Microbial Production of Value-added Products from Wood Hemicellulose Pre-hydrolysate

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Abstract

Hemicellulose, the second most abundant polymer in nature, has the great potential to be used for the production of biochemicals under the concept of biorefining. As hemicellulose is water-soluble, it can also be easily obtained by the pre-hydrolysis of wood prior to the pulping processes such as Kraft delignification or during the production of dissolving pulp. Utilization of these byproduct streams can play an important role in the development of a circular bioeconomy as it helps in maintaining the materials and resources for a long period instead of disposing it as waste. In the biochemical conversion platform for biorefineries, it is crucially important to use all fermentable sugars of lignocellulosic biomass including both hexose (C-6) and pentose (C-5) sugars.

The overall objective of this study was to produce bioplastic building blocks from hemicellulose streams. The specific objective of this work was to investigate the possibility of using wood-based hemicellulose for microbial production of value-added biochemicals including microbial oil, biopolyol and poly-γ-glutamic acid. The hemicellulose pre-hydrolysate used in this study was produced by a proprietary pretreatment process. Composition analysis of the hemicellulose pre-hydrolysate indicated that it had 143.89 ± 1.28 g/L of xylose along with smaller quantities of sugars like glucose and arabinose.

Bioconversion of hemicellulose sugar to microbial oil is one possible way to valorize this industrial side stream. An oleaginous yeast *Cryptococcus curvatus* (ATCC 20509) was selected for this bioconversion as it is known to accumulate high content of lipids and is able to grow on complex lignocellulosic hydrolysates/pre-hydrolysates even in the present of impurities. Initially, the effect of xylose concentration and carbon to nitrogen ratio were investigated in order to maximize the lipid accumulation. The robust yeast strain used was able to produce 13.78 g/L of cell biomass and 5.13 g/L of lipid after 164 hr of fermentation using poplar wood pre-hydrolysate without detoxification. The obtained microbial oil was characterized to identify its fatty acid profile. Oleic acid (45.86 ± 0.69 wt%) was found to be the main fatty acid present. This fermentation was scaled up in a batch bioreactor with 1 L capacity. 16.54 ± 0.65 g/L of cell biomass and 6.97 ± 0.58 g/L of lipid were obtained in the reactor which had better control of environmental conditions.

The microbial oil produced was then used as feedstock for production of bio-based polyol which has many applications in polymer industry and importantly serves as a precursor for polyurethane production. The bio-polyol was produced using a two-step approach: epoxidation followed by

ring-opening reaction. Lipase enzyme produced by *Candida antarctica* (Novozyme 435) and immobilized on acrylic resin was used as an unconventional catalyst for *in situ* epoxidation of microbial oil. 84.55 ± 1.80% conversion was achieved after 12 hours. Novozyme 435 was found to be very stable and can be reused up to 3 cycles efficiently. In the second step, Isopropanolamine was used to open the epoxy ring with the addition of hydroxyl group. Hydroxyl value and acid number of the microbial-based polyol were found to be 299.53 ± 1.24 mg KOH/g and 4.93 ± 1.07, respectively. Fourier transform infrared spectroscopy (FTIR) and nuclear magnetic resonance (NMR) were used for structural confirmation of produced bio-polyol. Production of biobased monomers from renewable materials using enzyme catalysts can be considered clean and leads to energy saving processes. Therefore, synthesis of renewable polymers via enzymatic polymerizations of biobased monomers provides an opportunity for achieving green polymers and a future sustainable polymer industry.

The production of another useful chemical, poly-γ-glutamic acid (PGA) was also studied. PGA is known to have a number of useful applications. In order to make this fermentation efficient, the detoxification of the pre-hydrolysate was carried out using a method previously developed in our lab. This detoxification method includes the combination of vacuum evaporation and solvent extraction procedures as a result of which acetic acid and hydroxy methyl furfural (HMF) were removed effectively with minimal loss of xylose. *Bacillus subtilis* (ATCC 23857), which is a glutamic acid-dependent strain, was used for this bioconversion. Initially, the concentration of pure xylose and L-glutamic acid were optimized by synthetic medium using response surface methodology (RSM). 65.40 g/L of xylose and 44.98 g/L of L-glutamic acid were found to be the optimal concentrations for maximum production of PGA. This strain was able to produce 12.93 ± 0.9 g/L of PGA after 96 hr of fermentation in pre-treated hemicellulose. Such studies on the production of PGA from renewable sources will contribute to further development of biorefineries and lead to commercial scale production of such products.

Overall, the findings of this dissertation will contribute to the utilization of available hemicellulose streams through fully biobased processes that can lead to the development of a successful economically feasible circular bioeconomy.

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I dedicate this thesis to

my mother

and my sister

Publications related to this thesis

1- **Samavi, M**., Uprety, B.K. and Rakshit, S., 2019. Bioconversion of Poplar Wood Hemicellulose Pre-hydrolysate to Microbial Oil Using *Cryptococcus curvatus*. *Applied biochemistry and biotechnology*, pp.1-12.

2- **Samavi, M**. and Rakshit, S., Utilization of Microbial Oil from Poplar wood Hemicellulose Pre-hydrolysate for the Production of Polyol using Chemo-enzymatic Epoxidation. *Biotechnology and Bioprocess Engineering*, 25, PP. 1-9.

3- Bioconversion of Poplar Wood Hemicellulose Pre-hydrolysate to Poly- γ -Glutamic acid using *Bacillus subtilis.* (preparation for submission)

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6- Amanna, R., Mahal, Z., Vieira, E.C.S., **Samavi, M.,** and Rakshit, S.K. Plastics: Towards a Circular Economy in “"Circular Bioeconomy: Current Developments and Future Outlook", *Elsevier Press* (in print).

List of Abbreviations

Acronym	Abbreviation
A_i	Atomic weight of iodine
A_0	Atomic weight of oxygen
AEFX	*Ammonia fiber explosion*
AIER	Acid ion exchange resin
AMP	Adenosine monophosphate
ARP	Ammonia recycle percolation
ATCC	American type culture collection
CALB	*Candida antarctica* lipase
CCD	Central composition design
C/N	Carbon to nitrogen ratio
DHA	Docosahexaenoic acid
EFA	Essential fatty acids
EPA	Eicosapentaenoic acid
FTIR	Fourier transform infrared spectroscopy
GC	Gas chromatography
GLA	Gamma linoleic acid
HMF	Hydroxy methyl furfural
HPHL	Hemicellulose pre-hydrolysate liquor
HPLC	High-performance liquid chromatography
IEA	International energy agency
IMP	Inosine monophosphate
IV	Iodine value
MO	Microbial oil
NADPH	Nicotinamide adenine dinucleotide phosphate hydrogen
NCC	Nanocrystalline cellulose
NMR	Nuclear magnetic resonance
NREL	National renewable energy lab

OO_{exp}	Experimental oxirane value
OO_{th}	Theorical oxirane value
PGA	Poly-γ-glutamic acid
PLA	Poly lactic acid
PU	Polyurethane
PUFA	Polyunsaturated fatty acid
RSM	Response surface methodology
SAA	Soaking aqueous ammonia
SCO	Single-cell oils
SFA	Saturated fatty acid
SP	Steam explosion pretreatment
TCA	Tricarboxylic acid
TGA	Tri-acylglycerides
UFA	Unsaturated fatty acid
VVM	Volume per volume minutes

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CHAPTER 1
INTRODUCTION

1 Introduction

1.1 General Overview

Steps are being taken around the world to move toward a more sustainable economy and gradually phasing out today's fossil-based economy. This is being planned due to the depletion of fossil resources, price volatility of these resources and various environmental issues associated with it. Researchers are seeking alternative solutions to mitigate climate change and reduce the consumption of fossil fuels. One promising solution to accommodate this transition is the development of biorefineries (Menon & Rao, 2012).

According to the International Energy Agency (IEA) Bioenergy Task 42 (Bell et al., 2014) ''Biorefining is the sustainable processing of biomass into a spectrum of marketable products and energy". In other words, the biorefinery is a network of facilities that integrates biomass conversion processes and equipment to produce transportation biofuels, power, and chemicals from all kinds of biomass (Cherubini, 2010).

A significant amount of research has focused on the production of biofuels under the concept of biorefinery (Badger, 2002; Thangaraj et al., 2018; Van Gerpen, 2005). Despite the remarkable development in this area in terms of investigating new feedstock, catalysts and improving the yield and productivity of biofuel production, currently, most of the existing biofuels are produced in single production chains using starch-based raw materials in competition with the food industry (Bušić et al., 2018). Hence, debates on "fuel vs food" continues. Moreover, as compared to fossil-based fuels the economic production of biofuels from cellulosic resources continues to be a challenge (Sarkar et al., 2012).

In the last few years, the European Commission has focused on the concept of the circular bioeconomy strategy and the related action plan for "closing the loop". This has brought about a new direction in biorefining options. A circular bioeconomy encourages a cascading use of biomass where higher value addition and efficient use of resources is the main focus. Within such systems production of fuels and energy out of biomass is usually referred to as "dead-end" of carbon as it releases the carbon into the atmosphere. Utilization of bioresources for energy leads to carbon accumulation in the atmosphere and is not encouraged. Under the new perspective, biorefineries need to focus on the enhanced production of biochemicals as compared to biofuels (Carus & Dammer, 2018).

Lignocellulosic biomass has great potential as a renewable feedstock to produce value-added chemicals. Lignocellulosic feedstocks have crucial advantages among other biomass supplies as they are the non-edible portion of the plant and therefore has no effect on the food industry. Use of wood biomass as a stable, carbon-storing and readily available lignocellulosic resource can lead to the development of a sustainable system (Isikgor & Becer, 2015). Currently the traditional forest products, including lumber, pulp and paper still constitute the major markets in the forestry sector. However, in order to develop an economical biorefinery diversification of products is necessary (Hurmekoski et al., 2018). This will bring more economic returns from the same amount of feedstock.

Wood like any other lignocellulosic biomass consists of carbohydrates and lignin which is about 80 % of the plant cell wall. Cellulose and hemicellulose are the main carbohydrates in the cell wall. The composition of the biomass varies based on the type of biomass. Softwood trees contain (%w/w) 45 - 50% of cellulose, 25 - 35% lignin, and 25 - 30% hemicelluloses. On the other hand, hardwood trees contains 40 - 55% cellulose, 18 - 25% lignin, and 25 - 30% hemicelluloses (Scheller & Ulvskov, 2010).

Cellulose (40-50% of lignocellulose biomass) is a long polysaccharide of glucose monomers which is the major part of the cell wall in plants. Its hydrophobicity as well as crystalline structure provide tensile strength and support to the plant cell. Hemicellulose, the second major component (20 - 35%) of lignocellulose biomass are (unlike cellulose) branched, heterogenous polymers of pentoses (xylose, arabinose), hexoses (mannose, glucose, galactose) and acetylated sugars. They have lower molecular weight compared to cellulose and branches with short lateral chains that are easily hydrolysed. Lignin (15 - 20%), the third major component, is a complex hydrophobic, cross-linked aromatic polymer. Lignin makes the pretreatment necessary. It interferes with hydrolysis process as well. It has a three-dimensional heterogeneous polycrystalline reticulated polymer, consisting of polyphenolic compounds (Shafiei et al., 2010). Significant research is in progress to investigate the application of lignin-derived chemicals in different industries (Feofilova & Mysyakina, 2016; Thakur et al., 2014). Lignin is currently being used as an energy source by direct combustion. Approximately, 50 million tons of lignin was extracted from the pulp and paper industry alone in 2010, however, only 2 % (1 million ton) was used in commercial productions like dispersing, flocculating or binding agents (Konduri & Fatehi, 2015). The remaining of lignin was burnt to generate energy through combustion (Smolarski, 2012).

Cellulose and hemicellulose can be broken down into monomeric fermentable sugars forming the hexose and pentose sugars which can form major resources for the microbial production of biofuels and biochemicals in a biorefinery.

According to National Renewable Energy Lab (NREL) concept of lignocellulosic biorefinery is based on two main pathways (Takkellapati et al., 2018). The first pathway is based on the biochemical conversion process which evolves around the sugar platform for fermentation products that was discussed above. The second pathway focuses on the thermochemical conversion process which mainly involves pyrolysis, combustion and liquefaction of lignocellulosic biomass. Biochemical conversion includes three main unit operations: pretreatment of the biomass, the (enzymatic) hydrolysis of the carbohydrates to fermentable sugars and finally the fermenting of these sugars by suitable microorganisms to the target molecules.

Cellulose, hemicellulose, and lignin form the plant cell wall with a highly ordered crystalline structure. Therefore, pretreatment is required to degrade such network structure and facilitates removing the lignin and recovery of fermentation sugars. There are different methods for pretreatment which will be discussed in chapter 3 in detail. Pretreatment processes usually separate lignocellulosic biomass into two fractions (a) solid wood residue rich in cellulose and lignin and (b) liquid fraction (hemicellulose or pre-hydrolysate) containing mostly pentose sugars and other components (Kumar & Sharma, 2017).

Cellulose can be hydrolyzed to obtain monomeric glucose which can be easily converted into ethanol by fermentation. However, the high cost of cellulosic ethanol due to the costs involved in its pretreatment, hydrolysis, and distillation has been always the main obstacle for biorefineries. Besides ethanol, cellulose has been the focus of research for its application as nanocrystalline cellulose (NCC) (Brinchi et al., 2013), textiles (Costa et al., 2013), pharmaceuticals (Shokri & Adibkia, 2013), etc.

Hemicellulose is an important but mostly underestimated renewable carbon source. It remains mostly unused, as many of the biotechnological important microorganisms are unable to break down hemicelluloses such as xylan or to metabolize xylose due to lack of related enzymes (Horlamus et al., 2019). However, hemicellulose as a natural water-soluble biopolymer has great potential for the production of biochemicals and bringing added value to the biorefinery. Also, an integrated process utilizing all components of biomass, generating near-zero waste and utilization of by-products will help in the development of a sustainable and economically feasible biorefinery.

For the bioconversion of hemicellulose, pretreatment is inevitable. Pretreatment allows the depolymerization of hemicellulose into sugar monomers and other products (Canilha et al., 2012) Based on the pretreatment method, operational condition, source and type of woody biomass hemicellulose pre-hydrolysate/hydrolysate contains oligosaccharides, lignin degradation products, sugar degradation products, and organic acids. Hence, detoxification is usually required to remove inhibitory products and enhance microbial growth (Parawira & Tekere, 2011). Based on the tolerance of microorganisms to the presence of inhibitory products, detoxified or non-detoxified hydrolysate/ pre-hydrolysate can be used as the carbon source in fermentation.

The bioconversion of hemicellulose pre-hydrolysate to microbial oil (MO), polyol and poly-γ-glutamic acid is the focus of this study.

1.2 Rationale

1. Utilization of hemicellulose as side streams and waste from ethanol production, textile, pulp, and paper industry can play an important role to boost the bioeconomy by maximizing the benefit from the biomass resource used. There is an increase in dissolving pulp production in Canada. While cellulose is manufactured using the suphite process, hemicellulose and lignin are also generated and only partially reused. The implementation of wood extraction prior to pulping (pre-hydrolysis), in Kraft milling processes with subsequent recovery of hemicellulose, has affected the operation of a conventional kraft pulp mill. Conversion of these waste streams is becoming a clear priority within the biorefinery concept.
2. It is crucially important to use all fermentable sugars of lignocellulosic biomass to make the biorefinery economically feasible. There is a need to focus on value-added bioproducts from wood-based hemicellulose. Besides, the value of products, materials, and resources is fixed by the production of biochemicals as compared to fuels which add to carbon in the atmosphere.
3. Biorefineries have high flexibility for the valorization of hemicellulose for environmental and economic development. It is crucially important to produce those biochemicals from hemicellulose that can serve as the starting material for a wide range of products. MO is one such product that can be used for fatty-acid derived chemicals and in the oleo-biochemical industry. Currently, vegetable oils are being used as a renewable source for

the production of many such chemicals. However, using vegetable oils for polymer production is dependent on the availability of arable lands for cultivation and climatic condition. They have a long production lifecycle and are costlier than fossil resources. Most importantly, it can lead to an increase in prices of cooking oils which adversely influences food security. Thus, the global oleochemical industry needs to focus on eco-friendly alternatives and low-cost raw materials which do not affect the food industry. MO with similar fatty acids composition to vegetable oils seems to be a promising feedstock to improve the production of fatty acids-derived oleochemicals.

4. MO obtained from oleaginous microbes have frequently been explored for their use as biodiesel feedstock. However, the circular bioeconomy concept encourages the production of chemicals where products can be used for as long as possible. In such a system, biodiesel would not be the preferred alternative. Diversifying applications of MO helps to move toward an economically feasible biorefinery. Microbial-based polyol produced from MO is a good product that can be used to demonstrate the application of this oil in the polymer industry.
5. Investigating microorganisms that can competently assimilate both C6 and C5 sugars is an important requirement for biochemical conversion in biorefineries as it utilizes all sugars present and prevents environmental issues if they are not utilized. Identification of pentose fermenting microorganisms will broaden the range of substrate available in wood-based biorefineries and lead to economic feasibility.

1.3 Objectives

Based on the above rationale the **overall objective** of this work was microbial production of value-added biochemicals from an underutilized hemicellulose-rich stream of the biomass-based industries. The **specific objectives** of this study were:

1. Production of MO from steam-treated Poplar wood hemicellulose pre-hydrolysate using an oleaginous microorganism
2. Demonstrating of MO-based polyol through chemo-enzymatic route.
3. Production of poly-glutamic acid from steam-treated Poplar wood hemicellulose pre-hydrolysate by optimizing the concentration of carbon source.

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CHAPTER 2
LITERATURE REVIEW

2.1 Introduction

2.2 Lignocellulosic Biorefineries

Biorefining is a general concept where biomass feedstocks are converted into a wide range of valuable products, similar to conventional refineries.

According to IEA biorefinery task 42, biorefineries can be classified to four groups; platforms (core intermediates such as syngas, lignin, pentose, and hexose carbohydrates), products (energy, chemicals), feedstock (biomass from agriculture, forestry, domestic industry) and process (thermochemical, chemical, biochemical and mechanical). Platforms are the most significant feature as they connect the biorefinery concept and target market (Bell et al., 2014).

Biorefinery platforms in turn can be divided into different categories (Table 2.1). Sugar platform (biochemical) are regarded as potential industrial biorefining processes (de Jong et al., 2012). Biochemical platform focuses on the fermentation of sugars extracted from lignocellulosic feedstocks. Lignocellulosic resources are expected to be promising due to their potential including a wide range of low-cost feedstock (Cheng & Wang, 2013; De Bhowmick et al., 2018).

Table 2.1. Most common biorefinery platforms (Carvalheiro et al., 2008; de Jong et al., 2012)

Platform	Feedstock	Product	Common Process
Sugar (Biochemical)	Starch, lignocellulosic biomass	Building block biochemicals	Fermentation, enzymatic hydrolysis, biotransformation
Syngas (Thermochemical)	Lignocellulosic biomass	Pyrolysis oil, gaseous fuels, liquid fuels	Pyrolysis, gasification
Biogas	Manure	Methane, ethanol, ammonia	Anaerobic digestion
Carbon-rich chain (Oil)	Alga oil, Plant-based oil, animal fat	Glycerol, biodiesel	Transesterification

All lignocellulosic biomass are mainly composed of cellulose, hemicellulose and lignin. These polymers form a hetero-matrix to different degrees depending on the type and source of the biomass. Table 2.2 shows the composition of cellulose, hemicellulose and lignin in various types of biomass.

Table 2.2. Composition (wt%) of cellulose, hemicellulose and lignin in various types of lignocellulosic biomass (Shafiei et al., 2015; Sjostrom, 1993).

	Cellulose (wt%)	Hemicellulose (wt%)	Lignin (wt%)
Softwood			
Pine	40 - 46	18 - 29	25 - 30
Spruce	40 - 46	21 - 31	27 - 29
Hardwood			
Birch	41- 49	21 - 32	21 - 22
Poplar	34 - 44	19 - 22	23 - 25
Aspen	46 - 50	18	18 - 23
Agricultural residues			
Wheat straw	33 - 50	24 - 36	9 - 17
Rice straw	28 - 47	19 - 25	10 - 25
Corncob	45	35	15

2.2.1 Cellulose

Cellulose is a linear chain of D-glucose linked together by β-(1 → 4) glycosidic bonds (Fig. 2.1). It is the most abundant polymer on earth with many beneficial properties such as hydrophilicity, biocompatibility, stereo-regularity, and reactive hydroxyl groups. Its polymer chains with highly crystalline structure result in its very stable properties. Cellulose as the main constituent of plant cell wall provides structural support. It exists in bacteria, fungi, and algae as well.

Cellulose can be hydrolyzed to produce monomeric glucose sugar which can be easily converted into other products through fermentation. The utilization of cellulose in various industries such as pulp and paper, textiles, pharmaceuticals, fuel, etc. have been studied (Karimi & Taherzadeh, 2016). This important polymer is still the focus of considerable research for better hydrolysis to glucose sugars, for the production of nanocrystalline cellulose with its numerous applications, etc (Kalia et al., 2011).

2.2.2 Hemicellulose

Unlike cellulose, hemicellulose is a heterogeneous polysaccharide. Hemicellulose consists of xylans, mannans, arabinans, galactans, glucuronoxylans, arabinoxylans, glucomannans and xyloglucans. The composition of hemicellulose varies with the species of plants. It is difficult to

separate the sugars in their single monomeric form. Among the key components of lignocellulosic biomass, hemicelluloses are the most thermo-chemically sensitive (Hendriks & Zeeman, 2009). Therefore, it is crucially important to carefully optimize the operational parameters in pretreatment as well as hydrolysis to avoid the formation of hemicellulose degradation products. Furfurals and hydroxymethyl furfurals (HMF) are such products that were reported to inhibit the microbial growth in the fermentation process (Palmqvist & Hahn-Hägerdal, 2000).

Both cellulose and hemicellulose can be broken down into simple sugars either enzymatically or by acid/alkali hydrolysis. The hexose sugars (mainly from cellulose) can easily be fermented by microorganisms while only a few microorganism strains can ferment the pentose sugars present in sugar mixtures obtained on the hydrolysis of hemicellulose. Considerable research is being carried out to genetically modify microorganisms to produce strains that will be capable of fermenting both glucose and xylose with high yields (Dien et al., 2003; Liu et al., 2015).

2.2.3 Lignin

Lignin is an amorphous polymer providing rigidity to the plant cell wall and resistance against microbial attack. It is insoluble in water to a large extent. Lignin is the third most abundant polymer in nature and consists of phenyl-propanoid precursors. The major chemical phenyl-propane units of lignin are made of syringyl, guaiacyl and p-hydroxy phenol which are linked together by a set of linkages in a complicated matrix. Lignin content varies in different plant species. For example, in softwood conifers, lignin represents 25 - 40 % of dry weight while lignin content is 18 - 25 % in hardwood. It is lower and to the extent of 10 - 20 % of the cell biomass dry weight in grasses and agricultural residues (Novo-Uzal et al., 2012). This complex cross-linked aromatic polymer interferes with the hydrolysis of cellulose. Therefore, lignocellulose must be pretreated to degrade the network structure of lignin and enhance the efficiency of cellulose utilization (Mosier et al., 2005).

2.3 Pretreatment

In order to overcome the lignocellulosic biomass recalcitrance prior to biological conversion, pretreatment is required. Pretreatment is key for subsequent enzymatic hydrolysis and fermentation steps to maximize the productivity of the desired product. The main features for effective pretreatment include (1) maximizing the accessibility of enzymes to appropriate substrates (2) avoiding the degradation of sugars (mainly hemicellulose) (3) minimizing the

formation of inhibitory products (4) recovery of lignin for conversion into valuable coproducts and (5) low capital and operational cost (Maurya et al., 2015).

Pretreatment technologies are generally classified into physical, chemical, biological and physicochemical pretreatment.

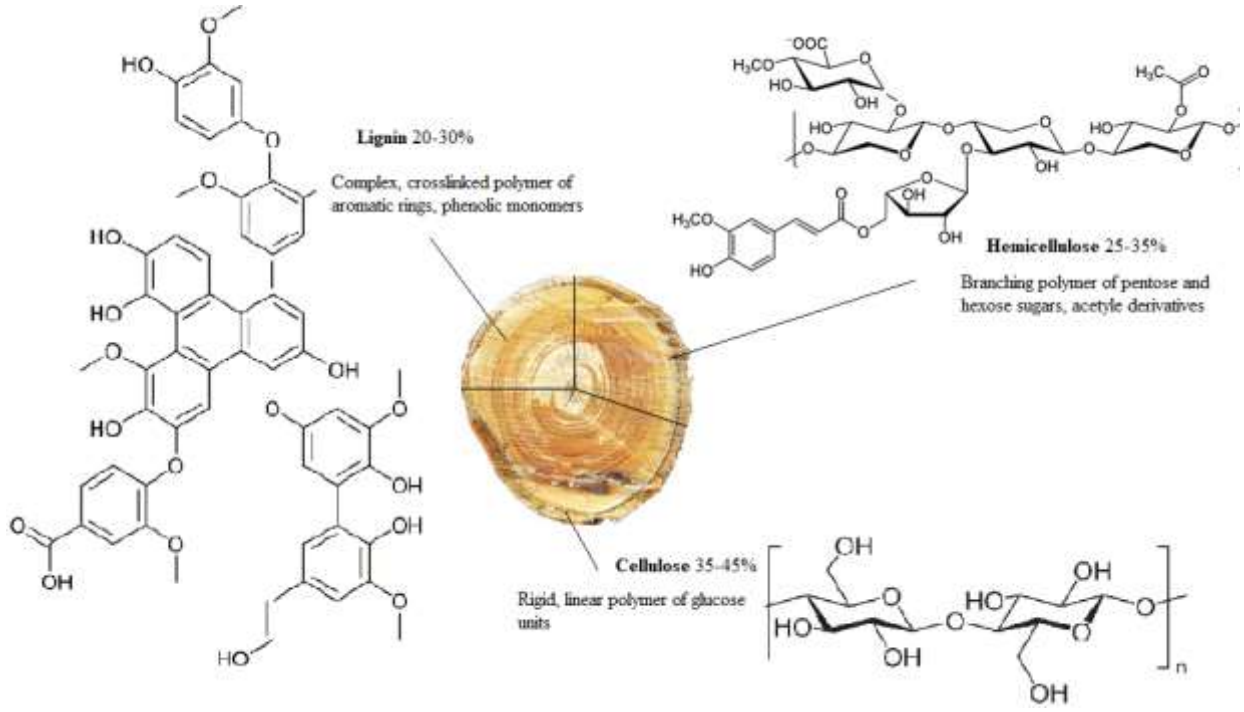

Fig. 2.1. Structure of components of lignocellulosic biomass.

2.3.1 Physical Pretreatment

2.3.1.1 Mechanical Size Reduction

Most lignocellulosic biomass requires mechanical processing for size reduction to increase the surface area and hence reaction with acid/alkali or enzyme. Mechanical splintering methods include dry crushing, wet crushing, chipping vibrating ball mill grinding and compression (Chen et al., 2017). This method is commonly carried out before other methods are applied to facilitate the subsequent process. Chipping reduces the biomass size to 10 - 30 mm while grinding and milling can reduce the particle size to 0.2 - 2 mm (Agbor et al., 2011).

The energy required for the mechanical processing of lignocellulosic biomass depends on both biomass characteristics and the final particle size. For instance, more energy requires for hardwood in comparison with agricultural residues (Cadoche & López, 1989).

2.3.1.2 Microwave Treatment

Microwave is an alternative to conventional heating. The microwave generates heat through direct interaction of the object and an applied electromagnetic field and it is suitable for wet biomass (Intanakul et al., 2003). It also alters the structure of cellulose and disrupts the silicified surface, degrades hemicellulose and partially removes lignin which finally leads to improving cellulose accessibility and reactivity. High temperatures (more than 160 ˚C) are required for microwave pretreatment on plant fibers (de Souza Moretti et al., 2014). Microwave-alkali-acid pretreatment was also been reported on bagasse. The result showed significant effect on lignin removal (Binod et al., 2012). Despite the high cost of this technology, the advantages include, (1) simple operation, (2), energy-efficient and, (3) short process time (Cheng et al., 2011).

2.3.2 Chemical Pretreatment

2.3.2.1 Acid Pretreatment

Acid pretreatment involves the use of concentrated and diluted acids to break down the linkage in lignocellulosic biomass. The use of inorganic acids (sulfuric, nitric, hydrochloric, and phosphoric acids) (Himmel et al., 1997; Wang et al., 2010; Zhang et al., 2007) and organic acids (formic, acetic, and propionic acids) have been reported (Aslanzadeh et al., 2014). However, sulfuric acid is the most commonly used. Acid pretreatment can be carried out either under low acid concentration (0.5-2 %) and high temperature (100 -240 ˚C) or under higher acid concentration (above 30%) and lower temperature (Taherzadeh & Karimi, 2008). Dilute sulfuric acid treatment has been commercialized for different types of biomass such as Poplar (Kumar & Wyman, 2009), spruce (softwood) (Shuai et al., 2010) and switchgrass (Li et al., 2010). High acid concentration has been given little attention due to its disadvantages including toxicity, corrosion of equipments and acid recovery (Yang & Wyman, 2009). The main use of acid pretreatment is fractionating the components of lignocellulosic biomass and removing the soluble hemicellulose (Zhang et al., 2007). The weaker links with the remaining lignin after pretreatment facilitates access to sugars for enzymes and microorganisms.

2.3.2.2 Alkaline Pretreatment

Alkaline pretreatment involves the use of bases, such as sodium, potassium, calcium, ammonium hydroxide and lime for modification of lignocellulosic biomass. Unlike acid and hydrothermal

processes, alkaline pretreatment is mainly used for its effective digestibility of cellulose and hemicellulose (Shafiei et al., 2015). During this pretreatment, a saponification reaction of ester bonds occurs. This leads to the disruption of crosslinks between hemicelluloses and the other components as a result of which the pore structures of lignocellulose are increased (Cheng et al., 2010b). Removing lignin and acetyl groups which inhibit the cellulose accessibility for enzymatic hydrolysis are the main advantages of this method (Chen et al., 2017).

Alkaline pretreatment can be carried out at relatively lower temperatures and does not require complex reactors (Balat et al., 2008). The long residence times required, ranging from hours to days, as well as the need for neutralization of the slurry are considered disadvantages of this method (Wan et al., 2011).

2.3.2.3 Organosolv Pretreatment

Organic solvents such as methanol, ethanol, ethylene glycol with or without acid catalysts (HCl, H_2SO_4) are used to extract the hemicellulose along with lignin and cellulose separately.

This pretreatment is especially efficient for lignocellulosic biomass with high lignin content as it is capable of breaking the internal lignin and hemicelluloses bonds. Besides, relatively pure lignin can be obtained as a by-product through this process (Wan et al., 2011) which is usually performed under high temperatures (100 - 250 ˚C). Since the acetylation makes the slurry acidic, the addition of acids is not necessary (Agbor et al., 2011). Solvents recovery is necessary through evaporation and condensation for further use to reduce the costs. This is also very important as the solvent may inhibit microbial growth, enzymatic hydrolysis, and fermentation or anaerobic digestion (Koo et al., 2012; Ostovareh et al., 2015).

2.3.3 Biological Treatment

Biological pretreatment involves the use of wood degrading microorganisms, including white, brown and soft-rot fungi as well as bacteria that degrade lignin and hemicellulose (Sun & Cheng, 2002). White-rot fungi were reported to be the most effective microorganism for bio-pretreatment of biomass (Sánchez, 2009). This process requires mild conditions and low cost. However, this method has been given little attention due to limitations such as long pretreatment times required for industrial processes.

Biological pretreatment could be effectively combined with other pretreatment methods. If the biomass has low lignin content biological pretreatment (Magnusson et al., 2008) is often recommended.

2.3.4 Physiochemical Pretreatments

2.3.4.1 Steam Explosion Pretreatment (SP)

This process involves heating the biomass using pressurized steam (160 - 270 ˚C, 0.69 - 4.83 MPa) for several seconds to a few minutes and then the system rapidly depressurized to atmospheric pressure when the condensed moisture evaporates. This makes the materials undergo an explosive decompression, as a result of which hemicellulose is solubilized in the liquid phase. The lignin structure is also transformed due to the high temperatures involved. The cellulose in the solid fraction becomes more accessible (Mood et al., 2013; Varga et al., 2004). Extensive research has been conducted on steam-explosion pretreatment which has been commercialized as well (Mosier et al., 2005; Shafiei et al., 2015).

Steam pretreatment also referred to as "Autohydrolysis". Since deacetylation reaction occurs during the pretreatment, acetic acid as an acid could hydrolysis the biomass hence using the term "autohydrolysis" (Baruah et al., 2018; Chandra et al., 2007). This method can be used in combination with acid treatment in order to improve hemicellulose hydrolysis during the pretreatment and cellulose digestibility later in the process (Ballesteros et al., 2006). This process has a lower environmental impact, and cost investment, fewer hazards of chemical reagent, and complete sugar recovery compared with other pretreatment methods (Avellar & Glasser, 1998).

2.3.4.2 Liquid Hot Water Pretreatment

This pretreatment method is similar to steam pretreatment; however, it uses water in the liquid state at elevated temperatures (160 - 220 ˚C) instead of steam. In this method, high pressure is required in order to keep the water in the liquid state. The hot water is in contact with biomass for about 15 min residence time (up to an hour) without the addition of any chemicals or catalysts. As compared to steam pretreatment there is no rapid decompression in liquid hot water pretreatment (Mood et al., 2013). Other names such as hydrothermolysis, solvolysis, hydrothermolysis, aqueous fractionation, and aquasolv have also been used to describe the use of liquid hot water for such purposes (Agbor et al., 2011)

This method of pretreatment enhances cellulose digestibility, sugar extraction and, pentose recovery while minimizing the formation of inhibitory products (Kim et al., 2008). The sugar-

enriched pre-hydrolysate can be directly fermented to produce bioethanol (Van Walsum et al., 1996). Liquid hot water pretreatment is reported to be capable of solubilizing biomass hemicellulose up to 80% (Laser et al., 2002). Cellulose digestibility increases through hemicelluloses removal.

2.3.4.3 Ammonia Pretreatment

Ammonia is considered to be an effective pretreatment reagent as it functions as a swelling reagent for lignocellulosic materials and has high selectivity for reactions with lignin (Wyman et al., 2005). Ammonia fiber explosion (AFEX) (Balan et al., 2009), ammonia recycle percolation (ARP) (Yoon et al., 1995) and soaking aqueous ammonia (SAA) (Kang et al., 2012) have also been reported as ammonia pretreatment methods with differences in operating condition.

The AFEX process is very similar to the steam explosion method during which biomass is subject to liquid ammonia (1- 2 kg of ammonia/kg of dry biomass), under the temperature of 90 - 100 ˚C and pressure of 1.72 - 2.06 MPa. The pressure released rapidly causing cleavage of lignin-carbohydrate complex and subsequent physical disruption of biomass fibers which enhances the digestibility of biomass.

The ARP method is another type of process utilizing ammonia. In this process, aqueous ammonia (10 - 15 wt %) passes through biomass at elevated temperatures (150 - 170 °C) after which the ammonia is recovered (Bals et al., 2010). ARP is followed by the separation and recycling of ammonia. Ammonia is relatively easy to recover due to its high volatility (Kumar & Wyman, 2009).

AAS is a modified version of AFEX using aqueous ammonia in a batch reactor. It is usually carried out at low temperatures (25 - 60 ˚C) and ambient pressure, reducing thus the energy consumption (Kim & Lee, 2005).

The summary of pretreatment methods is shown in Table 2.3. In our study, the starting material we used was Poplar biomass pretreated by a proprietary process using a high temperature extruder in combination with filters. This will be described in subsequent chapters.

2.4 Detoxification of Hemicellulose Hydrolysate/Pre-hydrolysate

As discussed above, acid/alkali thermochemical processes are required to break down the structure of lignocellulosic biomass and obtain fermentable sugars. On the other hand, the pretreatment or

hydrolysis processes result in the formation of toxin by-products that inhibit enzymatic hydrolysis and microbial fermentation.

Depending on the feedstock and the pretreatment method, the formation and concentration of inhibitory products from pretreatment and hydrolysis vary (Larsson et al., 1999). The substances

Table 2.3. Different pretreatment methods available and their relative advantages and disadvantages.

Pretreatment method		Advantage	Disadvantage
Physical	Mechanical splintered	Reduce particle size and cellulose crystallinity	No lignin and hemicellulose removal
	Microwave	Simple operation, Short time	High cost
Chemical	Concentrated acid	High glucose yield, Ambient temperatures	Corrosive
	Diluted acid	No recovery needed	High temperature, Formation of inhibitory products
	Organosolv	Causes lignin and hemicellulose hydrolysis	High cost
Physiochemical	Steam explosion	Hemicelluloses solubilization, Causes lignin transformation	High temperature and pressure
	Liquid hot water	Environmentally benign, Cost-effective	Formation of inhibitory compounds
	Ammonia fiber explosion	Increases accessible surface area, Low formation of inhibitors	Not efficient for raw materials with high lignin content, High cost of large amount of ammonia,
Biological		Degrades lignin and hemicellulose, Low energy consumption	Low rate

that inhibit the microbial growth include phenolic compounds and other aromatics, aliphatic acids, furan aldehydes, and inorganic ions. Phenolic compounds are lignin degradation products, while carbohydrates are the main source of furan aldehydes and aliphatic acids (Acetic acid, formic acid and, levulinic acid).

Acetic acid is mainly a result of the hydrolysis of acetyl groups of hemicellulose. However, formic acid and levulinic acid are being formed through thermochemical pretreatment/hydrolysis which involved acids (Ulbricht et al., 1984).

Degradation of pentose and hexose sugars results in the formation of furan aldehydes furfural and HMF, respectively. These chemicals are commonly found in lignocellulose hydrolysate/pre-hydrolysate. It is worth noting that, some of these degradation products such as levulinic acid, have a very high demand in the market (Bozell et al., 2000).

Therefore, successful removal and separation of these by-products are important as it can make a positive contribution to process economics. Detoxification methods have been proposed to transform inhibitors into inactive compounds or to reduce their concentration. Some of these methods are discussed below.

2.4.1 Biological Detoxification

Biological methods of treatment involve using enzymes or microorganisms that transform the toxic compounds present in the hydrolysate/pre-hydrolysate (López et al., 2004).

Usually, enzymes such as laccases and peroxides are involved for detoxification of hemicellulose hydrolysates. Specific enzymes of white-rot fungus *Trametes versicolor* modify the acidic and phenolic compounds in it. Oxidative polymerization of low-molecular-weight phenolic compounds was reported to be the possible mechanism of these enzymes (Jönsson et al., 1998).

The use of microorganisms has also been reported to selectively remove inhibitors from lignocellulose hydrolysate either directly with employing wild microorganisms (yeasts, fungi, bacteria) and/or recombinant microorganisms expressing laccase or peroxidases enzymes (Chandel et al., 2011a). A fungal isolate, *Coniochaeta ligniaria* (NRRL30616), was also reported to metabolize furfural, HMF, aromatic and aliphatic acids, and aldehydes present in corn stover hydrolysate (Nichols et al., 2008). Despite the effective results, the high cost of enzyme production and the time required for incubation are the main obstacles using this method for detoxification on a large scale (Parawira & Tekere, 2011).

2.4.2 Physical Detoxification

2.4.2.1 Vacuum Evaporation

Vacuum evaporation is a physical method that is suitable to remove acetic acid, furfural and other volatile compounds from hemicellulose hydrolysates. This method was used successfully in several studies for removing acetic acid and furans inhibitors. Approximately, 80% of acetic acid removal was reported from rice straw hydrolysate using vacuum evaporation (Coz et al., 2016). In

most cases, the detoxification by vacuum evaporation was carried out at below 70 ˚C. The main disadvantage of this method reported in the literature is the inability to remove the non-volatile toxic compounds at higher temperatures (Pittman, 2015).

2.4.3 Chemical Detoxification

2.4.3.1 Alkali or Reducing Agent

Alkali based methods include removing toxic compounds by applying alkali such as calcium hydroxide, ammonium hydroxide (Alriksson et al., 2005; Larsson et al., 1999).

However, over-liming (calcium hydroxide) is the commonly used detoxification method for pre-hydrolysate and dilute sulfuric acid-pretreated hydrolysate of lignocellulosic biomass. This low-cost method involves increasing the pH to 10 by adding $Ca(OH)_2$ and then reducing it to 6.5 with H_2SO_4 (Ranatunga et al., 2000).

This process was reported to effectively remove the volatile inhibitory compounds such as furfural and hydroxymethyl furfural (HMF) with sugar loss of approximately 10% (Chandel et al., 2011b). Sugar loss and affecting inhibitors by alkali are the main drawbacks of this method.

Using reducing agents such as dithionite, dithiothreitol, sulfite were reported as well. This *in situ* chemical detoxification helps to skip an extra step for detoxification (Alriksson et al., 2011).

2.4.4 Liquid-Solid Extraction

2.4.4.1 Activated Charcoal treatment

Activated charcoal is extensively used to remove compounds from the liquid phase by adsorption in order to purify or recover chemicals. Using this high capacity absorbent is known to be a cost-effective detoxification method without affecting levels of sugar in hydrolysate/pre-hydrolysate Different process variables such as pH, concentration of activated charcoal, contact time and temperature has a significant effect on the efficiency of the treatment (Prakasham et al., 2009).

It was reported that 27% of phenolic compounds were removed from rice straw hemicellulose hydrolysate with *hydrolysate*:*charcoal* ratio was 40 g/g (In, 2001).

Lack of recyclability of the activated charcoal is a major drawback for its usage in large scales (Pittman, 2015).

2.4.4.2 Ion-Exchange Resins

Anion exchange resins have been widely studied for their efficiency to remove the inhibitors from the hydrolysate efficiently. Both cationic and anionic resin can be used based on the inhibitors (Nilvebrant et al., 2001). Chandel et al (2007) have reported that 63.4% of furans (g/L) and 75.8% of total phenolic in sugarcane bagasse acid hydrolysate were removed using ion-exchange resin (Chandel et al., 2007).

Despite the advantages of using ion exchange resins including reusability and recyclability, the high cost associated with this method limits its application on an industrial scale in lignocellulosic derived products (Chandel et al., 2011a).

2.4.5 Liquid-Liquid Extraction

In this method, a solvent is used to remove the toxin chemicals. Important factors that need to be considered to select an effective solvent include partition coefficient, immiscibility and boiling point of evaporation (Datta et al., 2014).

Different solvents have been used to remove the inhibitors from lignocellulosic hydrolysate/pre-hydrolysate such as ethyl acetate (Cantarella et al., 2004), toluene, chloroform (Dalli et al., 2017), and hexane (Mateo et al., 2013). It has been found that low-molecular-weight phenolic compounds were the main toxin compounds in the ethyl acetate extract. This can be due to the high solubility of phenolic in ethyl acetate (Zhuang et al., 2009). Wilson et al showed that ethyl acetate extraction was more effective than using rotary evaporator by removing all the lignin-derivatives toxins, although, acetic acid was partially removed (Wilson et al., 1989).

2.4.6 Combined Methods

A single detoxification method may not be sufficient to remove different types of inhibitors from lignocellulosic, as each method is specific to certain types of compounds. In order to achieve better results a combination of two or more different methods can be used.

Rodrigues et al. (2001) studied the removal of volatile and non-volatile compounds from sugarcane bagasse hemicellulose hydrolysate using activated charcoal treatment either before or after vacuum evaporation. They reported that using activated charcoal before evaporation removed phenolic compounds effectively, though, acetic acid removal showed better performance using the treatment after evaporation (Rodrigues et al., 2001).

A unified detoxification technique with vacuum evaporation and solvent extraction have been recently developed in our lab with effective performance on removing acetic acid and furfural using toluene as solvent (Dalli et al., 2017)

2.5 Applications of Hemicellulose

In respect to biochemical conversion, it is crucially important to use all components of lignocellulosic biomass in order to make the biorefinery economically feasible

Extensive research has been carried out on cellulose in different areas (Badger, 2002; Kalia et al., 2011). The high cost of cellulosic ethanol has been always the main obstacle for biorefineries due to the costs involved in its pretreatment, hydrolysis, and detoxification of lignocellulosic hydrolysate (Hassan et al., 2018). Also, the production of fuels and energy out of biomass is usually referred to as a "dead-end" of carbon as it releases the carbon into the atmosphere. Circular bioeconomy encourages the cascading use of biomass, where energy uses come in the last place (Carus and Dammer, 2018).

There is a need to focus on value-added bioproducts out of all components of lignocellulosic biomass. Hemicellulose as a natural water-soluble polymer has a great potential for the production of biochemicals. Some of the possible products through bioconversion of xylose including those studied in this thesis are discussed below.

2.5.1 Xylitol

Xylitol is a pentahydroxy sugar alcohol with various applications in the food and pharmaceutical industries (Tamburini et al., 2015). The important property of xylitol is its lower calorific value (2.4 kcal/g) in comparison with sucrose (3.87 Kcal/g), hence its application as a sweetener in confectioneries and diets for diabetic patients (DuBois et al., 1991).

Xylitol can be produced both chemically and biologically from xylose on a commercial scale. The chemical route requires high production cost due to temperature and pressure involved for catalytic hydrogenation. Additionally, xylose in its pure form is needed for the chemical process which in turn increases the cost. However, the biotechnological method can use agricultural and forestry wastes that offer the economical production of xylitol (Ur-Rehman et al., 2015). Also, mild fermentation conditions such as low temperature, atmospheric pressure, and moderate pH are the other advantages of the biotechnological process (Rafiqul & Sakinah, 2013). The microbial process uses bacteria, fungi, and yeast for xylitol production from xylose or hemicellulos-rich

streams. Among these microorganisms, yeasts showed better performance in terms of yield and *Candida spp.* are considered as the best producers (Winkelhausen & Kuzmanova, 1998). Recombinant strains have also been reported to produce even higher yield as compared to wild type (Govinden et al., 2001).

Production of xylitol were reported using different lignocellulosic biomass such as rice straw (Huang et al., 2011), sugarcane bagasse (Dominguez et al., 1996), Poplar wood pre-hydrolysate (Dalli et al., 2017) and other hardwood (Parajó et al., 1996).

Currently, the major limitation in the fermentation process is downstream processing, where the product has to be separated from several metabolites.

2.5.2 Butanediol

2,3-Butanediol, also known as 2-3 butylene glycol (2,3-BD), is a valuable chemical with a broad range of applications in food, cosmetics, antifreeze agents, solvent, and as a precursor of many polymers and resins (Gao & Rehmann, 2014).

Utilization of synthetic pentose and hexose sugars and disaccharides such as sucrose and lactose were reported for biological production of 2,3 butanediol as a carbon source (Menon et al., 2010). However, recently renewable and low-cost resources such as lignocellulosic hydrolysate have attracted great attention due to their availability (Cai et al., 2016).

Butanediol is produced through a mixed acid-butanediol pathway which requires oxygen-limited conditions (Kosaric et al., 1992). Several microorganisms including *Aeromonas(Willetts, 1984)*, *Bacillus* (Perego et al., 2003)*, Paenibacillus* (Nakashimada et al., 2000), *Serratia*, *Aerobacter* (Kosaric & Velikonja, 1995), *Enterobacter* (Saha & Bothast, 1999) and also *Klebsiella* have been used to produce 2,3-butanediol. Among these, *Klebsiella* has been wildly used for 2,3-BDO production due to its ability to consume different types of substrate (Cheng et al., 2010a; Ji et al., 2008; Jiayang et al., 2006).

The main challenge associated with 2,3-BD production is purification and recovery from fermentation broth because of the high boiling point of 2,3-BD and its high affinity for water (Syu, 2001).

2.5.3 Microbial Lipid (ML)

ML, known as single-cell oils (SCO) are macromolecules constituted of carbon, hydrogen, oxygen with a polar head and a non-polar hydrophobic tail. Lipids are one of the main structural components of cells that act as barriers between the inner and outer parts of the cells to transport

the amino acid. Another function of lipid in the cell is that they are stored as carbon sources for the cell mainly in the form of Triacylglycerol (TAGs) and Free Fatty Acids (FFA) (Sorger & Daum, 2003). TAG and FFA are the major groups of lipids. FAs are groups of aliphatic chains with hydrophobicity that are normally activated in the form of acyl-CoA. TGA are molecules of glycerides where the three groups of hydroxyl glycerol are esterified by fatty acids.

MLs with similar fatty acid composition to vegetable oils are considered as a promising alternative for oil sources. These oils can be used as feedstock in biodiesel and bioplastic industry (Uprety et al., 2017b). Moreover, ML containing essential fatty acids (EFAs) such as gamma linoleic acid (GLA), eicosapentaenoic acid (EPA), arachidonic acid (ARA), and docosahexaenoic acid (DHA) are especially valuable in food applications (Das, 2006).

ML are produced by oleaginous microorganisms such as yeast, fungi, bacteria and algae. These microorganism can accumulate lipids more than 20% of their dried cell biomass (Ratledge & Wynn, 2002). Some strains are capable of accumulating a considerable amount of lipids. Up to 80% of cell biomass has been reported (Chatzifragkou et al., 2010). Other than TGAs and FAA, polar fractions (such as phospholipids, glycolipids), other neutral lipids (mono/di- acylglycerols and steryl-esters) and sterols present in the ML as well (Papanikolaou & Aggelis, 2011a). In general, through microbial growth on the hydrophilic substrate which refers to as "*de novo*" pathway, accumulated lipid contains higher quantities of TGA in comparison with hydrophobic substrates. Quantity of the component in the ML varies significantly depending on the microorganism and the growth environment. Oleaginous microorganisms were compared in Table 2.4. Among oleaginous microbes, fungi have attracted more attention as they can accumulate a large amounts of polyunsaturated fatty acids (PUFA) and pellet formation under submerged fermentation which facilitates the downstream separation process (Fakas et al., 2007; Fakas et al., 2009). In addition, short process cycle and easier scalability are beneficial characteristics that make oleaginous fungi very attractive for economical and sustainable production of biochemicals (Subramaniam et al., 2010).

Table 2.4. Comparison of different types of oleaginous microorganisms for production of microbial lipid (Chisti, 2007; Uprety et al., 2017c).

Microorganism	Advantages	Disadvantages
Microalga	High rate of growth Utilization of CO_2	Controlled temperature condition, High cost of production (land, maintenance), Phosphorus required for growth
Bacteria	High rate of growth, Easy culture condition	Accumulation of lipoid
Fungus (mold)	Robust against harsh growth condition, Accumulation of lipids rich in polyunsaturated fatty acids	Low productivity
Yeast	Accumulation of high quantities of lipid, Growth on broad range of substrate	Sensitive to presence of impurities

In oleaginous microorganisms, lipid accumulation and composition of lipids vary significantly depending on the species and even strains due to different genetic characteristics. Also, culture condition such as temperature, pH, culture time, etc. influences the lipid content and its composition (Patel et al., 2017). The lipid compositions of oleaginous microorganisms are shown in Table 2.5. Studies were reported on fatty acid compositional shift by changing the growth medium such as the addition of essential oils (Helal et al., 2007; Uprety & Rakshit, 2017) to change the metabolic flux of the fatty acid biosynthesis pathways. Hence, it is important to characterize the produced ML for lipid composition.

Table 2.5. Fatty acid composition of lipid obtained from different oleaginous microorganism (Meng et al., 2009).

Fatty acid (%w)	Microorganism			
	Microalga	**Bacteria**	**Fungi (mold)**	**Yeast**
C16:0	12-21	8-10	7-23	11-37
C16:1	55-57	10-11	1-6	1-6
C18:0	1-2	11-12	2-6	1-10
C18:1	58-60	25-28	19-81	28-66
C18:2	4-20	14-17	8-40	3-24
C18:3	14-30	-	4-42	1-3

2.5.3.1 Biosynthesis of lipid accumulation

The biochemistry of lipid build-up in yeast and fungus is similar, however, there are some differences for alga oil accumulation. (Zhu et al., 2016). Yeast and fungus metabolism require nutrient-starvation condition (usually nitrogen) and excess of carbon. Under such condition, proliferation continues until the limited nutrient is used up. Then they continue to consume the carbon source and direct it toward the production of lipids. Even though a hyperactive system of fatty acid biosynthesis does not exist in the oleaginous microorganisms, they are capable of producing a notable amount of acetyl-CoA which is the basic unit of fatty acid biosynthesis. Another important attribute of oleaginous yeasts and fungus is sufficient supply of nicotinamide adenine dinucleotide phosphate hydrogen (NADPH) reductant from malic acid or similar alternative NADPH generating sources (Papanikolaou & Aggelis, 2011a; Uprety et al., 2017c)

The cell converts adenosine monophosphate (AMP) into inosine monophosphate (IMP) and free ammonia within the cell in absence of nitrogen in the medium. This lowers the AMP in the cytosol and mitochondrion which in turn reduces the activity of isocitrate dehydrogenase. Noting that isocitrate dehydrogenase, involved in Kreps cycle, depends on AMP and its concentration is regulated by the activity of a related enzyme called AMP deaminase. Consequently, the excess amount of isocitrate accumulates which lead to a higher concentration of citric acid in the mitochondrion. This enters the cytosol and gets cleaved to acetyl-CoA and oxaloacetate. Therefore, the cell receives acetyl-CoA which enters the fatty acid biosynthesis pathway (Ratledge, 2004).

Several numbers of carbon sources were studied as a substrate for the production of ML from oleaginous microorganisms (Xu et al., 2013).

To reduce the cost of ML production, low-cost substrates are essential. Lignocellulosic sugar platform which follows "*de novo*" pathway is regarded as a potential feedstock for SCO production due to its advantages such as availability, and renewable feature (Qin et al., 2017).

Other than cellulose hydrolysate, a significant development in pentose-specific bioconversions has progressed to produce ML. Corncob acid hydrolysate, rich in xylose, was used to produce ML using *Lipomyces starkey.* This strain was able to consume both pentose and hexose sugar and accumulate 8.1 g/L of lipid after 8 days of fermentation (Huang et al., 2014). Other oleaginous yeasts were also reported with xylose consuming ability such as *Cryptococcus curvatus* ATCC 20509 (Yu et al., 2011). Metabolic engineering techniques developed to enhance the yield of this

bioconversion. Among these techniques overexpression of TAG biosynthesis enhancing enzymes, blocking competing pathways and regulation of TAG bypass routes showed better results due to better overexpression of associated enzymes (Liang & Jiang, 2013).

Hemicellulose-rich stream from different sources, mainly agricultural residues, including wheat straw (Yu et al., 2011), palm empty fruit bunches (Tampitak et al., 2015), wheat barn, corn stalk (Enshaeieh et al., 2013) and birch bark (Matsakas et al., 2017) have been investigated for the production of ML.

2.5.3.2 Application of Microbial Lipid

Biodiesel is the most commonly investigated product from ML which is regarded as an excellent alternative feedstock for the biodiesel industry as compared to plant-based oil (Sitepu et al., 2014). Although the production price of ML-based biodiesel has been estimated to be higher than conventional oils and fats (Huang et al., 2013a), a recent study shows that ML is economically competitive by improving the technology in optimized utilization of side and waste stream of biorefineries sectors (Soccol et al., 2017). Another environmentally friendly approach is the production of value-added non-fuel biochemicals from ML which will bring more economic values than biodiesel. More importantly, it fixed the carbon in the closed loop under the concept of a circular bioeconomy.

Polyols are important starting materials for the manufacture of polymers such as thermoplastic polyurethanes (PU) which has been extensively used as rigid and flexible foams, elastomers, adhesives, coatings, resins, fibers and other uses (Desroches et al., 2012). Polyols refer to compounds that contain multiple reactive hydroxyl groups in one molecule and their application varies based on the hydroxy value of produced polyols. polyols with a hydroxyl number of 300 – 650 mg KOH/g are found to be suitable for rigid polyurethanes (Veronese et al., 2011). Low hydroxyl value polyols have other applications in flexible foam and elastomers (Veronese et al., 2011; Wood, 1990).

Currently, polyols are mainly produced from fossil-based resources, though, attentions are attracted to more sustainable resources. Vegetable and plant-based oils as renewable sources were widely studied for the production of bio-based polyol (Li et al., 2015b). Fatty acids (Lligadas et al., 2010), fatty acid methyl esters (Petrović et al., 2012), crop residues (Aniceto et al., 2012; Hu et al., 2014), protein-based feedstocks (Mu et al., 2012) and crude glycerol (Luo et al., 2013) were also reported for the production of biobased-polyol.

Chemical modification is required to introduce the hydroxyl groups into their structure for the production of polyols due to lack of hydroxyl group in the fatty acid chain of ML (Fig 2.2). This modification occurs mainly at the carbon-carbon double bonds and/or ester linkages. The addition of hydroxyl groups to the fatty acid chains of oils can be done by using various methods including ozonolysis, thiol-ene coupling, transesterification and amidation, epoxidation followed by oxirane ring-opening and hydroformylation followed by hydrogenation (Pfister et al., 2011). However, the production of polyols by epoxidation and its subsequent a ring-opening reaction is the most common method investigated. During epoxidation an epoxy group is added to the double bonds present in the fatty acid chains of oils which results in more reactive groups. Fig. 2.2 shows the structure of oil-based polyol produced through epoxidation and ring-opening reaction.

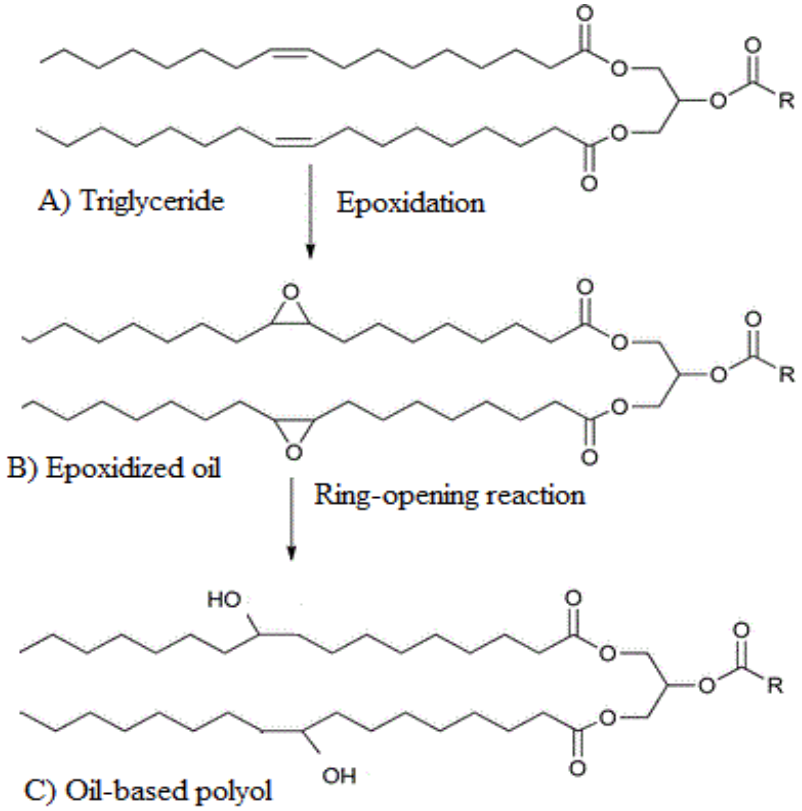

Fig. 2.2. Schematic illustration of microbial lipid-based polyol through epoxidation and ring-opening reaction.

Other than a precursor for PU production, epoxidized oil have a wide range of applications such as stabilizers and plastics plasticizers, solvent, lubricants, and additives and transformer fluids

(Milchert et al., 2015). In addition, it was reported that epoxidized oil can improve the mechanical (Gerbase et al., 2002), electric (Hernández-López et al., 2006), thermal (La Scala & Wool, 2005) properties of polymers and composites. This reaction can be carried out both chemically and enzymatically. The chemical route involves using catalysts such as strong sulfuric acid, acid ion exchange resin (AIER) and a metal catalyst. On the other hand, enzymatic epoxidation showed a selective performance under the milder reaction conditions (Tan & Chow, 2010). To obtain polyol epoxidation follows by a ring-opening reaction. Various polyols can be obtained by the opening of the epoxide group by different agents, which makes this method of polyol production attractive. Also, this method is simple in comparison with other methods reported, hence commercial products have become available on this method (Desroches et al., 2012).

2.5.4 Poly-γ-Glutamic Acid

Poly gamma glutamic acid (γ-PGA) is an extracellular biopolymer that polymerized via the α-amino and γ-carboxylic groups of D- and L-glutamic acid residues. As shown in Fig. 2.3 depending on the position of the amino group attached to the carboxyl group poly-α-glutamic acid (α-PGA), the isomer of PGA can be formed. Initially, microbial production of α-PGA was carried out using recombinant microbial technology (Buescher & Margaritis, 2007).

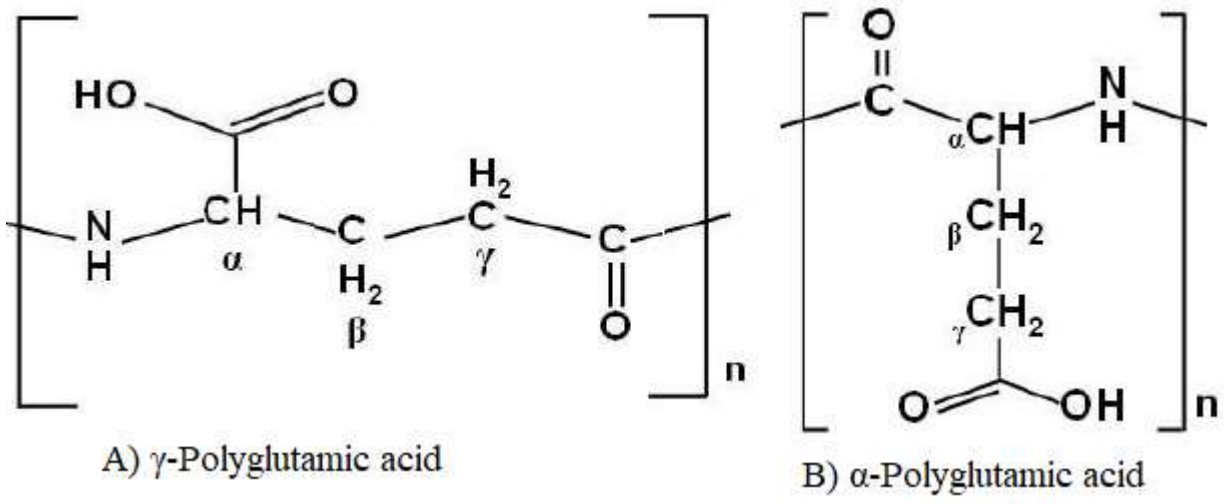

Fig. 2.3. Structure of A) γ-polyglutamic acid and B) α-polyglutamic acid (Buescher & Margaritis, 2007).

This biopolymer (γ-PGA) is water-soluble, biodegradable, edible and nontoxic toward humans and the environment. These beneficial characteristics make γ -PGA and its derivatives an applicable

polymer in a broad range of industrial fields such as food, cosmetics, medicine and water treatment (Sung et al., 2005).

γ-PGA is produced mainly by bacteria belonging to *Bacillus sp*., such as *B. licheniformis*, *B. subtilis*, *B. megaterium*, *B. pumilis*, *B. mojavensis* and *B. amyloliquefaciens*. It is known that PGA is synthesized in a ribosome-independent manner.

Based on the nutrient required, the PGA producing bacteria are classified into two groups as glutamic acid-dependent bacteria and glutamic acid-independent bacteria. The extent of research on the former group is more significant as the yield of fermentation using glutamic acid independent is low (Luo et al., 2016). Genetic engineered of both glutamate-dependent and independent producers have also been used to increase γ-PGA production (Ashiuchi et al., 2006; Cao et al., 2013; Feng et al., 2014).

Biosynthesis of γ-PGA has been studied and genes and enzymes involved in its synthesis have been reported. Fig. 2.4 shows the pathways for the synthesis of γ-PGA in *Bacillus* species via the tricarboxylic acid (TCA) cycle. The biosynthesis of γ-PGA occurs with L-glutamic acid units as the monomer. As mentioned earlier L-glutamic acid can be provided in two different ways either exogenous or endogenous. By the endogenous production of L-glutamic acid, a carbon source is converted to acetyl-CoA and TCA cycle intermediates. The ketoglutaric acid from the TCA cycle serves as a direct precursor of glutamic acid synthesis. On the other hand, during the exogeneous production of L-glutamic acid, a carbon source is converted to L-glutamine by the action of the enzyme glutamine synthase. Also, some of the L-glutamic acid is converted to the D-isomer through the action of glutamate racemase. Glutamine synthase is the precursor for γ-PGA (Sirisansaneeyakul et al., 2017).

In general, this biosynthesis can be divided into four distinct stages; racemization, polymerization, regulation, and degradation (Candela & Fouet, 2006).

The amount of L-glutamic acid added in the medium during the production of γ-PGA is dependent on the strain and other medium components. L-Glutamic acid which is known to act as a regulator or precursor through γ-PGA biosynthesis showed notable interactions with other medium components during the fermentation. Therefore, it is important to use the optimal concentration of L-glutamic acid as it is relatively costly to reduce the cost of the PGA production (Bajaj & Singhal, 2011).

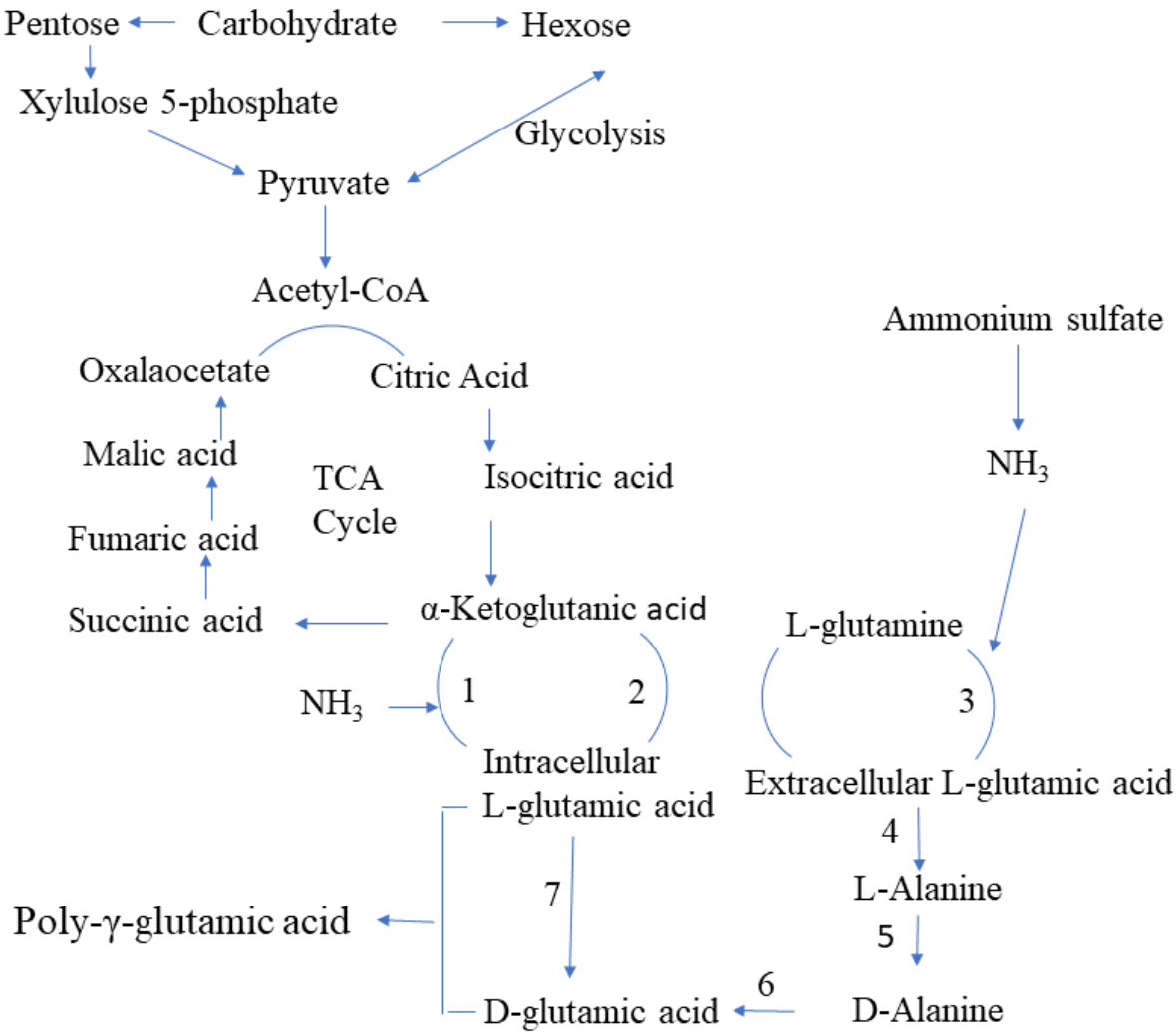

Fig. 2.4 Pathways for the synthesis of γ-PGA in *Bacillus* species via the tricarboxylic acid (TCA) cycle. *1*: glutamate dehydrogenase, *2:* glutamate 2-oxoglutarate aminotransferase, *3:* glutamine synthetase, *4:* l-glutamic acid: pyruvate aminotransferase, *5:* alanine racemase, *6:* d-glutamic acid: pyruvate aminotransferase, :*7* direction conversion, *8:* PGA synthetase.

Mostly pure sugars specifically glucose and glycerol were used as carbon source for production of γ-GPA (Sirisansaneeyakul et al., 2017). Recently different type of biomass such as cane molasses (Zhang et al., 2012), agro-industrial wastes (Tang et al., 2015b) , soybean residue (Wang et al., 2008) and rapeseed meal (Yao et al., 2012) have gain attention as low-cost carbon source in order to economical production of γ-GPA. There is a need to investigate a wider substrate spectrum for this bioconversion. Also, pentose-rich streams have been studied as well such as corncob fibers hydrolysate(Zhu et al., 2014) and rice straw (Tang et al., 2015a), though the number of reports is limited and requires further investigation.

The main constrains that limit the industrial production of γ-GPA are stereochemistry, wide range of yield and broth viscosity due to variety of *Bacillus* species. Also, using complex materials make the product isolation more difficult in the downstream process. The main methods for recovery of PGA from fermentation broth are: filtration and precipitation either by complex formation using metal cation such as Cu^{2+} or by reducing water solubility (Buescher & Margaritis, 2007). Complex formation approach is more selective as compared with reducing water solubility where co-precipitation of proteins and polysaccharides occurs inevitably. Though reducing water solubility, following by addition of ethanol to the supernatant, filtration and purification is being the most common practice for PGA recovery (Luo et al., 2016; Ogunleye et al., 2015).

Table 2.6. Applications of γ-PGA and its derivatives (Ogunleye et al., 2015; Pereira et al., 2012; Shih & Van, 2001).

Field	Application	Description
Medicine	Drug carrier	Improvement of anticancer drugs
	Curable biological adhesive	Substitution of fibrin
Cosmetic	Humectant	Enhancing quality of moisturizer, exfoliant and wrinkle-remover
Food industry	Thickener	Enhancing viscosity for fruit juice beverage
	Animal feed additives	Increasing egg-shells strength; decreasing body fat,
Bioremediation	Adsorption of heavy metals	Removal of heavy metals and radionuclides
	Biopolymer flocculant	Substitution for non-biodegradable flocculants such as polyacrylamide
Biochemical industry	Thermoplastics, fibers, films	Substitution for chemically synthesized, non-biodegradable plastics

2.5.5 Lactic Acid

Lactic acid is primarily used in the production of bioplastics such as poly-lactic acid (PLA), which is a promising biodegradable, biocompatible, and environmentally friendly alternative to plastics derived from petrochemicals. This organic acid is also widely used in food, cosmetic, pharmaceutical industries due to its attractive and valuable multi-function properties (Vijayakumar et al., 2008). Lactic acid has optical isomers, L-lactic, D-lactic, or DL-lactic, depending on the

microbial strain and fermentation condition (Carr et al., 2002). Currently, lactic acid is produced from edible crops such as corn starch on a large scale using various lactic acid bacteria. Recently, several lignocellulosic biomass such as wheat straw hydrolysate (Garde et al., 2002), soybean stalk (Xu et al., 2007), corncob molasses and (Wang et al., 2010) without competing with food security were investigated to produce lactic acid.

Lactobacillus spp. are mainly used for starch-based lactic acid industrial production, However, the majority of them are unable to assimilate C-5 sugars (Tsuji, 2002). Although some strains such as *Lactobacillus pentosus*, *L. brevis* and *Lactococcus lactis* are able to ferment pentose sugar, the yield is very low. With the phosphoketolase pathway, through which xylulose 5-phosphate is cleaved to glyceraldehyde 3-phosphate and acetyl-phosphate, the maximum theoretical yield of lactic acid is 0.6 g lactic acid per g xylose (Tanaka et al., 2002).

The biochemicals discussed above can be produced following biological routes from hemicellulose. It should be noted that the unique structure of hemicellulose as a branched heteropolymer containing several hydroxyl groups could be modified by many chemical reactions such as etherification, chemical crosslinking, surface modification, and esterification for the production of packing films, hydrogel oxygen barrier, and water-resistant coating (Farhat et al., 2017).

2.6 Challenges with Hemicellulose Utilization

Separation of hemicellulose is relatively easy as it is water-soluble. However, obtaining the hemicellulose sugar in pure form remains a challenging task. Because unlike cellulose, hemicellulose has a branched and complex chemical structure and it is difficult to get a monomer, for example, xylose in its pure form from the mixture. Different types of sugars are linked together and forms oligomers. It is challenging to recover the sugars in pure form using a cost-effective method (Pittman, 2015).

Another obstacle is the release of inhibitory compounds during the pretreatment process. Composition and concentration of these inhibitory products depend on the type of lignocellulosic biomass as well as pretreatment operating condition (Agbor et al., 2011). Some of the emerging pretreatment methods such as alkaline peroxide and AFEX generate solubilized and partially degraded hemicellulose. Though further treatment with enzyme, acid or alkali is needed to obtain fermentable sugars which lead to the formation of degradation products such as acetic

acid, furfural, and HMF. Improving the yields of xylose with low levels of byproducts is the key to improve the hemicellulose-based biorefinery (Froschauer et al., 2013; Li et al., 2013). Further research needs to be carried out on the development of efficient pretreatment methods, enzymes for hydrolyzing cellulose and hemicellulose on large scale.

Another main challenge in using lignocellulosic biomass including hemicellulose-rich stream as a feedstock is the heterogeneity of sugars present in the hydrolysate/pre-hydrolysate. It is important to utilize all sugars in order to maximize the yield. However, due to carbon metabolite repression microorganisms selectively consume hexose sugars as compared to pentose sugars. This sequential utilization of mixed sugars lowers the yield and productivity of fermentative productions (Zhu et al., 2014). Investigating microorganisms capable of fermenting hemicellulose sugars simultaneously seems to be necessary.

2.7 Conclusion

Hemicellulose, the second abundant carbohydrate fraction in lignocellulosic biomass, serves as an important resource for the production of biochemicals and generating added value in the circular bioeconomy. Bioconversion of hemicellulose sugars to biochemicals using a green process with a mild condition is crucially important for the establishment of a successful biorefinery.

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CHAPTER 3

OBJECTIVE 1

Bioconversion of Poplar Wood Hemicellulose Prehydrolysate to Microbial Oil Using *Cryptococcus curvatus*

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3 Bioconversion of Poplar Wood Hemicellulose Pre-hydrolysate to Microbial Oil Using *Cryptococcus curvatus*

3.1 Abstract

Poplar wood hemicellulose pre-hydrolysate was used for microbial oil production using an oleaginous microorganism *Cryptococcus curvatus*. Initially, the effect of substrate concentration and nitrogen content was investigated on synthetic media. Then Poplar wood pre-hydrolysate without detoxification was used as substrate in the fermentation. The result showed that this strain is capable of consuming both hexose and pentose sugars, a challenge in fermentation of hemicellulosic streams. It was able to accumulate 36.98% of lipid and the fermentation resulted in 13.78 g/L of cell biomass and 5.13 g/L of lipid under optimal conditions after 164 hours of fermentation. The lipid product obtained was characterized in terms of their fatty acid profiles. Overall this study shows that it is possible to produce microbial oil from a sustainable renewable feedstock like Poplar wood hemicellulose pre-hydrolysate. This robust strain used has the ability to grow on industrially produced hemicellulose which can help in the development of an integrated biorefinery, where all the three components of lignocellulosic biomass are utilized.

Key words: Microbial oil, Hemicellulose, Xylose, Forestry residues, *Cryptococcus curvatus*.

3.2 Introduction

Production of chemicals and fuels from biomass helps to mitigate climate change. A potential approach towards the development of a sustainable future is the use of wood biomass as a renewable, carbon-storing and readily available lignocellulosic resource. So, these substrates have to be used for production of biochemicals and biofuels along with other traditional forest products such as lumber or pulp. This diversification of products will bring more economic returns from the same amount of feedstock and make the biorefinery economically feasible. Demand for bio-based products is growing from 2% of the market in 2008 up to 22% in 2025 (Biddy et al., 2016). Hence it is crucially important to search for alternative sources and processes for value-added products.
Wood like other lignocellulosic biomass mainly consists of complex mixture of cellulose, hemicellulose, and lignin. Such a structure makes it highly recalcitrant. Biorefineries typically have to disrupt the plant cell-wall structure of lignocellulosic biomass by different methods of

pretreatment. Pretreatment involves the application of physical, chemical, thermal, biological or combinational methods which disrupt the complex structure of biomass (Pittman, 2015). Some methods like steam explosion and *ammonia fiber explosion* (AEFX) have been scaled up and currently are being used in industries (Agbor et al., 2011). Subsequent hydrolysis to obtain fermentable sugars is facilitated by pretreatment. The fermentable sugars derived from lignocellulosic biomass are mainly glucose from cellulose and xylose from hemicellulose. Significant amount of research carried out on lignin (Azadi et al., 2013; Thakur et al., 2014) and cellulose (Badger, 2002; Kalia et al., 2011). Also, the production of cellulosic ethanol has a number of bottleneck challenges including product value and energy input (Kang et al., 2014). The amount of work on hemicellulose is not so significant as compared to other two components. However, wood-based water soluble hemicellulose has considerable potential.

Recently hemicellulose hydrolysate has been the focus of some work for production of microbial oil (MO), also referred to as single-cell oils (SCO), using oleaginous yeasts. MOs have been studied for many years due to their important characteristics (Sitepu et al., 2014). They are a good source of poly-unsaturation fatty acids such as γ-linolenic acid (Fakas et al., 2007) eicosapentaenoic acid (EPA) (Guo & Ota, 2000) docosahexaenoic acid (DHA) (Wu et al., 2005). Also, as the long chain fatty acids in MO are similar to that present in conventional vegetable oils, they have become an important area of research focus as a promising feedstock for biodiesel as well as oleo-based biochemical industries (Sitepu et al., 2014; Uprety et al., 2017b). MO production does not require large arable lands, is not dependent on climatic conditions and has little effect on food security (Kumar et al., 2017). The price of the MO has been difficult to estimate due to the variation of feedstock, location and scale of the production (Koutinas et al., 2014; Parsons et al., 2019). US$ 1230-3000 per ton was reported as the cost of MO production (Huang et al., 2013a; Ratledge & Cohen, 2008). A recent study shows that by improving the technology microbial oil is economically competitive when compared to vegetable oil (Soccol et al., 2017). The cost of production is mainly influenced by carbon sources used in the fermentation (Fontanille et al., 2012). Hence, the use of low-cost hemicellulose-rich stream can serve as an important substrate for bioconversion to microbial oil on a large scale. Hemicellulose from agricultural residues like corn stover (Ruan et al., 2012), corncob (Huang et al., 2014), rice straw (Huang et al., 2009), palm empty fruit bunches (Tampitak et al., 2015) have been widely investigated during past years. Synthetic medium that are glucose-based (Khot et al., 2012) or xylose-based (Gao et

al., 2013) have been used to characterize the newly isolated strain or optimize the medium composition. Wood based hemicellulose has not been explored extensively as compared to agricultural residues, even though, it is a stable and abundant resource. Also, the challenge that the hemicellulosic stream contains both C-5 and C-6 based sugar has not been fully investigated.

Investigating oleaginous strains that are capable of consuming both glucose and xylose for a lipid-based lignocellulosic biorefinery will be very beneficial. Xylose, a major pentose sugar of hemicellulose, is generally not consumed by ethanol-producing yeast strains (Hector et al., 2008). Tanimura et al. (Tanimura et al., 2016) screened 1189 oleaginous yeast strains and reported that only 12 strains are capable of co-fermenting a sugar mixture including glucose, xylose and L-arabinose. *Pseudozyma hubeiensis IPM1-10* was reported to has the maximum sugar utilization rate (94.1%) and produce 1.56 g/L of lipid. Five oleaginous yeast strains, including, *Rhodotorula glutinis, Cryptococcus curvatus, Lipomyces starkeyi, Yarrowia lipolytica* and *Rhodosporidium toruloides* have been studied on wheat straw acid hydrolysate as substrate. The highest amount of cell biomass and lipid with concentration of 17.2 g/L and 5.8 g/L respectively were obtained by *Cryptococcus curvatus* on non-detoxified liquid hydrolysate (Yu et al., 2011). Huang et al (Huang et al., 2014) studied the growth of oleaginous yeast *Lipomyces starkeyi* on corncob hydrolysate. They reported dried cell weight of 17.2 g/L and lipid content of 37.8% of DCW after 8 days of fermentation.

In this study, a pre-hydrolysate of hemicellulose from Poplar wood has been used as a substrate to produce microbial oil using the oleaginous yeast *Cryptococcus curvatus*. Fermentation studies to determine substrate inhibition and carbon to nitrogen (C/N) ratio, an important process parameter in MO production was evaluated for lipid yield on synthetic sugar media initially. The effect of different C/N ratios on fatty acid profile were also monitored. To the best of our knowledge this is the first time *C. curvatus* has been grown on Poplar wood hemicellulose pre-hydrolysate. The use of an abundantly available renewable water-soluble renewable substrate and a robust oleaginous strain can make this bioconversion economically attractive.

3.3 Materials and Methods

3.3.1 Pre-hydrolysate Substrate

Hemicellulose pre-hydrolysate liquor (HPHL) obtained from Poplar wood was kindly donated by Green Field Global Inc., Chatham, Canada. The HPHL was obtained using a novel patented

process (Lehoux & Bradt, 2016). Briefly, the process involves a solid/fluid separation module and pretreatment method that operates at high temperature and pressure with variable processing and residence times. Steam treatment of Poplar wood chips through twin screw extruder results in two solid and aqueous streams. The former stream consists of cellulose and lignin and the latter is hemicellulose. This aqueous stream has been used as a substrate in this study.

3.3.2 Microorganism and Fermentation Condition

The oleaginous yeast *C. curvatus* (ATCC 20509), also known as *Trichosporon oleaginosus*, *Apiotrichum curvatum* or *Candida curvata* D (Bracharz et al., 2017) was used for fermentation. It was grown initially in medium containing 3 g/L yeast extract, 3 g/L malt extract, 5 g/L peptone, and 10 g/L xylose, pH 5.5 at 30˚C and 180 rpm for 24 hours as inoculum preparation step. The minimal medium containing (g/L) KH_2PO_4 2.7, Na_2 $HPO_4.12H_2O$ 0.95, $MgCl_2.6H_2O$ 0.2, yeast extract 0.1 was inoculated (10%) by seed inoculum. Ammonium chloride was used to adjust the C/N ratio. Subsequently the carbon source was either pure synthetic sugars (xylose, glucose and arabinose) or diluted PHL to the desired sugar concentration. The medium was supplemented with 10 ml/L of trace element consisted of (g per L of distilled water) $CaCl_2$. $2H_2O$ 4.0, $FeSO_4.7$ H_2O 0.55, Citric acid monohydrate 0.52, $ZnSO_4$. 7 H_2O 0.1, $MnSO_4$. H_2O 0.076 and 18M H_2SO_4 100 µL (Meesters et al., 1996). Shake flask fermentations were carried out in 125 ml flask with working volume of 50 ml. All sample analyses were carried out in duplicate and errors were presented as mean ± standard deviation.

3.3.3 Determination of Cell Biomass, Lipid Extraction and Methylation

The amount of cell biomass produced was determined by gravimetry method as follows: an aliquot of the fermentation broth was harvested by centrifugation at 4400 rpm for 10 min (model: Eppendorf 5702). The supernatant was used for sugar analysis and the wet cells were washed by distilled water, then dried at 80˚C overnight and finally weighted. Modified Bligh-Dyer method (Uprety et al., 2017a) developed in our lab was used to extract the lipid. Methylation was carried out by dissolving a lipid sample in 0.2 ml of toluene. In the next step, 1.5 ml of methanol and 0.3 ml of 8.0% (w/v) HCl in methanol were added respectively. The mixture was then vortexed and kept at 100 ˚C for 1 hour. One ml each of hexane and water were added to the tube after cooling down to room temperature. The hexane layer was separated by pasture pipet and used for GC

analysis (Ichihara & Fukubayashi, 2010). Sugar consumption, cell biomass and oil production were expressed as gram per liter of fermentation broth.

3.3.4 Determination of Total Solid and Sugar

Conventional oven method was used to determine the total solids (Sluiter et al., 2008). Sugars, organic acids and furfural concentration were analyzed by HPLC (Agilent Technologies 1260 Infinity). Analysis were carried out by Aminex HPX-87H ion exclusion column (300 mm - 7.8 mm) with a Refractive Index Detector (RID) at 50˚C. 5 mM H_2SO_4 at 0.5 mL min^{-1} flow rate was used as mobile phase.

3.3.5 Fatty Acid Composition Analysis

The fatty acid profiles of the lipids were obtained using a Gas chromatography (Thermo Scientific, Trace 1300 model, polar column TG WaxMS A; 30 m ×0.25 mm × 0.25 mm) equipped with FID detector. Methyl nonadecanoate (C19) was used as internal standard. 1µl of trans-esterified lipid samples were injected to GC with the following operating conditions. Flow rate of Helium as carrier gas at 1 ml/min, air 350 ml/ min, H_2 at 35 ml/min, and N_2 at 40 ml/min. Sample injection split ratio of 50:1. The temperature gradient in the oven 100˚C (0.25 min), 30˚C/min, 220˚C (0 min) and 10˚C/min, 250˚C (3 min).

3.3.6 Elemental Analysis

The nitrogen (N) and phosphorus (P) content of HPHL was determined by an Elementar Vario EL Cube analyzer (Langenselbold, Germany) at the Lakehead University Instrumentation Lab (LUIL). 25 mg of dried sample was transferred in the integrated carousel of the elemental analyzer and burned at 1200˚C for determining the nitrogen content.

3.4 Results and Discussion

3.4.1 Substrate Composition

HPHL provided by Greenfield Global as a result of pretreatment of Poplar wood was used in this study. The process does not involve any acid treatment. Therefore, the stream contains low levels of toxins or inhibitors such as acetic acid or furfural which are produced by most pretreatment processes (Agbor et al., 2011). The composition of concentrated HPHL is showed in Table 3.1 Xylose was found to be the main monosaccharaides in the HPHL. Glucose and arabinose are

present in the HPHL in relatively lower levels. The ratio of xylose to glucose is higher as compared to other hemicellulose-rich substrate reported for microbial oil production (Huang et al., 2014; Tampitak et al., 2015; Yu et al., 2011). The presence of 9.55 g L^{-1} acetic acid is due to high temperatures applied during the pretreatment process. Crude HPHL without detoxification has been used in fermentation, since by diluting the HPHL to desired sugar concentration (see next section) all other components including inhibitory get diluted to low level. Thus, the low concentration of inhibitory products in the HPHL substrate is beneficial. Moreover, *C. curvatus* is known to be able to grow on different types of lignocellulosic biomass, even in presence of inhibitory components such as acetic acid and hydroxy methyl furfural (HMF) to level of 3 g/L and 1 g/L, respectively (Bracharz et al., 2017; Yu et al., 2011). Low concentration of inhibitory products in HPHL enabled us to skip the detoxification step which is an important parameter in scaling up the overall process.

Table 3.1. Composition of steam treated Poplar wood pre-hydrolysate (HPHL) used in this study

Xylose (g/L)	143.89 ± 1.28
Glucose (g/L)	7.07 ± 1.53
Arabinose(g/L)	3.31 ± 1.06
Acetic Acid(g/L)	9.56 ± 0.89
HMF(g/L)	0.48 ± 0.35
Total Solid % (w/w)	19.25 ± 0.33
Nitrogen (wt %)	3.66 ± 0.03
Phosphorus (ppm)	56.19 ± 0.01
pH	2.81 ± 0.16
Density (g/mL)	1.04 ± 0.10

3.4.2 Effect of Substrate Concentration on Cell Biomass and Lipid Accumulation

C. curvatus was cultured in synthetic medium with various pure xylose concentrations in order to determine the effect of substrate inhibition in lipid accumulation with respect to cell growth. This

strain has been known for its ability to accumulate high intracellular concentration of lipid in the form of droplets (Bracharz et al., 2017). The fermentation results are given in Fig. 3.1 Maximum cell biomass was obtained after 7 days of fermentation at xylose concentration of 30 g/L above which substrate inhibits the growth. However, the highest accumulation of lipid of 3.57 g/L occurs with a concentration of 25 g/L of xylose with slightly less cell biomass. Considering the lipid yield, xylose concentration of 25g/L was selected for further experiments.

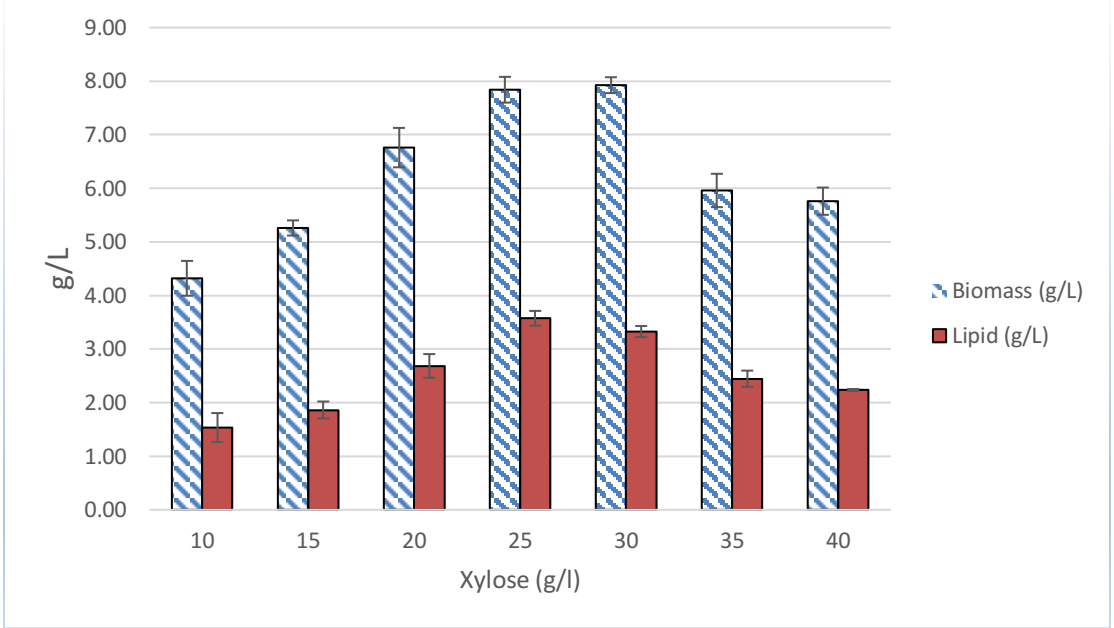

Fig. 3.1. Effect of substrate inhibition on *C. curvatus* grown on synthetic medium with different concentrations of pure xylose, C/N ratio: 100

3.4.3 Effects of C/N Ratio on Cell Biomass, Lipid Accumulation and Fatty Acid Profile

Based on the substrate, lipid biosynthesis in oleaginous yeasts follows two pathways “de novo” and “ex novo”. In the former, lipid accumulation is carried out on hydrophilic substrate such as lignocellulosic biomass (Jin et al., 2015), glycerol (Uprety et al., 2018), sewage sludge (Angerbauer et al., 2008) and food waste (Zhan et al., 2013). However, in ex novo lipid accumulation is carried out on hydrophobic substrates such as industrial fats (Papanikolaou & Aggelis, 2003). Usually de novo growth requires nitrogen-limited culture conditions (Papanikolaou & Aggelis, 2011b). Therefore C/N ratio of medium needs to be carefully controlled

to maximize the lipid yield. Different C/N molar ratios ranging from 50 to 300 were prepared in a synthetic medium, with similar HPHL sugar composition, containing (g/L) 25 xylose, 1.22 glucose and 0.57 arabinose. Nitrogen in the form of ammonium chloride was used to adjust different C/N ratios. Fig. 3.2 shows that the cell biomass increased as C/N ratio increases. At C/N ratio of 250 the maximum cell biomass of 9.06 g/L was obtained. However, lipid accumulation increased gradually and reached its maximum at C/N ratio of 200. Lipid concentration decreased for C/N ratios higher than 200. Also, separation of cell biomass from broth was difficult with centrifuge for C/N ratio above 200 and had to be carried out by filtration. Therefore, C/N ratio was kept at 200 for further experiments.

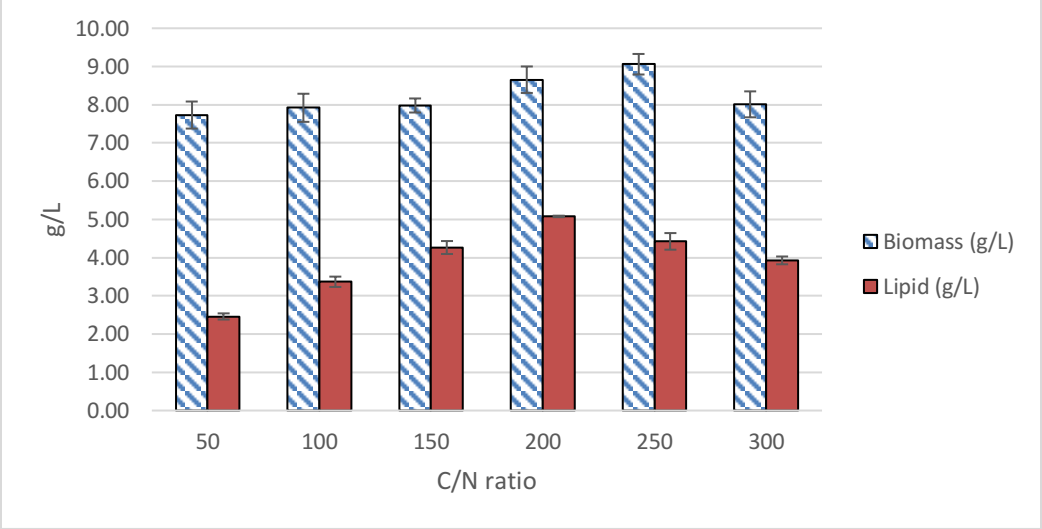

Fig. 3. 2. Effect of C/N ratio on lipid accumulation of *C. curvatus* grown on a synthetic medium with composition similar to steam treated poplar wood hemicellulose prehydrolysate (HPHL) (25 g/ L of xylose).

Based on our experience working with two different oleaginous microorganisms; *R. toruloides* (ATCC10788) (Uprety et al., 2018) and *C. curvatus* (ATCC20509), harvesting cell biomass after fermentation depends on the strain and the medium composition. For instance, *C. curvatus* produces loose clumps of cell biomass as compare to *R. toruloides* (dense aggregation pellet). Also, cell biomass separation is affected by media composition. The higher the C/N ratio, the harder the separation. The reason can be explained by the fact that high-lipid content cells move to the top layer (floating-cells) due to the high buoyancy property as illustrated in Fig. 3.3, whereas normal cells settle down to the bottom of the tube after centrifuge (Liu et al., 2015).

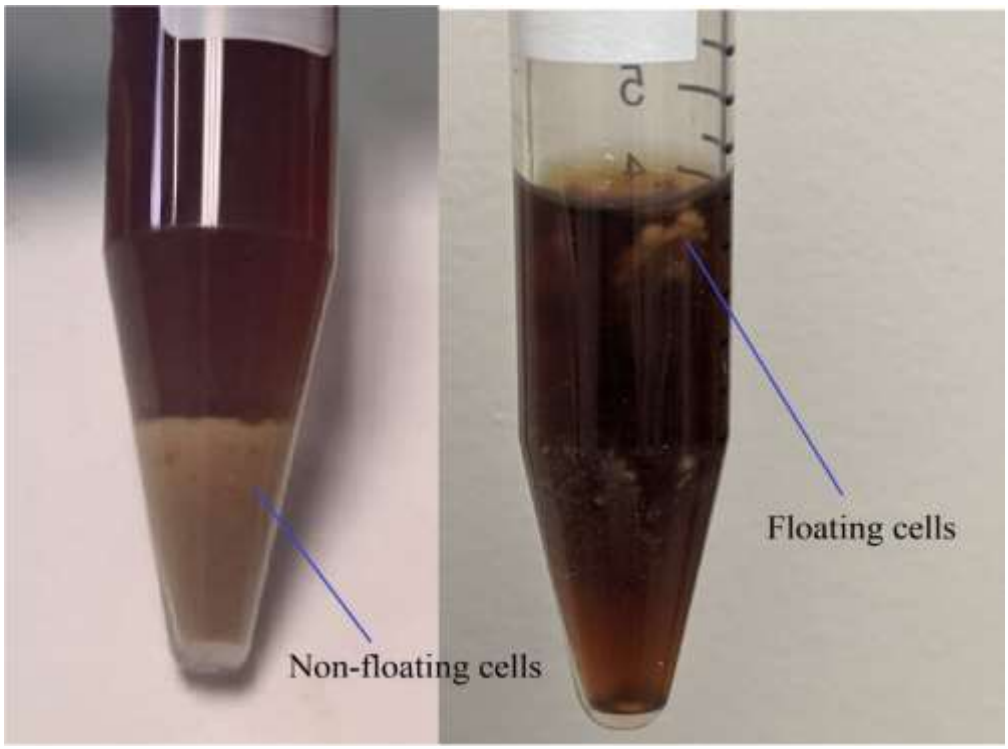

Fig. 3.3. High lipid content cells of *C. curvatus* (ATCC20509) and formation of floating cells (right) whereas settled cells (left).

Fatty acids profile of microbial oils varies from one microorganism to another one depending on the feedstock as well as growth condition (Patel et al., 2017). This in turn influences the properties of biodiesel or oleo-chemicals that are designed to be produced from obtained MOs (Samavi et al., 2016). Hence, the importance of screening fatty acid profile of product MOs. Table 3.2 shows an increasing trend in percentage of unsaturation (unsaturated fatty acid, UFA) of microbial oils obtained as C/N ratio increases. On the other hand, no significant change was observed in percentage of saturated fatty acid (SFA). Oleic acid (C18:1) is the main fatty acid presented in the oils with 31.82 – 47.12 wt.%. Oils rich in oleic acid are considered high quality raw materials for the production of bio-lubricants and biodiesel, due to their excellent properties including thermal and oxidative stability (McNutt, 2016; Park et al., 2008). The other main fatty acids present were palmitic (C16:0), stearic (C18:0), myristic (C14:0) and linoleic acids (C18:2), respectively.

Table 3.2. Effect of C/N ratio on fatty acid composition of microbial oil using *C. curvatus* on synthetic medium (SFA: Saturated Fatty Acid, UFA: Unsaturated Fatty Acid).

C/N Ratio	Fatty Acids						
	C14:0	C16:0	C18:0	C18:1	C18:2	SFA%	UFA%
50	13.71 ± 0.85	13.47 ± 0.83	8.05 ± 0.81	31.82 ± 1.32	7.45 ± 0.83	35.23	39.27
100	8.93 ± 1.48	19.28 ± 1.14	11.81 ± 1.21	42.67 ± 0.48	3.78 ± 0.86	39.99	46.45
150	8.90 ± 1.25	15.62 ± 0.82	13.15 ± 0.44	44.24 ± 1.03	7.53 ± 0.61	37.67	51.77
200	8.83 ± 0.31	17.66 ± 0.99	15.66 ± 0.98	42.86 ± 0.95	4.44 ± 0.93	42.15	47.30
250	4.25 ± 1.24	18.18 ± 1.36	17.06 ± 1.34	47.12 ± 1.00	4.35 ± 1.08	39.49	51.47

3.4.4 Cell Biomass and Lipid Accumulation in the Hemicellulose-based (HPHL) Medium

Following the experiments carried out with synthetic sugar-based media, the complex hemicellulose media was used. Cell growth, lipid accumulation and sugar consumption profile of *C. curvatus* grown on HPHL medium has been shown in Fig. 3.4 *C. curvatus* used both xylose and glucose, however, as expected glucose assimilation was found to be faster than xylose. As soon as glucose was used up, xylose intake speeded up. Considering the metabolic pathway of xylose in yeasts, xylose is firstly converted to xylulose 5-phosphate which is further metabolized through the phosphoketolase pathway or pentose phosphate pathway (Evans & Ratledge, 1984). It took almost 120 hours for all the xylose to be consumed. The amount of lipid was not considerable in the initial 2-4 days of fermentation. This is due to the de novo lipid metabolism as a result of which lipid synthesis starts after the essential nutrient (usually nitrogen) is used up in the medium. (Papanikolaou & Aggelis, 2011b). The lipid concentration increases in the second stage of the fermentation and reached to it maximum by end of 7^{th} day. Fig. 3.5 shows the morphology and lipid accumulation in *C. curvatus*. In general, the highest cell biomass of 13.87 g/L and lipid concentration of 5.13 g/L were obtained after 168 hours of fermentation. Lipid turn over, breaking down of the lipid by microorganisms to maintain their growth due to lack of nutrients, was observed on day 8^{th}. Similar result in terms of lipid turn over has been reported earlier (Meeuwse et al., 2011). As Fig 3.4 shows, all the sugar was consumed resulting in yield (Y_{xs}) of 0.19 g/g. In comparison with maximum theorical yield where glucose (0.32 g/g) and xylose (0.34 g/g) are used as substrates (Ratledge, 1988). 57.7% conversion was achieved using *C.curvatus* on HPHL.

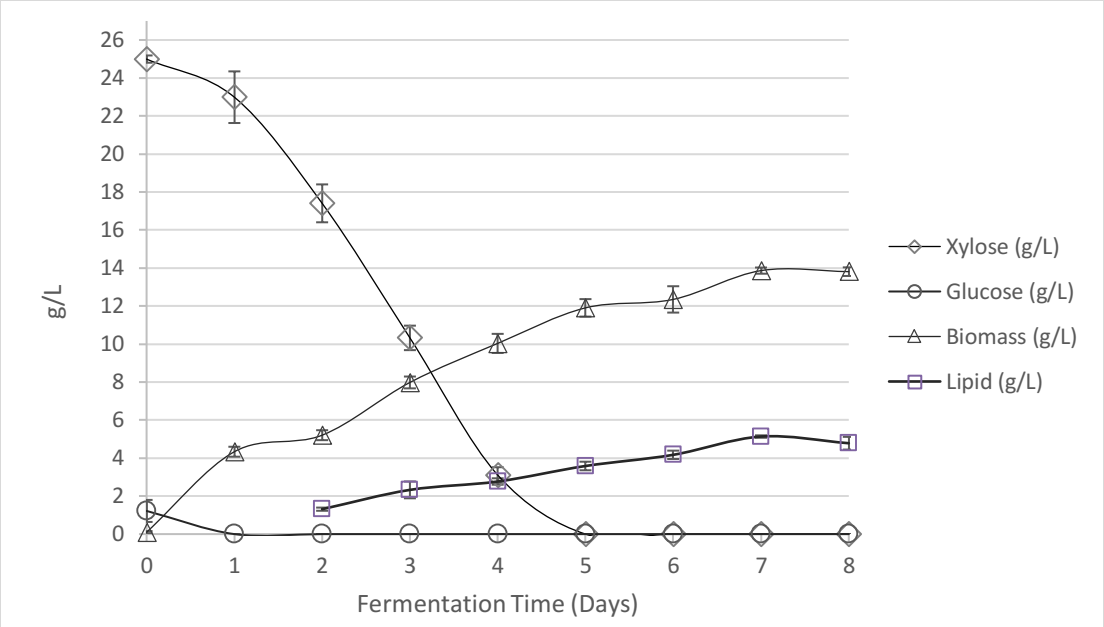

Fig. 3.4. Cell growth, lipid accumulation and sugar utilization by *C.curvatus* grown on steam treated Poplar wood hemicellulose pre-hydrolysate (HPHL).

Higher cell biomass with lower lipid content was obtained in HPHL-based medium as compared to synthetic medium. This may be explained by the fact that nitrogen content in the synthetic medium was carefully adjusted. The elemental analysis of HPHL showed 3.66 ± 0.03 weight percentage of nitrogen presents in the feedstock. This makes it difficult to adjust the desired C/N ratio. Interestingly, *C. curvatus* was able to produce 11.15 g/L of cell biomass with lipid content of 32.08% without adding trace elements or any nitrogen source other than yeast extract that was initially added through seed inoculation. This indicates that steam treated Poplar wood pre-hydrolysate contains enough nutrient for growth and lipid accumulation of *C. curvatus* which can be beneficial for lipid-based woody biorefinery.

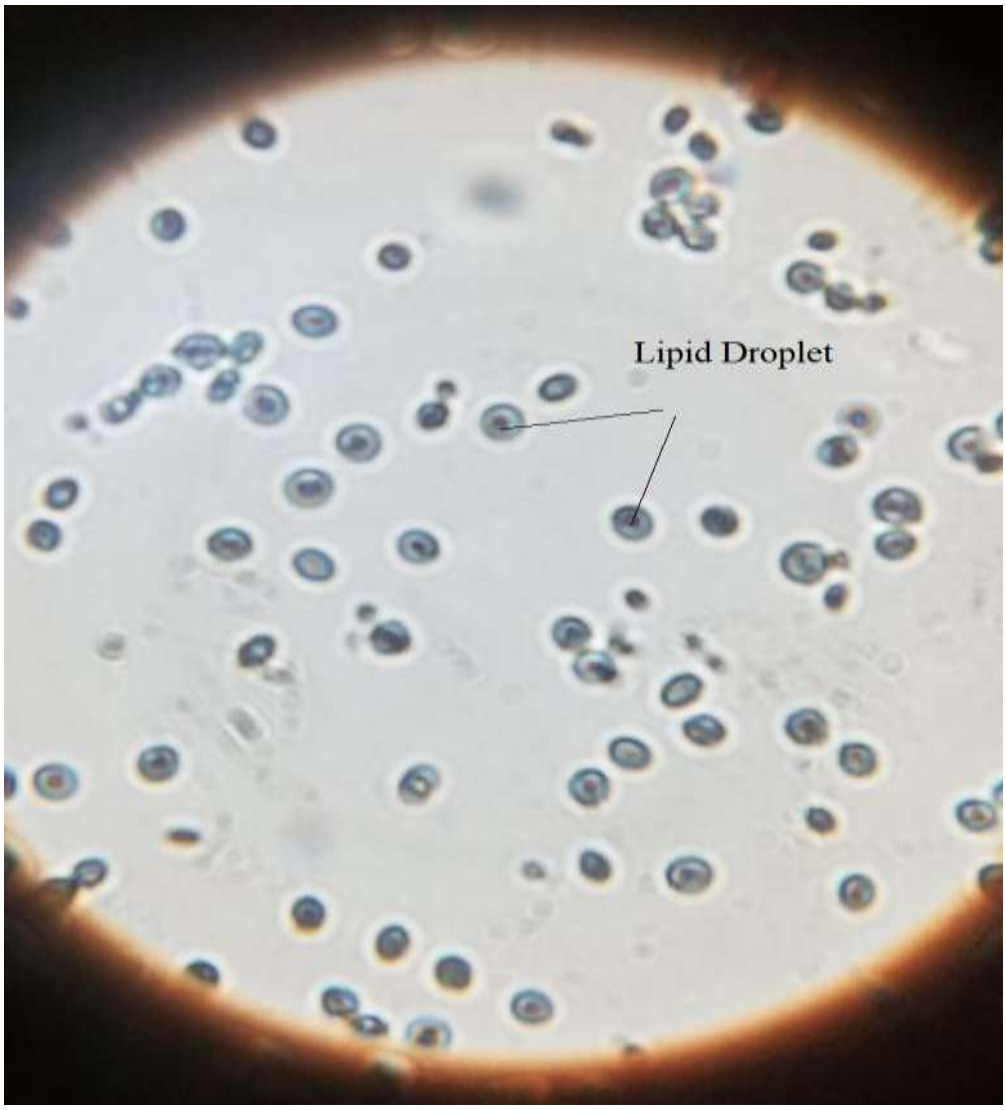

Fig. 3.5. Lipid droplet in *C.curvatus* grown on steam treated Poplar wood hemicellulose pre-hydrolysate (HPHL).

Our results have been compared with different oleaginous yeasts grown on hemicellulose biomass reported recently in Table 3.3 We can see that higher cell biomass and lipid concentration were obtained in comparison with sulfuric acid hydrolysate of birch bark a wood-based substrate (Huang et al., 2013b).

Fatty acids of MO from *C.curvatus* grown on HPHL medium was analyzed by GC. Results are similar to fatty acid composition of MO on synthetic medium discussed above. Oleic acid (45.86 ± 0.69 wt%), palmitic acid (15.67 ± 0.98 wt%), myristic acid (10.65 ± 0.86 wt%), linoleic acid (9.02 ± 0.86 wt%), and stearic acid (8.97 ± 1.16 wt%) were the main fatty acids in the oil,

respectively. In general, fatty acid composition of obtained MO is similar to vegetable oils. Therefore, it is regarded as a good alternative which can address the sustainability challenge of vegetable oil.

Table 3.3. Cell biomass and lipid obtained from different oleaginous yeasts using hemicellulosic biomass.

	Substrate	Fermentation medium	Detoxification method	Strain	Cell biomass (g/L)	Lipid (g/L)	Reference
1	Wheat straw	Dilute sulfuric acid hydrolysate	Non-detoxified	*C.curvatus*	17.2	5.8	(Yu et al., 2011)
			Over liming	*R. toruloides*	9.9	2.4	
			Non-detoxified	*L starkeyi*	14.7	2.4	
			Over liming	*Rhodotorula glutinis*	13.8	3.5	
2	Corncob	Dilute sulfuric acid hydrolysate	Over liming and activated carbon	*Trichosporon coremiiforme*	20.4	7.7	(Huang et al., 2013b)
3	Birch bark	Dilute sulfuric acid hydrolysate	Enzymatic detoxification and activated carbon	*R. Toruloides*	7.1	2.8	(Matsakas et al., 2017)
4	Palm empty fruit bunches	Dilute sulfuric acid hydrolysate	Over liming and activated carbon.	Candida tropicalis	6.81	2.73	(Tampitak et al., 2015)
5	wheat barn	Dilute sulfuric acid hydrolysate	-	*Rodotorula* 110	15.01	5.84	(Enshaeieh et al., 2013)
	Corn stalk				14.97	6.50	
6	Poplar wood	Steam treated pre-hydrolysate	Non-detoxified	C. Curvatus	13.78	5.13	This Work

3.5 Conclusion

Poplar wood hemicellulose pre-hydrolysate was used in this work to produce microbial lipid using *C. curvatus*. 13.78 g/L of cell biomass and 5.13 g/L of lipid were obtained. This result indicates that streams rich in hemicellulose sugars (containing high levels of C-5 xylose sugars) obtained from the pretreatment process of wood could be used to produce fermentative products like microbial oil and subsequently to other value-added products economically. The hemicellulose

stream contains low level of toxic compounds and enough nutrient for microbial growth of oleaginous yeasts. Oleaginous yeast *C. curvatus* with a remarkable xylose assimilating ability is a promising strain for lipid production. C/N ratio is an important parameter to obtain a high yield conversion. As the forest sector is one of the pillars for economic development in many parts of the world, production of value-added product from wood will bring more value to both the local and national economy. Utilization of hemicellulose along with cellulose and lignin will make this possible.

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CHAPTER 4

OBJECTIVE 2

Utilization of Microbial Oil from Poplar wood Hemicellulose Pre-hydrolysate for the Production of Polyol Using Chemo-enzymatic Epoxidation

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4 Utilization of Microbial Oil from Poplar Wood Hemicellulose Pre-hydrolysate for the Production of Polyol Using Chemo-enzymatic Epoxidation

4.1 Abstract

The aim of this work was to demonstrate the production of polyol from microbial oil using wood-based hemicellulose stream through a greener enzymatic process. Yeast biomass concentration of 16.54 ± 0.65 g/L and lipid concentration of 6.97 ± 0.58 g/L were obtained by batch fermentation of hemicellulose pre-hydrolysate of Poplar wood using an oleaginous yeast *C. curvatus*. The produced microbial oil was successfully converted to epoxidized oil catalyzed by lipase with 84.55 ± 1.80% conversion. The epoxidation followed by ring opening reaction to produce polyol with hydroxyl value of 299.53 ± 1.24 mg KOH/g. This showed the possible use of the hemicellulose stream of lignocellulosic biomass to microbial oil and its subsequent conversion to polyol, a precursor to polyurethane and a number of value-added products. This bio-based polyol could be used to substitute the conventional polyols.

Keywords: Hemicellulose, Microbial oil, Lipase, Oleaginous yeast, Biocatalyst, Biopolyol.

4.2 Introduction

Production of polyol from renewable resources has gain attention due to its numerous applications. Polyols are important starting materials for the manufacture of polymers such as polyurethane. Vegetable oils such as soybean, palm, canola and castor oil were investigated as alternatives for petroleum-based polyol (Miao et al., 2014). However, using vegetable oils for polymer production is dependent on the availability of arable lands for cultivation and climatic condition. Most importantly, it can lead to an increase in prices of cooking oils which adversely influences the food security (Liang & Jiang, 2013). Thus, global oleochemical industry needs to focus on eco-friendly alternatives and low-cost raw materials which do not affect the food industry. Also, using renewable resources from waste or side stream of other industries helps develop a circular bioeconomy.

Microbial oils (MO) with similar fatty acids composition to vegetable oils seem to be a promising feedstock to improve the production of oleochemicals (Adrio, 2017). These oils have a high content of oleic acid which is good for production of bio-lubricants, due to their excellent

properties, including thermal and oxidative stability. Currently, high-oleic acid content oils used for these purposes are derived from plants (McNutt, 2016).

MOs are produced by oleaginous yeasts which provide the platforms to develop sustainable production of oleochemicals as they synthetize and accumulate lipids mainly as triacylglycerides (TAGs) (Ratledge, 2004). These microorganisms are able to grow on a variety of carbon sources. As the cost of the fermentation products is mainly affected by the cost of the carbon source, it is crucially important to use low-cost substrates (Koutinas et al., 2014). Wood based lignocellulosic biomass has the potential to serve as an inexpensive, stable and sustainable feedstock to produce microbial oil and other value-added products. In order to compete with fossil-based industry all components of wood biomass need to be integrated. Utilization of hemicellulose, a natural water-soluble biopolymer, has the potential for the development of a sustainable system.

There are few earlier reports on the production of polyol from microbial oil using lignocellulosic biomass. Sugarcane molasses was used to produced microbial oil using *R.toruloides* in a fed-batch bioreactor. The oil obtained was then enzymatically converted to polyol with a conversion yield of approximately 85% (w/w) (Boviatsi et al.).

The objective of this study was the production of microbial oil from Poplar wood hemicellulose pre-hydrolysate rich in xylose and its further conversion to polyol using an environmentally friendly chemo-enzymatic epoxidation. To the best of our knowledge, this is the first time polyol has been produced from wood-based hemicellulose stream using *C. curvatus*.

4.3 Materials and Methods

4.3.1 Substrate

Hemicellulose pre-hydrolysate liquor (HPHL) obtained by pretreatment of Poplar wood was kindly donated by Greenfield Global Inc, Canada (Lehoux & Bradt, 2016). In their patented process wood chips are passed through a solid/fluid separation module with a two-step twin screw press. The module separates fluid (hemicellulose) from a slurry containing solids (cellulose and lignin) using compression by a screw press at high pressure. The aqueous stream containing hemicellulose was used in this study.

4.3.2 Microorganism and Fermentation

The oleaginous yeast *C. curvatus* (ATCC 20509) was used for fermentation of hemicellulose.

In order to prepare the seed culture, *C. curvatus* was grown in a medium containing 3 g/L yeast extract, 3 g/L malt extract, 5 g/L peptone, and 10 g/L xylose and maintained at pH 5.5 (Sartorius PB-11, pH meter), 30°C and 180 rpm for 24 hr. Then 10% (v/v) seed culture was inoculated into minimal medium containing (g/L) KH_2PO_4 2.7, $Na_2HPO_4 \cdot 12H_2O$ 0.95, $MgCl_2 \cdot 6H_2O$ 0.2, yeast extract 0.1. The carbon source was the sugars present in the HPHL. Ammonium chloride was used to adjust the carbon to nitrogen (C/N) ratio. The medium was supplemented with 10 mL/L of trace element consisted of (g in 1 L of distilled water) $CaCl_2 \cdot 2H_2O$ 4.0, $FeSO_4 \cdot 7H_2O$ 0.55, Citric acid monohydrate 0.52, $ZnSO_4 \cdot 7H_2O$ 0.1, $MnSO_4 \cdot H_2O$ 0.076 and 18 M H_2SO_4 100 µL (Meesters et al., 1996). A 1 L bioreactor (Sartorius, Biostat A) with working volume of 750 mL at 30°C, 300 rpm stirrer speed and 1.2 vvm aeration was used for fermentation. Automatic addition of 1 M NaOH or 1 M HCl maintained the pH of the media at 5.5. All chemicals and reagents used in this work were analytically pure and purchased from Fisher Scientific (Canada). All sample analysis were carried out in duplicate and errors were presented as mean ± standard deviation.

4.3.3 Determination of Cell Biomass, Lipid Extraction

Cell biomass was separated from fermentation broth using Sorvall RT1 Centrifuge (Thermo Fisher Scientific, Canada) with 4100 rpm and the quantity was determined by gravimetry method (Uprety et al., 2017a). Extraction of lipid from dry cell biomass was carried out according to Bligh-Dyer method with modification (Manirakiza et al., 2001). Cell biomass concentration and lipid yield are expressed as gram per liter of fermentation broth (g/L).

4.3.4 Polyol Production

Production of polyol from MO was carried out in two steps which involved epoxidation followed by a ring-opening reaction.

4.3.4.1. Epoxidation

Enzymatic epoxidation was selected to functionalize the double bond present in the oil. This reaction was catalyzed by the lipase B of *Candida antarctica* immobilized on acrylic resin (Novozym 435) that was purchased from Sigma-Aldrich, Ontario, Canada.

An aliquot of MO with iodine value (IV) of 81.05 ± 1.29 g I_2/100 g was mixed with 110 wt% of toluene and 8 wt% oleic acid at 40°C for 10 min to homogenize the mixture. Then 10 wt% enzyme was added and mixed for 10 more min. Sufficient hydrogen peroxide (35%) to set the molar ratio of hydrogen peroxide to double bond of 2, was added gradually to start the reaction over 30 min.

The reaction was allowed to run for 24 hr (Vlček & Petrović, 2006). The percentage conversion of oxirane was measured using the method described by Paquot (1979) that involves titration of the sample against bromide acetic solution. Iodine monochloride solution (Wijs method) was used to determine the IV number (Paquot, 1979).

4.3.4.2 Hydroxylation Reaction

Epoxidized MO (5 gr) and isopropanolamine, molar ratio of amino group to epoxy group was set at 2.5:1 (Miao et al., 2013), were insert into a 120-mL flask which was placed in the water bath at 80°C. The reaction was continued for 6 hr with magnetic stirring at 600 rpm. Acid and hydroxyl values of the obtained polyols were determined using the method described by Paquot (1979).

4.3.5 Analytical Analysis

4.3.5.1 Determination of Total Solid and Sugar

Sugars, acetic acid and hydroxy methyl furfural (HMF) concentration were analyzed using an HPLC (Agilent Technologies 1260 Infinity). Analysis were carried out by Aminex HPX-87H ion exclusion column (300 mm × 7.8 mm) with a Refractive Index Detector (RID) at 50 °C. 5 mM H_2SO_4 at 0.5 mL/min flow rate was used as mobile phase (Sluiter et al., 2006). Total solid were measured according to National Renewable Energy Laboratory (NREL) standard (Sluiter et al., 2008) which involves the following procedure: 10 mL of pre-hydrolysate was filtered through a 0.2 µm pore size filter. It was then transferred in a pre-dried aluminum foil dish and placed in 105°C drying oven (Isotemp, Fisher Scientific) for 6 hr. The dishes were weighted before and after drying. The percentage of total solids was calculated according to the following equation:

$$\%\text{Total Solid} = \frac{\text{Weight}_{\text{dry dish+dry sample}} - \text{weight}_{\text{dry dish}}}{\text{weight}_{\text{filtered sample}}} \times 100 \qquad (4\text{-}1)$$

4.3.5.2 Structural Analysis

FT-IR analysis of produced polyols were carried out using Bruker Tensor 37, Ettlingen, Germany, ATR accessory at Lakehead University Instrumentation Lab (LUIL). The analysis was conducted in transmittance mode in the range of 500 and 4000 cm^{-1} with a 4 cm^{-1} resolution, and 32 scans per sample. For ^{1}H NMR analysis, 5 mg of samples were dissolved in 1 mL of deuterated

chloroform and transferred into 5 mm ^{1}H NMR tubes and analyzed using INOVA-500 Varian-NMR instrument at Lakehead University Instrumentation Lab (LUIL).

4.4 Result and Discussion

4.4.1 Substrate Composition

Poplar hemicellulose pre-hydrolysate liquor (HPHL) was provided by Greenfield Global, one of the ethanol producers in Canada (Lehoux & Bradt, 2016). It was obtained by a novel solid/fluid separation module with two-step twin screw press as the pretreatment process. Details of HPHL composition has been discussed in section 3.4.1 in chapter 3.

4.4.2 Fermentation and Lipid production

The oleaginous yeast *C. curvatus* (ATCC 20509) was selected for the fermentation. Accumulation of high concentrations of intracellular lipid under nitrogen-starvation condition makes this strain very attractive. It is also known to grow on a variety of complex biomass hydrolysates (Bracharz et al., 2017). More importantly, the ability of this robust strain to grow even in the presence of fermentation inhibitors such as acetic acid and HMF enabled us to use the HPHL without detoxification. Diluting HPHL to desired sugar level, decreased the concentration of acetic acid and HMF present in the substrate as well. Acetic acid and HMF at levels of 3 g/L and 1 g/L, respectively do not inhibit the growth (Yu et al., 2011) of the yeast we used in our study.

Lipid biosynthesis in oleaginous yeasts on hydrophilic substrate (lignocellulosic biomass) is highly influenced by C/N ratio as it follows the "*de novo*" metabolite pathway (Papanikolaou & Aggelis, 2011a). In order to maximize the lipid yield, xylose concentration of 25 g/L and C/N ratio of 200 was used for batch fermentation. Details of these studies have been reported in sections 3.4.2 and 3.4.3 in chapter 3.

Fig. 4.1 illustrates the growth profile of *C. curvatus* using HPHL as substrate in a batch bioreactor. *C.curvatus* is able to consume both glucose and xylose showing that this promising strain can be used to produce microbial oil from hemicellulose hydrolysate/pre-hydrolysate in a microbial lipid-based biorefinery.

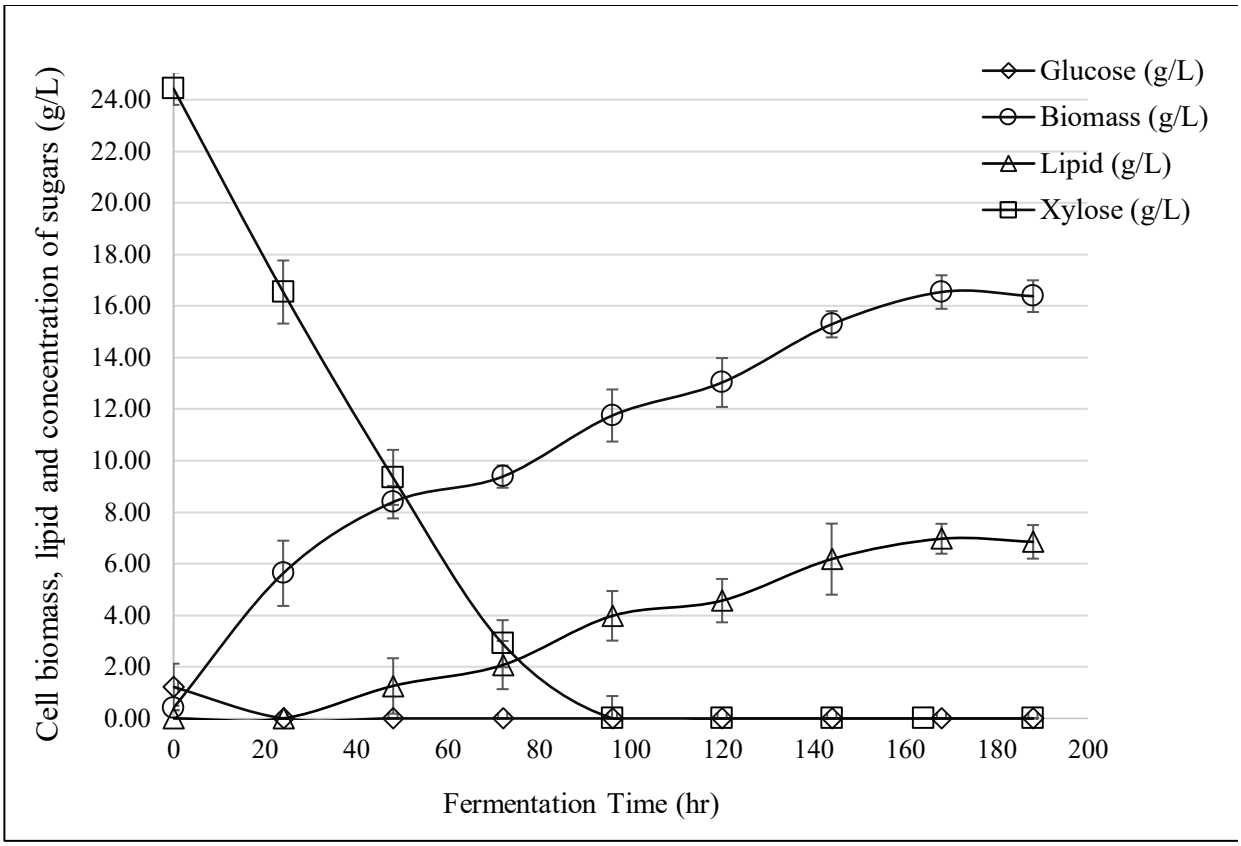

Fig. 4.1. Cell biomass, lipid accumulation, and sugar utilization by *C. curvatus* grown on steam treated Poplar wood hemicellulose pre-hydrolysate (HPHL) in a batch bioreactor used in this study to produce microbial oil. Initial xylose concentration was 25 g/L. The carbon to nitrogen (C/N) ratio was 200 with the combined sugar and nitrogen compounds present in the feed.

No significant lipid accumulation was observed during the first 70 hr of fermentation. Because *de novo* lipid biosynthesis is basically a secondary metabolism and lipid accumulation enhances only after essential nutrients are exhausted. The maximum cell biomass and lipid concentration of batch fermentation were 16.54 ± 0.65 and 6.97 ± 0.58 g/L, respectively after 168 hr of fermentation. 78.18% conversion was achieved using *C.curvatus* grown on HPHL in a batch fermenter as compared with maximum theorical yield where xylose is used as a sole carbon source (0.34 g/g). Table 4.1 compared the production of MO using HPHL in flask shake in our previous study with batch fermentation in this study. The increase of cell biomass and lipid accumulation in batch mode is a result of better pH control, improved oxygen supply and easier access to different growth factors throughout the media. In comparison with maximum theorical yield 76.47% conversion was achieved using *C.curvatus* on HPHL in a batch bioreactor.

Table 4.1. Comparison of total cell biomass and lipid concentration of *C. curvatus* grown on steam treated Poplar wood hemicellulose pre-hydrolysate (HPHL) in batch bioreactor and flask shake (chapter 3).

Fermentation	Biomass (g/L)	Lipid concentration (g/L)	Yield (g/g)	References
Flask shake	13.87	5.13	0.19	chapter 3
Batch reactor	16.54 ± 0.65	6.97 ± 0.58	0.26	This study

Initial xylose concentration was 25 g/L. The carbon to nitrogen (C/N) ratio was 200 with the combined sugar and nitrogen compounds present in the feed – 168 hr.
Analyses were performed in duplicate and errors were expressed as mean ± standard deviation.

Price of the microbial oil varies depending on the scale of the production, location as well as feedstock. Consequently, the price of the microbial oil-based biodiesel which is the most commonly studied product from microbial oils varies respectively. Over the course of time by development of technology in this area production of microbial oil-based biodiesel seems to be competitive in comparison with vegetable-oil based biodiesel. Soccol et al have shown a successful pilot-scale production of microbial oil biodiesel using sugarcane juice with price of US$ 0.76/L (Soccol et al., 2017).

However, production of fuels and energy is usually referred to as “dead end” of carbon as it releases the carbon into the atmosphere. Circular bioeconomy encourages the cascading use of biomass, where energy uses comes at the end (Carus & Dammer, 2018).

Therefore, there is a need to investigate other applications of microbial oil for value added products such as polyol which will bring more economical value from the same source. The only few reports on production of polyol from microbial oil used crude glycerol (Uprety et al., 2017b), confectionery waste (Papadaki et al., 2018) and sugarcane molasses (Boviatsi et al.). Using hemicellulose-rich stream for production of polyol will help to improve the circular bioeconomy.

4.4.3 Polyol Production – Enzymatic Epoxidation/ Ring-opening Reaction

In order to introduce hydroxyl group into oils’ structure, chemical modification is required at the double bonds and/or ester linkages. This can be done by different methods including epoxidation followed by oxirane ring opening reaction, ozonolysis, thiol-ene coupling, transesterification and amidation, hydroformylation followed by hydrogenation (Zhang et al., 2017). Two-step epoxidation and ring opening reaction is a common method which can be done in combined one step as well (Sharmin et al., 2007).

Epoxidation of the double bonds results in more reactive groups in the fatty acids. In this process (Fig. 4.2) an epoxy group is added to the double bonds present in the fatty acid chains of oils (Saurabh et al., 2011).

Epoxidized oils have a broad range of applications including stabilizers and plastics plasticizers, adhesive, paint diluents. More importantly, they are precursors for value-added products like glycol, polyol, polyurethane (Tan & Chow, 2010).

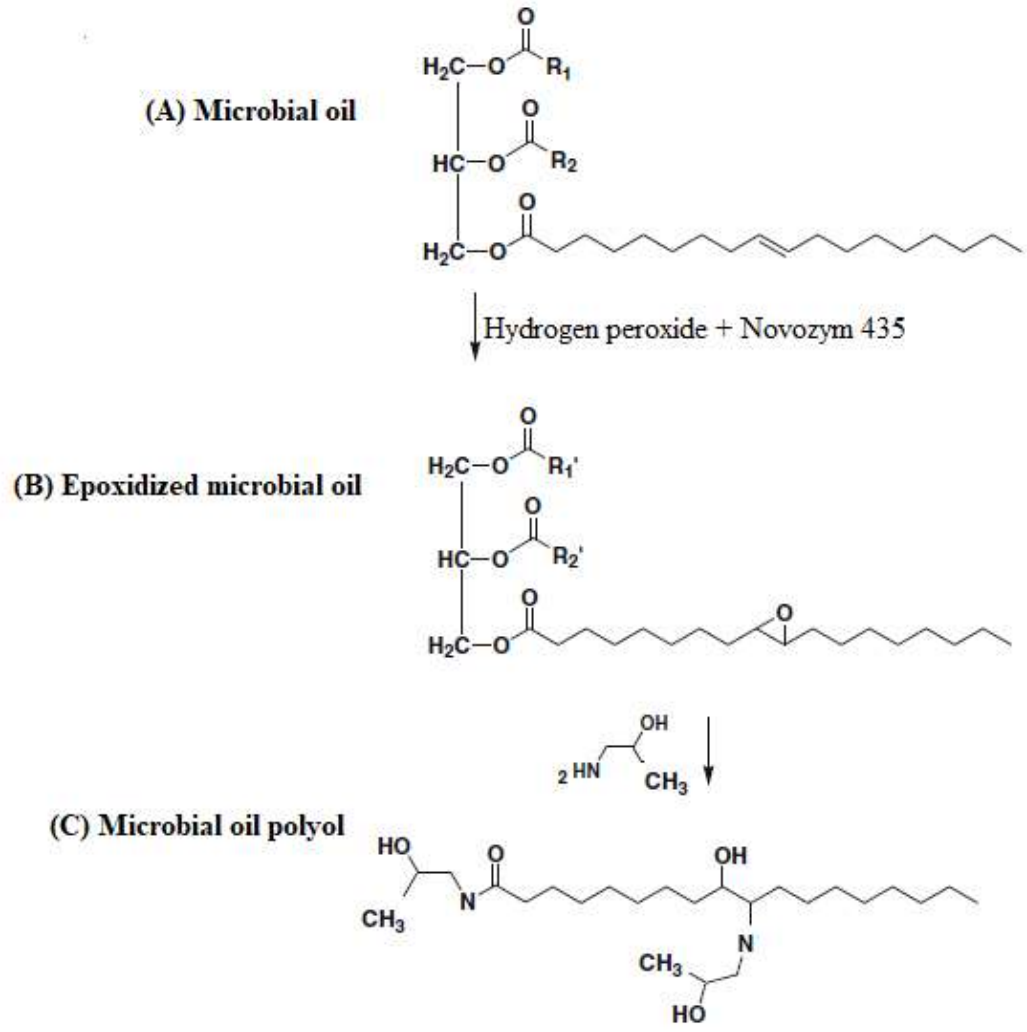

Fig. 4.2. Overall reaction scheme for the synthesis of microbial oil polyol from microbial oil using *C. curvatus* grown on steam treated Poplar wood hemicellulose pre-hydrolysate (HPHL) via enzymatic epoxidation and ring opening reaction. $R_1$and R_2 are the fatty acid chain.

This reaction was catalyzed by *Candida antarctica* Lipase B (CALB) immobilized on a macroporous support (Novozym 435). The peracid formed in an enzyme-catalyzed reaction epoxidizes the double bonds. This conversion can also be done chemically using strong sulfuric acid, acid ion exchange resin (AIER) and metal catalyst (Tan & Chow, 2010). The advantages of enzymatic epoxidation are selective performance of this biocatalyst as well as the milder reaction conditions required (Lin et al., 2011). As the enzyme requires the neutral environment, oleic acid as a weak acid was used which can also be converted to peracid. This in turn oxidizes the double bonds present in the MO and oleic acid itself (Vlček & Petrović, 2006).

Fig. 4.3 shows the oxirane content of the MO used in this work over the course of reaction. There was no significant change in oxirane value after 12 hr. This is in agreement of enzymatic epoxidation of vegetable oils that have been reported earlier (Lu et al., 2010; Miao et al., 2008). The oxirane values of MO at the end of 12 hr was found to be 4.11 ± 0.60 (% m/m). This indicates a conversion of 84.55 ± 1.80% in 100 gr of microbial oil according to equation (2) (Sun et al., 2011);

$$\text{Relative conversion to oxirane} = \frac{OO_{\text{exp}}}{OO_{\text{th}}} \times 100 \qquad (4\text{-}2)$$

$$OO_{\text{th}} = \left[\frac{\frac{IV_0}{2A_{\text{i}}}}{100 + \frac{IV_0}{2A_{\text{i}}}}\right] A_{\text{O}} \times 100 \qquad (4\text{-}3)$$

Where OO_{exp} is the experimental oxirane value obtained by titration and OO_{th} is the maximum theorical oxirane oxygen in 100 gr of microbial oil. IV_0 represents the initial iodine value of the MO, A_{i} and A_0 are atomic weight of iodine (126.9) and oxygen (16), respectively. In respect to xylose the yield of 0.23 yield (g epoxidized oil/g xylose) was achieved.

Lipases are among the most widely used enzymes in biocatalysis. The most common biological function of lipases is the hydrolysis of triglycerides. However, lipases catalyze various other reactions including triglyceride modification, biodiesel production, production and degradation of polymers (Ghaly et al., 2010; Hasan et al., 2006). The diverse reactions that lipases are able to catalyze are possible as they are relatively stable. All are able to function in organic solvents, aqueous media and ionic liquids. They are also able to perform in solvent-free systems (Milchert et al., 2015).

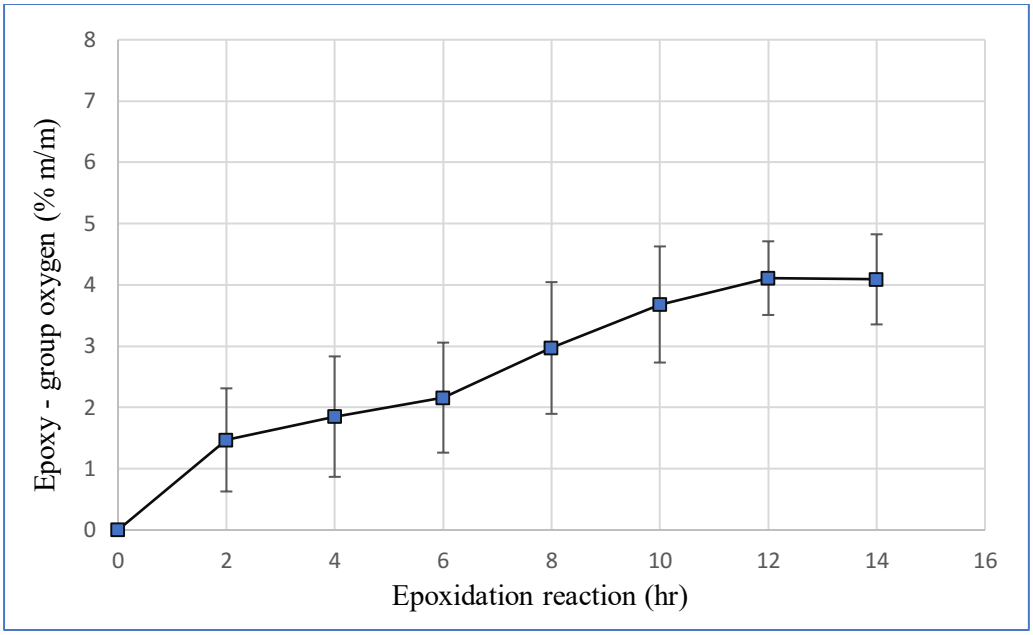

Fig. 4.3. Oxirane content of epoxidized microbial oil as a function of time during epoxidation reaction using Novozym 435 as catalyst.

In epoxidation the stability of the immobilized CALB is mainly affected by temperature and hydrogen peroxide. CALB is functional over a wide temperature range (20 – 90°C) (Chen et al., 2008). However, in the presence of hydrogen peroxide the activity of CALB decreases rapidly at 60°C as compared to 20°C (Törnvall et al., 2007). Increasing the temperature increases the rate of the reaction but results in some enzyme deactivation. Though, temperature below 50°C do not deactivate the enzymes and decompose the hydrogen peroxide (Sulciene et al., 2014). The undesirable side reactions of oxirane ring-opening during epoxidation can be substantially minimized by conducting the reaction at lower temperature with lipase catalysis (Milchert et al., 2015).

Even though 1mole of hydrogen peroxide is theoretically required to convert 1 mole of double bond, higher molar ratios are taken as hydrogen peroxide degrades spontaneously (Bajwa et al., 2016). In this study epoxidation at 40°C for 12 hr resulted in 84.55 ± 1.80% conversion.

Table 4.2. Reusability of lipase (Novozym 435) in enzymatic epoxidation of microbial oil using *C.curvatus* grown on Poplar wood hemicellulose pre-hydrolystae (HPHL).

Number of cycles	Epoxide conversion (%)
1	84.55 ± 1.80
2	79.93 ± 1.48
3	70.02 ± 0.86
4	45.68 ± 0.81

Analyses were performed in duplicate and errors were expressed as mean ± standard deviation.

Table 4.2 shows the reusability of lipase B. This enzyme can reuse for 3 cycles with 70.02 ± 0.86% conversion. This stability can be explained by interfacial activation mechanism of lipase. The active sites and their surrounding on lipase B are hydrophobic. These sites are covered by a kind of lid made of polypeptide chain with hydrophilic external face. This lid does not move in aqueous phase. Shifting the lid, known as open (active) form, exposes the active site to the media. This is when lipase are absorbed on MO (hydrophobic surface) in the interface of organic and aqueous phase in the reaction system (Ortiz et al., 2019). This unusual characteristic of lipase makes it a stable biocatalyst against high temperature and strong oxidant such as hydrogen peroxide.

Different active hydrogen-containing compounds such as alcohols, inorganic and organic acids, amines, water, and hydrogen can be used to introduce hydroxyl group by opening the epoxy ring. In general, ring-opening agents can be categorized into three main groups; alcohol, acid and hydrogen (Li et al., 2015a). Subsequently, polyols are formed with varying hydroxyl number, acid value and other properties. Using petroleum-based polyol production, polyols with hydroxyl number of 300 – 650 mg KOH/g are found to be suitable for rigid polyurethanes (Veronese et al., 2011). Low hydroxyl value polyols have other application in flexible foam and elastomers (Veronese et al., 2011; Wood, 1990). 5.8 g of polyol was obtained .The acid number for commercial grade polyol is usually less than 10 (Kiatsimkul et al., 2008). The acid number of produced polyol in our study was found to be 4.93 ± 1.07. This might be due to the formation of carboxylic acid and hydrolysis of the oil during epoxidation.

Table 4.3 compares the hydroxyl values of polyol produced and ring opening agent used in this work with some of the reports available in the literature. High hydroxyl number of 299.53 ± 1.24

mg KOH/g in our study is attributed to isopropanolamine which reacts with both epoxy group (epoxy ring opening) and ester linkage (amidation) present in the epoxidized oil. Therefore, the produced polyol is a mixture of polyether and polyester polyols.

Table 4.3. Comparison of different ring opening agents and hydroxyl values of polyols produced from plant-based oil (literature) and microbial oil used in this study.

Ring-opening agent	Oil	Hydroxyl Value[1] (mg KOH/g)	Reference
Alcohols			
Methanol	Jatropha	171 - 179	(Hazmi et al., 2013)
Methanol	Linseed oil	247.8	(Zlatanić et al., 2004)
Methanol	Sunflower	177.8	(Zlatanić et al., 2004)
Methanol	Canola	173.6	(Zlatanić et al., 2004)
1,2-Propanediol	Soybean	211-237	(Wang et al., 2009)
Isopropanolamine	Microbial Oil	299.53 ± 1.24	This study
Acids			
Formic acid	Soybean	104 - 162	(Monteavaro et al., 2005)
Phosphoric acid	Soybean	153 - 253	(Guo et al., 2007)
Hydrochloric acid	Soybean	197	(Guo et al., 2000)
Hydrobromic acid	Soybean	182	(Guo et al., 2000)
Hydrogen			
	Soybean	212–225	(Guo et al., 2000)

[1]The hydroxyl value is the number of miligrames of potassium hydroxyde required to neutralized the acetic acid capable of combining by acetylation with 1 g of polyol.

4.4.4 Structural Characterization

FTIR analysis was carried out to confirm the formation of epoxy and polyol in each reaction. Fig. 4.4 shows the FTIR of the produced MO, epoxidized oil and polyol. In Fig. 4.4(A), unsaturation present in the MO shows absorption spectra in the range of 3010 – 3100, 675 – 1000 and 1620 –

1680 cm^{-1} which are attributed to =C–H stretch, C–H bend, and C=C stretch, respectively. The disappearance and reduction of these peaks in the IR spectra of epoxidized oil and the absorption at about 901 cm^{-1} confirm the formation of epoxy group in the fatty acid chain. Strong hydroxyl absorption at about 3370 cm^{-1} in Fig. 4.4(C) shows the formation of polyol. The C-O-C (ether stretching) peak at 1070 – 1150 cm^{-1} and N-H bending at approximately 1570 cm^{-1} were observed as a result of amidation.

Fig. 4.5 illustrates the ^{1}H NMR analysis of the produced polyol for further confirmation of the structure. The proton signal at 5.36 ppm in Fig. 4.5(A) was attributed to the unsaturation present (alkene group) in the microbial oil. In Fig. 4.5(B) peaks within the range of 2.83 -2.93 ppm confirms the formation of epoxy group (Zhang et al., 2015). The proton in hydroxyl group appears at 2.22 ppm [-CH-(O<u>H</u>)]. Also, metheylen proton attached to hydroxyl group appeared within the range of 3.12 – 3.91 ppm (Abril-Milán et al., 2018). In Fig. 4.5(C), formation of the peak at about 5.92 ppm, while disappearing the bands at 4.17 – 4.29 ppm [C<u>H</u>$_2$O(OC-)-C<u>H</u>O(OC-)-C<u>H</u>$_2$O(OC-)] confirmed the amidation reaction (Acar et al., 2013).

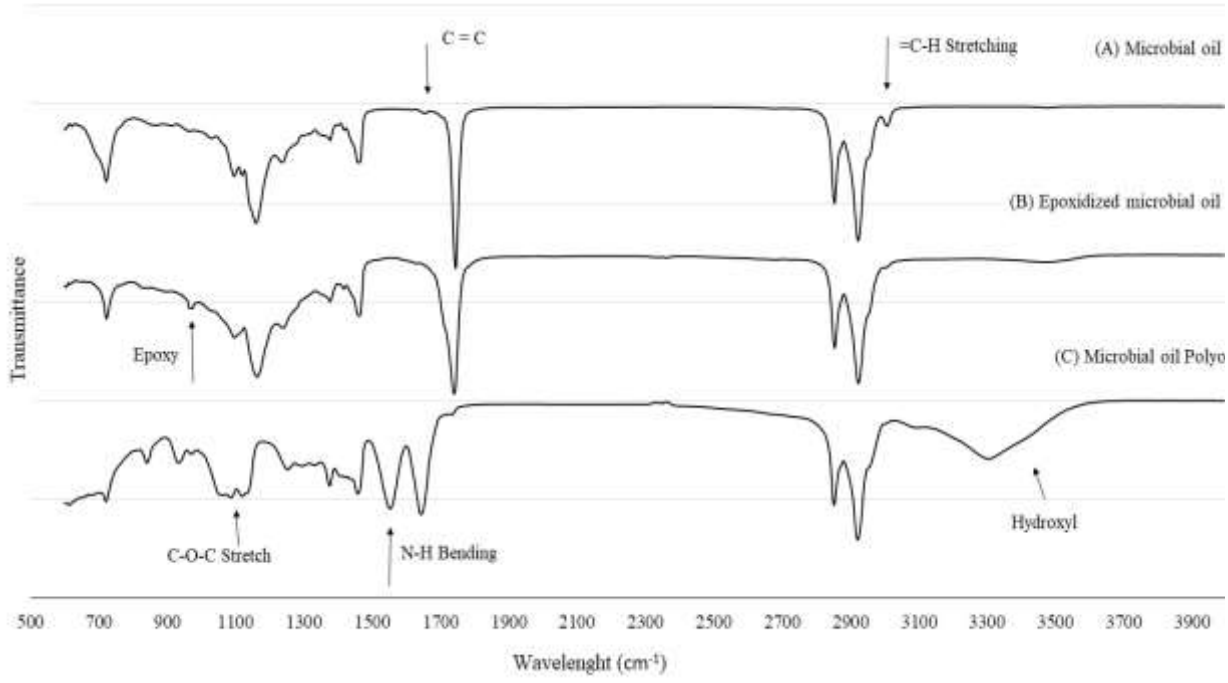

Fig. 4.4. IR spectra of (A) microbial oil (B) epoxidized microbial oil (C) microbial oil polyol produced in this study.

4.5 Conclusion

Successful conversion of produced microbial oil to polyol by chemoenzymatic method was demonstrated. *C. curvatus* was used as an oleaginous microorganism to produce microbial oil. 16.54 ± 0.65 g/L of yeast biomass and 6.97 ± 0.58 g/L of lipid were achieved by batch fermentation of hemicellulose pre-hydrolysate of Poplar wood. Isopropanolamine as a ring-opening agent involves in both epoxy group and ester linkage which resulted in producing microbial-based polyol with high hydroxyl number of 299.53 ± 1.24 mg KOH/g. This confirms the potential production of polyol from microbial oil using hemicellulosic industrial streams. This work provides a new route for the production of biopolymers from lignocellulosic biomass. Utilization of biobased sources through the development of integrated biorefinery can be considered as an important approach in eco-design which contribute to circular bioeconomy.

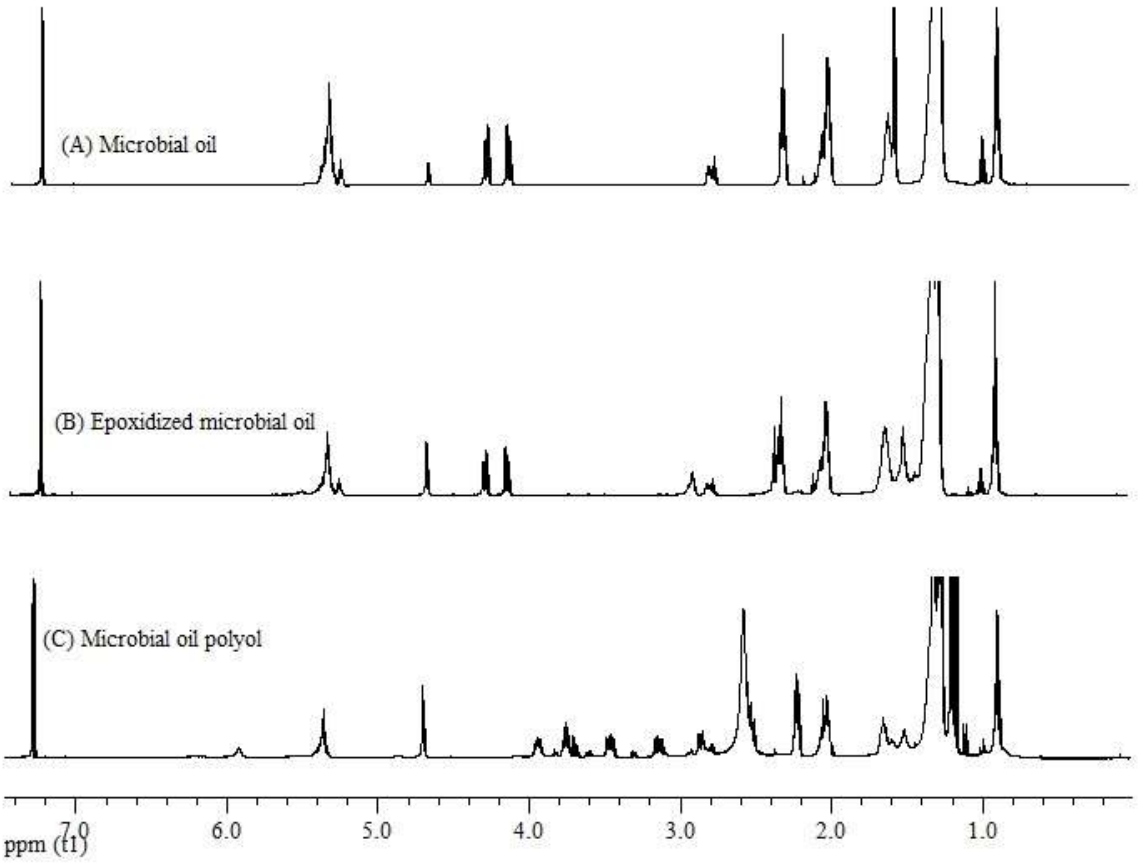

Fig. 4.5. ^{1}H NMR spectra of (A) microbial oil (B) epoxidized microbial oil (C) microbial oil polyol produced in this study.

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CHAPTER 5
OBJECTIVE 3

Bioconversion of Poplar Wood Hemicellulose Pre-hydrolysate to Poly- γ - Glutamic Acid Using *Bacillus subtilis*

This work is in process for submitting to the journal of " Biomass Conversion and Biorefinery".

5 Bioconversion of Poplar Wood Hemicellulose Prehydrolysate to Poly- γ - Glutamic Acid Using *Bacillus subtilis*

5.1 Abstract

Poly-γ-glutamic acid is a promising microbial polymer with a broad range of applications in wastewater treatment, agriculture and pharmaceutical industry. In this study the production of poly-γ-glutamic acid by *Bacillus subtilis* from Poplar wood hemicellulose pre-hydrolysate is reported for the first time. Initially, the optimal concentration of xylose and glutamic acid in the fermentation medium for the production of poly-γ-glutamic acid were obtained using response surface methodology on synthetic media and 21.74 g/L of poly-γ-glutamic acid was obtained. Based on these results, detoxified hemicellulose pre-hydrolysate was used as substrate in the fermentation. The results showed that this strain is capable of consuming both hexose and pentose sugars, which is normally a challenge in fermentation of hemicellulose streams. Concentration of 12.93 ± 0.9 g/L poly-γ-glutamic acid was produced after 96 hours of fermentation under optimized condition. This study showed that a hemicellulose-rich stream such as steam treated wood pre-hydrolysate could be used to produce poly-γ-glutamic acid and other value-added products beneficially using strains capable of growing on both hexose and pentose substrates.

Key words: Poly-γ-glutamic acid, Hemicellulose, Biopolymer, Xylose, *Bacillus subtilis.*

5.2 Introduction

Poly-γ-glutamic acid (γ-PGA) is a natural homo-polyamide that consists of D- and/or L-glutamic acid units via the gamma-amide linkage of monomeric glutamic acid units. This edible polymer is water soluble, biodegradable, and non-toxic toward humans and environment (Ogunleye et al., 2015). These remarkable characteristics make γ-PGA an attractive biopolymer with a broad range of applications including thickeners, hydrogels, flocculants, food additives, drug carriers and cosmetics (Shih & Van, 2001). Various strains of *Bacilli* are reported to be able to produce γ-PGA from a variety of carbon sources. Hexose sugars, glycerol and citrates have been used as substrates for this fermentation (Ashiuchi, 2013). The cost of γ-PGA like many other fermentation products mainly depends on the cost of the carbon source. Recent studies showed that using inexpensive

substrate such as lignocellulosic biomass for γ-PGA fermentation can reduce the carbon source cost approximately 42.5% - 84.2 % (Tang et al., 2015a; Zhang et al., 2012). Thus low-cost biobased waste streams are crucially important for the production of PGA on an industrial scale which meet the requirement of a circular bioeconomy (Carus & Dammer, 2018). Cane molasses (Zhang et al., 2012), soybean residue (Wang et al., 2008), rapeseed meal (Yao et al., 2012) were investigated as inexpensive substrates for production of γ-PGA. Wide range of substrate needs to be further investigated to make this bioconversion economically more attractive.

Wood-based hemicellulose is a stable, renewable and inexpensive resources that can be used for the production of biochemicals. Recently, research studies on xylose-specific bioconversions in predicted integrated biorefineries were carried out to enable the sustainable production of xylitol (Dalli et al., 2017), acetone, butanol, ethanol (Bellido et al., 2014) and microbial oil (Samavi et al., 2019). However, very few studies are available on production of γ-PGA from xylose or hemicellulose-rich stream.

Corncob fiber hydrolysate, rich in xylose, was used for the production of γ-PGA using *Bacillus subtillis* HB-1 (Zhu et al., 2014). 24.94 g/L of γ-PGA was obtained as a result of the batch fermentation. In another study, consumption of xylose and glucose by B. subtilis NX-2 was investigated in a co-fermentation reactor which helped to better understand and improve the yield of γ-PGA production using rice straw as a substrate with different fermentation mode (Tang et al., 2015a).

The present study focused on the potential utilization of wood-based hemicellulose pre-hydrolysate rich in xylose as a substrate to produce γ-PGA using *Bacillus subtilis.* Along with xylose, the optimal concentration of glutamic acid which serves as a precursor, was determined to maximize the γ-PGA concentration which can result in an economic process. To the best of our knowledge this is the first time that *Bacillus subtilis* has been grown on Poplar wood hemicellulose pre-hydrolysate which contains both C6 and C5 sugars for such an application.

5.3 Materials and Methods

5.3.1 Hemicellulose Pre-hyrdolysate

Hemicellulose pre-hydrolysate liquor (HPHL) was donated by Greenfield Global Inc., Ontario, Canada. Steam percolation pretreatment was used to obtain the HPHL from Poplar wood biomass (Lehoux & Bradt, 2016).

5.3.2 Detoxification of Hemicellulose Pre-hydrolysate

Vacuum evaporation followed by solvent extraction as a unified detoxification method was used to remove the fermentation inhibitors. This technique has been developed in our lab and reported earlier (Dalli et al., 2017). The method involves heating the HPHL to 65 ˚C, then dried in a vacuum rotary evaporator (Buchi Rotovap) at 65 °C until a viscose brown residue was obtained. Solvent extraction was then carried out with toluene as the organic solvent in a ratio (toluene: sugar residue) of 3:1. First sugar residue was mixed with toluene in a beaker for 5 min at room temperature. It was then transferred into a separating funnel and allowed to stand for 1 hr. The toluene phase was then discarded, and the detoxified hydrolysate was diluted with known amount of distilled water.

5.3.3 Bacterial Strains

Bacillus subtilis (ATCC 23857) were purchased from American Type Culture Collection (ATCC). It was grown on medium 415 (nutrient broth 8 g/L, potato extract 20 mL, distilled water 980 mL) for 24 hr in an Innova incubator shaker which was set at temperature of 26 °C and a speed of 150 rpm. The cultures were preserved on nutrient agar slant and in 15% (v/v) glycerol for short term and long-term storage at 4 °C and at −80 °C, respectively.

5.3.4 Inoculum Development and Production

Seed inoculum was prepared by growing *B.subtilis* on nutrient agar flask at 26 °C for 24 hr. The cells were suspended in saline solution and cell density was measured spectrophotometrically at 600 nm. The medium for fermentation consisted of (g/L): Citric acid 12, NH_4Cl 7, $MgSO_4.7H_2O$ 0.5, $FeCl_3.6H_2O$ 0.2, K_2HPO_4 0.5, $CaCl_2.2H_2O$ 0.15, and $MnSO_4.H_2O$ 0.2 (Leonard et al., 1958). Concentration of L-glutamic acid and synthetic xylose were set within the range of 10 – 60 g/L and 20 – 100 g/L, respectively for optimization. The fermentation was then carried out with HPHL as carbon source with optimized concentrations of L-glutamic acid and xylose. The pH (Sartorius PB-11, pH meter) was adjusted to 7 using 3M NaOH and 1M HCl. All media were sterilized by autoclaving at 121°C for 20 min at 15psi. The pH was readjusted after autoclave if needed.

The flasks were inoculated with 2% (v/v) of 24 hr-old inoculum and incubated in an orbital shaker (150 rpm) at 26 °C for 120 hrs and observed for maximum γ-PGA production. All chemicals and reagents used in this work were analytically pure and purchased from Fisher Scientific (Canada). All sample analysis were carried out in duplicate and errors were presented as mean ± standard deviation

5.3.5 Analytical Methods

5.3.5.1 Determination of Total Solid, Sugar and γ-PGA Concentration

Sugars, acetic acid and hydroxy methyl furfural (HMF) concentration were determined using an Agilent 1260 infinity HPLC equipped with Aminex ion-exchange Biorad column (HPX-87H, 300 × 7.8 mm) and a Refractive Index (RI) detector. γ-PGA concentration was measured by UV assay as described earlier (Zeng et al., 2012). Briefly, the absorbance of samples was measured by a UV/vis microplate spectrophotometer (BioRad xMark) at 216 nm against deionized water as blank. A standard curve was generated using commercially available γ-PGA purchased from Hangzhou Dayangchem Co., China. Sugars and γ-PGA concentration were expressed as gram per liter of fermentation broth. Total solid were measured according to National Renewable Energy Laboratory (NREL) standard (Sluiter et al., 2008) using conventional oven method. All sample analysis were carried out in duplicate and errors were presented as mean ± standard deviation.

5.3.5.2 Characterization of γ-PGA by FTIR spectroscopy

FT-IR analysis of sample was carried out using a Bruker Tensor 37, Ettlingen, Germany, ATR accessory at Lakehead University Instrumentation Lab (LUIL). The analysis was conducted in transmittance mode in the range of 500 and 4000 cm^{-1} with a 4 cm^{-1} resolution, and 32 scans per sample.

5.4 Result and Discussion

5.4.1 Substrate and Detoxification

Greenfield Global Inc. provided the Poplar wood hemicellulose pre-hydrolysate liquor (HPHL) which was obtained from a novel steam treated process (Lehoux & Bradt, 2016). The composition of the HPHL was quantified using HPLC to determine sugars and other components. Xylose was found to be the major sugar present in the HPHL (Table 1). Glucose and arabinose were present in much lower quantities compared to xylose. The pH of the HPHL used was found to be 2.81 ± 0.61, while the density was determined to be 1.04 ± 0.10 g/mL. The total solid content of HPHL was 19.25 ± 0.33% (w/w).

Because of presence of inhibitory by-products such as acetic acid, HPHL is unfavorable for fermentation. Therefore, the pre-hydrolysate was detoxified following the method developed in our lab using a unified detoxification technique with vacuum evaporation and toluene extraction. The initial and final concentrations of components present in HPHL before and after detoxification

are shown in Table 5.1. 90.06% of the acetic acid was successfully removed while 9.76% of xylose degraded during the process.

It was noted that even though the same pre-hydrolysate (same supplier) was used earlier in our lab (Dalli et al., 2017), the composition of HPHL was different from batch to batch which is one of the challenges in biorefinery. The composition of the cellulosic and hemicellulosic stream varies depending on the type of lignocellulosic biomass, location and operational condition of pretreatment and hydrolysis as well as different batches.

Table 5.1. Composition of hemicellulose pre-hydrolysate (HPHL) used in this study before and after detoxification by unified vacuum evaporation and solvent extraction technique.

Component	Initial concentration, g/L	Final concentration, g/L (after detoxification)
Xylose	143.89 ± 1.28	129.89 ± 163
Glucose	7.07 ± 1.53	4.30 ± 0.92
Arabinose	3.31 ± 1.06	1.60 ± 0.72
Acetic acid	9.56 ± 0.89	0.95 ± 0.54
HMF	0.48 ± 0.35	[1]ND

Sample analysis were carried out in duplicate and errors were presented as mean ± standard deviation.
1 Not detectable

5.4.2 Optimal Fermentation Condition with Synthetic Sugar Media

PGA-producing bacteria are generally divided into two groups: glutamic acid-dependent and glutamic acid-independent producers. The former strains have the potential to synthesize high level of γ-PGA by adding L-glutamic acid into the medium, as reported in *Bacillus subtilis* and *Bacillus licheniformis*. The *de novo* pathway of L-glutamic acid synthesis enables the latter category to produce γ-PGA in the absence of glutamic acid (Sirisansaneeyakul et al., 2017), however, the yield of γ-PGA production is relatively low (Luo et al., 2016). The function of glutamic acid varies in different strains. It was reported that glutamic acid can serve as a regulator (Kunioka, 1995) or can be assimilated and converted to γ-PGA (Ogawa et al., 1997).

Also, addition of large quantities of L-glutamic acid which shared up to 50% of the cost of raw materials (Zhang et al., 2012) lead to increase in the total cost of γ-PGA production (Cao et al.,

2011). The price of L-glutamic acid (or sodium salt of glutamate) were reported to be $1.52 – 9 /kg (Pal et al., 2016; Tang et al., 2015b). Therefore, the optimal concentration of L-glutamic acid needs to be investigated.

Table 5.2. Central composition design expressed with coded values along with response (observed result) of each set of experiment.

Experiment Number	X_1	X_2	γ-PGA (g/L)
1	1	1	18.32
2	-1.41	0	9.92
3	0	0	20.75
4	-1	-1	2.98
5	0	0	21.02
6	-1	1	14.33
7	0	1.41	20.06
8	1	-1	3.83
9	0	0	22.41
10	1.41	0	14.87
11	0	-1.41	1.35
12	0	0	21.19
13	0	0	22.07

X_1 is concentration of synthetic xylose.
X_2 is concentration of glutamic acid.

Response surface methodology (RSM), an experimental strategy for a multivariable system was used in order to determine the optimal fermentation condition. Central composite design (CCD) was applied to find out the optimal concentration of L-glutamic acid over a range of 10 – 60 g/L and xylose over a range of 20 – 100 g/L. The limit settings of low and high factor are coded as −1 and +1, and the midpoint is coded as 0. α is the distance of each axial point from the center and is

given by $2^{\frac{n}{4}}$ (for two factors n=2 and α=1.41). Table 5.2 shows the experimental design for RSM optimization in which γ-PGA production (concentration in g/L) was determined as a response. The relationship of independent variables (xylose and glutamic acid concentration are expressed as X_1, X_2 respectively) and the response (Y is γ-PGA concentration) was calculated by the full quadratic equation:

$$Y = a_0 + a_1X_1 + a_2X_2 + a_3X_1^2 + a_4X_2^2 + a_5X_1X_2 \qquad \text{Eq (5-1)}$$

Where $a_0$is a constant, a_1, a_2 are the linear coefficients, a_3, a_4 are the squared coefficients and a_5 is the interaction coefficient.

The R-squared was calculated to be 98.86%. P-value is an effective tool that shows the significance of each of the coefficients which in turn helps us to realize the mutual interactions between the variables. P-values less than 0.05 indicate model terms are significant. As shown in Table 5.3 the interaction of $X_1$and X_2 is negligible due to p-value of 0.19. According to the generated model concentration of glutamic acid influences the γ-PGA yield (response) more than xylose.

Table 5.3. P-values and coefficients of estimated quadratic model to optimize the γ-PGA production using central composition design.

	Coefficient	P-values	Std errors
Constant	22.29	0.00	0.48
Xylose (X_1)	0.0296	0.01	0.77
L-glutamic acid (X_2)	0.1307	0.00	0.77
Xylose × xylose	-0.001	0.00	0.82
L-glutamic acid × L-glutamic acid	-0.0012	0.00	0.82
Xylose × L-glutamic acid	1.5700	0.19	1.09

Surface plotting (Fig. 5.1) was used to illustrate the result of optimization. Optimal values of 65.40 g/L of xylose and 44.98 g/L of glutamic acid were found to be the optimal concentrations for fermentation to maximize the γ-PGA production. Statistically optimized levels were validated by a flask shake fermentation. The predicted response under the optimized conditions was 23.51g/L of γ-PGA which was only 7.55% different from the maximum γ-PGA concentration (21.74 g/L) observed at the validation experiment.

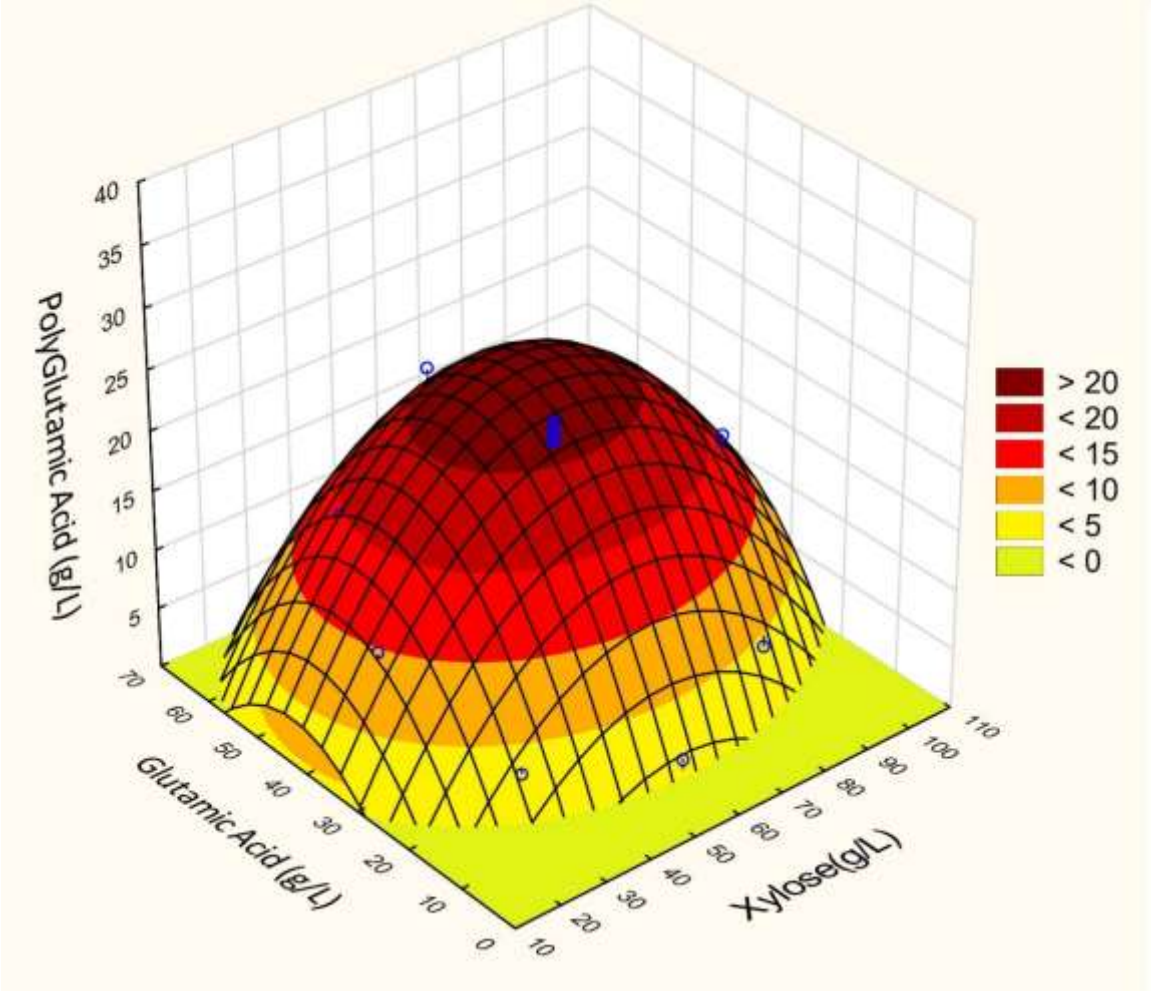

Fig. 5.1. Surface plot of γ-PGA production using central composition design illustrating the effect of glutamic acid and xylose concentration.

5.4.3 Production of γ-PGA in Hemicellulose-based (HPHL) Medium

Following the experiments carried out with synthetic sugar-based medium to optimize the sugar and glutamic acid concentration, the complex hemicellulose medium was used for fermentation under optimal media condition.

Cell growth, γ-PGA concentration, and sugar consumption profiles of the *B. subtilis* grown on HPHL medium are shown in Fig. 5.2. This strain utilizes both glucose and xylose present in the HPHL. *B. subtilis* is capable of consuming different sugars such as glucose, arabinose and xylose. Like other prokaryotes, *B. subtilis* has xylose metabolizing enzymes namely isomerase and xylulokinase (Zhu et al., 2014).

Glucose assimilation, not unexpectedly, was found to be faster than xylose as C6 sugars are consumed preferably (Kim et al., 2010). Even though xylose consumption started immediately (and not in pure diauxic manner), the low rate of xylose consumption leads to high fermentation time. Glucose which is lower in concentration was used up after 24 hr of fermentation. On the other hand, 120 hr of fermentation was required for 86.54% (g/L) of the initial xylose to be consumed. It should be noted that the wild-type *B. subtilis* usually is not able to utilize xylose rapidly as the sole carbon source due to the lack of an endogenous xylose specific transporter (Lindner et al., 1994) which in turn may affect the xylose consumption rate. Also, viscosity of the medium increases with γ-PGA production which can lead to mass transfer issues reducing both microbial growth and γ-PGA production (Richard & Margaritis, 2003). The amount of γ-PGA was not significant during the first 50 hr of fermentation and was available at reasonable levels late in the logarithmic growth phase, like a secondary metabolite product (Ishwar & Rekha, 2011). The γ-PGA concentration reached to its maximum of 12.93 ± 0.90 g/L with dry cell weight of 5.46 ± 0.82 g/L after 96 hr of fermentation. γ-PGA production decreased 40.52% as compare to synthetic sugar-based medium. This might be due to presence of inhibitors in HPHL and complexity of HPHL used.

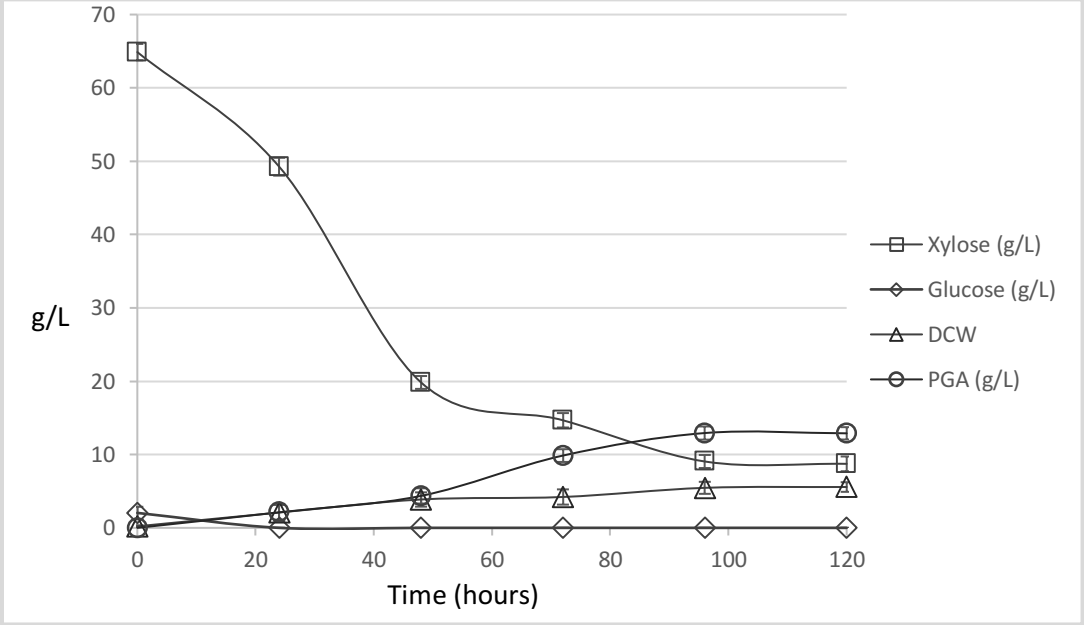

Fig. 5.2. Total cell biomass, γ-PGA and sugar concentration in flask shake fermentation of *B.subtilis* (ATCC 23857) grown on steam treated Poplar wood hemicellulose pre-hydrolysate (HPHL) used in this study under optimized condition.

5.4.4 Identification of PGA

It is important to identify the produced polymer as γ-PGA and hence, FT-IR spectroscopy was carried out to identify the functional group in the polymer structure. As shown in Fig. 5.3 the FT-IR absorption spectra of produced γ-PGA samples showed strong hydroxyl absorption at about 3370 cm^{-1}, carbonyl absorption at about 1400 cm^{-1} and C-N groups absorption peak at approximately 1070 cm-1. The N-H peaks observed at about 1590 cm^{-1} (Ho et al., 2006; Lee et al., 2018). The IR spectra of purified γ-PGA confirms the formation of functional groups in produced γ-PGA structure as discussed. The IR analysis of commercially available γ-PGA was also carried out for comparison and showed the similar result. However, due to hygroscopic nature of γ-PGA the spectra were noisy and is not shown in Fig. 5.3.

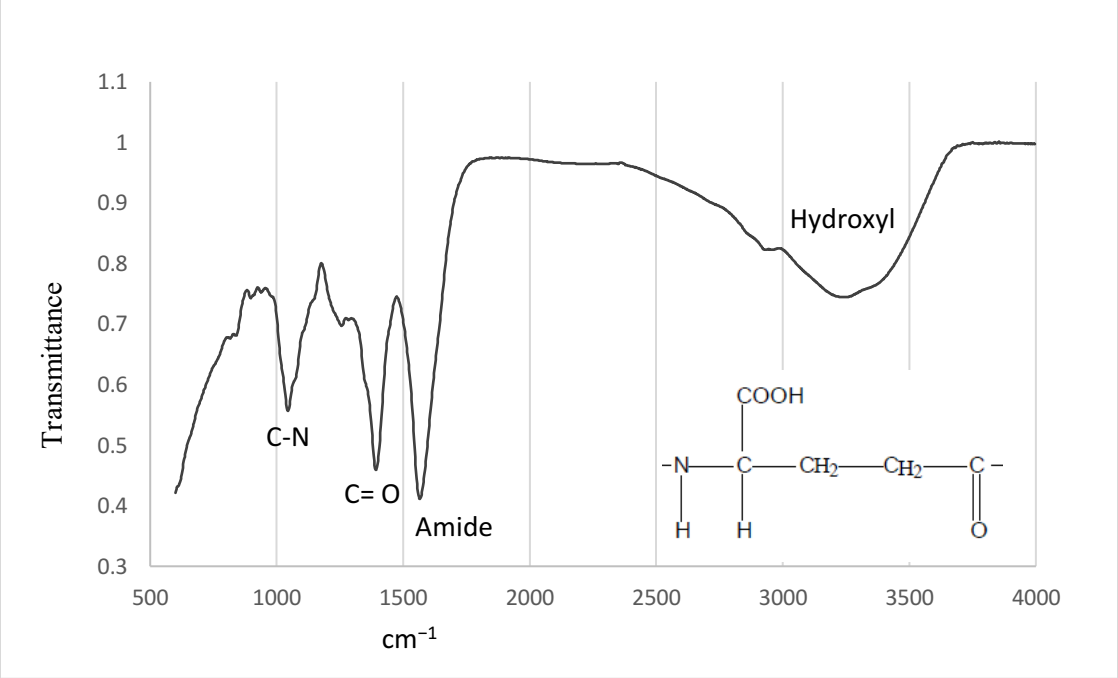

Fig. 5.3. FTIR spectra of purified γ-PGA produced by *Bacillus subtilis* (ATCC 23857) grown on steam treated Poplar wood hemicellulose pre-hydrolysate (HPHL) used in this study under optimized condition.

5.6 Conclusion

Steam treated Poplar wood hemicellulose pre-hydrolysate (HPHL) was successfully used to produce γ-PGA with a view to develop a low-cost production system. In order to maximize the γ-PGA production, concentration of xylose and L-glutamic acid were optimized using response surface methodology in synthetic medium and 21.74 g/L of poly-γ-glutamic acid was obtained. Under optimal condition 12.93 g/L of γ-PGA was obtained after 96 hours of fermentation using *B. subtilis* grown on HPHL. The results exploit the potential of wood-based hemicellulose as an abundant, stable and naturally water-soluble substrate for γ-PGA production. Such renewable and under-estimated resources can be used as a value-addition approach to develop a sustainable biorefinery.

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CHAPTER 6

Summary and Recommendation

6 Summary and Recommendation

6.1 Summary of the thesis

Lignocellulosic biomass has increasingly become regarded as an important resource with the potential to replace fossil-based products. This will be possible with integrated biorefineries that can make use of all three major components present in such residues

Hemicellulose-rich streams can be obtained from agriculture, forestry, pulp and paper and textile industries and can be used to produce a wide spectrum of products. Wood-based hemicellulose hydrolysate/pre-hydrolysate consisting of pentose and hexose sugars is an abundant water-soluble naturally occurring biopolymer. It has a great high potential to be used for production of bio-based chemicals. The fermentation of these C-5 carbohydrate streams can produce the products similar to six-carbon sugar stream. However, bioconversion of hemicellulose faces several limitations due to technical, biological and economic barriers. In order to overcome these challenges, innovative and efficient methods need to be developed.

In the present dissertation, bioconversion of pentose-rich stream to three value-added biochemicals namely microbial oil, biopolyol and poly-γ-glutamic acid were investigated.

The first objective of this thesis was production of microbial oil from Poplar wood hemicellulose pre-hydrolysate liquor. In our study pre-hydrolysate, which was provided by Green Field Global, a major ethanol producer in Canada, was used. The pre-hydrolysate was characterized to determine the sugars and organic acids present. The hemicellulose pre-hydrolysate was then used as the carbon source in fermentations using oleaginous microorganism *C. curvatus* (ATCC 20509) which is a robust strain capable of growing in presence of inhibitory compounds such as acetic acid and hydroxy-methyl-furfural (HMF). In order to maximize the lipid production xylose concentration and carbon to nitrogen (C/N) ratios were investigated in synthetic media using flask shake fermentations. The results showed that 25 g/L of xylose and C/N ratio of 200 maximized the lipid accumulation in *C. curvatus* growing on xylose as the sole carbon source. Bioconversion of hemicellulose pre-hydrolysate without detoxification to microbial oil under best xylose concentration and C/N ratio resulted in 13.87 g/L of cell biomass and 5.13 g/L of lipid after 168 hours of fermentation. The lipid obtained was characterized in terms of their fatty acid profiles. Oleic acid (45.86 ± 0.69 wt%), palmitic acid (15.67 ± 0.98 wt%), myristic acid (10.65 ± 0.86 wt%), linoleic acid (9.02 ± 0.86 wt%), and stearic acid (8.97 ± 1.16 wt%) were the main fatty acids

present in the oil. The High content of oleic acid makes the obtained microbial oil obtained an excellent raw material for production of bio-lubricants, biopolyol and other value-added products due to beneficial properties such as thermal and oxidative stability. Larger scale production of microbial oil was then carried out in 1 L bioreactor. The maximum cell biomass and lipid concentration of batch fermentation were 16.54 ± 0.65 and 6.97 ± 0.58 g/L, respectively after 168 hr of fermentation. 78.18% conversion was achieved using *C.curvatus* grown on hemicellulose pre-hydrolysate as compared with maximum theorical yield where xylose is used as a sole carbon source (0.34 g/g). Use of robust strains like *C. curvatus* (ATCC 20509) to produce microbial lipids from wood hemicellulose can brings profits to the forestry sectors as such processes can easily be integrated into existing plants.

The second objective of the thesis was to demonstrate the utilization of obtained microbial oil from the hemicellulose pre-hydrolysate for production of biopolyol through a green process using enzymes. In the first step the unsaturated binds present in the microbial oil was converted to epoxy group using *Candida antarctica* Lipase B (CALB) immobilized on a macroporous support. (Novozym 435) acts as a catalyst in the presence of hydrogen peroxide which acts as an oxygen donor. A conversion of 84.55% was achieved after 12 hr of reaction. In the second step, a ring opening agent, isopropanolamine was used to introduce hydroxyl group to the backbone structure of microbial oil. FTIR and ^{1}H NMR analysis confirmed the occurrence of ring opening reaction at epoxy and amidation at ester linkage. As a result, the final product obtained was mixture of both polyether and polyester polyols with hydroxyl value of 299.53 ± 1.24 mg KOH/g and acid number of 4.93 ± 1.07.

The third objective of the thesis involved bioconversion of hemicellulose pre-hydrolysate to poly-γ-glutamic acid (PGA) which is a water-soluble and biodegradable biopolymer with a broad range of application. *Bacillus subtilis* (ATCC 23857), which is a L-glutamic acid dependent strain, was selected for this fermentation. Concentration of xylose and L-glutamic acid were optimized to maximize the yield and lower the cost of this bioconversion by response surface methodology on synthetic medium. Optimal values of synthetic xylose and glutamic acid were found to be 65.40 g/L and 44.98 g/L, respectively. Hemicellulose were detoxified using vacuum evaporation followed by solvent extraction as a unified detoxification method to make the pre-hydrolysate amenable for fermentation. *B. subtilis* was able to consume both glucose and xylose and PGA

concentration reached to 12.93 ± 0.90 g/L with dry cell weight of 5.46 ± 0.82 g/L after 96 hr of fermentation.

The processes studied in this thesis can contribute to the development of the methodologies for the bioconversion of low value substrate hemicellulose streams to high value biochemicals which will contribute to develop a circular bioeconomy by utilizing a sugar rich stream which is often disposed as a waste.

6.2 Recommendation for Future Studies

Based on the results obtained from our study the following recommendations for future study are recommended:

In this study, we used hemicellulose pre-hydrolysate of Poplar wood obtained from a proprietary pretreatment method which is a side stream of ethanol production. The composition of this hemicellulose stream differed significantly from batch to batch. The extraction of hemicellulose stream from lignocellulosic biomass varies depending on the type of raw material, pretreatment and operation condition of the processes involved. This is one of the challenges of lignocellulosic-biorefineries which need to be further investigated. Studies can focus on different sources of wood-based hemicellulose such as soft-wood, hard-wood and mixture of hemicellulose-rich streams.

Presence of both hexose and pentose sugars in the hydrolysate/pre-hydrolysate lowers the productivity of fermentation as microorganism selectively consume C6 sugars. Further investigation on microorganism that are capable of consumes C6 and C5 sugars simultaneously is necessary to increase the productivity of the bioconversion.

In order to obtained specialized bioproducts such as biopolyol with specific characteristics, lipids from oleaginous yeasts can be tailored to contain specific type of fatty acid compositions. This will be of great interest to food, fuel and pharmaceutical industries.

High cost of pretreatment process and fermentation is one of the challenges that limit scaling up production. The detailed optimization as well as economic feasibility of such processes will need to be assessed by taking into account the contribution of such processes to the revenue of existing plants within integrated biorefineries.