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Production of xylitol and ethanol from lignocellulosics

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Abstract

Lignocelluloses as agricultural, industrial, forest residuals, food-processing waste or paper wastes have the potential to serve as low-cost and abundant source of raw materials for the production of different biochemicals which can serve as future energy sources. These potentially valuable materials consist of sugars and lignin that can be converted via biological pathway into many valuable bioproducts and biochemicals. Two valuable biochemicals that can be fermented from sugars derived from lignocellulosic biomass are xylitol and ethanol. Compared with glucose, which can be readily fermented by well studied yeast (*Saccharomyces cerevisiae*) and bacterial (*Zymomonas mobilis*) strains, xylose is more difficult to ferment because a lack of industrially suitable microorganism able to rapidly and efficiently metabolize xylose in presence of six carbon sugars. In order to keep biochemicals production cost at minimum, all the sugars naturally present in lignocellulosic biomass must be converted into biofuels and biochemicals. The need for a microorganism that can utilize all the sugars present in lignocellulosic biomass and to tolerate the inhibitory compounds generated during biomass pretreatment is therefore apparent.

One of the yeast which was identified in our labs as being capable of rapid assimilation and catabolism of five and six carbon sugars (arabinose, galactose, glucose, xylose and mannose) is *Rhodotorula mucilaginosa* strain PTD3, an endophytic yeast of hybrid poplar *Populus trichocarpa x deltoids*. PTD3 was found to be capable of producing xylitol from xylose, ethanol from glucose, galactose, and mannose, and arabitol from arabinose. Glucose-acclimated PTD3 produced enhanced yields of xylitol (67% of theoretical yield) from xylose and of ethanol (84, 86, and 94% of theoretical yield, respectively) from glucose, galactose, and mannose. Additionally, this yeast was capable of metabolizing high concentrations of mixed sugars (150

g/L), with high yields of xylitol (61% of theoretical yield) and ethanol (83% of theoretical yield). A 1:1 glucose:xylose ratio with 30 g/L of each during double sugar fermentation did not affect PTD3's ability to produce high yields of xylitol (65% of theoretical yield) and ethanol (92% of theoretical yield). Surprisingly, the highest yields of xylitol (76% of theoretical yield) and ethanol (100% of theoretical yield) were observed during fermentation of sugars present in the lignocellulosic hydrolysate obtained after steam pretreatment of a mixture of hybrid poplar and Douglas fir. Also, PTD3 demonstrated the ability to tolerate higher concentrations of inhibitors during xylitol and ethanol production compared to other yeasts described in the literature. Concentration of up to 5 g/L of furfural stimulated production of xylitol to 77% of theoretical yield (10% higher compared to the control) by PTD3. Xylitol yields produced by this yeast were not affected in a presence of 5-HMF at concentrations of up to 3 g/L. At higher concentrations of furfural and 5-HMF, xylitol and ethanol yields were negatively affected. The higher the concentration of acetic acid present in a media, the higher the ethanol yield approaching 99% of theoretical yield (15% higher compared to the control) was produced by the yeast. At all concentrations of acetic acid tested, xylitol yield was lowered. PTD3 was capable of metabolizing concentrations of 5, 15, and 5 g/L of furfural, 5-HMF, and acetic acid, respectively. The high xylitol and ethanol yields obtained and significant resistance to the toxicity of the inhibitors indicate the great potential of the tested strain as a realistic candidate for industrial scale biofuels and biochemicals production from lignocellulose.

After assessing these PTD3's abilities to ferment glucose and xylose and to tolerate the fermentation inhibitors, the next step was to determine the optimum pretreatment conditions which will cause the production of a unique concentration of fermentation inhibitors that would enhance fermentation of xylitol and ethanol by yeast *Candida guilliermondii*. Based on

concentrations of inhibitors present, hydrolysates obtained after steam pretreatment of five lignocellulosic feedstocks (mixed wood, hybrid poplar, *Arundo donax*, switchgrass, and sugarcane bagasse) were evaluated in terms of enhanced xylitol and ethanol production. In addition, for deeper understanding of the effect of process inhibitors on the overall xylitol and ethanol yields after fermentation, hybrid poplar feedstock was selected and pretreated at different severity conditions using steam pretreatment.

Customizing the steam pretreatment conditions for production of fermentation inhibitors at unique concentration can be used as a novel approach of improving xylose to xylitol and six carbon sugars to ethanol conversion by *Candida guilliermondii*.

Preface

This PhD thesis is submitted as the partial fulfillment of the requirements for the PhD degree at the University of Washington. The work included in the thesis was carried out at the School of Environmental and Forest Sciences, Bioresource Science and Engineering Department (Biofuels and Biochemicals Laboratory Group) at the University of Washington from March 2008 to December 2012 under the supervision of Professor Renata Bura.

The thesis consists of a general introduction that describes the theoretical background of the project with inclusion of the achieved results in relation to the published literature.

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Seattle, November 2012

Azra Vajzovic

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Bura R, Vajzovic A, Doty L. S. (2012) Novel endophytic yeast *Rhodotorula mucilaginosa* strain PTD3 I: production of xylitol and ethanol. J Ind Microbiol Biotechnol Vol 7: 1003-1011.....38

CHAPTER III

Vajzovic A, Bura R, Doty S (2012) Novel endophytic yeast *Rhodotorula mucilaginosa* strain PTD3 II: production of xylitol and ethanol in the presence of inhibitors. J Ind Microbiol Biotechnol 39:1453-1463.....67

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Schmitt E, Bura R, Gustafson R, Cooper J, Vajzovic A (2012) Converting lignocellulosic solid waste into ethanol for the State of Washington: An investigation of treatment technologies and environmental impacts. Bioresour Technol 104:400-409.....96

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List of Abbreviations

°C	degrees Celsius
α	alpha
β	beta
AFEX	ammonia fibre explosion treatment
anti	antibiotic
Ara	arabinose
CO ₂	carbon dioxide
CS	combined severity
EOH	ethanol
EtOH	ethanol
g	grams
Gal	galactose
Glu	glucose
h	hour
HCl	hydrochloric acid
H ₂ SO ₄	sulphuric acid
HMF	5-hydroxymethylfurfural
HPLC	high performance liquid chromatography
L	litre
Log Ro	severity factor
Man	mannose
mg	milligram
mL	millilitre
mm	millimetre
mM	millimolar concentration
NaOH	sodium hydroxide
nm	nanometer
OD	optical density
ODW	oven-dried weight
q _s	specific consumption/production rate
rpm	revolutions per minute
s	second
SHF	separate hydrolysis and fermentation
SO ₂	sulphur dioxide
SSF	simultaneous saccharification and fermentation
SSL	spent sulphite liquor
<i>t</i>	time
T	temperature
μL	microlitre
μm	micrometre
v/v	volume per volume
UV	ultraviolet light
Xyl	xylose
XOH	xylitol

YPD	culture media (1% yeast extract, 1% peptone, 1% glucose)
[YP/S]	product per unit substrate
[Y%T]	percent theoretical
YPX	culture media (1% yeast extract, 1% peptone, 1% xylose)

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To my children Zehra and Nedim

**The day you wonder
what mommy was doing those days**

CHAPTER 1

Introduction

1.1 Introduction

1.1.1 Biofuels and biochemicals trends and production

With the inevitable depletion of the world's oil supply, there has been an increasing worldwide interest in finding new renewable and biobased energy sources. Lignocellulosic materials such as agricultural residues, forestry, municipal wastes and other low-cost biomasses are an abundant and renewable source of sugar substrate that could be fermented to biofuels and biochemicals [12, 56]. Today, the first generation biofuels and biochemicals are produced from sugars, starches, and vegetable oils, but these production routes usually give rise to several issues. These routes compete with food industries and fertile land so their potential availability is limited by soil fertility and yields. The effective savings of CO₂ emissions and fossil energy consumption are limited by the high energy input required for crop cultivation and conversion [19].

Development of conversion technologies fed by renewable resources is seen as a promising option. Many technologies for renewable energy are already well-developed and competitive in the market. Emerging technologies include biorefinery complexes, where biomass is used as a renewable carbon-based source for the production of bioenergy and value-added products, including biofuels and biochemicals [18]. A commercial biorefinery requires high yield conversion of biomass feedstock to products that can be sold with good economic returns, while reducing fossil dependence. To make the future lignocellulosic bioconversion process more economically feasible, process yields need to improve. The whole process of converting lignocellulose to biochemicals consists of four major steps [56]: pretreatment, hydrolysis, fermentation, and product(s) recovery, (Figure 1.1).

The highly complex lignocellulosic matrix consisting of cellulose, hemicellulose and lignin forms a barrier against enzymes which prevents degradation of the biomass into its individual components. A pretreatment step is needed in order to alter biomass' rigid structure. Before enzymes can efficiently hydrolyse the sugar components of biomass, biomass needs to be pretreated to expose these sugars to enzymatic attack. Once the fiber structure is loosened up, the chemical constituents of the lignocellulose become available for extraction and /or separation. Ideally, the pretreatment of lignocellulosic biomass should lead to a reduction in particle size of the biomass, optimal recovery of hemicellulose-derived sugars in a monomeric form, and the production of an easily-hydrolysable water insoluble, cellulosic fraction. Cellulose and hemicellulose suspensions generated from the pretreatment are not directly fermentable, and have to be hydrolyzed to their constituent sugars before being further fermented into biochemicals.

Hydrolysis of cellulose to glucose can be achieved using either inorganic acids or cellulolytic enzymes. Enzymatic hydrolysis is accomplished by using commercially available enzymes, cellulases and hemicellulases, which are highly specific in the reactions they catalyzed. Chemical hydrolysis of biomass is relatively efficient and inexpensive; however, it generates fermentation inhibitors [66]. On the other hand, enzymatic hydrolysis, despite its relatively slow rate and high cost of enzymes, is a biocompatible and environmentally friendly option (as it avoids the use of corrosive chemicals) [13]. Once in monomeric form, the sugars derived from lignocellulosic hydrolysis can be fermented into biochemicals.

In order to keep bioconversion process at minimal cost, all the sugars naturally present in lignocellulosic biomass must be fermented into biofuels and biochemicals. The need for a microorganism that can utilize all the sugars present in lignocellulosic biomass and to tolerate the

inhibitory compounds generated during biomass pretreatment is therefore apparent. In order to develop optimum conditions for the efficient fermentation of lignocellulosic sugars, the influence of media composition and inoculum concentrations also need to be assessed. The maximum tolerable concentration of each inhibitor that can be present in hydrolysate without affecting the efficiency of the fermentative process is dependent on the microorganism utilized and its degree of adaptation, the fermentation process employed, and the simultaneous presence of other inhibitors. Establishing all these parameters and utilizing an adequate microorganism for sugar fermentation is of a great importance for the whole bioconversion of hydrolysates into various biochemicals.

The bioconversion of lignocellulose to biofuels and biochemicals generally result in the production of fermentation broths that contain moderate to low bioproducts concentrations [26]. These biochemicals can be recovered and separated from downstream processes. For example, the traditional ethanol recovery process includes the separation of the ethanol through distillation process, achieving 95% purity. This is followed by an azeotropic distillation step to dehydrate the ethanol even up to 99.9% [28]. Finally, the purified product is concentrated and diafiltered into the final formulation that can be used as a biochemical platform for other bioproducts.

A number of building blocks for high value bioproducts made using the platform of bioconversion from lignocellulosic sugars can be recovered. Of the twelve most promising of these reported by the U.S. Department of Energy, seven can currently be produced via biological pathways [109]. Those seven building blocks for high value products are: four carbon 1,4-diacids, 3-hydroxypropionic acid (3-HPA), aspartic acid, glutamic acid, itaconic acid, glycerol, sugar alcohols (xylitol and arabinitol). The remaining five are presently produced using chemical

pathways [109]. This dissertation presents xylitol and bioethanol fermentation as examples of biochemical production, but a wide variety of bioproducts can potentially be made from the sugars derived from lignocellulose.

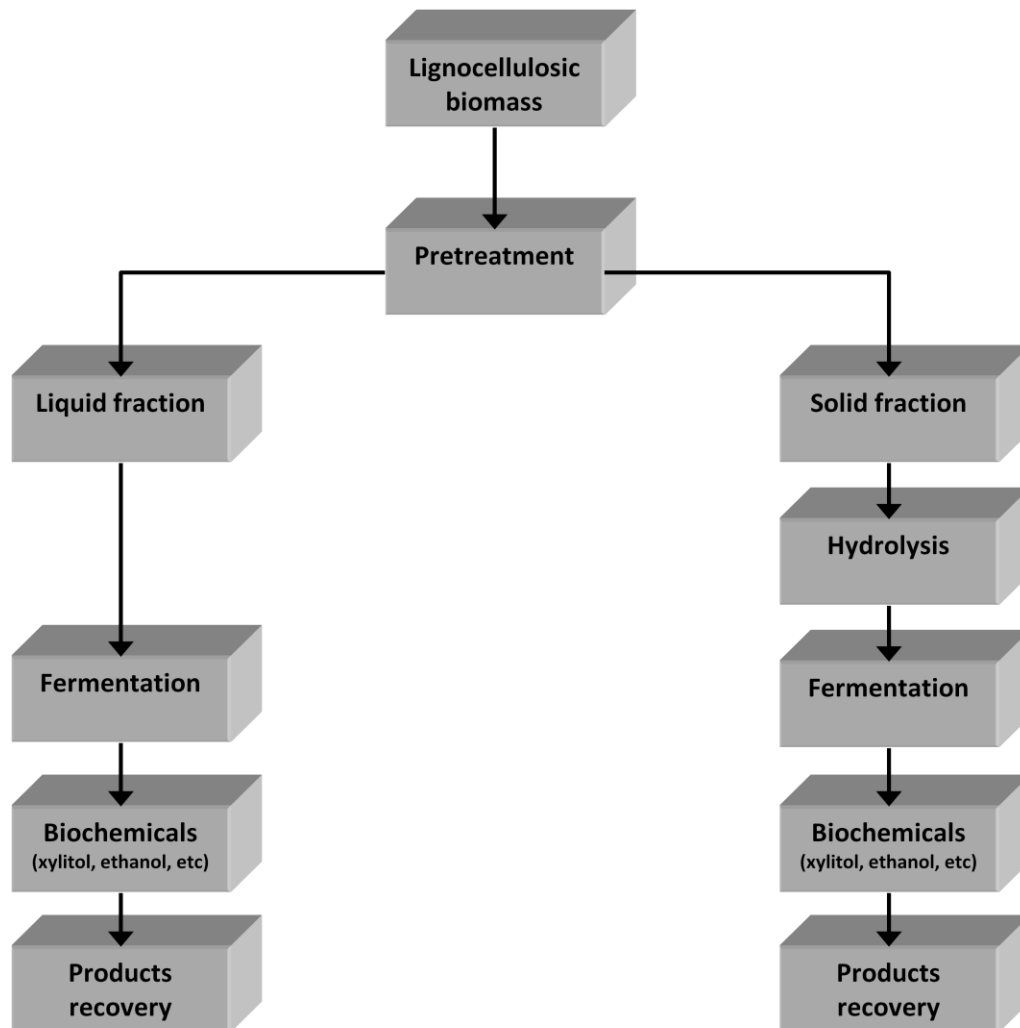


Figure 1.1. Simplified process flow diagram for the bioconversion of lignocellulosic biomass via steam explosion pretreatment to bioproducts. This process flow illustrates the separate hydrolysis and fermentation (SHF) process. These steps can alternatively be combined into one step or process known as simultaneous saccharification and fermentation (SSF).

1.1.2 Xylitol

Xylitol is a five-carbon sugar alcohol with an established commercial history as an alternative sweetener. It has recently drawn the attention of food and beverage manufacturers due to its low caloric value and potential use to reduce or control weight, leading to applications as a sweetener in chewing gums, mints, sweets and toothpaste [38]. It has also been utilized in the pharmaceutical industry due to its role in reduction of dental cavities [85]. Xylitol was discovered almost simultaneously by German and French chemists in the late 19th century, and was first popularized in Europe as a safe sweetener for people with diabetes that would not affect insulin levels [87]. Although xylitol is currently produced chemically by catalytic reduction of xylose, various microorganisms can convert xylose to xylitol by biological means. For over 30 years, considerable efforts have been focused on microbial production of xylitol from xylose [59, 112]. Xylose-fermenting yeasts, which reduce xylose to xylitol by the NAD(P)H dependent xylose reductase (XR), such as *Candida*, *Pachysolen* and *Debaryomyces* strains, have been tested [37, 39, 85, 112]. *Candida* yeasts in particular have been extensively studied with regards to their biotechnological application in the production of xylitol. Xylitol yields as high as 0.77 (g/g) for *Candida guilliermondii* and 0.85 (g/g) for *Candida tropicalis* have been reported by Barbosa [7] and Kwon [57], respectively. However, the yields depend on the type of microorganism employed and fermentation conditions (e.g. nutrients, oxygen, pH, and temperature) used during the conversion of sugars to xylitol. Establishing all experimental parameters and utilizing an appropriate microorganism for sugar fermentation is of great importance for the complete bioconversion of sugars into various biochemicals.

1.1.3 Bioethanol

Bioethanol is a form of renewable energy that can be produced from lignocellulosic feedstocks. It has been considered a sustainable alternative to fossil fuels that could alleviate the future dangers of increasing oil prices and greenhouse gas emissions [67]. Currently, ethanol is produced by the fermentation of crops like corn and sugar cane [40]. However, there has been considerable debate about how useful ethanol from food crops, such as, sugar cane, potato, manioc and corn will be in replacing gasoline. Increasing food prices, and limited to no benefit in energy and/or environmental compared to fossil fuels have brought the benefits into question [6]. Recent developments with cellulosic ethanol (bioethanol) production and commercialization may allay some of these concerns [14]. Recent studies have proven bioethanol is a viable liquid fuel for transportation, and renewable lignocellulosic biomass is an attractive feedstock for biofuel production by fermentation [67]. It can be made from agricultural residues, hardwoods, softwoods, municipal solid waste and other lignocellulosic feedstocks. One major technical constraint for commercializing the bioconversion process is the lack of microorganism able to efficiently ferment all the sugars naturally present in pretreated lignocellulosic composition [91]. Another limitation is microbial ability to endure process-derived inhibitory compounds found in the stream collected after pretreatment [91, 116]. Glucose fermenting microorganisms *Saccharomyces cerevisiae* and *Zymomonas mobilis* are unable to naturally ferment pentoses. There are genetically engineered strains to utilize both xylose and glucose and to convert them to ethanol by introducing genes encoding alcohol dehydrogenase and pyruvate decarboxylase for redirection of glycolytic fluxes to ethanol [3, 50]. However, common problems with genetically engineered ethanologens are their low tolerance to inhibitory compounds generated during pretreatment [24], instability of the plasmid carrying the

genes encoding xylose metabolism enzymes [54], and simultaneous fermentation of mixed sugars [77, 116]. The yeast *Pichia stipitis* and *Candida shehatae* are two very promising natural pentose (xylose) fermenting microorganisms, but their exploitation for ethanol production from xylose is restricted mainly by their slow rates of fermentation, low ethanol tolerance, and sensitivity towards inhibitory compounds present in the lignocellulosic hydrolysates [3, 90]. The need for a robust microorganism that can utilize all the sugars present in lignocellulosic biomass and to tolerate the inhibitory compounds generated during biomass pretreatment is therefore apparent.

1.1.4 Aim of the PhD study

The overall objective of this body of research is to determine all experimental parameters and an appropriate microorganism for sugar fermentation for the complete bioconversion of sugars into xylitol and ethanol as well as other various biochemicals. Specific research objectives are:

- 1.** To characterize the novel yeast *Rhodotorula mucilaginosa* strain PTD3 for utilization of sugars for xylitol and ethanol production in single, double and mixed sugars fermentation media in order to reveal its unique properties (Chapter 2).
- 2.** To conduct a systematic study of the effect of acetic acid, furfural and 5-HMF on the fermentation of both xylose and glucose to xylitol and ethanol, respectively, by *R. mucilaginosa* strain PTD3 (Chapter 3 and 4).
- 3.** To investigate the effect of different concentrations of fermentation inhibitors originating from different feedstock for enhanced xylitol and ethanol yields by *Candida guilliermondii*.

The further objective of this research is to test if the steam pretreatment conditions can be

customized for production of unique concentrations of fermentation inhibitors to enhance xylitol and ethanol production (Chapter 5).

In Chapter 1, several background sections are presented in order to put this research study into context. Section 1.1 provides a background and trends about bioconversion of lignocellulosic material into biochemicals and fuels. Section 1.2 discusses biomass chemistry and possible pretreatment methods and limitations. Section 1.3 describes steam explosion as a pretreatment of choice. Section 1.4 provides an overview of fermentation in terms of five and six carbon sugar fermentation and limitations in the presence of fermentation inhibitors. Section 1.5 introduces microorganisms used in this study in terms of metabolism of lignocellulosic sugars and fermentation of xylitol and bioethanol. Chapters 2, 3, and 4 contain publications regarding the *R. mucilaginosa* strain PTD3's ability to ferment synthetic pentoses and hexoses as well as sugar streams from pretreated lignocellulosic material despite the presence of the fermentation inhibitors with high xylitol and ethanol yields achieved. Chapter 5 contains a draft paper that will be submitted for publication regarding the use of fermentation inhibitors as indicators of steam pretreatment conditions for recovery of the hemicellulose and cellulose derived sugars in a hydrolysable and fermentable form.

1.2 Lignocellulose

In view of rising prices of crude oil due to increasing fuel demands, the need for alternative sources of bioenergy is expected to increase sharply in the coming years. Among potential alternative bioenergy resources, lignocellulosics have been identified as the prime source of biofuels and other value-added products [110]. Lignocelluloses as agricultural, industrial and forest residuals account for the majority of the total biomass present in the world [56]. To initiate

the production of industrially important products from lignocellulosic biomass, bioconversion of the lignocellulosic components into fermentable sugars is necessary.

Lignocellulosic biomass is a complex plant material composed primarily of the polysaccharides cellulose (35-50%) and hemicellulose (20-35%) [36, 41, 75, 111]. The third major component is lignin (10-25%), a phenolic polymer that provides structural strength to the plant as shown in Figure 1.2, and minor components of extractive (1%) [52, 90]. Depending on their species of origin, these constituent compounds are present in varying quantities among biomass types. For example, softwoods generally contain more lignin than hardwoods, while hardwood hemicellulose is highly acetylated [79, 100].

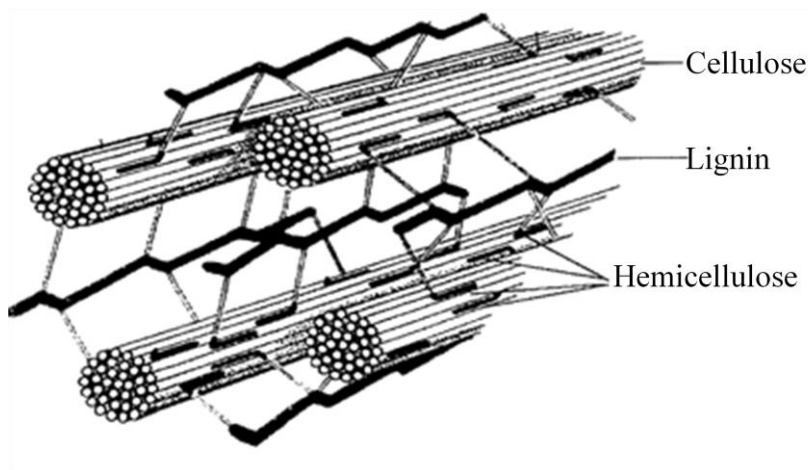


Figure 1.2. Illustration of the main components of lignocellulosic biomass[71].

1.2.1 Cellulose

The most abundant natural polysaccharide, cellulose is the target of bioethanol bioconversion processes [41, 75, 95]. Cellulose is a polymer composed of D-glucose subunits linearly linked by β -1,4 glucosidic bonds, with a high degree of polymerization and crystallinity, which makes the cellulose a hydrolysis-resistant molecule. It is because of this linkage that each individual

cellulose molecule forms a linear polymer [61, 95]. Bioconversion of biomass to biochemicals promotes the release of individual glucose units for microbial fermentation to bioproducts. The degree of polymerization (DP) of cellulose, or numbers of monomeric units in an oligomeric molecule, varies by feedstock [95]. Cellulose molecules with a DP less than 8 are considered water-soluble, while at higher DPs, they have a greater affinity for one another than for water [43]. The cellulose chain bristles with polar -OH groups. These groups are stabilized by rigidly arranged intra- and intermolecular hydrogen bonds with OH groups on adjacent chains, bundling the chains together. The chains also pack regularly in places to form hard, stable crystalline regions that give the bundled chains even more stability and strength, thus giving native cellulose a conformation that is difficult to degrade. The rigid structure of cellulose is recalcitrant to chemical and mechanical treatment, thus posing a challenge for the bioconversion process [21].

1.2.2 Hemicellulose

Hemicellulose is a highly branched heterogenous polymer. Similar to cellulose, it is also a polysaccharide, but its constituents sugars are both pentoses (arabinose and xylose) and hexoses (galactose, glucose, and mannose), and some uronic acids, although the exact composition varies with the origin of lignocellulosic material [77]. With a few exceptions, pentoses are generally not readily metabolized by naturally occurring yeast and bacteria [112]. Hemicellulose has a tendency of lower DP, a lower molecular weight than cellulose, and is a rather branched polymer. Its composition and DP varies widely across species [90]. Hardwood hemicellulose is mainly composed of xylans (15-30%) characterized by a β -1,4 linked xylose backbone, where many of its xylose units are acetylated at their C2 or C3 carbons. Side chains of α -1,2 linked 4-O-methylglucuronic acid units occur at every 6-11 xylose units. Mannans occur in only 3-5% of

hardwood by mass, and are characterized by β -1,4 linked backbone of mannose and glucose units with no side chains [95, 115]. In contrast, softwood hemicellulose is mostly composed of galactoglucomannans, comprised of a backbone of β -1,4 linked mannose and glucose units in 3:1 ratio. Acetyl groups are found on the C2 or C3 carbon of roughly every third backbone unit [35]. This backbone is branched with α -1,6 linked galactose side chains occurring on glucose units [21, 95]. Agricultural hemicelluloses contain mostly xylans that differ in composition depending on a species [90]. The frequency and composition of branches are dependent on the source of xylan [5]. Xylans can thus be categorized as linear homoxylan, arabinoxylan, glucuronoxylan, and glucuronoarabinoxylan [29]. For example, arabinoxylans have been identified as the main hemicellulose in corn stover as well as in wheat, rye, barley, oat, rice, and sorghum [13, 84]. Hemicellulose, unlike cellulose, has no crystalline structure, and is thus easier to hydrolyze [35, 45].

1.2.3 Lignin

The second most abundant organic compound within plant biomass is lignin, a highly heterogeneous complex polymer consisting of phenylpropane units [95]. Lignin composition again varies greatly between species, but its three main constituent phenolic ring structures are *p*-hydroxyphenyl, guaiacyl (containing one methoxyl group), and syringyl (containing two methoxyl groups), which are methoxylated and bound to one another through a complex network of carbon-carbon and ether bonds [1, 81]. Lignin acts as a binder among the holocellulose (cellulose and hemicellulose), all forming together a rather resistant structure that serves as a protection to lignocellulosic biomass, while also contributing to its flexibility [116]. The presence of lignin creates one of the biggest challenges to bioconversion process. Its degradation and removal are of critical importance in biomass utilization of woody plants, because the

presence of lignin limits hydrolytic enzyme access to cellulose and hemicellulose and its constituent sugars [118].

1.2.4 Extractives

Extractives are the compounds present in trees that can be solubilized (extracted) by organic solvents. They are found in higher concentrations in the bark of most woods and are generally considered to be biosynthesized in order to slow or prevent pathogen invasion. Their production is under strict genetic control, and some compounds are limited to individual species. Wood extractives consist mainly of lipids, phenolic compounds, terpenoids, fatty acids, resin acids, and waxes. Generally, the extractives content varies between 2% and 5%, but it can be as high as 15%. Though present in very small quantities, typically below 5%, extractives can still greatly affect certain properties of biomass, including its color, odor, and density [83].

Although the major components of the various types of biomass used for the bioconversion process are similar (cellulose, hemicellulose and lignin), each type of feedstock has different processing requirements, and corresponding potential biochemical yields, as a result of chemical and structural variations.

1.3 Pretreatment of lignocellulosic biomass

As previously described, cellulose and hemicellulose are tightly bound to lignin by hydrogen and some covalent bonds, which make lignocellulosic biomass rather resistant to break down to its constituent sugars [61]. In this regard, an additional pretreatment process is required to open up lignocellulosic structure and to increase accessibility of cellulose and hemicellulose fractions for subsequent hydrolysis. Several pretreatment methods have been developed to fractionate

biomass to cellulose, hemicelluloses and lignin in order to increase the accessibility of lignocellulosic sugars for the bioconversion process [74, 83]. Chemical, mechanical, and biological methods, or any combination of these, are utilized in lignocellulosic pretreatment.

The physical pretreatment techniques do not involve chemical application, and typical examples are milling, irradiation, steam explosion, and hydrothermolysis (high-temperature cooking) [47].

One of the most robust chemical-mechanical pretreatment methods of softwood, hardwood and agricultural residues is steam pretreatment. Steam explosion is a fusion of various pretreatment processes, combining both physical and chemical elements, causing the rupture of the wood cell wall structure, and hydrolysis and solubilisation of biomass components [86]. Steam explosion pretreatment with a addition of acid catalyst such as SO_2 and H_2SO_4 is considered a combination of physical and chemical pretreatment method [34, 74, 78]. Previous work has shown that SO_2 -catalysed steam explosion can successfully pretreat softwood [11, 16, 22, 92, 96, 97, 99, 103], hardwood [30, 68] and agricultural [33] residues as part of the overall bioconversion process. In addition, steam explosion is recognized as one the most cost effective pretreatments for lignocellulosic residues prior to enzymatic saccharification [23].

Chemical pretreatment techniques have received the most attention by far among all the categories of pretreatment methods and typical examples include dilute acid, alkali, solvent, ammonia, CO_2 , other chemicals, and pH-controlled hydrothermolysis [70]. One of the examples is ammonia fiber explosion (AFEX) pretreatment that yields optimal hydrolysis rates for pretreated lignocellulosics with close to theoretical yields at low enzyme loadings [74]. The major effect of the alkaline pretreatment is the removal of lignin from the biomass, thus improving, the reactivity of the remaining polysaccharides. Agricultural residues are well suited

for AFEX [46]. However, this method works only moderately well on hardwoods, and is not attractive for softwoods [104].

The last category of pretreatment methods, facing major techno-economic challenges, is biological pretreatment. This group comprises pretreatment techniques of applying lignin-solubilising microorganisms to render lignocellulosic materials amenable to enzymatic digestion [47]. For example, biological pretreatment of lignocellulose with white rot fungi is a promising technology for energy production from lignocellulose because of the advantages of low energy requirement and mild environmental conditions [117]. The difficulty in implementing this method at an industrial scale is primarily due to the relatively slow rate of the process and inability of the microorganisms to specifically solubilize and consume lignin in a distinct fashion from hemicellulose and cellulose [70].

Steam pretreatment, liquid hot water, acid, flow-through acid, lime, ammonia pretreatment (AFEX) are just a few among several developed pretreatment methods [55, 70, 74]. Each pretreatment has a specific effect on the cellulose, hemicellulose and lignin fraction, and should be selected with consideration of downstream processes.

The pretreatment severity has a negative influence on chemical composition of biomass. For instance, applying harsh conditions with extreme pHs and high temperatures will degrade biomass chemical components into by-products that become inhibitors for the subsequent processing. However, moderate pretreatment can be developed to avoid sugar degradation and toxic compounds formation during pretreatment. As a result, a whole slurry process without inter-stage washing/detoxification can be used, which can significantly reduce capital/operational cost. It should achieve some defibrillization without much sugar degradation, sufficient enough to open cell wall pores/channels. "Over pretreatment" may improve cellulose accessibility but

inevitably cause other chemical components degradation. The right degree of pretreatment is the key. One of the pretreatment methods used in this research study is steam explosion.

1.3.1 Steam explosion

After a review of pretreatments [32], steam explosion pretreatment was chosen due to its potential to fractionate a wide variety of biomass types, such as softwood, hardwood, and agricultural residues [13, 17, 33]. Chemically impregnated lignocellulosic biomass is conveyed into large vessels, and high-pressure steam is applied for a few minutes. At a set time, some steam is rapidly vented from the reactor to reduce the pressure, and the contents are discharged into a large vessel to flash cool the biomass. Biomass can be added to the reactor in either a wet or dry state while the reaction conditions remain the same with minimal extra time required to heat [70, 89]. After steam pretreatment two phases are collected: solid (mostly cellulose and lignin) fraction and liquid (mostly hemicellulosic sugars, acid soluble lignin, and fermentation inhibitors) lignocellulosic hydrolysate (Figure 1.1). The chemical and physical characteristics of liquid and solid phase depend on a type of feedstock and the pretreatment conditions used. For pretreatment of many biomass types such as softwoods and hardwoods, sulfur dioxide impregnation is necessary prior to steam pretreatment to improve hydrolysability of solids and aid hemicellulose removal [13].

Hemicellulose is thought to be hydrolyzed by the acetic and other acids released during steam explosion pretreatment. Steam explosion involves chemical effects and a reaction sequence of the type since acetic acid is generated from hydrolysis of acetyl groups associated with the hemicellulose may further catalyze hydrolysis and glucose or xylose degradation [48]. Water, itself, also acts as an acid at high temperatures [8, 9, 108]. The major chemical and physical

changes to lignocellulosic biomass by steam explosion are often attributed to the removal of hemicellulose. This improves the accessibility of the enzymes to the cellulose fibrils [72, 74].

Each feedstock needs different processing requirements due to chemical and structural variations. As mentioned previously, SO₂-catalyzed steam explosion at optimized conditions can provide a high recovery of hemicellulosic sugars and minimal production of fermentation inhibitors [103]. During SO₂-steam explosion pretreatment, there are three main process variables: temperature, time and SO₂ level. In order to maximize recovery yields for each fraction of pretreated wood (cellulose, hemicellulose and lignin), Overend [78] have proposed the introduction of a single factor, the severity. This factor, increases as a function of time t (min) and temperature T (°C), as follows:

$$R_o = t * e^{(T-100)/14.75} \quad (\text{Eq.1.1})$$

Chum et al. [20] introduced a third parameter, the environmental pH, into the equation above to describe the combined severity (CS):

$$CS = \log R_o - pH * \quad (\text{Eq.1.2})$$

*where pH is calculated after pretreatment.

A low severity can lead to incomplete fractionation of biomass, causing low sugar content in hydrolysis yields. Although, higher severity steam pretreatments result in more complete delignification and better hydrolysis yields which are desirable, but the inevitable effect of this is formation of numerous degradation products [77] that can inhibit subsequent microbial fermentation. After steam pretreatment, lignocellulosic hydrolysate can be directly fermented into biochemicals while solid phase needs to be hydrolyzed prior to the subsequent step (Figure 1.1).

1.4 Hydrolysis

The pretreatment processes are designed only to initiate the breakdown of the biomass structure and partially hydrolyse the carbohydrate polymers, making them accessible to enzymatic attack. There is obviously a great deal of interest in obtaining fermentable sugars from lignocellulosic biomass during the saccharification process for conversion to ethanol and value-added co-products. Hemicellulose and cellulose phases generated from lignocellulosic pretreatment are not directly fermentable into biochemicals; rather they have to be first hydrolyzed to their monomeric constituent sugars before being further fermented into bioproducts. Hydrolysis can be enzymatic or chemical. Enzymatic hydrolysis is achieved by the action of commercially available enzymes, cellulases and hemicellulases, which are highly specific in the reaction they catalyze [13].

Cellulases, perform a crucial task during saccharification by catalyzing the hydrolysis of cellulose to soluble and fermentable glucose. They are synthesized mainly by fungi and bacteria and are produced both aerobically and anaerobically. The aerobic mesophilic fungus *Trichoderma reesei* and its mutants have been the most intensively studied source of cellulases [27].

Hydrolysis of cellulose to glucose is carried out by cellulases, an enzyme mixture of three distinct classes of enzyme [65]:

- endoglucanases or 1,4- β -D-glucan-4-glucanohydrolases,
- exoglucanases, including 1,4- β -D-glucan glucanohydrolases (also known as cellodextrinases) and 1,4- β -D-glucan cellobiohydrolases (also known as cellobiohydrolases), and

- β -glucosidases or β -glucoside glucohydrolases.

Endoglucanases cut at random, internal amorphous sites in the cellulose polysaccharide chain, and generate oligosaccharides of varying lengths and consequently new chain ends [69]. Exoglucanases act on the reducing and nonreducing ends of cellulose polysaccharide chains, liberating either glucose (glucanohydrolases) or cellobiose (cellobiohydrolase) as major products [69]. Exoglucanases can also act on microcrystalline cellulose, presumably peeling cellulose chains from the microcrystalline structure [65]. β -Glucosidases hydrolyse soluble cellodextrins and cellobiose to glucose [10]. One explanation for this system of synergistic enzymatic activity is limitation of end-product inhibition. *Trichoderma reesei* is cultured industrially and its enzymes are separated and purified for use in large-scale hydrolysis [31, 106].

Hemicellulose is open to attack at intermediate positions along its long backbone with the release of oligomers made up of many sugar molecules that can be successively broken down to shorter-chained oligomers before single sugar molecules are formed [62]. A suitable cocktail of enzymes known collectively as hemicellulase can catalyze addition of water to hemicellulose with high specificity at modest temperatures, thus avoiding sugar degradation and resulting in high sugar yields [93]. Once in monomeric forms, all the sugars naturally present in lignocellulosic material can be fermented into a number of biochemicals by an adequate microorganism such as yeast or bacteria.

1.5 Fermentation

In order to achieve feasible bioconversion processes, all the fermentable sugars (arabinose, galactose, glucose, mannose, and xylose) derived from pretreatment of lignocellulosic biomass need to be converted into a number of biochemicals (e.g. ethanol and xylitol). Microorganisms

more readily assimilate and ferment glucose than xylose [112]. Although in small numbers, there are bacteria, yeasts and fungi capable of assimilating and fermenting xylose to xylitol, ethanol and other compounds [76]. To achieve high product yields in a fermentation process, it is a prerequisite to design an optimal nutrient medium and a set of optimal process operating conditions such as pH, temperature and oxygen requirements. Depending on the final products characteristics fermentation usually lasts 48-72 hours [107, 112]. Other products obtained during the lignocellulosic derived sugars to xylitol and ethanols are, for example, carbon dioxide (CO₂), glycerol, succinate, and acetate [61, 112, 113]. As mentioned previously, it is well known that more severe conditions during steam pretreatment will cause greater degradation of hemicellulose-derived sugars [77, 79, 94]. However, a high degree of severity is required to promote enzymatic hydrolysis of the cellulose fibres [44].

Two major groups of potential inhibitors have been found in the liquid fraction after pretreatment of lignocellulosic feedstock: process-derived inhibitors created during pretreatment (*e.g.*, lignin and sugar degradation products), and naturally-occurring inhibitors from the feedstock (*e.g.*, sterols, acetic and uronic acids and resin/fatty acids) [77, 80], all of which may cause adverse effects during fermentation [102]. For example, depending on the chemical composition of feedstock, primarily process derived inhibitors may be present in the water soluble stream (hydrolysate) after steam pretreatment, which can be minimized by careful tailoring of the severity of the pretreatment. Therefore, it should be possible to combine the water soluble and insoluble streams during consecutive hydrolysis and fermentation processes.

Simultaneous saccharification and fermentation (SSF) is an alternative to separate hydrolysis and fermentation (SHF). The SSF procedure is a one-stage process involving the enzymatic saccharification of cellulose and simultaneous fermentation of fermentable sugars by yeast or

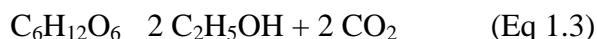
bacteria in a single reactor. SSF is advantageous over SHF due to the process integration obtained when hydrolysis and fermentation are performed in one reactor, which reduces capital cost, decreases saccharification and fermentation time, and most importantly reduces the possibility of end-product inhibition [98]. However, the main disadvantage of SSF is that it takes place under compromised conditions of temperature (around 37°C) and pH (around 5.5), which can have an effect on total yield [15]. The microorganisms and enzymes have to be matched with respect to temperature [42].

By contrast, in SHF, hydrolysis and fermentation are carried out as completely separate steps, which allows for each step to be performed at its optimum conditions for temperature and pH. However, a major disadvantage of SHF is that the accumulation of glucose and cellobiose during hydrolysis can lead to end-product inhibition [2, 111]. An ideal operating process would be flexible to allow for either method to be used in accordance to feedstock type and the final product characteristics.

1.5.1 Glucose fermentation

Ethanol fermentation with glucose substrate is one of the most studied of all the sugar fermentation reactions. The glucose metabolic reaction leading to ethanol fermentation has been studied extensively over the years. The metabolic reaction - or fermentation reaction - begins with the processing of glucose primarily by way of the Embden-Meyerhof pathway followed by the biochemical fermentation reaction, usually to ethanol, traditionally accomplished by a yeast strain *Saccharomyces cerevisiae* (Baker's or Brewer's yeast) [64]. The strain produces ethanol when grown at pH values between 3.5-6.0 at temperature of 30°C. The second ethanologenic microorganism commonly used for glucose fermentation is bacterial strain *Zymomonas mobilis* [88]. Both of these strains can convert glucose into ethanol at nearly the maximum yield of

theoretical of 0.51g-ethanol/g-glucose. However, these strains, unless genetically modified, are capable of fermenting only six carbon sugars which makes them less attractive for feasible bioconversion of lignocellulosic biomass into biochemicals process. The overall reaction of hexose fermentation to ethanol is:



1.5.2 Xylose fermentation

D-xylose is a five-carbon aldose (pentose, monosaccharide) that can be catabolized or metabolized into useful products by a variety of organisms. Xylitol is one of the products produced by fermentation of xylose. The xylose-fermenting yeasts such as *Candida*, *Pachysolen* and *Debaryomyces* strains reduce xylose to xylitol by the NAD(P)H dependant xylose reductase (XR) [37, 39, 85, 112]. The pathway is also called the “Xylose Reductase-Xylitol Dehydrogenase” or XR-XDH pathway presented in Figure 1.3. Xylose reductase (XR) and xylitol dehydrogenase (XDH) are the first two enzymes in this pathway. The XR is reducing D-xylose to xylitol using NADH or NADPH. Xylitol is then oxidized to D-xylulose by XDH, using the cofactor NAD. In the last step D-xylulose is phosphorylated by an ATP utilising kinase, XK, to result in D-xylulose-5-phosphate which is an intermediate of the pentose phosphate pathway [112]. The varying cofactors needed in this pathway and the degree of their availability for usage cause an imbalance that can result in an overproduction of xylitol byproduct or xylose metabolism pathway continues and feeds into the six carbon sugar pathway, contributing to the ethanol production [51]. The theoretical yield of xylitol is 91% of the starting xylose concentration [7].

Since, xylose is the main precursor to hemicellulose which is one of the main constituents of lignocellulosic biomass, it has to be converted into biochemicals (e.g., xylitol) in order to achieve

a feasible bioconversion process. The overall reaction of xylose fermentation to xylitol is indicated in the equation:

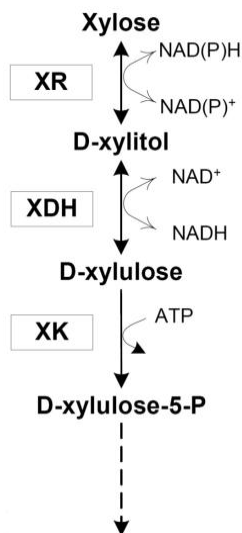


Figure 1.3. Metabolic pathway from xylose to xylitol in yeast.

A number of strains have been tested for conversion of xylose to xylitol. *Candida* yeasts in particular have been extensively studied with regards to their biotechnological application in the production of xylitol [37, 39, 85, 112]. Xylitol yields as high as 0.77 (g/g) for *C. guilliermondii* and 0.85 (g/g) for *C. tropicalis* have been reported by Barbosa [7] and Kwon [57], respectively. However, the yields depend on the type of microorganism employed and conditions for fermentation (nutrients, oxygen, pH, and temperature) used during the conversion of sugars to xylitol.

1.5.3 Lignocellulosic fermentation

Microorganisms producing high yields of biochemicals in laboratory media do not necessarily ferment pretreated lignocellulosic substrates efficiently due to the presence of inhibitory by-products liberated during pretreatment process [77]. Moreover, potential biochemical (e.g., xylitol and ethanol) producers have to be able to grow and produce biochemicals in pretreated lignocellulosic hydrolysates to make bioconversion process economically feasible [4, 75]. Due to the variation in their fermentation products and inhibitor tolerance, different microorganisms can yield dissimilar results under the same conditions [54, 73, 77, 116]. Therefore, the appropriate microorganisms must be selected in each individual case of fermentation, with regard to the sugars and inhibitors that are present, as well as desired product(s).

Rhodotorula mucilaginosa strain PTD3 and *Candida guilliermondii* are robust microorganisms that are capable of fermenting lignocellulosic hydrolysates with the higher xylitol and ethanol yields compared to the synthetic sugars control.

The studies presented in Chapters 2, 3, 4, and 5 demonstrated exceptional ability of PTD3 and *C. guilliermondii* fermenting lignocellulosic hydrolysates with the higher xylitol and ethanol yields by PTD3 and the higher ethanol yields by *C. guilliermondii* compared to the synthetic sugars control, despite the presence of fermentation inhibitors.

1.5.4 Fermentation inhibitors

As cellulose, hemicellulose and lignin decompose under the influence of the pretreatment, a multitude of organic compounds are released and formed that can inhibit downstream processes such as fermentation. The inhibitors affect the overall cell physiology and often decrease product yield [101]. Usually, no specific inhibitor is present at a sufficient level to be highly toxic, but a

combination of all inhibitory compounds present in the pretreated biomass is a reason for high hydrolysate toxicity [52]. As previously mentioned, inhibitory compounds in lignocellulosic liquid phase hydrolysate can be divided in two major groups based on their origin: naturally-occurring inhibitors from the feedstock such as weak acids (acetic, formic acid), and furan derivatives (furfural and 5-hydroxymethylfurfural) and phenolic compounds derived from sugars and lignin degradation, respectively [44, 79, 90].

1.5.4.1 Weak acids

The presence of sugar degradation products in hydrolysates often has an inhibitory effect on sugar fermentation process causing lower yields during the process [112]. For instance, acetic acid, once inside the cytoplasm, dissociates into hydronium and acetate ions, lowering intracellular pH [63]. In order to maintain intracellular pH, the plasma membrane ATPase pumps out protons at the expense of ATP hydrolysis [79]. In the presence of acetic acid, yeast can donate ATP to proton transport, ultimately restricting the availability of energy for cell growth. In the presence of high acid concentrations, ATP content gets depleted and results in cytoplasm acidification. The cell replicative ability and growth has been found to decrease linearly with decreasing intracellular pH [49]. No ATP means no energy and no cell growth; ultimately lower fermentation yields.

1.5.4.2 Furan derivatives

Other inhibitors, furfural and 5-hydroxymethylfurfural (5-HMF) are formed from degradation of xylose and glucose, respectively. They are metabolized by yeast to furfural alcohol and HMF-alcohol [79]. NADH-dependent alcohol dehydrogenase is believed to be responsible for this reduction. Since all NADH generated is used for furfural or 5-HMF reduction, glucose to ethanol process is greatly affected. This is because there is an increased acetaldehyde accumulation

inside the cell caused by an insufficient amount of NADH-dependent alcohol dehydrogenase available to reduce acetaldehyde to ethanol. Intracellular acetaldehyde accumulation is then considered to be the reason for the lag-phase in growth and ultimately lower yields produced in the presence of these inhibitors. Similar inhibitory mechanism is believed to be suggested for xylose to xylitol reduction in presence of these fermentation inhibitors [63, 94]. However, the effects of inhibitors on xylose to xylitol bioconversion have not been deeply investigated [82].

1.5.4.3 Phenolic compounds

Phenolic compounds are derived from partial break down of lignin which has an amorphous and highly irregular structure. During pretreatment, lignin is decomposed and releases monomers, dimers and polymers of aromatic units that have been identified as strongly inhibitory to yeasts [58]. Their inhibitory effect is due to loss of cell membrane integrity, thereby changing membrane permeability [79].

1.6 Rhodotorula mucilaginosa and Candida guilliermondii

One of the yeast identified in our labs, capable of rapid assimilation and catabolism of five and six carbon sugars (arabinose, galactose, glucose, xylose and mannose), is *Rhodotorula mucilaginosa* strain PTD3, an endophytic yeast of hybrid poplar *Populus trichocarpa x deltoids* [25, 114]. PTD3's stocks have been deposited at the Fungal Genetics Stock Center (<http://www.fgsc.net/>). PTD3 is the pink yeast strain known for its efficient performance in xylitol and ethanol production in the presence of several common fermentation inhibitors [105]. So far, investigation of xylitol production by yeasts has been limited to *Candida* and *Pichia* species, with few reports of studies of D-xylose metabolism in *Rhodotorula. spp.*

A second organism, *Candida guilliermondii*, a well known xylitol and ethanol producer, FTI-20037 (NRC 5578) was also utilized in this study to demonstrate cofermentation of xylitol alongside ethanol from lignocellulosic hydrolysates. This strain was obtained from the ATCC, a nonprofit biological resource center (BRC), Manassas, Virginia, [7, 59, 60]. *C. guilliermondii* converts xylose to xylitol via the Xylose Reductase-Xylitol Dehydrogenase [XR-XDH] pathway, presented in Figure 1.3. This yeast strain also concurrently ferments hexoses to ethanol. *C. guilliermondii* efficiently converts xylose in sugarcane bagasse to xylitol and is commonly considered for commercial production [63, 94]. There are many studies done about *C. guilliermondii* ability to tolerate inhibitors during bioconversion of lignocellulosic sugars. When evaluating the effect of acetic acid on xylose to xylitol bioconversion by *C. guilliermondii* from media augmented with synthetic sugars and acetic acid, Silva et al. [94] reported a reduction of 17% in cell growth and about 30% less xylitol yield when the fermentation medium contained more than 2g/L acetic acid. Kelly et al. [53] found that all three concentrations of furfural examined (0.5, 1 and 2 g/L) resulted in a moderately decreased growth rate of *C. guilliermondii*. At concentration of 2 g/L of furfural, the yeast produced 43% lower xylitol yield compared to the ‘no inhibitor’ control [53].

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CHAPTER 2

Novel endophytic yeast *Rhodotorula mucilaginosa* strain PTD3 I: Production of xylitol and ethanol

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I: Production of xylitol and ethanol

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Abstract

An endophytic yeast, *Rhodotorula mucilaginosa* strain PTD3 that was isolated from stems of hybrid poplar, was found to be capable of production of xylitol from xylose and ethanol from glucose, galactose, and mannose and arabitol from arabinose. The utilization of 30g/L of each of the five sugars during fermentation by PTD3 was studied in liquid batch cultures. Glucose-acclimated PTD3 produced enhanced yields of xylitol (67% of theoretical yield) from xylose and ethanol (84, 86, 94% of theoretical yield) from glucose, galactose and mannose, respectively. Additionally, this yeast was capable of metabolizing high concentrations of mixed sugars (150g/L), with high yields of xylitol 61% and ethanol 83% of theoretical yield. A 1:1 glucose:xylose ratio with 30g/L of each during double sugar fermentation did not affect PTD3's ability to produce high yields of xylitol (65% of theoretical yield) and ethanol (92% of theoretical yield). Surprisingly, the highest yields of xylitol (76% of theoretical yield) and ethanol (100% of theoretical yield) were observed during fermentation of sugars present in the lignocellulosic hydrolysate obtained after steam pretreatment of a mixture of hybrid poplar and Douglas-fir. PTD3 demonstrated an exceptional ability to ferment the hydrolysate, overcome hexose repression of xylose utilization with a short lag period of 10 hours and to tolerate sugar degradation products. In direct comparison PTD3 had higher xylitol yields from the mixed sugar hydrolysate compared to the widely studied and used xylitol producer, *Candida guilliermondii*.

Keywords: xylitol, ethanol, xylose, *Rhodotorula mucilaginosa*; *Candida guilliermondii*

2.1 Introduction

Different types of lignocellulosic biomass including agricultural, hardwood and softwood residues can potentially be converted into various value added products including biofuels and biochemicals. One of the products which can be obtained from biomass is xylitol. For over 30 years, considerable efforts have been focused on microbial production of xylitol from xylose [17, 35].

Xylitol is a five-carbon sugar alcohol with an established commercial history as an alternative sweetener. It has recently drawn the attention of food and drink manufacturers due to its low caloric value and thus the possibility of its use to reduce or control weight, leading to applications as a sweetener in chewing gums, mints, sweets and toothpaste [9]. It has also been utilized in the pharmaceutical industry due to its role in reduction of dental cavities [28]. Although xylitol is currently produced chemically by catalytic reduction of xylose, various microorganisms can convert xylose to xylitol by biological means. Several xylose-fermenting yeasts, which reduce xylose to xylitol by the NAD(P)H dependant xylose reductase (XR) such as *Candida*, *Pachysolen* and *Debaryomyces* strains have been tested [8, 10, 28, 35]. *Candida* yeasts in particular have been extensively studied with regards to their biotechnological application in the production of xylitol. Xylitol yields as high as 0.77 (g/g) for *C. guilliermondii* and 0.85 (g/g) for *C. tropicalis* have been reported by Barbosa [1] and Kwon [16], respectively. However, the yields depend on the type of microorganism employed and conditions for fermentation (nutrients, oxygen, pH, and temperature) used during the conversion of sugars to xylitol.

Establishing all experimental parameters and utilizing an appropriate microorganism for sugar fermentation is of great importance for the complete bioconversion of sugars into various

biochemicals. One yeast which was identified in our labs as being capable of rapid assimilation and catabolism of five and six carbon sugars (arabinose, galactose, glucose, xylose and mannose) is *Rhodotorula mucilaginosa* strain PTD3, an endophytic yeast of hybrid poplar *Populus trichocarpa x deltoides* [4, 36]. Since this is a novel, newly discovered yeast, very little is known about it and its behavior during bioconversion of lignocellulosic sugars to xylitol, ethanol and other co-products. Although studies have been done with other members of this yeast species for production of carotenoid pigments [20], lipid accumulation [13], and esterase activity [19], there has not yet been a detailed study conducted about *Rhodotorula mucilaginosa*'s ability to utilize a variety of sugars to produce xylitol and ethanol.

This is the first report on the bioconversion of xylose to xylitol, six carbon sugars to ethanol, and arabinose to arabitol by a newly discovered yeast *Rhodotorula mucilaginosa* strain PTD3 during synthetic single, double, and mixed sugars fermentation. The objective of this work is to characterize the novel yeast for utilization of sugars for xylitol and ethanol production in the single, double and mixed sugars fermentation media and to test the PTD3 strain in fermentation of hydrolysate from the pretreated lignocellulosic biomass in order to reveal its unique properties. The ultimate goal of our research regarding xylose utilization is to establish fermentation processes using both the hexose and pentose fractions of the hydrolysates obtained after steam pretreatment of lignocellulosic biomass to improve the feasibility of the bioconversion process.

2.2 Materials and methods

2.2.1 Yeast strains

Rhodotorula mucilaginosa strain PTD3, a pink yeast strain, was isolated from stems of hybrid poplar clone 184-402 (*Populus trichocarpa* × *P. deltoides*) from a greenhouse at the Oregon State University, Corvallis [36]. *Candida guilliermondii* FTI-20037 (NRC 5578) was obtained from the ATCC, a nonprofit biological resource center (BRC), Manassas, Virginia.

These strains were taken from -80°C stocks and maintained on YPG solid medium (10g/L yeast extract, 20g/L peptone, 20g/L glucose, and 18g/L agar, Difco, Becton Dickinson, MD) at 4°C and transferred to fresh plates on a weekly basis.

2.2.2 Culture media conditions

Cells were grown to high cell density in foam-plugged 1L Erlenmeyer flasks containing 500ml YP-sugar liquid media (10g/L yeast extract and 10g/L peptone, supplemented with 10g/L glucose) in an orbital shaker for 2 days at 30°C and 150 rpm, with concurrent transfer to fresh medium performed every 24 h. *C. guilliermondii* was pre-grown in a similar way but instead of glucose, xylose was utilized.

After 48 hours of growth, cell cultures were harvested, centrifuged, and decanted to yield cell pellets. Pellets were then washed three times with sterile distilled water and subsequently adjusted with sterile distilled water to a calculated concentration of 5g dry cell weight (DCW) per liter on a spectrophotometer (Shimadzu UV-1700, Columbia, MD) via standard curves relating 600nm absorbance to DCWL^{-1} (dry cell weight (DCW) per liter) concentration.

2.2.3 Carbohydrates and alcohols

Synthetic sugars (glucose, xylose, galactose, mannose, and arabinose) were obtained from Supelco, (Bellefonte, PA). Ethanol 4mg/ml, xylitol 5mg/ml, arabitol, and glycerol were obtained from Sigma–Aldrich, (St. Louis, MO).

2.2.4 Fermentations

2.2.4.1 Synthetic sugars

All fermentation experiments were performed three times with the appropriate controls that consisted of media lacking microorganism. Within each experiment, tests were conducted in triplicate in separate flasks. All media were sterilized by autoclaving. Solutions with sugars were filter-sterilized separately, and appropriate quantities added aseptically to the desired concentration to fermentation media.

Single sugar fermentations were performed in foam-plugged 125mL Erlenmeyer flasks (semi-aerobic) containing 1% (w/v) yeast extract, 1X Murashige and Skoog medium [21], and 3% (w/v) glucose or xylose with 50ml total volume. All fermentations were incubated at 30°C and maintained with continuous agitation (175 rpm), and initial pH value of ~ 6.0.

Double and mixed sugar fermentations were performed in a similar manner as single sugar fermentation with the following modification: for double sugar fermentations, media consisted of 3% (w/v) of each glucose and xylose while mixed sugar fermentations media contained 3% (w/v) of each sugar (arabinose, galactose, glucose, xylose and mannose). Sampling was aseptically performed at the time of inoculation and at specific time points thereafter. One milliliter aliquots were immediately centrifuged (14,000 rpm) for 4min at 4°C to yield cell-free supernatants,

which were then decanted and the supernatant was filtered by using a 0.22 μ m syringe filter (Restek Corp., Bellefonte, PA, U.S.) and then stored at -20°C until analysis.

2.2.4.2 The water soluble fraction (hydrolysate) fermentation

The mixture of hardwood (hybrid poplar) and softwood (Douglas-fir) chips (size 3/4x3/4x1/5 inch) with bark (60.0 % moisture content) was obtained from a University of Washington waste facility and stored at 4°C until use. The mixture was pre-pretreated by soaking in water overnight prior to SO₂-catalysed steam explosion. The detailed procedure of steam explosion experiments has been described previously by Ewanick [5]. Briefly, samples of 300g oven-dried weight (ODW) soaked chips were impregnated overnight with anhydrous SO₂ in plastic bags. The samples were then loaded, in 50g batches, into a preheated 2L steam gun in Gresham, Oregon and exploded at a temperature of 210°C, time 10 minutes and 3% (w/w) SO₂ concentration.

The water soluble fraction (hydrolysate) from steam explosion of the mixture of hardwoods and softwoods was recovered by filtration and kept at 4°C until use. The fermentation was performed in a similar manner as other fermentation experiments described earlier. The initial concentration of sugars present in the hydrolysate was arabinose (1.8g/L), galactose (2.7g/L), glucose (9g/L), xylose (7.6g/L) and mannose (9.2g/L) and the concentration of fermentation inhibitors was acetic acid (2.1g/L), 5-hydroxymethyl furfural (1.2g/L) and furfural (0.6g/L). A 1% (w/v) yeast extract and 1X Murashige and Skoog medium [21] were added to the hydrolysate fermented by PTD3 while 0.1% (w/v) yeast extract, 0.17% (w/v) yeast nitrogen base without amino acids and 0.5% (w/v) urea were added to the hydrolysate fermented by *C. guilliermondii*. The initial pH of the hydrolysate was adjusted to 6 prior to fermentation. The controls consisted of synthetic sugars at the same concentration as measured in the hydrolysate.

2.2.5 HPLC analysis

2.2.5.1 Monomeric sugars

The concentration of monomeric sugars (arabinose, galactose, glucose, xylose and mannose) was measured on a Dionex (Sunnyvale, CA) HPLC (ICS-3000) system equipped with an AS autosampler, ED electrochemical detector, dual pumps, and anion exchange column (Dionex, CarboPac PA1). Deionized water at 1 ml/min was used as an eluent, and post-column addition of 0.2 M NaOH at a flow rate of 0.5 ml/min ensured optimization of baseline stability and detector sensitivity. After each analysis, the column was reconditioned with 0.25 M NaOH. Twenty microliters of each sample were injected after filtration through a 0.22 μ m syringe filter (Restek Corp., Bellefonte, PA, U.S.). Standards were prepared containing sufficient arabinose, galactose, glucose, xylose, and mannose to encompass the same range of concentrations as the samples. Fucose (0.2g/L) was added to all samples and standards as an internal standard. The specific consumption rates were calculated based on the log-mean cell density

$$\frac{S - S_0}{X - X_0},$$

where S is the substrate or product, X is dry cell weight, and t is time [14].

2.2.5.2 Ethanol, xylitol and arabitol analysis

Ethanol, xylitol, arabitol and glycerol, were measured using refractive index detection on a Shimadzu Prominence LC. Separation of these compounds was achieved by an anion exchange column (REZEX RHM-Mono saccharide H⁺ (8%), Phenomenex, Inc., Torrance, CA, U.S.) with an isocratic mobile phase that consisted of 5mM H₂SO₄ at a flow rate of 0.6ml/min. The column oven temperature was maintained at a constant temperature of 63°C. Twenty microliters of each

sample were injected after being appropriately diluted in deionized water and filtered through a 0.22 μ m syringe filter (Restek Corp., Bellefonte, PA, U.S.). Standards were prepared and used to quantify the unknown samples.

The theoretical yield for ethanol production from glucose is 0.51g ethanol g⁻¹ glucose [23]. Ethanol yields and percent theoretical yields were calculated using the equations formulated by Keating [15]. The theoretical yield for xylitol production from glucose used was 0.91g xylitol g⁻¹ xylose [35]. Separation of arabinose, arabitol, and xylitol was achieved by an anion exchange column (REZEX RCM-Mono saccharide Ca⁺ (8%), Phenomenex, Inc., and Torrance, CA, U.S.) with isocratic mobile phase that consisted of HPLC grade water at a flow rate of 0.6 ml min⁻¹. The column oven temperature was maintained at 82°C. Twenty microliters of each sample were injected after being appropriately diluted in deionized water and filtered through a 0.22mm syringe filter (Restek Corp., Bellefonte, PA, U.S.).

It was assumed that all xylitol formed during the growth phase of the mixed sugar fermentations was derived from xylose and all arabitol formed during the same fermentation process was derived from arabinose. Cumulative xylitol (Y_{xylitol}; g xylitol produced g⁻¹ total xylose consumed) and arabitol (Y_{Arabitol}; g arabitol produced g⁻¹ total arabinose consumed) yields were calculated during and at the end-point of the fermentations. The specific production rates of xylitol from xylose, ethanol from galactose, glucose, and mannose, and arabitol from arabinose were calculated as described in the previous section.

2.3 Results and Discussion

2.3.1 Single synthetic sugar fermentation

The utilizations of five single sugars (xylose, glucose, arabinose, galactose and mannose) by *Rhodotorula mucilaginosa* strain PTD3 were studied in liquid batch cultures. In addition, the fermentation conditions for PTD3 (nitrogen, temperature, pH requirements and inoculum size) were tested previously as part of the preliminary investigation (data not shown). This is the first report on utilization of xylose to produce xylitol and six carbon sugars to produce ethanol by *R. mucilaginosa* strain PTD3.

Previous research with other xylose utilizers such as *Pachysolen tannophilus* and *Pichia stipitis* has shown that the activities of xylose reductase [XR] and xylitol dehydrogenase [XDH], two key enzymes in xylitol production, are induced in xylose pre-grown but not glucose-pre-grown yeasts [2]. Since *R. mucilaginosa* strain PTD3 has never been studied for production of xylitol from xylose and ethanol from six carbon sugars, during fermentation of xylose and glucose we tested yeast pregrown on glucose or xylose. It was shown that *R. mucilaginosa* pregrown on glucose converted xylose to xylitol and glucose to ethanol more efficiently (67% and 84% of theoretical yield, respectively) than when pregrown on xylose (59% and 64% of theoretical yield, respectively) with consumption and production rates approximately being twice as low (Table 1A and B, Table 2). Contrary to previous research [2], pre-growing on xylose did not stimulate xylose consumption and improve *R. mucilaginosa* xylitol fermentations (Table 2). Therefore, to test the utilization of galactose, mannose and arabinose, *R. mucilaginosa* was acclimated to glucose prior to fermentations. *R. mucilaginosa* exhibited varying responses to mannose, galactose and arabinose following acclimation to glucose. Ethanol yields of 94% and 86% of theoretical and arabitol yields of 29% of theoretical yield were observed respectively (Table 1A

and B). Of these sugars, the utilization of mannose was the most rapid, where complete consumption of 30g/L of this sugar required 26 hours (data not shown). This was followed by galactose (~50 hours). Only 29% of theoretical yield conversion of arabinose to arabitol occurred within 100 hours of fermentation (data not shown). The highest sugar consumption rate when PTD3 was pregrown on glucose was observed in glucose ($0.53 \text{ g g}^{-1} \text{ h}^{-1}$) and lowest in arabinose, ($0.06 \text{ g g}^{-1} \text{ h}^{-1}$) (Table 2). Among hexoses, the ethanol production rate was the highest from glucose ($0.27 \text{ g g}^{-1} \text{ h}^{-1}$) and lowest from galactose ($0.08 \text{ g g}^{-1} \text{ h}^{-1}$) (Table 2).

For this microorganism, with glucose, ethanol was the major fermentation product and glycerol concentration was negligible (0.2 g/L) (data not shown). Similarly, during metabolism of xylose by PTD3, xylitol was the main product and no ethanol was accumulated. Corresponding sugar utilization patterns and lower xylitol (55% of theoretical) yield were reported previously for *C. guilliermondii* by Lee [17]. During single sugar fermentation, the utilization of glucose was the most rapid; complete consumption of 20g/L of this sugar required 12 hours. This was followed by mannose (25 hours), xylose (36 hours) and galactose (42 hours) with similarity to PTD3's ethanol yields [17]. These results indicate PTD3's great ability to metabolize each available substrate with high xylitol and ethanol yields.

2.3.2 Double synthetic sugar fermentation

The ability of *R. mucilaginosa* to utilize and ferment concurrently glucose and xylose was studied. Since single sugar fermentation demonstrated that product yields were affected by acclimation conditions, during double sugar fermentation *R. mucilaginosa* was pregrown on glucose. For *R. mucilaginosa*, when glucose was present in the medium with xylose, a sequential pattern of utilization was observed, with glucose being consumed ahead of xylose (Figure 2.1). Utilization of glucose was not affected by xylose and commenced immediately (Figure 2.1).

However, xylose consumption was clearly affected by glucose and proceeded after lag period of 10 hours (Figure 2.1). This indicates the existence of a threshold above which glucose repression occurs as previously observed for *C. guilliermondii* and other yeast strains [17]. Double sugar fermentations indicated that *R. mucilaginosa* utilized xylose more slowly (75 hours) compared to single sugar fermentation (56 hours) (Figure 2.1). Indicative of the preference for glucose, the specific glucose consumption rate was 3 times higher ($0.51 \text{ g g}^{-1} \text{ h}^{-1}$) compared to the xylose rate ($0.16 \text{ g g}^{-1} \text{ h}^{-1}$) (Table 2). Xylose and glucose specific consumption and production rates for double sugar fermentation were lower compared to single sugar fermentation (Table 2). The complete xylose consumption by *R. mucilaginosa* yielded almost identical xylitol yields of 65% of theoretical yield compared to single sugars fermentation where 67% conversion of theoretical yield was achieved (Table 2.1.A and Figure 2.1). The complete consumption of glucose for single and double sugar fermentations occurred in 20 hours (Figure 2.1). However, the ethanol yield for double sugar fermentation (92% of theoretical yield) was 12% higher compared to single sugar fermentation (Table 2.1.B and Figure 2.1). The higher ethanol yield could be explained by the fact that PTD3 cell biomass was higher (3g/L more compared to the single sugar fermentation, data not shown) in double sugar fermentation. It is assumed that xylose was utilized for cell growth rather than for xylitol production.

In comparison, the other xylose-fermenting and xylitol-producing microbe *Candida guilliermondii* during double sugar fermentation of xylose and glucose had a xylitol yield of about 38% of theoretical yield and metabolized 20g/L of xylose within 70 hours [17]. Metabolism of 20g/L glucose started immediately and was completed in 10 hours with an ethanol yield of 25% of theoretical yield. PTD3 had higher yields of both, xylitol and ethanol compared to *C. guilliermondii*. However, PTD3 consumed xylose more slowly than *Candida*

guilliermondii. This is possibly due to the presence of a higher concentration of xylose (30g/L) compared to the media (20g/L) that was fermented by *C. guilliermondii*. Xylose is needed for the induction of xylose reductase and xylitol dehydrogenase and thus, high xylose concentration favors higher xylitol formation in yeasts [17, 35]. The PTD3's higher xylitol yield could be due to the concentration of yeast used and nutrients added to the fermentation media that were different for *Candida guilliermondii*. Ultimately, after assessing PTD3's co-fermentability of xylose with glucose with high yields, the next step was to study PTD3's performance in a mixed sugar fermentation composed of all five sugars that are naturally present in lignocellulosic hydrolysates.

2.3.3 Mixed synthetic sugar fermentation

For *R. mucilaginosa*, in the mixed sugar fermentation (arabinose, galactose, glucose, mannose, and xylose) a sequential pattern of utilization was observed, with glucose being consumed ahead of mannose, xylose, galactose and arabinose (Figure 2.2). Mixed sugar fermentations indicated that for *R. mucilaginosa*, xylose was utilized as fast as for double sugar fermentation (75 hours) and slower compared to single sugar fermentation (56 hours) (Figure 2.2). The complete xylose consumption by *R. mucilaginosa* yielded similar xylitol yields of 61% of theoretical compared to double (65%) and single sugars fermentation where 67% conversion of theoretical yield was achieved (Table 2.1.A and Figure 2.1).

The complete consumption of glucose from mixed sugars fermentation for PTD3 occurred in 26 hours, mannose 36 hours and galactose 74 hours. It took 6 hours longer for complete glucose metabolism for mixed sugar fermentation compared to single and double sugar fermentations (20 hours for both) (Table 2.1.B, Figure 2.1 and 2.2). However, the ethanol yield (83%) for all the six carbon sugars from mixed sugar fermentation was similar or smaller to single and double

sugar fermentation (84% and 92 of theoretical yield, respectively) (Table 2.1.B and Figure 2.1 and 2.2).

The utilization of mannose was not decreased but rather improved by the presence of the other sugars, while arabinose, galactose, glucose, and xylose were affected by being mixed together during mixed sugar fermentation compared to those found when they were the sole carbon source. The specific consumption rates of arabinose, galactose, glucose, and xylose (0.01, 0.16, 0.47, 0.16 $\text{gg}^{-1}\text{h}^{-1}$, respectively) were smaller compared to those found when they were the sole carbon source (0.06, 0.18, 0.53, 0.20 $\text{gg}^{-1}\text{h}^{-1}$, respectively) (Table 2). During mixed sugars fermentation PTD3 fully metabolized mannose within the same time period (27 hours) as during single sugar fermentation. Galactose was consumed completely, although at a rate slower (76 hours) than that when it was the sole carbon source (50 hours) while arabinose was not completely metabolized within 100 hours compared to single sugar fermentation (100 hours).

The ability of *Rhodotorula mucilaginosa* strain PTD3 to utilize and ferment xylose in the presence of another sugars showed similar behavior to *Candida tropicalis* with a xylitol yield of 69% of theoretical yield [29]. When glucose, mannose, and galactose were present in the medium mixed with xylose, a specific pattern of consumption was observed, with six carbon sugars being consumed ahead of xylose and arabinose. In culture of PTD3, the presence of xylose did not affect hexose utilization. The assimilation of glucose and mannose commenced immediately while that of xylose proceeded with a lag period of 12 hours, similar to double sugar fermentation. This indicates the existence of a threshold above which hexose repression occurs. This behavior has been seen with other yeast strains [2, 27]. Also, galactose consumption lagged for 24 hours. Although traditional microorganisms employed in ethanol fermentation exhibit preferences for hexose sugars, the mixture of glucose, galactose, and mannose presents a

metabolic obstacle to the efficient production of ethanol. This can be related to catabolite repression in which substrates are fermented sequentially. For example, galactose utilization markedly lags behind glucose and mannose consumption in the yeast *Saccharomyces cerevisiae* because of catabolite repression [7]. After assessing PTD3's fermentability of synthetic single, double and mixed sugars, it was necessary to test its ability to ferment streams collected after steam pretreatment of lignocellulosic substrates.

2.3.4 The water soluble fraction (hydrolysate) obtained after steam pretreated mixture of hardwoods and softwoods fermentation

In order to characterize *Rhodotorula mucilaginosa* strain PTD3's fermentability of lignocellulosic hydrolysates, the direct comparison with the well known xylitol and ethanol producer, *Candida guilliermondii* [11, 18, 32] was carried out. Hydrolysate obtained from a steam pretreated hardwood and softwood mixture was used as media for production of xylitol and ethanol by *Rhodotorula mucilaginosa* strain PTD3. The comparison of *Rhodotorula mucilaginosa* and *Candida guilliermondii* was done using the same lignocellulosic hydrolysate and parameters for both microorganisms. However, the lignocellulosic hydrolysate prepared for PTD3 was supplemented with different nutrients compared to the hydrolysate fermented by *C. guilliermondii* (as described in methods section). The synthetic mixed sugar controls were prepared using concentrations of each sugar found within the hydrolysate.

It was shown that PTD3 consumed xylose within 24 hours, and a high xylitol yield was obtained of 78% of theoretical yield (Figure 2.3.A), higher compared to synthetic sugars control 65% of theoretical yield (data not shown). *Candida guilliermondii* consumed xylose within 9 hours, and a high xylitol yield was obtained of 73% of theoretical yield (Figure 2.3.B), higher compared to

control 64% (data not shown). Xylose consumption commenced immediately by both microorganisms and no lag phase existed. The xylitol specific production rate, for PTD3 was higher ($0.04\text{gg}^{-1}\text{h}^{-1}$) compared to *C. guilliermondii* ($0.03\text{gg}^{-1}\text{h}^{-1}$) (data not shown) and based on the xylitol theoretical yields, the xylitol production for PTD3 was more pronounced.

During fermentation of the hydrolysate, glucose was consumed within 9 hours, and a high ethanol yield from all the six carbon sugars was obtained of 100% of theoretical yield (Figure 2.3.A), higher compared to the control 83% (data not shown). For *C. guilliermondii*, glucose was consumed within 4 hours, and a high ethanol yield was obtained of 100% (Figure 2.3.B), higher compared to control 66% (data not shown). The results from lignocellulosic hydrolysate were comparable with those attained in a synthetic medium and showed that lignocellulosic hydrolysate can be converted into xylitol and ethanol with approximately 15% higher yields. It is important to mention that the results were obtained without employing any detoxification methods such as yeast adaptation, neutralization and overliming, evaporation, solvent extraction, charcoal adsorption, biological treatment or the use of ion exchange resin [3, 22, 24, 34]. PTD3 demonstrated an ability to metabolize a variety of sugars coming even from lignocellulosic hydrolysates and producing higher xylitol yields compared to those of *Candida guilliermondii* reported in other studies. Conversion of 66% of theoretical yield of xylose to xylitol was shown by Silva [31] using *C. guilliermondii* from acid hydrolyzed hemicellulosic fractions of sugarcane bagasse and rice straw. Using the same strain, Felipe [6] reported xylitol yields of 29% of theoretical yield from hemicellulosic hydrolysate that was obtained by acid hydrolysis of eucalyptus chips.

It is noteworthy that PTD3 had a higher ethanol yield (100% of theoretical yield) from steam pretreated mixture of hardwood and softwood mixture compared to *Saccharomyces cerevisiae*,

Tembec Ltd strain, that had an ethanol yield of 74% of theoretical yield from steam pretreated Douglas-fir with 10% bark [30]. The total concentrations of six carbon sugars (31g/L) and xylose (3.4g/L) in hydrolysates that Robinson [30] tested was similar to the concentrations (21g/L) and (7.8g/L), respectively, found in the hydrolysate tested in our study. The concentrations of fermentation inhibitors, furfural and 5-HMF, that Robinson [30] reported were (0.3g/L and 1.4g/L, respectively) while in the water soluble stream we tested the concentrations of acetic acid (2.1g/L), furfural (0.6g/L) and 5-HMF(1.2g/L) were measured. The pretreatment conditions for both biomass types were similar, causing reduced generation of process-derived fermentation inhibitors. However, the difference was in the presence of 2.1 g/L of acetic acid in the hydrolysate that was tested in our study. This is due to the added presence of hardwoods in our feedstock. Hardwood hemicellulose is highly acetylated [25, 33] and thus acetic acid is produced by lignocellulose degradation.

One possible explanation for higher ethanol yield obtained by PTD3 could be that PTD3 is more robust, wild yeast compared to *S. cerevisiae* Tembec. Another is that the acetic acid present in the hydrolysate improved ethanol yields due to the yeast's need to maintain a neutral intracellular pH which is crucial for cell viability [12]. Low concentrations of acetic acid have been shown to have a stimulating effect on ethanol production by *S. cerevisiae* [26]. Thus, this could enhance the potential of this yeast for fermentation of hexose sugars in hydrolysates of lignocellulosic substrates.

In this study *R. mucilaginosa* strain PTD3 demonstrated the ability to assimilate all five sugars that are naturally present in lignocellulosic biomass and behaved similarly to the widely studied and used xylitol producer, *C. guilliermondii*. Although *R. mucilaginosa* strain PTD3 is a novel

yeast, it demonstrated great potential for future studies of the bioconversion of lignocellulosic hydrolysates to biochemicals.

2.4 Conclusions

R. mucilaginosa strain PTD3 was found capable of assimilating and fermenting xylose, glucose, galactose, mannose, and arabinose as a single and as well as mixed carbon source. This strain produced not only xylitol from xylose but ethanol and arabitol from hexoses and arabinose, respectively. When pregrown on glucose, PTD3's ability to metabolize sugars and produce xylitol and ethanol is enhanced. Xylitol and ethanol yields were not affected by a 1:1 ratio of xylose to glucose, resulting in repeated high theoretical yields, (65 and 92% respectively). Furthermore, the yeast exhibited the ability to ferment high concentrations of mixed sugars (150g/L). Additionally, the specific xylitol production rate was highest for double sugar ($0.10 \text{ g g}^{-1} \text{ h}^{-1}$) and for ethanol was highest for single sugar fermentation ($0.27 \text{ g g}^{-1} \text{ h}^{-1}$). Remarkably, this yeast performed best during fermentation of sugars coming from lignocellulosic hydrolysate, producing the highest yields of xylitol (76% of theoretical yield) and ethanol (100% of theoretical yield). Fermentation of the steam pretreated lignocellulosic hydrolysate served to illustrate PTD3's ability to utilize and ferment xylose in the presence of other sugars and to tolerate pretreatment degradation products.

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Figure Legends

Table 2.1.A. Maximum xylitol yields (product per unit substrate [YP/S] and percent theoretical [Y%T]) yields, arabitol yields and biomass accumulation during single sugar fermentation by *R. mucilaginosa* (*Rm*) pregrown on glucose or xylose. Standard deviation is indicated.

Table 2.1.B. Maximum ethanol yields (product per unit substrate [YP/S] and percent theoretical [Y%T]) yields and biomass accumulation during single sugar fermentation by *R. mucilaginosa* (*Rm*) pregrown on glucose or xylose. Standard deviation is indicated.

Table 2.2. The specific rates of sugar consumption and XOH, EOH and AOH production from synthetic sugars by *Rhodotorula mucilaginosa* (*Rm*) during single, double, and mixed sugar fermentation.

Figure 2.1. Sugar consumption and xylitol and ethanol production during double (glucose and xylose fermentation by *R. mucilaginosa* following acclimation to glucose. The error bars indicate standard deviation.

Figure 2.2. Sugar consumption and xylitol and ethanol production during mixed (arabinose, galactose, glucose, mannose, and xylose fermentation by *R. mucilaginosa* following acclimation to glucose. The error bars indicate standard deviation.

Figure 2.3. Sugar consumption and xylitol and ethanol production during fermentation of hydrolysates obtained during steam explosion of the mixture of softwoods and hardwoods by: (A) *R. mucilaginosa* following acclimation to glucose; (B) *C. guilliermondii* following acclimation to xylose. The error bars indicate standard deviation.

Table 2.1.A Maximum xylitol yields (product per unit substrate [YP/S] and percent theoretical [Y%T]) yields, arabitol yields and biomass accumulation during single sugar fermentation by *R. mucilaginosa* (*Rm*) pregrown on glucose or xylose. Standard deviation is indicated.

	$[XOH/AOH]^*$ YP/S (g/g)	$[XOH /AOH]^*$ $Y\%T$ (%)	$[Dry\ cell]_{max}$ (g/L)
Xyl (Glu-grown)	0.3 ± 0	67 ± 0.3	13.6 ± 0.1
Xyl (Xyl-grown)	0.3 ± 0	59 ± 0.1	11.8 ± 0.1
Ara (Glu-grown)	0.2 ± 0	29 ± 0.3	7.8 ± 0.2

* Xylitol (XOH) is the product of xylose and arabitol (AOH) is the product of arabinose fermentation.

Table 2.1.B Maximum ethanol yields (product per unit substrate [YP/S] and percent theoretical [Y%T]) yields and biomass accumulation during single sugar fermentation by *R. mucilaginosa* (*Rm*) pregrown on glucose or xylose. Standard deviation is indicated.

	$EOH\ YP/S$ (g/g)	$EOH\ Y\%T$ (%)	$[Dry\ cell]_{max}$ (g/L)
Glu (Glu-grown)	0.5 ± 0	84 ± 1	18.3 ± 0.1
Glu (Xyl-grown)	0.3 ± 0	64 ± 1	13.4 ± 0.2
Gal (Glu-grown)	0.4 ± 0	86 ± 1	16.2 ± 0.1
Man (Glu-grown)	0.5 ± 0	94 ± 1	16.1 ± 0.1

Table 2.2 The specific rates of sugar consumption and XOH, EOH and AOH production from synthetic sugars by *Rhodotorula mucilaginosa* (Rm) during single, double, and mixed sugar fermentation.

	<i>Single fermentation</i>		<i>Double fermentation</i>		<i>Mixed fermentation</i>	
<i>Fermentation parameters</i>	<i>Consumption²</i> (<i>gg⁻¹h⁻¹</i>)	<i>Production³</i> (<i>gg⁻¹h⁻¹</i>)	<i>Consumption²</i> (<i>gg⁻¹h⁻¹</i>)	<i>Production³</i> (<i>gg⁻¹h⁻¹</i>)	<i>Consumption²</i> (<i>gg⁻¹h⁻¹</i>)	<i>Production³</i> (<i>gg⁻¹h⁻¹</i>)
Xylose (Xyl¹)	0.20, (0.12 ¹)	0.09, (0.06 ¹)	0.16	0.10	0.16	0.07
Glucose (Xyl¹)	0.53, (0.37 ¹)	0.27, (0.12 ¹)	0.51	0.24	0.47	NC
Galactose	0.18	0.08	NA	NA	0.16	NC
Mannose	0.34	0.17	NA	NA	0.46	NC
Arabinose	0.06	0.02	NA	NA	0.01	0

The reported results are the average of triplicate studies with a deviation of $\leq 2\%$.

NA- not applicable

NC-not calculated. The ethanol production rate from each glucose, galactose, and mannose was not calculated due to the difficulty of knowing the exact concentration of ethanol produced from each sugar during mixed sugars fermentation.

¹ Production and consumption rates of PTD3 when pregrown on xylose, otherwise PTD3 was always pregrown on glucose.

² The specific rates of sugar consumption were calculated based on the log-mean dry cell density and the Δ substrate and Δ time.

³ The specific rates of xylitol from xylose, ethanol from galactose, glucose, and mannose, and arabitol from arabinose production were calculated based on the log-mean dry cell density and the product concentration and Δ time.

Figure 2.1 Sugar consumption and xylitol and ethanol production during double (glucose and xylose) fermentation by *R. mucilaginosa* following acclimation to glucose. The error bars indicate standard deviation.

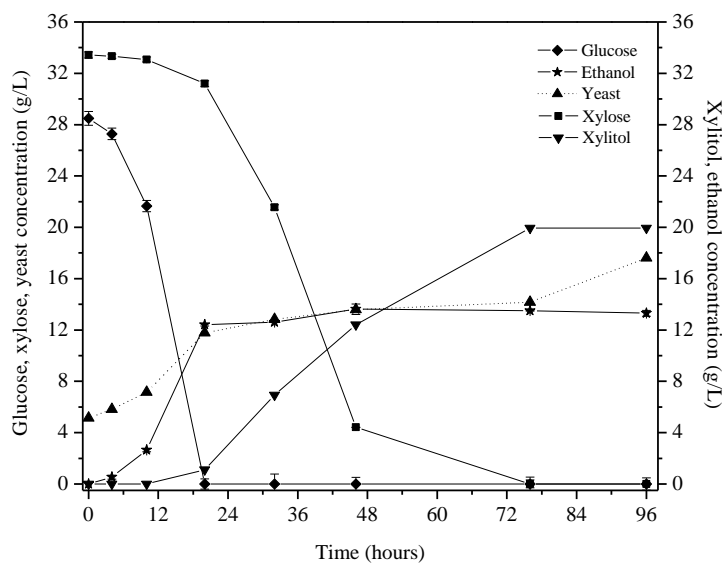


Figure 2.2 Sugar consumption and xylitol and ethanol production during mixed (arabinose, galactose, glucose, mannose, and xylose) fermentation by *R. mucilaginosa* following acclimation to glucose. The error bars indicate standard deviation.

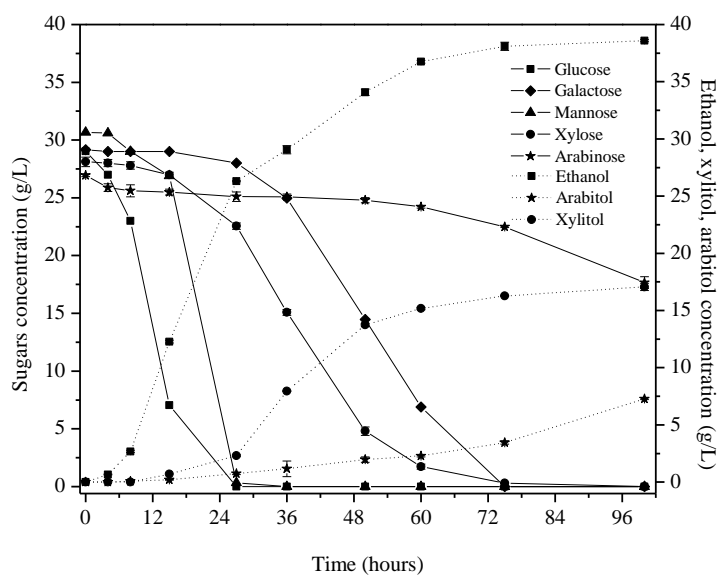
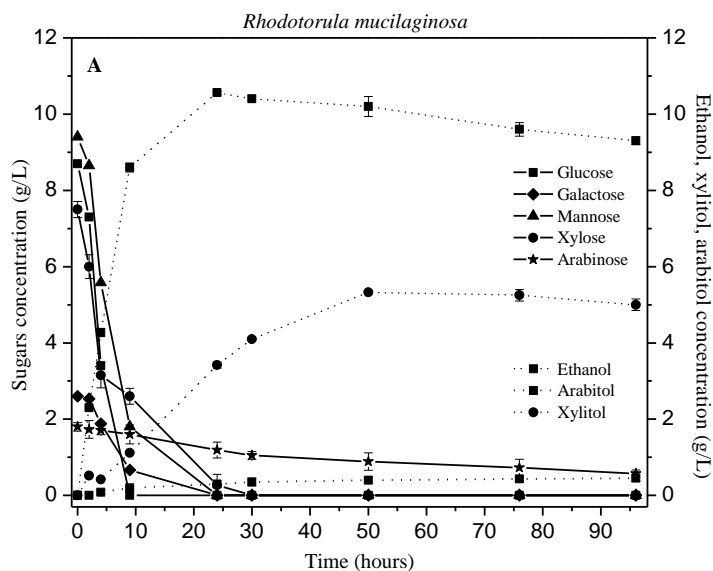
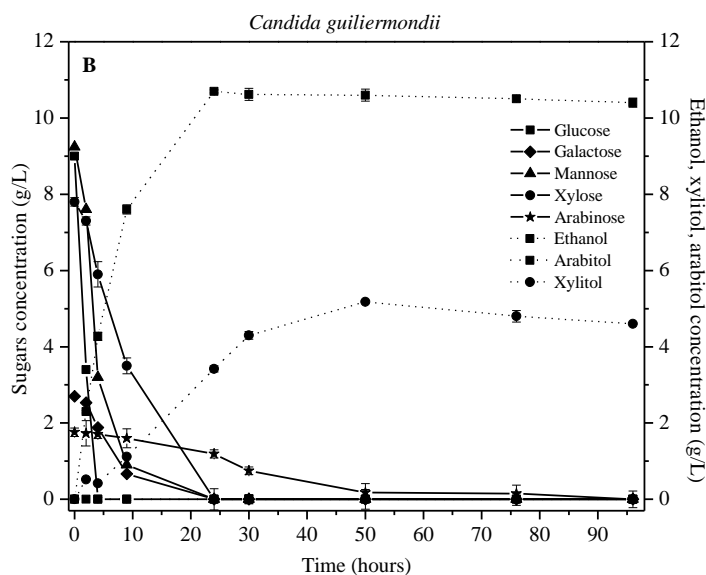


Figure 2.3 Sugar consumption and xylitol and ethanol production during fermentation of hydrolysates obtained during steam explosion of the mixture of softwoods and hardwoods by: (A) *R. mucilaginosa* following acclimation to glucose; (B) *C. guilliermondii* following acclimation to xylose. The error bars indicate standard deviation.



*Initial concentrations of acetic acid (2.1g/L), furfural (0.6g/L) and 5-HMF (1.2g/L).



*Initial concentrations of acetic acid (2.1g/L), furfural (0.6g/L) and HMF (1.2g/L).

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CHAPTER 3

**Novel endophytic yeast *Rhodotorula mucilaginosa* strain PTD3
II: Production of xylitol and ethanol in the presence of inhibitors**

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Novel endophytic yeast *Rhodotorula mucilaginosa* strain PTD3
II: Production of xylitol and ethanol in the presence of inhibitors

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Abstract

A systematic study was conducted characterizing the effect of furfural, 5-hydroxymethylfurfural (5-HMF), and acetic acid concentration on the production of xylitol and ethanol by a novel endophytic yeast, *Rhodotorula mucilaginosa* strain PTD3. The influence of different inhibitor concentrations on the growth and fermentation abilities of PTD3 cultivated in synthetic nutrient media containing 30 g/L xylose or glucose were measured during liquid batch cultures. Concentration of up to 5 g/L of furfural stimulated production of xylitol to 77% of theoretical yield (10% higher compared to the control) by PTD3. Xylitol yields produced by this yeast were not affected in a presence of 5-HMF at concentrations of up to 3 g/L. At higher concentrations of furfural and 5-HMF xylitol and ethanol yields were negatively affected. The higher the concentration of acetic acid present in a media, the higher the ethanol yield approaching 99% of theoretical yield (15% higher compared to the control) was produced by the yeast. At all concentrations of acetic acid tested, xylitol yield was lowered. PTD3 was capable of metabolizing concentrations of 5, 15, and 5 g/L of furfural, 5-HMF, and acetic acid, respectively. This yeast would be a potent candidate for the bioconversion of lignocellulosic sugars to biochemicals given that in the presence of low concentrations of inhibitors, its xylitol and ethanol yields are stimulated and it is capable of metabolizing pretreatment degradation products.

Keywords: acetic acid; furfural; 5-hydroxymethylfurfural (5-HMF); *Rhodotorula mucilaginosa*; xylitol;

3.1 Introduction

Rhodotorula mucilaginosa strain PTD3 ferments xylose to xylitol, six carbon sugars (galactose, glucose, and mannose) to ethanol, and arabinose to arabitol as it was characterized by Bura [1]. It was shown that, PTD3 is capable of rapid assimilation and catabolism of five and six carbon sugars as a single, double, and mixed carbon source. Bura [1] reported that this yeast performed best during fermentation of sugars coming from lignocellulosic hydrolysate, producing the highest yields of xylitol 76% of theoretical yield (10% higher compared to the synthetic sugar control) and ethanol 100% of theoretical yield (16% higher compared to the synthetic sugars control). This water soluble fraction (hydrolysate) was collected from steam explosion of the mixture of hardwood (hybrid poplar) and softwood (Douglas-fir) chips with bark. The concentrations of fermentation inhibitors present in the hydrolysate were acetic acid (2.1 g/L), 5-hydroxymethyl furfural (1.2 g/L) and furfural (0.6 g/L). Additionally, PTD3 demonstrated a huge potential for bioconversion of lignocellulosic rich urban waste into biochemicals [21]. The water soluble fractions of mixed waste paper, yard waste, and food waste were collected after pretreatment and assessed for their feasibility as media for effective fermentation to ethanol by PTD3 [21]. The ethanol yields from hexoses (glucose, mannose and galactose) for all the sugar streams tested were close to 100% of theoretical ethanol despite the presence of fermentation inhibitors [21]. Fermentation of the steam pretreated lignocellulosic hydrolysates, municipal solid waste, low grade mixed waste paper, and organic yard waste, served to illustrate PTD3's ability to utilize and ferment xylose in the presence of other sugars and to tolerate pretreatment degradation products. Since this is a novel yeast strain, very little is known about it and its behavior during bioconversion of lignocellulosic sugars to ethanol and other co-products,

especially in the presence of fermentation inhibitors that are generated during the pretreatment process of lignocellulosic biomass.

Lignocellulosic feedstock represents an abundant and inexpensive source of sugars which can be microbiologically converted to biochemicals. However, bioconversion of the sugars into biochemicals such as xylitol and ethanol using hydrolysates obtained after pretreatment of lignocellulosic materials is hindered by the presence of by-products liberated during the pretreatment process [15]. One problem associated with fermentation of such substrates is the presence of pretreatment-derived inhibitors which adversely affect microbial growth and fermentation [10, 25]. The common degradation products are furfural, 5-hydroxymethylfurfural (5-HMF), weak acids such as acetic acid, and phenolic compounds that are lignin degradation products. All of these inhibitors significantly affect the sugar fermentation process [16]. The concentration of each degradation product in hydrolysates, obtained after pretreatment, depends on the severity of the pretreatment conditions and of the feedstock undergoing the pretreatment method [15].

The presence of these sugar degradation products in hydrolysates and their inhibitory effect on sugar fermentation processes have been studied intensively [10, 25]. However, the effects of inhibitors on xylose to xylitol bioconversion have not been deeply investigated [19]. Pereira [19] reported that acetic acid, syringaldehyde, and ferulic acid are compounds that adversely affected metabolism of *Candida guilliermondii* (mainly cell growth) during conversion of xylose to xylitol. For example, xylose consumption, xylitol production, and cell growth were reduced by 13%, 18%, and 30%, respectively by the presence of acetic acid at concentration of 2.6 g/L compared to the control [19]. It was concluded that their toxic effect to *C. guilliermondii* was dependent on their concentration in the medium, with inhibition being more pronounced at

higher concentrations. However, it was stated that employing any detoxification methods of the fermentation medium was not necessary to obtain efficient conversion of xylose to xylitol by *C. guilliermondii* [19]. This suggests that sugar degradation products being present at small concentrations in a medium might not have an inhibitory affect on the whole bioconversion process. The maximum tolerable concentration of each inhibitor that can be present in hydrolysate without affecting the efficiency of the fermentative process is dependent on the microorganism utilized and its degree of adaptation, the fermentation process employed, and the simultaneous presence of other inhibitors [10, 25]. Establishing all these parameters and utilizing an adequate microorganism for sugar fermentation is of a great importance for the whole bioconversion of lignocellulosic derived hydrolysates into various biochemicals.

Knowledge regarding inhibitors and how to minimize their effects is of a great importance for a successful fermentation. Ultimately, after assessing PTD3's co-fermentability of xylose, the six carbon sugars, and sugar degradation products with high xylitol and ethanol yields, the next step was to study further PTD3's tolerance of higher concentrations of sugar degradation products. It was shown that PTD3 is capable of assimilating and fermenting xylose, glucose, galactose, mannose, and arabinose as a single and as well as mixed carbon source. Remarkably, this yeast performed best during fermentation of sugars coming from lignocellulosic hydrolysates despite the presence of fermentation inhibitors. Since PTD3 is novel yeast, not much is known about its fermentation abilities especially in the presence of inhibitors. Therefore, in order to learn the full potential of *Rhodotorula mucilaginosa* strain PTD3 for bioconversion of lignocellulosic hydrolysates to biochemicals, the objective of this research was a systematic study of the effect of acetic acid, furfural and 5-HMF on the fermentation of both xylose and glucose to xylitol and ethanol, respectively, by this yeast.

3.2 Materials and methods

3.2.1 Yeast strains

Rhodotorula mucilaginosa strain PTD3, a pink yeast strain, was isolated from stems of hybrid poplar 184-402 (*Populus trichocarpa* × *P. deltoides*) grown in greenhouse at the Oregon State University, Corvallis [26]. Throughout all fermentation experiments the *Rhodotorula mucilaginosa* strain PTD3 was used. The strain was taken from -80°C stocks and maintained on YPG solid medium (10 g/L yeast extract, 20 g/L peptone, 20 g/L glucose, and 18 g/L agar, Difco, Becton Dickinson, MD) at 4°C and transferred to fresh plates on a weekly basis.

3.2.2 Culture media conditions

Cells were grown to high cell density in foam-plugged 1L Erlenmeyer flasks containing 500 mL YP-sugar liquid media (10 g/L yeast extract and 10 g/L peptone, supplemented with 10 g/L glucose) in an orbital shaker for 2 days at 30°C and 150 rpm, with a transfer to fresh medium performed every 24h. After 48 hours of growth, cell cultures were harvested, centrifuged, and decanted to yield cell pellets. Pellets were then washed three times with sterile distilled water and subsequently adjusted with sterile distilled water to a calculated concentration of 5 g dry cell weight (DCW) per liter on a spectrophotometer (Shimadzu UV-1700, Columbia, MD) via standard curves relating 600nm absorbance to DCWL^{-1} (dry cell weight (DCW) per liter) concentration.

3.2.3 Carbohydrates, inhibitors and alcohols

Synthetic sugars (glucose, xylose, galactose, mannose, and arabinose), furfural, 5-HMF and acetic acid were obtained from Supelco, (Bellefonte, PA). Ethanol 4 mg/mL, xylitol 5 mg/mL, and glycerol were obtained from Sigma–Aldrich, (St. Louis, MO).

3.2.4 Fermentations

All fermentation experiments were performed three times with the appropriate controls that consisted of a media lacking the microorganism. Within each experiment, tests were conducted in triplicate in separate flasks. All media were sterilized by autoclaving. Solutions with sugars were filter-sterilized separately, and appropriate quantities added aseptically to the desired concentration in the fermentation media. Single sugar fermentations were performed in foam-plugged 125mL Erlenmeyer flasks (semi-aerobic) containing yeast extract-MS Murashige and Skoog medium [14] (1% (w/v) yeast extract, 1X MS, 3% (w/v) glucose or xylose) with 50mL total volume. All fermentations were incubated at 30°C and maintained with continuous agitation (175 rpm), and pH value of ~ 6.0. Sampling was aseptically performed at the time of inoculation and at specific time points thereafter. One milliliter aliquots were immediately centrifuged (14,000 rpm) for 4 min at 4°C to yield cell-free supernatants. These samples were then decanted and the supernatant was filtered by using a 0.22µm syringe filter (Restek Corp., Bellefonte, PA, U.S.) and then stored at -20°C until the analysis. For media requirement analysis, 1% (w/v) Bacto-peptone was used along with MS with or without yeast extract. Inhibitor-supplemented media was augmented with different concentrations of furfural (1, 1.5, 3, 5, 10 and 20 g/L), 5-HMF (1, 1.5, 3, 5, 10 and 15 g/L) or acetic acid (5, 10 and 20 g/L). The acetic acid-augmented media were adjusted to pH 6.0 prior to inoculation.

3.2.5 HPLC analysis

3.2.5.1 Monomeric sugars, inhibitors, ethanol, and xylitol analysis

The concentration of xylose, glucose, ethanol, xylitol, glycerol, acetic acid, furfural and 5-HMF were measured using high performance liquid chromatography refractive index detection on a Shimadzu Prominence LC [21]. Separation of those compounds was achieved by an anion exchange column (REZEX RHM-Mono saccharide H⁺ (8%), Phenomenex, Inc., Torrance, CA, U.S.) with isocratic mobile phase that consisted of 5 mM H₂SO₄ at a flow rate of 0.6 mL min⁻¹. The column oven temperature was maintained at a constant 63°C [21]. Samples were defrosted from -20°C and twenty microliters of each sample were injected after being appropriately diluted in deionized water and filtered through a 0.22 µm syringe filter (Restek Corp., Bellefonte, PA, U.S.). Standards were prepared and used to quantify the unknown samples.

The theoretical yield for ethanol production from glucose is 0.51 g ethanol g⁻¹ glucose [15]. Ethanol yields and percent theoretical yields were calculated using the equations formulated by [8]. The theoretical yield for xylitol production from xylose used was 0.91 g xylitol g⁻¹ xylose [25]. The specific consumption and production rates were calculated based on the log-mean cell density,

$\frac{S - S_0}{X - X_0}$, where S is the substrate or product, X is dry cell weight, and t is time [6]. Since

within each experiment tests were conducted in triplicates in separate flasks, the standard deviation was calculated between three samples using the excel's statistical function.

3.3 Results and Discussion

3.3.1 Furfural-Supplemented Fermentation

The effect of furfural at concentrations from 1 g/L to 20 g/L on the fermentation of xylose to xylitol and glucose to ethanol by *R. mucilaginosa* strain PTD3 was studied (Figure 3.1, Table 3.1A). Surprisingly, the higher the concentration of furfural up to 5 g/L, the higher the xylitol yield (10% higher compared to the furfural-free control, 67% of theoretical) that was produced by PTD3 (Figure 3.1E, Table 3.1A). Based on this we conclude that furfural at concentrations up to 5 g/L stimulated xylitol yield and to our knowledge this has not been reported in the literature. Kelly [9] found that in the presence of up to 3 g/L furfural, xylitol production rate by *C. guilliermondii* was reduced 43% of theoretical compared to the control. Not only can PTD3 produce a higher concentration of xylitol in presence of furfural at up to 5 g/L, but it also metabolized all the furfural (5 g/L) in solution within 12 hours (Figure 3.1C). The specific xylose consumption rate ($0.05 \text{ gg}^{-1}\text{h}^{-1}$) was the highest for up to 5 g/L of furfural (Table 3.1A). In the present study, the 30 g/L of xylose was completely consumed by PTD3 within 120 hours for concentrations of up to 5 g/L of furfural tested (Figure 3.1A). However, at the higher concentrations of furfural (10 g/L and 20 g/L), slower xylose consumption was observed compared to the control (Figure 3.1A). The specific production rate for xylitol was the highest and same for up to 3 g/L of furfural (Table 3.1A). Surprisingly enough, the specific consumption rate of furfural increased as the concentration of furfural increased from 1 g/L to 3 g/L and was the highest at 3 g/L. However, at concentrations above 3 g/L furfural, the specific consumption rate of furfural decreased significantly. This suggest that concentrations of up to 3 g/L furfural have rather a positive influence on conversion of xylose to xylitol by PTD3 while the higher concentrations adversely affect xylose metabolism and xylitol yield.

Indicative of the negative effect of higher concentrations of 10-20 g/L furfural, xylitol production was delayed and reduced to 1.2% of theoretical (Figure 3.1D) compared to control (Figure 3.1E, Table 3.1A). The consumption of furfural was also reduced as the concentration of furfural increased: 53% of 10 g/L and 27% of 20 g/L (Figure 3.1C). It is understood that yeast, during bioconversion of six carbon sugars to ethanol, metabolizes furfural to furfural alcohol [16]. NADH-dependent alcohol dehydrogenase is thought to be responsible for this reduction, causing the attenuated xylitol and ethanol yields in our study. Since all NADH generated is used for furfural reduction, the glucose to ethanol and likely also the xylose to xylitol process are greatly affected. This is because there is an increased acetaldehyde accumulation inside the cell caused by an insufficient amount of NADH-dependent alcohol dehydrogenase available in order to reduce acetaldehyde to ethanol. Intracellular acetaldehyde accumulation is then considered to be the reason for the lag-phase in growth and ultimately lower yields produced at the higher concentration of this inhibitor [9, 16].

Unlike xylitol yields, the presence of furfural negatively affected ethanol yields by PTD3 at all concentration tested. As the concentration of furfural in media increased (from 0 g/L to 20 g/L), glucose to ethanol yields by this strain decreased and even at the lowest concentration of furfural (1 g/L) tested, the ethanol yield (74% of theoretical) was already negatively affected compared to the furfural-free control (84% of theoretical) (Figure 3.1F, Table 3.1A). The specific glucose consumption and ethanol production rates ($0.12 \text{ g g}^{-1} \text{ h}^{-1}$ and $0.04 \text{ g g}^{-1} \text{ h}^{-1}$, respectively) were highest at 1 g/L of furfural (Table 3.1A). The yeast metabolized 30 g/L glucose in media with up to 5 g/L furfural within 50 hours but was slower compared to the control, while at 10 g/L and 20 g/L furfural glucose was not completely consumed (Figure 3.1B). Similar to xylose-augmented media, in glucose-supplemented media, PTD3 was able to completely metabolize up to 5 g/L of

furfural within 12 hours whereas consumption of 79% of 10 g/L and 29% of 20 g/L was observed (Figure 3.1D). The specific consumption rate of furfural was the highest at 3 g/L while afterwards was decreased significantly (Table 3.1A). A 5 g/L furfural resulted in a 72% and 50% inhibition of cellular growth of the microorganism in xylose and the glucose-supplemented fermentation media, respectively, compared to furfural-free media (data not shown). However, at concentrations above 5 g/L furfural no cellular growth was noted. Duarte [2] found that 0.5 g/L furfural decreased the specific growth rate of *Debaryomyces hansenii* CCMI 941, and at concentrations above 3.5 g/L furfural no cellular growth was observed. Olsson [15] reported that 2 g/L furfural resulted in a 90% and 99% inhibition of growth of *Saccharomyces cerevisiae* and *Pichia stipitis*, respectively. Unlike observed before, it was shown that furfural boosted the xylitol yields at lower concentrations of furfural with constant xylitol production and xylose consumption rates but negatively affected ethanol yields, glucose consumption and ethanol production rates by PTD3.

3.3.2 5-HMF-Supplemented Fermentation

Xylitol yield by *R. mucilaginosa* strain PTD3 was not affected for concentrations of up to 3 g/L of 5-HMF and was similar to the 5-HMF-free control (67% of theoretical) (Figure 3.2E, Table 3.1B). However, clearly xylitol yield was impaired by the presence of 5-HMF at higher concentrations (from 5 g/L to 15 g/L) tested (Figure 3.2E). Unlike with PTD3, Sanchez [20] reported that in the presence of up to 2 g/L of 5-HMF, growth inhibition of *C. guilliermondii* was noted, subsequently with drastically reduced xylitol yields compared to their controls. Note that the 30 g/L of xylose was completely metabolized by PTD3 at concentrations of up to 5 g/L 5-HMF, whereas consumption of 83% and 21% of the sugar at 10 g/L and 20 g/L of 5-HMF,

respectively, was observed (Figure 3.2A). Consequently, it resulted in lower xylitol yields by the yeast (63%, 45%, and 6%, respectively) (Figure 3.2E). The specific xylose consumption and xylitol production rates were decreasing as the concentration of 5-HMF increased (Table 3.1B). Although, PTD3 completely metabolized 5-HMF at all concentrations tested in xylose-supplemented media (Figure 3.3C), the highest 5-HMF consumption rate ($0.06 \text{ gg}^{-1}\text{h}^{-1}$) was for the lowest 5-HMF concentration tested 1 g/L (Table 3.1B). Similarly to the inhibition mechanism by furfural, during bioconversion of six carbon sugars to ethanol, yeast metabolizes 5-HMF to HMF-alcohol [9, 16]. NADPH-dependent alcohol dehydrogenase is understood to be responsible for this reduction. As such, the reduction of 5-HMF does not regenerate NAD^+ , and thus carbon is allocated to glycerol production (to produce NAD^+ and thus maintain overall redox balance) [16]. Since all NADPH, generated is used for 5-HMF reduction, it is believed that the xylose to xylitol and glucose to ethanol yields are adversely affected in our study.

As with xylose, 5-HMF had a similarly unfavorable effect on the glucose consumption and ethanol yields by the yeast. As the 5-HMF concentration increased from 1 g/L to 15 g/L, the corresponding ethanol yields by PTD3 decreased (80%, 78%, 73%, 63%, 58%, and 55% of theoretical, respectively) (Figure 3.2F, Table 3.1B). Similarly, Keating [7] noted that ethanol yield by *S. cerevisiae* was substantially lowered at a concentration of 4 g/L 5-HMF. In the presence of 5-HMF at all concentrations tested, 100% of glucose (30 g/L) was consumed by PTD3 (Figure 3.2B). However, the specific consumption rates of glucose and production rates of ethanol decreased as the concentration of 5-HMF tested increased (Table 3.1B). Additionally, the highest specific consumption rate of glucose and production rate of ethanol ($0.12 \text{ gg}^{-1}\text{h}^{-1}$ and $0.05 \text{ gg}^{-1}\text{h}^{-1}$) by this microorganism were at 1 g/L of 5-HMF and decreased as the concentration of 5-HMF present in media increased (Table 3.1B). PTD3 completely metabolized 5-HMF at all

concentrations tested in this media (Figure 3.3D). However, the specific 5-HMF consumption rate of 10 g/L 5-HMF in glucose media was the highest (Table 3.1B). 5-HMF caused a concentration-dependent decrease in yeast growth for both media types. A 15 g/L 5-HMF resulted in a 70% and 40% inhibition of cellular growth of PTD3 in xylose and the glucose-supplemented fermentation media, respectively, compared to 5-HMF-free media (data not shown). Based on these data we can conclude that PTD3 is capable of metabolizing 15 g/L 5-HMF and that at concentrations at up to 3 g/L 5-HMF does not affect xylitol yield, whereas the ethanol yield is inhibited at all concentrations of 5-HMF tested.

3.3.3 Acetic Acid-Supplemented Fermentation

In the presence of 5 g/L to 20 g/L, acetic acid negatively affected xylitol production from xylose by *Rhodotorula mucilaginosa* strain PTD3, whereas its presence enhanced glucose to ethanol bioconversion by this strain. It was shown that acetic acid enhanced production of ethanol by the yeast for all the concentrations tested. As the concentration of acetic acid in the media with xylose increased from 5 to 20 g/L, xylitol yields produced by PTD3 (lowest yield 32% of theoretical at 20 g/L acetic acid) decreased compared to the fermentation lacking acetic acid (67% of theoretical) (Figure 3.3E). The highest xylitol production rate ($0.06 \text{ g g}^{-1} \text{ h}^{-1}$) was at a concentration of 5 g/L of acetic acid (Table 3.1C). For PTD3 it took longer to consume 100% of 30 g/L xylose as the concentration of acetic acid increased (Figure 3.3A). The xylose consumption rate was the same ($0.03 \text{ g g}^{-1} \text{ h}^{-1}$) for all the concentrations of acetic acid tested (Table 3.1C). In xylose-supplemented fermentation, PTD3 metabolized completely only 5 g/L of acetic acid within 76 hours while it metabolized 72% of 10 g/L and 45% of 20 g/L of this inhibitor (Figure 3.3C). The exact mechanism of acetic acid inhibition of xylose to xylitol

bioconversion has not been thoroughly investigated [19]. It is believed that at high concentrations, acetic acid either jeopardizes the availability of ATP that is available for cell growth or interferes with xylose transport across the plasma membrane. Acetic acid was characterized as a powerful inhibitor of xylose metabolism of yeast cells but its inhibition to *C. guilliermondii* depended on its concentration [3, 4]. The negative effect of high concentrations of acetic acid on xylitol production by *C. guilliermondii* was also shown by Lima [12] and Silva [22]. Silva [22] found that *C. guilliermondii* in the presence of a concentration as low as 1 g/L of acetic acid favors conversion of xylose to xylitol while at concentrations higher than 3 g/L, xylose consumption and xylitol formation are inhibited. Lima [12] found that xylitol production by *C. guilliermondii* was not affected by the presence of acetic acid until the concentration tested reached 10 g/L. The lowest concentration of acetic acid used in this study was 5 g/L, therefore improvements of xylitol yields by PTD3 at low acetic acid concentrations were not observed.

The apparent lack of effect on the rate of glucose consumption and ethanol yield by PTD3 was surprising, especially with acetic acid concentrations of 10.0 g/L and 15.0 g/L, since [13] reported inhibition of cellular processes in *S. cerevisiae* at concentrations ranging from 0.5 to 9.0 g/L. Unlike xylitol production, ethanol production from 30 g/L glucose in the presence of acetic acid (from 5 g/L to 20 g/L) had theoretical yields of (85%, 88%, and 99% of theoretical, respectively) by this strain that were identical or higher compared to the fermentation lacking acetic acid (84% of theoretical) (Figure 3.3F). All 30 g/L of glucose was consumed by PTD3 between 14 to 20 hours for all the acetic acid concentrations tested (Figure 3.3B). During fermentation of glucose with acetic acid, only 5 g/L acetic acid was completely metabolized by PTD3 within 100 hours while consumption of 62% of 10 g/L and 41% of 20 g/L was observed (Figure 3.3D). The highest sugar consumption rate ($0.13\text{gg}^{-1}\text{h}^{-1}$) was noted when PTD3 was

fermenting glucose supplemented with 20 g/L acetic acid (Table 3.1C). The ethanol production rate ($0.06 \text{ gg}^{-1}\text{h}^{-1}$) was the highest in glucose supplemented with 10 g/L acetic acid (Table 3.1C). Similar to the other inhibitors, as the acetic acid concentration increased in fermentation media, the cellular growth of *R. mucilaginosa* strain PTD3 in both, xylose and glucose-augmented media, decreased. The highest concentration of acetic acid, 20 g/L, tested, resulted in a 33% and 22% inhibition of cellular growth of the yeast in xylose and the glucose-augmented fermentation media, respectively, compared to acetic acid-free media (data not shown). Silva [22] also observed that with the presence of acetic acid in the media, cell growth decreases. Its presence affects cell growth by increasing the adaptation lag time and decreasing or altering the growth rate [17]. For both glucose and xylose supplemented media, the consumption rate of acetic acid was the same ($0.01 \text{ gg}^{-1}\text{h}^{-1}$) for all the concentrations tested (Table 3.1C, Figure 3.3C and 3.3D).

The improved ethanol yields in the presence of acetic acid could be explained by the yeast's need to maintain a neutral intracellular pH which is crucial for cell viability [5]. In the presence of acetic acid, intracellular pH drops by dissociation of acetic acid into lipophobic acetate and protons, resulting in the drop in intracellular pH [11]. The pH is then neutralized at the expense of ATP hydrolysis by the plasma membrane ATPase. In order to maintain the intracellular pH, additional ATP must be generated and under anaerobic conditions this is accomplished by increased ethanol production at the expense of cellular growth [24]. The enhanced ethanol yields for the concentration of acetic acid up to 20 g/L suggest the possibility that the carbon normally diverted from the glycolytic intermediate dihydroxyacetone phosphate toward glycerol production was instead available for ethanol production, compensating for any inhibition of alcohol dehydrogenase (or other glycolytic enzyme) activity [16, 17]. Low concentrations of acetic acids have been shown to have a stimulating effect on ethanol production by *S. cerevisiae*

[18]. It was shown previously that acetic acid at concentrations up to 10 g/L can increase ethanol yield during fermentation of glucose by *S. cerevisiae* whereas higher concentrations of this compound decreased ethanol yields [17]. We have also demonstrated that even glucose to ethanol conversion by PTD3 was enhanced by the presence of a concentration as high as 20 g/L acetic acid.

In the presence of acetic acid at all concentrations, PTD3 was affected to the same extent positively for glucose to ethanol conversion and negatively for xylose to xylitol conversion. It was noted that the fermentation products, ethanol and xylitol, were also assimilated by PTD3 in all experiments conducted when the carbon source (glucose or xylose) was depleted and thus concurred with previous findings [23]. Although this strain of the *Rhodotorula mucilaginosa* strain PTD3 is a novel, not fully understood yeast, it demonstrated a great potential for future studies assessing its suitability in the bioconversion of lignocellulosic hydrolysates to biochemicals.

3.4 Conclusions

In this study we investigated the effect of inhibitors on the production of xylitol and ethanol by *Rhodotorula mucilaginosa* strain PTD3. Contrary to previous observations, it was shown that furfural boosted the xylitol yields at up to 5 g/L of furfural with constant xylitol production and xylose consumption rates, however, furfural negatively affected ethanol yields, glucose consumption and ethanol production rates by PTD3. 5-HMF at concentrations lower than 5 g/L increased or did not affect production of xylitol but lowered ethanol yields by this microorganism. Acetic acid, even at 20 g/L, stimulated ethanol yields for PTD3 while the opposite was observed for xylitol. PTD3 demonstrated the ability to tolerate higher

concentrations of inhibitors during xylitol and ethanol production compared to other yeasts described in the literature. The use of PTD3 can be proposed to selectively remove inhibitors from lignocellulosic hydrolysates to improve the fermentability, since it was capable of metabolizing concentrations of 5, 15, and 5 g/L of furfural, 5-HMF, and acetic acid, respectively. PTD3 demonstrated an exceptional ability to ferment the sugars in presence of sugar degradation products, and to tolerate and metabolize furfural, 5-HMF, and acetic acid. The implications of this work cannot be understated. By simply fermenting media containing certain concentration of fermentation inhibitors, the yield of xylitol and ethanol can be increased. PTD3 is a robust microorganism that is capable of fermenting lignocellulosic hydrolysates with the higher xylitol and ethanol yields compared to the control. This represents a promising means of increasing commercial ethanol and xylitol yields through simply monitoring and altering concentrations of fermentation inhibitors as they enter the process. Also, unlike reported before, this means that a detoxification step during bioconversion process is not necessary and PTD3 can be employed as detoxification agent due to its ability to metabolize the inhibitors. These findings have the potential to improve reproducibility of bench scale research and reduce costs at the industrial scale by not needing the detoxification step during bioconversion process and improving xylitol and ethanol yields.

Acknowledgements

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Figure Legends

Table 3.1 The specific rates of sugar consumption and XtOH and EtOH production from synthetic sugars by *Rhodotorula mucilaginosa* strain PTD3 in the presence of furfural (A), 5-HMF (B), acetic acid (C) during fermentation.

Figure 3.1 Xylose consumption (A), glucose consumption (B), furfural consumption in presence of xylose (C), furfural consumption in presence of glucose (D), xylitol production (E), and ethanol production (F) in furfural-augmented fermentation medium by *Rhodotorula mucilaginosa* strain PTD3.

The reported results are the average of triplicate studies with a deviation of $\leq 3\%$.

Figure 3.2 Xylose consumption (A), glucose consumption (B), 5-HMF consumption in presence of xylose (C), 5-HMF consumption in presence of glucose (D), xylitol production (E), and ethanol production (F) in 5-HMF-augmented fermentation medium by *Rhodotorula mucilaginosa* strain PTD3.

The reported results are the average of triplicate studies with a deviation of $\leq 2\%$.

Figure 3.3 Xylose consumption (A), glucose consumption (B), acetic acid consumption in presence of xylose (C), acetic acid consumption in presence of glucose (D), xylitol production (E), and ethanol production (F) in acetic acid-augmented fermentation medium by *Rhodotorula mucilaginosa* strain PTD3.

The reported results are the average of triplicate studies with a deviation of $\leq 4\%$.

Table 3.1 The specific rates of sugar consumption and XtOH and EtOH production from synthetic sugars by *Rhodotorula mucilaginosa* strain PTD3 in the presence of furfural (A), 5-HMF (B), acetic acid (C) during fermentation.

Table 3. 1 A

	<i>Furfural fermentation</i>						
Concentration	0 g/L	1 g/L	1.5 g/L	3 g/L	5 g/L	10 g/L	20 g/L
Fermentation parameters	<i>Consumption</i> ¹ / <i>Production</i> ² (gg ⁻¹ h ⁻¹)	<i>Consumption</i> ¹ / <i>Production</i> ² (gg ⁻¹ h ⁻¹)	<i>Consumption</i> ¹ / <i>Production</i> ² (gg ⁻¹ h ⁻¹)	<i>Consumption</i> ¹ / <i>Production</i> ² (gg ⁻¹ h ⁻¹)	<i>Consumption</i> ¹ / <i>Production</i> ² (gg ⁻¹ h ⁻¹)	<i>Consumption</i> ¹ / <i>Production</i> ² (gg ⁻¹ h ⁻¹)	<i>Consumption</i> ¹ / <i>Production</i> ² (gg ⁻¹ h ⁻¹)
Xylose	0.05	0.05	0.05	0.05	0.05	0	0
XtOH	0.03[67%]*	0.03 [66%]*	0.03 [69%]*	0.03 [74%]*	0.02 [77%]*	0 [1%]*	0 [1%]*
Inhibitor	---	0.08	0.12	0.24	0.09	0.01	0.01
Glucose	0.13	0.12	0.09	0.08	0.07	0.01	0
EtOH	0.06[84%]*	0.04 [74%]*	0.03 [71%]*	0.03 [68%]*	0.02 [67%]*	0 [5%]*	0 [3%]*
Inhibitor	---	0.06	0.09	0.18	0.08	0.02	0.01

The reported results are the average of triplicate studies with a deviation of $\leq 2\%$.

¹The specific rates of sugar consumption were calculated based on the log-mean dry cell density and the Δ substrate and Δ time.

²The specific rates of xylitol from xylose and ethanol from glucose, production were calculated based on the log-mean dry cell density and the product concentration and Δ time.

*The theoretical yield for xylitol and ethanol production from xylose and glucose, respectively for tested concentrations of furfural.

Table 3.1B

<i>5-HMF fermentation</i>							
Concentration	0 g/L	1 g/L	1.5 g/L	3 g/L	5 g/L	10 g/L	15 g/L
Fermentation parameters	<i>Consumption¹/</i> <i>Production²</i> <i>(gg⁻¹h⁻¹)</i>	<i>Consumption¹/</i> <i>Production²</i> <i>(gg⁻¹h⁻¹)</i>	<i>Consumption¹/</i> <i>Production²</i> <i>(gg⁻¹h⁻¹)</i>	<i>Consumption¹/</i> <i>Production²</i> <i>(gg⁻¹h⁻¹)</i>	<i>Consumption¹/</i> <i>Production²</i> <i>(gg⁻¹h⁻¹)</i>	<i>Consumption¹/</i> <i>Production²</i> <i>(gg⁻¹h⁻¹)</i>	<i>Consumption¹/</i> <i>Production²</i> <i>(gg⁻¹h⁻¹)</i>
Xylose	0.05	0.06	0.05	0.05	0.04	0.04	0.01
XtOH	0.03[67%]*	0.05 [67%]*	0.04 [68%]*	0.03 [70%]*	0.02 [63%]*	0.02 [45%]*	0 [6%]*
Inhibitor	---	0.1	0.09	0.08	0.08	0.05	0.03
Glucose	0.13	0.12	0.11	0.1	0.06	0.06	0.07
EtOH	0.06[84%]*	0.05 [80%]*	0.04 [78%]*	0.04 [73%]*	0.04 [63%]*	0.02 [58%]*	0.02 [55%]*
Inhibitor	---	0.1	0.1	0.07	0.06	0.14	0.07

The reported results are the average of triplicate studies with a deviation of $\leq 2\%$.

¹The specific rates of sugar consumption were calculated based on the log-mean dry cell density and the Δ substrate and Δ time.

²The specific rates of xylitol from xylose and ethanol from glucose, production were calculated based on the log-mean dry cell density and the product concentration and Δ time.

*The theoretical yield for xylitol and ethanol production from xylose and glucose, respectively for tested concentrations of 5-HMF.

Table 3.1C

	<i>Acetic acid fermentation</i>			
Concentration	0 g/L	5 g/L	10 g/L	20 g/L
Fermentation parameter	<i>Consumption¹/ Production²</i> (gg ⁻¹ h ⁻¹)	<i>Consumption¹/ Production²</i> (gg ⁻¹ h ⁻¹)	<i>Consumption¹/ Production²</i> (gg ⁻¹ h ⁻¹)	<i>Consumption¹/ Production²</i> (gg ⁻¹ h ⁻¹)
Xylose	0.05	0.03	0.03	0.03
XtOH	0.03[67%]*	0.02 [41%]*	0.01 [40%]*	0.01 [32%]*
Inhibitor	---	0.01	0.01	0.01
Glucose	0.13	0.11	0.12	0.13
EtOH	0.06[84%]*	0.05 [85%]*	0.06 [88%]*	0.03 [99%]*
Inhibitor	---	0.01	0.01	0.01

The reported results are the average of triplicate studies with a deviation of $\leq 2\%$.

¹The specific rates of sugar consumption were calculated based on the log-mean dry cell density and the Δ substrate and Δ time.

²The specific rates of xylitol from xylose and ethanol from glucose, production were calculated based on the log-mean dry cell density and the product concentration and Δ time.

*The theoretical yield for xylitol and ethanol production from xylose and glucose, respectively for tested concentrations of acetic acid.

Figure 3.1 Xylose consumption (A), glucose consumption (B), furfural consumption in presence of xylose (C), furfural consumption in presence of glucose (D), xylitol production (E), and ethanol production (F) in furfural-augmented fermentation medium by *R. mucilaginosa* strain PTD3. The reported results are the average of triplicate studies with a deviation of $\leq 3\%$.

Figure 3.1

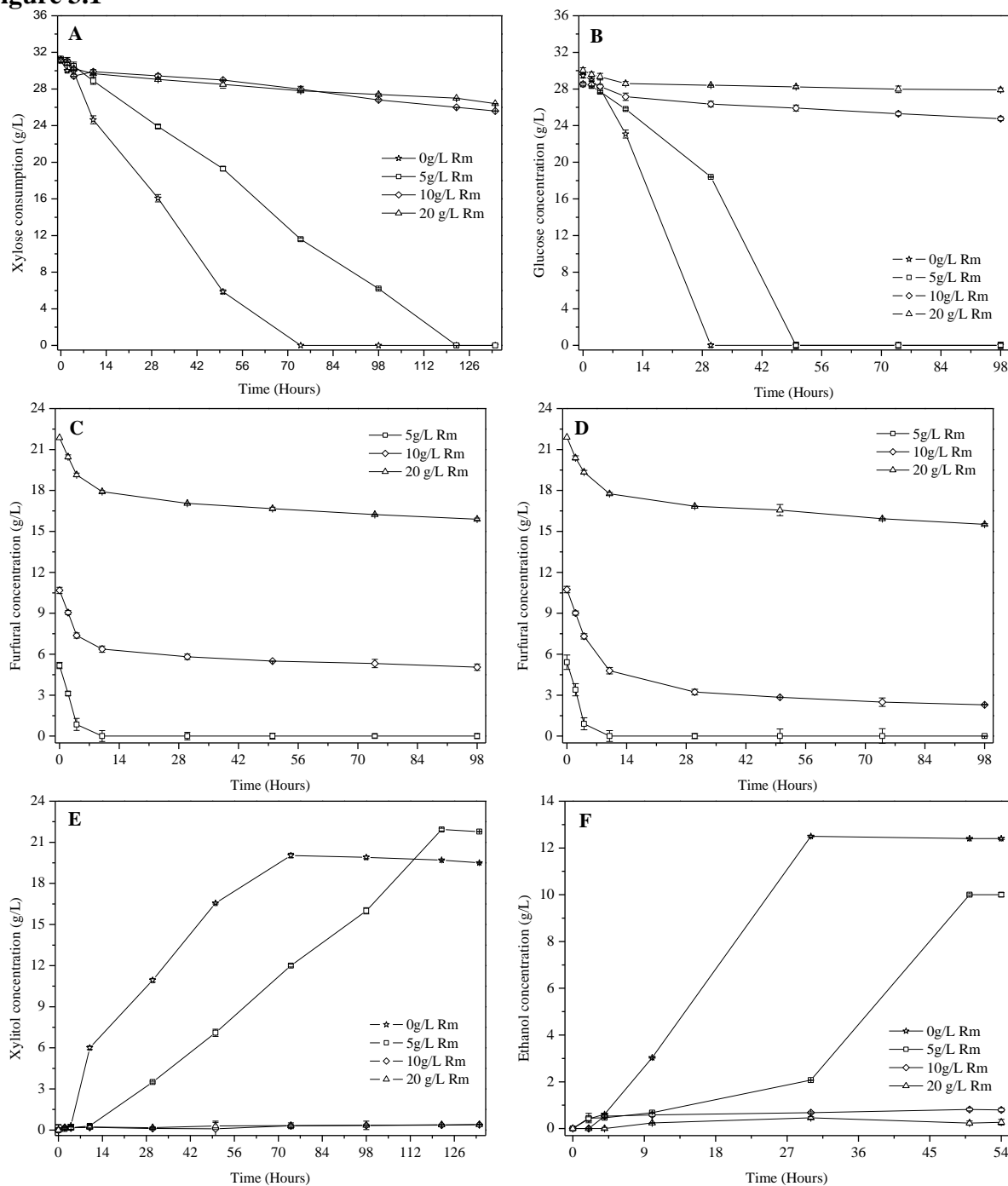


Figure 3.2 Xylose consumption (A), glucose consumption (B), 5-HMF consumption in presence of xylose (C), 5-HMF consumption in presence of glucose (D), xylitol production (E), and ethanol production (F) in 5-HMF-augmented fermentation medium by *R. mucilaginosa* strain PTD3. The reported results are the average of triplicate studies with a deviation of $\leq 2\%$.

Figure 3. 2

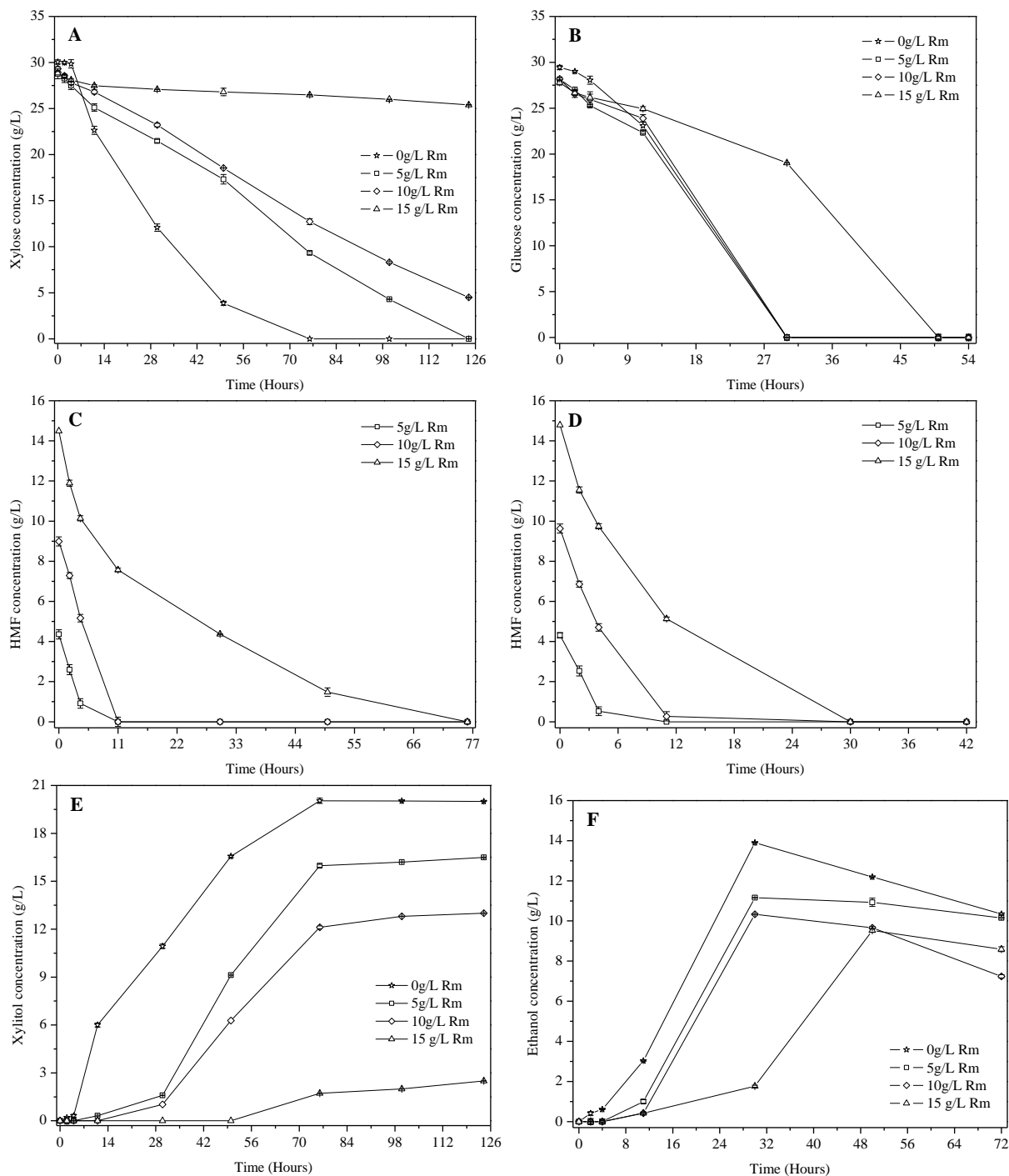
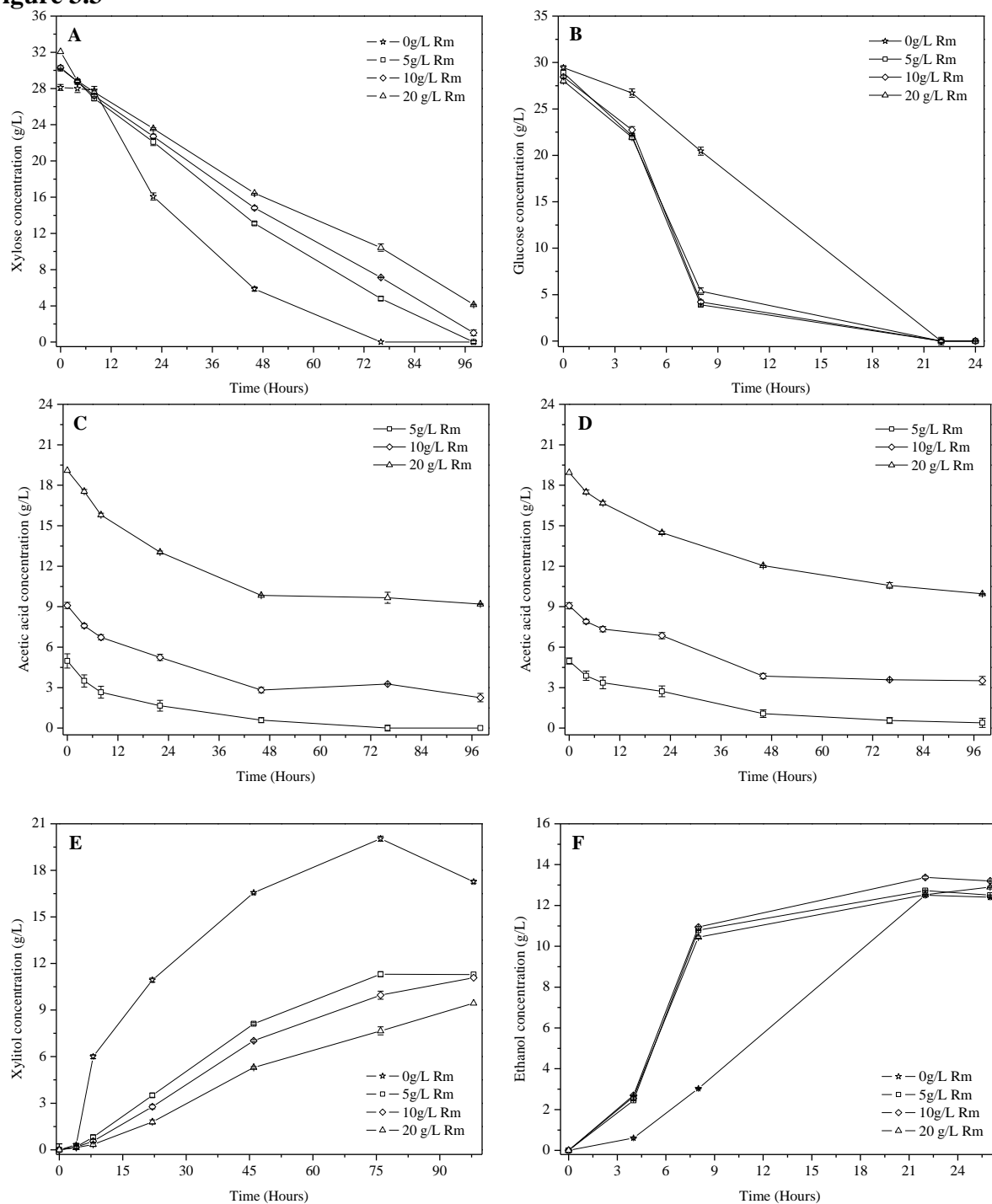


Figure 3.3 Xylose consumption (A), glucose consumption (B), acetic acid consumption in presence of xylose (C), acetic acid consumption in presence of glucose (D), xylitol production (E), and ethanol production (F) in acetic acid-augmented fermentation medium by *R. mucilaginosa* strain PTD3. The reported results are the average of triplicate studies with a deviation of $\leq 4\%$.

Figure 3.3



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CHAPTER 4

Converting Lignocellulosic Solid Waste into Ethanol for the State of Washington: An investigation of treatment technologies and environmental impacts

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Converting Lignocellulosic Solid Waste into Ethanol for the State of Washington: An investigation of treatment technologies and environmental impacts.

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Abstract

There is little research literature on the conversion of lignocellulosic rich waste streams to ethanol, and even fewer have investigated both the technical aspects and environmental impacts together. This study assessed technical and environmental challenges of converting three lignocellulosic waste streams to ethanol: Municipal Solid Waste (MSW), low grade Mixed Waste Paper (MWP), and organic Yard Waste (YW). Experimental results showed high conversion yields for all three streams using suitable conversion methods. Environmental impacts are highly dependent on conversion technology, and process conditions used. Life cycle assessment results showed that both chemicals production and waste collection are important factors to be included within a waste-to-ethanol study.

Keywords: Municipal Solid Waste; Life Cycle Assessment; Ethanol; Lignocellulosic; Pretreatment

4.1 Introduction

Many lignocellulosic materials, such as corn stover, hybrid poplar [8], lodgepole pine [13], have been investigated as potential feedstock for ethanol production, however, relatively little research has been done on the technical issues associated with converting lignocellulosic rich urban waste to ethanol. Shi [27] performed a techno-economical analysis of the conversion of municipal solid waste to ethanol. They obtained 79% glucan and 88% xylan recovery from alternative daily cover (ADC final, landfill mulch) after pretreatment of 1% H_2SO_4 at 140°C for 40 minutes and enzymatic hydrolysis supplemented with bovine serum albumin. Li [22] obtained 73% cellulose to glucose conversion after three-stage pretreatment comprised of dilute acid hydrolysis (1% H_2SO_4), followed by steam treatment (121°C, 15 minutes) and enzymatic hydrolysis of Municipal Solid Waste (MSW) composed of carrot, potato peelings, grass and newspaper. Ballesteros [4] achieved 30 g/L of ethanol (about 60% of maximum theoretical recovery) after thermal pretreatment (160°C, 30min) and Simultaneous Saccharification and Fermentation (SSF) of organic fraction of MSW. While few pretreatment processes have led to effective bioconversion of whitewood softwood to ethanol, such as organosolv [21]; dilute-acid [28]; and SO_2 -catalyzed steam explosion [13], none of the methods have proven to work with mixtures of forest residues (branches, needles, bark and softwood and hardwood chips).

In addition to the technical processing challenges of producing bioethanol from lignocellulosic feedstock, issues related to sustainability and environmental impacts, such as global warming, are major political and scientific concern. Various studies have shown that first-generation biofuels, such as corn-based ethanol, produce less harmful emissions while displacing transportation fuels derived from fossil fuels [14]. However, large amounts of chemical fertilizers used for crop production, increased water consumption, and increased greenhouse gas

(GHG) emissions released from indirect and direct land use changes may exceed the benefits from fossil fuel displacement [33]. Lignocellulosic rich urban waste streams are potential next generation feedstocks that may serve as low-cost, abundant feedstocks for the production of fuel ethanol, however, relatively little research has been performed on their potential environmental challenges [33]. Kalago [20] estimated that net GHG emissions from bioethanol from MSW were 65% lower than gasoline and 58% lower than corn ethanol, and required far less fossil fuel input than either gasoline or corn ethanol. Two studies recently published on the use of municipal solid waste for the production of ethanol in the State of California [10, 35], demonstrated a significant reduction in GHG emissions and fossil energy use compared to both corn ethanol and conventional gasoline.

In the State of Washington alone, over four million tons per year of lignocellulosic rich municipal solid waste is available for use as a biofuel feedstock [15]. More than half (55.3%) of the total waste surveyed within the State in 2009 was composed of lignocellulosic material [32]. In fact, nearly a third of the MSW was composed of: food waste (18.3%), leaves and grass (4.1%), mixed waste paper (6.7%) and cardboard (3.7%) [32]. Table 1 shows the characterization of Washington State municipal solid waste and estimated lignocellulosic content for some of these materials [6]. In addition to MSW, most major urban areas within the State collect separated recyclable and compostable materials that contain significant lignocellulosic fractions.

The goal of this research was to investigate lignocellulosic rich waste as a potential feedstock for a biomass to ethanol bioconversion process. For this study, lignocellulosic rich urban waste is divided into: yard waste (YW: mixture of hardwoods and softwoods with branches, bark, needles collected for composting), mixed waste paper (MWP: the lowest grade mixed paper waste collected for recycling) and municipal solid waste (MSW: food waste, such as, banana peels,

cereal, coffee grinds, canned corn, tomato juice and clean hygiene products destined for a landfill). These streams were investigated in two ways. First, an experimental analysis of the conversion of lignocellulosic rich waste components was performed using specific ethanol conversion techniques. Second, the results from the experimental analysis were used to perform a Life Cycle Assessment (LCA) of a hypothetical waste-to-ethanol production scenario for the State of Washington.

4.2 Materials and Methods

4.2.1 Experimental Methods

In this study first we pretreated synthetic municipal solids waste and the mixed waste paper using diluted sulfuric acid. The YW was pretreated by SO₂-catalysed steam explosion (Figure 4.1). After separations of liquid and solid mixtures, the water soluble fractions (WSF) were fermented to ethanol as shown in Figure 4.1. The water insoluble fractions (WIF) were first enzymatically hydrolyzed and then fermented to ethanol (Figure 4.1). All the experimental conditions, fractionation, hydrolysis and fermentation data/yields were inputted into the LCA model (Figure 4.2).

4.2.1.1 Pretreatment

The MWP was obtained from the Tacoma recycling facility owned by Weyerhaeuser and consisted of the lowest grade paper waste. A synthetic MSW was prepared in the lab due to the safety issues. It was composed of equal portions by weight of: banana peels, cereal, coffee grinds, canned corn, tomato juice and clean hygiene produces. Due to the potential decomposition of material, the MSW was kept at 4°C until use. The composition reflected the typical MSW

composition for Washington State. The MWP and MSW were pretreated by diluted sulfuric acid (4% (w/v)) at 60°C for 6 hours.

The YW, mixture of hardwood and softwood with branches, bark, needles (60.0 % moisture content) was obtained from the University of Washington waste facility and stored at 4°C until use. YW, which was composed of shrubbery cuttings, leaves, needles, tree limbs, and bark, was pretreated by soaking in water overnight prior to SO₂-catalysed steam explosion. The detailed procedure of steam explosion experiments have been described previously by Ewanick and Bura [12]. Briefly, samples of 300g oven-dried weight (ODW) were impregnated overnight with anhydrous SO₂ in plastic bags. The samples were then loaded, in 50g batches, into a preheated 2L steam gun in Gresham, Oregon and exploded at temperature of 210°C; time 10 minutes and 3% (w/w) SO₂ concentration.

The slurries from steam explosion of YW and dilute acid pretreatment of MWP and MSW were recovered, separated by filtration, divided into water soluble fractions (WSF) and water insoluble fractions (WIF) and kept at 4°C until use (Figure 4.1).

The WSF and WIF were analyzed as described below, and used to construct a complete mass balance of carbohydrates and lignin. WIFs were water-washed (with water equal to ten times the mass of solids) prior to analysis and saccharification (Figure 4.1).

4.2.1.2 Enzymatic hydrolysis

After the pretreatment, the steam or acid pretreated solids were enzymatically hydrolyzed at 2% consistency (w/v) solid concentration. The hydrolysis took place at 50°C with continuous agitation (150 rpm) for a period of up to 72 hours as previously described by Ewanick and Bura

[12]. Briefly, the solution was buffered at pH 4.8 with 0.05 M sodium acetate buffer and cellulase (Spezyme-CP, 26 FPU/mL, Sigma) was added at 20 FPU/g cellulose and supplemental beta-glucosidase (Novozym 188, 492 CBU/mL, Sigma) was added at 40 CBU/g cellulose.

The extent or yield of hydrolysis was expressed as the percentage of the theoretical glucose content in the feedstock at the start of hydrolysis that was recovered as monomeric glucose (i.e., the glucose yield). The determination of the theoretical glucose content of the feedstock was based on Klason analysis of the feedstock solids, and assumed all available glucose was present as cellulose. A conversion factor was applied in the calculation of the carbohydrate content to account for the hydration of the cellulose during cleavage [3].

4.2.1.3 Fermentation

WSFs obtained from each of the pretreatment and enzymatic hydrolysis steps were assessed for their efficiency during fermentation to ethanol by *Rhodotorula mucilaginosa*, without employing any detoxification steps as previously described by Vajzovic [31]. Briefly, *Rhodotorula mucilaginosa* strain PTD3 isolated from poplar trees [34] was streaked onto YPD agar plates and allowed to grow for 48 hours. The yeast strain has the ability to grow on five carbon sugars, including xylose and arabinose, and ferment hexoses to ethanol, xylose to xylitol and arabinose to arabitol [31].

Rhodotorula mucilaginosa strain was maintained on YPG solid medium (10 g/L yeast extract, 20 g/L peptone, 20 g/L glucose, and 18 g/L agar, Difco, Becton Dickinson, MD) at 4°C and transferred to fresh plates on a bimonthly basis. Cells were grown to high cell density in foam-plugged 1L Erlenmeyer flasks containing 500ml YP-sugar liquid media (10 g/L yeast extract and 10 g/L peptone, supplemented with 10 g/L glucose) in an orbital shaker for 2 days at 30°C and

150 rpm, with concurrent transfer to fresh medium performed every 24 h. Fermentations of the WSF were conducted in 125 mL flasks containing 50 mL medium pre-adjusted to pH 6.0 with 10 % (w/w) NaOH sodium hydroxide with 5 g/L of *Rhodotorula mucilaginosa*. Control fermentations were run in parallel using glucose-based media. The fermentation vessels were maintained at 30°C with continuous agitation (150 rpm). Samples (0.5 mL) were withdrawn aseptically by syringe, centrifuged for 5 min at $15000 \times g$ and 4°C and the supernatant was filtered by using a 0.45 mm syringe filter (Restek Corp., Bellefonte, PA, U.S.) and then stored at -20°C until analysis. Sugars and ethanol were determined periodically from the aliquot culture samples during the course of the fermentation. The relative ethanol yield, $Y_{\text{EtOH}} (Y_{\text{EtOH}}^{\text{ref}})^{-1}$ was defined as the ratio of the ethanol yield of the filtrate and the theoretical fermentation. The theoretical yield for ethanol production from glucose is 0.51 g ethanol g⁻¹ glucose. Each experiment was run in duplicate and the range values reported.

4.2.1.4 Instrumental Analysis

The concentration of monomeric sugars (arabinose, galactose, glucose, xylose and mannose) was measured on a Dionex (Sunnyvale, CA) HPLC (ICS-3000) system, as described previously by Ewanick and Bura [12]. Ethanol concentrations were determined using Shimadzu Prominence HPLC chromatograph (Shimadzu Corporation, Columbia, MD) as described previously by Ewanick and Bura [12]. Ash content of raw biomass samples was measured gravimetrically by heating 20-mesh-milled dry biomass to 550°C for 20 hours [12]. Solids were analyzed gravimetrically for lignin content, photometrically for soluble lignin, and by HPLC for carbohydrate content using the TAPPI method T-222 om-98 (TAPPI, 1998) as previously described by Ewanick and Bura [12]. Since the acid insoluble material included ash, the ash

content was subtracted from the total acid insoluble lignin amount. Monomeric and oligomeric soluble carbohydrates were determined as previously described Ewanick and Bura [12]. Oligomeric sugar was calculated by subtracting monomeric sugar content from total sugar content.

4.2.2 LCA Methods

The goal of the LCA was to identify potential environmental consequences associated with the conversion of three lignocellulosic rich solid waste streams to ethanol for the State of Washington. The LCA is attributional in nature, meaning only unit processes that can be directly attributed to the production of ethanol were included within the system boundaries [11], except where substitution is used to avoid allocation. The three waste streams considered are MSW, MWP, and YW. The conversion of waste materials to product can be considered a multifunctional process, providing two services: (1) making a product and (2) the management of waste [11]. Generally in waste management LCAs the functional unit is specified as the amount of waste managed (in tonnes) within the system over a specified time period [17]. However, since this LCA is concerned with the production of ethanol fuel the functional unit for each scenario is one liter of denatured ethanol fuel produced within Washington State. To fulfill the functional unit, the system boundaries of three ethanol production systems were defined well-to-tank (WTT) analysis for a transportation fuel, less consideration of fuel mixing. Furthermore, analyses of competing systems as a waste management strategy were not included for the sake of simplicity.

The system boundary is shown below in Figure 4.2. The model includes major aspects of the systems life cycle, including collection, intermediate processing, ethanol production, ancillary

chemicals production, electricity and fuel production, and residual waste management. Ethanol production is assumed to occur at a biorefinery within the State, and is then transported to a separate blending facility. Ancillary chemicals are produced and transported to the biorefinery for bioethanol production. Any residual waste throughout the system is sent to a landfill. Waste entering the system boundary does not include any prior inputs, or rather waste is considered to have “zero” burden associated with its production [17].

Life cycle inventory (LCI) data were collected to represent average industrial operations for total energy consumption (renewable and fossil), three greenhouse gases (GHG as CO₂, methane (CH₄), and nitrous oxide (N₂O)) and five additional pollutants (carbon monoxide (CO), nitrogen oxides (NO_x), particulate matter (PM as PM₁₀ and PM_{2.5}), sulfur oxides (SO_x), and non-methane volatile organic carbons (NMVOC)). The LCI model results were used to perform a life cycle impact assessment (LCIA) to determine contributions of these flows to total energy consumption, global warming potential (GWP – 100 year), acidification, smog formation, and total particulate matter. Characterization factors for the global warming potential (100 year) were obtained from the Intergovernmental Panel on Climate Change (IPCC) Fourth Assessment Report [19], while acidification and smog formation characterization factors were from U.S. EPA’s Tool for the Reduction and Assessment of Chemical and Other Environmental Impacts (TRACI 2) [5]. All unit process LCI data were gathered from publicly available sources to allow for complete transparency, and the deterministic results are compared to results from several relevant life-cycle studies of solid waste to ethanol production.

The following is a brief description of how unit process data was obtained for the LCA. Detail of the development of LCI data and major assumptions made within this study can be found in Schmitt [25].

4.2.2.1 Lignocellulosic Biorefinery

A model for a lignocellulosic biorefinery was created using ASPEN Plus software, based on previous work performed by researchers at National Renewable Energy Lab (NREL) for the conversion of corn stover to ethanol [1]. The model includes major unit processes for lignocellulosic ethanol production, such as: feedstock handling and separation, pretreatment, saccharification and co-fermentation, ethanol recovery, and facility utilities (i.e. co-heat and electricity generation, waste water treatment). The biorefinery is assumed to have separation equipment that removes and classifies non-lignocellulosic material prior to performing conversion. For MSW and MWP this includes equipment operation similar to that of a material recovery facility, which have energy requirements calculated outside the ASPEN model. Lignocellulosic compositions for the organic fraction of each feedstock and model inputs (i.e. reaction conditions, conversion rates, enzyme loading) were based in experimental work as described in Section 4.2.1 and shown in Table 2. Each feedstock was modeled separately with an input capacity of 98,040 kg-wet/hr (2,000 MT-wet/day) of lignocellulosic material, as was determined to be the minimal “nth” plant size capacity that is still economical [1]. For all three streams a separated hydrolysis and co-fermentation process were assumed. Ancillary chemicals, materials and energy input/output requirements, (i.e. sulfuric acid, hydrated lime, corn steep liquor (CSL), cellulase enzymes, ammonium phosphate, and denaturant) were determined from the ASPEN model. The net electricity required by the biorefinery is the amount of electricity produced from the combustion of suitable process conversion residuals minus total electricity consumed for processing. Residual wastes from feedstock separation and conversion processes (that are not combusted) are sent to landfill. Finally, assessment of co-products, such as gypsum and CO₂, is left for future work, including quantification of the amounts generated and the

related life cycle implications of potential uses and/or disposal, except for net electricity produced as will be discussed later.

4.2.2.2 Waste Collection and Processing

Collection of solid waste materials includes commercial collection by large heavy duty trucks, and self-hauling by passenger vehicles. In order to determine how much waste was collected commercially versus self-hauled, data was collected from counties within the State with high annual MSW generation ($>100,000$ MT-MSW/yr). From this data it was estimated that nearly 75% of MSW and recyclables were collected curbside, while only 7% of YW was collected by commercial vehicles. Furthermore, it was assumed that 55.3% of collected MSW contains lignocellulosic material [32], while 31% of recyclables contain the fraction of low grade mixed waste paper [9]. All yard waste collected is considered lignocellulosic (a composition of softwood and hardwood stems, leaves, and grass). A mechanistic model based on the work described in Schmitt [25] was used to calculate the total distance and fuel consumption of a commercial collection fleet, while emission factors for collection vehicles were taken from Agar [2]. Self-haul vehicles were assumed to be passenger vehicles with an average fuel efficiency of 25 mpg, and emission factors were from GREET 1.7 [29] for a passenger vehicle using conventional gasoline fuel. It is assumed that all collection and self-haul vehicles drop-off waste at an intermediate waste facility.

Two types of intermediate waste facilities were modeled in this assessment, transfer stations and material recovery facilities (MRF). Transfer stations (only used for MSW and YW in this study), are centralized facilities used to drop-off and compact waste loads, which are then transported by long-haul vehicles to a final waste processing facility. Equipment fuel and energy use was

estimated for a large transfer station requiring several front-end loaders and compactors. Associated air emissions for combustion of non-road diesel fuel in a front-end loader were based on U.S. EPA Non-Road model [30]. An MRF (only used for MWP in this study) is used to separate out individual recyclable streams into higher value product streams for recycling (e.g. glass, metals, plastics, and paper). Equipment used within an MRF includes: front-end loaders, bag breakers, conveyor belts, picking belts, trommel screens, ferrous magnets, eddy-current separators, hammer mill shredders, balers, and forklifts. Non-road vehicles, such as front-end loaders and forklifts, were assumed to consume diesel fuel. Emission factors for all non-road engine fuel combustion were based on U.S. EPA Non-Road model [30]. Both facilities used Washington grid electricity, and no construction or facility emissions were considered. Residual process waste from both facilities is sent to a landfill.

4.2.2.3 Ancillary Chemicals

Major chemicals used during the ethanol production process include: sulfuric acid, lime, diammonia phosphate, corn steep liquor, and cellulase enzymes. Corn steep liquor (CSL) is a byproduct from the steeping process of a corn wet-mill facility, and is used as a nutrient for the fermentation process. Process data for the wet-milling operations were taken from GREET 1.7 [29], and allocated for CSL based on energy partitioning of facility operations from the work performed by Galitsky [16]. Data from enzyme production developed by Sheehan [26] were used to model cellulase production. In the NREL model, diammonium phosphate (DAP) is used as a nitrogen source for the formation of the *Z. mobilis* bacteria during fermentation. Data for ammonia phosphate was used as a substitute for DAP. Sulfuric acid is used during pretreatment to enhance access to cellulose during hydrolysis; subsequently lime is used to neutralize the

acidity of the hydrolyzate. LCI data for sulfuric acid, lime, and ammonia phosphate (as substitute for diammonium phosphate) were adapted from GREET 1.7 [29]. Production of all ancillary chemicals are assumed to occur outside the State of Washington and assumed to be transported at an average distance of 500 km.

4.2.2.4 Electricity and Fossil Fuel

All electricity and fossil fuel energy production data were obtained from GREET 1.7 [29]. Electricity data were modeled using an average electricity fuel mix for Washington State: 72% hydro, 11.1% coal, 6.7% natural gas, 8.6% nuclear, 1.7% biomass, and 0.02% residual oil. Any net electricity produced at the biorefinery was assumed to avoid average Washington grid electricity. Electricity data for processes occurring outside the State of Washington were modeled using the U.S. average electricity mix. All transportation vehicles and transportation fuels were modeled in GREET 1.7 [29] using default values for U.S. average.

4.2.2.5 Waste Disposal

Residual waste from process inefficiencies and separation was disposed of at a landfill. No credits were assessed for any co-products produced during the separation of MSW and recyclable material. This could have a significant impact and is open for future work. Major landfills utilized by the State of Washington employ landfill gas to electricity technology, however, in this study landfill gas was assumed to be flared. The implications of this assumption were not assessed in this study.

The development of LCI data for landfill waste was broken down into three parts: landfill operations, landfill gas production, and leachate treatment. The model for landfill operations was

based on the work previously described by Schmitt [25]. This includes heavy-duty equipment operations for daily landfill covers: scraper, compactor, motor grader, track loader, track tractor, articulated truck, and water trucks. Emissions from major equipment operations were modeled using the Non-road model [30]. A mechanistic model for landfill gas production was developed based on the work previously described in Schmitt [25]. This model includes a number of parameters that were used to calculate Landfill gas production over a 100 year period, based on various stages in the waste decomposition. The model assumes a landfill gas collection system with an efficiency of 75% that operates over a 80 year period. LCI data for leachate treatment is based on the work described in Schmitt [25].

4.3 Results and Discussion

4.3.1 Experimental Results

We initially determined the carbohydrates and Klason lignin of the three original untreated solid waste streams (Table 4.2). The total polysaccharides content for YW, MSW, and MWP proved to be very high; 62%, 79% and 88% respectively, making these lignocellulosic rich urban wastes attractive materials for saccharification and fermentation processes. Glucose, followed by mannose and xylose were shown to be the most abundant components as determined by secondary acid hydrolysis of constituent polysaccharides. The ash content was determined to be highest for MWP, 6.9% and the lowest for MSW 3.1% (Table 4.2). The ash content of YW was 4.5% (Table 4.1). Lynd [23] have shown that the ash content of 44 paper sludges was as low as 2% and as high as 54%, which could present a challenge during pH adjustment with acid required prior to enzymatic hydrolysis. In addition, high ash content might be affecting the performance of hydrolysis and fermentation. However, in this study the ash content was low

(less than 7%) for all the feedstocks tested. The total lignin (Klason lignin) content was determined to be highest for YW, 34.6%, while for MSW it was 18.3% (Table 4.2). Similarly to Lynd [23] the total lignin content of MWP was very low 0.6% (Table 4.2). The yard waste contained high lignin content since lots of branches and bark (both of which are lignin rich) were visually present in the samples.

The concentration of monomeric and total sugars in the liquid fraction after acid hydrolysis of the pretreated waste samples is shown in Table 4.2. The total amount of sugars released during acid pretreatment of MSW and steam explosion pretreatment of YW were very high 25.5 g/L and 18.3 g/L, respectively, which makes these fractions very attractive for fermentation processes. The low sugar content in the water soluble stream following acid pretreatment of MWP (3.5 g/L) is a result of some of these sugars being removed in the pulping process.

Overall yield has been found to be the most important parameter when evaluating the production cost of bioethanol. During pretreatment of all three samples we recovered essentially all of the glucose and xylose 98-100% (Table 4.2). Not surprisingly, the greatest losses were of arabinose, followed by galactose, which concurs with previous findings [18]. It has been suggested that the high susceptibility of arabinosyl linkages to hydrolysis may be in part responsible for fragmentation and solubilisation of cell wall components in the lignocellulosic biomass, and thus formation of degradation products at elevated temperatures [7]. However, arabinose and galactose were minor sugars in original lignocellulosic rich urban biomass, thus the incomplete recovery does not greatly influence overall biomass to ethanol yield. Based on the complete glucose and xylose recoveries, it can be concluded that the conditions for pretreatment of all three waste streams were not too severe.

The cellulose to glucose and xylan to xylose conversions of MSW following enzymatic hydrolysis were very high, 89 and 87% respectively, making this material an excellent source for bioethanol production. We obtained very high cellulose to glucose conversion of 86% and moderate xylan to xylose conversion of 62% following hydrolysis of MWP proving that the lowest grade paper waste could be utilized for bioethanol production

The sequential steam explosion pretreatment of SO₂ soaked yard waste followed by enzymatic hydrolysis in the current study showed a 41% conversion of cellulose to monomeric glucose and 33% of xylan to xylose. The low conversions of sugars during enzymatic hydrolysis were not surprising since the original YW contained ~40% of lignin and the heterogeneous material contained softwood branches with bark and needles. Previous work has shown that SO₂-catalysed steam explosion can successfully pretreat softwood [13]; and hardwood residues [8] during the bioconversion process. However, due to their chemical characteristics (high guaiacyl lignin content), softwood residues have proven to be more recalcitrant toward enzymatic hydrolysis when using the optimum pretreatment conditions, allowing for maximum hemicellulose and cellulose recovery in a fermentable form [13]. Therefore, an additional delignification process is required in the bioconversion of softwood to ethanol prior to enzymatic hydrolysis, and consequently increasing the overall cost of the process.

The WSFs obtained after pretreatment and hydrolysis were assessed for their feasibility as media for effective fermentation to ethanol (Table 4.2). As expected, all the hexose sugars (glucose, galactose and mannose) liberated in the yard waste hydrolyzate were effectively used by *Rhodotorula mucilaginosa* during the fermentation process. The yeast grew well in the water soluble streams and showed similarity in growth and fermentative pattern with controls (data not shown). Ethanol yields from hexoses (glucose, mannose and galactose) for all the sugar streams

tested were close to 100% of theoretical ethanol (Table 4.2). A very high conversion rate of sugars to ethanol suggest that conditions of 210°C, 10 minutes and 3% SO₂ treatment for MSW, and 60°C, 2.5% H₂SO₄ treatment for MWP were optimal pretreatment conditions for fermentation process.

4.3.2 LCA Results

Experimental results for the conversion of three lignocellulosic waste streams to ethanol were used to develop an ASPEN model for a lignocellulosic biorefinery. The model results were used to develop LCI data for the production of ethanol (Table 4.3). The final yields for the three streams were 197 L/MT-wet MSW (includes all fractions of MSW, e.g. metals, plastic,...etc), 116 L/MT-wet recyclables (includes all fractions of recyclables), and 100 L/MT-wet yard waste. Yields are much higher based on the dry lignocellulosic matter of these materials, as 469 L/MT-dry, 413 L/MT-dry, and 264 L/MT-dry, for MSW, MWP, and YW, respectively. These yields range between 60 – 74% of the theoretical values. Similarly, Chester & Martin [10], assumed a baseline yield estimate of 75% of theoretical for MSW to ethanol, and ran a sensitivity analysis varying the yield between 17 – 90%. Their results indicated a linear relationship between fossil energy avoidance and yield. Lower yields were reported by Kalago [20] and Zhang [35], as their LCAs assumed a yield of 85 L/MT-wet MSW fluff. Since conversion yields are highly dependent upon specific process conditions, and feedstock characteristics (i.e. moisture content), it is assumed the yields defined within this research are reasonable.

Table 4.4 presents the LCIA results for the three feedstocks. Washington grid electricity was avoided in the MWP and YW models by co-product credits for electricity generation at the biorefinery. MSW has the largest total energy consumption, GWP and total PM emissions

among the feedstocks investigated. The LCIA GWP values approximately range from 0.9 – 1.2 kg CO₂-e per liter of ethanol. YW yields the lowest impact in four out of the five impact categories (total energy consumption, GWP, acidification, and total particulates), while MWP has the lowest smog formation. Results for all waste streams are within a magnitude of order of each other for most categories, except in total particulate matter where yard waste is three orders of magnitude lower than MSW and MWP. This is largely due to the electricity credit given to YW from power production at the biorefinery.

The LCIA results for total GWP and total energy consumption were compared with three other studies that published similar work (Figure 4.3). Kalogo [20] developed an LCA of MSW-to-ethanol using a dilute acid hydrolysis and gravity pressure vessel technology. The comparison uses results from Kalogo [20] both with and without the classification process. Chester and Martin [10] developed an Economic Input-Output LCA (EIO-LCA) using results from the NRELS dilute acid model [1]. Our comparison of results from Chester and Martin [10] only includes their results from classification, ethanol plants, and distribution to have a direct comparison of system boundaries. In addition, total energy consumption in Figure 4.3 for Chester and Martin [10] reflects fossil fuel energy only and where they made a distinction between fossil and biogenic GHG emissions, we do not. The work from Zhang [35] for MSW-to-ethanol was based on previous work from Kalogo [20]. Only the results from the MSW pathway are presented, and it should be noted that energy values for Zhang [35] are based off total petroleum consumption, not total energy. The comparison in Figure 4.3 qualitatively demonstrates that there is reasonable agreement between solid waste-to-ethanol LCA studies. Even though the four studies utilized various parameters (LCI data, system boundaries, conversion processes, model assumptions, ...etc.), the standard deviation between the four

studies is 4.4 MJ/L and 0.3 kg-CO₂e/L for total energy consumption and GWP, respectively. Based on the other works discussed above, we can conclude that waste-to-ethanol generally is more favorable than corn ethanol and petroleum.

A contribution analysis was performed by aggregating the unit process results into five major categories: Waste Collection and Processing (collection and intermediate facilities), Ethanol Production and Distribution, Chemicals Production (all ancillary chemicals production and transportation), Electricity Production (co-product from biorefinery), and Waste Disposal (residual waste sent to landfill). The contribution analysis was performed for four impact categories: total energy consumption, GWP potential, acidification, and smog air formation (Figures 4.4).

In previous studies, waste collection was ignored because it is part of an established waste management system already in place [10, 35]. In this study it was included and determined to be an important aspect of the waste-to-ethanol life cycle. Waste collection made up 13% of the total smog air formation impact for MSW, and was the second largest contributor to total energy, acidification, and smog-air formation for YW. Furthermore, it can be argued that it is important to include waste collection in waste-to-ethanol LCAs because of the potential impact producing commercial ethanol from waste would have on established waste systems. For instance, a 50 million gallon per year (gpy) biorefinery, as was assumed in Chester & Martin [10], in the State of Washington would require nearly a million metric tonnes of MSW. That is nearly 20% of the amount of MSW produced annually within the State for a single biorefinery. Furthermore, if several commercial scale nth plants are assumed, it is reasonable to believe that collection and intermediate waste processing would largely be diverted towards bioethanol production and not

waste management. Thus, waste collection is a directly attributable process that should be included in waste-to-ethanol LCAs.

Ancillary chemicals production was a significant contributor to all impact categories. This is in agreement with the findings from MacLean and Spatari [24], who determined that enzymes and chemicals make up 30% of both total fossil fuel input and GHG emissions for a similar process model. Nearly 90% of acidification for MSW (210 gH⁺e/L) and MWP (239 gH⁺e/L) was from sulfuric acid production alone. Lime and sulfuric acid production together made up nearly 25% of the total smog air formation for MSW, and 7.5 gNO_x-e/L for MWP. For total energy consumption lime production was 1.4 MJ/L, 1.6 MJ/L, and 0.07 MJ/L for MSW, MWP, and YW. Lime production was also a significant contributor to GWP for MSW and MWP, while denaturant and enzyme production were more dominant for YW. In general, impacts from chemicals production for YW were much smaller than that for MSW and MWP. This indicates that process conditions, pretreatment options in particular, have not only a major effect on process yields, but also environmental impacts. This is similar to the findings of MacLean and Spatari [24], who further investigated the sensitivity of fossil energy and GHG impacts from various process conditions, conversion technologies, and enzyme loadings. They found a much greater contribution from enzyme production than this study, ranging from 85 – 1600 g-CO₂e/L, whereas total GHG emissions from enzyme for this study range from 7 – 12 g-CO₂e/L. Clearly, this is an area (enzyme production in particular) that needs more research and greater access to specific industry data.

Biorefinery emissions (flue gas from fermentation and combustion from the boiler for heat and power) dominated GWP for all three feedstocks. If CO₂ emissions from the fermentation were captured, which is often done in processing, the contribution and overall GWP impact would be

much lower. Thus, the GWP can be considered a conservative estimate. For MWP and YW, approximately 18 MW of electricity was produced at the biorefinery (Table 4.3), which greatly reduced the total energy consumption for these feedstocks. It should also be noted that Washington state electricity largely comprised of hydro-electric power, and this avoidance would be much larger for fossil fuel dependent areas. Smog formation was dominated by the transportation of ethanol from the biorefinery to a blending facility, with NO_x emissions of 0.33 g/L from transportation vehicles. Impacts from residual waste processed at a landfill were minimal for all categories except GWP. This is expected as methane emissions from decomposing waste material are fairly large over a 100 year period.

4.4 Conclusion

Dilute acid hydrolysis is an effective pretreatment method for MWP and MSW allowing near theoretical yields during enzymatic hydrolysis, while steam pretreatment is a reasonable pretreatment method for YW, its yields were much lower (41%). Pretreated and hydrolyzed sugars of MSW, MWP, and YW were readily fermentable and high ethanol yields were obtained (100% of theoretical). Chemicals production and waste collection are important factors to be included within a waste-to-ethanol LCA. Environmental impacts are highly dependent on conversion technology, and process conditions for waste-to-ethanol LCAs. In general, research thus far suggests that bioethanol from waste would reduce impacts from current ethanol technologies.

Acknowledgements

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Figure Captions

Figure 4.1 Experimental procedure for solid waste streams to ethanol conversion.

Figure 4.2: System boundaries for Solid Waste-to-Ethanol LCA.

Figure 4.3: Comparison of total energy consumption and GWP potential for waste-to-ethanol LCA studies.

Figure 4.4: Contribution analysis for total energy consumption, global warming potential (GWP), acidification, and smog air formation for solid waste-to-ethanol.

Figure 4.1 Experimental procedure for solid waste streams to ethanol conversion.

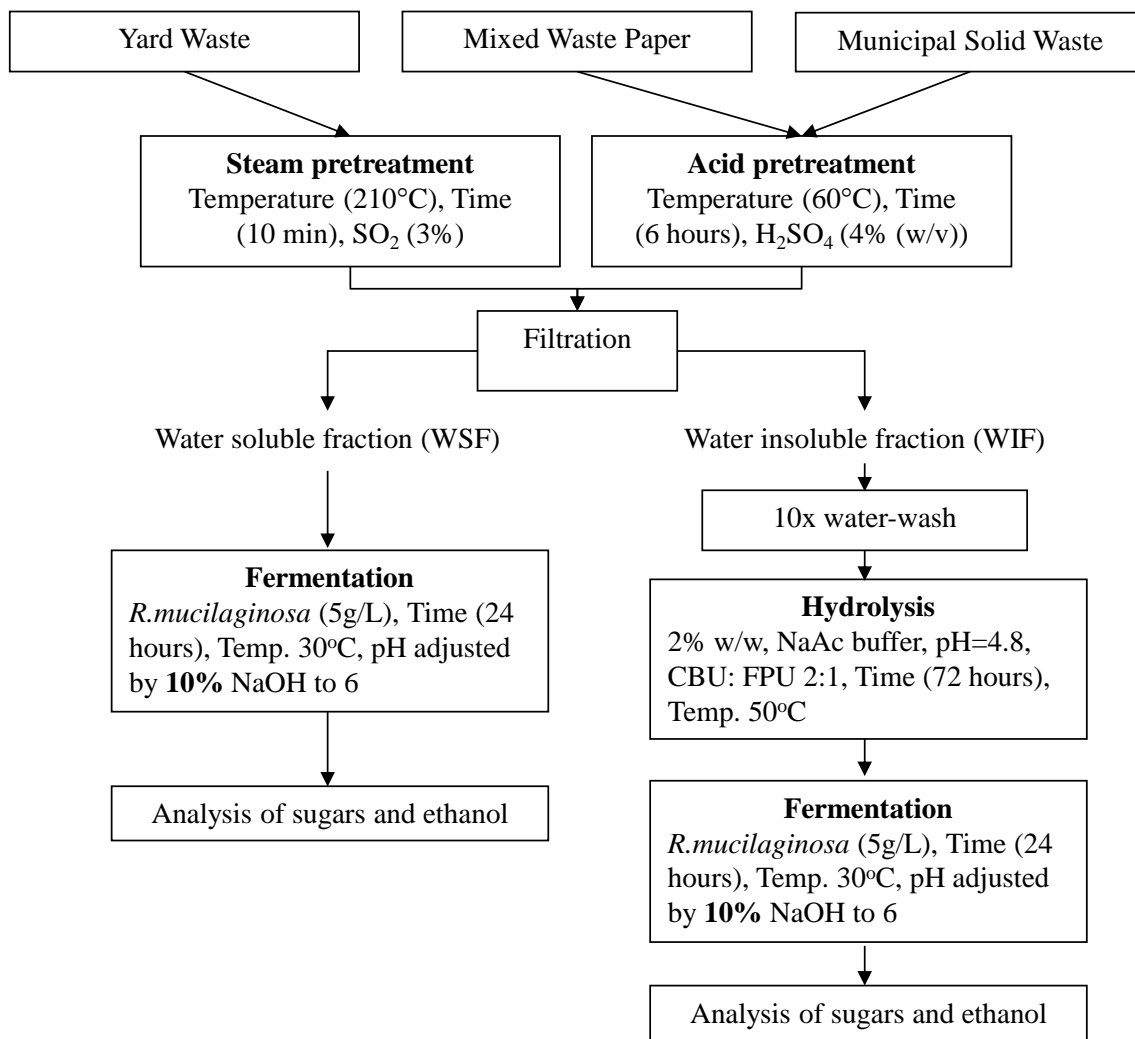


Figure 4.2: System boundaries for Solid Waste-to-Ethanol LCA

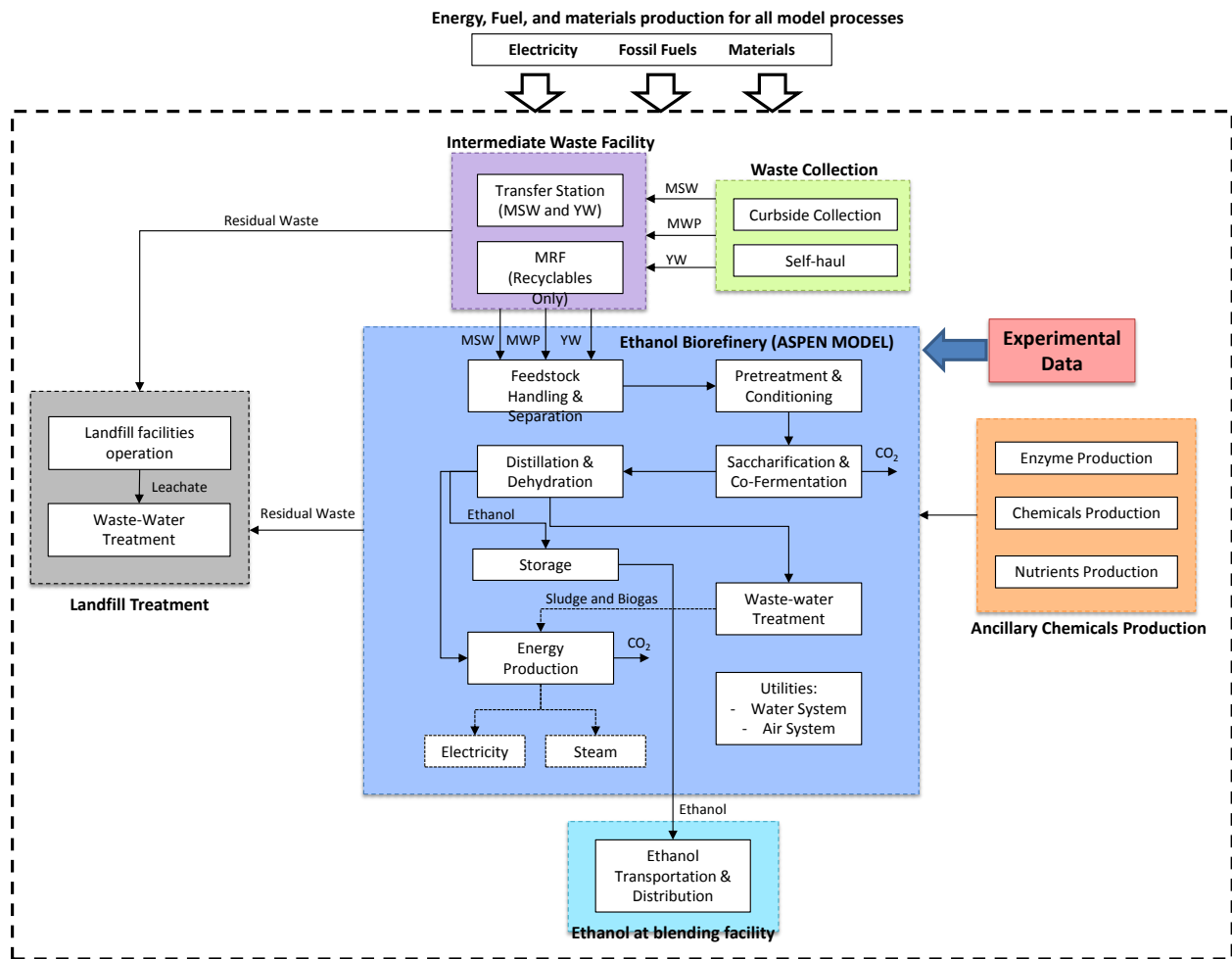


Figure 4.3: Comparison of total energy consumption and GWP potential for waste-to-ethanol LCA studies.

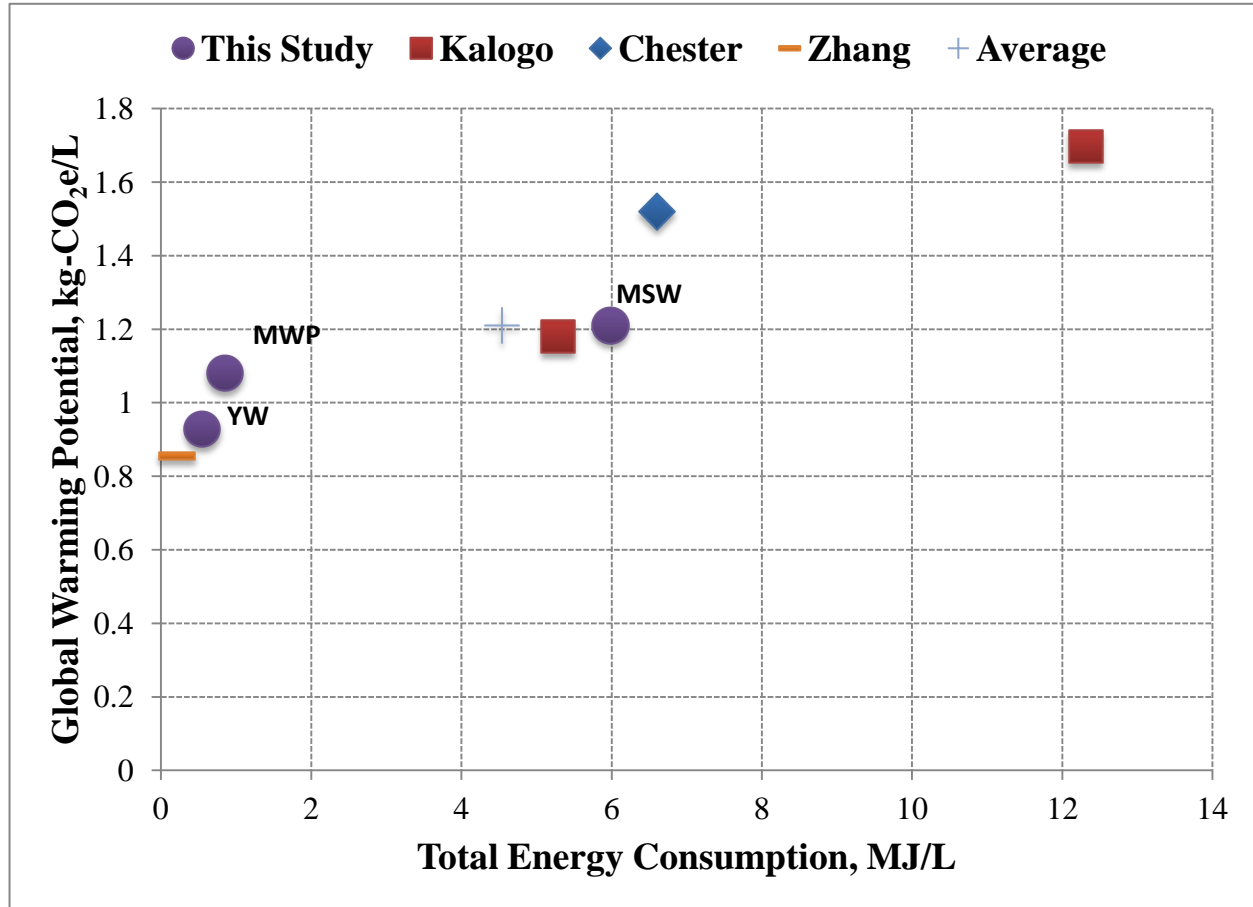


Figure 4.4: Contribution analysis for total energy consumption, global warming potential (GWP), acidification, and smog air formation for solid waste-to-ethanol.

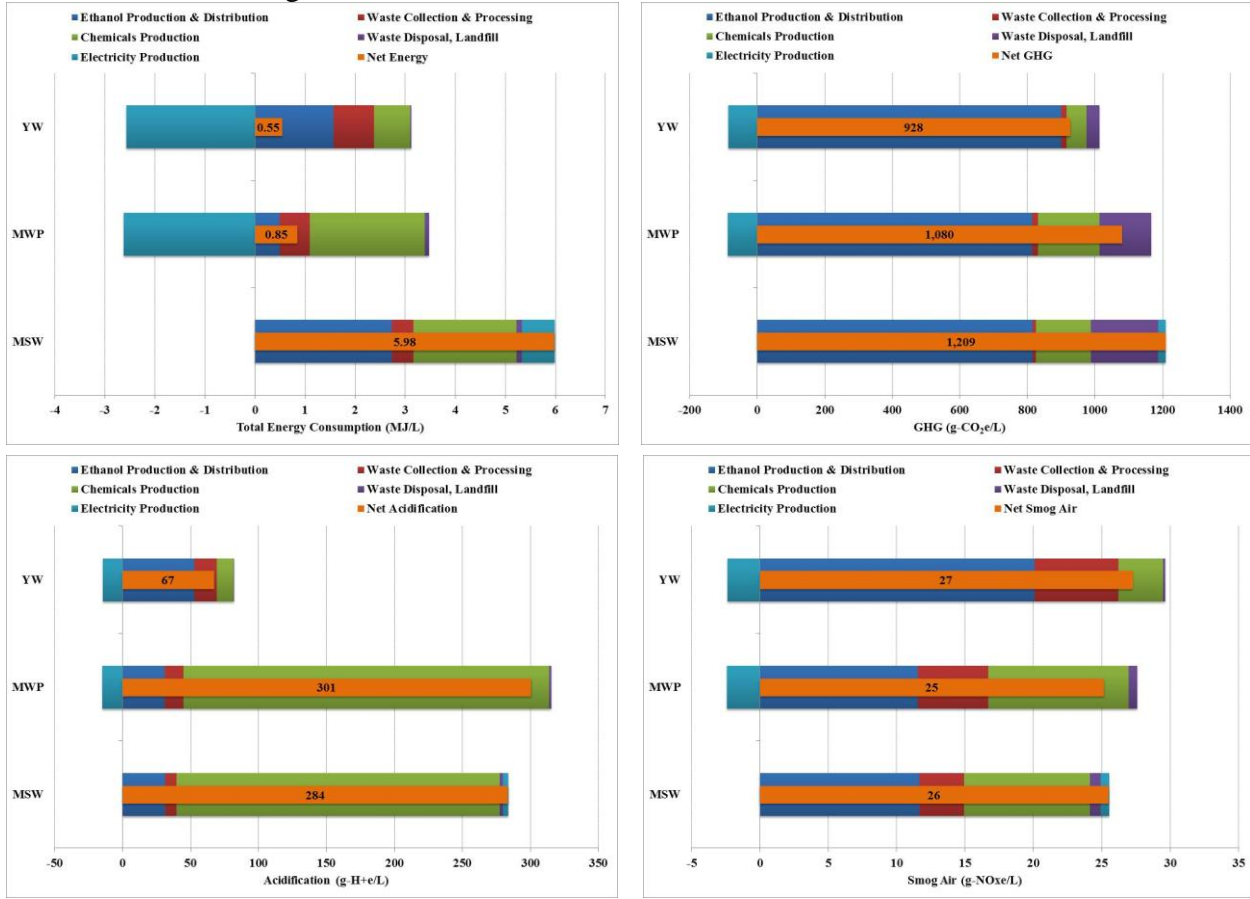


Table 4.1: Composition and characterization of major lignocellulosic components of municipal solid waste in the State of Washington. (Lignocellulosic composition from Barlaz [6]; and waste characterization fractions from Washington State Department of Ecology [32])

	Tons	%Total	Lignocellulosic Composition, % mass		
			Cellulose	Hemicellulose	Lignin
Newspaper	70,594	1.4%	48.5	9.0	23.9
OCC/Kraft Paper	3,894	0.1%	57.3	9.9	20.8
High-grade Paper	49,667	1.0%	87.4	8.4	2.3
Mixed/Low Grade Paper	81,068	1.6%	42.3	9.4	15.0
Compostable/Soiled Paper	201,801	4.1%	-	-	-
Paper/Other	552,600	11.1%	-	-	-
Leaves & Grass	203,909	4.1%	15.3	10.5	43.8
Food Waste	913,281	18.3%	55.4	7.2	11.4
Orther Organics	677,237	13.6%	-	-	-
Total Non-lignocellulosic	2,224,445	44.7%	-	-	-
Total Lignocellulosic	2,754,051	55.3%	51.2	11.9	15.2
Total Waste Collected	4,978,496	100.0%	-	-	-

Table 4.2: Bioconversion of municipal solids waste, paper waste and yard waste to ethanol; chemical composition of municipal solid waste, paper waste and yard waste solids (carbohydrates and lignin) (% weight); concentration of total and monomeric and oligomeric sugars (g/L) in WSFs after pretreatment (dilute acid or steam explosion); recovery of sugars after pretreatment expressed as g per 100g of sugars in the biomass; hydrolysability of pretreated WIFs; relative ethanol yield for the liquid fractions obtained after pretreatment (water soluble fraction) and enzymatic hydrolysis (water insoluble fraction) (%).

		MSW	MWP	YW
Chemical composition of biomass (%)	Arabinose	0.9	0.8	3.8
	Galactose	0.3	0.5	5.2
	Glucose	65.1	72.1	39.6
	Xylose	7.9	7.1	6.7
	Mannose	4.5	7	7.1
	Total Sugars	78.7	87.5	62.4
	Ash	3.1	6.9	4.5
	Total Lignin	18.3	0.6	34.6
Chemical composition WSFs (g/L) ^a	Arabinose	0.7 (0.8)	0.3 (0.3)	1.4 (1.8)
	Galactose	0.2 (0.9)	0.1 (0.2)	2.4 (3.5)
	Glucose	5.9 (22.3)	0.5 (1.5)	2.8 (4.4)
	Xylose	0.6 (1.1)	1.0 (1.1)	2.4 (3.2)
	Mannose	0.1 (0.4)	0.1 (0.4)	2.7 (5.4)
Sugar recovery after pretreatment (g/100g of sugar)	Arabinose	83.6	61.1	73.4
	Galactose	72	62.3	57.7
	Glucose	100	100	99.6
	Xylose	99.3	98.1	97.9
	Mannose	81.7	83.2	66.8
Hydrolysability of solids (WIFs) (%)	Cellulose to glucose conversion (%)	89	86	41
	Xylan to xylose conversion (%)	87	62	33
Ethanol yield (%)	Hexose to ethanol conversion of WSFs after pretreatment (%) ^b	100	99	99
	Hexose to ethanol conversion of WSFs after hydrolysis (%) ^c	100	100	100

^a numbers without the bracket correspond to concentration of monomeric sugars in WSFs after acid hydrolysis, numbers in the bracket correspond to concentration of total sugars (monomers and oligomers)

^b relative ethanol yield for the liquid fractions obtained after pretreatment

^c relative ethanol yield for the liquid fractions obtained after enzymatic hydrolysis

Table 4.3: LCI inputs from ASPEN model

Inputs		MSW	MWP	YW
Biomass Feedstock ^a	kg-dry/hr	78,432	93,138	39,216
Feedstock % Moisture Content		20%	5%	60%
Sulfuric Acid ^b	kg/dry-MT	92.9	92.9	-
Lime ^b	kg/dry-MT	77.7	78.2	8.8
Corn Steep Liquor (CSL) ^b	kg/dry-MT	15.5	15.5	15.8
Cellulase ^b	kg/dry-MT	23.9	12.8	13.0
DAP ^b	kg/dry-MT	1.9	1.9	2.0
LPG ^b	kg/dry-MT	15.4	13.5	8.6
Outputs				
Ethanol	kg/hr	29,010	30,376	8,162
Electricity Produced	kW	10,155	35,118	27,754
Electricity Consumed	kW	14,656	16,914	9,905
Net Electricity	kW	-4,501	18,204	17,849
CO2 Emissions	kg/hr	27,779	29,078	7,863
Theoretical Yield ^b	L/dry-MT	631	568	440
Model Final Yield ^b	L/dry-MT	469	413	264
% of Theoretical		74%	73%	60%
Annual Yield	ML/yr	1,790	94	31

a. Dry lignocellulosic content of waste feedstock

b. Based on dry lignocellulosic fraction of waste feedstock

Table 4.4: Life cycle impact assessment (LCIA) data for MSW, MWP, and YW. (The functional unit for each scenario is one liter of denatured ethanol fuel produced within WA State)

LCIA					
Category	Factor	Unit	MSW	MWP	YW
Total Energy Consumption	1	MJ	5.98E+00	8.52E-01	5.46E-01
Global Warming Potential (100 year)					
CO ₂	1	gCO ₂ -e	1.07E+03	9.71E+02	8.95E+02
CH ₄	25	gCO ₂ -e	1.41E+02	1.08E+02	3.20E+01
N ₂ O	298	gCO ₂ -e	1.97E+00	1.27E+00	1.44E+00
<i>Total</i>		gCO ₂ -e	1.21E+03	1.08E+03	9.28E+02
Acidification					
NO _x	61.3	gH+e	6.16E+01	6.09E+01	6.60E+01
SO _x	50.8	gH+e	2.22E+02	2.40E+02	1.23E+00
<i>Total</i>		gH+e	2.84E+02	3.01E+02	6.72E+01
Smog Air					
CH ₄	0.01	gNO _x -e	8.10E-02	6.23E-02	1.84E-02
N ₂ O	16.8	gNO _x -e	1.12E-01	7.15E-02	8.12E-02
NM VOC	3.6	gNO _x -e	3.54E-01	3.32E-01	3.91E-01
CO	0.06	gNO _x -e	5.62E-02	6.01E-02	5.80E-02
NO _x	24.8	gNO _x -e	2.49E+01	2.47E+01	2.67E+01
<i>Total</i>		gNO _x -e	2.55E+01	2.52E+01	2.73E+01
Particulate Matter					
PM10	1	gPM	2.06E-01	9.55E-02	-1.01E-02
PM2.5	1	gPM	7.03E-02	4.29E-02	1.04E-02
<i>Total</i>		gPM	2.76E-01	1.38E-01	3.03E-04

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CHAPTER 5

Enhanced xylitol and ethanol yields by fermentation inhibitors in steam pretreated hydrolysates

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Enhanced xylitol and ethanol yields by fermentation inhibitors in steam pretreated hydrolysates

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Abstract

A systematic study of the effects of low concentrations of fermentation inhibitors on the fermentation of xylose to xylitol and hexoses to ethanol by yeast *Candida guilliermondii* in the steam pretreated hydrolysates from sugarcane bagasse, hybrid poplar, switchgrass, yard waste and giant reed was conducted. The influence of different inhibitor concentrations on the growth and fermentation abilities of *C. guilliermondii* cultivated in lignocellulosic hydrolysates was measured during liquid batch cultures. In the presence of up to 8g/L of acetic acid along with furfural, 5-HMF, and phenolics in sugarcane bagasse hydrolysate, the ethanol and xylitol yields were boosted up to 20% compared to the control (100%). For all the steam pretreatment severities tested for hybrid poplar the ethanol yield of theoretical was enhanced as much as 22% compared to the control (100%). However, xylitol yield was negatively affected by the higher concentrations of inhibitors in hybrid poplar hydrolysates collected after six different steam pretreatment conditions. In fact, fermentation inhibitors are not necessarily harmful compounds. It was shown that acetic acid, furfural, 5-HMF, and phenolics at certain concentrations can be called rather enhancers than fermentation inhibitors.

Keywords: xylitol; ethanol; inhibitors; steam pretreatment; *Candida guilliermondii*

5.1 Introduction

A commercial biorefinery requires optimum conversion of biomass feedstock to products, which can be sold to give good economic returns and reduce our dependence on fossil fuels. Various feedstocks such as lignocellulosic biomass, agricultural and forest residues, food processing and paper waste can be utilized for the production of biofuels and biochemicals. Also these feedstocks are in abundance, and serve as a low-cost alternative

resource for renewable energy production [3, 20]. Conversion of lignocellulosics to biofuels and biochemicals consists of four major steps [20]: pretreatment, hydrolysis, fermentation, and product(s) recovery. Feedstock pretreatment is a key for successful bioconversion of lignocellulosics to biofuels and biochemicals [19, 26, 28].

Several different pretreatment methods can be used to facilitate the enzymatic hydrolysis of lignocellulosic material [8, 28]. One of the most thoroughly investigated methods is steam explosion pretreatment [5, 6, 10]. As has been shown previously, SO₂-catalyzed steam explosion at optimized conditions can provide a high recovery of hemicellulosic sugars and minimal production of fermentation inhibitors [47].

During SO₂-steam explosion pretreatment, there are three main process variables: temperature, time and SO₂ level. Each feedstock needs different processing requirements due to chemical and structural variations. SO₂-catalyzed steam explosion at optimized conditions can provide a high recovery of hemicellulosic sugars and minimal production of fermentation inhibitors [47]. A low severity can lead to incomplete fractionation of biomass, causing low sugar content in hydrolysis yields. Although, higher severity steam pretreatment results in a more complete delignification and better hydrolysis yields which is desirable, but the inevitable effect of this condition is formation of numerous degradation products [29]. These products will adversely affect biomass chemical components to produce by-products that become inhibitors for the subsequent processing.

One unavoidable effect of pretreatment is the generation of process-derived fermentation inhibitors, which are a consequence of high temperature and low pH used in the steam explosion process [29]. These inhibitory compounds adversely affect microbial growth and fermentation yields [19, 29, 50]. Two major groups of potential inhibitors have been found

in the liquid fraction after pretreatment of lignocellulosic feedstocks: process-derived inhibitors created during pretreatment (*e.g.*, lignin and sugar degradation products), and naturally-occurring inhibitors from the feedstock (*e.g.*, sterols, acetic and uronic acids and resin/fatty acids) [29, 35], all of which may have adverse effects during fermentation [46]. Chemical, physical, and biological methods can be used to remove inhibitors prior to the fermentation, which increases the fermentability [29]. Some of these include pH adjustment, pre-growing-adaptation of microorganisms to the unfavorable environment, steam stripping, an ion-exchange, an ion-exclusion, overliming, and organic solvents extraction [15, 21, 25, 49]. However, detoxification may not be necessary when low and unique concentrations of fermentation inhibitors are present in hydrolysates and when a fermenting organism with high inhibitor tolerance is used [2, 32]. For instance, complete fermentation of an acid hydrolysate of spruce, which was strongly inhibiting in batch fermentation has been achieved in fed-batch fermentation without any detoxification treatment [32, 45]. Fermentation inhibitors present at low concentrations, instead impose a stimulatory effect on final products yield and therefore their presence is somewhat desirable [3, 33, 40, 48]. These compounds may even lead to increased ethanol yield and productivity due to uncoupling by the presence of weak acids [34, 44], or due to decreased glycerol production in the presence of furfural [33]. Therefore, pretreatment degradation products are not necessarily always inhibitory compounds as long as their concentration is lower than a threshold concentration that negatively affects the process [22].

In lignocellulosic hydrolysates, the concentration of sugars as well as the concentration of pretreatment by-products depends on pretreatment conditions [22]. The understanding of the influence of pretreatment conditions on sugar release and production of degradation

products can aid in optimizing the pretreatment conditions for enhanced final products yields. Therefore, the objective of this study is to investigate the effect of different concentrations of fermentation inhibitors originating from five different steam pretreated feedstocks for enhanced xylitol and ethanol yields by *Candida guilliermondii*. Furthermore, the overarching objective is to test if the steam pretreatment conditions can be tailored for production of unique concentrations of fermentation inhibitors to enhance xylitol and ethanol production.

5.2. Materials and methods

5.2.1 Yeast strain

Candida guilliermondii FTI-20037 (NRC 5578) was obtained from the ATCC, a nonprofit biological resource center (BRC), Manassas, Virginia. This strain was taken from -80°C stocks and maintained on YPG solid medium (10g/L yeast extract, 20g/L peptone, 20g/L glucose, and 18g/L agar, Difco, Becton Dickinson, MD) at 4°C and transferred to fresh plates on a weekly basis.

5.2.2 Culture media conditions

Cells were grown to high cell density in foam-plugged 1L Erlenmeyer flasks containing 500ml YP-sugar liquid media (10g/L yeast extract and 10g/L peptone, supplemented with 10g/L xylose) in an orbital shaker for 2 days at 30°C and 150 rpm, with concurrent transfer to fresh medium performed every 24 h. After 48 hours of growth, cell cultures were harvested, centrifuged, and decanted to yield cell pellets. Pellets were then washed three times with sterile distilled water and subsequently adjusted with sterile distilled water to a calculated concentration of 5g dry cell weight (DCW) per liter on a spectrophotometer

(Shimadzu UV-1700, Columbia, MD) via standard curves relating 600nm absorbance to DCWL^{-1} (dry cell weight (DCW) per liter) concentration.

5.2.3 Carbohydrates and alcohols

Synthetic sugars (glucose, xylose, galactose, mannose, and arabinose) were obtained from Supelco, (Bellefonte, PA). Ethanol 4mg/ml, xylitol 5mg/ml, arabitol, and glycerol were obtained from Sigma–Aldrich, (St. Louis, MO).

5.2.4 Fermentation

5.2.4.1 The water soluble fraction (hydrolysate) fermentation

Lignocellulosic hydrolysates from hybrid poplar at 200°C, 5 min, 3% SO_2 [5], giant reed (*Arundo donax*) at 190°C, 5 min, 3% SO_2 [4], switch grass at 195°C, 7.5 min, 3% SO_2 [9], sugarcane bagasse at 205°C, 10 min, 3% SO_2 [9], mixture of hardwood (hybrid poplar) and softwood (Douglas-fir) chips at 210°C, 10 min, 3% SO_2 [41] were steam pretreated, collected, and fermented into xylitol and ethanol exactly as previously described by Bura [3].

Furthermore, optimization of steam pretreatment conditions for production of unique concentrations of fermentation inhibitors for improved xylitol and ethanol yield, hybrid poplar chips (screened to approximately 5mm thickness and 1-3 cm length and width) without bark (50% moisture content) were obtained from Forest Concepts (Auburn WA) facility and stored at -20°C until use. The detailed procedure of steam explosion experiments has been described previously by Ewanick [9]. Briefly, half of samples of 800g oven-dried weight (ODW) hybrid poplar chips were impregnated overnight with

anhydrous SO₂ in plastic bags. The samples were then loaded, in 400g batches, into a preheated, 2.7 L UW steam gun, manufactured by Aurora Technical, Savona BC and exploded in the order: three samples impregnated with 3% (w/w) SO₂ concentration were exploded for 5 minutes at temperature of 190°C, 195°C, and 200°C. The other three samples were exploded without SO₂ at 205°C and 212°C for 10 min, and 212°C for 15 min reaction time.

The water soluble fractions (hydrolysates) from steam explosion of the hybrid poplar chips at six different conditions were recovered by filtration and kept at 4°C until use. The liquid fraction, along with the wash fraction, was analyzed for monomeric, and oligomeric carbohydrates, acetic acid, HMF and furfural. The solid fraction was analyzed for carbohydrates, lignin and acetate groups. Monomeric and oligomeric soluble carbohydrates were determined as previously described [9]. Oligomeric sugars were calculated by subtracting monomeric sugars content from total sugars content. The fermentation process was performed in a similar manner as the fermentation experiments described by Bura [3]. The initial concentration of sugars (glucose and xylose) present in the hydrolysate was brought up to 30g/L. Solutions with sugars were filter-sterilized separately, and appropriate quantities added aseptically to the desired concentration to the hydrolysates fermentation media. A 0.1% (w/v) yeast extract, 0.17 % (w/v) yeast nitrogen base without amino acids and 5% (w/v) urea were added to the hydrolysates. The initial pH of the hydrolysates was adjusted to 6 prior to fermentation. The controls consisted of synthetic sugars at the same concentration as measured in the hydrolysates. The detailed procedure of sampling procedure has been described previously by Bura [3] and Vajzovic [48].

5.2.6 HPLC analysis

5.2.6.1 Monomeric sugars

The concentration of monomeric sugars (arabinose, galactose, glucose, xylose and mannose) was measured on a Dionex (Sunnyvale, CA) HPLC (ICS-3000) system equipped with an AS autosampler, ED electrochemical detector, dual pumps, and anion exchange column (Dionex, CarboPac PA1). Deionized water at 1 ml/min was used as an eluent, and post-column addition of 0.2 M NaOH at a flow rate of 0.5 ml/min ensured optimization of baseline stability and detector sensitivity. After each analysis, the column was reconditioned with 0.25 M NaOH. Twenty microliters of each sample were injected after filtration through a 0.22 μ m syringe filter (Restek Corp., Bellefonte, PA, U.S.). Standards were prepared containing sufficient arabinose, galactose, glucose, xylose, and mannose to encompass the same range of concentrations as the samples. Fucose (0.2g/L) was added to all samples and standards as an internal standard.

5.2.6.2 Ethanol, xylitol, acetic acid, furfural, 5-HMF, and phenolics analysis

Ethanol, xylitol, acetic acid, furfural, and 5-HMF were measured using refractive index detection on a Shimadzu Prominence LC. Separation of these compounds was achieved by an anion exchange column (REZEX RHM-Mono saccharide H⁺ (8%), Phenomenex, Inc., Torrance, CA, U.S.) with an isocratic mobile phase that consisted of 5mM H₂SO₄ at a flow rate of 0.6ml/min. The column oven temperature was maintained at a constant temperature of 63°C. Twenty microliters of each sample were injected after being appropriately diluted in deionized water and filtered through a 0.22 μ m syringe filter (Restek Corp., Bellefonte, PA, U.S.). Standards were prepared and used to quantify the unknown samples. The Folin–

Ciocalteu (F–C) assay was used as a standardized method for approximating the total phenolics concentrations in the hydrolysates, using gallic acid as a standard [1]. Folin Ciocalteu reagent and gallic acid were purchased from Sigma. The samples were analyzed by determining the absorbance of each solution at 765 nm against the blank and absorbance vs. concentration were plotted.

The theoretical yield for ethanol production from glucose is 0.51g ethanol g⁻¹ glucose [29]. Ethanol yields and percent theoretical yields were calculated using the equations formulated by Keating [17] . The theoretical yield for xylitol production from glucose used was 0.91g xylitol g⁻¹ xylose [50]. It was assumed that all xylitol formed during the growth phase of the mixed sugar fermentations was derived from xylose. Cumulative xylitol (Y_{xylitol}; g xylitol produced g⁻¹ total xylose consumed) yields were calculated during and at the end-point of the fermentations. The specific consumption and production rates were calculated based on the log-mean cell density,

$$\frac{S - S_0}{X - X_0}, \text{ where } S \text{ is the substrate or product, } X \text{ is dry cell weight, and } t \text{ is time [16].}$$

Since within each experiment, tests were conducted in triplicate in separate flasks, the standard deviation was calculated between three samples using the excel's statistical function.

5.2.6 Severity factor

Steam pretreatment impregnated with SO₂ as well as uncatalyzed reaction were evaluated with the severity factor correlation, often used for the evaluation of the explosion process and to describe the lignin reduction and xylan solubilization [30]. Equation 1 describes the severity factor of the pretreatment which increases as a function of time t (min) and temperature T (°C), as follows:

$$R_o = te^{(T-100)/14.75} \quad (1)$$

The combined severity (CS) factor is calculated based on the severity factor $\log(R_o)$ (Equation 1), and the pH after pretreatment, through Equation 2 [7, 37] where the pH is measured after the pretreatment:

$$CS = \log R_o - pH \quad (2)$$

5.3. Results and Discussion

5.3.1 The water soluble fractions (hydrolysates) obtained after steam pretreatment of five different feedstock fermentation

Based on concentrations of fermentation inhibitors present, lignocellulosic hydrolysates obtained after steam pretreatment of five lignocellulosic feedstocks (mixed wood, hybrid poplar, giant reed, switchgrass, and sugarcane bagasse) were evaluated for enhanced xylitol and ethanol production by strain *Candida guilliermondii*. Two major groups of potential inhibitors have been found in the liquid fraction after pretreatment of the lignocellulosic feedstock. The feedstock-inherited (acetic acid) and those process-derived (furfural, 5-HMF, and phenolics) inhibitors were present in all liquid fractions fermented (Table 5.1).

According to Table 5.1, the concentration of acetic acid ranged from 1g/L to 8.2g/L and was the highest in giant reed hydrolysate. The concentration of furfural ranged from 0.3g/L to 3.4g/L, 5-HMF from 0.1g/L to 0.5g/L, and phenolics from 1.3g/L to 3.3g/L. All the highest inhibitors concentrations measured were noted in giant reed hydrolysate. The ethanol and xylitol yields were measured as high as 120% and 88% of theoretical compared to the controls, 100% and 67%, respectively (Figure 5.2.E and Table 5.1).

The concentrations of fermentation inhibitors were dependent on applied pretreatment condition and the type of feedstock used. For example, in mixed wood hydrolysate, the concentrations of all the inhibitors were the lowest in comparison to the other hydrolysates (Table 5.1). Since all five hydrolysates originated from different feedstock and were pretreated at different conditions, the chemical composition of the hydrolysates has shown that the higher the concentration of acetic acid up to 5g/L measured in lignocellulosic hydrolysate, resulted in higher ethanol yield of theoretical produced as compared to the control (Table 5.1, Figure 5.1, Figure 5.2).

Vajzovic [48] and Bura [3] previously reported that unique concentrations of fermentation inhibitors tend to have a stimulatory effect on xylitol and ethanol yields by *Rhodotorula mucilaginosa* strain PTD3. The ethanol yield of theoretical was up to 20% (sugarcane bagasse) higher compared to the control (100%) (Figure 5.1 and Figure 5.2.E). Ethanol production in excess with respect to this stoichiometry, was ascribed to enhanced xylose fermentation in presence of acetic acid [42].

As the hydrolysates contain more inhibitors, the decreased growth rates and the increased glucose and xylose uptake by *C. guilliermondii* possibly reflects the extra amount of energy required for proton transport through the plasma membrane. The intracellular pH becomes unbalanced due to the heterogeneous acetate/acetic acid distribution between the inside and the outside of the yeast cell [39, 43]. The data presented in this work lead to the conclusion that the presence of acetic acid in the culture medium can favor xylose metabolism of *C. guilliermondii* through an increase in the activities of xylose reductase [XR] and xylitol dehydrogenase [XDH]. The driving force for this phenomenon could be the result of an

increase in ATP concentration inside the cell, which would favor cross membrane proton transport. As a consequence, the internal cell pH would be maintained near neutrality [24, 48].

In attempts to explain the conversion of xylose to ethanol by known reactions, it is generally accepted that the initial steps involve sequential reduction to xylitol and oxidation to convert xylitol to xylulose [23, 50]. Xylulose kinase then catalyzes the formation of xylulose-5-phosphate, which undergoes rearrangements catalyzed by transketolase and transaldolase to form hexose phosphate [13, 14, 23]. Finally, hexose phosphate is converted to ethanol by the glycolytic pathway [23, 50]. Considering that ethanol yield increased by 20% in sugarcane bagasse compared to the control (Table 5.1 and Figure 5.2.E), we speculate that a fraction of xylitol is converted into xylulose due to the simultaneous increase of XR and XDH activities. In giant reed hydrolysate, the ethanol yield by *C. guilliermondii* was not enhanced at acetic acid concentrations of 8.2g/L and was similar to the control (100% of theoretical) (Figure 5.1, Figure 5.2.C, Table 5.1 and Table 5.3A). This signals that the concentration of acetic acid was approaching a threshold concentration point, beyond which, the lag-phase in growth would occur and ultimately lower ethanol yields.

Similarly, in the presence of acetic acid, fermentation of xylitol was enhanced up to 20% for concentrations up to 5g/L of acetic acid (Table 5.1, Figure 5.1, and Figure 5.2). However, fermentation of xylose to xylitol from hydrolysate originated from pretreated giant reed was negatively affected by the presence of acetic acid at 8g/L (Figure 5.1 and Figure 5.2.C). Vajzovic [48] reported that the ethanol yield by *Rhodotorula mucilaginosa* strain PTD3 was enhanced even in the presence of the highest concentration of 20g/L of

acetic acid tested while xylitol yield was lowered even at the lowest concentration of 5g/L of acetic acid tested. In the current study the highest furfural (3.4g/L), 5-HMF (0.5g/L), and phenolics (3.3g/L) measured in giant reed hydrolysate (Table 5.1), contributed to the lowered xylitol yield due to the synergism and cumulative concentration effects of these compounds. Indicative of the negative effect of higher concentrations of acetic acid, furfural, 5-HMF, and phenolics, the xylitol yield in giant reed hydrolysate was reduced to 41% of theoretical compared to inhibitors-free control (67%) (Figure 5.1, Figure 5.2.C, and Table 5.1). The specific xylitol and ethanol production rate as well as the specific xylose and glucose consumption rate dropped (Table 5.3A). However, the effects of inhibitors on xylose to xylitol bioconversion have not been deeply investigated [36].

It is understood that yeast, during bioconversion of six carbon sugars to ethanol, metabolizes furfural to furfural alcohol [31]. NADH-dependent alcohol dehydrogenase is thought to be responsible for this reduction, causing the attenuated xylitol and ethanol yields in our study. Since all NADH generated is used for furfural reduction, the glucose to ethanol and also the xylose to xylitol process are greatly affected. This is because there is an increased acetaldehyde accumulation inside the cell caused by an insufficient amount of NADH-dependent alcohol dehydrogenase available in order to reduce acetaldehyde to ethanol [18, 31]. Intracellular acetaldehyde accumulation is therefore considered to be the reason for the lag-phase in growth and ultimately resulting in lower yields at the higher concentration of this inhibitor in giant reed hydrolysate. Similarly to the inhibition mechanism by furfural, yeast metabolizes HMF to HMF-alcohol [18, 31]. Likewise, NADPH-dependent alcohol dehydrogenase is understood to be responsible for this reduction. As such, the reduction of HMF does not regenerate NAD^+ , and thus carbon is

allocated to glycerol production (to produce NAD^+ and thus maintain overall redox balance) [31].

In spite of the presence of all four inhibitory compounds in the other hydrolysates (mixed, hybrid poplar, switchgrass, and sugarcane bagasse) there was no synergistic effect observed by combining inhibitors, and in contrast, the combinations were enhancing both, xylitol and ethanol yields. This is because the total concentration of all the inhibitors was below the inhibitory threshold concentration by which the cell growth rate and the kinetics of product formation by *C. guilliermondii* were not affected (Figure 5.1, Figure 5.2, Table 5.1, and Table 5.3A). Hence, in this study certain concentrations of acetic acid, furfural, 5-HMF, and phenolics have been shown to exert a stimulating effect on the xylitol and ethanol yields by *C. guilliermondii* up to 20% in sugarcane bagasse. Contrary to previous observations [31, 36], it was shown that fermentation inhibitors present at certain concentrations in the lignocellulosic hydrolysate boosted the xylitol and ethanol yields by *C. guilliermondii* (Figure 5.1, Figure 5.2, Table 5.1, and Table 5.3A).

5.3.2 The water soluble fraction (hydrolysate) obtained after steam pretreated hybrid poplar fermentation

Systematic screening of six different steam pretreatment conditions for production of acceptable concentration range of fermentation inhibitors in hybrid poplar hydrolysate for enhanced xylitol and ethanol yields was conducted. The effects of pretreatment conditions on the composition of the liquid fractions obtained from hybrid poplar were studied (Table 5.2 and Table 5.3B). For conditions 2 and 3 (Table 5.2 and Table 5.3B), the pretreatment reaction time is different while the other operational conditions were the same.

As the combined severity (CS) factor increased, concentrations of acetic acid and phenolics increased (conditions 1-3) and plateaued for conditions (4-6) with higher CS factors. As the CS factors increased, the concentration of furfural and 5-HMF increased linearly for both uncatalyzed and SO₂-catalyzed pretreatment. Ethanol yields (up to 47% more, as compared to the control) by *C. guilliermondii* were enhanced by the presence of the fermentation inhibitors for all the concentrations tested (Table 5.2). As the CS factors increased, the specific sugars (glucose and xylose) consumption and specific production (xylitol and ethanol) rates decreased for all the pretreatment conditions tested (Table 5.3B). Clearly, xylitol yield was impaired by the inhibitory compounds for all the conditions tested. Drop in xylitol yields was especially pronounced in hydrolysates collected after SO₂-catalyzed steam pretreatment with the lowest xylitol yields (22% of theoretical) compared to the control (67% of theoretical).

In addition, it was observed that as the CS factor was increasing, the ethanol and xylitol yields in hydrolysates obtained from uncatalyzed steam pretreatment (conditions 1-3) were decreasing (Table 5.2). Similarly, the impregnation of the material with SO₂ resulted in increased formation of inhibitors and thus lowered ethanol and xylitol yields, when compared to a not-catalyzed pretreatment (conditions 4-6), (Table 5.2). Eventhough, the specific production rate of ethanol decreased as the CS factor increased, the ethanol yield was still stimulated by the presence of fermentation inhibitors and was higher compared to the control (Table 5.2, Table 5.3B). The lowest ethanol yield observed was 22% higher compared to the control (100% of theoretical). As seen in the previous section, this behavior can be explained by xylose contributing to the ethanol yield in the presence of the fermentation inhibitors. Nevertheless, the concentration of fermentation inhibitors matters.

At lower pretreatment severities, the recovery of hemicellulosic sugars is high and the least amount of inhibitory compounds are produced [11, 28]. However, within the high-range of pretreatment severities, soluble sugars derived from plant polysaccharides are partially lost as degradation by-products [29]. In general, the overall carbohydrate yield decreases sharply when temperature increases, whereas higher yields of lignin condensation and pentosan dehydration are observed at longer reaction times [11, 19, 28, 30, 38]. In addition, the use of a catalyst in steam pretreatment can elevate production of process derived inhibitors due to the improved recovery of the hemicellulose-derived sugars [28, 30]. According to Garrote and Parajo [12] even at lower pretreatment severities part of the acetyl esters are removed as acetic acid, catalyzing the xylan depolymerization. Our study showed that acetyl bonds were completely hydrolyzed through SO₂ catalyzed pretreatment under a high severity condition, resulting in the highest concentrations of acetic acid (Table 5.2) and the same as in untreated biomass (data not shown). Nonetheless, acetic acid was detected even under low severity conditions with or without SO₂ catalyst (Table 5.2).

The major chemical and physical changes to lignocellulosic biomass with catalyzed steam explosion are often attributed to the elevated removal of hemicellulose. This is due to the SO₂ penetrating deeper into the fibers, removing more acetyl groups, thus causing more sugar release [30]. It improves the accessibility of the enzymes to the cellulose fibrils [27, 28]. In conclusion, the impregnation of the material with SO₂ resulted in increased formation of fermentation inhibitors, when compared to a non-catalyzed pretreatment of the same feedstock (conditions 4-6) (Table 5.2). Based on these data sets we can conclude that the severity of the pretreatment influenced the concentration of process-derived

fermentation inhibitors and those unique concentrations of the inhibitors positively influenced ethanol and negatively affected xylitol yields by *C. guilliermondii*.

5.4 Conclusions

In this study we investigated the effect of inhibitors on the production of xylitol and ethanol by *Candida guilliermondii*. Contrary to previous observations, it was shown that certain concentrations of acetic acid, furfural, 5-HMF, and phenolics boosted the xylitol and ethanol yields in hydrolysates from five different steam pretreated feedstocks. In the presence of up to 2.1g/L acetic acid enhanced ethanol yields up to 20% of theoretical in mixed wood, switchgrass and sugarcane bagasse hydrolysates compared to the control (67%). Unlike other similar studies, our study demonstrated that acetic acid boosted the xylitol yields at up to 5 g/L of this inhibitor with constant xylitol production and xylose consumption rates. Tailoring of the steam pretreatment conditions of hybrid poplar for enhanced ethanol and xylitol yields generated a range of fermentation inhibitors that was acceptable for enhanced ethanol yield only. However, the customized conditions were not an optimal for enhancing xylitol yield. Ethanol yields (up to 47% more compared to the control) were enhanced by the presence of acetic acid and the other fermentation inhibitors for all the concentrations tested. Nevertheless, a 10% drop in xylitol yield was already noticed even at the lowest acetic acid concentration (1.6g/g) or (4.8g/L) tested in hydrolysate collected after uncatalyzed steam pretreatment compared to the control (67% of theoretical). In this study we present a novel approach of optimizing the steam pretreatment conditions to generate a low concentration of fermentation inhibitors to improve hexoses to ethanol and xylose to xylitol yields by *C. guilliermondii*. This approach will allow us in the

future to find the optimum concentration of inhibitors to increase ethanol and xylitol yields during fermentation of hexoses and pentoses. Provided that the inhibitors in lignocellulosic hydrolysates are identified, the fermentation process can be improved in several ways. This study demonstrated that acetic acid, furfural, 5-HMF, and phenolics at certain concentrations can be called rather enhancers than fermentation inhibitors.

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Figure Legends

Figure 5.1A. Xylitol production from five steam pretreated lignocellulosic hydrolysates by *C. guilliermondii*.

Figure 5.1B. Ethanol production from five steam pretreated lignocellulosic hydrolysates by *C. guilliermondii*.

Figure 5.2 Sugar consumption and xylitol and ethanol production during fermentation of hydrolysates obtained during steam explosion of: (A) the mixture of softwoods and hardwoods, (B), hybrid poplar, (C) giant reed, (D) switchgrass, and (E) sugarcane bagasse by *C. guilliermondii* following acclimation to xylose. The error bars indicate standard deviation.

Table 5.1. Process variables, xylitol and ethanol yields, and chemical composition in hydrolysates obtained by steam pretreatment of mixed wood (hybrid poplar and Douglas-fir chips), hybrid poplar, giant reed, switchgrass, and sugarcane bagasse. The reported results are the average of triplicate studies with a deviation of $\leq 2\%$.

Table 5.2. Process variables, xylitol and ethanol yields, combined severity, and chemical composition in hydrolysates obtained by steam pretreatment of hybrid poplar chips. The reported results are the average of triplicate studies with a deviation of $\leq 3\%$.

Table 5.3A. The specific rates of glucose and xylose consumption and XOH and EOH production during fermentation of different hydrolysates by *Candida guilliermondii*.

Table 5.3B. The specific rates of glucose and xylose consumption and XOH and EOH production during fermentation of hybrid poplar hydrolysates by *Candida guilliermondii*.

Figure 5.1A. Xylitol production from five steam pretreated lignocellulosic hydrolysates by *C. guilliermondi*.

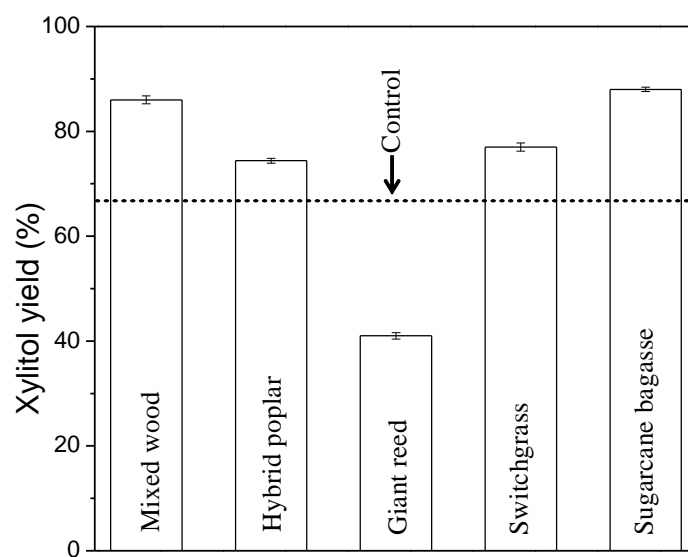


Figure 5.1B. Ethanol production from five steam pretreated lignocellulosic hydrolysates by *C. guilliermondi*.

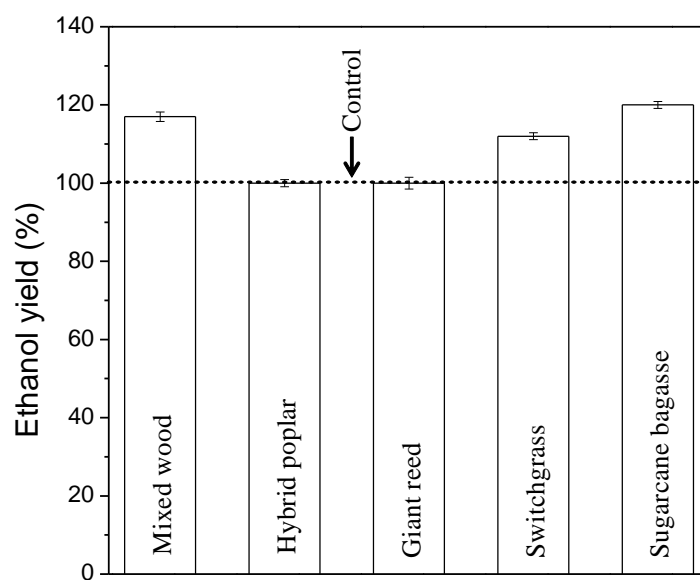
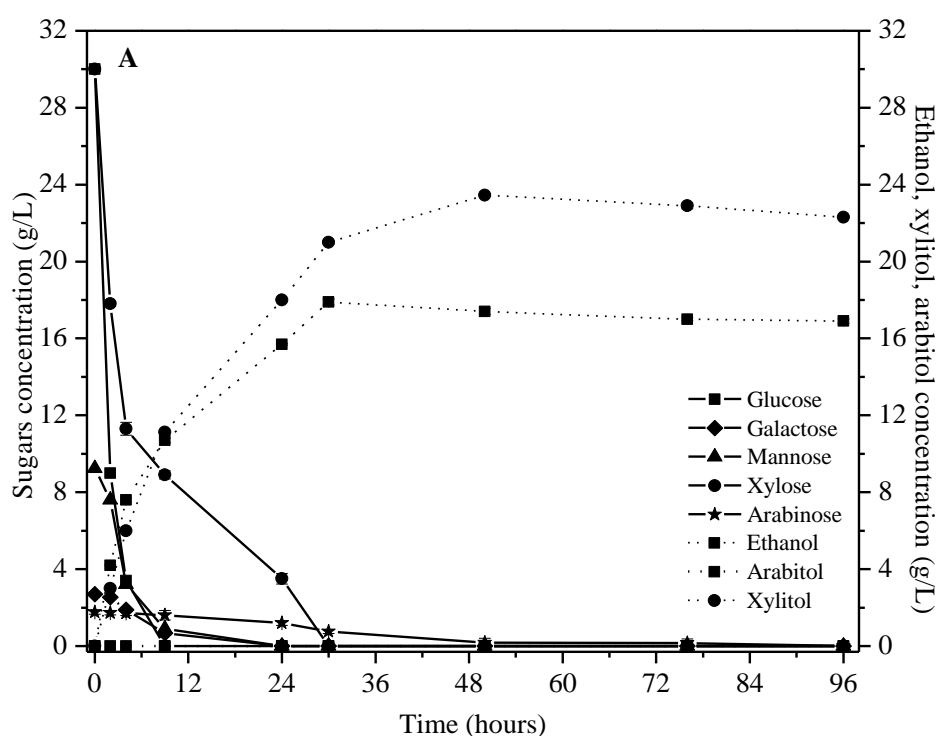
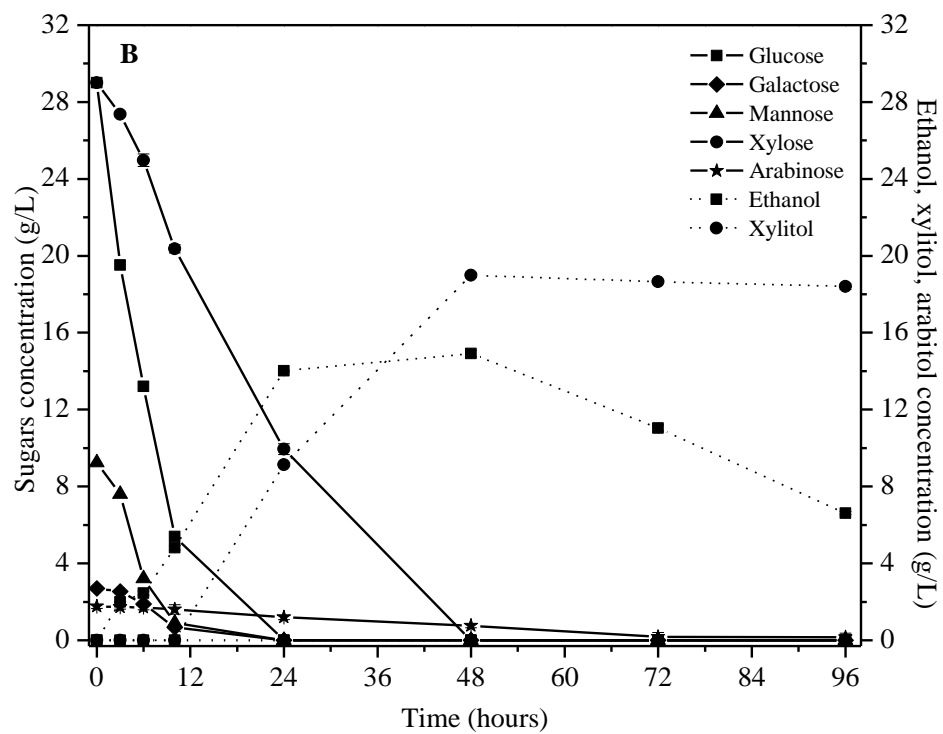
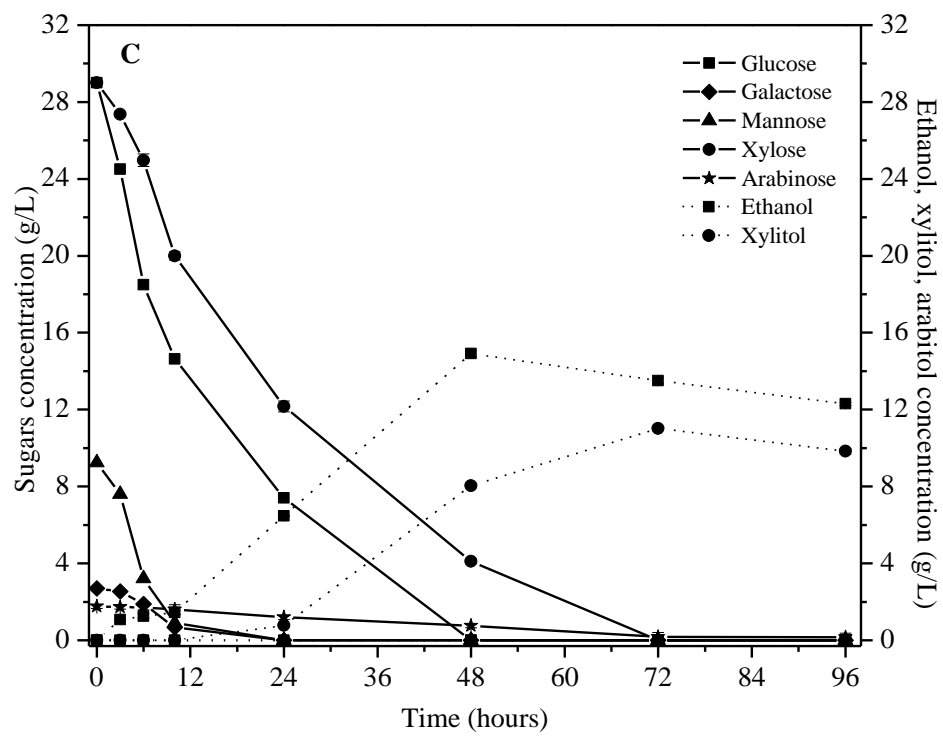
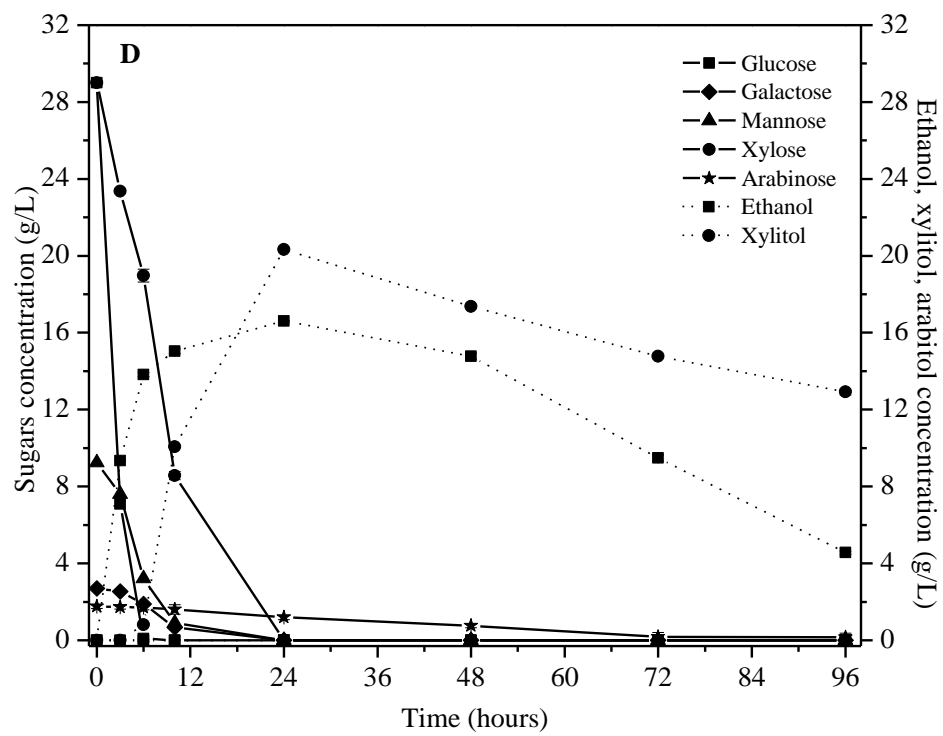


Figure 5.2 Sugar consumption and xylitol and ethanol production during fermentation of hydrolysates obtained during steam explosion of: (A) the mixture of softwoods and hardwoods, (B), hybrid poplar, (C) giant reed, (D) switchgrass, and (E) sugarcane bagasse by *C. guilliermondii* following acclimation to xylose. The error bars indicate standard deviation.









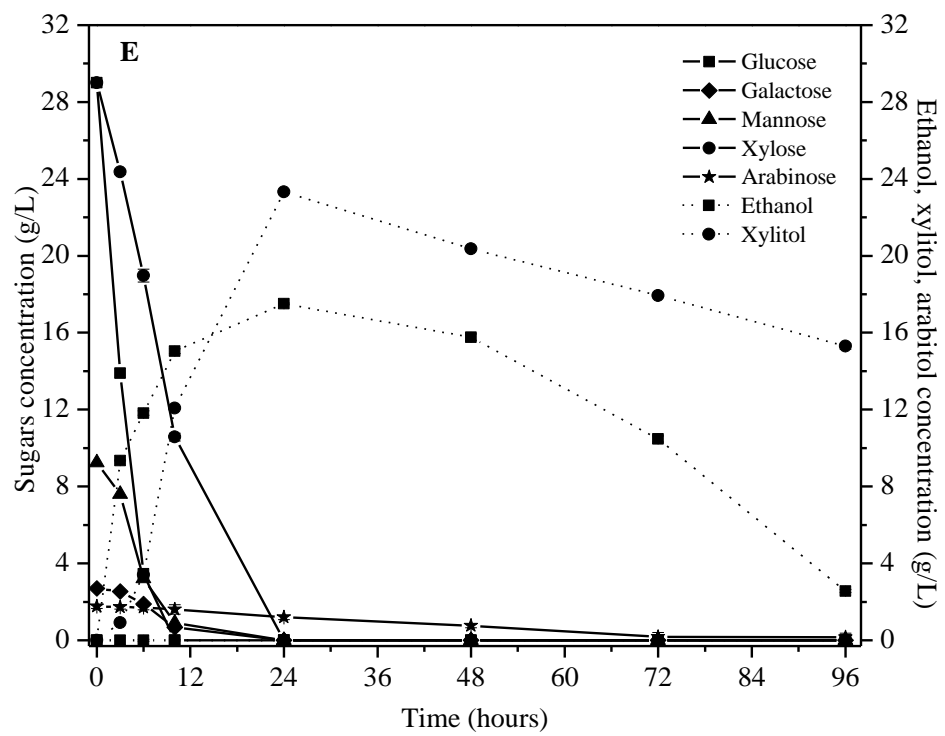


Table 5.1. Process variables, xylitol and ethanol yields, and chemical composition in hydrolysates obtained by steam pretreatment of mixed wood (hybrid poplar and Douglas-fir chips), hybrid poplar, giant reed, switchgrass, and sugarcane bagasse. The reported results are the average of triplicate studies with a deviation of $\leq 2\%$.

<i>Sample</i>	<i>Acetic acid</i> (g/L)	<i>Furfural</i> (g/L)	<i>5-HMF</i> (g/L)	<i>Phenolics</i> (g/L)	<i>[XOH]</i> Y%T (%)	<i>[EOH]</i> Y%T (%)
Mixed wood	1.0	0.3	0.1	1.3	86 \pm 0.8	117 \pm 1.1
Switchgrass	1.0	0.3	0.1	1.5	77 \pm 0.5	112 \pm 1.0
Sugarcane bagasse	2.1	0.8	0.3	1.7	88 \pm 0.6	120 \pm 0.8
Hybrid poplar	4.9	0.8	0.4	2.6	74 \pm 0.9	100 \pm 1.0
Giant reed	8.2	3.4	0.5	3.3	41 \pm 1.0	100 \pm 1.1
Control	0.0	0.0	0.0	0.0	67 \pm 1.0	100 \pm 0.5

Table 5.2. Process variables, xylitol and ethanol yields, combined severity, and chemical composition in hydrolysates obtained by steam pretreatment of hybrid poplar chips. The reported results are the average of triplicate studies with a deviation of $\leq 3\%$.

<i>Sample</i>	<i>Temperature</i> (°C)	<i>Time</i> (minutes)	<i>SO₂</i> (% w/w)	<i>CS*</i>	<i>Acetic acid</i> (g/g)	<i>Furfural</i> (g/g)	<i>5-HMF</i> (g/g)	<i>Phenolics</i> (g/g)	<i>[XOH]</i> <i>Y%T (%)</i>	<i>[EOH]</i> <i>Y%T (%)</i>
1	205	10	0	0.6	1.6	0.7	0.2	0.8	57 ± 0.5	147 ± 1.1
2	212	10	0	1.0	2.6	1.2	0.3	1.0	51 ± 0.9	142 ± 1.0
3	212	15	0	1.3	3.5	2.0	0.6	1.1	34 ± 0.5	122 ± 1.2
4	190	5	3	1.9	3.6	0.9	0.2	1.4	42 ± 0.7	138 ± 1.0
5	195	5	3	2.0	3.7	1.3	0.3	1.4	24 ± 0.8	132 ± 1.3
6	200	5	3	2.1	3.8	1.7	0.4	1.5	22 ± 0.4	128 ± 1.0
Control	NA	NA	NA	NA	0.0	0.0	0.0	0.0	67 ± 1.0	100 ± 0.5

*The combined severity factor is calculated based on the severity factor $\log(R_0)$ (Equation 1), and the pH after pretreatment, through Equation 2 where the pH is measured after the pretreatment.

Table 5.3A. The specific rates of glucose and xylose consumption and XOH and EOH production during fermentation of different hydrolysates by *Candida guilliermondii*.

Feedstock	Control	Mixed wood	Hybrid poplar	<i>Arundo donax</i>	Switchgrass	Sugarcane bagasse
Fermentation parameters	$\text{Consumption}^1 / \text{Production}^2$ ($\text{gg}^{-1}\text{h}^{-1}$)	$\text{Consumption}^1 / \text{Production}^2$ ($\text{gg}^{-1}\text{h}^{-1}$)	$\text{Consumption}^1 / \text{Production}^2$ ($\text{gg}^{-1}\text{h}^{-1}$)	$\text{Consumption}^1 / \text{Production}^2$ ($\text{gg}^{-1}\text{h}^{-1}$)	$\text{Consumption}^1 / \text{Production}^2$ ($\text{gg}^{-1}\text{h}^{-1}$)	$\text{Consumption}^1 / \text{Production}^2$ ($\text{gg}^{-1}\text{h}^{-1}$)
Xylose	0.05	0.11	0.07	0.04	0.10	0.11
XOH	0.03	0.09	0.03	0.02	0.08	0.09
Glucose	0.13	0.43	0.13	0.07	0.23	0.25
EOH	0.06	0.08	0.03	0.04	0.06	0.06

The reported results are the average of triplicate studies with a deviation of $\leq 2\%$.

¹The specific rates of sugar consumption were calculated based on the log-mean dry cell density and the Δ substrate and Δ time.

²The specific rates of xylitol from xylose and ethanol from glucose, production were calculated based on the log-mean dry cell density and the product concentration and Δ time.

Table 5.3B. The specific rates of glucose and xylose consumption and XOH and EOH production during fermentation of hybrid poplar hydrolysates by *Candida guilliermondii*.

	<i>Hybrid poplar hydrolysate</i>						
Condition	Control	205°C, 10'	212°C, 10'	212°C, 15'	190°C, 3%SO ₂	195°C, 3%SO ₂	200°C, 3%SO ₂
Fermentation parameters	Consumption ¹ / Production ² (gg ⁻¹ h ⁻¹)	Consumption ¹ / Production ² (gg ⁻¹ h ⁻¹)	Consumption ¹ / Production ² (gg ⁻¹ h ⁻¹)	Consumption ¹ / Production ² (gg ⁻¹ h ⁻¹)	Consumption ¹ / Production ² (gg ⁻¹ h ⁻¹)	Consumption ¹ / Production ² (gg ⁻¹ h ⁻¹)	Consumption ¹ / Production ² (gg ⁻¹ h ⁻¹)
Xylose	0.05	0.08	0.06	0.04	0.1	0.06	0.06
XOH	0.03	0.04	0.04	0.02	0.04	0.01	0.01
Glucose	0.13	0.36	0.22	0.09	0.27	0.11	0.11
EOH	0.06	0.12	0.06	0.04	0.09	0.06	0.05

The reported results are the average of triplicate studies with a deviation of $\leq 2\%$.

¹The specific rates of sugar consumption were calculated based on the log-mean dry cell density and the Δ substrate and Δ time.

²The specific rates of xylitol from xylose and ethanol from glucose, production were calculated based on the log-mean dry cell density and the product concentration and Δ time.

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CHAPTER 6

Conclusions and future work

6.1 Conclusions

An overall hypothesis in this thesis is that steam pretreated lignocellulosic hydrolysate can be efficiently fermented into xylitol and ethanol without prior detoxification. While an undetoxified medium certainly implies limitations, it appears that an appropriate choice of pretratment conditions and microorganism can still allow for acceptable substrate utilisation and satisfactory yields of biochemicals, xylitol and ethanol. Concluding remarks for objectives 1, 2, and 3 are:

1. It is well established that for succesful and feasible bioconversion of biomass to biochemicals procees the choice of strain will be of the greatest importance for a process involving fermentation of lignocellulosic hydrolysate, or of any medium with a similar profile of toxicity.
 - *R. mucilaginosa* strain PTD3 was found capable of assimilating and fermenting xylose, glucose, galactose, mannose, and arabinose as a single and as well as mixed carbon source.
 - This strain produced not only xylitol from xylose but ethanol from hexoses. Xylitol and ethanol yields were not affected by a 1:1 ratio of xylose to glucose, resulting in repeated high theoretical yields, (65 and 92% respectively).
 - Furthermore, the yeast exhibited the ability to ferment high concentrations of mixed sugars (150g/L).
 - Noteworthy, this yeast performed best during fermentation of sugars coming from lignocellulosic hydrolysate, producing the highest yields of xylitol (76% of theoretical yield) and ethanol (100% of theoretical yield).
 - Fermentation of the steam pretreated lignocellulosic hydrolysate served to illustrate PTD3's ability to utilize and ferment sugars derived from steam pretreated mixture of

hardwood (hybrid poplar) and softwood (Douglas-fir) chips and to tolerate pretreatment degradation products (Chapter 2).

2. The use of PTD3 can be proposed to selectively remove inhibitors from lignocellulosic hydrolysates to improve the fermentability, since it was capable of metabolizing concentrations of 5, 15, and 5 g/L of furfural, 5-HMF, and acetic acid, respectively.
 - Contrary to previous observations, it was shown that furfural boosted the xylitol yields at up to 5 g/L of furfural with constant xylitol production and xylose consumption rates, however, furfural negatively affected ethanol yields, glucose consumption and ethanol production rates by PTD3. 5-HMF at concentrations lower than 5 g/L increased or did not affect production of xylitol but lowered ethanol yields by this microorganism. Acetic acid, even at 20 g/L, stimulated ethanol yields for PTD3 while the opposite was observed for xylitol.
 - PTD3 demonstrated an exceptional ability to ferment the sugars in presence of sugar degradation products, and to tolerate and metabolize higher concentrations of furfural, 5-HMF, and acetic acid compared to other yeasts described in the literature.
 - The implications of this work cannot be understated. This represents a promising means of increasing commercial ethanol and xylitol yields through simply monitoring and altering concentrations of fermentation inhibitors as they enter the process. Also, unlike reported before, this emphasizes that a detoxification step during bioconversion process is not necessary and PTD3 can be employed as detoxification agent due to its ability to metabolize the inhibitors (Chapter 3 and 4).
3. A crucial determining element of any bioconversion process is an appropriate choice of pre-treatment conditions and feedstock that can affect subsequent processes.

- This study investigated the effect of inhibitors on enhanced production of xylitol and ethanol by *Candida guilliermondii*.
- It was shown that certain concentrations of acetic acid, furfural, 5-HMF, and phenolics boosted the xylitol and ethanol yields in hydrolysates from five different steam pretreated feedstocks. As the concentration of acetic acid increased (approximately up to 2.1g/L) , the ethanol and xylitol yields were enhanced up to 20% of theoretical in mixed wood, switchgrass and sugarcane bagasse hydrolysates compared to the controls.
- Tailoring of the steam pretreatment conditions of hybrid poplar showed that for all the conditions employed, the ethanol yield was up to 50% enhanced by *C. guilliermondii*. However, the opposite was discovered for xylitol fermentation.

In this study, it was demonstrated that quantifying the concentrations of fermentation inhibitors generated during the steam pretreatment can be a guidance for designing the pretreatment conditions to enhance final product yields (Chapter 5).

6.2 Future work

Throughout this study, the possibility of using lignocellulosic biomass to demonstrate the technical and economical bioconversion of biomass to xylitol and ethanol has been a main focal point. However, there are several unanswered questions in this area. Future experiments, described below, should help to reach the ultimate goal of one day establishing a commercially viable bioconversion of lignocellulosic material to xylitol and ethanol process.

1. Investigation of yeast strain adaptation to lignocellulosic hydrolysate prior to fermentation to improve acclimatization of the yeast to inhibitory compounds within lignocellulosic hydrolysate.

2. Studying the tailoring of the pretreatment conditions for the other lignocellulosic feedstock (i.e. sugarcane bagasse, corn stover, wheat straw)
3. Scaling up the sugars fermentation from shaker flask level to a bioreactor and boosting xylitol and ethanol production to make a compatible process to industrial-scale operation. Already established biofuels and bioenergy lab at the University of Washington for the bioconversion of lignocellulosic biomass to biofuels and biochemicals will allow the use of larger scale fermentors (5 L, New Brunswick[®]), with pH and oxygen probes.
4. Development of continuous fermentation processes with cell re-cycling for prolonged periods of exponential growth without lag phase in order to have a more time efficient bioconversion process with higher yields.
5. Utilizing Raman spectroscopy for online measurements of xylitol and ethanol production by yeast *Candida guilliermondii*.
6. Techno-economic and Life Cycle Analyses (LCA) of the proposed bioconversion process of lignocellulosic hydrolysates to xylitol and bioethanol would benefit decisions regarding industrial-scale operation.

Vita

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EDUCATION

- 2008–Present PhD program at School of Environmental and Forest Sciences, Bioresource Science and Engineering, University of Washington (graduating in December, 2012, thesis title: "Enhanced production of xylitol and ethanol", mentored by Professor Renata Bura)
- 2000-2002 Bachelor of Science Degree, major Biochemistry, University of Washington
- 1998-2000 Associate of Arts Degree, major Pre-dental, Highline Community College
- 1997-1998 Certified dental assistant, Highline Community College

SCHOOLRASHIPS/FELLOWSHIPS/AWARDS

1. Xi Sigma Pi Alpha University of Washington Research Grant Award for 2009 (\$1000).
2. Bioenergy IGERT fellowship 2009-2011, the National Science Foundation's flagship interdisciplinary training program, focused on developing renewable energy solutions for Northwest Native American Tribes (\$60,000).
3. Graduate Student Travel Award for 2010 (\$500).
4. Denman Professorship in Bioresource Science Engineering, University of Washington for 2011-2012 (\$30,000).

PUBLICATIONS

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PRESENTATION

1. Bura, R and Vajzovic, A. Biological production of xylitol and ethanol by novel naturally occurring yeast. IGERT PI Meeting, June 2012, Washington, DC.
2. Vajzovic A. and Bura R. Novel approach of improving xylose to xylitol conversion by *Candida guilliermondii*. International Conference on Renewable Resources & Biorefineries , June, 2011 Bruges, Belgium.
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5. Vajzovic A, Bura R, Doty S.L. Biological production of ethanol and xylitol by novel naturally occurring yeast. International Conference on Renewable Resources & Biorefineries , June, 2010 Dusseldorf, Germany.
6. Vajzovic A. Bura R, Doty S.L, Kohlmeier K. Increased tolerance of inhibitors during biological production of ethanol and xylitol by a novel yeast. Washington Pulp and Paper Foundation annual conference. May, 2010, Seattle, Washington.

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8. Vajzovic A, Bura R, Doty S.L, Kohlmeier K. A sweet solution to fuel troubles. The Symposium on Biotechnology for Fuels and Chemicals, May, 2009, San Francisco, California.
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PATENT

1. UW TechTransfer accepted our Record of Invention (ROI) and formally accepted it as a patent application in October 2008. ROI Title: Naturally occurring yeast strains for xylitol production, UW TechTransfer Reference No. 8107D.