Université du Québec

INRS Institut national de la recherche scientifique

UNIVERSITÉ DU QUÉBEC
INSTITUT NATIONAL DE LA RECHERCHE SCIENTIFIQUE
CENTRE EAU TERRE ENVIRONNEMENT

Bioproduction of fumaric acid and its derivatives using agro-industrial residues as feedstock

Par

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(M. Res., Brewing Science; M. Sc., Applied Biomolecular Technology)

Thèse présentée pour l'obtention du grade de
Philosophiae Doctor (Ph.D.)
en sciences de l'Eau

Jury d'évaluation

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Dédicace

This thesis is dedicated to my mom and friends for their unconditional support and love.

REMERCIEMENTS

I would like to express my gratitude to my Ph.D. supervisor, Professor Satinder Kaur Brar for her guidance, suggestions and valuable feedback during my doctoral training. Her constant guidance and support, has helped me immensely in being organized and outside the box thinking. This allowed me to work with more freedom and passion. I want to equally express my regards to my project co-supervisor, Dr. Tarek Rouissi who helped me in solving essential problems with his support, valuable suggestions and ideas.

I would also like to thank my examiners, Prof. Riadh Hammami, Prof. Mohammed Aider, Prof. Yann Le Bihan whose suggestions and recommendations helped in improving the quality of my thesis. I am sincerely thankful to TEDGIEER for financing the project work. I would also like to thank Prof. Andre St-Hilaire and Prof. Patrice Couture for their assistance with the administrative works.

I would like to thank all the administrative and laboratory personnel from INRS, especially Stephane Moise and Sebastian Duval for providing the instrument training as well as analysis of experimental samples. I would like to thank my colleagues who supported me throughout my Ph.D. study, especially, Dr. Krishnamoorthy Hegde, Dr. Joanna Lecka, Dr. Bikash Tiwari, Dr. Pratik Kumar, Dr. Gayatri Suresh, Dr. Agnieszka Cupryś, Dr. Rama Pulicharla, Dr. Linson Lonappan, Dr. Mehrdad Taheran, Dr. Naghdi Mitra, Dr. Vinayak Pachapur, Dr. Lalit Kumar, Carlos Osorio, Rahul Saini, Mariana Valdez, Saba Miri and Mohammad Reza. A special thanks are in order to Oumaima El-Hachimi and Mona Chaali for their enormous help with the French translation of my thesis. It would be incomplete without thanking my intern, Karen Villegas-Dominguez, who assisted me during the project.

Finally, I would like to give my sincerest thanks to my parents, my sibblings and the extended family for their support and motivation.

RÉSUMÉ

L'acide fumarique (AF) est un produit métabolique microbien qui a été identifié comme une importante plate-forme chimique pour les les dérivées des hydrates de carbone par le ministère américain de l'énergie (DOE). La présence d'un groupe dicarboxylique en plus de la double liaison rend l'acide fumarique modifiable chimiquement et utilisable comme monomère pour la synthèse de polymères biodégradables. À l'heure actuelle, la demande mondiale en AF est assurée uniquement par la conversion chimique de l'anhydride maléique, dérivé du pétrole en acide maléique, en acide fumarique, ce qui entraîne la libération de gaz toxiques et de gaz à effet de serre qui polluent l'environnement et contribuent au réchauffement de la planète. L'augmentation prévue de la demande et la pollution qui en découle ne feront qu'aggraver les impacts négatifs sur l'environnement. Pour atténuer ces effets, la bioproduction de l'acide fumarique a fait l'objet de cette étude. La bioproduction constitue donc une voie de synthèse alternative, durable et respectueuse de l'environnement qui permet la fixation et la récupération du carbone. Dans la présente étude, différents résidus agro-industriels, tels que les boues d'ultrafiltration de marc de pomme et le marc de pomme, ainsi que de la biomasse lignocellulosique, ont été utilisés comme substrat pour la production d'AF par fermentation submergée en utilisant le champignon *R. oryzae*. L'acide fumarique ainsi produit a été ensuite converti en ses esters correspondants à l'aide d'une stratégie de bioconversion qui utilise des enzymes, telles que la lipase et l'estérase, pour catalyser la conversion. De plus, la biomasse résiduelle a été soumise à une extraction alcaline pour récupérer du chitosane et, à cet effet, les conditions optimales de l'irradiation par micro-ondes ont été identifiées. La production d'AF est associée à la morphologie du champignon et l'adoption de la stratégie d'immobilisation nous permet de surmonter cette limitation et avoir un meilleur contrôle du processus. Cet effet a été étudié dans ce projet de recherche. Parmi les matériaux

étudiés, la mousse de polystyrène s'est avérée être le matériau de support idéal pour l'immobilisation de R. oryzae. *De plus, la réduction de la taille du matériau de support a conduit à une amélioration significative du rendement en AF (27 g/L contre 19 g/L) ainsi qu'à une réduction considérable de la phase de latence de la production d'AF*. Afin d'améliorer encore la production d'AF, la fermentation en mode fed-batch a été effectuée avec un apport discontinu de mélasse comme nourriture. *Cela a permis de tripler le rendement en AF à l'échelle de la fiole et de l'augmenter à l'échelle du fermenteur pour obtenir un titre AF de 12 g/l.*

Dans le but d'améliorer encore la compétitivité économique du processus de bioproduction, la possibilité d'extraire le chitosan à partir de la biomasse fongique a été également étudiée. À cet effet, les conditions optimales des paramètres opératoires (puissance et de durée de chauffage) de de l'extraction assistée par micro-ondes (MAE) du chitosane ont été identifiées *comme étant 300W (puissance) et 22 minutes (durée de chauffage). Le chitosane obtenu dans ces conditions optimales a montré un degré plus élevé de désacétylation (94,6 %), un poids moléculaire plus élevé (127,6 kDa), un rendement plus élevé de 13,43 % (p/p) et une consommation d'énergie plus faible que le chitosane extrait par voie chimique standard.* Ces résultats ont un impact positif et améliore la compétitivité économique du bioprocessus en permettant ainsi un processus de bioproduction circulaire.

Mots clés : *Acide fumarique, Esters fumarates, Bioproduction, Immobilisation, Bioconversion, R. oryzae, Biomasse lignocellulosique, Chitosane, Extraction assistée par micro-ondes*

ABSTRACT

Fumaric acid (FA), is a microbial metabolic product that has been identified as an important carbohydrate-derived platform chemical by the US Department of Energy (DOE). The presence of the dicarboxylic group in addition to the double bond renders FA amenable to chemical modifications and suitable for use as a monomer for the synthesis of biodegradable polymers. At present, the global demand for FA is solely fulfilled via chemical conversion of petroleum-derived maleic anhydride to maleic acid and thereafter to fumaric acid, leading to the release of toxic as well as greenhouse gases causing environmental pollution and contributes to global warming. The expected future increase in demand and the associated pollution will further add to the stresses on the environment. To alleviate the associated stress on the environment bioproduction of fumaric acid is being considered. Hence, offering an attractive alternate sustainable and environmentally friendly means of synthesis that allows for carbon fixation and recovery.

In the current investigation, different agro-industrial residues, such as apple pomace ultrafiltration sludge and apple pomace, and lignocellulosic biomass, were used as a substrate for FA production through submerged fermentation employing *R. oryzae*. The fumaric so produced will then be converted to its corresponding esters using a bioconversion strategy that utilizes enzymes, such as lipase and esterase, to catalyze the conversion. Additionally, the recovered biomass was subjected to alkaline extraction of chitosan and to this effect the optimal conditions of the more efficient microwave irradiation were identified.

FA production is associated with fungal morphology and the adoption of the strategy of immobilization allows us to overcome this limitation and allow for better process control and was investigated. Polystyrene foam was found to be the ideal support material for the immobilization

of *R. oryzae* among the materials investigated. *Additionally, the reduction in the size of the support material led to a significant improvement in FA yield (27 g/L against 19 g/L) as well as a considerable reduction in the lag-phase of FA production*. To further improve the quantity of FA obtained, operating the fermentation in fed-batch mode was considered with intermittent supplementation of molasses as feed. *This led to an additional three-fold increase in FA yield at flask scale and scale-up in fermenter provided for a FA titer of 12 g/L.*

To further improve the economic competitiveness of the bioproduction process the possibility of chitosan extraction from the fungal biomass was investigated. To this effect, the optimal conditions of power and duration of heating for microwave-assisted extraction (MAE) of chitosan from *R. oryzae* biomass were identified. *The optimal conditions were identified to be 300W (power) and 22 minutes (duration of heating). Chitosan obtained under the optimal conditions of microwave extraction showed a higher degree of deacetylation (94.6 %), higher molecular weight (127.6 kDa), higher yield of 13.43 % (w/w) and lower energy consumption*. These have a profound impact on the economic competitiveness of the bioprocess as well as allow for a circular bioproduction process.

Keywords: Fumaric acid, Fumarate esters, Bioproduction, Immobilization, Bioconversion, *R. oryzae*, Lignocellulosic biomass, Chitosan, Microwave-assisted extraction

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7. **Sebastian, J.,** Rouissi, T., Brar, S.K., Biobased dimethyl fumarate and monomethyl fumarate synthesis. (Under review Process Biochemistry)

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LISTE DES ABRÉVIATIONS

AIM	Alkali insoluble material
APUS	Apple pomace ultra-filtration sludge
AP	Apple pomace
ARS	Agricultural Research Services
ATP	Adenosine triphosphate
BOD	Biological oxygen demand
BPE	Breakeven price
CO_2	Carbon dioxide
CO	Carbon monoxide
COD	Chemical oxygen demand
DDA	Degree of deacetylation
$d.H_2O$	Distilled water
DMF	dimethyl fumarate
DNA	Deoxyribonucleic acid
DNS	3,5-Dinitrosalicylic acid
DO	Dissolved oxygen
DOE	US Department of Energy
FA	Fumaric Acid
FAEs	Fumaric Acid Esters
FCI	Fixed capital investment
FDA	Food and Drug Administration
FMCG	Fast-moving consumable goods
FT-IR	Fourier-transform infrared spectroscopy
fum	fumarase
GHGs	Greenhouse gases
GRAS	Generally regarded as safe

Kg	Kilogram
ldh	lactate dehydrogenase
MAE	Microwave-assisted extraction
MMF	mono-methyl fumarate
mdh	malate dehydrogenase
NaOH	Sodium hydroxide
NRRL	Northern Regional Research Lab
PDA	Potato dextrose agar
PDB	Potato dextrose broth
PVP	polyvinyl pyridine
pyc	pyruvate carboxylase
rpm	Revolutions per minute
SEM	Scanning electron microscopy
RSM	Response surface methodology
vvm	Vessel volumes per minute
TCA	Tricaboxylic acid
TCI	Total capital investment
TEA	Techno-economic anlaysis
WCI	Working capital investment

CHAPTER ONE: SYNTHÈSE

PARTIE 1. INTRODUCTION

Depuis longtemps, les micro-organismes sont exploités pour fabriquer divers produits allant de produits chimiques, comme l'éthanol et les acides organiques, aux produits de consommation, comme le pain, le yaourt et le fromage. L'acide fumarique (AF), également appelé acide (E)-2-butènedioïque ou acide trans-1,2-éthylène dicarboxylique, est l'un de ces produits métaboliques microbiens qui ont été identifiés comme une plate-forme chimique par le ministère américain de l'énergie (DOE) en 2004 (Bozell & Petersen, 2010; Choi et al., 2015). Il s'agit d'une importante plate-forme chimique dérivée des hydrates de carbone, un intermédiaire clé du métabolisme microbien, avec plusieurs applications dans la synthèse de produits chimiques biosourcés et la production de polymères. La présence d'un groupe dicarboxylique et d'une double liaison rend les AF susceptibles d'être modifiés chimiquement et de servir de monomère pour la synthèse de polymères biodégradables (Roa Engel et al., 2008).

L'acide fumarique est principalement utilisé comme additif et acidulant alimentaire et dans la production de résines de papier, de résines de polyester insaturé et de plastifiants (Rodríguez-López et al., 2012; Xu et al. 2012). Plus récemment, les dérivés de l'acide folique, en particulier les esters d'acide fumarique (EAF), ont trouvé leur application comme produit chimique important avec un large éventail d'applications biomédicales, telles que le traitement du psoriasis et de la sclérose et le matériau de support pour l'ingénierie tissulaire. Des études de niveau clinique sur les effets pharmacologiques de l'acide fumarique et de ses dérivés esters (EAF) ont confirmé leur efficacité. Les esters méthyliques de l'acide fumarique, le fumarate de mono-méthyle (MMF) et le fumarate de diméthyle (DMF) ont même été autorisés en Europe pour le traitement du psoriasis vulgaire sévère (Ermis et al., 2013; Reich et al., 2009). La récente approbation par la FDA du DMF (Tecfidera) pour le traitement des formes récurrentes de la sclérose en plaques prouve l'importance

des AF et des EAF. De même, les EAF, en particulier les esters éthyliques, ont trouvé leur application dans l'ingénierie tissulaire en tant qu'échafaudage ainsi que dans l'administration de médicaments (Jansen et al., 2010; Jansen et al., 2012).

Actuellement, la demande mondiale d'AF est uniquement assurée par la conversion chimique de l'anhydride maléique dérivé du pétrole en acide maléique, puis en acide fumarique. L'anhydride maléique est obtenu par oxydation du butane ou du benzène en présence du pyrophosphate de vanadyle comme catalyseur (Roa Engel et al., 2008; Rodríguez-López et al., 2012). Ce procédé chimique médié par un catalyseur permet d'obtenir un rendement élevé en alcool furfurylique (112 % p/p) à partir d'anhydride maléique (Felthouse et al., 2001; Roa Engel et al. 2008). Cependant, ce procédé conduit également à la formation d'un gaz toxique, le monoxyde de carbone, et d'un gaz à effet de serre, le dioxyde de carbone, en tant que sous-produits, ce qui entraîne une pollution environnementale et contribue au réchauffement climatique (Das et al., 2016a). De même, la synthèse des EAF repose sur des processus chimiques pour la conversion de l'anhydride maléique en ester fumarate.

La demande mondiale en AF devrait passer à plus de 300 kilotonnes d'ici 2020, contre 225,2 kilotonnes (2012). Cette augmentation de la demande est principalement alimentée par une hausse de la demande de l'acide utilisé dans l'industrie alimentaire et des boissons, qui représente plus de 35 % de la consommation mondiale (Papadaki et al., 2017; www.grandviewresearch.com; www.ihsmarkit.com). En outre, l'identification récente de ses propriétés pharmaceutiques peut conduire à une utilisation accrue de l'AF et de ses dérivés, en particulier des EAF, dans l'industrie pharmaceutique, comme nous l'avons vu précédemment. Cela ne fera qu'accroître la demande mondiale en cet acide. De plus, le prix élevé du pétrole brut, son épuisement et l'accent mis

récemment sur la chimie verte pour le développement durable ont nécessité l'adoption de moyens alternatifs pour la production d'AF et de ses dérivés (Papadaki et al., 2017; Xu et al. 2012).

La bioproduction microbienne d'AF constitue une alternative intéressante pour répondre aux futures demandes de cette importante plate-forme chimique. Le passage à une stratégie de bioproduction permet un développement durable en réduisant la dépendance aux produits pétroliers ainsi que l'impact environnemental négatif associé. Des études portant sur plusieurs espèces de champignons ont conclu que l'espèce *Rhizopus* était le meilleur producteur naturel d'acide fumarique. De plus, l'adoption d'une stratégie de bioproduction permet la fixation et la récupération du dioxyde de carbone, notamment lorsque des résidus végètaux sont utilisés. L'adoption d'une stratégie de bioproduction permet d'utiliser de la biomasse renouvelable ainsi que des résidus industriels pour la production. Cela permet de réduire considérablement les coûts de production et d'améliorer ainsi la faisabilité. D’ailleurs, la faible solubilité de l'acide fumarique, 5-7 g/L (à température ambiante), permet de le récupérer facilement par simple précipitation après acidification. Par conséquent, aucune stratégie spécifique n'est nécessaire pour sa récupération (Roa Engel et al., 2008; Sauer et al., 2008).

Compte tenu de l'importance de l'AF en tant que produit chimique de base et de sa récupération facile, le développement d'une stratégie de bioproduction est un enjeu capital pour répondre à la future demande. L'utilisation du *Rhizopus* sp., pour sa bioproduction permet d’exploiter des résidus agro-industriels en raison de ses besoins minimes en nutriments, ce qui permet de valoriser les résidus agro-industriels et de capturer le carbone qui, autrement, aurait entraîné une augmentation des émissions de gaz à effet de serre. Une valeur ajoutée supplémentaire au processus de bioraffinage peut être rendue possible en permettant la conversion de l'acide fumarique obtenu en ester de fumarate, un dérivé de haute valeur et de haute demande. La bioconversion des AF en

EAF n'a pas été rapportée jusqu'à présent. Par conséquent, le développement d'une stratégie de bioproduction permettant la conversion de l'acide fumarique en ester de fumarate peut avoir un impact positif sur la faisabilité du processus de bioraffinage.

Dans cette étude, différents résidus agro-industriels, tels que les boues d'ultrafiltration de marc de pomme et le marc de pomme, ainsi que la biomasse lignocellulosique, ont été utilisés comme substrat pour la bioproduction d'AF à l'aide de la fermentation médiée par *R. oryzae.* L'utilisation de différentes stratégies, telles que l'immobilisation des champignons et la fermentation en mode fed-batch, ont été étudiées pour améliorer la production d'AF. De plus, afin d'améliorer la compétitivité économique ainsi que l'aspect écologique du processus de bioproduction, l'extraction du chitosane à partir de la biomasse fongique assistée par micro-ondes et la bioconversion enzymatique des AF en EAF ont été étudiées. La combinaison de toutes ces stratégies devrait permettre le développement d'un processus de bioraffinage hautement compétent et respectueux de l'environnement.

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PARTIE 2. REVUE DE LITTÉRATURE

Les micro-organismes peuvent produire une gamme variée de produits. Ces produits du métabolisme microbien, tels que les acides organiques, ont un large éventail d'applications et peuvent être utilisés dans notre vie quotidienne. Depuis longtemps, l'être humain a eu recours aux micro-organismes pour produire divers produits allant des produits chimiques, comme l'éthanol et les acides organiques, aux produits de consommation, comme le pain, le yaourt et le fromage. L'acide fumarique (AF), également appelé acide (E)-2-butènedioïque ou acide trans-1,2-éthylène dicarboxylique, est l'un de ces produits métaboliques microbiens qui ont été identifiés comme une plate-forme chimique par le ministère américain de l'énergie (DOE) en 2004 (Bozell & Petersen, 2010; Choi et al., 2015). Il s'agit d'une importante plate-forme chimique dérivée des hydrates de carbone, un intermédiaire clé du métabolisme microbien, avec plusieurs applications dans la synthèse de produits chimiques biologiques et la production de polymères. Ces diverses applications sont dues à la présence de groupes dicarboxyliques. Cela permet aux AF de se prêter à des modifications chimiques et de s'utiliser comme monomère pour la synthèse de polymères biodégradables (Roa Engel et al., 2008).

L'AF est principalement utilisé comme additif et acidulant alimentaire et dans la production de résines de papier, de résines de polyester insaturé et de plastifiants (Rodríguez-López et al., 2012; Xu et al., 2013). L'application de l'acide fumarique, en général, a été présentée en détail par Das et al. (2016 a, b) et les diverses applications industrielles de l'acide comme résine, plastifiant et composant pour la synthèse de polymères ont été examinées par Doscher et al. (1941). Plus récemment, les dérivés de l'acide fumarique, en particulier les esters de l'acide fumarique (EAF), ont trouvé leur application comme produit chimique important avec un large éventail d'applications biomédicales, telles que le traitement du psoriasis et de la sclérose et le matériau de

support pour l'ingénierie tissulaire. Des études à l'échelle clinique portant sur les effets pharmacologiques de l'acide fumarique et de ses dérivés esters (EAF) ont confirmé leur efficacité. Les esters méthyliques de l'acide fumarique, les esters du fumarate de mono-méthyle (MMF) et du fumarate de diméthyle (DMF), ont même été autorisés en Europe pour le traitement du psoriasis vulgaire sévère (Ermis et al., 2013; Reich et al., 2009). La récente approbation par la FDA du DMF (Tecfidera) pour le traitement des formes récurrentes de la sclérose en plaques prouve l'importance des AF et des EAF. De même, les EAF, en particulier les esters éthyliques, ont trouvé leur application dans l'ingénierie tissulaire en tant qu'échafaudage ainsi que dans l'administration de médicaments (Jansen et al., 2010; Jansen et al., 2012). La revue de littérature publié par Das et al. (2016) fournit des informations complètes sur les diverses applications médicales de l'acide fumarique et de ses dérivés (Das et al., 2016).

Les diverses applications des AF ont alimenté la demande en acide dicarboxylique, qui est assurée par synthèse chimique à partir de produits pétroliers. L'accent mis sur la transition vers une économie biosourcée a suscité un regain d'intérêt pour le développement de stratégies de bioproduction d'AF. L'AF est un intermédiaire clé de la voie métabolique pour un groupe diversifié de micro-organismes, ce qui a conduit à l'étude approfondie de ces micro-organismes comme source potentielle pour la production d'AF. Certaines souches fongiques, comme *Rhizopus oryzae*, ont été identifiées comme surproducteurs d'AF et comme source potentielle d'AF. Cette revue de littérature aborde les différentes stratégies d'amélioration des souches étudiées pour optimiser la production d'AF par *R. oryzae* ainsi que les stratégies de génie métabolique et génétique étudiées pour parvenir à une production microbienne en utilisant des non producteurs d'AF. Ces recherches n'ont pas abouti à la bioproduction commerciale d'AF et les raisons plausibles en sont fournies. En

outre, les progrès des techniques de génie métabolique nous ont fourni de nouveaux outils et des pistes pour l'utilisation de ces stratégies pour la bioproduction d'AF et de ses dérivés.

2.2. Synthèse actuelle de l'acide fumarique

À l'heure actuelle, la demande mondiale d'acide fumarique est uniquement assurée par les méthodes de synthèse pétrochimiques représentées dans la figure 1.1 (Roa Engel et al., 2008; Rodríguez-López et al., 2012). Il est issu de l'isomérisation de l'acide maléique obtenu par hydrolyse de l'anhydride maléique. L'anhydride maléique est obtenu par oxydation du butane ou du benzène en présence d'un catalyseur, le pyrophosphate de vanadyle (Roa Engel et al., 2008; Rodríguez-López et al., 2012). La conversion médiée par le catalyseur est la seule technique possible pour la conversion sélective du butane en acide maléique (Felthouse et al., 2001). Ce procédé chimique médié par un catalyseur permet d'obtenir un rendement élevé d'AF (112 % p/p) à partir d'anhydride maléique (Felthouse et al., 2001; Roa Engel et al., 2008). Cependant, ce procédé conduit également à la formation de gaz toxique, le monoxyde de carbone, et de gaz à effet de serre, le dioxyde de carbone, en tant que sous-produits, ce qui pollue l’environnement et contribue au réchauffement climatique (Das et al., 2016). La demande mondiale d'AF devrait augmenter à plus de 300 kilotonnes d'ici 2020, contre 225,2 kilotonnes (2012). Cette augmentation de la demande est principalement alimentée par une hausse de la demande de l'acide utilisé dans l'industrie alimentaire et des boissons, qui représente plus de 35% de la consommation mondiale (Papadaki et al., 2017; www.grandviewresearch.com; www.ihsmarkit.com). En outre, l'AF est un intermédiaire important pour la synthèse de produits comestibles, tels que l'acide L-malique et l'acide L-aspartique. L'augmentation de la demande d'édulcorants artificiels, tels que l'aspartame, dans les boissons et les aliments, va encore accroître la demande mondiale d'AF et de ses dérivés (Goldberg et al., 2006). En outre, l'identification récente de ses propriétés pharmaceutiques peut

conduire à une utilisation accrue de l'AF et de ses dérivés dans l'industrie pharmaceutique, comme nous l'avons vu précédemment. Cela ne fera qu'accroître la demande mondiale de cet acide. En outre, le prix élevé du pétrole brut, son épuisement et l'accent mis récemment sur la chimie verte pour le développement durable, ont nécessité l'adoption de moyens alternatifs pour la production d'AF (Papadaki et al., 2017; Xu et al., 2012).

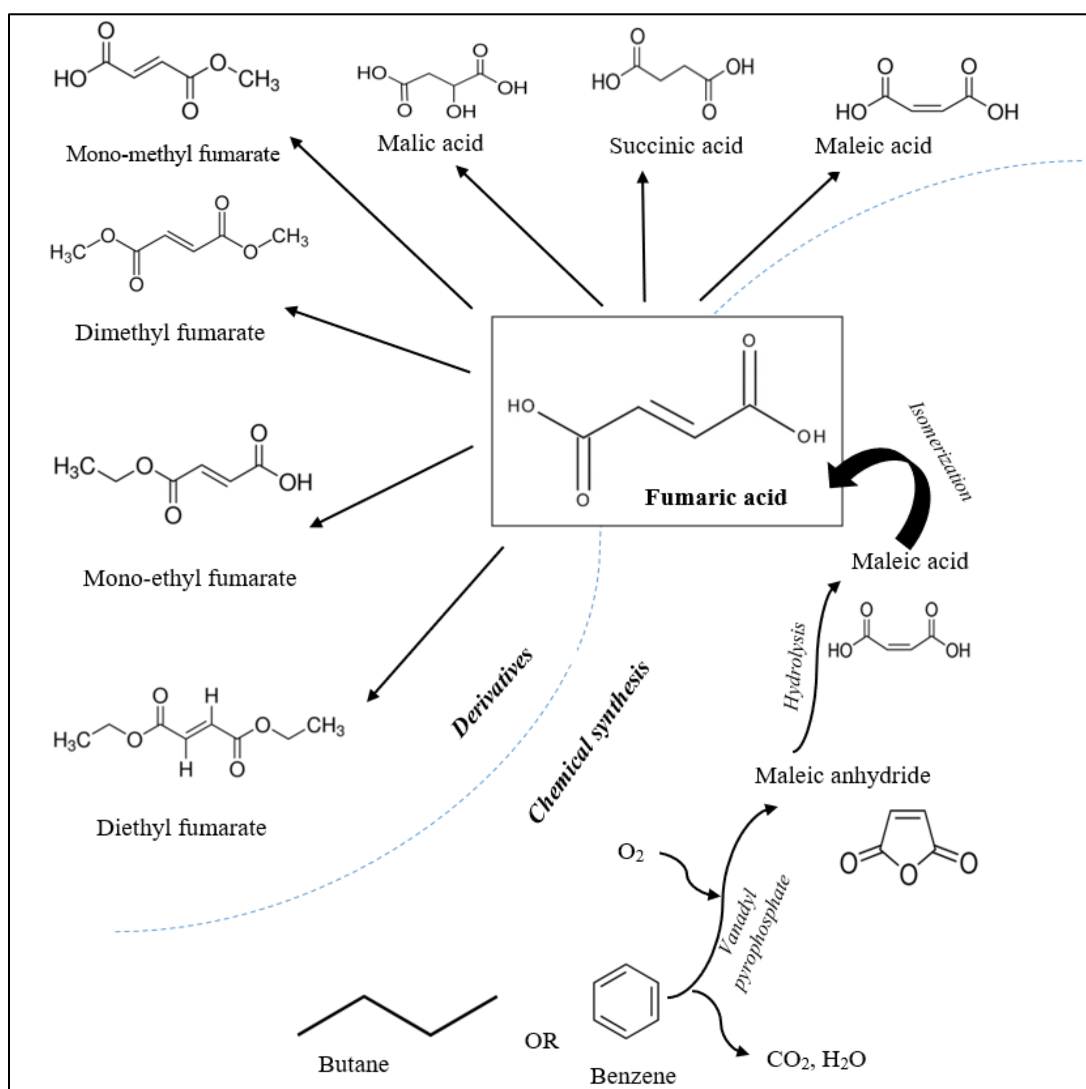

Figure 1. 1. Chemical fumaric acid synthesis and its derivatives (Sebastian et al. 2019)

<u>Déduction 1</u> : L'acide fumarique est un acide organique essentiel qui trouve des applications dans divers domaines. La bioproduction de l'acide fumarique est hautement souhaitable. De plus, la matière première utilisée pour la production doit être renouvelable et bon marché pour rendre le processus compétitif en termes de coûts et respectueux de l'environnement.

2.3. Bioproduction d'acide fumarique

La bioproduction microbienne de la substance biochimique dérivée des hydrates de carbone constitue une alternative attrayante qui garantit le développement durable en réduisant la dépendance aux combustibles fossiles. En outre, l'utilisation de matières premières renouvelables permet de récupérer du carbone précieux. L'AF est l'un de ces composés synthétisés par de nombreux microorganismes, notamment les microorganismes aérobies, en tant que composé intermédiaire au cours du cycle TCA (Das et al., 2016; Lee et al., 2011). Le champignon *Rhizopus nigricans*, a été identifiée pour sa capacité à synthétiser les AF dès 1911. Plus tard, des études impliquant plusieurs espèces fongiques ont conclu que l'espèce *Rhizopus* sp. était le meilleur producteur d'acide fumarique. Xu et al. (2013) ont fourni un résumé de l'historique de la bioproduction d'AF. De plus, l'adoption de la stratégie de bioproduction permet la fixation et la récupération du dioxyde de carbone, notamment lorsque des résidus industriels organiques sont utilisés. Cependant, l'avènement de moyens pétrochimiques moins coûteux pour la synthèse de l'acide fumarique a conduit à l'abandon de la bioproduction d'AF. Des rendements plus élevés en AF sont obtenus avec les procédés chimiques (112 % p/p contre 85 % p/p par voie fermentaire en utilisant le glucose comme source de carbone). Aussi, le coût de production plus faible et le meilleur contrôle des conditions de production étaient en faveur de l'utilisation généralisée du procédé chimique pour la production d'AF (Roa Engel et al., 2008). La récente augmentation du

prix des produits pétroliers, l'importante croissance des stratégies de production à faible empreinte carbone et l'approche de la chimie verte pour le développement durable, ont entraîné un regain d'intérêt pour la production d'acide fumarique par voie fermentaire (Papadaki et al., 2017; Xu et al., 2013). De plus, l'adoption de la stratégie de bioproduction permet d'utiliser la biomasse renouvelable ainsi que les résidus industriels pour la production. Cela permet de réduire considérablement les coûts de production et d'améliorer ainsi la faisabilité. De plus, la faible solubilité de l'acide fumarique, 5-7 g/L (à température ambiante), permet de le récupérer facilement par simple précipitation après acidification. Aucune stratégie spécialisée n'est donc nécessaire pour la récupération de l'acide fumarique (Roa Engel et al., 2011; Sauer et al., 2008).

2.3.1 Voie métabolique de la synthèse de l'acide fumarique

L'acide fumarique est un intermédiaire important du cycle de l'acide tricarboxylique (TCA) ou cycle de Krebs. Deux voies métaboliques différentes sont impliquées dans la synthèse de l'acide fumarique, à savoir le cycle TCA oxydatif et la voie TCA réductive. La fixation du CO_2 et la conversion du pyruvate en oxaloacétate se produisent au cours du cycle TCA. La fixation réductrice du CO_2 catalysée par l'enzyme pyruvate carboxylase est la principale raison de l'accumulation d'acide fumarique pendant la fermentation, ce qui a été observé dans le cas des espèces de *Rhizopus* sp. (Xu et al., 2013). Le cycle réducteur a un rendement théorique maximal de 2 moles d'AF par mole de glucose consommé et 2 moles de CO2 fixé. La dépendance à l'égard de la carboxylation réductrice du pyruvate, en tant que seule voie, ne conduirait à aucune formation d'ATP. Or, cet ATP est nécessaire à l'entretien des cellules et influe donc sur leur croissance globale. Le cycle TCA est donc actif pendant la production d'AF et le rendement fumarique théorique complet n'est pas atteint (Das et al., 2016; Roa Engel et al., 2008). L'adoption de stratégies visant à limiter la croissance fongique a le potentiel d'assurer une production optimale

d'acide fumarique. La voie métabolique et les enzymes impliquées dans les étapes clés sont représentées dans la Figure 1.2 et un résumé des voies métaboliques impliquées dans la production microbienne d'acide fumarique est fourni dans les revues de Engel et al. (2008) et Xu et al. (2013). De plus, il a été observé que le métabolisme des acides aminés et des acides gras, ainsi que l'activation de la voie du glyoxylate, peuvent avoir un impact potentiel sur l'accumulation des AF (Li et al., 2014; Yu et al., 2012).

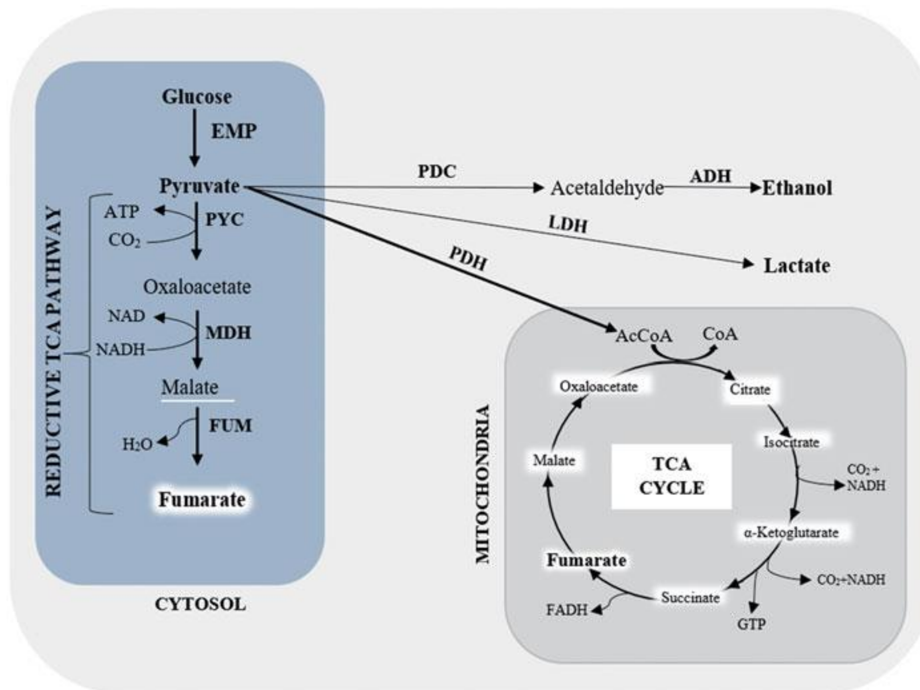

Figure 1. 2. Overview of metabolic pathway and enzymes involved in the fumaric acid synthesis in R. oryzae. EMP stands for Embden–Meyerhof–Parnas pathway or glycolysis. The enzymes involved in the various steps of the pathway are PDC pyruvate decarboxylase (EC 4.1.1.1); ADH—alcohol dehydrogenase (EC 1.1.1.1); LDH—lactate dehydrogenase (EC 1.1.1.27); PDH—PYRUVATE dehydrogenase (EC 1.2.4.1); PYC—pyruvate carboxylase (EC 6.4.1.1); MDH—malate dehydrogenase (EC 1.1.1.37) and FUM—fumarase (EC 4.2.1.2) (Sebastian et al. 2019).

2.3.2 Production d'acide fumarique par *Rhizopus* sp.

Les champignons ont été utilisés pour produire une vaste gamme de composés et de produits consommables. Les champignons filamenteux multicellulaires sont largement utilisés pour

produire des aliments fermentés, des métabolites secondaires et des enzymes industrielles. *Rhizopus* sp. est un exemple de champignon filamenteux, appartenant à la famille des "Mucoraceae", qui est utilisé pour produire un large éventail de composés allant des produits alimentaires fermentés aux produits chimiques de plate-forme tels que l'acide fumarique (Lee et al., 2011; Skory & Ibrahim, 2007; Wakai et al., 2017).

Rhizopus oryzae, généralement considéré comme sûr (GRAS), a généralement été employé pour la production d'AF par fermentation à des valeurs de pH neutres pour produire des sels de fumarate (Roa Engel et al., 2011). L'espèce fongique est subdivisée en deux groupes (Type I et Type II) en fonction des différences génétiques et phénotypiques. Le type I accumule l'acide lactique alors que le type II synthétise principalement l'acide fumarique en présence de sucres fermentescibles. La principale différence entre ces deux types est que le type I contient deux gènes de lactate déshydrogénase (ldh), *ldhA* et *ldhB*, alors que le type II ne possède qu'un seul des gènes ldhB. L'enzyme lactate déshydrogénase, LdhA, codée par le gène *ldhA* permet la conversion de l'acide pyruvique en acide lactique alors que l'enzyme codée par le gène *ldhB* possède une capacité réductrice insuffisante. Cette capacité réduite permet la conversion de l'excès de pyruvate en acide fumarique ou en éthanol (Abe et al., 2017; Skory & Ibrahim, 2007). Le tableau 1.1 présente un résumé des études de production d'acide fumarique réalisées avec *R. oryzae*.

Certaines des limitations de la bioproduction de l'acide fumarique à l'aide de souches fongiques peuvent être surmontées en effectuant la fermentation à un pH plus faible. La fermentation à pH plus faible réduit la quantité d'agent neutralisant nécessaire pour maintenir un pH proche de la neutralité, ainsi que la quantité de sel générée pendant la récupération de l'acide fumarique à partir du bouillon fermenté. De plus, l'acide fumarique non dissocié ainsi formé peut être récupéré par cristallisation à partir du bouillon en raison de la faible solubilité de l'acide fumarique. En outre,

la récupération directe de l'acide fumarique à partir du bouillon en utilisant des adsorbants, tels que la résine de polyvinyl pyridine (PVP) et l'Amberlite IRA-900, serait possible (Xu et al., 2013). Cette stratégie de récupération de l'acide fumarique à l'aide d'adsorbants a été utilisée avec succès par Cao et al. (1996) pour récupérer de l'acide fumarique de haute pureté après désorption et acidification. À cet effet, une durée réduite du contrôle du pH et une fermentation à pH 5 ont été proposées (Roa Engel et al., 2011). Il a été observé que l'arrêt du contrôle du pH à 90 heures au lieu de 120 heures n'entraînait pas d'effet drastique sur la production d'AF. L'inconvénient de cette stratégie est qu'un contrôle du pH est nécessaire pendant les étapes initiales de la fermentation, même si la consommation d'agents neutralisants est réduite.

De plus, il a été observé que la morphologie du champignon pendant la fermentation a un impact majeur sur la production d'AF et a été étudié de manière approfondie par Zhou et al (2011) et Engel et al (2011)(Roa Engel et al., 2011; Zhou et al., 2011). Ces derniers ont observé que le diamètre des granules fongiques jouait un rôle clé dans la productivité de l'acide fumarique. Zhou et al. (2011) ont conclu que le diamètre petit des granulés permettait un meilleur transfert de masse ainsi qu'un contact avec une plus grande quantité de champignons en croissance active présents à la surface. En outre, Engel et al (2011) ont observé qu'un environnement anaérobie était présent au cœur du granulé fongique. Par conséquent, les granulés fongiques de plus petit diamètre sont préférés, ce qui nécessite l'optimisation des conditions, telles que la vitesse d'agitation, les conditions de pH, la température et le volume, pour obtenir le diamètre optimal du granulé. Cette dépendance vis-à-vis de la morphologie fongique ajoute encore de la complexité à la bioproduction fongique de l'acide fumarique. À cet effet, l'immobilisation du champignon sur du support solide a été proposée pour réduire l'effet de la morphologie des granules et s'est avérée efficace pour augmenter la productivité ainsi que le rendement en AF, comme le montre le tableau 1.1 (Cao et

al., 1996; Das et al., 2015a; Gu et al., 2013). De plus, une réduction de la durée de la fermentation, conduisant à une augmentation de la productivité, de 144 heures à 24 heures a été observée par Gu et al, (2013). Un rendement en acide fumarique de 32,03 g/L a été obtenu après immobilisation du champignon *R. arrhizus* sur du filet pendant l'étude.

Table 1. 1. Summary of fumaric acid production studies carried out using R. oryzae

Carbon source	Fermenter	Fumaric acid concentration	Productivity (g/L/h)	Reference
Glucose	Stirred tank	56.2 g/L	0.7	(Fu et al., 2009)
Glucose	Stirred tank	41.1 g/L	0.37	(Huang et al., 2010)
Glucose	Stirred tank	32.1 g/L	0.32	(Kang et al., 2010)
Glucose	Stirred tank	30.2 g/L	0.19	(Roa Engel et al., 2011)
Xylose	Shake flask	28.4 g/L	-	(Wen et al., 2013)
Xylose	Shake flask (immobilization)	45.3 g/L	-	(Liu et al., 2015)
Glucose/Xylose	Shake flask	46.7 g/L	-	(Liu et al., 2017a)
Corn straw	Shake Flask	27.8 g/L	0.33	(Xu et al., 2010)
Cornstarch	Shake flask (Simultaneous saccharification fermentation)	44.1 g/L	0.53	(Deng et al., 2012)
Cornstarch	Shake Flask	45.0 g/L	0.55	(Huang et al., 2010)
Brewery wastewater	Shake Flask	31.3 g/L	-	(Das & Brar, 2014)
Apple juice extraction waste	Shake Flask	25.2 g/L	0.35	(Das et al., 2015b)
Diary manure	Stirred tank	31.0 g/L	0.32	(Liao et al., 2008)
Apple pomace	Solid state-Rotating drum fermenter	138 g/kg dry weight	-	(Das et al., 2015b)
Glucose/Crude glycerol	Shake flask	22.81 g/L	0.34	(Zhou et al., 2014)
Synthetic medium	Immobilized fungi	32.03 g/L	1.33	(Gu et al., 2013)
Synthetic medium	Immobilized fungi	40.13 g/L	0.32	(Naude & Nicol, 2017)
Synthetic medium	Immobilized fungi	30.3 g/L	0.21	(Liu et al., 2017b)
Brewery wastewater	Immobilized fungi	43.67 g/L	1.21	(Das et al., 2015a)
Synthetic medium	Immobilized fungi-fed-batch	85 g/L	4.25	(Cao et al., 1996)

2.3.3 Limites de la production de l'AF à par *Rhizopus oryzae*

La production d'AF par l'intermédiaire de *R. oryzae* est généralement conduite à un pH proche de la neutralité. La production de l'acide pendant la fermentation entraîne une baisse significative du pH de 6 à 4 dans les 48 heures de fermentation. Cette baisse du pH entraîne un ralentissement du taux de production, qui finit par s'arrêter complètement. Pour éviter cette baisse de pH, l'acide fumarique est transformé en sels de fumarate en utilisant des neutralisants, tels que le carbonate de calcium et le carbonate de sodium. L'acide fumarique est ensuite récupéré par précipitation, en raison de sa faible solubilité, après acidification. Le besoin de neutralisants et d'acides durant l'étape de récupération entraîne une consommation élevée de bases et d'acides ainsi que la production de grandes quantités de sels. Le carbonate de calcium est couramment utilisé comme neutralisant, mais l'insolubilité du sel de calcium entraîne une forte consommation d'énergie et des difficultés techniques, telles qu'une viscosité accrue et une consommation d'énergie associée (Das & Brar, 2014; Gangl et al., 1990; Roa Engel et al., 2011). Par conséquent, le carbonate de sodium est proposé comme agent neutralisant parce que le sel de sodium du fumarate est soluble, mais une comparaison complète par Gangl et al (1990) a montré que l'utilisation du carbonate de calcium donnait un rendement plus élevé en AF. Ils ont étudié les avantages et les inconvénients de l'utilisation du carbonate de calcium et du carbonate de sodium comme agent neutralisant (Gangl et al 1990).

Déduction 2 : Rhizopus oryzae est le microorganisme idéal pour la bioproduction d'AF. Les limites de la bioproduction médiée par R. oryzae peuvent être surmontées en immobilisant les champignons et le carbonate de calcium est l'agent neutralisant idéal.

2.4. Génie génétique et métabolique pour améliorer le rendement en acide fumarique et surmonter les limites de la bioproduction par *R. oryzae*

Pour surmonter les complexités de la production d'acide fumarique à l'aide de *R. oryzae*, la modification génétique de micro-organismes, tels que *R. oryzae*, *Escherichia coli*, *Saccharomyces cerevisiae* et *Torulopsis glabrata*, pour accumuler l'acide fumarique a été proposée. Le faible rendement en'acide fumarique dans des conditions de faible pH (3-5), dû à une croissance fongique réduite, ainsi que la complexité de la production d'acide fumarique à l'aide de souches fongiques ont incité les chercheurs à étudier la possibilité d'utiliser d'autres micro-organismes pour la production de l'acide fumarique. Les développements des techniques de génie génétique, telles que la transformation génétique et la surexpression de gènes homologues, comme *fum*, *pyc* et *mdh*, et le génie métabolique, ont fourni les outils nécessaires pour améliorer la production microbienne d'acides organiques, comme l'acide malique, l'acide succinique et l'acide fumarique (Meussen et al., 2012; Wakai et al., 2017). Les stratégies d'amélioration des souches reposent principalement sur l'augmentation des rendements des produits, améliorant ainsi la faisabilité de la production commerciale. Ces stratégies d'amélioration des souches et le rendement correspondant en acide fumarique des nouvelles souches sont présentés dans le tableau 1.2.

Table 1. 2. Random mutagenesis of R. oryzae and fumaric acid concentrations obtained (R. oryzae reassigned as R. arrhizus, (Dolatabadi et al., 2014))

Fungal strain	Mutagen	Fumaric acid titer (g/L)	Yield (g/g)	Reference
		Pure culture		
***R. arrhizus* NRRL 2582**	-	103	0.79	(Rhodes et al., 1962)
***R. arrhizus* NRRL1526**	-	98	0.82	(Kenealy et al., 1986)
		Mutant		
***R. oryzae* RUR709**	UV and γ-ray mutagenesis	32.1	0.45	(Kang et al., 2010)
***R. oryzae* ZD-35**	UV irradiation	57.4	0.67	(Huang et al., 2010)

***R. oryzae* ME-F01**	UV and nitrosoguanidine	52.7	-	(Fu et al., 2010)
***R. oryzae* ME-F12**	Nitrogen ion implantation	44.1	0.44	(Deng et al., 2012)
***Rhizopus oryzae* FM19**	Femtosecond laser irradiation	49.4	0.56	(Yu et al., 2012)

De plus, il a été observé que l'activation de la voie du glyoxylate dans *E. coli* a permis à la souche transformée d'accumuler de l'AF (Li et al., 2014; Song et al., 2013). Par conséquent, cette réorientation de la voie métabolique en conjonction avec le cycle TCA réducteur inhérent peut être introduite dans *R. oryzae* pour améliorer la production d'AF. Cette possibilité de transformation n'a pas été rapportée. Cette réorientation via la voie du glyoxylate a le potentiel d'améliorer l'accumulation d'AF dans la souche fongique. Il a été signalé que la voie du glyoxylate est reliée à la virulence fongique (Lorenz & Fink, 2001). Par conséquent, une compréhension complète du risque sous-jacent de l'amélioration de la virulence fongique, s'il existe, doit être étudiée de manière approfondie avant d'envisager toute application commerciale de la souche transformée.

De plus, des modifications métaboliques similaires à celles décrites par Chen et al. (2015) peuvent être étudiées pour *R. oryzae*. Ces modifications ont permis d'augmenter les teneurs en AF chez *T. glabrata*. Par conséquent, l'introduction d'enzymes métaboliques impliquées dans le métabolisme de l'azote et des purines en plus de celles du métabolisme du carbone peut être envisagée pour améliorer le rendement en fumarate. De plus, l'introduction d'un transporteur d'acide dicarboxylique C4- efficace, tel que SpMAE1 et DcuB, qui s'est avéré améliorer la production de fumarate de manière significative peut être envisagée (Chen et al., 2015). La tolérance aux acides de *T. glabrata*, ainsi que la production de fumarate, ont été améliorées par la délétion du gène qui code pour l'adénylosuccinate synthase (*ade12*). De même, cette modification peut être étudiée pour améliorer la tolérance à l'acide de *R. oryzae*. Cette modification aura un impact considérable sur

la faisabilité des stratégies commerciales de bioproduction d'AF vu le coût élevé associé à l'utilisation de l'agent neutralisant ainsi que la récupération des AF à partir du complexe de fumarate formé par l'utilisation d'un agent neutralisant.

***Déduction 3*: Le micro-organisme issu du génie génétique n'a pas réussi à produire des titres d'AF plus élevés. Par conséquent, les souches indigènes de R. oryzae peuvent être utilisées pour la bioproduction.**

2.5 Valeur ajoutée à l'acide fumarique par la conversion en esters d'acide fumarique

L'une des principales limitations liées à la bioproduction de l’acide fumarique à l'échelle industrielle est la faible valeur marchande de l'acide fumarique. Pour surmonter cette limitation, l'acide fumarique peut être converti en esters d'acide fumarique, un ingrédient pharmaceutique actif à haute valeur marchande.

2.5.1 Synthèse des esters de fumarate

Le dérivé de l'acide fumarique, l'ester d'acide fumarique (EAF), a récemment été identifié comme un composé important en raison de ses diverses applications dans les industries pharmaceutique et plastique (Das et al., 2016). La bioconversion in-situ de l'acide fumarique en forme d’ester nous a fourni une alternative attrayante pour la synthèse verte des esters. Les méthodes actuelles de synthèse industrielle des esters sont non sélectives et consomment beaucoup d'énergie. Le plus souvent, ces procédés commerciaux se basent sur l'estérification chimique directe. Ces réactions d'estérification à haute température, médiées par des catalyseurs inorganiques, transforment un acide organique en esters en présence d'un alcool (Pacheco et al., 2014). En raison des inconvénients des procédés chimiques, tels que la non-sélectivité et la forte consommation

d'énergie, la biosynthèse a été proposée comme une alternative appropriée. De plus, l'utilisation d'une température élevée conduit à la formation d'allergènes ainsi que de substances cancérigènes (Gumel et al., 2011). La conversion in situ de l'acide fumarique, un produit chimique de base de faible valeur, en formes d'esters de grande valeur peut augmenter considérablement la faisabilité de la stratégie de bioproduction.

Table 1. 3. Difference in the price of commercial fumaric acid and fumaric acid esters (https://www.chemicalbook.com/ProductIndex_EN.aspx; https://www.pharmacompass.com/)

Chemicals	Quantity (in Grams)	Price (in USD)
Fumaric acid	1000	1.00
Monomethyl fumarate	1000	1,136.00
Monoethyl fumarate	25	60.50
Dimethyl fumarate	1000	169.00
Diethyl fumarate	100	15.00

2.5.2 Bioproduction d'ester de fumarate

Des stratégies de bioproduction d'esters d'acides organiques ont été précédemment rapportées et ces conversions enzymatiques reposent principalement sur l'utilisation d'une enzyme lipase comme biocatalyseur (de Meneses et al., 2019; Escandell et al., 2015; Martins et al., 2013; Yan et al., 2014). Les lipases sont les enzymes les plus utilisées pour la biosynthèse d'un groupe diversifié de composés pharmaceutiques. Ces enzymes ont une excellente stabilité, même en présence de solvants organiques. D'où leur application dans la biosynthèse de divers composés. Cette application des enzymes dans la synthèse d'un ensemble diversifié de composés pharmaceutiques ainsi que de composés aromatiques a été examinée en détail par Carvalho et al. (2015) et Almeida et al. (2017) respectivement.

R. oryzae est un producteur connu de lipases et est largement utilisé pour des applications industrielles (Salah et al., 2006; Witt et al., 2015). Par conséquent, il serait possible de convertir biologiquement les AF produits en EAF en induisant la synthèse des lipases, soit par modification génétique, soit par induction de la synthèse. L'alcool nécessaire à la conversion des AG en EAF peut être fourni de l'extérieur. La production d'éthanol pendant la fermentation de la production d'AF a été rapportée (Das et al., 2015a). L'induction de cette synthèse d'éthanol ne serait pas recommandée car le détournement des sucres vers la synthèse d'éthanol peut conduire à une réduction de la production d'AF. Ce moyen de bio-conversion à médiation enzymatique des AF en EAF n'a pas été rapporté jusqu'à présent et constitue un moyen alternatif attrayant et respectueux de l'environnement pour produire des EAF.

***Déduction 4*: La conversion de l'acide fumarique en ester de fumarate peut avoir un impact profond sur la compétitivité économique du processus de bioproduction. La bioconversion de l'acide fumarique en ester de fumarate n'a pas été rapportée.**

2.6. Chitosane comme sous-produit de la biomasse du champignon *R. oryzae*

En outre, le sous-produit de la fermentation de l'AF est la biomasse fongique générée, qui peut être utilisée comme source de chitosane fongique. Le champ d'application et l'intérêt de ces composés à haute valeur ajoutée sont abordés dans les sections suivantes.

Les biopolymères naturels sont biocompatibles, biodégradables, bioactifs et renouvelables, car ils sont obtenus à partir de sources renouvelables. Ces qualités présentes dans les biopolymères les ont rendus hautement souhaitables pour une grande variété d'applications industrielles. La capacité et la flexibilité des biopolymères naturels à subir des modifications chimiques et enzymatiques ont créé des dérivés qui posent diverses applications. Le chitosane est l'un de ces bio-polymères,

composé de fragments de D-glucosamine, qui a diverses applications. Le chitosane est un polymère cationique pseudo-naturel dérivé de la chitine (composée de β-(1-4)-N-acétyl-D-glucosamine), après la désacétylation chimique. En raison de sa nature cationique, le chistosane est adapté pour de nombreuses applications qui utilisent cette caractéristique unique comme la récupération et la purification des protéines. En plus, le chitosane, soluble dans des solutions aqueuses, peut être utilisé dans diverses applications sous forme de solutions, de gels, de films ou de fibres. À l'état solide, le chitosane est de nature semi-cristalline et présente une morphologie polymorphe (Ravi Kumar, 2000; Rinaudo, 2006).

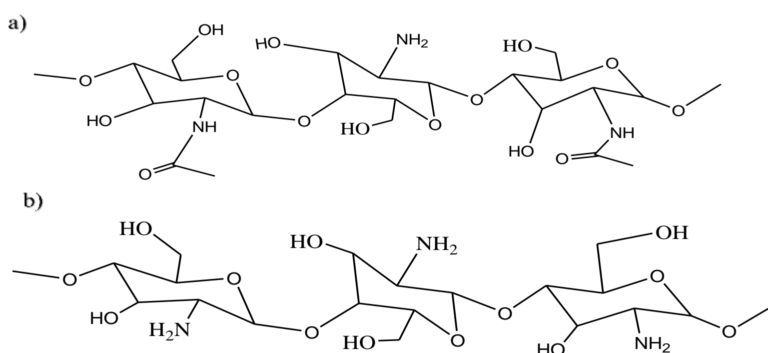

Figure 1. 3. Structure of chitin (a) and chitosan (b)

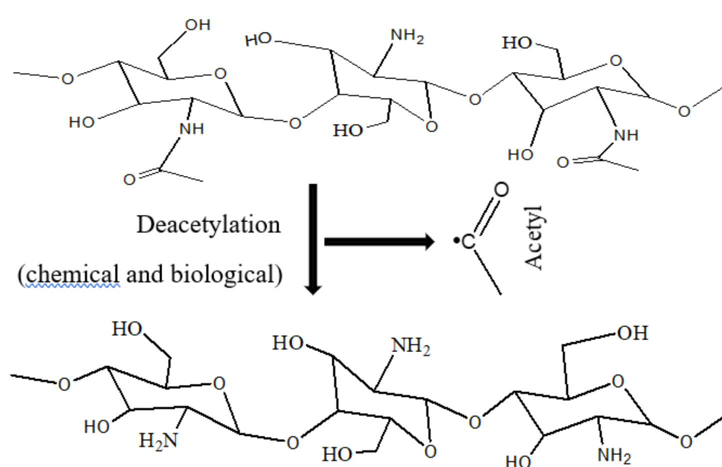

Figure 1. 4. Conversion of chitin to chitosan

Le chitosane est le seul polymère cationique pseudo-naturel et il a démontré avoir beaucoup d’applications dans de nombreuses industries, telles que les industries alimentaires (agent de conservation, antimicrobien, revêtement, antioxydant), la cosmétologie (additifs capillaires, lotions, crèmes pour le visage et le corps), la biotechnologie (émulsifiant, chélateur, floculant), l'agriculture (fongicide, films, modificateur de sol, éliciteur), et ses applications en pharmacologie et en médecine (tissus, fibres, organes artificiels, administration de médicaments et d'ADN, membranes) (Knorr & Klein, 1986; Mourya & Inamdar, 2008; Rajeshkumar et al., 2009).

2.6.1. Production commerciale actuelle de chitosane et ses limites

Vu que la chitine et le chitosane commercialisés sont extraits de coquilles de crabes et de crevettes, ils sont considérés comme le deuxième polymère le plus abondant. La chitine et le chitosane sont extraits des crustacés par un traitement acide pour dissoudre le carbonate de calcium suivi d'une extraction alcaline pour solubiliser les protéines. Par la suite, une étape de décoloration est requise, laquelle est souvent ajoutée pour éliminer les pigments restants et obtenir un produit incolore. Ces traitements doivent être adaptés à chaque source de chitine en raison des différences trouvés dans l'ultrastructure des matériaux initiaux. Le plus important dérivé de la chitine, le chitosane, est obtenu par désacétylation partielle dans des conditions alcalines. Ces étapes de production, à partir de coquilles de crabes et de crevettes, produisent, pendant le processus d'extraction, de grandes quantités d'effluents hautement alcalins et acides, riches en déchets protéiniques. Ce processus d’extraction peut prendre jusqu'à 2 jours. L'approche chimique actuelle pour la production de chitosane, à partir de chitine provenant de la coquille des crustacés, limite son utilisation, car le chitosane obtenu présente des incohérences au niveau des propriétés physiques et chimiques, telles que le poids moléculaire et le degré de désacétylation. Par ailleurs, la dépendance à utiliser les crustacés comme source principale de chitosane et la technique utilisée dans la production

commerciale entraîne une pollution environnementale importante (Amorim et al., 2001; Chatterjee et al., 2005; Kumari & Rath, 2014; Rinaudo, 2006).

Le chitosane est actuellement produit à partir de coquilles de crabes et de crevettes qui sont rejetées par l'industrie de transformation basée aux États-Unis et au Japon. La production de chitosan, à partir de déchets de coquille, est économique et durable dans une certaine mesure, mais elle n'est pas respectueuse de l'environnement car elle produit de grandes quantités d'effluents nocifs. Environ 6,3 kg de HCl et 1,8 kg de NaOH sont nécessaires pour la production de chaque kilogramme de chitosan désacétylé à 70 %, en plus de l'azote et de l'eau utilisés pour le traitement et le refroidissement. Ces exigences peuvent augmenter de manière significative le prix du chitosane, en plus de produire une quantité importante d'effluents toxiques riche en matières organiques, qui s'élève actuellement à 1,000 - 1,500 dollars US comme cité par Sigma-Aldrich. D'après les travaux réalisés par les chercheurs du Central Institute of Fisheries Technology, en Inde, la fraction moyenne de déchets solides chitineux des crustacés capturés en Inde se situe entre 60 et 80 000 tonnes. Il a été observé que les déchets de crevettes et de squilles sèches contenaient 23% et 15% de chitine, respectivement (Ravi Kumar, 2000).

2.6.2. Synthèse verte du chitosane

La quantité requise de produits chimiques, les effluents toxiques produits et le temps requis pour l'extraction de chitosane à partir de déchets de crustacés, les problèmes de variabilité et de pureté du chitosane extrait à partir de déchets de crustacés, ont nécessité le développement de moyens alternatifs de production de chitosane, soit l'utilisation du chitosane fongique ou soit par l'utilisation de nouvelles méthodes d'extraction modernes. Le chitosane fongiques est de qualité constante avec d'excellentes propriétés. De plus, l'extraction du chitosane, à partir de sources fongiques, exige une quantité moindre d'ingrédients toxiques lesquels, finalement, produiront

moins de déchets difficile à traiter (Dhillon et al., 2012). Le processus de production de chitosane, à partir de sources fongiques, est d'une durée plus courte et demande une quantité d'énergie plus faible. L'importance de ces avantages est discutée en détail dans la section suivante.

L'utilisation de moyens d'extraction biologiques est plus efficace et plus respectueuse de l'environnement que les méthodes d'extraction chimiques. Les micro-organismes, tels que *Lactobacillus* sp., *Pseudomonas aeruginosa*, *Bacillus licheniformis*, *Bacillus subtilis* et *Bacillus cereus*, peuvent, par les voies de la déprotéinisation et de la déminéralisation, réduire le nombre de produits chimiques nécessaires pour l'extraction du chitosane, diminuant ainsi la quantité d'effluents toxiques générés. Les moyens biologiques d'extraction peuvent être réalisés en utilisant des enzymes pures comme la trypsine et l'alcalase, pour la déprotéinisation qui est suivie d'une déminéralisation à l'aide de l'acide lactique. Le degré de déprotéinisation et de déminéralisation peut varier entre 60-95% et 70-95%, respectivement, en fonction des moyens biologiques utilisés dans les étapes. En plus des moyens biologiques de déprotéinisation et de déminéralisation, la désacétylation peut également être réalisée par l'utilisation de l'enzyme désacétylase obtenue à partir de souches fongiques telles que *R. oryzae* ou par biotransformation de la chitine en chitosane, à partir du champignon en présence de la chitine obtenue par les déchets de crustacés (Dhillon et al., 2012; Kaur & Dhillon, 2013; Synowiecki & Al-Khateeb, 2003). Ces moyens de production de chitosane ont un faible impact sur l'environnement. Cependant, la méthode de co-culture doit être optimisée avant de l'appliquer à l'échelle commerciale.

L'avènement de l'extraction assistée par micro-ondes a révolutionné l'extraction chimique. L'utilisation du chauffage par micro-ondes à la place du chauffage conventionnel a le potentiel de réduire le temps d'extraction du chitosane à quelques minutes au lieu d'heures ou de jours. L'utilisation des micro-ondes est un moyen plus efficace, plus respectueux de l'environnement et

plus économique. Lors d'un chauffage conventionnel, les réactifs sont activés lentement, de manière non uniforme, tandis que le chauffage par micro-ondes se produit au niveau moléculaire, entraînant une augmentation rapide et uniforme de la température. La méthode conventionnelle d'extraction du chitosane est lente et consomme beaucoup d'énergie. Par conséquent, la technologie des micro-ondes est une alternative avantageuse. Par exemple, dans la production de chitosane par micro-ondes, le temps de désacétylation a été réduit considérablement à quelques minutes pour atteindre le même degré de désacétylation que les méthodes traditionnelles. En outre, l'utilisation du chauffage par micro-ondes, à la place du chauffage conventionnel, pendant les étapes de déprotéinisation et de déminéralisation a également montré une grande réduction du temps qui passe d'une journée à quelques heures. En plus, il a été observé que l'utilisation de l'extraction assistée par micro-ondes a produit du chitosane avec un faible poids moléculaire et une cristallinité plus élevée (Alishahi et al., 2011; El Knidri et al., 2016; Mahdy Samar et al., 2013; Sagheer et al., 2009). Le passage du chauffage conventionnel à une technique d'extraction plus efficace peut conduire à une réduction du nombre de produits chimiques utilisés, et à une diminution de l’utilisation d’énergie, ce qui conduit à une synthèse plus écologique.

2.6.2.1 Chitosane fongique et son importance

La chitine est un composant important de la paroi cellulaire des champignons et des levures, ainsi que d'autres organismes. La présence de chitine dans la paroi cellulaire des champignons a été signalée pour la première fois chez *Mucor rouxii* par Hopkins en 1929. Ce n'est que plus tard, en 1954, que la présence de chitosane naturel dans les hyphes et les sporangiophores de *Phycomyces blakesleeanus* a été identifiée, pour la première fois, par Kreger. L'intérêt pour la production microbienne de chitosane a été encouragé, par Bartnicki-Garcia et Nickerson (1962), du fait que le chitosane est le composant le plus abondant dans la paroi cellulaire du champignon *Mucor*

rouxii. Ils ont observé que même si le champignon présente du dimorphisme, du filamenteux et du levurien, le chitosane est le composant principal des parois cellulaires avec des concentrations de 32,7% et 27,9% de la composition totale de la paroi cellulaire, respectivement. Cette observation a permis à d'autres d'étudier la possibilité d'isoler le chitosane à partir d'autres espèces fongiques, en vue d'une production commerciale, ainsi que d'utiliser les mycéliums résiduels de cultures fongiques industrielles pour l'isolement du chitosane (Bartnicki-Garcia & Nickerson, 1962; Davis & Bartnicki-Garcia, 1984; Muzzarelli et al., 1980). Les propriétés physiques, chimiques et biologiques uniques ainsi que la polyvalence de la chitine et du chitosane ont conduit à l'identification d'une pléthore d'applications pour ce polymère, ce qui le rend important.

Le chitosane est généralement extrait de la chitine qui est un déchet de l'industrie de transformation de fruits de mer (Kumar, 2000). Cependant, il a des propriétés physicochimiques hétérogènes et incohérentes puisque les approvisionnements en déchets de fruits de mer sont sujets à des variations saisonnières. Par conséquent, plusieurs levures et champignons filamenteux qui contiennent de la chitine et du chitosane dans leur paroi cellulaire, par exemple, *Schizosaccharomyces pombe*, *Candida albicans*, *Saccharomyces cerevisiae*, *Mucor rouxii*, *Phycomyces blakesleeanus*, *Coprinus cinereus*, *Neurospora crassa*, *Trichoderma reesei*, *Rhizopus* sp., *Absidia* sp, *Mucor* sp., *Mortierella isabelina* et *Lentinus edodes*, ont été étudiées pour la production de chitosane (Amorim et al., 2001; Chatterjee et al., 2005; Chatterjee et al., 2008; Knorr & Klein, 1986; Streit et al., 2009; Suntornsuk et al., 2002; White et al., 1979; Yokoi et al., 1998). Ces micro-organismes peuvent être facilement cultivés dans des nutriments simples et utilisés comme source alternative de chitosane. De plus, le chitosane obtenu à partir de ces micro-organismes présente une meilleure uniformité en termes de poids moléculaire et de désacétylation. Il est exempt d'allergènes susceptibles de provoquer des chocs anaphylactiques potentiellement

dangereux (Suntornsuk et al., 2002). La production de chitosane à partir de champignons, lesquels ne nécessite pas d'étapes de déminéralisation, de déprotéinisation, de décoloration, constitue une voie alternative intéressante pour obtenir du chitosane d'une qualité constante. Les progrès actuels de la technologie de la fermentation fournissent un moyen alternatif de production écologique du polymère. Ils seront discutés dans les sections suivantes.

2.6.2.2 Production actuelle de chitosane fongique

La procédure standard pour la production du chitosane fongique consiste en une première étape de lavage à l'eau distillée. Ce mycélium est ensuite homogénéisé puis passé à l'autoclave en présence de NaOH 1N (Biomass : NaOH::1:20 v/v). Les mycéliums fongiques traités aux alcalis sont ensuite filtrés et le surnageant est rejeté. Le matériel insoluble en milieu alcalin (MIA) est recueilli et lavé avec de l'éthanol et de l'eau distillée. Le MIA est homogénéisé après en présence de 2 % d'acide acétique, le ratio de MIA:acide acétique étant de 1:100. Il est ensuite centrifugé et le surnageant est recueilli. Le chitosan présent dans le surnageant est ensuite précipité en ajustant le pH à 8,5. Le précipité est lavé, centrifugé puis lyophilisé (Arcidiacono & Kaplan, 1992). Cette extraction a été légèrement modifiée en augmentant la quantité d'hydroxyde de sodium utilisée afin d'obtenir une matière insoluble en milieu alcalin. La méthode modifiée utilise un ratio de biomasse:NaOH de 1:40 pour obtenir la MIA (Hu et al., 2004).

2.6.2.3 Biomasse de *Rhizopus oryzae* comme source de chitosane

R. oryzae est un champignon filamenteux qui appartient à la division des Zygomycota. Les souches appartenant à cette espèce de champignon sont généralement considérées comme sûres (GRAS). C'est l'une des souches fongiques qui est généralement utilisée pour la production de produits chimiques plate-forme importants sur le plan industriel, tels que l'acide L-lactique, l'acide

fumarique et l'éthanol, à des concentrations élevées, en plus d'une grande variété d'enzymes commerciales, telles que l'amylase, la xylanase, la pectinase et la cellulase. Le champignon est également utilisé dans la production de produits alimentaires fermentés traditionnels, comme le tempeh, célèbre en Indonésie et en Malaisie, ainsi que de boissons alcoolisées. Ce champignon ne nécessite que des milieux simples pour sa croissance et peut se développer à des températures allant de 25ºC à 45ºC et à des pH allant de 4 à 9, mais elle nécessite une humidité plus élevée (Cantabrana et al., 2015; Meussen et al., 2012). Ces propriétés la rendent idéale pour la production de chitosane fongique, soit à partir du mycélium résiduel, soit en cultivant le champignon dans des milieux simples. Le tableau 1.4 présente un résumé des recherches sur l'utilisation potentielle de cette souche fongique pour la production de chitosane.

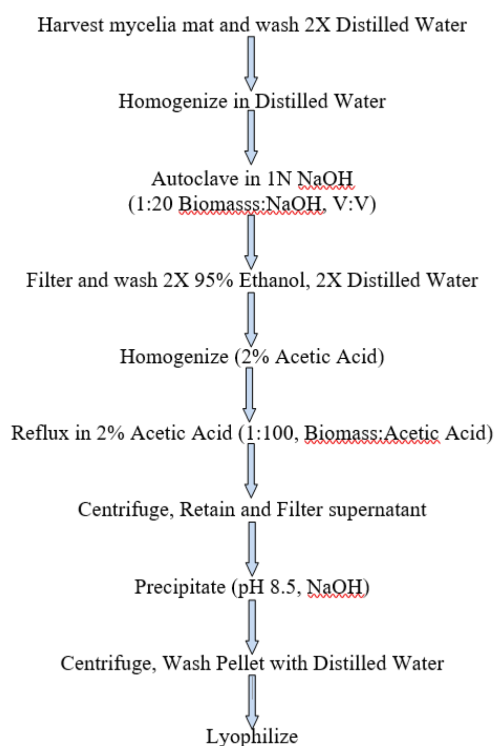

Figure 1. 5. Flowchart for the extraction of chitosan from fungal mycelia (adapted from Arcidiacono and Kaplan, 1992)

Le travail initial qui a étudié la faisabilité de l'utilisation des mycéliums de *R. oryzae* comme source possible de chitosane a été effectué par Hang (1990). Un rendement en chitosane de 700mg/L a été obtenu en cultivant ce champignon sur du riz simple dans des conditions de fermentation submergée. Ce travail a montré que le *R. oryzae* peut être utilisé comme une source de chitosan fongique. De la même manière, *M. rouxii*. Tan et al. (1996) ont observé que la concentration la plus élevée de chitosane était obtenue pendant la phase exponentielle tardive de croissance, en cultivant ce champignon sur un milieu synthétique dans des conditions d'immersion. Une observation similaire a été faite aussi dans l'étude d'autres souches fongiques (Arcidiacono & Kaplan, 1992; Nwe & Stevens, 2004; Tan, 1996). Les observations faites soulignent l'importance d'identifier la période de la phase de croissance qui produit la plus grande concentration de chitosane dans les conditions de fermentation et le milieu utilisé pour la production.

Table 1. 4. Studies on using Rhizopus oryzae in chitosan production and chitosan yield

Culture media/ fermentation type	Chitosan yield/ Molecular weight	Degree of acetylation/ deacetylation*	Reference
Potato dextrose broth/ Submerged fermentation	138 mg/g dry weight/6.9x10^4 Da	87.9 ± 2.1*	(Pochanavanich & Suntornsuk, 2002)
Whey medium with plant growth hormones/ Submerged fermentation	0.749-1.131 g/L of medium/ 120-270 kDa	87.2-87.5±0.5*	(Chatterjee et al., 2008)
Synthetic medium/ Submerged fermentation	55.70±8.53 and 43.98±3.05mg/200ml substrate	_	(Tan et al., 1996) *R. oryzae* 0602, *R. oryzae* 0263
Soybean and mungbean residues/ Solid state fermentation	4.3g/kg of soybean residue	_	(Suntornsuk et al., 2002)
Rice medium/ Submerged fermentation	700mg/L	_	(Hang, 1990)

Pochanavanich et Suntornsuk (2002) ont réussi à obtenir le rendement le plus élevé de chitosane qui est de 138 mg/g de mycélium sec en utilisant *R. oryzae* cultivé dans dans un milieu à base de dextrose et de pomme de terre. Le poids moléculaire et le degré d'acétylation du chitosane obtenu

étaient de $6{,}9 \times 10^4$ Da et de 87,9%, respectivement. Cette étude a porté quatre espèces de champignons filamenteux (Pochanavanich & Suntornsuk, 2002).

La capacité de *R. oryzae* à se développer dans des milieux simples, spécialement dans des résidus industriels et agricoles, a été prouvée par de multiples études. Au cours de ces études, il a été possible d'obtenir du chitosane de haute qualité avec un bon rendement. Chatterjee et al. (2008) a été en mesure de produire du chitosane, qui possède différentes propriétés comme le poids moléculaire et le degré d'acétylation, en cultivant *R. oryzae* sur un milieu à base de lactosérum supplémenté avec différentes hormones végétales. Le poids moléculaire du chitosane obtenu variait de 120 à 270 kDa et le degré de désacétylation était de 87%. Un rendement en chitosane de 5,63g/kg de milieu et un degré de désacétylation de 90% ont été obtenus en cultivant ce champignon sur de la paille de riz (Khalaf, 2004). La capacité du champignon à se développer sur des résidus et à produire du chitosane a été démontrée par Suntornsuk et son équipe. Ils ont utilisé des résidus de soja et de haricot mungo comme milieu pour la production de chitosane. Ils ont réussi à obtenir des rendements de 4,3 g/kg de résidus de soja et de 1,6 g/kg de résidus de haricot mungo (Suntornsuk et al., 2002). Ces résultats et la possibilité d'utiliser la biomasse mycélienne laissée après la production d'enzymes commerciales et de produits chimiques de base font de *R. oryzae* une source idéale de chitosane fongique.

***INFERENCE 5*: La biomasse de Rhizopus oryzae générée pendant la fermentation de l'AF est un sous-produit très important car elle peut être utilisée comme source de chitosan. Le développement d'une méthode d'extraction plus écologique peut contribuer à rendre le procédé de bioproduction plus écologique.**

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PARTIE 3: PROBLÉMATIQUE

La revue de littérature sur la production et les applications des AF, des EAF et du chitosan a suggéré la nécessité de poursuivre les recherches pour rendre le processus de bioraffinage plus durable et économiquement compétitif. Le besoin de résoudre les problèmes techniques suivants a été déduit :

3.1. Augmentation de la demande et l'impact environnemental associé aux méthodes chimiques de synthèse actuelles de l'acide fumarique

La demande mondiale d'acide fumarique devrait passer de 225,2 kilotonnes (2012) à plus de 340 kilotonnes d'ici 2022. Cette augmentation est incitée par l'élévation dela demande de l'acide utilisé dans l'industrie alimentaire et des boissons, la synthèse de produits comestibles, tels que l'acide L-malique et l'acide L-aspartique, et l'identification récente des propriétés pharmaceutiques de l'AF et de ses dérivés. La demande mondiale actuelle d'AF est uniquement satisfaite par les **méthodes pétrochimiques de synthèse** par isomérisation de l'acide maléique obtenu par hydrolyse de l'anhydride maléique. L'anhydride maléique est obtenu par oxydation du butane ou du benzène en présence d'un catalyseur, étant le pyrophosphate de vanadyle. Cette méthode de synthèse chimique pétrochimique conduit à la formation de **gaz toxiques** tel que le monoxyde de carbone, et de **gaz à effet de serre** comme le dioxyde de carbone, en tant que sous-produits, ce qui entraîne une pollution de l'environnement et contribue au réchauffement de la planète. La dépendance à l'égard de la méthode de synthèse chimique pour répondre à la demande croissante peut aggraver l'impact négatif sur l'environnement. *Par conséquent, un autre processus de production respectueux de l'environnement est nécessaire pour répondre à la demande croissante.*

3.2. Durabilité et augmentation du coût de production

La voie pétrochimique de synthèse des AF utilise des **ressources non renouvelables** pour sa synthèse. Cette dépendance à l'égard des produits pétrochimiques rend le **processus non durable**. Il est donc essentiel de trouver une autre méthode de synthèse durable pour répondre à la demande croissante d'AF. En outre, l'augmentation des prix du pétrole a entraîné une augmentation correspondante du prix de la principale matière première, l'anhydride maléique, pour la synthèse chimique de l'AF. Cette augmentation du prix de la matière première se répercutera sur le prix de l'AF à l'avenir en raison de la diminution des réserves de combustibles fossiles. *L'utilisation d'une stratégie de bioproduction pour répondre à la demande future d'AF offre un moyen alternatif durable pour la synthèse de la plate-forme chimique. Le passage à une stratégie de bioproduction permettra d'utiliser des ressources renouvelables comme matière première pour la synthèse des acides gras.*

3.3. Augmentation des déchets agro-industriels et alimentaires et leur valorisation pour l'économie circulaire

La dépendance accrue à l'égard des aliments transformés a entraîné la production de quantités considérables de déchets provenant des industries de biens de consommation à rotation rapide (BCTR). Par exemple, la province de Québec se classe au 10e rang en Amérique du Nord pour la quantité de déchets agro-industriels, comme les déchets générés par les industries de la transformation du jus de pomme. La plupart de ces déchets sont riches en matières organiques et ont des teneurs **élevées en DBO et en DCO**. Cela les rend très sensibles à la dégradation microbienne. L'élimination et la gestion inappropriées de ces déchets peuvent entraîner une augmentation des émissions de gaz à effet de serre (GES) ainsi que des effets néfastes sur la santé.

L'importance accrue et la transition vers le concept **d'économie circulaire**, ont rendu nécessaire le développement de stratégies pour la **valorisation de ces déchets organiques**. Ainsi, les déchets deviennent des matières premières pour la production de produits, tels que des produits chimiques de base, des composés aromatiques et des biocarburants. Les progrès des innovations biotechnologiques, notamment dans le domaine de la fermentation, ont ouvert de nouvelles voies pour la valorisation des résidus agro-industriels et alimentaires. *À cet effet, les boues d'ultra-filtration de marc de pomme (APUS) peuvent être utilisées comme matière première pour la production d'AF. Cette valorisation des APUS, riches en contenu organique, permettra de capturer du carbone précieux et d'éviter le rejet de gaz à effet de serre.*

3.4. Amélioration du rendement en acide fumarique en utilisant une combinaison d'immobilisation fongique sur un support synthétique et de fermentation submergée en mode fed-batch

Le rendement en AF a été observé fortement dépendant **de la morphologie** du champignon pendant la fermentation. Le diamètre des granules fongiques a été observé ayant un rôle clé dans la productivité de l'acide fumarique. Cette complexité associée à la morphologie pourrait être surmontée par l'utilisation de ***R. oryzae*** **immobilisé**. *À cet effet, des supports neutres synthétiques peuvent être utilisés pour l'immobilisation et permettent d'utiliser des déchets plastiques facilement disponibles comme support, n'entraînant ainsi aucun coût supplémentaire lié à l'immobilisation*. De plus, l'utilisation de champignons immobilisés permet de séparer facilement la biomasse fongique du milieu de fermentation, ce qui rend le traitement en aval moins fastidieux. Cette biomasse fongique peut ensuite être utilisée pour l'extraction du chitosan. En outre, cela permet de faciliter le fonctionnement de la fermentation, que ce soit en mode discontinu ou continu.

3.5. Traitement en aval de l'acide fumarique et des produits à haute valeur ajoutée par conversion en esters de fumarate ainsi que par extraction de sous-produits pour améliorer la compétitivité économique du processus de bioraffinage

L'ester d'acide fumarique (EAF), le dérivé de l'acide fumarique, a récemment été identifié comme un dérivé important en raison de ses diverses applications dans l'industrie pharmaceutique et des résines. La conversion in situ ou ex situ des AF en EAF permettrait **d'ajouter de la valeur** aux AF obtenus par fermentation. Les différences de prix des AF (coût faible) et des EAF (coût élevé) peuvent avoir un impact profond sur la **compétitivité économique** du processus de bioraffinage. Aussi, l'un des principaux sous-produits du processus de bioproduction est la production de biomasse fongique. Cette biomasse peut être utilisée comme source de chitosan, un produit de grande valeur. *Cette production concomitante de plusieurs produits de grande valeur à partir du même processus de bioraffinage peut renforcer la compétitivité du processus*. En outre, le développement d'une stratégie de bioconversion pour la synthèse de l'ester de fumarate ainsi que le développement d'un processus plus efficace pour l'extraction du chitosane de la biomasse fongique peuvent ajouter aux références écologiques du processus de bioraffinage.

PARTIE 4. HYPOTHÈSE

Le présent travail de recherche comprend les hypothèses suivantes:

1) Hypothèse 1 pour le problème 3.1: La méthode actuelle de production d'AF, issue de la pétrochimie, n'est pas durable et a des effets néfastes sur l'environnement. Les AF sont naturellement produits par divers micro-organismes dans le cadre de leur activité métabolique. Par conséquent, le passage à la bioproduction d'AF pour satisfaire la demande future de cet acide organique devrait permettre d'atténuer les problèmes associés à la technique de production actuelle. À cet effet, l'espèce fongique *Rhizopus oryzae* a été identifiée comme un surproducteur naturel d'AF. *Par conséquent, le passage à un processus de bioproduction utilisant ce champignon pour répondre à la demande d'AF serait meilleure pour la protection de l'environnement.*

2) Hypothèse 2 pour le problème 3.2/3.3: La souche fongique *R. oryzae* a un besoin minimal en nutriments et peut être cultivée sur des résidus agro-industriels, une ressource renouvelable. Ainsi, les résidus agro-industriels, tels que l'APUS, les mélasses et la biomasse lignocellulosique saccharifiée, peuvent être utilisés comme matière première pour la production d'AF sans apport important de nutriments. L'utilisation de matières premières renouvelables à faible coût pour la production d'AF, par fermentation, peut avoir un impact profond sur la compétitivité économique du processus de bioproduction. *Cette transition vers la bioproduction permettra de réduire la dépendance à l'égard des combustibles fossiles pour la production d'une importante plate-forme chimique. Ainsi, le processus sera durable et respectueux de l'environnement*. De plus, elle permet de valoriser les résidus organiques et donc de séquestrer du carbone précieux qui, autrement, aurait été perdu dans l'environnement et aurait contribué aux émissions de gaz à effet de serre. Ainsi, il adhère au concept de bioéconomie circulaire, une étape clé pour le développement durable.

3) Hypothèse 3 pour le problème 3.4: Le rendement en AF a été observé dépendant de la morphologie de la croissance fongique dans le milieu de fermentation. Le contrôle de la morphologie de la croissance fongique au cours de la fermentation peut être très fastidieux et entraîner des incohérences dans le rendement en AF. *Par conséquent, l'immobilisation de R. oryzae sur un support approprié pourrait être réalisée pour surmonter cette difficulté*. La stratégie d'immobilisation facilite la prévention des limitations de diffusion et permet un transfert de masse plus élevé. *Cela peut conduire à un taux plus élevé de conversion du substrat en AF*. Elle permet également de réduire la viscosité du bouillon de fermentation en réduisant la présence de mycéliums libres dans le milieu de fermentation, ce qui permet de réduire la consommation d'énergie. De plus, l'utilisation de champignons immobilisés pour la fermentation permet de récupérer facilement la biomasse fongique, ce qui rend le processus en aval moins fastidieux et évite d'avoir à utiliser des équipements coûteux pour traiter le bouillon et récupérer le produit.

4) Hypothèse 4 pour le problème 3.5: Les AF sont des produits de faible valeur avec un grand volume par rapport à certains de leurs dérivés tels que les EAF. Ces EAF ont, ces dernières années, trouvé des applications dans de nombreuses industries, notamment l'industrie pharmaceutique et l'industrie des polymères. La demande pour les formes esters d'AF devrait croître parallèlement à la croissance de la demande d'acide fumarique. *Ainsi, en convertissant les AF en EAF in-situ ou ex-situ, la faisabilité et la compétitivité du processus de bioproduction peuvent être profondément améliorées*. La bioconversion à médiation enzymatique d'acides organiques en leur forme ester a été rapportée mais pas pour les AF. Des enzymes, telles que la lipase, pourraient être utilisées pour permettre la bioconversion des AG en EAF. *La mise au point d'une méthode de bioconversion peut renforcer l'aspect écologique du processus de bioraffinage et améliorer sa faisabilité économique.*

5) Hypothèse 5 pour le problème 3.5: L'un des principaux sous-produits du processus de bioproduction est la génération de biomasse fongique. La biomasse de *R. oryzae* a été identifiée pour donner de grandes quantités de chitosan, un produit de grande valeur et de faible volume. Actuellement, le chitosan est obtenu par extraction alcaline à partir de résidus de crustacés, ce qui entraîne la production d'effluents toxiques. La transition vers le chitosan fongique est bénéfique car elle réduit considérablement les effets néfastes sur l'environnement. *A cet effet, la biomasse fongique obtenue après fermentation peut être utilisée pour la production de chitosan*. Cette production de produits supplémentaires de grande valeur et de forte demande peut améliorer profondément la faisabilité du processus de bio-production. Le développement d'une méthode d'extraction plus efficace, telle que l'extraction assistée par micro-ondes, à partir de la biomasse fongique est souhaitable car elle permet de réduire la consommation d'énergie.

PARTIE 5. OBJECTIFS

L'objectif général de la présente étude est de mettre en œuvre les concepts de bioraffinage, via la production fermentative de l'AF par *Rhizopus oryzae* suivie d'une conversion en dérivés de haute valeur. Ce processus de bioraffinage permet l'utilisation de ressources renouvelables, telles que les résidus agro-industriels et la biomasse lignocellulosique, comme matière première. D'où sa valorisation pour atteindre l'objectif d'avoir une économie circulaire. Les objectifs spécifiques suivants ont été dérivés de la revue de la littérature:

Objectif 1: Étudier l'utilisation de résidus agro-industriels, APUS, comme substrat pour la production d'AF par fermentation submergée en utilisant *R. oryzae*.

Objectif 2: Immobiliser *R. oryzae 1526* sur des supports synthétiques et étudier son impact sur l'amélioration de la production d'AF. Des supports synthétiques peu coûteux et facilement disponibles ont été utilisés pour l'immobilisation de *R. oryzae*.

Objectif 3: Évaluer l'impact de la réduction de la taille du support d'immobilisation sur le rendement en AF et la durée de la fermentation, et étudier l'effet de la supplémentation en mélasse sur les titres d'AF.

Objectif 4: Augmenter la production d'acide fumarique sur une plus grande échelle en mode fed-batch dans des fermenteurs à l'échelle du laboratoire.

Objectif 5: Étudier l'utilisation de la biomasse lignocellulosique saccharifiée comme matière première pour la production d'AF par fermentation submergée en utilisant *R. oryzae*.

Objectif 6: Évaluer la conversion des AF à faible coût en dérivés de grande valeur, les EAF, soit ex-situ soit in-situ via une conversion chimique ou biologique.

Objectif 7: Développer une méthode d'extraction assistée par micro-ondes plus efficace pour l'extraction du chitosan de la biomasse de *R. oryzae*.

Objectif 8: Évaluer la compétitivité économique du processus de bioraffinage

PARTIE 6. ORIGINALITÉ

L'étude actuelle intègre les points d'originalité suivants :

6.1 L'immobilisation de R. oryzae sur des supports synthétiques à faible coût, tels que des mousses de polystyrène, des mousses de polyuréthane et des éponges de polyester, pour la production d'AF en utilisant des résidus agro-industriels comme matière première. La fermentation réalisée à l'aide de champignons immobilisés a le potentiel d'améliorer les titres d'acide fumarique.

6.2 L'utilisation de *R. oryzae* immobilisé pour la production d'AF à l'échelle du fermenteur, exploité en fed-batch, en utilisant des résidus agro-industriels comme matière première pour améliorer la concentration en acide fumarique.

6.3 La production fermentaire d'AF par R. oryzae à partir de biomasse lignocellulosique, telle que le panic erige, panic raide et la fibre de chanvre, n'a pas été rapportée dans la littérature.

6.4 L'identification des conditions optimales pour la conversion chimique ex-situ des AF obtenus par fermentation en EAF. Cette valeur ajoutée pourrait améliorer la compétitivité technico-économique du processus de bioraffinage.

6.5 L'extraction assistée par micro-ondes du chitosan de la biomasse fongique n'a pas été rapportée jusqu'à présent. Les conditions optimales d'énergie et de durée du traitement par micro-ondes pour l'extraction du chitosan de la biomasse de R. oryzae NRRL 1526 ont été identifiées. Cette extraction d'un sous-produit de haute valeur pourrait augmenter la compétitivité du processus de bioproduction.

Les travaux précédents sur la bioproduction se sont principalement concentrés uniquement sur la production d'acide fumarique par fermentation. Cependant, la possibilité d'améliorer la

compétitivité du bioprocessus par une valeur ajoutée, fournie par la conversion des AF en EAF, et l'extraction d'un sous-produit de grande valeur, le chitosan, à partir de la biomasse résiduelle n'ont pas été étudiées. Ainsi, l'originalité globale de la recherche actuelle est la suivante : ***" Production améliorée d'acide fumarique à partir de ressources renouvelables et conversion en dérivés de haute valeur (fumarate esters) ainsi que l'extraction de chitosan comme sous-produit "***.

PARTIE 7 : SOMMAIRE DES DIFFÉRENTS VOLETS DE RECHERCHE EFFECTUÉS DANS CETTE ÉTUDE

Les résultats de cette étude sont présentés dans les chapitres suivants et un bref résumé des résultats est fourni dans cette section. Le deuxième chapitre de cette thèse discute de la bioproduction de l'acide fumarique en utilisant la biomasse agro-industrielle et lignocellulosique comme matière première. Dans une perspective de valorisation des sous-produits issus de la bioproduction de l'acide fumarique, le troisième chapitre présente l'apport de valeur ajoutée du chitosane obtenu à partir de la biomasse du *Rhizopus oryzae* en utilisant une nouvelle méthode d'extraction. Par la suite, la compétitivité économique et l'impact de l'ajout de l'acide fumarique ainsi que celui du chitosane sont discutés dans le chapitre 4 de la thèse.

7.1 Sélection des matières premières pour la bioproduction d'acide fumarique (Chapitre 2)

Pour surmonter la dépendance de l'industrie de production de l'acide fumarique à l'égard des ressources pétrolières qui s'épuisent au fur et à mesure, des matières premières renouvelables telles que la biomasse agro-industrielle et lignocellulosique ont été testées comme milieu de culture. De plus, des stratégies de production telles que le recours à l'immobilisation de *Rhizopus oryzae* et le et à la fermentation en mode fed-batch ont été utilisées pour améliorer la production de l'acide fumarique. Les résultats de ces études sont résumés dans les sections qui suivent.

7.1.1 Production de l'acide fumarique en utilisant un mode de fermentation alternatif par *Rhizopus oryzae* immobilisé - une stratégie de production plus écologique (Chapitre 2, Partie 1)

L'immobilisation de *Rhizopus oryzae* sur des supports solides a été proposée pour pallier aux difficultés opérationnelles associées à la fermentation mycélienne libre. Les billes de mousse de polystyrène se sont avérées être le support idéal pour l'immobilisation de *Rhizopus oryzae*. La fermentation effectuée en utilisant *R. oryzae* immobilisée, sur des billes de mousse de polystyrène de 0,1-0,3 cm, a conduit à une amélioration significative de la production d'acide fumarique (27 g/L contre 19 g/L). Une amélioration similaire de la concentration finale en acide fumarique, 7,9 g/L contre 6,3 g/L, a été observée lorsque les boues d'ultrafiltration de résidus de pomme ont été directement utilisées comme matière première. La supplémentation du bouillon de fermentation avec de la mélasse a permis de tripler la concentrationen acide fumarique pour atteindre de 5,1 g/L.

7.1.2 Bioproduction de l'acide fumarique dans des fermenteurs à l'échelle du laboratoire en utilisant des résidus agro-industriels comme matière première et du *Rhizopus oryzae* immobilisé (Chapitre 2, Partie 2)

Après avoir identifié le matériau de support idéal pour l'immobilisation de *R. oryzae* et le mode de fermentation optimal, une étude de mise en échelle a été faite en utilisant un fermenteur de 4 litres. Cette étude de mise en échelle a permis de quadrupler la concentration finale de l'acide fumarique, pour atteindre une valeur de presque 12 g/L, en utilisant l'APUS comme matière première principale et la mélasse comme supplément. Cet ajout de supplément complémentaire a permis d'augmenter la concentration du milieu en sucre. Ainsi, la fermentation en mode fed-batch ainsi que l'immobilisation ont permis d'améliorer le rendement et la productivité. La fermentation sans

immobilisation en mode batch et fed-batch n'a donné que des concentrations en acide fumarique de 2,9 g/L et 7,7 g/L respectivement.

7.1.3 *Miscanthus* sp. - une biomasse lignocellulosique pérenne comme matière première pour la bioproduction d'acide fumarique (Chapitre 2, Partie 3)

Dans cette section, la possibilité d'utiliser la biomasse lignocellulosique comme matière première pour la production d'acide fumarique a été étudiée. *Miscanthus* sp., une herbe pérenne qui a été identifié comme une matière première lignocellulosique idéale pour la production d'acide fumarique. Le prétraitement des *Miscanthus* sp., a donné 39,5 g/L de sucre réducteur avec une conversion enzymatique de 79 % de la biomasse obtenue après prétraitement alcalin. Une concentration d'acide fumarique de 8-9 g/L a été obtenue lors de l'utilisation de l'hydrolysat de *Miscanthus* sp. comme matière première par fermentation en utilisant trois souches de *R. oryzae* (NRRl 1526, NRRL 6400, NRRL 2582). La souche fongique *R. oryzae* NRRL 1526 a été identifiée comme étant la meilleure en raison de sa productivité la plus élevée.

7.2 Valeur ajoutée à l'acide fumarique: valorisation des sous-produits de fermentation (Chapitre 3)

L'une des principales limitations qui empêchent la bioproduction à l'échelle industrielle de l'acide fumarique est sa faible valeur marchande de 1 $ (kg^{-1}). Par conséquent, pour améliorer la compétitivité économique et la faisabilité, l'apport de valeur ajoutée par la conversion d'AF biosourcés en EAF a été proposé. En outre, l'extraction du chitosane fongique de la biomasse fongique, un sous-produit de la fermentation médiée par *R. oryzae*, pourrait améliorer la compétitivité économique. À cet effet, l'extraction par micro-ondes a été étudiée. Les résultats de

ces recherches visant à améliorer la compétitivité économique sont résumés dans les sous-sections suivantes.

7.2.1 Synthèse du fumarate de diméthyle et du fumarate de monométhyle d'origine biologique (Chapitre 3, Partie 1)

La valeur ajoutée obtenue par conversion de l'acide fumarique en esters d'acide fumarique (EAFs) peut avoir un grand impact positif sur la compétitivité technico-économique du bioprocédé développé. Les EAF sont des ingrédients pharmaceutiques actifs ayant des applications dans le traitement de la sclérose en plaques et du psoriasis. Les conditions optimales pour la synthèse des esters ont été identifiées via une méthodologie de surface de réponse. Les conditions optimales pour l'obtention du fumarate de diméthyle (DMF) ont été identifiées comme étant 111 minutes, 98 °C et une concentration d'acide sulfurique de 9,7 % (v/v). D'autre part, l'utilisation de conditions modérées a favorisé la synthèse du fumarate de monométhyle (FMM). Les conditions optimales ont été identifiées comme étant 30 minutes, 55,5 °C et 2 % (v/v) d'acide sulfurique, pour la synthèse du FMM. L'utilisation de conditions optimales a permis la conversion de 99,2 % l'acide fumarique d'origine biologique en EAF.

7.2.2 Extraction assistée par micro-ondes du chitosane de la biomasse de *Rhizopus oryzae* NRRL 1526 (Chapitre 3, Partie 2)

La biomasse fongique est l'un des sous-produits de la bioproduction d'acide fumarique par *R. oryzae*. Cette biomasse peut être utilisée pour isoler du chitosane fongique. Les conditions optimales d'extraction assistée par micro-ondes (EAM) du chitosane à partir de la biomasse de *R. oryzae* NRRL 1526 ont été identifiées comme étant 300W et 22 minutes. Le chitosane ainsi obtenu a montré un degré de désacétylation plus élevé que celui du chitosane obtenu par les méthodes

d'extraction conventionnelles (94,6 % contre 90,6 %). Le poids moléculaire ainsi que la cristallinité se sont avérés être comparables De plus, l'EAM a permis de récupérer une quantité plus importante de chitosane comparé au méthodes d'extraction conventionnelles, 13,43% (≈2 fois) contre 6,67% (w/ w).

7.3 Analyse technico-économique du procédé de bioproduction de l'acide fumarique (Chapitre 4)

La commercialisation d'un procédé biotechnologique dépend fortement de la rentabilité technico-économique lorsqu'il est comparé par rapport au procédé basé sur la conversion chimique. Le prix d'équilibre (PE) de l'acide fumarique a été calculé à \$ 2,304 (kg^{-1}) pour un procédé de bioproduction conçu pour la production d'acide succinique. En raison des propriétés uniques de l'acide fumarique, certaines opérations unitaires ont pu être supprimées. Cela a conduit à une réduction des PE à \$1,775 (kg^{-1}). De plus, l'optimisation du processus de bioproduction, en augmentant le rendement et en réduisant le total des investissements en capital (TCI), a le potentiel de réduire encore plus les PE pour atteindre un prix de \$ 1,5 (kg^{-1}).

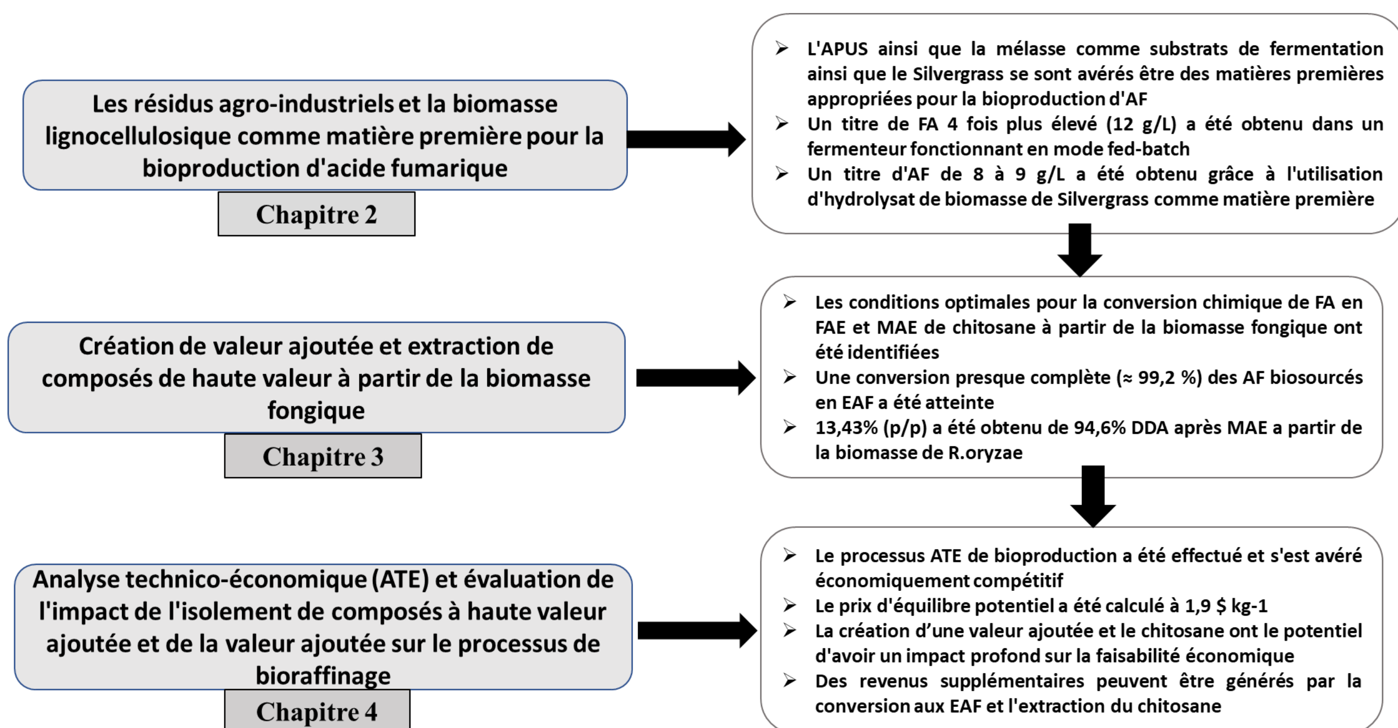

Figure 1. 6. Schematic of objectives (left) and a brief summary (right)

CHAPTER 2: Screening of feedstocks for fumaric acid bioproduction

PART 1

Fumaric acid production using alternate fermentation mode by immobilized *Rhizopus oryzae*- a greener production strategy

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Chemosphere 281 (2021) 130858; DOI 10.1016/j.chemosphere.2021.130858

Résumé

Ce travail de recherche a pour objectif d'étudier l'impact de l'utilisation de *Rhizopus oryzae* NRRL 1526 immobilisée pour la bioproduction d'acide fumarique à partir des résidus agro-industriels comme matière première. L'utilisation de ses résidus agro-industriels, une matière première renouvelable, pour la production d'un produit chimique de base biologique rend le processus compétitif en termes de coûts et plus écologique en empêchant la libération de carbone organique assimilable dans l'environnement, réduisant ainsi la production de gaz à effet de serre. L'immobilisation de *R. oryzae* a été proposée précédemment pour atténuer les difficultés opérationnelles rencontrées lors de la fermentation fongique mycélienne libre. À cet effet, trois matériaux de déchets synthétiques, à savoir la mousse de polystyrène, l'éponge de polyester et la mousse de polyuréthane, ont été étudiés pour leur convenabilité à la bioproduction d'acide fumarique. La mousse de polystyrène a été identifiée comme le matériau de support le plus approprié pour l'immobilisation ainsi que pour la production d'acide fumarique. En plus de la réduction considérable de la phase de latence (de 48 à 24 h), la réduction de la taille du matériau de support, des cubes de 1 cm aux billes de 0,1-0,3 cm, a conduit à une amélioration de 42% de la production d'acide fumarique (27 g/L contre 19 g/L). La culture de *R. oryzae* immobilisée par des billes de mousse de polystyrène sur des boues d'ultrafiltration de résidus de pomme comme seule charge d'alimentation a permis d'obtenir un titre final d'acide fumarique de 7,9 g/L alors que la fermentation mycélienne libre n'a donné que 6,3 g/L. En ajoutant les mélasses, un résidu agro-industriel, comme alimentation aux boues de résidus de pomme durant la fermentation en mode alimentation intermittente, une augmentation de trois fois (1,7 g/L à 5,1 g/L) de la production d'acide fumarique a été obtenue.

Mots clés: Résidu agro-industriel; acide fumarique; *Rhizopus oryzae*; immobilisation; polystyrène

Abstract

The current work investigates the impact of using immobilized *Rhizopus oryzae* NRRL 1526 for bioproduction of fumaric acid using agro-industrial residues as feedstock. This use of agro-industrial residues, a renewable feedstock, for the production of bio-based platform chemical makes the process cost-competitive as well as greener by preventing the release of assimilable organic carbon to the environment, thereby reducing the generation of greenhouse gases. Immobilization of *R. oryzae* has been proposed previously to alleviate operational difficulties confronted during free mycelial fungal fermentation. To this effect, three synthetic refuse materials namely polystyrene foam, polyester sponge and polyurethane foam were investigated for their suitability towards fumaric acid bioproduction. Polystyrene foam was identified as the most suitable support material for immobilization as well as fumaric acid production. In addition to the considerable reduction in the lag-phase (from 48 to 24 hours) the reduction in the size of the support material from cubes of 1 cm to beads of 0.1-0.3 cm led to a 42 % improvement in fumaric acid production (27 g/L against 19 g/L). Growing the polystyrene foam bead immobilized *R. oryzae* on apple pomace ultrafiltration sludge as sole feedstock yielded a final fumaric acid titer of 7.9 g/L whereas free mycelial fermentation yielded 6.3 g/L. Moreover, upon operating the fermentation with intermittent feeding, a three-fold increase (1.7 g/L to 5.1 g/L) in fumaric acid production was obtained upon supplementation of the apple pomace sludge media with molasses, an agro-industrial residue, as feed.

Keywords: Agro-industrial residue; fumaric acid; *Rhizopus oryzae*; immobilization; polystyrene

Introduction

Fumaric acid (FA) is a naturally occurring organic acid and a key intermediate of the TCA (Tricarboxylic acid cycle) metabolic pathway. FA along with the other two four-carbon dicarboxylic acids (malic and succinic acid), has been identified as key biomass-based value-added chemical (Guo et al., 2020; Swart et al., 2020). The presence of a double bond in addition to the carboxylic group, in trans configuration, renders fumaric acid with unique properties and suitable for polymer manufacture. The polymer so obtained has been reported to be non-toxic and harder than those derived from maleic anhydride (Martin-Dominguez et al., 2018; Zhou et al., 2011). Currently, the production of fumaric acid relies on petrochemically derived maleic anhydride, adding to the adverse environmental impact (Ilica et al., 2018). Therefore, the switch to the bioproduction of fumaric acid provides a sustainable and eco-friendly alternative to petroleum-based production (Deng & Aita, 2018; Papadaki et al., 2018).

The bioproduction of FA via fermentation has usually been carried out with *Rhizopus oryzae* around neutral pH values (Roa Engel et al., 2008). The fungal species has established itself as the dominant microbial FA producer and the attempts to use genetically modified organisms have failed to yield FA concentration achieved by the fungal species (Sebastian et al., 2019). The fungi tend to grow in clumps or pellets when cultivated in a stirred-tank bioreactor and it has been observed that the optimal production of FA is highly dependent on the mycelial pellet size formed during fermentation. Additionally, these pellet sizes have to be below a certain critical value to prevent oxygen limitation (Ilica et al., 2018; Zhou et al., 2011). Under such oxygen diffusion limitation conditions ethanol production, as the main by-product, is observed even under aerobic conditions (Roa Engel et al., 2011). Despite numerous studies to optimize pellet size for optimal FA production as well as reduced ethanol formation, the elimination of ethanol formation has not

been reported (Naude & Nicol, 2017). Moreover, the control of the pellet size for optimal fumaric acid production in a stirred-tank reactor is difficult and has been a major obstacle for the commercialization of fumaric acid bio-production (Liu et al., 2017). The culturing of *Rhizopus* in a stirred-tank reactor often leads to the formation of different morphologies, thereby hindering fumaric acid production and operational problems. These operational difficulties include impeller impediment, agitation blade fouling, increase in viscosity and blocking of sampling ports among others (Liu et al., 2017; Zhang et al., 2008).

To overcome these adversities, the immobilization of fungus on different carriers to produce FA has been investigated and has been proven to be efficient due to good mass and oxygen transfer (Das et al., 2015; Naude & Nicol, 2017). Fungi have the inherent ability to attach and propagate on a wide variety of organic and inorganic support materials. This ability of the filamentous fungi to adhere to suitable inert substrates can be exploited to overcome the fore-mentioned bioprocess difficulties. These immobilized fungi are in direct contact with the substrate, thereby probably reducing or eliminating mass transfer problems associated with suspended mycelial cultures. The use of immobilized fungi for the bio-production of organic acids, such as fumaric acid, as well as enzymes has been investigated and has been proven to be effective (Das et al., 2015; Liu et al., 2017; Mussatto et al., 2012). Additionally, the use of immobilization strategy allows for the re-use of the fungi in subsequent fermentations, operating the fermenters in fed-batch or continuous fermentation mode and unexacting recovery of fungal mycelia which in turn makes downstream processing of fermented broth less challenging (A. Abdelmajeed, 2012; Karagoz et al., 2019).

Most of the research so far has relied on the use of pure glucose, sugars obtained from lignocellulosic biomass hydrolysate and starchy material as feedstock, as presented in recent reviews on fumaric acid bioproduction (Guo et al., 2020; Sebastian et al., 2019). The work

discussed here aims to evaluate the use of agro-industrial residues as a direct feedstock for fumaric acid production as well as strategies to improve fumaric acid titers obtained during fermentation. The direct use of agro-industrial residues as feedstock for production can profoundly affect the feasibility of the bioproduction process, as it eliminates the need for pre-treatment and saccharification, which is the case when lignocellulosic biomass is used as feedstock. Additionally, fungal mycelial immobilization was adopted as a strategy to alleviate the issues of the dependence of fumaric acid production on fungal morphology as well as problems associated with free mycelial fermentation. To this effect, the best synthetic refuse support material for immobilization of *Rhizopus oryzae* NRRL 1526 was identified and the effect of this immobilization on fumaric acid production on synthetic media and apple pomace ultrafiltration sludge (APUS), an agro-industrial residue, was evaluated. The adoption of the fed-batch mode to further improve fumaric acid titer was also investigated.

Materials and methodology

Fungal strain and culture conditions for spore suspension preparation

R. oryzae NRRL 1526 was obtained from the agricultural research service (ARS) culture collection. The spore suspension for inoculation was obtained by culturing the fungi on potato dextrose agar (PDA) plates incubated at 30 ± 1 °C for 96 hours. Spores were recovered by washing the PDA plates with sterilized distilled water and filtration of the suspension through sterile cotton wool to remove fungal mycelium and stored at 4± 1 °C. The concentration of the spore stock solution was maintained at 1×10^7 spores per mL.

Synthetic fungal growth and fumaric acid production medium

The growth medium is composed of (in g L^{-1}) 5 glucose, 2 urea, 0.6 KH_2PO_4, 0.25 $MgSO_4 \cdot 7H_2O$ and 0.088 $ZnSO_4.7H_2O$. Glucose and urea were sterilized separately from the rest of the components to prevent the Maillard reaction. The fumaric acid production medium was made up of (in g L^{-1}) 100 glucose, 0.6 KH_2PO_4, 0.25 $MgSO_4$, 0.088 $ZnSO_4 \cdot 7H_2O$ and 1 yeast extract. Distilled water was used and all media were sterilized in an autoclave at 121 °C for 20 min (Naude & Nicol, 2017). Calcium carbonate (50 g/L) was used in the production medium to neutralize fumaric acid produced during fermentation (Gangl et al., 1990).

Selection of support for immobilization and fermentation conditions for immobilization

Three different readily available, used as packaging and sealing materials, refuse synthetic support materials, polyurethane foam, polyester foam and polystyrene foam, were studied for their suitability for *R. oryzae* immobilization and fumaric acid production. Cubes of support materials of approx. 1 cm size was prepared, weighed, washed with sterile water, followed by 70% (v/v) ethanol, subjected to UV sterilization and left for drying at room temperature in the safety cabinet.

The fungi were pre-grown on the support material in a 500 mL Erlenmeyer flask containing 100ml of sterilized growth medium. The flasks containing 25 support materials were incubated at 30 °C and 150 rpm. Spores from fungi cultured on 4% PDA were inoculated to achieve a final concentration of 1 $x10^6$ spore per Liter. Following the immobilization of the fungus on the support material after 72 hours, they were then transferred into the production medium for fumaric acid production after washing with sterile water (Das & Brar, 2014; Naude & Nicol, 2017).

Fermentation conditions for fumaric acid production

Fumaric acid production was performed in a 500 mL flask containing 100 mL production medium and 10 immobilized support particles per flask at 30 °C and 200 rpm. The ideal support material, selected based on the fumaric acid concentration achieved in the synthetic fermentation medium, was then used for fumaric acid production on apple pomace ultrafiltration sludge (APUS).

APUS composed of 30 g/L total suspended solids (TSS) was used to investigate the production of fumaric acid at the lab scale. The initial pH of the sludge (2.8) was adjusted to achieve a final pH of 6 and fermentation performed at 30 °C and 150 rpm for 7 days. Calcium carbonate (50 g/L) was used in the production sludge medium to neutralize fumaric acid produced during fermentation. Fed-batch flask scale fermentations using APUS as feedstock was also studied to identify the ideal feed supplement to improve fumaric production individually. About 50 mL APUS, 12 mL brewery wort residues (La Barberie, Quebec) and 6 mL of 10% (v/v) molasses solution were used as feed. The fermenting medium was supplemented with the feeds separately every 24 hours and this corresponded to an addition of 1.5 g of reducing sugar.

Sampling and analytical techniques

Scanning electron microscopy (SEM) imaging was carried out, using Zeiss Evo®50 Smart SEM system between 5 kV and 15 kV, to investigate the biofilm formation over the immobilization support. Samples were collected regularly during the fumaric acid production fermentation. Dinitrosalicyclic acid (DNS) assay was performed to quantify reducing sugar concentration during fumaric acid. The fumaric acid produced was recovered by acidification with 5N sulfuric acid at 90 °C (Das & Brar, 2014) and quantified using the spectrophotometric method as described by

Das and Brar (2014). UV-VIS Cary 50 Spectrophotometer was used for the spectrophotometric quantifications of total reducing sugar and fumaric acid.

Statistical Analysis

Statistica software (Version 13.3) was used for the analysis of experimental data to determine the statistically significant differences (p-values < 0.05) among the experimental data.

Results and Discussion

Selection of appropriate synthetic immobilization support

Three different readily available synthetic refuse support materials, polystyrene foam, polyester sponge and polyurethane foam were investigated for their suitability as support for the immobilization of fumaric acid-producer *R. oryzae* NRRL 1526. These synthetic support materials are readily used as packaging and insulating materials. The immobilization of the fungus, as well as evaluation of their suitability for fumaric acid production, was performed on synthetic media. Pre-immobilized *R. oryzae* on supports (cubes of 1cm), by growing on synthetic growth media, was added into the synthetic production media and concentrations of fumaric acid produced as well as reducing sugar consumed quantified. Polystyrene foam was observed to be the ideal support material for fungal immobilization and fumaric acid production (Figure 2.1.1) and provided a fumaric acid titer of 19 ± 2.6 g/L which is similar (p-value = 0.1) to that of free mycelial fermentation after 240 hours of fermentation. This fumaric acid concentration obtained was considerably higher than14.2 and 10.3 g/L, which was obtained for fermentations when *R. oryzae* was immobilized on polyester and polyurethane supports, respectively (p- value = 0.03).

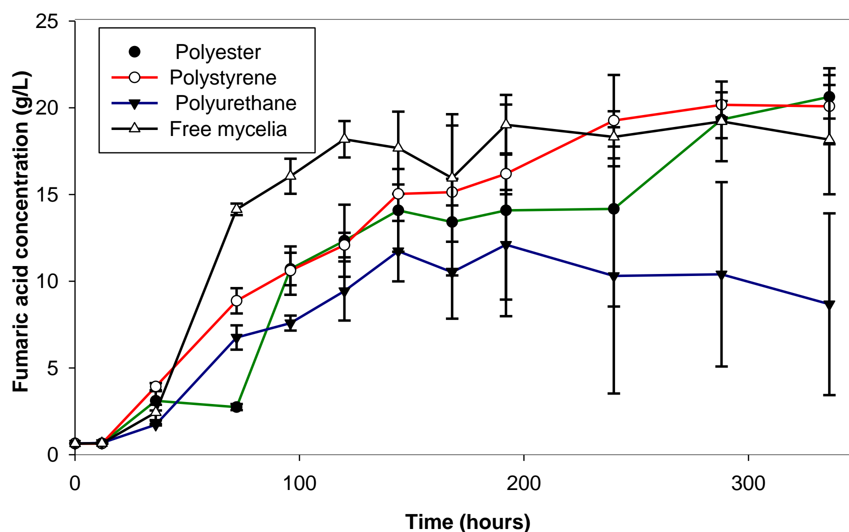

Figure 2. 1. 1. Comparison of fumaric concentration produced during fermentation on synthetic media using different immobilization support materials

A notable difference in the lag-phase of fumaric production was observed and was found to be lower than that of free mycelial fermentation (control), in case of fermentations performed using *R. oryzae* immobilized on polystyrene foam and polyester sponge. This observation is in accordance with the most general observations that immobilization leads to a reduction in the lag phase of production (Dolejš et al., 2019; Hussain et al., 2015; Kourkoutas et al., 2004; Vasconcelos et al., 2004). Such a reduction in lag phase was not observed in the case of fermentation performed using polyurethane foam as support material. Moreover, the maximum fumaric acid titer obtained (while using polyurethane foam as support material), 12 ± 3.2 g/L, was observed to be profoundly lower than those achieved when polystyrene foam and polyester sponge were used as support material. The maximum fumaric acid titer of nearly 20 ± 0.8 g/L was achieved in the case of both these fermentations. This titer was similar to that achieved for free mycelial fermentation (19 ± 2.2 g/L). These observations were not in accordance with that of Petruccioli et al. (1996) and they concluded polyurethane sponge to be suitable material for fumaric acid production. This contrast

in observation may be attributed to the difference in the material used, as polyurethane foam (used as insulation), which is more brittle, was used in the current study rather than a sponge. Additionally, the final fumaric acid concentration was achieved earlier for fermentation performed using polystyrene foam as support material than when the polyester sponge was used (240 hours vs 336 hours). Hence, polystyrene foam was identified to be the ideal support material for fungal immobilization for fumaric acid production. The use of polystyrene foam, used as packaging material, as support material for fungal immobilization and fumaric acid production has not been reported so far. Therefore, this provides a new avenue for the valorization of the readily available refuse packaging material. The use of polypropylene tube as support material for *R. oryzae* immobilization and fumaric acid production has been reported previously. The formation of a thin biofilm of the fungus on the polypropylene tube was achieved and fumaric acid titers of 30-40 g/L were obtained during fermentation (Naude & Nicol, 2017).

Effect of reduction immobilization support size on fumaric acid

It was observed that even though a similar fumaric acid titer was achieved by the use of polystyrene foam as immobilization support, the fumaric acid productivity of 0.08 $gL^{-1}h^{-1}$ was still lower than that of free mycelial fermentation (0.16 $gL^{-1}h^{-1}$). To overcome this reduction in the rate of fumaric acid production, the size of fungal immobilization support was reduced and the effect of this on fumaric acid production was investigated. To this effect, polystyrene foam cubes (1cm) were broken down into individual beads of size ranging from 0.1 to 0.3 cm and used as immobilization support. *R. oryzae* immobilized on these polystyrene beads were then used to evaluate fumaric acid production.

The reduction in the size of immobilization support was found to be highly effective in improving fumaric acid titer as well as further reduce the initial lag phase of fumaric acid production (Figure

2.1.2). The use of *R. oryzae* immobilized on polystyrene foam beads for fermentation yielded a fumaric acid titer of 27 ± 1.1 g/L (refer fig. 2.1.2). This titer was significantly higher than the maximum titers of 19 ± 1.8 g/L and 20 ± 1.2 g/L achieved for free mycelial fermentation and fermentation with fungus immobilized on polystyrene cubes respectively. Moreover, the use of fungus immobilized on polystyrene beads led to improved productivity (at 120 hours) of 0.19 ($gL^{-1}h^{-1}$) when compared to 0.16 ($gL^{-1}h^{-1}$) and 0.10 ($gL^{-1}h^{-1}$) of free mycelial and polystyrene cube fermentation, respectively. The use of polystyrene beads improved the surface area further enhancing the fumaric acid titer. This increase in the area provides more surface for fungal biofilm to attach and be in contact with the substrate. Similarly, improvement in fumaric acid production upon an increase in surface area for fungal immobilization was observed by Das et al. (2014). It was observed that an increase in surface area of immobilization from 4 cm^2 to 25 cm^2 led to an increase in fumaric titer from 28.3 g/L to 41.5 g/L (Das & Brar, 2014). A similar observation was made by Gu et al. (2013) as well and nearly a three-fold increase in FA production was observed when the surface area of the immobilization support used for the study was increased from 25 cm^2 to 150 cm^2 (Gu et al., 2013). The increase in surface area as a result of breaking each cube of polystyrene into individual beads provides for an additional surface for fungal attachment and growth. This might lead to a reduction in the thickness of the fungal mycelia bound to the immobilization support and hence, might be improving mass transfer compared to polystyrene cubes. The formation of thicker biofilm does not support aerobic processes such as FA production, due to oxygen and nutrition limitations as reported previously (Skory et al., 1998; Wang et al., 2010).

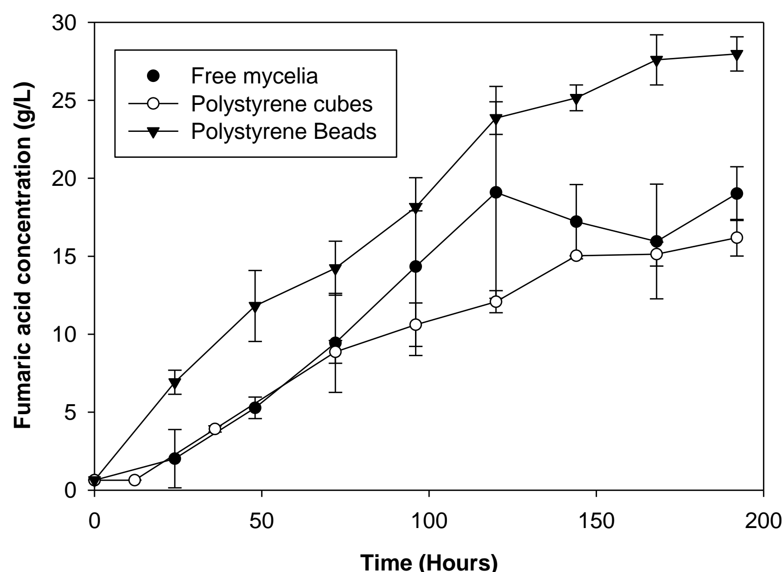

Figure 2. 1. 2. Comparison of fumaric acid production between polystyrene beads (0.1-0.3 cm), polystyrene cubes (1 cm) and free mycelia.

Fumaric acid production using apple pomace ultrafiltration sludge

Post identification of polystyrene beads as the ideal support for fumaric acid production. *R. oryzae* immobilized on polystyrene beads were used for fumaric acid production using apple sludge as fermentation media. The results of this study are represented in Figure 2.1.3. It was observed that the use of immobilized beads was able to profoundly reduce the lag phase as observed for synthetic production media. The fumaric acid titer of 5.2 ± 1 g/L was obtained in the first 48 hours of fermentation performed using fungal mycelia immobilized beads whereas the free mycelial fermentation yielded a fumaric concentration of 3.3 ± 0.1 g/L. This profound difference in fumaric acid production can be attributed to the higher rate of reducing sugar utilization by the immobilized fungi during the first 48 hours of fermentation (represented in Figure 2.1.3 b). A similar pattern was observed in the final fumaric acid titer achieved towards the end of fermentation. Maximum fumaric acid titers of 7.9 ± 0.6 g/L and 6.3 ± 0.9 g/L were achieved during the fermentations carried

out using immobilized fungi and free mycelial fermentation, respectively. The SEM images (Figure 2.1.4) of the fresh polystyrene foam beads and that obtained after fermentation showed the proliferation of fungal mycelia on the beads.

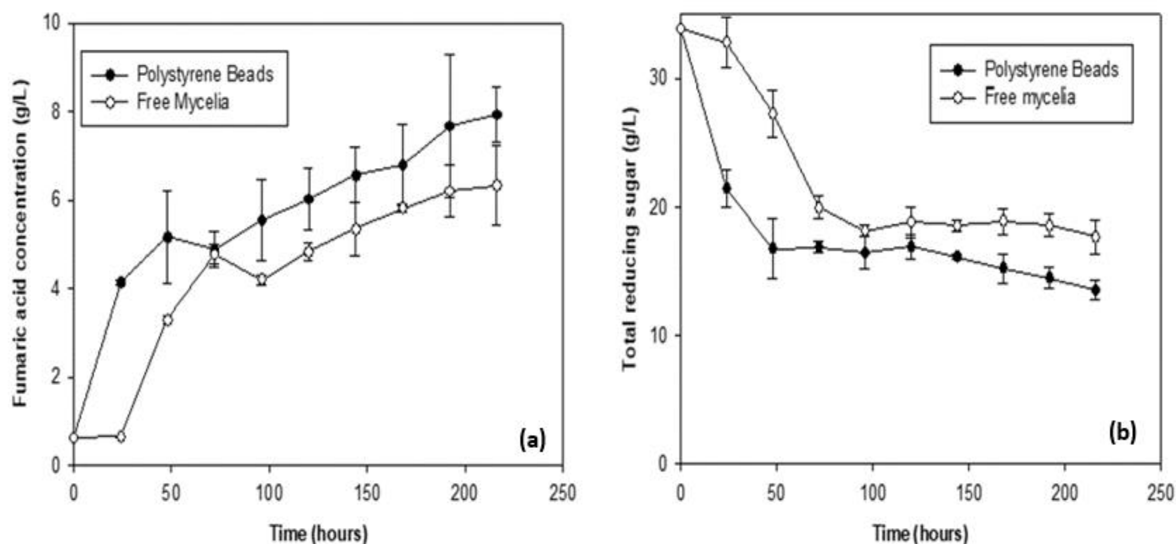

Figure 2. 1. 3. Fumaric production (a) and total reducing sugar utilization (b) during fermentation on apple pomace ultrafiltration sludge using R. oryzae immobilized on polystyrene beads

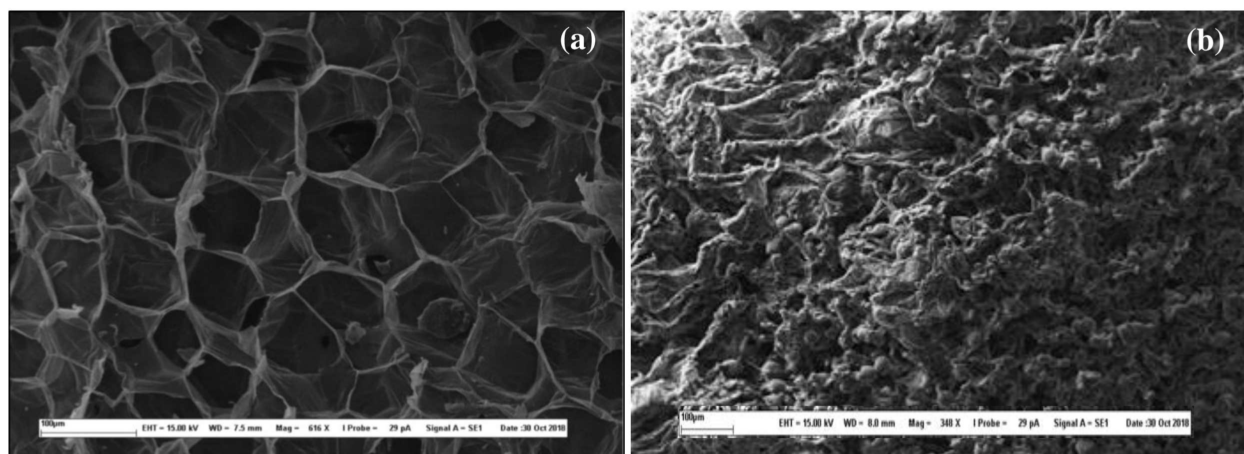

Figure 2. 1. 4. SEM images of polystyrene beads before immobilization (a); and after fermentation in apple pomace ultrafiltration sludge (b)

Fed-batch fermentation at flask scale using *R. oryzae* immobilized beads for fumaric acid production using apple pomace ultrafiltration sludge as feedstock

The drastic decrease in reducing sugar concentration during the first 48 hours of fermentation followed by a nearly stationary sugar utilization (Figure 2.1.3 b), prompted to investigate the possibility of the use of the fed-batch system of fermentation. These investigations were carried out at the flask scale to identify the appropriate feed among apple pomace ultrafiltration sludge, brewery wort residues and molasses, for FA production. The results of this investigation are presented in Figure 2.1.5. It was observed that supplementation of fermentation media with 10% (v/v) molasses solution was able to improve maximum fumaric acid concentration obtained by 3 folds, from 1.7 ± 0.05 g/L to 5.4 ± 0.1 g/L, after 84 hours. Improvement in maximum fumaric acid titer was also observed in the case of fermentation where APUS was used as a supplement to achieve a titer of 2.7 ± 1 g/L. All the fermentation followed a similar pattern during the first 24 hours of fermentation, followed by an increase in fumaric acid production due to feed supplementation (at 24 hours, 48 hours and 72 hours) in case of fermentation with apple pomace ultrafiltration sludge and molasses supplementation. However, the supplementation with brewery wort failed to improve fumaric acid and a continuous decrease in fumaric acid titer was observed following supplementation at 24 hours. The exact reason for this decrease in fumaric acid titer is not clear but may be due to the high nitrogen content of brewery wort and supplementation might have led to an accumulation of nitrogen in the fermentation media which in turn led to inhibition of fumaric acid production. Ideally, for optimal fumaric acid production, a high C/N ratio is preferred and the results obtained here further support previously reported observations (Roa Engel et al., 2008; Xu et al., 2012) .

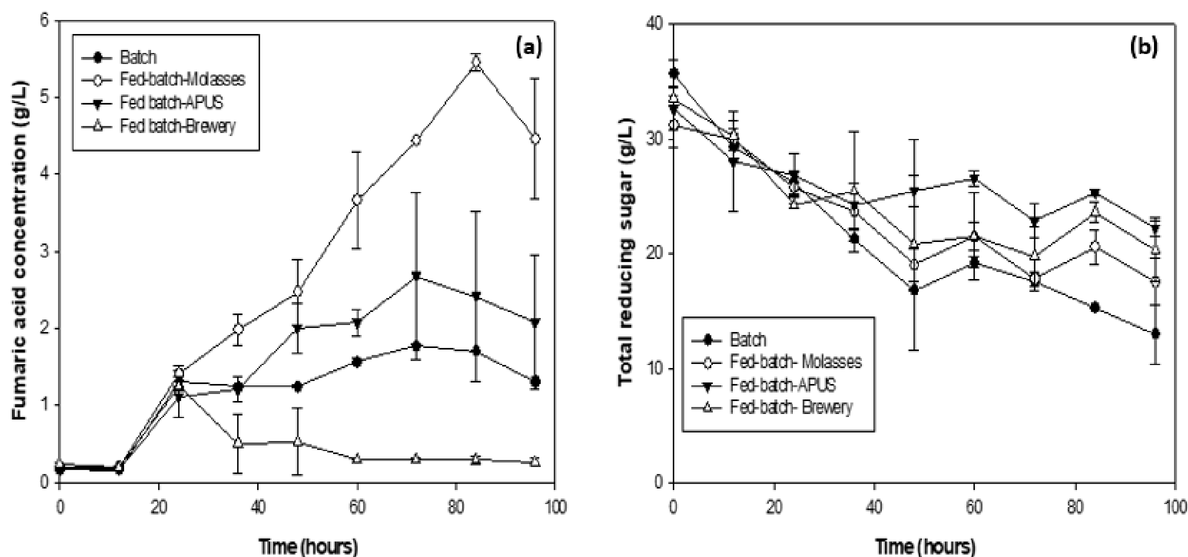

Figure 2. 1. 5. Fumaric production (a) and total reducing sugar utilization (b) during fermentation on apple pomace ultrafiltration sludge operated on fed-batch mode using R. oryzae immobilized on polystyrene beads

The improvement in the fumaric acid titer obtained, afforded by the use of molasses as feed, further signifies the importance of having a high C/N ratio for fumaric acid fermentation. The strategy of using multiple agro-industrial residues with varying degrees of C/N ratio for fumaric acid production has been investigated here and has been observed to be effective in further improving fumaric acid production. The supplementation of APUS (lower C/N ratio) with molasses (high C/N ratio) as intermittent feed was able to improve fumaric acid production profoundly. Such a strategy of using multiple waste streams can be scaled up further and adopted for the production of fumaric acid as well as other organic acids.

Scope, scalability and future work

The work presented here aims at identifying the best synthetic inert support material for *R. oryzae* immobilization as well as the best fermentation strategy for fumaric acid bioproduction using agro-industrial residues as feedstock. Such direct use of residues, without additional modification requirements, can have a significant impact on the economic feasibility and cost competitiveness

of the biorefining process. In this particular case, APUS and molasses were used but other food/agro-industrial wastewater, such as starchy material processing plant and the non-alcoholic sugar-sweetened beverage industry, can also be used for the bioproduction process (Comelli et al., 2015; De Grazia et al., 2017; Kot et al., 2020; Luo et al., 2018; Zhou et al., 2011). The channeling of such carbon-rich residues towards a biorefining process would allow for sequestration of carbon which otherwise would have led to the release of greenhouse gases as is the case with the current commercial production. Therefore, the development of a biorefining process renders the commercial production of fumaric acid sustainable and environment-friendly.

The intermittent feeding during fermentation has shown to be effective to improve fumaric acid production but to have a valid comparison between the feeds investigated, the concentration of molasses added was intentionally kept low. The promising result of a three-fold increase in fumaric acid production using molasses as feed points us in the right direction for our future work, targeted to develop a process for fumaric acid production using agro-industrial residues as feedstock. A higher fumaric acid titer is essential for the feasibility of the process and this might be achieved by increasing the concentration of molasses provided as intermittent feed as well as better control over parameters, such as dissolved oxygen and agitation, afforded by the use of fermenters during scale-up. Hence, adding to the economic feasibility and scalability of the process.

Additionally, the use of immobilized fungi allows for the easy recovery of as well as the operation of fermentation of multiple fed-batch or continuous fermentation without the need for fresh fungal seed cultures. The lack of fungal mycelia in the fermentation broth makes further downstream processing for product recovery easier. Furthermore, the recovered fungal biomass can be used as the raw material for fungal chitosan extraction, a high-value product (Ghormade et al., 2017; Sebastian et al., 2020). This combination of multiple products, in this case, a high-volume low-

value product (FA) and low volume high-value by-product (chitosan), can have a profound impact on the economic feasibility and cost competitiveness of the bioproduction process.

Conclusion

The ideal synthetic support material among, polystyrene foam, polyurethane foam and polyester sponge, for fumaric acid production using immobilized *R. oryzae* was identified and evaluated for its suitability. Polystyrene foam was found to be the ideal support material and a considerable reduction in the lag phase was observed. Furthermore, the reduction in the size of the support material, from cubes of 1 cm to polystyrene foam beads of 0.1-0.3 cm, led to a significant improvement in fumaric acid production (27 g/L against 19 g/L). A similar improvement in final fumaric acid titer, 7.9 g/L versus 6.3 g/L, was observed when apple pomace ultrafiltration sludge was directly used as feedstock and a three-fold increase in fumaric acid production was obtained upon supplementation with molasses. These results have shown that the direct use of certain agro-industrial results is potentially feasible and has laid the foundation for future research into fumaric acid production in stirred-tank reactors. Hence, providing for a platform to develop an economically feasible and sustainable biorefining process for the production of a key platform chemical.

Acknowledgments

The authors are sincerely thankful for the financial support of NSERC (Discovery Grants Program) and the Ministère des Relations Internationales du Québec (coopération Paraná-Québec 2010–2012). Likewise, support from James and Joanne Love Chair in Environmental Engineering at York University is also appreciated. The views and opinions expressed in this review are those of the authors.

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LINK-1

LINK: The flask scale studies showed that the immobilization of *Rhizopus oryzae* and molasses supplementation during fermentation was able to increase fumaric acid concentration achieved during fermentation. To further improve the fumaric acid titer the flask scale fermentation was scaled up in 4 L bench scale fermenter. The fermentation was operated in fed-batch mode using APUS as the primary feedstock and molasses (25% v/v) as the feed.

PART 2

Fumaric acid bioproduction in bench scale fermenters using agro-industrial residues as feedstock and immobilized *Rhizopus oryzae*

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Waste and Biomass Valorization (Submitted)

Résumé

Le présent travail étudie l'utilisation de ressources renouvelables, telles que les boues d'ultrafiltration de marc de pomme et les mélasses, comme matière première pour la bioproduction d'acide fumarique dans un fermenteur à échelle réduite. Les stratégies d'immobilisation et le fonctionnement de la fermentation en mode fed-batch ont été étudiés pour leur effet sur le rendement et la productivité de l'acide fumarique. Rhizopus oryzae immobilisé sur des billes de mousse de polystyrène a été utilisé dans cette étude. Cette immobilisation et le fonctionnement de la fermentation en mode fed-batch ont permis de multiplier par 4 les titres d'acide fumarique pour atteindre une concentration de près de 12 g/L. Les résultats montrent que les résidus agro-industriels peuvent être utilisés efficacement pour la bioproduction d'acide

Mots clés: Résidus agro-industriels, bioproduction, acide fumarique, immobilisation, *Rhizopus oryzae*

Abstract

The present work investigates the use of renewable resources, such as apple pomace ultrafiltration sludge and molasses, as feedstock for fumaric acid bioproduction in bench scale fermenter. Strategies of immobilization and operation of the fermentation in fed-batch mode were investigated for their effect on fumaric yield and productivity. *Rhizopus oryzae* immobilized on polystyrene foam beads was used in this study. This immobilization and the operation of the fermentation in fed-batch mode led to a 4-fold increase in fumaric acid titers to achieve a concentration of nearly 12 g/L. The results show that the agro-industrial residues can be utilized effectively for fumaric acid bioproduction in fermenters. Hence, providing a sustainable alternative to production of a key biobased organic acid.

Keywords: Agro-industrial residues, bioproduction, fumaric acid, immobilization, *Rhizopus oryzae*

Introduction

Fumaric acid (FA), referred as (E)-2-butenedioic acid or trans-1,2-ethylene dicarboxylic acid, is a natural organic acid and an intermediate of the Tricarboxylic acid (TCA) metabolic pathway. This organic acid along with other organic acids, such as succinic acid and malic acid, has been identified as key biobased platform chemical by the US Department of Energy (DOE) in 2004 (Choi et al., 2015; Bozell and Petersen, 2010). The properties, such as presence of a double bond in addition to the dicarboxylic group and its low solubility, has rendered fumaric acid amenable to modifications and recovery less tedious (Sebastian et al., 2019; Martin-Dominguez et al., 2018). The current industrial production of fumaric acid relies on depleting petroleum resource as the primary feedstock. This process involves the catalytic chemical conversion of petroleum derived benzene or butane to maleic anhydride followed by hydrolysis to maleic acid. The maleic acid so obtained is subjected to isomerization to fumaric acid. The process of chemical synthesis leads to the release of toxic greenhouse gases, such as carbon monoxide and carbon dioxide, thereby adversely impacting the environment and contribute to global warming (Rodriguez-Lopez et al., 2012; Felthouse et al., 2001).

The diverse applications of fumaric acid range from use as an acidulant in the food industry as well as the use of its derivatives, fumaric acid esters (FAEs), in the pharmaceutical industry (Das et al., 2016). These applications have led to an increased demand for the organic acid and the reliance on the chemical production process to meet this demand can further the adverse environmental impact. Hence, a shift to bioproduction is essential to meet the future demand. The strategy of bioproduction allows for the use of carbohydrates as the feedstock and relies on fermentation of the sugars by the fungi *Rhizopus oryzae*, a natural over producer of the organic acid (Lee et al., 2011). In addition, the fungus has simple nutrient requirements and therefore

allows for the use of renewable industrial organic residues as the raw material for bioproduction. Thus, ensuring sustainable development as well as capture of carbon that otherwise would have been lost to the environment, adding to the greenhouse gas (GHG) effect.

The work here investigated the use of agro-industrial residues as the feedstock in combination for fumaric acid production in fermenters. Apple pomace ultrafiltration sludge (APUS), a carbon and nitrogen rich residue of the apple juice industry, and molasses, a carbon rich residue of the sugar industry, were used for fumaric acid bioproduction. Previous work (at flask scale) by our group had identified that these substrates are suitable for fumaric acid production (Sebastian et al., 2021). In the current work, the strategy was further scaled up in bench scale fermenter of 4 L to improve the fumaric acid yield.

Materials and methodology

Fungal spore suspension preparation

Lyophilized spores of the fungus *R. oryzae* NRRL 1526 was obtained from the agricultural research service (ARS) culture collection. The spores were revived by overnight culturing in potato dextrose broth (PDB) at 30 ± 1 °C. The suspension was then cultured on potato dextrose agar (PDA) plates at 30 ± 1 °C for 96 hours. The spore suspension was prepared by filtration of the liquid obtained after washing the plates with sterilized distilled water and stored at 4 ± 1 °C.

Inoculum preparation and immobilization of *R. oryzae*

The inoculum for free mycelial fermentation was prepared by culturing 1% (v/v) of spore suspension in growth medium composed of 5 glucose, 2 yeast extract, 0.6 KH_2PO_4, 0.25 $MgSO_4 \cdot 7H_2O$ and 0.088 $ZnSO_4.7H_2O$ (gL^{-1}) for 48 hours at 30 ± 1 °C. The preparation of the inoculum for the immobilized fermentation was as follows: 200 polystyrene foam cubes, a readily

available packaging waste, of 1 cm in dimension were broke down to individual beads and used as the support material for immobilization. The sterile beads were prepared by washing with 70% (v/v) ethanol, followed by UV sterilization and dried at room temperature in the laminar hood. The beads were then transferred to 100 ml growth medium (in 500 ml flasks), followed by the addition of spore suspension and cultured at 30 ± 1 °C for 48 hours. After immobilization, the beads were washed with sterilized water to remove free mycelia and transferred to the fermenter. A 5% (v/v) inoculum size was used for the free mycelial fumaric acid production fermentation.

Fumaric acid production in 4 L fermenter

The fermentation was performed in Minifors fermenter (INFORS HT, Basel, Switzerland) containing 2.5 L of production media at 30 ± 1 °C and aeration of 1 vvm. When the fermentation was performed in batch mode APUS was provided as the substrate for fumaric acid production. 100ml of 25 % (v/v) of molasses solution was provided as feed after 24 hours, upon operation of the fermentation in fed-batch mode. 25 g/L of calcium carbonate was used to maintain the fermentation pH at 6.

Analytical techniques

Samples were taken at regular intervals and the following analyses performed.

Reducing sugar and fumaric acid quantification

Spectrophotometric quantification of total reducing sugar and fumaric acid was performed using a UV-VIS Cary 50 Spectrophotometer. The total reducing sugar concentration of the samples collected was quantified using 3,5-dinitrosalicylic acid (DNS) assay. The fumaric acid was recovered by acidification of the samples at 90°C. 5N sulphuric acid was used for the acidification and the fumaric acid quantified as per the method described by Das and Brar (2014).

Fourier transform infrared (FT-IR) spectrometric analysis

Nicolet iS50 FT-IR equipped with Smart™ iTX accessory and OMNIC FT-IR software (ThermoFisher Co., Boston, MA, USA) was used to record the FT-IR spectrum of the fumaric acid obtained in the middle infrared region (4000 cm^{-1} to 400 cm^{-1}). The spectrum was recorded in absorbance mode with a resolution of 4 cm^{-1} in and 16 scans at room temperature.

Statistical Analysis

The data is presented as mean value of duplicates along with the standard deviation.

Results and Discussion

Fermentation mediated by immobilized of *R. oryzae* has been adopted as a strategy to improve the fumaric yield, overcome the operational difficulties during industrial scale fermentation as well as the impact of mycelial pellet size on fumaric acid production (Ilica et al., 2018; Naude & Nicol, 2017; Zhou et al., 2011). It has also been observed that the mycelial pellet size needs to be optimal to prevent oxygen limitations and under such conditions higher degree of ethanol is produced as the main by-product (Roa Engel et al., 2011). Therefore, to overcome these problems, polystyrene foam bead was identified as the suitable immobilization support material in our previous work (at flask scale). The immobilized fungi mediated fermentation provided for a three-fold increased fumaric acid titers when the APUS media was supplemented with molasses (Sebastian et al., 2021). Most often the immobilized fungi mediated fermentation require the use of specialized bioreactors for fumaric acid production as designed by Naude and Nicol (2017). The strategy of immobilization on polystyrene beads or similar materials would allow for the use of regular fermenters for fumaric acid bioproduction and was investigated in the present work with the fumaric acid titers obtained presented in Figure 2.2.1.

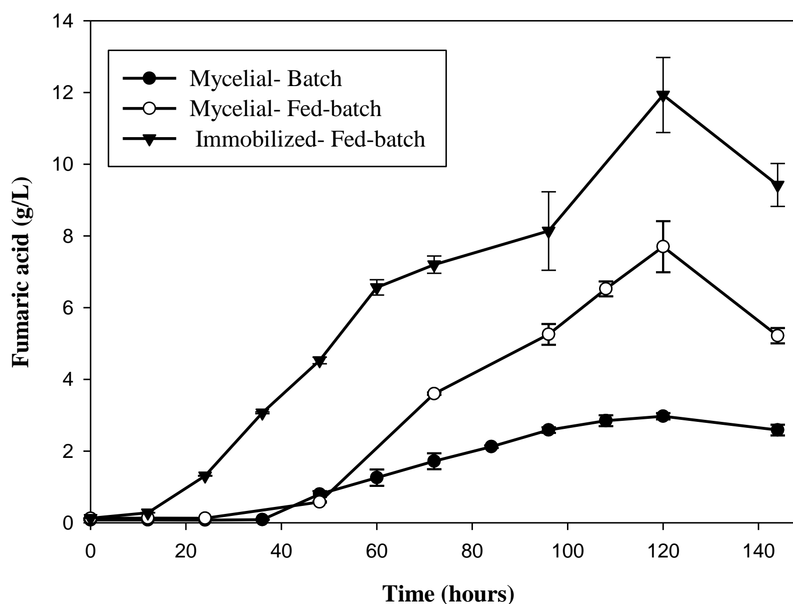

Figure 2. 2. 1. Difference in fumaric acid concentrations achieved during fermentations operated in batch and fed-batch mode

The free mycelial fermentation when operated in batch mode, with APUS as the substrate, yielded a maximum fumaric acid titer of 2.9 ± 0.1 g/L. This fumaric acid titer was in itself a seventy-percentage improvement over the concentration obtained at flask scale in our previous work. The supplementation of the free mycelial fermentation with molasses solution (25 %, v/v) led to a 2.5-fold increase and a concentration of 7.7 ± 0.7 g/L was achieved. However, a lag phase of nearly 36 hours in fumaric acid production was also observed. The fumaric acid concentration achieved during the free mycelial fermentation was improved further by carrying out the fermentation using the immobilized fungi. The immobilization led to an increased fumaric acid titer of 11.9 ± 1.05 g/L and this corresponds to an additional fifty-five-percentage improvement over the concentration obtained for the free mycelial fermentation. This fumaric acid titer corrersponds to a four-fold improvement over the free-mycelial fermentation performed using APUS as the sole substrate. Moreover, the lag of fumaric acid production was reduced significantly to nearly 12 hours from

36 hours (for free mycelial fed-batch). This was further confirmed by calculating the productivity and yield for the first 24 hours of fermentation (Table 2.2.1). Additionally, it was observed that volumetric productivity was improved profoundly during the later stages by immobilizing the fungi and operating the fermentation in fed-batch mode.

Table 2. 2. 1. Comparison of fumaric acid yield and productivity during fermentation

Fermentation mode	Time (Hours)	Fumaric acid produced (g/L)	Productivity ($gL^{-1}h^{-1}$)	Yield (g/g_{TRS})
Free mycelial-Batch	0 - 24	0.0	0.0	0.0
	48 - 120	2.9 ± 0.1	0.04	0.62
Free mycelial-Fed batch	0 - 24	0.0	0.0	0.0
	48 - 120	7.7 ± 0.7	0.064	0.42
Immobilized- Fed batch	0 - 24	1.31 ± 0.01	0.055	0.43
	48 - 120	10.6 ± 1.05	0.15	0.66

The observation of drastic improvement in the fumaric acid titers and its productivity as well as yield was in accordance to that of previous investigations. However, the previous investigations were performed at flask scale and the increased titers were attributed to the increased surface area and reduced biofilm thickness afforded by the use of supports to immobilize the fungus (Das et al., 2015; Gu et al., 2013). The use of polystyrene beads allows for increased surface area for fungal attachment and growth, thereby leading to a thinning of the fungal biofilm. Hence, potentially providing for a better oxygen and nutrient transfer. It has been observed that the formation of a thick biofilm causes oxygen and nutrient limitations (Wang et al., 2010; Skory et al., 1998). The FT-IR spectroscopy showed that the fumaric acid obtained via fermentation of the agro-industrial residues was highly similar to the commercial fumaric acid (represented in Figure

2.2.2). Thus, yielding a sustainable bioproduction process using renewable residues as the feedstock.

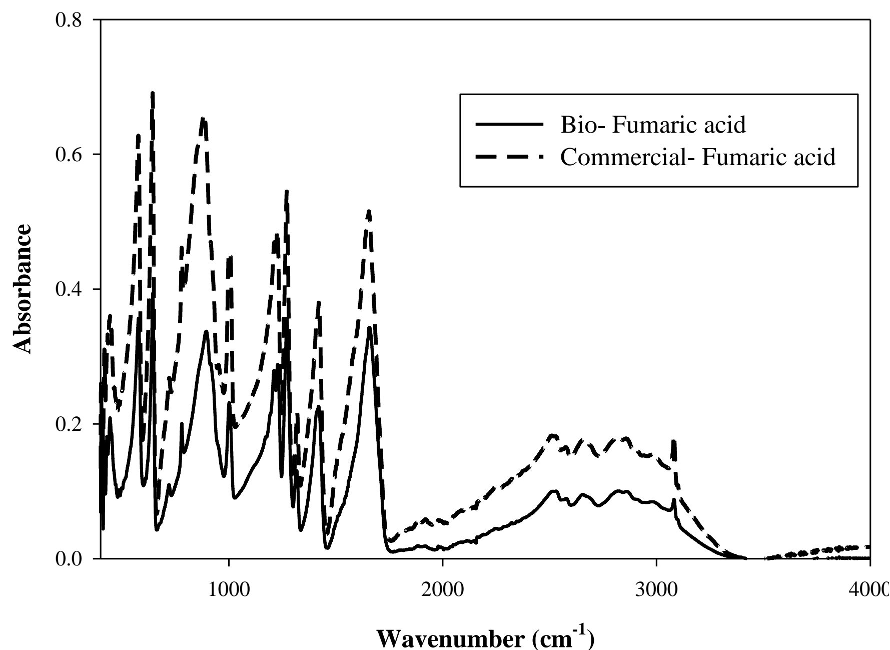

Figure 2. 2. 2. Biobased fumaric acid and commercial fumaric acid FT-IR spectrum

Conclusion

The current investigation in fermenter using immobilized *R. oryzae* has shown that renewable feedstocks such as agro-industrial sources can be used effectively for fumaric acid bioproduction. The fungus immobilization led to a four-fold increase in fumaric acid titer to achieve a concentration of nearly 12 g/L. Moreover, immobilization afforded a significant reduction in lag phase of production from 36 hours to nearly 12 hours. The operation of fermentation in fed-batch mode as well as immobilization improved both yield and productivity. Hence, providing a sustainable strategy for bioproduction of fumaric acid.

Acknowledgments

The authors are sincerely thankful for the financial support of NSERC (Discovery Grants Program) and the Ministère des Relations Internationales du Québec (coopération Paraná-Québec 2010–2012). Likewise, support from James and Joanne Love Chair in Environmental Engineering at York University is also appreciated. The views and opinions expressed in this review are those of the authors.

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LINK -2

LINK: The use of industrial residues might lead to restriction of the use of the biobased fumaric acid for pharmaceutical applications. To this effect, the use of high biomass yielding grasses and hemp has been proposed and evaluated in the next sub-objective.

PART 3

Miscanthus sp. – a perennial lignocellulosic biomass as feedstock for fumaric acid bioproduction

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Industrial Crops and Products, 175, 114248. doi.org/10.1016/j.indcrop.2021.114248

Résumé

Le travail présenté étudie l'utilisation potentielle de la biomasse lignocellulosique comme matière première pour la production d'acide fumarique ainsi que l'identification de la souche idéale de *Rhizopus oryzae* pour la bioproduction. A cet effet, des graminées pérennes, le silvergrass et le switchgrass, ainsi que le chanvre ont été soumis à un prétraitement alcalin et à une saccharification enzymatique. L'hydrolysat ainsi obtenu a été utilisé comme matière première pour la production d'acide fumarique. Le silvergrass a été identifié comme la biomasse lignocellulosique idéale en raison de sa concentration plus élevée en sucres réducteurs de 39,5 g/L ainsi que de son rendement de 0,79 g/g de biomasse prétraitée. L'utilisation de l'hydrolysat pour la production d'acide fumarique, par trois souches de *R. oryzae*, à savoir NRRL 1526, NRRL 6400 et NRRL 2582, a permis d'obtenir des concentrations d'acide biosynthétique de 8 à 9 g/L. Parmi elles, la souche NRRL 1526 a été identifiée comme la meilleure souche pour la bioproduction de l'acide organique, offrant ainsi une nouvelle avenue durable pour la production d'acide fumarique.

Mots clés: Lignocellulose; acide fumarique; bioproduction; lignite; bioraffinage

Abstract

The work presented here investigates the potential use of lignocellulosic biomass as the feedstock for fumaric acid production as well as identify the ideal *Rhizopus oryzae* strain for bioproduction. To this effect, alkaline pretreatment of the biomass from perennial grasses, silvergrass and switchgrass, and hemp was performed followed by enzymatic saccharification. The hydrolysate so obtained was used as the feedstock for fumaric acid production. Silvergrass was identified as the ideal lignocellulosic biomass due its higher reducing sugar concentration of 39.5 g/L as well as a yield of 0.79 g/g of pretreated biomass. The use of the hydrolysate for fumaric acid production, by three *R. oryzae* strains namely NRRL 1526, NRRL 6400 and NRRL 2582, yielded biobased acid concentrations of 8 – 9 g/L. Among them the strain NRRL 1526 was identified as the best strain for the bioproduction of the organic acid, thereby providing a new sustainable avenue for fumaric acid production.

Keywords: Lignocellulose; fumaric acid; bioproduction; silvergrass; biorefining

Introduction

Fossil fuel, especially crude oil, acts as the main raw material for the production of a vast variety of chemicals and this dependence on an exhaustible resource is both unsustainable as well as detrimental to the environment. Hence, to overcome these drawbacks of the current industrial production alternate environmentally friendly platform chemicals have been proposed. One such green platform chemical that was identified by the US Department of Energy was fumaric acid (FA) (Dorsam et al., 2017). The unique properties afforded by the presence of a double bond in addition to the two carboxylic groups makes the C-4 acid suitable for the synthesis of numerous other chemicals as well as polymers. Additionally, the fungal species *Rhizopus oryzae* has been identified as a natural over-producer of fumaric acid via the reductive TCA metabolic pathway. The current production of fumaric acid is unsustainable as well as cause detrimental impacts on the environment as it relies on depleting petroleum resources (Rao Engel et al., 2011). Hence, the alternate strategy of bioproduction provides for a sustainable and eco-friendly process to satisfy the future demands for a key organic acid.

The processes of bioproduction of fumaric acid developed so far mainly relies on the use of glucose as the carbon source (Naude and Nicol, 2017; Gu et al., 2013; Xu et al., 2013). This dependence on the carbon source has a profound effect on the feasibility of the bioproduction strategy and has been one of its key limitations. Hence, the use of alternate carbon sources is highly desirable and to this effect and lignocellulosic biomass provides an attractive alternative. This shift to lignocellulosic biomass as feedstock for fumaric acid bioproduction has the potential to greatly improve the feasibility of the process as well as not contributing to the "food or fuel" debate (Ochsenreither et al., 2014).

Lignocellulosic biomass, made up of cellulose, hemicellulose and lignin, is the most abundant renewable resource with lignin and lignin-derived inhibitors considered to be the key component that hinders enzymatic hydrolysis as well as microbial growth (Dörsam et al., 2017; Karunanithy and Muthukumarappan, 2011). Pre-treatment of lignocellulosic material is essential to disrupt the cell wall and improve accessibility for enzymatic or microbial break down of cellulose and hemicellulose. This can be achieved by either physico-chemical (dilute acid, alkali, organosolv, steam explosion) or biological (microbial and enzymatic) methods. Among these pretreatment strategies dilute acid, alkali and steam explosion have been explored extensively (Kapoor et al., 2015; Kumar et al., 2009). Severing of the ester bonds in hemicellulose, lignin and hemicellulose solubilization and decrystallization of cellulose and partial solubilization of hemicellulose, can be achieved by alkali treatment. On the other hand, dilute acidic treatment leads to the loss of rigidity, hemicelluloses solubilization and hexose and pentose sugars separation (Brodeur et al., 2011; Pu et al., 2013; Silverstein et al., 2007). Additionally, acidic treatment is effective in cleavage of cellulose fibers to release glucose and hence can be used for the direct release of sugars from cellulosic fibers (Palme et al., 2016; Lacerda et al., 2013).

The shift towards the use of lignocellulosic biomass as the feedstock for biofuel and biochemical production would require large quantities of the biomass. For example, 408–870 Mg day^{-1} of biomass would be required to produce 75 ML per year ethanol (Digman et al., 2010). A similar biomass requirement can be envisaged for bioproduction of platform chemicals. To this effect, perennial plants, such as switchgrass (*Panicum virgatum*) and silvergrass (*Miscanthus* sp.), can be used as feedstock. These grass varieties are fast growing and yield 10–30 tonnes of dry matter/hectare (Sun et al., 2011; Guo et al., 2008). Moreover, some of these are considered as invasive species. Hence, offer an attractive alternative to the use of hardwood and softwood as the

renewable biomass source. Additionally, in recent years there has been an increased interest in the use of the annual crop hemp (*Cannabis sativa*) as a bioenergy source (Prade et al., 2011). The high land use efficiency provides for the potential use of these renewable biomasses for bioproduction of platform chemicals such as fumaric acid.

To this effect, the suitability of the biomass for platform chemical bioproduction needs to be investigated. In the present paper, the lignocellulosic biomass (silvergrass, switchgrass and hemp fiber) was subjected to alkaline pretreatment for delignification and further subjected to enzymatic saccharification. The biomass hydrolysate thus obtained was used as feedstock for fumaric acid production using three strains of the fungi *Rhizopus oryzae* (NRRL 1526, NRRL 6400 and NRRL 2582) and the best strain for bioproduction using lignocellulosic biomass as feedstock was identified.

Materials and methods

Fungal strains and spore suspension preparation

Lyophilized spores of the three natural FA over-producing strains of *R. oryzae*, namely NRRL 1526, NRRL 2582, NRRL 6400, was received from the culture collection of agricultural research service (ARS). Fungal suspension cultures of the strains prepared by transferring the lyophilized spores to potato dextrose broth (PDB), followed by incubation overnight at 30 ± 1 °C. The suspension was then transferred on to potato dextrose agar (PDA) plates and incubated at 30 ± 1 °C for 96 hours. The PDA plates were washed with sterilized distilled water and the suspension filtered through sterile cotton wool to obtain the spore stock suspension. The spore concentration of the suspension was maintained at 1×10^7 spores per mL and stored at 4 °C.

Biomass hydrolysate preparation

Alkaline pre-treatment of lignocellulosic biomass

Dried lignocellulosic biomass, silver grass, switchgrass and hemp fiber, was milled in a domestic blender. The biomass was then subjected to delignification under alkaline conditions at 120 ℃ for 30 minutes. About 2% NaOH (w/v) and biomass to alkali ratio of 1:10 (w/v) were used for delignification. The pretreated biomass was then recovered from the slurry at 12,000 x g for 15 min, washed to neutral pH using deionized water and dried at 60 °C.

Enzymatic saccharification

The enzymes, Cellic-Ctec (cellulase blend) and Viscozyme-L, purchased from Sigma Aldrich was used for the saccharification of the alkali pre-treated biomass. Cellic-Ctec (cellulase blend):Viscozyme-L enzyme mixture (1:1) and a biomass loading ratio of 1:20 was used for saccharification. The activity of the enzyme mixture was 40 FPU/mL. About 50mM citrate buffer (pH 4.8) was used for the saccharification carried out at 50 ℃ for 24 hours. The reaction mixture was then centrifuged at 12,000 x g, and hydrolysate obtained stored at -20 ℃. The ideal biomass was then identified based on total reducing sugar (TRS) yield. This biomass hydrolysate was then used as the feedstock for FA production.

FA production fermentation and identification of best *R. oryzae* strain

The inoculum for FA production was obtained by culturing the spores on synthetic media, made up of 5 glucose, 2 urea, 0.6 KH_2PO4, 0.25 $MgSO_4{\cdot}7H_2O$ and 0.088 $ZnSO_4.7H_2O$ (g/L), at 30 °C, pH 6 and 200 rpm for 24 hours. The pre-inoculum so obtained was then used to produce the inoculum by transferring 5 % (v/v) to the biomass hydrolysate, containing 2 yeast extract, 0.6 KH_2PO_4, 0.25 $MgSO_4{\cdot}7H_2O$ and 0.088 $ZnSO_4.7H_2O$ (g/L), and cultured for 24 hours. Fumaric

acid production was performed (in duplicates) in 500 ml Erlenmeyer flasks containing 100 ml production medium at 30 °C, pH 6 and 200 rpm. This production media was made up of biomass hydrolysate supplemented with 0.6 KH_2PO_4, 0.25 $MgSO_4 \cdot 7H_2O$, 0.088 $ZnSO_4.7H_2O$, 0.5 yeast extract and 25 calcium carbonate (g/L). Analysis of variance (ANOVA) on the quantity of FA produced during fermentation by the three strains of *R. oryzae* was performed to determine the ideal strain based on the *p*-value obtained.

Sampling and analytical techniques

Samples of biomass hydrolysate and fermentation were collected and the total reducing sugar and fumaric acid concentration quantified as follows.

Total reducing sugar (TRS)

The concentration of TRS was quantified using di-nitrosalicyclic acid (DNS) assay for the samples obtained following alkaline pretreatment and enzymatic saccharification as well as during the fermentation. The yield of TRS obtained during the sequential pre-treatment of biomass represented using the equation 1 below:

$$\text{TRS yield} = (\text{TRS concentration (g/ml)} * \text{volume (ml)}) / \text{Weight of biomass (g)} \qquad \text{Equ 1}$$

Fumaric acid quantification

The fermentation samples collected at regular intervals was acidified with 5 N sulfuric acid at 90 °C. The fumaric acid so released quantified using the spectrophotometric method provided by Das and Brar (2014).

FTIR analysis

FTIR spectra in the absorbance mode for the middle infrared (4000 cm^{-1} – 400 cm^{-1}) was recorded with a resolution of 4 cm^{-1} and 16 scans. Nicolet iS50 FT-IR equipped with Smart™ iTX accessory and OMNIC FT-IR software (ThermoFisher Co., Boston, MA, USA) was used to obtain the spectra. The FT-IR spectra of biobased fumaric acid was compared against commercial fumaric acid (ACROS Organics, Geel, Belgium) as well as lignocellulosic biomass against biomass obtained after alkaline pre-treatment.

Statistical analysis

Minitab 19 was used to perform ANOVA on the experimental data. The experiments were conducted in duplicates and the mean values presented with the standard deviation.

Results and discussion

Alkaline pretreatment of lignocellulosic biomass

Lignocellulosic biomasses, made up of cellulose, hemicellulose and lignin, are recalcitrant in nature. Hence, pretreatment of lignocellulosic biomass is performed to remove lignin and hemicellulose and reduce the crystallinity of cellulose, thereby allowing for better release of sugars by hydrolysis (Brodeur et al., 2011; Kumar et al., 2009; Yang and Wyman, 2008). With these objectives in mind, the lignocellulosic biomasses used in the current study was subjected to alkaline pretreatment in presence of 2% (w/v) sodium hydroxide at 120 °C. Sodium hydroxide is the most widely used alkali for biomass pretreatment (Karunanithy and Muthukumarappan, 2011). This alkali treatment causes the degradation of ester and glycosidic linkages leading to lignin and hemicellulose removal, cellulose swelling and it's partial decrystallization. Thus, allowing for better accessibility for the enzymes to cellulose and hemicellulose (Cheng et al., 2010; Brodeur et

al., 2011). The percentage biomass recovered after NaOH mediated alkaline pretreatment is represented in Figure 2.3.1.

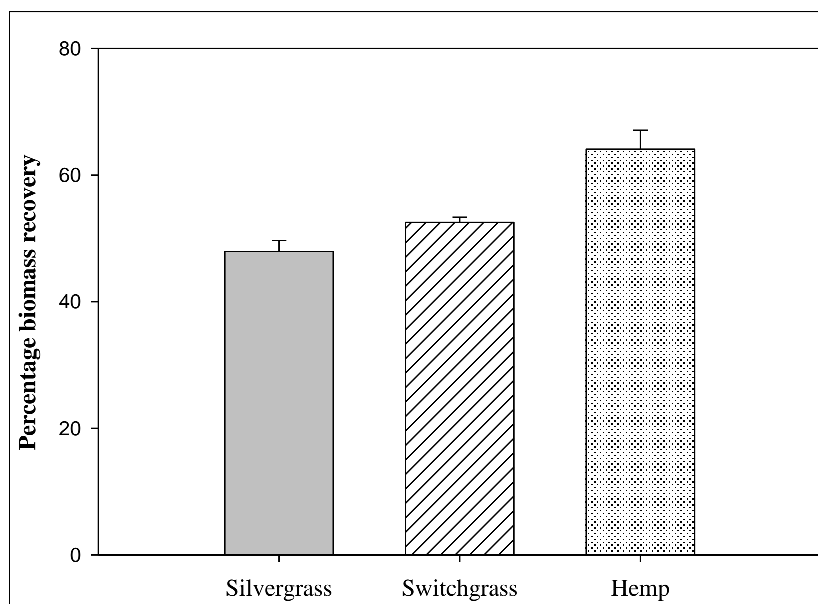

Figure 2. 3. 1. Percentage biomass recovered after alkaline pretreatment

Highest degree of solid loss was obtained for silvergrass whereas the least amount of solid loss was observed for hemp. A 52.07 ± 1.7 % (w/w) degree of delignification and hemicellulose removal was obtained for silvergrass whereas 47.5 ± 0.8 % (w/w) and 35.9 ± 3.0 % (w/w) was observed for switchgrass and hemp respectively. The biomass recovery of 48 % (w/w) after alkaline pretreatment is close to the cellulose content of 44.5 % (w/w) reported for silvergrass. Also, the degree of delignification and hemicellulose removal was observed to be in accordance to the combined lignin and hemicellulose (26.2 % hemicellulose, 26.5 % lignin) content reported for switchgrass (Loow et al., 2016; Kang et al., 2013). It was also observed that the percentage biomass recovered after the pretreatment to be within the range of cellulose content of 53-91% reported for hemp fiber (Liu et al., 2017). Additionally, this biomass recovery was close to the

cellulose content of 64.2 % reported previously (Zommere et al., 2013). Furthermore, the solid loss of 47.5 % obtained for switchgrass in the current study was significantly higher than the 25 % reported by Isci et al., 2008. On the other hand, this obtained solid loss was within the range of 25% and 57 % obtained for microwave assisted pretreatment of switchgrass in presence 1% NaOH (for 5 min) and 3 % NaOH (for 20 min) respectively (Keshwani and Cheng, 2010).

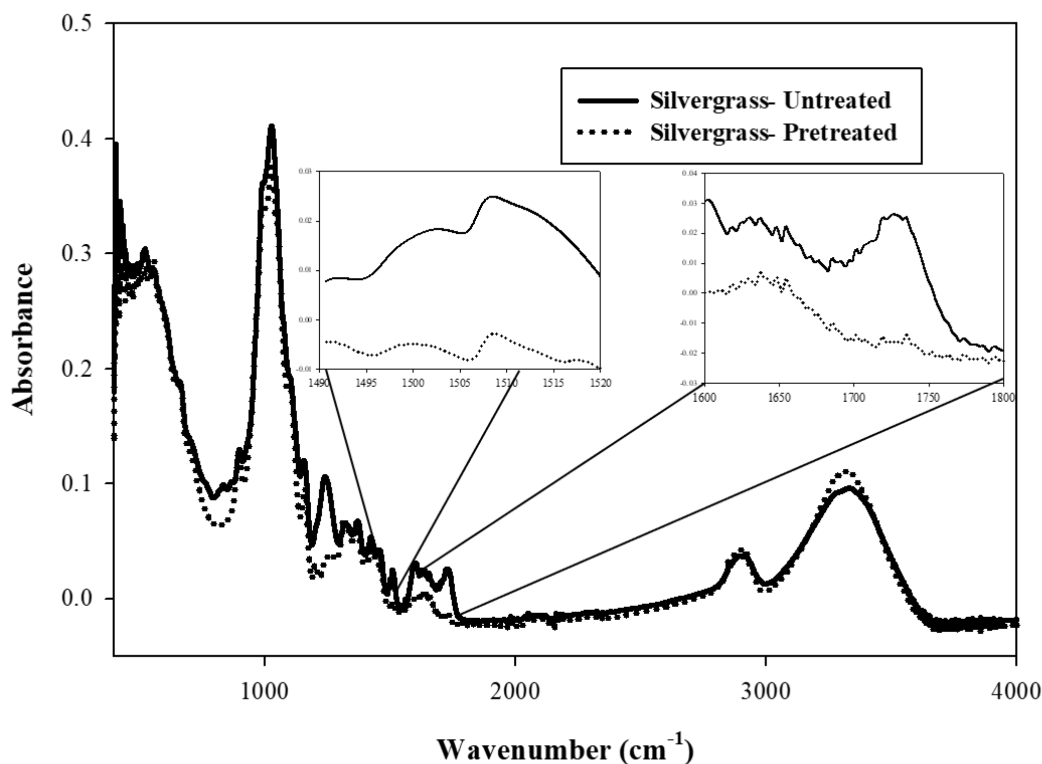

Figure 2. 3. 2. FTIR spectrum of silvergrass- untreated and pretreated

The effectiveness of the alkaline pretreatment of the lignocellulosic biomass was further confirmed by subjecting the pretreated biomass to FT-IR analysis and presented in Figure 2.3.2. The FT-IR spectrum showed the typical absorption peaks at 3300 cm^{-1}, 2900 cm^{-1}, 1610 cm^{-1} and 1420 cm^{-1} corresponding to cellulose. Profound decrease in the intensities were observed at peaks 1510 cm^{-1}, corresponding to aromatic skeletal vibration, and1730 cm^{-1}, corresponding to the carbonyl group contained in hemicellulose (Wawro et al., 2019; Shi et al., 2012). These observations confirm

effective delignification and hemicellulose removal from the silvergrass biomass during the alkaline treatment.

Enzymatic saccharification and selection of biomass hydrolysate for fumaric acid production

The solids recovered after pretreatment of biomass was subjected to enzymatic saccharification and the concentration of reducing sugar and yields presented in the Table 2.3.1. TRS concentrations of 39.5 g/L was obtained for silvergrass which was higher than the 34.5 g/L and 30.3 g/L obtained for switchgrass and hemp respectively. Additionally, silvergrass provided for the highest sugar yield (0.79, $g/g_{pretreated\ biomass}$) from the biomass recovered after alkaline pretreatment. Hence, silvergrass was chosen as the feedstock for fumaric acid bioproduction. A comparison of the TRS yields from untreated biomass showed that silvergrass and hemp provided for a similar yield, 0.380 and 0.388 ($g/g_{lignocellulose}$) respectively. The lowest yield was obtained when switchgrass was subjected to enzymatic saccharification.

The TRS sugar concentration of 39.5 g/L achieved for silvergrass is similar to the maximum glucose concentration (40 g/L) achieved during simultaneous saccharification and fermentation of silvergrass biomass subjected to alkaline pretreatment (Gao et al., 2020). Also, this concentration was higher than the maximum TRS concentration achieved (22.4 g/L) by microbial digestion of cellulose obtained after pretreatment of silvergrass (Kriger et al., 2020). Moreover, the yield of 0.38 ($g/g_{lignocellulose}$) obtained is similar to the 0.41 ($g/g_{lignocellulose}$) reported for silvergrass (Yasuda et al., 2012). A similar observation was also made in the case of hemp fiber, where the sugar concentrations obtained in the current study were in accordance to previously reported values (Wawro et al., 2020; Gunnarsson et al., 2015). On the contrary, the sugar recovery of 0.32 $g/g_{lignocellulose}$ is lower than the range of 35 – 45 % reported for switchgrass (Karunanithy and Muthukumarappan, 2011).

Table 2. 3. 1 Total reducing sugar concentration and yield after enzymatic saccharification of pretreated biomass

Pretreated Biomass	Total reducing sugar (g/L)	Yield ($g/g_{pretreated\ biomass}$)	Yield ($g/g_{lignocellulose}$)
Silvergrass	39.5 ± 0.3	0.791	0.380
Switchgrass	34.5 ± 0.03	0.690	0.318
Hemp	30.3 ± 0.5	0.606	0.388

Fumaric acid production and selection of ideal strain of *R. oryzae*

Three natural fumaric acid overproducer strains of *R. oryzae* were compared for their fumaric acid producing capacity on silvergrass biomass hydrolysate. The fumaric acid production and total reducing sugar consumption is presented in Figure 2.3.3 and the comparison of the yield/productivity presented in Table 2.3.2. The fermentation performed using *R. oryzae* NRRL 1526 yielded a maximum fumaric acid titer of 8.95 ± 0.05 (g/L) after 48 hours of fermentation (p value = 0.00). While the fermentations with NRRL 6400 and NRRL 2582 yielded maximum fumaric acid titers of 8.07 ± 0.42 g/L (after 72 hours, $p < 0.05$) and 9.26 ± 0.1 g/L (after 120 hours, $p < 0.05$) respectively. The ANOVA performed on this concentration confirmed that these values were significantly different (p value = 0.03). *R. oryzae* NRRL 1526 was identified as the best strain for fumaric acid production following comparison of the productivity and yield corresponding to the maximum fumaric acid concentration achieved during fementation. The strain provided for a productivity of 0.186 $gl^{-1}h^{-1}$ and yield of 0.529 g/g reducing sugar consumed. On the other hand, the corresponding yields and productivity for the other two strains were, 0.413 g/g and 0.112 $gl^{-1}h^{-1}$ (for NRRL 6400) and 0.321 g/g and 0.077 $gl^{-1}h^{-1}$ (for NRRL 2582), respectively. Such a comparison of *R. oryzae* strains and the identification of the best strain as well as the use of silvergrass biomass hydrolysate as feedstock for fumaric acid production has not been reported to the best of our knowledge.

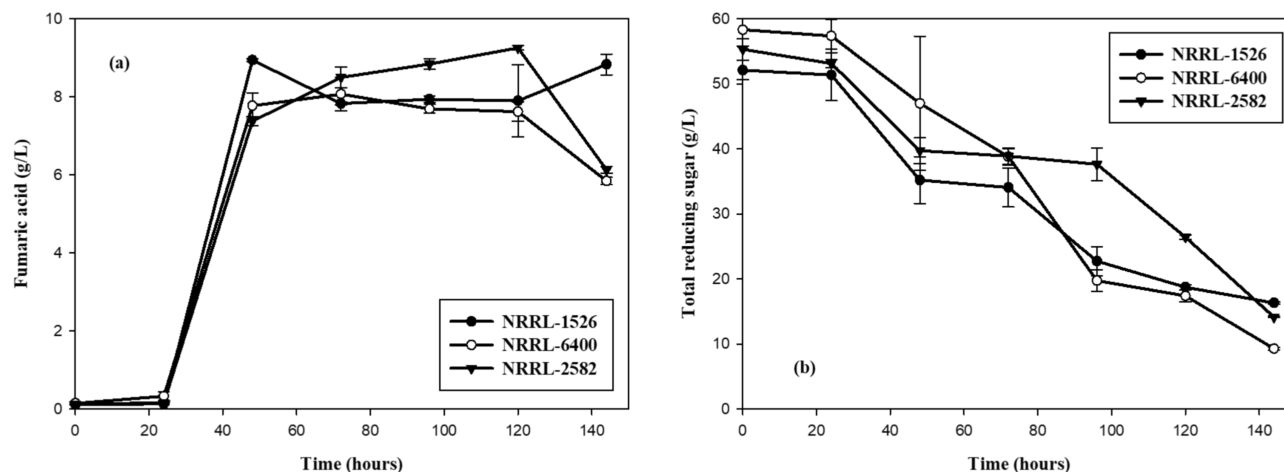

Figure 2. 3. 3.. Fumaric acid production (a) and total reducing sugar utilization (b) during fermentation using silvergrass hydrolysate as feedstock

Table 2. 3. 2 Comparison of maximum fumaric acid concentration, the corresponding productivity and yield

***Rhizopus oryzae* strain**	**Fumaric acid (g/L)**	**Productivity ($gL^{-1}h^{-1}$)**	**Yield ($g/g_{reducing\ sugar}$)**
NRRL 1526	8.95 ± 0.05	0.186	0.529
NRRL 6400	8.07 ± 0.42	0.112	0.413
NRRL 2582	9.26 ± 0.1	0.077	0.321

Lignocellulosic biomass has not yet been widely used as a feedstock for fumaric acid production when compared to bioethanol production. The observations of the current study show that perennial lignocellulosic biomass, such as silvergrass, has the potential to be used as feedstock for fumaric acid bioproduction. Although, the fumaric acid titers obtained in the current study was observed to be lower than the most of the previous studies where *R. oryzae* was used, as summarized by Ilica et al. (2019), but the productivity obtained were comparable to some of the previous works (Ilica et al., 2019). The fumaric acid titer obtained was considerably lower than the 23.94 g/L obtained upon the use of a mutated strain of *Rhizopus* RH-7-13-807 but comparable to 13.23 g/L obtained for the original strain. The researchers used food waste hydrolysate, by hot

water extraction, as the feedstock (Fan et al., 2020). Liao et al. (2008) reported fumaric acid titers ranging from 7.4 – 4.9 g/L upon the use of dairy manure hydrolysate as substrate. This titer was further enhanced by supplementation with pure glucose (Liao et al., 2008). A similar approach of supplementation or performing the fermentation in a fed-batch mode can be used to further improve the fumaric acid titers obtained in the current study. Such an approach has been observed to be effective in a previous work by our group, where apple juice industry residue was supplemented with sugar industry residue (molasses) (Sebastian et al., 2021). Fumaric acid concentrations ranging from 2 – 12.7 g/L was reported when cassava baggase enzymatic hydrolysate was used as substrate (Carta et al., 1999).

Conclusion

Silvergrass was identified as the ideal lignocellulosic feedstock for fumaric acid production, when compared to switchgrass and hemp, as it yielded 39.5 g/L reducing sugar with a 79 % enzymatic conversion of the biomass obtained after alkali pretreatment. The FT-IR spectrum revealed that this alkali treatment was effective in the removal of lignin and hemicellulose. About 8-9 g/L of fumaric acid titer was obtained upon the use of silvergrass hydrolysate as feedstock for bioproduction of the organic acid. Moreover, the *R. oryzae* NRRL 1526 was identified as the best strain for its higher productivity and yield. Hence, this provided for a sustainable and environmentally friendly biorefining alternative for fumaric acid production.

Acknowledgement

The authors are sincerely thankful for the financial support of NSERC (Discovery Grants Program) and the Ministère des Relations Internationales du Québec (coopération Paraná-Québec 2010–

2012). Likewise, support from James and Joanne Love Chair in Environmental Engineering is also appreciated. The views and opinions expressed in this review are those of the authors.

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CHAPTER 3: Value addition and extraction of high value compound from the fungal biomass

LINK -3

LINK: The investigations in Chapter 2 had shown that high fumaric acid yields can be achieved during fermentation using renewable resources as feedstock. However, one of the restrictions to the industrial scale bioproduction is the low market value, $ 1 kg^{-1}, of fumaric acid. Hence, value addition by conversion of fumaric acid to fumaric acid esters have been proposed. This value addition could have a profound impact on the economic competitiveness and feasibility of the bioproduction process due to the high market price for the esters. Furthermore, one of the key by-products of fumaric bioproduction is the generation of *Rhizopus oryzae* biomass. This biomass could be used as source of fungal chitosan which is compound of high market value. The combined use of strategies of value addition and extraction of high value compound could have a profound impact on the bioproduction process.

PART 1

Biobased dimethyl fumarate and monomethyl fumarate synthesis

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Process Biochemistry (Under review)

Résumé

Les esters d'acide fumarique (EAF) tels que le fumarate de monométhyle (FMM) et le fumarate de diméthyle (DMF) sont des ingrédients pharmaceutiques actifs (API) utilisés dans le traitement du psoriasis et de la sclérose en plaques. Le présent travail étudie l'utilisation d'acide fumarique biosourcé pour la production d'EAF. L'effet du temps, de la température et de l'acide sulfurique comme catalyseur sur la conversion de l'acide fumarique en EAF a été élucidé. Les conditions optimales pour la synthèse des EAF ont été identifiées, en utilisant la méthodologie de surface de réponse, comme étant une incubation de 111 minutes avec une concentration d'acide sulfurique de 9,7% (v/v) à 98 °C. L'utilisation d'acide fumarique biosourcé permet d'utiliser des ressources renouvelables comme matière première primaire pour la synthèse des EAF à la place des ressources pétrolières en voie d'épuisement. Cette valeur ajoutée peut avoir un impact positif sur la faisabilité technico-économique d'un procédé de bioproduction d'acide fumarique, et contribuer à rendre la synthèse d'un API durable

Mots clés: Fumarate de diméthyle; fumarate de monométhyle; acide fumarique; biosourcé; esters d'acide fumarique

Abstract

Fumaric acid esters (FAEs) such as monomethyl fumarate (MMF) and dimethyl fumarate (DMF) are active pharmaceutical ingredients (API) used in the treatment of psoriasis and multiple sclerosis. The present work investigates the use of biobased fumaric acid for the production of FAEs. The effect of time, temperature, and sulphuric acid as a catalyzer on the conversion of fumaric acid to FAEs was elucidated. The optimal conditions for DMF synthesis were identified, using response surface methodology, to be 111 minutes incubation using 9.7% (v/v) sulphuric acid concentration at 98 °C. The use of biobased fumaric acid allows the use of renewable resources as the primary feedstock for the synthesis of FAEs in place of depleting petroleum resource. This value addition can have positive impact on the technoeconomic feasibility of a process towards bioproduction of fumaric acid, as well as contribute to rendering the synthesis of an API sustainable.

Keywords: Dimethyl fumarate; monomethyl fumarate; fumaric acid; biobased; fumaric acid esters

Introduction

Fumaric acid esters (FAEs), monomethyl fumarate (MMF) and dimethyl fumarate (DMF) are methyl esters of fumaric acid, a naturally occurring organic acid. In recent years, these compounds have garnered a huge interest due to their pharmaceutical applications for the treatment of psoriasis and multiple sclerosis (Lima et al., 2020; Sator et al., 2019; Landeck et al., 2018). DMF was first introduced in the market in 1994, Fumaderm®, and has been the primary drug for the treatment of psoriasis (Lehmann et al., 2007). Following the identification of its effectiveness against multiple sclerosis, an autoimmune disorder, Tecfidera® was launched for the treatment of relapsing-remitting multiple sclerosis (RRMS) in adults (Dede et al., 2021; Smith et al., 2017; Mills et al., 2018).

The DMF provided as the drug is metabolized to MMF and hence considered to be the active compound (Angiari & O'Neill, 2018; Nibbering et al., 1993). The exact mechanism of action of FAEs is not known but considered immunomodulatory. However, it is stipulated that the administration of DMF and MMF causes reduced expression of micro-RNA-21 which is essential for the production of pathogenic cells (Lima et al., 2020). Additionally, it has been reported that the immunomodulatory properties of DMF are rendered by the inhibition of glycolysis via post-transcriptional modification of glyceraldehyde 3-phosphate dehydrogenase (GAPDH), thereby blocking immune cell activation (Angiari & O'Neill, 2018). In addition to its use as an immune modulator, FAEs have been identified as a key ingredient in the manufacturing of scaffolds for tissue engineering applications due to their flexibility, cross-linking nature and biodegradability (Das et al., 2016).

The multitude of applications for FAEs has led to a surge in the demand for the chemical. The current manufacturing process involves the catalytic conversion of maleic anhydride or dimethyl

maleate or fumaric acid to the ester form via isomerization or Fisher esterification (Love and Bennett, 2017). This reliance on the petroleum-derived maleic anhydride for the production of FAEs to meet the future demand is unsustainable due to depleting petroleum resources. Moreover, the production of maleic anhydride and fumaric acid from petroleum leads to toxic as well as greenhouse gas emissions (Ilica et al., 2018; Roa Engel et al., 2011). In this sense, the shift to the use of biobased fumaric acid as a renewable feedstock for the production FAEs has the potential to render the production process sustainable and environmentally friendly.

In recent years, there has been a revival of interest in bioproduction of fumaric acid especially using renewable feedstocks. One of the main microorganism that has been identified as a natural over producer of the acid is the fungal species Rhizopus oryzae (Roa Engel et al., 2008). This shift towards biobased fumaric acid would allow for the use of renewable resources, such as lignocellulosic biomass and agro-industrial residues, as the primary feedstock for the synthesis of FAEs. Nonetheless, the conversion of this bio-derived fumaric acid to its ester forms has not been studied further so far. Therefore, alleviating the detrimental effects as well as the limitations of the current strategies of FAE production.

The work performed here investigates the use of biobased fumaric acid as feedstock for the production of FAEs. To this purpose, the effect of temperature, time and acid concentration was tested on the conversion of fumaric acid to FAEs. The optimal conditions for the conversion were identified using response surface methodology (RSM) and validated using biobased fumaric acid. The value additon afforded by this conversion has the potential to profoundly impact the economic competitiveness of the fumaric acid bioproduction.

Materials and methods

FAEs synthesis experimental design and data analysis

Fumaric acid solution (0.25 M) and sulphuric acid (0.45 M) were prepared in methanol. Commercial fumaric acid (ACROS Organics, Geel, Belgium) was used to identify the optimal condition for the synthesis of FAEs.

Design expert software (Version 13) was used for the design of the experiment and analysis of the experimental data. The optimal conditions of conversion were identified using Box-Behnken design (three central points), with independent variables of time, temperature and sulphuric acid concentration. Moreover, analysis of variance (ANOVA) was performed to determine the effect of independent variables. RSM analysis using the regression model (Equation 1) was used to obtain the contour plots for the dependent variables of MMF and DMF.

$$y = \beta_0 + \sum_{i=1} \beta_i X_i + \sum \beta_{ii} X_i^2 + \sum_{i=1} \sum_{j=i+1} \beta_{ij} X_{ij} \qquad \text{Equation 1}$$

where, y is the predicted response, β_0 is the model intercept; β_i and β_{ij} are the coefficients of linear terms; β_{ii} is the coefficient of the quadratic term; X_i and X_j are the independent variables. Reaction mixtures were prepared and subjected to the conditions as per the experimental design, with the experiments performed in duplicates.

Fungal strain and culture conditions for spore suspension preparation

The lyophilized spores *R. oryzae* NRRL 1526 received from Northern Regional Research Lab (NRRL) was revived and cultured on potato dextrose agar (PDA) plates at 30 ± 1 °C for 96 hours. Thereafter, the filtered spore suspension stock was prepared by washing the plates with sterile water and stored at 4 ± 1 °C with a spore concentration of 1×10^7 spores per mL

Inoculum preparation, fumaric acid production and recovery

The inoculum was prepared by using a growth medium composed of (in g L^{-1}) 5 glucose, 2 urea, 0.6 KH_2PO_4, 0.25 $MgSO_4·7H_2O$ and 0.088 $ZnSO_4.7H_2O$ and incubation at 30 ± 1 °C for 24 hours, 200 rpm. 5 % (v/v) of the inoculum was transferred to the production media composed of 100 glucose, 0.6 KH_2PO_4, 0.25 $MgSO_4$, 0.088 $ZnSO_4·7H_2O$, 1 yeast extract and 50 calcium carbonate (in g L^{-1}) (Naude and Nicol, 2017; Gangl et al., 1990). The flasks were then incubated at 30 ± 1 °C for 1 week with the agitation of 200 rpm. The fumaric acid formed was recovered by acidifying the media with 5 N sulphuric acid at 95 °C. The calcium sulphate formed was removed by centrifugation at 8000 × g for 5 mins, and the supernatant was then cooled to 10 °C to allow for the crystallization of fumaric acid. Lastly, fumaric acid crystals were recovered via centrifugation at 8000 × g for 10 mins and dried at 60 °C.

High-performance liquid chromatography for DMF and MMF quantification

A Spectra system (Thermo Scientific, Massachusetts, USA) was used for the quantification of the esters. Acclaim organic acid column (150 mm length x 4 mm internal diameter, and 5μm pore size) was used at 35°C. As a mobile phase, an aqueous solution of methanesulfonic acid 2.5mM (in water): Acetonitrile, in a ratio of 90:10, was used, with a flow rate of 0.7 mL/min in isocratic mode and 40 μL of total injection volume. A UV detector (210 nm) was used to identify and quantify the FAEs according to the peak area and retention time of their respective standards.

FT-IR spectrophotometry

FT-IR spectra, in the absorbance mode, was recorded in the middle infrared region (4000 cm^{-1} to 400 cm^{-1}) with a resolution of 4 cm^{-1} for 16 scans at room temperature. The FT-IR spectrum of

the biobased DMF was obtained using Nicolet iS50 FT-IR equipped with Smart™ iTX accessory and OMNIC FT-IR software (ThermoFisher Co., Boston, MA, USA).

Results and discussion

Statistical studies and identification of optimal conditions for FAE of synthesis

The effect of temperature, time and sulphuric acid concentration, ranging from 50 – 100 °C, 30 – 120 minutes, 2 – 10 % (v/v) respectively, on FAE synthesis, were investigated. The optimal conditions for the maximum conversion of fumaric acid to its ester form was identified using Box-Behnken design. The experimental data was analyzed to elucidate the effect of the parameters on ester synthesis. The optimal conditions for conversion were identified based on the desirability value, as represented in Figure 3.1.1 and Figure 3.1.2. The results of ANOVA and the interactions are represented in Table 3.1.1.

Table 3. 1. 1. Effect of independent variables (A- Time; B- Sulphuric acid concentration; C- Temperature) on dependent variables (DMF and MMF concentration) (Significant values: $p < 0.05$)

Model	Dimethyl fumarate		Monomethyl fumarate	
	p	Effect	*p*	Effect
A	0.0014	5.590	0.2754	-2.862
B	0.0019	5.318	0.1563	-3.940
C	< 0.0001	10.798	0.0202	-8.061
AB	0.4723	-0.917	0.9767	0.096
AC	0.2147	1.702	0.7163	-1.221
BC	0.1397	-2.127	0.5125	-2.264
A^2	0.1116	-2.849	0.4941	-2.885
B^2	0.4001	-1.273	0.5374	2.429
C^2	0.0577	-3.143	0.8496	-0.678

Effect of parameters and optimal condition for monomethyl fumarate synthesis

Table 3.1.2 shows the effect of temperature, reaction time and sulfuric acid as a catalyzer on DMF and MMF conversion. Overall, the conversion of fumaric acid to MMF was affected primarily by temperature and the highest concentration of 27.8 g/L was obtained when conditions of 30 minutes,

75 °C and 2% sulphuric acid were used. Likewise, the result showed that an increase in temperature and sulphuric acid concentration led to a decrease in the conversion of fumaric acid to MMF. The experimental data showed that the use of lower temperatures of 50 °C and 75 °C contributed positively to the conversion of fumaric acid to MMF rather than DMF.

The ANOVA performed on the experimental data, presented in Table 3.1.2, based on Box-Behnken design showed that only temperature had a significant effect on the conversion of fumaric acid to MMF ($p = 0.02$). This effect was observed to be linear whereas sulphuric acid concentration and duration of treatment had no significant effect on MMF synthesis, p values 0.15 and 0.27 respectively. Moreover, a good degree of correlation was observed between the experimental data and the predicted response ($R^2 = 0.766$). Therefore, such a strategy of using lower temperatures for conversion can be adopted to channel fumaric acid towards MMF during esterification. The optimal conditions (55.5 °C, 2% (v/v) of sulphuric acid, and 30 minutes) for conversion have been represented in Figure 3.1.1 (a).

Table 3. 1. 2. DMF and MMF conversion using commercial fumaric acid

Treatments	Time (mins)	Sulfuric acid (% v/v)	Temperature (°C)	Dimethyl fumarate (g/L)	Monomethyl fumarate (g/L)
1	30	2	75	8.15	27.81
2	120	2	75	22.77	12.29
3	30	10	75	24.46	7.73
4	120	10	75	33.29	0.69
5	30	5	50	4.75	17.78
6	120	5	50	11.92	19.39
7	30	5	100	21.93	2.76
8	120	5	100	36.95	0.72
9	60	2	50	2.12	14.07
10	60	10	50	15.24	19.07

11	60	2	100	28.79	5.60
12	60	10	100	32.62	0.62
13	60	5	75	20.47	8.86
14	60	5	75	23.00	9.53
15	60	5	75	24.39	9.32

Effect of parameters and optimal condition for dimethyl fumarate synthesis

The use of conditions of 120 minutes, 5 % sulphuric acid concentration and 120 °C temperature yielded the highest concentration of DMF (36.9 g/L). The use of high temperature, sulphuric acid concentration and incubation promoted the conversion of fumaric acid to DMF. A near-complete conversion of fumaric acid to DMF was achieved upon the use of these conditions. The temperature was identified as the most important parameter that affects DMF synthesis, with a *p* value less than 0.0001, following ANOVA to interpret the effect of experimental conditions on DMF synthesis. This interaction was observed to be linear and to an extend quadratic ($p = 0.058$). Even though the temperature was the most important parameter, the other parameters of time and sulphuric acid concentration also had a significant impact on the conversion. The *p* values were calculated to be 0.0014 and 0.0019 for time and sulphuric acid respectively. Additionally, a high degree of correlation was observed for the experimental data and the predicted response ($R^2 =$ 0.98). The contour plot of the optimal conditions represented in Figure 3.1.1 (b).

The predicted optimal conditions following RSM analysis were identified to be 111 minutes, 9.7 % (v/v) and 98 ° C. This optimal temperature is close to the 105 °C identified as the suitable temperature for 99% conversion of fumaric acid to dimethyl fumarate by Lima et al., (2020) but the duration of incubation was significantly higher than the 16 minutes reported (Lima et al., 2020). This profound difference may be attributed to the continuous flow reactor of low volume used by

them whereas a single pot system was used in the current work. The low reaction volume distributed over a long tube would provide for a better heat transfer, thereby enabling better conversion of fumaric acid to DMF. The optimal duration of incubation for DMF identified using RSM analysis was significantly lower than the 8 hours used for the synthesis in a previous work (Tachibana et al., 2010).

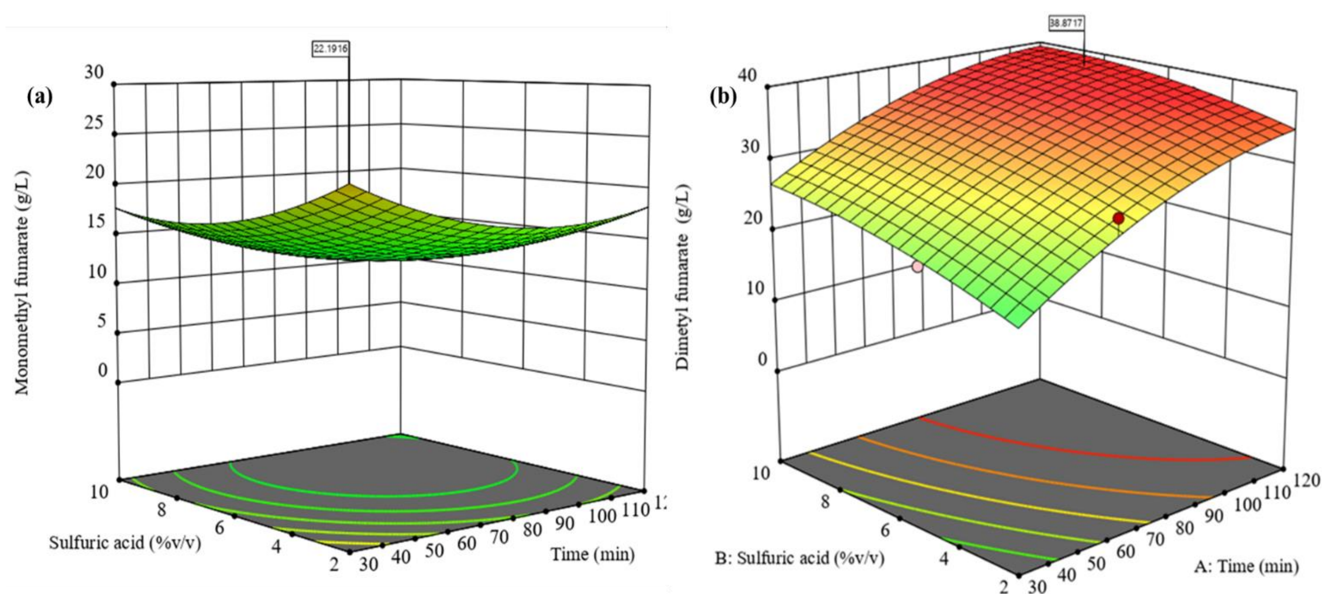

Figure 3. 1. 1. Desirability contour plot (a) Monomethyl fumarate (b) Dimethyl fumarate

Fumaric acid production and validation of optimal conditions

The validation of the optimal conditions predicted was performed using biobased fumaric acid as the feedstock for MMF and DMF synthesis. Fumaric acid titer of 19 ± 2.2 g/L was obtained by culturing *R. oryzae* on synthetic media and recovered following acidification and precipitation at 10 °C. The low solubility of fumaric acid, 5 – 7 g/L, renders its recovery less tedious (Sebastian et al., 2019). The FT-IR analysis of the bioderived fumaric acid showed a similar chemical makeup to commercial fumaric acid obtained via the chemical conversion process (Appendix Figure D5).

The validation was performed accordingly with the optimal conditions identified in previous sections for MMF and DMF, respectively. Results of the validation experiment are presented in Table 3.1.4, where a conversion efficiency of 35.9% was obtained with 12.9 g/L of MMF. This concentration is profoundly lower than the predicted concentration of 22.2 g/L. Also, some of the treatments (Table 3.1.2) yielded higher MMF concentrations than those obtained for the validation. Lower temperature ranges were used for these treatments which is in accordance with the identification that temperature is the most important parameter for conversion of fumaric acid to MMF ($p = 0.02$). Therefore, further optimization of the conditions, especially temperature, might need to be performed to achieve maximum conversion of fumaric acid to MMF.

Table 3. 1. 3. Results of validation experiment performed using biobased fumaric acid

MMF validation	**Predicted concentration (g/L) ***	**Experimental concentration (g/L)**	**Monomethyl fumarate conversion (%)**	**Dimethyl fumarate conversion (%)**	**Residual Fumaric acid (%)**
	22.19	12.95	35.85	2.95	61.19
DMF validation	**Predicted concentration (g/L) ****	**Experimental concentration (g/L)**	**Dimethyl fumarate conversion (%)**	**Monomethyl fumarate conversion (%)**	**Residual Fumaric acid (%)**
	38.80	34.93	96.33	2.86	0.79

* Confidence interval for MMF synthesis – 6.55 g/L to 37.83 g/L

** Confidence interval for DMF synthesis – 32.1 g/L to 45.5 g/L

The experiment to validate the optimal conditions yielded DMF concentration of 34.9 g/L which is close to the predicted concentration of 38.80 g/L and within the confidence intervals predicted. This yield corresponded to a conversion of 96.3 % and only 0.79% fumaric acid was left unconverted during the reaction. 2.9% of the fumaric acid was converted to MMF as well. When both esters are considered, 99.2% of the fumaric acid was converted to FAEs. These results show

that a near-complete conversion of fumaric acid to FAEs can be achieved by using the optimal conditions identified in the present work. Thus, allowing for the conversion of a low-value bioproduct to a high-value chemical of high demand. FT-IR analysis allows for the comparison of the chemical makeup of compounds. To this effect, FT-IR analysis was performed on the DMF obtained and compared against commercially available DMF to confirm the quality of the biobased product. The FT-IR spectroscopy, represented in Figure 3.1.2, revealed that the biobased DMF is highly similar to the commercial DMF. Hence, validating that the shift towards the use of biobased fumaric acid for fumarate ester synthesis is highly feasible and provide for a sustainable alternative.

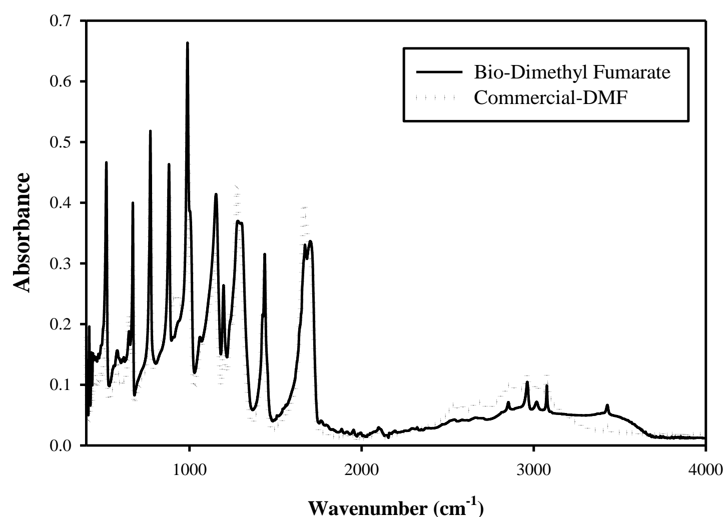

Figure 3. 1. 2. Biobased DMF FT-IR spectrum showing high degree of similarity to commercial DMF

Scope, limitations and future works

Bioproduction of fumaric acid has been studied extensively in the past decade and reviewed extensively by our group and other researchers (Guo et al., 2020; Sebastian et al., 2019; Ilica et al., 2018). Some of these studies have been presented in Table 3.1.4. These studies have shown that high concentrations of fumaric acid can be obtained during fermentation. However, the key

limitations of bioproduction at an industrial scale is the low market value of fumaric acid and therefore rendering the process uncompetitive against the chemical process. Value addition by conversion of fumaric acid to FAEs can aid in overcoming this limitation of the bioproduction process. The main reason for this is the vast difference in the average market price of fumaric acid and its esters. The average market price of fumaric acid is 1 USD/kg whereas that of DMF is 168 USD/kg and MMF is 1, 100 USD/kg (https://www.pharmacompass.com/). Thus, the economic competitiveness of the bioproduction process can be profoundly impacted. Moreover, this shift allows for the use of renewable resources, such as agro-industrial residues or lignocellulosic biomass, as the primary feedstock rendering the process sustainable.

Table 3. 1. 4. Fumaric acid concentrations and productivity achieved during studies using different carbon source (adapted from Sebastian et al., 2019)

Carbon source	Fermenter	Fumaric acid concentration	Productivity (g/L/h)	Reference
Glucose	Stirred tank	56.2 g/L	0.7	Fu et al., 2010
Glucose	Stirred tank	32.1 g/L	0.32	Kang et al., 2010
Glucose	Stirred tank	30.2 g/L	0.19	Roa Engel, 2011
Corn straw	Shake Flask	27.8 g/L	0.33	Xu et al., 2010
Cornstarch	Shake flask	44.1 g/L	0.53	Deng et al., 2012
Diary manure	Stirred tank	31.0 g/L	0.32	Liao et al., 2008
Cornstarch	Shake Flask	45.0 g/L	0.55	Huang et al., 2010
Synthetic medium	Immobilized fungi	32.03 g/L	1.33	Gu et al., 2013
Synthetic medium	Immobilized fungi	40.13 g/L	0.32	Naude and Nicol, 2017

One of the key limitations of the process discussed here is the catalyst used for the conversion. Sulphuric acid was used as a catalyst in the current study but the recovery of the acid after conversion is tedious. To this effect, the use of solid catalysts, such as heterogeneous catalyst, similar to that used by Dedè et al. (2021) can be investigated. They used a 63:37 mixture of Si-propylsulfonic acid SCX-2/Silica GE60 as a catalyst for the conversion of fumaric acid to DMF, with a conversion of 98.32% and >99.5% purity (Dedè et al., 2021). Furthermore, the optimal incubation time of 111 minutes identified in the current study can be improved further by the use

of continuous flow reactors, similar to that used by other research groups (Dedè et al., 2021; Lima et al., 2020). In recent years, continuous flow reactors have garnered huge interest in the production of active pharmaceutical ingredients (API) (Bogdan & Dombrowski, 2019). The shift from one pot system, used in the current study, to continuous flow systems would allow for a continuous conversion of fumaric acid to its esters.

Conclusion

The optimal condition for biobased fumaric acid conversion to its ester derivatives, DMF and MMF, was identified. 111 minutes, 98 °C and 9.7 % (v/v) sulphuric acid, for DMF synthesis, and 30 minutes, 55.5 °C and 2 % (v/v) sulphuric acid, for MMF synthesis. The use of optimal conditions yielded a 99.2 % conversion of fumaric acid to its esters. This shift towards the use of bioderived fumaric acid for DMF and MMF synthesis allows for sustainable production of key active pharmaceutical ingredients and has the potential to improve the economic competitiveness of fumaric acid bioproduction.

Acknowledgement

The authors are sincerely thankful for the financial support of NSERC (Discovery Grants Program) and the Ministère des Relations Internationales du Québec (coopération Paraná-Québec 2010–2012). Likewise, support from James and Joanne Love Chair in Environmental Engineering is also appreciated. The views and opinions expressed in this review are those of the authors.

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LINK -4

LINK: The previous work had shown that a near complete conversion of fumaric acid to its esters is possible thereby showing that the value addition can achieved. To further improve the economic competitiveness of the bioproduction process the extraction of chitosan from the fungal biomass is proposed. To this effect, the use microwave assisted extraction with considerably lesser energy consumption has been investigated here.

PART 2

Microwave-assisted extraction of chitosan from *Rhizopus oryzae* NRRL 1526 biomass

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Carbohydrate Polymers 219 (2019) 431–440; DOI 10.1016/j.carbpol.2019.05.047

Résumé

L'extraction assistée par micro-ondes (EMA) du chitosan à partir de la biomasse fongique séchée de *Rhizopus oryzae* NRRL1526, obtenue par culture dans un milieu de Bouillon Dextrose de Pomme de terre (PDB), a été réalisée et les conditions optimales requises ont été identifiées à l'aide d'une analyse statistique pour la première fois dans cette étude. Cette extraction assistée par micro-ondes (EMA) a été comparée à la méthode conventionnelle d'extraction du chitosan assistée par autoclave. Le plan expérimental factoriel complet a été utilisé pour étudier l'impact des paramètres de fonctionnement de l'EMA, la puissance des micro-ondes (100 W-500 W) et la durée (10 min-30 min), sur le rendement en matière insoluble alcaline (MIA), le rendement en chitosane et le degré de désacétylation (DDA). L'effet des conditions de fonctionnement a ensuite été évalué en utilisant une analyse factorielle complète des données et la condition optimale pour le EMA du chitosan a été identifiée en utilisant la méthodologie de surface de réponse comme étant 300W et 22 min. Cette condition optimale identifiée a ensuite été évaluée et le chitosan obtenu a été caractérisé. Un rendement plus élevé de chitosan de 13,43 ± 0,3 % (g/g) de la biomasse fongique a été obtenu par rapport à celui obtenu de la biomasse sèche, soit de 6,67 % ± 0,3 % (g/g), pour le processus d'extraction conventionnel. L'EMA a donné un chitosane avec un degré de désacétylation plus élevé, 94,6 ± 0,9% contre 90,6 ± 0,5% (chauffage conventionnel), cependant le poids moléculaire a été observé comme étant similaire à celui obtenu en utilisant le chauffage conventionnel en autoclave. On a observé que l'EMA du chitosane produisait une plus grande quantité de chitosane par rapport au processus d'extraction conventionnel et que le chitosane obtenu présentait un plus haut degré de désacétylation ainsi qu'un poids moléculaire plus élevé. La consommation d'énergie plus faible de 0,11 kW h pour la MAE (5 kW h pour le processus conventionnel) et la réduction concomitante de la facture énergétique de 50 cents à 1,1 cents, en

plus des résultats ci-dessus, montrent que l'irradiation par micro-ondes est un moyen plus efficace et plus respectueux de l'environnement pour obtenir du chitosan à partir de la biomasse fongique.

Mots clés: Chitosan, micro-ondes, extraction, biomasse fongique, *R. oryzae*, degré de désacétylation

Abstract

Microwave-assisted extraction (MAE) of chitosan from dried fungal biomass of *Rhizopus* oryzae NRRL1526, obtained by culturing on potato dextrose broth (PDB), was performed and the optimal conditions required were identified using statistical analysis for the first time in this study. This microwave-assisted extraction (MAE) was compared against the conventional autoclave assisted method of chitosan extraction. The full factorial experimental design was used to investigate the impact of operating parameters of MAE, microwave power (100 W- 500 W), and duration (10 min – 30 min), on alkaline insoluble material (AIM) yield, chitosan yield, and degree of deacetylation (DDA). The effect of operating conditions was then evaluated using full factorial data analysis and optimum condition for MAE of chitosan was identified using response surface methodology to be 300 W and 22 minutes. This optimum condition identified was then further evaluated and the chitosan obtained characterized. Higher chitosan yield of 13.43 ± 0.3% (w/w) of fungal biomass was obtained when compared to that obtained, 6.67% ± 0.3% (w/w) of dry biomass, for the conventional extraction process. MAE yielded chitosan of higher degree of deacetylation, 94.6 ± 0.9% against 90.6 ± 0.5% (conventional heating), but the molecular weight was observed to be similar to that obtained by using conventional autoclave heating. MAE of chitosan was observed to yield a higher quantity of chitosan when compared to conventional extraction process and obtained chitosan exhibited a higher degree of deacetylation as well as molecular weight. The lower energy consumption of 0.11 kWh for MAE (5 kWh for conventional process) and the concomitant reduction in the energy bill to 1.1 cents from 50 cents, in addition to the above results, show that microwave irradiation is a more efficient and environment-friendly means to obtain chitosan from fungal biomass.

Keywords: Chitosan, microwave, extraction, fungal biomass, *R. oryzae*, degree of deacetylation

Introduction

Chitosan is the only pseudo-natural cationic polymer, made up of N-glucosamine moieties, derived from chitin that is widely distributed in nature in organisms ranging from yeast to marine organisms, such as crustaceans (Rinaudo, 2006). The deacetylated form of chitin (considered to be the second most abundant polysaccharide after cellulose) possesses important properties, such as versatile biological activity, excellent biocompatibility, complete biodegradability, and low toxicity. These unique properties of the biopolymer have fueled its demand for applications in diverse fields such as wastewater treatment, pharmaceuticals, food and beverage, cosmetics and agricultural. Moreover, it has been awarded GRAS (Generally Regarded as Safe) status by the United State Food and Drug Association (US FDA) (Knor and Klein, 1986; Rinaudo, 2006; Maurya and Inamdar, 2008; Rajeshkumar et al., 2009).

The current production of chitosan from chitin present in shellfish waste, which can take up to 2 days, involves stages of demineralization, deproteinization, and decolorization, using large quantities of toxic chemicals, prior to alkaline deacetylation of chitin to chitosan. The chitosan so obtained show inconsistencies in both physical and chemical properties, such as protein contamination, degree of deacetylation and variations rendered due to seasonal variations, which limits its application (Knor and Klein, 1986; Amorim et al., 2001; Chatterjee et al., 2005; Kumari and Rath, 2014). This dependence on crustaceans causes environmental pollution due to the generation of large quantities of highly alkaline and acidic effluents rich in proteinaceous waste materials. These limitations have necessitated the need for alternate means of chitosan production by the switch to fungal chitosan.

Chitin and chitosan are an integral part of the fungal cell wall of certain fungi especially those belonging to the class Zygomycetes (Nwe and Stevens, 2004). Several yeasts and fungal species,

such as *Candida albicans*, *Saccharomyces cerevisiae, Mucor rouxii*, *Cunninghamella elegans*, *Gongronella butleri*, *Phycomyces blakesleeanus*, *Rhizopus* spp., and *Absidia* spp., have been previously investigated for chitosan production (White et al., 1979; Knor & Klein, 1986; Muzarelli et al., 1994; Yokoi et al., 1998; Amorim et al., 2001; Suntornsuk et al., 2002; Chatterjee et al., 2005; Chatterjee et al., 2008; Streit et al., 2009). The chitosan obtained from fungal strains shows better uniformity in molecular weight, low polydispersity index and degree of deacetylation (Suntornsuk et al., 2002; Brown et al., 2016). Moreover, extraction of chitosan from fungal sources require less amount of chemicals as only mild conditions are needed for extraction and do not require stages, such as demineralization and depigmentation. Hence, producing a lesser amount of toxic waste can be considered as a greener alternative (Dhillon et al., 2012). The main limitation of the fungal chitosan production is low biomass production. Hence, an efficient extraction technique is required to obtain maximum chitosan yield from the available biomass.

Conventional alkaline extraction of chitosan is performed at high temperature and hence, consume a lot of energy and requires a lot of time. To this effect, the adoption of microwave heating in place of conventional heating can reduce the chitosan extraction time from hours to minutes to attain the same degree of deacetylation as traditional methods (Sagheer et al., 2009; Alishahi et al., 2011; Mahdy Samar et al., 2013; El Knidri et al., 2016). The use of microwave irradiation is a more efficient and environment-friendly technique to extract animal chitosan as observed by Samar et al. (2013) and El Knidri et al. (2016). During conventional heating, the reactants are slowly activated, not uniformly, whereas microwave heating happens at molecular level leading to a uniform rapid rise in temperature (Mahdy Samar et al., 2013; El Knidri et al., 2016). Hence, microwave assisted extraction may be adopted for effective extraction of chitosan from fungal biomass.

The microwave-assisted extraction of chitosan from the shellfish waste have been studied (Sagheer et al., 2009; Alishahi et al., 2011; Mahdy Samar et al., 2013; El Knidri et al., 2016;) but microwave assisted extraction from fungal biomass has not been reported so far. In this study, optimization of microwave-assisted extraction of the biopolymer from *R. oryzae* NRRL 1526 biomass, using full factorial experimental design and data analysis, was performed. In recent years there has been a revived interest in bio-production of commodity chemicals, especially dicarboxylic acids, via *R. oryzae* mediated fermentation (Meussen et al., 2012; Martin-Dominguez et al., 2018). Moreover, the fungus is readily used in the food industry, as well as the known producer of enzymes, such as lipase, protease, and pectinase, due to its generally regarded as safe (GRAS) status (Ghosh and Ray, 2011; Cantabrana et al., 2015). The simultaneous extraction of chitosan from the residual *R. oryzae* biomass after fermentation can have a profound impact on feasibility of a bio-production process of commodity chemicals. In fact, in the closed loop concept, if the residual biomass can be obtained after recovery of primary bioproduct and used to co-extract another important product, it justifies the carbon-neutral approach of bio-production process.

The work performed here investigates the possibility of adopting microwave assisted extraction as an efficient alternative to conventional autoclave mediated extraction of fungal chitosan. This use of more efficient heating source, afforded by the use of microwave, might be able to improve the chitosan yield from fungal biomass. Moreover, the identification of optimal conditions of microwave heating should be able to reduce the amount of energy consumed for fungal chitosan extraction as well as provide higher chitosan yield due to improved heating efficiency.

Materials and methods

Fungal strain, culture conditions and biomass harvesting for optimization of microwave extraction conditions

R. oryzae NRRL 1526 was obtained from ARS (Agricultural Research Service, US) culture collection. The inoculum was prepared by growing the fungus on potato dextrose agar (PDA) (Nutri-Bact, Quebec, Canada) plates at 30 ± 1 °C for 3 days (Chatterjee et al., 2005). The potato dextrose broth (PDB) culture media (Nutri-Bact, Quebec, Canada) were inoculated with one 1cm × 1cm mycelium covered agar block and incubated at 30 ± 1°C for 2 weeks (Chatterjee et al., 2005; Suntornsuk et al., 2002). The mycelial mat obtained was harvested, rinsed with deionized water, frozen and freeze-dried at -55 °C (CoolSafe, SCANVAC) as it was faster than drying at high temperature as well as easier to crumble in a blender and hence, no data about yield after drying was determined.

Conventional alkali extraction of chitosan

The extraction of chitosan was carried out, in duplicates, by a modified method described by Suntornsuk et al. (2002). The freeze-dried mycelia were finely ground with a blender, suspended with 1 N NaOH (Fisher Chemicals, New Jersey, USA) solution (1:50; w/v) and autoclaved at 121 °C for 15 min. The alkali-insoluble fraction was collected after centrifugation at 8000× g for 15 min, washed with distilled water and recentrifuged until neutral pH was obtained. The residues obtained after washing was dried and alkali insoluble material (AIM) quantified. AIM was further extracted in 2% acetic acid (1:40; w/v) (Fisher Chemicals, New Jersey, USA) at 95±1 °C for 8 hours and the slurry obtained centrifuged at 8000 × g for 15 min and the acid-insoluble fraction discarded. The pH of the supernatant obtained was adjusted to pH 10 with 2 N NaOH. The solution was centrifuged at 12,000× g for 15 min. The precipitated chitosan was then washed with distilled water and centrifuged and the step was repeated with 95% ethanol (1:20; w/v), followed by acetone (1:20; w/v). The chitosan so obtained was freeze-dried at -55 °C (CoolSafe, SCANVAC).

Microwave-assisted extraction experimental design and data analysis

Statistica software (Version 13.3) was used for the design of experiment as well as analysis of experimental data. Full factorial experimental design, with independent variables power and duration of microwave heating (2 factors at 3 levels), was used to identify the optimal conditions for MAE. The microwave energy and duration of heating used for the study ranged from 100-500 W and 10-30 min respectively (with concentration of NaOH kept constant at 1N). Analysis of variance on the experimental data obtained was performed to determine the effect of power and duration of heating on the dependent variables of AIM yield, chitosan yield and degree of deacetylation. The contour plots obtained following RSM analysis using the regression model given by Eq (1) was used to determine the optimal conditions of MAE of chitosan from *R. oryzae* biomass.

$$y = \beta_0 + \sum_{i=1} \beta_i X_i + \sum_{i=1} \beta_{ii} X_i^2 + \sum \sum_{j=i+1} \beta_{ij} X_{ij} \qquad \text{Eq 1}$$

where, y is the predicted response, β_0 is the model intercept; β_i and β_{ij} are the coefficients of linear terms; β_{ii} is the coefficient of the quadratic term; X_i and X_j are the independent variables.

The freeze-dried mycelia were finely ground with a blender, suspended with 1 N NaOH solution (1:50; w/v) and subjected to the different microwave conditions (in duplicates), with the aid of Perkin Elmer Multiwave microwave sample preparation system (Anton Paar GmbH, Strasse, Austria). The alkali-insoluble fraction was collected after centrifugation at 8,000 x g for 15 min, washed with distilled water and recentrifuged until neutral pH was obtained. The residues obtained after washing was freeze-dried (CoolSafe, SCANVAC) and alkali insoluble material (AIM) quantified. AIM was further extracted in 2% acetic acid (1:40; w/v) at 95 °C for 8 hours and the slurry obtained centrifuged at 8,000 x g for 15 min and the acid-insoluble fraction discarded. The pH of the supernatant obtained was adjusted to pH 10 with 2 N NaOH. The solution was

centrifuged at 8,000×g for 15 min. Precipitated chitosan was then washed with distilled water, 95% ethanol (1:20; w/v) and acetone (1:20; w/v), respectively, and freeze-dried at -55 °C (CoolSafe, SCANVAC). The schematic of the experiment represented in Figure 3.2.1.

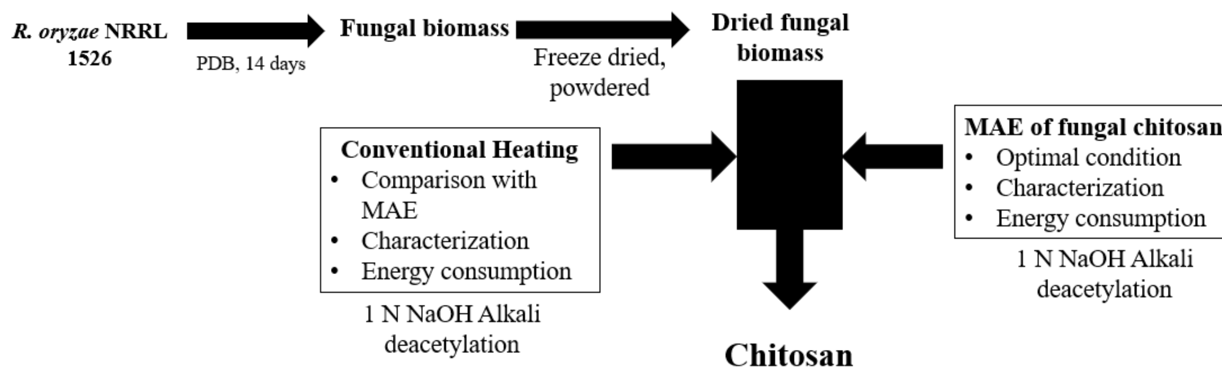

Figure 3. 2. 1. Schematic of the experiment

Characterization of fungal chitosan

The determination of the properties of the fungal chitosan, such as DDA and viscosity average molecular weight, discussed below was performed in duplicates.

FT-IR studies and determination of the degree of deacetylation (DDA)

FTIR spectra were recorded in the middle infrared (4000 cm^{-1} to 400 cm^{-1}) with a resolution of 4 cm^{-1} in the absorbance mode for 16 scans at room temperature. The various powdered mycelial chitosan samples were subjected to FTIR analysis, equipped with Nicolet iS50 FT-IR with Smart™ iTX accessory and OMNIC FT-IR software (ThermoFisher Co., Boston, MA, USA). The FT-IR spectra obtained was compared against commercial crab chitosan of DDA 95% and molecular weight of 100-300 kDa (ACROS Organics, Geel, Belgium). The DD was calculated by measuring the absorbance ratio of A_{1655} and A_{3450}. The amide I band at 1655 cm^{-1} and the hydroxyl group absorption band at 3450 cm^{-1} were used as internal reference. The equation, proposed by

Czechwska-Biskup et al. (2012), used for determination of the degree of deacetylation is as follows using Equation 2:

$$\text{Degree of deacetylation (DDA)} = 100 - [((A_{1655}/A_{3450}) \times 100)/1.33] \quad \text{Eq 2}$$

Determination of degree of deacetylation by potentiometric titration

The degree of deacetylation was determined by potentiometric titration, according to the method of Czechowska-Biskup et al. (2004). 0.02 g of dried fungal chitosan, derived using optimum MAE condition as well as conventional autoclave extraction, was dissolved in 20 ml 0.1 M acetic acid. Titration of this solution with a 0.1 M NaOH solution was performed and a pH curve with two inflection points obtained. The first derivative of the pH curve was determined and plotted to precisely identify the volumes of 0.1 M NaOH used corresponding to the two inflections points. The degree of deacetylation (DDA) of the chitosan samples was then determined, using the volume information, according to the formula (Czechowska-Biskup et al., 2004):

$$\text{Degree of deacetylation (DDA)} = 2.03\times ((V_2-V_1)/(m+0.0042(V_2-V_1))) \quad \text{Eq 3}$$

where m is the weight of chitosan sample; V_2, V_1 is the volumes of 0.1 M sodium hydroxide solution corresponding to the deflection points.

Molecular weight determination

Extracted chitosan solutions, conventional, as well as microwave extracted, of varying concentrations ranging from 0.0625 to 0.5% (w/v), were prepared in 2% acetic acid (Fisher Scientific, New Hampshire, USA), stirred for 24 hours at room temperature and filtered through 0.45 μm glass filters (Fisher Scientific, New Hampshire, USA) to remove insoluble fractions (Chatterjee et al., 2005; Vaingankar and Juvekar, 2014). The viscosity of the solutions, in

duplicates, was determined at 25°C using Fungilab rotational viscometer (Fungilab, Barcelona, Spain). The reduced viscosity (η_{red}) was calculated using Equations below (4-6) and plotted against concentration (C, g/100ml). The intrinsic viscosity ([η]) was obtained by extrapolating the curve to Y-axis.

Relative viscosity, $\eta_{relative}$ = viscosity of solution/viscosity of solvent Eq 4

Specific viscosity, $\eta_{sp} = \eta_{relative} - 1$ Eq 5

Reduced viscosity, $\eta_{red} = \eta_{sp}/C$ Eq 6

The intrinsic viscosity obtained was then used to calculate the viscosity-average molecular weight (M_v) of chitosan from the viscosity-molecular weight equation (Sagheer et al., 2009):

$$[\eta] = 0.078 * M_v^{0.76} \quad \text{Eq 7}$$

Crystallinity index determination using X-ray powder diffractometry (XRD)

The XRD measurements on powdered chitosan samples, commercial animal derived chitosan and fungal chitosan obtained via MAE as well as conventional autoclave extraction, were carried out to determine their crystal structure. The diffractogram was obtained using benchtop X-ray diffractometer (AERIS, MalvernPANalytical, Malvern, UK). The diffractometer was equipped with Cu kα radiation (λ=1.5406 Å) and operated at 40 kV and 8 mA. A continuous scan between 2θ angles of 5° of 40° was carried out with a step size of 0.022° and a scan time of 46.9 s. The crystallinity index (I_{CR}) was calculated using the following equation (Sangheer et al., 2009; Knidri et al., 2016):

$$\text{Crystallinity index } (I_{CR}) = (I_{110} - I_{am})/I_{110} \times 100 \quad \text{Eq 8}$$

where I_{am} is the intensity of amorphous diffraction at 2θ≅16° and I_{110} is the maximum intensity at 2θ≅20°.

Energy consumption

Electrical power consumed during microwave assisted chitosan extraction as well as during conventional method of autoclave extraction from fungal biomass is determined by Equation 9 (Laadila, et al., 2017).

$$E = (P \times t)/1000 \quad \text{Eq 9}$$

where E is electric energy consumed during the operation of microwave digester (kWh); P is power (W) and t is time (hour).

Results and Discussion

Statistical studies and identification of optimal conditions for MAE of chitosan

The effect of microwave power (X_1) and time of incubation (X_2), ranging from 100-500 Watts and 10-30 minutes respectively, were studied to identify the optimal conditions for maximum chitosan extraction. Full factorial data analysis was performed to obtain an understanding of the effect of each parameter on chitosan extraction and represented in Table 3.2.1. The optimum values of independent variables were obtained by applying desirability function (d) to the results of the dependable variables (AIM yield, chitosan yield and degree of deacetylation) given in Table 3.2.2 for the best agreement. This was followed by the determination of the global desirability function (D) to determine the optimum condition for microwave assisted chitosan extraction. These results are represented in Figure 3.2.2 and the global desirability illustrated in Figure 3.2.4. The predicted responses are transformed into dimensionless desirability function (d_{AIM}, $d_{Chitosan}$, and d_{DDA}). The objective of this study was to obtain maximum chitosan yield with high DDA and therefore only a single function was used.

$$d_{AIM} = [(\text{AIM yield} - A) / (B - A)], \text{ where } d_{AIM} = 0 \text{ (if AIM} < A) \text{ and} \quad \text{Eq 10}$$

$$d_{AIM} = 1 \text{ (if AIM} > B)$$

$d_{Chitosan}$ = [(Chitosan yield - A) / (B - A)], where $d_{Chitosan}$ = 0 (if chitosan yield < A) and $d_{Chitosan}$ = 1 (if chitosan yield > B) Eq 11

d_{DDA} = [(DDA - A) / (B - A)], where d_{DDA} = 0 (if DDA < A) and d_{DDA} = 1 (if DDA > B) Eq 12

where; A and B are the lowest and highest yields obtained for the corresponding response.

The global desirability (*D*) was obtained by combining the desirability of the individual response, i.e., d_{AIM}, $d_{Chitosan,}$ and d_{DDA} as described by the Equation below:

$$D = (d_{AIM} \times d_{Chitosan} \times d_{DDA})^{1/3} \quad \text{Eq 13}$$

When *D* > 0, all responses are within the desirable ranges and when *D* is close to 1 signifies that the combination of the different independent variables (Power and Time) was globally optimum.

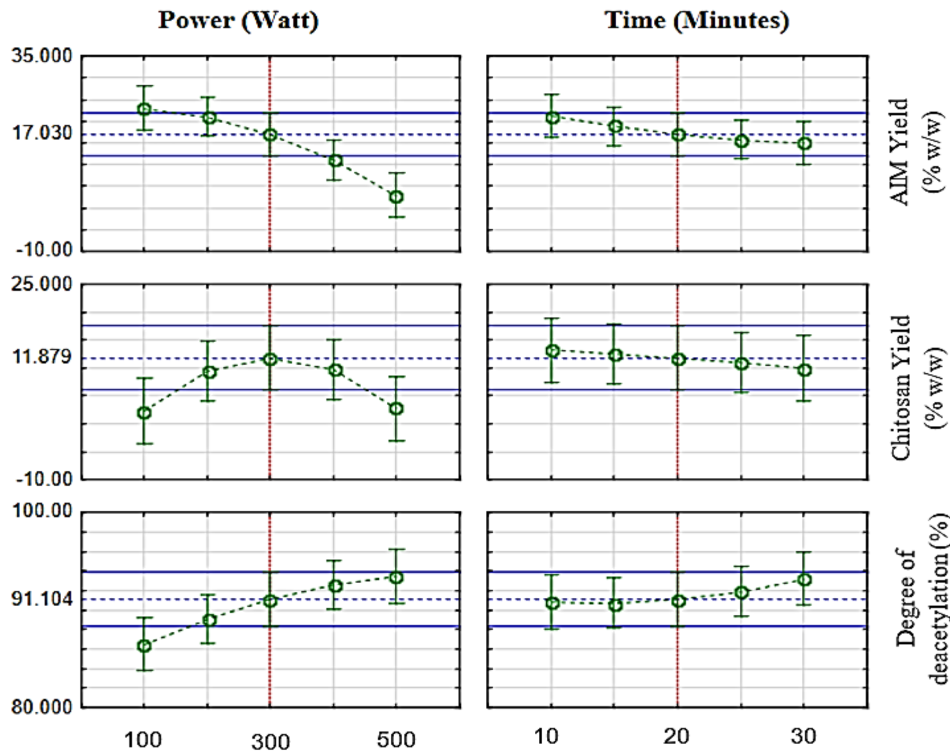

Figure 3. 2. 2. Predicted profiles of dependent variables time (AIM yield, chitosan yield and degree of deacetylation)

The results of ANOVA analysis and interactions are represented in Table 3.2.1. Investigation of the effect of the factors, power, and duration of incubation, showed that microwave power had a significant effect on AIM production ($p = 0.001$) whereas duration of microwave treatment had an only slight effect on AIM yield ($p = 0.04$). Figure 3.2.3 (a) provides the contour plot that shows the impact of time and microwave power on AIM extraction. Moreover, it was concluded that only power had a linear and quadratic effect on AIM extraction but this effect was not completely independent of the duration of microwave treatment ($p = 0.20$) as can be observed with the p-value for the linear effect. The experimental data have a high degree of correlation with the predicted response ($R^2 = 0.982$).

The AIM obtained was then subjected to chitosan extraction. The statistical analysis (ANOVA) of the data showed that microwave power only had an impact on the quantity of chitosan extracted from the AIM ($p = 0.01$). The contour plot of the effect of power and time on chitosan yield is provided in Figure 3.2.3 (b) and the experimental data had a lower degree of correlation with predicted response ($R^2 = 0.918$). The degree of deacetylation is one of the key parameters that determine the conversion of chitin to chitosan. The effect of different levels of microwave power and duration of the treatment on the degree of deacetylation was investigated. This analysis showed that only power had a significant effect on the degree deacetylation ($p = 0.005$) but the interactive effect of power and time was significant ($p = 0.04$). The experimental data showed a good correlation with the predicted response ($R^2 = 0.962$) and the contour plot of the effect of power and time on DDA is represented in Figure 3.2.3 (c).

Table 3. 2. 1. Effect of independent variables (X_1- Power; X_2- Time) on dependent variables (AIM yield; Chitosan yield; Degree of deacetylation) (Significant values: $p < 0.05$)

Model	AIM yield (% w/w)		Chitosan yield (% w/w)		Degree of deacetylation (%)	
	p	Effect	*p*	Effect	*p*	Effect
X_1	0.001346	-20.020	0.886201	0.3133	0.005187	6.9400
X_1^2	0.072485	4.0367	0.012715	9.3433	0.256050	1.1433
X_2	0.037689	-6.1067	0.209808	-3.2033	0.088072	2.3533
X_2^2	0.532541	-1.0433	0.866767	0.31833	0.339012	-0.9267
X_1X_2	0.199119	-3.4450	0.217709	-3.8350	0.036723	-4.1600

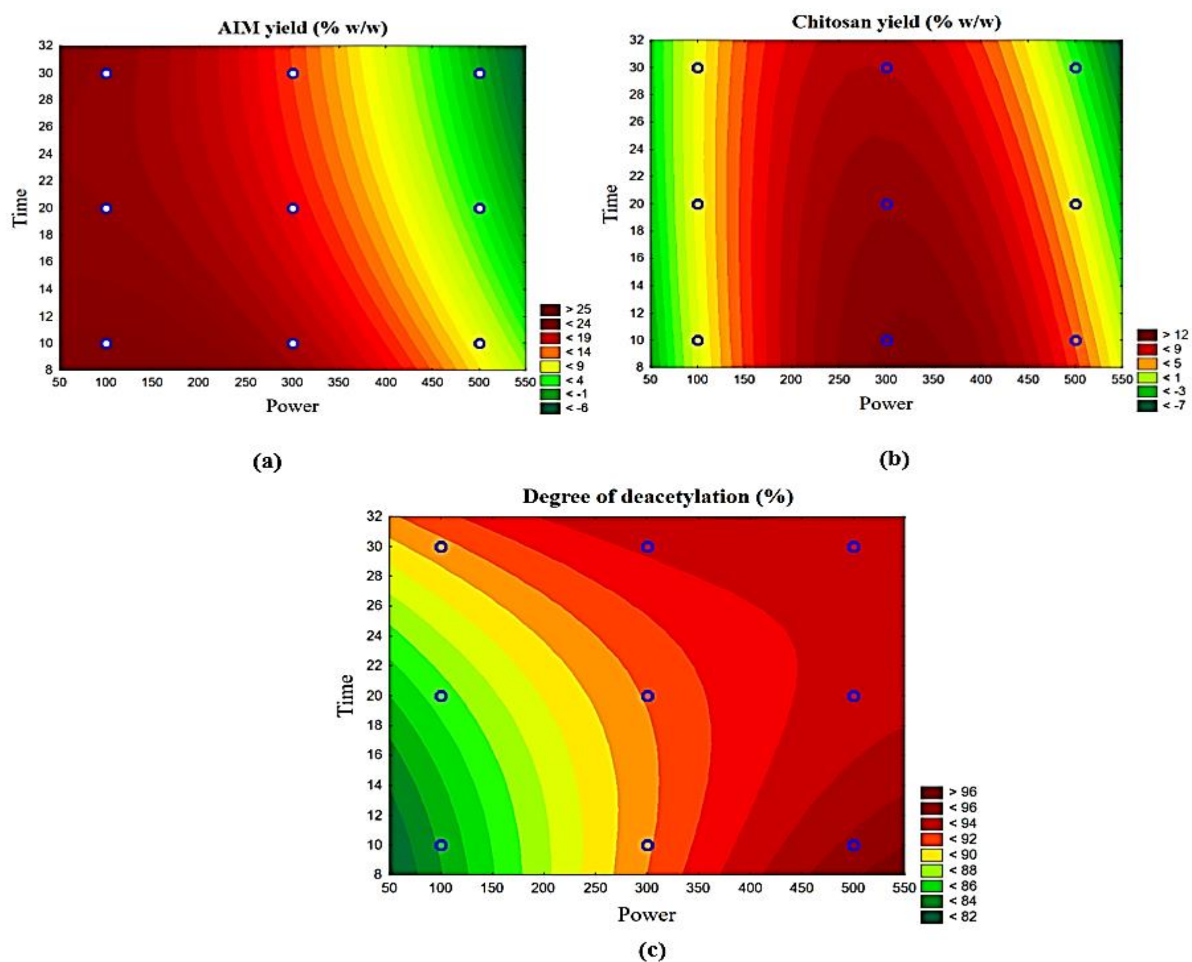

Figure 3. 2. 3. Contour plots of the effect of Power (Watt) and Time (minutes) on AIM yield (a), chitosan yield (b) and degree of deacetylation (DDA) (c)

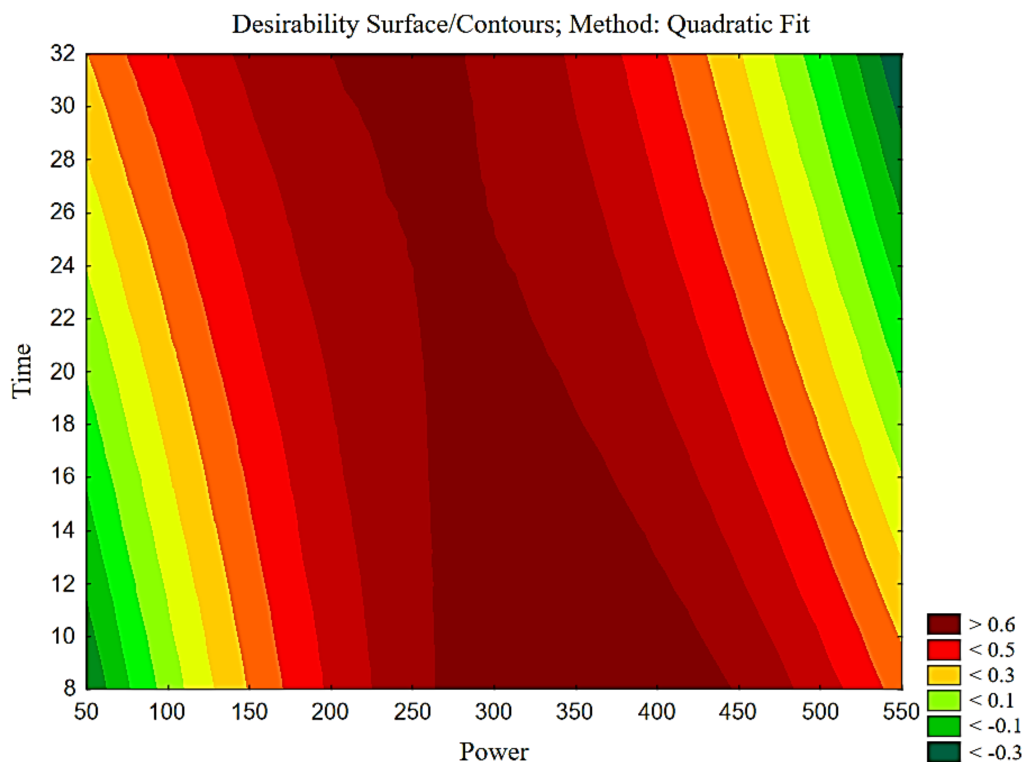

Figure 3. 2. 4. Global Desirability contour plot

Microwave extraction of chitosan and optimization

Power and duration of microwave treatment were chosen as the variables for the study. Previous studies have used, most commonly, 1N NaOH for the extraction of chitosan from the fungal biomass (Crestini et al., 1996; Tan et al., 1996; Pochanavanich and Suntornsuk, 2002; Suntornsuk et al., 2002; Hu et al., 2004; Chatterjee et al., 2005; Suntornsuk and Kleekayai, 2011; Vaingankar and Juvekar, 2014). Hence, this concentration was kept constant during the study and conventional autoclave assisted extraction was used for comparison of the results obtained during microwave extraction. The maximum temperature achieved during different conditions of extraction were 85°C, 200°C, 260°C and 121°C for 100W, 300W, 500W (30 minutes exposure to microwave) and autoclaving, respectively. The quantity and yield of AIM and chitosan obtained during the different

extraction conditions and the degree of deacetylation are presented in Table 3.2.2. The yields of chitosan and AIM presented in this study are represented with respect to unit biomass dry weight. The use of least microwave power (100W) gave the highest alkali insoluble material (AIM) yield, 23% (w/w), albeit the lowest chitosan yield of 2.1% w/w. This might be due to the low temperature (50°C – 85°C) achieved during the low power condition (100W) lead to the only partial conversion of fungal biomass to chitosan. The AIM primarily might be made up of undigested hydrated fungal biomass. On the other hand, the use of highest microwave power (500W) gave the least AIM yield, 3.12 % (w/w), but average chitosan yield, 2.6% (w/w), was slightly better than that obtained during the lowest power conditions. High temperatures (200°C - 260°C) achieved in this condition might have led to complete digestion of fungal biomass in the presence of 1N NaOH. The exact reason for this reduction in both AIM as well as chitosan yield is not clearly understood and might be due to degradation of chitosan. The observed drastic decrease in both AIM and chitosan yield, represented in Table 3.2.2, should be able to support this hypothesis. Moreover, the use of microwave has been studied to accelerate the degradation of high molecular weight chitosan to low molecular weight chitosan (Li et al., 2012). Highest chitosan yield was obtained when moderate microwave power condition (300W, 20 minutes) was used and the yield under said conditions were higher, 14.43 ± 3.9% (w/w) of dry biomass versus 6.67 ± 0.3% (w/w) of biomass than the conventional autoclave method of chitosan extraction.

Table 3. 2. 2. AIM yield, chitosan yield and degree of deacetylation

Power (W)	**Time (mins)**	**AIM yield (% w/w)**	**Chitosan yield (% w/w)**	**Degree of deacetylation (%)**
100	10	24.63±3.3	1.69±0.4	84.31±0.7
	20	24.57±7.8	2.13±0.4	87.18±0.3
	30	21.89±3.1	2.67±0.7	89.84±0.1

300	10	20.64±0.2	12.23±1.4	90.10±1.9
	20	17.84±3.3	14.43±3.9	90.65±0.7
	30	14.69±2.3	8.33±0.5	94.42±1.3
500	10	10.03±0.1	6.87±0.5	95.87±0.9
	20	0.60±4.2	0.38±0.5	93.20±0.6
	30	0.40±3.5	0.18±0.1	93.08±0.5
Conventional	20	22.09±2.3	6.67±0.3	90.62±0.5

[1]

The maximum chitosan yield of 14.4 % (w/w) of biomass, under microwave extraction condition, was found to be higher than most of the previous studies that used conventional autoclave assisted chitosan extraction. This yield is similar to 14% (w/w) reported for *R. oryzae* (Pochanavanich and Suntornsuk, 2002) and higher than 11.9 % (w/w) obtained from *R. oryzae* grown on deproteinized whey medium (Chatterjee et al., 2008) but lower than the 21% (w/w) of biomass content reported for *Gongronella butleri* CCT4274 (Streit et al., 2009). Chitosan yield of 4.3 g/kg of the substrate was obtained by culturing *R. oryzae* TISTR3189 on soybean residues during solid-state fermentation (Suntornsuk et al., 2002). Additionally, chitosan yields of up to 406 and 700 mg/l in corn and rice media, respectively, have been obtained (Hang et al., 1990). This lack of uniformity in the units, as well as lack of information on total biomass yield or the fermentation media volume, makes it tough to compare chitosan yields with previous studies. The chitosan yield in this study was calculated against biomass obtained. Most of the researchers used g/L as the unit for the quantity of chitosan obtained. Since chitosan is extracted from the fungal biomass obtained, it is probably best to express chitosan yield as a function of biomass obtained rather than the amount of media used. The fungal biomass obtained can be expressed as a function of the quantity of media used.

[1] Each value is the mean of two independent replicate

The chitosan yield obtained during this study was found to be higher than those obtained for *R. arrhizus* during the study carried out by Berger et al., (2014). They obtained a maximum yield of 49.31 mg/g. The chitosan yield of 6.67% using conventional autoclaving technique is in agreement with the 7.1% obtained for *R. delemar* (Miyoshi et al., 1992) as well as 6.0 to 7.7% obtained for *Mucor rouxii* (Chatterjee et al., 2005). The maximum chitosan yield, 14.43 % (w/w), obtained during this study is higher than the 10.7% and 7.3% reported for *Absidia coerulea* 14076 and *Mucor rouxii*, respectively (Rane and Hoover, 1993; Synowiecki and Al-Khateeb, 1997)

AIM yield of 22.09% obtained by the use of conventional autoclave extraction is in accordance with that reported for *R. oryzae* (Hu et al., 2004). Hu et al., (2004) screened 33 different strains of fungus for chitosan production. They reported AIM percentage yield of 19.9% for *R. oryzae* but the chitosan yield obtained was lower than that obtained in this study. This profound difference in chitosan yield, 14.43% (w/w) against 0.9% (w/w), may be probably due to the difference in the media used. PDB was used for this study whereas PGY salt broth, made up of 20 (g/L) glucose, 10 (g/L) peptone, 1 (g/L) yeast extract, 5 (g/L) ammonium sulfate, 1 g di-potassium hydrogen orthophosphate, 1 (g/L) sodium chloride, 5 (g/L) magnesium sulphate-7-hydrate, 0.1 g calcium chloride-2-hydrate, was used by Hu et al. (2004). It has been reported that nitrogen content and type of nitrogen source had an impact on fungal biomass production, chitosan yield and molecular weight of extracted chitosan (Jiang et al., 2011). Both temperature and the duration of exposure has been observed to affect chitosan yield from mushrooms (Kanan, et al., 2010). Hence, the higher temperature of nearly 160°C achieved under optimum conditions of microwave extraction probably explains the high yield observed in this study.

Moreover, it was observed that the degree of deacetylation of the chitosan extracted in this study was higher than that obtained by Hu et al., (2004) for *R. oryzae*. They obtained chitosan of 67.3%

degree to deacetylation whereas chitosan of varying degree of deacetylation ranging from 84%-95% was obtained during this study under various MAE conditions. The degree of deacetylation obtained corresponds to that obtained for *R. oryzae* by Khalaf (2004) as well as similar to that observed by Kleekayai and Suntornsuk (2011) and Pochanavanich and Suntornsuk (2002). The highest degree of deacetylation of 95% obtained points to the probable effective conversion of chitin to chitosan, in the presence of NaOH, by using higher microwave power.

The optimum condition for microwave extraction of chitosan and its characterization

The theoretical optimal conditions of microwave extraction conditions were 300 W of microwave power and 22 minutes, represented in Figure 3.2.3. Fungal biomass was subjected to these optimal conditions of microwave extraction and the chitosan yield obtained evaluated and characterized. This optimal condition of microwave extraction gave a chitosan yield of 13.43% ± 0.3% (w/w) and 94.58 ± 0.9% degree of deacetylation using FT-IR analysis. The obtained degree of deacetylation was further confirmed by potentiometric titration and was found to be 97.3% (and 83.9% for conventional heating) (Supplementary data 2). The chitosan yield is similar to the highest chitosan yield represented in Table 3.2.2 but was observed to have a higher degree of deacetylation (94.58% vs 90.6%). This increase in the degree of deacetylation rendered by the use of optimal conditions (duration and energy) of microwave heating was statistically confirmed via analysis of variance, which yielded a p-value of 0.005. Moreover, chitosan of higher degree of deacetylation was obtained via microwave heating (optimum conditions) when compared to conventional heating and was found to be significantly higher (p-value= 0.003). Hence, the optimal condition of (300 W, 22 min) yielded a similar quantity of chitosan but of the higher degree of deacetylation. A similar study that investigated the optimization of microwave extraction conditions for chitosan extractions from *Aspergillur niger* was performed by Hesheng et al. (2001).

They used ethanol in combination with alkali whereas this study used alkaline treatment alone for deacetylation (Hesheng et al., 2001). Moreover, the optimum condition identified (480 W, 20 min) was higher than the optimum condition presented in Table 3.2.3. Additionally, in the previous study, the chitosan obtained was of a lower degree of deacetylation (88%) compared to that obtained (94%) in the current study. Additionally, statistical analysis and determination of optimum condition using response surface methodology were performed in the current study and no such study was performed in the previous study. The yields (AIM and chitosan) and properties of chitosan under optimal conditions of MAE are represented in Table 3.2.3. The purity of the chitosan obtained was not investigated as defined potato dextrose broth (PDB) was used as the media for culturing *R. oryzae*. Hence, the chances of presence of recalcitrant impurities are minimal. Moreover, the use of high temperature and 1 N NaOH for alkaline extraction of chitosan and deacetylation will lead to degradation of proteins as well as sugars.

Table 3. 2. 3. Comparison of chitosan obtained MAE and conventional extraction.

			Optimal MAE conditions			
Power (W)	Time (min)	AIM yield (% w/w)	Chitosan yield (% w/w)	Degree of deacetylation (%)		Molecular weight (kDa)
300 (170°C)	22	26.11 ± 4.5	13.43 ± 0.3	FT-IR calculation	94.58 ± 0.9[b]	127.58 ± 33.9[a]
				Potentiometric titration	97.28 ± 0.1[d]	
			Conventional extraction			
Condition	Time	AIM yield (% w/w)	Chitosan yield (% w/w)	Degree of deacetylation (%)		Molecular weight (kDa)
121°C	20	22.09 ± 2.3	6.67 ± 0.3	FT-IR calculation	90.62 ± 0.5[c]	109 ± 20.5[a]
				Potentiometric titration	83.88± 0.1[e]	

[2]

[2] Each value is the mean of two independent replicate
[a] *p*-value >0.05
[b,c] *p*-value <0.05 (DDA calculated from FT-IR data)
[d,e] *p*-value <0.05 (DDA calculated using potentiometric titration)

Microwave-assisted extraction of chitosan from marine crustacean waste has been studied (Sagheer et al., 2009; El Knidri et al., 2016) but not studied for the extraction from fungal biomass. The reduction in the time required to perform deacetylation from hour to minutes was observed in these studies. A similar reduction in the time required for deacetylation was observed wherein the maximum chitosan yield of 14.43% (w/w) of biomass, with DDA 90.6%, was obtained upon microwave irradiation at 300W for only 20 minutes whereas chitosan yield of 13.43 %, with DDA 94.5%, was obtained under optimum conditions of 300W and 22 minutes. It was observed by Sagheer et al. (2009) that increase in duration of heating lead to an increase in the degree of deacetylation during initial stages of conversion, which plateaus off following a further increase in duration of heating. The rapid increase in temperature achieved via microwave heating due to dielectric heating effect at the molecular level (https://wiki.anton-paar.com/en/microwave-assisted-synthesis/), most likely helps to achieve a greater degree of deacetylation. Additionally, the reduction in wall effect provided by the use of microwave heating, wherein the walls of the container are heated first followed by gradual increase in the temperature of the reaction medium, leads to a rapid rise in temperature of the reaction medium. This most probably allowed for the effective conversion of chitin to chitosan via removal of the acetyl group from the N-acetylglucosamine monomer moieties that make up chitin.

Moreover, chitosan obtained by MAE (127.58 kDa) was observed to have a comparable molecular weight (p-value 0.58) to that obtained via the conventional autoclaving process (109.08 kDa). This, observation is not in accordance with that made by El Knidri et al. (2016) and Sagheer et al. (2009). They observed an increase in molecular weight of chitosan extracted from crustaceans using microwave heating. Sagheer et al. (2009) observed an increase in intrinsic viscosity with duration of microwave heating followed by a decrease in the viscosity. Additionally, microwave assisted

heating has also been proposed as a technique for obtaining low molecular weight chitosan (Li et al, 2012). Hence, the optimal conditions of microwave heating used in this study might not have led to the degradation of the fungal chitosan and yielded chitosan of molecular weight similar to conventional heating. The molecular weights obtained in this study for fungal chitosan, 109 kDA (conventional) and 127 kDA (MAE), is within the range reported for *R. oryzae*, 80 – 128 kDA, by Kleekayai and Suntornsuk (2011) and similar to 120 kDa reported by Chatterjee et al. (2008).

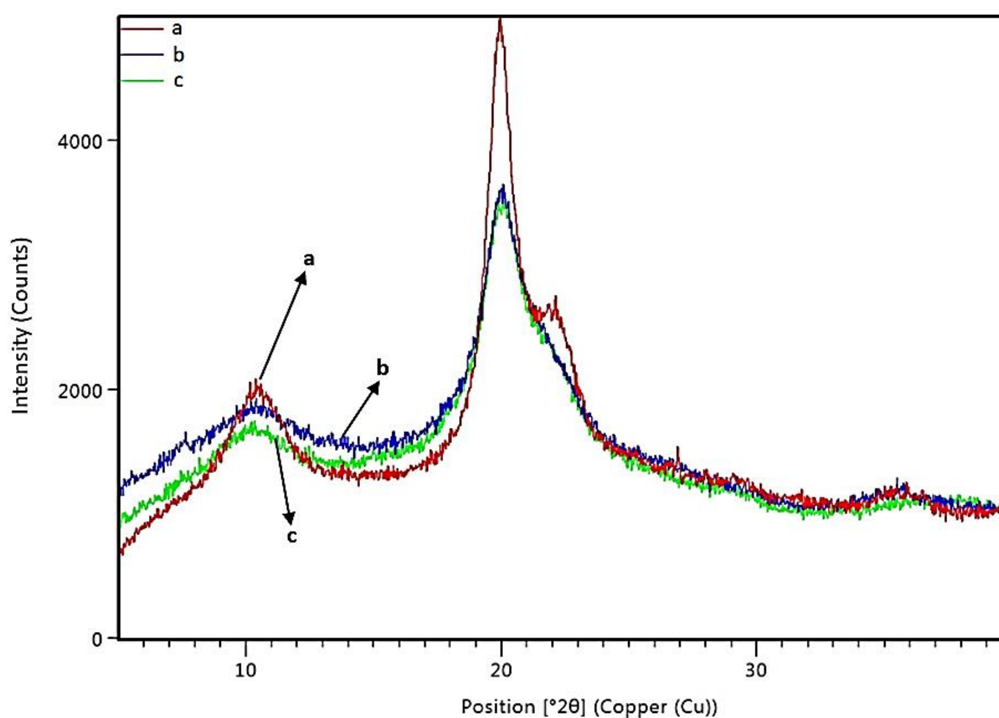

Figure 3. 2. 5. XRD pattern of animal-derived commercial chitosan (a), conventional autoclave assisted extracted fungal chitosan (b) and microwave-assisted extracted chitosan (c)

The X-ray diffraction study of the crystal structure, presented in Figure 3.2.5, showed that the adoption of MAE did not have a profound impact on the crystal structure of the extracted fungal chitosan. The diffraction pattern of chitosan extracted from *R. oryzae* was similar to commercial animal derived chitosan. Both fungal chitosan extracted by conventional heating and microwave

showed the characteristic two distinct peaks corresponding to 2θ angles of 10° and 20° (Knidri et al., 2016). The calculation of crystallinity index from the diffractogram showed that the crystalline structure of a microwave and conventionally extracted chitosan were similar (0.56 for conventional; 0.57 for microwave). This observation is in accordance with that of Knidri et al. (2016). They observed a similar crystalline structure for the microwave as well as conventional heating extracted chitosan.

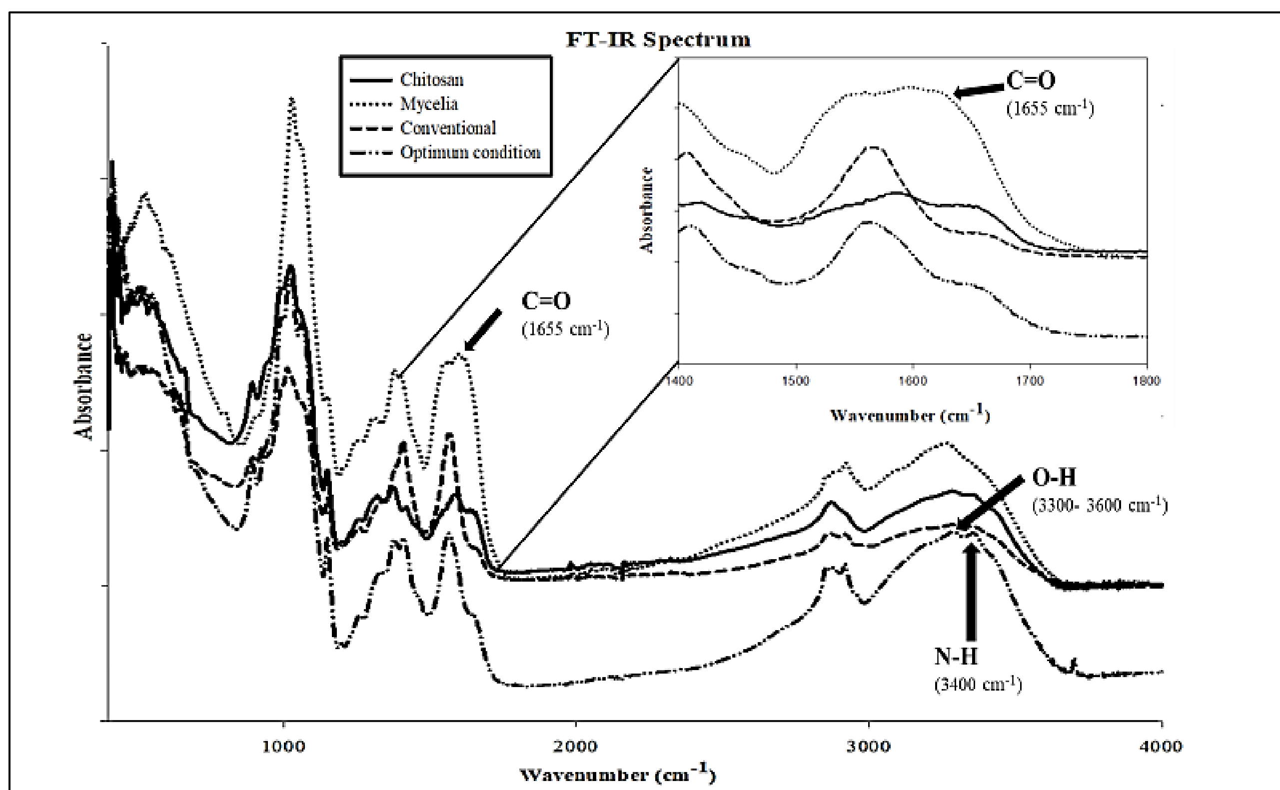

Figure 3. 2. 6. FT-IR spectrum of commercial crab chitosan, fungal mycelia and chitosan obtained after extraction (conventional and optimum microwave condition identified)

The FT-IR spectrum of chitosan samples along with fungal mycelia is represented in Figure 3.2.6. The FT-IR spectrum of fungal chitosan extracted by microwave irradiation and conventional heating show a high degree of similarity to that of commercial animal derived chitosan. The fungal mycelia show a different pattern compared to chitosan sample, especially around absorbance band 1654 cm^{-1}, which corresponds to amide I. Absorbance corresponding to the bands 3450 cm^{-1},

which corresponds to OH stretching, and 1655 cm^{-1} was used to calculate the degree of deacetylation (Czechwska-Biskup et al. 2012; Knidri et al., 2016). The reduction in the absorbance corresponding to the band 1655 cm^{-1} shows the effectiveness of the deacetylation process.

Microwave-assisted extraction (MAE) and pressurized liquid extraction (PLE) were compared to obtain polysaccharides, (particularly biologically active β-glucans) from the fruiting bodies of mushrooms *Pleurotus ostreatus* and *Ganoderma lucidum* by Smiderle et al. (2017). They observed that temperature had a stronger influence on the extraction than time (Smiderle et al., 2017). A similar observation was made during this study. It was observed that an increase in power from 100W to 300W, with a corresponding increase in temperature from 85 °C to 200 °C, led to higher chitosan yield. Microwave irradiation as a tool for DNA extraction has been investigated and these studies have shown that microwave treatment is an efficient and reliable technique for extraction of DNA from fungal biomass for use in PCR (Tendulkar et al., 2003; Zhang et al., 2010). The suitability of the use of microwave irradiation for biological polysaccharide isolation was further proven by the study performed by Xu et al. 2018. They optimized the microwave extraction process for isolation of biological polysaccharide from *Eucommia ulmoides* Oliver leaf. The biological polysaccharide so obtained was characterized and investigated for its antioxidant properties. It was concluded that the optimized microwave extraction condition provided 2.9-fold higher polysaccharide yield than that of the conventional heat reflux extraction method (Xu et al., 2018). Hence, microwave extraction is a promising technique for the extraction of valuable biomolecules.

Energy Calculations

The main benefit of adoption of microwave-assisted extraction is the significantly reduced energy consumption. The conventional autoclave method consumes a larger amount of energy to achieve

the temperature required for effective deacetylation of fungal chitin and deproteination. For instance, the autoclave used for the study uses a 6000 W heater that needs to be operated for 50 minutes to attain and maintain a temperature of 121 °C. This accounts for energy consumption of 5 kWh and amounts to 50 cents per run (assuming 10 cents/ kWh for medium scale classification of electricity supply) (https://www.hydro.mb.ca). On the other hand, the efficient heating achieved by microwave leads to the energy consumption of 0.11 kWh operating under optimal conditions (300 W, 22 minutes) identified in this study. This energy consumption only amounts to 1.1 cents per run for the microwave digester used for the study. The considerable difference in this operational cost associated with chitosan extraction can have a profound impact on the feasibility of a commercial establishment aimed at fungal chitosan production. Affordable industrial microwave systems are available but they need to be optimized or modified for chitosan extraction prior to commercial production. Hence, MAE of chitosan provides an attractive alternative to conventional fungal chitosan extraction.

Conclusion

The study here identified the optimal conditions of microwave-assisted extraction (MAE) of chitosan from the biomass obtained by culturing *R. oryzae* NRRL1526 to be 300 W and 22 minutes. The chitosan obtained under the optimal conditions of MAE, compared to conventional heating, showed a higher degree of deacetylation (94.6% against 90.6%). However, the molecular weight as well as crystalline structure were found to be similar. Additionally, it was observed that MAE of chitosan from fungal biomass yielded a higher amount of chitosan when compared to conventional heating, 13.43 % (≈2-fold) versus 6.67 % (w/w) of fungal biomass. This increase in chitosan yield in addition to the significant reduction in the energy bill, 1.1 cents against 50 cents for conventional heating, can have a profound impact on the feasibility and attractiveness of

commercial production of fungal chitosan. The use of microwave-assisted heating in the current study has shown to improve chitosan yield as well as reduce energy consumption when compared to conventional heating. Thus, MAE-optimized extraction of chitosan from fungal biomass can be an attractive and feasible alternative to animal chitosan.

Acknowledgments

The authors are sincerely thankful for the financial support of NSERC (Discovery Grants Program) and the Ministère des Relations Internationales du Québec (coopération Paraná-Québec 2010–2012). The views and opinions expressed in this review are those of the authors.

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CHAPTER 4: Techno-economic analysis (TEA) and evaluation of the impact of high value compound isolation and value addition on the biorefining process

LINK-5

LINK: The adoption of the fumaric acid bioproduction at industrial scale is highly dependent on the techno-economic competitiveness of the process against the conventional process. To this effect, the economic competitiveness of the fumaric acid produced was assessed and determined. Moreover, the impact of the value addition and high value compound extraction from the fungal biomass on the gross revenue generation was determined.

Techno-economic analysis of fumaric acid bioproduction – prospect of value addition and by-product isolation

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Résumé

L'épuisement des réserves pétrolières a nécessité le développement de procédés alternatifs respectueux de l'environnement pour la synthèse de l'acide fumarique, un produit chimique de base. La fermentation de ressources renouvelables, telles que la biomasse lignocellulosique et les résidus agro-industriels, a été identifiée comme une alternative durable à la synthèse chimique. L'étude actuelle visait à examiner la compétitivité technico-économique du processus de bioproduction envisagé pour la production d'acide fumarique. Le prix d'équilibre (PE) pour la production d'acide fumarique a été trouvé à \$ 2,304 (kg^{-1}) en utilisant le sucre de canne comme matière première. Ce PE a été réduit à \$ 1,916 (kg^{-1}) par l'utilisation de résidus agro-industriels comme matière première ainsi que par la suppression de certaines opérations unitaires permises par les propriétés uniques de l'acide fumarique. Une réduction supplémentaire de la PE est envisagée par l'optimisation du processus ainsi que par l'isolement des sous-produits et l'ajout de valeur par la conversion de l'acide fumarique en esters d'acide fumarique.

Mots clés: Acide fumarique, bioproduction, résidu agro-industriel, prix d'équilibre, sous-produit

Abstract

The depletion of petroleum reserves has necessitated the development of alternate environmentally friendly processes for the synthesis of fumaric acid, a bulk platform chemical. Fermentation of renewable resources, such as lignocellulosic biomass and agro-industrial residues, has been identified as a sustainable alternative to chemical synthesis. The current study aimed to investigate the techno-economic competitiveness of the bioproduction process envisaged for fumaric acid production. The breakeven price (BEP) for fumaric acid production was found to be \$ 2.304 (kg^{-1}) using cane sugar as feedstock. This BEP was reduced to \$ 1.916 (kg^{-1}) by use of agro-industrial residues as feedstock as well as removal of certain unit operations afforded by the unique properties of fumaric acid. Further reduction in the BEP is envisaged by further optimization of the process as well as by-product isolation and value addition by conversion of fumaric acid to fumaric acid esters.

Keywords: Fumaric acid, bioproduction, agro-industrial residue, breakeven price, by-product

Introduction

Fumaric acid is a key di-carboxylic organic acid that has been identified by the US department of energy (DOE) as a key biobased platform chemical. The unique properties of the organic acid have rendered it with diverse applications (Choi et al., 2015; Bozell and Petersen, 2010). Some of these include, as acidulant in food industry, manufacture of paper resin as well as unsaturated polyester resins, as plasticizer and in green chemistry. Additionally, the organic acid and its derivatives, especially fumaric acid esters (FAEs), have been identified to effective in the diseases such as psoriasis and multiple sclerosis. These diverse applications have fueled an increase in the demand for the organic acid (Roa Engel et al., 2008). Currently, this demand is fulfilled by the catalytic chemical conversion of petroleum derived benzene to fumaric acid. This chemical conversion leads to adverse effect on the environment by the release of carbon dioxide and carbon monoxide. The depletion of the oil reserves and the emphasis towards sustainable has led to an interest in alternate sustainable strategies for the production of fumaric acid (Sebastian et al, 2019).

Being a key intermediate of the microbial metabolism, the demand for fumaric acid can satisfied by microbial fermentation. Some strains of the fungus, *Rhizopus oryzae,* have been identified as a natural overproducer of the acid. Therefore, the fungi can be utilized for the bioproduction of fumaric acid (Xu et al., 2013). This shift towards a bioproduction strategy allows for reducing the adverse environmental effect associated with fumaric acid synthesis. Moreover, the simple nutrient requirements of the fungus allow for the use of renewable resources as the primary feedstock for the production. Renewable feedstocks such as agro-industrial residues and lignocellulosic biomass can be used for production. Thus, providing for a sustainable strategy towards fumaric acid production as well as adding to the concept of circular bioeconomy.

However, the adoption of the strategy of bioproduction of fumaric acid will highly dependent on the techno-economic competitiveness of the process. To this effect, an initial techno-analysis of a process to produce 30 kt yr^{-1} of fumaric acid using agro-industrial residues as feedstock was performed. The use of these carbon rich residues generated by industrial scale activity has the potential to profoundly impact the economic competitiveness of the bioproduction process. This also allows for the valorization of the residues thereby enabling us to achieve the goal of a sustainable circular bio-economy. Moreover, the potential impact of value addition and by-product synthesis on the overall bioproduction process have been elucidated in this section.

Process description

The proposed fumaric acid bioproduction plant is presented in Figure 4.1 and is composed of upstream fumaric acid fermentation and downstream processing to obtain purified fumaric acid. The upstream process starts with the sterilization of the substrate and preparation of the seed culture. This was followed by transfer to the fermenter for production of fumaric acid at 30 °C, pH 6 and calcium carbonate as the neutralizing agent. The fermented broth will be filtered to recover the fungal biomass and then acidified at 95 °C to release fumaric acid from the calcium fumarate complex. The low solubility (5 – 7 g/L) of fumaric acid allows for easy crystallization without the need for concentrating the broth by evaporation. Following crystallization at 20 °C, the fumaric acid is recovered by the use of a rotary vacuum filter and dried.

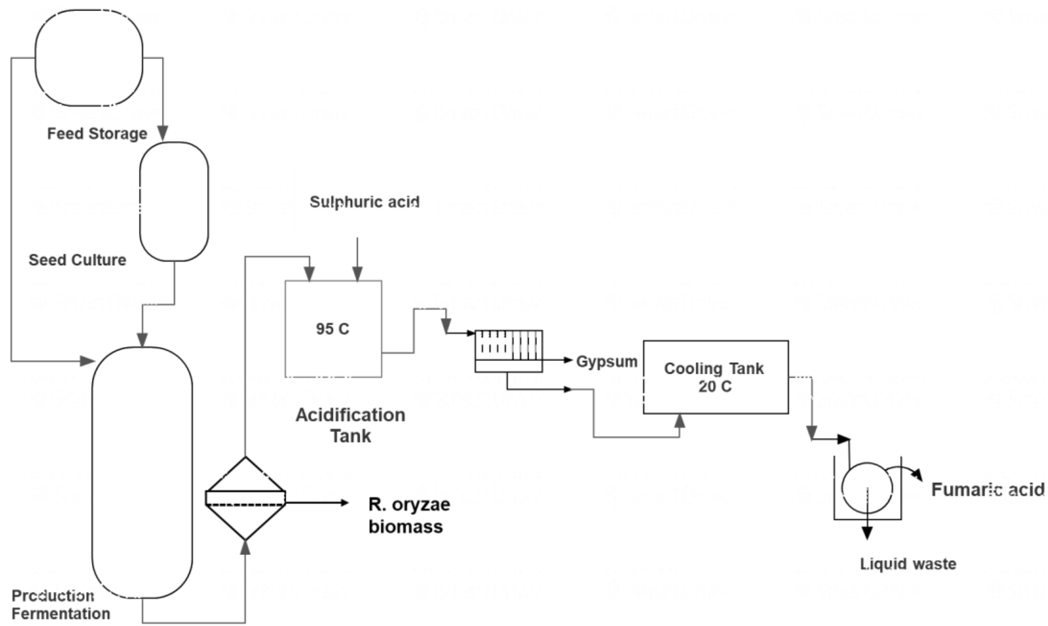

Figure 4. 1. Process flowchart for fumaric acid production via fermentation

Assumptions and economic evaluation

Stainless steel, unless specified otherwise, was used for the construction of the bioproduction process. The assumptions and the cost evaluations reported by Efe et al. (2013) were used, so as to elucidate the benefits of fumaric acid bioproduction using agro-industrial residues and to determine breakeven pricing (BEP). This breakeven pricing was calculated by considering 1 year of design, 2 years of construction, 10 years of operation and zero net present value at 10 % discount rate. Depreciation was assumed as 10 % of the fixed capital investment (FCI) and the salvage value was not included. The equipment cost, labour cost, working capital investment (WCI) and the total capital investment (TCI) were considered to be same as that reported by Efe et al. (2013) for succinic acid bioproduction.

Techno-economic analysis of fumaric acid bioproduction using agro-industrial residue as feedstock

The techno economic analysis of succinic acid bioproduction performed by Efe et al. (2013) provided for a model equation (Eq 1) to calculate BEP. Also, they identified via sensitivity analysis the main parameters that affected BEP to be succinic acid yield ($Y_{succ/S}$), sugar price (C_S), total capital investment (TCI) and energy cost (C_{energy}). The process flowchart of the proposed succinic acid bioproduction is represented in Figure 4.2. Three cases were considered and BEP was calculated for them. Cane sugar was used as substrate in first case (Case 1) whereas agro-industrial residue was used in the second case (Case 2). Fumaric acid has a considerably lower solubility than other organic and this unique property allows for removal of certain unit operations during the downstream processing. The impact of this, along with the use of agro-industrial residues, on BEP was determined in the third case (Case 3).

$$BEP\ (\$/\ Kg) = 0.3370 + 1.382{*}C_S - 0.1753{*}Y_{succ/S} + 0.0421{*}C_{energy} + 1.083{*}10^{-8}{*}TCI \qquad \text{Eq. 1}$$

This equation may be used for the calculation of the BEP for fumaric acid bioproduction, as they are closely related organic acid. During microbial metabolism fumaric acid is produced as a result of dehydrogenation of succinic acid. The BEP for fumaric acid bioproduction was obtained to be $ 2.304 ($\$/ kg^{-1}$), for the same parameters with sucrose as feedstock but a fumaric acid yield ($Y_{FA/S}$) of 0.5 (g/g). However, this BEP can be profoundly reduced by the use of agro-industrial residues such as apple pomace ultrafiltration sludge and molasses as feedstock. This use of residues as feedstock might lead to reduction in the working capital investment required for the bioproduction and in turn the total capital investment. The revised BEP for fumaric acid production, with

consideration that the cost associated with feedstock (C_S) to be negligible, was obtained to be $ 1.903 (kg^{-1}).

Table 4. 1 Capital investment for organic acid bioproduction

Cost Type	Case 1 ($)	Case 2 ($)	Case 3 ($)
Equipment purchase cost	38,561,237	38,561,237	38,561,237
Installed equipment cost	55,277,912	55,277,912	55,277,912
Fixed capital investment	149,702,352	149,702,352	149,702,352
Working capital	3,750,000	2,882,282	2,203,092
Total capital investment	153,452,352	143,040,184	134,889,900
Breakeven pricing ($/ kg)*	2.304	1.903	1.775

* The calculation of breakeven pricing provided in Appendix F

One main of the main drawback of succinic acid is its highly solubility of 58 g/L. Hence, requires energy intensive unit operations such as evaporators for concentrating the acid obtained after fermentation (Figure 4.1). These evaporators account for 64 % of the total energy cost. The low solubility of fumaric acid (5 – 7 g/L) offers a unique advantage over succinic acid. Due to this low solubility, the use of unit operations such as evaporators can be eliminated from the process and lead to a reduction in the associated TCI. This led to a further reduction in the BEP to 1.775 ($/ kg). Further reduction in the BEP can be achieved by increasing the fumaric acid titer obtained during fermentation. This increase in the titer would lead to reduced number of fermenters required as well as the concomitant reduction in TCI. Since, TCI has the most impact on the BEP, the use of an existing production system for fumaric acid production can also have profound impact on the BEP. The use of such strategies to reduce the TCI led to a reduction of BEP for succinic acid from 2.08 ($/ kg) to 1.48 ($/ kg) (Efe et al., 2013). Similar, degree of decrease in the TCI can be envisaged for fumaric acid bioproduction thereby leading to development of an economically competitive bioproduction process for fumaric acid production.

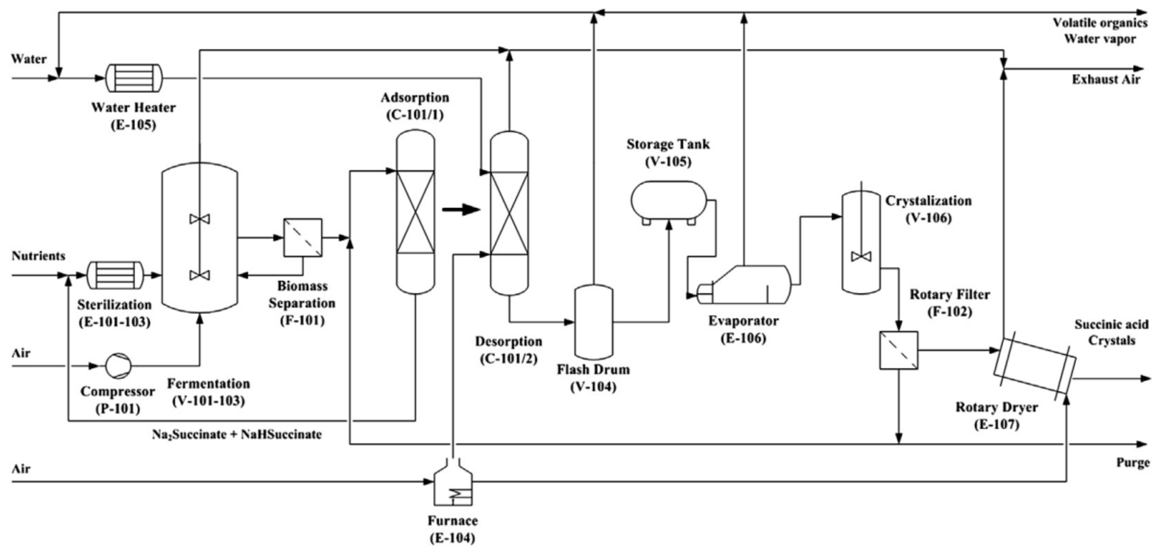

Figure 4. 2. Process flow diagram of succinic acid bioproduction (Efe et al., 2013)

Revenue generation via by-product isolation and value addition

One of the key by-products of the fumaric acid production process is the generation of the fungal biomass. The utilization of this biomass for fungal chitosan production provides for additional revenue generation that has the potential to profoundly impact the BEP for fumaric acid. Assuming a biomass concentration of 30 % of fumaric acid concentration, the 30 kt yr^{-1} fumaric acid production process can provide for a fungal biomass of 9 kt yr^{-1}. The use of MAE assisted extraction has been reported to chitosan yield of 13.4 % (g/g) (Sebastian et al., 2019). Hence, 1.2 kt yr^{-1} of chitosan can be produced as a by-product by the same bioproduction process. This amount to a revenue generation of $ 12 million per year, which is close to 9% of the TCI that has been calculated for the proposed bioproduction process. The extraction of this high value product together with the use of efficient extraction techniques, such as the microwave assisted extraction, would also be desired to further the economic competitiveness of the bioproduction process.

Additionally, value addition by conversion of obtained fumaric acid to fumaric acid esters (FAEs) can be highly beneficial. A near complete conversion of fumaric acid to its ester form (99.2 %) is

afforded by the use of a chemical conversion process. The significant difference in the prices, 1 dollar (fumaric acid) versus above 100 dollars (FAEs), makes this value addition highly desirable. Hence, the adoption of this value addition to even 10 % of the 30 kt fumaric acid produced per year, would amount to a gross revenue generation of $ 300 million per year. This revenue generated is twice the amount of the TCI that has been calculated for the proposed fumaric acid bioproduction process. Therefore, the development of such an integrated bioproduction process with bioproduction of fumaric acid, followed by value added conversion to FAEs, and extraction of chitosan from the by-product (fungal biomass) should be envisaged in the future. The additional revenue generated via the isolation of a high value compound from the by-product as well as value addition has the potential to profoundly affect the investment return rate.

Conclusion

The techno economic analysis of the proposed fumaric acid bioproduction process has confirmed the economic competitiveness of the process. BEP for fumaric acid was calculated to be 2.304 ($/ kg) for a bioproduction process designed for succinic acid production. Fumaric acid has ten-time lower solubility than succinic acid. Hence, certain unit operations could be removed from the process leading to a decrease in the TCI. This reduction in the TCI provided for a reduction in BEP to 1.775 ($/ kg). The additional optimization of the bioproduction process, by increasing yield and reducing TCI, has the potential to reduce BEP even further to achieve a price of 1.5 ($/ kg). Moreover, the isolation of chitosan as by-product as well as value addition by conversion of fumaric acid FAEs can increase the revenue generated. This increased revenue generation has been envisaged to further reduce the BEP.

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CONCLUSIONS ET RECOMMANDATIONS

CONCLUSIONS

Les conclusions suivantes peuvent être tirées a partir de ce projet de recherche :

8.1 La mousse de polystyrène s'est avérée être le support idéal pour l'immobilisation de *Rhizopus oryzae* pour la production d'acide fumarique. La fermentation avec le *R. oryzae* immobilisé a permis de réduire considérablement la phase de latence. De plus, la réduction de la taille du matériau de support, passant de cubes de 1 cm à des billes de mousse de polystyrène de 0,1-0,3 cm, a conduit à une amélioration significative de la production d'acide fumarique (27 g/L contre 19 g/L). Une amélioration similaire de la concentration finale en acide fumarique, 7,9 g/L contre 6,3 g/L, a été observée lorsque les boues d'ultrafiltration de résidus de pomme ont été directement utilisées comme matière première. La supplémentation du bouillon de fermentation avec de la mélasse a permis de tripler la concentrationen acide fumarique.

8.2 Après avoir identifié le support d'immobilisation idéal pour *R. oryzae* et le mode de fermentation, la fermentation a été mise en échelle en utilisant un fermenteur de 4 litres pour augmenter la production de l’acide fumarique. Cette étude de mise en l'échelle a permis de quadrupler la concentration d'acide fumarique, pour atteindre une concentration d'acide fumarique de près de 12 g/L, en utilisant l'APUS et la mélasse comme substrats. La fermentation en mode fed-batch ainsi que l'immobilisation ont amélioré le rendement et la productivité. Des concentrations d'acide fumarique de 2,9 g/L et 7,7 g/L ont été obtenus pour les fermentations mycéliennes libres réalisées en mode batch et fed-batch.

8.3 La possibilité d'utiliser la biomasse lignocellulosique comme matière première pour la production d'acide fumarique a été étudiée. *Miscanthus* sp., une herbe pérenne, a été identifiée

comme la matière première lignocellulosique idéale pour la production d'acide fumarique, comparé au panic erigé et au chanvre. Le prétraitement du *Miscanthus* sp. a permis d'obtenir 39,5 g/l de sucre réducteur avec une conversion enzymatique de 79 % de la biomasse obtenue après prétraitement alcalin. Le spectre FTIR a révélé que ce traitement alcalin était efficace pour éliminer la lignine et l'hémicellulose. Une concentration d'acide fumarique de 8-9 g/L a été obtenue en utilisant l'hydrolysat du *Miscanthus* sp. (Silvergrass) comme matière première. De plus, la souche fongique *R. oryzae* NRRL 1526 a été identifiée comme la meilleure souche pour sa productivité et son rendement supérieurs.

8.4 La valeur ajoutée par la conversion de l'acide fumarique en esters d'acide fumarique (EAF) peut avoir un impact profond sur la compétitivité technico-économique du processus de bioproduction. Les EAF sont des ingrédients pharmaceutiques actifs ayant des applications dans le traitement de la sclérose en plaques et du psoriasis. À cet effet, la condition optimale pour la conversion de l'acide fumarique en ses dérivés esters, le DMF et le MMF, a été identifiée en utilisant l'analyse RSM. Les conditions optimales ont été identifiées comme étant 111 minutes, 98 °C et 9,7 % (v/v) d'acide sulfurique, pour la synthèse du DMF, et 30 minutes, 55,5 °C et 2 % (v/v) d'acide sulfurique, pour la synthèse du MMF. L'utilisation de conditions optimales a permis de convertir à 99,2 % l'acide fumarique d'origine biologique en ses esters. Cette évolution vers l'utilisation d'acide fumarique d'origine biologique pour la synthèse du DMF et du MMF permet d’avoir une production durable d'ingrédients pharmaceutiques actifs essentiels.

8.5. La biomasse fongique est l'un des principaux sous-produits de la bioproduction d'acide fumarique induite par *R. oryzae* qui peut être utilisée pour l’obtention du chitosane fongique. À cet effet, les conditions optimales d’extraction assistée par micro-ondes (EAM)dans cette étude étaient de 300W et 22min. Le chitosane obtenu dans ses conditions optimales de l'EAM, comparé

au chitosane obtenu par le procédé conventiel (chauffage et traitement acido-basique) se caractérise par un degré de désacétylation plus élevé (94,6% contre 90,6%). Cependant, le poids moléculaire ainsi que la cristallinité se sont avérés être similaires. Il a été également observé que le EAM a permis l'obtention d'une plus grande quantité de chitosane par rapport au procédé conventionnel, 13,43% (≈2 fois) contre 6,67% (w/ w). Cette augmentation du rendement en chitosane, en plus de la réduction significative de la facture énergétique, 1,1 cents contre 50 cents pour le chauffage conventionnel, peut avoir un impact positif sur la faisabilité et l'attractivité de la production commerciale de chitosane fongique.

8.6 La faisabilité et l'opportunité d'une bioproduction dépendent fortement de la compétitivité technico-économique du procédé par rapport à la conversion chimique de la matière première dérivée du pétrole en acide fumarique. L'analyse technico-économique du procédé de bioproduction d'acide fumarique proposé a confirmé la compétitivité économique du procédé de bioproduction. Le prix d'équilibre (BEP) de l'acide fumarique a été calculé à $ 2,304 (kg^{-1}) pour un procédé de bioproduction conçu pour la production d'acide succinique. L'acide fumarique est dix fois moins soluble que l'acide succinique. Par conséquent, certaines opérations unitaires pourraient être supprimées du procédé, ce qui entraînerait une diminution de l'investissement total en capital (ITC). Cette réduction de l'investissement total en capital a permis de réduire les MPE à $ 1,775 (kg^{-1}). L'optimisation supplémentaire du processus de bioproduction, en augmentant le rendement et en réduisant le TCI, a le potentiel de réduire encore plus le BEP pour atteindre un prix de $ 1,5 (kg^{-1}). De plus, l'isolement du chitosane comme sous-produit ainsi que l'ajout de valeur par la conversion des EAF d'acide fumarique peuvent augmenter les revenus générés. Cette augmentation des revenus générés a été envisagée pour réduire davantage les BEP.

RECOMMENDATIONS

Sur la base des résultats obtenus dans cette étude, les recommandations suivantes sont à considérer:

1. L'étude à l'échelle du laboratoire a montré que la fermentation en mode fed-batch était efficace pour améliorer la concentrationen acide fumarique. Cependant, une optimisation plus avancée des conditions opérationnelles est essentielle pour améliorer davantage les la production d'acide fumarique ainsi que l'identification des conditions optimales à utiliser pour une productionà l'échelle industrielle.

2. Des travaux supplémentaires sont essentiels pour améliorer la concentration en sucre obtenue lors de la préparation de l'hydrolysat à partir de la biomasse lignocellulosique. À cet effet, des enzymes cellulases à haut rendement peuvent être utilisées et les conditions de prétraitement de la biomasse lignocellulosique doivent être optimisées.

3. La production d'acide fumarique peut être amélioré via l'optimisation des conditions de fermentation et le choix convenable du support d'immobilisation.

4. Une conversion chimique a été utilisée pour la synthèse des esters de fumarate mais une bioconversion enzymatique pourrait être possible. L'étude utilisant la lipase n'a pas permis la synthèse d'esters d'acide fumarique. Cependant, d'autres enzymes peuvent être utilisées pour la synthèse des esters et doivent être étudiées.

5. Une analyse technico-économique plus détaillée de la bioproduction d'acide fumarique doit être réalisée pour mieux dresser sa compétitivité et sa faisabilité. Les effets de l'intégration du chitosan fongique comme sous-produit doivent être considérés. De plus, l'analyse technico-économique intégrant l'utilisation de la biomasse lignocellulosique comme matière première pour la production d'acide fumarique doit être étudiée.

APPENDIX

APPENDIX A (Supplementary Data- Chapter 2 Part 1)

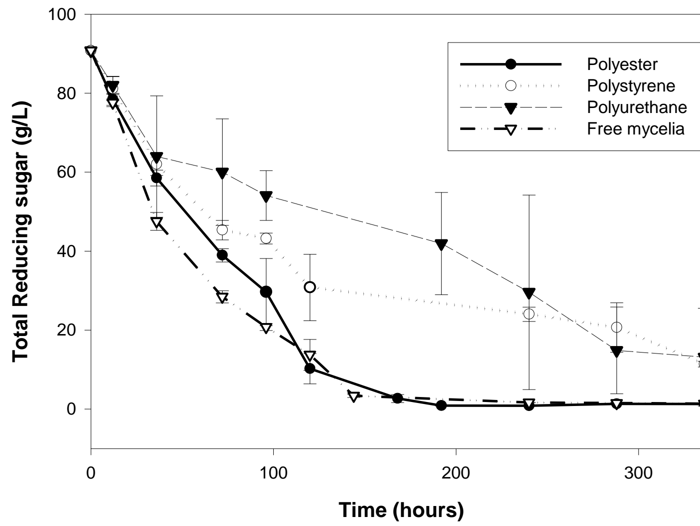

Figure A1: Comparison of total reducing sugar consumption during fermentation using different immobilization support materials

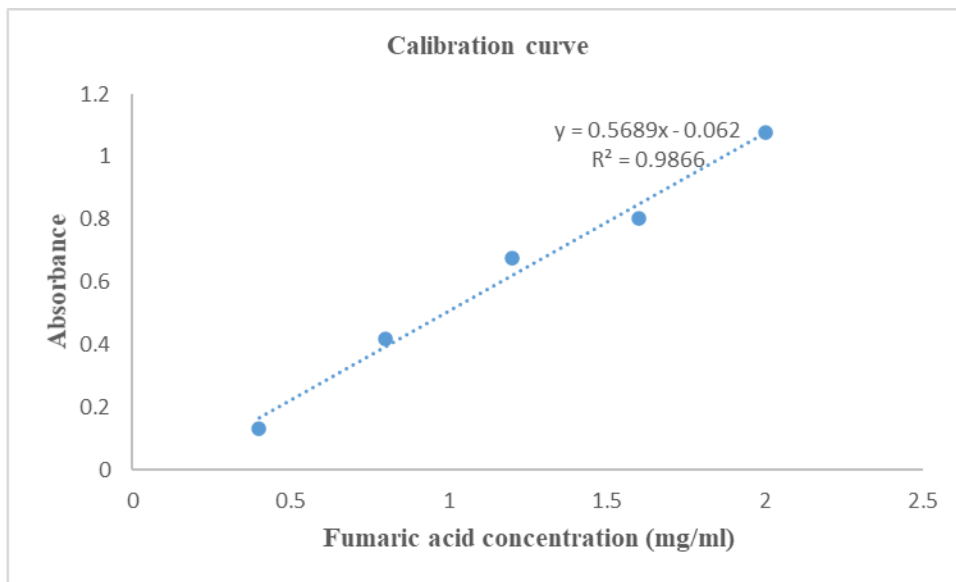

Figure A2: Fumaric acid calibration curve

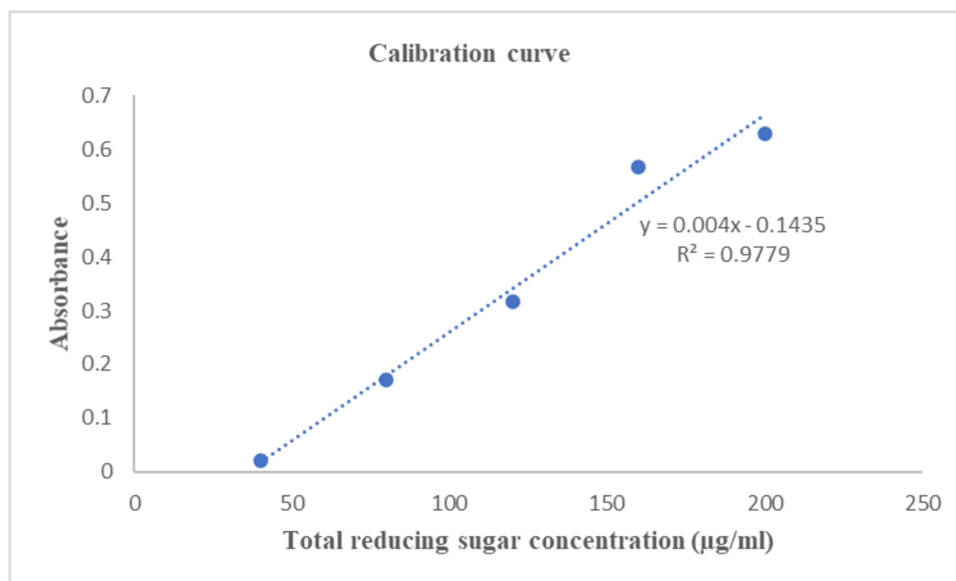

Figure A3: Total reducing sugar calibration curve

APPENDIX B (Supplementary Data- Chapter 2 Part 2)

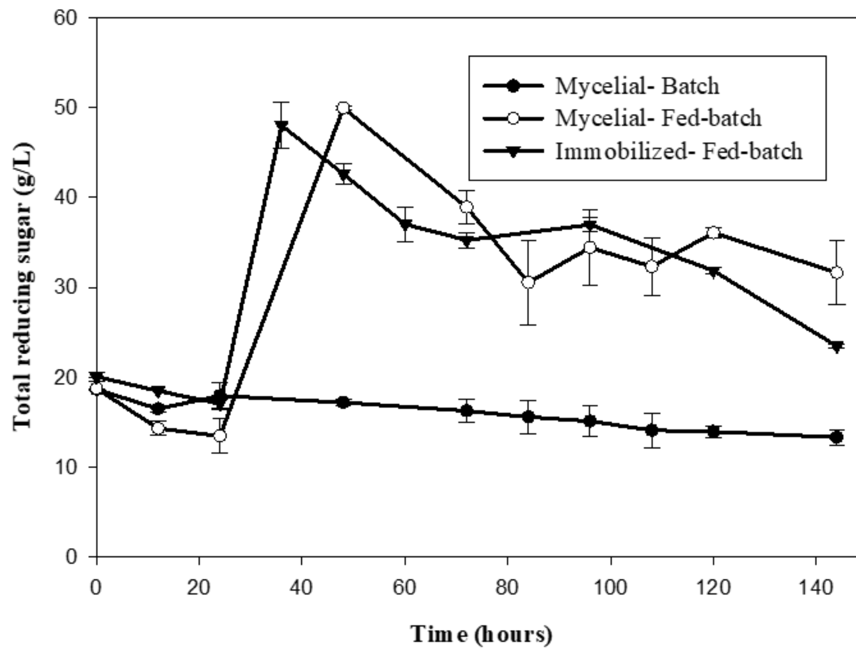

Figure B1: Comparison of the total reducing sugar consumed during fermentation operated in batch and fed-batch mode

APPENDIX C (Supplementary Data- Chapter 2 Part 3)

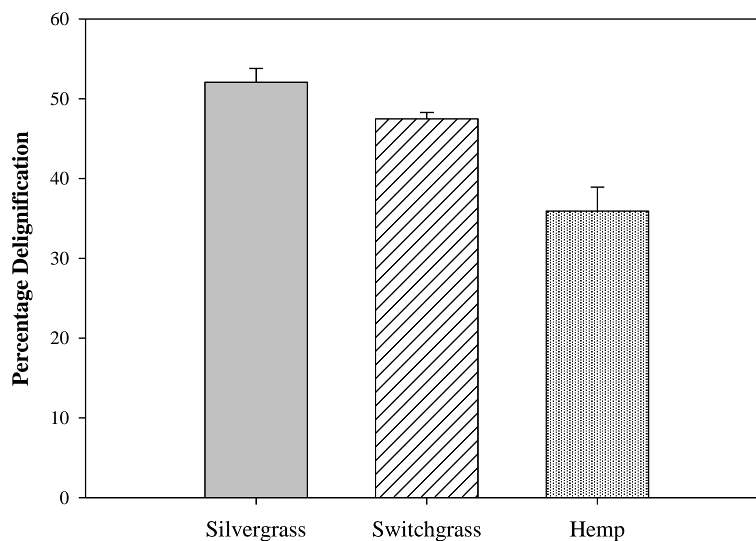

Figure C1: Percentage delignification of lignocellulosic biomass after alkaline pretreatment

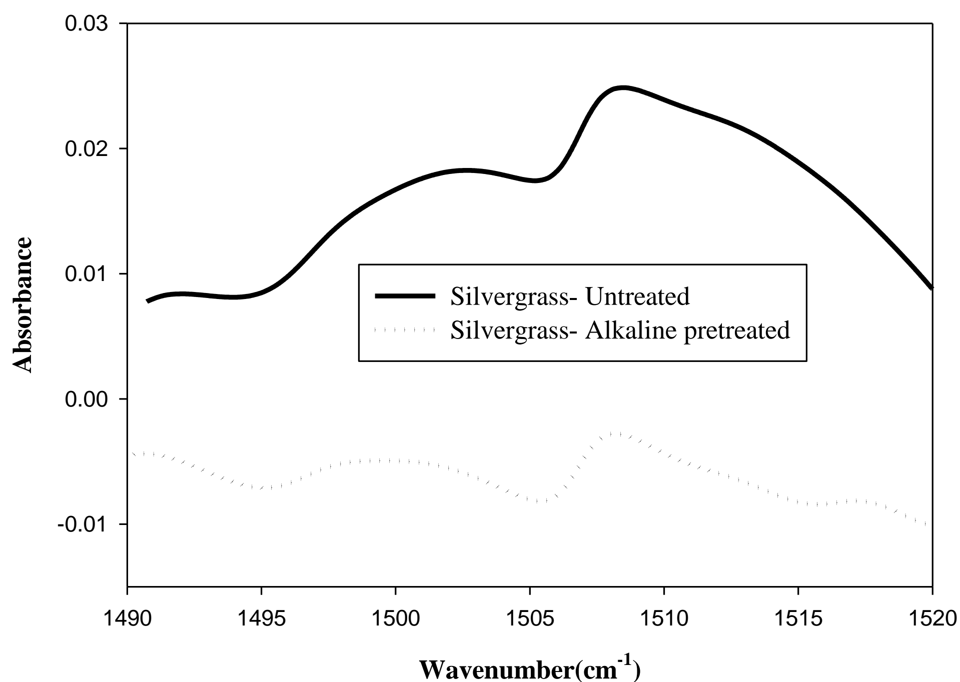

Figure C2: FT-IR spectrum between 1490 cm^{-1} – 1520 cm^{-1} showming intensity reduction at 1510 cm^{-1} corresponding aromatic group present in lignin

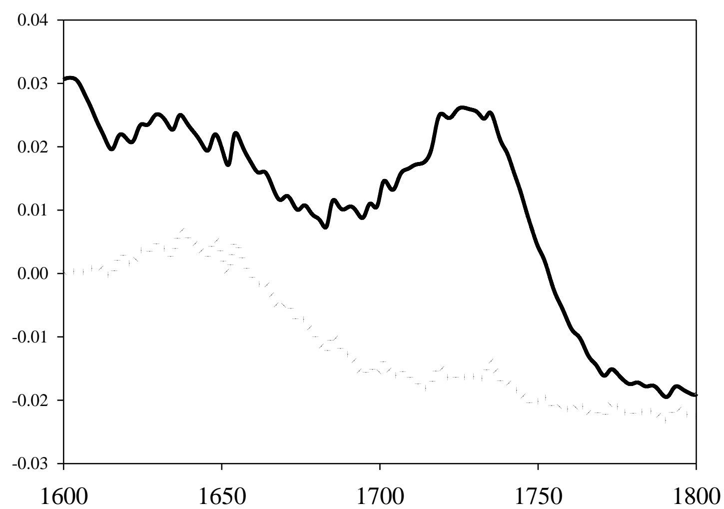

Figure C3: FT-IR spectrum between 1600 cm^{-1} – 1800 cm^{-1} showming intensity reduction at 1730 cm^{-1} corresponding carbonyl group present in hemicellulose (Solid Line- Untreated biomass; Dotted line- Alkaline pre-treated biomass)

APPENDIX D (Supplementary Data- Chapter 3 Part 1)

Calibration Report

Method: C:\ChromQuest\Methods\fumarates20210331.met
Print Time: 20/04/2021 4:02:59 PM
User: System
Instrument: HPLC-UV (Offline)

DMF (UV2000-210nm)
Average RF: 721039. RF StDev: 109142. RF %RSD: 15.1367
Scaling: None LSQ Weighting: None Force Through Zero: Off
Replicate Mode: Replace
Fit Type: Quadratic
y = -1857.97x^2 + 817045.x - 106443.
Goodness of fit (r^2): 0.999982

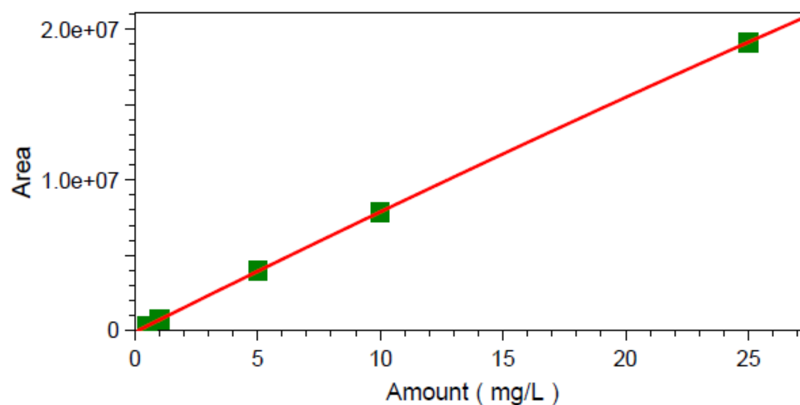

Figure D1: Calibration report for dimethyl fumarate (DMF) using quantification using HPLC

Calibration Report

Method: C:\ChromQuest\Methods\fumarates20210331.met
Print Time: 20/04/2021 4:02:12 PM
User: System
Instrument: HPLC-UV (Offline)

MMF (UV2000-210nm)
Average RF: 558651. RF StDev: 33225.3 RF %RSD: 5.94742
Scaling: None LSQ Weighting: None Force Through Zero: Off
Replicate Mode: Replace
Fit Type: Quadratic
y = -1040.65x^2 + 565976.x + 18470.6
Goodness of fit (r^2): 0.999950

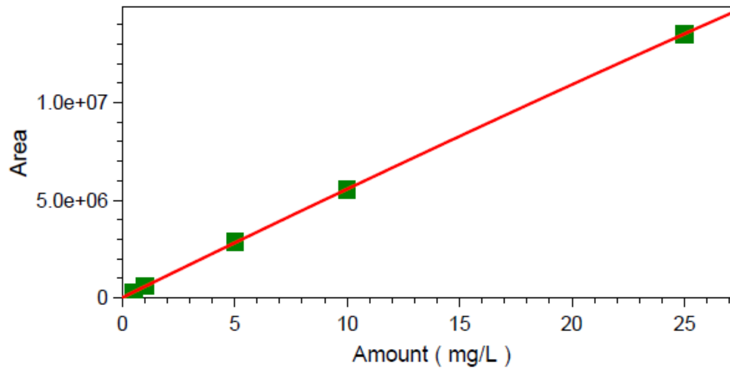

Figure D2: Calibration report for monomethyl fumarate (MMF) using quantification using HPLC

Calibration Report

Method: C:\ChromQuest\Methods\fumarates20210331.met
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User: System
Instrument: HPLC-UV (Offline)

fumaric acid (UV2000-210nm)
Average RF: 790380. RF StDev: 106963. RF %RSD: 13.5331
Scaling: None LSQ Weighting: None Force Through Zero: Off
Replicate Mode: Replace
Fit Type: Quadratic
y = -490.799x^2 + 704777.x + 143910.
Goodness of fit (r^2): 0.999990

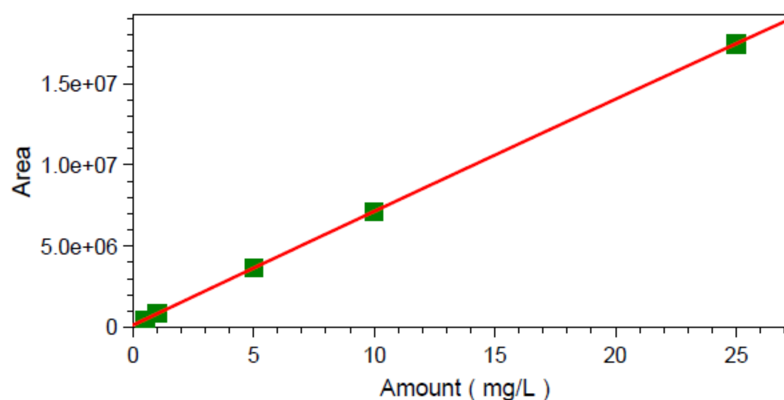


Figure D3: Calibration report for fumaric acid quantification using HPLC

Calibration Report

Sequence : C:\ChromQuest\Sequence\Fumarates20210413.seq
User : System
Printed : 20/04/2021 3:58:42 PM

UV2000-210nm

Compound	Old RT (Min)	New RT (Min)	RF Average	RF %RSD	RF %RSD Limit	Status
fumaric acid	4.598	4.317	790379.680	13.5331	0.00	Passed
MMF	6.827	6.093	558650.980	5.9474	0.00	Passed
DMF	12.317	10.513	721039.180	15.1367	0.00	Passed

Figure D4: Calibration report for the fumaric acid esters with their corresponding retention time

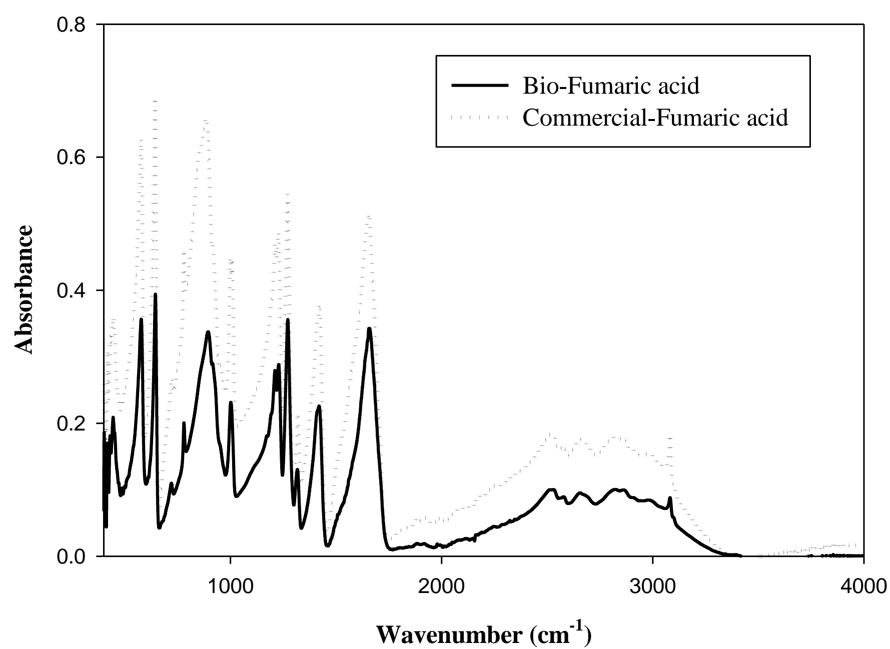


Figure D5: FT-IR spectrum of biobased fumaric acid and commercial fumaric acid

APPENDIX E (Supplementary Data- Chapter 3 Part 2)

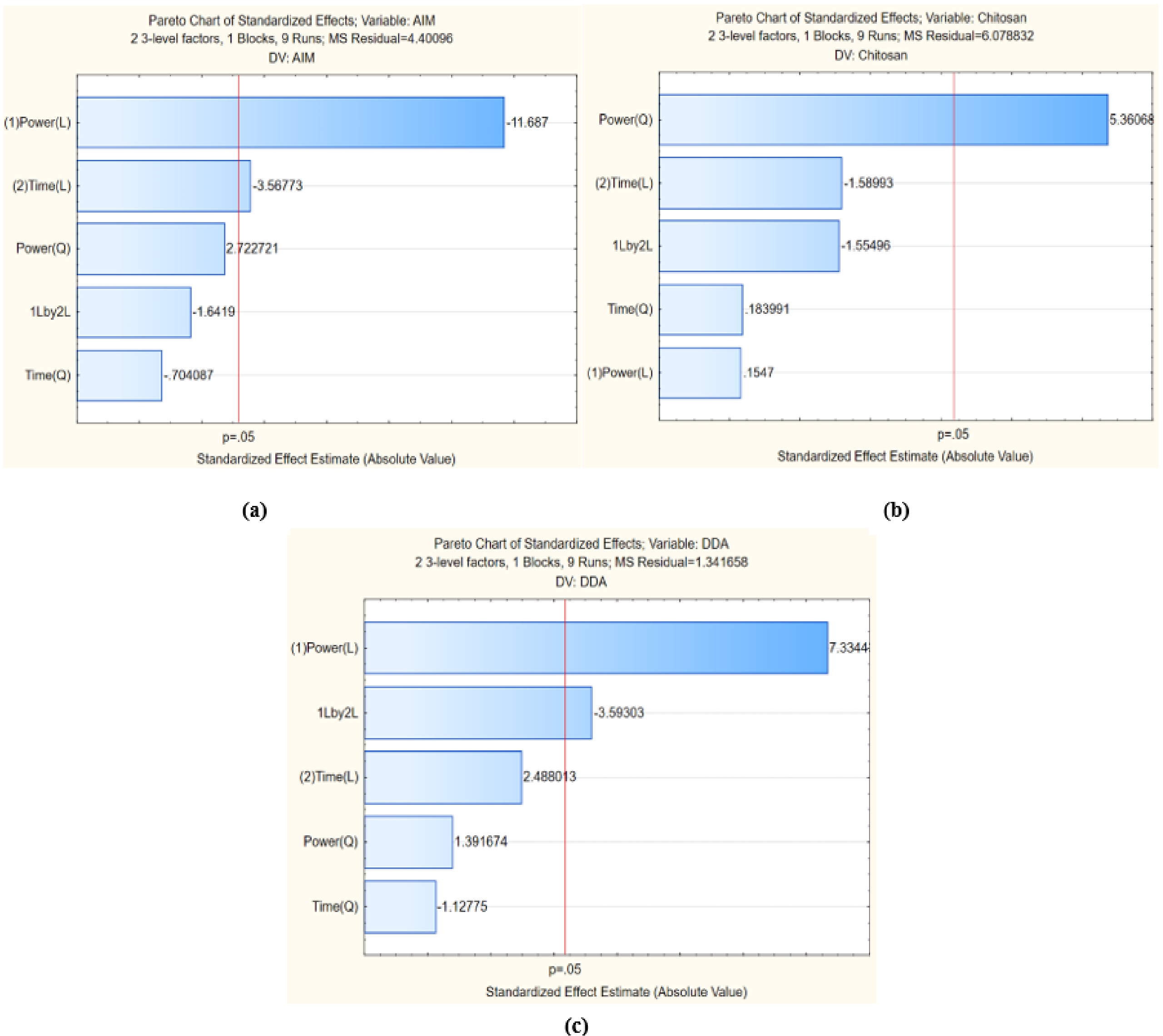
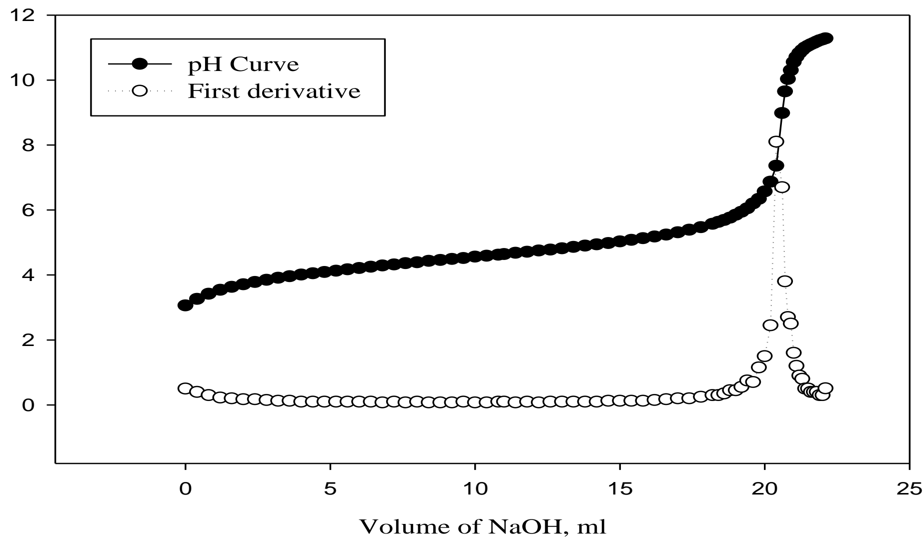


Figure E1: Pareto Plot that shows the effect of Power and Time on AIM yield (a), Chitosan yield (b) and Degree of deacetylation (c)



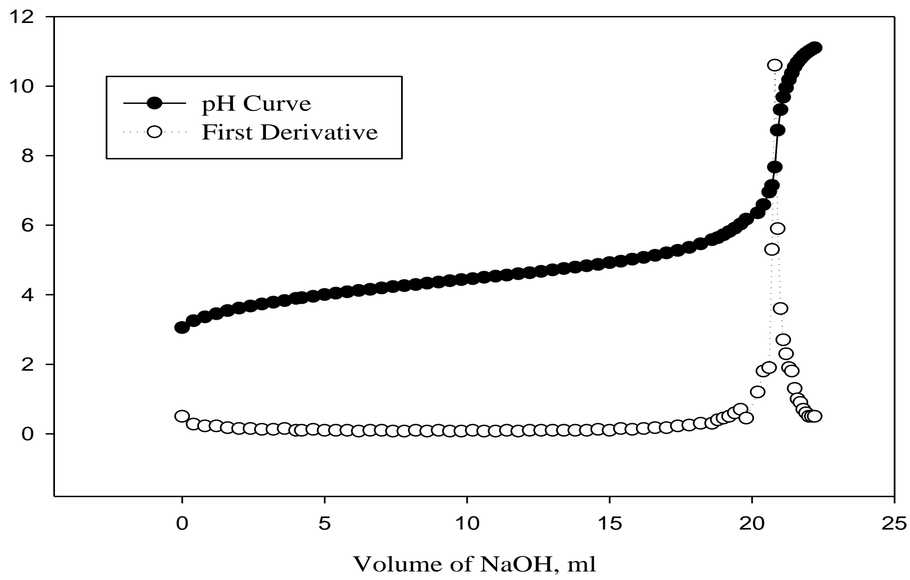


Figure E2: pH curve and first derivative of chitosan obtained by conventional heating (a); and microwave extraction (b)

APPENDIX F (Supplementary data- Chapter 4)

Table F1: Value of parameters used for the calculation of breakeven price (BEP)

Cases	Carbon source cost, C_s ($/kg)	Yield, $Y_{FA/sugar}$ (g/g)	Energy cost, C_{energy} ($ GJ^{-1})	Total capital investment, TCI ($)
1	Sucrose, 0.212	0.5	2.5	153,452,352
2	AIR*, 0.0	0.5	2.5	143,040,184
3	AIR*, 0.0	0.5	1.5	134,889,900

*AIR- Agro-industrial residues

<u>Case – 1</u>

Carbon source – Sucrose

BEP ($/kg) = $0.3370 + 1.382*C_S - 0.1753*Y_{succ/S} + 0.0421*C_{energy} + 1.083*10^{-8}*TCI$

$= 0.3370 + 1.382*0.212 - 0.1753*0.5 + 0.0421*2.5 + 1.083*10^{-8}*1.534*10^{8}$

$= 2.304$

<u>Case -2</u>

Carbon source - Agro-industrial residues

BEP ($/kg) = $0.3370 + 1.382*C_S - 0.1753*Y_{succ/S} + 0.0421*C_{energy} + 1.083*10^{-8}*TCI$

$= 0.3370 + 1.382*0 - 0.1753*0.5 + 0.0421*2.5 + 1.083*10^{-8}*1.431*10^{8}$

$= 2.011$

<u>Case - 3</u>

Carbon source - Agro-industrial residues

Energy cost – 60% reduction in energy cost as a result of no evaporator required due fumaric acid low solubility

BEP ($/kg) = $0.3370 + 1.382*C_S - 0.1753*Y_{succ/S} + 0.0421*C_{energy} + 1.083*10^{-8}*TCI$

$= 0.3370 + 1.382*0 - 0.1753*0.5 + 0.0421*1.5 + 1.083*10^{-8}*1.350*10^{8}$

$= 1.775$