield of chitosan by R.oryzae – 4.3 g/kg (soyabean residues), 1.6 g/kg (moongbean residues)	121 °C/15 min-1N NaOH 95 °C/ 8 h- 2 % (v/v) acetic acid	-	Agricultural residues can be used as economical substrates	Suntornsuk et al., 2002

Abbreviations: DD-degree of deacetylation; DW-dry weight; RS-reducing sugars

Table 4.1. 4 Different techniques employed for the determination of physico-chemical characteristics and properties affected by these characteristics.

Characteristics	Property affected by characteristic	Determination techniques [References]
DA	Analgesic, antimicrobial, antichloresterolemic, antioxidant, adsorption, biodegradibility, biocompatibility, mucoadhesion, and hemostatic	IR Spectroscopy [Roberts 1992; Brugnerotto et al., 2001; Van de Velde and Kiekens, 2004; Kasaai, 2008] NMR spectroscopy (^{1}HNMR and ^{13}CNMR) [Varum et al., 1991; Raymond et al., 1993; Duarte et al 2001; Van de Velde and Kiekens, 2004; Lebouc et al., 2005; Kasaai, 2010] 1st derivative UV-spectroscopy [Muzzarelli and Rocchetti, 1985; Wu and Zivanovic, 2008] Conductometric and potentiometric titration [Raymond et al., 1993; Jiang et al., 2003] Differential scanning calorimetry [Guinesi and Cavalheiro, 2006] FTIR spectroscopy [Duarte et al 2002; Van de Velde and Kiekens, 2004;
Average Mw or Mw distribution	Antimicrobial, antichloresterolemic, antioxidant, biodegradability mucoadhesion and hemostatic	Gel-permeation chromatography [Brugnerotto et al., 2001] Viscometry [Rinaudo et al., 1993] Light scattering [Terbojevich and Cosani, 1997]
Crystallinity	Antimicrobial, antichloresterolemic, and haemostatic	X-ray diffraction [Roberts 1992; Yen et al., 2009]
Protein	Antimicrobial, antichloresterolemic, and haemostatic	[Lowry et al., 1951; Bradford, 1976]
Moisture and ash content		Gravimetric analysis [ASTM, 2001]

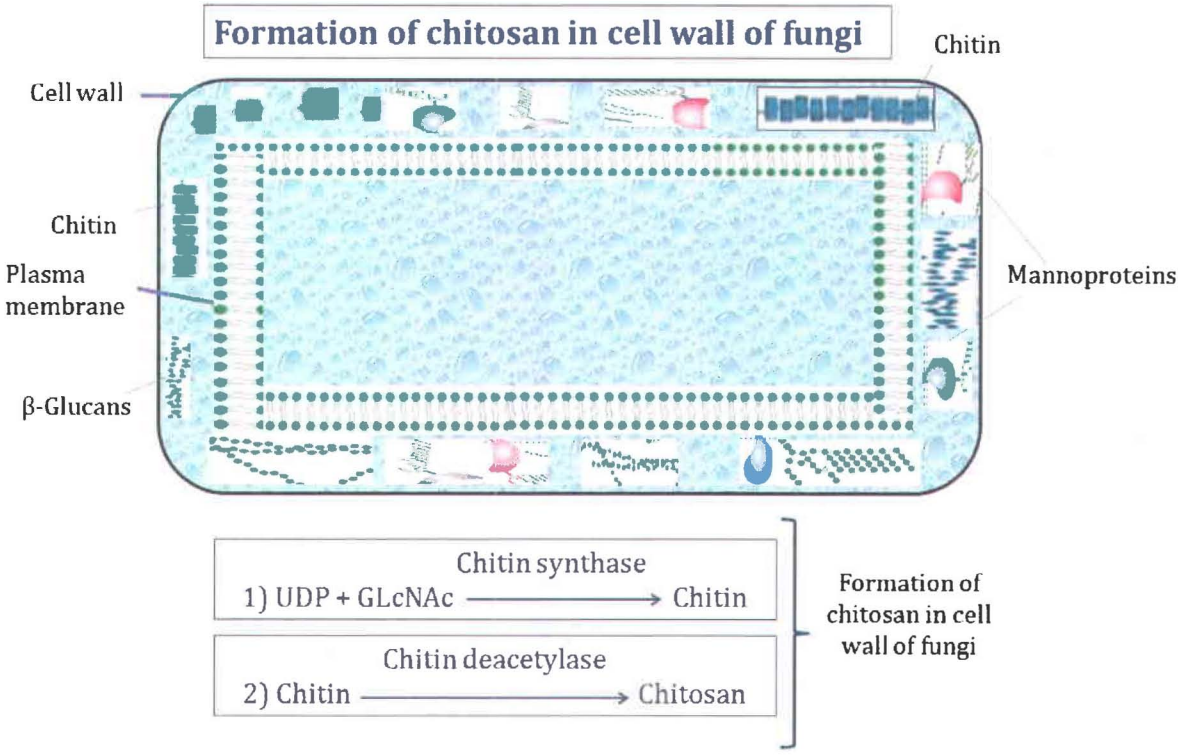


Figure 4.1. 1 Diagrammatic representation of chitosan formation in fungal cell wall

Abbreviations: GlcNAc - N-acetylglucosamine; UDP-Glc-NAc - uridino-di phospho *N* acetylglucosamine (The fungal cell organelles are not shown for clarity)

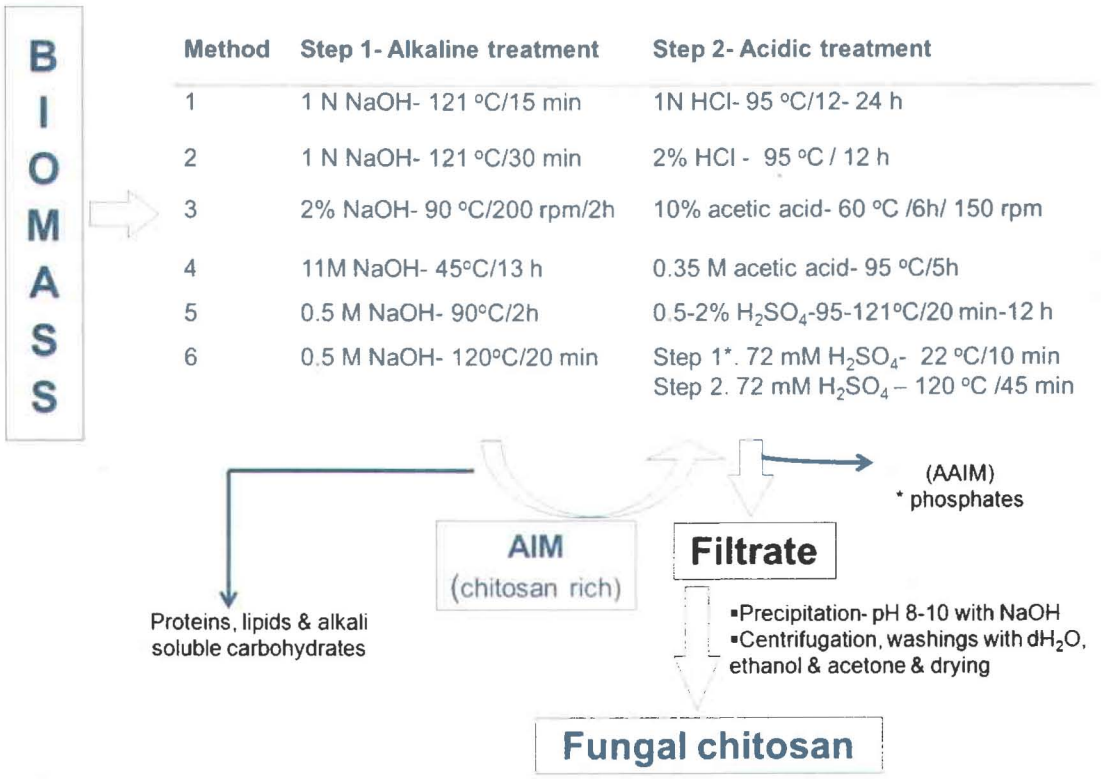


Figure 4.1. 2 Different methods employed for the extraction of chitosan from the fungal mycelium

Abbreviations: AIM- alkali insoluble material; AAIM- acid-and alkali-insoluble material

References: White et al., 1979; Rane and Hoover, 1993; Synowiecki and Al-Khateeb, 1997; Nwe and Stevens, 2002a; Zamani et al., 2007; Zamani et al., 2010

CO-PRODUCT (CHITOSAN) EXTRACTION FROM WASTE FUNGAL BIOMASS DERIVED FROM CITRIC ACID BIOSYNTHESIS

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RÉSUME

Pour le but d'explorer la possibilité d'un coproduit d'extraction (chitosane CTS) du mycélium fongique des déchets résultant pendant la production d'acide citrique (AC), la présente étude a été réalisée. La biosynthèse de l'AC a été réalisée par fermentation à l'état solide et immergée dans des fermenteurs de l'échelle du laboratoire à l'aide de marc de pomme (AP) et des boues de pomme ultrafiltres (APS) par *Aspergillus Niger NRRL* 567. La production de l'AC est de 294 ± 13 g / kg sec AP et 40,3 ± 2 g/L. la production des APS a été réalisée grâce aux SSF et SmF après une période d'incubation de 120 h et 132 h, respectivement. La biomasse fongique des déchets résultant lors de la production de l'AC a été utilisée pour l'extraction de CTS en utilisant des conditions ambiantes. Les CTS extractibles ont été trouvé à être plus élevés dans le contrôle avec 6,40% et 5,13% de la biomasse sèche, respectivement avec le mycélium fongique résultant de la SSF et SmF. Toutefois, les CTS extractibles ont été trouvés à être plus faibles dans le traitement avec des inducteurs. Le degré de désacétylation de CTS a été varié de 78 à 86% pour la biomasse fongique obtenue à partir de SSF et SmF avec différents inducteurs. La viscosité de CTS fongiques a été jugée allant de 1.02 à 1.18 $Pa.s^{-1}$ qui est considérablement inférieur à celui de CTS commerciale de la coquille de crabe. L'étude a montré la possibilité de co-extraction de CTS de qualité supérieure provenant de la biomasse fongique des déchets résultant de divers champignons des industries biotechnologiques, y compris l'AC.

Mots clés: marc de pomme, boues de pulpe de pomme, ultrafiltration; acide citrique; chitosane; fermentation en milieu solide; fermentation submergée.

ABSTRACT

Exploring the possibility of co-product (chitosan, CTS) extraction from waste fungal mycelium resulting during citric acid (CA) production, the present study was carried out. CA biosynthesis was carried out through solid-state and submerged fermentation in lab scale fermenters using apple pomace (AP) and apple pomace ultrafiltration sludge (APS) by *Aspergillus niger* NRRL 567. CA production of 294±13 g/kg dried AP and 40.3±2 g/L APS was achieved through SSF and SmF after 120 h and 132 h incubation period, respectively. The resulting waste fungal biomass during CA production was used for CTS extraction using ambient conditions. Extractable CTS was found to be higher in control with 6.40% and 5.13% of dried biomass, respectively with the fungal mycelium resulting from the SSF and SmF. However, the extractable CTS was found to be lower in the treatments with inducers. Degree of deacetylation of the CTS was ranged from 78-86 % for fungal biomass obtained from SSF and SmF with different inducers. The viscosity of fungal CTS was found to be ranging from 1.02-1.18 $Pa.s^{-1}$ which is considerably lower than the commercial crab shell CTS. The study indicated the possibility of co-extraction of superior quality CTS from waste fungal biomass resulting from various fungal-based biotechnological industries including CA.

Key words: Apple pomace; apple pomace ultrafiltration sludge; *Aspergillus niger* NRRL-567; citric acid; chitosan; solid-state fermentation; submerged fermentation.

INTRODUCTION

There is an increasing demand of the biomolecules having non-toxic, biodegradable, biocompatible and environmentally safe nature. The natural occurrence of citric acid (CA) as metabolic intermediate product in mostly all organisms and chitosan (CTS) in some fungal strains assures their non-toxic nature. CA has been emerged as one of the important multifarious platform chemical having great potential in the food, pharmaceuticals, cosmetics, detergents and agricultural sectors. It is acknowledged worldwide as a GRAS (generally recognized as safe), as approved by the Joint FAO/WHO Expert Committee on Food Additives. Recently, CA has been researched for an array of advanced applications, such as in biomedicine industry for synthesis of biopolymers for drug delivery, culturing of wide variety of human cell lines; nanotechnology, for bioremediation of heavy metals from soil and metal ore mines and for making water-based wood preservatives [Soccol et al., 2006; Ashkan et al., 2010; Dhillon et al., 2010; Guillermo et al., 2010; Forest Products Laboratory]. CA market has been under tremendous pressure for the past few years and continues to fluctuate with reduction in prices. High raw materials and energy costs has transformed the lucrative CA production sector into an unprofitable market. The search for inexpensive substrates as an alternative to high cost substrates is vital to reduce the production cost of CA.

The other viable option to stabilize the CA markets is the extraction of co-product, CTS from waste fungal mycelium resulting after CA production. CTS is a natural biopolymer having unique polycationic, chelating and film forming properties due to the presence of active amino and hydroxyl functional groups. Owing to these unique properties, CTS is widely used in diverse fields ranging from medicine, nano-science, food and chemical engineering, pharmaceutical, cosmetics, nutrition and agriculture. CTS is an active ingredient used for the formation of different nanoparticles (NPs) which find potential applications in many areas, such as silver NPs having applications against emerging antibiotic resistance (Wei et al., 2009). CTS also exhibits an array of biological properties, such as antimicrobial activity, induced disease resistance in plants and diverse stimulating or inhibiting properties towards a number of human cell lines (Abd-El-Kareem et al., 2006). Recently, CTS is widely used for lipid absorption, lowering of serum cholesterol, cell and enzyme immobilizer, as a drug carrier, material for production of contact lenses, or eye bandages, permeability control agent, as adhesive, chromatographic support, paper-strengthening agents, antimicrobial compounds, seed

coats and flocculating and chelating agents in wastewater treatments (Crestini et al., 1996; Yokoi et al., 1998; Shahidi et al., 1999; Cetinus & Oztop, 2003; Gil et al., 2004).

Traditionally, CTS is mainly derived from naturally occurring crustacean chitin, such as crab and shrimp shell by *N*-deacetylation using harsh chemical treatments (Knorr, 1991). However, the CTS obtained by such treatments suffer some inconsistencies, such as protein contamination, inconsistent levels of deacetylation and molecular weight which resulted in variable physico-chemical characteristics of chitosan. There are some additional problems, such as environmental issues due to use of toxic chemicals, limitation of seafood shell supply, geographical limitation, seasonal variation and high cost (Crestini et al., 1996). Therefore, biological approaches for the synthesis of CTS need to be explored as the novel green route. Recent advances in fermentation technology suggest the solution for many of these problems, mainly by culturing fungi (Rane & Hoover, 1993; Chiang et al., 2003; Kucera, 2004). The mycelia of various fungi including *Zygomycetes, Ascomycetes, Basidiomycetes* and *Deutermoycetes*, are alternative promising sources of chitin and CTS (Ruiz-Herrera et al., 1992; Hon, 1996; Pochanavanich & Suntornsuk, 2002). A fungal cells wall contains up to 50% chitin as compared to crustacean shells which contain 14-27% on dry biomass basis (Zamani et al., 2007). In this perspective, production and purification of CTS from cell walls of fungi grown under controlled conditions offers greater potential for highly consistent product.

Aspergillus strains are widely used for the industrial production of CA among other carboxylic acids, antibiotics and in other fermentative products. Generally, the mycelium waste is discarded to the sewage treatment system or burnt. The alkali-insoluble cell-wall residue of the *A niger* biomass consists mainly of chitin, CTS and β-glucans. Fungal biomass waste of the CA and other industries can become free and abundant alternative source of CTS. Moreover, the fungal CTS can have unique properties compared with those derived from crustaceans, such as: 1) free of allergenic shrimp protein and; 2) the molecular weight and degree of deacetylation (DD) of fungal CTS can be controlled by varying the fermentation conditions (Arcidiacono & Kaplan 1992, Stevens et al. 1997).

In this study, we have considered the possibility of extraction of important co-product, CTS from *A. niger* NRRL-567 waste biomass produced during CA production. In this context, the bidentate approach was applied as follow: 1) the economical production of CA from Apple pomace (AP) and apple pomace sludge (APS) under solid-state

fermentation (SSF) and submerged fermentation conditions and; 2) extraction of co-product, CTS from resulting waste fungal biomass. The characterization of the properties of the produced CTS was also investigated. As per the published literature, so far to the best of our knowledge, there is no study reported on the utilization of fruit processing industry waste for the combined bioproduction of CA and co-extraction of CTS from waste fungal biomass.

MATERIALS AND METHODS

Microorganisms and inoculum preparation

Aspergillus niger NRRL-567 was procured from Agricultural Research Services (ARS) culture collection, IL-USA and cultivated as freeze-dried form. The culture conditions, maintenance of fungus and inoculum production are described in Dhillon et al. (2011a; 2012)

Substrate procurement and pretreatment

Apple pomace (AP) and Apple pomace ultrafiltration sludge (APS) from (Lassonde Inc., Rougemont, Montreal, Canada), was selected as fermentation substrate for citric acid production. AP was already supplemented with rice husk (1% w/w), as a common practise of supplementing the apples with rice husk during the extraction of juice for a better hold on the apples in the industry during meshing and filtration. AP was completely dried at 50±1 °C in a hot air oven till constant weight, grounded and was passed through sieves to get the desired particle size of 1.7 mm to 2.0 mm which was used for this study.

APS is the liquid sludge obtained after the ultrafiltration of crude juice. The apples are mashed in the initial step of processing and the solid waste is separated, and the crude juice so obtained is again filtered twice through ultrafiltration system which leaves apple pomace sludge accounting for 5-10% of total processed apples. APS contains high macro- and micronutrient contents (total carbon 51.9 g/l, total nitrogen 2.94 g/l, carbohydrates 66.0±1.7 g/l, lipids 5.9±0.3 g/l, protein 33.75±2.0 g/l, among others) (Dhillon et al. 2011b). For experimental purpose, the desired suspended solids (SS) concentrations of 25 g/L were made up by adding distilled water. The pH was adjusted to 3.5±0.1.

Solid-state fermentation

Solid-state CA fermentation was performed in a 12-L rotating drum type bioreactor, Terrafor (Infors HT, Switzerland). Approximately, 3 kg of rehydrated AP (moisture 75%, v/w) was sterilized in autoclave (121 ± 1 °C, 30 min) and was transferred into the sterilized bioreactor under aseptic condition. After transferring the substrate into the bioreactor the sterilization was done again to ensure complete decontamination. After cooling, the substrate was supplemented with 3% (v/w) of inducers, ethanol and methanol, respectively. The initial pH (3.5 ± 0.1) was taken as such without any adjustment. The inoculation was done with the spore suspension having 1×10^7 spores/g substrate. For final moisture content of 75% (v/w), the volume of inducers and spore suspension was also taken into account. The fermentation was carried out in a controlled environment at 30 ± 1 °C, intermittent agitation rate of 2 rpm (for 1 h after every 12 h) and aeration rate of 1 vvm. The fermentation was carried out till 144 h, and the samples were harvested after every 24 h under aseptic conditions for CA and total spore count analysis. CA was extracted from a solution prepared with 1 g of a fermented sample macerated with 15 ml of distilled water and shaken for 20 min in an incubator shaker at 200 rpm at 25±1 °C. The supernatant was filtered through glass wool for removal of solid substrate and fungal mycelia. About 100 µl sample was taken in Eppendorf tubes for viability (total spore count) assay using haemocytometer and the remaining sample was centrifuged (Sorvall RC 5C plus by Equi-Lab Inc., Québec, Canada) at 9000 × g for 20 min and the clear supernatant was analyzed for CA concentration.

Submerged fermentation (SmF)

All the experiments were performed in a 7.5 l capacity fermenter with 4.5 l working volume (Labfors, HT Bottmingen, Switzerland). APS having 25 g/l suspended solids (SS) concentration was used as a substrate for CA bioproduction. APS was sterilized at 121±1 °C for 30 min. After sterilization, the substrate was supplemented with inducers (3% (v/v) ethanol and methanol) in different treatment. The medium was inoculated with 10% (v/v) inoculum concentration. Temperature and pH of the fermentation medium was controlled at 30±1 °C and 3.5, respectively. To maintain dissolved oxygen concentration above 20% saturation (critical oxygen concentration), the medium was agitated at a speed of 300 rpm, and the air flow rate was automatically controlled using a computer

controlled system. Samples were withdrawn from the fermenter at 24 h intervals for the CA and biomass estimation.

Analytical techniques

The pH of the substrates was measured with the pH-meter equipped with glass electrode. The total solids content of APS was estimated by drying the 25 mL sample in an oven at 100±5 °C to constant weight. For experimental purpose, the required total solids concentrations were made with distilled water. The moisture of the substrate was analyzed using a moisture analyzer (HR-83 Halogen; Mettler Toledo, Switzerland). In order to measure the amount of living fungal biomass in solid-state cultivation, viability assay was used as an indicator. Total spores were counted microscopically using Naubeur chamber. CA concentrations were determined gravimetrically by the modified pyridine-acetic anhydride method (Marier and Boulet, 1958). CA concentrations were expressed as grams per g/kg of dried AP and g/L for APS.

Chitosan extraction

Chitosan extraction was carried out by a modified method of Zamani et al. (2010) as described in Figure 4.2.1. The whole solid-state fermented biomass and biomass resulting from SmF was dried at 50 °C till constant weight and grounded. Powdered biomass was treated with 0.1% (v/w) Tween-80 solution in the ratio 1:10 (g dried biomass: mL Tween-80 solution) at incubated for 15 min at 50±1 °C and 200 rpm. Then 0.5 % (v/w) NaOH solution in the ratio 1:25 (g dried biomass: mL alkaline solution) was added and the contents were autoclaved at 121±1 °C for 30 min. Alkali-insoluble material (AIM) were collected after 3 washings with distilled water and centrifuging at 9000 × g for 20 min till neutral pH. AIM obtained was dried in an oven at 50 °C. In the first step of acidic treatment the dried AIM was treated with 72 mM H_2SO_4 in the ratio 1:40 (g dried AIM: ml acidic solution) at 22 °C for 15 min. The contents were centrifuged at 9000 × g for 15 min. In the second step of acidic treatment, 100 mM H_2SO_4 (1:40) was added in the solids resulting from first step and autoclaved at 121±1 °C for 45 min. The contents was hot vacuum filtered and the pH of the supernatant adjusted to 9-10. The contents were centrifuging at 9000 × g for 15 min followed by washings with acetone and

ethanol. The pellets obtained was lyophilized (ScanVac Cool Safe, LaboGene) and CTS obtained in the form of white powder.

Chitosan Characterization

Deacetylation

The extent of CTS deacetylation was determined by titration with 0.01M NaOH (Donald & Hayes, 1988). The method involved hydrolysing the acetyl groups present in CTS with 0.01 M NaOH and converting the salt to acetate, which was evaporated as an azeotrope with water and titrated. The acetyl percentage was determined from the Equation 1:

$$\%acetyl = \frac{V \times 0.04305}{W} \quad \textbf{(1)}$$

where V is the corrected volume of NaOH and W is the weight of the sample. DD of CTS was calculated by using the Equation 2:

$$\%deacetylation = 100 - \%acetyl \quad \textbf{(2)}$$

Viscosity

The viscosity of 1% CTS in 1% acetic acid solution was determined using a Brookfield digital Rheometer (Model DV-III, Brook Engineering laboratories, Inc., Stoughton, MA) with SC-34 (small sample adapter) spindle at 25°C. The gaps between spindle and sample chamber were 4.830 mm for SC-34 to adapt to the analysed samples of CTS.

Statistical analysis

CA and CTS values presented are an average of three replicates along with the standard deviation (±SD). Database was subjected to an analysis of variance (ANOVA), and multiple range tests among data were carried out using the Statistical Analysis System Software (STATGRAPHICS Centurion, XV trial version 15.1.02 year 2006, Stat Point, Inc., USA), and the results which have $p<0.05$ were considered as significant.

RESULTS AND DISCUSSION

Citric acid fermentation

Fig. 4.2.1 A and 4.2.2 A illustrates the CA production trends in SSF and SmF and biomass production thorough submerged fermentation through *A. niger* NRRL-567. CA production of 182.83±8.20, 252.8±12.14 and 294.19±13.22 g/kg dried AP, respectively in control, inducers, 3% (v/w) EtOH and 3% (v/w) MeOH, respectively through SSF after 120 h incubation period. Similarly, in SmF CA production of 18.4±1.24, 33.3±1.70 and 40.34±1.98 g/L APS was achieved in control and inducers, 3% (v/w) EtOH and 3% (v/w) MeOH, respectively after 132 h of fermentation time.

The viability of *A. niger* NRRL-567 in SSF and biomass production thorough SmF through is given in Fig. 4.2.1 B and 4.2.2 B. The viability is given as the total spore count representing colony forming units/gram fermented substrate (CFUs/g). The viability data indicates that the spore count was initially decreased till 48 h due to the active growth of fungus during the log phase. Later on increase in the spore count was observed after 48-72 h fermentation period due to the less availability of substrates due to fungus growth and it was increased till the end of fermentation. The spore count was significantly higher in the control as compared to the treatments with inducers. The lower alcohols inhibit sporulation during SSF. During SmF, the biomass (g/L) obtained in the control (12.6±0.9) was higher than the treatments with inducers. However, the CA production was significantly ($p<0.05$) higher in the treatments with inducers. Studies have reported that a higher production of CA can be achieved only under the conditions of limited biomass production (Couto and Sanroman 2006).

Tran et al. (1998) compared the different types of SSF systems, such as flask fermentation, tray fermentation and rotating drum bioreactor fermentation. They concluded that flask level fermentation gives higher production of CA 14 g/100 g dry fermented pineapple waste supplemented with 3% (v/w) MeOH after 3 days. However, in tray and rotating drum bioreactor the yield was lower at 8 g/100 g dry fermented pineapple waste in the presence of 3% (v/w) MeOH. As opposed to the previous studies conducted by other researchers, it is evident that higher CA production was obtained by using AP through SSF. The present study also showed that MeOH has more enhancing effect on CA production than EtOH as an inducer. The supplementation of EtOH and

MeOH in the liquid medium resulted in the pellet morphology instead of filamentous growth during the fermentation which is considered best for the higher CA production (Dhillon et al., 2010).. The present study showed that expensive media is not required for supplementation of inexpensive agro-industrial wastes which are rich in carbon and other vital nutrients required for CA production.

In solid-state culturing at laboratory scale, CA is generally produced in Erlenmeyer flasks (Hang and Woodams 1984; 1985; Hang et al., 1987; Shankranand and Lonsane 1994a; 1994b; Lu et al., 1995). Flasks are convenient and easy to handle for investigation in the laboratory scale. It is possible to use separate flasks which can be removed daily for analysis without disrupting other samples. However, in large bioreactor, the removal of samples can disrupt fungal mycelia which may adversely affect growth of fungus resulting in low yield of CA. Bioreactor design and handing operation for CA production requires much more sophisticated controls.

Chitosan extraction from waste fungal biomass

Fig. 4.2.3 illustrates the AIM and CTS yield obtained from *A. niger* waste mycelium resulting from CA fermentations. Higher AIM and CTS production of 45.60%, 33.55%, 32.20% and 6.40%, 5.41%, 3.93% of dried biomass, respectively in control and inducers EtOH and MeOH, respectively were obtained with the fungal mycelium resulting from the SSF. However, as compared to SSF the mycelium resulting from the SmF rendered lower AIM and CTS production of 11.5%, 9.53%, 6.4% and 5.13%, 4.46%, 2.95%, respectively in control, EtOH and MeOH. Higher AIM fraction obtained with fungal biomass resulting from SSF was mainly because the whole fermented AP was taken for CTS extraction. In SSF, it was difficult to separate the fungal mycelium, as it penetrates into the solid substrates.

CTS extraction from SSF biomass was found to be higher than the biomass obtained through SmF. This can be attributed to the fact the fungi grows more efficiently in SSF. As evident from the present results, although the supplementation of lower alcohols resulted in higher CA production but the extractable chitosan yield declined might be due to the cell wall permeability enhancing effect of lower alcohols (Dhillon et al., 2011c). Under SSF, the percentage of AIM obtained can be considered as a fungal growth parameter since it has been reported to be mainly constituted by mycelium growth (Di

Lenia *et al.*, 1994). The late exponential phase is considered best for the higher yield of extractable CTS and also the higher CA production phase.

The DD of the CTS was observed to be 78-86 % for different treatments, such as fungal biomass obtained from SSF and SmF with different inducers. The DD being the highest for CTS obtained from control biomass without inducer. The DD of CTS is an important parameter affecting its physico-chemical properties. In fact, the large positive charge density due to the high DD makes fungal CTS unique for industrial applications, particularly as a coagulation agent in physical and chemical waste-treatment systems, as a chelating and clarifying agent in the food industry, and as an antimicrobial agent. The viscosity of fungal CTS (1% w/v) was found to be ranging from 1.02-1.18 $Pa.s^{-1}$ which is considerably lower than the commercial high molecular weight (310-375 kDa) crab shell CTS from Sigma with viscosity of 1.120 centipoise. Results were similar to those reported by Shimahara et al. (1989) and Pochanavanich and Suntornsuck (2002). This means that the molecular weight may be lower than that of crab shell CTS. Thus, fungal CTS could have potential medical and agricultural applications (Pochanavanich & Suntornsuck, 2002). With further optimization of the extraction parameters CTS ranging from low molecular weight to medium molecular weight can be easily prepared.

Maghsoodi et al. (2008) investigated the effect of different nitrogen sources on the amount of CTS produced by *A. niger* PTCC 5012. The authors utilized naturally occurring waste substrates, such as soyabean, corn seed and canola residue as substrates for culturing *A. niger* and concluded that it produces the highest amount of CTS (17±0.9 g/kg of dry substrate) with soya bean residues having moisture content 37% and nitrogen content of 8.4±0.3% after 12 days incubation period. However, corn seed residues having 1.9±0.4% of nitrogen content resulted in very lower amount of CTS. Recently, Maghsoodi et al. (2009) studied the effect of glucose supplementation on CTS production in SmF of *A. niger* BBRC 20004. The addition of 8% glucose in sobouro dextrose broth resulted in the highest production of CTS 0.912 g/L as compared to 0.845 g/L without glucose after 12 days of cultivation. Although, the CTS production from *Aspergillus* waste biomass is lower than few other studies, however, the present study utilized the negative cost waste biomass resulting from CA production which renders it more economical than from the fungal biomass obtained by culturing them on costly substrates.

Aspergillus strains are widely employed for the production of CA and other biotechnological and pharmaceutical products on industrial scale. Moreover, due to the increasing demand of CA, its annual worldwide production is estimated to be 1.7 million tons which will result in 0.34 million tons of *A. niger* mycelium waste per annum and furthermore increasing at an annual growth rate of 5% (Wu et al., 2005; Dhillon et al., 2010). However, for the past few years, this profitable market has been under tremendous pressure and continues to swing with reduction in prices due to high energy and raw materials costs. This mandates an obvious need to develop some integrative technology to utilize the unlimited waste mycelium resulting from CA industries for co-product, CTS extraction which will also help in the stabilization of CA prices. The waste fungal biomass of CA or other biotechnological industries can become free and rich alternative sources of CTS beside the traditional industrial source - shellfish waste materials. Moreover, the fungal CTS can have unique properties as compared with those derived from crustaceans. Due to the abundant and inexpensive availability, the waste mycelia could be viewed as the possible source of industrial production of CTS. The alkali-insoluble cell-wall residue of the *A. niger* biomass consists mainly of CTS, chitin and β-glucans, with a significant prevalence of (1 3)-β-D-glucan.

CONCLUSION

Co-extraction of CTS from waste fungal biomass resulting from CA production is hereby proposed as an economical and eco-friendly alternative to the CTS derived from crab and shrimp shells. The proposed CTS extraction process which is operated at ambient conditions eliminates the necessity of usage of large excess of caustic alkali solutions for obtaining CTS from crustacean shells. The use of fungal biomass resulting from SSF gave higher amount CTS. The extractable CTS was also observed to be higher in control as compared to treatments supplemented with inducers in both SSF and SmF. This technique provides a new economical and efficient route, suitable for direct scaling up to large scale industrial production for high-quality CTS.

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LIST OF ABBREVIATIONS

AAIM	acid-and alkali insoluble material
AIM	alkali soluble material
AP	apple pomace
APS	apple pomace ultrafiltration sludge
CA	citric acid
CTS	Chitosan
DD	degree of deacetylation
EtOH	Ethanol
MeOH	Methanol
SmF	submerged fermentation
SSF	solid-state fermentation

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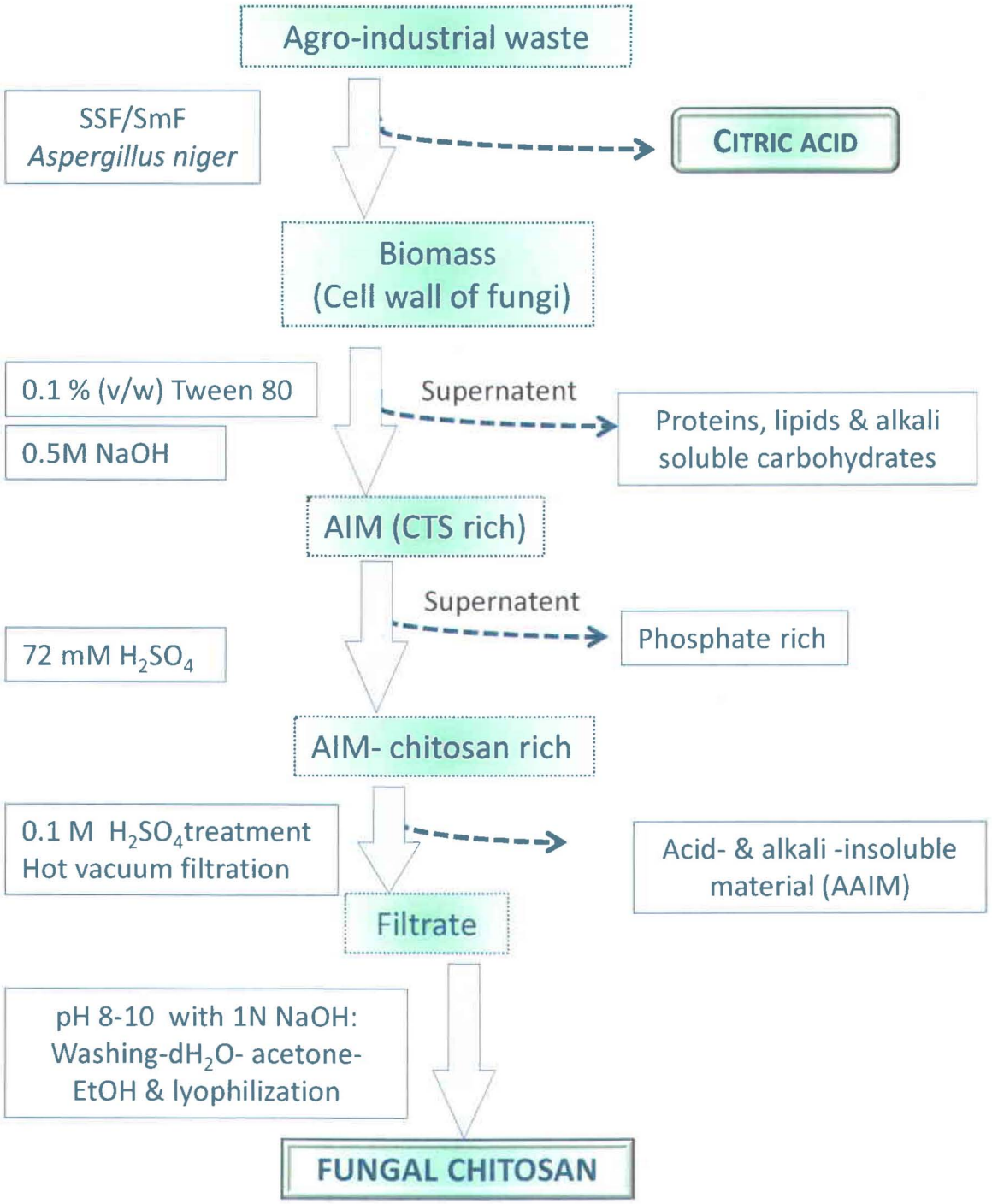


Figure 4.2. 1 Flow chart of the integrated process of CA production by *A. niger* and co- extraction CTS from waste mycelium.

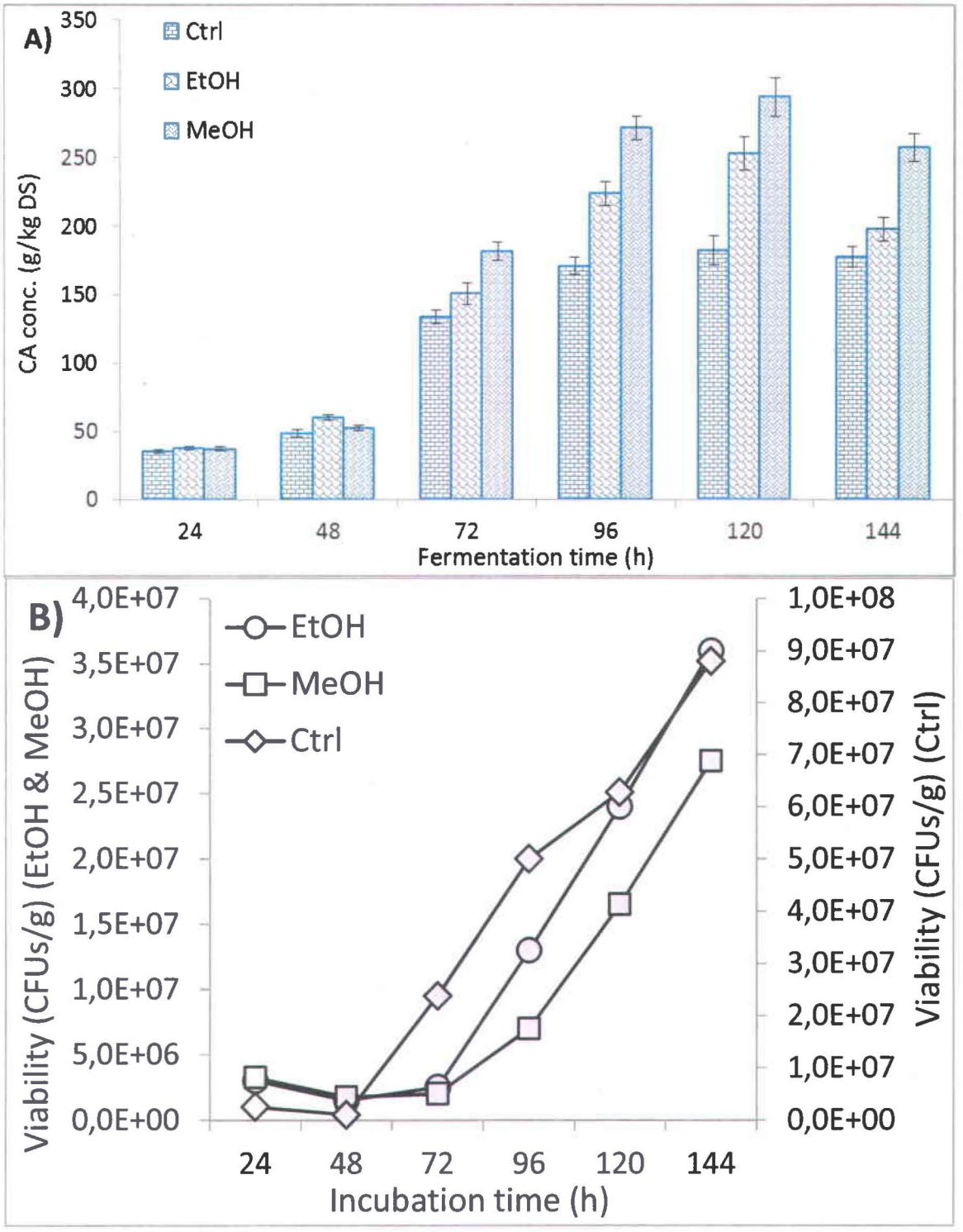


Figure 4.2. 2 (A) CA production by SSF using apple pomace (AP) as substrate and; (B) fungal viability in terms of total spore count (CFUs/g) fermented substrate

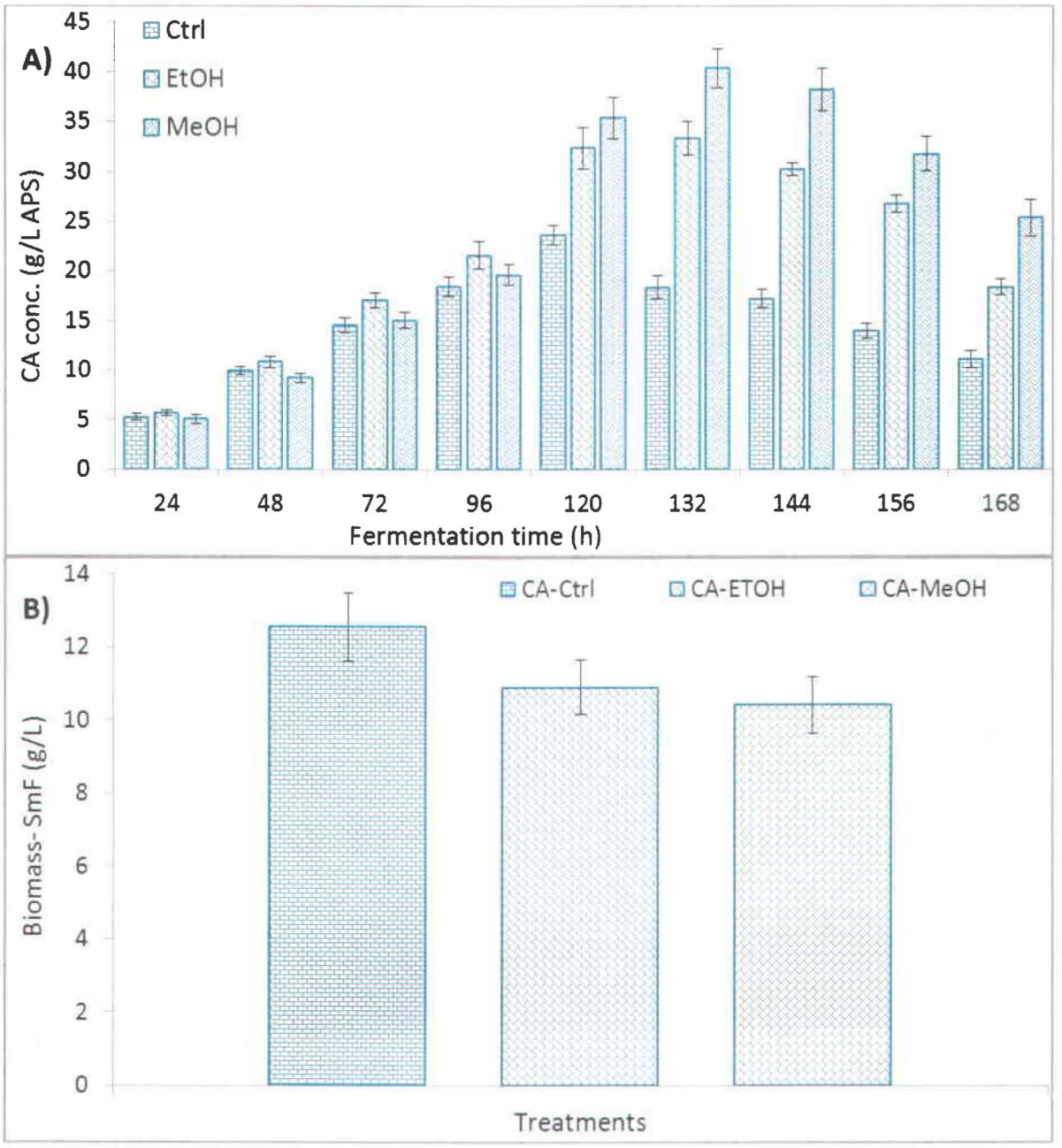


Figure 4.2. 3 (A) CA production through SmF using APS as substrate and; (B) fungal biomass after 132 h of fermentation

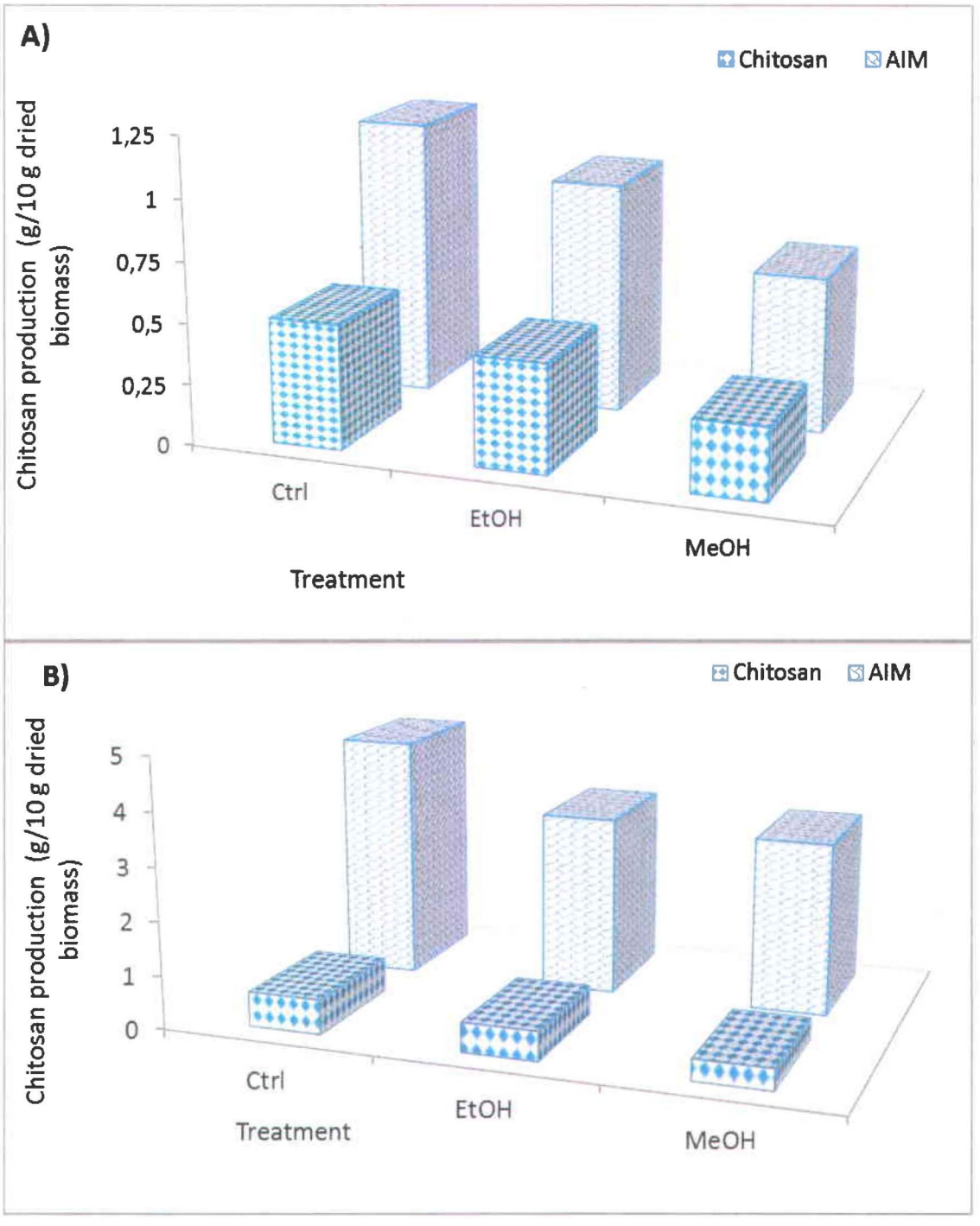


Figure 4.2. 4 Chitosan production trends using *A. niger* NRRL-567 mycelium resulting from (A) submerged CA fermentation using apple pomace ultrafiltration sludge (APS) after 132 h and; (B) solid-state CA fermentation using apple pomace (AP) as substrate after 120 h of fermentation.

PARTIE 5.

APPLICATIONS OF BIOMATERIALS RESULTING FROM DIFFERENT WASTE STREAM DURING CITRIC ACID FERMENTATION

CITRIC ACID: AN EMERGING SUBSTRATE FOR THE FORMATION OF BIOPOLYMERS

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NOVEL BIOMATERIALS DERIVED FROM CITRIC ACID FERMENTATION AS BIOSORBENTS FOR REMOVAL OF METALS FROM WASTE CCA WOOD LEACHATES

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RÉSUME

Dans cette étude, le potentiel de différents types de déchets de biomatériaux (BM) résultants suite à la fermentation de l'acide citrique (AC) et l'extraction de chitosane a partir d'*Aspergillus Niger* ont été évalués dans la bio remédiation et l'enlèvement des métaux toxiques dans les solutions aqueuses et de rejets de lixiviats contaminés par l'arséniate de cuivre chromaté (ACC). La fermentation des déchets de pomme (AP) et les déchets résultant des boues ultrafiltrées de pulpe de pomme (APS), en milieu solide et en culture submergée, respectivement aboutit à la production de CA 294±13 g/kg de substrat sec (5 jours) et 40.3±2 g/l APS (6 jours). Après la production de CA, la biomasse fongique des déchets a été utilisée comme une source d'extraction de chitosane (CTS). Les déchets différents des biomatériaux résultants suite à l'extraction de l'AC et de CTS, telles que la biomasse fongique, l'alcali insoluble (AIM), de l'acide et à l'alcali insoluble (AAIM) ont été testés pour leur capacité à éliminer les As, Cu et Cr partir de solutions aqueuses et le CCA à partir de lixiviats des déchets de bois. L'effet de différents paramètres tels que la concentration de biosorbate, la concentration en métal et le temps de contact ont été étudiés. La remise en forme des données des modèles d'adsorption de Freundlich et de Langmuir a été étudiée en utilisant la technique des lots d'adsorption. Parmi les tests d'adsorption isothermes, le test isotherme de Langmuir a donné le meilleur ajustement avec les coefficients de corrélation (R^2) de trois métaux différents, allant de 0.9539 à 0.9973 avec une valeur moyenne de 0.9819, suivie de test de Freundlich (moyenne 0.9783). Par conséquent, cette étude démontre que les déchets BM résultants pendant production de l'acide citrique (AC) et l'extraction de CTS et qui sont des polluants environnementaux pourraient être utilisés pour adsorber les métaux lourds des eaux usées contaminées et permet ainsi d'atteindre la propreté en affaiblissant les nuisances environnementales causées par les déchets.

Mots-clés: *Aspergillus niger*; biosorbants, l'alcali insoluble, de l'acide et à l'alcali insoluble; marc de pomme, chromé, bois arséniate de cuivre, la fermentation

ABSTRACT

In this study, the potential of different waste biomaterials (BMs) following citric acid (CA) fermentation and chitosan extraction using *Aspergillus niger* were evaluated for bioremediation of toxic metals from aqueous solutions and from leachates of discarded chromated copper arsenate (CCA) woods under different conditions. Fermentation of apple pomace (AP) and apple pomace ultrafiltration sludge (APS) under solid-state fermentation and submerged fermentation, respectively resulted in CA production of 294±13 g/kg dry substrate (5 days) and 40.3±2 g/L APS (6 days) (Dhillon et al., 2012, 2013). The waste fungal biomass following CA production was used as a source of chitosan (CTS) extraction. The different resulting waste BMs during the CA and CTS extraction, such as fungal biomass (living and dead), alkali insoluble material (AIM), acid and alkali insoluble material (AAIM) were screened for their potential to remove As, Cu and Cr from aqueous solutions and finally from waste CCA wood leachates. The effect of different parameters such as biosorbate concentration, metal concentration and contact time were also investigated. The fitness of biosorption data for Freundlich and Langmuir adsorption models was investigated by employing batch adsorption technique. Among the adsorption isotherm tested, Langmuir isotherm gave the best fit with correlation coefficients (R^2) value of three different metals (SSF biomass) ranging from 0.89-0.97; 0.96-0.99 and 0.76-0.95 for As, Cr and Cu, respectively. Similarly, the significant removal of metals (> 60% in leachate 2) from waste CCA wood leachate was achieved with the different BMs. Therefore, this study demonstrates that resulting waste BMs during CA production and CTS extraction serving as environmental pollutants could be used to adsorb heavy metals from contaminated waste waters and achieve cleanliness thereby abating environmental nuisance caused by the these wastes.

Keywords: *Aspergillus niger* biomass; biosorbents, alkali insoluble material; acid and alkali insoluble material; apple industry waste; CCA woods, fermentation

INTRODUCTION

The presence of certain heavy metals in various environmental sectors is of major concern because of their toxicity, non-biodegradable nature and risk to human, animal and plant life. The most widely used formulation for wood preservation since the 1970s is chromated copper arsenate (CCA). However, discarded CCA-treated woods pose serious environmental and health challenges to flora and fauna. The chemicals used for wood preservation are highly toxic to the organisms and they may be harmful, if discharged in to the environment. According to American Wood Preservation Agency, CCA type C is the most commonly used CCA formulation, and is comprised of 19% copper(II) oxide (CuO_2), 50% chromium(VI) oxide (CrO_3) and 31% arsenic(V) oxide (As_2O_5). The complexion mechanism of CCA into the wood is carried out by the reduction of chromate (Bull, 2001) that leads to the formation of Cr(III)/As(V) cluster, Cr(III) and Cu(II) complex with the wood components as well as hydroxide compounds (Bull, 2001; Nico et al., 2004). Arsenic and hexavalent chromium are highly toxic to the environment including humans. Various studies have revealed that leaching of metals results from in-service CCA treated woods (Stilwell and Graetz, 2001; Solo-Gabriele et al., 2003; Townsend et al., 2003; Khan et al., 2006). The discarded CCA-treated wood contains high metal concentrations (Cooper et al., 2001). As governmental organizations have described treated wood material as non-hazardous waste, it is frequently dumped into landfills where it is susceptible to metal-leaching and dispersion (Jambeck et al., 2007). The quantity of metals leached from CCA-treated wood can generally exceed the toxicity guidelines generally used for hazardous waste identification (Townsend et al., 2004). Various other studies also demonstrated the potential of arsenic release from CCA-treated wood wastes in construction and demolition landfills or municipal landfills (Jambeck et al., 2004; Khan et al., 2006). Considering currently in-service CCA-treated wood and expected service life-time, about 2.5 million m^3 of CCA-treated wood waste would be generated in Canada by 2020 and over 9 million m^3 in USA by 2015 (Cooper, 2003). In view of the huge quantity of generated waste wood, it is imperative to develop new ecologically safe CCA-treated wood waste management and recycling strategies to avoid the accumulation of wood wastes in landfill sites, resulting in dispersion of contaminants in the environment. Although various, conventional physical and chemical techniques for removal of chromium ions from effluents, such as reverse osmosis, ion exchange, reduction, precipitation, adsorption, solvent extraction, and lime coagulation are preferred choice (Sudha and Abraham, 2001; Selvi et al., 2001). However, these

techniques are highly expensive, ineffective at lower metal concentrations, and environmentally hostile as they generate a large amount of toxic sludge, which has to be disposed in further steps. Recently, biosorption has emerged as a potential alternative green technology to the existing conventional physico-chemical methods for removal of heavy metals. It eliminates the need for huge sludge handling and utilizes biomaterials (BMs) that are cheaper and readily available. Different type of BMs, such as fungal, bacterial, yeast, moss, aquatic plants and algal biomass have been investigated with the aim of finding more efficient or cost-effective metal-removal biosorbents (Chang et al., 1997; Niu et al., 1993; Fang et al., 2006). Studies have been reported on the use of *R. nigricans* (Sudha and Abraham, 2001) as adsorbents for the removal of Cr(III) and Cr(VI) from aqueous solutions.

However, no information is available on the use of waste biomass resulting from citric acid (CA) fermentation and during chitosan (CTS) extraction from waste mycelium as an adsorbent for the removal of toxic metals from waste CCA woods. Fungal biomass is produced in huge quantities by various biotechnological and pharmaceutical industries, such as citric acid. Fungal cell walls contain chitin along with other components. The utilization of this chitin rich source for its transformation to important biopolymer, chitosan (CTS) offers numerous economic and environmental benefits. The remaining waste biomass after treatment of fungal mycelium with dilute alkali and acids for CTS extraction is a kind of activated BMs. It provides a cost effective solution for biosorption of toxic metals from waste CCA woods. Moreover, fungal biomass have numerous advantages over traditional sorbents, such as regeneration and metal recovery potentiality, lesser volume of chemical and/or biological sludge to be disposed, higher efficiency in dilute effluents and have large surface area to volume ratio. The fungal biomass possesses high metal binding capacities due to the presence of polysaccharides, proteins or lipids present on the surface of their cell walls. The cell walls contain some functional groups, such as amino, hydroxyl, carboxyl and sulfate, which can act as binding sites for metals. The non-viable form has been proposed as potential biosorbents as they are essentially dead materials and require no nutrition to maintain the biomass.

The main aim of the present investigation is the maximum utilization of wastes resulting during fermentation and to develop a cost-effective and ecologically safe process for biosorption of CCA wood leachates. In brief, the objectives are: 1) chitosan (CTS)

extraction from the resulting waste fungal mycelium from CA fermentation through solid state and liquid fermentation with *Aspergillus niger* NRRL 567; 2) screening of different BMs resulting from CA and CTS extraction process for finding potential biosorbent for removal of metals from aqueous solution spiked with As, Cr and Cu under different conditions; 3) kinetics of metals removal using selected BMs to establish the mechanism and; 4) real world applications of selected BMs along with few commercial biopolymers (chitin , chitosans and citric acid) for removal of metals from discarded CCA wood leachates.

MATERIALS AND METHODS

Microorganisms and chemicals

A laboratory strain of *A. niger* (NRRL# 567) was routinely maintained on potato dextrose agar (PDA) plates and was stored at 4±1 °C with routine transfers in a cycle of 8 weeks. The culture conditions and maintenance of fungus have been already described in Dhillon et al. (2011a). The spore suspension was prepared by the method already described by Dhillons et al. (2011b) and spore suspension having spore count of 1×10^7 spores/mL was used as an inoculum in SSF of AP. Similarly, the inoculum for SmF was prepared by culturing *A. niger* on PDB for 36 h at 30±1 °C and 200 rpm. All the chemicals used for ICP were of trace metal grade. Stock metal solutions, As_2O_3, CrO_3, and Cu_2O (Sigma Aldrich) having concentration of 1000 ppm were prepared in 4% HNO_3. Final metal solutions after biosorption were made to 2% (v/v) HNO_3. Chitin and chitosan [low molecular weight chitosans (LMWCs) with MW of 190-310 kDa, DD 82%, and viscosity 522 cps and medium molecular weight chitosan (MMWCs) with MW of 310-375 kDa, DD 77%, and viscosity 1,120 cps] were purchased from (Sigma Aldrich), chitosan (high molecular weight chitosan (HMWCs) having MW of 600-800 kDa, degree of deacetylation (DD) >90%, and viscosity 200-500 mPa·s) and citric acid were purchased from Fischer scientific, Quebec, Canada.

Fungal citric acid fermentation through SSF and SmF

The solid-state CA fermentation by *A. niger* using apple pomace (AP) as a substrate and solid support was carried out in a 12-L rotating drum type bioreactor, Terrafor (Infors HT, Switzerland) (Dhillon et al., 2013). Similarly, SmF of apple pomace ultrafiltration sludge

(APS) was performed in a 7.5 L capacity fermenter with 4.5 L working volume (Labfors, HT Bottmingen, Switzerland) (Dhillon et al., 2012). The BMs resulting during the previous CA production studies were used in the present study.

BMs preparation during chitosan extraction from waste fungal biomass followed by CA production

The waste *A. niger* mycelium resulting from CA production from previous studies was used for CTS extraction. In case of SSF, the whole fermented AP after CA extraction was used for CTS extraction, as it was not possible to separate mycelium from AP. The method for CTS extraction is described in Fig 5.2.1. The waste byproducts resulting during CA fermentation and CTS extraction from waste fungal mycelium were used as biosorbents for removal of toxic metals from CCA wood leachates. BMs having live fungal biomass were used as such without drying. BMs with dead fungal biomass were prepared by autoclaving the biomass and oven drying it. BMs during CTS extraction process, such as alkali insoluble material (AIM) and acid and alkali insoluble material (AAIM) were prepared according to the protocol described in Fig 5.2.1. AIM and AAIM was dried at 40-45±1 °C in hot air oven.

Characterization of BMs by scanning electron microscopy (SEM)

Different BMs, such as waste fungal mycelium resulting from SSF and SMF, AIM and AAIM fractions obtained during CTS extraction were characterized using SEM (Model: Carl Zeiss EVO® 50 smart SEM system) Preparation of samples for SEM monographs was performed by mounting the powdered samples on aluminum stubs and coated with gold using a SPI™ sputter coater module to increase conductivity and thus to minimize sample charge up. The morphology of the synthesized ZnO NPs was examined by a SEM with a working distance (WD) 20 mm, secondary electrode 1 (SE 1) detector and EHT 10 kV.

Metal removal efficiency of different BMs from aqueous solutions and CCA wood leachates

Different BMs used for biosorption of metals from aqueous solutions spiked with different metals are given in Table 5.2.1. The removal performance of metals (As Cr and Cu) by

different BMs was determined by measuring the residual concentrations of metals over time. Batch experiments were carried out in Erlenmeyer flasks. The metal solutions were prepared by dissolving the exact quantities of analytical quality CrO_3 (Aldrich Chemical Company), As_2O_3 (Sigma-Aldrich) and Cu_2O (MAT) in ultrapure water. Each solution was acidified with concentrated HNO_3 (Martin et al. 1994).

Initially, screening of BMs (2.5 g/L) was performed using metal solution (50 mg/l of each metal) in an Erlenmeyer flask and incubated at 25±1 °C and 175 rpm for 24 h. The samples were vacuum filtered through a Whatman (Fischer) filter paper having pore diameter of 0.45 microns. The filtrate was collected in tubes of 50 ml and was made to 2% HNO_3 and ICP analysis was carried out after sufficient dilution. After initial screening, one potential BMs resulting from both SSF and SmF was selected and was used for further biosorption experiments.

In order to evaluate the kinetics of metal removal, the biosorption was carried out with varied concentration of best BMs (2.5, 5.0, 7.5 and 10 g/L) and fixed metal concentration (50 mg/L each metal). The samples were withdrawn after every 3 h till 48 h. Metal concentrations were measured using emission spectrometry (Inductively Coupled Plasma) ICP-AES (Varian Vista AX-model). Quality control was performed with certified liquid samples (Multi Element Standards, Catalog # 900-Q30-002, SCP Science, Lasalle, Canada) and a solution of yttrium 1 mg/L was used as internal standard. The amount of metals adsorbed per g of biomass, Qe (mg/g) was calculated using the Eq. 1:

$$Qe = (Ci - Ce)\frac{V}{M} \qquad [1]$$

Where, *Ci* and *Ce* are the initial and final concentrations of metals in solution, respectively and are expressed in mg/L. *V* is the volume in liters of the solution and *M* represents the mass of biomass (g).

The best concentration of BMs was used further for biosorption from aqueous solution with varied metal concentrations of 50, 100, 150, 200, 250, 350 and 500 mg/L. The samples were taken after 24 h and analyzed for residual metals after biosorption. Finally, the BMs along with some commercial biopolymers (chitin, chitosans and citric acid) were used for the removal of As, Cr and Cu from the CCA wood leachates

resulting through leaching process as described in Fig 5.2.2. Leaching of metals from discarded CCA wood chips was carried out using the following conditions: 300 g wet mass was taken and 2 L of 0.4 N H_2SO_4 was added and incubated at 75-80±1 °C (Janin et al., 2009). Under these optimized conditions, three leaching steps were performed each for 2 h period.

Batch kinetic and isotherm studies

Langmuir and Freundlich models were used to describe the adsorption isotherm. The linear forms of the Langmuir isotherm (Langmuir, 1918) are respectively represented by Equations 2 and 3.

$$Q_e = \frac{Q_m K_L C_e}{1 + K_L C_e} \qquad [2]$$

$$\frac{1}{Qe} = \frac{1}{K_L . C_e . Q_m} + \frac{1}{Q_m} \qquad [3]$$

Where, Qe is the amount of metal adsorbed on the surface of the support at equilibrium (mg/g). Qm represents the maximum amount of adsorbate (mg/g) and K_L constant relative to the energy of adsorption (L/g). The graphical representation of the variation of the ratio (Qe) versus (Ce) gives rise to lines from which the theoretical values, Qm and K_L are calculated using the slopes and intercepts.

Linear forms of the Freundlich isotherm (Freundlich, 1906) are given by Eq. 4 and 5.

$$Qe = K_F (Ce)^{\frac{1}{n}} \qquad [4]$$

$$LnQ_e = LnK_F + \frac{1}{n} LnC_e \qquad [5]$$

Where, K_F is the Freundlich constant and n is a factor relating to the strength of adsorption, also called heterogeneity factor. The graphical representation of the variation of Ln (Qe) based Ln (Ce) leads to straight regressions from which n and the theoretical values, K_F are calculated. This is an equation of the form y = ax+b, where the y-intercept of the corresponding regression line equals Ln K_F and the slope of this straight line is equal to 1/n.

Analytical Methods

The total solid content of APS was estimated by drying 25 ml sample in an oven at 105±1 °C to constant weight. The pH of the substrate was measured using a pH meter (EcoMet, Istek, Seoul, South Korea) equipped with glass electrode. Metal concentrations were measured using ICP-AES (Varian, model Vista-AX, Canada). The standard metal solutions used for metal analysis in ICP were purchased from Plasma CAL, Canada. Quality controls was performed with certified liquid samples (multi-element standard, catalogue number 900-Q30-100, PlasmaCAL, Canada) to ensure conformity of the equipment.

Statistical Analysis

CA values presented are an average of three replicates along with the standard deviation (±SD). Database was subjected to an analysis of variance (ANOVA), using the Statistical Analysis System Software (STATGRAPHICS Centurion, XV trial version 15.1.02 year 2006, Stat Point, Inc., USA), and the results which have $p<0.05$ were considered as significant.

RESULTS AND DISCUSSION

Citric acid production through SSF and SmF

Apple industry waste was used for the production of CA owing to the presence of high carbon content and other vital nutrients required for culturing fungi (Dhillon et al., 2012, 2013). The solid-state fermentation of AP resulted in CA production of 294± 13.2 g/kg dry substrate after 5 days of incubation. Similarly, submerged fermented of APS rendered CA production of 40.3±1.9 g/L after 6 days of fermentation.

Characterization and screening of different BMs for the removal of toxic metals (As, Cr and Cu)

Scanning electron micrographs of different BMs resulting during CA fermentation and CTS extraction are provided in Figure 5.2.3 and 5.2.4. As evident from the micrographs, in case of BMs obtained through SSF, the fermented AP, AIM and AAIM fraction due to the pretreatment (CA fermentation or NaOH/acid treatment during CTS extraction) resulted in activated biomass as compared to AP powder. Similarly, in case of BMs resulting from SmF, AIM and AAIM fractions seemed more activated than the fungal biomass. The activated biomass became more porous and has enhanced surface area making it apt for biosorption of metals.

The effect of inducers (methanol and ethanol) during CA production, the effect of CTS extraction treatment [alkali insoluble material (AIM) and the acid-and alkali insoluble material (AAIM)] on BMs and the effects of viable and dead biomass as BMs were evaluated for their ability to simultaneously remove the toxic metals from aqueous solutions. The initial concentration of As, Cr and Cu (50 mg/L each) and a constant mass (2.5 g/L) of the BMs resulting from SSF, SmF and during CTS extraction were used in screening experiments. Monitoring of residual As, Cr and Cu was performed by ICP after 24 h. Table 5.2.3 shows the percentage removal and adsorption capacity per unit mass of metal. Overall, the percentage of removal of metals As, Cr and Cu varies between 8 and 59.31%.

Living biomass (control) obtained by SSF gives a higher percentage removal of the three metals as compared with other BMs with 53.25, 59.31 and 25.73 % removal of As, Cr and Cu, respectively. While BMs resulting from SmF living biomass (MeOH) gives better results with a 56.32, 57.92 and 34.07 %, respectively for As, Cr and Cu as compared to other BMs resulting from SmF. Due to their higher metal removal (%), living fungal biomass resulting from SSF (control) and SmF with MeOH as inducer, respectively was selected for further kinetic studies.

Evaluation of kinetic parameters of the solid and liquid biomass

Influence of the concentration of BMs (g/L) on biosorption of metals over time

The kinetics data of SSF and SmF biomass during batch experiments as a function of time is provided in Table 5.2.4 and Fig 5.2.6 and 5.2.7. The influence of the initial concentration of two BMs (2.5, 5.0, 7.5 and 10 g/L metal solution) [SSF (control; live biomass) and SmF (MeOH; live biomass) on their adsorption capacity has been studied with solution containing the three metals selected (50 mg/L). As evident from data, the increasing the contact time leads to improved treatment performance. Overall, the ability of biosorption decreases for each metal after equilibrium.

The BMs resulting from SSF (control, living biomass) resulted in the biosorption values as follows: As (49.2 % removal rate with biomass concentration of 2.5 g/L) compared with Cr and Cu (55.9 % removal rate with biomass concentration of 10 g/L and 35.84 % removal rate with biomass concentration of 10 g/L), respectively. The biosorption capacity of As ranges from 0.20- 0.67 mg/g for the respective concentrations of biomass with 2.5, 5.0, 7.5 and 10 mg/L of metal solution. The biosorption capacity of Cr varied from 0.28-0.88 mg/g, a removal rate of 50.6-55.9 % for the respective concentrations of 5.0, 7.5 and 10 mg/L. Similarly, biosorption capacity of Cu varied from 0.18-0.57 mg/g and removal rate of 27.9-35.8 % for biomass concentrations of 2.5, 5.0, 7.5 and 10 g/L metal solution. These results can be compared with those of Ncibi et al. (2008), who studied the biosorption of chromium (vi) by a biomass of *Posidonia oceanica* (L.) Mediterranean: and obtained the same trend. The biosorption capacity was optimized by the amount of biosorbent and the initial concentration of metals by the authors. As evident from the results, the removal rate obtained for different biomass concentrations is almost similar in both cases. There was no significant difference ($p<0.05$) in the removal rate for different biomass concentrations i.e. 2.5, 5.0, 7.5 and 10 g/L of metal solution. The slight increase in the percentage removal of each metal with rising biomass concentration is mainly due to an increase in the number of active sites of adsorption on the surface of the biomass.

Modelling of adsorption isotherms

The results of the three metals adsorbed on SSF biomass (control, live) and SmF biomass (MeOH, live) were fitted to Langmuir and Freundlich models. The results and parameters of the Langmuir and Freundlich isotherms and the correlation coefficient R^2 are summarized in Table 5.2.4. Values of correlation coefficients (R^2) of three different metals were higher for the Langmuir isotherm (ranging from 0.76-0.99 for SSF and 0.97-0.99 for SmF) than the Freundlich isotherm, which means that the Langmuir isotherm better represents the adsorption process of As, Cu and Cr by SSF control biomass. This could be explained by uniform distribution of active sites on the surface of the biomass. Moreover, the biomass was more porous and possessed enhanced surface area as evident from the SEM micrographs also. Similar results were reported by Tazerouti and Amrani (2010) work on the adsorption of Cr (VI) by activated lignin. There is also a better adsorbability of Cr compared to As and Cu. In particular, the maximum capacity Qm values are 5.76, 5.16 and 4.71 mg/g respectively for Cr, Cu and As.

Biosorption capacities of BMs with varied initial concentration of metals

Figure 5.2.5 provides the removal rate (%) as a function of varied concentration of metals with BMs resulting from both SSF and SmF. The removal rate achieved with BMs (SSF, control live biomass) ranges from 23.70- 53.0, 25-54.17 and 21.18- 49.25 %, respectively for As Cr and Cu after 24 h of incubation. Higher removal rate for As was achieved at 150 mg/L and for Cr and Cu at 50 mg/L of initial metal concentration. At 150 mg/L initial concentration of As removal rate significantly differs as compared to 50 and 100 mg/L of initial metal concentration respectively. However there was no significant difference observed with 50, 100 and 150 mg/L initial concentration of metal solution for Cr and Cu respectively. While using 350 and 500 mg/L initial concentration of metals, significant decrease in removal rate was observed.

The BMs resulting from SmF (MeOH, live biomass) ends up with removal rate of 22.9-43.4, 21.8-44.9 and 19.4- 43.5 %, respectively for As Cr and Cu after 24 h of incubation. Higher removal efficiency was obtained with 50 mg/L of initial metal concentration for all metals. No significant difference was observed until 200 mg/L initial concentration of metal solution for As, Cr and Cu, respectively. However, above 200 mg/L initial concentration of metals, significant decrease in removal rate was observed for all

metals. The decrease in the metal biosorption can be due to the fact that the higher concentrations of metals proved toxic to the living biomass. In case of dead BMs, the decline in % metal removal rate is probably caused by the saturation of some adsorption sites. The rate of biosorption is a function of the initial concentration of metal ions, which makes it an important factor to be considered for effective biosorption. As clear from Figure 5.2.5, the data reveal that capacity of biosorbent was almost same with the initial metal concentration up to 200 mg/l. The decrease in biosorption above 200 mg/l indicates that surface saturation was dependent on the initial metal ion concentrations. At low matal concentrations biosorbent sites take up the available metal more quickly. However, at higher concentrations, metal ions need to diffuse to the biomass surface by intraparticle diffusion and greatly hydrolyzed ions will diffuse at a slower rate (Horsefall and Spiff, 2005). This also showed the high efficiency of BMs for metal removal from dilute solution which is the main disadvantage with conventional methods which shows inadequate efficiencies at low metal concentrations (chemical precipitation, lime coagulation, ion exchange, reverse osmosis and solvent extraction).

Practical application of BMs: biosorption of toxic metals from CCA wood leachates

The biosorption capacities and removal of different metals (As, Cr and Cu) from waste CCA wood leachates is shown in Fig. 5.2.8 A, B, C, D, E, and F. The selected BMs based on biosorption capacities from aqueous solutions and some selected commercial biopolymers, such as chitin, different molecular weight chitosans and citric acid were applied for the biosorption and removal of toxic metals from discarded CCA wood leachates. Three types of leachates were obtained from waste CCA woods based on the leaching process described in Fig 5.2.2.

As evident from Fig. 5.2.8, the biosorption values obtained for As were as follows: leachate 1 (Qe- 0.87-4.7 mg/g; removal rate of 11.23-48.65%); leachates 2 (Qe- 3.36-14.65 mg/g; removal rate of nearly 60-66 %) and leachate 3 (Qe- 1.53-9.22 mg/g with removal rate 34.17- 51.52%), respectively. Higher removal rate for As was obtained with the citric acid treatments for leachate 1, no significant difference ($p<0.05$) for As removal in leachate 2 and above 40% for all treatments for leachate 3, except chitin (10 g/l) and CA (5g/l) concentration. Similarly, the higher removal rate of Cr (>55 and 65% for leachate 2 and 3) and Cu (>60% for leachate 2) was achieved in all treatments. Higher

removal rates obtained in leachate 2 could be due to the concentration of metals (As- 266.3; Cr-275.7 and Cu- 157.1 mg/L respectively) in leachate 2. Leachate 3 contains significantly higher concentration of all metals (As- 869.0; Cr- 904.7 and Cu- 582.7 mg/L, respectively) resulting in the poor biosorption capacity with different BMs. Lower removal rate of metals with SSF (treatment 1) and SmF biomass (treatment 2) in leachate 1 may be due to the fact that high concentration of metals proved toxic to living fungal biomass resulting in poor removal rate of metals. Similar results were obtained in the experiment with different metal concentrations where higher removal efficiency was achieved in aqueous solutions spiked with metal concentrations in the range of 150-200 mg/L each.

Heavy metals can be removed by several ways, e.g., precipitation and sorption. Various substances, such as activated carbon, natural and synthetic zeolites, and clay minerals have been used as adsorbents for the removal of heavy metals from water and wastewater. In the recent years the adsorption of heavy metals by a variety of substances has been the subject of many studies. Biosorption has emerged as the front line mechanism for removal of metals as an economical alternative to other high cost techniques. Activated carbon is the most popular adsorbent and has been used with great success (Yang and Al-duri, 2001). In fact, large number of other low-cost adsorbents has been utilized for metals removal, such as bentonite and perlite for the adsorption of trivalent chromium from aqueous solutions was reported (Chakir et al., 2002). However, the adsorption of metals by activated carbon is more complex than for organic compounds because the ionic charges affect their removal rates from solution. Metal ion adsorption by activated carbon varies with the chemical properties of adsorbate, temperature, pH, ionic strength of the liquid phase, etc. Many activated carbons are commercially available at higher costs but few are selective for heavy metals. Due to the high cost of carbon for water treatment, a search for novel biosorbents is sought. Wastewater treatments require vast quantities of activated carbon, so improved and tailor-made biosorbents are attractive alternatives for these demanding applications. Such BMs should be easily available, economically feasible, and readily and quantitatively regenerated chemically. In this context, the present study demonstrated the potential of BMs resulting from the fermentation of apple industry waste for biosorption of heavy metals from aqueous solutions and waste CCA wood leachates. In totality, it meets the biorefinery approach as different waste streams

generated during bioproduction of CA as a platform chemical transformed to value-added products.

CONCLUSIONS

The waste biomass produced by solid state and submerged CA fermentation of AP and APS and the wastes resulting during CTS extraction from fungal biomass were investigated for biosorption of toxic metals (As, Cr and Cu) from aqueous solutions and waste CCA wood leachates. CA production achieved during SSF was 294± 13.2 g/kg dry AP and through SmF was 40.3±1.9 g/L of APS. Based on the screening experiments, SSF biomass (control, live) and SmF biomass (MeOH, live), respectively were selected as potential biomaterials for further kinetic studies during biosorption of metals (As, Cr and Cu). Modeling isotherms showed that Langmuir model satisfactorily described the adsorption process. It has been shown that the percentage of metal removal at equilibrium can be optimized by further pretreatment of wastes, such as particle size optimization. Similarly, the significant removal of metals from waste CCA wood leachates was achieved using different BMs. Taking into account all the results from this study, the waste derived novel BMs resulting through CA fermentation of apple industry waste could be used as an adsorbent for the removal of As, Cr and Cu in contaminated waste waters.

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ABBREVIATIONS

AIM	alkali insoluble material
AAIM	alkali and acid insoluble material
AP	apple pomace
APS	apple pomace ultrafiltration sludge

BMs	Biomaterials
CA	citric acid
CTS	Chitosan
EtOH	Ethanol
MeOH	Methanol
SEM	scanning electron microscope
SmF	submerged fermentation
SSF	solid-state fermentation
SS	suspended solids

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Table 5.2. 1 Different types of biomaterials used for biosorption of metals

S. No.	Type of biomaterials	Step details	Fungal mycelium type in biomass
Biomaterials resulting from SSF of AP			
1	AP–without fermentation	-	No mycelium
2	AP (ctrl)- fermented	No CA extraction	Live
3	AP (EtOH)- fermented	No CA extraction	Live
4	AP (MeOH)- fermented	No CA extraction	Live
5	AP (Ctrl)- fermented	After CA extraction	Dead
6	AP (EtOH)- fermented	After CA extraction	Dead
7	AP (MeOH)- fermented	After CA extraction	Dead
8	AIM (Ctrl	CTS extraction	Dead
9	AIM (EtOH)	CTS extraction	Dead
10	AIM (MeOH)	CTS extraction	Dead
11	AAIM (Ctrl)	CTS extraction	Dead
12	AAIM (EtOH)	CTS extraction	Dead
13	AAIM (MeOH)	CTS extraction	Dead
Biomaterials resulting from SmF of APS			
1	APS- Ctrl	After CA extraction	Dead
2	APS- EtOH	After CA extraction	Dead
3	APS- MeOH	After CA extraction	Dead
4	APS- Ctrl	After CA extraction	Live
5	APS- EtOH	After CA extraction	Live
6	APS- MeOH	After CA extraction	Live
7	AIM (Ctrl)	CTS extraction	Dead
8	AIM (EtOH)	CTS extraction	Dead
9	AIM (MeOH)	CTS extraction	Dead
10	AAIM (Ctrl)	CTS extraction	Dead
11	AAIM (EtOH)	CTS extraction	Dead
12	AAIM (MeOH)	CTS extraction	Dead

Abbreviations: AIM- alkali insoluble material; AAIM- acid and alkali insoluble material; AP- apple pomace; APS- apple pomace ultrafiltration sludge; CA- citric acid; CTS- chitosan; Ctrl- control; EtOH- ethanol; MeOH- methanol

Table 5.2. 2 Metal concentrations in initial CCA wood (dry basis) and different leachates

Metals	Initial metals in CCA woods (dry wood basis)	Leachate 1	Leachate 2	Leachate 3
As (mg/L)	6842 mg/kg	869.0	266.3	70.97
Cr (mg/L)	7805 mg/kg	904.7	275.7	75.16
Cu (mg/L)	4536 mg/kg	582.7	157.1	41.83

Abbreviations: CCA- chromated, copper arsenate treated woods

Table 5.2. 3 The amount of adsorption [Qe (mg/g)] and removal percentage of metals (As, Cr, Cu) during screening studies by the BMs resulting from solid-state and liquid fermentation of CA and chitosan extraction process

Biomaterials and Treatments		As		Cr		Cu	
		Qe (mg/g)	removal (%)	Qe (mg/g)	removal (%)	Qe (mg/g)	removal (%)
Biomass (SSF)							
Apple pomace	**Non-fermented**	2.11	10.43	3.12	14.77	2.24	10.23
Biomass – living	**Control**	10.75	53.25	12.54	59.31	5.63	25.73
	EtOH	9.00	44.57	10.62	50.21	2.23	10.20
	MeOH	9,45	46.80	10.88	51.44	2.95	13.49
Biomass-dead	**Control**	3.71	18.36	4.66	22.06	4.21	19.24
	EtOH	3.19	15.81	4.05	19.16	3.56	16.29
	MeOH	3.53	17.53	4.35	20.58	3.93	17.99
AIM	**Control**	2.67	13.21	4.08	19.30	3.14	14.34
	EtOH	2.93	14.51	4.41	20.87	3.41	15.58
	MeOH	2.75	13.63	4.05	19.16	3.20	14.65
AAIM	**Control**	2.95	14.61	3.97	18.78	2.92	13.37
	EtOH	3.44	17.04	4.67	22.09	3.67	16.77
	MeOH	3.50	17.36	4.46	21.10	3.69	16.89
Biomass (SmF)							
Biomass-dead	**Control**	0.39	11.19	0.48	15.84	0.36	10.53
	EtOH	0.29	8.41	0.41	13.44	0.31	8.93
	MeOH	0.41	11.75	0.46	15.38	0.39	11.25
Biomass – live	**Control**	1.63	47.20	1.53	50.66	0.67	19.44
	EtOH	1.79	51.84	1.64	54.36	0.87	25.27
	MeOH	1.95	56.32	1.75	57.92	1.17	34.07
AIM	**Control**	1.58	45.68	1.72	56.86	0.44	12.75
	EtOH	1.55	44.87	1.74	57.63	0.52	15.10
	MeOH	1.69	48.72	1.77	58.40	0.59	17.21
AAIM	**Control**	0.76	21.96	0.82	27.27	0.75	21.86
	EtOH	0.69	19.80	0.69	22.97	0.68	19.94
	MeOH	0.59	16.94	0.64	21.01	0.56	16.37

Table 5.2. 4 Values of parameters of Langmuir and Freundlich adsorption during kinetics studies with SSF biomass (control, live) and SmF biomass (MeOH, live)

Metals	Langmuir constants			Freundlich constants		
	Qm (cal.) ($mg \cdot g^{-1}$)	R^2	K_L ($L \cdot mg^{-1}$)	K_F ($L \cdot g^{-1)}$	N	R^2
SSF biomass (control, live)						
Concentration 2.5 g/L						
As	0.011	0.8894	0.204	484.2	0.104	0.9229
Cr	0.162	0.9613	0.382	3.270	0.396	0.9900
Cu	0.006	0.8621	0.216	1316	0.085	0.9050
Concentration 5 g/L						
As	0.015	0.9716	0.221	105.1	0.128	0.9929
Cr	0.091	0.9924	0.398	2.199	0.426	0.9981
Cu	0.010	0.9526	0.237	160.2	0.111	0.9882
Concentration 7.5 g/L						
As	0.004	0.8958	0.2025	121.1	0.119	0.9240
Cr	0.062	0.9622	0.403	1.805	0.432	0.9913
Cu	0.014	0.75811	0.286	162.4	0.106	0.8734
Concentration 10 g/L						
As	0.012	0.9464	0.241	19.186	0.170	0.9863
Cr	0.049	0.9561	0.416	1.488	0.456	0.9896
Cu	0.009	0.8954	0.263	16.91	0.163	0.9748
SmF biomass (MeOH, live)						
Concentration 2.5 g/L						
As	0.203	0.9879	0.351	3.589	0.422	0.9969
Cr	0.224	0.9907	0.455	2.356	0.505	0.9977
Cu	0.224	0.9887	0.441	7.471	0.497	0.9972
Concentration 5 g/L						
As	0.084	0.9773	0.327	3.589	0.422	0.9941
Cr	0.099	0.9854	0.429	2.356	0.505	0.9963
Cu	0.099	0.9854	0.417	7.472	0.497	0.9963
Concentration 7.5 g/L						
As	0.067	0.9706	0.348	2.263	0.418	0.9924
Cr	0.077	0.9781	0.461	1.433	0.516	0.9944
Cu	0.075	0.9845	0.441	1.524	0.498	0.9960
Concentration 10 g/L						

Metals	Langmuir constants			Freundlich constants		
	Qm (cal.) ($mg \cdot g^{-1}$)	R^2	K_L ($L \cdot mg^{-1}$)	K_F ($L \cdot g^{-1)}$	N	R^2
As	0.044	0.9854	0.333	2.266	0.385	0.9962
Cr	0.054	0.9901	0.446	1.333	0.489	0.9975
Cu	0.053	0.9897	0.431	1.398	0.480	0.9974

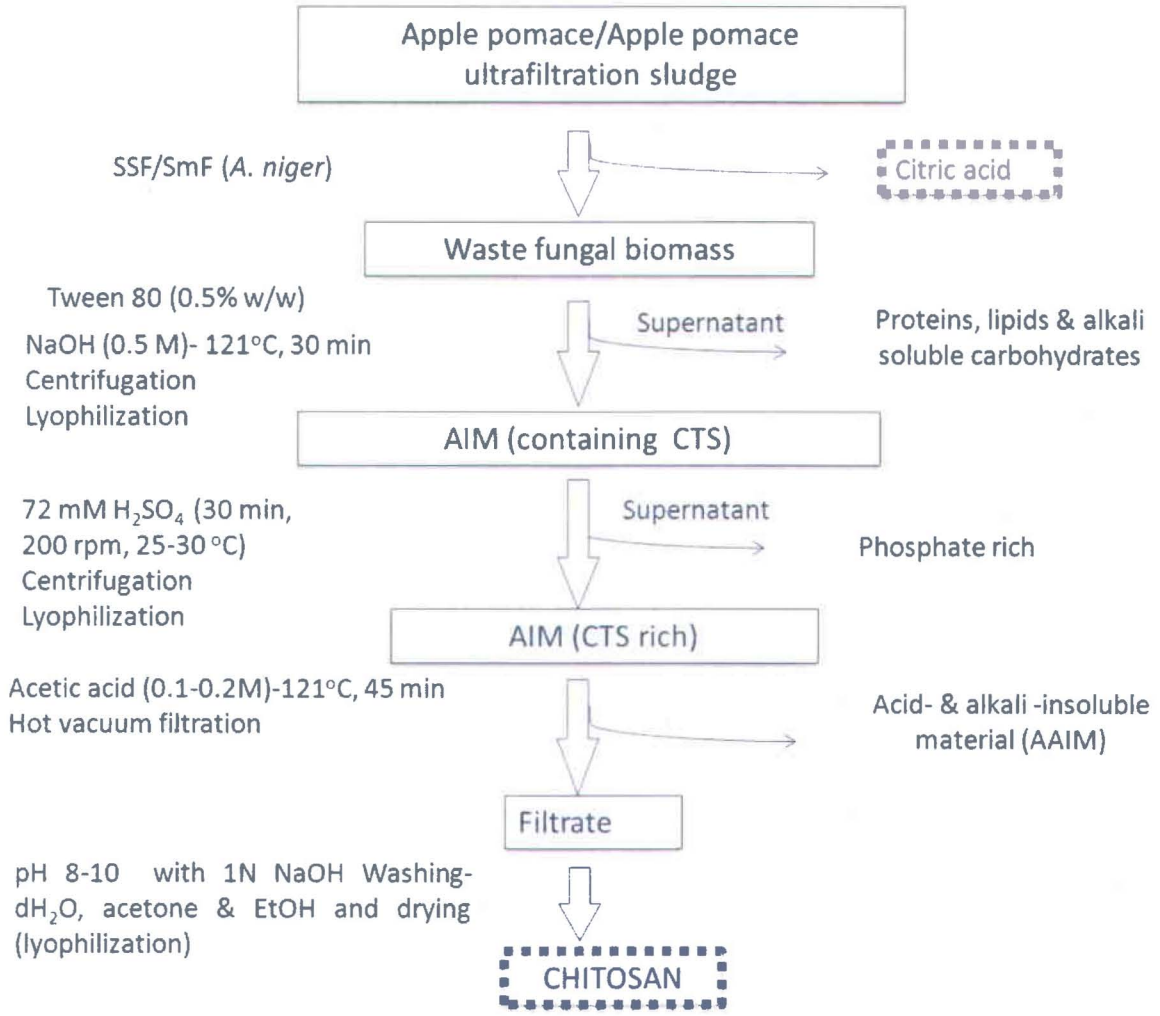


Figure 5.2. 1 Flowchart of the CA production by *A. niger* followed by CTS extraction from waste fungal mycelium.

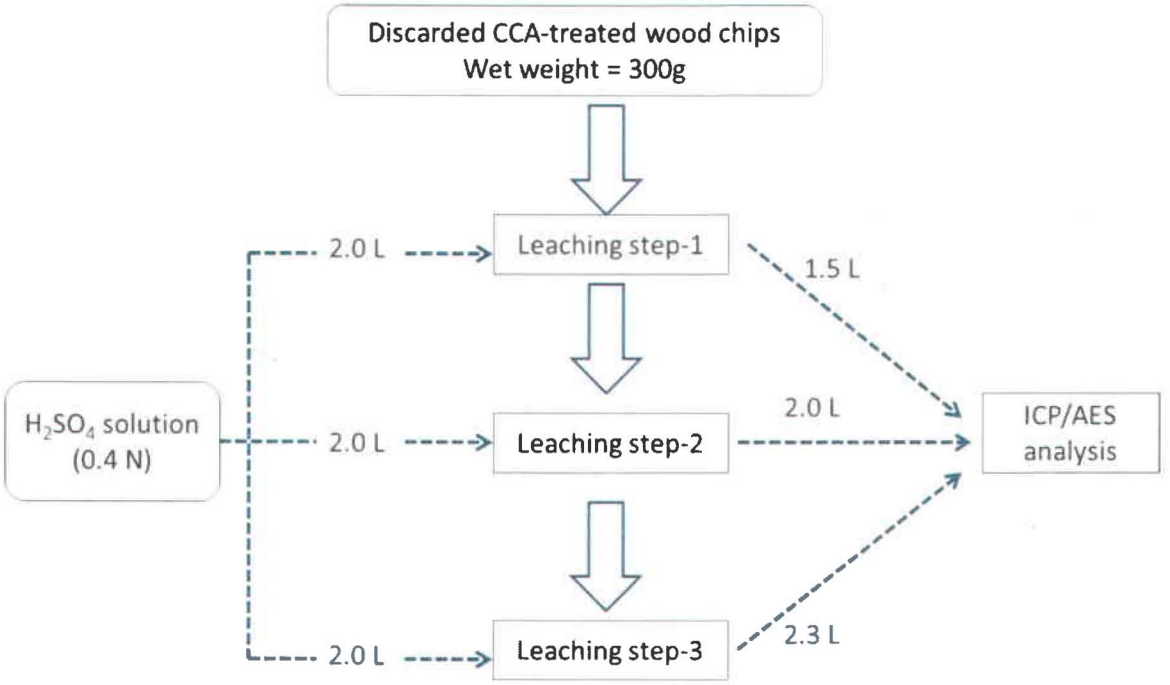


Figure 5.2. 2 Flowchart showing the leaching of metals from discarded CCA wood chips by H_2SO_4.

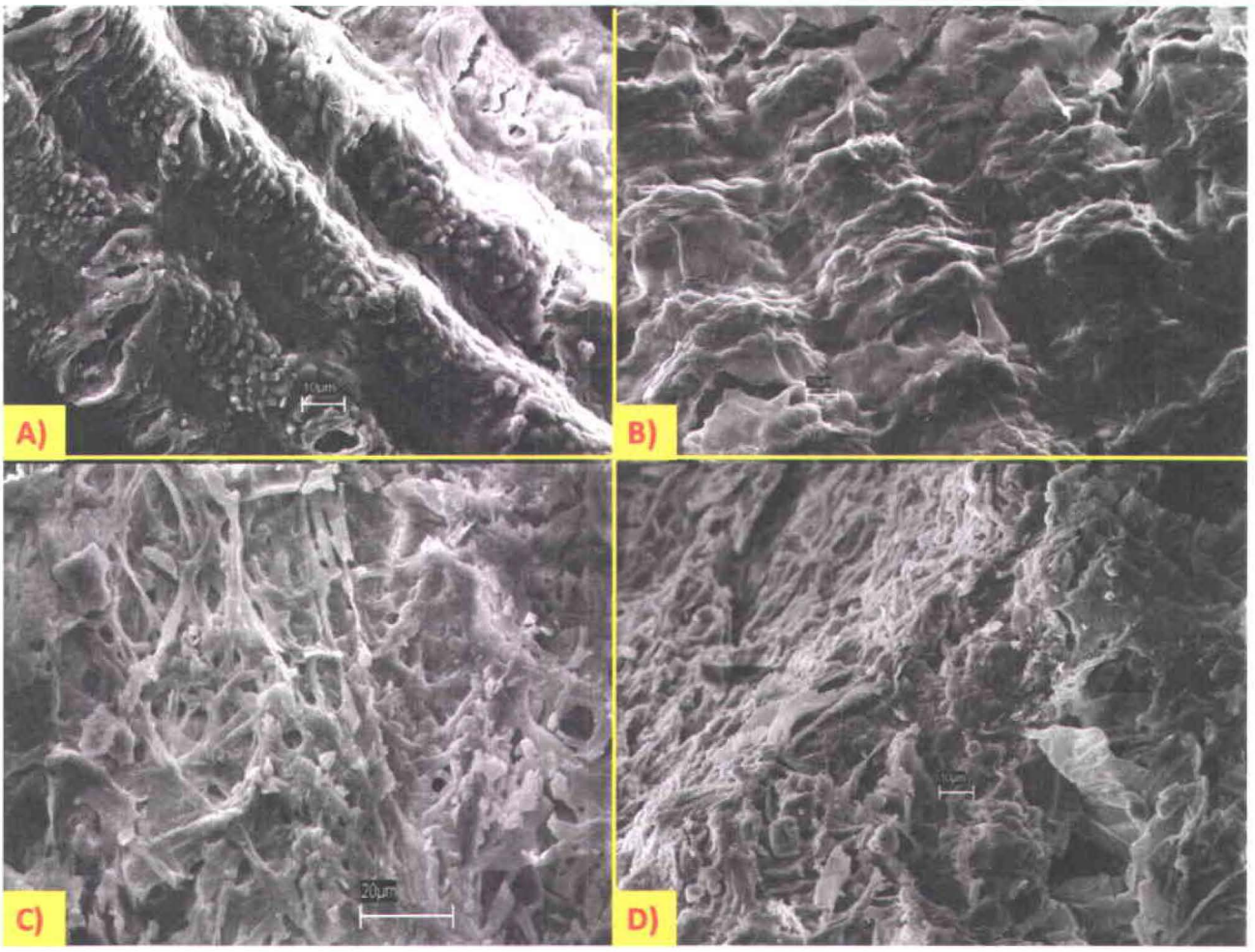


Figure 5.2. 3 Scanning electron micrographs (× 2K) of: A) AP powdered (1-2 mm); B) AP fermented using SSF; C) AIM fraction during chitosan from fermented AP using SSF and; D) AAIM fraction (after acetic acid [0.1 M] extraction) during chitosan from fermented AP using SSF

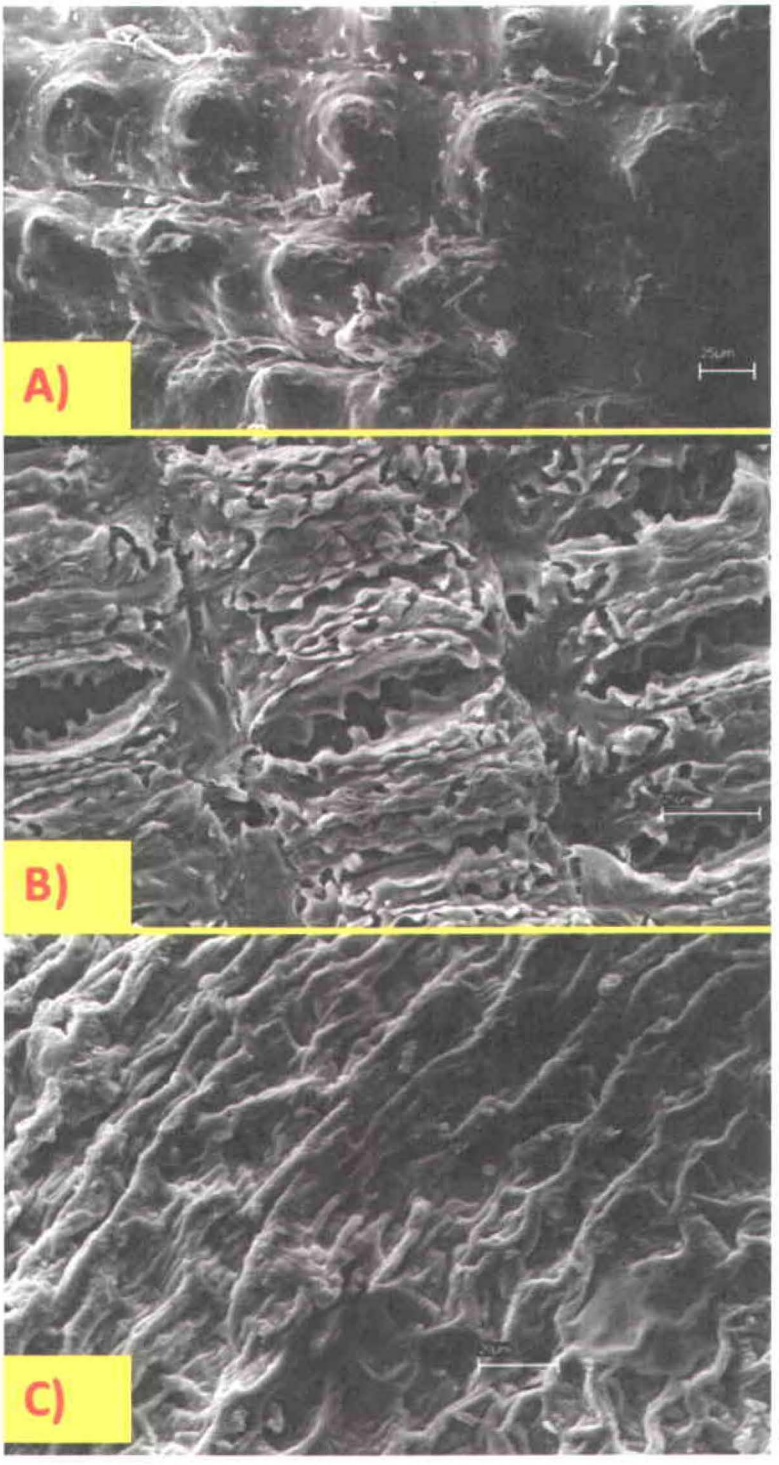


Figure 5.2. 4 Scanning electron micrographs of: A) *Aspergillus niger* biomass -SmF (× 1k); B) AIM solid fraction during chitosan from *A. niger* biomass using SmF (APS)- 6 days (2k) and; C) AAIM fraction (after acetic acid [0.1 M] extraction) during chitosan using SmF (2 k)

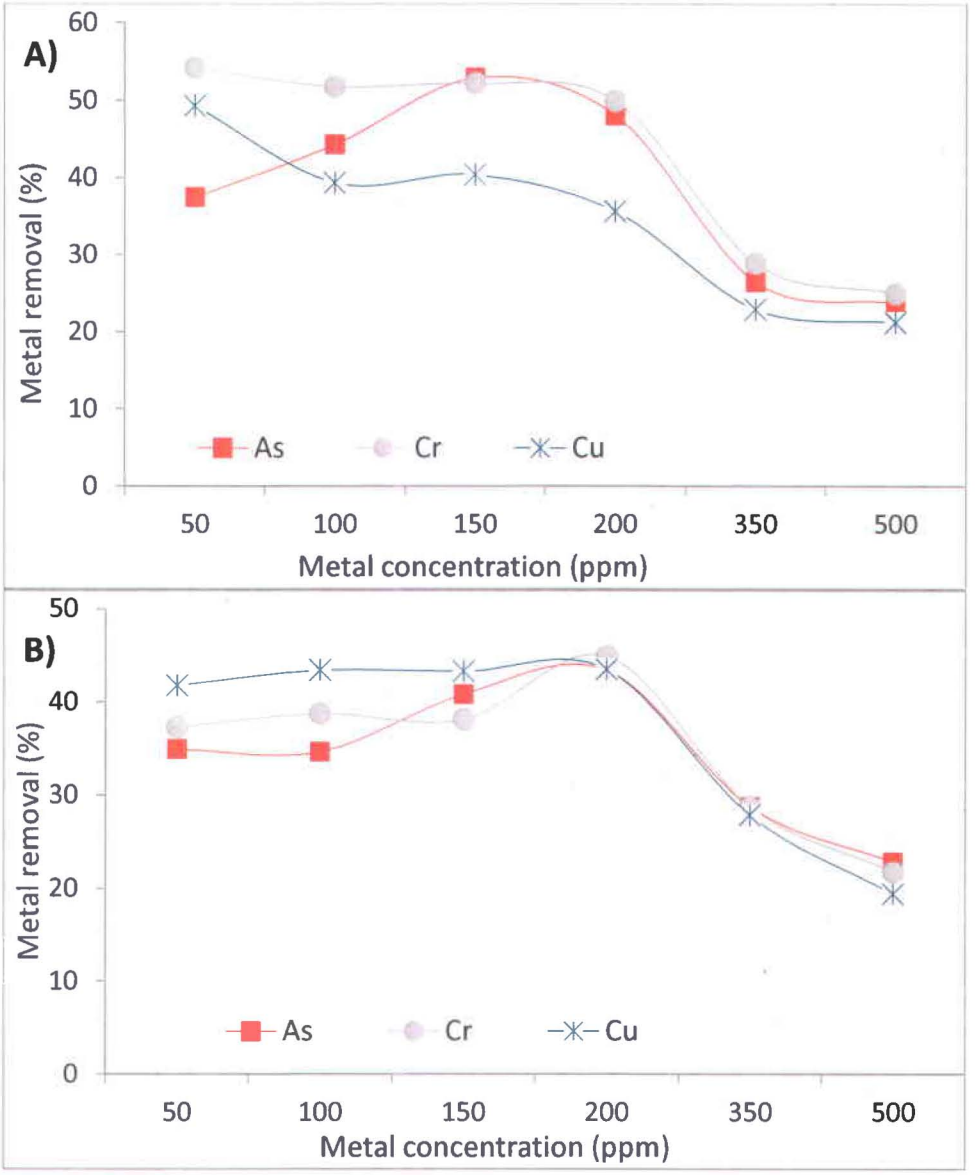
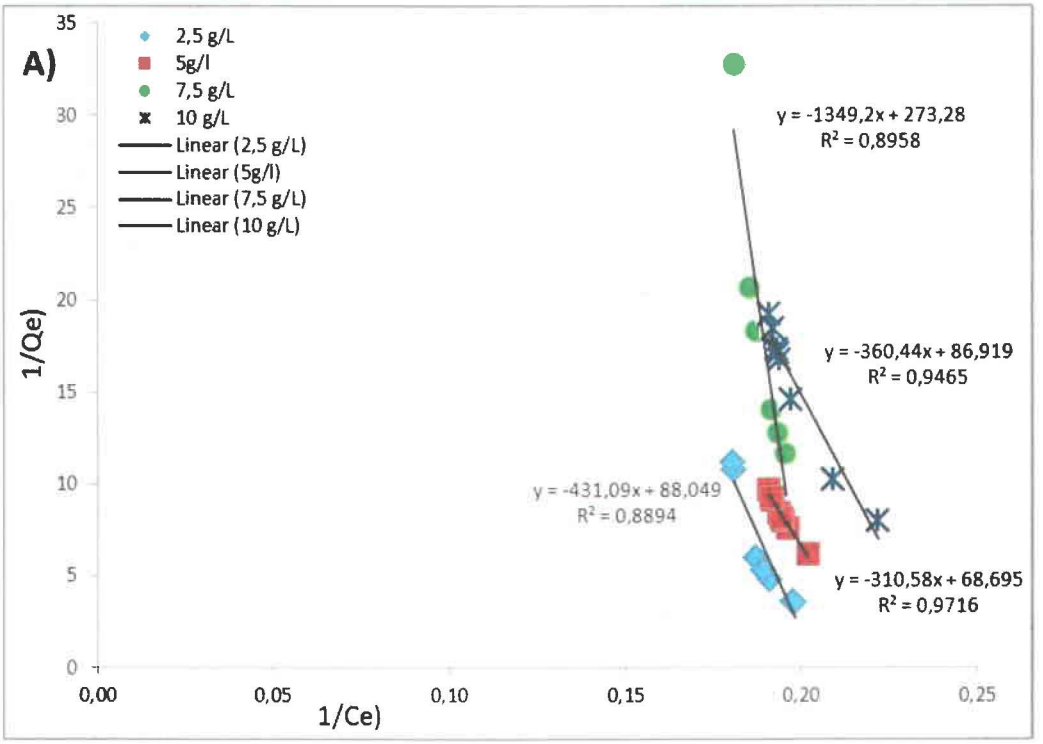
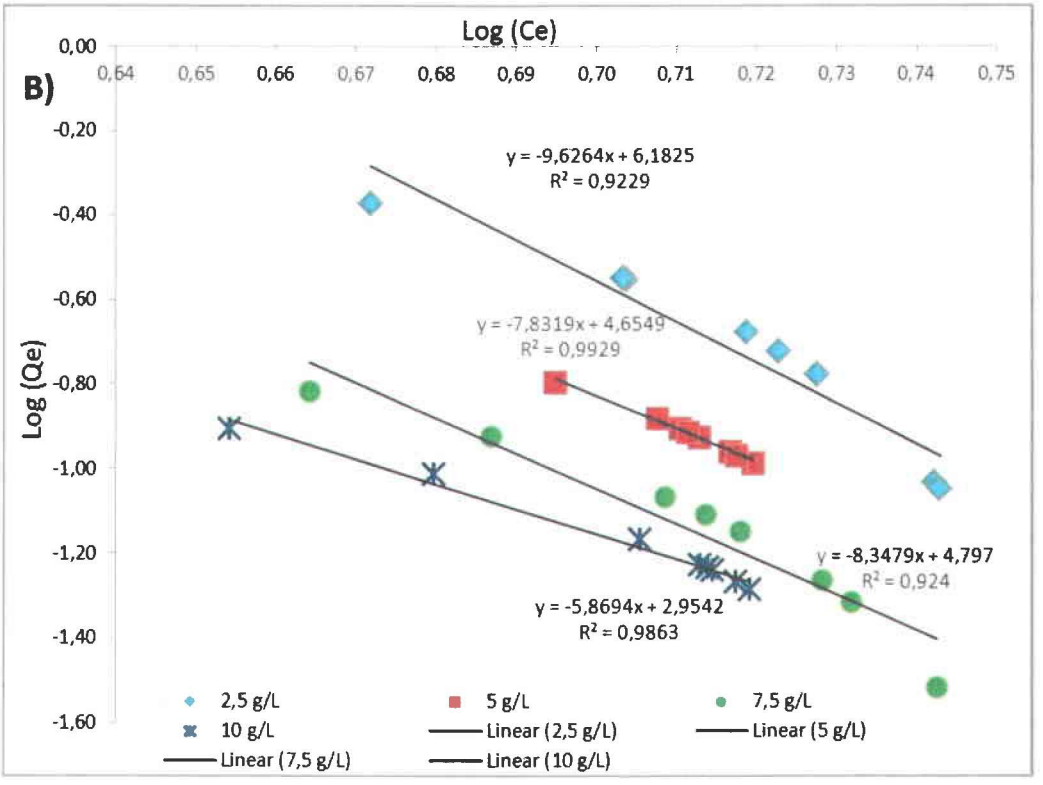
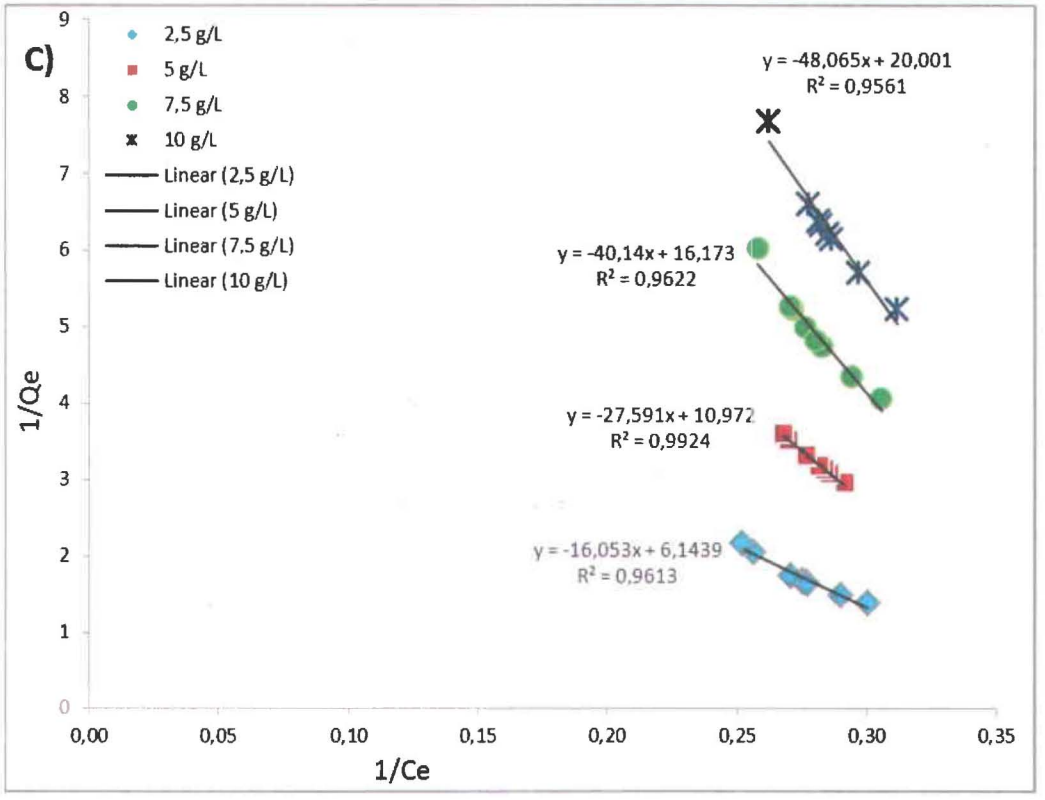
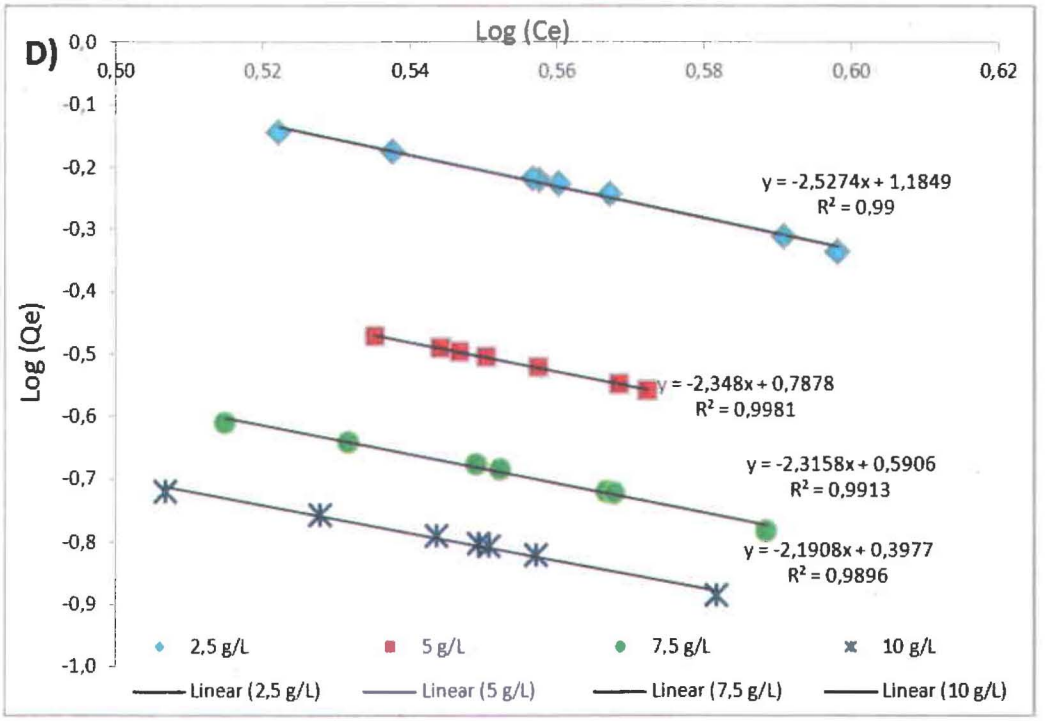


Figure 5.2. 5 Removal of metals (As, Cr and Cu) aqueous solution using: (A) AP live biomass (control) (2.5 g/L) resulting from SSF after 24 h incubation period and; (B) APS live biomass (MeOH)- 2.5 g/L) resulting from SmF after 18 h incubation period.


A)
2,5 g/L
5g/l
7,5 g/L
10 g/L
Linear (2,5 g/L)
Linear (5g/l)
Linear (7,5 g/L)
Linear (10 g/L)
y = -1349,2x + 273,28
R² = 0,8958
y = -360,44x + 86,919
R² = 0,9465
y = -431,09x + 88,049
R² = 0,8894
y = -310,58x + 68,695
R² = 0,9716
1/Qe)
1/Ce)
35
30
25
20
15
10
5
0
0,00
0,05
0,10
0,15
0,20
0,25


B)
Log (Ce)
Log (Qe)
y = -9,6264x + 6,1825
R² = 0,9229
y = -7,8319x + 4,6549
R² = 0,9929
y = -8,3479x + 4,797
R² = 0,924
y = -5,8694x + 2,9542
R² = 0,9863
2,5 g/L
5 g/L
7,5 g/L
10 g/L
Linear (2,5 g/L)
Linear (5 g/L)
Linear (7,5 g/L)
Linear (10 g/L)
0,00
-0,20
-0,40
-0,60
-0,80
-1,00
-1,20
-1,40
-1,60
0,64
0,65
0,66
0,67
0,68
0,69
0,70
0,71
0,72
0,73
0,74
0,75


C)
2,5 g/L
5 g/L
7,5 g/L
10 g/L
Linear (2,5 g/L)
Linear (5 g/L)
Linear (7,5 g/L)
Linear (10 g/L)
y = -48,065x + 20,001
R² = 0,9561
y = -40,14x + 16,173
R² = 0,9622
y = -27,591x + 10,972
R² = 0,9924
y = -16,053x + 6,1439
R² = 0,9613
1/Qe
1/Ce
0
1
2
3
4
5
6
7
8
9
0,00
0,05
0,10
0,15
0,20
0,25
0,30
0,35


D)
Log (Ce)
Log (Qe)
y = -2,5274x + 1,1849
R² = 0,99
y = -2,348x + 0,7878
R² = 0,9981
y = -2,3158x + 0,5906
R² = 0,9913
y = -2,1908x + 0,3977
R² = 0,9896
2,5 g/L
5 g/L
7,5 g/L
10 g/L
Linear (2,5 g/L)
Linear (5 g/L)
Linear (7,5 g/L)
Linear (10 g/L)
0,0
-0,1
-0,2
-0,3
-0,4
-0,5
-0,6
-0,7
-0,8
-0,9
-1,0
0,50
0,52
0,54
0,56
0,58
0,60
0,62

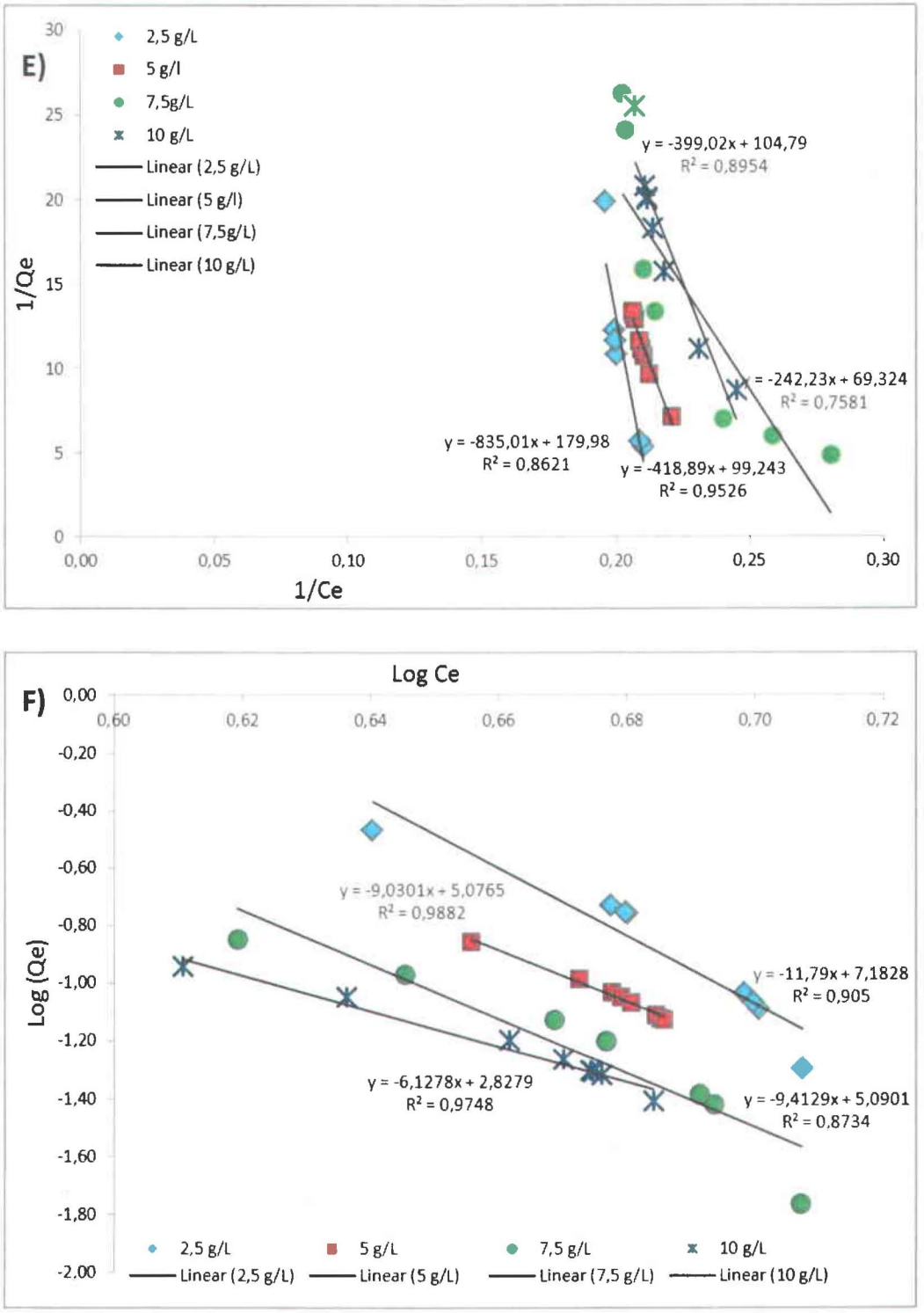
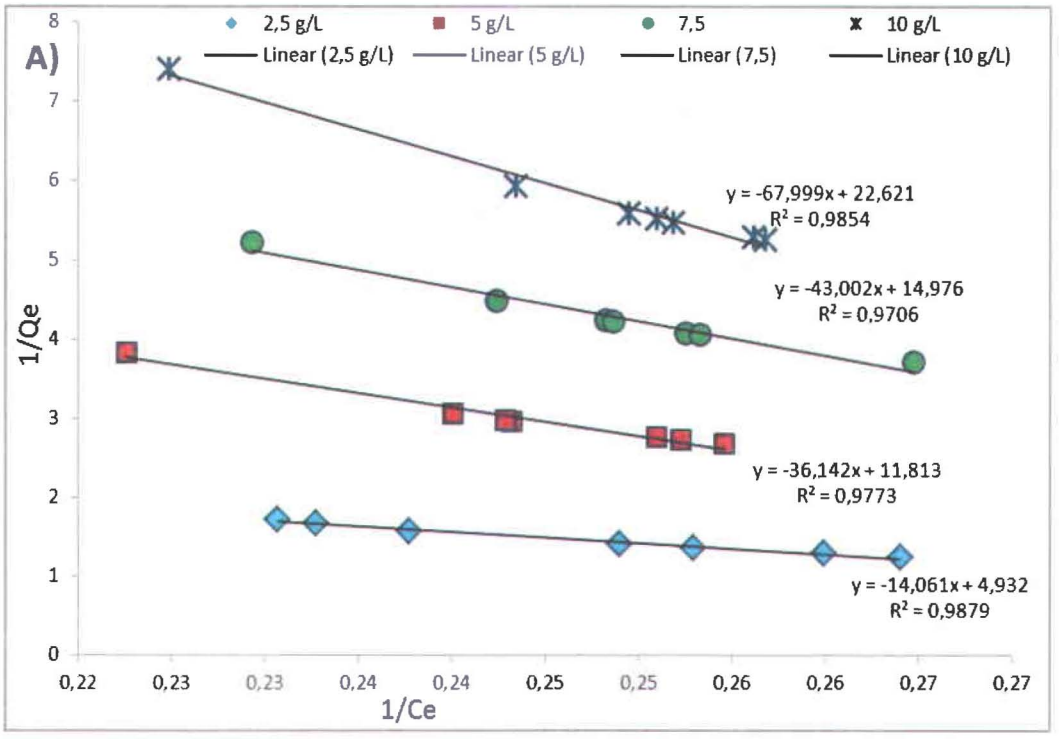
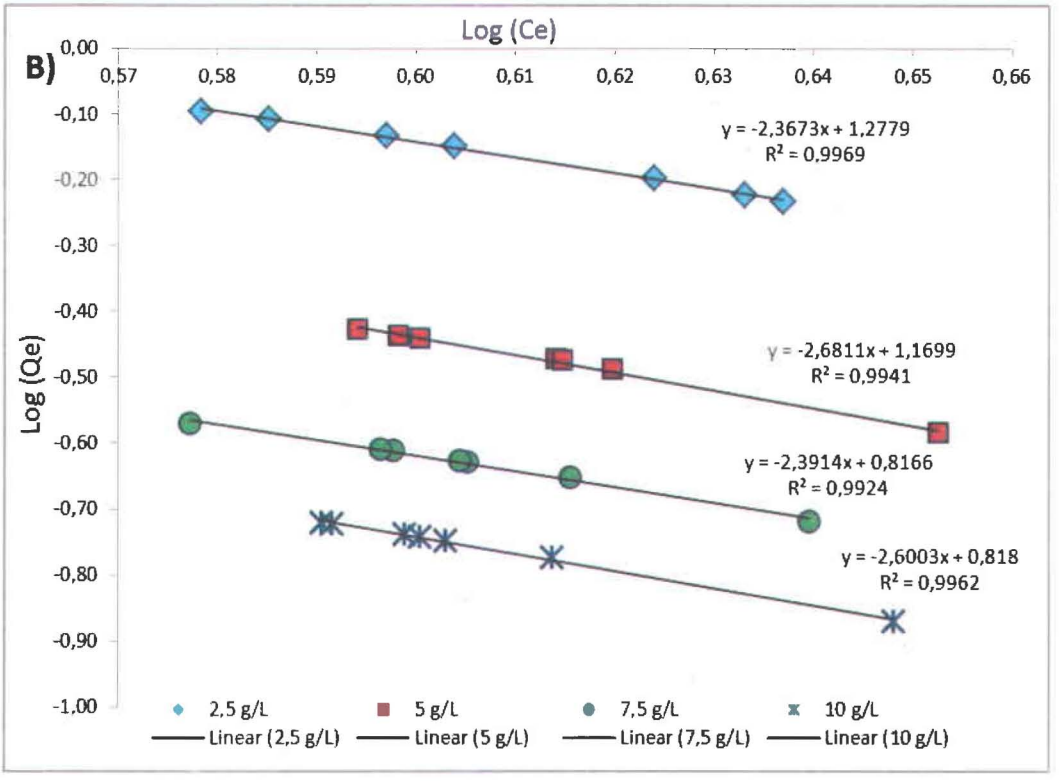
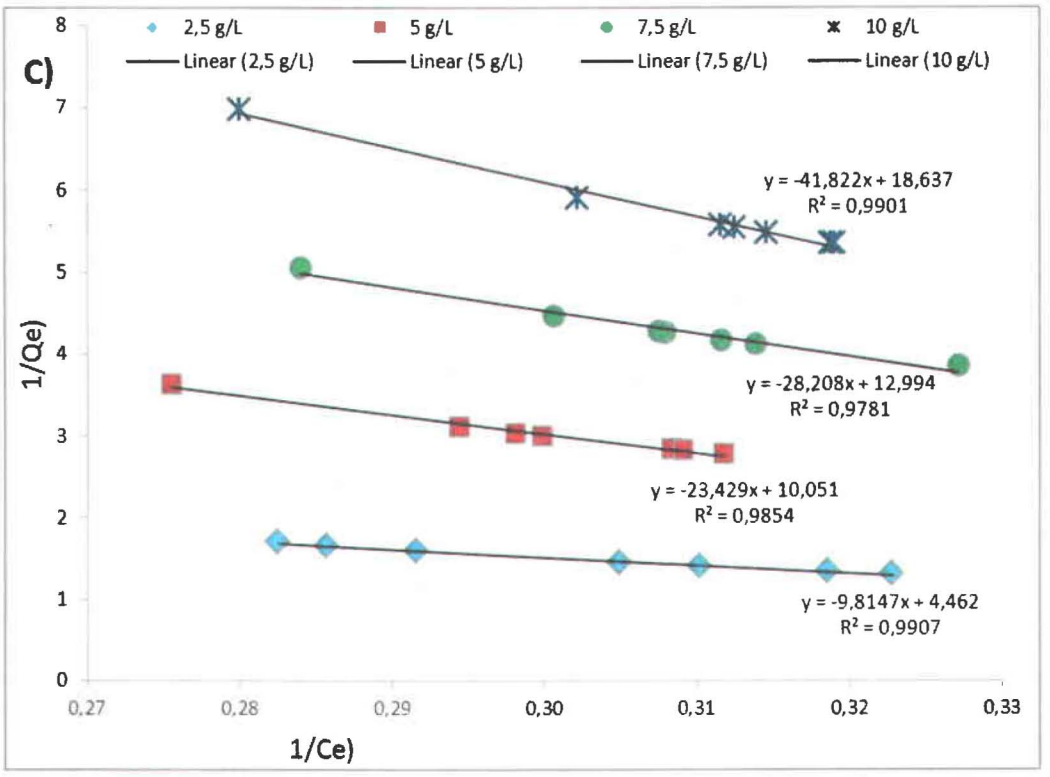
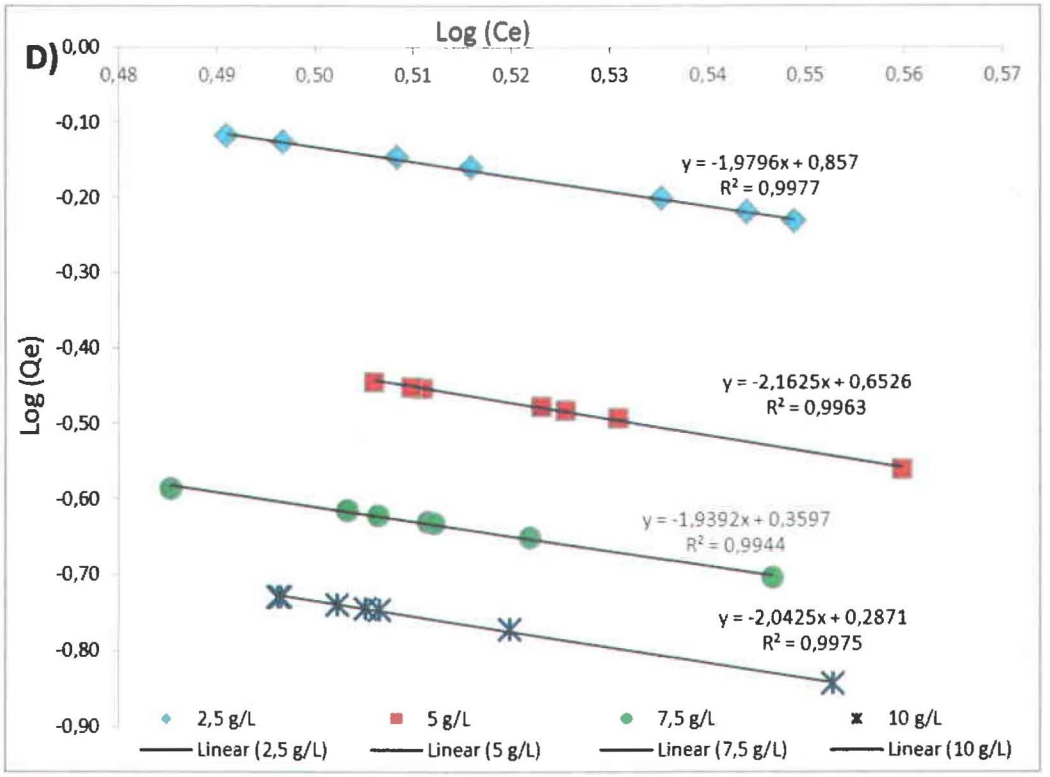


Figure 5.2. 6 Adsorption isotherm (Langmuir and Freundlich) modelling related to the biosorption of: A & B) arsenic; C & D) chromium and; E & F) copper onto biomass SSF (Control, live) (temperature 25±1 °C, 175 rpm).


A)
2,5 g/L
5 g/L
7,5
10 g/L
Linear (2,5 g/L)
Linear (5 g/L)
Linear (7,5)
Linear (10 g/L)
y = -67,999x + 22,621
R² = 0,9854
y = -43,002x + 14,976
R² = 0,9706
y = -36,142x + 11,813
R² = 0,9773
y = -14,061x + 4,932
R² = 0,9879
1/Qe
1/Ce
0
1
2
3
4
5
6
7
8
0,22
0,23
0,23
0,24
0,24
0,25
0,25
0,26
0,26
0,27
0,27


B)
Log (Ce)
Log (Qe)
y = -2,3673x + 1,2779
R² = 0,9969
y = -2,6811x + 1,1699
R² = 0,9941
y = -2,3914x + 0,8166
R² = 0,9924
y = -2,6003x + 0,818
R² = 0,9962
2,5 g/L
5 g/L
7,5 g/L
10 g/L
Linear (2,5 g/L)
Linear (5 g/L)
Linear (7,5 g/L)
Linear (10 g/L)
0,00
-0,10
-0,20
-0,30
-0,40
-0,50
-0,60
-0,70
-0,80
-0,90
-1,00
0,57
0,58
0,59
0,60
0,61
0,62
0,63
0,64
0,65
0,66


C)
2,5 g/L
5 g/L
7,5 g/L
10 g/L
Linear (2,5 g/L)
Linear (5 g/L)
Linear (7,5 g/L)
Linear (10 g/L)
y = -41,822x + 18,637
R² = 0,9901
y = -28,208x + 12,994
R² = 0,9781
y = -23,429x + 10,051
R² = 0,9854
y = -9,8147x + 4,462
R² = 0,9907
1/Qe)
1/Ce)
0
1
2
3
4
5
6
7
8
0,27
0,28
0,29
0,30
0,31
0,32
0,33


D)
Log (Ce)
Log (Qe)
y = -1,9796x + 0,857
R² = 0,9977
y = -2,1625x + 0,6526
R² = 0,9963
y = -1,9392x + 0,3597
R² = 0,9944
y = -2,0425x + 0,2871
R² = 0,9975
2,5 g/L
5 g/L
7,5 g/L
10 g/L
Linear (2,5 g/L)
Linear (5 g/L)
Linear (7,5 g/L)
Linear (10 g/L)
0,00
-0,10
-0,20
-0,30
-0,40
-0,50
-0,60
-0,70
-0,80
-0,90
0,48
0,49
0,50
0,51
0,52
0,53
0,54
0,55
0,56
0,57

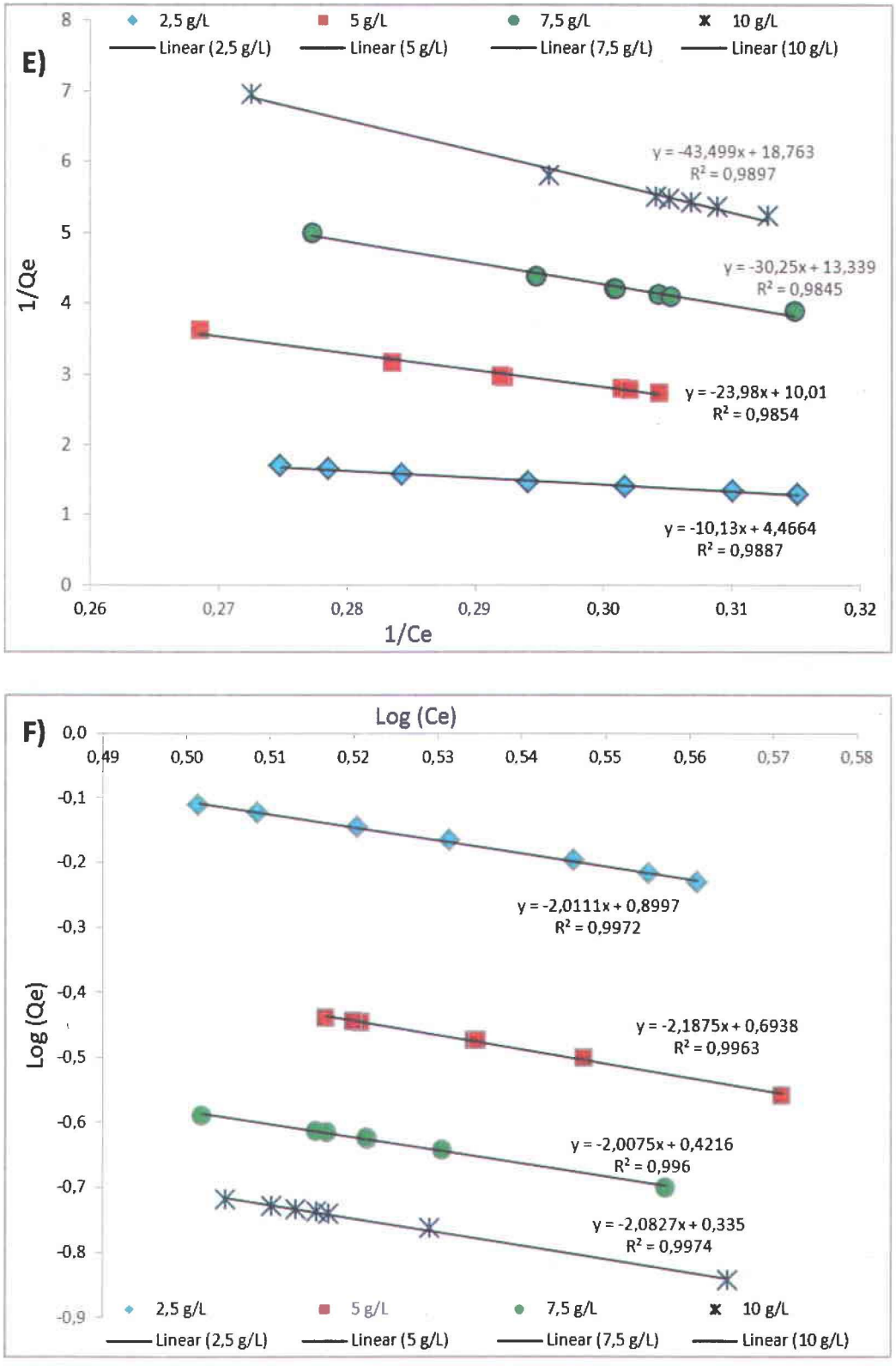


Figure 5.2. 7 Adsorption isotherm (Langmuir and Freundlich) modelling related to the biosorption of: A & B) arsenic; C & D) chromium and; E & F) copper onto biomass SmF (MeOH, live)

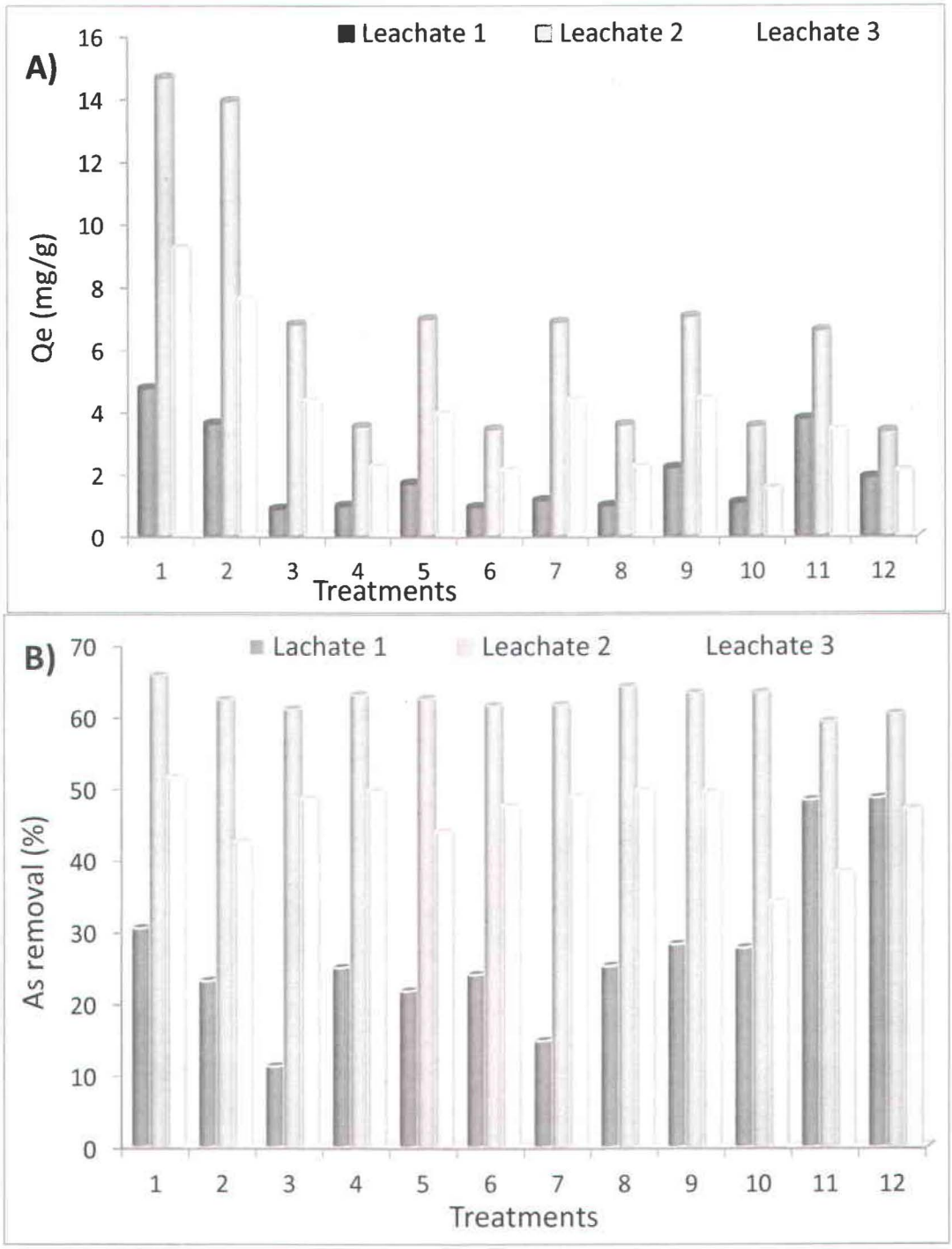

A)
Leachate 1
Leachate 2
Leachate 3
Qe (mg/g)
16
14
12
10
8
6
4
2
0
1 2 3 4 5 6 7 8 9 10 11 12
Treatments
B)
Lachate 1
Leachate 2
Leachate 3
As removal (%)
70
60
50
40
30
20
10
0
1 2 3 4 5 6 7 8 9 10 11 12
Treatments

Figure 8

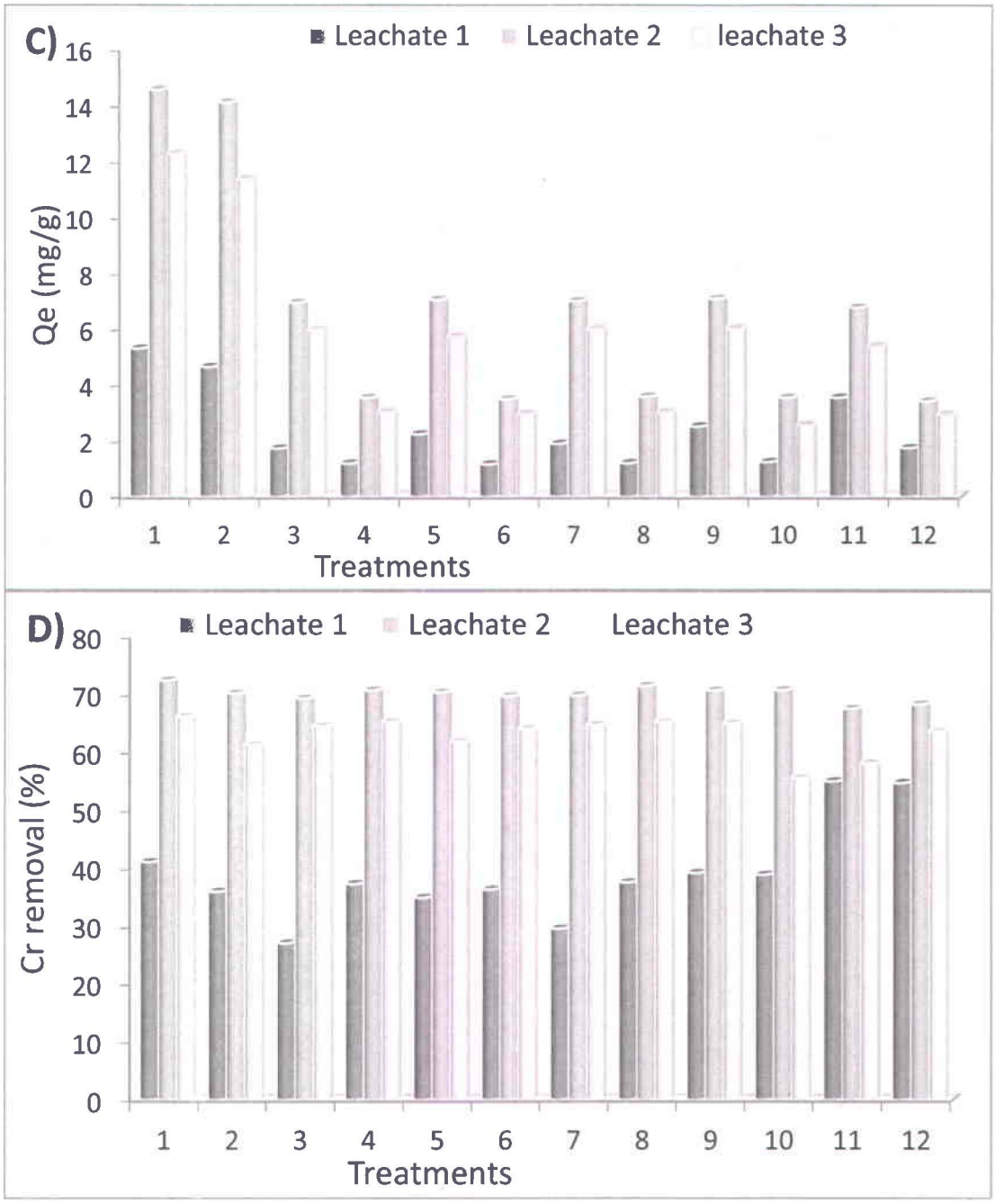

C)
Leachate 1
Leachate 2
leachate 3
Qe (mg/g)
0
2
4
6
8
10
12
14
16
1
2
3
4
5
6
7
8
9
10
11
12
Treatments
D)
Leachate 1
Leachate 2
Leachate 3
Cr removal (%)
0
10
20
30
40
50
60
70
80
1
2
3
4
5
6
7
8
9
10
11
12
Treatments

Figure 8

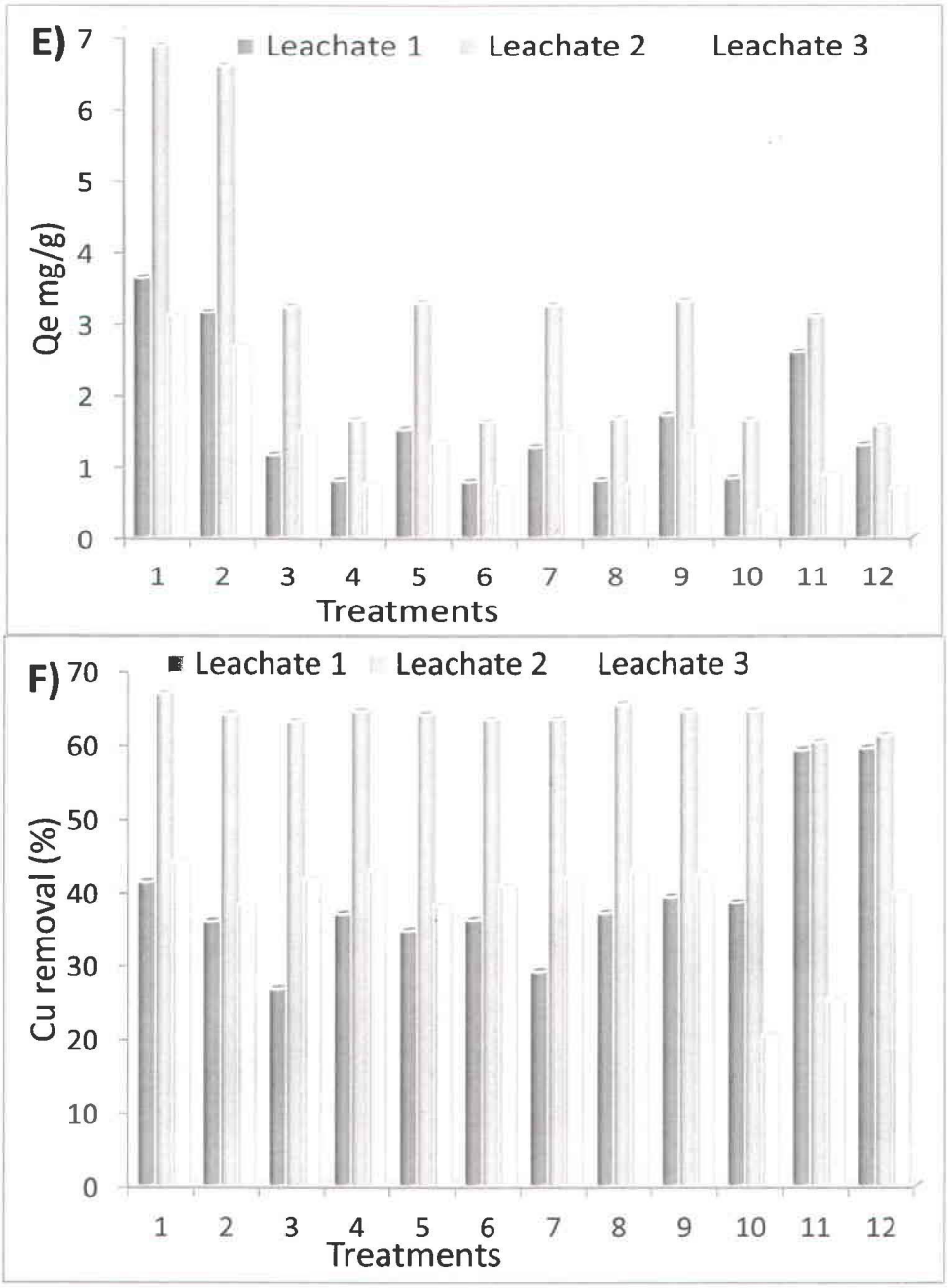


Figure 5.2. 8 Metal biosorption capacity, Qe (mg/g) and removal rate (%) of different metals from waste CCA wood leachates using different BMs: (A & B) As, (C & D) Cr and: (E & F) Cu.

Treatments: 1) SSF biomass (control, live- 2.5 mg /L); 2) SmF biomass (MeOH, live- 2.5 mg/L); 3) low molecular weight chitosan (LMWCs) 5 mg/L; 4) low molecular weight chitosan (LMWCs) 10 mg/L; 5) medium molecular weight chitosan (MMWCs) 5 mg/L; 6) medium molecular weight chitosan (MMWCs) 5 mg/L; 7) high molecular weight chitosan (HMWCs) 5 mg/L; 8) high molecular weight chitosan (HMWCs) 5 mg/L; 9) chitin 5 mg/L; 10) chitin 10 mg/L; 11) citric acid- 5mg/L and; 12) citric acid- 10 mg/L.

FACILE SYNTHESIS OF CHITOSAN-BASED ZINC OXIDE NANOPARTICLES BY NANO SPRAY DRYING PROCESS AND EVALUATION OF THEIR ANTIMICROBIAL AND ANTIBIOFILM POTENTIAL

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RÉSUME

Les différents plans de la présente enquête; la synthèse et la caractérisation facile de chitosane (CTS) à base d'oxyde de zinc (ZnO) des nanoparticules (NPs), et leurs applications antimicrobiennes et antibiofilm ont été étudiées contre les bactéries pathogènes. Zn NPs ont été synthétisés par le nano processus ; la pulvérisation et la méthode de précipitation à l'aide de divers composés organiques (acide citrique, glycérine, amidon et du lactosérum) qui sont utilisés en tant que stabilisateurs. La caractérisation détaillée des ZnO NPs a été réalisée à l'aide de spectroscopie d'UV-Vis, diffusion dynamique de la lumière (DLS) analyse granulométrique, mesures de potentiel zêta et la microscopie électronique à balayage (MEB). Ces outils ont confirmé la fabrication des IP avec des différentes formes et tailles. Le test antibactérien de la synthèse des ZnO-CTS NPs a été réalisée à la fois dans le milieu de croissance liquide et solide contre les bactéries pathogènes (*Candida albicans, Micrococcus luteus* et *Staphylococcus aureus*). La croissance bactérienne a été suivie par mesure de la densité optique de la solution de culture et l'estimation des unités formant colonies (UFC) sur un milieu solide. L'activité de la formation antibiofilm a également été évaluée. Le NPs-ZnO-CTS a montré un potentiel prometteur en tant qu'agent antimicrobien et antibiofilm.

Mots clés: antimicrobiens; antibiofilm; chitosane; nanoparticules; voie sèche par pulvérisation nano

ABSTRACT

The present investigation deals with the facile synthesis and characterization of chitosan (CTS)-based zinc oxide (ZnO) nanoparticles (NPs) and their antimicrobial and biofilm inhibition activities against pathogenic bacteria. ZnO-CTS NPs were synthesized by nano spray process and precipitation method using various organic compounds (citric acid, glycerol, starch and whey) as stabilizers. The detailed characterization of the NPs was carried out using UV-Vis spectroscopy, dynamic light scattering (DLS) particle size analysis, zeta potential measurements and scanning electron microscopy (SEM), which confirmed the fabrication of NPs with different shapes and sizes. Antimicrobial assay of synthesized ZnO-CTS NPs was carried out against pathogenic bacteria (*Candida albicans, Micrococcus luteus* and *Staphylococcus aureus*). The bacterial growth was monitored by measuring the optical density of the culture solution. The significant inhibition of growth was observed for both *M. luteus* with OD (600 nm) of 0.502±0.093 units (ZnO-CTS-CA NPs, at a dose of 0.156 mg/ml) as compared to control with OD of 1.904±0.432 units and *S. aureus* with OD (600 nm) of 0.289±009 units (ZnO-CTS-CA NPs at a concentration 0.156 mg/ml) as compared to control with OD of 1.578±0.342. ZnO-CTS-based NPs also showed significant biofilm inhibition activity ($p<0.05$). The study demonstrated the potential of ZnO-CTS NPs as antimicrobial and antibiofilm agents.

Key words: Antimicrobial; antibiofilm; chitosan; nanoparticles; nano spray dryer

INTRODUCTION

Metal oxide nanoparticles (NPs), such as zinc oxide (ZnO) have been found to exhibit interesting properties, such as large surface-to-volume ratio, high surface reaction activity, high catalytic efficiency, and strong adsorption ability (Singh, et al., 2007; Wei et al., 2006; Wang, et al., 2006) that make them potential candidates for various applications. Zinc oxide is an organic material with several advantages, such as wide band gap (3.34 eV), semiconductor with a high exciton binding energy (60meV), high isoelectric point (9.5), and fast electron transfer kinetics as well as having a unique combination of properties (Cheng et al., 2006; Wahab et al., 2009). All these features suggest the feasibility of ZnO for synthesis of special ZnO nanostructures. Nano ZnO can be synthesized in many forms: rods, wires, whiskers, belts, bipods, tetrapods, tubes, flowers, propellers, bridges, and cages (Zhang et al., 2002; Ayudhya et al., 2006; Bitenc et al., 2009). Nanostructured ZnO has great potential for many practical applications, such as dye sensitized solar cells, piezoelectric transducers, UV-light emitters, chemical and gas sensors, and transparent conductive coating (Ozgur et al. 2005). It also exhibits intense ultraviolet absorption and can potentially be utilized as UV-shielding materials and antibacterial agents (Kim and Osterloh 2005). ZnO NPs have been prepared by techniques, such as the sol-gel method (Hubbard et al., 2006; Lee et al., 2003), precipitation (Wang and Muhammed, 1999.], hydrothermal synthesis [Xu et al., 2004), and spray pyrolysis (Tani et al., 2002).

Recently, hybrid materials based on chitosan (CTS) have been developed, including conducting polymers, metal NPs, and oxide agents, due to excellent properties of individual components and outstanding simultaneous synergistic effects (Li et al., 2010). Currently, the research on the combination of CTS and metal oxide has focused on titanium dioxide (TiO_2), as it has excellent photocatalytic performance and is stable in acidic and alkaline solvents. In parallel with TiO_2, ZnO also has similar band-gap and antibacterial activity (Li et al., 2010). The cationic nature of CTS makes it possible to adhere to the negatively charged surface, such as bacterial cell membranes. CTS is soluble in dilute acidic solutions and is able to interact with polyanions to form complexes and gels. CTS is innocuous and possess antibacterial and antifungal properties (Agnihotri et al., 2004; Kim and Rajapakse, 2005). CTS has tremendous ability to form metal complexes with Zn metal (Muzzarelli and Sipos, 1971; Muzzarelli and Tubertini, 1969). Owing to the presence of amine and hydroxyl groups on CTS,

currently, ZnO-CTS complex attracted great interest for its potential use as UV protector and medicament.

However, due to the extremely high reactivity, the initially formed ZnO NPs tend to react rapidly with the surrounding media or agglomerate, resulting in the formation of much larger (micrometer to millimeter scale) particles or flocs. In the process, they rapidly lose their bioactivity. In order to prepare more stable and active ZnO NPs, studies have been carried out either by the fabrication on the surface of NPs by organoalkoxysilane (Posthumus et al., 2004) or by grafted polymers (Tang et al., 2006). However, only few studies have been carried out on the surface modification of NPs to create a stable dispersion in aqueous medium. Various organic compounds, such as citric acid (CA), glycerol, starch and whey powder (contains lactose moiety), among others can be used as capping agents. These water-soluble organic compounds serve as a stabilizer and dispersant that prevents the resultant NPs from agglomeration, thereby prolonging their reactivity and maintaining the physical integrity. Moreover, these organic compounds are safe and innocuous. CA is also considered as GRAS and it possesses antimicrobial activity as well.

The present work was carried out with the following objectives: (1) facile synthesis and characterization of CTS-based ZnO NPs with different stabilizers using nano spray drying and precipitation method. Various organic compounds, such as CA, glycerol, starch and whey powder were used as capping agents to stabilize the NPs formulation and; (2) application of ZnO-CTS NPs as antimicrobial and antibiofilm forming agents against pathogenic bacteria.

MATERIALS AND METHODS

Chemicals

Yeast Peptone Glucose (YPG) medium, tryptic soya broth and agar were purchased from Fischer scientific. Zinc oxide (ZnO) and CA were supplied by Sigma-Aldrich (Ontario, Canada). Chitosan (molecular weight- 600-800 kDa and degree of deacetylation >90% and viscosity 200-500 mPa·s) and starch (soluble, ACS grade) was purchased from Fisher Scientific (Ontario, Canada). Whey powder (whey permeate) was purchased from Agropur Cooperative, Québec, Canada. Milli-Q water was prepared in the laboratory using a Milli-Q/Milli-RO Millipore system (Milford, MA, USA). Glycerol was purchased from Labmat Inc. Québec, Canada. The composition or propoerties of whey

powder was as follows: Carbohydrates (mainly as lactose) > 82.0 %; protein ≤ 3.5 %; fat <0.3 %; ash content 8.0-9.0 %; moisture < 5.0 %; pH (10 % solution) 6.1 and titrable acidity of 0.12 %.

Preparation of CTS-based ZnO NPs

Nano spray drying method

The facile synthesis of CTS-based nanoparticles was carried out using nano spray dryer (B-90 BUCHI, Switzerland). NPs were prepared by dissolving 0.75 g of ZnO powder in 100 ml of 1% (v/v) acetic acid. This led to the dissociation of ZnO to zinc cations. To this solution, 1 g of CTS was added. After fully dissolving the CTS, different organic compounds [1% (w/v) CA; 0.5% (w/v) whey powder; 0.5% (w/v) soluble starch and 0.5% (w/v) glycerol] were added as stabilizers in various treatments. For comparison, control of ZnO NPs and ZnO-CTS NPs were prepared in the absence of stabilizer following similar procedure. The process conditions were as follows: spray 100%, inlet and head temperature was set at 120 °C. The pump speed was adjusted on standard mode of 1 and gas pressure was adjusted at 40-45 hPa which corresponds to a gas flow of around 150-160 l/min. The NPs were collected using particle collection paper.

Precipitation method

The similar procedure as nano spray dryer was followed and after making the solution containing ZnO, CTS in the absence or presence of stabilizer, the mixture was sonicated for 30 min. With magnetic stirring, 1M NaOH drop by drop was added until the solution attained pH 10. The samples were heated in water bath at 80 °C for about 3 h followed by washing with distilled water several times and lyophilization using a freeze dryer (ScanVac Cool Safe, LaboGene, Lynge, Denmark).

Analytical measurement

Viscosity of different samples was measured by using a rotational viscometer Brookefield DVII PRO+ (Brookfield Engineering Laboratories, Inc., Stoughton, MA, USA) equipped with Rheocalc32 software. The viscosity data acquisition and analysis was carried out using Rheocalc V2.6 software, (B.E.A.V.I.S. – Brookfield Engineering Advanced Viscometer Instruction Set). All measurements were performed at 25±1 °C,

36.69 s^{-1} shear rate and viscosity was referred to as "apparent viscosity". pH of the different metal oxide solutions were measured with the pH meter equipped with glass electrode.

Characterization of NPs

UV-Vis Spectrum

Absorption spectra of the NPs were recorded using a UV-Vis spectrophotometer (UV 0811 M136, Varian, Australia) at 200-600 nm. The optical path length of the cuvettes used in all the experiments was 1 cm. The absorption spectra were recorded at room temperature.

Size and Zeta potential measurements

The mean size and size distribution and zeta potential measurements of the ZnO-CTS NPs were performed by Nano Zetasizer (Nano series, Malvern instruments Ltd., Worcestershire, UK) equipped with multipurpose titrator (MPT-2).

Scanning Electron Microscope (SEM)

The morphology of the synthesized ZnO NPs was examined by a scanning electron microscope (Model: Carl Zeiss EVO® 50 smart SEM) with a magnification of 10.0 K X, working distance (WD) 20 mm, secondary electrode 1 (SE 1) detector and EHT 10 kV. All the samples were coated with gold before SEM testing.

Assessment of *in vitro* antimicrobial and antibiofilm activity of ZnO-CTS NPs

The fungal strain, *Candida albicans* CCRI 10345, and bacterial strains, *Micrococcus luteus* CCRI 16025 and *Staphylococcus aureus* CCRI 20630 were used in this study to assess antimicrobial and antibiofilm activity of ZnO-CTS-based NP formulations. The microbial strains were cultured for 48 h using Yeast Peptone Glucose (YPG) medium agar plates (*C. albicans*) and tryptic soya agar plates (*M. Luteus and S. aureus*).

These strains were selected due to the following reasons:

- *Candida albicans* is a causal agent of opportunistic oral and genital infections in humans (Ryan and Ray, 2004). Systemic fungal infections (fungemias) by *C. albicans* have emerged as major causes of morbidity and mortality in immuno-compromised patients. *C. albicans* forms biofilms on the surface of implantable medical devices, as a consequence hospital-acquired infections by *C. albicans* have become a cause of major health concerns.

- *Micrococcus luteus* is a Gram-positive, spherical, saprotrophic bacterium that belongs to the family Micrococcaceae. *M. luteus* is found in soil, dust, water and air, and as part of the normal flora of the mammalian skin. The bacterium also colonizes the human mouth, mucosae, oropharynx and upper respiratory tract. *Micrococcus luteus* shows higher potential for biofilm formation.

- *Staphylococcus aureus* is a facultative anaerobic Gram-positive coccal pathogenic bacterium belonging to Staphylococcaceae family. *S. aureus* is the prevalent cause of food intoxication and can cause a wide range of illnesses, from minor skin infections, such as pimples, impetigo, boils (furuncles),cellulitis folliculitis carbuncles, scalded skin syndrome, and abscesses, to life-threatening diseases such as pneumonia, meningitis, osteomyelitis, endocarditis, bacteremia, and sepsis. Its incidence ranges from skin, soft tissue, respiratory, bone, joint, endovascular to wound infections. It is one of the five most common causes of nosocomial infections and is often the cause of postsurgical wound infections. (Bowersox, 1999).

Qualitative antimicrobial assay

The *in vitro* qualitative screening of the different ZnO-CTS NPs was performed using agar diffusion method. The filter paper discs (5 mm diameter) were dipped in each filter sterilized tested sample (NPs concentration of 5 mg/ml) and were placed in Petri dishes with yeast extract, peptone and glucose (YPG) (for fungi) and trypic soya agar (for bacteria) medium previously seeded with the bacterial inocula. The inoculated plates were incubated for 24 h at 30±2 °C. Antimicrobial activity was assessed by measuring the inhibition zone diameter (mm). Based on the qualitative assay results, only the bacterial strains susceptible to tested samples have been further tested in the quantitative assay using liquid medium.

Quantitative antimicrobial assay of the minimal inhibitory concentration

The quantitative assay was performed in liquid medium with 2-fold serial dilutions using susceptible strains assessed through qualitative assays and was performed in 96 well plates. The assay was performed with serial binary dilutions of the tested compounds (ranging between 5 and 0.01 mg/ml) using TSB medium. The assay was performed using 200 µl volume of nutrient medium and each well was inoculated with 20 µl inocula. The microplates were incubated at 30±2 °C for 24 h. The antimicrobial activity was quantified by measuring the optical density at 600 nm and minimum inhibitory concentration (MICs) were read as the last concentration of the tested compounds, which inhibited the microbial growth.

Antibiofilm activity

The antibiofilm activity was performed using the microtiter method. For this purpose, the microbial strains have been grown in the presence of twofold serial dilution of the tested compounds performed in liquid nutrient medium distributed in 96-well plates. In brief, 200 µl medium with known concentrations of tested sample was inoculated with 20 µl bacterial suspension and incubated for 24 h at 30±2 °C for bacterial strains. At the end of the incubation time, the plastic wells were emptied, washed three times with phosphate buffer saline (PBS), fixed with cold methanol, and stained with 1% violet crystal solution for 30 min. The biofilm formed on plastic wells was resuspended in 30 % acetic acid. The intensity of the colored suspension was assessed by measuring the absorbance at 492 nm (Limban et al., 2011). The last concentration of the tested compound that inhibited the development of microbial biofilm on the plastic wells was considered as the MICs of the biofilm development and was also expressed in mg/ml (Olar, et al., 2010). The quantitative antimicrobial assay and biofilm inhibition assay was performed in triplicates and values are given as average± standard deviation (SD).

RESULTS AND DISCUSSION

ZnO-CTS-based NPs synthesis and characterization

UV-Vis spectra

Figure 5.3.1 A and B shows the UV-Vis obtained for different ZnO-CTS NPs prepared through nano spray drying and precipitation method. The results indicated the formation

of ZnO-CTS NPs. The synthesis of ZnO-CTS NPs are clearly evident, as the excitation absorption peak was observed due to ZnO-CTS NPs at 273 nm, which lies much below the bandgap wavelength of 388 nm (E_g = 3.2 eV) of ZnO. It was observed that absorption peaks of ZnO NPs prepared in the presence of stabilizers were prominent as compared to ZnO-CTS NPs without stabilizers. The absorption peak for a suspension of NPs is broader and is determined by the distribution of particle size. In the smaller wavelength range, particles with smaller size contribute more and at the region of absorbance maximum, all particles contribute to the absorbance (Pesika et al., 2003). Therefore, the average particle size of ZnO-CTS NPs observed in the presence of stabilizers was lower as compared to the ZnO NPs prepared in the absence of stabilizers.

Size and Zeta potential

The mean size and size distribution of various ZnO-CTS NPs with different stabilizers and synthesized through different methods are provided in Table 5.3.2. As evident from the Table the mean size of the ZnO-CTS NPs synthesized through nano spray dying method ranged between 93.2- 402.5 nm. The mean size of ZnO-CTS-glycerol NPs (402.5 nm) were even larger than the control (ZnO nano, 215.4 nm). The reason for this may be due to the aggregation of NPs which affects their mean size. The size distribution is also highly variable with peak 1 (1196 nm with intensity of nearly 85 %) and peak 2 (9.289 nm with intensity of 14.4 %) This indicates that the concentration of ZnO, CTS and glycerol needs to be optimized to get discrete, uniform and dispersed NPs. In all other treatments, there is not much variation between the mean size and size distribution.

The mean size of ZnO-CTS NPs with different stabilizers and synthesized through precipitation lies between 99.28- 603.1 nm. The ZnO-CTS followed by ZnO-CTS-starch exhibits the large size NPs with diameter of 358.7 and 285.1 nm, respectively. The ZnO-CTS NPs formulation showed some crosslinking or aggregation which impacts their mean size and size distribution. There was not much variation between the mean size and size distribution in other treatments. The results obtained in the present study indicates the possibility that NPs obtained can be further tailored with specific size, uniformity and highly dispersed nature befitted for specific applications through optimization of different variables, such as concentration of metal, CTS and stabilizers.

The zeta potential values obtained for different ZnO-CTS NPs prepared through nano spray drying and precipitation method are presented in Figure 5.3.2 A and B. The zeta potential value of -7.54, -12.87, -1.9, -24.72 and -35.54 mV were calculated for ZnO, ZnO-CTS, ZnO-CTS-glycerol, ZnO-CTS-starch and ZnO-CTS-whey powder NPs synthesized through nano spray drying method, respectively. Similarly, NPs fabricated via precipitation method resulted in zeta potential values of -17.92, -2.68, -37.3, 1.5, -12.76 and -10.9 mV for ZnO, ZnO-CTS, ZnO-CTS-CA, ZnO-CTS-glycerol, ZnO-CTS-starch and ZnO-CTS-whey powder NPs, respectively. Higher zeta potential value was observed for NPs prepared in the presence of different stabilizer except for ZnO-CTS-glycerol NPs in both methods. Higher zeta potential values can be due to the higher intensity of smaller size NPs in the medium with different stabilizers. Generally, the particles come closer and flocculation (aggregation of particles to form large size particle) takes place at low zeta potential and vice versa. Based on DLVO (Derjaguin, Landau, Verwey, and Overbeek) theory, a higher (negative) value of zeta potential is expected to result in larger electrostatic repulsion within the particles, leading to a higher shear sensitivity between particles and, consequently, smaller particle sizes.

SEM of NPs formed through nano spray drying and precipitation method

The scanning electron micrographs for chitosan and ZnO-CTS NPs prepared by nano spray drying and precipitation method using different stabilizers are given in Fig 5.3.3 and 5.3.4. As evident from the micrographs, the NPs with different size and shapes (circular, elliptical and nano rods) were synthesized depending on the different treatments and methods.

As evident from the results, ZnO NPs synthesized by the nano spray drying method were agglomerated (Fig. 5.3.3 B). It can be inferred that in the absence of CTS and stabilizer, the resultant ZnO NPs do not appear as discrete nanoscale particles and form much bulk dendritic floc-like structures with varying density. This type of aggregation was mainly due to the high surface energy of ZnO NPs (Tang et al., 2006) leading to formation of bulk structures with lower reactivity. On contrary, CTS-based ZnO NPs (Fig 5.3.3 C) was observed to be more distinct and uniform as compared to ZnO NPs. The ZnO-CTS- CA NPs were not realizable during the study. The reason for this can be the formation of viscous solution indicative of some polymer formation due to the presence of various functional groups on both CA and CTS resulting in some interaction between

them. The nozzle of the nano spray dryer was blocked with viscous solution of ZnO-CTS-CA and resulted in the formation of thin film on the nozzle exit. ZnO-CTS-glycerol NPs was also found to be less discrete and elliptical type. ZnO-CTS-starch and ZnO-CTS-whey stabilized NPs were observed to be circular in shape. ZnO-CTS-starch NPs were porous structures whereas ZnO-CTS-whey NPs were found to be more discrete and well dispersed as compared to ZnO-CTS-starch NPs. The presence of whey contributed to the steric hindrance between ZnO-CTS NPs and prevented their aggregation and thus maintained the high surface area and reactivity of the particle.

In comparison, ZnO NPs formed by precipitation technique (Fig. 5.3.4) were discrete and less variable as compared to ZnO NPs formed through nano spray drying method. ZnO-CTS NPs formed by precipitation were circular but agglomerated. ZnO-CTS-CA NPs were found to be uniform, distinct and very well dispersed. ZnO-CTS-glycerol NPs appeared to be bulk dendritic floc like structures with non- discrete appearance. On the other hand, ZnO-CTS-starch and ZnO-CTS-whey NPs were found to possess rod like aggregated structures. Studies demonstrated the multifunctional role of citrate ions in the NPs synthesis process, such as acting as a reducing agent, a stabilizer, and a complex agent (Ji et al., 2007; Jiang et al., 2009). Ji et al.(2007) suggested that the citrate ions act as reducing agents, stabilizers, and pH mediators in the synthesis of gold NPs. Various studies have shown that the growth of silver nanocrystals in solution is sensitive to the presence of CA or sodium citrate. However, the precise role of citrate ions still remains dubious. For instance, studies demonstrated that the citrate ions act as stabilizers in an efficient synthesis method for generating mono dispersed silver nanoprisms through illumination of visible light (Jin et al., 2001; 2003).

APPLICATIONS OF ZNO-CTS NPS

Antimicrobial activity

Qualitative antimicrobial assay

The results of qualitative antimicrobial assay using ZnO-CTS NPs is provided in Table 5.3.3. As evident from the inhibition zone diameter measurements, it can be inferred that the ZnO-CTS NPs were more effective towards *M. leteus* and *S. aureus* as compared to fungal strain *C. albicans*. Based on the qualitative antimicrobial assay results, *M. leteus*

and *S. aureus* were further selected for quantitative assay and biofilm inhibiting activity testing.

Quantitative antimicrobial assay

The antimicrobial activity of ZnO-CTS NPs against *M. luteus* and *S. aureus* is shown in Fig 5.3.5 and 5.3.6. The significant inhibition of growth was observed for *M. luteus* with OD (600 nm) of 0.502±0.093 units (with ZnO-CTS-CA NPs prepared through precipitation method and at a dose of 0.156 mg/ml) and 0.508±0.078 units (with ZnO-CTS NPs prepared through precipitation method and at a dose of 0.156 mg/ml) as compared to control with OD of 1.904±0.432 units. No significant (p>0.05) differences in *M. luteus* growth inhibition was observed with ZnO-CTS NPs prepared by 2 different methods, namely nanospray drying and precipitation method.

Significant growth inhibition of *S. aureus* was also observed. The final OD (600 nm) obtained after 24 h incubation time was 0.289±009 units (ZnO-CTS-CA NPs prepared by precipitation method) and OD of 0.423±0.05 units (with ZnO-CTS-whey powder NPs prepared through nano-spray drying method) as compared to control with OD of 1.578±0.342. ZnO-CTS CA NPs prepared by precipitation method showed promising results with nearly 82 % growth inhibition at concentration of 0.156 mg/ml. ZnO-CTS NPs prepared by 2 different methods, namely nanospray drying and precipitation method exert significant (p< 0.05) difference in growth inhibition of *S. aureus* with maximum inhibitory effect shown by ZnO-CTS-CA NPs prepared by precipitation method. Contrary to ZnO bulk powder and CTS powder, ZnO-CTS NPS showed maximum inhibition at lower concentrations (mg/ml) against both *M. luteus* and *S. aureus*. As the concentration of ZnO-CTS NPS was increased, lower growth inhibition was observed. The bulk ZnO and CTS powder showed maximum growth inhibition at higher concentrations of 5 mg/ml that was significantly lower as compared to ZnO NPs.

An important aspect of the use of ZnO as an antibacterial agent is its non-toxic nature towards human cells which is also the prerequisite for any antimicrobial agent (Nair et al., 2009). ZnO is currently being investigated as an antibacterial agent in both micro-scale and nano-scale formulations. Various studies have demonstrated that ZnO NPs showed antibacterial activity (Yamamoto, 2001; Adamas et al., 2006; Brayner et al., 2006; Colon et al., 2006; Jeng and Swanson, 2006; Yang and Xie, 2006; Reddy et al.,

2007; Zhang et al., 2007). The antimicrobial activities displayed by ZnO NPs were greater as compared to micro particles (Yamamoto, 2001), as also evident from this study. The exact mechanisms of the antibacterial action have not yet been clearly elucidated. However, studies advocated various mechanisms, such as the role of reactive oxygen species (ROS) generated on the surface of the particles (Sawai et al., 1997, 1998), zinc ion release (Yang and Xie, 2006), membrane dysfunction (Yang and Xie, 2006; Zhang et al., 2007), and NPs internalization (Brayner et al., 2006).

Antibiofilm activity

The biofilm inhibition activity of ZnO-CTS NPs against *M. luteus* and *S. aureus* is shown in Fig 5.3.7 and 5.3.8. ZnO-CTS-based NPs prepared through nano spray dying and precipitation methods showed significant biofilm inhibition activity. The results indicated that both in the case of *M. luteus* biofilm, maximum inhibition was achieved with ZnO-CTS-CA NPs and ZnO-CTS-glycerol NPs at a lower concentration of 0.156 mg/ml. Similarly, maximum *S. aureus* biofilm inhibition was obtained with ZnO-CTS-CA (prepared by precipitation method) NPs and ZnO-CTS-glycerol (prepared by nano spray drying method) NPs at a lower concentration of 0.156 mg/ml.

ZnO-CTS NPs showed higher biofilm inhibition activity against both bacterial strains as compared to micro particles of ZnO and CTS. Higher biofilm inhibition activity was achieved with lower concentration (mg/ml) of NPs as compared to micro particles (ZnO and CTS powder) which exerted higher activity at higher concentrations of 5 mg/ml. No significant differences with respect to *M. luteus* and *S aureus* biofilms inhibition were observed with NPs prepared by different methods.

M. luteus and *S. aureus* represent two of the most common opportunistic pathogens, due to their frequent incidence in the aetiology of the community-acquired and nosocomial infections and to their high rates of natural and acquired resistance to different antimicrobial agents including the widely used antibiotics. The clinical incidence of these bacterial strains is augmented owing to their ability to colonize various industrial settings and the cellular as well as the inert substrates, such as medical devices. Both of these strains are responsible for the formation of biofilms on medical devices and are associated with various chronic infections which are very hard to treat and often are responsible for severe outcome (Donlan and Costerton et al., 2002). Formation of

biofilms offers inherent resistance to antimicrobial agents which are the root of many persistent and chronic bacterial infections (Costerton et al., 1999). The genetic resistance of different microbial strains to various antimicrobial agents is increased when one microorganism is found growing in a biofilm (Davey and O'Toole, 2000). Antimicrobial resistance is a trait characteristic of most biofilm microorganisms and it has been speculated that biofilms are the causative agent of up to 65% of total bacterial infections (De Kievit et al., 2001). Biofilms are believed to become recalcitrant to antimicrobial attack through a number of different mechanisms. Giving the increasing risk of antimicrobial resistance, research is focusing on the discovery of compounds that inhibits the biofilm formation by various pathogenic bacterial strains.

In the present study, the inhibitory effect on biofilm could be explained by the potential inhibitory effect of the ZnO NPs on the bacterial exopolysaccharides secretion. Moreover, both ZnO and CTS showed antimicrobial properties. These results are accounting for the potential use of ZnO-CTS NPs in the prevention of the bacterial biofilm development on prosthetic devices, as well as for the design of new antiseptics and disinfectants with efficient protective action against bacterial colonization of tissue and inert surfaces. Further, different types of formulations can be developed for these NPs through encapsulation, powder and hydrogels to suit different application needs.

CONCLUSIONS

The study demonstrated the facile synthesis of ZnO-CTS nanoparticles stabilized with different organic compounds. The synthesis of NPs was carried out by two different methods: 1) nano spray drying and; 2) precipitation method. The size distribution analysis, UV-Vis spectrum and SEM monographs of ZnO-CTS NPs indicated that NPs prepared in the presence of stabilizers agglomerated less and dispersed better in water as compared to ZnO and ZnO-CTS NPs without stabilizers. Both the synthesis methods were simple and were devoid of any chemical usage. However, the ratios of ZnO, CTS and stabilizer needs to be further optimized for uniform size, shape and better dispersed NPs. The antimicrobial and antibiofilm of the fabricated ZnO-CTS NPs activity was examined. In this study, the antimicrobial activity and the potential inhibitory effect of the NPs was tested on the ability of *M. luteus* and *S. aureus* clinical strains to colonize the inert substratum, quantified by the biofilm inhibition activity. The study indicated the promising potential of ZnO-CTS NPs as antimicrobial and antibiofilm agents.

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Table 5.3. 1 Different physico-chemical parameters of ZnO-CTS solution supplemented with different compounds

Exp.	Treatment	pH (±0.1)	Viscosity (mPa.s)
1	ZnO (nano) Ctrl	5.60	-
2	ZnO + CTS	6.00	1.02
3	ZnO +CTS + CA	3.92	4.48
4	ZnO + CTS + glycerol	4.95	1.16
5	ZnO + CTS + starch soluble	5.38	1.12
6	ZnO + CTS + whey powder	5.55	1.06

Quantity of different components used for NPs synthesis: CA (1%); CTS (1%); glycerol (0.5%); starch soluble (0.5 %); whey powder (0.5 %); ZnO (0.75%)

All the NPs were prepared in 1% (v/v) acetic acids.

Table 5.3. 2 The mean size and size distribution measurements of the different ZnO-CTS NPs

Sample	Z-average (nm)	PDI	Peak 1	Intensity (%)	Peak 2	Intensity (%)	Peak 3	Intensity (%)
Nano spray drying method								
ZnO (nano) Ctrl	215.4	0.540	186.4	76.2	228.3	23.0	-	-
ZnO + CTS	167.2	0.639	148.9	92.5	268.4	7.5	-	-
ZnO + CTS + glycerol	402.5	0.892	1196.0	85%	9.289	14.4	-	-
ZnO + CTS + starch soluble	93.2	0.372	121.1	93.3	215.5	6.7	-	-
ZnO +CTS +whey powder	98.43	0.296	139.2	100%	-	-		
Precipitation method								
ZnO (nano) Ctrl	603.1	0.891	156.9	83.8	32.27	16.2	-	-
ZnO + CTS	358.7	0.569	278.4	69.7	43.22	24.6	5.647	5.7
ZnO +CTS + CA	99.28	0.639	110.28	52.8	62.20	46.5	2.27	0.6
ZnO + CTS + glycerol	162.7	0.424	217.2	72.1	29.47	17.2	8.344	9.4
ZnO + CTS + starch soluble	285.1	0.535	344.2	65.4	55.5	17.0	13.95	10.5
ZnO +CTS +whey powder	166.5	0.539	285.5	93.1	104.1	6.9	-	-

Abbreviations: CA-citric acid; CTS- chitosan; PDI-poly dispersity index

Table 5.3. 3 Qualitative antimicrobial assay using agar diffusion method

Tested samples	**Inhibition zone diameter (mm)**		
(5mg/ml)	***C. albicans***	***M. luteus***	***S. aureus***
Control			
ZnO Bulk powder	< 0.10	< 0.10	< 0.10
CTS	0.10	0.15	0.10
NPs synthesized by nano spray drying technique			
ZnO nano	0.15	0.20	0.20
ZnO-CTS	0.2	0.20	0.25
ZnO-CTS-glycerol	0.2	0.30	0.30
ZnO-CTS-starch	0.2	0.25	0.35
ZnO-CTS-whey powder	0.2	0.30	0.25
NPs synthesized by precipitation technique			
ZnO nano	1.5	2.0	0.25
ZnO-CTS	2.0	2.5	0.30
ZnO-CTS-CA	2.0	0.40	0.45
ZnO-CTS-glycerol	1.5	0.25	0.25
ZnO-CTS-starch	<2.0	0.25	0.30
ZnO-CTS-whey powder	2.0	0.25	0.25

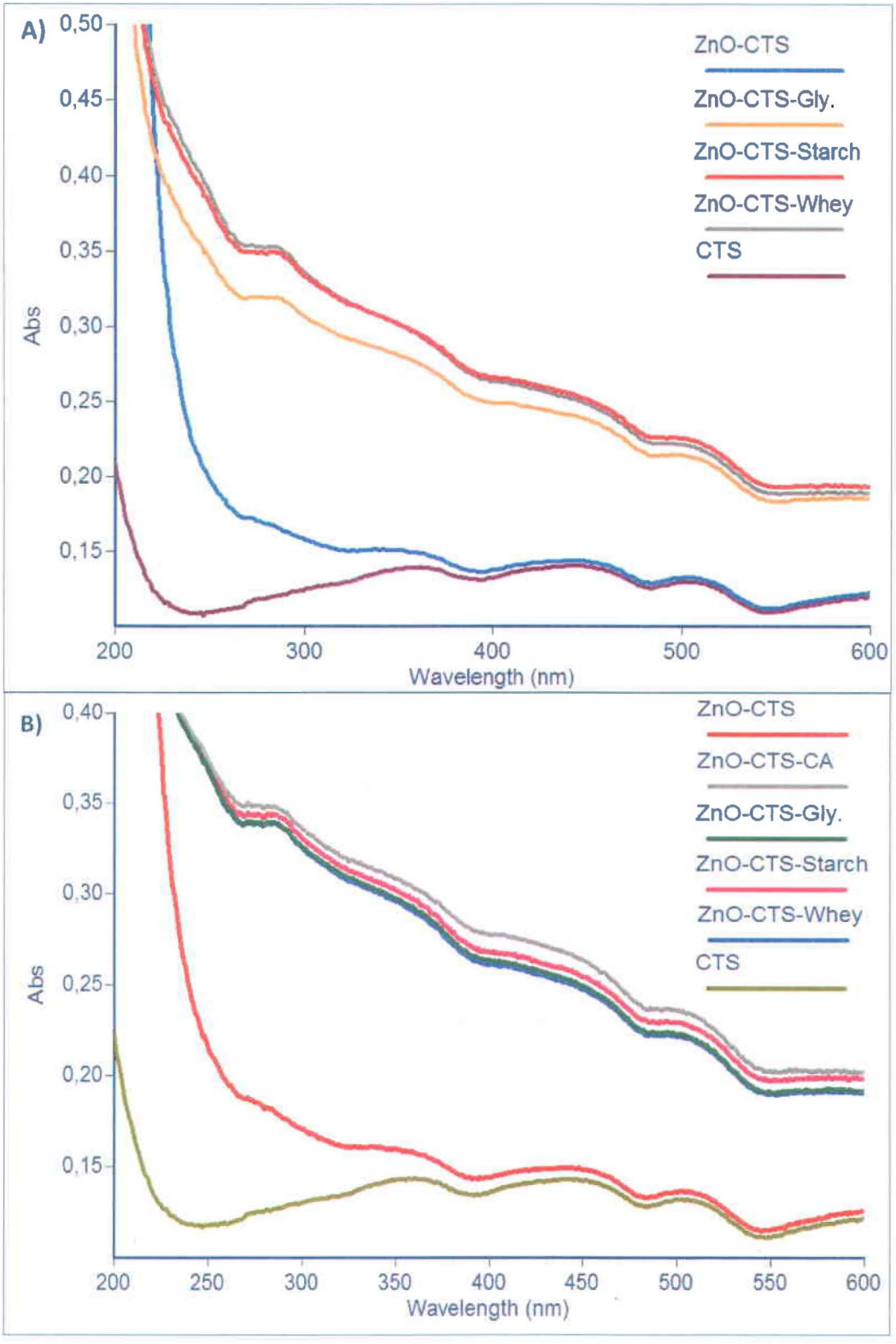


Figure 5.3. 1 UV-Vis spectra of the different ZnO-CTS NPs synthesized by: A) nano spray drying and; B) precipitation method

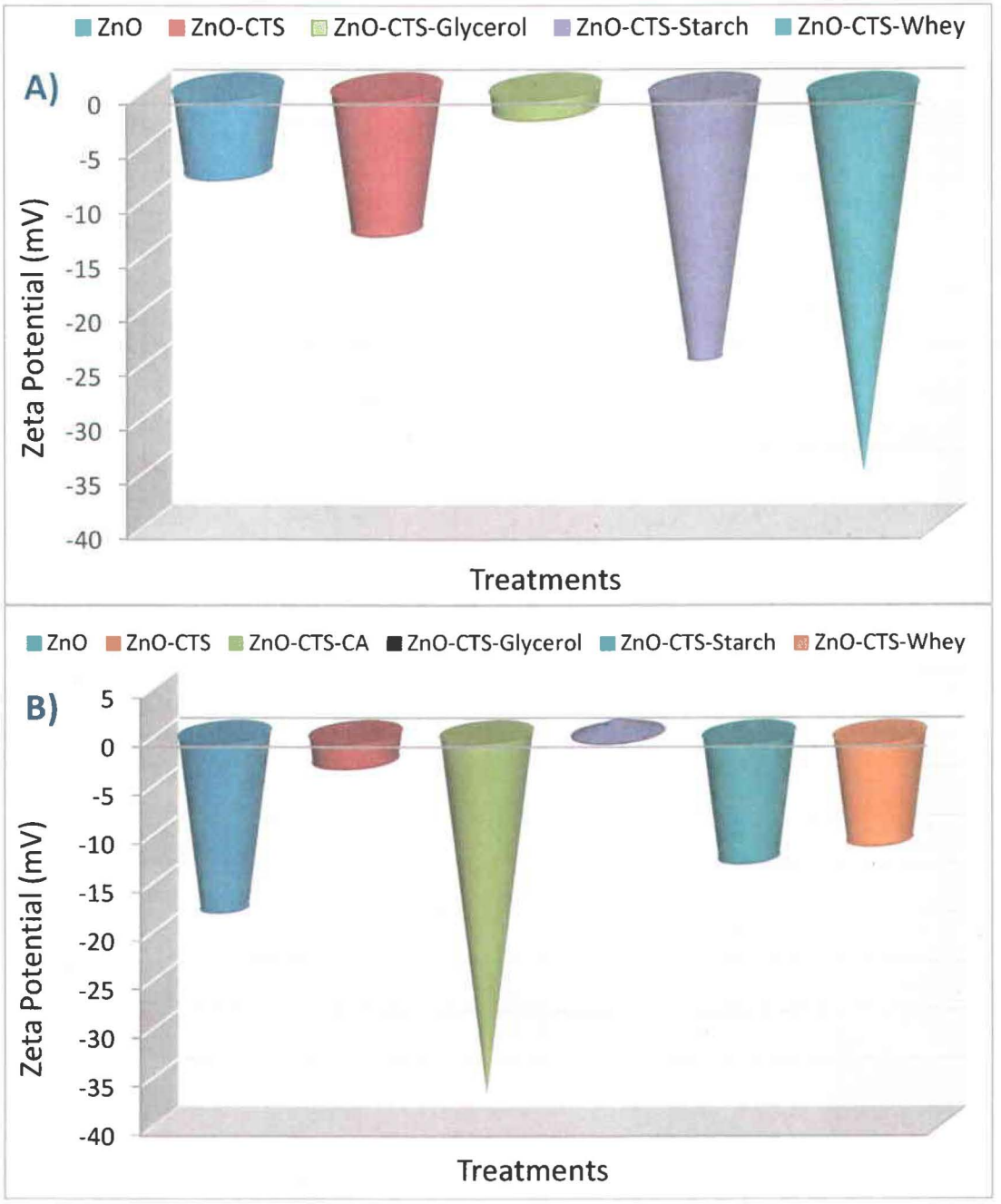


Figure 5.3. 2 Zeta potential measurements of different ZnO-CTS NPs prepared by: A) nano spray drying and; B) precipitation method

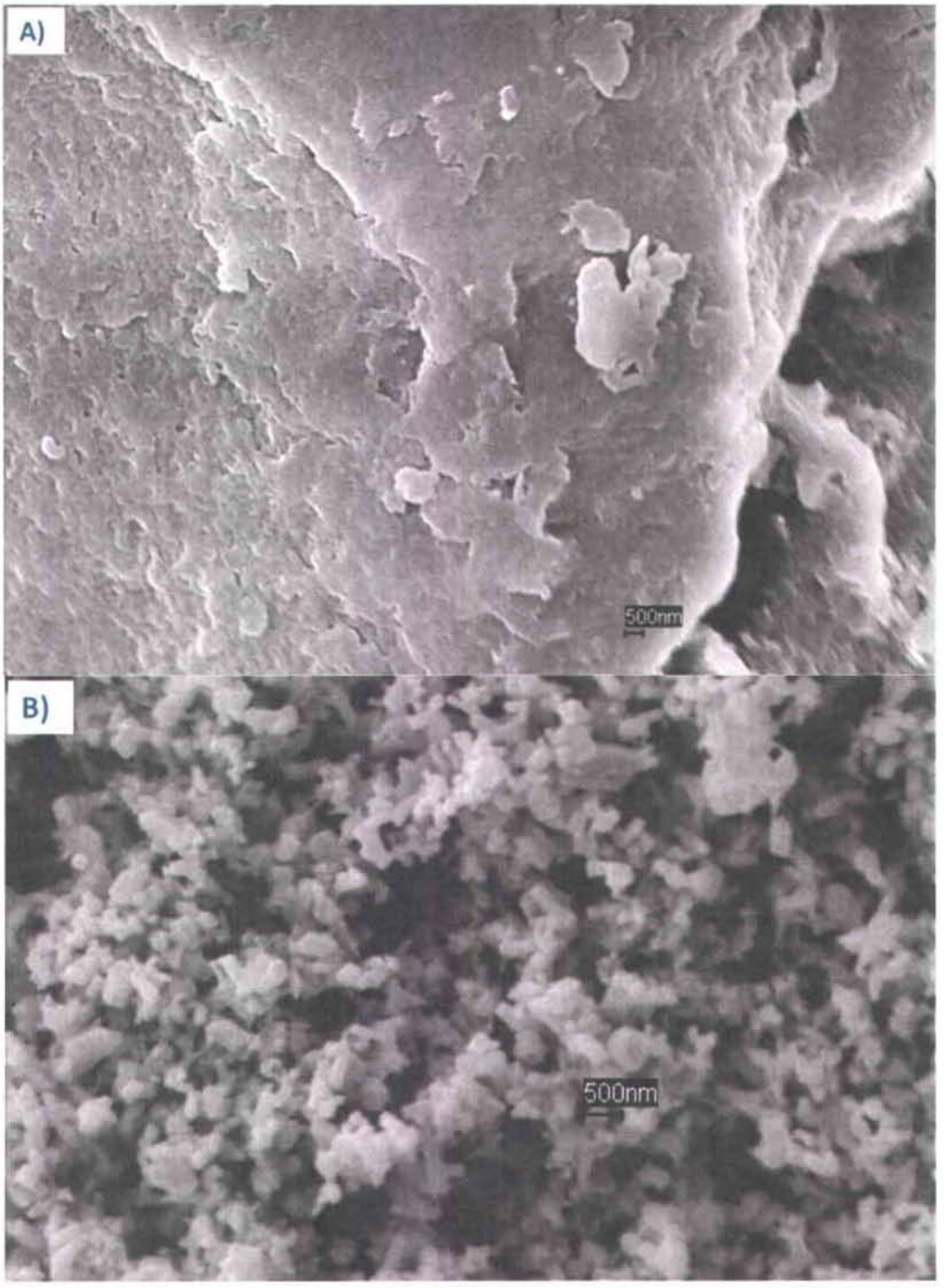


Figure 3

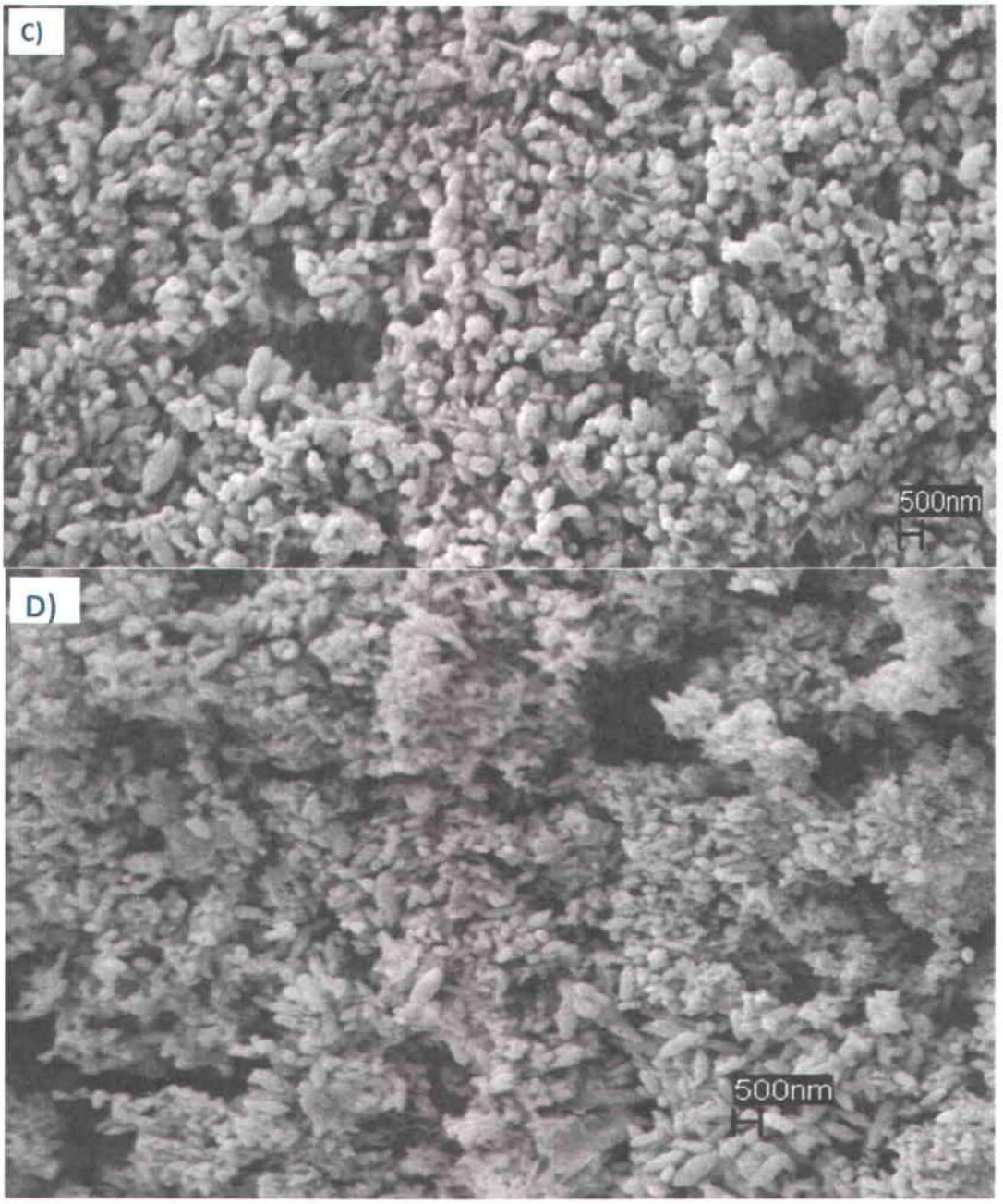


Figure 3

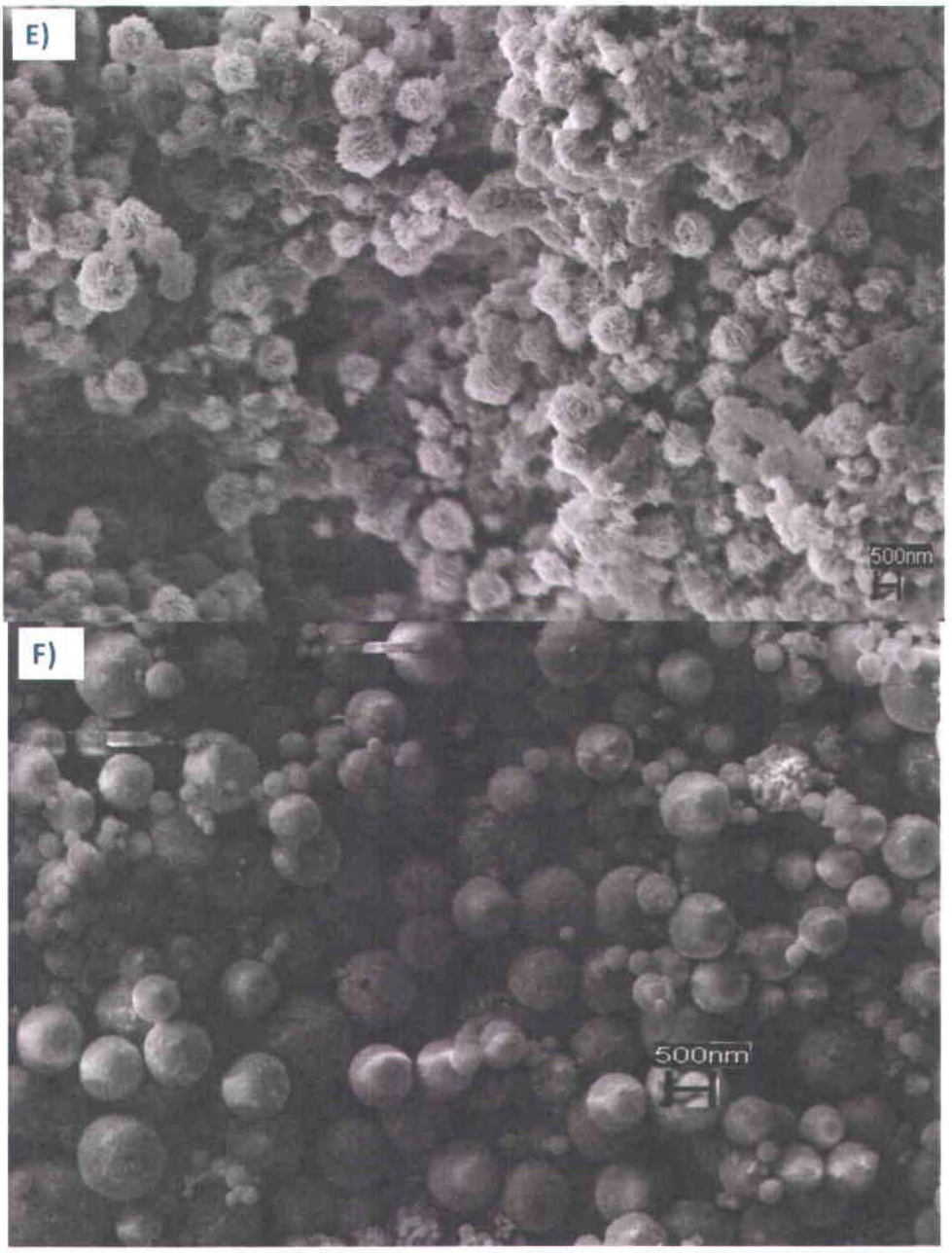


Figure 5.3. 3 SEM micrographs of: (a) chitosan (powder) and different NPs prepared through nano spray drying process: (b) ZnO (nano); (c) ZnO-CTS; (d) ZnO-CTS-glycerol; (e) ZnO-CTS-starch and; (f) ZnO-CTS-whey powder

All the SEM images were taken at 10 K magnification.

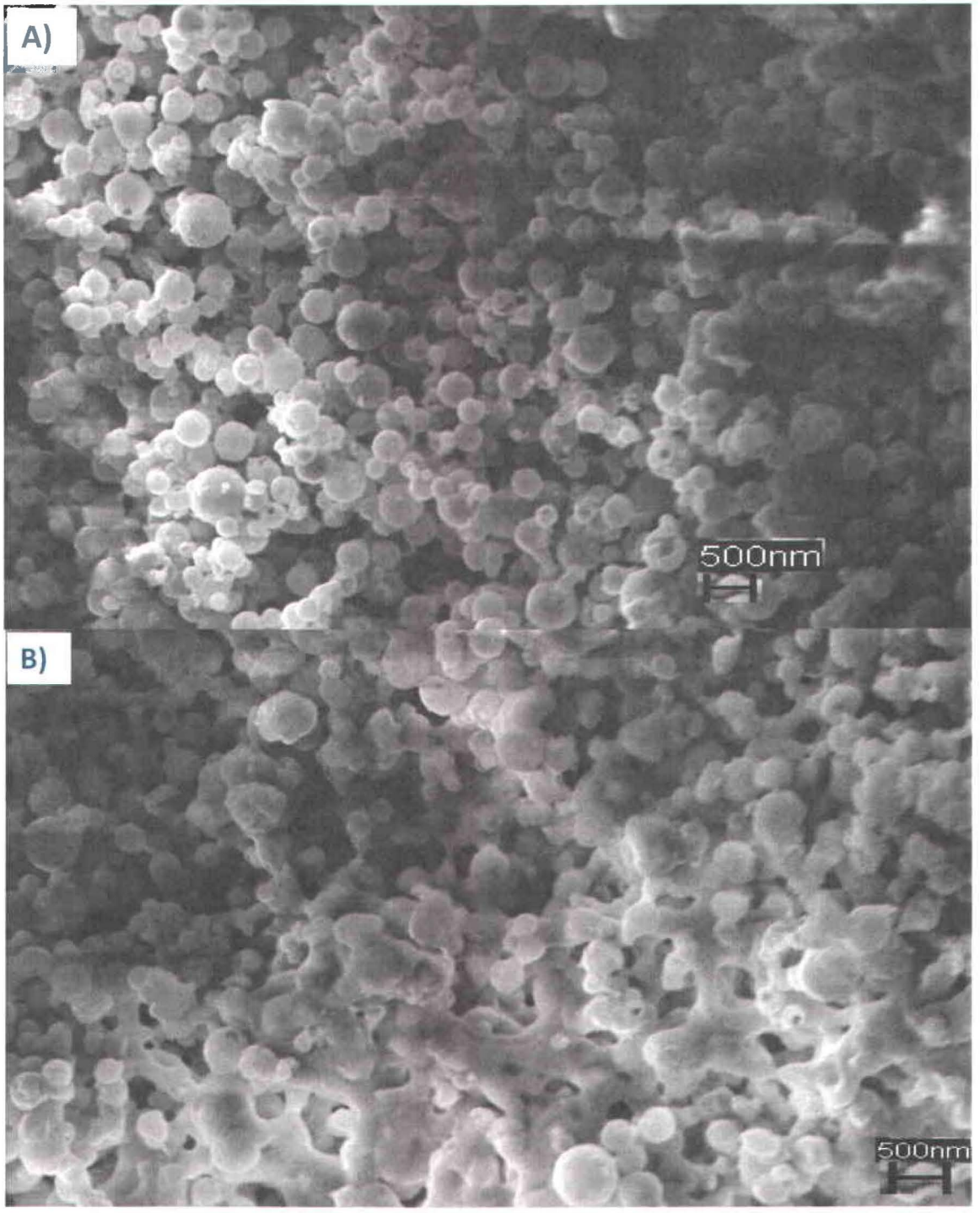


Figure 4

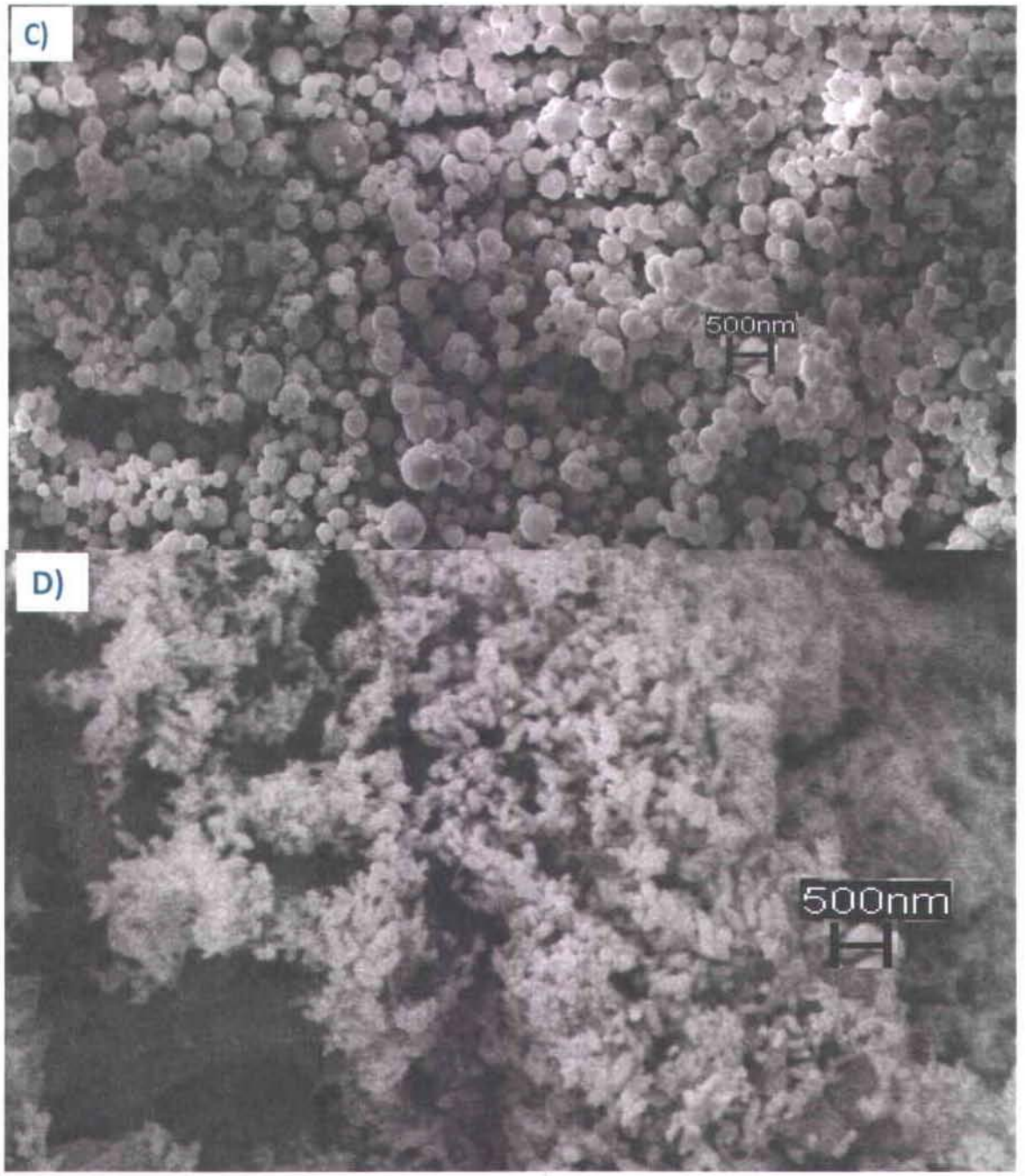


Figure 4

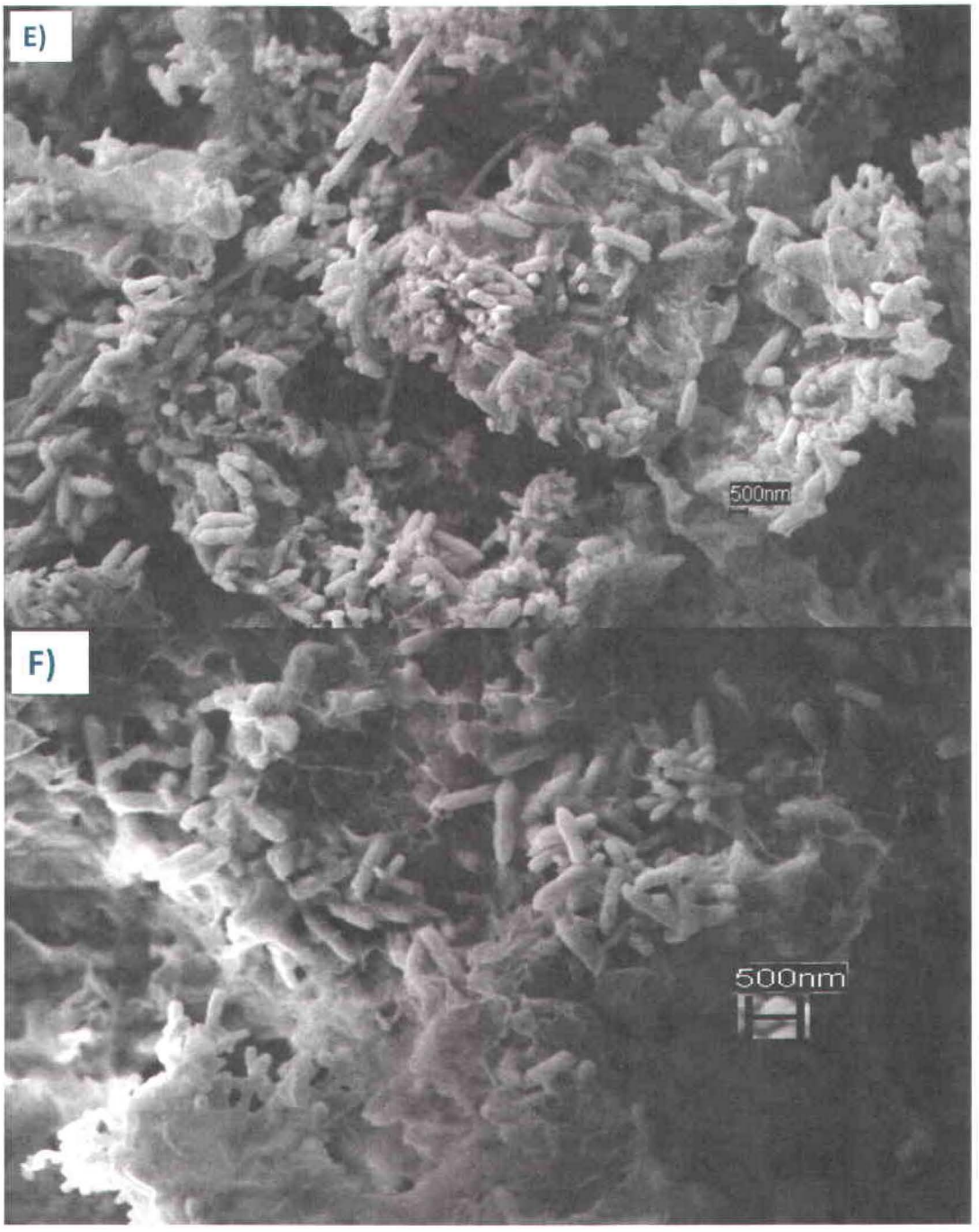


Figure 5.3. 4 SEM micrographs of different NPs prepared through chemical method: (a) ZnO (nano); (b) ZnO-CTS; (c) ZnO-CTS-CA; (d) ZnO-CTS-glycerol; (e) ZnO-CTS-starch and; (f) ZnO-CTS-whey powder

All the SEM images were taken at 10 K magnification.

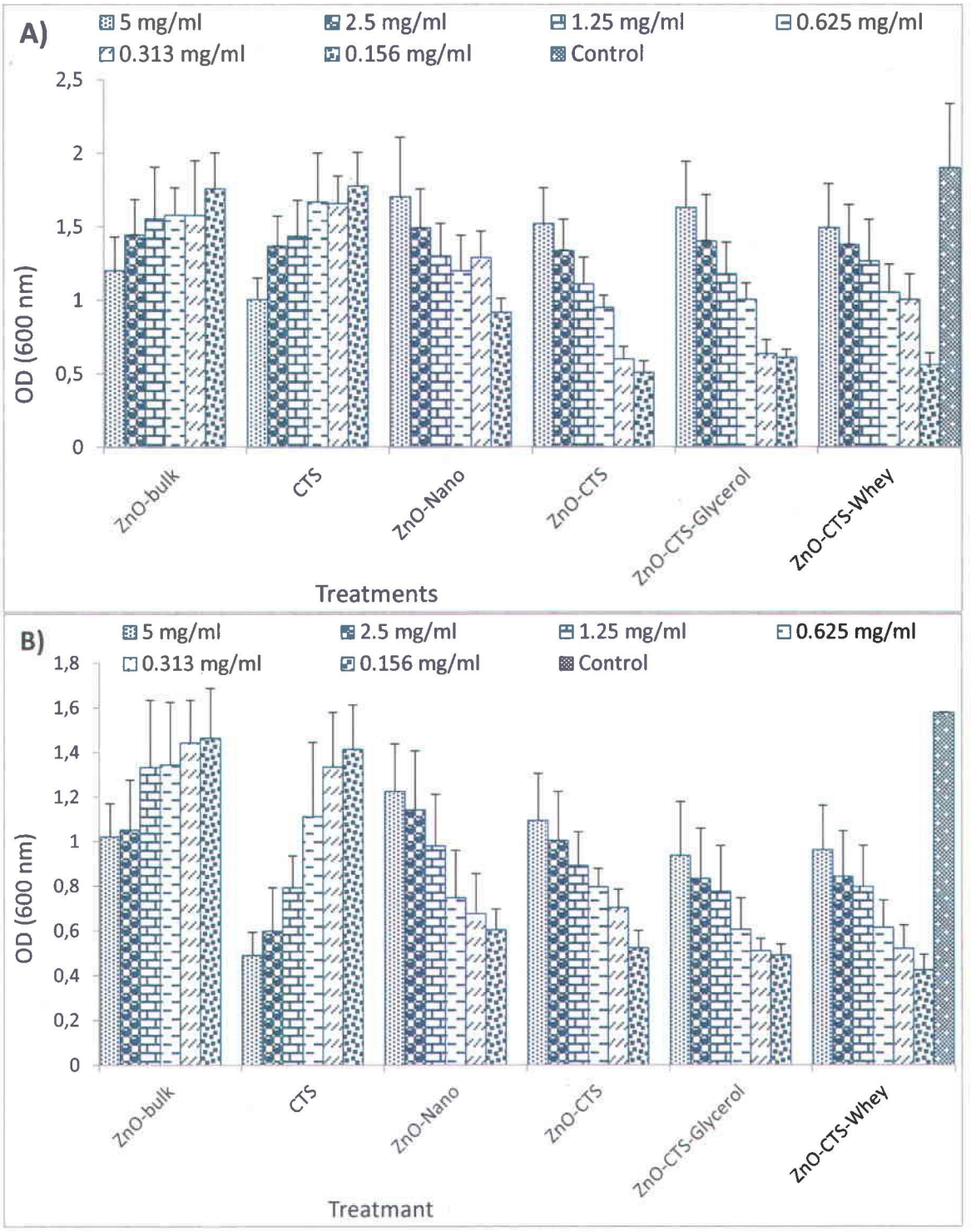


Figure 5.3. 5 Antimicrobial activity of ZnO-CTS-based NPs prepared by nanospray drying method against: (A) *Micrococcus luteus* and; (B) *Staphylococcus aureus*

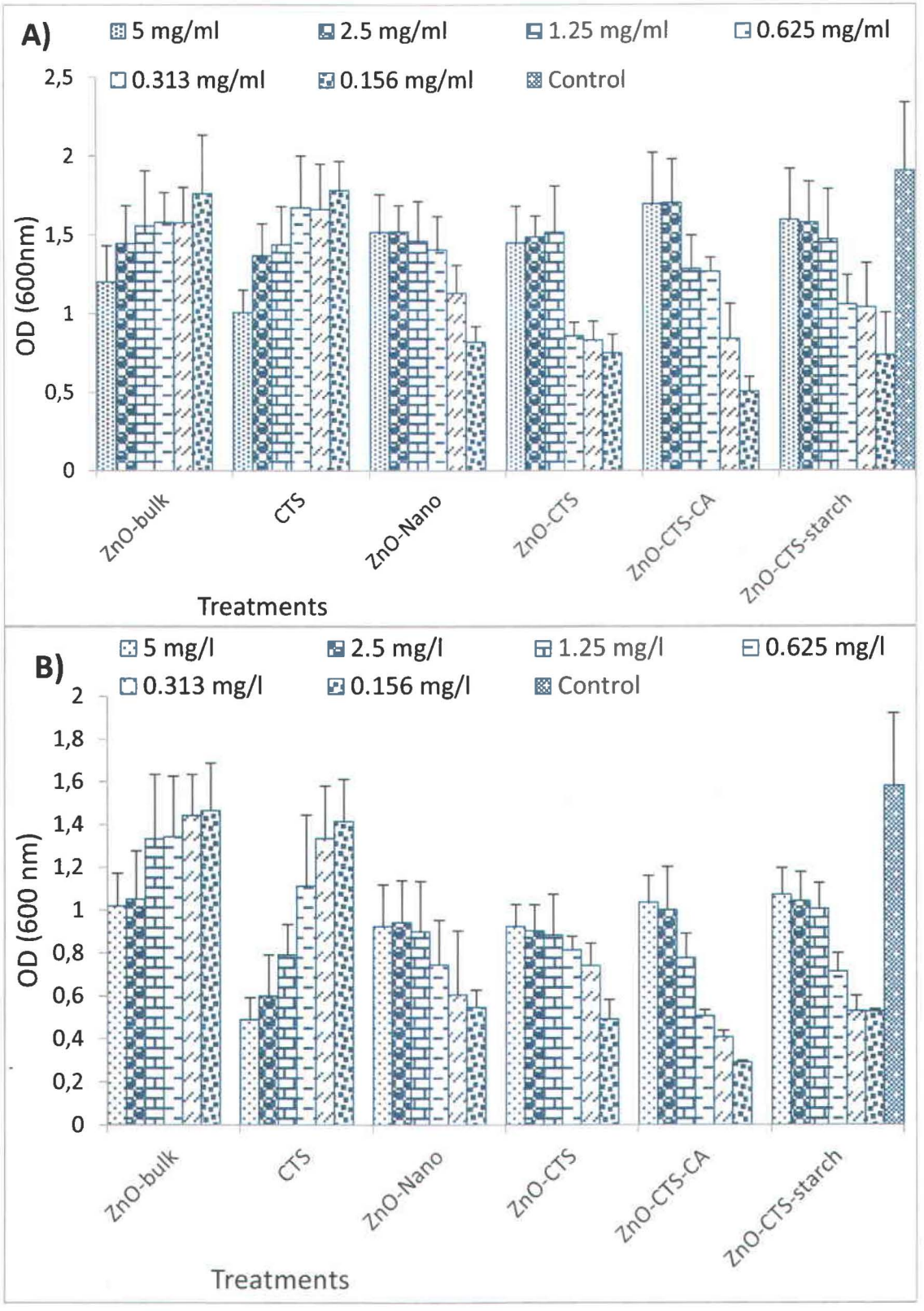


Figure 5.3. 6 Antimicrobial activity of ZnO-CTS-based NPs prepared by precipitation method against: (A) *Micrococcus luteus* and; (B) *Staphylococcus aureus*

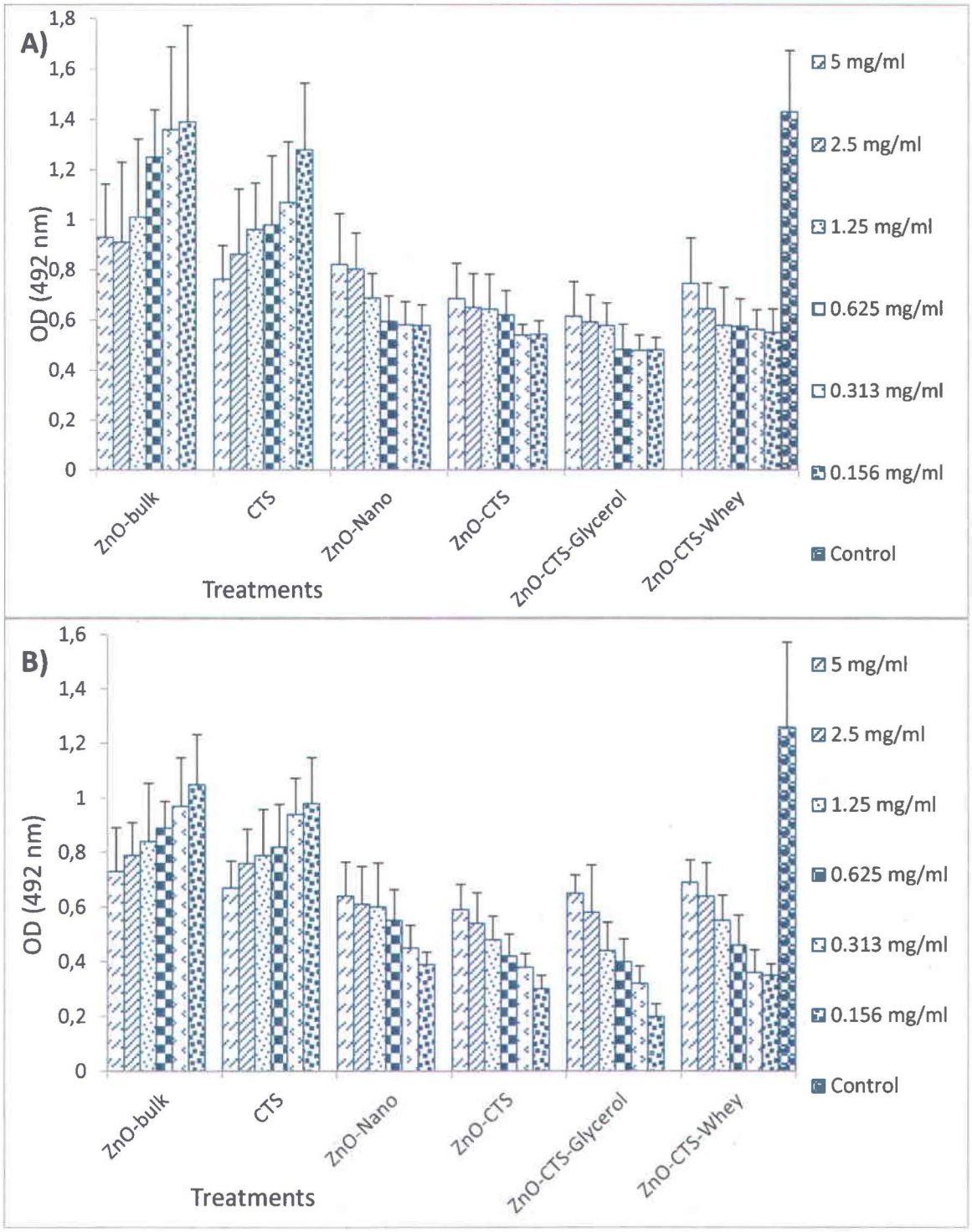


Figure 5.3. 7 Biofilm inhibition activity of ZnO-CTS-based NPs prepared by nanospray drying method against: (A) *Micrococcus luteus* and; (B) *Staphylococcus aureus*

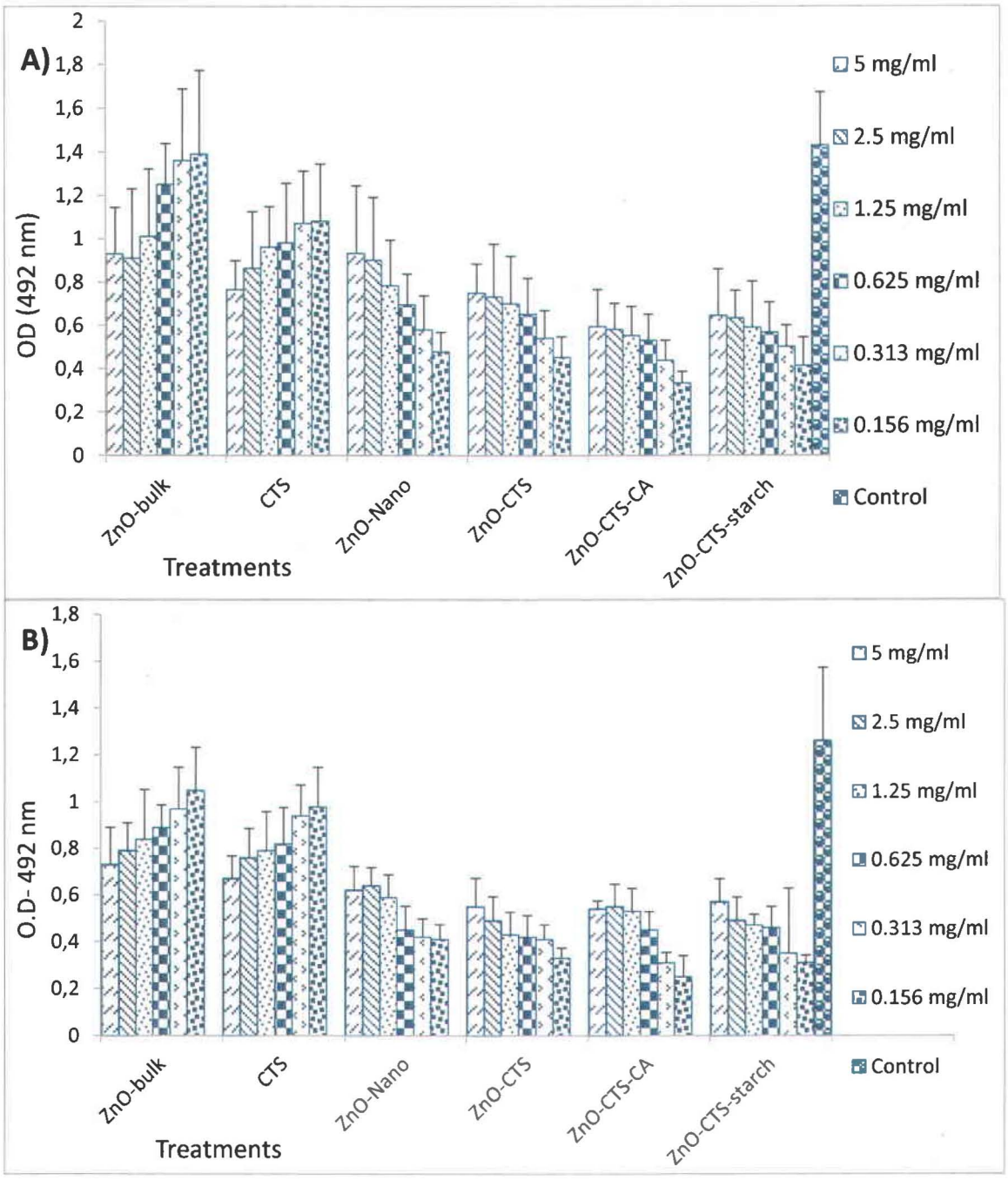


Figure 5.3. 8 Biofilm inhibition of ZnO-CTS-based NPs prepared by precipitation method against: (A) *Micrococcus luteus* and; (B) *Staphylococcus aureus*

PARTIE 6. CONCLUSIONS ET RECOMMENDATIONS

CONCLUSIONS

From the research work accomplished as part of the doctoral research, following conclusions can be drawn:

[1]. The screening studies demonstrated the potential of AP for CA production through SSF by *A. niger* NRRL 567 as compared to other substrates and with *A. niger* NRRL 2001. Higher CA production of 127.9±4.3 g/kg and 115.8±3.8 g/kg of dry substrate, respectively using AP supplemented with 3% EtOH (v/w) and 4% MeOH (v/w).

[2]. Apple pomace ultrafiltration sludge proved to be the best substrate in submerged CA fermentation by *A. niger* NRRL 567 of the different liquid agro-industrial wastes chosen. CA production of 18.2±0.4 g/l and 13.9±0.4 g/l of APS were attained after addition of 3% EtOH (v/v) and 4% MeOH (v/v).

[3]. The response surface optimization of SS concentration and inducer concentration resulted in higher CA production of 132.31±4.06 g/kg (96 h), 303.34±9.67 g/kg (120 h) and 187.96±7.69 g/kg (120 h) of dry AP with 75% (v/w) initial moisture in control and along with two inducers [3% (v/w) MeOH and 3% (v/w) EtOH] through solid-state tray by *A. niger* NRRL 567. The fermentation efficiency of 82.89 % and 50.80 %, respectively was achieved with MeOH and EtOH as inducers depending upon total carbon utilization after 120 h incubation period.

[4]. The fermentation parameter optimization in SmF through RSM resulted in 44.9g/100 g and 37.9 g/100 g SS, respectively using APS having 25 g/l SS concentration and inducers (3% (v/v) MeOH and EtOH) in flasks.

[5]. Higher citric acid bioproduction (220.6 ± 13.9 g/kg dry solids, DS) were achieved with optimum conditions of 3% (v/v) methanol, intermittent agitation of 1 h after every 12 h at 2 rpm and 1 vvm of aeration rate and 120 h incubation time through solid-state CA fermentation in a 12-L rotating drum type bioreactor. Further through extraction

optimization, CA extraction of 294.19 g/kg DS was achieved at optimum conditions: extraction time of 20 min, agitation rate of 200 rpm and extractant volume of 15 ml.

[6]. Scale up of the submerged CA fermentation of the screened liquid substrate (APS) based on RSM in 7.5 L capacity fermentor with 4.5 L working volume resulted in 23.67±1.0 g/l (120 h) and 40.34±1.98 g/l APS (132 h), respectively in control and 3% MeOH (v/v) by *A. niger* NRRL 567.

[7]. Co-product chitosan was found to be higher in control with 6.40% and 5.13% of dried biomass, respectively with the fungal mycelium resulting from the SSF and SmF. However, the extractable CTS was found to be lower in the treatments with inducers. Degree of deacetylation of the CTS was ranged from 78-86 % for fungal biomass obtained from SSF and SmF with different inducers. The viscosity of fungal CTS was found to be ranging from 1.02-1.18 $Pa.s^{-1}$ which is considerably lower than the commercial crab shell CTS.

[8]. The applications of different waste BMs resulting during the CA and CTS extraction provedpromising for the biosorption of toxic metals (As, Cr and Cu) from waste CCA wood leachates. Langmuir isotherm gave the best fit with correlation coefficients (R^2) value of three different metals (SSF biomass) ranging from 0.89-0.97; 0.96-0.99 and 0.76-0.95 for As, Cr and Cu, respectively. Similarly, the significant removal of metals (> 60% in leachate 2) from waste CCA wood leachate was achieved.

[9]. ZnO-CTS-based NPs stabilized with different organic compounds showed promising antimicrobial and biofilm inhibition potential against pathogenic bacteria, *Micrococcus luteus* and *Staphylococcus aureus*.

RECOMMENDATIONS

Future research on value addition of apple industry waste for citric acid production could advantageously consider the following recommendations:

[1]. Apple industry wastes could be used as promising negative cost substrates for citric acid production.

[2]. Solid-state tray fermentation proved to be a simple and cheap alternative for small scale production of citric acid from AP

[3]. Citric acid production economics could be improved through the integrated process of extraction of co-product chitosan from waste fungal mycelium.

[4]. Different waste streams resulting through this integrated process can be used as potential biosorbents for removal of metalsfrom contaminated wastewaters.

[5]. Citric acid production process could be scaled up using apple pomace sludge and examine the feasibility of feasibility of integrated process of chitosan extraction from waste fungal mycelium.

ANNEXES

ANNEXE I

Données: Utilization of different agro-industrial wastes for sustainable bioproduction of citric acid by *Aspergillus niger*

Table 1 Apple production and estimated waste generation in Canada and worldwide

Producer	**Apple product (tonnes)**	**Production/year**	**Estimated production of waste (tonnes) (25-30% AP and 5-10 % APS)**
Canada	455, 361	2009	113,84 to 136, 61 (AP) 22,768 to 45,536 (APS)
Québec	116, 088	2009	(29, 022 to 34, 826) (AP) 5,804 to 11,609 (APS)
World	69, 603,640	2008	17, 400,910 to 20, 881, 092 (AP) 3, 480, 182 to 6, 960, 364 (APS)

Figure 1 A & B Viability of fungus cultivated on wastes (apple pomace, brewery spent grain, citrus waste and sphagnum peat moss) through solid-state fermentation by (A) *Aspergilllus niger* NRRL 567; (B) *Aspergilllus niger* NRRL 2001.

Substrates	**Fermentation time (h)**					
	24	**48**	**72**	**96**	**120**	**144**
A)	***Aspergilllus niger* NRRL 567**					
AP	1.35E+07	2.30E+07	1.30E+08	1.55E+08	1.60E+08	1.52E+08
BSG	5.00E+06	1.83E+07	9.30E+07	2.08E+08	3.94E+08	3.81E+08
CW	5.50E+06	5.00E+06	4.50E+06	4.75E+06	4.75E+06	5.75E+06
SPM	5.00E+06	4.00E+06	3.75E+06	4.00E+06	4.75E+06	6.50E+06
B)	***Aspergilllus niger* NRRL 2001**					
AP	6.75E+06	1.05E+07	2.25E+07	4.18E+07	9.00E+07	8.70E+07
BSG	5.25E+06	1.23E+07	1.04E+08	3.02E+08	2.63E+08	2.44E+08
CW	5.50E+06	4.75E+06	4.50E+06	4.75E+06	5.25E+06	6.00E+06
SPM	5.25E+06	5.00E+06	4.75E+06	5.00E+06	5.00E+06	700E+06

Abbreviations: AP-apple pomace; BSG-brewery spent grain; cfu-colony forming units; CW- citrus waste; SPM-sphagnum peat moss

Figure 2 A & B. Viability of fungus cultivated on wastes (apple pomace ultrafiltration sludge 1 and 2, municipal secondary sludge, starch industry water and lactoserum)) through submerged fermentation by (A) *Aspergilllus niger* NRRL 567; (B) *Aspergilllus niger* NRRL 2001

Substrates	**Fermentation time (h)**					
	24	**48**	**72**	**96**	**120**	**144**
A) *A. niger* 567						
APS 1	1.25E+05	1.25E+05	1.25E+05	3.13E+05	1.19E+06	1.69E+06
APS 2	1.88E+05	1.25E+05	6.25E+04	3.75E+05	1.38E+06	1.75E+06
MSS	1.25E+05	1.88E+05	1.25E+05	2.50E+05	1.00E+06	1.88E+06
SIW	1.25E+05	1.25E+05	1.88E+05	3.75E+05	1.38E+06	2.00E+06
LS	1.88E+05	1.88E+05	1.88E+05	3.13E+05	1.56E+06	2.06E+06
B) *A. niger* 2001						
APS 1	1.88E+05	1.25E+05	1.25E+05	4.38E+05	1.38E+06	1.75E+06
APS 2	1.25E+05	6.25E+04	1.25E+05	5.00E+05	1.56E+06	2.06E+06
MSS	1.88E+05	1.25E+05	6.25E+04	3.13E+05	1.38E+06	1.94E+06
SIW	1.88E+05	1.25E+05	1.25E+05	3.13E+05	1.81E+06	2.19E+06
LS	1.88E+05	1.25E+05	2.50E+05	3.75E+05	2.00E+06	2.31E+06

Abbreviations: APS 1- apple pomace ultrafiltration sludge; APS 2- apple pomace ultrafiltration sludge; cfu-colony forming units; SIW-starch industry water; LS-lactoserum; MSS- municipal secondary sludge

Figure 3 (A) Solid-state CA fermentation using AP and; (B) submerged CA fermentation using APS-1 by *Aspergilllus niger* NRRL 567

A)	Substrates	Fermentation time (h)					
		24	48	72	96	120	144
1	AP- control	27.20±1.03	43.23±1.97	67.31±2.34	90.23±3.32	76.20±2.65	64.59±1.97
2	AP-MeOH 3%	25.30±1.10	47.31±1.78	70.99±2.38	97.20±3.45	80.18±2.69	72.36±2.09
3	AP-MeOH 4%	25.79±0.97	50.81±1.8	74.51±2.57	115.77±3.89	96.90±2.30	74.70±2.38
4	AP-EtOH 3%	26.77±1.21	55.62±1.92	78.04±2.21	127.94±4.34	109.21±4.29	79.11±2.51
5	AP-EtOH 4%	24.56±0.97	47.99±1.34	68.96±2.45	97.82±2.79	80.25±2.67	67.00±2.81
6	AP-MeOH+EtOH*	25.60±1.08	44.77±1.78	62.86±2.33	91.77±2.58	62.55±2.34	45.61±1.98
7	SPM- control	11.65±0.89	24.99±1.23	35.80±1.76	45.57±2.59	35.71±2.07	28.95±1.35
8	SPM-MeOH 4%	10.84±1.09	28.93±1.20	40.54±1.53	58.63±2.89	38.28±2.34	31.02±1.31

B)	Substrates	Fermentation time (h)						
		24	48	72	96	120	144	168
1	APS 1- control	4.95±0.25	6.03±0.27	7.97±0.29	8.33±0.27	9.39±0.30	7.68±0.36	5.28±0.20
2	APS 1-MeOH 3%	4.78±0.21	6.15±0.27	7.19±0.28	7.99±0.29	10.74±0.38	12.38±0.39	8.57±0.24
3	APS 1-MeOH 4%	4.85±0.29	6.81±0.27	7.31±0.37	8.36±0.32	11.38±0.37	13.94±0.37	10.10±0.27
4	APS 1-EtOH 3%	4.85±0.27	6.08±0.25	7.21±0.36	11.03±0.38	15.75±0.40	18.23±0.43	16.12±0.35
5	APS 1-EtOH 4%	4.70±0.27	6.06±0.28	7.12±0.38	9.81±0.36	14.50±0.41	14.57±0.34	11.01±0.30
6	APS 1-MeOH+EtOH*	4.31±0.19	5.50±0.20	6.53±0.37	7.67±0.33	9.67±0.33	7.82±0.39	4.80±0.22

Abbreviations: AP, apple pomace; APS-1, apple pomace ultrafiltration sludge; CA, citric acid; SPM-sphagnum peat moss

*In treatment with combined effect of inducers, 2% (v/v) EtOH and MeOH was added.

Figure 4 Viability of *Aspergilllus niger* NRRL 567 cultivated on **(A)** apple pomace and sphagnum peat moss through solid-state fermentation **(B)** apple pomace ultrafiltration sludge 1 through submerged fermentation

A)		Fermentation time (h)						
S. No	**Substrates**	**12**	**24**	**48**	**72**	**96**	**120**	**144**
1	AP- control	8.44E+06	2.63E+06	2.63E+06	1.76E+07	2.74E+07	4.65E+07	4.89E+07
2	AP-MeOH 3%	3.13E+06	1.13E+06	3.75E+05	1.50E+06	2.25E+06	2.44E+06	2.44E+06
3	AP-MeOH 4%	3.44E+06	7.50E+05	3.75E+05	2.25E+06	2.06E+06	2.44E+06	2.06E+06
4	AP-EtOH 3%	2.19E+06	5.63E+05	5.63E+05	1.69E+06	1.88E+06	2.81E+06	3.00E+06
5	AP-EtOH 4%	1.88E+06	7.50E+05	1.88E+05	1.69E+06	2.06E+06	2.25E+06	2.44E+06
6	AP-MeOH+EtOH*	1.56E+06	5.63E+05	3.75E+05	2.25E+06	2.44E+06	2.81E+06	3.38E+06
7	SPM- control	2.50E+06	1.13E+06	1.88E+06	3.39E+07	4.33E+07	5.14E+07	7.69E+07
8	SPM-MeOH 4%	2.19E+06	9.38E+05	7.50E+05	2.25E+06	2.44E+06	3.00E+06	3.56E+06

B)		Fermentation time (h)							
S. No	**Substrates**	**12**	**24**	**48**	**72**	**96**	**120**	**144**	**168**
1	APS 1- control	3.75E+05	3.13E+05	1.25E+05	2.50E+05	5.00E+05	8.13E+05	9.38E+05	1.13E+06
2	APS 1-MeOH 3%	3.13E+05	2.50E+05	1.88E+05	1.88E+05	2.50E+05	3.13E+05	4.38E+05	5.00E+05
3	APS 1-MeOH 4%	4.38E+05	1.88E+05	1.25E+05	1.25E+05	1.88E+05	2.50E+05	3.13E+05	4.38E+05
4	APS 1-EtOH 3%	3.75E+05	2.50E+05	1.88E+05	1.25E+05	1.88E+05	2.50E+05	3.13E+05	3.75E+05
5	APS 1-EtOH 4%	3.75E+05	2.50E+05	6.25E+04	1.88E+05	2.50E+05	3.75E+05	4.38E+05	5.00E+05
6	APS 1-MeOH+EtOH*	3.13E+05	1.88E+05	1.88E+05	2.50E+05	4.38E+05	6.88E+05	8.13E+05	1.00E+06

Abbreviations: AP- apple pomace; SPM- sphagnum peat moss; APS 1- apple pomace ultrafiltration sludge; cfu-colony forming units;

*In treatment with combined methanol and ethanol, 2% (v/w) of each was added

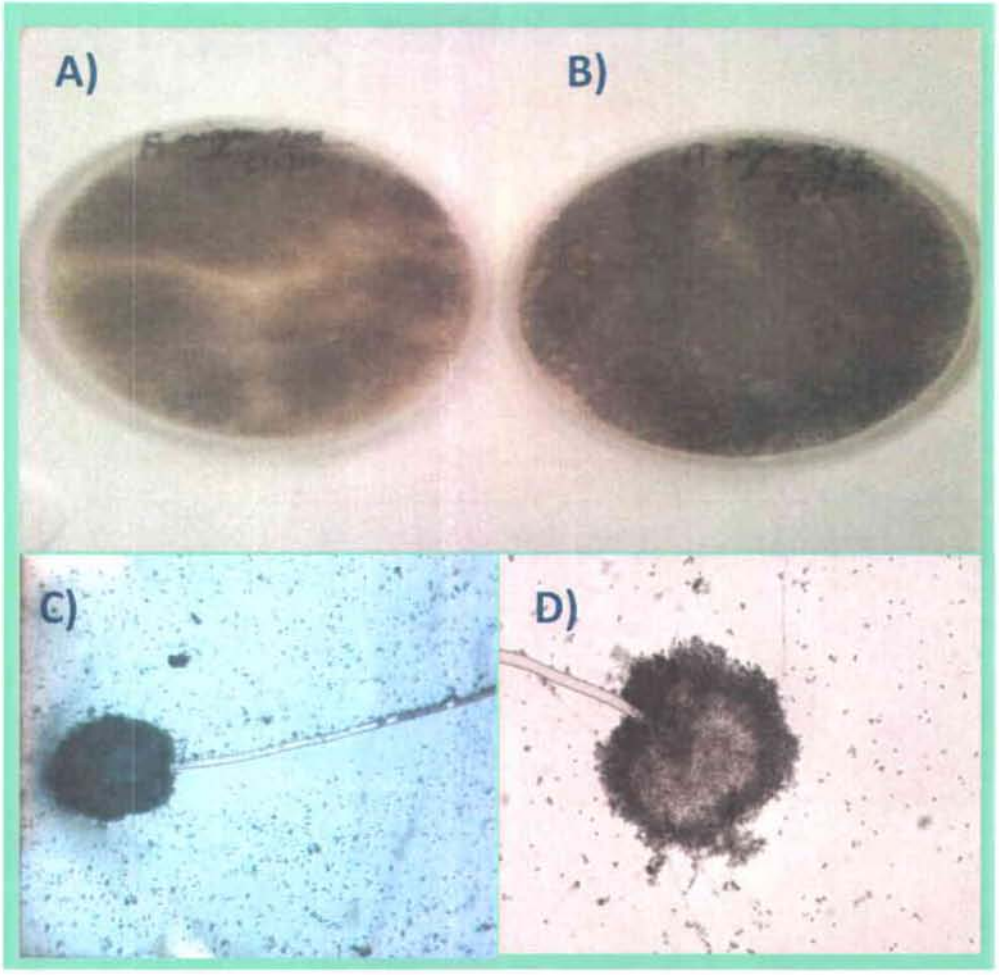


Figure *Aspergillus niger* grown on potato dextrose agar plates: (A) *A. niger* NRRL 2001; (B) *A. niger* NRRL 567 and (C & D) their hyphae with spores.

ANNEXE II

Données: Enhanced solid-state citric acid bioproduction using apple pomace waste through response surface methodology

Figure 4 Viability of *Aspergilllus niger* NRRL 567 cultivated on apple pomace through solid-state fermentation supplemented with inducer: A) ethanol and; B) methanol

A)	Moisture (%)	EtOH %	Fermentation time (h)						
S.No.			24	48	72	96	120	144	168
1	70.00	2.00	3.75E+06	9.38E+05	2.44E+06	1.88E+07	7.11E+07	1.38E+08	2.40E+08
2	70.00	4.00	5.81E+06	1.13E+06	7.50E+05	2.06E+06	3.38E+06	8.81E+06	3.34E+07
3	70.00	2.00	4.69E+06	9.38E+05	1.31E+06	5.25E+06	1.67E+07	1.99E+07	2.96E+07
4	80.00	4.00	6.00E+06	1.69E+06	1.13E+06	1.50E+06	2.63E+06	7.50E+06	1.71E+07
5	80.00	3.00	3.94E+06	1.50E+06	5.63E+05	1.31E+06	2.06E+06	5.78E+07	1.47E+08
6	67.92893	3.00	5.06E+06	1.88E+06	1.13E+06	1.50E+06	2.25E+06	8.06E+06	2.53E+07
7	82.07107	1.585782	5.81E+06	1.31E+06	2.63E+06	1.46E+07	4.14E+07	8.93E+07	1.25E+08
8	75.00	4.414214	4.13E+06	1.13E+06	5.63E+05	1.13E+06	2.25E+06	6.38E+06	1.37E+07
9	75.00	3.00	3.75E+06	5.63E+05	9.38E+05	1.69E+06	5.06E+06	3.71E+07	1.45E+08

B)	Moisture (%)	EtOH %	Fermentation time (h)						
S.No			24	48	72	96	120	144	168
1	70.00	2.00	4.31E+06	2.25E+06	1.31E+06	2.25E+06	1.41E+07	1.48E+07	3.08E+07
2	70.00	4.00	5.81E+06	2.44E+06	1.50E+06	1.50E+06	2.81E+06	3.38E+06	4.31E+06
3	70.00	2.00	5.25E+06	2.25E+06	1.69E+06	1.13E+06	1.31E+06	6.56E+06	9.94E+06
4	80.00	4.00	6.00E+06	1.50E+06	9.38E+05	1.31E+06	1.13E+06	1.50E+06	2.25E+06
5	80.00	3.00	4.31E+06	1.31E+06	1.13E+06	1.31E+06	1.50E+06	4.13E+06	5.44E+06
6	67.92893	3.00	4.88E+06	1.88E+06	1.13E+06	9.38E+05	1.13E+06	1.88E+06	2.63E+06
7	82.07107	1.585782	5.81E+06	1.31E+06	2.06E+06	1.74E+07	2.72E+07	3.24E+07	4.26E+07
8	75.00	4.414214	3.94E+06	1.31E+06	1.13E+06	2.81E+06	2.81E+06	5.25E+06	9.56E+06
9	75.00	3.00	3.94E+06	9.38E+05	1.13E+06	2.63E+06	2.63E+06	5.25E+06	6.19E+06

ANNEXE III

Données: Apple pomace ultrafiltration sludge- a novel substrate for fungal bioproduction of citric acid: Optimization studies

Figure 4 Viability of *A. niger* NRRL 567 cultivated on apple pomace through solid-state fermentation supplemented with inducer: (A) ethanol and (B) methanol.

A)	Treatments		Fermentation time (h)						
S.No.	X_1	X_2	24	48	72	96	120	144	168
1	30.00	2.00	3.75E+05	2.60E+05	1.35E+05	1.88E+05	3.13E+05	3.65E+05	4.48E+05
2	30.00	4.00	3.75E+05	1.88E+05	1.25E+05	1.25E+05	1.88E+05	2.50E+05	5.00E+05
3	50.00	2.00	2.50E+05	1.88E+05	1.25E+05	1.88E+05	3.13E+05	3.75E+05	4.38E+05
4	50.00	4.00	3.75E+05	1.25E+05	1.25E+05	1.88E+05	2.50E+05	3.75E+05	5.00E+05
5	25.86	3.00	1.88E+05	1.25E+05	1.25E+05	2.50E+05	3.13E+05	3.75E+05	4.38E+05
6	54.14	3.00	4.38E+05	1.88E+05	1.88E+05	2.50E+05	3.75E+05	8.13E+05	9.38E+05
7	40.00	1.59	3.13E+05	1.88E+05	1.88E+05	1.25E+05	2.50E+05	3.75E+05	4.38E+05
8	40.00	4.41	3.75E+05	3.13E+05	1.88E+05	2.50E+05	3.13E+05	3.75E+05	4.38E+05
9	40.00	3.00	2.50E+05	1.25E+05	1.25E+05	1.88E+05	2.50E+05	3.13E+05	4.38E+05
10	40.00	3.00	3.13E+05	1.25E+05	1.25E+05	1.25E+05	2.50E+05	3.75E+05	4.38E+05
B)	Treatments		Fermentation time (h)						
S. No.	X_3	X_4	24	48	72	96	120	144	168
1	30.00	2.00	3.13E+05	2.50E+05	1.25E+05	2.50E+05	3.13E+05	3.75E+05	4.38E+05
2	30.00	4.00	3.75E+05	1.72E+05	1.23E+05	1.88E+05	2.50E+05	3.13E+05	3.75E+05
3	50.00	2.00	3.13E+05	2.50E+05	1.88E+05	1.90E+05	2.50E+05	3.13E+05	3.75E+05
4	50.00	4.00	3.13E+05	2.50E+05	1.88E+05	2.50E+05	3.75E+05	5.00E+05	5.63E+05
5	25.86	3.00	2.50E+05	1.88E+05	1.25E+05	1.88E+05	2.50E+05	3.13E+05	3.75E+05
6	54.14	3.00	4.38E+05	1.88E+05	1.25E+05	1.88E+05	3.13E+05	4.38E+05	6.25E+05
7	40.00	1.59	3.75E+05	2.50E+05	1.88E+05	2.50E+05	3.13E+05	3.75E+05	4.38E+05
8	40.00	4.41	5.00E+05	3.75E+05	2.50E+05	3.13E+05	3.13E+05	3.75E+05	4.38E+05
9	40.00	3.00	3.13E+05	1.88E+05	1.25E+05	1.88E+05	2.50E+05	3.13E+05	3.75E+05
10	40.00	3.00	3.13E+05	1.88E+05	1.88E+05	2.50E+05	3.13E+05	3.75E+05	4.38E+05

Abbreviations: $X_1$and X_2 represents variables i.e. total solids concentration and inducer (ethanol) concentration for experiment set A)

X_3 and X_4 variables i.e. total solids concentration and inducer (methanol) concentration for experiment set B)

ANNEXE IV

Données: Bioproduction and extraction optimization of citric acid from *Aspergillus niger* by rotating drum type solid-state bioreactor

Figure 1 (A) Citric acid (CA) production (g/kg dry substrate) and (B) viability trend of *A. niger* NRRL 567 during solid-state fermentation using apple pomace (AP) as a substrate (mositure 75% v/w)

S.No.	Treatments	Fermentation time (h)					
		24	48	72	96	120	144
A)	**Citric acid (CA) production**						
1	Control	26.47±1.22	36.70±2.56	100.9±5.22	128.68±6.34	137.47±10.66	133.77±7.45
2	EtOH- 3%	28.43±1.43	45.51±1.98	113.50±7.80	168.18±8.78	190.1±12.34	148.85±8.55
3	MeOH 3%	28.14±1.50	39.66±2.05	136.76±6.45	204.07±8.90	220.64±13.89	193.57±10.23
B)	**Viability trend of *A. niger* NRRL 567**						
1	Control	2.50E+06	1.00E+06	2.38E+07	5.00E+07	6.28E+07	8.80E+07
2	EtOH- 3%	3.00E+06	1.50E+06	2.50E+06	1.30E+07	2.40E+07	3.60E+07
3	MeOH 3%	3.25E+06	1.75E+06	2.00E+06	7.00E+06	1.65E+07	2.75E+07

ANNEXE V

Données: Rheological studies during submerged citric acid fermentation by *Aspergillus niger* in stirred fermenter using apple pomace ultrafiltration sludge

Figure 1 Citric acid (CA) and viability (CFUs/ml) during SmF by *Aspergillus niger* NRRL-567 on apple pomace ultrafiltration sludge (A) control and; (B) inducer methanol (MeOH)- 3% (v/v) concentration

Time (h)	Biomass g/L		Viability MPN method		CA (g/100 g SS)	
	Control	MeOH	Control	MeOH	Control	MeOH
0	0	0	982	789	0	0
12	1.47	1.23	10010	14000	4.49	3.99
24	2.66	2.31	1470000	165000	5.25	5.07
30	3.78	3.02	10000000	7980000	6.99	5.45
36	3.98	3.44	11600000	9780000	8.40	7.28
42	4.55	3.91	19000000	18100000	9.05	8.34
48	5.78	4.69	38900000	24500000	9.94	9.20
54	6.87	6.23	6.63E+07	55600000	11.62	11.77
60	7.45	7.45	8.64E+07	81940000	12.27	12.51
66	9.67	7.88	1.85E+08	92800000	12.56	13.06
72	10.87	8.42	2.76E+08	2.48E+08	14.55	15.06
84	12.72	9.89	3.89E+08	3.65E+08	16.76	16.91
90	14.98	10.77	4.1E+08	3.92E+08	17.50	17.90
96	16.23	12.9	4.37E+08	3.98E+08	18.42	19.64
102	16.78	13.02	5.01E+08	4.14E+08	19.27	22.17
108	14.5	14.21	5.89E+08	3.79E+08	20.70	24.45
114	12.05	12.32	5.57E+08	3.65E+08	23.54	30.90
120	11.92	11.5	4.92E+08	3.43E+08	23.67	35.38
132	10	10.55	4.38E+08	2.48E+08	18.41	40.34
144	9.12	10.12	3.6E+08	1.73E+08	17.25	38.22
156	8.24	10	3.11E+08	1.42E+08	13.96	31.79
168	7.9	9.12	2.73E+08	1E+08	11.10	25.36

Figure 2. Changes in (A) viscosity and; (B) DO (%) profiles during citric acid (CA) fermentation by *Aspergillus niger* NRRL-567 with control apple pomace ultrafiltration sludge and with inducer methanol (MeOH) in a concentration of 3% (v/v).

Time (h)	DO (%)		Viscosity (10^{-3} kg m^{-1} s^{-2})	
	Ctrl	MeOH	Ctrl	MeOH
0	100.0	96	2.86	1.78
6	96.0	79.12	-	-
12	79.12	77.88	3.52	1.92
18	77.88	75.20	-	-
24	75.20	70.32	3.84	2.02
30	70.32	60.12	-	-
36	60.12	57.12	7.51	3.88
42	57.12	52.56	-	-
48	52.56	48.52	9.05	6.50
54	48.52	47.20	-	-
60	47.20	48.00	9.54	10.04
66	48.00	49.72	-	-
72	49.72	56.68	11.98	12.90
78	56.68	56.16	-	-
84	56.16	59.20	16.26	17.80
90	59.20	69.96	-	-
96	69.96	76.08	22.0	16.98
102	76.08	77.80	-	-
108	77.80	79.28	18.81	14.38
114	79.28	87.24	-	-
120	87.24	89.68	14.49	9.12
126	89.68	10.00	-	-
132	100.0	100.0	11.22	5.28
138	100.0	100.0	-	-
144	100.0	100.0	10.91	3.12
150	100.0	100.0	-	-
156	100.0	100.0	8.49	2.52
162	100.0	100.0	-	-
168	100.0	100.0	7.02	2.08

ANNEXE VI

Données: Novel biomaterials resulting from citric acid fermentation process as biosorbents: removal of toxic metals from discarded chromated, copper arsenate (CCA) treated wood leachates

Table 4 Values of parameters of Langmuir and Freundlich adsorption during kinetics studies with SSF biomass (control, live) and SmF biomass (MeOH, live)

Supplementary data- SSF KINETICS

Metal	Concentration of biomass/metal solution (g/l)			
	2.5	**5.0**	**7.5**	**10**
	Langmuir Isotherm			
As	y = -431.09x + 88.049 R^2 = 0.8894	y = -310.58x + 68.695 R^2 = 0.9716	y = -1349.2x + 273.28 R^2 = 0.8958	y = -360.48x + 86.926 R^2 = 0.9464
Cr	y = -16.053x + 6.1439 R^2 = 0.9613	y = -27.591x + 10.972 R^2 = 0.9924	y = -40.14x + 16.173 R^2 = 0.9622	y = -48.065x + 20.001 R^2 = 0.9561
Cu	y = -835.01x + 179.98 R^2 = 0.8621	y = -418.89x + 99.243 R^2 = 0.9526	y = -242.23x + 69.324 R^2 = 0.7581	y = -399.02x + 104.79 R^2 = 0.8954
	Freundlich isotherm			
As	y = -9.6264x + 6.1825 R^2 = 0.9229	y = -7.8319x + 4.6549 R^2 = 0.9929	y = -8.3479x + 4.797 R^2 = 0.924	y = -5.8694x + 2.9542 R^2 = 0.9863
Cr	y = -2.5274x + 1.1849 R^2 = 0.99	y = -2.348x + 0.7878 R^2 = 0.9981	y = -2.3158x + 0.5906 R^2 = 0.9913	y = -2.1908x + 0.3977 R^2 = 0.9896
Cu	y = -11.79x + 7.1828 R^2 = 0.905	y = -9.0301x + 5.0765 R^2 = 0.9882	y = -9.4129x + 5.0901 R^2 = 0.8734	y = -6.1278x + 2.8279 R^2 = 0.9748

SMF KINETICS

Metal	Concentration of biomass/metal solution (g/l)			
	2.5	5.0	7.5	10
Langmuir isotherm				
As	y = -14.061x + 4.932 R^2 = 0.9879	y = -36.142x + 11.813 R^2 = 0.9773	y = -43.002x + 14.976 R^2 = 0.9706	y = -67.999x + 22.621 R^2 = 0.9854
Cr	y = -9.8147x + 4.462 R^2 = 0.9907	y = -23.429x + 10.051 R^2 = 0.9854	y = -28.208x + 12.994 R^2 = 0.9781	y = -41.822x + 18.637 R^2 = 0.9901
Cu	y = -10.13x + 4.466 R^2 = 0.9887	y = -23.98x + 10.01 R^2 = 0.9854	y = -30.25x + 13.339 R^2 = 0.9845	y = -43.499x + 18.763 R^2 = 0.9897
Frendlich isotherm				
As	y = -2.3673x + 1.2779 R^2 = 0.9969	y = -2.6811x + 1.1699 R^2 = 0.9941	y = -2.3914x + 0.8166 R^2 = 0.9924	y= -2.6003x + 0.818 R^2 = 0.9962
Cr	y = -1.9796x + 0.857 R^2 = 0.9977	y = -2.1625x + 0.6526 R^2 = 0.9963	y = -1.9392x + 0.3597 R^2 = 0.9944	y = -2.0425x + 0.2871 R^2 = 0.9975
Cu	y = -2.0111x + 0.8997 R^2 = 0.9972	y = -2.1875x + 0.6938 R^2 = 0.9963	y = -2.0075x + 0.4216 R^2 = 0.996	y = -2.0827x + 0.335 R^2 = 0.9974

Figure 6 Adsorption isotherm (Langmuir and Freundlich) modelling related to the biosorption of: A & B) arsenic; C & D) chromium and; E & F) copper onto biomass SSF (Control, live) (temperature 25±1 °C, 175 rpm).

Arsenic removal as a function of time – Biomass concentration 2.5 g/L of metal solution

Time (h)	Ci	Ce	Qe (mg/g)	log Qe	log Ce	1/Ce	1/Qe
3	5.757	5.341	0.17	-0.78	0.73	0.19	6.01
6	5.757	5.057	0.28	-0.55	0.70	0.20	3.57
9	5.757	5.233	0.21	-0.68	0.72	0.19	4.77
12	5.757	5.282	0.19	-0.72	0.72	0.19	5.26
15	5.757	5.525	0.09	-1.03	0.74	0.18	10.78
18	5.757	5.533	0.09	-1.05	0.74	0.18	11.16
21	5.757	5.05	0.28	-0.55	0.70	0.20	3.54
24	5.734	4.331	0.56	-0.25	0.64	0.23	1.78
30	5.734	4.064	0.67	-0.18	0.61	0.25	1.50
36	5.757	4.697	0.42	-0.37	0.67	0.21	2.36

Arsenic removal as a function of time – Biomass concentration 5.0 g/L of metal solution

Time (h)	Ci	Ce	Qe (mg/g)	log Qe	log Ce	1/Ce	1/Qe
3	5.757	5.243	0.10	-0.99	0.72	0.19	9.73
6	5.757	5.163	0.12	-0.93	0.71	0.19	8.42
9	5.757	5.101	0.13	-0.88	0.71	0.20	7.62
12	5.757	5.134	0.12	-0.90	0.71	0.19	8.03
15	5.757	5.146	0.12	-0.91	0.71	0.19	8.18
18	5.757	5.209	0.11	-0.96	0.72	0.19	9.12
21	5.757	5.218	0.11	-0.97	0.72	0.19	9.28
24	5.734	4.093	0.33	-0.48	0.61	0.24	3.05
30	5.734	4.026	0.34	-0.47	0.60	0.25	2.93
36	5.757	4.954	0.16	-0.79	0.69	0.20	6.23

Arsenic removal as a function of time – Biomass concentration 7.5 g/L of metal solution

Time (h)	Ci	Ce	Qe (mg/g)	log Qe	log Ce	1/Ce	1/Qe
3	5.757	5.225	0.07	-1.15	0.72	0.19	14.10
6	5.757	5.394	0.05	-1.32	0.73	0.19	20.66
9	5.757	5.113	0.09	-1.07	0.71	0.20	11.65
12	5.757	5.349	0.05	-1.26	0.73	0.19	18.38
15	5.757	5.528	0.03	-1.52	0.74	0.18	32.75
18	5.757	5.172	0.08	-1.11	0.71	0.19	12.82
21	5.757	4.615	0.15	-0.82	0.66	0.22	6.57
24	5.734	4.039	0.23	-0.65	0.61	0.25	4.42
30	5.734	4.054	0.22	-0.65	0.61	0.25	4.46
36	5.757	4.863	0.12	-0.92	0.69	0.21	8.39

Arsenic removal as a function of time – Biomass concentration 10.0 g/L of metal solution

Time (h)	Ci	Ce	Qe (mg/g)	log Qe	log Ce	1/Ce	1/Qe
3	5.757	5.163	0.06	-1.23	0.71	0.19	16.84
6	5.757	5.217	0.05	-1.27	0.72	0.19	18.52
9	5.757	5.182	0.06	-1.24	0.71	0.19	17.39
12	5.757	5.174	0.06	-1.23	0.71	0.19	17.15
15	5.757	5.074	0.07	-1.17	0.71	0.20	14.64
18	5.757	5.238	0.05	-1.28	0.72	0.19	19.27
21	5.757	4.51	0.12	-0.90	0.65	0.22	8.02
24	5.734	3.884	0.19	-0.73	0.59	0.26	5.41
30	5.734	3.741	0.20	-0.70	0.57	0.27	5.02
36	5.757	4.783	0.10	-1.01	0.68	0.21	10.27

Chromium removal as a function of time – Biomass concentration 2.5 g/L of metal solution

Time (h)	Ci	Ce	Qe (mg/g)	log Qe	log Ce	1/Ce	1/Qe
3	5.12	3.634	0.59	-0.23	0.56	0.28	1.68
6	5.12	3.449	0.67	-0.17	0.54	0.29	1.50
9	5.12	3.612	0.60	-0.22	0.56	0.28	1.66
12	5.12	3.693	0.57	-0.24	0.57	0.27	1.75
15	5.12	3.899	0.49	-0.31	0.59	0.26	2.05
18	5.12	3.966	0.46	-0.34	0.60	0.25	2.17
21	5.12	3.605	0.61	-0.22	0.56	0.28	1.65
24	5.00	2.972	0.81	-0.09	0.47	0.34	1.23
30	5.00	2.804	0.88	-0.06	0.45	0.36	1.14
36	5.12	3.329	0.72	-0.14	0.52	0.30	1.40

Chromium removal as a function of time – Biomass concentration 5.0 g/L of metal solution

Time (h)	Ci	Ce	Qe (mg/g)	log Qe	log Ce	1/Ce	1/Qe
3	5.12	3.43	0.34	-0.47	0.54	0.29	2.96
6	5.12	3.501	0.32	-0.49	0.54	0.29	3.09
9	5.12	3.522	0.32	-0.50	0.55	0.28	3.13
12	5.12	3.551	0.31	-0.50	0.55	0.28	3.19
15	5.12	3.61	0.30	-0.52	0.56	0.28	3.31
18	5.12	3.703	0.28	-0.55	0.57	0.27	3.53
21	5.12	3.735	0.28	-0.56	0.57	0.27	3.61
24	5.00	2.823	0.44	-0.36	0.45	0.35	2.29
30	5.00	2.808	0.44	-0.36	0.45	0.36	2.28
36	5.12	3.611	0.30	-0.52	0.56	0.28	3.31

Chromium removal as a function of time – Biomass concentration 7.5 g/L of metal solution

Time (h)	Ci	Ce	Qe (mg/g)	log Qe	log Ce	1/Ce	1/Qe
3	5.12	3.401	0.23	-0.64	0.53	0.29	4.36
6	5.12	3.62	0.20	-0.70	0.56	0.28	5.00
9	5.12	3.54	0.21	-0.68	0.55	0.28	4.75
12	5.12	3.688	0.19	-0.72	0.57	0.27	5.24
15	5.12	3.877	0.17	-0.78	0.59	0.26	6.03
18	5.12	3.697	0.19	-0.72	0.57	0.27	5.27
21	5.12	3.273	0.25	-0.61	0.51	0.31	4.06
24	5.00	2.885	0.28	-0.55	0.46	0.35	3.54
30	5.00	2.869	0.21	-0.67	0.46	0.35	4.68
36	5.12	3.568	0.21	-0.68	0.55	0.28	4.83

Chromium removal as a function of time – Biomass concentration 10.0 g/L of metal solution

Time (h)	Ci	Ce	Qe (mg/g)	log Qe	log Ce	1/Ce	1/Qe
3	5.12	3.371	0.17	-0.76	0.53	0.30	5.72
6	5.12	3.512	0.16	-0.79	0.55	0.28	6.22
9	5.12	3.554	0.16	-0.81	0.55	0.28	6.39
12	5.12	3.608	0.15	-0.82	0.56	0.28	6.61
15	5.12	3.543	0.16	-0.80	0.55	0.28	6.34
18	5.12	3.817	0.13	-0.89	0.58	0.26	7.67
21	5.12	3.212	0.19	-0.72	0.51	0.31	5.24
24	5.00	2.707	0.23	-0.64	0.43	0.37	4.35
30	5.00	2.645	0.31	-0.50	0.42	0.38	3.18
36	5.12	3.497	0.16	-0.79	0.54	0.29	6.16

Copper removal as a function of time – Biomass concentration 2.5 g/L of metal solution

Time (h)	Ci	Ce	Qe (mg/g)	log Qe	log Ce	1/Ce	1/Qe
3	5.22	5.098	0.05	-1.30	0.71	0.20	19.84
6	5.22	4.758	0.19	-0.73	0.68	0.21	5.36
9	5.22	4.993	0.09	-1.03	0.70	0.20	10.82
12	5.22	5.02	0.08	-1.09	0.70	0.20	12.25
15	5.22	4.787	0.17	-0.76	0.68	0.21	5.72
18	5.22	5.009	0.09	-1.07	0.70	0.20	11.63
21	5.22	4.783	0.18	-0.75	0.68	0.21	5.67
24	5.11	3.925	0.47	-0.33	0.59	0.25	2.12
30	5.11	3.681	0.57	-0.24	0.57	0.27	1.75
36	5.22	4.369	0.34	-0.47	0.64	0.23	2.92

Copper removal as a function of time – Biomass concentration 5.0 g/L of metal solution

Time (h)	Ci	Ce	Qe (mg/g)	log Qe	log Ce	1/Ce	1/Qe
3	5.22	4.845	0.08	-1.12	0.69	0.21	13.19
6	5.22	4.76	0.09	-1.03	0.68	0.21	10.78
9	5.22	4.706	0.10	-0.98	0.67	0.21	9.65
12	5.22	4.777	0.09	-1.05	0.68	0.21	11.19
15	5.22	4.794	0.09	-1.07	0.68	0.21	11.63
18	5.22	4.837	0.08	-1.11	0.68	0.21	12.92
21	5.22	4.85	0.07	-1.13	0.69	0.21	13.37
24	5.11	3.624	0.30	-0.53	0.56	0.28	3.37
30	5.11	3.553	0.31	-0.51	0.55	0.28	3.22
36	5.22	4.526	0.14	-0.86	0.66	0.22	7.16

Copper removal as a function of time – Biomass concentration 7.5 g/L of metal solution							
Time (h)	**Ci**	**Ce**	**Qe (mg/g)**	**log Qe**	**log Ce**	**1/Ce**	**1/Qe**
3	5.22	4.752	0.06	-1.20	0.68	0.21	15.89
6	5.22	4.939	0.04	-1.42	0.69	0.20	26.32
9	5.22	4.663	0.07	-1.13	0.67	0.21	13.37
12	5.22	4.913	0.04	-1.38	0.69	0.20	24.12
15	5.22	5.095	0.02	-1.76	0.71	0.20	58.14
18	5.22	4.752	0.06	-1.20	0.68	0.21	15.89
21	5.22	4.162	0.14	-0.85	0.62	0.24	7.06
24	5.11	3.865	0.17	-0.78	0.59	0.26	6.04
30	5.11	3.566	0.21	-0.69	0.55	0.28	4.87
36	5.22	4.421	0.11	-0.97	0.65	0.23	9.34

Copper removal as a function of time – Biomass concentration 10.0 g/L of metal solution							
Time (h)	**Ci**	**Ce**	**Qe (mg/g)**	**log Qe**	**log Ce**	**1/Ce**	**1/Qe**
3	5.22	4.679	0.05	-1.26	0.67	0.21	18.35
6	5.22	4.724	0.05	-1.30	0.67	0.21	20.00
9	5.22	4.728	0.05	-1.30	0.67	0.21	20.16
12	5.22	4.744	0.05	-1.32	0.68	0.21	20.83
15	5.22	4.59	0.06	-1.20	0.66	0.22	15.77
18	5.22	4.832	0.04	-1.41	0.68	0.21	25.51
21	5.22	4.08	0.11	-0.94	0.61	0.25	8.74
24	5.11	3.402	0.17	-0.77	0.53	0.29	5.87
30	5.11	3.276	0.18	-0.74	0.52	0.31	5.46
36	5.22	4.328	0.09	-1.05	0.64	0.23	11.16

Figure 7 Adsorption isotherm (Langmuir and Freundlich) modelling related to the biosorption of: A & B) arsenic; C & D) chromium and; E & F) copper onto biomass SmF (MeOH, live)

Arsenic removal as a function of time – Biomass concentration 2.5 g/L of metal solution

Time (h)	Ci	Ce	Qe (mg/g)	log Qe	log Ce	1/Ce	1/Qe
3	5.796	5.72	0.03	-1.52	0.76	0.17	32.89
6	5.796	5.409	0.15	-0.81	0.73	0.18	6.46
9	5.796	5.603	0.08	-1.11	0.75	0.18	12.95
12	5.796	4.335	0.58	-0.23	0.64	0.23	1.71
15	5.796	4.297	0.60	-0.22	0.63	0.23	1.67
18	5.796	4.207	0.64	-0.20	0.62	0.24	1.57
21	5.796	3.954	0.74	-0.13	0.60	0.25	1.36
24	5.796	3.848	0.78	-0.11	0.59	0.26	1.28
30	5.796	3.788	0.80	-0.10	0.58	0.26	1.25
36	5.796	4.017	0.71	-0.15	0.60	0.25	1.41

Arsenic removal as a function of time – Biomass concentration 5.0 g/L of metal solution

Time (h)	Ci	Ce	Qe (mg/g)	log Qe	log Ce	1/Ce	1/Qe
3	5.796	5.727	0.01	-1.86	0.76	0.17	72.46
6	5.796	5.45	0.07	-1.16	0.74	0.18	14.45
9	5.796	5.264	0.11	-0.97	0.72	0.19	9.40
12	5.796	4.493	0.26	-0.58	0.65	0.22	3.84
15	5.796	4.112	0.34	-0.47	0.61	0.24	2.97
18	5.796	4.118	0.34	-0.47	0.61	0.24	2.98
21	5.796	3.985	0.36	-0.44	0.60	0.25	2.76
24	5.796	3.928	0.37	-0.43	0.59	0.25	2.68
30	5.796	3.965	0.37	-0.44	0.60	0.25	2.73
36	5.796	4.166	0.33	-0.49	0.62	0.24	3.07

Arsenic removal as a function of time – Biomass concentration 7.5 g/L of metal solution

Time (h)	Ci	Ce	Qe (mg/g)	log Qe	log Ce	1/Ce	1/Qe
3	5.796	5.53	0.04	-1.45	0.74	0.18	28.20
6	5.796	5.411	0.05	-1.29	0.73	0.18	19.48
9	5.796	5.166	0.08	-1.08	0.71	0.19	11.90
12	5.796	4.361	0.19	-0.72	0.64	0.23	5.23
15	5.796	4.029	0.24	-0.63	0.61	0.25	4.24
18	5.796	4.126	0.22	-0.65	0.62	0.24	4.49
21	5.796	4.022	0.24	-0.63	0.60	0.25	4.23
24	5.796	3.96	0.24	-0.61	0.60	0.25	4.08
30	5.796	3.948	0.25	-0.61	0.60	0.25	4.06
36	5.796	3.778	0.27	-0.57	0.58	0.26	3.72

Arsenic removal as a function of time – Biomass concentration 10.0 g/L of metal solution

Time (h)	Ci	Ce	Qe (mg/g)	log Qe	log Ce	1/Ce	1/Qe
3	5.796	5.522	0.03	-1.56	0.74	0.18	36.50
6	5.796	5.267	0.05	-1.28	0.72	0.19	18.90
9	5.796	4.277	0.15	-0.82	0.63	0.23	6.58
12	5.796	4.447	0.13	-0.87	0.65	0.22	7.41
15	5.796	4.009	0.18	-0.75	0.60	0.25	5.60
18	5.796	3.904	0.19	-0.72	0.59	0.26	5.29
21	5.796	3.985	0.18	-0.74	0.60	0.25	5.52
24	5.796	4.108	0.17	-0.77	0.61	0.24	5.92
30	5.796	3.971	0.18	-0.74	0.60	0.25	5.48
36	5.796	3.895	0.19	-0.72	0.59	0.26	5.26

Chromium removal as a function of time – Biomass concentration 2.5 g/L of metal solution

Time (h)	Ci	Ce	Qe (mg/g)	log Qe	log Ce	1/Ce	1/Qe
3	5.004	4.832	0.07	-1.16	0.68	0.21	14.53
6	5.004	4.486	0.21	-0.68	0.65	0.22	4.83
9	5.004	4.68	0.13	-0.89	0.67	0.21	7.72
12	5.004	3.539	0.59	-0.23	0.55	0.28	1.71
15	5.004	3.5	0.60	-0.22	0.54	0.29	1.66
18	5.004	3.43	0.63	-0.20	0.54	0.29	1.59
21	5.004	3.224	0.71	-0.15	0.51	0.31	1.40
24	5.004	3.139	0.75	-0.13	0.50	0.32	1.34
30	5.004	3.098	0.76	-0.12	0.49	0.32	1.31
36	5.004	3.28	0.69	-0.16	0.52	0.30	1.45

Chromium removal as a function of time – Biomass concentration 5.0 g/L of metal solution

Time (h)	Ci	Ce	Qe (mg/g)	log Qe	log Ce	1/Ce	1/Qe
3	5.004	4.673	0.07	-1.18	0.67	0.21	15.11
6	5.004	4.492	0.10	-0.99	0.65	0.22	9.77
9	5.004	4.351	0.13	-0.88	0.64	0.23	7.66
12	5.004	3.629	0.28	-0.56	0.56	0.28	3.64
15	5.004	3.335	0.33	-0.48	0.52	0.30	3.00
18	5.004	3.354	0.33	-0.48	0.53	0.30	3.03
21	5.004	3.243	0.35	-0.45	0.51	0.31	2.84
24	5.004	3.207	0.36	-0.44	0.51	0.31	2.78
30	5.004	3.235	0.35	-0.45	0.51	0.31	2.83
36	5.004	3.396	0.32	-0.49	0.53	0.29	3.11

Chromium removal as a function of time – Biomass concentration 7.5 g/L of metal solution

Time (h)	Ci	Ce	Qe (mg/g)	log Qe	log Ce	1/Ce	1/Qe
3	5.004	4.506	0.07	-1.18	0.65	0.22	15.06
6	5.004	4.432	0.08	-1.12	0.65	0.23	13.11
9	5.004	4.168	0.11	-0.95	0.62	0.24	8.97
12	5.004	3.521	0.20	-0.70	0.55	0.28	5.06
15	5.004	3.247	0.23	-0.63	0.51	0.31	4.27
18	5.004	3.326	0.22	-0.65	0.52	0.30	4.47
21	5.004	3.252	0.23	-0.63	0.51	0.31	4.28
24	5.004	3.209	0.24	-0.62	0.51	0.31	4.18
30	5.004	3.186	0.24	-0.62	0.50	0.31	4.13
36	5.004	3.057	0.26	-0.59	0.49	0.33	3.85

Chromium removal as a function of time – Biomass concentration 10.0 g/L of metal solution

Time (h)	Ci	Ce	Qe (mg/g)	log Qe	log Ce	1/Ce	1/Qe
3	5.004	4.461	0.05	-1.27	0.65	0.22	18.42
6	5.004	4.296	0.07	-1.15	0.63	0.23	14.12
9	5.004	3.43	0.16	-0.80	0.54	0.29	6.35
12	5.004	3.571	0.14	-0.84	0.55	0.28	6.98
15	5.004	3.21	0.18	-0.75	0.51	0.31	5.57
18	5.004	3.137	0.19	-0.73	0.50	0.32	5.36
21	5.004	3.2	0.18	-0.74	0.51	0.31	5.54
24	5.004	3.31	0.17	-0.77	0.52	0.30	5.90
30	5.004	3.179	0.18	-0.74	0.50	0.31	5.48
36	5.004	3.135	0.19	-0.73	0.50	0.32	5.35

Copper removal as a function of time – Biomass concentration 2.5 g/L of metal solution

Time (h)	Ci	Ce	Qe (mg/g)	log Qe	log Ce	1/Ce	1/Qe
3	5.106	4.975	0.05	-1.28	0.70	0.20	19.08
6	5.106	4.641	0.19	-0.73	0.67	0.22	5.38
9	5.106	4.814	0.12	-0.93	0.68	0.21	8.56
12	5.106	3.639	0.59	-0.23	0.56	0.27	1.70
15	5.106	3.59	0.61	-0.22	0.56	0.28	1.65
18	5.106	3.518	0.64	-0.20	0.55	0.28	1.57
21	5.106	3.315	0.72	-0.14	0.52	0.30	1.40
24	5.106	3.225	0.75	-0.12	0.51	0.31	1.33
30	5.106	3.173	0.77	-0.11	0.50	0.32	1.29
36	5.106	3.4	0.68	-0.17	0.53	0.29	1.47

Copper removal as a function of time – Biomass concentration 5.0 g/L of metal solution

Time (h)	Ci	Ce	Qe (mg/g)	log Qe	log Ce	1/Ce	1/Qe
3	5.106	4.825	0.06	-1.25	0.68	0.21	17.79
6	5.106	4.637	0.09	-1.03	0.67	0.22	10.66
9	5.106	4.483	0.12	-0.90	0.65	0.22	8.03
12	5.106	3.724	0.28	-0.56	0.57	0.27	3.62
15	5.106	3.422	0.34	-0.47	0.53	0.29	2.97
18	5.106	3.425	0.34	-0.47	0.53	0.29	2.97
21	5.106	3.318	0.36	-0.45	0.52	0.30	2.80
24	5.106	3.286	0.36	-0.44	0.52	0.30	2.75
30	5.106	3.311	0.36	-0.44	0.52	0.30	2.79
36	5.106	3.527	0.32	-0.50	0.55	0.28	3.17

Copper removal as a function of time – Biomass concentration 7.5 g/L of metal solution

Time (h)	Ci	Ce	Qe (mg/g)	log Qe	log Ce	1/Ce	1/Qe
3	5.106	4.654	0.06	-1.22	0.67	0.21	16.59
6	5.106	4.583	0.07	-1.16	0.66	0.22	14.34
9	5.106	4.295	0.11	-0.97	0.63	0.23	9.25
12	5.106	3.607	0.20	-0.70	0.56	0.28	5.00
15	5.106	3.324	0.24	-0.62	0.52	0.30	4.21
18	5.106	3.393	0.23	-0.64	0.53	0.29	4.38
21	5.106	3.323	0.24	-0.62	0.52	0.30	4.21
24	5.106	3.287	0.24	-0.62	0.52	0.30	4.12
30	5.106	3.277	0.24	-0.61	0.52	0.31	4.10
36	5.106	3.176	0.26	-0.59	0.50	0.31	3.89

Copper removal as a function of time – Biomass concentration 10.0 g/L of metal solution

Time (h)	Ci	Ce	Qe (mg/g)	log Qe	log Ce	1/Ce	1/Qe
3	5.106	4.607	0.05	-1.30	0.66	0.22	20.04
6	5.106	4.424	0.07	-1.17	0.65	0.23	14.66
9	5.106	3.52	0.16	-0.80	0.55	0.28	6.31
12	5.106	3.669	0.14	-0.84	0.56	0.27	6.96
15	5.106	3.289	0.18	-0.74	0.52	0.30	5.50
18	5.106	3.197	0.19	-0.72	0.50	0.31	5.24
21	5.106	3.278	0.18	-0.74	0.52	0.31	5.47
24	5.106	3.381	0.17	-0.76	0.53	0.30	5.80
30	5.106	3.238	0.19	-0.73	0.51	0.31	5.35
36	5.106	3.259	0.18	-0.73	0.51	0.31	5.41

ANNEXE VII

Données: Facile synthesis of chitosan-based Zinc oxide nanoparticles stabilized with different organic compounds and evaluation of their antimicrobial and antibiofilm activity

Figure 2 Zeta potential measurements of different ZnO-CTS NPs prepared by: A) nano spray drying and; B) precipitation method

S.No.	Treatments	Nano spray drying	Chemical synthesis method
1	ZnO	-7.54	-17.92
2	ZnO-CTS	-12.87	-2.68
3	ZnO-CTS-CA	-	-37.3
4	ZnO-CTS-Glycerol	-1.9	1.5
5	ZnO-CTS-Starch	-24.72	-12.76
6	ZnO-CTS-Whey	-35.54	-10.9