Efficient production of platform [sic] organic acids from ligocellulosic and algal biomass carbohydrates

Shao Heng
University of Toledo

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A Dissertation

entitled

Efficient Production of Plat-form Organic Acids from Ligocellulosic and Algal Biomass Carbohydrates

by

Heng Shao

Submitted to the Graduate Faculty as partial fulfillment of the requirements for the

Doctor of Philosophy Degree in Biomedical Engineering

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May 2015
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Lignocellulosic biomass, such as agricultural and forest residues, is an inexpensive feedstock for bio-based products. Cost-effective production of bio-based products from lignocellulosic biomass requires simple conversion steps to break down carbohydrates to component mono-saccharides, and fermentation and/or chemical conversion of the sugars to final products.

Lactic acid is one potential value-added product that could be produced economically from lignocellulosic biomass, if both the hexose and pentose sugars - derived from the cellulose and hemicellulose fractions, respectively - can be utilized completely with high efficiency. However, most natural lactic acid bacteria (LAB) cannot utilize xylose efficiently: the isomerization of xylose to xylulose in the phosphoketolase (PK) pathway constitutes a bottleneck step. Fortunately, it is possible to overcome this bottleneck via exogenous isomerization of xylose, thus allowing the microorganism to utilize xylulose as a viable alternative substrate for xylose. In this study, it has been demonstrated that this new approach could significantly improve the lactic acid yield. *Lactobacillus pentosus* and *Lactobacillus casei* (subspecies *rhamnosus*) were used in the
fermentation of hexose, xylose, and xylulose to lactic acid. With *L. pentosus*, no preferential utilization of xylulose over xylose was seen, when both sugars were present in the medium. Sodium tetraborate and isomerization buffers, added to the fermentation broth to promote exogenous isomerization of xylose, strongly inhibited the growth of *L. pentosus*, which, in turn, led to poor utilization of xylulose. In contrast, with *L. casei* more robust growth and superior lactic acid yield were achieved from both glucose and xylulose, following exogenous isomerization with negligible xylose left at the end of fermentation. These results confirmed that, unlike *L. pentosus*, the exogenous isomerization additives do not inhibit *L. casei* and it is possible to maximize the utilization of both C6 and C5 sugars for lactic acid production by *L. casei* via the approach proposed in this study.

In addition to lactic acid, succinic acid is a very important intermediary chemical building block that could constitute a viable alternative for petroleum-based bulk chemical precursors. Bio-based succinic acid produced from lignocellulosic biomass via microbial fermentation of the carbohydrate-derived sugars has the potential to reduce the cost of the product. However, inhibitors generated during the pretreatment and saccharification of biomass, especially lignin-derived phenolic compounds, could adversely affect the growth of succinic acid-producing microbes. *Actinobacillus succinogenes* - a promising strain that could be utilized for commercial succinic acid production - is strongly inhibited by the toxic compounds generated during pretreatment.

A number of detoxification methods of the biomass hydrolyzate have been developed to alleviate the impact of these inhibitors on the downstream fermentation step. Traditional detoxification methods such as adsorption of inhibitory compounds on
activated charcoal or ion-exchange resins could lead to significant sugar loss. In addition, the high cost of these adsorbents and the complicated methods of their regeneration limit their application on an industrial scale. Developing a cost-effective method for removal of the fermentation inhibitors from the hydrolyzate is vital for successful large-scale bio-succinic acid production. The findings of this study indicate that liquid-liquid reactive extraction (LLRE) with trioctylphosphine oxide (TOPO) and trioctylamine (TOA) as the reactive-extraction agents, under low pH conditions (pH<4), could remove most of the acidic inhibitors (for example, phenolic compounds, acetic acid and levulinic acid) and furans efficiently from the hydrolyzate, without sugar loss. Following extraction of the inhibitors, the organic extraction phase could be regenerated by contacting the medium with a base-solution into which the inhibitors are stripped. We compared the detoxification effectiveness of solid phase adsorbents vis-à-vis LLRE by evaluating the succinic acid production by fermentation, following “conditioning of the hydrolyzate” by both the methods. Succinic acid yields and productivities seen in fermentations with the hydrolyzate conditioned using LLRE with TOPO or TOA were nearly the same as those with the control media (i.e., model sugar solutions without the inhibitory compounds).

Another unique feature of LLRE is that, unlike solid phase adsorption, it can be implemented for conditioning the whole (heterogeneous slurry of) “pretreated biomass”, prior to depolymerization of polysaccharides by enzymatic hydrolysis. Performing “conditioning” ahead of saccharification would not only improve succinic acid production during fermentation, but also could improve the sugar yields during enzymatic hydrolysis by mitigating any inhibitory effect of the pretreatment-derived toxins on the enzymes.
In addition to lignocellulose, carbohydrate-rich microalgae are also an extremely promising feedstock for bio-succinic acid production. In general, carbohydrates (polysaccharides), proteins, pigments and other valuable nutrients present in microalgae are ideal sources that can sustain and promote microbial fermentation processes targeted towards the production of biofuels and organic acids. Moreover, microalgae can be cultivated in non-arable land and/or in low quality water. In addition, the lignin-free structure of microalgae could potentially allow for the application of milder pretreatment during its deconstruction, resulting in lower fermentation inhibitor generation.

In this study, some inexpensive commercially available enzymes were used to digest the chemical bonds between the glycoprotein in the cell wall and the polysaccharides of microalgae, which enabled the release of intracellular lipids and sugars. After removing the lipids using solvent extraction, the residue of the microalgae and the sugars remaining in the solution were successfully used as carbon and nitrogen sources for A. succinogenes fermentation for producing succinic acid. As such, the new process for fractionating microalgae developed in this study could significantly reduce the production costs of lipids and other bio-based products, because it allows the maximum utilization of every component in the micro-algal biomass.
Acknowledgements

It was an unforgettable experience to me during my journey as a Ph.D. student at UT. Now I look back and the past six years I spent here has become an exciting memory that I will definitely recall for the rest of my life.

My special thanks go to my wife and my parents, who have given me their love and bless continuously. It is their nurturing and support that laid the foundation of my accomplishments today.

I would like to express my gratitude to my advisors, Drs. Patricia Relue, Sasidhar Varanasi and Sridhar Viamajala, and also my committee members, Drs. Stephen Callaway and Randall Ruch, for giving me the opportunity to work on the project of biofuels and chemicals from lignocellulosic biomass. As my mentors, their ideas and advices always opened up my minds. It is there guidance and support that made my research smooth and accomplished.

Last but not least, our lab coordinator, Tammy Phares and my colleague Dawei Yuan, Bin Li, Peng Zhang, Kelly Marbough and Agasteswar Vadlamani, I want to thank them all for their help and support.

My research was supported by Department of Energy, Office of Biomass Program (GO-18163); Ohio Third Frontier Advanced Energy Program (AEP08-044); Suganit Systems, Inc.; and University of Toledo.
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Chapter 1

Overview

1.1 Background

Rising petrochemical prices and an increased interest in reducing carbon dioxide (CO₂) emissions have prompted the development of biofuels and the production of bio-based chemicals. Countries around the world are using various types of biofuels for energy sources as alternatives to fossil fuels. Although biofuels based on corn and similar crops have been commercialized, their production has been blamed for the global increase in food prices over the last decade [1]. The new generation of biofuels derives from agricultural waste or algal biomass. These new biomass crops can be cultivated on non-arable land or in low quality water (e.g. industrial wastewater), which do not compete with the space used for producing food resources, therefore solving the problem of using farmland to grow fuel instead of food. In recent years, efforts have also been directed towards producing biofuels from “genetically engineered” plants or biomass, as genetic manipulation has the potential to significantly enhance the productivity and selectivity of native strains to meet the large-scale production needs of targeted molecules. For example, Synthetic Genomics (SGI) and Exxon Mobile have initiated major
collaboration efforts to develop superior strains of algae with genomic technology for cost effective biofuel production [2].

Table 1.1 illustrates the global market for a few important chemicals that are traditionally derived from petroleum and for which bio-based production routes have been developed. Biomass-based production routes could significantly reduce the cost of these products and improve environmental sustainability. The selling price of bio-based products is generally higher than biofuels (see Table 1-1), and a bio-refinery in which fuels and value-added products are produced together is likely to have better economic viability. Bio-lactic and succinic acids are two organic acids that have been recognized as platform molecules suitable for the production of important materials and value-added compounds.

Table 1.1 Bio-based product market in 2012

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<th>Butanol</th>
<th>Lactic acid</th>
<th>Succinic acid</th>
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<td>Price ($/ton)</td>
<td>455</td>
<td>1230</td>
<td>1300-1600</td>
<td>2000-3000</td>
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<tr>
<td>Global Market</td>
<td>61,000,000</td>
<td>3,000,000</td>
<td>300,000</td>
<td>30,000-50,000</td>
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Lactic acid is traditionally used in food industry as a food preservative and as a curing or flavoring agent. It is also extensively used in cosmetic, textile and pharmaceutical industries [7]. Currently, demand for lactic acid as a feedstock for the production of the biopolymer poly-lactic acid (PLA) is also rapidly increasing. PLA is an innovative biodegradable plastic with various applications, especially in the medical field for manufacturing products such as surgical sutures, orthopedic implants and drug delivery systems. It also finds application in disposable consumer products. The
biodegradability of PLA could significantly alleviate the disposal problems associated with conventional plastics.

Lactic acid can be produced either by chemical synthesis or by microbial fermentation. Lactic acid displays optical isomerism (L-lactic acid and R-lactic acid), with optically pure isomers being more desirable as a platform molecules. Chemical synthesis from petrochemical sources leads to a racemic mixture, whereas microbial fermentation produces L-lactic acid [8]. Microbial lactic acid fermentation (bio-lactic acid) offers a number of additional advantages over chemical synthesis. First, bio-based lactic acid can be produced from renewable biomass, especially the low-cost lignocellulosic biomass. Second, fermentations can be carried out at mild operating conditions (low temperatures and atmospheric pressure) requiring less energy consumption. In addition, lactic acid produced via microbial fermentation can generate optically pure lactic acid, if an appropriate strain is selected [9]. The pure isomers, L-lactic acid or D-lactic acid have more value than the mixtures since each isomer has its unique physical properties with specific commercial applications [10]. Currently, almost all lactic acid produced globally is manufactured via microbial fermentation, using glucose from starch as the carbon source. Of late, the focus has, however, shifted to using the sugars derived from the lignocellulosic feedstocks (C6 and C5 sugars), instead of starch-derived glucose, in light of the plentiful availability of lignocellulosic biomass and the improved process economics afforded by the efficient utilization of lignocelluloses [10].

Another important chemical compound, succinic acid, topped the list in a report identifying 12 chemicals that could be produced from sugars through microbial
fermentation, released by U.S. Department of Energy (DOE) in 2004 [11]. Succinic acid is a four-carbon molecule that could serve as a platform chemical for the production of various high-value derivatives, including 1,4-butanediol (1,4-BDO), ethylene diamine disuccinate, tetrahydrofuran (THF) and adipic acid [12] (Figure 1-1). With various new applications, including use in biodegradable polymers, in food additives as an acidic regulator, and in the pharmaceutical industry as a synthetic precursor, the demand for succinic acid is expected to increase considerably. Current global succinic acid production is 30,000 to 50,000 tons per year with prices ranging from $2000 to 3000 per ton, and the market is expected to reach 100,000 tons per year by 2015 [13].

In the past, succinic acid was produced using chemical processes, mainly paraffin oxidation, catalytic hydrogenation, and the electroreduction of maleic acid or maleic anhydride [14]. However, these processes are complicated, expensive, and may have negative environmental implications. As a result, there is a growing interest in discovering more economical and environmentally friendly production techniques, and bacterial fermentation of biomass-derived carbohydrates to succinic acid is one method that is receiving increased attention [15]. In bio-based succinic acid production, use of inexpensive feedstocks and optimization of the pretreatment process of these feedstocks are key issues to be addressed in order to reduce the production costs.
Figure 1-1 various products that are derived from the platform molecule: succinic acid [11]

1.1.1 Biomass structure

Lignocellulosic as well as micro-algal biomass are abundant carbon sources for the production of biofuels or bioorganic acids. Lignocellulosic biomass has a complex structure composed of cellulose (40–50%), hemicellulose (25–35%), and lignin (15–20%) [16]; the relative proportion of these major components varies based on both the plant species and the plant tissue utilized. Cellulose and hemicellulose are two major polysaccharides that can be hydrolyzed to monosaccharaides, which can be fermented into various bio-based products. Cellulose is made up of linear glucose subunits linked by β-(1–4)-glycosidic bonds. The cellulose chains usually are aligned in the form of highly crystalline bundles due to extensive hydrogen bonding among individual chains [17]. Hemicellulose is a heteropolymer with highly-branched shorter chains that contains hexoses (D-glucose, D-galactose, and D-mannose), pentoses (D-xylose and L-arabinose)
and uronic acids. However, in contrast to cellulose, hemicellulose could be hydrolyzed easily to its constituent monosaccharaides due to its random, amorphous structure with minimal crystallinity [18].

Microalgae is typically composed of some or all of the following four principal biochemical classes of molecules: carbohydrates, proteins, nucleic acids, and lipids. With respect to carbohydrates, different classes of microalgae produce specific types of polysaccharides. Starch consisting mainly of amylose is the carbohydrate energy store found in most algae. For example, the green alga *Tetraselmis suecica* accumulates 11% and 47% of its dry weight as starch in nutrient replete and deplete conditions, respectively [19]. *Red algae* synthesize a carbohydrate polymer known as floridean starch consisting mostly of amylose, and a commonly found polysaccharide in lots of algal species is *chrysolaminarin*, a linear polymer of β(1/3) and β(1/6) linked glucose units [20].

Microalgae cells differ from lignocellulosic biomass in that they are single-cell organisms that are buoyant, eliminating the need for structural biopolymers, such as hemicellulose and lignin, which are essential components of supporting-tissue in land-based plants. Besides carbohydrates, proteins (6-52% of dry microalgae biomass) play an essential role in varied functions ranging from catalysis of metabolic reactions to providing structural support [21, 22]. Currently, protein-rich microalgae are considered a potential source of animal feed and also find application in the development of cosmetic products [22]. Microalgae also has a varied proportion of lipid (normally 7 to 23% of dry microalgae biomass, but could be much higher) based on the species and cultivation conditions [23]. Solazyme, Inc (South San Francisco, CA) stated that the lipid content of
their microalgae reached over 80% via a heterotrophic cultivation process [24]. These lipids usually act as energy reserves (triglycerides) or membrane structural components (mostly composed by phospholipids and glycolipids).

For cost-effective production of bio-based products and fuels from microalgal biomass, a complete utilization of all the algal components is desired. In addition, the energy requirements of the conversion processes should be competitive to petroleum-based processes. With some microalgae, carbohydrate-rich microalgal residues remaining after lipid extraction could directly serve as carbon and nutrient sources for producing value-added compounds via microbial fermentation methods; however, some mild pretreatment and/or enzymatic digestion of the cells might be needed to improve product yield [25].

1.1.2 Pretreatment of lignocellulosic and microalgal biomass

Typically, lignocellulosic as well as microalgal biomass would require some pretreatment to open their structure and make the polysaccharides more accessible for hydrolytic depolymerization by task-specific enzyme cocktails (saccharification). However, the severity of the pretreatment applied may differ due to the differences in the structures of the two types of feedstocks. First, most of the carbohydrates in microalgae are starch-like (non-structural) polysaccharides rather than the structural cellulose found in lignocellulosic biomass. Second, there is no lignin structure in microalgal biomass. Therefore, it can be expected that milder conditions in the chemical pretreatment and enzymatic hydrolysis processes would be sufficient to break down these biopolymers in microalgal biomass into fermentable sugars [12].
For lignocellulosic biomass, various pretreatment techniques, including dilute acid pretreatment, ionic-liquid pretreatment, ammonia fiber explosion, chemical treatment, biological treatment, and steam explosion, can be applied to deconstruct the structure of cellulosic biomass to increase the exposure of cellulose to the external environment before employing acids or enzymes to break down the cellulose into its constituent sugars [11]. Dilute acid pretreatment is effective, when conducted at relatively high acid concentrations of 1 - 4% (w/w), in deconstructing low-lignin lignocellulosic biomass [13]. During the past two decades, extensive efforts were made to optimize the dilute acid pretreatment technology that included optimization of acid-loading, operating temperature, and incubation time to achieve a high sugar recovery with minimum energy input [14]. Although with an increase in severity of operating conditions (in an appropriate range), the sugar yield can be significantly improved, the concentration of inhibitory compounds could also rise [15]. Newer pretreatment methods, such as pretreatment with ionic liquids, have been developed recently [16]. Certain classes of ionic liquids have been shown to be effective solvents for cellulose and lignocellulose. These liquids sufficiently de-crystallize cellulose and increase the hydrolysable surface area to permit enzymatic hydrolysis at high solid loadings, short residence times (less than 24 hours) and low enzyme concentrations [17]. Nevertheless, ionic liquid pretreatment is not a commercially viable technology unless several issues are addressed, notably reduction in water consumption and efficient ionic liquid recycling.

Following the pretreatment process, “enzyme hydrolysis” is widely used to depolymerize cellulose and hemicellulose into their constituent sugars [18]. Enzyme loading and concentration can profoundly affect the saccharification costs, and “enzyme
“hydrolysis” has been identified as the bottleneck to the overall cost of producing sugars from lignocellulosic biomass [19]. Use of high temperatures (of up to 180 °C) during dilute acid pretreatment could permit lower enzyme loadings, as the higher temperatures are more effective in disrupting biomass structure [14]; however, more sugar-degradation compounds are also generated. Also, carrying out the pretreatment at higher biomass loading (up to 30% (w/v)) could reduce the pretreatment cost significantly, since higher concentration systems can process the same amount of dry biomass with less capital cost [20]. Using 30% (w/v) of the biomass loading during pretreatment would typically result in over 150 g/L of total sugar concentration in the hydrolyzate. However, at this concentration levels, most microorganisms would likely experience product inhibition before metabolizing all available sugar (> 100 g/L usually leads to substrate inhibition) [21]. In addition, the high loading of biomass could generate solid-like slurries that increase the viscosity of the medium, requiring special design of pumps and mixers used in moving and mixing the slurries during pretreatment [16]. Moreover, it has been observed that high biomass loadings would adversely affect the delignification and sugar yield [16]. Therefore, high severity pretreatment conditions are required to reduce the negative impact of high biomass loading, which, in turn, could increase the chance of generation of additional sugar and lignin degradation products, as already noted. These compounds could inhibit the downstream fermentation processes and complicate the separation of the desired-product from the fermentation broth, necessitating the inclusion of a “conditioning step” to remove the inhibitors prior to fermentation. Several methods have been proposed in literature for “conditioning” the hydrolyzate including over-liming, adsorption on activated carbon, and ion-exchange resins [22-24].
When using micro-algal biomass, the pretreatment process becomes substantially simpler. Several studies used dilute acid, mechanical disruption or ultrasonic-methods to disintegrate the cell-wall structure for the extraction of oil or release of carbohydrates [25] [26, 27]. The carbohydrates in micro-algal biomass could be hydrolyzed to monomers by enzymes after pretreatment. It has been shown that use of immobilized *cellulases* to digest microalgae's cell walls led to appreciable sugar yields [28]. In general, the pretreatment conditions used with micro-algal biomass are milder compared to the conditions required with lignocellulosic biomass, due to the less rigid cell-wall structure of the microalgae. Accordingly, fewer inhibitory compounds are generated during the pretreatment of micro-algae [29].

1.1.3 Lactic acid fermentation

Lactic acid (2-hydroxypropanoic acid) is a natural organic acid long used in food and non-food industries, including the cosmetic and pharmaceutical industries, in the production of oxygenated chemicals, plant growth regulators, and special chemical intermediates. The demand for lactic acid has increased considerably due to its wide range of applications. As already noted, currently lactic acid can be produced either by chemical synthesis or by microbial fermentation. Chemical synthesis using petrochemical resources always results in a mixture of L-lactic and D-lactic acid, which requires a costly separation process. In contrast, microbial lactic acid fermentation offers numerous advantages, including the utilization of renewable carbohydrate biomass, a low production temperature, decreased energy consumption, and the production of optically pure lactic acid through the selection of an appropriate strain [10].
There are two types of Lactic acid bacteria (LABs) according to their fermentation patterns and products: homo-fermentative and hetero-fermentative [30]. Homo-fermentative LABs degrade hexose via glycolysis pathway, producing 2 moles lactic acid from 1 mole of hexose, leaving minimum byproduct [31]. Hetero-fermentative bacteria metabolize the substrate via phosphoketolase (PK) pathway, where one mole of glucose is further cleaved into one mole of ribulose-5-phosphate and one mole of CO₂ after dehydrogenation. After that, the xylulose-5-phosphate produced from ribulose-5-phosphate splits into glyceraldehyde phosphate (GAP) and acetyl-phosphate. Then the GAP enters the same pathway as homo-fermentation and leads to 1 mole lactic acid from per mole of GAP, while the acetyl-phosphate is converted to acetic acid or ethanol via PK pathway [30]. From Figure 1-2, it can be noted that, through homo-fermentation, a higher lactic acid yield and less byproduct formation can be achieved. Several researchers have studied lactic acid production via homo-fermentation by certain LAB strains when hexose was used as the sole substrate [32-34]. However, only a few species of LAB possess the glycolitic pathway for the metabolism of pentose sugars. The fermentation of pentoses by LABs, in general, generated acetic acid or ethanol [31], which make extra ATP that is energy-favorable to the strain [35] (see Figure 1-2).

As stated before, lignocellulosic biomass-derived sugar monomers are a mixture of both hexoses (e.g. glucose) and pentoses (e.g. xylose and arabinose) after the pretreatment and saccharification steps. Therefore, it is crucial to convert both hexoses and pentoses efficiently to lactic acid via homo-fermentation using appropriate strain to maximize overall yield of lactic acid.
In this study, selected types of LABs have been tested with model hexose and pentose sugars and their mixtures. A new strategy is developed for diverting more pentose sugar toward conversion to lactic acid instead of acetic acid and ethanol. In addition, the performance of the selected strains, in combination with this new strategy, has been evaluated with poplar hydrolyzates generated by “ionic-liquid pretreatment” (followed by enzymatic-saccharification). Ionic-liquid pretreatment, unlike dilute-acid pretreatment, provides hydrolyzates containing a mixture of hexose and pentose sugars, and hence permits the evaluation of the efficacy of LAB strains in converting both C6 and C5 sugars to lactic acid. The results are discussed in Chapter 2.
Figure 1-2  Pathways for lactic acid production from lignocellulose-derived sugars (glucose, xylose, and arabinose) by lactic acid bacteria [10].
1.1.4 Succinic acid fermentation

Currently several bacterial strains, in particular Actinobacillus succinogenes, Anaerobiospirillum succiniciproducens, and Mannheimia succiniciproducens, have been recognized as efficient producers of succinic acid and are being studied most intensively [36]. These natural bacterial species contain the pathway for converting phosphoenolpyruvate (PEP) to succinate. In some of these strains, succinic acid pathways have been demonstrated by nuclear magnetic resonance (NMR) and gas chromatography-mass spectrometry (GC-MS) analyses of $^{13}$C-labeled metabolic product isotopes (Figure 1-3) [37]. Although some genetically modified organisms (GMOs) have been developed that could produce even higher yields of succinic acid, through clever engineering, their stability in the continuous fermentation process and safety concerns, such as potential health and environmental risks, are likely to limit their large-scale application [38].
In this study, *A. succinogenes* 130 Z (ATCC55618) is the strain under investigation. The strain, isolated from bovine rumen, shows a distinctive ability to produce a relatively large amount of succinic acid from a broad range of carbon sources,
such as arabinose, cellobiose, fructose, galactose, glucose, lactose, maltose, mannitol, mannose, sorbitol, sucrose, xylose, and salicin, under anaerobic conditions [40]. Unlike *E. coli* or *A. succiniciproducens*, *A. succinogenes* is a moderate osmophile that tolerates a high concentration of sugars, which is beneficial for fermentation [41]. In addition, because of the ability of *A. succinogenes* to utilize both C6 and C5 sugars, numerous feedstocks, in addition to corn kernels, could serve as sources of carbon for producing succinic acid, including a number of lignocellulosic agricultural residues such as wheat straw, switch grass, and poplar [42].

Furthermore, microalgae are also potential feedstock for succinic acid production. In addition to utilizing the micro-algal-lipids for biodiesel production, microalgae carbohydrates can also be converted to various kinds of bio-based chemicals, such as succinic acid [43]. The advantages of using microalgae rather than terrestrial plants as feedstock for succinic acid production are that there is no requirement of fertile soil and no need to draw upon valuable and often scarce supplies of fresh water. Moreover, the fixation and storage of CO₂ into the biomass while the microalgae grow mitigates the greenhouse gases [44] emissions to the atmosphere. Researchers have successfully used microalgae as the carbon source in lactic acid fermentation after lipid extraction [27]. Moreover, several studies have also demonstrated the utilization of algal biomass as the renewable energy feedstock in the production of bio-ethanol or bio-hydrogen [26, 45].

As indicated in Figure 1-3, McKinlay *et al.* [37, 39] have determined the metabolic pathway of involved in the production of succinic acid by *A. succinogenes*. Succinic acid is produced as an intermediate of the tricarboxylic acid cycle and as one of the fermentation products of anaerobic metabolism. In the process of succinic acid
production using this strain, two moles of nicotinamide adenine dinucleotide hydride (NADH) are required for every mole of succinic acid formed, and the NADH level is insufficient. Consequently, some byproducts, such as acetate and formate, which are formed without NADH consumption, are synthesized to maintain the intracellular redox balance [46]. Researchers are exploring the effects of redox potential on succinic acid formation in A. succinogenes to seek pathways for higher succinic acid yield and less byproducts formation. Van der Werf et al. tested the effects of feeding hydrogen and carbohydrate sources with different reduction levels on succinate production [47], while Park and Zeikus explored the effects of adding electrons to the fermentation process using electricity in a microbial fuel cell [48]. McKinlay and Vieille attempted to change the flux distribution in A. succinogenes 130Z through the addition of hydrogen [49], and Li et al. have investigated the effects of oxidation-reduction potential (ORP) regulation on key enzyme activity, the distribution of metabolites flux and NADH/NAD\(^+\) ratios [50]. Majority of these studies were focusing on the mechanism of “redox potential regulation” in the cell to discover methods of improving succinic acid yield via controlling this parameter. It was found that with ORP level adjusted at -350mV, a high succinic acid yield (1.28 mol mol\(^{-1}\)) and a high mole ratio of succinic acid to acetic acid (2.02) can be achieved [50].

Besides redox potential, numerous other factors could affect succinic acid fermentation. For example, temperature, pH control, and the nutrients in the media could significantly influence the succinic acid yield and growth of the strain [51]. In addition, the fermentation inhibitors generated from pretreatment process would also influence the productivity of succinic acid [15, 22]. The most significant inhibitors affecting succinic
acid fermentation are the sugar degradation products and lignin derivatives [22, 24]. As indicated in the results of this study, the inhibitors created by sugar degradation (furans) and lignin degradation (phenolic compounds) affect the growth of the bacteria even at very low concentrations (from 1 to 5 g/L). To avoid this and to ensure robust growth of the strain, certain conditioning processes, described in the following section, are required.

1.1.5 Inhibitors from pretreatment and detoxification

During the thermochemical pretreatment, degradation of hemicellulose leads to the generation of aliphatic acids (acetic, formic, and levulinic acids) and furan derivatives: furfural and 5-hydroxymethylfurfural (HMF). Previous studies established that there is no obvious inhibition to yeast growth from aliphatic acids at concentrations <100 mM [52]. Luckily, in most of hydrolysates, the total aliphatic acid concentration is normally under 100 mM, which is unlikely to impact the growth of strain [53]. Furans have also been tested in the context of bioethanol production via fermentation using yeast strains and found to inhibit the growth of yeast and decrease ethanol yield and productivity [53]. Studies have shown the high hydrophobicity of furans to compromise yeast cell-membrane integrity leading to extensive membrane disruption/leakage, which eventually will cause reduction in cell replication rate, ATP production, and consequently lower ethanol production [54]. Moreover, the disintegration of lignin during pretreatment of biomass leads to formation of a variety of phenolic compounds [24] (e.g. syringaldehyde and syringone, and vanillic acid, vanillone, and vanildehyde). 4-Hydroxybenzoic acid, ferulic acid, and guaiacol are among the most common lignin-derived inhibitors observed in dilute-acid pretreated lignocellulose hydrolysates [55]. They have a severe negative effect on the cell membrane and lead to the dissipation of proton/ion gradients across the
membrane. This, in turn, affects the key enzymes in the metabolic pathway and DNA/RNA replication, compromising the cell growth and sugar assimilation [15]. Thus, phenolic inhibitors have been shown to adversely affect the fermentation performance of a wide range of microbes [56], including A. succinogenes [22] and S. cerevisiae [57].

Dilute-acid hydrolysis is a common method used to pretreat the lignocellulosic biomass. The flow diagram in Figure 1-4 illustrates a cost effective process for dilute-acid hydrolysis pretreatment and conditioning procedure designed by National Renewable Energy Laboratory (NREL) [3]. Hydrolysis reactions are catalyzed using dilute sulfuric acid and heat from steam. The first stage in pretreatment involves a horizontal screw-feed reactor with a short residence time (5 to 10 minutes). The second stage features a lower temperature/longer residence time “oligomer conversion” step that converts most of the xylose oligomers leaving the first stage to monomeric xylose without generating significant additional degradation products. After passing through the pretreatment reactors, the hydrolysate slurry is flash-cooled, vaporizing a large amount of water along with some of the acetic acid and furfural. The flash vapor is condensed and sent to the wastewater treatment area. The hydrolysate slurry is cooled by dilution with water and sent to a conditioning reactor where overliming is usually performed [3].

Overliming involves the addition of lime to increase the pH, followed by pH adjustment to a value suitable for microbe growth. Overliming is an efficient and inexpensive method of treating hydrolysate to remove furans, phenols, and aliphatic acids. However, the formation of a calcium sulfate precipitate (gypsum) is a drawback of this process as the separation of the precipitate from the product is necessary [58].
Ammonia conditioning could be an alternative method for replacing overliming that can eliminate sugar losses and gypsum disposal costs. Unlike overliming, ammonia is soluble in water, therefore the entire hydrolysate slurry may be treated at once without a solid-liquid separation step [3].

![Simplified flow diagram of the pretreatment and conditioning process](image)

Figure 1-4 Simplified flow diagram of the pretreatment and conditioning process [3]

Besides overliming, solid-phase adsorption has also been investigated for the detoxification of lignocellulosic hydrolysate [22, 59]. The common solid phase used included activated charcoal, ion-exchange resins or polymeric resins [24]. These solid phase material could bind specific inhibitors based on their physical properties. Nevertheless, one major disadvantage of solid-phase adsorption is that they cannot be implemented with the whole slurry before enzymatic hydrolysis, since it is difficult to separate the solid phase from biomass residue after the detoxification step. Therefore, the inhibitors present in the pre-hydrolysate (e.g., pretreated biomass) could inhibit the activity of saccharifying-enzymes and could lead to a reduction in overall sugar recovery. In addition, some solid phase material such as activated charcoal has a poor selectivity for the inhibitory compounds, and significant sugar adsorption also takes place along
with the inhibitors. All of these disadvantages limited their application on an industrial scale [59].

Liquid-liquid reactive extraction (LLRE) is a standard unit operation to remove phenols and other inhibitors from the aqueous phase [60]. In contrast to solid phase adsorption, it can be implemented with the whole slurry and does not lead to sugar losses. Recently, Burghoff and Haan used the trioctylphosphine oxide (TOPO) blend: Cyanex 923 (which is a mixture of four trialkylphosphine oxides), to extract phenol from the aqueous phase. TOPO has very low solubility in the aqueous phase (<10 mg/L at 25 °C) and can be dissolved easily in water-insoluble solvents, such as hexane [61]. Phosphorous-bonded oxygen donor extractants like TOPO contain a phosphoryl group that is a strong Brønsted base capable of accepting proton donors, such as carboxylic acids or phenol. In low-pH solutions, the un-ionized acids easily flow into the organic phase and bind the phosphoryl oxide group, forming a hydrogen bond in the organic phase. The inhibitors present in the hydrolysate, such as phenolic compounds, aliphatic acid and furans, can dissolve in the TOPO-hexane phase, and could be extracted without affecting the sugar in the hydrolyzate. Trioctylamine (TOA), which has a structure and properties similar to those of TOPO but costs less, can also be used as an extractant in two-phase acid extraction [62].

The choice of the detoxification method highly depends on the types and concentrations of inhibitors in the hydrolysate and the tolerance levels of microorganism to these inhibitors [24]. For example, pretreatment using ionic liquids generate fewer inhibitorsthan dilute acid pretreatment. Therefore, the best detoxification method for IL
pretreated hydrolysate could be different from dilute acid pretreated hydrolysate, although the biomass could be same.

In microalgal biomass, there is no lignin unlike in terrestrial plants. The cell wall is, hence, easier to break down, and so a relatively milder pretreatment could be applied. Accordingly, with microalgae no lignin derivatives but only trace quantities of furans are generated during the pretreatment. Therefore, when using microalgae as feedstock in succinic acid production, detoxification may not even be needed.

1.1.6 Cultivation and utilization of microalgae biomass

Microalgae is receiving increasing attention worldwide as an alternative and renewable energy production source. Microalgae can be utilized to produce various types of biofuels, including biodiesel, bio-syngas, bio-oil, bio-ethanol, and bio-hydrogen through different conversion processes [63]. Besides fuels, many microorganisms are able to digest the carbohydrates portion of the microalgae, allowing it to be converted to high-value organic acids, such as succinic and lactic acid [27]. However, a number of challenges still need to be overcome before large-scale production of microalgal feedstocks is achieved, which renders algae-based bio-refineries a rather long-term prospect [63]. Presently, the low productivity of microalgae under phototrophic cultivation, the contamination control, high cost of harvesting and pretreatment are the major hurdle to bring the cost of algal biomass down [64]. With current estimated algae-based fuels price ranging from $300 to $2600 per barrel (crude oil price less than $80 per barrel in 2014), significant technology breakthroughs are required before algal-refineries can compete with petro-refineries [65].
Generally, algae-based biorefining involves four major steps: (i) algal cultivation, (ii) algae harvesting, (iii) pretreatment to disrupt the algal cell wall structure and lipid extraction and (iv) conversion (through chemical/bio-chemical methods) of the residual carbohydrates to various products. Algae can be grown in open or closed cultivation systems [64]. Open ponds (a typical open system) are cheap to build and operate, but the loss of productivity due to varying climate conditions and competition from predators would be a challenge that needs to be dealt with before industrial scale implementation of these systems becomes feasible. Conversely, the closed systems (e.g. photobioreactors) can be controlled better with a higher algal cell growth rate, but the cost of production is much higher and could prove to be an impediment in way of commercialization of these systems – particularly, if fuel is the final product of interest [64].

The harvesting of microalgae is a crucial process which accounts for 20-30% of the total algal fuel production cost [66]. One ton of water must be processed to obtain 1 kg of algal biomass, if algal suspensions are harvested from a starting concentration of 1 g/L. In many cases, the concentrations are even lower (<0.5 g/L), particularly if the cultivation is done in an open pond. Separating such large amounts of water requires huge energy expenditures. Usually, harvesting of microalgae is carried out as a two-step process. First, dilute micro-algal suspensions are concentrated to a 2-7 wt% via sedimentation or flocculation. In the second step, called thickening, further concentration is achieved via centrifugation or filtration. Thickening is more energy intensive than the first step due to the high-energy demand of centrifuges [67].

Depending on the type of biomass, following harvesting, pretreatment may be required to disrupt the cell wall of the microalgae and release the lipids and carbohydrates before oil extraction. The cell-wall disruption and lipid
extraction is carried out via physical, chemical, or biological methods and through solvent extractions. Because removing water beyond 10 to 30 wt% of biomass is energy intensive, a lipid extraction technique that can be applied to a wet feedstock could save a significant amount of energy [68]. Many pretreatment methods have been investigated, including microwave application, sonication, bead beating, autoclaving [69], grinding, osmotic shock, homogenization, freeze-drying and the addition of 10% (w/v) sodium chloride (NaCl) [70].

After lipid extraction, the residual microalgal biomass remains rich in carbohydrates (polysaccharides), proteins, pigments and other valuable nutrients. These materials are ideal carbon and nitrogen sources for the microbial fermentation methods typically employed for producing biofuels and organic acids. Clearly, to optimize the overall process economics, the process should maximize the utilization of all the components in the microalgal biomass, and use of these residues could significantly improve the economics. In addition, manufacturers should also strive to minimize the energy involved in each step of the process to realize a cost-effective algae-based biofuel/production scheme.

### 1.2 Objectives and Significance

#### 1.2.1 Explore the lactic acid production from lignocellulose-derived sugars

Few microorganism can utilize both hexoses and pentoses and convert them to lactic acid. Advances in the pretreatment and saccharification methods of lignocellulosic biomass have reduced the cost of production of lignocellulose-derived sugars, *i.e.*, glucose and xylose, which could serve as feedstocks for bio-based lactic acid production.
Ability to use both C6 and C5 sugars simultaneously and converting them both to lactic acid at high yields would be ideal for economical manufacture of bio-based lactic acid.

In this study, xylose isomerization, a technique that could allow the low-cost conversion of xylose into xylulose, was incorporated in lactic acid production. Xylulose, which LAB may find easier to use than xylose, would significantly improve the efficiency of the fermentation process.

The investigation focused on two types of LABs used in lactic acid fermentation, *L. pentosus* and *L. casei*. Lactic acid production was assessed with model sugars and ionic-liquid pretreated hydrolysate from poplar biomass, which contains both glucose and xylose. In addition, the effectiveness of the isomerization process was tested with both types of LABs to compare the differences in their performance. A detailed discussion of the results follows in Chapter 2

1.2.2 Identify the inhibitors of the succinic acid fermentation process from the lignocellulosic biomass hydrolysate

The inhibitors generated during hydrothermal and chemical pretreatment include furans (furfurals and 5-hydroxymethylfurfural (5-HMF)), phenolics (such as syringaldehyde, vanillin, and ferulic acid), weak acids (including acetic acid, levulinic acid, and formic acid), raw material extractives (acidic resins, and tannic and terpene acids), and heavy metal ions (iron, chromium, nickel, and copper) (see Figure 1-5) [56] [15]. In actual hydrolysate, not all of these inhibitors are present simultaneously. The quantity and types of inhibitors are strongly dependent on the feedstock, pretreatment method and conditions [53].
The hydrolysates used in this research were derived from “ionic-liquid pretreatment” and “dilute acid pretreatment”. Both the types of hydrolysates were well studied and have great potential for commercial applications. During the course of this study, inhibitors in the hydrolysate were identified using various techniques, including high-performance liquid chromatography (HPLC), GC-MS before quantifying the concentrations of those inhibitors. To establish the toxicity of the inhibitors, different concentration for each inhibitor has been tested in the succinic acid fermentation with pure sugar along with well-designed control runs. This protocol established benchmarks for susceptibility of culture to inhibitors and also allowed development of strain-specific inhibitor removal strategies. The results were discussed in the Chapter 3.
1.2.3 Compare the efficiency of different detoxification methods on hydrolysates derived from diluted-acid and ionic-liquid pretreatments

Traditional methods of inhibitor removal include treatment with activated carbon or ion-exchange resins [22]. Although these methods remove large molecules, such as the lignin derivatives efficiently, they also remove a considerable amount of sugar (up to 10%) [56]. Liquid-liquid reactive extraction could be an alternative detoxification method. This method has been applied in the recovery of organic acids, such as lactic and succinic acids [62]. When the extraction agents [61] (tri-n-octylphosphine oxide (TOPO) and tri-n-octylamine (TOA)) are added to the organic phase, these molecules are able to bind to the acid compounds, such as phenols or organic acids in the hydrolyzate. The inhibitors are extracted into the organic phase via a complex formation between the extracting agents and the inhibitors. Subsequently, when the organic phase containing the complexed-toxins is contacted with a strongly basic aqueous phase, the complex will unbind and the inhibitors are released to the stripping phase, which allows the organic phase to be regenerated. The advantage of this method is that the inhibitors are removed from the hydrolyzate efficiently without any sugar loss. To improve the efficiency of this process, a “cyclic-batch LLRE process”, that would permit the tandem operation of the two steps of the removal of the inhibitors and the regeneration of organic phase, has been developed.

The LLRE process (with tri-n-octylphosphine oxide (TOPO) and tri-n-octylamine (TOA) as extraction agents) was evaluated against the solid-phase adsorption methods including activated charcoal, ion-exchange resin, and AMBERLITE™ XAD™ (a
polymeric adsorbent) to assess their relative effectiveness in detoxifying the hydrolyztes for succinic acid production. The results were discussed in the Chapter 3.

1.2.4 Use of microalgae biomass as the carbon and nitrogen source for succinic acid production

As stated before, microalgae offers a number of advantages compared to lignocellulosic biomass and has the potential to be the next generation feedstock for bio-based products. A complete utilization of carbohydrates and proteins in the microalgae as carbon and nitrogen sources, respectively, for microbial fermentations after the extraction of lipids from microalgae could be a viable approach, especially with some strains of microalgae containing large portion of polysaccharides. It has been reported that some algal species could accumulate up to 50% starch [71], which is an ideal carbon source for succinic acid fermentation. In addition, protein-rich microalgae have been shown to be a nitrogen source for ethanol fermentation. [72]. We propose here that microalgae could serve as both carbon and nitrogen sources for the succinic acid fermentation process. This would significantly reduce the cost of the fermentation because no external nitrogen source, such as yeast extract, is needed. In this study, a less energy intensive, novel enzymatic hydrolysis process was developed to disrupt the algal cell wall and release the carbon and nitrogen sources in a form assimilable by the microorganism A. succinogenes. A number of low cost and commercially-available enzymes were evaluated for this process. In addition, the parameters including enzyme loading, temperature, and pH have been optimized to achieve maximum sugar recovery. Moreover, microalgae, after enzymatic hydrolysis, has been used in the succinic acid fermentation with or without supplementation with external nitrogen source. These results were discussed in Chapter 4.
1.3 Organization of the Dissertation

Chapter Two describes lactic acid fermentation, using lignocellulose-derived hexoses and pentoses, by two types of lactic acid bacteria (LAB), *L. pentosus*, and *L. casei*. To improve the utilization of the pentose, xylose was isomerized to xylulose, a sugar isomer that is more readily utilized by LABs. Although the borate used in the isomerization process strongly inhibited the growth of *L. pentosus*, *L. casei*, the other strain tested, was not affected by the borate or the buffer solution. Both strains showed improved conversion from xylulose to lactic acid in comparison to the conversion from xylose to lactic acid. The fermentation performance was evaluated using both model sugars and the hydrolysates.

*A. succinogenes*, which is discussed in Chapter Three, differs from LABs and is one of the succinic acid producing strains that can utilize both hexoses and pentoses. One of the obstacles encountered in using this strain is that some toxic chemical compounds generated from the lignocellulosic biomass pretreatment process significantly inhibited succinic acid fermentation. In this study, an efficient detoxification method based on liquid-liquid reactive extraction was developed and tested. This method was found to be effective for removal of inhibitors without sugar loss. As a result, fermentation after detoxification improved in comparison to untreated hydrolysate. A comparative assessment with other methods of detoxification was also performed.

In Chapter Four, enzymatic hydrolysis of microalgal biomass and succinic acid fermentation from microalgae-derived sugars is described. In this study, cell wall disruption, lipids, and sugar extraction, the fermentation of carbohydrates to succinic acid using dissolved N from the disrupted microalgae cells were investigated.
Chapter Five provides suggestions for future research in this area.
Chapter 2

Lactic acid fermentation from lignocellulosic biomass: strategies for utilizing both C6 and C5 sugars

2.1 Introduction

Lactic acid serves as a building block for the production of the industrially important chemical ethyl lactate and the biodegradable polyester polylactic acid (PLA) that has seen increasing use as a plastic biomaterial, as well as in the food, cosmetic and pharmaceutical industries. Lactic acid is currently produced primarily from the carbohydrate fermentation of corn starch sugars by lactic acid bacteria (LAB) or fungi, which generally follow the Embden-Meyerhof-Parnas (EMP) pathway for hexose sugars (e.g., glucose) with a theoretical yield at two moles of lactic acid comes from one model of glucose (1 g lactic acid produced/g glucose consumed) while the homofermentative LAB converts glucose almost exclusively into lactic acid. Some heterofermentative LABs catabolize glucose into ethanol, acetate and CO₂ as well as lactic acid. In addition,
a few LAB can also convert xylose to lactate via the phosphoketolase (PK) pathway [73-76] with a theoretical yield at one mole of lactic acid from one mole of xylose (0.6 g lactic acid produced/g xylose consumed).

Lignocellulosic biomass may serve as an alternative sustainable and possibly more cost-effective feedstock for the production of lactic acid in the future, especially if the utilization of pentose sugars (comprising the hemicellulose portion of the lignocellulosic biomass), xylose in particular, can be incorporated. However, only a few non-GMO LABs can convert xylose to lactate [77]. Previous efforts aimed at utilizing lignocellulosic biomass sugars for lactic acid production involved the use of *Lactobacillus pentosus* and *Lactobacillus casei* subsp. *rhamnosus* [74, 75]. Garde et al. applied two strains, *Lactobacillus pentosus* and *Lactobacillus brevis*, as lactic acid producers from wheat straw hydrolysate with mixed sugars of glucose and xylose. Respectively, 88% and 95% of the theoretical yield of lactic acid was obtained along with large quantities of acetate or ethanol as byproducts [76]. The primary limitation of these LAB strains is their slower rate of lactic acid production from xylose than from glucose [78]. We hypothesize that one of the rate-limiting biochemical reactions in lactate production is the isomerization of xylose to xylulose in the first step of the PK pathway. Two strategies can be undertaken to bypass this rate-limiting step. One is the use of genetically modified microorganisms with additional xylose isomerase encoded by *Piromyces sp* [79]. The other option is to isomerize the xylose to xylulose externally. A 2014 market analysis showed that approximately 85% of lactic acid is used in food and food-related applications [80]. Therefore, using lactic acid produced by GMOs, especially in food-related industries, could cause great public safety concerns [81].
Taking on the second strategy would allow for more complete fermentation of hydrolysate sugars without using GMO strains. Recently, remarkable progress has been made by incorporating the isomerization from xylose to xylulose into bioethanol production from lignocellulosic biomass [82]. Xylose isomerase (EC 5.3.1.5) (XI) was found to be the ideal candidate to convert the xylose to xylulose extracellularly. Traditionally, XI was widely consumed in the food industry [83], and it can be acquired at a low cost for the isomerization of xylose to xylulose and subsequent fermentation to lactic acid, which is used to catalyze the isomerization of D-glucose and D-xylose to D-fructose and D-xylulose, respectively. These enzymes are intracellularly produced by Streptomyces and Bacillus species [84]. Although commercially utilized for the isomerization of glucose, the $K_m$ values of the enzymes for xylose are at least one order of magnitude higher than those for glucose [85]. Thus, xylose is the more preferred substrate for the enzymes, and, when used on biomass, the production rates of xylulose will likely be much higher than the isomerization of glucose to fructose [86].

In isomerization with XI, the reaction is reversible [87], but the equilibrium does not favor xylulose formation with a molar ratio of xylose: xylulose only at 6:1 even at an optimum pH between 6.0 and 7.5. [88]. In order to shift this equilibrium to generate more xylulose, we need to reduce the xylulose present in the reaction. Borate can bind with the xylulose to form a complex at a neutral pH. Therefore, if borate were added to the isomerization reaction, the complex formation between borate and xylulose would lead to a reduction of xylulose monomer concentration, which would shift the equilibrium to more xylulose conversion [88]. In this study, we have devised a scheme for the efficient extracellular isomerization of xylose to xylulose. This approach has the advantage of
controlling the rate as well as the equilibrium associated with the isomerization step, thereby eliminating one of the bottlenecks in the PK pathway of native LABs. However, this approach assumes that xylulose uptake would be rapid and not rate-limiting. If successful, this approach would enable the use of the well-tested LABs used in corn starch conversion with lignocellulosic sugars.

2.2 Materials and Methods

2.2.1 Enzymes and chemicals

Gensweet® IGI-HF (275 U/g) was a gift from Genencor International, Inc (Palo Alto, CA). This immobilized glucose isomerase is produced from a genetically modified strain of *Streptomyces rubiginosus* and catalyzes the isomerization of glucose to fructose and xylose to xylulose. The Gensweet® pellets were dry, tan-to-brown, cylindrical-shaped granules with a diameter of approximately 0.3-1.2 mm. Gensweet® IGI-HF was stored at 4°C until use.

Additional chemicals, including xylose, sodium tetraborate, citric acid, sodium citrate, and tris(hydroxymethyl)aminomethane (Tris) were all purchased from Sigma Aldrich (St. Louis, MO).

The pure xylose and hydrolysate were filtered with a 0.22 μm filter (Corning Life Science, Amsterdam, Netherlands) and transferred to 250 mL Erlenmeyer flasks. The pH of the solution was adjusted to 7.5 and Gensweet® pellets were added. The stirred media was isomerized for 6 h with or without borate at 50°C
2.2.2 Isomerization of xylose to xylulose

Isomerization of xylose using unaltered Gensweet® pellets was performed by using 30 g/L xylose. The reaction medium includes 30 g/L xylose, 50 mM borax, and 18 g/L Gensweet® IGI-HF prepared in 50 mM Tris-HCl buffer (pH 7.5). The isomerization was carried out at 34°C in 1 L shake flasks agitated at 130 rpm using incubator shaker (New Brunswick Enfield, CT) until equilibrium was reached (about 24 h). The solution was then used to prepare fermentation media containing xylulose. [88].

2.2.3 Production of lignocellulosic biomass hydrolysate

Ionic liquid pretreatment followed by enzymatic saccharification was used to produce biomass hydrolysate [89]. Briefly, dried poplar wood was ground (-20/+80 mesh sized particles), and 25 g was incubated with 225 g of 1-ethyl-3-methylimidazolium acetate (EMIM Ac) at 120°C for 1 h in an oil bath. The treated biomass mixture was washed thoroughly with deionized water. The washed solids were added to a 0.05 M Na-citrate solution (served as buffer, pH 4.8) at a loading of 7.5% (w/w). Spezyme CP (15 FPU/g glucan) and Novozyme 188 (60 FPU/g glucan) were then added and saccharification was carried out for 24 h at 50°C. To mimic a higher saccharification biomass loading of 15% (w/w), the poplar hydrolysate was concentrated two-fold by evaporation on a hot plate at 80°C for 4 h. The resulting hydrolysates were filtered through a 0.22 mm pore-size filter and stored at 4 °C until use.

2.2.4 Microorganisms and fermentation

Fermentation was carried out anaerobically in 250-mL Erlenmeyer flasks with a working volume of 50 mL. Sterilized nitrogen gas was infused into the flasks for one
minute to push the air out. Under such conditions, the system is not strictly anaerobic; there is a chance that the oxygen can diffuse back into the flasks. However, this condition was also carried out by other investigators while conducting lactic acid fermentation with \textit{L. casei} [90], \textit{Lactobacillus brevis}, \textit{Lactobacillus pentosus}, and \textit{Lactobacillus spicheri} [91]. Previous studies found, for \textit{Lactobacillus plantarum} (one strain has a very close genotype and highly similar phenotypes to \textit{Lactobacillus pentosus} [92]), that there is no significant difference in lactic acid production under aerobic or anaerobic conditions [93]. Additionally, \textit{Lactobacillus casei} was also used in lactic acid fermentation with a micro-aerobic sequencing batch reactor with a high lactic acid concentration produced [94]. Therefore, we also conducted lactic acid fermentation with Erlenmeyer flasks to simplify the process and operation. An incubator shaker (New Brunswick Scientific Co., NJ) was used to maintain culture temperature (34° C) and to provide agitation (135 rpm). In fermentations where pH was controlled, excess calcium carbonate (~6 g/L) was added to the flask. Once new acid was released, protons will react with CO$_3^{2-}$ to generate HCO$_3^-$ or H$_2$CO$_3$. Therefore, the pH will not decrease during the process. Previous studies evaluated several neutralizing agents including CaCO$_3$, NaOH, ammoniacal solution, and NaHCO$_3$ on lactic acid fermentation with \textit{R. oryzae}. The results showed that using CaCO$_3$ as a neutralizing agent had the highest lactic acid productivity and yield since using either NaOH or ammoniacal solution would cause mycelium morphology of the strain, leading to a reduction of growth [95]. In addition, compared with other carbonate salts (e.g., Na$_2$CO$_3$ or NaHCO$_3$), CaCO$_3$ could make lactic acid precipitate in the form of calcium lactate, and facilitate the downstream separation and extraction of lactic acid [96].
Samples were collected periodically, and a nitrogen sweep was used to maintain the anaerobic environment at each time of the sampling.

### 2.2.5 Analytical Techniques

Samples collected from the experiments were diluted three-fold in ultrapure water and filtered through a 0.22 μm filter. Calibration standards for glucose, xylose, xylulose, lactic acid and acetic acid were prepared in a similar manner. All samples were analyzed on an Agilent 1100 HPLC system equipped with a refractive index detector (RID). A Shodex SH1101 column maintained at 50 or 65°C was used for chromatographic separation of the analytes. Filtered 5 mM sulfuric acid was used as the mobile phase with a flow rate at 0.5 ml/min. The carbon balance was calculated based on the sum of all carbon concentration (g/L) in the measured compounds (substrates and products) at each time of sampling. Carbon balance concentration was calculated via following equation:

\[
\text{Carbon balance concentration (g/L)} = \sum \frac{12 \times C_s \times N_s}{M_s} + \sum \frac{12 \times C_p \times N_p}{M_p}
\]

- \(C_s\): concentration of substrate (g/L)
- \(C_p\): concentration of product (g/L)
- \(N_s\): number of carbon atom in the substrate molecule
- \(N_p\): number of carbon atom in the product molecule
- \(M_s\): Molecule weight of substrate
- \(M_p\): Molecule weight of product
2.3 Results and Discussion

2.3.1 Effect of pH control on lactic acid fermentation (Batch)

In lactic acid fermentation, a low pH has an inhibitory effect on cellular metabolism and lactic acid production. The majority of LAB cannot grow below pH 4 [97]. In batch lactic acid fermentations, the constant production of acid reduces the pH of the medium to values below 4 since the pKₐ of lactic acid is 3.78. Therefore, a base must be added to neutralize the produced acids and to reduce the inhibitory effects of low pH.

In this study, excessive calcium carbonate was added to the medium as a buffer to control the pH during the fermentation at around ‘neutral,’ which is the optimum pH for the growth of the LABs tested. Previous studies indicate that using this method for pH control is simple and effective for batch lactic acid fermentation as an alternative method for continuous pH-adjusting by system [95, 98]. In addition, when CaCO₃ was added, CO₂ was continuously released, which could create semi-anaerobic conditions that are optimal for this process [98]. In this method, CaCO₃ needs to be added initially without any automatic monitoring or control modules required. As controls, a parallel set of fermentations were performed without any addition of the carbonate.
Figure 2-1 Glucose (30 g/l) fermented by L. pentosus. Results are shown with (A) and without (B) CaCO$_3$ added for pH control.
As shown in Figure 2-1, for *L. pentosus*, elimination of the pH drop associated with acid formation using CaCO$_3$ leads to a significant improvement in the rate of glucose conversion to lactic acid. Acetic acid formation was also lower in the pH-controlled fermentations. While pH was not controlled with a neutralizing agent such as CaCO$_3$, the final pH at the end of the fermentation dropped to around 2.5. Under these conditions, there is a significant delay in the utilization of substrate and the production of lactic acid (Figure 2-1 B). This delay not only reduces the productivity of lactic acid, but also increases the chances of contamination. Therefore, pH control appears to be critical for lactic acid fermentation. In media containing excess CaCO$_3$, the pH at the beginning and end of fermentations was measured to be approximately 6.0 (Figure 2-1 A). Another LAB, *L. casei*, was also tested with the same media and fermentation conditions as *L. pentosus*. It was found that *L. casei* could convert glucose to lactic acid at a much higher rate (over 95% g/g) than *L. pentosus* (Figure 2-2).
Figure 2-2 Glucose (30 g/l) fermented by L. casei. CaCO$_3$ was used to maintain the pH at 6.

2.3.2 Lactic acid fermentation from pentose

Since xylose is the most abundant pentose present in the lignocellulosic biomass hydrolysate, studies were performed to assess its utilization by L. pentosus and L. casei and its fermentation to lactic acid. As shown in Figure 2-3, fermentation of xylose by L. pentosus produces equal quantities of lactic acid and acetic acid. In addition, all the xylose supplied for fermentation was not utilized, suggesting that fermentation had stalled at xylose concentrations of approximately 10 g/L. For L. pentosus, previous studies indicated that the strain has a yield of 0.48 and 0.51 g lactic acid per gram of sugar consumed while corn cobs and barley bran husks were hydrolyzed, respectively. Both hydrolysate from these lignocellulosic biomass contained over 40 g/L of pentose (xylose and arabinose) and less than 6 g/L glucose, which takes up to 100 hours to
completely utilize all sugars [99]. These results demonstrate that *L. pentosus* can convert pentose to lactic acid at around 0.5 g/g yield, but the conversion rate is much slower than lactic acid conversion from glucose by *L. casei*. However, the results showed that *L. casei* was not able to utilize any xylose, which was not a surprise because other studies also found that *L. casei* lacks the metabolic pathway to utilize xylose [4].
Figure 2-3 Xylose (30 g/l) fermented by *L. pentosus* (A) and *L. casei* (B). CaCO$_3$ was used to maintain the pH at 6.
Next, we tested both strains for the utilization of xylulose. Since pure xylulose is very expensive to obtain, we isomerized the xylose to xylulose ourselves. As stated before, borate and some Tris buffer (to maintain the pH at 7.5) need to be added to help the enzyme reaction reach the maximum conversion ratio (with converted xylulose concentration: initial xylose concentration over 90%). Although the reaction can be processed without borate, only 30% of the xylulose conversion was obtained. Without borate, the isomerization equilibrium under optimum conditions (neutral pH) does not favor xylulose formation (xylose:xylulose ~6:1) [15]. However, *L. pentosus* was strongly inhibited by the borate and Tris buffer. A control experiment was conducted for pure glucose and borate, which demonstrates a strong inhibition with *L. pentosus* but no inhibition with *L. casei* (Figure 2-4). Therefore, for *L. pentosus*, in order to allow it to grow robustly, we could only test it with a medium with a maximum xylulose conversion at 30% without any borate or Tris buffer present. Luckily, *L. casei* could survive and grow robustly in the presence of the borate and Tris buffer. This allows us to perform the isomerization with the borate, which enables 90% of the xylose to be converted to xylulose. Furthermore, the isomerized media with 30% and 90% xylulose conversion were used for lactic acid fermentation by *L. pentosus* and *L. casei*, respectively.
Figure 2-4 Glucose (30 g/l) fermented by *L. casei* and *L. pentosus* with 50 mM borax prepared in 50 mM Tris-HCl buffer (pH 7.5)
Figure 2-5 Xylose partially isomerized to xylulose (30 g/L total sugar) and fermented by *L. pentosus* (A) and *L. casei* (B). CaCO$_3$ was used to maintain the pH at 6.
In the results, *L. pentosus* could utilize both xylose and xylulose at a very slow rate with less lactic acid produced (Figure 2-55(A)). In addition, there was no preference for either the xylose or xylulose as the substrate. *L. casei* was not inhibited by borate or Tris and utilized nearly 50% of xylulose (Figure 2-55(B)), converting it to a 50:50 mass ratio of lactic and acetic acids. The xylose was not utilized at all by *L. casei*. It can be concluded that, while xylose and xylulose are present at the same time, *L. casei* has a strong preference for utilizing xylulose than *L. pentosus*.

### 2.3.3 Lactic acid fermentation in the lignocellulosic hydrolysate

After the pretreatment, saccharification and partial evaporation of water, the concentrations of glucose and xylose in the hydrolysate were approximately 40 g/L and 8 g/L, respectively. In comparison with pure sugar fermentations (Figures 2-2, 2-3, and 2-5), the total sugar concentration was higher (~50 g/L), and therefore greater LA and AA production is expected. With glucose and xylose mixed together, *L. pentosus* utilized glucose first and a large amount of LA was produced. However, only a little xylose was consumed. Previous studies also indicate that *L. pentosus* utilizes hexose first before pentose when mixed sugar is the substrate in LA fermentation [100]. The enzymes used for the conversion of xylose are inducible, which causes a lag time before these enzymes for assimilation appear in the presence of xylose [101]. In addition, xylose will also result in the accumulation of intracellular intermediates such as fructose 1, 6-diaphosphate, which inhibit the enzyme activity for xylose metabolism [102]. In addition, the lower utilization of xylose might be due to the products (LA and acetic acid) inhibition. Buyonda’s team showed that xylose can be fully utilized when the substrate loading is low in the LA fermentation by *L. pentosus*. While the substrate loading increases, there
are 10 g/L of xylose that cannot be utilized even though fermentation lasts 100 hours with
60 g/L of lactic acid and 20 g/L of acetic acid present in the medium [100]. This result is
also in agreement with the report by Moldes’ team [103]. Further work needs to be done
to prove this hypothesis by fermenting xylose with the addition of different amounts of
LA present initially.
Figure 2-6 Saccharified poplar hydrolysate (~50 g/l total sugar) fermented by *L. pentosus* (A) and *L. casei* (B). CaCO$_3$ was used to maintain the pH at 6.
Next, we also isomerized the sugars in the hydrolysate and used this medium as the fermentation broth to test both of the strains. Again, the medium for *L. pentosus* was isomerized without the borate, which contained much less xylulose than the medium for *L. casei*. As is shown in Figure 2-77, *L. pentosus* was not able to utilize most of the xylose, but it was able to consume the xylulose. The *L. casei* could only utilize the xylulose, but was not able to utilize xylose.
Figure 2-7 Saccharified poplar hydrolysate (~50 g/l total sugar) partially isomerized without borate and fermented by *L. pentosus* (A), isomerized with borate and fermented by *L. casei* (B). CaCO$_3$ was used to maintain the pH at 6.
Table 2.1 Summarizes the lactic acid fermentation by *L. pentosus* and *L. casei* with sugars and poplar hydrolysate. The yield of lactic acid and the lactic acid to acetic acid ratio has been compared.

<table>
<thead>
<tr>
<th>Sugar</th>
<th>Glucose</th>
<th>Xylose</th>
<th>Xylose/xylulose w/ borate</th>
<th>Xylose/xylulose w/o borate</th>
<th>Hydrolysate Isomerized hydrolysate</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>L. pentosus</em></td>
<td>0.56</td>
<td>0.26</td>
<td>0</td>
<td>0.19</td>
<td>0.56</td>
</tr>
<tr>
<td><em>L. casei</em></td>
<td>0.95</td>
<td>0.06</td>
<td>0.28</td>
<td>Not done</td>
<td>0.65</td>
</tr>
</tbody>
</table>

The above table summarizes the utilization of different carbon sources by *L. pentosus* and *L. casei*. *L. pentosus* could use both glucose and xylose, and produced a LA yield of 0.56 g/g and 0.26 g/g. In addition, the lag time for pentose utilization is relatively long due to the fact that the enzyme for xylose assimilation was not a specific transporter [100]. We tried to use xylulose to bypass the hypothesis rate-limiting metabolic pathway via isomerization extracellularly, but it required the presence of borate to obtain a higher xylulose conversion result. With 30% of xylulose conversion, *L. pentosus* had not shown a preference for utilizing xylose or xylulose, with both of the sugars consumed together at a very low rate with a poor LA yield of 0.19 g/g, which is even worse than xylose. This result indicated that extracellular isomerization is not a good way to improve xylose utilization by *L. pentosus*. In the fermentation of poplar hydrolysate, mixed glucose and xylose were present together in the medium. *L. pentosus* utilized glucose first and then used xylose partially, which led to a LA yield of 0.56 g/g. This result demonstrated that *L. pentosus* prefers to utilize glucose via the EMP pathway, and then utilize xylose via the PK pathway.
While fermenting the isomerized hydrolysate with glucose, xylose, and xylulose, similar to the isomerized xylose medium, *L. pentosus* did not show a preference for xylulose. After glucose was consumed completely, both xylose and xylulose were not utilized well, with a LA yield (0.57 g/g) from total sugar similar to the unisomerized hydrolysate. Therefore, it can be concluded that using isomerization extracellularly will not improve the utilization of pentose by *L. pentosus*. *L. casei* could convert glucose to lactic acid at a very high yield (0.95 g/g), which contributes to its homofermentative pathway that completely converts one mole of glucose to two moles of lactic acid with a very small amount of acetic acid generated [104]. However, *L. casei* can hardly utilize xylose. The hypothesis (that we bypass the rate-limiting step that converts the xylose and xylulose) can be carried out by extracellular isomerization. With the presence of borate, the equilibrium shifts to high xylulose generation. Fortunately, unlike *L. pentosus*, this strain is not affected by borate in the fermentation. In the fermentation of an isomerized medium with 90% xylulose and 10% xylose as substrate, a LA yield of 0.28 g/g was obtained. This is higher than the one obtained from *L. pentosus*. In the fermentation of the mixed glucose and xylose from poplar hydrolysate, *L. casei* produced 65% of the lactic acid from the sugar added with most of xylose left unutilized. But while the hydrolysate was isomerized with 90% of the xylose converted to xylulose, the complete consumption of glucose and xylulose was achieved with a LA yield of over 71%, which is the best among mixed sugar. In conclusion, *L. casei* is a more robust strain that is able to convert both glucose and xylose to lactic acid at a high yield using extracellular isomerization.
2.4 Conclusions

Lactic acid fermentation is a cost-effective process required to utilize both glucose and xylose, the major sugar component of lignocellulosic biomass. Two strains were tested in this study to evaluate the utilization of both sugars. Extracellular isomerization can be an alternative method to bypass the rate-limiting step to improve the utilization of xylose. However, *L. pentosus* showed no preference in the selection of xylose or xylulose as the substrate, with slow lactic acid productivity from both sugars. In addition, *L. pentosus* was strongly inhibited while the isomerization catalyst borate was present in the fermentation, which limited the use of this strategy. *L. casei* was unable to ferment xylose, but it was able to utilize xylulose very well. Moreover, *L. casei* can grow robustly with the presence of borate. The high LA yield obtained after the utilization of most mixed sugar from isomerized poplar hydrolysate suggests that *L. casei* could be a viable organism for producing LA from lignocellulosic biomass hydrolysates. This process could be improved if more complete utilization of mixed sugars can be achieved by coupling the fermentation with a simultaneous isomerization and reactive extraction (SIRE) process. This low-cost process enables 97% of the xylulose to be converted from xylose with no presence of borate in the recovered medium, which reduces the risk of inhibiting microorganisms and the cost of the downstream product separation process [105]. With further optimization of this process, co-fermentation of all major hydrolysate sugars with *L. casei* should be possible.
Chapter 3

Detoxification of dilute-acid and ionic-liquid pretreated wheat straw hydrolysate to improve succinic acid fermentation

3.1 Introduction

Unsustainable use of fossil fuel resources and widespread interest in reducing CO$_2$ emissions have prompted the development of bio-based chemical production using renewable resources. Since substrate cost significantly impacts the production price of bio-based products, investigation has recently begun on the use of economically-attractive substrates such as lignocellulosic materials. When processing lignocellulosic biomass to obtain a sugar-rich hydrolysate, a maximized sugar release from the carbohydrate portion and minimized sugar and lignin degradation is the ideal result. Typically, the conversion includes chemical and/or thermal pretreatment to destabilize the biomass structure followed by enzymatic hydrolysis to break down carbohydrates, made more readily digestible during pretreatment, to their component monosaccharides (glucose and xylose).
During most biomass pretreatment processes, harsh conditions, like high temperature and high pressure, are adopted. The type and severity of pretreatment directly impacts sugar recovery. Dilute acid hydrolysis is a fast and traditional method of disrupting the lignocellulosic structure and partially solubilizing polysaccharides to release sugars. In addition to the sugars, this process forms aliphatic acids (acetic, formic, and levulinic acids), furans (furfural and 5-hydroxymethylfurfural, HMF), and phenolic compounds (i.e. vanillin). Ionic-liquid pretreatment was developed as an alternative approach to deconstruction with the specific objectives of disrupting the biomass structure to improve enzymatic hydrolysis while minimizing degradation product generation [106, 107]. This method can be conducted under relatively low temperatures (90°C to 130°C), leading to less degradation of monosaccharides, and consequently minimum inhibitory product formation compared to dilute-acid pretreatment [108, 109]. However, inhibitors such as phenolic compounds and acetic acid, which come from lignin breakdown during the pretreatment process [53], are still produced.

The by-products from sugar and lignin degradation not only impact the conversion of sugars to products, especially while these inhibitors are present in the microbial fermentation, but also the efficiency of the preceding enzymatic saccharification. Although pretreatment conditions can be optimized to generate both a high yield of fermentable sugars and lower concentrations of inhibitors, these inhibitors are often present at inhibitory concentrations in the hydrolysate [110, 111].

Phenolic compounds are often potent inhibitors to microbial growth. Phenolics are known to increase biological membrane fluidity and decrease cellular integrity, which impacts key enzymes and disrupts DNA/RNA replication, thereby adversely affecting
microbial fermentation [112, 113]. The low molecular weight phenolic compounds (LMWPCs) vanillic, syringic, p-coumaric, and ferulic acids and guiacol are among the most common lignin-derived phenolic inhibitors observed in lignocellulosic hydrolysates; these phenolics have been shown to adversely affect the fermentation performance of *Saccharomyces cerevisiae* [114, 115] and mutated strains of *Clostridium beijerinckii* [116]. Hodge found that the phenolic compounds present in acid-pretreated hydrolysate from a softwood biomass are one of the major inhibitors to the production of succinic acid by mutated *Escherichia coli* [111]. The mechanism of the inhibition by phenolic compounds has not been elucidated, although some researchers suggest that these compounds increase cell membrane fluidity. Increased membrane fluidity may increase membrane permeability [112] and cause leakage of cellular contents [117]. Phenolic compounds also inhibit enzymatic hydrolysis; a ratio of 4 mg vanillin to 1 mg enzyme reduces the rate of cellulose hydrolysis by 50%, which is economically unacceptable in industrial production [118, 119].

In addition, furans, as one class of sugar degradation product, exert toxic effect to many organisms. It has been reported that furfural at 1 g/l negatively affects cell growth of *S. kluyveri* [120], reducing maximum cell dry weight by approximately 20%. Delgenes *et al.* [121] tested furan inhibition to *S. cerevisiae* and found that 1 g/L of furfural or HMF reduced maximum cell concentration by 81% and 65%, respectively. Wikandari *et al.* [122] tested the inhibition of furfural and HMF on an isolated yeast and found that 1 g/L of furfural or HMF decreased the ethanol yields by 26 and 18%, respectively. The hydrophobicity of the furans results in compromised membrane integrity which
eventually causes a reduction in cell replication rate, ATP production, and consequently lower ethanol production [54].

Moreover, organic acids such as formic, acetic, and levulinic acid could be present in the hydrolysate, which in higher concentrations (> 0.2 M) also contribute to inhibition of *S. cerevisiae* [110]. Combination of these inhibitors can produce a synergic effect which completely inhibits microbial fermentation [123]. A recent study found that HMF caused a notable synergistic effect with levulinic acid and several phenolic compounds, although these compounds showed no inhibitory effect individually (Zha et al. 2014). Therefore, many of these inhibitors may need to be reduced in concentration to maximize the efficiency of both enzymatic hydrolysis and fermentation.

Fortunately, these inhibitors can be removed through different methods including overliming [114] and solid-phase adsorption by activated carbon [111, 124] and ion-exchange resins [125]. However, these treatments have drawbacks or challenges associated with their implementation for hydrolysate detoxification. For overliming, the difficulties include slurrying the lime at a high concentration, controlling lime addition, disposal of the filter cake, and equipment scaling [126]. Recent work has found that 30–40% of calcium can remain soluble after overliming, leading to problems with equipment scaling [111]. Using solid-phase adsorption to remove inhibitors in the hydrolysate is expensive due to the high cost of the solid adsorbent and the processes required for its regeneration. Currently, industrial-scale methods for regeneration of granular activated carbon include steam and thermal or chemical treatment, drawbacks of which include high energy input and approximately 10% loss for each thermal regeneration cycle; powdered activated carbon cannot be regenerated [127]. Moreover, a significant sugar
loss is reported when activated carbon is used as a detoxification adsorbent [128]. Other adsorbents, such as ion-exchange and polymeric resins, are far more expensive for detoxification treatment [129]. Finally, it is difficult to incorporate solid-phase detoxification methods into the lignocellulosic biomass deconstruction process. If solid-phase detoxification is conducted before saccharification, the solid adsorbent cannot be separated from the solid biomass residue. Conversely, conducting solid-phase detoxification after saccharification and removal of non-carbohydrate solids (1) does not prevent inhibition of the saccharification enzymes used during enzymatic hydrolysis and (2) increases the adsorption of released sugars. These difficulties limit the adoption of solid-phase adsorption for hydrolysate detoxification.

Liquid-liquid reactive extraction (LLRE) may be a desirable alternative method for the detoxification of lignocellulosic biomass hydrolysate. In this two-phase separation process, extractant agents in the organic phase react with the inhibitors in the aqueous biomass phase to form a complex that is soluble in the organic phase. Common extractant agents for acidic compound removal include: (1) carbon-bonded oxygen-donor molecules (including inert aliphatic, aromatic, and substituted hydrocarbons); (2) phosphorus-bonded oxygen-bearing compounds; and (3) high molecule weight aliphatic amines [130]. These extractant agents contain a functional group that is a strong Brønsted base capable of accepting proton donors such as carboxylic acids or phenolic compounds [131]. Phenolic compounds and aliphatic acids present in the lignocellulosic biomass hydrolysate can bind reversibly with the extractant. Other inhibitors, such as furans, may also have some affinity toward the extractant and will also partition into the organic phase based on their relative solubilities between the two phases. The sugar monomers,
which are non-acidic and highly water soluble, remain exclusively in the aqueous phase. LLRE has been used to remove organic acids and furans from DA-pretreated corn stover hydrolysate. Using an organic phase of 30% (v/v) tri-n-octylamine (TOA) in octanol and kerosene, 45.7% of 5-hydroxymethylfurfural and 100% of furfural were successfully removed from the hydrolysate [132]. However, LLRE has not been systematically evaluated for its ability to remove all types of inhibitors commonly found in lignocellulosic biomass hydrolysate; in addition, the long-term recyclability of the organic phase for repeated detoxification has not been evaluated.

We have evaluated the toxicity of individual furans and phenolics found in lignocellulosic biomass hydrolysates on the growth and succinic acid production of A. succinogenes, a model organism for anaerobic C5 and C6 sugar fermentation. Wheat straw hydrolysates produced via dilute-acid and ionic liquid pretreatment methods were evaluated for inhibitor content and detoxified via solid-phase and liquid-phase methods. For solid-phase detoxification methods, activated carbon, ion-exchange resin, and polymeric resin were used to remove inhibitors after enzymatic saccharification. Liquid-liquid extraction and LLRE were used to detoxify the biomass slurry after pretreatment but prior to saccharification. Microbial growth and yields of succinic acid were measured and compared between different conditioned hydrolysates. Detoxification by LLRE, which showed both the best broad-range inhibitor removal and maximization of succinic acid production, was also implemented in a cyclic batch processing configuration to reuse and recycle the organic phase for sequential detoxification of multiple batches of hydrolysate.
3.2 Materials and Methods

3.2.1 Chemicals and reagents

The Folin–Ciocalteu reagent, Bacto™ tryptic soy broth (TSB) (Becton Dickinson, Franklin Lakes, NJ) were purchased from Fisher Scientific (Waltham, MA). The phenolic compounds (p-coumaric acid, ferulic acid, vanillin, caffeic acid and syringic acid), hexane, liquid-phase extractants (tri-n-octylphosphine oxide (TOPO) and tri-n-octylamine (TOA)), organic acids (succinic acid, acetic acid, formic acid and levulinic acid), fermentation media (glucose, xylose, arabinose, phosphate salts, biotin and yeast extract), solid phase adsorbents (activated carbon, Amberlyst® A21 resin, and AMBERLITE® XAD4 polymeric resin) and all other chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

The saccharification enzyme Spezyme CP (Trichoderma reesei cellulase) was a kind gift from Genencor (Palo Alto, CA). Novozyme 188 (β-glucosidase) was purchased from Sigma (St. Louis, MO, Cat. No. C6150).

IL pretreated wheat straw hydrolysate was a kind gift from SuGanit Systems, Inc (Toledo, OH).

3.2.2 Biomass characterization

Milled wheat straw was obtained from SuGanit Systems, Inc (Toledo, OH). The sugar content of the biomass was determined by NREL LAP (Determination of Structural Carbohydrates and Lignin in Biomass, NREL). The carbohydrate content of the biomass was 36.7% glucan, 24.4% xylan and 1.2% arabinan.
3.2.3 Dilute-acid (DA) pretreatment of wheat straw biomass

DA-pretreatment was conducted using methods as described in the literature [14]. A 10 g sample of the dry milled wheat straw biomass feedstock mesh sized from -20 to +80 was presoaked at room temperature in 1 wt% dilute sulfuric acid solution at 15 wt% solids loading for at least 4 h. The presoaked slurry was transferred to a 300 ml Parr reactor (Parr Instrument Company, Moline, IL). The temperature was set at 140 °C with the impeller rotated at a speed of 100 rpm for 40 minutes.

For detoxification by LLRE or LLE, the biomass was diluted to 13 wt% by addition of DI water, and the pH was adjusted to 2.0 with 2M NaOH. This slurry concentration results in a hydrolysate total sugar concentration of approximately 60 g/l after enzymatic hydrolysis. The biomass slurry was mixed directly with the organic phase for detoxification. For solid-phase detoxification, sodium citrate was added to 0.05 M, the biomass loading was adjusted to 13 wt% with DI water, and the pH was adjusted to 4.8 with 2 M NaOH in preparation for enzymatic saccharification.

3.2.4 Ionic liquid (IL) pretreatment of wheat straw biomass

The ionic-liquid pretreated wheat straw hydrolysate was obtained from SuGanit Systems, Inc (Toledo, OH). The method used was that of Dadi et al. [107]. In this process, 5 g of dry milled wheat straw biomass feedstock mesh sized from -20 to +80 were added to an autoclaveable vial with 95 g of the ionic liquid 1-ethyl-3-methyl imidazolium acetate (EMIMAc) The biomass slurry was incubated at 120°C on a temperature controlled hotplate for 15 min with vigorous stirring using a magnetic stir bar at 800 rpm. Following swelling of the biomass, deionized (DI) water was used as a displacement
solvent to separate ionic liquid from the biomass. A volume of 250-400 ml of DI water was added to the biomass/IL slurry; the sample was briefly mixed, centrifuged and the supernatant was removed. This washing process was repeated two more times until the supernatant appeared visually clear; the biomass was then washed with 0.01 M sodium citrate buffer. The biomass was prepared for saccharification by addition of 0.05 M sodium citrate buffer (pH 4.8) to a biomass slurry concentration of 12 wt%. Due to the low concentration of inhibitors produced via IL-pretreatment, both liquid-phase and solid-phase detoxification methods were applied after enzymatic saccharification.

3.2.5 Enzymatic saccharification of pre-treated wheat straw

The DA--pretreated biomass samples were processed in one of two ways depending on the method of inhibitor removal employed. For liquid-phase detoxification methods, the pretreated biomass slurry was detoxified prior to enzymatic saccharification. For solid-phase detoxification methods the pretreated biomass was saccharified, and the biomass hydrolysate was filtered to remove any remaining solids prior to inhibitor removal. For IL-pretreated biomass, all methods of detoxification were conducted on the filtered, saccharified biomass hydrolysate.

The enzymatic saccharification procedure used was based on a protocol from NREL [133]. Following pretreatment (or liquid-phase detoxification), a 13 wt% (12 wt% for IL-pretreated biomass) biomass slurry in 0.05 M sodium citrate buffer was pH-adjusted to 4.8 with 2 M NaOH. An enzyme cocktail consisting of 15 FPU/g glucan Spezyme CP, (Trichoderma reesei cellulase, Genencor, Palo Alto, CA), and 30 CBU/g glucan Novozyme 188, (β-glucosidase, Sigma (St. Louis, MO, Cat. No. C6150) was added to the biomass slurry; enzyme loadings were based on the glucan content of the
untreated biomass. The hydrolysis was conducted in a water bath orbital shaker (New Brunswick, Enfield, CT) at 50 °C and 130 rpm for 72 hours.

To prepare the hydrolysate for detoxification or fermentation experiments, the hydrolysate was centrifuged at 10,000 rpm for 10 min and then filtered with a 0.22 μm filter to remove any solid particulate. The filtered hydrolysate was stored at 4°C until use.

3.2.6 Detoxification methods

3.2.6.1 Liquid phase detoxification of DA-pretreated biomass slurry

The DA-pretreated biomass slurry (13 wt%, pH 2, 100 ml) was transferred to a 500 ml screw capped bottle. The organic phase (100 ml) – hexane, TOA in hexane or TOPO in hexane – was added to the slurry for detoxification. To maximize bacterial growth and succinic acid production, the concentration of extraction agent (TOA, TOPO) used for LLRE detoxification was varied over a range of 10-200 g/l. The bottle containing the organic phase and biomass slurry was capped tightly and the mixture was agitated at 200 rpm in an orbital shaker (New Brunswick, Enfield, CT) at 30 °C for 2 hours until inhibitor extraction reached equilibrium. To recover the organic phase, the slurry was centrifuged at 6000 rpm for 20 min and the organic phase was removed with a separation funnel. The detoxified biomass slurry was buffered by adding solid sodium citrate to 0.05 M and then adjusted to pH 4.8 with 2 M NaOH for enzymatic saccharification.
3.2.6.2 Liquid phase detoxification of IL-pretreated biomass hydrolysate

For IL-pretreated biomass, the hydrolysate generated during enzymatic saccharification was centrifuged at 10,000 rpm for 10 min and then filtered with a 0.22 μm filter to remove any solid particulate. The hydrolysate, pH-adjusted to 2.0 with 6 M HCl, was detoxified by addition of an equal volume of organic phase. The hydrolysate/organic phase mixture was agitated vigorously at 200 rpm in an orbital shaker at 30 °C for 2 hours until inhibitor extraction reached equilibrium. To recover the organic phase, the slurry was centrifuged at 6000 rpm for 20 min and the organic phase was removed with a separation funnel.

3.2.6.3 Solid-phase detoxification of hydrolysates

All solid-phase detoxification was performed on saccharified, filtered hydrolysates. Following saccharification, the inhibitor-containing hydrolysates were filtered and the pH was adjusted to 2.0 with 6 M HCl. The solid-phase loading was optimized for the DA-pretreated hydrolysate by determining the mass needed to maximize succinic acid yield in the fermentation; the optimized mass of adsorbent was also used for detoxification of the IL-pretreated hydrolysate. Three solid-phase adsorption materials were evaluated for detoxification efficacy: activated carbon, Amberlyst® A21 ion-exchange resin, and AMBERLITE® XAD4 polymeric resin. The solid-phase adsorbent was added to the biomass hydrolysate and the mixture was agitated at 200 rpm at 30 °C for 2 hours until equilibrium adsorption was attained. The solid-phase adsorbant material was then separated from the detoxified hydrolysate by filtration with Whatman No.1 filter paper. The hydrolysate was then pH adjusted to 7.0 with 2M
NaOH and filtered through a 0.22 μm filter for use in fermentation. The filtered hydrolysate was stored at 4°C until use.

3.2.7 Fermentation

3.2.7.1 Microorganisms and inoculum preparation

*Actinobacillus succinogenes* type strain 130Z (ATCC 55618) was purchased from the American Type Culture Collection (ATCC, Manassas, VA).

In all the fermentations, media was inoculated from a second generation liquid culture. The second generation liquid culture was produced by inoculating a colony from the second generation agar slant tube in 100 ml of TSB media. The culture was grown aerobically in a 250 mL shake flask at 37 °C with agitation at 130 rpm until the concentration, as measured by optical density at 660 nm (OD$_{660}$) reached the desired level. The theoretical OD$_{660}$ of the culture was determined by measuring the OD$_{660}$ on a diluted culture sample and multiplying the absorbance by the dilution factor; an OD$_{660}$ of 1.0 represents a dry weight of 567 mg bacteria/L. The theoretical OD$_{660}$ used in these experiments was 4.45±0.13, reached after approximately 24 hrs in culture. The second generation liquid culture was stored at -80 °C until use.

3.2.7.2 Fermentation and microbial growth experiments

Fermentation was carried out anaerobically in 158 mL serum vials. MgCO$_3$ (~4 g) was added to each vial prior to autoclaving and served as a solid-phase pH buffer during the fermentation. The complete fermentation media consisted of either biomass hydrolysate or model sugar solution (35 g/L glucose and 27 g/L xylose) supplemented
with 2 mg/L biotin; 10 mM Naphos (1.24 g/L Na₂HPO₄ and 0.74 g/L NaH₂PO₄ give a pH=6.8); 4 g/L Na₂CO₃, and 10 g/L yeast extract. For control experiments testing the effect of inhibitor concentration on microbial growth and succinic acid production, inhibitors were added to the complete model sugar fermentation medium in varying concentrations. The medium components, inoculum, and fermentation samples were transferred into and out of the vials by sterile syringe; the vial headspace was filled with sterile carbon dioxide to maintain anaerobic conditions. Fermentation was initiated by addition of 4 vol% (volume inoculum to total working volume) of the second generation bacteria inoculum (OD₆₆₀ of 4.45±0.13) thawed at room temperature; the total working volume of these fermentations was 50 ml. The fermentation vials were maintained at 37 °C in a shaker incubator with continuous 130 rpm agitation. The fermentations were conducted for 48 hours with 1 mL samples collected at 0, 4, 8, 12, 24, 36 and 48 hr. Bacteria concentration was determined using OD₆₆₀ absorption on fermentation samples after treating with 6 M HCl to dissolve any solid MgCO₃ in the sample.

### 3.2.8 Analytical methods

Samples were analyzed for sugars, organic acids and furans (furfural and HMF) via HPLC using an Agilent 1100 HPLC system equipped with a Shodex SH1011 ion exchange column and a refractive index (RI) detector. Samples and standards were processed using a mobile phase of 5 mM sulfuric acid at 0.6 ml/min; the column temperature was 65°C, and the detector temperature was 35°C.

Phenolic compounds and standards were analyzed using the same HPLC with a ZORBAX SB-C18 column (Agilent, Santa Clara, CA) and UV detection at 280 nm. The mobile phase (0.7 ml/min) was a gradient of two phases: (A) acetonitrile and (B) water
with 0.1% (v/v) glacial acetic acid. The gradient (v/v) applied was: 0-6 min – 20% A + 80% B; 7-9 min – 40% A + 60% B; and 10-20 min – 20% A + 80% B. Both the column and detector temperatures were set at 35°C.

Total phenolic compounds in the hydrolysate were quantified using the Folin–Ciocalteu method [134] with vanillin as the calibration standard. The Folin-Ciocalteu reagent contains a specific redox reagent that will form a blue colored complex with phenolic compounds. The blue complex has a maximum absorption in the region of 750 nm, and absorption is proportional to the total quantity of phenolic compounds present that can be detected spectrophotometrically at a wavelength of 750 nm [135].

All experiments (except production of IL-pretreated biomass hydrolysate) were performed at least in duplicate, and values are reported as mean ± standard deviation.

### 3.3 Result and discussion

After enzymatic saccharification, both the dilute-acid (DA) and ionic-liquid (IL) pretreated hydrolysates had a total sugar concentration of approximately 60 g/L. The DA-pretreated hydrolysate contained a substantial amount of phenolics (4.73 g/L) and furans (0.08 g/L of HMF and 0.14 g/L of furfural) as well as organic acids (see Table 3.1).

The most-concentrated low molecule weight phenolic compound (LMWPC) detected was ferulic acid, which represented 40% of the total phenolic compounds. Compared to the DA-pretreated hydrolysate, the IL-pretreated hydrolysate had significantly lower total phenolics and much lower concentrations of furans and levulinic acid, a rehydration product of HMF (see Table 3.1).
Table 3.1 Composition of wheat straw hydrolysate prior to detoxification. The values in the table for dilute acid pretreatment are based on measurements obtained from two hydrolysates; measurements for IL pretreated hydrolysate are from a single hydrolysate.

<table>
<thead>
<tr>
<th>Component</th>
<th>Dilute-acid pretreated hydrolysate (g/L)</th>
<th>Ionic-liquid pretreated hydrolysate (g/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sugars</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucose</td>
<td>34.64±1.45</td>
<td>36.92</td>
</tr>
<tr>
<td>Xylose</td>
<td>25.56±0.36</td>
<td>23.54</td>
</tr>
<tr>
<td>Arabinose</td>
<td>1.13±0.05</td>
<td>0.99</td>
</tr>
<tr>
<td>Sugar degradation products</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Furfural</td>
<td>0.14±0.01</td>
<td>0.03</td>
</tr>
<tr>
<td>HMF</td>
<td>0.08±0.02</td>
<td>0.04</td>
</tr>
<tr>
<td>Levulinic acid</td>
<td>0.71±0.08</td>
<td>0.03</td>
</tr>
<tr>
<td>Formic acid</td>
<td>1.09±0.17</td>
<td>0.76</td>
</tr>
<tr>
<td>Acetic acid</td>
<td>1.98±0.13</td>
<td>1.58</td>
</tr>
<tr>
<td>Lignin degradation products</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total phenolics</td>
<td>4.73±0.43</td>
<td>0.84</td>
</tr>
<tr>
<td>Caffeic acid</td>
<td>0.11±0.01</td>
<td>0.02</td>
</tr>
<tr>
<td>p-Coumaric acid</td>
<td>0.12±0.01</td>
<td>0.03</td>
</tr>
<tr>
<td>Ferulic acid</td>
<td>1.88±0.09</td>
<td>0.31</td>
</tr>
<tr>
<td>Syringic acid</td>
<td>0.21±0.01</td>
<td>0.02</td>
</tr>
<tr>
<td>Vanilllin</td>
<td>0.32±0.02</td>
<td>0.02</td>
</tr>
</tbody>
</table>
3.3.1 Effects of major contaminants in the hydrolysates on fermentation outcomes

To better understand the potential impact of the phenolic compounds on fermentation, the major phenolic inhibitors present in the hydrolysates were evaluated for their impact on bacteria growth and succinic acid yield of *A. succinogenes* (Figure 3-1). Using model sugar solutions spiked with the low molecular weight phenolic compounds (LMWPCs) at 1 and 2 g/l, the most inhibitory of the compounds tested was ferulic acid. Ferulic acid at 1 g/l reduced the maximum bacteria concentration by 45% and the succinic acid yield by 82%; this is of considerable concern for the DA-pretreated hydrolysate which contains ferulic acid at 1.88 g/l. While none of the other LMWPCs are present in either hydrolysate at concentrations near 1 g/l, it remains a concern that the synergistic effects of multiple inhibitory compounds in the hydrolysate may result in lower fermentation yields than expected based on the inhibition associated with the individual compounds.
Figure 3-1 (A) Relative maximum bacteria concentration and (B) relative succinic acid yield after 24 hour fermentation by *A. succinogenes* with 1 g/l or 2 g/l phenolic acids added to the culture broth. Results shown are relative to those obtained from an inoculum grown in simulated hydrolysate containing 27 g/L xylose and 35 g/L glucose with no inhibitors. In the control culture, the maximum bacteria concentration reached was 4.45 g/l and the succinic acid (SA) yield was 0.75 g SA/g sugar. Theoretical yield for SA production is 0.75 g SA/g sugar.
The concentration-dependent inhibition of *A. succinogenes* growth and succinic acid production was also evaluated for sugar degradation products. Both furfural and HMF showed similar inhibition, with a 50% reduction in maximum bacteria concentration and > 55% reduction in succinic acid production at approximately 4 g/l (Figure 3-2).
Figure 3-2 (A) Relative maximum bacteria concentration and (B) relative succinic acid yield after 24 hour fermentation by *A. succinogenes* with different concentration of furfural or HMF added to the culture broth. Results shown are relative to those obtained from an inoculum grown in simulated hydrolysate containing 27 g/L xylose and 35 g/L glucose with no inhibitors. In the control culture, the maximum bacteria concentration reached was 4.45 g/l and the succinic acid (SA) yield was 0.75 g SA/g sugar.

Compared to phenolics and furans, *A. succinogenes* is significantly less sensitive to inhibition by the organic acids produced in the hydrolysates. Lin et al. [136] determined that inhibition of succinic acid production by formic acid or acetic acid occurred with this organism at an IC$_{50}$ of 8 g/l for formic acid and 20 g/l for acetic acid, concentrations 5-fold higher than present in our hydrolysates (Table 3-1). In our experiments, levulinic acid at 10 g/l resulted in a 50% reduction in maximum cell concentration of *A. succinogenes* (data not shown).

While the concentrations of the organic acids are relatively low in both hydrolysates, a synergic inhibitory effect has been observed when furans and/or furan-derivatives are present with phenolic compounds [113]. One recent study indicated that HMF and syringaldehyde were individually toxic towards the fermenting yeast at 1.0 g/l, and similar inhibition occurred if their combined concentrations were 1 g/l [137]. Therefore, when multiple inhibitors are present, the toxic effect may be more closely related to the total toxin load.
3.3.2 Detoxification of the DA-pretreated hydrolysate for maximum succinic acid production

Theoretically, detoxification methods can inserted in the biomass deconstruction process prior anywhere prior to fermentation. However, if contaminants are removed prior to enzymatic saccharification, sugar losses may be reduced as less soluble sugars are present at this step. In addition, if fermentation inhibitors are also inhibitory to the saccharification enzymes, higher saccharification yield may be achieved. For solid-phase detoxification methods, recovery and separation of the adsorbent from other solids in the biomass slurry is not feasible. Hence, we have used different strategies for detoxification depending on pretreatment method (dilute acid or ionic liquid) and detoxification phase (solid or liquid phase) as shown in Figure 3-3. Solid-phase detoxification methods were used only on saccharified, filtered hydrolysates. For liquid-phase detoxification, the presence of solids does not interfere with either the detoxification or the solvent recovery process. Thus, we applied liquid-phase detoxification methods to the DA-pretreated biomass slurry prior to enzymatic saccharification. Since the IL-pretreatment process generated significantly less inhibitors than DA-pretreatment, all detoxification methods were applied to the saccharified, filtered IL-pretreated hydrolysate.
Figure 3-3 Relative succinic acid yield of the dilute-acid and ionic-liquid pretreated wheat straw hydrolysate with different conditioning methods. Relative succinic acid yield represents the succinic acid yield in the culture relative to the theoretical yield. SA

All detoxification procedures were optimized for the removal of contaminants from the DA-pretreated biomass since this hydrolysate had higher concentrations of contaminating compounds. Sugar loss, maximum bacteria concentration, and succinic acid yields were measured for all methods as a function of the adsorbent/extractant loading, with the goal of establishing detoxification parameters for each method that maximized succinic acid production. Results for the solid-phase detoxification methods are shown in Figure 3-4.
Activated carbon (w/v)

Relative maximum bacteria concentration
Relative succinic acid yield
Sugar loss

Ion exchange A21 resin (w/v)

Relative maximum bacteria concentration
Relative succinic acid yield
Sugar loss
Figure 3-4 Sugar loss, maximum bacteria concentration, and succinic acid production for each of the solid-phase detoxification methods applied to dilute-acid pretreated wheat straw hydrolysate. Sugar losses are due to non-specific adsorption; fermentation results are expressed relative to those of a model hydrolysate with similar sugar concentrations (27 g/L xylose and 35 g/L glucose). Results are shown for different loadings of (A) activated carbon and (B) Amberlyst® A21 resin; and (C) XAD-4 resins. The best succinic acid production was achieved with loadings of 5 wt% of activated carbon; 10 wt% of Amberlyst® A21 resin; and 10 wt% of AMBERLITE® XAD-4 resin.

Succinic acid production for all three solid-phase methods (activated carbon, Amberlyst® A21 ion-exchange resin, and AMBERLITE® XAD-4 polymeric resin) went through a maximum due to a positive correlation between sugar adsorption and solid-phase adsorbent loading. The liquid-phase detoxification methods (Figure 3-5) had minimal sugar losses and hence succinic acid levels plateaued with increasing extractant concentration.
Figure 3-5 Sugar loss, maximum bacteria concentration, and succinic acid production for each of the liquid-phase detoxification methods applied to dilute-acid pretreated wheat
straw slurry prior to enzymatic hydrolysis. Fermentation results are expressed relative to the yield of a model hydrolysate with similar sugar concentrations (27 g/L xylose and 35 g/L glucose). Results are shown for different loadings of (A) TOPO and (B) TOA in hexane for LLRE. The best succinic acid production was achieved with loading of 100 g/L of TOPO or 100 g/L of TOA in hexane for LLRE

Figure 3-6 Removal efficiency of phenolics, furans, and organic acids from wheat straw hydrolysate produced using (A) dilute-acid and (B) ionic-liquid pretreatment. For dilute-acid pretreatment, liquid-phase detoxification methods were applied prior to enzymatic saccharification. For all others, detoxification was performed on the filtered hydrolysate.

The maximum succinic acid yields achieved with each detoxification method are summarized in Figure 3-6. Data shown are relative succinic acid (SA) yield, where the SA yield in each experiment (g SA/g sugar) is normalized to the theoretical yield (0.75 g SA/g sugar); fermentations with model hydrolysate solutions all reached theoretical SA
yield. Liquid-phase reactive extraction methods outperformed all of the other detoxification methods tested, with relative succinic acid yields of greater than 96%. For the solid-phase detoxification methods, the relative succinic acid yield under conditions of maximum SA production was highest for AMBERLITE® XAD-4, followed by Amberlyst® A21 and activated carbon. When comparing inhibitor removal, activated carbon removed more total phenolics and furans than any other method, but also removed a higher percentage of the hydrolysate sugars (>15%, Figure 3-7 A), resulting in lower succinic acid yield. The ion-exchange and polymeric resins, while having dramatically different levels of contaminant removal for total phenolics, furans, and organic acids, adsorb considerably less hydrolysate sugar than activated carbon and have high relative SA yields (>89%). As indicated previously by our experiments on inhibitor toxicity to succinic acid production by *A. succinogenes*, the LMWPCs found in our DA-pretreated hydrolysate are the most-inhibitory of the contaminants present. Figure 3-8 shows that the ion-exchange resin, polymeric resin, and both LLRE methods were particularly effective at removing caffeic, p-coumaric, ferulic and syringic acids, whereas activated carbon and hexane extraction were less effective. Since total removal of LMWPCs tracks the SA production results, these compounds appear to be the most important inhibitors to remove from the hydrolysate; however, the overall reduction in inhibitor burden may also favorably impact the SA production.
Figure 3-7 Removal efficiency of phenolics, furans, and organic acids from wheat straw hydrolysate produced using (A, C) dilute-acid and (B,
D) ionic-liquid pretreatment. Detoxification was conducted using the conditions that maximized SA production. Liquid-phase detoxification methods were applied to the DA-pretreated biomass slurry prior to enzymatic saccharification. All other detoxifications were performed on the saccharified, filtered hydrolysates.

Figure 3-8 Removal efficiency of low molecule weight phenolic compounds (LMWPCs) and total phenolic compounds by the different detoxification methods. Detoxification was conducted as described in Figure 3-7.

As shown in the Figure 3-2, the solid phase and liquid phase detoxification methods were applied at different points in the biomass deconstruction process. Solid phase methods were applied to saccharified hydrolysates, whereas LLRE was applied...
to the DA-pretreated biomass slurry prior to enzymatic saccharification. If inhibitor removal also impacts saccharification efficiency, then LLRE detoxification prior to enzymatic saccharification should result in increased sugar release. Hence, we measured the kinetics of saccharification for the DA-pretreated biomass slurry with and without liquid-phase detoxification. As shown in Figure 3-8, detoxification of the slurry by either of the LLRE methods improved the saccharification efficiency, with an additional 5.5 g/L sugars (11% more glucose, 5% more xylose/arabinose) released after 72 hrs in the conditioned hydrolysates. Thus, in addition to having higher SA yield due to effective removal of LMWPCs (Figure 3-7) and negligible sugar loss during the detoxification (Figure 3-6), the SA production is actually higher due to improved enzymatic hydrolysis and increased sugar basis in the hydrolysate.
Figure 3-9 Impact of detoxification on sugar release from dilute acid-pretreated wheat straw during the enzymatic hydrolysis. LLRE detoxification results in a 10% increase in sugar yield for (A) glucose and (B) xylose and arabinose, relative to the undetoxified hydrolysate.
The IL-pretreated hydrolysate had much lower initial inhibitor concentrations and, even without any detoxification, was able to produce SA at near 70% of the control culture level (Figure 3-6). The IL-pretreated hydrolysate was detoxified after saccharification using the same methods as optimized for the DA-pretreated hydrolysate; the succinic acid yields for this hydrolysate for all methods were equal to or better than those achieved with the DA-pretreated hydrolysate, as expected. For the LLRE methods, the actual SA production (g) is higher in the DA-pretreated hydrolysate than in the IL-pretreated hydrolysate due to the improved sugar release during the enzymatic saccharification; this increase is SA production is not reflected in the yield (g SA/g sugar). Presumably, lower loadings of the solid-phase adsorbents could be used to reduce the inhibitor concentrations to acceptable levels in the IL-pretreated hydrolysate with correspondingly lower sugar losses and higher SA yields than shown here. Thus, the detoxification method implementation for the solid-phase methods is highly-dependent on the composition and concentration of the inhibitor compounds present.

### 3.3.3 Continuous detoxification of DA-pretreated hydrolysate with LLRE

Reactive extraction is a reversible complexation between the extracting agent (TOA or TOPO used here) in the organic phase and the un-ionized inhibitor in the hydrolysate. Since the weakly-acidic inhibitors will be un-ionized at low pH [131], detoxification of the biomass slurry immediately following DA-pretreatment (pH below 2) is ideal. The predominant LMWPCs have pKa’s near 4.75 or higher [138], allowing their effective complexation and removal from the hydrolysate. The contaminants, once sequestered in the organic phase, can be stripped out by contacting the organic phase with a strongly basic aqueous phase. Under these
conditions, the contaminants ionize fully and transfer to the polar aqueous strip solution [131].

Unlike solid-phase detoxification methods, the liquid-phase reactive extraction methods evaluated here cannot be “over-applied”. Thus, if hydrolysate composition varies significantly, LLRE methods using conditions optimized for high contaminant loadings can be used without concern for loss of sugars that would occur with solid-phase detoxification methods. In addition, since the liquid-liquid reactive extraction method allows easy separation of the contaminant-laden organic phase from the hydrolysate, it is well-suited for use in the design of a sustainable process design for hydrolysate detoxification. To determine if reuse and recycle of the organic extraction phase is feasible for removing inhibitors from multiple batches of hydrolysate, it is necessary to determine if repeated extraction and stripping cycles will result in loss of reactive extraction efficiency and reduction in product yield. Thus, we have designed a cyclic batch process that simultaneously detoxifies the hydrolysate and regenerates the organic extraction phase. The process schematic is shown in Figure 3-10 and the experimental set-up is pictured in Figure 3-11. For each batch of biomass slurry processed, the same organic (200 g/l TOPO in hexane) and stripping (water, maintained at pH 10 by addition of 10 M NaOH as needed) phases were used. The organic phase was recirculated continuously for 30 min with two pumps at 30 ml/min; half of the organic phase was in contact with the hydrolysate or the strip solution at all times. The inhibitors were extracted to the organic phase on the detoxification side and transferred to the stripping solution on the regeneration side. Equal volumes (100 ml) of all three phases were used.
Figure 3-10 Schematic design for continuous hydrolysate detoxification and organic phase regeneration. In the experimental set-up, the organic phase (200 g/l TOPO in hexane) was recirculated between the hydrolysate and stripping phases at 30 ml/min for 30 min. Equal volumes (100 ml) of hydrolysate, organic extraction phase, and stripping solution were used. After 30 min, the pumps were stopped, the hydrolysate was separated from the organic phase using a separation funnel, and a fresh batch of hydrolysate was contacted with the organic phase.
We have evaluated the performance of this cyclic-batch process for the detoxification of 10 identical batches of DA-pretreated biomass slurry processed sequentially. As shown in Figure 9, there is little change in the removal efficiency of the total phenolics and furans over the course of the 10 cycles. Levulinic acid is the only compound whose removal efficiency drops significantly, but the 15% decrease in removal corresponds to only 0.1 g/l. All of the LMWPCs (caffeic p-coumaric, ferulic and syringic acids and vanillin) in the hydrolysate are removed at a level of over 98% for every cycle of the cyclic-batch detoxification process.

Figure 3-11 Photo of the experimental set-up used for cyclic batch detoxification of hydrolysate. All batches were processed at room temperature (~19 °C)
Figure 3-12 Results for inhibitor removal in the cyclic batch conditioning of DA-pretreated wheat straw biomass slurry using LLRE with 200 g/l TOPO in hexane. After each 30 min cycle, the detoxified biomass slurry was removed and analyzed for inhibitor concentration. 100 ml of biomass slurry was used for each cycle. The same organic phase and receiving phase were used for all cycles. The initial concentration of contaminants in the slurry was: (A) total phenolics 4.93g/L; furfural 0.15 g/L;
HMF 0.09 g/L; levulinic acid 0.73 g/L; and (B) caffeic acid 0.12 g/L; p-coumaric acid 0.13 g/L; ferulic acid 1.99 g/L; syringic acid 0.22 g/L; and vanillin 0.33 g/L

3.4 Conclusion

Inhibitors, particularly low molecular weight phenolic compounds, can have a more

The selection of detoxification method is highly dependent on the properties and amount of the inhibitors present in the hydrolysate as well as the sensitivity of the organism used in fermentation. Phenolics, furans and organic acid are considered to be the most-inhibitory compounds present in hydrolysate and their presence can have negative impacts on both enzymatic saccharification and fermentation. We have shown that the selection of the best conditioning method for a particular hydrolysate must be determined based on product yield as opposed to inhibitor removal as some detrimental effect on organism growth and product yield than others. We have also shown that liquid-liquid reactive extraction (LLRE) using TOPO is an easily-implementable detoxification methods that can be used for detoxification of either biomass slurry or saccharified hydrolysate with negligible sugar losses. By implementing LLRE prior to enzymatic saccharification, LLRE improves saccharification yield, leading to improved biomass utilization and higher succinic acid production. We have also demonstrated a cyclic-batch method for hydrolysate detoxification that incorporates simultaneous reactive extraction phase regeneration. This process has been demonstrated for detoxification of 10 batches of hydrolysate with little change is detoxification efficiency between batches.
Chapter 4

Succinic acid fermentation from algal biomass carbohydrates: utilization digested algal protein as a nitrogen source

4.1 Introduction

Microalgae has recently received increasing attention as a promising feedstock for bio-based products with economic and environmental benefits [139]. Unlike terrestrial crops (e.g., corn), microalgae can be cultivated on low-quality land, using wastewater and waste nutrients. In addition, its unicellular structure without lignin allows relatively milder pretreatment methods to break down the cell wall and release carbohydrates, such as mechanical disruption, thermolysis, microwaves, lasers, and sonication [140]. Although these chemical or physical methods are less severe, resulting in fewer inhibitors, all are highly energy-intensive and require greater equipment or maintenance costs; if applied to bio-based products (e.g., biofuels), a negative net energy output may occur, which is obviously unsustainable [141]. Therefore, further studies focus on alternative methods, with more consideration on energy consumption and overall cost evaluation to achieve several objectives: (1)
disrupt cell walls, (2) extract lipids, (3) release and hydrolyze polysaccharides to monomer sugars, and (4) release and hydrolyze proteins for a nitrogen source for fermentation.

As stated in Chapter One, microalgae is formed by four major components: carbohydrates, proteins, nucleic acids, and lipids. The carbohydrates in microalgae are composed of a mixture of neutral sugars, amino sugars, and uronic acids, and the amount of each composition is based on the species and growth conditions [142]. Protein-rich algae such as *Spirulina* contain up to 70% of protein, mainly composed of amino acid and peptides [143]. Lipids, mainly simple fatty acids and triglycerides, serve both as energy reserves and structural components (membranes) of the cell. In addition, vitamins, pigments, minerals, and other valuable trace elements are also present in the algae biomass [144]. All of these components can be utilized in various applications if recovered. Previously, marine algae were mainly used in lipid production for biodiesel [141] or its proteins for animal feed [144]. Recently, for a better economic output, algae growers (e.g., Solozyme and Sapphire Energy) have focused on producing a broader range of bio-based chemicals from organic acid (e.g., lactic acid) [27] to high-end nutrient and cosmetic products (e.g., astaxanthin or fucoxanthin) [145].

For starch-rich microalgae, cultures might be directly usable as feedstock in fermentation to various bio-based products (e.g., succinic acid), although pretreatment and/or enzymatic digestion could also be necessary to the carbohydrate polymers to monomeric sugars [146]. Traditional pretreatments for terrestrial lignocellulosic biomass use chemicals (acid or base) with a severe hydrothermal condition to achieve a high recovery of sugars. This is because lignin, a compact recalcitrant polymer consisting of aryl-groups, surrounds the carbohydrate part of the plant fibers, and
must be removed/unpacked by pretreatment in order to provide accessibility to the carbohydrates. However, it may not be necessary to apply the same level of severity to the algal biomass, since microalgae do not have lignin.

Although a milder pretreatment condition would reduce the energy input, a higher temperature of 140°C to 160°C and a sulfuric acid concentration from 1% to 3% v/v are required to give good sugar yield if no enzyme digestion is applied thereafter [26]. Under such conditions, the cost of energy consumption and equipment maintenance would be high. Enzyme hydrolysis can be conducted at lower temperatures (room temperature to 60°C) with fewer byproducts generated and no corrosion issues, and is a great candidate to replace the traditional pretreatment methods. However, the overall economic viability of enzyme-based processes is subject to the cost and ease of enzyme production. Commercially available enzymes, such as fungal α-amylase and protease, are manufactured with lower costs and are widely applied in the food industry. These enzymes are desired candidates for algae digestion. A previous study used α-amylase and glucoamylase to digest microalgae biomass for ethanol production by *S. cerevisiae* S288C via two steps: liquefaction by thermostable α-amylase at 90°C abd pH 6.0, then saccharification at 55°C and pH 4.5 [147]. The result also indicated that α-amylase shows protease activity that can hydrolyze the glycoprotein, the major structure in the microalgae cell wall. In the enzyme production by fungi, the product is not always pure; some α-amylase and glucoamylase blended in the protease [148]. While these enzymes are mixed as a cocktail for the digestion of algae, a synergistic effect might occur to drastically reduce the amount of each enzyme required.

Digestion by the enzyme not only releases sugar and lipids, but also protein, which can be an indispensable nutrient for the growth and metabolism of
microorganism cells in the fermentation to bio-based products. Some studies proved that nitrogen source feeding (ammonium, free amino acid, urea, or yeast extract) can increase the fermentation rate and improve the growth of cells [149]. Conversely, a lack of nitrogen nutrition will lead to the reduction in the succinic acid [150] or ethanol [151] formation rate and yield. In industrial processes, an additional nitrogen source feeding is necessary and costs a considerable portion of the total succinic acid cost. Previous studies used cheaper nitrogen sources, such as spent brewer’s yeast [152] or corn steep liquor [153] in succinic acid fermentation. In addition, microalgae biomass, as one cheaper nitrogen source, has been used in ethanol fermentation by *Saccharomyces cerevisiae* [146]. However, microalgae as the nitrogen source in succinic acid fermentation has not been evaluated. Presently, the protein portion of microalgae are mainly used as one byproduct in the low-profit field, such as animal feedstock or fertilizer [146]. One study has shown that the protein portion in the varied species of microalgae ranges from 6% to 71% (w% of dry matter) [144]. These proteins can be derived to the free amino acid during the appropriate conversion process, which is an ideal nitrogen source in fermentation. In general, the free amino acids (free amino nitrogen and peptides) are the better nitrogen source in comparison to the ureas, because the urea must be degraded further to ammonia in two steps, which cannot be utilized directly [154]. Therefore, protein-rich microalgae biomass could serve as an alternative low-cost nitrogen source in succinic acid fermentation.

In this study, microalgae biomass was hydrolyzed by enzyme treatment with different conditions and levels. As with the last chapter, *Actinobacillus succinogenes* was also used as a model microorganism to evaluate the effects of the utilization of the carbon source and nitrogen source from the microalgae biomass.
4.2 Materials and Methods

4.2.1 Chemicals and reagent

Bacto™ tryptic soy broth (TSB) (Becton Dickinson, Franklin Lakes, NJ) were purchased from Fisher Scientific (Waltham, MA). Fermentation media (glucose, xylose, arabinose, phosphate salts, biotin and yeast extract) and all other chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

The enzyme used in the saccharification: α-amylase (Aspergillus oryzae, 28.75 U/mg, Cat No.10065), glucoamylase or amyloglucosidase (Aspergillus niger, 30 U/mL, Cat No. A7095) and protease (Aspergillus oryzae, 500 U/mL, Cat No. P6110) were purchased from Sigma (St. Louis, MO).

Microalgae biomass used in the enzymatic hydrolysis for sugar recovery and nitrogen source for fermentation are kind gift from AlgEvolve (Corvallis, MN).

Two microalgae biomass used for lipids extraction: Chlorella sp. SLA-04 and Schizochitrium limacium SR21 were cultivated by Vadlamani, Agasteswar, who is the research partner in our research group.

4.2.2 Determination of composition of microalgae biomass

The sugar content of microalgae was determined according to standard National Renewable Energy Laboratory (NREL) analytical procedure (Determination of total carbohydrates in algal biomass) [155]. The lipid content of microalgae was determined according to standard National Renewable Energy Laboratory (NREL) analytical procedure (Determination of Total Lipids as Fatty Acid Methyl Esters (FAME)) [44]
4.2.3 Enzymatic hydrolysis of dried microalgae

Enzymatic hydrolysis experiments were conducted using protease, α-amylase, and glucoamylase, and combinations. A mixed culture of lipid-lean algal biomass (lipid content <5% (w/w)) was obtained from a commercial wastewater treatment facility (AlgEvolve, Corvallis, MN). The biomass had a carbohydrate content of 24.5% (w/w). The digestion experiments were performed at a solid loading of 16% (w/v) with 50 mL working volume. Citric acid was added to 50 mM in each serum bottle that contained a microalgae solution. The pH was then adjusted to a specific pH in each serum bottle using 10 M NaOH solution. For enzymatic loading, different amounts of α-amylase (α-amylase stock was 28.75 units/mg), amyloglucosidase (300 AGUnits/mL), and protease (stock of 500 Units/mL) were added to the solution in each bottle. For unit definitions, 1 unit of α-amylase is the amount of enzyme that liberates 1 μmol maltose per minute at pH 6.0 and 25 °C, 1 Novo amyloglucosidase unit (AGU) is the amount of enzyme that cleaves 1 μmol of maltose per min at pH 4.3 and 25 °C, and 1 unit of protease is the amount of enzyme that hydrolyzes 1 μmol of L-leucine-p-nitroaniline per min.

Enzymatic hydrolysis reactions were performed in sterilized rubber-capped serum vial bottles for 6 h in a temperature-controlled air bath shaker at 55 °C with agitation at 200 rpm.

4.2.4 Lipid recovery from lipid-rich microalgae biomass

For lipid recovery and analysis, a 3:2 (v/v) mixture of hexane/iso-propanol was used. About 0.5 mL of the solvent mixture was added to 300 μL of the digested samples, and extraction was carried out at 90 °C for 30 min. Lipids in the extraction
solvent were analyzed and quantified using a gas chromatograph (GC) connected with a flame ionization detector (FID).

**4.2.5 Optimization of the temperature, pH, and loading amount of enzymes in enzymatic hydrolysis of microalgae biomass**

Based on the enzymatic hydrolysis method, we tried to optimize the parameters applied in this method, including pH, temperature, and enzyme loading. For pH and temperature optimization, we fixed the loading of the total biomass amount at 1.6 g in a 10 mL buffer solution, glucoamylase loading at 3.75 U/g, α-amylase loading at 312.5 U/g, and protease loading at 62.5 U/g. The statistical software package Design-Expert 8.0 (trial version) was used to design the experimental conditions. The total number of experiments with two factors was 12. The design matrix with two variables (temperature (A) and pH (B)) with random levels generated by the software is presented in Error! Reference source not found.. The process performance was evaluated by analyzing the total sugar yield after 1 h enzymatic hydrolysis. In optimization, the response can be related to the chosen factors with linear or quadratic models. A quadratic model, which includes a linear model, is given as follows:

\[ Y_{\text{yield}} = \alpha_0 + \alpha_1 \cdot A + \alpha_2 \cdot B + \alpha_3 \cdot A \cdot B + \alpha_4 \cdot A^2 + \alpha_5 \cdot B^2 \]

Where \( Y_{\text{yield}} \) is the predicted response; \( \alpha_0 \) is the intercept; \( \alpha_1 \) and \( \alpha_2 \), linear coefficients; \( \alpha_3 \), interaction coefficients; \( \alpha_4 \) and \( \alpha_5 \), squared coefficients. Data were processed for this equation using the Design-Expert 8.0 program including analysis of variance (ANOVA) to obtain the interactive effects between the process variables and the response. The quality of fit of the polynomial model was expressed by the
coefficient of determination $R^2$, and its statistical significance was checked with the $F$-test in the same program.

Table 4.1 Design matrix for optimizing the pH and temperature for enzymatic hydrolysis of the microalgae biomass

<table>
<thead>
<tr>
<th>Experiment Runs</th>
<th>Factor 1 A: Temperature °C</th>
<th>Factor 2 B: pH</th>
<th>Response 1 Sugar yield %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>30</td>
<td>2.5</td>
<td>17.8</td>
</tr>
<tr>
<td>2</td>
<td>30</td>
<td>3.0</td>
<td>32.9</td>
</tr>
<tr>
<td>3</td>
<td>30</td>
<td>4.5</td>
<td>43.6</td>
</tr>
<tr>
<td>4</td>
<td>30</td>
<td>6.0</td>
<td>36.8</td>
</tr>
<tr>
<td>5</td>
<td>35</td>
<td>2.5</td>
<td>16.7</td>
</tr>
<tr>
<td>6</td>
<td>35</td>
<td>4.0</td>
<td>38.4</td>
</tr>
<tr>
<td>7</td>
<td>40</td>
<td>4.0</td>
<td>44.0</td>
</tr>
<tr>
<td>8</td>
<td>40</td>
<td>6.0</td>
<td>42.8</td>
</tr>
<tr>
<td>9</td>
<td>60</td>
<td>2.5</td>
<td>16.4</td>
</tr>
<tr>
<td>10</td>
<td>60</td>
<td>4.5</td>
<td>49.0</td>
</tr>
<tr>
<td>11</td>
<td>60</td>
<td>5.0</td>
<td>46.4</td>
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<tr>
<td>12</td>
<td>60</td>
<td>6.0</td>
<td>44.7</td>
</tr>
</tbody>
</table>

Similarly to the previous study for pH and temperature, we fixed the loading amount for protease, and used different levels for loading $\alpha$-amylase and glucoamylase. The total number of experiments with two factors with five levels each was 25. The design matrix with two variables ($\alpha$-amylase (A) and glucoamylase (B)) with five levels generated by the software is presented in Error! Reference source not found.. The process performance was evaluated by analyzing the total sugar yield after 1 h enzymatic hydrolysis. In optimization, the response can be related to the chosen factors with linear or quadratic models. A quadratic model, which also includes a linear model, is given as follows:

$$Y_{\text{yield}} = \beta_0 + \beta_1 \ast A + \beta_2 \ast B + \beta_3 \ast A \ast B + \beta_4 \ast A^2 + \beta_5 \ast B^2$$

Where $Y_{\text{yield}}$ is the predicted response; $\beta_0$ is the intercept; $\beta_1$ and $\beta_2$, linear coefficients; $\beta_3$, interaction coefficient; $\beta_4$ and $\beta_5$, squared coefficients. The data were
processed for this equation using the Design-Expert 8.0 program including analysis of variance (ANOVA) to obtain the interactive effects between the process variables and the response. The quality of fit of the polynomial model was expressed by the coefficient of determination \( R^2 \), and the statistical significance was checked with the F-test in the same program.

Table 4.2 Design matrix for optimizing the loading amount of α-amylase and glucoamylase for enzymatic hydrolysis of the microalgae biomass

<table>
<thead>
<tr>
<th>Run</th>
<th>Factor 1 A: Amylase (kUnits)</th>
<th>Factor 2 B: Glucoamylase (kUnits)</th>
</tr>
</thead>
<tbody>
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</tr>
<tr>
<td>2</td>
<td>468.75</td>
<td>2.8125</td>
</tr>
<tr>
<td>3</td>
<td>312.5</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>625</td>
<td>3.75</td>
</tr>
<tr>
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</tr>
<tr>
<td>6</td>
<td>0</td>
<td>3.75</td>
</tr>
<tr>
<td>7</td>
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<td>1.875</td>
</tr>
<tr>
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<td>625</td>
<td>2.8125</td>
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<td>156.25</td>
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<tr>
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<td>625</td>
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</tr>
<tr>
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<tr>
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<tr>
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<tr>
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<td>0.9375</td>
</tr>
<tr>
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<td>0.9375</td>
</tr>
<tr>
<td>25</td>
<td>312.5</td>
<td>3.75</td>
</tr>
</tbody>
</table>

4.2.6 Enzyme separation with SDS-PAGE

Dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) was used to separate protease, α-amylase, and glucoamylase. Individual enzymes were loaded
on Any kD Mini-PROTEAN TGX precast gels (Bio-Rad, Hercules, CA, US) using Tris/Glycine buffer (Bio-Rad). Initial dilutions of the enzyme were diluted to 1% (v/v) in the 5X Laemmli Buffer (Bio-Rad) before boiling at 90 °C for 5 min. Prestained Kaleidoscope Standards (Bio-Rad, Hercules, CA, US) were loaded together with the samples. Enzyme samples were applied per lane and were processed for 60 min at 110 V. Once the process was completed, the gel was stained with Coomassie stain overnight and destained with a solution of 70% DI water, 20% methanol, and 10% acetic acid until the protein bands were clear.

4.2.7 Microorganisms

*Actinobacillus succinogenes* type strain 130Z (ATCC 55618) was purchased from the American Type Culture Collection (ATCC, Manassas, VA). A single colony was selected from the primary ATCC agar plate and cultured in Tryptic soy broth media (Bacto, Franklin Lakes, NJ) in a 250 mL Erlenmeyer flask with 100 ml working volume at 37 °C for 24 h. An aliquot from this culture was streaked on an agar slant tube to produce the second generation of the strain. In all fermentations, the media were inoculated from a second-generation liquid culture. The second-generation liquid culture was produced by inoculating a colony from the second-generation agar slant tube in 100 ml of Tryptic soy broth media in a 250 mL shake flask at 37 °C until the OD$_{660}$ reached 4.45±0.13 (approximately 24 h).

4.2.8 Preparation for the fermentation media

After the 1 to 6 h enzymatic hydrolysis, the algae biomass hydrolysate samples were cooled to room temperature. For the medium with microalgae solids, the hydrolysate was directly used as the medium after the pH was adjusted to neutral with 2 M NaOH. For the medium without microalgae solids, the hydrolysate were
transferred to centrifuge tubes and centrifuged at 5000 rpm for 10 min; the clarified supernatant solutions were transferred back to the serum vials for fermentation after the pH was adjusted to neutral with 2 M NaOH. To verify whether the nitrogen content in the microalgae could be used as the nitrogen source in fermentation, various amounts of external nitrogen source in the form of yeast extract (YE) at 2 g/L were added to the medium before fermentation. As controls, model sugar solutions with the same sugar concentration as the microalgae biomass hydrolysate and 2, 5, or 10 g/L of YE were also prepared for fermentation.

4.2.9 Fermentation

Fermentation was carried out anaerobically in 158 mL serum vials. MgCO\(_3\) (about 4 g) was added to each vial before autoclaving and served as a solid phase pH buffer during fermentation. The complete fermentation media consisted of either biomass hydrolysate or model sugar solution (40 g/L glucose and 20 g/L xylose) supplemented with 2 mg/L biotin; 10 mM Naphos (1.24 g/L Na\(_2\)HPO\(_4\) and 0.74 g/L NaH\(_2\)PO\(_4\) give pH=6.8); 4 g/L Na\(_2\)CO\(_3\), and 10 g/L yeast extract. For the control experiments that tested the effect of the inhibitor concentration on microbial growth and succinic acid production, inhibitors were added to the complete model sugar fermentation medium in varying concentrations. The medium components, inoculum, and fermentation samples were transferred into and out of the vials with a sterile syringe; the vial headspace was filled with sterile carbon dioxide. Fermentation was initiated by the addition of 4 vol% (volume inoculum to total working volume) of the bacteria inoculum (OD\(_{660}\) of 4.45±0.13); the total working volume of these fermentations was 50 ml. The fermentation vials were maintained at 37 °C in a shaker.
incubator with continuous 130 rpm agitation. Fermentation was conducted for 48 h with 1 mL samples collected at 0, 4, 8, 12, 24, 36, and 48 h.

### 4.2.10 Analytical techniques

Samples collected from the fermentation experiments were diluted ten-fold in ultrapure water and filtered through 0.22 µm syringe filters. Calibration standards for glucose, xylose, succinic acid, acetic acid, and ethanol were prepared in a similar manner. All samples were analyzed via HPLC using an Agilent 1100 HPLC system equipped with a Shodex SH1011 ion exchange column and a refractive index (RI) detector. Samples were processed using a mobile phase of 5 mM sulfuric acid at 0.6 ml/min; the column temperature was 65 °C, and the detector temperature was 35 °C.

### 4.3 Results

#### 4.3.1 Composition analysis of microalgae biomass

<table>
<thead>
<tr>
<th>Composition</th>
<th>Microalgae (From AlgEvolve)</th>
<th>Chlorella sp. SLA-04</th>
<th>Schizochitrium limacium SR21</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total sugar</td>
<td>24.6%</td>
<td>19.2%</td>
<td>12.1%</td>
</tr>
<tr>
<td>Lipids</td>
<td>4.2%</td>
<td>35.0%</td>
<td>44.0%</td>
</tr>
</tbody>
</table>

The composition of the microalgae biomass has been determined according to the Laboratory analytical procedure by NREL. The microalgae from AlgEvolve has nearly 25% of sugar content, but has very small quantity of lipids. The two other species *Chlorella sp. SLA-04* and *Schizochitrium limacium* SR21 has a significant amount of lipids along with relatively low sugar content.
4.3.2 Enzymatic digestion with α-amylase, amyloglucosidase, and protease

To determine if addition of α-amylase (in combination with glucoamylase) results in higher recovery of monomeric sugars, experiments were performed at α-amylase loadings between 0.5 and 15 kU. About 150 U of glucoamylase was also added to the solution. The results of these experiments (Error! Reference source not found.) showed that at α-amylase loading below 5 kU, less than 60% of the biomass carbohydrates were converted to monomeric sugars. However, at α-amylase loading of 15 kU, nearly 90% of the polysaccharides were hydrolyzed to sugars. These results show that by adjusting the levels of α-amylase and glucoamylase appropriately, nearly complete hydrolysis of algal polysaccharides is possible. In all cases, hydrolysis was complete within 2 h. Longer enzymatic hydrolysis did not improve the sugar yield under these conditions.

![Graph showing sugar yield in enzyme hydrolysis over time for different α-amylase loadings](image)
Figure 4-1 Algae biomass enzymatic hydrolysis (saccharification) with various loadings of α-amylase from *Aspergillus oryzae* and fixed glucoamylase (150 AGU) pH=4.5 and 55 °C. Results based on 16% (w/v) microalgae biomass loading.

In addition, to evaluate the effect of each enzyme or different combination of these three enzymes in enzymatic hydrolysis, one or a mixture of the enzymes were added to the reaction. As was shown in Error! Reference source not found., the protease showed the ability to break the cell wall and release a significant amount of carbohydrates, which could be digested by the α-amylase and glucoamylase in the sugar monomers. However, α-amylase and protease combined do not significantly increase the sugar yield compared to using protease only. Without glucoamylase, the sugar yield dropped significantly. Protease might have some mixture of α-amylase but does not contain glucoamylase. Later, this hypothesis was proved by running the three enzymes on SDS-PAGE (Error! Reference source not found.). Therefore, protease from fungi such as *A. oryzae*, if not purified, might contain sufficient α-amylase to support carbohydrate hydrolysis.
Figure 4-2 Effect of enzyme cocktail composition on sugar release. Enzymatic hydrolysis of 16% (w/v) microalgae biomass solution after 2 h with various combinations of α-amylase, amyloglucosidase, and protease enzymes.
4.3.3 Optimized condition in enzymatic hydrolysis

Enzyme activity is normally affected by temperature and pH. In addition, the loading amount determines the reaction rate and final yield. To find the best condition for this enzymatic hydrolysis process, we ran experiments with different temperatures and pH.
The response data were analyzed in the Design-Expert software. The application of RSM yielded the following regression equation, which is an empirical relationship between succinic acid and the test variables in coded units:

\[ Y_{yield} = 44.89 + 1.2 \times A + 11.92 \times B + 2.4 \times A \times B - 0.88 \times A^2 - 14.64 \times B^2 \]

Where \( Y_{yield} \) is the succinic acid produced as a function of temperature \((A)\), pH \((B)\). The statistical significance of this equation was checked with the \( F \) test, and the ANOVA for the response surface quadratic model is shown in Error! Reference source not found.4. The model \( F \) of 35.69 and probability \((P)>F \) (0.0002) indicated that the model terms were significant. The \( F \) test and the corresponding \( P \) values along with the parameters were estimated. The smaller the \( P \) value, the larger the significance of the corresponding coefficient. The estimated parameters and the corresponding \( P \) values suggest that, among the independent variables, temperature \((A)\) has a much larger \( P \) value (0.30) than pH \((B)\) (<0.0001), which indicates that pH has more significant effect on the sugar yield.
The result is shown in Error! Reference source not found., which suggests that the highest yield was obtained at pH 4.5 and the temperature has little effect on the yield, even at a temperature near room temperature (30 °C).

Figure 4-4 Optimization of the temperature and pH in the enzymatic hydrolysis of microalgae biomass. Mixed culture was obtained from a wastewater treatment process (from AlgEvolve LLC. Total biomass amount = 1.6g; glucoamylase loading = 3.75U/g; α-amylase loading = 312.5U/g; protease loading = 62.5 U/g.

Next, we tested the impact of sugar recovery by adding different amounts of each enzyme. First, we fixed the loading of glucoamylase (18.75U/g), and loaded different amounts of protease. The results in Error! Reference source not found. show that the protease loaded at 187.5 Units/g of microalgae biomass was enough to obtain a good sugar yield. In this experiment, we did not add any α-amylase because the protease contained sufficient α-amylase for digestion.
Figure 4-5 Effect of different loading of protease in the enzymatic hydrolysis of microalgae biomass. Total biomass loading was at 8 g with fixed glucoamylase loading at 18.75 U/g biomass and variable protease loading from 0 to 375U/g biomass. No amylase was added in this test.

In addition, we evaluated the activities of two other enzymes. We used pure protease without a mixture of α-amylase. We fixed the protease (without α-amylase activity) loading at 62.5 U/g biomass and loaded different amounts of α-amylase and glucoamylase. Error! Reference source not found. shows that the loading amount of α-amylase has much more of an impact than glucoamylase, which is almost linearly dependent on the sugar yield. The equation we obtained is as follows:

\[
Y_{yield} = 47.56 + 32.41 \times A + 9.97 \times B + 9.51 \times A \times B - 7.79 \times A^2 - 0.057 \times B^2
\]

Where \(Y_{yield}\) is the succinic acid produced as a function of α-amylase (A) and glucoamylase (B). The statistical significance of this equation was checked with the F test, and the ANOVA for the response surface quadratic model is shown in Error! Reference source not found.5. The model F of 128.57 and probability less than 0.0001 indicated that the model terms were significant. The F test and the corresponding P values along with the parameters were estimated. The estimated
parameters and the corresponding $P$ values suggest that, among the independent variables, α-amylase (A) has a much larger $F$ value (555.16) than glucoamylase (B) (52.52), which indicates that the amount of α-amylase has a more significant effect on sugar yield.

Table 4.5 ANOVA for response surface quadratic model for optimizing α-amylase and glucoamylase in the enzymatic hydrolysis of microalgae biomass

<table>
<thead>
<tr>
<th>Source</th>
<th>Sum of Squares</th>
<th>df</th>
<th>Mean Square</th>
<th>F Value</th>
<th>p-value Prob &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Model</td>
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<td>5</td>
<td>3040.4</td>
<td>128.6</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>A-Amylase</td>
<td>13128.5</td>
<td>1</td>
<td>13128.5</td>
<td>555.2</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>B-Glucoamylase</td>
<td>1242.0</td>
<td>1</td>
<td>1242.0</td>
<td>52.5</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>AB</td>
<td>565.5</td>
<td>1</td>
<td>565.5</td>
<td>23.9</td>
<td>0.0001</td>
</tr>
<tr>
<td>A$^2$</td>
<td>265.8</td>
<td>1</td>
<td>265.8</td>
<td>11.2</td>
<td>0.0033</td>
</tr>
<tr>
<td>B$^2$</td>
<td>0.0</td>
<td>1</td>
<td>0.0</td>
<td>0.0</td>
<td>0.9806</td>
</tr>
<tr>
<td>Residual</td>
<td>449.3</td>
<td>19</td>
<td>23.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cor Total</td>
<td>15651.1</td>
<td>24</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 4-6 Optimization of the loading of different amounts of enzymes in the enzymatic hydrolysis of wet microalgae biomass. Total biomass loading at 1.6 g and fixed protease (without amylase activity) loading at 62.5 U/g biomass was applied. Variable α-amylase loading from 0 to 625 U/g biomass and variable glucoamylase loading from 0 to 3.75 U/g biomass was applied in this optimization.

4.3.4 Enzymatic hydrolysis and extraction of lipids using dry algae

From Error! Reference source not found., it can be observed that the rate and extent of lipid released from both algae materials (Chlorella sp. SLA-04 and Schizochitrium limacium SR21, obtained from ATCC (MYA 1381)) is similar in treatments with protease alone as well as in treatments with a mixture of protease and amylases. For SLA-04, >85% of the lipid (measured as fatty acid methyl ester, FAME) contained in the biomass was released (and extracted) as a result of the enzymatic
treatments. For SR-21, >72% of the cellular lipid was released. The enzyme-free control treatments did not release any extractable lipids.

**Error! Reference source not found.** shows that at least a portion of the lipids released during digestion separate into an oil phase and can be separated without solvent extraction. **Error! Reference source not found.** shows that the majority of the lipids recovered after extraction were triglycerides.

Figure 4-7 Fraction of lipid extractable after enzymatic treatment of (a) SLA-04 and (b) SR-21 after enzymatic digestion with protease (open bars) or a mixture of protease and amylases (filled bars). The horizontal dashed line indicates the total FAME content of the biomass samples: 35% (w/w) for SLA-04 and 44% (w/w) for SR21.
Figure 4-8 Photograph showing lipid droplets that formed on the surface of the water phase in enzymatically digested SLA-04.

Figure 4-9 Example GC chromatograms of solvent extracts obtained after digestion and recovery of lipids from (a) SLA-04 and (b) SR-21 showing that most of the recovered lipids were triglycerides (retention time >30 min).
After the enzyme treatment, solubilized monomeric sugar concentrations were measured in the digested slurries to determine if carbohydrate hydrolysis also occurred during the treatments. Figure 4-10 shows that treatments with protease alone as well as treatments with a mixture of protease and amylases resulted in the release of monomeric sugars from both algal biomass samples (SLA-04 and SR-21). However, more sugar was released in the treatments that contained amylase. Glucose was the major sugar recovered along with smaller amounts of xylose.

![Figure 4-10](image)

**Figure 4-10** Concentrations of solubilized sugars released from (a) SLA-04 and (b) SR-21 after enzymatic digestion with protease (open bars) or a mixture of protease and amylases (filled bars).

### 4.3.5 SA fermentation of microalgaes biomass hydrolysate

In this study, the hydrolysate was generated from digested microalgaes biomass (160 g/L). Nearly 40 g/L of the total sugar monomers (glucose and galactose) were released with more than 95% of sugar recovery. These sugar monomers are the ideal carbon source in the fermentation. Since the total protein content in the microalgaes is up to 55% of its dry weight, once released in the media, this partition could be an ideal nitrogen source for fermentation. Protease used in the enzymatic hydrolysis helps break certain peptide bonds; thus, the amino acids or small peptides can be
released and dissolved in the media. Although the protease might break down proteins only partially since there were still large amounts of microalgae solid residues composed of protein present after the hydrolysis, which indicated that the protein digestion was incomplete. These insoluble particles could not pass through the cell membrane of the microbe so they could not be utilized. Determining the quantity of the digested nitrogen from microalgae biomass will be important to appropriately utilize it in fermentation in the future. Therefore, we used Actinobacillus succinogenes as a model microorganism for succinic acid (SA) fermentation with carbon and nitrogen source both enzymatically digested from microalgae biomass. We selected the yeast extract (a widely used nitrogen source in microbial fermentation) as the control, and compared the performance of SA fermentation between it and digested microalgae biomass as the nitrogen source. Fermentation with or without microalgae biomass solid residue was also conducted to evaluate whether more nitrogen source was released during fermentation. As shown in Error! Reference source not found. and Error! Reference source not found., external supplementation of the hydrolysate with extra yeast extract (additional 2 g/L) showed no significant difference in SA fermentation compared to fermentation with digested microalgae biomass only. The SA production from the microalgae hydrolysate is similar to the model sugar control fermentation containing 10 g/L yeast extract as the nitrogen source; thus, the nitrogen and micronutrients available in the hydrolysate itself were comparable to those provided by the yeast extract. For SA fermentation of the microalgae biomass hydrolysate, no external nitrogen source or other nutrient is necessary. The SA production and yield were slightly lower in fermentation with a solid residue. This is because the solid residue was not sterilized with filtration; some
A mixed culture was involved in producing other products (lactic acid was found in the fermentation products).

Figure 4-11 Succinic acid fermentation from microalgae biomass: (A) Fermentation with the dried microalgae biomass after enzymatic hydrolysis with or without the solid residue (B) Fermentation with the model sugar with supplemental yeast extract.
In addition, the nitrogen source in the microalgae biomass might be not only enough to support the conversion of the sugar it contained but also could support the conversion of an extra sugar supplement externally. If it works, cheaper carbon sources (e.g., from corn or lignocellulosic biomass) could be blended with hydrolyzed microalgae biomass for feedstock to reduce the cost of producing bio-based products. Therefore, we investigated SA fermentation with various amounts of digested microalgae biomass as the nitrogen source in fermentation. First, we fixed the total sugar concentration (sugar added externally and sugars released from the microalgae biomass), and then added different amounts of the digested microalgae biomass. Next, we fixed the loading amount of the digested microalgae biomass but with different initial total sugar concentrations (sugar added externally and sugars released from the microalgae biomass).
Figure 4-13 SA fermentation with fixed sugar concentration (60 g/L) and different amounts of loading of microalgae biomass (10 to 160 g/L). Fermentation did not include the microalgae solid residue.

Figure 4-14 SA fermentation with fixed amounts of loading of microalgae biomass (40 g/L) and different initial sugar concentrations (10 to 60 g/L). Fermentation did not include the microalgae solid residue.
Error! Reference source not found. shows that only with high loading of microalgae biomass up to 160 g/L is the SA fermentation yield above 70%, which is close to the SA yield with the 10 g/L yeast extract as the nitrogen source. Although less microalgae biomass was loaded, the SA yield decreased significantly, which is likely due to the lack of sufficient nitrogen sources. In Error! Reference source not found., 40 g/L of microalgae biomass was loaded with different concentrations of pure sugar. Although the sugar concentration was below 20 g/L, there is no significant difference between the microalgae biomass (40 g/L) and yeast extract (10 g/L) as the nitrogen source. However, when the initial sugar concentration increased to 40 g/L or even higher, the nitrogen source from the microalgae (40 g/L) would not support the demand for a good SA yield in SA fermentation. This phenomenon suggested that microalgae biomass would release nitrogen content partially in enzymatic hydrolysis, which could be used as the nitrogen source in SA fermentation. The nitrogen content released will be enough to support SA fermentation from carbohydrate released from the microalgae biomass itself. However, supplemented with an external carbon source, the nitrogen source from microalgae biomass is not sufficient to support a good SA yield in fermentation.

We found that if the microalgae biomass were hydrothermolyzed via autoclave at 121 °C for 30 min, more nitrogen content was released and that significantly reduced the microalgae biomass loading (Error! Reference source not found.).
Figure 4-15 Comparison of succinic acid fermentation with or without autoclave treatment using the microalgae biomass as the nitrogen source.

To understand the utilization of the nitrogen source in fermentation, the total soluble nitrogen concentration was analyzed. Total dissolved nitrogen (TDN) consists of two fractions: an inorganic fraction, composed of ammonium (NH$_4^+$), nitrate (NO$_3^-$), and nitrite (NO$_2^-$), and an organic fraction (i.e., dissolved organic nitrogen (DON), the composition of which is unknown but can include amino acids, proteins, urea, and humic and fulvic acids [156]. Persulfate oxidation is the most commonly used method that promotes efficient hydrolysis and oxidation of most nitrogenous compounds, resulting in nitrate ions, which could be determined with the spectrophotometer after colorimetric reaction.

In Error! Reference source not found. (A), the soluble total nitrogen concentration was analyzed. With a different initial sugar concentration but fixed microalgae biomass loading, nearly all of the nitrogen content was depleted at a similar consumption rate. Less soluble nitrogen content was observed when yeast extract was used, which means the yeast extract contained more nitrogen that was
easier to be utilized by the microbe. In the microalgae biomass, some soluble nitrogen might not be consumed by the microbe, which was still left in the solution. In Error! Reference source not found. (B), use of higher loading of microalgae biomass releases more nitrogen in the media, which might be excessive for the microbe to consume in fermentation. Therefore, the results showed some soluble nitrogen not utilized in the solution when 160 g/L of the microalgae biomass was loaded. In contrast, when less microalgae biomass was loaded, most of the total soluble nitrogen content was utilized. The nitrogen left in the media was not sufficient to support the metabolism of the strain to continue growing and produce succinic acid. Therefore, a lower succinic acid yield was observed (Error! Reference source not found.).
Figure 4-16 Total soluble nitrogen content in the succinic acid fermentation using microalgae biomass as the nitrogen source (A) different initial sugar concentrations (B) different amounts of loading of microalgae biomass.
4.4 Discussion and Summary

Microalgae have recently been considered as a third-generation feedstock for biofuel production [157]; the focal fuel in such processes is biodiesel. However, for microalgae species that have lipid and carbohydrate content, developing a method that could cost-effectively maximum utilize most of the components would be significant for the production process. Traditionally, people use a physical, chemical, or biological pretreatment to break the cell walls of microalgae to release the lipids and carbohydrates. However, the higher energy input from the high temperature and pressure or the cost of equipment and operations add great cost to this process. In addition, harsh conditions may lead to the decomposition of the sugars into unwanted compounds that inhibit the fermentation process [158]. Therefore, in this study, we tried to use enzymatic hydrolysis, which is an environmentally benign process and can obtain higher glucose yields without producing inhibitory products. The enzymes used in this process were produced from acid fungi (A. oryzae or A. niger), which are very cheap compared to the cellulose applied for the lignocellulosic biomass. In the results, the sugar yield was more than 90% with optimized loading of the enzymes and appropriate temperature and pH. For lipids-rich microalgae, the lipid extraction could be more than 80%. The process could be conducted even at room temperature without a significant decrease in efficiency, which would save a large amount of energy input in this process. Therefore, this novel method could be a potential cost-effective process for treating a lipid-rich or carbohydrate-rich microalgae biomass. Several process options are possible depending upon the biochemical composition of the algal biomass.

Error! Reference source not found. shows a two-stage process where protease is used alone in the first stage for cell disruption and recovery of lipids. After
treatment with the extraction solvent, the aqueous phase is treated with additional amylases, if necessary, to break down additional polysaccharides into monomeric sugars. The lipids are recovered from the organic phase through evaporation. The aqueous phase could be filtered to recover protein-rich residues while leaving behind the soluble sugars, protein, and other cellular material for fermentation to products such as alcohols or organic acids. Alternately, the whole slurry could be fermented.

**Error! Reference source not found.** shows a one-stage process that incorporates simultaneous treatment with a mixture of protease and amylase. In this process, cell digestion and polysaccharide hydrolysis are completed in a single step. The subsequent recovery and conversion pathways are similar to those shown for the two-stage process.

**Error! Reference source not found.** shows a one-stage process for algal material that is rich in carbohydrates but does not contain significant amounts of lipids. In this case, treatment with amylases alone or in combination with proteases releases cellular polysaccharides as monomeric sugars. Thermal treatments could also be used to reduce enzyme loading.
Figure 4-17: Two stage enzymatic hydrolysis of lipid-rich microalgae using proteases and amylases. The block flow diagram shows the process for production of sugar-rich hydrolysate and lipid from lipid-containing microalgae biomass with **two stages** enzymatic hydrolysis. Recovered lipids may be used for production of fuels and other oleochemicals while released sugars may be fermented (with or without solids separation) to bio-products such as alcohols, organic acids or methane.
Figure 4-18: One stage enzymatic hydrolysis of lipid-rich microalgae using a mixture of aproteases and amylases. The block flow diagram shows the process for the production of sugar-rich hydrolyzate and lipids from lipid-containing microalgae biomass with one stage enzymatic hydrolysis. Recovered lipids may be used for production of fuels and other oleochemicals while released sugars may be fermented (with or without solids separation) to bio-products such as alcohols, organic acids or methane.
Figure 4-19: Enzymatic hydrolysis of lipid-lean microalgae using protease alone or in combination with amylases. Following enzyme treatment, the hydrolyzate may be fermented (with or without prior separation of insoluble solids) to bio-products such as alcohols, organic acids or methane.
Chapter 5

Future Directions

5.1 Improve lactic acid fermentation from hydrolysate with higher sugar concentration

As described in chapter 2, xylose can be partially utilized by \textit{L. pentosus} and xylulose can be utilized by both \textit{L. pentosus} and \textit{L. casei}. Although extracellular isomerization is possible to provide higher conversion (over 90\%) from xylose to xylulose with the presence of borate, \textit{L. pentosus} is very sensitive to borate and the Tris buffer, which means it has difficulties fermenting in the hydrolysate directly after isomerization with borate. Without borate, only 30\% of the xylulose can be converted, so the majority of the remaining xylose is still unable to be utilized by \textit{L. casei}.

To overcome this obstacle, fermentation from a media with a higher xylulose/xylose ratio without borate is preferred. Bin Li, my colleague in our group, tried to isomerize and extract sugars at the same time, which resulted in a media with isomerized sugar but no borate. This novel strategy for simultaneous-isomerization and reactive-extraction (SIRE), where the isomerization of aldose sugars and the reactive-extraction of ketoses occur concurrently in the same vessel, was published by the same group conducting the present study [159]. In this method, the conversion of xylose to xylulose was occurred in one container with isomerase at the optimum pH.
One organic phase contained the extraction agents capable of binding the sugar at the optimum pH, which allows the sugars to transfer into the organic phase. A stripping solution at low pH was then used to collect and concentrate sugar (over 95% purity of ketose sugar can be achieved) while in contact with the organic phase. Finally, a purified isomerized sugar media can be obtained, which is ready to be fed into the lactic acid fermentation by either *L. pentosus* or *L. casei* without any inhibition. To learn more about the SIRE process, please refer to my colleague Bin Li’s paper and thesis.

### 5.2 Improve the performance of succinic acid fermentation

Chapter 3 focused on the detoxification of the hydrolysate and improved the succinic acid fermentation. The experiment conducted in this study for the detoxification setup is actually a two-step process. First, we mixed the hydrolysate with the organic phase to remove the inhibitors from the hydrolysate. Second, we mixed the organic phase with the receiving phase to regenerate the organic phase. In order to reduce the cost and improve the efficiency of the detoxification, a setup incorporating a supporting liquid membrane was designed, as shown in Figure 5-1, which combines both steps into one. A liquid membrane is formed by a thin layer of organic phase (usually with dissolved reagents) between two aqueous phases of different compositions. This thin layer of organic phase can be immobilized onto a suitable inert microporous support, which, when interposed between two aqueous solutions, is termed a supported liquid membrane (SLM). In this three-phase extraction technique, analytes are extracted from a continuously flowing aqueous sample through an organic liquid phase into another, usually temporally stagnant, aqueous phase. The salient features of the supported liquid membrane (SLM)
technique include simultaneous extraction and stripping, low solvent inventory, process economy, high efficiency, less extractant consumption, and low operating costs [160].

The SLM can also be installed in a hollow fiber module. The outer cell of the module is a single, nonporous material, through which the solution inside cannot be transported. Inside the shell, many thin fibers are packed in neat rows (Figure 5-2). The organic phase passes through the fibers, and the receiving phase through the shell side, with the help of pumps [161]. The supporting liquid membrane comprised of hollow fiber types provides a high interfacial surface area for achieving maximum inhibitor flux.

Figure 5-1 Schematic diagram of supported liquid membrane (SLM) in membrane separation process.
Figure 5-2 Hollow fiber supported liquid membranes (HFSLM), extra flow membrane contactor [161].

5.3 Analyze the composition of nitrogen source utilized in the fermentation

In Chapter 4, it was discussed the carbohydrate and lipid extraction and bio-based product fermentation from the microalgae biomass. During fermentation, the dissolved nitrogen compounds after the enzymatic digestion could serve as the nitrogen source for the fermentation by microorganism. For example, in the ethanol fermentation by yeast, free amino acids feeding can boost ethanol yield and reduce glycerol formation by inhibiting the intracellular amino acid synthesis from glucose and ammonia. Conversely, a lack of free amino acids will result in more intracellular amino acid synthesis, consuming more glucose and generating surplus NADH, which can lead to an increase in glycerol formation and a reduction of ethanol production [72, 162]. Similar to yeast, this phenomenon might also occur in succinic acid fermentation by *A. succinogenes*. While amino acids are insufficient in the medium, it would lead to a sharp reduction of glucose during the fermentation, but also to a surplus of NADH, as well as have a greater impact to the metabolic pathway via C3
or C4 (See chapter 1.3), which results in a different succinic acid yield. Therefore, determination the appropriate amount of the free amino acid and other soluble nitrogen source required for succinic acid fermentation would potentially assist us in finding a way to improve succinic acid yield. In addition, it has been revealed through studies that using acid hydrothermal treated microalgae biomass would generate six times the amount of soluble nitrogen than the one without the treatment, which resulted in a higher succinic acid yield in the fermentation. Free of harsh conditions, the proteins or polypeptides would not be broken down into monomers, which can be utilized by the microbes. However, a server condition would also lead to higher energy input and the generation of inhibitors. An optimization of treatment conditions is required to generate an ideal nitrogen source portion for each specific strain in the fermentation with a cost-effective process.

In Chapter 4, we used the fungi enzymes to digest the cell wall and release the carbohydrates and lipids. In the future, the enzymatic hydrolysis might not be separate but “in-situ.” This means we would be able to cultivate those enzyme production fungi directly in the algae biomass media. Fungi can not only synthesize the enzymes to breakdown the cell wall as the first step of an invasion, but it can also produce a great number of bio-based products including organic acids, biofuels, and even proteins [163] by utilizing the sugars and proteins in the microalgae as the second step. If this process could be achieved, it would allow the whole process to be simplified into a single step, which would significantly reduce the cost of production. Some investigators used *A. niger* to produce these enzymes with solid-state fermentation from the soybean meal medium [154] without further pretreatment or enzymatic digestion. The protein (enzymes) as the fermentation product has a much higher value than the biofuel or organic acid, which could make it very promising if microalgae
could successfully be used as the feedstock.
References


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