Utilization of Wood Based Hemicellulose Pre-hydrolysate for The Production of High Value Added Platform Chemicals

Submitted by
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Abstract

The immense benefits of using renewable resources are because they can reduce global warming and other environmental issues which directly affect life on earth. Forest residues serve as potential renewable resources which can substitute the fossil based products with biobased products. In order to produce such renewable biobased products, biorefining concepts are needed to develop innovative and effective processes. However, biorefineries are at early stages of development and are directed towards making marketable products from forest residues. Development of efficient and economically viable methods is necessary. This thesis contributes to this endeavor by developing novel and efficient methods to produce valuable platform chemicals from a wood based resource, hemicellulose. In these studies, hemicellulose obtained from the poplar wood was used to produce xylitol, succinic acid and levulinic acid. They were listed among the top twelve chemicals which have the potential for high market demand, by the Department of Energy (DOE), US.

The hemicellulose prehydrolysate used in this study was produced by a proprietary pretreatment process. As a pre-requirement to this study, I have analyzed its composition. Acid hydrolysis of the prehydrolysate resulted in a concentrated xylose rich hydrolysate. However, xylose undergoes several side reactions and results in undesired byproducts. In order to avoid the formation of these byproducts, the acid concentration was optimized to obtain a xylose rich hydrolysate with relatively low amounts of inhibitors. This hydrolysate was used in all the processes studied in this thesis.

Microbial fermentation of the hydrolysates is inhibited by impurities in the media. In order to make the fermentation efficient, the detoxification (purification) of the hydrolysate has to be much effective. Therefore, a detoxification method was developed to remove the impurities such as furfural and acetic acid with minimal loss of xylose. This detoxification method includes the combination of vacuum evaporation and solvent extraction procedures. The fermentation of the hydrolysate detoxified with the developed process found to produce high yields of xylitol (0.59 g/g).

Along with the hydrolysis and detoxification procedures, the conversion method was also improved further by using immobilized yeast strains. In these experiments, two yeast strains

(*Candida guilliermondii* and *Candida tropicalis*) were used to produce xylitol. *Candida tropicalis* strain used in this study is a new strain isolated from a decaying wood biomass. The yield (0.92 g/g) and productivities (0.88 g/L/h) of xylitol obtained with the immobilized form of this yeast were significant and found to be higher than those reported in literature. It is evident that the hydrolysis, detoxification and fermentation methods developed are efficient in improving the yields of xylitol from hemicellulose prehydrolysates.

Subsequently, the hydrolysed hemicellulose was used for the production of succinic acid (SA) by chemical routes. It was found that an important byproduct, furfural, formed during the acid hydrolysis of hemicellulose, can be converted to succinic acid. Therefore, a simple and facile method was developed to convert hemicellulose to furfural which was subsequently converted to succinic acid. Several challenges such as preventing humin formation, determination of an ideal solvent for furfural extraction etc. were addressed in this work. A heterogeneous acid catalyst, Amberlyst 15 used in this study provides an advantage of recycling. The ratio of catalyst to the substrate was also investigated to determine the optimum amount of catalyst loading. A biphasic system was developed for simultaneous production, separation and oxidation of furfural to succinic acid. These studies demonstrate the conversion of the hydrolysate into furfural and then to succinic acid with acceptable yields (49%) in the biphasic system.

Another platform chemical, levulinic acid, was also studied for its production from hemicellulose. Levulinic acid can be produced by the acid catalysed reaction of furfuryl alcohol (FA), which can be produced from the hemicellulose derived furfural. In our study, ethanol was found to be a better solvent for the conversion of furfuryl alcohol to the ester of levulinic acid, ethyl levulinate (EL). A homogeneous acid (sulfuric acid) and a heterogeneous acid (Amberlyst 15) catalysts were compared in these studies and it was found that both are effective in ethanol than in water. In order to understand the conversion process, to improve and control the reaction, it necessary to know the actual reaction mechanism. The main focus of this study was to determine the reaction mechanism of this conversion process in the presence of sulfuric acid in ethanol. The intermediates were isolated and identified using nuclear magnetic resonance (NMR) spectroscopy and gas chromatography mass spectroscopy (GCMS). Though several intermediates were found, four intermediates could be identified based on GCMS and NMR data. Based on the interpreted

intermediates, a reaction mechanism for the conversion of FA to EL was proposed. Besides, the molar yield of ethyl levulinate obtained from FA was found to be 85 – 90 % in ethanol.

Based on the results obtained in this study, it is apparent that the high value products studied in this thesis can be produced from a cheap renewable resource, hemicellulose. The contribution of such processes, if bolted on the existing processes, can make the overall integrated biorefining process economically feasible.

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I dedicate this thesis to my supervisor, Prof. Sudip Kumar Rakshit!

List of Abbreviations

Acronym	Abbreviation
AFEX	Ammonia Fiber Explosion
ARP	Ammonia Recycling Percolation
DOE	Department of Energy
EL	Ethyl levulinate
FA	Furfuryl alcohol
FDCA	Furan di-carboxylic acid
FL	Furfural
GCMS	Gas Chromatography Mass Spectroscopy
HMF	Hydroxymethyl furfural
HPLC	High performance Liquid Chromatography
IUPAC	International union of Physical and Applied Chemistry
LA	Levulinic acid
NMR	Nuclear Magnetic Resonance
PEF	Pulsed Electric-Field
PHL	Prehydrolysate
RF	Response Factor
RID	Refractive Index Detector
SA	Succinic acid
TLC	Thin Layer Chromatography
VMS	Vogel's Minimal Salt
VWD	Variable Wavelength Detector
YEP	Yeast Extract Peptone
YEPX	Yeast Extract Peptone Xylose

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Table of Contents

Outline of the contents of the thesis

Chapter 1 *Introduction*

An introduction about renewable biomass resources with an emphasis on the hemicellulose prehydrolysate liquor and its latest market trends are presented. The rationale of the work, the overall and specific research objectives of the thesis are also stated.

Chapter 2 *Literature Review*

Recent progress of the work done in the area of hemicellulose utilization is discussed. The importance of pretreatment of biomass and the conversion of hydrolysate into value added products is reviewed. This chapter provides an insight into the immense amount of research carried out on microbial production of xylitol for the past two decades. However, the production of levulinic acid and succinic acid from the lignocellulosic biomass are relatively new concepts and are at their early stages of development. The production of platform chemicals from hemicellulose streams and the knowledge gaps to be filled to develop efficient processes are discussed. This review has been published as a chapter in a book titled "Lignocellulose – Biotechnology, chemical composition and future prospects".

Chapter 3 *Development of pretreatment and acid hydrolysis of hemicellulose to produce toxin-free of xylose*

This chapter reports the original research work carried out in our laboratory to develop an efficient acid hydrolysis and detoxification techniques to make the hemicellulose hyfrolysates favorable for fermentation. The optimization of acid concentration is necessary to reduce the formation of byproducts (toxins in case of fermentation) such as furfural, acetic acid and humins. A dilute sulfuric acid method was determined and found to produce low quantities of microbial growth inhibitors. A novel approach for detoxifying the hydrolysate was reported to remove most of the inhibitors from the hydrolysate. These processes were proven to be efficient by fermenting the detoxified hydrolysate using *Candida* strain which showed higher yields of xylitol than those reported in literature. The results are discussed with reference to previous studies in this area and emphasized the benefits of the proposed methods. This work has been reported in the journal "Biomass and Bioenergy".

Chapter 4 *Enhanced production of xylitol from wood hemicellulose prehydrolysate using various strains*

In this chapter, the fermentation methods were optimized and immobilization of yeast cells were reported to enhance the xylitol production from wood prehydrolysate treated with the pre-fermentation techniques described in previous chapter. These methods were also evaluated on different substrates, sugarcane bagasse hydrolysates, in order to compare the results. A new microbial strain of *Candida* was used in the fermentation of the hydrolysates and found to produce the highest yields with immobilized strains compared to literature. A part of this work is published in the journal "Applied Biochemistry and Biotechnology".

Chapter 5 *An oxidative synthesis of succinic acid from the renewable hemicellulosic pentose sugars using heterogeneous acid catalyst*

In this chapter, we have reported the methods developed to produce a very important platform chemical, succinic acid from wood hemicellulose prehydrolysate. Unlike various literature reports, we have demonstrated the succinic acid synthesis from pentose based sugar, xylose in hemicellulose. Furfural is the major precursor to produce succinic acid in this process. Several challenges like furfural separation, solvent determination, effect of acid catalyst have been discussed and answered. A multiple-step process and a novel biphasic system was developed to synthesize succinic acid from hemicellulose. Simulation of the developed processes were done using the software Aspen Plus Version 8.4 and studied the energy efficiency and economic analysis. A manuscript of this work has been submitted to the journal "Industrial and Engineering Chemistry Research".

Chapter 6 *Synthesis and mechanistic studies of levulinic acid from the wood hemicellulose prehydrolysate using heterogeneous acid catalyst and the determination of byproducts*

Production of another important platform chemical, levulinic acid, from wood prehydrolysate was demonstrated in this chapter. The production of levulinic acid from a hemicellulose derived furfuryl alcohol was studied. The novelty of this chapter lies in the mechanistic studies and the identification of the intermediates and byproducts of this process. Besides, using a heterogeneous catalyst, Amberlyst 15 provides an advantage of possible recycling and reuse. This work has been submitted to the journal "Sustainable Chemistry and Engineering".

Chapter 7 *Summary and Recommendations*

The overall conclusions that can be drawn from this thesis is presented in this chapter. Recommendations for future work and the importance of the studies in this thesis were also suggested.

CHAPTER 1

INTRODUCTION

1 Introduction

The use of wood based renewable resources for the production of useful chemicals instead of fossil based reserves will help mitigate climate change. The synthesis report of Inter-governmental Panel on Climate Change (IPCC) strongly recommends phasing out the use of fossil fuels. They anticipate 90% increase in pollution, if we continue at the present level of emissions [1]. This will result in extreme weather conditions and related disasters.

Biorefineries are sustainable alternatives for the production of high demand platform chemicals from renewable resources. Lignocellulosic biomass has a great potential to serve as a renewable feedstock to produce value-added chemicals and platform chemicals which can serve as a precursor to a number of bulk chemicals and polymers. Therefore, there is considerable research attention to produce a wide range of valuable products from wood resources. The principal aim of a biorefinery is to use renewable resources such as lignocellulosic biomass in a sustainable and cost-effective way to produce products which are commonly produced from fossil resources.

Lignocellulose of any type of biomass is mainly composed of cellulose, hemicellulose and lignin (Figure 1.1). However, specific compositions vary depends on the type of biomass, e.g. softwoods (gymnosperms), the hardwoods (angiosperms), agriculture residue (wheat straw, bagasse, rice husks, corn cobs etc.) (Table 1.1). Detailed studies by Fengel and Grosser (1975) revealed that the stem wood portion of softwood trees contains 45-50% of cellulose, 25-35% lignin, and 25-30% hemicelluloses [2]. On the other hand, the stem-wood portion of hardwood trees contains 40-55% cellulose, 18-25% lignin, and 25-30% hemicelluloses [3].

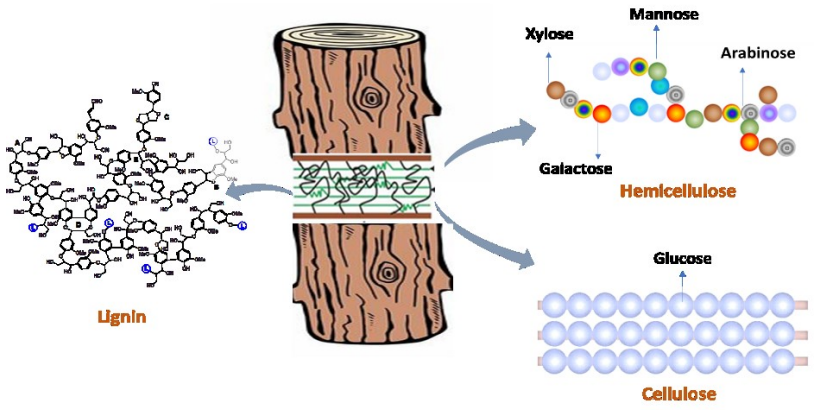

Figure 1.1. Graphical representation of major carbohydrate and phenolic components of the stem wood of forest biomass

Cellulose is a major part of the primary cell wall in plants. It is a long polysaccharide of glucose monomers. It is hydrophobic in nature, thus, helps in water transportation and provides tensile strength to the plant cell. Lignin is an integral part of middle lamella of plant fibers. The concentration of lignin diminishes as we go deep into the fiber. It gives mechanical strength to plant tissues, water impermeability to the secondary cell walls and prevents the collapse of water transporting elements. Unlike cellulose, hemicellulose is a branched polysaccharide of several sugars in the primary cell wall and exists in a complex matrix along with cellulose and pectin.

Table 1.1. Composition of cellulose, hemicellulose and lignin in various types of biomass**.**

Types of Biomass	Lignocellulosic substrate	Cellulose (%)	Hemicellulose (%)	Lignin (%)
Forest biomass	Hardwood stems	40-55	24-20	18-25
	Softwood stems	45-50	25-30	25-35
	Leaves	15-20	80-85	0
Energy crops	Empty fruit bunch	41	24	21.2
	Switch grass	45	31.4	12
Agricultural residues	Corncobs	45	35	15
	Barley straw	33-40	20-35	8-17
	Corn stover	39-42	22-28	18-22
	Nut shells	25-30	25-30	30-40
	Wheat straw	30	50	15

A pretreatment process is required to separate the three main components (cellulose, hemicellulose and lignin) of biomass before they can be used in biorefineries [4-8]. It is one of the major bottlenecks of the effective use of renewable biomass residues for the production of biofuels and various platform chemicals. Several well-established pretreatment techniques were reported in literature to separate the lignocellulosic components [9]. These processes are discussed in detail in the *literature review*. The Pretreatment processes usually attempt to separate lignocellulosic biomass into two fractions (a) pretreated solid wood residue rich in cellulose and lignin and (b) liquid fraction (hemicellulose or prehydrolyzate) containing hemicellulosic sugars (pentoses) and other components (Figure 1.2).

Cellulose in the solid fraction can be hydrolyzed to obtain a solution of glucose sugar which can be easily converted into ethanol by fermentation. Though cellulosic ethanol has been the focus of research and a host of other products, economics need to be improved by cost effective processes [10]. The utilization of cellulose in various industries like pulp and paper, textiles (rayon, cellophane), pharmaceuticals (fillers in drugs), fuel (ethanol, butanol), and many others have been studied extensively [11]. The other component of the solid residue, lignin, a complex phenolic polymer, is currently used as an energy source by direct combustion. In 2010, nearly 50 million tonnes of lignin was extracted from the pulp and paper industry alone, but only 2 % (1 million ton) was used for producing commercial products such as dispersing, flocculating or binding agents [12, 13]. The rest of the lignin was burnt to produce combustion energy [14]. However, considerable research is being carried out to make use of this biopolymer in various applications [15, 16].

One possible way to make biorefining of lignocellulosics economical is to use the liquid stream of pretreated wood, hemicellulose, which is rich in carbohydrate sugars. It can potentially be used to produce various value added products by integrating with the existing plants. We have recently presented the importance of such integration in biorefining processes, in order to compete successfully with the fossil fuel industry [17]. From Table 1.1, it is evident that for every 1 ton of biomass, an average of 450 kg of hemicellulose (prehydrolysate) is obtained from the pretreatment process. In hardwood species, ~440-530 kg glucose and 110-240 kg of xylose per metric tonne of wood can be obtained. Theoretically, it is possible to produce 510 kg of ethanol per tonne of hexose or xylose [18]. However, this is not in practice due to the high costs involved in pretreatment and hydrolysis of biomass. Also, 90% yield of ethanol can be achieved using hexose fraction (glucose), while using pentose fraction (hemicellulose) the fermentative yield is as low as 40-50% of the theoretical yield [19, 20].

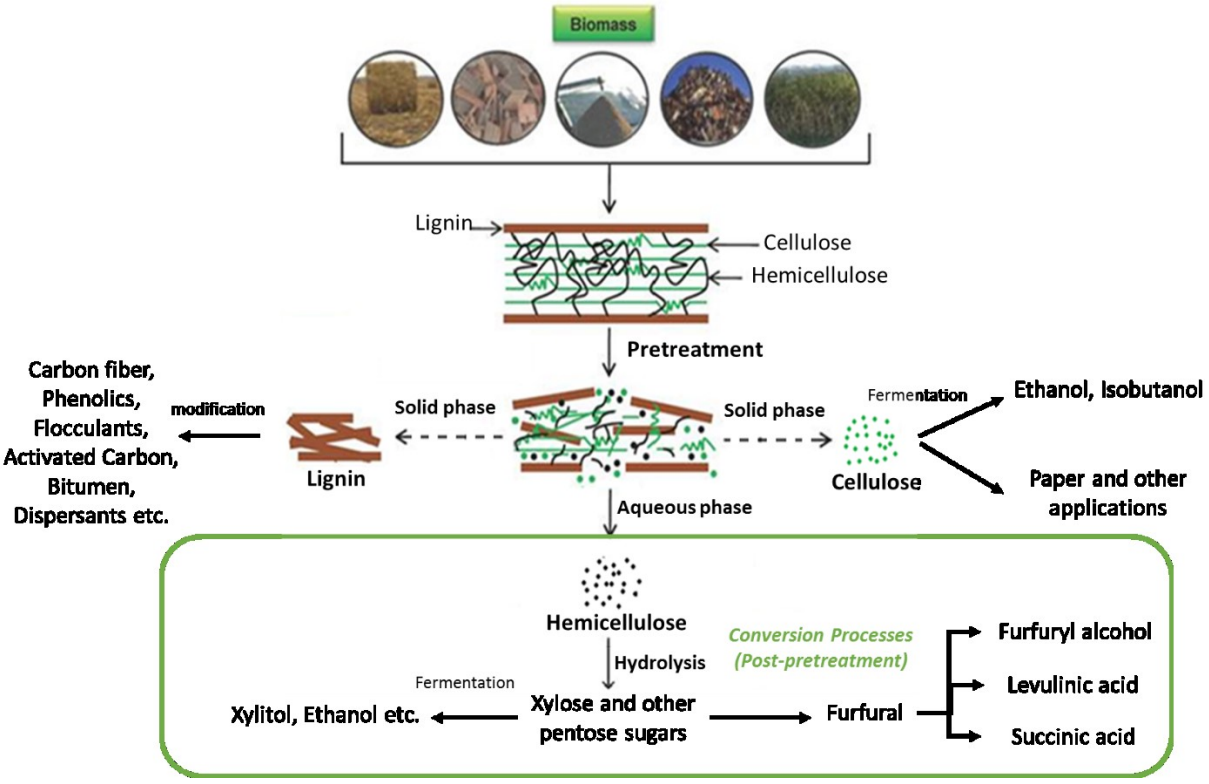

Figure 1.2. Schematic representation of the separation of components in the renewable biomass. Partially adopted from Dashtban et al. (2009) [21].

Depending upon the pretreatment method, the hemicellulose prehydrolysate contains various oligosaccharides, lignin degradation products (phenolic compounds), sugar degradation products (furan derivatives) and organic acids (formic acid and acetic acid). The applications of prehydrolysate are limited due to its complex composition of sugars. The sugars from the prehydrolysate mixture need to be separated to their pure form, before they can be used. Several processes in literature are discussed in subsequent chapters. These carbohydrate rich fractions, mainly containing xylan which needs to be further hydrolyzed (acidic or enzymatic process) to obtain monomeric sugars. These sugars have considerable potential to produce various useful products like platform chemicals, fuels and biologically active compounds [22]. Typically, with current technology, using pure xylose or the sugars from hemicellulose to produce ethanol will not be profitable. Instead, glucose, xylose or other sugars can be purified and marketed. Currently, pure glucose and xylose have a market price of $300/ton and $200/ton, respectively (Listed price on Alibaba website). The other preferred option is to convert the sugars present in pretreated fractions to produce other value added products.

According to the Department of Energy (DOE) US, the top twelve platform chemicals that have a high demand in the market and that have high potential to be produced from renewable resources include succinic acid, levulinic acid, fumaric acid, maleic acid, 2,5-furandicarboxylic acid (FDCA), hydroxypropanoic acid, glucaric acid, xylitol, glycerol, aspartic acid, itaconic acid, 3-hydroxybutyrolactone and glutamic acid (DOE, US 2004). The production of xylitol (by fermentation), levulinic acid and succinic acid (by chemical methods) is the focus of this study.

1.1 Rationale of the thesis

This thesis aims to demonstrate the value addition of hemicellulose, the low value byproduct streams of biomass based industries, by converting them into high value chemicals in efficient processes.

i) The upstream processes such as hydrolysis and detoxification of hemicellulose have significant effect on the fermentative production of xylitol. These processes divide hemicellulose into a xylose rich stream and a furan based stream (predominantly furfural). Current literature reports show several methods to hydrolyze and detoxify the hemicellulose, but the conditions are severe and there is a significant loss in sugars. Therefore, an efficient methodology for these processes were developed and optimized to make the hemicellulose hydrolysate more favorable for further usage – Reported in Chapter 3.

ii) Though the xylose rich hydrolysates are amenable to microbial growth, the metabolic rates are lower in xylose consumption compared to glucose consumption. Therefore, the fermentation methods are not so competent with traditional chemical conversion of xylose to xylitol due to their low yields. New methods have to be developed to make the xylose fermentation efficient and productive. Therefore, we have developed a fermentation method with immobilized yeast cells and identified a *Candida* yeast strain which was found to produce high yields and productivities of xylitol – Reported in Chapter 4.

iii) Being an important building block for various chemicals, fuels, biopolymers etc., succinic acid (SA) has come under remarkable research focus for production from renewable resources. In recent years, several reports have been published in the literature about the microbial production of SA using *Actinobacillus succinogenes* [23] *Mannheimia succiniciproducens* [24] and *Anaerobiospirillum succiniciproducens* [25] on substrates like hexose, pentoses and glycerol, respectively. Succinic acid production from maleic acid via chemical routes is followed

extensively in industries using heterogeneous metal catalysts like Pd/C and Zn/Hg, as high yields are obtained [26].

In Canada, BioAmber Inc. started to produce bio-succinic acid from corn starch. Nonetheless, using starch based resources can have significant impact on food costs and security. Hence, several researchers are exploring alternate routes for the production of SA from low value substrates to reduce the overall production costs involved, reduce use of fossil fuels and environment pollution. In this thesis, we have developed methods to convert the hemicellulose prehydrolysate to succinic acid without a metal catalyst. Furfural is a major intermediate in this process as well, but its production and separation is challenging. We have done extensive studies to determine a suitable solvent to separate out the furfural and avoid unwanted side reactions. A novel biphasic system was designed to produce succinic acid in acceptable yields. This demonstrates the high value addition to the hemicellulose by producing succinic acid in acceptable yields and productivity. So far, this is the first report to show the succinic acid production from hemicellulose. The effect of catalyst on the reaction process was also evaluated. It was reported in chapter 5.

iv) Levulinic acid is one of such important platform chemicals listed by DOE, US, as mentioned earlier. Current industrial production of levulinic acid involves the acid hydrolysis of glucose into 5-hydroxymethyl furfural (5-HMF) which will be dehydrated to afford levulinic acid. However, the conversion of pentose sugars into levulinic acid has been underexploited. The furfural obtained from acid hydrolysis of hemicellulose has high potential in producing various chemicals including levulinic acid. Furfural can be reduced to furfuryl alcohol which can be hydrolysed to levulinic acid. In recent years, this procedure has gained considerable research attention as few procedures are available in literature to convert furfuryl alcohol to levulinic acid. However, the total conversion process has not been explored sufficiently. It is necessary to understand the reaction nature and characteristics to initiate the process and obtain high yields of product. In this thesis, we have studied the reaction mechanism by separating and identifying the intermediates during the reaction. Also, the byproducts formed during the production of levulinic acid from furfuryl alcohol were characterized and identified. This work is reported in chapter 6.

The whole study was done using a hemicellulose hydrolysate of poplar wood which was obtained from a proprietary pretreatment process.

1.2 Objectives of the whole study

Based on the above rationale, the overall objective of this thesis is to produce the high value chemicals (Xylitol, succinic acid, levulinic acid,) from the low value renewable hemicellulose from lignocellulosic biomass.

The specific objectives were:

a) To develop a mild treatment method to depolymerize the oligo- and polysaccharides in hemicellulose streams to monosaccharides with low byproduct formation and subsequently separate the sugars and other byproducts in an effective way.

b) To determine optimum conditions for the enhanced xylitol production from the detoxified wood hydrolysate through microbial fermentation using free and immobilized yeast cells.

c) To develop and demonstrate an efficient method to produce succinic acid from the crude wood prehydrolysate using heterogeneous acid catalyst.

d) To perform mechanistic studies and identify byproducts during levulinic acid synthesis from hemicellulose derived furfuryl alcohol using homogeneous and heterogeneous acid catalysts.

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CHAPTER 2
LITERATURE REVIEW

The literature review has been published as a book chapter in the book entitled "*Lignocellulose: Biotechnology, chemical composition and future prospects*" by Nova Publishers.[1]

My Contribution: Responsible for all the literature survey and the draft write up.

[1] Dalli, S. S. & Rakshit, S. K. (2015). Utilization of hemicelluloses from lignocellulosic biomass-potential products. *In: Lignocellulose,* Pp. 85-113, Pittman, K. L. (ed.) Nova publishers Inc., New York.

2 Literature Review

2.1 Hemicellulose

The term hemicellulose was coined by Schulze [1] to represent heterogeneous β (1→3) and (1→4) linked polysaccharides (Figure 2.1) other than cellulose and pectin in plants. There are similarities in structure between the plant polymers, hemicellulose and pectin. Some reports suggest that β (1→3) linked glucose residues should not be considered as hemicellulose [2]. Hemicelluloses are biologically synthesized by glycosyltransferases located in Golgi membranes. But the synthesis of some of its contents such as xylans and glucans is not very clear and remains unknown [2]. Hemicelluloses interact with celluloses, and in some species with lignin and play a vital role in strengthening the plant cell wall. Due to its solubility in water, it can be easily separated during the early stages of pre-treatment processes.

Unlike cellulose, hemicellulose is a heterogeneous polysaccharide and it is difficult to separate as single monomeric sugar in its pure form. Hemicellulose comprise of xylans, mannans, arabinans, galactans, glucuronoxylans, arabinoxylans, glucomannans, xyloglucans. The composition of hemicellulose differs widely between different species of plants (Figure 2.1). Typically, it ranges from 20-30% of wood in a dry basis. The descending order of the amount of sugars in hardwood hemicellulose is xylose, mannose, glucose, galactose, arabinose, rhamnose. However, in softwood hemicellulose, mannose concentration would be higher than xylose.

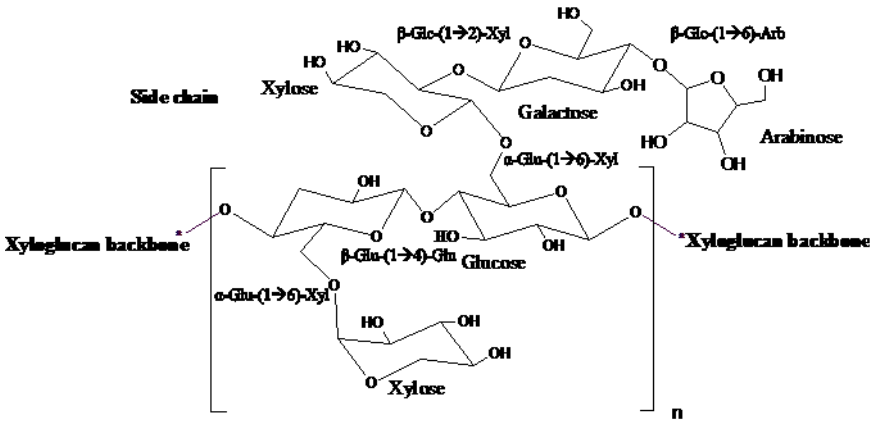

Figure 2.1. Schematic representation of the sugars linked to each other in hemicellulose.

According to literature, some of the components of the hemicellulose have significant function in plants, if extracted to pure form (Table 2.1). For example, arabinoxylan chiefly serves as reservoir

of large amounts of ferulic acid and other phenolic compounds in plants and protects them against pathogens. It has antioxidant properties when extracted from hemicellulose [3]. Glucomannan is typically used as a dietary fiber, food additives, emulsifier, thickener and weight loss supplement. It is sold as nutritional supplements for constipation [4], obesity, acne vulgaris [5] and type 2 diabetes [6].

Table 2.1. List of polymer components of hemicellulose and their composition in cell wall

Polymer components	**Composition of polymers**	**Linkages**	**Proportion in the cell wall (%w/w)**	**References**
Xylan	O-acetyl-4-O-methyl glucuronoxylan, and arabino-4-O-methyl glucuronoxylan	β (1→4)	10 - 35	[2]
Glucuronoxylan	Glucuronic acid with D-xylopyranosyl	β (1→2) β (1→4)	20 - 30	[7]
Glucurono-arabinoxylan	Glucuronic acid, Arabinose with Xylan	β (1→2) β (2→3) β (1→4)	10 – 25	[2, 8]
Glucomannan	Acetylated mannose and glucose	β (1→4)	2 - 5	[2]
Xyloglucan	Glucan with xylose side chains	β (1→4) α (1→6)	20-25	[9]

Hemicellulose structure and composition vary depending on its source such as hardwood, softwood and agricultural residues. Detailed composition of hemicelluloses present in softwood and hardwood are summarized in Table 2.2. Hemicelluloses are matrix polysaccharides which include xylan, glucuronoxylan, arabinoxylan, glucomannan, and xyloglucan. Unlike cellulose, these polysaccharides contain repetitive units of different sugar units such as xylose, glucose, galactose, mannose, rhamnose and arabinose in addition to glacto or glucounronic acid. Different hemicellulosic polysaccharides have different hydrolysis rates and may yield different amounts of monomeric and oligomeric sugars under the same process conditions. The proportions of the monomeric sugars present in the hemicelluloses are (a) softwoods: mannose > xylose > glucose > galactose > arabinose [10], (b) hardwoods: xylose > mannose > glucose > galactose > arabinose >

rhamnose [10]. In most of the hardwood hemicellulose, xylose is the major monomeric sugar among the total carbohydrates present in hemicellulose. Hemicellulose can be converted to several valued products by hydrolysis and subsequent conversion of its constituents. However, it has to be separated from cellulose, lignin and the other components of lignocellulose before it can be used further.

Table 2.2. Composition of hemicellulose in softwood and hardwood.

Type of wood	Type of hemicellulose	content (%) in wood	Composition			DP*
			Units	Linkage	Molar ratios	
Softwood	Galacto-glucomannan	5-8	β-D-Man*p*	1 → 4	3.0	100
			β-D-Glc*p*	1 → 4	1.0	
			α-D-Gal*p*	1 → 6	1.0	
			Acetyl		1.0	
	(Galacto)-glucomannan	10-15	β-D-Man*p*	1 → 4	4.0	100
			β-D-Glc*p*	1 → 4	1.0	
			α-D-Gal*p*	1 → 6	0.1	
			Acetyl		1.0	
	Arabino-glucuronoxylan	7-10	β-D-Xyl*p*	1 → 4	10.0	100
			4−O−Me−α-D−Glc*pA*	1 → 2	2.0	
			α-L-Araf	1 → 3	1.3	
Hardwood	Glucuronoxylan	15-30	β-D-Xyl*p*	1 → 4	10.0	200
			4−O−Me−α-D−Glc*pA*	1 → 2	1.0	
			Acetyl		7.0	
	Glucomannan	2-5	β-D-Man*p*	1 → 4	1-2	200
			β-D-Glc*p*	1 → 4	1.0	

Due to high quantities of xylose and mannose present in hemicellulose, current research is intensified towards the utilization of these sugars for the production of high value compounds. According to European Union report in 2007, the market volume of hemicellulose in the Europe alone was 848.9 million euros and a total of 153.5 million euros in U.S, China, Switzerland, Argentina and Japan [11]. According to these reports the potential use of hemicellulose could lead to high revenues.

In plants, polysaccharides (i.e., cellulose and hemicellulose) form a complex matrix with lignin and are thus protected from pathogens [12]. Due to the recalcitrance of lignin it is difficult to access

the polysaccharide sugars for chemical modifications or to treat with enzymes for the production of desired products. However, the yields of these products are based on purity of the raw material. Therefore, all sugars from lignocellulose ideally need to be separated in their pure form either as monosaccharides or oligosaccharides. This is achieved by a stepwise process in biorefineries or bio-based industries including pretreatment, detoxification, hydrolysis, fermentation and product purification. Pretreatment process break the complex matrix of sugars and lignin in lignocellulose and make them accessible for the conversion into various high value added chemicals [13].

2.2 Pretreatment techniques to extract hemicellulose

Pretreatment techniques can be broadly categorized as thermal, chemical or physical methods. Ideally, they have to be designed to separate the cell wall components such as cellulose, hemicellulose and lignin. The principle aim of such Successful pretreatment method should meet some requirements such as to break the lignin barrier to access the carbohydrates, improve the formation of monomeric sugars subsequently, avoid byproduct formation to reduce inhibition in further process and should be economical [14]. Some of the pretreatment methods are discussed below.

2.2.1 Hydrothermal Pretreatment

This is a type of physico-chemical pretreatment and is a widely used effective method for the extraction on hemicelluloses from biomass [15]. As the name indicates this process involves water or steam and heat with or without pressure. Though it is considered to be most effective with mineral acids such as sulfuric acid [16], the disposal of waste acid is regarded as harmful to the environment. Therefore, it is most widely used with water or steam under pressure which is considered as ecofriendly [17]. Temperature maintenance plays a prominent role in this process. Hydrolysis does not occur below 100°C [18], while pyrolysis occurs above 220°C [19]. Therefore, it is necessary to maintain the temperature within the range of 100-200ºC. In this process hemicellulose dissolves in aqueous phase and can be separated further but some portion of it forms weak acids such as furfural and hydroxymethyl furfural which acts as inhibitors in subsequent processes [17]. Lignin and some other insoluble material form solid residues which may affect the rate of hydrolysis [20].

a. **Autohydrolysis:** In this process, only water is used under high pressure (more than saturation point) and temperatures (200ºC). While no catalyst is involved in this process,

hydronium ions in water acts as a catalyst initially to separate the biomass components. Later hydrogen ions from acetic acid which are obtained from the depolymerization of hemicellulose, enhances the rate of reaction [21]. Though this process hydrolyses all the lignocellulosic biomass components, hemicellulose is the primary substance in the hydrolysate liquor with a separable solid residue of cellulose and lignin [22]. Due to the high pressure and temperatures, hemicellulose can be hydrolysed in the same reactor to form monosaccharides along with some byproducts such as furfural. This is an ecofriendly method [18] which works under mild pH conditions, reduces corrosion rate [23] and does not need neutralization of acids. Though this method is relatively cheap, the formation of byproducts such as furfural and hydroxymethyl furfural lead to difficulties in further processing.

b. **Steam explosion:** In this process, lignocellulosic biomass is heated in steam under high pressure of around 20-50 bar at high temperatures (210-290°C) for few minutes before pressure is quickly released through a nozzle causing an explosion of biomass [21]. The steam under pressure enters into the inter and intra molecular spaces in biomass and when the pressure is released, steam evaporates immediately resulting in the disintegration of biomass due to instantaneous pressure drop [24]. There are various proposals to describe the mechanism of steam explosion in the literature. One study considers it as shear and decompression effect [25], another suggests that it is a chemical mechanism [26]. Many reports say that it is a thermomechanochemical process because of the steam (thermo) involved under high pressure which creates shear forces (mechano) and result in breakage of glycosidic bonds (chemical) between monosaccharides [21, 26]. Hemicellulose can be easily separated as it dissolves in the hydrolysate liquor, whereas cellulose and lignin forms a precipitate.

c. **Steam extrusion:** Steam extrusion with a twin-screw extruder is an important physical pretreatment attempted. It is significant because of the advantages of low production cost and low generation of byproducts. Under high temperature (100 – 125^{o}C), wet lignocellulosic biomass is forced through the screw extruders which are the main elements of this process for the biomass degradation. The essential parameters to be controlled in this process are temperature, type of screw extruder (single or twin) and speed of screw rotation (25 -125 rpm) [27]. Greenfield ethanol Inc. had patented this technology of using

twin screw extruders which works at high pressures (20,000 psi) with inbuilt metal filters designed to capture solid particles of different sizes and separate them from the liquid hemicellulose prehydrolysate. This technique facilitates the separation of 90% hemicellulose stream out of lignocellulosic wood biomass [28].

2.2.2 **Wet Oxidation**

Biomass can be pretreated using water and air without any other additional catalysts. Pressurized air or oxygen at a temperature of 150 – 320 ^{o}C is typically used for wet (air) oxidation [29]. Organic compounds suspended in water get oxidized by oxygen and get depolymerized to monomeric components. However, the capital cost involved in pressurizing gas or air is also high. Therefore other oxidizers, like Na_2CO_3 have been used for the conversion of biomass to monosaccharides [21]. In addition, a huge amount of short chain carboxylic acids are released [29, 30]. These acids can act as inhibitors in further processes. Therefore, this process is unlikely to be employed in industries.

2.2.3 **Acid Hydrolysis**

Since the 19th century, sulfuric acid, phosphoric acid, hydrochloric acid, nitric acid and trifluoroacetic acid have been used for the acid catalyzed biomass hydrolysis. Temperature and acid concentration play a prominent role in this process [21]. Higher acid concentration is preferred because of its instantaneous degradation of biomass at lower temperatures. Though it hydrolyzes the biomass efficiently with less byproducts, it is not considered environmentally safe due to the disposal of strong acids as waste [31]. It is very expensive to recover or neutralize the concentrated acid after hydrolysis. Another demerit of using concentrate acids is corrosion of equipment [32]. Therefore, dilute acid (approximately 4%) is being used widely for various biomass feedstocks [33]. Dilute acid hydrolysis is performed under high temperatures (120-210^{o}C) to disintegrate the biomass and recovers approximately 85-95% hemicellulose in hydrolysate liquor. The use of dilute acid provides various benefits in contrast to using concentrated acid such as reduction of equipment corrosion and the disposal of dilute acids cause less harm to the environment [33]. Hemicellulose usually obtained in the aqueous solution has low level of byproducts in it and can then be used for subsequent fermentation process.

2.2.4 **Alkaline Pretreatments**

Unlike acid and hydrothermal processes, alkaline pretreatment is mainly used for its effective digestibility of cellulose and hemicellulose. Aqueous ammonia, sodium carbonate and hydroxides of sodium, potassium or calcium (lime) are mainly used in this process. Lime and NaOH are commonly used together at moderate temperature (150^{o}C) and takes less time for the process [34]. It is reported that the efficiency of this process can be increased substantially with the help of ultrasonication [35]. The phenolic compounds can be recovered and used for various applications [36]. Alkaline peroxide is another type of alkaline pretreatment where oxygen or hydrogen peroxide (1-3%) is added to biomass and catalyzed by lime or NaOH [21]. It gives better results in removing the lignin from the polysaccharides. One report suggest that alkaline peroxide pretreatment provides better delignification than acid hydrolysis but is less efficient than NaOH hydrolysis [37]. In addition to the above, other alkaline pretreatments including Ammonia Fiber Explosion (AFEX) and Ammonia Recycling Percolation (ARP) are also reported. In AFEX, liquid ammonia is added to biomass at a moderate temperature ranging from 40-140^{o}C and under high pressure (250-300 psi) and the reaction is carried out for shorter time. It is stated that approximately 100% theoretical yield of glucose could be achieved from most of the hardwoods rather than from softwoods [38, 39]. In ARP, aqueous ammonia of 10-15% is recycled in flow through mode into biomass using a column reactor. This process is more effective in hardwoods and corn stovers than in softwoods and provides high delignification and moderate hemicellulose solubilization of approximately 40-60% [21].

2.2.5 **Organosolv Pretreatment**

In this process, organic solvents (methanol, ethanol, ethylene glycol) with or without acid catalysts (HCl, H_2SO_4) are used to extract most of the hemicellulose from biomass along with lignin and cellulose in separate streams. If this pretreatment process is performed under high temperatures (185 – 210^{o}C), addition of acids is not necessary because deacetylation from the sugars make the medium acidic. However, it is reported that this process is more effective when the acids are added externally [40]. It is necessary to remove the solvents from the biomass after the pretreatment process because they inhibit the microbial growth in the fermentation process. The organic solvents used in this process can be recovered easily as most of them are volatile and can be separated by distillation. Unlike other pretreatment processes, lignin dissolves in the organic phase along with other mono and polysaccharides of hemicellulose, with cellulose as solid residue. As

lignin is mostly dissolved in the organic phase, it can be recovered as a fine precipitate by flashing the pulping liquor to atmospheric pressure, followed by rapid dilution with water. It is easy to recover hemicellulose due to its better solubility in the aqueous phase which is separated instantly from the organic phase. A high yield of xylose is an asset of this method [40]. However, the demerits of this process include the high usage of organic solvents and formation of clumps of lignin while washing the pretreated biomass with water. Therefore, recovery of sugars often becomes a cumbersome and costly process.

2.2.6 **Pulsed Electric Field Pretreatment**

Pulsed-electric-field (PEF) pretreatment involves application of a short burst of high voltage (5-20 kV/cm) to a sample placed between two electrodes [14]. PEF pretreatment can have serious effects on the structure of plant tissues. When high intensity electric potential is applied to its breaks the plant cell wall or ruptures the plant tissues. The electric field pulses are applied in the form of exponential-decay or square waves [31]. This creates permanent pores in the cell membrane and hence facilitate the entry of acids or enzymes used to break down the cellulose into its constituent sugars. This process is mainly used to help the enzymes (in biological treatments) or chemical reactants (in chemical modifications) to transport through the plant tissues and degrade the cellwall constituents. Higher electric potential helps in breaking the bonds between wood components and make them more available for further treatments. The significant benefits of PEF are low energy use (very short pulse time ~100μs), ambient conditions and no complex equipment required since no movement is involved [41]. Kumar et al. (2011) have performed a model experiment by using a neutral red dye in place of a catalyst and reported that usage of this pretreatment method made the plant cell to uptake the catalysts or reactants in the medium faster than the fresh plant cells [42]. Therefore, it has been proven that pulsed electric field pretreatment gives better results in separation of the plant biomass constituents into separate streams.

2.3 **Detoxification of hemicellulose hydrolyzate**

After pretreatment of lignocellulosic biomass, along with high amount of monomeric sugars, some byproducts such as phenolics, other aromatics, aliphatic acids, furan derived compounds and inorganic ions are produced [43]. These compounds act as microbial growth inhibitors in the fermentation process. According to Rehman et al. (2013), there are four ways to decrease the affect of inhibitors on fermentation process to obtain better yields of desired products [44]. They are (i)

efficient hydrolysis of biomass by reducing the byproduct formation; (ii) purification or detoxification of the hydrolysate using different techniques; (iii) usage of metabolically engineered microorganisms; and (iv) chemical conversion of byproducts into nontoxic compounds [44]. The cost of detoxification using different techniques is relatively lower than the other procedures.

For the past two decades, various detoxification methods have been reported in literature. Some of them are enzymatic detoxification [45], alkali treatment [46], solvent extraction [47], anion and cation exchange resins, activated charcoal [48], heating and evaporation [49]. According to various works in the literature, detoxification methods using charcoal and ion exchange resin are effective so far [50, 51]. In this section merits and demerits of all the detoxification procedures were discussed.

2.3.1 **Enzyme detoxification**

Enzymes such as Laccases and peroxides are typically used for the detoxification of hemicellulose hydrolysates [44]. These specific enzymes from white-rot fungus *Trametes versicolor* change the composition of the hydrolysate by modifying the acidic and phenolic compounds in it [52]. According to Mussatto et al. (2004), the mechanism of such detoxification might follow oxidative polymerization of phenolics. These enzymes can be used after extracting them from the organism or the microorganism also can be used directly. Zhang et al. (2013) reported the highest biological detoxification rates using a metabolically engineered *Enterobacter* sp. FDS8, which decreased the furfural and hydroxymethyl furfural at a rate of 0.54 g L^{-1} h^{-1} and 0.12 g L^{-1} h^{-1}, respectively. The total sugar loss was 5% and reported that the recyclability of whole cells is at least 5 times without losing the capability of detoxification [53]. Although the biological detoxification produces good results, it is not cost competitive in large scale compared to physical or chemical detoxification methods as the cost of production of enzymes is higher where as in using the whole cells, downstream processing involves high cost and labor.

2.3.2 **Alkaline treatment**

Treatment of hydrolysate with alkaline solutions were believed to improve the fermentability of sugars in the production of high value added products [46]. A well studied alkaline solution is $Ca(OH)_2$ in a method called overliming. There are several studies on overliming of the hydrolysate before fermentation in the literature [46, 54-56]. In this method, addition of calcium hydroxide increases the pH of the hydrolysate to 7.0 which is reduced to 5.5 by the addition of H_3PO_4 to

make the hydrolysate feasible for fermentation [54]. Significant research has been done on overliming, though the exact mechanism of it is not explained accurately [43]. One of the widely accepted explanations is the effect of overliming lies in the precipitation of toxins [57]. Later it was explored that the effect of detoxification was due to chemical conversion of toxic compounds rather only precipitation [58] based on sodium hydroxide treatment which did not resulted precipitation. Besides overliming, sodium hydroxide and ammonium hydroxide were also used together in the detoxification process which showed comparatively less degradation of sugars with better removal of toxins [46]. However, there is no significant differences were observed between calcium hydroxide, sodium hydroxide and ammonium hydroxide except the formation of precipitate was not observed in the later two alkaline solutions. Major drawback associated with the alkaline detoxification is that not only inhibitors affected by the alkali used, but also sugars could degrade and results in huge loss of sugars and subsequently reduces the yields of desired products. However, some of the efficient alkali treatments could reduce the loss of sugars as reported in the literature [43].

2.3.3 **Solvent extraction**

For the past few years, several organic solvents were evaluated for the better removal of toxins from the hydrolysate. However, some of the solvents such as ethyl acetate and acetone showed better results in detoxification with minute loss of sugars. According to Wilson et al. (1989), ethyl acetate extraction of inhibitors from the hydrolysate resulted93% increase in ethanol production by *P. stipitis* [59]. Due to the high solubility of phenolics produced during the pretreatment of hydrolysate in ethyl acetate, it has been chosen as an effective solvent for this method in several studies [60]. In a separate study, Mateo et al. (2013) have studied two different solvents other than ethyl acetate, chloroform and hexane in different proportions. Their study also suggests that ethyl acetate is a better solvent than others which was able to extract nearly 50% of the total phenolics and 57% of total furan based compounds [48]. This method presents little loss of sugars with moderate removal of inhibitors. An extended research is needed to explore the possibilities of removing inhibitors in high amounts.

2.3.4 **Ion exchange resins**

Anion exchange resins have been widely studied for their efficiency to remove most of the inhibitors from the hydrolysate. The reusability and recyclability is remarkable and hence

suggested for batch scale usage as the overall production cost would reduce [50]. In a study conducted by Mancilha et al. (2003) with sodium ion form of resins (ion-exchange A 103 S and A 860 S) showed 99% and 100% removal of furfural and acetic acid, respectively with 94 – 100% recovery of xylose [61]. The fermentation of the resulted hydrolysate showed high yield (0.41 g g^{-1}) of xylitol. Vithanage et al. (2015) studied the effect of Amberlite IRA 400 Chloride form ion-exchange resin which resulted in a significant increase (348%) in the fermentative production of xylitol by *C. guilliermondii.* In a separate study, Nilvebrant et al. (2001) compared three types of ion exchange resins i.e., anion, cation and no charge, and concluded that the efficiency of the resins are in order of anion < cation < no charge. The costs involved in ion exchange resins are the main drawbacks for their usage in industrial scale [50].

2.3.5 **Activated charcoal**

This detoxification method is one of the generally used techniques to purify the hydrolysate due to its less costs and labor involved [62]. Various factors such as pH, temperature, incubation time has significant effect on the efficiency of the treatment. According to Villarreal et al., (2005) most of the phenolic compounds are removed in this treatment at acidic pH whereas organic acids are removed at alkaline pH [50]. In a study conducted by Kamal et al., (2011), usage of 2.5% of activated charcoal for the detoxification of sago trunk cortex hydrolysate for 60 minutes yielded maximum xylitol concentration of 19.53 g L^{-1}. However, the efficacy of the detoxification using charcoal is relatively less than some of the other detoxification techniques like ion exchange [50]. Lack of recyclability of the activated charcoal is a major drawback for its usage in large scales.

2.3.6 **Evaporation**

Concentrating the hydrolysate through evaporation removes most of the volatile compounds leaving sugar moieties in the hydrolysate. However, due to the high boiling points of major inhibitors such as acetic acid, furfural and hydroxymethyl furfural (118^{o}C, 162^{o}C and 116^{o}C, respectively), it is not recommended to boil the hydrolysate to such higher temperatures as the sugar might caramelizes and results in loss of sugar. Therefore, evaporation of volatile compounds must be carried under vacuum to reduce the loss of sugars. The vacuum evaporation results in drastic removal of furfural from the hydrolysate whereas acetic acid volatility increases with the decrease in pH [63, 64]. The major drawbacks reported in the literature are the increase in

hydroxymethyl furfural during the vacuum evaporation and inability to remove the non-volatile toxic compounds at 70ºC.

Current research includes the development of new detoxification techniques to obtain 'toxins-free' hydrolysate along with combining two or more of the aforementioned techniques. We have demonstrated a novel detoxification technique developed in Chapter 3 [65].

2.4 Applications of hemicellulose

Though the hemicellulose extraction is technically feasible, the costs involved in its pretreatment, hydrolysis and detoxification has always been major bottlenecks to the process. The potential of using lignocellulosic biomass to produce ethanol would range between 313-390 L per ton of pretreated biomass [66]. With an actual market price of 0.38 USD per liter, the value of ethanol would be at a range of 150-187 USD per ton of biomass processed. The cost of producing cellulosic ethanol is high because of pretreatment and hydrolysis of cellulose. Besides, depending on location, the feedstock itself is expensive. There is a need to get added value out of the other components of lignocellulosic biomass, namely hemicellulose. This thesis provides extensive information about the structural, functional properties, and potential market value of hemicellulose and some products.

According to a report from the Department of Energy (DOE), US [67] cellulose from lignocellulosic biomass have been studied extensively for the production of several products. Even though hemicellulose has been a research focus for many years [68], it has got importance for the production of crude oil derived products. Since the xylose is a major component of hemicellulose, researchers focus their efforts on potential products from xylose. Some of the possible products from xylose studied in this thesis are discussed below.

2.4.1 Xylitol (*IUPAC name: (2R,3R,4S)-Pentane-1,2,3,4,5-pentol*)

Xylitol is an optically inactive sugar alcohol (C5 polyol) (Fig. 2.2). It is a naturally occurring product in many fruits and vegetables to levels of approximately 1 % of dry weight [69]. The optical inactivity enables the conversion between D and L configurations (For e.g. D-Xylose to L-Arabinose) [70]. The major beneficial property of xylitol is its lower calorific value (2.4 kcal/g) compared to sucrose (3.87 Kcal/g) though it has the same sweetening power [71]. Therefore, it is used as a sweetener in confectionaries and as a sugar replacement in diets for diabetic patients

because of its insulin independent metabolism [72]. In addition to its sweetening property, it is biologically active and well known as an anti-carcinogen and prevents tooth decay and ear infections in children. Due to these advantageous properties, it has high demand globally.

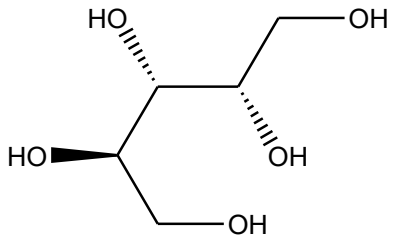

Figure 2.2. Structure of xyiltol.

Xylitol is produced by many industries worldwide. These include Xylitol Canada in North America, Danisco, a subsidiary of DuPont, Futaste Co. Ltd. in China etc. It is readily available in consumer markets under different brand names like Xyla, Xylitol Canada and Xivia, Danisco. In China and the US, the source of xylitol is glucose mainly obtained from corn cobs. Some companies like Xylitol Canada and Danisco use hardwood hemicelluloses and dissolved pulp respectively [73] to produce xylitol through chemical processes. Worldwide annual market for xylitol is estimated to be about $340 million with a good potential growth in the future [62].

Typically, xylose is catalytically hydrogenated in the presence of a metal catalyst (Ni) at 80-140°C temperature and 50 atm pressure to produce xylitol. Some recent reports show that hydrogenation can be carried out by using other catalysts like Ru [74, 75]. Though it produces moderate yields of xylitol, chemical processes have certain drawbacks such as need for initial purification of xylose often which can be expensive, high temperature and pressure conditions etc. Therefore, it is not economical to employ for the conversion of xylose in hemicelluloses to xylitol by the route.

Alternatively, microbial fermentation of xylose to produce xylitol has a number of potential benefits. For the last two decades, production of xylitol from various feedstock such as rice straw, sugarcane bagasse and hardwoods had been reported by many researchers [69, 70, 76]. Mild fermentation conditions such as low temperature, atmospheric pressure and moderate pH (5.0-7.0) are the advantages of such processes. Bacteria, fungi and yeast have been tested for xylitol production from xylose. However, they were reported as less efficient producers of xylitol than yeasts. *Candida spp.* are considered as the best producers [77]. Though the wild type strains give high yields (70-80%), recombinant strains reportedly produce better yields of xylitol (85-90%)

[78]. These recombinant strains are not used at industrial scale as yet. The major disadvantage of the fermentation process is the downstream processing as the product has to be separated from a number of metabolites.

Other researchers have explored the possibility of using enzymes for the conversion of xylose to xylitol. Though yields are higher (90-96%) in such enzyme catalyzed processes, it is not economically viable due to the high cost of enzymes [79]. Researchers are more focused on fermentation processes to make it more feasible than chemical process for the industrial production. The idea is to produce xylitol at high concentrations, so that downstream processing costs are reduced. This is one of the specific objectives of our study.

2.4.2 Furfural (*IUPAC name: Furan 2 carbaldehyde*)

Furfural (Fig. 2.3) is an important chemical used to synthesize various high value products like FDCA, furfuryl alcohol, gamma-valerolactone etc. At present, pentose sugars are converted to furfural using acid catalysts like sulfuric acid. China is the largest furfural producer with approximately 70% of the global production [80]. Westpro is one of the leading companies producing furfural using fixed bed reactors and continuous dynamic refining (David Tin Win 2005).

Major limitations in the furfural production from hemicellulose include low yields (~50%), unwanted side reactions like resinification, furfural polymerization resulting humins (unwanted byproducts) and decomposition [81]. Though, some of the methods like steam stripping are available to separate furfural from the aqueous mixture to avoid humins, high time and energy requirements make it difficult to be used at large scale [81]. Some procedures reported in literature indicate that supercritical fluids that can quickly separate the furfural and avoid unwanted side reactions [82].

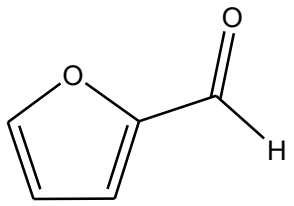

Figure 2.3. Structure of Furfural.

Besides, the use of strong mineral acid catalysts is also found to be a major limitation, as it causes corrosion and raises environment issues. According to Guenic et al. (2016), HCl, H_2SO_4, and H_3PO_4 are widely used in pretreatment and hydrolysis of the hemicellulose [83]. However, recent research advances in developing novel catalysts can overcome these limitations. Amberlyst, Nafion and some acid resins are showing promising results for the production of furfural. Another important limitation is the formation of humins which result from the reaction of xylose and furfural during the acid hydrolysis. Extensive research is being carried out to avoid the formation of humins and obtain the high yields of furfural [83]. Hence, an efficient method of acid hydrolysis has to be developed in order to avoid the formation of humins, to overcome the environment issues due to the use of mineral acids and to separate the furfural in downstream processing without decomposition. Like other byproducts, furfural has a very high potential to be converted into a number of useful chemicals such as gamma-valerolactone, furan dicarboxylic acid (FDCA), levulinic acid, succinic acid, etc. The second objective of this thesis was to produce furfural in high yields by overcoming the limitations and further convert it into levulinic acid and succinic acid. The specific limitations of the production of these products are given separately in their respective sections below.

2.4.3 Levulinic Acid (*IUPAC name: 4-Oxopentanoic acid*)

Levulinic acid also known as 4-oxopentanoic acid (Figure 2.4) is an organic compound which was initially obtained by heating sucrose in presence of acid (HCl). Usually acid hydrolysis of carbohydrates produce 5-HMF which is then hydrolysed to form an equimolar mixture of levulinic acid and formic acid [84]. It is a versatile compound which can be used in food, agriculture, pharmaceuticals and polymer industries. It is also used in the manufacture of polymers like nylon and plastics [85] and as an intermediate for some medical supplies [86]. Some valuable chemicals such as γ-valerolactone, ethyl levulinate and 2-methyltetrahydrofuran can also be synthesized from levulinic acid [87]. The market price of levulinic acid is approximately US$ 5-8 per kg with an estimated demand of 20,000 kilo tons by the year 2020. It is estimated that the annual market value of levulinic acid in agricultural pesticide industry and pharmaceuticals will grow by 6% and 5.4% respectively, in the period 2014-2020 [88].

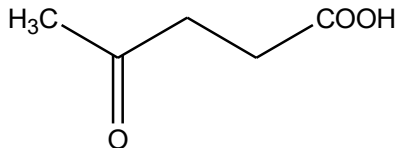

Figure 2.4. Structure of Levulinic acid.

Though various studies have suggested different methods to produce levulinic acid from hexose sugars, the yield is moderate. It had been reported that theoretical yield of levulinic acid is 64-72% depending on the type of sugars used (hexoses, sucrose etc.). The practical yield is two thirds of the theoretical yield [80]. Typically, strong acids such as hydrochloric acid, sulfuric acid and phosphoric acid are used in the acid hydrolysis of glucose to produce levulinic acid. Recently, Muranaka et al. (2014) have reported that usage of ionic liquids in the process would yield higher concentrations of levulinic acid [87]. Usually phosphoric acid and hydrochloric acids can convert 48.2 mol% and 59.1 mol% of cellulose to levulinic acid, respectively. When ionic liquids are used cellulose is dissolved in the aqueous phase and this enhances the conversion rate to 72.9 mol% [87].

There are a few reports available in the literature for the conversion of pentose sugars (xylose etc.) to levulinic acid. The direct conversion of xylose to levulinic acid using solid catalyst Amberlyst 70, hydrogenation catalyst Pd/Al_2O_3 in presence of acid and methanol as a medium has been reported [89]. Methanol reacts with xylose and forms methyl xylosides and prevents it from getting converted to xylitol. These methyl xylosides convert to furfural in presence of Amberlyst 70. Furfural is then hydrogenated by Pd/Al_2O_3 to furfuryl alcohol which produce levulinic acid over Amberlyst 70 [89]. However, the yields of LA were not significant despite the use of costly catalysts. The low yields of LA are due to the formation of other byproducts from various side reactions in the medium. In another report by Chamnankid et al., 2014, levulinic acid was successfully produced from xylose using alkaline treated zeolite Y in hot compressed water. The maximum yield observed was 30% [90]. Dumesic et al. (2013) have patented a conversion process of xylose along with glucose to levulinic acid by using γ-valerolactone as solvent. They have reported a range of experiments by varying the concentrations of acid, substrate, solvent and changing the duration of reaction time which yielded levulinic acid in a range of about 25-60% [91].

Levulinic acid can also be produced from pentose derived furan compounds like furfural, 5-hydroxymethyl furfural and furfuryl alcohol have been recently explored using various catalysts [89, 92]. However, very few of these methods are considered inexpensive and ecofriendly, besides being produced in very low yields of LA. For e.g., Xun Hu et al. reported the production of methyl levulinate (22.9%) and LA (3.5%) from furfural using 70 bar H_2 at 165 ^oC in methanol as solvent [93]. In another study conducted by Gorbanev et al. (2011), LA of 25% yield was achieved from 5-hydroxymethyl furfural (5-HMF) at 2.5 bar O_2 in presence of $Ru(OH)x/Al_2O_3$ catalyst [94]. According to Gonzalez M et al. (2012), conversion of furfuryl alcohol to alkyl levulinate can be obtained in acceptable yields using Amberlyst 15 as an acid catalyst in ethanol [95]. Developing novel catalysts and using appropriate solvents potentially avoid the formation of byproducts. For instance, Zhang et al. (2011), have reported 93 % yield of n-butyl levulinate from FA using a novel organic-inorganic hybrid solid acid catalyst, $[MIMBS]3PW_{12}O_{40}$ after 12 h of conversion reaction under nitrogen atmosphere [96].

Though the yields are moderate, more novel techniques and methods have to be developed to convert xylose (pentose carbohydrates) to levulinic acid in an attractive and cost effective manner. There is a need for more facile methods to produce levulinic acid in high yields [96]. The developed methods must be studied thoroughly to understand the reaction processes of the formation of levulinic acid from xylose or its derivatives. In this thesis, as one of the objectives, we have studied the reaction mechanism of one of the chemical processes to convert furfuryl alcohol, a xylose derivative into levulinic ester (ethyl levulinate).

2.4.4 Succinic Acid (*IUPAC name: Butanedioic acid*)

Succinic acid (Fig. 2.5) is one of the top 12 value added building block chemicals listed by the U.S. Department of Energy [67]. Its chemical structure is similar to maleic acid except that it has an unsaturated (double) bond (Figure 2.5) and is therefore considered as a potential replacement to the latter. Succinic acid has various industrial applications in pharmaceutical, food and other industries. It can be used as plasticizer for polymers, alkyl resins, coatings, deodorant, antidote, flavouring agent, for protein gelatination, as an adhesive etc. [97].

Currently, succinic acid is commercially produced through petrochemical routes by catalytic hydrogenation of maleic anhydride using catalysts such as Pd/C and Zn/Hg. According to Weastra market research, the market volume of succinic acid in 2011 was approximately 40,000 MT out of

which approximately 95-97% is produced through petrochemical route with a pricing of US$ 2,400/MT to 2,600/MT [98]. Biobased succinic acid production was estimated to have 1,150 MT market volume with a market price of approximately US$ 2,860/MT to 3,000/MT. Some of the companies producing succinic acid through petrochemical routes are DSM, Gadiv Petrochemical Industries, Mitsubishi Chemicals, Kawasaki Kasel Company, Nippon Shokubai, Anquing Hexing chemicals, Lixing Chemicals, Anhui Sunsing Chemicals. Some of biobased succinic acid producing companies are BioAmber (350 MT volume) U.S.A, Succinity (500 MT) Germany, Riverdia (300 MT) Netherlands [98]. The bio-based succinic acid is produced from starch based raw material. Bio-Amber, for example, is producing succinic acid from corn starch [99].

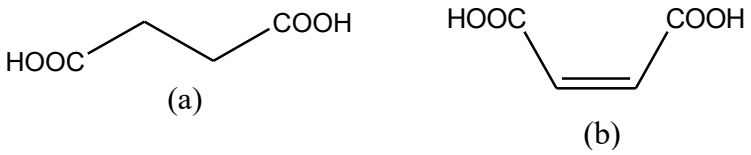

Figure 2.5. Structures of (a) succinic acid and (b) Maleic acid.

The main aim of some researchers and companies developing methods to use sugars like glucose, xylose etc. in the biomass instead of petrochemicals, is to reduce the usage of fossil fuels and make the commodities economical and sustainable. In order to make biosuccinic acid commercially acceptable, the yield has to 100% (w/w) with a production rate of 3 g/L/h and titers up to 250 g/L [100]. The DOE, US, has recently suggested that the production cost for biosuccinic acid through bioprocess should be US$ 0.48/kg [101]. At the moment, due to the high costs in biosuccinic acid production, it is not yet competitive to the petrochemical process [102].

Biosuccinic acid can be produced through fermentation of renewable biomass, mainly glucose based. Most widely used strains are *Anaerobacillus siccinogens, Anaerobiospirillum succiniproducens, Mannheimia succiniproducense* and *Escherichia coli* [101]. Anaerobic bacterium, *Anaerobiospirillum succiniciproducens*, has been considered one of the best succinic acid producers from glucose [103]. Fermentation of wheat produced 27 g/L SA with the byproducts, acetic acid and formic acid [104]. Few microorganisms, such as *B. succiniproducens* DD1 or *A. succinogens* are able to produce SA from glycerol [105]. Even though high yields were reported (1.02 g SA/g glycerol), several obstacles have to be surpassed to turn the process economically viable, in particular low production rates (0.094 g SA/L h) [106]. As previous reports

suggest, these microbes when cultured anaerobically in a medium containing 6.5 g/L of glycerol, produces as high as 133% of succinic acid [107].

Cellulose in lignocellulosic biomass was found to be a potential substrate for the production biosuccinic acid. However, these microbes cannot consume the polymeric form of glucose, cellulose. Therefore, it has to be degraded to glucose either chemically or enzymatically before subjecting for fermentation. Due to low yields of chemical process, enzymatic hydrolysis of cellulose gained attention. However, the costs of such process hamper the production of succinic acid at large scale. Therefore, one of the alternatives to reduce the costs is to combine the hemicellulose stream containing pentose sugars with the cellulose and make it amenable for succinic acid production. The fermentation of such raw material is receiving increased attention as it is renewable and reduce carbon footprint compared to chemical processes [102]. However, very few microorganisms have the ability to consume pentose sugars and produce the desired products. Therefore, it is necessary to develop efficient methods to convert pentose sugars into succinic acid either chemical or biologically.

As discussed earlier, xylose in hemicellulose can be converted to furfural which can be subsequently oxidized to succinic acid. Recently, Choudary et al. (2015) have reported a successful procedure for producing SA from furfural and hydroxylmethyl furfural (HMF) using hydrogen peroxide and a heterogeneous catalyst, Amberlyst 15 in aqueous medium [108]. However, the furfural and HMF used was the commercial grade and performed in a minute bench scale (3 mL reactor). This method must be optimized and developed for higher scales and to be make it plausible to convert hemicellulose directly into succinic acid. Very few reports are available in the literature for the production of succinic acid from renewable resources. Therefore, an intense research is needed to convert the renewable resources like hemicellulose to succinic acid using ‘green’ solvents and sustainable catalysts via ecofriendly routes. Therefore, as one of the objectives in this study, we have developed a method to produce succinic acid from hemicellulose using renewable acid catalyst.

2.4.5 Other products

Several other platform chemicals like furmaric acid, furan-2,5-dicarboxylic acid (FDCA), 3,3-hydroxypropionic acid, glutamic acid, itaconic acid and glycolic acid were also listed in the potential chemicals from hemicellulose. Conventionally, all the above chemicals are produced

from fossil based resources and recently, starch based glucose [109-112]. However, using lignocellulosic hemicellulose for these products has been gaining attention because of its potential to be converted easily to the above-mentioned chemicals. It has many advantages such as renewability, greenhouse gas mitigation and its ecofriendly nature. However, utilization of hemicellulose is challenging due to the limitations involved with the current methodologies.

2.5 Challenges with hemicellulose utilization

There are some challenges that have to be overcome before we can fully utilize the C5 sugars in lignocelluloses. These include efficient separation, detoxification (removal of inhibitors or byproducts), isolation of sugars and product recovery processes. Though various technologies are being developed to overcome these limitations, it has not been commercialized to industrial scale as yet. Interactive research has to be done for the conversion of hemicelluloses to produce high value added products in order to meet the market demand at acceptable costs.

Unlike cellulose, hemicellulose has a complex chemical structure wherein different types of sugars (xylose, glucose and mannose) are linked each other in a branched format to form different types of hemicellulose oligomers (xylan, glucuronoxylan, arabinoxylan, glucomannan, xyloglucan). Therefore, it is a challenging task to produce a specific sugar monomers (like xylose, arabinose etc.) from hemicellulose, partly due to its chemical and physical properties [113].

Hemicellulose is soluble in hot water, unlike the other major components of lignocellulosic biomass, cellulose and lignin. It can thus be separated relatively easily, but in pure form which remains a challenging task which lacks efficient technology. The separated hemicellulose fraction can be used as fermentation substrates to produce bioethanol or other value added products. This requires the hemicellulose to be depolymerized to monosaccharides by pretreating with acid or alkali. Though there are many pretreatment techniques developed for the separation of hemicellulose, none has been implemented at larger scale for the production of pure hemicellulose. Developing an efficient and economical pretreatment method, remains a challenge particularly in scaled up processes.

The most widely used method is dilute acid treatment, which produces many by-products which in turn act as microbial growth inhibitors. Additionally, equipment corrosion and environment hazard are the key limitations to such process if acid is used for hydrolysis. Carmen et al. (2012), have reported the usage of ionic liquids to separate hemicellulose selectively and quantitatively

[114]. However, it is costlier and limited to lab scale separation. Besides, the complex composition of the sugars present in hemicellulose yields a mixture of sugars with variable concentrations depending on the biomass [115]. Improving the yields of xylose from hemicellulose with low levels of byproducts is the key to the development of acceptable processes [116].

Hemicellulosic hydrolysate sugars can be converted to platform chemicals using enzymatic, chemical or microbial processes. The products of these processes include biopolymers, biofilms which are used in food and dietary industries. Some hemicellulose fractions can even have the potential to be pharmacologically active [117]. There are several naturally existing microorganisms that can use pentose sugars as carbon source in the production of enzyme and value added products, for example *Pichia stipitis, Candida shehatae,* and *Pachysolen tannophilus* etc. However, the conversion rate of pentose sugar into platform chemicals is relatively poor when compared to hexose sugars because xylose metabolism rates are lower in the microorganisms.

Bacteria, fungi, and yeast have similar xylose metabolic pathways with significant differences in transport, regulation, cofactor and the products of pyruvate fermentation [118]. Microorganisms like yeasts can metabolize glucose by following glycolysis pathway and produces pyruvate which gets degraded to acetaldehyde and CO_2 by pyruvate decarboxylase. A notable step in yeast fermentation is its ability to convert acetaldehyde to ethanol is remarkably different from other fungi and bacteria. Yeasts cannot metabolize xylose or arabinose due to lack of enzymes like xylose dehydrogenase or arabinose dehydrogenase [119]. Therefore, many companies and academic researchers are focused on developing genetically modified organisms that can ferment pentose sugars into either alcohol or product of interest. For example, some of the studied species include *Saccharomyces cerevisiae, Escherichia coli, Zymomonas mobilis* etc.

An example for the use of genetically modified yeast strains on hemicellulose is the production of ethanol from xylose using *Saccharomyces cerevisiae*. Yeast can be modified to ferment xylose to ethanol by inducing the pentose phosphate pathway enzymes and creating favourable conditions to grow. Kuyper et al. (2005) were observed that ethanol yield is higher with xylulose as carbon source than xylose, therefore by converting xylose into xylulose by using xylose isomerase or xylose isomerase producing microorganism, higher yields of ethanol can be obtained. Kuyper et. al (2005) were able to prepare a yeast strain by over expressing the genes coded for the xylose

isomerase enzyme for the production of xylulose and achieved 0.42 g of ethanol/ g of xylose by optimizing the conditions [120].

There are several other factors that limits the utilization of xylose present in the prehydrolyzate, such as inhibitors in the prehydrolysate which hinder the production of desired products by fermentation. The major inhibitors are sugar degradation products, furfural, 5-HMF and phenolics. These inhibitors are cytotoxic and affect the growth of microorganisms [121]. Besides, substrate (feed) at higher concentration inhibits enzyme activity. For example, in conversion of xylose to xylitol, the higher substrate concentration inhibits the enzymatic conversion to xylitol. Therefore, in this case, development of optimum fed batch technology is carried out allowing the possibility of keeping xylose levels below inhibitory levels in order to achieve high yield of xylitol [122].

The byproducts (furfural, furfuryl alcohol, hydroxymethyl furfural) obtained during the pretreatment and hydrolysis can also be used to convert into various chemicals such as levulinic acid, succinic acid, 2-methyltetrahydrofuran etc. However, with the existing technology, chemical conversion of the hemicellulose byproducts is not found to be feasible and efficient to produce high value chemicals. Therefore, these byproducts, if produced in large quantities, can be converted into various products using different type of chemical catalysts. For example, furfural can be converted into succinic acid, methyl tetrahydrofuran, FDCA etc. However, the chemical conversion processes also have various limitations such as substrate purity, unwanted side reactions, costs of catalysts, lack of suitable solvent and environmental effects. Such challenges involved with the production of succinic acid and levulinic acid, the focus of this study, are discussed in the separate sections of each of these products above.

Therefore, extensive research is required to overcome all these limitations to make use of hemicellulose from lignocellulosic biomass. Hemicellulose streams are generated during the pretreatment of any lignocellulosic residue and needs to be utilized in parallel processes along with the utilization of the cellulosic and lignin fractions. Efficient technologies and methods have to be developed at large scales within the context of such integrated biorefining processes, to make it economically feasible and add to the overall profit of biomass industries.

2.6 References

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CHAPTER 3

OBJECTIVE 1

This work is has been re-submitted with minor revisions for publication to the journal entitled "Biomass and Bioenergy, Elsevier Press".

My Contribution

All the experimental design, data interpretation and most of the write up.

Co-author's contribution

Misha Patel – Helped in some experimental work and the collection of some data.

3 Development of acid hydrolysis and detoxification methods to produce toxin-free hemicellulose hydrolysates to improve microbial production of xylitol

3.1 Abstract

A major bottleneck to the utilization of hemicellulose streams obtained on pretreatment of lignocellulosic residues is the toxins formed during the hydrolysis of the pentose polymers. Low acid (1.75 % (w/w) and 120 ºC) hydrolysis yielded a 3-fold increase in xylose concentration with low byproduct formation. Efficient detoxification method using vacuum evaporation and solvent extraction techniques removed the major inhibitors, acetic acid (80 %) and furfural (98 %) with little loss of sugars. The effectiveness of hydrolysis and subsequent detoxification was ascertained by the fermentation of detoxified hydrolysate with *Candida guilliermondii.* The xylitol concentration and yield obtained was 28.78 g L^{-1} and 0.59 g g^{-1} respectively, in 37 h. The productivity of 0.81 g L^{-1} h^{-1}, the highest reported from wood prehydrolysates, demonstrates that the upstream processing methods are effective. Wood prehydrolysates treated in this way could be used to produce fermentative products like xylitol and other value added products economically and beneficially.

Keywords

Hemicellulose prehydrolysate; acid hydrolysis; detoxification; fermentation; xylitol; *Candida guilliermondii*.

3.2 Introduction

The use of wood based resources for the production of useful chemicals instead of fossil based resources will help mitigate climate change. Lignocellulosic biomass has the potential to serve as a renewable feedstock to produce value-added chemicals and precursor platform chemicals. The utilization of cellulose in various industries like pulp and paper, textiles (rayon, cellophane), pharmaceuticals (fillers in drugs), fuel (ethanol, butanol), and many others have been studied extensively [1]. However, in order to make biorefining of lignocellulosics economical, the other components of wood such as hemicelluloses and lignin need to be integrated and utilized to produce value added products. We have recently presented the importance of such integration in biorefining processes, in order to compete successfully with the fossil fuel industry [2].

There is considerable potential for use of the hemicellulose stream which contains a variety of pentose sugars to produce various useful products like platform chemicals, fuels and biologically active compounds [3]. Hemicelluloses from corn cob, sugarcane bagasse, wheat straw, switch grass, spent grains and barley have been used as carbon sources to produce some products like xylitol, a non-fermentable sugar alcohol, through microbial fermentation [4]. Since xylose is the major monomeric sugar in hemicellulose, it is relatively easy to produce xylitol, which has good commercial value with an estimated annual market of US $537 million, from this sugar [5].

Current industrial production of xylitol involves catalytic hydrogenation of xylose at high temperatures (80-140^{o}C) and pressure (50 atm). Some examples of catalysts used in this process are Nickel (Ni) and Ruthenium (Ru) [6, 7]. The major drawback of this process is in upstream processing, as the substrate (xylose) needs to be in pure form requiring detoxification and removal of impurities along with other sugars. This purification process of xylose increases the cost of production of xylitol [4]. Microbial fermentation of xylose to produce xylitol does not need such high substrate purity and thus gained attention for its economical and ecofriendly nature. Therefore, the hemicellulose prehydrolysates of different types of biomass can be used for the production of xylitol using various microbes in the fermentation process. Though many researchers report the use of various microbes that produce xylitol [8, 9], the microbial route has not been commercialized yet. This is because these microbial methods are not able to compete with the current industrial production of xylitol through chemical processes in the production and commercial aspects. As the use of such renewable resources have many advantages, considerable efforts on producing xylitol from hemicelluloses by biological routes is being carried out, in order to develop an efficient method that can be commercialized.

Renewable resources such as poplar wood prehydrolysate, byproduct stream rich in hemicellulosic sugars mainly obtained from the pretreatment process of pulp and paper industries, can also be used to produce xylitol. There are many pretreatment techniques reported in literature about the production of hemicellulose prehydrolysates from various renewable lignocellulosic resources [10]. Xylose sugar present in all these prehydrolysate streams exist as poly and oligo-saccharides, and need to be hydrolysed to release the xylose monomers. Sulfuric acid is commonly used catalyst in acid hydrolysis of prehydrolysate. However, the usage of high acid concentrations is not environmentally acceptable and also produce large amounts of inhibitory by-products. In the first part of this study, an efficient acid hydrolysis method was optimized to produce high

concentration of fermentable monomers using low acid concentration. During acid hydrolysis, the formation of some inhibitors like acetic acid, furfural and other phenolics is unavoidable. These substances limit microbial growth and production of valuable metabolites [10]. Attempts have been made to remove these inhibitors through detoxification methods such as ion exchange chromatography [11], solvent extraction, evaporation [12], membrane distillation [13] and adsorption techniques [14]. In the second part of this study, a detoxification method using vacuum evaporation and solvent extraction was attempted to reduce the concentration of inhibitors to the levels that did not result in inhibition of the fermentation by microorganisms. The effectiveness of the hydrolysis and detoxification process was demonstrated by the enhanced production of xylitol by fermentation using *Candida guilliermondii* and the detoxified hydrolysate as substrate. This paper presents an efficient process to utilize the poplar hemicellulose prehydrolysate stream to produce xylitol in high yields and productivity. The effectiveness of the low acid hydrolysis and subsequent efficient detoxification on xylitol production indicates that these monomeric sugar streams could be used for other fermentation products as well.

3.3 Materials and methods

3.3.1 Prehydrolysate substrate

3.3.1.1 Composition analysis

Hemicellulose prehydrolysate liquor (PHL) of poplar wood was provided by Green Field Ethanol Inc. (Chatham, ON, Canada). The crude liquid PHL was obtained using a two stage pretreatment process including a steam percolation system [15]. The poplar wood chips were passed through twin extruders in presence of steam and the three major components (cellulose, hemicellulose, lignin) of lignocellulose were separated in two different streams viz. solid (cellulose and lignin) and aqueous streams (hemicelluloses). The latter crude PHL stream was used as starting material in our experiments.

3.3.1.2 Determination of total solids

The total dissolved and undissolved solids were determined by following the standard NREL procedure recommended by the Department of Energy (US) using a conventional oven method [16]. 1 mL of PHL was weighed in a pre-heated aluminum dish and the sample was oven dried for 6 hours at 105 ^{o}C. The weight of the sample was then recorded and the total solid content was determined by using the formula given in TAPPI standards.

$$\%\ Total\ Solids = \frac{weight\ of\ dry\ pan\ plus\ dry\ sample - weight\ of\ dry\ pan}{weight\ of\ liquid\ sample} x100$$

For the determination of total dissolved solids, another sample of PHL was centrifuged at 4400 rpm for 30 minutes and then filtered through a 0.2 µm filter (Millex® filter unit) to remove the minute solid particles present in the PHL and then the supernatant was dried overnight in oven at 105 ºC. The filtered PHL was stored at 4 ºC for further experiments.

3.3.2 Acid hydrolysis

Crude PHL (150 mL) was hydrolysed with various concentrations of sulfuric acid ranging from 1.0 – 2.5 % (w/w) at 120 ºC for 2 h to determine the lowest possible concentration for higher polysaccharide conversion and simultaneous low inhibitor formation. The hydrolysate was then centrifuged and vacuum filtered to remove the traces of black solid particles called 'humins' formed due to the reaction between furfural and xylose at high temperatures [17]. The resulted hydrolysate was then neutralized with 3N NaOH, before being used for further experiments.

3.3.3 Detoxification of hydrolysate

Acid hydrolysis of PHL results in rise of monomeric xylose amounts along with organic acids (e.g. acetic acid), furfural and phenolic compounds. It is necessary to remove these byproducts which act as inhibitors in the microbial fermentation of the hydrolysate. In this study, the detoxification methods of ion exchange resin technique and a method involving vacuum evaporation and solvent extraction which was developed in our laboratory were compared.

3.3.3.1 Ion Exchange resin based detoxification

In this study, Amberlite IRA 400 (Cl^-) resin was initially used to remove inhibitors. In order to regenerate the chloride ions on the resin, it was treated with 1M HCl and left soaked in it overnight [18]. A column (50 x 3 cm) was packed with the HCl treated resin and washed with water to remove free chloride ions unassociated with the resin. The hydrolysate was then added to the column and allowed to react for 30 minutes. The collected samples were subsequently analyzed for sugars, acetic acid and furfural using an HPLC. The column was washed with water and the resin was regenerated with 1M HCl and stored for further use.

3.3.3.2 A unified detoxification technique with vacuum evaporation and solvent extraction

Vacuum evaporation using a Buchi rotovap has been done to remove acetic acid and furfural from the hydrolysate at lower temperature. The hydrolysate (150 mL) was heated to 65 ^{o}C as it was found to be an optimum temperature beyond which humins formation was observed. The vapor pressure of acetic acid and furfural at 65 ^{o}C are 175 mbar and 50 mbar, respectively [19]. Therefore, the vacuum was created in the system at 65 ^{o}C using Buchi rotovap and the pressure was reduced slowly from 175 mbar to 50 mbar until the inhibitors were removed and a brown residue was obtained in the flask. The residue contains xylose with minor amounts of some phenolic compound. This process took 2.5 – 3 h depending on the mixture to remove the aqueous phase from the round bottomed flask. The removal of solvent from the hydrolysate resulted in a dark brown sugar residue in the flask. In order to remove trace organic compounds like phenolics present in the residue which are non-volatile at these conditions, solvent extraction was carried out. Various organic solvents such as toluene, hexane, dichloromethane, acetone, ethanol, methanol and ethylacetate were tested for the relative solubility of phenolic compounds and the sugars. A 125 mL separating funnel was used to extract sugar from non-polar solvents. For the polar solvents, the solubility of the known amount of xylose was evaluated using HPLC. The resulted detoxified hydrolysate was then diluted with a known amount of distilled water to obtain the required concentration of sugars at which fermentation experiments were subsequently carried out.

3.3.3.3 Inoculum preparation and fermentation experiments

Candida guilliermondii FTI 20037 strain was selected for the fermentation experiments as it is known to be one of the best xylitol producers among yeasts [9, 20, 21]. It was grown and maintained in a medium containing 10 g L^{-1} yeast extract, 20 g L^{-1} peptone and 20 g L^{-1} xylose (YEPX media). Pure xylose medium, crude PHL and the hydrolysate obtained from acid hydrolysis before and after detoxification were used as carbon sources for the yeast in separate shake flask fermentation experiments.

In an initial set of fermentation experiments with pure xylose medium, the concentration was varied in the range of 25 g L^{-1} to 100 g L^{-1} in order to determine yields, productivities, effects of substrate (xylose) inhibition on the microbial growth and consumption rates of xylose by microbes. In the second set of experiments, fermentation of crude prehydrolysate liquor was done. In order to enhance the availability of xylose in the PHL liquor, it was subjected to acid hydrolysis

and used directly in fermentation broths without detoxification, with xylose concentrations of 25 g L^{-1}, 50 g L^{-1} and 75 g L^{-1} in a third set of experiments. In a fourth set of experiments the hydrolysate detoxified by two different methods were also subjected to fermentation. The fermentation broths of hydrolysate detoxified by the ion exchange resin contained 25 g L^{-1}, 50 g L^{-1}, and 75 g L^{-1} of xylose, while the fermentation broths of hydrolysate detoxified by evaporation contained 25 g L^{-1} and 50 g L^{-1} of xylose. These were done for the comparison of the various factors such as substrate inhibition, and the effects of toxic compounds in the hydrolysate. Finally, the yields and productivities were calculated and based on the high yields, all the fermentation experiments were compared.

All the required materials were sterilized separately in an autoclave at 121 ºC and 15 psi for 15 minutes before use. The fermentation media were prepared to have a composition of 80 % (v/v) of a carbon source in the form of suitably diluted hydrolysates or xylose solutions, 8 % (v/v) of YEP media, and for the better growth of microbes 2 % (v/v) of 50X Vogel's Minimal Salt (VMS) media was supplemented. Higher concentration of nutrients would reduce the xylitol production [22] and hence these nutrients are supplemented in limited quantities.

Candida guilliermondii FTI 20037 inoculum grown in yeast extract peptone xylose (YEPX) media for 12 hours and containing approximately 1 x 10^7 cells mL^{-1} was added to each fermentation broth to a level of 10 % (v/v), which was found to. The fermentation was conducted in shaken and static conditions and was found that static conditions do not show any detectable xylitol in the media even after 36 hours. Therefore, all experiments were performed in a shaking incubator. All batches were adjusted to a pH of 5.5 and maintained at 32 ºC in an incubator shaker with an agitation speed of 150 rpm for 120 hours. All experiments were performed in duplicates. 1 mL samples were collected at various time intervals for the analysis of xylose, xylitol and acetic acid. Each sample was centrifuged at 8000 rpm at 10 ºC for 15 minutes to separate the biomass, and then the supernatant liquid was filtered through 0.2 µm filters (Millex® filter unit) to remove any trace particles. The samples were then analyzed using an HPLC. The residual biomass was washed twice with water to remove any traces of sugar and other compounds and dried overnight in a preheated oven at 105 ºC to determine the biomass dry weight.

3.4 Analytical methods

The filtered samples were analyzed using an HPLC (Agilent Technologies 1260 Infinity) for sugar, organic acids and furfural concentrations. Aminex HPX-87H ion exclusion column (300mm

x 7.8mm) along with a Refractive Index Detector (RID) at 50 ºC was used for sugar and organic acid analysis with a mobile phase of 5 mM H_2SO_4 at 0.5 mL min^{-1} flow rate. Agilent Poroshell 120 EC-C18 column (4.6 x 50 mm) along with a Variable Wavelength Detector (VWD) was used to detect furfural at 280 nm with a newly developed gradient method for the analyses of furfural and phenolics. The column temperature was maintained at 60 ºC and, a mixture of 1 % acetic acid and methanol was used as a mobile phase at a flow rate of 0.5 mL min^{-1}.

3.5 Results and discussion

3.5.1 Characterization of the hemicellulose prehydrolysate substrate (PHL)

The pretreatment of lignocellulosic residues leads to a hemicellulose stream which can be potentially be used for the production of a number of value added products. GreenField Ethanol Inc. provided the poplar hemicellulose prehydrolysate liquor (PHL) which was obtained from a novel two stage steam percolation pretreatment process [15]. The concentrated PHL supplied was stored in a freezer at -20 ºC for future use. The composition of the PHL was analysed using HPLC to determine sugar and other components quantitatively (Table 3.1). The PHL contained some amount of acetic acid which was formed due to the high temperatures used during the pretreatment process [23]. The pH of the crude PHL used was found to be 3.56, while the density was determined to be 1.03 g mL^{-1}. It was found that the total solid content of the prehydrolysate liquor was 15.31 % (w/w), out of which 15.04 % (w/w) is made up of only dissolved solids.

Table 3.1. Composition of the hemicellulose prehydrolysate used in this study

Component	Concentration (g/L)
Xylo-oligosaccharides	52.30
Xylose	31.97
Glucose	2.11
Arabinose	3.18
Acetic acid	2.37
Hydroxy methylfurfural	1.02
Furfural	0.35

3.5.2 Acid hydrolysis and detoxification of hydrolysate

The polymers in the hemicellulose stream have to be hydrolysed to sugars that can be converted to other products using fermentative and chemical routes. PHL was hydrolysed using sulfuric acid as a catalyst to degrade xylo-oligosaccharides to xylose monomers. During this process, by-products like acetic acid and furfural were also formed in significant quantities. Hence, the acid concentration, temperature and time were varied in order to get optimum conditions that produced high levels of xylose with low amounts of inhibitory by-products. Though the optimum conditions show better results, the amount of inhibitors formed are adequate to inhibit the microbial growth in case of fermentation. Therefore, a unified method of detoxification which includes both vacuum evaporation and solvent extraction together was developed to detoxify the hydrolysate.

Hence an efficient hydrolysis and detoxification has to be carried out in such a way that maximum amount of monomeric sugar is released and most of the inhibitors are removed without the sugar loss from the medium.

3.5.3 Effect of acid concentration in acid hydrolysis of PHL

Sulfuric acid concentration in the range of 1 to 2.5 % (w/w) was used in these experiments as beyond the level of acidity the formation of humins was observed. Preliminary experiments with 1 %, 2 % and 2.5 % (w/w) of sulfuric acid at 120 ^{o}C are shown in Fig. 3.1. It was found that the reaction with 1 % (w/w) of sulfuric acid resulted in very low concentrations of xylose whereas 2 % and 2.5 % (w/w) of sulfuric acid resulted in comparatively higher concentrations of xylose. From the figure, we can see that the furfural and acetic acid concentrations show similar patterns, higher the acid concentration, higher the formation of furfural and acetic acid, respectively. Though there is not much difference between 2 % and 2.5 % (w/w) sulfuric acid concentration on the production of xylose and acetic acid, the quantity of the furfural produced under these conditions differs significantly.

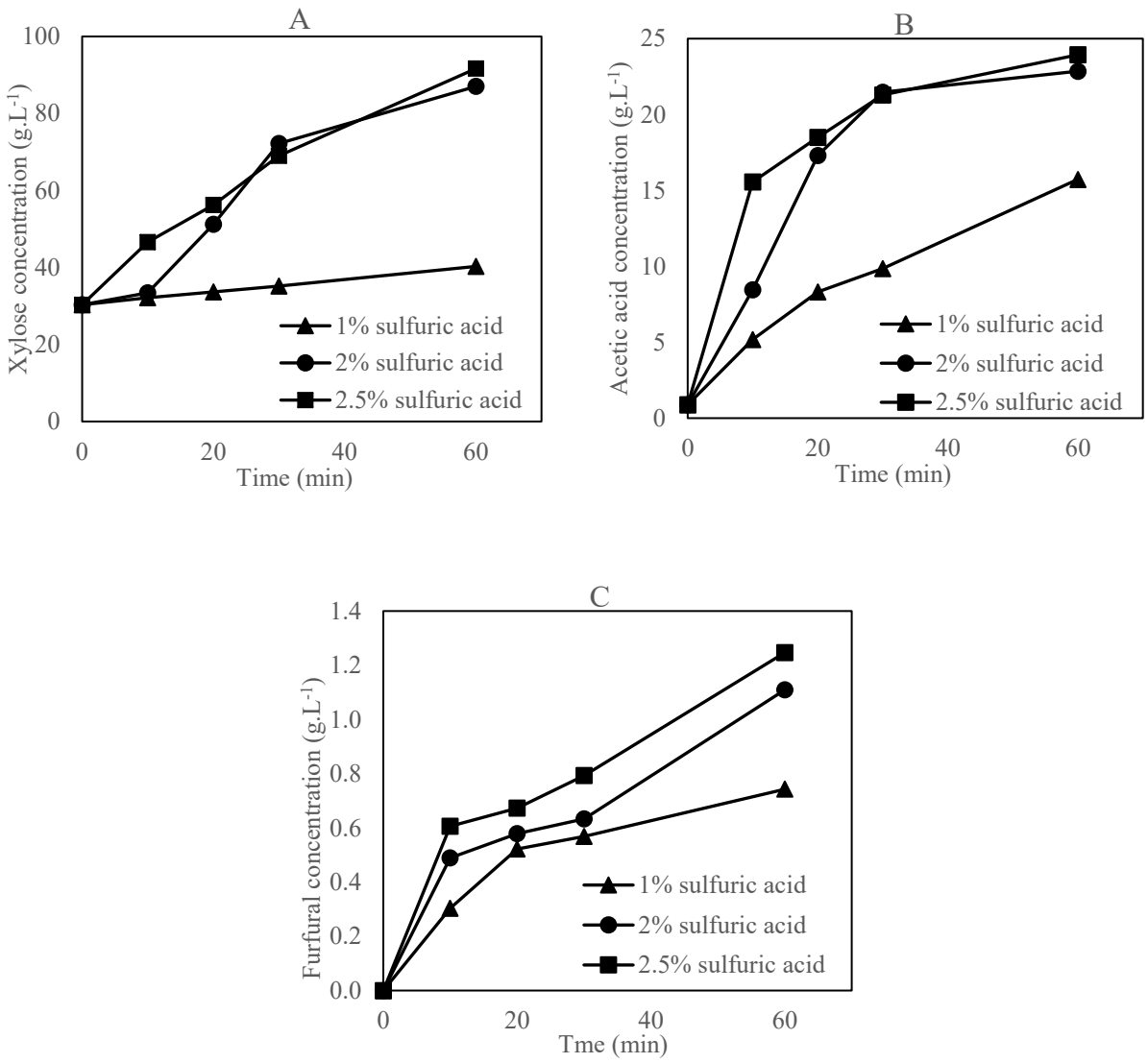


Figure 3.1. Comparison of (A) xylose, (B) acetic acid and (C) furfural concentrations of the hydrolysate after acid hydrolysis at 120 ^{o}C for 1 hour with 1 % (w/w) H_2SO_4, 2 % (w/w) H_2SO_4 and 2.5 % (w/w) H_2SO_4.

Based on these preliminary results, acid concentration range was narrowed down to find out lowest possible acid concentration for the higher conversion of polysaccharides with low by-product formation. Since the concentration of 1 % (w/w) is too low to convert xylo-oligomers to xylose efficiently, a higher acid concentration should be used. An acid concentration of 2.5 % (w/w) showed substantial increase of inhibitory by-products concentration with a xylose concentration similar to 2 % (w/w) sulfuric acid. Therefore, three additional concentrations, 1.5 %, 1.75 % and 2.2 % (w/w) were chosen for further analysis of acid hydrolysis for an extended period of 120 minutes.

As shown in Fig. 3.2, sulfuric acid concentration of 1.5 % (w/w) provides lower amounts of acetic acid and furfural where as 1.75 % and 2.2 % (w/w) show similar amounts. However, at the end of 120 minutes, 1.75 % (w/w) of sulfuric acid treatment was found to form more xylose than the other two concentrations with moderate byproduct formation. Therefore, 1.75 % (w/w) of sulfuric acid is recommended for the acid hydrolysis of PHL as good conversion of xylo-oligosaccharides to xylose with relatively low amounts of inhibitory by-products is obtained.

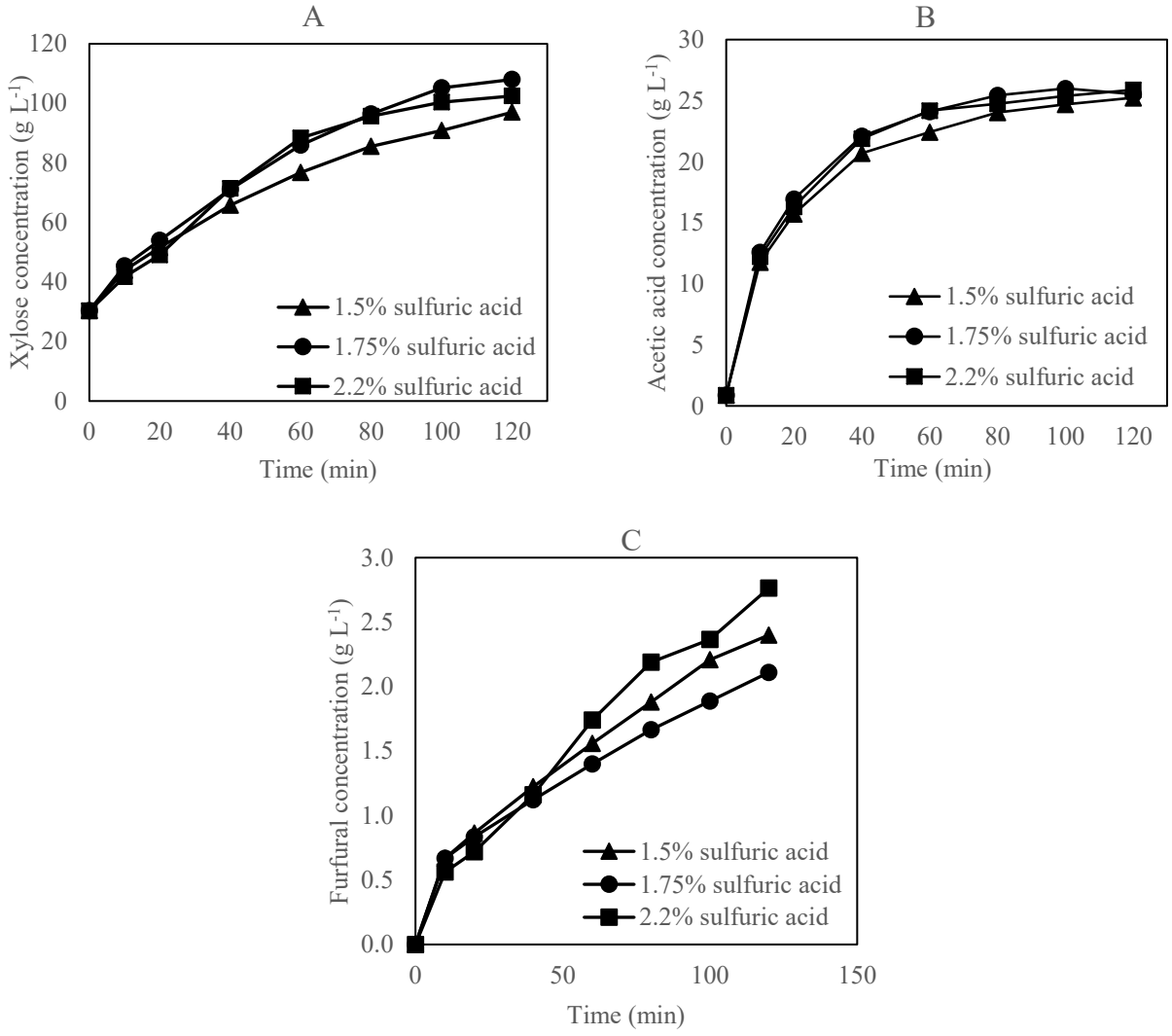


Figure 3.2. Comparison of (A) xylose, (B) acetic acid and (C) furfural concentrations of the hydrolysate after acid hydrolysis at 120 °C for 2 hours with 1.5 % (w/w) H_2SO_4, 1.75 % (w/w) H_2SO_4, 2.2 % (w/w) H_2SO_4.

Though the optimum acid hydrolysis condition shows better results with high amount of xylose (108.48 g L^{-1}), the concentration of inhibitors such as acetic acid (24.52 g L^{-1}) and furfural (3.11 g

L^{-1}) formed are adequate to slightly inhibit the microbial growth during fermentation. This was confirmed by carrying out fermentation of non-detoxified hydrolysate. It was discussed in further sections. A unified method of detoxification which includes both vacuum evaporation and solvent extraction together was developed to detoxify the hydrolysate.

3.5.4 Detoxification of the hydrolysate

The major inhibitory by-products (acetic acid and furfural) formed during acid hydrolysis act as inhibitors in fermentation experiments. Acetic acid results from the breakdown of acetyl groups on monomers and oligomers of xylose, and the furfural forms due to dehydration of xylose [24]. There are various detoxification methods reported in literature to remove these by-products from the hydrolysate such as activated charcoal, anion and cation ion-exchange resins, alkali treatment such as calcium oxide (over liming), enzymatic detoxification, evaporation and solvent extraction techniques [12-14]. Although Amberlite ion exchange resin technique was earlier suggested for hydrolysate detoxification [21], it was not found to be appropriate in these experiments as only 44.5 % of acetic acid and 60.7 % of furfural was removed through this method, while there was also a simultaneous loss of 31.54 % of xylose. It is necessary to remove these by-products efficiently from the hydrolysate as the fermentation of non-detoxified and Amberlite resin treated hydrolysate show no microbial growth.

Hence, a unified detoxification method of vacuum evaporation and solvent extraction was developed by optimizing pressure and temperature. Boiling the hydrolysate at high temperatures could result in loss of xylose due to its decomposition to furfural and subsequent formation of humins due to the simultaneous interaction of furfural and xylose [17]. Therefore, it is essential to optimize the temperature at which significant amount of acetic acid and furfural removal can be achieved without decomposing xylose. As mentioned in the materials and methods section, the conditions of vacuum evaporation have been studied. At 65 ^{o}C, humins formation was not observed and the decomposition of xylose was minimum. Hence, it was found to be appropriate to keep the temperature at this optimum level. Since the boiling points of acetic acid and furfural are 118 ^{o}C and 161.7 ^{o}C respectively at atmospheric pressure, they cannot be removed by a simple evaporation at the chosen temperature. Therefore, vacuum has been applied to the system in order to lower the boiling points and removed the inhibitors.

Even after acetic acid and furfural were removed substantially, the presence of phenolic compounds formed during the acid hydrolysis could still hinder fermentation. A number of solvents (see materials and methods section) were tested for their solubility of these phenolic compounds. However, most of them were found to solubilize the sugars also. Only toluene removed most of the phenolics with very low solubility of the pentose sugars. This solvent is thus recommended for removal of the residual phenolic inhibitors.

The initial and final concentrations of xylose, acetic acid and furfural of both the detoxification techniques were compared in Table 3.2. The hydrolysate which was detoxified by the unified technique with evaporation under reduced pressure and solvent extraction with Toluene was found to be much more effective. With this method, 80 % of the acetic acid and 98.84 % of the furfural was removed, with a loss of 5.6 % of xylose. The loss of xylose can be due to its partial degradation into furfural during the process. However, the amount of inhibitors removed with this method found to be higher than other reports in literature [25-27].

Table 3.2. Concentration of xylose and major inhibitors in crude prehydrolysate (PHL), acid hydrolysed hydrolysate (with 1.75% (w/w) H_2SO_4 at 120 ^oC), and detoxified hydrolysate by resin adsorption and unified vacuum evaporation and solvent extraction techniques.

Component	Initial PHL concentration ($g\ L^{-1}$)	Final concentration after acid hydrolysis ($g\ L^{-1}$)	Detoxification	
			Final concentration after Amberlite resin treatment ($g\ L^{-1}$)	Final concentration following unified detoxification ($g\ L^{-1}$)
Xylose	31.97	108.48	74.26	102.4
Acetic acid	2.37	24.52	13.60	4.90
Furfural	0.35	2.11	1.22	0.03

In the studies conducted by Villareal et al. (2006), conventional evaporation was able to remove 97 % of furfural and 63% of acetic acid at high temperature. However, higher temperatures resulted in polymerization of furfural as well [25]. Therefore, in such cases, the loss of sugar would be higher and reduces the desired fermentation product. According to Mateo et al. (2013), using

activated charcoal for the detoxification of hydrolysate removes 87 – 97% of furfural [26]. In a study conducted by Lee et al. (2011) using activated charcoal, 93% furfural and as low as 14% acetic acid was removed [28]. Another study by Seo et al. (2005) using charcoal shows 92% removal of total phenolics from the hydrolysate [27].Therefore, it can be inferred that acetic acid and some other phenolics cannot be removed in high amounts with activated charcoal alone [21]. Moreover, the loss of sugars with these methods are also significant. Detoxification of hydrolysate using anion exchange resins was also extensively studied in literature [29]. A study by Vithanage et al. (2015) show that 50 – 80% acetic acid and 100% phenolics were removed from the hydrolysate [21]. However, they also reported that the method removes xylose sugar substantially (70 – 80%). Table 3.3 compares the results obtained in this study with literature results. Therefore, the detoxification method reported in our study is efficient in removing the inhibitory compounds without loss of sugars and makes the hydrolysate more amenable for fermentation as shown in the following sections.

Table 3.3. Comparison of the results obtained in this study with several literature reports

Substrate	Detoxification method	Xylose (%)	Furfural (%)	Acetic acid (%)	References
Spruce hydrolysate	Sodium sulfite	6	0	1	[21]
	Cation exchanger, pH 5.5	0	4	7	
	NaOH	6	8	2	
	NH_4OH	7	10	0	
	Activated charcoal	5	94	28	
Eucalyptus wood hydrolysate	Solvent extraction (EtOAc)	-	84	-	[12]
Sugarcane bagasse hydrolysate	Over liming	14	59	-	[29]
Poplar wood hydrolysate	**Over liming**	30	38	19	This study
	Anion exchanger, pH 5.5	26	60	44	This study
	Unified method (Vacuum	5	98	80	This study

	evaporation & solvent extraction)

3.5.5 Fermentation of treated PHL for the production of xylitol

The effectiveness of the hydrolysis and detoxification was then determined using the fermentation process for the conversion of xylose in detoxified hydrolysate to xylitol. A series of fermentation experiments using different substrates, such as pure xylose, acid hydrolyzed and detoxified hydrolysates were compared.

3.5.5.1 Effect of substrate concentration in fermentation of pure xylose medium

Mediums containing four different xylose (commercial grade) concentrations ranging from 25 - 100 g L^{-1} were used in the fermentation experiments with *Candida guilliermondii* FTI 20037 to determine the effect of substrate inhibition on microbial growth. The comparison of the fermentation results at these concentrations based on the highest yield after 18 hours of fermentation are given in Table 3.4. The xylose consumption rates were found to be similar in all the broths, S_1 to S_4 (25, 50, 75 and 100 g L^{-1}) and the production of xylitol increased with an increase in the xylose concentration (Fig. 3.3a). The consumption of xylose and simultaneous production of xylitol was observed within 3 - 4 hours of inoculation in broths S_1 to S_3, without any lag in microbial growth and xylitol production. However, a slight lag in the production of xylitol was observed in the broth containing 100 g L^{-1} and higher xylose. Therefore, substrate inhibition on the growth of microbes seems to occur at these higher xylose concentrations. This was further confirmed with the fermentation experiments carried out with higher xylose concentrations. It was observed that as the xylose concentrations were increased, the microbial growth and xylitol production was very slow in the initial hours and took longer time to provide moderate levels of xylitol.

Table 3.4. Fermentation results using pure xylose media (S_1 to S_4), the pre-hydrolysate liquor medium (P), the crude hydrolysate media (H), the hydrolysate media detoxified by resin (HR_1, HR_2, HR_3), and the hydrolysate media purified by the unified detoxification method (HE_1 and HE_2) based on the highest yield during fermentation carried out at 32 °C.

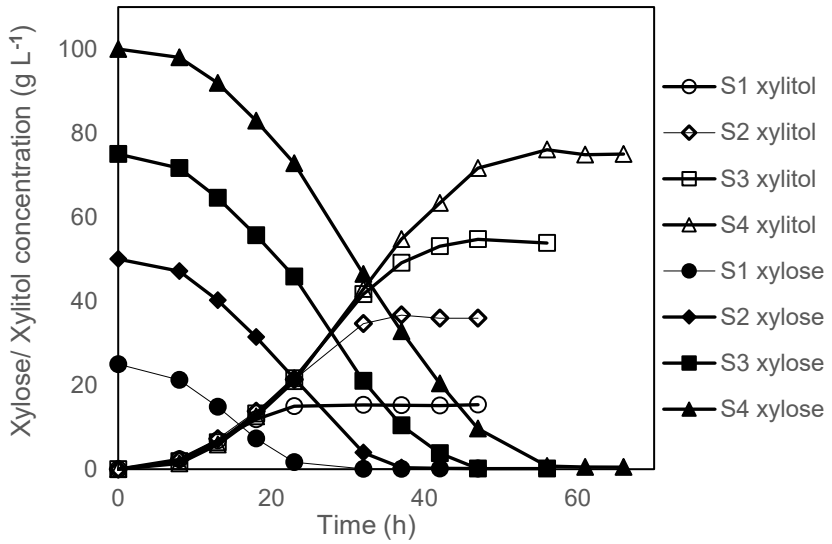
Sample	Initial xylose concentration (g L^{-1})	Fermentation time (h)	Xylose consumed (%)	Xylitol concentration produced (g L^{-1})	Xylitol yield (g g^{-1})
Pure xylose mediums					
S_1	25.30	24	84.33	15.01	0.63
S_2	50.82	32	79.65	34.67	0.73
S_3	75.04	32	61.94	41.63	0.75
S_4	100.81	37	56.43	54.77	0.79
Crude pre-hydrolysate					
P	13.02	20	80.94	5.08	0.460
Crude hydrolysate (non-detoxified)					
H	81.6	48	ND	ND	ND
Detoxified hydrolysate using resin					
HR_1	25.63	35	83.05	1.30	0.040
HR_2	50.42	75	70.28	3.46	0.060
HR_3	75.85	69	71.55	2.87	0.056
Detoxified hydrolysate through a unified method					
HE_1	25.24	24	76.93	12.34	0.44
HE_2	50.31	37	80.44	28.34	0.59

S = pure xylose; P = prehydrolysate; H = acid hydrolysed hydrolysate; HR = hydrolysate treated with resin; HE = hydrolysate detoxified through unified method; ND = Not Detected

The effect of substrate concentration on the microbial growth was also evaluated in terms of dry weight of the biomass. In Fig. 3.3b, dry biomass weights of mediums containing up to 100 g L^{-1} xylose were compared. The growth of the microbes appeared to be still increasing in the 75 and 100 g L^{-1} batches, while the dry weight of biomass became constant after 24 and 48 h in 25 and 50 g L^{-1} batches, respectively. In this set of data, the substrate inhibition was not significant and did not affect the microbial growth. However, in the fermentation broths containing higher xylose concentrations, microbial growth was completely inhibited in the first few hours of fermentation. Therefore, it was evident that initial substrate concentration and subsequently formed product influence the growth pattern of *Candida guilliermondii*. This indicates and confirms again that with pure xylose concentrations greater than 100 g L^{-1} in a batch, significantly

lowers the cell growth rates and consequently reduces the xylitol production, as it is a growth associated product. However, media containing 50 and 75 g L-1 xylose found to be optimum levels for the better xylitol production with effective microbial growth.

a)



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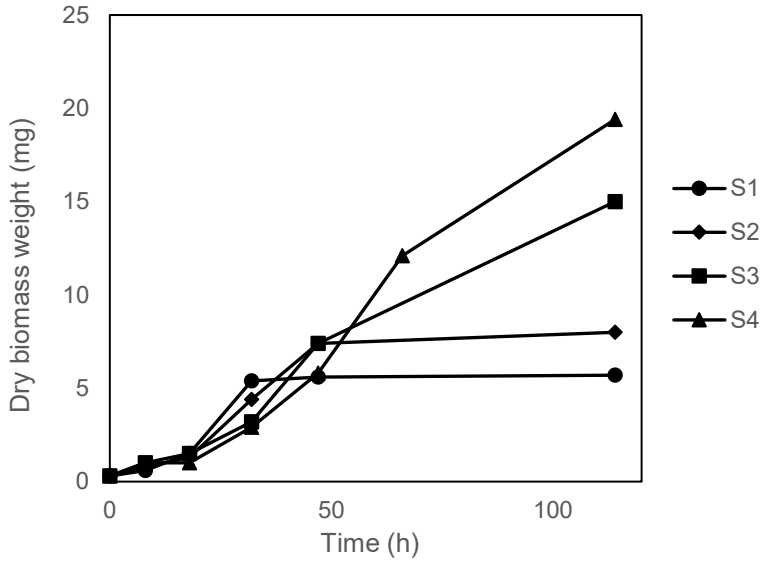

Figure 3.3. The profiles of xylose and xylitol concentrations (A) and microbial biomass (B) during the fermentation with initial pure xylose concentrations of 25 g L^{-1} (S1), 50 g L^{-1} (S2), 75 g L^{-1} (S3) and 100 g L^{-1} (S) using *C. guilliermondii* at 32 ^{o}C.

3.5.5.2 Prehydrolysate fermentation

Prehydrolysate (PHL) with an initial xylose concentration of 13.02 g L^{-1} was used in the fermentation experiments with *C. guilliermondii.* Though the xylose concentration of PHL is 31.97 g L^{-1}, addition of YEPX media and Vogel Salts resulted the decrease in its concentration in the fermentation broth. The highest xylitol yield observed was 0.46 g g^{-1} with a productivity of 0.26 g L^{-1} h^{-1} (Table 3.3). Due to the low concentrations of acetic acid (0.88 g L^{-1}) and furfural (0.62 g L^{-1}) in PHL, their inhibition effect on microbial growth was not observed. However, as the concentration of xylose was also low, amount of biomass and product formation was also trivial. Apparently, maximum xylitol concentrations reached in shorter times due to low availability of xylose sugar. This indicates that the oligo- and poly-saccharides in hemicellulose has to be hydrolyzed to generate more fermentable sugars in order to enhance xylitol production.

3.5.5.3 Non-detoxified hydrolysate fermentation and effect of inhibitors

The crude PHL was thus hydrolysed using H_2SO_4 to breakdown the xylo-oligosaccharides to xylose monosaccharides (as discussed in previous section) which resulted in an increase in the xylose concentration from 31.97 g L^{-1} to 108.48 g L^{-1}. After acid hydrolysis of pre-hydrolysate liquor, there is a substantial increase in the concentration of xylose. However, inhibitors such as organic acids and phenolic compounds were expected in it as discussed earlier. These inhibitors were expected to have a significant impact on fermentation. In order to confirm the extent of inhibition, the hydrolysate obtained after acid hydrolysis was initially used for fermentation directly, without detoxification. The pH of hydrolysate was adjusted to 4.5 with 3 N NaOH and diluted to the required substrate (xylose) concentration. Following sterilization, the hydrolysate was supplemented with other nutrients for the fermentation experiments and adjusted the pH to 5.5. Despite maintaining the same conditions as all the other experiments, no microbial growth or xylitol production was observed until 48 hours of fermentation. This can be attributed to the high concentration of inhibitors produced during the hydrolysis. In order to make use of the xylose present in the hydrolysed sugar solution, these inhibitors needed to be removed.

3.5.5.4 Fermentation of detoxified hydrolysate obtained using ion exchange resin

The hydrolysate was detoxified using two different methods to reduce inhibitor concentrations to very low levels. In the first experiment, an Amberlite resin was used for the detoxification as per the procedure discussed in the previous sections. The detoxified hydrolysate

obtained using this method was used in 3 different fermentation experiments with initial xylose concentrations of 25 g L^{-1}, 50 g L^{-1} and 75 g L^{-1}. Due to the inefficient removal of inhibitors, the observed xylitol yields for these three concentrations were 0.040, 0.060, 0.056 g g^{-1} with volumetric productivities of 0.038, 0.047, 0.043 g L^{-1} h^{-1}, respectively, as seen in Table 2. These low yields and productivities are because of the combined inhibition effect on microbial growth by the substrate, acetic acid and furfural concentrations which were retained even after the detoxification. It should be noted here that while the resin was able to remove some of the inhibitors, it simultaneously removed some of the xylose sugar as well (31.54 % loss). Therefore, an efficient detoxification method is necessary to intensify the removal of inhibitors while preventing the loss of sugars.

3.5.5.5 Fermentation of detoxified hydrolysate obtained using a unified method of vacuum evaporation and solvent extraction

The detoxified hydrolysate obtained by vacuum evaporation and solvent extraction, as discussed in previous section, contained a higher concentration of sugars and trace amounts of inhibitors. As mentioned earlier, this was done under reduced pressure so that the heat requirement was lower and did not affect the sugar present in the system. The fermentation experiments with this detoxified hydrolysate resulted in a significantly higher xylitol yield than the hydrolysate purified through the resin (Table 3.3). This confirms that it is necessary to employ an efficient detoxification method to remove inhibitors from the hydrolysate medium in order to have efficient usage of xylose in the medium to obtain high yields of xylitol.

The hydrolysate obtained from the above procedure was diluted to various xylose concentrations such as 25, 50, 75 and 100 g L^{-1} and used in the fermentation. Due to the higher substrate inhibition, the mediums with the xylose concentrations of 75 and 100 g L^{-1} produced low yields of xylitol. However, 25 and 50 g L^{-1} (HE_1 and HE_2 respectively) show better yields and closer results to the pure xylose fermentation. The fermentation was then carried out for 60 h using the *Candida guilliermondii* strain with same conditions as in other previous experiments and the profiles of xylose and xylitol concentrations are shown in Fig. 3.4. The xylitol yield obtained in HE_1 was 0.44 g g^{-1} with a maximum productivity of 0.34 g L^{-1} h^{-1} at 24 hours whereas in HE_2 the yield was 0.59 g g^{-1} with a highest productivity of 0.81 g L^{-1} h^{-1} at 37 hours. These results are found to be better than the results obtained from different detoxification techniques reported in literature.

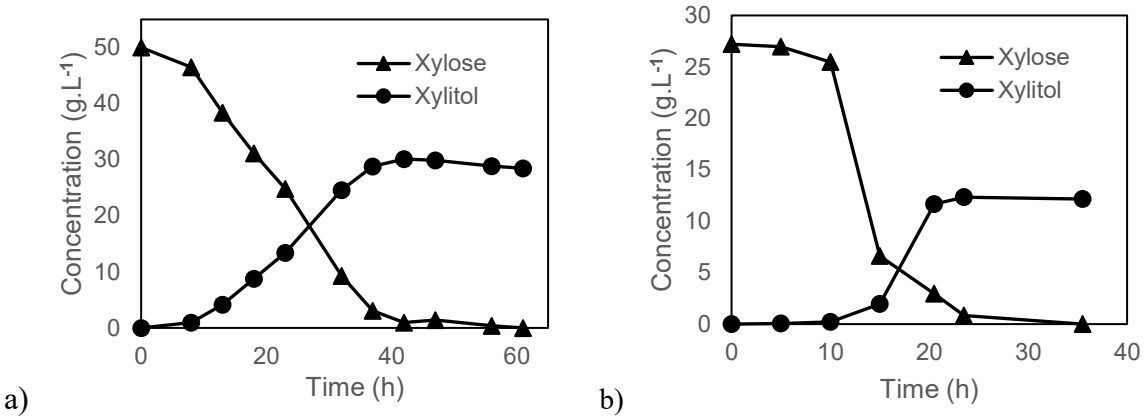

Figure 3.4. The Profiles of xylose and xylitol concentrations of the fermentation experiments using *C. guilliermondii* and detoxified hydrolysates with initial xylose concentration of 25 g L^{-1} (a) and 50 g L^{-1} (b) at 32 °C.

According to Canilha et al. (2008), fermentation of wheat straw detoxified by over liming resulted in 0.59 g L^{-1} h^{-1} productivity of xylitol using *C. guilliermondii* as biocatalyst [30]. In another study by Tada et al. (2004), charcoal pellet adsorption detoxification of corncob hydrolysate was fermented with *C. magnoliae* and found to produce 0.75 g g^{-1} xylitol [31]. It is one of the highest yields reported in literature, but the productivity (0.52 g L^{-1} h^{-1}) was relatively low than our results. Ping et al. (2012) have reported that high xylitol yield (0.7 g g^{-1}) was obtained from non-detoxified corncob hydrolysate with a productivity of 0.46 g L^{-1} h^{-1} [32]. Thus, the upstream methods we adapted and developed increase xylitol yield and productivity in the reported process.

Since the hydrolysate was detoxified efficiently through the unified vacuum evaporation and solvent extraction technique, it resulted in the hydrolysate with very minimal amount of acetic acid and furfural (0.5 % and 0.003 %, respectively in the hydrolysate). The results of the hydrolysate fermentation were found to be very similar to the fermentation using pure xylose mediums with the same concentration. These results show that it is possible to make good use of the hemicellulose stream obtained from poplar wood, following an effective and environmentally safe acid hydrolysis with low acid concentration, efficient detoxification and an optimized fermentation processes to produce xylitol. This method can be suitably optimized for other lignocellulosic feedstocks and the detoxification of hemicellulosic hydrolysates for the production of xylitol and other commercially beneficial products.

3.6 Conclusions

Low acid (1.75 % (w/w) H_2SO_4) hydrolysis, followed by efficient removal of inhibitors (80 % acetic acid and 98.84 % furfural) with very little loss of xylose is reported. The effectiveness of these methods were confirmed by the good xylitol yield (0.59 g g^{-1}) and concentration (28.78 g L^{-1}) obtained by fermentation using *C. guilliermondii.* The volumetric productivity of 0.81 g L^{-1} h^{-1} is the highest productivity reported so far using hemicellulose prehydrolysate and is close to the results obtained in pure xylose fermentation. The monomeric sugars obtained by such upstream processes can be used for the production of other value added products.

3.7 References

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CHAPTER 4
OBJECTIVE 2

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My contribution

All the experimental design, work and write up. Part of this work was done during a short-term visit to University of Sao Paulo, Brazil.

(Mitacs Globalink Research Award)

Co-author's contribution

Prof. Dr. Silvio Silverio da Silva – Co-supervised this project at University of Sao Paulo, Brazil.

Bijaya K. Uprety – Helped in some experimental work.

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4 Enhanced Production of Xylitol from Poplar Wood Hydrolysates Through a Sustainable Process Using Immobilized New Strain *Candida tropicalis* UFMG BX 12-a

4.1 Abstract

A new strain, *Candida tropicalis* UFMG BX 12-a was found to produce higher yields of xylitol on poplar wood hemicellulose hydrolysate. The hemicellulose hydrolysate liquor was detoxified using an efficient method we developed, involving vacuum evaporation and solvent separation of inhibitors which made the hydrolysate free of toxins while retaining high concentrations of fermentable sugars. The effect of the detoxification method on the fermentation was also reported and compared to well-known methods reported in literature. In this study, the new strain *Candida tropicalis* UFMG BX 12-a was used on the detoxified hydrolysate to produce xylitol. It was also compared to *Candida guilliermondii* FTI 20037, which has been reported one of the best strains for fermentative production of xylitol. To further improve the efficiency of the fermentation process, these strains were immobilized in calcium alginate beads. The yield (0.92 g g^{-1}) and productivity (0.88 g L^{-1} h^{-1}) obtained by fermenting the wood hydrolysate detoxified by our new detoxification technique using an immobilized new *Candida* strain, were found to be higher than the values reported in literature.

4.2 Introduction

Xylitol, a xylose based sugar alcohol, is one of the top twelve chemicals listed by the US Department of Energy as high valued chemicals in the global market [1]. Currently, xylitol is produced through chemical catalytic routes in industrial scale. Though such process produce xylitol in acceptable yields (97%) [2], high upstream processing costs and environmental effects result in the search for alternate fermentative routes. Hemicellulose prehydrolysate from different types of biomass have been studied for the microbial production of xylitol. Corn cob [3], sugarcane bagasse [4], wheat straw [5], rice straw [6] and wood hemicellulose streams [7] have been extensively studied using various yeast and recombinant strains to determine their industrial feasibility for the production of xylitol. Yeast strains such as *Candida guilliermondii, Candida tropicalis, Debaryomyces hansenii* and recombinant *E.coli* have been widely reported in the literature [7-11].

Acid hydrolysis of hemicellulose prehydrolysate has to be carried out to obtain its monomer constituents in which xylose occupies the major part. Therefore, it enhances the xylose concentration in the resultant hydrolysate liquor. Byproducts such as furfural, acetic acid, 5-hydroxymethyl furfural (HMF) etc. formed during the acid hydrolysis act as microbial growth inhibitors. Major constraints involved in fermentative production of xylitol include the removal of these toxins from the hydrolysate and an efficient microbe to produce xylitol in high yields [12]. Several detoxification techniques were reported in the literature to make the hydrolysate more amenable for the fermentation [13-15]. According to few reports, anion exchange resins are efficient and effectively remove the inhibitors compared to other techniques [12, 16]. However, the high cost of the resins and the loss of sugars from the hydrolysate during detoxification make their use uneconomical at a large scale [17].

According to Jonsson et al. (2013), overliming is one of the efficient detoxification processes to remove inhibitors from the hydrolysate [18]. Based on the different studies in literature, overliming can reduce the furfural content in a range of 18 -70 % depending on the conditions (temperature and time) used. However, the loss of sugar was also significant within a range of 1-68 % [18]. Li et al. (2011) have studied the fermentation of hemicellulose hydrolysate which was detoxified by vacuum evaporation. A significant increase (12 %) in the xylitol production was observed in their study [8]. Zhuang et al. (2009) have studied the use of organic solvents such as ether and ethyl acetate to evaluate the detoxification effect on fermentation [15]. The liquid-liquid extraction method reported by them have resulted in removal of 74 % furfural and 63 % acetic acid from the hydrolysate with 23 % loss of sugar [15]. Many of the techniques reported in literature have not been able to remove the toxins completely. Therefore, it is necessary to develop a better and efficient technique to remove the microbial growth inhibitors in the hydrolysate to the maximum. An integrated technique reported in this article provides a good example for the requirement of such efficient processes.

Many studies in literature have reported the production of xylitol under various fermentative conditions using different microbial strains and types of biomass [3-7]. Most of the reports claimed that *C. guilliermondii* strain shows better xylitol yields and productivities [7, 19]. According to Hernandez-Perez et al. (2015), *C. guilliermondii* was able to produce 36 g L^{-1} of xylitol with a high productivity of 0.75 g L^{-1} h^{-1} when grown on 57 g L^{-1} xylose [20]. In the study conducted by

Silva C.J.S.M. et al. (2006), *C. guilliermondii* on rice straw hemicellulose produced 45.4 g L^{-1} of xylitol with 1.01 g $L^{-1}h^{-1}$ productivity [21]. Therefore, considering the high productivity of *C.guilliermondii* strain, we compared *C. guilliermondii* FTI 20037 to our new strain, *Candida tropicalis* UFMG BX 12-a, in our fermentation studies.

Microbial cell concentration in the fermentation medium has significant effect on the production of xylitol [4]. Immobilization of the microbial cells is one technique through which high cell concentrations can be obtained. It helps to attain high fermentation rates due to the high concentration of entrapped cell [22] and benefits the fermentation process by reducing the operation costs involved in downstream processing. In addition, the efficiency of the fermentation can also be improved as the immobilized cells can be reused further. Reusability of these biocatalysts reduces the production costs further [4, 23]. Entrapment method using calcium alginate is one of the common techniques used to immobilize the microbial cells [4]. Studies conducted by Shyamkumar et al. (2014) on immobilization of cells suggest that the fermentation processes can be enhanced with use of cell entrapment technique and the costs involved in downstream processes can be reduced [24]. According to Milessi et al. (2015), using immobilized yeast cells makes the fermentation process to overcome the barrier of low economic feasibility [25]. Antunes et al. (2016) have studied the immobilization systems with various concentrations of sodium alginate and calcium chloride to determine the optimum concentration to form strong beads with yeast cells [26]. According to their studies, encapsulation of yeast cells is efficient among various methods of cell immobilization reported in literature and is widely used method due to its reproducibility and simple procedures involved [25, 27]. Based on these studies, we have used cell entrapment technique in calcium alginate and used in fermentation further.

In this study, we present an efficient detoxification method by integrating vacuum evaporation and solvent extraction. It was used to remove toxins from the acid hydrolysed wood hydrolysate with low sugar loss. A new strain, *Candida tropicalis* UFMG BX 12-a, entrapped in calcium alginate beads was used to ferment the detoxified hydrolysate to produce xylitol in high yields.

4.3 Material and Methods

4.3.1 Acid hydrolysis and detoxification of prehydrolysate

Poplar wood hemicellulose prehydrolysate used as a substrate in this study was provided by Greenfield Ethanol Inc., Canada. Steam percolation pretreatment was used to obtain the hemicellulose prehydrolysate from poplar wood biomass. It was reported elsewhere [28]. The resultant wood prehydrolysate was hydrolysed using dilute sulfuric acid of 1.8 % (w/w) at 120°C for 4 h. The hydrolysate was then detoxified by vacuum evaporation and solvent extraction techniques developed at our laboratory to reduce the fermentation inhibiting compounds furfural, acetic acid and 5-HMF. Vacuum evaporation was performed at constant temperature (65°C) and a ramped vacuum in the range of 250 mbar to 175 mbar was applied to remove acetic acid and furfural along with aqueous phase. Other phenolics and trace amounts of furfural left in the dark brown residue of the hydrolysate were removed by washing with acetone [29]. The detoxified hydrolysate was then stored at 4°C before subsequent use. Standard xylose and acetic acid, obtained from Sigma Aldrich were used to prepare the synthetic hydrolysate medium which served as control.

4.3.2 Microbial inoculum preparation

Candida tropicalis UFMG BX 12-a isolated from decaying wood biomass, was kindly provided by Prof. Carlos A. Rosa from Federal University of Minas Gerais, Brazil. The yeast was maintained on malt extract (3% w/v) agar (1.5% w/v) slants at 4°C before being used in this study. *Candida guilliermondii* FTI 20037 was also used simultaneously to compare the results obtained from the new strain. *C. guilliermondii* was a kind gift from Dr. Hung Lee (University of Guelph, ON, Canada) and was maintained on yeast extract (1% w/v), peptone (2% w/v), agar (2% w/v), and D-glucose (1% w/v) slants at 4°C [30]. A loopful of both yeasts from the slants were inoculated into separate Erlenmeyer flasks containing 75 ml of yeast extract (1% w/v), peptone (2% w/v), xylose (1% w/v) media. The pH was adjusted to 5.5 and the flasks were incubated for 24 hours in a shaker at 32°C and 200 rpm [31]. The culture broth was then centrifuged to separate the yeast cells from the broth. The recovered yeast cells were washed and immobilized further.

4.3.3 Cell culture immobilization

The *C. guilliermondii* and *C. tropicalis* strains were immobilized separately by entrapment technique in calcium alginate beads using methods reported in literature [26]. A 7% solution of sodium salt of alginic acid from brown algae (Sigma Aldrich) was mixed with an equal amounts of yeast cells suspended in sterilized distilled water with a cell concentration of 10^7 cells/mL. The mixture was then added to a sterilized solution of calcium chloride dihydrate (11 g L^{-1}) in a dropwise manner using a syringe under sterile conditions. Spherical gel beads of calcium alginate with encapsulated *C. guilliermondii* and *C. tropicalis* were produced separately (Fig. 4.1). The immobilized cell cultures were left in solutions of calcium chloride overnight at 4°C to strengthen the bead structure. The gel beads were washed thoroughly with sterile water, before being used in fermentation experiments.

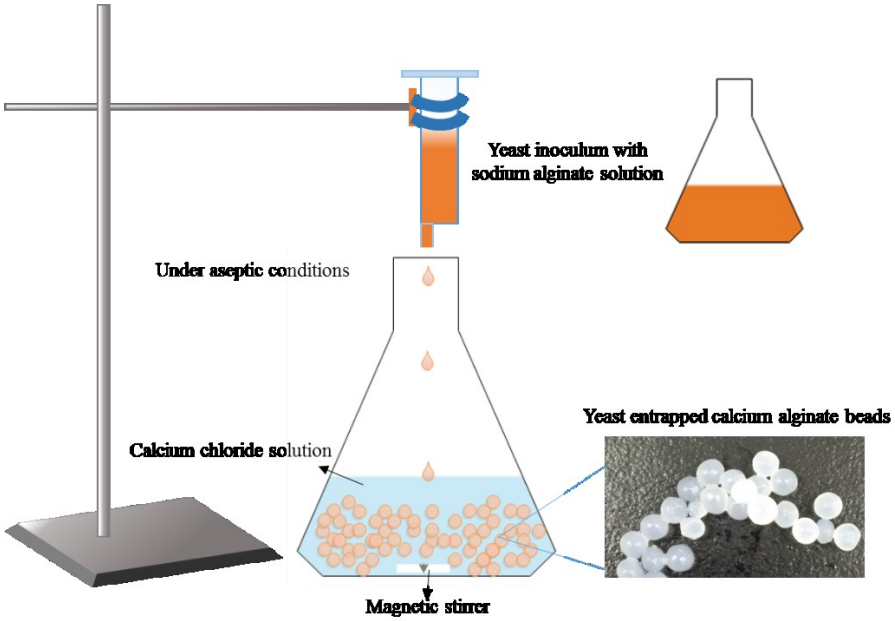

Figure 4.1. Illustration of yeast entrapment by the calcium alginate bead immobilization process

4.3.4 Fermentation process

The detoxified wood hydrolysate was fermented with both the yeast strains separately. The fermentation media were composed of hydrolysates (50 g L^{-1}) and Vogel's Minimal Salt Media (2%) (composition: $Na_3.2\ H_2O$, KH_2PO_4 (anhydrous), NH_4NO_3 (anhydrous), $MgSO_4.7\ H_2O$, $CaCl_2.2\ H_2O$). Immobilized yeasts were packed in separate column (3 cm x 40 cm) and the

hydrolysate was passed through them at a flow rate of 5 ml/min. The hydrolysate media was kept in a 1L bioreactor (750 mL working volume) with agitation speed of 150 rpm and circulated through the immobilized cell column at 32°C for 4 days (Fig. 4.2). Samples were taken at various time intervals and analyzed by a high performance liquid chromatography (HPLC) to determine the substrate consumption and xylitol production levels.

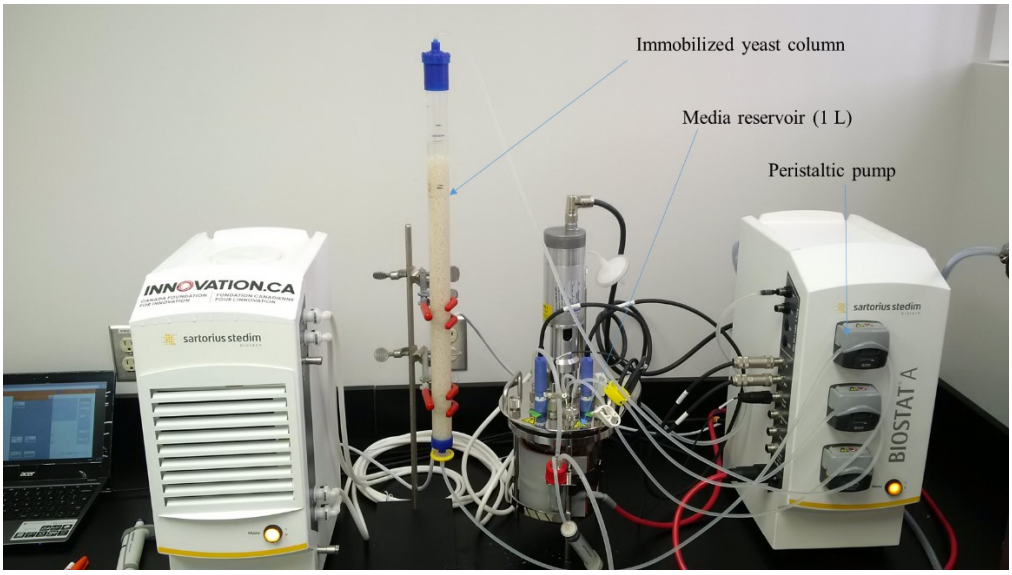

Figure 4.2. Experimental setup of the fermentation of the hydrolysates and control media with immobilized cells packed in a separate column

4.4 Analytical methods

All samples including pretreated biomass, acid hydrolysed, detoxified hydrolysate and fermented samples were analyzed using an HPLC (Agilent Technologies 1260 Infinity). Sugars and acetic acid content were analyzed using Bio-Rad Aminex HPX-87H ion exclusion column (300mm x 7.8mm) and Refractive Index Detector (RID) with a mobile phase of 5 mM H_2SO_4 at 0.6 mL/min flow rate. Other phenolics were analysed using Agilent Poroshell 120 EC-C18 column (4.6 x 50 mm) along with a Variable Wavelength Detector (VWD) at 280 nm with a mobile phase of (90:10) methanol and 1% acetic acid at a flow rate of 0.6 mL/min.

4.5 Results and Discussion

Steam percolation of poplar wood resulted in a solid (cellulose and lignin) and a liquid prehydrolysate (hemicellulose) stream. These streams were separated by following a patented technology developed at GreenField Ethanol Inc. [28]. Initially, 52 L hemicellulose prehydrolysate liquid stream was obtained which was then concentrated to 8 L for our use.

4.5.1 Composition of prehydrolysate and hydrolysate

The prehydrolysate was hydrolysed using sulfuric acid to degrade the poly- and oligo-saccharides to monomeric pentose sugars. Xylose concentration after the acid hydrolysis was found to be nearly doubled along with a significant increase in acetic acid (4.2 times) and furfural (10 times). Due to the increase in phenolic components like furfural which inhibit microbial growth, the hydrolysate obtained after the acid hydrolysis is unfavorable for fermentation. Therefore, the hydrolysate was purified by following an efficient detoxification method developed in our lab. The composition of the hydrolysate in all stages (pretreatment, acid hydrolysis, detoxification) were characterized using an HPLC (Table 4.1).

Table 4.1. The composition of poplar wood hemicellulose at all stages (prehydrolysate, acid hydrolyzed, detoxified hydrolysates) and the % loss of each component after detoxification.

Component	Prehydrolysate ($g\ L^{-1}$)	Acid hydrolyzed hydrolysate ($g\ L^{-1}$)	Detoxified hydrolysate ($g\ L^{-1}$)	% loss
Xylose	31.97	58	54.76	5.6
Acetic acid	2.37	9.97	1.25	87.5
Furfural	0.35	3.56	0.10	97.4
Glucose	2.11	1.64	1.14	30.5
Arabinose	3.18	2.11	1.47	30.4
Hydroxymethyl furfural	1.02	1.85	ND	100

ND - Non detectable.

After detoxification, it was found that vacuum evaporation with subsequent solvent extraction efficiently removed acetic acid (87.5%) and furfural (97.4%) with minimal loss of xylose (5.6%). It was found that the detoxification method described in this study has given better results than other methods reported in literature. For instance, according to Zhuang et al. (2009), only 74% of furfural and 63% of acetic acid was removed using a resin and ethyl acetate solvent [15]. In another

study by Martinez et al. (2001), overliming of hydrolysate resulted in 69% decrease in furfural [32].

4.5.2 Fermentation of the hydrolysate using immobilized yeast cells

Fermentation of non-detoxified hydrolysate (pH adjusted to 5.5) obtained from acid hydrolysis of prehydrolysate showed neither microbial growth nor production of xylitol due to the presence of high concentration of microbial growth inhibitors (acetic acid, furfural and other phenolics). Therefore, the hydrolysate was detoxified using an efficient detoxification method before using it for fermentation. The pH of the detoxified hydrolysate was adjusted to 5.5 and fermented using two types of *Candida* strains (*C. tropicalis* and *C. guilliermondii*) immobilized in calcium alginate beads in separate experiments until the substrate was fully consumed. Xylose is the major carbon substrate for microbial growth as the amount of other sugars are relatively low. The xylose and acetic acid composition in the wood hydrolysate was mimicked and a synthetic hydrolysate medium was prepared. It was considered as control (W_c) for the fermentation experiments. The initial xylose concentration in the hydrolysate and the control media was maintained at 50 $g.L^{-1}$. However, the fermentation results obtained from two yeasts were distinct from each other. The comparison of the fermentation results of the hydrolysate and the control media helps in determining the effect of other components on the microbial xylitol production.

Fermentation of the hydrolysate (W_h) and control media (W_c) using *C. tropicalis*:

The detoxified wood hydrolysate (W_h) was fermented using immobilized *C. tropicalis* strain for 4 days along with the control media (W_c). Fig. 4.3 shows the profile of xylose consumption and xylitol production in both the hydrolysate and control media. It was observed that the consumption of xylose in W_h was similar to that of in its control (W_c). It is in contrast to the fermentation results of non-detoxified hydrolysate where no microbial growth was observed in 4 days. This indicates that the inhibitors in the detoxified hydrolysate were so low that microbial growth was not affected. Therefore, it is evident that the detoxification used for wood hydrolysate was efficient in removing most of the inhibitors from the hydrolysate. The maximum xylitol concentration in W_h was achieved at the same time as in its control (W_c). *C. tropicalis* fermentation of W_h resulted in 0.92 $g\ g^{-1}$ yield and 0.88 $g\ L^{-1}\ h^{-1}$ productivity within 48 hours. The comparison of yield and productivities of the hydrolysate and control media was shown in Fig. 4.4. These values are 96% of yield and 95.6% of productivity of the control fermentation results.

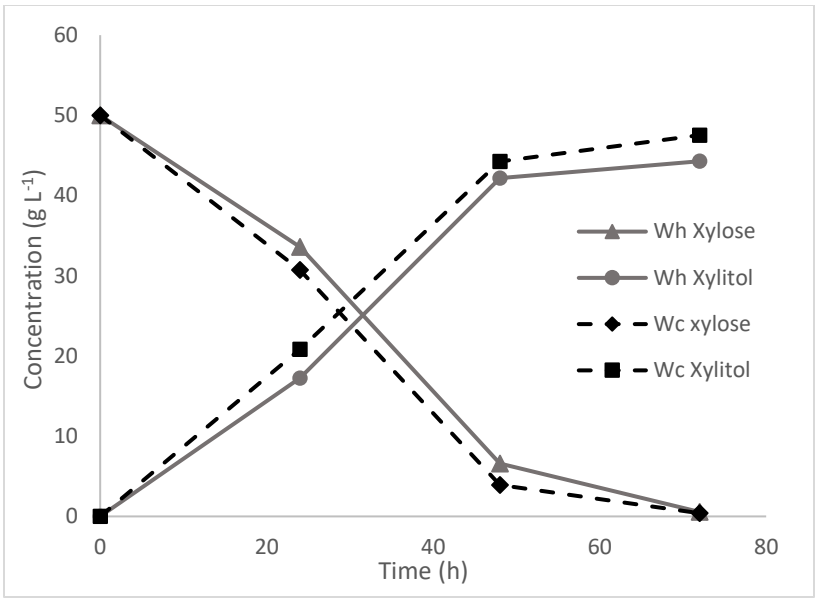

Figure 4.3. Fermentation profile of detoxified wood hydrolysate (W_h) and its control medium (W_c) using *Candida tropicalis* UFMG BX 12-a in terms of xylitol production and xylose consumptions.

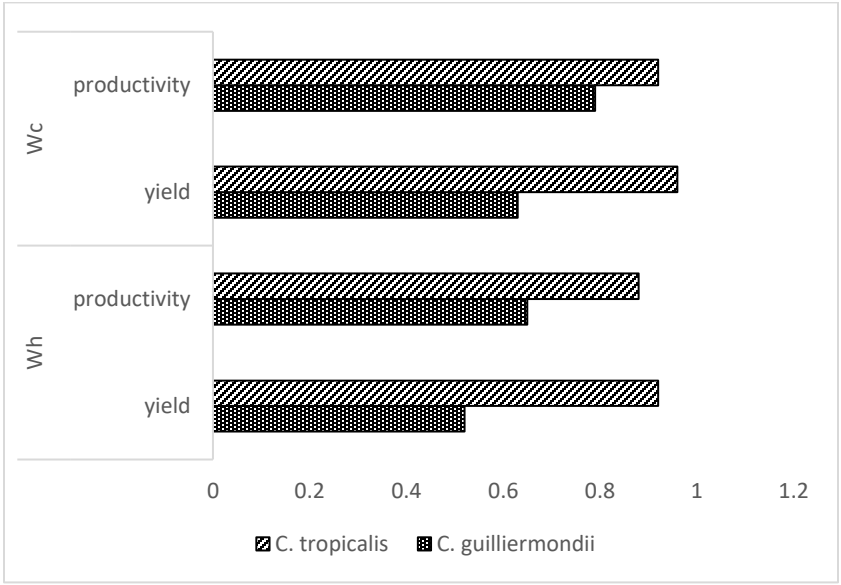

Figure 4.4. Comparison of xylitol yields and productivities of the fermentation of wood hydrolysates (W_h) and its control media (W_c) using *Candida tropicalis* and *Candida guilliermondii*.

Fermentation of the hydrolysate (W_h) and control media (W_c) using *C. guilliermondii*:

As described in the earlier sections, *C. guilliermondii* is one of best xylitol producers reported in the literature [7]. Therefore, we used it in the fermentation of the same hydrolysate and control

media to compare it to the capability of *C. tropicalis* strain, in producing xylitol. In the fermentation profile of *C. guilliermondii*, xylose consumption rate in hydrolysate and control media was found to be similar (Fig. 4.5). However, the xylitol yield (0.52 g g^{-1}) and productivity (0.65 g L^{-1} h^{-1}) obtained with *C. guilliermondii* is less than the results obtained with *C. tropicalis*. Though the maximum xylitol concentration (29.2 g L^{-1}) was observed in 48 hours of fermentation, it is less than the concentrations accumulated in the fermentation with *C. tropicalis*. In the fermentation of wood hydrolysate, the microbial growth rate was higher in case of *C. guilliermondii* with maximum uptake of xylose and relatively low production xylitol. It can be observed that the yield (0.92 g g^{-1}) obtained by *C.tropicalis* on the detoxified wood hydrolysate (W_h) was higher than the yield (0.52 g g^{-1}) obtained by *C.guilliermondii.* It is the highest yield obtained when compared to similar experiments reported in the literature (Table 4.2).

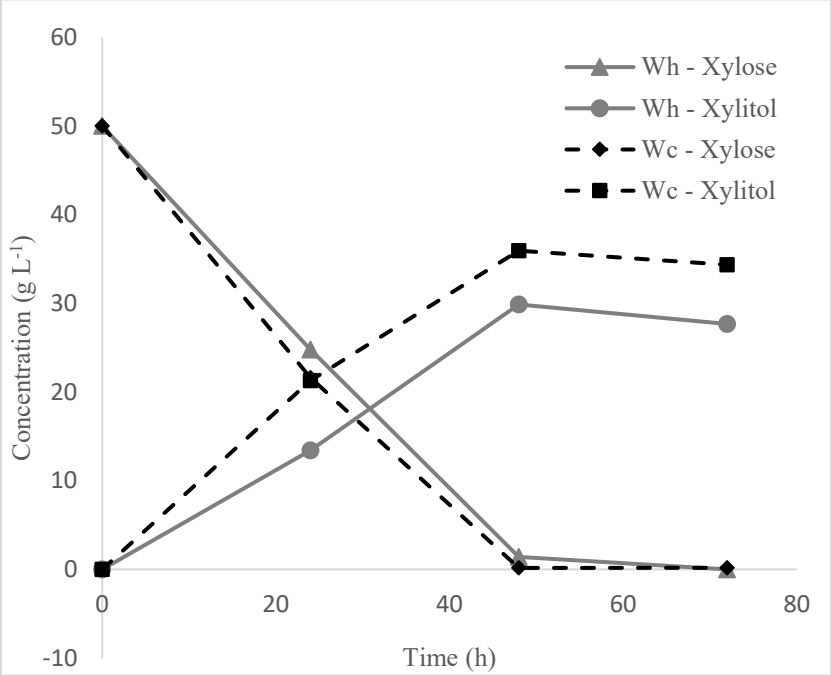

Figure 4.5. Fermentation profile of detoxified wood hydrolysate (W_h) and its control medium (W_c) using *Candida guilliermondii* FTI 20037 in terms of xylitol production and xylose consumption.

Table 4.2. Comparison of xylitol production from various detoxified and non-detoxified hemicellulosic feedstocks using various strains.

Microorganism	Feedstock	Detoxification method	Xylitol production			Reference
			concentration ($g\ L^{-1}$)	Yield ($g\ g^{-1}$)	Productivity ($g\ L^{-1}\ h^{-1}$)	
C. tropicalis CCTCC M2012462	Corncob hydrolysate	No detoxification	38.8	0.7	0.46	Ping et al. (2013) [3]
Candida guilliermondii FTI 20037	wheat straw	overliming	30.5	0.42	0.59	Canilha et al. (2008) [5]
C. magnoliae FERMP-16522	Corncob hydrolysate	Charcoal pellet adsorption	18.7	0.75	0.52	Tada et al. (2004) [33]
Candida mogii NRRL Y-17032	Rice straw	alkaline treatment	NA	0.71	0.46	Mayerhoff et al. (1997) [6]
C. tropicalis BCRC 20520	Hard wood hydrolysate	Activated carbon treatment and cation exchange resin	32.3	0.73	0.54	Ding et al. (2006) [34]
Candida tropicalis As 2.1776	corncob hydrolysate	overliming	58.3	0.74	0.61	Li et al. (2012) [8]
Candida guilliermondii FTI 20037	poplar wood hydrolysate	Vacuum evaporation followed by solvent extraction	29.2	0.52	0.65	This study
Candida tropicalis UFMG BX 12-a	poplar wood hydrolysate	Vacuum evaporation followed by solvent extraction	27.8	0.91	0.88	This study

4.5.3 Effect of Detoxification on fermentation

Both yeast strains used in this study showed notably high xylitol yields and productivities in the fermentation of detoxified wood hydrolysate when compared to the control media (Table 4.3). It was assumed that complete consumption of xylose in less time denotes faster microbial growth

rate. Therefore, it can be explained that efficient detoxification makes the microbe multiply rapidly and produce high amount of xylitol by faster xylose intake. The maximum xylitol production from the detoxified wood hydrolysate by using *C. tropicalis* and *C. guilliermondii* are compared in Table 4.3 with the fermentation results of their respective control media. It is evident from these experiments, that the components present in the hydrolysate like acetic acid and furfural (besides xylose) have significant effect on microbial production of xylitol.

Table 4.3. Fermentation results of wood hydrolysate (Wh) and its control media (W_c) using *C. tropicalis* and *C. guilliermondii.* The initial xylose concentration in both hydrolysate and control media was 50 g L^{-1}.

Yeast	Media	Time (h)	Xylose conc. (g L^{-1})	% Xylose consumed	Xylitol conc. (g L^{-1})	Xylitol yield (g g^{-1})	Xylitol Productivity (g L^{-1} h^{-1})
C. tropicalis	Wood hydrolysate control (W_c)	24	30.74	39.13	20.83	1.08	0.87
		48	3.92	92.32	44.26	0.96	0.92
		72	0.4	99.22	47.53	0.95	0.66
	Wood hydrolysate (W_h)	24	33.58	32.84	17.25	1.05	0.72
		48	6.58	86.84	42.17	0.92	0.88
		72	0.56	98.86	44.28	0.82	0.62
C. guilliermondii	Wood hydrolysate control (W_c)	24	21.62	50.78	21.28	0.74	0.95
		48	0.164	85.59	35.93	0.63	0.79
		72	0.163	85.58	34.34	0.57	0.50
	Wood hydrolysate (W_h)	24	24.7	45.74	13.27	0.51	0.59
		48	0.95	83.83	29.23	0.52	0.65
		72	0.4	85.12	27.3	0.38	0.4

The effect of our new detoxification method and the fermentation results obtained using *C. tropicalis* UFMG BX 12-a strain, in our study, was proven better by comparing with various studies reported in literature (Table 4.2). It is observed that detoxification has a significant effect

in xylitol production. In a study conducted by Canilha et al. [5], fermentation of wheat straw detoxified by overliming, and using *C. guilliermondii* resulted in xylitol productivity of 0.59 g L^{-1} h^{-1} which is similar to the productivity obtained in this work using same strain on different hydrolysate. However, using our new strain on the wood hydrolysate, the xylitol productivity was enhanced (1.5 times) to 0.88 g L^{-1} h^{-1} with high yields (0.92 g g^{-1}). Another study by Tada et al.[33], involved charcoal pellet adsorption detoxification of corncob hydrolysate which was fermented by *C. magnoliae* strain to produce xylitol with the yield of 0.75 g g^{-1} which is one of the highest yields reported in literature, but the productivity (0.52 g L^{-1} h^{-1}) was lower than our results. This can be due to the presence of inhibitors in the hydrolysate, which result in longer lag phase of microbial growth. Therefore, longer fermentation time results in lower productivity even though the yields are higher. It can be observed in the study conducted by Ping et al.[3], fermentation of non-detoxified corn cob hydrolysate using *C. tropicalis* strain resulted in high yield (0.7 g g^{-1}) but with low productivity (0.46 g L^{-1} h^{-1}). According to Li et al. (2012), high productivity of xylitol (0.61 g L^{-1} h^{-1}) can be achieved by detoxifying the corncob hydrolysate through overliming and subsequent fermentation using *C. tropicalis* strain [8]. However, a different strain of *C.tropicalis* in this study has shown higher productivities and better yields from the wood substrate detoxified by an integrated vacuum evaporation and solvent extraction techniques.

4.6 Conclusions

A new strain, *Candida tropicalis* UFMG BX 12-a, was used in fermenting wood hydrolysate, detoxified by a new detoxification method reported in this study. Vacuum evaporation and organic solvent extraction were combined to obtain the hydrolysate almost free of microbial growth inhibitors. It was observed that 97% of furfural and 87.5% acetic acid was removed from the acid hydrolysed wood hydrolysate. Fermentation of the detoxified wood hydrolysate has shown better xylitol production. Hence, it is necessary to remove the toxins or reduce the concentration of the inhibitors to the concentrations of 0.1-1 g L^{-1} in the hydrolysates to obtain higher levels of xylitol that are close to theoretical values. The fermentation of the detoxified wood hydrolysate using immobilized *Candida tropicalis* UFMG BX 12-a has shown high yield (0.92 g g^{-1}) and productivity (0.88 g L^{-1} h^{-1}) when compared to the reports in literature using various strains. A comparative study was also conducted by using an immobilized *C. guilliermondii* FTI 20037 strain on the same hydrolysate to prove the efficiency of the new strain in producing higher xylitol yields.

The usage of immobilized yeast also offers the reusability in further fermentation processes and make the xylitol production sustainable. This study also provides a good insight into the necessity of developing efficient upstream processes to produce xylitol in high levels from renewable biomass.

4.7 References

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CHAPTER 5

OBJECTIVE 3

A manuscript reporting this work has been submitted to the journal “Industrial and engineering Chemistry Research, ACS Press”.

5 Oxidative synthesis of succinic acid from renewable hemicellulosic pentose sugars using heterogeneous acid catalysts

5.1 Abstract

Novel approaches for the conversion of biomass based hemicellulose prehydrolysate to high value succinic acid (SA) has been investigated and reported in this chapter. A vital intermediate in this process is furfural (FL) which can be oxidized to produce succinic acid. The aim of this study was to produce succinic acid in acceptable yields from hemicellulose using a heterogeneous acid catalyst, Amberlyst 15 and hydrogen peroxide. Production of furfural in acceptable yields is a limiting step in such processes for a number of reasons. We have also demonstrated two methods using insitu extraction of furfural to an organic phase as it is produced. This resulted in an efficient method for producing succinic acid from hemicellulose with the molar yields of succinic acid from furfural ranging from 49 – 52% (mole $_{SA}$/mole $_{FL}$). In this study, the effect of the acid catalysts was also determined and found that 50 $mg_{cat}/mmol_{FL}$ is optimum to obtain 100% FA conversion in less time. Simultaneous production, extraction and conversion of furfural to succinic acid is the basis of the methods developed.

5.2 Introduction

Succinic acid (SA) was identified by the Department of Energy, US [1], as a platform chemical that will have a very high market in the coming years. It plays a major role as a building block in synthesis of several polymers [2, 3]. It has several applications in food, cosmetics, pharmaceuticals, biopolymers, polyesters, polyurethane, plasticizers and fine chemical industries [4-8]. In 2007, a market of 15 billion USD was projected for the chemicals which can be synthesized from succinic acid [9]. However, it has failed to reach such growth in production due to the high costs involved in its production of succinic acid. In 2015, the global production of succinic acid was 58.5 kilotons [10] and it is projected to reach 251.3 kilotons worth 701.0 million USD by 2021 [10].

Conventional industrial production of succinic acid involves the chemical conversion of maleic acid using heterogeneous metal catalysts like Pd/C and Zn/Hg [5]. Though the yields of succinic acid were high, concerns of the use of petroleum based resources and expensive catalysts motivate researchers to look for alternative raw materials. Renewable substrates like agricultural and forest based residues have high potential for the production of succinic acid. However, its production

from renewable raw material is not carried out in industrial level due to the low yields which makes its production costly. Statistical studies indicate that the US, for example, produces approximately 1 billion tons of inedible biomass from forests and agricultural lands [11]. Therefore, several researchers are exploring alternate routes for the production of SA from low value substrates to reduce the overall production costs involved.

Microbial fermentation of various substrates like hexose, pentoses and glycerol using *Actinobacillus succinogenes* [12], *Mannheimia succiniciproducens* [13] and *Anaerobiospirillum succiniciproducens* [14] have been reported [12, 15]. Though fermentative yields are high, the downstream processing costs limit the use of these methods. BioAmber is one of the recently established industries for the production of biosuccinic acid. However, its production is from corn starch [16] and not from inedible biomass like cellulose. Succinic acid produced from fermentation was estimated to cost 2.2 USD per kilogram with a production level of 5000 tons per year [17]. However, it has been projected that the price would drop to 0.55 USD if the production level increases to 75000 tons per year [17].

Alternative routes reported in literature for the production of succinic acid include the oxidation of 1,4-butanediol [18], carbonylation of ethylene glycol [3], hydrogenation of fumaric acid in presence of Ru catalyst [19], oxidation of levulinic acid [20] and condensation of acetonitrile to produce butanedinitrile which can be subsequently hydrolyzed to succinic acid [21]. Most of these routes involve metal catalysts to carry out the reactions. Recent studies have shown that succinic acid can also be produced from furfural using a chemical conversion pathway.

Extensive research has been done in the oxidative conversion of furan derivatives to various products using various metal catalysts [22]. Production of maleic acid [23], fumaric acid [7], furanone[24] and furandicarboxylic [25] acid from furfural has been reported in literature. An effective methodology reported by Choudhary et al. (2013) for the synthesis of succinic acid from pure furfural using hydrogen peroxide [5]. They have reported that Amberlyst-15 is an efficient replacement for the homogeneous acid catalyst in the oxidation of furfural in water. Amberlyst-15 is a sulfonated polystyrene based ion-exchange resin with 4.7 mmol/g acidity [26]. It has a similar effect as sulfuric acid (H_2SO_4) in the synthesis of carboxylic acids from furan derivatives [5, 26, 27]. The heterogeneous catalyst, Amberlyst-15 has an advantage because it exists in solid phase and can be recycled easily for the oxidation reactions of furan derivatives like furfural,

hydroxymethyl furfural, furoic acid etc. These furan derivatives are usually obtained from hexose and pentose sugars of edible and inedible crops. Recently, research has been focussed on the utilization of inedible renewable resources to produce chemicals. However, limited information is available in literature for the use of renewable resources such as hemicellulose prehydrolysate from agriculture or forest residue for the production of carboxylic acids such as succinic acid.

Xylose in hemicellulose can be converted to furfural which can then be converted to succinic acid [5]. The major drawback involved in the direct conversion of xylose to succinic acid is that furfural, an intermediate in this process, polymerizes and undergoes side reactions to form undesired products. It is important to avoid these side reactions during such conversions without much loss in the substrate. Avoiding the side reactions and the conversion of furfural to succinic acid in acceptable yields was the focus of this study.

5.3 Material and Methods

5.3.1 Substrate and standards

Hemicellulose prehydrolysate was obtained from GreenField Ethanol Inc., Canada. They had produced it from poplar wood chips using a patented pretreatment process [28]. The standards of furfural and succinic acid were purchased from Sigma Aldrich. Xylose, hydrogen peroxide, sulfuric acid and organic solvents viz toluene, ethylacetate, chloroform were purchased from Fisher Scientific. All the chemicals and solvents were used without further purification.

5.3.2 Experimental procedure

Two methods were developed and studied for the production of succinic acid from hemicellulose prehydrolysate.

5.3.2.1 Method – I: Production and separation of furfural and subsequent conversion to succinic acid

In this method, the hemicellulose prehydrolysate was hydrolysed to obtain furfural. The hydrolysis was optimized to determine the ideal acid concentration and conditions (temperature and pressure) to obtain furfural in high amounts. The furfural formed in the hydrolysate liquor was extracted using organic solvents. Organic solvents such as chloroform, ethylacetate, and toluene, were evaluated for their ability to extract furfural from aqueous phase. The best solvent, based on furfural extractability, was used in the conversion studies. Fig.5.1 shows the outline of method I and the details of the process are explained step wise in the following sections.

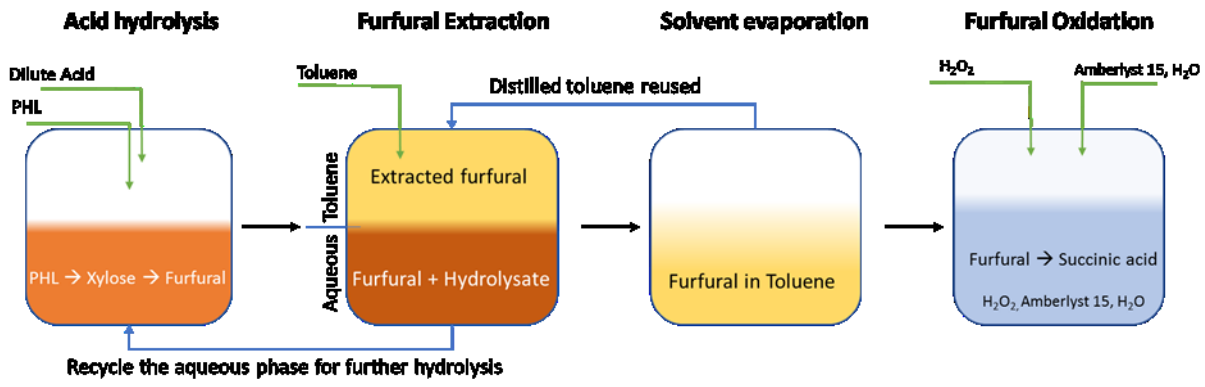


Figure 5.1. Schematic representation of production of furfural from hemicellulose prehydrolsate (PHL) and subsequent oxidation to succinic acid.

a) Hydrolysis of hemicellulose prehydrolysate

Acid hydrolysis was done using dilute sulfuric acid at different pressure conditions. Several reports in literature suggest that yield of furfural is higher when the hydrolysis is done under pressure (For e.g. in a Parr reactor) [29]. Therefore, acid hydrolysis was carried out under pressure and at atmospheric pressure to obtain furfural and precursor (xylose) of furfural. Sulfuric acid (10% w/w) was added to the prehydrolysate (50 mL) in a 250 mL Erlenmeyer flask. The contents of the flask were mixed well and placed in an autoclave maintained at 15 psi and temperature to 121ºC for 15 mins. In another experiment at atmospheric pressure, the reaction mixture was taken in a 250 mL round bottomed flask and placed in an oil bath. The round bottomed flask was connected to a condenser to prevent the solvent from evaporation. Then the temperature of oil bath was maintained at 120ºC and the reaction mixture refluxed with stirring for 4 h. Sulfuric acid concentration of 10% and 2% (w/w) were used in separate hydrolysis experiments (fig. 5.2). After the hydrolysis, flask was cooled down, excess sulfuric acid was neutralized to a pH of 6.5 – 7.0. Hydrolysate samples were diluted 20 times with distilled water for the composition analysis using an HPLC.

b) Separation of furfural from the hydrolysed prehydrolysate (hydrolysate)

The aqueous hydrolysate was mixed with equal volume of organic solvent. The immiscible mixture of two phases (organic/aqueous) was taken in a separating funnel and mixed well to extract furfural into the organic phase. The two phases were allowed to stand and the aqueous phase was then separated from the organic phase. The aqueous hydrolysate was washed three times with

organic solvent in order to extract furfural completely. All fractions of the organic layer extractants were combined and then dried with anhydrous sodium sulfate which absorbs water molecules dissolved in the organic phase. The organic solution with furfural was then analyzed using a GC-FID to determine the concentrations of furfural. Subsequently, the organic solvent was evaporated in a rotary evaporator under vacuum and furfural was obtained as a residue and was used for the synthesis of succinic acid.

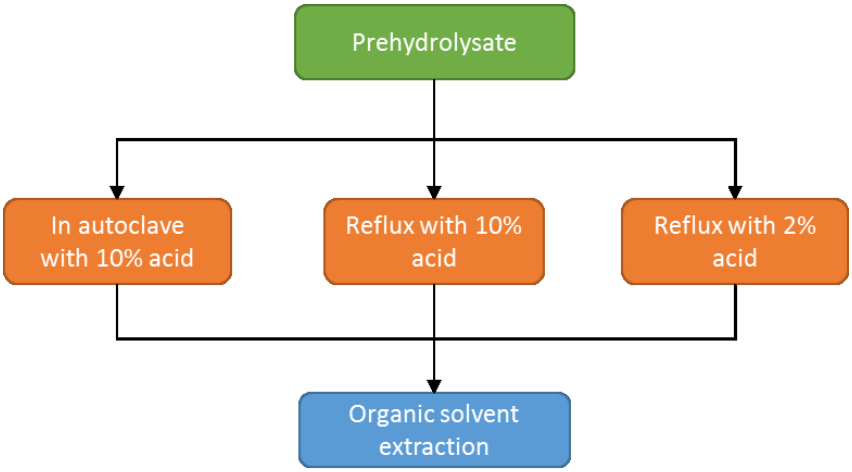


Figure 5.2. Alternate conditions of acid hydrolysis of prehydrolysate with different acid concentrations before solvent extraction of furfural.

c) Synthesis of succinic acid by the oxidation of furfural

The oxidation of the separated furfural residue was carried out in a round bottomed flask attached to a condenser. Furfural (1 mmol) produced from previous step was added to a flask containing water (50 mL) and mixed well. The heterogeneous acid catalyst Amberlyst 15 (50 mg) and the oxidizing agent, hydrogen peroxide (4 mmoles) were then added and the mixture was stirred. The round bottomed flask containing the reaction mixture was attached to the condenser and placed in an oil bath. The temperature of the oil bath was increased to make the reaction temperature reach 80^{o}C and maintained throughout the reaction for 24 h. These conditions were adopted from the studies conducted by Choudhary et al (2013) on the oxidation of furan derivatives using hydrogen peroxide [5]. At the end of reaction, the reaction mixture was diluted 10 times and the catalyst was filtered out. The recovered catalyst was reused after washing it with water a couple of times and drying overnight. The water in product solution was evaporated under vacuum. The residue was dissolved in minimum amount of water (3 mL) and filtered. The resultant solution was kept in

refrigerator for the succinic acid to crystallize. The solid product was filtered and dried under vacuum to obtain white crystalline solid. The resultant products were analyzed using HPLC and NMR techniques.

5.3.2.2 Method II: Simultaneous production, separation and oxidation of furfural

In this method, a biphasic system of organic and aqueous solvent was used to produce furfural and succinic acid. Based on the results obtained in the previous method, an ideal organic solvent (toluene) was used in this method to separate furfural from aqueous layer simultaneously while it is produced. However, during succinic acid synthesis, the organic solvent serves as a reservoir of furfural which continuously supplies furfural to the aqueous layer. (Fig. 5.3) shows a schematic representation of this method and the details of the procedure are given below.

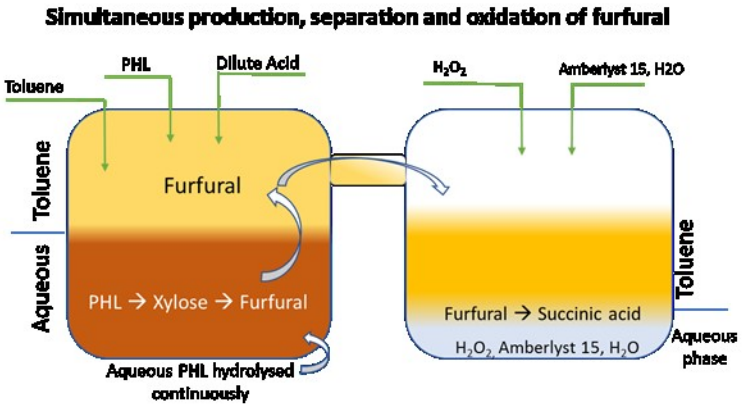


Figure 5.3. Schematic representation of simultaneous production, separation and oxidation of furfural to produce succinic acid.

a) <u>Production of furfural from prehydrolysate in biphasic system</u>

The aqueous hemicellulose prehydrolysate (50 mL) was added to toluene (125 mL) in a round bottomed flask at room temperature resulting in a two-phase system. Sulfuric acid (2% w/ w $_{\text{aqueous layer}}$) was carefully added to the biphasic system while the reaction mixture was stirring. The round bottomed flask was attached to a reflux condenser and placed in an oil bath. The temperature of the oil bath was increased to maintain the inside temperature of round bottomed flask at 100 ºC and maintained for 4 hours. The system was then cooled down to room temperature. The aqueous layer (Aq.-I) separated out from toluene phase. The toluene solution was used for the synthesis of succinic acid. The aqueous layer containing unconverted xylose was hydrolysed again until all the xylose present was converted.

b) Succinic acid synthesis in biphasic system

The toluene phase of solution obtained was mixed with 10% (v/v) deionized water, Amberlyst 15 (50 mg) and hydrogen peroxide (4 mmole). The temperature was then increased to 80 ^{o}C and maintained for 24 h. After the reaction, the aqueous layer (Aq.-II) and the catalyst, Amberlyst 15 were separated from organic phase. The aqueous layer was concentrated in rotary evaporator and filtered any undissolved particles. The resultant solution was kept in a refrigerator to crystallize the succinic acid. The crystallized product was analyzed using an NMR to confirm the product. The toluene phase was analyzed for the residual furfural using a GC-FID. Once most of the furfural in toluene was converted, it was distilled to obtain relatively pure toluene and reused for subsequent batches of experiments.

5.4 Analytical techniques

5.4.1 HPLC

The composition of hemicellulose prehydrolysate and aqueous layers (Aq. I and II) separated were analyzed using an HPLC (Agilent Technologies 1260 Infinity) with Bio-Rad Aminex HPX-87H ion exclusion column (300mm x 7.8mm) and a Refractive Index Detector (RID). The mobile phase used in this method was 5 mM H_2SO_4 with a flow rate of 0.5 mL/min at 50 ^{o}C. The instrument was calibrated with standards of varying concentration and the response factor (RF) obtained for the standards was used to calculate the concentrations of the products formed.

5.4.2 GC-FID

Furfural in toluene was analyzed using a Thermo-scientific GC (Trace 1300 series) with Flame Ionization Detector (FID) and a capillary column (Trace Gold -TG-WAXMS A) (30 m length, 0.25 mm internal diameter, and 0.25 μm film thickness of cross linked polyethylene glycol). A new method was developed to analyze furfural with low retention time. A ramped flow rate of the carrier gas was used with initial flow rate of 5 mL/min for 0.74 min and subsequently reduced and maintained at 4 mL/min until the end of the run. The temperatures of the oven and the detector were maintained at 200^{o}C and the inlet temperature was maintained at 250^{o}C. Split mode (split flow: 200; split ratio: 40) injection was used in the analysis. Initially, the GC was calibrated with standards at different concentrations. Response factor (RF) of the standard furfural was obtained by the equation: [Peak area] = RF[standard concentration]. The obtained RF was used to determine the unknown concentration of furfural in the samples obtained during the reaction.

5.4.3 NMR

The purified products were analyzed and confirmed by using NMR spectroscopy ("Varian-NMR" Inova-500 MHz) by elucidating their structure with ^{1}H NMR and ^{13}C NMR spectra. Deuterated water and chloroform were used to dissolve the products for the NMR analysis.

5.5 Results and Discussion

Traditionally, succinic acid is produced at large scale using either metal catalyzed conversion of maleic acid or fermentation of glucose. However, xylose obtained from the pretreatment of prehydrolysate also has high potential to be converted into succinic acid via furfural. Very few reports in literature discuss the conversion of furfural to succinic acid [5, 30]. However, succinic acid production from hemicellulose found to be very challenging because of several constraints associated with furfural production and separation. As described earlier, furfural is formed by the loss of 3 water molecules from xylose in presence of acid. During the hydrolysis, a polymerization reaction results in loss of furfural by converting into unwanted byproducts. One way to inhibit such reactions is to separate furfural from the aqueous media intermittently. A common technique used to separate furfural is distillation. However, it forms an azeotrope with water when it reaches the level of 35% (wt/wt) in the aqueous solution and cannot be concentrated by distillation. Hence, the production of SA from hemicellulose requires an alternative approach. In order to reduce the cost of production, taking into account the challenges involved, a new approach is necessary to produce SA. In the following sections, we have extensively discussed the novel methods we developed to produce succinic acid from hemicellulose prehydrolysate.

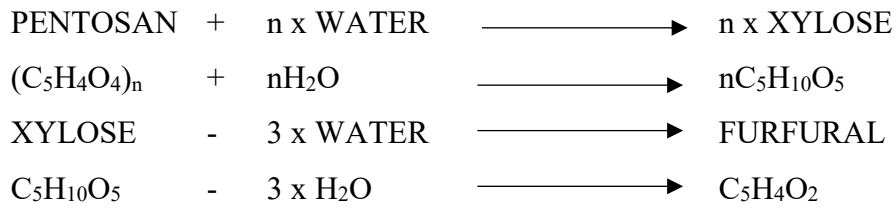
5.5.1 Method I: Production of furfural, its separation and subsequent conversion to succinic acid

In this method, prehydrolysate was hydrolysed to get furfural which was then separated by an organic solvent. The extracted furfural was then purified and oxidized to succinic acid in different conditions. The details of each step along with the parameters and solvents studied are discussed in detail in the following sections.

5.5.1.1 Hydrolysis of hemicellulose to furfural

In the production of succinic acid from hemicellulose prehydrolysate, furfural plays an important role as the precursor of succinic acid. Hence, it is essential to produce furfural in good quantities which is subsequently converted to succinic acid. The major sugar component of hemicellulose,

i.e., xylose, dehydrates to form furfural in the presence of acid catalysts. Various acid catalysts have been studied and discussed in literature for the conversion of xylose to furfural [31, 32, 33]. These reports indicate that sulfuric acid facilitates efficient hydrolysis. Therefore, it has been chosen to produce furfural from the hemicellulose prehydrolysate. Several processes and methodologies of acid hydrolysis have been invented and developed in the past and well discussed in literature [31]. In this study, we used sulfuric acid, a common acid catalyst used in the production of furfural from xylose. Due to environmental concerns, it is always recommended to use dilute sulfuric acid at low dilutions. According to British Pharmacopoeia, any concentration below 10% (w/v) of sulfuric acid is considered as dilute [34]. We used dilute sulfuric acid from 2 - 10% (w/w) to compare the furfural production capability. As discussed in chapter 3, sulfuric acid concentration below 2% yields relatively lower yields of furfural (see Table 5.1). The reactions occur in the prehydrolysate during acid hydrolysis is shown in the Scheme 5.1.



Scheme 5.1. Schematic representation of reactions take place during acid hydrolysis of prehydrolysate.

Table 5.1 shows that the hydrolysis of prehydrolysate using 10% acid and 2% acid yielded 1.28 g/L and 6.0 g/L furfural, respectively. The furfural yield from the former with higher levels of sulfuric acid was lower because the furfural formed was converted by different side reactions to form other products like humins. The amount of humins observed in 10% acid hydrolysis was 33.3 g/L, whereas in experiments with 2% acid, the humin content was only 6.6 g/L. Therefore, it is evident that the reaction mixtures with a high acid concentration results in the polymerization of furfural to insoluble resins. However, some reports suggest that production of furfural is much effective under pressure [29]. Therefore, we used an autoclave for the acid hydrolysis of prehydrolysate at 15 psi pressure and 121°C temperature with 10% sulfuric acid. We have observed that furfural concentration increased to 5.91 g/L with 33 g/L of humins. Even with 2% sulfuric acid, the humin formation was found to be relatively high. Though the acid hydrolysis under high pressure increase the yields of furfural, it also induces the humin formation, substantially which results in furfural loss. Therefore, by performing the acid hydrolysis at atmospheric pressure, the

polymerization of furfural can be slowed down and it is possible to obtain relatively higher levels of furfural.

For the subsequent experiments, the acid concentration was lowered to 2% and hydrolysed the prehydrolysate under reflux at 120^{o}C. As expected, it resulted in lower amounts of humins (6.6 g/L) but high amounts of furfural (6 g/L). Therefore, it is evident that by decreasing the acid concentration, the furfural polymerization was suppressed. Due to the slow process of the side reaction, furfural was accumulated and resulted in acceptable yields.

These results were observed from the acid hydrolysis reactions which were carried out for 4 h. The xylose (96 g/L) obtained in the hydrolysis can be treated with acid repeatedly to convert it completely to furfural. However, continuous acid treatment is not recommended because after 5 - 6 h with this acid concentration, furfural found to decrease due to the polymerization with xylose. Therefore, separating aqueous xylose solution from furfural after 4 h of acid hydrolysis is recommended to avoid polymerization reaction with furfural. Subsequently, the separated xylose can be hydrolysed again to produce more furfural.

Table 5.1. The concentrations of xylose, furfural and humins obtained in the acid hydrolysis of prehydrolysate after 4 h using different acid concentrations.

	10% acid hydrolysis at atmospheric pressure	10% acid hydrolysis in autoclave	1% acid hydrolysis	1.5% acid hydrolysis	2% acid hydrolysis	2.5% acid hydrolysis
Xylose (g/L)	76	75	84	80	96	79
Furfural (g/L)	1.28	5.91	4.16	5.8	6.0	6.24
Humins (g/L)	33.3	33.2	2.7	4.8	6.6	7.8

5.5.1.2 Furfural separation

In most of the literature in this area, the major challenges reported for the acid hydrolysis of hemicellulose are i) separation of furfural ii) the formation of humins due to polymerization of furfural with xylose. Steam distillation is a common and widely used technique in industries to separate furfural from an aqueous phase. However, separation by such method was found to be challenging as it forms azeotropic mixture with 35% of furfural and 65% of water by weight in the

solution at 370 K under atmospheric pressure [35]. Therefore, it is still difficult to separate all the furfural from the aqueous phase.

Other novel techniques are also being explored to separate furfural from aqueous phase without any loss. Recently, Song et al (2015) reported a gas stripping assisted vapour permeation (GSVP) method and studied its energy efficiency [36]. Adsorption on polymeric resins [37], pervaporation using hydroxy-terminated butadiene polyurethane membranes [38] and a patented technology using organic acids [39] are some of the recent developments in furfural separation. However, they are not feasible at large scale.

Another technique is the extraction using an immiscible organic solvent having high furfural solubility that is capable to extract furfural from aqueous phase. A biphasic system with a good organic solvent can be used during acid hydrolysis. This can be beneficial as the furfural produced in the hydrolysate can immediately be transferred to the organic phase and prevents the side reactions or polymerization. We have studied this method and determined a suitable solvent for furfural extraction. Several non-polar solvents were evaluated for their solubility of furfural and extractability from water. Simulation studies were also conducted using Aspen Plus software to determine the mutual solubility of organic phase, aqueous phase and furfural.

Solvent determination to extract furfural

A good organic solvent can greatly enhance the extraction of furfural from the aqueous phase without interfering in the reaction. For this purpose, three solvents, chloroform, ethylacetate and toluene were evaluated for the solubility and extractability of furfural from aqueous phase (Fig. 5.5). It was found that furfural is highly soluble in all the solvents used in this study. However, the extractability differs in each case. Three sets of aqueous furfural solutions (1 mL) were prepared with amounts ranging from 10 to 100 mg in water. However, the maximum solubility of furfural was found to be 72 – 75 mg/mL. The vials with more than 75 mg of furfural resulted in two phases with excess undissolved furfural.

The aqueous solution with dissolved furfural was taken to examine the extractability of organic solvents. The organic solvents (1 mL) under study were added to the aqueous furfural solution. The final concentration of furfural in organic solvent after extraction was analyzed using a GC-FID. Fig.5.4 shows the concentration of furfural found in the organic solvents after extraction from

aqueous layer. The extractability (%) was calculated from the concentrations of furfural in organic solvents obtained from GC results. From the Fig.5.4 it is evident that toluene extracted 80 – 85 % of furfural from aqueous phase at all concentrations. The extractability of the solvents was found to be in the order of toluene > Chloroform > Ethylacetate. However, 100% extraction of furfural was not observed in either case because of the mutual solubility of the solvents (water, organic solvent and furfural) present in the system resulting in two phase ternary systems. From the graph (Fig. 5.4), it is evident that toluene extractability was almost constant with various furfural concentrations. However, other solvents seem to be losing their extractability. This can be attributed that with the change in concentrations of three phases (organic solvent/water/furfural), some of the organic solvent was lost in the extraction and resulting in less furfural concentration during the analysis.

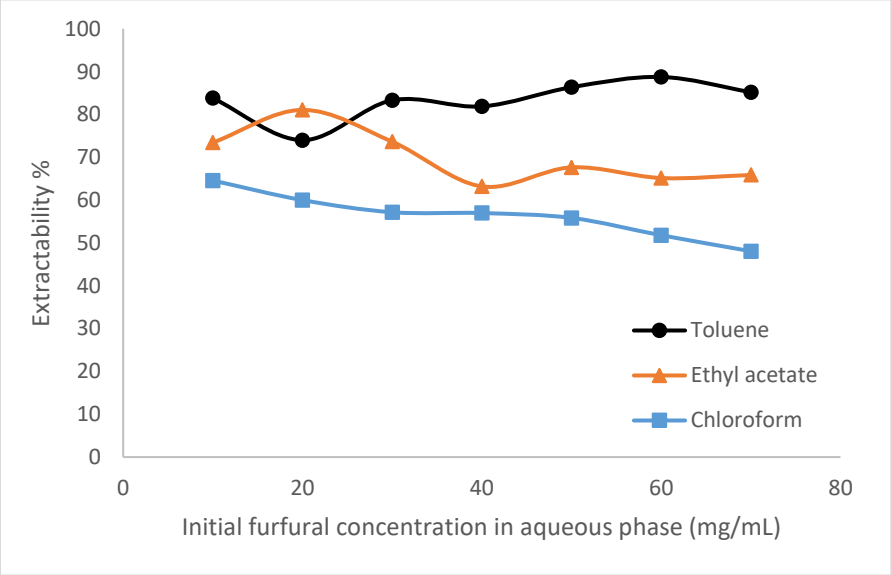


Figure 5.4. Graphical representation of extractability of the organic solvents to extract from aqueous phase (O/A ratio = 1:1).

The mutual solubility of the organic solvent and water was evaluated by plotting ternary diagrams using Aspen Plus software (version 8.4). Ethylacetate and toluene were evaluated as they found to solubilize furfural in high quantities. Each ternary diagram (Fig. 5.5 and 5.6) represents the mutual solubility of the aqueous, organic and furfural in the system. In both the ternary diagrams, the regions outside the envelop are single phase regions while inside the envelop are two phase regions. The equilibrium solubility curves which form the envelop are shown in blue color and separates the two-phase regions from the single-phase regions. Three tie lines which are

connecting the two equilibrium solubility curves are shown in black, red, green and magenta colors. The vertices of the triangle represent pure components. The sides of triangle connecting any of two vertices represent mixture of two components. In the ternary diagrams, the left side of the triangle represents mixture of organic solvent and furfural in a single-phase region and illustrates organic layer and furfural are completely miscible in each other. The base of the triangle represents the miscibility of water and organic solvent. In fig. 5.5 a, it is clearly shown that water solubility in toluene phase is very low (~1%) whereas the solubility of toluene in water is also negligible (zoomed in fig. 5.5 b). However, in fig. 5.6 a, the solubility of water in ethyl acetate was found to be much higher (~22.5%) whereas ethylacetate was also slightly soluble in water (Fig. 5.6 b) which is relatively higher when compared to toluene.

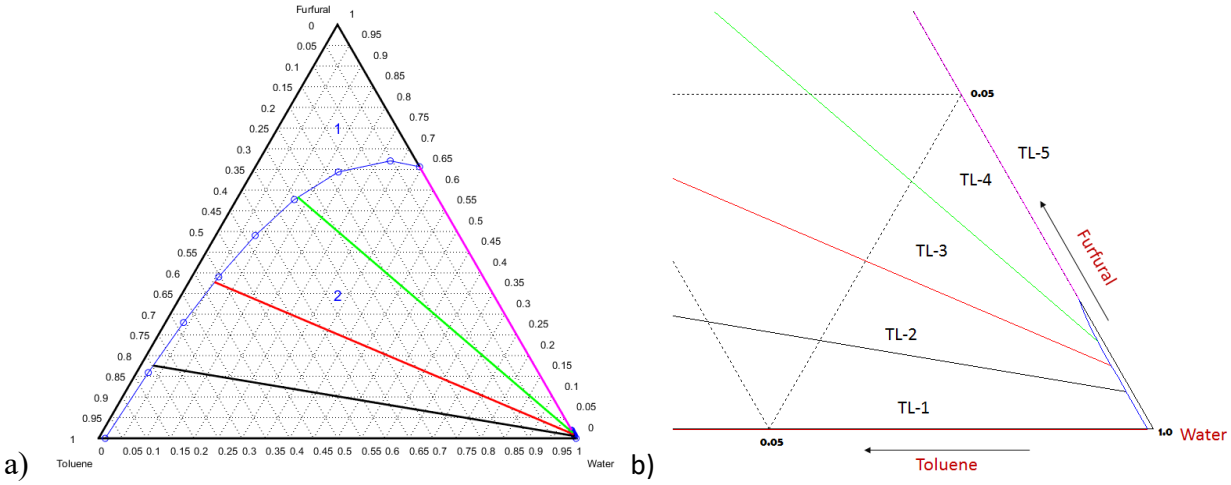


Figure 5.5. a) Ternary diagram of liquid-liquid phase of toluene/water/furfural system simulation. b) Zoomed view of the solubility of toluene in water.

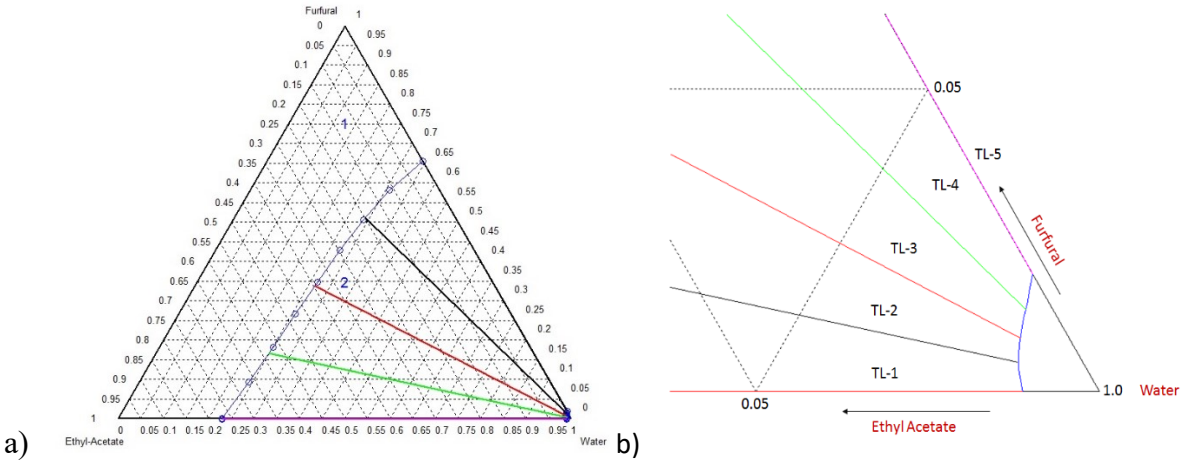


Figure 5.6. a) Ternary Liquid-Liquid phase diagram of water/furfural/ethylacetate system. b) Zoomed view of the solubility of ethyl acetate in water.

From these data, it is found that water is less soluble in toluene than in ethyl acetate. Therefore, the use of toluene as an organic solvent was found to be the best for extraction of furfural from the aqueous phase and used in the rest of this study. The aqueous solution with furfural after acid hydrolysis of hemicellulose was washed with toluene 3 times to extract all the furfural in subsequent experiments. The toluene phase was found to extract 6 g of furfural per liter of hemicellulose prehydrolysate. For the succinic acid synthesis in this method, toluene was evaporated under vacuum to obtain pure furfural which was then oxidized further.

5.5.1.3 Succinic acid synthesis

Furfural obtained from the previous step was oxidized to succinic acid using hydrogen peroxide in presence of Amberlyst 15. In this method, the oxidation was directly carried out in water. Choudhary et al. (2013) have studied the effect of various concentrations of acid and hydrogen peroxide on the oxidation of furan derivatives [30]. In their study, the mole ratio of hydrogen peroxide to furfural required for high yields of carboxylic acids was 4:1. Higher or lower concentrations of hydrogen peroxide results low yields of succinic acid. Therefore, in our study, we have used hydrogen peroxide 4 times to the mole fraction of furfural obtained in previous step.

An acid catalyst must be used with hydrogen peroxide to produce succinic acid. Hydrogen peroxide alone oxidizes the furfural present in aqueous phase and produces furoic acid. But, in presence of acid catalyst, hydrogen peroxide selectively yields succinic acid from furfural [5]. Sulfonic acid functional group on Amberlyst 15 is mainly responsible for succinic acid selectivity during the oxidation of furfural. Studies on the effect of various acid homogeneous acid catalysts like p-tosylic acid, hydrochloric acid, sulfuric acid, and heterogeneous acid catalysts like Amberlyst 15, Nafion NR50, Nafion SAC-13, γ- $Al2O_3$, Nb_2O_5, ZrO_2 has been reported elsewhere [30]. Based on their report, Amberlyst 15 was chosen for our study as it has shown good catalytic activity and can be reused.

The effect of amount of Amberlyst 15 in the reaction system was determined to study the variation in the yield of succinic acid and furfural conversion. A range of Amberlyst 15 catalyst amount (10 – 50 mg/ mmol furfural) were used to determine their effect on succinic acid synthesis. It was observed that furfural is getting converted in the first few hours of reaction while the succinic acid

forms later. From the fig.5.7, it is evident that 50 mg of Amberlyst 15 per each mmol of furfural was converting 100% of furfural within 4 h of reaction time. It shows that higher the catalyst loading, faster the conversion of furfural. Lower amounts of Amberlyst 15 found to convert furfural much later than 50 mg of catalyst. Hence, that amount was chosen to carry out the reaction to synthesize succinic acid from furfural.

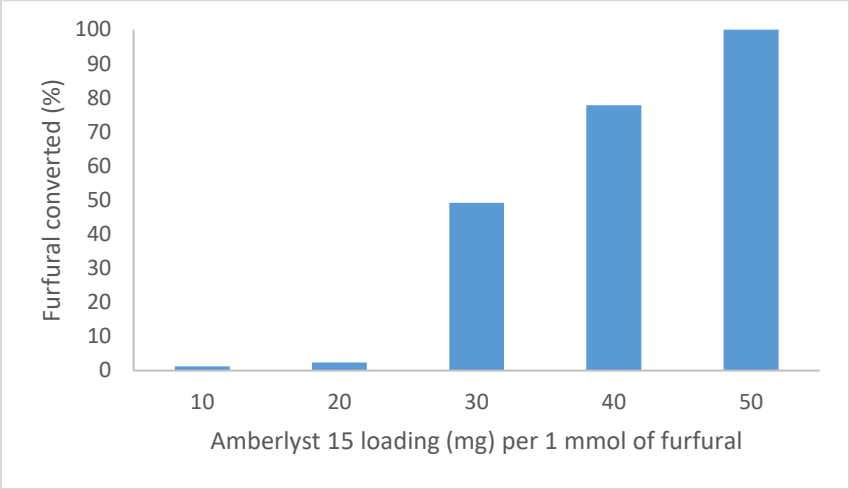


Figure 5.7. Graphical representation of effect of catalyst loading in the oxidation of furfural at 4 hours of reaction time.

The reaction of furfural oxidation to succinic acid was monitored and few samples were taken during the reaction. Each sample was extracted with toluene to remove furfural from the aqueous phase. Succinic acid is highly polar and insoluble in organic solvents like toluene. Therefore, the aqueous phase was analyzed for succinic acid content using HPLC whereas the toluene phase was analyzed using GC-FID for furfural content. The yield % at different times are shown in fig.5.9. At the end of reaction, the final yield of succinic acid was found to be 52.4 % (mole $_{product}$/mole $_{substrate}$) at 48 h (fig.5.9). Though the furfural gets consumed within 4 h of reaction, the succinic acid yield was low. It may be due to the formation of reaction intermediates (not identified) which were converted to succinic acid later.

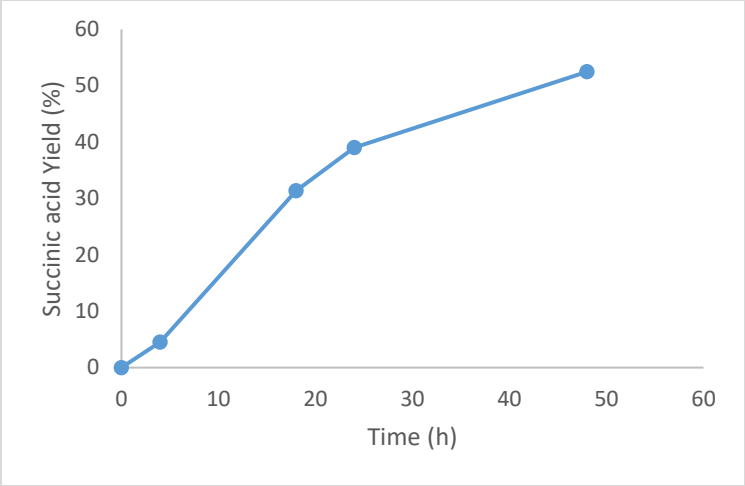
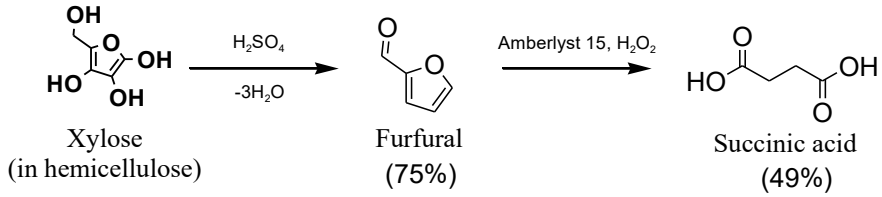


Figure 5.8. Succinic acid yield profile as a function of time in the conversion method I.

Based on the solvent extraction, we have developed and studied a biphasic system to reduce the steps involved in succinic acid production and eventually obtain high yields.

5.5.2 Method II: Succinic acid synthesis from furfural using two-solvent (biphasic) system

A two-solvent system was developed in which the hydrolysis of hemicellulose prehydrolysate with simultaneous furfural production and separation was demonstrated (Fig.5.3). Frufrual produced in aqueous phase was found to be migrated to the organic phase. Therefore, furfural in the organic layer was subsequently converted in to succinic acid in the same system. The two stages (Scheme 5.2), hemicellulose to furfural conversion and furfural to succinic acid conversion is discussed in the following section.

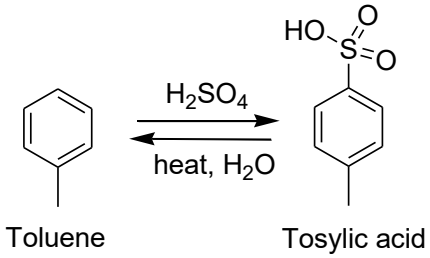


Scheme 5.2: Overall reaction in the production of succinic acid from hemicellulose

a) Hemicellulose to furfural

In a biphasic system of water and toluene, the furfural produced in the aqueous layer will be immediately transferred to toluene and avoids the formation of humins and furfural polymerization. This also allows quick furfural separation.

Xylose in aqueous prehydrolysate is converted into furfural with the help of an acid catalyst, sulfuric acid. In situ, sulfuric acid reacts with toluene used in the biphasic system and is converted to tosylic acid (Scheme 5.3) which is evident from the formation of a thick slurry immediately after addition of sulfuric acid to the biphasic system. However, hydrolysis is not affected because tosylic acid itself acts as a strong organic acid which is capable of carrying out the acid hydrolysis. Moreover, it was observed that when the reaction medium is heated to the required temperature, tosylic acid reverts back to toluene and sulfuric acid in presence of water and the biphasic system is reformed after 40 – 60 minutes of reaction. Therefore, hydrolysis of hemicellulosic xylan polymer is facilitated in the biphasic system along with simultaneous conversion of xylose to furfural in aqueous layer. Subsequently, furfural produced in the aqueous layer is rapidly transferred to the toluene layer. This was monitored by analyzing the reaction sample in GC-FID. The final concentration of furfural obtained in toluene phase after the reaction was 1.3 g/L after 4 h of reaction.



Scheme: 5.3. Conversion of toluene to tosylic acid in presence of acid.

b) Oxidation of furfural to succinic acid

Furfural obtained in toluene phase was converted to succinic acid in presence of hydrogen peroxide and Amberlyst 15. However, this process takes place in the aqueous phase as hydrogen peroxide is miscible in water and immiscible in toluene. Therefore, addition of water to the toluene phase with furfural is necessary to facilitate the oxidation of furfural in presence of acid catalyst. Though the volume of water (10 mL) is much less than toluene (50mL), it is sufficient enough to solubilize succinic acid even the yield reach 100%. According to the Institute for Occupational Safety and Health of the German Social Accident Insurance database, the maximum solubility of succinic acid in water is 58 mg/mL at room temperature [40]. Therefore, we used 10 mL water so that succinic acid is not saturated in the aqueous phase.

As mentioned earlier, furfural oxidation takes place in the aqueous phase. Though furfural selectively dissolves in toluene, it is also dissolved in small amounts of water present in toluene phase. Therefore, the partial amount of furfural dissolved in aqueous phase of the biphasic system gets oxidized to succinic acid. Due to the imbalance of furfural equilibrium in two phases during the reaction, furfural tends to transfer into the aqueous phase continuously. Simultaneously, hydrogen peroxide in presence of acid catalyst in aqueous phase oxidizes the transferred furfural. The volumetric ratio of aqueous phase to toluene phase chosen for this reaction seems to be ideal as total furfural (>99%) in toluene found to be transferred and converted. It was confirmed with GC analysis of the organic layer. Succinic acid produced in aqueous phase does not dissolve in toluene and remain in aqueous phase as it is highly polar and insoluble in organic solvents. The final product, succinic acid was quantified using an HPLC.

The final yield of succinic acid from furfural was found to be 49.7% in 24 h. It was observed that in the biphasic system, the reaction was found to be much faster than previous method and achieved similar yields were achieved in less time. Therefore, continuous slow addition of furfural, instead adding all together, is necessary to increase the reaction rates. In this case, toluene acts as a reservoir for furfural and continuously supplies furfural to the aqueous phase where the oxidation takes place. Therefore, the developed biphasic system found to have advantages for production and synthesis of succinic acid from hemicellulose prehydrolysate.

5.6 Conclusions

Hemicellulose prehydrolysate was converted to furfural using an optimized acid hydrolysis method with 2% sulfuric acid at 100ºC. An ideal organic solvent (toluene) for furfural extraction from aqueous phase was determined and used in the biphasic reaction systems. Acid hydrolysis of hemicellulose produces furfural which was separated immediately by using an organic solvent (toluene) in the reaction system. This avoids the unwanted reactions which results in resinification of furfural. The developed method found to be efficient in converting hemicellulose prehydrolysate to succinic acid using hydrogen peroxide (1:4 furfural to H_2O_2 mole ratio) and Amberlyst 15 (50 mg) as catalysts. The effect of acid catalyst loading was determined and observed that higher the catalyst loading, faster the furfural conversion. However, in our study, 50 mg of Amberlyst 15 per mmol of furfural was found to convert 100% furfural in 4 h of reaction time. Heterogeneous catalyst used in this study also has an advantage as it can be recycled and reused. The molar yields

of succinic acid obtained from furfural was 39 % via method I whereas 49% via method II in 24 hours.

The biphasic system used in this study facilitates simultaneous production, separation and oxidation of furfural to produce succinic acid. The solvent, toluene used in this method helps bypass the polymerization reaction to the high extent and directs the oxidation selectively towards succinic acid. An advantage with the insolubility of the produced succinic acid in the solvent toluene helps easy separation after the reaction. This study shows the potential for the utilization of low value hemicellulose prehydrolysate to produce high value succinic acid.

5.7 References

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CHAPTER 6
OBJECTIVE 4

The manuscript of this work has been submitted to the journal "Sustainable Chemistry and Engineering, ACS Press"

My Contribution

Experimental design, experimental work and write up

Co-author's contribution

Dr. Justin Jiang, Department of Chemistry, Lakehead University – Experimental design suggestions and co-supervision on proposing reaction mechanism and interpretation of the instrumentation data

6 Synthesis and mechanistic studies of levulinic acid from hemicellulose derived furfuryl alcohol using heterogeneous acid catalyst and the determination of byproducts

6.1 Abstract

Furfuryl alcohol was converted to ethyl levulinate and levulinic acid in ethanol and water, respectively, using sulfuric acid (homogeneous acid catalyst) and Amberlyst 15 (heterogeneous acid catalyst). A reaction mechanism of the conversion of furfuryl alcohol to ethyl levulinate in presence of sulfuric acid was investigated using gas chromatography-mass spectroscopy (GC-MS) and nuclear magnetic resonance (NMR) spectroscopy. We have identified several intermediates and confirmed four molecules from the GC-MS and NMR data and the structural elucidation of these intermediates was explained. A feasible reaction pathway in different routes was proposed based on the identified intermediates. Furthermore, it was proven that the production of ethyl levulinate from furfuryl alcohol in ethanol takes place in different pathways which eventually lead to ethyl levulinate. The conversion yield of furfuryl alcohol to ethyl levulinate in ethanol was 86 - 90%, which was higher than the conversion yield of furfuryl alcohol to levulinic acid in water (18-23%).

6.2 Introduction

The conversion of glucose to carboxylic acids like levulinic acid, one of the top twelve bio-derived value-added chemicals reported by the Department of Energy (DOE), US [1], via chemical or biological pathways is well studied [2-4]. Large amounts of glucose can be obtained by hydrolysis of cellulose present in lignocellulosic biomass. However, this reaction has been a major bottleneck to the use of monomers due to its recalcitrant nature and high costs involved in the enzymatic hydrolysis [3].

On the other hand, hemicellulose can be easily hydrolyzed to obtain pentose monomers, predominantly xylose. Therefore, the usage of hemicellulose as a source of xylose, that can be easily converted into various products like xylitol, furfural, furfuryl alcohol, γ-valerolactone, levulinic acid, succinic acid etc is being explored [5]. Upon acid hydrolysis, xylose in hemicellulose loses three water molecules to produce furfural which in turn gets reduced to furfuryl alcohol and other derivatives (Fig. 6.1). Conversion of furfural to furfuryl alcohol requires a

catalyst to bring about hydrogenation reaction. Several studies have been reported in literature for such conversions using homogeneous and heterogeneous catalysts [6, 7]. Generally, FA is produced via dehydration of xylose followed by hydrogenation of furfural. Perez et al. (2014) have reported one-step production of FA from hemicellulose using a dual catalyst composed on Pt/SiO_2, sulfated ZrO_2 and acid catalysts [8]. Gurbuz et al. (2012) have described a biphasic reaction system to produce FA from hemicellulose [9]. Another study by Sirotori et al. (2014) demonstrated a one-step synthesis of furfuryl alcohol from xylose using a solid acid-base catalyst (Cr_2O_3/ Hydrotalcite) [10].

The conversion of these pentose sugars in hemicellulose along with hexose sugars into such value-added products would lead to the better utilization of the components of renewable lignocellulosic biomass **(Fig. 6.1)**. Conversion of lignocellulosic sugars with high product yields could result in low cost processes with the complete utilization of biomass polysaccharides (cellulosic and hemicellulosic) [10]. One such platform chemical, levulinic acid, which has potential to be produced from hemicellulose is discussed in this chapter.

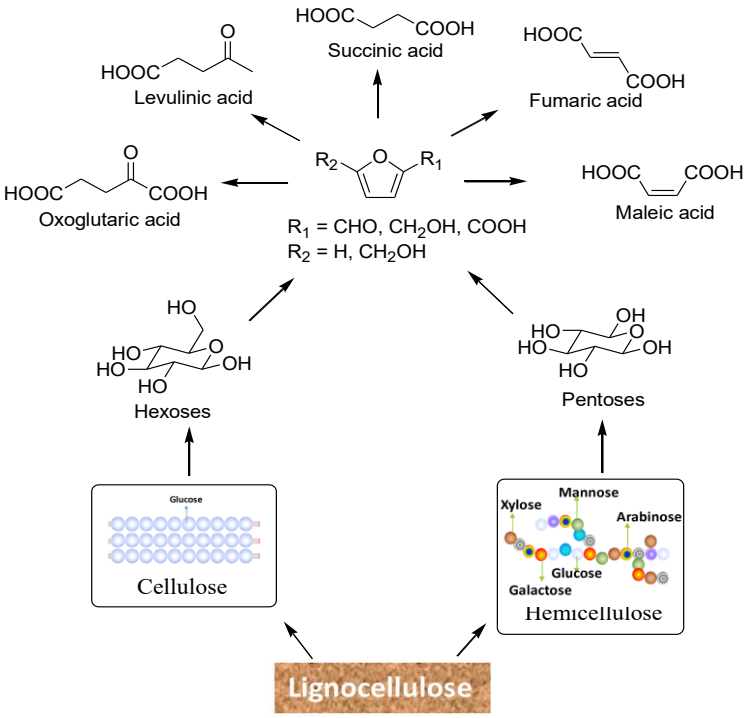


Figure 6.1: Schematic representation of the lignocellulose potential to produce various carboxylic acids.

Levulinic acid has two different functional groups (reactive sites) which makes it ideal for production of various derivatives used in food, pharmaceuticals, agriculture and cosmetics industries [4, 5, 11]. The calcium derivative of LA is considered to be a potential supplement for calcium in food [11]. In agriculture, δ-amino-levulinic acid has been used as a green (harmless) and biodegradable weedicide [12]. A derivative of levulinic acid, γ-valerolactone is an important ingredient used in perfume industry and also blended with gasoline due to its high energy content [13].

Production of levulinic acid from biomass derived furan derivatives like furfural, 5-hydroxymethyl furfural and furfuryl alcohol have been recently explored using various catalysts [14, 15]. However, these methods require high temperature and pressure besides being produced in very low yields of LA. For e.g., Xun Hu et al. (2014) reported the production of methyl levulinate (22.9%) and LA (3.5%) from furfural using 70 bar H_2 at 165 ^oC in methanol as solvent [16]. In another study conducted by Gorbanev et al., LA of 25% yield was achieved from 5-hydroxymethyl furfural (5-HMF) at 2.5 bar O_2 in the presence of $Ru(OH)x/Al_2O_3$ catalyst.[17] According to Gonzalez M et al. (2012), conversion of furfuryl alcohol to alkyl levulinate can be obtained in good yields using Amberlyst 15 as an acid catalyst in ethanol [15]. Attempts have been made to produce LA directly from xylose using Amberlyst 70 and hydrogenation catalyst Pd/Al_2O_3 in methanol medium [14]. However, the yields of LA were not significant despite the use of costly catalysts. The low yields of LA are due to the formation of other byproducts from various side reactions in the medium. Developing novel catalysts and using appropriate solvents have the potential to avoid the formation of byproducts. For instance, Zhang et al. (2011), have reported 93 % yield of n-butyl levulinate from FA using a novel organic-inorganic hybrid solid acid catalyst, $[MIMBS]_3PW_{12}O_{40}$ after 12 h of conversion reaction under nitrogen atmosphere [18].

Very few reports in literature have studied the mechanism and formation of products in the conversion of FA to levulinic acid [15]. Some reports suggest the formation of byproducts such as alkylated furans during the conversion of furfuryl alcohol into levulinic acid [15, 19].

Maldonado et al. (2012) have studied and proposed a reaction mechanism for FA – EL conversion in presence of Amberlyst 15 acid catalyst [15]. They have identified 2 intermediates (Ethyl Methyl Furan and 4,5,5-triethoxypentan-2-one) which were analogous to the conversion. Though the

reaction mechanism proposed by them discusses about the reaction between ethanol and FA, other side reactions in the medium which leads to ethyl levulinate formation were not identified. Besides, the reaction mechanism of FA – EL conversion in presence of H_2SO_4 was also not studied in literature. In this study, we analyzed major intermediates and byproducts formed during the conversion of furfuryl alcohol in presence of heterogeneous and homogeneous acid catalysts and two different solvents. Intermittent analysis has been done in short intervals during 6 h of reaction time to determine the reaction mechanism. In order to understand the mechanism of sulfuric acid mediated reaction and the side reactions which leads to the formation of EL, we have isolated the intermediates formed throughout the reaction and studied them exclusively.

6.3 Experimental Section

6.3.1 Materials

The reactants furfuryl alcohol (FA) and the heterogeneous acid catalyst, Amberlyst 15 were obtained from Sigma-Aldrich. The homogeneous acid catalyst, sulfuric acid was obtained from Fisher Scientific. All chemicals used in this study were of analytical grade without further purification.

6.3.2 Experimental Procedure

6.3.2.1 Reaction conditions

The conversion of furfuryl alcohol was studied in two solvents, water and ethanol, separately with two different type of acid catalysts, homogeneous (H_2SO_4) and heterogeneous (Amberlyst-15). In the experiments with homogeneous acid catalyst, H_2SO_4 (0.58 mmol, 0.06 % v/v) was added to a round bottomed flask containing the aqueous or alcohol solution (50 mL) of furfuryl alcohol (0.49 g, 5 mmol). A heterogeneous acid catalyst, Amberlyst 15 (0.25 g) was added instead of sulfuric acid in separate experiments. The round bottomed flask was attached to the condenser and placed in an oil bath. The temperature was increased to 100 ^oC in case of water and 80 ^oC in the case of ethanol and the reaction mixture was continuously stirred. Samples of the reaction mixture were taken in time intervals of 10 min. until 90 min. to analyze the product and byproducts formed. At the end of the reaction, the product mixture was cooled down and filtered to remove the catalyst, in case of Amberlyst 15. The filtered heterogeneous catalyst was washed with distilled water and reused. The pH of the product mixture was adjusted to 7 by adding 1 M sodium bicarbonate and then filtered to remove any solids. The solvent was evaporated under reduced pressure in rotary

evaporator. The resultant residue was dissolved in known amount of water and analyzed using an HPLC to determine the composition.

6.3.2.2 Isolation of intermediates

After 1 h of the reaction, the catalyst, Amberlyst 15 was removed from the reaction medium and the pH was adjusted to 7 by the addition of sodium bicarbonate. The solvent was then evaporated from the product mixture and the residue was dissolved in known amount of water. The aqueous solution of the final residue was extracted with ethylacetate twice. The combined organic solution was dried with sodium sulfate to remove the traces of water and a clear solution was obtained. Ethylacetate was then evaporated under reduced pressure and the residue was subjected to column chromatography to separate the intermediates. Hexane/ Ethylacetate (4:1) was used as the mobile phase to separate these intermediates. The fractions collected from the column chromatography were tested for the isolation of intermediates using Thin Layer Chromatography (TLC). The fractions containing pure compounds were combined and the solvent was evaporated. The residue was dried in vacuo and dissolved in deuterated chloroform ("100%" ≥ 99.96%, Sigma Aldrich) for analysis in the NMR.

6.4 Analytical Methods

6.4.1 Gas Chromatography-Flame Ionization Detector (GC-FID)

Furfuryl alcohol (FA) was analyzed using a Thermo-scientific GC (Trace 1300 series) with Flame Ionization Detector (FID) and a capillary column (Trace Gold -TG-WAXMS A) (30 m length, 0.25 mm internal diameter, and 0.25 μm film thickness of cross linked polyethylene glycol). A new method was developed to analyze FA with low retention time. A ramped flow rate of the carrier gas was passed through the column with initial flow rate of 5 mL/min for 0.74 min and later reduced and maintained at 4 mL/min until the end of the run. The temperatures of the oven and the detector were maintained at 200ºC and the inlet temperature was maintained 250ºC. Split mode (split flow: 200; split ratio: 40) injection was used in the analysis. Initially, the GC was calibrated with standards at different concentrations. Response factor (RF) of the standard FA was obtained by the equation: [Peak area] = RF[standard concentration]. The obtained RF was used to determine the unknown concentration of FA in the samples obtained during the reaction.

6.4.2 Gas Chromatography-Mass Spectrometer (GCMS)

The intermediates isolated were analyzed using a Varian model 450 gas chromatography (made in The Netherlands) coupled with a Varian model 300-mass spectrometer quadrupole (made in US) equipped with Factor Four capillary column (VF-5 ms, 30 m x 0.25 mm ID, DF ¼ 0.25 mm). Helium was used as the carrier gas with a flow rate of 1.0 mL/min. It was operated in a splitless mode with an autosampler injecting the samples at a temperature of 270 ^{o}C. Initially, the column temperature was maintained at 50^{o}C for 2 min. then increased to 155 ^{o}C at a rate of 8 ^{o}C/ min. It was then increased to 275 ^{o}C at a rate of 40 ^{o}C/min. and maintained for 9 min. Electron ionization (EI) conditions were maintained at an ionization energy of 70 eV and ion source at 200 ^{o}C. The range of mass scan was set to 40 – 500 m/z. The temperature of GCMS interface was set at 266 ^{o}C. The resulted spectra were analyzed using Varian MS workstation version 6.

6.4.3 High Performance Liquid Chromatography (HPLC)

Products were analyzed using an HPLC (Agilent Technologies 1260 Infinity) with Bio-Rad Aminex HPX-87H ion exclusion column (300mm x 7.8mm) and a Refractive Index Detector (RID). The mobile phase used in this method was 5 mM H_2SO_4 with a flow rate of 0.5 mL/min at 50 ^{o}C. The instrument was calibrated with the standards of varying concentration and the response factor (RF) obtained for the standards was used to calculate the concentrations of the products formed.

6.4.4 Nuclear Magnetic Resonance (NMR) Spectroscopy

The purified products were analyzed and confirmed by using NMR spectroscopy ("Varian-NMR" Inova-500 MHz) by elucidating their structure with ^{1}H NMR and ^{13}C NMR spectra. Deuterated chloroform was used to dissolve the products for the NMR analysis.

6.5 Results and Discussion

Initially, furfuryl alcohol conversion into levulinic acid or ester was studied with water and ethanol as solvents using two different catalysts. Sulfuric acid and Amberlyst 15 were used as a homogeneous acid and heterogeneous acid catalysts respectively, in both the solvents. Both the catalysts were studied as they were found to be efficient in converting FA to levulinic acid/ester in literature [19, 20]. According to J. P. Lange et al. (2009), H_2SO_4 is the most efficient acid catalyst compared to macroreticular resins, gel resins and zeolites for the conversion of FA [19].

In their study, sulfuric acid produced higher yields of ethyl levulinate (EL) than Amberlyst 36 but Amberlyst 15 showed similar EL yields as H_2SO_4 [19]. However, the ratio (g_{cat}/g_{FA}) of catalyst to the substrate (FA) was the same for all the catalysts used in their study. It is important to consider the acidic strength of the catalysts when comparing their effect.

For example, the effect of 2 g of H_2SO_4 is certainly not equal to the effect of 2 g of Amberlyst 15 on furfuryl alcohol conversion. The acidic strength of sulfuric acid is very high (Ka = 10^3) compared to many other acids. Each mole of sulfuric acid generates 2 moles of hydrogen ions. Therefore, the approximate calculated acidic strength of sulfuric acid is 20 mmol H^+/g. However, Amberlyst 15 has relatively less acidic strength of 4.5 mmol H^+/g (as mentioned on the product bottle, by Sigma). Therefore, sulfuric acid was diluted accordingly to make the acidic strength equivalent to the Amberlyst 15 and used in our experiments. This helped to compare their catalytic activity in the experiments. A comparison of the acidic strengths of some solid acid catalysts can be found in Table 6.1. The experimental conditions used in our study are moderate and facile compared to some literature reports where high pressure was applied during the reaction [19]. In our study, the reactions were carried out under atmospheric pressure and at relatively low temperature.

Table 6.1: Acidic strength of various macroreticular acidic resins. Adapted from Gao X. et al. (2015) [20]

Macroreticular resin	Acidity*, mmol/g
Amberlyst 36	5.4
Amberlyst 732	4.5
Amberlyst 15	4.7
H-mordenite	1.88
Amberlyst 21	1.3
ZSM-5	1.19

*Acidity of the resins were provided by manufacturers

6.5.1 Experiments with homogeneous acid catalyst, Sulfuric acid (H_2SO_4)

Diluted H_2SO_4 served as the homogeneous acid catalyst in these reactions for the conversion of FA into open chain carboxylic acids. The reaction carried out in water showed relatively high

amounts of solid particles as the reaction progressed. The quantitative analysis of the residual product indicated that a mixture of compounds including levulinic acid (LA) with a yield of 18% was present (Scheme 1). Other components were not quantified but assumed to be intermediates or other potential byproducts such as furoic acid and formaldehyde. The formation of solid particles was due to the favourable conditions for the polymerization of furfuryl alcohol in water. This eventually reduced the yield of levulinic acid. Though the concentration of sulfuric acid used in this study is very low (0.06%), the formation of solid particles due to polymerization is significant.

On the other hand, using sulfuric acid in ethanol for FA conversion did not show much solid by the end of the reaction. The yield of ethyl levulinate (EL) obtained in sulfuric acid/ethanol medium is 90.3% with >99% of FA conversion. Therefore, it is evident that in ethanol, sulfuric acid selectively synthesized ethyl levulinate. It can be assumed that the side reaction occurring in water has also been diverted toward ethyl levulinate formation. This has been proven and explained in the following reaction mechanism section. The GC-MS data of the residual product was shown in Fig. 6.2. The *m/z* ratio of ethyl levulinate is 144.1 and its fragments have *m/z* ratio of 99.0 and 45.0. The *m/z* ratio of 129.1 in the spectra represents a fragment resulted from the molecular ion losing a methyl group (M-15).

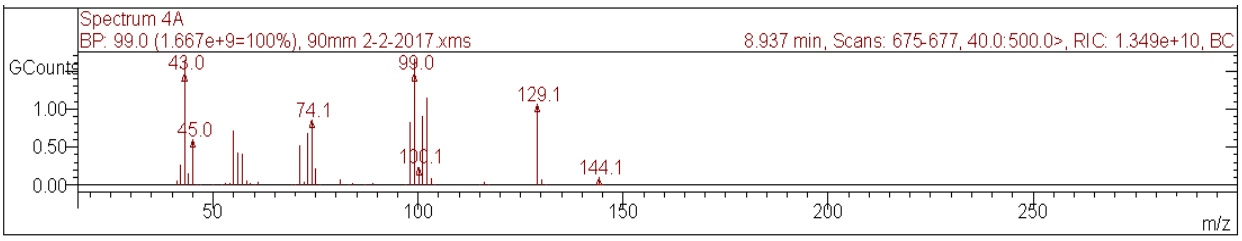
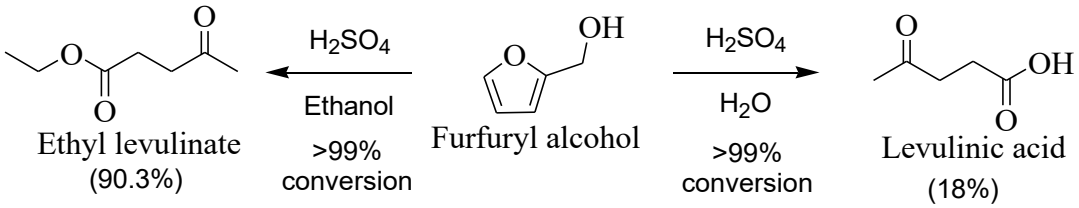


Figure 6.2: Mass spectrum of Ethyl levulinate obtained from FA – EL conversion catalyzed by H_2SO_4.

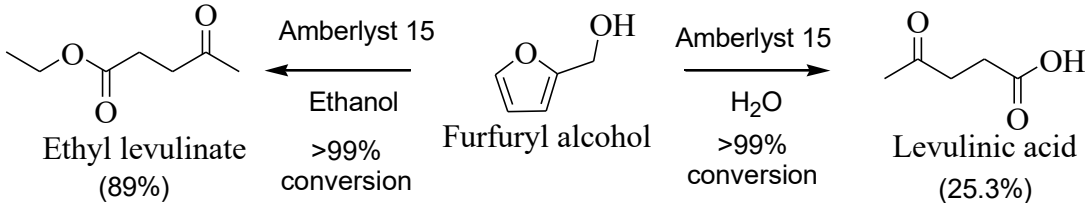


Scheme 6.1. Furfuryl alcohol conversion in presence of sulfuric acid in ethanol and water media.

6.5.2 Experiments with heterogeneous acid catalyst, Amberlyst 15

In these experiments, Amberlyst 15 have an acidic strength equivalent to sulfuric acid used in the previous experiments. Amberlyst 15 was also used in both water and ethanol solvents to determine its activity in producing levulinic acid or ester, respectively. Unlike H_2SO_4, Amberlyst 15 in water did not show any solids during the reaction and no residue was observed at the end of reaction except that of the solid catalyst itself. The HPLC analysis of the filtered product mixture showed 2.08 g/L of levulinic acid with 25.3 % molar yield. It can be observed that the yield of levulinic acid with Amberlyst 15 is higher than the yield with sulfuric acid in water. Therefore, compared to sulfuric acid, Amberlyst 15 was found to result in low levels of furfuryl alcohol polymerization and did not produce insoluble solids during the reaction. However, the HPLC analysis of the product mixture show that other intermediates or byproducts are present.

On the other hand, Amberlyst 15, when used with ethanol as solvent did not favour the polymerization. Therefore, no solids were observed at the end of reaction. Besides, Amberlyst 15 was found to selectively drive the reaction towards the formation of levulinic ester, ethyl levulinate (EL) in ethanol. The product was quantified and found to have 10.3 g/L of EL. The molar yield of EL was found to be in the range of 84 – 89 % from the replicates of the reaction (Scheme 6.2). The NMR spectral data for EL obtained in this experiment are as follows: ^{1}H NMR (500 MHz, $CDCl_3$, TMS): δ= 1.25 (t, J = 7.0 Hz, 3H), 2.19 (s, 3H), 2.57 (t, J = 7.0 Hz, 2H), 2.75 (t, J = 6.5 Hz, 2H), 4.11 – 4.15 (q J = 7.0 Hz, 2H) (Fig. 6.3); ^{13}C NMR (500 MHz, $CDCl_3$ TMS): δ = 14.17, 28.01, 29.90, 37.95, 60.63, 171.77, 206.73 (Fig. 6.4). The yield of ethyl levulinate in Amberlyst 15/ ethanol is similar to the yield obtained in sulfuric acid/ ethanol medium.



Scheme 6.2: Furfuryl alcohol conversion using Amberlyst 15 in ethanol and water medium

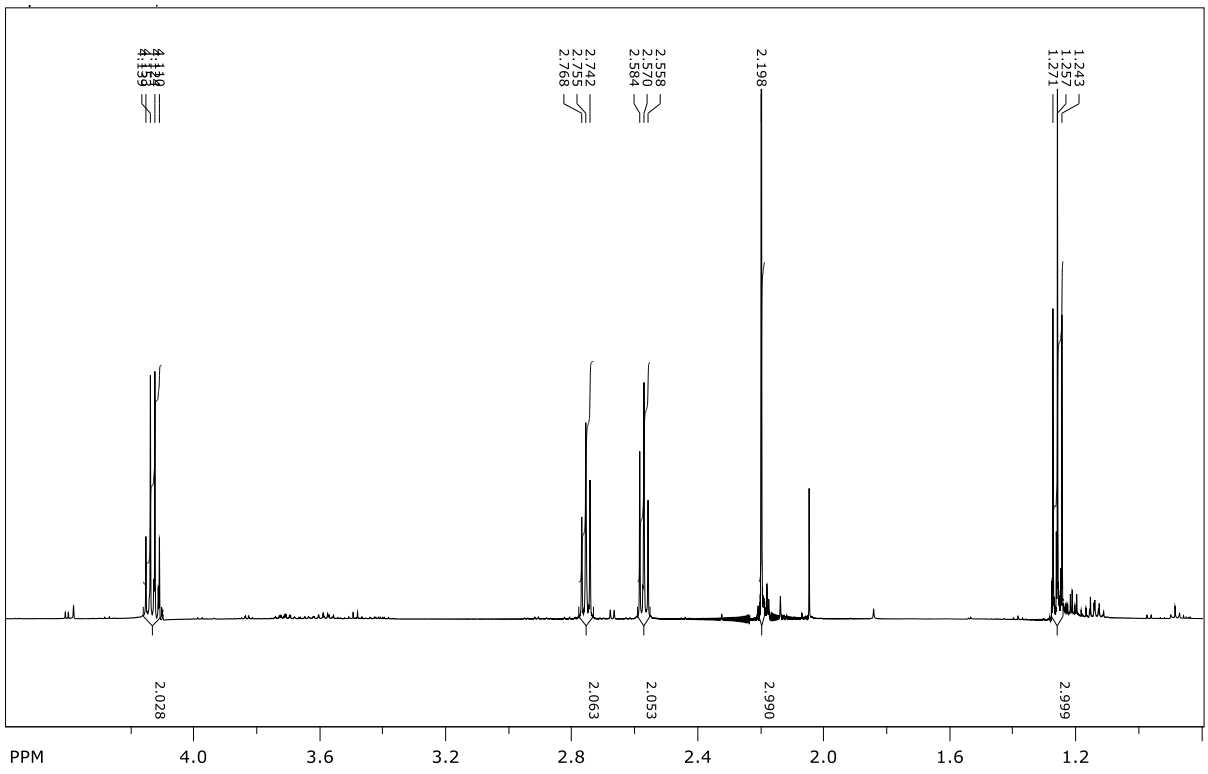


Figure 6.3. Proton NMR of ethyl levulinate

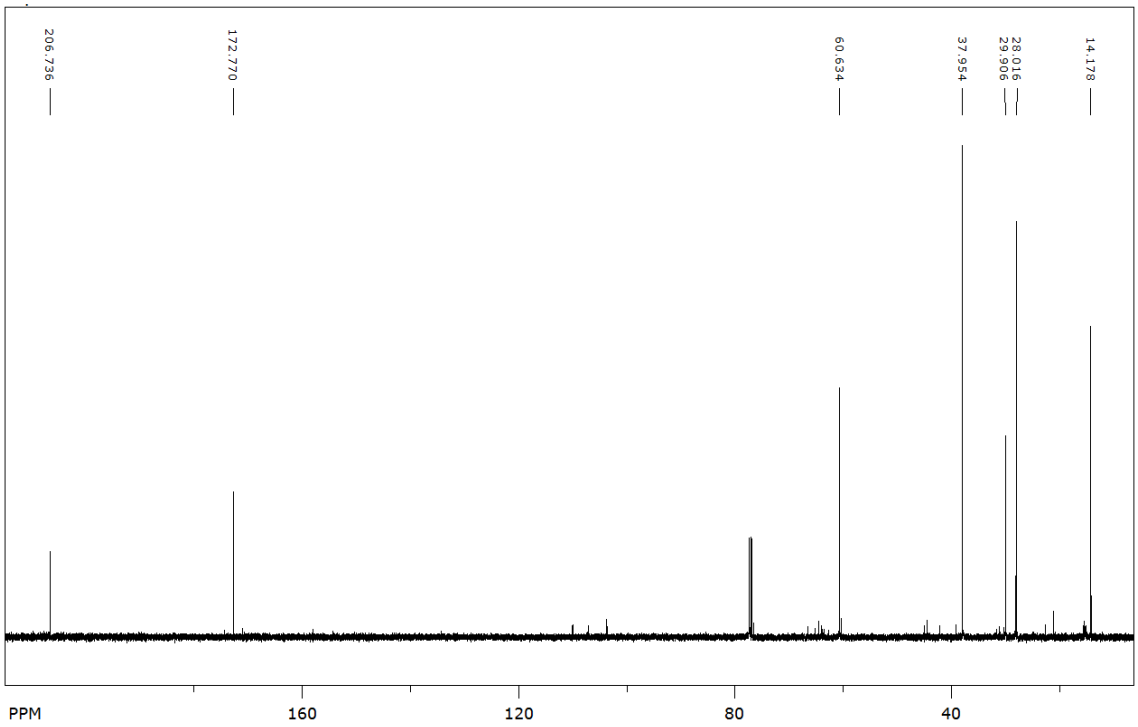


Figure 6.4. ^{13}C NMR of ethyl levulinate

6.5.3 Interpretation of reaction mechanism

Even though the acidic strengths of both the catalysts (Amberlyst 15 and H_2SO_4) are same in ethanol and water, the yield of EL in ethanol was much higher than the yield of LA in water with >99% conversion of furfuryl alcohol in both cases. This suggests that water facilitates other side reactions like polymerization of FA whereas ethanol selectively facilitates ethyl levulinate synthesis. It can be speculated from the products in both cases that the conversion mechanisms involved in the two solvents are different from each other. In order to understand the significant difference in product yields with the change in solvents, it is important to learn the reaction mechanism of FA to EL.

The reaction was monitored at a frequent time intervals (i.e., every 10 min.) to observe the formation of intermediates. From the GC analysis, it was found that the number of intermediates were increased with the time as did ethyl levulinate concentration in the reaction medium. Subsequently, the intermediates were consumed and the concentration of EL increased (Fig. 6.5). However, some of the intermediates were found to be stable and did not form ethyl levulinate. The reaction was stopped when high concentrations of such intermediates were formed. Most of the observed intermediates were found to be unstable as they decompose with time. However, some of the unstable intermediates could be depicted from the GCMS data.

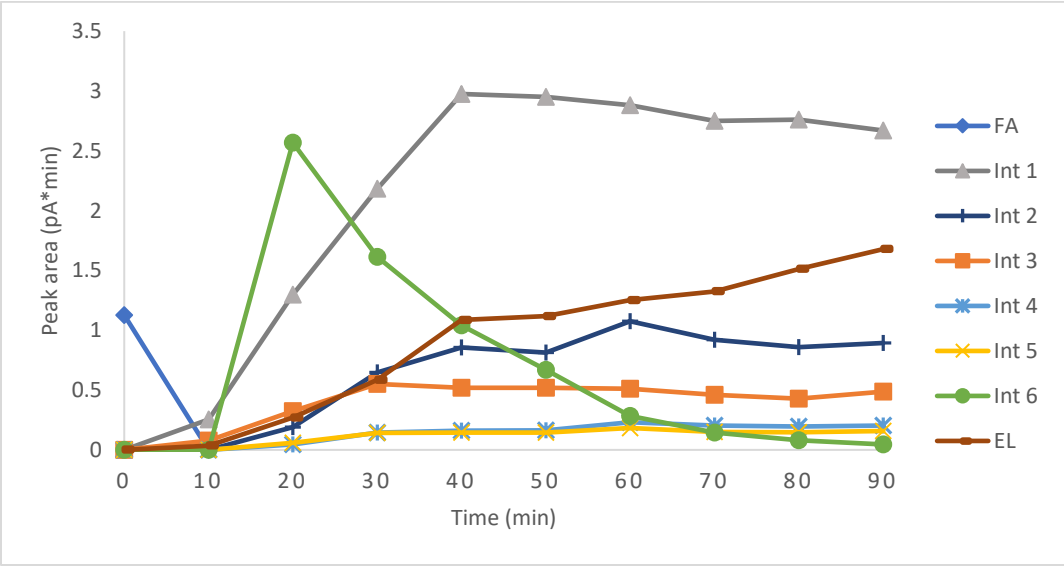


Figure 6.5. The profile of furfuryl alcohol, intermediates and ethyl levulinate with respect to reaction time until 90 min. (*Int – Intermediate; FA – Furfuryl alcohol; EL – Ethyl levulinate)*

The mass spectrum of intermediate-1 shown in Fig. 6.6 indicates that furfuryl alcohol reacted with ethanol forming ethoxy furfuryl alcohol (Species 2A). The fragmentation pattern and the fragments corresponding to the mass spectrum of species 2A were shown in Scheme 6.3. This intermediate was found to form immediately after the reaction was started. In presence of acid catalyst, species 2A tends to lose a water molecule and forms species 3A. The possible structure of the species 3A molecule was given in the scheme of mechanism. This compound was confirmed based on the mass spectrum as shown in Fig. 6.7. The peaks obtained in the mass spectrum were identified as MS (3), MS (4) and MS (5) fragments as shown in the scheme 6.4. A fragmentation pattern was proposed based on these fragments. These intermediates were found to be very unstable and could not be isolated for NMR studies.

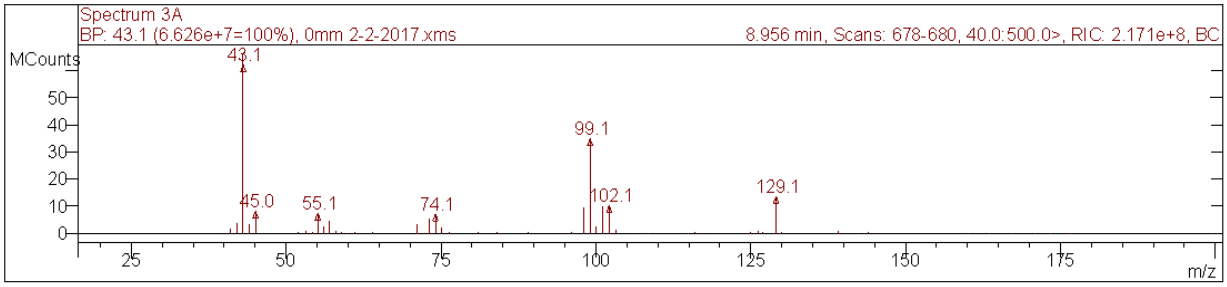
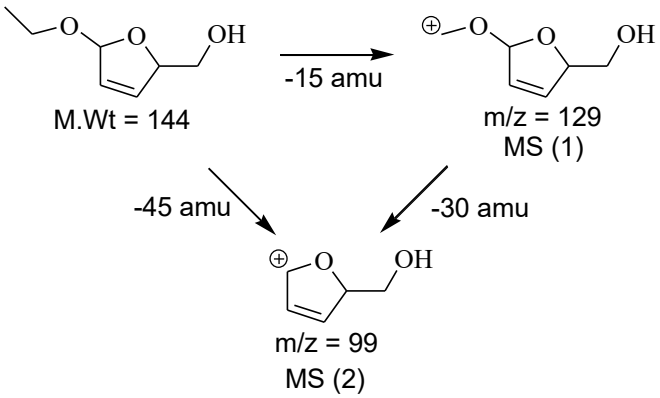


Figure 6.6. Mass spectrum of intermediate-1



Scheme 6.3. Proposed fragmentation pattern of Intermediate-1 based on the mass spectrum

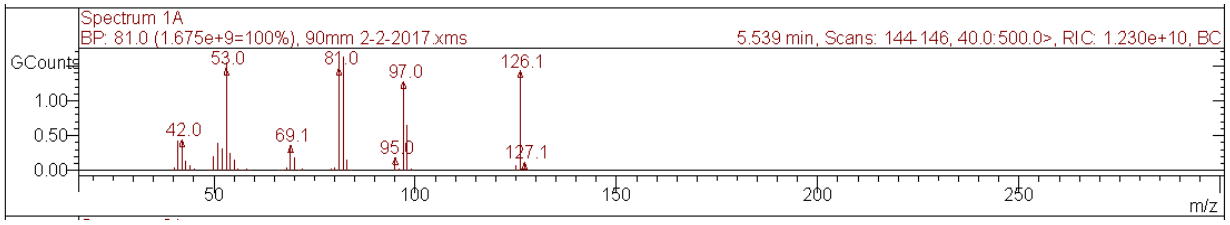
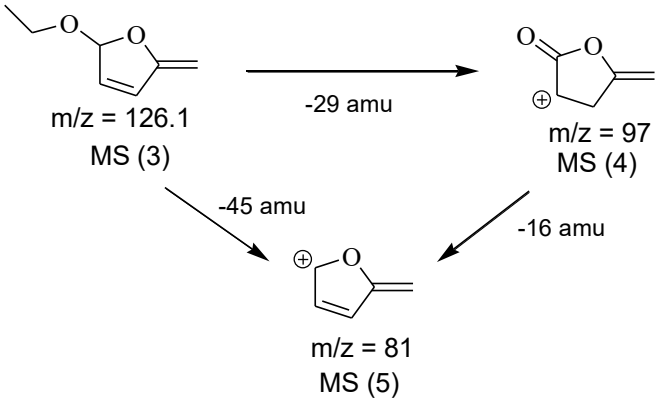


Figure 6.7. Mass spectrum of intermediate 2

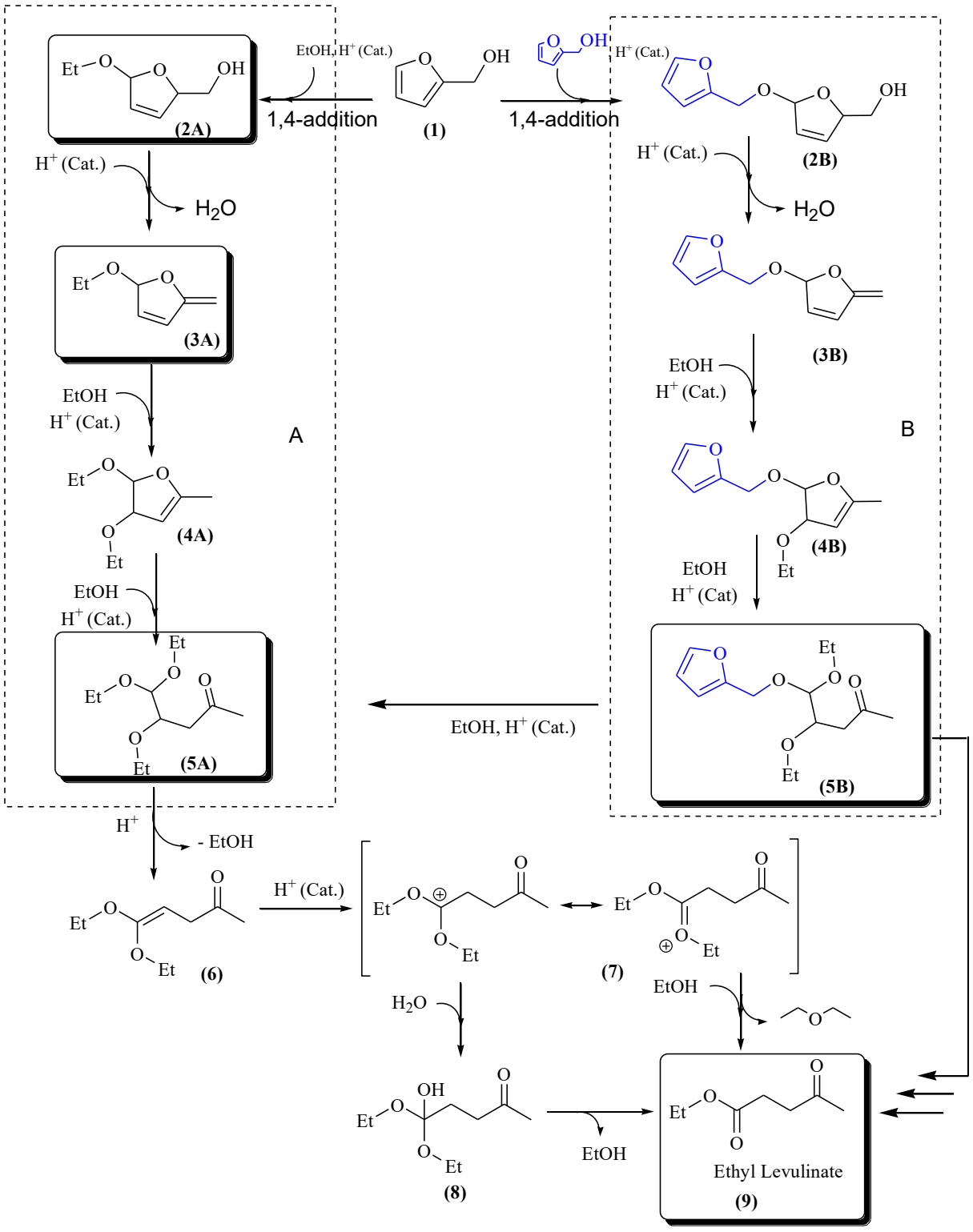


Scheme 6.4. Proposed fragmentation pattern of Intermediate-2 (species 3A) based on the mass spectrum

6.5.3.1 Structural elucidation of intermediates using NMR

Though, we were able to isolate 6 intermediates from the reaction mixture, only two were pure and stable enough to be analyzed using an NMR. The structures of these two intermediates were elucidated using the proton and carbon spectra and found to be analogous to the furfuryl alcohol conversion. They are represented as species 5A (4,5,5-triethoxy pentanone) and 5B (4,5-diethoxy-6-(furan-2-yl-methoxy)-pentanone) in the proposed reaction mechanism (Scheme 6.5). The NMR spectra of the species 5A are as follows: ^{1}H NMR (500 MHz, $CDCl_3$) δ = 1.15 (t, J = 7.0 Hz, 6H), 1.261 (t, J = 7.0 Hz, 3H), 2.174 (s, 3H), 2.667 (d, J = 6, 2H), 3.59 (m, 2H), 3.71 (m, 4H), 3.84 (m, 1H), 4.400 (d, J = 5, 1H); ^{13}C NMR (500 MHz, $CDCl_3$) δ = 15.288, 15.375, 15.600, 31.133, 44.409, 63.944, 63.972, 66.610, 79.599, 103.758, 207.293. The graphical NMR spectra of this data can be found in Fig. 6.8 and 6.9. This intermediate was found to be similar to the intermediate in Amberlyst catalyzed reaction reported by Maldonado et al. 2012. It is also similar to the methyl substituted molecule suggested by Horvat et al. 1985 [21]. However, in ethanol medium, a reaction between two furfuryl alcohol molecules is also possible.

We have identified an intermediate with a furfuryl moiety which was represented as species 5B. This intermediate was found to have two chiral centers and has four possible stereoisomers (two pairs of enantiomers). The NMR spectra of species 5B have shown the presence of two diastereomers in approximately 1:1 ratio. The NMR data of 5B as a mixture of two diastereomers are as follows: ^{1}H NMR (500 MHz, $CDCl_3$) δ = 1.15 (t, J = 7.0 Hz, 3H), 1.20 (t, J = 7.0 Hz, 3H), 1.25 (t, J = 7.0 Hz, 3H), 1.29 (t, J = 7.0 Hz, 3H), 2.14 (s, 3H), 2.15 (s, 3H), 2.43 (d, J = 10.0, 15.0 Hz, 2H), 2.57-2.67 (m, 2H), 2.71 – 2.82 (m, 2H), 2.90 (dd, J = 10.0, 15.0 Hz, 1H), 3.42 (m, 2H), 3.50 (m, 2H), 3.65 (m, 2H), 3.74 (d, J = 7.5 Hz, 1H), 3.73 - 3.82 (m, 2H), 3.85 - 3.92 (m, 2H), 3.94 (m, 2H), 4.08 (d, J = 10.0, 1H), 4.92 (d, J = 5.5 Hz, 1H), 5.19 (d, J = 5.0 Hz, 1H), 5.73 – 5.79 (m, J = 7.5 Hz, 1H), 5.73 - 5.79 (m, 1H), 5.91 (d, J = 6.0 Hz, 1H), 5.92 (d, J = 6.0 Hz, 1H), 6.00 (d, J = 5.5 Hz, 1H), 6.01 (d, J = 5.5 Hz, 1H) (Fig. 6.10 and 6.11); ^{13}C NMR (500 MHz, $CDCl_3$) δ = 15.067, 15.232, 15.455, 15.471, 30.175, 30.239, 37.810, 39.539, 45.382, 47.727, 63.838, 64.120, 64.183, 64.336, 75.188, 75.243, 95.627, 96.927, 104.397, 108.208, 108.243, 108.635, 127.170, 127.654, 133.318, 135.193, 207.060, 208.265 (Fig. 6.12). The furfuryl moiety present in this intermediate suggests that the reaction takes place between two furfuryl alcohol molecules.



Scheme 6.5: Plausible reaction pathway for the conversion of furfuryl alcohol to ethyl levulinate in ethanol in presence of sulfuric acid. The intermediates isolated and identified are represented in boxes and the species proposed in the mechanism are in parenthesis.

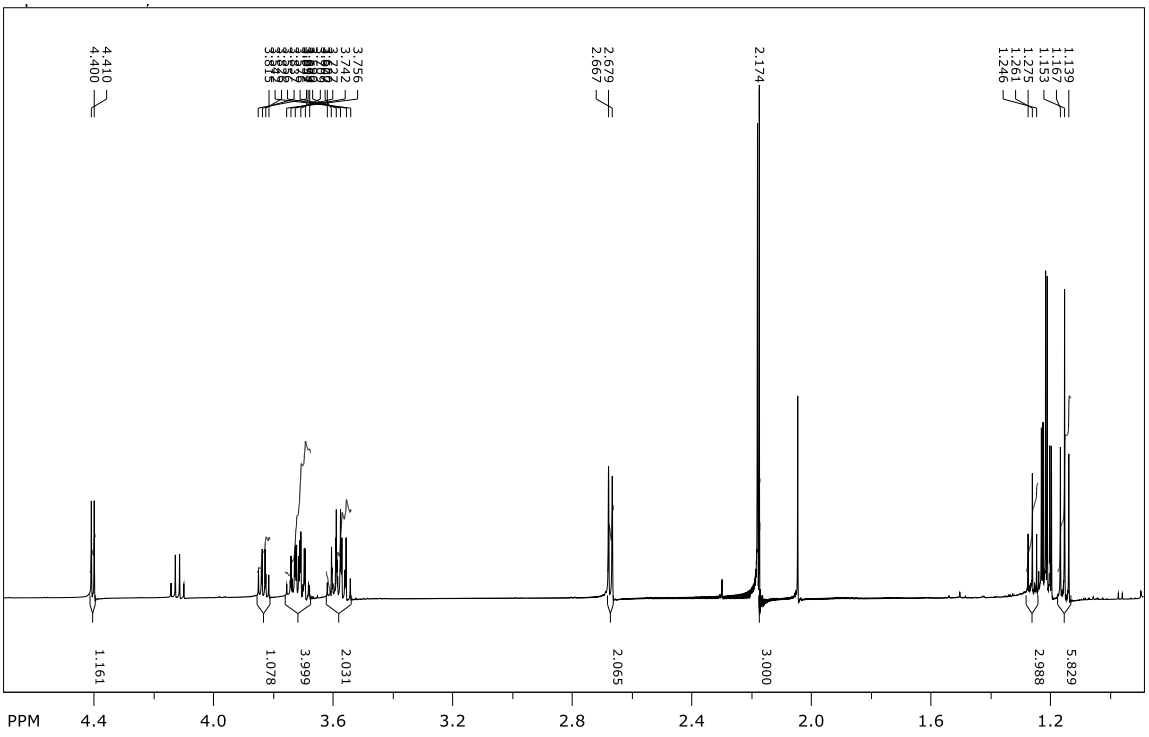


Figure 6.8: Proton NMR spectrum of species 5A obtained from FA – EL conversion in presence of sulfuric acid.

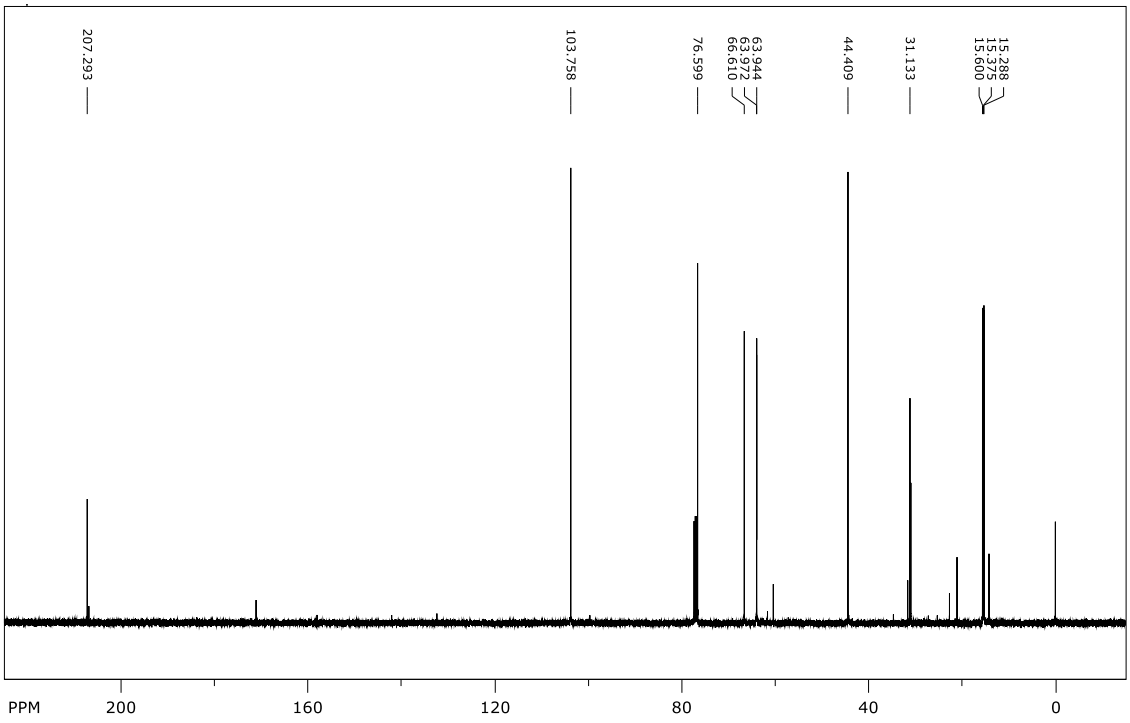


Figure 6.9: Carbon NMR spectrum of species 5A obtained from FA – EL conversion in presence of sulfuric acid.

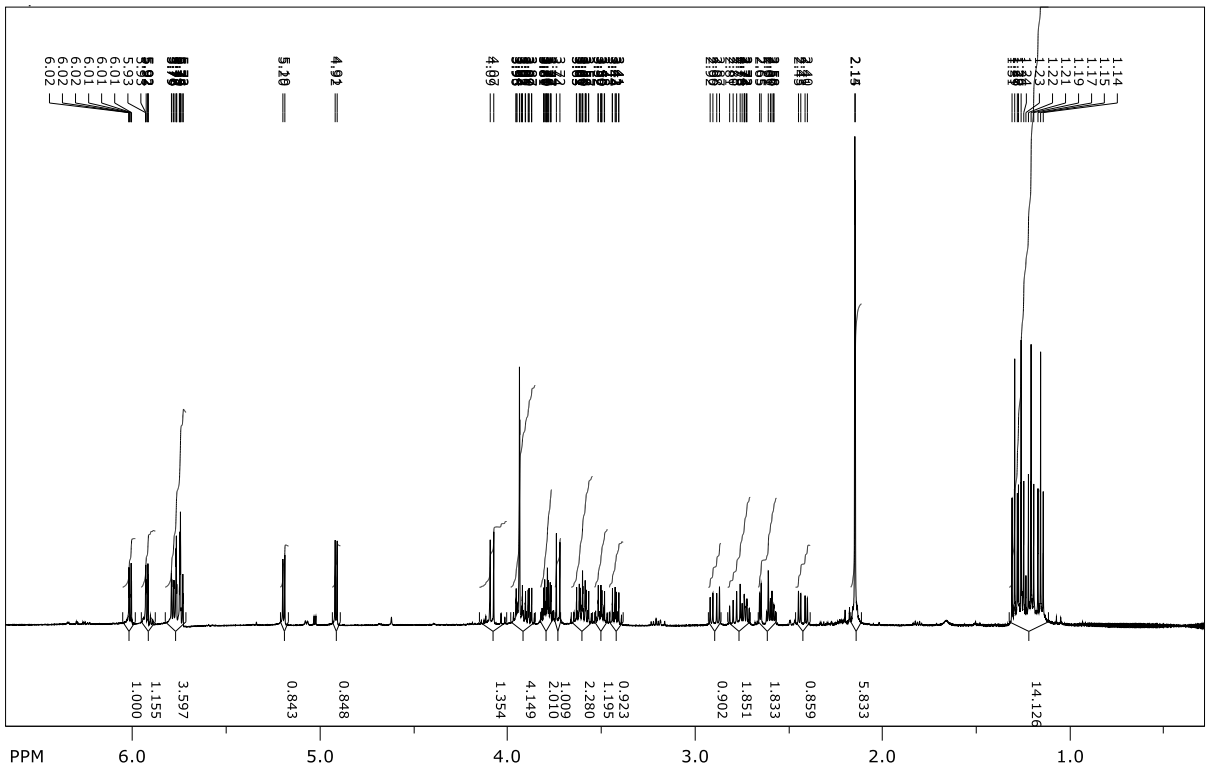


Figure 6.10: Proton NMR spectrum of species 5B obtained from FA – EL conversion in presence of sulfuric acid.

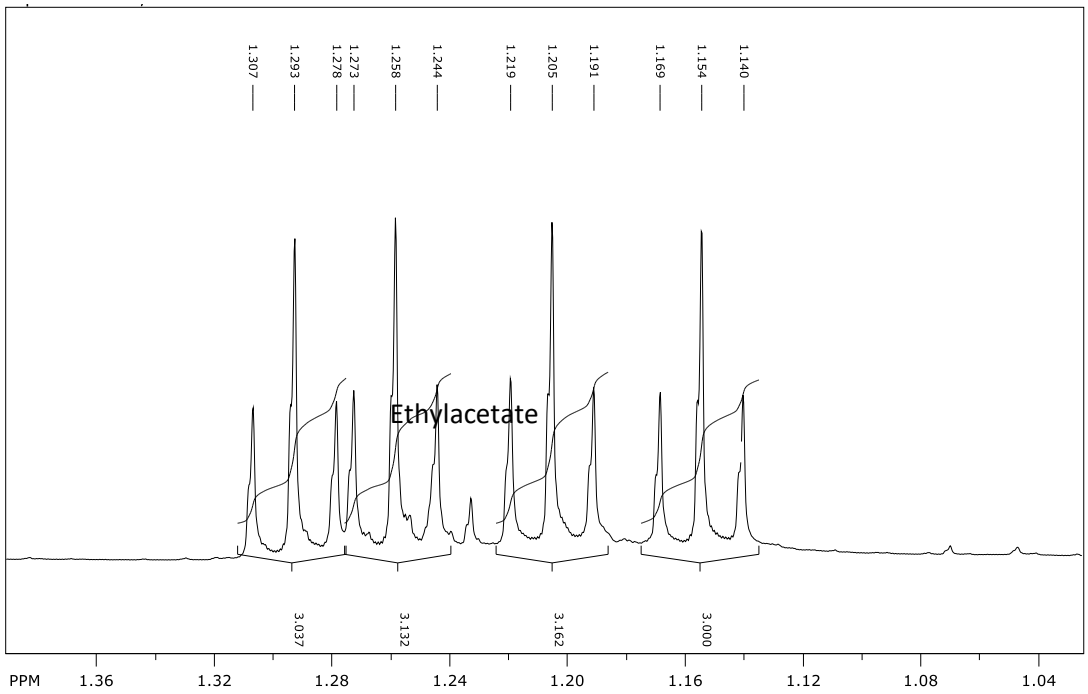


Figure 6.11. Zoomed in of 1H NMR of species 5B at 1.04 – 1.36 ppm

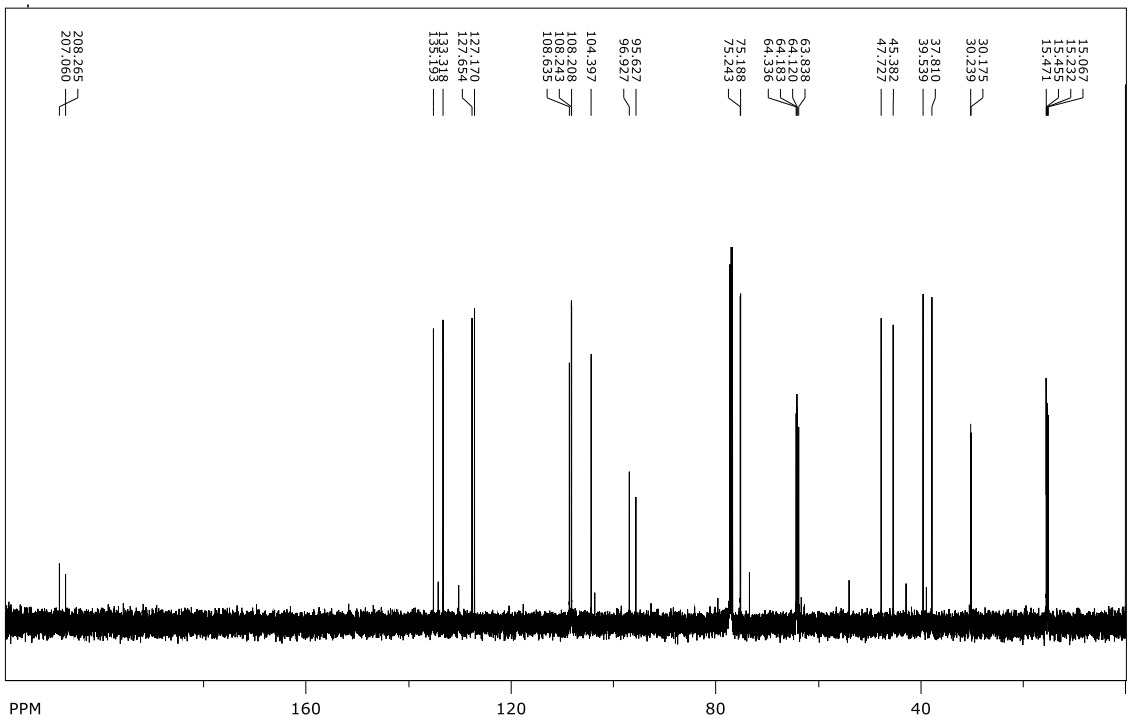


Figure 6.12: Carbon NMR of species 5B obtained from FA – EL conversion in presence of sulfuric acid.

The two intermediates (species 5A and 5B), described above, are vital in the proposed mechanism because each of them suggests a different pathway. Both the species have a common 5-carbon backbone with two ethoxy substituents at C4 and C5 positions (scheme 6.5). It was observed that the substituent on C5 position in both the species are distinct from each other. However, it was observed that species 5A was produced in high amount when compared to the 5B. It is because ethanol is more abundant than furfuryl alcohol in the reaction medium. Therefore, it can be depicted that the formation of 5A and 5B took place in separate pathways. Based on this inference, we have proposed two different pathways in the reaction mechanism.

In the beginning of the reaction, furfuryl alcohol reacts with either ethanol or another furfuryl alcohol molecule in the presence of an acid catalyst. The alcohol undergoes 1,4-addition reaction to produce species 2A or 2B which subsequently loses a water molecule to form 3A or 3B, respectively. As discussed in the earlier sections, species 2A and 3A were identified and determined using GC-MS data. As shown in the scheme 4, these species react with alcohol molecules to afford species 5A or 5B which were determined using NMR spectra. Though

precursors 2B – 4B of species 5B were not detected, it was believed that the formation of species 5B would follow a similar reaction pathway as the formation of 5A. Furthermore, both species 5A and 5B were eventually converted into ethyl levulinate as the final product in several steps. 5A underwent an elimination reaction (→ 6) followed by a partial hydrolysis reaction through the oxocarbonium ion (7) to provide 8, which lost an ethanol to give ethyl levulinate 9. Alternatively, the oxocarbonium ion 7 reacted with an ethanol to produce ethyl levulinate 9 and generated a diethyl ether molecule, which was in accordance with the studies done by Maldonado et al. [15]. Species 5B could be readily converted to 5A through a trans-acetalization reaction, in which the furfuryl group was replaced by an ethyl group due to the presence of large amount of ethanol in the reaction medium. In fact, it is possible that species 5B could follow the same reaction pathway as 5A did (5A → 6 → 7 → 8 → 9), leading to ethyl levulinate, assuming that at any stage of the mechanism the furfuryl group was replaced by an ethyl group through a trans-acetalization or trans-esterification reaction.

On the other hand, in the presence of water instead of ethanol, the species resulted from the reaction between two furfuryl alcohol molecules cannot be converted to levulinic acid as there is no nucleophile present in the medium that is stronger than furfuryl alcohol to replace the furfuryl moiety on the species. Therefore, it could lead to the formation of other byproducts such as dimer or polymer with furfuryl alcohol moieties. Therefore, most of the furfuryl alcohol undergoes such side reactions and leaving small amounts of FA to proceed towards the formation of levulinic acid. This can be attributed to the low yields of levulinic acid in water as medium. Hence, it is proven again that alcoholysis of furfuryl alcohol yields high amount of levulinate by converting the byproduct species obtained in pathway B to the species in pathway A.

6.6 Conclusions

In the present study, the conversion reaction of FA to EL was carried out through a facile method with moderate temperature (80ºC) and at atmospheric pressure using a homogeneous acid catalyst (sulfuric acid) and a heterogeneous acid catalyst (Amberlyst 15). The reactions were carried out in water and ethanol to determine the yields and products. We have found that the solvent, ethanol selectively produces ethyl levulinate by diverting the side reactions towards the desired product. However, water facilitates several side reactions which results in low yields of LA. The alcoholysis of furfuryl alcohol in presence of sulfuric acid was studied in detail and we were able to

successfully identify the intermediates formed during the reaction. The reaction mechanism for the same was proposed by elucidating the structures of intermediates using GCMS and NMR. According to the experimental data obtained in this study, it was concluded that the conversion of FA – EL occurs in different pathways with a yield of 90.3%. Two pathways (Pathway A and B) were proposed which were found to occur parallel to each other. In ethanol, the species obtained in pathway B can be converted to the species in pathway A at any stage of reaction due to abundance of ethanol. Based on the intermediates identified in the pathway B, it is concluded that, unlike in ethanol, the byproducts formed in water cannot be converted into levulinic acid as there is no nucleophile stronger than furfuryl alcohol. This results in high amounts of unwanted byproducts and low yields of levulinic acid (23%). The proposed reaction mechanism of FA – EL conversion in sulfuric acid/ethanol medium confirmed by the analytical results of GCMS, ^{1}H and ^{13}C NMR is different from the reaction mechanism in Amberlyst 15/ ethanol medium proposed in literature.

6.7 References

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Chapter 7
Summary and Recommendations

7 Summary and Recommendations

7.1 Summary of the thesis

Lignocellulosic hemicellulose has a high potential to be used as a renewable resource for the production of several fossil based products. However, in the conversion of hemicellulose, the current biorefineries face several limitations due to low yields, high production costs and lack of appropriate proven methods. In order to overcome these bottlenecks, innovative and efficient methods need to be developed. In the present thesis, several efficient methods were developed to produce three platform chemicals, xylitol, succinic acid and levulinic acid. The bottlenecks involved in all the processes, such as formation of unwanted byproducts, loss of product, separation techniques, acid concentration, temperature, pressure and reaction conditions were addressed and resolved by optimizing and improvising the methods.

The first objective of this thesis was to hydrolyse the hemicellulose prehydrolysate and make it amenable for further use. In our study, it was observed that higher acid concentrations resulting in high levels humins and byproducts. Therefore, various acid concentrations were investigated and it was found that 1.75% (w/w) of sulfuric acid results in relatively low byproducts and humins with xylose rich hydrolysate. In order to make the hydrolysate much favorable for microbial growth, the byproducts were removed from the hydrolysate. An efficient detoxification method was developed using vacuum evaporation and solvent extraction procedures to detoxify the hydrolysate. This procedure was found to be very effective and removed 80% of acetic acid and 98.8% furfural with very little loss of xylose. The effectiveness of these methods was confirmed by fermenting the hydrolysate using *C. guilliermondii.* It was observed that the xylitol yield was 0.59 g g^{-1} with a concentration of 28.78 g L^{-1}. The volumetric productivity of 0.81 g L^{-1} h^{-1} is the highest reported so far using hemicellulose prehydrolysates and is close to the results obtained in pure xylose fermentation. The acid hydrolysis and detoxification methods developed were proven to be efficient in making the hydrolysate fermentable.

The second objective of the thesis involves the improvisation of fermentation methods to produce xylitol with high yields. The hydrolysate obtained in previous method was fermented with two different strains of candida species. An immobilization technique was integrated with the fermentation method to make it efficient as recycling of the biocatalysts (immobilized yeast cells) was possible. A new strain, *Candida tropicalis* UFMG BX 12-a in its immobilized form has shown

xylitol yields of 0.92 g g^{-1} with a productivity of 0.88 g L^{-1} h^{-1}. These are the highest known results obtained using immobilized yeast strains compared to the reports in literature. A comparative study was also conducted by using an immobilized *C. guilliermondii* FTI 20037 strain on the same hydrolysate to prove the efficiency of the new strain in producing higher xylitol yields. The usage of immobilized yeast also offers the reusability in further fermentation processes and make the xylitol production sustainable.

The third objective of the thesis was to demonstrate the production of succinic acid from hemicellulose. A novel biphasic system of toluene and aqueous hydrolysate was developed in this process. This facilitates the furfural formed in the aqueous phase to be transfered immediately to the toluene phase and avoids the polymerization of furfural. The furfural obtained in the toluene phase was directly oxidized to succinic acid. The succinic acid yield obtained in this process was 49% from furfural. The method developed here is very effective in preventing the furfural loss during the process. It also found to be beneficial in separating succinic acid from the reaction media. The heterogeneous acid catalyst, Amberlyst 15 used in this study can be recycled and used for further conversions. The ideal catalyst/substrate ratio found in this study was found to be 50 mg/ mmol furfural because it converted 100% furfural in 4 h of reaction time. Using such low amounts of catalysts and recyclability substantially reduce the costs involved in SA production. This method has high potential for scale up and in order to valorize hemicellulose.

The fourth and last objective of this thesis shows the production of levulinic acid from furfuryl alcohol (FA) along with the reaction mechanism. Two catalysts, sulfuric acid and Amberlyst 15 were found to be efficient in ethanol in converting FA to ethyl levulinate (EL) which can subsequently hydrolysed to levulinic acid. The study of reaction mechanism provides information on the potential side reactions which results in several byproducts. An important intermediate found in the mechanism (intermediate 4) is responsible for furfuryl alcohol polymerization in water medium. This intermediate form due to the reaction between two furfuryl alcohol molecules. It was found that ethanol is responsible to drive this intermediate to form ethyl levulinate. However, in presence of water, this intermediate leads to a stable byproduct. Due to this, the yield of levulinic acid in water as medium found to be much low (18% – 25%) whereas it was much higher in ethanol (85% – 90%). It was also proven that FA – EL conversion takes place by different pathways and the solvent plays an important role in driving these intermediates to the desired product.

The above described processes contribute to the development of the methodologies to convert low value substrate hemiceullulose to high value platform chemicals. These methods are found to be efficient in lab scale.

7.2 Recommendations for future studies

Based on this study we would like to make a few recommendations for further investigation and follow up.

In this study, we used hemicellulose hydrolysate of poplar wood obtained from a proprietary pretreatment method. In order to make these processes successful, it is important to integrate an appropriate pretreatment process into existing plants including pulp and paper mills. The extraction of the hemicellulose stream from these mills will vary, depending on the type of raw material used and the process followed etc. The hemicellulose extraction can be an independent process or obtained by precipitation of lignin from black liquor. As higher yields were obtained in the production of all these products attempted (xylitol, succinic acid and levulinic acid), it is necessary to scale up these studies to further enhance production and study its feasibility.

The economic feasibility of production of such processes will be different with standalone mills. The contribution of such processes to the revenue of existing bioproduct mills will need to be done with care. The reduction of market prices of product like succinic acid when large number of industries enter the market has also to be considered.