Biotransformation of biomass waste residues into value-added chemicals using filamentous fungi

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Abstract

Use of biomass as feedstock for the production of carbon-based fuels and chemicals represents an essential component of the global strategy to attain sustainable and circular industrial economies. Current strategic objectives for biomass conversion technologies focus on enhancing cost competitiveness through, for example, the use of cheaper feedstocks (e.g. lignocellulosic wastes). However, adoption of lignocellulosic biomass as substrate will only be possible after addressing the inefficiencies and costs which stem from the additional processing necessitated by the compositional complexity of lignocellulosic materials. Nature, wherein the recycling of lignocellulosics is an essential ecological process, may offer strategies for engineers and scientists to emulate.

Fungi, particularly filamentous fungi, are well-known for their role as material recyclers in the natural carbon cycles and, as such, are intriguing for use in biomass-conversion, or biorefining processes. Application of filamentous fungi in biorefineries was therefore examined in a comprehensive literature review (Chapter 2). From this review, it may be concluded that while no single strain of fungi, indeed no microorganism, may practically perform all the process steps (i.e. from pre-processing of raw biomass to accumulation of desired products), the complete valorization of biomass is possible by employing and/or combining multiple fungal strains at specific steps in the process, and by targeting multiple products. Concerning the products of fungal biotransformations, the number of potential products is proportional to the number of metabolites associated with a given organism; and these metabolic products remain largely underexplored or altogether unidentified. Therefore, a method for screening and evaluating a large number of products that may be obtained from fungal biotransformation of various biomass feedstocks was developed as part of the study described in Chapter 3. The products of the biotransformations were identified using non-targeted ultra-performance liquid chromatography coupled with quadrupole-time-of-flight electrospray ionization mass spectrometry and evaluated using the multivariate statistical tools commonly employed in the field of metabolomics. Identified among the products were valuable chemicals, such as those described in two recent US Department of Energy surveys, including furans and organic acids. In addition to these “top chemical opportunities”, substantial production of bio-pigments was also observed. Of the feedstocks investigated, a simulated food-waste feedstock was associated

with the highest pigment production and was thus further investigated for maximal pigment production (Chapter 4). To that end, an optimized extraction method was employed and, following this, a novel strategy was explored involving co-culturing of multiple strains of fungi together in a single conversion reaction. It was found that co-culturing the pigment-producing fungus with a specific second species elicited significantly more pigment production vis-à-vis the pigment-producing species alone.

2,5-furandicarboxylic acid (FDCA), a compound of industrial interest for use as a replacement for terephthalic acid in polymer production, was also identified among the fungal biotransformation products of apple pomace feedstock. Since FDCA is mainly produced via oxidation of 5-hydroxymethylfurfural (HMF), itself a carbohydrate-derived product, the process of using filamentous fungi for converting HMF into FDCA was more closely investigated (Chapter 5). Several industrially relevant strains of filamentous fungi, including the strain used throughout this project (*Talaromyces* sp. NRRL 2120), were screened for oxidative activity on HMF. It was found that multiple strains of fungi readily performed one oxidation on HMF (i.e. converted HMF into its acid counterpart) but accumulated very little, if any, FDCA under the given reaction conditions. To enable additional oxidation steps, a chemo-biocatalytic cascade involving the best-performing fungal strain coupled with an enzyme/mediator system was devised. This system enabled full conversion of HMF into FDCA and achieved the highest yield and productivity for any system involving filamentous-fungal whole-cells. Following this, the ability to reuse the whole-cell biocatalysts for multiple reactions was explored via encapsulation of the filamentous fungal whole cells (Chapter 6). Here it was found that encapsulation of the fungal cells in Ca-alginate beads enabled the biocatalysts to be reliably recycled for up to nine reaction cycles.

Résumé

L'utilisation de la biomasse comme matière première pour la production de carburants et de produits chimiques représente un élément essentiel de la stratégie mondiale visant à mettre en place des économies industrielles durables et circulaires. Les objectifs actuels à la réalisation des technologies de conversion de la biomasse sont axés sur l'amélioration de la compétitivité des coûts, notamment par l'utilisation de matières premières moins coûteuses (par exemple, les déchets lignocellulosiques). Cependant, l'utilisation de produits lignocellulosiques ne sera possible qu'après avoir résolu les inefficacités et les coûts dus aux étapes additionnelles de transformation requises en raison de la complexité compositionnelle des matières lignocellulosiques. La nature, où le recyclage des matières lignocellulosiques est un processus écologique essentiel, peut offrir des modèles de stratégies que les ingénieurs et les scientifiques pourront imiter.

Les mycètes, en particulier les mycètes filamenteux, sont bien connus pour leur rôle de recycleurs de matériaux dans les cycles naturels du carbone et, à ce titre, ils sont d'intéressants candidats à prendre en considération dans les processus de conversion de la biomasse (bioraffinage). L'application des mycètes filamenteux dans les bioraffineries a donc été examinée dans le cadre d'une revue exhaustive de la littérature (Chapitre 2). Cette revue littéraire permet de conclure que, si aucune souche de mycète, voire aucun micro-organisme, ne peut réaliser à lui seul toutes les étapes de la conversion de la biomasse (du prétraitement de la biomasse brute à l'accumulation des produits souhaités), la valorisation complète de la biomasse est possible en employant et/ou en combinant plusieurs souches fongiques à des étapes spécifiques du processus et en ciblant plusieurs produits. En ce qui concerne les produits des biotransformations fongiques, le nombre de produits potentiels est proportionnel au nombre de métabolites associés à un organisme donné, et ces produits métaboliques restent largement sous-explorés ou totalement non identifiés. Par conséquent, une méthode de criblage et d'évaluation du grand nombre de produits pouvant être obtenus par la biotransformation fongique de matières premières de la biomasse a été mise au point dans le cadre de l'étude décrite au chapitre 3. Les produits issus des biotransformations ont été identifiés à l'aide d'une chromatographie liquide ultra-performante non ciblée couplée à une spectrométrie de masse par électronébulisation à temps de vol quadripolaire et évalués à l'aide d'outils statistiques multivariés couramment utilisés

dans le domaine de la métabolomique. Parmi les produits identifiés, on retrouve des produits chimiques extrêmement intéressants tels que ceux décrits dans deux études récentes du ministère américains de l'énergie, notamment les furanes et les acides organiques. En plus de ces composés, une production substantielle de biopigments a également été observée. Parmi les matières premières étudiées, la simulation de la fermentation des déchets alimentaires a été associée à la production de pigments la plus élevée et a donc fait l'objet d'une étude plus approfondie en vue d'une production accrue de pigments (chapitre 4). À cette fin, une nouvelle méthode d'extraction optimisée a d'abord été employée, puis une nouvelle stratégie a été explorée, impliquant la co-culture de plusieurs souches de champignons dans une seule réaction de conversion. Il s'est avéré que la co-culture du champignon producteur de pigments, *Talaromyces* sp. NRRL 2120 avec une seconde espèce particulière permettait de doubler la production de pigments.

L'acide furandicarboxylique (FDCA), une molécule d'intérêt industriel utilisé pour remplacer l'acide téréphtalique dans la production de polymères, a également été identifié parmi les produits de biotransformation fongique, en particulier de la matière première du marc de pomme. Le FDCA étant principalement produit par l'oxydation de 5-hydroxymethylfurfural (HMF), un produit dérivé des hydrates de carbone, le processus d'utilisation de champignons filamenteux pour convertir le HMF en FDCA a été étudié de plus près (chapitre 5). Plusieurs souches de mycètes d'intérêt industriel ont été examinées pour leur activité oxydante sur le HMF. Il a été constaté que plusieurs souches de champignons effectuaient facilement une oxydation sur le HMF mais accumulaient très peu, voire pas du tout, de FDCA dans les conditions de réaction données. Pour permettre des étapes d'oxydation supplémentaires, une cascade chimio-biocatalytique impliquant la souche fongique la plus performante couplée à un système enzyme/médiateur a été conçue. Ce système a permis la conversion complète du HMF en FDCA et a atteint le rendement et la productivité les plus élevés de tous les systèmes impliquant des cellules entières de champignons filamenteux. Ensuite, l'encapsulation des cellules entières fongiques filamenteuses a été étudiée afin de permettre le recyclage du biocatalyseur à cellules entières (chapitre 6). Il a été constaté que l'encapsulation des cellules entières fongiques dans des billes de Ca-alginate permettait de recycler les biocatalyseurs de manière fiable jusqu'à neuf cycles de réaction.

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List of Abbreviations

Abbreviation	Details
ADH	Aldehyde Dehydrogenase
AP	Apple pomace
AUC	Area under the curve
bbl.	Barrel
BGC	Biosynthetic gene cluster
BSTFA	*N*,*O*-Bis(trimethylsilyl)trifluoroacetamide
CBP	Consolidated Bioprocess(ing)
CAD	Cis-Aconitate Decarboxylase
CMCase	Carboxy Methyl Cellulase
CC	Corn cob
DFA	Detergent fiber analysis
DFF	2,5-diformylfuran
DSM	Dry starting matter
FC	Fold change
FDCA	2,5-furandicarboxylic acid
FFCA	2-formyl-5-furancarboxylic acid
FTIR-ATR	Fourier-transform infrared spectroscopy-attenuated total reflectance
GC-MS	Gas Chromatography-Mass Spectrometry
gds	Grams dry substrate
GHG	Greenhouse Gas
HCA	Hierarchical clustering analysis
HMF	5-hydroxymethylfurfural
HMFCA	5-hydroxymethyl-2-furan carboxylic acid
HPLC	High Performance Liquid Chromatography
HW	Hardwood

IA	Itaconic Acid
IU	International Unit
KA	Kojic Acid
LA	Levulinic Acid
LiP	Lignin Peroxidase
MDH	NAD-Malate Dehydrogenase
MESP	Minimum Ethanol Selling Price
MnP	Manganese Peroxidase
MonAzP	*Monascus* azaphilone pigment
MSM	Mineral Salt Media
NAD(H)	Nicotinamide Adenine Dinucleotide
PC	Principal component
PCA	Principal component analysis
PDA	Potato dextrose agar
PDB	Potato dextrose broth
PDC	Pyruvate Decarboxylase
PDO	1,3-propanediol
PFK-1	Phosphofructokinase-1
PLA	Polylactic Acid
QC	Quality control
ROC	Receiver operating curve
ROI	Return on Investment
SEM	Scanning electron microscope
SFW	Simulated food waste
SHMM	Spent-Hydrolysate Model Medium
SmF	Submerged Fermentation
SSF	Solid-State Fermentation
TCA	Tricarboxylic Acid (cycle)
TEMPO	(2,2,6,6-tetramethylpiperidin-1-yl)oxidanyl
TGA	Thermogravimetric analysis

U	Enzyme Unit
UPLC-QTOF-ESI-MS	Ultra-performance liquid chromatography coupled with quadrupole-time-of-flight electrospray ionization mass spectrometry
USDA	US Department of Agriculture
US DOE	US Department of Energy

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Contribution of Authors

This thesis is submitted in the form of original papers published, accepted or to be submitted for publication to international and peer-reviewed scientific journals. The candidate, Derek Troiano, was responsible for designing and conducting the experiments, handling the data analysis, as well as the preparation of the manuscript and thesis. Dr. Valérie Orsat and Dr. Marie-Josée Dumont, respectively the supervisor and co-supervisor of the candidate as well as Professor and Associate Professor in the Department of Bioresource Engineering of McGill University, contributed to all aspects of this research work. Both provided assistance in the form of planning and directing research, editing and reviewing the thesis and manuscripts before submission for publication. Felicity Meyer, an MSc student in the Department of Bioresource Engineering of McGill University conducted a portion of the data analysis and was listed as a co-author on the fifth paper. The details of the papers that have been published, accepted for publication, or submitted for publication are provided below:

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Chapter 1: Introduction

1.1. General Introduction

The accelerated consumption of fossil resources beginning in the 19th century has enabled rapid technological progress but has, at the same time, wreaked environmental havoc (e.g. carbon emissions and accumulation of plastic waste), and exacerbated unequal global resource distribution.[1] While renewable energy is available through a variety of non-carbon-based technologies (e.g. solar, hydro, wind, tidal, and nuclear), the use of carbon-based feedstock is nonetheless unavoidable for the production of certain materials and chemicals.[2] Technologies which use CO_2 to meet the global carbon needs are still in infancy, and thus biomass represents the current focus of research.[2a, 3] Full realization of biomass processing operations (i.e. biorefineries) will require government policy support, unfavorable fossil fuel prices, as well as the reduction of biomass processing input costs and maximization of biorefinery profitability.[4] Concerning the latter, one strategy for minimizing input costs is the use of local waste stream residues (e.g. agricultural, forestry, and post-consumer residues) as feedstock.[5] Advantages associated with such feedstocks may include the obviation of additional land, water, fertilizer, etc. for its production, no competition for use in the food and feed economy, and reduction of costs associated with transportation.[6] On the other hand, unlike simple carbohydrate feedstocks, these waste-biomass residues require additional processing prior to conversion into final products due to the recalcitrance of the lignocellulosic composition.[7] These additional steps can ultimately represent an excess of 45% of the total operation cost.[8] Exploration of more technically and economically efficient methods for converting lignocellulosic biomass is therefore one of the primary biorefinery research focal points.

In nature, the complete degradation of lignocellulosics and subsequent reintegration of the resulting substituents back into the carbon cycle is accomplished mainly by communities of microbial organisms.[9] While the communities involved in material recycling are complex and comprise many domains and kingdoms of life, there are perhaps none more synonymous with such processes than the members of the kingdom of fungi and, specifically, the filamentous fungi.[9] These prolific organisms, for whom the defining feature is the hypha, are found in virtually every eco-system on earth, from Antarctica to the inside of airplane fuel tanks, where

they may be found degrading organic matter and secreting myriad enzymes, organic acids, and secondary metabolites into their environment.[10] Humans have been exploiting filamentous fungi for millennia, for example. in the production of fermented foods and beverages (e.g. blue cheeses, tempeh, oncom, miso, soy sauce, sake, etc.) as well as, more recently, for the production of medicinal compounds (e.g. antibiotics, immunosuppressants, inhibitors, etc.).[10a] Future applications of filamentous fungi will involve exploiting together their abilities for breaking down varied and complex organic matter and for producing a wide variety of chemicals.

Given the parallels between the processes which take place in the biorefinery and those on the forest floor, the filamentous fungi are singularly appealing for use in addressing the current challenges hampering biorefinery development. Though filamentous fungi are already widely employed in the commercial production of enzymes and certain organic acids (e.g. citric acid), their application in operational biorefineries remains limited in scope; i.e. they are primarily only used for production of enzymes used in biomass pre-processing and hydrolysis.[11] This reflects, among other things, a knowledge gap between filamentous fungi and long-established processes involving biotech favorite organisms (e.g. *E. coli*, yeasts, *Pseudomonas*, etc.). Therefore, filamentous fungi should be explored for production of a wider variety of target compounds using feedstocks relevant to biorefinery processes (i.e. biomass or biomass-derivatives). Moreover, designing processes that more closely resemble natural systems (i.e. solid-state fermentation) may represent the key to realizing the potential of filamentous fungi as microbial cell factories in the context of biorefineries. A "one-pot" consolidated bioprocess, wherein the processing, hydrolysis, and conversion of raw biomass feedstock occurs simultaneously, represents, if made technically and economically viable, an ideal biorefining process and one that filamentous fungi are uniquely equipped to realize.

1.2. Hypotheses

The principal hypotheses that have been examined in this work are:

1. Non-targeted LC-MS coupled with multivariate statistical analysis may be applied for molecular screening in the development of biomass conversion processes involving filamentous fungi.

2. Filamentous fungi may be used in novel processes for upgrading waste-residues into valuable chemicals.
3. Co-culturing of multiple species of filamentous fungi can enhance processes for conversion of biomass into valuable products.
4. The oxidative enzymes common in filamentous fungi can make them effective whole-cell oxidative biocatalysts in conversion of biomass-derived substrates.

1.3. Study Objectives

The objective of this work is to employ filamentous fungi in the conversion of biomass residues into commodity and platform chemicals. This includes application of filamentous fungi directly to unprocessed biomass (i.e. solid state fermented) as well as to biomass-derived substrate (i.e. the well-known product of acid-hydrolysis of biomass 5-hydroxymethylfurfural). To that end, the specific study objectives were:

1. To review the current state of filamentous-fungal-based processes employed in biomass conversion (Chapter 2)
2. To employ an underexplored strain of filamentous fungi, *Talaromyces* sp. NRRL 2120, in solid state-fermentation of four biomass feedstocks (with a diverse spectrum of compositions) and analyze the fermentation products using LC-MS coupled with multivariate statistical analysis (Chapter 3)
3. From the data set generated in Objective 2, to identify valuable products and enhance their acquisition. As part of this, to investigate fungal co-culturing as a viable strategy for enhanced productivity (Chapter 4)
4. To employ a simple substrate derived from biomass and investigate the potential for filamentous fungi in bio-catalytic upgrading of said substrate (Chapter 5)
5. To explore novel strategies for enhancing filamentous-fungal whole-cell biocatalytic reactions (Chapter 6)

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Chapter 2. Literature Review: Status of filamentous fungi in integrated biorefineries

2.1. Abstract

Biorefinery operations may be made more cost competitive through use of cheaper feedstocks (e.g. lignocellulosic wastes) and integration of multiple revenue streams. Current methods for converting lignocellulosics into valuable products are inefficient and expensive due to the complexity of the feedstock. By looking to nature, however, these expensive and complex processes may be consolidated into a simple, cheap and environmentally benign process. The findings of this review demonstrate that the diversity of filamentous fungi is such that each step of the biorefining process may be catalyzed by a number of different species. While no single microorganism may catalyze all the process steps, by combining certain strains and targeting multiple products, the complete valorization of biomass may be achieved. This review describes in detail the variety of valuable chemical products that filamentous fungi produce, the variety of substrates used to produce these chemicals, and methods for maximizing production. The technical and economic findings herein demonstrate the means by which a successful integrated biorefinery model may be devised using filamentous fungi.

2.2. Introduction

In the search for alternatives to fossil-based resources, biomass stands out as the only renewable source of fixed carbon.[12] Biomass can be converted into power/heat, transportation fuels, and chemical feedstocks.[13] Power, heat, and biofuels suffer from low return on investment (ROI) as petroleum is still a much cheaper option. However, the production of bio-based chemicals can provide added incentives in the form of additional revenue streams for biomass handling operations.[14] The United States Department of Energy (U.S. DOE) has identified fifteen ideal target molecules for biorefinery research and development based on criteria which include industrial scale viability, market potential and versatility of the molecule as a platform for derivative products.[14] Current forecasts estimate a compound annual growth rate for bioproduct markets of 20% over the next decade and a market share for both biofuels and biomaterials of around 17% to 30% by 2050.[15] While the trajectory of bioproduct value will be

heavily influenced by government regulations and fossil fuel prices, the chief strategy for realizing the success of biorefinery products will be reducing production costs by minimizing input costs and maximizing profitability.[4] To this end, adoption of waste materials (e.g. crop and forest residues, municipal waste, animal manures, etc.) as feedstock has great potential to reduce both input costs and potential negative socio-environmental impacts vis-à-vis first-generation biomass feedstock (e.g. glucose and starch).[16] Waste biomass requires no additional land, water, fertilizer, etc. for its production and thus there are no adverse effects on the human or animal food economies.[6a] Furthermore, using waste materials as feedstock espouses the principles of a circular economy, i.e. extending resource functionality and thereby increasing resource use efficiency and diminishing generation of waste.[17]

Converting raw biomass into valuable chemicals involves a pretreatment step, necessitated by the recalcitrance of lignocellulosic components, followed by a processing step.[7] Currently employed methods for pretreating biomass often represent more than 45% of the total operation cost due to inefficiency and material expense.[8] The pretreatment and processing steps can be biological, chemical, thermal, or mechanical.[7a, 18] Thermochemical methods, involving heat and/or catalysts for example, while efficient, require expensive equipment, intensive energy consumption, and involve the use of corrosive chemicals.[7a] This limits industrial applications and contributes to environmental pollution.[7a] Biological methods (e.g. use of microbial derived enzymes) possess advantages over chemical methods as the biological treatment components are biodegradable, have lower heat requirements, and possess higher specificity in the targeted conversions.[19] The main drawbacks of biological pretreatments are that the processes are time consuming and the price of pure enzymes can be relatively expensive.[7a] For example, price estimates for the cellulase enzyme range from around 4-20 $/kg.[20] In ethanol production, the cost of enzyme can represent from 0.40 $/gallon ethanol up to around 1.50 $/gallon ethanol.[20] Many current industrial processes use some combination of thermochemical or biological pretreatment, and thermochemical or biological processing. Recently, the concept of using only enzymes or biological organisms for the entire process, known as white biotechnology[21], has proven to be a comparatively effective strategy. In a collaboration between the companies DSM and Roquette, a white biotechnological process was implemented for the production of succinic acid, one of the target molecules identified in the U.S. DOE report.[14, 22] Life cycle analysis revealed 50% less greenhouse gas emission (GHG) vis-à-vis chemical production methods.[22]

With respect to the white biotechnological approach of converting biomass into valuable chemicals, fungi are in a unique position to offer utility at each stage of the process. Fungi, especially filamentous fungi, produce a myriad of extracellular enzymes in high abundance that may be used to break down polymeric biomass into its monomeric constituents. These constituents may then be converted, by other species of fungi or in some cases the same species, into a multitude of valuable products. A number of reviews exist which include discussion of fungal products and enzymes that are relevant to biorefineries.[23] The novelty of this review is the demonstration technically and economically that a process comprised entirely of filamentous fungi can conceivably be adopted to boost the economic competitiveness of a biorefinery. The aim is to present extensive details of the chemical/biochemical properties of relevant substrates and key metabolic pathways of fungi juxtaposed with empirical data of process optimization. This review provides an overview of the valuable products produced by fungi, strategies for maximizing this production, and how these processes may be combined in the context of an integrated biorefinery. The findings of this review will be relevant for consideration in making biorefinery processes more environmentally friendly and cost-effective.

2.3. Overview of Filamentous Fungi: Ecology and Metabolism

The kingdom of fungi is comprised of eukaryotic organisms distinguished by chitinous cell walls. These organisms serve a vital role as recyclers of organic material and with an estimate of over 1 million different species, fungi represent an extremely diverse kingdom morphologically, physiologically, and ecologically.[24] A distinct type of fungi, filamentous fungi, are distinguished by a mycelium composed of septate hyphae, or branching filaments that are divided into distinct sections.[24c, 25] Additionally, filamentous fungi are characterized by a high capacity for producing extracellular enzymes and organic acids. This quality, as will be shown, has been exploited by mankind for millennia and is increasing in importance.

2.3.1. Enzyme Production

Fungi are heterotrophic and obtain sustenance by hydrolyzing complex material into simple molecules for uptake and use in biosynthesis and energy production.[24a, 24c, 26]

Filamentous fungi exist in a myriad of environments and need to be able to process diverse and complex substrates as well as compete with a large variety of other microorganisms. The composition of substrates varies significantly as a function of the source, while it mainly consists of starches or lignocellulosic polymers such as cellulose, hemicellulose, and lignin.[12c, 27] Starch is primarily comprised of a varying amount of two polyglucans: amylose, comprised of linear chains of 1-4 α-D-glucose residues; and amylopectin, comprised of short 1-4 α-D-glucose residues heavily branched with 1-6 α-D-linkages at the branch points.[28] Enzymes involved in the breakdown of starch into glucose include the endo-amylases (e.g. α-amylase) and exo-amylases (e.g. β-amylase and γ-amylase), which respectively act on the internal and terminal 1-4 α-D-glucosidic bonds; as well as debranching enzymes (e.g. isoamylase and pullulanase), which act on the 1-6 α-D-glucosidic linkages (Fig. 1a).[29] Cellulose is comprised of β(1→4) linked α-D-glucopyranose which forms a crystalline structure *via* inter- and intra-molecular hydrogen bonding and van der Waals forces (Fig. 1b).[12b] The enzymes required to degrade cellulose to glucose include: endoglucanase, which acts on the internal β-1,4-glucosidic bonds; exoglucanase, which acts on the reducing and non-reducing ends of the cellulose polymer; and β-glucosidase, which catalyzes the hydrolysis of soluble cellobiose (a β-1,4-glucose disaccharide to glucose).[30] The composition of hemicellulose (Fig. 1c) depends on the plant species and specific plant structure it comes from, but most commonly consists of a linear backbone containing β(1→4) linked D-xylanopyranosal residues, which forms an interconnected matrix with cellulose through hydrogen bonds and van der Waals forces.[12b, 27b, 31] Complete hydrolysis of hemicellulose into its constituent sugars (e.g. xylose and arabinose) requires xylanase, β-xylosidase and a multitude of other enzymes including arabinosidase, glucuronidase and esterase.[12b, 27b, 30b, 31-32] Lastly, lignin is a highly cross-linked polymer and comprises of substituted phenylpropene units connected to both cellulose and hemicellulose *via* hydrogen bonds, ionic interactions, and ester and ether linkages.[12b, 27b] While the enzymatic breakdown of lignin into food for microorganisms is still not completely understood, it is known that enzymes such as laccase, peroxidase, and oxidase are involved in its oxidative degradation.[33] Notably, all enzymes needed to degrade starch, cellulose, hemicellulose, and lignin, are produced by various filamentous fungi which break down complex polymeric substrate into simple molecules for consumption.

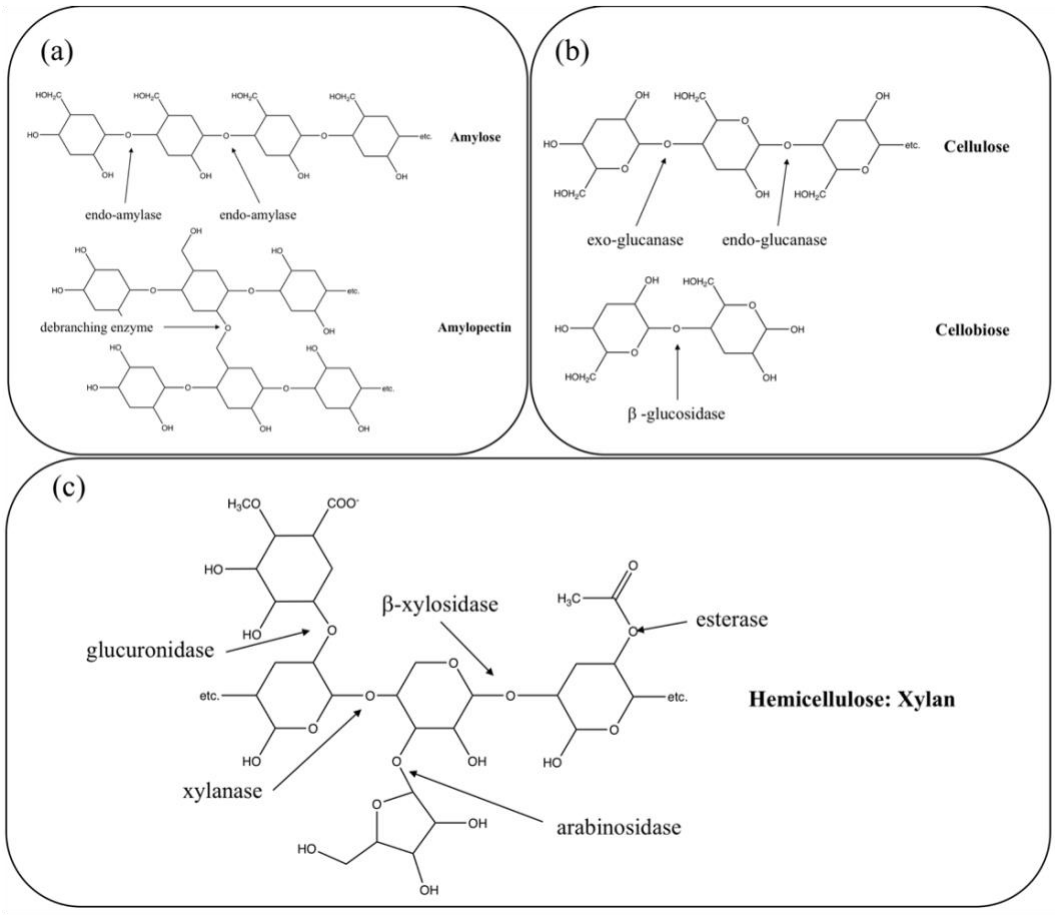

Fig. 2.1. Enzymatic activity involved in the breakdown of the components of (a) starch, (b) cellulose and (c) hemicellulose.

2.3.2. Metabolic Pathways

Simple molecules, such as glucose, are typically first converted to pyruvate and subsequently enter into certain metabolic or biosynthetic pathways depending on environmental conditions. Chemicals such as ethanol and lactic acid are derived *via* fungal fermentation while others such as citric and succinic acid come from the tricarboxylic acid (TCA) cycle. Additionally, valuable chemical products from fungi are not limited to fermentation or the TCA cycle but may be derived from a variety of pathways (Fig. 2). The proclivity of filamentous fungi for organic acid production may come from a lack of regulation under laboratory settings, as these organisms may not, in natural environments, encounter such high sugar concentrations

as in bioreactors.[34] Alternatively, high acid secretion may give advantages to fungi by inhibiting the growth of competitors or by acting as chelating agents in environments that have low or insoluble metal concentrations.[34] The variety of organic acids may then reflect the variety of competition strategies.[34]

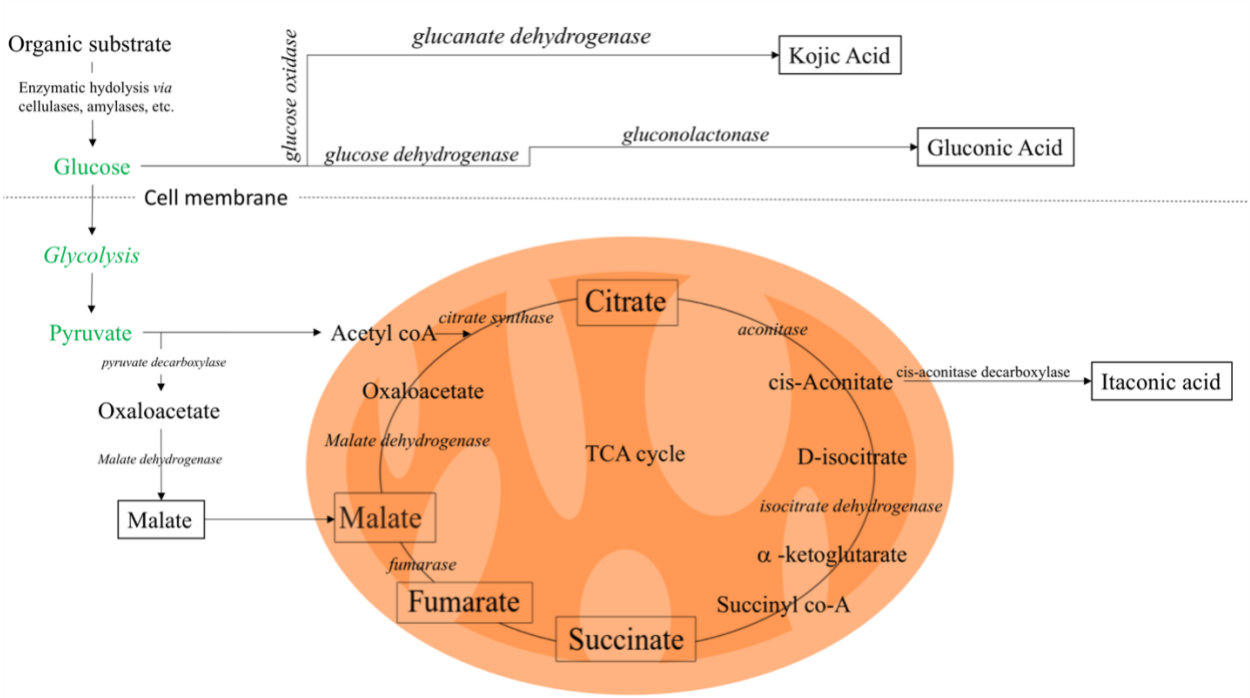

Fig. 2.2. Overview of general fungal metabolism, including and emphasizing the pathways of the organic chemicals discussed in this review.

2.4. Filamentous Fungi in the Production of Valuable Chemicals

The first examples of industrial biotechnological products were the organic acids, citric and fumaric, as well as the antibiotic penicillin, which were produced by the filamentous fungi *A. niger*, *R. orzyae* and *P. chrysogenum* respectively.[35] At present, citric acid and gluconic acid are the two biggest commercial fungal products in terms of production volume, and both are produced *via* fermentation of glucose or sucrose by *A. niger*.[34] Of the molecules identified by the U.S. DOE report, 70% were organic acids and about half can be derived from fungi as summarized in Table 1 and Table 2.[36] While *E. coli* and yeast are popular organisms for use as cell factories, the diversity of substrates that these organisms are able to use is inferior to that of

filamentous fungi. For example, virtually all microorganisms can ferment six carbon sugars such as glucose, but filamentous fungi are exceptional in their capability of also using five carbon sugars such as xylose and arabinose. This ability is a consequence of the highly diverse environments filamentous fungi inhabit, and this quality will prove to become increasingly relevant as chemical and fuel production shifts from fossil fuel feedstock to biomass feedstock.

Table 2.1. Comparison of major organic chemical products derived from various filamentous fungi and other industrially relevant microbes. **Note: Yields not included for comparison due to lack of reported sugar content for various lignocellulosic materials*

***Patent; **Solid-state; ***Mixed bacterial culture including: *L. delbrueckii* subsp lactic ATCC 12315, *L. casei* NRRL B-1445, *L. delbrueckii* NRRL B-445, *L. helveticus* NRRL B-1937 and *L. casei* NRRL B-1922; ****Immobilized enzyme; *****Not filamentous fungi**

Citric Acid			
Organism	**Carbon Source**	**Production**	**Source**
A. niger B60	Cane Sugar	26.0 g/L	[37]*
A. niger A60 NRRL 2270	Beet Molasses	105 g/L	[38]
A. niger UMIP 2564.04	Beet Molasses	114 g/L	[39]
A. niger NRRL 567	Apple Pomace	883 g/kg	[40]
A. niger SIIM M288	Corn Stover	100.04 g/L	[41]
A. niger LPB B6	Citric Pulp**	617 g/kg	[42]
A. niger NRRL 567	Apple Pomace	9.00 +/- 3 g/L	[43]
A. niger NRRL 567	Apple Pomace**	128 g/kg	[43]
A. niger NRRL 567	Brewery Spent Grain**	14.0 g/kg	[43]
A. niger NRRL 2001	Citrus Waste**	63.6 g/kg	[43]
A. niger	Rice Extract	14.4 g/L	[44]
A. niger	Potato Extract	1.47 g/L	[44]
A. niger EB-12	Molasses Sugar and Chicken Feather Peptone	68.8 g/L	[45]
A. niger NRRL 2001	Apple Pomace + Rice Husk**	364 g/kg	[46]
A. niger NRRL 567	Sphagnum Peat Moss**	124 g/kg	[47]
A. niger NRRL 2001	Corn Husk**	259 g/kg	[48]
A. niger MTCC 282	Banana Peel**	180 g/kg	[49]
A. niger CFTRI 30	Coffee Husk**	150 g/kg	[50]
A. niger LPB-21	Cassava Bagasse**	347 g/kg	[50]
A. niger CECT 2090	Orange Peel**	193 mg/g	[51]
A. niger CECT 2090	Orange Peel	9.20 g/L	[52]
A. niger LPB21	Cassava Bagasse**	309 g/kg	[53]
A. niger PTCC 5010	Date Pulp**	168 g/kg	[54]
A. niger NRRL 599	Orange Peel Press Liquor**	730 g/kg	[55]
Gluconic Acid			

Organism	Carbon Source	Production	Source
*A. niger*****	Glucose	200 g/L	[56]∗
A. niger IAM 2094	Glucose	550 g/L	[57]
*Aureobasidium pullulans******	Glucose	375 g/L	[58]
A. niger ATCC 10577	Dry Fig**	685 g/kg	[59]
A. niger ORS4.410	Glucose	85.9 g/L	[60]
A. niger ORS4.410	Glucose**	107 g/L	[60]
A. niger	Breadfruit Hydrolysate	110 g/L	[61]
A. niger SIIM M276	Corn Stover	76.7 g/L	[62]
A. niger ITCC 5483	Tea Waste	58.4 g/L	[63]
A. niger ITCC 5483	Tea Waste**	82.2 g/L	[63]
A. niger NCIM 530	Raw Golden Syrup	85.2 g/L	[64]
Itaconic Acid			
Organism	**Carbon Source**	**Production**	**Source**
A. terreus 10020	Peeled Sugarcane Pressmud**	55.0% w/w	[65]∗
A. terreus TN-484	Glucose	82.0 g/L	[66]
A. terreus DSM 23081	Glucose	90.0 g/L	[67]
A. Terreus TN-484	Corn Starch	62.0 g/L	[68]
A. terreus NRRL 1960	Glucose	130 g/L	[69]
A. terreus NRRL 1961	Xylose	38.9 g/L	[70]
A. terreus NRRL 1961	Arabinose	34.8 g/L	[70]
A. terreus NRRL 1961	Glucose + Xylose + Arabinose	33.2 g/L	[70]
A. terreus 1971	Mannose	36.4 g/L	[70]
A. terreus DSM 23081	Crude Wheat Chaff Hydrolysate (not detoxified)	23.3 g/L	[71]
A. terreus DSM 23081	Glucose	160 g/L	[72]
A. terreus CICC40205	Wheat Bran Hydrolysate	49.7 g/L	[73]
A. terreus AS32811	Bran and Corncob**	63.5% w/w	[74]
A. terreus TN484-M1	Sago Starch Hydrolysate	48.2 g/L	[75]
Malic Acid			
Organism	**Carbon Source**	**Production**	**Source**
R. arrhizus NRRL	Glucose	26.1 g/L	[76]∗
A. flavus ATCC 13697	Xylose	19.4 g/L	[77]∗
A. flavus ATCC 13697	Starch	24.4 g/L	[77]∗
A. flavus ATCC 13697	Cane Molasses	30.6 g/L	[77]∗
A. flavus ATCC 13697	Glycerol	35.4 g/L	[77]∗
A. oryzae NRRL3488	Glucose	30.3 g/L	[78]
A. oryzae NRRL3488	Glucose	154 g/L	[79]
A. flavus ATCC 13697	Glucose	113 g/L	[80]
A. flavus ATCC 13697	Thin Stillage	10.2 g/L	[81]
A. niger ATCC 9142	Thin Stillage	17.0 g/L	[81]
A. niger ATCC 9143	Crude Glycerol	77.4 g/L	[82]
*Zygosaccharomyces rouxii******	Glucose	75.0 g/L	[83]
S. cerevisiae RWB525*****	Glucose	59.0 g/L	[84]
Kojic Acid			

Organism	Carbon Source	Production	Source
A. oryzae BCRC 30010	Glucose	83.5 g/L	[85]
A. oryzae var. *effusus* NRC14	Glucose	49.5 g/L	[86]
A. oryzae RMS2	Wheat Bran and Sesame Oil Cake**	106 g/kg	[87]
A. oryzae M866	Glucose	40.4 g/L	[88]
A. oryzae NRRL 484	Glucose	83.0 g/L	[89]
A. oryzae MK107-39	Continuous Glucose	117 g/L	[90]
A. oryzae 124A	Glucose	44.2 g/L	[91]
A. flavus Link 44-1	Pineapple Waste**	0.415 g/L	[92]
A. flavus Link 44-1	Glucose	39.9 g/L	[93]
A. flavus NRRL 626	Glycerol + Corn Steep Liquor	29.0 g/L	[94]
A. flavus var. *columnaris*	Rice Fragment	21.2 g/L	[95]
A. flavus	Glucose	53.5 g/L	[95]
A. flavus	*M. calabura* Fruits	88.8 g/L	[96]
A. flavus HAK1	Malt Extract Sucrose	15.5 g/L	[97]
A. parasiticus	Glucose	34.4 g/L	[98]
Lactic Acid			
Organism	**Carbon Source**	**Production**	**Source**
LBM5***	Glucose	99.2 g/L	[99]*
R. oryzae NRRL 395	Glucose	105 g/L	[100]
R. oryzae GY 18	Xylose	54.2 g/L	[101]
R. oryzae GY 18	Glucose	115 g/L	[101]
R. oryzae GY 18	Corncob Hydrolysate	355 g/kg	[101]
R. oryzae RQ4015	Glucose	121 g/L	[102]
R. oryzae RQ4015	Xylose	75.0 g/L	[102]
R. oryzae NRRL 395	Glucose**	137 g/L	[103]
R. oryzae ATCC 52311	Glucose	83.0 g/L	[104]
R. oryzae NRRL 395	Cassava Pulp**	463 mg/g	[105]
R. *oryzae* NBRC 5384	Glucose	145 g/L	[106]
R. oryzae NBRC 5384	Fed Batch Glucose	231 g/L	[106]
R. microsporus DMKU 33	Cassava Starch**	119 g/L	[107]
Lactobacillus pentosus FL0421*****	Corn Stover**	92.3 g/L	[108]
Bacillus coagulans LA204*****	Straw**	97.6 g/L	[109]
P. acidilactici TY112*****	Corn Stover**	105 g/L	[110]
Bacillus sp. 2-6*****	Fed Batch Glucose	182 g/L	[111]
B. subtilis DA12*****	Glucose	535 mM (48.0 g/L)	[112]
Fumaric Acid			
Organism	**Carbon Source**	**Production**	**Source**
R. arrhizus NRRL 1526	Glucose	135 g/L	[76]*
R. arrhizus 2582	Glucose	100 g/L	[113]
R. arrhizus NRRL 2582	Soybean Cake Hydrolysate	40.0 g/L	[114]
R. arrhizus RH7-13	Food Waste (Liquid Fraction)	32.68 g/L	[115]

R. arrhizus ATCC 13310	Lyophilized Orange Peels with Grape Must**	31.9 g/kg	[116]
R. arrhizus RH-7-13-9#	Glucose and Xylose	46.8 g/L	[117]
R. arrhizus RH-7-13-9#	Glucose	30.3 g/L	[118]
R. oryzae ATCC 20344	Glucose	85.0 g/L	[119]
R. oryzae ME-F12	Corn Starch	44.1 g/L	[120]
R. oryzae ATCC 20344	Wheat Bran	20.2 g/L	[121]
R. oryzae 1526	Apple Pomace**	138 g/kg	[122]
Citric Acid			
Organism	**Carbon Source**	**Production**	**Source**
A. niger B60	Cane Sugar	26.0 g/L	[37]*
A. niger A60 NRRL 2270	Beet Molasses	105 g/L	[38]
A. niger UMIP 2564.04	Beet Molasses	114 g/L	[39]
A. niger NRRL 567	Apple Pomace	883 g/kg	[40]
A. niger SIIM M288	Corn Stover	100.04 g/L	[41]
A. niger LPB B6	Citric Pulp**	617 g/kg	[42]
A. niger NRRL 567	Apple Pomace	9.00 +/- 3 g/L	[43]
A. niger NRRL 567	Apple Pomace**	128 g/kg	[43]
A. niger NRRL 567	Brewery Spent Grain**	14.0 g/kg	[43]
A. niger NRRL 2001	Citrus Waste**	63.6 g/kg	[43]
A. niger	Rice Extract	14.4 g/L	[44]
A. niger	Potato Extract	1.47 g/L	[44]
A. niger EB-12	Molasses Sugar and Chicken Feather Peptone	68.8 g/L	[45]
A. niger NRRL 2001	Apple Pomace + Rice Husk**	364 g/kg	[46]
A. niger NRRL 567	Sphagnum Peat Moss**	124 g/kg	[47]
A. niger NRRL 2001	Corn Husk**	259 g/kg	[48]
A. niger MTCC 282	Banana Peel**	180 g/kg	[49]
A. niger CFTRI 30	Coffee Husk**	150 g/kg	[50]
A. niger LPB-21	Cassava Bagasse**	347 g/kg	[50]
A. niger CECT 2090	Orange Peel**	193 mg/g	[51]
A. niger CECT 2090	Orange Peel	9.20 g/L	[52]
A. niger LPB21	Cassava Bagasse**	309 g/kg	[53]
A. niger PTCC 5010	Date Pulp**	168 g/kg	[54]
A. niger NRRL 599	Orange Peel Press Liquor**	730 g/kg	[55]
Gluconic Acid			
Organism	**Carbon Source**	**Production**	**Source**
*A. niger*****	Glucose	200 g/L	[56]*
A. niger IAM 2094	Glucose	550 g/L	[57]
*Aureobasidium pullulans*****	Glucose	375 g/L	[58]
A. niger ATCC 10577	Dry Fig**	685 g/kg	[59]
A. niger ORS4.410	Glucose	85.9 g/L	[60]
A. niger ORS4.410	Glucose**	107 g/L	[60]
A. niger	Breadfruit Hydrolysate	110 g/L	[61]
A. niger SIIM M276	Corn Stover	76.7 g/L	[62]

A. niger ITCC 5483	Tea Waste	58.4 g/L	[63]
A. niger ITCC 5483	Tea Waste**	82.2 g/L	[63]
A. niger NCIM 530	Raw Golden Syrup	85.2 g/L	[64]
Itaconic Acid			
Organism	**Carbon Source**	**Production**	**Source**
A. terreus 10020	Peeled Sugarcane Pressmud**	55.0% w/w	[65]*
A. terreus TN-484	Glucose	82.0 g/L	[66]
A. terreus DSM 23081	Glucose	90.0 g/L	[67]
A. Terreus TN-484	Corn Starch	62.0 g/L	[68]
A. terreus NRRL 1960	Glucose	130 g/L	[69]
A. terreus NRRL 1961	Xylose	38.9 g/L	[70]
A. terreus NRRL 1961	Arabinose	34.8 g/L	[70]
A. terreus NRRL 1961	Glucose + Xylose + Arabinose	33.2 g/L	[70]
A. terreus 1971	Mannose	36.4 g/L	[70]
A. terreus DSM 23081	Crude Wheat Chaff Hydrolysate (not detoxified)	23.3 g/L	[71]
A. terreus DSM 23081	Glucose	160 g/L	[72]
A. terreus CICC40205	Wheat Bran Hydrolysate	49.7 g/L	[73]
A. terreus AS32811	Bran and Corncob**	63.5% w/w	[74]
A. terreus TN484-M1	Sago Starch Hydrolysate	48.2 g/L	[75]
Malic Acid			
Organism	**Carbon Source**	**Production**	**Source**
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*Zygosaccharomyces rouxii*****	Glucose	75.0 g/L	[83]
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A. flavus	Glucose	53.5 g/L	[95]
A. flavus	*M. calabura* Fruits	88.8 g/L	[96]
A. flavus HAK1	Malt Extract Sucrose	15.5 g/L	[97]
A. parasiticus	Glucose	34.4 g/L	[98]
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Organism	**Carbon Source**	**Production**	**Source**
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R. oryzae GY 18	Xylose	54.2 g/L	[101]
R. oryzae GY 18	Glucose	115 g/L	[101]
R. oryzae GY 18	Corncob Hydrolysate	355 g/kg	[101]
R. oryzae RQ4015	Glucose	121 g/L	[102]
R. oryzae RQ4015	Xylose	75.0 g/L	[102]
R. oryzae NRRL 395	Glucose**	137 g/L	[103]
R. oryzae ATCC 52311	Glucose	83.0 g/L	[104]
R. oryzae NRRL 395	Cassava Pulp**	463 mg/g	[105]
R. *oryzae* NBRC 5384	Glucose	145 g/L	[106]
R. oryzae NBRC 5384	Fed Batch Glucose	231 g/L	[106]
R. microsporus DMKU 33	Cassava Starch**	119 g/L	[107]
Lactobacillus pentosus FL0421*****	Corn Stover**	92.3 g/L	[108]
Bacillus coagulans LA204*****	Straw**	97.6 g/L	[109]
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Bacillus sp. 2-6*****	Fed Batch Glucose	182 g/L	[111]
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Organism	**Carbon Source**	**Production**	**Source**
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R. arrhizus 2582	Glucose	100 g/L	[113]
R. arrhizus NRRL 2582	Soybean Cake Hydrolysate	40.0 g/L	[114]
R. arrhizus RH7-13	Food Waste (Liquid Fraction)	32.68 g/L	[115]
R. arrhizus ATCC 13310	Lyophilized Orange Peels with Grape Must**	31.9 g/kg	[116]
R. arrhizus RH-7-13-9#	Glucose and Xylose	46.8 g/L	[117]
R. arrhizus RH-7-13-9#	Glucose	30.3 g/L	[118]
R. oryzae ATCC 20344	Glucose	85.0 g/L	[119]
R. oryzae ME-F12	Corn Starch	44.1 g/L	[120]
R. oryzae ATCC 20344	Wheat Bran	20.2 g/L	[121]
R. oryzae 1526	Apple Pomace**	138 g/kg	[122]

Table 2.2. Detailed summary of some processes involved in relatively high organic acid production.

Organism	Substrate	Conditions	Product	Results	Source
A. niger UMIP 2564.04	**Beet molasses** pre-treated with 15 g/L calcium phosphate,	UV mutated *A. niger*; **Submerged** fermentation at 30° C for 152 hours	Citric Acid	114 g/L ; 70.9% yield	[39]
A. niger ORS4.410	120 g/dm^3 of HCl pretreated **sugarcane bagasse** (70% moisture)	**Solid-state** at 30° C for 12 days	Gluconic Acid	107 g/L ; 94.7% yield	[60]
A. terreus DSM 23081	180 g/L **glucose**	**Submerged** at 35° C for 6.7 days; initial pH 3.1 raised to 3.4 after 2 days	Itaconic Acid	160 g/L ; 46% yield	[72]
A. oryzae NRRL 3488	160 g/L **glucose**; 9 g/L Bacto peptone	Mutant strain SaMF2103a-68 with overexpression of C4T318 transporter, *pyc* and *mdh*; **Submerged** at 34° C for 164 hours	Malic Acid	154 g/L	[79]
A. flavus isolated from soil	50 g ***Muntingia calabura*** **fruits**; 0.5 g Peptone	**Surface** fermentation at 29° C for 28 days	Kojic Acid	88.8 g/L	[96]
R. oryzae NBRC 5384	150 g/L **glucose**	**Submerged**; immobilization of *in situ* mycelial cells in sponge-like cubic particle (polyurethane foam); 37° C; intermittent addition of glucose at 100 g/L	Lactic Acid	280 g/L ; 92.5% yield	[106]
R. oryzae ATCC 20344	109 g/L **Glucose**	**Submerged** at 30° C for 24 hours in rotary biofilm contactor coupled with an adsorption column	Fumaric Acid	93.0 g/L ; 85% yield	[119]

In addition to organic acids and other metabolites, filamentous fungi such as *Aspergillus* and *Trichoderma* are the primary industrial producers of enzymes used in biomass conversion as shown in Table 3. The preference for filamentous fungi comes from superior enzyme productivity as compared to yeast and bacteria, high enzyme activity at neutral pH values, and enzymatic thermal stability.[123] As in fermentation, filamentous fungi may use a broad range of carbon sources, including pentose sugars, for enzyme production.[124] Even wood, among the most difficult lignocellulosic materials to degrade, can be effectively degraded by enzymes produced by filamentous fungi species such as *Trichoderma reesei* and *Phanerochaete*

chrysosporium.[125] Agricultural residues including sugarcane bagasse, straw and corn stover as well as spent hydrolysates are extremely attractive and inexpensive feedstocks for the production of chemicals. Since these substrates contain large amounts of pentose sugars, including xylose and arabinose, filamentous fungi may be better equipped for processing such substrates as compared with the current biotech favorites *E. coli* and yeast.[124]

Table 2.3. Comparison of enzyme production in filamentous fungi and other industrially relevant microbes (organized by substrate).

***Semi Solid-state Fermentation; **Solid-state Fermentation; ***Not Filamentous Fungi**

Starch				
Organism	**Substrate**	**Enzyme**	**Production**	**Source**
*B. amyloliquefaciens****	Potato Starchy Waste	α-amylase	98.4 U/mL	[126]
A. oryzae S2	Starch**	α-amylase	22100 U/g	[127]
*B. amyloliquefaciens****	Potato Starchy Waste	β-xylosidase	0.52 U/mL	[126]
A. niger	Corn Flour	β-xylosidase	346 U/mL	[128]
*B. amyloliquefaciens****	Potato Starchy Waste	γ-amylase (amyloglucosidase)	1.60 U/mL	[126]
A. niger NRRL 3122	Starch	Amyloglucosidase	886 U/g	[129]
R. oryzae PR7	Oat and Arum**	Isoamylase	7.10 U/mL	[130]
E. coli MDS42***	Glycerol	Isoamylase	23,000 U/mL	[131]
M. purpureus ATCC 16365	Bakery Waste**	Glucoamylase	8.00 U/g	[132]
Cellulose				
Organism	**Substrate**	**Enzyme**	**Production**	**Source**
T. reesei QMY-1	Wheat Straw**	Cellulase	17.2 IU/mL; 430 IU/g	[133]
B. vallismortis RG-07***	Sugarcane Bagasse	Cellulase	4110 U/mL	[134]
T. reesei Vib-1	Wheat Bran and Cellulose	CMCase (carboxy methyl cellulase)	6.22 IU/mL	[135]
T. reesei RUT C30	Horticultural Waste Powder**	CMCase (carboxy methyl cellulase)	90.5 U/g	[136]
T. reesei RUT C30	Wheat Bran**	Endoglucanase	15.0 U/mL	[137]
A. niger MTCC 7956	Wheat Bran**	Endoglucanase	6.77 U/mL	[137]
A. niger MTCC 7956	Wheat Bran	β-xylosidase	2.84 U/mL	[137]
A. niger RCKH-3	Wheat Bran**	β-xylosidase	87.6 IU/g	[138]
Hemicellulose				
Organism	**Substrate**	**Enzyme**	**Production**	**Source**
T. reesei QMY-1	Wheat Straw**	Xylanase	540 IU/mL	[133]
P. methanolica	1% Oat Spelts Xylan	Xylanase	71,700 U/mL	[139]
A. brasiliensis BLf1	Rice Husk	β-xylosidase	28.1 U/g	[140]
P. oxalicum RGXyl-1	Corn Stover	β-xylosidase	15.1 IU/mL	[141]
T. reesei RUT C30	Horticultural Waste Powder**	β-xylosidase	10.4 U/g	[136]
A. niger NCH-189	Defatted Copra	Mannanase	27.4 U/mL	[142]
A. nidulans	Guar Gum	Mannanase	108 U/mL	[143]
Lignin				
Organism	**Substrate**	**Enzyme**	**Production**	**Source**
P. chrysosporium-IBL-03	Wheat Straw Waste	Lignin Peroxidase	1220 U/mL	[144]

P. chrysosporium BKM-F-1767 (ATCC 24725)	Grape Seeds*	Lignin Peroxidase	1620 U/L	[145]
P. chrysosporium-IBL-03	Wheat Straw Waste	Manganese Peroxidase	994 U/mL	[144]
P. radiata	Wheat Straw Waste	Manganese Peroxidase	6.90 U/g	[146]
T. aurantiacus	Oats Straw**	Laccase	15.5 IU/g	[147]
Unspecified White rot fungus	Ground Nut Shell**	Laccase	384 U/g	[148]
Protein				
Organism	**Substrate**	**Enzyme**	**Production**	**Source**
A. oryzae	Sunflower Meal**	Protease	400 U/g	[149]
A. oryzae	Rapeseed Meal**	Protease	728 U/g	[150]
A. oryzae	Palm Kernel Cake**	Protease	319 U/g	[151]
A. oryzae	Wheat Pieces	Protease	173 U/g	[152]
A. oryzae NRRL 1808	Wheat Bran**	Protease	31.2 U/g	[153]
A. oryzae NRRL 1808	Wheat Bran	Protease	8.70 U/g	[153]
M. purpureus ATCC 16365	Bakery Waste**	Protease	117 U/g	[132]
B. licheniformis 21415***	Extracted Soybean Cake and Dextrin	Protease	29,554 U/mL	[154]

2.4.1. *Aspergillus*

Aspergillus is one of the most commercially utilized filamentous fungus and microbe in general. It is a common and widespread genus identifiable by the morphology of the conidiophore, the structure that bears the asexual spores.[155] Though used by humans for millennia in various fermented foods and beverages, *Aspergillus* was first named in 1729 after a religious device, the asperges, which the conidiophore resembled.[155-156] While members of the genus are plant and animal pathogens, causing aspergillosis in immunosuppressed humans, these fungi also have important roles in natural ecosystems. Moreover, *Aspergillus* produce commercially valuable extracellular enzymes, organic acids, secondary metabolites, and serve as a model organism in fundamental scientific research.[155, 157] An example of a well-known *Aspergillus* product, originally derived from *A. terreus*, is lovastatin, which reduces cholesterol and has been developed by Merck into the profitable drug Mevacor.[157]

2.4.1.1. *Aspergillus niger*

Aspergillus niger is the largest fungal producer of organic acids, as it is the commercially preferred route for the production of citric and gluconic acids, in addition to being a popular source of cellulase, amylase and pectinase among other enzymes.[25, 34, 123, 158]

Over 1.5 million tons of citric acid is produced annually and that volume is increasing by 5% each year.[159] Around 75% of the citric acid is used in the food and beverage industry, though citric acid is also used in cleaning products, in the pharmaceutical industry as a buffering agent, and as a metal chelator in soaps and detergents.[25, 34, 160] Citric acid was originally extracted from fruits, principally lemons; however, the production shifted by the 1920s to derivation from filamentous fungi.[25] In fungal citric acid production, sugars such as glucose are transported across the cell membrane into the cytosol *via* transporters. Each sugar molecule enters glycolysis during which it is converted to two molecules of pyruvate.[34] One molecule of pyruvate is decarboxylated in the cytosol *via* mitochondrial pyruvate decarboxylase to form oxaloacetate, while the other is decarboxylated *via* pyruvate dehydrogenase to form acetyl-CoA.[34] Oxaloacetate is transported to the mitochondria *via* malate, where it condenses with acetyl-CoA *via* citrate synthase to form citrate. Citrate is then either converted to cis-aconitate *via* aconitase, which will continue to be iteratively oxidized for production of NADH in the TCA cycle, or the citrate is transported out of the cell.[34] Substantial accumulation of citric acid generally occurs through deactivation of aconitase and isocitrate dehydrogenase.[160] Citrate itself is known to inhibit glycolysis, specifically the enzyme phosphofructokinase-1 (PFK1), and thus needs to be counteracted in order to promote continual citric acid production and accumulation. Addition of Zn^{2+}, Mg^{2+}, NH_4^+ and fructose-2,6-biphosphate helps activate PFK1 and maintain glycolysis.[160] Additionally, manganese deficiency promotes inhibition of anaerobic and TCA cycle enzymes, with the exception of citrate synthase, thus causing a flux of citric acid accumulation.[161]

Industrial production of citrate using *A. niger* reportedly achieves concentrations exceeding 200 g/L with typical yields and productivities of 80% and 0.7-1.0 g/L/h respectively.[35, 162] Current research focuses on making the process cheaper through improved efficiency and use of cheaper feedstock (e.g. agro-waste materials). Mutagenesis is a widespread method for generating microbial strains with improved citric acid production. Ozdal and Kurbanoglu used ethidium bromide to generate mutants that were able to raise titers from 31 g citric acid/L sugar beet molasses of the parent strain to 46 g/L.[45] These researchers further increased this production to 69 g/L through optimization of the fermentation media. Lotfy, Ghanem *et al.* used ultraviolet light exposure to produce mutants that improved the wild-type titer of 24 g citric acid/L sucrose media to 96 g/L of the mutant.[39] This strain was then tested

with agro-waste materials, including beet molasses which demonstrated a production of 98 g/L. Treatment of the beet molasses with either methanol, sulfuric acid or calcium phosphate further increased the production to 100 g/L, 109 g/L, and 114 g/L, respectively. Low molecular weight alcohols, e.g. methanol, are commonly used in fermentation to counteract inhibition caused by heavy metals that may be present, and also to facilitate citric acid permeability through the *A. niger* membrane.[42] Hang and Woodams found that addition of methanol to the fermentation media improved the citric acid yield from 259 g/kg apple pomace, using no methanol, to 883 g/kg at 4% methanol.[40] Dhillon, Brar *et al*. showed 4% methanol addition improved solid-state fermentation (SSF) of sphagnum peat moss from 45 g/kg to 57 g/kg and submerged fermentation (SmF) of apple pomace from 10 g/L to 18 g/L.[43] Rodrigues, de Souza Vandenberghe *et al*. showed that the addition of 4% methanol improved citric acid production from sugarcane molasses from about 260 g/kg to 460 g/kg.[42] This production reached 617 g/kg when the group used a UV mutant. However, the effects of low molecular weight alcohols are not always so dramatic. For example, Zhou, Meng *et al*. found that methanol only slightly changed citric acid accumulation.[41] Instead, Zhou, Meng *et al*. demonstrated that through bio-detoxification of the substrate using the fungus *A. resinae* ZN1, the production could be increased from 85 g/L corn stover hydrolysate to 100 g/L with a yield of 94.1%. Some studies have found that citric acid production was optimal when a specific redox potential profile, comprised of two maxima (260 and 280 mV) and two minima (180 and 80 mV), was observed over the course of the fermentation.[38] Berovič, Rošelj *et al*. demonstrated that by manipulating the aeration and agitation (increasing both to raise the redox potential and reducing both to lower it) the desired redox potential profile could be contrived and a production of 105 g/L beet molasses could be attained.

While a pH of around 2 has been found to be ideal for citric acid production, higher pH's can promote gluconic acid production.[161] Unlike citric acid production, where glucose is metabolized within the organism, in gluconic acid production, an extracellular enzyme called glucose oxidase is responsible for catalyzing glucose to gluconic acid.[34] A possible ecological function of this activity is that removal of glucose from the environment provides a competitive advantage to *A. niger* which, under the right conditions, can convert nearly 100% of glucose to gluconic acid.[34, 163] In the mechanism, glucose oxidase, which is localized to the cell wall, catalyzes β-D-glucopyranose to D-glucono-1,5 lactone and hydrogen peroxide.[163] The hydrogen

peroxide is catalyzed *via* catalase to water and oxygen, and the D-glucono-1,5 lactone is hydrolyzed to gluconic acid either spontaneously or *via* gluconolactonase.[163]

The conversion of glucose to gluconic acid simply involves oxidation of the C1 aldehyde group to a carbonyl group. Although chemical, electrochemical and bio-electrochemical methods exist, yields are lower than with fungal production and therefore fungal production is the preferred industrial method.[163-164] Yields in excess of 95% gluconic acid from glucose have been reported since at least the 1930s.[165] Current research therefore focuses on improving productivity while reducing costs. One patent[56] demonstrates the use of immobilized glucose oxidase and catalase to convert 1000 kg of glucose over the course of 100 runs using the same 100 mL of enzyme. While on average this method produces titers reaching at least 200 g/L, the cost of pure enzymes is relatively expensive. Fungal mycelium is much cheaper, and its employment is becoming increasingly cost-competitive through repeated use of the same mycelia. Sakurai, Hang *et al*. showed that by periodic additions of 150 g glucose/L to a fermentation broth, *A. niger* produced up to 550 g gluconic acid/L.[57] However, it was observed that productivity slowed after a concentration of 300 g/L was exceeded. Based on this observation, Sakurai, Hang *et al*. attempted to reuse the fungi over multiple runs, ceasing each run once 300 g/L was reached. Following this method, the same mycelia could be reused for about four runs. To expand on this, Sakurai, Hang *et al*. subsequently showed that by adding glucose at concentrations of 100 g/L, immobilizing the mycelia in non-woven fabric, and ceasing the run once 220 g gluconic acid/L was reached, the production using the same mycelia could be performed up to 14 times. Many supplementations have also been explored to improve gluconic acid production. Sharma, Vivekanand *et al*. for example found that, after optimizing moisture content, incubation temperature and aeration rate, the addition of 0.5% yeast extract increased the titer from 76 g/L to 82 g/L.[63] Additionally, as in citric acid production, addition of methanol has been shown to improve production.[59] Roukas showed that the addition of 6% methanol enhanced the gluconic acid titer from 490 g/kg substrate to 685 g/kg. SSF represents a potentially cheaper approach to organic acid production and apropos of this Singh, Jain *et al*. demonstrated that SSF of sugarcane bagasse produced a better titer and yield (107 g/L and 94.7% respectively) than SmF (89 g/L and 75.5% respectively), surface fermentation (92 g/L and 90.7%), and semi solid-state fermentation (89 g/L and 75.5%).[60] Studies exploring other fungi include reports of the yeast-like fungus *Aureobasidium pullulans* producing 375 g/L using a

cross-over filtration system.[58] With annual production of around 100,000 metric tons, gluconic acid is used in the manufacturing of metal, leather, food, pharmaceuticals, in detergents as a sequestering agent, and as an additive to improve cement hardening.[34, 163-164]

2.4.1.2. *Aspergillus terreus*

Aspergillus terreus is a soil dwelling pathogenic fungus distributed worldwide.[166] It is a commercially significant species in its production of itaconic acid (IA), a chemical with a wide range of applications including in the polymer industry.[34, 167] Often described as citric acid production in the presence of cis-aconitate decarboxylase (CAD), IA is believed to be produced from the decarboxylation of cis-aconitate, a TCA intermediate between citrate and iso-citrate, *via* CAD.[167a] As in citric acid production, glucose is first converted into two molecules of pyruvate in the glycolysis pathway, which are converted into acetyl-CoA and oxaloacetate, and combined in the first step of the TCA cycle to yield citrate.[167a, 168] Citrate is then dehydrated to cis-aconitate *via* aconitase, which is then decarboxylyzed *via* CAD to form IA.[34]

Though originally discovered as a product of pyrolytic distillation of citric acid, production of IA was observed in *Aspergillus* species as early as 1932. A patent described in 1945 demonstrated the first use of SmF for IA production, which claimed yields around 50% greater than those from distillation.[169] By 1955, Pfizer Co. Inc. was producing IA industrially using SmF and this remains the most commonly employed commercial production method today.[167a, 170] In 1962 another Pfizer patent demonstrated yields of 70% using the cheap substrate beet molasses.[171]

Similar to citric acid production, maximization of IA production has been accomplished through nutrient limitation which obstructs oxidative phosphorylation and produces a high flux of glycolysis.[167a] Karaffa, Díaz *et al*. demonstrated that with manganese concentration in the fermentation media kept below 5 μg/L, 130 g IA/L glucose media could be achieved with yields of 90%.[69] Kuenz, Gallenmüller *et al*. investigated the importance of oxygen supply and phosphate concentration. An adequate oxygen supply, in the form of flask shaking, was shown to improve production from 23 g/L in 20 days to 65 g/L in 10 days.[67] By then increasing the phosphate concentration from 0.04 g/L to 0.16 g/L, the product concentration further increased to 90 g/L. Hevekerl, Kuenz *et al*. demonstrated the importance of pH control during fermentation.

If the pH was controlled and maintained at a value of 3, 146 g/L could be achieved after 2 days of fermentation.[172] This was a 66% increase as compared with no pH control. In a subsequent study, the production was further increased to 160 g/L by targetting a pH of 3.4 instead of 3.[72]

The annual production of IA is around 40,000 tons at a price of 1.5-2$/kg. In order to be competitive with petrochemical-derived products, the U.S. DOE estimates that the price must be cut in half.[167a] Since the production of IA is already close to the theoretical limit, one of the best strategies moving forward is to use cheaper substrates.[167a] *A. terreus* possesses the ability to use a multitude of sugars as substrate including saccharose, lactose, glycerol and xylose.[173] *A. terreus* can also use citric acid as a substrate, which is about a tenth of the price of IA.[174]

2.4.1.3. *Aspergillus oryzae*

Aspergillus oryzae, also called "koji-kin" in Japanese, has been used for millennia in the production of fermentation products.[156, 164] Along with certain other *Aspergillus* species called koji molds, these fungi release amylases that break down rice starch in order to make products including saké, miso, amazake, shouchu, shoyu (soy sauce), and mirin.[155, 175] *A. orzyae* is the primary source for industrial production of kojic acid (KA), which is used in cosmetics and as a precursor to flavor enhancers including maltol and ethyl maltol.[155, 163] KA is a growth inhibitor of bacteria, other fungi and viruses.[163] It inhibits catecholase activity of tyrosinase, an essential enzyme in the biosynthesis of melanin, which makes it popular for use in cosmetics as a skin whitening agent.[155, 163]

While KA may be chemically derived from pyranoid 3,2-enolones, industrial production exclusively uses filamentous fungi which achieve yields of 70-90% depending on the carbon source.[163-164, 176] Many different fungi have been shown to be effective KA producers, including *A. oryzae*'s near genetic twin *A. flavus*. Some of the highest KA production and yields were observed using *A. flavus* but, because of the toxicity of *A. flavus*, other species are preferred.[164] In one screening of five *Aspergillus* species, including *oryzae* and *flavus*, *A. parasiticus* outperformed all strains in terms of KA production.[98] In a different screening involving 278 different fungal isolates, 135 of which were *Aspergillus*, a strain of *A. flavus* outperformed all others and, under optimized conditions, produced KA concentrations reaching 54 g/L.[95] And in a more recent study in which 43 fungal isolates were screened, including *A.*

flavus and *A. parasiticus*, the highest production was observed in an *A. oryzae* strain (44 g/L glucose media).[91] This strain also showed relatively good production when given sugarcane molasses as a carbon source (29 g/L).[91] In the same study, a different *A. oryzae* strain produced 32 g/L when given starch while the best *A. flavus* strain produced 38 g/L and 13 g/L when given glucose or starch respectively.

To maximize production of KA, Ariff, Salleh *et al*. demonstrated that close control of aeration (80% dissolved oxygen during growth phase followed by 20% during production phase) resulted in a two-fold increase in production using *A. flavus* as compared with unregulated fermentation.[177] Devi, Vijayalakshmi *et al*. reported KA production reaching 889 g/L of calabura fruits using *A. flavus* after optimizing fermentation parameters including substrate concentration, temperature, phosphate concentration, etc.[96] Numerous studies showed an improvement in production through immobilization of the fungal cells. Kwak and Rhee immobilized *A. oryzae* in calcium alginate gel to ultimately produce 83 g/L, while free cells only reached a concentration of 25 g/L.[89] Liu, Yu *et al*. reached a production exceeding 84 g/L when using *A. oryzae* immobilized on plastic composite support.[85] Mutation of select strains has also been shown to be an effective method for realizing high production levels of KA. Yan, Tang *et al*. produced a mutant capable of achieving 1.7 times higher concentrations of KA than the parent strain while, in another study, Futamura, Okabe *et al*. produced a mutant strain achieving 7.7 times higher concentrations than the parent strain.[88, 90] Conditions for the latter mutant strain were optimized and, when continuously fed glucose, the strain was able to achieve concentrations of 117 g/L.[90]

The production of KA is similar to that of citric acid and IA in terms of working sugar and phosphate concentrations. As such, by simply changing other fermentation parameters, e.g. stirring or aeration rate, strains producing citric or IA can be shifted to KA production.[163] Despite long time usage and the simple nature of the glucose conversion in the KA reaction (one oxidation and two dehydrations), the pathway is not yet fully understood.[163, 175a] The proposed pathway involves direct conversion of glucose *via* glucose dehydrogenase to D-gluconic acid which is then converted to KA by gluconate dehydrogenase.[163, 175a]

Other beneficial characteristics of *A. oryzae* include an ability to use a variety of carbon sources (e.g. mannose, galactose, xylose, arabinose, sorbitol, acetate, ethanol and glycerol) and an ability to produce high amounts of other organic acids (e.g. malic acid).[155-156] It is also a

prolific producer of protease enzymes for which it can use a variety of appealing feedstocks including sunflower meal, rapeseed meal, palm kernel cake, wheat bran, and bakery waste.[132, 149-153]

2.4.1.4. *Aspergillus flavus*

Aspergillus flavus is a pathogenic fungus almost genetically identical to *A. oryzae*.[178] It is a hardy and abundant species, mostly existing as a saprophyte in soil.[178] *A. flavus* possesses an exceptional ability to accumulate L-malic acid. This commercially important product is mostly used as an acidulant in foods and beverages but can also be used in the synthesis of a biodegradable polymer, polymalic acid.[80, 179] In the past, malic acid was produced *via* extraction from apple juice.[80] Today it is produced either by catalytic hydration of maleic or fumaric acid at high temperature, or by enzymatic transformation of fumaric acid.[80, 179] While biological/enzymatic methods yield optically pure products (synthetic chemical pathways do not), these methods are more expensive.[80] However, as petroleum based starting materials increase in price, fermentation may become a more economic means of production.

Malic acid is an essential component of cellular metabolism but generally accumulates at a much lower rate than citric or fumaric acid and therefore has not been produced commercially *via* microbes.[80] In *A. flavus*, there are two pathways leading to the production of L-malic acid that, as enzyme activity monitoring would suggest, operate simultaneously during the acid production phase of the fungal organism's life cycle.[80] While malic acid is an intermediate of the TCA cycle, ^{13}C NMR studies suggest that a separate, reductive pathway is primarily responsible for most malic acid accumulation in *A. flavus*.[80] C NMR detects the ^{13}C isotope of carbon, which comprises about 1.1% of naturally abundant carbon.[180] Thus by incubating *A. flavus* in a medium of glucose with labeled anomeric carbon (1-^{13}C), the researchers could demonstrate that this anomeric carbon was only incorporated in the C3 of L-malic acid.[80] From this the researchers were able to conclude that L-malic acid must be synthesized from oxaloacetate.[80] This pathway begins, like many, with pyruvate in the cytosol, which is converted into oxaloacetate *via* pyruvate carboxylase.[181] Oxaloacetate is then reversibly converted to malate *via* NAD-malate dehydrogenase (MDH).[181]

Battat *et al*. were able to optimize the production of L-malic acid using *A. flavus* to reach 113 g/L after 190 hours with a productivity of 0.59 g/L/h.[80] This was accomplished by fine tuning fermentation conditions including the agitation rate and the concentrations of Fe^{2+} ion, phosphate, and neutralizing agent.[80] While *A. flavus* possesses extraordinary malic acid accumulating properties, it is also known to produce aflatoxins, which are toxic and carcinogenic.[182] Since malic acid is predominantly used in food, this precludes the use of *A. flavus* for production.[80] Alternatively, some reports demonstrate that strains of *A. oryzae* and *A. niger* achieve competitive or even better production than *A. flavus*. Knuf, Nookaew *et al*. demonstrated that *A. oryzae* was able to produce relatively high titers (>30 g/L) at productivity levels about equal with *A. flavus* (0.58 g/L/h).[78] Iyyappan, Bharathiraja *et al*. generated a mutant of *A. niger* using methanol, that was reported to produce 77 g malic acid/L.[82] Brown, Bashkirova *et al*. genetically engineered a strain of *A. oryzae* with upregulation of genes encoding pyruvate carboxylase, malate dehydrogenase and a C4-dicarboxylic acid transporter.[79] This mutant was able to achieve production of 154 g/L (upregulation of only the transporter gene resulted in production of 122 g/L).[79] Thus, these organisms may be used instead of the aflatoxin producing *A. flavus,* should economic conditions become favorable for malic acid production *via* fermentation.

Aspergillus flavus has also shown potential for use in the valorization process of lignin, a component of lignocellulose that is otherwise mostly burned for heat.[183] *A. flavus* has demonstrated the ability to convert aromatic carboxylic acids, which are common and abundant products of lignin in biomass processing facilities, into corresponding alcohols *via* oxidative decarboxylation or carboxyl reduction mechanisms.[183] *A. flavus* could convert a diverse number of starting compounds at great rates (78%-100%), with great selectivity (alcohols were the only biotransformation products) and with high substrate tolerance (standard performance up to 8 g vanillic acid/L).[183]

2.4.2. *Rhizopus*

Rhizopus species are common and widespread soil dwelling saprotrophs that grow quickly and, as such, are early colonizers of an environment.[184] These fungi are industrial

producers of enzymes, organic acids, fermented foods, biodiesel, alcohols, esters, polymers, volatile compounds, and have been used in cancer research.[184]

In *Rhizopus*, all fermentable carbon sources are metabolized to pyruvate.[185] Subsequent carbon flow is influenced by dissolved oxygen in the medium; i.e. under anaerobic conditions, the direction of carbon flow is favored toward the formation of ethanol, while under aerobic conditions with excess of carbon substrate, the direction of the flow is favored toward organic acid production.[185] Under anaerobic conditions, pyruvate is largely converted to acetaldehyde *via* pyruvate decarboxylase (PDC) and is then converted to ethanol *via* aldehyde dehydrogenase (ADH). Under aerobic conditions, the genes for PDH and ADH are down regulated and pyruvate is largely converted to lactic acid *via* a single NAD^+ dependent step involving L-lactate dehydrogenase.[184b, 185]

With an annual production of 74 billion gallons, ethanol is a molecule of premier economic importance.[185] While the current benchmark for ethanol production is with *Saccharomyces cerevisiae*, there are a number of advantages in using a filamentous fungus including *R. oryzae*, which has been reported to produce yields of ethanol and possess ethanol tolerance levels comparable to yeast (yields of 0.5 g/g glucose and tolerance of over 20% v/v ethanol).[185-186] For example, *S. cerevisiae* is unable to use pentose sugars, which are present in hemicellulose hydrolysate, while *R. oryzae* is able to grow on a myriad of substrates including xylose, glycerol, lactic acid, glucose, mannose, fructose, cellobiose, fatty acids, oils and ethanol.[185] Additionally, *R. oryzae* has low growth requirements, is able to tolerate inhibitors present in the acid hydrolysates of lignocellulosic biomass, can use cellulose and hemicellulose directly (though slowly), can withstand high sugar concentrations (in excess of 100 g/L) and can grow in a wide range of temperatures (up to 40° C) and pHs (4 to 9).[185] There is a large body of literature focused on bio-ethanol production; however, the scope of this review focuses on organic acids and enzymes.

R. oryzae strains are typically lactic acid producers, fumaric acid producers, or producers of both [184b]. With an annual production of 100,000 tons, lactic acid is used primarily in the food industry as a preservative, flavor enhancer and acidulant, but it is also used in the manufacture of oxygenated chemicals, biodegradable solvents (e.g., ethyl lactate), and polylactic acid (PLA).[34, 185] Interest in PLA has increased steadily due to its biodegradability, environmental friendliness, and its potential to functionally replace many of the most common fossil fuel

derived plastics, see Fig. 3.[187] Lactic acid can be synthesized chemically through a number of processes including hydrolysis of lactonitrile by strong acids. However, none of these routes are technically economically viable and thus industrial production of lactic acid is most commonly achieved *via* microbial fermentation.[188] There is much literature focusing on bacterial fermentation for lactic acid production, but fungi offer a number of advantages over bacteria including reduced costs, from cheaper medium requirements and less complicated product purification, as well as optically pure products and absence of byproduct accumulation.[184b, 188-189]

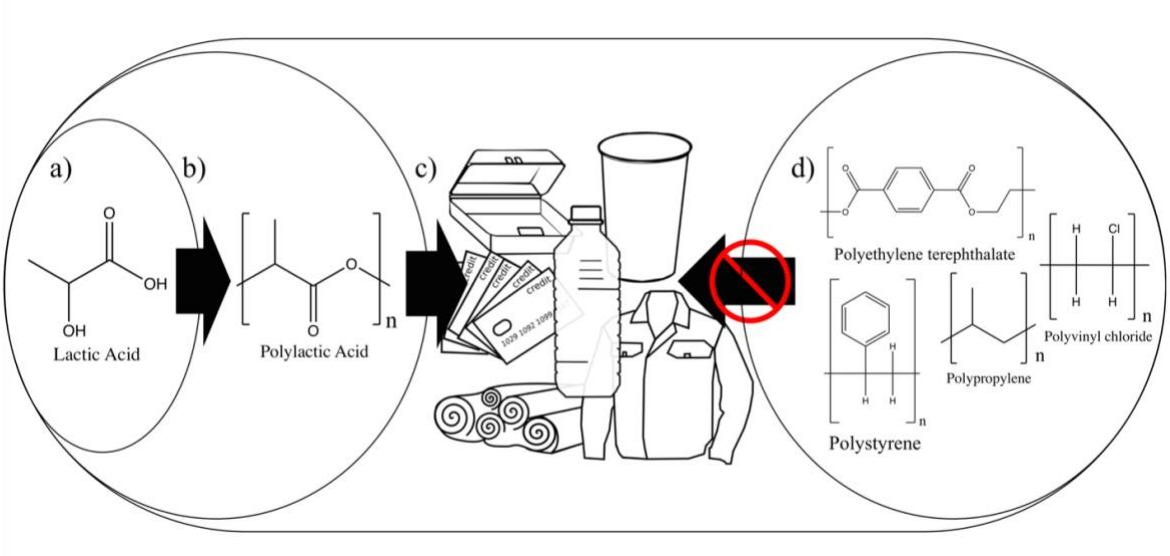

Fig. 2.3. The microbial product lactic acid **(a)** may be used to generate polylactic acid **(b)** which itself may be used in production of textiles/fabrics, food and liquid containers, building materials, and much more **(c)**; in serving as a building block for production of these materials, polylactic acid will replace many common fossil fuel feedstocks including polyethylene terephthalate, polystyrene, polypropylene, and polyvinyl chloride**(d)**.

Soccol, Marin *et al*. demonstrated that *R. oryzae* was able to produce up to 137 g/L in SSF of glucose with bagasse solid support.[103] This was superior to the production using SmF in a flask or fermenter which achieved 89 g/L and 93 g/L respectively.[103] Yamane and Tanake immobilized *R. oryzae* cells within sponge-like particles made of polyurethane foam and produced 145 g/L (or 175 g/L as anhydrous calcium lactate) at 1.42 g/L/h from 150 g/L glucose, and 231 g/L (or 280 g/L as anhydrous calcium lactate) at 1.83 g/L/h in fed-batch cultivation.[106]

Park, Kosaki *et al*. demonstrated the importance of *R. oryzae* morphology on lactic acid production. In this study, poly(ethylene oxide) was added to a culture after 12 hours in an air-lift bioreactor containing mineral supports.[100] This induced the cultures to adopt a cotton-like mycelial morphology and increased production up to 105 g/L (87% yield) as compared with 43 g/L of the control.[100] Guo, Yan *et al*. screened 100 different strains of *R. oryzae* for an optimal lactic acid producer, and optimized conditions for maximum production using the selected strain. The selected strain (*R. oryzae* GY18) was able to produce: 46-115 g/L lactic acid when using glucose concentrations from 60-160 g/L; 15-69 g lactic acid/L using 20-100 g xylose/L; and 370 g/kg of corncob hydrolysate.[101] Wang, Li *et al*. used low-energy ion beam irradiation to create a mutant strain of *R. oryzae* (RQ4015) that was able to produce up to 121 g/L lactic acid at 3.36 g/L/h (10% and 46.7% respectively better than the parent strain) using 150 g/L glucose.[102] This strain was also able to produce 74 g/L lactic acid using 100 g xylose/L.[102]

Fumaric acid was traditionally produced *via Rhizopus* but is now synthesized through cheaper chemical synthesis methods including isomerization of malic acid (or maleic anhydride) catalyzed *via* benzene.[163, 190] However, like with malic acid, as petroleum-based materials become more expensive, enzymatic fumaric acid production may reemerge as a widely employed method. With an annual production of 90,000 tons, fumaric acid is mainly used in the food industry as an acidulant, but it is also used for the industrial production of malic acid, production of aspartame, jet printing inks, plastic surface coating, paper sizing, preparation of unsaturated polyester and alkyd resins, used as an optical bleaching agent, and in psoriasis treatment among other things.[163, 185]

In *Rhizopus*, fumaric acid is an intermediate in the TCA cycle and is formed *via* two pathways: (i) the normal oxidative pathway of the TCA cycle in which succinate is oxidized *via* succinate dehydrogenase to form fumaric acid; and (ii) a reductive pathway in which CO_2 is condensed with pyruvate *via* pyruvate carboxylase to form oxaloacetate, which is reduced to malate and then to fumarate.[163, 191] A patent from Rhode, Lagoda *et al*. in 1962 identified the necessity for continuous neutralization of fumaric acid for optimal yields.[113] Rhodes, Lagoda *et al*. were able to acquire 100 g/L fumaric acid from *R. arrhizus* with a 90% yield after 60 hours maintaining 0.8 g calcium carbonate/L.[113] However, calcium carbonate makes the fermentation more difficult to handle.[113] To avoid this, Cao, Du *et al*. report using a rotary biofilm contactor coupled with an adsorption column.[119] In this design, *R. oryzae* attaches to plastic discs that

alternate between nitrogen rich (during growth period) and nitrogen poor (during non-growth period) conditions.[119] Subsequently, any fumaric acid produced would be removed by the adsorption column. This ultimately led to production of 85 g/L fumaric acid from 100 g/L glucose in 20 hours. As in fungal production of many other organic acids, dissolved oxygen content in the media has been showed to play an important role in the production of fumaric acid. A patent from Lorraine B. Ling in 1989 demonstrated that 80% dissolved oxygen content led to a production of 135 g/L after 70 hours using *R. arrhizus*, while dissolved oxygen levels <5% only produced 39 g/L after the same amount of time (100% dissolved oxygen produced 72 g/L).[76] While most wild strains of *R. arrhizus* do not use xylose as a carbon source, a strain isolated by Liu Wang *et al*. produced 45 g fumaric acid/L using 80 g xylose/L when immobilized in a mesh of printing and dying chemical fiber that, in a previous study, was shown to reduce fermentation time by 83.3%.[117, 192] As an alternative to the chemical fiber, loofah fiber, a natural, biodegradable, renewable polymer, was also found to be a suitable carrier for immobilized *R. arrhizus*, enabling production of 30 g/L using glucose.[118]

Rhizopus is also an industrial source of enzymes. *R. oryzae*, for example, is a prolific producer of amylases (e.g. β-, gluco and isoamylase) and lipases, which have both traditionally been used as food aids but recently have been used in biotechnology for the production of fuels (e.g. biodiesel), fine chemicals, pharmaceuticals, agrochemicals, perfumes, flavors and bio-surfactants.[184b] In addition to amylase and lipase, other enzymes found in *R. oryzae* cultures include cellulases, hemicellulases, proteases, urease, ribonuclease, pectate lyase, and polygalacturonase.[184b] The genome of *R. oryzae* provides insight into the types of enzymes it may be optimized to produce. Most prevalent are genes coding for enzymes involved in degradation of storage polysaccharides (e.g. galactomannan and starch) and of backbone molecules in structural polysaccharides (e.g. cellulose and pectin).[184a] There are also chitinolytic and β-1,3-gluconolytic systems which are used in the breakdown of cell wall material, probably used in defense against competing fungi or in recycling cell wall material left over from former fungal colonizers.[184a]

2.4.3. *Trichoderma*

The genus *Trichoderma* is comprised of soil dwelling species found worldwide in decaying cellulosic material as well as in the rhizosphere of plants, where these fungi are known to help protect the plant against pathogens, enhance root growth, and improve uptake and use of nutrients.[193] *Trichoderma* are characterized by rapid growth and successful colonization of diverse habitats.[193a] These filamentous fungi can thrive in rich and complex tropical rainforest soil as well as sterile biological fermenters. Various species of *Trichoderma* have been isolated from sources ranging from cockroaches to marine muscles, shellfish and termite guts.[193a] First identified in 1794, *Trichoderma* has now found use in bio-remediation, recycling of waste materials, biocontrol through bio-fungicides and antibiotics, and commercial production of enzymes.[193a, 194] Enzymes from *Trichoderma* are used in varied applications such as to improve brewing processes (β-glucanases), for maceration of fruits in fruit juice production (pectinases, cellulases, hemicellulases), and as feed additive in livestock farming (xylanases). Because of the widespread use of these enzymes, the cellulase system of *Trichoderma* is probably the most widely studied cellulase system.[193a, 194]

Trichoderma are a successful genus due to a high capacity for secreting enzymes and antibiotic metabolites, which include lytic and proteolytic enzymes, ABC transporter membrane pumps, diffusible or volatile metabolites in addition to polyketides, pyrones, terpenes, peptides and metabolites derived from amino acids.[193a] *Trichoderma reesei* represents the premier industrially employed species of the genus.[124, 193a] *T. reesei* is used in heterologous protein expression and is the most important cellulase producer worldwide. This species is known to produce titers of 100 g cellulase/L using a broad range of carbon sources that include agricultural and industrial byproducts.[124, 193a] Additionally, *T. reesei,* which primarily produces exo- and endoglucanases with little β-glucosidase activity, and *A. niger*, which mostly produces β-glucosidase, represent the most commonly used fungi for production of enzymes that are used in the hydrolysis and saccharification of lignocellulosic materials.[195]

Enzyme production in *Trichoderma* can be induced by substrates including lactose, sophorose, xylobiose, D-xylose, and L-sorbose, though these carbon sources are expensive and thus require high investment costs.[124, 195b] However, *T. reesei* has also shown the ability to use lignocellulosic carbon sources for production of cellulases, amylases, hemicellulases, lignin

degrading enzymes, peptidases, proteinases, and transport proteins.[196] For example, spent hydrolysate model medium (SHMM) and manure were found to induce high production of cellulases and xylanolytic enzymes.[124, 197]

Enzymatic production in *T. reesei* is transcriptionally regulated and heavily dependent on the carbon source.[193a] As such, titers and abundances are quite variable due to the complex nature of lignocellulosic materials.[124, 194, 196] *T. reesei* has been reported to produce at least four endo-1,4-β-xylanases, two β-xylosidases, two endo-1,4-β-D-glucan cellobiohydrolases, five endo-1,4-β-D-glucan-4-glucanohydrolases, and two β-D-glucosidases as well as β-mannanase, β-mannosidase, α-L-arabinofuranosidase, α-galactosidase, acetylxylan esterases, and laccases.[198] Despite the widespread use of *T. reesei* for enzyme production, sequencing of the genome reveals that other species within the genus have as much as 2000 more genes than *T. reesei* that encode cellulolytic and hemicellulolytic enzymes.[193a] Other studies confirm that *T. reesei* produces a relatively smaller number of cellulases and hemicellulases when compared with other plant cell wall degrading fungi, the caveat being that the production is heavily dependent on the medium.[124] Other species within the genus may therefore be further explored for potential adaptation to the industrial production of enzymes. *T. harzianum*, for example, has been reported to express β-glucosidase, β-xylosidase, pectinase and α-amylase during SSF of wheat bran.[194, 199] Additionally, *T. harzianum* was found to produce the antibiotic decalactone, 6-pentyl-α-pyrone, from substrates including castor cake and espresso coffee grounds.[199b]

2.5. Application, Techno-economics, and Policy

2.5.1. Application and Techno-economic Analyses

Techno-economic analyses have long established the viability of bio-based processes (i.e. fermentation, enzymes, etc.) in the production of many fuels and bulk chemicals (e.g. 1,3-propanediol (PDO), polytrimethylene terephthalate, and succinic acid).[21] However, the competitiveness of petrochemicals has impaired investment in such processes.[200] This issue extends, and to a greater extent, when considering lignocellulosics as feedstock. A 2011 report released on behalf of the National Academy of Sciences concludes that cellulosic-based fuels are only likely to be competitive in the presence of very high petroleum prices (>191$/bbl.) and/or a

regulatory environment marked by high carbon price.[201] It is worth noting though that this report fails to include projections for biorefineries that incorporate multiple revenue streams. This type of approach would be analogous to that of integrated petrochemical refineries where 7 or 8% of crude oil is dedicated to chemical production which results in 25 to 35% of annual profit.[202] In a DuPont/Tate and Lyle commissioned study analyzing PDO, the projected ROI following a conventional fossil fuel route was 11% while production from biomass showed just 3%.[202] However, by integrating chemical and fuel production in the same facility, the projected ROI for a biomass based route jumped to 20%.[202]

In a similar way, filamentous fungi can be used to integrate additional revenue streams. For example, in a starch-based ethanol facility, the thin stillage waste stream was valorized using various fungal species (*N. intermedia*, *A oryzae* and *Rhizopus* sp.) into animal feed and additional ethanol.[203] Furthermore, coupling lignocellulosic feedstocks to such processes would help relieve the high capital costs and investment risks associated with lignocellulosic-based processes. One study of such a system demonstrated a required capital investment of 77 million USD which, after 20 years, would yield a net present value of 162 million USD.[200] Additionally, organic acids are appealing value-added products in lignocellulosic valorization processes. For example, it was found that, across various scenarios, the lowest long-term minimum ethanol selling price (MESP), 1.98 $/gallon, would result from pairing a recombinant ethanol producing yeast with an organic acid producing fungus (*F. oxysporum*).[204] The near-term base case involving only ethanol production from yeast resulted in 3.35 $/gallon.[204] Even in a process involving *F. oxysporum* alone, the consolidated bioprocess (CBP) production of ethanol and organic acids was predicted to yield a competitive long-term MESP of 2.10 $/gallon (if ethanol tolerance of the strain could be improved).[204]

2.5.2. Policy

In general, the socio-environmental goals of bioeconomy policy must include regional development, waste management, support for sustainability, and reduction of GHG emissions, pollution, and pressure on land and marine resources.[205] In planning construction of biorefineries, government policy should facilitate studies on the type of biomass (e.g. crop residues, municipal solid waste, food waste, etc.) and its origin, quantities, recyclability, and

sustainability in particular areas.[205] These studies will help determine the type of biorefinery and where to build it as well as which products to make, how much to make, and which processes to use for production.[205] Additionally, depending on the type of biomass, integration with existing regulation (e.g. waste management, health, environment) will be necessary.

While many governments do have published bioeconomy strategies, most attention is paid to the biomass feedstock and not to the role of biotechnology.[206] Numerous startups already employ biotechnology to make renewable fuels, chemicals, and materials but most startups lack the resources and infrastructure for fundamental research.[206] Larger biotechnology or chemical companies, which do have such resources, may lack the incentive, particularly with uncertain government policy, to use these resources for research in biotechnology applications to fuel and chemical production.[206] It is through public-private partnerships that confidence can be established in government commitment to the bioeconomy.[206]

To address issues of reproducibility, reliability and predictability caused by the large inherent variability in bioprocesses, government policy must aid in developing the standards of good practice for the industry. According to research for the British Standards Institution, these standards contribute to a large portion of annual productivity growth.[206] Standards are used by companies as management systems and as a means to reduce risk in novel processes, accelerate routes to market, facilitate trade, and to improve consumer, investor, and regulator confidence.[206] Additionally, policy must promote training in areas relating to the new job opportunities that will support the bioeconomy. Policy must incentivize technical training in relevant skills and, at the level of higher education, encourage cross disciplinary studies which incorporate computer science and engineering approaches into biology curricula.

2.6. Future Prospects

2.6.1. Consolidated Bioprocessing

The process of deriving chemicals from lignocellulosic material usually involves several steps: (i) pretreatment of material to separate lignin and free the holocellulose for hydrolysis; (ii) hydrolysis of polysaccharides to obtain fermentable sugars; (iii) fermentation; and (iv) separation

of products.[207] Simplification of this scheme and integration of as many steps as possible, referred to as CBP, can serve as a successful strategy to reduce capital and processing costs.[208]

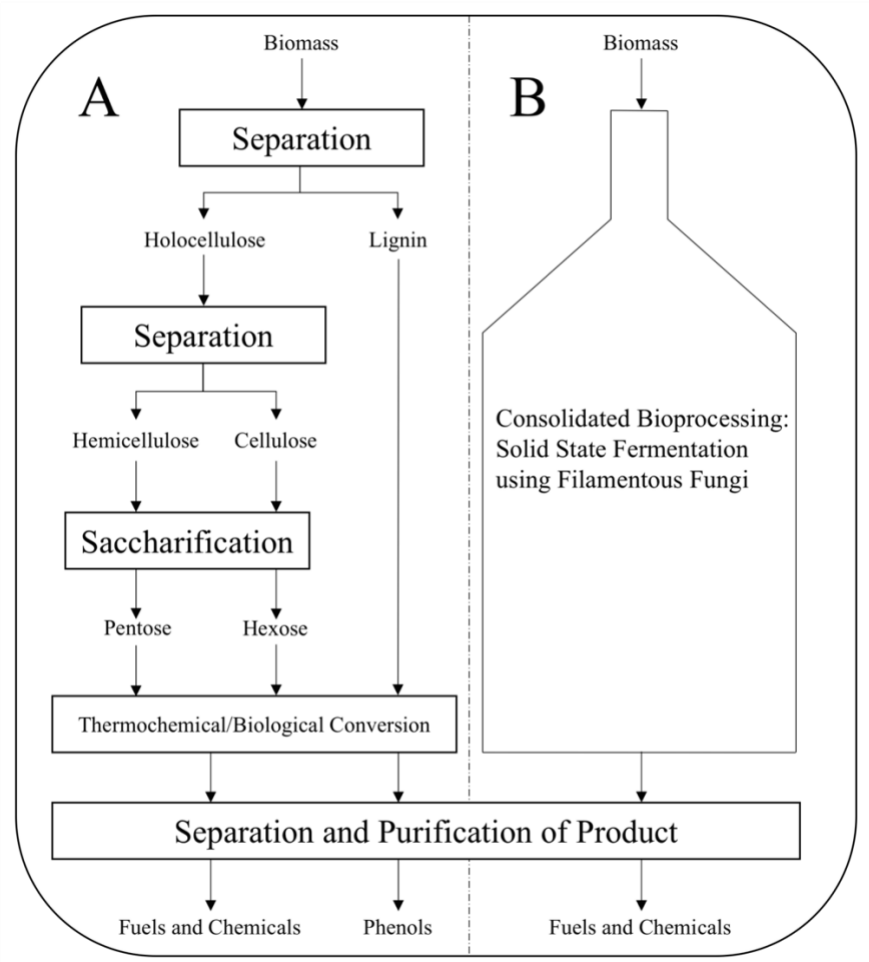

Fig. 2.4. (A) represents a typical process flow of raw biomass to final products while (B) represents a theoretical consolidated bioprocess, in this scenario using fermentation via filamentous fungi, where many of the original process steps are condensed into one step.

Pretreatment, hydrolysis and fermentation may all be consolidated into a single step *via* SSF, as shown in Fig. 4, where the waste material is directly fermented without any processing. Intact lignocellulosic material is, in nature, primarily degraded by SSF, which is technically defined as fermentation in absence or near absence of free water.[125a, 209] For example, microorganisms, especially fungal cultures, produce comparatively high titers of cellulase under conditions which are similar to the natural environment.[209] While SSF is one of the oldest

technologies humans have applied (in fermentation of food and for composting and ensiling), SmF became popular due to ease of handling and better monitoring capability.[210] Almost all industrial bioproduct production schemes, including for enzymes and commodity chemicals, currently use SmF.[209-211] However, recent research demonstrates that adopting SSF can, for many processes, result in a 10-fold reduction of costs.[209-211] Additional major reported advantages of SSF over SmF include higher product titers and yields, reduction in water consumption and waste generation, technical simplicity, low capital investment, low energy requirement, and better product recovery.[123b, 209-210, 212] *T. reesei*, for example, is known to be generally deficient in β-glucosidase activity, but in SSF it showed increased β-glucosidase levels and increased hydrolytic potential vis-à-vis liquid fermentation.[30a]

2.6.2. Complete Valorization of Lignocellulosic Material

While the utility of sugars derived from the holocellulose component has been extensively explored for the production of valuable chemicals, lignin, after being separated, is traditionally just burnt for energy.[213] However, lignin, which is the only renewable and abundant polymer containing aromatic rings as building blocks, may contribute an estimated $13 billion in revenue to biorefinery operations if used for purposes other than heat.[213-214] For example, vanillin, the main component of vanilla flavoring, may be derived from lignin. Currently, around 1% of vanilla flavoring comes from the vanilla bean pod of the tropical vanilla orchid (priced between 1000-4000$/kg) while the remainder (priced around 10-15$/kg) is mainly derived from the petroleum product guaiacol.[215] However, there is growing demand for both "natural" and cheap vanilla flavoring.[215] One of the most successful strategies achieving this involves microbial fermentation.[216] In fact, the largest global producers of vanillin, Solvay and Evora, are scaling up "natural" vanillin production using microbial fermentation of ferulic acid (another lignin derivative).[216] Though most of the literature focuses on bacterial fermentation for vanillin production, filamentous fungi have also shown promising utility here including a reported ability for using whole lignocellulosic substrates.[217] Numerous other building block chemicals may be derived from lignin including ethanol and other alcohols, dimethyl ether, hydrocarbons, phenols, and dimethyl sulfoxide. Without depolymerization, lignin has potential for use in the production of carbon fiber.[213]

It has already been widely demonstrated that numerous microbes can degrade lignin under aerobic and anaerobic conditions.[207] While the process of lignin biodegradation has been extensively studied in the filamentous white-rot fungi *Phanerochaete chrysosporium*, which catalyzes the degradation with extracellular oxidative enzymes in a H_2O_2 dependent process, the entire process is not yet fully understood and the heretofore identified enzymes may not even be directly involved.[218] Other enzymes may therefore need to be identified. The main enzymes so far identified to be responsible for lignin degradation include lignin peroxidase (LiP), manganese peroxidase (MnP) and the copper-containing phenoloxidase known as laccase.[33c] These ligninolytic enzymes have already demonstrated utility in the food industry, pulp and paper industry, textile industry, and as biocatalysts.[33c] Currently, the main challenges in moving forward with microbial lignin degradation is that ligninolytic enzyme producing microbes only produce small amounts of these enzymes.[211] Thus, in addition to gaining a full understanding of the complete mechanism, more research is needed on improving ligninolytic enzyme production. The latter is an area of focus in molecular engineering research and recent advances demonstrate improved expression, activity, and stability of ligninolytic enzymes.[219] Future work will include research into ancestral ligninolytic proteins to gain insight into the functional evolution of the ligninolytic consortium.[219] Other research will involve engineering a ligninolytic secretome into yeast for CBP production of ethanol from lignocellulosic materials.[219] It is to be seen whether the metabolic burden and expression constraints will prove to be limiting factors.[219]

2.6.3. Mixed Culture Fermentation

Effective use of lignocellulosic materials require, due to complex composition, multiple enzymes for pretreatment/saccharification including ligninolytic, cellulolytic, and xylanolytic enzymes.[211] While numerous reviews exist demonstrating that every step in the lignocellulosic conversion process may be catalyzed by a known organism, no single natural microorganism possesses all the features needed for the complete process.[207, 220] Certain fungi, for example, possess superior hydrolytic enzyme production capacity while others give high product yields. Most natural ecological processes are symbiotic or competitive and, as such, fungal consortia resembling the natural metabolic synergies found in nature, may be excellent for production of a

myriad of lignocellulolytic enzymes and value-added molecules in a single reactor within a biorefinery.[211, 221] This would simultaneously address the two most important issues facing lignocellulosic biomass conversion processes; i.e. the need to overcome the recalcitrance of the material and to reduce the number of steps involved in biorefining. Co-culturing is already widely used in SmF production of antibiotics, enzymes, fermented food and beverages, composting and conversion of wastewater sludge, but the advantages of co-culturing may be exploited even further if applied to SSF.[211] Current industrial process involving co-culture SSF include the anaerobic digestion of organic materials to produce methane and, more recently, investigations have been made into using co-cultures to produce hydrogen, ethanol, and biodegradable plastics.[207]

To develop a co-culture system: first, individual strains are identified that possess the desired properties; next compatibility of the strains is tested; and then performance is measured.[211] Currently, the variety of research on mixed cultures applied to lignocellulose conversion is limited and the anticipated challenges moving forward involve controlling the consortium as well as finding matching fermentation conditions for multiple species.[208] Successful examples, summarized in Table 4, include a combination of *Trichoderma* and a pair of yeast species that were used to simultaneously saccharify non-detoxified dilute acid pre-treated wheat straw slurry, resulting in yields of 10 g ethanol/L.[208] Additionally, the white rot fungi *Phanerochaete*, known to only produce small amounts of extracellular enzymes and thereby precluding use in industrial application, showed higher laccase production when co-cultured with *Trichoderma*.[211]

Table 2.4. Examples of results using mixed microbial cultures.

Organisms	Substrate	Product	Amount	Source
T. reesei & *L. pentosus*	Cellulose + Xylose	Lactic Acid	54.6 g/L	[222]
T. reesei & *L. pentosus*	Beech Wood	Lactic Acid	19.8 g/L	[222]
Rhizopus sp. MK-96-1196 & *A. thermophilus* ATCC 24622	Corn Cob	Lactic Acid	24 g/L	[223]
2 strains of *A. niger*	Bran and Cottonseed Powder	Glucoamylase	13, 400 U/g	[224]
A. niger & *F. monoliforme*	Wheat bran, Oat Straw and Beetroot Press	α-amylase	250 IU/g	[225]

2 strains of *A. niger*	Bran and Cottonseed Powder	Cellulase	996 U/g	[224]
A. niger BC-1 & *T. reesei* C-30 (ATCC 56765)	Rice Straw & Wheat Bran	CMCase	131 IU/g	[226]
Thermoascus aurantiacus & *A. niger*	Oats Straw	β-glucosidase	298 IU/g	[147]
A. niger & *A. oryzae*	Wheat Bran	β-glucosidase	2975 +/- 5.3 U/g/min	[227]
A. niger BC-1 & *T. reesei* C-30 (ATCC 56765)	Rice Straw & Wheat Bran	Xylanase	3110 IU/g	[226]
A. niger BC-1 & *T. reesei* C-30 (ATCC 56765)	Sugarcane Bagasse + Black Gram Husk	Xylanase	8205+/- 168 IU/g	[228]
2 strains of *A. niger*	Bran and Cottonseed powder	Hemicellulase	15,900 U/g	[224]
2 strains of *A. niger*	Bran and Cottonseed powder	Pectinase	7,620 U/g	[224]
2 strains of *A. niger*	Bran and Cottonseed Powder	Acidic Proteinase	5,580 U/g	[224]
A. *niger* & *T. versicolor*	Wheat Bran, Oat Straw and Beetroot Press	Laccase	97,600 IU/g	[225]
T. maxima & *P. carneus*	Glucose and Peptone	MnP	1230 U/L	[229]

2.6.4. Exploration of Other Target Chemicals

Many reviews are available on CBP for production of biofuels using yeast paired with a lignocellulose hydrolyzing fungus (e.g. *Trichoderma*)[220, 230], but few discuss the potential to apply CBP to production of other valuable chemicals and/or use of other organisms. A good place to begin consideration for new research is by looking at the other chemicals listed on the U.S. DOE's report, e.g. levulinic acid (LA). LA is continually ranked high among important biorefinery target products and is used to produce numerous valuable commodities including succinic acid, resins, polymers, herbicides, pharmaceuticals, flavoring agents, plasticizers, anti-freeze agents and biofuels/oxygenated fuel additives.[12b, 231] Heretofore the majority of research on LA production from biomass has focused on using mineral acid (in particular, Brønsted acids) catalysts though these processes are challenged with expensive raw material, low yields, high equipment cost, problematic recovery and handling, and significant amounts of waste that contributes to environmental pollution.[12b, 231] All of these challenges may be addressed by adoption of a CBP scheme. For example, it has been reported that the filamentous fungi *P. purpurogenum* produces LA when given corn cob, wheat bran, or rice husks as a substrate, with

production up to 46.1 mg/g.[26] This production may be tested with other substrates, synergistic microbes, optimized parameters, or additional co-products. Other products of interest obtained from *P. purpurogenum* include various enzymes, phenolic compounds, and bio-colorants.[232] Bio-colorants, which are used in human and animal food as well as by the pharmaceutical industry, are increasingly researched as value-added chemicals and filamentous fungi, including also *Monascus purpureus* and *Blakeslea trispora*, are emerging as premier producers of bio-colorants.[132, 233] With the goal of adding as much value as possible to biorefinery operations, it is important to explore many different target chemicals and novel, cheap, and environmentally benign ways of extraction.

2.7. Concluding Remarks

The bio-economy and circular economy are interrelated visions, among many, of a more secure and sustainable future for humanity's resources. Biorefineries represent an essential component in realizing both of these concepts. The future of biorefineries involves adopting cheaper feedstock sources such as municipal and agricultural waste materials. Furthermore, it will be essential to completely valorize the biomass source with minimum loss of energy and mass. Biological methods of conversion have the potential to meet the technical, economic, and environmental objectives of biorefinery operations. This review has described the capacity of various filamentous fungi to catalyze each step involved in the complete conversion of raw biomass to valuable products. Numerous fungal products already show promise in replacing fossil fuel-based feedstocks for production of fuels, polymers, and chemicals. The future will involve improving the cost competitiveness of these processes, optimizing feedstock choice and enhancing productivity, and in testing the heretofore underexplored fungal products for industrial applications.

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Connecting Statement 1

As presented in Chapter 2, filamentous fungi possess diverse utility in biorefining processes. They produce myriad enzymes that make them appealing for use in processing raw biomass and are also known to produce numerous commodity and specialty chemicals. Despite the sizeable amount of such information reported to date, the vast majority of potential products from filamentous fungi (and microorganisms in general) remain underreported or not yet described. This represents a key knowledge gap that has important implications in the development of processes for, and proliferation of biorefineries. For biorefineries, in order to make best use of locally available feedstocks (especially local waste residues), the choices of which feedstock to use and which product to target are key components of the design process. Thus, knowledge of all potential products into which a given locally available feedstock may be transformed would be immensely valuable.

In Chapter 3, the tools commonly employed in the field of metabolomics are applied in a novel methodology for screening the products of filamentous fungal solid-state fermentation of feedstocks representative of locally available biomass residues. This chapter is based on a manuscript prepared for publication with Dr. Valérie Orsat and Dr. Marie-Josée Dumont as co-authors.

Chapter 3. Non-targeted screening and multivariate analysis of waste stream biomass conversion products

3.1. Abstract

Derivation of energy and materials from local waste streams may contribute to the obviation of the unsustainable linear fossil-based material economy. To that end, knowledge of products that may be derived from these feedstock streams must be expanded. When considering bio-based feedstock conversion methods (e.g. fermentation, whole-cell/enzymatic catalysis, etc.), techniques from metabolomics may be excellent for the comprehensive screening of underexplored feedstocks and/or underexplored bioconversion platforms. In this study, the compositions of various residues representative of local municipal organic waste streams were characterized and, subsequently, each residue underwent solid-state fermentation with a filamentous fungus (*Talaromyces* sp. NRRL 2120). Non-targeted ultra-performance liquid chromatography coupled with quadrupole-time-of-flight electrospray ionization mass spectrometry in combination with multivariate analysis was applied to analyze the products of the fermentations. The product profile was observed to vary substantially with different residues (as well as between fermented and non-fermented samples of the same residue). Between 3647-7895 features were identified in each residue, with between 1823-2618 unique and statistically significant metabolites associated with specific fermented residues. Among these metabolites were many "top opportunity" chemicals, as identified by the US Department of Energy, including furans and organic acids.

3.2. Introduction

Biorefineries, wherein renewable biomass feedstocks are converted into carbon-based fuels and materials, represent the bioeconomy analog to the petro-chemical refinery. Industrial scale products, including fuels and building block chemicals (e.g. hydrogen, syngas, methane, organic acids, polyhydroxyalkanoates, etc.), are currently produced in biorefineries using thermochemical (e.g. pyrolysis, gasification, chemo-catalysis, etc.) and/or biological methods (e.g. fermentation, whole-cell/enzymatic catalysis, etc.).[234] Of the variety of feedstocks

available to biorefineries, local waste stream residues (e.g. agricultural, forestry, and post-consumer residues) may be associated with numerous advantages in terms of cost and sustainability.[5] Additionally, biorefineries may benefit from being designed on a more local scale (reducing costs and emissions associated with transportation among much else) and contributing to a more circular material economy by closing material loops of the processes from which the feedstocks are generated. While the number of oxygenated products that may be derived from biomass is large (untenably so), the variety of products and conversion technologies available to biorefineries is limited. This issue is further exacerbated when attempting to make use of highly variable local waste-streams. Petrochemical refineries on the other hand, have benefited (almost exclusively so) from the amount of research to date focusing on highly reduced fossil-based hydrocarbons as opposed to highly oxygenated carbohydrate-based materials.[202] The closing of this knowledge gap represents one objective for the advancement of biorefineries.

In the case of biological-based conversion of feedstocks (which offer a number of advantages vis-à-vis thermochemical conversion including biodegradability of treatment components, lower heat requirements, etc.), and specifically whole-cell conversions, the number of potential products is proportional to the number of metabolites associated with a given organism which remain largely underexplored or altogether unidentified.[234b] We propose here that the application of techniques utilized in the burgeoning "-omics" fields may serve to rapidly and efficiently close the knowledge gap between petro- and bio- processes as well as enable biorefineries to more effectively use locally availably feedstocks. Specifically, techniques in metabolomics (defined as the comprehensive and quantitative study of the entire set of small molecules in a biological sample) may be ideally suited for this purpose.[235] Metabolomic methods have been widely applied for the purpose of biochemical pathway elucidation, drug discovery, biomarker identification as well as in bioprocess optimization (e.g. in strain development, parameter optimization, as well as in comparing biomass composition for use in, for example, production of biomass pellets).[236] However, to the best of the authors' knowledge, such techniques have not yet been applied in elucidating the products of biomass conversion processes.

In this study, four different residues, representative of local municipal organic waste streams, were processed via biological-based conversion, the products of which were catalogued

and analyzed using multivariate statistical techniques. The biomass residues included corn cob, apple pomace, hardwood, and dog food. Dog food was selected due to its similar fat, protein and fiber content as that of general food waste and, as such, will be referred to in this study as simulated food waste (SFW).[237] The other three residues were selected in order to explore feedstocks with high sugar content and low structural polymer content (apple pomace), low sugar content and high structural polymer content (corn cob), and very high structural polymer content (hardwood). By way of a brief overview, first the composition of each biomass residue was characterized using thermogravimetric analysis (TGA) and detergent fiber analyses (DFA). The residues were fermented using a filamentous fungus (*Talaromyces* sp. NRRL 2120) and, following fermentation, water extracts from each culture were analyzed using ultra-performance liquid chromatography coupled with quadrupole-time-of-flight electrospray ionization mass spectrometry (UPLC-QTOF-ESI-MS). Lastly, the metabolic data was investigated using multivariate analyses.

3.3. Experimental

3.3.1. Feedstock

Corn (yellow), apples (Cortland), hardwood (maple) chips, and SFW (Pedigree Vitality) were acquired. The kernels were removed from the corn and the remaining cob was cut into small pieces. The corn cob pieces, the SFW, and the hardwood chips were separately ground using a blender (Breville) and then fine ground using a coffee grinder (Black and Decker). The apples were juiced (Breville) in order to obtain the pomace. The corn cob, apple pomace, SFW, and hardwood chips were dried in an oven at 70 ºC until weight stabilization.

3.3.2. Compositional Analyses

TGA (TA instruments Q50) was used to determine the moisture and ash content of each residue. After undergoing the preparatory steps described above, each residue in triplicate (11.63 ± 4.8 mg for each residue except apple pomace for which an average of 54.73 mg was used) was

heated under a nitrogen atmosphere at a previously optimized heating rate (5 ºC/min for apple pomace and 20 ºC/min for each other residue; Figure S1 in Appendix A) until reaching 800 ºC.

To determine polysaccharide and lignin content, each residue was lyophilized (Labconco), fine ground using a coffee grinder (Black and Decker) and passed through a 0.5 mm sieve. The samples then underwent acid, neutral, and lignin detergent fiber analyses (DFA) using a fiber analyzer (ANKOM) following standard, manufacturer-provided protocols.[238] For analysis of fermented feedstock, the homogenized and dried residual solids (see *Extractions*) underwent the same procedures described above for DFA.

3.3.3. Organism

Talaromyces sp. NRRL 2120, obtained as *Penicillium purpurogenum* (NRRL 2120), was acquired as dry mycelia from the USDA. Note: certain strains of *Penicillium* have been transferred and combined into *Talaromyces*.[239] The organism was revived in liquid potato dextrose (PD) broth (BD Difco) and incubated at 25 ºC while shaken at 100 rpm. After 7 days, mycelium from the liquid culture (0.15 g) was used to inoculate a potato dextrose agar (PDA) plate, which was then incubated at 28 ºC. After 7 days, sterile water was added to the plate to extract spores. Mycelium grown on solid media was used for analysis using a phase contrast microscope (Olympus CH-2) and a scanning electron microscope (Hitachi TM3000).

3.3.4. Solid State Fermentation

Five g of each dry residue were added in repeats of ten to 125 mL Erlenmeyer flasks. For the apple pomace samples, 2 g of wood chips were also added for the purposes of bulking in order to prevent anoxia. All samples were then autoclaved. After autoclaving, 15 mL of sterile salt solution (4 g/L KH_2PO_4, 1.6 g/L $(NH_4)_2SO_4$, and 1 g/L $MgSO_4$) and 0.2 mL of spore solution ($3x10^6$ spores) were added to half the samples of each residue type (the spore solution was not added to the remaining half which served as the non-fermented controls). The samples were then incubated at 28 ºC for 15 days.

3.3.5. Extractions

Following fermentation, 50 mL of distilled water were added to each sample and the samples were shaken at 140 rpm for 30 minutes. The liquid was decanted into falcon tubes which were then centrifuged at 3000 x g for 60 minutes. The residual solid material remaining after separation from the aqueous fraction was homogenized (Fisherbrand Homogenizer 850) and then dried in an oven at 70 ºC until weight stabilization (note: for the apple pomace samples, as much of the hardwood chips as possible were removed prior to homogenization). Following centrifugation, the aqueous fraction was filtered (0.2 μm) and used for measuring pH as well as for liquid chromatography. Five pooled quality control (QC) samples were also prepared by combining 1 mL aliquots from each sample.

3.3.6. Non-targeted metabolite analysis by UPLC-QTOF-ESI-MS

Liquid chromatography coupled with mass spectrometry is the most widely employed method in metabolomic profiling.[240] Here, metabolite profiling of the water extracts was conducted using an Agilent UPLC 1290 coupled to an Agilent 6545 QTOF-ESI-MS. A poroshell120 EC-C_{18} 2.7 μm, 3x5mm guard column and 3x100mm analytical column were used. LCMS was run in negative ion mode and the specific LC parameters were as follows: 10 μL injection volume, 0.25 mL/min flow rate, a gradient elution of mobile A (5 mM ammonium acetate in water) and mobile B (MeOH/AcN 1:1) at 1% mobile B from 0 to 1 min, ramp to 100% mobile B from 1 to 5 min, hold at 100% from 5 to 8 min, and return to 1% B from 8 min to 10 min. The MS parameters were as follows: negative ionization mode, 175 ºC gas temperature, 10 mL/min drying gas, 30 psi nebulizer, 150 ºC sheath gas temperature, 12 mL/min sheath gas flow, 4000 V capillary, 2000 V nozzle voltage, 100 V fragmentor, and 50 V skimmer.

3.3.7. Data processing and statistical analysis

Raw data files acquired from the UPLC-QTOF-ESI-MS analysis were imported into Agilent Profinder software for pre-processing. The metabolites were identified by accurate mass, MS spectra, and the metabolomic database “Metlin_Metabolites” (score >80). Each

detected feature (i.e. an entity for which one can assign a neutral mass, retention time, and abundance) was considered in the count of total features. The peak table resulting from the pre-processing step was imported into R studio for further processing and statistical analysis.

Pre-treatment of the data set involved removal of features which were detected in <80% of repeats (i.e. peak threshold of 80%). Prior to normalization, it was necessary to add small values equal to peak areas of 1 to the peak area value for each feature in order to account for zeros in the data set to allow for subsequent log transformations.[241] Normalization of the data involved transformation ($\log_{10}$), centering, and scaling (root mean squared).

The principal components (PCs) of the data set were calculated via singular value decomposition. The first three principal components from each sample type were used for data visualization. The separation of the first principal component (PC1) of each sample type from one another was evaluated using receiver operating curve (ROC) analysis and quantitatively compared using the area under the curve (AUC). For hierarchical clustering analysis (HCA), the means of each normalized metabolite peak area across each sample type were used. HCA was performed using Ward's method (Lance—Williams dissimilarity update formula) with Pearson correlation distance and visualized using a heatmap with dendrograms.

The fold change (FC) in abundance was calculated for each metabolite by dividing the means of the normalized peak areas for the fermented samples by the corresponding mean peak areas of the non-fermented samples. P-values were calculated for this change in abundance using an unpaired, two-tailed t-test assuming homoscedastic data. The -log(p-values) were plotted against the $\log_2$(FC) to visualize the statistically significant changes in abundance (i.e. volcano plots). The metabolites which exhibited $\log_2(FC) \geq 2$ and $P \leq 0.05$ were distinguished for further discussion.

3.4. Results and Discussion

3.4.1. Biomass composition and fermentation

Since fermentation processes are influenced by variations in biomass feedstock properties, characterization of composition is essential for ensuring comparability and repeatability.[242] The results of the TGA and DFA are summarized in Figure 3.1(A-B). It was

revealed that the residues used in this study varied in composition from what is typical in reports from the literature.[243] For example, the corn cob and hardwood residues possessed higher ash content and much lower lignin and cellulose content than what has been reported elsewhere.[243a-d] The apple pomace also had a much lower lignin content than expected which may be attributable to the heterogeneity of apple pomace due to the presence of, for example, seeds.[243e] While plant compositions within the same species should not differ much, differences such as those observed here may arise from different growing conditions, age of the plants, harvesting times, proximities to sources of pollution, agronomic practices, etc.[244]

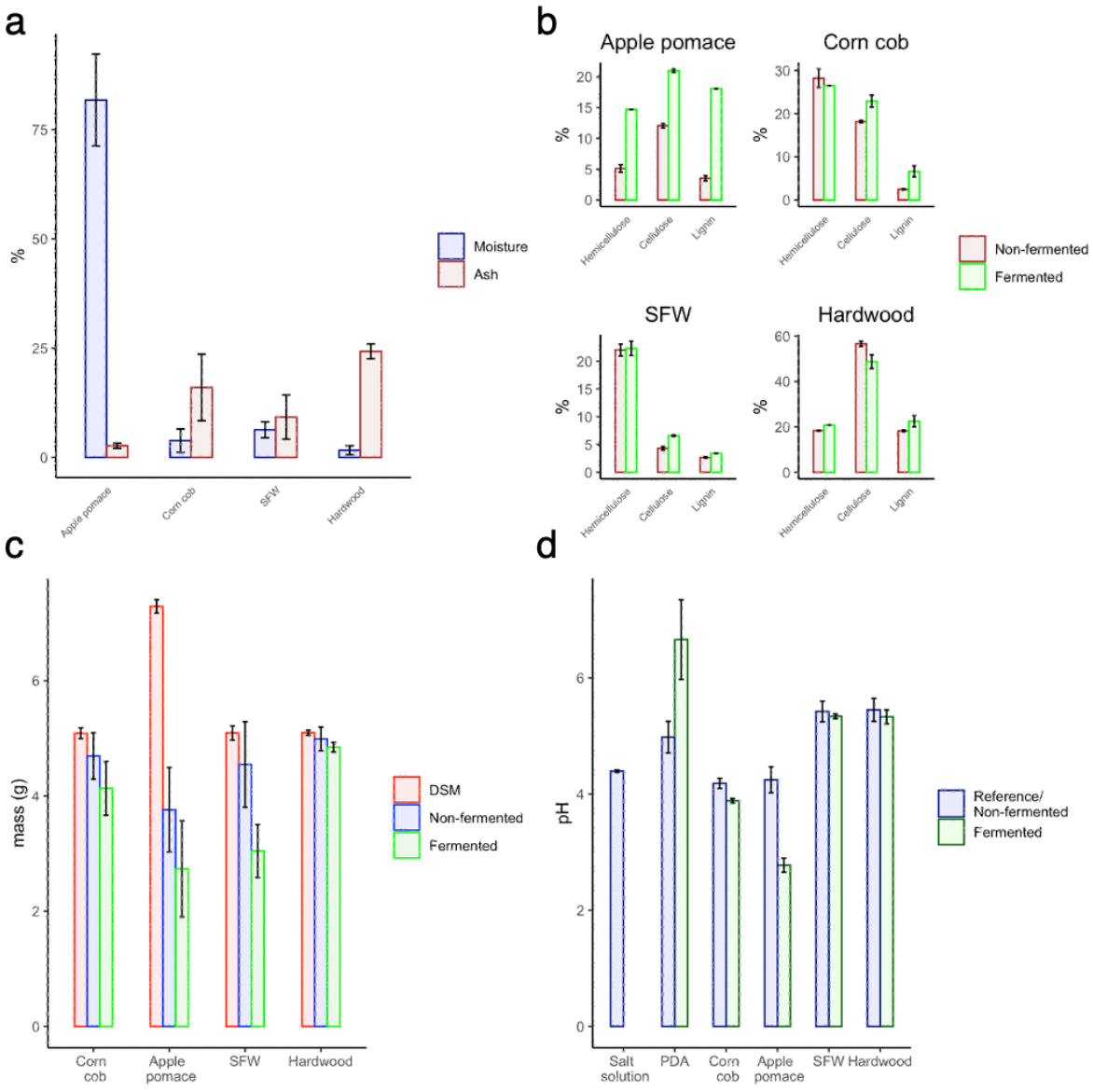


Figure 3.1. **a)** Summary of results of thermogravimetric analysis; **b)** Summary of results of acid detergent fiber, neutral detergent fiber, and lignin detergent fiber analyses for the non-fermented (red) and fermented (green) biomass residues; **c)** The change in dry mass of each biomass residue before (red) and after (blue for non-fermented and green for fermented samples) 15 day incubation (note: apple pomace substrate includes 5 grams apple pomace plus 2 grams wood chips for bulking); **d)** Comparison of the aqueous extract pH for non-fermented (blue) and fermented samples (green) for each residue (including PDA).

In general, valorization of biomass residues involves first separating the biomass into various components (e.g. in the case of lignocellulose, lignin must be separated from holocellulose), next converting the polymeric material into simple sugars that can be transformed into products (e.g. fermented into ethanol), and then finally isolating the products. Solid-state fermentation (SSF) of the raw biomass feedstocks enables integration of these steps into a much simpler conversion process (also referred to as a consolidated bioprocess). While many commonly used organisms in biotechnology (e.g. yeasts and *E. coli*) cannot break down complex polymeric material or utilize all of the monomeric sugars that are thus generated (e.g. pentose sugars), filamentous fungi are unparalleled in their capacity for both.[11a, 234b] Thus for simplicity, a filamentous fungus was chosen for this study in order to convert the raw biomass residues without the need for preprocessing the feedstock (e.g. hydrolysis or saccharification). The specific filamentous fungus, *Talaromyces* sp. NRRL 2120 (the basionym for *Penicillium purpurogenum*), was selected because it has shown ability to convert biomass residues into valuable products including phenolic compounds and platform chemicals (in addition to demonstrating a capacity for high enzyme and pigment production) but is nonetheless an underexplored species.[26, 232]

Following inoculation of each solid and unprocessed biomass residue, it was observed that the fungi grew quickest on the apple pomace, with mycelia appearing after 36 hours, followed by SFW, on which it appeared after 48 hours. Fungal growth appeared on the wood chips after 72 hours and then finally on corn cob after 7 days. Following 15 days of fermentation, water was added to each culture in order to extract the metabolites. This aqueous fraction was then separated from the residual solids which were subsequently homogenized and dried. The fibrous composition of the fermented solids was analyzed and compared to that of the non-fermented biomass described earlier (Figure 3.1B). In general, there was an increase in the relative amount of hemicellulose, cellulose, and lignin (with one exception) as a result of the fungal fermentation. This is consistent with the expectation that the organism preferentially consumed the non-polysaccharide components of a given feedstock (e.g. the monosaccharide, lipid, and protein components). The one exception to the general increase in relative polysaccharide content was observed for hardwood fermentation where the relative cellulose content decreased. This may be attributable to the fact that, unlike the other residues, hardwood comprises nearly 100% polysaccharides, and thus cellulose was likely the primary source for

meeting the organism's carbon needs. It is also worth mentioning that, in the case of the fermented apple pomace, the large increase in relative polysaccharide content (especially with respect to the lignin content) is likely exaggerated due to the presence of residual bulking material (i.e. hardwood chips).

The mass change during fermentation (i.e. how much substrate was consumed and converted into CO_2 and/or water-soluble metabolites) as well as the pH change (reflective of the fungal production of acids) as a result of fermentation may be seen in Figure 3.1(C-D). The largest mass changes were observed for the apple pomace and SFW residues. Though the measurements taken for apple pomace showed high variability (likely due to the high initial moisture content), the general trend in comparing the data across residues is consistent with the understanding that samples with higher holocellulose and lower carbohydrate content were converted more slowly than those with lower holocellulose and higher carbohydrate content. A slight decrease in pH was observed for each biomass residue fermentation (an increase in pH was observed for growth on PDA) with largest pH drop observed in the fermentation of apple pomace.

3.4.2. Metabolomic data visualization

The aqueous extracts were analyzed using a non-targeted, or global, approach of ultra-performance liquid chromatography coupled with quadrupole-time-of-flight electrospray ionization mass spectrometry (UPLC-QTOF-ESI-MS). In contrast with targeted methods, which typically quantify a number of predefined metabolites, non-targeted methods measure as many metabolites as possible (quantifying hundreds or thousands depending on the extraction method) with no a priori selection and then compare the metabolites between samples without bias.[240] Across the biomass residue samples in this study (i.e. in both fermented and non-fermented samples for a given biomass residue), there were 6124, 3647, 7895, 6675 entities (or features) with unique mass and retention times identified in corn cob, apple pomace, SFW, and hardwood samples respectively (5971 were identified in PDA). To transform large data sets such as this one into functional knowledge, effective computational methods are required.[245] Principal component analysis (PCA) is a widely applied method for reducing the dimensionality of data with minimal information loss.[246] The products of PCA are variables (i.e. principal

components; PCs) which are linear functions of variables in the original data set which maximize variance and are uncorrelated with each other.[246-247] Thus, each sample type in this study (i.e. non-fermented or fermented biomass residue) may be visualized in terms of the metabolites which contributed the most to the data variance. As can be seen in Figure 3.2, there was good grouping of PCs based on residue type as well as non-fermented or fermented samples. The coefficients of variation calculated for the QC samples were 2.8%, 6.0%, and 2.9% for PC1, PC2, and PC3 respectively.

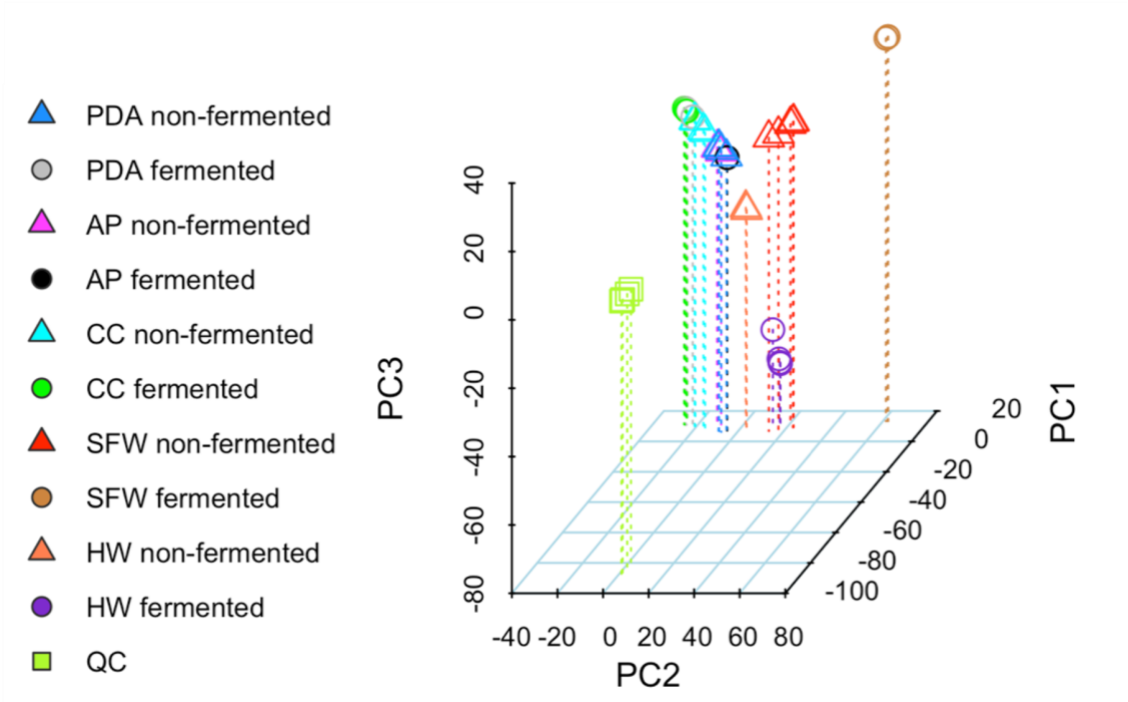


Figure 3.2. Scores plot from PCA of LC-MS data set for non-fermented and fermented PDA, apple pomace (AP), corn cob (CC), SFW, and hardwood (HW). Non-fermented vs fermented samples are distinguished by tetrahedral or spherical shapes respectively (QC samples are cubical).

For the data set as a whole, the first three PCs accounted for ~45% of the data variability, with the first two (PC1 and PC2) accounting for ~35% (Figure S2; Appendix A). The relatively low variability attributable to the first two or three PCs may be interpreted to be indicative of a large number of different metabolites contributing to the variance across samples. Altogether,

the PCA results show that the metabolic profiles were consistent within a sample type but distinct from one another.

The quality of the separation between PC groupings was evaluated using ROC analysis (see Table S1; Appendix A). The average AUC value across all samples was determined to be 0.97 (with a value of 1 considered perfect separation), though the individual values ranged from 0.6-1.[248] The PC groupings of the fermented SFW and both the fermented and non-fermented Hardwood samples were largely separated from one another (AUC values of 1 with respect to all other samples). On the other hand, the separation between apple pomace and PDA samples (both non-fermented and fermented) was less pronounced. Closer inspection reveals low but acceptable separation between fermented PDA and non-fermented apple pomace (AUC = 0.68) and good separation (AUC > 0.84) for all other PDA and apple pomace samples with respect to one another. The lowest separation between fermented and non-fermented samples of the same residue was identified in corn cob (AUC = 0.8). Lastly, non-fermented SFW showed close grouping with both non-fermented corn cob (AUC = 0.76) and fermented corn cob (AUC = 0.6). While the proximity of the apple pomace and non-fermented PDA samples may be related to the high amount of carbohydrates present in these samples, the proximity of corn cob and fermented PDA samples is somewhat more enigmatic.

To support the observations derived from the PCA results, clustering was also performed on the data set. Clustering is another widely used method for transforming expression data into functional knowledge and involves subdividing a set of items (in this case metabolites) in such a way that similar items fall into the same cluster, whereas dissimilar items fall into different clusters. [236b, 245, 249] Specifically, hierarchical clustering analysis (HCA) was applied in this study. These results are depicted in the form of two dendrograms (one for the sample types and the other for metabolites) and may be seen in Figure 3.3 along with an expression (or metabolite abundance) heatmap. The root node of a dendrogram represents the whole data set while each leaf node represents either a sample type or metabolite (row vs column).[249b] The height of the intermediate nodes describes the extent to which each pair of data points and/or clusters are similar to one another.[249b]

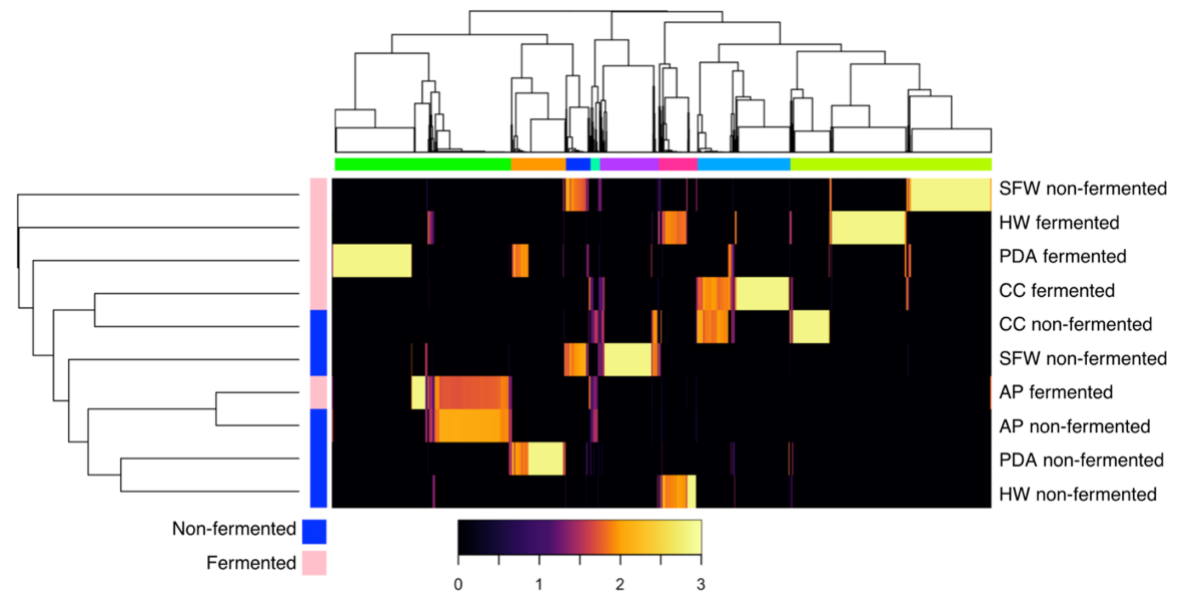


Figure 3.3. Hierarchical clustering analysis with heatmap for the metabolic profiles of non-fermented and fermented PDA, apple pomace (AP), corn cob (CC), SFW, and hardwood (HW).

Looking first at the dendrogram for the sample types, observations consistent with those from the PCA results may be made. That is, the non-fermented along with the fermented apple pomace and, to a lesser extent, the fermented corn cob samples showed the highest similarity with one another. The highest similarity of any pair of samples was observed for the fermented and non-fermented apple pomace samples which in turn showed high similarity to the non-fermented PDA sample as well as to the non-fermented hardwood sample. With the exception of the corn cob samples, which showed high similarity to one another (though not to the PDA control and thus differing somewhat from the PCA results), the remaining samples all showed high dissimilarity from one another and with the cluster which contained the majority of the non-fermented samples. Therefore, while many metabolites were common and in relatively similar proportions across the non-fermented residues (mainly polysaccharides and sugar-derived compounds), with the exception of fermented apple pomace, the metabolites were much more varied in fermented samples with respect to both the non-fermented samples and each other.

Turning to the metabolite dendrogram, the large number of metabolites in the data set represents a challenge for interpretation. Thus, the optimal number of clusters was calculated using the “elbow” method which identifies the optimal number of clusters as the number above

which the total within-cluster sum of square (i.e. total intra-cluster variation) would not be (much) further minimized.[250] In this case, 8 was determined to be the ideal number of clusters (Figure S3; Appendix A). Thus, there were 8 clusters of metabolites which tended to show consistent abundance levels depending on the sample type. In referring to the heatmap, this distinction is likely mostly attributable to the large amounts of metabolites which are highly abundant in few sample types but not detected at all in the rest.

3.4.3. Differentially abundant metabolites

As is common in identifying differentially expressed genes in microarray experiments, the log ratio (log_2) of the fermented samples and the non-fermented samples was used here to evaluate the magnitude of change in concentration of the metabolites in the data set.[251] Then to determine which changes in metabolite concentration were statistically significant, a t-test was performed. The number of metabolites which changed in concentration with a p-value ≤ 0.05 were 2168, 1876, 2618, and 1775 for corn cob, apple pomace, SFW, and hardwood respectively (1823 for PDA). Of these metabolites, those that increased log_2 fold-change ≥ 2 in concentration numbered 821, 233, 1643, and 869 for the same samples (1149 for PDA). To summarize these results, volcano plots (i.e. the $-log_{10}$ transformed p-values plotted against the log_2 fold changes) were generated for each biomass residue as may be seen in Figure 3.4.[251] Each of the volcano plots presented an unusual shape at the extremes, which may be attributable (similar to what was observed in the heatmap) to the high number of metabolites which were present in large amounts in one condition but not present at all in the other (i.e. only present in non-fermented or fermented samples for a given biomass residue).

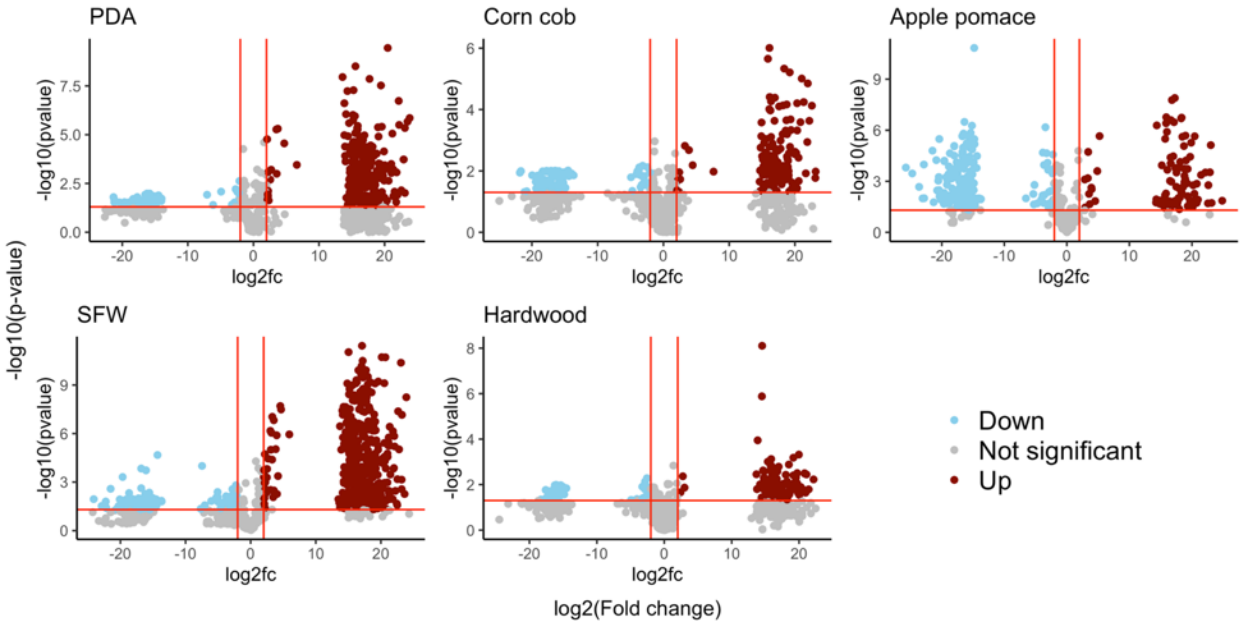


Figure 3.4. Volcano plot (i.e. $\log_{10}$(p-value) vs $\log_2$(fold change)) for each feature identified in the biomass residue fermentations and growth on PDA.

Of the metabolites which increased in abundance (≥ 2 $\log_2$ fold change) and were statistically significant ($P \leq 0.05$), which may be seen in the top right panels of the volcano plots, many were common across many or all fermentations of the different residues. These commonly identified metabolites are summarized using a Venn diagram and, as may be seen in Figure 3.5, the majority of the metabolites for each residue type were unique. There were varying amounts of metabolites common across 2-4 of the residues and thirty metabolites were identified common to all the biomass residue fermentations and PDA. All thirty commonly identified metabolites were cyclic or polycyclic compounds which included terpenoids, phthalates, various aromatic acids, esters, and aldehydes, etc.

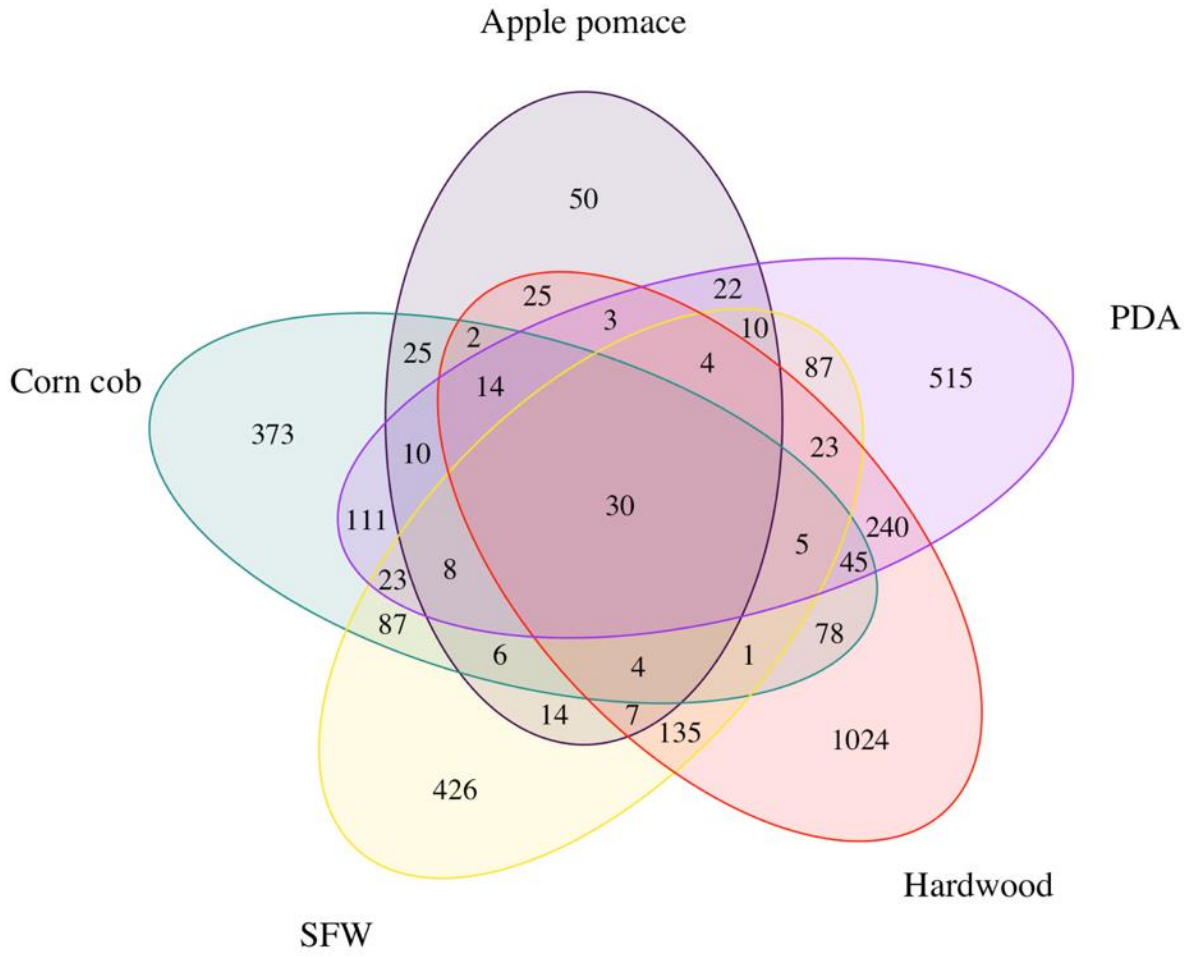


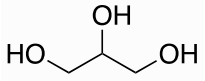
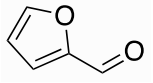
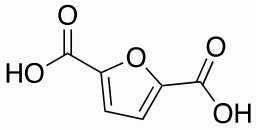
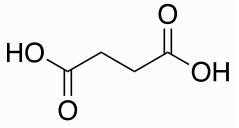
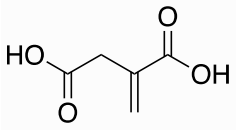
Figure 3.5. Venn diagram summarizing the number of metabolites ($log_2FC \geq 2$ and $P \leq 0.05$) common across fermented residues.

3.4.4. Valuable chemical identification

Many industrially relevant chemicals were identified among the metabolites which increased significantly in abundance in the fermented biomass samples. These molecules include ketones, polyols, sugar alcohols, organic acids (e.g. sugar-, carboxylic, dicarboxylic-, α-hydroxy-, etc.), furans, etc. Without knowing virtually every single possible metabolic product that may be derived from multiple feedstocks, process designers for biorefineries already face a plethora of well-known and commonly targeted product compounds. Several attempts have been

made to address such challenges including the US DOE's 2004 and 2010 "top chemical opportunities" guides for bio-based product research.[14] Numerous criteria factor into the selection of compounds identified as "top opportunities" including economics, industrial viability, market size, as well as consideration of known processes and the ability of a given compound to serve as a platform chemical for the production of derivatives.[14] Thus, these top chemical opportunities (which include alcohols, furans and organic acids) were used as the primary search targets to guide in the analysis of the molecules present in the residue fermentations. As may be seen in Table 1, several such chemicals were identified in the data set, with at least one found in each biomass residue.

Table 3.1. Summary of US DOE top chemical opportunities that were detected in each fermentation water extract. The values displayed are the log fold change.

Platform Chemical		Feedstock	Log_2FC	p-value
Glycerol		Corn cob	15.0	$8.95 \cdot 10^{-7}$
Furfural		Corn cob	19.6	$9.97 \cdot 10^{-4}$
2,5-furandicarboxylic acid		Apple pomace	3.9	$2.18 \cdot 10^{-4}$
Succinic acid		SFW	18.1	$1.16 \cdot 10^{-2}$
Itaconic acid		Hardwood	15.5	$1.05 \cdot 10^{-2}$

Filamentous fungi, including those belonging to the *Penicillium*/*Talaromyces* genus, are well known carboxylic acid producers and thus, considering also the extraction and LCMS analytical methods, the large representation of carboxylic acids among the products is unsurprising.[234b] Succinic acid is identified as a "top chemical opportunity" due to its

potential to replace petroleum-derived maleic anhydride and to serve as a precursor for known petrochemical products such as 1,4-butanediol, tetrahydrofuran, γ-butyrolactone, or various pyrrolidinone derivatives.[14, 252] Like succinic acid, the appeal of itaconic acid lies in its ability to serve as a substitute for current petrochemical-derived compounds used in diverse applications such as fibers, coatings, adhesives, thickeners, binders, artificial glass, or as bioactive compounds in agriculture, pharmacy, and medicine.[253] 2,5-furandicarboxylic acid (FDCA) is a molecule currently receiving large amounts of attention due to its enormous potential impact in the production of bio-based polymers which can replace, for example, polyethylene plastics.[254] The non-carboxylic acid products included furfural and glycerol, both of which have potential to serve as platforms for biofuel and/or bio-based chemical production.[255]

To expand the search for top chemical opportunities, one useful approach may be to search for families of compounds which are distinguished according to shared functionalities. By way of example, FDCA, succinic and itaconic acids all share similar functionality in that they each possess two carboxylic acid functional groups and therefore may serve as useful platforms in (but certainly not limited to) the production of polyesters. Indeed, these three compounds are already being explored for such purposes.[254, 256] Other dicarboxylic acids that were identified ($\log_2 FC \geq 2$, $P \leq 0.05$) as fermentation products of the biomass residues in this study included oxalic acid (identified in corn cob and SFW), malonic acid (corn cob), glutaric acid (corn cob, SFW, and hardwood), adipic acid (SFW and Hardwood), etc. Though not featured among the US DOE top chemical opportunities, these acids have economic and industrial importance in their own right. For example, adipic acid is one of the most widely used dicarboxylic acids in the textiles and plastics industry as a precursor to nylon.[257] Thus, to realize the full benefits of data mining metabolomes in search of platform chemicals, more effective and comprehensive search methods must be developed.

3.5. Conclusion

Repurposing materials considered waste in the context of the linear industrial economy, such as organic residues, is essential for transitioning to a circular economy. In this study, techniques from metabolomics were applied, to the best of the authors' knowledge, for the first

time as a means of exploring and comparing bioconversion products from a variety of organic residues representative of typical local waste streams (including food waste). In this process, the residues were first characterized using TGA and DFA and then converted via fungal fermentation. The composition of the fermented solids was compared with that of the non-fermented residues and the water-soluble fermentation products were identified using UPLC-QTOF/ESI-MS. These products were then analyzed using multivariate statistical techniques. Among the fermentation products were a variety of compounds identified as "top chemical opportunities" including furans (e.g. FDCA and furfural) and organic acids.

3.6. Appendix A. Supporting information for: Non-targeted screening and multivariate analysis of waste stream biomass conversion products

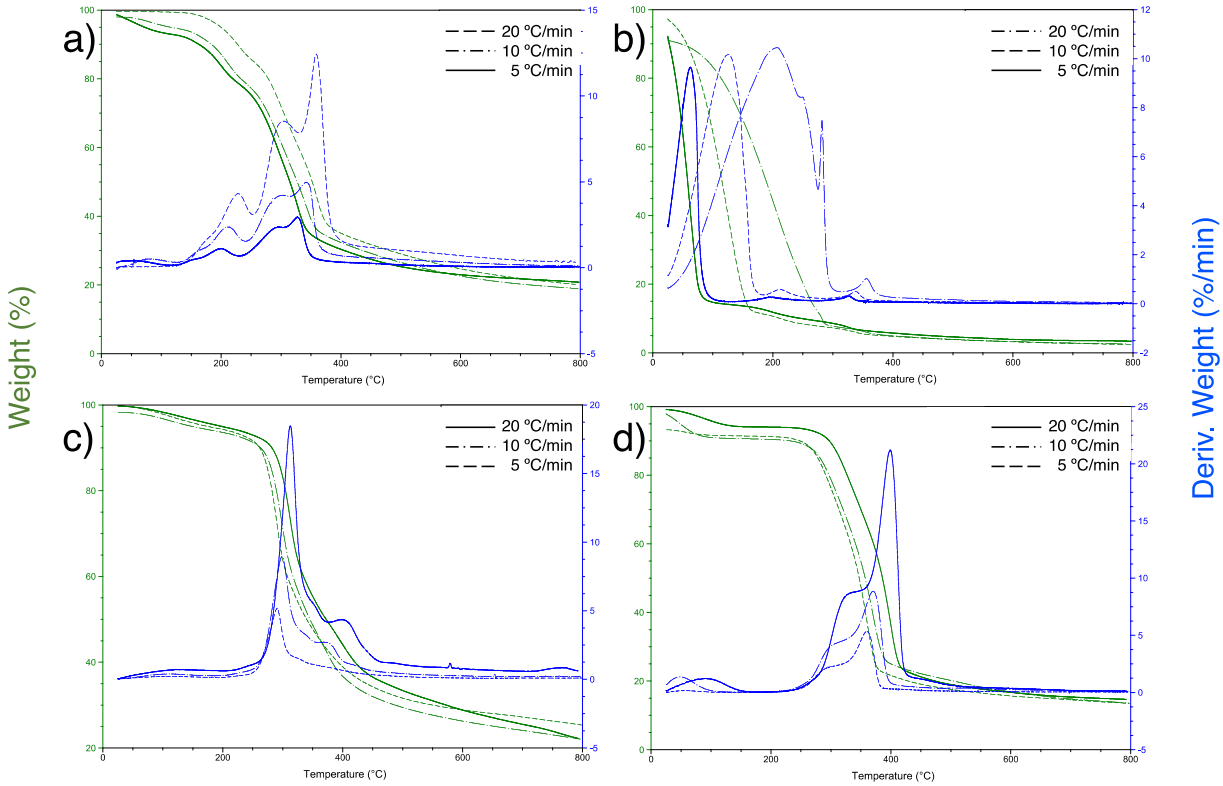


Figure S1. Method optimization for thermogravimetric analysis. Results of change in weight at heating rates of 20, 10, and 5 ºC/min are shown for: a) corn cob; b) apple pomace; c) simulated food waste; d) hardwood.

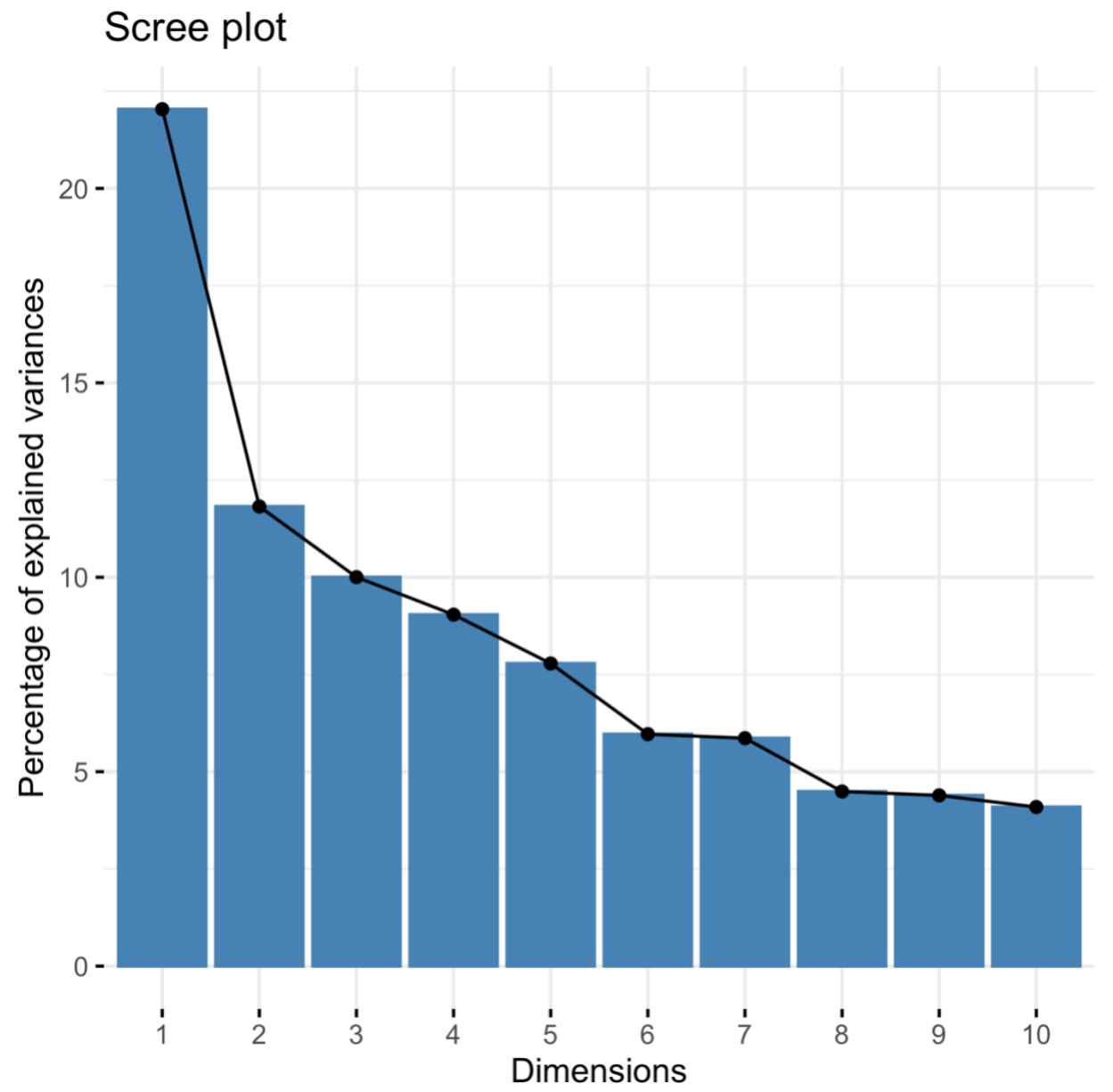


Figure S2. Scree plot from principal component analysis of metabolic data set.

Table S1: AUC values for ROC analysis of PCs of each sample.

	PDA NF	PDA F	AP NF	AP F	CC NF	CC F	SFW NF	SFW F	HW NF	HW F	QC
PDA NF		0.96	0.84	1	1	1	1	1	1	1	1
PDA F	0.96		0.68	1	1	1	1	1	1	1	1
AP NF	0.84	0.68		0.9	1	1	1	1	1	1	1
AP F	1	1	0.9		1	1	1	1	1	1	1
CC NF	1	1	1	1		0.8	0.76	1	1	1	1
CC F	1	1	1	1	0.8		0.6	1	1	1	1
SFW NF	1	1	1	1	0.76	0.6		1	1	1	1
SFW F	1	1	1	1	1	1	1		1	1	1
HW NF	1	1	1	1	1	1	1	1		1	1
HW F	1	1	1	1	1	1	1	1	1		1
QC	1	1	1	1	1	1	1	1	1	1	

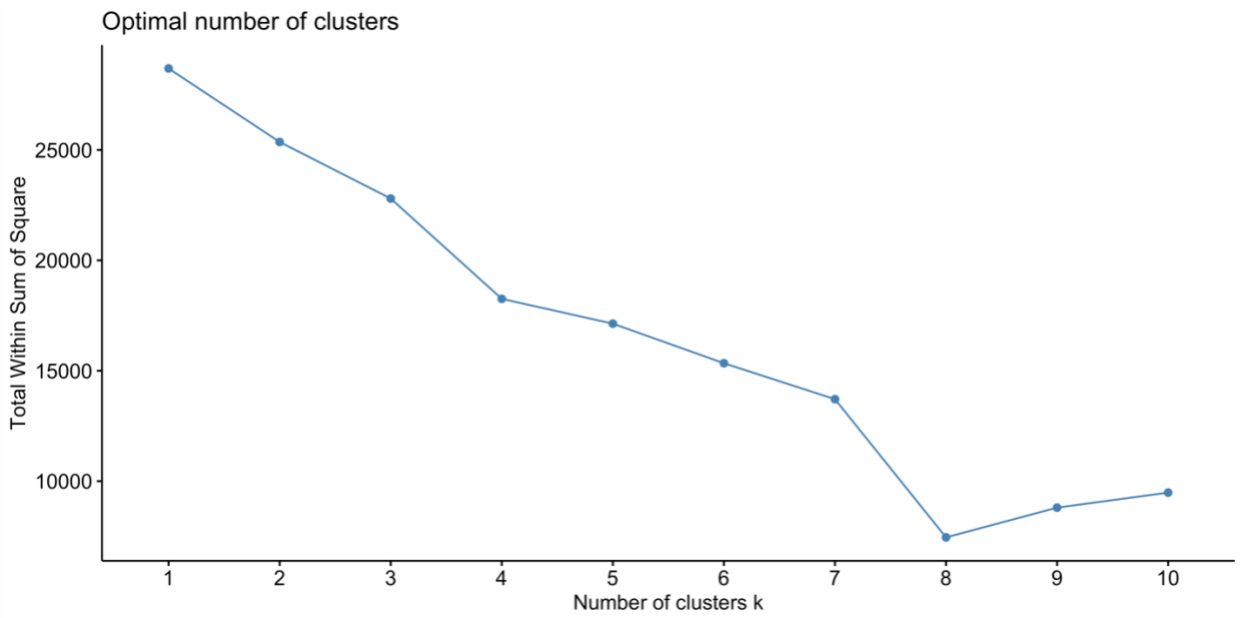


Figure S3. Optimal number of clusters as revealed by the elbow method

3.7. References

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Connecting Statement 2

While the main objective of the study described in Chapter 3 was to develop an identification process that may be useful for general biomass application, a key outcome was the identification of numerous valuable products of the solid-state fermentation of the specific biomass residues investigated. Among the commercially valuable products identified were bio-pigments; specifically, red, orange, and yellow azaphilone pigments. Though not explicitly described in Chapter 3, these bio-pigments were observed to accumulate in large amounts over the course of the study especially in the fermentations of a simulated food waste feedstock (i.e. a commercial dog food). Maximizing production of bio-pigment from the fermentation of simulated food waste was therefore the next objective.

To that end, Chapter 4 describes an investigation into an optimized pigment extraction technique as well as a novel fungal co-culturing methodology for eliciting higher pigment production. This chapter is based on an article accepted for publication in *Applied Microbiology and Biotechnology* with Dr. Valérie Orsat and Dr. Marie-Josée Dumont as co-authors of this paper.

Chapter 4. Solid-state Co-culture Fermentation of Simulated Food Waste with Filamentous Fungi for Enhanced Production of Bio-pigments

4.1. Abstract

The use of waste stream residues as feedstock for material production simultaneously helps reduce dependence on fossil-based resources and to shift toward a circular economy. This study explores the conversion of food waste into valuable chemicals, namely bio-pigments. Here, a simulated food waste feedstock was converted into pigments via solid-state fermentation with the filamentous fungus *Talaromyces albobiverticillius* (NRRL 2120). Pigments including monascorubrin, rubropunctatin, and 7-(2-hydroxyethyl)-monascorubramine were identified as products of the fermentation via ultra-performance liquid chromatography coupled with quadrupole-time-of-flight electrospray ionization mass spectrometry. Pigments were obtained at concentrations of 32.5, 20.9, and 22.4 AU/gram dry substrate for pigments absorbing at 400, 475, and 500 nm, respectively. Pigment production was further enhanced by co-culturing *T. albobiverticillius* with *Trichoderma reesei* (NRRL 3652), and ultimately yielded 63.8, 35.6, and 43.6 AU/gds at the same respective wavelengths. This represents the highest reported production of pigments via solid-state fermentation of a non-supplemented waste stream feedstock.

4.2. Introduction

Repurposing materials considered waste in the context of the linear industrial economy, such as organic residues, is essential for transitioning to a circular economy. Each year, North Americans generate around 265 million metric tons of organic waste (1500 kg per person), of which, 168 million tons comprise discarded food matter.[258] This food waste fraction alone, which represents 278$ billion dollars of value and 217 trillion kilocalories, results in 193 million tons of greenhouse gas emissions (CO_2 equivalent) for the landfill life-cycle and occupies 38.6 million m^3 of space.[258b] Furthermore, the production of this ultimately discarded food required 18 thousand billion liters of water, 22.1 million hectares of cropland, 4 million metric tons of fertilizer, and 10^{19} joules of energy.[258b] With an increasingly urgent need for sustainable

feedstocks to fulfill global carbon needs (i.e. production of carbon-based fuels and materials), food waste represents an appealing option in that it would simultaneously help reduce dependence on fossil-based resources while also contributing to sustainable waste management and the transition toward a circular industrial economy.

Here, food waste is explored as a feedstock for the production of colorants. Colorants (i.e. dyes and pigments), which are almost entirely derived from petrochemicals on the industrial scale, represent a global market of around $27 billion.[259] With diverse applications (e.g. in textiles, food, paints, cosmetics, paper, coatings, plastics, construction, glass, automotive, and printing inks), the adoption of colorants derived from more sustainable feedstocks and produced using more environmentally benign methods, would substantially advance the transition toward a more sustainable industrial economy.[259a] In general, before raw biomass may be converted into commercial products, it must undergo fractionation and saccharification. Solid-state fermentation (SSF) of raw biomass feedstock may enable integration of these pre-processing steps, referred to as consolidated bioprocessing, and in-turn possesses economic benefits.[208, 260] Filamentous fungi, many of which are known to be prolific pigments producers, are unparalleled in their capacity for breaking down complex polymeric material and thus ideally suited for such processes.[11a, 234b]

Compounds derived from fungi have been used by humans since ancient times with documented use of filamentous fungal colorants in food dating at least to the first century.[261] Fungal pigments are typically secreted as secondary metabolites (i.e. metabolites not involved in primary metabolism) and are broadly classified as carotenoids (aliphatic polyene chains with light-absorbing conjugated double bonds) and polyketides. Polyketides, a structurally and functionally diverse group of molecules produced by myriad and disparate organisms, are generally characterized as containing many carbonyls and alcohols separated by methylene carbons.[232a, 261a, 262] Classes of polyketides include anthraquinones, flavonoid pigments, naphthalenes, tetracyclines, tropolones, naphthoquinones, and azaphilones amongst others.[261a] With respect to fungal pigments, the most widely utilized and researched are the azaphilones which are a class characterized as having a highly oxygenated pyrano-quinone bicyclic core, usually known as isochromene, and a chiral quaternary structure.[261a, 263] Azaphilone pigments are known to be produced from a variety of ascomycetous and basidiomycetous fungi (the source of the coloring in the fungal fruiting bodies and mycelia) but are most well known in the genera

Monascus. Currently, *Monascus* azaphilone pigments (MonAzPs) are employed, mainly in East Asia, as traditional food additives in, for example, sausages, fermented bean curd, red rice wine as well as in preservation of dry meat and fish products.[261b] However, commercial application of these species outside of Asia, particularly for use as food ingredients, is hampered due to the production of harmful mycotoxins including citrinin, an azaphilone and the most well-known mycotoxin.[263-264]

Certain members of the *Talaromyces* genus, on the other hand, are known to produce *Monascus* compound homologues without production of harmful mycotoxins.[239c, 265] Unfortunately reports of SSF pigment production by *Talaromyces* sp. (commonly referred to by their *Penicillium* basionyms) have shown limited productivity.[239a, 239b, 262a] Given that the variety and quantity of azaphilone pigments are highly dependent on substrate components, the aim of this study was to first determine if the composition typical of food waste is conducive to pigment production with a non-toxin producing strain of *Penicillium/Talaromyces*.[261b] Following this, a novel strategy for enhancing this pigment production involving a fungal co-culture system was employed and ultimately achieved the highest SSF pigment production from a non-supplemented waste stream feedstock.

4.3 Experimental

4.3.1. Feedstock Analysis

SFW (Pedigree Vitality+ Original Roasted Chicken and Vegetable Flavour 2kg) was acquired, ground and fine ground using a blender and coffee grinder respectively, then dried in an oven at 70 ºC until weight stabilization. Thermogravimetric analysis (TGA; TA instruments Q50) was used to determine the moisture and ash content. After undergoing the preparatory steps described above, each sample in triplicate (21.8 ± 4.3 mg) was heated under a nitrogen atmosphere at a previously optimized heating rate (20 ºC/min) until reaching 800 ºC. Detergent fiber analyses (DFA) were performed to determine polysaccharide and lignin contents. The SFW was freeze dried (Labconco), then fine ground using a coffee grinder, and then passed through a 0.5 mm sieve. The samples then underwent acid, neutral, and lignin detergent fiber analyses using a fiber analyzer (ANKOM) following standard, manufacturer-provided

protocols.[238] For analysis of fermented feedstock, the homogenized and dried residual solids (see *Pigment Extraction*) underwent the same procedures described above for TGA and DFA.

4.3.2. Basic organism cultivation and solid-state fermentation

Talaromyces albobiverticillius (NRRL 2120; originally identified as *Penicillium purpurogenum*) and *Trichoderma reesei* (NRRL 3652) were acquired as dry mycelia from the United States Department of Agriculture ARS Culture Collection. Note: certain strains of *Penicillium* have been transferred and combined into *Talaromyces*.[239] The organisms were revived in liquid potato dextrose (PD) broth (BD Difco) and incubated at 25 ºC while shaken at 100 rpm. After 7 days, mycelia from the liquid cultures (~0.15 g) were used to inoculate potato dextrose agar (PDA) plates, which were then incubated at 28 ºC. After 7 days, sterile water was added to the plate to extract spores. Mycelia grown on solid media was used for analysis using a phase contrast microscope (Olympus CH-2) and a scanning electron microscope (SEM; Hitachi TM3000). For the former, samples were prepared following the slide culture technique.[266] For the latter, a cork borer was used to extract 1 cm diameter plugs from the cultures and the mycelia was carefully separated from the PDA using a razor. This slice of mycelia was then frozen and freeze dried before SEM analysis.

Five grams of dry substrate, prepared as described above (see *Feedstock analysis*), were added to 125 mL Erlenmeyer flasks then autoclaved. After autoclaving, 15 mL of a sterile salt solution (4 g/L KH_2PO_4, 1.6 g/L $(NH_4)_2SO_4$, and 1 g/L $MgSO_4$) were added to each sample which was then mixed. To inoculate the solid media, spores were extracted from 7-day old *T. albobiverticillius* PDA cultures using sterile water. Inoculum of $3x10^6$ spores (0.2 mL) was added to half the flasks (non-inoculated flasks were used as controls). All flasks, prepared in at least duplicate, were statically incubated at 28 ºC for up to 15 days.

4.3.3. Co-culture screening and fermentation

A cork borer was used to extract 1 cm diameter plugs from 7-day old PDA cultures of *T. albobiverticillius* and *T. reesei*. 5 replicates of PDA plates containing *T. albobiverticillius* with *T. reesei* (seeded at a distance of 4.0 ± 0.44 cm from one another) were prepared and monitored

for growth. Single cultures were also prepared as controls. All cultures were statically incubated at 28 ºC for 21 days. Following 21 days of incubation, mycelia from the cultures was analyzed using SEM as described above.

For co-culture solid-state fermentation, 1 cm diameter plugs from 7-day old PDA cultures of *T. albobiverticillius* and *T. reesei* were seeded on opposite sides in the same 125 mL Erlenmeyer flask containing 5 g of substrate prepared as described above (*Basic organism cultivation and solid-state fermentation*). Single cultures were also prepared as controls. All flasks, prepared in at least duplicate, were statically incubated at 28 ºC for 15 days.

4.3.4. Pigment Extraction

For the basic extraction method, 50 mL of distilled water were added to each flask following the 15-day incubation. The flasks were shaken at 140 rpm and 30 ºC for 30 minutes, following which the contents were transferred into falcon tubes and centrifuged at 3000 × g for 60 minutes. The supernatant was then filtered (0.2 μm) and used for measuring pH, spectrophotometric analysis, and quadrupole-time-of-flight electrospray ionization mass spectrometry (UPLC-QTOF-ESI-MS) analysis. The residual solids were homogenized (Fisherbrand Homogenizer 850) and then dried in an oven at 70 ºC until weight stabilization.

For the optimized extraction method, which was employed in subsequent rounds of fermentation, 50 mL of 70% aqueous ethanol was added to each sample after the 15-day fermentation period. The flasks were placed in an ultrasonic bath for 30 min at room temperature, then shaken at 180 rpm and 30 ºC for 60 min. The contents were transferred into falcon tubes and centrifuged at 11,500 x g for 20 min. Following this, the supernatant was collected, filtered, and used for spectrophotometric analysis.

4.3.5. Pigment Analysis

Water and aqueous ethanol extracts were analyzed using a spectrophotometer (Biochrom Ultrospec 1000) for absorbance at 400 nm, 475 nm, and 500 nm wavelengths which are the typical absorption wavelengths for yellow, orange, and red pigments respectively (note: the physiology of the human eye is such that the observed colors of the pigments are the

complementary colors of the color wavelengths absorbed by the pigments).[262a, 267] The values were expressed as the means of the absorbance per gram dry substrate (gds) ± standard deviations. The absorbances were compared with standard absorbance curves of the commercial pigments β-carotene and betanin (red beet extract). These standard curves, which comprised absorbance of a given pigment at 400, 475, and 500 nm plotted against concentration of the pigment, were used to calculate the effective equivalent amount (in mg of the two commercial pigments) that may be obtained from a given fermentation extraction. To determine mass yields for the pigment material, the extracts were dried using a rotovap (BÜCHI) and the yields were calculated on the basis of mass of dried solid product per gds ± standard deviations.

Water extracts were also analyzed using an Agilent UPLC 1290 coupled to an Agilent 6545 QTOF-ESI-MS. A poroshell120 EC-C_{18} 2.7 μm, 3x5mm guard column and 3x100mm analytical column were used. The LCMS was run in positive ion mode and the specific LC parameters were as follows: 10 μL injection volume, 0.4 mL/min flow rate, a gradient elution of mobile A (5 mM ammonium acetate in water with 0.1% formic acid) and mobile B (AcN with 5 mM acetate and 0.1% formic acid) at 75% mobile B from 0 to 1 min, ramp to 85% mobile B from 1 to 5 min, ramp to 95% B from 5 to 10 min, ramp to 100% B from 10-13 min, return to 75% B from 13 min to 15 min. The MS parameters were as follows: positive ionization mode, 175 ºC gas temperature, 10 mL/min drying gas, 30 psi nebulizer, 350 ºC sheath gas temperature, 12 mL/min sheath gas flow, 4000 V capillary, 2000 V nozzle voltage, 200 V fragmentor, and 50 V skimmer. Suspect screening was performed using Agilent Profinder software with the database "Metlin_Metabolites" (score >80).

4.4. Results

In summarizing the findings of numerous studies on the average composition of food waste from various municipalities around the world (including in China, Denmark, Greece, India, Japan, Spain, and the US), the typical composition of food waste may broadly be described as comprising about one tenth each ash and fiber (lignin, cellulose, and hemicellulose), about one sixth each fat and protein, and about two fifths carbohydrates (starch and sugar).[268] Dog food is commonly used as a simulated food waste (SFW) in studies which model, for example, composting of food waste.[237, 269] It possesses a similar fat, protein, and fiber

composition as that of general food waste and also offers compositional consistency from batch to batch.[237] Thus, dog food, referred to as SFW throughout this study, was chosen as the substrate for exploring conversion of food waste into pigments. The nutritional information label reports this substrate to comprise at least 21% crude protein, 11% crude fat, and 4% crude fiber. In the lab, the composition of the SFW was further elucidated using TGA and DFA. The results of these analyses are summarized in Figure 1a. TGA revealed that the feedstock contained 24% ash and ~1% moisture (note: this is the moisture content of the feedstock as it was used in SSF, for which it was first oven dried), while the DFA identified the fiber content to comprise, on a dry matter basis, of mainly hemicellulose (22%) and, to a lesser extent, cellulose, and lignin (4.3 and 2.7%, respectively).

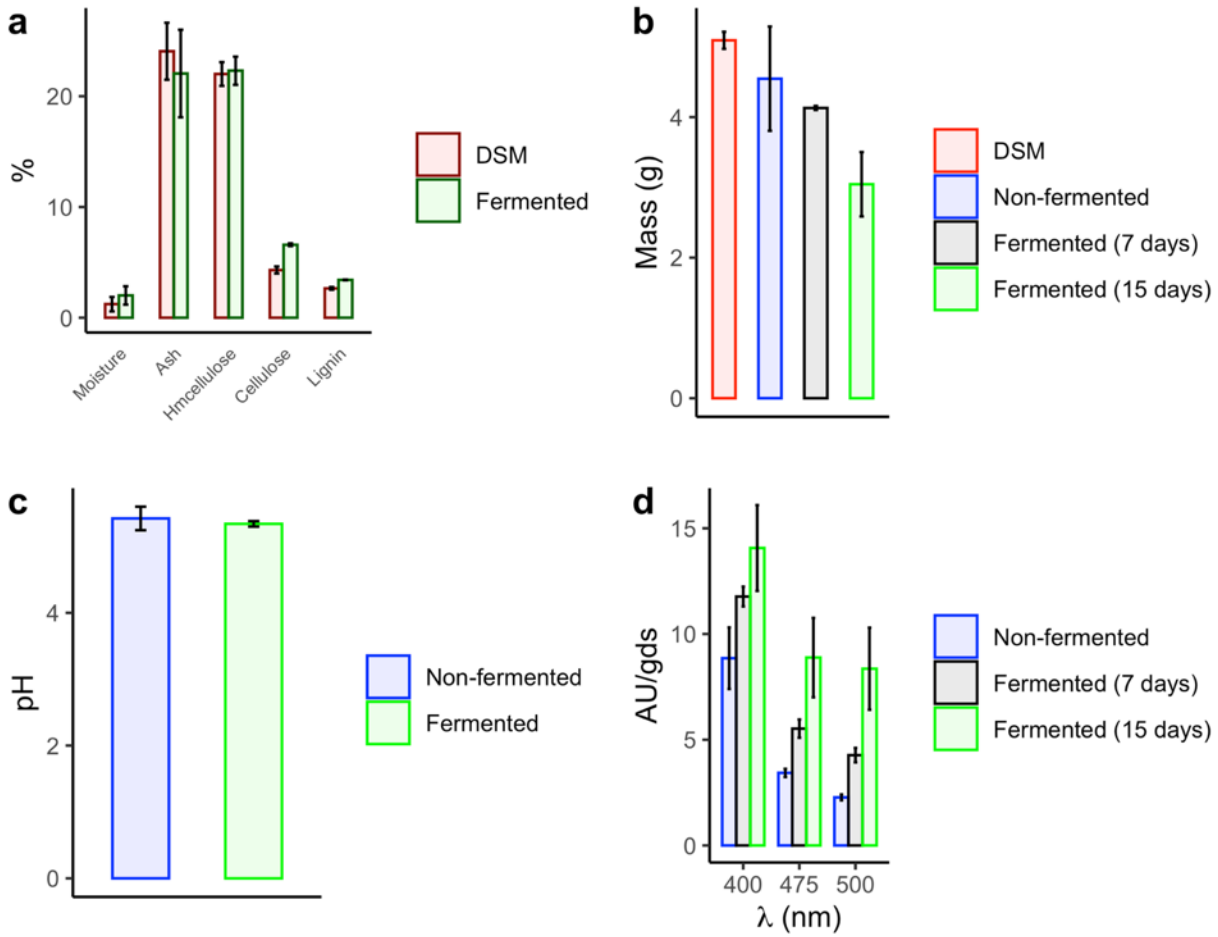

Figure 4.1. **a)** Summary of results of thermogravimetric, acid detergent fiber, neutral detergent fiber, and lignin detergent fiber analyses of SFW dry starting matter (DSM) and fermented SFW. Note: The values from detergent fiber analyses (hemicellulose, cellulose, and lignin) are on a dry matter basis; **b)**

Evaluation of mass loss associated with fermentation as revealed by comparison of SFW DSM, non-fermented SFW (15 day incubation), and fermented SFW (7- and 15-day incubations); **c)** Comparison of pH between water extracts from non-fermented and fermented SFW (both 15 day incubations); **d)** Pigments obtained from water extracts of non-fermented SFW (15 day incubation), and fermented SFW (7- and 15-day incubations) measured for absorbance at 400, 475, and 500 nm.

Prior to SSF, *T. albobiverticillius* was cultivated in liquid and solid potato dextrose cultures. In the liquid culture, the fungus grew in the form of mycelial pellets exhibiting a distinct red pigmentation; on the solid media, the fungus grew as a mycelial mat with the same distinct red coloring being displayed on the reverse while bluish gray on the obverse (Figure S1). Following inoculation, mycelia first appeared on SFW within 48 hours. The non-exposed samples, or controls, did not show any growth throughout the study. Following a 15-day incubation, the cultures were harvested (cultures incubated for 7 days were also analyzed for reference). The residual solids were separated, homogenized, dried, weighed, and analyzed for compositional changes via TGA and DFA. In Figure 1a, it may be seen that the relative amount of polysaccharide content (i.e. hemicellulose, cellulose, and lignin) increased slightly, suggesting that the organism may have more readily consumed the non-polysaccharide components of the SFW (i.e. the lipid, protein, and monosaccharide components). The aqueous fractions from the fermentation harvest were measured for pH comparison and pigment production, see Figure 1b-d. From Figure 1(b-c), it may be seen that the SFW decreased in mass by around 33% without a large change in the pH. This suggests that the SFW feedstock does not elicit the excretion of large quantities of organic acids from the fungus.

Pigment production on SFW was apparent, as can be seen in Figure S2, and quantified using the water extracts following 15-days of fermentation to be 14.1, 8.9, and 8.4 AU/grams dry substrate (gds) for yellow, orange, and red pigments (400, 475, and 500 nm), respectively (Figure 1d). The mass yield of the pigment material was determined to be 96.3 ± 13 mg/gds (see Figure 2a). For reference, standard curves of the widely used and commercially available pigments β-carotene and betanin were generated (see Figure S4a-b). Based on these curves, it was estimated that each 50 mL extract contained the effective equivalent amount of red pigment (in terms of absorbance at 500 nm) as 98 mg β-carotene and 240 mg of betanin (Figure 2b-c).

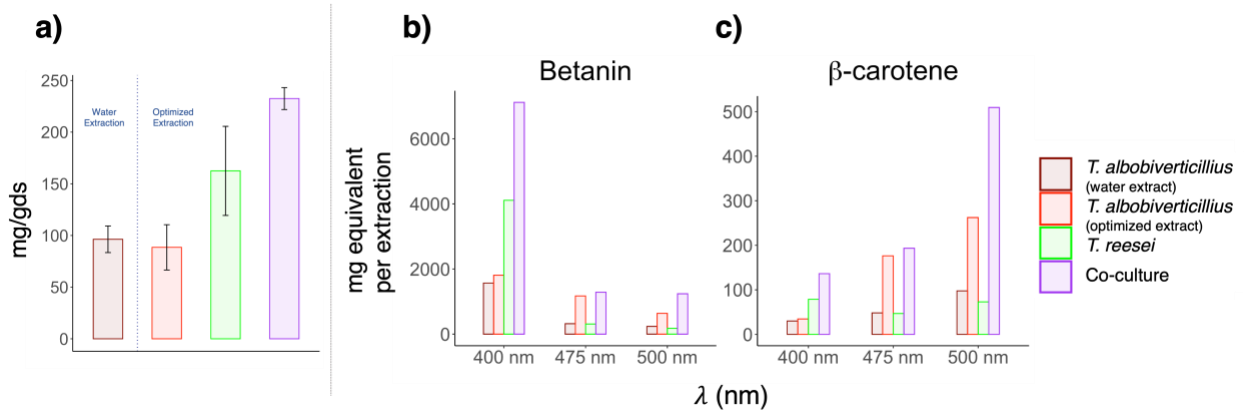

Figure 4.2. **a)** The mass yields of the heterogenous pigment product obtained from (left to right) the water (basic) extraction of SFW fermentation with *T. albobiverticillius* and the 70% aqueous ethanol (optimized) extraction of SFW fermentation with *T. albobiverticillius*, *T. reesei*, and *T. albobiverticillius* with *T. reesei* (co-culture); **b)** the equivalent amount of β-carotene in each 50 mL extraction based on absorbance; **c)** the equivalent amount of betanin in each 50 mL extraction based on absorbance.

The identities of the pigments were elucidated using quadrupole-time-of-flight electrospray ionization mass spectrometry (UPLC-QTOF-ESI-MS). Notably, citrinin was not identified among the products. As illustrated in Figure 4.3, numerous "classical" MonAzPs were identified (e.g. the orange pigments monascorubrin and rubropunctatin) in addition to the red pigment molecule 7-(2-hydroxyethyl)-monascorubramine (also referred to as PP-R) which are all considered stage IV MonAzPs.[264c] The stage IV MonAzPs are derived from the conserved trunk MonAzP biosynthetic pathway which has been elucidated in numerous species of *Monascus* as well as *Talaromyces*.[264c] Briefly, this biosynthetic pathway involves formation of an acylated pyran ring system generated from polyketide synthase and fatty acid synthase-derived intermediates which subsequently undergoes a series of intramolecular reactions before conversion into either the yellow pigments monascin and ankaflavin (the main pathway) or the orange pigments rubropunctatin or monascorubrin (the shunt pathway).[261b, 264c] The orange pigments may then be transformed into the "classical" red pigments rubropunctamine and monascorubramine via non-enzymatic reactions with amines.[261b] In the case of 7-(2-hydroxyethyl)-monascorubramine, first the *trans* C10(11) double bond of monascorubrin is converted to a *cis* double bond to generate (10Z)-monascorubrin (also referred to as PP-Y) which then undergoes *O*-to-*N* replacement with an amine.[264c, 270]

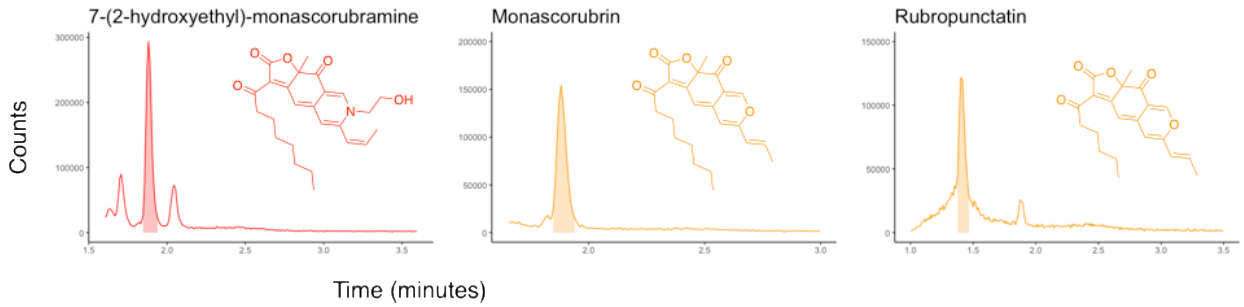

Figure 4.3. Chromatograms and affiliated structures of *Monascus* pigments identified in the fermentation water extracts.

Since water is not the ideal extraction solvent for pigments, the fermentations were repeated in order to apply an optimized extraction method reported elsewhere in literature.[262a] With this method, which includes the use of 70% aqueous ethanol instead of water for the extracting solvent as well as an ultrasonication step, pigments were obtained at concentrations of 32.5, 20.9, and 22.4 AU/gds for pigments absorbing at 400, 475, and 500 nm respectively (Figure 4.4). Though the mass yield (referring to Figure 4.2a) did not increase, the relative abundance of pigments increased substantially. This level of production is comparable with (indeed generally higher than) what is reported in other studies on fungal conversion of waste biomass substrate which typically ranges from 10-30 AU/gds (though higher pigment production may be elicited via supplementation of the media with, for example, glucose according to some studies).[262a, 264b, 271] The relatively good production of specifically the red pigments may be attributable to the substrate's protein content. Azaphilones are characterized as having high reactivity with nitrogen sources (the source of their name is derived from the high affinity of the 4H-pyran nucleus to undergo substitution with primary amines to form the corresponding vinylogous γ-pyridones) including proteins and amino acids and turn red in the presence of primary amines due to an exchange of the pyran oxygen for nitrogen (e.g. by forming 7-(2-hydroxyethyl)-monascorubramine).[261a, 263, 272]

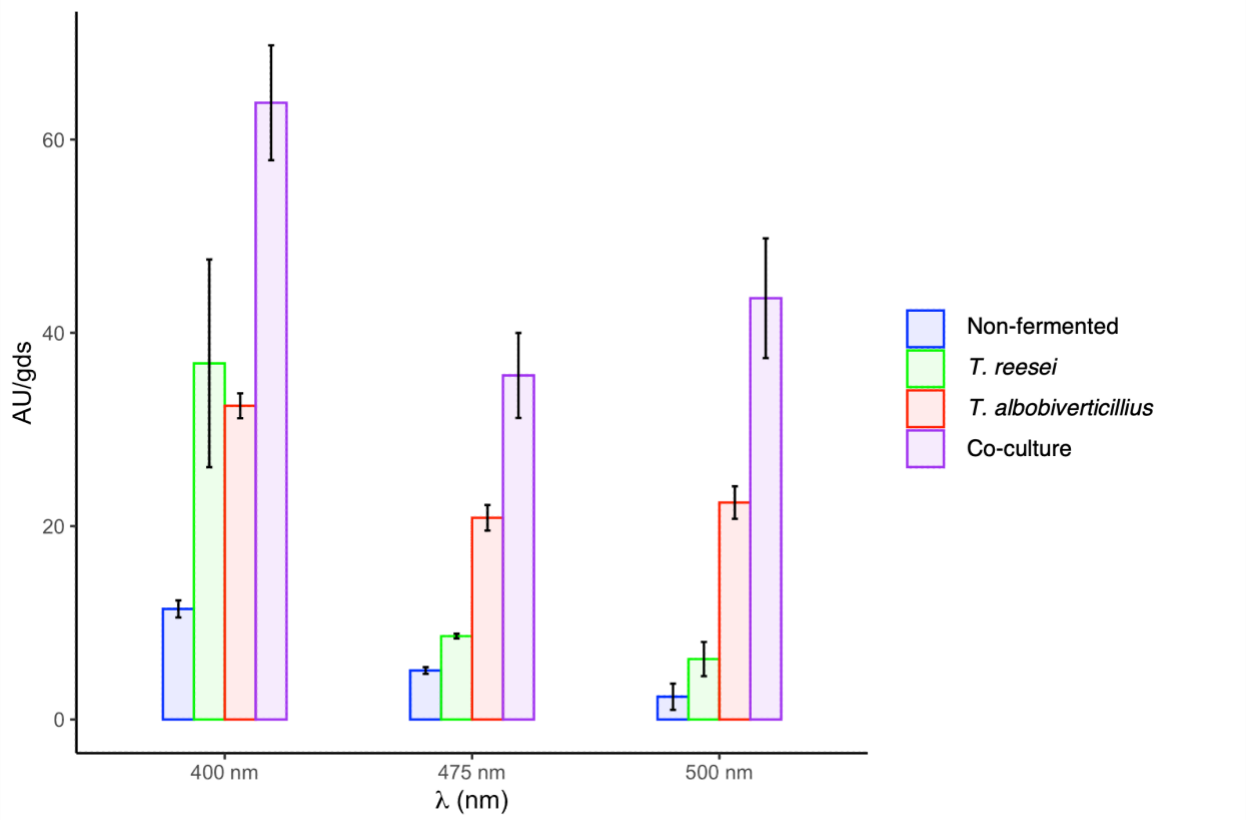

Figure 4.4. Pigments obtained using optimized extraction method. Absorbance at 400, 475, and 500 nm is shown for non-fermented SFW extracts and SFW fermented with *T. reesei*, *T. albobiverticillius*, and a co-culture of *T. albobiverticillius* with *T. reesei*.

Building on the optimized extract procedure, the next objective was to enhance pigment production. Most studies exploring strategies for enhancing pigment production have focused on optimizing process conditions including temperature, pH, agitation, etc.[273] To the best of our knowledge, no studies have explored the use of fungal co-cultures in the SSF production of MonAzPs. Such systems exploit the millions of years of microbial co-evolution where such secondary metabolites were used as chemical signals for communication relating to habitat defense, or competitor growth inhibition.[274] Co-culturing of microorganisms activates/alters secondary metabolite production and has been widely applied in enhancing productivity of various metabolites (e.g. carotenoids, organic acids, proteins, etc.) as well as in eliciting production of novel metabolites including, extensively, polyketides.[211, 234b, 274-275] Additionally, given that solid substrate fermentation in nature is carried out simultaneously by multiple species of fungi and bacteria, it may elicit better biomass and secondary metabolite production as well as

proper utilization of substrate in engineered bioprocesses.[276] Interestingly, one study demonstrated that submerged co-culture fermentation of a strain of *Penicillium* with a strain of yeast (*Candida tropicalis*) elicited production of a red pigment that was not observed in either strain on its own.[277]

To develop a co-culture system, which is not to be conflated with a mixed culture wherein unspecified microbes may be cultured under septic conditions, specific individual strains are first identified which possess the desired properties, then tested for compatibility, and lastly measured for performance.[211, 275c] *Trichoderma reesei* is one of the most commonly employed species for pairing with other fungi in co-culture processes and is also known to produce yellow pigments (though in industrial settings, in which *T. reesei* is primarily employed for enzyme production, this pigment production is considered undesirable as it must be removed during downstream processing).[278] For these reasons, *T. reesei* was selected for exploration in the enhancement of pigment production in *T. albobiverticillius*.

From Figure 5 it may be seen that the PDA co-culture of *T. albobiverticillius* and *T. reesei* reached an apparent deadlock after about one week. Due to its quicker growth rate, *T. reesei* secured for itself more of the plate but did not apparently invade or intermingle with the growth of *T. albobiverticillius*. This was confirmed with SEM imaging of the cultures (Figure S3). The effect of microbial pairing on each species, in terms of morphology and physiology, may be described generally as antagonistic, indifferent, or favorable (though, more specific, systematic descriptions have also been proposed).[211, 221, 279] In this case, the growth relationship between *T. albobiverticillius* and *T. reesei* was characterized as indifferent and therefore concluded to be suitable for further analysis in co-culture for SSF pigment production.

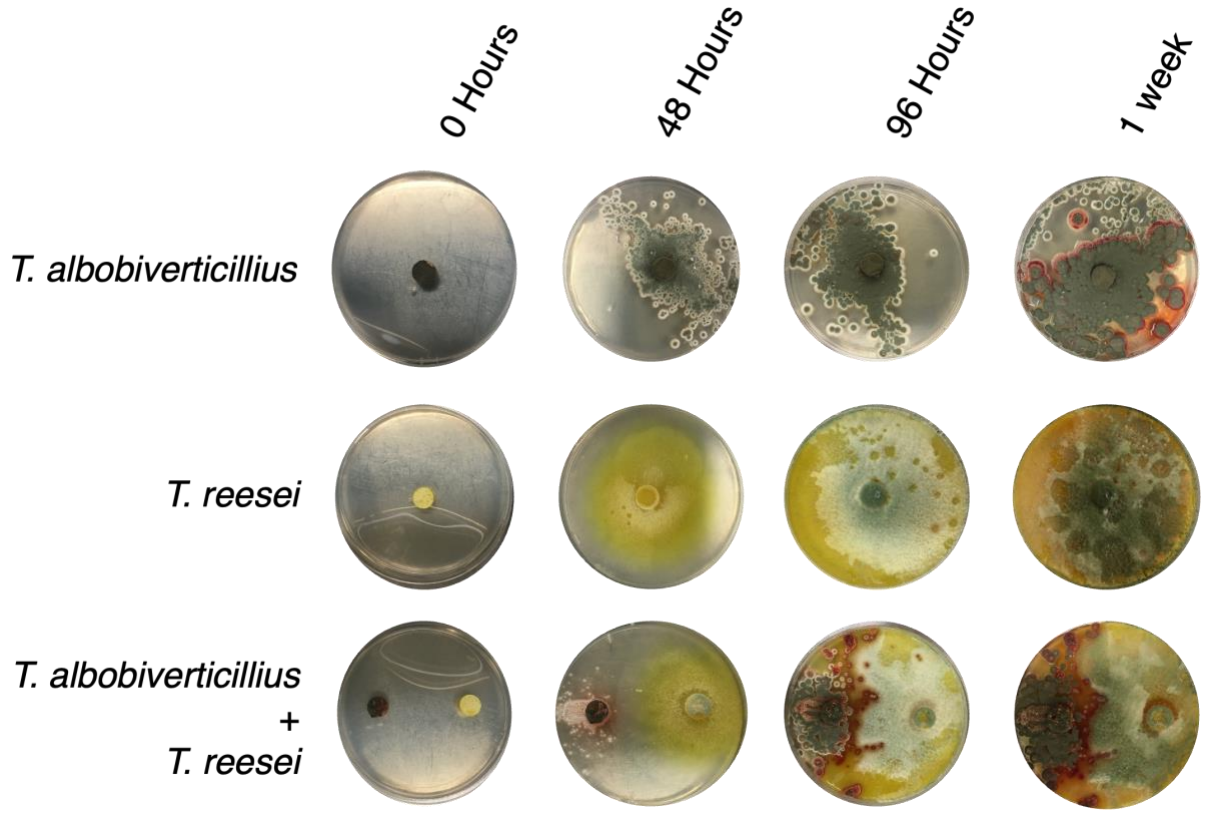

Figure 4.5. Obverse view of growth on PDA over 7 days of: single cultures of *T. albobiverticillius* (top) and *T. reesei* (middle); co-cultures of *T. albobiverticillius* + *T. reesei* (bottom).

The two fungi were then co-cultured on SFW, the results for which are illustrated in Figure 3 (*T. reesei* was also screened as a single culture on SFW as a reference). On its own, *T. reesei* was found to be a good producer, indeed better than *T. albobiverticillius*, of pigments which absorb at 400 nm (36.8 AU/gds) but produced little that absorbed at 475 and 500 nm. The co-culture resulted in 63.8, 35.6, and 43.6 AU/gds at 400, 475, and 500 nm, respectively. This represents a 73% increase in pigment production at 400 nm over *T. reesei* (97% higher than *T. albobiverticillius*) as well as a 71% and 94% increase at 475 and 500 nm, respectively, for *T. albobiverticillius*. Referring to the β-carotene and betanin standard curves (see Figure S4), it was estimated that each 50 mL co-culture extract contained the equivalent amount of red pigment (in terms of absorbance at 500 nm) as >500 mg β-carotene and 1.2 g of betanin (Figure 2b-c). Lastly, the mass yield, as may be seen in Figure 2a, also increased substantially with co-culture. For every gram of dry SFW substrate, >230 mg of dry pigment product was obtained.

4.5. Discussion

The enhanced pigment production is related to the activation of the biosynthetic gene clusters (BGCs) associated with the production, regulation, and transport of azaphilone pigments (e.g. the *mrpig* genes in *M. ruber* M7).[261b] However, the specific azaphilone pigment synthetic pathway has not yet been elucidated in *T. albobiverticillius*, thus more work will be necessary to understand the precise molecular mechanisms. In general, the transcriptional and epigenetic activation of BGCs associated with secondary metabolites is dependent on the developmental stage of the producing fungus and/or is a consequence of environmental stimuli including carbon and nitrogen sources, temperature, light, pH, amino acids in the environment, reactive oxygen species, hypoxic conditions, biofilm formation, iron availability, and stimuli derived from other organisms.[274, 280]

Functionally, azaphilones are widely known to have antifungal and antimicrobial activity.[261a, 263] Therefore, this increase in azaphilone pigment production in co-culture may be attributed to the stimulation of fungal defense. Even though in the PDA co-cultures the fungi did not out-compete one or another, they were not apparently complementary (one characteristic of which is inter-mingling growth) and the apparent mutual inhibition is likely a result of the production of defensive metabolites including azaphilones.[275c, 279a] To understand the specific metabolites involved in the interaction of *T. albobiverticillius* and *T. reesei*, future work may draw from recent studies employing high-throughput chemical (e.g. LC-MS, NMR, etc.) and multivariate statistical (e.g. PCA, PLS, HCA, etc.) analyses to identify the metabolic changes in co-cultured fungi.[281] Several such studies have already investigated co-cultured strains of *Trichoderma* and *Talaromyces*, though focusing on different species: *Trichoderma harzianum* and *Talaromyces pinophilus* (*Penicillium pinophilum*).[282] The results from these studies show that when co-cultured, *T. harzianum* and *T. pinophilus* significantly increase production of metabolites associated with fungal defense (e.g. antibiotics and siderophores).[282b, 282c] Worth noting also is the accumulation of red pigment which was observed to occur in the co-cultures but not in any single cultures.[282c] Such results may be interpreted to support the mechanism for increased pigment production in *T. albobiverticillius* co-cultured with *T. reesei* as being related

to fungal defense. To confirm this, metabolomic studies focusing specifically on *T. albobiverticillius* and *T. reesei* interactions will be needed.

Use of various agro-industrial wastes as substrate in SSF for the production of pigments has been widely applied (Table 1).[262a, 264b, 271b, 283] Though most reports involve *Monascus*, *Talaromyces* represents an appealing alternative due to the reported absence of mycotoxins (e.g. citrinin) that are associated with *Monascus*.[262a] While available reports of SSF pigment production by *Talaromyces* have shown limited productivity, here it is shown that food waste may be an effective feedstock for eliciting high pigment production with *Talaromyces* sp. NRRL 2120.[262a] The results described herein may also demonstrate particularly broad applicability vis-à-vis studies that use specific food wastes (e.g. the substrates shown in Table 1) which may be more appropriate in specific industrial partnerships, specific regions, or may require additional processing of the food waste streams. Additionally, this study has shown that the observed high pigment production of *Talaromyces* sp. NRRL 2120 may be further enhanced by co-culturing with *T. reesei*.

Table 4.1. Summary of brief literature survey of fungal pigment production from SSF of biomass residues.

Organism	Substrate	Pigment	Concentration (Absorbance units/ mass dry substrate)	Source
M. purpureus	Jackfruit seed + monosodium glutamate	Yellow (413 nm)	25.5	[271b]
		Red (484 nm)	30.8	
M. purpureus	Orange peel	Yellow (400 nm)	9.0	[262a]
		Orange (470 nm)	3.5	
		Red (500 nm)	2.5	
A. carbonarius	Pomegranate pulp	Yellow (400 nm)	61.8	[283b]
T. albobiverticillius	Simulated food waste	Yellow (400 nm)	32.5	This study
		Orange (475 nm)	20.9	
		Red (500 nm)	22.4	

T. reesei		Yellow (400 nm)	36.8	
T. albobiverticillius + *T. reesei*		Yellow (400 nm)	63.8	
		Orange (475 nm)	35.6	
		Red (500 nm)	43.6	

In summary, productivity of pigments absorbing at the yellow, orange, and red wavelengths from simulated food waste feedstock was substantially improved through the employment of a filamentous fungal co-culture solid-state fermentation system. Since red and yellow hues have traditionally been the most extensively used food colorants, there is substantial economic potential for such a process. With increasing demand for natural pigments, particularly as food ingredients, the findings here offer one appealing potential use for food waste.

4.6. Appendix B. Supporting information for: Solid-state Co-culture Fermentation of Simulated Food Waste with Filamentous Fungi for Enhanced Production of Bio-pigments

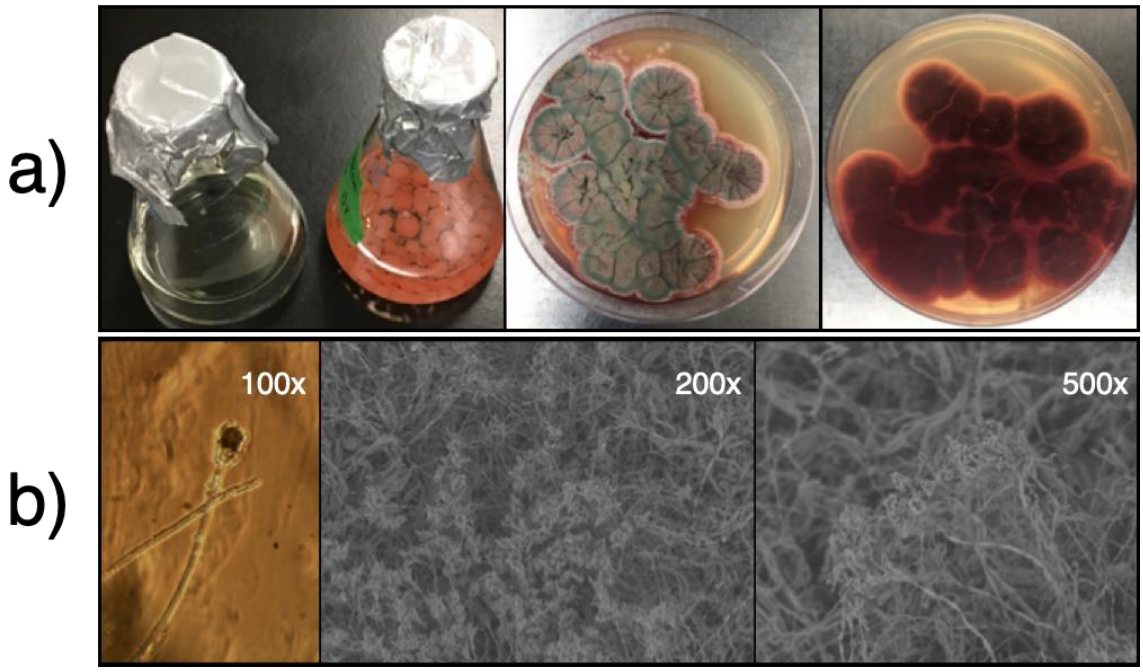

Figure S1. Colonial and microscopic images of *T. albobiverticillius*: **a)** (left to right) Liquid broth prior to and 7 days after inoculation with *T. albobiverticillius*; 7 day old PDA cultures of *T. albobiverticillius* as

viewed from the obverse and reverse; **b)** (left to right) Conidiophore of *T. albobiverticillius* visualized using phase contrast microscopy (100x); Conidiophores of *T. albobiverticillius* visualized using scanning electron microscopy at 200x and 500x.

Figure S2. Simulated food waste prior to (left) and 15 days after (right) inoculation with spores of *T. albobiverticillius.*

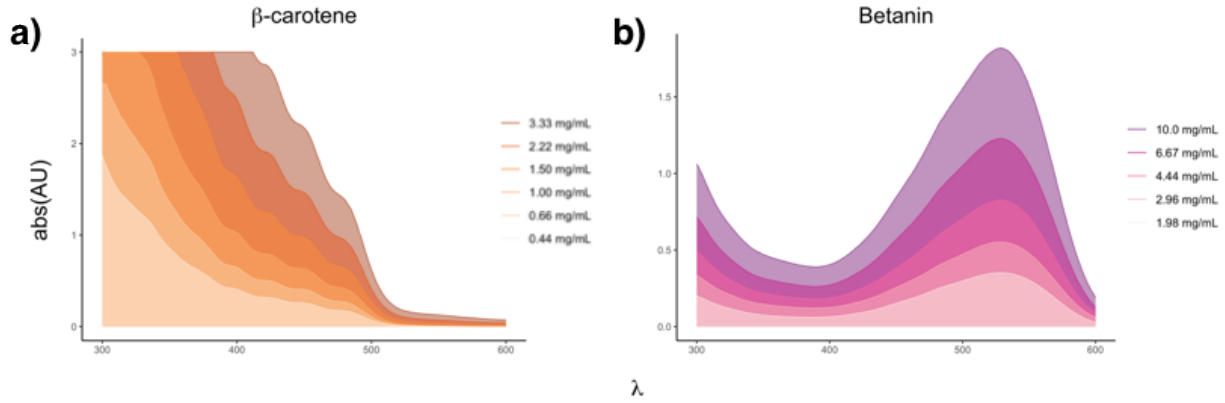

Figure S3. **a)** Standard curve for β-carotene absorbance in the wavelength(λ) range of 300-600; **b)** Standard curve for betanin (red beat extract) absorbance in the wavelength(λ) range of 300-600 nm.

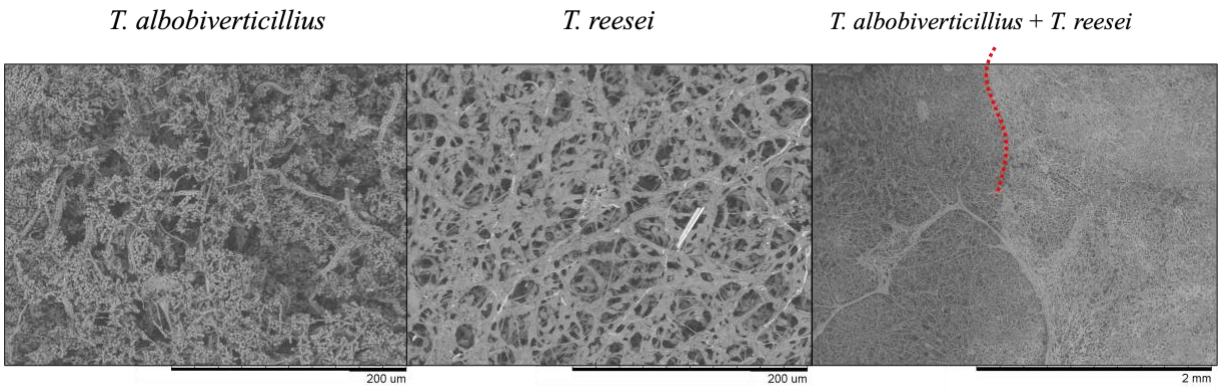

Figure S4. SEM images of mycelia taken from the co-culture screening experiments. Experimental controls comprising single cultures of *T. albobiverticillius* (left) and *T. reesei* (middle); Co-culture of *T. albobiverticillius* with *T. reesei* (right). Note: the dotted red line denotes the interface of the two fungi.

4.7. References

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Connecting Statement 3

From the results described in Chapter 3, the platform chemical 2,5-furandicarboxylic acid (FDCA) was another interesting product of the fungal fermentation of biomass, specifically when apple pomace was used as feedstock. FDCA possesses great industrial potential due to its use as a precursor in the production of bioplastics (e.g. polyethylene furanoate). Microbial production of this compound from biomass feedstock is the focus of much current research but is limited primarily to the use of bacteria. Very few publications have described the use of filamentous fungi despite the potential advantages that their use may offer. The results described in Chapter 3 suggest that at least one additional strain of filamentous fungus has the ability to accumulate this potentially important product, though it is likely not so rare among the filamentous fungi which are well-known for their oxidizing enzymes (the main path for production of FDCA is oxidation of the carbohydrate-derived compound 5-hydroxymethylfurfural (HMF))

Chapter 5 therefore involves, for the first time, the screening of five industrially relevant strains of filamentous fungi in the conversion of HMF into FDCA. An optimized process which includes the use of an enzyme/mediator system is ultimately developed. This chapter is based on an article published in *Bioresource Technology* with Dr. Valérie Orsat and Dr. Marie-Josée Dumont as co-authors of this paper.

Chapter 5: Use of filamentous fungi as biocatalysts in the oxidation of 5-(hydroxymethyl)furfural (HMF)

5.1. Abstract

The objective of this study was to explore the use of filamentous fungi as oxidative biocatalysts. To that end, filamentous fungal whole-cells, comprising five different species were employed in the oxidation of 5-(hydroxymethyl)furfural (HMF). Two species (*A. niger* and *T. reesei*), which demonstrated superior HMF conversion and product accumulation, were further evaluated for growth on alternative substrates (e.g. pentoses) as well as for use in a chemo-biocatalytic reaction system. Concerning the latter, the two whole-cell biocatalysts were coupled with laccase/TEMPO in a one-pot reaction designed to enable catalysis of the three oxidative steps necessary to convert HMF into 2,5-furandicarboxylic acid (FDCA), a compound with immense potential in the production of sustainable and eco-friendly polymers. Ultimately, the optimal one-pot chemo-biocatalytic cascade system, comprising 1 g/L *T. reesei* whole cells coupled with 2.5 mM laccase and 20 mol% TEMPO, achieved a molar yield of 88% after 80 hours.

5.2. Introduction

The field of industrial biocatalysis is relatively young but rapidly expanding. This trend coincides with, and is related to, the transition toward a bioeconomy; i.e. an economy in which material goods are derived from renewable bio-based feedstocks and converted into final products using, ideally, environmentally friendly, bio-based technologies. Unlike typical thermo-chemical catalysis, biocatalysis (i.e. use of enzymatic or whole-cell catalysts) typically occurs under mild reaction conditions (e.g. ambient temperature and pressure and mild pH), in an aqueous environment and, in the case of catalytic oxidations, often with use of molecular oxygen for the oxidant.[284] As enzymes and cells are inherently biodegradable, they may be cleaner and more eco-efficient than stoichiometric reagents and metal catalysts while also possessing high (and often perfect) chemo-, regio-, and stereoselectivities reliable even for fine chemicals with complex structures and oxidation-sensitive functional groups.[284-285] With 85% of industrial

chemical processes being catalytic, the expansion of biocatalysis has potential to make an enormous impact on the sustainability of industrial processes (currently only 3% of industrial catalytic processes are biocatalytic).[286]

Among biocatalysts, enzymes offer high reaction efficiency and control while whole-cell biocatalysts are often more robust, benefitting from a protective barrier against the stress of aeration, reactive substrates or products as well as benefitting from endogenous cofactors and enzymes which perpetuate catalytic pathways (which otherwise require expensive stoichiometric reagents) and catabolize inhibitory by-products (e.g. the common by-product of oxidation reactions H_2O_2).[284b] Several studies employ enzymes and whole-cell biocatalysts together in "one-pot" reactions to exploit the advantages of both, while minimizing their respective disadvantages.[287] Additionally, the approach of engineering whole-cells to contain desired enzyme cascades is increasingly reported.[288] While enzymes are derived from myriad sources, most currently employed whole-cell biocatalysts are comprised of bacteria or yeast. The under-represented filamentous fungi possess unique characteristics which may offer long term benefits for use generally as whole-cell biocatalysts and, as such, deserve more in-depth exploration. With respect to the characteristics considered key in the development of whole-cell biocatalysts, filamentous fungi are particularly robust (i.e. tolerant of wide ranging process conditions and resistant to contamination by other microorganisms) and accept a broad scope of carbon sources which may include those that are inexpensive, non-competitive with food or feed production, and/or comprise waste residues.[289] Fungi are anticipated to play an essential role in the global sustainable future including in the conversion of biomass and waste residues, pollution mitigation, enhancement of agriculture, and in the production of sustainable bulk and specialty chemicals.[11a, 290]

There is perhaps no material more urgently in need of replacement with a renewable and biodegradable alternative than plastic. To that end, 2,5-furandicarboxylic acid (FDCA) is a promising bio-based alternative for the production of biodegradable polyesters and, as such, has been identified by the US Department of Energy (US DOE) as a "top opportunity" for the future chemical industry.[291] The importance of FDCA resides in its prospect as a replacement for oil-derived terephthalic acid in the production of polyesters (e.g. the biodegradable polyethylene furandicarboxylate).[292] Given its potential impact, FDCA was chosen as the target product for this study.

FDCA is mainly synthesized from 5-(hydroxymethyl)furfural (HMF), another US DOE "top opportunity".[292c] Since HMF contains an alcohol group and an aldehyde group, synthesis of FDCA from HMF requires three consecutive oxidation steps that can proceed in one of two ways.[292c] In the first pathway, the aldehyde group is preferentially oxidized to a carboxyl group to form 5-hydroxymethyl-2-furan carboxylic acid (HMFCA).[292c] The alcohol group of HMFCA is then oxidized to an aldehyde group, yielding 2-formyl-5-furancarboxylic acid (FFCA), which is further oxidized to FDCA.[292c] In the second pathway, the alcohol group of HMF is preferentially oxidized to an aldehyde group to form 2,5-diformylfuran (DFF), following which the aldehyde groups of DFF are sequentially oxidized to carboxyl groups to yield FDCA via FFCA.[292c]

Aldehydes, including HMF, can damage cellular components such as proteins, nucleic acids, and organelles through the formation of reactive oxygen species and by inhibiting essential metabolic enzymes.[291] Thus, many organisms produce enzymes (e.g. aldehyde dehydrogenases) to convert these aldehydes into less toxic forms (e.g. an alcohol or acid).[291] These enzymes make the organisms which produce them, as well as the enzymes themselves in isolated form, appealing catalysts in the conversion of HMF to HMFCA and FFCA to FDCA.[287b, 291] Conversion of furans has long been reported in bacteria and fungi alike, including (extensively) conversion of HMF into HMFCA.[291, 293] Moreover, the biological pathways involved in HMF oxidation (which include FDCA as an end-product or intermediate in subsequent metabolic pathways) have been elucidated.[291] Reports suggest that the gene sequences involved in this HMF catabolic pathway are limited to a relatively small group of bacteria.[291, 294] However, more recently, similar pathways containing analogous genes have been shown to be widespread in filamentous fungi.[293, 295] Moreover, conversion of HMF into FDCA has been reported for species including *Aspergillus*, *Penicillium*, and *Pleurotus*.[289a, 294, 296] To the best of the authors' knowledge, the highest molar yield of FDCA from HMF using filamentous fungi (67% after 336 hours under optimized conditions) was observed in a strain of *A. flavus* isolated from the soil of an acid pretreatment liquor disposal site for a biofuel pilot plant.[294]

In this study, five untested species of filamentous fungi -*A. niger*, *P. chrysosporium*, *P. purpurogenum* (the basionym for *Talaromyces albobiverticillius*), *R. oryzae*, and *T. reesei*– were investigated for their ability to oxidize HMF.[239c] These species were selected due to pre-

existing widespread industrial application and/or reports in literature which demonstrate their relevant potential (their taxonomic relation to other key fungal species may be seen in Figure 5.1).[234b] Unlike in other related studies, these strains were not acquired from, or habituated to, HMF-enriched soil. Thus, this study aims to explore a process which can either be applied generally among readily available species of filamentous fungi or be adopted to further enhance processes which involve optimized strains. In addition to representing the first such report involving these five specific species, this study also includes the first report of a chemo-biocatalytic reaction system incorporating any species of filamentous fungi.

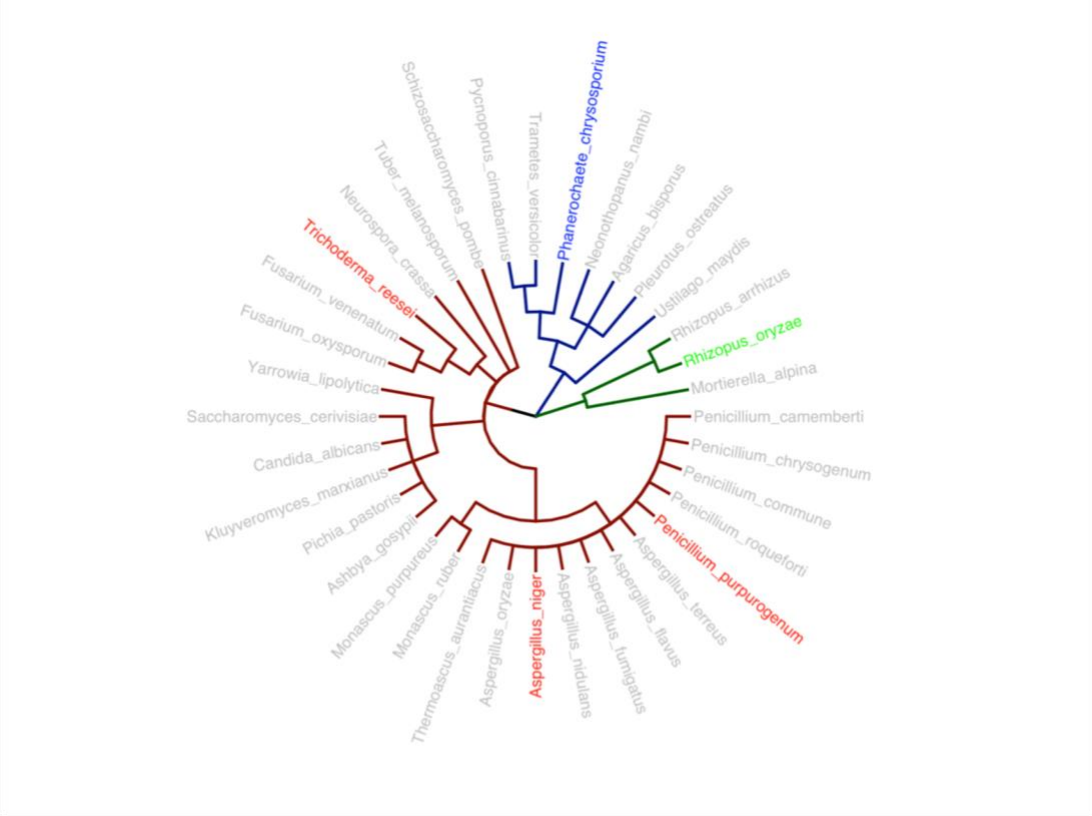


Figure 5.1. Dendrogram displaying the organisms used in the present study as they relate to various fungal organisms with high importance in industry, commerce, agriculture, human health, and the environment.

5.3. Materials and Methods

5.3.1 Reagents

The following reagents were acquired from Sigma Aldrich: HMF, HMFCA, FFCA, FDCA, Laccase (*Trametes versicolor*), (2,2,6,6-tetramethylpiperidin-1-yl)oxidanyl (TEMPO), and HPLC grade water.

Many of the reactions described below took place in mineral salt media (MSM) which is comprised of the following in HPLC grade water: 0.2 g/L $MgSO_4 \cdot 7H_2O$, 0.02 g/L $CaCl_2 \cdot 2H_2O$, 0.5 g/L K_2HPO_4, 0.5g/L KH_2PO_4, 0.5 NH_4Cl, and 10 mL/L trace element solution (300 mg/L $FeSO_4 \cdot 7H_2O$, 50 mg/L $MnSO_4 \cdot H_2O$, 106 mg/L $CoCl_2 \cdot 6H_2O$, 34 mg/L $Na_2MoO_4 \cdot 2H_2O$, 40 mg/L $ZnSO_4 \cdot 7H_2O$, 50 mg/L $CuSO_4 \cdot 5H_2O$).[294, 297]

5.3.2 Organism cultivation

Five species of filamentous fungi -*Aspergillus niger* (NRRL 567), *Phanerochaete chrysosporium* (NRRL 6370), *Penicillium purpurogenum* (NRRL 2120), *Rhizopus oryzae* (NRRL 1526), and *Trichoderma reesei* (NRRL 3652)- were acquired from the USDA and cultivated on potato dextrose agar (PDA) for 5 days at 30 °C. Sterile water was used to extract spores from each culture. The concentration of spores was adjusted to $1x10^6$ spores/mL. 1 mL of spore solution was used to inoculate PDA starter plates (incubated at 30 °C for 5 days) as well as 100 mL potato dextrose broth (PDB) cultures. The PDB cultures were incubated for 72 hours at 30 °C and 200 rpm on a flask shaker.

5.3.3 Effect of substrate components on fungal growth

To determine the effect of HMF on fungal growth, each organism was grown on solid media enriched with various concentrations of HMF. PDA plates were prepared with the following concentrations of HMF: 0, 1, 1.5, and 2 g/L (0, 8, 12, and 16 mM). Plates comprised of 1.5% agar and 1 g/L HMF in MSM (i.e. HMF and agar as sole carbon sources) were also prepared. A cork-borer was used to transfer a plug (1 cm diameter) from a starter culture to a

test plate. Each growth condition was repeated at least twice. The growth rate of each organism was measured with respect to the diameter of the colony and the measurements are reported as the means of the diameters ± standard deviations. The IC_{50} of each organism was estimated to be the HMF concentration at which the growth rate of the organism was 50% relative to that on PDA with 0 g/L HMF.

To compare the ability of fungi to grow on alternative substrates, two specified organisms were grown on solid and liquid media according to the following recipes adapted from standard recipes for Sabouraud media (sans peptone). Solid media was prepared as the following: 40 g/L specified sugar in MSM; 15 g/L agar in MSM. Liquid media was prepared as the following: 40 g/L specified sugar in MSM. For controls, both solid and liquid media were prepared with 0 g/L sugar.

5.3.4 General procedure for biocatalytic oxidation of HMF

Biocatalytic reaction solutions were prepared as follows: 20 mL from PDB culture (following the 3-day incubation as described above) was collected and centrifuged at 3000 × g for 30 minutes. The supernatant was decanted, and the process was repeated. Following the second centrifugation/supernatant decantation, the remaining biomass pellet was used to inoculate 50 mL of MSM spiked with 1 g/L HMF. The amount of biomass used was ~3 grams wet mass (gwm), giving each reaction a concentration of ~60 mg whole-cell biocatalyst/mL. The reactions were performed in at least duplicate. The solutions were incubated at 30 °C and 200 rpm. ~1 mL of supernatant was removed at regular intervals, filtered (0.2 μm pore size), and analyzed using HPLC and GC-MS. The values were expressed as the means of the analyte concentration (calculated using standard curves) ± standard deviations. To analyze intracellular content, an amount of unfiltered reaction solution was centrifuged at 10,000 × g for 30 minutes. The supernatant was discarded, and the remaining biomass pellet was homogenized (Fisherbrand Homogenizer 850). ~1 mL of homogenate was taken, filtered (0.2 μm pore size), and analyzed using HPLC and GC-MS.

5.3.5 One-pot chemo-biocatalytic system using laccase/TEMPO

Following the same method described above, a reaction system was constructed which also included a specified mol% TEMPO and 2.5 mM laccase in 50 mL MSM with whole-cell biocatalyst and HMF. To control for laccase/TEMPO activity, a separate system was prepared with the same reaction conditions (20 mol% TEMPO) minus the whole-cell biocatalyst. Each reaction was performed in at least duplicate. ~1 mL of supernatant was removed at regular intervals, filtered (0.2 μm pore size), and analyzed using HPLC and GC-MS. The values were expressed as the means of the analyte concentration (calculated using standard curves) ± standard deviations.

5.3.6 Box-Behnken optimization

A Box-Behnken design was used to evaluate the influence of and optimize three reaction parameters in the conversion of HMF to HMFCA: amount of whole-cell biocatalyst, incubation temperature, and shaker rotational speed. The whole-cell biocatalyst was acquired as described above, and the quantities of biocatalyst tested were 1, 3, and 5 gwm. The incubation temperatures tested were 20 (room temperature), 30, and 40 °C. The different rotational speeds tested were 100, 150, and 200 rpm. The concentration of HMF and reaction time were held constant for all experiments at 1 g/L and 20 hours respectively. After 20 hours, ~1 mL of supernatant was removed, filtered (0.2 μm pore size), and analyzed using HPLC. A total of 15 experiments were conducted. All statistical analyses were performed using JMP 14 software and R studio.[298]

5.3.7 HPLC analysis

All HPLC analysis was performed on an Agilent 1260. For samples generated from the screening experiments, components were analyzed using an Agilent Hi-Plex Ca (Duo), 8 μm, 300 x 6.5 mm column with Agilent Hi-Plex guard column. The specific LC parameters were as follows: 5 μL injection volume, 0.5 mL/min flow rate, 254 nm detection wavelength, an isocratic elution of 90:10 HPLC grade water: acetonitrile with 0.1% formic acid as mobile phase for 33-45

minutes. For samples generated from the optimization experiments and subsequent experiments, components were analyzed using an Agilent ZORBAX Eclipse plus C18, 3.5 μm, 4.6 × 100 mm column with Agilent ZORBAX Reliance Cartridge guard column. The specific LC parameters were as follows: 6 μL injection volume, 0.8 mL/min flow rate, 274 nm detection wavelength, an isocratic elution of 100% HPLC grade water as mobile phase for 5 minutes plus 1-minute post-run. All HPLC data analysis was performed using Agilent technologies OpenLAB CDS ChemStation software. Analyte concentrations were calculated based on standard curves using pure reagents.

5.3.8 GC-MS analysis

Following HPLC analysis, the samples were lyophilized. For derivatization, the lyophylate was resuspended in 750 μL each of pyridine and BSTFA and then incubated overnight at 70 °C. For, non-derivatized samples, the lyophylate was resuspended in methanol. For samples with unknown composition (i.e. in product identification), both derivatized and non-derivatized repeats were analyzed. An Agilent 6890 GC system coupled with 5973 MS system was used. The GC parameters were as follows: Agilent HP-5 30m x 0.25mm x 0.25 m column; helium as the carrier gas; 70 °C initial temp; 280 °C max temp; 20 °C/min ramp. For data analysis, Agilent MSD Productivity ChemStation software was used with peak identification performed using the NIST02 Mass Spectral Library.

5.4 Results and Discussion

5.4.1 Effect of HMF on fungal growth

The growth rate of each organism on PDA, PDA spiked with HMF, and MSM/agar spiked with 1 g/L HMF is summarized in Figure 5.2a. The majority of PDA (without HMF) plates were completely covered (maximum diameter of 8.5 cm) in mycelia within 24-48 hours (the exceptions being one *A. niger* repeat and all the *P. purpurogenum* repeats). Hence, the following growth rates were calculated based on fungal colony diameter at the 48-hour time point. When cultivated on PDA spiked with 1 g/L HMF, the growth rate for *A. niger*, *P.*

chrysosporium, and *T. reesei* decreased by ~50% (decreasing from 3.4, 4.3, and 4 cm/day to 1.65, 2.4, 2 cm/day respectively). From this data, the IC_{50} for *A. niger*, *P. chrysosporium*, and *T. reesei* was estimated to be around 1 g/L HMF, which is similar to reports for other filamentous fungi.[293-294] Above 1 g/L HMF, the growth rates for *A. niger* and *T. reesei* diminished more slowly with increasing HMF concentration while the growth rate for *P. chrysosporium* continued to decrease considerably with each increasing HMF concentration. At the 48-hour time point, *P. purpurogenum* showed no clear trend in the relation between growth rate and HMF concentration. However, using the growth rate calculated up to the 5-day time point, the IC_{50} for *P. purpurogenum* may be estimated to be 1.5 g/L HMF in PDA (growth rates of 1.22 and 0.69 cm/day for 0 and 1.5 g/L HMF in PDA respectively). While *P. purpurogenum* had a relatively high tolerance to HMF, it also had an overall much slower growth rate than all the other tested organisms as well as high variability (similar to *A. niger* in this latter respect). Of all tested species, *R. oryzae* showed the highest tolerance, with IC_{50} estimated to be greater than (but close to) 2 g/L.

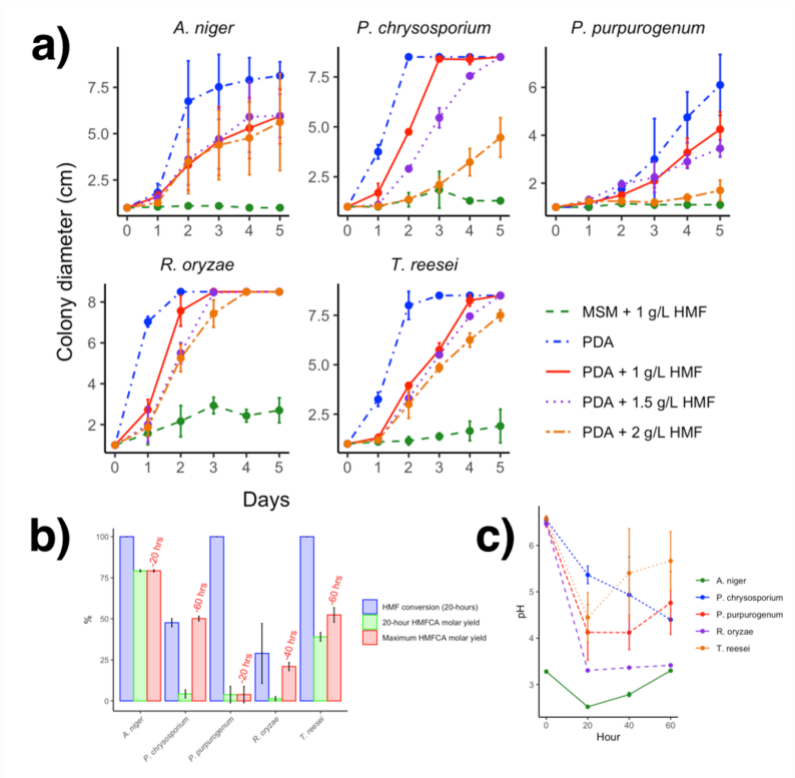


Figure 5.2. **a)** Growth rate of *A. niger* (top left), *P. chrysosporium* (top middle), *P. purpurogenum* (top right), *R. oryzae* (bottom left), and *T. reesei* (bottom middle) on PDA spiked with 0, 1, 1.5, and 2 g/L

HMF as well as MSM/agar spiked with 1 g/L HMF; **b)** Biocatalytic conversion of 1 g/L HMF by (from left to right) *A. niger*, *P. chrysosporium*, *P. purpurogenum*, *R. oryzae*, and *T. reesei*. Shown are the values for percentage of HMF converted after 20 hours, the HMFCA molar % yield after 20 hours, and the maximum molar % yield of HMFCA with the associated time point above; **c)** Supernatant pH change over 60-hour period for fungal whole-cell bioconversion of 1 g/L HMF.

The largest effects on growth were observed on the MSM/agar plates with 1 g/L HMF. With HMF and agar as sole carbon sources, each organism appeared to initially expand into the media and grow, albeit at a greatly reduced rate vis-à-vis the growth rate on PDA or PDA with HMF. However, after peak growth at ~5 days, each organism began to recede and eventually die (i.e. was visibly desiccated). While filamentous fungi (e.g. *Aspergillus* sp.) are reported to be able to utilize agar as a sole source of carbon, there also exists an "agar-inhibition" effect on growth when weak catabolite repressors (e.g. organic acids) are also present as a main carbon source.[299] From these results, it may be concluded that these species of filamentous fungi are able to handle the toxic aldehyde HMF, and thus likely show chemical reactivity to HMF, but perhaps do not consume it and utilize it for cellular metabolism. These characteristics allow consideration of these species for use as bioconversion catalysts.

5.4.2 Screening for oxidative activity on HMF

To better understand the activity of the fungi on HMF, the fungi were incubated in 50 mL MSM liquid cultures spiked with 1 g/L HMF. To prepare the whole-cell biocatalysts, each fungal species was first cultivated in PDB at a rotational rate of 200 rpm. This elicits a different morphology in the fungi than the typical mycelial mat associated with solid state growth. While *P. purpurogenum*, *R. oryzae*, and *T. reesei* grew as diffuse colonies, *A. niger* and *P. chrysosporium* grew in the form of mycelial pellets. These specific morphologies of each fungus are used in all subsequent biocatalytic reactions.

Following addition of the whole-cell biocatalysts, the supernatant of each reaction solution was sampled every 20 hours for a period of 60 hours, see Figure 5.2b. With the exception of *R. oryzae*, each species was able to convert all the HMF within 60 hours. After 20 hours, the same, single product with the same LC retention time was detected in each sample

(see Appendix C). Using GCMS, the identity of this product was confirmed to be, as expected, HMFCA (see Appendix C). For *P. purpurogenum* and *A. niger*, product levels began to decrease following the 20-hour time point, though this was much more pronounced in the case of *P. purpurogenum*. Moreover, in addition to only accumulating small amounts of product by the 20-hour time point, the *P. purpurogenum* reaction supernatant contained no detectable product by the 40-hour time point. Since it appeared that HMF was being consumed, with little or no product secretion, both the supernatant and intracellular contents of the *P. purpurogenum* reactions were analyzed by GC-MS. The major compound found in the cell homogenate (cells used from 60-hours reaction) was identified as furan acetic acid (see Appendix C). This compound was also detected in small amounts in the supernatant of *P. purpurogenum* and in that of the other organisms. Further investigation will be required to determine if this compound is an intermediate in the HMF catabolic pathways of these organisms. Finally, as may be seen in Figure 5.2c, there appeared to be a correlation between pH and conversion of HMF. This is expected given the formation of carboxylic acid products but does not explain the drop observed in the reaction solution containing *P. purpurogenum* (which, as mentioned, did not form significant product) or the later increase in pH observed in the reaction solutions containing *T. reesei*. In the case of *A. niger*, the organism very quickly acidified the unbuffered media (which is typical for the species) as evident in the much lower initial pH vis-à-vis the other fungi.[300] Subsequently, the increasing and then decreasing levels of carboxylic acid product correlate to the further decrease in pH over the following 20 hours and then the increase at each later time point.

These results suggest that *A. niger*, *T. reesei*, and, to a lesser extent, *P. chrysosporium* readily oxidize HMF into HMFCA but show very little further oxidative activity (FDCA was only detected in small amounts in the supernatants of *A. niger* after 20 hours as well as *P. chrysosporium* and *T. reesei* after 60 hours; (see Appendix C). This accords with the understanding that organisms seek to neutralize the harmful effects of aldehydes and, once accomplished, there is no apparent need to continue to modify the compound.

5.4.3 Growth on Pentose Substrate

Based on HMF conversion efficiency and HMFCA productivity, *A. niger* and *T. reesei* were identified as promising candidates for continued evaluation as biocatalysts. One notable characteristic of filamentous fungi is their ability to accept a wide variety of carbon sources including pentose sugars.[289b] The option to produce biocatalysts grown from pentose (e.g. xylose and arabinose) would represent an advantage in terms of process sustainability since pentose sugars, which are the main components of hemicellulose, comprise around a third of plant biomass.[301] Biocatalyst growth could thus represent a valorization stream for the hemicellulose fraction of lignocellulosic biomass while obviating the need for glucose (and the high latent energy content associated with the utilities required for its production) or other hexose substrates (which compete for other uses in other process streams thus driving up the price).

Here, *A. niger* and *T. reesei* were evaluated for ability to utilize pentose-based substrates vis-à-vis dextrose-based substrates including commercial PDA and PDB. As may be seen in Figure 5.3a, of the solid media, both organisms grew best on PDA. Interestingly, while among the non-commercial media tested, dextrose served as a good carbon source, xylose and arabinose elicited the highest growth rates for *A. niger* and *T. reesei* respectively. Of the liquid media, *A. niger* produced the most biomass using dextrose-based media (dextrose in MSM being the best though somewhat more variable than PDB), with a decrease of about ~50% when substituting for xylose and with very little biomass produced when given arabinose. In the case of *T. reesei*, though the absolute amount of biomass obtained was much smaller, xylose proved to be the best overall carbon source for growth. Furthermore, the amount of biomass obtained using arabinose was greater than was obtained from *A. niger*. Though in general *A. niger* was superior in terms of biomass production, *T. reesei* appeared more versatile with respect to carbon source and better equipped to grow on pentose-based media. No organism was able to grow using media containing only MSM.

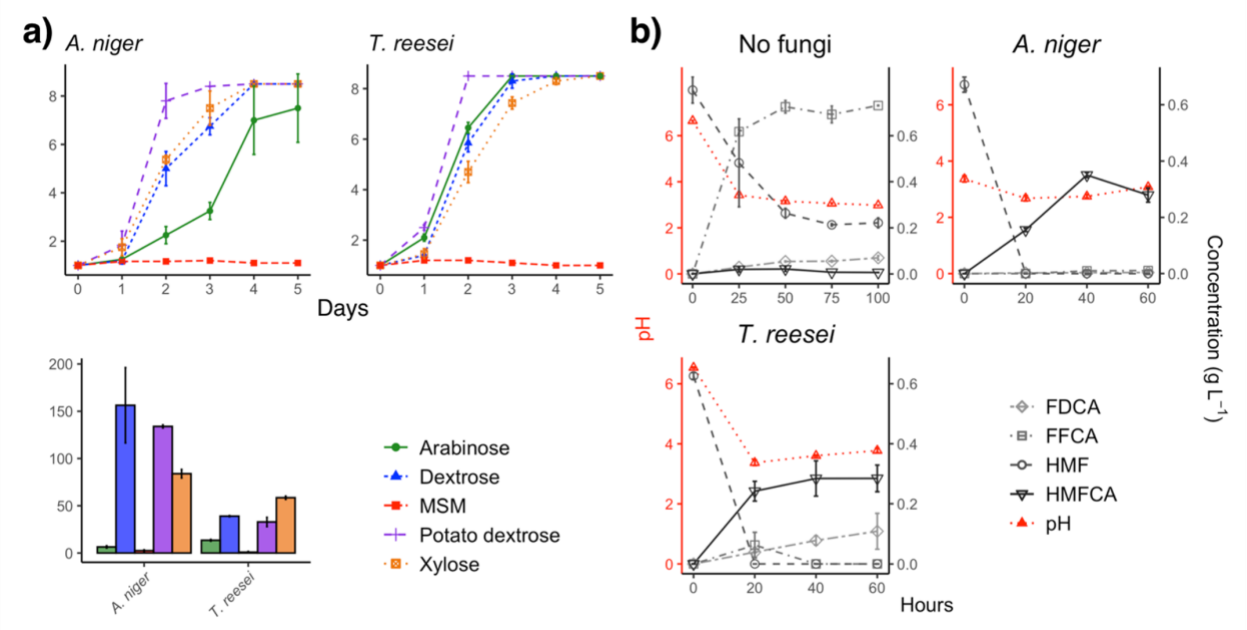


Figure 5.3. **a)** (top) Comparison of *A. niger* and *T. reesei* growth on growth over five-day period on solid media with pentose or dextrose carbon sources; (bottom) Comparison of the amount of biomass acquired following 72-hour incubation in liquid media containing pentose or dextrose carbon sources; **b)** Conversion of 1 g/L HMF using laccase/TEMPO systems; shown are the rates of HMF conversion and product accumulation along with the pH trend for: (top left) laccase/TEMPO alone; (top right) laccase/TEMPO coupled with *A. niger*; and (bottom left) laccase/TEMPO coupled with *T. reesei.*

5.4.4 One-pot chemo-biocatalytic reaction

The slow accumulation of FDCA in *T. reesei* (as well as in *P. chrysosporium*) is consistent with what has been reported elsewhere for other species of filamentous fungi.[294] In *A. niger*, it was observed that oxidative products of HMF, HMFCA and FDCA (FFCA was not detected), accumulated to maximum levels within 20 hours after which point product concentrations decreased. These results, along with other such data available from literature, suggest that the ability to convert HMF into FDCA varies across filamentous fungal species and indeed studies demonstrate that the genomes of certain species of filamentous fungi, including those of the *Aspergillus* genus, contain a greater number of the genes associated with HMF catabolism.[293] Additionally, some studies involve use of field isolates acquired from HMF-enriched soils which may possess different genetic and metabolomic activity (e.g. upregulation

of genes involved in oxidation of HMFCA and FFCA, upregulation of genes associated with effluxion of FDCA into the environment, and/or downregulation of the genes associated with FDCA catabolism).[294] It would be useful to establish methods which enable more substantial FDCA production capabilities in otherwise incapable species of filamentous fungi, particularly in those already exhibiting widespread industrial use.

Continuing with *A. niger* and *T. reesei*, the next goal was to investigate how to both increase the rate of FDCA accumulation as well as ensure continued product accumulation. The combination of the fungal enzyme laccase coupled with the nitroxyl radical (2,2,6,6-tetramethylpiperidin-1-yl)oxidanyl (TEMPO) is known to catalyze oxidation of alcohol and aldehyde functional groups including the hydroxymethyl group of HMFCA.[287] Since HMFCA was the product which accumulated the most, a system was developed which coupled *A. niger* or *T. reesei* with laccase/TEMPO. The laccase/TEMPO pair alone was able to oxidize HMF and accumulate FFCA with small amounts of FDCA (see Figure 5.3b). Here, conditions conducive to fungal whole-cell catalysis (e.g. neutral pH) were employed which may explain the relative lack of HMF conversion since laccase/TEMPO systems typically employ a low pH. The accumulation of FFCA, on the other hand, is commonly reported in such systems and is believed to be related to the low hydration degree of FFCA.[284a, 287b] Nevertheless, the desired result was observed in that little HMFCA was detected, suggesting rapid conversion into FFCA, under conditions suitable for fungal whole-cell catalysis. Reaction solutions containing, separately, *A. niger* and *T. reesei* (which on their own showed very little activity toward HMFCA) were combined with laccase and 20 mol% TEMPO. As may be seen in Figure 5.3b, the addition of laccase/TEMPO did not help increase FDCA accumulation in *A. niger* and showed an inhibitory effect on the accumulation of HMFCA. This is likely related to the known toxic effect TEMPO has on cells (reported for mammalian cells).[302] On the other hand, *T. reesei* appeared to be more tolerant to the presence of TEMPO and the reaction benefitted accordingly. FDCA was observed to accumulate in *T. reesei* supernatant after 20 hours and roughly doubled every additional 20 hours. The identity of FDCA was confirmed using GC-MS (see Appendix C).

5.4.5 Reaction Optimization

Oxidative biocatalytic reaction conditions were next optimized for *T. reesei*, chosen due to its superior performance in both HMFCA production as well as in the laccase/TEMPO system. First, HMFCA production was optimized using a Box-Behnken design which included 15 sets of experiments; the amount of biocatalyst (i.e. *T. reesei* biomass; X1), incubation temperature (X2), and rotational speed (X3) were tested at three levels. The concentration of substrate (1 g/L HMF) and incubation time (20 hours) were held constant for all reactions. Table 5.1 presents the design matrix of the coded variables together with the experimental results in terms of the HMFCA yield. Contour plots of the HMFCA yields, presented in Figure 5.4a, are shown in terms of biocatalyst amount and temperature, biocatalyst amount and rotational speed, and temperature and rotational speed.

Table 5.1. Box-Behnken experimental design and HMFCA yield from 1 g/L HMF in 20 hours by *T. reesei*

Trial	Variable			HMFCA
	X1 (g)	X2 (ºC)	X3 (rpm)	yield (%)
1	1.14 ± 0.07 (-)	22.5 (-)	150 (0)	48.12
2	5.04 ± 0.29 (+)	22.5 (-)	150 (0)	17.31
3	1.14 ± 0.07 (-)	40 (+)	150 (0)	19.05
4	5.04 ± 0.29 (+)	40 (+)	150 (0)	35.57
5	1.14 ± 0.07 (-)	30 (0)	100 (-)	61.66
6	5.04 ± 0.29 (+)	30 (0)	100 (-)	29.23
7	1.14 ± 0.07 (-)	30 (0)	200 (+)	57.81
8	5.04 ± 0.29 (+)	30 (0)	200 (+)	31.32
9	2.84 ± 0.19 (0)	22.5 (-)	100 (-)	35.98
10	2.84 ± 0.19 (0)	40 (+)	100 (-)	41.45
11	2.84 ± 0.19 (0)	22.5 (-)	200 (+)	33.26
12	2.84 ± 0.19 (0)	40 (+)	200 (+)	38.65

13	2.84 ± 0.19 (0)	30 (0)	150 (0)	43.94
14	2.84 ± 0.19 (0)	30 (0)	150 (0)	41.71
15	2.84 ± 0.19 (0)	30 (0)	150 (0)	43.23

The statistical analysis of the Box-Behnken results produced an acceptable model ($P < 0.05$) with the amount of biocatalyst and temperature representing the variables exhibiting the largest influence over HMFCA yield. While the % of HMF converted did vary across reaction conditions, the model of the correlation was poor. Nonetheless, the average conversion % was very high across all conditions (~92 ± 0.2%).

Together, Table 1 and Figure 5.4a show that higher levels of biocatalyst concentration support relatively low levels of HMFCA yield while basal temperature conditions are associated with higher levels of HMFCA yield. Basal rotational speed was a minimum for HMFCA yield though the statistical analysis suggests that rotational speed has the smallest impact of the three variables on HMFCA yield. The model generated from this data would predict optimal conditions outside the experimental space, thus the conditions within the experimental space which showed the highest HMFCA yield (i.e. ~1 g biocatalyst incubated at 30 °C and 100 rpm) were taken as the optimized method for the following experiments. Using these optimized parameters, reaction solution pH was next explored (see Figure 5.4b) which revealed the neutral starting pH of 7 to be the best.

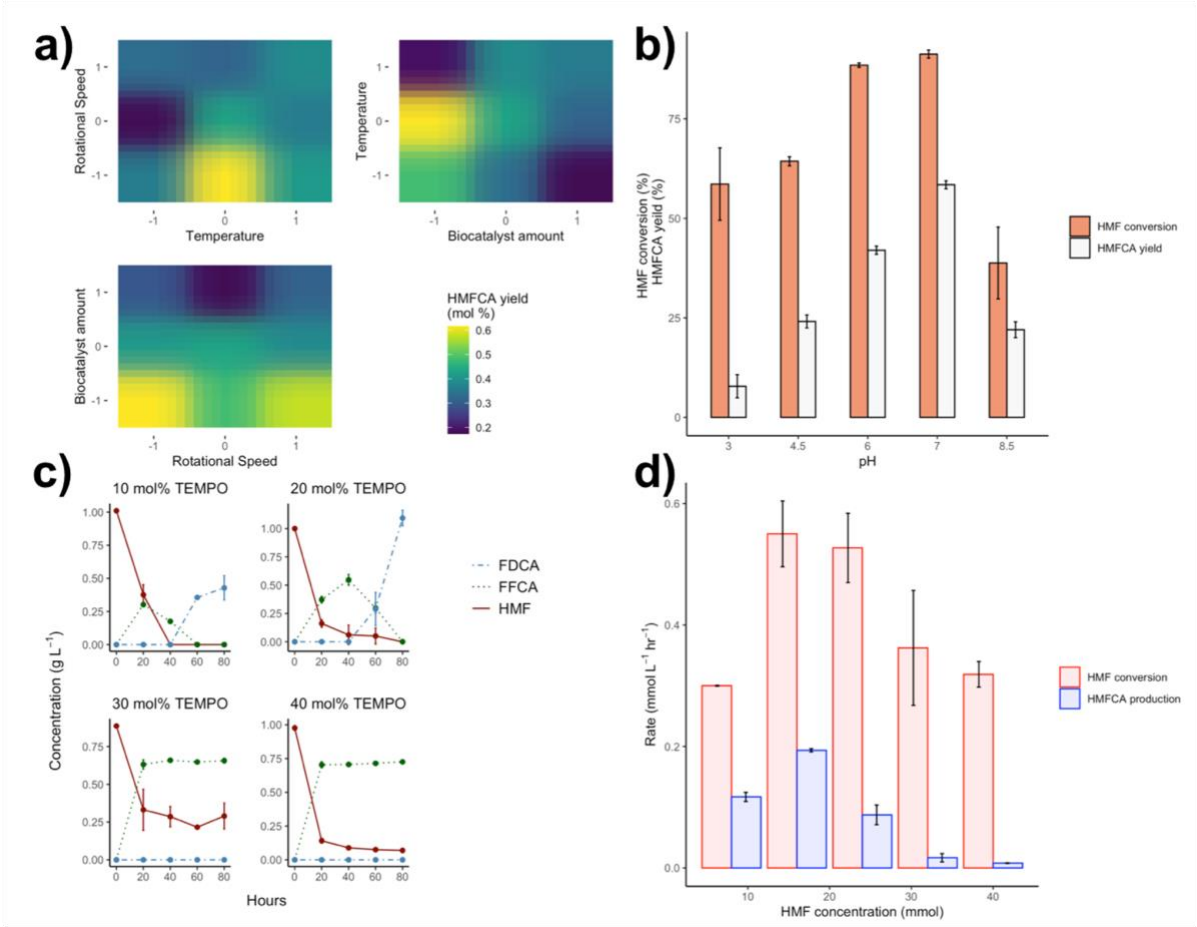


Figure 5.4. **a)** Contour plots showing the interaction of biomass and temperature (top left), temperature and rotational speed (top right), rotational speed and biomass (bottom); **b)** HMF conversion and HMFCA yield after 20 hours in 1 g/L HMF reaction solutions with pH ranging from 3-8.5; **c)** One-pot chemo-biocatalytic oxidation of HMF using *T. reesei* coupled with laccase/TEMPO. Shown are the rates of HMF conversion and product formation at varying mol% of TEMPO; **d)** Effect of increasing HMF starting concentration on conversion efficiency and HMFCA production.

Next, reaction solutions were tested which contained *T. reesei* combined with laccase and 10-40 mol% TEMPO under the previously optimized conditions. As may be seen in Figure 5.4c, solutions with 10-20 mol% TEMPO demonstrated complete conversion of HMF as well as the accumulation of both FFCA (after 20 hours) and FDCA (after 60 hours) with detection of little to no HMFCA. Higher TEMPO mol% (30 mol% and above) was associated with inhibition of the whole-cell catalysis which was evident in the accumulation of FFCA and the inability to convert 100% of the HMF. At the optimal TEMPO concentration (20 mol%), a titer of ~1.1 g/L FDCA

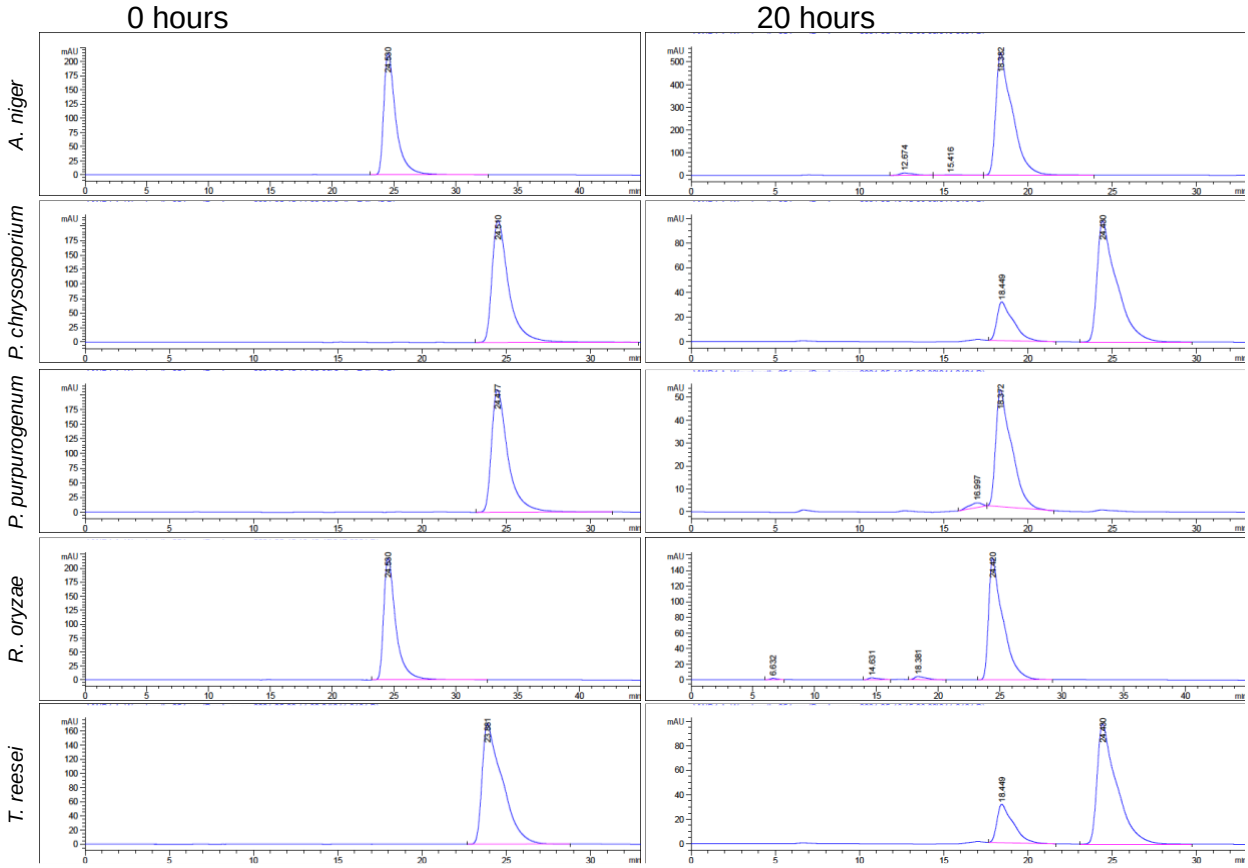
(88% yield) was reached after 80 hours. The rate of FDCA accumulation slowed considerably after this point, only reaching a yield of ~90% after an additional 20 hours (following which the reaction was stopped). This molar yield of FDCA (~90%) represents the highest reported to date for a reaction system involving filamentous fungi with the next highest reported in one study involving *A. flavus* (67% yield after 14 days).[294] Since the reaction in this mentioned study involved only *A. flavus* whole-cells, it may stand to reason that the addition of laccase/TEMPO could further enhance filamentous fungi which exhibit such intrinsic FDCA productivity.

With an FDCA space time yield from this one-pot chemo-biocatalytic system of ~0.01 g $L^{-1}h^{-1}$, the main focal point for future work with fungal whole-cell biocatalysts should be on improving productivity; the competitive range for biocatalytic reactions is considered at 0.001-0.3 kg L^{-1} h^{-1} (with 1-10 kg L^{-1} h^{-1} reported for heterogenous catalysts).[303] One way to achieve this involves increasing the substrate loading concentration for the system. Here, it was found that the HMF conversion efficiency and HMFCA productivity began to decline when substrate concentrations exceeded 16 mM (Figure 5.4d). Future work may focus on strain development for achieving higher substrate loading tolerances. Such work may benefit from recent advances in the genetic engineering of filamentous fungi.[304] Additionally, immobilization of enzymes, whole-cells, and certain chemical components (e.g. TEMPO) have also been proven effective methods for improving yields and productivity.[284a, 305] With all this taken together, there is great opportunity to develop a biocatalytic system involving filamentous fungi which thus incorporates the intrinsic benefits of fungi in a process that is economically viable and industrially applicable.

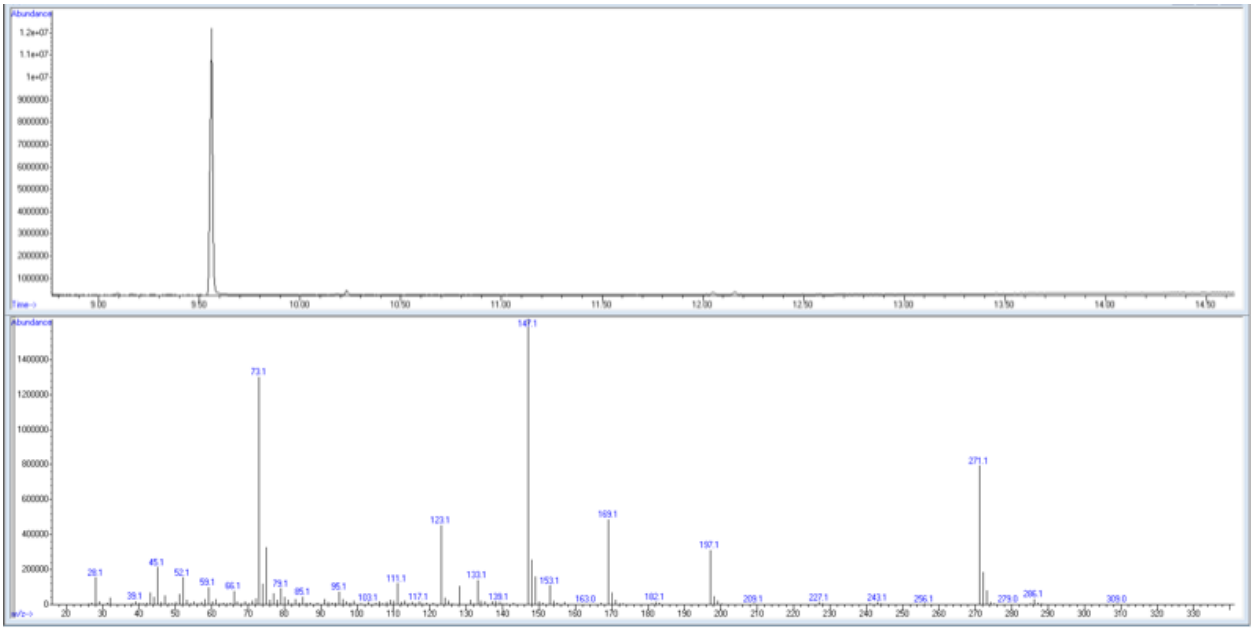
5.5. Conclusion

The results reported herein include (1) the identification of several filamentous fungal strains which demonstrate the ability to convert HMF into HMFCA as well as (2) the development of a method for bio-oxidative conversion of HMF into FDCA using filamentous fungal whole-cells coupled with laccase/TEMPO. The optimized one-pot chemo-biocatalytic cascade which comprised 1 g/L *T. reesei* whole cells coupled with 2.5 mM laccase and 20 mol% TEMPO achieved a molar yield of 88% after 80 hours. These results represent the highest molar and space-time yields reported to date for any system involving filamentous fungi.

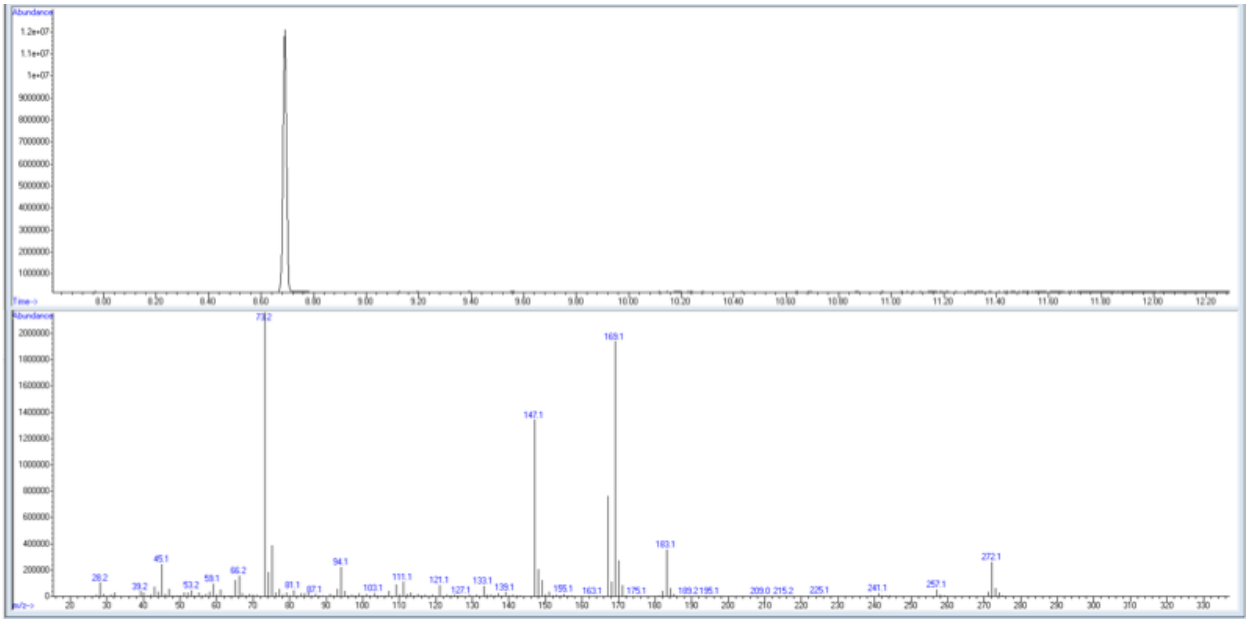
5.6. Appendix C. Supporting information for: Use of filamentous fungi as biocatalysts in the oxidation of 5-(hydroxymethyl)furfural (HMF)



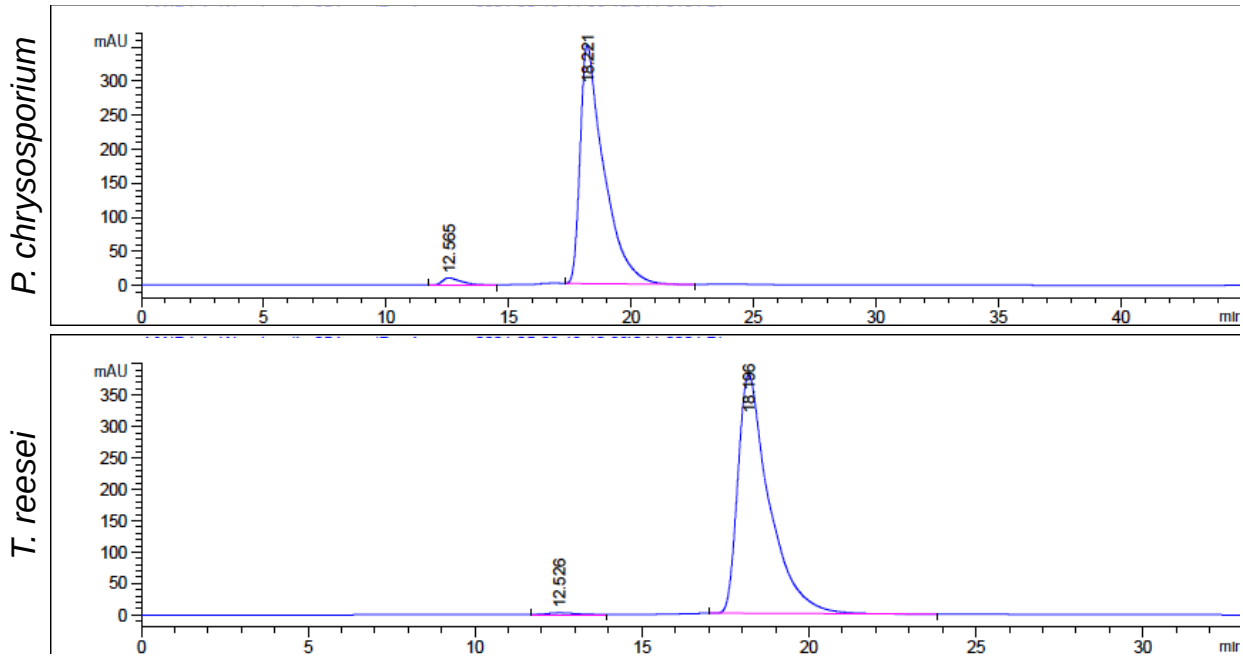
HPLC chromatograms at time zero (left) and after twenty hours of reaction (right) for the supernatant of 1 g/L HMF solutions with (in order from top to bottom) *A. niger*, *P. chrysosporium* , *P. purpurogenum* *R. oryzae*, and *T. reesei*. Note the different time scale when comparing chromatograms.



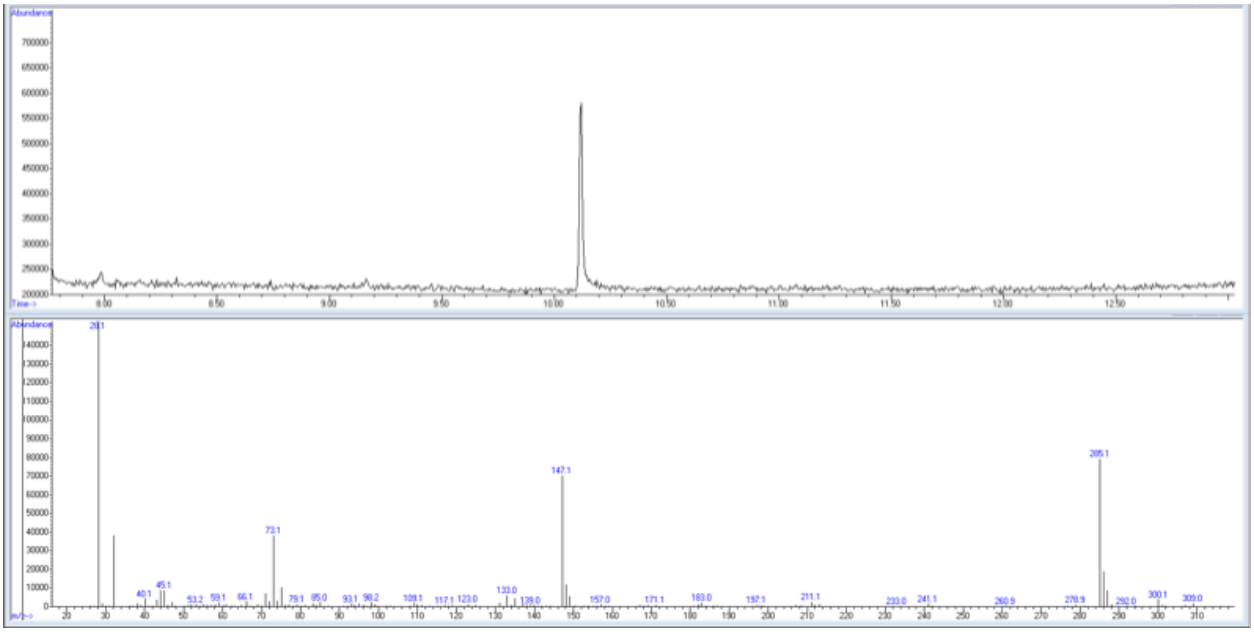
GC chromatogram and electron ionization mass spectrum of main peak from supernatant of 1 g/L HMF reaction solution with *T. reesei* whole-cells at 20-hour time point. Library cross-reference identified this peak as 2-Furancarboxylic acid, 5-[((trimethylsilyl)oxy)methyl]-,trimethyl ester (the derivatized form of 5-hydroxymethyl-2-furan carboxylic acid). Note: the same peak was detected as the main component in all five supernatants.



GC chromatogram and electron ionization mass spectrum of main peak from *P. purpurogenum* cell homogenate of 1 g/L HMF reaction solution. Library cross-reference identified this peak as 2-Furanacetic acid, alpha-[(trimethylsilyl)oxy]-,trimethyl ester (the derivatized form of Furan acetic acid)



HPLC chromatograms of the supernatant from 1 g/L HMF solutions of *P. chrysosporium (*top) and *T. reesei* (bottom) following 60 hours of reaction. The main peak (RT ~ 18.2 minutes) was identified to be HMFCA while the smaller peak (RT ~ 12.5 minutes) was identified to be FDCA. Note the different time scale when comparing chromatograms.



GC chromatogram and electron ionization mass spectrum of main peak from supernatant of 1 g/L HMF reaction solution with *T. reesei* whole-cells coupled with laccase/TEMPO. Library cross-reference identified this peak as 2,5-Furandicarboxylic acid, bis[trimethylsilyl] ester (the derivatized form of 2,5-furandicarboxylic acid).

5.7. References

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Connecting Statement 4

Chapter 5 described the ability of multiple filamentous fungal strains to perform biocatalytic oxidation reactions. One strain in particular, *Trichoderma reesei*, outperformed the other strains in reaction efficiency and other areas related to beneficial process characteristics (e.g. growth on pentose substrate, tolerance to TEMPO, etc.). One way to further enhance both the economics and sustainability of a potential process involving *T. reesei* as whole-cell biocatalysts is to the enable these biocatalysts to be recycled across multiple reaction cycles.

To that end, Chapter 6 examines the technique of encapsulation of *T. reesei* whole-cells in Ca-alginate beads in order to enable their reuse over multiple reaction cycles. This chapter is based on an article published in *Biomass and Bioenergy* with Dr. Valérie Orsat, Dr. Marie-Josée Dumont, and Felicity Meyer as co-authors of this paper.

Chapter 6. Recyclable immobilized *Trichoderma reesei* whole-cells for the catalytic oxidation of 5-hydroxymethylfurfural

6.1. Abstract

Filamentous fungal whole-cells have recently demonstrated utility in oxidizing aldehyde substrate into carboxylic acid products. In this study, immobilization of filamentous fungal-whole cells was explored in the oxidation of 5-hydroxymethylfurfural into 5-hydroxymethyl-2-furan carboxylic acid (HMFCA). To that end, resting filamentous fungal whole-cells (*Trichoderma reesei*) were encapsulated in Ca-alginate beads to enable recyclability. It was found that encapsulation enabled the biocatalysts to be reliably recycled for up to nine reaction cycles. After 9 cycles, >290 mg of HMFCA was acquired at a molar yield of ~60%. By the tenth cycle, very little product was formed due to degradation of the immobilization matrix. Analysis of the biocatalysts via scanning electron microscopy revealed the formation of large pores while analysis via Fourier-transform infrared spectroscopy-attenuated total reflectance demonstrated depolymerization of the alginate matrix.

6.2. Introduction

For the eco-friendly conversion of bio-based substrates into commodity chemicals, biocatalysis (i.e. use of enzymes and whole-cells) offers a number of benefits vis-à-vis thermochemical reactions.[234c] Primarily, these benefits relate to the mild reaction conditions (e.g. low temperature, neutral pH, etc.) employed in biocatalytic processes. Although, biocatalysts are not consumed as part of the reaction or incorporated in the final product, they may be difficult to recover following a reaction and often end up as waste at the end of a reaction. Immobilization of biocatalysts enables easy recovery in addition to myriad other benefits.[306]

5-hydroxymethylfurfural (HMF) represents one of the premier bio-based chemicals due to its capacity to serve as a platform for the production of a variety of fuels and chemicals.[231, 307] Ample reviews cover the conversion of biomass to HMF[231, 308] as well as the catalytic upgrading of HMF including both well-established practices[309] and more recent innovations.[310]

Among the numerous possible derivatives of HMF, the oxidation product 5-hydroxymethyl-2-furan carboxylic acid (HMFCA) may serve in the production of polyesters as well as potentially in human health (e.g. it has been reported to show antitumor activity and to act as an interleukin inhibitor).[311] Numerous bacterial whole-cell-based biocatalytic systems (e.g. *Escherichia coli*, *Glucanobacter oxydans*, and *Comamonas testosteroni*) have been reported to oxidize HMF to HMFCA with high yields (90-98%) and excellent productivities (as high as 10 g L^{-1} hr^{-1} in the initial 6 hours of one system involving *G. oxydans* DSM 50049).[311-312] Additionally, the utility of filamentous fungi in oxidative biocatalysis of HMF into HMFCA has been recently demonstrated.[313] Filamentous fungi are otherwise underexplored in such applications but may offer potential long-term benefits to process sustainability and economics due to their robustness and their ability to accept a broad scope of inexpensive and sustainable carbon sources.[289, 314] Building on previous work, immobilization of filamentous fungi was explored in order to enable recyclability of the whole-cell biocatalysts and thereby enhance process sustainability.

Immobilized filamentous fungi have long been employed for a number of applications including production of organic acids and enzymes, as well in detoxification.[315] The vast majority of procedures involve suspension of fungal spores in the immobilization matrix followed by incubation in production media where growth and product formation occur simultaneously. This methodology has shown positive results but is not ideal for reactions which require resting cells or in reactions involving non-sporulating fungi. There is one study which demonstrated the use of mycelial fragments first grown on solid media and then macerated and encapsulated before employment in a biocatalytic reaction but, to the best of our knowledge, no studies exist which employ resting fungal cells grown first in agitated liquid culture.[316] Filamentous fungi grown in such conditions have distinct morphology from those grown in solid-state.[317] Here, the filamentous fungus *Trichoderma reesei* was grown in agitated liquid culture, immobilized in calcium alginate beads, and then evaluated for use as a bio-oxidative catalyst in the conversion of HMF into HMFCA.

6.3. Materials and Methods

6.3.1. Materials and Reagents

The following reagents were acquired from SigmaAldrich: HMF, HMFCA, and sodium alginate from brown algae.

6.3.2. Organism cultivation

Trichoderma reesei (NRRL 3652) was acquired from the USDA and cultivated on potato dextrose agar (PDA) for 5 days at 30 °C. Sterile water was used to extract spores from each culture. The concentration of spores was adjusted to $1x10^6$ spores/mL 1 mL of spore solution was used to inoculate 100 mL potato dextrose broth (PDB) cultures. The PDB cultures were incubated for 72 hours at 30 °C and 200 rpm.

6.3.3. Immobilized Biocatalyst

Following the 3-day incubation, the PDB cultures were centrifuged at 3000 × g for 30 minutes. The supernatant was decanted, and the process was repeated. Following the second centrifugation/supernatant decantation, the remaining biomass pellet was used for immobilization.

To prepare the immobilized biocatalyst, 1 g sodium alginate and a specified amount of biomass from liquid culture (2 or 6 g cell wet mass (gwm)) were mixed together in 25 mL of sterile water. The viscous substance was then transferred to a 10 mL syringe, from which it was slowly expelled drop-wise into a 0.1 M solution of $CaCl_2$. The beads were allowed to harden for at least ten minutes before multiple rounds of washing with sterile water. These beads were then used as the immobilized biocatalyst in all following reactions.

6.3.4. Oxidation of HMF

In general, a specified amount of immobilized biocatalyst (0.15 or 0.4 g biocatalyst/mL reaction solution) was used to inoculate 50 mL of mineral salt media (MSM) spiked with 1 g/L HMF. See supporting information (SI) for composition of the MSM. The reactions were performed in at least duplicate. The solutions were incubated at 30 °C and 200 rpm. The aforementioned reaction conditions as well as pH and reaction time were previously optimized and reported elsewhere.[313] ~1 mL of supernatant was removed at regular intervals, filtered (0.2 μm pore size), and analyzed using HPLC. See SI for specific equipment, operating, and analytical parameters. The values were expressed as the means of the analyte concentration (calculated using standard curves) ± standard deviations.

The immobilized biocatalyst recipe was crudely optimized for fungal cell concentration within the immobilization matrix, as well as for the quantity of immobilized biocatalyst used per reaction. This was performed following a two-level full factorial design comprising 4 experiments. T. reesei cell concentration was tested at 0.08 or 0.25 gwm/mL immobilization matrix. The concentration of immobilized matrix was tested at 0.15 or 0.4 g/mL reaction solution.

6.3.5. Biocatalyst structural analysis

The surface properties of the immobilized biocatalysts were analyzed using Fourier-transform infrared spectroscopy-attenuated total reflectance (FTIR-ATR; Thermo Scientific) and a scanning electron microscope (SEM; Hitachi TM3000). For both, samples were first freeze dried (Labconco) and then either directly analyzed (for SEM) or ground to a fine powder prior to analysis (for FTIR-ATR). FTIR spectra were analyzed using OMNIC v8.3 software.

6.4. Results and Discussion

Encapsulation within alginate-based matrices is among the most common methods for immobilizing whole-cells.[318] In this study, filamentous fungal whole-cells were immobilized *in situ* by mixing fungal biomass in a solution of alginic acid and then extruding this mixture into a

solution containing a divalent cation crosslinker (i.e. Ca^{2+}). This resulted in a dispersion containing macro-sized beads roughly 1-2 mm in diameter. The beads (i.e. the immobilized biocatalysts) were then transferred to a reaction solution and incubated for 20 hours. Two reaction conditions, the concentration of *T. reesei* cells in the immobilization matrix and the quantity of immobilized biocatalyst beads per reaction, were optimized. As may be seen in Figure 6.1a, all immobilization conditions, with the exception of the low cell concentration and low bead quantity, were associated with relatively high levels of product accumulation (0.32-0.36 g/L). However, these levels of product accumulation represent a decrease of ~20-30% as compared with reports of reactions (non-optimized) involving free *T. reesei* cells.[313] The diminished product accumulation may be due to hampered diffusion of substrate, oxygen, products, and inhibitory by-products. Exploring different sources of alginate may be one strategy to address some of these issues since different organisms and/or tissues are associated with different mannuronic (M) and guluronic (G) acid contents which are directly related to diffusivity through the polymer (i.e. higher G content is associated with higher polymer rigidity and lower diffusivity).[319]

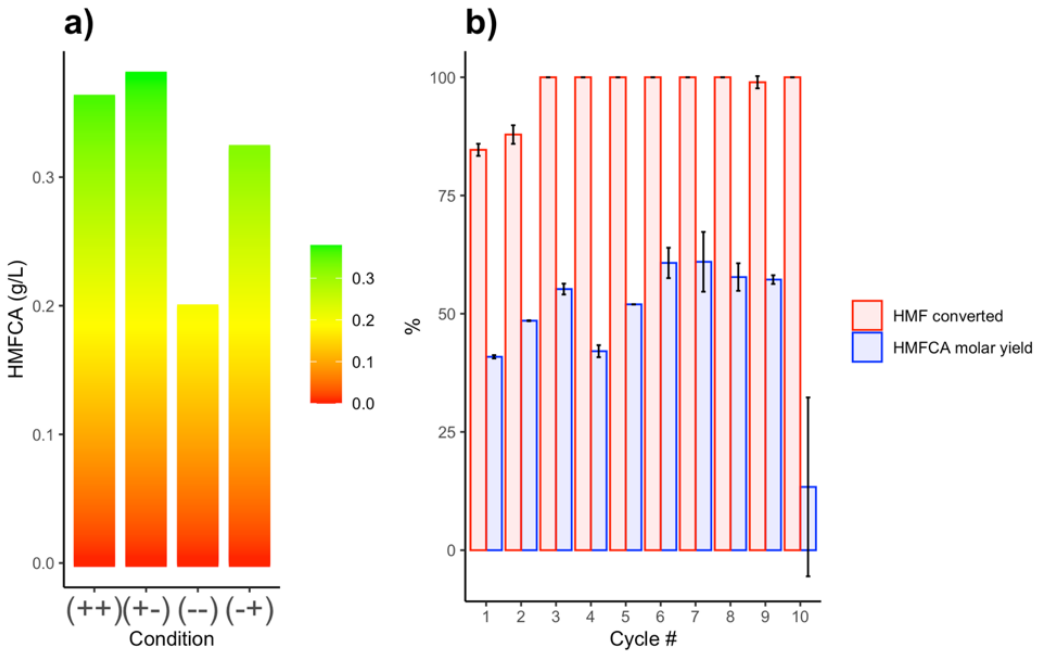

Figure 6.1. **a)** HFCA accumulation after 20 hours in solution containing 1 g/L HMF and immobilized *T. reesei* whole-cells at high bead with high cell concentration (++), high bead with low cell concentration

(+-), low bead with low cell concentration (--), and low bead with high cell concentration (-+); **b)** Results of the HMF conversion using the immobilized *T. reesei* whole-cells across 10 reuses. The red bars, or the left bar in each pair, display the molar percent of HMF converted in each 20-hour cycle while the blue bars, or the bars on the right in each pair, displays the molar percent yield of HMFCA after each 20-hour cycle.

Next, the recyclability of the immobilized catalysts was tested. Here, the biocatalysts were again incubated in reaction solution for 20 hours. After 20 hours, the biocatalysts were separated from the reaction solution and added to a fresh reaction solution for another 20-hour cycle. This was repeated for ten cycles. It was found that replacement in a fresh reaction solution was associated with equal or greater biocatalytic activity with respect to the first cycle (see Figure 6.1b). This supports the premise that product accumulation is hampered due to the resulting drop in pH and by-product formation (e.g. H_2O_2). Interestingly, substrate conversion was observed to improve with subsequent catalyst reuse and product accumulation was observed to reach maximum levels at later cycles. This may be attributed to the degradation of the gel matrix and thus the increasing interaction between the cells and the substrate. The degradation of the matrix appeared to reach a critical point after 9 cycles as evidenced in the sharp decrease in product formation in the tenth cycle. Moreover, product formation in the tenth cycle showed high variability across replicates. It appears that between the ninth and tenth cycles, the mass transfer benefits of the more permeable matrix are outweighed by the concomitant loss of cellular material from the matrix (thus preventing recycling). After 9 cycles, >290 mg of HMFCA was acquired (an overall molar yield of ~60%). The modest HMFCA yield vis-a-vis the use of metal nanoparticles [320] or certain bacterial whole-cell biocatalysts [311-312] (all exceeding 90%) is offset by the recyclability of the biocatalysts which leads to good product turnover. Moreover, when considered alongside the advantages associated with the use of filamentous fungi (e.g. robustness, inexpensive growth requirements, etc.), the performance reported herein supports the candidacy of filamentous fungal whole-cell biocatalysts (particularly *T. reesei*) for further development through, for example, metabolic engineering and/or novel process engineering strategies.

The surface of the catalyst was investigated using SEM and FTIR. From the SEM images displayed in Figure 6.2, the formation of pores ranging from 100-500 μm in diameter may be seen after the alginate beads were reused for 10 cycles. In comparing the FTIR spectra

of unused and 10x recycled beads (see Figure 6.3), many of the signature peaks of alginate (see Figure S1; Appendix D) were retained with a few key changes. The formation of a medium shoulder in the 10x recycled beads at 1716 cm^{-1} may be associated with C=O stretching of free carboxyl groups accumulating due to gel disintegration.[321] A new peak at 1244 cm^{-1} of the 10x beads is likely due to the C-O bond of alcohol moieties. Both samples displayed a broad peak associated with -O-H stretching at ~3300-3000 cm^{-1} which can be partially attributed to bound water in the system. However, a slight broadening of this peak likely indicates additional -O-H stretching of the available alcohol groups. While many of the spectral bands were retained, it should be noted that slight peak shifts were observed, which could be attributed to an increase in interactions with ions present in the solution as the polymer breaks down and chain stiffness is relaxed, allowing the M and G residues to interact more freely with their ionic environment.

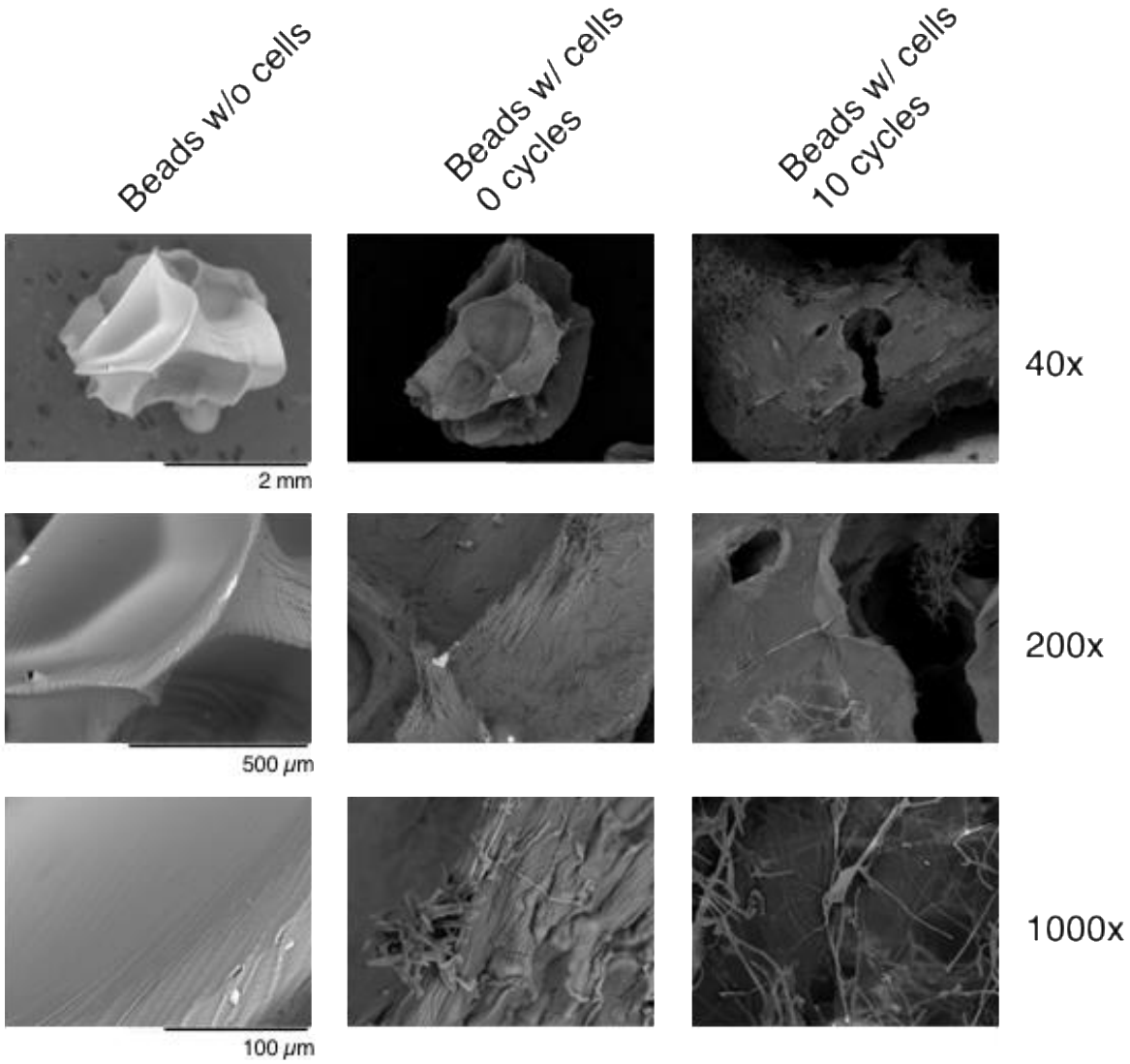

Figure 6.2. SEM images at increasing magnification of the surfaces of calcium alginate beads without any cells (first column) and the immobilized biocatalysts prior to any reactions and after 10 reuses (second and third column respectively).

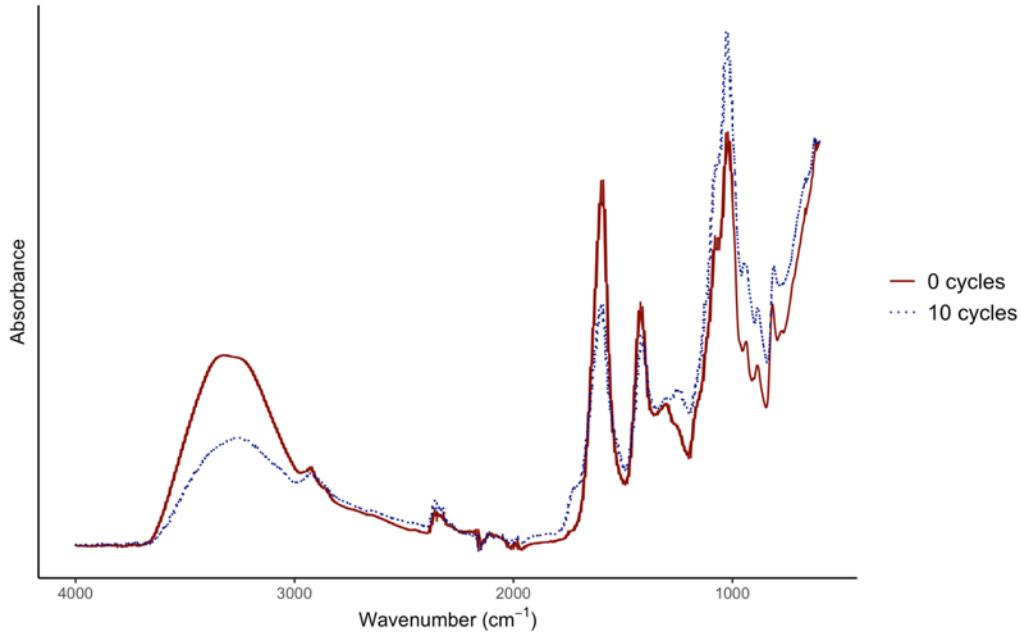

Figure 6.3. FTIR profile of the immobilized biocatalysts prior to any reactions and after 10 reuses (red solid line and blue dashed line respectively).

It appeared that alginate chain breakdown occurred slowly throughout cycling and reached a critical point of reduced polymeric integrity during the 10th cycle, causing the encapsulation system to break down. Solubilization of the high molecular weight alginate polymers and leakage of the entrapped material is caused by degradation of the Ca^{2+} cross-linked matrix.[322] This may occur by removal of the Ca^{2+} ions by chelating agents including organic acids, by a high concentration of other ions, proton catalyzed hydrolysis, and/or from enzymatic stress.[322] The nature of the biocatalytic system used in this study is such that each of these factors likely contributed to the degradation of the alginate beads.

6.5. Conclusions

Encapsulation of filamentous fungal whole-cells in calcium alginate beads was shown to enable recycling of the biocatalyst with minimal loss of activity for up to 9 cycles. After 9 cycles, >290 mg of HMFCA was acquired (corresponding to a molar yield of ~60%). Future work should focus on fungal strain evolution for enhanced substrate and by-product tolerance as

well as optimization of the composition of the immobilization matrix and its durability and functionality.

6.6. Appendix D. Supporting information for: Recyclable immobilized *Trichoderma reesei* whole-cells for the catalytic oxidation of 5-hydroxymethylfurfural

Composition of the mineral salt media employed in the biocatalytic reactions

1 g/L HMF, 0.2 g/L $MgSO_4 \bullet 7H_2O$, 0.02 g/L $CaCl_2 \bullet 2H_2O$, 0.5 g/L K_2HPO_4, 0.5g/L KH_2PO_4, 0.5 NH_4Cl, and 10 mL/L trace element solution (300 mg/L $FeSO_4 \bullet 7H_2O$, 50 mg/L $MnSO_4 \bullet H_2O$, 106 mg/L $CoCl_2 \bullet 6H_2O$, 34 mg/L $Na_2MoO_4 \cdot 2H_2O$, 40 mg/L $ZnSO_4 \bullet 7H_2O$, 50 mg/L $CuSO_4 \bullet 5H_2O$)

Parameters employed in high-performance liquid chromatography

All HPLC analysis was performed on an Agilent 1260. Components were analyzed using an Agilent ZORBAX Eclipse plus C18, 3.5 μm, 4.6 × 100 mm column with Agilent ZORBAX Reliance Cartridge guard column. The specific LC parameters were as follows: 6 μL injection volume, 0.8 mL/min flow rate, 274 nm detection wavelength, an isocratic elution of 100% HPLC grade water as mobile phase for 5 minutes plus 1-minute post-run. All HPLC data analysis was performed using Agilent technologies OpenLAB CDS ChemStation software. Analyte concentrations were calculated based on standard curves using pure reagents.

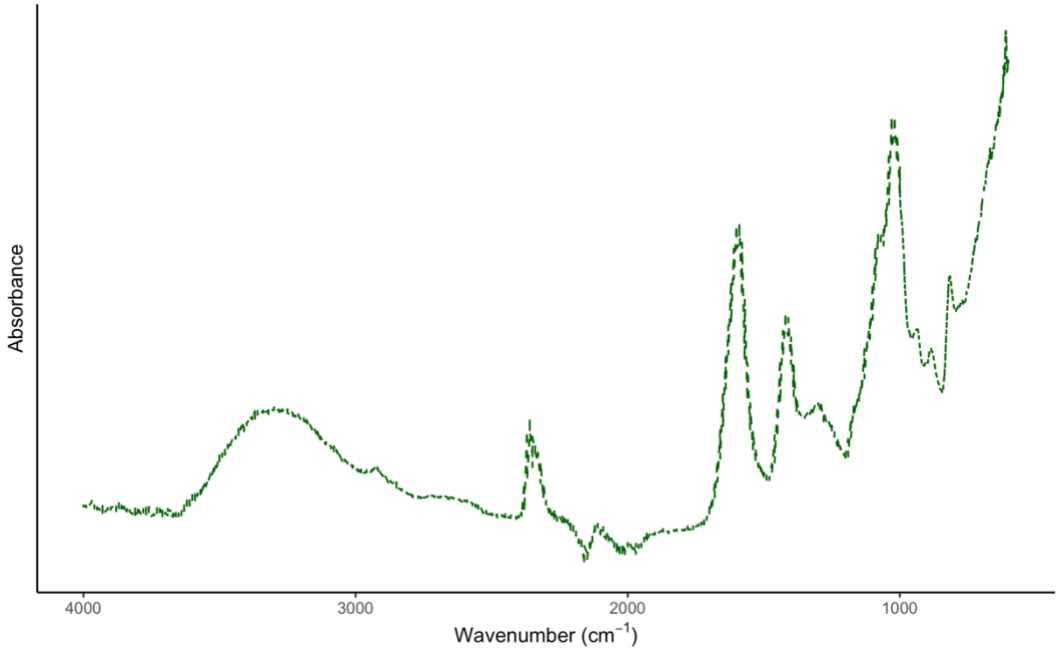

Figure S1. FTIR spectra for Ca-alginate beads without cells.

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Chapter 7: General Conclusions

7.1. General conclusions and summary

This thesis examined the application of filamentous fungi in the upgrading of biomass and biomass-derived feedstocks. Filamentous fungi possess numerous advantages vis-à-vis more commonly employed biomass processes (i.e. thermochemical and/or non-filamentous fungal bio-based conversions) and, as such, deserve continued exploration for use in biomass-based technologies. Specifically, filamentous fungi are known to degrade myriad substrates and produce myriad valuable chemicals. Here, it was shown that a specific strain of *Penicillium*/*Talaromyces* was able to degrade, albeit at different efficiencies, different biomass residues with widely varying compositions and, as an outcome of the degradation, to excrete chemicals with potential industrial application (Chapter 3). Two products identified from this process were investigated further. First, the production of biopigments from simulated food waste was enhanced (Chapter 4). This was accomplished by employing a novel strategy involving the co-culturing of multiple strains of fungi together in one fermentation reaction. This ultimately elicited a near doubling of pigment production from the primary producing fungal strain.

The second chemical, which was first identified in the results described in Chapter 3 and then investigated further, was FDCA. To that end, in Chapter 5, filamentous fungi were screened for their ability to oxidize the biomass-derived compound HMF (the primary substrate for production of FDCA). Several strains demonstrated the ability to oxidize HMF into HMF acid and no further. However, incorporation of an enzyme/mediator system with the whole-cells of *Trichoderma reesei* enabled the full oxidation pathway (i.e. HMF to FDCA). Following this, the whole-cells of *T. reesei* were encapsulated in Ca-alginate beads in order to enable recyclability (Chapter 6). This technique was found to enable reuse of the whole-cell biocatalysts for up to nine reaction cycles.

7.2. Contributions to knowledge

Filamentous fungi are expected to play an important role in the circular bio-economy due to their natural role as material recyclers. This work has helped to advance the realization of such applications by making the folowing contributions to knowledge:

1. A methodology was established for comprehensively screening and evaluating the products of filamentous fungal solid-state fermentation of waste biomass residues.
2. A novel strategy involving the co-culturing of two filamentous fungal strains was described wherein significant enhancement of biopigment production may be achieved.
3. Several strains of filamentous fungi were identified for the first time as having the ability to oxidize HMF into HMFCA.
4. A novel chemo-biocatalytic cascade was described to achieve complete oxidation of HMF into FDCA.
5. Encapsulation of filamentous fungal whole-cells, investigated here for the first time using filamentous fungal mycelia grown in liquid culture, was shown to enable the reuse of the biocatalysts for up to nine reaction cycles.

7.3. Recommendations for future work

Based on the work performed in this thesis, certain avenues for further advances in this line of research are proposed:

1. The primary product of the methodology described in Chapter 3 was a large data set containing identities of all products of the bio-conversion of various residues. While statistical methods were useful in removing insignificant compounds from the data set, it was still necessary to perform identification of the products of industrial or commercial interest manually. This approach is inefficient and hampers expanding the scope of such methodologies so as to include multiple organisms, process variables, etc. An efficient technique, likely sourced from bioinformatics, should be developed for

quickly distinguishing products of interest from those which have no industrial or commercial importance.

2. The co-culturing of multiple strains of filamentous fungi was revealed to be an effective method for eliciting higher productivity in pigment-producing fungi (Chapter 4). If these fungal-derived biopigments are to be used as natural colorants in food and textiles, their safety must be confirmed and, to that end, should be tested for toxicity toward mammalian cells.
3. The results described in Chapter 5 suggest that the ability to oxidize HMF may be relatively common among filamentous fungi. Future directions from here should be two-fold. First, more strains of fungi should be screened for oxidative activity on HMF. Second, but concurrently with the first strategy, a wider variety of industrially important aldehyde substrates (e.g. benzaldehyde) should be investigated using selected filamentous fungal whole-cell biocatalysts (e.g. *T. reesei* and *A. niger*).
4. Encapsulation of *T. reesei* in Ca-alginate beads, as described in Chapter 6, enabled recyclability of the whole-cell biocatalysts for up to nine HMF oxidation reactions. As in (4), a wider variety of filamentous fungal strains as well as aldehyde substrates should be tested in encapsulated fungal whole-cell reaction systems. Additionally, the ecapsulated biocatalysts should be employed in the chemo-biocatalytic cascade described in Chapter 5. This may, for example, provide improved tolerance toward TEMPO which exhibited an inhibitory effect on the free whole-cell biocatalysts (especially *A. niger*).

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