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PRODUCTION ACCRUE D'ACIDE FUMARIQUE PAR FERMENTATION DE DÉCHETS AGRO-INDUSTRIELS

Par

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DÉDICACE

This thesis is dedicated to my parents Chandra Kumar Das and Tarinibala Das

for their love, sacrifices and endless support

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Résumé

L'acide fumarique (AF) a été identifié comme étant l'un des dix meilleurs produits chimiques de construction modulaire qui peut être produit par fermentation submergée et par fermentation à l'état solide à partir de différents déchets de organiques (la plupart d'origine agro-industrielle). Il a été établi que le coût du substrat (presque 40-60% du coût total) est la principale contribution économique au coût total de production dans la fermentation du AF. Normalement, l'AF est produit commercialement à partir de l'anhydride maléique. Toutefois, en raison de la hausse du prix de l'anhydride maléique (60-70% du coût total de production), les chercheurs ont semblé être plutôt en AFveur d'une production biologique (fermentation) du AF. Comparée à celle des autres champignons filamenteux, la souche *Rhizopus oryzae* 1526 (famille: Mucoraceae) est utilisée comme principal producteur de AF en raison de ses faibles besoins nutritionnels. Dans l'étude de la production d'AF par la technique d'immobilisation, les coûts des matériaux de support d'immobilisation peuvent représenter de 60 à 70% du coût total du processus et donc l'utilisation de matériaux compatibles de faible coût pour des applications peut être une très bonne option à envisager. Dans les études portant sur la production d'AF par fermentation, le $CaCO_3$ est utilisé pour maintenir un pH proche de la neutralité (6.0) pour favoriser une production maximale d'AF. Une source rentable et durable de $CaCO_3$ peut être une option efficace. De plus, l'utilisation de nanoparticules de $CaCO_3$ et d'irradiation par micro-ondes (en anglais: MWI) permet de réduire la viscosité du milieu de culture et le temps de récupération du AF. La détermination spectrophotométrique du AF dans un échantillon de bouillon fermenté n'est pas une approche méthodologique courante. Ainsi, le développement d'une méthode spectrophotométrique simple et rapide pour l'estimation de la production d'AF dans des échantillons de bouillons fermentés peut être une option intéressante à envisager.

Dans cette étude, une procédure colorimétrique rapide et efficace a été développée pour la quantification du AF présent dans les échantillons de bouillon fermenté. Différents déchets agro-industriels, à savoir les eaux usées de brasserie (en anglais: BW) et les boues d'ultrafiltration de jus de pomme (en anglais: APS), les déchets solides de jus de pomme mélangés aux pelures de riz (en anglais: AP) et des déchets solides de pâtes et papiers (en anglais: PPSW), ont été testés et optimisés pour améliorer la production de AF par fermentation en utilisant le *R. oryzae* 1526 (*R. oryzae*). Une stratégie d'immobilisation rentable a été adoptée pour une production accrue d'AF. L'impact des différentes concentrations de micro et de nanoparticules de ZnO, Fe_3O_4 et MnO_2 sur la production d'AF a également été étudié. Enfin, l'application des nanoparticules de carbonate de calcium (en anglais: CCNPs) et l'irradiation par micro-ondes (en anglais: MWI) ont été étudiées dans la production et la récupération du AF, respectivement.

Pour les BW, en utlisant les les conditions optimales de croissance (pH 6, 25 °C, agitation à 200 rpm, 5% (v/v) d'inoculum, 25 g/L concentration en matières solides totales de 25 g/L, et un diamètre de granule de 0,47 ± 0,04 mm), la concentration la plus élevée de AF atteinte a été de 31,3 ± 2,8 g/L.

Dans l'étude de l'immobilisation sur un chiffon de mousseline (en anglais: MC), les niveaux de production et la productivité volumétrique de AF ont été nettement augmentés, passant de 30,56 ± 1,40 g/L à 43,67 ± 0,32 g/L et 0.424 g/(L h) à 1.21 g/(L,h), comparativement à la fermentation acellulaire. Dans une autre étude comprenant les déchets de coquilles d'œufs de poule (en anglais: EGS), les biofilms (formés sur les EGS) obtenus par fermentation submergée ont nettement amélioré la production et la productivité volumétrique de l'acide fumarique de 30,23 ± 1,23 g/L à 47,22 ± 0,77 g/L et de 0.419/(L,h) à 1.657 g/(L,h), respectivement par rapport aux cellules libres. Les EGS servent également de source de $CaCO_3$.

L'étude comprenant les PPSW a entraîné de bons taux de production de AF par fermentation submergée et par fermentation à l'état solide avec différentes gammes de taille de particules (1,7 mm < x ≤ 3,35 mm, 850 µm < x ≤ 1,7 mm, 300 µm < x ≤ 850 µm, 75 µm < x ≤ 300 µm et 33 µm < x ≤ 75 µm). Dans la fermentation submergée, un maximum de 23,47 ± 0,70 g/L de AF a été obtenu avec une taille de particules de 33 µm < x ≤ 75 µm. La fermentation à l'état solide avec une taille de particules de 75 µm < x ≤ 300 µm a abouti à une plus forte production de AF (41.45 g/kg de poids sec de PPSW) après 21 jours.

Pour l'étude de la fermentation submergée avec les APSU, les paramètres optimaux pour la production de AF (25,2 ± 1,0 g/L, 0,350 g/(L,h)) soit une concentration de 40 g/L en solides totaux de APSU, un pH de 6,0, une température de 30 °C, agitation à 200 rpm et un temps d'incubation de 72h. La fermentation à l'état solide a permis une production de 52 ± 3 g d'AF par kg en poids sec d'AP avec les conditions optimisées.

Les expériences réalisées avec les AP dans le fermenteur à tambour rotatif à l'état solide à l'échelle laboratoire ont abouti à une concentration d'AF de 138 ± 9 g par kg de poids sec d'AP selon les conditions optimisées. La teneur totale en phénol des AP a considérablement augmenté, passant de 185 ± 10.5 à 345 ± 8.5 mg/g de lyophilisat.

L'étude de l'impact des différentes concentrations (200-1000 µg/ml) de micro- et de nanoparticules de ZnO, Fe_3O_4 et MnO_2 sur la production d'AF a montré que les micro et les nanoparticules de Fe_3O_4 sont les particules les plus biocompatibles avec le champignon *R.oryzae*.

L'application de CCNPs a permis a réduit le temps de neutralisation du AF d'environ 160 secondes. De plus, les CCNPs ont amélioré la productivité volumétrique de AF, qui est

passée de 0,47 g/(L,h) à 0,74 g/(L,h). Les viscosités des CCNPs se sont avérées être inférieures à celles des microparticules de carbonate de calcium (en anglais: CCMPs). Une période de chauffage de 10 ± 1 min par MWI a été jugée suffisant pour la récupération d'AF et ce temps est beaucoup plus faible que le temps de chauffage classique de 28 ± 1 min.

ABSTRACT

Fumaric acid (FA) has been identified as one of the top ten building block chemicals that can be produced by submerged and solid state fermentation from different waste materials (mostly of agro-industrial origin). It has been established that substrate cost (almost 40-60% of the total cost) is the major economic input to the total production cost in FA fermentation. Normally, FA is solely being produced commercially from maleic anhydride. However, due to the rising price of maleic anhydride (60-70% of the total production cost), fermentation route is being preferred. Owing to its low nutritional requirements, *Rhizopus oryzae 1526* (family: Mucoraceae) strain is used as the main producer of FA. In the immobilization based FA production study, the immobilization support material costs can range from 60-70% of the total process cost and thus exploration of low cost and compatible materials can be a very good option. In the fermentation based FA production studies, $CaCO_3$ is used to maintain a pH around neutral (6.0) for maximum FA production. A cheap and sustainable source of $CaCO_3$ can be an effective option. Moreover, applications of $CaCO_3$ nanoparticles and microwave irradiation (MWI) can reduce the broth viscosity and recovery time of FA, respectively. Spectrophotometric determination of FA in fermented broth sample is not a common methodological approach. Thus, development of a simple and rapid spectrophotometric method for FA estimation in fermented broth samples can be an interesting option to investigate.

In the present investigation, a time and cost effective colorimetric procedure was developed for the quantification of FA in the fermented broth samples. Different agro-industrial wastes *viz.* brewery wastewater (BW) and apple pomace ultrafiltration sludge (APS), apple pomace with rice husk (AP) and pulp and paper solid waste (PPSW) were screened and optimized for the enhanced production of FA through fermentation employing *R. oryzae 1526* (*R. oryzae*). Cost-effective immobilization technique was used for enhanced FA production. Impact of different concentrations of micro- and nanoparticles of ZnO, Fe_3O_4 and MnO_2 on FA production was also investigated. Finally, application of calcium carbonate nanoparticles (CCNPs) and microwave irradiation (MWI) in FA production and recovery, respectively, were investigated.

For BW, with all the optimized growth conditions (pH 6, 25 °C, 200 rpm, 5% (v/v) inoculum size, 25 g/L total solids concentration, and pellet diameter of 0.465 ± 0.04 mm), the highest concentration of FA achieved was 31.3 ± 2.77 g/L.

In the immobilization study with muslin cloth (MC), production level and volumetric productivity of FA were markedly increased from 30.56 ± 1.40 g/L to 43.67 ± 0.32 g/L and

0.424 g/(L h) to 1.21 g/(L h) as compared to free-cell fermentation. In another study with hen's egg shells (EGS), as compared to free-cell, biofilms (formed on EGS) mediated submerged fermentation markedly enhanced the production and volumetric productivity of fumaric acid from 30.23 ± 1.23 g/L to 47.22 ± 0.77 g/L and 0.419/(L h) to 1.657 g/(L h), respectively. EGS also served the purpose of source of $CaCO_3$.

The study with PPSW resulted in good productivities of FA for submerged and solid state fermentation with different particle ranges (1.7 mm < x ≤ 3.35 mm, 850 µm < x ≤ 1.7 mm, 300 µm < x ≤ 850 µm, 75 µm < x ≤ 300 µm and 33 µm < x ≤ 75 µm). In submerged fermentation with, a maximum of 23.47 ± 0.70 g/L of FA was obtained with 33 µm < x ≤ 75 µm. Solid state fermentation with 75 µm < x ≤ 300 µm particle size resulted in highest FA production (41.45 g/kg dry weight of PPSW) after 21 days.

For submerged fermentation study with APSU, 40 g/L of total solids concentration of APUS, pH 6.0, 30 ^{o}C, 200 rpm flask shaking speed and 72 h of incubation were found to be optimum for FA production (25.2 ± 1.0 g/L, 0.350 g/(L h)). Solid state fermentation resulted in 52 ± 2.67 g FA per kg dry weight of AP under all optimized conditions.

The experiments carried out with AP in rotating drum type solid-state bench scale fermenter resulted in FA concentration of 138 ± 9.11 g per kg dry weight of AP at all optimized conditions. Total phenolic content of AP was considerably increased from 185 ± 10.5 mg/g to 345 ± 8.5 mg/g of lyophilizate.

The investigation on the impact of different concentrations (200-1000 µg/mL) of micro- and nanoparticles of ZnO, Fe_3O_4 and MnO_2 on FA production showed Fe_3O_4 micro- and nanoparticles to be the most biocompatible to the fungus *R.oryzae*.

The application of CCNPs reduced FA neutralization timing by around 160 seconds. Moreover, CCNPs enhanced the volumetric productivity of FA from 0.47 g/(L h) to 0.74 g/(L h). Viscosities of CCNPs were found to be lower than calcium carbonate microparticles (CCMPs). Under MWI heating, 10 ± 1 min was found to be sufficient for recovery of FA and this was much lower than conventional heating timing of 28 ± 1min.

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TABLE DES MATIÈRES

LISTE DES TABLEAUX

LISTE DES FIGURES

LISTE DES RÉACTIONS

LISTE DES ABRÉVIATIONS

AM	autoimmune myocarditis
AP	apple pomace
APHA	American public health association
APUS	apple pomace ultrafiltration sludge
ARS	agricultural research services
ART	antiretroviral therapy
AWWA	American water works association
BAPO	bisacrylphosphrine oxide
BDL	below detection limit
BDW	biomass dry weight
BG	Biogen Idec
BOD	biological oxygen demand
BSF	solid-state bench scale fermenter
BW	brewery wastewater
cAMP	cyclic AMP
CCL_2	chemokine ligand 2
CCMPs	calcium carbonate microparticles
CCNPs	calcium carbonate nanoparticles
CNS	central nervous system
CNTs	carbon nanotubes
CO	carbon monoxide
CO_2	carbon dioxide
COD	chemical oxygen demand
CONFIRM	comparator and an oral fumarate in relapsing-remitting multiple sclerosis
CyaA	adenylate cyclase
DA	dark agouti

DA	diacrylate
DEF	diethylfumarate
DEFINE	determination of the efficacy and safety of oral fumarate in relapsing-
DEP	diepoxide
DI	deionized water
DME	dimethylethyl
DMF	dimethylfumarate
DNA	deoxyribonucleic acid
DV	divinyl
EAM	experimental autoimmune myocarditis
EFSA	European food safety authority
EGS	eggshells
EHF	ethylhydrogen fumarate
ESM	egg shell membrane
FA	fumaric acid
FAEs	fumaric acid esters
FAO	food and agriculture organization
FBA	flux balance analysis
FDA	food and drug administration
FUM	fumarase
FUM1	fumarase enzyme gene
GHGs	greenhouse gases
GPCR	G protein-coupled receptors
GSH	glutathione
GSM	glucose salts medium
GTPase	guanosine triphosphatese
HAND	HIV-associated neurocognitive disorders
HCl	hydrochloric acid

HIV	human immunodeficiency virus
HO-1	heme-oxygenase 1
HPLC	high performance liquid chromatography
Hsp90	heat shock protein 90
IC50	half maximal inhibitory concentration
ICP	Inductively coupled plasma
ICR	imprinting control region
IDF	insoluble dietary fibre
MAA	methacrylic acid
MC	moisture content
mcp-1	monocyte chemotactic protein-1
MCs	muslin cloths
MDH	malate dehydrogenase,
MEE	monoethylester
mHA	microhydroxyapatite
MHF	methylhydrogenfumarate
MMF	monomethylfumarate
MOG-EAE	artificially induced experimental autoimmune encephalomyelitis
mPEGA	methoxy poly(ethylene glycol) monoacrylate
MPs	microparticles
MRI	magnetic resonance imaging
MS	multiple sclerosis
MWI	microwave irradiation
NA	not applicable
NF-kB	nuclear factor kappa B
NFN	5-nitrofuran naphthyridine
nHA	nanohydroxyapatite
nm	nanometer

NPs	nanoparticles
NVDP	N-vinyl pyrrolidone
OD	optical density
OPF	oligo (poly (ethylene glycol) fumarate)
PCLF	poly (caprolactone fumarate)
PDA	potato dextrose agar
PDI	polydispersity index
PEG-DMA	poly(ethylene glycol)-dimethacrylate
PEGF	poly(ethylene glycol fumarate).
PEPC	phosphoenolpyruvate carboxylase
PF-co-EG	poly (propylene fumarate-co-ethylene glycol)
PGRs	plant growth regulators
PKA	protein kinase A
PLGA	poly(DL-lactic-co-glycolic acid)
PPF	poly (propylene fumarate
PPSW	pulp and paper solid waste
PTM	phloroglucinol triglycidyl methacrylate
PYC	pyruvate carboxylase
R.oryzae 1526	*Rhizopus oryzae 1526*
RRMS	remitting multiple sclerosis
RGD	arginine-glycine-aspartic acid
RNA	ribonucleic acid
rpm	revolutions per minute
RRMS	relapsing-remitting multiple sclerosis
SDF	soluble dietary fibre
SEM	scanning electron microscope
SIW	starch industry waste
SmF	submerged fermentation

SPK	spitzenkorper
SPR	specific fumaric acid production rate
SSF	solid state fermentation
TAA	thioacetamide
TC	total carbohydrates
TCA	tricarboxylic cycle
TDF	total dietary fibre
TNFa	tumor necrosis factor alpha
TOC	total organic carbon
TON	total organic nitrogen
TPC	total phenolic content
TSC	total solid concentration
WHC	water holding capacity
WPCF	water pollution control federation
β-TCP	β-tricalcium phosphate

CHAPITRE 1
SYNTHÈSE

PARTIE I: HISTOIRE ET GÉNÉRALITÉS SUR L'ACIDE FUMARIQUE - REVUE DE LA LITTÉRATURE

L'acide fumarique (AF) est un acide dicarboxylique d'origine naturelle à quatre carbones, de formule moléculaire $C_4H_4O_4$. C'est un produit intermédiaire clé du cycle de l'acide tricarboxylique (TCA) ou cycle de Krebs pour tous les organismes aérobies. Albert Szent-Györgyi (1893-1986, Hongrie) a découvert la catalyse de l'AF lors de son étude (y compris sur la vitamine C) sur le processus de combustion cellulaire (cycle de Krebs) pour lequel il a reçu le Prix Nobel de physiologie ou de médecine en 1937 (www.nobelprize.org). L'importance de l'AF comme plate-forme chimique a été établie après des recherches datant d'une dizaine d'années sur différents aspects. Normalement, l'AF est uniquement produit commercialement à partir de l'anhydride maléique ($C_4H_2O_3$). Cependant, suite à la hausse du prix de l'anhydride maléique (60-70% du coût total de production de l'AF) et la croissance des problèmes environnementaux, tels que la détérioration de l'environnement mondial causé par l'exploitation de produits pétroliers, encouragerait la production d'AF à partir de biomasse renouvelables sous des conditions de production modérés comme la fermentation (Anonyme, 2007). En effet, l'AF a été identifié comme étant l'un des dix meilleurs produits chimiques polyvalente qui peuvent être produits à partir de glucides par conversion biologique (Werpy & Petersen, 2004). Ceci a son importance en regard de principes " dévalorisation des lhydrates de carbone" et de developement de "bio raffineries". Ile récent regain d'intérêt pour la production biologique de l'AF est également influencé par la prise de conscience, de plus en plus forte, de l'importance de la biosécurité dans les secteurs de l'alimentation et des produits laitiers. Le benzène est utilisé en tant que matière première pour la production de l'anhydride maléique et il est aussi connu pour son potential cancérigène. Tout ceci a généré une recherche d'alternatives sécuritaires pour la production de FA (Kang *et al*., 2010).

l'AF est un intermédiaire de grande valeur dans la préparation de produits comestibles, tels que l'acide L-malique et de l'acide L-aspartique. La demande mondiale de d'AF et de ses dérivés est de plus en plus importante d'année en année (Goldberg *et al*., 2006). Selon un rapport de 2014, le volume du marché actuel de l'AF est d'environ 240 000 tonnes et serait de 350 000 tonnes en 2020 (www.grandviewresearch.com). La capacité de l'AF à être converti en produits pharmaceutiques et d'agir comme matériau de départ dans des réactions de polymérisation et d'estérification a conduit le Département américain de l'Énergie à désigner l'AF comme étant l'un des 10 meilleurs produits chimiques derives de biomasses avec un potentiel imports économiques uniiques (Xu *et al*., 2012a). De manière conventionnelle, l'AF est largement utilisé dans l'industrie alimentaire (comme un acidulant

contrôlant la croissance des micro-organismes, tout en permettant de réguler le pH, et d'améliorer la saveur) et dans l'industrie alimentaire pour les animaux (comme agent antibactérien et substance physiologiquement active) (Yang *et al*., 2011). La nourriture et les boissons ont représenté 33% de la consommation mondiale de FA en 2009, suivies par des papiers colophane (20,0%), les résines de polyester insaturées (18,6%), et des résines alkydes (12,3%) (IHS Chemical. Avril 2010). En plus des utilisations classiques, l'AF et ses dérivés esters (en anglais: FAEs) ont été étudiés pour un certain nombre de nouvelles applications dans la domaine des sciences biomédicales (Das *et al*., 2015a). Il convient de mentionner que la reconnaissance récente de la formulation de FAE Tecfidera (fumarate de diméthyle) pour traiter des adultes humains porteurs de formes récurrentes de sclérose en plaques par l'U.S. Food and Drug Administration (FDA, États-Unis) a été une réalisation importante (www.fda.gov). Les champs d'applications du FA se sont considérablement élargis suite à des recherches de pointe sur ses différentes propriétés.

La demande toujours croissante pour l'AF exige la mise aupoint de procédés de fermentation alternatifs utilisant des matières premières peu coûteuses, tels que les déchets agro-industriels. Des recherches ont été menées sur la production de FA à partir de copeaux de bois, de fumier de bovins laitiers, de glycérol brut et de biomasse lignocellulosique, comme la paille de maïs (Xu *et al*., 2012b;.. Roa Engel *et al*., 2008). Parmi les différents microorganismes testés pour la production de FA, le *Rhizopus oryzae 1526* (appelé *R. oryzae* par la suite) (famille des Mucoraceae) a été identifié comme étant l'une des souches les pls performantes pour produire du FA en raison de ses faibles besoins nutritionnels (Xu *et al*., 2012a ; Oda *et al*., 2003; Roa Engel *et al*., 2008). Récemment, on a mis en évidence une relation quantitative directe entre la morphologie de granules et une production accrue de FA avec cette souche (Zhou *et al*., 2011). En dehors de cette constatation, il est prouvé, que la formation de granules fongiques peut bénéficier à la production de FA enelles réduisant la viscosité moyenne, en réutilisant de la biomasse fongique et en augmentant l'efficacité des transferts de chaleur et d'oxygène (Li *et al*., 2000; Rodriguez Porcel *et al.*, 2005) .Toutefois, il est difficile de tirer des conclusions fermes de l'optimisation des paramètres à partir des différentes études réalisées sur la morphologie des champignons produisant le FA. Avec chaque nouvelle composition du milieu, les paramètres doivent être optimisés pour contrôler la morphologie de la souche fongique. Le défi est d'obtenir des granules de tailles réduites. Il existe toujours le problème de la morphologie des granules car elle peut conduire à la limitation de la diffusion des nutriments et des l'oxygène dans le bouillon de culture ainsi qu' à une réduction de la production de FA. Pour surmonter ces problèmes, une approche d'immobilisation a été adoptée pour améliorer la production de FA (Kautola *et al*., 1989; Petruccioli *et al*., 1996;. Gu *et al*., 2013.). Les mycéliums fongiques

immobilisés ont souvent des caractéristiques avantageuses, telles que la réduction ou l'élimination des problèmes de transfert de masse associés aux cultures de cellules en suspension couramment utilisées (Vassilev & Vassileva, 1992). Toutefois, les coûts des matériaux de support de la technique d'immobilisation peuvent faire varier de 60-70% le coût total du processus de production de FA. L'étude de matériaux compatibles utilisés dans des applications en technique d'immobilisation, ayant un faible coût est importante pour une production rentable de FA.

Dans la plupart des études réalisées sur la production de FA par fermentation, le carbonate de calcium ($CaCO_3$) est utilisé comme neutralisant pour maintenir un pH proche de la neutralité (6,0) afin de maximiser la production de FA. Après des décennies de recherche sur différents agents neutralisants [tels que, $CaCO_3$, Na_2CO_3, $NaHCO_3$, $(NH_4)_2CO_3$, et $Ca(OH)_2$)], le $CaCO_3$ s'est avéré être l'agent de neutralisation le plus efficace pour la production commerciale de FA (Xu *et al*., 2012a). Cependant, l'addition de $CaCO_3$ est non stœchiométrique, ce qui conduit à la consommation d'une grande quantité d'acides minéraux (HCl, H_2SO_4, etc.). De plus, de l'eau et des déchets de $CaSO_4$ sont produits en proportion lors de la récupération de FA. En outre, des mycéliums fongiques forment des hyphes qui se mêlent souvent au produit de fermentation de la phase soluble faiblement aqueuse, le fumarate de calcium ($CaC_4H_2O_4$) et le $CaCO_3$ solide inutilisé. Cela conduit à l'augmentation de la viscosité du bouillon de culture fermenté qui provoque des problèmes opérationnels, telles que les transferts de masse, la production de chaleur et le transfert d'oxygène qui ralentissent la production de FA. L'utilisation de $CaCO_3$ est inévitable pour récupérer l'AF à un pH bas (3,5), ce qui menerait à la diminution de la production de FA (Roa Angel, 2010). La diminution de la concentration de $CaCO_3$ et la simplification des traitements de récupération de FA sont un défi pour l'obtentionde FA par fermentation. En ce qui concerne le coût de $CaCO_3$, une source potentielle (à faible coût et durable) de $CaCO_3$ peut avoir un très bon impact sur les coûts du processus global de production de l'AF à grande échelle.

Une autre question importante pour la production de FA est la mise au point d'une méthode simple, rentable et rapide pour sa détermination spectrophotométrique. En général, l'AF présent dans un échantillon de bouillon de culture fermenté est quantifié en employant un équipement d'analyse sophistiqué, tel que l'HPLC. La littérature révisée sur la production de FA par fermentation montre que la présence de FA a été déterminée par la formation de fumarate mercureux insoluble dans l'acide nitrique à 5% en tant que méthode pour la quantification exacte de FA (Olander, 1929). Ainsi, le développement d'une méthode de

spectrophotométrie simple et rapide pour l'estimation de AF présent dans des échantillons de bouillon de culture fermenté peut être une option intéressante à étudier.

Ainsi, en prenant en considération tous les aspects importants du FA précédemment mentionnés, les études sur la pertinence de résidus et de sous-produits issus de différentes industries basées au Canada (jus de pomme, brasserie, pâte et papier) pour la production de FA par fermentation (submergée et à l'état-solide, SmF et SSF respectivement) peuvent certainement apporter une valeur ajoutée. L'amélioration de la production de FA par l'optimisation de la morphologie des champignos et par une stratégie rentable d'immobilisation en utilisant ces déchets industriels comme milieux de fermentation serait une approche intéressante. De plus, le développement d'une méthode spectrophotométrique simple et rapide pour l'estimation de l'AF, la réduction de la consommation et du coût $CaCO_3$ ainsi que la conception de nouvelles stratégies pour simplifier et raccourcir le traitement en aval de FA, peuvent représenter des résultats significatifs.

Pour une discussion détaillée sur la production et les applications du FA, le chapitre du livre (Das *et al*., 2015a) et la revue (Das *et al*., 2015b) présentés dans les chapitres 2 et 3 de cette thèse, , peuvent être consultés.

PARTIE II: PROBLÉMATIQUE

La littérature sur la production et sur divers aspects de l'application du FA a suggéré un plus grand effort de recherche afin de rendre la production de FA plus verte et économique. Nous en avons déduit les problèmes techniques suivants:

2.1. Risques associés aux voies chimiques de la production d'acide fumarique

Bien que l'AF ($C_4H_4O_4$) soit uniquement produit commercialement par l'isomérisation de l'acide maléique ($C_4H_4O_4$) obtenue à partir de l'hydrolyse de l'anhydride maléique, qui à son tour est produit à partir de l'oxydation du butane (C_4H_{10}) ou du benzène (C_6H_{16}), les voies chimiques imposent des facteurs de risque pour la santé humaine et l'environnement comme indiqué ci-dessous:

(a) La détérioration de l'environnement mondial causée par l'exploitation de produits pétroliers incluant le butane, la matière première pour la production de FA.

(b) La production de monoxyde de carbone (CO) et de dioxyde de carbone (CO_2) comme principaux sous produits de la réaction (Felthouse *et al.*, 2001).

(c) La prise de conscience des dangers que peuvent présenter certains additifs alimentaires sur la santé peut affecter l'utilisation de FA issu de la synthèse chimique dans un proche avenir.

(d) Le benzène est utilisé comme matière première pour la production de l'anhydride maléique, et est connu pour être cancérigène. Ce constat ouvre la voie à la recherche d'une alternative plus sécuritaire pour la production de FA (Kang *et al*., 2010).

2.2. Facteur coût et demande croissante d'acide fumarique

Comme le prix du pétrole augmente assez rapidement, l'anhydride maléique étant un dérivé du pétrole a vu son prix également augmenté. En outre, la demande toujours croissante pour des dérivés de FA exige l'utilisation de procédés de fermentation alternatifs utilisant des matières premières peu coûteuses, tels que certains déchets agro-industriels. Cela nécessite une action urgente. La production durable de FA à partir de matières premières à faible coût est fortement recommandée.

L'analyse des coûts de la production de FA via la pétrochimie et par des voies de fermentation a été examinée par Roa Engel *et al*. (2008). L'utilisation de matières premières à faibles coûts pour la production de FA par fermentation pourrait compenser les rendements plus élevés de la production pétrochimique de l'anhydride maléique donc la voie de fermentation pourrait devenir une alternative économiquement viable pour la production de FA à l'avenir.

2.3. Problème de la réduction des déchets agro-industriels et agro-alimentaires ainsi que leur valorisation

Étant donné l'escalade de la demande des consommateurs pour les produits alimentaires, d'énormes quantités (plusieurs tonnes par jour) de déchets agro-industriels et agro-alimentaires sont générées dans le monde chaque année. Par exemple, au Canada, une grande quantité de déchets solides (25-30%) et liquides (5-10% de boues) est issue de la transformation des fruits. La province de Québec est elle-même classée 10^{e} en Amérique du Nord pour la masse totale de ses déchets agro-industriels. Les industries de fabrication des jus de pomme, du papier et des brasseries génèrent des quantités importantes de déchets solides (pulpe de pommes, boues de pâtes et papier, drêche de brasserie, etc.) et de déchets liquides (par exemple, les boues d'ultrafiltration de jus de pomme, les eaux usées de brasserie (en anglais: BW)). Ces déchets sont riches en glucides et autres micro- et macro- nutriments ayant une teneur élevée en humidité et une charge organique facilement biodégradable (DBO (en anglais: BOD) élevée.

De même, les coquilles d'œufs (en anglais: EGS) sont l'un des bio déchets alimentaires générés en milliers de tonnes par an à travers le monde. Par exemple, le Mexique produit environ 480 000 tonnes de coquilles d'œufs par an (soit 1315 tonnes par jour) (Rivera *et al*., 1999). En 2007, la production du marché canadien d'œufs a atteint 521,1 millions de douzaines, dont 25% ont servi à l'industrie de la transformation des aliments (œufs pour être cassés). Cela représente 1,9 milliards d'œufs en coquille qui sont cassés dans les usines de transformation des aliments. Toujours en 2007, le Canada a produit 70,2 millions d'œufs à couver, 45% en Ontario et 19,2% au Québec, ce qui représente 13,5 millions d'œufs à couver produits au Québec cette année. Par conséquent, le Québec le deuxième plus grand producteur d'œufs au Canada, après l'Ontario (www.regioacton.ca).

En poids, environ 11% du poids total d'un œuf est représenté par sa coquille (EGS), ce qui est significatif en termes de déchets. En général, les EGS ne sont pas utilisés pour aucun produit à valeur ajoutée à l'exception de certaines applications comme engrais ou dans la nutrition humaine et/ou animale. Les EGS sont riches en carbonate de calcium ($CaCO_3$) (environ 94%) et contiennent également du phosphate de calcium (1%), de la matière organique (4%) et du carbonate de magnésium (1%), ce qui en fait un sous-produit important et souhaitable à valeur ajoutée. La disparition et la bonne gestion de ces déchets constituent une préoccupation à travers le monde. Décharger ces déchets dans l'environnement peut avoir de nombreux effets nocifs, tels que: (a) la production de gaz à effet de serre (en anglais: GHGs); (b) des pollutions secondaires (émission d'odeur causée par une attaque microbienne, contamination de l'eau souterraine) et (c) fournir un substrat et un abri aux micro-organismes et divers vecteurs qui peuvent entraîner des épidémies pour les populations environnantes. L'un des facteurs incontournables des coûts associés à la disparition des déchets est le transport et la main-d'œuvre engagés par les sociétés qui se traduisent par une hausse de prix pour les produits alimentaires. De plus, de nombreux pays développés ont comme objectif de diminuer le volume des déchets organiques et inorganiques et d'encourager leur valorisation par recyclage ou réutilisation.

Au Canada, la croissance des industries agro-alimentaires a généré une énorme quantité de déchets solides (25-30%) et liquides (5-10% de boues) issus de la transformation des fruits. Chaque année, des milliers de tonnes de déchets solides de jus de pomme (en anglais: AP) et des boues d'ultrafiltration de jus de pommes (en anglais: APUS) sont générés par les industries de transformation de pomme au Canada. Selon un rapport canadien publié en 2014, la production totale de pommes en 2013 était d'environ 382 millions de kg dont 28% était originaire de la province du Québec (la première étant l'Ontario avec 40%) (www.hortcouncil.ca). Le Canada, avec une forte demande pour les produits à base de

pommes, génère une grande quantité de biomasse de déchets de AP et leur élimination plus sécuritaire est un défi en raison de considérations environnementales (Gasara *et al*., 2011a). Par ailleurs, la production de déchets de papier d'origine industrielle tels que les déchets solides de pâtes et papiers (en anglais: PPSW) est encore très élevée au Canada. Il y a environ 130 entreprises industrielles de pâtes et de papier au Canada, pour la plupart située dans les provinces de Québec, de Colombie-Britannique et d'Ontario. Plus d'un tiers du total des déchets du Canada sont des PPSW et seulement un quart de ceux-ci et des cartons sont recyclés (www.ec.gc.ca). Ces industries subissent des pertes en raison du traitement des déchets et du coût de transport des rejets dans les sites d'enfouissement; elles sont affectées par les nouvelles tendances de la réglementation en termes d'interdiction de mise en décharge des déchets bio-solides (Dhillon *et al*., 2011).

Avec l'avènement d'innovations biotechnologiques, principalement dans le domaine de la technologie de la fermentation, de nouvelles pistes ont été ouvertes pour une meilleure valorisation des déchets agro-industriels *et al*imentaires. À cet égard, les AP, APUS, BW etc. peuvent servir à la production de FA par fermentation microbienne. Cela devrait rendre la production de FA plus économique (coûts de substrat inférieurs de 60-70%) et contribuer à la réduction des déchets (c.-à-d. plus de respect de l'environnement). De même, la valorisation des EGS comme source potentielle de $CaCO_3$ pour une application dans la production de FA peut diminuer de façon significative les coûts de production totaux de FA.

2.4. Défis dans le traitement en amont de la production d'acide fumarique par fermentation

(a) Aucune méthode spectrophotométrique simple et rapide n'est disponible pour l'estimation des teneurs en FA présentes dans le bouillon de culture fermenté.

(b) La production de FA par SmF avec la souche fongique *R. oryzae* requiert d'une optimisation des conditions de fermentation (pH, température, vitesse de rotation, durée, etc.) pour chaque nouvelle source de carbone utilisée. Il n'existe pas de conditions de SmF optimisées pour une production accrue de FA.

(c) Étant donné que la morphologie des granules de la souche fongique inoculée joue un rôle critique dans la production de FA par SmF, son optimisation a été un défi pour chaque nouvelle source de carbone utilisée, tout en gardant la souche fongique (*R. oryzae*) fixe.

(d) La nécessité d'utiliser le $CaCO_3$ comme agent neutralisant est inévitable. Toutefois, aucun rapport n'était disponible sur le coût et la source la plus écologique de cet ingrédient important pour la production de FA par fermentation.

(e) Aucune recherche active n'avait été effectuée sur l'étude de matériaux peu coûteux à utiliser comme support d'immobilisation pour la souche fongique pour la production de FA par SmF.

(f) L'application de la SSF pour la production de FA était un domaine de recherche peu exploré par rapport à la SmF. En effet, aucune information n'a été trouvée sur la production à grande échelle de FA par SSF.

(g) L'utilisation de la nanotechnologie dans la production par fermentation de FAde FA n'a jamais été tentée. Ainsi, il était intéressant d'explorer cette voie pour une une éventuelle application de cette technologie dans le domaine de la production de FA par SmF.

2.5. Problématique du traitement en aval et de la récupération de l'acide fumarique

Même après plusieurs années de recherche active sur le traitement en aval de FAde FA, le problème demeurait encore dans le développement d'une méthode de récupération facile et rentable de FA de FA à partir du bouillon fermenté. Un des écueils majeurs a été l'ajout non stœchiométrique de $CaCO_3$ comme agent neutralisant, ce qui a eu comme effet d'abaisser les rendements de FA. De plus, ceci a généré de grandes quantités de déchets (e.g, $CaSO_4$) provenant de la réaction entre un acide minéral (H_2SO_4 ou HCl), $CaCO_3$ non réactif et le fumarate de calcium ($CaC_4H_2O_4$). En outre, le chauffage du bouillon fermenté à haute température jusqu'à ce qu'il devienne clair est un processus qui consomme du temps et de l'énergie. Aborder ces deux problèmes en faisant appel à des nouvelles technologies (par exemple les nanotechnologies et la technologie de micro-ondes) a été une bonne voie de solutions ouvrant sur de nombreuses possibilités. Ceci a été étudié dans le cadre de ce travail de recherche.

PARTIE III: HYPOTHÈSES, OBJECTIFS ET ORIGINALITÉ DE LA RECHERCHE

3.1. HYPOTHÈSES

Ce travail de recherche comprend les hypothèses suivantes:

1. Comme la littérature le suggère, différentes espèces fongiques de *Rhizopus* produisant de FAont été cultivées avec succès à partir de déchets organiques, sans supplément d'aucun nutriment et ont abouti à une bonne qualité de produits de FA. Les déchets agro-industriels, tels que AP, APUS, BW, PPSW sont riches en glucides et autres nutriments essentiels. Ces déchets de production ou transformation peuvent être utilisés en tant que substrats pour la production de FA par SmF et SSF sans aucun ajout d'autre supplément nutritionnel afin de faire croître la souche fongique *R. oryzae* utilisée dans la présente recherche.

2. La simple comparaison des voies pétrochimiques et des voies de fermentation pour la production de FA suggère que le faible coût des matières premières utilisées dans les procédés de fermentation peut compenser les hauts rendements de la production pétrochimique de FA à partir de l'anhydride maléique. La fermentation peut devenir une alternative économiquement viable pour la production de FA.

3. La souche fongique *R. oryzae* est très sensible à divers facteurs physico-chimiques tels que le pH, la température, le temps d'incubation, la concentration totale en solides (g/L), la nature des substrats utilisés, le volume d'inoculum utilisé (v/v) de FA. L'optimisation de toutes les conditions de fermentation peut améliorer la production de FA.

4. Il existe maintenant un consensus adopté par tous que la formation de granules fongiques peut être bénéfique à la fermentation de FA. Ces granules réduisent la viscosité moyenne, améliorent la réutilisation de la biomasse fongique, et augmentent l'efficacité des transferts de masse, de chaleur et d'oxygène. Tous ces paramètres conduisent à une production plus élevée de FA. Cependant, on ne peut tirer de conclusions solides concernant les paramètres optimisés rapportés par différentes études. Ainsi, à chaque nouvelle composition du milieu de culture, les paramètres doivent être optimisés pour contrôler la morphologie de la souche fongique. Par conséquent, la souche fongique et les déchets agro-industriels (AP, APUS, BW et PPSW) utilisés dans la présente étude peuvent entraîner une production accrue de FA grâce à l'optimisation des conditions de fermentation pour la formation de granules.

5. Les filaments fongiques supportent mal la chaleur, l'oxygène et le transferts de masse durant la fermentation. De plus, il est difficile de contrôler la taille des pastilles fongiques et cela peut conduire à la limitation de la diffusion dans le bouillon de culture et engendrer une production réduite de FA. Pour surmonter ces difficultés, une approche d'immobilisation peut être développée pour améliorer la production de FA. L'immobilisation facilite les transferts de gaz, de chaleur et de masse à travers le mycélium qui se développe sous forme de biofilm; on devrait donc obtenir une conversion plus élevée du substrat dans le produit désiré. Les cellules immobilisées sur des supports par adhésion sont en contact direct avec la phase liquide contenant les substrats, même si la cellule et les phases liquides sont superficiellement séparées. Ceci réduit ou élimine les problèmes de transfert de masse associés aux cultures de cellules en suspension couramment utilisées. En utilisant le support et les géométries appropriés, un ratio volume/surface élevé peut être maintenu.

6. Le coût des matériaux des supports d'immobilisation peut représenter entre 60 et 70% du coût total du processus de FA. Ainsi, pour rendre la production de FA plus durable et rentable, le coût des matériaux d'immobilisation doit être minimisé. L'utilisation de matériaux compatibles à faible coût pour l'immobilisation peut être une très bonne option.

7. L'application de nanoparticules de $CaCO_3$ (CCNPs) comme agent neutralisant dans la production de FA peut fournir plus de surface pour les molécules de FA pour les réactions chimiques et donc réduire le temps de réaction de neutralisation entre le FA et les CCNPs. De plus, les CCNPs peuvent réduire la viscosité du bouillon de culture par rapport aux micro particules de $CaCO_3$ (CCMPs).

8. Le produit de fermentation et les molécules de fumarate de calcium ($CaC_4H_2O_4$), interagissent étroitement avec le mycélium opaque de biomasse fongique du bouillon de culture et ceci est un facteur important dans la consommation totale d'énergie thermique pendant le processus de récupération de FA. L'amélioration de la dissolution par chauffage aux micro-ondes (en anglais: MWI) est un concept innovant qui peut en outre accélérer la dissolution et augmenter la solubilité d'une substance. La MWI est une voie écologique et efficace pour obtenir un chauffage plus rapide et un taux de dissolution plus élevé. L'application de la MWI peut rendre le processus de récupération de FA plus efficace en termes de temps et moins coûteux en terme d'énergie.

9. Comme la littérature le suggère, les EGS pourraient être expérimentés également comme support d'immobilisation pour les champignons et ce serait une nouvelle

approche à explorer pourla production de FA par fermentation submergée (SmF). De plus, les EGS sont une source très riche en $CaCO_3$ et la production de FA nécessite l'utilisation de $CaCO_3$ comme agent neutralisant et source de CO_2 (pour la synthèse de l'acide oxaloacétique dans le cycle de tricarboxylique et la voie de carboxylation réductrice). Il est donc pertinent d'explorer l'utilisation des EGS pour la production par SmF de FA.

10. Comme la SSF offre une productivité plus élevée par rapport à la SmF, l'application d'AP et de déchets de pâte et papier (PP) pour améliorer la production de FA par SSF est justifiée.

11. La détermination spectrophotométrique de FA dans l'échantillon de bouillon fermenté n'est pas une approche méthodologique commune. Un équipement d'analyse sophistiqué, tel que l'HPLC, est généralement utilisée pour l'estimation des teneurs en FA. La littérature portant sur la production par fermentationde FA montre que la présence d'acide fumarique a été déterminée semi-quantitativement par la formation de fumarate mercurique insoluble dans l'acide nitrique à 5% et qu'elle est la méthode utilisée pour l'obtention d'une quantification exacte de FA. Ainsi, le développement d'une méthode de spectrophotométrie simple et rapide pour l'estimation des teneurs en FA présent dans des échantillons de bouillon fermenté peut être une option intéressante à explorer pour simplifier le dosage quantitatif.

12. La morphologie du champignon *R. oryzae* est influencée par la présence de métaux à l'etat traces tels que Zn^{2+}, Fe^{2+} et Mn^{2+}. Les caractéristiques morphologiques de la souche affectent le profil de production des produits fermentés dont le FA. La présence et les concentrations de Zn^{2+}, Fe^{2+} et Mn^{2+} dans le milieu de culture sont importantes étant donné que ces trois éléments ont démontré leur influence sur la formation des granules pour différentes espèces de *Rhizopus*. Cependant, des études utilisant des nanoparticules (NPs) de Zn^{2+}, Fe^{2+} et Mn^{2+} n'ont pas encore été réalisées avec *R. oryzae.*

3.2. OBJECTIFS

L'objectif global de cette étude est la mise en œuvre des concepts d'«économie de glucides» et de «Bio-raffinerie» pour la production par fermentation de FA par SmF et SSF. Les principaux objectifs sont axés sur l'optimisation de la production de FA en présence de *R. oryzae* en utilisant des résidus des agro-industries et des industries alimentaires, à faible coût. Les objectifs spécifiques suivants ont été élaborés à partir de la revue de la littérature:

1. Le développement d'une méthode d'analyse spectrophotométrique rapide et efficace pour l'estimation des teneurs en FA présent dans le bouillon fermenté.

2. L'utilisation de différents déchets agro-industriels (BW, PPSW, APUS) comme milieux de fermentation pour la production accrue de FA par SmF en utilisant une souche fongique de *R. oryzae*. L'optimisation des paramètres opératoires (pH, température, vitesse d'agitation, concentration totale en solides et proportion de l'inoculum) sera effectuée pour obtenir une bonne morphologie de granules et une plus grande quantité de FA.

3. L'application de la technique d'immobilisation pour améliorer la production de FA. Des matériaux à faibles coûts et facilement disponibles seront utilisés pour immobiliser *R. oryzae* afin d'améliorer l'économie du procédé.

4. Afin de réduire ultérieurement les coûts de production totaux de FA, les EGS seront utilisés comme source potentielle de $CaCO_3$ (94%) dans la production de l'AF. Les EGS seront également testés comme support d'immobilisation pour le champignon afin d'améliorer la production de FA.

5. L'utilisation des CCMPs et des CCNPs comme agents de neutralisation dans la production de FA et l'analyse comparative des temps de réaction pour la neutralisation du bouillon de culture, la réduction de la viscosité du milieu et l'augmentation des rendements en FA.

6. L'utilisation de la méthode MWI dans le processus de récupération de FA. Une étude comparative du temps de réaction et de la consommation de chaleur sera effectuée par rapportà la méthodologie classique.

7. L'utilisation de la SSF pour la production de FA en utilisant les AP et PPSW sera utilisée. Les paramètres opératoires seront optimisés.

8. La production à grande échelle de l'AF par SSF en utilisant les AP sera évaluée.

9. L'étude des effets des NPs de ZnO, Fe_3O_4 et MnO_2 sur la morphologie du champignon *R. oryzae* et la production de FA sera effectuée.

3.3. ORIGINALITÉ

La présente étude comporte les points originaux:

1. Jusqu'à présent, les BW et APUS n'ont pas été utilisés pour la biosynthèse de l'AF par SmF avec la souche fongique *R. oryzae*.

2. Il n'existe pas de méthode spectrophotométrique publiée, simple et rapide pour la quantification de FA présent dans des échantillons de bouillon de culture fermenté.

3. Bien que la technique d'immobilisation pour la production de FA soit une voie déjà explorée, le facteur de rentabilité n'a pas été étudié dans ces études. L'étude de divers matériaux efficaces et peu coûteux, tels que chiffons de mousseline (en anglais: MC) n'a jamais été réalisée pour l'immobilisation de la souche fongique *R. oryzae* en vue de la production de FA par SmF.

4. Dans les études d'immobilisation pour la production de FA, les EGS n'ont jamais encore été utilisées comme matériau d'immobilisation; aucun rapport sur l'application des EGS comme source d'agent neutralisant ($CaCO_3$) dans la production de FA n'a été signalé avant ce travail de recherche.

5. L'application de CCNPs comme agent neutralisant dans la production de FA est une approche innovante intégrant la nanotechnologie et l'ingénierie biochimique.

6. L'amélioration de la dissolution du FA par la méthode MWI est un concept innovant jamais appliqué aux procédés de récupération de FA par fermentation.

7. Considérant que les rapports sur la production de FA par SSF sont rares, la production de FA à partir de JP et de PP par SSF en utilisant le *R. oryzae* est une approche innovante.

8. La production de FA à l'échelle pilote sur substrats solides et dans un fermenteur à tambour rotatif (BSF) et l'étude des différents mécanismes sous-jacents n'ont jamais été réalisées avant ce travail de recherche.

9. Aucune étude antérieure n'a été faite pour observer et mesurer les effets des NPs de ZnO, Fe_3O_4 et MnO_2 sur la morphologie du champignon *R. oryzae* champignon et la production de FA par fermentation.

Dans l'ensemble, l'originalité de la recherche proposée est, **"L'amélioration de la production d'acide fumarique à partir de déchets agro-industriels par fermentation submergée et substrats solides avec la mise en œuvre de différentes techniques dans une optique de réduction des coûts de production"**.

PARTIE IV. SOMMAIRE DES DIFFÉRENTS VOLETS DE RECHERCHE EFFECTUÉS DANS CETTE ÉTUDE

Un aperçu simplifié de ce travail de recherche est présenté à la Figure 1.1. De plus, des études spécifiques menées dans le cadre de cette thèse ont été classées en chapitres énoncés ci-dessous:

4.1. Les aspects de la production et des applications (conventionnelles et biomédicales) de l'acide fumarique (Chapitre 2, un chapitre de livre et un article de revue publiés)

4.2. L'utilisation des eaux usées de brasseries pour la production d'acide fumarique par fermentation submergée en milieu liquide et le développement d'une méthode spectrophotométrique simple et rapide pour la détermination des teneurs en acide fumarique (Chapitre 3, un article de recherche publié)

4.3. La production d'acide fumarique par des techniques rentables et durables (Chapitre 4, partie I et partie II, deux articles de recherche publiés)

4.4. L'utilisation de déchets solides de pâte et de papier pour la production d'acide fumarique par fermentation submergée et en milieu solide (Chapitre 5, un article de recherche publié)

4.5. L'utilisation de déchets liquides et solides de l'industrie de transformation des pommes pour la production d'acide fumarique par fermentation submergée et en milieu solide (Chapitre 6, partie I et partie II, deux articles de recherche publiés)

4.6. Les effets de différentes nanoparticules métalliques sur la germination et la morphologie du champignon *Rhizopus oryzae* 1526 et leur impact sur la production d'acide fumarique (Chapitre 7, un article de recherche publié)

4.7. Les applications de nanoparticules de carbonate de calcium et du chauffage par micro-ondes dans le traitement en amont et en aval de l'acide fumarique (Chapitre 8, un article de recherche soumis)

4.1. Aspects de la production et des applications (conventionnelles et biomédicales) de l'acide fumarique (Chapitre 2)

4.1.1. Aspects de la production et des applications de l'acide fumarique (Chapitre 2, Partie I, un chapitre de livre publié)

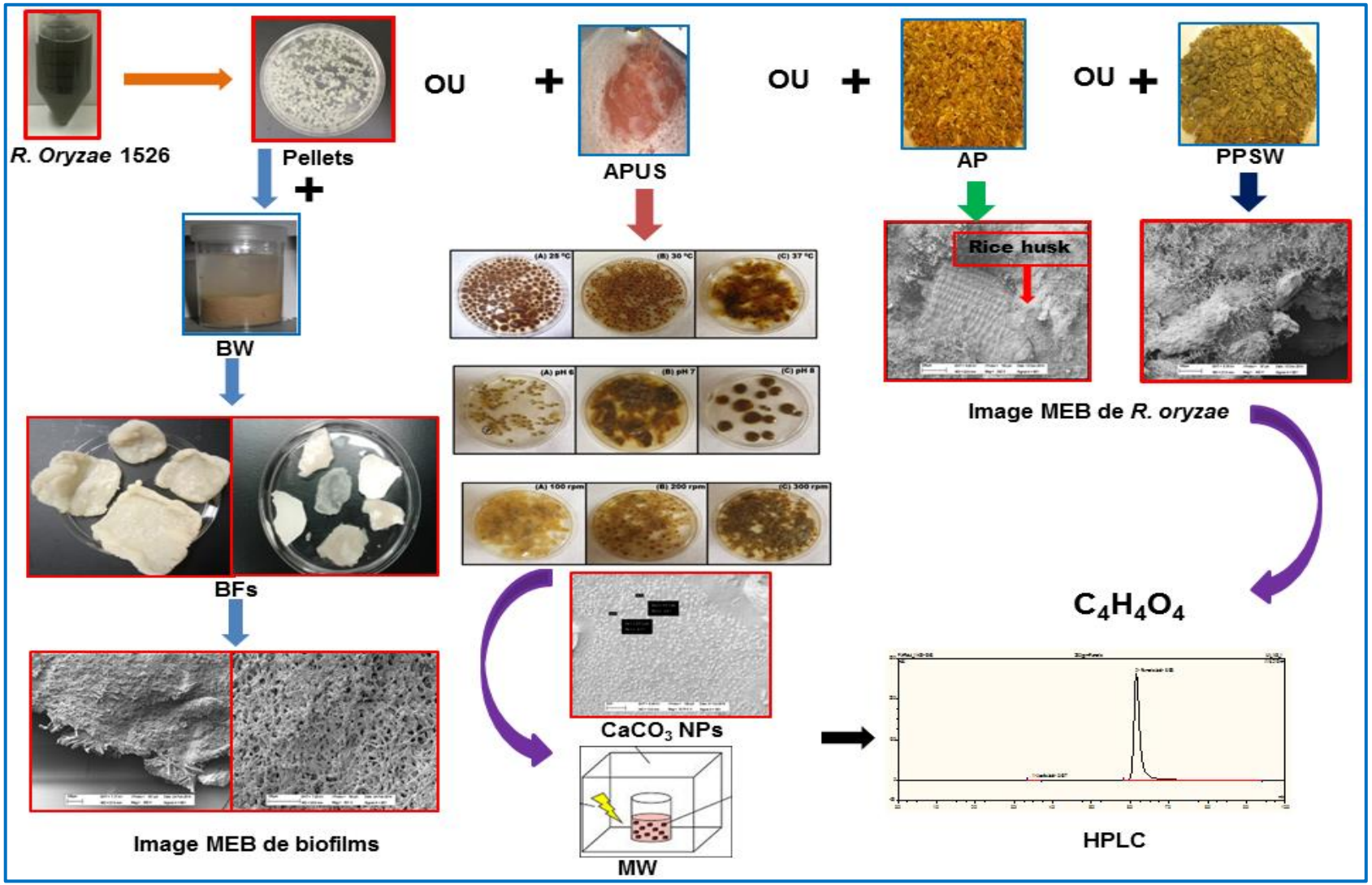

Figure 1.1 : Représentation schématique de l'ensemble des conclusions de la présente recherche

La documentation disponible sur les applications et la production de FAa fait l'objet d'un forum de discussions mais s'est limitée à une approche traditionnelle. Nous sommes d'avis que les problèmes clés liés à la production de FA doivent être abordés avec de nouvelles propositions et les progrès accomplis dans les applications de FA doivent être pris en considération. En outre, aucune donnée n'est disponible sur les facteurs génétiques qui mettent en exacte corrélation la production de FA à la morphogenèse fongique dans des conditions submergées. La littérature suggère que l'immobilisation fongique sur un support solide peut améliorer la production de FA. Cependant, le coût des matériaux utilisés comme support d'immobilisation peut représenter de 60 à 70% du coût total du procédé. L'utilisation de nouveaux matériaux à moindres coûts pour l'immobilisation peut rendre le processus de production de FA par fermentation intéressant d'un point de vue économique.

La fermentation à l'état solide (SSF) pour la production de FA est un domaine inexploré et celle-ci offre une productivité plus élevée que la SmF. L'expérimentation de nouveaux déchets solides pour la SSF est une bonne plate-forme pour élargir le spectre de production de FA. La production à faibles coûts de FAà l'échelle commerciale est encore un défi à cause du traitement en aval et des problèmes inhérents à la biomasse du champignon filamenteux. Une solution possible au problème susmentionné est l'application de nanoparticules de $CaCO_3$ au lieu de la poudre (de taille microscopique) utilisée généralement. Dans le traitement d'aval du FA, la technologie de chauffage par micro-ondes (MWI), qui est une voie écologique et efficace d'élever plus rapidement la température et d'accélérer lavitesse de dissolution, présente un champ d'application très intéressant pour la récupération rapide de FA à partir du bouillon de culture. En dehors des pratiques usuelles, le FA et ses dérivés esters (FAEs) ont été explorés pour des applications plus innovantes et prometteuses dans les sciences de la santé en particulier.

4.1.2. Progrès récents dans les applications biomédicales de l'acide fumarique et ses dérivés esters: Les alternatives thérapeutiques multiples (Chapitre 2, Partie II, un article de revue publié)

Des recherches cliniques sur les effets pharmacologiques de l'AF et de ses dérivés esters (en anglais: FAEs) ont confirmé leurs propriétés curatives pour le psoriasis humain, pour la forme rémittente de sclérose en plaques (FRSP, en anglais: RRMS), pour les troubles neurocognitifs associés au VIH (en anglais: HIV) et aussi pour l'agent du VIH responsable de la maladie avec de hauts degrés d'efficacité. De plus, les FAEs ont été étudiés pour des applications en ingénierie du tissu osseux (constructions orthopédiques) et comme composant de vecteurs de médicaments. Des efforts ont été faits pour étudier la pharmacocinétique des différents FAEs dans le corps humain. Plusieurs études in vivo

menées sur des rats et des souris ont montré l'effet inhibiteur du FAsur la cancérogénèse de différentes origines. Ces études ont fourni des indices importants sur la nouvelle application probable du FAcomme un agent anti-cancérigène. Cependant, jusqu'à maintenant, la littérature collective sur les applications biomédicales du FAet des FAEs n'a pas été abondante et suffisamment documentée.

4.2. Utilisation des eaux usées de brasseries industrielles pour la production d'acide fumarique en fermentation submergée et le développement d'une méthode spectrophotométrique simple, rapide et peu coûteuse pour la détermination de l'acide fumarique (Chapitre 3)

Dans cette étude, les eaux usées de brasseries (en anglais: BW) ont été utilisées comme substrat pour la fermentation submergée (SmF) et l'optimisation des paramètres a été réalisée afin d'améliorer la production de FA. La souche de champignon *Rhizopus oryzae* NRRL 1526 a été utilisée comme biocatalyseur. Dans cette étude, les conditions du procédé de SmF (concentrations en solides totaux, pH, température, vitesse d'agitation , température et concentrations de l'inoculum) ont été optimisées. La morphologie des granules cellulaires de *R. oryzae* a été étudiée par microscopie électronique à balayage (en anglais: SEM). Une méthode spectrophotométrique a également été développée pour une quantification rapide et peu coûteuse de FA des teneurs en FA présent dans les échantillons de bouillon fermenté. Avec toutes les conditions optimisées (pH 6, 25 °C, 200 tours par minute, 5% (v/v) de volume d'inoculum, 25 g/L de solides totaux et un diamètre de pastille fongique de 0,47 ± 0,04 mm), la plus forte concentration d'acide fumarique obtenue a été de 31,3 ± 2,8 g/L.

4.3. Production d'acide fumarique par des techniques rentables et durables (Chapitre 4, partie I et partie II, deux articles de recherche publiés)

4.3.1. Un chiffon de mousseline et des eaux usées de brasserie pour améliorer la bio-production de l'acide fumarique (Chapitre 4, partie I, un article de recherche publié)

Dans la présente étude, un "chiffon de mousseline " (en anglais: MC) a été utilisé comme support solide pour le champignon *R. oryzae* et les BW ont été utilisés comme source de carbone pour la production de FA par SmF. Pour optimiser les paramètres de production de FA pour des cellules libres et immobilisées, quatre tailles différentes de MC (longueur x largeur = 2 x 2 cm^2, 3 x 3 cm^2, 4 x 4 cm^2 et 5 x 5 cm^2) et quatre concentrations de spores (1.0, 1.5, 2.5 et 3 x 10^6 par mL) ont été testées. Les morphologies des granules cellulaires et le biofilm de *R. oryzae* ont été caractérisés par microscopie électronique à balayage (en anglais: SEM). Sous des conditions de croissance de 30 °C et 200 tours par minute, la concentration la plus élevée de FA obtenue a été de 30,6 ± 1,40 g/L 72 h de SmF avec des

cellules libres. La concentration la plus élevée de FA (41,5 ± 0,85 g/L) avec cellules immobilisées a été obtenue avec un MC de 25 cm^2. Pour la taille de MC optimisée à 25 cm^2, la concentration optimum de spores était de 1,5 x 10^6 par mL. Les taux spécifiques de production d'acide fumarique (en anglais: SPRs) ont également été estimés (g/g h). Le SPR pour les cellules libres était de 3,395 g/g.h après 72 h de SmF. Pour la SmF immobilisée, le SPR a augmenté progressivement par des MC de 4 cm^2 (0,522 g/(g.h)) à 25 cm^2 (3,496 g/(g.h)). L'analyse par SEM des biofilms de *R. oryzae* développée pour les MCs de tailles différentes a confirmé la croissance uniforme du mycélium sur les deux faces des MCs.

4.3.2. Application de coquilles d'œuf comme surface d'immobilisation et une source de $CaCO_3$ dans l'amélioration de la bio-production d'acide fumarique à partir d'eaux usées de brasserie et de milieux de sels de glucose basique (Chapitre 4, partie II, un article de recherche publié)

Dans la présente étude, des coquilles d'œuf ont été utilisées (en anglais: EGS) pour l'immobilisation de *R. oryzae* en vue de la production de FA par SmF et également comme agent neutralisant. Les BW et le milieu de base de glucose-sel (en anglais: GSM) ont été testés individuellement pour des essaies d'optimisation du nombre de EGS, des concentrations de spores, du temps d'incubation pour l'immobilisation, de la vitesse d'agitation du ballon et du nombre de biofilms (1, 2, 3 et 4). Ces derniers ont été alors optimisés pour une meilleure production de FA à la fois avec les BW et les GSM. L'analyse minérale des BW a été réalisée avec la technique de spectroscopie au plasma à couplage inductif (en anglais: ICP). Le microscope électronique à balayage a été utilisé pour étudier la morphologie de la surface du biofilm. Les résultats ont confirmé les effets du nombre et de l'épaisseur de biofilm pour la production de FA. Les EGS au nombre de trois, une concentration de 1,0 x 10^6 spores par mL, 24 h de temps d'incubation, 150 tours par minute d'agitation en flaconont été optimisés pour la formation de biofilms avec les BW. Par rapport aux cellules libres, les biofilms obtenus par SmF en utilisant les BW ont manifestement amélioré la production et la productivité volumétrique d'acide fumarique de 30,23 ± 1,23 à 47,22 ± 0,77 g/L (c'est-à-dire près de 56%) et de 0,419 à 1,657 g/(g h) (c'est-à-dire par 3,95 fois), respectivement.

4.4. Utilisation des déchets solides de pâtes et papiers pour la production d'acide fumarique par fermentation submergée et fermentation à l'état solide (chapitre 5, un article de recherche publié)

Dans cette étude, les DSPP (en anglais: PPSW) ont été utilisés comme substrats solides et liquides pour la production de FA par SmF et SSF. La souche fongique *R. oryzae* a été

utilisée comme biocatalyseur. La caractérisation physico-chimique des PPSW a été effectuée avant leur utilisationcomme substrat. Le traitement mécanique a conduit à différentes tailles de particules (1,7 mm < x ≤ 3.35 mm, 850 µm < x ≤ 1,7 mm, 300 µm < x ≤ 850 µm, 75 µm < x ≤ 300 µm et 33 µm < x ≤ 75 µm) de PPSW. Les particules de PPSW de toutes tailles ont été prétraitées par hydrolyse acide dans un four micro-ondes avantla fermentation submergée (en anglais: SmF). Les hydrolysats ont également été testés pour la production de FA. Le prétraitement mécanique (réduction de la taille) des PPSW a facilité la libération des sucres. Une plus forte concentration de glucose et de xylose a été obtenue lorsque la taille des particules a été réduite. L'hydrolyse des particules de PPSW de taille comprises entre 33 µm < x ≤ 75 µm a permis de produire 11,2 ± 0,8 g /L de glucose et 20,22 ± 0,85 g/L de xylose. Dans l'étude de la SmF, pour une concentration fixée de 5% p/v en solides totaux de PPSW, la production de FA est passée de 12,24 à 23,47 g/L avec une diminution de la taille des particules. La productivité volumétrique de FA a également considérablement augmentée et est passée de 0,127 à 0,488 g/(L.h), avec des plateaux en plastique (longueur x largeur x hauteur = 35 cm × 22 cm × 11 cm) utilisés comme bioréacteurs pour la fermentation à l'état solide (en anglais: SSF). Des tailles de particules de 850 µm < x ≤ 300 µm ont été optimisées pour la production de FA par SSF. À la fin des 21 jours de SSF, la concentration de FA a atteint 41,65 g/kg de DSPP sec pour cette gamme de taille de particules. L'analyse au microscopie électronique à balayage des mycéliums de *R. oryzae* cultivés sur les particules de PPSW a révélé une pénétration du mycélium fongique en profondeur dans le substrat et une croissance dense du mycélium sur toute la surface des particules.

4.5. Utilisation des déchets liquides et solides d'usines de transformation de la pommes pour la production d'acide fumarique par fermentation submergée et en milieu solide (Chapitre 6, partie I et partie II, deux articles de recherche publiés)

4.5.1. Approche par fermentation vers l'optimisation de la biosynthèse directe d'acide fumarique avec le *Rhizopus oryzae* 1526 en utilisant des résidus d'usines de transformation de la pomme (Chapitre 6, partie I, un article de recherche publié)

Dans cette étude, les boues d'ultrafiltration de jus de pomme (en anglais: APUS) ont été utilisées comme substrat pour la production par SmF de FA et la souche fongique *R. oryzae* a été utilisée comme biocatalyseur. Quatre différents paramètres à savoir la concentration en solides totaux du substrat, le pH, la vitesse d'agitation du flacon et la température d'incubation ont été optimisés pour une production accrue de FA. Pour l'étude de la SSF, des plateaux en plastique (longueur x largeur x hauteur = 35 cm × 22 cm × 11 cm) ont été utilisés comme bioréacteurs. La photographie numérique a été utilisée pour l'étude

morphologique du champignon *R. oryzae*. Pour la SmF, avec toutes les conditions optimisées (40 g/L en solides totaux, 30 ^{o}C, pH 6 et 200 tours par minute), la concentration de FA atteinte était de 25,2 ± 1,0 g/L avec une productivité volumétrique de 0,350 g/(L.h). Dans l'étude de la fermentation à l'état solide (en anglais: SSF), après 14 jours d'incubation, la production maximale atteinte a été de 52 ± 2 g/kg avec 50% d'humidité (p/p). L'analyse par microscopie électronique à balayage de l'échantillon de SSF a montré la croissance du mycélium de *R. oryzae* sur la surface des particules d'AP. L'analyse du contenu phénolique et l'évolution de sa concentration avant et après la SSF a confirmé la biodégradation des polysaccharides (cellulose et hémicellulose) lié à la lignine du JP par le *R. oryzae* pour l'acquisition de la nutrition.

4.5.2. Bioconversion de résidus solides de jus de pommes en acide fumarique dans un fermenteur à tambour rotatif et l'étude des différents mécanismes sous-jacents (Chapitre 6, partie II, un article de recherche publié)

Un fermenteur de laboratoire (stérilisable in-situ) en milieu solide à Tambour rotatif (en anglais: BSF) a été utilisé pour l'étude de la SSF et des différents mécanismes impliqués dansla bio-conversion des AP en FA. Trois modes de fonctionnement (rotation continue, rotation intermittente et mode statique) du BSF ont été testés pour la production de FA. L'estimation des fibres alimentaires totales (en anglais: TDF), des fibres alimentaires insolubles (en anglais: IDFI) et des fibres alimentaires solubles (en anglais: SDF) d'échantillons de AP séchés fermentés et non fermentés a été réalisée. La capacité de rétention d'eau (en anglais: WHC) de AP et le contenu phénolique total (en anglais: TPC) ont été estimés à l'aide de méthodes classiques. Les conclusions générales sur les effets du taux d'humidité (en anglais: MC) et le mode de rotation du BSF sur la production de FA ont suggéré que 50% du MC et une rotation continue (2 tours par minute) étaient des paramètres optimums pour une production accrue de FAde FA (138 ± 9, g/kg à la fin des 14 jours). La WHC de l'AP était aux alentours de 8,12 ± 1,25 g d'eau par gramme de poids sec d'AP. L'analyse de la composition en fibres des AP a montré des changements dans le contenu des FAT, des FAI et des FAS avant et après la SSF. L'augmentation du nombre de spores (de 5×10^4 à $3,2 \times 10^8$ spores par g d'échantillons séchés d'AP) durant la SSF a indiqué que l'AP serait une bonne source de carbone et de micronutriments. Par rapport au glucose et au saccharose, le fructose a été consommé très rapidement par *R. oryzae*. Les résultats ont confirmé que le champignon convertit biologiquement les sucres et les biomasses lignocellulosiques présents dans les AP en FA dans des conditions optimales de SSF.

4.6. Effets de différentes nanoparticules métalliques sur la germination et la morphologie du champignon *Rhizopus oryzae* 1526 et leur impact surla production d'acide fumarique (Chapitre 7, un article de recherche publié)

L'influence de faibles concentrations de cations métalliques à savoir Zn^{2+}, Fe^{2+} et Mn^{2+} sur la germination des spores, la morphologie de *R. oryzae* et la production de FA ont été étudiées dans des milieux de base glucose-sel. Les microparticules (en anglais: MPs) et les nanoparticules (en anglais: NPs) de ces trois éléments ont été testées à différentes concentrations. À la plus faible concentration appliquée (200 µg/mL), le pourcentage de germination de spores est presque identique (96 à 98%) aux témoins. Des concentrations plus élevées (> 200 µg/mL) de MPs et de NPs ont causé l'inhibition de la germination des spores. Les champignons ont montré des changements dans la taille des granules (mm) et dans la morphologie (à partir de granules de mycélium en suspension) à différentes concentrations de MPs et NPs. Pour les MPs, la production de FA a également été influencée par des micro et nanoparticules de ZnO, Fe_3O_4 et MnO_2. Parmi les trois NPs, les NPs de ZnO ont entraîné de plus faible production de FA (5,22 ± 1,45 g/L) à la concentration la plus faible appliquée de 200 µg/mL. À cette concentration de NPs, la productivité de FA pour les NPs de Fe_3O_4 et MnO_2 était de 36 ± 2,3 et de 18,8 ± 1,8 g/L, respectivement. Les résultats de la présente étude ont confirmé l'influence du type de formulations (nano ou micro) de ZnO, Fe_3O_4 et MnO_2 sur la morphologie du champignon *R. oryzae* et sur la production de FA. Le champignon *R. oryzae* a montré des effets liés à la taille des particules ajoutées (nano et microparticules) sur l'absorption des ions de Zn^{2+}, Fe^{2+} et Mn^{2+} au cours de la croissance du mycelium et de la SmF.

4.7. Effets de nanoparticules de carbonate de calcium et du chauffage aux micro-ondes dans le traitement en amont et en aval de l'acide fumarique (Chapitre 8, un article de recherche soumis)

L'application des nanoparticules de carbonate de calcium (an anglais: CCNPs) comme agent de neutralisation dans la production de FA par SmF a été étudiée. De plus, le chauffage par micro-ondes (an anglais: MWI) a été utilisé comme une alternative aux méthodes classiques de chauffage, pour une récupération rapide de FA à partir du bouillon de culture. Un milieu de base (glucose-sel) a été utilisé pour les études de la SMF. L'analyse en MEB a montré des CCNPs grossièrement sphériques avec une structure solide dense. L'analyse nano zetasizer a révélé une distribution uniforme de taille des CCNPs avec une taille moyenne de 190 ± 20 nm. La réaction de neutralisation entre le FA et les microparticules de carbonate de calcium (en anglais: CCMPs) a pris environ 350 ± 19 s et le pH a atteint 6,2. Lorsque les tests ont été effectués avec les CCNPs, les variations de pH

ont été beaucoup plus rapides et un pH stable de 6,23 a été atteint après 190 ± 12 s. Nous n'avons pas noté d'effet de la taille du CC sur la viscosité du produit de réaction. Dans les 12 premières heures de la SmF avec les CCNPs, l'élévation de la viscosité a été d'environ 0,6 cP, valeur beaucoup plus élevé que 0,17 cP, valeur obtenue avec les CCMPs. Dans les 12-24 h de la SmF, la productivité volumétrique de FA a été augmentée de 0,47 g/(L.h) à 0,74 g/(L.h). Les concentrations les plus élevées de FA (67± 2 et 66,9 ± 2,7 g/L, respectivement) obtenues avec les CCMPs et les CCNPs, étaient presque identiques et ont suggéré que le rendement métabolique du champignon utilisé, *R. oryzae*, a été affectée par les CCNPs. Les conditions de chauffage MWI, un minimum de 10 ± 1 minutes était nécessaire pour une récupération maximale de FA. Par rapport à la durée de chauffage requis (28 ± 2 min) pour les procédés classiques de récupération de FA, la méthode MWI a permis de réduire le temps de près de 2,8 fois sans affecter le rendement de récupération de FA.

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END OF CHAPTER 1

CHAPTER 2

FUMARIC ACID: PRODUCTION AND APPLICATION (CONVENTIONAL AND BIO-MEDICAL) ASPECTS

PART I

FUMARIC ACID: PRODUCTION AND APPLICATION ASPECTS

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Résumé

Les récents progrès réalisés dans le domaine des transformations industrielles ont contribué à la réévaluation des procédés chimiques de production de fumarique acide (FA). La recherche active sur l'économie du carbone et les concepts de bio raffinage a donné un nouvel élan à la production biologique d'acide fumarique. Les techniques d'immobilisation, ainsi que des études d'ingénierie génétique et métabolique ont été explorées en vue d'améliorer la production de FA. Toutefois, pour les traitements d'aval, les limitates actuelles n'ont pas été repoussées ou atténuées par les nouvelles percées microbiologiques. La pertinence de différents facteurs moléculaires ou mécaniques en rapport à la morphogénèse des souches fongiques produisant l'acide fumarique doit être analysée. Un aperçu en profondeur des diverses applications de l'acide fumarique et leur évaluation dans un scénario de production industrielle sont importants. Dans ce chapitre, les aspects de la production et de l'utilisation de FA ont été revus en mettant l'accent sur les percées et les résultats qui nous sont apparus les plus prometteurs.

Mots clés: acide fumarique; *Rhizopus oryzae* 1526; fermentation submergée; morphologie; immobilisation; agents neutralisants; ingénierie génétique; ingénierie métabolique; traitement en aval, applications diverses.

Abstract

The recent progress made in the applied field has helped in re-evaluating fumaric acid (FA) beyond the scope of a platform chemical. Active research on carbon economy and bio-refinery concepts has speeded up the biological production of fumaric acid. Immobilization technique, genetic and metabolic engineering strategies have been explored for enhanced FA production. However, in the downstream processing, prevailing bottlenecks have not been well addressed with new methodological concept. Relevancy of different molecular factors/mechanisms to the morphogenesis of fumaric acid producing fungal strains needed to be analyzed. A deep insight into the minute details of various applications of fumaric acid and evaluating them together with production scenario is important. In this book chapter, production and application aspects of FA has been re-addressed with updated status and relevant discussions put forward on the future line of development of some of the important findings made so far.

Keywords: Fumaric acid, *Rhizopus oryzae 1526*; submerged fermentation; morphology, immobilization; neutralizing agents; genetic engineering; metabolic engineering; downstream processing, diverse applications.

Introduction

Fumaric acid (FA) originally derived its name after the plant, *Fumaria officinalis* (family: Papaveraceae), from which this organic acid was isolated first time (Roa Engel *et al.*, 2008). Some other common names of FA are allomaleic acid, boletic acid, lichenic acid and tumaric acid. The plant is an herb with its pink colored flowers that appears from April to October in the northern hemisphere (Figure 2.1.1). The plant is well known for its medicinal uses and considered as the major source of FA.

FA is a multifunctional chemical intermediate that find applications in nearly every field of industrial chemistry. Each molecule contains two acid carbonyl groups and a double bond in α, β position (Figure 2.1.2). Physical constants for FA including solid and liquid properties are given in Table 2.1.1. FA is a geometric isomer (trans configuration) of another dicarboxylic organic acid 'malic acid' that exists in the cis configuration. Aqueous dissociation constants and solubility of the two acids show variations attributable to geometric isomer effects (Felthouse *et al*., 2001). In the recent time, FA has been revisited with novel approaches in its production and application domain. Cost effective biological production of FA through fermentation and newer strategies for its enhanced production and easy downstream processing have been extensively studied in the last few decades. Newly emerging experimental evidences on the efficacy and safety of using FA and its ester derivatives in diverse fields has opened new avenues for this multifaceted platform chemical. This book chapter reviews and summarizes the recent progresses made on FA research outcomes.

Production routes of fumaric acid

Fumaric acid biosynthesis: Metabolic pathways

In all aerobic organisms, FA biosynthesis occurs via two different metabolic pathways namely: (1) Tricarboxylic Acid Cycle (TCA) or Krebs Cycle and; (2) Reductive Carboxylation Pathway. Albert Szent-Gyorgyi (1893-1986, Hungary) discovered FA catalysis during his study (including on vitamin C) on cellular combustion process (TCA cycle) for which he was awarded Noble Prize in Physiology or Medicine in 1937 (www.nobelprize.org). TCA involves CO_2 fixation coupled with the conversion of pyruvate to oxaloacetate, the precursor to malate and fumarate (Fig 2.1.3). Reductive CO_2 fixation catalyzed by the enzyme pyruvate carboxylase under aerobic conditions explains the high molar yields in FA production. This CO_2 fixation led to oxaloacetic acid formation that eventually converted into FA (Fig 2.1.4). The reductive CO_2 fixation pathway is attributed to the experimental yield of 140% over theoretical molar yield of 100% caused by only TCA (Reaction 2.1.1). However, the source of the additional CO_2 for fixation needs to be provided from outside during FA production. In

the fermentative FA production approach, this is achieved by the addition of $CaCO_3$ that reacts with FA and releases CO_2. A detailed discussion on this reaction mechanism is given in the section 'Fermentative production of fumaric acid'.

Petrochemical route of fumaric acid production

At present, FA is solely being produced commercially by the isomerization of maleic acid obtained from hydrolysis of maleic anhydride, which in turn is produced from oxidation of benzene as presented in reaction 2.1.2 and reaction 2.1.3. The catalyst 'vanadyl pyrophosphate' [$(VO)_2P_2O_7$] is used for the production of butane from maleic anhydride. The catalyst is synthesized by the reaction of vanadium (V) oxide and phosphoric acid followed by the heat mediated water elimination from the intermediate product vanadyl hydrogen phosphate, $VOHPO_40.5H_2O$ (reaction 2.1.4). Extensive studies have been done to understand and improve the butane-to-maleic anhydride conversion process and more than 225 U. S. patents issued on this technology since 1980. All these efforts are mostly designed to simplify the complexity of this conversion reaction. The process involves a 14-electron oxidation that occurs exclusively on the surface of the catalyst vanadyl pyrophosphate' [$(VO)_2P_2O_7$]. This catalyst is the only commercially viable system that selectively produces maleic anhydride from butane (Felthouse *et al*., 2001). The butane oxidation reaction to produce maleic anhydride is very exothermic. The main reaction by-products are carbon monoxide (CO) and carbon dioxide (CO_2). Stoichiometry and heats of reaction for the three principal steps are presented in reaction 2.1.5.

Fermentative production of fumaric acid

Pertaining to the depletion of conventional oil and the deterioration of the global environment, many platform chemicals are being produced from renewable biomass under moderate process conditions, such as submerged fermentation (SmF). FA is one of the best examples of platform chemicals. Currently, the annual production of FA is estimated to be 240,000 tons, and the projected market volume is 350,000 tons by 2020 (www.grandviewresearch.com). FA is solely being produced commercially by the isomerization of maleic acid obtained from hydrolysis of maleic anhydride, which in turn is produced from oxidation of benzene. However, due to the rising cost of the main raw material i.e. maleic anhydride (60-70% of the total production cost), researchers seemed to be more inclined towards biological production (fermentation) of FA. In addition, the recent renewed interest in biological production of FA is also influenced by the growing awareness in food and dairy safety. FA has been identified as one of the top ten building block chemicals that can be produced from sugars via biological conversion (Werpy *et al*., 2004). The recent tendency of transition from a fossil fuel- based economy to a bio-based economy

has strongly promoted the sustainable production of FA using renewable carbon sources. Moreover, fermentation based production of FA has been given more priority as it does not carry the toxicity risk element associated with chemically produced once. The filamentous fungus, *Rhizopus oryzae* (to be called as *R. oryzae* thereafter) (family: Mucoraceae) is used as the main producer of FA owing to its low nutritional requirements (Xu *et al*., 2012 a; Oda *et al*., 2003; Roa Engel *et al*., 2008). It is worth mentioning that in the early 1940s, FA was manufactured by fermentation employing *R.oryzae* on a commercial scale by the American multinational pharmaceutical company 'Pfizer' with an annual production rate of about 4000 tones (Roa Engel *et al*., 2008). However, the company discontinued the fermentation based production of FA as chemical synthesis became economically more attractive. Although, some other companies, such as Changmao Biochemistry and Jiangsu Jiecheng Bioengineering of China have taken appreciable initiatives for microbial FA production and industrialization, globalization of such effort is far away from the required pace (Xu *et al*., 2012 a). With the increasing awareness of low cost carbon option for the synthesis of value-added product, low cost carbon sources of agro-industrial origin have been explored as substrate for FA production with a good productivity. Investigations on FA production from woodchips, dairy manure, crude glycerol and lignocellulosic biomass, such as corn straw exhibited high product yield of FA (Xu *et al*., 2010; Zhou *et al*., 2014). Thus, in FA production, the upstream domain (availability of low cost carbon sources and easy processing) strongly supports the concept of "carbohydrate economy" and "biorefinery". However, low cost production of FA at commercial scale is still challenged by the downstream processing domain and inherent problems of filamentous fungus for commercialization.

Substrate selection

Since it has been established that substrate cost (about 40-60%) is the major economic input of the total production cost in FA fermentation and process economics, researchers are experimenting to design more economical and eco-friendly strategies for FA production. In the recent time, production of FA through SmF from different waste materials has gained tremendous importance.

Low cost carbon sources of agro-industrial origin have been explored as substrate for FA production with higher production (21-45 g/L). Investigation on FA production from wood chips, daily manures, crude glycerol and lignocellulosic biomass, such as corn straw has exhibited good product spectrum (Table 2.1.2). With the increasing research on low cost carbon option for the synthesis of value-added products, more such substrates are being considered at large scale. However, the suitability of a novel carbon source for FA production has to be experimented at individual level. Process control and corresponding FA

producing fungal responses are very specific for a combination of new carbon source and the fungal strain being used. Thus, a detailed investigation with each new carbon source for FA production is imperative. This is an interesting area of research with enough scope for economic and eco-friendly production of FA.

Fungal strains *vs.* fumaric acid production

The fungal species and the strains commonly selected for FA production has a strong and decade-old research background. In 1911, Felix Ehrlich discovered the ability of filamentous fungal species *Rhizopus nigricans* to produce FA. In 1938, more investigation was made by Foster and Waksman on another 41 strains from 8 genera of Mucorales order. They identified 4 genera *viz*. *Rhizopus, Mucor, Cunninghamella* and *Circinella* that can produce FA (Foster *et al.*, 1939). Later, many fungal species belonging to the genus *Rhizopus* (family: Mucoraceae) were identified as the best FA producers and received industrial attention. The genus gained more importance as many companies employed different species of this genus for FA production at commercial level (Table 2.1.3). After more active research on the compatibility of *Rhizopus* species for FA production from different carbon sources, four species are commonly selected for the fermentative production of FA. These species are *R. nigricans, R. formosa, R. arrhizus*, and *R. oryzae*. A good production spectrum of FA was achieved with these species as shown in Table 2.1.2. However, among the four species, *R. oryzae* is preferred over the other three due to its simple nutrient requirements and high productivity (4.25 g/L h for glucose). After the 1990s, *R. oryzae* has been the frontliner in the production of FA (Xu *et al*., 2012a). Among different strains of *R. oryzae* tested for FA production, the strain NRRL 1526 is one of the best strains (Oda *et al*., 2003). Moreover, it is now a well-established fact that fungal pellet morphology plays key role in the enhanced production of FA (Li *et al*., 2000; Rodriguez *et al*., 2005). The morphology of *R. oryzae* 1526 could be programmed to different forms (pellets, suspended mycelia or mycelial clumps) by the optimization of growth conditions (fermentation environment) and production of FA could be enhanced (Zhou *et al*., 2011). Thus, *R. oryzae* species has distinctive technical advantages over the other species for application in FA production.

Selection of neutralizing agent

It is now a well-known fact that in *Rhizopus*-mediated FA production, the pH value of FA production medium drops down (e.g., from 5 to 2) quickly in the first 20 to 24 h after inoculation due to production of FA. The consequence is the strong inhibitory effect on the growth of *R. oryzae* and FA production. This necessitates the addition of a neutralizing agent that will make complex with FA and thus maintaining the pH level at optimum for the growth

of *R. oryzae* and FA production. After decades of research on different neutralizing agents (e.g., $CaCO_3$, Na_2CO_3, $NaHCO_3$, $(NH_4)_2CO_3$, and Ca $(OH)_2$), calcium carbonate was found to be the most efficient neutralizing agent in the commercial level production of FA (Xu *et al*., 2012). The justifications made are: (a) FA yield and volumetric productivity were found to be lower for other neutralizing agents than $CaCO_3$; (b) accumulation of byproducts, such as malic acid and ethanol, was higher with other neutralizing agents; and (c) $CaCO_3$ can supply CO_2 that can be used for the formation of oxaloacetate in the reductive carboxylation pathway of fumaric acid biosynthesis (Figure 2.1.4). A comparative account of the technical advantages/disadvantages of using different neutralizing agents has been summarized in Table 2.1.4.

Role of medium composition

In FA production through SmF, addition of a limiting amount of nitrogen source (e.g. urea, $(NH_4)_2SO_4$) starves the microbial cells for biomass and produces more FA. As nitrogen is required for the biosynthesis of important macromolecules such as DNA, RNA and proteins; fermentation medium starved in nitrogen results in more metabolic activities rather than fungal growth. In one such study, Ding *et al*., (2011) observed higher FA production (from 14.4 to 40.3 g/L) when urea concentration was lowered from 2.0 to 0.1 g/L. FA production has also been found to be affected by the type of N-source. In 1989, Ling and Ng showed that selection between organic or inorganic N-source can increase or decrease the FA production and fungal biomass. Organic N-source such as yeast extract supported more fungal growth, whereas inorganic source such as $(NH_4)_2SO_4$ caused enhanced FA production (Ling and Ng, 1989). Attempt has been made with N-free medium for enhanced FA production. However, after certain time of SmF, fungal cells lost their activities. As an alternative to N-source starvation, FA production under phosphorus starved condition has also been successfully experimented (Riscaldati *et al*., 2000). Another key point in fermentative FA production is the maintenance of a high carbon (C) to nitrogen (N) ratio in the medium. It is observed that a high C/N ratio of 120 to 250 (w/w) helps in converting about 60-70% of medium glucose to FA (Magnuson and Lasure, 2004). However, higher glucose concentration (10 %, w/v) can cause inhibition in FA production. Apart from the N and C sources, trace metal ions (Mg^{2+}, Zn^{2+}, Fe^{2+}) at proper concentration are essential for the growth and metabolic activities of FA producing fungal strains. Trace metals, such as Zn, Fe and Mn have significant influence on the morphology of the filamentous fungi. Most of these studies have been carried out for many fungal model organisms (Foster *et al*., 1939; Couri *et al*., 2003; Papagianni, 2004). These studies confirmed that the concentration of trace elements in the media composition affected the morphology of filamentous fungus and finally influenced product yield during submerged fermentation. The filamentous fungi can

have different morphological forms, such as dispersed and/or free filaments, clump and pellets as a response of applied concentration of trace elements. Moreover, metal ions, such as Zn^{2+} and Fe^{2+} act as cofactors or activators for many cellular enzymes involved in catabolism and biosynthesis of macromolecules (DNA and RNA). For the Rhizopus species of filamentous fungi, morphology is highly influenced by these trace elements, and morphological features finally affect the fermentation. Presence of Mn, Zn and Fe in culture medium and their concentrations are significant as these three elements were shown to influence pellet formation for different *Rhizopus* species (Xu *et al*., 2012; Liao *et al*., 2007; Liao *et al*., 2008, Foster *et al.*, 1938). Absence or supplementations of these trace metals in growth medium showed specific effects in terms of pellet features and growth behavior.

Role of fungal morphology

It has been claimed that there exists a direct quantitative relation between the fungal pellet morphology and enhanced production of FA (Zhou *et al*., 2011). Formation of fungal pellets can benefit the fermentation as they reduce the medium viscosity and also has the advantages of not wrapping into the impeller of fermenter, reuse of fungal biomass, and more mass and oxygen transfers (Li *et al*., 2000; Rodriguez *et al*., 2005). In many recently conducted studies, it is claimed that tuning of morphology of the fungal strain *R. oryzae* can work as a decisive factor in the overall performance of the fungus for FA production (Liao *et al*., 2007; Liao *et al*., 2008). These investigations finally led to the conclusion that formation of pellet morphology by *R. oryzae* is very important to enhance the production of FA in SmF. In turn, the pellet formation could be programmed by changing the growth conditions (pH, temperature, rpm, total solid concentration, inoculum volume etc.). However, the optimized parameters from different studies cannot be drawn into a strong conclusion. Hence, with every new medium composition, parameters need to be optimized to control the morphology of the fungal strain. The recent trend on the development of efficient strategies for pellet formation with a reduced diameter and claim for enhanced production of FA is an important parameter that can be projected for more studies with new carbon sources. Fungal morphology is a phenotypic property. The development of a phenotype with a specific morphology is always the result of property. The development of a phenotype with a specific morphology is always the result of the genotype and the environment, a relationship that can be formulated as:

$$Phenotype = Genotype \times Environment \ (P = G \times E)$$

Where E= carbon source, pH, temperature, carbon/nitrogen ratio etc.

The interdependence between fungal morphology and other properties can be summarized in Fig 2.1.5. Optimization of growth conditions (fermentation environment) for a new

combination of carbon source and a fungal strain is a big challenge for enhancing the product yield of FA. Strategies for enhanced production of fumaric acid

Genetic and Metabolic Engineering

Microbial strains have been considered for genetic /metabolic engineering mechanisms with the purpose of achieving a better product feature of FA. However, as compared to other approaches, the number of reports on genetic modification for strain improvement is scanty. A summary of the different metabolic and genetic engineering approaches for fumaric acid production is presented in Table 2.1.5. In 1992, Kaclíková *et al*. experimented with a mitochondrial fumarase deficient strain of *Saccharomyces cerevisiae* and studied the FA production profile (Kaclíková *et al*., 1992). The strain was selected through in vitro biochemical assay for the fumarase enzyme. Glucose was fermented into FA and extracellular accumulation was detected. As the strain was deficient in the mitochondrial fumarase enzyme, FA was not converted into malic acid. At optimum growth condition, only 12% glucose was converted into FA and highest concentration detected was 0.5 g/L. Acidic pH of the environment might have caused the lower production of FA. In another study, effects of overexpression of the gene for pyruvate carboxylase (PYC) was observed taking *Pichia pastoris* as model (Wu *et al*., 2011)). In the reductive TCA cycle pathway of FA production, this enzyme is the key for FA production through Acetyl CoA $\longrightarrow$ Oxaloacetate Malate $\longrightarrow$ Fumarate pathway and accounts for 200 % theoretical production of FA from glucose (*i.e.* 2 moles FA from 1 mole glucose). Overexpression caused more accumulation of the intermediate products but FA production was not increased by much as compared to control. FA production enhanced from 38.71 mg/L to 40.05 mg/L following overexpression of the gene. In a similar study by Xu *et al*., the reductive pathway enzymes *viz.* malate dehydrogenase (MDH) and fumarase (FUM) were heterologously expressed in the cytosol of *S. cerevisiae* by genetic modifications (Xu *et al*., 2012b). Originally, the genes for the two enzymes were selected from the FA producing filamentous fungal strain *R. oryzae* NRRL1526 (ATCC 10260). Expression of the endogenous PYC of the recombinant yeast strain was up-regulated. The resultant effect was the significantly higher yields of fumarate in the glucose medium as compared to the control strain empty vector. FA production was increased by 488.9% (from 0.54 ± 0.04 g/L to 3.18 ± 0.15 g /L). However, this enhancement in FA production was much lower than as achieved with *Rhizopus* strains. The reasons stated for such lower production were the low level of PYC and more carbon flux towards ethanol production in the engineered strain. In a more detailed investigation, Zhang *et al*. studied the effects of overexpression of PYC and phosphoenolpyruvate carboxylase (PEPC) enzyme genes (pyc and pepc) on FA biosynthesis employing the mutant strain *R. oryzae* M16 (Zhang *et al*., 2012). This was the first study on metabolic engineering guided improved

FA production. Two most significant findings of the study were (a) PYC activity in pyc transformants increased from 56% to 83% and (b) PEPC activity in the pepc transformants increased from 3 to 6 mU/mg as compared to the wild type strain *R. oryzae* 99880. The pepc transformant increased fumaric acid production from glucose in batch fermentation by 26% (0.78 g/g glucose *vs.* 0.62 g/g for the wild type). However, pertaining to the formation of cell pellets with larger diameter and oxygen deficient, pyc transformants resulted in more accumulation of ethanol instead of FA production. In a different approach, Xu *et al*. developed an in silico method of FA production by direct fermentation using metabolically engineered *S. cerevisiae* (Xu *et al*., 2012 c). Flux balance analysis (FBA) mathematical model was used for the genome scale reconstructions. Based on literature survey, metabolic networking was designed for the fumarase defects or deletion and thus fumarase enzyme gene (FUM1) was targeted. The fum1-deleted mutant produced a maximum of 610 ± 31 mg/L (yield of 0.018 moles of FA per mole of glucose) FA after 120 h of incubation. Under same growth conditions, the original iND750 model of *S. cerevisiae* did not produce FA confirming the deletion effect. In addition, the study also concluded that FUM1 deletion had no significant influence on the growth rate of the mutant as compared to the iND750 (0.954/h and 0.973/h respectively). The findings by Xu *et al*. revealed the predictive capability of in silico guided metabolic engineering for FA production using *S. cerevisiae*.

The summary of the FA production by genetic and metabolic engineering approach has been tabulated below. As compared to other options such as immobilization, pellet morphology control, FA production level obtained from genetically engineered strains is much lower. Although, genetic modification of microbial strain is a potentially useful strategy to improve productivity and yield of commercially important product, the outcome of the research efforts made so far in this direction for FA production is not satisfactory. This becomes apparent when higher FA productivity obtained with different *Rhizopus* species is considered. More research inputs towards genetic or metabolic modifications of *Rhizopus* species can lead to improved FA production and this has to be a cost effective strategy too.

Immobilization of fungal mycelium

Due to the complex morphologies of filamentous fungi (*Rhizopus* species) used for FA production, large-scale FA fermentation production is a challenging task. The fungal filaments are technically not supportive of heat, oxygen and mass transfer during fermentation. Moreover, size control of fungal pellets is difficult and thus can lead to diffusion limitation in the broth and results in reduced production. To overcome these difficulties, immobilization approach was adapted for enhanced production of FA. A summary of the different immobilization based studies for fumaric acid production is presented in Table 2.1.6. Kautola and Linko (1989), studied immobilized *Rhizopus arrhizus* TKK 204-1-1a cells using polyurethane foam. The highest FA concentration obtained was 16.4 g/L. In another study, production of FA was experimented with the immobilized *Rhizopus arrhizus* NRRL 1526 on polyurethane sponge and reached the highest FA concentration of 12.3 g/L (Petruccioli *et al*., 1996). Recently, a novel immobilization method was designed using a combination of net and wire. With the immobilized *Rhizopus arrhizus* RH-07-13 mycelium used in this method, the fermentation time for FA production was reduced to 83.3% compared with free-cell fermentation (Gu *et al*., 2013). All these baseline information are very important for scale-up studies of FA production with immobilized fungus. However, compared with the number of reports generated on different experimental elements (such as, carbon source, fungal growth conditions, fungal strain, bio-reactor type, among others) associated with FA fermentation production, studies of immobilization based FA production are yet to receive much attention. For making the production of FA more sustainable and cost-effective, economic input for immobilizing material should be minimized. Exploration of low cost and compatible materials for application in immobilization can be a very good option.

Molecular biology of fungal morphogenesis *vs.* fumaric acid production

Despite impressive amounts of research, the molecular mechanisms involved in the regulation of fungal morphogenesis are inadequately defined. Most of the contemporary research findings have well interpreted the variations in FA product features in response to fungal morphogenesis. However, no data is available on the genetic factors exactly correlating FA production to fungal morphogenesis in submerged conditions. Contrarily, detailed investigation on the different genetic factors for morphogenesis was successfully carried out for the model filamentous fungus *Aspergillus niger* (to be called as *A .niger* thereafter). Induced by the variations in manganese ion (Mn^{2+}) concentration, different genes with tentatively assigned function were identified for switching in between filamentous and pelleted growth states of *A. niger*. The authors suggested considering the genetic factors

involved in the morphogenesis of *A. niger* for other filamentous fungi (Dai *et al*., 2004). In general, morphogenesis is controlled by some common regulatory factors in all filamentous fungi. Extensive studies have been carried out on the molecular signaling pathways of morphogenesis for revealing fungal pathogenesis and growth behavior in response to external stimuli. In particular, progress made towards the understanding of molecular basis of hyphal morphogenesis in the most widely recognized model fungus *Aspergillus nidulans* (*A. nidulans*) is categorically true for other filamentous fungi (Todd *et al*., 2007 a, c; Osmani *et al*., 2006; Szewczyk *et al*., 2006). Thus, scientific insight into previous findings on the genetic aspects of fungal morphogenesis and discussion on the possible molecular mechanism/genetic blueprint involved in the morphogenesis of filamentous strains used for FA production will be of great importance.

Basically, fungal morphogenesis can be discussed considering spore germination and filamentation stages. In the common methodology of FA fermentation, spores (mitospores or sporangiospores) of the fungal strains are first grown into pelletized mycelium in growth medium during pre-culture and the obtained pellets are used as inoculum for fermentation medium. During germination, the dormancy of fungal spore is broken through different sequential processes, such as spore re-hydration, initiation of translation, resumption of metabolic activity, and isotropic expansion of the cell surface. Spore germination is followed by formation of germ tube and lead to hyphal growth. Extensive research has been done on the model filamentous fungal species A. nidulans for exploring the underlying molecular mechanisms /factors in the germination process (Table 2.1.7).

Involvement of G protein-coupled receptors (GPCR), adenylate cyclase (CyaA), cyclic AMP (cAMP), protein kinase A (PKA) and Ras signaling pathway in the germination stages were confirmed from many studies (Chang *et al*., 2004; Cheng *et al*., 2001; Fillinger *et al*., 2002) (Table 2.1.7). Mutant spores were used for better conclusion on these aspects (Osherov *et al.*, 2000, 2001). It is pertinent to think that the same factors/mechanisms are responsible for frontline FA producer, such as *R. oryzae* strains when it is considered that *A. nidulans* represents the best model filamentous fungus for efficient analysis of gene function. Although, in the taxonomic hierarchy, *A. nidulans* (Phylum: Ascomycota) represents higher form of fungus as compared to *R. oryzae* (Phylum: Zygomycota), the spore germination stages are controlled by the basic functional genes conserved in spore producing fungal species. However, there still exists scope for good research as spore germination is highly susceptible to any change in the growth conditions (such as source of carbon, medium pH, incubation temperature, flash agitation speed) being maintained. For post germination stage, the studies carried out on two different model filamentous fungi well revealed the molecular biology for hyphal growth (Table 2.1.7). Role of GPCR, Spitzenkorper (SPK), Endocytosis

and Ras GTPase, RasB, TeaR in controlling the different steps of hyphal extension were confirmed from the molecular level investigations (Fortwendel *et al*., 2005; Rittenour *et al*., 2009). Apart from the well explored normal morphogenetic molecular events of model filamentous fungus, the effects of external stimuli such as changes in the temperature and pH during growth (pre-culture and fermentation) and fungal responses have been explored as well. For temperature sensitivity, heat shock protein 90 (Hsp90) are recognized as the most common domain for response to change in external temperature for many microorganisms including fungi (Shapiro *et al*., 2009). In case of pH, fungi have a common and well conserved molecular approach to respond to change in the external pH known as 'Internal pH Homeostatic System' that basically maintains pH under high alkaline and acidic stress conditions (Caddick *et al*., 1986; Arts *et al*., 2003). Recently, PacC mediated pH responsive signal-transduction pathway has been explored in different model fungi (Penalva *et al*., 2008). However, such approach is yet to be made for *R. oryzae*.

The overall concept developed on the molecular domain of morphogenesis of different model filamentous fungi so far can be a strong supporting element for exploring *R.oryzae* or other FA producing fungi with a novel approach for correlating FA production to fungal morphology at molecular level.

Downstream processing of fumaric acid

The downstream domain of microbial production of FA has imposed the major economical barrier to entry commercial trade for this multifaceted organic acid. As compared to the well-established downstream strategies for different fermented organic acids such as lactic acid, succinic acid and citric acid, separation techniques for FA from the fermented broth have not met the conditions for scaling up in both economical and technical aspects. Although, different new downstream techniques have been applied for easy recovery of FA from the fermented broth, the separation efficiencies of these processes are lower and technically more complex.

Conventionally, FA recovery from broth is done with mineral acid (H_2SO_4 or HCl) + heat assisted precipitation method. Different neutralizing agents (e.g., $CaCO_3$, Na_2CO_3, $NaHCO_3$, $(NH_4)_2CO_3$, and $Ca(OH)_2$) have been applied in microbial FA production for pH maintenance. However, after decades of research on different neutralizing agents, $CaCO_3$ has been established to be the most efficient neutralizing agent in the commercial level production of FA (Xu *et al*., 2012). The justifications made were: (a) FA yield and volumetric productivity were found to be lower for other neutralizing agents than $CaCO_3$; (b) accumulation of by-products, such as malic acid and ethanol, was higher with other neutralizing agents; and (c) $CaCO_3$ can supply CO_2 that can be used for the formation of

oxaloacetate in the reductive carboxylation pathway of fumaric acid biosynthesis. In $CaCO_3$ neutralized FA production, two different conventional precipitation methods are used: (A) high temperature heating and acidification method and; (B) simultaneous heating and acidification method (Figure 2.1.6 (A) and 2.1.6 (B)). Both methods consume large volume of mineral acids and water and end up in dead fungal biomass. A technical comparison of the two methods leads to a mutual feedback in their pros and cons (Table 2.1.8). In Method (A), although more energy is required to obtain a high temperature (160 ^{o}C) but not limited to the detection of higher concentration of FA (< 50 g/L) as in the case of Method (B). Both method produce $CaSO_4$ as wastes in large amount. In most of the fermentation based FA production studies, excess amount of $CaCO_3$ is used to maintain a pH around neutral value (6.0) for maximum FA production (Xu *et al*., 2012 a). The addition of $CaCO_3$ is not stoichiometric. However, in the fermented broth, the chemical reaction between FA ($C_4H_4O_4$) and $CaCO_3$ must follow a stoichiometric ratio which can be represented by reactions 2.1.6.

Thus, for each molecule of FA produced, one molecule of $CaCO_3$ is consumed during fermentation and form one molecule of calcium fumarate ($CaC_4H_2O_4$). For example, 20 g/L (200 mM) of $CaCO_3$ will neutralize approximately 23.2 g/L (200 mM) of fumaric acid. Practically, it is not feasible to maintain a stoichiometric addition of $CaCO_3$ to the broth due to unknown concentration of FA. Thus, to avoid the drop in pH, addition of excess amount of $CaCO_3$ is always preferred and it leads to complications in the downstream processing of FA. The fungal mycelia form interlocking hyphae and are often mingled with the feebly aqueous soluble precipitated fermentation product $CaC_4H_2O_4$ (21g/L) and unused solid $CaCO_3$. This leads to the viscosity increment of the fermented broth that causes operational problems, such as mass, heat and oxygen transfer and slows down FA production. Moreover, being sparingly soluble in water, $CaCO_3$ cause the dispersion problem in the broth (Gang *et al*. 19990). There is a scope of further research on this aspect of FA production through submerged fermentation.

Application aspects of fumaric acid

Conventionally, FA is mostly used in the feed industry as an antibacterial agent. Food and beverages accounted for 33% of world consumption of FA, followed by rosin paper sizes (20.0 %), unsaturated polyester resins (18.6%), and alkyd resins (12.3%) (3). Moreover, FA and its ester derivatives (FAEs) have been explored with a number of newer applications in the fields of medical science (such as neurology, immunology and dermatology) veterinary science (reduction of CH_4 emission upto 70% & improvement in feeding efficiency in dairy and poultry industries) and bio- nanotechnology (drug delivery and tissue engineering) (Yang *et al*. 2011; Mrowietz *et al*., 2005; Temenoff *et al*., 2007; Bayaru *et al*., 2001; Shao *et al*., 2013; Rohokale *et al*., 2014, Sharma *et al*., 2012). Recently, U.S. Food and Drug

Administration (FDA, US) has approved the FAE 'dimethyl fumarate' (DMF) for the treatment of human adults with relapsing forms of multiple sclerosis (www.fda.gov). The details of the bio-medical applications of FA and FAEs are addressed in the part II of this chapter. Following are the some of the most important conventional and newer applications of FA.

Uses of fumaric acid in food industry

FA is the least expensive of the food grade acids. It has been used as a nutritional additive and acidulant in various forms in the food and farming industries without untoward effects since 1946. It is the strongest tasting food acidulant that can control the growth of microorganisms, adjust pH and enhance flavors (Yang *et al*., 2011). The hydrophobic nature of FA results in persistent, long lasting sourness and flavor impact. In food products with pH greater than 4.5, minimal amount of FA is added for buffering the pH. Being a low molecular weight molecule, FA has more buffering capacity than other food acids at pH near 3.0. Moreover, because of its strength, less amount is required when compared to other organic food acids, therefore reducing costs per unit weight (www.thechemco.com). As per IHS chemical report, 2010, food and beverages accounted for around 33% of world consumption of FA in 2009. This can be mainly attributed to safety issues of FA application and growing consumption of nutritional bars HIS, Chemical, April 2010). Moreover, FA acts as an intermediate product in the preparation of other organic acid such as L-malic acid and L-aspartic acid. With more consumption of these organic acids for use in sweeteners, beverages and heath drinks, the production of FA demand is growing every year (Goldberg *et al*., 2006).

Dairy and poultry applications

As part of normal digestive process, domesticated livestock, such as cattle, sheep, buffalo, goats and camels produce large amounts of methane (CH_4) gas. In addition, the stored manures of these animals also cause CH_4 production. It is estimated, CH_4 alone represents around 9% of the different greenhouse gases (others are carbon dioxide, nitrous oxide and fluorinated gases) on a global basis. In US, enteric fermentation and manure management contributes 23% and 9% of CH_4 emissions, respectively (www.epa.gov). The growing awareness of global warming has necessitated the designing of methane reduction strategies including changes in the animal feeding practices. Several in vitro studies have shown the potential use of FA in ruminant diets to reduce enteric methane emissions. Carro and Ranilla (2003), reported that adding fumarate (from 0 to 10 mM) to concentrate feeds in batch culture decreased CH_4 production by up to 5%. Similarly, Iwamoto *et al*. (1999), reported that when up to 30 mM of fumarate was added to ruminal inoculum in batch cultures, CH_4 production decreased by about 10%. Valdes *et al*. (1999), also observed that adding sodium fumarate (from 0 to 10 mM) to a diet containing 50% hay and 30% barley

grain decreased CH_4 production by 5 to 6% in batch culture, which was then confirmed using the rumen simulation technique. In an in vivo study, Bayaru *et al*. (2001), added 20 g/kg of dry matter intake of FA (approximately 18 mM) to a sorghum silage diet fed to cattle and observed a 23% reduction in CH_4. Apart from the eco-friendly benefit of FA, another important aspect explored for FA in poultry industry is the effect of FA on the feeding efficiency in broilers and laying hens. Many studies have reported the improvement in feeding efficiency of broilers when different concentrations of FA were mixed in their feeds (Skinner *et al*., 1991). The broilers gained weights without affecting the feed consumption rate. FA has also been successfully tested as an alternative to conventional antibiotic growth promoters and showed significant improvement in feed to gain ratio (Luckstadt *et al.*, 2011). Achieving a low feed to gain ratio with the application of FA in diet is an easily accessible option for the broiler farmers as far as FA cost is concerned.

Application in resin industry

Resin industry extensively uses FA for manufacturing resins of different chemical nature. This industry consumes around 56% of the total FA produced annually (90,000 ton/year) with a splitting profile of: paper resins (35%), alkyd resins (6%) and unsaturated polyester resins (15%). Presence of carbon-carbon double bond (>C=C<) and two carboxylic groups makes FA chemically suitable for polymerization and esterification reactions (www.the-innovation-group.com). This property of FA has been exploited for the manufacture of unsaturated polyester resins at commercial scale. A simple condensation reaction between FA and polyhydric alcohol produce polyester resin (reaction 2.1.7). An infinite number of polyesters are theoretically possible by making changes in type or proportion of the polyhydric alcohol (www.nzic.org.nz). FA resins are more resistant to chemical corrosions with more durability and harder in its structure compared to those manufactured from maleic anhydride. However, at commercial level, maleic anhydride is economically more attractive as it is less expensive than FA by around 10% (www.chemweek.com). Thus, FA resins are manufactured for specific applications not covered by maleic anhydride based resins. Being biodegradable and biocompatible polymer, FA based resins are safer for environmental exposures. Another added advantage of using FA in resin synthesis is that macromers of FA are highly unsaturated and therefore can cross-link by themselves or with a cross-linking agent to form polymer networks and provide a novel type of polymer-based products (Shao *et al*., 2013).

Application in green chemistry: as Beckmann rearrangement promoter

The ever growing awareness on the environmental pollution caused by the application of organic solvents used for different synthesis reactions has provoked researcher to go for solvent-free chemistry and replacement of conventional acid catalysts with eco-friendly acid catalyst. Beckmann rearrangement is an example of such reaction that involves acid-induced rearrangement of oximes to give amides. Different acid catalysts (trifluromethanesulfonic, chlorosulfonic, sulfamic and p-toluenesulfonic acids) are conventionally used in this reaction. However, toxicity of these acid catalysts has been a major drawback. Very recently, FA has been reported as one of the highly efficient promoters of the Beckmann rearrangement (from benzophenone oxime Ia to benzamide IIa) under solvent-free conditions and thermal and microwave irradiation (Rohokale *et al*., 2014). The study included seven organic acids (fumaric, citric, oxalic, tartaric, malic, succinic and malonic acid). This is a welcome approach. Application of FA as a green catalyst is significant. Many organic chemical reactions such as esterification and aldol reactions are acid catalyzed. There is a scope for exploiting FA as a green acid catalyst for those commercially important chemical reactions.

Future perspectives and challenges

As suggested by the literature review on the production and application aspects of FA, the biological route of FA is preferred over the chemical one and scope of FA application is widened after active research on different properties of FA. The two facts: (1) Fermentation based production of FA has been given more priority as it does not carry the toxicity risk element as associated with chemically produced ones and; (2) pertaining to the rising cost of the main raw material maleic anhydride (60-70% of the total production cost of chemical method) and increasing awareness of low cost carbon option for the synthesis of value added product and environmental pollution, exploration of novel cheap and sustainable carbon source as fermentation medium for FA production is highly recommended. This will be a value addition approach to many industrial wastes. Moreover, selection of wastes with pollution concerns (such as pulp and paper wastes) can have the extra benefit of an eco-friendly approach (waste reduction). Apart from the carbon source, tuning of the morphological features of FA producing fungi (*Rhizopus* species) against the applied environmental conditions for enhanced production of FA is major issue to be addressed with every new source of carbon for a particular fungal strain. As seen from the literature, the production spectrum of FA varies with different carbon sources, fungal strains and types of fermentors. Thus, there is a good scope of trying novel carbon sources for SmF production of FA and optimize conditions for higher production of FA. This is an interesting area of research with enough scope for economic and eco-friendly production of FA. Literature also

suggested that fungal immobilization on a solid support can enhance FA production. However, immobilization support material costs can range from 60 - 70% of the total process cost. Exploration for new low/no cost materials for immobilization can balance the process economics of FA production. Solid state fermentation (SSF) for FA production is an unexplored area and SSF offers higher productivity over SmF. Experimentation with novel solid waste materials for SSF is good platform for achieving higher production spectrum of FA. In the downstream domain, there still exist problems with the conventional FA recovery procedures. Non-stoichiometric addition of $CaCO_3$ during SmF and its negative effects on FA production and recovery need further research inputs. Although, different new downstream techniques have been applied for easy recovery of FA from the fermented broth, use of excessive amount of $CaCO_3$ has not been eliminated from these strategies.

Abbreviations: FA= fumaric acid, SmF= submerged fermentation, SSF= solid state fermentation, MDH= malate dehydrogenase, FUM= fumarase, PYC= pyruvate carboxylase, PEPC= phosphoenolpyruvate carboxylase, FBA= flux balance analysis, FUM1= fumarase enzyme gene, GPCR= G protein-coupled receptors, CyaA= adenylate cyclase, cAMP= cyclic AMP, PKA= protein kinase A, Hsp90= heat shock protein 90, SPK= spitzenkorper, FAEs= fumaric acid esters, DMF= dimethyl fumarate.

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Table 2.1.1: Physical constants and solubility of fumaric acid (Arkema Global Product Strategy (GPS) safety summary, 2012).

Property	**Values**
Category	Organic acid
Molecular formula	$C_4H_4O_4$
IUPAC ID	(E)-Butenedioic acid
Melting point	287 ^oC
Boiling point	290 ^oC
Density	1.64 g/cm³
Solubility	Water = 6.3 gL^{-1} (at 25 ^oC) Acetone = 17 gL^{-1} (at 29.7 ^oC) Ethanol (95%) = 54.5 (at 29.7 ^oC) Chloroform = 0.2 gL-1 (at 25 ^oC) Carbon tetrachloride = 0.27 gL^{-1} (at 25 ^oC) 2-butenenitrile = 0.34 gL^{-1} (at 50 ^oC) Diethyl ether = 7.1 gL^{-1} (at 25 ^oC)
E number	E297
pKa values	3.03 and 4.44
Heat of formation	-811.03 kJ/mol
Free energy of formation	-655.63 kJ/mol
Heat of combustion	-1334.7
Heat of hydrogenation	-130.3 kJ/mol
Heat capacity	0.1418 kJ/mol
Heat of sublimation	123.6 kJ/mol
Dipole moment,	8.17
Crystalline form	Monoclinic, Prismatic, Needles or Leaflets

Table 2.1.2: Literature summary on fumaric acid production from different waste carbon sources.

Carbon source	***Rhizopus* species used**	**Fermenter**	**FA concentration (g/L)**	**FA productivity (g/L.h)**	**Yield (g/g)**	**References**
Corn straw	*Rhizopus oryzae* ME-F12 (mutant)	Shake flask	27.79	0.33	0.35	Xu *et al.*, 2010
Corn starch	*Rhizopus oryzae*	Shake flask	71.9	0.50	0.60	Moresi *et al.*, 1992
Dairy manure	*Rhizopus oryzae*	Stirred tank	31.0	0.322	0.31	Liao *et al.*, 2008
Cassava bagasse	*Rhizopus formosa*	Shake flask	21.3	Not mentioned	0.34	Carta *et al*,. 1999
Molasses	*Rhizopus nigricans*	Fluidized bed	17.5	0.36	0.36	Petruccioli *et al.* 1996
Crude glycerol	*Rhizopus arrhizus* RH-07-13 (mutant)	Shake flask	22.81	0.158	0.346	Zhou *et al.*, 2014
Potato flour	*Rhizopus nigricans*	Shake flask	43.5	0.42	0.58	Moresi *et al.* 1991
Xylose	*Rhizopus nigricans*	Shake flask	15.3	0.07	0.23	Kautola and Linko, 1989
Wood chips hydrolysate	*Rhizopus arrhizus*	Shake flask	5.085	Not mentioned	0.089	Woiciechowski *et al.*, 2001

Table 2.1.3: The commercial application of *Rhizopus* species for fumaric acid production.

Company	***Rhizopus* species**	**Year**	**References**
Pfizer	*R. nigrican, R. arrhizus*	1943	Kane and Amann, 1943; Roa Engel *et al*., 2008
National Distillers and Chemical Corporation	*Rhizopus*	1958	Lubowitz and La Roe, 1958
Du Pont	*Rhizopus*	1986	Goldberg and Stieglitz, 1986; Ling and Ng, 1989

Table 2.1.4: Different neutralizing agents used in the fermentative production of fumaric acid and their advantages/disadvantages of applications.

Neutralizing Agents	Advantages	Disadvantages
Calcium carbonate ($CaCO_3$)	FA yield and volumetric productivity is always high	Low solubility of calcium fumarate in water (15.6 g/L at 25^{o} C), more consumption of energy and mineral acids in the downstream processing,
Na_2CO_3,$NaHCO_3$, $(NH4)_2CO_3$ or $Ca(OH)_2$	Fumarate salts are easily soluble in water, downstream processing is free of heating, fungal biomass could be reused, waste mycelia could be sold as an animal feed or for chitosan/chitin extraction	FA yield and volumetric productivity is always lower

Table 2.1.5: Different metabolic and genetic engineering approaches for fumaric acid production.

Microbial strain used	Mechanism	FA concentration (g/L)	Reference
S. cerevisiae	Mitochondrial fumarase deficient mutant	0.5	Kaclíková *et al*. 1992
Pichia pastoris	Overexpression of the gene for pyruvate carboxylase	0.04	Wu *et al*. 2011
S. cerevisiae	Overexpression of malate dehydrogenase and fumarase genes	3.18	Xu *et al*. 2012b
R. oryzae M16	Overexpression of pyruvate carboxylase and phosphoenolpyruvate carboxylase	0.78 g/g glucose	Zhang *et al*. 2012
S. cerevisiae	Deletion of fumarase gene (*FUM1*)	0.61	Xu at el. 2012c

Table 2.1.6: Different immobilization based studies for fumaric acid production.

Microbial strain used	**Material used for immobilization**	**FA concentration (g/L)**	**Reference**
Rhizopus arrhizus TKK 204-1-1a	Polyurethane foam	16.4	Kautola and Linko (1989)
Rhizopus arrhizus NRRL 1526	Polyurethane sponge	12.3	Petruccioli *et al.*, 1996
Rhizopus arrhizus RH-07-13	Combination of net and wire	More volumetric productivity (83.3% reduction in fermentation time)	Gu *et al.*, 2013

Table 2.1.7: The important molecular mechanisms/factors involved in the morphogenesis of some model filamentous fungi and their relevancies for the fumaric acid producing fungal strains.

Important parameter	Molecular mechanisms/factors	Model filamentous fungus	Remarks	Reference
Spore germination	G protein-coupled receptor (GPCR), adenylate cyclase (CyaA), cyclic AMP (cAMP), protein kinase A (PKA), Ras signaling pathway	*Aspergillus nidulans*	Pre-culture (pellet formation) of *R.oryzae* spores and effects of growth conditions are of prime concern for these molecular factors/mechanisms	Chang *et al*. (2004), Cheng *et al*. (2001), Fillinger *et al*. (2002), Osherov & May (2000, 2001).
Hyphal growth	GPCR, Spitzenkorper (SPK), Endocytosis	*Aspergillus nidulans*	Important for the study of morphogenesis as a response to growth	Fortwendel *et al*. (2005), Rittenour *et al*. (2009).
	Ras GTPase, RasB, TeaR		Conditions during both pre-culture and fermentation stages	
Effects of Temperature	Heat shock protein 90 (Hsp90)	*Candida albicans*	Can be predicted for *R. oryzae* also it is th most common domain for response to change in external temperature.	Shapiro *et al*. (2009)
Effects of pH	Internal pH homeostatic system, transcription factor PacC	*Aspergillus nidulans*	The acid tolerance mechanism of *Rhizopus* sp. during fumaric acid production remains uncharacterized Involvement of PacC is needed to be investigated	Caddick *et al*. (1986), Arts & Penalva (2003), Penalva *et al*.(2008)

Table 2.1.8: Different neutralizing agents used in the fermentative production of fumaric acid and their advantages/disadvantages of applications.

Parameter	Method A	Method B
Consumption of energy	More Heat Energy Consumption	Less Heat Energy Consumption
Production $CaSO_4$ (gypsum) as unwanted by-product	Yes	Yes
Too much consumption of mineral acid	Yes	Yes
Fumaric acid detection limit	Applicable to detection of any concentration range of fumaric acid	Not applicable if fumaric acid concentration is less than 50 g/L
Reuse of fungal biomass	No	No

Table 2.1.9: Application safety summary of fumaric acid.

Application parameter	References
Purity criteria for animal application: Min. 99.5% by wt.	European Food Safety Authority (EFSA), Joint FAO/WHO Expert Committee on Food Additives (EC, 1990, 2000)
Application guidelines: Fumaric acid is authorized as technological additives for use in all animal species with no limitation of age and no maximum content	
Toxicological Data: (a) Oral, rat, LD50 = 10,700 mg/kg (b) Skin, rabbit, LD50 = 20,000 mg/kg (c) Intraperitoneal, rat LDLo = 587 mg/kg	Lewis and Sax's Sr., 1992

Figure 2.1.1: Image of a *Fumaria officinalis* plant (source: www.planetepassion.eu)

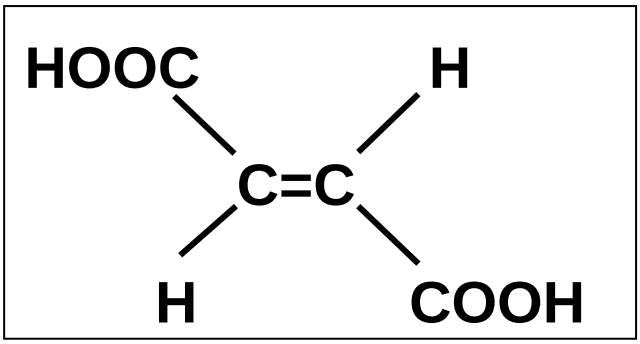


Figure 2.1.2: Chemical structure of fumaric acid

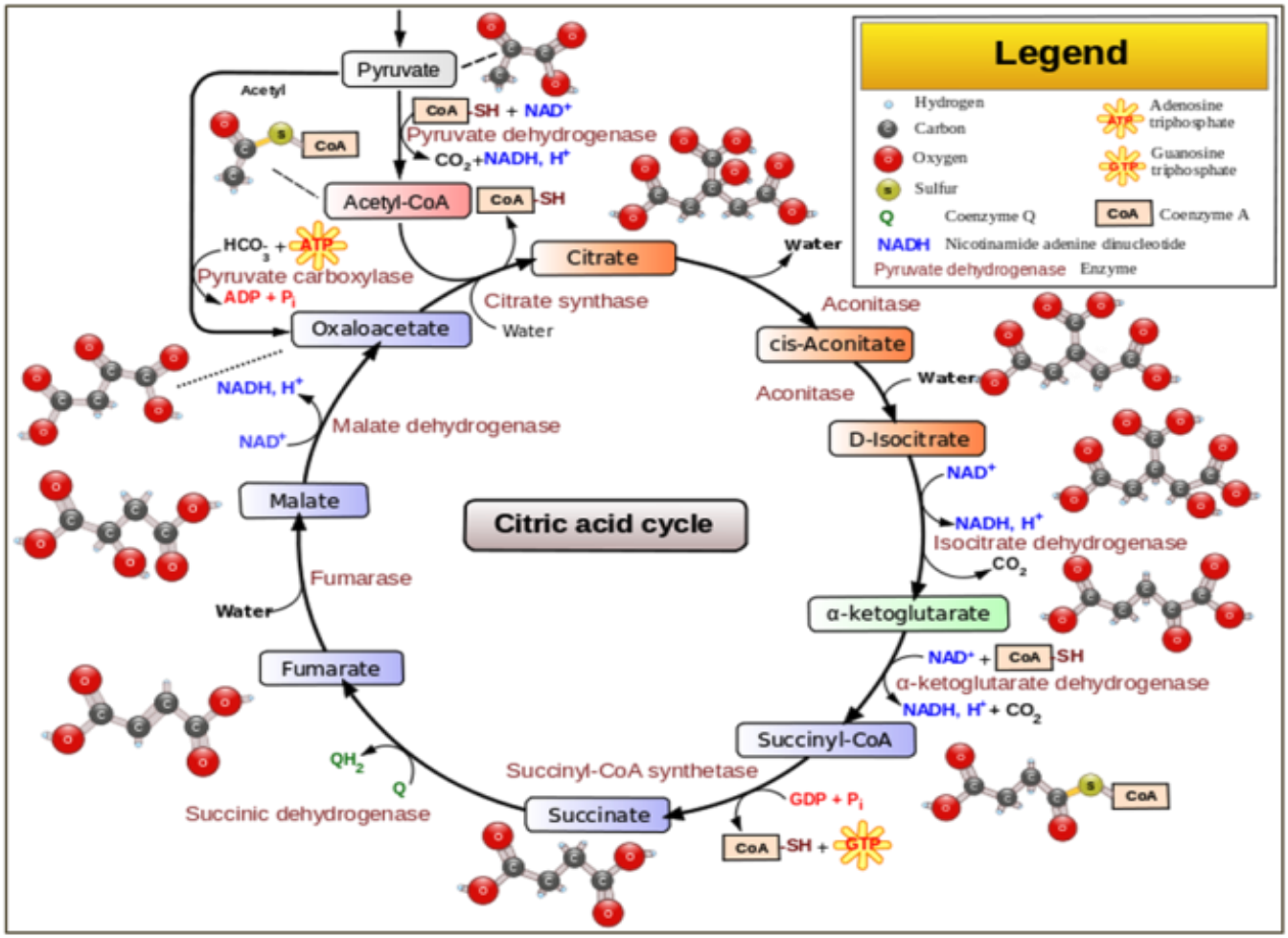


Figure 2.1.3: Tricarboxylic acid (TCA) cycle pathway of fumaric acid biosynthesis (source: www. wikipedia.org)

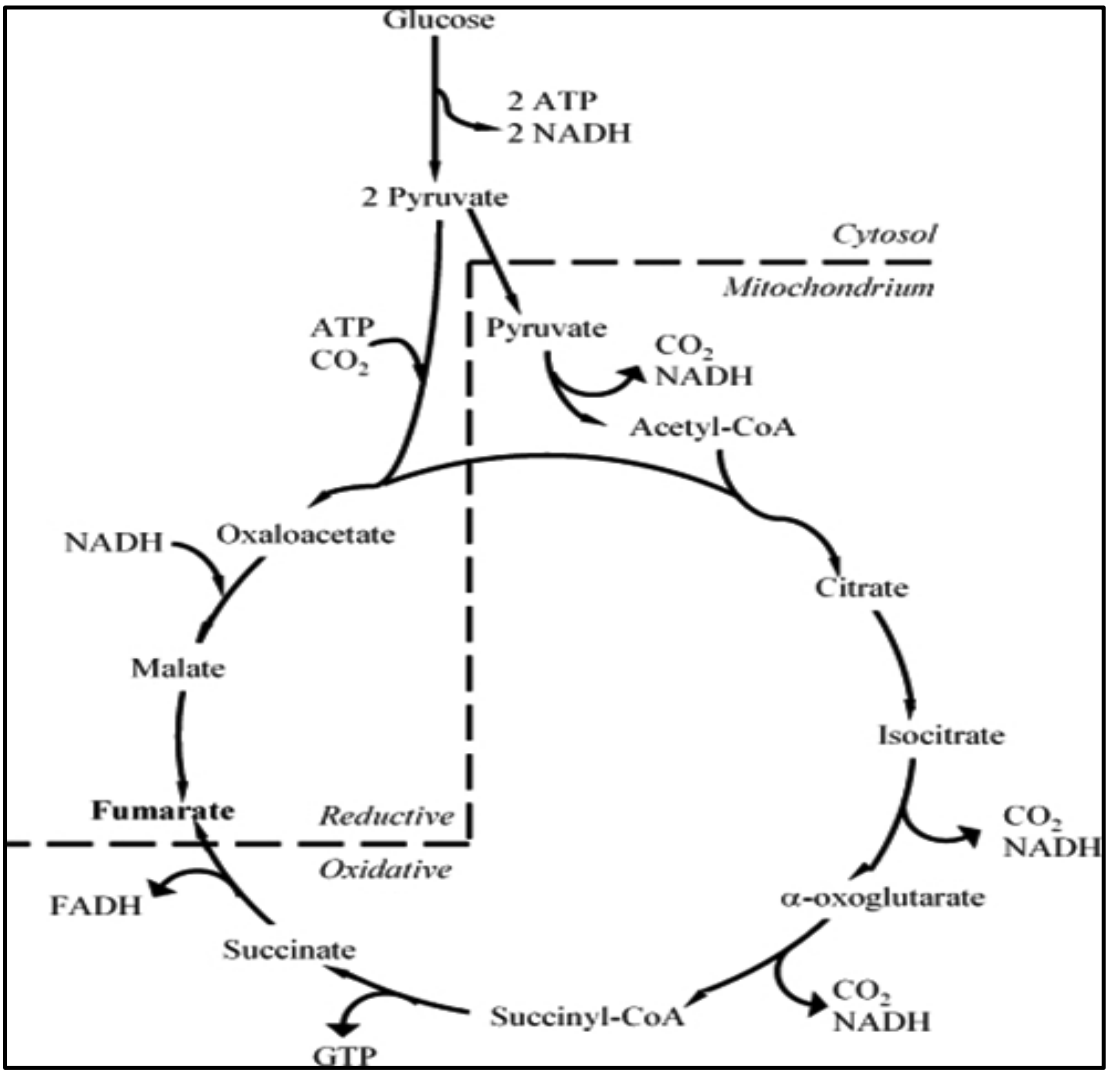


Figure 2.1.4: Reductive carboxylation pathway of fumaric acid biosynthesis (adapted from Roa Engel *et al.,* 2008).

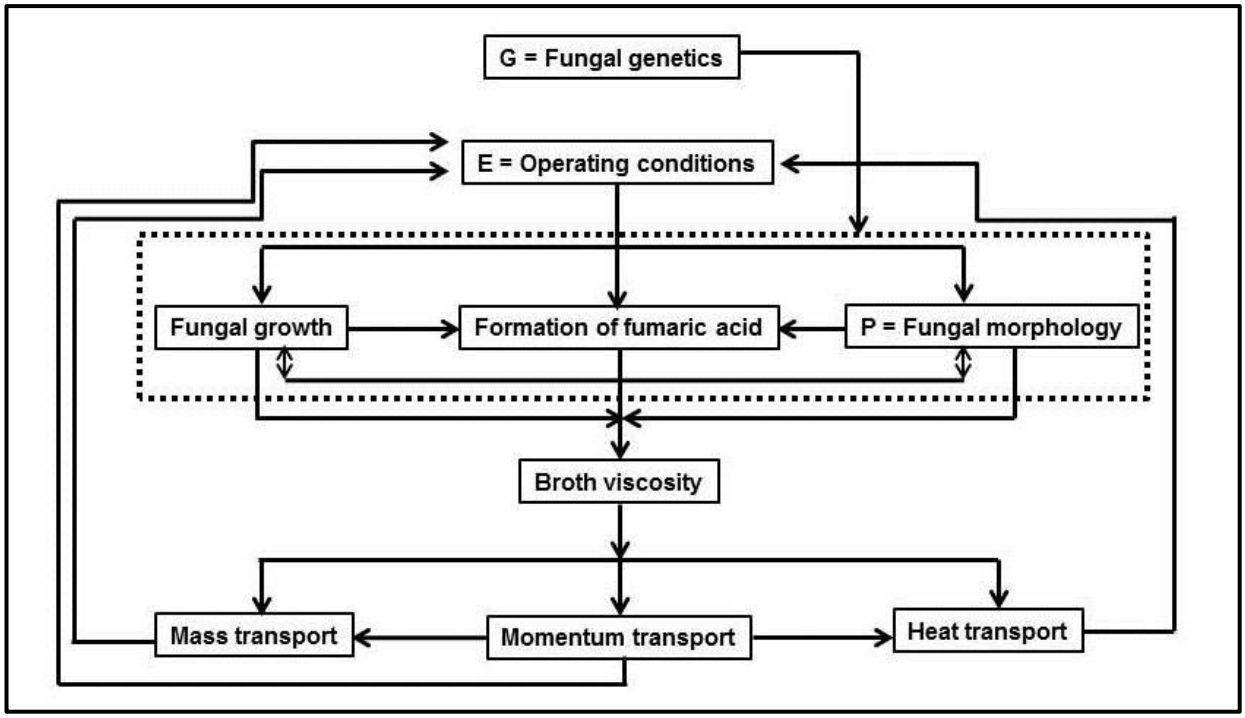


Figure 2.1.5: The mutual dependence of fungal morphology, fermentation conditions and fungal genetics and the overall influence on fumaric acid production.

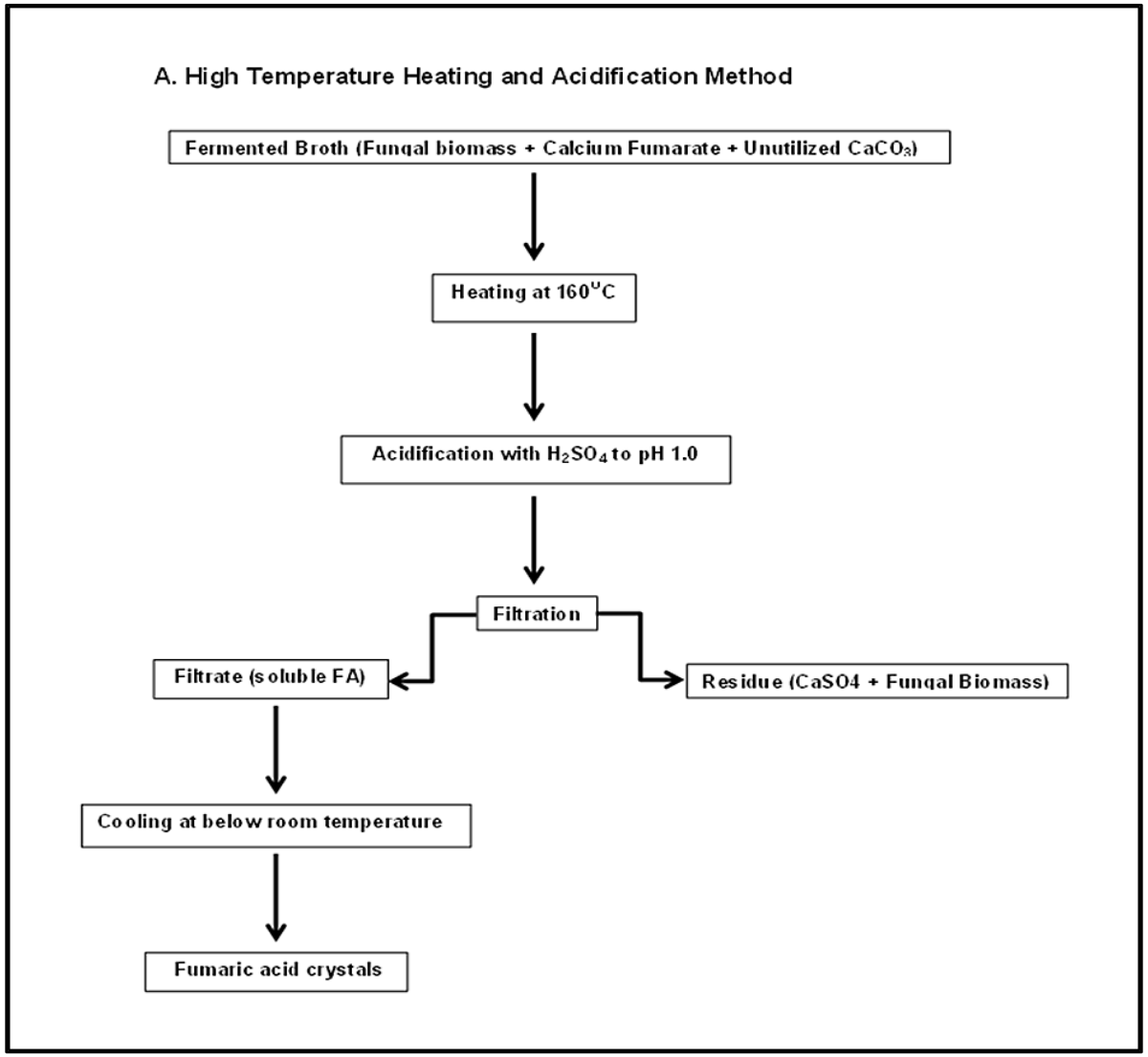


Figure 2.1.6 (A): High temperature heating and acidification method of fumaric acid recovery from fermented broth.

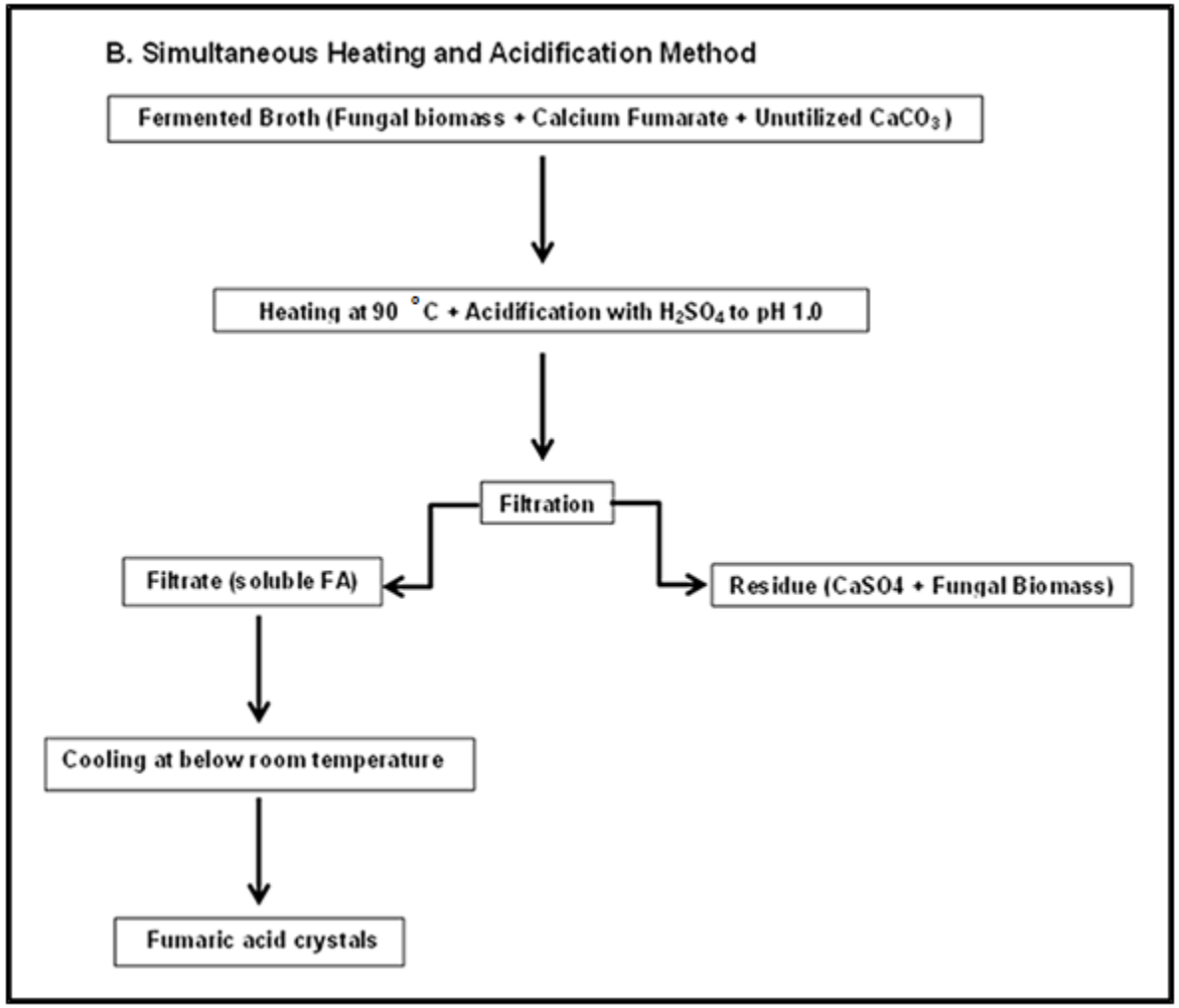


Figure 2.1.6 (B): Simultaneous heating and acidification method of fumaric acid recovery from fermented broth.

$C_6H_{12}O_6 + 2CO_2$ (from $CaCO_3$) $\longrightarrow$ $2C_4H_4O_4 + 2H_2O$ **Eq. (1)**

Glucose Fumaric acid

Reaction 2.1.1: Experimental yield of fumaric acid in TCA cycle and reductive carboxylation pathways.

$C_4H_{10} + 3.5O_2 \xrightarrow{\text{vanadyl pyrophosphate, } (VO)_2P_2O_7} C_4H_2O_3 + 4H_2O$ **Eq. (2)**

Butane Maleic anhydride

Reaction 2.1.2: Conversion of butane into maleic anhydride.

$C_4H_2O_3 \xrightarrow{\text{Hydrolysis}} C_4H_4O_4 \xrightarrow{\text{Isomerization}} C_4H_4O_4$ **Eq. (3)**

Maleic anhydride Maleic acid Fumaric acid

Reaction 2.1.3: Conversion of maleic anhydride into fumaric acid.

$V_2O_5 + H_3PO_4 \xrightarrow{\text{Reducing agent + HCl + } H_2O}$

$V_2O_5 + H_3PO_4 \xrightarrow{\text{Reducing agent + isobutyl alcohol}}$

$VOHPO_4.0.5H_2O \xrightarrow{\Delta} (VO)_2P_2O_7$ **Eq. (4)**

Vanadyl hydrogen phosphate

Reaction 2.1.4: Steps in the chemical synthesis of vanadyl pyrophosphate [$(VO)_2P_2O_7$] catalyst.

$C_4H_{10} + 3.5\ O_2 \longrightarrow C_4H_2O_3 + 4\ H_2O\ \Delta H = -1236$ kJ/mol **Eq. (5)**

$C_4H_{10} + 6.5\ O_2 \longrightarrow 4\ CO_2 + 5\ H_2O \quad \Delta H = -2656\ kJ/mol$ **Eq. (6)**

$C_4H_{10} + 4.5\ O_2 \longrightarrow 4\ CO + 5\ H_2O \quad \Delta H = -1521\ kJ/mol$ **Eq. (7)**

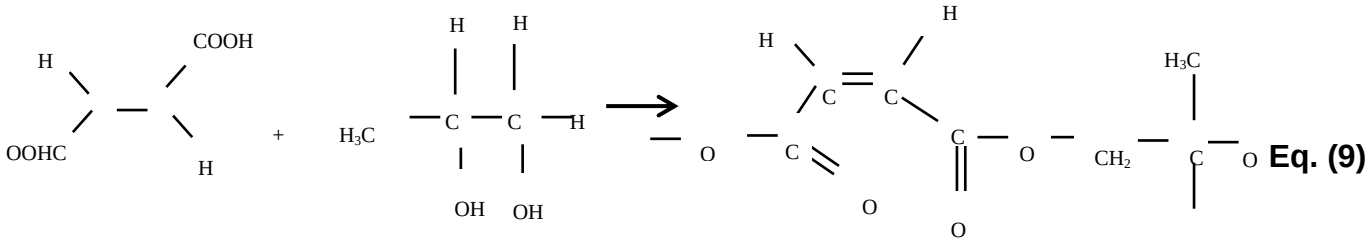
Butane

Reaction 2.1.5: Oxidation reactions of butane for conversion into maleic anhydride and by-products (Felthouse *et al*., 2001).

$CaCO_3 + C_4H_4O_4 \longrightarrow CaC_4H_2O_4 + H_2CO_3$ **Eq. (8)**

Fumaric acid Calcium fumarate

Reaction 2.1.6: The chemical reaction between fumaric acid and calcium carbonate**.**



Eq. (9)

Fumaric acid Propylene glycol Poly (propylene fumarate)

Reaction 2.1.7: Production of poly (propylene fumarate) resin.

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PART II

Recent Advances in the Biomedical Applications of Fumaric Acid and its Ester Derivatives: The Multifaceted Alternative Therapeutics

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Résumé

Plusieurs éléments de preuve ont démontré le potentiel des applications biomédicales de l'acide fumarique (AF) et de ses dérivés esters contre de nombreuses maladies humaines. Les esters d'acide fumarique (en anglais: FAEs) ont été autorisés pour le traitement du psoriasis, une maladie auto-immune. Biogen Idec Inc. a confirmé l'innocuité et l'efficacité de la formulation de FAEs (BG-12) pour le traitement de la SEP-RR (forme récurrente-rémittente de la sclérose en plaques) (en anglais: RRMS). Il a été mis en évidence qu'une autre formulation des FAEs, le FDM (en anglais: DMF) a pu permettre de de se réduire, dans des conditions cardiaques inflammatoires, telles que la myocardite auto-immune, l'ischémie et la reperfusion. Il a également été signalé que le DMF pourrait avoir un potentiel neuroprotecteur contre les troubles neurocognitifs associés au VIH (en anglais: HAND). Plusieurs études in vivo menées sur des rats et des souris ont montré les effets inhibiteurs de l'acide fumarique sur la cancérogenèse de diverses origines. De plus, les FAEs sont apparus comme étant un ingrédient important de la matrice dans la fabrication de matrices biodégradables pour des applications d'ingénierie tissulaire. Les vecteurs de livraison de médicaments composés de FAEs ont montré des résultats prometteurs dans le transport de certaines molécules médicamenteuses importantes. En dehors de ces applications, de nombreuses études sur les FAEs ont révélé de nouveaux potentiels thérapeutiques pour des applications cliniques. Cependant, jusqu'à présent, ces diverses informations n'ont pas été écrites dans un compte-rendu collectif ni analysées dans les moindres détails. Le but de cet article est d'examiner l'avancement accompli dans les applications biomédicales de FA et des FAEs et de se concentrer sur les études cliniques et l'interprétation des effets bénéfiques de FA et des FAEs au niveau moléculaire.

Mots clés: Esters de l'acide fumarique; immuno modulateur; tecfidera; ingénierie tissulaire; anti-cancer

Abstract

Several lines of evidence have demonstrated the potential biomedical applications of fumaric acid (FA) and its ester derivatives against many human disease conditions. Fumaric acid esters (FAEs) have been licensed for the systemic treatment of the immune-mediated disease psoriasis. Biogen Idec Inc. announced about the safety and efficacy of the formulation FAE (BG-12) for treating RRMS (relapsing-remitting multiple sclerosis). Another FAE formulation DMF (dimethyl fumarate) was found to be capable of reduction in inflammatory cardiac conditions, such as autoimmune myocarditis and ischemia and reperfusion. DMF has also been reported to be effective as a potential neuroprotectant against the HIV-associated neurocognitive disorders (HAND). Many *in vivo* studies carried out on rat and mice models indicated inhibitory effects of fumaric acid on carcinogenesis of different origins. Moreover, FAEs has emerged as an important matrix ingredient in the fabrication of biodegradable scaffolds for tissue engineering applications. Drug delivery vehicles composed of FAEs have shown promising results in delivering some leading drug molecules. Apart from these specific applications and findings, many more studies on FAEs have revealed new therapeutic potentials with the scope of clinical applications. However, until now, this scattered vital information has not been written into a collective account and analyzed for minute details. The aim of this paper is to review the advancement made in the biomedical application of FA and FAEs and to focus on the clinical investigation and molecular interpretation of the beneficial effects of FA and FAEs.

Keywords: Fumaric acid esters; immunomodulatory; tecfidera; tissue engineering; anti-cancer

Introduction

Fumaric acid (FA) is a well-known intermediate product of Tricarboxylic Acid Cycle (TCA) or Krebs cycle in all aerobic organisms. As a platform chemical, FA has wider applications in food and resin industries, dairy and poultry sector and in green chemistry (Yang *et al*., 2011; Goldberg *et al*., 2006; Rohakale *et al*., 2014) Apart from these conventional uses, FA has recently regained tremendous importance as a parent molecule for the synthesis of high value derivatives for biomedical applications. Decade old research carried out at clinical level on the pharmacological effects of FA and its ester derivatives (FAEs) confirmed their curative properties with high efficacy. Many recent studies have shown the usefulness of FA and FAEs in different fields, such as tissue engineering, drug delivery, cancer research, neurology, cardiology and immunology. Worth mentioning, FA and FAEs have been used as an important ingredient in designing the formulations of many drug delivery vehicles and biomaterial based scaffolds for bone tissue engineering applications (Temenoff *et al*, 2007). Moreover, the recent recognition of FAEs formulation 'Tecfidera' (dimethyl fumarate) to treat human adults with relapsing forms of multiple sclerosis by the U.S. Food and Drug Administration (FDA, US) is a significant achievement (www.fda.gov) . Undoubtedly, the scope of FA and FAEs application is widened after active research on different properties of these molecules. With the growing interest of experimenting FA and FAEs for different purposes, more reports are getting piled up on its novel applications. These studies have significantly contributed towards establishing FAEs as drug molecules with high efficacy and safety. Extensive research has been carried out on many FAEs based drug formulations for application in the remedies of human diseases. Some studies have been accomplished after successful human trials, while others are *in vitro*or *in vivo* stages. Some of the experimental findings on FA and FAEs are not followed by sequential studies and thus were not summarized into a written account with conclusive evidence. On the contrary, conclusions made from many progressive studies carried out on the potential applications of FAEs for curing some human diseases, need special attention. However, until now, collective literature on the biomedical applications of FA and FAEs has not been well documented. In fact, there is a timely need to go through the major breakthrough of FA and FAEs biomedical applications and put forward relevant discussions on the future line of development of some of the important findings made so far. This review article encompasses most of the recent findings on the biomedical applications of FA and FAEs and closely analyzes the general acceptance and severity of these molecules in part on their safety, side effects and future prospects. Few human disorders of clinical importance were taken into account for FAEs applicability. Applications of FA and FAEs in drug delivery, tissue engineering and cancer

research were enriched with recent findings and analyzed for minute details. An overall view of the diverse field of applications of FA and FAEs is shown in Figure 2.2.1.

FA and FAEs as potential therapeutics

This section discusses the sequential findings on the curative property of FA and FAEs from different studies at *in vitro*, *in vivo* and human trial levels with more recent updates and analyzes the clinical correlations. An overview is summarized in Table 2.2.1

FAEs in treating Psoriasis: Key findings so far

Psoriasis is a hyperplastic skin disorder caused by the activation of alternative and pathological pathway of keratinocytes regenerative maturation (Gniadecki, 1998). The disease is characterized by the hyperproliferation of keratinocytes and infiltration of inflammatory cells, including dendritic cells, T-helper (Th) cells and granulocytes into the skin (Cai *et al*., 2012). Th17 and Th1 cells use different receptors, such as chemokine receptor type 5 and 6 (CCR6, CCR5), cutaneous leucocyte-associated (CLA) and lymphocyte function-associated antigen 1 (LFA-1) receptors and migrates into skin (Ghoreschi *et al*., 2007; Nestle *et al*., 2009; Leonardi *et al*., 2008; Belge *et al*., 2014). Psoriasis attacks the skin and joints, and causes red patches with itchiness and scaling. Keratins 6 and 16 are the markers of hyperproliferative abnormal keratinocytes. Epidermal growth factors (EGF), such as transforming growth factor α (TGFa) and amphiregulin are overexpressed and some lymphokines and cytokines that normally act as growth inhibitors of keratinocytes start to stimulate the growth of stem keratinocytes (Gniadecki, 1998).

Active research on the curative properties of FAEs against psoriasis is many decade old. The first instance was reported in 1959, when the German chemist, Walter Schweckendiek, suffering from psoriasis self-experimented and cured himself with FAEs (Schweckendiek *et al*., 1959) In the last few decades, many clinical studies with new formulations of FAEs have been carried out and the outcome strongly supported the role of FAEs as a potential alternative to the contemporarily available conventional therapies, such as PUVA (Psoralen and long-wave ultraviolet light), methotrexate, anthralin and glucocorticosteroids. Dr. Helmut Christ, a pioneer physician in the field of antipsoriatic treatment remarkably succeeded in curing nearly 4000 psoriatic patients with monoethylester (MEEs) and dimethyl esters (DMEs) formulations of FA in different clinics of Switzerland and Netherlands. Different formulations (oral administration, ointment, and lotion) of MMEs and DMEs of FA were recommended for patience with different severity of the diseases. The prognosis, definitive courses of the formulations and side effects were clearly mentioned by Dr. Helmut (Cathcart

et al., 1990). In 1994, a mixture of dimethyl fumarate (DMF) and Ca, Mg and Zn salts of the corresponding monoetylfumarate (MEF) and ethylhydrogen fumarate (EHF) was registered by German drug administration agency 'Bundesinstitut für Arzneimittel und Medizinprodukte' (BfArM) as Fumaderm® initial (low-strength tablets) and Fumaderm® (high-strength tablets) for the systemic treatment of severe psoriasis. The American global biotechnology company 'Biogen' funded the clinical study of Fumaderm® formulations. Since its official registration, Fumaderm® has become the number one drug for the systemic therapy of psoriasis in Germany (Mrowietz *et al*., 2005). The efficacy and safety of these formulations has been subjected to many clinical trials from time to time. Altmeyer and his group conducted a multicenter, randomized and double-blind study on 100 cases of psoriatic patients (Altmeyer *et al*., 1994). Two formulations (low-strength and high-strength) of DMF and monomethylhydrogenfumarate (MMHF) as calcium, magnesium, zinc salts were orally administered to the patients for a maximum of 16 weeks with a placebo control for each dose. Based on Psoriasis Area and Severity Index (PASI), remission indexes (RI) for different drug groups were estimated. A total of 71.3% of treated patients showed positive responses with different RI, while 18% did not show response and rest 10.2% showed deterioration. No noticeable changes occurred in the normal profile of laboratory test parameters of the treated patients. Adverse events, such as nausea, diarrhea and gastrointestinal complaints occurred several times in one patient. It was concluded that DHF and MMHF were effective for persons suffering from labile or pustular psoriasis, unresponsive to common modalities but not recommended for stable chronic plaque psoriasis. However, this study did not include long term follow-up periods and thus therapeutic effects and safety of long-term uses of these ester formulations were not known. Although, some more clinical studies with 2-3 years of follow-up periods were carried out by some research groups (Mrowietz *et al*., 1998; Kolbach *et al*., 1992; Thio *et al*., 1995), but clinical trials of longer systematic therapy with FAEs was lacking. In 2003, Hoefnagel *et al*., carried out a clinical study on the safety of long-term uses of DMF and Ca, Mg and Zn salts of the corresponding MEF in severe psoriasis (Hoefnagel *et al*., 2003). The experiment was carried out on 66 patients and 12 of them were monitored for a maximum of 14 years. This was a very important observation for safety assessment of FAEs against chronic form of psoriasis. The long term uses of FAEs caused mild subjective adverse events, such as flushing, tiredness, stomach complaints, nausea and diarrhea. Lymphocytopenia, transient eosinophilia and moderate liver enzyme elevations were also observed among the treated patients. Considering the efficacy and lack of serious side effects from prolonged uses of FAEs, use of FAEs for chronic form of psoriasis was strongly recommended. Several more clinical studies have been carried out in the recent time and the results obtained reconfirmed

the efficacy of FAEs in treating psoriasis (Brewer *et al*., 2007; Reich *et al*., 2009; Wain *et al*., 2010; Harries *et al*. 2005; Ismail *et al*., 2014). The conclusive mechanism of antipsoriatic action of FAEs formulations proposed is the systemic and targeted (i.e. no effect on the secretion of Th-1 cytokine interleukins IL-2 and interferon IFN-γ) induction of Th-2 cytokines interleukins such as IL-4 and IL-5 (Ockenfels *et al*., 1998; Jong *et al*., 1996). Down regulation of type 1 cytokines has also been shown to be associated with antipsoriatic action of FAEs (Litjens *et al*., 2003). *In vitro* study with hyperproliferative HaCaT keratinocytes cell line showed high efficacy of DMF as antiproliferative agent (Sebok *et al*., 1994a, b). Although reports on secondary or non-subjective adverse events of FAEs are scanty, it is noteworthy to mention about such clinical findings for a better conclusion on the safety of FAEs in treating psoriasis. Stühlinger *et al*., observed reversible nephrotoxic effects (haematuria, proteinuria and tubular-interstitial renal damage) after two weeks of local and oral administration of FAEs in two patients suffering from psoriasis guttata since childhood (Stuhlinger *et al*., 1990). Renal damage was also diagnosed in psoriatic patients undergoing treatment with Fumaderm®. A dose dependent secretion of urinary β2-microglobulin indicated for the early renal dysfunction after prolonged therapy with FAEs formulation (Haring *et al*., 2011). Few clinical studies with FA as antipsoriatic agent were diagnosed with reversible renal damages and osteomalacia (Fliegner *et al*., 1992; Raschka *et al*., 1999).

Overall, the progress made in the clinical studies with FAEs as antipsoriatic drug is remarkable. The clinical feedback on beneficial effects of FAEs for treating psoriasis is quite supportive in declaring this new line of treatment as a potential alternative to the contemporarily available therapeutics for curing psoriasis.

FAEs for Multiple Sclerosis: A journey to Tecfidera

Multiple sclerosis (MS) is an autoimmune disease that typically affects the white matter of the central nervous system, but both tissue damage demyelination and axonal damage are observed. The French neurologist Jean-Martin Charcot (1825-1893) was the first person to recognize MS as a distinct disease in 1868 (Clanet, 2008). The severe consequence of MS is the disruption in the normal nervous conduction and leads to the development of wide range of signs and symptoms including physical, mental and often present motor disabilities and sometimes even cognitive impairment (Compston 2002, 2008). MS might occur in relapsing or progressive forms. The global scenario of MS is of serious concern. As of 2015, 2,300,000 people are affected globally with rates varying widely in different regions of the world and among various populations. MS is more common among woman than men and people in the age range of 20-50 are highly prone to MS (www.msif.org/global-ms-news).

Recently, FA esters (FAE), a group of simple structured compounds, have been evaluated as a potential treatment for relapsing-remitting MS (RRMS). Several lines of evidence have demonstrated FAE to be an orally available option for the immunomodulatory treatment of patients with RRMS. The concept that FAE could be applied for curing RRMS developed on the basis of immunomodulatory efficacy and safety of oral FAE against psoriasis. As RRMS is also an inflammatory disease, researcher predicted about the possible healing property of FAE for RRMS. Schimrigk *et al*. (2006) first experimented on the efficacy of Fumaderm® against 10 patients suffering from RRMS (Schimrigk *et al*., 2006). He used Gadolinium (Gd^+) based contrast enhancing agent for Magnetic Resonance Imaging (MRI) investigation on the brain leisons caused by RRMS. There was significant reduction of Gd^+ lesions after the first treatment phase (720 mg/d, 6 weeks) that also persisted during the second treatment phase even with only half of the dose (360 mg/d, 48 weeks). Immunological investigations revealed a higher apoptosis rate of CD4+ lymphocytes which indicated less inflammatory responses. There were some mild and reversible side effects, such as gastrointestinal symptoms and flushing. In another study by Schilling *et al*. (2006), FAE showed promising results against the myelin oligodendrocyte glycoprotein (MOG) peptide (amino acid 35-55) induced experimental autoimmune encephalomyelitis (EAE) animal model mimicking many aspects of MS. The major findings were the significant reduction of microglia and macrophages, significant preservation of myelin and axonal integrity (Schilling *et al*., 2006). In 2008, a phase II human trial study was carried out by Kappos *et al*. with a second generation FA derivative known as BG-12. Clinically, the study was a multicenter, double-blind, placebo-controlled that included 257 patients in 10 countries and was designed to evaluate the safety, efficacy, and dose-ranging. This new generation FAE contained DMF as an enteric-coated and developed by Fumapharm AG and Biogen Idec Inc. The MRI analysis of the Gd^+ enhanced T1- weighted scans and T2-weighted scans revealed a statistically significant reduction of 69% in the mean number of lesions compared to the placebo group. The overall outcome of the study was the promising short-term efficacy and safety profile of BG-12 (Anon, 2005; Wakkee *et al*., 2007). To further confirm the long-term safety and efficacy of the application of BG-12, a large scale phase III study programme was launched in Europe and North America in 2008. Named as DEFINE (determination of the efficacy and safety of oral fumarate in relapsing- remitting MS) and CONFIRM (comparator and an oral fumarate in relapsing-remitting MS), the proposed massive study included international, multicenter, two-year randomized, double-blind, placebo-controlled, dose comparison studies (Moharregh-Khiabani *et al*., 2009). Recently, Biogen Idec Inc. have announced that their FAE, DMF (BG-12), has met the primary and secondary end points of a global Phase III trial under the DEFINE programme for treating RRMS. As the company claims, when given twice daily, BG-

12 cut the relapse rate by 44 percent at two years compared with a placebo. When given three times a day, it cut the relapse rate by 51 percent (www.biogenidec.com). On March 27, 2013 the Food and Drug Administration of US (FDA, US) announced that it had approved BG-12 to be marketed under the brand name Tecfidera™ (www.fda.gov). The safety concern of DMF and Techfidera in treating MS has been recently addressed. Patients with long-term use of DMF were diagnosed with the rare and serious brain infection called progressive multifocal leukoencephalopathy (PML). The first case of PML was diagnosed in 2010. A psoriatic patient who was receiving DMF therapy for about 4 years developed PML. Discontinuation of DMF therapy improved the patient's clinical condition. It was concluded that the long-term use of DMF increased the risk of PML due to immunodeficiency (Ermis *et al*., 2013). In 2012, a woman suffering from MS and undergoing treatment with Psorinovo (DMF + copper gluconate) was found PML positive (van Oosten *et al*., 1950). Long-term use of DMF has been identified as the causative factor for adverse effects, such as leukopenia and lymphopenia and these two clinical conditions have been claimed to be the primary risk factors for PML (Rosenkranz *et al*., 2015). Very recently, Khatri *et al*. (2015) studied the effect of Tecfidera™ on lymphocyte count, CD4 and CD8 counts in 256 MS patients. After 12 month long study, it was concluded that Tecfidera™ has immunosuppressive effects and lowered the lymphocyte count, CD4 and CD8 counts. The clinical findings of this study strongly suggested that Tecfidera™ therapy should not be continued to MS patients with leukocyte counts below 3000/µL (3×10^9 /L) or lymphocyte counts below 500/µL (0.5×10^9/L). Moreover, it was also advised that MS patients seropositive for John Cunningham virus (JCV) that opportunistically causes PML in immunodificient person should also be considered for discontinuation of Tecfidera™ therapy (Khatri *et al*., 2015). In a more recent study, Tecfidera™ has been found to cause reduction in the lymphocyte CD4+ and CD8+ T subsets counts (Spencer *et al*., 2015). Following these clinical findings, it is now a general recommendation that frequent laboratory testing for lymphocyte counts should be performed for MS patients receiving Tecfidera™ therapy. The severity of negative effects of Tecfidera™ therapy became pronounced as Biogen Idec notified about the death of a MS patient who developed PML after long-term use of Tecfidera™. Following this incidence, FDA issued safety concern warnings on the use of Tecfidera™ in November 2014 (www.fda.gov). Recently, the absolute and comparative efficacy and safety of DMF for MS treatment has been reviewed and published by Cochrane Collaboration. The Cochrane MS and rare diseases of the central nervous system group published database of systematic reviews on DMF for MS (Xu *et al*., 2014). It is a very good effort for making aware the MS patients and health care professionals of new clinical findings of DMF therapy.

The decade old extensive research and progressive outcomes on the curative property of FAE for MS is highly appreciable and could be considered as the remarkable contribution towards curative neuroscience. Being the parent molecule of these FAE formulations, FA can be considered as the point source for developing new generation drug molecules for MS. The chemical property of FA makes the synthesis and modifications of FAE easy and feasible. FA itself not being a high cost biomolecule, the overall impact on the expenditure for MS medications could be well controlled. Moreover, the clinical trial feedbacks of DMF and Tecfidera™ therapies suggest the need for further investigation on the molecular events leading to adverse effects and clinical condition, such as PML.

Inflammatory cardiac conditions and FAEs

Beneficial effects of FAEs have recently been experimented against some inflammatory cardiac conditions, such as autoimmune myocarditis (AM) and ischemia and reperfusion. Autoimmune myocarditis (AM) is a major inflammatory disorder of the myocardium muscle that leads to physiological and anatomical abnormalities of human hearts. Viral (such as parvovirus B19 and coxsackie B) or non-viral (such as Streptococcal and Mycoplasma) infections can cause the AM (www.myocarditisfoundation.org). The proven curative property of FAEs for other inflammatory disorders, such as MS and psoriasis led researchers to experiment with FAEs for treating AM. In the early part of this research domain, Loewe *et al*. showed that DMF at an applied concentration of ≤ 70μM can inhibit the expression of the endothelial adhesion molecule CD62E of human umbilical vein endothelial cells (HUVEC). The inhibition was caused by the interference on TNF-α induced activation of CD62E in a NF-κB dependent signaling pathway (Loewe *et al*., 2011). In another study with DMF on HUVEC cell line, DMF was shown to inhibit NF-κB induced mRNA and protein expression and TNF-induced DNA binding of NF-κB proteins at the level of nuclear entry (Loewe *et al*., 2002). The convincing basis that FAEs can downregulate the expression of nuclear factor-κB (NF-κB) and tumor necrosis factor-α (TNF-α) and activation of NF-κB and TNF-α have been shown to be associated with AM and ischemia and reperfusion conditions, more experimentation with FAEs against these inflammatory cardiac conditions were carried out. In 2008, Milenkovic´ *et al*. applied DMF against the myosin-induced experimental autoimmune myocarditis (EAM) on Dark Agouti (DA) rats. DMF was found to be capable of attenuating the development of EAM as manifested by the reduction in disease incidence and severity of inflammation and necrosis of myocardium. There was a marked decrease in the secretion of TNF-α during the early stage treatment (during the first 10 days after disease induction). DMF was also effective when applied even after 10 days of first clinical observations of EAM (Milenkovic *et al*., 2008). Findings of this *in vivo* study were significant

for initializing the human trials of FAEs. More research in this direction can prove the efficacy and safety of FAEs in treating AM as compared to the existing well-known AM drugs, such as corticosteroids and azathioprine. Myocardial damage following ischemia and reperfusion involves activation of NF-κB signaling that regulates genes of proinflammatory cytokine molecules (Kis *et al*., 2003). From an *in vivo* study carried out on endothelial heart cells of Sprague Dawley rats, it was concluded that inhibition of nuclear entry of NF-κB by could be achieved with DMF (10 mg/kg body weight) and myocardial infarct size after ischemia and reperfusion was significantly reduced (Meili-Butz *et al*., 2008). This new line of evidence can aid in developing DMF as a potential alternative drug molecule for curing myocardial infarct injury following ischemia and reperfusion in near future.

FAEs as neuroprotectant

Very recently, DMF has been identified as a potential neuroprotectant against the HIV-associated neurocognitive disorders (HAND) and also as HIV disease-modifying agent (Cross *et al*., 2011). The study demonstrated the inhibitory actions of DMF in the key steps of development of HAND pathogenesis through distinct effects on HIV replication and macrophage-mediated neurotoxin production. Both HIV replication and release of neurotoxins by macrophages were suppressed by the application of DMF on HIV-infected human monocyte-derived macrophages. The proposed mechanisms for the dual functions of DMF were based on the inhibition of NF-kB nuclear translocation and signaling that contribute to the suppression of HIV replication, and induction of heme oxygenase-1 (HO-1), which is associated with decreased neurotoxin release. Moreover, the study also concluded that DMF and its primary metabolite monomethylfumarate (MMF) can reduce the chemokine ligand 2(CCL2) or monocyte chemotactic protein-1 (mcp-1) induced chemotaxis in human monocytes. This was a strong indication of DMF efficiency for decreasing the recruitment of activated monocytes to the CNS in response to inflammatory mediators. The overall findings of the study suggested DMF to be used as an adjunctive neuroprotectant and HIV disease modifier in antiretroviral therapy (ART) treated individuals. It was proposed that DMF should be considered a relevant therapeutic candidate for neurological disorders and other complications of HIV-infection mediated by monocyte and macrophage inflammation. Further deep insight into the action mechanisms of DMF and MMF in healing the HAND can establish these FAE molecules as front-line remedies. The broad spectrum beneficial effects of FAEs in HIV therapy has recently been reviewed by Gill and Kolson with more emphasis on immunomodulatory and antioxidant actions of DMF and MMF (Gill *et al*., 2013). The role of DMF and MMF in protection against neurodegeneration and associated cognitive dysfunction in HIV patients has been attributed to their inducing act on overexpression of

heme oxygenase-1 (HO-1) gene that promotes antiinflammatory and antioxidative cellular state (Gill *et al*., 2014). Human trials of DMF and MMF in HIV infected patients can lead to conclusive evidence in declaring these FAEs as new therapeutic molecules for clinical applications.

More therapeutic potentials of FAEs

Apart from the specific findings on the curative properties of FA and FAEs, there have been more studies with these molecules with further scope of novel clinical applications. Some of these studies better explained the role of FA and FAEs in curing inflammatory disorders at molecular level and was correlated with the overall performance. However, due to the lack of followed up studies, some of the new findings can be considered as discrete evidence. This section analyzes some important findings on the beneficial effects of FAEs and discusses on the clinical correlations.

Despite the fact that both DMF and MHF are now the key therapeutic molecules for curing psoriasis vulgaris, pharmacodynamics effects were found to be better for MHF. In a study carried out by Schmidt *et al*., both DMF and MHF were conjugated with glutathione (GSH) and plasma samples were analyzed for their metabolic fates. At near physiological pH (7.4), DMF conjugated more rapidly with GSH as compared to MHF and produced R,R- or the R,S-configured diastereomeric 2-(S-glutathionyl)-succinic acid dimethyl esters. This indicated a slow interaction between MHF and GSH and interpreted as the cause of long occurrence in the plasma and thus was more effective for oral administration in psoriasis treatment (Schmidt *et al*., 2007). In a different *in vivo* study on mice, the neuroprotective role of DMF was correlated to the transcription factor nuclear factor (erythroid-derived 2)-related factor 2 (Nfr2) mediated antioxidant pathway. The oxidative stress-induced cell death in MS was shown to be inhibited by anti-oxidant responses (accumulation of NADP (H) quinoline oxidoreductase-1) triggered by DMF (Linker *et al*., 2011). The study revealed the therapeutic role of DMF as anti-oxidant in curing MS. In a more recent *in vitro* study with DMF and DEF, Nfr2 activation was correlated with the accumulation of another transcription factor hypoxia-inducible transcription factor 1 alpha (HIF-1α) that promoted the release of vascular endothelial growth factor (VEGF) in a cell type dependent manner (Wiesner *et al*., 2013). HIF-1α activation and VEGF release occurred only in astrocytes; while stimulation of Nfr2 was triggered in all other studied cell types (primary astrocytes, neurons and oligodendrocytes) except microglia. Specific studies on dendritic cell differentiation and granulocytes revealed the beneficial actions of FAEs in curing psoriasis (Zhu *et al*., 2001; Nibbering *et al*., 1993). The baseline information that MMF can inhibit dendritic cell

differentiation and can act as agonistic against granulocytes, were confirmed from this study and can be correlated with FAEs therapeutic efficacy in antipsoriatic therapy. DMF inhibitory action on chemokine production by the keratinocytes was another ground of explanation using FAEs formulation in the treatment of psoriasis (Stoof *et al*., 2001). Possible involvement of kerotinocytes hydroxy-carboxylic acid receptor 2 (HCA2, GPR109A) in FAEs mediated skin flushing reaction has been recently reviewed and emphasized on further investigation for conclusive evidence (Hanson *et al*., 2012). Some more therapeutic potential of FAEs were revealed from two different studies on herpetic stromal keratitis (HSK) and non-responsive alopecia areata (aa). Based on the fact that FAEs can induce the release of Th-2 cytokines interleukins such as IL-4 and IL-5, DMF (15 mg/kg body weight) was found to significantly improve the HSK in mice and viral infection of cornea was eliminated (Heiligenhaus *et al*., 2005). In another single clinical investigation, 10 patients with resistant aa were given oral doses of Fumaderm (360, 600 and 720 mg/day) and monitored for six months. Out of 10 treated patients, six responded positively with different remission results. Three of them showed almost complete remission, while others either had partial or moderate remission. This was the first ever study on the therapeutic use of FAEs against aa. Inhibition in the keratinocytes proliferation by controlling cytokine release from hair follicles was explained as the reason for positive response of FAEs against aa (Venten *et al*., 2006).

The newly emerging benefits of FAEs in biomedical applications show the versatile nature of these molecules with scope for clinical studies. More follow-up study with long term use can lead to consensus view on the possible applications of FAEs for above mentioned *in vivo* or clinical cases.

FAEs based scaffolds for tissue engineering applications

Biodegradable polymeric matrix based scaffolds for tissue engineering applications have emerged as a promising and advantageous candidate in the recent time. Among these scaffolds, matrices with FAEs as an ingredient are more preferred for their flexibility, cross-linked nature and biodegradability. Different biodegradable polyesters of FAEs have recently been explored for their possible clinical applications in tissue engineering. The leading examples are poly (propylene fumarate) (PPF), poly(e-caprolactone fumarate) (PCLF), (propylene fumarate)-diacrylate (PPF-DA), poly(hexamethylene carbonate fumarate) (PHCF), oligo (poly (ethylene glycol) fumarate) (OPF), poly (propylene fumarate-co-ethylene glycol) (P (PF-co-EG)) and poly (propylene glycol-co-fumaric acid). Among these, PPF and PCLF have gained tremendous importance for application in bone tissue engineering (orthopedic scaffolds). For a better overview on the recent progress made in this direction, the major findings have been summarized in Table 2.2.2. Extensive study on the different

physicochemical properties of PPF has been carried out (Wang *et al*., 2006). The findings are of key importance for designing novel PPF based scaffolds. Chemical cross linking, injectability and easy degradability are some of the unique features of PPF that make it frontline matrix over other FEAs based formulations. PCLF has the advantages of injectability and easily parameter controllable features over its physical and mechanical properties (Elfick *et al*., 2002). Different approaches have been made for enhancing the suitability of these polymer materials for scaffold designing. Fabrication of nanocomposite by reinforcement of PPF with different moieties has been carried out by many research groups. Compressive mechanical strength of PPF was significantly improved upon incorporation of inorganic filler particles, such as β-tricalcium phosphate (Peter *et al*., 1998). In another study, nanocomposite of PPF-PPF-DA with higher flexural modulus and highly interconnected pores was designed by nanoreinforcement with surface-modified carboxylate alumoxane nanoparticles (Horch *et al*., 2004). PPF was also reinforced with carbon nanotubes (single and multi-walled, one and two-dimensional), graphene oxide nano-platelets and graphene oxide nanoribbons (single and multi-walled). The nanocomposite exhibited excellent biocompatibility in terms of cell viability (82-97%) as confirmed from the cytotoxicity assay carried out on the NIH3T3 mouse fibroblasts cell line (Farshid *et al*., 2013). The combination of PPF- PPF-DA has been exploited in designing rigid and biodegradable scaffolds with highly interconnected pores, such as poly high internal phase emulsions (PolyHIPEs) (Christenson *et al*., 2007). These microporous monoliths have the advantage of pore size control. Stereolithography (STL) based three-dimensional (3D) scaffolds have been designed from a combination of PPF, diethyl fumarate (DEF) and bisacrylphosphrine oxide (BAPO) with controlled microstructures (Lee *et al*., 2007). In a similar effort, a micro-STL based 3D PPF/ (DEF) scaffold was designed with biomimetic apatite coating and arginine-glycine-aspartic acid (RGD) peptide enriched surface and tested in MC3T3-E1 pre-osteoblasts cell line (Lan *et al*., 2009). The biocompatibility of the cells was remarkably enhanced by the biomimetic apatite coating. Scaffold for trabecular bone replacement application purpose with advantages of compressive mechanical strength, low viscous and easy handling was fabricated by Fisher *et al*. by copolymerization of PPF with DEF (Fisher *et al*., 2002). Divinyl and diepoxide terminated PPF oligomers with a solid particulate base of calcium carbonate and tricalcium phosphate exhibited high strength (30-129 MPa) with added advantages of less water absorption and slow biodegradation. This formulation has very good scope of bone cementing application such as bone fracture repairing (Domb *et al*., 1996). For enabling angiogenesis in condition like femoral head osteonecrosis, cementation has been a successfully implemented improvised technique. In such an effort, Chang *et al*. designed injectable and angiogenic formulation of PPF + calcium

phosphate that has advantages in clinical application over poly(methyl methacrylate) (PMMA) (Chang *et al*., 2010). Many more studies on designing PPF based scaffolds with injectability and higher mechanical strengths for orthopedic applications have been carried out (Cai *et al*., 2010; Timmer *et al*., 2003; Payne *et al*., 2002a, b; He *et al*., 2000; Jayabalan *et al*., 2001) PPF based scaffolds with localized drug release property was designed by incorporating microparticles of poly(DL-lactic-co-glycolic acid) and poly(ethylene glycol) into PPF scaffold (Hedberga *et al*., 2005). In a more specific study, PPF was crosslinked to poly(ethylene glycol) and the resultant hydrogel was tested for platelet adhesion and aggregation behavior. The radiolabeled platelets showed lower tendency of adherence as compared to glass and silicon rubber controls. The hydrogel formulation was suggested for application in cardiovascular implant designing (Suggs *et al*., 1999).Very recently, a 3D scaffold for tissue engineering application has been fabricated by exploiting the resorbable property of PPF (Childers *et al*., 2015). This 3D printable PPF based scaffold has wide range of controllable parameters, such as porosity and geometry that are decisive features in designing a best suitable scaffold. PCLF has also been experimented for micro and nano composite design with successful modulation in mechanical properties. Composite based on PCLF and micro or nano hydroxyapatite (HA), N-vinyl pyrrolidone (NVP), polypyrrole and methacrylic acid have proven better mechanical and morphological suitability for bone tissue engineering and nerve regeneration applications (Runge *et al*., 2010; Farokhi *et al*., 2012; Shafieyan *et al*., 2011; Wang *et al*., 2006). As compared to the number of reports on PPF and PCLF based experimentation, studies on FA based scaffold or nanocomposite designing is very scanty. Recently, a nanocomposite of FA functionalized AgCl/TiO_2 support was synthesized and therapeutic application was evaluated by antibacterial activity (Desai *et al*., 2013).

The extensive research on the potential use of FAEs in purposely engineered and constructed biocompatible and biodegradable scaffolds for tissue engineering applications constitute an evolving approach. The non-toxic nature and chemical properties of FAE has eased the fabrication of different scaffolds with required technical features. Being a naturally occurring organic acid in plants and animals, FA has advantages of inherent biocompatibility and the esters made from it have shown excellent biodegradability. More research inputs are required for the safety assessment of FAEs based scaffolds before going to clinical application.

FAEs in drug delivery application

Many nano-drug carriers have recently been fabricated using FAEs as one of the components. Fluocinolone acetonide-loaded injectable PPF matrix with a maximum of 400 days *in vitro*sustained release study was carried out by Ueda *et al* (Ueda *et al*., 2007). The formulation exhibited good drug loading capacity (5%) and could be experimented for the long term controlled release of other intraocular drug. Biodegradable PCLF nanoparticles have been used for encapsulation and delivery of the leading anti-cancer drug doxorubicin HCl (Shokri *et al*., 2011). In another study, MEE was cross-linked to poly (trimethylene carbonate) for drug (vitamin B12) delivery application (Jansen *et al*., 2010). Microsphere composed of poly(fumaric-co-sebacic) was used for the delivery of dicumarol (an anticoagulant drug) and increased bioavailability was achieved as compared to unencapsulated drug (Chickering *et al*., 1996). Although, reports on the use of FAEs as component in drug delivery vehicles are scanty, the outcome of these studies is very significant. Further research inputs can reveal the applicability of FAEs for more model drugs in near future.

Pharmacokinetics of FAEs

Compared to the extensive clinical studies carried out about the pharmacodynamics of FAEs, clinical investigation about pharmacokinetics of FAEs is scanty. Efforts have been made to uncover the fate of FAEs, such as DMF, MMF and MEF in human body. The available data provides strong evidence of the metabolism of FAEs. In 1999, Mrowietz *et al*. in their review article, mentioned about *in vivo* study of pharmacokinetics of FAEs by Joshi *et al*. (personal communication) (Mrowietz *et al*., 1999). The study indicated that DMF was rapidly (half-life of 12 min) hydrolyzed into MMF by the action of cellular esterase enzymes. MMF was further metabolized into fumaric acid and later into H_2O and CO_2 by Krebs cycle. The study also confirmed no binding behavior of DMF and 50% binding of MMF with serum proteins. The half-life of MMF was found to be 36 h with a T_{max} of 5-6 h. In an *in vitro* study by Werdenberg *et al*. on the first-pass effect or presystemic metabolism and intestinal absorption of FAEs, DMF was found to be completely metabolized in the intestinal tissue (Werdenberg *et al*., 2003). The study considered a homologous series of mono- and diesters of FA that exhibited lipophilicity (of the hydrocarbon chains) dependent intestinal permeability. More lipophilic FAEs exhibited increased intestinal permeability as compared to less lipophilic ones. Interestingly, presystemic metabolism rate of the FAEs was enhanced by the ester chain length and this finding led to the conclusion that increased intestinal permeability of the more lipophilic FAEs were counter-balanced by the presystemic

metabolism. In 2004, Litjens *et al*. performed *in vitro*study on the hydrolysis of DMF, MMF and MEF with simulated body fluids (pH 1 for stomach and pH 8 for small intestine) (Litjens *et al*., 2004a). The study re-confirmed that DMF hydrolysis occurs in small intestine and the metabolite MMF is no more hydrolyzed and thus easily detectable in blood circulation after oral administration of FAEs. MMF and MEF were found to be tolerant to both acidic (pH 1) and alkaline (pH 8) simulated fluids. However, at 7.4 pH, DMF was not hydrolyzed to MMF. Analysis of the serum and whole blood samples confirmed the involvement of lymphocytes/monocytes in the metabolism of DMF into MMF. Another in-depth analysis of DMF metabolism in human blood samples was carried out by Litjens *et al*. (2004b). The experiments included 10 healthy volunteers that received tablets of Fumaraat 120® (120 mg DMF + 95 mg Ca-MEF) after overnight fasting and standardized breakfast. One week of wash-out period was maintained in-between the two types of administration. Serum concentration of MMF increased with time in 8 participants who received oral dose of the drug after overnight fasting. An increased lag-time was observed in the peak serum concentration of MMF in the fed individuals suggesting lower absorption of the drug. The authors concluded that fumarate formulation should be administered to the recipient before meal for easy absorption and metabolism. As the study was not performed on psoriatic or MS patients, authors emphasized further clinical investigation on the applicability of their findings. Recently, Rostami-Yazdi *et al*. has shown that major portion of the orally administered DMF is not hydrolyzed into MMF as suggested by previous clinical studies (Rostami-Yazdi *et al*., 2009). The results of this *in vivo* investigation uncovered new metabolic fate of DMF as confirmed from the urine sample analysis of the psoriatic patience. Fumaderm (240 mg of DMF) was orally administered to the patients under fasting conditions. Liquid chromatography/mass spectrometry (LC-MS) analysis of urine samples showed the presence of the N-acetyl-S-(1, 2-dimethoxycarbonylethyl) cysteine (NAC-DMS)). The concentration of NAC-DMS detected after 6 h. of drug administration was about 54.4 ± 9.5 µM. NAC-DMS is the mercapturic acid of DMF. From previous literature [60], authors proposed that DMF reacted with cellular glutathione (GSH) faster than MMF and formed S-(1, 2-dimethoxycarbonylethyl) glutathione (GS-DMS). For MMF the products were mixture of S-(1-carboxy-2-methoxycarbonylethyl) glutathione and S (2-carboxy-1-methoxycarbonylethyl) glutathione. The metabolism of GS-DMS into NAC-DMS was catalyzed by cysteine-containing enzymes (cysteinyl-glycinedipeptidase and cysteine-S-conjugate-N-acetyltransferase). The conclusion drawn from this vital *in vivo* study was that DMF reacted with intracellular GSH of immune cells and this induced release of anti-inflammatory cytokines. Metabolism of GS-DMS into NAC-DMS was indicative of GSH consumption by DMF based therapy. More evidence on the absorption of DMF without

hydrolyzed into MMF came from the first ever clinical-case based study on psoriatic patients carried out by Rostami-Yazdi *et al*. (2010). Fumaderm® (240 mg DMF + 190 mg MEF] was orally administered to the three patients after 10 h of fasting and with a gap period of 24 h from the last dose of regular medication of Fumaderm®. HPLC analysis of the plasma samples was performed to detect DMF and MMF. The study concluded that: (a) only DMF part of Fumaderm® formulation technically fulfilled the criterion of Lipinski's rule of five; (b) bioavailability of DMF was much higher as compared to MEF and MMF and; (c) DMF was used up completely by the first-pass effect or presystemic metabolism.

The *in vitro* and *in vivo* studies about the pharmacokinetics of FAEs carried out by different research groups have generated vital information on the efficacy of FAEs in treating psoriasis and MS. It is expected that more clinical trials involving larger number of healthy volunteers or patients will help to reach a consensus on the metabolic fate of FAEs in human body. Pharmacokinetics of FAEs used in other inflammatory diseases, tissue engineering and drug delivery is yet to receive much attention as most of these studies have not reached clinical trial phase.

Anti-cancer property of FA and DMF

Many *in vivo* studies carried out on rat and mice models indicated the inhibitory effects of FA on carcinogenesis of different origins. These studies provided important clues on the probable novel application of FA as an anti-cancer agent. The most important findings of different experiments designed for exploring the anti-cancer property of FA during different time periods have been discussed henceforth.

In 1976, Kuroda *et al*. experimented with the crude ethanolic extract of the plant Capsella bursa-pastoris for inhibiting the solid growth of Ehrlich tumor on ICR (Imprinting Control Region) mice models (Kuroda *et al*., 1976). The intraperitoneal injection (0.14 g/kg/day) of the Capsella bursa-pastoris extract (CBP) caused 50 to 80% growth inhibition of Ehrlich solid tumor in the ICR mice. The tumor lumps in the treated mice showed multifocal necroses and the infiltration of host fibrous tissue cells. The researcher isolated and crystallized the active compound from the crude extract and identified as FA. The purified compound (FA) was found to be more effective in inhibiting the growth of Ehrlich solid tumor at a dose of 10 mg/kg/day with a half-maximal inhibitory concentration (IC_{50}) value of 266 mg/kg. In a similar study, this plant derived FA was also found to reduce markedly the growth and viability of Ehrlich, MH134, and L1210 mouse tumor cells in culture at concentration 1.2 mg/mL (Kuroda *et al*., 1981). A different study with male ICR/JCL mice suggested the strong inhibitory effect of FA on the forestomach and lung carcinogenesis induced by 5-nitrofuran

naphthyridine (NFN) derivative (Kuroda *et al*., 1982). Diet containing 1% FA suppressed the NFN-induced stomach and lung carcinogenesis. Anti-carcinogenic effect of FA was also manifested when applied against the 3'-methyl-4-(dimethylamino) azobenzene (3'-Me-DAB) fed male Donryu strain rats (Kuroda *et al*., 1986). The liver carcinogenesis induced by 3'-Me-DAB was markedly suppressed by FA when given a dose of 1% and 0.025% in diet and drinking water, respectively for 53 to 69 weeks. Rate of DNA synthesis was markedly enhanced in the hepatocytes of the FA fed rats suggesting a faster proliferation of the liver cells counteracting the carcinogenic effects of 3'-Me-DAB. Histopathological observations of the distribution of subcellular organelles of the FA fed rat hepatocytes were found to be normal. In another study, FA was found to be with even more inhibitory action when tested against a group of male Donryu rats fed with thioacetamide (TAA) (Akao *et al*., 1990). Rats fed FA at 1% in a basal diet after ingestion of TAA (first group) and TAA plus a supplement of 1% FA (second group) in the diet, showed no sign of hepatic carcinomas. Anti-carcinogenic effects of the FA were also studied for tumors of the esophagus, forestomach, tongue, throat, brain and kidney in two different rat models (Bespalov *et al*., 1992). Induced by N-methyl-N-benzylnitrosamine (for esophagus, forestomach, tongue) and N-ethyl-N-nitrosourea (for brain and kidney), FA counteracted the carcinogenic activity when fed at the dose of 1 g/L at the post-initiation stage of the carcinogenesis and resulted in marked reduction in esophageal papilloma, brain glioma and mesenchymal tumors of the kidney. Recently, DMF induced apoptosis mechanism has been revealed from experiment carried out on different hematopoietic cell lines (Tsubaki *et al*., 2014). The study confirmed the inhibition action of DMF on nuclear factor-kB (NF-kB) p65 nuclear translocation and suppression of expression of other two factors B-cell lymphoma extra-large (Bcl-xL) and X-linked inhibitor of apoptosis (XIAP). This was strong evidence on the anticancer property of DMF manifested at molecular level.

The experimental findings on the strong anti-carcinogenic property of FA could be used as the baseline information coming out of basic research. Compared to the other novel efforts being made for adding more value to this platform chemical, exploration of FA and FAEs with strong inhibitory effects on tumor development is of prime concern when worldwide cancer fatality scenario is concerned. The experiments conducted on the anti-carcinogenic property of FA included different mouse or rat models with various types of carcinogenesis induced by an array of compounds. This indicated the high efficacy and safety of FA as a potential anti-carcinogenic agent. Although FA or DMF is far from emerging as a common candidate for anti-carcinogenic applications, the results of the *in vitro* and *in vivo* studies made so far strongly demand an extensive research input to establish FA and DMF as a novel anti-carcinogenic agent.

Fumaria officinalis: Natural source of FA

Fumaric acid derived its name after the plant Fumaria officinalis, from which this organic acid was isolated first time (Roa Engel *et al*., 2008). The plant is very well known for its medicinal uses. It is good source of bitter principles (plant molecules with bitter taste) and alkaloids, such as fumarine and protoppine (Launert, 1989). Very recently, this plant has been positively tested in rats for hepatoprotective activity. In this study, crude ethanolic extract prepared from aerial parts of the plant was applied on the CCl_4 induced liver damage in rats. It is claimed that alkaloids present in the aerial parts of the plant possess antioxidant property that acted against the free radicals generated by CCl_4 (Sharma, 2012). As mentioned before, FA itself was found with anti-carcinogenic effects in many *in vitro* and *in vivo* studies. The hepatoprotective activity might be caused by the presence of FA in the plant extract. Further fractionation of the crude extract can resolve the issue and can provide more strong evidence for the hepatoprotective activity of FA. From medicinal chemistry prospective, it is very significant that one plant molecule already known for its multiple therapeutic actions in its pure form (commercially available) also exhibited the same activity from its source of origin. This is irrespective of the plant Fumaria officinalis as FA source. All plants that go through the normal TCA cycle will produce FA. Thus, it is pertinent to think about the possible role of FA in every medicinally important plant with known common action as exhibited by FA when applied pure. Such novel approaches can re-evaluate FA for more application in natural product chemistry.

Concluding remarks and future perspectives

Fumaric acid ester formulations tested in different biological assays (*in vitro*, *in vivo* and human trials) has provided vital information on the efficacy and safe application against inflammatory disorders, such as multiple sclerosis, psoriasis and autoimmune myocarditis. Recognition of dimethyl fumarate for the treatment of multiple sclerosis is a key success. The findings that dimethyl fumarate and monomethyl fumarate can provide protection against neurodegeneration and associated cognitive dysfunction in HIV patients are very significant. Application of fumaric acid esters as monomer for constructing injectable and biodegradable scaffolds materials for use in bone tissue engineering has been very successful. Similarly, incorporation of fumaric acid esters as a component in designing drug delivery vehicles has exhibited good results. The outcome of the intensive research on anti-cancer property of fumaric acid tested against induced carcinogenesis by an array of compounds is interesting and worth of attention. Revealing of the mechanism of anti-tumor effect induced by dimethyl fumarate is very encouraging and provides a strong platform for

carrying out further investigation on anti-cancer property of other fumaric acid esters. Being a natural source of fumaric acid, the plant *Fumaria officinalis* is yet to be fully explored for its medicinal values.

Molecular level studies being carried out on the emerging benefits of fumaric acid and its ester derivatives have successfully interpreted most of the therapeutic action of different applications. In the recent time, many research groups actively working on the different aspects of these molecules have contributed with new evidences with a scope of clinical application. However, apart from the two significant recognitions, the formulations of Fumaderm and Techfidera for human uses, the other findings are yet to be practiced at clinical level. Unrevealing of diverse therapeutic potentials of fumaric acid and its ester derivatives has opened a new avenue for developing alternative therapies for human disorders. The initial findings on possible applications of these molecules in bone tissue engineering and drug delivery is quite supportive of human trials. Further sequential studies might establish theses multifaceted molecules as default modalities in clinical applications.

Abbreviations: Please refer to Table 2.2.1 and Table 2.2.2.

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Table 2.2.1: Representative examples of fumaric acid and fumaric acid esters formulations tested (*in vitro, in vivo* and human trials) for different curative properties.

Formulation	Targeted application	Mode of action	Level of study	References
BG-12®	Multiple sclerosis treatment	Immunomodulatory	Human trial	Temenoff *et al*., 2007
DMF (Fumaderm®)	Psoriasis treatment	Immunomodulatory	Human trial	Mrowietz *et al*., 2005
DMF	Autoimmune myocarditis treatment	Immunomodulatory	*In vivo*	Milenkovic *et al*., 2008
DMF	Predicted application for treatment of ischemic heart disease	Inhibits nuclear entry of Nuclear Factor-κB (NF-κB) in heart endothelial cells	*In vivo*	Meili-Butz *et al*., 2008
MMF, DMF	HIV-associated neurocognitive disorders corrections	Immunomodulatory	*In vivo*	Cross *et al*., 2011
DEF	To induce different signaling pathways in cell types from the central nervous system	Stimulation of astrocytic vascular endothelial growth factor (VEGF) expression through hypoxia inducible factor -1 alpha (HIF-1α) and nuclear factor erythroid-derived 2-like 2 (Nrf2)	*In vitro*	Wiesner *et al*., 2013
DMF (Ca-MHF)	Possible application in the treatment of severe psoriasis	Inhibition of monocyte derived dendritic cell differentiation	*In vitro*	Zhu *et al*., 2001
MMF	Treatment for psoriatic skin lesions	Anti-inflammatory effects on granulocytes and anti-proliferative effects on keratinocytes	*In vitro*	Nibbering *et al*., 1993
DMF	Improvement of herpetic stromal keratitis	Systemic induction of T helper 2 cytokines	*In vivo*	Heiligenhaus *et al*., 2005
Fumaderm®	Treatment of therapy-resistant	Immunomodulatory	Human trial	Venten *et al*., 2006

Formulation	Targeted application	Mode of action	Level of study	References
(FA-DME of calcium, magnesium, and zinc)	alopecia areata		(non-placebo controlled)	
FA	As anti-carcinogenic agent	Not specified	*In vivo*	Kuroda *et al*., 1976
FA	For anti-intoxication of kidney and liver cells	Inhibition of the changes of subcellular components and organelle in the liver and kidney induced by mitomycin C	*In vivo*	Kuroda *et al*., 1981
FA	For enhanced DNA synthesis	Counteraction of the toxicity caused by mitomycin C or aflatoxin B1in liver cells	*In vivo*	Kuroda *et al*., 1982

Abbreviations: DMF: Dimethylfumarate; MMF: Monomethylfumarate; FA: Fumaric acid; DME: Dimethylethyl; MHF: Methylhydrogenfumarate; DEF: Diethylfumarate; BG: Biogen Idec.).

Table 2.2.2: Representative examples of fumaric acid esters based scaffolds for tissue engineering application with their formulations and advantages.

Fumaric acid polyesters	Cross-linked polymers and molecules	Advantages	Application in tissue engineering	References
PPF	β-TCP	Compressive mechanical strength	Orthopedic scaffolds	Peter *et al*., 1998
	PPF +DA + carboxylate alumoxane nanoparticles	Higher flexural modulus scaffolds with highly interconnected pores	Orthopedic scaffolds	Horch *et al*., 2004
	CNTs	Less toxicity	Orthopedic scaffolds	Farshid *et al*., 2013
	PPF + DA	Synthesis of poly high internal phase emulsions	Diverse applications	Christenson *et al*., 2007
	DEF + BAPO	Controlled microstructures	Diverse applications	Lee *et al*., 2007
	DEF + biomimetic apatite coating + RGD peptide	3D and enhanced biocompatibility	Orthopedic scaffolds	Lan *et al*., 2009
	DEF	Compressive mechanical strength, low viscous and easy handling	Trabecular bone replacement	Fisher *et al*., 2002

	DV and DEP terminated PPF + $CaCO_3$ + $Ca_3(PO_4)_2$	High strength (30 - 129 MPa), less water absorption and slow biodegradation	Bone cement application	Domb *et al*., 1996
	$Ca_3(PO_4)_2$	Angiogenic and injectable	In the treatment of osteonecrosis of femoral head	Chang *et al*., 2010
	mPEGA	Increased surface hydrophilicity, lower surface frictional coefficient and protein absorption, better cell attachment, proliferation and differentiation	Orthopedic scaffolds	Cai *et al*., 2010
	PPF + DA	Increased mechanical strength, lower shrinkage property and controlled biodegradation	Orthopedic implants	Timmer *et al*., 2003a, b
	Gelatin microparticles	Injectability, encapsulated viability of cells, long term cell proliferation and differentiation	Orthopedic scaffolds	Payne *et al*., 2002a,b
	PEG-DMA + β-TCP	Injectability with enhanced compressive modulus	Orthopedic scaffolds	He *et al*., 2002

	PTM	Injectability and partial biodegradation	Orthopedic scaffolds	Jayabalan *et al*., 2001
	Microparticles of PLGA and PEG	Scaffold with drug delivery potential	For delivery of bioactive molecules	Hedberga *et al*., 2005
	PEG	Low platelet adhesion and aggregation	Cardiovascular implant	Suggs *et al*., 1999
PCLF	Polypyrrole	Good electric conductivity and biocompatibility	Nerve regeneration	Childers *et al*., 2015
	NVDP + nHA	High E-modulus and good biocompatibility with osteoinduction and osteoconduction	Orthopedic scaffolds	Runge *et al*., 2010
	mHA + MAA	High E-modulus and cytocompatible	Orthopedic scaffolds	Farokhi *et al*., 2012
	PEGF	Injectable, self-crosslinkable and photocrosslinkable	Diverse application	Shafieyan *et al*., 2011

Abbreviations: PPF**:** Poly (propylene fumarate); β-TCP: β-tricalcium phosphate; DA: Diacrylate CNTs: Carbon nanotubes; DEF: Diethyl fumarate; BAPO: Bisacrylphosphrine oxide; RGD: Arginine-glycine-aspartic acid; DV: Divinyl; DEP: Diepoxide; mPEGA: Methoxy poly(ethylene glycol) monoacrylate; PEG-DMA: Poly(ethylene glycol)-dimethacrylate; PTM: Phloroglucinol triglycidyl methacrylate; PLGA:

Poly(DL-lactic-co-glycolic acid); PCLF: Poly(caprolactone fumarate); NVDP: N-vinyl pyrrolidone; nHA: Nanohydroxyapatite; mHA: Microhydroxyapatite; MAA: Methacrylic acid; PEGF: Poly(ethylene glycol fumarate).

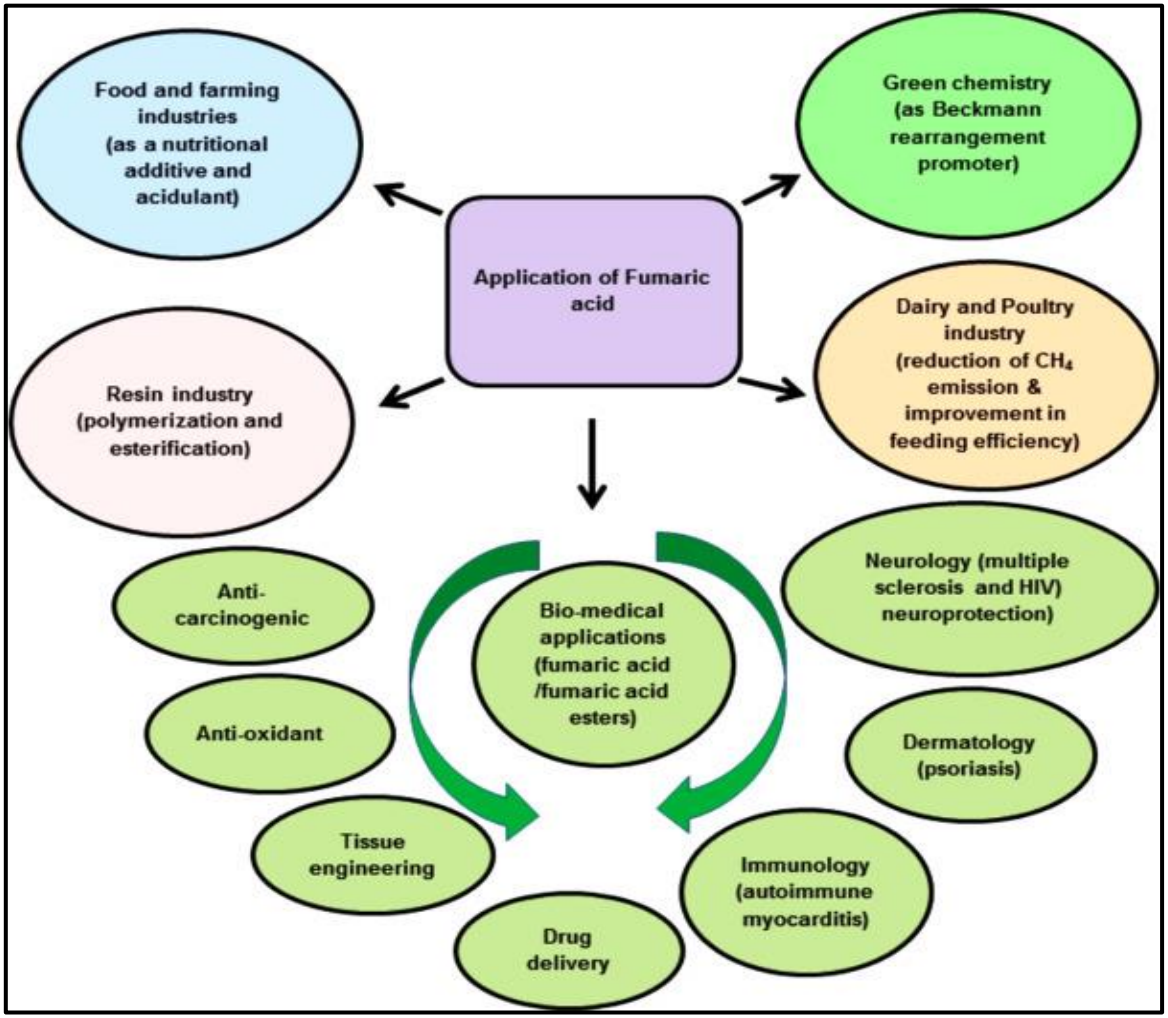

Figure 2.2.1: Diverse application fields of fumaric acid and fumaric acid esters.

END OF CHAPTER 2

CHAPTER 3

BREWERY WASTEWATER FOR FUMARIC ACID PRODUCTION AND DEVELOPMENT OF SPECTROPHOTOMETRIC METHOD

ENHANCED FUMARIC ACID PRODUCTION FROM BREWERY WASTEWATER AND INSIGHT INTO THE MORPHOLOGY OF *Rhizopus oryzae* 1526

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Résumé

Ce travail de recherche explore la possibilité de l'utilisation potentielle des eaux usées de brasserie (en anglais: BW) comme nouveau substrat pour la production de l'acide fumarique (FA) en utilisant la souche de champignon filamenteux *Rhizopus oryzae* 1526 (*R. oryzae*) par fermentation submergée. Les effets de différents paramètres, tels que la concentration ensolides totaux, le pH de fermentation, la température d'incubation, la vitesse d'agitation et la taille de l'inoculum ont été étudiés sur les morphologies fongiques. Différentes formes morphologiques (amas de mycélium, mycélium en suspension, granules solides/poilus) de *R. oryzae* 1526 ont été obtenues pour différentes conditions opératoires (pH de fermentation, températures d'incubation, vitesses d'agitation, et tailles de l'inoculum). Parmi toutes les morphologies obtenues, la forme de granules s'est trouvée être le plus favorable pour améliorer la production de FA pour les différents paramètres étudiés. Une étude en microscopie électronique à balayage a été réalisée pour explorer les morphologies fines des granules formées sous toutes les conditions optimisées. Avec toutes les conditions optimales de croissance (pH 6, 25 °C, 200 tours par minute, 5% (v/v) de volume de l'inoculum, 25 g/L en solides totaux, et un diamètre de granule de 0,47 ± 0,04 mm), la concentration la plus élevée de FA atteinte était de 31,3 ± 2,8 g/L. Les résultats ont démontré que les BW pourraient être utilisées comme substrat pour la souche fongique *R. oryzae* en fermentation submergée pour la production de FA.

Mots clés: Acide fumarique; eaux usées de brasserie; 460 nm; *Rhizopus oryzae* 1526; microscopie électronique à balayage

Abstract

The present research work explores brewery wastewater (BW) as a novel substrate for fumaric acid (FA) production employing the filamentous fungal strain *Rhizopus oryzae* 1526 (*R. oryzae*) through submerged fermentation. The effects of different parameters such as substrate total solid concentrations, fermentation pHs, incubation temperatures, flask shaking speeds, and inoculum sizes on the fungal morphologies were investigated. Different morphological forms (mycelium clumps, suspended mycelium, and solid/hairy pellets) of *R. oryzae 1526* were obtained at different applied fermentation pH, incubation temperature, flask shaking speed, and inoculum size. Among all the obtained morphologies, pellet morphology was found to be the most favorable for enhanced production of FA for different studied parameters. Scanning electron microscopic investigation was done to reveal the detailed morphologies of the pellets formed under all optimized conditions. With all the optimized growth conditions (pH 6, 25 °C, 200 rpm, 5 % (v/v) inoculum size, 25 g/L total solid concentration, and pellet diameter of 0.465 ± 0.04 mm), the highest concentration of FA achieved was 31.3 ± 2.77 g/L. The results demonstrated that BW could be used as a good substrate for the fungal strain *R. oryzae* in submerged fermentation for the production of FA.

Keywords: Fumaric acid, Brewery wastewater, 460 nm, *Rhizopus oryzae* 1526, scanning electron microscope

Introduction

Many platform chemicals are being produced from renewable biomass under moderate process condition pertaining to the depletion of conventional oil and the deterioration of the global environment. Fumaric acid (FA) and its derivatives are examples of such chemicals. The ability of FA to be converted into pharmaceutical products and act as starting material for polymerization and esterification reactions has led the US Department of Energy to designate FA among the top 12 biomass building block chemicals with potential to significantly enhance the economy (Xu *et al*., 2012). FA functions as an acidulant and controls the growth of microorganisms, adjusts pH, and enhances flavors (Yang *et al*., 2011). As an important platform chemical, FA is a valuable intermediate in the preparation of edible products, such as L-malic acid and L-aspartic acid, and with the increasing market share of L-aspartic acid and L-malic acid in sweeteners, beverages, and other health food areas, the worldwide demand for FA and its derivatives is growing each year (Goldberg *et al*., 2006). Currently, the annual production of FA is estimated to be 12,000 t, and the projected market volume is 200,000 t (Sauer *et al*., 2008). Additionally, FA is widely used in the feed industry as an antibacterial agent and a physiologically active substance (Mrowietz *et al*., 1999). World growth prospects for FA in food and beverages are significant. The main factors behind this growth are food safety, desire for convenience, new beverage and food introduction, and growing consumption of nutritional bars (including cereal, sports, and energy bars), particularly in North America, Europe, and Asia. Food and beverages accounted for 33% of world consumption of FA in 2009, followed by rosin paper sizes (20.0%), unsaturated polyester resins (18.6%), and alkyd resins (12.3%) (IHS Chemical. April 2010). Moreover, two potentially new applications for FA are (a) as a medicine to treat psoriasis, and (b) as a supplement in cattle feed that reduce the methane emission up to 70% (Beauchemin *et al*., 2006). Very recently, FA has been explored for many novel applications in the biomedical field. A number of synthetic biodegradable and injectable scaffold materials based on FA for an assortment of tissue engineering applications have been designed that can also be tailored for particular applications, ranging from cell encapsulation to gene delivery (Temenoff *et al*., 2008). In the recent time, production of FA employing *Rhizopus oryzae* through submerged fermentation from different waste materials has gained tremendous importance. Low-cost carbon sources of agro-industrial origin have been explored as substrate for FA production with a good productivity. Investigation on FA production from woodchips, dairy manure, and lignocellulosic biomass such as corn straw had exhibited good product features (Xu *et al*., 2010). With the increasing awareness of low-cost carbon option for the synthesis of value-added product, more of such substrates are

being considered at large scale. In support of the "carbohydrate economy" principles, production of FA, the high value platform chemical from agro-industrial biomass is also very important in "biorefinery" prospects. Meanwhile, the brewing industry holds a strategic economic position with an annual world beer production exceeding 1.34×10^9 hL in 2002. The brewing process uses large volumes of water in a number of different batch-type operations in processing raw materials to the final beer product. Water is a very substantial ingredient of beer, composing of 90–95% of beer by mass (Olajire *et al*., 2012). An efficient brewery uses between 4 and 7 L of water to produce 1 L of beer for the brewing, rinsing, and cooling processes (European Commission, 2006). Thus, a large amount of brewery wastewater (BW) is discharged to the drains as effluent. This water must be disposed of or safely treated for reuse, which is often costly and problematic for most breweries. The BW contains biological contaminants (0.7–2.1 kg of BOD/barrel) (Olajire *et al*., 2012). The main solid wastes are spent grains, yeast, spent hops, and diatomaceous earth. BW is not toxic, does not usually contain appreciable quantities of heavy metals, and is easily biodegradable (Brewers of Europe, 2002). BW can be a good source of nutrition for microorganism and bioproduction of a platform chemical which is important from both carbohydrate economy and biorefinery prospects.

In the present investigation, *R. oryzae* NRRL 1526, the one of the best filamentous fungus strain for fumaric acid (FA) production, was employed against BW. Recently, it has been claimed that there exists a direct quantitative relation between the pellet morphology and enhanced production of FA with this strain (Zhou *et al*., 2011). Apart from this specific finding, it is now a consensus view that formation of fungal pellets can benefit the fermentation as they reduce the medium viscosity and also has the advantages of not wrapping into the impeller of fermenter, reuse of fungal biomass, and more mass and oxygen transfers (Li *et al*., 2000; Rodriguez *et al*., 2005). However, the optimized parameters from different studies cannot be drawn into a strong conclusion. Hence, with every new medium composition, parameters need to be optimized to control the morphology of the fungal strain. The recent trend on the development of efficient strategies for pellet formation with a reduced diameter and claim for enhanced production of FA highly encouraged us to carry out the present investigation. Moreover, the spectrophotometric method adopted for the quantification of FA in the present approach further eased the FA determination procedure compared with conventional analytical methods such as high-performance liquid chromatography (HPLC). Thus, the present work encompasses the economic, eco-friendly, and methodological advantages in the production of FA.

Materials and methods

Microbial culture

Rhizopus oryzae NRRL 1526 (to be called *R. oryzae* thereafter) was procured from Agricultural Research Services (ARS) culture collection, IL, USA. The obtained strain was first cultured on a potato dextrose agar (PDA) slant at 37 ± 1 °C for a maximum of 4 days to form spores. For spore inoculum preparation, spores were further propagated on PDA plated (90 mm) at 37 ± 1 °C for 72 h. The agar plates were washed with sterile distilled water and filtered through sterile cotton wool to obtain a spore suspension free of mycelial contamination. The suspended spores were maintained at 4 °C for future use. For long-time storage, the spore suspension was placed in 20% glycerol solution at −80 °C. The cultures were renewed every 4 weeks. After counting with a haemocytometer, the spore concentration of the suspension was controlled to 1×10^7 spores/mL and used for inoculation.

Culture media

Two different media were applied in this study. For pre-culture of *R. oryzae*, glucose basic salt medium was used, while for fumaric acid production, BW was exploited.

Composition of pre-culture medium and growth condition

Pre-culture medium consisted of (g/L) glucose 50, urea 2, KH_2PO_4 0.6, $MgSO_4 \cdot 7H_2O$ 0.5, $ZnSO_4 \cdot 7H_2O$ 0.11, and $FeSO_4 \cdot 7H_2O$ 0.0088. The medium pH (4.6) was not adjusted, otherwise mentioned. The medium was sterilized in two parts to avoid the Maillard reaction between the carbonyl group (>C=O) of glucose and amino group ($-NH_2$) of urea. One part without the glucose was heat-sterilized (20 min, 15 lb, 121 ± 1 °C), while glucose was sterilized separately (20 min, 15 lb, 110 ± 1 °C). Sterilized medium was used for pre-culture of *R. oryzae* to obtain the pelletized seed. Medium was inoculated with the spore suspension (2%, v/v). Pre-cultures were carried out in 250-mL Erlenmeyer flasks with a final medium volume of 50 mL under the growth conditions of 30 °C and 200 rpm for 24 h. The pre-cultured *R. oryzae* was used as inoculum for the fermentation of BW at a concentration of 10% (v/v).

Procurement of brewery wastewater and application for submerged fermentation (smf) for fumaric acid production

Brewery wastewater was utilized as fermentation medium to evaluate its potential for FA production. BW was procured from a local brewery industry (La Barberie, Quebec, Canada). To avoid microbial decay, BW was stored at 4 ± 1 °C for a maximum of 2 weeks before fermentation. For compositional data of BW, the work of Dhillon *et al*. was cited (Dhillon *et al*., 2012). To prepare for the fermentation, 135 mL of BW was dispensed in a 500-mL Erlenmeyer flask and heat-sterilized (20 min, 15 lb, 121 ± 1 °C). Sterilized BW was inoculated with 15 mL (10%, v/v) of pre-cultured fungus (pellets + mycelium) giving a final volume of 150 mL. Flasks were incubated at 30 °C and 200 rpm in a shaker incubator for 3 days. Different growth conditions were applied for *R. oryzae* in the fermentation process. Based on previous findings, the parameters chosen for the optimum production of FA were pH (4-10), rpm (100, 150, 200, 250, and 300), temperature (25 °C and 37 °C), total solid concentration (TSC) (10-40 g/L) of BW, and percentage (2.5, 5, 10, and 20%) of pre-cultured inoculum. Effects of variations in the values of each parameter were co-related with FA production. The fermentation time was maintained at 72 h for all experiments.

Neutralizing agent

Calcium carbonate ($CaCO_3$) was used as neutralizing agent in the fermentation medium (BW) at a fixed concentration of 40 g/L.

Analytical methods

Fumaric acid concentration

FA was quantified spectrophotometrically by the modified method of Marshall, Orten, and Smith (Marshall *et al*., 1949). Briefly, the modified method is as follows:

Chemicals

Anhydrous fumaric acid (FA), pyridine, anhydrous $CuSO_4$, gum ghatti, anhydrous citric acid, NH_4OH, and sodium diethyldithiocarbamate. All the chemicals used were of analytical grade and purchased from Fisher Scientific, Canada.

Working solutions

FA (1 mg/mL), pyridine (0.5%, v/v), $CuSO_4$ (20%, w/v), gum ghatti (2%, w/v), citric acid (20%, w/v), NH_4OH (10%, v/v), sodium diethyldithiocarbamate (0.2%, v/v), and copper-pyridyl reagent (20 mL of 20% $CuSO_4$ + 8 mL of pyridine).

The standard curve of FA

Different volumes (200, 400, 600, 800, and 1000 µL) of FA were transferred to five different test tubes. To each test tube, 50 µL of copper-pyridyl reagent was added and left for 1-2 min. Development of turbidity indicated the formation of copper-pyridine-fumarate complex. To this, another 500 µL of copper-pyridyl reagent was added and vortexed. The tubes were left at 4 °C for atleast 15 min for incubation. The content of each test tube was then transferred into microcentrifuge tubes and subjected to centrifugation at 2000 rpm for 3 min. The supernatant was removed, and to each of pellet left, 2 mL cold (kept at 4 °C) pyridine solution was added and centrifuged at 2000 rpm for 3 min. Pellet recovered were dissolved in 1 mL of citric acid solution. This was followed by the addition of 1 mL of NH_4OH solution and mixed properly. To this mixture solution, 200 µL of gum ghatti and 1 mL of diethyldithiocarbamate solution were added. The solutions were left for color development. After 4-5 min, all the solution developed a light blue color (formation of fumarate-copper-diethyldithiocarbamate complex). The transmittance of the solutions was measured at λ_{max} = 460 nm in a 96-well plat reader. The OD (optical density) values were plotted against fumaric acid concentrations, and a standard curve was constructed.

Sample analysis for FA estimation

Concentration of FA for different broth samples were quantified taking the standard curve of FA as reference. The concentrations were expressed in grams per liter of BW.

Sample blank

A mixture of citric acid, NH_4OH, gum ghatti, and diethyldithiocarbamate in the same proportion as used for the standard curve was taken as “sample blank”.As mentioned before, the procedure mentioned in the original protocol was technically improvised to make it more time and cost effective. A detailed comparison of the original and modified protocol has been presented in Table 3.1.

Downstream processing for fumaric acid recovery

To recover FA from the insoluble calcium fumarate, fermented broth was heated at 90 °C with simultaneous acidification (5 N, H_2SO_4) until clear. Finally, the broth was centrifuged (8000 × g,

10 min), and the supernatant containing the FA was collected and analyzed spectrophotometrically at λ_{max} = 460. The precipitate (fungal biomass + $CaSO_4$) was further processed for dry mass determination.

Biomass dry weight

Biomass dry weight (BDW) was measured by washing the mycelia three times with distilled water and then allowed to dry at 60 ± 1 °C until a constant weight was achieved.

Morphological study

The morphological patterns displayed by *R. oryzae* with the variations of growth conditions during fermentation were studied with digital photography and electron microscopy. After recovering the samples from culture, they were repeatedly washed with copious amount of water and photographed with a digital camera (Canon PC 1585). The fungal pellets obtained at the optimized growth conditions were further considered for scanning electron microscopic (SEM, Carl Zeiss EVO® 50) analysis to have a highly magnified view of the surface morphology. To prepare for SEM, cleaned fungal pellets were air-dried on a microscopic glass slide at isolated position overnight at room temperature (25 ± 1 °C). Dried samples were directly mounted on a SEM grid and sputter coated (SPI Module Sputter Coater) with gold before SEM analysis. Both size and shape were analyzed to confirm the parameter effects on *R. oryzae* morphology.

Statistical analysis

Data are represented as mean ± SD of three independent experiments. Correlations were considered significant at $P < 0.05$ for different applied parameters.

Results and discussion

The advantages of selecting the strain *R. oryzae* for fumaric acid production

The fungal species and the strain selected for the present study has a strong and decade-old research background. Many fungal species belonging to the genus *Rhizopus* were identified as the best FA producers and received industrial attention. The most important

were *R. nigricans, R. formosa, R. arrhizus*, and *R. oryzae*. However, among the four species, *R. oryzae* was preferred over the other three due to its simple nutrient requirements and high productivity (4.25 g/(L h)). After the 1990s, *R. oryzae* has been the frontliner in the production of FA (Xu *et al*., 2012). Among different strains of *R. oryzae* tested for FA production, the strain NRRL 1526 is one of the best strains (Oda *et al*., 2003). Thus, selection of *R. oryzae* 1526 for FA production meets technical requirements for a scientific investigation on FA production.

The Brewery wastewater as substrate for *R. oryzae*

Brewery wastewater (BW) is the waste sludge produced by any brewery industry in large volumes. Recently, BW has been screened as the potential substrate for the production of citric acid (Dhillon *et al*., 2012). In this study, compositional analysis of BW showed it to be rich in carbon and other vital nutrients required for culturing fungi. The results of the study suggested that BW could be a good and cheap biomass source for FA fermentation. So far, this agro-industrial waste has not been exploited for FA production.

Selection of neutralizing agent

It is now a well-known fact that in Rhizopus-mediated FA production, the pH value of FA production medium drops down (e.g., from 5 to 2) quickly in the first 20 to 24 h after inoculation due to production of FA. The consequence is the strong inhibitory effect on the growth of R. oryzae and FA production. This necessitates the addition of a neutralizing agent that will make complex with FA and thus maintaining the pH level at optimum for the growth of R. oryzae and FA production. After decades of research on different neutralizing agents (e.g., $CaCO_3$, Na_2CO_3, $NaHCO_3$, $(NH_4)_2CO_3$, and Ca $(OH)_2$), calcium carbonate was found to be the most efficient neutralizing agent in the commercial level production of FA (Xu *et al*., 2012). The justifications made were (a) FA yield and volumetric productivity were found to be lower for other neutralizing agents than $CaCO_3$; (b) accumulation of byproducts, such as malic acid and ethanol, was higher with other neutralizing agents; and (c) $CaCO_3$ can supply CO_2 that can be used for the formation of oxaloacetate in the tricarboxylic acid (TCA) cycle. Thus, selection of $CaCO_3$ as a neutralizing agent makes sure that the present study does not compromise with the growth of *R. oryzae* and production of FA.

Downstream processing

In FA production, depending on the type of neutralizing agent being used, downstream processing of the fermented broth is performed with different methods. In case the

neutralizing agent is $CaCO_3$, the downstream methods employ mineral acids (HCl or H_2SO_4) and heat energy for the recovery of FA from the feebly soluble calcium fumarate ($CaC_4H_2O_4$) formed during fermentation. In one method, heating of broth at high temperature (160 °C) followed by acidification (pH 1.0) is performed for FA recovery (Gangl *et al*., 1990). However, the approach consumes a large amount of energy to maintain the high temperature and also requires especially durable heating equipment, acidification and heating of broth at moderate temperature (60-90 °C) for FA recovery. No special heating equipment is required in this method (Xu *et al*., 2012). Therefore, in the present study, low heating strategy was adapted to make the FA production more economically attractive.

The improvised spectrophotometric method for fumaric acid quantification

All the quantification of FA was performed spectrophotometrically following the method of Marshall, Orten, and Smith. However, some technical improvisations were made in the original protocol that did not cause any significant difference in the measured values of FA. The technical comparison of the procedure mentioned in the original work and followed in the present investigation is shown in Table 3.1. The outcome of the technical improvisation of the existing technique could be evaluated in terms of cost (volumetric reduction of reagents used) and time effectiveness (total estimation time). However, the chemical requirement was the same in both cases. As can be summarized from Table 3.1, there was an almost fourfold decrease in the total time of estimation in the improvised technique. Practically, there was no drastic change in the FA concentration determined following both procedures. Previously, there was no report on the spectrophotometric determination of FA in biological sample. The method being followed in the present investigation could be a good alternative to conventional methodological approach such as HPLC for concentration measurement of FA. Literature on fermentation-based production of FA shows that the presence of (only qualitative) fumaric acid was determined by the formation of insoluble mercurous fumarate in 5 % nitric acid as no method for exact quantification of FA was available (Olander, 1929). This might have urged many researchers to develop a reliable colorimetric method for the determination of FA concentration in biological samples. However, probably pertaining to the rapid development in the modernization and sophistication of analytical instruments such as HPLC, this method of FA estimation was not explored anymore. The present investigation highlighted and improvised a method important in both economical and analytical prospective.

The parameters chosen for the present study and their effects

The parameters taken into consideration for the present investigation are very important from the point of view that *R. oryzae* is highly susceptible to these parameters and has a direct impact on the FA production. In many recently conducted studies, it is claimed that tuning of morphology of this fungal strain can work as a decisive factor in the overall performance of the fungus for FA production (Zhou *et al*., 2011). These investigations finally led to the conclusion that formation of pellet morphology by *R. oryzae* is very important to enhance the production of FA in SmF. In turn, the pellet formation could be programmed by changing the growth conditions. Based on those important previous findings on the morphological behaviors of *R. oryzae*, five different parameters (pH, temperature, rpm, total solid concentration, and volume of pre-culture) were studied. To start with, growth condition parameters of pH5, 30 °C, and 200 rpm were initially maintained for SmF. Under these growth conditions, *R. oryzae* 1526 performed best in terms of FA production (Zhou *et al*., 2011). For BW as novel substrate, process optimization was carried out for achieving the fungal pellets of smaller diameter and higher production of FA.

Effects of total solid concentration of BW

Different total solid concentrations were tested to optimize for the maximum production of FA. With the increase of TSC, there was a corresponding increase in the body dry weight of *R. oryzae*, but FA production was lowered (Figure 3.1). The inverse relation between TSC versus FA concentration was due to the broth rheology. The broth viscosity was highly prone to TSC and fungal morphology (Dhillon *et al*., 2012). The TSC concentration supporting the pellet formation has provided the double benefits of lower viscosity and more production of FA, while formation of suspended mycelium increased the viscosity. TSC concentration of 25 g/L was considered to be the optimum one as fungal pellets and higher production FA were obtained.

Role of production medium (BW) pH

The BW has an original pH of 3.5 (Dhillon *et al*., 2012). However, there is an agreement in the literature that *R. oryzae* grows well within the pH range of 4-9 (Meussen *et al*., 2012). Thus, the BW pH was adjusted from 3.5 to the required values (4, 5, 6, 7, 8, and 9) before the SmF. The effects of various pH on FA production and morphology of *R. oryzae* were very interesting. FA production was highest (14.16 ± 1.9 g/L) at pH 6. From pH 4–6, there was a gradual increase in FA production, but it started declining at pH 7 and continued up to pH 8 (Figure 3.2 (A)). The starting pH (4) resulted in a very lower production (3.7 ± 1.16 g/L)

of FA. Beyond the pH value of 8, in fact, there was no growth of R. oryzae in the fermentation medium.

The fungus exhibited an array of distinctive morphological patterns corresponding to different pH of BW (Figure 3.2 (B)). Mycelium clumps, suspended mycelium and pellets, the common morphological forms of fungus, were manifested as a response to the variations in the pH values. However, the pellet types were not the same in their shape and size. Hairy and less aggregated pellets were dominantly formed at pH 5. The pattern changed into solid and non-aggregated type at pH 6. While reaching pH 7, formation of even more aggregated pellets was favored. The clump and suspended mycelium were formed at pH 4 and 8, respectively. The highest pH (9) value applied did not support the growth of *R. oryzae* in the fermentation medium although the incubation was continued for 120 h.

The production of FA and morphological behavior are highly interrelated. Many previous studies confirmed that pellet morphology was the most suitable form for FA production (Zhou *et al*., 2011). In the case of hairy pellets, shaving off the hairs by the hydrodynamic force (during flask shaking) reseeds into more mycelial growth and leads to damage and deactivation of both reseeded mycelia and the pellets. This initiates the aging and vacuolation of the fungal hyphae and finally leads to activity reduction of both pellet and reseeded mycelia (Cui *et al*., 1998). Thus, the production of FA was influenced by the morphological forms of *R. oryzae*, while fermentation medium pH triggered the morphological changes under the growth conditions of 200 rpm, 30 °C, and 25 g/L of TSC. Substrate pH value of 6 supported the highest production of FA and formation of solid pellet by *R. oryzae* as well. Reconsidering the previously investigated best growth conditions (200 rpm, 30 °C, pH 5) for the strain *R. oryzae* for highest production of FA, it can be concluded that with novel substrate (BW in the present study), the optimum growth conditions for the same fungal strain varied considerably which can be mainly attributed to the medium composition (Zhou *et al*., 2011).

Mechanical force or shaking speed effect

The optimized pH 6 (section “Role of Production Medium (BW) pH”) was further put into the investigation of mechanical force effects on the FA production and morphology of *R. oryzae* 1526. Different shaking speeds (100, 150, 200, 250, and 300 rpm) were tested. The other growth conditions were 30 °C and 25 g/L of TSC. The effects on FA production and *R. oryzae* morphology are shown in Figure 3.3 (A) and Figure 3.3 (B), respectively. FA

production was highest (11.8 ± 1 g/L) at 200 rpm, while at other applied shaking speed, the production level was lowered down considerably. As it can be seen from Figure 3.3 (B), pellet morphology was reproduced at 200 rpm as obtained under same growth conditions (pH 6, 200 rpm, and 30 °C) discussed in section "Role of Production Medium (BW) pH". The hydrodynamic effects on morphology and subsequent control on FA production were quite obvious. Suspended mycelium was formed at 100, 150, and 250 rpm shaking speeds. At the highest applied speed (300 rpm), there was no proliferation of the pre-cultured inoculum in BW.

It is a well-known fact that in SmF, agitation is used to achieve uniform gas dispersion, homogenization, and interphase mass and heat transfer. The hydrodynamic force generated at various rpm speed can have a direct impact on the overall morphology of *R. oryzae*. Strong agitation can lead to fungal deactivation due to the shaving off of hyphae, while lower speed can disturb the oxygen, heat, and mass transfer (Cui *et al*., 1998). Teng *et al*. also mentioned about the formation of distinctive morphologies as a response of shear forces (Teng *et al*., 2009). It is obvious that at lower agitation speed, the pre-cultured fungus consisting of mixed morphologies of pellets and mycelium do not become separated from each other, and their further growth leads to suspended mycelium which is actually the agglomeration. The hydrodynamic force generated out of the agitation actually dissipates specific free energy that has a control (proportional relation with an exponent of −0.25) on the fungal mean hyphal length (Cui *et al*., 1997). Thus, with the increase of flask shaking speed, dissipation of specific free energy will increase exponentially and cause shorter hyphal length of loose mycelium which is less protected from the shearing forces and becomes less active (Cui *et al*., 1998). Moreover, formation of pellets itself is supportive of more protection against shearing stress and less prone to deactivation. Consequently, more number of hyphae will be functional and metabolic activity will be higher. Thus, the moderate value (200) of shaking speed supported the pellet formation of *R. oryzae* that resulted in more production of FA compared with other applied speeds.

Variation in SmF temperature

The fermentation experiment was also carried out at two more different temperatures, namely, 37 and 25 °C. Recently, it was reported that pellet diameter could be controlled by varying the pre-culture temperature for *R. oryzae* 1526 strain. With the increase of incubation temperature (> 30 °C), pellet diameter became smaller and beyond 30 °C, the pellets with larger diameter were formed (Zhou *et al*., 2011). In the present investigation, the

approach was not made at pre-culture stage but during SmF. As already discussed, in the present investigation, pellets were obtained both at pre-culture and fermentation stages. For SmF, the growth conditions optimized for pellet formation and higher production of FA were pH 6, 30 °C, and 200 rpm. Investigation on the possible role of SmF temperature in *R. oryzae* morphology and thus influencing the production level of FA was important.

In the experimental procedure, SmF was carried out at 25 and 37 °C while keeping the other growth conditions (200 rpm, pH 6, and 25 g/L of TSC) constant. After 72 h of SmF, the fungal biomass exhibited two distinct morphologies. The experiment carried out at 25 °C produced solid pellets with a reduced diameter (1 mm, Figure 3.4) compared with pellets obtained at 30 °C (3-5 mm, Figure 3.2 (B) and Figure 3.3 (B)). The other experiment designed for 37 °C resulted in suspended mycelium (Figure 3.4). The important outcome of this experiment was the marked increase in the production level of FA. The FA concentration reached 23.66 ± 2.1 g/L in 72 h of SmF. This is almost 10 g/L more as compared with the maximum concentration (14.16 ± 1.9 g/L) of FA obtained at 30 °C, 200 rpm, and pH 6. The FA concentration for 37 °C was very low (2.88 ± 1.33 g/L).

Many previous studies proved that fungal pellet formation was affected by incubation temperature. To be more specific, the approach of temperature lowering can induce pellet formation (Braun *et al*., 1991). In another study by Schugerl *et al*., various morphological forms were observed in the incubation temperature range of 25-35 °C (Schugerl *et al*., 1998). The conclusion made from these temperature-dependent investigations was that at higher temperature when the oxygen supply of the cells was inadequate, pellets were transformed into filamentous mycelium. Thus, the findings of the present study are in agreement with the general acceptance of temperature versus fungal morphology relationship.

Effect of inoculum volume

With the optimized growth conditions (pH 6, 200 rpm, 25 °C, 25 g/L TSC), SmF was also tested for the probable effects of the pre-cultured inoculum size (%, v/v) on FA production and morphology of *R. oryzae*. Although most of the SmFs involving filamentous fungus apply 10% (v/v) of pre-culture for inoculating the production medium, variation in inoculum size can generate important information on the change in FA production and morphology of *R. oryzae*.

Four different inoculum concentrations (2.5, 5, 10, and 20% v/v) were tested in the study. At 2.5 % (v/v) inoculum concentration, FA production was very low (2.17 g/L) and a mixed type (pellet + mycelium) of morphology were observed. The next higher concentration (5%, v/v) resulted in a marked increase in the FA production (31.3 ± 2.77 g/L) and produced fungal pellets with a highly reduced diameter (Figure 3.5) which was later confirmed from SEM analysis (Figure 3.6). At 10% (v/v), the results were almost repeated as obtained earlier with the growth conditions of pH 6, 200 rpm, and 25 °C. On reaching the highest concentration (20%, v/v), there was a massive fungal mycelium growth (12 g/L BDW) with negligible amount (0.892 g/L) of FA production.

The critical role played by vegetative inoculum volume in the development of fungal morphology and relation to metabolite production in the fermentation culture was investigated previously. In one such study, fungal morphology was manipulated by means of inoculum amount, and corresponding product level was estimated (Papagianni, *et al*., 2004). The inoculum volume that supported the pellet morphology resulted in more activity of the targeted product, while with filamentous morphology, the scenario was just opposite. Although most of the studies on fungal inoculum effects are spore-type based, some of the basic concepts can also be extended for vegetative inoculum. For instance, whether it is of spore or vegetative origin, higher population (large volume of inoculum) always ends up with either big-sized fungal pellets or suspended mycelium due to agglomeration (Nielsen *et al*., 1995; Yanagita *et al*., 1963; Vecht-Lifshitz *et al*., 1989). In the present case, 5 % (v/v) inoculum caused the formation of fungal pellets with reduced diameter and increased the FA production level to 31.3 ± 2.77 g/L.

Morphological studies

The pellet morphology of *R. oryzae* was further analyzed using SEM. Many conventional measurement tools (microphotography, ruler-based scaling, and others) are applied for the measurement of fungal pellet diameter. However, accuracy and qualitative aspects are not within the scope of these techniques. A high-quality imaging system such as SEM can reveal both quantitative (diameter of pellet and hyphae) and qualitative (pellet shape, surface topology, and hyphal texture) minute details of fungal pellets. For these reasons, optimized pellet samples were subjected to SEM analysis.

The pellets formed under the growth conditions of pH 6, 200 rpm, 25 °C, and 10% (v/v) inoculum size were observed under the high magnification and resolution states of SEM. The pellets were found to be roughly spherical and almost of uniform sizes (1 ± 0.15 mm,

Figure 3.3, Figures 3.6 (A) and 3.6 (B)). The edges were very sharp without mycelial protrusions from the pellet peripheries and thus ending in solid boundaries. The mycelium compactness was very high, and the hyphal average diameter was measured to be approximately 3.5 ± 0.5 µm (Figure 3.6 (C)).

To have a comparative account on the size and shape of the pellets obtained under all the optimized growth conditions (pH 6, 200 rpm, 25 °C, and 5% (v/v) inoculum size), SEM analysis was also carried out for those pellets. As shown in the Figures 3.3, 3.6 (D) and 3.6 (E), the pellets almost maintained uniformity in their sizes and shapes. The pellet diameter measured was 0.440 ± 0.05 mm. The reduction (~50%) in pellet diameter was very significant in terms of FA production. Hyphal diameter remained almost unchanged (3 ± 0.8 µm, Figure 3.6 (F)). The structural intactness with reduced diameter (from 1 to 0.4 mm) regulated by the inoculum volume was an important finding of the present study.

From the process optimization experiments done in this study, the best growth conditions for *R. oryzae* 1526 could be summarized as pH 6, 200 rpm, 25 °C, 5% (v/v) of inoculum size, and TSC of 25 g/L. With these optimized conditions, the maximum concentration of FA obtained was 31.3 ± 2.77 g/L. Considering that FA was being produced from a waste biomass without any supplementation of nutrients, the FA production level was quite high.

Conclusions

A novel combination between BW and *R. oryzae* 1526 was applied for the production of FA. Parameters including fermentation pH, temperature, shaking speed, inoculum size, and total solid concentration of BW were optimized for higher production of FA. The highest concentration of FA obtained in this study was 31.3 ± 2.77 g/L. A growth condition of pH 6, 25 °C, 200 rpm, 5% (v/v) inoculum size, and 25 g/L of TSC supported for the enhanced production of FA. Spectrophotometric determination of FA was done in this study. The used substrate served the purpose of macronutrient and micronutrient for *R. oryzae* 1526.

Abbreviations

BW= brewery wastewater, BDW= biomass dry weight, SEM= scanning electron microscope, FA= fumaric acid, rpm = revolution per minute, OD= optical density

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Table 3.1: Technical comparison of the procedures followed in the original and improvised protocol for the colorimetric determination of fumaric acid.

Experimental step	Original protocol	Modified protocol
Preparation of reagents	Pyridine (0.5%) $CuSO_4$ (20%) Gum Ghatti (2%) Citric acid (20%) NH_4OH (10%) Sodium diethyldithiocarbamate (0.2%) Copper-pyridyl reagent (20 mL of $CuSO_4$ + 8 mL of pyridine)	No change
Preparation of Gum Ghatti solution	A wire screen containing 20 g of gum ghatti is suspended just below the surface of one litre of water in a glass cylinder. After standing for 24 h, the liquid is strained through a clean cloth.	No need of wire screen and standing for 24 h. Gum ghatti powder was directly mixed with water, vortexed vigorously, filtered (whatmann 3) and used.
Cooling of copper-pyridyl-fumarate complex	The copper-pyridyl-fumarate complex was put at 4 ^oC for 1h	The incubation time could be reduced to 0.25h (15 min)
Centrifugation of copper-pyridyl-fumarate complex after cooling	Centrifuged at 200 rpm for 15 min	Reduced to 3 min at 2000 rpm
Dissolving copper-pyridyl-fumarate precipitates in 20% citric acid	A total volume of 10 (1 + 5 + 4) mL of citric acid (20%) was used to dissolve the complex in a sequence of three steps	1 mL was enough to dissolve the complex completely in one step.
Dissolved copper-pyridyl-fumarate precipitates + NH_4OH + Gum Ghatti + sodium diethyldithiocarbamate	The reaction mixture was left undisturbed for 4 min for color development.	No change
Measurement of transmittance of the final reaction mixture	at 460 nm	No change
Total time required	60 + 15 + 3 + 4 = 82 min	15 + 3 + 4 = 22 min

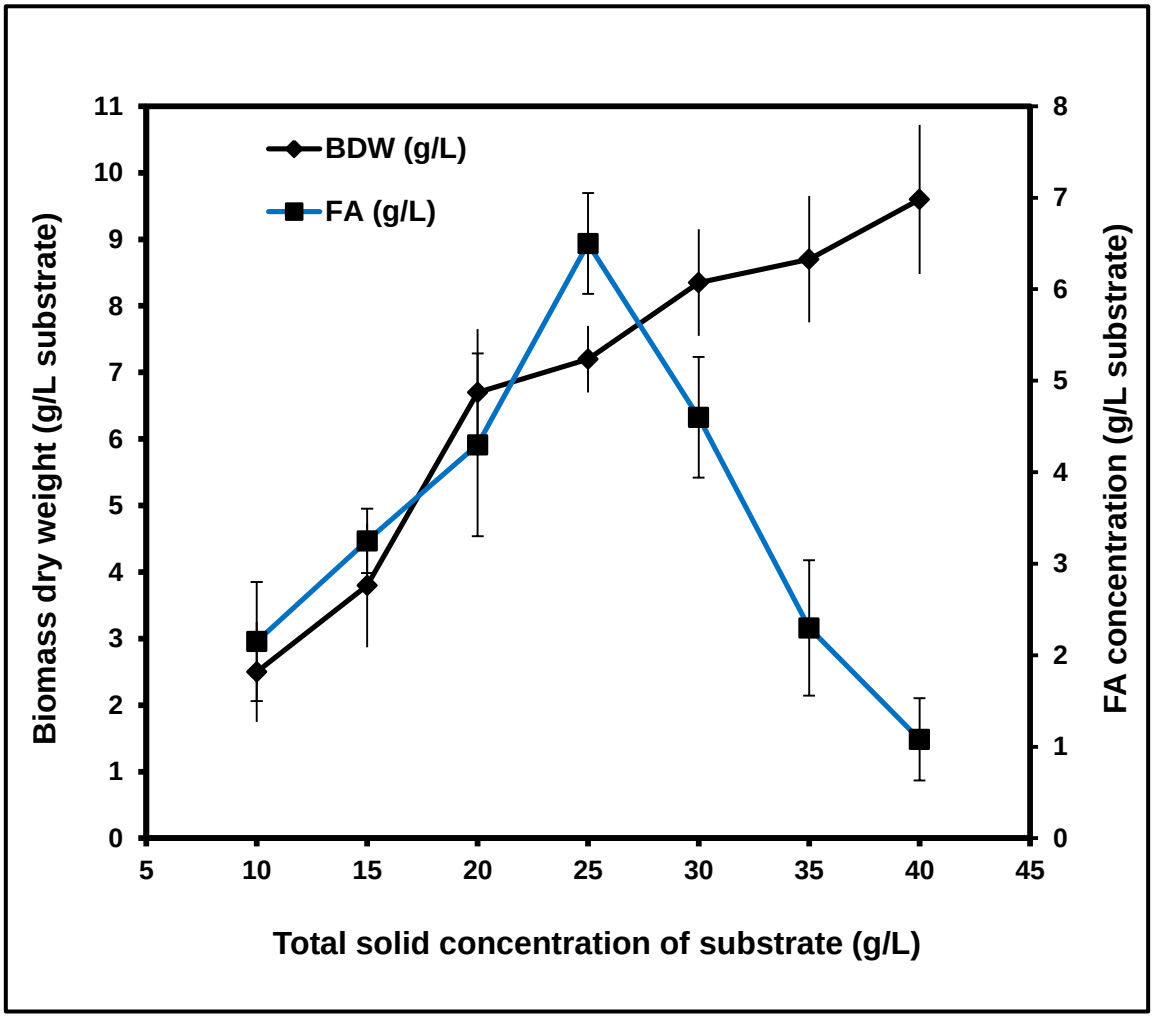

Figure 3.1: Effect of substrate total solid concentration (g/L) on biomass dry weight (BDW) of *R. oryzae* and fumaric acid (FA) production.

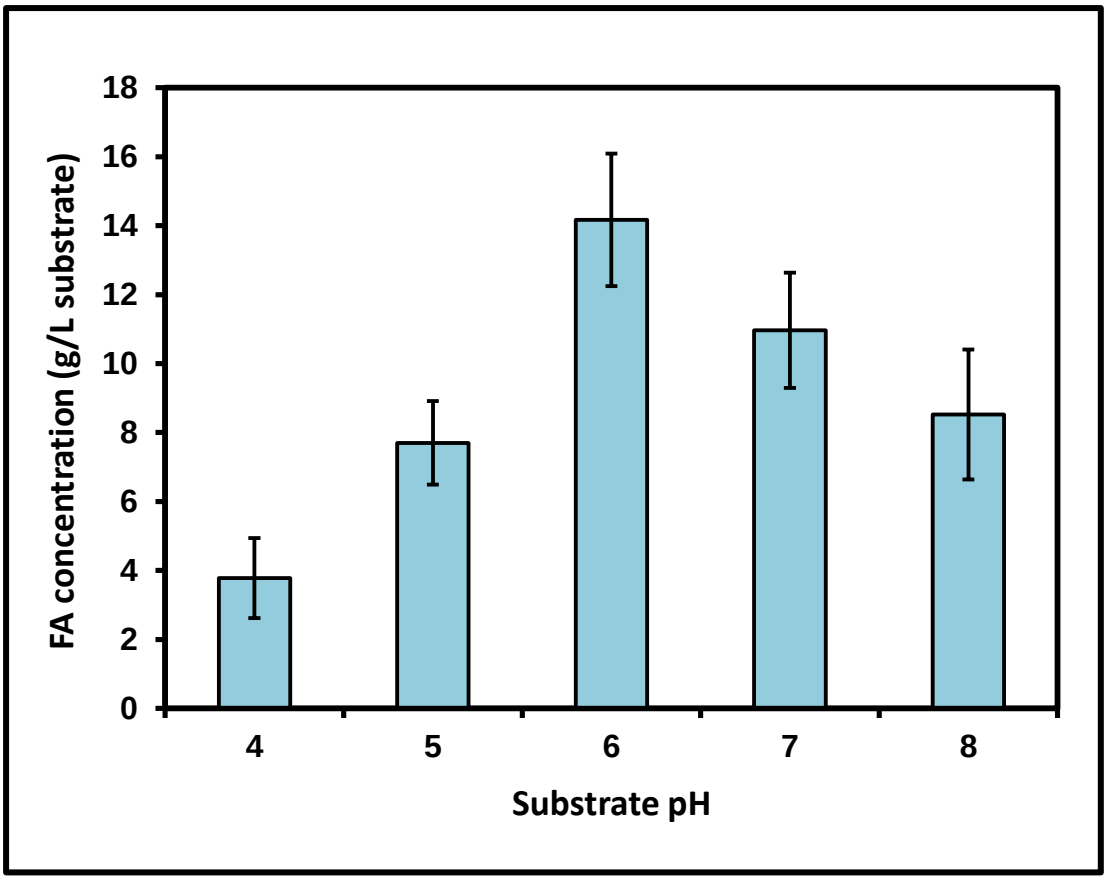

Figure 3.2 (A): Change in fumaric acid concentration (g/L of substrate) with the variations (4, 5, 6, 7 and 8) of fermentation pH.

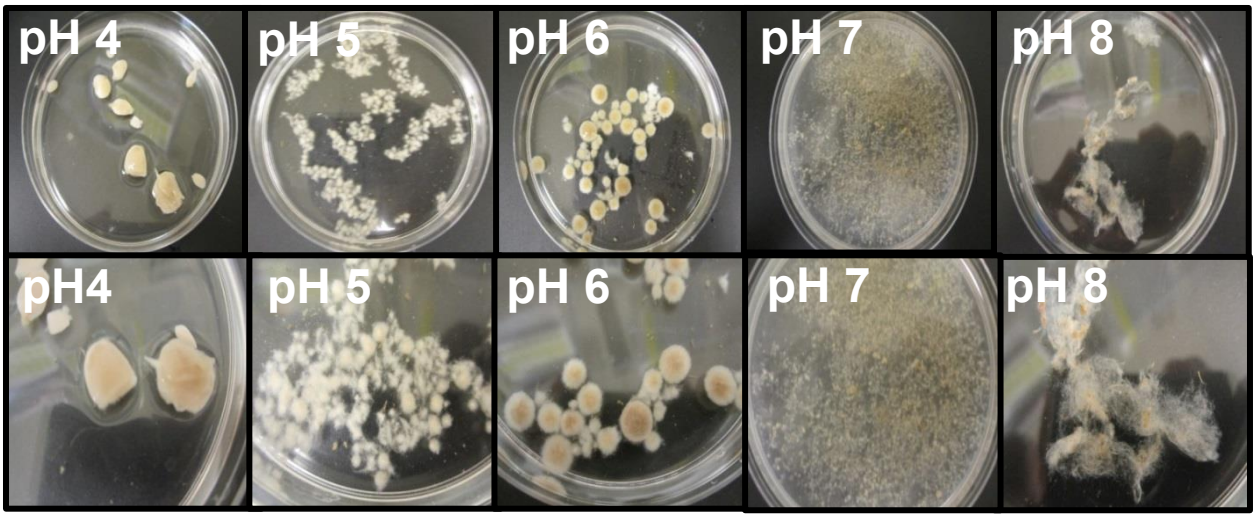

Figure 3.2 (B): Different morphological forms of *R. oryzae* obtained at different fermentation pH. Below each figure is seen a corresponding close view of the morphological forms.

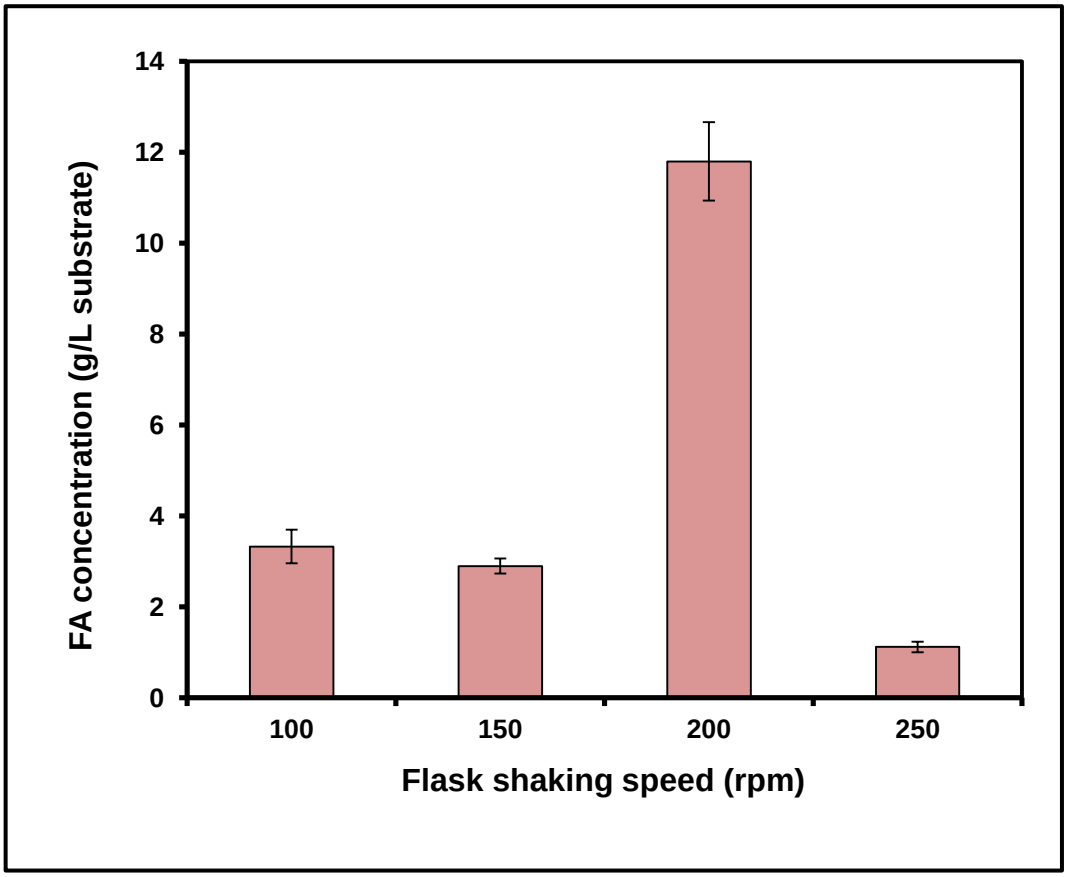

Figure 3.3 (A): Effect of flask shaking speed on fumaric acid concentration during fermentation.

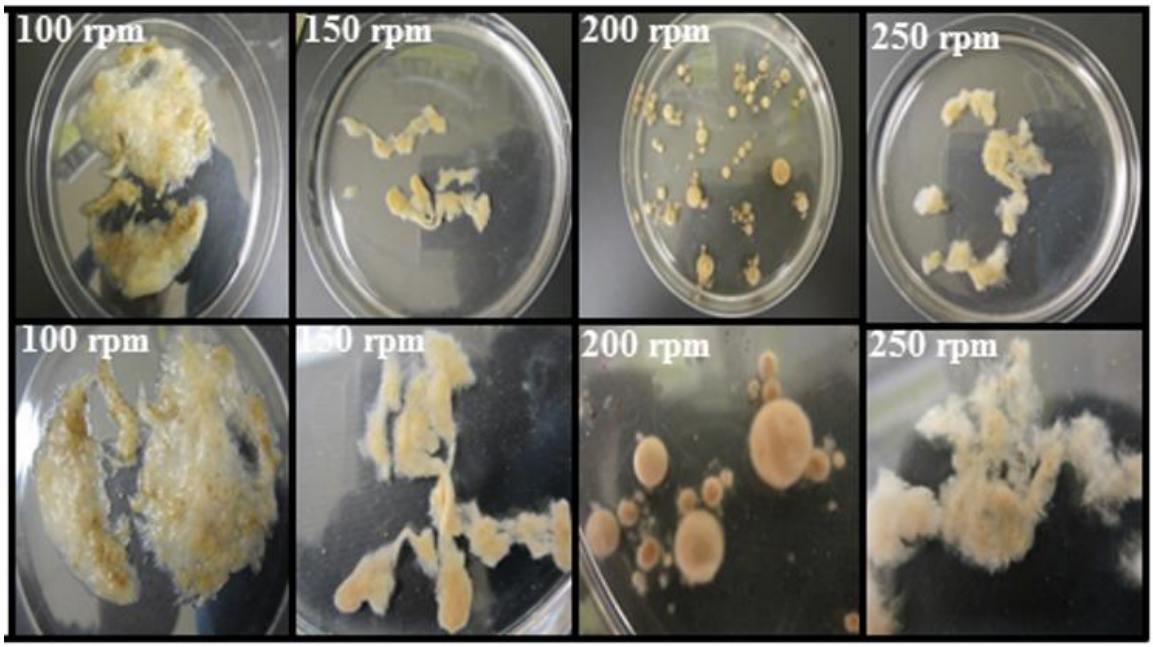

Figure 3.3 (B): Morphological responses of *R. oryzae* to the change in flask shaking speed during fermentation. Below each figure is seen a corresponding close view of the morphological forms.

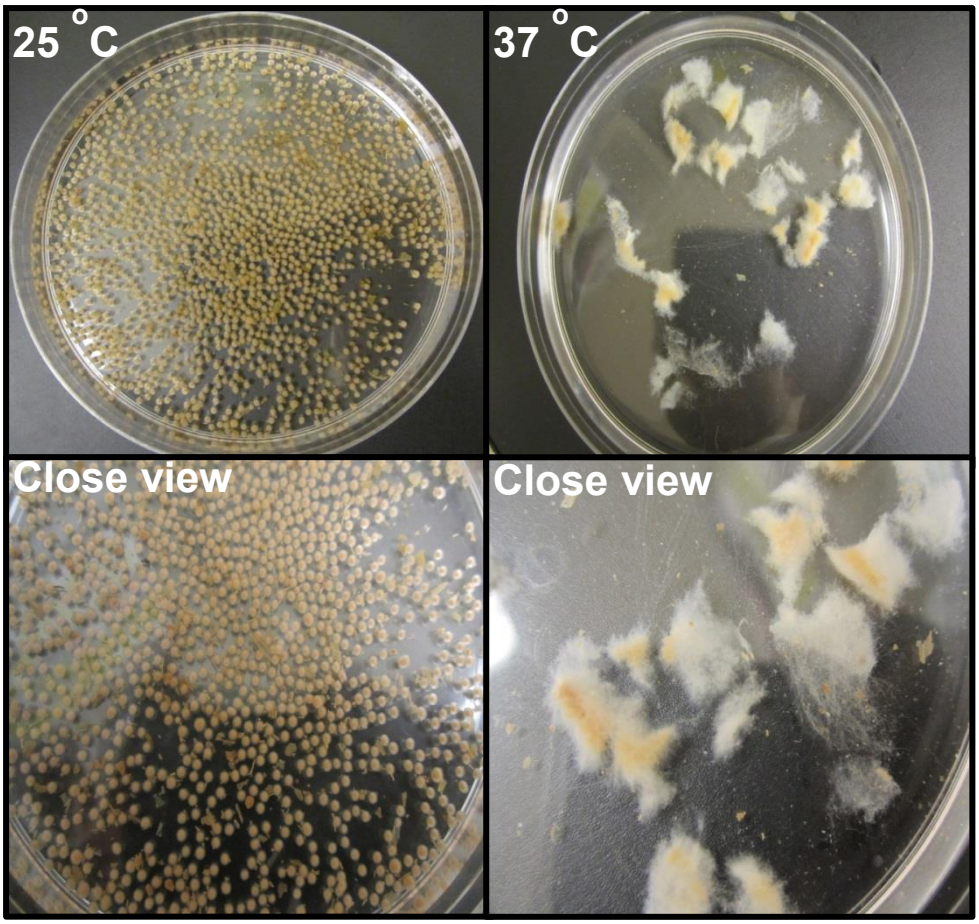

Figure 3.4: Two distinct forms of morphologies of R.oryzae 1526 obtained at 25 ^{o}C and 37 ^{o}C of fermentation temperature.

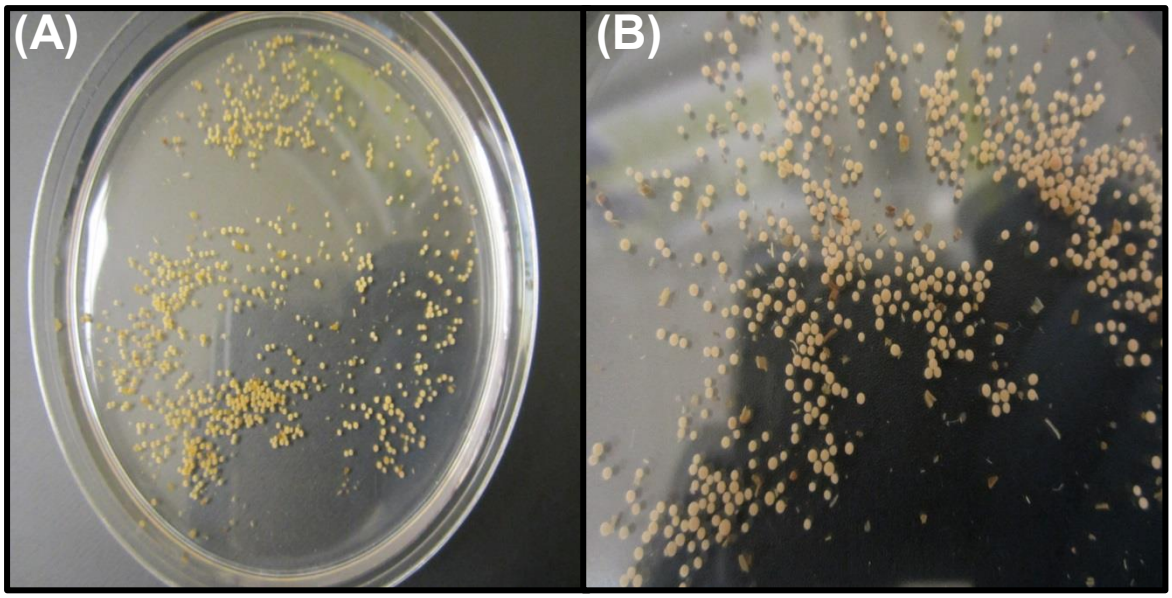

Figure 3.5: (A) Pellets of *R.oryzae* formed at 5% (*v/v*) of vegetative inoculum size **(B)** close view of the formed pellets.

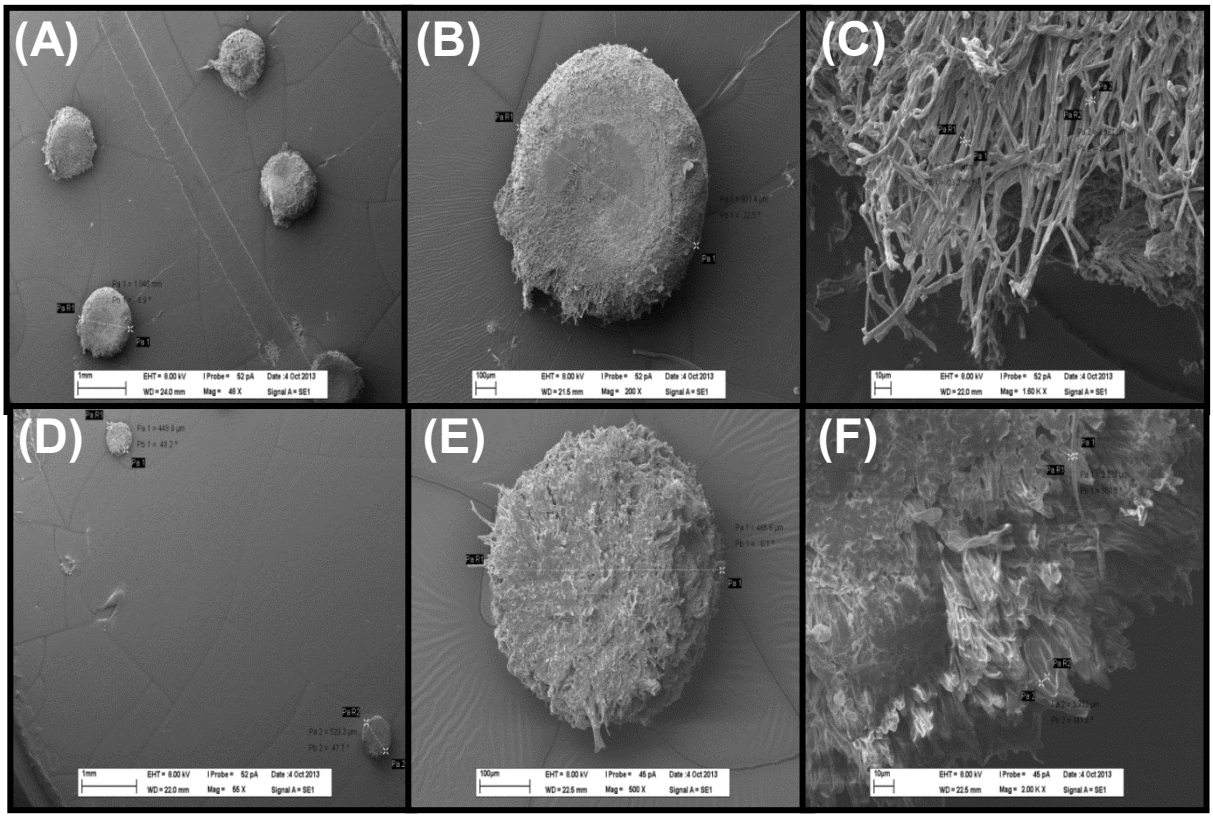

Figure 3.6: Scanning electron micrograph images of the pellets of *R.oryzae* obtained under different growth conditions. **(A) (B) (C)** represent the overall morphology, size and hyphal close view of the pellets formed at pH 6, 200 rpm, 25 ^{o}C, 10% (*v/v*) inoculum size and 25 g/L total solid concentration, respectively. **(D) (E) (F)** represent the overall morphology, size and hyphal close view of the pellets formed at pH 6, 200 rpm, 25 ^{o}C, 5% (*v/v*) inoculum size and 25 g/L total solid concentration, respectively.

END OF CHAPTER 3

CHAPTER 4

PRODUCTION OF FUMARIC ACID BY COST-EFFECTIVE AND SUSTAINABLE IMMOBILIZATION TECHNIQUES

PART I

ENHANCED FUMARIC ACID PRODUCTION FROM BREWERY WASTEWATER BY IMMOBILIZATION TECHNIQUE

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Résumé

L'amélioration de la production d''acide fumarique (AF) par immobilisation de souches de champignons filamenteux sur différents supports solides a déjà été rapportée. Cependant, les "eaux usées de brasserie" (en anglais: BW), comme milieu de fermentation riche en carbone et l'utilisation d'un chiffon de mousseline (en anglais: MC) comme dispositif d'immobilisation pour la souche de champignon *Rhizopus oryzae* 1526 (*R. oryzae*) n'ont jamais été étudiées auparavant pour la production de FA. Dans ce travail, une production accrue de FA par la technique d'immobilisation a été obtenue avec une nouvelle combinaison de BW, de MC et de *R. oryzae*. Une surface de MC de 25 cm^2 et une concentration de 1.5 × 10^6 par mL de spores se sont avéré être les paramètres optimaux pour maximiser la production de FA. Le niveau de production et de productivité volumétrique de FA ont été nettement augmentés, passant de 30,56 ± 1,40 g/L à 43,67 ± 0,32 g/L et de 0,424 g/(L.h)) à 1.21 g/(L h)), respectivement, pour la fermentation submergée avec cellules immobilisées par rapport à la fermentation submergée avec cellules libres. Toutefois, les taux de production spécifiques de FA pour les cellules libres et les 25 cm^2 de MC se sont avérés pratiquement identiques (3,39 g/(g.h) et 3.49 g/(g.h)) respectivement). Les études par microscopie électronique à balayage du champignon immobilisé ont confirmé qu'il y avait une bonne fixation des hyphes fongiques au MC. Les résultats ont démontré que les BW et le MC pourraient être utilisés pour la production accrue de FA par fermentation submergée.

Mots clés: Acide fumarique; eaux usées de brasserie; chiffon de mousseline; immobilisation; productivité; microscope électronique à balayage

Abstract

Enhanced fumaric acid (FA) production by immobilization of filamentous fungal strains on different solid supports has been reported previously. However, the carbon-rich agro-industrial waste biomass 'brewery wastewater' (BW) as fermentation medium and muslin cloth (MC) as immobilizing device for the fungal strain *Rhizopus oryzae* 1526 (*R. oryzae*) have never been investigated before. In the present research work, enhanced production of FA by an immobilization technique was carried out with a novel combination of BW, MC and *R. oryzae*. MC area of 25 cm^2 and 1.5 × 10^6 per mL spore concentration were found optimal for the highest production of FA. Production level and volumetric productivity of FA were markedly increased from 30.56 ± 1.40 g/L to 43.67 ± 0.32 g/L and 0.424 g/(L h) to 1.21 g/(L h) for immobilized submerged fermentation compared with free-cell fermentation, respectively. However, the specific FA production rates for free-cell and 25 cm^2 MC were found to be comparable (3.39 g/(g h) and 3.49 g/(g h) respectively). Scanning electron microscope studies of the immobilized fungus confirmed the good attachment of the fungal hyphae to the MC. Results demonstrated that BW and MC could be used for the enhanced production of FA through submerged fermentation.

Keywords: Fumaric acid; brewery wastewater; muslin cloth; immobilization; productivity; scanning electron microscope

Introduction

Fungi can attach to a wide variety of organic and inorganic materials. Adhesion of fungal spores and/or hyphae to the substrate is the prerequisite for developmental structures, such as fungal biofilms. This ability of fungi significantly contributes to the prevalent problems, such as material deterioration or biofouling (Lugauskas *et al*., 2003; Barratt *et al*., 2003). However, the ability of filamentous fungi to adhere to inert substrates can also be exploited. Immobilized mycelia often have advantageous features compared with mycelial suspensions (Vassilev *et al*., 1992). Cells immobilized on surfaces by adhesion are in direct contact with the liquid phase containing the substrates, even though the cell and the liquid phases are distinctly separated. This reduces or eliminates mass transfer problems associated with the commonly used suspended cell cultures (D'Souza *et al*., 1998). Using appropriate support and geometries, a high surface to volume ratio can be maintained.

Fumaric acid (FA) is a naturally occurring four-carbon dicarboxylic acid with the molecular formula $C_4H_4O_4$. FA is well known as an intermediate product of the tricarboxylic acid cycle (TCA) or Krebs Cycle in all aerobic organisms. It is commonly used as a food acidulant and beverage ingredient (Yang *et al*., 2005). As an important platform chemical, FA is a valuable intermediate in the preparation of edible products, such as L-malic acid and L-aspartic acid (Goldberg *et al*., 2006). Moreover, FA can be polymerized to produce synthetic resins and biodegradable polymers (Roa Engel *et al*., 2010). The fermentation route for FA production has received a lot of attention because the chemical method has proven to be hazardous to the environment and economically not feasible. Among the different microorganisms tested for FA production, *Rhizopus oryzae* 1526 (*R. oryzae*) has been identified as the best FA producing strain (Zhou *et al*., 2011). However, due to the complex morphology of this filamentous fungus, large-scale FA fermentation production is a challenging task. The filaments are technically not supportive of heat, oxygen and mass transfer during fermentation. Moreover, size control of fungal pellets is difficult and thus can lead to diffusion limitation in the broth and results in reduced production (Wang *et al*., 2010). To overcome these difficulties, an immobilization approach was adapted for enhanced production of FA. Kautola and Linko (1989) studied immobilized *Rhizopus arrhizus* TKK 204-1-1a cells using polyurethane foam. The highest FA concentration obtained was 16.4 g/L (Kautola *et al*., 1989). In another study, production of FA was experimented with the immobilized *Rhizopus arrhizus* NRRL 1526 on polyurethane sponge and reached the highest FA concentration of 12.3 g/L (Petruccioli *et al*., 1996). Very recently, a novel immobilization method was designed using a combination of net and wire. With the immobilized cells, the fermentation

time for FA production was reduced to 83.3% compared with free-cell fermentation (Gu *et al*., 2013). All this baseline information is very important for scale-up studies of FA production with immobilized fungus. However, compared with the number of reports generated on different experimental elements (such as, carbon source, fungal growth conditions, fungal strain, bio-reactor type, among others) associated with FA fermentation production, studies of immobilization based FA production are yet to receive much attention.

Recently, we reported the production of FA through submerged fermentation (SmF) employing the carbon-rich waste biomass 'brewery wastewater' (BW) sourced from brewery industry (Das *et al*., 2014). The fungal strain *R. oryzae* was used for the study and growth conditions were optimized to obtain pellet morphology. The highest concentration of FA achieved was 31.3 ± 2.77 g/L. The present research work was carried out to further investigate the possibility that immobilization of this fungal strain could really enhance the production of FA. We used with a 100% cotton made, 'Muslin cloth' (MC) as solid support for the fungus. This cotton fabric is non-toxic to the fungal strain *R. oryzae* and chemically inert to the product. Tay and Yang used cotton cloth for immobilization of Rhizopus oryzae NRRL 395 for L-lactic acid production in a rotating fibrous bed bioreactor (Tay *et al*., 2002). However, no report on the application of this cotton cloth for immobilizing *Rhizopus oryzae* 1526 for FA production was found.

Materials and methods

Microbial culture

Rhizopus oryzae NRRL 1526 was procured from Agricultural Research Services (ARS) culture collection, IL, USA. For spore production, the strain was first grown on a potato dextrose agar (PDA) slant at 37 ± 1 °C for a maximum of 4 days. Spore inoculum was prepared by propagating (spread plate method) the spores on PDA plates (90 mm) at 37 ± 1 °C for 72 h. Spores were collected in sterile distilled water after filtration through sterile cotton wool to remove fungal mycelium. The spore suspension was maintained at 4 °C for regular use and at −80 °C for long time storage after adding 20% glycerol solution. Stock spore concentration was maintained at 1×10^8 spores per mL and used for inoculation.

Different culture media used in the present study

Two different culture media were used in the present study. Glucose-basic salts medium was used as pre-culture medium for producing pelletized seed or immobilized *R. oryzae*. In the SmF of FA production, BW was used as a fermentation medium.

Preparation of pre-culture medium

The chemical composition of the pre-culture medium was (g/L): glucose 50, urea 2, KH_2PO_4 0.6, $MgSO_4 \cdot 7H_2O$ 0.5, $ZnSO_4 \cdot 7H_2O$ 0.11 and $FeSO_4 \cdot 7H_2O$ 0.0088. The medium final pH (4.6) was not adjusted unless specifically indicated. Pre-culture medium was prepared in two parts: (a) glucose; and (b) urea + salts. Both parts were heat sterilized (20 min, 15 psi, 121 ± 1 °C) separately to avoid the Maillard reaction between the carbonyl group (>C=O) of glucose and amino group ($-NH_2$) of urea under heating condition. After cooling to room temperature, the two parts were mixed together inside a laminar hood and used for the pre-culture of *R. oryzae*.

Procurement of brewery wastewater and application in SmF

Brewery waste was procured from a local brewery (La Barberie, Quebec, Canada). To avoid microbial decay, BW was stored at 4 ± 1 °C for a maximum of 2 weeks before fermentation. The original pH (3.5) of BW was adjusted to 6.0 before SmF and a final total solid concentration (TSC) of 25 g/L was maintained throughout the study. BW was autoclaved (20min, 15 psi, 121 ± 1 °C) before use for SmF.

Pre-culture preparation and free-cells fermentations

Pre-cultures were carried out by inoculating 50mL of pre-culture medium with 2% (v/v) spore suspension in 250 mL Erlenmeyer flasks and incubating at 30 °C and 200 rpm for 24 h. Of the 50 mL cell pellet suspension obtained, 7.9 mL (final inoculum concentration 5%, v/v) was transferred into 500 mL Erlenmeyer flasks containing 142.1 mL of BW. The inoculated flasks were incubated at 25 °C, in a rotary shaker at 200 rpm for 96 h.

Immobilization of *R. oryzae* on muslin cloth and SmF

Muslin cloths (MCs) were cut into pieces of four different sizes. Average weights of cut MC pieces of each size type were measured. Prior to the immobilization step, all cut MC pieces were heat sterilized. To prepare for inoculation, sterile MCs were put into 250 mL

Erlenmeyer flasks containing 49 mL of pre-culture medium and inoculated with 1 mL of the stock spore suspension (2% v/v final spore concentration). After incubation at 30 °C, 200 rpm for 24 h in a rotary shaker, the growth medium was removed and MC pieces with immobilized mycelia were washed with copious amount of sterile distilled with water for three times. Washed MCs were then transferred into the 500 mL flasks containing 150 mL of sterilized production medium (BW) and $CaCO_3$. SmF was carried out at 25 °C in a rotary shaker at 200 rpm for 96 h.

Neutralizing agent

Calcium carbonate (CaCO3) was used as a neutralizing agent in the fermentation medium (BW). 7.5 g of sterilized $CaCO_3$ was added to 150 mL of BW (i.e. 50 g/L) to maintain a pH of 6.0 during SmF.

Downstream processing for fumaric acid recovery

The fermented broth (free-cell or immobilization SmF) containing the water insoluble calcium fumarate ($CaC_4H_2O_4$) was heated at 90 °C with simultaneous acidification (5 N, H_2SO_4) until clear (Dang *et al*., 2009). The broth of free-cell SmF was centrifuged (8000 × g, 10 min, 20 °C) to collect the supernatant for FA analysis. For immobilization SmF, after heating and acidification of the fermented broth, MCs with biofilms were removed from the treated broth before centrifugation.

Biomass dry weight (BDW) of cell pellets and immobilized mycelium

The cell pellets and immobilized mycelium formed at the end of pre-culture were recovered and washed twice with copious amounts of distilled water and dried at 95 °C until a constant weight was achieved (Yin *et al*., 1998). All BDWs were expressed in g/L.

Analytical method

FA was quantified spectrophotometrically by the modified method of Das *et al*. (2014).

Morphological studies

The morphologies of cell pellets and immobilized mycelium were studied with digital photography (Canon PC 1585) and scanning electron microscopy (SEM, Carl Zeiss EVO® 50). For SEM analysis, small cut pieces of immobilized mycelia were air-dried at room

temperature (25 ± 1 °C). Dried samples were directly mounted on a SEM grid and sputter coated (SPI Module Sputter Coater) with gold before SEM analysis.

Optimization of different parameters

Different sets of experiments were designed for optimizing the parameters of maximum FA production for free-cell and immobilization SmF. The following parameters were considered in the present study.

Effects of MC size

Four different sizes (length × breadth= 2 × 2 cm^2, 3 × 3 cm^2, 4 × 4 cm^2 and 5 × 5 cm^2) of MC were used for the immobilization of *R. oryzae*.

Effects of spore concentration

With the optimized size of MC for maximum FA production, four different spore concentrations (1.0, 1.5, 2.5 and 3 × 10^6) were tested for any effects on the biofilm formation and FA production.

Statistical analysis

Data are represented as mean±SD of three independent experiments. Correlations were considered significant at $P < 0.05$ for different applied parameters.

Results and discussion

Biomass dry weights of pre-cultured free-cell and immobilized mycelium

The spores inoculated into the pre-culture medium underwent morphogenesis for 24 h incubation at 200 rpm and 30 ^oC. Free cell pellets were formed in the flasks without MCs, while in the flasks with MC, fungal spores were immobilized onto the surfaces of MCs and formed mycelia (Figure 4.1 (A), (B) and (C)). The three sequential phases, namely germination, adherence and full confluence of mycelium on the immobilizing device (MCs) were completed in 24 h of incubation time in growth medium. The BDW obtained for the pre-cultured free-cell pellets was 4.60 ± 0.424 g/L. For the immobilized mycelium, there was an increase in BDW with larger surface area of MCs (Table 4.1.1). The BDWs obtained at 4, 9 and 16 cm^2 of MCs area were lower than that of cell pellets. After 24 h incubation with MCs, not all the sporangiospores were actually attached to MCs of 4, 9, and 16 cm^2 area. Some unattached sporangiospores germinated in the liquid pre-culture medium and were grown

into suspended mycelium. This was visible at the end of the pre-culture incubation. However, the MC with highest surface area (25 cm^2) well accommodated the sporangiospores and led to a surplus BDW compared with free-cell BDW. No mycelial growth was observed in the medium after incubation.

SmF with free-cell

In the first set of experiments, pre-cultured cell pellets (5%, v/v) were tested for FA production under the growth conditions 30 ^oC, 200 rpm for 96 h. In our previous study, it was found that under these culture conditions, cell pellets of *R. oryzae* produced a maximum of 31.3 ± 2.77 g/L of FA from BW in 72 h (Das *et al*., 2014). This was reproduced in the present study and the highest FA concentration of 30.56 ± 1.40 g/L was achieved after 72 h of SmF (Figure 4.1.2). For the cell pellet, inoculum size of 5% (v/v) used for free-cell SmF had a corresponding BDW of 0.22 ± 0.029 (g/L) (Table 4.1.2). To correlate BDW and FA production, SmF with free cells was inferred to be more supportive than immobilized SmF for FA production. However, as mentioned before, 5% (v/v) cell pellet inoculum size was optimized in the previous study that covered many parameters for highest FA production. Thus, 0.22 ± 0.029 (g/L) BDW of free cell pellets cannot be extrapolated for further enhanced production of FA by switching into the higher inoculum size as it only led to large-sized fungal pellets or suspended mycelium due to agglomeration (Das *et al*., 2014) This concept is also well supported by the literature (Nielsen *et al*., 1995; Yanagita *et al*., 1963; Vecht-Lifshitz *et al*., 1989). Moreover, other morphological forms (suspended mycelium or mycelial clumps) of *R. oryzae* are technically not suitable for higher FA production (Zhou *et al*., 2011).

Immobilization SmF and effects of muslin cloth size

In the immobilization SmF, the production of FA varied with incubation time and surface area of the MCs under growth conditions 30 ^oC, 200 rpm for 96 h (Figure 4.1.3). For the MC with 4 cm^2 area, the highest concentration (28.32 ± 0.615 g/L) of FA was achieved after 48 h of SmF. For the next higher surface area of 9 cm^2, the FA concentration was increased to 34.46 ± 1.160 g /L for the same time of SmF. The highest concentration (41.54 ± 0.846 g/L) was obtained with MC of 25 cm^2. The SmF time required for maximum production of FA was markedly decreased from 48 h (for 4 and 9 cm^2 of MCs) to 36 h (for 16 and 25 cm^2). This resulted in almost 2 times the volumetric productivity (from 0.59 to 1.153 g/(L h), Table 4.1.2) of FA. For all applied MCs, the immobilized mycelium grew as biofilm and well supported FA

production. In general, biofilm has the added advantage of easy mass, heat and gas transfer compared with free cell SmF (Li *et al*., 2000; Rodriguez Porcel *et al*., 2005). In the present study, the variations in FA production and volumetric productivity can be explained based on fungal physiology and broth rheology. With more surface area of MC, fungal biofilm became thinner compared with MCs with smaller surface area. The biofilms developed on 4 and 25 cm^2 MC area reached a thickness of around 2.5 and 1.0 mm at the end of 60 and 48 h of SmF, respectively. This is also evident from the changes in BDW for 4 and 25 cm^2 MC after SmF. There was about 55% and 5% increase in the BDW for 4 and 25 cm^2 MC, respectively (Table 4.1.2). The difference in biofilm thickness might have caused minimum FA production for 4 cm^2 MC. Due to oxygen and nutrition limitations, thicker biofilm does not support aerobic processes such as FA production (Skory *et al*., 1998). Thus, under the growth conditions 25 ^oC, 200 rpm and BW pH of 6, the 25 cm^2 area biofilm produced maximum FA (41.54 ± 0.846 g/L) in 36 h of SmF with volumetric productivity of 1.153 g/(L h).

In the present study, all the immobilization experiments on muslin cloths (MCs) for the fungus *R. oryzae* were carried out in 250 mL Erlenmeyer flasks (Pyrex, USA) with an inner diameter of about 6.5 cm. The surface area of 25 cm^2 was found to be the maximum allowable size for the square shaped MCs with sufficient space to interact with spore inoculated pre-culture medium and for efficient immobilization. Although, the next higher surface area, i.e. 36 cm^2 (6 cm × 6 cm) was tested in 250 mL flasks, it required folded MCs during shaking and no full confluence of mycelial grew on them (data not shown). However, MCs of 36 cm^2 or higher surface area can be experimented in Erlenmeyer flasks (Pyrex, USA) of higher volume, such as 500 mL (inner diameter 8.5 cm). As the study considered only 50 mL total volume for both free and immobilized pre-culture, immobilization surface area of more than 25 cm^2 was not within the scope of the present investigation.

Effects of spore concentration

The final spore concentration used for the preparation of free-cell and immobilized inoculum studies was 2×10^6 per mL from a stock of 10^8 per mL. With the optimized MC size of 25 cm^2, the four different spore concentrations applied resulted in different BDW for the immobilized mycelia (Table 4.1.3). There was a corresponding increase and decrease in the BDW of immobilized mycelium with applied higher and lower final spore concentration as compared with 2×10^6 per mL. It is anticipated that higher spore concentration will result in a dense network of hyphae and more biomass. For the two higher spore concentrations (2.5×10^6 and 3.0×10^6 per mL), more spores were immobilized onto the 25 cm^2 surface area.

After inoculation into the BW for SmF, the immobilized mycelium grew into thicker biofilms after 36 h of incubation. The changes in BDW for 2.5×10^6 and 3.0×10^6 per mL spore concentrations were estimated to be around 27% and 26%, respectively (Table 4.1.3). Regarding the greater consumption of nutrients from BW for biomass growth, metabolic activity of the thicker biofilms was less than that obtained at spore concentration 2.0×10^6 per mL. This caused the lowest FA concentration of 29.88 ± 0.66 g/L at the highest applied spore concentration of 3.0×10^6 (Table 4.1.3). Gu *et al*. (2013) observed a similar effect of higher spore concentration on FA production in an immobilization study. For lower applied spore concentrations (1.0×10^6 and 1.5×10^6 spores per mL), there was a corresponding decrease in the BDWs of the immobilized mycelium. For 1.5×10^6 per mL spore concentration, there was a slight increase in FA concentration (43.67 ± 0.32 g/L) compared with 2.0×10^6 spores per mL concentration obtained after 36 h of SmF. However, further lowering of spore concentration (1.0×10^6 spores per mL) significantly lowered FA production to 23.44 ± 0.78 g/L after 36 h of SmF. The enhancement of FA concentration at 1.5×10^6 per mL spore concentration was an indication of less dense biomass in the biofilm facilitating widespread easy access to nutrients and oxygen. However, there was no significant change in the biofilm thickness (1.0 mm) from that obtained with 2.0×10^6 spores per mL concentration. Less compact mycelia can simply be held responsible for variation in FA production. When spore concentration was further lowered to 1.0×10^6 per mL, FA production was affected negatively due to sub-optimal biomass developed on the 25 cm^2 MC, which led to lower production of FA after 36 h of SmF. The variation in fungal BDW at different applied spore concentrations and its effects on mass and oxygen transfer in the broth controlled the production of FA (Wang *et al*., 2010).

For a comparative study, the optimized spore concentration of 1.5×10^6 per mL was repeated for free-cell SmF under the same growth conditions. After pre-culture, the BDW obtained for 5% (v/v) was 0.18 5 ± 0.029 g/L. This BDW value was lower to that obtained at 2.0×10^6 per mL spore concentration and can be related to the lower cell pellet number. In the SmF study, the majority of cell pellets were transformed into suspended mycelium morphology and led to a lower concentration (21.33 ± 2.34 g/L) of FA after 72 h fermentation.

Thus, for the optimized MC size of 25 cm^2 and spore concentration of 1.5×10^6 per mL, the highest FA concentration and volumetric productivity achieved were 43.67 ± 0.32 and 1.21 g/(L h).

Specific fumaric acid production rate for free-cell and immobilized SmF – comparative analysis

Specific fumaric acid production rates (SPRs) were estimated for a comparative analysis and better conclusion on the efficiencies of free-cell and MC based immobilized method for FA production. The values of SPR were calculated as grams of FA produced per gram of fungal biomass per hour (g/(g h)) of SmF.

The SPR for free-cell SmF was 3.395 g/(g h) at 72 of SmF, and represented high metabolic activity of the fungal pellets. For the immobilized SmF for all MC sizes, SPR increased gradually from 4 cm2 (0.522 g/(g h)) to 25 cm^2 (3.496 g/(g h)) as shown in Figure 4.1.4. Although, SPRs of free-cell and 25 cm^2 MC suggested the same level of metabolic activity, the difference in the highest level of FA production (g/L) can be ascribed to the limitation in the BDW (maximum 5% v/v of pellet inoculum) for free-cell SmF. Immobilization of *R. oryzae* led to a high pre-cultured BDW (5.90 ± 0.35 g/L) with metabolically active mycelium and produced more FA (41.54 ± 0.846 g/L) compared with free-cell SmF (30.56 ± 1.40 g/L). This significant enhancement in the production of FA was the technical advantage of the immobilized SmF over the free-cell SmF and also an indication of the compatibility between MC support and *R. oryzae*. In the spore concentration effect study using MC 25 cm^2, SPR increased from 0.813 to 1.05 g/(g h) for the two lower spore concentrations of 1.0×10^6 and 1.5×10^6 per mL, respectively (Figure 4.1.5). In contrast, SPR was reduced to 0.571 and 0.404 g/(g h) for the higher applied spore concentrations (2.5×10^6 and 3.0×10^6 per mL), respectively. The differences in the SPR and FA production level for 1.5×10^6 and 2.0×10^6 per mL spore concentrations were the interesting findings of the present study. Although, at 2.0×10^6 per mL spore concentration SPR reached the highest value of 3.496 g/(g h), the FA production level was slightly lower than that obtained with 1.5×10^6 per mL. As mentioned earlier, spore concentration influences the fungal mycelial compactness, which contributes to mass, heat and oxygen transfer rates in SmF. Pre-cultured immobilized mycelium with 1.5×10^6 per mL spore concentration might have resulted in a less dense mycelium network compared with the spore concentration of 2.0×10^6 and led to slightly higher production of FA. However, the change in the BDW was higher (1.15 g/L) for 1.5×10^6 per mL spore concentration and thus a lower SPR (1.05 g/(g h)) was obtained compared with 2.0×10^6 per mL spore concentration.

Scanning electron microscopic studies of immobilized mycelium

The spores inoculated into the growth medium with MCs developed into dense mycelia on the cotton solid supports (Figure 4.1.6 (A), (B), (C) and (D)). As can be seen from the SEM images, the mycelium of *R. oryzae* grew uniformly on the surfaces of MCs of various sizes. Both upper and lower surfaces of MCs were covered by mycelium. A magnified view of the mycelial growth shows fine detail of the hyphal arrangement on the MCs (Figure 4.1.6 (E), (F), (G) and (H)).

Conclusions

Successful immobilization of the mycelium of the fungal strain *Rhizopus oryzae* 1526 on cotton fabric MC enhanced FA production and volumetric productivity compared with free-cell pellet SmF. Increase in the production and volumetric productivity of 43% and by 2.85 times, respectively, were very significant findings. A highest SPR of 3.496 g/(g h) was achieved for 25 cm^2 MC at 72 h of SmF. BW served the purpose of both micro and macronutrients for the growth of the fungus without needing any supplements. Being an agro-industrial renewable waste biomass, the present study was an important value addition effort for BW. The results obtained strongly suggest that the combination of MC and BW could be exploited for the production of other organic acids.

Abbreviations

BW= brewery wastewater, BDW= biomass dry weight FA= fumaric acid, MC= muslin cloth, rpm= rotation per minutes, SmF= submerged fermentation, OD= optical density, SPR= specific fumaric acid production rate.

Acknowledgements

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Zhou Z, Du G, Hua Z, Zhou J, & Chen J (2011) Optimization of fumaric acid production by *Rhizopus delemar* based on the morphology formation. *Bioresource Technology* 102(20):9345 - 9349.

Table 4.1.1: Biomass dry weights of pre-cultured cell pellets and immobilized mycelium after 24 h of growth at 30 °C and 200 rpm.

Free/Immobilized	Area of muslin cloth (cm^2)	Pre-cultured biomass dry weight (g/L)
Immobilized	4	2.04 ± 0.21
	9	3.38 ± 0.29
	16	3.88 ± 0.18
	25	5.90 ± 0.35
Free cell pellets	Not applicable	4.60 ± 0.424

Table 4.1.2: A comparative account of the biomass dry weights, fumaric acid production and volumetric productivity obtained for cell pellets and immobilized mycelium.

Pre-cultured biomass(free or immobilized)	Pre-cultured Biomass dry weight (g/L)	Biomass dry weight after SmF (g/L)	Fumaric acid concentration (g/L)	Time of SmF (h)	Fumaric acid volumetric productivity (g/(L h))
Cell pellets (5%, *v/v*, optimized)	0.22 ± 0.029	0.345 ± 0.05	30.56 ± 1.40	72	0.424
4 cm^2	2.04 ± 0.21	3.17 ± 0.17	28.32 ± 0.615	48	0.59
9 cm^2	3.38 ± 0.29	4.1 ± 0.23	34.46 ± 1.16	48	0.717
16 cm^2	3.88 ± 0.18	4.35 ± 0.33	38.86 ± 0.979	36	1.079
25 cm^2	5.90 ± 0.35	6.23 ±.12	41.54 ± 0.846	36	1.153

Table 4.1.3: Variations in biomass dry weights, fumaric acid production and volumetric productivity against different applied spore concentrations for 25 cm^2 size immobilizing device.

Spore concentration (per mL)	Pre-cultured biomass dry weight (g/L)	Biomass dry weight after SmF (g/L)	Time of SmF(h)	Fumaric acid concentration (g/L)	Fumaric acid volumetric productivity (g/(L h))
1.0×10^6	4.8 ± 0.44	5.6 ± 0.33	36	23.44 ± 0.78	0.652
1.5×10^6	5.2 ± 0.57	6.35 ± 0.66		43.67 ± 0.32	1.21
2.0×10^6	5.90 ± 0.35	6.23 ± .12		41.54 ± 0.846	1.153
2.5×10^6	6.38 ± 0.37	8.12 ±.59		35.8 ± 0.54	0.994
3.0×10^6	7.8 ± 0.29	9.85 ±.43		29.88 ± 0.66	0.83

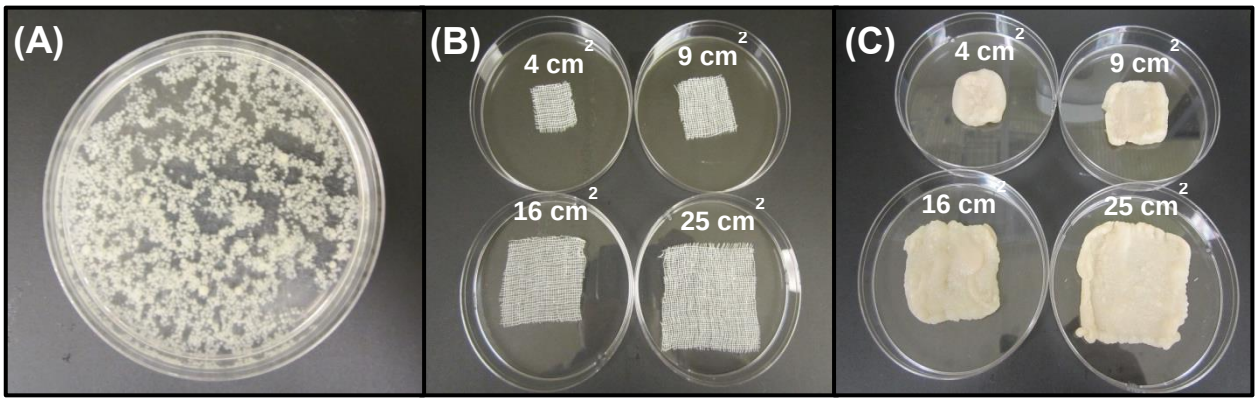

Figure 4.1.1: (A) Pre-cultured cell pellets of *Rhizopus oryzae* 1526 at 30 °C, 200 rpm for 24 h; (B) muslin cloth pieces of different sizes used for immobilization of *Rhizopus oryzae* 1526; and (C) immobilized mycelium of *Rhizopus oryzae* 1526 on muslin cloths of different sizes after incubating at 30 °C, 200 rpm for 24 h.

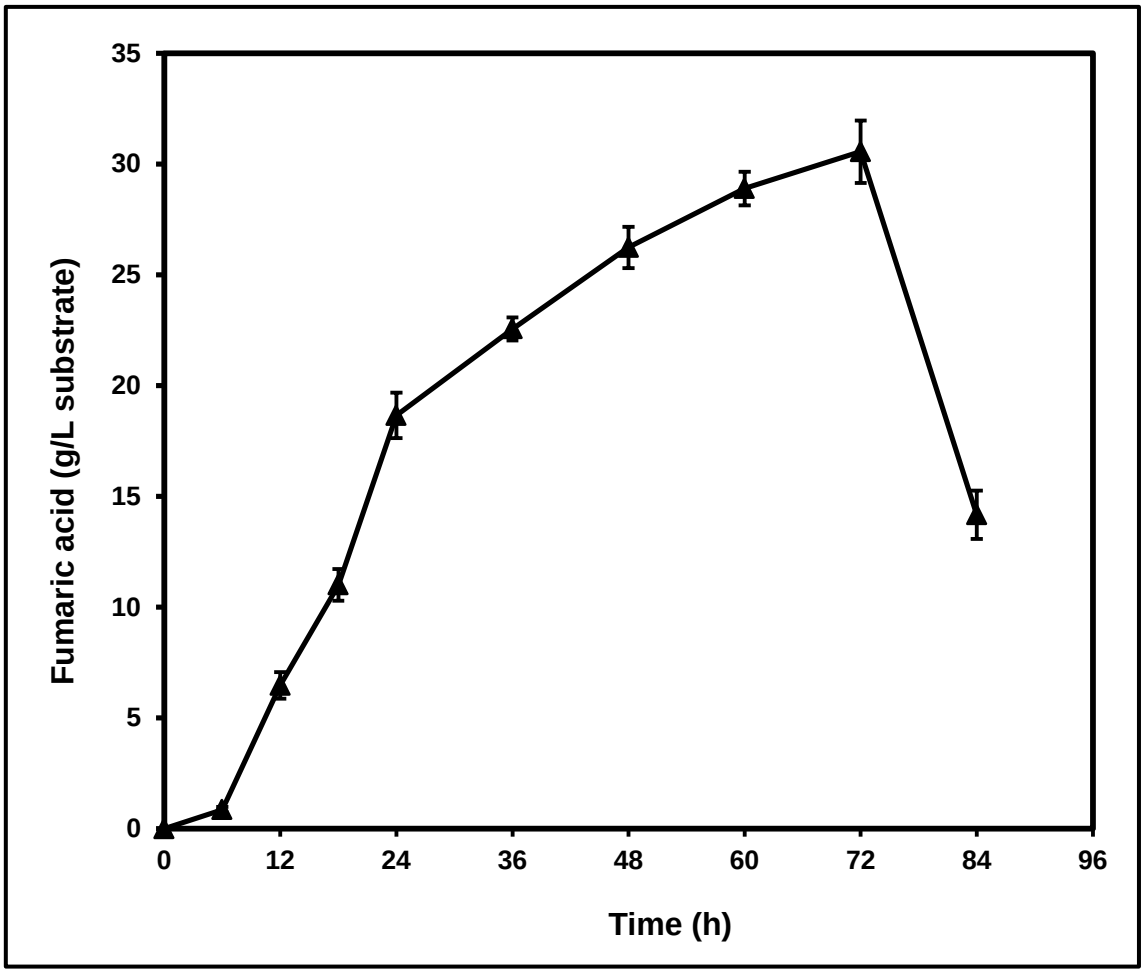

Figure 4.1.2: Production profile of fumaric acid in a free-cell submerged fermentation with brewery wastewater and *Rhizopus oryzae* 1526 at 25 °C and 200 rpm.

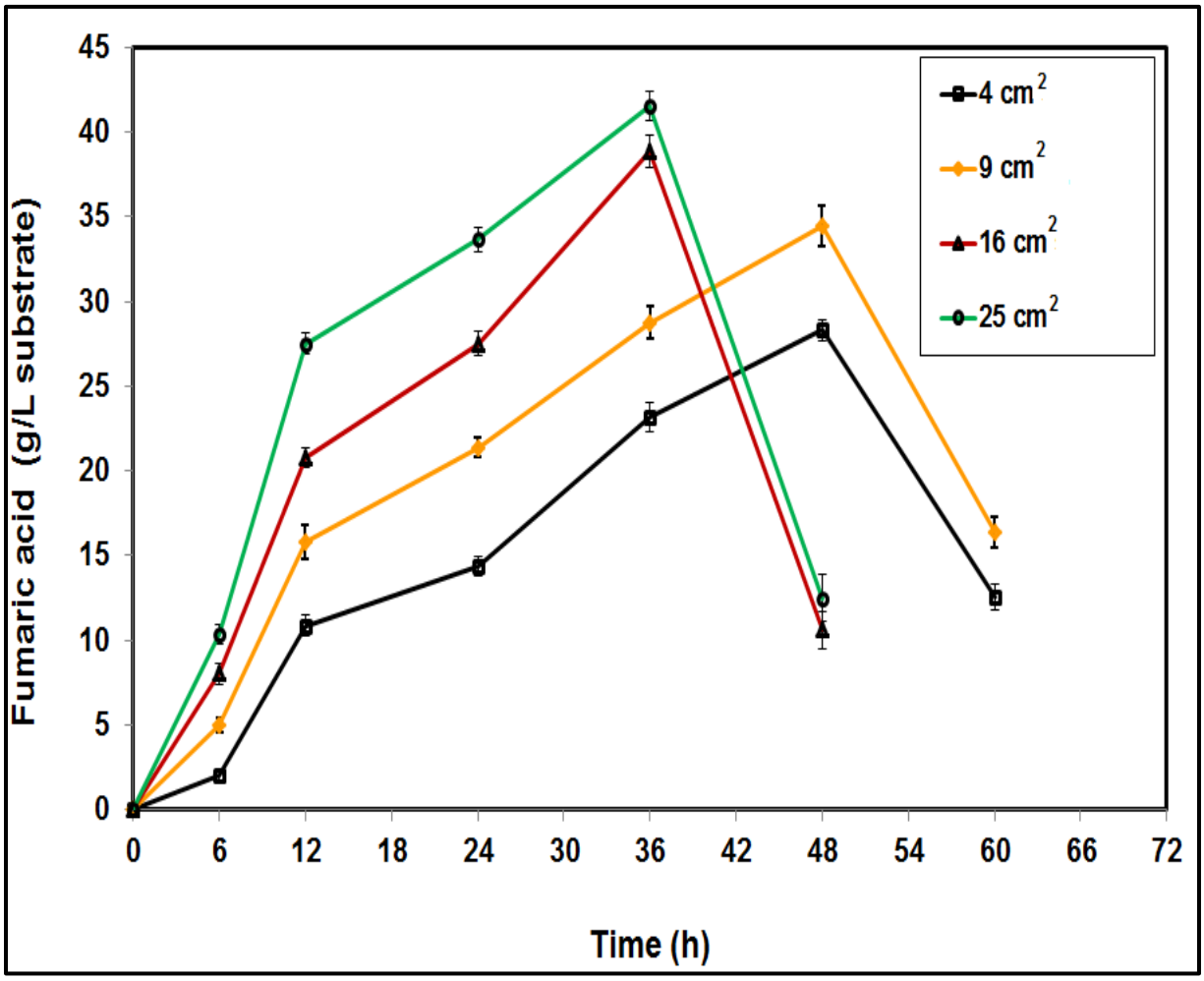

Figure 4.1.3: Effects of the size of muslin cloths on the production profile of fumaric acid in immobilized submerged fermentation with brewery wastewater and *Rhizopus oryzae* 1526 at 25 °C and 200 rpm.

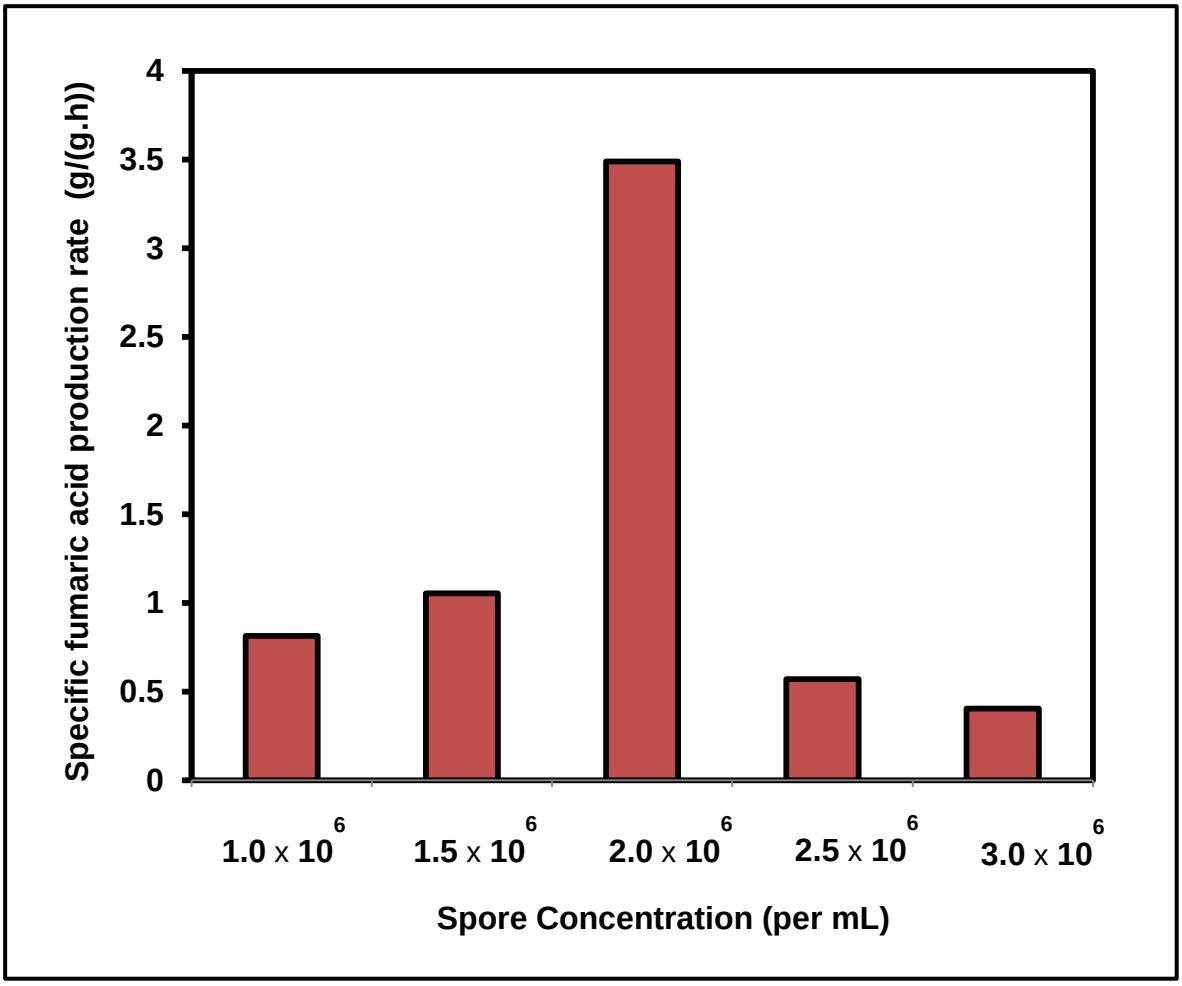

Figure 4.1.4: Specific fumaric acid production rates (g/(g h) for free-cell and immobilized SmF at 25 °C, 200 rpm and 2.0 × 10^6 per mL spore concentration.

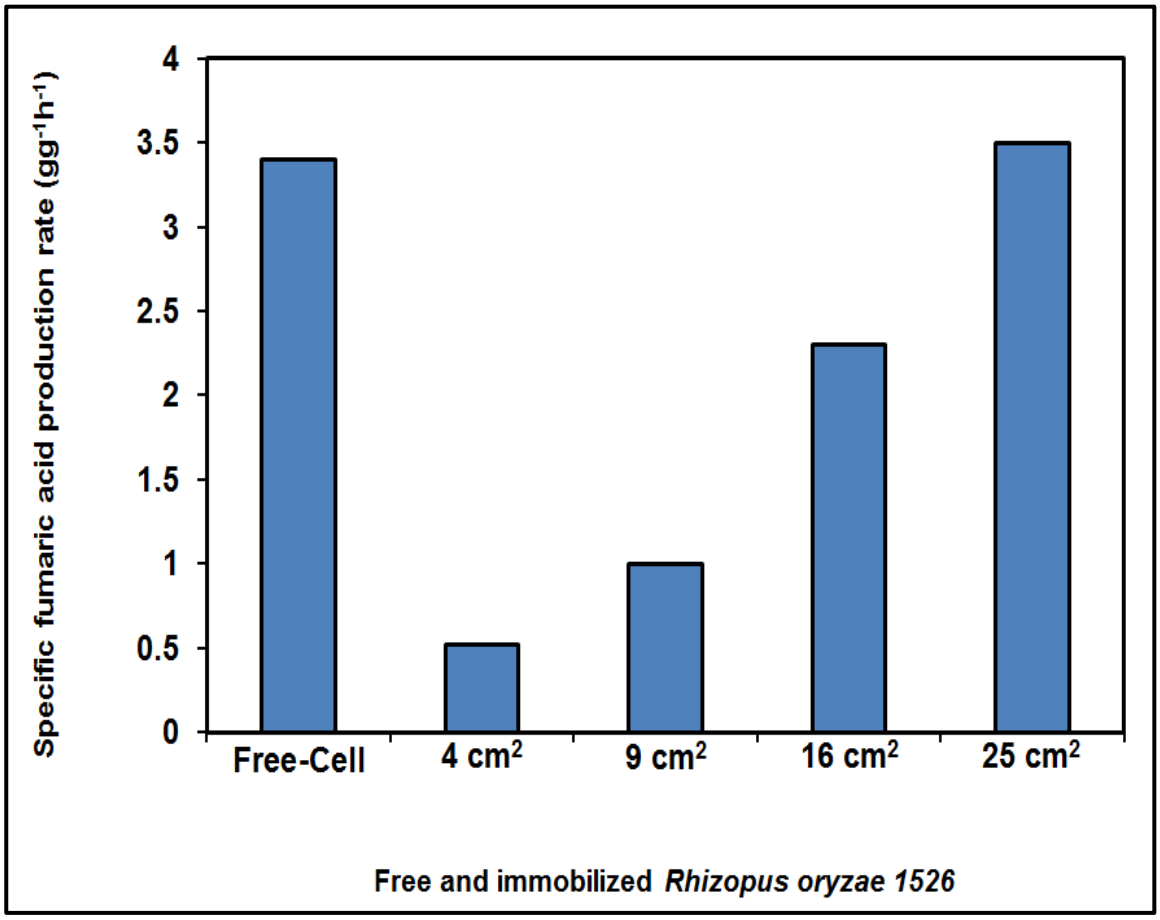

Figure 4.1.5: Specific fumaric acid production rates (g/(g h)) obtained for 25 cm^2 area muslin cloth at different spore concentrations at 25 ^oC and 200 rpm.

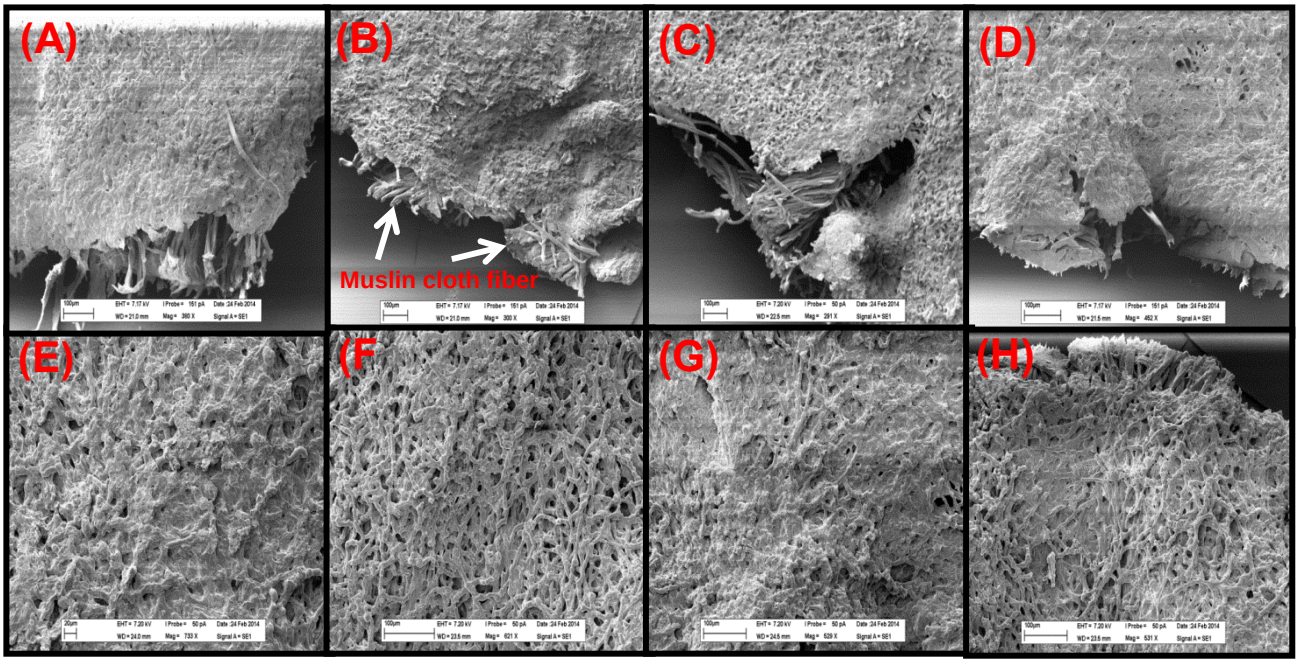

Figure 4.1.6: Scanning electron microscopy images of immobilized mycelium of *Rhizopus oryzae* 1526 on (A) 4 cm^2 (B) 9 cm^2 (C) 16 cm^2 and (D) 25 cm^2 areas of muslin cloths. Close views of the same are seen in the images (E), (F), (G) and (H).

PART II

VALORIZATION OF EGG SHELL BIOWASTE AND BREWERY WASTEWATER FOR THE ENHANCED PRODUCTION OF FUMARIC ACID

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Résumé

Deux résidus agro-industriels différents, à savoir les coquilles d'œufs (en anglais: EGS) et les eaux usées de brasserie (en anglais: BW) ont été utilisées pour la production améliorée de FA avec l'adoption d'une stratégie d'immobilisation. Les EGS ont été utilisés à la fois comme source de $CaCO_3$ et comme support d'immobilisation, tandis que les BW ont été utilisées comme milieu de fermentation pour la production de FA en régime submergée. Pour vérifier la valeur nutritive du milieu de fermentation, une analyse de la composition des BW a été réalisée. Le champignon filamenteux, *Rhizopus oryzae* 1526 (*R. oryzae*) a été immobilisé sur les surfaces des EGS et s'est développé en biofilms fins (environ 1 mm d'épaisseur). Les biofilms ont été utilisés pour la production de FA après optimisation des paramètres de fermentation (25 g/L de concentration en solides totaux de BW, 30 °C, 24h de taux d'incubation , une vitesse d'agitation de 150 rpm). Les mycéliums immobilisés ont également été cultivés dans un milieu de base glucose-sel. Pour la formation de biofilms, différents paramètres ont été optimisés, à savoir le nombre d'EGS, la concentration en spores (1, 0 × 10^6 par mL), le temps d'incubation (24 h) et la vitesse d'agitation (150 tours par minute). Par rapport aux cellules libres, la fermentation submergée en présence de biofilms a nettement amélioré la production et la productivité volumétrique de FA qui sont passées de 30,23 ± 1,23 g/L à 47,22 ± 0,77 g/L (soit 56%) et de 0,419 g/(L h) à 1.657 g/(L.h) (soit une augmentation d'un facteur de 3,95), respectivement. L'application des EGS, avec deux fonctionnalités (source de $CaCO_3$ et dispositif d'immobilisation) pour la production de FA, s'est avérée une nouvelle approche de valorisation ayant des avantages économiques et écologiques.

Mots clés: Acide fumarique; eaux usées de brasserie; coquilles d'œufs; carbonate de calcium; immobilisation; biofilm.

Abstract

Two different industrial wastes, namely egg shells (EGS) and brewery wastewater (BW) were valorized for the enhanced production of FA by adopting immobilization strategy. EGS were used both as a source of $CaCO_3$ and as immobilizing support, while BW was used as fermentation medium for FA production through submerged fermentation. To check the suitability as fermentation medium, compositional analysis of BW was carried out. The filamentous fungus, *Rhizopus oryzae* 1526 (*R. oryzae*) was immobilized on EGS surfaces that developed into thin (ca. 1 mm) biofilms. Biofilms were used for FA production at the optimized fermentation parameters (25 g/L total solids concentration of BW, 30 ^{o}C of incubation and 150 rpm). Immobilized mycelia were also experimented with glucose salt medium for enhanced FA production. For biofilm formation, different parameters *viz.* number of EGS for immobilization (three), spore concentration (1.0×10^6 per mL), incubation time (24 h) and flask shaking speed (150 rpm) were optimized. As compared to free-cell, biofilms mediated submerged fermentation markedly enhanced the production and volumetric productivity of FA from 30.23 ± 1.23 g/L to 47.22 ± 0.77 g/L (i.e. by 56%) and 0.419 g/(L h) to 1.657 g/(L h) (i.e. by 3.95 times), respectively. The application of EGS with dual functions (source of $CaCO_3$ and immobilization device) for FA production was a new approach of valorization with economic and ecological benefits.

Keywords: Fumaric acid; brewery wastewater; egg shells; calcium carbonate; immobilization; biofilm

Introduction

Production of fumaric acid (FA) through fermentation route by exploiting different agro-industrial waste materials has recently gained tremendous importance. Pertaining to the fact that fermentation technology is now proven to be a good alternative and eco-friendly approach as compared to chemical methods, more such efforts are being implemented using different low cost and sustainable carbon sources. Investigation on FA production from woodchips, dairy manures, crude glycerol, brewery wastewater and lignocellulosic biomass, such as corn straw has exhibited good product features (Xu *et al*., 2010; Das *et al*., 2014; Zhou *et al*., 2014) Apart from the production domain, recent progress made in the application part, in particular, different drug formulations made from FA and fumaric acid esters (FAEs) for the treatment of human diseases are of great importance. For instance, U.S. Food and Drug Administration (FDA) have recently approved dimethyl fumarate (DMF) to treat human adults with relapsing forms of multiple sclerosis (www.fda.gov). Moreover, FA has a widespread field of applications with emerging benefits (Yang *et al*., 2011; Mrowietz *et al*., 2005; Temenoff *et al*., 2007; Kanda *et al*., 2001; Shao *et al*., 2013; Rohokale *et al*., 2014; Sharma *et al*., 2012). With the increasing demand for FA, new strategies must be adopted for the enhanced production of this multifaceted organic acid.

Fungal immobilization on different solid substrates has been a common and effective approach for the enhanced production of FA. Previously, different FA producing fungal strains have been immobilized for biofilm formation that caused higher production (g/L) and volumetric productivity (g/(L h)) of FA as compared to free cell fermentation (Kautola, *et al*., 1989; Petruccioli *et al*., 1996; Gu *et al*., 2013). Nevertheless, the immobilization support material costs can range from 60 to 70 % of the total process cost (personal communication). Thus, for making the production of FA more sustainable and cost-effective, economic input for immobilizing material should be minimized. In this regard, bio-waste materials could be a good option. Worth mentioning, eggshells can be exploited for immobilization of fungi for FA production.

Eggshells (EGS) are one of the biowaste generated in million tons per day worldwide. For instance, Mexico itself produces approximately 480,000 tons of eggshells per year (i.e. 1315 tons daily). By weight, around 11% of the total weight of an egg is represented by its EGS,

which is worthy of attention in terms of waste generated per egg ratio. In general, EGS are not valorized for any value-added products except some reports on its application as fertilizer or conversion into foodstuff for man and animal uses. In chemical composition, EGS are rich in calcium carbonate ($CaCO_3$) content (~94 %) and also contains calcium phosphate (1 %), organic matter (4 %) and magnesium carbonate (1%) (Rivera *et al*., 1999). There are number of reports on the application of EGS as immobilizing agent for different enzymes and heavy metals. Makkar *et al*. immobilized b-galactosidase on ground EGS by crosslinking with glutaraldehyde (Makkar *et al*., 1983). In another study, goat liver catalase was immobilized on hen EGS (other solid supports used were alumina, gelatin and polyacrylamide) that exhibited better stability, higher affinity for H_2O_2 and less sensitive to inhibition by formaldehyde and sodium azide compared to the soluble enzyme (Chatterjee *et al*., 1990). Oil contaminated with heavy metals cadmium and lead was remediated by the application of EGS for immobilization (Ok *et al*., 2010).

As the literature suggests, EGS could be experimented as immobilizing device for fungi too and it will be a new approach if applied for FA production through submerged fermentation (SmF). In addition, the two facts that: (a) EGS is a very rich source of $CaCO_3$ and (b) production of FA requires $CaCO_3$ as neutralizing agent and CO_2 source (for the synthesis of oxaloacetic acid in tri-carboxylic cycle and reductive carboxylation pathway), thus it is pertinent to think about the application of EGS in SmF production of FA. Recently, we have reported the enhanced production of FA using brewery industry wastewater as the fermentation medium by employing the fungal strain, *Rhizopus oryzae* 1526 (to be addressed as *R. oryzae* hereafter). The fungus was immobilized on 100% cotton made muslin cloths that caused higher production (from 30.56 to 43.67 g/L) and volumetric productivity (from 0.424 to 1.21 g/(L h)) of FA as compared to free-cell SmF (Das *et al*., 2015). In the present investigation, a more cost-effective approach was made for FA production. The immobilizing agent, EGS, being a biowaste material is free of any-cost and served the dual purpose of immobilizing support and source of $CaCO_3$. In immobilization based FA production study, EGS has never been exploited before as an immobilization material; while report on the application of EGS as a source of neutralizing agent ($CaCO_3$) in FA production was nil until this investigation.

Materials and methods

Materials

The FA producing fungal strain, *Rhizopus oryzae* 1526 was procured from Agricultural Research Services (ARS) culture collection, IL, USA. The fermentation medium, brewery wastewater (BW) was procured from a local brewery industry (La Barberie, Quebec, Canada). It is estimated that for the production of 1 L of beer, 3-10 L of waste effluent is generated depending on the production and specific water usage after fermentation step (Braeken *et al*., 2004). EGS were collected from domestic wastes. All the chemicals used were of analytical grade and purchased from Fisher Scientific (Ottawa, Ontario, Canada).

Methods

Preparation of Microbial culture

The lyophilized strain was initially revived on a potato dextrose agar (PDA) slant at 37 ± 1 ^{o}C for a maximum of 4 days for sporulation. To prepare the spore suspension for inoculation, collected spores were first propagated (spread plate method) on PDA plates (90 mm) and incubated at 37 ± 1 ^{o}C for 72 h. Spores were collected by washing the PDA plates with sterilized distilled water (d.H_2O) and filtration of the suspension through sterile cotton wool to remove fungal mycelium. For regular use, spore suspension was stored at 4 ^{o}C and at -80 ^{o}C for long time storage after adding 20% glycerol solution. The spore stock concentration was maintained at 1×10^8 spores per mL.

Media preparation and sterilization

Glucose salts medium (GSM) was used for pre-culture (cell pellets and biofilm) and FA production. The composition of GSM for pre-culture was in g/L: 50 glucose 50, urea 2, KH_2PO_4 0.6, $MgSO_4 \cdot 7H_2O$ 0.5, $ZnSO_4 \cdot 7H_2O$ 0.11 and $FeSO_4 \cdot 7H_2O$ 0.0088. For FA production, the glucose and urea concentration were maintained at 80 and 0.2 g/L, respectively. GSM was steam sterilized (20 min, 15 psi, 121 ± 1 ^{o}C) before use. To avoid the Maillard reaction between the carbonyl group (>C=O) of glucose and amino group (-NH_2) of urea under heating condition, the GSM was sterilized in two parts (glucose, urea + salts) and then aseptically mixed after cooling at room temperature. BW (source: La Barberie, Quebec, Canada) was used as FA production medium. The original pH (3.5) of BW was adjusted to 6.0 before application in FA production. The final total solids concentration (TSC) of BW was maintained at 25 g/L. BW was heat

sterilized before use in FA production. All the glassware used in the present investigation was heat sterilized prior to their use for culture and fermentation purposes.

Processing of egg shells

Prior to the application for immobilization of *R. oryzae*, EGS were first reduced to small arc shaped portions (approximately of L = 4 cm, B = 2 cm), washed with copious amount of distilled water (d.H_2O), air dried and heat sterilized (20 min, 15 psi, 121 ± 1 ^oC). For the application of EGS as neutralizing agent, air dried small EGS pieces were crushed and grinded by using a mortar and pestle to obtain $CaCO_3$ powder. EGS derived $CaCO_3$ powder was heat sterilized (20 min, 15 psi, 121 ± 1 ^oC) before use in FA production.

Preparation of pre-culture of *Rhizopus oryzae*

Pre-culture of *R. oryzae* was prepared according to the procedure of Das *et al*. (2014). Briefly, 50 mL of GSM (pre-culture medium) was transferred into a 250 mL Erlenmeyer flask and aseptically inoculated with 2% (v/v) spore suspension and left for incubation at 30 ^oC and 200 rpm for 24 h.

Free-Cell and immobilized submerged fermentation (SmF) of *R. oryzae*

To prepare for free-cell SmF, the pre-cultured cell pellets were used to inoculate GSM or BW for SmF. An inoculum volume of 7.9 mL was transferred into a 500 mL Erlenmeyer flask containing 142.1 mL of GSM or BW (5%, v/v) under aseptic conditions. The inoculated flasks were incubated at 25 ^oC, in a rotary shaker at 200 rpm for 96 h. In the immobilization procedure, small pieces of EGS were first transferred into 250 mL Erlenmeyer flasks containing 50 mL of pre-culture GSM, then aseptically inoculated with 2% (v/v) spore suspension and left for incubation at 30 ^oC and 200 rpm for 24 h. No prior measurement of EGS weight was done for calculating biomass dry weight (BDW) of the immobilized *R. oryzae*. For SmF, immobilized mycelia on EGS were aseptically taken out from the pre-culture medium and washed with copious amounts of d.H_2O and transferred into a 500 mL flask containing 150 mL of GSM or BW. SmF was carried out at 25 ^oC in a rotary shaker at 150 rpm for 96 h.

Neutralizing agent (Eggshell Derived $CaCO_3$)

Sterilized EGS powder was added to GSM or BW at a final concentration of 55 g/L for maintaining SmF pH at 6.0 ± 0.5. As EGS contained 94 % of $CaCO_3$, thus 55 g of EGS powder actually contributed to approximately 50 g/L of $CaCO_3$.

Determination of biomass dry weight (BDW)

BDW of pre-cultured cell pellets, immobilized mycelia and biofilms were determined as per the previous methods with some required modifications (Yin *et al*., 1998). After immobilization, fungal mycelia were removed from the EGS, washed and dried at 60 ^{o}C. All BDW were expressed in g/L.

Downstream processing

Fermented BW was treated with simultaneous heating and acidification (90 ^{o}C, 5 N H_2SO_4) until clear and later centrifuged (8000 × g, 10 min, 20 ^{o}C) to collect the supernatant for FA analysis (Dang *et al*., 2009). Biofilms were removed from the treated BW before centrifugation.

Analytical methods

Fumaric acid was quantified by high-performance liquid chromatography (HPLC) (System: DIONEX DX500) with an Acclaim OA, 5 µm, (4.6 × 150 mm) column with a refractive index detector (PDA-100 DIONEX, UV, 210 nm). The mobile phase was 2.5 mM methanesulfonic acid (CH_3SO_3H) at a flow rate of 1 mL/min and column temperature of 30 ^{o}C. Ethanol concentration was quantified by gas chromatography (GC) (7890B, Agilent, Technologies, USA) with a flow of 1 mL/min over the ZB-WAX plus column (30 m × 0.25 mm, 0.25 µm film thickness) using flame ionization detector (FID) with a helium carrier gas and a temperature profile of 150-250 ^{o}C at 16 min run time.

Compositional analysis of brewery wastewater

The physicochemical characterization of BW was carried out as per guidelines of American Public Health Association (APHA), American Water Works Association (AWWA) and Water Pollution Control Federation (WPCF) published in 2005, Washington DC (APHA, AWWA, WPCF, 2005). The estimation of total organic carbon (TOC) and total organic nitrogen (TON) of BW was done using a carbon: hydrogen: nitrogen: sulphur (C:H:N:S) analyzer (Make: LECO Corporation, USA, Model: CHNS-932). Samples were analyzed in triplicates and

results were expressed as mean ± standard deviation (SD). Elemental analysis of BW was carried out with Inductively Coupled Plasma (ICP) Spectroscopic Technique. For ICP analysis, 5 mL of BW was transferred to a Teflon vial and 5 mL of HNO_3 was added. Autoclave digestion (17 psi, 120 ± 1 ^oC, 2 h) was performed to get a clear solution for analysis. The clear solution was transferred to a vial and made up to 50 mL with Milli-Q water. The prepared sample was then analyzed with ICP-AES (Make: Varian, Model: Vista AX). Multi-element calibration standards preparation was done prior to sample analysis.

Morphological investigations

Scanning electron microscopy (SEM, Carl Zeiss EVO) was used to investigate of surface morphology of the biofilm. The sample preparation for SEM study was followed as mentioned by Das *et al*. (2015).

Parameters optimization

The following parameters were optimized in the present investigation.

Number of EGS pieces for immobilization

The maximum number of EGS that can be utilized in one batch of immobilization in a 250 mL flask was optimized with the conditions of confluent biofilms on each piece of EGS and no mechanical damages to EGS and biofilms.

Spore concentration

Effect of spore concentration on mycelium immobilization and fungal biomass was studied. Six different spore concentrations (0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 × 10^6 spores per mL) were applied and optimized for the best immobilized mycelium.

Incubation time for immobilization

The incubation time (h) for the immobilization of fungal mycelium on EGS was optimized. Immobilization behaviors were studied for four different incubation periods (12, 24, 36 and 48 h) and optimized for the full confluence of biofilms on EGS surfaces with minimum thickness.

Flask shaking speed for immobilization

Three different shaking speeds (100, 150, and 200 rpm) were experimented for the immobilization of the fungus on the EGS surfaces and optimized with best biofilm formed without causing any mechanical damage to the used EGS.

SmF and biofilm optimization

Different number of biofilms (1, 2, 3 and 4) was employed to study the effect of immobilization on the production rate (g/L), volumetric productivity (g/(L h)) and specific production rate (g/(g h)) of FA as compared to the free-cell fermentation.

Statistical analysis

Data are represented as mean ± SD of three independent experiments in all cases. Correlations were considered significant at $P > 0.1$ for different studied parameters.

Results and discussion

Compositional analysis of brewery wastewater

Compositional analysis of BW showed it to be rich in carbon and other micro and macro nutrients required for culturing *R. oryzae* (Table 4.2.1). Many metal ions, including Mg^{2+}, Zn^{2+} and Fe^{2+} are vital for the growth of R. oryzae and also play an important role in FA production (Foster *et al*., 1938). The nutritional content of BW supported the growth of the fungus and subsequently metabolized the carbon source into FA.

Selection of egg shells for immobilization and source of $CaCO_3$

The formation of *R. oryzae* biofilms on EGS surfaces suggested a good compatibility (attachment, adsorption, germination and others) between the sporangiospores and EGS. A good quality hen's egg contains 5.5 g of $CaCO_3$ (94% of the dry EGS, on average) and as a biowaste, EGS has an unlimited access in any part of the world (www.afn.org). Considering the fact that 20 g of $CaCO_3$ can neutralize maximum of 23.2 g of FA, 147.35 tons of EGS could be utilized for the production of 1 ton of FA. Thus, making use of EGS as a potential source of $CaCO_3$ is a good way of valorization in terms of ecological (pollution control) and economic (cost reduction in FA production) aspects. As $CaCO_3$ has been found to be the best neutralizing agent for achieving maximum product yield of FA (Xu *et al*., 2012), a very

low cost source (EGS) with almost no processing step for dual (immobilizing device and source of $CaCO_3$) applications is an important and pertinent valorization approach for EGS.

Optimization of number of egg shells for immobilization

Maximum number of EGS pieces to be inserted into a 250 mL Erlenmeyer flask containing 50 mL of pre-culture medium was an important parameter for optimization. Effect of EGS number on biofilm formation process and the thickness attained by biofilms was well observed (Table 4.2.2). With the minimum of one EGS piece, very thick (ca. 2.5-3.0 mm) biofilm was formed after 24 h of incubation. Not all the fungal spores were well accommodated by the limited surface area of a single EGS and thus grew into free fungal mycelium in the medium. This was almost repeated when two EGS pieces were used but with a less thick (ca. 2 mm) biofilm and free mycelium. Subsequently, higher number (three) of EGS pieces caused thin (ca. 1 mm) biofilms and left no traces of free mycelium. Further increase in the EGS number (four) led to incomplete biofilm formation on each EGS and there was some free mycelium. At 2×10^6 per mL spore concentration, the optimum required surface area for spore attachment and subsequent formation of thin biofilms were provided by three egg shell pieces. Moreover, more than three egg shell pieces caused steric hindrance inside the 6.5 cm diameter flask and inhibited the fungal growth into the biofilms. Thus, for immobilization, maximum of three pieces of EGS was considered to be the optimum.

Biofilm formation versus spore concentration, incubation time and flask shaking speed

As fungal spore concentration has a strong influence on its biomass, it was important to optimize the final effective spore concentration for obtaining thin biofilms of *R. oryzae* immobilized on EGS surfaces. Five different spore concentrations (0.5, 1.0, 1.5. 2.0 and 2.5 $\times 10^6$ spores per mL) were tried in the present study keeping other parameters fixed including optimized number of EGS. Thickness of biofilms was controlled by the spore concentrations and maximum number of spores that could be accommodated and grown into the thin biofilms on the optimized number EGS pieces exhibited a strong correlation (Table 4.2.3). Thin fungal biofilms are preferred for a better heat, mass and oxygen transfer throughout the medium during SmF and achieving the same is an important and challenging task for any biofilm based fermentation production study (Li *et al*., 2000; Rodriguez *et al*., 2005). From the observed results for different spore concentration, it was inferred that 1.0 ×

10^6 per mL spore concentration produced the thinnest (~1 mm) biofilm. Spore concentration lower or higher than this value either resulted in no formation of biofilms or other technical difficulties, such as growth of massive free mycelium in the pre-culture medium. A combination of 1.0×10^6 per mL spore concentration and three EGS pieces was found to be technically compatible for the present study.

Subsequent to spore concentration, optimization of minimum incubation time for biofilm formation was carried out. An incubation time profile of 12, 24, 36 and 48 h was studied keeping other experimental parameters fixed (Table 4.2.4). The incubation period of 12 h was found not to be sufficient for biofilm formation as there was no visual manifestation on the EGS surfaces. At the end of 24 h, thin (~ 1 mm) biofilms developed on all the three EGS pieces and there was no mycelial growth in the medium. From 24 to 36 h, there were no marked changes on the biofilm thickness. However, further continuation of the incubation up to 48 h led to the mycelium formation in the medium. This newly emerged mycelium might have been originated from the reproductive development (formation of sporangiospores) of the biofilm mycelia that dispersed into the medium and grew into mycelia. Such developed mycelia were of no interest for the present study and could easily be eliminated if incubation was not to continue after 24 h and considered to be the optimum incubation time for biofilm formation.

As per the experimental design of the present investigation, the flask shaking speed (200 rpm) previously optimized for both pre-culture and SmF was reconsidered for optimization. To see if flask shaking speed can influence the biofilm formation, three different speeds (100, 150, and 200) were tried (Table 4.2.5). Starting at 100 rpm, the attached fungal spores generated only non-confluent biofilms on EGS surfaces. Reaching to 150 rpm, morphogenesis of the spores resulted in thin and confluent biofilms and no suspended mycelia were observed in the media. The speed of 200 rpm caused uplifting movement of the EGS with biofilm intake. EGS started colliding randomly against the flask wall. For the adsorption and attachment of sporangiospores onto the surfaces of EGS, a minimum flask speed was required so that spores can uniformly come in contact with the immobilizing support. Technically, the minimum speed should not cause any mechanical damage to EGS and biofilms. In the optimization of the other parameters (EGS number, spore concentration and incubation time), although 200 rpm did not exhibit any negative impacts (such cracking of EGS, detachment of biofilms from EGS surfaces), still it was pertinent to obtain gentle

shaking of the flask with no chances of uplifting movement of EGS. Thus, 150 rpm was best suited for the present investigation.

Optimization of free-cell SmF conditions and limitations

The parameters (25 g/L total solids concentration of BW, 30 ^{o}C, 200 rpm, 96 h and 5%, v/v inoculum) used for the free-cell SmF for FA production were optimized in our previous study (Das *et al*., 2014). Under these optimized growth conditions, the maximum FA production reached 31.3 ± 2.77 g/L. In the present study, FA concentration obtained in the free-cell SmF using BW was 30.23 ± 1.23 g/L that confirmed the reproducibility of the experimental design (Figure 4.2.1 (A)). However, under the same SmF conditions, FA concentration reached 37.22 ± 0.9 g/L with GSM in 66 h of incubation (Figure 4.2.1 (B)). More refined carbon source, such as GSM helped in the accelerated consumption of glucose and easy conversion into FA with more volumetric productivity (0.563 g/(L h)) as compared to the study with BW (0.419 g/(L h)). As mentioned in the introduction section, the strong motivation behind carrying out the present study was to cross the barrier of free-cell SmF in FA production employing BW. Considering the general acceptance that fungal biomass has a strong correlation with FA product features, free-cell SmF involved less biomass (0.208 ± 0.06 g/L, Table 4.2.6) and was beyond the scope of further increase both in biomass and FA production. As confirmed from our previous study, more than 5% v/v inoculum size resulted in non-pelleted morphologies of *R. oryzae* and considerably reduced the FA production level (Das *et al*., 2014). As immobilization can accommodate more fungal biomass, it was interesting to see the effects of optimized parameters on FA product features in synthetic medium (GSM) and BW

SmF with biofilms and product features of fumaric acid

The biofilms of *R. oryzae* developed on EGS were used as inoculum for SmF for FA production in GSM and BW. To evaluate the efficiency of biofilm for FA production as compared to free-cell in BW, SmF was carried out in three different sets of experiments (one biofilm, two biofilms and three biofilms) under same growth conditions (25 g/L TSC of BW, 30 ^{o}C and 150 rpm). Starting with a single biofilm, the FA concentration at 60 h of SmF reached up to 39.11 ± 1.45 g/L and then declined sharply at 72 h (Figure 4.2.1 (A)). As compared to free-cell SmF, there was a considerable increment of 8.88 g in FA production. With GSM, single biofilm enhanced FA production up to 43.26 ± 0.88 g/L within 54 h of incubation (Figure 4.2.1 (B)). In the SmF with two biofilms for BW, the production level of FA further increased up to 45.66 ± 0.98 g/L and required incubation time was reduced to 48 h.

The enhancement was not proportionate in terms of quantity of biofilm numbers. This was further confirmed when three biofilms were used for SmF and FA production reached to maximum of 47.22 ± 0.77 g/L. A similar tendency was also observed with GSM as the highest FA concentration reached up to 49.22 ± 0.65 g/L for two biofilms and then declined to 41.2 ± 0.94 g/L with three biofilms. The lower production of FA with three biofilms for GSM suggested that two biofilms was optimum for enhancing the FA production as compared to three biofilms required for BW (Figure 4.2.1 (A) and (B)).The overall results demonstrated that the fungal biomass of single biofilm was metabolically very active and converted BW into FA in shorter time (54 and 60 h). However, further increase in the biomass (two and three biofilms) did not induce higher FA production but reduced the SmF time. With more number of biofilms in the fermentation medium (GSM or BW), metabolic activity of fungal mycelia was reduced and resulted in the growth of biomass instead of FA production. As compared to SmF with single biofilm, changes in the biomass before and after SmF was more in the case of two and three biofilms mediated SmF for BW (Table 4.2.6). A similar change in BDW was also observed in the study with GSM (Table 4.2.6). Application of increased biomass caused speedy consumption of nutrients and conversion into FA. This caused the reduction in SmF time as compared to free cell or with single biofilm. However, higher biomass has the disadvantages as it can affect the fungal morphology and subsequently the FA production (Wang *et al*., 2010). This was well manifested in SmF with two and three biofilms as they developed cotton like layers that might have caused limitations of oxygen and nutrients to fungal cells and changed the metabolic activities. Finally, it led to lower production of FA as compared to SmF with single biofilm. As oxygen is crucial for FA production, limitation to this can cause metabolic shifting towards more ethanol production (Skory *et al*., 1998)

In both free-cell and biofilm mediated SmF studies, ethanol was detected as by product. For BW, maximum ethanol concentration detected for free-cell SmF was 5.2 ± 0.26 g/L. With single biofilm, this declined to 2.42 ± 0.19 g/L. The ethanol concentration reached up to 7.96 ± 0.21 and 8.7 ± 0.25 g/L for two and three biofilms, respectively (Figure 4.2.2 (A)). More fungal biomass in the form of biofilm and easy access to oxygen accelerated the conversion of BW into FA. However, with more number of biofilms (two and three), anaerobic fermentation was favored as the ethanol concentration enhanced further with simultaneous reduction in FA production (Skory *et al*., 1998). The results obtained with GSM further supported the relation between fungal biomass versus ethanol production in SmF studies (Figure 4.2.2 (B)). However, peak ethanol concentrations obtained with free-cell or any

number of biofilms, were slightly less than that achieved with BW. GSM being a more refined media, oxygen transfer is always expected to be better as compared to a medium, such as BW with solid contents. This affected the aerobic and anaerobic processes of FA and ethanol production, respectively (Table 4.2.7).

The comparative study of volumetric productivity (g/(L h)) and specific fumaric acid production rate (g/(g h)) of free-cell and biofilm mediated SmF correlated the production profile of FA with biomass concentration and metabolic activity of *R. oryzae*. In case of BW, the volumetric productivity increased from free-cell to biofilm mediated SmF and was attributed to the reduction in fermentation time and more production of FA (Figure 4.2.3 (A); Table 4.2.6). A highest volumetric productivity (1.675 g/(L h)) was achieved for SmF with three biofilms. However, the specific fumaric acid production rate considerably decreased from 3.44 to 0.679 g/(g h) for SmF with free-cell and two biofilms respectively. Although more biomass was used in the form of biofilm, pertaining to the lower metabolic activity of the fungal cells (caused by morphological changes), production level of FA was not increased correspondingly. This was also seen for SmF with three biofilms as the enhancement in specific production rate was only 0.108 g/(g h). In the study with GSM, highest volumetric productivity (1.716 g/(L h)) was achieved with three biofilms and specific production rate was also decreased from 6.26 g/(g h) (free-cell SmF) to 0.536 g/(L h) (SmF with three biofilms) as shown in Figure 4.2.3 (B).

The immobilization of *R. oryzae* on EGS and their application in SmF enhanced the production level of FA as compared to free-cell fermentation. For BW, it increased by 56% (from 30.23 to 47. 22 g/L) which is considerably high. For GSM, the increment in FA production was 32.2%. However, it should be noted that FA production with free-cell SmF with GSM was higher (37.22 ± 0.9 g/L) to the value achieved with BW (30.23 ± 1.23 g/L). It suggested that immobilization strategy was more effective toThis new strategy of immobilization helped in reaching a higher concentration of FA (47. 22 g/L) as compared to the results (43.67 g/L) obtained with muslin cloth immobilization in our previous study (Das *et al*., 2015)

Scanning electron microscopic analysis of biofilm

SEM analysis of a thin biofilm (obtained with all optimized parameters) revealed the morphological details of the fungal mycelia. The biofilm was formed by uniformly grown and packed mycelia with well confluence and proliferation (Figure 4.2.4 (A), (B)). The different

steps of immobilization process, such as adsorption, germination and proliferation into thin biofilm were well supported by the EGS surfaces for the sporangiospores of R. oryzae.

Conclusions

The findings of the present investigation highlighted potential applications of two industrial wastes. Production of fumaric acid from brewery waste and its enhancement with the intervention of egg shells based immobilization techniques was an interesting finding. The two matrices, production level (g/L) and volumetric productivity (g/(L h)) of fumaric acid were considerably enhanced by 56% and 3.95 times respectively as compared to free-cell submerged fermentation. The experiments done with glucose medium also suggested the effectiveness of the present immobilization strategy for FA enhancement. The novel application of egg shells with dual purposes of neutralizing agent and immobilizing device was a very cost-effective approach. Being recognized as one of the major unwanted biowaste with pollution concerns, valorization of egg shells for fumaric acid production has ecological aspects too.

Abbreviations

FA= Fumaric acid, EGS = Egg shells, GSM= Glucose salts medium, BW= Brewery wastewater, ESM= Egg shell membrane, rpm= Rotation per minute, BDW= Biomass dry weight, SmF= Submerged fermentation, PDA= Potato dextrose agar, DMF= Dimethyl fumarate, TSC =Total solid concentration, SEM =Scanning electron microscope

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Table 4.2.1: Compositional details of brewery wastewater.

Total solid (g/L)	Total carbon (g/L)	Total nitrogen (g/L)	Total carbohydrate	Total protein (g/L)	Elements with wavelengths	Elemental concentration (mg/L)
58.22 ± 4.8	36.43 ± 3.6	2.15 ± 0.0.25	31.22 ± 4.6	14.68 ± 3.8	Zn 213.857	69.22
					Mg 279.553	82.17
					Mn 260.568	7.85
					Cu 327.395	11.45
					Fe 259.940	15.20
					Al 167.019	192.41
					Ca 317.933	1116.77
					Cd 214.439	0.011
					Co 238.892	0.08
					Cr 267.716	0.011
					K 769.897	315.52
					Mo 202.032	0.0192
					Na 589.592	192.71
					Ni 231.604	0.05
					P 178.222	24.6708
					Pb 182.143	0.0289
					S 180.669	156.14
					Si 250.690	25.31375

Table 4.2.2: Optimization of number of egg shell pieces for immobilization.

Parameter	Number	Biofilm formation	Other fixed parameters	Optimized data
Number of egg shells pieces in 250 mL flask (inner diameter of 6.5 cm)	1	Very thick biofilm (ca. 2.5-3 mm) and large amount of suspended mycelia in the medium	Incubation time: 24 h, Flask shaking speed: 200 rpm, Incubation temperature: 30 ^{o}C, Spore concentration: 2 × 10^{6} per mL	3 no of egg shell pieces
	2	Less thick biofilm (ca. 2 mm) and less amount of suspended mycelia in the medium		
	3	Thin biofilm (ca. 1mm) and no fungal mycelia in the medium		
	4	Biofilms partially developed on the EGS and some traces of fungal mycelia the media		

Table 4.2.3: Application of different spore concentrations and corresponding changes in biofilm growth.

Spore concentration (per mL)	Biofilm formation	Other fixed parameters	Optimized data
0.5×10^6	No biofilm formation but random mycelial growth on EGS surfaces	Incubation time: 24 h, Flask shaking speed: 200 rpm, Incubation temperature: 30 ^oC, Number of egg shell pieces: 3	1.0×10^6 per mL spore concentration
1.0×10^6	Very thin (< 1 mm) biofilm and no traces of suspended mycelia in the media		
1.5×10^6	No marked changes from what was observed in 1.0×10^6		
2.0×10^6	Thin biofilm (ca. 1mm) and no traces of fungal mycelia in the medium		
2.5×10^6	Relatively much thicker biofilms (ca. 2-3 mm) and some visible free mycelia in the medium		
3.0×10^6	EGS with thick biofilms were mingled with the massive free mycelium grown in the medium		

Table 4.2.4: Effects of incubation time on biofilm formation

Incubation time (h)	Biofilm formation	Other fixed parameters	Optimized data
12	No biofilm formation	Flask shaking speed: 200 rpm, Incubation temperature: 30 ^{o}C, Number of egg shell pieces: 3, Spore concentration: 1.0 × 10^{6} per mL	24 h incubation time
24	Formation of uniform and thin biofilms and no traces of mycelia in the medium		
36	No marked changes from what was observed in 24 h		
48	Mycelial growth in the medium that mingled with the biofilms		

Table 4.2.5: Effects of flask shaking speeds on biofilm formation.

Flask shaking speed (rpm)	Biofilm formation	Other fixed parameters	Optimized data
100	Biofilms were formed but not confluent and left some mycelium in the medium	Incubation temperature: 30 ^{o}C, Number of egg shell pieces: 3, Spore concentration: 1.0 × 10^{6} per mL, Incubation time : 24 h	150 rpm of flask shaking speed
150	Thin and confluent biofilms were formed with no traces of free mycelia in the medium		
200	Thin and confluent biofilms were formed as in 150 rpm speed, but caused uplifting movement of EGS and also collision against the flask wall		

Table 4.2.6: Relation between the biomass dry weight of *R. oryzae* 1526 and product features of fumaric acid obtained with brewery wastewater.

Biomass	**Initial biomass dry weight (g/L)**	**Biomass dry weight (g/L) after SmF**	**Fumaric acid production (g/L)**	**Time of SmF (h)**	**Fumaric acid volumetric productivity (g/(L h))**
Cell pellets (5%, v/v, optimized**),** Ref: Das *et al*., 2014a	0.208 ± 0.06	0.330 ± 0.035	30.23 ± 1.23	72	0.419
One biofilm	0.44 ± 0.027	0.722 ± 0.037	39.11 ± 1.45	60	0.651
Two biofilms	0.850 ± 0.032	2.25 ± 0.24	45.66 ± 0.98	48	0.951
Three biofilms	1.28 ± 0.055	3.78 ± 0.032	47.22 ± 0.77	24	1.657

Table 4.2.7: Relation between the biomass dry weight of *R. oryzae* 1526 and product features of fumaric acid obtained with glucose salts medium.

Biomass	Initial biomass dry weight (g/L)	Biomass dry weight (g/L) after SmF	Fumaric acid production (g/L)	Time of SmF (h)	Fumaric acid volumetric productivity (g/(L h))
Cell pellets (5%, v/v)	0.225 ± 0.011	0.315 ± 0.015	37.22 ± 0.9	66	0.563
One biofilm	0.451 ± 0.018	0.680 ± 0.053	43.26 ± 0.88	54	0.801
Two biofilms	0.838 ± 0.030	1.95 ± 0.165	49.22 ± 0.65	36	1.36
Three biofilms	1.45 ± 0.055	4.65 ± 0..180	41.2 ± 0.94	24	1.716

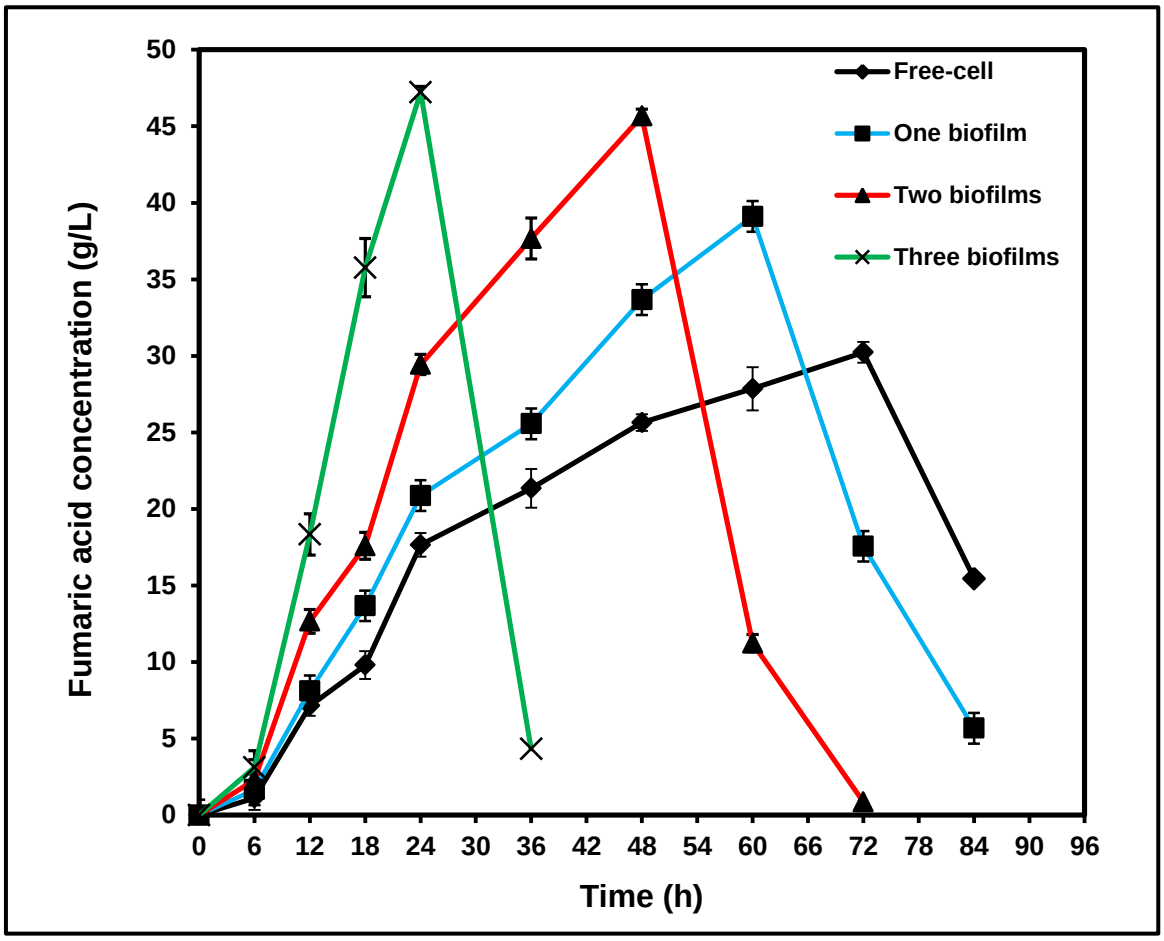

Figure 4.2.1(A): Production profile of fumaric acid obtained with free cells and biofilms of *R. oryzae* 1526 through submerged fermentation with brewery wastewater.

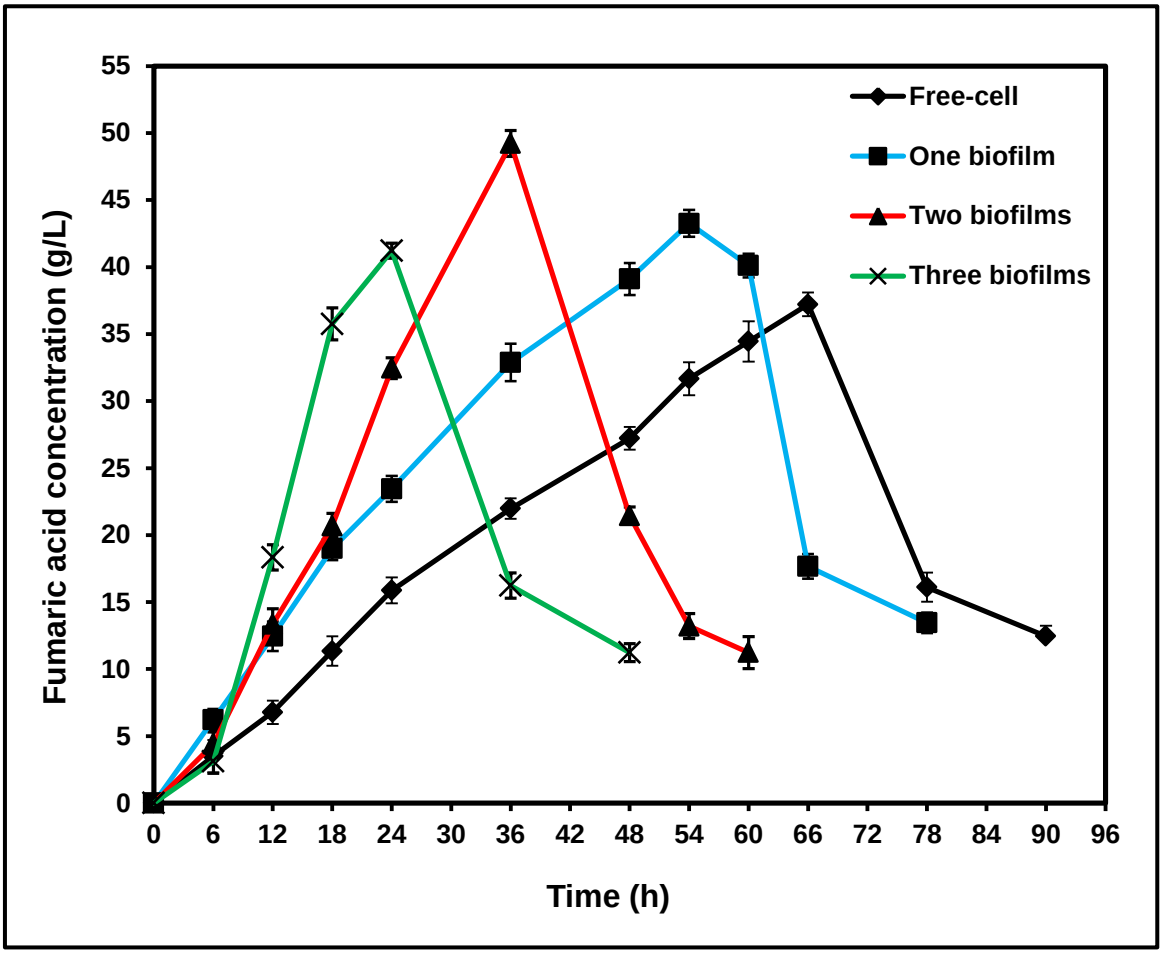

Figure 4.2.1(B): Production profile of fumaric acid obtained with free cells and biofilms of *R. oryzae* 1526 through submerged fermentation with glucose salt medium.

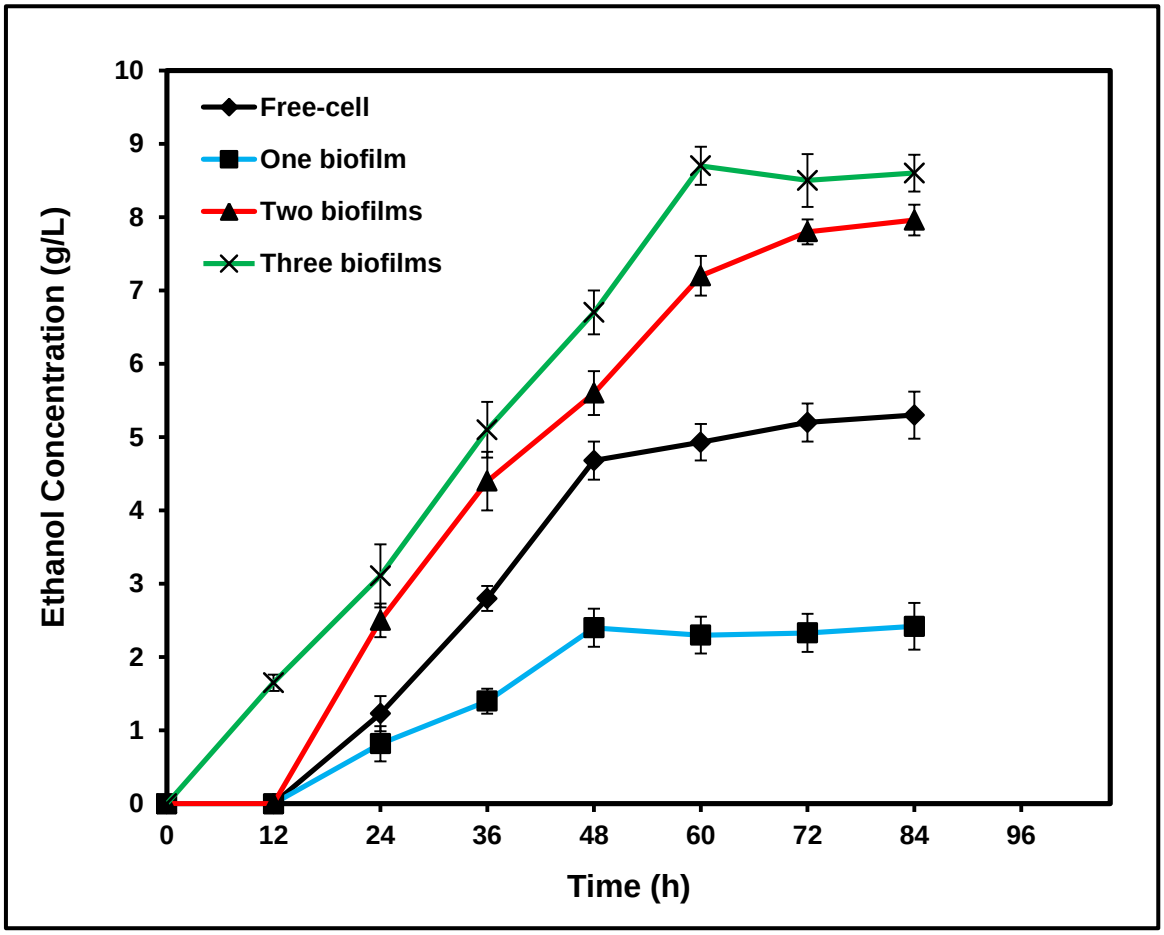

Figure 4.2.2 (A): Production profile of ethanol obtained with free cells and biofilms of *R. oryzae* 1526 through submerged fermentation with brewery wastewater.

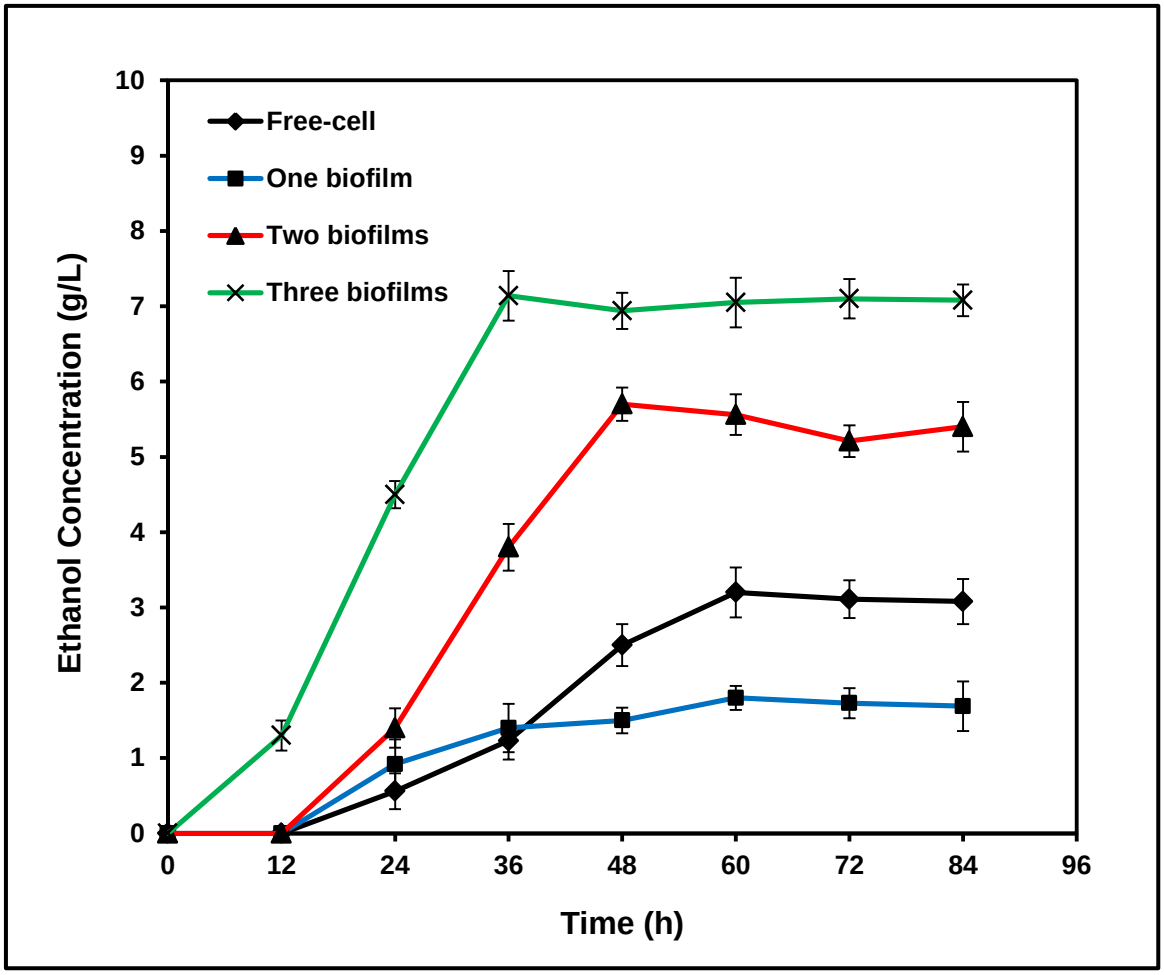

Figure 4.2.2 (B): Production profile of ethanol obtained with free cells and biofilms of *R. oryzae* 1526 through submerged fermentation with glucose salt medium.

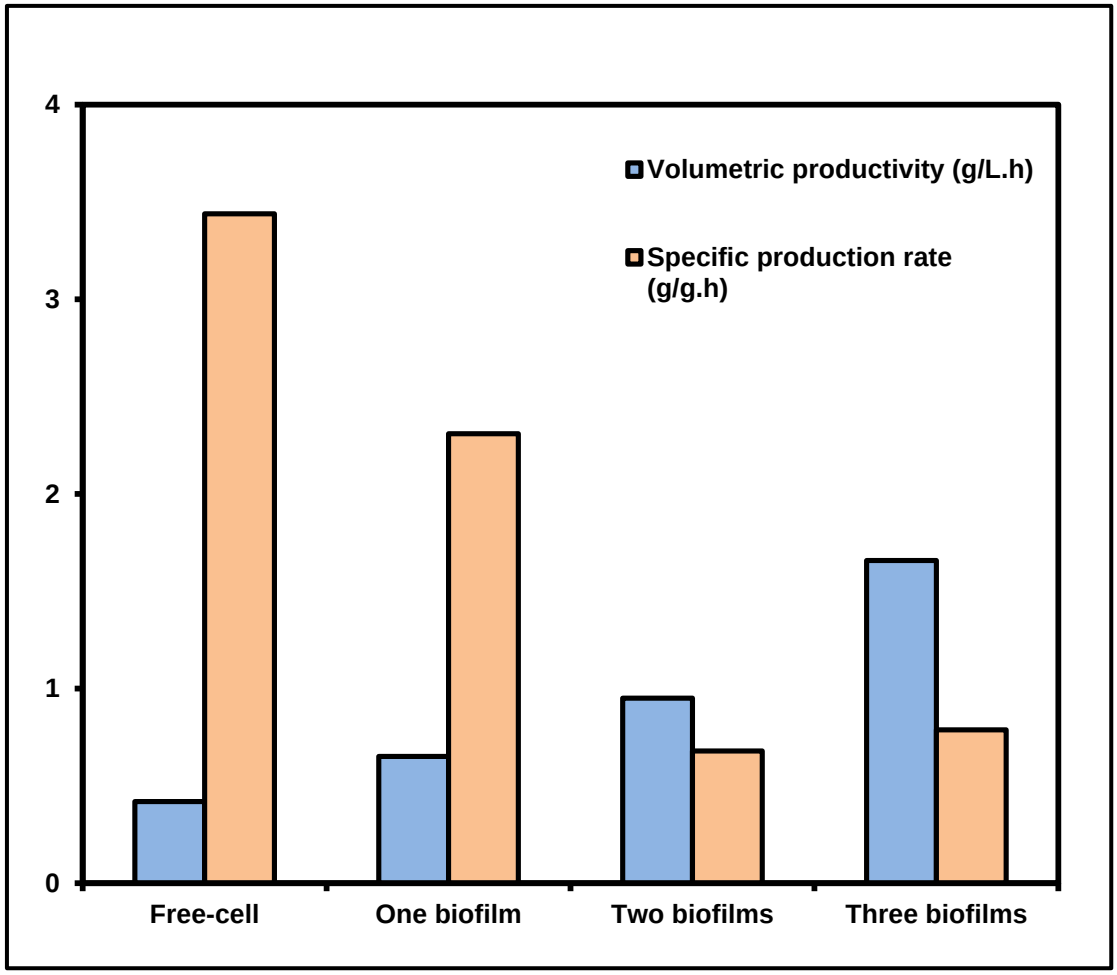

Figure 4.2.3 (A): Volumetric productivities and specific production rates of fumaric acid obtained with free-cell and biofilms of *R. oryzae* 1526 through submerged fermentation (A) with brewery wastewater.

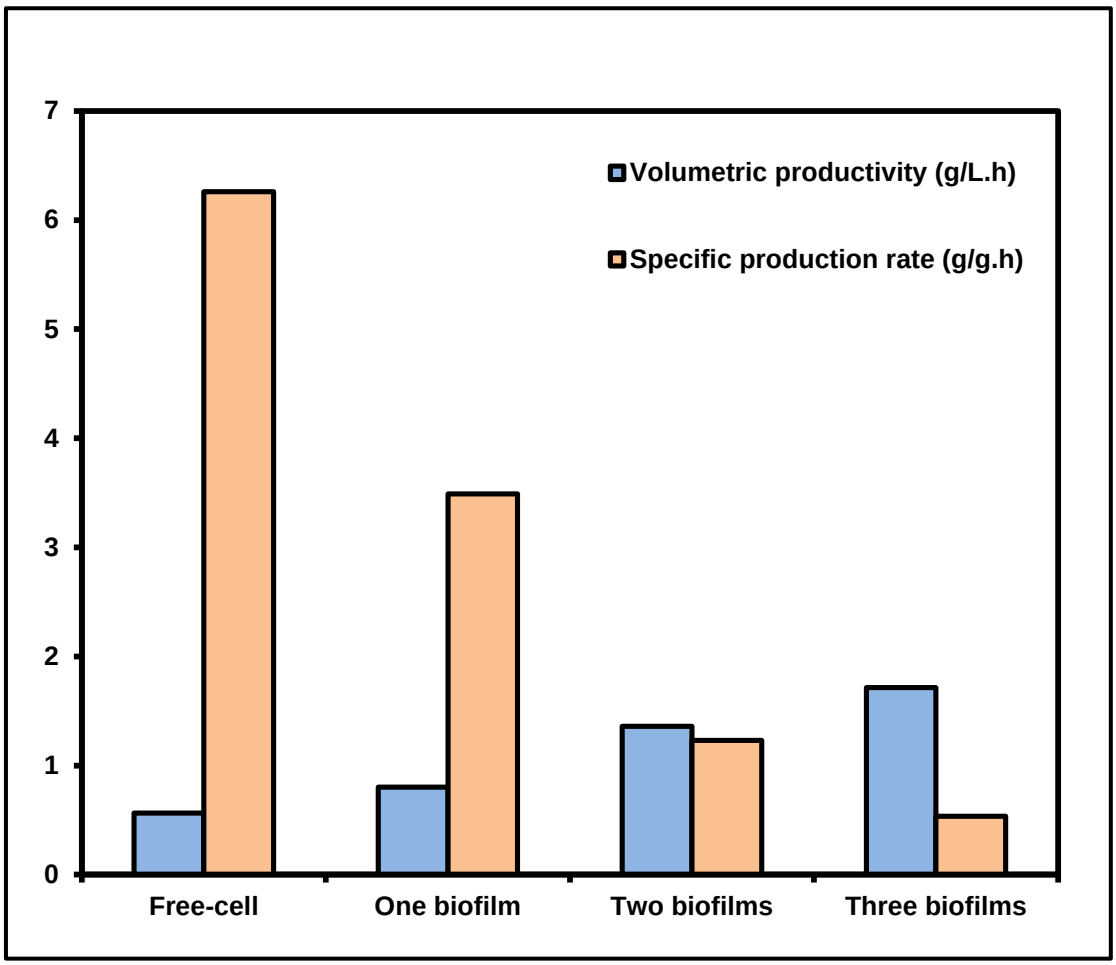

Figure 4.2.3 (B): Volumetric productivities and specific production rates of fumaric acid obtained with free-cell and biofilms of *R. oryzae* 1526 through submerged fermentation with glucose salt medium.

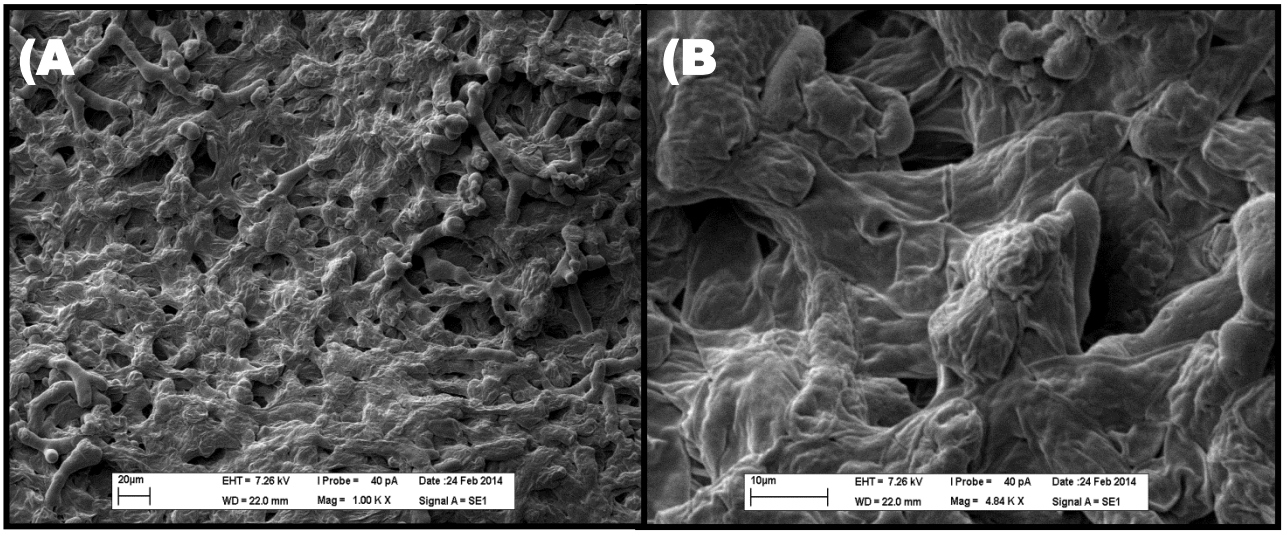

Figure 4.2.4 (A) Scanning electron micrograph of the surface morphology of *R. oryzae* 1526 biofilm; **(B)** a close view of the fungal mycelia.

END OF CHAPTER 4

CHAPTER 5

APPLICATION OF PULP AND PAPER SOLID WASTE FOR ENHANCED FUMARIC ACID PRODUCTION

POTENTIAL USE OF PULP AND PAPER SOLID WASTE FOR THE BIO PRODUCTION OF FUMARIC ACID THROUGH SUBMERGED AND SOLID STATE FERMENTATION

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Résumé

Les résidus solides de pâtes et papiers (en anglais: PPSW) provenant de l'industrie de transformation du bois, ont été utilisés pour la production de fumarique acide (FA) à travers la fermentation submergée et la fermentation à l'état solide en utilisant le champignon filamenteux *Rhizopus oryzae* 1526 (*R. oryzae*). La caractérisation physico-chimique, le pH et l'analyse de la teneur en humidité des PPSW ont été suivis. Le prétraitement des PPSW par désintégration mécanique a permis d'obtenir des particules de différentes tailles (1,7 mm < x ≤ 3,35 mm, 850 µm < x ≤ 1,7 mm, 300 µm < x ≤ 850 µm, 75 µm < x ≤ 300 µm et 33 µm < x ≤ 75 µm). En fermentation submergée, un maximum de 23,47 ± 0,70 g/L de FA a été obtenu avec des tailles allant de 33 µm < x ≤ 75 µm sous des conditions de fermentation optimales (30 °C, 200 rpm, 5% d'inoculum pré-cultivé (v/v) et 48 h d'incubation). La mesure de la viscosité et l'analyse des sous-produits du bouillon fermenté ont été effectuées. L'hydrolyse de particules comprises entre 33 µm et 75 µm effectuée par chauffage aux micro-ondes et ajout d'acide phosphorique, a donné un hydrolysat ayant une teneur maximale en glucose (11,2 ± 0,8 g/L) et en xylose (20,22 ± 0,85 g/L). La fermentation submergée avec cet hydrolysat a confirmé l'utilisation du xylose à la fois pour la production de FA et pour la croissance fongique. La fermentation à l'état solide avec des tailles de particules comprises entre 75 µm < x ≤ 300 µm a abouti à une plus grande production de FA (41,45 g/kg de poids sec de PPSW) après 21 jours. La microscopie électronique à balayage a révélé les caractéristiques morphologiques du champignon cultivé sur les particules. Les résultats de la présente étude ont confirmé que les PPSW comme source de carbone et d'oligo-éléments pour le champignon *R. oryzae* ainsi que son efficacité dans la bioconversion de FA pendant la fermentation. La bio production de FA à partir des PPSW à faible coût, est une approche novatrice potentiellement intéressante pour les industries de pâtes et papier.

Mots clés: Déchets solides de pâtes et papiers, acide fumarique, réduction de la taille, hydrolyse, micro-ondes, fermentation

Abstract

Pulp and paper solid waste (PPSW) originating from paper industry, was experimented for the production of fumaric acid (FA) through submerged and solid state fermentation by utilizing the filamentous fungus *Rhizopus oryzae* 1526 (*R. oryzae*). Physicochemical characterization, pH and moisture content analysis of PPSW were carried out. Pre-treatment of PPSW by size reduction resulted in particles of different size ranges (1.7 mm < x ≤ 3.35 mm, 850 µm < x ≤ 1.7 mm, 300 µm < x ≤ 850 µm, 75 µm < x ≤ 300 µm and 33 µm < x ≤ 75 µm). In submerged fermentation with all particle size ranges, a maximum of 23.47 ± 0.70 g/L of FA was obtained with 33 µm < x ≤ 75 µm under the fermentation conditions of 30 ^{o}C, 200 rpm, 5% pre-cultured inoculum (v/v) and at 48 h. Viscosity measurement and analysis of by-product of the fermented broths were performed. Microwave-phosphoric acid mediated hydrolysis of 33 µm < x ≤ 75 µm particle size produced hydrolysate with maximum glucose (11.2 ± 0.8 g/L) and xylose (20.22 ± 0.85 g/L) contents. Submerged fermentation with this hydrolysate confirmed the utilization of xylose for both FA production and fungal growth. Solid state fermentation with 75 µm < x ≤ 300 µm particle size resulted in highest FA production (41.45 g/kg dry weight of PPSW) after 21 days. Scanning electron microscopy revealed the morphological features of the fungus grown on the particles. The results of the present study confirmed the utilization of PPSW as a source of carbon and trace elements by the fungus *R. oryzae* and also the bioconversion into FA during fermentation. FA being a high value platform chemical, its bioproduction from the low cost PPSW, is a value addition approach.

Keywords: Pulp and paper solid waste, fumaric acid, size reduction, hydrolysis, microwave, fermentation

Introduction

To meet the escalating demand for paper based products; huge amounts (several tonnes per day) of pulp and paper solid waste (PPSW) are produced by paper mills worldwide every year. In fact, PPSW has been found to be the 3rd largest industrial polluter of air, water and soil (www.theworldcounts.com). Proper disposal of PPSW has become a big environmental challenge in many countries, including Canada. There are approximately 130 pulp and paper industry establishments in Canada, mostly located in the provinces of Quebec, British Columbia and Ontario. More than 1/3 of Canada's total waste is PPSW and only 1/4 of PPSW and paperboard is recycled (www.ec.gc.ca). Apart from Canada, the proper disposal and management PPSW is a serious global problem (Oral *et al*., 2005; Abou-Elela *et al*., 2006). PPSW accounts for 25% of landfill waste and 33% of municipal waste (www.theworldcounts.com). Although different conventional strategies, such as landfilling, composting, incineration and recycling are being adopted for the management of PPSW, they are not found to be safe for environmental and human health (Fikru, 2014). In terms of microbial susceptibility, PPSW is a very good source of carbon (energy), vital micro and macro nutrients (minerals), high moisture content (60-70%) and easily biodegradable organic load (high BOD and COD values). Dumping of these wastes in the environment can have direct adverse effects on the environment; worth mentioning are the generation of greenhouse gases (GHGs) and secondary pollution (emission of foul smell caused by microbial putrefaction and contamination of groundwater). Rotten PPSW emits methane (CH_4) gas, which has a global warming potential 72 times greater than CO_2 over a 20 year period (www.globalwarming-forecasts.com). PPSW can also provide food and shelter to microorganisms and disease vectors (e.g. rats, insects etc.) that can cause epidemics in nearby localities. Moreover, because of the unavoidable associated cost factor (transportation and labour charges), landfilling option is no more considered profitable by paper industries. The extra cost demands for price rise in the paper industry based products. Another alarming issue in PPSW landfilling is the new trends in regulations, imposed in terms of ban on landfilling of the waste biosolids in different continents (www.epa.gov; Monte *et al*., 2009; Testa *et al*., 2014). Thus, new alternatives of safer disposal of PPSW with the intervention of modern techniques can contribute to the solution of this global problem. On the contrary, valorization of PPSW for the production of high value product without much economic inputs will be a welcome approach.

With the advent of biotechnological innovations, mainly in the area of fermentation technology, many new avenues have been opened for the proper valorization of different

industrial wastes (Kajaste, 2014). In this regard, PPSW can be subjected to valorization for fumaric acid (FA) production through microbial fermentation. As compared to refined carbon sources such as glucose, PPSW is a low cost carbon source and its utilization can slash the overall fermentative production cost of FA. Recalling the fact that biological FA production was sought to replace the petrochemical route, search for new waste biomass with the potential of serving as a good source of carbon, micro and macro nutrients is actively going on. Moreover, the worldwide demand for FA and its derivatives is growing each year (Goldberg *et al*., 2006). In 2014, the global FA market demand was around 240, 000 t and the projected market volume is 350,000 t by 2020 (www.grandviewresearch.com). In addition to the conventional uses, FA and its ester derivatives (FAEs) have been explored with a number of newer applications in diverse fields (Yang *et al*., 2011b; Mrowietz and Asadullah, 2005). The ever increasing demand for FA mandates the use of alternative fermentation processes using inexpensive raw materials, such as agro-industrial wastes through fermentation. Investigations on FA production from woodchips, dairy manure, crude glycerol, brewery wastewater and lignocellulosic biomass, such as corn straw exhibited high product yield of FA (Xu *et al*., 2010; Zhou *et al*., 2014; Das *et al*., 2015). As literature suggests, different FA producing fungal species of Rhizopus have been successfully grown on wastes raw materials without supplement of any nutrients and resulted in good FA product spectrum. The comparison of petrochemical and fermentation routes for FA production suggests that the lower raw material cost of the fermentative production might compensate the higher yields of the petrochemical production from maleic anhydride, and fermentation may become an economically viable alternative (Roa Engel *et al*., 2008). The fungal strain, *Rhizopus oryzae* 1526 (to be abbreviated as *R. oryzae* afterwards) is highly susceptible to various physico-chemical factors, such as pH, temperature, incubation time, total solids concentration (g/L) of the used substrate, inoculum volume size (v/v) and has a direct impact on the FA production (Xu *et al*., 2012). Screening of a novel carbon source for FA production has to be experimented at individual level. Process control and corresponding fungal (FA producing) responses are very specific for a combination of new carbon source and the fungal strain being used. Optimization of all the fermentation conditions can enhance FA production.

Thus, taking all the aforesaid important aspects of FA into consideration, investigations on the suitability of PPSW for improved FA production through fermentation (submerged and solid-state) were carried out.

Materials and methods

Materials

The FA producing fungal strain *R. oryzae* NRRL 1526 was procured from Agricultural Research Services (ARS) culture collection, IL, USA. The PPSW was procured from Resolute Forest Products, Montreal (Quebec) Canada. All the chemicals used were of analytical grade and purchased from Fisher Scientific (Ottawa, Ontario, Canada).

Methods

Microbial culture and media preparation

The procured fungal strain was first cultured on potato dextrose agar (PDA) slant at 37 ± 1 ^{o}C for a maximum of 4 days. Spread plate method was used to prepare spore inoculum by propagating the spores on PDA plates (90 mm) at 37 ± 1 ^{o}C for 72 h. Mycelium free spores were collected in sterile distilled water after filtration through sterile cotton wool. For regular use, the spore suspension was maintained at 4 ^{o}C. Spores were also preserved at -80 ^{o}C after adding 20% glycerol solution for long term use. A stock of 1×10^{8} spores per mL was maintained and used for inoculation. Glucose-basic salts medium (g/L: glucose 50, urea 2, KH_2PO_4 0.6, $MgSO_4 \cdot 7H_2O$ 0.5, $ZnSO_4 \cdot 7H_2O$ 0.11 and $FeSO_4 \cdot 7H_2O$ 0.0088) was used for preparing the pre-culture of *R. oryzae*. The medium final pH (4.6) was not adjusted, unless specifically indicated. To avoid Maillard reaction, pre-culture medium was prepared in two parts: (a) glucose; and (b) urea + salts and were heat sterilized (20 min, 103421.3594 Pa, 121 ± 1 ^{o}C) separately. Room temperature cooled media components were mixed together inside a laminar flow hood and used for the pre-culture of *R. oryzae*. Pre-culture of *R. oryzae* was prepared by inoculating 50 mL of pre-culture medium with 2% (v/v) spore suspension in a 250 mL Erlenmeyer flask and then incubating at 30 ^{o}C, 200 rpm for 24 h.

Physicochemical characterization of dried pulp and paper solid waste

The estimation of total organic carbon (TOC) and total organic nitrogen (TON) of dried PPSW was done using a carbon:hydrogen:nitrogen:sulphur (C:H:N:S) analyzer (LECO Corporation, USA, Model: CHNS-932). The ash, extractives and acid-insoluble contents of the PPSW was determined using American Society for Testing and Materials (ASTM) standard methods (ASTM Method E 1756-95, ASTM Method E 1755-95 and ASTM Method E 1690-95). Determination of total carbohydrates of the extractive free PPSW was done

following the Anthrone method (Hedge *et al*., 1962). For pH measurement, 1 g of PPSW was mixed in 10 mL of distilled water using magnetic stirrer, and the pH was recorded using a pH-meter equipped with glass electrode. The moisture content (MC) of PPSW was analyzed by oven-dry method (Reeb and Milota, 1999). About 5 g of PPSW sample was allowed to dry at 60 ± 1 ^{o}C until a constant weight was achieved. The MC in PPSW was calculated as per the following formula:

$$\mathrm{MC} = \frac{\text{Initial weight} - \text{Oven dry weight}}{\text{Initial weight}} \times 100 \qquad (1)$$

Size reduction and hydrolysis of PPSW

Oven dried PPSW was ground using an electrical grinder and then sieved through a set of USA standard test sieves (140, 200, 50, 20, 12 and 6 mesh) (ASTM, E-II specification) to obtain the initial particle size ranges: 1.7 mm < x ≤ 3.35 mm, 850 µm < x ≤ 1.7 µm, 300 µm < x ≤ 850 µm, 75 µm < x ≤ 300 µm and 33 µm < x ≤ 75 µm. The ash, extractives and acid-insoluble contents of each of the PPSW particles size range was determined using ASTM standard methods (ASTM Method E 1756-95, ASTM Method E 1755-95 and ASTM Method E 1690-95). Total carbohydrates content of the extractive free particles was determined by Anthrone method (Hedge *et al*., 1962). PPSW of each particle size were used as substrate for SmF and SSF. PPSW particles of all size range were pre-treated with microwave (Microwave sample preparation system, Perkin Elmer, Model: Par physica) mediated acid hydrolysis before application as fermentation medium. For hydrolysis, the method of Orozco *et al*. (2007) was followed with some modifications (Orozco *et al*., 2007). Briefly, PPSW of each particle size was mixed with 2.5% (v/v) of phosphoric acid (H_3PO_4) with a liquid/solid ratio of 10:1 (w/w). The hydrolysis reaction conditions were 175 ^{o}C for 5 min under constant power input of 1000 W and 3 × 10^6 Pa pressure. The hydrolyzed samples were centrifuged (9000 × g, 10 min and 25 ^{o}C), collected supernatants were pH adjusted (5.0) with NaOH solution (10 N) and paper filtered (Whatman # 44). Finally, filtrates were syringe filtered (0.45 mm) and analyzed for glucose and xylose concentrations by LC/MS/MS.

Submerged fermentation (SmF) and solid state fermentation (SSF)

Dried and ground PPSW (5%, w/v) of different initial particle size ranges: 1.7 mm < x ≤ 3.35 mm, 850 µm < x ≤ 1.7 mm, 300 µm < x ≤ 850 µm, 75 µm < x ≤ 300 µm and 33 µm < x ≤ 75 µm, were used as SmF substrate for FA production. To prepare for SmF, the pre-cultured cell pellets of *R. oryzae* were used to inoculate SmF media. An inoculum volume of 7.9 mL (5%, v/v) was transferred into a 500 mL Erlenmeyer flask containing sterilized SmF medium (PPSW + d.H_2O) under aseptic conditions. The final volume was maintained at 150 mL. The inoculated flasks were incubated in a rotary shaker at 30 ^oC, 200 rpm for 96 h. The same procedure was repeated for SmF with hydrolysate supplemented with basic salts (g/L: urea 2, KH_2PO_4 0.6, $MgSO_4 \cdot 7H_2O$ 0.5, $ZnSO_4 \cdot 7H_2O$ 0.11 and $FeSO_4 \cdot 7H_2O$ 0.0088) under same growth conditions as applied for PPSW. All SmF media were heat sterilized (20 min, 103421.35 Pa, 121 ± 1 ^oC) before use. During SmF, sterilized calcium carbonate ($CaCO_3$) was used as a neutralizing agent at a concentration of 50 g/L. Fermented broth was treated with simultaneous heating and acidification (90 ^oC, 5 N H_2SO_4) until clear and later centrifuged (8000 × g, 10 min, 20 ^oC) to collect the supernatant for FA analysis (Dang *et al*., 2009). Biomass dry weight (BDW) of R. oryzae mycelia from SmF broth was determined as per the previous methods with some required modifications (Yin *et al*., 1998). Briefly, the fungal biomass (pellets or suspended mycelia) recovered from the fermented broth was first placed on a standard test sieve (the same sieves used for PPSW sizing) and then washed with copious amount of d.H_2O and shaken in between, until fungal biomass becomes free of unutilized PPSW particles. The choice of the sieve was done based on the PPSW particle size range being used for SmF. The PPSW particle free fungal biomass was oven dried at 60 ± 1 ^oC, until a constant weight was achieved. All BDW was expressed in g/L.

For SSF, dried and ground PPSW particles of different size ranges were used as solid medium for SSF. Plastic trays (length × breadth × height = 35 cm × 22 cm × 11 cm) were used as bioreactor for SSF experiments. Moisture of the solid medium (250 g for each experiment) was adjusted to 70% with d.H_2O and then sterilized by autoclaving (20 min, 103421.35 Pa, 121 ± 1 ^oC) before inoculation. Sterilized and cooled medium was inoculated with spore suspension having 1 × 10^7 spores per g dry substrate aseptically, mixed gently with a glass rod until homogenization and incubated at 30 ± 1 ^oC for a maximum of 21 days (d). For pH maintenance, calcium carbonate ($CaCO_3$) was used as a neutralizing agent during SSF. 20 g of sterilized $CaCO_3$ was mixed with inoculated SSF media after 3 d of incubation.

For sampling in SSF, 5 g of a wet sample was collected from each tray at 7 d, 14 d and 21 d of incubation period under aseptic conditions. The solid samples were further processed by dispensing in 50 mL sterilized d.H_2O and by placing in a magnetic stirrer with hot plates (300 rpm, 50 ^oC) for 120 min. The supernatant was filtered through glass wool for removal of solid substrate and fungal mycelia. The filtered supernatant was downstream processed as done for SmF samples and FA concentration was estimated using HPLC.

As a control experiment, a plastic tray containing 250 g of PPSW with 70% adjusted MC was incubated at 30 ± 1 ^oC and monitored for change in moisture level by measuring the lost amount of water at every 12 h time interval. This control experiment was performed for each set with different particle size ranges. Based on the control experiment results, the moisture of the sample was re-adjusted to 70% MC by adding required volume of sterilized d.H_2O under aseptic conditions.

Analytical methods

Elemental analysis of PPSW was carried with an Inductively Coupled Plasma (ICP) Spectroscopic Technique. For ICP analysis, a representative 1-2 g (dry weight) of PPSW was digested with repeated additions of one part of nitric acid (HNO_3) and 3 parts hydrogen peroxide (H_2O_2) by volume. Hydrochloric acid (HCl) was added to the digestate and then refluxed. The digestate was then diluted to a final volume of 100 mL with deionized water. The prepared sample was then analyzed with ICP-AES (Varian, Model: Vista AX).

Sample preparation for FA estimation was performed following the method of Zhou *et al*. (2000). FA was quantified by HPLC (System: DIONEX DX500) with an Acclaim OA, 5 mm (4.6 × 150 mm) column with a refractive index detector (PDA-100 DIONEX, UV, 210 nm). The mobile phase was 2.5 mM methanesulfonic acid (CH_3SO_3H) at a flow rate of 1 mL/min and column temperature of 30 ^oC. Liquid chromatography -mass spectroscopy (LC/MS/MS) (Thermo TSQ Quantum equipped with an Electrospray Ionization (ESI)) analysis was done for glucose and xylose estimation in the concentrated hydrolysates with an analytical column (Zorbax Carbohydrate 4.6 mm × 150 mm; 5 mm, Agilent). The mobile phase used was 75% Acetonitrile; 0.1% NH_4OH; 25% Water and 0.1% NH_4OH. Injection volume was 10 mL and Glucose- D2 and D-(+)-Xylose (Sigma) were used as the internal standards. Ethanol concentration was quantified by gas chromatography (GC) (7890B, Agilent, Technologies,

USA) with a flow of 1 mL/min over the ZB-WAX plus column (30 m × 0.25 mm, 0.25 mm film thickness) using flame ionization detector (FID) detector with a helium carrier gas and a temperature profile of 150-250 ^{o}C at 16 min run time. Viscosity measurement of the fermented broth was carried out by a rotational viscometer (Fungilab, Premium Series, NY, USA). L1 spindle was used with a sample cup volume of 30 mL.

Process optimization in submerged and solid state fermentation

The following parameters were optimized in the SmF study for enhanced FA production.

(a) Effect of PPSW particle size on fumaric acid product features

PPSW of different initial particle size ranges: 1.7 mm < x ≤ 3.35 mm, 850 µm < x ≤ 1.7 mm, 300 µm < x ≤ 850 µm, 75 µm < x ≤ 300 µm and 33 µm < x ≤ 75 µm, were used as SmF substrate.

(b) Effect of PPSW particle size on the morphology of R. oryzae

All the particle size ranges applied for SmF was monitored for pellet morphology formation and correlated with FA production. For SSF, following parameters were optimized in the SSF study for enhanced FA production.

For SSF, following parameters were optimized in the SSF study for enhanced FA production.

(a) Effect of PPSW particle size

PPSW raw sample and of different initial particle size ranges: 1.7 mm < x ≤ 3.35 mm, 850 µm < x ≤1.7 mm, 300 µm < x ≤ 850 µm, 75 µm < x ≤ 300 µm and 33 µm < x ≤ 75 µm were used as SSF substrates.

(b) SSF time

Three different incubation periods viz. 7 d, 14 d and 21 d were considered for SSF for all initial particle size ranges of PPSW.

Morphological studies

Digital photography (Canon PC 1585) and scanning electron microscopy (SEM, Carl Zeiss EVO® 50) were used for the morphological studies of the fungus R. oryzae. To prepare samples for SEM, PPSW particles were directly mounted on a SEM grid, sputter coated (SPI Module Sputter Coater) with gold and then analyzed.

Statistical analysis

Data are represented as mean ± SD of three independent experiments. Correlations were considered significant at $P < 0.05$ for different applied parameters.

Results and discussion

Physicochemical characterization of pulp and paper solid waste

The elemental analysis of PPSW revealed the presence of different trace metals required for the growth of *R. oryzae* (Table 5.1). Metal ions, such as Mg^{2+}, Zn^{2+} and Fe^{2+} act as cofactors or activators for many cellular enzymes involved in catabolism and biosynthesis of macromolecules (DNA and RNA). Fungal morphology is highly influenced by these trace elements and morphological features finally affect the fermentation. Presence of Manganese (Mn), Zinc (Zn) and Iron (Fe) in PPSW were significant as these three elements were shown to influence pellet formation (Zhou *et al*., 2000; Liao *et al*., 2007; Foster *et al*., 1938). The TOC and TON were found to be 410.16 ± 0.3 and 31.9 ± 0.07 g/kg of PPSW, respectively. The extractives and ash content were estimated to be 16.67 ± 1.2% and 9.7 ± 0.65 respectively. The acid-insoluble residue (AIR) content was found to be 26.63 ± 1.23% and mostly represent lignin. The total carbohydrate (TC) of extractive free PPSW biomass was estimated to be 31.55 ± 1.65%. The PPSW procured from Resolute Forest Products is a woody waste biomass of the two softwood plants viz. spruce and fir that are used for paper production. In general, woody biomass might have 60-80% of TC (Fengel *et al*., 1989). PPSW being a waste biomass, the estimated value of TC was well justified. The results of compositional analysis of PPSW biomass was not summative but confirmed the availability of carbohydrate content for fermentation. The original pH and MC of PPSW were found to be 6.8 and 66% respectively. The pH was supportive of the growth of *R. oryzae* and thus no prior pH adjustment was required before application in SmF and SSF. The compositional

analysis of PPSW demonstrated a nutrient rich medium appropriate for fermentative production of FA.

Pre-treatment of pulp and paper solid waste

Lignocellulosic biomass could be pre-treated with different methods before application as fermentation substrate. Physical or chemical modes of pre-treatments are carried out before hydrolysis of lignocellulosic materials for an easy conversion into fermentable sugars (Broder *et al*., 2001; Badger, 2002; Orozco *et al*., 2007). However, influence of structural features of such substrate on facilitating the hydrolysis has not been well explored for FA producing fungus. Size reduction of lignocellulosic materials can have significant impact on the performance of *R. oryzae* in FA production. In the present investigation, PPSW was considered for initial size reduction pretreatment before being applied as FA producing medium. Grinding and sieving of the dried PPSW resulted in an initial particle size ranges: 1.7 mm < x ≤ 3.35 mm, 850 µm < x ≤ 1.7 mm, 300 µm < x ≤ 850 µm, 75 µm < x ≤ 300 µm and 33 µm < x ≤ 75 µm and were used as substrates for SmF and SSF. Sieved fractions exhibited heterogeneous distribution in ash, extractive, AIR and TC contents (Table 5.2). For 1.70 mm < x ≤ 3.35 mm sized particles, ash and extractive contents decreased to 8.2 ± 0.65 and 11.2 ± 1.35%, respectively. However, with further decrease in the size range (850 µm < x ≤ 1.7 mm, 300 µm < x ≤ 850 µm, 75 µm < x ≤ 300 µm and 33 µm < x ≤ 75 µm), minor changes were observed in the ash and extractive contents. Elemental analysis of the sieved PPSW particles confirmed the intake of the trace elements required for the growth of the fungus *R. oryzae* (data not shown). Mechanical grinding may cause the removal of non-structural material from PPSW biomass and thus reduced the extractive content. It was interesting to find an increase in TC content with decrease in PPSW particle size range. At 33 µm < x ≤ 75 µm particle size range, a maximum of 40.22 ± 2.5% of TC was obtained. This was a clear indication that the mechanical pre-treatment (size reduction) of PPSW feedstock facilitated more release of sugars. Redistribution of nutrient content is possible with change in the size of solid biomass and this could be held responsible for the increase in TC content of the sieved PPSW particles (Liu, 2011).

Microwave-phosphoric acid (H_3PO_4) mediated hydrolysis of PPSW

The effect of particle size on hydrolysis products (glucose and xylose) was well manifested. As seen in Table 5.3, higher concentration of glucose and xylose was obtained as particle size decreased. The hydrolysis of 33 µm < x ≤ 75 µm sized PPSW particles produced

around 11.2 ± 0.8 g/L of glucose and 20.22 ± 0.85 g/L of xylose. As compared to the amount of glucose (2.4 ± 0.5 g/L) and xylose (3.57 ± 0.3 g/L) obtained after hydrolysis of the raw PPSW feedstock, the enhancement in sugar concentration was a positive correlation of the mechanical pre-treatment of PPSW. Under same hydrolysis conditions (175 ^{o}C, 5 min of MWI, 1000 W, 3×10^6 Pa), the variation in glucose and xylose concentrations (g/L) might be due to the higher surface area of the small sized particles for interaction with the acid catalyst and MWI. Previously, it was shown that substrate particle size influenced the performance of enzymatic hydrolysis. Particles of smaller size with higher surface area facilitated catalytic activity and consequent conversion rate into simple sugar (glucose) was higher as compared to large sized particles. Moreover, mechanical grinding might have acted more strongly on the surfaces of the small sized particles and led to reduction of cellulose crystallinity making the surfaces more amorphous and easy for hydrolysis (Dasari *et al*., 2007; Millett *et al*., 1976; Fan *et al*., 1982). However, as compared to such enzymatic approach, combination of diluted acid and MWI applied in the present study, considerably reduced the time of hydrolysis. MWI is a known technology for enhancing rate reaction and product diffusion; while for hydrolysis of lignocellulosic biomass, dilute acid has advantages over concentrated acid (Orozco *et al*., 2007).

Submerged fermentation with PPSW

R. oryzae strains are capable of producing an array of intracellular and extracellular cellulolytic, hemicellulolytic, pectinolytic and amylolytic enzymes that can convert lignocellulosic biomass such as PPSW into fermentable sugars (Ghosh *et al*., 2011). Five different initial particle size ranges: 1.7 mm < x ≤ 3.35 mm, 850 µm < x ≤ 1.7 mm, 300 µm < x ≤ 850 µm, 75 µm < x ≤ 300 µm and 33 µm < x ≤ 75 µm of PPSW were experimented for FA production through SmF. The product features of FA obtained at different particle size ranges are represented in Figure 5.1 and Table 5.4. For a fixed total solid concentration (5%, w/v) of PPSW, production of FA increased from 12.24 to 23.47 g/L with decrease in particle size ranges. The volumetric productivity of FA was also considerably enhanced from 0.127 to 0.488 g/(L h). The reduction in SmF time from 96 to 48 h suggested that rapid assimilation of nutrients was brought about by the hydrolytic activity on the smaller particles by the enzymes of the fungus, *R. oryzae*. This ultimately facilitated early conversion of fermentable sugars into FA. Higher particle size favoured increased biomass yield (0.533 ± 0.085 g BDW/g dry PSW). Yield for FA was highest (0.570 g FA/g dry PSW) for the smallest particle size as compared to the largest one (0.406 g FA/g dry PSW).

The effects of initial particle size range on the FA product features can well be elaborated on the basis of different aspects. In general, smaller particles have larger surface area per unit volume and the surface area available on the larger particles is less. For a substrate to be converted into some product by enzymatic actions, more interactions are possible with larger surface area at initial stages (Dasari *et al*., 2007). The cellulolytic enzyme profile released by the fungus *R. oryzae* acted faster on the small sized particles and thus caused a faster conversion into FA during SmF. This shortened SmF time and enhanced volumetric productivity of FA. Moreover, it is a well-established fact that the ratio of crystalline and amorphous fractions determines the hydrolysis efficiency for a lignocellulosic material (Yang *et al*., 2011a; Fan *et al*., 1980; Lee *et al*., 1983). More crystallinity delays enzymatic conversion into product. Reduction in crystallinity and an increase in amorphous nature at the surface by mechanical grinding can have significant impact on the enzymatic hydrolysis of lignocellulosic biomass. In the present study, particle size reduction from 1.70 mm < x ≤ 3.35 mm to 33 µm < x ≤ 75 µm, might have changed the fungal enzyme kinetics (reducing the long residence time) that finally led to enhanced production profile of FA.

Moreover, broth viscosity played an important role in the FA product features. As shown in Table 5. 5, at the staring of SmF (0 h), the viscosity obtained for different particle size ranges clearly suggested a relationship between particle size and broth viscosity. There was increment in the broth viscosity with increased particle size. With smallest particle size, the initial viscosity was 0.0315 ± 0.0018 Pa·s; while for the largest one it was higher (0.0517 ± 0.0024 Pa·s). Considering the fact that the control experiments ($CaCO_3$ + 5% w/v PPSW + d.H_2O) contained all components except the inoculum (5% pre-cultured cell pellets), the difference in the initial viscosities was caused by the PPSW particles sizes. In rheological studies with solid particles, the broth viscosity can change with particle size at a fixed solid concentration. This can be attributed to: (a) small particles generate larger inter-particle free space that helps in lowering viscosity and; (b) due to the greater inertia of interaction between larger particles, more energy is required to momentarily accelerate or retard the larger particles. This energy accounts for the extra viscosity (Clarke, 1967; Marc, 2011). Reduction in the broth viscosity facilitated homogenization, and interphase mass, oxygen and heat transfer throughout the broth. In particular, being an aerobic process, FA production is influenced by oxygen supply within the broth. However, the final broth viscosity (96 h) was also influenced by fungal morphology (discussed in the following paragraph).

Another obvious reason for the enhanced viscosity and changes in FA product features was the morphological forms of R. oryzae obtained with different particle size ranges as shown in Figure 5.2 and Figure 5.3. Highly hairy-pellets were observed in SmF with the particle size range of 1.7 mm < x 3.35 mm, 850 µm < x ≤1.7 µm, 300 µm < x ≤ 850 µm and 75 µm < x ≤ 300 µm) (Figure 2 (A) and 2 (B)). For the smallest particle size range (33 mm < x ≤ 75 mm) applied, discrete solid pellets (<1 mm in diameter) were obtained (Figure 3 (A) and 3 (B)). The difference in morphological forms caused the changes in the broth viscosity and thus ended with higher (0.377 ± 0.012 Pa·s for hairy pellets) and lower (0.0674 ± 0.005 Pa·s for solid pellets) viscosities respectively. It is an established fact that as compared to the fungal pellets morphology, hairy or suspended mycelia increase broth viscosity (Li *et al*., 2000; Rodriguez Porcel *et al*., 2005). Although, the reason behind such morphological change is not exactly known, still it can be ascertained that formation of solid pellets is only driven by the smaller particle size factor as all other growth conditions (pH, rpm, temperature, TSC and inoculum volume) applied during SmF were same for the particle size range studied.

Highest biomass yield (0.533 ± 0.085 g BDW/g dry PSW) was achieved with the largest particle size and considerably decreased (0.278 ± 0.046 g BDW/g dry PSW) at the smallest particle size range (33 µm < x ≤ 75 µm). Production of more fungal biomass with hairy pellets is a common phenomenon in filamentous based SmF. During flask shaking, the hairs from the pellets are shaved off by the hydrodynamic force and reseeds into more mycelial growth. This leads to damage and deactivation of both reseeded mycelia and the pellets and consequently all such pellets suffer from ageing and vacuolation of the fungal hyphae (Cui *et al*., 1998). The activity of pellet and reseeded mycelia are highly reduced and thus affects the SmF performance. The reduction in the values of FA product features and more biomass yield was controlled by the morphological forms of *R. oryzae* during SmF.

Production of ethanol as by-product in the SmF with different particle size ranges suggested a supportive role of the small sized PPSW particles towards aerobic process such as FA production as compared to bigger ones. As it can be seen from Figure 5.4, after 96 h of SmF, ethanol production was 8.5 ± 0.35 g/L for 1.7 mm < x ≤ 3.35 mm sized PPSW and considerably lowered to 1.28 ± 0.097 g/L as particle size range decreased to 33 mm < x ≤ 75 mm. Increase in particle size favoured anaerobic fermentation and ethanol concentration enhanced further with simultaneous reduction in FA production (Figure 5.1 and Figure 5.4).

Hairy pellet morphology and high viscosity of the SmF broth of largest particle size caused lack of oxygen supply within the broth and led to more ethanol production (Skory *et al*., 1998). With decrease in the particle size, viscosity also decreased and it facilitated more oxygen supply within the broth and thus favoured FA production.

Submerged fermentation with hydrolysates

To find out the exact carbon source for FA production and biomass growth of *R. oryzae*, hydrolysate of 33 µm < x ≤ 75 µm was experimented as substrate for SmF. The hydrolysate contained around 11 g/L of glucose and 20 g/L of xylose. During 84 h SmF, utilization of glucose and xylose and conversion into FA or fungal biomass varied with time. Production of FA was initially supported by hydrolysate glucose and then became xylose dependent. FA production reached to a maximum of 15.2 ± 1.23 g/L at the end of 72 of SmF time with a volumetric productivity of 0.21 g/(L h) (Figure 5.5 (A)). However, as compared to glucose, utilization of xylose was slower and mostly used for fungal growth. At the end of 36 h of SmF, the residual glucose concentration was found to be 0.85 ± 0.1 g/L, indicating fast glucose consumption by *R. oryzae* (Figure 5.5 (B)). It was interesting to observe that production of FA was continued from 36 to 72 h of SmF at the expense of xylose only and this was confirmed from the decrease in the residual xylose concentration in the broth. The fungal biomass increased considerably upto 8.1 g/L after 84 h SmF and the increment in FA production from 36 to 84 h SmF was around 8.3 g/L. It suggested that xylose was utilized for both FA production and fungal biomass growth. Fast consumption of hexose sugars is a normal tendency of natural microorganisms, while utilization of xylose for fungal growth is a well-established fact (Xu *et al*., 2010). Previous studies showed utilization of xylose for organic acid production by *R. oryzae* (Kautola *et al*., 1989; Mass *et al*., 2006, 2008). However, as compared to the pure substrates used in these studies, utilization of hexose and pentose sugars from a mixture substrate (glucose + xylose) by *R. oryzae* for bioconversion into FA and fungal biomass was an important outcome.

Solid state fermentation with PPSW

The different initial particle size ranges: 1.7 mm < x ≤ 3.35 mm, 850 µm < x ≤ 1.7 µm, 300 µm < x ≤ 850 µm, 75 µm < x ≤ 300 µm and 33 µm < x ≤ 75 µm of PPSW used as SSF substrate showed variations in FA profile with changes in SSF time and particle size (Figure 5.6). There was no FA production by *R. oryzae* in the first 7 d of incubation for 1.7 mm < x ≤

3.35 mm and 850 µm < x ≤ 1.7 µm particle size range. For the particle size ranges of 300 µm < x ≤ 850 µm, 850 µm < x ≤ 300 µm and 33 µm < x ≤ 75 µm, FA concentrations were 1.65, 3.25 and 5.43 g/kg of dry PPSW respectively. These preliminary results indicated a basic tendency of more FA production with the decrease of particle size. However, this was not a conclusive observation as FA production was not supported by the higher particle size ranges. To further confirm, how the particle size and SSF time may influence the FA production, incubation was continued for another phase of 7d. At the end of 14 d of SSF, all particle size ranges produced FA. The inverse relation of particle size vs. FA production continued upto the size range of 850 µm < x 300 µm. A highest FA concentration of 27.66 g/kg of dry PPSW was found for 850 µm < x ≤ 300 µm. FA production was lowered as size range reached to 33 µm < x ≤ 75 µm. At this stage, it was difficult to explain such deviation in FA production with the applied lowest size range. For conclusive evidence, SSF was continued until 21 d. FA production profile similar to obtained at 14 d, was observed at 21 d of SSF. FA concentration increased from 27.66 to 41.65 g/kg of dry PPSW for the particle size range of 850 µm < x ≤ 300 µm; while production level remained almost same (16.34 & 18.22 g/kg of dry PPSW) for 33 µm < x ≤ 75 µm. From the results of 7 d, 14 d and 21d of SSF, it was concluded that: (a) the growth conditions maintained during SSF supported FA production; (b) particle size ranges affected considerably the FA production level and size range of 850 µm < x ≤ 300 µm was found to be the optimized one; and (c) smallest particle size range (33 µm < x 75 µm) negatively affected the FA production. Beyond 21 d of SSF, FA production level changed marginally for all the applied particle size ranges.

The effects of PPSW particle size on FA production during SSF can be well explained with the perception of interactions of filamentous fungus with the solid substrate. In general, small particle size with larger surface area is desired for an easy initial access to fungal mycelia for interactions. However, for a smooth heat, mass and oxygen transfer, packing density and void space of a solid substrate should be optimum and all these factors are primarily governed by the substrate particle size. Too small particles may result in substrate agglomeration and thus retard fungal proliferation and finally product level (Krishna, 2005). With regard to these facts, it was concluded that the particle size range of 850 µm < x ≤ 300 µm well supported the growth of *R. oryzae* and made SSF biophysicochemical environment favourable for FA production. It was quite possible that SSF with particle size range of 33 µm < x ≤ 75 µm caused agglomeration with the maintained 70% MC and led to poor growth of *R. oryzae*. This finally lowered the FA production level during SSF.

SEM analysis

The SEM analysis of the *R. oryzae* mycelia grown on the PPSW particles revealed morphological details. As seen in Figure 5.7 (A), the fungal mycelia penetrated deep into the substrate and dense mycelial growth occurred all over the particle surface (i.e. confluent). A closed view displayed the mycelial compactness (Figure 5.7 (B)). The SEM analysis confirmed the suitability of PPSW as supporting material for the filamentous fungus *R. oryzae*.

PPSW vs other waste carbon sources used for FA production

A comparative view of FA production profile from different waste carbon sources is presented in Table 6. The successful production of FA confirmed the fact that this waste biomass can be a good carbon source for FA producing fungal strains. However, based on the selected carbon source, methodology and used fungal strain, the FA production profile varied. Among all waste carbon sources tested, corn starch led to higher FA production (71.9 g/L), volumetric productivity of 0.5 g/(L h) and product yield of 0.6 g/g. The study on corn straw carried out by Xu *et al*. (2010) resulted in the highest production, volumetric productivity and product yield (27.79 g/L, 0.33 g/(L h) and 0.35 g/g respectively) of FA for any lignocellulosic biomasses used for FA production. The production profile achieved in the present study confirmed the suitability of PPSW as a good carbon source for FA production. The volumetric productivity of 0.488 g/(L h) and product yield of 0.57 g/g of present study is comparable to the SmF results of corn starch. The FA production (23.47 g/L) level was either higher or in the concentration range obtained with majority of other waste carbon sources.

Conclusions

A strategy of enhanced fumaric acid production by utilization of industrial paper solid waste was developed through submerged and solid state fermentation techniques. Particle size of the waste considerably influenced the fumaric acid production and sugar release. Fumaric acid concentration increased from 12.24 ± 0.534 g/L to 23.47 ± 0.70 g/L as particle size decreased in the range of 1.7 mm < x ≤ 3.35 mm, 850 µm < x ≤ 1.7 mm, 300 µm < x ≤ 850 µm, 75 µm < x ≤ 300 µm and 33 µm < x ≤ 75 µm. The volumetric productivity was enhanced from 0.127 to 0.488 g/(L h). The combination of microwave technology and phosphoric acid facilitated fast hydrolysis of the waste with high yields of glucose and xylose in short reaction times. Hydrolysate of 33 µm < x ≤ 75 µm particle sized when applied as

substrate, confirmed the utilization of xylose for fumaric acid production apart from fungal growth. Pellet morphology of *R. oryzae* played important role in fumaric acid enhancement in submerged fermentation. Particle size also affected the fumaric acid production in solid state fermentation. A maximum of 41.65 g fumaric acid per kg of dry weight of the solid was obtained with the particle size range of 850 μm < x ≤ 300 μm. The scanning electron microscopic analysis of the solid state fermented particle confirmed the close interactions with the fungus. The results of the present study suggest for similar utilization of the pulp and paper solid waste for cost effective production of other organic acids.

Abbreviations

TC= total carbohydrates, DW= dry weight, PPSW= pulp and paper solid waste, TOC= total organic carbon, TON= total organic nitrogen, BDL= below detection limit,

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Table 5.1: Compositional details of pulp and paper solid waste (PPSW)

TOC (g/kg DW of PPSW)	TON (g/kg DW of PPSW)	TC (%)	Extractives (%)	Ash (%)	Acid-insoluble residue (%)	Elemental concentration (mg/kg DW of PPSW)
410.16 ± 0.3	31.9 ± 0.7	31.55 ± 1.65	16.67 ± 1.2	9.7 ± 0.65	26.63 ± 1.23	K (1789.19) Mg (977.47) Na (2261.87) Mn (2040.62) As (5.66) Se (BDL) Ca (5868.57) Co (332.76) Cu (11.96) Fe (4305.95) Ni (7.75) P (3745.26) Zn (23.65)

Abbreviations: TC: Total carbohydrates; DW: Dry weight; PPSW: Pulp and paper solid waste; TOC: Total organic carbon; TON: Total organic nitrogen; BDL: Below detection limit

Table 5.2: Compositional details of pulp and paper solid waste (PPSW) sieved particles of different particle size ranges.

PPSW particle size range	TC (%)	Extractives (%)	Ash (%)	Acid-insoluble residue (%)
1.70 mm < $x \leq$ 3.35 mm	32.5 ± 0.8	11.2 ± 1.35	8.2 ± .65	22.4 ± 2.3
850 µm < $x \leq$ 1.70 mm	32.3±1.23	10.8 ± 1.8	7.6 ± 1.4	21.8 ± 1.8
300 µm < $x \leq$ 850 µm	36.8 ± 0.7	10.67 ± 1.15	7.7 ± 0.5	22.5 ± 1.5
85 µm < $x \leq$ 300 µm	37.11 ± 1.2	10.5 ± 0.54	7.5 ± 0.7	17.65 ± 2.3
33 µm < $x \leq$ 75 µm	40.22 ± 2.5	10.3 ± 0.34	7.2 ± 0.4	15.43 ± 1.1

Abbreviations: TC: Total carbohydrates; DW: Dry weight; PPSW: Pulp and paper solid waste)

Table 5.3: Glucose and xylose concentrations obtained for different hydrolysates after microwave-phosphoric acid (H_3PO_4) mediated hydrolysis of pulp and paper solid waste (PPSW) sieved particles of different size ranges.

Hydrolysate	Glucose (g/L)	Xylose (g/L)
Raw PPSW	2.4 ± 0.5	3.57 ± 0.3
1.70 mm < $x \leq$ 3.35 mm	3.5 ± 0.15	3.8 ± 0.15
850 µm < $x \leq$ 1.70 mm	4.2 ± 0.8	4.5 ± 0.4
300 µm < $x \leq$ 850 µm	5.8 ± 0.6	6.2 ± 0.7
85 µm < $x \leq$ 300 µm	10.0 ± 0.5	16.8 ± 0.9
33 µm < $x \leq$ 75µm	11.2 ± 0.8	20.22 ± 0.85

Table 5.4: Particles size effects of pulp and paper solid waste (PPPSW) on fumaric acid product features and fungal biomass in submerged fermentation.

Particle size	FA concentration (g/L)	SmF time (h)	Volumetric productivity (g/(L h))	Biomass yield after SmF (g BDW/g dry PPSW)	FA yield after SmF (g FA/g dry PPSW)
1.70 mm < x ≤ 3.35 mm	12.24 ± 0.534	96	0.127	0.533 ± 0.085	0.406
850 µm < *x* ≤ 1.70 mm	14.69 ± 0.228	96	0.153	0.514 ± 0.076	0.417
300 µm < *x* ≤ 850 µm	16.77 ± 1.27	84	0.199	0.459 ± 0.066	0.451
85 µm < *x* ≤ 300 µm	19.48 ± 0.869	60	0.324	0.402 ± 0.059	0.497
33 µm < *x* ≤ 75µm	23.47 ± 0.70	48	0.488	0.278 ± 0.046	0.570

Abbreviations: BDW: Biomass dry weight; PPSW: Pulp and paper solid waste; SmF: Submerged fermentation

Table 5.5: Viscosities obtained at different pulp and paper solid waste (PPSW) particle sized substrates before and after submerged fermentation.

SmF Substrate	Initial viscosity (0 h) (Pa·s)	Final viscosity (96 h) (Pa·s)
1.70 mm < $x \leq$ 3.35 mm	Control: 0.0499 ± 0.0025 Sample: 0.0517 ± 0.0024	Control: 0.0503 ± 0.0018 Sample: 0.377 ± 0.012
850 µm < $x \leq$ 1.70 mm	Control: 0.0455 ± 0.0028 Sample: 0.0468 ± 0.0015	Control: 0.0462 ± 0.0018 Sample: 0.218 ± 0.008
300 µm < $x \leq$ 850 µm	Control: 0.0432 ± 0.0011 Sample: 0.0445 ± 0.0012	Control: 0.0423 ± 0.0025 Sample: 0.166.86 ± 0.011
85 µm < $x \leq$ 300 µm	Control: 0.0415 ± 0.0012 Sample: 0.0412 ± 0.0013	Control: 0.0428 ± 0.0016 Sample: 0.111 ± 0.009
33 µm < $x \leq$ 75µm	Control: 0.0328 ± 0.0022 Sample: 0.0315 ± 0.0018	Control: 0.0308 ±0.0011 Sample: 0.0674 ± 0.005

Table 5.6: A comparative account of fumaric acid production profile achieved with different waste carbon sources.

Carbon source	***Rhizopus* species used**	**Fermenter**	**FA concentration (g/L)**	**FA productivity (g/(L h))**	**Yield (g/g)**	**References**
Corn straw	*Rhizopus oryzae* ME-F12 (mutant)	Shake flask	27.79	0.33	0.35	Xu *et al.*, 2010
Corn starch	*Rhizopus oryzae*	Shake flask	71.9	0.50	0.60	Moresi *et al.*, 1992
Dairy manure	*Rhizopus oryzae*	Stirred tank	31.0	0.322	0.31	Liao *et al*., 2008)
Cassava bagasse	*Rhizopus formosa*	Shake flask	21.3	Not mentioned	0.34	Carta *et al,.* 1999
Molasses	*Rhizopus nigricans*	Fluidized bed	17.5	0.36	0.36	Petruccioli *et al.* 1996
Crude glycerol	*Rhizopus arrhizus* RH-07-13 (mutant)	Shake flask	22.81	0.158	0.346	Zhou *et al.*, 2014
Potato flour	*Rhizopus nigricans*	Shake flask	43.5	0.42	0.58	Moresi *et al.* 1991
Xylose	*Rhizopus nigricans*	Shake flask	15.3	0.07	0.23	(Kautola and Linko, 1989)
Wood chips hydrolysate	*Rhizopus arrhizus*	Shake flask	5.085	Not mentioned	0.089	Woiciechowski *et al.*, 2001
Pulp and paper solid waste	*Rhizopus Oryzae 1526*	Shake flask	23.47	0.488	0.570	Present study

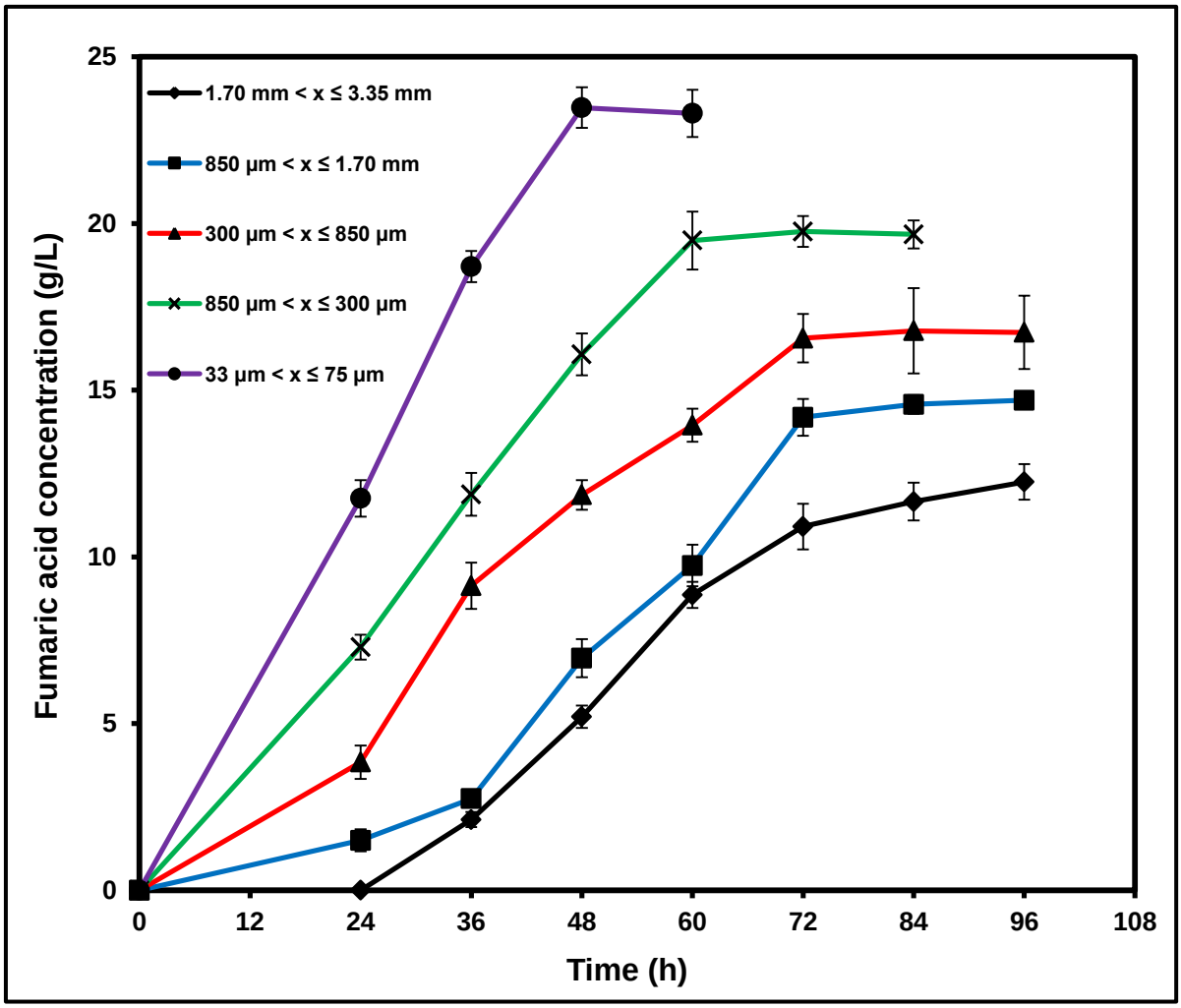

Figure 5.1: Effects of the paper solid waste particle size on the production profile of fumaric acid in submerged fermentation condition.

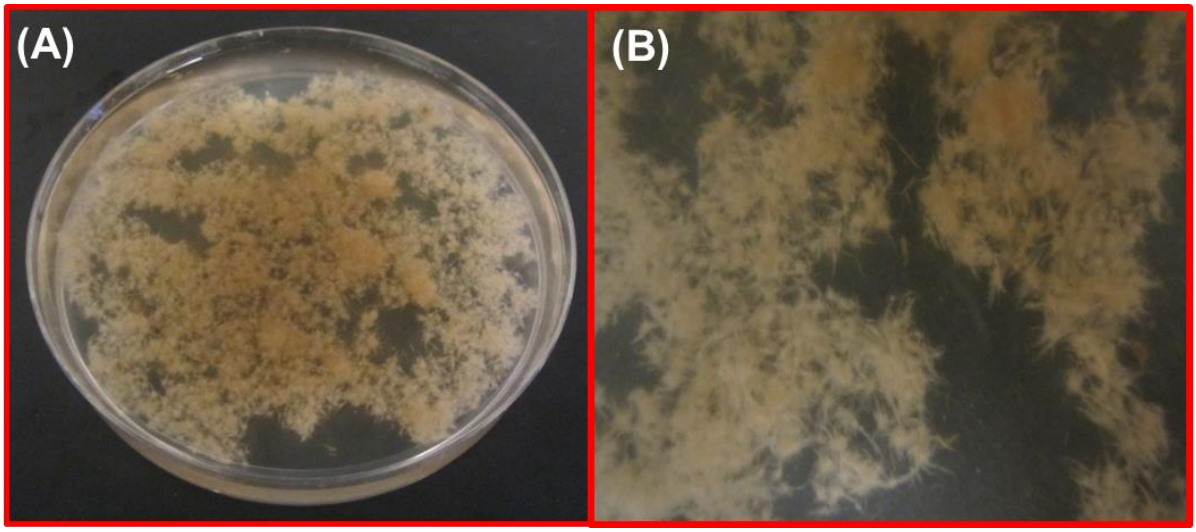

Figure 5.2: (A) A representative digital image of the hairy pellets morphology of *R. oryzae* obtained with PPSW particle size range of 1.7 mm < x ≤ 3.35 mm, 850 µm < x ≤ 1.7 mm, 300 µm < x ≤ 850 µm and 75 µm < x ≤ 300 µm **(B)** A close view of the hairy pellets.

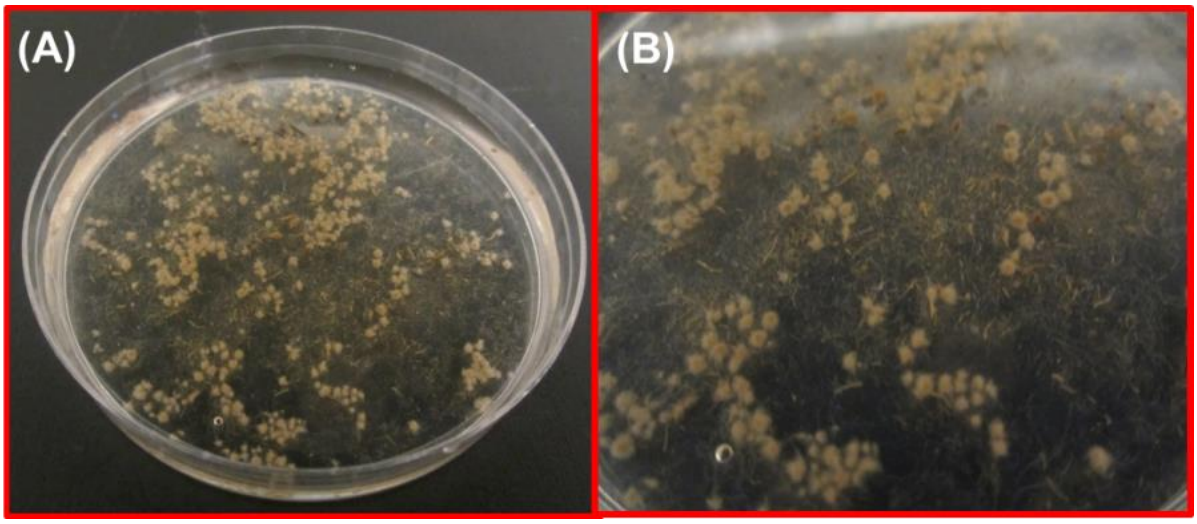

Figure 5.3: (A) A representative digital image of the solid discrete pellets morphology of *R. oryzae* obtained with pulp and paper solid waste (PPSW) particle size range of 33 µm < x ≤ 75 µm **(B)** A close view of the solid pellets.

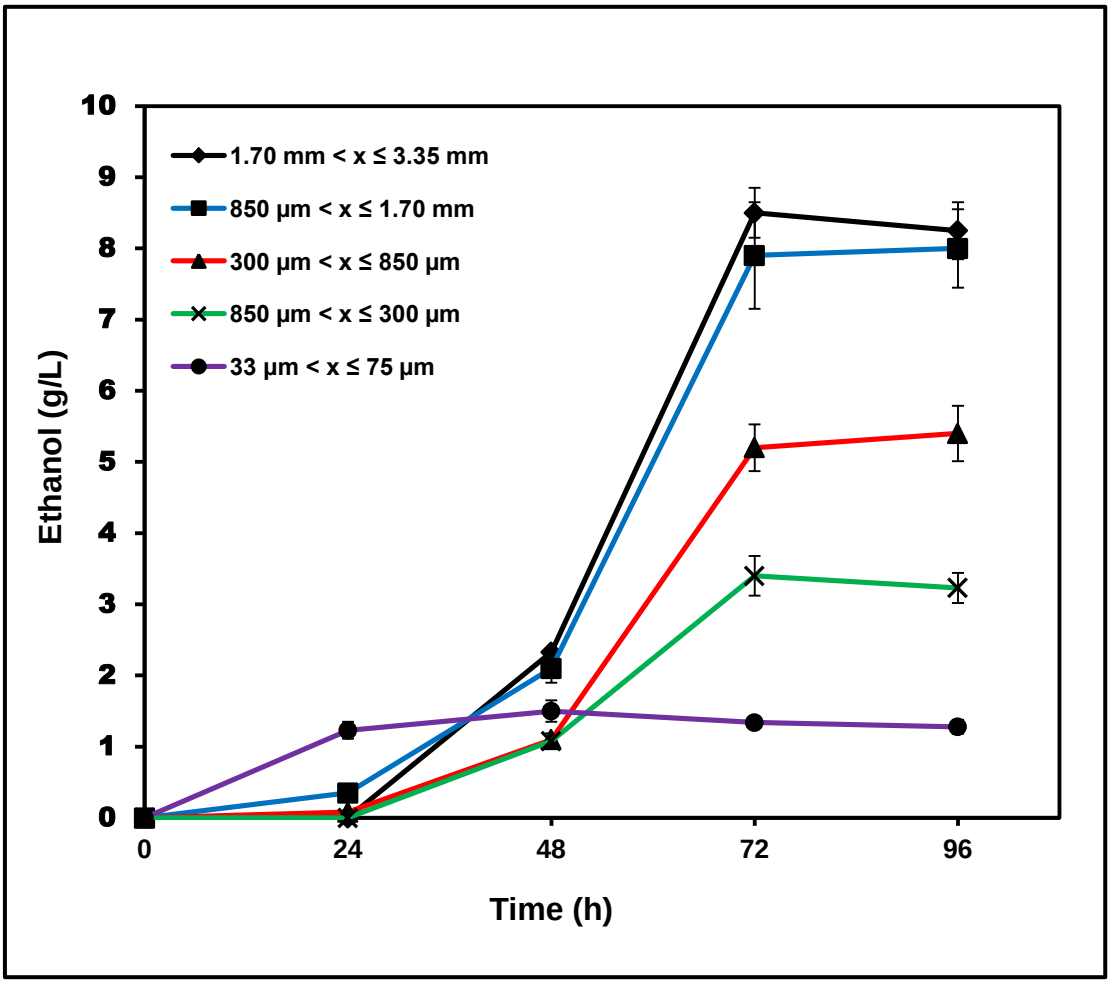

Figure 5.4: Ethanol production profile during submerged fermentation with pulp and paper solid waste (PPSW) of different particle size range.

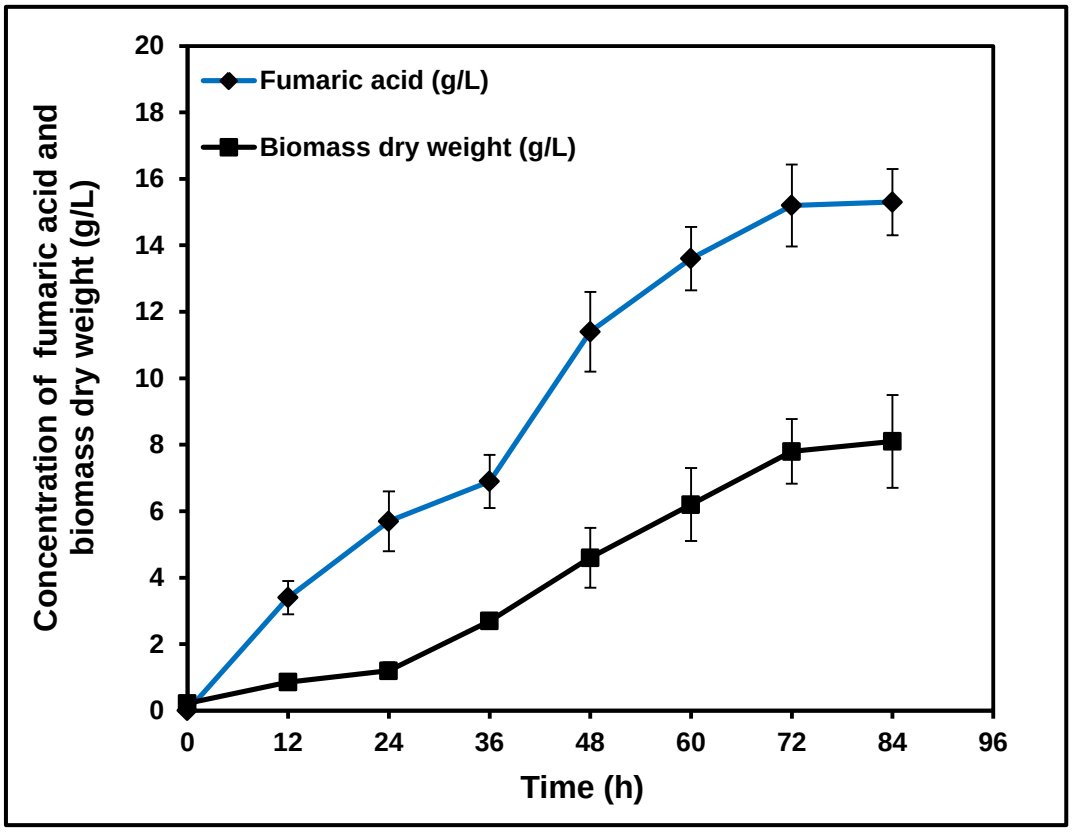

Figure 5.5 (A): Production of fumaric acid and biomass growth of *R. oryzae* during submerged fermentation with 33 µm < x ≤ 75 µm hydrolysate.

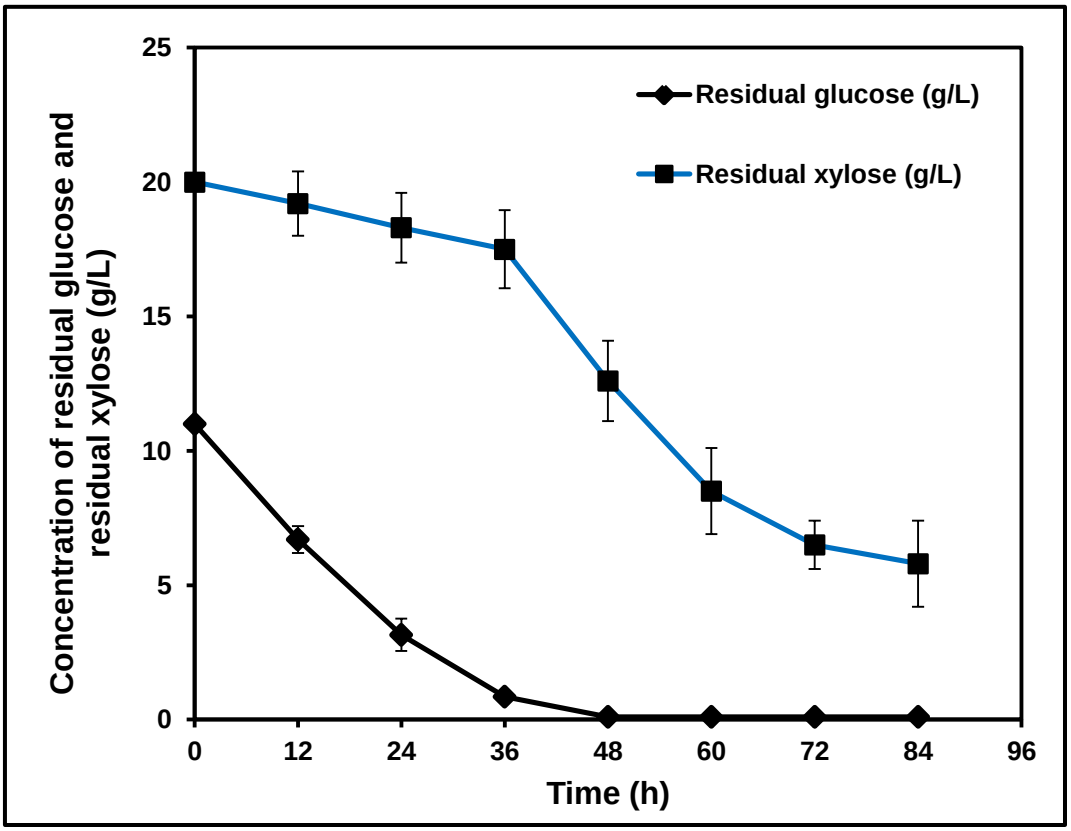

Figure 5.5 (B) Residual glucose and xylose concentrations at different time intervals of submerged fermentation with 33 µm < x ≤ 75 µm hydrolysate.

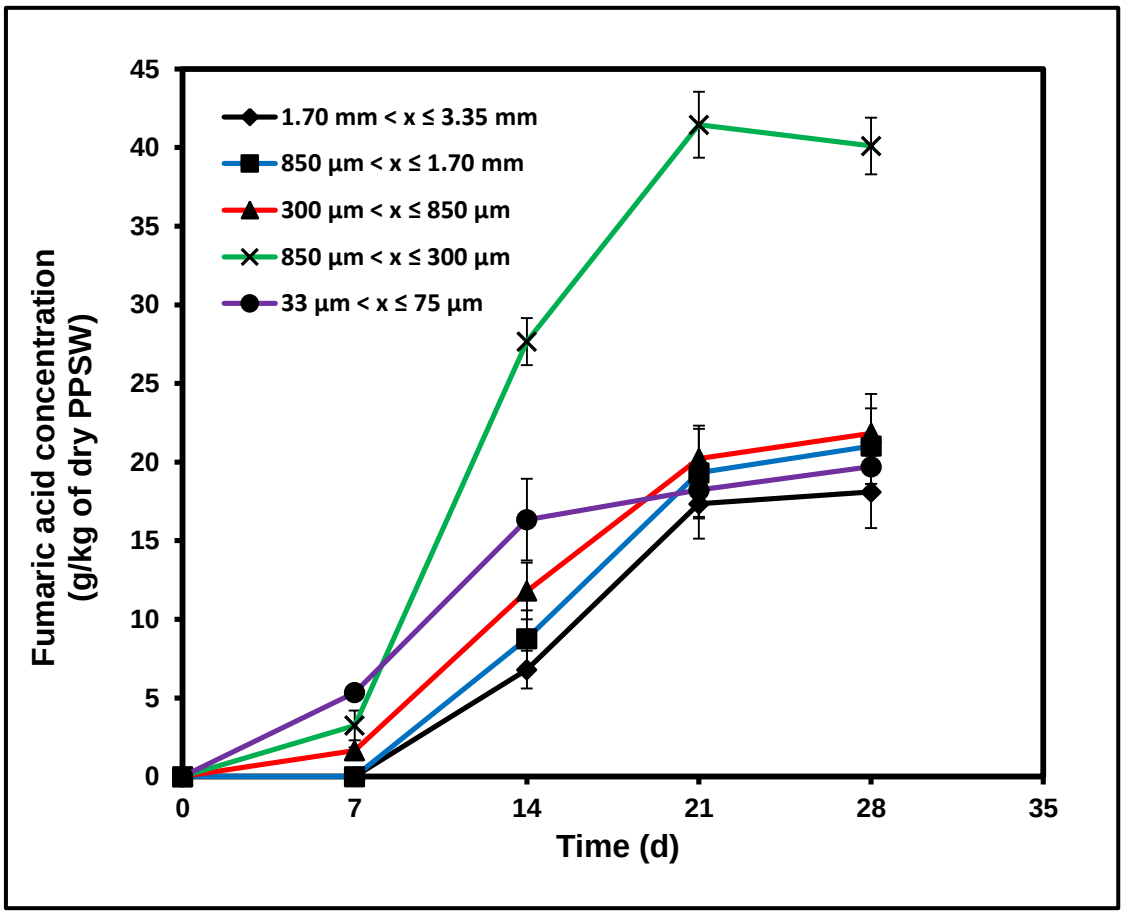

Figure 5.6: Effects of the pulp and paper solid waste (PPSW) particle size on the production profile of fumaric acid in solid state fermentation condition.

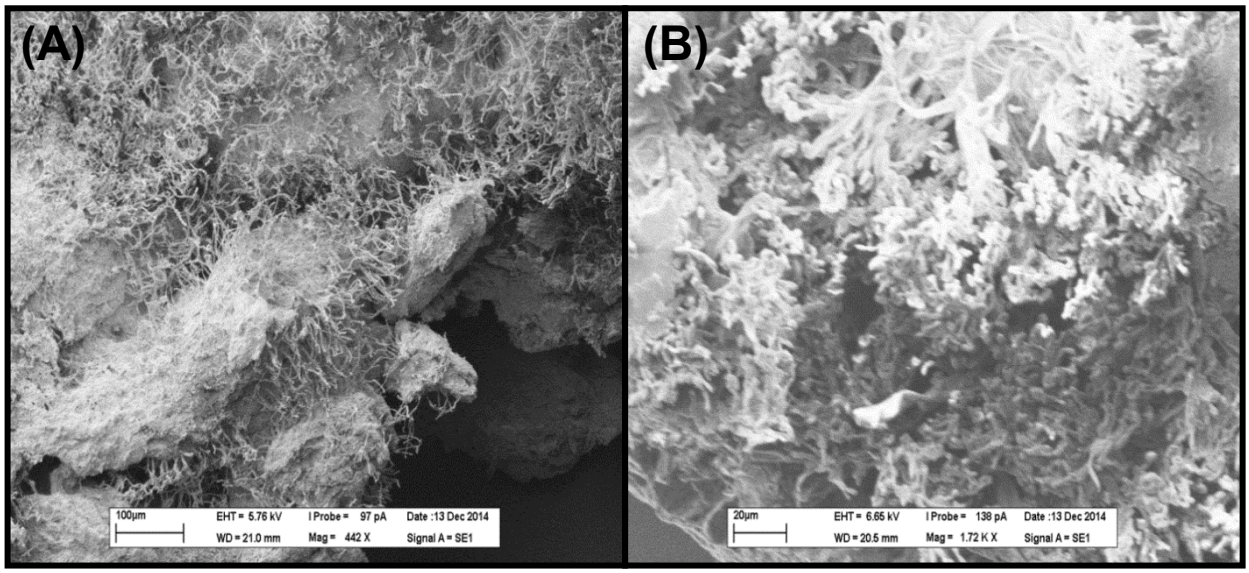

Figure 5.7: (A) SEM image of the mycelia of *R. oryzae* grown on paper solid waste (PPSW) particle during solid state fermentation **(B)** A close view of the mycelia.

END OF CHAPTER 5

CHAPTER 6

APPLICATION OF APPLE INDUSTRY LIQUID AND SOLID WASTES FOR FUMARIC ACID PRODUCTION THROUGH SUBMERGED AND SOLID-STATE FERMENTATIONS

PART I

A FERMENTATIVE APPROACH TOWARDS OPTIMIZING DIRECTED BIOSYNTHESIS OF FUMARIC ACID BY *Rhizopus oryzae* 1526 UTILIZING APPLE INDUSTRY WASTE BIOMASS

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Résumé

Cette recherche porte sur la bio production d'acide fumarique (AF) à partir de boues d'ultrafiltration de jus de pommes (en anglais: APUS) et de résidus solides résultant de la productionde jus de pommes (en anglais: AP). Le champignon filamenteux *Rhizopus oryzae* 1526 (*R. oryzae*) a été utilisé comme biocatalyseur et l'impact de sa morphologie sur la production de FA a été analysé en détails. Pour la fermentation submergée, nous avons observé que 40 g/L de solides totaux de APUS, qu'un pH de 6,0, 30 °C, qu'une vitesse d'agitation de 200 rpmet qu'un temps d'incubation de 72 heures soient des paramètres optimaux pour la production de FA de FA (25,2 ± 1,0 g/L, 0,350 g/(L.h)). La viscosité du bouillon (cP), la réduction des sucres résiduels (g/L) et de l'éthanol (g/L) produits en tant que sous-produits, ont également été analysés. Des plateaux en plastique ont été utilisés pour la fermentation à l'état solide sous un taux d'humidité et une période d'incubation optimisés. Près de 52 ± 3 g de FA par kg de poids sec AP ont été obtenus. Les changements dans la teneur phénolique totale (mg/g de poids sec de AP) ont été surveillés à intervalles réguliers. L'utilisation des APUS et des AP pour la bio-synthèse de FA, produit chimique à haute valeur ajoutée, par la souche fongique *R. oryzae* a permis de mettre en évidence l'importance du contrôle physiologique et morphologique des champignons sur la production de cet acide par fermentation.

Mots clés: Déchets solides de jus de pommes, boues d'ultrafiltration de jus de pommes, fermentation, morphologie, sucre réducteur, viscosité.

Abstract

The present research account deals with the bioproduction of fumaric acid (FA) from apple pomace ultrafiltration sludge (APUS) and apple pomace (AP) through fermentation. The filamentous fungus *Rhizopus oryzae* 1526 (*R. oryzae*) was used as a biocatalyst and its morphological impact on FA production was analyzed in detail. For submerged fermentation, 40 g/L of total solids concentration of APUS, pH 6.0, 30 ^{o}C, 200 rpm flask shaking speed and 72 h of incubation were found to be optimum for FA production (25.2 ± 1.0 g/L, 0.350 g/(L h)). Broth viscosity (cP), residual reducing sugar (g/L) and ethanol (g/L) produced as by-product, were also analyzed. Plastic trays were used for solid state fermentation and at optimized level of moisture and incubation period, 52 ± 2.67 g FA per kg dry weight of AP was obtained. Changes in the total phenolic content (mg/g dry weight of AP) were monitored at regular intervals. Utilization of APUS and AP for the directed synthesis of the high value platform chemical FA by the fungal strain *R. oryzae* was an excellent display of fungal physiological and morphological control over a fermentative product.

Keywords: Apple pomace**,** apple pomace ultrafiltration sludge**,** fermentation**,** morphology**,** reducing sugar**,** viscosity.

Introduction

The recent trend of safer production of high value platform chemicals through biological means, such as fermentation technology, has opened a new avenue for pertinent utilization of different agro-industrial waste biomass. Among those explored with this approach, fumaric acid (FA) stands as one of the frontliner. The worldwide demand for FA and its derivatives is growing each year (Goldberg *et al*. 2006). As per the report 2014, the global FA market demand was around 240,000 t and the projected market volume is 350,000 t by 2020 (www.grandviewresearch.com). Different fungal strains have been experimented for FA production from waste biomass and the obtained results exhibited variation in FA productivity (Moresi *et al*. 1991; Moresi *et al*. 1992; Woiciechowski *et al*. 1996; Carta *et al*. 1999; Xu *et al*. 2010; Das *et al*., 2014; Zhou *et al*. 2014; Das *et al*. 2015a, 2015b). To make biological FA production economically viable and compensate for the higher yield of petrochemical route (benzene to maleic acid through maleic anhydride and isomerization of maleic acid), search for new waste biomass with the potential of serving as a good source of carbon, micro and macro nutrients is actively going on (Roa Engel *et al*. 2008; Xu *et al*. 2012). In this regard, the waste by-products of apple industry can be a good choice for FA production through fermentation.

Apple (*Malus domestica* Borkh. syn. *Malus pumila* Mill.) based products, such as apple juice and cider are very good sources of phenolics and antioxidants (Candrawinata *et al*. 2014). In a typical apple juice making process, pressing of the apples and separation of the liquid leaves a solid residue commonly called as the apple pomace (AP). On an average, AP represents 20-35% of the original fruits (Carson *et al*. 1994; Suarez *et al*. 2010). Looking at the worldwide expanding market volume of apple production (76.37 million tons, www.faostat.fao.org), a large amount of AP is produced by the apple industries and safe disposal of it has become a challenging task. Physicochemical characterization of AP has shown it to be a very good source of nutrients for microorganisms (Gullon *et al*. 2007; Vendruscolo *et al*. 2008; Shalini & Gupta 2010; Dhillon *et al*. 2011). High water content (70-75% moisture) and easily fermentable forms of sugars make AP highly prone to microbial attack and thus unsafe for environmental disposal. Management of AP waste imposes substantial extra cost on the apple juice producing industries. Although, in some parts of the world, AP is considered as waste food resource and fed to animals, this is not the common practice in majority of apple producing countries. Alternatively, AP is dumped on open land area and this causes environmental pollution. With respect to the specific interest of the present study, apple production in Canada is considerably high. As per the Canadian

horticulture report published in 2014, the total apple production in 2013 was around 382 million kg and 28% of it was from the Quebec province itself (first was Ontario with 40%) (www.hortcouncil.ca). In Canada with high demand for apple based products, a large amount of AP biomass is generated and its safer disposal is being challenged by environmental concerns (Gasara *et al*. 2011a). Another apple industry waste by-product is the apple pomace ultrafiltration sludge (APUS) that originates after clarification of apple juice. APUS has also been previously characterized for its nutritional value details and shown to be a good source of carbon and other essential elements for microorganisms (Dhillon *et al*. 2011, 2012). Application of AP and APUS as substrates for solid state fermentation (SSF) and submerged fermentation (SmF) in many previous studies confirmed their suitability as carbon source for different microorganisms (Hang & Woodams 1986, 1994, 1995; Dhillon *et al*. 2011, 2012; Gassara *et al*. 2011b; Parmar & Rupasinghe 2013). The product profiles achieved with AP or APUS as substrate for different targeted products were excellent and comparable to synthetic media.

After decade old research on the compatibility of *Rhizopus* species for fermentative FA production from different carbon sources, the species viz. *Rhizopus oryzae*, *Rhizopus formosa*, *Rhizopus arrhizus* and *Rhizopus nigricans* were found to be good FA producers through fermentation (Kane & Amann 1943; Goldberg & Stieglitz 1986; Ling & Ng 1989; Roa Engel *et al*. 2008). However, due to simple nutrient requirements and higher productivity, the species *R. oryzae* is preferred over the other three species (Liao *et al*. 2008). After 1990s, R. oryzae has been the frontliner in the production of FA (Xu *et al*. 2012). To be more specific in strain selection, it is worth mentioning that among the different strains of *R. oryzae* tested for FA production, the strain NRRL 1526 is one of the best strains (Oda *et al*. 2003).

However, until this report, no prior study has been made for FA production using APUS and AP through fermentation employing the filamentous fungus *Rhizopus oryzae* 1526 (to be called *R. oryzae* thereafter). Optimization of SmF or SSF operating conditions for enhanced FA production is a challenging task with new substrate. Moreover, this FA producing fungal strain is well-known for its morphological display under various applied conditions that influence the fermentation process and thus product yields (Morin & Ward 1990; Liao *et al*. 2007, 2008; Zhou *et al*. 2011; Das & Brar 2014). Control over the formation of different morphological forms is an important and decisive step in fermentative FA production through SmF. The present study optimizes the operating conditions for SmF and SSF for FA production from APUS and AP.

Materials and methods

Materials

The fungal strain, *Rhizopus oryzae* 1526 was procured from Agricultural Research Services (ARS) culture collection, IL, USA. The apple industry waste biomass viz. apple pomace ultrafiltration sludge (APUS) and apple pomace (AP) with rice husk were procured from Lassonde Inc., Rougemont, Montreal, Canada. All the chemicals used in the present study were of analytical grade and purchased from Fisher Scientific (Ottawa, Ontario, Canada).

Methods

Culture and maintenance of *Rhizopus oryzae*

The procured fungal strain was first grown on potato dextrose agar (PDA) slant at 37 ± 1 ^{o}C for 4 d and then propagated on petri dishes with PDA. After an incubation period of 72 h at 37 ± 1 ^{o}C, PDA plates with fully grown and sporulated fungus were washed with distilled water (d.H_2O) and sporangiospores were collected by filtering through glass wool. Spore suspension was maintained at 1×10^8 spores/mL and stored at 4 ^{o}C for regular use. For long time storage, spore suspension was mixed with 20% glycerol and kept at 80 ^{o}C.

Preparation of pre-culture medium and inoculum

To grow the fungus for inoculum (pre-culture) preparation, glucose salts medium (g/L: 50 glucose, 2 urea, KH_2PO_4 0.6, $MgSO_4 \cdot 7H_2O$ 0.5, $ZnSO_4 \cdot 7H_2O$ 0.11 and $FeSO_4 \cdot 7H_2O$ 0.0088) was used. The medium was heat sterilized (20 min, 15 psi, 121 ± 1 ^{o}C) following the standard procedure to avoid Maillard reaction between the carbonyl group (>C=O) of glucose and amino group (-NH_2) of urea. Pre-culture inoculum of *Rhizopus oryzae* 1526 was prepared as mentioned before following the method of Das *et al.* (2014). Briefly, Erlenmeyer flasks containing 50 mL of sterilized pre-culture medium were aseptically inoculated with the sporangiospores (2%, v/v) and incubated at 30 ^{o}C and 200 rpm for 24 h.

Submerged fermentation

To prepare for SmF, Erlenmeyer flasks containing 142.1 mL of sterilized APUS were inoculated with pre-cultured cell pellets (5 %, v/v, i.e. 7.9 mL) and incubated at 25 ^{o}C, in a rotary shaker at 200 rpm for 72 h. As neutralizing agent, sterilized calcium carbonate ($CaCO_3$) was used at 50 g/L during SmF. The original pH (2.8) of APUS was adjusted to 6.0 before SmF. Downstream processing of the fermented broth samples were treated following

the method of Dang *et al*. (2009). Briefly, samples were treated with simultaneous heating and acidification (90 ^{o}C, 5 N H_2SO_4) until clear and later centrifuged (8000 × g, 10 min, 20 ^{o}C) and supernatants were collected. FA concentration (g/L) in the supernatants was estimated using HPLC. For estimation of biomass dry weight of *Rhizopus oryzae*, mycelia were collected by centrifugation, washed with copious amount of d.H_2O and oven dried at 60 ± 1 ^{o}C until a constant weight was obtained. All biomass dry weight was expressed in g/L.

Solid state fermentation

Plastic trays (length × breadth × height = 35 cm × 22 cm × 11 cm) were used as bioreactors for SSF. Oven dried AP was moisture adjusted (w/w) with d.H_2O, heat sterilized (20 min, 15 psi, 121 ± 1 ^{o}C) and cooled before aseptically inoculated with sporangiospores of *Rhizopus oryzae* 1526. Around 250 g of dried AP was taken for each bioreactor experiment and spore concentration was maintained at 1 × 10^7 spores/g dry weight of AP. Plastic trays were incubated in a static incubator at 30 ± 1 ^{o}C for a maximum of 21 days (d). After 72 h of incubation, 20 g of sterilized $CaCO_3$ was added to AP. The moisture content of AP was maintained by adding required amount of sterilized d.H_2O. For FA estimation, 5 g of fermented AP was collected from each tray at 5 d, 7 d, 9 d, 12 d, 14 d, 18 d, and 21 d of incubation period under aseptic conditions. Collected samples were mixed with sterilized d.H_2O at 1:10 solid to liquid ratio and then kept in a shaking incubator at 200 rpm at 30 ^{o}C for 1 h. The samples were then filtered through glass wool for removal of solid residues and fungal mycelia. Downstream processing of the filtrates was carried out following same steps as for SmF samples and FA concentration was estimated using HPLC

Process optimization

For enhanced production of FA, the following parameters were studied for SmF and SSF.

(A) For SmF: Four different important parameters *viz*. total solids concentration (10-50 g/L) of APUS, pH of APUS (6, 7, and 8) flask shaking speed (100, 200, and 300 rpm) during SmF and incubation temperature of SmF (25 ^{o}C, 30 ^{o}C, and 37 ^{o}C) were optimized.

(B) For SSF: The moisture content (40, 50, and 60 %) of AP and incubation time of SSF (5 d, 7 d, 10 d, 12 d, and 14 d) was optimized.

Analytical techniques

For FA estimation, broth samples were prepared following the method of Zhou *et al*. (2000) and later quantified by HPLC. The specification of HPLC was as follows: System: DIONEX DX500, with an Acclaim OA, 5 mm, (4.6 × 150 mm) column with a refractive index detector (PDA-100 DIONEX, UV, 210 nm). The mobile phase was 2.5 mM methanesulfonic acid (CH_3SO_3H) at a flow rate of 1 mL min1 and column temperature of 30 ^oC. The quantification of ethanol in the SmF broth samples was carried out using gas chromatography (GC) (7890B, GC-Agilent Technologies, Santa Clara, CA, USA) equipped with flame ionization (detector) FID. The column specification was ZB-WAX plus column (30 m × 0.25 mm, 0.25 mm film thickness). Helium was used as carrier gas and flow rate was maintained at 1 mL/min with total flow duration of 16 min. The initial oven temperature was set at 35 ^oC later increased to 85 ^oC at 10 ^oC/min and to 200 ^oC at 25 ^oC/min. The amount of total reducing sugar of APUS and fermented broth samples were estimated by dinitrosalicylic acid method using glucose as standard. APUS and broth samples were centrifuged (9000 × g, 10 min and 25 ^oC) and the collected supernatants were paper filtered (Whatman#44). Finally, filtrates were syringe-filtered (0.45 mm) and analyzed for total reducing sugar. The estimation of total solids concentration of APUS was calculated following standard procedure (APHA, AWWA, and WPCF 2005) and expressed in g/L. A rotational viscometer (Fungilab, Premium Series, NY, USA) was used for the viscosity measurement of the SmF broth samples. L1 spindle was used with a sample cup volume of 30 mL. The analysis of moisture content of SSF samples was analyzed by oven-dry method (Reeb & Milota 1999). About 5 g of SSF sample was allowed to dry at 60 ± 1 ^oC, until a constant weight was achieved. The moisture content in AP was calculated as per the following formula:

$$\mathrm{MC} = \frac{\text{Initial weight} - \text{Oven dry weight}}{\text{Initial weight}} \times 100 \qquad (1)$$

The phenolic content in the SSF samples were extracted following the method of Schmidt *et al*. (2014) with required modification. Briefly, 5 g of SSF sample was mixed with 30 mL of methanol and kept under shaking condition for 1 h at 200 rpm and 25 ± 1 ^oC. The sample was then filtered (Whatman#4) and the filtrate was washed with copious amount of hexane until the impurities (fatty acid, waxes etc.) are removed. The methanolic extract was subjected to rota-evaporation (BUCHI, Switzerland) at 45 ± 1 ^oC under reduced pressure and the concentrated extracted was resuspended in 10 mL of sterilized d.H_2O and kept in an ultrasonication bath for 15 min. The sample was then clarified by adding 5mL each of 0.1 M

$ZnSO_4$ and 0.1 M $Ba(OH)_2$. The mixture was allowed to stand for 30 min and then centrifuged (15 min, 20 ± 1 ^oC, 4000 × g). The collected supernatants were lyophilized (ScanVac CoolSafe Freeze Drying) and its phenolic content was estimated by Foline-Ciocalteau method. Gallic acid was used to set up the standard curve and OD were measured at λ_{max} =765 nm using a 96 well plate reader (BioTek Instruments, Epoch, USA). The morphological studies of *Rhizopus oryzae* 1526 were performed using digital photography (Canon PC 1585) and scanning electron microscopy (SEM, Carl Zeiss EVO 50). To prepare for SEM, SSF samples were directly mounted on SEM grids and sputter coated (SPI Module Sputter Coater) with gold for 120 s under vacuum conditions and later analyzed.

Statistical analysis

Data are represented as mean ± SD of three independent experiments. Correlations were considered significant at $P < 0.05$ for different applied parameters.

Results and discussion

Submerged fermentation and process optimization

The results of the SmF studies under different applied parameters confirmed the production of FA by *Rhizopus oryzae* 1526 from APUS. The fungus exhibited good growth and metabolic activities upon consumption of nutrients present in APUS. Previously, our laboratory carried out experiments on the potential application of APUS as SmF substrate for the production of high value chemicals, such as citric acid (Dhillon *et al*. 2011, 2012). Physico-chemical characterization of APUS showed the presence of high carbon content and other essential micro/macro nutrients for many microorganisms (Dhillon *et al*. 2012). Thus, selection of APUS as SmF substrate for FA production was pertinent.

To find out the maximum total solid concentration of APUS that could be utilized for FA production, a total solid concentration range of 10-50 g/L was applied and observed for changes in FA production and biomass dry weight. The results are shown in Figure 6.1.1. With the change in total solid concentration from 10 to 40 g/L, production level of FA increased from 1.5 ± 0.15 to 13.6 ± 0.45 g/L in 72 h of SmF at 25 ^oC, 200 rpm and SmF pH of 6 with a volumetric productivity of 0.188 g/(L h). The biomass dry weight also increased from 1.38 ± 0.2 to 7.85 ± 0.96 g/L. At 45 g/L of total solid concentration, production of FA considerably decreased to 4.5 ± 0.3 g/L, while biomass dry weight continued rising to 9.7 ±

0.9 g/L. Further increase in total solid concentration resulted in fungal growth but not FA production. The correlations between total solid concentration vs FA and total solid concentration vs biomass dry weight led to the conclusion that 40 g/L of total solid concentration was optimum for FA production. Substrate inhibition at total solid concentration > 40 g/L can be attributed to the lower production of FA. Previous studies on this aspect exhibited similar correlations between total solid concentration of SmF substrate, FA production and biomass dry weight of the used fungal strains (Xu *et al*. 2010; Das *et al*., 2014).

Apart from total solid concentration, other applied parameters (pH, flask shaking speed and SmF temperature) also influenced FA production. As shown in Figure 6.1.2, changes in the SmF temperature caused variation in the morphological forms of the fungus. As compared to the large pellets (3-5 mm diameter) obtained at 25 ^{o}C, small pellets (1-2 mm diameter) were formed at 30 ^{o}C (Figures 2 (A) (B)). At 37 ^{o}C, the fungus formed suspended mycelia (Figure 2 (C)). Previous study on the effect of temperature on the morphology of *Aspergillus awamori* in fermentation cultures supported the findings of the present investigation (Schugerl *et al*. 1998). The fungus *A. awamori* formed pellet at 25^{o}C-30 ^{o}C and suspended mycelia at 35 ^{o}C fermentation temperature range. The authors proposed that inadequate supply of oxygen to fungal pellets at higher temperature caused transformation into suspended mycelia. Braun & Vecht-Lifshitz (1991) have reviewed these aspects and concluded that lowering of fermentation temperature can induce pellet morphology in filamentous fungi. As it can be seen in Figure 6.1.3, the changes in SmF pH also triggered different morphological display of *R. oryzae*. At pH 6, small pellets (1-2 mm diameter) were formed (Figure 6.1.3 (A)), while pH 7 and 8 resulted in dense suspended mycelia and large pellets (6-10 mm diameter) (Figures 6.1.3 (B) (C), respectively. SmF medium pH affects the cell wall structure and surface properties of fungal hyphae and causes morphological changes (Pirt *et al*., 1959; Metz *et al*., 1977). Moreover, medium pH favouring pellet or suspended mycelia formation might be specific for a particular fungal strain and SmF product (Galbraith *et al*., 1969; Carsen *et al*. 1994; Das *et al*., 2014). The flask shaking speed of SmF was changed from 200 to 100 rpm and 200-300 rpm and observed for any change in morphological display of the fungus. The morphological responses of the fungus to different flask shaking speeds are shown in Figure 6.1.4. Dense suspended mycelia and small pellets (1-2 mm diameter) were formed at 100 and 200 rpm, respectively (Figures 6.1.4 (A) (B)). Increase in flask shaking speed from 200 to 300 rpm resulted in mixed morphology of large pellets (3-4 mm diameter) and mycelia (Figures 6.1.4 (C)). The morphological responses to different flask shaking speeds can be attributed to the

agglomeration of pre-cultured pellets, hydrodynamic effect on the fungal hyphae and dissipation of specific free energy during shaking. At 100 rpm flask speed, the pre-cultured pellets inoculum did not separate from each other (agglomeration) and further incubation resulted in dense suspended mycelia (Das *et al*., 2014). Previously, it was proposed that the hydrodynamic force generated by the flask shaking can shave off the outer zone of fungal pellets and flask speed determines the severity of shaving. The shaved off hyphae are reseeded into loose mycelia and if continuously exposed to hydrodynamic force, these loose mycelia start forming hyphal vacuoles which lowers the hyphal activity (Cui *et al*. 1998). Moreover, the fungal mean hyphal length was found to proportionally (with an exponent of 0.25) change to the specific energy dissipation rate during shaking (Dion *et al*., 1959; Ayazi Shamplou *et al*. 1994; Cui *et al*. 1997; Teng *et al*. 2009). Thus, higher flask shaking speed (300 rpm) increased the specific free energy dissipation rate and resulted in shorter hyphae and less compact mycelium. The moderate flask shaking speed (200 rpm) did not cause agglomeration or shaving off the hyphae and helped in the formation of small pellet for enhanced FA production.

The changes in the morphological forms of the fungus under different applied parameters caused variation in the production profile of FA as presented in Figure 6.1.5. The maximum FA production achieved at 30 ^{o}C was 27.5 ± 1.54 g/L and it decreased to 10.85 ± 0.58 g/L at 37 ^{o}C (Figure 6.1.5 (A)). The volumetric productivities at 30 and 37 ^{o}C were 0.381 and 0.146 g/(L h) respectively. Fungal morphology plays a decisive role in the fermented FA production. Pellet morphology is more supportive than suspended mycelia for easy mass, oxygen and heat transfer within the broth (Li *et al*. 2000; Rodriguez Porcel *et al*. 2005; Zhou *et al*. 2011). As FA production is an aerobic process, small pellets do not suffer from hypoxic condition in their core zone and it affects the overall metabolic activity of the fungus (Papagianni, 2004; Xu *et al*. 2012). Thus, 30 ^{o}C SmF temperatures that resulted in small pellets (1-2 mm diameter) favoured higher FA production as compared to the large pellets (3-5 mm diameter) and suspended mycelia formed at 25 and 37 ^{o}C respectively. For different applied SmF media pH, production of FA reached upto 26.22 ± 2.5 g/L at pH 6 with a volumetric productivity of 0.364 g/(L h) (Figure 6.1.5 (B)). This FA concentration and volumetric productivity were approximately the same that was obtained with optimized total solid concentration (40 g/L) and SmF incubation temperature (30 ^{o}C). However, FA production considerably decreased for pH 7 and pH 8. Only 7.7 ± 1.3 g/L and 5.6 ± 0.75 g/L of FA were produced at pH 7 and pH 8, respectively (Figure 6.1.5 (B)). The lower FA production can be the resultant outcome of morphological influence on SmF performance and pH tolerance of *R. oryzae*. It is of general acceptance that as compared to small fungal

pellets, large fungal pellets have higher oxygen mass transfer resistance through the boundary layer on the liquid side of the gas-liquid interface and this causes insufficient oxygen and nutrient transport supply into the core zone of the large pellets and finally lead to lower efficiency and productivity of the fungus (Zhou 1999; Roa Engel *et al*. 2008). Thus, at pH 8, larger pellets (6-10 mm diameter) produced less FA acid as compared to the small pellets (1-2 mm diameter) formed at pH 6. Moreover, fungal dense suspended mycelia or clumps usually suffer from hypoxic conditions that result in low levels of product formation and similar situation might have caused the lower FA production by the dense suspended mycelia formed at pH 6 (Xu *et al*. 2012). The maximum FA production achieved at 100 rpm flask shaking speed was 18.6 ± 1.3 g/L in 84 h of SmF (Figure 6.1.5 (C)). The SmF time increased from 72 h to 84 h for FA production and thus volumetric productivity was lowered (0.221 g/(L h)). Increase in flask shaking speed from 200 to 300 rpm lowered FA concentration to 14.6 ± 1.2 g/L (Figure 6.1.5 (C)). Under all optimized conditions (40 g/L total solid concentration, 30 ^{o}C and pH 6 and 200 rpm flask shaking speed), SmF reproduced small pellets and the FA concentration achieved was 25.2 ± 1.0 g/L (Figure 6.1.5 (D)) with a volumetric productivity of 0.350 g/(L h).

Ethanol as by-product of SmF

Ethanol was detected as by-product in the broth samples. The variation in ethanol production under different SmF conditions is shown in Figure 6.1.6. Under all optimized conditions (40 g/L total solid concentration, 30 ^{o}C, 200 rpm and pH 6), production of ethanol was 2.8 ± 0.5 g/L in 60 h of SmF (Figure 6.1.6 (A)). These SmF conditions favoured aerobic process such as FA production (24.3 ± 1.6 g/L) rather than anaerobic (ethanol production) and indicated that oxygen and nutrient supply to fungal biomass were sufficient. Small pellets were reproduced under these SmF conditions that favoured more FA production. Changes in SmF temperature, pH and flask shaking speeds caused variation in ethanol concentrations. Under SmF conditions of 45 g/L total solid concentration, 37 ^{o}C, pH 8 and 300 rpm, the ethanol production increased to 8.6 ± 0.75 g/L within 48 h of SmF (Figure 6.1.6 (B)). FA concentration reached 7.8 ± 0.8 g/L in 84 h of SmF time. The condition resulted in the formation of suspended mycelia that caused limitation in oxygen supply to fungal biomass. As discussed earlier, all these applied parameters when experimented individually, did not support the production of FA. The obvious reasons for higher ethanol production under these SmF conditions were: (a) being an aerobic process, oxygen was crucial for FA and hypoxic condition caused metabolic shift to anaerobic process, such as ethanol production; and (b) SmF parameters that support the formation of fungal suspended mycelia in the broth caused oxygen depletion and lowered the production of FA (Gu *et al*. 2013).

These results also suggested that the optimum SmF conditions for ethanol production using *R. oryzae* are not supportive of FA production.

Conversion of APUS into FA

To find out the real carbon source for *Rhizopus oryzae*, estimation of total reducing sugar (g/L) was performed before and after the SmF. The experiment was carried out under optimized SmF conditions (40 g/L total solid concentration, 30 ^{o}C, 200 rpm, pH 6, and 72 h). The variations in total reducing sugar and FA concentration during SmF are shown in Figure 6.1.7. It was interesting to observe that the fungus utilized the initially available total reducing sugar (11 g/L) in the first 36 h of SmF with a respective FA concentration of 8.9 ± 0.95 g/L. At 36 h, the residual total reducing sugar was 1.4 ± 0.15 g/L. After 36 h, there was an increase in total reducing sugar from 1.4 ± 0.15 to 3.4 ± 0.25 g/L upto 60 h of SmF and FA production reached upto 18.3 ± 0.43 g/L. After 72 h of SmF, total reducing sugar decreased to 0.25 ± 0.015 g/L and FA production was 24.6 ± 0.5 g/L. The rise in total reducing sugar concentration after 36 h of SmF indicated the bioconversion of the APUS solids components rich in cellulose and hemicellulose into fermentable sugars. As *R. oryzae* strains are well-known for producing intracellular and extracellular cellulolytic, hemicellulolytic, pectinolytic and amylolytic enzymes; conversion of APUS into sugars was quite feasible (Ghosh *et al*., 2011). Although estimation of residual total solid concentration of SmF broth samples was attempted, still the unused $CaCO_3$ that precipitated along with the residual solids of APUS unavoidably interfered in the analysis of residual total reducing sugar.

Viscosity of fermented broth samples

Measurement of viscosities of the broth samples obtained under different applied SmF conditions suggested supportive and non-supportive broth conditions for enhanced FA production along with definitive morphological impacts on viscosity. Viscosities of the fermented broth samples obtained under different SmF conditions of FA production are presented in Table 6.1.1. The initial viscosities of the SmF samples at 0 h were around 45 cP and the final viscosities at 72 h were found to be affected by the applied SmF conditions. Under all optimized conditions (40 g/L total reducing sugar, 30 ^{o}C, 200 rpm and pH 6), the final viscosity increased from 45.5 ± 1.5 to 82.8 ± 2.3 cP. The maximum viscosity of 215 ± 2.8 cP was reached under SmF conditions of 40 g/L total reducing sugar, 30 ^{o}C, 100 rpm and pH 6. Overall, higher viscosities were recorded in the broth samples either with suspended mycelia or large pellets. It is well-known that fermentation broth rheology can play a decisive role in the overall performance of the fermentation process (Papagianni,

2004). Moreover, fungal morphology can affect the broth viscosity that finally influences the fermentative production, especially the aerobic processes, such as FA production (Li *et al*. 2000; Rodriguez Porcel *et al*. 2005). In the present study, relative higher production of FA was achieved with the small pellet as compared the suspended mycelia or large pellets and this was in accord with the general acceptance of broth rheology vs fungal morphology correlations.

Solid state fermentation and process optimization

In the present study, potential of AP as solid substrate for FA production was experimented through SSF. AP is rich source of carbon and essential micro and macro nutrients for different microorganisms. It has been utilized for the production of different high value chemicals through fermentation process (Dhillon *et al*. 2011; Vastrad *et al*., 2011; Parmar *et al*., 2013).

The SSF carried out with AP in the plastic trays resulted in FA production. The SSF conditions comprised: 30 ± 1 ^{o}C, 50% moisture content (w/w) for 21 d. The production profile of FA is shown in Figure 6.1.8. After 5 d of incubation, the FA concentration reached 7.5 ± 0.87 g/kg dry weight AP. The enhancement in FA production occurred from 5 to 14 d of incubation. At the end of 14 d, the maximum FA production achieved was 52 ± 2.1 g/kg. The SSF experiment was continued up to 21 d and monitored for FA production. However, from 14 to 21 d SSF time, concentration of FA did not increase further. The SSF experiments carried out with 40% and 60 % moisture content did not give higher productivity of FA. At 40 % moisture content, the maximum FA production achieved was 23.44 ± 2.4 g/kg at 20 d of incubation. Lower moisture content in SSF substrate can cause nutrient transport problem and also lower the microbial enzyme activities that inhibits substrate to product conversion process (Moo-Young *et al*. 1983; Lonsane *et al*. 1985). This might have delayed the FA production with an extra SSF time of 6 d as compared to 50% moisture content. The FA production achieved with 60 % moisture content was around 38.54 ± 2.8 g/kg of AP at the end of 14 d of SmF. Although it was difficult to pinpoint the exact reason for lower FA production at 60%, but the most probable reasons were the partial or complete hypoxic condition developed in the surroundings of fungal mycelia grown on the AP and agglomeration of AP particles (Gowthaman, 2001). Thus, a moisture content of 50 % (w/w) and 14 d of incubation time were found to be the optimum conditions for enhanced FA production for the present study.

SEM analysis of the SSF sample (14 d) showed the growth of the mycelia of Rhizopus oryzae on the AP particle surface (Figures 6.1.9 (A) (B)). The fungal hyphae grew inside the AP particles and acquired nutrition for FA production.

Phenolic content in SSF samples

AP is known to have high content of phenolic content and methanol is the best solvent for its extraction (Candrawinata *et al*. 2014). SSF carried out at the optimum conditions (50% moisture content, 30 ^{o}C and 14 d) were subjected to phenolic content analysis and the changes in its concentration is shown in Figure 6.1.10. In SSF perspective, bioconversion of lignin into phenols by filamentous fungus is an indication of utilization and biodegradation of a solid substrate containing lignin in its structure (Schmidt *et al*. 2014). The AP used in this study was shown to have high (~23%, w/w) lignin content (Dhillon *et al*. 2011). The phenolic content of AP before SSF was found to be around 1.35 ± 0.15 mg/g dry weight. More phenolic content was detected in the fermented samples as SmF time increased. At the end of 7 d, the phenolic content was around 1.85 ± 0.12 mg/g. It further increased to 2.2 ± 0.18 mg/g at the end of 9 d. The highest phenolic content was found to be around 2.45 ± 0.19 mg/g at the end of 14 d of SSF. The 81.5% enhancement in the phenolic content of fermented AP after SSF strongly suggested the biodegradation of the polysaccharide (cellulose and hemicellulose) bound lignin by *Rhizopus oryzae* for acquiring nutrients (Schmidt *et al*. 2014).

Potential of APUS and AP biomass for FA production

Different waste biomasses have been successfully exploited for FA production with higher productivity. These efforts well supported the concept of carbohydrate-economy and biorefinery for FA production. The representative examples of different waste carbon sources used and the respective FA product profiles obtained have been summarized in Table 6.1.2. The FA productivity (25.2 g/L) obtained in the present study with APUS was in the same range as obtained with some other waste carbon sources through SmF (Carta *et al*. 1999; Xu *et al*. 2010; Zhou *et al*. 2014). For AP, the FA concentration of 52 g/kg AP was higher than all other waste carbon sources used so far, except for corn starch (71.9 g/L) (Moresi *et al*. 1992). The productivity comparison suggested APUS and AP to be very good waste carbon sources for the utilization of FA producing fungal strains.

Conclusions

The present study confirmed the fermentative production of fumaric acid by the fungus, *Rhizopus oryzae* 1526 from apple pomace and apple pomace ultrafiltration sludge. Under the optimized submerged fermentation conditions (40 g/L total solids concentration, pH 6, 30 ^{o}C, 200 rpm and 72 h) the fumaric acid concentration reached 25.2 ± 1.0 g/L with a volumetric productivity of 0.350 g/(L h). Small sized fungal pellets favoured higher fumaric acid production as compared to large sized fungal pellets. Reducing sugar and solid content present in apple pomace ultrafiltration sludge was converted to fumaric acid by the fungus during the submerged fermentation. For solid state fermentation, 52 ± 2.1 g fumaric acid per kg dry weight of apple pomace (with 50% moisture content) was obtained after 14 d of incubation. Estimation of total phenol of apple pomace before and after fermentation indicated biodegradation and utilization of apple pomace by the fungus. Scanning electron microscopy analysis confirmed hyphal growth inside the apple pomace.

Abbreviations

FA = fumaric acid, AP= apple pomace, APUS = apple pomace ultrafiltration sludge, SmF = submerged fermentation, SSF = solid state fermentation

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Table 6.1.1: Viscosities of the fermented broth samples obtained under different submerged fermentation conditions of fumaric acid production.

SmF conditions	Initial viscosity (cP) (0 h)	Final viscosity (cP) (72 h)
40 g/L TSC, 30 ^{o}C, 200 rpm and pH 6	45.5 ± 1.5	82.8 ± 2.3
40 g/L TSC, 25 ^{o}C, 200 rpm and pH 6	44.8 ± 1.6	95.77 ± 1.2
40 g/L TSC, 37 ^{o}C, 200 rpm and pH 6	45.3 ± 0.95	137.8 ± 2.7
40 g/L TSC, 30 ^{o}C, 200 rpm and pH 7	44.5 ± 1.34	126.7 ± 1.8
40 g/L TSC, 30 ^{o}C, 200 rpm and pH 8	45.2 ± 1.1.5	90.3 ± 2.1
40 g/L TSC, 30 ^{o}C, 100 rpm and pH 6	45.8 ± 1.5	215 ± 2.8
40 g/L TSC, 30 ^{o}C, 300 rpm and pH 6	44.74 ± 1.0	180 ± 2.5

Table 6.1.2: Production profile of fumaric acid obtained with different waste biomass.

Carbon source	*Rhizopus* species used	Fermenter	FA (g/L)	FA productivity (g/(L h))	Yield (g/g)	References
Xylose	*Rhizopus nigricans*	Shake flask	15.3	0.07	0.23	Kautola and Linko, 1989
Potato flour	*Rhizopus nigricans*	Shake flask	43.5	0.42	0.58	Moresi *et al*. 1991
Corn starch	*Rhizopus oryzae*	Shake flask	71.9	0.50	0.60	Moresi *et al*. 1992
Molasses	*Rhizopus nigricans*	Fluidized bed	17.5	0.36	0.36	Petruccioli *et al*. 1996
Cassava bagasse	*Rhizopus formosa*	Shake flask	21.3	NM	0.34	Carta *et al*. 1999
Wood chips hydrolysate	*Rhizopus arrhizus*	Shake flask	5.085	NM	0.089	Woiciechowski et al. 2001
Dairy manure	*Rhizopus oryzae*	Stirred tank	31.0	0.322	0.31	Liao *et al*. 2008
Corn straw	*Rhizopus oryzae* ME-F12 (mutant)	Shake flask	27.79	0.33	0.35	Xu *et al*. 2010
Crude glycerol	*Rhizopus arrhizus* RH-07-13 (mutant)	Shake flask	22.81	0.158	0.346	Zhou *et al*. 2014
Brewery wastewater	*Rhizopus oryzae 1526*	Shake flask	31.3, 43.67, 47.22	0.434, 1.21, 1.675	NM	Das *et al*. 2014, 2015a and 2015b
Apple pomace ultrafiltration sludge	*Rhizopus oryzae 1526*	Shake flask	25.2	0.350	NM	Present study
Apple pomace	*Rhizopus oryzae 1526*	Plastic trays	52/kg apple pomace	NA	0.052	Present study

Abbreviations: FA = fumaric acid, NM= not mentioned, NA= not applicable

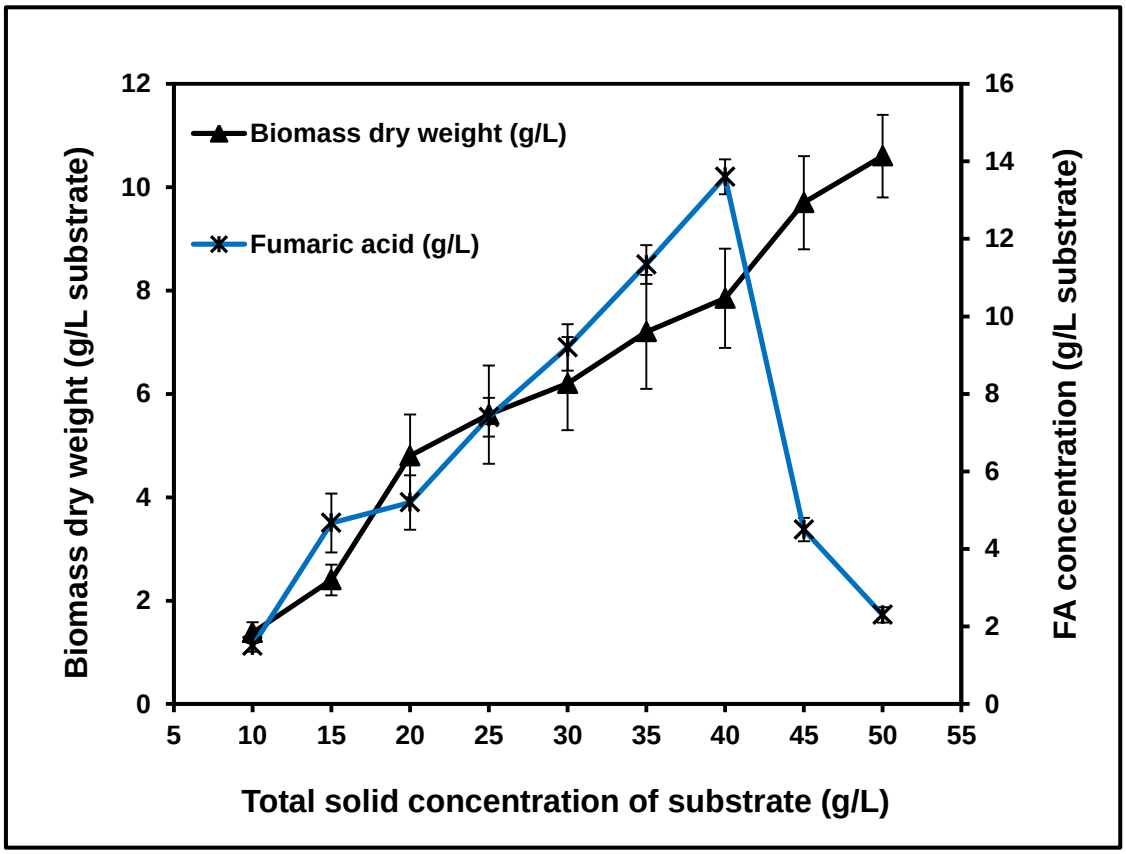

Figure 6.1.1: Changes in the biomass dry weight (g/L) of *R. oryzae* and fumaric acid concentration (g/L) with variation in the total solids concentration (g/L) of apple pomace ultrafiltration sludge.

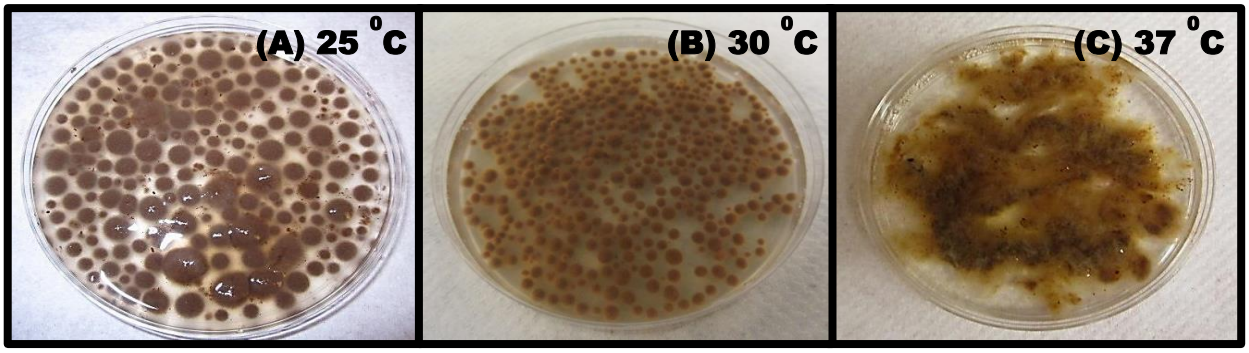

Figure 6.1.2: Representative digital images of the morphological forms of *R. oryzae* obtained at **(A)** 25 ^{o}C **(B)** 30 ^{o}C, and **(C)** 37 ^{o}C of submerged fermentation with apple pomace ultrafiltration sludge.

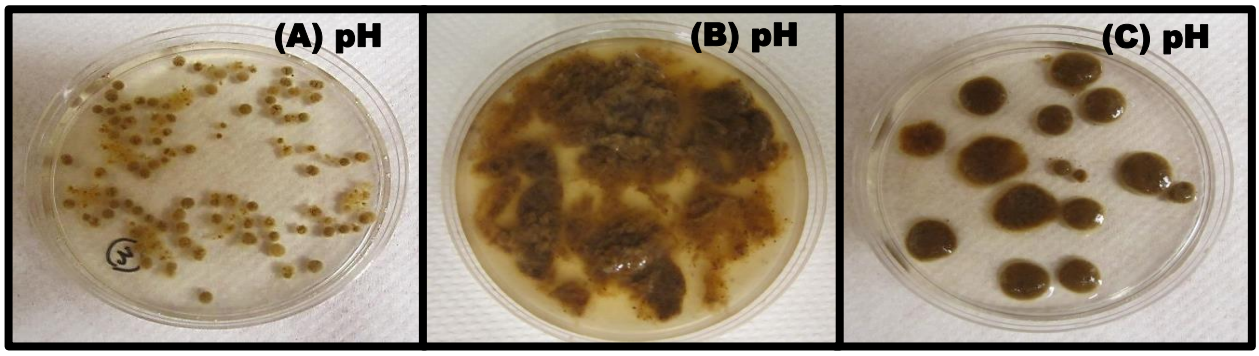

Figure 6.1.3: Representative digital images of the morphological forms of *R. oryzae* obtained at **(A)** pH 6 **(B)** pH 7, and **(C)** pH 8 of apple pomace ultrafiltration sludge during submerged fermentation with *R. oryzae*.

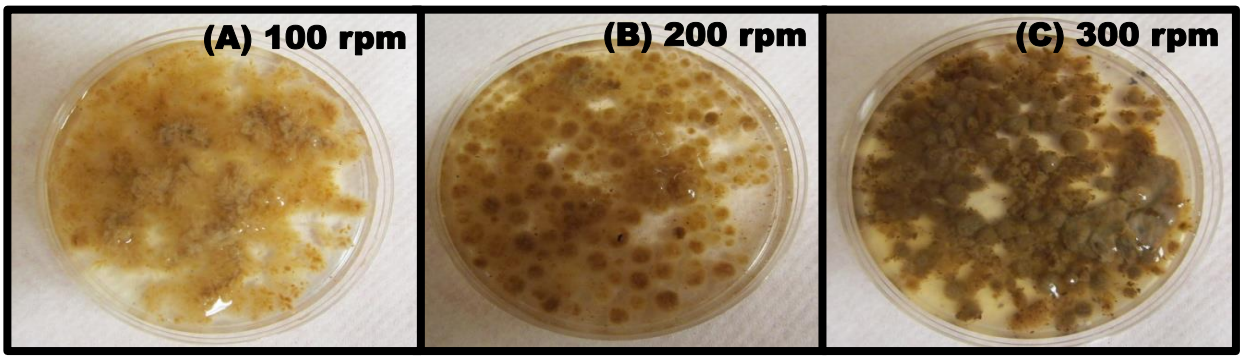

Figure 6.1.4: Representative digital images of the morphological forms of *R. oryzae* obtained at **(A)** 100 rpm **(B)** 200 rpm, and **(C)** 300 rpm flask shaking speed during submerged fermentation with apple pomace ultrafiltration sludge and *R. oryzae*.

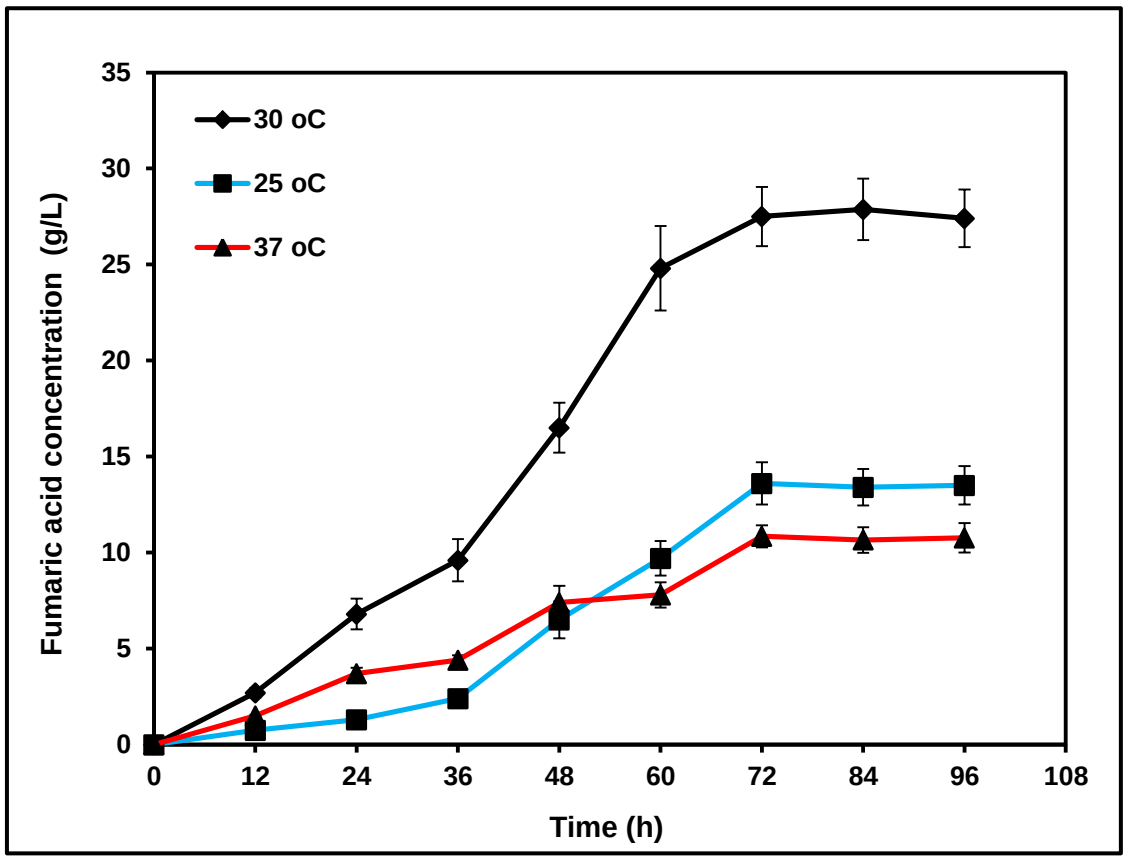

Figure 6.1.5 (A): Changes in the production profile of fumaric acid with variation in temperature of submerged fermentation with apple pomace ultrafiltration sludge and *R. oryzae*.

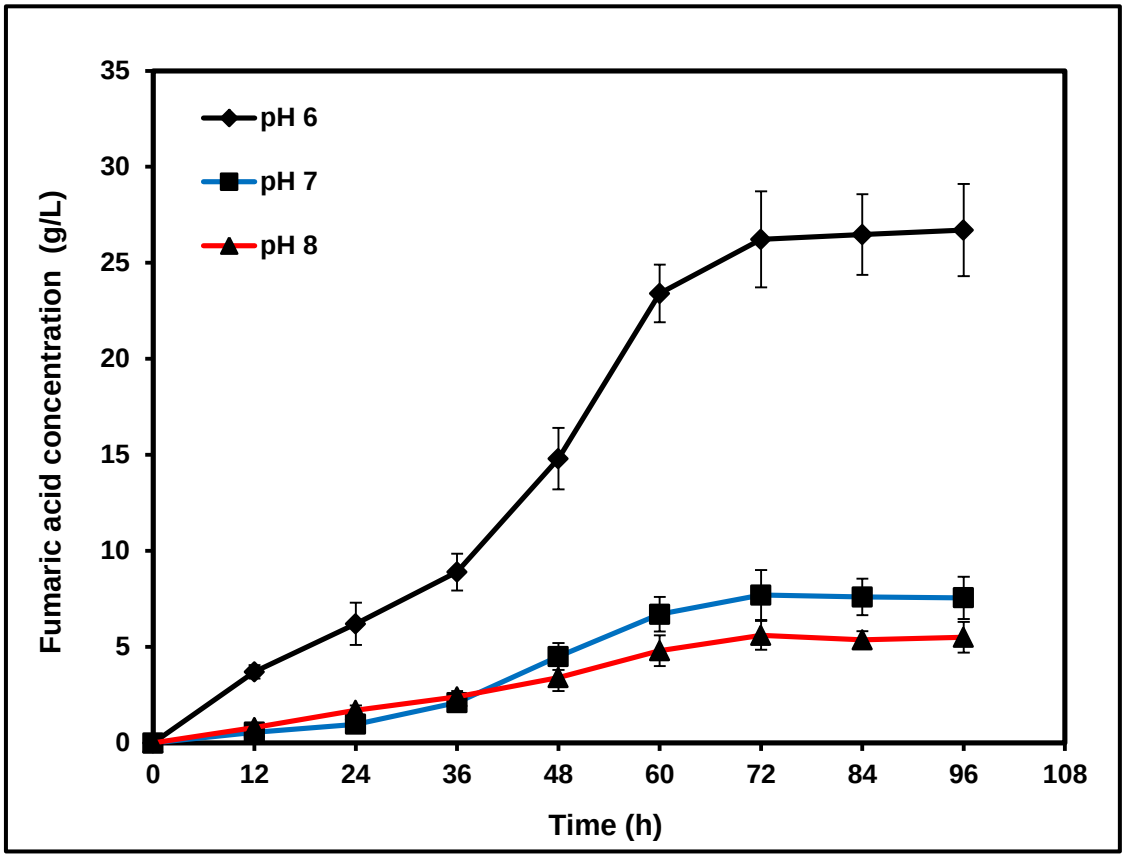

Figure 6.1.5 (B): Changes in the production profile of fumaric acid with variation in pH of submerged fermentation with apple pomace ultrafiltration sludge and *R. oryzae*.

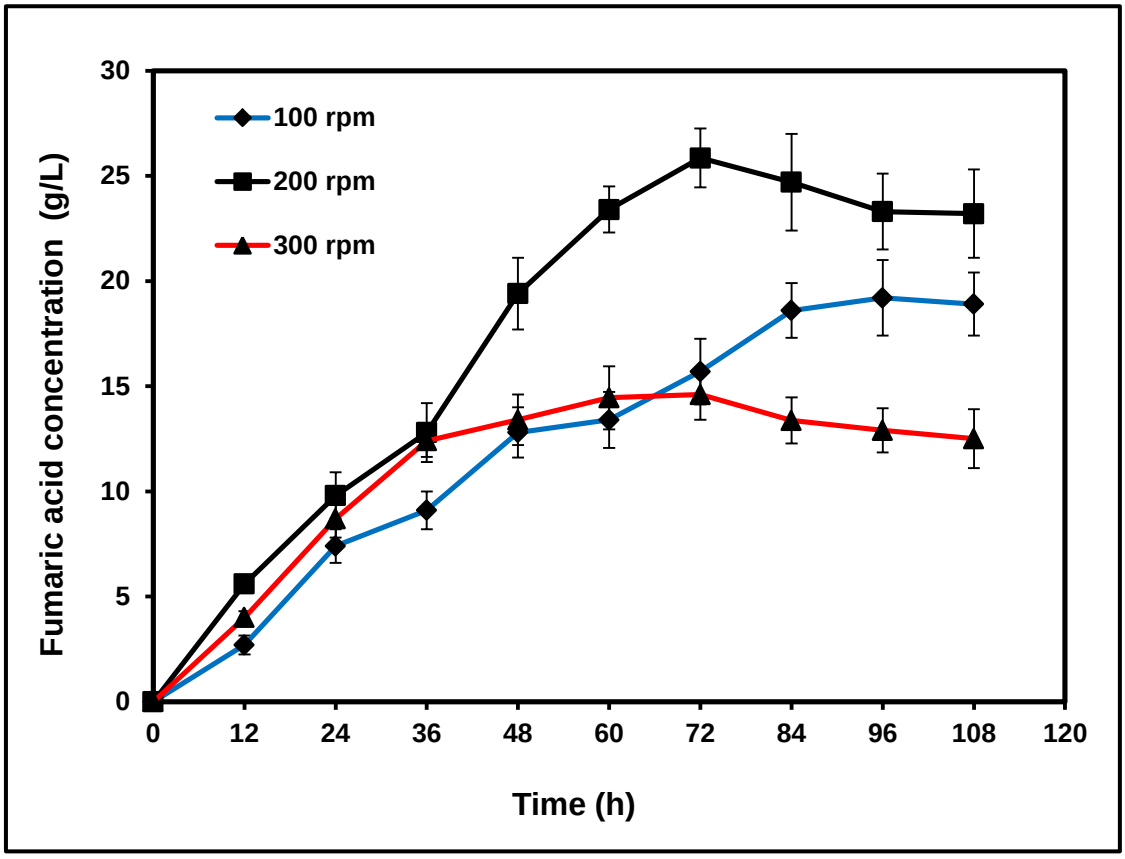

Figure 6.1.5 (C): Changes in the production profile of fumaric acid with variation in flask shaking speed of submerged fermentation with apple pomace ultrafiltration sludge and *R. oryzae*.

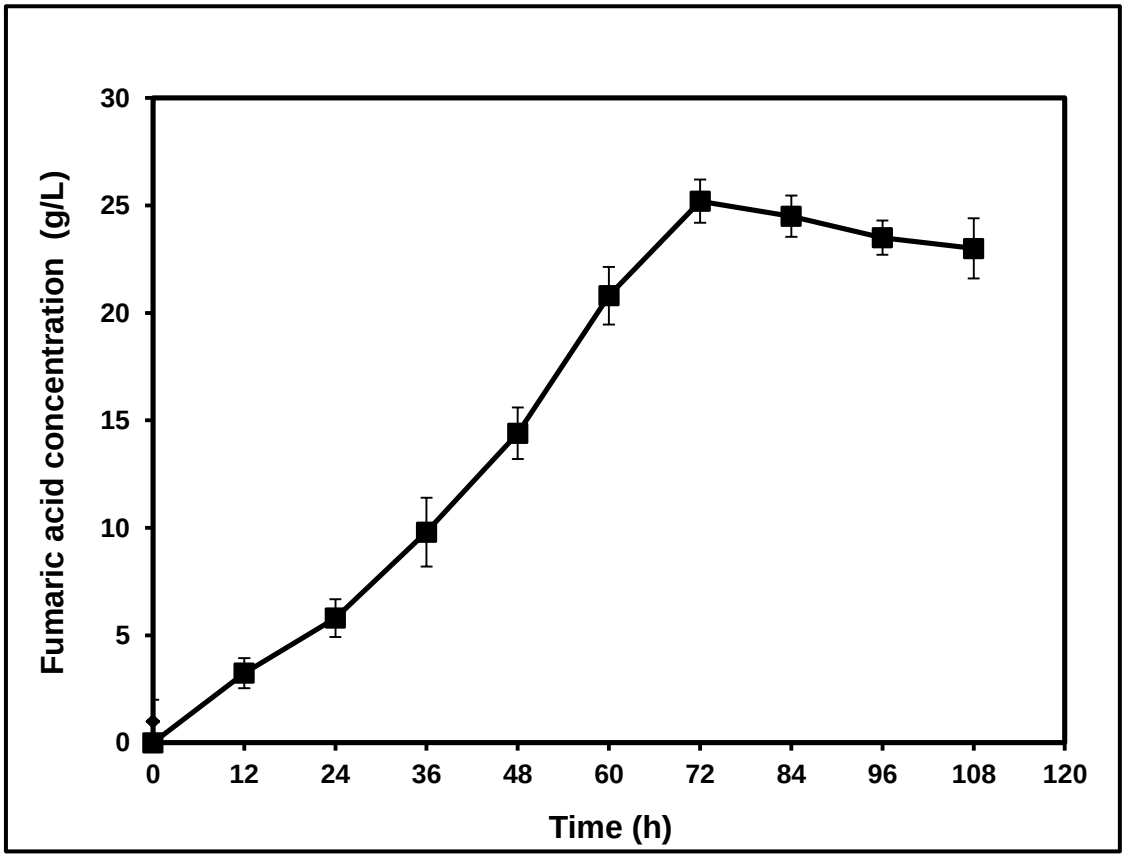

Figure 6.1.5 (D): Fumaric acid production under all optimized conditions (40 g/L total solid concentration, 30 ^{o}C and pH 6 and 200 rpm flask shaking speed.

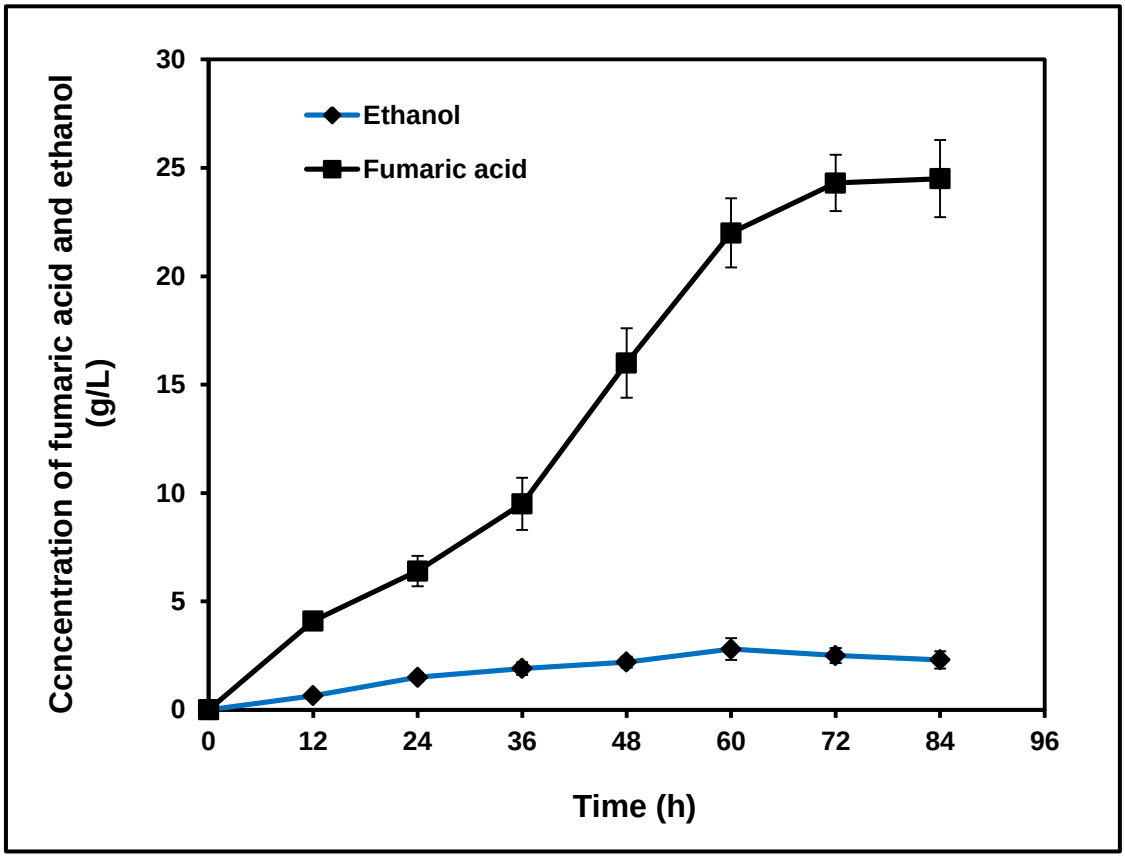

Figure 6.1.6 (A): Production profile of fumaric acid and ethanol under submerged fermentation conditions of 40 g/L total solid concentration, 30 ^{o}C, pH 6 and 200 rpm.

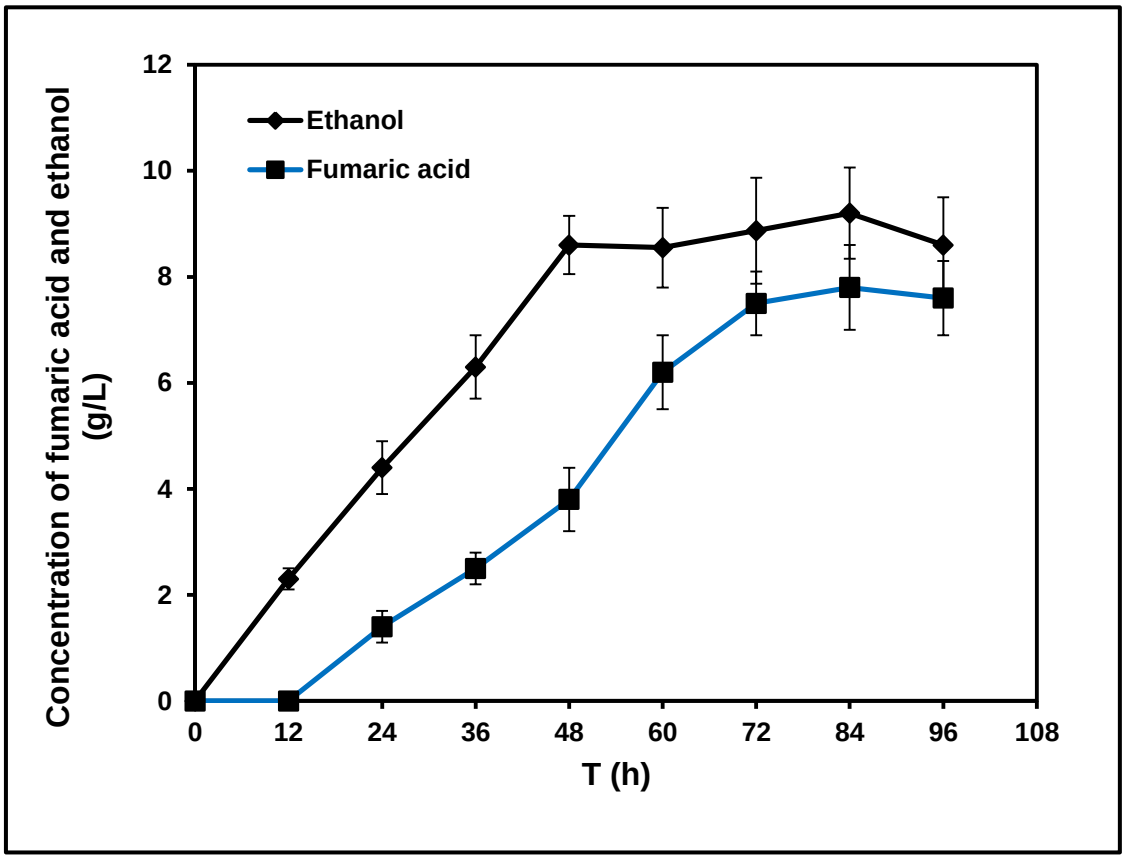

Figure 6.1.6 (B): Production profile of fumaric acid and ethanol under submerged fermentation conditions of 45 g/L total solid concentration, 37 ^{o}C, pH 8 and 300 rpm.

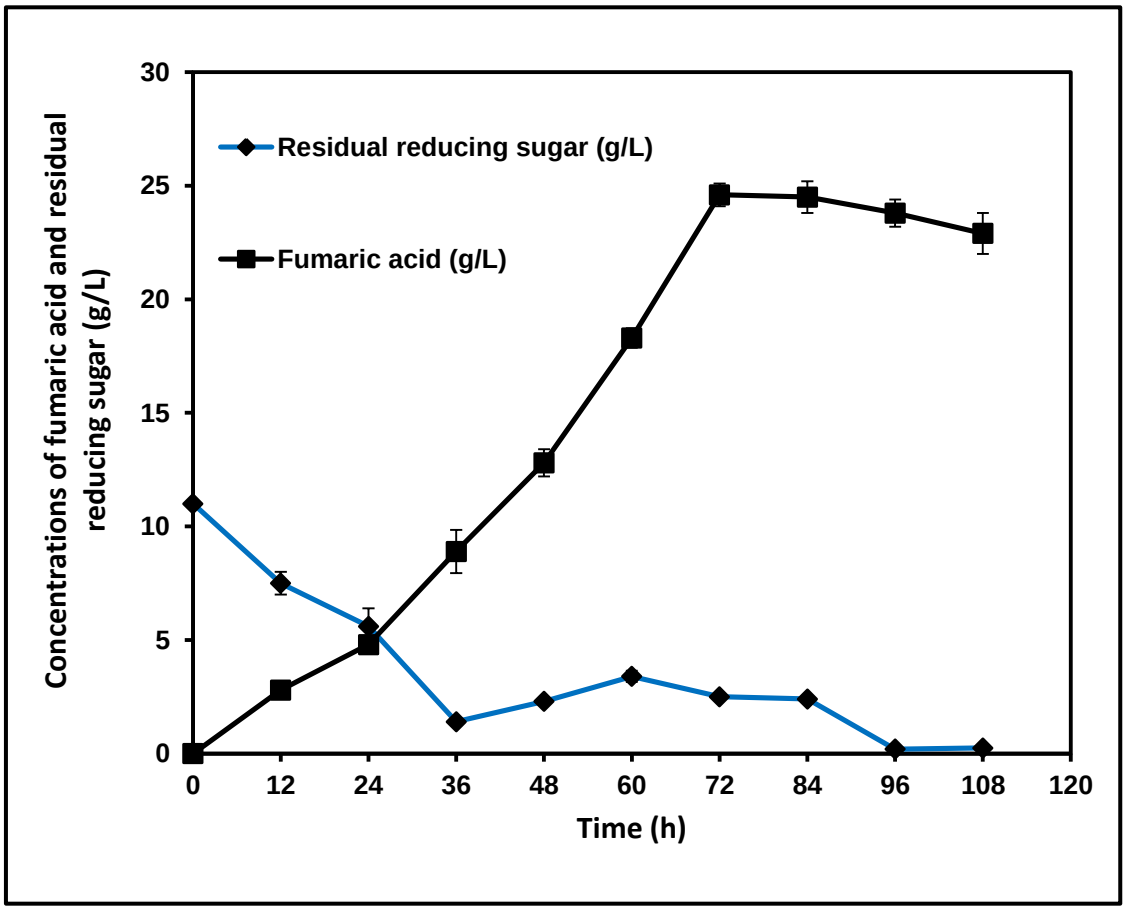

Figure 6.1.7: Changes in the concentration of residual reducing sugar (g/L) and fumaric acid (g/L) during submerged fermentation with apple pomace ultrafiltration sludge and *R. oryzae*

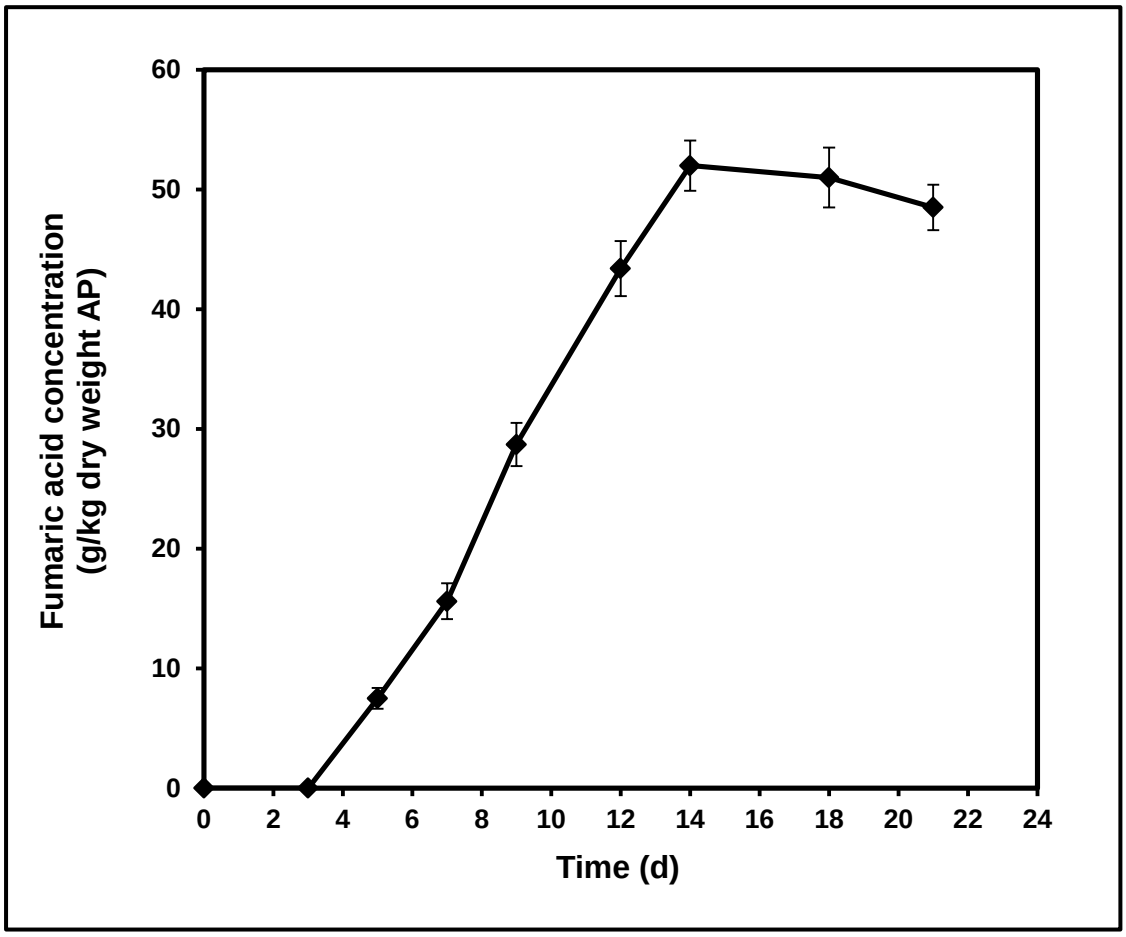

Figure 6.1.8: Production profile of fumaric acid obtained with apple pomace and *R. oryzae*

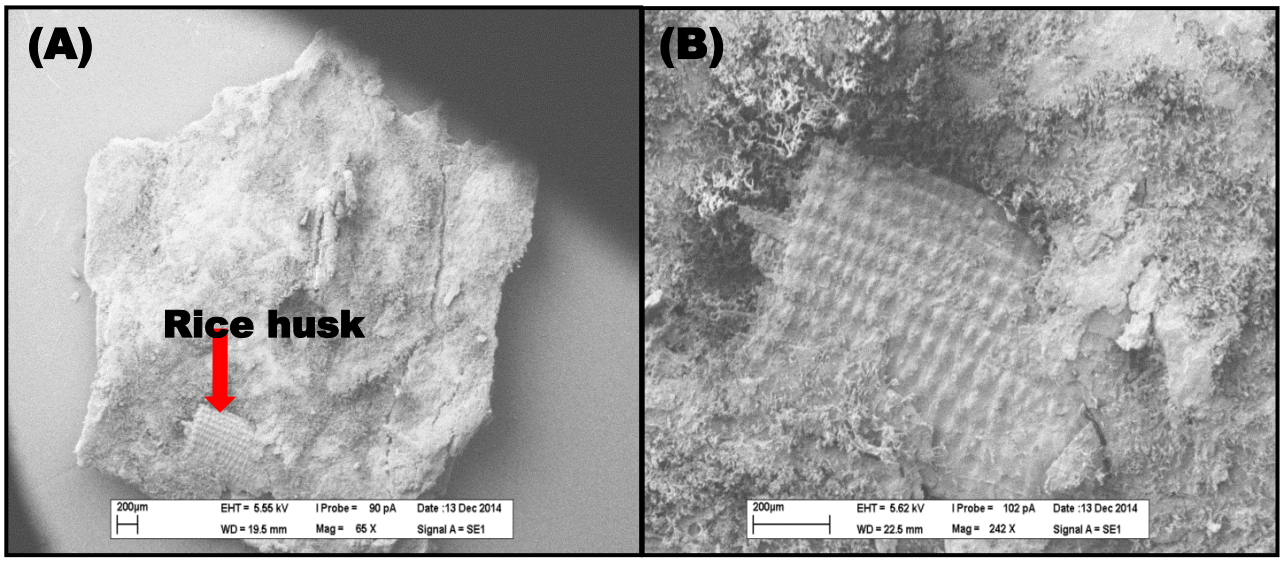

Figure 6.1.9: (A) SEM micrograph of a small piece of solid state fermentation apple pomace sample showing mycelia of *R. oryzae* interspersed with rice husk **(B)** A close view of the sample.

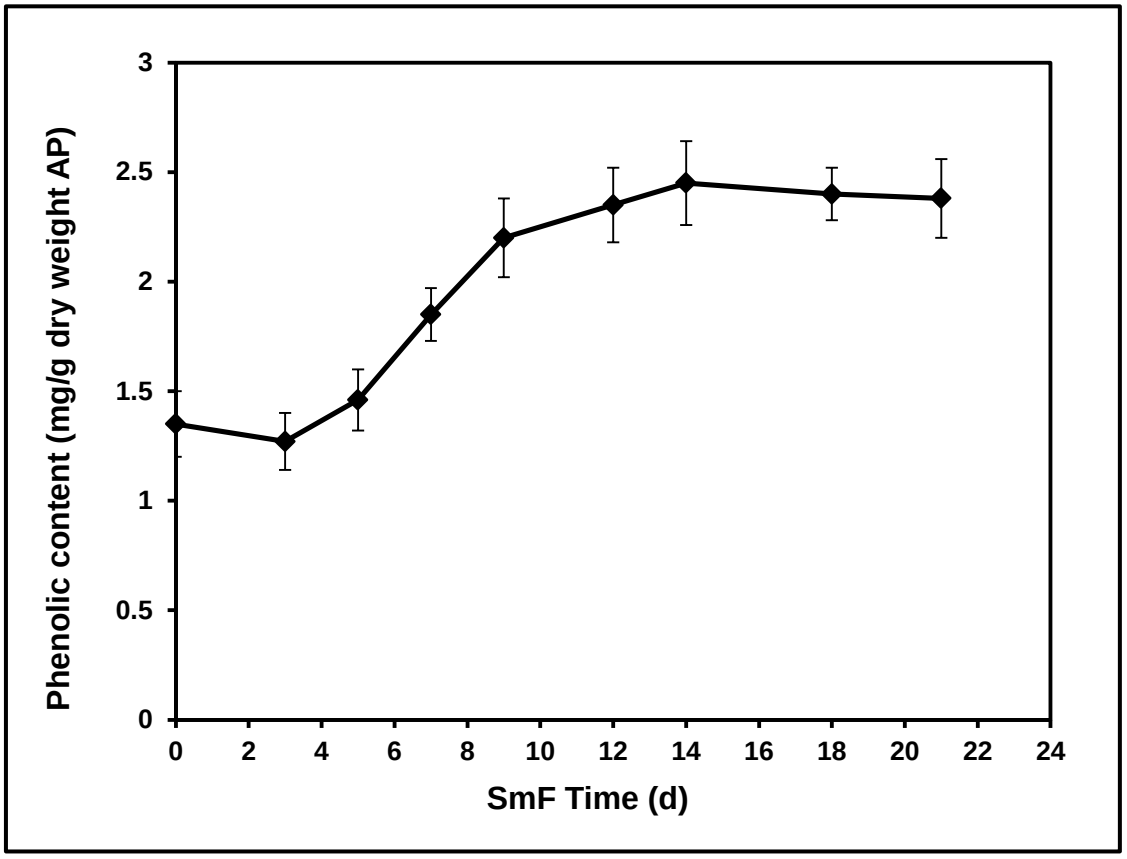

Figure 6.1.10: Changes in the phenolic content (mg/g dry weight) of apple pomace sample during solid state fermentation.

PART II

BIO-CONVERSION OF APPLE POMACE INTO FUMARIC ACID IN A ROTATING DRUM TYPE SOLID-STATE BENCH SCALE FERMENTER AND STUDY OF THE DIFFERENT UNDERLYING MECHANISMS

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Résumé

L'utilisation de résidus solides de l'industrie de la transformation des pommes, comme la pulpe de pommes (en anglais: AP), pour la production d'acide fumarique (en anglais: FA) en présence d'un fermenteur de laboratoire à tambour pour la bio-transformation de substrats solides a été étudiée dans des conditions optimisées et sous différents régimes d'agitation. La souche du champignon filamenteux, *Rhizopus oryzae* 1526 (*R*. *oryzae*) a été utilisée dans cette étude. La fermentation en milieu solide a été effectuée en rotation continue, en rotation intermittente et en mode statique dans le fermenteur, pour un maximum de 21 jours. Deux différentes teneurs en humidité (70% et 50%, p/p) des AP ont été appliquées pour chaque lot. La plus forte concentration de FA (138 ± 9,11 g par kg de poids sec des AP) a été atteinte à 50% d'humidité et sous rotation continue après 14 jours. Le contrôle de la viabilité du champignon a révélé le maintien d'un nombre élevé de spores ($2,74 \times 10^8$ par g d'AP secs) lors de la fermentation. L'analyse de la composition en fibres des AP a confirmé la conversion des fibres alimentaires insolubles en fibres alimentaires solubles ainsi que l'utilisation de fibres alimentaires pour la production d'AF. La teneur totale en phénol des AP a été considérablement augmentée (augmentation de l'ordre de 86%), passant de 185 ± 10,5 mg à 345 ± 8.5 mg/g de lyophilisat après 18 jours de fermentation. L'analyse par chromatographie LC/MS/MS a confirmé la consommation des glucides solubles (glucose, fructose, saccharose) présents dans des AP par le champignon au cours de la fermentation. La présence de différents composés phénoliques et l'évolution de leur contenu dans le milieu fermentaire ont également été confirmées par l'analyse LC/MS/MS. Deux autres conditions d'opération ont permis de produire 82 ± 6,8 g et 58 ± 8,5 g de FA par kg de poids sec des AP, respectivement après 18 jours de fermentation.

Mots clés: déchets solides de jus de pommes, fermentation à l'état solide, fermenteur à tambour rotatif à l'état solide à l'échelle du laboratoire, teneur en humidité, sucres réducteurs; contenu phénolique, fibres des déchets solides de jus de pommes.

Abstract

Utilization of apple industry solid waste, apple pomace (AP) for the production of fumaric acid (FA) using a rotating drum type solid-state bench scale fermenter was studied under optimized conditions and different mechanisms underlying the conversion were investigated. The filamentous fungal strain, *Rhizopus oryzae* 1526 (*R. oryzae*) was used in the study. The solid-state fermentation was carried out in continuous rotation, intermittent rotation and static mode of the fermenter operations for a maximum of 21 days. Two different moisture contents (70% and 50%, w/w) of AP were applied for each batch. The highest FA concentration (138 ± 9.11 g per kg dry weight of AP) was achieved at 50% moisture content and under continuous rotation after 14 days. Viability checking of the fungus showed maintenance of a high cell count (2.74×10^8 spores per g dry AP) during fermentation. Analysis of AP fibre composition confirmed the conversion of insoluble dietary fibers into soluble dietary fibers and utilization of the dietary fibres for FA production. Total phenolic content of AP was considerably increased from 185 ± 10.5 mg to 345 ± 8.5 mg/g lyophilizate (by around 86%) after 18 days of fermentation. LC/MS/MS analysis confirmed the consumption of sugars (glucose fructose and sucrose) present in AP by the fungus during fermentation. The presence of different phenolic compounds and changes in their content after fermentation was also confirmed by LC/MS/MS analysis. Two other operating conditions produced 82 ± 6.8 g and 58 ± 8.5 g of FA per kg dry weight of AP, respectively after 18 days of fermentation.

Keywords: Apple pomace, solid state fermentation, rotating drum type solid-state bench scale fermenter, moisture content, reducing sugars; phenolic content, apple pommace fibres.

Introduction

In recent times, advancement in biotechnological approaches and utilization of the natural ability of microorganisms has enabled the bioconversion of different waste biomass into value added products. The global message of developing a sustainable and green option for each chemically produced product has been very successful for many platform chemicals (Sheldon *et al*. 2014) Although, the number of reports in this research area are piling up at a great pace, scale-up studies are scarce and this might be caused by the unfeasibility constraints generated as a result of experimental volumetric expansion from laboratory to industrial scale. Among the different biotechnological processes, fermentation has been the most explored and successful option for the bioproduction of many high-value chemicals. In this regard, the recent progress made in the biological production of 'fumaric acid' (FA), the organic acid with diverse applications is worthy of attention (Qu *et al*., 2012; Yang *et al*., 2011; Roa Engel *et al*., 2008). FA and its ester derivatives have emerged as a multifaceted molecule with newer applications in bio-medical field with previous conventional uses (Mrowietz *et al*., 2005; Moharregh-Khiabani *et al*., 2009; Shokri *et al*., 2011; Goldberg *et al*., 2006; Das *et al*., 2015a). The growing demand of FA can be well understood by looking at its current market volume statistics. The report published in 2014 stated that the current market volume of FA is around 240 000 t and the projected market volume is 350 000 t by 2020 (www.grandviewresearch.com). Biological route of FA production over chemical method is being preferred due to human and environmental health issues (Roa Engel *et al*., 2008; Kang *et al*., 2011). In fact, FA has been identified as one of the top ten value-added chemical that can be produced from sugars by means of biological conversion (Werpy *et al*., 2004). In the last four decades, lots of efforts have been made to produce FA from different waste carbon sources employing various microorganisms in particular, different strains of the filamentous fungus *Rhizopus oryzae* (Yang *et al*., 2011; Das *et al*., 2015b). Bibliographic data shows that most of the fermentation studies carried out so far on FA production were submerged fermentation (SmF); while experimentation on solid state fermentation (SSF) based FA production is yet to draw much attention. SSF has many technical advantages over SmF and this issue has been extensively reviewed by experts (Krishna *et al*., 2005; Sargantanis *et al*., 1993; Raghavarao *et al*., 2003). However, finding a suitable solid substrate with good potential of serving the purposes of carbon, micro and macro nutrients is vital for SSF. Moreover, for every new combination of a solid substrate and microbial strain, extensive study on process optimization is required. In this regard, the apple industry origin waste solid biomass 'apple pomace' (AP), can be considered as SSF substrate. The AP is the left over solid residue after apples are pressed for juice collection and separated from the liquid. Although, AP is an apple industry waste by-product, it represents around 20–35% of

the original fruits (Carson *et al*., 1994; Suarez *et al*., 2010). AP is shown to be a very good source of nutrients for microorganisms and this also make AP highly prone to microbial attack and thus unsafe for environmental disposal (Dhillon *et al*., 2011; Gullon *et al*., 2007; Shalini *et al*., 2010; Vendruscolo *et al*., 2008; Gassara *et al*., 2011). High apple production in Canada (around 382 million kg as of 2014 report) and demand for apple juice makes AP production unavoidable (www.hortcouncil.ca). Looking at the possibility of utilizing AP as a good carbon source for FA producing fungal strain such as *Rhizopus oryzae* 1526 AP was considered as a solid substrate for FA production in a rotating drum type solid-state bench scale fermenter. The selection of *R. oryzae* 1526 for FA production was made on the basis of important sequential findings of decade old research on FA production ability of this fungal strain. In the 1950s and 1980s the genus *Rhizopus* gained tremendous importance as different species of this genus were found to capable of producing FA at commercial level. The companies 'National Distillers and Chemical Corporation' (1958) and 'Du Pont' (1986) manufactured FA employing species of *Rhizopus*.(Lubowitz *et al*., 1958; Goldberg *et al*., 1986; Ling *et al*., 1989). For species selection, advantage of using *Rhizopus oryzae* over other FA producing species (such as *R. nigricans, R. formosa* and *R. arrhizus*) is supported by previous findings. It has been shown that Rhizopus oryzae has simple nutritional requirement for its growth and metabolism and this facilitates the utilization of low cost carbon sources. Moreover, *Rhizopus oryzae* species resulted in the highest productivity (4.25 g/(g h)) of FA in a rotary reactor (Xu *et al*., 2012).These research outcomes have established *R. oryzae* as the frontliner in the production of FA. To be specific in strain selection, it's worth mentioning that among different strains of *R. oryzae* tested for FA production, the strain NRRL 1526 is one of the best strains (Oda *et al*., 2003). Thus, considering the general acceptance, technical feasibility and advantages over other FA producing species and strains, the fungal strain *Rhizopus oryzae* 1526 (to be called *R. oryzae* hereafter) was selected for the present research work. To the best of author's knowledge, no prior study with rotating drum type solid-state bench scale fermenter has been carried out for FA production and this implies the novelty elements of the present study.

Materials and methods

Materials

The fungal strain used for FA production was *Rhizopus oryzae* 1526 and was procured from Agricultural Research Services (ARS) culture collection, IL, USA. The apple industry waste biomass with rice husk was procured from Lassonde Inc., Rougemont, Montreal, Canada. All

the chemicals used in the present study were of analytical grade and purchased from Fisher Scientific (Ottawa, Ontario, Canada).

Culture of *Rhizopus oryzae* 1526

The procured fungal strain was revived on potato dextrose agar (PDA) slant after incubating at 37 ± 1 ^{o}C for 4 days (d) and then propagated on Petri dishes with PDA. The inoculated PDA Petri dishes were incubated for a period of 72 h at 37 ± 1 ^{o}C. After 3 d of incubation, the fully grown and sporulated fungus was washed with distilled water (d.H_2O) and the sporangiospores were collected by filtering through glass wool. The stock of the spore suspension was maintained at 1 × 10^8 spores per mL and stored at 4 ± 1 ^{o}C for regular uses. For long time storage, spore suspension was mixed with 20% glycerol and kept at -80 ^{o}C.

Rotating drum type solid-state bench scale fermenter: operating conditions

An in situ sterilizable solid-state bench scale fermenter (BSF) of 12 kg capacity (Model: Terrafor-IS, Make: Infors HT, Switzerland) with maximum 3-4 kg working volume was used for the present study. To prepare for each batch of SSF, operating conditions of BSF was set as follows: (a) aeration rate of 1 vvm (rotameter controllable); (b) maximum operating pressure: +2.0 bar; (c) feed 1 pump (condensate line return to vessel) at 10%; (d) feed 2 pump connected to reagent bottle (filled with sterilized water) with the T-piece and manually controlled; (e) push valves of condensate return lines were opened and; (f) operating temperature (for inoculation) was set at 30 ± 1 ^{o}C.

Substrate preparation for solid-state fermentation

To prepare for SSF, oven dried AP (with 6-10% of moisture content; MC%) was first moisture adjusted (50 and 70%, w/w) by homogenously mixing with distilled water (d.H_2O). For each batch, 1 kg (dry weight) of AP was used. In situ sterilization of MC adjusted AP was carried out at 121 ± 1 ^{o}C for 20 min. Push valves of condensate return lines were closed during sterilization.

A test run was done to see the change in the MC of the AP substrate before and after sterilization. Sterilized AP was allowed to cool down to the inoculation temperature of 30 ± 1 ^{o}C. Before inoculation, pH of sterilized AP was adjusted to 5.5– 6.0 by aseptically adding required amount of sterilized $CaCO_3$ powder and rotating the BSF continuously for at least 12 h at 5 rpm. Well mixed AP samples were collected through the sample collection port (Tri-

Clamp port) under aseptic condition (flaming method) and analyzed for changes in MC and pH. If required, MC and pH was further adjusted before inoculation.

Inoculation and solid-state fermentation

After cooling down to inoculation temperature (30 ± 1 ^{o}C), AP was inoculated with *R. oryzae* spores at a concentration of 1×10^{7} spores per g dry AP. The inoculation was done following standard procedure for BSF. Briefly, spore suspension was filled in sterilized syringe and pierced through the septum (piercing membrane) in the 19 mm port of the BSF under aseptic conditions (flaming method) and later set to SSF conditions. For optimization of FA production, BSF was run under different SSF conditions for a maximum of 21 d. Two parameters, *viz*. MC of

AP and mode of rotation of BSF were considered. Three modes of operation (continuous rotation, intermittent rotation and static mode) of BSF were tested for FA production. In the continuous mode of operation, 2 rpm for counter-clockwise for 12 h and 2 rpm clockwise for the next every 12 h was followed. For the intermittent rotation, programme was set at 2 rpm for 60 min counter-clockwise and at 2 rpm for 60 min clockwise, after every 12 h. AP adjusted with 50% and 70% (w/w) of MC were used for each run of SSF with different modes of operation.

Sample collection and downstream processing

For FA estimation, 5 g of fermented samples was collected through the Tri-Clamp port of BSF under aseptic condition (flaming method) at 3 d, 5 d, 7 d, 9 d, 12 d, 14 d, 16 d, 18 d and 21 d intervals of SSF. Sterilized d.H_2O at 1: 10 solid to liquid ratio was mixed with the collected samples and then kept in a shaking incubator at 200 rpm at 30 ± 1 ^{o}C for 1 h. For the removal of solid residues and fungal mycelia from the mixture, samples were filtered through glass wool. Downstream processing of the fermented broth samples was carried out following the standard method (Dang *et al*., 2009). Briefly, filtered samples were treated with simultaneous heating and acidification (90 ± 1 ^{o}C, 5 N H_2SO_4) until clear and later centrifuged (8000 × g, 10 min, 20 ± 1 ^{o}C) and supernatants were collected. FA concentrations in the supernatant were estimated by using high performance liquid chromatography (HPLC) technique and the FA concentrations were expressed in g per kg dry apple pomace.

Analytical techniques

Estimation of total dietary fibre (TDF), insoluble dietary fibre (IDF) and soluble dietary fibre (SDF) of unfermented and fermented dried AP samples were carried out following the AOAC (Association of Official Analytical Chemists) methods (Horwitz *et al*., 2000). To determine the water holding capacity (WHC) of AP, the previously mentioned method was followed with some modifications (Chen *et al*., 1988). Briefly, 5 grams of dried AP was vigorously mixed with 100 mL of d.H_2O for 10

min and then centrifuged at 8000 × g for 15 min at 20 ± 1 ^oC. The supernatant was carefully discarded and the centrifuge bottles with AP pellets were kept inverted until free water droplets stopped running from the AP pellets. Finally, the MC of the AP pellets was determined following standard method and WHC (g/g) was accordingly calculated (Reeb *et al*., 1999). For pH measurement, 2 g fermented AP sample was mixed with 20 mL of d.H_2O and kept for vortexing for 10 min and filtered through filter paper (Whatman # 4). Filtrate was analyzed for pH measurement using a pH meter equipped with glass electrode (EcoMet P25). Oven-dry method was used for the analysis of moisture content (MC) of AP samples (raw and fermented) (Goldberg *et al*., 1986). Briefly, about 5 g of AP sample was allowed to dry at 60 ± 1 ^oC until a constant weight was achieved. The MC in PPSW was calculated as per eqn. (1):

$$\mathrm{MC} = \frac{\text{Initial weight} - \text{Oven dry weight}}{\text{Initial weight}} \times 100 \qquad (1)$$

For the extraction of phenolic compounds from the AP (raw and fermented) samples, the procedure of previously mentioned method was followed (Schmidt *et al*., 2014). The phenolic content was estimated by Folin–Ciocalteu method taking gallic acid as standard. Optical density (OD) was measured at λ_{max} = 765 nm using a 96 well plate reader (BioTek Instruments, Epoch, USA). For the identification and estimation of different phenolic compounds, liquid chromatography-mass spectroscopy (LC/MS-MS) analysis was performed. To prepare sample for LC/ MS-MS analysis, lyophilized phenolic extract was first resuspended in water and methanol (1:1) and later analyzed (Schmidt *et al*., 2014).

To prepare samples for sugar (glucose, fructose and sucrose) consumption analysis, 5 g of oven-dried (for both raw and fermented) sample was mixed with 30 mL of d.H_2O and kept under shaking condition for 1 h at 200 rpm and 25 ± 1 ^oC. The samples were then

centrifuged (8000 × g for 15 min at 25 ± 1 ^{o}C) and collected supernatants were checked for pH (5.0) and if required, the pH was adjusted with NaOH solution (10 N) and paper filtered (Whatman # 44). Finally filtrates were syringe-filtered (0.45 mm) and analyzed for sugars using LC/MS-MS method. For viability check of *R. oryzae* at different time intervals of SSF, around 100 mL sample (prepared for downstream processing for FA estimation) was taken and total spore count (per gram dry AP) was done using a haemocytometer.

The quantification of FA in the downstream processed AP samples was carried out by HPLC technique. Sample preparation for HPLC analysis was carried out following standard procedure (Zhou *et al*., 2000). The specification of the HPLC used was as follows: System: DIONEX DX500, with an Acclaim OA, 5 mm, (4.6 × 150 mm) column with a refractive index detector (PDA-100 DIONEX, UV, 210 nm). The mobile phase was 2.5 mM methanesulfonic acid (CH_3SO_3H) at a flow rate of 1 mL/min and column temperature of 30 ± 1 ^{o}C. For the identification and estimation of sugars (glucose, fructose and sucrose) and different phenolic compounds (gallic acid, ferulic acid, 4-amino-benzoic acid, vanillic acid and vanillin) in the fermented AP samples, LC/MS/ MS technique was employed. The technical details of the LC/ MS/MS instrument used for the analysis were: (a) for sugar estimation: Thermo TSQ Quantum model, equipped with an Electrospray Ionization (ESI) in negative ion mode; Zorbax Carbohydrate (4.6 mm × 150 mm; 5 mm, Agilent) analytical column; 75% acetonitrile; 0.1% NH_4OH; 25% water and 0.1% NH4OH mobile phase and 10 mL injection volume. Glucose-D2, D-(-)-fructose and sucrose (all from Sigma) was used as the internal standards; (b) for phenolic compound estimation: Thermo TSQ Quantum model, equipped with an Electrospray Ionization (ESI) in negative ion mode, Thermo Scientific Beta- Basic C18 LC column (100 mm × 2.1 mm; 3 mm); mobile phase of methanol and acidified water (0.1% acetic acid) at a ratio of 17.5 : 82.5; flow rate of 0.3 mL/min and 20 mL injection volume. Gallic acid, ferulic acid, 4-amino-benzoic acid, vanillic acid and vanillin (all from Sigma) were used as internal standards

Statistical analysis

Data are represented as mean ± SD of three independent experiments. Correlations were considered significant at $P < 0.05$ for different applied parameters.

Results and discussion

Effects of AP moisture content and BSF mode of rotations on FA production

The FA concentrations (g per kg dry AP) achieved with 70% (w/ w) and 50% (w/w) MC of AP is shown in Figure 6.2.1 and Figure 6.2.2, respectively. The FA concentration varied under continuous rotation, intermittent rotation and static mode of BSF. At 70% (w/w) of MC and under continuous rotation, the FA concentration reached up to 22.6 ± 3.5 g/kg at the end of 5 d of SSF. From 5 d onwards, production of FA started decreasing and at the end of 12 d of SSF, only 4.5 ± 1.8/kg of FA was recorded. With 50% (w/w), there was a continuous increase in the FA concentration from 5 d to 14 d of SSF and then became static onwards. At the end of the 14 d of SSF, FA concentration reached 138 ± 9.1 g/kg. In comparison to the highest FA concentration (22.6 ± 3.5 g/kg) achieved with 70% (w/w), the FA production was enhanced by about 6.1 folds. In the intermittent mode of rotation, similar trend of effects of MC and mode of rotation of BSF on FA production was observed. AP with 70% (w/w), FA concentration started increasing on 5 d onwards and reached up to 31 ± 3.1 g/kg at the end of 9 d of SSF and declined thereafter. For 50% (w/w) MC, the production level of FA was 82 ± 6.8 g/kg at the end of 18 d of SSF. In the static mode of rotation of BSF, with 70% (w/w) of AP, FA concentration reached 17.3 ± 3.5 g/kg at the end of 12 d of SSF. When experimented for 50% (w/w) MC, the FA production was increased to 58 ± 8.5 g/kg after 18 d of SSF. The MC of SSF substrate is a fundamental parameter for controlling the growth and metabolism of filamentous fungi. In general, a MC that allows to maintain the conditions: (a) no free-moving water and (b) air as the continuous phase are considered suitable for SSF (Krishna *et al*., 2005; Sargantanis *et al*., 1993; Raghavarao *et al*., 2003). These conditions are met by optimization studies on each new combination of SSF substrate and fungal strain. In the present SSF study with AP and *R. oryzae*, the production profile of FA changed as responses to the changes made in the MC of AP. Apart from MC, mode of operations (continuous rotation, intermittent rotation and static mode) of BSF also influenced the FA production level. Thus, it will be pertinent to elaborate the obtained results for each combination of MC and mode of operations of BSF. The variations in the FA production profiles under continuous mode of rotation of BSF can be well explained with some basic principles of SSF. It was observed that, under continuous mode of rotation, AP with 70% (w/w) formed agglomerates of different sizes (4–5 cm) and shapes after 5 d of SSF. Most of the agglomerates were roughly spherical shaped and highly compacted, while others were of irregular shapes. Only small fractions of AP were left without agglomeration. However, this was not the case with 50% (w/w) MC of AP under continuous mode of rotation of BSF. No

agglomerates were formed up to 18 d of SSF and before this time period, FA reached its maximum concentration 138 ± 9.1 g/kg at the end of 14 d of SSF. Although, it was difficult to pinpoint the exact contributing factor for the agglomeration of AP, still high MC of AP and continuous rotation might have helped in the process. To rule out the possibility of any influence of speed (rpm) and direction (clockwise and anticlockwise) of rotation of BSF on agglomeration of AP, apart from 2 rpm, three more different speeds (1, 3 and 4 rpm) at clockwise or anticlockwise directions, were experimented with 70% (w/w) MC. In all applied speeds, agglomeration of AP could not be avoided. Direction of BSF rotation did not show any impact on the agglomeration of AP. In either clockwise or anticlockwise direction of BSF, there was agglomeration with 1, 3 and 4 rpm speeds. Under all such applied conditions, AP resulted in lower FA production (data not shown). The continuous rotation mode of BSF selected for the present study was intended for the proper mixing of the substrates with $CaCO_3$ (pH maintenance) and aeration throughout the substrate during SSF. Worth mentioning, the design of the BSF allowed aeration only on the top layer of the solid substrate and the approximate height of the 1 kg AP inside the vessel of BSF was around 11-12 cm. Penetration of air to the middle and lower portions of AP layer of this height, was not possible without rotation. FA being an aerobic process, without proper supply of air, production level is considerably affected (Gu *et al*., 2013). The agglomeration of AP under continuous rotation might be the consequence of high MC as it is of general acceptance that high MC of SSF substrate can cause particle agglomeration along with limiting the air transfer (Krishna *et al*., 2005). This became evident as agglomeration of AP was not caused at 50% (w/w) MC of AP under continuous mode of rotation. It was obvious that agglomerates being highly compacted were not supportive of air supply and fungal growth inside and thus lowered FA production. Although, AP formed smaller agglomerates in the intermittent mode of rotation with 70% (w/w), much of the AP was le• as free biomass as compared to continuous mode of rotation. The 2.64 folds enhancement in FA concentration for 50% (w/w) MC could be attributed to non-agglomeration behaviour of AP. However, 82 ± 6.8 g/kg of FA concentration was achieved at the end of 18 d of SSF and this was much longer incubation compared to with 70% (w/w). It was obvious that 50% (w/w) MC supported higher FA production but intermittent mode of rotation might have supported air supply less efficiently as compared to continuous mode of rotation and this delayed the process of FA production. Under static conditions, air supply will be sufficient only on the top part of AP and cannot reach most portions of the 11-12 cm height AP bed. This caused least FA production as compared to continuous and intermittent mode of BSF rotations for both applied MCs. To ensure that estimation of FA was carried out for the whole AP, the substrate was first mixed with a sterilized iron rod through the Tri-Clamp port of BSF under aseptic condition (flaming

method) and samples were collected for FA estimation. The variation in FA concentrations with 50% (w/w) and 70% (w/w) MC may be due to the limitation of air supply at higher MC compared to lower MC of AP. The overall findings on the effects of MC and BSF mode of rotation on FA production suggested that 50% MC and continuous rotation (2 rpm) were optimum for enhanced FA production with AP and *R. oryzae*.

Water holding capacity (WHC) of AP

The WHC of AP was found to be around 8.12 ± 1.25 g water per gram of AP dry weight. This value is comparable to previous findings (Chen *et al*., 1988; Sudha *et al*., 2007). The higher WHC of AP showed AP fibres to be good water binder. For the long-term maintenance of MC by a solid substrate, higher WHC is important. As SSF represents a condition of no free-moving water, WHC actually is the maximum water content for the dissolution of solutes (nutrients) in aqueous phase throughout the substrate (Chen, 2013; Geravais, 2008). In the present study, the spore germination, propagation and sporulation of the fungus *R. oryzae* were supported under SSF conditions. These fungal processes used sufficient amount of water held by AP. Moreover, agglomeration of AP at 70% MC was also caused by the high WHC of AP. High WHC and collision of AP particles during the rotational motion of the drum of BSF led to adhesion of AP particles and hence formation of agglomerates. This was also supportive of the interstitial liquid induced agglomeration of solid particles (Derksen *et al*., 2011).

Sugar consumption and FA production

To find out the exact carbon source for the fungus *R. oryzae* during SSF, concentrations (mg per g dried AP) of sugar molecules (glucose, fructose and sucrose) in the raw (before SSF) and fermented AP samples were estimated. The residual concentrations (mg/g) of these three sugar molecules at different time intervals of SSF are shown in Figure 6.2.3 (A). The initial concentrations of glucose, fructose and sucrose were estimated to be 128 ± 5.6, 270 ± 7.5 and 35 ± 4.5 mg/g of dried AP samples, respectively. Fructose was consumed very fast and at the end of 7 d of SSF, the residual concentration of fructose was found to be 19 ± 3.5 mg/g. After same SSF time (7 d), the residual glucose concentration was estimated to be 8.6 ± 1.5 mg/g. At the end of 12 d of SSF, the residual fructose concentration decreased to 4.5 ± 1.4 mg/g. In Figure 6.2.3 (A), it was important to observe that residual glucose concentration suddenly increased from 8.6 ± 1.5 to 36 ± 2.8 mg/g after 7 d of SSF and was brought down from 36 ± 2.8 to 5 ± 1.1 mg/g after 14 d. Consumption of sucrose was slowest, because the microorganisms are favorable to use glucose and fructose first. Sucrose reached its

minimum residual concentration of 3.2 ± 1.2 mg/g after 14 d of SSF. To have a better understanding of the preferential consumption of the sugar molecules under SSF conditions, sugar consumption rates (mg per g dried AP per h) were estimated for glucose, fructose and sucrose. The results are displayed in Figure 6.2.3 (B). At the end of 3 d, glucose consumption rate was around 0.72 ± 0.1 mg/(g h). For fructose, consumption rate was much higher (2.08 ± 0.2 mg/(g h)) in the first 3 d of SSF. As mentioned before, after 7 d of SSF, glucose concentration increased from 8.6 ± 1.5 to 36 ± 2.8 mg/g and this corresponded to the negative value (-0.57 ± 0.1 mg/(g h)) of glucose consumption rate recorded after 9 d of SSF. At the end of 5 d, fructose consumption rate decreased to 0.89 ± 0.1 mg/(g h) and in between 5 d to 7 d, it was slightly increased from 0.89 ±0.15 to 1.2 ± 0.2 mg/(g h). A similar change in consumption rate was also observed for glucose. From 9 d onwards, consumption rate of glucose and fructose started decreasing until 16 d and 12 d respectively and then remained almost unchanged. For sucrose, the consumption rate was slower (0.1 ± 0.05 mg/(g h)) in the first 5 d and increased to 0.34 ± 0.1 mg/(g h) after 7 d of SSF. At the end of 9 d, consumption rate considerably decreased to 0.014 ± 0.01 mg/(g h) and maintained negligible changes onwards. Overall, fructose consumption rate was highest among the three sugar moieties followed by glucose and sucrose. During SSF under continuous rotation with 50% MC, the fungus utilized the sugar molecules in a preferential manner. This is a well-known fact that microorganisms prefer hexose sugars for their metabolic activities (Xu *et al*., 2010). In the present study, the fungus consumed the easily available hexose sugar moieties (glucose and fructose) and bioconverted it into FA. As mentioned earlier, under continuous rotation with 50% MC, FA concentration reached the highest concentration of 138 ± 9.1 g/kg at the end of 14 d of SSF. This was quite supportive, if residual concentrations of glucose and fructose at the end of 14 d are considered. The increase in glucose concentration from 8.6 ± 1.5 to 36 ± 2.8 mg/g after 7 d of SSF can be attributed to the hydrolysis of cellulose and hemicellulose moieties into fermentable sugars, such as glucose. The fungus, *R. oryzae* is known to produce different intracellular and extracellular cellulolytic, hemicellulolytic, pectinolytic and amylolytic enzymes that can help in accessing the alternative carbon sources for metabolic activity and growth (Ghosh *et al*., 2011). Fibre compositional analysis of AP samples before and after SSF confirmed the utilization AP dietary fibres for FA production by the fungus. Discussion on this analysis has been detailed in the proceeding sub-section. In the present study, once the easily available hexose sugars were exhausted, the fungus started to feed on the structural fibres of AP. After 14 d of SSF, the residual glucose concentration was brought down suggesting utilization of both free and lignocellulose derived glucose for bioconversion into FA. Estimation of sugar moieties in the

fermented AP samples confirmed the utilization and bioconversion of available carbon sources in AP into FA during SSF.

Analysis of fibre composition of AP

The fibre compositional analysis of AP showed changes in the content of TDF, IDF and SDF before and after SSF. The pre- and post-SSF estimated values of TDF, IDF and SDF are presented in Table 6.2.1. Before SSF, the TDF, IDF and SDF contents were around 39.4 ± 1.5, 32.5 ± 0.7 and 6.80 ± 0.65 (% w/w), respectively. After 21 d of SSF (under continuous rotation with 50% MC) the TDF, IDF and SDF contents were estimated to be 19.15 ± 1.2, 17.35 ± 1.3 and 2.3 ± 0.45 (% w/w), respectively. The decrease in TDF was an interesting finding of the present study. It further confirmed the hydrolysis of cellulose and hemicellulose of AP by the activities of the enzymes released by the fungus *R. oryzae* during SSF. The monosaccharides derived were utilized as carbon source and bioconverted into FA. The decrease in IDF after SSF suggested solubilization of IDF constituents (cellulose and hemicellulose) by the fungal enzymatic action. This initially increased the SDF content but as the fungus started utilizing these sugar reserves SDF was almost exhausted after 18 d of SSF. As mentioned earlier, the glucose concentration was enhanced from 8.6 ± 1.5 to 36 ± 2.8 mg/g after 7 d of SSF which further supported the increase in SDF during SSF.

Viability check of *R. oryzae* during SSF

The viability check of *R. oryzae* was carried out at different time intervals of SSF (under continuous rotation with 50% MC) and the obtained results are presented in Figure 6.2.4. The inoculum contained a spore count of 1×10^7 spores per g dried AP samples. In the first 3 d of SSF, the spore count decreased to 5×10^4 spores per g dried AP samples. After 3 d of SSF, the spore count considerably increased and reached 3.2×10^8 spores per g dried AP samples at end of 7 d of SSF. From 7 d to 12 d of incubation, the spore count remained almost unchanged. Towards the last phase (12 d to 16 d) of SSF, spore count decreased to 2.74×10^8 spores per g dried AP samples.The initial decrease in the spore count from 1×10^7 to 5×10^4 spores per g dried AP samples during 0 d to 3 d of SSF was caused by the germination, mycelial propagation and adaption to applied fermentation conditions (colonization) of the fungus (Dhillon *et al*., 2011). However, the increase in the spore count during SSF suggested AP to be a good source of carbon and micronutrients for the fungus that supported sporulation, germination and then mycelial propagation. FA production

profile recorded 3 d onwards also supported the higher metabolic activities of the fungus (Figure 6.2.4).

Phenolic content and profiling

As presented in Table 6.2.1., the TPC of AP was considerably increased (by around 86%) from 185 ± 10.5 to 345 ± 8.5 mg/g lyophilizate after 18 d of SSF under continuous rotation with 50% MC. LC/MS/MS profiling of AP samples showed the presence of some of the important phenolic acids viz. gallic acid, ferulic acid, 4- amino-benzoic acid and vanillic acid and the phenolic aldehyde compound vanillin, as presented in Table 6.2.2. Among the different phenolic acids, gallic acid, ferulic acid and vanillic acid displayed considerable increase in their content after 18 d of SSF. The increase in the contents for gallic acid, ferulic acid and vanillic were by around 6.2 folds (from 6.2 ± 1.3 to 38.56 ± 3.6 mg/g lyophilizate), 6 folds (from 13.54 ± 2.7 to 82.5 ± 5.6 mg per g lyophilizate) and 5.8 folds (from 7.23 ± 1.4 to 42.5 ± 3.8 mg/g lyophilizate), respectively. 4-Amino-benzoic acid content was almost doubled (from 3.4± 1.1 to 6.5 ± 1.4 mg/g lyophilizate), while vanillin content changed only marginally (from 1.8 ± 0.35 to 2.3 ± 0.45 mg/g lyophilizate).

Quantitative estimation of TPC before and after SSF confirmed the efficacy of the fungus *R. oryzae* in degrading AP under the applied SSF conditions. About 86% increase in total phenolic content after 18 d of SSF strongly suggested the release of different phenolics (free, esterified and insoluble-bound) from the hydrolysable lignin and cellulose of AP (Schmidt *et al*., 2014). During SSF, the fungus utilized lignocellulosic moieties for nutrition and this caused the cleavage of the bonds of phenolics with lignin and cellulose. For this purpose, filamentous fungi including *R. oryzae* use extracellular carbohydrolisases and ligninolytic oxidative system for the degradation of phenyl ring and it causes higher TPC. In order to grow and sporulate, *R. oryzae* degraded lignin of AP during SSF and released ferulic acid (a constituent of lignin) (S´anchez-Moreno *et al*., 1998; Martins *et al*., 2011). As the SSF was carried out under aerobic conditions, there was a higher chance that most of the vanillin present in AP was oxidized into vanillic acid during SSF. As the initial vanillic acid content was lower, the enhancement in its content was the result of bioconversion of high vanillin (increased from 7 d to 12 d of SSF, data not shown) into vanillic acid. The estimation of TPC and profiling of phenolic compounds thus confirmed the biodegradation of lignocellulosic moieties of AP by the fungus, *R. oryzae* for its nutritional purpose.

Conclusions

From the present optimization study on the fumaric acid production using the rotating drum type solid-state bench scale bioreactor, it was confirmed that apple pomace worked as the sole source of carbon, micro and macro nutrients for the growth and reproduction of the fungal strain, *Rhizopus oryzae* 1526. The fungus bio-converted the sugars and lignocellulosic biomasses present in apple pomace into fumaric acid under optimum fermentation conditions. The maximum fumaric acid concentration (138 ± 9.1 g/kg at the end of 14 days) was obtained under continuous mode of rotation of the fermentor and with 50% moisture content (w/w) of apple pomace. Sugar consumption analysis confirmed the utilization of glucose, fructose and sucrose by the fungus for fumaric acid production. Analysis of apple pomace fibre composition also confirmed the conversion of insoluble dietary fiber into soluble dietary fiber and utilization of the dietary fibre for fumaric acid production. Viability check during fermentation showed maintenance of a high cell count of 2.74×10^8 spores/g dry apple pomace samples at end of 16 days. Total phenolic content analysis strongly suggested for biodegradation of lignin and cellulose moieties by the fungus during fermentation. LC/MS/MS profiling of AP samples showed the presence of different phenolic constituents (gallic acid, ferulic acid, vanillic acid, vanilli and 4-amino-benzoic acid) and changes in their content after fermentation. Enhancement in ferulic acid content confirmed lignin degradation, while higher content of vanillic acid suggested the maintenance of an aerobic condition during the solid-state fermentation. The outcome of the present investigation ensures the scale-up application of apple pomace for fumaric acid bioproduction.

Abbreviations

FA= fumaric acid, AP= apple pomace, SmF= submerged fermentation, SSF= solid state fermentation, BSF= solid-state bench scale fermenter, TDF= total dietary fibre, IDF= insoluble dietary fibre, SDF = soluble dietary fibre, WHC= water holding capacity, OD= optical density, TPC= total phenolic content, MC= moisture content.

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Table 6.2.1: Fibre composition (dry weight basis) and total phenolic content of apple pomace samples before and after solid state fermentation.

Apple pomace sample	Total dietary fibre %(*w/w*)	Insoluble dietary fiber %(*w/w*)	Soluble dietary Fiber %(*w/w*)	Total phenolic content (mg/g lyophilizate)
Before fermentation	39.4 ± 1.5	32.5 ± 0.7	6.80 ± 0.65	185 ± 10.5
After fermentation	19.15 ± 1.2	17.35 ± 1.3	2.3 ± 0.45	345 ± 8.5

Table 6.2.2: Phenolic composition of apple pomace before and after solid state fermentation.

Compound	Concentration (mg/g lyophilizate) before solid state fermentation	Concentration (mg/g lyophilizate) after solid state fermentation
Gallic acid	6.2 ± 1.3	38.56 ± 3.6
Vanillin	1.8 ± 0.35	2.3 ± 0.45
Ferulic acid	13.54 ± 2.7	82.5 ± 5.6
4-amino – benzoic acid	3.4 ± 1.1	6.5 ± 1.4
Vanillic acid	7.23 ± 1.4	42.5 ± 3.8

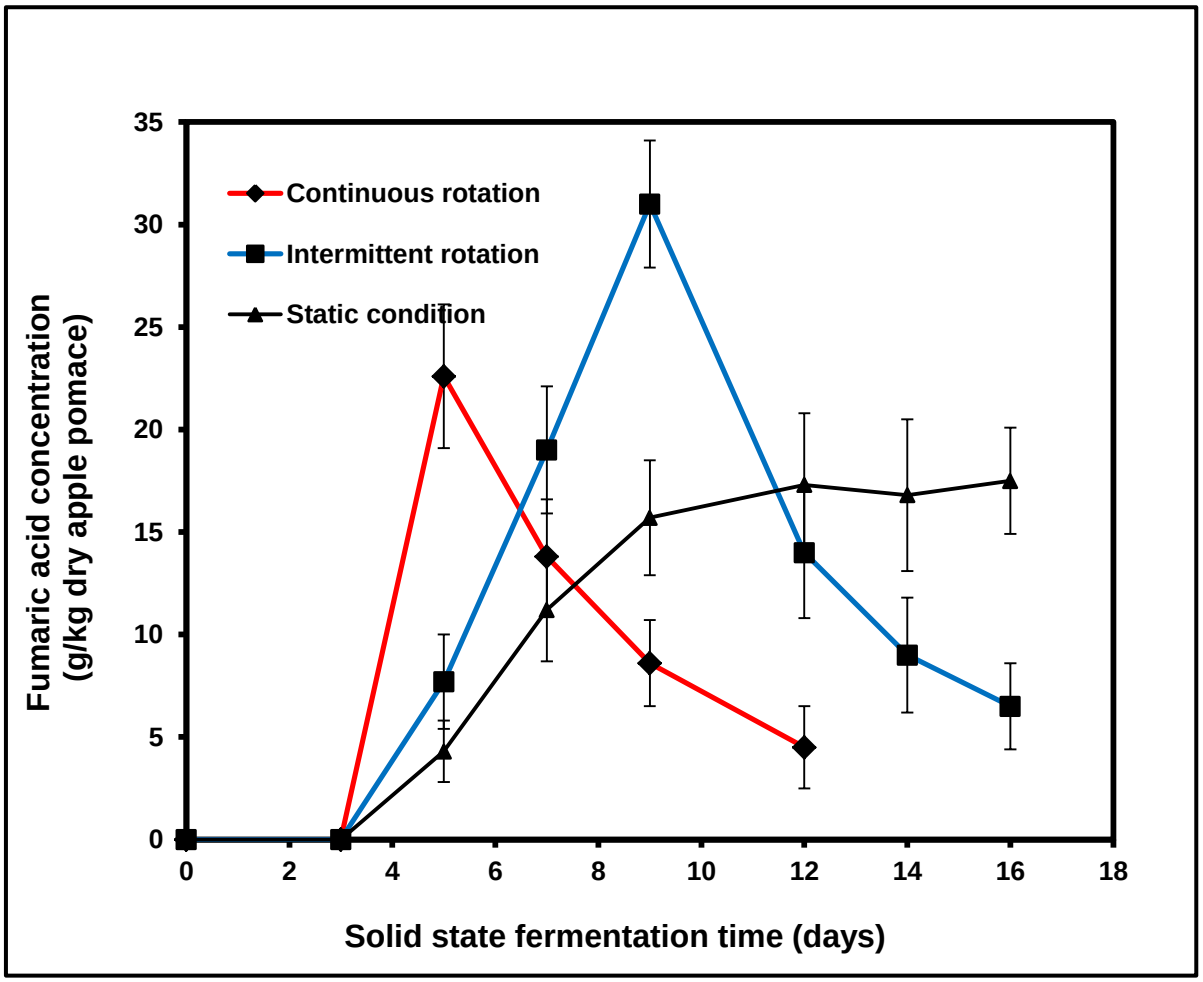

Figure 6.2.1: The fumaric acid concentrations (g/kg dry apple pomace) achieved with 70% (*w/w*) of moisture content of apple pomace under continuous rotation, intermittent rotation and static mode of the bench scale fermenter.

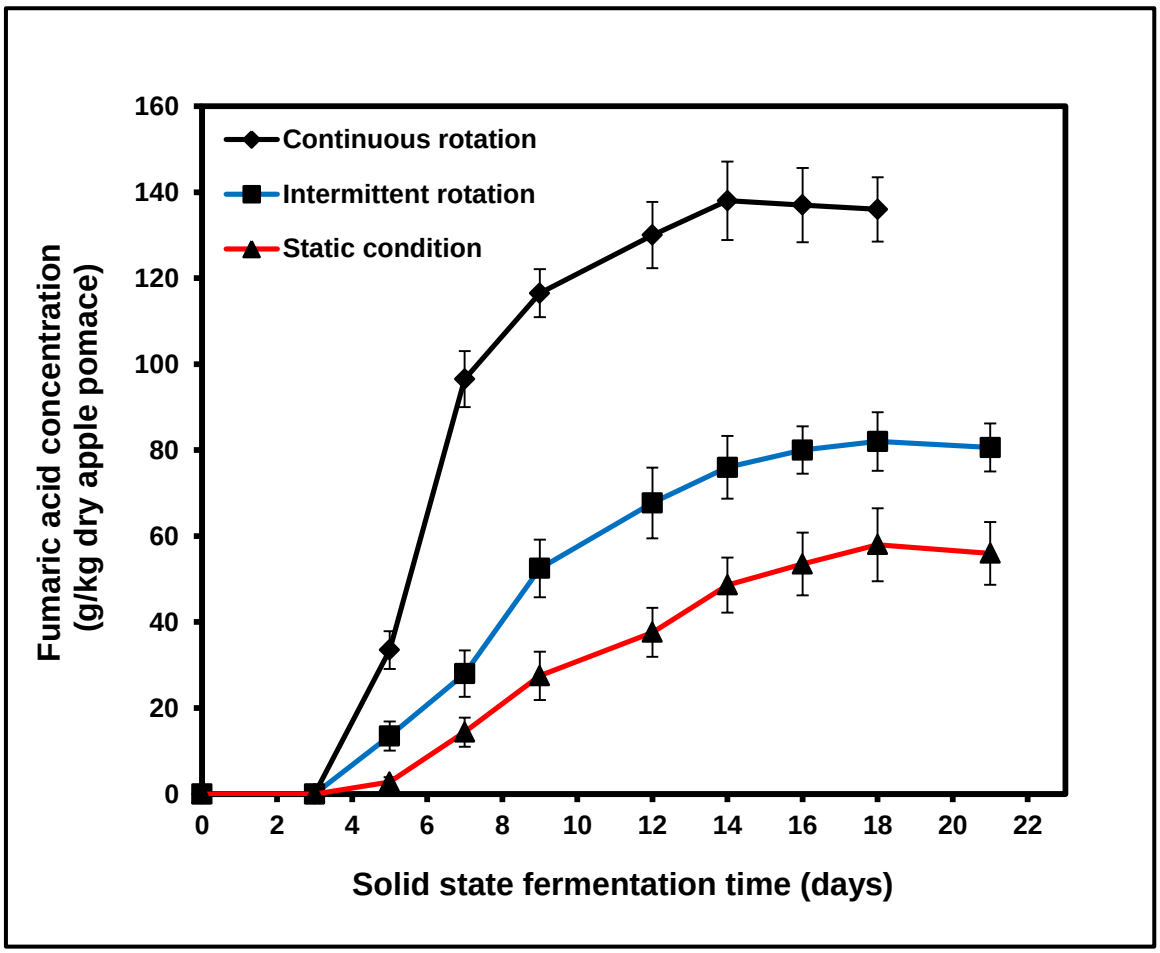

Figure 6.2.2: The fumaric acid concentrations (g/kg dry apple pomace) achieved with 50% (w/w) of moisture content of apple pomace under continuous rotation, intermittent rotation and static mode of the bench scale fermenter.

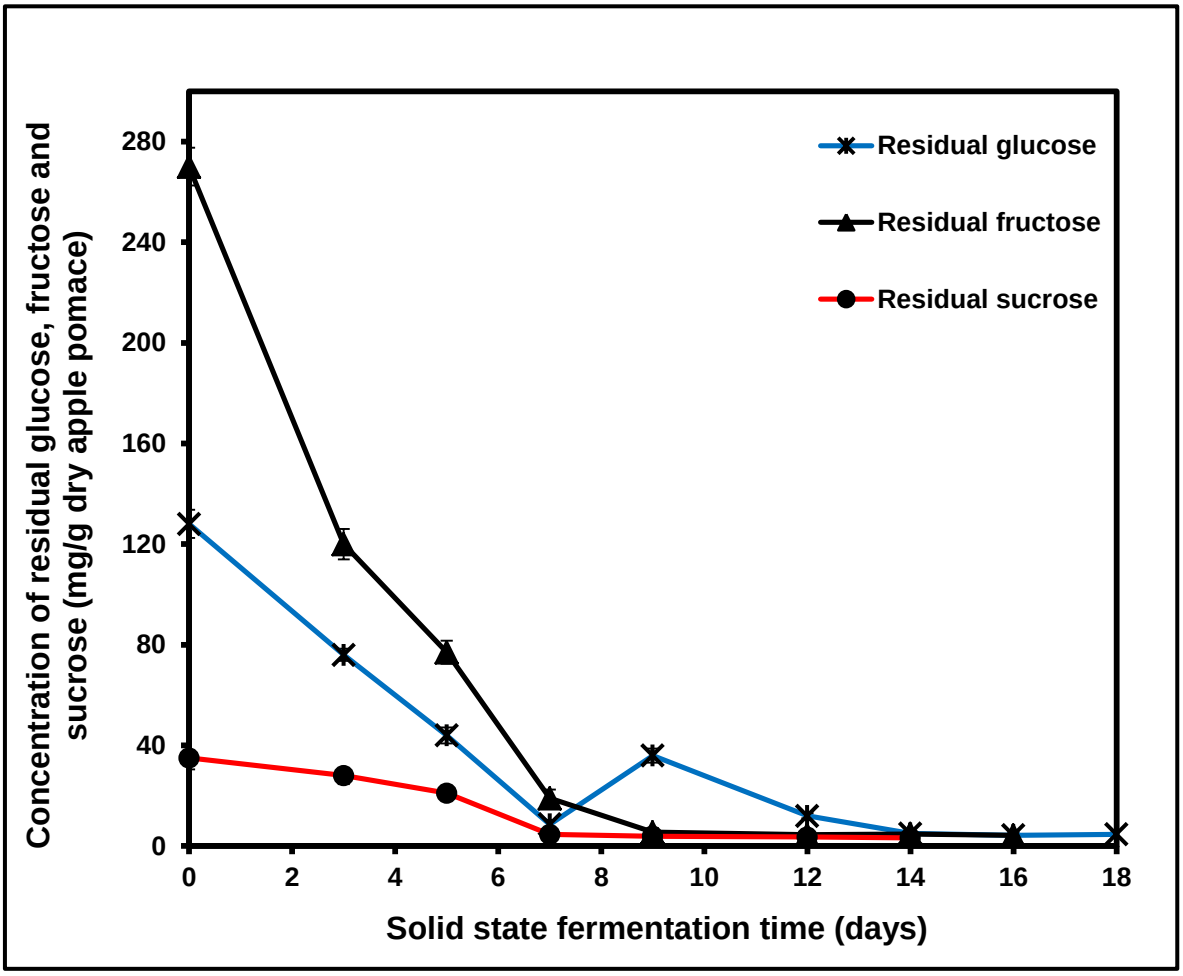

Figure 6.2.3 (A) Changes in the concentrations (mg/g dry apple pomace) of residual glucose, fructose and sucrose during solid state fermentation under continuous rotation and with 50% (w/w) of moisture content of apple pomace.

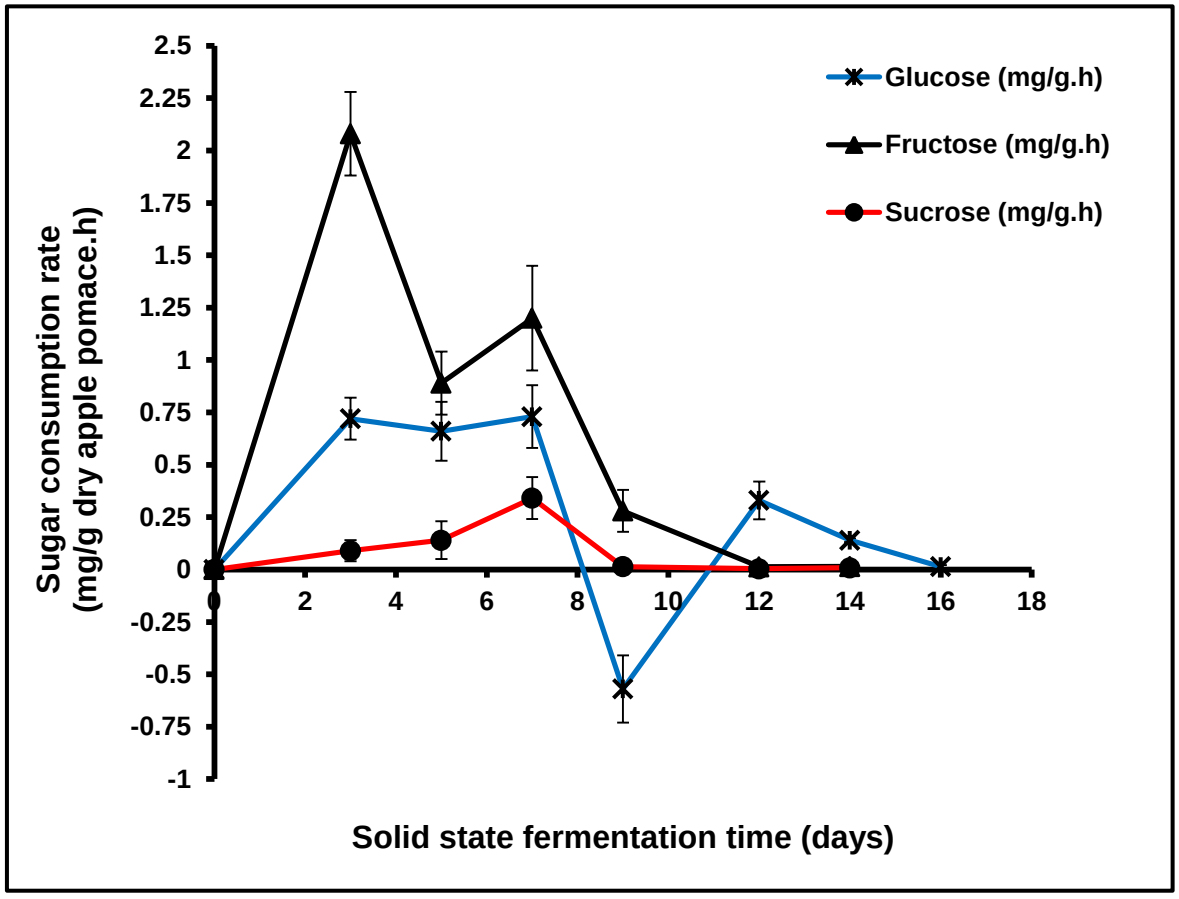

Figure 6.2.3 (B): Changes in the consumption rate (mg/(g h) dry apple pomace) of glucose, fructose and sucrose, during solid state fermentation under continuous rotation and with 50% (w/w) of moisture content of apple pomace.

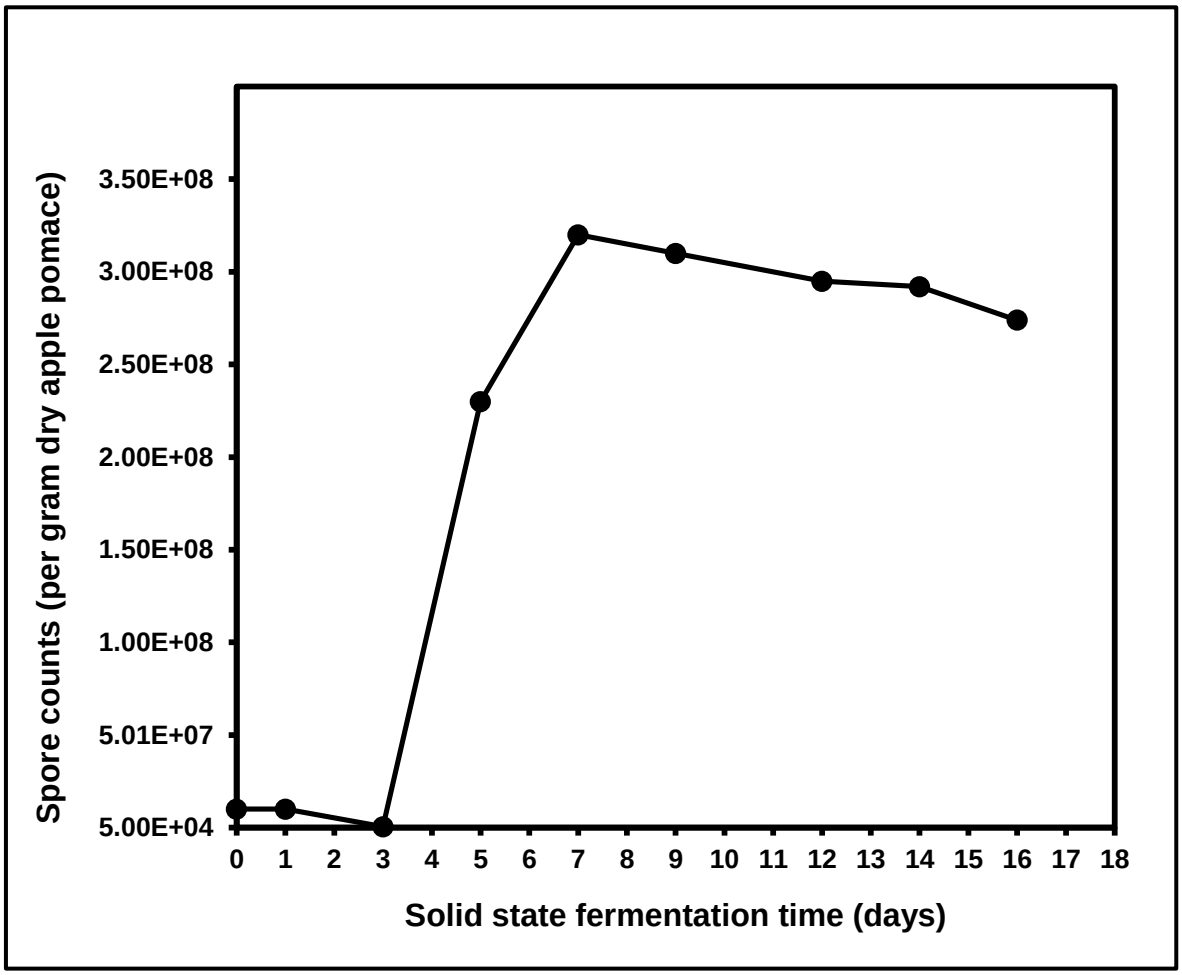

Figure 6.2.4: Changes in the spore count (per gram dry apple pomace) of the fungus *R. oryzae* during solid state fermentation under continuous rotation and with 50% (w/w) of moisture content of apple pomace.

END OF CHAPTER 6

CHAPTER 7

EFFECTS OF VARIOUS METALLIC NANOPARTICLES ON FUMARIC ACID PRODUCTION

EFFECTS OF DIFFERENT METALLIC NANOPARTICLES ON GERMINATION AND MORPHOLOGY OF THE FUNGUS *Rhizopus oryzae* 1526 AND CHANGES IN THE PRODUCTION OF FUMARIC ACID

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Résumé

La présente recherche a été effectuée pour étudier l'impact des différentes concentrations (200-1000 µg/mL) des micro et nanoparticules de ZnO, Fe_3O_4 et MnO_2 sur la germination des spores et la morphologie du champignon *Rhizopus oryzae* 1526 (*R. oryzae*) produisant l'acide fumarique (AF, en anglais FA). Le profil de production de FA a également été étudié en réponse aux micro- et nano-formes de ces composés. Le milieu de base glucose-sel a été utilisé pour la croissance et la production de FA. Différentes conditions d'incubation ont été appliquées pour la croissance de ce champignon (30°C, 200 rpm d'agitation et 24 h d'incubation) et la production de FA (30 °C, 200 rpm et 72 h). Des essais témoins ont été réalisés sans les micro- et nano-formes des composés inorganiques. En présence de 200 µg/mL de micro et de nanoparticules de ZnO, Fe_3O_4 et MnO_2, le pourcentage de germination des spores obtenu est presque identique (96-98%), aux essais témoins, à l'exception des microparticules de MnO_2 (85%). Les concentrations plus élevées (> 200 µg/mL) de micro et de nanoparticules ont provoqué une inhibition de la germination des spores. Le champignon a montré des changements dans la taille et dans la morphologie des granules à différentes concentrations de particules. La production de FA a également été influencée par les micro et les nanoparticules de ZnO, Fe_3O_4 et MnO_2. Globalement, Fe_3O_4 a été jugé comme étant la plus biocompatible à la fois sous la forme micro et nano. Les résultats de ce travail de recherche suggèrent que les trois cations essentiels (Zn^{2+}, $Fe^{2+/3+}$ et Mn^{2+}) peuvent avoir des effets stimulateurs ou inhibiteurs différents sur le champignon lorsqu'ils sont utilisés sous formes de micro ou de nanoparticles.

Mots clés: *Rhizopus oryzae* 1526, microparticules, nanoparticules, germination, morphologie, acide fumarique.

Abstract

The present study was undertaken to investigate the impact of different concentrations (200-1000 µg/mL) of micro- and nanoparticles of ZnO, Fe_3O_4 and MnO_2 on spore germination and morphology of the fumaric acid (FA)-producing fungus *Rhizopus oryzae* 1526 (*R. oryzae*). Production profile of FA was also investigated as a response to micro- and nano-forms of these compounds. Glucose-salts media was used for the growth and FA production. Different incubation conditions were applied for growth (30 °C, 200 rpm and 24 h) and FA production (30 °C, 200 rpm and 72 h). Control experiments were designed without the micro- and nano-forms of the inorganic compounds. At 200 µg/mL of micro- and nanoparticles of ZnO, Fe_3O_4 and MnO_2, percent of spore germination obtained were almost the same (96-98%) as control experiments, except for microparticles of MnO_2 (85%). Higher concentrations (> 200 µg/mL) of the micro- and nanoparticles caused inhibition in spore germination. The fungus exhibited changes in the pellet size and morphology at different concentrations of the particles. FA production was also influenced by micro- and nanoparticles of ZnO, Fe_3O_4 and MnO_2. Overall, Fe_3O_4 was found to be the most biocompatible in both micro and nanoform. Findings of the present research work suggested that the three essential cations (Zn^{2+}, $Fe^{2+/3+}$ and Mn^{2+}) can have different impacts on the fungus when applied as micro- and nanoformulations.

Keywords: *Rhizopus oryzae* 1526, microparticles, nanoparticles, germination, morphology, fumaric acid.

Introduction

Influences of trace metals cations such as Zn^{2+}, Fe^{2+} and Mn^{2+} on the morphology of filamentous fungi have been studied for many fungal model organisms (Foster *et al*., 1939; Couri *et al*., 2003; Papagianni *et al*., 2004). These studies confirmed that the changes in the concentration of these trace elements in the culture media can affect the morphology (dispersed and/or free filaments, clump and pellets) of filamentous fungi. Moreover, these metal ions act as cofactors or activators for many cellular enzymes involved in catabolism and biosynthesis of macromolecules (DNA and RNA). For the *Rhizopus* species of filamentous fungi, morphology is highly influenced by these trace elements and morphological features finally affect the production profile of fermented products (Das *et al*., 2014; Das *et al*., 2015a, b). Presence of Zn^{2+}, Fe^{2+} and Mn^{2+} in culture medium and their concentrations are significant as these three elements were shown to influence pellet formation for different *Rhizopus* species (Foster *et al*., 1939; Xu *et al*., 2012; Liao *et al*., 2007). Absence or supplementations of these trace metals in growth medium showed specific effects in terms of pellet features and growth behaviour. However, studies using the nanoparticles (NPs) of Mn^{2+}, Zn^{2+} and Fe^{2+} cations have not been performed earlier with Rhizopus species. ZnO NPs have great potential as an effective fungicide in controlling diseases and previous studies on the fungus Rhizopus stolonifer showed significant inhibition in the germination of spores (Wani *et al*., 2012). ZnO can compete well with commercial antifungal feed additives, such as probiotic, propionic acid and clove oil (Shah *et al*., 2010). Fe_3O_4 NPs have gained more attention due to their special uses as antibacterial, antifungal and antiviral agent (Nabawy *et al*., 20140). Although reports on MnO_2 NPs as antibacterial agents are available, its antifungal property has not been well explored.

In the present investigation, ZnO, Fe_3O_4 and MnO_2 NPs were investigated for their effects on the growth (germination and morphology) of the filamentous fungal strain *Rhizopus oryzae* 1526 (to be called *R. oryzae* hereafter). For a comparative study, microparticles (MP) of Mn^{2+}, Zn^{2+} and Fe^{2+} cations were also used. Recently, the fungal strain *R. oryzae* has gained tremendous importance due to its higher efficiency in the production of the organic acid, fumaric acid (FA), through fermentation. Commercial importance of *Rhizopus* species for FA production has been reviewed by different experts and many strains have been patented for industrial production (Xu *et al*., 2012; Roa Engel *et al*., 2008; Kane *et al*., 1943; Lubowitz *et al*., 1958; Goldberg *et al*., 1986; Ling *et al*., 1989). It is a consensus view that this fungal strain is the frontline producer of FA using submerged fermentation (SmF) conditions (Oda *et al*., 2003). The product, FA and its ester derivatives (FAEs) have been recently explored for newer biomedical applications for treating human diseases, such as multiple sclerosis, psoriasis and cardiac inflammatory conditions (Mrowietz *et al*., 2005; Belge *et al*., 2014;

Loewe *et al*., 2011). Moreover, FA and FAEs have found wider applications in tissue engineering and drug delivery (Temenoff *et al*., 2007; Horch *et al*., 2004; Desai *et al*., 2013; Skokri *et al*., 2011). For human safety, production of FA through biological routes, such as SmF has been emphasized over chemical route (Roa Engel *et al*., 2008; Kang *et al*., 2010). Literature data shows that SmF conditions of 30 °C, 200 rpm for 24 h are optimum for the growth of this fungus in glucose-basic salts medium and results in small (1-2-mm size) pellets. These pellets are used for inoculating production medium for FA production under SmF conditions of 30 °C, 200 rpm for 72 h. Authors have performed extensive studies on these aspects and literature also supports these growth conditions as optimum (Das *et al*., 2014; Das *et al*., 2015a, b; Gu *et al*., 2013). Exposing the fungus to MPs and NPs of Mn^{2+}, Zn^{2+} and Fe^{2+} cations during optimal growth (30 °C, 200 rpm for 24 h) and production (30 °C, 200 rpm for 72 h) conditions can generate important data on the effects of these cations on the physiological processes (germination, mycelia development and FA production) of the fungus. Taking all aforementioned aspects into consideration, toxicity effects of the MPs and NPs were investigated on the fungal strain *R. oryzae*. The novelties of this study can be summarized as follows: (a) the first ever study on the toxicity effects of MPs and NPs of Mn^{2+}, Zn^{2+} and Fe^{2+} cations on the fungal strain *R. oryzae*; (b) growth of *R. oryzae* and FA production profile were investigated against different applied concentration ranges of MPs and NPs of Mn^{2+}, Zn^{2+} and Fe^{2+} cations and (c) a conclusive evidence on the size effects of the same metallic cations applied as MPs or NPs on the fungal morphology and production efficiency.

Materials and methods

Materials

The fungal strain, *R. oryzae* NRRL 1526 was procured from the Agricultural Research Services (ARS) culture collection IL, USA. All the chemicals (glucose-basic salts medium composition, $ZnSO_4 \cdot 7H_2O$, NaOH, $FeCl_3$, $FeCl_2$, $MnSO_4$, C_2MnO_4, ZnO, Fe_3O_4 and MnO_2) used were of analytical grade and purchased from Fisher Scientific (Ottawa, Ontario, Canada).

Microbial culture and media preparation

The procured fungal strain was first cultured on a potato dextrose agar (PDA) slant at 37 ± 1 °C for a maximum of 4 days. Spread plate method was used to prepare spore inoculum by propagating the spores on PDA plates (90 mm) at 37 ± 1 °C for 72 h. Mycelium free spores were collected in sterile distilled water (dH_2O) after filtration through sterile cotton wool. For regular use, the spore suspension was maintained at 4 °C. Spores were also preserved at

−80 °C after adding 20% glycerol solution for long-term use. A stock of 1×10^8 spores/mL was maintained and used for inoculation. For the growth of *R. oryzae*, glucose-basic salts medium (g/L: glucose 50, urea 2, KH_2PO_4 0.6, $MgSO_4{\cdot}7H_2O$ 0.5, $ZnSO_4{\cdot}$ $7H_2O$ 0.11 and $FeSO_4{\cdot}7H_2O$ 0.0088) was used. For FA production, the glucose and urea concentration were maintained at 80 and 0.2 g/L, respectively. The medium final pH (4.6) was not adjusted, unless specifically indicated. To avoid Maillard reaction, growth medium was prepared in two components: (a) glucose and (b) urea + salts and were heat sterilized (20 min, 15 psi, 121 ± 1 °C) separately. Room temperature cooled media components were mixed together inside a laminar hood and used for the pre-culture of *R. oryzae*. Culture of *R. oryzae* was prepared by inoculating 50 mL of growth medium with 2%(v/v) spore suspension in a 250-mL Erlenmeyer flask and then incubating at 30 °C, 200 rpm for 24 h.

Submerged fermentation production of FA

For SmF-based FA production, 2.63 mL (final inoculum concentration 5%, v/v) of cell pellets of *R. oryzae* was transferred into 50-mL Erlenmeyer flasks containing 47.37 mL of FA production medium. The inoculated flasks were incubated at 30 °C, in a rotary shaker at 200 rpm for 72 h. Sterilized calcium carbonate ($CaCO_3$) was used as a neutralizing agent at 70 g/L.

Preparation of different metallic nanoparticles

ZnO, Fe_3O_4 and MnO_2 NPs were prepared by following the methods previously mentioned with some modification (Kumar *et al*., 2013; Hyeon, 2002; Kumar *et al*., 2013). The preparation steps are briefed below.

Preparation of ZnO nanoparticles: To 100 mL of 1 M zinc sulphate aqueous solution, 40 mL 2 M sodium hydroxide solution was slowly added dropwise under vigorous stirring (700 rpm) condition and the stirring was continued for 12 h. The obtained precipitate was first filtered and then washed thoroughly with dH_2O. The sample was ground to fine powder using an agate mortar. The powder obtained from the above method was finally calcined in a muffle furnace at 500 °C for 4 h. ZnO NPs powder hence obtained was used for the present study.

Preparation of Fe_3O_4 nanoparticles: Co-precipitation method was used to prepare the Fe_3O_4 NPs. An amount of 32.44 g of ferric chloride and 12.675 g of ferrous chloride was dissolved in 100 mL of dH_2O with a molar ratio of 2:1. The mixture solution was then stirred and heated to 60 °C. As magnetite (Fe_3O_4) is sensitive to oxidation (transformation to maghemite i.e. Fe_2O_3), N_2 gas was continuously purged through the mixture solution followed by slow addition of 50 mL of 2.5 M NaOH keeping the reaction temperature at 60

°C. The reaction mixture was left undisturbed for 30 min. The black-coloured reaction product (Fe_3O_4 NPs) was then removed from the solution by magnetic separation and washed with dH_2O and ethanol, respectively. Finally, collected Fe_3O_4 NPs were re-dispersed in dH_2O and lyophilized and kept for future use.

Preparation of MnO_2 nanoparticles: MnO_2 NPs were prepared by co-precipitation method. About 50 mL and 0.2 M concentration of both manganese sulphate and manganese oxalate aqueous solutions were mixed under magnetic stirring conditions at 60 °C. The pH of the mixture solution was adjusted to 12 with NaOH. The resultant solution was left under same reaction condition for another 2 h. The brown-coloured reaction product was collected by filtration and then washed three times with ethanol. The precipitate was oven dried at 80 °C overnight. Dried sample was calcined in a muffle furnace at 500 °C for 4 h. The MnO_2 NPs powder, hence, produced was used for the present study.

Characterization of the synthesized nanoparticles

Morphological characteristics of ZnO, Fe_3O_4 and MnO_2 NPs prepared during this study were investigated by a scanning electron microscope (Zeiss EVO® 50 Smart SEM system). Size of the prepared nanoparticles was measured by zetasizer nanoZS (Malvern instruments Ltd., UK). For size measurement, the nanoparticles were dispersed in Milli-Q water.

Treatment of *R. Oryzae* with ZnO, Fe_3O_4, MnO_2 MPs and NPs

Different concentrations (200-1000 µg/mL) of ZnO, Fe_3O_4 and MnO_2 MPs and NPs were applied in the growth medium of the fungus *R. oryzae*, and incubated at 30 °C, 200 rpm for 24 h. The fungus was also treated with the ZnO, Fe_3O_4 and MnO_2 MPs and NPs at 200-1000 µg/mL concentrations range during SmF-based FA production. The SmF conditions were maintained at 30 °C, 200 rpm for 72 h. Before application, MPs and NPs of ZnO, Fe_3O_4, MnO_2 were heat sterilized (20 min, 15 psi, 121 ± 1 °C). Control experiments were designed without the MPs and NPs of ZnO, Fe_3O_4 and MnO_2 for the growth and FA production media.

Morphological studies

The morphology of *R. oryzae* obtained with different NPs was studied with digital photography (Canon PC 1585).

Percent spore germination

To count the percent spore germination, the following formula was used:

$$\text{Percent spore germination} = \frac{\text{Number of spore germinated}}{\text{Total number of spore examined}} \times 100 \quad (1)$$

High-performance liquid chromatographic (HPLC) analysis of FA

Quantification of FA in the fermented broth samples was carried out by HPLC analysis. The method of Zhou *et al*. (2000) was followed for HPLC sample preparation. The technical details of the HPLC were as follows: System: DIONEX DX500, with an Acclaim OA, 5 µm, (4.6 × 150 mm) column with a refractive index detector (PDA-100 DIONEX, UV, 210 nm). 2.5mMmethanesulfonic acid (CH_3SO_3H) at a flow rate of 1 mL/min was used as the mobile phase and column temperature was 30 °C.

Statistical analysis

Data are represented as mean ± SD of three independent experiments. Correlations were considered significant at $P < 0.05$ for different applied parameters.

Results and discussion

Culture medium and growth conditions of *R. Oryzae*

The culture medium and the growth conditions (30 °C, 200 rpm for 24 h) chosen for the fungus *R. oryzae* are very common and optimized parameters for achieving small-sized cell pellets. In many previous studies, these conditions were applied during the pre-culturing step (inoculum preparation) of the fungus for fermentation purpose (Das *et al*., 2014; Das *et al*., 2015a, b). Moreover, the FA production medium with high carbon to nitrogen (C:N= 400:1) and fermentation conditions (30 °C, 200 rpm for 72 h) used in this study is well explored. To study the effects of newly introduced factors (MPs and NPs of ZnO, Fe_3O_4 and MnO_2) into the growth or production media, this parameter setup was considered to be optimum for the present study. The pellets morphology obtained under these conditions represented the results of control (without MPs and NPs) experiments. These pellets were used as inoculum for the SmF-based FA production. Variations in FA production profile were correlated with the changes in the initial pellet morphology of the inoculum after being exposed to MPs and NPs of ZnO, Fe_3O_4 and MnO_2.

Choosing ZnO, Fe_3O_4 and MnO_2 for the present study

Presence of Zn^{2+}, Fe^{2+} and Mn^{2+} ions in the growth medium is essential for *R. oryzae*, and they play an important role in shaping the morphological forms and pellet diameter (Foster *et al*., 1939). Changes in the concentrations of these ions were found to alter the growth and morphology of the fungus. However, it was not explored whether NPs containing these three elements (ZnO, Fe_3O_4 and MnO_2) might have an influence on the morphology of the fungus. Moreover, finding the concentration tolerability of the fungus to these NPs is also an important factor. It is also pertinent to study the effects of MPs of ZnO, Fe_3O_4 and MnO_2 on the growth of the fungus. As this fungal strain is the frontline FA producer, changes in the morphology and FA production after treatment with MPs of ZnO, Fe_3O_4 and MnO_2 is an interesting area to investigate. Thus, relevancies with some general findings of previous studies and requirement of investigation in a new research area highly encouraged us to carry out the present study.

Characterization of ZnO, Fe_3O_4 and MnO_2 nanoparticles

Shape, size and aggregation of the NPs were displayed by SEM analysis. For ZnO NPs, mean size was around 158 ± 10 nm and roughly of spherical shape (Figure 7.1). The mean size of Fe_3O_4 NPs was around 296 ± 10 nm and revealed the abundance of spherical particles (Figure 7.2). For MnO_2, a mean size of 249 ± 22 nm was obtained and particles were of polymorphic nature (Figure 7.3). The size distribution spectra of the prepared NPs are displayed in Figure 7.4.

Effects of ZnO, Fe_3O_4 and MnO_2 NPs on the growth of *R. Oryzae*

ZnO, Fe_3O_4 and MnO_2 NPs displayed different effects on spore germination and morphology of *R. oryzae*. In the applied concentration range from 200-1000 µg/mL of the NPs, the changes in pellet size and germination behaviour was qualitatively accessible. As shown in Figure 7.5, at the lowest applied concentration (200 µg/mL) of ZnO NPs, the germination of the sporangiospores in the growth medium was 96.7 ± 0.8% and comparable to the results obtained in the control experiment (98.5 ± 1.0). The next higher concentration (400 µg/mL) of ZnO NPs caused inhibition in spore germination (78 ± 2.5%), and at 600 µg/mL, it reached the minimum (33 ± 1.8%). No germination occurred beyond the 600 µg/mL concentration of ZnO NPs. The NPs treated spores resulted in pellet morphology at 200-600 µg/mL ZnO concentrations with variation in the pellet diameter as shown in Figure 7.6 (A) to (C). At 200 µg/mL, the average pellet diameter was < 1 mm (Figure 7.6 (A)). The pellets obtained at 400 µg/mL were of mostly large sized (ca. 2-3 mm on average) and some spores

germinated into mycelial clumps (Figure 7.6 (B)). At 600 µg/mL concentration of ZnO NPs, bigger pellets (4-5 mm) were formed (Figure 7.6 (C)). The overall effects of the different applied concentrations of ZnO NPs can be explained on the basis of antimycotic effect of ZnO and possible role of Zn in morphology of the fungus *R. oryzae*. The applications of ZnO NPs as antibacterial, antifungal and anti-odour are well-established (Wani *et al*., 2002). In the present study, inhibition of spore germination with increased ZnO NPs concentration was well manifested. ZnO NPs at 600 µg/mL was found to be the threshold concentration for the fungus as no germination of the spores occurred beyond this concentration. ZnO NPs caused changes in the pellet diameter and sizes of the pellets increased with higher concentration of the NPs. Zn^{2+} cations are vital for the fungus and affect the cellular mechanisms of fungal growth and physiology (Couri *et al*., 2003). At 200 µg/mL, the average pellet diameter was < 1 mm and this was smaller for the controlled ones (ca. 1-2 mm). The assimilation of media components and growth of the fungus has been found to be controlled by the concentration of Zn^{2+} cations (Hughes *et al*., 1989). The morphological shift from smaller to bigger pellets as a response to concentration gradient of ZnO NPs might be a Zn^{2+} cations stress-tolerating strategy of the fungus. It is well-known that larger fungal pellets suffer from shortage of required nutrients at their central region and this leads to a less metabolically active conditions in the fungal pellets (Papagianni *et al*., 2004). In the present study, higher concentration of ZnO NPs caused bigger pellets, thus indicating retraction from metabolically active stage. Assimilated nutritents were utilized for more biomass production (bigger pellets) to resist the stress condition of high ZnO NPs concentration. However, the antifungal property of ZnO as NPs was very much effective as germination percentage was considerably reduced. Thus, the present study confirmed the antifungal effects of ZnO against the fungus, *R. oryzae*, with new information on morphological impacts.

The effects of Fe_3O_4 NPs on spore germination and pellet diameter of *R. oryzae* at different applied concentrations (200-1000 µg/mL) are shown in Figure 7.5 and Figure 7.7. In the concentration range from 200-600 µg/mL, spore germination percentage and pellet diameter were comparable to the controlled ones. The spore germination was nearly 98% and pellet diameter was in the range of 1-2 mm (Figure 7.7 (A) to (C)). At 800 µg/mL, the germination was inhibited and reduced to 65.2 ± 1.8%. The pellet diameter was increased (ca. 4-5 mm) and mycelial clumps were formed (Figure 7.7 (D)). At 1000 µg/mL, there was no germination at all. The role of Fe^{2+} ions in the growth and metabolic activities of model filamentous fungi has been explored earlier (Couri *et al*., 2003; Friedrich *et al*., 1990). Basically, Fe^{2+} acts as cofactor for many enzymes and is essential for biomass production and also influences the fungal morphology. The present study with Fe_3O_4 NPs showed a higher tolerance level of Fe (600 µg/ mL) as there was no evidence of changes in percent of spore germination and

pellets diameter as compared to control experiment. As compared to ZnO NPs, the stress imposed by Fe_3O_4 NPs was less responsive. Retaining of almost same pellet diameters up to 600 µg/mL and no considerable change in biomass production suggested for unchanged metabolic activities of *R. oryzae*. The influence of Fe_3O_4 NPs on spore germination, pellet diameter and morphological forms (pellets to mycelial clumps) at higher concentration (> 600 µg/mL) was a cumulative effect and was difficult to pinpoint the particular role of Fe_3O_4 NPs.

The MnO NPs exhibited different effects on the germination and pellet diameter of *R. oryzae* as shown in Figure 7.5 and Figure 7.8. At 200 µg/mL, spore germination was reduced to 92.5 ± 1.8% with formation of some mycelial clumps and pellet obtained were of 2-3 mm in diameter (Figure 7.8 (A)). The next higher concentration of 400 µg/mL considerably inhibited spore germination and brought it down to about 40.5 ± 2.3%. At this concentration, some pellets with larger diameter (4-5 mm) were formed (Figure 7.8 (B)). Spore germination was almost completely inhibited (8.6 ± 1.2%) at the next higher concentration (400 µg/mL) of MnO_2 NPs (Figure 7.8 (C)) and above this concentration, there was no growth. Mn^{2+} plays an important role in the normal activities of many enzymes involved in anabolism and growth phase of filamentous fungi, such as *Aspergillus niger* (Couri *et al*., 2003; Kisser *et al*., 1980). Moreover, its role in protein synthesis in this model fungus has also been investigated earlier (Kubicek *et al*., 1939; Yang *et al*., 2009). The results of the present study showed that MnO_2 NPs influenced the spore germination and pellet diameter of *R. oryzae* at different applied concentrations. Mn^{2+} is not an ingredient in the commonly used growth medium for *R. oryzae*, but supplementation of this in the form of NPs was more available for the fungus. At 200 µg/mL MnO_2 NPs, the spore germination and pellet features almost matched results of the control experiments and this indicated the tolerability of the fungus against Mn^{2+} cations. The next two higher concentrations (400 and 600 µg/mL) were found to be inhibitory, and this may be attributed to the strategic adaptation of the fungus towards ionic stress.

Effects of ZnO, Fe_3O_4 and MnO_2 MPs on the growth of *R. oryzae*

The effects of ZnO, Fe_3O_4 and MnO_2 MPs on spore germination and morphology of *R. oryzae* were found to be different as compared to the NPs. The spore germination profile obtained against the applied concentration range of 200-1000 µg/ mL for each type of MPs is presented in Figure 7.9. At 200 µg/mL concentration, the spore germination obtained for ZnO, Fe_3O_4 and MnO_2 MPs were 97.5 ± 0.8, 98.2 ± 0.78 and 85 ± 1.67%, respectively. As compared to the percent spore germination (92.5 ± 1.8%) recorded for MnO_2 NPs, MPs of MnO_2 inhibited spore germination. In the next higher concentration range (400-1000 µg/mL), ZnO, Fe_3O_4 and MnO_2 MPs exhibited different effects on spore germination. For ZnO MPs,

spore germination was completely inhibited at 800 µg/mL. Interestingly, Fe_3O_4 microparticles maintained high percent spore germination (95.7%) up to 1000 µg/mL. For MnO_2 MPs, spore germination was brought down to 36% at 1000 µg/mL concentration. The comparison of the germination inhibition effects of MPs and NPs of ZnO, Fe_3O_4 and MnO_2 clearly indicated the toxic nature of Zn^{2+}, $Fe^{2+/3+}$ and Mn^{2+} cations when applied as nanoparticles. As compared to complete germination inhibition at 800 µg/mL for NPs of Fe_3O_4, MPs were found to be tolerable up to 1000 µg/mL. Similarly, contrary to the complete germination inhibition recorded at 800 µg/mL for MnO_2 NPs, 1000 µg/mL concentration of MnO_2 MPs reduced germination inhibition up to 40 %. ZnO did not affect spore germination when applied as MPs. Increased tolerability of the fungus towards MPs of Fe_3O_4 and MnO_2 was an indication of size-dependent toxicity of these compounds. The morphology of the fungus, *R. oryzae* changed against the different applied concentrations of ZnO, Fe_3O_4 and MnO_2 MPs as presented in Figure 7.10, 7.11 and 7.12, respectively. For ZnO MPs, small pellets (ca. 1 mm) were formed at 200 µg/ mL (Figure 7.10 (A)) and changed to hairy aggregated pellets as concentration reached to 400 µg/mL (Figure 7.10 (B)). At next higher concentrations (600 µg/mL), germinated spores did not develop into particular morphological form. At 800 µg/mL concentration, there was no growth as spore germination was completely inhibited. For Fe_3O_4 MPs, irrespective of the applied concentration, highly dense suspended mycelia were formed. The mycelia formed at 200 and 1000 µg/mL are presented in Figure 7.11 (A) and (B), respectively. Similar morphologies were formed at 400, 600 and 800 µg/mL concentrations of Fe_3O_4 MPs (data is not shown). The light blackish hue of the suspended mycelia was imparted by the unconsumed Fe_3O_4 MPs. For MnO_2 MPs, mixed morphologies (pellets and suspended mycelia) were formed at 200, 400 and 600 µg/mL concentrations (Figure 7.12 (A) to (C)). At 800 and 1000 µg/mL concentrations, dense suspended mycelia were formed (Figure 7.12 (D) and (E)). Likewise in Fe_3O_4 microparticles, the blackish colour of the pellets and suspended mycelia were imparted by the unconsumed MnO_2 microparticles. The morphological responses to ZnO, Fe_3O_4 and MnO_2 MPs differed from those obtained with NPs at respective concentrations. Higher tolerance of *R. oryzae* to Fe^{2+} and Mn^{2+} ions applied as MPs and changes in the morphological forms suggested adaptability of the fungus towards ionic stress.

Effects of ZnO, Fe_3O_4 and MnO_2 NPs and MPs on FA production

As *R. oryzae* is well-known for morphological directed biosynthesis of FA under different applied SmF conditions, it was interesting to investigate the effects of ZnO, Fe_3O_4 and MnO_2 MPs and NPs on SmF-based FA production. For the control SmF experiment, FA concentration reached 68.45 ± 2.5 g/L at the end of 72 h of incubation. The SmF production

profile obtained for FA after exposure to the different concentrations (200-1000 µg/mL) of ZnO, Fe_3O_4 and MnO_2 NPs is presented in Figure 7.13 (A). Among the three NPs, ZnO NPs caused the minimum FA production (5.22 ± 1.45 g/L) at the lowest applied concentration of 200 µg/mL. At this NPs concentration, the FA productivity for Fe_3O_4 and MnO_2 NPs were 36 ± 2.3 and 18.75 ± 1.8 g/L, respectively. The inhibitory effect of higher applied concentrations of the NPs on FA production was displayed by all the three type of NPs. At 1000 µg/mL NPs concentration, FA production (19.8 ± 2.45 g/L) was obtained only for Fe_3O_4 NPs and for the rest two type NPs (ZnO and MnO_2 NPs), the fungal growth was completely inhibited resulting in no FA production. Considering the inhibitory effects of the NPs on FA production during SmF, the order of ZnO > MnO_2 > Fe_3O_4 NPs was considered conclusive. For the MPs of ZnO, Fe_3O_4 and MnO_2, a similar trend for inhibitory effects was monitored. The results are presented in Figure 7.13 (B). However, as compared to MnO_2 and Fe_3O_4 NPs, the FA production at 200 µg/mL concentration of MnO_2 and Fe_3O_4 MPs was higher (23.11 ± 2.5 and 42 ± 3.7 g/L, respectively). At 1000 µg/mL concentration of Fe_3O_4 MPs, FA production (32.5 ± 3.5 g/L) was higher as compared to Fe_3O_4 NPs. The morphological forms obtained during SmF were mostly repetitive of those displayed in the growth media experiments (data not shown).

Metal ion tolerance by means of extracellular metal ion chelation and cell wall binding by fungi is an adaptive strategy to protect the metal-sensitive cellular targets. This adaptive tolerance behaviour of fungi makes them good adsorbent for heavy metals (Yang *et al*., 2009; Anahid *et al*., 2011; Valix *et al*., 2003). Previous study with *Rhizopus arrhizus* species confirmed the uptake of different metal ions by the biomass and it was proposed that electrostatic attraction is involved in uptake of metal cations (Tobin *et al*., 1984). In general, fungi use different receptor or functional groups for the uptake of metal ions (Bartnicki-Garcia *et al*., 1962; Tsezos*et al*., 1982a, b). However, study on size-dependent (MPs and NPs) variation in metal ions uptake has been not carried out so far. Variations in spore germination percentage, morphological behaviour and FA production profile obtained for one particular applied concentration of MPs and NPs of ZnO, Fe_3O_4 and MnO_2 suggested that the uptake mechanisms and internalized concentrations of Zn^{2+}, Fe^{2+} and Mn^{2+} ions by *R. oryzae* were not the same. Being a strong inhibitor of fungal growth and fungal spore germination (Somers, 1961) uptake of Zn^{2+} might not have been influenced by the size variation (MPs or NPs). However, enhanced tolerance of the fungus to Fe^{2+} and Mn^{2+} ions as manifested with MPs indicated for more uptake of these two ions with less toxicity. From the effects of ZnO, Fe_3O_4 and MnO_2 NPs and MPs on FA production, it was difficult to correlate the FA production profile with the fungal morphology for each applied concentration of MPs and NPs of Zn^{2+}, Fe^{2+} and Mn^{2+} ions. However, at all applied concentrations, FA production

was lower as compared to control experiments. This suggested that the fungus was able to produce FA under ionic stress conditions, but normal metabolic activities were not maintained. Further research on the cellular level events can reveal the role played by the size dependent uptake of Zn^{2+}, Fe^{2+} and Mn^{2+} ions and their effects on FA production.

Conclusions

The present study confirmed the effects of ZnO, Fe_3O_4 and MnO_2 micro- and nanoparticles on spore germination and morphology of the fungus, *R. oryzae*. The FA production efficiency of the fungus was also influenced by ZnO, Fe_3O_4 and MnO_2 micro- and nanoparticles. Among the three nanoparticles applied, Fe_3O_4 was found to be most biocompatible (65.2% spore germination at 800 µg/mL). Contrary to this, MnO_2 exhibited the least tolerance (8.2 % spore germination at 600 µg/mL). However, the lowest applied concentration (200 µg/mL) of all the three nanoparticles displayed a good biocompatibility in terms of germination. Microparticles of Fe_3O_4 also displayed least toxicity at the highest applied concentration (95.7% spore germination at 1000 µg/mL). This was an important outcome of this study as at 1000 µg/mL Fe3O4 nanoparticle concentration, spore germination was completely inhibited. Similarly, MnO_2 microparticles were found to be less toxic than MnO_2 nanoparticles. Micro- and nanoparticles of ZnO, Fe_3O_4 and MnO_2 caused variation in the morphology of *R. oryzae* in both growth and submerged fermentation conditions. Production profile of fumaric acid was influenced by the type of formulation (nano or micro) applied for ZnO, Fe_3O_4 and MnO_2. ZnO was found to inhibit the FA production and this was irrespective of micro- or nanoformulation. Fe_3O_4 and MnO_2 microparticles were supportive of FA production as compared to nanoformulations. The higher concentrations of Zn^{2+}, Fe^{2+} and Mn^{2+} ions induced different stress-tolerating mechanisms in the fungus and responses were manifested as changes in spore germination, morphology and fumaric acid production. Overall, the fungus R. oryzae exhibited size-dependent effects (nano- or microparticles) on the uptake of Zn^{2+}, Fe^{2+} and Mn^{2+} ions.

Abbreviations

FA= fumaric acid, SmF= submerged fermentation, NPs= nanoparticles, MPs= microparticles.

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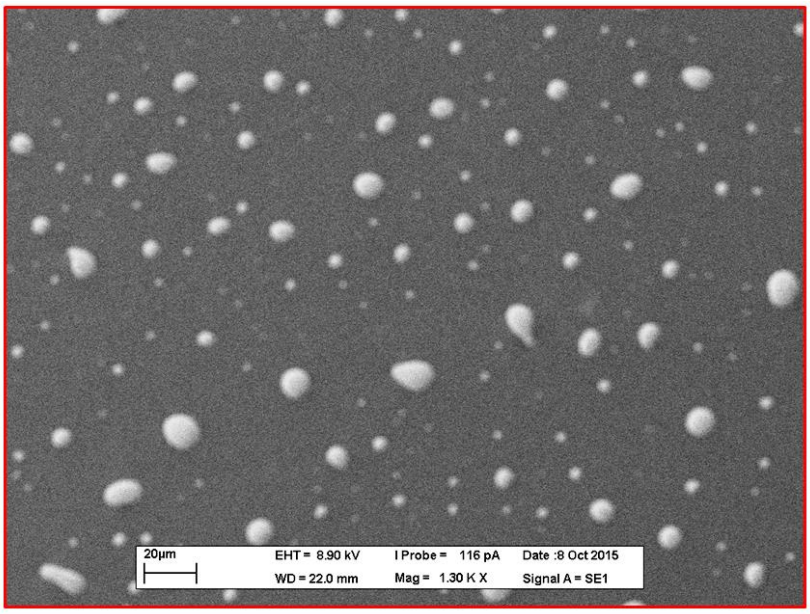

Figure 7.1: SEM image of ZnO nanoparticles prepared in the present study.

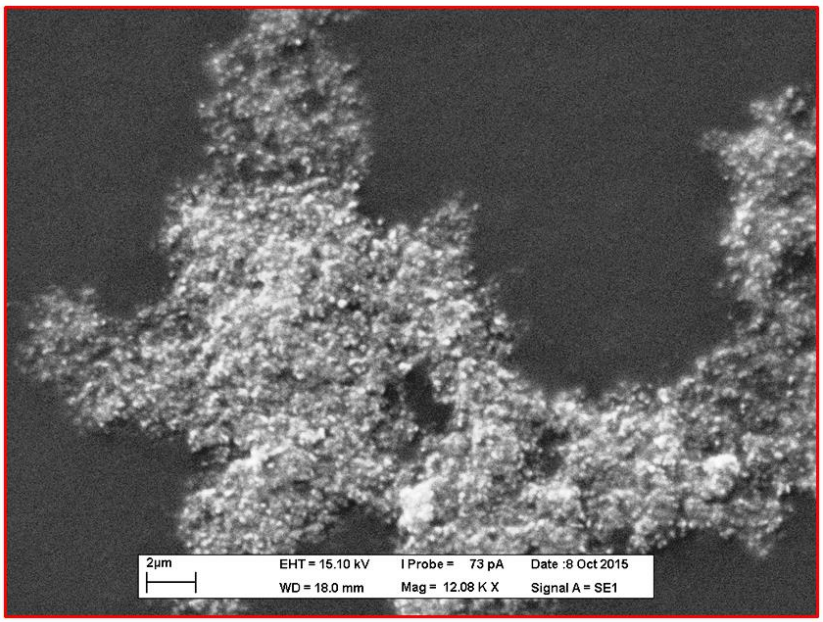

Figure 7.2: SEM image of Fe_3O_4 nanoparticles prepared in the present study.

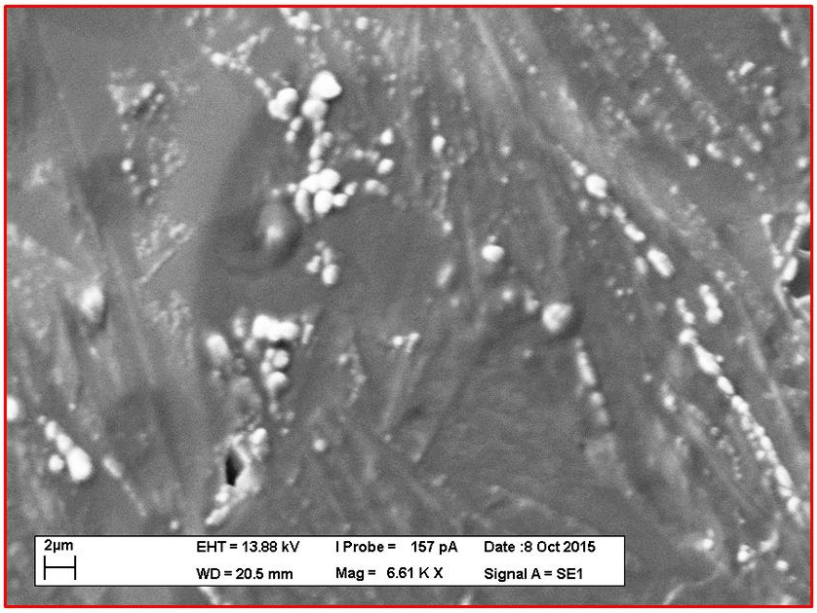

Figure 7.3: SEM image of MnO_2nanoparticles prepared in the present study.

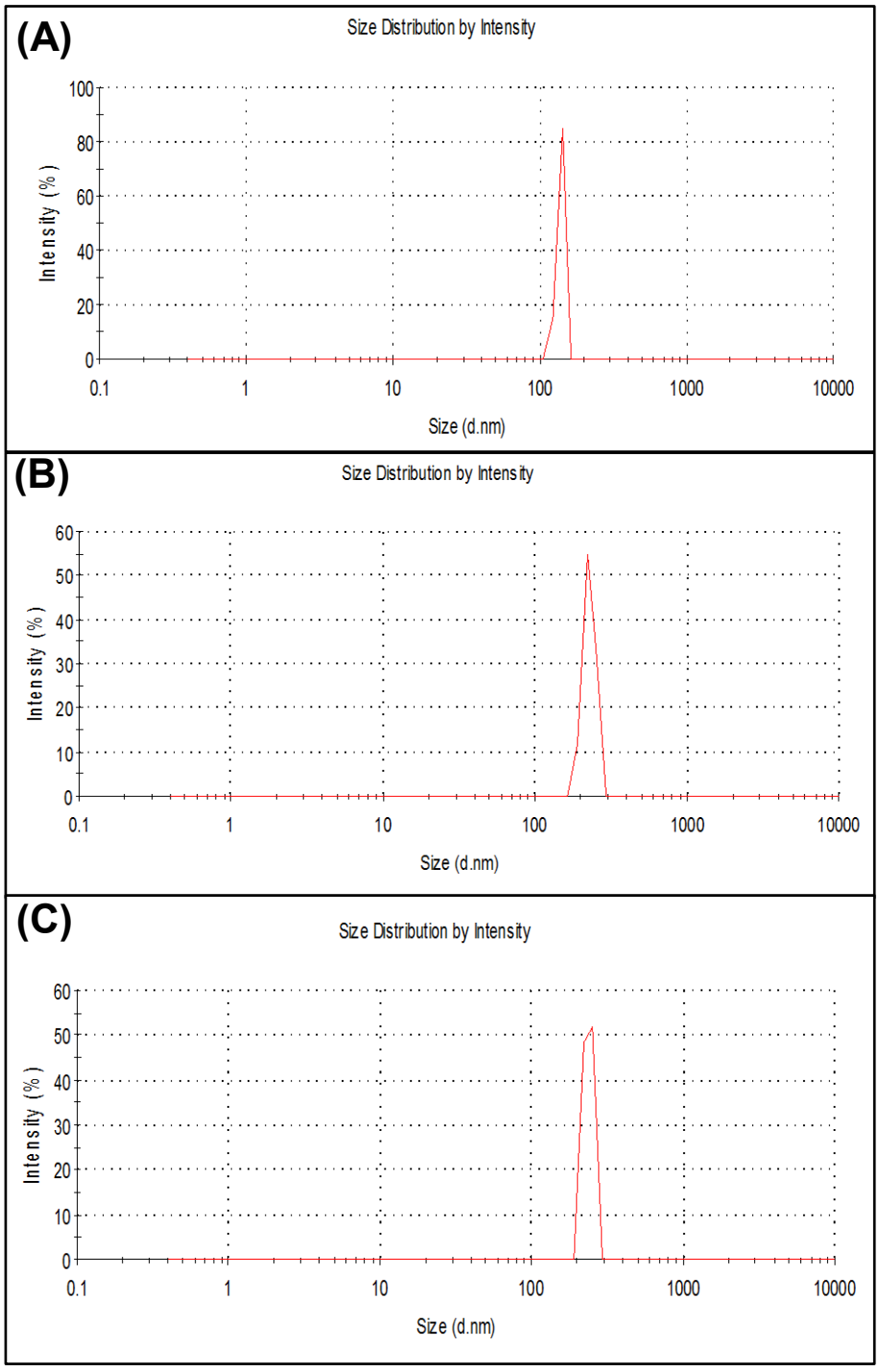

Figure 7.4: Size distribution of nanoparticles prepared in the present study **(A)** ZnO **(B)** Fe_3O_4 and **(C)** MnO_2

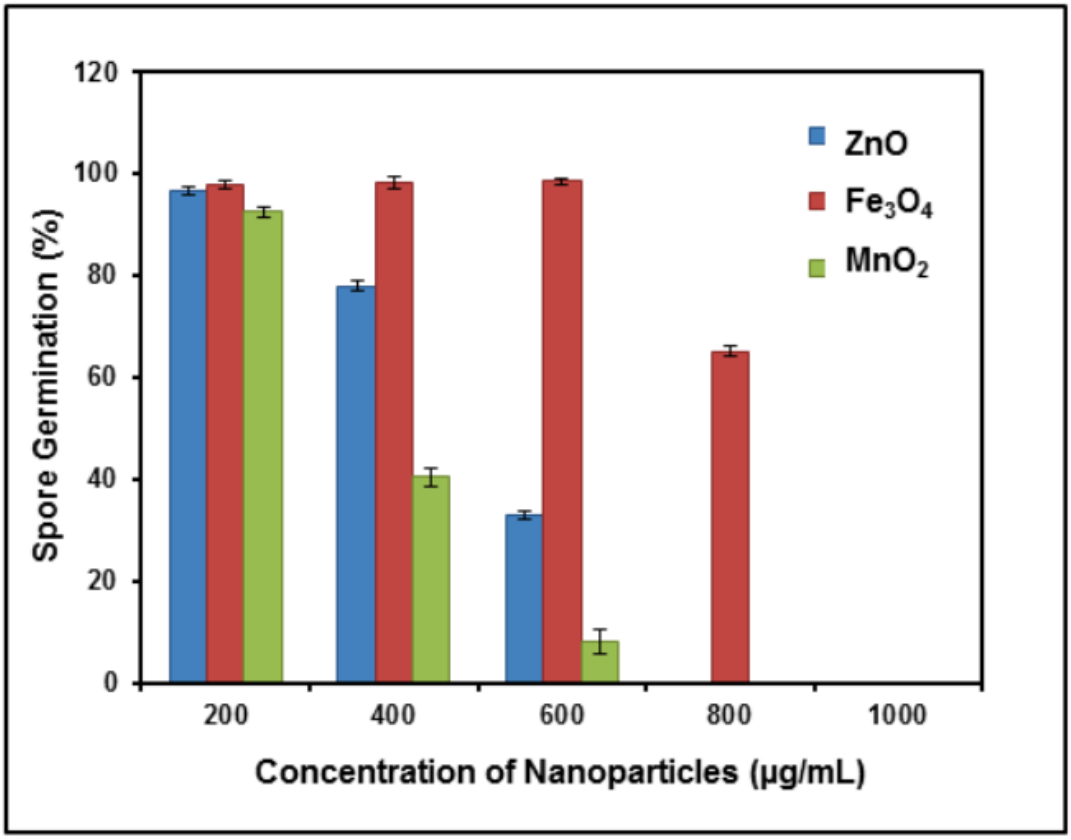

Figure 7.5: Percent spore germination of *R. oryzae* at different concentrations of ZnO, Fe_3O_4 and MnO_2 nanoparticles.

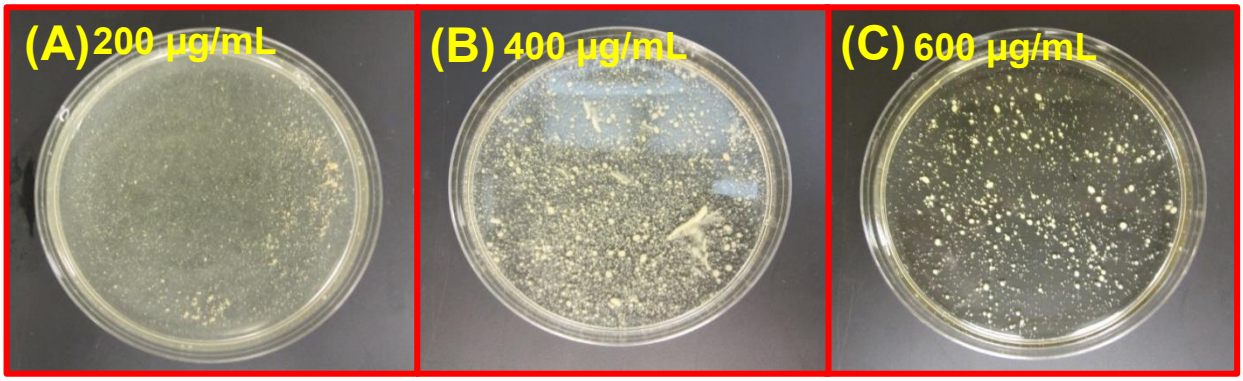

Figure 7.6: Effects of ZnO nanoparticles on the morphology of *R. oryzae* at the concentration of **(A)** 200 µg/mL **(B)** 400 µg/mL and **(C)** 600 µg/mL.

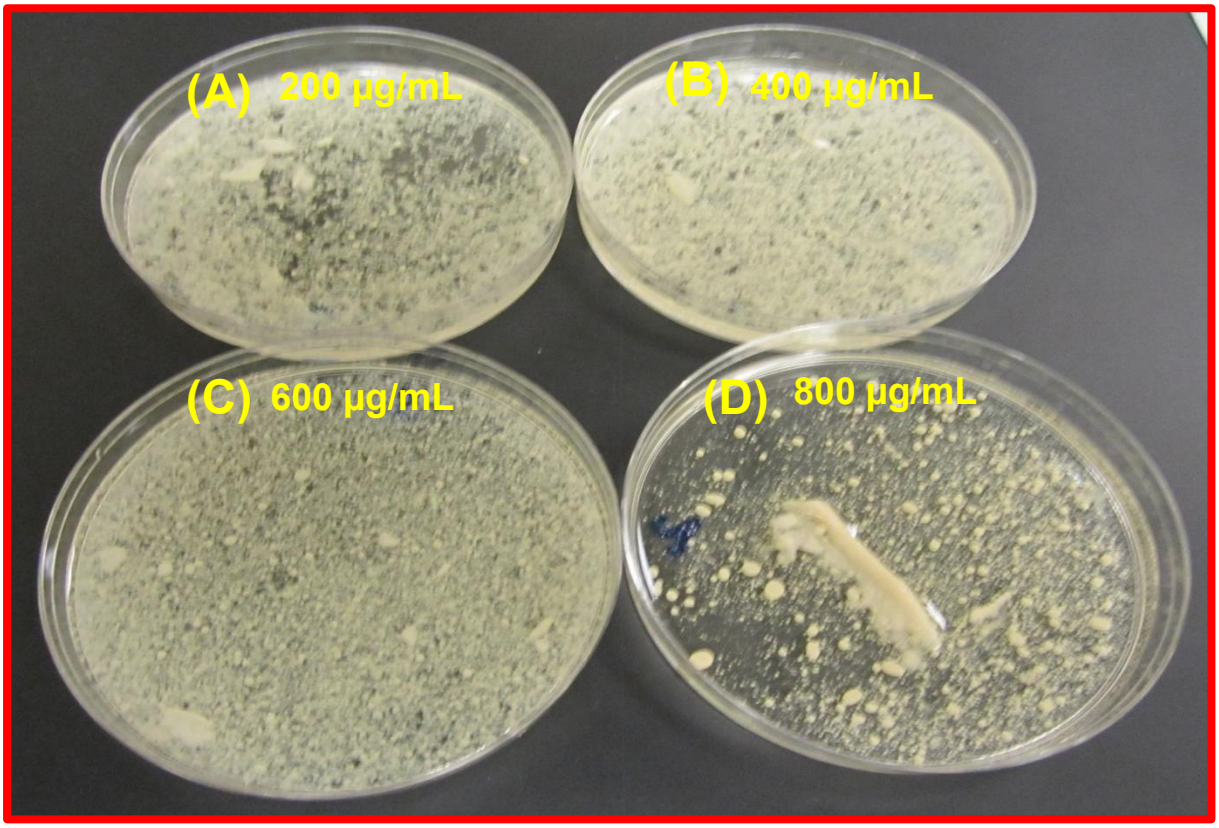

Figure 7.7: Effects of Fe_3O_4 nanoparticles on the morphology of *R. oryzae* at the concentration of **(a)** 200 µg/mL **(b)** 400 µg/mL **(c)** 600 µg/mL and **(d)** 800 µg/mL.

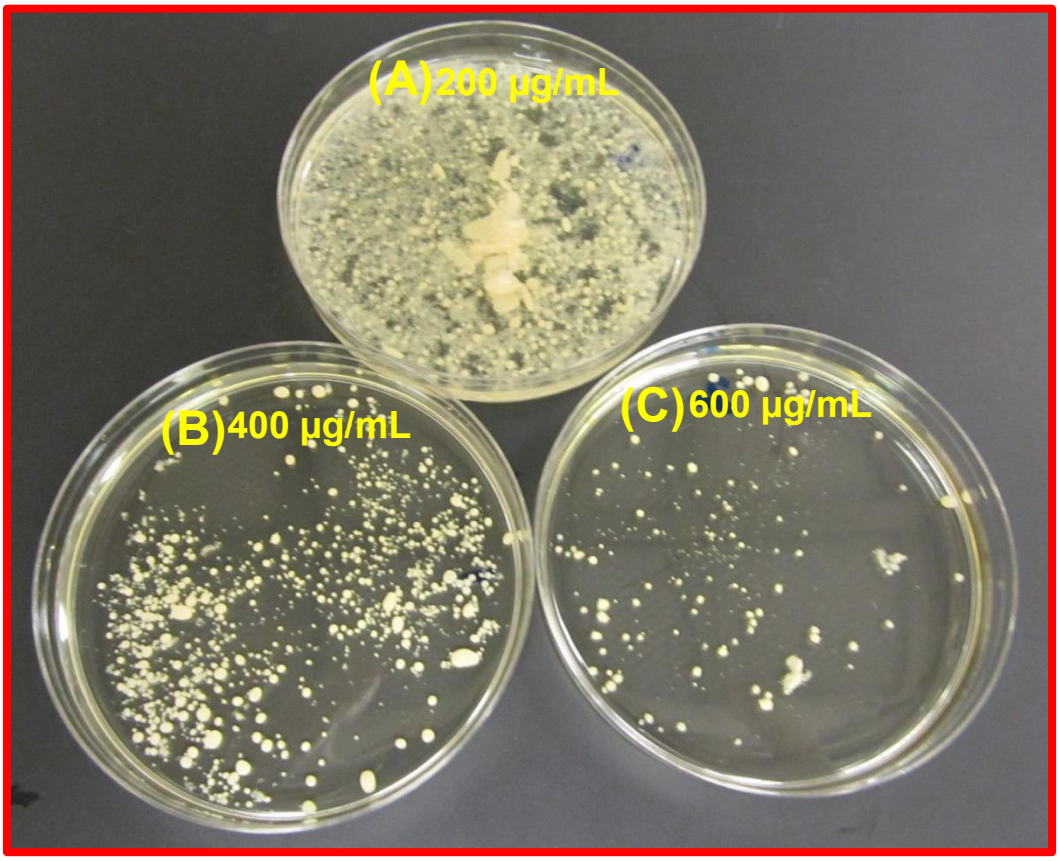

Figure 7.8: Effects of MnO_2 nanoparticles on the morphology of *R. oryzae* at the concentration of **(A)** 200 µg/mL **(B)** 400 µg/mL and **(C)** 600 µg/mL.

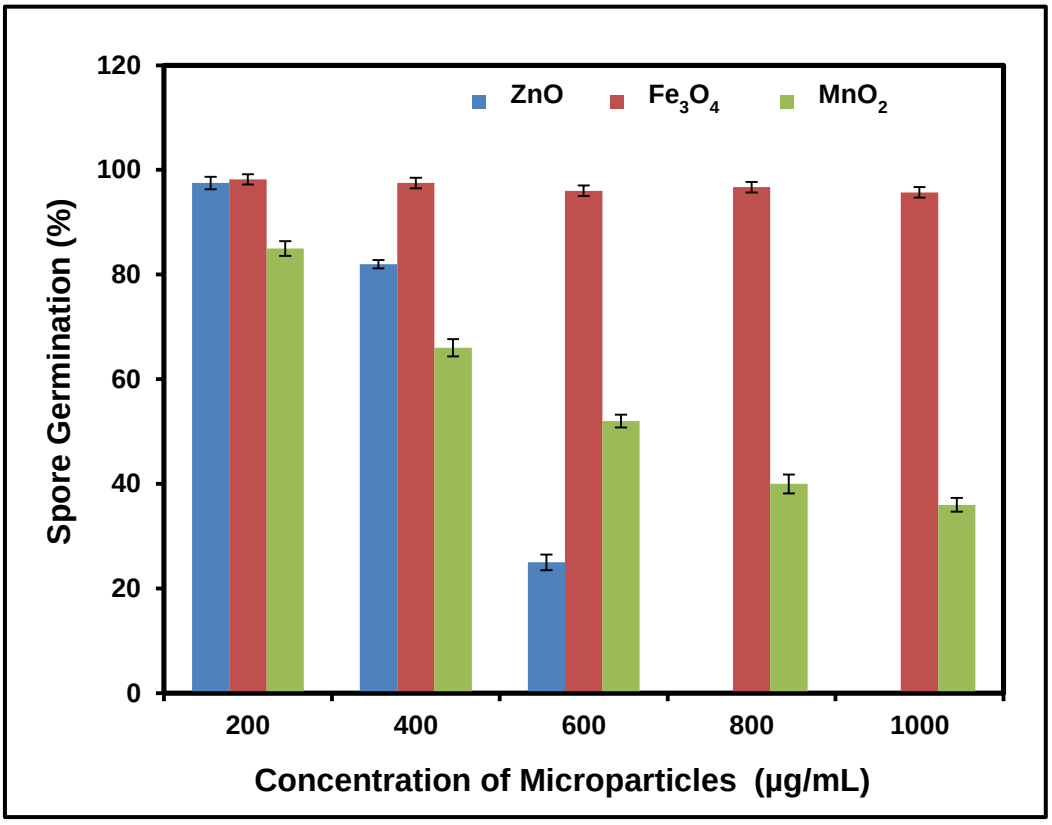

Figure 7.9: Percent spore germination of *R. oryzae* at different concentrations of ZnO, Fe_3O_4 and MnO_2 microparticles.

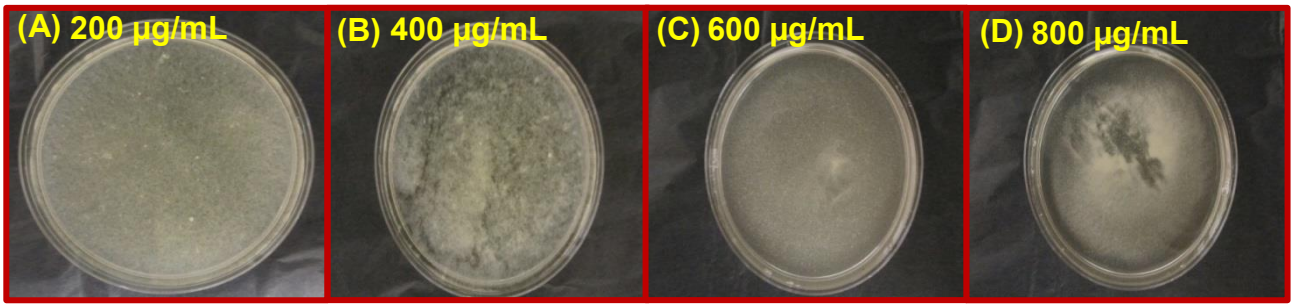

Figure 7.10: Effects of ZnO microparticles on the morphology of *R. oryzae* at the concentration of **(A)** 200 µg/mL **(B)** 400 µg/mL **(C)** 600 µg/mL and **(D)** 800 µg/mL.

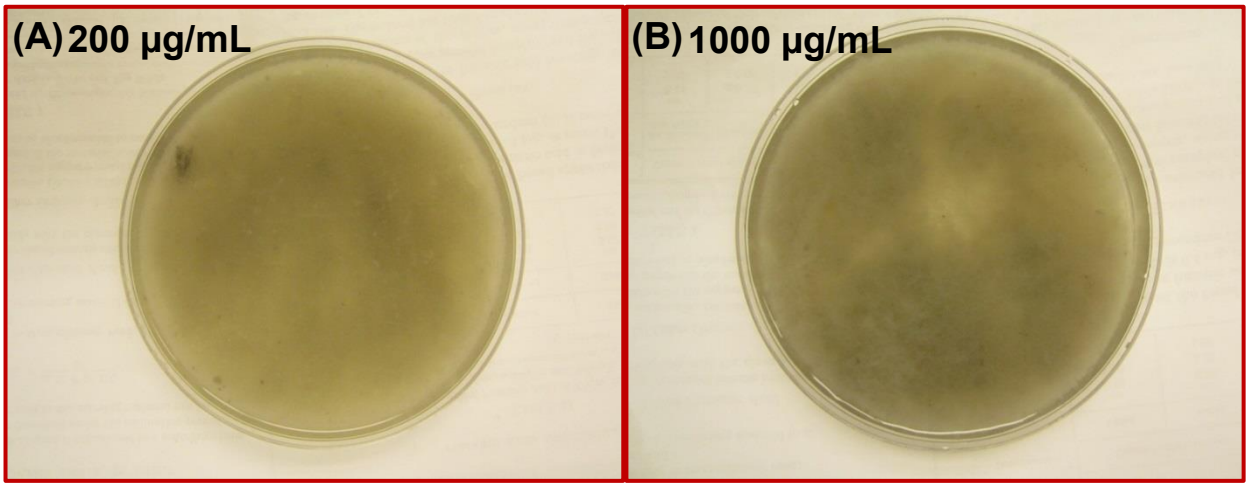

Figure 7.11: Effects of Fe_3O_4 microparticles on the morphology of *R. oryzae* at the concentration of **(A)** 200 µg/mL and **(B)** 1000 µg/mL.

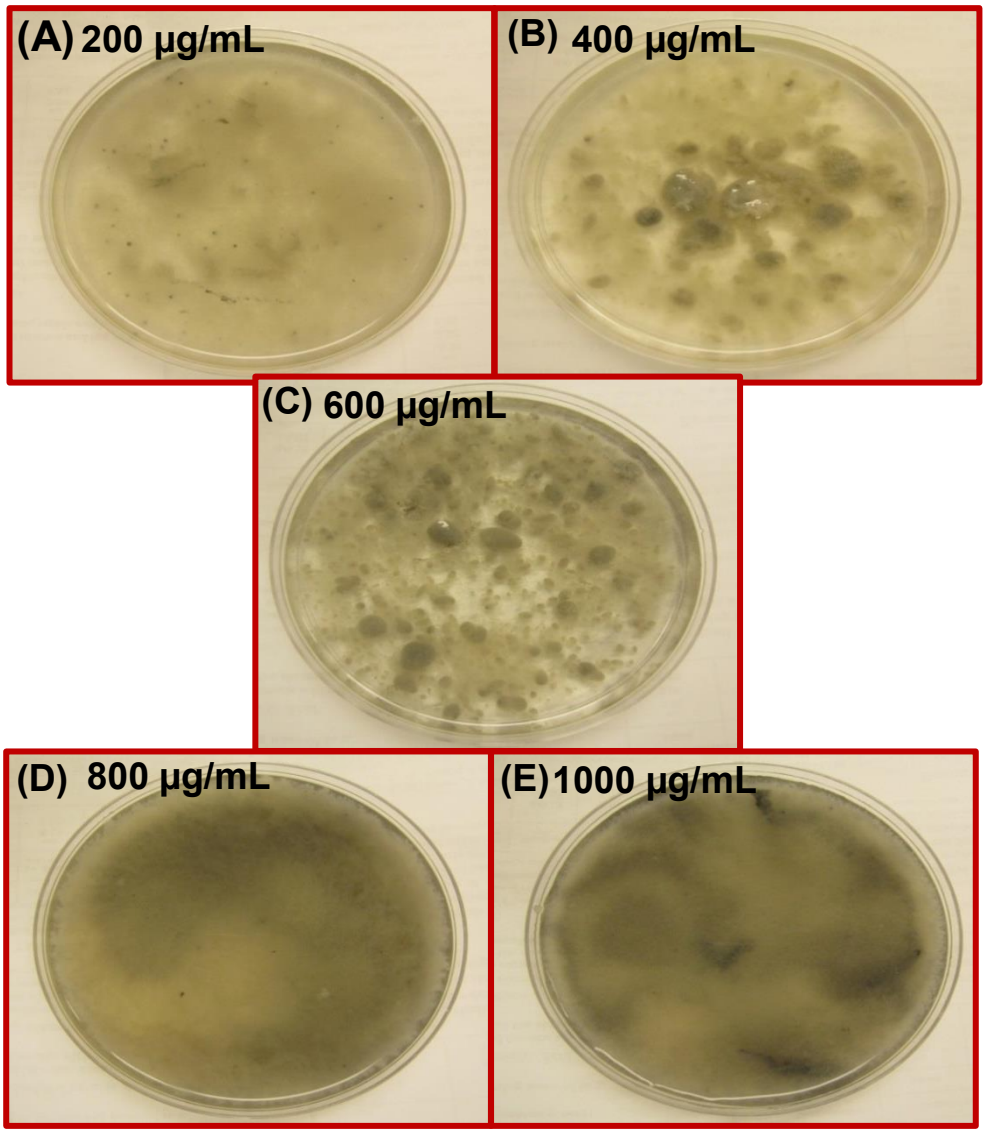

Figure 7.12: Effects of MnO_2 microparticles on the morphology of *R. oryzae* at the concentration of **(A)** 200 µg/mL **(B)** 400 µg/mL **(C)** 600 µg/mL **(D)** 800 µg/mL and **(E)** 1000 µg/mL.

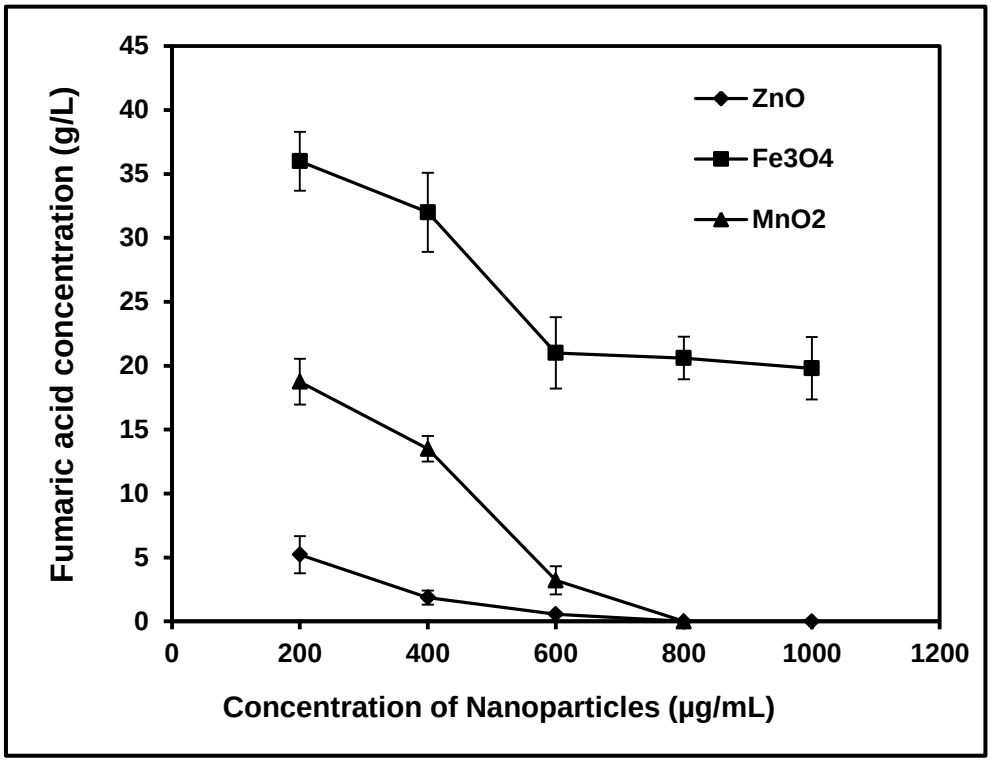

Figure 7.13 (A) : Effects of different concentrations of nanoparticles of ZnO, Fe_3O_4 and MnO_2 on the production profile of fumaric acid under submerged fermentation conditions of 30 °C, 200 rpm and 72 h with *R. oryzae* and glucose-basic salt medium.

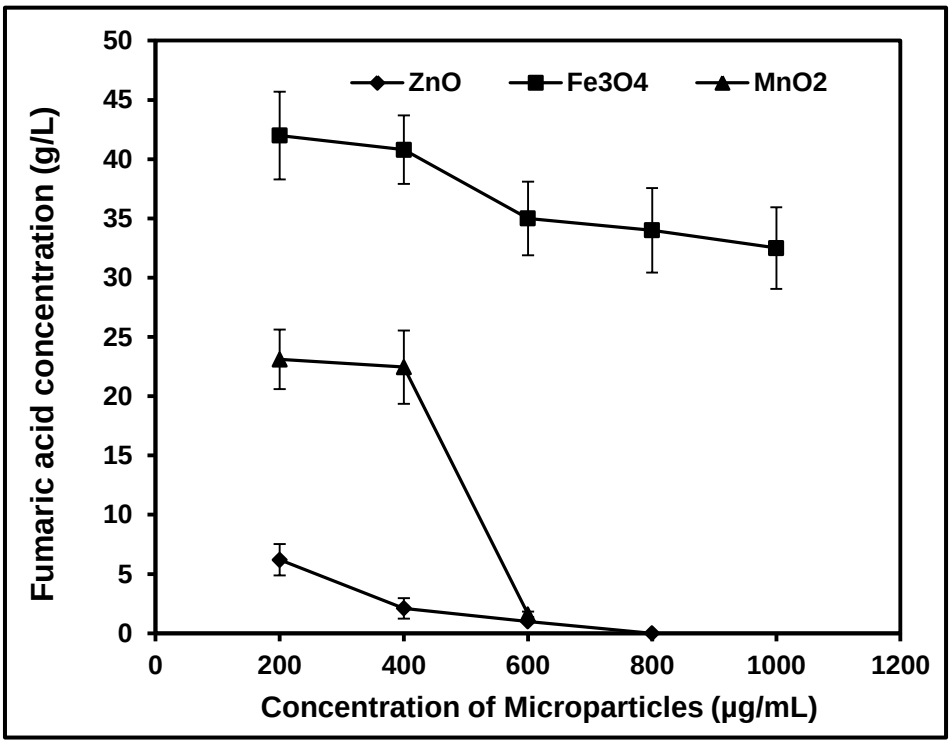

Figure 7.14 (B): Effects of different concentrations of microparticles of ZnO, Fe_3O_4 and MnO_2 on the production profile of fumaric acid under submerged fermentation conditions of 30 °C, 200 rpm and 72 h with *R. oryzae* and glucose-basic salt medium.

END OF CHAPTER 7

CHAPTER 8

APPLICATIONS OF CALCIUM CARBONATE NANOPARTICLES AND MICROWAVE IRRADIATION IN FUMARIC ACID PRODUCTION

APPLICATION OF CALCIUM CARBONATE NANOPARTICLES AND MICROWAVE IRRADIATION IN THE SUBMERGED FERMENTATION PRODUCTION AND RECOVERY OF FUMARIC ACID: A NOVEL APPROACH

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Résumé

Le but de la présente étude était d'explorer l'application éventuelle de nanoparticules de carbonate de calcium (CCNPs) et l'irradiation de micro-ondes (MWI) dans de l'acide fumarique (FA) la production et la récupération, respectivement. La souche fongique *Rhizopus oryzae* 1526 a été utilisé comme biocatalyseur pour la production FA. milieu de sels basiques de glucose a été utilisé comme milieu de fermentation. Balayage au microscope électronique à balayage (MEB) L'analyse de CCNPs affiche les formes sphériques, tandis que la mesure de Zetasizer a montré CCNPs se situer autour de 190 ± 20 nm. L'analyse IRTF de CCNPs a confirmé la composition chimique. Analyse BET a confirmé des surfaces spécifiques plus élevées de CCNPs (11,95 ± 0,03 m^2/g) par rapport à des microparticules de carbonate de calcium (CCMPs) (3,51 ± 0,02 m^2/g). FA temps de neutralisation pour CCNPs était beaucoup plus faible que CCMPs (190 et 350 secondes, respectivement). CCNPs amélioré la productivité volumétrique de FA de 0,47 g / (L h) à 0,74 g / (L h). A 20, 40 et 60 g/L concentrations et à 25 ^oC, viscosités de CCNPs se sont avérés inférieurs à CCMPs respectifs. En outre, CCNPs ne présente aucune toxicité envers les champignons. La production FA CC obtenue avec les micro et CCNPs étaient 67,34 ± 2 g/L et 66,92 ± 2,7 g/L, respectivement. Sous chauffage MWI, 10 ± 1 min a été jugé suffisant pour la récupération de la FA, ce qui est beaucoup plus faible que le moment de chauffage classique de 28 ± 1 min.

Mots clés: Acide fumarique, nanoparticules de carbonate de calcium, viscosité; productivité volumétrique, irradiation par micro-ondes.

Abstract

The aim of the present study was to explore the possible application of calcium carbonate nanoparticles (CCNPs) and microwave irradiation (MWI) in fumaric acid (FA) production and recovery, respectively. The fungal strain *Rhizopus oryzae* 1526 was employed as the biocatalyst for FA production. Glucose-basic salts medium was used as fermentation medium. Scanning electron microscopic (SEM) analysis of CCNPs displayed the spherical shapes, while zetasizer measurement showed CCNPs to be around 190 ± 20 nm in size. FTIR analysis of CCNPs confirmed the chemical composition. BET analysis confirmed higher specific surface areas of CCNPs (11.95 ± 0.03 m^2/g) compared to calcium carbonate microparticles (CCMPs) (3.51 ± 0.02 m^2/g). FA neutralization timing for CCNPs was much lower than CCMPs (190 and 350 seconds, respectively). CCNPs enhanced the volumetric productivity of FA from 0.47 g/(L h) to 0.74 g/(L h). At 20, 40 and 60 g/L concentrations and at 25 ^oC, viscosities of CCNPs were found to be lower than respective CCMPs. Moreover, CCNPs did not exhibit any toxicity towards the fungus. FA production obtained with CC micro and CCNPs were 67.34 ± 2 g/L and 66.92 ± 2.7 g/L, respectively. Under MWI heating, 10 ± 1 min was found to be sufficient for recovery of FA and this was much lower than conventional heating timing of 28 ± 1min.

Keywords: Fumaric acid, calcium carbonate nanoparticles, viscosity; volumetric productivity, microwave irradiation.

Introduction

Fumaric acid (FA) [IUPAC ID: (E)-Butenedioic acid] is an organic acid with diverse applications in the field of food, resin and dairy industries (Doscher *et al*., 1941; Yang *et al*., 2011; Goldberg *et al*., 2006). Because of the non-hygroscopic and non-toxic properties and higher buffering capacity than other food acids, FA is preferably used as acidulant and nutritive additive in food industry (Yang *et al*., 2011). The chemical structure makes FA suitable for polymerization and esterification reactions and this has led to the extensive application of FA in resin industry. About 56% of the total annual production of FA is used in resin industry for the production of paper resins, alkyd resins and unsaturated polyester resins (www.the-innovation-group.com, www.nzic.org.nz). In the dairy industry, FA has been recommended for use in ruminant diet for reducing enteric methane (CH_4) emission (Bayaru *et al*., 2001; Carro *et al*., 2003). Moreover, FA has been recently experimented as highly efficient promoters of the Beckmann rearrangement (from benzophenone oxime Ia to benzamide IIa) (Rohokale *et al*., 2014). In the production domain, FA production through submerged fermentation (SmF) has emerged as the most explored biological alternative to chemical route. Active research on different aspects of upstream and downstream processing of fermentative FA production has uncovered the key factors that contribute to the higher yield of this multifaceted organic acid (Xu *et al*., 2012a; Roa Engel *et al*., 2008). Apart from the safety issues of chemically derived FA, newly explored bio-medical applications of FA and its ester derivatives has necessitated the biological production of FA ((Das *et al*., 2015a, Kang *et al*., 2010). In the last two decades, different research groups have explored the cost-effective FA production through SmF by introducing new concepts, such as carbon-economy and white biotechnology. Low cost carbon sources have been utilized for FA production. Different strains of the filamentous fungus *Rhizopus oryzae* have been employed as the most efficient biocatalysts in SmF based FA production (Xu *et al*., 2012a; Roa Engel *et al*., 2008). Strategies, such as immobilization of fungal strains on different solid supports, genetic engineering and metabolic shift in Tricarboxylic cycle have been experimented for enhanced FA production under SmF conditions (Zhang *et al*., 2012; Xu *et al*., 2012b; Das *et al*., 2015b; Gu *et al*., 2013). The overall progress made on the upstream processing of fermentative FA production is remarkable. However, fermentative approach of FA production is yet to address the bottlenecks in its downstream processing domain. This issue has been highlighted by different experts from time to time (Xu *et al*., 2012a; Roa Engel *et al*., 2008). Efforts have been made for easy recovery of FA from fermentation broth. In a study by Zhang *et al*., activated carbon was used as the adsorbent for FA recovery from the fermentation broth and later desorbed into acetone. The FA recovery yield was found to be around 93% (Zhang *et al*., 2014a). In another study, a fixed

bed column of amberlite ion exchange resin (IRA900) was applied with a stirred-tank bioreactor for intermittent in situ FA recovery from the fermentation broth. Compared to the results of the control experiment (without adsorption), this approach enhanced the recovery yield and productivity of FA by 25% and 59%, respectively (Zhang *et al*., 2014b). Techniques such as nanofiltration and bipolar electrodialysis have also been applied for the recovery of FA from model solutions and real fermentation broth. The combination of nanofiltration (nanoporous ceramic membrane) with bipolar electrodialysis (membrane PC 200bip and the anion-exchange membrane PC 200D) efficiently recovered and concentrated FA from the fermentation broth (Wozniak *et al*., 2014).

To the specific interest of the present study, application of calcium carbonate (to be called as CC hereafter) as a neutralizing agent in SmF based FA production is unavoidable. There has been serious concern regarding the disadvantages of using CC over other neutralizing agents, such as Na_2CO_3, $NaHCO_3$, $(NH_4)_2CO_3$, and $Ca(OH)_2$. However, pertaining to the higher yield of FA, CC has been established as the default neutralizing agent in FA production (Xu *et al*., 2012a). Lower solubility and higher viscosity of CC slurries causes problems in the heat, mass and oxygen transfer throughout the broth during SmF. Non-stoichiometric addition of CC for neutralization of FA and maintenance of broth pH near to 6-7 is a regular practice during SmF based FA production. The fermentation product 'calcium fumarate' ($CaC_4H_2O_4$) has low water solubility (15.6 g/L) and this makes fermentation broth even more viscous (Xu *et al*., 2012a). $CaC_4H_2O_4$ has commercial application as calcium fortifier in beverages. Studies have been carried out on the relationship between the water solubility of $CaC_4H_2O_4$ and pH with different acidulants (www.bartek.ca). By stoichiometric ratio, 20 g of CC can neutralize 23.2 g of FA and this is irrespective of any SmF conditions. As FA concentration (g/L) in the SmF broth is not predictable, this stoichiometric ratio cannot be followed and this necessitates the addition of extra CC during SmF. Sticking to this principle, it might be possible to change the physical state of the CC but not the required concentration (g/L). Application of CC nanoparticles (CCNPs) instead of CC microparticles (CCMPs) can be an interesting and noble approach to experiment with. Nano dimension imparts new physical properties lacking in the bulk form (including micro size). Higher surface to volume ratio is one of the common advantages of nanoform over their micro-sized counterparts. For CCNPs, apart from higher surface area, lower viscosity and increased rate of FA neutralization might be the technical advantages over CCMPs. There has been no prior study in this sense but studies have been carried out on the advantages of using small sized CC in acid neutralizing kinetic studies (Notari *et al*., 1965; Fusi *et al*., 2012; Nogami *et al*., 1962).

Apart from the CC associated bottlenecks of FA production, the fermentation products $CaC_4H_2O_4$ needs attention for easy recovery of FA. Conventionally, $CaC_4H_2O_4$ in broth is simultaneously heated (around at 80 ^oC) and acidified (mineral acids such as HCl or H_2SO_4) that release FA from $CaC_4H_2O_4$ and also increases the solubility of FA in the broth. However, this method is time consuming. The dissolution of FA into water depends on uniform heating and rate of heating. In this regard, microwave irradiation (MWI) can be a good option for time-effective recovery of FA from the broth. MWI is an electromagnetic spectrum with a frequency in the range of 300 MHz to 300 GHz. For a variety of inorganic synthesis and biomedical applications, MWI is a well proven and widely accepted processing technology (Thiebaut *et al*., 1993; Correa *et al*., 1998). As compared to general heating treatment, a rapid and homogenous heating can easily be achieved by employing MWI even in materials exhibiting low heat conductivity as the transfer of energy does not rely on heat diffusion (Kushare *et al*., 2012; Papadimitriou *et al*., 2008). On the other hand, dissolution enhancement is an innovative concept which additionally accelerates the solubility and dissolution(Papadimitriou *et al*., 2008; Patel *et al*., 2008). However, a good dielectric property is required for excellent efficiency of heat transfer (Bradshaw *et al*., 1998). Water (in the fermented broth) represents the solvent with a dielectric constant value of 80.37 (at 25 ^oC) and the major byproduct 'ethanol' formed during FA fermentation has a dielectric constant value of 24.3 (at 25 ^oC) (Maryott *et al*., 1951). Both solvents can thus certainly enhance the MWI efficacy of heating the fermented broth in a shortened time. Moreover, acidic condition favors MWI heating. Considering the bottlenecks in the production and recovery of FA, CCNPs and MWI were thus investigated in the present study.

Material and methods

Materials

The fungal strain, *Rhizopus oryzae* NRRL 1526 (to be called *R. oryzae* hereafter) was procured from Agricultural Research Services (ARS) culture collection, IL, USA. The inorganic salts ($CaCl_2$, Na_2CO_3, KH_2PO_4, $MgSO_4{\cdot}7H_2O$, $ZnSO_4{\cdot}\ 7H_2O$, $FeSO_4{\cdot}7H_2O$ and $CaCO_3$), urea and dextrose (dextrorotatory glucose) used in this study were of analytical grade (with min 99.5% purity) and purchased from Fisher Scientific (Ottawa, Ontario, Canada).

Culture and maintenance of *Rhizopus oryzae* 1526

For cultural practices (revival, sub-culturing and regular maintenance) of *R. oryzae*, the procedure mentioned by Das *et al*. (2014) was followed. Briefly, potato dextrose agar (PDA)

medium and incubation conditions of 37 ± 1 °C for 4 days were used for revival. Sporangiospores were collected by spread plate method after incubating 37 ± 1 °C for 72 h. Mycelium free spore suspension was kept at 4 °C and the glycerol stock (20%) was preserved at −80 °C. For inoculation, a stock of 1×10^8 spores/mL was maintained.

Media and inoculum preparation

Glucose-basic salts medium consisting of glucose (50 g/L), urea (2 g/L), KH_2PO_4 (0.6 g/L), $MgSO_4{\cdot}7H_2O$ (0.5 g/L), $ZnSO_4{\cdot}7H_2O$ (0.11g/L) and $FeSO_4{\cdot}7H_2O$ (0.0088 g/L) was used for spore germination. The medium final pH (4.6) was not adjusted, unless specifically indicated. To avoid Maillard reaction, the two media components: (a) glucose; and (b) urea + salts, were heat sterilized (20 min, 15 psi, 121 ± 1 °C) separately and mixed aseptically at room temperature. For SmF medium preparation, the same procedure was followed but with a higher carbon (80 g/L glucose) to nitrogen (0.2 g/L urea) ratio (C: N = 400:1) was maintained.

Around 50 mL of sterilized growth medium was inoculated (2%, v/v) with sporangiospores of *R. oryzae* in a 250-mL Erlenmeyer flask and then incubated at 30 °C, 200 rpm for 24 h.

SmF conditions

Around 7.9 mL (final inoculum concentration 5%, v/v) of cell pellet inoculum was transferred into 500 mL Erlenmeyer flasks containing 142.1mL of SmF medium. The inoculated flasks were incubated at 25 °C, in a rotary shaker at 200 rpm for a maximum of 84 h. Heat sterilized CCMPs (5-10 µm) and CCNPs (190 ± 20 nm) were used as neutralizing agents at 60 g/L.

Preparation of CCNPs

The CCNPs were prepared following the method of Ueno *et al*. with some modifications (Ueno *et al*., 2005). Briefly, 6.50 mL of 5 M $CaCl_2$ and 25 mL of 1 M Na_2CO_3 were mixed and stirred vigorously for 20 min, followed by addition of 50 mL of d.H_2O. The large CCNPs were first discarded by precipitation without centrifugation. The suspension was centrifuged (34, 000 × g, 10 min) and the supernatant and precipitate (CCNPs) was separated. The prepared CCNPs were then dispersed in d.H_2O and freeze-dried into powder form for further application in SmF.

Analytical methods

Physicochemical characterization of the prepared CCNPs was carried out by different instrumental analysis. The size and shape of the CCNPs was investigated by a scanning electron microscope (Zeiss EVO® 50 Smart SEM system). For average size (diameter) measurement, zetasizer nanoZS (Malvern instruments Ltd., UK) was employed. Fourier transform infrared (FTIR) analysis of the CCNPs was carried with a Nicolet™ iS™50 spectrophotometer (Thermo Scientific) with He-Ne laser, DTGS detector and KBR beamsplitter with a built-in Miracle -ATR (Diamond). The ATR-FTIR spectra of the CCNPs samples were collected and analyzed using OMNIC Specta™ software. The Brunauer-Emmett-Teller (BET) surface area analysis of CCMPs and CCNPs samples was carried out with a Quantachrome, AUTOSORB-1 series model instrument. The changes in the pHs of FA solution after addition of CC (CCNPs or CCMPs) were recorded as a function of time (seconds, s) and compared for CCNPs and CCMPs. Viscosities of the CC slurries, $CaC_4H_2O_4$ solutions and broth samples were measured by a rotational viscometer (Fungilab, Premium Series, NY, USA). L1 spindle was used with a sample cup volume of 30 mL. Viscosities were expressed in the unit of cP. Acidified broth samples were irradiated for different time durations (2–20 minutes) at the maximum power output (900 W) of the microwave oven. For comparision, conventional heating method was followed for FA recovery (Dang *et al*., 2009). HPLC analysis was carried out for quantification of FA in the fermented broth samples. For HPLC sample preparation, the method of Zhou *et al*. was followed (Zhou *et al*., 2000). The technical details of the HPLC were as follows: System: DIONEX DX500, with an Acclaim OA, 5 µm, (4.6 × 150 mm) column with a refractive index detector (PDA-100 DIONEX, UV, 210 nm). 2.5mM methanesulfonic acid (CH_3SO_3H) at a flow rate of 1 mL/min was used as the mobile phase and column temperature was 30 °C.

Statistical analysis

Data are represented as mean ± SD of three independent experiments. Correlations were considered significant at $p < 0.05$.

Results and discussion

Characterization of CCNPs

SEM analysis displayed roughly spherical CCNPs with solid dense structure. A SEM image of the CCNPs is shown in Figure 8.1. As no stabilizing agent was used during the preparation of CCNPs, aggregation of CCNPs was observed after drying of the sample on SEM grid. The size distribution curve of CCNPs originating from the zetasizer nano analysis

indicated uniform size distribution of particles with a mean size of 190 ± 20 nm as shown in Figure 8.2. FTIR analysis of CCNPs sample showed the three distinctive infrared transmission bands corresponding to their chemical composition. The results are displayed in Figure 8.3. The infrared bands at 1452 cm^{-1}, 871 cm^{-1} and 714 cm^{-1} wavenumbers corresponded to the asymmetric stretching vibration (v_3), asymmetric stretching vibration (v_2) and symmetric stretching vibration (v_4) of CO_3, respectively; and also confirmed the calcite crystalline state of CCNPs (Foran *et al*., 2013; Rodriguez-Blanco et a l., 2011; Legodi *et al*., 2001). The BET analysis confirmed the larger specific surface area of CCNPs as compared CCMPs. The specific surface areas measured for CCMPs and CCNPs were 3.51 ± 0.02 m^2/g and 11.95 ± 0.03 m^2/g respectively. Previously, it has been shown that changes in the specific surface area of CCNPs depend on preparation method and concentration of the inorganic salts ($CaCl_2$ and Na_2CO_3 in the present study) (Bang *et al*., 2012).

Measurement of pH of FA solutions

Solubility of FA in water changes with temperature and this in turn, controls the final pH of the solution. In the present study, different amounts (g/L) of FA were dissolved in water at 25 °C and the corresponding pH was measured. The results are presented in Table 8.1. The solubility of FA in water was around 6.1 g/L at 25 ^oC and it corresponded to the pH of 2.1 ± 0.2. Lower concentration (3.05 g/L) increased the pH to 2.4 ± 0.3. However, above 6.1 g/L FA, the pH remained almost unchanged. These results were in accordance with the literature on FA properties (Lange *et al*., 1930). After reaching the maximum solubility of 6.1 g/L at 25 ^oC, the pH was not affected by further addition of FA. At half (3.05 g/L) of the maximum soluble concentration, less production of H^+ ions from the dissociation of FA caused higher pH (2.4 ± 0.3). This basic information was extrapolated for the pH *vs*. FA production under SmF conditions.

Formation of $CaC_4H_2O_4$ and reaction time

The reaction between CC and FA can be represented as follows in Equation (1):

$$CaCO_3 + C_4H_4O_4 \longrightarrow CaC_4H_2O_4 + H_2O + CO_2 \qquad (1)$$

This reaction confirms that 20 g (200 mM) of $CaCO_3$ (CCNPs or CCMPs) neutralizes approximately 23.2 g (200 mM) of FA. In the present study, CC was used as CCMPs and CCNPs against FA at above mentioned stoichiometric ratio. The time (s) required for change in the pH (2.5) of FA aqueous solution was recorded and the results are shown in Figure 8.4. The neutralization reaction between FA and CCMPs took around 350 ± 19 s and the pH reached to 6.2. Thereafter, changes in pH were negligible and indicated completion of reaction. When experimented with CCNPs, the changes in pH were rapid and a stable pH of

6.23 was achieved after 190 ± 12 s. Thus, in comparision to CCMPs, the neutralization reaction time for CCNPs was reduced by 160 s. Particle size of neutralizing agent can have impacts on the reaction time. Previously, it has been shown that when applied as antacids; acid-consuming capacity and acid neutralizing velocity are influenced by the available specific surface area of the particles of CC. As the specific surface areas of CCNPs (11.95 ± 0.03 m^2/g) were larger to CCMPs (3.51 ± 0.02 m^2/g), the FA neutralization reaction was faster (Notari *et al*., 1965; Fusi *et al*., 2012; Nogami *et al*., 1962).

Viscosity measurement

Measurements of viscosities (cP) of different concentrations of the CCMPs and CCNPs slurries were carried at 25 ^oC and the results are displayed in Table 8.2. The concentrations (20, 40 and 60 g/L) of CC was selected on the basis of the stoichiometric ratio to FA. For CCMPs, the viscosity was 1.39 ± 0.12 cP at 20 g/L and increased with further increase in CC concentration. At 60 g/L, viscosity reached 1.9 ± 0.11 cP. Compared to CCMPs, the viscosities of CCNPs slurries at same corresponding concentrations were lower. At 20 g/L of CCNPs concentration, the viscosity was 1.15 ± 0.1cP and at the next two applied concentrations (40 and 60 g/L), the viscosities recorded were 1.48 ± 0.15 cP and 1.72 ± 0.1 cP, respectively. The lowering of viscosity of CCNPs slurries can be discussed on the ground of general acceptance of solid particle properties vs. viscosity. It has been shown that small particles generate larger inter-particle free space that helps in lowering viscosity. For larger particles, due to the greater inertia of interaction, more energy is required to momentarily accelerate or retard. This energy accounts for the extra viscosity (Clarke, 1967; Marc, 2011). Moreover, recently it has been proven that CCNPs can enhance surface hydrophobicity and decreases the yield stress and viscosity (Xu *et al*. 2013). Based on these theories, it can be ascertained that lowering of viscosity of CCNPs slurries, as was found in the present study, was pertinent.

Apart from CCMPs and CCNPs slurries, viscosities were also measured for the reaction products (i.e. calcium fumarate, $CaC_4H_2O_4$) formed after the reactions between different applied concentrations of CCMPs and CCNPs and FA in a stoichiometric ratio. As shown in Table 8.2, the reaction product $CaC_4H_2O_4$ formed from the reaction between 20 g/L of CCMPs + 23.2 g/L of FA showed a viscosity of 2.2 ± 0.28 cP at 25 ^oC. At double stoichiometric concentrations of CCMPs and FA, the viscosity increased to 2.67 ± 0.21 cP. For the reaction product of 20 g/L of CCNPs + 23.2 g/L of FA, the viscosity recorded was 2.18 ± 0.19 cP and increased to 2.58 ± 0.26 cP at 40 g/L of CCNPs + 46.4 g/L of FA stoichiometric ratio. Overall, there was no effect of the size of CC on the viscosity of the reaction product. Previous study confirmed that the maximum solubility of $CaC_4H_2O_4$ in water at 25 ^oC was 1.56 grams per 100 grams of water (Weiss *et al*., 1923). Thus, the viscosities

measured for $CaC_4H_2O_4$ in water at 25 ^oC actually represented the rheological behaviour of $CaC_4H_2O_4$ with a solubility of 15.6 g/L in water solvent. The formation of $CaC_4H_2O_4$ in water at 25 ^oC was found to be irrespective of the size (MPs and NPs) of CC. As pH lowering, viscosity increase/decrease and formation of $CaC_4H_2O_4$ in water at 25 ^oC during FA production should follow the same stoichiometric ratio, the information on the viscosities of $CaC_4H_2O_4$ solutions was vital before real SmF application of CCNPs.

SmF with CCMPs and CCNPs and effects on FA production

It is a consensual view that broth viscosity can influence the productivity of FA during SmF (Papagianni, 2004). Previous studies have confirmed that CC slurries (concentration dependent) increase broth viscosity which leads to oxygen transfer problem throughout the broth (Xu *et al*., 2012a; Roa Engel *et al*., 2008). In an aerobic process such as FA production, high viscosity of CC slurry can lower FA production. Meanwhile, pellet morphology of FA producing strains is considered as the most favourable form that allows easy mass, heat and oxygen transfer during SmF. Thus, SmF with pellet rules out morphological interference in FA production. To practically correlate all these vital factors to FA production profile under real SmF conditions, viscosities of different samples were measured before and during SmF. The obtained results are shown in Table 8.3. The glucose basic-salt medium with around 80 g/L of glucose showed viscosity of 1.25 ± 0.12 cP at 25 ^oC. This value was in accordance with previous findings (Converti *et al*., 1999). After addition of 5% pre-cultured cell pellets (inoculum), the viscosity of the medium changed marginally. However, mixing of CCMPs (60 g/L) considerably increased the viscosity to 3.27 ± 0.18 cP. In case of CCNPs, mixing of same amount to the medium increased the viscosity upto 2.98 ± 0.1 cP. In the first 24 h of SmF, the viscosity changes were monitored after every 12 h. For CCMPs, the initial (0 h) viscosity lowered from 3.27 ± 0.18 to 3.1 ± 0.08 cP after 12 h of SmF. It further decreased to 2.28 ± 0.2 cP after 24 h of SmF. From 24-48 h, the viscosity increased again and reached 2.41 ± 0.18 cP. There was a marginal increase (0.14 cP) from 48-72 h. CCNPs also showed similar changes of viscosity during SmF. However, in the first 12 h of SmF with CCNPs, the change in the viscosity was around 0.6 cP and this was much higher than 0.17 cP, as was obtained with CCMPs. The decrease in the viscosity during zero to 12 h of SmF for both CCMPs and CCNPs was caused by the consumption of CC by FA. This trend continued upto 24 h of SmF. After reacting with FA, CCMPs and CCNPs formed $CaC_4H_2O_4$ and this product has higher solubility (15.6 g/L) as compared to CCMPs or CCNPs (6.1 g/L) at 25 ^oC. Further accumulation of $CaC_4H_2O_4$ caused viscosity increase from 24-72 of SmF for both CCMPs and CCNPs.

The production profile of FA obtained with glucose basic-salt medium and CCMPs and CCNPs, is shown in Figure 8.5. In the first 12 h of SmF, the concentrations of FA were 6.44 ± 1.23 and 5.6 ± 1.8 g/L for CCMPs and CCNPs, respectively. From 12-24 h of SmF, as compared to FA concentration (11.34 ± 2.4 g/L) obtained with CCMPs, production of FA was higher (17.89 ± 3.3 g/L) with CCNPs. This corresponded to the increase of volumetric productivity from 0.47 g/(L h) to 0.74 g/(L h). From 24-36 h of SmF, the FA product yields for CCMPs and CCNPs were almost similar (around 12.33 and 13.11 g/L, respectively). However, the most productive phase of FA was in between 36-48 h that resulted in around 31.55 and 30.65 g/L of FA for CCMPs and CCNPs, respectively. Overall, FA production was found to be higher from 12-60 h of SmF with CCNPs. The application of CCNPs as neutralizing agent in the SmF production of FA has not been carried out earlier and thus discussion needs be made for higher FA yield with CCNPs can only be referred to the other supporting experimental results obtained during the present investigation. As shown earlier, CCNPs slurries were found to be less viscous to CCMPs slurries at same applied concentration (60 g/L) and temperature (25 ^{o}C). As FA production is influenced by the broth viscosity during the entire time frame of SmF, there was high possibility that FA production was favoured by the use of CCNPs. Moreover, as the highest FA concentrations (67.34 ± 2 and 66.92 ± 2.7 g/L, respectively) obtained with CCMPs and CCNPs were almost same, it strongly suggested that the metabolic performances of the used fungus R. oryzae 1526, was not influenced by the replacement of CCMPs with CCNPs. It also ruled out any associated nano-toxicity of CCNPs on the fungus or in other words, application of CCNPs was safe for this fungus mediated FA production through SmF technology. Nanoparticle concentrations such as 60 g/L used in the form of CCNPs, was an exceptionally higher concentration to be tolerated by any microorganisms including *R. oryzae* 1526. From the FA production profiles obtained with CCMPs and CCNPs, it was concluded that volumetric productivity of FA was enhanced by CCNPs, but not the final FA concentration.

MWI vs conventional heating in FA downstream processing

The two well-known FA recovery methods proposed by Gang *et al*. (1990) and Dang *et al*. (2009) make use of heat energy and CC neutralizing properties of mineral acids (such as HCl or H_2SO_4). Acidification neutralizes unused CC, while heating increases the solubility of both FA and $CaC_4H_2O_4$. Technically, both methodologies facilitate the separation of soluble FA from the solid mass (fungal biomass and $CaSO_4$). However, due to the technical advantages such as no need of special heating equipment and lower consumption of heat energy, the FA recovery method of Dang *et al*. (2009) is mostly followed. In the present study, MWI was intended for application in FA recovery. For a comparative study, fermented broth samples were processed through conventional method (Dang *et al*. 2009) and studied

parameters were recorded. The results are presented in Table 8.4. SmF broth samples from both CCMPs and CCNPs neutralizing agents mediated SmF of FA production were considered for this investigation. The broth samples were also heated under MWI and required time was monitored. For both conventional and MWI heating, acidification and pH adjustment were similar. For MWI heating, an experiment was carried out on the optimization of minimum time required for maximum FA recovery from the broth. A time range of 2-20 min was applied and correlated with the recovered FA concentration (g/L) (data not shown). From this optimization study, it was concluded that under MWI heating conditions, a minimum of 10 ± 1 minute was required for maximum FA recovery. Although, the minimum MWI heating time required for the visual manifestation of broth clarity was around 6-7 min, this resulted in lower FA recovery for both CCMPs and CCNPs (data not shown). Thus, on an average, 10 min MWI heating time was found to be optimum for maximum recovery of FA (65 ± 2.56 g/L and 68.5 ± 1.7 g/L for CCMP and CCNPs, respectively). The comparative outcome of this study confirmed the lower time consuming FA recovery through MWI. The heating time was almost lowered by 2.8 folds without affecting the recovery of FA. This was conclusive for both CCMPs and CCNPs. The results also suggested that application of CCNPs in FA production did not influence the downstream processing of FA under conventional or MWI heating conditions. With higher or lower broth volumes, heating time will be changed accordingly. It was concluded that irrespective of CCMPs and CCNPs, the well-known technical advantages of MWI over conventional heating resulted in FA recovery in lesser time. A rapid and homogenous heating of the broth and dissolution enhancement of FA under MWI, shortened the FA recovery time by almost 2.8 folds (Kushare *et al*., 2012; Papadimitriou *et al*., 2008; Papadimitriou *et al*., 2008; Patel *et al*., 2008). MWI energy heated the broth samples directly, while under conventional heating condition, heat energy first had to conduct through the walls of the Erlenmeyer flasks containing the broth samples. It delayed the heating of the broth samples. In general, MWI heating takes place through ionic conduction and dipole rotation (Papadimitriou *et al*., 2008). In the present study, presence of polar groups such as –COOH and >C=O (in FA) and –OH (in water) in the broth samples accelerated (rotational alignment) the conversion of MWI into heat energy. Moreover, the high dielectric constant value (80.37, at 25 ^{o}C) of water enhanced the MWI efficacy of heating the fermented broth in a shortened time.

Conclusions

Application of calcium carbonate nanoparticles was found to be advantageous over micro form of calcium carbonate during submerged production of fumaric acid. Fumaric acid neutralization timing for calcium carbonate nanoparticles was much lower than

microparticles (190 and 350 s, respectively). Higher specific surface area of calcium carbonate nanoparticles (11.95 ± 0.03 m^2/g) compared to micro form (3.51 ± 0.02 m^2/g), caused faster consumption of fumaric acid and resulted in the lowering of neutralization timing. Calcium carbonate nanoparticles enhanced the volumetric productivity (from 0.47 g/L h to 0.74 g/L h) of fumaric acid in the first 12-24 h of fermentation. Nanoformulation of calcium carbonate did not exhibit toxicity towards the fungus *Rhizopus oryzae* 1526. Microwave irradiation shortened the time required for the maximum recovery of fumaric acid from 28 to 10 min.

Abbreviations

CCMPs= calcium carbonate microparticles, CCNPs= calcium carbonate nanoparticles, FA= fumaric acid, NA= not applicable

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Table 8.1: pH of different fumaric acid aqueous solutions measured at 25 ^{o}C.

Fumaric acid concentration (g/L)	pH
3.05	2.4 ± 0.3
6.1	2.1 ± 0.2
10	2.2 ± 0.1
20	2.1 ± 0.1
30	2.1± 0.2

Table 8.2: Viscosities of different samples of calcium carbonate measured at 25 ^{o}C.

Sample analyzed	Viscosity (cP)
Water	0.85 ± 0.08
CCMPs (20 g/L)	1.39 ± 0.12
CCMPs (40 g/L)	1.7 ± 0. 18
CCMPs (60 g/L)	1.9 ± 0.11
CCNPs (20 g/L)	1.15 ± 0.1
CCNPs (40 g/L)	1.48 ± 0.15
CCNPs (60 g/L)	1.72 ± 0.1
Calcium fumarate (20 g/L of CCMPs + 23.2 g/L of FA)	2.2 ± 0.28
Calcium fumarate (40 g/L of CCMPs + 46.4 g/L of FA)	2.67 ± 0.21
Calcium fumarate (20 g/L of CCNPs + 23.2 g/L of FA)	2.18 ± 0.19
Calcium fumarate (40 g/L of CCNPs + 46.4 g/L of FA)	2.58 ± 0.26

Abbreviations: CCMPs, Calcium carbonate microparticles; CCNPs, Calcium carbonate nanoparticles; FA, Fumaric acid.

Table 8.3: Viscosities of different samples at various time intervals (h) of submerged fermentation

Sample analyzed	Viscosity (cP)				
	0 h	**12 h**	**24 h**	**48 h**	**72 h**
Glucose basic salt medium	1.25 ± 0.12	NA	NA	NA	NA
Glucose basic salt medium + 5% (v/v) cell pellets	1.28 ± 0.05	NA	NA	NA	NA
Glucose basic salt medium + 5% (v/v) cell pellets + CCMPs (60 g/L)	3.27 ± 0.18	3.1 ± 0.08	2.28 ± 0.2	2.41 ± 0.18	2.55 ± 0.13
Glucose basic salt medium + 5% (v/v) cell pellets + CCNPs (60 g/L)	2.98 ± 0.1	2.38 ± 0.19	1.65 ± 0.23	1.95 ± 0.14	2.11 ± 0.15

Abbreviations: NA, Not applicable; CCMPs, Calcium carbonate microparticles; CCNPs, Calcium carbonate nanoparticles.

Table 8.4: Comparision of the conventional and microwave irradiation methods applied for fumaric acid downstream processing.

Parameter studied	**Conventional method**		**Microwave Irradiation**	
	Broth with CCMPs	Broth with CCNPs	Broth with CCMPs	Broth with CCNPs
Heating temperature (^oC)	80 ± 1 ^oC	80 ± 1 ^oC	95 ± 1 ^oC	95 ± 1 ^oC
H_2SO_4 concentration	5 N	5 N	5N	5N
Final pH	1.0 ± 0.1	1.0 ± 0.1	1.0 ± 0.1	1.0 ± 0.1
Heating time required (min)	28 ± 2 (until clear)	27 ± 2 (until clear)	10 ± 1 (until clear)	10 ± 1 (until clear)
FA (g/L) recovered	67.55 ± 2	66.45 ± 2.7	65 ± 2.56	68.5 ± 1.7

Abbreviations: CCMPs, Calcium carbonate microparticles; CCNPs, Calcium carbonate nanoparticles.

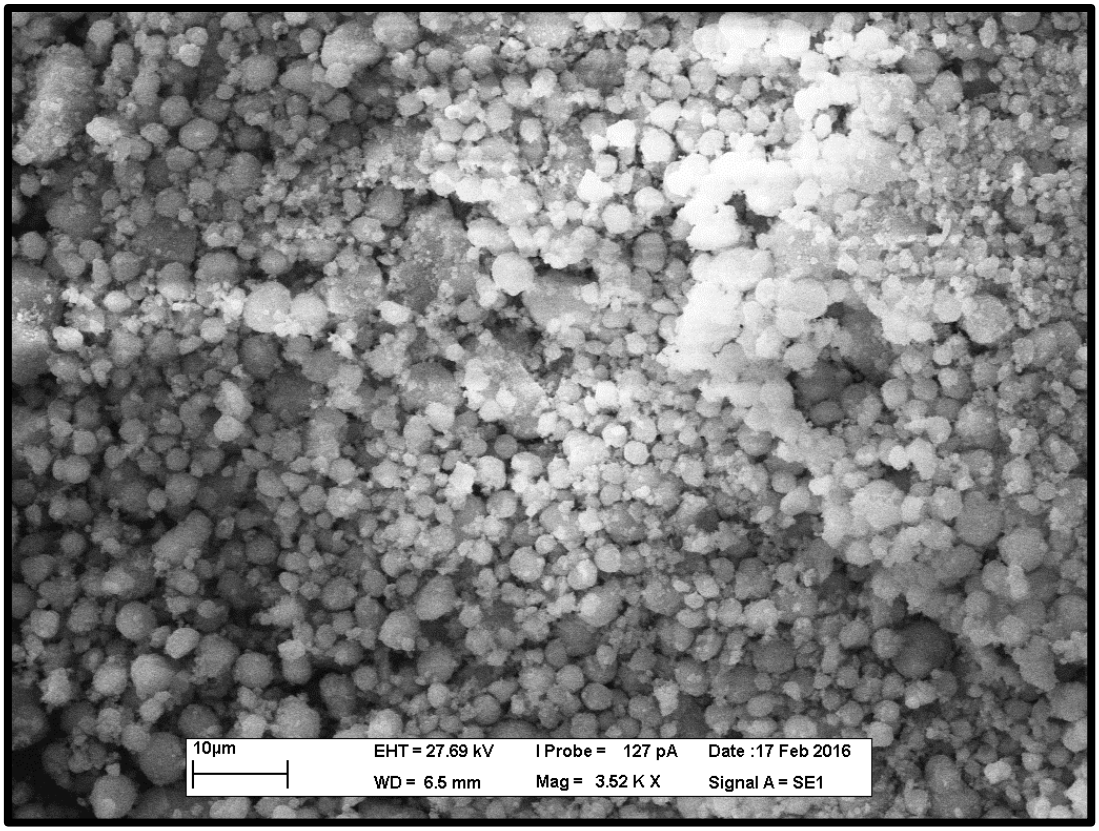


Figure 8.1: SEM micrograph of calcium carbonate nanoparticles prepared in the present study.

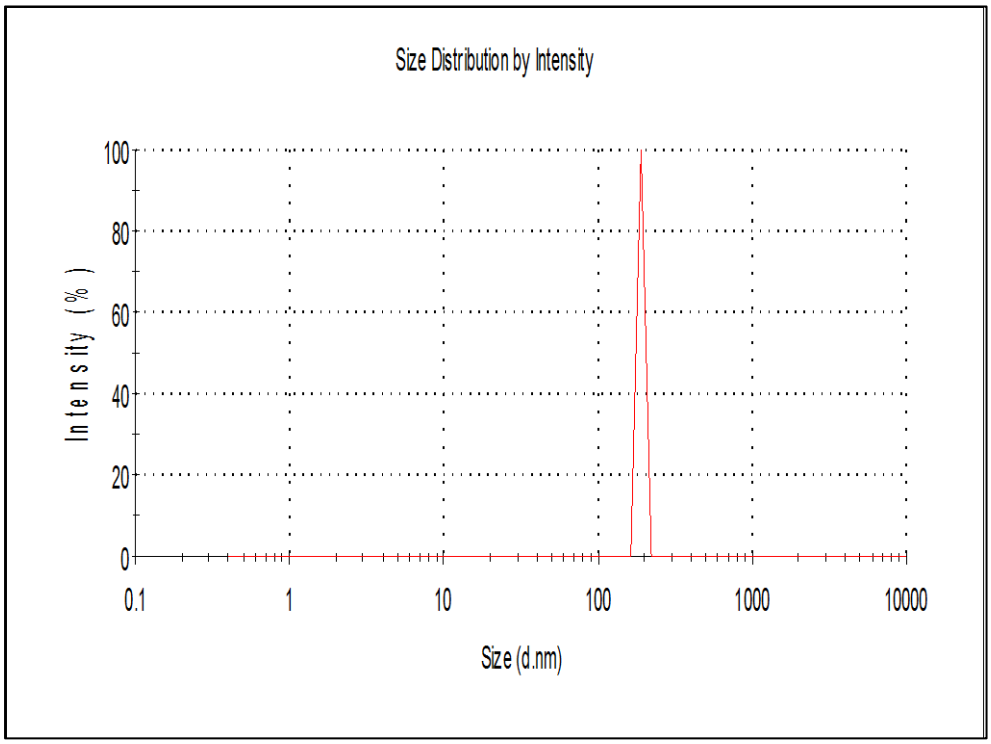


Figure 8.2: Size distribution curve of calcium carbonate nanoparticles prepared in the present study.

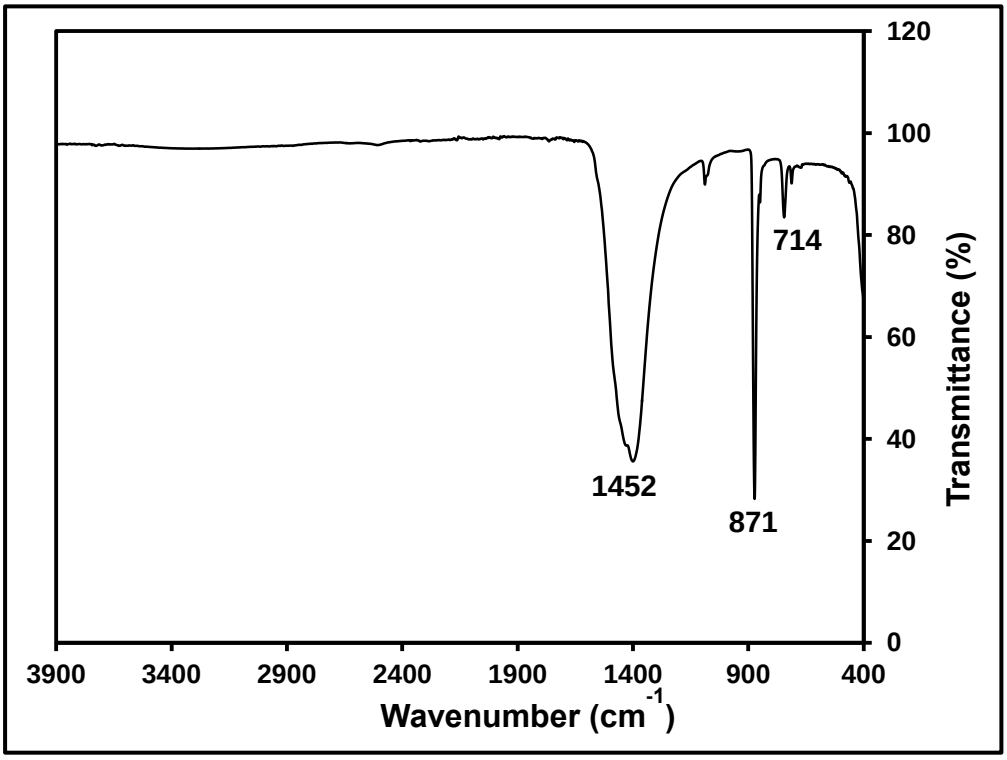


Figure 8.3: ATR-FTIR spectrum for $CaCO_3$ nanoparticles prepared in the present study.

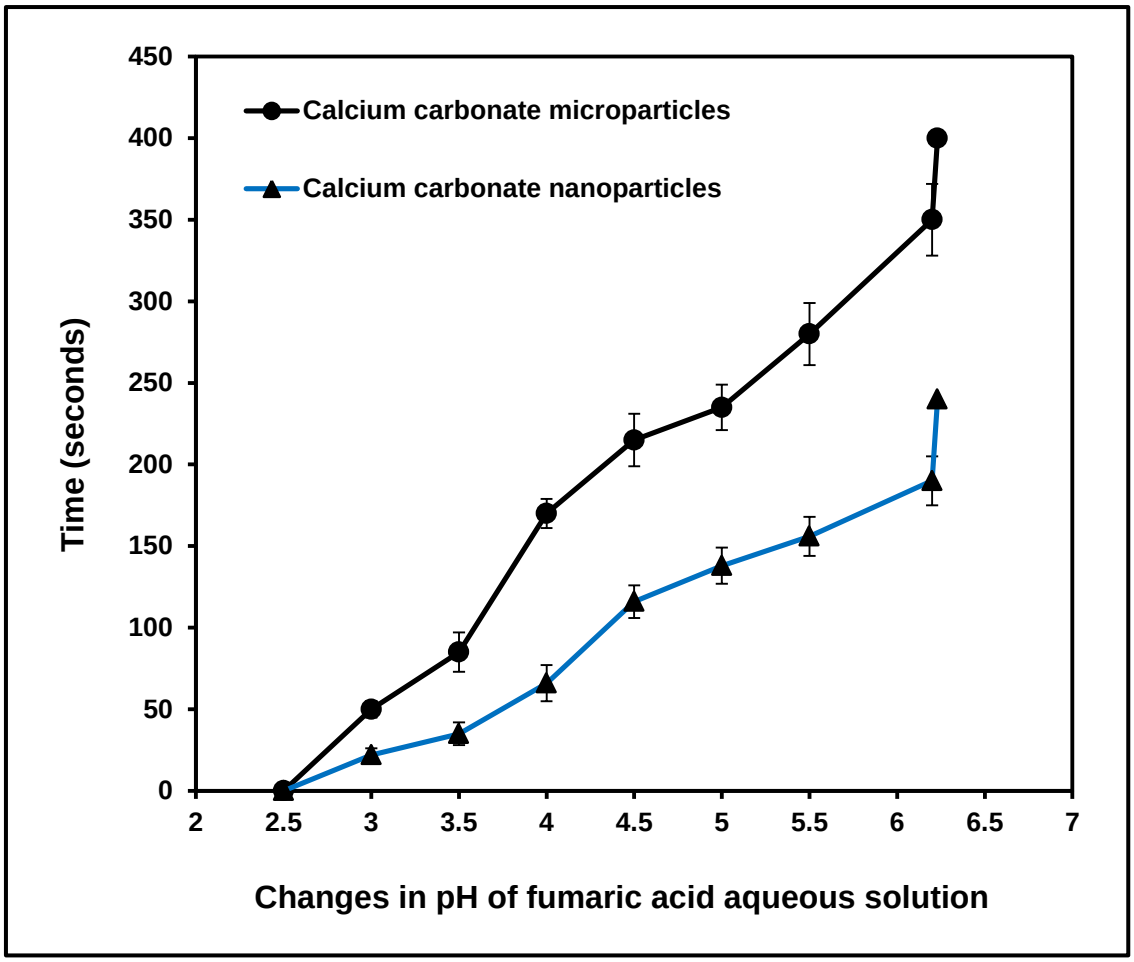


Figure 8.4 Effect of $CaCO_3$ particle size on the neutralization time (seconds) of fumaric acid aqueous solution.

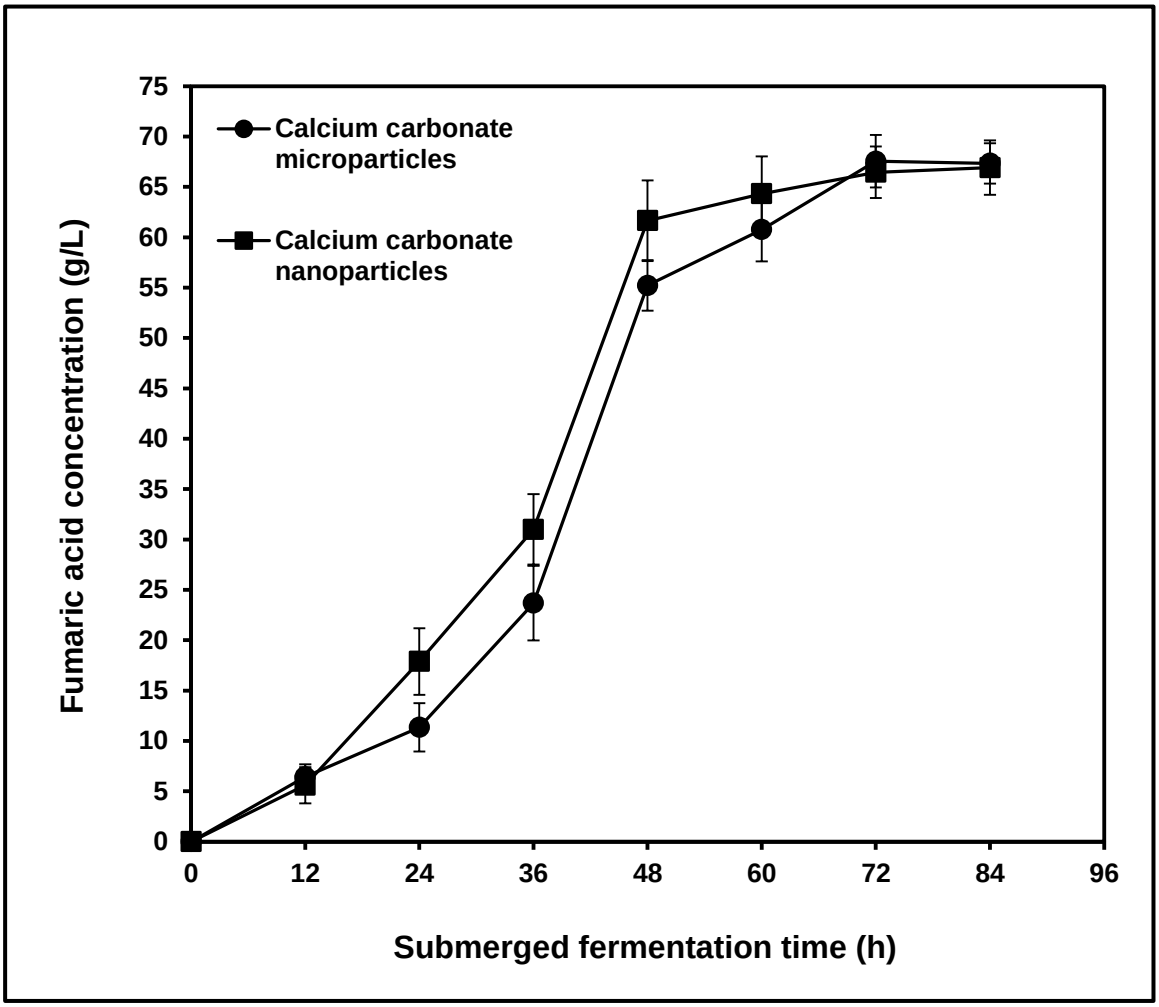


Figure 8.5: Production profile of fumaric acid under submerged fermentation conditions of 25 ^{o}C, 200 rpm and 84 h with $CaCO_3$ micro and nanoparticles as neutralizing agent.

END OF CHAPTER 8

CHAPITRE 9
CONCLUSIONS ET RECOMMANDATIONS

Conclusions

Les conclusions suivantes peuvent être tirées à partir de ce travail de recherche

1. Le développement d'une méthode spectrophotométrique simplifiéepour la quantification de l'acide fumarique est un résultat important de cette étude. La technique simplifiée a été évaluée avec succès en termes de coûts (réduction volumétrique des réactifs utilisés) et d'efficacité de temps (temps d'estimation total). Il y avait une diminution de presque 75% du temps total d'estimation (réduction de 82 min à 22 min) par rapport à la technique originale.Il n'y a pas eu de variation signidicative de la concentration d'acide fumarique par la méthode originale et et par la méthode modifiée. De plus, l'analyse statistique (détermination de la valeur p) de la concentration de FA (g/L) obtenue par la méthode spectrophotométrique et l'HPLC a confirmé l'exactitude de la méthode spectrophotométrique en termes d'efficacité de temps et de rentabilité pour la quantification de l'acide fumarique.

2. Les résultats ont démontré que les eaux usées de brasserie (BW) pourraient être utilisées comme substrat pour la production d'acide fumarique par la souche fongique *R. oryzae* en fermentation submergée. Les BW ont répondu aux besoins en micro- et des macronutriments pour la croissance et la multiplication du champignon sans avoir besoin de suppléments. La culture de *R. oryzae* sur les eaux usées de brasserie dans des conditions de fermentation optimisées (pH 6, 25 °C, 200 rpm, 5% (v/v de volume de l'inoculum, et 25 g/L de solides totaux et diamètre des granules de 0,47 ± 0,04 mm), a permis d'obtenir 31,3 ± 2,8 g/L de FA. Les champignons ont montré des variations morphologiques (e.g., les petites et grandes granules, mycélium en suspension, etc.) comme réponses aux changements dans les conditions appliquées de SmF et cela a entrainé des fluctuations dans la production de FA. L'analyse par microscopie électronique à balayage a permis une caractérisation plus poussée des granules fongiques et des mycéliums.

3. Dans l'étude de la fermentation submergée (SmF) réalisée en présence d'un support de mousseline (en anglais: MC), l'immobilisation réussie du mycélium de la souche fongique de l'espèce *R. oryzae* sur des MCs, a permis d'améliorer la production et la productivité volumétrique de FA par rapport à la fermentation submergée sans support d'immobilisation. Les niveaux de production et de productivité volumétrique de FA ont nettement augmenté, passant de 30,56 ± 1,40 à 43,67 ± 0,32 g/L et de 0,42 à 1,21 g/L.h pour la fermentation submergée immobilisée par rapport à la fermentation sans support. La surface de 25 cm^2 de MC et une concentration de spores de 1,5 x 10^6 par mL s'avèrent optimales pour une

production accrue d'acide fumarique. La microscopie électronique à balayage a confirmé l'uniformité de la formation d'un biofilm sur les surfaces de MC.

4. Dans l'étude réalisée en présence de coquilles d'œufs, les résultats ont mis en évidence les applications potentielles de deux résidus industriels. La production d'acide fumarique à partir de déchets de brasserie et son amélioration avec l'inclusion de coquilles d'œufs pouvant servir de support d'immobilisation, a été une découverte intéressante. Pour la formation de biofilms, différents paramètres à savoir, le nombre de coquilles d'œuf pour l'immobilisation (trois), la concentration de spores ($1,0 \times 10^6$ par mL), le temps d'incubation (24 h) et la vitesse d'agitation du flacon (150 tours par minute) ont été optimisés. Par rapport aux cellules en suspension, les biofilms obtenus par fermentation submergée ont permis d'améliorer nettement la production et la productivité volumétrique d'acide fumarique de 30,23 ± 1,23 à 47,22 ± 0,77 à g/L (augmentation de 56%) et de 0,419 à 1,657 g/L.h (par 3,95 fois), respectivement. Les coquilles d'œuf ont également servi comme agent neutralisant ($CaCO_3$) pendant la SmF. L'analyse par microscopie électronique à balayage a confirmé l'uniformité de la formation du biofilm sur les surfaces des coquilles d'œufs (en anglais: EGS). La nouvelle application des coquilles d'œufs, avec comme double objectif d'être des agents de neutralisation et des supports d'immobilisation, a été une approche très prometteuse.

5. Les résidus solides de pâtes et papiers (en anglais: PPSW) ont permis la croissance du champignon *R. oryzae* sans supplémentation de nutriments. Le champignon a produit de l'acide fumarique pendant la fermentation submergée de particules solides. Le prétraitement des PPSW par réduction de la taille a abouti à l'amélioration de la production d'acide fumarique (23,47 ± 0,70 g/L) dans les conditions de fermentation optimale (30 °C, 200 rpm, 5% d'inoculum pré-cultivé (v/v) et 48 h d'incubation). L' hydrolyse effectuée en présence d'acide phosphorique et un chauffage aux micro-ondes avec des particules de taille allant de $33\ \mu m < x \leq 75\ \mu m$ ont produit un hydrolysat avec une teneur en glucose (11,2 ± 0,8 g/L) et en xylose (20,22 ± 0,85 g/L) maximales. La fermentation submergée avec cet hydrolysat a confirmé l'utilisation de xylose à la fois pour la production et la croissance fongique de l'AF. La fermentation en milieu solide avec des tailles de particules allant de $75\ \mu m < x \leq 300\ \mu m$ a abouti à des productions de FA plus élevées (41,45 g/kg par poids sec) après 21 jours. La microscopie électronique à balayage a permis de mettre en évidence certaines caractéristiques morphologiques du champignon cultivé sur des particules de PPSW.

6. Les résidus de l'industrie de transformation de la pomme, à savoir les boues d'ultrafiltration des jus de pommes (an anglais: APUS) et les rejets solides de jus de pommes (en anglais: AP), ont été utilisés comme sources de carbone, de micro et de macro-nutriments pour le *R. oryzae*. Le champignon a transformé biologiquement les APUS et les AP en acide fumarique lors des fermentations submergées et des fermentations en milieu solide. Pour la fermentation submergée, une concentration en solides totaux d'APUS de 40 g/L, un pH de 6,0, une température de 30 °C, une vitesse d'agitation de 200 rpm et un temps d'incubation de 72 h se sont avérés être optimaux permettant une amélioration de la production de FA et de la productivité volumétrique (25,2 ± 1,0 g/L et 0,350 g/(L.h), respectivement). Le champignon a montré des variations morphologiques (petites et grandes granules et du mycélium en suspension) en fonction des différentes conditions de fermentation submergée appliquées. Une réduction de sucre et du contenu en solides des APUS ont permis de conclure à la capacité du champignon et de transformer ce substrat en acide fumarique lors de la fermentation submergée. Pour la fermentation en milieu solide, à un niveau optimisé d'humidité (50%, p / p) et une période d'incubation (14 jours), 52 ± 2, 7 g d'acide fumarique par kg de poids sec de AP ont été obtenu. L'amélioration de 81,5% de la teneur en composés phénoliques d'AP fermenté après la fermentation à l'état solide a fortement suggéré une biodégradation des polysaccharides (cellulose et hémicellulose) liés à la lignine par le *R. oryzae* comme source de nutriments. La microscopie électronique à balayage a permis de mettre en évidence les caractéristiques morphologiques du champignon cultivé sur les particules de AP.

7. Dans la production pilote d'acide fumarique (en anglais: FA) avec des résidus solides de jus de pommes (en anglais: AP) dans un fermenteur à tambour rotatif (an anglais: BSF), le champignon *R. oryzae* a utilisé le AP et l'a transformé biologiquement en FA. En mode de rotation continu du BSF pendant 14 jours, la concentration de FA la plus élevée obtenue était de 138 ± 9,11 g par kg de poids sec d'AP avec un taux d'humidité de 50% d'AP. Le contrôle de la viabilité du champignon a montré le maintien d'un nombre élevé de spores (2.74×10^8 par g sec d'AP) pendant la fermentation. L'analyse de la composition en fibres de l'AP a confirmé la conversion de fibres alimentaires insolubles en fibres alimentaires solubles et l'utilisation des fibres alimentaires pour la production de FA. La teneur totale en composés phénoliques de l'AP a été considérablement augmentée (d'environ 86%) de 185 ± 10,5 mg à 345 ± 8,5 mg par g de lyophilisat après 18 jours. L'analyse par chromatographie en phase liquide couplée à la spectrométrie de masse LC/MS/MS a confirmé la consommation des sucres (fructose, glucose et saccharose) présents dans le AP par le champignon au cours de la fermentation. La présence de différents composés phénoliques

et l'évolution de leur contenu, après la fermentation, a également été confirmée par l'analyse LC/MS/MS. Les deux autres modes de fonctionnement (intermittents et statiques) du BSF ont permis de produire 82 ± 6,8 g et 58 ± 8,5 g de FA de FA par kg de poids sec d'AP, respectivement après 18 jours de fermentation. Les résultats de la présente étude permettent d'envisager de nouvelles applications à grande échelle des AP pour la production de FA.

8. L'étude des trois NPs, ZnO, Fe_3O_4 et MnO_2 a confirmé leur effet sur la production d'acide fumarique (AF) et sur la morphologie du champignon *R. oryzae*. Parmi les trois NPs, ZnO a donné une production minimale de FA (5,22 ± 1,45 g/L) avec la concentration la plus faible de 200 µg/mL. À cette concentration de NPs, les productivités de FA pour les NPs de Fe_3O_4 et MnO_2 étaient de 36 ± 2,3 g/L et 18,8 ± 1,8 g/L, respectivement. À la concentration de NPs la plus élevée soit 1000 µg/mL, la production de FA (19,8 ± 2,45 g/L) a été obtenue seulement pour les NPs de Fe_3O_4. Les effets inhibiteurs des NPs sur la production de FA se manifestaient dans l'ordre suivant: ZnO > MnO_2 > Fe_3O_4. Cependant, par rapport aux NPs de MnO_2 et Fe_3O_4, la production de FA à 200 µg/mL, pour les microparticules (MPs) de MnO_2 et Fe_3O_4, était la plus élevée (23,1 ± 2,5 g/L et 42 ± 3,7 g/L, respectivement). À 1000 µg/mL de concentration en MPs de Fe_3O_4, la production de FA (32,5 ± 3,5 g/L) était plus élevée par rapport aux NPs de Fe_3O_4. Le champignon a affiché différentes formes morphologiques (e.g. grandes et petites granules, mycélium en suspension, etc.) et ce pour différentes concentrations de NPs et de MPs de ZnO, Fe_3O_4 et MnO_2.

9. L'application des nanoparticules de carbonate de calcium (CCNPs) a amélioré la productivité volumétrique d'acide fumarique (en anglais: FA) de 0,47 g/L h à 0,74 g/(L.h) Le remplacement des microparticules de carbonate de calcium (CCMPs) par les CCNPs a permis de réduire le temps de réaction de neutralisation de FA de 350 secondes à 190 secondes. Aux concentrations de 20, 40 et 60 g/L et à 25 ^{o}C, les viscosités de CCNPs ont été moins élevées que les CCMPs. En outre, les CCNPs n'ont présenté aucune toxicité envers le champignon *R. oryzae*. Les productions de FA obtenues avec les CCMPs et les CCNPs était presque identiques (67,34 ± 2 g/L et 66,92 ± 2,7 g/L, respectivement). Une période de chauffage par micro-ondes de 10 ± 1 min a été jugée suffisante pour la récupération de FA; ce traitement est beaucoup plus court que le temps de chauffage classique de 28 ± 2 min.

Recommendations futures

À partir des résultats présentés dans cette thèse, les recommandations suivantes peuvent être données pour la continuité de la recherche:

1. La morphologie du champignon *Rhizopus oryzae* 1526 joue un rôle crucial dans la fermentation submergée pour la production d'acide fumarique (AF). Quelles que soient les sources de carbone utilisées, l'optimisation des conditions de fermentation doit être dirigée vers l'obtention d'une morphologie de granules adaptée et de préférence, des petites granules ayant un diamètre <1 mm.

2. Les résidus solides de pâtes et papiers (en anglais: PPSW) devraient être étudiés plus en profondeur pour une production à grande échelle de FA. Comparés aux résidus des industries de transformation de pomme et des brasseries, les PPSW conviennent tout particulièrement pour la production de FA ou d'autres acides organiques étant donné leur grande disponibilité, et leur statut actuel comme pollueur (3ème plus grand pollueur industriel de l'air, de l'eau et du sol).

3. La biologie moléculaire pourrait fournir des outils très utiles pour étudier la morphogenèse du champignon *Rhizopus oryzae* 1526 sous différents paramètres de production en fermentation submergée de FA. *Rhizopus oryzae* 1526 pourrait devenir un organisme modèle d'espèces et de souches fongiques filamenteuses produisant de l'acide fumarique.

4. En considérant la consommation mondiale annuelle de bière (1.34×10^9 hL) et le fait que les industries de brasserie utilisent entre 4 et 7 litres d'eau pour produire 1 litre de bière, il devient évident qu'une grande quantité de BW est produite à l'échelle de la planète. Les résultats des études actuelles utilisant les BW ont fortement suggéré qu'il y avait un très bon potentiel pour la production à grande échelle d'acide fumarique à partir de BW. Bien que les résultats obtenus avec les boues d'ultrafiltration de jus de pomme (en anglais: APUS) ont suggéré, pour une utilisation efficace de ces derniers, une concentration jusqu'à 40 g/L en solides totaux par rapport aux BW (jusqu'à 25 g/L), la productivité de FA a été plus faible pour les APUS. Étant donné que ce résultat comparatif a indiqué une meilleure compatibilité (métabolique) du champignon *Rhizopus oryzae* 1526 avec les BW, des études à grande échelle sont recommandées sur ces effluents de brasserie plutôt que sur les APUS.

5. Les résidus solides de jus de pomme peuvent faire l'objet d'essais à grande échelle pour la production d'acide fumarique par fermentation à l'état solide.

6. L'immobilisation du champignon *Rhizopus oryzae* 1526 sur un tissu de mousseline pour la production de FA mérite d'être explorée à plus grande échelle.

8. Le champignon *Rhizopus oryzae* 1526 pourrait encore être cultivé en présence d'autres nanoparticules métalliques (par exemple, l'or, l'argent, le platine, etc.) pour explorer diverses influences métaboliques et morphologiques et les corréler à la production de FA. La biologie moléculaire pourrait fournir des outils intéressants pour réaliser ces études.

9. L'isolement et la purification de FA obtenu par fermentation devront faire l'objet de recherches plus poussées pour démontrer la faisabilité et la supériorité éventuelle de l'approche biologique par rapport aux procédés chimiques.

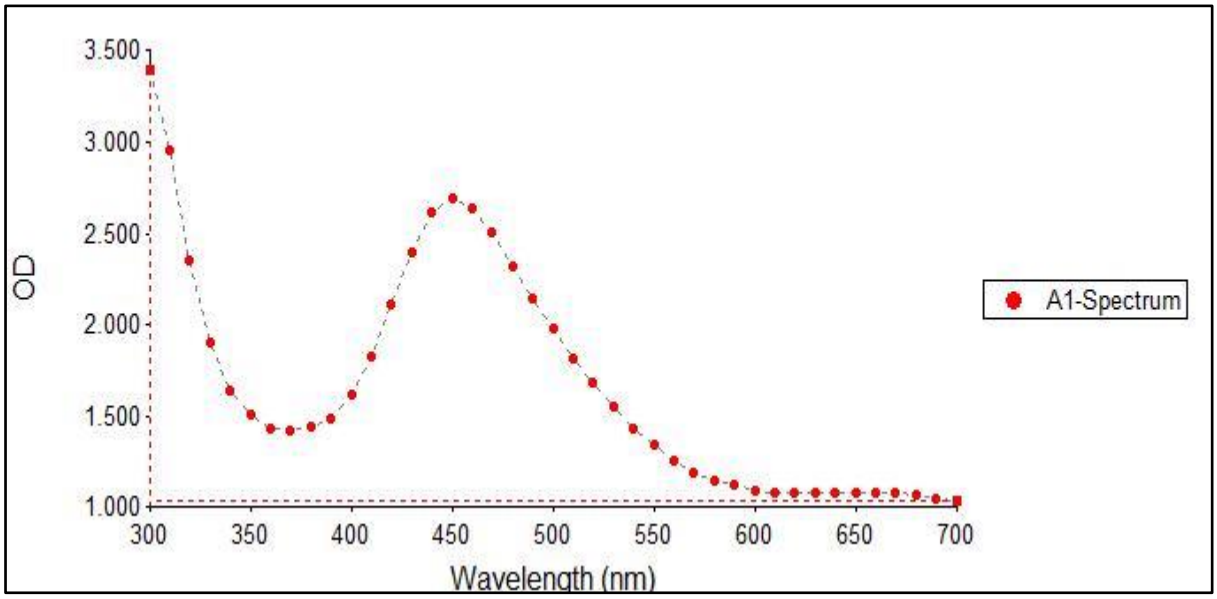
END OF CHAPTER 9

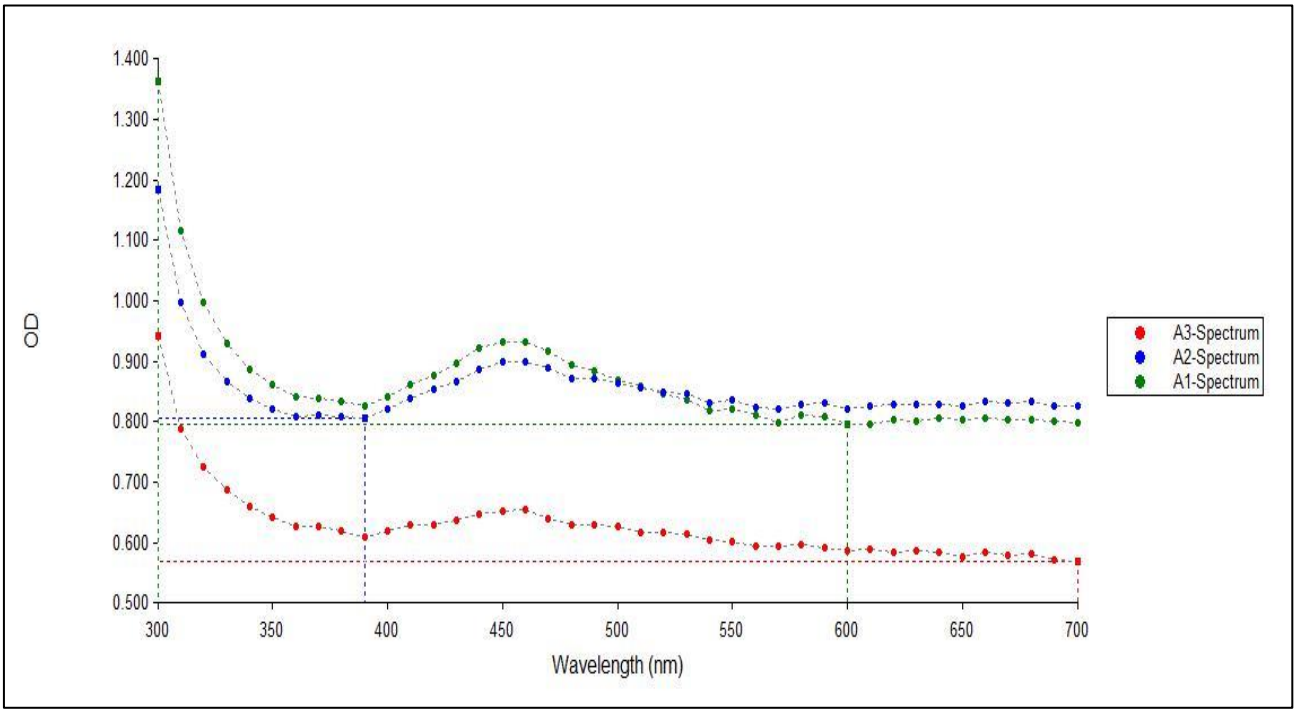
ANNEXES

ANNEXE I

Data: The spectrophotometric (UV-Vis) scanning of fumarate-copper-diethyldithiocarbamate complex for deterimination of λ_{max} (nm).

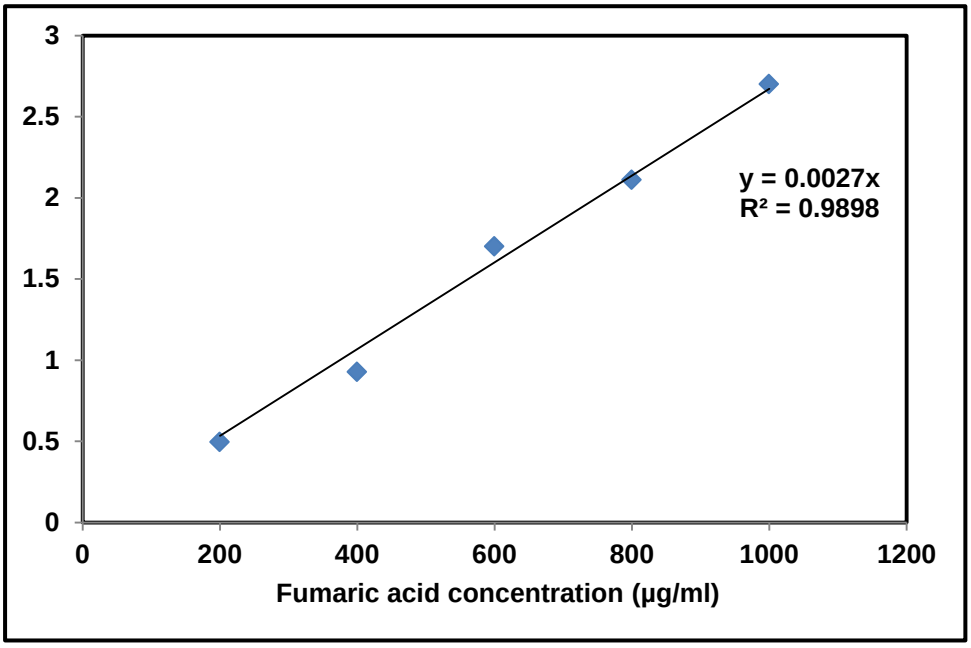
Table 3.1: Technical comparison of the procedures followed in the original and improvised protocol for the colorimetric determination of fumaric acid.

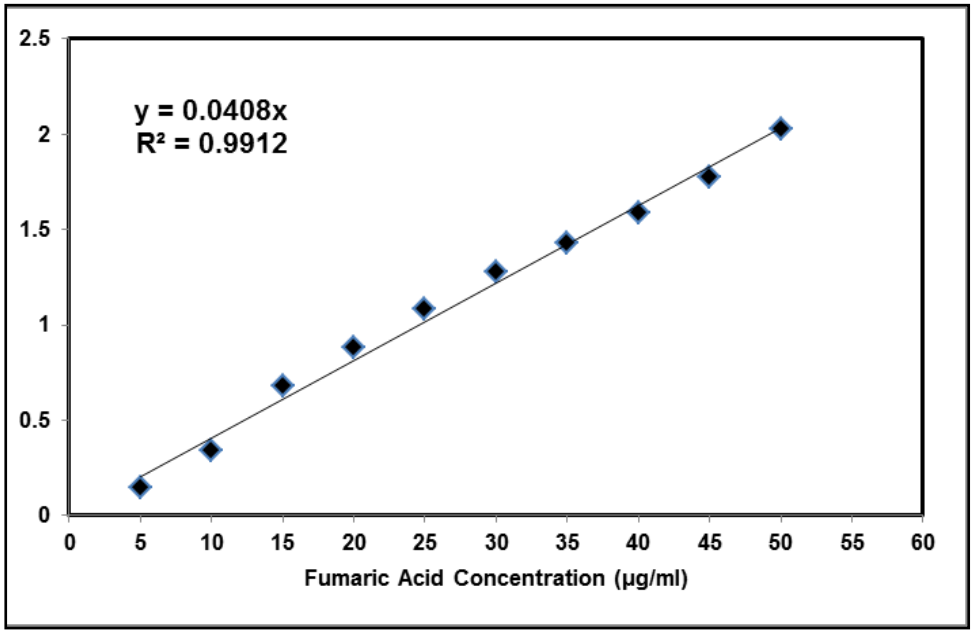




Data: Standard curves (at higher and lower concentration range) of fumaric acid OD measured at λ_{max} = 460 nm.

Table 3.1: Technical comparison of the procedures followed in the original and improvised protocol for the colorimetric determination of fumaric acid.





ANNEXE II

Data: Determination of fumaric acid specific production rate (g/(g h)) for free-cell and immobilized mycelium of *R. oryzae* 1526 on musclin cloth of different surface area and at different spore concentrations at 25 °C, 200 rpm.

Figure 4.1.4: Specific fumaric acid production rates (g/(g h) for free-cell and immobilized SmF at 25 °C, 200 rpm and 2.0×10^6 per mL spore concentration.

Free or immobilized mycelium	Fumaric acid concentration (g/L)	Diference in biomass dry weight (g/L)	Time of submerged fermentation (h)	Fumaric acid specific production rate (g/(g h))
Free-Cell	30.56	0.125	72	3.39
4 cm^2	28.32	1.13	48	0.52
9 cm^2	34.46	0.72	48	0.99
16 cm^2	38.86	0.47	36	2.29
25 cm^2	41.54	0.33	36	3.49

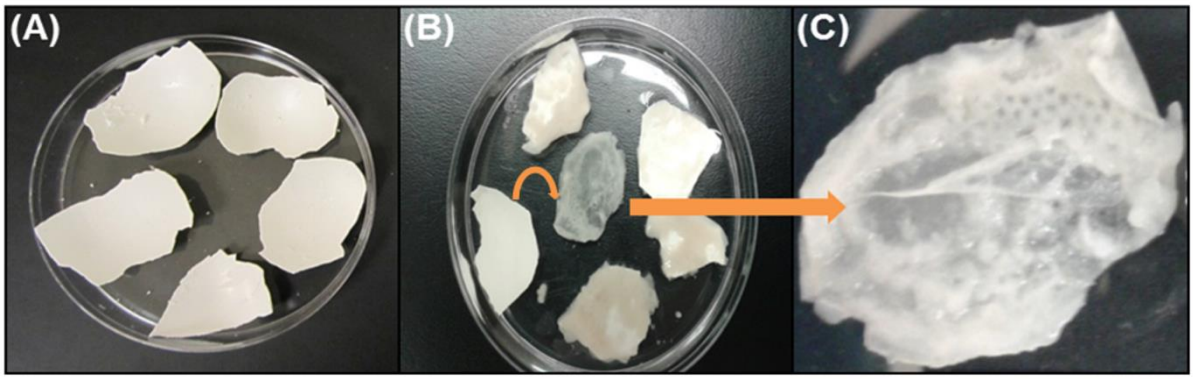
Figure 4.1.5: Specific fumaric acid production rates (g/(g h)) obtained for 25 cm^2 area muslin cloth at different spore concentrations at 25 ^oC and 200 rpm.

Spore Conc (per mL)	Fumaric acid concentration (g/L)	Diference in biomass dry weight (g/L)	Time of submerged fermentation (h)	Fumaric acid specific production rate (g/(g h))
$1.0x10^6$	23.44	0.8	36	0.81
$1.5x10^6$	43.67	1.15	36	1.05
$2.0X10^6$	41.54	0.33	36	3.49
$2.5x10^6$	35.8	1.74	36	0.57
$3.0x10^6$	29.88	2.05	36	0.40

ANNEXE III

Data: (A) Egg shell pieces used for immobilization **(B)** Egg shells with biofilms of *R. oryzae* 1526 grown on their surfaces **(C)** One single fungal biofilm retracted from egg shell surface.

(For chapter 4, Part II)



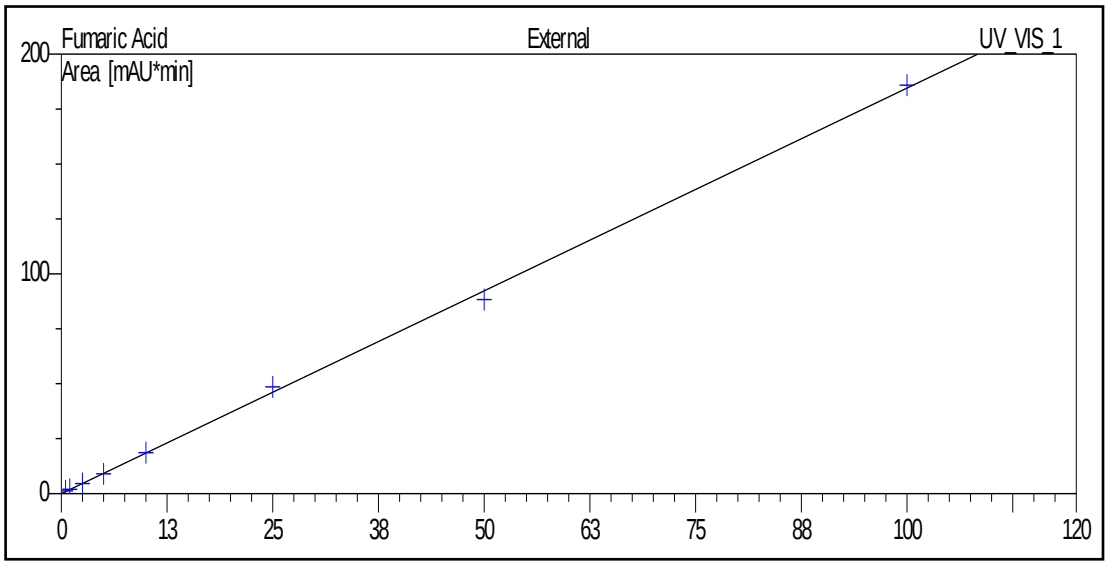
Data: (A) Powdered egg shells used as a source of $CaCO_3$ for fumaric acid production **(B)** A close view of the egg shell powder.

(For chapter 4, Part II)

ANNEXE IV

Data: Fumaric acid standard curve for HPLC analysis of fermented broth samples.

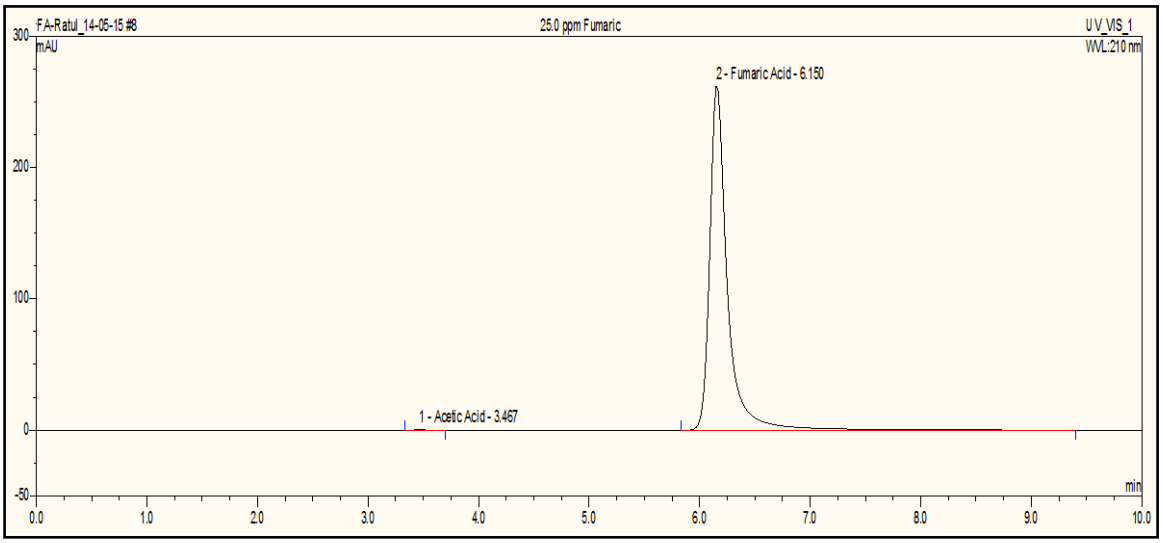
(A representative example of the fumaric acid standard curve prepared for Chapter 4, Part II. Similar methodology was follwed for Chapter 5, Chapter 6, Chapter 7 and Chapter 8. As the number of analyzed fermented broth samples were too high and modifications were required in the preparation of standard curve for the detection of lower or higher concentrations fumaric acid, standard curve varied accordingly.



ANNEXE V

Data: HPLC **c**hromatogram of fumaric acid.

(A representative example of the HPLC chromatogram of fumaric acid obtained for the fermented broth sample)

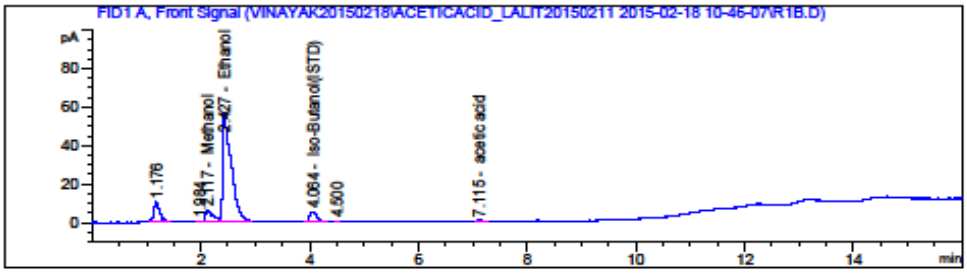


ANNEXE VI

Data: GC **c**hromatogram of ethanol.

(A representative example of the GC chromatogram of ethanol obtained for the fermented broth sample)

```
Data File C:\CHEM32\...\VINAYAK20150218\ACETICACID_LALIT20150211 2015-02-18 10-46-07\R1B.D
Sample Name: R1b

    ======================================================================
    Acq. Operator    : SYSTEM                          Seq. Line :  10
    Acq. Instrument  : GC7890B                          Location : Vial 108
    Injection Date   : 18/02/2015 1:59:27 PM                 Inj :   1
                                                       Inj Volume : 0.7 µl
    Acq. Method      : C:\CHEM32\1\DATA\VINAYAK20150218\ACETICACID_LALIT20150211 2015-02-18 10-46-07
                       \ALCOOL.M
    Last changed     : 18/02/2015 10:46:08 AM by SYSTEM
    Analysis Method  : C:\CHEM32\1\METHODS\VINAYAKCALCMEOHETOH.M
    Last changed     : 18/02/2015 1:17:27 PM by SYSTEM
                       (modified after loading)
    Method Info      : Acides gras volatils
```

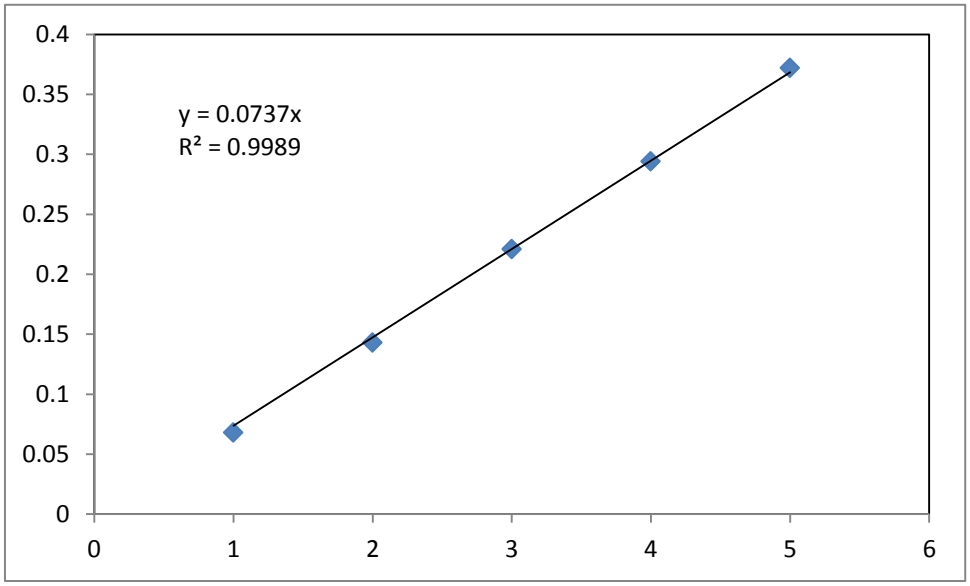



```
    ======================================================================
                          Internal Standard Report
    ======================================================================

    Sorted By             :      Signal
    Calib. Data Modified  :      18/02/2015 4:32:25 PM
    Multiplier            :      1.0000
    Dilution              :      1.0000
    Do not use Multiplier & Dilution Factor with ISTDs
    Sample ISTD Information:
    ISTD  ISTD Amount   Name
      #     [ppm]
    ----|-------------|-------------------------
      1    142.00000   Iso-Butanol(ISTD)


    Signal 1: FID1 A, Front Signal

    RetTime  Type  ISTD    Area     Amt/Area    Amount   Grp   Name
     [min]         used  [pA*s]      ratio      [ppm]
    -------|------|----|----------|----------|----------|--|------------------
      2.117 VV        1   60.01361    3.20300  554.75238    Methanol
      2.427 VB        1  620.44733    1.28878 2307.67916    Ethanol
      4.064 BB  +I    1   49.20342    1.00000  142.00000    Iso-Butanol(ISTD)
      7.115 BB        1    4.76537    7.75861  106.70222    acetic acid
      8.193           1     -          -          -         butiric acid

    Totals without ISTD(s) :                2969.13377

GC7890B 18/02/2015 4:45:31 PM SYSTEM                              Page   1 of 2
```

ANNEXE VII

Data: Standard curve of glucose ((λ_{max} = 540 nm) used for the estimation total reducing sugar in BW, APUS and fermented broth samples by DNS method.

(A representative example)



ANNEXE VIII

Data: Standard curve of gallic acid (λ_{max} = 765 nm) preapred for the estimation of total phenolic contents of AP and PPSW and SSF samples of the respective substrates.

(A representative example)