CONVERSION OF WOODY BIOMASS TO POLYHYDROXYBUTYRATE

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Presented in Partial Fulfillment of the Requirements for the

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Authorization to Submit Dissertation

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Abstract

This project focused on the conversion of wood to the bioplastic polyhydroxybutyrate (PHB) that was successfully achieved. The first step of the conversion of wood involved a hot-water pretreatment process followed by enzymatic hydrolysis to release the monosaccharides (hydrolysates) from the lignocellulosic structure. The process was optimized using a response surface methodology (RSM). This method was proved to be efficient to convert 95% of total carbohydrates into monosaccharides (sugars) which also generated limited amount of fermentation inhibitors such as acetic acid (<1.5 g/L), furfural (<0.1 g/L) and hydroxymethylfufural (HMF) (<0.1 g/L).

The bioconversion of wood hydrolysates to PHB was successfully performed using mixed microbial cultures (MMCs). Through the optimization of fed-batch experiments using a statistical design of experiments, several bioreactors could finally reach the steady states with reasonable PHB yields. The maximum PHB accumulated was 27% cell dry weight with a yield of 0.32 g/g (g PHB produced per g sugars consumed). Microbial community analysis was done at genus level, by next generation genomic sequencing, and the results showed that MMCs presented a community evolution in different bioreactors and during operational period. Several characterized genera belonged to *Alphaproteobacteria* and *Betaproteobacteria* classes such as *Rhizobium, Sphingobium, Paracoccus,* and *Amaricoccus* which have been characterized as PHA-storing microorganisms.

PHB Polymer characterization (chemical structure, PHB content modelling by FTIR, molecular weight, thermal and mechanical properties) had been evaluated for isolated PHB derived from MMC and compared with isolated pure culture PHB and a commercial PHB. Results showed that the properties of MMC PHB were similar to isolated pure culture and commercial PHB, which proved that MMC PHB was a good replacement for pure culture PHB.

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List of Abbreviations

AFEX: Ammonia Fiber Explosion

ATR: Attenuated Total Reflection

ADF: Aerobic Dynamic Feeding

CDW: Cell Dry Weight

COD: Chemical Oxygen Demand

DH: Dehydrogenase

DSC: Differential Scanning Calorimetry

DMA: Dynamic Mechanical Analysis

EDTA: Ethylenediamine Tetra Acetic Acid

FTIR: Fourier Transform Infrared Spectroscopy

FF: Feast And Famine

GAOs: Glycogen Accumulating Organisms

GC-MS: Gas Chromatography-Mass Spectrometry

GPC: Gel Permeation Chromatography

GLM: Generalized Linear Model

GCMS: Gas Chromatography-Mass Spectrometry

HMF: Hydroxymethylfurfural

HA: Hydroxyalkanoate

HB: Hydroxybutyrate

HV: Hydroxyvalerate

HHX: Hydroxyhexanoate

HHP: Hydroxyheptanoate

HPLC: High Performance Liquid Chromatography

ILs: Ionic Liquids

MCL: Medium Chain Length

Mw: Weight Average Molecular Weight

MMCs: Mixed Microbial Cultures

OD: Optical Density

OLR: Organic Loading Rate

PHA: Polyhydroxyalkanoate

PHB: Polyhydroxybutyrate

PP: Polypropylene

PAOs: Polyphosphate Accumulating Organisms

PDH: Pyruvate Dehydrogenase

PEG: Polyethyleneglycol

PL: Pre-liquor

RSM: Response Surface Methodology

RMSEP: Root Mean Square Error Of Prediction

SSF: Simultaneous Saccharification And Fermentation

SCL: Short Chain Length

SDS: Sodium Dodecyl Sulfate

S/G: Syringyl/Guaiacyl

SRT: Solid Retention Time

SEC: Size Exclusion Chromatography

TCA: Tricarboxylic Acid Cycle

TGA: Thermogravimetric Analysis

TSS: Total Suspended Solids

TS: Total Sugar

T_m: Melting Temperature

T_g: Glass Transition Temperature

T_c: Cold Melting Point

X_c: Degree Of Crystallinity

Chapter 1 Introduction and Literature Review

1.1 Introduction

Woody biomass can be converted to value-added products and chemicals by using either the thermochemical (e.g. gasification or pyrolysis) route or the fermentation route (Galbe and Zacchi, 2012). This project focuses on converting woody biomass to bioplastics using the fermentation route by means of microorganisms. Woody biomass is a rich and renewable carbon source in the form of carbohydrates which can be used as intermediate chemicals for further bioconversion. However, woody biomass is unlike food based resources which contain limited nutrition such as nitrogen, protein and mineral salts making it very challenging to convert to other chemicals through microbial fermentation route. Lignocellulosic material is defined as biomass containing, primarily cellulose, hemicellulose, and lignin and based on its composition is a recalcitrant material (Fengel and Wegener, 1984). The technique for conversion of lignocellulosic biomass to fuel ethanol has been developed and is currently being commercialized. However, cellulosic ethanol has economic barriers to overcome and the price of natural gas and crude oil to increase to be economically competitive (Nagle et al., 2002; Kim et al., 2009). Therefore, upgrading the conversion of cellulosic biomass to higher value biobased plastics, such as polyhydroxyalkanoates (PHAs), are of higher value compared to cellulosic ethanol. PHA is a class of biosynthesized polyester, stored intra-cellularly in bacteria, and made up of hydroxycarboxylic acid repeating unit. The cost of the carbon substrate reportedly contributes more than 40% of the production cost of PHA (Choi and Lee, 1997; Reis et al., 2003; Coats et al., 2007). The use of inexpensive renewable agricultural and forestry materials such as woody biomass as feed stocks could be a tremendous advantage to the economics of PHA production (Keenan et al., 2006; Sathesh Prabu and Murugesan, 2011).

Hybrid poplar is a short rotation fast growing wood species with low lignin content and has been highlighted as a good biomass resource for fuel and chemical production (Kim et al., 2011; Hamelinck et al., 2005). In order to break down cellulose crystalline structure a pretreatment process involving high temperatures, long reaction times and addition of

catalysts is required to make it more accessible to cellulase enzymes (Kim et al., 2011). Enzymatic hydrolysis is the most common method for converting woody biomass to fermentable sugars. Compared with acid hydrolysis, enzymatic hydrolysis yields are limited and contain no or low levels of fermentation inhibitors, such as furfural, and the hydrolysates do not need to be neutralized and/or detoxified (Kim et al., 2009). A relatively mild hot-water pretreatment with controlled pH has been shown to improve enzymatic digestibility of lignocellulosic biomass (Mosier et al., 2005; Kim et al., 2009). Acetic acid and other organic acids are released from hemicelluloses during hydrothermal treatment which help auto-catalyze hemicellulose hydrolysis and disrupt cellulose and lignin structure. To obtain fermentable sugars from wood efficiently, a hot-water pretreatment in conjunction with enzymatic hydrolysis was used and the released fermentation inhibitors compounds were quantified to evaluate possibility for further bioconversions.

Currently, commercial PHAs can be produced from different bacterial genera including *Azetobacter*, *Bacillus*, *Alcaligens*, *Pseudomonas*, *Rhizobium* and *Rhodosprillum*. These PHAs are fully biodegradable polymers and can be used alternatively to petrochemical plastics (Anderson and Dawes, 1990). Polyhydroxybutyrate (PHB) is a well characterized homopolymer in PHA family, made up of 3-hydroxybutyrate (3HB) units, and possess physical properties similar to polypropylene (PP) and is completely biodegradable and biocompatible under aerobic and anaerobic environment (Tripathi et al., 2012). PHB can be synthesized from refined carbon sources such as glucose, sucrose, acetic acid and glycerol. Inexpensive carbon sources are currently studied to reduce the production cost of PHB. Wood hydrolysates (fermentable sugars), for example, has been studied for the production of PHB using pure cultures by several research groups (Silva et al., 2004; Pan et al., 2012; Radhika and Murugesan, 2012; Davis et al., 2013). However, the utilization of both mixed microbial culture and wood hydrolysates to make PHB has not been studied. Therefore, understanding the fate of carbohydrates in the biosynthetic pathway for PHB production is critical converting woody biomass to bioplastics by mixed cultures. Mixed microbial culture was used in priority for the production of PHB which is financially attractive in comparison to pure culture PHB production (Serafim et al., 2008). Using a mixed culture does not

require equipment sterilization and routine reactor maintenance, so the cost of operation can be reduced greatly (Reddy and Mohan, 2012).

1.2 Literature Review

1.2.1 Conversion of Lignocellulosic Materials into Fermentable Sugars

The production of liquid fuels as well as chemicals from renewable resources is gained increasing interest world-wide. Renewable materials such as starch- or sugar-containing materials, e.g., corn, wheat or sugarcane are already exploited in many places for the production of ethanol. The industrial processes for this are well established and the techniques are mature. However, more non-food renewable materials have been focused on the production of fuels, such as forest and agricultural biomass, e.g., hard- and soft-wood, sugarcane bagasse and straws (Galbe and Zacchi, 2012). Lignocellulosic materials, for example, differ from one species to another. The major components (cellulose, hemicellulose and lignin) differ in content and composition within a plant cell wall, between plants of the same species and between plant species (Figure 1.1). Cellulose is the main component of plant cell walls consisting of linear chains of β-(1→4) linked glucose units and have both intra- and inter-chain hydrogen bonding make it rigid, have a crystalline structure, insoluble in most common solvents (e.g. water), also very resistant to enzymatic hydrolysis. Hemicellulose consists of short and highly branched chains, in hard-woods, *O*-acetyl-4-*O*-methylglucuronxylan (i.e. a β-(1→4) linked xylan with 4-*O*-methyl glucuronic acid branches and acetyl side groups), while in softwoods it is a mixture of an *O*-acetyl-galactoglucomannan and arabino-4-*O*-methylglucuronxylan. Lignin is the major non-cabohydrate component in lignocellulosic materials comprising a highly complex, three-dimensional polyphenolic compound. It is closely attached to the cellulose and hemicelluloses and is responsible for the remarkable strength of plants (Fengel and Wegener, 1984). Lignin content in different species for hardwoods, softwood and grasses can vary greatly from 15-40% (Sarkanen and Ludwid, 1971) which influences the plant structure (e.g. frequency of β-*O*-4 linkages) and make it resistant to break down in different extent. The complex and highly crystalline structure of lignocellulosic materials make them hard to be

hydrolyzed by either acid or enzyme. Therefore, the first step conversion of woody biomass to fermentable sugars usually needs a pretreatment process to break down the crystalline structure of cellulose and loosen the encapsulating lignin structure (Chiaramonti et al., 2012).

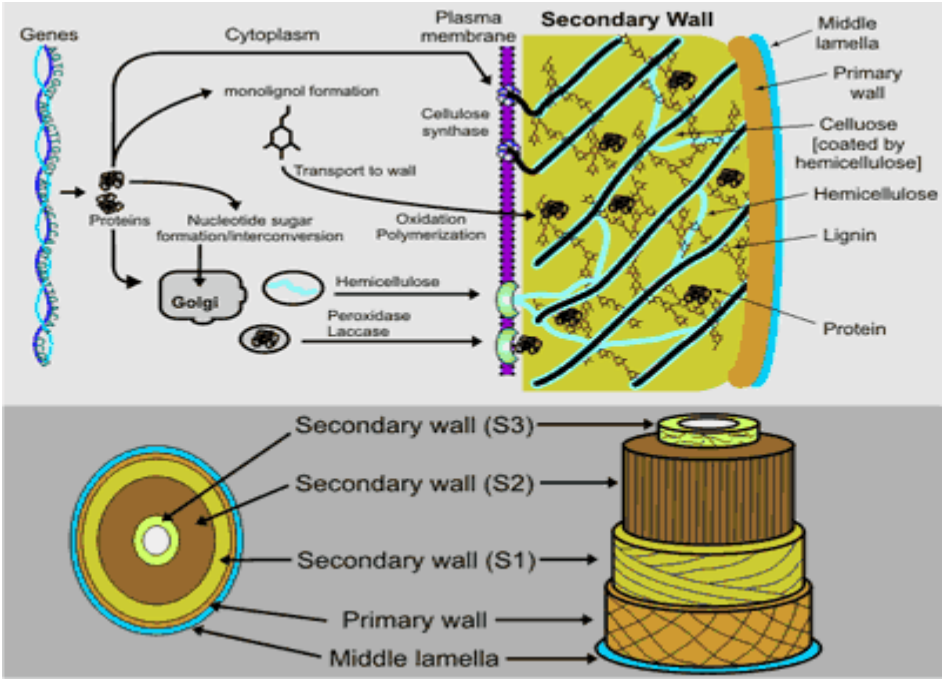


Figure 1.1 Representation of the secondary cell wall in wood (Howard, 2007)

1.2.1.1 Pretreatment Technologies

Many pretreatment technologies have been studies for different types of biomass (e.g. molasses, sugar beet pulp, cheese whey, sugarcane residues, vegetable oil, straws and wood). These pretreatment methods can be categorized into groups, which including biological, physical, physical-chemical, chemical pretreatments (Fang et al., 2013).

1.2.1.1.1 Biological Pretreatment

Biological pretreatment means the utilization of enzymes or microorganisms on lignocellulosic materials that disrupts the cell wall to facilitate enzymatic hydrolysis or further bioconversion (Lee, 1997). The reaction time for biological pretreatment is much

longer than other pretreatment methods. Therefore, it is considered as a preventative factor for industrial use. However, in recent years, the interest has increased and new studies have been performed involving other types of microorganisms, e.g., brown rot fungi, which can improve yields for subsequent enzymatic hydrolysis (Ray et al., 2010). It also been found that biological pretreatment can be used as a first step, followed by some other methods which can be conducted at lower severity (Galbe and Zacchi, 2012).

1.2.1.1.2 Physical Pretreatment

Physical pretreatment includes chipping, grinding and milling to reduce the particle size and increase the accessible surface area of the biomass (Chiaramonti et al., 2012). Mild torrefaction is a newly developed method which improves the grindability of fibrous materials, thus reducing the energy required for grinding the biomass and makes the biomass more accessible to enzymes (Chiaramonti et al., 2010). The yield of fermentable sugars after torrefaction is higher than untreated biomass, even if still lower than steam exploded biomass, but it is a promising pretreatment method and needs more research in this field (Chiaramonti et al., 2012).

1.2.1.1.3 Physical-Chemical Pretreatment

This category comprises methods that combines a physical and a chemical effect like steam pretreatment with addition of a catalyst. The methods include steam explosion (SO_2, CO_2 added steam explosion) (Clark et al. 1989), autohydrolysis, hydrothermolysis and ammonia fiber explosion (AFEX) (Chiaramonti et al., 2012; Fang et al., 2013). The mechanism of steam explosion is similar to dilute acid hydrolysis, but conducted at much higher dry weight and in steam rather than a hot liquid (Brownell et al., 1986). Typically, lignocellulosic material is treated at temperature range from 160 to 240^{o}C for 1-20 min. Also, a catalyst may be added to impregnate the material. Most wood species and agricultural residues release organic acids (mainly acetic acid) when they are thermally treated in an aqueous environment which causing autohydrolysis of hemicellulose take place (Galbe and Zacchi, 2012). SO_2 can be added to steam explosion to improve the recovery of both

cellulose and hemicellulose (Clark et al. 1989). SO_2 acid catalyst addition is typically in the range of 1-5% mass fraction on dry wood and the sugar yield can be as high as 98% (Eklund et al., 1995). However, the formation of degradation products inhibitors of fermentation is unavoidable (Fchutt et al., 2013). As regards to pretreat corn-stover with a small amount of SO_2, the overall sugar yields achieved almost the theoretical value: glucose yield resulted in 90%, but xylose yield was not as high as the dilute sulfuric acid process (Taherzadeh and Karimi, 2008). CO_2 has molecular dimension comparable to water and ammonia and can penetrate into the accessible pores of the feedstock under pressure. By pressurizing biomass with CO_2 (sub- or super-critical) followed by an explosive release of CO_2 pressure allows the disruption of the cellulosic structure and increasing the accessible surface area of the substrate for enzymatic hydrolysis (Chiaramonti et al., 2012). Several works have been carried out to investigate the possibility of using CO_2 in the presence of water, supercritical CO_2 which can efficiently improve the enzymatic digestibility of wood close to 100% (Park et al., 2001; Chiaramonti et al., 2012). Park et al. (2001) reported that using supercritical CO_2 (16 MPa, 90 min at 50^{o}C) and enzymatic hydrolysis together applied on cellulose gave a conversion close to 100%. The main drawback to the diffusion of CO_2 explosion is the high cost. However, Zheng et al. (1998) found that CO_2 explosion was more cost-effective than ammonia fiber explosion (AFEX) and did not cause the formation of inhibitory compounds as could occur in steam explosion.

Autohydrolysis is a relatively mild type of acid hydrolysis since the starting pH is almost neutral and the pH at the end of the process is 3.5-4.0 depending on how much acid is released (Yu et al., 2010). Hydrothermal pretreatment is similar to steam explosion which occurs in liquid hot water rather than using the steam (Galbe and Zacchi, 2012). Hydrothermolysis mainly results in a mixture of solubilized oligosaccharides and the concentration is usually low. Therefore, further treatment or hydrolysis is needed to obtain high yields of monosaccharides (Griebl et al., 2006). Ammonia fiber explosion (AFEX) uses ammonia instead of water at elevated pressures and it is an alkaline pretreatment where the material is treated for 10-60 min at about 100^{o}C or less (Teymouri et al., 2005). Similar to other alkaline methods, the resulting dissolved material after pretreatment is in oligomeric form which requires further hydrolysis of the substrate so it can be fermented (Griebl et al.,

2006). Due to the content of oligomers and polymers, the pretreated liquid has a water-holding capacity and ability to form gels which may lead to biodegradable polymer materials, e.g., barrier films and capsules for controlled release of drugs (Galbe and Zacchi, 2012). The major advantage of AFEX is that no formation of inhibitory products and no addition of water wash is needed (Chiaramonti et al., 2012). However, ammonia is used when the process needs to be recycled to make it both environmentally and economically feasible (Iyer et al., 1996). AFEX is more effective on low-lignin containing material, such as hardwood and agricultural residues, but is less effective for softwood (Chiaramonti et al., 2012; Galbe and Zacchi, 2012).

1.2.1.1.4 Chemical Pretreatment

Chemical pretreatment involves the use of acid, alkaline, organic solvent and ionic liquids (ILs) to the process. Acid hydrolysis can be performed either at low temperature with concentrated acid or at high temperature with diluted acid. The acid used needs to be recycled and the hydrolysate needs to be neutralized with alkaline. Dilute acid hydrolysis is more popular today and the most common method used among chemical pretreatments (Taherzadeh and Karimi, 2008). Hydrochloric and sulfuric acids are typically diluted from 1-5% and used at a temperature range of 120-170^{o}C for 10-90 min (Hu et al., 2010; Chiaramonti et al., 2012). Typically, when the reaction time is longer than 1 h at 120^{o}C on barley husks, xylose concentration decreases due to degradation (Cruz et al., 2002). Hu et al. (2010) had reported that the yield of xylose of sugar maple wood can reach up to 161 g/L by using 6.2% dilute sulfuric acid pretreatment at atmospheric pressure (95^{o}C, 50 min). The major disadvantage for acid hydrolysis is the formation of fermentation inhibitors such as carboxylic acids, furans and phenolic compounds. Therefore, a low pH pretreatment usually needs to be optimized to produce low levels of inhibitors (Hsu, 1996). Acid pretreatment allows for high yield of sugars but the cost are usually higher than other processes such as steam explosion or AFEX (Taherzadeh and Karimi, 2008). This is due to the neutralization of pH which is necessary for the downstream enzymatic hydrolysis and fermentation steps.

Alkaline pretreatments involve the use of NaOH, $Ca(OH)_2$ or ammonia to remove lignin and partial removal of hemicellulose which will increase the accessibility of cellulose to an enzyme (Chiaramonti et al., 2012; Galbe and Zacchi, 2012). The digestibility of NaOH-treated hardwood increased from 14 to 55% with the decrease of lignin content by 24-55% (Chiaramonti et al., 2012). Alkaline pretreatment is usually operated at low temperature, long reaction time and high base concentration which have been shown to be the most effective method for cleaving ester bonds between lignin, hemicellulose and cellulose while avoiding depolymerization of the hemicellulose polymers (Gaspar et al., 2007). Kim and Holtzapple (2006) pretreated corn stover with excess $Ca(OH)_2$ in non-oxidative and oxidative conditions (25-55^{o}C). Both conditions removed around 90% of the acetyl groups at the temperatures studied. Delignification is highly depended on temperature and the presence of oxygen. Lignin and hemicellulose were selectively removed or solubilized, but cellulose was not affected by lime pretreatment at mild temperatures (25-55^{o}C). Kong et al. (1992) found that alkaline pretreatment removed acetyl groups from hemicellulose (mainly xylan), thereby reducing the steric hindrance of hydrolytic enzymes and greatly enhancing carbohydrate digestibility.

In the organosolv process, the suitable solvents employed are methanol, ethanol, acetone, ethylene, glycol and tetrahydrofurfuryl alcohol (Chiaramonti et al., 2012). Due to the cost of the solvents, ethanol and methanol are the preferable candidates. The process solvents used in the process need to be collected from the reactor, evaporated, condensed and recycled to reduce the operational cost and avoid inhibitory effects on enzymatic hydrolysis (Galbe and Zacchi, 2012). Pan et al. (2006) applied the organosolv process using extraction with aqueous ethanol for the conversion of poplar to ethanol. The process resulted in the fractionation of poplar chips into cellulose-rich solids fraction, ethanol organosolvation lignin fraction and water-soluble fraction containing hemicellulosic sugars and sugar breakdown products. The yield of glucose after 48 h enzymatic hydrolysis was 90% to the total cellulose.

The use of ionic liquids (ILs) as solvents for pretreatment of lignocellulosic materials has received much attention recently. ILs are salts, typically composed of large organic cations

and small inorganic anions, which exist as liquids at relatively low temperatures (often at room temperature). Their solvation properties can be varied by adjusting the anion and the alkyl constituents of the cation (Hayes, 2009). ILs are generally called “green” solvents since no toxic or explosive gases are formed. Carbohydrates and lignin can be simultaneously dissolved in ILs such as 1-butyl-3 methylimidazolium cation $[C4mim]^+$. As a result, the intricate network of non-covalent interactions among biomass polymers of cellulose, hemicellulose, and lignin is effectively disrupted with limited formation of degradation products. For the large-scale application of ILs, the toxicity to enzymes and fermentation microorganisms must be investigated (Yang and Wyman, 2008). Since ILs would degrade the sugars, the ILs residues must be removed after treatment (Alvira et al., 2010).

1.2.1.2 Hydrolysis

Acids and enzymatic hydrolysis are the most ready used approaches in recent applications for bioconversion of lignocellulosic biomass to value-added bioproducts (Figure 1.2) (Viikari et al., 2012; Newman et al., 2013). Acid hydrolysis can be either performed in one single stage at higher concentration or in separated stages (e.g. 2 stages: a pretreatment and a hydrolysis stage) at relatively lower concentration (Wyman et al., 2005). The yield of acid hydrolysis is usually high. Yat et al. (2008) reported using dilute acid pretreatment and enzymatic hydrolysis on four wood species (aspen, balsam fir, bass wood and red maple) and switchgrass gave a final xylose yield of 70%, glucose yield of 94% and 60% for the other sugars. Although, acid pretreatment can significant improve the yield of further cellulose hydrolysis, its cost is usually higher than other physicochemical pretreatment processes. The major drawback of acid hydrolysis is the release of unavoidable fermentation inhibitors, such as furfural, hydroxymethylfurfural (HMF), acetic acid and phenolic compounds. Also, the recycling of the acid used and neutralization with alkaline processes must take serious consideration to make the method economically feasible.

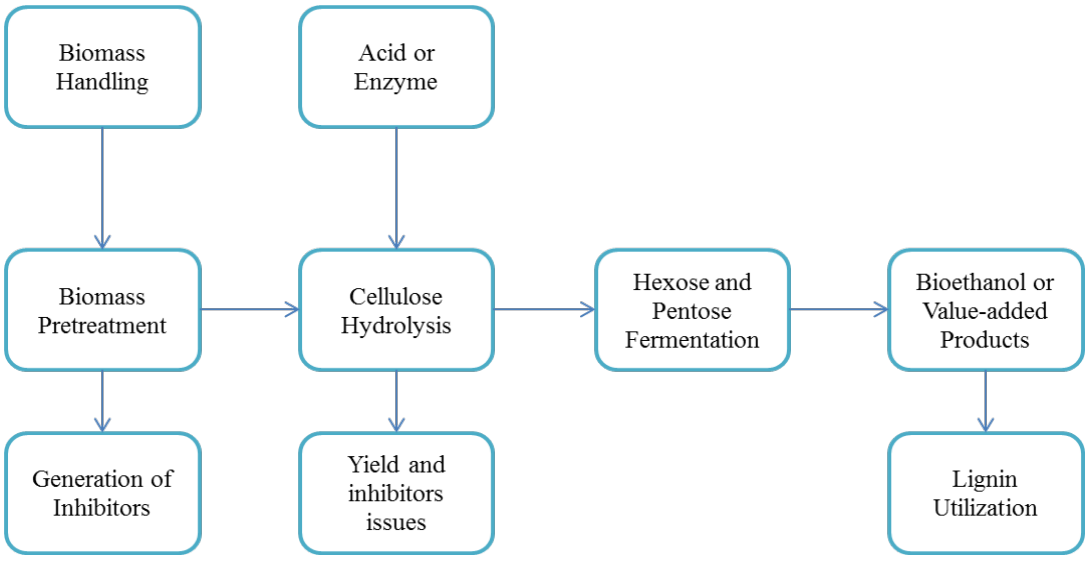


Figure 1.2 Biological conversion of lignocellulosic biomass to bioproducts

Enzymatic hydrolysis is the most popular and widely used technique to release fermentable sugars from lignocellulosic materials. The end product is relatively pure with no or limited inhibitors which make it a good hydrolysis method especially when further fermentation or bioconversion is required (Kim et al., 2009). However, the reaction time of enzymatic hydrolysis is usually long compared to acid hydrolysis. Research is now focused on enzyme selection and enzyme loading to reduce the process cost (Newman et al., 2013). Kim et al. (2009) showed that a hot-water pretreatment at 200^{o}C, 10 min and 15% (wt/vol) slurry of hybrid poplar followed by enzymatic hydrolysis (15 FPU for 120 h) gave a 62% yield for pentose and 54% yield for glucose. The fermentation inhibitors (furfural, HMF and acetic acid) generated were less than 1 g/L in total. Therefore, the pretreatment using hot water followed by enzymatic hydrolysis for the saccharification process is another promising technology (Kumar et al., 2009; Kim et al., 2009; Yu et al., 2010). The residence time is usually from 10 to 30 min at temperature range of 180-230^{o}C. In this pretreatment, approximately 60% total biomass is expected to dissolve with more than 20% cellulose, 30-60% lignin and all the hemicellulose. As a result, the enzymatic hydrolysis is expected to release more than 90% of total carbohydrates with limited or no fermentation inhibited compounds.

A novel type of hydrolysis is call simultaneous saccharification and fermentation (SSF) which performs enzymatic hydrolysis and fermentation in one reactor (Brethauer and Wyman, 2010). In an SSF system, substrate usually constantly fed to the reactor and a flow of fermentation product broth is discharged to keep the working volume constant. Furthermore, the retention time is kept long enough to achieve a steady state of operation. The SSF process has been successfully used in ethanol production from corn and lignocellulosic materials (Brethauer and Wyman, 2010). South et al. (1993) reported a SSF system using sulfuric acid pretreated hardwood with a cellulose feed of 61 g/L, an enzyme loading of 12 FPU/g, and a dilution rate of 0.02 /h, an ethanol concentration of 21 g/L was reached, corresponding to a conversion of 60% and a productivity of 0.41 g/Lh. Studies on PHB production using sugars from broken rice with *cupriavidus necator* in a fed-batch fermentor obtained 38% PHB in the cell dry weight (CDW) after 58 h fermentation (Ugwu et al., 2012).

1.2.2 Pure Culture PHA Synthesis

1.2.2.1 Production History

Accumulation of PHB in the bacterium *Bacillus megaterium* was first reported in 1926 (Prieto, 2007). By the end of 1950s, PHB was demonstrated as an intracellular carbon and energy reserve polymer in gram negative bacteria (Sudesh et al., 2000). Initially, the 3HB unit was thought to be the only hydroxyalkanoate (HA) constituent that forms the building block for this microbial reserve polymer (Sudesh et al., 2000). In 1974, 3-hydroxyvalerate (3HV) and 3-hydroxyhexanoate (3HHX) were found in chloroform extracts of activated sewage sludge (Wallen and Rohwedder, 1974). After a decade, it has been reported that *Bacillus megaterium* cells accumulated a polymer which consisted of 95% 3HB, 3% 3-hydroxyheptanoate (3HHP), 2% of an 8-carbon HA and trace amounts of three other HA compounds (Findlay and White, 1983). Since the homopolymer of PHB is a brittle material with limited applications, the incorporation of a second monomer unit can significantly improve its properties and widen the applications. In the 1980s, research interests lead to the synthesis and characterization of a copolymer consisting 3HB and 3HV (Sudesh et al., 2000).

At the same time, it has been determined that both gram negative and gram positive bacteria synthesize PHA. These bacteria included aerobic (cyanobacteria) and anaerobic (non-sulfur and sulfur purple bacteria) photosynthetic bacteria, as well as some archaebacteria (Anderson and Dawes, 1990). To date, the PHA family of polyesters has a number of approximately 125 different HA units (Sudesh et al., 2000). Figure 1.3 shows the chemical structures of PHAs.

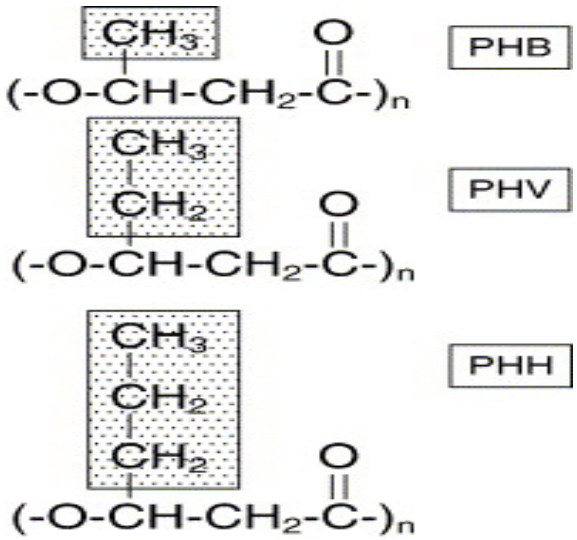


Figure 1.3 Structures of PHAs. The pendant R groups (shaded boxes) varied in chain length from one carbon to over 14 carbons. Structure shown here are PHB (R=CH_3), PHV (R=CH_2CH_3), PHH (R=$CH_2CH_2CH_3$) (Suriyamongkol et al., 2007)

The next stage of PHA studies focused on the cloning and characterization of genes involved in the biosynthesis of these polymers so that the biosynthetic pathways were better understood at the genetic level (Anderson and Dawes, 1990). By the end of 1980s, the genes coding for enzymes in the PHA biosynthesis were already cloned from *Ralstonia eutropha* and the genes were also active in *Escherichia coli* (Peoples and Sinskey, 1989). Three enzymes have been identified to regulate PHA synthesis from acetyl-CoA and were designated as PHA synthase (Figure 1.4). To date, about 38 PHA synthase genes from more than 32 different bacteria have been cloned. Also, the determination of tertiary and quaternary structures of PHA synthase help the understanding of the catalytic mechanisms, substrate specificities of the group of enzymes and the factors influence the molecular

weight of PHA produced. At present, the studies of PHA biosynthesis is focused on the manipulation of PHA synthase and other enzymes involved in the biosynthetic pathway (Sudesh et al., 2000).

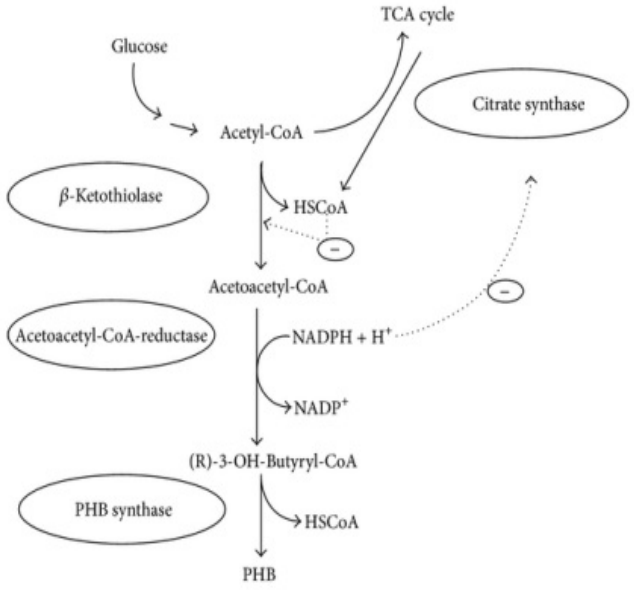


Figure 1.4 PHB biosynthetic pathway from sugars

1.2.2.2 Pure Strains

Pure culture PHA synthesis is the main method for commercial PHA production which utilizing well defined microbial species to intercellular accumulating the polymer. Many bacteria species which are in the family *Halobactericeae* of *Archaea*, synthesize PHAs. The list of such microorganisms is growing and currently contains more than 300 organisms (Anderson and Dawes, 1990; Braunegg et al., 1998; Ciesielski et al., 2006; Suriyamongkol et al., 2007). Table 1.1 summarizes pure PHA synthesis strains with their preferable carbon sources, polymer type and final PHA content (% cell dry weight, CDW). *Ralstonia eutropha* (formerly called *Alcaligenes eutrophus* or *Cupriavidus necator*) has been the most commonly used strain for both industrial and laboratorial production of PHB and PHBV. The strain was able to grow to a high cell density >200 g/L containing over 80% PHB CDW when fed analytical grade glucose (Chen, 2009). However, when glucose was fed together

with propionate, 160 g/L cell dry weight with over 75% PHBV was produced over 48 h of growth (Chen, 2009). When renewable carbon sources were used on *R. eutropha* for PHB production, the cell growth was usually much lower (<20 g/L) than pure feed-stocks. The accumulated PHB could reach 60% CDW. For example, Radhika and Murugesan (2012) reported that *Cupriavidus necator* produced PHB fed water hyacinth hydrolysates in a fermenter gave 12 g/L CDW containing 60% PHB. PHA synthase in *R. eutropha* reacts with a narrow range of substrates which accumulate C_3-C_5 chain lengths and prefers C_4-substrates (Steinbuchel and Schlegel, 1991).

Escherichia coli has been found to be another very important industrial strain other than *R. eutropha* due to its simplicity of growth condition (Chen, 2009). *E. coli* is not a natural PHA producer, but this bacterium can be used as an appropriate host (after gene manipulation) for generating high yields of the biopolymer because of its fast growth and ease with which it can be lysed (Suriyamongkol et al., 2007). *E. coli* can accumulate more than 80% PHA CDW when fed glucose, while only 45% PHA CDW yield when fed sucrose (Suriyamongkol et al., 2007; Chen, 2009; Arifin et al., 2011). *Alcaligenes lactus* is similar to *R. eutropha* in terms of PHA production, which can use both glucose and sucrose as carbon sources (Chen, 2009; Wang et al., 2013). The optimal strain to produce PHB using sucrose as carbon source is *A. lactus* though it accumulates less PHB than using glucose (Wang et al., 2012). However, Yezza et al. (2007) had reported that using renewable feedstock maple sap (sucrose-rich) with *A. lactus* for PHB production showed higher cell growth and PHB content than using pure sucrose. Even though the CDWs from sap and sucrose were 4.4 and 2.9 g/L, the PHB contents were 77.6 and 74.1%, respectively. Therefore, *A. lactus* is a good candidate for PHA production using both pure and renewable resources. Other sugar preferable strains are *Bacillus spp.* and *Bacillus megaterium,* which can synthesize various PHAs with 3HB, 3HV and 4-hydroxybutyrate (4HB) polymer units from structurally unrelated carbon sources such as fructose, sucrose and gluconate (Valappil et al., 2007; Bora, 2012). *B. megaterium* is also a good candidate using high concentration of crude glycerol (by-product of biodiesel) for PHB production. Naranjo et al. (2013) has reported using crude glycerol (initial concentration 20 g/L) for PHB production which gave a final CDW of 7.7 g/L with a maximum PHB content of 62% CDW.

Aeromonas hydrophila is used for large scale production of random copolymer poly-3-hydroxybutyrate-co-3-hydroxyhexanoate (PHB-co-HH). Final cell weight of 50 g/L was achieved containing 50% PHA CDW (Chen, 2009). *Pseudomonas putida* is a typical strain using alkanoic or fatty acids as carbon sources to produce PHA (Chen, 2009; Suriyamongkol et al., 2007; Gumel et al., 2013). It can produce more than 60% medium-chain-length (mcl) PHA and the wide choice of substrates and the low substrate specificity of PHA synthase PhaC enable *P. putida* synthesize over 100 monomer types of PHA structures. However, *P. putida* are not able to directly convert triacylglycerides like various plant oil (Ashby et al., 2001). Moreover, it cannot convert structurally unrelated carbon sources like sugars to precursors for mcl-PHA synthesis (Muhr et al., 2013).

The use of lignocellulosic hydrolysates to produce PHB has been gained interests recently. *Burkholeria cepacia* is a gram-negative bacterium capable of producing short-chain-length (scl) PHAs including PHB as well as copolymer PHBV when provided with monomer precursors such as levulinic acid, which is also an inhibitor to cell growth of bacteria (Keenan et al., 2006). It is also a widely used bioremediation agents due to their ability to degrade polyaromatic hydrocarbons, polychlorinated biphenyls, furans and lignin monomers (Pan et al. 2012). *B. cepacia* has been reported to synthesis PHB from lignocellulosic materials such as wheat straw, surgarcane bagasse and hemicellulose hydrolysates which showed a PHB CDW yield >50% (Silva et al., 2004; Pan et al., 2012; Teresa Cesario et al., 2014).

Mixed cultures system containing two (or more) pure microbial strains have also been reported in literature. For example, Tohyama et al. (1999, 2002) had studied on converting glucose to lactate by *Lactobacillus delbrueckii* and the lactate was converted to PHB by *R. eutropha* in one fermenter. Tanaka et al. (1995) employed a two-stage system where xylose was converted first to lactate by *Lactococcus lactis* and the lactate was converted to PHB by *R. eutropha.*

Table 1.1 PHA synthesis strains

Strain	Carbon source	PHA type	Final PHA (% CDW)	References
Ralstonia eutropha	Glucose	PHB	> 80	Chen, 2009
Ralstonia eutropha	Fatty acids	PHBV, P3HB4HB	> 80	Chen, 2009
Escherichia coli	Glucose	PHB	> 80	Chen, 2009; Suriyamongkol et al., 2007
Escherichia coli	Sucrose	PHB	> 45	Arifin et al., 2011
Alcaligenes lactus	Glucose	PHB	> 75	Chen, 2009
Alcaligenes lactus	Sucrose	PHB	> 45	Wang et al., 2012; Yezza et al., 2007
Aeromonas hydrophila	Lauric acid	PHBHHx	> 50	Chen, 2009
Pseudomonas putida	Fatty acids	mcl PHA	> 60	Chen, 2009; Suriyamongkol et al., 2007; Gumel et al., 2013
Bacillus spp.	Sucrose, Fructose, Gluconate	PHB	> 40	Chen, 2009; Valappil et al., 2007
Bacillus spp.	Glucose	PHB	> 60	Bora, 2013
Cupriavidus necator	Glucose, Sucrose, Fructose	PHB	> 30	Zahari et al., 2012; Radhika and Murugesan, 2012
Bacillus megaterium	Fructose	PHB	> 55	Bora, 2013
Bacillus megaterium	glycerol	PHB	> 60	Shahid et al., 2013; Naranjo et al., 2013
Burkholeria cepacia	hydrolysates (glucose, xylose)	PHB	> 60	Silva et al., 2004; Pan et al., 2012; Teresa Cesario et al., 2014

1.2.3 Mixed Culture PHA Synthesis

1.2.3.1 Feed Stocks

Mixed culture production of PHA using wastewater activated sludge is a financially attractive proposition in comparison to pure culture PHA production (Serafim et al., 2008). Using a mixed culture does not require equipment sterilization and routine reactor maintenance, so the cost of operation can be reduced greatly. Moreover, using mixed culture is a feasible approach to use waste or renewable materials for PHA production due to a simpler process control with non-sterile conditions (Reddy and Mohan, 2012). It allows for a wide variety of substrates to be used due to the presence of several PHA producing microorganisms (Table 1.2). Pure substrates had been studied on mixed culture by several researches, such as acetate (Chua et al., 2003; Johnson et al., 2010; You et al., 2011),

glucose (You et al., 2011; Reddy and Mohan, 2012), sodium acetate (Liu et al., 2011), volatile fatty acid mixtures (Dionisi et al., 2004). In order to commercialize PHA, much effort has been made to reduce the production cost through the development of bacterial strains and more efficient fermentation and recovery processes (Grothe et al., 1999). Several waste streams had been reported using mixed culture to produce PHA, including food waste (Rhu et al., 2003; Kumar et al., 2004), sugar cane molasses (Albuquerque et al., 2007/2011), palm oil (Md Din et al., 2006) and olive oil mill effluent (Beccari et al., 2009), paper mill effluent (Bengtsson et al., 2008), pyrolysis by-products (Moita and Lemos, 2012), municipal sludge (Coats et al., 2007; Mengmeng et al., 2008; Morgan-Sagastume et al., 2010; Coats et al., 2011), and crude glycerol (Dobroth et al., 2011; Naranjo et al., 2013). Aspects relevant to use organic waste to produce PHA may be of interest, including evaluating the effects of non-VFA organic matter and high levels of organics and nutrients on the phenotypic environment inductive to PHA accumulation (Morgan-Sagastume et al., 2010).

Table 1.2 Feed stocks for mixed culture PHA synthesis

Substrates	PHA type	Final PHA content (%)	References
Pure acetic acid	PHB	30-60	Chua et al., 2003; Johnson et al., 2010; You et al., 2011
Pure glucose	PHB	40-50	You et al., 2011; Reddy and Mohan, 2012
Pure sodium acetate	PHB	67	Liu et al., 2011
Pure VFAs mixture	PHBV	50	Dionisi et al., 2004
Food waste	PHB	60	Rhu et al., 2003; Kumar et al., 2004
Sugar cane molasses	PHBV	50-77	Albuquerque et al., 2007/2011
Palm oil	PHBV	44	Md Din et al., 2006
Olive oil mill effluent	PHBV	50	Beccari et al., 2009
Paper mill effluent	PHBV	48	Bengtsson et al., 2008
Pyrolysis by-products	PHBV	10	Moita and Lemos, 2012
Municipal sludge	PHBV	20-50	Coats et al., 2007; Mengmeng et al., 2008; Morgan-Sagastume et al., 2010; Coats et al., 2011
Crude glycerol	PHB	60	Dobroth et al., 2011; Naranjo et al., 2013

1.2.3.2 Mechanisms

1.2.3.2.1 Nutrients Limitation

Similar to most pure culture PHA synthesis, mixed culture PHA synthesis can be performed under nutrients limited conditions, such as nitrogen, phosphorus and oxygen (Sudesh et al., 2000). It has been found that PHA can be synthesized under unbalanced conditions with full aeration where the microorganisms can take up organic substrates by obtaining energy through oxidative degradation of some part of the substrates (Satoh et al., 1998). Punrattanasin (2001) showed that fully aerobic (AE) condition with nitrogen (N) and phosphorus (P) limitations were the optimum conditions for PHA production when the laboratory prepared mixtures of organics were used as the substrate. However, a fully AE condition with combinations of N, P, and potassium (K) limitations were better for PHA production when a high acetic acid industrial wastewater was the substrate. Limitation or partial limitation of a single nutrient, either N or P, as used for commercial production utilizing pure culture, did not promote significant PHA production in activated sludge biomass compared to the simultaneous limitation of two or more nutrients. A maximum cellular PHA accumulation of 70% TSS was obtained under fully AE conditions with multiple alternating periods of growth and N and P limitations (Punrattanasin, 2001). Other than nutrients limitations, the amount of oxygen input is another key parameter in this PHA synthesis mechanism. If the supply of oxygen is sufficient, the microorganism may be able to get enough energy for assimilative activities such as the production of protein, glycogen and other cellular components while taking up the organic substrates. However, oxygen must be controlled to let the microorganism grow and accumulate PHA simultaneously (Salehizadeh and Van loosdrecht, 2004).

1.2.3.2.2 PAO/GAO System

Two types of microorganisms are capable to anaerobic store of carbon source in mixed culture: polyphosphate accumulating organisms (PAOs) and glycogen accumulating organisms (GAOs). PAOs are probably the most widely recognized for producing storage

polymers. The whole competitive advantage for these organism is based on their capacity to utilize the energy stored as Poly-P to store exogenous substrate in the form of PHA when there is no electron acceptor (oxygen or nitrate) available for energy generation (Salehizadeh and Van loosdrecht, 2004). GAOs can store fermented substrates (e.g. glucose) inside the cell rather than excreting them. These organisms can also use internal stored glycogen for fermentation to PHB. The energy released in the glycolysis is subsequently used to accumulate fermentation products (e.g. acetate) in the form of PHB (Salehizadeh and Van loosdrecht, 2004). The type of carbon source is highly critical for the PAO/GAO system, since it dictates the metabolism of PAOs and GAOs communities. For example, Wang et al. (2009) suggested that acetate might be a better substrate than glucose for PAOs because its conversion to PHA requires energy and reducing powers that are conveniently provided by polyphosphate transformations. However, the conversion of glucose by GAOs yields large amounts of energy by itself, so the role of polyphosphate is minimized. The type of PHA polymer production is dependent on the type of carbon substrate. The different polymers are formed to allow the cells to balance the redox equivalents produced and needed in conversion of substrate to PHA (Salehizadeh and Van Loosdrecht, 2004). Liu et al. (1994) and Satoh et al. (1998) had demonstrated the existence of HV formation in polyphosphate-independent anaerobic-aerobic activated sludge. The glycogen in the fermentation is converted to PHA through propionyl-CoA in order to satisfy the redox balance (Figure 1.5). The enzymes of intermediary cell metabolism are still active under anaerobic conditions due to the rapid change between aerobic and anaerobic conditions. This is why PHV can be formed when acetate is the sole carbon source for PAOs (Pereira et al., 1996). Although activated sludge accumulates PHA under anaerobic-aerobic conditions, there is no guarantee that this operation is best for enrichment of PHA accumulating microorganisms. It had been reported by Saito et al. (1995) that sludge accumulated more PHB under aerobic conditions than anaerobic conditions.

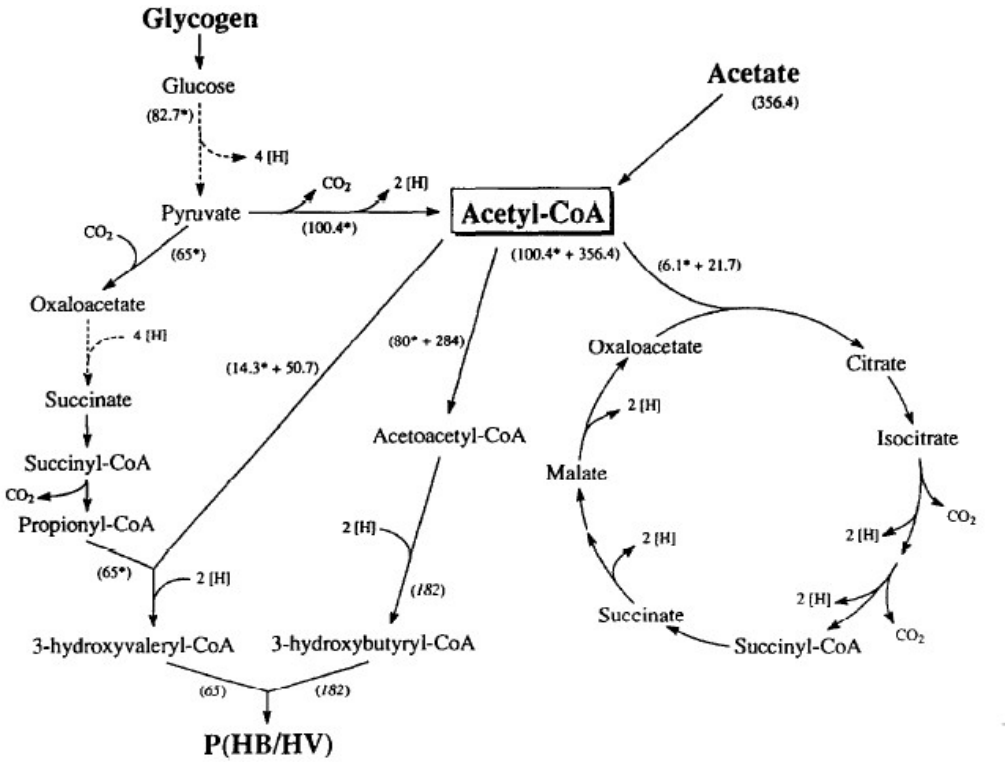


Figure 1.5 Metabolic model for conversion of acetate and glycogen to PHBV by activated sludge (Pereira, et al., 1996)

1.2.3.2.3 Feast-Famine Process

Recent mixed culture studies have focused on employing a feast-famine process for PHA production (Chua et al., 2003; Liu et al., 2011; Albuquerque et al., 2007, 2011). When the external substrate is available called feast period and no external substrate is available called famine period. At this unbalance growth, the growth of biomass and the storage of polymer occur simultaneously when an excess of external substrate presents. After the external substrate is consumed, stored polymer can be used as carbon and energy source for microorganisms. It was proposed that the absence of external substrate during famine period cause a decrease in the amount of RNA and enzymes needed for cell growth. If the microorganisms are spiked with an excess carbon after the famine period, the amount of enzymes available in the cells is lower than that required to reach the maximum growth rate, thus the storage of polymer becoming dominant phenomena (Daiger and Grady, 1982). This is the most probable mechanism for storing PHA by mixed culture subjected to aerobic

feast-famine process (Daiger and Grady, 1982; Serafim et al., 2008). This process promotes the conversion of carbon substrates to PHA directly and not to glycogen or other intracellular materials (Salehizadeh and Van Loosdrecht, 2004). Culture selection with a high capacity for PHA accumulation is one of the important challenges in mixed culture process. A two-stage process had been used by many authors on mixed microbial culture (Beccari et al., 1998; Dionisi et al., 2004; Lemos et al., 2006; Serafim et al., 2008). The first stage aims to get microbial enrichment where all the selected microorganisms have a high PHA storage capacity which was in terms of culture selection. The second stage was to accumulate PHA in higher cell content. It is worth noting that the operation of culture selection reactor should be optimized to obtain homogeneous population with high and stable storage capacity rather than to maximize PHA content in one reactor (Serafim et al., 2008).

1.2.4 PHA Metabolic Pathways

There are four major pathways plus several other pathways for the synthesis of PHAs found to date. These pathways in detail have been reported elsewhere (Khanna and Srivastava, 2005; Chen, 2010). The first type is a well-studied pathway using sugar as carbon source to synthesis PHB by *R. eutrophus*. The second type involves β-oxidation for PHA synthesis through fatty acids. The third type involves malonyl-CoA-ACP transacylase (FabD) and 3-hydroxyacyl-ACP-CoA transferase (encoded by PhaG). Substrates are converted to 3-hydroxyacyl-ACP which can then form 3-hydroxyacyl-CoA and thus PHA. Pathway four uses NADH-dependent acetoacetyl-CoA reductase to oxidize 3-hydroxybutyryl-CoA. A high ratio of NADPH to $NADP^+$ could enhance the delivery of the reductant to nitrogenase in *Rhizobium* (Cicer) sp. strain CC 1192 (Chen, 2010). This could also favor the reduction of acetoacetyl-CoA for PHB synthesis. The other pathways used for the synthesis of alternative copolymers, such as, the synthesis of homopolymer P4HB by *Clostridium kluyveri* and *A. hydrophila* 4AK4 (Laycock et al., 2014). Two major types of biosynthetic pathways, carbohydrates and fatty acids pathways will be reviewed in details.

1.2.4.1 Carbohydrate Pathway

PHB synthesized from sugars is considered the simplest biosynthetic pathway (Figure 1.6). The process starts with glycolysis of the sugar to pyruvate which is later converted to acetyl-CoA via pyruvate dehydrogenase (PDH) oxidation pathway. Then two molecules of acetyl-CoA are condensed to yield acetoacetyl-CoA, coupling with the oxidation of NADH to NAD^+. The next step is the reduction of acetoacetyl-CoA to hydroxybutyryl-CoA. In the presence of PHB synthase (PhaC), hydroxybutyryl-CoA is polymerized into PHB polyester. This PHB biosynthetic pathway competes for acetyl-CoA with other metabolic pathways such as the tricarboxylic acid cycle (TCA) and fatty acid biosynthesis (Chen et al., 2012). Therefore, the control of acetyl-CoA is the key feature of PHB synthetic pathway. Acetyl-CoA can be regulated by oxygen limitation which is the cause of PHB accumulation during growth phase. In oxygen limitation, when $NADH/NAD^+$ ratio increases, citrate synthase and isocitrate dehydrogenase are inhibited by NADH, in consequence, acetyl-CoA doesn't enter the TCA cycle at the same rate and it is converted to acetoacetyl-CoA by 3-ketothiolase (the first enzyme of PHB biosynthesis) which is inhibited by CoA (Anderson and Dawes, 1990; Nath et al., 2008; Tripathi et al., 2013). However, oxygen limitation needs to be controlled between 2 and 3 mg/L for PHA synthesis (Yezza et al., 2007). Extremely low dissolved oxygen, specifically less than 1 mg/L, makes the metabolic pathway toward fatty acids synthesis (Tohyama et al., 2000).

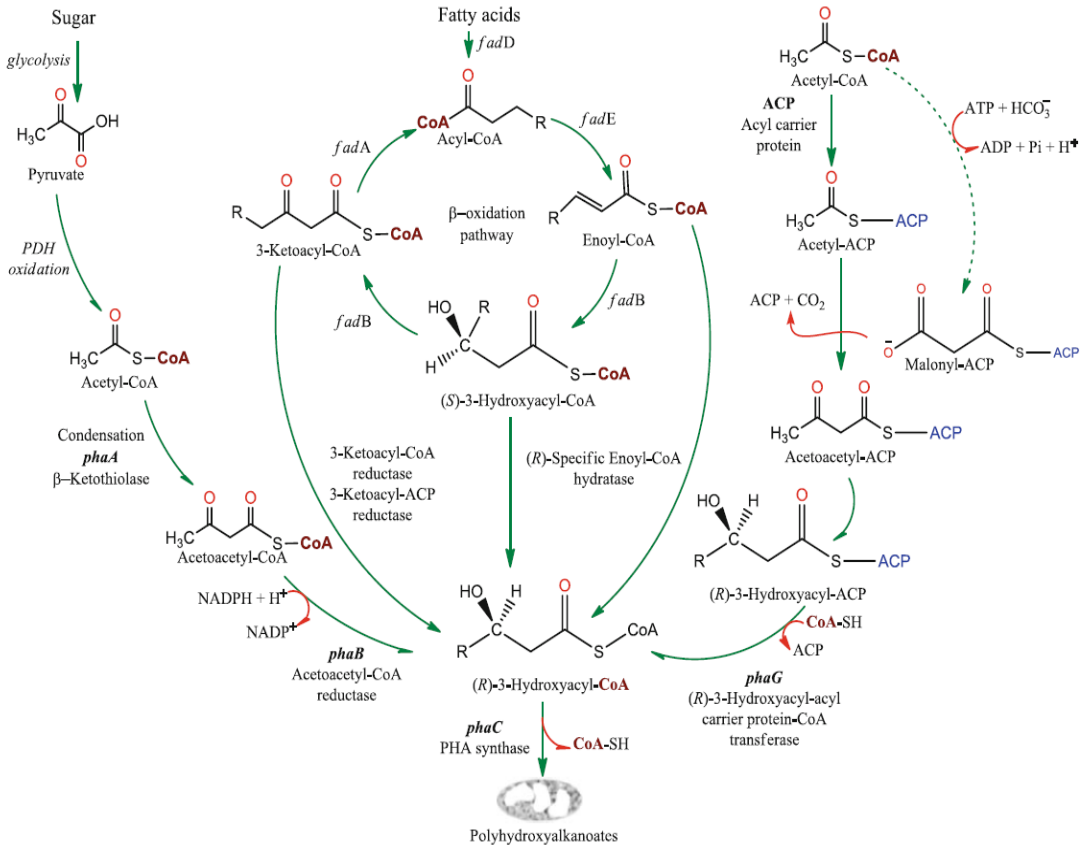


Figure 1.6 PHA biosynthesis from sugar catabolism, fatty acid β-oxidation and intermediary pathways (Gumel et al., 2012)

1.2.4.2 Fatty Acids Pathway

Fatty acids are the other types of carbon sources for various PHAs production via β-oxidation synthetic pathway. Medium-chain-length (mcl) PHAs from fatty acids or alkanoic acids can be synthesized by this pathway using *P. oleovorans* and *P. fragii* bacteria strains. The intermediates of fatty acid β-oxidation pathway include enoyl-CoA, 3-ketoacyl-CoA and 3-hydroxyacyl-CoA, which can serve as precursors of mcl-PHA synthesis (Suriyamongkol et al., 2007). The PHA synthase (phaC) in *Pseudomonas* make mcl-PHA polyesters from 3-hydroxyacyl-CoA. The cost of the monomer precursor (e.g. fatty acids) is high. Therefore, current PHA research on pure culture metabolic engineering focuses on producing monomers from structurally unrelated carbon sources such as glucose to synthesize mcl-PHA (Li et al., 2010; Davis et al., 2013). Engineering strain *E. coli* was

selected for mcl-PHA production from structurally unrelated carbon sources. The carbon source (e.g. glucose) was first converted to fatty acids by Acyl-ACP thioesterases via fatty acid biosynthesis, the fatty acid was then further converted to PHA via β-oxidation (Suriyamongkol et al., 2007; Agnew et al., 2012). Alternatively, mcl-PHA can be produced from renewable or waste feed stocks enriched with free fatty acids (e.g. palm and soybean oil). Pradella et al. (2012) showed that soybean oil could be used as a substrate for PHB production via β-oxidation synthetic pathway and the final PHB content was 81% with a productivity of 2.5 g/Lh. Therefore, any suitable fatty acid or carbohydrate sources can be used for PHA production.

1.2.5 PHA Characterization

1.2.5.1 Chemical Structures

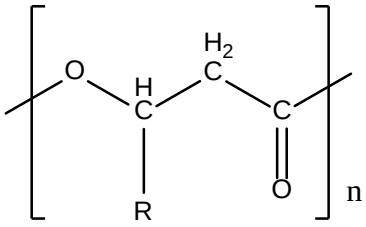
Since the chemical structure and monomer composition of a polymer are the most important factors influencing its material properties, a short list of common monomer units for the PHAs family and the utilization of spectroscopic methods for determining of the polymer chemical structure will be reviewed in this section.

1.2.5.1.1 Monomer Units

PHAs are polyesters of HAs with the general unit structure shown in Figure 1.7. The monomer composition of PHAs depends on the nature of the carbon source and the microorganisms used. Based on the numerous monomers in PHA chains, they can be divided into two groups. The short-chain-length (scl-PHA) usually consists of 3-5 carbon atoms and medium-chain-length (mcl-PHA) which consists of 6-14 carbon atoms (Anderson and Dawes, 1990). This difference is mainly due to the substrate specificity of the PHA synthases that can accept 3HAs of a certain range of carbon length.

The most common representative for scl-PHA is PHB and its copolymers with hydroxyvalerate. PHB is a simplest PHA structure with a methyl group as the pendent R-

unit (Figure 1.7) and has a perfectly isotactic structure with only the R-configuration. PHB isolated from bacteria possesses 55-80% crystallinity, while the molecules within the bacterial cells are amorphous and exist as water insoluble inclusions (Sudesh et al., 2000). The mcl-PHA can be synthesized by *Pseudomonas* species when using aliphatic hydrocarbons like n-alkane, n-alkanoate, or n-alkanol serve as feed stocks resulting in a random copolymer containing a 3-hydroxyalkanoate unit as the major component, with a carbon chain length equivalent to that of the growth substrates (Lagaveen et al., 1988). Chemical structure of PHA can be determined by gas chromatography-mass spectrometry (GC-MS). One method involved methanolysis has been reported by many people. It used acidified (e.g. H_2SO_4) methanol and chloroform with an internal standard (e.g. benzoic acid) to heat digesting the biomass. Then the methyl-ester derivatives were recovered and analyzed by GC-MS (Braunegg et al., 1978). Other methods found in literature use 3:1 butanol to concentrated (37%) HCl to hydrolyze and derivatize the biomass. Esters of the PHA, carbohydrates converted to levulinic acid and long chain microbial fatty acids were extracted into hexane for GC-MS analysis (Werker et al., 2008).



n varies from 600 to 35000

R=H	Poly-3-hydroxypropionate
R=CH_3	Poly-3-hydroxybutyrate
R=CH_2CH_3	Poly-3-hydroxyvalerate
R= $(CH_2)_2CH_3$	Poly-3-hydroxyhexanoate
R= $(CH_2)_3CH_3$	Poly-3-hydroxyoctanoate
R=$(CH_2)_8CH_3$	Poly-3-hydroxydodecanoate

Figure 1.7 Structure of PHA

1.2.5.1.2 Spectroscopic Methods

Fourier transform infrared (FTIR) spectroscopy has been used to probe the structure of PHAs and determine the composition of PHBV (HB/HV ratio) copolymer (Valappil et al., 2007). Hong et al. (1999) had shown characteristic absorption bands at 1728 cm^{-1} (carbonyl ester group) and 1282 cm^{-1} (-CH group) of PHB. The FTIR spectral C-H (2900 cm^{-1}) and C-C bands (977 cm^{-1}) of PHBV copolymer are sensitive to the polymer structure. The C-H bands are well resolved and therefore useful for compositional analysis. Holden and Bloembergen (1986) have found that the large variation of intensity change between different HV compositions would make FTIR spectroscopy a feasible tool to quantitatively determine the composition of the copolymer. Moreover, FTIR spectroscopy has also been used for determination of PHA content (cell dry weight basis) in the biomass by modeling the spectra with PHA reference data with the aid of chemometric tool (Arcos-Hernandez, 2010). In addition, FTIR spectroscopy can be used for determination of degree crystallinity of PHA polymers. Padermshoke et al. (2004) had reported using attenuated total reflection (ATR) to study the crystallization behavior of P(HB-co-HHX) and PHB. The C=O stretch band at 1720 cm^{-1} was shown to gradually decrease to a broad band at 1731 cm^{-1} with increasing temperature. The peak at 1720 cm^{-1} was assigned to the crystalline phase, while the peak at 1731 cm^{-1} was assigned to the amorphous phase. The IR bands at 1185, 1278 and 1724 cm^{-1} were found to be sensitive to the degree of crystallinity of PHA (Bayari and Severcan, 2005).

Near-infrared (NIR) spectroscopy has been applied to in-line monitoring the thermal degradation behavior of PHA. Several band assignments showed strong variations related to the solid state PHA stretching, which included C-H methyl (CH_3) first overtone at 1690 cm^{-1}, CH_3 first overtone at 1720 cm^{-1} , C=O second overtone at 1950 cm^{-1} , and C=O + CH_3 combination at 2120 cm^{-1} (Montano-Herrera et al., 2013). ^{1}H NMR is a common method used for determination of HB and HV composition in the PHBV copolymer. ^{13}C NMR is a traditional method to determine chemical structure of a polymer which can also be used for compositional and distributional analysis of copolymer PHBV. Diad and triad sequence

analysis is used to assess the extent of deviation of the copolymer composition from statistically random (Bernoullian) compositional distribution. The parameter D is given by:

$$D = \frac{FBBFVV}{FBVFVB}$$

The FBB, FVV, FBV and FVB can be assigned to BB, VV, BV and VB diad sequences of PHBV, respectively, the mole fraction of HV units in the polymer can be calculated based on the relative peak areas of the split multiplets (Bluhm et al., 1986). The D value for statistically random copolymers is 1.0. Nonrandom copolymers will have D values greater or less than 1.0, with blocky and alternating copolymers having D and R values far greater than 1.0 and very close to 0, respectively (Zagar and Krzan, 2009).

1.2.5.2 PHA Recovery

Many recovery methods include solvent extraction, mechanical disruption, chemical digestion, enzymatic digestion and supercritical fluids have been used to recover PHA from the biomass (Tamer et al., 1998). The extraction of polymer using organic solvent seems to be the most common approach and has been reported to have more advantages than other PHA extraction methods (Kunasundari and Sudesh, 2011). Chloroform/methanol, chloroform/hexane and dichloromethane/hexane are typical solvent systems for PHA extraction. Using solvent extraction can not only improve the cellular membrane permeability and subsequent solubilization of the polymer without degrading it, but prevents gram-negative bacterial endotoxin (lipopolysaccharides) coextraction with the polymer as well as improving the quality for biomedical applications (Jacquel et al., 2008; Naranjo et al., 2013). The process usually starts with drying (typically freeze-dred) of the biomass, followed by a solvent wash (e.g. acetone) and vacuum dried before extracted with chloroform. Cold methanol is generally used to precipitate the polymer from chloroform (Kunasundari and Sudesh, 2011). In some cases, dichloromethane has also been used for the extraction instead of chloroform (Chardron et al., 2010).

Mechanical disruption by ultrasonication of cells in combination with solvent extraction have been used and reported to effectively reduce the chemicals used and polymer degradation (Penloglou et al., 2012). Use of chemicals to digest the cells has been reported

by some studies (Tamer et al., 1998; Yang et al., 2011). The chemicals include detergents such as sodium dodecylsulfate (SDS), ethylenediamine tetra-acetic acid (EDTA) and alkali (NaOH). It has been studied that chemical digestion gives a higher purity (>99%) of PHA polymer than solvent extraction (>70%) but with a higher cost (Gumel et al., 2013). Bacteria contain a significant proportion of their biomass as DNA in the cell homogenate which can be quite viscous and difficult to process. Thus, enzymes such as bezonase (a commercial nuclease) may be added to the cell broth to digesting DNA and processing through certain mechanical cell disruption devices (Horowitz and Brennan, 2010). Usually, the PHA pellet recovered from the digested cells needs to be further treated with ozone or peroxide to remove contaminants (Horowitz and Brennan, 2010). Supercritical fluids, particularly supercritical CO_2, have attracted attention for PHA recovery since they are inexpensive, readily available, no toxic residue, low reactivity, nonflammable and has a moderate temperature (30^oC) and pressure (7 MPa) (Jacquel et al., 2008). Supercritical fluids can recover 86-99% PHA CDW from the biomass (Gumel et al., 2013).

1.2.5.3 Molecular Weight

The molecular weight distribution is responsible for the end-use properties of PHAs which is determined by the types of microorganisms, feed stocks and fermentation and cultivation conditions (van der Walle et al., 2001). It is known that the molecular weight of PHA synthesized biologically is much higher than obtained chemically (Chen, 2010). Generally, the weight average molecular weight (M_w) of PHA ranges between 200,000 and 3,000,000 g/mol (Byrom, 1994). The mechanical properties of PHA deteriorate when the M_w is lower than 300,000 g/mol (Laycock et al., 2014). In most cases, the molecular weight of PHA is determined by size exclusion chromatography (SEC) using polystyrene standards to provide a universal calibration curve.

The increased polydispersity (M_w/M_n) is a good indicator of polymer accumulation (e.g. from 1.5 to 2.0). These results could be due to either chain transfer reactions or depolymerase activity during granule formation or to the random decay of active synthase molecules (Koizumi et al., 1995). There are two approaches to control the molecular weight

of PHA during polymer synthesis. The first involves control of the level and time expression of PHA synthase. A lower molecular weight is obtained when high levels of synthase expression are induced while a higher molecular weight results when low levels of synthase are expressed (Sim et al., 1997). However, the molecular weight of PHB produced by recombinant *R. eutropha* was found to be independent of PHA synthase activity (Kichise et al., 1999). The second method is to add chain transfer agent of polyethyleneglycol (PEG) to the fermentation medium to effectively lower the molecular weight of PHA (Abe et al., 1994). Helm et al. (2008) has reported that a potassium deficiency can lead to the synthesis of a high M_w PHB (>3,000,000 g/mol) in a methane-utilizing mixed culture. It also been reported by other studies that as the carbon source concentration increases, so the average M_w decreases (Kusaka et al., 1997; Laycock et al., 2014).

1.2.5.4 Thermal Properties

Most PHAs are semi-crystalline polymers so that their glass transition temperature (T_g) of the amorphous phase and the melting temperature (T_m) of the crystalline phase determine their thermal and mechanical properties (Anderson and Dawes, 1990). A list of PHB and PHA copolymers T_m and T_g are given in Table 1.3. Some scl-PHA like PHB is typical thermoplastic polymer, which becomes fluid above its T_m's. PHB has been reported to show a relatively high T_m of about 170^{o}C and a T_g of approximately 6^{o}C (Sudesh et al., 2000; van der Walle et al., 2001). PHB can achieve a high degree of crystallinity (X_c), usually between 50-80% depending on processing conditions (e.g. cooling rate) and molecular weight (Bluhm et al., 1986). The relatively high T_g temperature and crystallinity contribute to their brittleness and limit their applications. There are three major reasons for the brittleness of PHB. First is the secondary crystallization of the amorphous phase during storage at ambient condition. Second is the T_g of PHB is below room temperature. Third is the low nucleation density of PHB which make it large spherulites exhibit inter-spherulitic cracks (El-Hadi et al., 2002).

Incorporating hydroxyvalerate (HV) units into PHB to form a co-polymer will decrease both the T_m and T_g and making it more flexible (Table 1.3). For example, PHBV, containing up

to 28 mol %HV, has a T_m of about 102^{o}C and a T_g of -8^{o}C. However, the copolymers are slow to reach crystallization equilibrium and they crystallize slowly to form large crystals (Gunaratne and Shanks, 2005). Some copolymers have much low T_g (-26-4^{o}C) and T_m (45-170^{o}C) values (Van der Walle et al., 2001; Li et al., 2011). To decrease the crystallinity of PHB, some studies have reported that the addition of nucleating agents or plasticizers will increase the number of small spherulites. Thus, the molecular motion can be enhanced and T_g is lowered so that high elongation at break and high degree of flexibility can be achieved (El-Hadi et al., 2002).

In DSC thermograms, the melting peak(s) can be observed during the heating process. However, there may be multiple melting peaks observed because of different crystalline polymorphs present in the sample or recrystallization from the melt. Such behavior is commonly observed for semi-crystalline polymers and has been reported in PHB (Barham et al., 1984). The lower temperature peak is the melting peak of crystals, called cold melting point (T_c), while the higher one is the melting peak of crystals recrystallized during the heating process in the DSC measurement (Yoshie et al., 1995).

DSC and X-ray diffraction have been performed to examine the crystallization behavior of PHAs. In DSC, the melting enthalpy is determined from the thermogram which allows for the calculation of the degree of crystallinity. The percent crystallinity is reported by normalizing the observed heat of fusion to that of a 100 % crystalline sample of the same polymer (Blaine, 1994). It is assumed for PHB with 100% crystallinity has a melting enthalpy of 132 J/g (Serafim et al., 2008). Barham et al. (1984) also reported a melting enthalpy of 146 J/g for PHB with 100% of crystallinity. X-ray diffraction is a traditional technique used to determine crystallinity of a wide range of materials, and the crystallinity of PHA is calculated by measuring the crystal area to the total area in the diffractogram (Holden and Bloembergen, 1986).

1.2.5.5 Mechanical Properties

The mechanical properties of PHA include the elongation at break, tensile strength and Young's modulus are the major concerns for determining PHA applications. As it is discussed above, the brittleness of PHB make it a big obstacle to practical application and much research effort has been made to manipulating these mechanical properties. It has been reported that solvent casting PHB film and its copolymers have very fine spherulitic morphology because of low crystallization temperature and resulting high nucleation density, which can result in a higher elongation to break and impact strength (Holmes, 1988).

Table 1.3 Properties of PHB and copolymers (Li et al., 2011)

Sample	3HB mol%	3HV mol%	3HHp mol%	3HHx mol%	T_m (°C)	T_c (°C)	T_g (°C)	M_n ($\times 10^5$)	M_w/M_n	Young's modulus (GPa)	Tensile strength (MPa)	Elongation at break (%)
P(3HB) homopolymer	100	0	0	0	171.8	41.2	3.1	–	–	1.47	18	3
Random copolymer	4.4	46.5	49.1	0	–	–	–26.4	–	–	–	–	–
Random copolymer	72.9	13.1	14	0	–	–	–7.3	1.27	2.9	0.13	7.0	462
Block copolymer	71.5	10.2	16.5	0	170.6	50.6	–23.6,3.5	0.52	8.7	0.37	7.5	63
Blend	71.5	10.2	16.5	0	166.5	54.3	–20.0,4.4	0.81	4.4	0.26	5.3	24
PHBHHx	88	0	0	12	–	–	–	–	–	0.28	7.0	400
P(3HV) homopolymer	0	100	0	0	106.2	57.9	–15.7	–	–	0.39	6.6	3.5

The most common way to improving the mechanical properties of PHB is to synthesize copolymers such as P(3HB-co-3HV). Compared to PHB, P(3HB-co-3HV) has decreased stiffness and brittleness, higher elongation to break and increased tensile strength and toughness (Van der Walle et al., 2001). This improvement is due to the incorporation of HV unit to the P(3HB) lattice which significantly depress the melting point, the heat of fusion of the HB crystal and the fold surface free energy (Orts et al., 1990). Annealing is another solution to improve the polymer mechanical properties. It has been reported by de Koning (1995) that the elongation to break can be increased up to 130% when P(HB-co-12% HV) treated with heat after 3 months. The degree of molecular segmental motion the amorphous portion of PHA can achieve is strongly influenced by the difference between the T_g and the end-use temperature. Therefore, even at the same crystallinity, a polymer with a lower T_g should be more flexible (Chen, 2010). The mechanical properties of mcl-PHA copolymers

such as P(3HB-co-3HHx) and P(3HB-co-4HB) has been characterized more flexible than P(3HB-co-3HV) copolymer (Doi et al., 1995; Chanprateep et al., 2010).

A number of studies have researched the effect of block copolymer on the mechanical and rheological properties of PHA. It has been found that block copolymer has better mechanical properties than random copolymer (Laycock et al., 2014). This is because the block copolymers are covalently link polymers which the rate and degree of crystallization are less than the random copolymers (McChalicher et al., 2007). Li et al. (2011) used ethanol fractionation to remove random copolymer and retain the block copolymer in a mixture of materials comprising a blocky copolymeric component containing 70% P(3HB) as one block and the random copolymer P(3HV-co-3HHp) as another block. A comparison was made with homopolymer P(3HB), random copolymer as well as the blend of P(3HB), P(3HV) and P(3HHp). Although the elongation to break of the block copolymer is lower than random copolymers, both the tensile strength and Young's modulus are significant higher than random or blend copolymers (Table 1.3).

1.2.5.6 PHA Additives and Blends

The use of additives is a common approach to improve the PHA properties. Nucleating agents, for example, can accelerate the rate of crystallization and can help limit the secondary crystallization (El-Hadi et al., 2002). The nucleation rate of PHA copolymer is extremely low so the addition of nucleating agents can facilitate processing (Volova, 2004). A large number of nucleating agents have been tried with PHA polymers such as boron nitride, saccharin, lignin, terbium oxide, lanthanum oxide, cyclodextrin, phthalimide and orotic acid (El-Hadi et al., 2002; Kai et al., 2004; Volova, 2004; Laycock et al., 2014). The use of rubbery additives such as hyperbranched polymers, chlorinated polyethylene, polychloroprene and poly(butylacrylate) were found to be effective for improving the toughness of PHAs (Laycock et al., 2014). Plasticizers can also have a dramatic effect on T_g with improvements in ductility and crystallization kinetics at expense of lowering softening points (Choi and Park, 2004). Fillers such as calcium carbonate, china clay and nano-sized fillers have also been studied. It has been reported that the mechanical properties, thermal

properties and biodegradability should be all improved if good integration between filler and polymer matrix performed (Bordes et al., 2009). Since high HV content and most mcl-PHA have low crystallization rates and their tendency to soften and lose their coherence at very low temperature (40^{o}C) make processing difficult, crosslinking such as the addition of peroxide has been reported as one solution (de Koning et al., 1994).

The mechanical properties of PHA-PHA blends have been reported by some studies. Barham and Organ (1994) showed that the blends of P(3HB) with random copolymer P(3HB-co-3HV) (containing 18.4 mol% HV and 19.7 mol% HV, respectively) were prepared using s single screw extruder followed by hot melt pressing. The fresh samples showed higher fracture toughness than controlled copolymer. However, the improvements were found to be lost during the aging process. The blending of PHA with other polymeric materials such as polyethylene oxide, polyvinyl alcohol, polyvinylphenol, polyvinyl acetate, polylactic acid, polyethylene succinate and polypropylene carbonate is an approach for modifying end-use properties (Yang et al., 2012). The use of multilayer and fiber reinforced composites is also a good approach to improve the materials properties while lower the cost (Ha and Cho, 2002).

1.2.6 PHA Applications

1.2.6.1 Applications

Table 1.4 shows commercially produced PHAs and their applications. With diverse properties of PHA, it can be used as bioplastics, fibers, biomedical materials and drug delivery carriers. Injection molded and extruded PHA products cover a wide range of consumer applications, including cutlery, packaging, agriculture mulch films, personal care, office supplies, golf pins, toys and various household wares (Chen, 2009). PHA can also be used as fibers with high tensile strength and can be used in automobile carpets, dental floss and cigarette filters and non-woven applications (Shen et al., 2009). Because of PHA's good performance in terms of biocompatibility and absorbability in human tissue, it can be used in the medical field including tissue engineering, wound healing, cardiovascular, orthopaedics

and drug delivery. PHA suture, artificial esophagus and artificial blood vessels are currently offered as commercial products (Jin, 2007).

Table 1.4 Applications of PHAs (Chen, 2009)

Applications	Examples
Packaging industry	All packaging materials that are used for a short period of time, including food utensils, films, daily consumables, electronic appliances et al.
Printing & photographic industry	PHA are polyesters that can be easily stained.
Other bulk chemicals	Heat sensitive adhesives, latex, smart gels. PHA nonwoven matrices cab be used to remove facial oils.
Block copolymerization	PHA can be changed into PHA diols for block copolymerization with other polymers.
Plastic processing	PHA can be used as processing aids for plastic processing.
Textile industry	Like nylons, PHA can be processed into fibers.
Fine chemical industry	PHA monomers are all chiral R-forms, and can be used as chiral starting materials for the synthesis of antibiotics and other fine chemicals.
Medical implant biomaterials	PHA have biodegradability and biocompatibility and can be developed into medical implant materials. PHA can also be turned into drug controlled release matrices.
Medical	PHA monomers, especially R3HB have therapeutic effects on Alzheimer's and Parkinson's diseases, osteoporosis and even memory improvement.
Healthy food additives	PHA oligomers can be used as food supplements for obtaining ketone bodies.
Industrial microbiology	The PHA synthesis operon can be used as a metabolic regulator or resistance enhancer to improve the performances of industrial microbial strains.
Biofuels or fuel additives	PHA can be hydrolyzed to form hydroxyalkanoate methyl esters that are combustible.
Protein purification	PHA granule binding proteins phasin or PhaP are used to purify recombinant proteins.
Specific drug delivery	Coexpression of PhaP and specific ligands can help achieve specific targeting to diseased tissues.

1.2.6.2 Worldwide PHA Producing Companies

A number of companies have attempted to produce PHAs at an industrial scale since the 1980s and are listed in Table 1.5 (Chen, 2009). Chemie Linz AG Austria has a successful large scale production of PHB. The copolymer PHBV has been commercially produced by ICI, UK (which was bought out by Monsanto) and TianAn, China. The copolymer P(HBHHx) is produced by the joint venture between Tsinghua University, Korea Advanced Institute of Science and Technology and Procter & Gamble. In 2009 Metabolix (USA) and Tianjin Green Bioscience (China) produced each 50,000 and 10,000 tons/year of PHA,

respectively. With growing global PHA production new applications for the use of PHA are expected to rise.

Table 1.5 Worldwide PHA producing companies (Chen, 2009)

Company	Types of PHA	Production scale (t/a)	Period	Applications
ICI, UK	PHBV	300	1980s to 1990s	Packaging
Chemie Linz, Austria	PHB	20–100	1980s	Packaging & drug delivery
btF, Austria	PHB	20–100	1990s	Packaging & drug delivery
Biomers, Germany	PHB	Unknown	1990s to present	Packaging & drug delivery
BASF, Germany	PHB, PHBV	Pilot scale	1980s to 2005	Blending with Ecoflex
Metabolix, USA	Several PHA	Unknown	1980s to present	Packaging
Tepha, USA	Several PHA	PHA medical implants	1990s to present	Medical bio-implants
ADM, USA (with Metabolix)	Several PHA	50	2005 to present	Raw materials
P&G, USA	Several PHA	Contract manufacture	1980s to 2005	Packaging
Monsanto, USA	PHB, PHBV	Plant PHA production	1990s	Raw materials
Meredian, USA	Several PHA	10	2007 to present	Raw materials
Kaneka, Japan (with P&G)	Several PHA	Unknown	1990s to present	Packaging
Mitsubishi, Japan	PHB	10	1990s	Packaging
Biocycles, Brazil	PHB	100	1990s to present	Raw materials
Bio-On, Italy	PHA	10	2008 to present	Raw materials
Zhejiang Tian An, China	PHBV	2000	1990s to present	Raw materials
Jiangmen Biotech Ctr, China	PHBHHx	Unknown	1990s	Raw materials
Yikeman, Shandong, China	PHA	3000	2008 to present	Raw materials
Tianjin Northern Food, China	PHB	Pilot scale	1990s	Raw materials
Shantou Lianyi Biotech, China	Several PHA	Pilot scale	1990s to 2005	Packaging and medical
Jiang Su Nan Tian, China	PHB	Pilot scale	1990s to present	Raw materials
Shenzhen O'Bioer, China	Several PHA	Unknown	2004 to present	Unclear
Tianjin Green Bio-Science	P3HB4HB	10	2004 to present	Raw materials & packaging
Shandong Lukang, China	Several PHA	Pilot scale	2005 to present	Raw materials & medical

1.3 Project Objectives

The overall objective of this project is to advance our understanding on how to utilize wood to generate fermentable sugars then to produce bioplastics, specifically PHB. The following flow chart gives a full view of detailed tasks of this project. Hybrid Poplar, a hardwood species grown regionally as a bioenergy feedstock was used as raw material. Biomass composition analysis was done ahead to determine the chemical components (cellulose, hemicellulose, lignin, extractives, ash) of the raw material. A Response Surface Methodology (RSM) was chosen to determine the optimal hot-water pretreatment condition and the yield of fermentable sugars from enzymatic hydrolysis was used as the response variable in the experimental design. In order to release large quantity of sugars, a scale-up of hot-water pretreatment and enzymatic hydrolysis was conducted. The synthesis of PHB was started at this stage with high yield and nontoxic sugars. A 2^3 factorial design has been done to evaluate the cell growth, PHB yield and substrate consumption of PHB synthesis. Microbial community analysis was conducted on the mixed cultures to give a good examination for the distribution of PHB synthesis related microbes. Pure culture PHB synthesis was conducted under sterile conditions with nutrition modification media by *Burkholderia cepacia* (ATCC 17759) and compared to the mixed culture system. A comparison of polymer properties (chemical structure, thermal, and mechanical properties) was tested between PHB synthesized from mixed and pure culture.

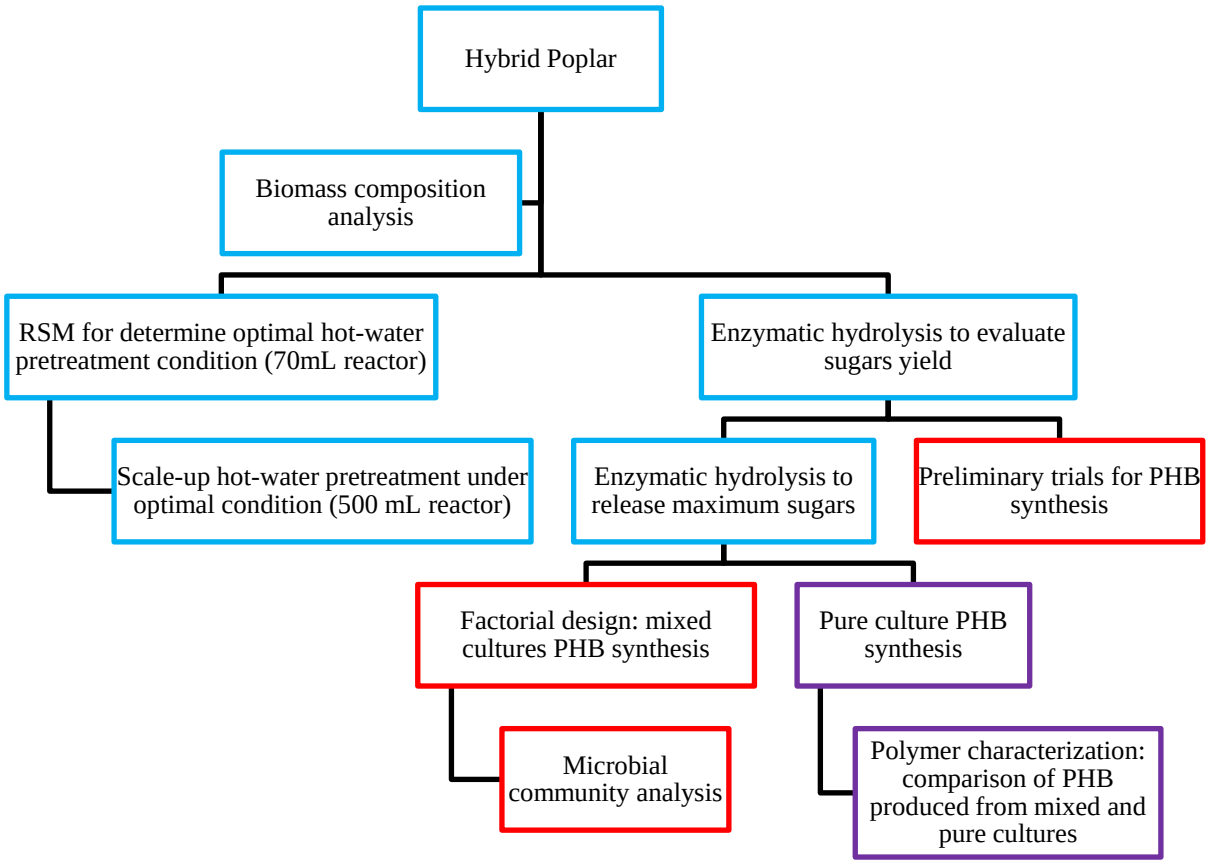


Figure 1.8 Flow diagram of the dissertation project (blue-chapter 2; red-chapter 3; purple-chapter 4)

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Chapter 2 Production of Fermentable Sugars from Hybrid Poplar: Response Surface Optimization of a Hot-Water Pretreatment and Subsequent Enzymatic Hydrolysis

2.1 Abstract

In this study, the target was the generation of fermentable monosaccharides from hardwoods that can be converted into value-added chemicals, such as polyhydroxybutyrates (PHB). Poplar flour was subjected to a hot-water pretreatment. The pretreatment conditions were optimized using a response surface methodology (RSM) on a 2^3 full central composites design by varying temperature, reaction time and solid loading. The optimal pretreatment condition for producing sugars was 200^{o}C, 22 min and 20% solid loading. After pretreatment the solid residue was treated with a commercial cellulase/xylanase preparation and released sugars quantified. The total sugars yield was applied as response variable to the RSM. A maximum yield of 96% sugars was obtained. Potential fermentation inhibitors were also detected at low concentrations (acetic acid, furfural, hydroxymethylfurfural). Sugar degradation during storage and autoclave has also been tested for further utilization.

2.2 Introduction

In the United States Pacific Northwest (PNW), hybrid poplar has been cultivated as an energy crop and gone from the research and developmental stage into a commercial enterprise occupying about 25,000 ha with a biomass growth rate up to 20 x 10^3 kg/ha/y (500,000 tonnes/y) on 6-8 year rotations (Stanton et al., 2002). A significant amount of hybrid poplar saw mill residues is available (for example, the GreenWood Resources mill can process 190,000 m^3/y (Berguson et al. 2010)) and can be converted into biofuels and/or biomaterials and this would benefit the nation's energy security and economy by displacing imported petroleum (Keenan et al., 2006). The technique for conversion of lignocellulosic biomass to fuel ethanol has been well developed. Cellulosic ethanol, however, has economic barriers to overcome to be economically competitive (Nagle et al., 2002; Kim et al., 2009).

Therefore, upgrading cellulosic biomass, after bioconversion, to higher value products such as PHB (polyhydroxybutyrate, a biodegradable polymer produced intracellularly by a wide variety of microbes) would gain better commercial value compared to cellulosic ethanol. The cost of the carbon substrate reportedly contributes more than 40% of the production cost of PHB (Choi and Lee, 1997; Reis et al., 2003; Coats et al., 2007). So by using inexpensive renewable energy crop feed stocks (e.g. hybrid poplar) could be a tremendous advantage to the economics of PHB production (Keenan et al., 2006; Sathesh-Prabu and Murugesan, 2011).

Hybrid poplar is a fast growing hardwood with low lignin (~20%) and high carbohydrate (cellulose plus hemicellulose) contents and has been highlighted as a good biomass resource for chemical production (Hamelinck et al., 2005; Kim et al., 2011). Cellulose is present in poplar (~50%) and is a semi-crystalline polymer comprised of D-glucose units linked by β-*O*-4 linkages (Fengel and Wegener, 1989). The major proportion of cellulose exists in the crystalline form. However, cellulose is more susceptible to degradation in its amorphous form (Kumar et al., 2009). Thus, breaking down cellulose crystalline structure to make it more accessible to cellulase enzymes usually requires pretreatment with heat, long reaction time and addition of catalysts (Clark et al. 1989; Kim et al., 2011). Xylose is the main constituent of hardwood hemicellulose, as acetyl-4-*O*-methylglucuronoxylan (Manju and Chadha, 2011). Recently, studies showed that xylose can be obtained via a pretreatment process using dilute sulfuric acid (Hu et al., 2010; Yamaguchi and Aoyama, 2010).

Extractives and solubilized lignin from poplar may act as fermentation inhibitors, so that a detoxification step such as over-liming, ion exchange and extraction is necessary (Kumar et al., 2009; Vila et al., 2012). A pretreatment process can not only depolymerize lignin, but also remove some lignin (Mok and Antal, 1992; Weil et al., 1997). Enzymatic hydrolysis is the most common method for converting woody biomass to sugars. Compared with acid hydrolysis, enzymatic hydrolysis yields limited amount or even no fermentation inhibitors such as furfural and it does not need neutralization and detoxification (Kim et al., 2009). The only disadvantage of enzymatic hydrolysis is longer reaction time required for releasing

sugars. However, enzymatic hydrolysis is a better choice if further fermentation or bioconversions are required to produce value-added chemicals (Kim et al., 2009).

Hot-water pretreatment with controlled pH has been shown to improve enzymatic digestibility of lignocellulosic biomass (Mosier et al., 2005; Kim et al., 2009; Wang et al., 2012). Acetic acid and other organic acids are released from the hemicelluloses, which help autocatalyze hemicellulose hydrolysis and disrupt cellulose and lignin structure. The pH of the pretreatment liquor needs to be kept between 4 and 7 to minimize decomposition of sugars (Kim et al., 2009). Furfural and 5-hydroxymethylfurfural (5-HMF) are the two major sugar degradation products from hot-water pretreatment. When the concentration of furfural is greater than 2 g/L, it is significantly toxic for fermentation microbes (Kim et al., 2009). The present combination of acetate and sugar degradation products would also give strong inhibition effects in normal fermentation processes (Pan et al., 2012). However, PHB can be biosynthesized from acetate by PHA producing bacteria (Chua et al., 2003; Johnson et al., 2010; You et al., 2011).

Research on polyhydroxyalkanoates (PHA) has been increasing rapidly in recent decades due to the rise of petroleum prices as well as environmental concerns related to plastic pollution. Polyhydroxybutyrate (PHB) is the most studied homopolymer in the PHA family which can be used as an alternative for polypropylene (Lee, 1996). PHB can be produced from waste or renewable resources with the advantage of its biodegradability and biocompatibility. Lee (1996) reviewed that using hemicelluloses hydrolysates for the production of PHB would cost $0.34/kg PHB to yield 0.20 g PHB/ g substrate, which was much cheaper than using pure acetate ($1.56/kg PHB) as a substrate. Several reports are available for the production of PHB using plant hydrolysates and pure microbial strains, including bagasse hydrolysates by *Ralstonia eutropha* (Yu and Stahl, 2008), water hyacinth hydrolysates by *Cupriavidus necator* (Radhika and Murugesan, 2012) and hydrolyzed rice straw by *Bacillus firmus* (Sindhu et al., 2013). To the best of our knowledge, there is no report on using wood hydrolysates and mixed cultures for the production of PHB.

For the purpose of scale-up or industrial production, determining the optimal pretreatment conditions by using statistical approach is important. The experimental design works for variety of species, chemical reagents, temperature and reactor features. The aim of this study was to find optimal conditions to obtain total sugars (mainly glucose and xylose) via a hot-water pretreatment followed by enzymatic hydrolysis. A response surface methodology (RSM) was chosen to determine the optimal pretreatment conditions for total sugars yield in the hydrolysates. Temperature, reaction time and solid loading were the three variables tested in this design. Sugar degradation tests have also been performed to evaluate the utilization of hydrolysates for further PHB biosynthesis.

2.3 Materials and Methods

2.3.1 Raw Materials

Hybrid poplar (Potlatch Corp., ID, USA) was milled to <1 mm using a Wiley mill (Thomas Scientific, NJ, USA), dried and stored in sealed plastic bags (moisture content of 5.4%). Chemical composition analysis (ash, extractives, Klason lignin and neutral carbohydrate composition) of raw and residual materials was determined using procedures described by Jain et al., 2010.

2.3.2 Hot-Water Pretreatment

Pretreatment was conducted in a 75 mL pressure reactor, (Figure 2.1, Model 4740, Parr Instrument Co., IL, USA) connected to a temperature controlled block heater built in-house. Wood meal (5.0 g, oven dry basis) was introduced to the reactor to which water was added giving a solid loading range from 13.2 to 46.8% and sealed. The reaction temperature ranged from 160 to 210^{o}C. A 2^3 full factorial design for temperature, time and solid loading was conducted (Table 2.1). An additional temperature probe was used for monitoring the temperature of the reactor vessel. The reaction vessel took 5, 10 and 15 min to reach 170, 185 and 200^{o}C, respectively. Timing was started when the vessel reached the desired temperature. After pretreatment, the vessel was placed in an ice-water bath to quench the

reaction. The pretreated samples then were washed with hot-water (Milli-Q, 150 mL, 90^{o}C) (Nagle et al., 2002; Kim et al., 2009) to extract out the sugars and acids generated, centrifuged (10,000 rpm) to separate the solid and liquid fractions. The liquid fraction was named pre-liquor (PL) and pH was measured. After sampling 1 mL from the PL for inhibitor compounds and 5 mL for sugars determination, the PL together with the solid residue collected were used for enzymatic hydrolysis trials.

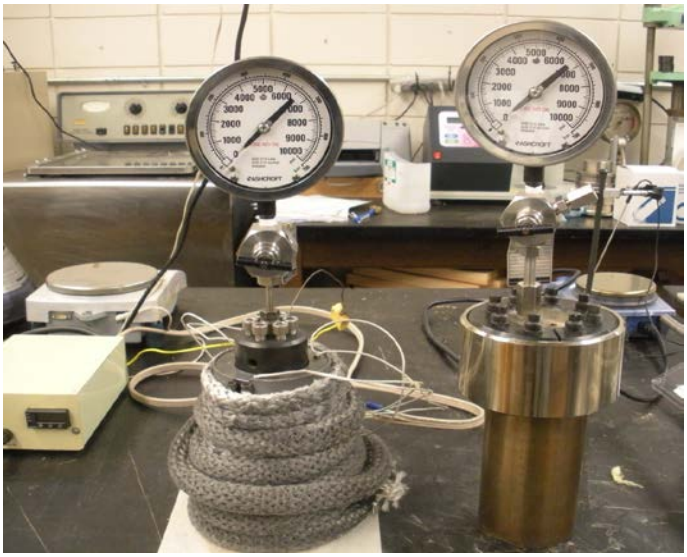


Figure 2.1 High pressure pretreatment reactors: 75 mL (left) and 500 mL (right)

A 500 mL pressure reactor (Figure 2.1, Model 4740, Parr Instrument Co., IL, USA) was used for wood pretreatment under optimal condition determined by the experimental design. Wood (50 g) was added into the reactor to produce larger quantities of pretreated solids to generate sufficient amounts of sugars for further PHB biosynthesis.

Table 2.1 Pretreatment 2^3 factorial experimental design varying on reaction temperature, time, and solid loading

	Variables			Coded levels		
Experiment No.	Temp. (^{o}C)	Time (min)	Solid Loading (%)	Temp. (^{o}C)	Time (min)	Solid Loading (%)
	X1	X2	X3	X1	X2	X3
1	170	10	20	-1	-1	-1
2	200	10	20	1	-1	-1
3	170	30	20	-1	1	-1
4	200	30	20	1	1	-1
5	170	10	40	-1	-1	1
6	200	10	40	1	-1	1
7	170	30	40	-1	1	1
8	200	30	40	1	1	1
9	160	20	30	-1.68	0	0
10	210	20	30	1.68	0	0
11	185	3.2	30	0	-1.68	0
12	185	36.8	30	0	1.68	0
13	185	20	13.2	0	0	-1.68
14	185	20	46.8	0	0	1.68
15	185	20	30	0	0	0
16	185	20	30	0	0	0
17	185	20	30	0	0	0
18	185	20	30	0	0	0
19	185	20	30	0	0	0
20	185	20	30	0	0	0

2.3.3 Enzymatic Hydrolysis

Enzymatic saccharification was done on the mixture of pretreated solid and PL following the LAP method 009 (Brown and Torget, 1996). The hydrolysis was conducted in either 250 (small pretreatment: 5 g wood) or 2000 mL (large pretreatment: 50 g wood) Erlenmeyer flasks in an oil bath for 3 days at 50^{o}C, and with magnetic stirring (200 rpm). The enzyme loading was based on solution weight % (100 x g enzyme solution/g wood) for experimental design samples was 5% (0.2 mL Novozyme HTec2, 1.209 g/mL, Novozymes North America Inc, NC, USA). Samples were taken every 24 h to determine sugar content by High Performance Liquid Chromatography (HPLC).

2.3.4 Experimental Design

A RSM was used to determine the optimal pretreatment condition for producing maximum total reducing sugars. The method has been described in several studies (Perez et al., 2008; Kim et al., 2009; Lee et al., 2009; Dai and McDonald, 2013). The design was based on a 2^3 full factorial central composite design (CCD) and was conducted using Design Expert v8.0 software (Stat-Ease, Inc. MN, USA). The experimental conditions with corresponding codes are listed in Table 2.1. The three variables were temperature, reaction time and solid loading with 6 repeated experiments in the central point (185^{o}C, 20 min, 30% loading). All the three variables were coded to real independent variables. The independent variables were calculated as (condition of the run-condition at central point) / step change of the variable. Therefore, the coded value were X1 (temp-185)/15, X2 (time-20)/10, and X3 (solid-30)/10.

2.3.5 Analytical Methods

Sugars were quantified by HPLC using two Rezex RPM columns in series (7.8 mm x 30 cm, Phenomenex, Torrance, CA) and a Waters HPLC (Waters, Milford, MA) equipped with differential refractive index detector (ERC-5710, ERMA), on elution with water (0.5 mL/min) at 85^{o}C. Aliquot portions of hydrolysates (6 mL) were centrifuged and the supernatant (5 mL) was transferred to a test tube containing inositol as an internal standard (1 mL, 0.5 mg/mL), mixed, deionized (column containing Amberlite IR-120 H^+ (0.5 mL) and Amberlite IRA35 OH^- (0.5 mL) resins) and filtered (0.45 µm).

Acetic acid, furfural and 5-hydroxymethylfurfural (5-HMF) were quantified by HPLC using a Rezex ROA organic acid column (7.8 mm x 30 cm, Phenomenex, Torrance, CA) and a Waters HPLC (Waters, Milford, MA) equipped with differential refractive index detector (ERC-5710, ERMA), on elution with 0.005 N aqueous H_2SO_4 (0.5 mL/min) at 65^{o}C. An aliquot portion of hydrolysate (1 mL) was taken and filtered (0.45 µm) into an HPLC vial.

The total reducing sugar yield (%) for each sample was calculated as following equation (1). Since the maximum sugar yield was detected after 3 days hydrolysis, the third day total sugar yield was used in the response surface optimization analysis.

$$(1)\ \text{Total sugar yield (\%)} = \frac{\text{sum of sugars concentrations } \left(\frac{\text{mg}}{\text{mL}}\right) \times \text{H2O volume 150 (mL)} \times 100\%}{\text{wood dry weight 5000 (mg)}}$$

Lignin syringyl/guaiacyl (S/G) ratio was determined by FTIR spectroscopy (Avatar 380, ThermoNicolet) using a ZnSe ATR probe. The spectrum was baseline adjusted and ATR corrected using the Omnic v7.0 software. The S/G ratio was calculated based on absorption intensities of bands at 1460 to 1512 cm^{-1} and 1221 to 1270 cm^{-1} (Chen and McClure, 2000).

2.3.6 Sugar Degradation Test

Enzymatic hydrolysate was diluted into various concentrations (5, 10, 15, 20, 30 g/L), and kept in Nalgene centrifuge bottles (300 mL). At each individual concentration, the pH of the hydrolysates were adjusted to either 3.8, 4.8, or 6.0 and stored in the fridge. Sugar concentrations after enzymatic hydrolysis and after 5 days storage were analyzed by HPLC to observe any sugar degradation occur during storage. In order to develop sterilized sugar feedstock for pure culture PHB synthesis, the hydrolysate was autoclaved at 121^{o}C for 20 min. The initial (5, 10, 15, 20, 30 g/L), after autoclaved and 5 d storage sugar concentrations were also analyzed by HPLC.

2.4 Results and Discussion

2.4.1 Analysis of Components in Pretreated Liquor (PL) and Enzymatic Hydrolysates

Chemical composition analysis of the hybrid poplar was comprised of 49% glucan, 21% xylan, 1.5% galactan, 1.0% arabinan, 2.5% mannan, 22% Klason lignin, 2% extractives and 0.8% ash. Table 2.2 shows all component levels for each experiment in this study. In both PL and enzymatic hydrolysates five neutral reducing sugars (glucose, xylose, galactose,

arabinose and mannose) were measured by HPLC. Acetic acid, furfural and 5-HMF from PL were determined to evaluate pretreatment efficiency and fermentation inhibition. The pH of PL is shown in Table 2.2, which ranged between 3.67 and 4.83 depending on the pretreatment condition. There was no or little neutralization needed for subsequent enzymatic hydrolysis at this pH range. Therefore, the pH was not adjusted for all enzymatic hydrolysis in the DOE. Also, without pH adjustment sugar yield from enzymatic hydrolysis can be directly used by RSM which would give a better evaluation on the pretreatment efficiency compared to pH adjusted solid fraction. During the pretreatment, acetyl groups from poplar were released as acetic acid thus lowering the pH of the solids which can act as acid catalyst for hydrolyzing the hemicelluloses (Fengel and Wegener, 1989; Weil et al., 1997; Kim et al., 2009). Acetic acid concentrations in all PL were less than 1.4 g/L (Table 2.2) and below the level of 5 g/L at which it could act as an inhibitor for fermentation (Delgenes et al., 1996; Takahashi et al., 1999; Hu et al., 2010; Kim et al., 2011). The highest acetic acid level was observed at a pretreatment temperature of 200^{o}C for 30 min. This suggests that pretreatment temperature and reaction time were important factors. Compared with an acid pretreatment, a hot-water pretreatment generates much less acetic acid during the process (Kim et al., 2009). Moreover, a hot-water wash step can help reduce acetic acid and other inhibitors generated from pretreatment (Nagle et al., 2002; Kim et al., 2009).

The pH in all experiments was around 4 together with low acetic acid concentrations observed would result in limited sugar degradation. The concentrations of furfural and 5-HMF are given in Table 2.2. At all 20 conditions, 5-HMF was either not detected at all or present at less than 0.02 g/L. Furfural was found in some severe pretreatment conditions at less than 0.08 g/L. Considering the extremely low inhibitor levels, both the PL and solid fraction were used for enzymatic hydrolysis and the hydrolysates was further used as feedstock for PHB production. By doing this, the solubilized xylo-oligosaccharides in the PL can be further hydrolyzed to monosaccharides by a hemicellulase enzyme. Thus, an increasing total sugars yield would be expected after enzymatic hydrolysis and raw materials can also be properly utilized.

In the PL, xylose was the main sugar while low levels of other sugars could be detected. However, glucose was the major sugar released followed by xylose after enzymatic hydrolysis. Experiment # 2 (200^{o}C, 10 min, 20% solid loading) gave the highest total sugar yield (73%). While experiment #11 (185^{o}C, 3.2 min, 30% solid loading) gave the lowest sugar yield (41%). This result indicated that experiment #11 was not a severe pretreatment condition due to short reaction time (3.2 min). Since the concentrations of all potential inhibitors (acetic acid, furfural, 5-HMF) were very low and therefore can be neglected. This study was targeting to use the total sugar yield as a response variable by RSM which was also the major feedstock for further bioconversion.

Table 2.2 Analysis of components of pretreated liquor and enzymatic hydrolysates

	Pre-liquor					Enzymatic hydrolysates			
Stds runs	Total sugars[a] (%)	pH	Acetic acid (g/L)	Furfural (g/L)	5-HMF (g/L)	Glucose (g/L)	Xylose (g/L)	Other sugars[b] (g/L)	Total sugars[a] (%)
1	0.67	4.54	0.27	0	0	10.28	4.08	0.08	46.32
2	8.43	3.97	0.72	0.0016	0	14.5	6.6	3.1	72.58
3	3.75	4.42	0.52	0	0	10.95	5.2	2.23	55.13
4	6.28	3.71	1.37	0.0828	0.0223	14.38	6.31	2.9	70.76
5	1.35	4.8	0.1	0	0	9.89	4.06	0.69	43.91
6	5.16	3.87	0.61	0	0	14.29	6.32	2.61	69.65
7	0.84	4.26	0.1	0	0	10.63	5.16	2.05	53.52
8	4.6	3.67	1.03	0.0691	0	13.05	5.96	2.52	64.58
9	0.9	4.83	0.02	0	0	10.01	4.12	0.74	44.62
10	1.59	3.74	0.81	0.003	0.0023	13.15	5.77	1.89	62.44
11	1.29	4.48	0	0	0	9.15	3.52	1.07	41.23
12	1.26	4.19	0.58	0	0	13.11	5.83	1.48	61.25
13	5.13	3.82	0.34	0	0	14.46	6.26	2.88	70.81
14	0.97	3.94	0.07	0	0	13.02	5.58	1.61	55.67
15	2.66	3.9	0.47	0	0	13.17	6.12	2.48	65.32
16	3.02	3.9	0.48	0	0	13.08	6.06	2.31	64.34
17	1.89	4.03	0.51	0	0	13.11	6.03	2.41	64.64
18	2.13	3.94	0.53	0	0	13.02	5.85	2.22	63.28
19	2.25	4.02	0.54	0	0	12.89	5.79	2.29	62.9
20	3.12	4.05	0.55	0	0	12.76	5.92	2.04	62.17

Total sugars[a]: Sum of glucose, xylose, galactose, arabinose and mannose to the dry wood.

Other sugars[b]: Sum of galactose, arabinose and mannose.

2.4.2 Response Surface Methodology (RSM) for Producing Fermentable Sugars

Before determining the optimization pretreatment condition, a RSM model was conducted using 20 experiments (Table 2.1) and Table 2.2 (total sugar yield, %). Figure 2.2A is a three-dimensional (3D) plot that modeled the pretreatment conditions for total sugar yield in a curved surface with the optimal sugar yield at fixed variable (solid loading, 30%). This is a direct view of the data generated from this experimental design. Since 30% solid loading was the central point in the design, it was selected to be the fixed variable and the other two more significant variables (based on ANOVA results) were displayed in the response surface. From Figure 2.2A, the highest surface occurred at 200^{o}C and a reaction time around 20 min.

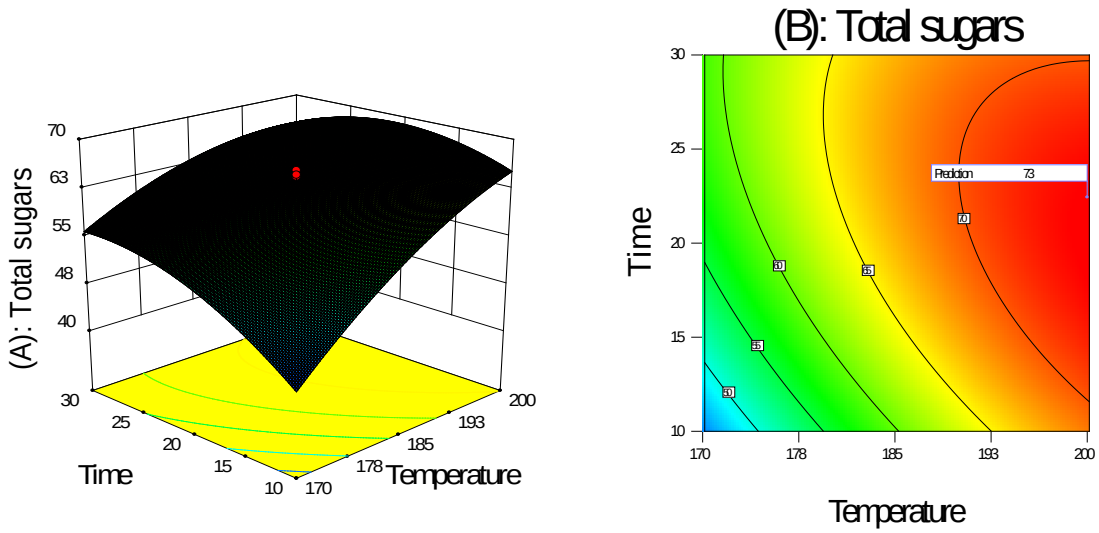


Figure 2.2 (A): Three-dimensional plot of RSM for total sugars yield based on 2^3 CCD (solid loading was set to 30%); (B): Contour plot of RSM for optimization of total sugars yield (the maximum predicted response was 73% of total sugars yield at 200^{o}C, 22 min, 20% solid loading)

The modeling result is shown in equation (2):

(2) $Y = a0 + a1 \times A + a2 \times B + a3 \times C + a1^2 \times A^2 + a2^2 \times B^2$

Table 2.3 shows the ANOVA results and corresponding coefficients which established the model in Equation (2). Temperature (A) was shown to be the most significant variable, followed by time (B) in the 2nd order and solid loading (C) was significant. The model also included the second order term of temperature and time based on their significance (α=0.05). The R-square of the model was 0.86, which was acceptable to give a decent prediction on total sugar yield with appropriate pretreatment condition.

The optimization of total sugar yield was conducted based on the model generated in equation (2). The 2D contour plot (Figure 2.2B) gave the optimization result based on the quadratic model. The maximum predicted response was 73% (dry wood basis) of total sugar yield (pretreated at 200^{o}C, 22 min, 20% solid loading). Kim et al. (2009) have reported that optimal liquid hot water pretreatment condition on hybrid poplar was 200^{o}C, 10 min, 15% slurry which gave 67% (dry wood basis) total monomeric sugar yield (glucose and xylose). Yu et al. (2010) have reported a two-step (first step: 180^{o}C, 20 min; second step: 200^{o}C, 20 min) liquid hot water pretreatment of *Eucalyptus grandis* which yielded 96.6% (carbohydrates basis) total sugars. Compared to their results, this study was advanced in several aspects, including high sugar recovery, shorter reaction time, higher solids loading, low inhibitors concentrations, and no buffering chemicals introduced to the enzymatic hydrolysates.

Table 2.3 Analysis of variance for total sugars yield (quadratic model)

Source	Sum of squares	DF	Mean square	F value	Prob > F	Coefficient	
Model	1498.16	9	166.462	7.1088	0.0025	a0	63.64
A-Temperature	864.54	1	864.54	36.9204	0.0001	a1	7.96
B-Time	149.595	1	149.595	6.3885	0.03	a2	3.31
C-Solid Loading	118.769	1	118.769	5.072	0.048	a3	-2.95
AB	80.0745	1	80.0745	3.4196	0.0942	a12	-3.16
AC	3.2385	1	3.2385	0.1383	0.7177	a13	-0.64
BC	0.7503	1	0.7503	0.032	0.8615	a23	-0.31
A2	107.848	1	107.848	4.6057	0.0574	a12	-2.74
B2	181.133	1	181.133	7.7353	0.0194	a22	-3.55
C2	3.906	1	3.906	0.1668	0.6916	a32	0.52
Residual	234.164	10	23.4164				
Lack of Fit	227.122	5	45.4245				
Pure Error	7.0412	5	1.4082				
Corrected total	1732.33	19					

R^2=0.86, adjusted R^2=0.74.

2.4.3 Analysis of Biomass Composition on Optimal Condition

Repeated experiments on the optimal pretreatment condition (200^{o}C, 22 min, 20%) were done to support the model. The average total sugar yield after 3 days enzymatic hydrolysis was 72% which almost achieved the theoretical maximum yield (75%). So this model was confirmed to be reliable for determining the pretreatment condition. Figure 2.3A shows the composition analysis of pretreated and enzymatic hydrolysis samples. The total carbohydrate content was 67.85% after pretreatment, which means only 7% carbohydrates was hydrolyzed into monosaccharides (mainly xylose). Lignin was also removed after pretreatment and content decreased from 22 to 18.3%. This observation was higher than those reported by Yu et al. (2010) for *E. grandis* lignin content from 26.2 to 17.6% after a two-step liquid hot water pretreatment. Lignin removal during pretreatment can help disrupt wood structure and make it more accessible to enzymes. However, lignin removal is highly dependent on wood species, lignin syringyl/guaiacyl (S/G) ratio and pretreatment conditions (temperature, time) (Santos et al., 2012). The reactivity of lignin is directly related to the S/G ratio and the higher the S/G, the less condensed of lignin that would be easier to be

removed during pretreatment (Santos et al., 2012). The lignin S/G ratio of 1.2 for hybrid poplar residual lignin was determined by FTIR, which is lower than other fast growing poplar species (S/G: 1.8-2.0) (Davison et al., 2006; Meng et al., 2012). Also, severe conditions such as higher temperature and longer reaction time would remove more lignin after pretreatment (Yu et al., 2010; Santos et al., 2012). Therefore, the pretreatment condition (200^{o}C, 22 min, 20%) developed in this study can be considered a mild process which was expected to solubilize less lignin and generate less fermentation inhibitors. There were 7.65% total carbohydrates remaining after 3 days enzymatic hydrolysis (Figure 2.3A) which was 3% more than the model predicted (4%) due to error of model prediction. This was still acceptable considering the high 93% total sugars yield. The hydrolysate residual solids contained about 16% Klason lignin which was reduced from pretreated solid since enzymatic hydrolysis would also remove a portion of lignin (Obama et al., 2012).

Additional experiments were performed under the optimal conditions using a larger 500 mL reactor to generate sufficient quantities of fermentable sugars. Biomass composition analysis for the large scale pretreatment experiment is shown in Figure 2.3B. Both pretreated and enzymatic residual solids gave similar results compared with the small scale pretreatment. More carbohydrates and lignin were removed upon pretreatment because a slightly longer reaction time was required for heating and cooling the larger reactor. However, acetic acid was detected at a higher level (1.5 g/L) than using the small reactor (0.8 g/L), which was not considered an inhibitor for fermentation at <5 g/L (Delgenes et al., 1996; Takahashi et al., 1999; Hu et al., 2010; Kim et al., 2011).

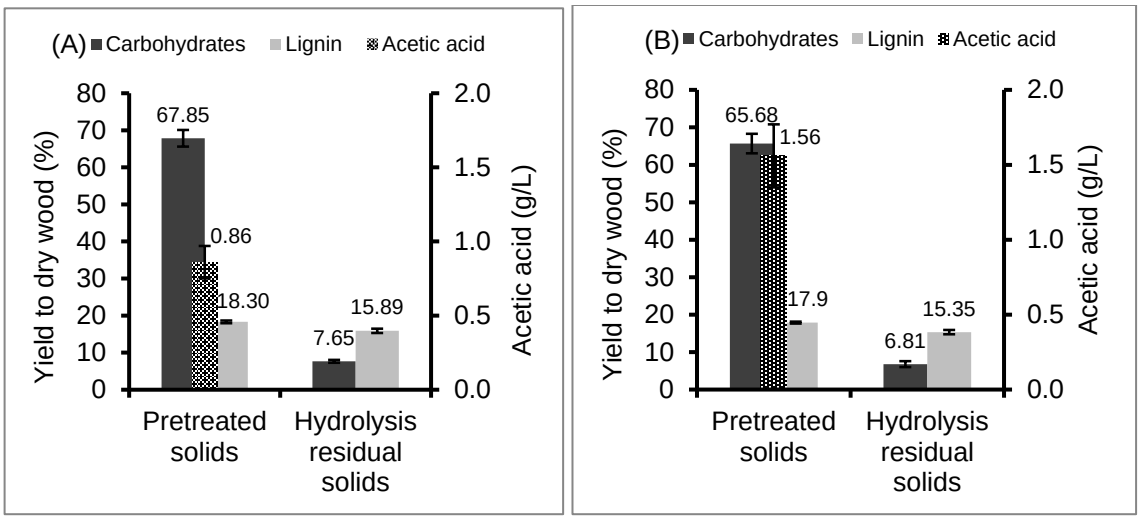


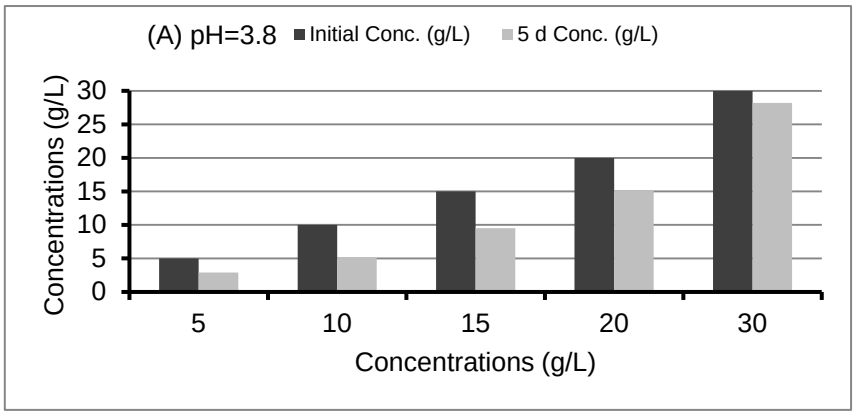
Figure 2.3 Biomass composition analysis for pretreated and residual solids under optimal pretreatment condition (A) experimental scale reactor (75 mL) (B) large scale reactor (500 mL)

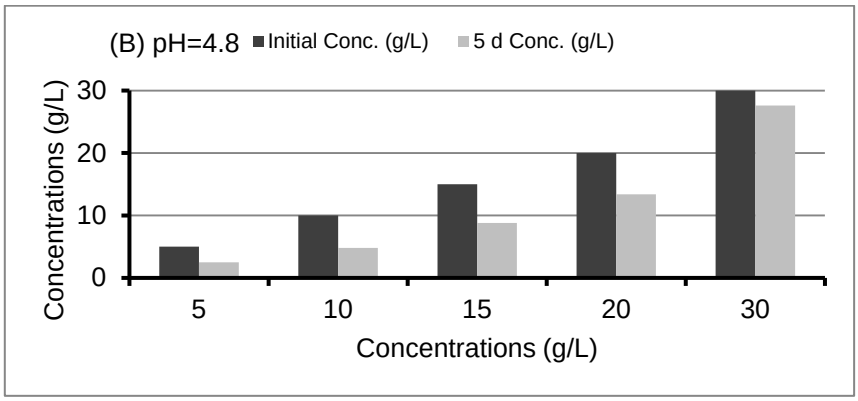
2.4.4 Sugar Degradation Test

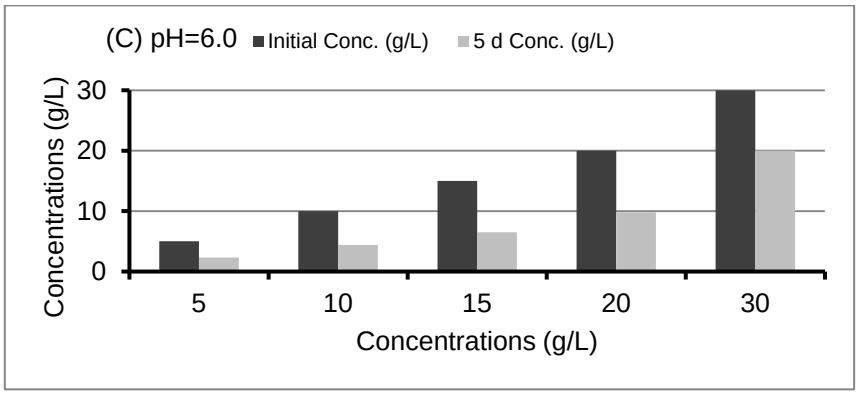
Sugar degradation was evaluated based on the comparison of the concentrations of initial and stored (5d) sugars after enzymatic hydrolysis and the results are shown in Figure 2.4. At pH 3.8 (Figure 2.4A), the concentrations of 5d stored sugars decreased by about a half of the initial concentrations at a high dilution level (5, 10 g/L), while it dropped to less than 2 g/L at a high initial concentration (30 g/L, undiluted). A similar sugar degradation trend was observed at pH 4.8 (Figure 2.4B). However, at near neutral pH (6.0), the stored sugars degraded to about 2/3rd of the initial concentration at 30 g/L and even more degradation was observed upon dilution (5, 10, 15, 20 g/L). Therefore, the hydrolysate was relatively stable at low pH and high initial concentration for storage. Similar results have been found by Kjellstrand et al. (2004) in that the best glucose storage condition after heat sterilization was at 20^{o}C and pH 3.2. Furthermore, significant sugar degradation was detected at a pH over 5.0.

In order to develop feedstock for pure culture PHB synthesis, autoclave was also performed on the wood hydyolysates to detect any sugar degradation. The results are shown in Figure

2.4D. About 2 g/L total sugar degradation occurred after autoclaving at different initial concentrations (5, 10, 15, 20, 30 g/L) and were not able to be stabilized after 5d storage. Therefore, sterilization at 121^{o}C (autoclave condition) should be avoided to minimize thermal degradation of sugars. Instead of autoclaving, heat sterilization at lower temperature (70-80 ^{o}C) is preferred for wood hydrolysates to avoid sugar degradation during both the heating and storage processes (Teresa Cesario et al., 2014).







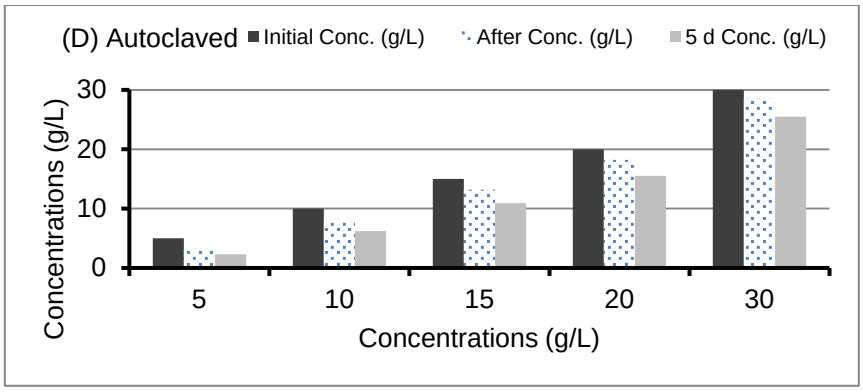


Figure 2.4 Sugar degradation tests under different conditions: (A) pH adjusted to 3.8; (B) pH adjusted to 4.8; (C) pH adjusted to 6.0 before storage for 5 days; and (D) autoclaved hydrolysate

2.5 Conclusions

A hot-water pretreatment on hybrid poplar was successfully achieved to produce a substrate that can be hydrolyzed using a cellulase preparation. The optimized pretreatment condition (temperature 200^{o}C, time 22 min, solid loading 20%) was determined using a RSM. The final total sugars yield of 72% was achieved with low levels of fermentation inhibitors. Large amount of fermentable sugars was produced for further subsequent bioconversion into bioproducts. Sugar degradation tests showed that the enzymatic hydrolysates need to be kept at a high initial concentration and low pH.

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Chapter 3 Polyhydroxybutyrate Biosynthesis from Hybrid Poplar Hydrolysates by Mixed Microbial Cultures

3.1 Abstract

The aim of this study was to investigate the potential of using wood hydrolysates (enzymatically hydrolyzed from hybrid poplar) as substrate to produce polyhydroxybutyrate (PHB) by mixed microbial cultures (MMCs). The optimal operational conditions for fed-batch bioreactors were 4 d solid retention time with an organic loading rate of 2.5 g/Ld. The maximum PHB accumulated was 27% of cell dry weight (CDW) with a yield of 0.32 g/g (g PHB produced per g sugars consumed). Microbial community analysis was done at the genus level by 16S rRNA sequencing and community evolution was observed among different samples and initial seed. *Actinobacteria*, *Alpha-* and *Beta-proteobacteria* were found to be the dominant groups in all the bioreactors. Several PHB-storing microorganisms were characterized belonging to *Alpha-* and *Betaproteobacteria*.

3.2 Introduction

Petroleum-based plastics have been widely used in modern life-style, including food packaging, communication, transportation, clothes, shelter and health care (Chen, 2009). Due to their large amount of consumption, plastics present a serious disposal problem in the landfills causing by their very slow rate of degradation. Therefore, bio-based and biodegradable plastics are gained a lot of interest and being developed in order to be suitable alternatives for petroleum-based plastics. Polyhydroxyalkanoates (PHAs) are biodegradable and biocompatible thermoplastics that can be synthesized by various microbial strains under unbalanced growth conditions such as the presence of excess carbon source and limitation of at least one essential nutrient (e.g. phosphorous, nitrogen, magnesium or oxygen) (Anderson and Dawes, 1990; Kim et al., 1994; Chen, 2010). These polymeric chains are stored in the cytoplasm as granules and function as carbon and energy storage materials (Findlay and White, 1983).

Most of carbon sources used for PHA production are noble sources, such as pure carbohydrates (glucose, sucrose), alkanes and fatty acids. To reduce the production cost, inexpensive carbon sources such as industrial by-products including crude glycerol (Dobroth et al., 2011; Naranjo et al., 2013), cheese whey (Pais et al., 2009) or agricultural residues like sugar cane bagasse (Silva et al., 2004; Yu and Stahl, 2008), sawdust or forest biomass (Keenan et al., 2006) have been used as substrates. This approach has the advantage of converting waste materials into value-added bioproducts. The production of PHA must be a sustainable process and should rely on using fewer raw materials and not compete with food based substrates (Queiros et al., 2014). Therefore, the use of lignocellulosic biomass would be a more suitable substrate and possibly cost effective for PHA production (Keenan et al., 2006).

Lignocellulosic materials mainly consist of cellulose, hemicellulose and lignin. Cellulose and hemicellulose are excellent carbon source to be used in different biological processes after hydrolysis to fermentable sugars. Cellulose is a highly crystalline polymer of β-D-glucopyranose units. Hardwood hemicellulose is a branched polysaccharide that consists of pentoses, mainly xylose and 4-*O*-methylglucuronic acid (Fengel and Wegener, 1984). Cellulose and hemicellulose are embedded in a complex lignin matrix which acts as a binder, impermeable and resistant to microbial attack. Therefore, a pretreatment process is needed to break down the crystalline structure and make the biomass more accessible to subsequence enzymatic hydrolysis. Hybrid poplar is a short rotation fast growing hardwood species with low lignin content and has been used as a good raw material for biofuel and other chemicals production (Hamelinck et al., 2005; Kim et al., 2011). A mild hot-water pretreatment with controlled pH has been shown to improve enzymatic digestibility (Mosier et al., 2005; Kim et al., 2009) which was used in this work to produce fermentable sugars for PHB production.

Most of the technologies for microbial PHA production are not economically competitive with synthetic plastics production. The use of mixed microbial cultures (MMCs) has been gained a lot of interests to produce PHA with low costs due to lower sterility needs and lower equipment controls (Queiros et al., 2014). Moreover, MMCs can utilize a wide range

of inexpensive substrates, including industrial and urban waste streams (Coats et al., 2011) and agro-forestry waste (Albuquerque et al., 2010). Culture selection with a high PHA storage capacity is one of the challenges in PHA production process using mixed cultures. An aerobic dynamic feeding (ADF) also called feast and famine (FF) process is designed to use mixed cultures for PHA production. It works under conditions in which growth is restricted by either an external nutrient or an internal factor. During this process, substrate uptake is mainly driven towards PHA accumulation. The absence of the external substrate for a considerable period of time causes a decrease in the amount of enzymes required for cell growth. Following such a starvation period, if the microbial culture is enriched with an excess of carbon source, the enzymes available in the cells is lower than that needed to reach maximum growth rate, thus the storage becomes the dominant phenomena (Salehizadeh and van Loosdrecht, 2004). Most studies have reported PHA production using MMCs in two separate stages, culture selection and PHA accumulation. Many bacteria produce PHA under certain nutrient depleted conditions (Anderson and Dawes, 1990). Therefore, it is necessary to study the appropriate substrate load, nitrogen and phosphorous concentrations and oxygen limitation of the bioreactors to understand what mechanism driven PHA production.

PHA-storing populations should be evaluated by analyzing the microbial community in order to follow its evolution, identify the producer and determine their relative abundance (Queiros et al., 2014). Microbial characterization needs to be done with different operational conditions allowing for a better understanding of changes in the performance of MMCs that could vary with the same substrate feeding. Several studies have identified the PHA-storing microorganisms. In these reports, most found organisms belong to *Alphaproteobacteria*, *Betaproteobacteria* and *Gammaproteobacteria* classes and some of them were already identified at the genus level. According to these reports, the differences observed in the relative abundance of each genus could be explained based on the type of volatile fatty acids (VFAs) used (Lemos et al., 2008; Jiang et al., 2011). The genera *Amaricoccus*, *Thauera* and *Azoarcus* were found to be dominant in the bioreactors where acetic acid and propionic acid were used as substrate (Lemos et al., 2008). *Plasticicumulans acidivorans* and *Thauera selenatis* were observed in a selected MMCs system fed lactic acid (Jiang et al., 2011). Another work showed a phylogenetic profile of *Firmicutes* (71%) and *Proteobacteria* (28%)

in a PHA accumulation and waste treatment reactor fed municipal waste water (Reddy and Mohan, 2012).

To the author's knowledge, no studies have been found using both MMCs and wood hydrolysates for PHB production. The major objective for this study is to successfully synthesis PHB from wood hydrolysates as a carbon substrate by MMCs. A proposed PHB synthetic mechanism was explained through the optimization of production yield with varied operational conditions (such as initial substrate load and solid retention time (SRT)) achieved by a factorial design. The microbial community was identified at genus level to determine possible PHB-accumulating bacteria.

3.3 Materials and Methods

3.3.1 Culture Preparation

MMC (activated sludge) was seeded from waste water treatment plant (Moscow, ID, USA) and cultured for 20 h with aeration at room temperature in nutrient medium. The composition of the medium used for culture cultivation was (g/L): 2 g KH_2PO_4, 0.6 g Na_2HPO_4, 1 g $MgSO_4$, 0.1 g $CaCl_2$, 0.1 g $CaCO_3$, 1 g NH_4Cl, 2.5 g yeast extract, 5 g Bacto peptone, 10 g hybrid poplar enzymatic hydrolysate (30 g/L × 0.333 L), and 0.667 L Milli-Q water. Medium pH was adjusted to 7.0 before 1 L mixed seed was added to it. The initial mixed liquor suspended solids (MLSS) was 2.65 g/L, which was increased to 9.10 g/L after 20 h cultivation.

3.3.2 Carbon Source

The hybrid poplar hydrolysates were prepared from a hot-water pretreatment followed by enzymatic hydrolysis. Detailed methods can be found in section 2.3.2-3. The initial total sugar concentration after enzymatic hydrolysis was 30 g/L, pH 4.8 (with 2 g/L variation per batch of pretreatment). The hydrolysates and lignin mixture were heated for stabilization in a hot water bath at 80^{o}C for 30 min, then cooled down to room temperature to separate

liquid and solid fraction (lignin) by filtration using glass fiber filter paper (FisherBrand G6). The filtered hydrolysates were stored in plastic bottles and stored in a fridge.

3.3.3 Optimization of PHB Production

Continuous fed-batch bioreactors were conducted in 1 L glass beakers with working volume (WV) of 0.8 L for PHB production based on a factorial design (Table 3.1). The bioreactors were set up on a magnetic stirrer (300 rpm) at room temperature (~ 25^{o}C) (Figure 3.1). Solid retention time (SRT) and sugar content in the initial feed were two designed parameters and the organic loading rate (OLR) was calculated for each batch based on the SRT, sugars content and working volume. The initial inoculation to each bioreactor was 2.8 g/L of cultivated culture. All reactors were cycled every 24 h with initial pH adjusted to 7.0 using 2 M NaOH and full aeration (0.5-6 mg/L changed during reaction period). NH_4Cl was used as the N source which was fed at 0.4 g/Ld. Phosphorus (KH_2PO_4) was fed at 0.25 g/L every SRT cycle. Foaming was controlled with an infrequent addition of Antifoam A concentrate (Sigma Aldrich, St. Louis, MO, USA). Sampling was conducted after 3 SRT's to make sure the bioreactors had stabilized. Data analysis used SAS v9.3.

Table 3.1 Experimental design for optimization of PHB production

ID	SRT (d)	Sugars (g/L)	OLR (g/Ld)	WV (L)	Cycle (h)
A	4	10	2.5	0.8	24
B	6	10	1.7	0.8	24
C	8	10	1.3	0.8	24
D	4	20	5	0.8	24
E	6	20	3.3	0.8	24
F	8	20	2.5	0.8	24
G	4	30	7.5	0.8	24
H	6	30	5	0.8	24
I	8	30	3.8	0.8	24
J	2	10	5	0.8	24
K	2	20	10	0.8	24
L	2	30	15	0.8	24

SRT: solid retention time (d)

Sugars (S): total sugar concentration in the hydrolysates (g/L)

OLR: organic loading rate (g/Ld)

WV: working volume (L)

Figure 3.1 Continues fed-batch bioreactors set-up

3.3.4 Analytical Methods

3.3.4.1 pH, Dissolved Oxygen and Optical Density

pH and dissolved oxygen (DO) were measured in the bioreactors using an RDO optical dissolved oxygen meter (Thermo Scientific 4 star) with a RDO optical sensor and a low maintenance gel-filled pH electrode. Optical density (OD) was measured immediately after the sample was taken at 665 nm versus a water blank on a UV/VIS spectrophotometer (Beckman DU series 640).

3.3.4.2 Total Suspended Solids

Mixed biomass and liquor samples were separated into two factions (biomass and substrates) by centrifugation at 3500 rpm for 10 min. The biomass (pellet) fraction was lyophilized and the total suspended solid (TSS) was determined gravimetrically.

3.3.4.3 PHB Content

PHB content was determined according to the methods described by Dobroth et al. (2011) and Braunegg et al. (1978). Biomass was methanolyzed at 100°C in 2% H_2SO_4 in methanol (2 mL) and chloroform (2 mL containing 0.25 mg/mL benzoic acid internal standard) for 4 h. The methyl-ester derivatives were recovered and analyzed by GC-MS on a PolarisQ iontrap (Thermofinnigan) instrument. Separation was achieved on a ZB1 capillary column (30 m x 0.25 mm, Phenomenex) with a temperature program 40°C (1 min) to 200°C at 5°C/min. The compounds were confirmed by retention time and mass spectral matching with known standards as methyl ester derivatives.

3.3.4.4 Total Sugar Content

Total sugar (TS) content was quantified by HPLC using two Rezex RPM columns in series (7.8 mm x 30 cm, Phenomenex, Torrance, CA) and a Waters HPLC (Waters, Milford, MA)

equipped with differential refractive index detector (Waters 2414), on elution with water (0.5 mL/min) at 85^{o}C. Aliquot portions of hydrolysates (6 mL) were centrifuged and the supernatant (5 mL) was transferred to a test tube containing inositol as an internal standard (1 mL, 0.5 mg/mL), mixed, deionized (column containing Amberlite IR-120 H^+ (0.5 mL) and Amberlite IRA35 OH^- (0.5 mL) resins) and filtered (0.45 μm). Mixed standards (glucose, xylose, galactose, arabinose and mannose) were prepared for the quantification of samples.

3.3.4.5 Chemical Oxygen Demand, Ammonia, and Phosphorus

Centrifuged and filtered (0.22 μm nylon syringe filter, Fisher Scientific) bioreactor samples (2 ml) was added to the digestion test tube containing dichromate reagent with chemical oxygen demand (COD) range 20-1500 mg/L and the mixture digested for 2 h at 150^{o}C then cooled to room temperature. The absorbance at 620 nm was measured on a UV/VIS spectrophotometer (Beckman DU series 640). A standard curve was prepared by using Hach standard COD solution (0-1100 mg/L) (Reactor digestion method 8000, Hach, Loveland, Colorado, USA). Ammonium nitrogen was measured based on EPA Method 350.2. Total Phosphorus was measured based on EPA Method 365.3.

3.3.4.6 Microbial Community Analysis

Isolation of Bacterial Genomic DNA

The seed culture and bioreactor biomass samples were thawed on ice and vortexed independently to ensure that a homogeneous solution was obtained. 250 μL of each sample was gently mixed with 100 μL of lytic enzyme cocktail (50 μL lysozyme 500 kU mL^{-1}, 6 μL mutanolysin 25 kU mL^{-1}, 4 μL lysostaphin 3000 kU mL^{-1}, and 41 μL mixture of 10 mM Tris-HCl and 50 mM EDTA pH 8.0) and incubated at 37°C for 1 h in a dry heat block. All samples were then transferred to a bead beating tubes contains 750 mg of zirconia-silica beads (Φ 0.1 mm) and placed in Mini-BeadBeater-96 at room temperature for 1 min at 2100 rpm followed by a brief centrifugation. The isolation of bacterial genomic DNA was performed with a QIAamp DNA Mini kit (Qiagen Inc., USA) according to the

manufacturer's protocol. The isolated DNA was examined with 1% agarose gel stained with ethidium bromide in 0.5x TAE buffer and quantified with QuantiFluor dsDNA kit (Promega, Inc.) on Turner TBS-380 mini-fluorometer (Turner BioSystems, USA) and verified with Agilent DNA 1000 kit on Agilent Bioanalyzer 2100 according to manufacturer's recommendations.

PCR amplification and Illumina sequencing

The bacterial 16S rRNA gene was amplified using primers that flanked the variable regions 1 and 3 (Escherichia coli positions 27F-534R). Generations of sample amplicons were performed using a double round of PCR and dual indexing on the Illumina MiSeq. The first round of PCR extracts the targeted V1-V3 regions. The second round of PCR attaches the sample barcode and sequencing adapters. The PCR primer sequences are described as 27F primer (27F-YM1: ACACTGACGACATGGTTCTACAGTAGAGTTTGATCCTGGCTCAG; 27F-YM2: ACACTGACGACATGGTTCTACA*C*GTAGAGTTTGATCATGGCTCAG; 27F-YM3: ACACTGACGACATGGTTCTACA*AC*GTAGAGTTTGATTCTGGCTCAG; 27F-YM4: ACACTGACGACATGGTTCTACA*AC*GTAGAGTTTGATTCTGGCTCAG; 27F-Bif: ACACTGACGACATGGTTCTACA*GTAC*GTAGGGTTCGATTCTGGCTCAG; 27F-Bor: ACACTGACGACATGGTTCTACA*CGTAC*GTAGAGTTTGATCCTGGCTTAG; 27F-Chl: ACACTGACGACATGGTTCTACA*ACGTAC*GTAGAATTTGATCTTGGTTCAG), 534R primer (534R-1: TACGGTAGCAGAGACTTGGTCTCCATTACCGCGGCTGCTGG; 534R-2: TACGGTAGCAGAGACTTGGTCT*G*CCATTACCGCGGCTGCTGG; 534R-3: TACGGTAGCAGAGACTTGGTCT*TG*CCATTACCGCGGCTGCTGG; 534R-4: TACGGTAGCAGAGACTTGGTCT*ATG*CCATTACCGCGGCTGCTGG; 534R-5: TACGGTAGCAGAGACTTGGTCT*CATG*CCATTACCGCGGCTGCTGG; 534R-6: TACGGTAGCAGAGACTTGGTCT*TCATG*CCATTACCGCGGCTGCTGG; 534R-7: TACGGTAGCAGAGACTTGGTCT*ATCATG*CCATTACCGCGGCTGCTGG) and adapter primers (P7-CS2: CAAGCAGAAGACGGCATACGAGAT*NNNNNNNN*TACGGTAGCAGAGACTTGGTCT; P5-CS1:

AATGATACGGCGACCACCGAGATCTACAC*NNNNNNNN*ACACTGACGACATGGTTCTACA), where the underlined sequences are universal sequences CS1 (ACACTGACGACATGGTTCTACA) and CS2 (TACGGTAGCAGAGACTTGGTCT). Bold Sequences denote the universal 16S rRNA primers 27F and 534R, and seven different 27F primer sequences were used in order to capture the greatest number of taxa. The bold, italized bases were added to the template specific primers in order to introduce variability of base calls during Illumina sequencing. The adapter primers included the Illumina specific sequences P7 and P5 for dual indexing where the unique sequence barcodes were denoted by the 8 italicized N's. This allowed us to sequence the amplicons from many samples simultaneously using relatively few barcoded adapter primers, and afterwards assign each sequence to the sample they were obtained from (40 P7-CS2 primers and 40 P5-CS1 primers enable 800 unique samples identifiers).

The concentrations of amplicons were determined using a picogreen assay and a Fluorometer (SpectraMax GeminiXPS 96-well plate reader) and then pooled in equal amounts (~100 ng) into a single tube. The amplicon pool was then cleaned to remove short undesirable fragments from the following procedure. First the pool is size selected using AMPure beads (Beckman Coulter), the product was then ran on a 1% gel, gel cut and column purified (Qiagen MinElute PCR purification kit), and size selected again with AMPure beads. To determine the final quality we PCR amplified the resulting amplicon pool with Illumina adaptor specific primers and ran the PCR product on a DNA 1000 chip for the Agilent 2100 Bioanalyzer. The final amplicon pool was deemed acceptable only if no short fragments were identified after PCR, otherwise the procedure was repeated again. The cleaned amplicon pool is then quantified using the KAPA 454 library quantification kit (KAPA Biosciences) and the Applied Biosystems StepOne plus real-time PCR system. Finally, sequences were obtained using an Illumina MiSeq paired-end 300bp protocol (Illumina, Inc., San Diego, CA).

Sequence analysis and taxonomic classification

Raw DNA sequence reads from the Illumina MiSeq were demultiplexed and classified in the following manner. The custom python application dbcAmplicons

(https://github.com/msettles/dbcAmplicons) was used to identify and assign reads by both expected barcode and primer sequences. Barcodes were allowed to have at most 1 mismatch (hamming distance) and primers were allowed to have at most 4 mismatches (Levenshtein distance) as long as the final 4 bases of the primer matched the target sequence perfectly. Reads were then trimmed of their primer sequence and merged into a single amplicon sequence using the application flash (Magoc and Salzberg, 2011). Finally, the RDP Bayesian classifier was used to assign sequences to phylotypes (Wang et al., 2007). Reads were assigned to the first RDP taxonomic level with a bootstrap score >=50.

3.4 Results and Discussion

3.4.1 PHB Production in Continues Fed-Batch Bioreactors

3.4.1.1 Feb-Batch PHB Production in One Operational Cycle

Growth, PHB accumulation and substrates consumption in one operational cycle (24 h) at different OLR are shown in Figure 3.2 (A: OLR=2.5 g/Ld; B: OLR=3.3 g/Ld). The TSS and OD showed a similar trend in which both increased during the operational cycle and dropped slightly at the end of cycle which gave a continuous cell growth in the fed-batch reactors. Higher OLR (Figure 3.2 B1) had a higher TSS (3.5-4.0 g/L) as compared to lower OLR (Figure 3.2 A1, 3.0-3.5 g/L). However, PHB accumulation showed an opposite trend and was higher in reactor A (Figure 3.2 A1: >20% CDW) and lower in reactor E (Figure 3.2 B1: <15% CDW). Similar results were found in other bioreactors with different OLR (data not shown). These findings indicate that PHB production does not follow an exact linear relationship with external carbon loading while cell growth does increase with OLR.

Substrate consumption was determined by the change of concentrations of TS, COD, NH_3 and P during one cycle. Both TS and COD decreased during the production cycle to the same extent for reactor A with TS fully depleted (Figure 3.2 A2). In reactor E (Figure 3.2 B2), the TS was depleted but not for COD which means some soluble COD may still exist as non-sugar content. NH_3 and P were consumed but not depleted during the cycle since a

certain amount of nutrients (NH_3 and P) were added to the bioreactors periodically. It has been reported that low P concentration showed good PHA accumulation rather than complete P deficiency (Reddy and Mohan, 2012). A minimal level of internal phosphate is essential for PHA accumulation. Low concentration of both NH_3 and P are favorable for the enhancement of PHB production. High NH_3 and P concentrations lead to protein synthesis, while phosphate deprivation shows reduction of protein synthesis rate and diverts towards PHA accumulation (Panda et al., 2006). Under balanced growth conditions CoASH levels are high and PHA synthesis gets inhibited. However, when growth is limited by an essential nutrient other than the carbon and energy source, this reduces the complexity of the metabolism that leads to a unidirectional path such as PHA synthesis. Furthermore, the NADH level increases resulting in the inhibition of enzymes in the TCA cycle, such as, citrate synthase and isocitrate dehydrogenase. This leads to the accumulation of acetyl-CoA which relieves the inhibition exerted by CoASH, leading to PHB formation (Dawes, 1986).

The TS contains about 70% hexose (mainly glucose) and 30% pentose (mainly xylose) sugars. The MMCs was able to consume both hexose and pentose sugars during one operational cycle, but at different rates. Xylose was consumed only when glucose was depleted (data not shown), therefore the consumption rate for xylose was much lower than glucose. It was reported by some studies (Lopes et al., 2011; Teresa Cesario et al., 2014) that high pentose concentration would significantly inhibit cell growth and PHB accumulation. Thus, it is crucial that both increase the total carbon up-take by the cells and avoid pentose accumulation in the broth which may cause inhibition effect to PHB production. Based on the results shown in Figure 3.2, both cell growth and PHB production can be achieved and stabilized at an initial sugar loading <3.3 g/Ld without pentose accumulation in the fed-batch cultures.

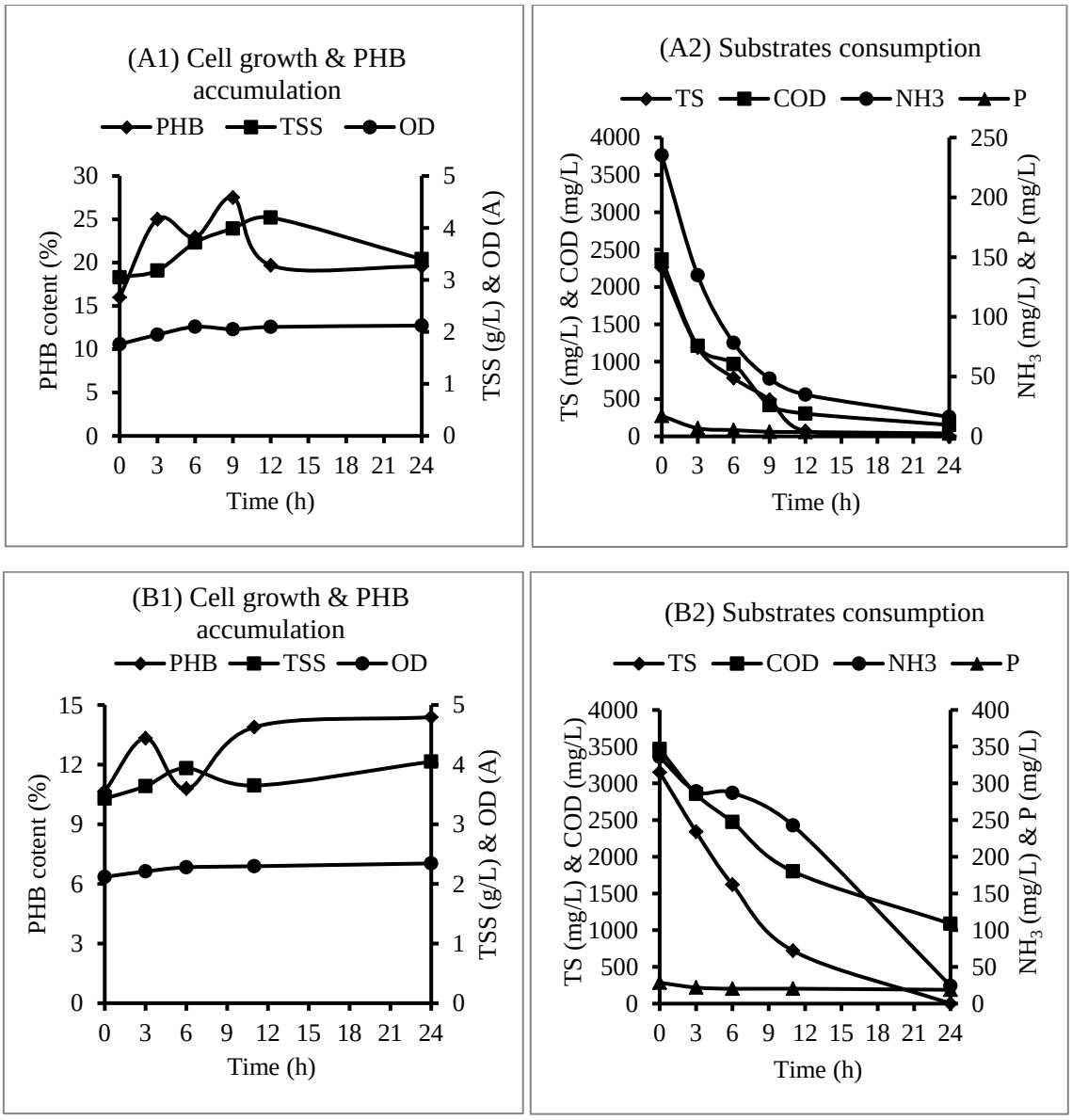

Figure 3.2 Growth, PHB production and substrate consumption by MMCs at different OLR over one operational cycle: (A) reactor A: 2.5 g/Ld (B) reactor E: 3.3 g/Ld

Since the external carbon source, TS, was completely depleted and resupplied during one operational cycle, where a famine period was observed followed by excess carbon period, a feast-famine process was proposed in these fed-batch cultures. However, the internal accumulated carbon PHB was not depleted during the cycle which did not fulfill the evidence of a typical feast-famine process. Besides feast-famine mechanism, a nutrient limited pattern for PHB synthesis was also observed. Dehydrogenase (DH) is an intracellular

enzyme involved in the oxidation-reduction reactions occurring in the cell which is the main enzyme of the glycolytic pathway and converts glucose to pyruvate then to acetyl-CoA. It was shown by Reddy and Mohan (2012) that limitation of N and P gave higher DH activity which resulted in higher carbohydrate removal and higher PHA accumulation. They had also found out that another key enzyme phosphatase showed a decrease trend of activity while substrate utilization gave a good explanation of carbohydrate consumption during the cycle. Since both the nutrients limitation and substrate consumption regulated the change of enzyme activity, PHB accumulation was driven under this unbalanced condition. Moreover, when the external substrate was depleted in the broth, there was still enough enzyme activity (e.g. DH) transferring intermediates such as pyruvate and acetyl-CoA into PHB which could synthesize the biopolymer throughout the cycle. Therefore, a combination of feast-famine and nutrient limitation metabolic pathway is proposed for this fed-batch PHB production by MMCs.

3.4.1.2 Feb-Batch Studies for PHB Production

The optimization of PHB production in fed-batch MMCs had been performed using a factorial design. Two designed parameters, SRT and TS, in the feed stock together with the corresponding OLR are shown in Table 3.2. The distribution of PHB content (%CDW) by reactor ID was evaluated and shown in a box-plot (Figure 3.3). The four bioreactors, D, G, K, and L with high OLR (>5 g/ld) and TS (>20 g/L) did not synthesize PHB. Reactor C designed with the lowest OLR (1.3 g/Ld) accumulated less than 5% PHB. Reactor H and J also produced less than 5% PHB since they had a high OLR (5 g/Ld) and short SRT (2 d), respectively. The four reactors, B, E, F, and I, produced around 10% PHB and had an OLR <3.8 g/Ld. The most productive reactor A was designed with relatively mild operational conditions (SRT=4 d, S=10 g/L, OLR=2.5 g/Ld) which was able to accumulate as high as 27% PHB in CDW. A mixture of analytical grade pure glucose and xylose (5 g/Ld and 10 g/Ld) had also been used for MMCs PHB production (data not shown), but no PHB was synthesized and sugar accumulation was observed. Based on the results, PHB was only successfully synthesized at a low OLR (<5 g/Ld) and with a SRT of 4-6 d. At higher OLR,

the sugars were accumulated in the broth which caused an inhibition to PHB production/accumulation.

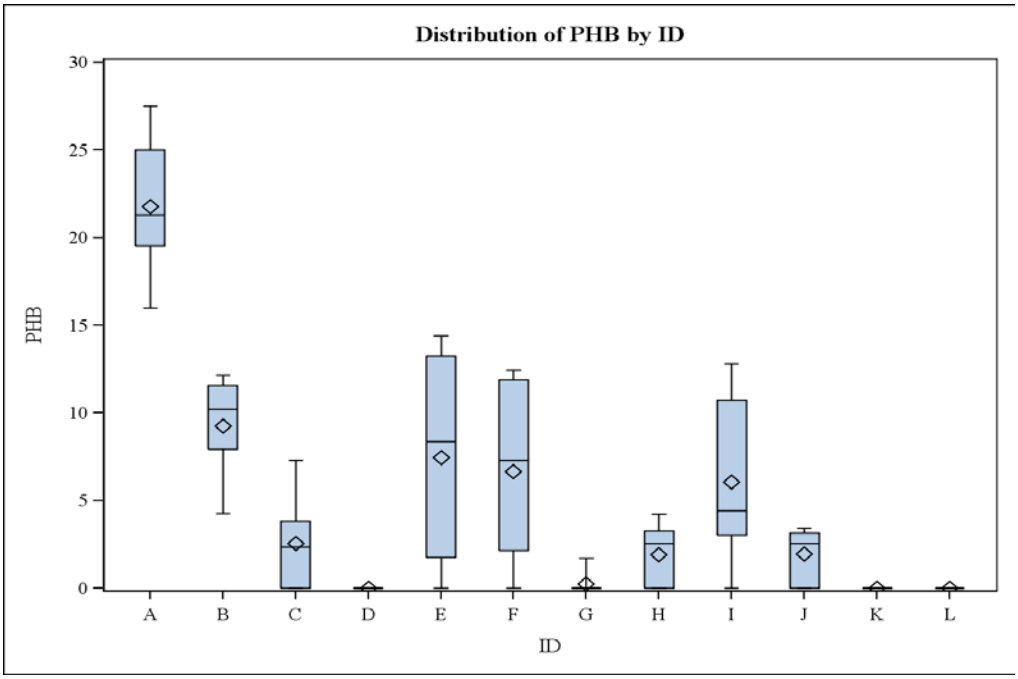

Figure 3.3 Profile of PHB production (%CDW) in the 12 bioreactors

The biomass growth and substrates consumption for one non-PHB producing reactor (G) is shown in Figure 3.4. In this reactor, as well as reactors D, K and L, the cell growth in terms of TSS and OD was increased during one operational cycle. However, no PHB accumulation observed in the cells. Since no pH and oxygen control were done during the operational cycle, this may due to the low DO level in the system which will drive the microorganisms to produce volatile fatty acids via fermentation instead of PHB synthesis. The pH values (4.32-5.65) were also much lower in this reactor compared to those PHB producing reactors (pH>7.0). Acetic acid was found in the reactor substrate as well (data not shown) which may be the reason for low pH and supports that the reactor was behaving as an anaerobic fermenter. The placement of an additional air sparger in reactor G did not increase the DO and pH values and without any PHB accumulation. Therefore, the MMCs at higher OLR when fed wood hydrolysates produce fatty acids. TS was consumed during the operational

cycle (Figure 3.4 A2) but not depleted and xylose (a pentose) was found to be present which may cause an inhibitory effect by its accumulation.

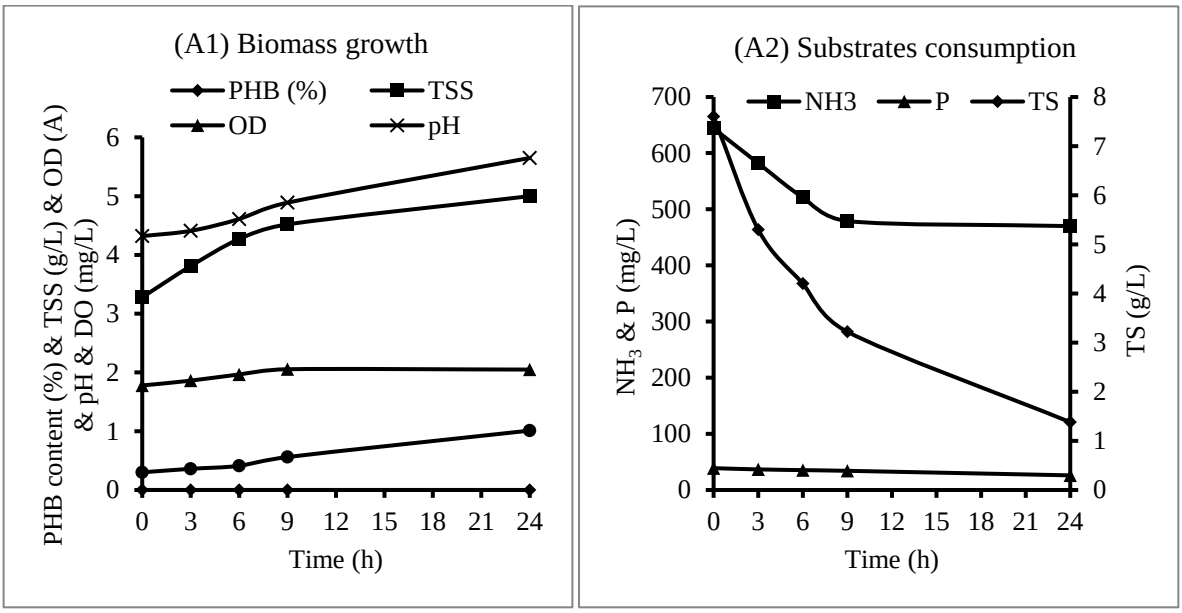

Figure 3.4 Biomass growth (A1) and substrates consumption (A2) for non-PHB producing reactor G

In order to study the potential of wood hydrolysates as a renewable carbon source for PHB production, the effect of different OLR with the final cell growths and PHB yields were evaluated (Table 3.2). Maximum TSS was obtained in high OLR and long SRT reactors such as I (5.5 g/L). However, higher OLR with shorter SRT (2 d) reactors, such as J and K grew less biomass compared to the others. Maximum PHB yield (based on g total sugar consumed) was also observed in reactor A (0.32 g/g) and this result was better than PHB grown on straw hydrolysates on *Burkholderia cepacia* by Teresa Cesario et al. (2014) at 0.25 g/g. However, the average PHB production rate (0.068 g PHB/g TS/d) for reactor A was 10 times lower than a study using pure glucose for PHB synthesis by *Alcaligenes eutrophus* (Henderson and Jones, 1997). Another study used renewable sugars from oil palm to produce PHB with *Cupriavidus necator* CCUG 52238 and gave a maximum PHB yield of 0.20 g/g (Zahari et al., 2012). Several of our bioreactors (B, C, E, F, and I) were of comparable performance to studies using pure cultures which shows that this approach of using MMCs to synthesize PHB with wood hydrolysates promising. Based on current results,

the PHB accumulation by MMCs were not substrate-dependent. It was observed that by increasing the OLR up to 5 g/Ld did not drive PHB accumulation in cells due to the high initial sugar loading. Also, lower pH (<5) and DO (<1 mg/L) were observed for these non-PHB producing reactors (D, G, K and L) (Table 3.2). Therefore, we concluded that these were anaerobic fermenters instead of arerobic bioreactors which undergo fatty acids synthetic pathway.

Table 3.2 Cultivation parameters for PHB production by MMCs

ID	SRT (d)	S (g/L)	OLR (g/Ld)	WV (L)	Cycle (h)	pH range	DO range (mg/L)	Max TSS (g/L)	Max PHB content (%)	PHB yield (g/g)	Average PHB production rate (g/g/d)
A	4	10	2.5	0.8	24	6.5-7.7	3.69-6.65	4.20	27.51	0.32	0.068
B	6	10	1.7	0.8	24	6.9-8.3	4.50-6.66	3.79	12.16	0.16	0.027
C	8	10	1.3	0.8	24	6.8-8.2	3.05-6.94	3.66	7.31	0.24	0.006
D	4	20	5.0	0.8	24	4.2-5.8	0.90-1.50	4.30	0.00	0.00	0.000
E	6	20	3.3	0.8	24	7.0-8.0	2.80-5.40	4.39	14.39	0.12	0.029
F	8	20	2.5	0.8	24	7.0-8.0	3.03-5.42	4.06	12.44	0.14	0.018
G	4	30	7.5	0.8	24	4.3-5.6	0.30-1.00	4.57	1.70	0.02	0.000
H	6	30	5.0	0.8	24	5.0-6.1	0.52-2.10	3.84	4.24	0.05	0.003
I	8	30	3.8	0.8	24	6.9-8.2	3.52-6.13	5.59	12.81	0.24	0.021
J	2	10	5.0	0.8	24	4.3-5.1	0.29-0.53	2.20	3.43	0.01	0.001
K	2	20	10.0	0.8	24	4.4-5.2	0.17-1.01	2.19	0.00	0.00	0.000
L	2	30	15.0	0.8	24	4.2-5.3	0.65-1.27	4.07	0.00	0.00	0.000

SRT: solid retention time (d)

Sugars (S): total sugar concentration in the hydrolysates (g/L)

OLR: organic loading rate (g/Ld)

WV: working volume (L)

Max TSS: maximum total suspended solids (g/L)

Max PHB content: maximum PHB content in cell dry weight (%)

Max PHB conc.: maximum PHB concentration produced (g/L)

PHB yield: g per PHB yield per g total sugars consumed (g/g)

Average PHB production rate: g per PHB yield per g total sugars consumed per day

3.4.1.3 Study of Long Chain Fatty Acids Synthesis by Full Nutrients Condition

When PHB was detected in the cell, long chain fatty acids (LCFAs, C15-C18) which formed the microbial cellular components were also detected by GC-MS (Table 3.3). The three major fatty acids were palmitic acid (C16), oleic acid (C18:1) and stearic acid (C18). These cellular components were found regardless of how much PHB was accumulated. However, the higher the nutrients loading (C, NH_3, P) the more the fatty acid content was observed in the cellular biomass. This is supportive evidence that using full nutrient conditions was good for cell growth but not for PHB accumulation (Albuquerque et al., 2010; Reddy and Mohan, 2012).

Table 3.3 Type of LCFAs in microbial cellular components

Retention Time (min)	Long chain fatty acids as their methyl ester derivative
32.8-33.3	Palmitic acid (C16, major)
37.8	Oleic acid (C18, unsaturated)
38.0	Stearic acid (C18, saturated)
35.0	C17 (minor)
30.7	C15 (minor)

A full nutrient condition experiment (sugar loading 40 g/Ld, C:N=20:1) was performed in a fed-batch bioreactor (12 h) and no PHB and sugar accumulation were detected by GC-MS, however, high levels of LCFAs were found in the biomass samples. Methanolysis was applied to quantify the concentration of LCFAs in the biomass and the results are shown in Figure 3.5. TSS was increased after 24 h and kept consistent (3.6-4.6 g /L). LCFAs started to accumulate after 12 h and reached the maximum concentration of 700 mg/L after 60 h. The growth trend of LCFAs was similar to TSS which indicates that the LCFAs represent a major cellular component in the biomass. The pH dropped from 7.0 (adjusted) to 5.0 and remained at 5.0 during the operational period and DO was less than 1 mg/L. This condition had been reported to be suitable for LCFAs accumulation rather than PHA production (Tohyama et al., 2000).

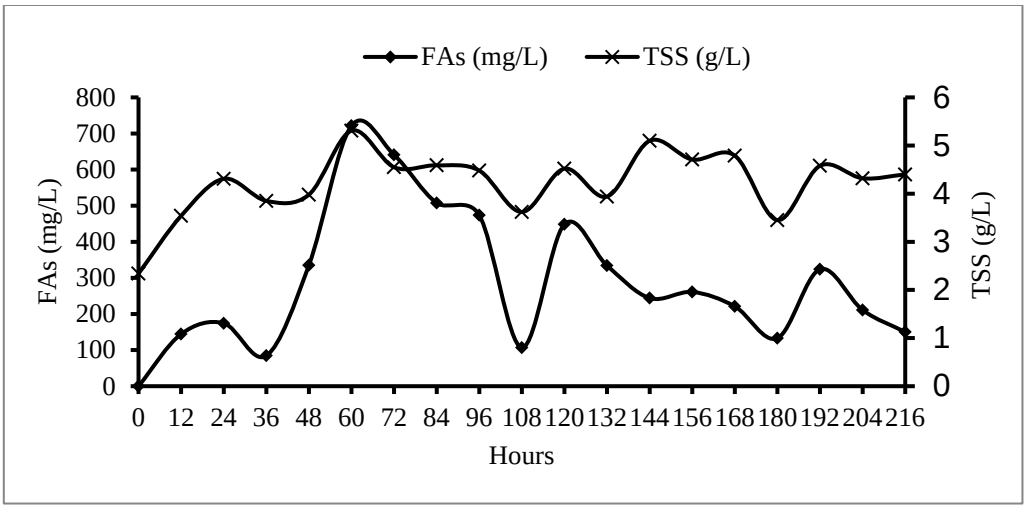

Figure 3.5 Long chain fatty acid concentration and total suspended solids for fed-batch bioreactor

3.4.2 Factorial Design for PHB Production

In this study, the effects of each cultivation parameters were evaluated through a generalized linear model (GLM). The PHB content was applied as response variable to the model. The X variables included pH, dissolved oxygen (DO), TSS, TS, NH_3, Phosphorus (Phos), COD, and OD and their interactions. Each parameter was determined through random sampling and total of 111 samples were taken for the 12 bioreactors. The design variable SRT and initial sugar concentration in feed stock (S) was also included in the GLM to test the design effect.

The analysis of variance (ANOVA) results for PHB content is presented in Table 3.4 with both type I and type III mean square errors. The two design variables (SRT and S) and their interaction term (determine the OLR) were significant at $P<0.05$ level for both types which means the PHB accumulation was highly dependent on the SRT and initial sugar loading in these fed-batch experiments. TSS and OD were significant variables and both showed strong positive effects on PHB accumulation. These results indicate that polymer accumulation and cell growth occurred simultaneously, where higher PHB accumulation in cells must have enough biomass growing in terms of TSS and OD.

Table 3.4 Analysis of variance (ANOVA) of cultivation parameters

Source	Parameter Estimate	Type I Mean Square Error	F Value	Pr > F
SRT	-3.9578	111.769	10.87	0.0014
S	-1.1032	417.0772	40.57	<.0001
pH	0.2042	271.0424	26.36	<.0001
DO	0.234	65.5687	6.38	0.0132
TSS	2.6554	909.1321	88.42	<.0001
TS	0.6183	0.0245	0	0.9612
NH3	-19.18	259.4787	25.24	<.0001
Phos	-31.4891	141.9531	13.81	0.0003
COD	0.0802	2.0174	0.2	0.6588
OD	13.611	518.617	50.44	<.0001
SRT*S	0.1378	351.5017	34.19	<.0001
NH3*Phos	85.6915	3.2087	0.31	0.5777
TS*NH3	1.0391	35.3281	3.44	0.0668
Source	Parameter Estimate	Type III Mean Square Error	F Value	Pr > F
SRT	-3.9578	581.9122	56.6	<.0001
S	-1.1032	391.2687	38.06	<.0001
pH	0.2042	0.569647	0.06	0.8144
DO	0.234	10.76616	1.05	0.3087
TSS	2.6554	164.325	15.98	0.0001
TS	0.6183	15.40345	1.5	0.2239
NH3	-19.18	118.0125	11.48	0.001
Phos	-31.4891	4.010669	0.39	0.5337
COD	0.0802	1.07579	0.1	0.747
OD	13.611	302.7759	29.45	<.0001
SRT*S	0.1378	318.7975	31.01	<.0001
NH3*Phos	85.6915	2.395754	0.23	0.6304
TS*NH3	1.0391	35.32814	3.44	0.0668

R^2=0.76, Sum of residuals=0.

Since pH, DO and Phos were not significant in type III error and therefore were not included in the final model. Also, based on previous analysis, pH and DO were two factors determining whether fermentation or PHB biosynthesis processes occurred. NH_3 showed strong negative effect on PHB content which supports the nutrients limitation mechanism for PHB production using wood hydrolysates by MMCs. No significant effects (linear relationship) were found for TS and COD which were also reasonable because PHB can still accumulate in cells even when total sugars were depleted in the broth. Therefore,

conclusions can be made that PHB accumulation was only dependent on the initial sugar loading (S and SRT*S) but not associated with total sugars concentration in the reactor. Since the interaction terms of NH_3*Phos and TS*NH_3 were not significant and they should not be counted in the final model.

Based on the ANOVA results, a predicted model was generated (type III) for PHB content (%CDW) which was useful for further reference as given below:

PHB (%CDW) = - 3.96SRT - 1.10S + 2.66TSS - 19.18NH_3 + 13.61OD + 0.14SRT*S

3.4.3 Microbial Community Analysis

With the purpose of understanding how the composition of the microbial community selected in the fed-batch and sub-cultured reactors, 16S rRNA sequencing analysis had been performed. The operation of reactors is shown in Table 3.5. Initial seed was from activated sludge and cultured with full nutrients before inoculated to reactor A and B. SA was a side reactor of A which inoculated from A and operated continuously with feeding. WA was seeded from SA and operated as same as reactor A (Figure 3.6).

Table 3.5 Seed and PHB reactors operation for microbial community analysis

ID	PHB (%)	Description
Seed	0	Activated sludge from Moscow waste water treatment plant
A	13	Sugars 2.5 g/L/d, 7 h growth, 24 h cycle, SRT 4 d
B	9	Sugars 1.7 g/L/d, 7 h growth, 24 h cycle, SRT 6 d
SA	17	Side stream of reactor A, continues feeding every 12 h
WA	15	Seeded from SA, operated same as reactor A

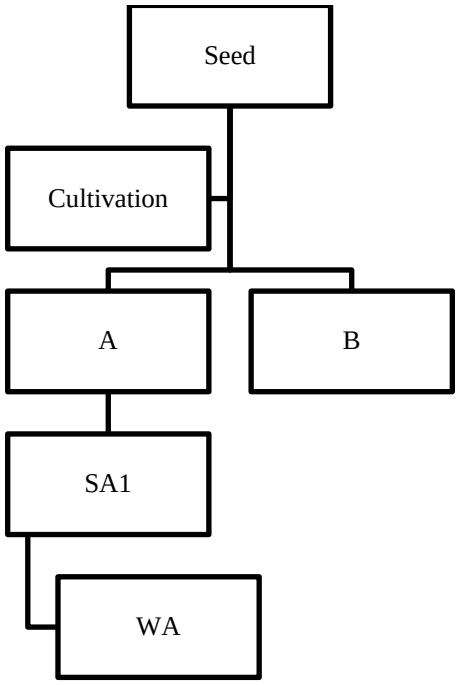

Figure 3.6 Reactors seeding sequence for microbial community analysis

The raw genomics data of taxa identified by illumina 16S rRNA sequencing is given in Appendix 1. The number of total sequence reads for the seed and bioreactors A, B, SA and WA were respectivey, 5474, 44405, 70572, 10525, and 62776. The taxa documented were >1% across all the 5 samples analyzed for community evolution and clustering. The taxonomical classification is shown in Figure 3.7 where both the reactor and taxon were classified based on the similarity of each other. The cluster analysis showed that reactor A and B had the most similarity in terms of bacterial distribution and were so different from the initial seed. This indicated that a significant microbial community shift was occurring after seeding in the fed-batch reactors. Reactor SA had similar bacteria distribution to reactor A since it had been inoculated from A. However, WA, the reactor operated for the longest period (more than two months), showed a significant difference from the first two running bioreactors A and B which means a bacterial community evolution was also occurring during the operational period. The bacterial taxa were ranked based on their average density (standardized z-score) among the 5 samples from low to high. The others (unidentified taxa) showed the highest density than the other taxa. From the heatmap plot (Figure 3.7), it was clear that the bacterial distribution of taxa in these reactors were

different. To better characterize the taxa and their evolution among the samples, a higher level of taxon names and counts were given by grouping them into belonged classes which are shown in Table 3.6 and Figure 3.8.

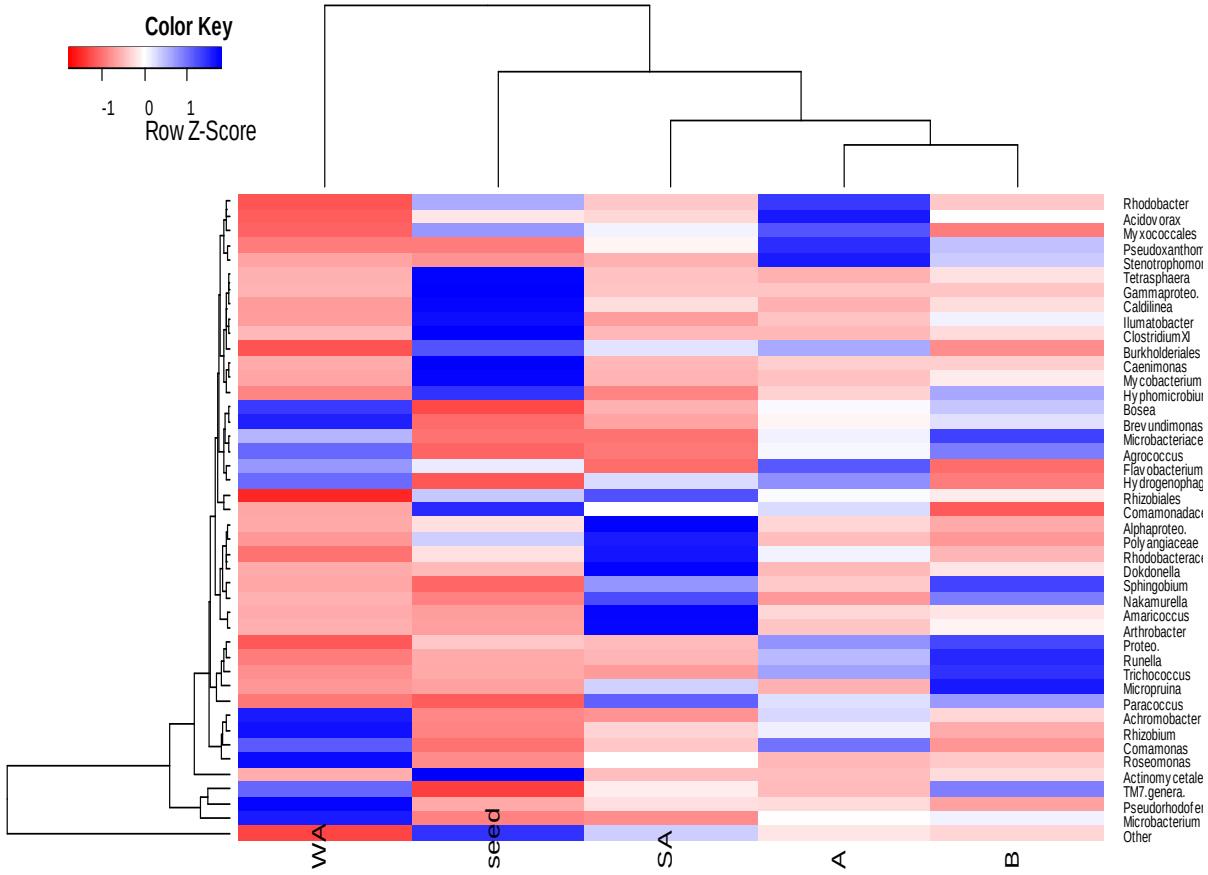

Figure 3.7 Heatmap.2 for microbial community clustering analysis of the seed and PHB bioreactors A, B, WA and SA

Table 3.6 shows the full name of the taxa and their class and counts in the reactor samples. *Actinobacteria* and other *Proteobacteria* (including *Alpha-*, *Beta-*, *Delta-*, *Gamma-Proteobacteria*) were the dominant species among all samples while only two genera belonged to *Firmicutes*. This result was quite opposite to the work reported by Reddy and Mohan (2012) where the phylogenetic profile of 16S rRNA sequencing showed the

dominant two phylum were *Firmicutes* (71.4%) and *Proteobacteria* (28.6%) using designed synthetic wastewater as substrate. Another study used synthetic wastewater as substrate under an anaerobic/aerobic process for PHB synthesis which showed 5 dominant genera present by pyrosequncing of the 16S rRNA gene: *Bacteroidetes sp.*, *Acinetobacter sp.*, *Betaproteobacteria sp.*, *Gammaproteobacteria sp.*, *Arcobacter sp.* and *Bacillus sp.* (Liu et al., 2013). However, they also stated that the bacterial community for PHB synthesis was more diverse than the 5 genera mentioned above, such as *Alphaproteobacteria*, *Actinobacteria* and *Acidobacteria*. The group of others (unclassified) taxa (<0.1%) in the initial seed was 2.2% and it dropped to 0.9% for reactor A, 1.1% for B and 0.8% for SA. However, it decreased to only 0.4% in reactor WA which was operating continuously for more than two months. These results show evidence of microbial community evolution in the different bioreactors during an operational period.

Table 3.6 Microbial taxon class and counts in the seed and PHB reactor samples

Taxon Name	Class	Seed	A	B	SA	WA
Actinomycetales	*Actinobacteria*	0.119	0.006	0.017	0.006	0.001
Agrococcus	*Actinobacteria*	0.000	0.008	0.014	0.001	0.015
Arthrobacter	*Actinobacteria*	0.000	0.005	0.011	0.045	0.002
Ilumatobacter	*Actinobacteria*	0.009	0.001	0.003	0.000	0.000
Microbacteriaceae	*Actinobacteria*	0.001	0.009	0.018	0.001	0.012
Microbacterium	*Actinobacteria*	0.003	0.065	0.074	0.009	0.180
Micropruina	*Actinobacteria*	0.005	0.007	0.044	0.022	0.004
Mycobacterium	*Actinobacteria*	0.024	0.002	0.005	0.001	0.000
Nakamurella	*Actinobacteria*	0.001	0.002	0.012	0.014	0.003
Tetrasphaera	*Actinobacteria*	0.021	0.000	0.003	0.001	0.000
Alphaproteobacteria	*Alphaproteobacteria*	0.009	0.008	0.004	0.035	0.004
Amaricoccus	*Alphaproteobacteria*	0.001	0.009	0.011	0.050	0.003
Bosea	*Alphaproteobacteria*	0.001	0.012	0.015	0.007	0.023
Brevundimonas	*Alphaproteobacteria*	0.000	0.010	0.013	0.004	0.027
Hyphomicrobium	*Alphaproteobacteria*	0.019	0.006	0.013	0.002	0.002
Paracoccus	*Alphaproteobacteria*	0.005	0.033	0.043	0.051	0.009
Rhizobiales	*Alphaproteobacteria*	0.025	0.021	0.019	0.034	0.004
Rhizobium	*Alphaproteobacteria*	0.002	0.023	0.008	0.014	0.056
Rhodobacteraceae	*Alphaproteobacteria*	0.009	0.011	0.007	0.021	0.004
Rhodobacter	*Alphaproteobacteria*	0.017	0.021	0.012	0.012	0.008
Roseomonas	*Alphaproteobacteria*	0.001	0.006	0.008	0.014	0.042
Sphingobium	*Alphaproteobacteria*	0.001	0.007	0.022	0.017	0.005
Achromobacter	*Betaproteobacteria*	0.000	0.028	0.014	0.002	0.061
Acidovorax	*Betaproteobacteria*	0.011	0.030	0.013	0.010	0.001
Burkholderiales_incertae_sedis	*Betaproteobacteria*	0.013	0.010	0.003	0.008	0.001
Caenimonas	*Betaproteobacteria*	0.029	0.004	0.004	0.002	0.001
Comamonadaceae	*Betaproteobacteria*	0.036	0.022	0.006	0.019	0.012
Comamonas	*Betaproteobacteria*	0.001	0.034	0.005	0.011	0.037
Hydrogenophaga	*Betaproteobacteria*	0.001	0.016	0.003	0.012	0.018
Pseudorhodoferax	*Betaproteobacteria*	0.007	0.024	0.004	0.025	0.122
Myxococcales	*Deltaproteobacteria*	0.020	0.025	0.002	0.013	0.000
Polyangiaceae	*Deltaproteobacteria*	0.013	0.004	0.001	0.027	0.001
Dokdonella	*Gammaproteobacteria*	0.002	0.002	0.005	0.024	0.001
Gammaproteobacteria	*Gammaproteobacteria*	0.020	0.001	0.001	0.001	0.000
Pseudoxanthomonas	*Gammaproteobacteria*	0.001	0.024	0.014	0.009	0.001
Stenotrophomonas	*Gammaproteobacteria*	0.000	0.023	0.011	0.002	0.001
Clostridium XI	*Firmicutes*	0.009	0.000	0.001	0.000	0.000
Trichococcus	*Firmicutes*	0.004	0.033	0.051	0.002	0.000
Flavobacterium	*Flavobacteria*	0.012	0.019	0.004	0.004	0.016
TM7_genera_incertae_sedis	*TM7*	0.002	0.029	0.073	0.040	0.078
Counts > 1%		0.454	0.600	0.591	0.572	0.755
0.1% < Counts < 1%		0.524	0.391	0.398	0.420	0.241
Others (<0.1%)		0.022	0.009	0.011	0.008	0.004

The evolution of the microbial community within the 5 samples is shown in Figure 3.8. It can be seen that *Actinobacteria*, *Alphaproteobacteria* and *Betaproteobacteria* were three dominant groups across all the samples including the seed. *Actinobacteria* remained quite consistent around 20% in the seed, reactor B and reactor WA, while it reduced to about 10% in reactors A and SA. *Alphaproteobacteria* was present and showed a significant increase in the four reactors compared to the initial seed, especially in reactor SA. SA was a continuously fed (without wasting) reactor which grew more *Alphaproteobacteria* through the feeding strategy employed. The percentage of *Betaproteobacteria* was much higher in reactors A and WA as compared to reactors B and SA which may due to the higher OLR in A and WA. Thus, the *Betaproteobacteria* may favor being grown on the wood hydrolysates. Moreover, *Burkholderiales_incertae_sedis* is a genus that has a lineage to *Burkholdia cepacia* which has been reported to utilize wood hydrolysates to produce PHB (Silva et al., 2004; Pan et al., 2012; Teresa Cesario et al., 2014) and both of them belonged to the *Betaproteobacteria* class. Therefore, further isolation work from MMCs can be focused on the *Betaproteobacteria* in order to use wood hydrolysates as substrate for producing PHB.

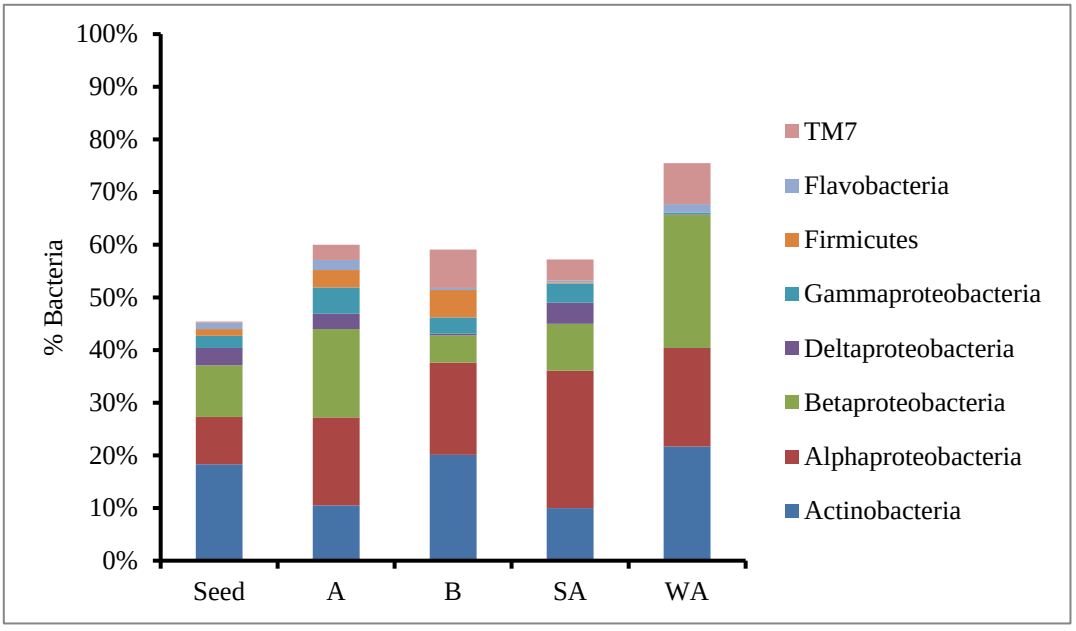

Figure 3.8 Bacterial community evolution from the seed to the different bioreactors

Delta- and *Gammaproteobacteria* were two classes which were present in low levels in all the samples and therefore little information could be inferred on their abilities to utilize wood hydrolysates. The phylum *Firmicutes* was depleted in bioreactors SA and WA, where PHB could still be accumulated. Thus, the *Firmicutes* may not responsible for polymer accumulation in cells. *Flavobacteria* is a class widely present in water and soil and this genus is commonly found in activated sludge (Park et al., 2007; Nielsen et al. 2009). *Flavobacteria* was present a very low levels across all the samples. *TM7_genera_incertae_sedis* was the most interesting genera because it only presented 0.2% in the seed but increased to 3% in reactor A, 7% in reactor B, 4% in reactor SA and 8% in reactor WA. It also gave the highest growth in terms of percentage compared to other genuses. *TM7* phylum in wastewater activated sludge is consistent with specialized protein hydrolyzing organisms, a function that links them to protein turnover (Ibarbalz et al., 2013). However, this group of organisms has not yet been reported to be able to accumulate PHA (Liu et al., 2013).

The maximum PHB accumulation of 17% was found in reactor SA in which the dominant group was *Alphaproteobacteria.* Therefore, *Alphaproteobacteria* was proposed to be another PHB storage related group. Some genera from *Alphaproteobacteria* such as *Rhizobium, Sphingobium, Paracoccus,* and *Amaricoccus* have already been characterized as PHA-storing microorganisms (Anderson and Dawes, 1990; Lemos et al., 2008; Pisco et al., 2009; Liu et al., 2013) and these genera were also found in this work (Table 3.6). The characterization of MMCs fed wood hydrolysates was done at the genus level and several potential PHB-storing organisms in both genus and class levels were highlighted here. However, a complete characterization of all genera is required for future work by modeling the bioreactor in time series with the aid of statistical analysis. Nevertheless, this was the first time that microbial community analysis of MMCs was done in genus level in PHB production reactors fed wood hydrolysates.

3.5 Conclusions

The operation of fed-batch bioreactors fed wood hydrolysates were able to adapt and successfully accumulated PHB (maximum yield of 27% CDW) by using the MMCs. Microbial community analysis showed that MMCs presented a community evolution in different bioreactors and during operational period. Several characterized genera belonged to *Alphaproteobacteria* and *Betaproteobacteria* classes were PHB-accumulated microorganisms. The results in this work gave a proof of concept study using wood hydrolysates to synthesize PHB by MMCs which can be considered as a promising step for the biorefinery of lignocellulosic materials.

3.6 Acknowledgements

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Chapter 4 Polyhydroxybutyrate Characterization

4.1 Abstract

Polyhydroxybutyrate (PHB) have been biosynthesized from either mixed microbial cultures or pure culture (*Burkholderia cepacia* ATCC 17759) fed hybrid poplar hydrolysates (monosaccharides) using a fed-batch reactor. The PHB biopolymers were isolated from the microbial biomass by chloroform extraction and subsequently characterized. The chemical structure of PHB was identified using Fourier transform infrared (FTIR) spectroscopy and PHB content (%CDW) was determined by gas chromatography-mass spectrometry (GC-MS) as methyl ester derivatives. The isolated PHB samples were characterized at carbonyl ester band (1735 cm^{-1}). In addition, a predicted partial least squares (PLS) regression model was generated between full range IR spectra and GC-MS reference data with a R^2 of 0.88 and a root mean square error of prediction (RMSEP) 2%. The weight average molecular weight (M_w) determined by size exclusion chromatography (SEC) for isolated MMC PHB and pure culture PHB were 377,000 and 353,000 g/mol, respectively and smaller than commercial PHB of 1,500,000 g/mol. Thermal degradation, thermal and mechanical properties were determined by thermogravimetric analysis (TGA), differential scanning calorimetry (DSC) and tensile testing, respectively. The thermal degradation temperature for isolated PHBs all showed small variations between samples and the value was 213^oC. The melting temperature (T_m), glass transition temperature (T_g) and degree of crystallinity (X_c) for isolated PHB samples were similar at about 169^oC, 2^oC and 61%, respectively. The tensile strength, Young's modulus and elongation at break for isolated MMC PHB were 10.3 MPa, 0.4 GPa and 3% and for isolated pure culture PHB were 10.7 MPa, 0.6 GPa and 4.3%, respectively.

4.2 Introduction

Polyhydroxybutyrate (PHB), a naturally abundant polyhydroxyalkanoate (PHA), was first isolated and characterized as lipids by Lemoigne (1925-1927) (Prieto, 2007). Later, it was proved to be thermoplastic. Compared to synthetic polymers, PHB have the fundamental advantage of being renewable resources not dependent on the supply of petroleum. Moreover, it is biodegradable which can be completely digested and metabolized by a wide variety of bacteria and fungi (Krupp and Jewell, 1992). However, cheaper biodegradable plastics, such as starch-based thermoplastics, are available. The most important feature of PHB may be its hydrophobicity, which makes it exceed their biodegradable competitors in moisture resistance (de Koning, 1995). Over the past years, PHB as polymeric material has been developed into applications in plastics, medical implants, printing and packaging materials (Chen, 2009).

PHB isolated from microorganisms is a semi-crystalline material with crystallinity values in the range of 55-80% (de Koning, 1995). However, the polymer molecules within the bacteria are in their amorphous phase and exist as water-insoluble inclusions (Misra et al., 2006). Various extraction methods (Gumel et al., 2013) have been developed to preserve the native state of PHB so that it can increase the applicability of the polymer for biomedical applications. PHB crystals usually show a lamellar morphology and form spherulites when crystallized from the melt into bulk materials. PHB is a generally stiff and rigid material and has tensile strength comparable to polypropylene. Although PHB has mechanical properties comparable to those synthetically produced polyesters such as polylactides, the relatively high brittleness of crystalline PHB is still a disadvantage. It has been reported that the mechanical properties of PHB can be improved by the addition of plasticizers (Choi and Park, 2004). PHB blends with other degradable polymers also lead to higher flexibility and elongation at break (Barham and Organ, 1994; Yang et al., 2012). The properties of PHB can vary from sample to sample due to different production organisms, fermentation strategies, types of feed stock, extraction methods and sample preparation methods used (Doi et al., 1995).

FTIR spectroscopy has been used to probe PHB chemical structure. Specifically, the carbonyl band (ester linkage, C=O stretch) at 1720 cm^{-1}, is characteristic of PHB. FTIR spectroscopy has also been used for determination of PHB content (%CDW) in the biomass by modeling the spectra with PHA reference data with the aid of chemometric tool (Arcos-Hernandez, 2010). PHB molecular weight has been shown to influence polymer properties (Doi et al., 1995). The weight average molecular weights (M_w) of PHB range between 150,000 to 1,000,000 g/mol (Van der Walle et al., 2001). The T_m and T_g has been reported in a range of 160-177^oC and -4-15^oC, respectively (Misra et al., 2006). Tensile strength and tensile modulus and elongation at break have been reported between 15 and 40 MPa, 0.5 and 3.5 GPa and 1 and 6%, respectively (Van der Walle et al., 2001). Since PHB polymer properties (such as M_w and thermal/mechanical properties) reported in literature varied a lot due to different production methods, it is necessary to characterize the synthesized polymer which would help establish possible applications for the use of the bioplastic.

This study was aimed at characterizing the isolated PHB (derived from the wood hydrolysates bioreactor) biopolymer for potential applications. The diagram of PHB characterization in this study is shown in Figure 4.1. The isolated PHB was chemically characterized by a combination of FTIR spectroscopy, SEC, TGA and GC-MS analysis. The thermal and mechanical properties were examined by DSC and tensile testing, respectively.

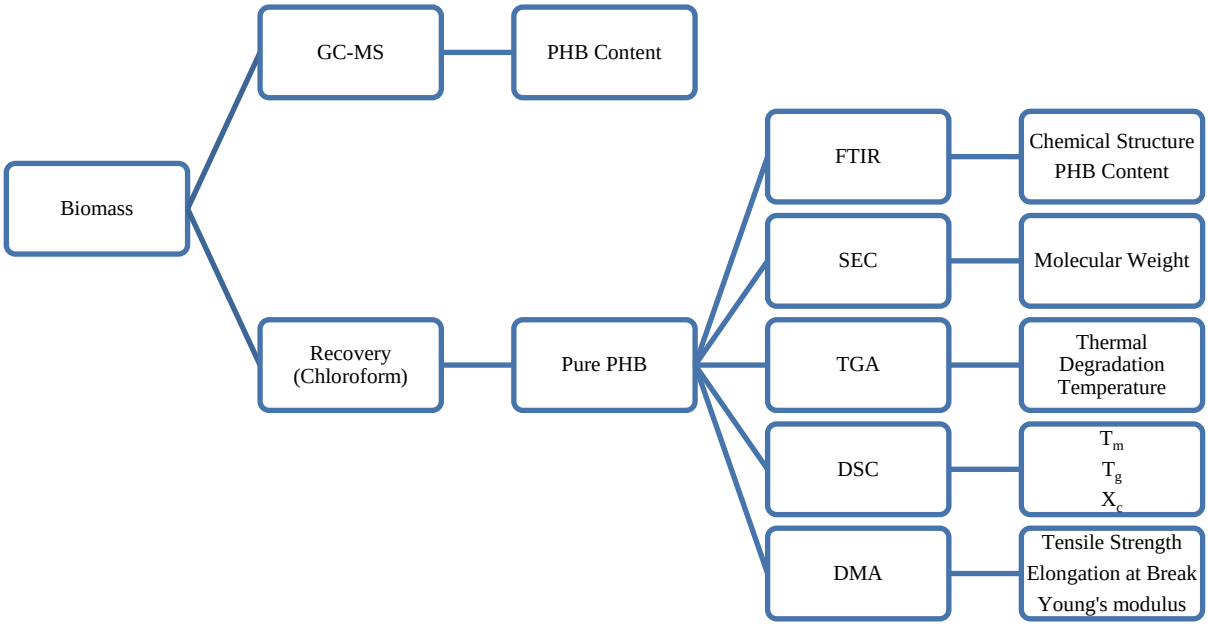

Figure 4.1 PHB characterization diagram

4.3 Materials and Methods

4.3.1 PHA Recovery

4.3.1.1 Biomass Collecting and Bioreactor Operation

Mixed Microbial Cultures (MMC) Samples

Biomass samples were collected from the waste of the factorial design bioreactors periodically when cycled. The samples were centrifuged, the supernatant discarded and the pellet washed with DI water and placed in the freezer. The biomass samples then were freeze-dried (Labconco -50^{o}C) to obtain a dry cellular mass for subsequent characterization. The operation parameters of these bioreactors are outlined in Chapter 3.3.3.

Pure Culture Samples

The bacteria *Burkholderia cepacia* ATCC 17759 used for pure culture PHB synthesis was obtained from American Type Culture Collection, Manassas, VA, USA. *B. cepacia* was revived using ATCC broth media #3 at 26^{o}C for 48 h. Culture was maintained in nutrient agar plate (ATCC agar media #3). Glycerol stock for the original bacteria was prepared and stored in the freezer. Media for culture cultivation was (g/L): NH_4Cl 0.8, beef extract 0.1, KH_2PO_4 1.0, Na_2HPO_4 0.6, $MgSO_4$ 1.0, hybrid poplar hydrolysates 10.0. Media for PHB production in fed-batch experiment was (g/L): NH_4Cl 4.0, KH_2PO_4 2.0, $MgSO_4$ 1.0, citric acid 1.0. All media had been autoclaved at 121^{o}C for 40 min. The carbon source (hydrolysates) was pasteurized separately by heating at 90^{o}C for 30 min to avoid sugar degradation.

Fed-batch reactor experiments were carried out in a 2 L Erlenmeyer flask with a working volume of 1 L. The pH was controlled from 6.5 to 7.2 with 2M NaOH during the operational cycle. The set-point of dissolved oxygen was 2 mg/L and remained uncontrolled during operational cycle. The inoculum 100 mL (10%, v/v) was prepared in a 500 mL Erlenmeyer flask containing 80 mL cultivation media. The reactor was initial fed with 20 g/L hydrolysates (667 mL), 0.93 g/L N and 0.47 g/L P (233 mL production media). The reactor was cycled every 24 h with 5 g/L (167 mL) hydrolysates and 33 mL PHB production media and run for at least 3 SRTs. The waste volume (200 mL) was taken for polymer characterization.

4.3.1.2 Polymer Extraction and Purification

PHB extraction and purification details can be found in Hu et al. (2013). Lyophilized biomass was pretreated with boiling acetone for 30 min and the acetone extract was discarded. The biomass was then transferred to a round bottom flask (500 mL), to which $CHCl_3$ (50 in volumes) was added. The biomass slurry was extracted in boiling $CHCl_3$ (45^{o}C) under constant stirring for 20 h. The obtained $CHCl_3$ extract was separated from residual biomass by filtration. PHB was precipitated by addition of five volumes of cold petroleum ether (boiling point range 35-60^{o}C) under constant stirring, recovered by

centrifugation (3500 rpm, 10 min), and vacuum dried until constant weight. Further purification was done by dissolving the isolated polymer in $CHCl_3$, filtered with glass fiber filter (Fisher Scientific G6) and dried. A commercial PHB sample used in this work was from Sigma-Aldrich (Poly[(R)-3-hydroxybutyric acid], 363502).

4.3.2 Fourier Transform Infrared (FTIR) Spectroscopy

Both isolated PHB polymers and PHB-contained biomass were analyzed by FTIR spectroscopy (Avatar 380, ThermoNicolet) using a ZnSe ATR probe. The spectrum was baseline adjusted and ATR corrected using the Omnic v9.0 software (Thermoscientific). PHB %CDW was determined by GC-MS and the method can be found in Chapter 3.3.4.3. Data analysis and model calibration was performed using the unscrambler X10.3 software (Camo).

4.3.3 Size Exclusion Chromatography (SEC)

The weight average molecular weight (M_w) of purified PHB was determined by SEC. Separation was achieved using a Jordi mixed bed DVB column (7.8 mm x 30 cm) at 40^{o}C on elution with $CHCl_3$ at 0.5 mL/min and detected by differential refractive index (Waters model 2414). Data analysis was manually performed using universal analysis with narrow polystyrene standards (PS 13,000 g/mol, PS 50,000 g/mol, PS 99,000 g/mol, PS 240,000 g/mol, PS 2,000,000 g/mol) (Temyanko et al., 2001).

4.3.4 Thermogravimetric Analysis (TGA)

Thermal stability of isolated PHB was determined by thermogravimetric analysis (TGA) (Perkin Elmer TGA-7) under nitrogen (30 mL/min) from 50 to 900^{o}C at a heating rate of 20^{o}C/min.

4.3.5 Differential Scanning Calorimetry (DSC)

Thermal analysis was conducted by differential scanning calorimetry (DSC) on a TA Instrument Q200 device equipped with refrigerated cooling. The melting temperature (T_m), glass transition temperature (T_g), and degree of crystallinity (X_c) were determined. Test procedures included first heating the sample to 190^{o}C to destroy any prethermal history, then cooling to -50^{o}C at a rate of 50^{o}C/min, and finally, reheating to 190^{o}C at a rate of 10^{o}C/min for the determination of thermal transitions. The T_m was recorded as the peak maximum of the crystalline melting peak. The T_g was recorded as the inflection point in the thermogram (Sudesh et al., 2000). The X_c was calculated by dividing fusion enthalpy by 146 J/g for 100% crystalline PHB (Barham et al., 1984). Data analysis was performed using the Universal Analysis software (TA instruments).

4.3.6 Tensile properties

Tensile properties of isolated PHB film were determined on a dynamical mechanical analyzer instrument (TA Instruments model Q800). Samples were prepared from solvent ($CHCl_3$) film-casting to typical thickness of 0.06-0.08 mm and cut into strips of 25 mm x 5 mm. Measurements were performed with a force ramp from 1 to 18 N at 1 N/min. Tensile strength, Young's modulus and elongation at break were obtained from stress-strain curve using the Universal Analysis software (TA instruments) analysis package.

4.4 Results and Discussion

4.4.1 FTIR Characterization

4.4.1.1 Chemical Structure Identification

FTIR spectroscopy was used for chemical structure identification of isolated PHB from PHB-containing biomass. The spectroscopic band assignments for PHB and biomass are summarized in Table 4.1. FTIR spectra of commercial PHB (Aldrich), isolated PHB from mixed microbial cultures (MMC) and isolated PHB from pure culture (*B. cepacia*) are shown in Figure 4.2. The spectra all reveal similar bands that have been previously associated with the presence of PHB in pure cultures (Kansiz et al., 2000/2007) at about 1730 cm^{-1}, as well as in the range from 1200 to 900 cm^{-1}. For example, Misra et al. (2000) reported key PHB bands at 1724 cm^{-1} and strong bands at 1280-1300 cm^{-1} and Hong et al. (1999) reported that a band at between 1728 cm^{-1} and 1744 cm^{-1} was characteristic of PHB. The exact location of the bands is known to vary with the crystallinity of the PHB and with the polymer chain length (Hong et al., 1999). Bands present at 1637 cm^{-1} and at 1536 cm^{-1} have been reported as Amide Band I and Amide Band II, respectively, which are associated with cellular proteins (Kansiz et al., 2000).

Table 4.1 IR absorption bands for PHB and biomass (Helm and Naumann, 1995; Arcos-Hernandez et al., 2010)

Wavenumber (cm^{-1})	Band assignment
~1735	C=O ester from PHB, lipids and fatty acids
~1650	C=O amides associated with proteins
~1540	N-H amides associated with proteins
~1455	CH_3 and CH_2 of proteins
~1398	CH_3 and CH_2 of proteins, C-O of COO^- groups
~1242	P=O phosphodiester backbone of nucleic acids
~1080	P=O phosphodiester backbone of nucleic acids
1200-900	C-O-C polysaccharides

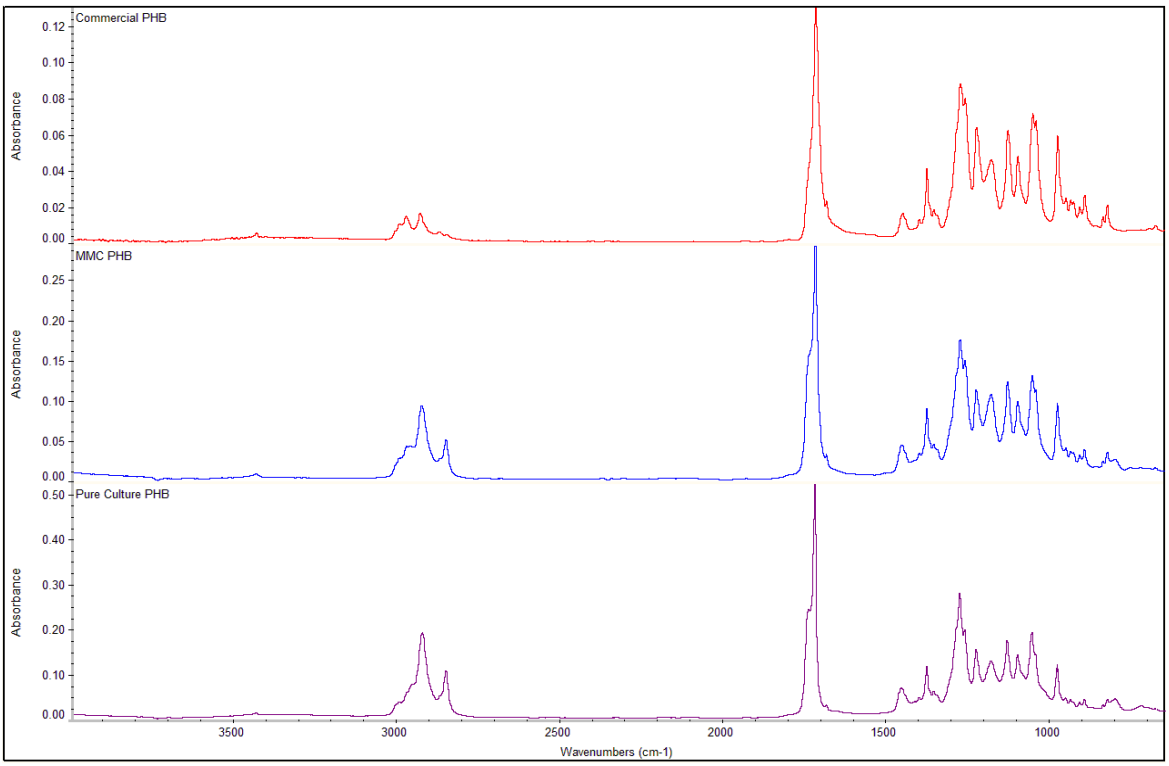

Figure 4.2 FTIR spectra of commercial PHB (top), isolated PHB from mixed microbial cultures (MMC) (middle) and isolated PHB from pure culture (*B. cepacia*) (bottom)

4.4.1.2 PHB Content Characterization by Multivariate Analysis

FTIR has been used for predicting PHB content (%CDW) in the MMC biomass in KBr pellets (Arcos-Hernandez et al., 2010) and applied this approach using the attenuated total reflection (ATR) probe in this study. A sample size of 21 was used with a PHB content range from 0 to 23%. Full range of spectra (1869 variables) after baseline and ATR correction was used as X variable and PHB content determined by GC-MS was used as Y variable. A partial least squares (PLS) regression model was generated between X and Y (Figure 4.3). The model produced 4 factors which can account for 90% variances in the data set (Figure 4.3 Explained Variance), where factor 1 explained 79% of X, 15% of Y and factor 2 explained 19% of X, 25% of Y (Figure 4.3 Scores Plot). From the regression coefficients plot, it is clear to see that band at 1730 cm^{-1} gives the strongest positive effect on the PHB content which again proves the band at 1730 cm^{-1} (C=O) was a characteristic band for PHB. Other positive effects can be found at 1390 cm^{-1} and 1450 cm^{-1} which were

associated with CH_2 and CH_3 stretches. Both amide bands at 1637 cm^{-1} and 1540 cm^{-1} show significant negative effects on PHB content indicating protein content was not responsible for PHB content in the cell. The final model was obtained from the predicted vs. reference plot in which the R^2 = 0.88 was obtained for model calibration (blue) and R^2 = 0.74 for model validation (red) plots. A root mean square error of prediction (RMSEP) of 2% was produced by the PLS regression model which gave a 2% potential error when predicting new sample set using this model. A better model calibration could be expected if the thickness of biomass samples was better controlled or by using KBr pellets in the transmission mode (Holden and Bloembergen, 1986). Finally, this study showed that FTIR-ATR spectroscopy in conjunction with multivariate analysis was a successful and robust method to quantify intracellular PHB in mixed cultures.

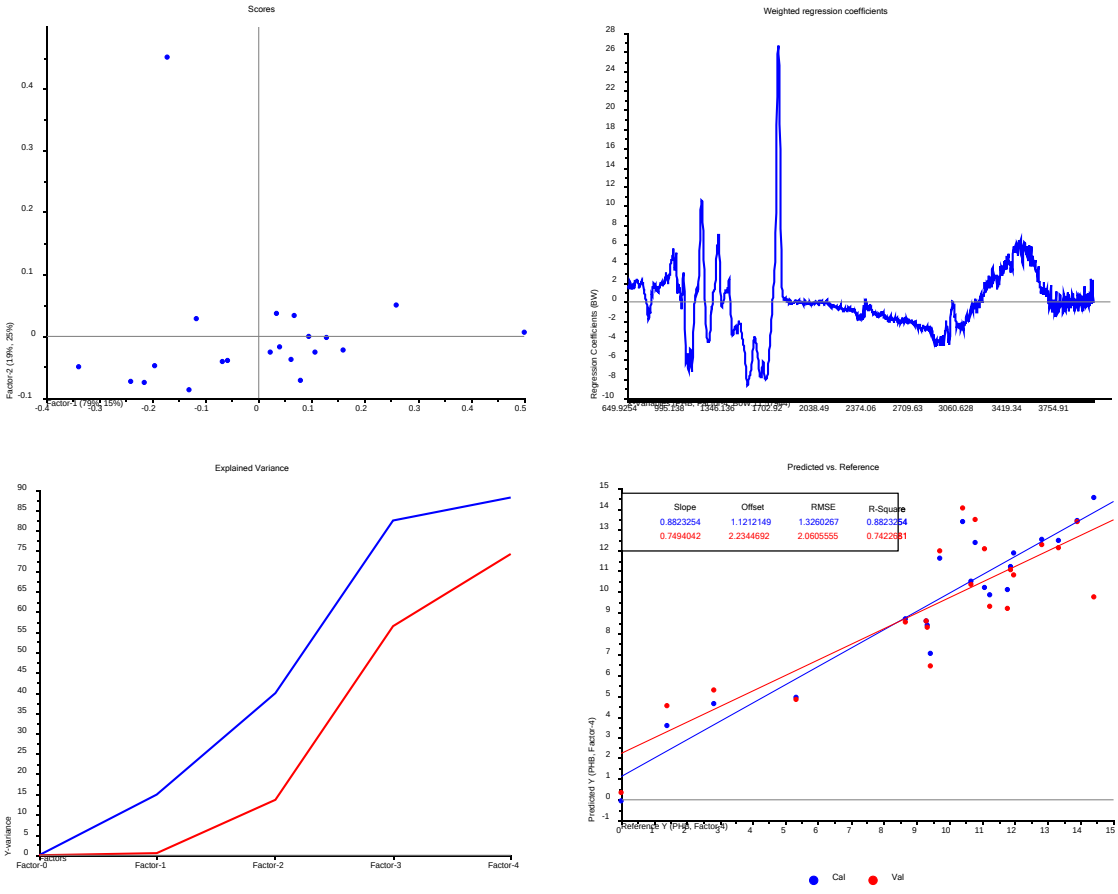

Figure 4.3 Partial least squares (PLS) model for predicting PHB content (%CDW) in MMC biomass from FTIR-ATR spectra

4.4.2 Molecular Weight Determination by SEC

The M_w of isolated MMC PHB and pure culture PHB was 377,000 and 353,000 g/mol, respectively (Table 4.2). Hu et al. (2013) reported isolated PHB derived from a crude glycerol fed bioreactor using MMCs had a M_w of 300,000 g/mol. Yezza et al (2007) gave a M_w of 310,000 g/mol for isolated PHB derived from maple sap by *A. latus* (ATCC 29714). However, some studies had reported the M_w values more than 600,000 g/mol for isolated PHB produced from pure cultures (Laycock et al., 2014). Therefore, the M_w of isolated PHB varies greatly with the type of microorganism, feed stock, production strategy and polymer extraction technique (Doi et al., 1995; Yezza et al., 2007). The M_w of polymers is believed to be one of the most important factors affecting their physicochemical and mechanical properties and thus their application (Van der Walle et al., 2001; Laycock et al., 2014). The relatively high molecular weight of PHB measured in this study suggested that the biopolymer had a degree of polymerization suitable for commercial utilization. Cox (1994) reported that the mechanical properties of PHB decreased significantly below a M_w of 400,000 g/mol and the material was quite brittle below 200,000 g/mol.

Table 4.2 Weight average molecular weight (M_w) and thermo degradation temperatures for commercial PHB, isolated MMC PHB and pure culture PHB

Sample	M_w (g/mol)	T_{onset} (^oC)	Weight begin (%)	T_{onset} end (^oC)	Weight end (%)
Commercial PHB	1,510,000	215	98	284	4
MMC PHB	377,000	214	99	255	16
Pure culture PHB	353,000	212	98	263	17

4.4.3 Thermo Degradation Analysis by TGA

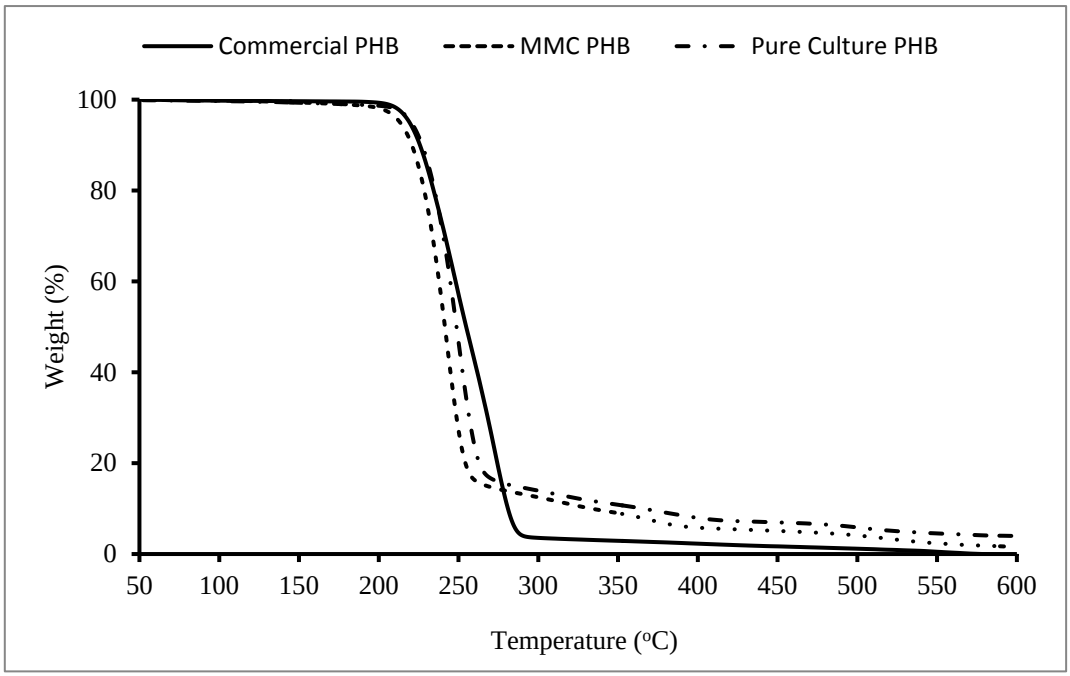

Figure 4.4 Thermogravimetric analysis of commercial PHB, isolated MMC PHB and isolated pure culture PHB

TGA thermograms of isolated PHB samples together with commercial PHB are shown in Figure 4.4. The TGA results are given in Table 4.2. All the three samples showed a similar starting degradation temperature at 213^{o}C where about 98% total weight presented. After this starting temperature, all the PHB samples showed a rapid thermal degradation between 212 and 284^{o}C. However, the end degradation temperatures for the three samples were different. Commercial PHB presented the highest end degradation temperature at 284^{o}C with 4% residual content left in the weight. MMC PHB and pure culture PHB showed similar degradation profile where the end degradation temperature was 255^{o}C with 16% residual content and 263^{o}C with 17% residual content, respectively. This result illustrates that the isolated PHB produced either from MMC or pure culture exhibited similar thermal stability and both are above the melting point of PHB. Therefore, the high decomposition temperature of isolated PHB makes them suitable for polymer processing.

4.4.4 Thermal Characterization by DSC

DSC is an extremely useful tool for the characterization of PHA materials, particularly in light of the significance of crystallization with respect to polymer properties and also the impact melting temperature (T_m), glass transition temperature (T_g) on polymer processing and thermal stability, etc (Laycock et al., 2014). Therefore, the isolated PHB samples were evaluated by DSC and typical DSC thermograms with one heating and cooling cycle on a comparison of commercial PHB, MMC PHB and pure culture PHB are shown in Figure 4.5. The thermogram of PHB shows two peaks, one is the endothermic peak and the other is the exothermic peak which represent the heat absorbance of melting of PHB crystallites and heat released during melt-crystallization of PHB, respectively. Since PHB is a semi-crystalline polymer, a relatively large crystallization peak (X_c) is able to be observed which makes DSC an adequate tool to determine crystallinity of PHB (Barham et al., 1984). The T_m and T_g of PHB were also determined by DSC (Figure 4.5). All the three samples shows similar trend in the thermogram indicates that the thermal properties of isolated PHB derived from this work are comparable to commercial PHB.

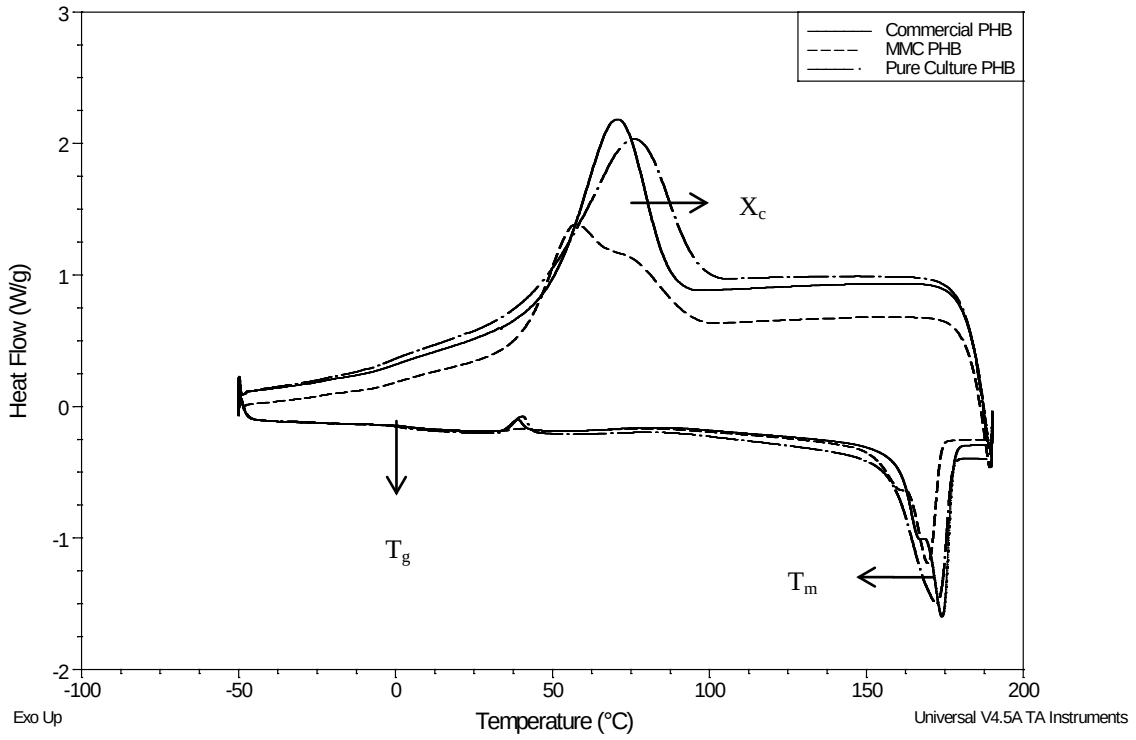

Figure 4.5 DSC thermograms (one heating and cooling cycle) of commercial PHB, isolated MMC PHB and isolated pure culture PHB

The T_m, T_g and X_c of commercial PHB, isolated MMC PHB and isolated pure culture PHB are shown in Figure 4.6. The average T_m of commercial PHB was the highest of 174^oC compared to MMC PHB 170^oC and pure culture PHB of 168^oC which was comparable at 171^oC to Li et al. (2011). The T_m was also in the range of 160-180^oC reported in literature (Laycock et al., 2014). The average T_g of isolated PHB from MMC was 2.3^oC and from pure culture was 1.8^oC, respectively, which was lower than commercial PHB of 6.5^oC. The high T_g value for the commercial PHB may be due to its high M_w (Lee, 1996). The crystallinity (X_c) for commercial PHB, isolated MMC PHB and pure culture PHB was similar to each other at 66%, 59% and 64%, respectively. The values were comparable to other works reported previously on PHB from 55 to 80% (Van der Walle et al., 2001). The

thermal properties of isolated PHB from MMC and pure culture were comparable to commercial PHB which make them both applicable for commercial utilization.

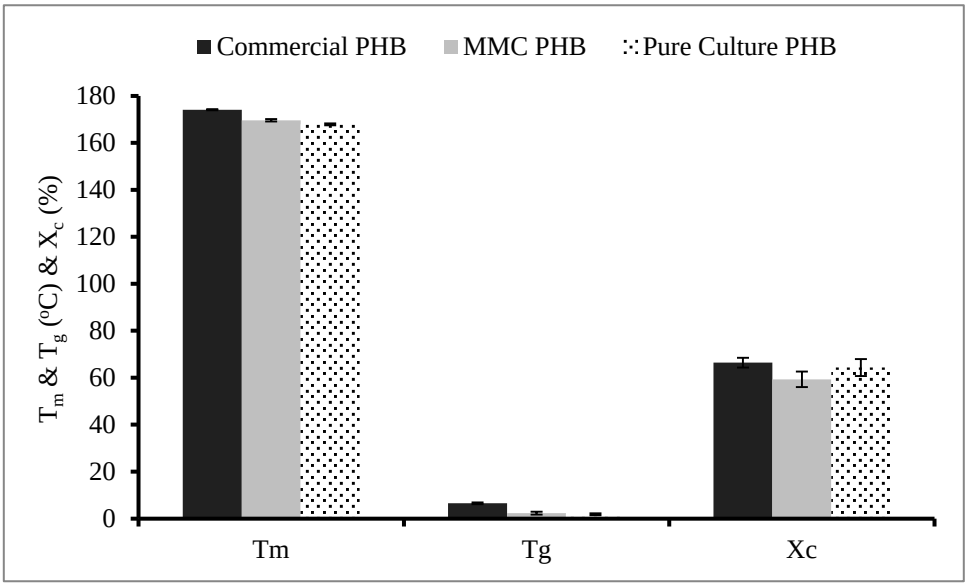

Figure 4.6 Thermal properties (T_m, T_g, X_c) of commercial PHB, isolated MMC PHB and isolated pure culture PHB

4.4.5 Mechanical Characterization by DMA

The tensile stress-strain curves of cast films from commercial PHB, isolated MMC PHB and isolated pure culture PHB are shown in Figure 4.7. The tensile strength of commercial PHB was much higher than isolated PHBs. The isolated PHB samples were shown to have a greater elongation at break values (strain of 3.0-4.5%) than the commercial PHB sample (2%). Tensile properties of the three samples are shown in Figure 4.8. The tensile strength and Young's modulus for isolated MMC PHB were 10.3 MPa and 0.4 GPa and for isolated pure culture PHB were 10.7 MPa and 0.6 GPa, respectively, which were lower than that of commercial PHB (tensile strength of 18.1 MPa and Young's modulus of 1.47 GPa). This was most likely attributable to the lower molecular weight of isolated PHB (350-370 KDa) than the commercial PHB (1500 KDa).

The M_w reported here was slightly low, and there was little variability in the thermal properties and mechanical properties. The tensile strength and Young's modulus was lower than generally reported for PHB (Laycock et al., 2014). This could be because of the lower M_w compared to commercial samples or because there was some minor contaminant or copolymeric component.

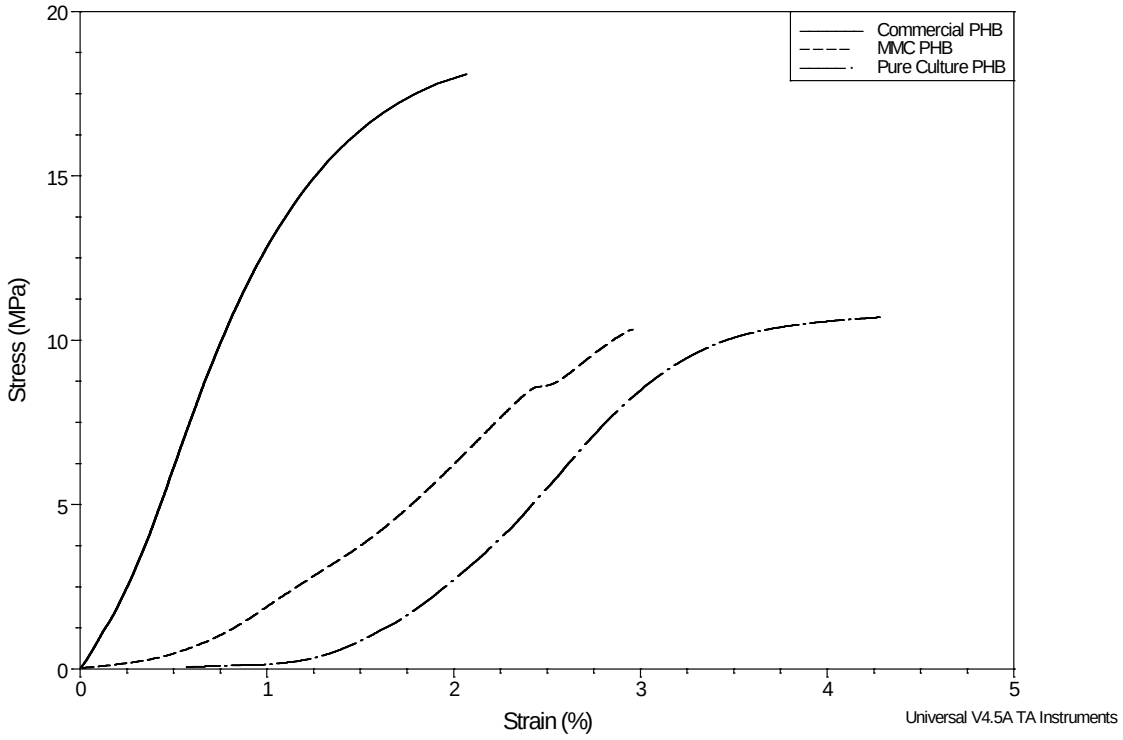

Figure 4.7 Tensile stress-strain curves of commercial PHB, isolated MMC PHB and isolated pure culture PHB

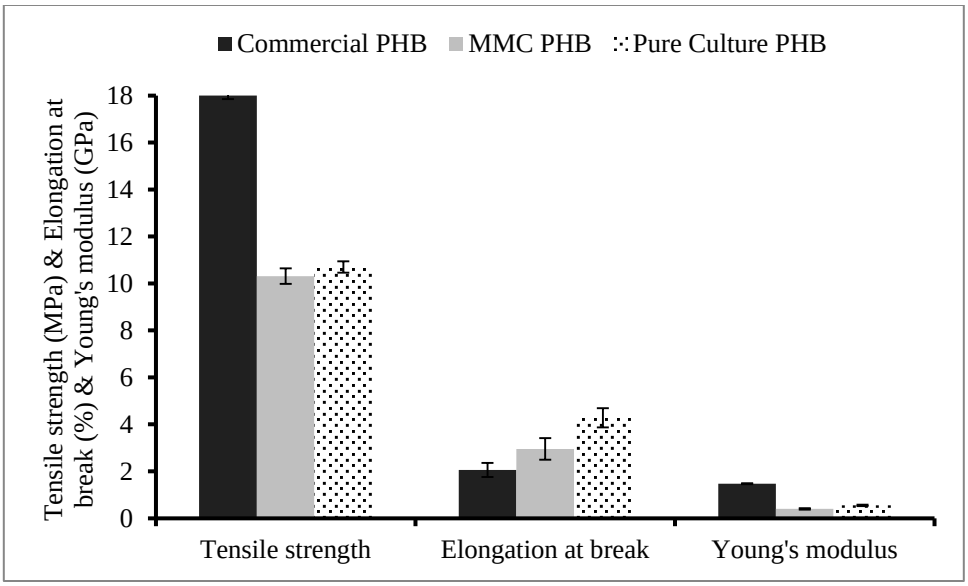

Figure 4.8 Tensile properties (tensile strength, elongation at break and Young's modulus) of commercial PHB, isolated MMC PHB and isolated pure culture PHB

4.5 Conclusions

In this study, the isolated PHB from MMC and pure culture were characterized and compared to commercial PHB. FT-IR spectroscopy was successfully used to identify the chemical structure of PHB and predict PHB content (%CDW) in the biomass. Thermal degradation, mechanical and thermal properties were tested which had similar performances to commercial PHB. However, the molecular weights of isolated PHBs were 4-5 times lower than commerical PHB. All these findings show that the polymer properties are inter-influencing each other and the final properties of the isolated biopolymer can be manipulated by the biosynthetic process where carbon source and microorganism varies.

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Chapter 5 Conclusions and Future Works

5.1 Conclusions

In this project, the conversion of wood meal to bioplastic polyhydroxybutyrate was successfully achieved. In order to release fermentable sugars from wood efficiently and make the sugars usable for further bioconversion, several key aspects need to be solved out ahead. Many pretreatment and hydrolysis methods have been reviewed for the ability to release sugars from wood (Galbe and Zacchi, 2012; Chiaramonti et al., 2012). Here, a high efficiency, less energy and chemicals input and more economically feasible together with limited or even no fermentation inhibitors generated method was needed. Therefore, the first step of conversion involved a hot-water pretreatment process followed by enzymatic hydrolysis to release the monosaccharides from lignocellulosic structure. This method was proved to be efficient to convert 95% of total carbohydrates into monomer sugars which also generated limited amount of inhibitor compounds such as acetic acid (<1.5 g/L), furfural (<0.1 g/L) and hydroxymethylfufural (HMF) (<0.1 g/L). By removing the ethanol specific portions of a biomass-to-ethanol process model, an economic analysis of a mixed sugar stream from biomass has been developed by McAloon et al. (2000). The analysis shows that a dilute mixed-sugar stream from lignocellulosic biomass is estimated to carry a required transfer price of 16.5¢/kg (7.5¢/lb) in the near-term and can be reduced to 7.05¢/kg (3.2¢/lb) with continued research and industry development. That research needs to involve improved pretreatment yields as well as increase cellulase specific activity and thermotolerance. If the lignin stream can be sold, the sugar transfer price can be reduced by 2.4¢/kg (1.1¢/lb) in the base case and 0.7¢/kg (0.3¢/lb) in the 2010 / Large Capacity case (Ruth and Wooley, 2000).

The bioconversion of wood hydrolysates to PHB was successfully performed using mixed microbial cultures (MMCs). To author's best knowledge, this is the first time that MMCs was used to utilize wood hydrolysates for PHB biosynthesis. Through the optimization of fed-batch experiments, several bioreactors could finally reach the steady states with reasonable PHB yields. A combine of feast-famine and nutrient limitation metabolic pathway was proposed for this fed-batch PHB production by MMCs. This was because both

mechanisms were observed in the fed-batch studies. First, the external carbon source TS was completely depleted and resupplied during one operational cycle, where a famine period was observed followed by excess carbon period. Second, PHB can still be accumulated when external TS depleted indicated that either the key enzyme dehydrogenase (DH) or phosphatase related to PHB formation was at suitable level so that higher carbohydrate removal and higher PHB accumulation was observed. These enzymes were regulated through the limitation of N and P (Reddy and Mohan, 2012). Therefore, PHB accumulation was driven under this unbalanced condition.

The maximum PHB accumulated was 27% (CDW) with a yield of 0.32 g/g (g PHB produced per g sugars consumed) which was comparable to other pure culture works reported (Zahari et al., 2012; Teresa Cesario et al., 2014). The utilization of wood hydrolysates had been reported to reduce 50% of PHB production cost (Keenan et al., 2006; Van-Thuoc et al., 2007). Lee (1996) reviewed that using hemicelluloses hydrolysates for the production of PHB would cost $0.34/kg PHB to yield 0.20 g PHB/ g substrate, which was much cheaper than using pure acetate ($1.56/kg PHB) as a substrate. Microbial community analysis showed that MMCs presented a community evolution in different bioreactors and during operational period. Several characterized genera belonged to *Alphaproteobacteria* and *Betaproteobacteria* classes were PHB-accumulated microorganisms. Other works using MMC had reported significantly different compositions of the microbial community. However, the carbon feed stocks were quite different using either synthetic wastewater or volatile fatty acids (Reddy and Mohan, 2012; Queiros et al., 2014). Therefore, the microbial community was characterized highly dependent on types of feed stocks and biosynthetic mechanism. All the works done above gave a proof of concept study using wood hydrolysates to biosynthesize PHB by MMCs which can be considered as a promising step for the biorefinery of lignocellulosic materials.

Since biopolymer properties vary from sample to sample, isolated MMC PHBs were characterized for determining their physical, chemical, thermal and mechanical properties which gave suggestions for potential applications. Since isolated PHBs samples were extracted from conbined biomass taken from different bioreactors during different

operational period, the values determined by SEC were in average for each culture broth. The actual M_w may have significant variations between different batches of studies. Therefore, one of the further studies can focus on the properties tunning (e.g. M_w) by these fed-batch experiments. The properties of MMC PHB were compared with isolated pure culture PHB and commercial PHB, and similar performances can be achieved for all these three samples. Therefore, the MMC PHB was proved to be a good substitute for pure culture PHB or even commercial PHB.

5.2 Future Works

This work was preliminary exploration using MMC and wood hydrolysates for PHB production. Potential future works may include the following aspects.

1. Scale-up

To scale-up the bioreactor, more works will be needed to develop such as the control of pH and dissolved oxygen level presented in the system, enzyme activities during the metabolic pathway and a fully characterization of PHB-accumulating organisms. Meanwhile, the utilization of pure culture PHB production needs further investigations on how nutrients composition influence PHB accumulation and potential inhibitor effects on the culture.

2. Economic and engineering analysis

A valorization analysis for a comparison of MMC and pure culture PHB production using wood hydrolysates may be of further interests.

3. Large scale sample characterization and process assessment

In order to test the polymer properties in real application, large sample specimen are highly recommended. Also, real molded products are expected and the production process should be evaluated.

4. The control of polymer properties

The tune of polymer properties will be of great interests such as the control of molecular weight. The studies of the variation of microorganisms, feed stocks and bioreactor operation may have significant effect on final polymer properties.

5. Improving the chemomechanical properties of PHB

To improve the chemomechanical properties of PHB and enlarge its application areas, two orientations can be considered for further investigations. First, odd number HV precursor (e.g. propionic acid) can be introduced to the sugar feed stock for a random or block copolymer polyhydroxybutyrate-*co*-hydroxyvalerate (PHBV) production by either MMC or pure culture (Pederson et al., 2006). Second, the study of PHB blends with other low molecular weight thermoplastics or other PHA copolymers (e.g. PHBV) will be necessary to improve the properties of PHB (Barham and Organ, 1994; Yang et al., 2012).

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Appendix 1 Taxa identified by illumina 16S rRNA sequencing

Taxon Name	Level	Seed	A	B	SA	WA
Acetobacter	genus	0.001	0.001	0.000	0.001	0.000
Acetobacteraceae	family	0.001	0.001	0.000	0.002	0.001
Achromobacter	genus	0.000	0.028	0.014	0.002	0.061
Acidimicrobiales	order	0.002	0.000	0.000	0.000	0.000
Acidovorax	genus	0.011	0.030	0.013	0.010	0.001
Acinetobacter	genus	0.001	0.000	0.000	0.000	0.000
Actinobacteria	class	0.001	0.000	0.000	0.000	0.000
Actinobaculum	genus	0.002	0.002	0.002	0.000	0.000
Actinomycetaceae	family	0.001	0.000	0.000	0.000	0.000
Actinomycetales	order	0.119	0.006	0.017	0.006	0.001
Aeromicrobium	genus	0.000	0.000	0.000	0.000	0.000
Aeromonas	genus	0.001	0.000	0.000	0.000	0.000
Afipia	genus	0.002	0.001	0.002	0.002	0.001
Agaricicola	genus	0.000	0.000	0.000	0.000	0.003
Agrococcus	genus	0.000	0.008	0.014	0.001	0.015
Albidiferax	genus	0.002	0.001	0.000	0.001	0.000
Alcaligenaceae	family	0.000	0.001	0.000	0.000	0.001
Alicycliphilus	genus	0.000	0.001	0.000	0.000	0.000
Alphaproteobacteria	class	0.009	0.008	0.004	0.035	0.004
Amaricoccus	genus	0.001	0.009	0.011	0.050	0.003
Aminobacter	genus	0.001	0.000	0.000	0.000	0.000
Anaerolineaceae	family	0.002	0.000	0.000	0.001	0.000
Ancylobacter	genus	0.000	0.001	0.001	0.001	0.001
Angustibacter	genus	0.002	0.000	0.000	0.000	0.000
Aquabacterium	genus	0.003	0.002	0.001	0.002	0.000
Aquamicrobium	genus	0.000	0.000	0.000	0.000	0.002
Aquiflexum	genus	0.004	0.000	0.000	0.000	0.000
Aquimonas	genus	0.001	0.001	0.004	0.002	0.000
Aquincola	genus	0.001	0.013	0.002	0.003	0.000
Armatimonadetes_gp5	genus	0.001	0.000	0.001	0.000	0.000
Armatimonas/Armatimonadetes_gp1	genus	0.000	0.001	0.000	0.002	0.000
Arsenicicoccus	genus	0.000	0.000	0.001	0.000	0.000
Arthrobacter	genus	0.000	0.005	0.011	0.045	0.002
Aspromonas	genus	0.000	0.000	0.000	0.000	0.000
Aurantimonas	genus	0.000	0.000	0.000	0.000	0.000
Azohydromonas	genus	0.008	0.002	0.001	0.012	0.000
Azospira	genus	0.003	0.000	0.000	0.000	0.000
Azospirillum	genus	0.000	0.002	0.003	0.001	0.000

Bacillus	genus	0.000	0.002	0.004	0.001	0.000
Bacteria	domain	0.020	0.005	0.017	0.016	0.002
Bacteriovorax	genus	0.000	0.001	0.001	0.000	0.000
Bacteroidetes	phylum	0.003	0.001	0.000	0.002	0.000
Balneimonas	genus	0.000	0.000	0.001	0.000	0.000
Bauldia	genus	0.001	0.000	0.000	0.001	0.000
Bdellovibrio	genus	0.002	0.013	0.009	0.003	0.000
Bdellovibrionales	order	0.000	0.000	0.000	0.002	0.000
Beijerinckia	genus	0.000	0.001	0.000	0.000	0.000
Beijerinckiaceae	family	0.001	0.000	0.001	0.000	0.000
Bellilinea	genus	0.000	0.000	0.000	0.000	0.000
Betaproteobacteria	class	0.007	0.004	0.003	0.002	0.000
Bifidobacterium	genus	0.001	0.000	0.000	0.000	0.000
Blastochloris	genus	0.001	0.001	0.001	0.000	0.000
Blastomonas	genus	0.000	0.000	0.000	0.001	0.000
Blautia	genus	0.001	0.000	0.000	0.000	0.000
Bosea	genus	0.001	0.012	0.015	0.007	0.023
Bradyrhizobiaceae	family	0.001	0.001	0.001	0.001	0.000
Bradyrhizobium	genus	0.002	0.001	0.000	0.000	0.000
Brevundimonas	genus	0.000	0.010	0.013	0.004	0.027
Brooklawnia	genus	0.001	0.000	0.001	0.000	0.001
Burkholderiales	order	0.007	0.006	0.006	0.011	0.003
Burkholderiales_incertae_sedis	family	0.013	0.010	0.003	0.008	0.001
Byssovorax	genus	0.002	0.001	0.000	0.006	0.000
Caenimonas	genus	0.029	0.004	0.004	0.002	0.001
Caldilinea	genus	0.016	0.001	0.003	0.003	0.000
Catellibacterium	genus	0.001	0.002	0.005	0.002	0.000
Caulobacter	genus	0.003	0.001	0.001	0.004	0.003
Caulobacteraceae	family	0.000	0.000	0.000	0.000	0.000
Cellulomonas	genus	0.000	0.002	0.005	0.000	0.001
Cellulosimicrobium	genus	0.000	0.001	0.002	0.000	0.000
Cellvibrio	genus	0.000	0.000	0.000	0.001	0.000
Cesiribacter	genus	0.001	0.001	0.004	0.001	0.000
Chelativorans	genus	0.000	0.000	0.000	0.000	0.000
Chitinophaga	genus	0.000	0.001	0.000	0.000	0.000
Chitinophagaceae	family	0.003	0.004	0.004	0.006	0.004
Chloroflexaceae	family	0.007	0.001	0.002	0.008	0.000
Chloroflexi	phylum	0.002	0.000	0.001	0.005	0.000
Chlorophyta	genus	0.000	0.000	0.000	0.000	0.001
Chondromyces	genus	0.003	0.000	0.000	0.002	0.000
Chromatiales	order	0.001	0.000	0.000	0.000	0.000
Chryseobacterium	genus	0.000	0.000	0.000	0.000	0.048
Citrobacter	genus	0.000	0.002	0.002	0.000	0.000

Cloacibacterium	genus	0.001	0.001	0.000	0.000	0.000
Clostridiales	order	0.001	0.000	0.000	0.000	0.000
Clostridium sensu stricto	genus	0.002	0.000	0.000	0.000	0.000
Clostridium XI	genus	0.009	0.000	0.001	0.000	0.000
Clostridium XIX	genus	0.000	0.000	0.000	0.000	0.000
Collinsella	genus	0.001	0.000	0.000	0.000	0.000
Comamonadaceae	family	0.036	0.022	0.006	0.019	0.012
Comamonas	genus	0.001	0.034	0.005	0.011	0.037
Conexibacter	genus	0.002	0.000	0.001	0.000	0.000
Corynebacterium	genus	0.000	0.000	0.000	0.000	0.000
Cryomorphaceae	family	0.003	0.002	0.001	0.001	0.000
Cupriavidus	genus	0.000	0.001	0.000	0.000	0.000
Curtobacterium	genus	0.000	0.000	0.000	0.000	0.001
Curvibacter	genus	0.005	0.003	0.001	0.008	0.002
Cystobacteraceae	family	0.000	0.001	0.000	0.001	0.000
Cytophagaceae	family	0.002	0.002	0.001	0.001	0.002
Daeguia	genus	0.000	0.000	0.000	0.000	0.000
Dechloromonas	genus	0.016	0.000	0.000	0.000	0.000
Defluviicoccus	genus	0.006	0.002	0.002	0.001	0.000
Delftia	genus	0.000	0.005	0.000	0.000	0.001
Deltaproteobacteria	class	0.007	0.007	0.008	0.008	0.000
Demequina	genus	0.001	0.000	0.000	0.000	0.000
Dermatophilaceae	family	0.000	0.000	0.001	0.002	0.000
Derxia	genus	0.011	0.002	0.002	0.001	0.000
Devosia	genus	0.002	0.004	0.009	0.009	0.020
Diaphorobacter	genus	0.002	0.017	0.004	0.003	0.000
Dokdonella	genus	0.002	0.002	0.005	0.024	0.001
Dongia	genus	0.001	0.001	0.000	0.002	0.000
Duganella	genus	0.000	0.001	0.000	0.000	0.000
Dyadobacter	genus	0.000	0.001	0.001	0.001	0.001
Emticicia	genus	0.000	0.000	0.000	0.001	0.000
Enhygromyxa	genus	0.000	0.001	0.000	0.002	0.000
Ensifer	genus	0.000	0.003	0.002	0.000	0.004
Enterobacter	genus	0.000	0.000	0.000	0.000	0.000
Enterobacteriaceae	family	0.000	0.000	0.000	0.000	0.000
Enterococcus	genus	0.000	0.000	0.001	0.002	0.000
Exiguobacterium	genus	0.000	0.000	0.000	0.000	0.000
Ferribacterium	genus	0.001	0.000	0.000	0.000	0.000
Ferrithrix	genus	0.001	0.000	0.000	0.000	0.000
Ferruginibacter	genus	0.004	0.010	0.007	0.002	0.000
Fibrisoma	genus	0.000	0.000	0.000	0.006	0.000
Filimonas	genus	0.000	0.000	0.000	0.001	0.010
Filomicrobium	genus	0.001	0.000	0.001	0.000	0.000

Flammeovirgaceae	family	0.000	0.000	0.000	0.000	0.001
Flavisolibacter	genus	0.000	0.000	0.000	0.000	0.000
Flavitalea	genus	0.000	0.000	0.000	0.000	0.000
Flavobacteriaceae	family	0.002	0.002	0.001	0.003	0.001
Flavobacteriales	order	0.004	0.001	0.002	0.001	0.000
Flavobacterium	genus	0.012	0.019	0.004	0.004	0.016
Flectobacillus	genus	0.000	0.000	0.000	0.031	0.003
Fluviicola	genus	0.000	0.000	0.000	0.000	0.000
Gammaproteobacteria	class	0.020	0.001	0.001	0.001	0.000
Geminicoccus	genus	0.001	0.001	0.001	0.003	0.000
Gemmata	genus	0.005	0.001	0.002	0.004	0.000
Gemmatimonas	genus	0.000	0.000	0.000	0.001	0.000
Gemmobacter	genus	0.001	0.000	0.000	0.002	0.000
Giesbergeria	genus	0.009	0.000	0.000	0.000	0.000
Gordonia	genus	0.001	0.000	0.003	0.000	0.000
Gp10	genus	0.000	0.000	0.000	0.000	0.000
Gp16	genus	0.001	0.000	0.000	0.000	0.000
Gp4	genus	0.001	0.002	0.007	0.000	0.000
Gp6	genus	0.001	0.000	0.000	0.000	0.000
Haematobacter	genus	0.000	0.000	0.000	0.000	0.001
Haliangium	genus	0.002	0.000	0.000	0.000	0.000
Haliea	genus	0.001	0.000	0.000	0.000	0.000
Haliscomenobacter	genus	0.008	0.001	0.004	0.002	0.000
Haloferula	genus	0.000	0.000	0.001	0.001	0.000
Herbaspirillum	genus	0.000	0.003	0.000	0.000	0.000
Hoeflea	genus	0.000	0.000	0.000	0.000	0.000
Humibacter	genus	0.000	0.000	0.000	0.000	0.001
Hyalangium	genus	0.000	0.003	0.005	0.001	0.000
Hydrogenophaga	genus	0.001	0.016	0.003	0.012	0.018
Hyphomicrobiaceae	family	0.003	0.001	0.002	0.000	0.000
Hyphomicrobium	genus	0.019	0.006	0.013	0.002	0.002
Hyphomonadaceae	family	0.000	0.000	0.000	0.000	0.000
Iamia	genus	0.001	0.000	0.000	0.000	0.000
Ideonella	genus	0.025	0.007	0.002	0.007	0.000
Ilumatobacter	genus	0.009	0.001	0.003	0.000	0.000
Inhella	genus	0.002	0.001	0.000	0.000	0.000
Intrasporangiaceae	family	0.001	0.000	0.000	0.000	0.000
Janthinobacterium	genus	0.000	0.001	0.000	0.000	0.000
Kaistia	genus	0.000	0.005	0.001	0.002	0.001
Ketogulonicigenium	genus	0.000	0.000	0.000	0.002	0.000
Kineosphaera	genus	0.000	0.000	0.000	0.000	0.000
Kinneretia	genus	0.000	0.003	0.000	0.000	0.000
Klebsiella	genus	0.000	0.001	0.000	0.000	0.000

Kofleria	genus	0.011	0.003	0.001	0.008	0.000
Labrys	genus	0.000	0.000	0.000	0.000	0.000
Lachnospiracea_incertae_sedis	genus	0.001	0.000	0.000	0.000	0.000
Lachnospiraceae	family	0.001	0.000	0.000	0.000	0.000
Lactobacillus	genus	0.000	0.000	0.000	0.001	0.000
Lactococcus	genus	0.001	0.001	0.001	0.000	0.000
Larkinella	genus	0.000	0.001	0.001	0.001	0.000
Leadbetterella	genus	0.000	0.001	0.002	0.002	0.019
Legionella	genus	0.000	0.001	0.000	0.000	0.000
Leifsonia	genus	0.001	0.002	0.011	0.001	0.000
Leucobacter	genus	0.001	0.006	0.022	0.001	0.005
Limnohabitans	genus	0.006	0.000	0.000	0.000	0.000
Luteimonas	genus	0.000	0.002	0.002	0.001	0.000
Luteococcus	genus	0.001	0.001	0.001	0.000	0.000
Luteolibacter	genus	0.000	0.001	0.003	0.000	0.000
Lysinibacillus	genus	0.000	0.000	0.001	0.000	0.000
Lysobacter	genus	0.000	0.000	0.001	0.000	0.000
Marmoricola	genus	0.001	0.000	0.000	0.000	0.000
Massilia	genus	0.000	0.001	0.000	0.000	0.000
Meganema	genus	0.001	0.000	0.000	0.000	0.000
Mesorhizobium	genus	0.002	0.002	0.003	0.000	0.000
Methylocapsa	genus	0.000	0.001	0.002	0.000	0.000
Methylococcus	genus	0.000	0.000	0.001	0.000	0.000
Methylocystis	genus	0.003	0.001	0.001	0.000	0.000
Methyloversatilis	genus	0.001	0.000	0.000	0.000	0.000
Microbacteriaceae	family	0.001	0.009	0.018	0.001	0.012
Microbacterium	genus	0.003	0.065	0.074	0.009	0.180
Micrococcaceae	family	0.000	0.000	0.001	0.004	0.000
Microlunatus	genus	0.001	0.000	0.001	0.000	0.000
Micropruina	genus	0.005	0.007	0.044	0.022	0.004
Microvirga	genus	0.000	0.001	0.002	0.000	0.000
Miniimonas	genus	0.000	0.000	0.001	0.000	0.000
Mitsuaria	genus	0.000	0.001	0.000	0.000	0.000
Mobilicoccus	genus	0.000	0.000	0.001	0.005	0.001
Mucilaginibacter	genus	0.000	0.000	0.000	0.000	0.000
Mycobacterium	genus	0.024	0.002	0.005	0.001	0.000
Myxococcales	order	0.020	0.025	0.002	0.013	0.000
Nakamurella	genus	0.001	0.002	0.012	0.014	0.003
Nannocystaceae	family	0.000	0.001	0.000	0.001	0.000
Nannocystis	genus	0.000	0.005	0.001	0.002	0.000
Niabella	genus	0.000	0.000	0.002	0.001	0.003
Nitrobacter	genus	0.001	0.000	0.000	0.000	0.000
Nitrosomonas	genus	0.001	0.000	0.000	0.000	0.000

Nitrospira	genus	0.021	0.000	0.000	0.000	0.000
Nocardioidaceae	family	0.001	0.000	0.000	0.000	0.000
Nocardioides	genus	0.008	0.000	0.003	0.001	0.000
Novosphingobium	genus	0.000	0.024	0.000	0.003	0.000
Nubsella	genus	0.000	0.000	0.000	0.000	0.001
Ochrobactrum	genus	0.000	0.005	0.005	0.000	0.001
Ohtaekwangia	genus	0.002	0.001	0.000	0.006	0.000
Opitutus	genus	0.000	0.000	0.000	0.003	0.000
Orientia	genus	0.001	0.000	0.000	0.000	0.000
Ornithinibacter	genus	0.000	0.000	0.000	0.000	0.000
Oscillibacter	genus	0.001	0.000	0.000	0.000	0.000
Ottowia	genus	0.000	0.000	0.001	0.000	0.000
Parabacteroides	genus	0.000	0.000	0.000	0.000	0.000
Paracoccus	genus	0.005	0.033	0.043	0.051	0.009
Pasteuria	genus	0.002	0.000	0.002	0.000	0.000
Pedobacter	genus	0.000	0.002	0.000	0.002	0.004
Pedomicrobium	genus	0.003	0.001	0.001	0.001	0.000
Pelomonas	genus	0.000	0.002	0.000	0.001	0.002
Persicitalea	genus	0.000	0.013	0.002	0.000	0.001
Phaselicystis	genus	0.000	0.000	0.000	0.002	0.000
Phenylobacterium	genus	0.001	0.001	0.001	0.000	0.003
Phycisphaera	genus	0.004	0.001	0.002	0.001	0.000
Phyllobacteriaceae	family	0.001	0.001	0.002	0.001	0.000
Phyllobacterium	genus	0.000	0.000	0.001	0.000	0.000
Pigmentiphaga	genus	0.000	0.000	0.000	0.000	0.001
Pimelobacter	genus	0.002	0.000	0.001	0.000	0.000
Piscicoccus	genus	0.000	0.000	0.001	0.000	0.000
Piscinibacter	genus	0.001	0.000	0.000	0.000	0.000
Planctomyces	genus	0.003	0.000	0.002	0.000	0.000
Planctomycetaceae	family	0.003	0.001	0.001	0.000	0.000
Planococcaceae_incertae_sedis	genus	0.000	0.000	0.000	0.000	0.000
Plantibacter	genus	0.000	0.000	0.000	0.000	0.001
Polaromonas	genus	0.000	0.000	0.000	0.000	0.000
Polyangiaceae	family	0.013	0.004	0.001	0.027	0.001
Propionibacteriaceae	family	0.002	0.002	0.010	0.002	0.000
Propionicimonas	genus	0.001	0.001	0.003	0.001	0.000
Propionivibrio	genus	0.035	0.001	0.000	0.000	0.000
Prosthecobacter	genus	0.001	0.001	0.001	0.003	0.000
Proteobacteria	phylum	0.012	0.027	0.034	0.011	0.002
Pseudochrobactrum	genus	0.000	0.001	0.002	0.000	0.000
Pseudoclavibacter	genus	0.001	0.000	0.001	0.000	0.000
Pseudomonadaceae	family	0.000	0.000	0.000	0.000	0.000
Pseudomonas	genus	0.000	0.003	0.001	0.007	0.008

Pseudorhodoferax	genus	0.007	0.024	0.004	0.025	0.122
Pseudoxanthomonas	genus	0.001	0.024	0.014	0.009	0.001
Ramlibacter	genus	0.001	0.000	0.000	0.000	0.000
Raoultella	genus	0.000	0.000	0.000	0.000	0.000
Rarobacter	genus	0.000	0.000	0.001	0.000	0.000
Rathayibacter	genus	0.000	0.000	0.000	0.001	0.007
Rhizobacter	genus	0.006	0.002	0.001	0.001	0.000
Rhizobiaceae	family	0.000	0.000	0.000	0.001	0.001
Rhizobiales	order	0.025	0.021	0.019	0.034	0.004
Rhizobium	genus	0.002	0.023	0.008	0.014	0.056
Rhizomicrobium	genus	0.001	0.000	0.000	0.001	0.000
Rhodobacter	genus	0.017	0.021	0.012	0.012	0.008
Rhodobacteraceae	family	0.009	0.011	0.007	0.021	0.004
Rhodococcus	genus	0.000	0.000	0.001	0.000	0.000
Rhodocyclaceae	family	0.011	0.001	0.000	0.000	0.000
Rhodoferax	genus	0.014	0.002	0.000	0.000	0.000
Rhodopirellula	genus	0.000	0.000	0.000	0.000	0.000
Rhodospirillaceae	family	0.002	0.001	0.001	0.001	0.000
Rhodospirillales	order	0.001	0.001	0.000	0.002	0.000
Rhodovarius	genus	0.000	0.000	0.000	0.008	0.001
Riemerella	genus	0.000	0.000	0.000	0.000	0.003
Rivibacter	genus	0.000	0.000	0.000	0.002	0.000
Roseicyclus	genus	0.000	0.001	0.001	0.000	0.000
Roseiflexus	genus	0.001	0.000	0.000	0.001	0.000
Roseomonas	genus	0.001	0.006	0.008	0.014	0.042
Rubrivivax	genus	0.001	0.001	0.000	0.001	0.000
Rudaea	genus	0.000	0.000	0.000	0.000	0.000
Ruminococcaceae	family	0.001	0.000	0.000	0.000	0.000
Runella	genus	0.005	0.019	0.032	0.006	0.001
Salana	genus	0.000	0.000	0.001	0.000	0.000
Sanguibacter	genus	0.000	0.000	0.001	0.000	0.000
Saprospiraceae	family	0.001	0.000	0.001	0.000	0.000
Schlegelella	genus	0.001	0.000	0.000	0.000	0.000
Schlesneria	genus	0.002	0.000	0.001	0.000	0.000
Sediminibacterium	genus	0.000	0.000	0.000	0.000	0.000
Segetibacter	genus	0.000	0.000	0.000	0.002	0.000
Shinella	genus	0.001	0.002	0.005	0.002	0.015
Simplicispira	genus	0.001	0.001	0.000	0.000	0.000
Singulisphaera	genus	0.000	0.001	0.001	0.001	0.000
Sinorhizobium	genus	0.000	0.000	0.000	0.000	0.000
Siphonobacter	genus	0.000	0.000	0.000	0.000	0.001
Skermania	genus	0.001	0.000	0.000	0.000	0.000
Solibacillus	genus	0.000	0.000	0.001	0.000	0.000

Sorangium	genus	0.005	0.001	0.000	0.006	0.000
Spartobacteria_genera_incertae_sedis	genus	0.000	0.001	0.000	0.001	0.000
Sphaerobacter	genus	0.000	0.000	0.000	0.000	0.000
Sphaerotilus	genus	0.000	0.007	0.001	0.001	0.000
Sphingobacteriales	order	0.006	0.009	0.002	0.008	0.000
Sphingobacterium	genus	0.000	0.002	0.000	0.000	0.006
Sphingobium	genus	0.001	0.007	0.022	0.017	0.005
Sphingomonadaceae	family	0.001	0.001	0.002	0.002	0.000
Sphingomonadales	order	0.000	0.000	0.000	0.000	0.000
Sphingomonas	genus	0.002	0.001	0.003	0.003	0.001
Sphingopyxis	genus	0.002	0.000	0.000	0.000	0.000
Spirosoma	genus	0.000	0.000	0.000	0.001	0.000
Sporichthya	genus	0.001	0.000	0.000	0.000	0.000
SR1_genera_incertae_sedis	genus	0.000	0.000	0.000	0.000	0.000
Stella	genus	0.000	0.001	0.000	0.005	0.000
Stenotrophomonas	genus	0.000	0.023	0.011	0.002	0.001
Steroidobacter	genus	0.001	0.000	0.000	0.000	0.000
Streptobacillus	genus	0.001	0.000	0.000	0.000	0.000
Streptococcus	genus	0.001	0.000	0.000	0.002	0.000
Subdivision3_genera_incertae_sedis	genus	0.003	0.000	0.000	0.000	0.000
Subdoligranulum	genus	0.001	0.000	0.000	0.000	0.000
Sulfuritalea	genus	0.011	0.000	0.000	0.000	0.000
Terrimonas	genus	0.003	0.002	0.001	0.002	0.000
Tessaracoccus	genus	0.004	0.004	0.011	0.000	0.000
Tetrasphaera	genus	0.021	0.000	0.003	0.001	0.000
Thauera	genus	0.002	0.000	0.000	0.000	0.000
Thermomonas	genus	0.002	0.002	0.003	0.001	0.000
Thiobacter	genus	0.002	0.000	0.000	0.000	0.000
TM7_genera_incertae_sedis	genus	0.002	0.029	0.073	0.040	0.078
Trichococcus	genus	0.004	0.033	0.051	0.002	0.000
Turicibacter	genus	0.001	0.000	0.000	0.000	0.000
Turneriella	genus	0.000	0.000	0.000	0.001	0.000
Uruburuella	genus	0.002	0.000	0.000	0.000	0.000
Variovorax	genus	0.000	0.003	0.000	0.019	0.004
Vasilyevaea	genus	0.000	0.004	0.003	0.008	0.002
Verrucomicrobium	genus	0.000	0.001	0.000	0.006	0.000
Vitreoscilla	genus	0.001	0.000	0.000	0.000	0.000
Wandonia	genus	0.000	0.000	0.001	0.001	0.000
Wautersiella	genus	0.000	0.000	0.000	0.000	0.000
Xanthobacter	genus	0.000	0.002	0.002	0.004	0.002
Xanthobacteraceae	family	0.000	0.000	0.000	0.000	0.000
Xanthomonadaceae	family	0.004	0.001	0.003	0.005	0.000
Xenophilus	genus	0.000	0.001	0.000	0.002	0.001

Zavarzinella	genus	0.005	0.000	0.001	0.001	0.000
Zhihengliuella	genus	0.000	0.000	0.000	0.001	0.000
Zoogloea	genus	0.005	0.000	0.000	0.001	0.000
Unknown	Root	0.001	0.000	0.001	0.000	0.000
Others		0.022	0.009	0.011	0.008	0.004
Sample Counts		54743	44405	70572	10525	62776