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Adding Value to Lignocellulosic Biorefinery: Efficient Process Development of Lignocellulosic Biomass Conversion into Polyhydroxybutyrate

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ADDING VALUE TO LIGNOCELLULOSIC BIOREFINERY: EFFICIENT PROCESS DEVELOPMENT OF LIGNOCELLULOSIC BIOMASS CONVERSION INTO POLYHYDROXYBUTYRATE

by

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ADDING VALUE TO LIGNOCELLULOSIC BIOREFINERY: EFFICIENT PROCESS DEVELOPMENT OF LIGNOCELLULOSIC BIOMASS CONVERSION INTO POLYHYDROXYBUTYRATE

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Polyhydroxybutyrate (PHB) is bacteria synthesized polymer that has comparable mechanical properties as petroleum-based plastics and high biocompatibility. Current commercial PHB production process is not cost effective. Raw materials make up about 50% of the production cost. Lignocellulosic biomass are cheap, abundant feedstocks that can be converted into PHB to add profit and sustainability to lignocellulosic biorefinery. Lignocellulosic biorefinery upstream process produces polymeric sugar rich stream and lignin-enriched stream. Polymeric sugars are then hydrolyzed into a sugar stream with glucose, xyloseand arabinose mainly present. To the best of the author's knowledge, limited studies have been done on sugar mixture conversion into PHB. For lignin, previous research achieved a PHB production ranging from 0.13 to 1 g/L, which is too low to be economical. The primary objectives of this research were twofold: (1) process development of polymeric sugars conversion into PHB, with a focus on sugar mixture conversion into PHB by *Burkholderia sacchari* DSM 17165, and (2) process development of lignin into PHB by *Cupriavidus necator* DSM 545, with a focus on enhancing PHB production using various types of supplements. For sugar mixture conversion into PHB, first, shake flask (250 mL) scale statistical experimental design and modeling were performed to optimize sugar mixture ratio and process variables for maximal PHB production; second, bioreactor scale (3L) fed-batch cultivation was conducted to produce PHB from simulated corn fiber sugar mixture. The highest PHB production reached 67 g/L for 4:2:1 (glucose:xylose:arabinose) mixture at 41 h

corresponding to an accumulation of 77% of cell dry weight. Corresponding sugar conversion efficiency and productivity were 0.33 g PHB/g sugar consumed and 1.6 g/L/h, respectively, which are comparable to or higher than most previous studies. For lignin conversion into PHB, first, shake scale (250 mL) study achieved 10-fold increase (0.2 to 2.1 g/L) in PHB production by optimizing supplement formulations with Plackett-Burman and central composite designs. Second, fed-batch cultivation at bioreactor scale (1.7 L) were conducted to enhance PHB production to 4.5 g/L. This is the highest PHB production from lignin that the author has been aware of in the literature.

司马迁：究天人之际，穷古今之变，成一家之言。

季羡林： 要求知识面广，大概没有人反对。因为，不管你探究的范围多么窄狭，多么专门，只有在知识广博的基础上，你的眼光才能放远，你的研究才能深入。这样说已经近于常识，不必再做过多的论证了。我想在这里强调一点，这就是，我们从事人文科学和社会科学研究的人，应该学一点科学技术知识，能够精通一门自然科学，那就更好。

To Sima, Qian (司马迁), Ji, Xianlin (季羡林) and my family

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CHAPTER I

1. Research objectives and brief description of studies

The overall objective was to develop an efficient process for PHB production from lignocellulosic biomass. This objective was decomposed into two supporting objectives: (1) Optimizing PHB production from sugar mixture of lignocellulosic biomass by *Burkholderia sacchari* DSM 17165 and (2) Enhancing PHB production from lignin with supplements by *Cupriavidus necator* DSM 545. These two supporting objectives were realized by the following studies.

i) **Statistical design and modeling to optimize sugar mixture ratio and process variable for PHB maximal production at shake flask scale (chapter III):** Upstream processing of lignocellulosic feedstock generated a sugar stream with glucose, xylose and arabinose being the main sugars (Aristidou and Penttila, 2000; Cesario et al., 2014). However, how ternary sugar ratio affects PHB production by *B. sacchari* DSM 17165 and how sugar mixture ratio interacts with other process variables have not been studied before to the best of the author's knowledge. An advanced experimental design called combined mixture-process design was employed to build a statistical model and then to derive the optimal conditions that led to maximum PHB production. This study was performed in the 250 ml shake

flask scale. The model could provide references for optimizing PHB production for different types of sugar mixture streams.

ii) **Statistical design and modeling to enhance PHB production from lignin with supplements at shake flask scale (chapter IV):** In previous studies, multiple enzymes and chemicals have been widely studied in their roles in lignin structural and molecular changes and low molecular aromatic compound conversion (Beckham et al., 2016; Chen and Wan, 2017a; Linger et al., 2014; Paliwal et al., 2012). In this study, oxidative enzymes (laccase and aryl alcohol oxidase (AAO)), and their mediators (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS) and hydroxybenzotriazole (HOBT)), surfactants (Aerosil R816 silica nanoparticle and Tween 80) and DMSO were added as supplements to see if PHB production can be enhanced. Plackett-Burman design, powerful in screening many variables with small number of experimental runs, was used in the screening study. Central composite design, one of the most commonly used designs under response surface methodology, was used to find the optimal conditions that leads to maximal PHB production.

iii) **Non-destructive determination of PHB production from APL using flow cytometry (chapter V):** Traditional PHB quantification method is methonolysis at 105 °C and resultant compound quantification using gas chromatography-mass spectrometer (GC-MS). This method is time consuming and requires the use of organic solvents. A quick and non-destructive method to determine PHB production from APL is important to monitor bacterial cell and/or PHB accumulation during fermentation. On the other hand, APL has insoluble

substances and is dark, which makes quantitation of cells through visible light absorbance difficult. Flow cytometry with fluorescence staining was used to count cells and quantitate PHB content through fluorescence intensity. PHB granules were stained by certain dyes (for example, Nile Red) and exhibited fluorescence in flow cytometry. However, the insoluble particles in APL may interfere with bacterial cells. A sample preparation method was developed using Nile Red staining and flow cytometry to quantify bacterial cells and PHB concentration. A linear relationship was constructed to predict PHB concentration based on fluorescence intensity acquired from a flow cytometer.

iv) **Fed-batch cultivation to produce PHB from simulated sugar mixture at bioreactor scale (chapter VI):** After obtaining the optimal conditions for sugar mixture conversion into PHB at shake flask scale in chapter III, it is necessary to study PHB production from sugar mixture at bioreactor scale. Previous studies have studied PHB production from wheat straw sugar mixture and xylose rich sugar mixtures at bioreactor scale (Cesario et al., 2014; Raposo et al., 2017). However, bioreactor scale production of PHB from corn fiber derived sugar stream was not studied before. PHB productions from three different types of simulated corn fiber sugar mixtures were demonstrated at bioreactor scale (3 L) under fed-batch mode. Cell and PHB production, cell and PHB productivity, and sugar conversion efficiencies into cell and PHB were evaluated.

v) **Fed-batch lignin conversion into PHB at bioreactor level (chapter VII)**: After obtaining the optimal conditions for lignin conversion into PHB at shake flask scale in chapter IV, it is necessary to study PHB production from lignin at bioreactor

scale. First, lignin conversion into PHB under the same optimal conditions derived from chapter IV was performed at 1.3 L bioreactor scale. To further enhance PHB production, fed-batch cultivation under two different feeding strategies were applied: single pulse feeding of 300 mL medium and 4 pulses feeding of 75 mL medium each time. PHB production and productivity were evaluated.

1.1. References

Aristidou, A., Penttila, M. 2000. Metabolic engineering applications to renewable resource utilization. Curr. Opin. Biotechnol., 11(2), 187-198.

Beckham, G.T., Johnson, C.W., Karp, E.M., Salvachua, D., Vardon, D.R. 2016. Opportunities and challenges in biological lignin valorization. Curr. Opin. Biotechnol., 42, 40-53.

Cesario, M.T., Raposo, R.S., De Almeida, M.C.M., Van Keulen, F., Ferreira, B.S., Da Fonseca, M.M.R. 2014. Enhanced bioproduction of poly-3-hydroxybutyrate from wheat straw lignocellulosic hydrolysates. New Biotechnol., 31(1), 104-113.

Chen, Z., Wan, C. 2017a. Biological valorization strategies for converting lignin into fuels and chemicals. Renew. Sust. Energ. Rev., 73, 610-621.

Linger, J.G., Vardon, D.R., Guarnieri, M.T., Karp, E.M., Hunsinger, G.B., Franden, M.A., Johnson, C.W., Chupka, G., Strathmann, T.J., Pienkos, P.T. 2014. Lignin valorization through integrated biological funneling and chemical catalysis. Proc. Natl. Acad. Sci., 111(33), 12013-12018.

Paliwal, R., Rawat, A.P., Rawat, M., Rai, J.P.N. 2012. Bioligninolysis: Recent updates for biotechnological solution. Appl. Biochem. Biotechnol., 167(7), 1865-1889.

Raposo, R.S., De Almeida, M.C.M., De Oliveira, M.d.C.M., Da Fonseca, M.M., Cesario, M.T. 2017. A *Burkholderia sacchari* cell factory: production of poly-3-hydroxybutyrate, xylitol and xylonic acid from xylose-rich sugar mixtures. New Biotechnol., 34, 12-22.

CHAPTER II

2. Literature review: Towards cost-effective PHA production: recent advances in feedstocks, strains and process development

Abstract

Polyhydroxyalkanoates (PHAs) have been actively studied in academia and industry for their properties comparable to petroleum-derived plastics and high biocompatibility. However, the major limitation for commercialization is their high cost. Feedstock costs, especially carbon costs, account for the majority of the final cost. Adapting cheap feedstocks for PHA production and associated strain and process development are critical for a cost-effective PHA production. In this study, waste materials from different sources, particularly lignocellulosic biomass, were proposed as suitable feedstocks for PHA production. Strains involving in the conversion of these feedstocks into PHA were reviewed. Newly isolated strains were emphasized. Related process development, including the factors that affect PHA production and fermentation modes, was elaborated upon.

2.1. Introduction

Due to their environment-friendly and sustainable nature, bioplastics have been of great interest to partially replace synthetic plastics from petroleum for the last few decades. As one of the three types of fully degradable bioplastics (thermoplastic starch, polylactides and

polyhydroxyalkanoates) (Endres and Siebert-Raths, 2011; Rohrbeck et al., 2013), microbial produced polyhydroxyalkanoates (PHAs) are a unique family of polyesters that can be produced by more than 300 bacteria and a wide range of archaea (Bernd, 2003). These polyesters function as storage compounds under microbial unbalanced growth when essential nutrients such as nitrogen, phosphorus and oxygen are limited and carbon is excessive (Anderson and Dawes, 1990; Gasser et al., 2014; Zou and Chen, 2007). PHAs deposit within cells as spherical intracellular inclusions and can reach a concentration of 90% of dry cell weight in some bacteria (Spiekermann et al., 1999; Sudesh et al., 2000). There are over 150 constituents of PHAs exhibiting varying physicochemical properties that have been identified by varying the monomer compositions (Chen and Wu, 2005). Detailed physical properties comparisons between different PHAs and conventional petroleum-based polymers can be found in Anjum et al. (2016). PHAs have demonstrated for many applications, such as packaging containers, bottles, wrapping films, bags, fibers and medical devices such as surgical pins, bone screws and controlled drug delivery carriers (Glazer and Nikaido, 2007; Tanaka et al., 2004). PHAs were used as biofuels or fuel additives to develop hydroxyl-alkanoate methyl esters (HAMEs) to enhance the combustion heat of ethanol (Wang et al., 2010; Zhang et al., 2009). The most common PHAs are poly(3-hydroxybutyrate) (PHB), poly(3-hydroxybutyratre-co-3-polyhydroxyvalerate) and poly(3-hydroxyoctanoate-co-3-polyhydroxyhexanoate), with PHB as the most common (Laycock et al., 2013). A degradability study conducted by Singh et al. (2013) showed PHB synthesized by *Bacillus subtilis* was completely degraded after 30 days in compost with 25% moisture.

In recent years, PHAs have gained much attention in both research and industry. Without any doubts, they are valuable materials with versatile properties (Mozejko-Ciesielska and Kiewisz, 2016). The major drawback for PHAs is that they are not cost competitive compared to plastics

from petroleum. For example, commercial PHB produced by microbes is still around 3 times more expensive than petroleum-based plastics. In 2018, the price of PHB was around $3.50/kg (Aramvash et al., 2018), while the prices of petroleum-based plastics such as polyethylene and polypropylene were around $1.20–$1.30/kg (Platts, 2019). Reviews on PHA production at commercial scale, commercial PHA manufacturers and their production capacity can be found in Snell et al. (2015), Anjum et al. (2016) and Dietrich et al. (2017a).

The final costs of PHAs mainly depend on the price of feedstocks added as a carbon source for microbial growth (Mozejko-Ciesielska and Kiewisz, 2016). Raw materials make up about 50% of the production cost in which carbon sources account for 70-80% of the total cost (Choi and Lee, 1999; Khanna and Srivastava, 2005; Mozumder et al., 2015). Cheap and readily available feedstocks such as industrial, agricultural, municipal and food-based wastes that are rich in carbon are desirable feedstocks for PHA production. Particularly, wastewater treatment incorporated with PHA production has been studied intensively, but compositions of wastewater vary greatly. For example, an analysis based on experimental results at laboratory scale and detailed data from German waste water treatment plants showed that 20% and 120% of current biopolymer production could be produced at Germany and EU treatment plants, respectively (Pittmann and Steinmetz, 2016). Another type of cheap feedstock are lignocellulosic biomass feedstocks. These feedstocks are abundant, cheap and sustainable, do not compete with the human food chain and can be used as the major carbon sources for PHB production (Saratale et al., 2019). Lignocellulose feedstocks have a relatively consistent quality. Recent advances in PHA production by multiple strains using waste materials and lignocellulosic feedstocks are reviewed in this study.

Another major limitation for the industrial production of PHAs is to maintain optimal bacterial growth conditions and to maximize PHA titer, accumulation in cells and productivity.

Process development associated with the aforementioned cheap feedstocks are discussed in this study. Some waste feedstocks and lignocellulosic feedstocks require pretreatment processes for the carbon sources to be available for PHA conversion. Related pretreatment processes are briefly discussed. Specifically, fed-batch cultivation strategies are the most commonly used fermentation strategy to create nutrient limitation conditions, divert more carbon sources into PHA formation, and enhance cell density and PHA productivity. The advances of downstream processing for PHA manufacturing will not be discussed here, the reader can refer to Kosseva and Rusbandi (2018). Because PHB is the most common PHA, PHB production is emphasized in this study.

2.2. Feedstocks and strains

2.2.1 Waste feedstocks

Wastes from industrial, agricultural, municipal, food and other sources are produced in massive amounts. Conversion of these wastes into PHA would create profit and solve waste disposal problems. As shown in Fig. 2.1, there are three major pathways for PHA synthesis (pathway I, acetyl-CoA to 3-hydroxybutyryl-CoA, pathway II, fatty acid degradation and pathway III, fatty acid biosynthesis) (Anjum et al., 2016; Philip et al., 2007). Pathway I and III use sugars as a carbon source and pathway III uses fatty acids as a carbon source (Anjum et al., 2016). Therefore, sugar and/or fatty acid containing wastes could be the best candidates for PHA production (Rodriguez-Perez et al., 2018). Based on the pathways, Gomez Cardozo et al. (2016) defined two types of wastes: sugar waste (i.e. wastes rich in simple carbohydrates such as glucose, sucrose, fructose, saccharose or stachyose) and fatty waste (i.e. wastes with high contents in fatty acids such as myristic, palmitic, stearic, oleic, linoleic, linolenic butyric, acetic or propionic

acids). PHA yields were around 0.6–0.8 g/g for fatty substrates, and around 0.3–0.4 g/g for sugar substrates (Cruz et al., 2016).

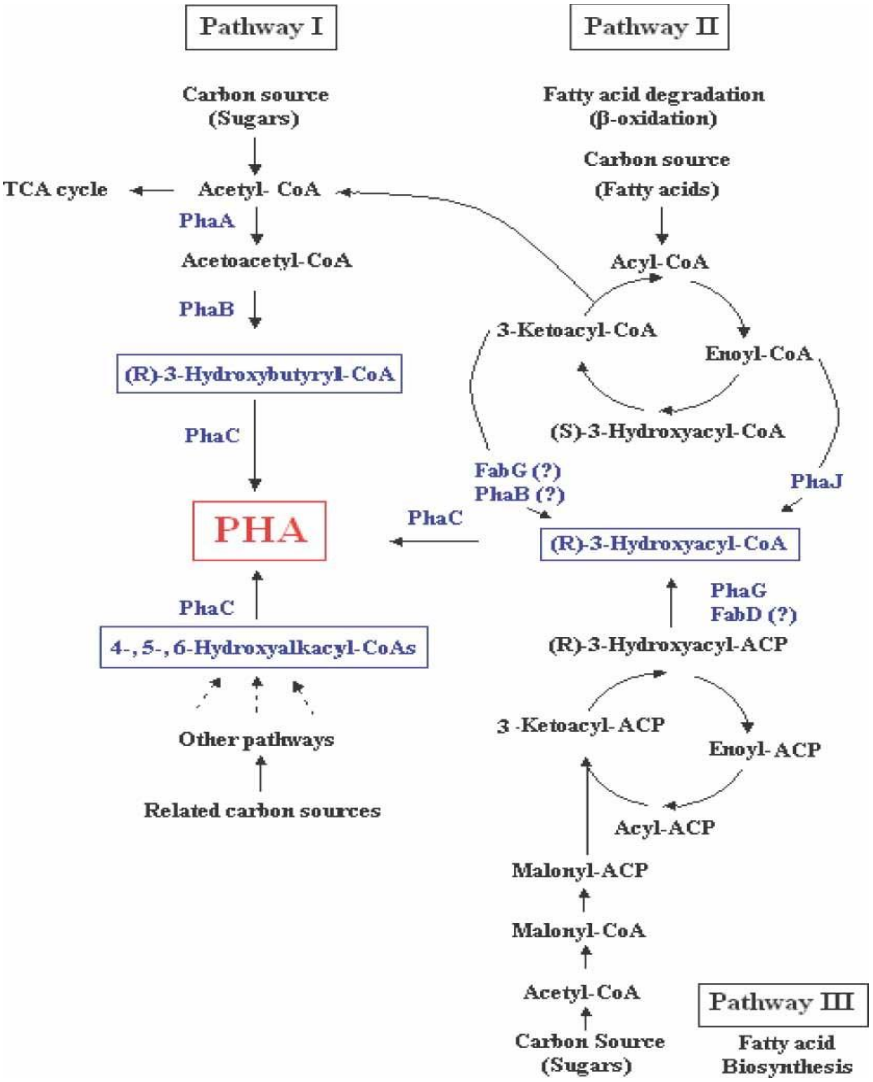

Fig. 2.1. PHA biosynthetic pathways (Philip et al., 2007).

Specifically, in recent years, PHAs have been produced from cheese whey (Colombo et al., 2016; Pais et al., 2016), municipal wastewater and solid waste (Bengtsson et al., 2017; Korkakaki et al., 2016a; Morgan-Sagastume et al., 2015), spend coffee grounds (Obruca et al., 2014b), waste cooking oil (Sharma et al., 2017; Tufail et al., 2017; Valentino et al., 2011; Walsh et al., 2015), cane molasses (Shasaltaneh et al., 2013), agro-industrial waste (Elain et al., 2016), phenol (Zhang

et al., 2018), food waste (Ravindran and Jaiswal, 2016), non-food crops such as ryegrass (Kataria et al., 2018) and sweetwater, a by-product from sugar cane refining (Yatim et al., 2017). There are a few reviews discussing feedstocks that can be converted into PHA depending on their origin and physical forms. For example, PHA production feedstocks including industrial byproducts, waste fats and oils, lignocellulosic raw materials, agricultural and household waste materials, glycerol, sugars and wastewater were discussed in Anjum et al. (2016). Pakalapati et al. (2018) mainly focused on reviewing agricultural, industrial and food–derived waste conversion into PHA. Depending on their origin, the waste streams have been classified as agro-alimentary, industrial not agro-alimentary, food, urban wastes and synthetic substrate (Rodriguez-Perez et al., 2018).

Some feedstocks are good candidates and can be easily transformed into readily consumable carbon, i.e. simple carbohydrates or volatile fatty acids (VFAs) such as acetate, propionate, butyrate or lactate, for PHA production only after necessary physicochemical or biologic pretreatments. Pretreatments to adjust waste to an appropriate feed stream is highly suggested by Rodriguez-Perez et al. (2018). The outcomes of pretreatment include increasing carbon sources availability, diluting the concentration of organic matter, regulating pH, controlling temperature, sterilizing waste materials, removing suspended solids and minimizing potential inhibitory effects on PHA production strains (Rodriguez-Perez et al., 2018). For example, for physicochemical pretreatment, mild acid pretreatment (pH 3-5) was mostly applied to dairy effluent, paper mill effluent and sugar cane molasses to improve PHA production (Gomaa, 2014; Munir et al., 2015; Rathika et al., 2018). Cane molasses pretreated with H_2SO_4 led to a significant increase (~50%) of PHA production and biomass of *B. subtilis* and *Escherichia coli* (Gomaa, 2014). In terms of biological pretreatment, mixed hydrolytic bacterial culture was used to raw waste materials and PHA production increased by almost 4 fold (Kumar et al., 2016b). For olive

oil mill waste, acidogenic fermentation increased VFA concentration for enhanced PHA production (Campanari et al., 2017).

2.2.2 Lignocellulosic feedstocks

Lignocellulosic feedstocks are generally considered as the most abundant organic materials on earth with estimated global quantities at about 80,000 Mt (Obruca et al., 2015). For most strains, pretreatment and hydrolysis processes are required prior to using the feedstocks for PHA production. Efficient pretreatment processes involving physicochemical, alkaline and biological reactions and subsequent hydrolysis on different lignocellulosic feedstocks have achieved high sugar yield for biofuel production for the last decade (Ding et al., 2018; Ding et al., 2019; Frederick et al., 2016; Li, 2015; Li et al., 2018a; Li et al., 2010; Liu et al., 2015; Ramachandriya et al., 2013; Socha et al., 2014; Suryawati et al., 2009). After pretreatment and hydrolysis, the carbon stream contains sugars with glucose, xylose and arabinose as the main sugars (Aristidou and Penttila, 2000; Cesario et al., 2014), organic acids (levulinic acid, lactic acid, formic acid and acetic acid), microbial inhibitors (furfural and hydroxymethylfurfural (HMF)), and lignin derivatives (i.e. vanillin, phenols). The carbon streams after these pretreatments can also be used for PHA production. In other words, PHA production can be incorporated into biofuel production. For example, pentoses that are not utilized for biofuel production, organic acid and lignin derivatives can be converted into PHA by certain microorganisms. However, there are a few challenges for conversion of carbon streams from lignocellulosic feedstocks into PHA, such as catabolite repression in terms of sugar conversion, and that most organic acids, inhibitors and lignin derivatives are inhibitory to most PHA-producing strains. Recent advances of how to address these challenges are stated below.

Most previous studies on lignocellulosic biomass conversion to PHA have focused on sugars, specifically on glucose or glucose and xylose (Brodin et al., 2017; Cesario et al., 2014; Dai et al., 2015; Pan et al., 2012a; Raposo et al., 2017). Due to catabolite repression, glucose is consumed quickly and other sugars accumulate to a high concentration that may inhibit the growth of microorganisms. Experiments were conducted to evaluate the sugars consumption rate and statistical models were derived to find the optimal sugar mixture ratio. For softwood hemicellulose hydrolysate fermented by *Paraburkholderia sacchari* to produce PHB, sugars were depleted at different rates in the following order: glucose, mannose, xylose, galactose, and arabinose (Dietrich et al., 2019). Genetic engineering approaches were attempted to eliminate catabolite repression. Using engineered *E. coli*, a glucose/xylose switching strategy (glucose was used for cell growth at the initial stage and xylose was used for polymer production at the feeding stage) was employed to enhance poly(lactate-*co*-3-hydroxybutyrate) production. The enhanced polymer production may be caused by channeling of the metabolic flux from acetyl-CoA towards polymer production over into the tricarboxylic acid cycle in the xylose-fed cultures (Hori et al., 2019). A *E. coli ndh* knockout strain was found to simultaneously utilize glucose and xylose to accumulate PHB 1.9-fold higher than the control strain (Huo et al., 2017).

Except for levulinic acid, most organic acids, inhibitors and lignin derivatives, especially furfural and HMF, are inhibitory for most PHA-producing organisms (Dietrich et al., 2017b). Levulinic acid obtained after acid pretreatment resulted in synthesis of poly-3HB-3HV synthesis by *Burkholderia sacchari*, and levulinic acid was preferred over xylose or glucose (Ashby et al., 2018). Levulinic acid can also induce synthesis of poly-3HB-3HV by *Pseudomonas putida* (Martin and Prather, 2009) and *C. necator* (Jaremko and Yu, 2011; Kachrimanidou et al., 2014). For other compounds, detoxification is necessary. Detoxification significantly promoted PHA production

from spent coffee grounds by *Haloferax halophila* (Kovalcik et al., 2018). An alternative way to minimize inhibitor content is to apply a short period (<2 min, 190-200 °*C*) of liquid hot water/dilute acid pretreatment (Laser et al., 2002). The PHA-producing bacterium *H. mediterranei* was found to tolerate polyphenols present in medium containing 25% olive mill wastewater (Alsafadi and Al-Mashaqbeh, 2017). Applying high initial cell density inoculum could overcome growth inhibition (Dietrich et al., 2017b). Some strains can even metabolize these inhibitory compounds at a relatively low level. For softwood hemicellulose hydrolysates, all potential inhibitors (i.e. volatile fatty acids and furans) except phenols were metabolized by *P. sacchari* (Dietrich et al., 2017b). However, for *Burkholderia* sp. F24, the metabolites of HMF and furfural (HMF is converted to hydroxymethyl-furoic acid and furfural to 2-furoic acid) are still inhibitory (Lopes et al., 2014). From a different view, these inhibitory compounds can be extracted and become valuable ingredients. For example, oils and phenolics extracted from spent coffee ground were proposed by Kovalcik et al. (2018) to be used for active substances in cosmetic formulation (Ribeiro et al., 2013) and UVB absorbent in sunscreens (Marto et al., 2016) .

Lignin valorization into PHA has attracted more attention in recent years. Aromatic compounds in lignin can be ring-opened by bacteria via the β-ketoadipate pathway and converted to PHA from acetyl-CoA through fatty acid synthesis. Alkaline pretreatment is preferred as a fractionation method because it can preserve over 90% of sugars in solids and over 50% lignin is dissolved in alkaline pretreatment liquor (APL) (Kim and Holtzapple, 2005; Xu and Cheng, 2011). PHA/PHB production from APL/lignin derivatives ranged from 0.13 to 1.0 g/L (Berezina et al., 2015; Linger et al., 2014; Liu et al., 2017; Shi et al., 2017; Si et al., 2018; Wang et al., 2018). It should be noted that in these studies, APL was obtained from combinatorial H_2SO_4 pretreatment followed by NaOH pretreatment, which is more efficient than single alkaline pretreatment, and

PHB production reached 1.0 g/L (Liu et al., 2018). However, such PHB/PHA concentrations are still too low to be economical.

2.2.3 Strains

An detailed list of microorganisms that can produce PHAs can be found in Anjum et al. (2016). In recent years, many newly isolated strains have been reported. Special strains, including strains that directly degrade cellulose, or metabolize non-regular carbon sources such as CO_2 and methane, are reviewed below. The dominant microorganisms present in mixed microbial culture for wastewater treatment, and halophilic microorganisms that are tolerant to high salinity are also mentioned. *Burkholderia* species that can metabolize sugar mixtures, grow and produce PHA quickly, and accumulate high PHA in cells, are emphasized.

There are many newly isolated PHA-producing strains found in recent years. *Rhodococcus equi*, isolated from fertile soil, converted palm kernel oil into PHB (Altaee et al., 2017). *Microbacterium* sp. produced PHA from molasses, whey and ground sesame (Osman et al., 2016). *B. pumilus* converted cow dung into PHB (Devi et al., 2018). *Lysinibacillus* sp. was isolated and produced P(3HB-co- 3-hydroxydodecanoate-co-3-hydroxytetradecanoate) from glucose for the first time (Mohapatra et al., 2016). *Wickerhamomyces anomalus* growing on sugar molasses and palm oil produced PHA (Ojha and Das, 2018). *Pandoraea* sp. was able to convert crude glycerol from biodiesel production into PHA (De Paula et al., 2017). Cyanobacteria were widely studied for accumulation of PHB in photoautotrophic systems (Carpine et al., 2018; Gupta et al., 2013; Singh et al., 2017). At large scale, PHA production was conducted by cyanobacteria from the effluent gases of a power plant (Troschl et al., 2017) and from German and European municipal wastewater treatment plants (Pittmann and Steinmetz, 2017). Archaea can also produce PHB from

various substrates (Danis et al., 2015; Hermann-Krauss et al., 2013). There are some strains that can produce PHA directly from nonpretreated cellulose/cellulosic biomass. *Saccharophagus degradans* was used to convert cellulose directly into PHA (Sawant et al., 2017). Avicel or tequila bagasse was used as the sole carbon source fed to *S. degradans* to produce PHA in one study (Munoz et al., 2008). An isolate from termite gut identified as *B. cereus* was found to degrade cellulose and produce PHA (Cibichakravarthy et al., 2017). These strains have the potential to eliminate the pretreatment and hydrolysis for lignocellulosic substrates.

Some other strains do not consume regular carbon sources like sugar or fatty carbon sources for PHA synthesis. For example, some are able to fix CO2, such as microalgae *Chlorella minutissima*, *Synechococcus subsalsus*, and *Spirulina* sp. (Costa et al., 2018), cyanobacteria (Troschl et al., 2017), *B. cereus* (Maheshwari et al., 2018), and chemolithotrophic oleaginous bacteria *Serratia* sp. (Kumar et al., 2016a). A methanotroph *Methylocystis hirsuta*, could consume methane and produce PHB simultaneously under continuous operation (Garcia-Perez et al., 2018).

Wastewater contains many complex substrates; thus, the strategy is to enrich the substrates with PHA-storing microorganisms (microbial mixed culture) for feedstocks treatment and PHA production. Mixed cultures have the advantage that they do not need sterile conditions; therefore, reducing cost, and they are better than pure cultures to adapt to complex substrates (Reis et al., 2011). Also, mixed culture from activated sludge for producing PHA has the ability to tolerate toxic substrates such as phenol (Zhang et al., 2018). Research identifying the dominant strains in mixed microbial cultures is active. For a mixed microbial culture converting wood hydrolysates into PHB, actinobacteria and α- and β-proteobacteria were dominant groups in bioreactors (Dai et al., 2015). *Comamonas, Azoarcus* and *Thauera* were the dominant PHA-accumulating bacteria

genera (Fra-Vazquez et al., 2019). For a mixed culture converting wastewater into PHA, the dominate phyla were Proteobacteria, Acidobacteria and Firmicutes (Amulya et al., 2016). The genera of *Oceanicella*, *Piscicoccus* and *Vibrio* were found as PHA accumulating bacteria in glucose-enriched mixed microbial cultures (Cui et al., 2016). Mainly four classes of bacteria, *γ-Proteobacteria*, *Cellvibrio* sp., an uncultured bacterium and *Pseudomonas* sp., were identified in the final stable system for PHA production in a microbial consortium from waste activated sludge (Huang et al., 2016).

Many wastewater plants produce high salinity wastewater. Halophilic microorganisms were widely studied for their ability to tolerate high salt conditions (Stanley et al., 2018). Another unique ability of *Halomonas* sp. are that they can grow in non-sterile media (Chen et al., 2017; Yin et al., 2015). Under non-sterile conditions, 83 g/L cell dry weight containing 61% P(3HB-*co*-4HB) was achieved in 48 h in a 1,000-L pilot fermentor with a productivity of 1.04 g/L/h (Chen et al., 2017). However, too high salt concentration inhibits cell growth and PHA production. The optimal salt concentration for both cellular growth and PHB production was 45 g/L for *B. megaterium* (Rodriguez-Contreras et al., 2016).

It is important to mention that *Burkholderia* species are important PHA producing strains. *B. sacchari* is a hexose and pentose-consuming bacterium that naturally grows to high cell densities and accumulates up to 75% of its cell dry weight as PHAs (Gomez et al., 1996a) . *B. sacchari*'s specific growth rate is also faster than the commonly studied PHA-accumulating *C. necator* (Gomez et al., 1996b), and it has been shown that *B. sacchari* can produce PHA copolymers with processing properties superior to polyhydroxybutyrate (PHB) by changing the carbon source (Mendonca et al., 2014). Fine-tuning of PHA composition under fed-batch conditions was obtained by adding 4-hydroxybutyrate precursors to sucrose as the substrates

(Miranda De Sousa Dias et al., 2017). A few studies have demonstrated that this strain can produce PHB >100 g/L using lignocellulosic feedstock hydrolysates (Cesario et al., 2014; Raposo et al., 2017).

2.3. Process development

2.3.1 Factors that affect PHA production

PHA pathways have been understood very well, but there are still aspects not known in terms of the physiology of PHA-producing microbes. Optimization of culture is important for empirical study, especially for large scale processes in industry. The full composition of the media are vital for PHA production. Concentration of trace metals, i.e. Fe, B, Cu, Mn, Mo, Zn, and salinity were found to highly affect PHA production yield (Alsafadi and Al-Mashaqbeh, 2017; Chen et al., 2015; Palmeiro-Sanchez et al., 2016). Culture conditions include carbon, nitrogen, phosphate source and concentration, dissolved oxygen, pH, the ratios of carbon to nitrogen and phosphate, trace elements and supplements levels can affect PHB titer and productivity. Supplements refer to substances that are not essential to microbial growth, but may enhance PHA production greatly.

Carbon to nitrogen (C/N) and carbon to phosphate (C/P) ratios are the most important factors that affect PHA content in cells and monomer composition in PHAs. Directing carbon metabolic flow towards PHA instead of cellular mass by limiting nitrogen is one of the most convenient culture strategies. The appropriate C/N ratio differs depending on the substrate and the microorganisms applied. The maximum PHB content was 89 wt% after 7.6 h under ammonium starvation, 77 wt% after 9.3 h under ammonium limitation and 69 wt% after 4.4 h under ammonium excess, for γ-proteobacterium dominant culture growing on synthetic medium

(Johnson et al., 2010). PHA content of 65% w/w in *C. necator* was achieved with C/N ratio of 20 when growing on dilute acid pretreated sugarcane bagasse (Yu and Stahl, 2008). The C/N ratio was optimized to 42-48 using *C. necator* to transform plant oils into PHA and the copolymers had 3HB as the major components (88-98 mol%) (Lee et al., 2008).

A minimum concentration of nutrients is necessary for PHA production and accumulation, but a too high nutrient concentration causes inhibition (Colombo et al., 2016). It is noticeable that waste substrates available for PHA production contain varying levels of nutrients. In the case of PHA production from municipal wastewater using activated sludge, substrate specific uptake rates were found to be dependent on the cellular P content and PHA production capacity is lost when the C/P ratio is over 300. Among potassium, manganese and nitrogen limitation for PHB produced by *M. hirsuta*, nitrogen starvation resulted in the highest PHB content (Garcia-Perez et al., 2018). No PHA was produced using a complete or magnesium-deprived medium using *P. aeruginosa* growing on fatty acid (Impallomeni et al., 2018). For PHA produced from pineapple peel waste by *C. necator*, the best fermentation conditions were pH 9, C/N: 11, C/P: 6 and a fermentation time of 60 h (Vega-Castro et al., 2016). The ratios of C/N and C/P determined the monomer compositions of PHAs (Valentino et al., 2015). Therefore, only some waste streams that have an appropriate C/N/P ratio can be used without the addition of nutrients (N and or P) and monomer composition in PHA can be regulated by adjusting the C/N and C/P ratios.

Supplements may enhance PHA production significantly. For *C. necator*, Luria broth (LB) and Bonnarme's media, both supplemented with 10 g/L sodium glutamate, were found to be the most efficient media that enhanced overall PHA productivity 33 times comparing to traditional cultivation methods (Berezina, 2013). Cell debris generated from PHA recovery increased cell growth and PHA synthesis remarkably since the cell debris can be readily assimilated by the cells

as the nutrients, besides, a large amount of aqueous waste was avoided (Yu, 2012). NaCl up to 8 g/L was used as external stress factor to enhance PHB yield; however, NaCl concentration at 16 g/L was found to inhibit production of PHB (Azizi et al., 2017). For lignin conversion into PHA, supplements are vital to enhance PHA production. Oxidative enzymes (laccase, aryl alcohol oxidase and lignin peroxidase), mediators (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS) and hydroxybenzotriazole (HOBT)), and surfactants, such as Tween-80, were found to improve PHA production by different bacteria (Li et al., 2019a; Liu et al., 2018; Xie et al., 2015; Zhao et al., 2016).

Dissolved oxygen (DO) control was mostly limited for enhancement of PHA production. Nath et al. (2008) reported an increase in PHB production by 0.8-fold by limiting DO levels. They proposed that the decreased DO shifted the flux of acetyl-CoA towards PHB accumulation as opposed to the TCA (tricarboxylic acid) cycle. Multiple studies maintained DO at 20% to enhance PHA production and DO was mostly maintained by adjusting agitation speed and aeration rate (Cesario et al., 2014; Hermann-Krauss et al., 2013; Kachrimanidou et al., 2016). Kim (2000) achieved higher PHB concentration, PHB content and productivity from *Azotobacter chroococcum* with oxygen limitation than without oxygen limitation. Controlling agitation speed can be used as a control strategy when hexoses and pentoses are both present in the substrate medium. For *B. sacchari*, when the stirring speed was kept high at 1200 rpm, only glucose was consumed; when the stirring speed was kept low at 900 rpm, xylose and arabinose consumption was triggered (Cesario et al., 2014).

For most microbes producing PHA, the favorable pH is around 7.0, though *B. megaterium*, had an optimal pH of 9 for dairy and sea water conversion into PHA (RamKumar Pandian et al., 2010). Neutral pH operation has been shown to induce higher dehydrogenase enzyme activity and

substrate degradation than acidic and alkaline pH at microaerophilic environment, which correlated well with higher PHA production. Neutral pH showed better PHA synthesis (56%) than basic (44%) and acidic (28%) redox microenvironments (Amulya et al., 2016). The optimum pH was 7.0 for P(3HB-co-3HV) copolymer synthesized by *C. necator*. The copolymer content dropped dramatically to 40% when the initial pH was 6.5 and to 60% when the initial pH was 8.5 (Lee et al., 2008). For wheat straw hydrolysate fermentation by *B. sacchari*, the pH was adjusted to be 6.8 (Cesario et al., 2014). Acidic pH conditions (4.5-5.8) were found to completely suppress *B. cereus* sporulation (Kominek and Halvorson, 1965; Valappil et al., 2007), which was shown to cause low PHB productivity in the genus *Bacillus* (Wu et al., 2001). Mixed microbial cultures can have higher tolerance for a wide pH range. For PHA production from cheese whey using mixed microbial culture, fluctuating pH led to PHAs of different compositions (Gouveia et al., 2017). Montiel-Jarillo et al. (2017) demonstrated that enrichment of a mixed microbial culture without pH control (pH ranging 8.8 to 9.2, averaged pH: 9.0) was feasible to accumulate PHA.

Optimization research has been active for maximal PHA production. At shake flask scale, advanced experimental design were used to optimize media formulations and growth conditions. Mixture design to study media formulation (Li et al., 2019b; Yang et al., 2010), Plackett-Burman design to screen important factors (Berekaa and Al Issa, 2016; Li et al., 2019a; Sabapathy et al., 2019; Zihayat et al., 2019) and response surface methodology (central composite and Box-Behnken design) to find optimal conditions (Das and Grover, 2018; Dey and Rangarajan, 2017; Kumar et al., 2016a; Li et al., 2019a; Ojha and Das, 2018; Sabapathy et al., 2019) were widely used. Under most of the cases, multiple inhibitors are present and form a mixture. Mixture design was employed to evaluate how an inhibitor mixture affected *P. sacchari* growth and PHB production (Dietrich et al., 2017b). At bioreactor scale, for kinetics study on bacterial growth and

PHA production, mostly modified logistic equations are fitted to experimental data. Commonly applied models include Monod, Contois, Herbert, and Luedeking–Piret type kinetics models (Das and Grover, 2018; Divyashree et al., 2009; Gaudy et al., 1971; Raje and Srivastava, 1998; Sakthiselvan and Madhumathi, 2018; Sonnleitner et al., 1979). Subsequently, numerical analyses are applied to find the optimal point for maximum PHA titer and productivity.

Because PHAs are intracellular products, coproduction with other extracellular products are proposed in a multi-purpose biorefinery platform. For example, PHA and polyphenols, volatile fatty acids, biogas were co-produced from red grape pomace (Martinez et al., 2016). Co-production with ethanol and hydrogen seems promising as they do not compete for the same metabolites (Kumar and Kim, 2018). Co-production of PHA and rhamnolipids was realized using *B. thailandensis* from sunflower cooking oil (Kourmentza et al., 2018). A *B. sacchari* cell factory was proposed to produce PHB, xylitol and xylonic acid from xylose-rich sugar mixtures (Raposo et al., 2017). A review of co-production of value-added products along with PHA can be found in Li et al. (2017) and Kumar and Kim (2018). PHA production can be incorporated into biofuel and microalgae lipids production (Koller et al., 2017). An integrative approach was proposed to produce hydrogen and PHA from mixed culture (Patel et al., 2016). Hemicellulose hydrolysates and crude glycerol can be produced as by-products on a large scale during various second generation biofuels' production (Jiang et al., 2016). These by-products can be converted into PHA. This would create the potential to offset production cost of biofuels and reduce the overall production cost of PHAs.

2.3.2 Fermentation strategy

Fed-batch strategy has been observed to be an efficient strategy to improve PHA concentration and PHA content in cells on different strains. For substrate cost reduction, using 60-Brix syrup and fed-batch cultivation yielded a 77% cost reduction compared with using refined cane sugar and batch cultivation when producing PHB using *Azohydromonas lata* (Wisuthiphaet and Napathorn, 2016). Most fed-batch strategies focus on carbon source feeding. Stanley et al. (2018) used salt tolerant strain *H. venusta* and studied several feeding strategies including pH-based fed-batch and variants of pulse feeding (glucose as carbon source). PHA content 88.12% of the dry cell weight was achieved with a high concentration (~ 100 g/L) single pulse method. A net 8.65-fold increase in PHA concentration using this feeding strategy was realized when compared to batch studies (Stanley et al., 2018). pH-based feeding caused most of the carbon flux to divert towards biomass formation (PHA content only 39%). Similarly, for PHA production from acid-pretreated red algae using *B. megaterium*, pulse feeding led to higher cell dry weight and PHA production compared to pH-stat feeding (Alkotaini et al., 2016). Fed-batch strategy is also applied to carbon and nitrogen concurrent feeding. Feeding both carbon and nitrogen during fed-batch mode is found to be optimal compared to only supplying carbon or nitrogen (Norhafini et al., 2019). Feeding carbon and nitrogen both twice in a pulse feeding mode was found to be the most optimal strategy achieving 92 wt% accumulation of the total biomass, with the highest PHA concentration (46 g/L) and yield ($Y_{p/x}$) of 11.5 g/g. Norhafini et al. (2019) proposed the strategy of keeping C/N ratio constantly at the optimal ratio during the active PHA-producing phase and feeding *C. malaysiensis* at 36 and 72 h.

Similar to the idea of pulse feeding of a carbon source, as the most widely applied strategy in wastewater treatment for PHA production with microbial mixed culture selection, feast-famine

strategy could greatly enrich PHA producing bacteria and is regarded as the state of the art (Fradinho et al., 2016; Morgan-Sagastume et al., 2015). The feast-famine strategy consists in the intermittent feeding of the substrate, where the external substrate or oxygen is taken up and accumulated intracellularly as PHA (feast phase), followed by phases without substrate or oxygen addition that favors cell growth on storage products (famine phase), thus creating a selection pressure for organisms capable of storing PHA (Reis et al., 2003). During the period of starvation (famine phase), bacteria with the ability of polymer-storage gained a selection advantage as they are able to use the stored polymers as a carbon and energy source. Cyclic feast-famine regimes consist of repeatedly alternating disposability (feast phase) or absence of substrate (famine phase) aerobic dynamic feast/famine feeding strategy in a pulse wise mode of substrate addition. Under a feast-famine regime, enrichment of PHA-producers was established on low VFA-containing streams (Korkakaki et al., 2016b). DO limitation was incorporated into the feast-famine strategy to perform an aerobic feast and anoxic famine regime to enhance PHA production and reduce oxygen use (Basset et al., 2016).

There are also a few studies doing solid state fermentation and continuous fermentation, which are important at large scale in industry. For solid state fermentation, PHA was produced from tapioca industry waste, palm jaggery and horse gram flour by *B. megaterium* (Sathiyanarayanan et al., 2013), soy cake and sugarcane molasses by *C. necator* (Oliveira et al., 2007), polyurethane foams by *B. sphaericus* (Jena et al., 2015). Continuous production of PHB is an efficient strategy to reduce production cost and improve sustainability in terms of life cycle assessment (Lopar et al., 2013). Tan et al. (2011) implemented a 14-day unsterile and continuous production of PHB by *Halomonas* TD01. Two fermenters were used where the first one was intended to accumulate cell mass and the second was used to promote PHB fermentation with

nitrogen deficient medium. As a result, PHB conversion efficiency was more than 50% in the second fermenter compared with less than 30% in the first fermenter. Overall, a high PHB productivity (0.26 g/L/h) was achieved (Tan et al., 2011). Hafuka et al. (2011) developed a continuous-feeding regimen and PHB was produced over 259 h (8 draw-fill cycles), but the PHB concentration and content decreased with increasing operation time.

Mixed microbial culture is a promising fermentation mode for PHA production from wastewater. In mixed microbial cultures, controlling the substrate concentration, maintaining oxygen flow rate and control of the N and P concentration will increase PHA storage in cells (Din et al., 2012). A few studies proved mixed cultures are better than pure cultures. For example, Ashby et al. (2005) used mixed cultures (*P. corrugate* and *P. oleovorans*), glycerol as substrate, reported high content of PHA and maximum utilization of substrate when both of the strains are used compared to monocultures of each species. Kourmentza et al. (2009) used both mixed cultures and pure cultures for PHA production and reported that mixed cultures were more efficient in terms of better final yields and PHA and faster accumulation rates than individual cultures.

2.4. Conclusion and future perspectives

PHAs are promising substitutes for petrochemical-derived plastics due to their mechanical properties and high biocompatibilty. The realization of a cost-effective PHA production relies on the microorganisms applied, feedstocks, substrates, feeding strategies, and culture conditions. Using cheap feedstocks as substrates can reduce the product cost and realize the commercialization of PHA at large scale. Most waste feedstocks have complex compositions. Pretreatment of waste materials to make readily consumable carbon is the key for successful conversion of waste feedstocks into PHA. Mixed microbial culture is promising for complex feedstocks and feasible

to operate at unsterile conditions and large scale. PHA production can be incorporated into biofuel production from lignocellulosic feedstocks to offset the production cost for both PHA and biofuel production. Under the multi-purpose biorefinery platform, coproduction of various valuable products, especially extracellular products that can be separated from PHA easily, increase the profit of large scale PHA manufacturing. Process development associated with complex feedstocks such as waste feedstocks and lignocellulosic feedstocks requires more research. Most PHA production research has been performed at small scale, which is unlike commercial PHA manufacturing. Strain stability such as thermo-tolerance and osmosis resistance should be considered. More work should be done to scale up successful lab-scale processes followed by life cycle and economic assessment to evaluate process feasibility. Continuous fermentation and mixed microbial culture for specific feedstocks should be studied more.

2.5. References

Alkotaini, B., Koo, H., Kim, B.S. 2016. Production of polyhydroxyalkanoates by batch and fed-batch cultivations of *Bacillus megaterium* from acid-treated red algae. Korean J. Chem. Eng., 33(5), 1669-1673.

Alsafadi, D., Al-Mashaqbeh, O. 2017. A one-stage cultivation process for the production of poly-3-(hydroxybutyrate-co-hydroxyvalerate) from olive mill wastewater by *Haloferax mediterranei*. New Biotechnol., 34, 47-53.

Altaee, N., El-Hiti, G.A., Fahdil, A., Sudesh, K., Yousif, E. 2017. Screening and evaluation of poly (3-hydroxybutyrate) with *Rhodococcus equi* using different carbon sources. Arab. J. Sci. Eng., 42(6), 2371-2379.

Amulya, K., Reddy, M.V., Rohit, M., Mohan, S.V. 2016. Wastewater as renewable feedstock for bioplastics production: understanding the role of reactor microenvironment and system pH. J. Clean. Prod., 112, 4618-4627.

Anderson, A.J., Dawes, E.A. 1990. Occurrence, metabolism, metabolic role, and industrial uses of bacterial polyhydroxyalkanoates. Microbiol. Rev., 54(4), 450-472.

Anjum, A., Zuber, M., Zia, K.M., Noreen, A., Anjum, M.N., Tabasum, S. 2016. Microbial production of polyhydroxyalkanoates (PHAs) and its copolymers: A review of recent advancements. Int. J. Biol. Macromol., 89, 161-174.

Aramvash, A., Moazzeni Zavareh, F., Gholami Banadkuki, N. 2018. Comparison of different solvents for extraction of polyhydroxybutyrate from *Cupriavidus necator*. Eng. Life Sci., 18(1), 20-28.

Aristidou, A., Penttila, M. 2000. Metabolic engineering applications to renewable resource utilization. Curr. Opin. Biotechnol., 11(2), 187-198.

Ashby, R.D., Solaiman, D.K., Foglia, T.A. 2005. Synthesis of short-/medium-chain-length poly (hydroxyalkanoate) blends by mixed culture fermentation of glycerol. Biomacromolecules, 6(4), 2106-2112.

Ashby, R.D., Solaiman, D.K.Y., Nunez, A., Strahan, G.D., Johnston, D.B. 2018. *Burkholderia sacchari* DSM 17165: A source of compositionally-tunable block-copolymeric short-chain poly(hydroxyalkanoates) from xylose and levulinic acid. Bioresour. Technol., 253, 333-342.

Azizi, N., Najafpour, G., Younesi, H. 2017. Acid pretreatment and enzymatic saccharification of brown seaweed for polyhydroxybutyrate (PHB) production using *Cupriavidus necator*. Int. J. Biol. Macromol., 101, 1029-1040.

Basset, N., Katsou, E., Frison, N., Malamis, S., Dosta, J., Fatone, F. 2016. Integrating the selection of PHA storing biomass and nitrogen removal via nitrite in the main wastewater treatment line. Bioresour. Technol., 200, 820-829.

Bengtsson, S., Karlsson, A., Alexandersson, T., Quadri, L., Hjort, M., Johansson, P., Morgan-Sagastume, F., Anterrieu, S., Arcos-Hernandez, M., Karabegovic, L. 2017. A process for polyhydroxyalkanoate (PHA) production from municipal wastewater treatment with biological carbon and nitrogen removal demonstrated at pilot-scale. New Biotechnol., 35, 42-53.

Berekaa, M.M., Al Issa, A.M. 2016. Enhanced production of polyhydroxybutyrate (phb) from agro-industrial wastes: fed-batch cultivation and statistical media optimization. J. Microbiol. Biotechnol. Food Sci., 5(6), 606.

Berezina, N. 2013. Novel approach for productivity enhancement of polyhydroxyalkanoates (PHA) production by *Cupriavidus necator* DSM 545. New Biotechnol., 30(2), 192-195.

Berezina, N., Yada, B., Lefebvre, R. 2015. From organic pollutants to bioplastics: insights into the bioremediation of aromatic compounds by *Cupriavidus necator*. New Biotechnol., 32(1), 47-53.

Bernd, H. 2003. Polyester synthases: natural catalysts for plastics. Biochem. J., 376(1), 15-33.

Brodin, M., Vallejos, M., Opedal, M.T., Area, M.C., Chinga-Carrasco, G. 2017. Lignocellulosics as sustainable resources for production of bioplastics–A review. J. Clean. Prod., 162, 646-664.

Campanari, S., Augelletti, F., Rossetti, S., Sciubba, F., Villano, M., Majone, M. 2017. Enhancing a multi-stage process for olive oil mill wastewater valorization towards polyhydroxyalkanoates and biogas production. Chem. Eng. J., 317, 280-289.

Carpine, R., Raganati, F., Olivieri, G., Hellingwerf, K.J., Pollio, A., Salatino, P., Marzocchella, A. 2018. Poly-β-hydroxybutyrate (PHB) production by *Synechocystis* PCC6803 from CO2: Model development. Algal Res., 29, 49-60.

Cesario, M.T., Raposo, R.S., De Almeida, M.C.M., Van Keulen, F., Ferreira, B.S., Da Fonseca, M.M.R. 2014. Enhanced bioproduction of poly-3-hydroxybutyrate from wheat straw lignocellulosic hydrolysates. New Biotechnol., 31(1), 104-113.

Chen, G.-Q., Wu, Q. 2005. Microbial production and applications of chiral hydroxyalkanoates. Appl. Microbiol. Biotechnol., 67(5), 592-599.

Chen, X., Yin, J., Ye, J., Zhang, H., Che, X., Ma, Y., Li, M., Wu, L.-P., Chen, G.-Q. 2017. Engineering *Halomonas bluephagenesis* TD01 for non-sterile production of poly (3-hydroxybutyrate-co-4-hydroxybutyrate). Bioresour. Technol., 244, 534-541.

Chen, Z., Huang, L., Wen, Q., Guo, Z. 2015. Efficient polyhydroxyalkanoate (PHA) accumulation by a new continuous feeding mode in three-stage mixed microbial culture (MMC) PHA production process. J. Biotechnol., 209, 68-75.

Choi, J., Lee, S.Y. 1999. Factors affecting the economics of polyhydroxyalkanoate production by bacterial fermentation. Appl. Microbiol. Biotechnol., 51(1), 13-21.

Cibichakravarthy, B., Abinaya, S., Prabagaran, S.R. 2017. Syntrophic association of termite gut bacterial symbionts with bifunctional characteristics of cellulose degrading and polyhydroxyalkanoate producing bacteria. Int. J. Biol. Macromol., 103, 613-620.

Colombo, B., Sciarria, T.P., Reis, M., Scaglia, B., Adani, F. 2016. Polyhydroxyalkanoates (PHAs) production from fermented cheese whey by using a mixed microbial culture. Bioresour. Technol., 218, 692-699.

Costa, S.S., Miranda, A.L., Andrade, B.B., De Jesus Assis, D., Souza, C.O., De Morais, M.G., Costa, J.A.V., Druzian, J.I. 2018. Influence of nitrogen on growth, biomass composition, production, and properties of polyhydroxyalkanoates (PHAs) by microalgae. Int. J. Biol. Macromol., 116, 552-562.

Cruz, M.V., Freitas, F., Paiva, A., Mano, F., Dionisio, M., Ramos, A.M., Reis, M.A. 2016. Valorization of fatty acids-containing wastes and byproducts into short-and medium-chain length polyhydroxyalkanoates. New Biotechnol., 33(1), 206-215.

Cui, Y.-W., Zhang, H.-Y., Lu, P.-F., Peng, Y.-Z. 2016. Effects of carbon sources on the enrichment of halophilic polyhydroxyalkanoate-storing mixed microbial culture in an aerobic dynamic feeding process. Sci. Rep., 6, 30766.

Dai, J., Gliniewicz, K., Settles, M.L., Coats, E.R., McDonald, A.G. 2015. Influence of organic loading rate and solid retention time on polyhydroxybutyrate production from hybrid poplar hydrolysates using mixed microbial cultures. Bioresour. Technol., 175, 23-33.

Danis, O., Ogan, A., Tatlican, P., Attar, A., Cakmakci, E., Mertoglu, B., Birbir, M. 2015. Preparation of poly (3-hydroxybutyrate-co-hydroxyvalerate) films from halophilic archaea and their potential use in drug delivery. Extremophiles, 19(2), 515-524.

Das, M., Grover, A. 2018. Fermentation optimization and mathematical modeling of glycerol-based microbial poly(3-hydroxybutyrate) production. Process Biochem., 71, 1-11.

De Paula, F.C., Kakazu, S., De Paula, C.B.C., Gomez, J.G.C., Contiero, J. 2017. Polyhydroxyalkanoate production from crude glycerol by newly isolated *Pandoraea* sp. J. King Saud. Univ. Sci., 29(2), 166-173.

Devi, N.C., Mazumder, P.B., Bhattacharjee, A. 2018. Statistical optimization of polyhydroxybutyrate production by *Bacillus pumilus* H9 using cow dung as a cheap carbon source by response surface methodology. J. Polym. Environ., 26(8), 3159-3167.

Dey, P., Rangarajan, V. 2017. Improved fed-batch production of high-purity PHB (poly-3 hydroxy butyrate) by *Cupriavidus necator* (MTCC 1472) from sucrose-based cheap substrates under response surface-optimized conditions. 3 Biotech, 7(5), 310.

Dietrich, K., Dumont, M.-J., Del Rio, L.F., Orsat, V. 2017a. Producing PHAs in the bioeconomy-Towards a sustainable bioplastic. Sustain. Prod. Consump., 9, 58-70.

Dietrich, K., Dumont, M.-J., Orsat, V., Del Rio, L.F. 2019. Consumption of sugars and inhibitors of softwood hemicellulose hydrolysates as carbon sources for polyhydroxybutyrate (PHB) production with *Paraburkholderia sacchari* IPT 101. Cellulose, 1-14.

Dietrich, K., Dumont, M.-J., Schwinghamer, T., Orsat, V., Del Rio, L.F. 2017b. Model study to assess softwood hemicellulose hydrolysates as the carbon source for PHB production in *Paraburkholderia sacchari* IPT 101. Biomacromolecules, 19(1), 188-200.

Din, M.F.M., Mohanadoss, P., Ujang, Z., Van Loosdrecht, M., Yunus, S.M., Chelliapan, S., Zambare, V., Olsson, G. 2012. Development of Bio-PORec® system for polyhydroxyalkanoates (PHA) production and its storage in mixed cultures of palm oil mill effluent (POME). Bioresour. Technol., 124, 208-216.

Ding, C., Li, M., Hu, Y. 2018. High-activity production of xylanase by *Pichia stipitis*: Purification, characterization, kinetic evaluation and xylooligosaccharides production. Int. J. Biol. Macromol., 117, 72-77.

Ding, C., Wang, X., Li, M. 2019. Evaluation of six white-rot fungal pretreatments on corn stover for the production of cellulolytic and ligninolytic enzymes, reducing sugars, and ethanol. Appl. Microbiol. Biotechnol., 1-12.

Divyashree, M.S., Rastogi, N.K., Shamala, T.R. 2009. A simple kinetic model for growth and biosynthesis of polyhydroxyalkanoate in *Bacillus flexus*. New Biotechnol., 26(1), 92-98.

Elain, A., Le Grand, A., Corre, Y.-M., Le Fellic, M., Hachet, N., Le Tilly, V., Loulergue, P., Audic, J.-L., Bruzaud, S. 2016. Valorization of local agro-industrial processing waters as growth media for polyhydroxyalkanoates (PHA) production. Ind. Crops Prod., 80, 1-5.

Endres, H.-J., Siebert-Raths, A. 2011. End-of-life options for biopolymers. in: *Engineering biopolymers: Markets, manufacturing, properties and applications*, Vol. 71148, Hanser Publications. Cincinnati.

Fra-Vazquez, A., Santorio, S., Palmeiro-Sanchez, T., Val del Rio, A., Mosquera-Corral, A. 2019. PHA accumulation of a mixed microbial culture co-exists with ammonia partial nitritation. Chem. Eng. J., 360, 1255-1261.

Fradinho, J.C., Reis, M.A.M., Oehmen, A. 2016. Beyond feast and famine: Selecting a PHA accumulating photosynthetic mixed culture in a permanent feast regime. Water Res., 105, 421-428.

Frederick, N., Li, M., Carrier, D.J., Buser, M.D., Wilkins, M.R. 2016. Switchgrass storage effects on the recovery of carbohydrates after liquid hot water pretreatment and enzymatic hydrolysis. AIMS Bioeng., 3(3), 389-399.

Garcia-Perez, T., Lopez, J.C., Passos, F., Lebrero, R., Revah, S., Munoz, R. 2018. Simultaneous methane abatement and PHB production by *Methylocystis hirsuta* in a novel gas-recycling bubble column bioreactor. Chem. Eng. J., 334, 691-697.

Gasser, E., Ballmann, P., Droge, S., Bohn, J., Konig, H. 2014. Microbial production of biopolymers from the renewable resource wheat straw. J. Appl. Microbiol., 117(4), 1035-1044.

Gaudy, A.F., Obayashi, A., Gaudy, E.T. 1971. Control of growth rate by initial substrate concentration at values below maximum rate. Appl. Environ. Microbiol., 22(6), 1041-1047.

Glazer, A.N., Nikaido, H. 2007. *Microbial biotechnology: fundamentals of applied microbiology*. *Second ed*. Cambridge University Press, New York.

Gomaa, E.Z. 2014. Production of polyhydroxyalkanoates (PHAs) by *Bacillus subtilis* and *Escherichia coli* grown on cane molasses fortified with ethanol. Braz. Arch. Biol. Technol., 57(1), 145-154.

Gomez Cardozo, J.R., Mora Martinez, A.L., Yepes Perez, M., Correa Londono, G.A. 2016. Production and characterization of polyhydroxyalkanoates and native microorganisms synthesized from fatty waste. Int. J. Polym. Sci., 2016.

Gomez, J., Rodrigues, M., Alli, R., Torres, B., Netto, C.B., Oliveira, M., Da Silva, L. 1996a. Evaluation of soil gram-negative bacteria yielding polyhydroxyalkanoic acids from carbohydrates and propionic acid. Appl. Microbiol. Biotechnol., 45(6), 785-791.

Gomez, J.G.C., Rodrigues, M.F.A., Alli, R.C.P., Torres, B.B., Netto, C.L.B., Oliveira, M.S., daSilva, L.F. 1996b. Evaluation of soil gram-negative bacteria yielding polyhydroxyalkanoic acids from carbohydrates and propionic acid. Appl Microbiol Biot, 45(6), 785-791.

Gouveia, A.R., Freitas, E.B., Galinha, C.F., Carvalho, G., Duque, A.F., Reis, M.A.M. 2017. Dynamic change of pH in acidogenic fermentation of cheese whey towards polyhydroxyalkanoates production: Impact on performance and microbial population. New Biotechnol., 37, 108-116.

Gupta, V., Ratha, S.K., Sood, A., Chaudhary, V., Prasanna, R. 2013. New insights into the biodiversity and applications of cyanobacteria (blue-green algae)-Prospects and challenges. Algal Res., 2(2), 79-97.

Hafuka, A., Sakaida, K., Satoh, H., Takahashi, M., Watanabe, Y., Okabe, S. 2011. Effect of feeding regimens on polyhydroxybutyrate production from food wastes by *Cupriavidus necator*. Bioresour. Technol., 102(3), 3551-3553.

Hermann-Krauss, C., Koller, M., Muhr, A., Fasl, H., Stelzer, F., Braunegg, G. 2013. Archaeal production of polyhydroxyalkanoate (PHA) co-and terpolyesters from biodiesel industry-derived by-products. Archaea, 2013.

Hori, C., Yamazaki, T., Ribordy, G., Takisawa, K., Matsumoto, K.i., Ooi, T., Zinn, M., Taguchi, S. 2019. High-cell density culture of poly(lactate-co-3-hydroxybutyrate)-producing Escherichia coli by using glucose/xylose-switching fed-batch jar fermentation. J. Biosci. Bioeng., 127(6), 721-725.

Huang, L., Liu, C., Liu, Y., Jia, X. 2016. The composition analysis and preliminary cultivation optimization of a PHA-producing microbial consortium with xylose as a sole carbon source. Waste Manag., 52, 77-85.

Huo, G., Zhu, Y., Liu, Q., Tao, R., Diao, N., Wang, Z., Chen, T. 2017. Metabolic engineering of an *E. coli* ndh knockout strain for PHB production from mixed glucose–xylose feedstock. J. Chem. Technol. Biotechnol., 92(10), 2739-2745.

Impallomeni, G., Ballistreri, A., Carnemolla, G.M., Rizzo, M.G., Nicolo, M.S., Guglielmino, S.P.P. 2018. Biosynthesis and structural characterization of polyhydroxyalkanoates produced by

Pseudomonas aeruginosa ATCC 27853 from long odd-chain fatty acids. Int. J. Biol. Macromol., 108, 608-614.

Jaremko, M., Yu, J. 2011. The initial metabolic conversion of levulinic acid in *Cupriavidus necator*. J. Biotechnol., 155(3), 293-298.

Jena, J., Kumar, R., Dixit, A., Pandey, S., Das, T. 2015. Evaluation of simultaneous nutrient and COD removal with polyhydroxybutyrate (PHB) accumulation using mixed microbial consortia under anoxic condition and their bioinformatics analysis. PLoS ONE, 10(2), e0116230.

Jiang, G., Hill, D., Kowalczuk, M., Johnston, B., Adamus, G., Irorere, V., Radecka, I. 2016. Carbon sources for polyhydroxyalkanoates and an integrated biorefinery. Int. J. Mol. Sci., 17(7), 1157.

Johnson, K., Kleerebezem, R., van Loosdrecht, M.C.M. 2010. Influence of ammonium on the accumulation of polyhydroxybutyrate (PHB) in aerobic open mixed cultures. J. Biotechnol., 147(2), 73-79.

Kachrimanidou, V., Kopsahelis, N., Papanikolaou, S., Kookos, I.K., De Bruyn, M., Clark, J.H., Koutinas, A.A. 2014. Sunflower-based biorefinery: Poly (3-hydroxybutyrate) and poly (3-hydroxybutyrate-co-3-hydroxyvalerate) production from crude glycerol, sunflower meal and levulinic acid. Bioresour. Technol., 172, 121-130.

Kachrimanidou, V., Kopsahelis, N., Vlysidis, A., Papanikolaou, S., Kookos, I.K., Monje Martínez, B., Escrig Rondan, M.C., Koutinas, A.A. 2016. Downstream separation of poly(hydroxyalkanoates) using crude enzyme consortia produced via solid state fermentation integrated in a biorefinery concept. Food Bioprod. Process., 100, 323-334.

Kataria, R., Woods, T., Casey, W., Cerrone, F., Davis, R., O'Connor, K., Ruhal, R., Babu, R. 2018. Surfactant-mediated hydrothermal pretreatment of Ryegrass followed by enzymatic saccharification for polyhydroxyalkanoate production. Ind. Crops Prod., 111, 625-632.

Khanna, S., Srivastava, A.K. 2005. Recent advances in microbial polyhydroxyalkanoates. Process Biochem., 40(2), 607-619.

Kim, B.S. 2000. Production of poly (3-hydroxybutyrate) from inexpensive substrates. Enzyme Microb. Technol., 27(10), 774-777.

Kim, S., Holtzapple, M.T. 2005. Lime pretreatment and enzymatic hydrolysis of corn stover. Bioresour. Technol., 96(18), 1994-2006.

Koller, M., Marsalek, L., De Sousa Dias, M.M., Braunegg, G. 2017. Producing microbial polyhydroxyalkanoate (PHA) biopolyesters in a sustainable manner. New Biotechnol., 37, 24-38.

Kominek, L.A., Halvorson, H.O. 1965. Metabolism of poly-β-hydroxybutyrate and acetoin in *Bacillus cereus*. J. Bacteriol., 90(5), 1251-1259.

Korkakaki, E., Mulders, M., Veeken, A., Rozendal, R., Van Loosdrecht, M.C., Kleerebezem, R. 2016a. PHA production from the organic fraction of municipal solid waste (OFMSW): overcoming the inhibitory matrix. Water Res., 96, 74-83.

Korkakaki, E., Van Loosdrecht, M.C., Kleerebezem, R. 2016b. Survival of the fastest: Selective removal of the side population for enhanced PHA production in a mixed substrate enrichment. Bioresour. Technol., 216, 1022-1029.

Kosseva, M.R., Rusbandi, E. 2018. Trends in the biomanufacture of polyhydroxyalkanoates with focus on downstream processing. Int. J. Biol. Macromol., 107, 762-778.

Kourmentza, C., Costa, J., Azevedo, Z., Servin, C., Grandfils, C., De Freitas, V., Reis, M.A.M. 2018. *Burkholderia thailandensis* as a microbial cell factory for the bioconversion of used cooking oil to polyhydroxyalkanoates and rhamnolipids. Bioresour. Technol., 247, 829-837.

Kourmentza, C., Ntaikou, I., Kornaros, M., Lyberatos, G. 2009. Production of PHAs from mixed and pure cultures of *Pseudomonas* sp. using short-chain fatty acids as carbon source under nitrogen limitation. Desalination, 248(1-3), 723-732.

Kovalcik, A., Kucera, D., Matouskova, P., Pernicova, I., Obruca, S., Kalina, M., Enev, V., Marova, I. 2018. Influence of removal of microbial inhibitors on PHA production from spent coffee grounds employing *Halomonas halophila*. J. Environ. Chem. Eng., 6(2), 3495-3501.

Kumar, M., Gupta, A., Thakur, I.S. 2016a. Carbon dioxide sequestration by chemolithotrophic oleaginous bacteria for production and optimization of polyhydroxyalkanoate. Bioresour. Technol., 213, 249-256.

Kumar, P., Kim, B.S. 2018. Valorization of polyhydroxyalkanoates production process by co-synthesis of value-added products. Bioresour. Technol.

Kumar, P., Ray, S., Kalia, V.C. 2016b. Production of co-polymers of polyhydroxyalkanoates by regulating the hydrolysis of biowastes. Bioresour. Technol., 200, 413-419.

Laser, M., Schulman, D., Allen, S.G., Lichwa, J., Antal, M.J., Lynd, L.R. 2002. A comparison of liquid hot water and steam pretreatments of sugar cane bagasse for bioconversion to ethanol. Bioresour. Technol., 81(1), 33-44.

Laycock, B., Halley, P., Pratt, S., Werker, A., Lant, P. 2013. The chemomechanical properties of microbial polyhydroxyalkanoates. Prog. Polym. Sci., 38(3), 536-583.

Lee, W.-H., Loo, C.-Y., Nomura, C.T., Sudesh, K. 2008. Biosynthesis of polyhydroxyalkanoate copolymers from mixtures of plant oils and 3-hydroxyvalerate precursors. Bioresour. Technol., 99(15), 6844-6851.

Li, M. 2015. White rot fungi *Pleurotus ostreatus* pretreatment on switchgrass to enhance enzymatic hydrolysis and ethanol production. in: *Biosystems and Agricultural Engineering*, Vol. Masters thesis, Oklahoma State University. Stillwater, Oklahoma, pp. 178.

Li, M., Eskridge, K., Liu, E., Wilkins, M. 2019a. Enhancement of polyhydroxybutyrate (PHB) production by 10-fold from alkaline pretreatment liquor with an oxidative enzyme-mediator-surfactant system under Plackett-Burman and central composite designs. Bioresour. Technol., 281, 99-106.

Li, M., Eskridge, K.M., Wilkins, M.R. 2019b. Optimization of polyhydroxybutyrate production by experimental design of combined ternary mixture (glucose, xylose and arabinose) and process variables (sugar concentration, molar C: N ratio). Bioprocess Biosystems Eng., 1-12.

Li, M., Marek, S.M., Peng, J., Liu, Z., Wilkins, M.R. 2018a. Effect of moisture content and inoculum size on cell wall composition and ethanol yield from switchgrass after solid-state *Pleurotus ostreatus* treatment. Trans. ASABE, 61(6), 1997-2006.

Li, T., Elhadi, D., Chen, G.-Q. 2017. Co-production of microbial polyhydroxyalkanoates with other chemicals. Metab. Eng., 43, 29-36.

Li, X., Kim, T.H., Nghiem, N.P. 2010. Bioethanol production from corn stover using aqueous ammonia pretreatment and two-phase simultaneous saccharification and fermentation (TPSSF). Bioresour. Technol., 101(15), 5910-5916.

Linger, J.G., Vardon, D.R., Guarnieri, M.T., Karp, E.M., Hunsinger, G.B., Franden, M.A., Johnson, C.W., Chupka, G., Strathmann, T.J., Pienkos, P.T. 2014. Lignin valorization through integrated biological funneling and chemical catalysis. Proc. Natl. Acad. Sci., 111(33), 12013-12018.

Liu, K., Atiyeh, H.K., Pardo-Planas, O., Ezeji, T.C., Ujor, V., Overton, J.C., Berning, K., Wilkins, M.R., Tanner, R.S. 2015. Butanol production from hydrothermolysis-pretreated switchgrass: quantification of inhibitors and detoxification of hydrolyzate. Bioresour. Technol., 189, 292-301.

Liu, Z.-H., Olson, M.L., Shinde, S., Wang, X., Hao, N., Yoo, C.G., Bhagia, S., Dunlap, J.R., Pu, Y., Kao, K.C. 2017. Synergistic maximization of the carbohydrate output and lignin processability by combinatorial pretreatment. Green Chem., 19(20), 4939-4955.

Liu, Z.-H., Xie, S., Lin, F., Jin, M., Yuan, J.S. 2018. Combinatorial pretreatment and fermentation optimization enabled a record yield on lignin bioconversion. Biotechnol. Biofuels, 11(1), 21.

Lopar, M., Vrana Spoljaric, I., Atlic, A., Koller, M., Braunegg, G., Horvat, P. 2013. Five-step continuous production of PHB analyzed by elementary flux, modes, yield space analysis and high structured metabolic model. Biochem. Eng. J., 79, 57-70.

Lopes, M.S.G., Gomez, J.G.C., Taciro, M.K., Mendonça, T.T., Silva, L.F. 2014. Polyhydroxyalkanoate biosynthesis and simultaneous remotion of organic inhibitors from sugarcane bagasse hydrolysate by *Burkholderia* sp. J. Ind. Microbiol. Biotechnol., 41(9), 1353-1363.

Maheshwari, N., Kumar, M., Thakur, I.S., Srivastava, S. 2018. Production, process optimization and molecular characterization of polyhydroxyalkanoate (PHA) by CO_2 sequestering *B. cereus* SS105. Bioresour. Technol., 254, 75-82.

Martin, C.H., Prather, K.L.J. 2009. High-titer production of monomeric hydroxyvalerates from levulinic acid in *Pseudomonas putida*. J. Biotechnol., 139(1), 61-67.

Martinez, G.A., Rebecchi, S., Decorti, D., Domingos, J.M., Natolino, A., Del Rio, D., Bertin, L., Da Porto, C., Fava, F. 2016. Towards multi-purpose biorefinery platforms for the valorisation of red grape pomace: production of polyphenols, volatile fatty acids, polyhydroxyalkanoates and biogas. Green Chem., 18(1), 261-270.

Marto, J., Gouveia, L., Chiari, B., Paiva, A., Isaac, V., Pinto, P., Simoes, P., Almeida, A., Ribeiro, H. 2016. The green generation of sunscreens: Using coffee industrial sub-products. Ind. Crops Prod., 80, 93-100.

Mendonca, T., Gomez, J., Buffoni, E., Rodriguez, R., Schripsema, J., Lopes, M., Silva, L. 2014. Exploring the potential of Burkholderia sacchari to produce polyhydroxyalkanoates. J Appl Microbiol, 116(4), 815-829.

Miranda De Sousa Dias, M., Koller, M., Puppi, D., Morelli, A., Chiellini, F., Braunegg, G. 2017. Fed-batch synthesis of Poly (3-hydroxybutyrate) and poly (3-hydroxybutyrate-co-4-hydroxybutyrate) from sucrose and 4-hydroxybutyrate precursors by *Burkholderia sacchari* strain DSM 17165. Bioeng., 4(2), 36.

Mohapatra, S., Samantaray, D.P., Samantaray, S.M., Mishra, B.B., Das, S., Majumdar, S., Pradhan, S.K., Rath, S.N., Rath, C.C., Akthar, J., Achary, K.G. 2016. Structural and thermal characterization of PHAs produced by *Lysinibacillus* sp. through submerged fermentation process. Int. J. Biol. Macromol., 93, 1161-1167.

Montiel-Jarillo, G., Carrera, J., Suarez-Ojeda, M.E. 2017. Enrichment of a mixed microbial culture for polyhydroxyalkanoates production: Effect of pH and N and P concentrations. ScTEn, 583, 300-307.

Morgan-Sagastume, F., Hjort, M., Cirne, D., Gerardin, F., Lacroix, S., Gaval, G., Karabegovic, L., Alexandersson, T., Johansson, P., Karlsson, A. 2015. Integrated production of

polyhydroxyalkanoates (PHAs) with municipal wastewater and sludge treatment at pilot scale. Bioresour. Technol., 181, 78-89.

Mozejko-Ciesielska, J., Kiewisz, R. 2016. Bacterial polyhydroxyalkanoates: Still fabulous? Microbiol. Res., 192, 271-282.

Mozumder, M.S.I., Garcia-Gonzalez, L., De Wever, H., Volcke, E.I.P. 2015. Effect of sodium accumulation on heterotrophic growth and polyhydroxybutyrate (PHB) production by *Cupriavidus necator*. Bioresour. Technol., 191, 213-218.

Munir, S., Iqbal, S., Jamil, N. 2015. Polyhydroxyalkanoates (PHA) production using paper mill wastewater as carbon source in comparison with glucose. J. Pure Appl. Microbiol., 9, 453-460.

Munoz, A., Esteban, L., Riley, M.R. 2008. Utilization of cellulosic waste from tequila bagasse and production of polyhydroxyalkanoate (PHA) bioplastics by *Saccharophagus degradans*. Biotechnol. Bioeng., 100(5), 882-888.

Nath, A., Dixit, M., Bandiya, A., Chavda, S., Desai, A.J. 2008. Enhanced PHB production and scale up studies using cheese whey in fed batch culture of *Methylobacterium* sp. ZP24. Bioresour. Technol., 99(13), 5749-5755.

Norhafini, H., Huong, K.-H., Amirul, A.A. 2019. High PHA density fed-batch cultivation strategies for 4HB-rich P(3HB-co-4HB) copolymer production by transformant *Cupriavidus malaysiensis* USMAA1020. Int. J. Biol. Macromol., 125, 1024-1032.

Obruca, S., Benesova, P., Marsalek, L., Marova, I. 2015. Use of lignocellulosic materials for PHA production. Chem. Biochem. Eng. Q., 29(2), 135-144.

Obruca, S., Petrik, S., Benesova, P., Svoboda, Z., Eremka, L., Marova, I. 2014b. Utilization of oil extracted from spent coffee grounds for sustainable production of polyhydroxyalkanoates. Appl. Microbiol. Biotechnol., 98(13), 5883-5890.

Ojha, N., Das, N. 2018. A statistical approach to optimize the production of polyhydroxyalkanoates from *Wickerhamomyces anomalus* VIT-NN01 using response surface methodology. Int. J. Biol. Macromol., 107, 2157-2170.

Oliveira, F.C., Dias, M.L., Castilho, L.R., Freire, D.M. 2007. Characterization of poly (3-hydroxybutyrate) produced by *Cupriavidus necator* in solid-state fermentation. Bioresour. Technol., 98(3), 633-638.

Osman, Y., Elrazak, A.A., Khater, W. 2016. Bioprocess optimization of microbial biopolymer production. J. Biobased Mater. Bioenergy, 10(2), 119-128.

Pais, J., Serafim, L.S., Freitas, F., Reis, M.A. 2016. Conversion of cheese whey into poly (3-hydroxybutyrate-co-3-hydroxyvalerate) by *Haloferax mediterranei*. New Biotechnol., 33(1), 224-230.

Pakalapati, H., Chang, C.-K., Show, P.L., Arumugasamy, S.K., Lan, J.C.-W. 2018. Development of polyhydroxyalkanoates production from waste feedstocks and applications. J. Biosci. Bioeng., 126(3), 282-292.

Palmeiro-Sanchez, T., Fra-Vazquez, A., Rey-Martinez, N., Campos, J., Mosquera-Corral, A. 2016. Transient concentrations of NaCl affect the PHA accumulation in mixed microbial culture. J. Hazard. Mater., 306, 332-339.

Pan, W., Nomura, C.T., Nakas, J.P. 2012a. Estimation of inhibitory effects of hemicellulosic wood hydrolysate inhibitors on PHA production by *Burkholderia cepacia* ATCC 17759 using response surface methodology. Bioresour. Technol., 125, 275-282.

Patel, S.K., Lee, J.-K., Kalia, V.C. 2016. Integrative approach for producing hydrogen and polyhydroxyalkanoate from mixed wastes of biological origin. Indian J. Microbiol., 56(3), 293-300.

Philip, S., Keshavarz, T., Roy, I. 2007. Polyhydroxyalkanoates: biodegradable polymers with a range of applications. J. Chem. Technol. Biotechnol., 82(3), 233-247.

Pittmann, T., Steinmetz, H. 2017. Polyhydroxyalkanoate production on waste water treatment plants: Process scheme, operating conditions and potential analysis for German and European municipal waste water treatment plants. Bioeng., 4(2), 54.

Pittmann, T., Steinmetz, H. 2016. Potential for polyhydroxyalkanoate production on German or European municipal waste water treatment plants. Bioresour. Technol., 214, 9-15.

Platts, S.P.G. 2019. S&P Global Platts Petrochemical Index (PGPI), Vol. 2019.

Raje, P., Srivastava, A.K. 1998. Updated mathematical model and fed-batch strategies for poly-β-hydroxybutyrate (PHB) production by *Alcaligenes eutrophus*. Bioresour. Technol., 64(3), 185-192.

Ramachandriya, K.D., Wilkins, M., Atiyeh, H.K., Dunford, N.T., Hiziroglu, S. 2013. Effect of high dry solids loading on enzymatic hydrolysis of acid bisulfite pretreated Eastern redcedar. Bioresour. Technol., 147, 168-176.

RamKumar Pandian, S., Deepak, V., Kalishwaralal, K., Rameshkumar, N., Jeyaraj, M., Gurunathan, S. 2010. Optimization and fed-batch production of PHB utilizing dairy waste and sea water as nutrient sources by *Bacillus megaterium* SRKP-3. Bioresour. Technol., 101(2), 705-711.

Raposo, R.S., De Almeida, M.C.M., De Oliveira, M.d.C.M., Da Fonseca, M.M., Cesario, M.T. 2017. A *Burkholderia sacchari* cell factory: production of poly-3-hydroxybutyrate, xylitol and xylonic acid from xylose-rich sugar mixtures. New Biotechnol., 34, 12-22.

Rathika, R., Janaki, V., Shanthi, K., Kamala-Kannan, S. 2018. Bioconversion of agro-industrial effluents for polyhydroxyalkanoates production using *Bacillus subtilis* RS1. Int. J. Environ. Sci. Technol. (Tehran), 1-10.

Ravindran, R., Jaiswal, A.K. 2016. Exploitation of food industry waste for high-value products. Trends Biotechnol., 34(1), 58-69.

Reis, M., Albuquerque, M., Villano, M., Majone, M. 2011. Mixed culture processes for polyhydroxyalkanoate production from agro-industrial surplus/wastes as feedstocks. Second ed. in: *Comp. Biotechnol.*, (Ed.) M. Moo-Young.

Reis, M., Serafim, L., Lemos, P., Ramos, A., Aguiar, F., Van Loosdrecht, M. 2003. Production of polyhydroxyalkanoates by mixed microbial cultures. Bioprocess Biosystems Eng., 25(6), 377-385.

Ribeiro, H., Marto, J., Raposo, S., Agapito, M., Isaac, V., Chiari, B.G., Lisboa, P.F., Paiva, A., Barreiros, S., Simoes, P. 2013. From coffee industry waste materials to skin ‐ friendly products with improved skin fat levels. Eur. J. Lipid Sci. Technol., 115(3), 330-336.

Rodriguez-Contreras, A., Koller, M., Braunegg, G., Marques-Calvo, M.S. 2016. Poly[(R)-3-hydroxybutyrate] production under different salinity conditions by a novel *Bacillus megaterium* strain. New Biotechnol., 33(1), 73-77.

Rodriguez-Perez, S., Serrano, A., Pantion, A.A., Alonso-Farinas, B. 2018. Challenges of scaling-up PHA production from waste streams. A review. J. Environ. Manage., 205, 215-230.

Rohrbeck, M., Körsten, S., Fischer, C.B., Wehner, S., Kessler, B. 2013. Diamond-like carbon coating of a pure bioplastic foil. TSF, 545, 558-563.

Sabapathy, P.C., Devaraj, S., Parthipan, A., Kathirvel, P. 2019. Polyhydroxyalkanoate production from statistically optimized media using rice mill effluent as sustainable substrate with an analysis on the biopolymer's degradation potential. Int. J. Biol. Macromol., 126, 977-986.

Sakthiselvan, P., Madhumathi, R. 2018. Kinetic evaluation on cell growth and biosynthesis of polyhydroxybutyrate (PHB) by *Bacillus safensis* EBT1 from sugarcane bagasse. Eng. Agri. Envir. Food, 11(3), 145-152.

Saratale, R.G., Saratale, G.D., Cho, S.K., Kim, D.S., Ghodake, G.S., Kadam, A., Kumar, G., Bharagava, R.N., Banu, R., Shin, H.S. 2019. Pretreatment of kenaf (*Hibiscus cannabinus* L.) biomass feedstock for polyhydroxybutyrate (PHB) production and characterization. Bioresour. Technol., 282, 75-80.

Sathiyanarayanan, G., Kiran, G.S., Selvin, J., Saibaba, G. 2013. Optimization of polyhydroxybutyrate production by marine *Bacillus megaterium* MSBN04 under solid state culture. Int. J. Biol. Macromol., 60, 253-261.

Sawant, S.S., Tran, T.K., Salunke, B.K., Kim, B.S. 2017. Potential of *Saccharophagus degradans* for production of polyhydroxyalkanoates using cellulose. Process Biochem., 57, 50-56.

Sharma, P.K., Munir, R.I., de Kievit, T., Levin, D.B. 2017. Synthesis of polyhydroxyalkanoates (PHAs) from vegetable oils and free fatty acids by wild-type and mutant strains of *Pseudomonas chlororaphis*. Can. J. Microbiol., 63(12), 1009-1024.

Shasaltaneh, M.D., Moosavi-Nejad, Z., Gharavi, S., Fooladi, J. 2013. Cane molasses as a source of precursors in the bioproduction of tryptophan by *Bacillus subtilis*. Iran. J. Microbio., 5(3), 285.

Shi, Y., Yan, X., Li, Q., Wang, X., Xie, S., Chai, L., Yuan, J. 2017. Directed bioconversion of kraft lignin to polyhydroxyalkanoate by *Cupriavidus basilensis* B-8 without any pretreatment. Process Biochem., 52, 238-242.

Si, M., Yan, X., Liu, M., Shi, M., Wang, Z., Wang, S., Zhang, J., Gao, C., Chai, L., Shi, Y. 2018. In situ lignin bioconversion promotes complete carbohydrate conversion of rice straw by *Cupriavidus basilensis* B-8. ACS Sustain. Chem. Eng., 6(6), 7969-7978.

Singh, A.K., Sharma, L., Mallick, N., Mala, J. 2017. Progress and challenges in producing polyhydroxyalkanoate biopolymers from cyanobacteria. J. Appl. Phycol., 29(3), 1213-1232.

Singh, G., Kumari, A., Mittal, A., Yadav, A., Aggarwal, N.K. 2013. Poly β-hydroxybutyrate production by *Bacillus subtilis* NG220 using sugar industry waste water. BioMed Res. Int., 2013.

Snell, K.D., Singh, V., Brumbley, S.M. 2015. Production of novel biopolymers in plants: recent technological advances and future prospects. Curr. Opin. Biotechnol., 32, 68-75.

Socha, A.M., Parthasarathi, R., Shi, J., Pattathil, S., Whyte, D., Bergeron, M., George, A., Tran, K., Stavila, V., Venkatachalam, S. 2014. Efficient biomass pretreatment using ionic liquids derived from lignin and hemicellulose. Proc. Natl. Acad. Sci., 111(35), E3587-E3595.

Sonnleitner, B., Heinzle, E., Braunegg, G., Lafferty, R. 1979. Formal kinetics of poly-β-hydroxybutyric acid (PHB) production in *Alcaligenes eutrophus* H 16 and *Mycoplana rubra* R 14 with respect to the dissolved oxygen tension in ammonium-limited batch cultures. Eur. J. Appl. Microbiol. Biotechnol., 7(1), 1-10.

Spiekermann, P., Rehm, B.H., Kalscheuer, R., Baumeister, D., Steinbuchel, A. 1999. A sensitive, viable-colony staining method using Nile red for direct screening of bacteria that

accumulate polyhydroxyalkanoic acids and other lipid storage compounds. Arch. Microbiol., 171(2), 73-80.

Stanley, A., Kumar, H.P., Mutturi, S., Vijayendra, S.N. 2018. Fed-batch strategies for production of PHA using a native isolate of *Halomonas venusta* KT832796 strain. Appl. Biochem. Biotechnol., 184(3), 935-952.

Sudesh, K., Abe, H., Doi, Y. 2000. Synthesis, structure and properties of polyhydroxyalkanoates: biological polyesters. Prog. Polym. Sci., 25(10), 1503-1555.

Suryawati, L., Wilkins, M.R., Bellmer, D.D., Huhnke, R.L., Maness, N.O., Banat, I.M. 2009. Effect of hydrothermolysis process conditions on pretreated switchgrass composition and ethanol yield by SSF with *Kluyveromyces marxianus* IMB4. Process Biochem., 44(5), 540-545.

Tan, D., Xue, Y.-S., Aibaidula, G., Chen, G.-Q. 2011. Unsterile and continuous production of polyhydroxybutyrate by *Halomonas* TD01. Bioresour. Technol., 102(17), 8130-8136.

Tanaka, T., Iwata, T., Aoyagi, Y., Doi, Y. 2004. Processing strong fibers of microbial biodegradable aliphatic polyesters-drawing and annealing conditions of polyhydroxyalkanoates fibers. Text. Res. J., 74(11), 1031.

Troschl, C., Meixner, K., Drosg, B. 2017. Cyanobacterial PHA production—Review of recent advances and a summary of three years' working experience running a pilot plant. Bioeng., 4(2), 26.

Tufail, S., Munir, S., Jamil, N. 2017. Variation analysis of bacterial polyhydroxyalkanoates production using saturated and unsaturated hydrocarbons. Braz. J. Microbiol., 48(4), 629-636.

Valappil, S.P., Misra, S.K., Boccaccini, A.R., Keshavarz, T., Bucke, C., Roy, I. 2007. Large-scale production and efficient recovery of PHB with desirable material properties, from the newly characterised *Bacillus cereus* SPV. J. Biotechnol., 132(3), 251-258.

Valentino, F., Karabegovic, L., Majone, M., Morgan-Sagastume, F., Werker, A. 2015. Polyhydroxyalkanoate (PHA) storage within a mixed-culture biomass with simultaneous growth as a function of accumulation substrate nitrogen and phosphorus levels. Water Res., 77, 49-63.

Valentino, F., Villano, M., Bertin, L., Beccari, M., Majone, M. 2011. Olive oil wastewater as a renewable resource for production of polyhydroxyalkanoates. Rew. Polym. Synth. Process. Technol., 175-219.

Vega-Castro, O., Contreras-Calderon, J., Leon, E., Segura, A., Arias, M., Perez, L., Sobral, P.J.A. 2016. Characterization of a polyhydroxyalkanoate obtained from pineapple peel waste using *Ralsthonia eutropha*. J. Biotechnol., 231, 232-238.

Walsh, M., O'Connor, K., Babu, R., Woods, T., Kenny, S. 2015. Plant oils and products of their hydrolysis as substrates for polyhydroxyalkanoate synthesis. Chem. Biochem. Eng. Q., 29(2), 123-133.

Wang, S.Y., Wang, Z., Liu, M.M., Xu, Y., Zhang, X.J., Chen, G.-Q. 2010. Properties of a new gasoline oxygenate blend component: 3-Hydroxybutyrate methyl ester produced from bacterial poly-3-hydroxybutyrate. Biomass Bioenergy, 34(8), 1216-1222.

Wang, X., Lin, L., Dong, J., Ling, J., Wang, W., Wang, H., Zhang, Z., Yu, X. 2018. Simultaneous improvements of cell growth and polyhydroxyalkanoate production in *Pseudomonas* from a lignin derivative for lignin consolidated bioprocessing. Appl. Environ. Microbiol., AEM. 01469-18.

Wisuthiphaet, N., Napathorn, S.C. 2016. Optimization of the use of products from the cane sugar industry for poly (3-hydroxybutyrate) production by *Azohydromonas lata* DSM 1123 in fed-batch cultivation. Process Biochem., 51(3), 352-361.

Wu, Q., Huang, H., Hu, G., Chen, J., Ho, K., Chen, G.-Q. 2001. Production of poly-3-hydroxybutyrate by *Bacillus* sp. JMa5 cultivated in molasses media. Antonie Van Leeuwenhoek, 80(2), 111-118.

Xie, S., Qin, X., Cheng, Y., Laskar, D., Qiao, W., Sun, S., Reyes, L.H., Wang, X., Dai, S.Y., Sattler, S.E. 2015. Simultaneous conversion of all cell wall components by an oleaginous fungus without chemi-physical pretreatment. Green Chem., 17(3), 1657-1667.

Xu, J., Cheng, J.J. 2011. Pretreatment of switchgrass for sugar production with the combination of sodium hydroxide and lime. Bioresour. Technol., 102(4), 3861-3868.

Yang, Y.-H., Brigham, C.J., Budde, C.F., Boccazzi, P., Willis, L.B., Hassan, M.A., Yusof, Z.A.M., Rha, C., Sinskey, A.J. 2010. Optimization of growth media components for polyhydroxyalkanoate (PHA) production from organic acids by *Ralstonia eutropha*. Appl. Microbiol. Biotechnol., 87(6), 2037-2045.

Yatim, A.F.M., Syafiq, I.M., Huong, K.H., Amirul, A.-A.A., Effendy, A.W.M., Bhubalan, K. 2017. Bioconversion of novel and renewable agro-industry by-products into a biodegradable poly (3-hydroxybutyrate) by marine *Bacillus megaterium* UMTKB-1 strain. Biotechnol., 98(2), 141-151.

Yin, J., Chen, J.-C., Wu, Q., Chen, G.-Q. 2015. Halophiles, coming stars for industrial biotechnology. Biotechnol. Adv., 33(7), 1433-1442.

Yu, J. 2012. Using cell debris generated from PHA recovery for enhanced cell growth and biopolyester formation, Google Patents.

Yu, J., Stahl, H. 2008. Microbial utilization and biopolyester synthesis of bagasse hydrolysates. Bioresour. Technol., 99(17), 8042-8048.

Zhang, X., Luo, R., Wang, Z., Deng, Y., Chen, G.-Q. 2009. Application of (R)-3-hydroxyalkanoate methyl esters derived from microbial polyhydroxyalkanoates as novel biofuels. Biomacromolecules, 10(4), 707-711.

Zhang, Y., Wusiman, A., Liu, X., Wan, C., Lee, D.-J., Tay, J. 2018. Polyhydroxyalkanoates (PHA) production from phenol in an acclimated consortium: Batch study and impacts of operational conditions. J. Biotechnol., 267, 36-44.

Zhao, C., Xie, S., Pu, Y., Zhang, R., Huang, F., Ragauskas, A.J., Yuan, J.S. 2016. Synergistic enzymatic and microbial lignin conversion. Green Chem., 18(5), 1306-1312.

Zihayat, B., Shakibaie, M., Sabouri-Shahrbabak, S., Doostmohammadi, M., Ameri, A., Adeli-Sardou, M., Forootanfar, H. 2019. Medium optimization for polyhydroxyalkanoate production by *Pseudomonas pseudoalcaligenes* strain Te using D-optimal design. Biocatal. Agr. Biotechnol., 18, 101001.

Zou, X.H., Chen, G.Q. 2007. Metabolic engineering for microbial production and applications of copolyesters consisting of 3-hydroxybutyrate and medium-chain-length 3 - hydroxyalkanoates. Macromol. Biosci., 7(2), 174-182.

CHAPTER III

3. Optimization of polyhydroxybutyrate production by experimental design of combined ternary mixture (glucose, xylose and arabinose) and process variables (sugar concentration, molar C:N ratio)

Abstract

Polyhydroxybutyrate (PHB) is a biodegradable plastic that is comparable with some petroleum based plastics in terms of mechanical properties. Conversion of lignocellulosic feedstocks to PHB could make a lignocellulosic biorefinery more profitable and sustainable. Glucose, xylose and arabinose are the main monomer sugars derived from pretreatment and hydrolysis of herbaceous feedstocks. Burkholderia sacchari DSM 17165 is a bacterium strain that can convert all three sugars into PHB. However, the effects of three sugar ratio in mixture, sugar concentration, molar C:N ratio and their interactions on PHB production have not been studied before. In this study, a combined mixture-process experimental design was employed. A seven-run mixture design for sugar ratio combined with a 32 full factorial design for process variables was performed to optimize PHB production. A polynomial model combining mixture and process variables was built based on experimental data and optimum conditions for different types of sugar streams were derived and validated. Highest PHB production (3.81 g/L) was achieved with

arabinose-only culture under sugar concentration of 25.54 g/L and molar C:N ratio of 74.35. Results provide references for manipulation of sugar mixture and process control to maximize PHB production.

3.1. Introduction

Polyhydroxybutyrate (PHB) is a biodegradable polymer consisting of 3-hydroxybutyrate that can reach a degree of polymerization of 2,000. Due to its mechanical properties that are comparable to many petroleum-based plastics, PHB has been produced in industry for decades (Aramvash et al., 2018). PHB is also widely applied in the pharmaceutical industry due to its biocompatibility (Koller, 2018). One of the major approaches for PHB production is through microbial cultivation (Tokiwa and Calabia, 2004). PHB is a product of unbalanced growth in bacteria and is used as an energy and carbon storage material (Kessler and Witholt, 2001). Recent research showed accumulation of PHB in photoautotrophic systems such as cyanobacteria (Carpine et al., 2018; Gupta et al., 2013; Singh et al., 2017). However, commercial PHB produced by microbes is still around 3 times expensive than petroleum-based plastics. Recent price of PHB was around 3.5 USD/kg (Aramvash et al., 2018), 3 times expensive than petroleum-based plastics such as polyethylene and polypropylene, which is around 1.2–1.3 USD/kg (Platts, 2019). Raw materials make up about 50% of the production cost (Choi and Lee, 1999; Khanna and Srivastava, 2005; Mozumder et al., 2015). Lignocellulosic biomass feedstocks are abundant, cheap and sustainable, do not compete with the human food chain and can be used as the major carbon sources for PHB production (Saratale et al., 2019). Some expensive precursors, such as levulinic acid (4-ketovaleric acid), a precursor substrate to produce co-polyesters containing both 3-hydroxybutyric acid (3HB) and 3-hydroxyvaleric acid (3HV) (Gorenflo et al., 2001), can also be derived from hydrothermolysis pretreatment of lignocellulosic feedstocks. One characteristic of lignocellulosic

feedstocks is that they have a high molar carbon to nitrogen (C:N) ratio (47-57:1) (Al-Kaisi and Guzman, 2013), which makes them suitable substrates for unbalanced bacterial growth and accumulation of PHB.

Carbon forms in lignocellulose biomass are mainly polysaccharides (cellulose and hemicellulose) and the aromatic polymer lignin (Saratale et al., 2013). In a lignocellulosic biomass refinery, cellulose and hemicellulose can be hydrolyzed into a sugar stream consisting of monomer sugars including glucose, xylose, galactose, arabinose and mannose. For the last decade, upstream pretreatment and hydrolysis technologies have successfully produce a sugar yield over 90% from multiple lignocellulosic biomass and pretreatment methods (Hamalainen et al., 2016b; He et al., 2017; Heng et al., 2017; Liu et al., 2015; Obruca et al., 2014a; Roberto et al., 1995; Saha et al., 2015; Saratale et al., 2018a; Saratale et al., 2018b; Silva et al., 2004). For commercial PHB production, it is best to convert all of these sugars into PHB. In a lignocellulosic biorefinery, glucose, xylose and arabinose are the main sugars present (Aristidou and Penttila, 2000; Cesario et al., 2014) and the ratios of these three sugars in sugar streams can vary greatly depending on upstream lignocellulosic biomass sources and pretreatment methods (Table 3.1). Sugar streams containing sugar mixtures can also be acquired from existing commercial lignocellulosic fuel production facilities. For example, Silva et al. (2014) proposed the production of PHB associated with ethanol production from lignocellulose by converting sugars remaining after glucose conversion to ethanol (rich in xylose and arabinose) to PHB. As a PHB accumulating bacterium isolated from the soil of a sugar-cane plantation in Brazil, *Burkholderida sacchari* DSM 17165 is capable of converting glucose, xylose and arabinose into PHB (Bramer et al., 2001; Cesario et al., 2014). Glucose is converted into PHB via acetyl-CoA by the Entner Doudoroff pathway. Xylose and arabinose are converted into PHB via acetyl-CoA through the pentose phosphate pathway

(Lopes et al., 2011). In fact, previous studies have attempted to produce PHB from defined glucose, xylose and arabinose ternary mixtures and lignocellulosic feedstock hydrolysates (Cesario et al., 2014; Saratale and Oh, 2015). For the effect of sugar mixture, *B. sacchari* DSM 17165 was found to prefer glucose over arabinose and xylose for metabolism and PHB accumulation due to carbon catabolite repression (Lopes et al., 2011). Previous studies showed process variables such as initial sugar concentration and C:N ratio are important factors affecting PHB production (Choi and Lee, 1999). However, knowledge on how ternary sugar ratio affects PHB production and how sugar mixture interacts with other process variables to affect PHB production were not much to the best of our knowledge. It is necessary to understand how sugar ratio and process variables affect PHB production.

B. sacchari DSM 17165 was used in this study to produce PHB from ternary sugar mixtures (glucose, xylose and arabinose). The overall goal of this study is to enhance PHB production by optimizing the conditions of sugar mixture and two process variables (initial sugar concentration and molar C:N ratio). Seven-run mixture design is a good statistical design to study the effect of mixture component ratio on response variables (Cornell, 2011). The mixture design was combined with a 3^2 full factorial design for process variables to evaluate the interaction effect of sugar mixture ratio and process variables. The objectives of this study were 1) to study the effect of sugar mixture ratio, sugar concentration and molar C:N ratio on PHB production; 2) to construct a response surface model through regression on sugar mixture ratio and process variables; and 3) to obtain the optimum conditions leading to maximum PHB production for different types of sugar streams based on the model. To the best of the author`s knowledge, this is the first study looking into the effect of sugar ratio, sugar concentration and C:N ratio on PHB production using a combined mixture-process experimental design. The models obtained in this study can be applied

to manipulate sugar streams from different sources and process variables to maximize PHB production. In the context of lignocellulosic ethanol and fuel production, multiple research studies have been done to engineer strains that can co-ferment three sugars (Aristidou and Penttila, 2000; Wisselink et al., 2009; Xiao et al., 2011), however, the process control of sugar ratio was not well studied. The approach in this study may be extended to manufacture other fermentation products such as lignocellulosic fuels using mixed sugars as the carbon source.

3.2 Materials and methods

3.2.1 Microorganism and inoculum

B. sacchari DSM 17165 in the form of dried pellets was purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). *B. sacchari* DSM 17165 was revitalized and sub-cultured with seed medium (meat extract 3 g/L, meat peptone 5 g/L, glucose 30 g/L) for at least two generations until the OD reached 10 at 24 h. *B. sacchari* culture was conducted at 29 ℃ at 250 rpm in 20 mL working medium in 250 mL Kimax baffled flasks with silicone sponge closure throughout the study unless specified. *B. sacchari* culture of OD 10 was preserved at -80 ℃ with 30% glycerol in 2 mL aliquots and used for pre-culture preparation. One aliquot of *B. sacchari* culture was inoculated to seed medium and *B. sacchari* was cultured to obtain late exponential phase culture with an optical density (OD) of 8-10. Then, *B. sacchari* culture was washed with sterile 0.89% sodium chloride solution twice and used as inoculum. Inoculum was added to each flask to achieve an initial OD of 2.

3.2.2 PHB production

B. sacchari DSM 17165 in the PHB production medium contained alternatively glucose, xylose or arabinose as the carbon source in varying concentrations described later, $(NH_4)_2SO_4$ as

the nitrogen source in varying concentrations based on varying molar C:N ratio described later, salts ($Na_2HPO_4 \cdot 2H_2O$, 2.9 g/L; KH_2PO_4, 1.5 g/L; $MgSO_4 \cdot 7H_2O$, 0.2 g/L; $CaCl_2 \cdot 2\ H_2O$: 0.01 g/L; $Fe(NH_4)$ citrate: 0.06 g/L) and trace elements (1 mL/L) as described previously in Ramsay et al. (1990). The $MgSO_4 \cdot 7H_2O$, $CaCl_2 \cdot 2H_2O$ and $Fe(NH_4)$ citrate solutions were autoclaved separately. PHB production was conducted at 29 °C at 250 rpm as described in Section 3.2.1, but with 15 mL working volume in the same 250 mL flask and cultivation was stopped at 72 h for PHB quantification as described in Section 3.2.4.

For the experimental design, PHB production was conducted in three stages. First, single variable experiments (Fig. 3.1) were conducted to evaluate the effect of two process variables (sugar concentration and C:N ratio) using a "one factor at a time "approach on four sugar mixtures. The four mixtures were denoted as glucose, xylose, arabinose and equal (Fig. 3.1). Glucose, xylose and arabinose mean only glucose, xylose or arabinose were present. Equal means that three sugars were of equal concentration in the mixture. Initial sugar concentrations varied from 10 to 50 g/L with 10 g/L increments at a fixed molar C:N ratio of 50 (Fig. 3.1a); molar C:N ratios ranged from 20 to 100 with increments of 20 at a fixed sugar concentration of 30 g/L (Fig. 3.1b). Two flasks per treatment were run each time for each treatment condition. Second, based on the results of the single variable experiments, a combined mixture-process variable design was employed (Fig. 3.2). The mixture design structure consisting of 7 runs is shown in Figs. 3.2a and c. For process variables, a 9 run (3^2) full factorial (Fig. 3.2b) was applied to study the effect of initial sugar concentrations (10, 20 and 40 g/L) and C:N ratio (40, 60 and 80). By combining a mixture design and a process variables design, there were a total of 63 (7*9) treatment combinations (Fig. 3. 2c). Again, two flasks per treatment condition were run. A total of 126 flasks were run in three batches due to the limitation of incubator space. Each batch contained 21 treatment conditions (42 flasks)

and was associated with one of the three sugar concentrations. The 21 treatment conditions were assigned to 42 flasks with two flasks per treatment randomly. Experimental data from the combined mixture-process design (Table 3.2) was used to derive the polynomial model described in Section 3.2.3. Third, using the model derived from the second stage, optimum conditions leading to the maximal PHB production was derived and validated by running an additional set of experiments under the optimum condition. Validation experiments were also run in two flasks per condition.

3.2.3 Response surface and optimum conditions

3.2.3.1 Polynomial model fitting

A special cubic model (Equation 3.1) proposed by Scheffe (1958) was used to fit the 7-run mixture design, and a quadratic linear polynomial model (Neter et al., 1996) (Equation 3.2) was used to fit the full factorial design (Cornell, 2011). Equation 3.1 has 7 terms and equation 3.2 has 9 terms. The combined model (Equation 3.3) is a result of multiplication of Equations 3.1 and 3.2 (Cornell, 2011). The combined model contained 63 terms. The random effect of batch was ignored. Model reduction was performed manually and lack of fit was checked for the reduced model.

$$Y_M = b_A A + b_B B + b_C C + b_{AB} AB + b_{AC} AC + b_{BC} BC + b_{ABC} ABC \quad (3.1)$$

$$Y_P = b_0 + b_D D + b_E E + b_{DE} DE + b_{D^2} D^2 + b_{E^2} E^2 + b_{D^2E} D^2 E + b_{DE^2} DE^2 + b_{D^2E^2} D^2 E^2 \quad (3.2)$$

$$Y = Y_M * Y_P \quad (3.3)$$

where A, B and C denote glucose, xylose and arabinose in coded level, respectively, and D and E denote sugar concentration and molar C:N ratio, respectively.

The combined polynomial Equation 3.3 was implemented using GLM procedure in SAS 9.3 (SAS Institute Inc., Cary, NC). Analysis of variance (ANOVA) and testing of model adequacy

used p-values < 0.05 as statistically significant. Response surface plots of sugar mixture and full factorial process variables to exhibit interaction effects were plotted using Design Expert 11 (Stat-Ease, Inc., Minneapolis, MN).

3.2.3.2 Optimization by sequential quadratic programming and validation experiment

Sequential quadratic programming (SQP) and global search methods have been used in multiple previous research studies to obtain optimal conditions from response surface models (Chen et al., 2005; Liou et al., 2018; Nocedal and Wright, 2006; Sreeraj et al., 2013). SQP is a type of quasi-newton optimization method that can identify optimum solution with fast convergence (Nocedal and Wright, 2006). Its disadvantage is that it may identify a local optimum instead of the global (Rentizelas and Tatsiopoulos, 2010). In order to search for the global optimum, the concept of global search optimization procedure (Ugray et al., 2007) was combined with the SQP method. Both the SQP and global search were implemented using the Global Optimization toolbox of MATLAB 2014a (MathWorks, Inc., Natick, MA, USA). Sequential quadratic programming was performed using build-in function "optimoptions" with "fmincon" and "sqp" algorithm. Global search was conducted using function "createOptimProblem" with "GlobalSearch" algorithm. The model obtained from Section 3.2.3.1 was formulated as an objective function that was optimized to obtain the maximum PHB production and the corresponding optimum conditions. The function "fmincon" for SQP is used to find the minimum value of a polynomial equation. Therefore, -1 times Y in Equation 3.3 was used as the objective function. The steps of using SQP and global search to find the optimum were expressed below.

Step one: set objective function (fun): $fun = -Y$. Y is from Equation 3.3.

Step two: set constraints for independent variables. The basic constraints are: $A + B + C = 1; 10 \leq D \leq 40;\ 40 \leq E \leq 80$. These constraints are from the experimental setup for combined mixture-process design. One of the objectives of this study was to use the polynomial model to derive optimum conditions for different types of sugar streams. Therefore, additional constraints can be applied to the mixture components A, B and C based on different types of sugar streams. In this study, four types of sugar mixtures were defined as corn stover, corn fiber, hemicellulose rich and global maximum. For corn stover type sugar stream, xylose concentration is approximately one-half of glucose concentration and these two sugars are the dominant sugars (Saha et al., 2005; Saha et al., 2016). Additional constraints for corn stover type sugar stream are: $0.5 \leq A \leq 0.7;\ 0.15 \leq B \leq 0.3;\ 0.05 \leq C \leq 0.2$. For corn fiber type sugar streams, glucose, xylose and arabinose are approximately in equal proportions with one of them usually less than other two depending on feedstock sources and pretreatment method (Gaspar et al., 2007; Noureddini and Byun, 2010; Samala et al., 2012). Additional constraints for corn fiber type sugar stream are: $0.4 \leq A \leq 0.6;\ 0.1 \leq B \leq 0.3;\ 0.3 \leq C \leq 0.5$. Hemicellulose rich sugar streams are high in xylose with other sugars in much smaller amounts compared to xylose (Pan et al., 2012a). Additional constraints for hemicellulose rich sugar stream are: $0.05 \leq A \leq 0.15;\ 0.55 \leq B \leq 0.7;\ 0.2 \leq C \leq 0.35$. These constraints for mixtures were set based on the literature data in Table 3.1. Global maximum means no additional constraints were set for mixtures.

Step three: apply SQP and global search to find the maximum PHB production and corresponding optimal conditions.

After obtaining the optimal conditions, validation experiments were performed by running an additional set of experiments using the optimal conditions.

3.2.4 PHB quantification by gas chromatography-mass spectrometer (GCMS)

PHB concentration was determined according to the methods described by Dai et al. (2015) and Braunegg et al. (1978). Dried cell biomass was methanolyzed at 105 °C in an acidified methanol (5% H_2SO_4, v/v, 2 mL) and chloroform (2 mL) mixture for 3 h. Sodium hydroxybutyrate (Sigma-Aldrich, MO, USA) was used as an external standard. After 3 h, 1 mL deionized water (18.2 MΩ-cm) was added to the mixture and vortexed. The methyl-ester derivatives were extracted from the lower chloroform layer and passed through a column containing anhydrous sodium sulfate before being injected into a GC-MS.

The GC-MS was equipped with a Trace 1310 Gas Chromatograph, ISQ QD single quadrupole mass spectrometer (ThermoScientific, Waltham, MA, USA) and TG-5MS (30 m x 0.25 mm ID x 0.25 film) capillary column (ThermoFisher Scientific, Waltham, MA, USA). The temperature profile was held at 60 °C for 5 min, increased to 220 °C at a rate of 30 °C/min and held at 220 °C for 5 min. The injector temperature was 230 °C, and the injection mode was splitless (1 µL). The pressure of the carrier gas (helium) was 142 kPa (18 psi). MS transfer line temperature was 250 °C, ion source temperature was 220 °C and ionization mode was electron ionization. The ionization energy was 70 eV and selective ion monitoring (SIM) mode was used. Methyl-ester derivative was confirmed with SIM at 43, 71, and 74. Solvent delay was 3 min. Each chromatogram was analyzed with Chromeleon 7 chromatography studio (ThermoFisher Scientific, Waltham, MA, USA).

3.3 Results and discussion

3.3.1 Single variable experimental results

Single variable experimental results are shown in Fig. 3.1. For the effect of sugar concentration on PHB production, molar C:N ratio was held at 50 (Fig. 3.1a). The effects of sugar concentration on glucose and arabinose only mixtures were similar; for both glucose and arabinose only mixtures, highest PHB productions were observed with sugar concentration 20 g/L. For xylose only mixture, PHB production decreased with increasing sugar concentration and highest PHB production was observed with sugar concentration 10 g/L. This implied that high xylose concentration caused much higher stress than the other sugars in terms of PHB production. For the equal mixture, highest PHB productions were observed with sugar concentration 40 g/L. To study the effect of molar C:N ratio, sugar concentration was held at 30 g/L (Fig. 3.1b). The highest PHB production for glucose only mixtures were observed when the ratio was 40. PHB production for xylose only mixtures were much lower compared with other sugar mixtures across all molar C:N ratios. Since the xylose only mixture produced the highest PHB with sugar concentration of 10 g/L (Fig. 3.1a), it is expected that the high sugar concentration (30 g/L) used in the experiments to study the C:N ratio caused stress and resulted in low PHB production (Fig. 3.1b). The arabinose-only mixture had the highest PHB when the C:N ratio was 80. The equal mixture had the highest PHB when the C:N ratio was 60. Based on the first stage experimental results, the levels of sugar concentrations were set as 10, 20 and 40 g/L and the levels of molar C:N ratio were set as 40, 60 and 80 for next step combined mixture-process variable design (Fig. 3.2).

3.3.2 Combined mixture-process variable results and polynomial model fitting

Combined mixture-process experimental design and observed experimental values for 63 treatment combinations are shown in Table 3.2. The highest PHB production in Table 3.2 was 3.67 g/L, which is 20% higher than the highest PHB produciton (3.07 g/L for equal mixture at sugar cocentration 40 g/L and C:N ratio 50) in Fig. 3.1. The combined mixture-process variable design clearly achived higher PHB production than single variable expeirmental design.

For model fitting, initially, a full model containing 63 terms with zero degrees of freedom for lack of fit was fit to the experimental data in Table 3.2. The full model showed a coefficient of determination (R^2) of 0.93. Variable reduction was performed manually by dropping terms that were insignificant from the highest order. The *p*-value for the term of interaction between ABC and D^2E^2 in Table 3.3 was > 0.05. Therefore, this interaction term was dropped and lack of fit was checked to be 0.93. If any more terms were dropped, the lack of fit became significant and R^2 dropped dramatically (from 0.93 to 0.7). Therefore, a reduced model with 62 terms (only dropping interaction term $ABCD^2E^2$) was used as the final model (Table 3.3). The parameter estimates and modeling fitting results for the final model are shown in Table 3.3. The final model had a highly significant *p*-value (<0.0001) and non-significant lack of fit (*p*-value = 0.566 > 0.05), indicating the final model was adequate to explain the experimental data. The coefficient of determination (R^2 =0.93) of the final model demonstrated good correlation between experimental and predicted values of the response.

3.3.3 Effect of mixture, process variables and their interaction

The response surface plots of 7 mixtures based on the final model in Table 3.3 are presented in Fig. 3.3. In Table 3.2, it is noteworthy that at sugar concentrations of 10 g/L, PHB production

of xylose only mixture was greater than that of glucose only mixture for all molar C:N ratios and greater than that of arabinose only mixture for the molar C:N ratio of 80. Similarly, xylose only mixture was found to produce more PHB than glucose at sugar concentration 10 g/L, but with a molar C:N ratio of 19 (Cesario et al., 2014). In Figs. 3.3a and 3.3d for process conditions (10, 40) (sugar concentration in g/L, molar C:N ratio) and (10, 60), arabinose only mixture had the highest PHB production and the mixture of glucose and xylose at equivalent concentrations led to higher PHB production than glucose or xylose-only cultures. At 10 g/L, when the molar C:N ratio increased to 80, more PHB was produced using xylose-only mixture than the mixture of glucose and xylose at equivalent concentrations. For sugar concentrations of 10 and 20 g/L, the trend of PHB production affected by the mixture was similar to that of Cesario et al. (2014), but PHB concentrations were lower in this study, which was probably due to the use of yeast extract in Cesario et al. (2014).

Trends of process conditions (10, 40), (20, 60) and (20, 80) (Figs. 3.3a, 3.3e and 3.3h) were similar, in which an additional increment of arabinose in mixture increased PHB production more than an additional increment of glucose or xylose. In fact, arabinose only mixture produced the highest PHB among all process conditions except for process conditions (10, 80), (20, 40) and (40, 60). The reason that arabinose produced higher PHB than glucose remians unknow and reuqires furthur research. At process conditions (10, 80), (20, 40) and (40, 60), highest PHB productions were obtained with xylose-only mixture, mixture of glucose and xylose at equivalent concentrations and glucose-only mixtures, respectively. For sugar concentration at 40 g/L, the lowest concentration of PHB was produced in xylose-only mixture among all mixtures. Similarly, in the study of Cesario et al. (2014), PHB volumetric productivities were found to be 0.13, 0.08 and 0.09 g/(L* h) for glucose, xylose and arabinose-only cultures, respectively. In this study, *B.*

sacchari produced more PHB in arabinose or glucose-rich mixtures than in xylose-rich mixtures at high sugar concentration (40 g/L) (Figs. 3.3c, 3.3f and 3.3i).

PHB is an energy storage product triggered by nitrogen limitation. The effect of molar C:N ratio on different sugar mixtures differed. For most previous studies applying nitrogen limitation for PHB production, the molar C:N ratio ranged from 20 to 60 for glucose and arabinose rich sugar streams (Cesario et al., 2014; Garcia-Torreiro et al., 2016; Kalaiyezhini and Ramachandran, 2015; Raposo et al., 2017; Rodriguez-Contreras et al., 2015). Based on the results of this study, for xylose rich streams from such as rice straw or corn cob, the molar C:N ratio should be increased to 80 and the sugar concentration should be low at around 10 g/L since PHB concentrations were much higher when xylose concentration was at 10 g/L and molar C:N ratio was increased to 80 (Table 3.2 and Figs. 3.3a, 3.3d and 3.3g). Increasing the molar C:N ratio can increase PHB accumulation in bacteria cells, but high molar C:N ratios limit cell growth (Kalaiyezhini and Ramachandran, 2015). This may explain that for some mixtures in Table 3.2, PHB production at the molar C:N ratio 60 was higher than the other two ratios (40 and 80). High molar C:N ratio also decreases the PHB formation rate (Choi and Lee, 1999), therefore, increasing the fermentation time. Future work should be performed to study PHB production kinetics in terms of the effect of molar C:N ratio for certain sugar mixtures.

3.3.4 Optimum condition and validation results

Optimum PHB conditions and the corresponding PHB production are shown in Table 3.4. For four types of sugar mixtures, validation results from the model were close to the experimental results based on the optimum conditions derived from the model, indicating the model described in Table 3.3 is valid to predict PHB production from varied sugar mixtures and process variables.

The global maximum (the greatest PHB production) was found to be 3.815 g/L using arabinose-only culture at a sugar concentration of 25.54 g/L and a C:N ratio of 74.34. This is in accordance with the plots shown in Figs. 3.3e and 3h that show arabinose only culture led to the highest PHB production with sugar concentration of 20 g/L and C:N ratio at 60 and 80. The response surface plots for the arabinose only culture with process variables changing are shown in Fig. 3.4. It is in accordance with the optimal condition of arabinose concentration of 25.54 g/L and a C:N ratio of 74.34 (Fig. 3.4a). However, the surface in Fig. 3.4a looks flat when increasing the C:N ratio from 74.34 to 80. Another response surface plot with the C:N ratio changing from 60 to 85 is shown in Fig. 3.4b. It is clear to see that PHB production starts to decrease after C:N ratio of 74.34.

Corn fiber type sugar stream produced higher PHB than corn stover type sugar stream and hemicellulose rich sugar stream produced the lowest PHB. For different sugar stream in Table 3.1, it is expected that PHB production from highest to lowest will be in the following order: corn fiber, wheat bran and spend coffee ground > corn stover, switchgrass, sugarcane bagasse, softwood, hardwood and rich husk > rice straw and corn cob. For either corn stover or corn fiber type sugar streams, only around 2.0 g/L PHB was produced from 40 g/L sugar concentration, which implies that large amounts of sugar were left after the fermentation. It is also expected that large amounts of sugars were left for other two types of sugar streams (hemicellulose rich and global maximum). Future work should be conducted to recover the sugars and improve the sugar conversion to PHB. Application of fermentation strategy such as fed-batch may also increase cell and PHB production

3.4 Conclusion

In this study, the effects of sugar mixture, sugar concentrations and carbon to nitrogen ratio on PHB production were studied and a well-fitting polynomial model was built. Optimum

conditions for four typical types of sugar streams were derived for maximum PHB production. Validation results were close to predicted values based on the optimum conditions. At low sugar concentration (10 g/L), greater PHB concentrations were produced using xylose than using glucose. At high sugar concentration (40 g/L), glucose and arabinose dominant cultures produced higher PHB than xylose dominant culture. These results can provide reference of manipulating certain sugar amounts in different sugar mixture stream and associated process variables to maximize PHB production.

Table 3.1 Sugar mixture (glucose, xylose and arabinose) proportions reported in previous literature.

Feedstocks	Pretreatment method	Glucose, %	Xylose, %	Arabinose, %	References
Corn stover	White rot fungi	65	23	2	Saha et al. (2016)
Wheat bran	Dilute sulfuric acid	43	35	22	Liu et al. (2010)
Rice straw*	Dilute sulfuric acid	21	67	12	Roberto et al. (1995)
Spend coffee ground	Dilute sulfuric acid	7	NA	51	Obruca et al. (2014a)
Sugarcane bagasse*	Dilute sulfuric acid	42	57	1	Silva et al. (2004)
Rice husk	Alkaline (NAOH)	80	15	5	Heng et al. (2017)
Swtichgrass	Hythermolysis	96	3	1	Liu et al. (2015)
Eastern red cedar (hardwood)	Acid bisulfite	96	3	1	Liu et al. (2015)
Spruce (softwood)	Steam explosion	71.5	8	1	Hamalainen et al. (2016a)
Corn stover	Dilute sulfuric acid	56	37	6	Saha et al. (2015)
Corn cob	Dilute oxalic acid	22	71	6	He et al. (2017)
Corn fiber	Hydrothermolysis	32	40	27	This lab

Note: NA means not applicable, for feedstocks labeled with *, sugar streams were from the liquid fractions after pretreatment. In the literature, some of the sugar mixtures were reported as concentrations (g/L) in the original literature, they were converted to percentage for comparison. Summation of three sugar mixtures were not 100 due to the presence of galactose and mannose.

Table 3.2. Experimental values of 63 treatment combinations from the combined mixture-process experimental design

Mixture proportions			Process variable settings (sugar conc. (g/L), molar C/N))								
Glucose	Xylose	Arabinose	10, 40	10, 60	10, 80	20, 40	20, 60	20, 80	40, 40	40, 60	40, 80
1	0	0	0.48 (0.11)	0.81 (0.07)	0.35 (0.05)	1.72 (0.29)	1.16 (0.29)	0.64 (0.12)	0.94 (0.19)	2.17 (0.21)	1.97 (0.10)
0	1	0	1.53 (0.06)	1.92 (0.53)	2.68 (0.57)	0.86 (0.07)	1.16 (0.15)	1.29 (0.12)	0.57 (0.09)	0.49 (0.01)	1.02 (0.28)
0	0	1	3.67 (0.46)	2.52 (0.02)	2.21 (0.05)	1.82 (0.33)	3.43 (0.01)	3.53 (0.37)	2.07 (0.11)	1.64 (0.02)	2.42 (0.26)
0.5	0.5	0	2.04 (1.22)	2.16 (0.77)	0.80 (0.54)	2.24 (0.18)	1.01 (0.06)	1.20 (0.24)	1.49 (0.19)	2.06 (0.12)	1.12 (0.10)
0.5	0	0.5	2.74 (0.12)	1.20 (0.28)	0.44 (0.03)	1.16 (0.02)	2.05 (0.18)	0.73 (0.05)	2.33 (0.02)	1.59 (0.19)	1.80 (0.41)
0	0.5	0.5	1.93 (0.15)	1.63 (0.16)	1.31 (0.19)	1.41 (0.17)	2.21 (0.08)	1.09 (0.24)	1.89 (0.08)	1.94 (0.10)	1.18 (0.23)
0.333333	0.333333	0.333333	1.66 (0.00)	2.23 (0.29)	0.83 (0.36)	1.36 (0.18)	1.66 (0.02)	2.78 (0.32)	1.86 (0.18)	2.06 (0.24)	2.25 (0.06)

Note: values are shown as mean (standard deviation) of two replicates.

Table 3.3. Paramter estimates and model fitting for combined mixture-process final model

Model Parameter	Coefficient								
	Interaction with Intercept (b_0)	Interaction with D	Interaction with E	Interaction with DE	Interaction with D^2	Interaction with E^2	Interaction with D^2E	Interaction with DE^2	Interaction with D^2E^2
A	-14.2754466	1.4999318	0.4394633	-0.0418529	-0.0321835	-0.0032142	0.0009211	0.0002875	-0.0000063
B	6.5382201	-0.5804532	-0.1794871	0.0191352	0.0120048	0.0019903	-0.0004156	-0.0001899	0.000004
C	36.9968533	-3.5407457	-1.0404525	0.1096012	0.0695478	0.0071714	-0.0021995	-0.0007671	0.0000157
AB	-82.6510675	9.0799541	3.3936149	-0.3462563	-0.1819441	-0.0305147	0.0068859	0.0029773	-0.0000589
AC	95.7301327	9.5095409	-2.9266359	-0.3462563	0.1932354	0.0213655	-0.0060154	-0.0022423	0.0000459
BC	-59.6108006	6.0281791	2.0819316	-0.2183860	-0.1267103	-0.0192235	0.0047436	0.0020088	-0.0000437
ABC	-342.8622676	16.124429	10.6557613	-0.4570364	-0.1667196	-0.0722796	0.0034943	0.0024145	drop

Model fitting	P value
Model	<0.0001
Lack of fit	0.566
R^2	0.93

Note: A, B and C denote glucose, xylose and arabinose in coded level, D and E denote sugar concentration and molar carbon to C: N ratio. Drop means the highest order term (interaction between ABC and D^2E^2) was dropped.

Table 3.4. Optimal conditions and validation results for different types of sugar streams

Defined sugar streams	Glucose	Xylose	Arabinose	Sugar conc. (g/L)	Molar C/N	Experimental PHB Conc. (g/L)	Predicted PHB Conc. (g/L)
Corn stover	0.5	0.3	0.2	40	69.97	2.085 (0.136)	2.153 (0.636)
Corn fiber	0.4	0.2	0.4	40	80	2.210 (0.011)	2.310 (0.671)
Hemicellulose rich	0.15	0.65	0.2	25.54	58.77	1.783 (0.001)	1.370 (0.641)
Global maximum	0	0	1	25.54	74.35	3.813 (0.601)	3.974 (0.675)

Note: Experimental results are reported in the form of mean (standard deviation) (n=2), predicted values are reported in the form of mean (95% confident interval).

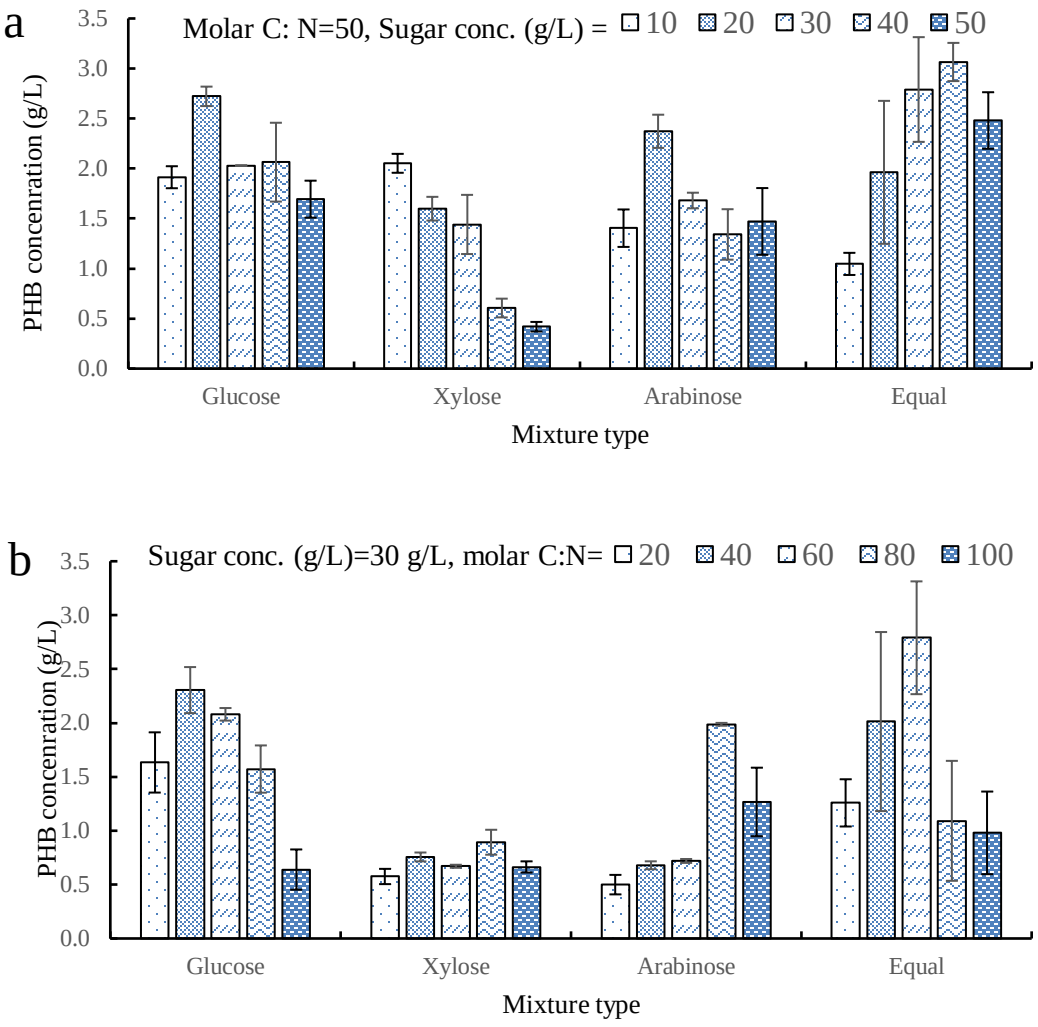

Fig. 3.1. Effect of sugar concentration (a) and molar C:N ratio (b) on PHB production on four sugar mixtures.

a

Mixture Run #	Components Glucose (A)	Xylose (B)	Arabinose (C)
1	1	0	0
2	0	1	0
3	0	0	1
4	1/2	1/2	0
5	1/2	0	1/2
6	0	1/2	1/2
7	1/3	1/3	1/3

b

9- run Process variables	Initial sugar conc. (g/L) (D)	Molar C: N (E)
(-1, -1)	10	40
(-1, 0)	10	60
(-1, +1)	10	80
(0, -1)	20	40
(0, 0)	20	60
(0, +1)	20	80
(1, -1)	40	40
(1, 0)	40	60
(1, +1)	40	80

c

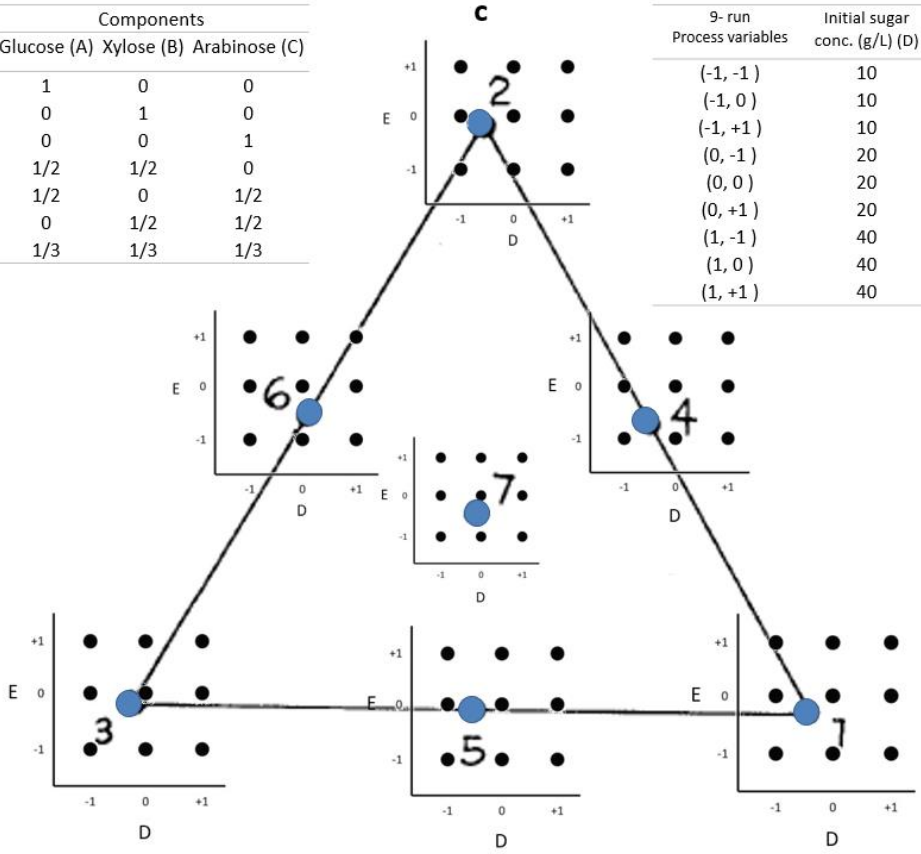

Fig. 3.2 Response illustration of combined mixture-process experimental design.

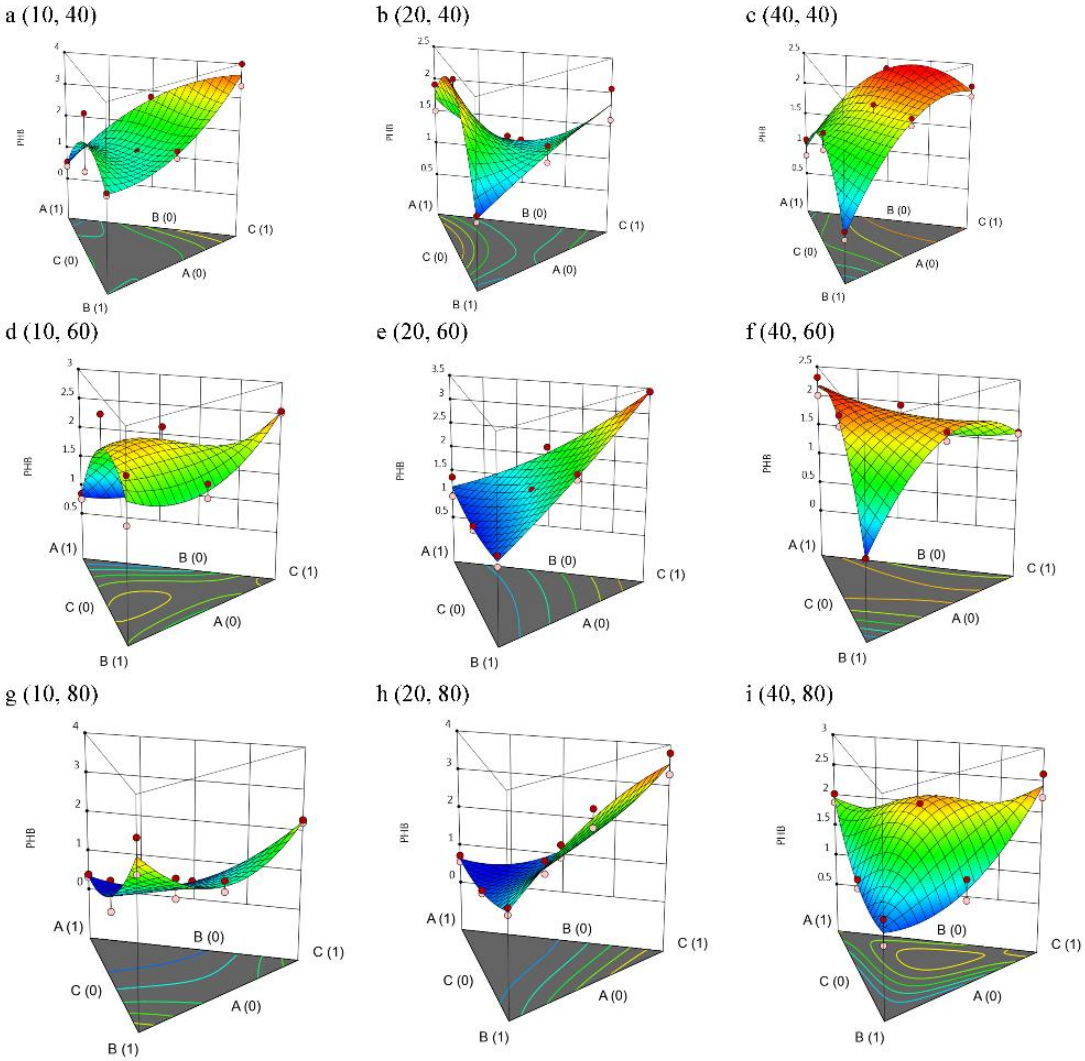

Fig. 3.3 Response surface plots (a-i) for ternary mixture combining with process variables treatment combinations.

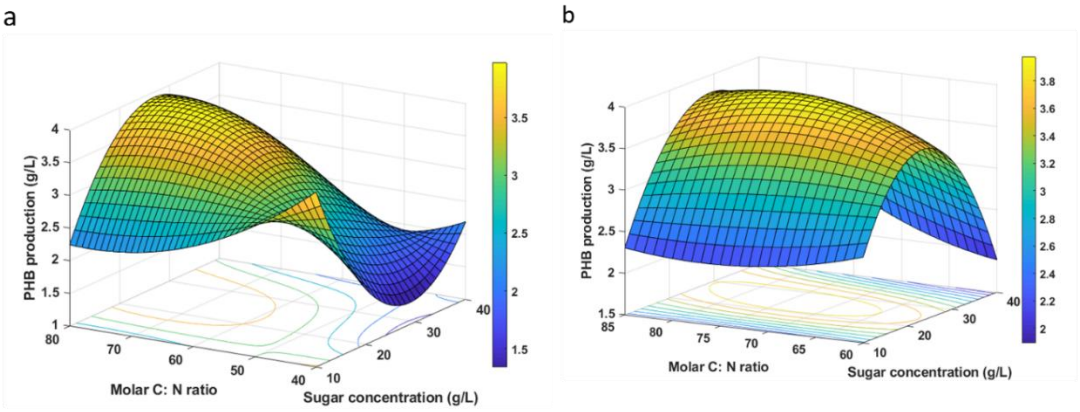

Fig. 3.4 Response surface plot affected by two process variables (sugar concentration and C:N ratio) for arabinose only sugar mixture.

3.5 References

Al-Kaisi, M.M., Guzman, J.G. 2013. Effects of tillage and nitrogen rate on decomposition of transgenic Bt and near-isogenic non-Bt maize residue. Soil Tillage Res., 129, 32-39.

Aramvash, A., Moazzeni Zavareh, F., Gholami Banadkuki, N. 2018. Comparison of different solvents for extraction of polyhydroxybutyrate from *Cupriavidus necator*. Eng. Life Sci., 18(1), 20-28.

Aristidou, A., Penttila, M. 2000. Metabolic engineering applications to renewable resource utilization. Curr. Opin. Biotechnol., 11(2), 187-198.

Bramer, C.O., Vandamme, P., Da Silva, L.F., Gomez, J., Steinbuchel, A. 2001. Polyhydroxyalkanoate-accumulating bacterium isolated from soil of a sugar-cane plantation in Brazil. Int. J. Syst. Evol. Microbiol., 51(5), 1709-1713.

Braunegg, G., Sonnleitner, B., Lafferty, R. 1978. A rapid gas chromatographic method for the determination of poly-β-hydroxybutyric acid in microbial biomass. Eur. J. Appl. Microbiol. Biotechnol., 6(1), 29-37.

Carpine, R., Raganati, F., Olivieri, G., Hellingwerf, K.J., Pollio, A., Salatino, P., Marzocchella, A. 2018. Poly-β-hydroxybutyrate (PHB) production by *Synechocystis* PCC6803 from CO2: Model development. Algal Res., 29, 49-60.

Cesario, M.T., Raposo, R.S., De Almeida, M.C.M., Van Keulen, F., Ferreira, B.S., Da Fonseca, M.M.R. 2014. Enhanced bioproduction of poly-3-hydroxybutyrate from wheat straw lignocellulosic hydrolysates. New Biotechnol., 31(1), 104-113.

Chen, M.-J., Chen, K.-N., Lin, C.-W. 2005. Optimization on response surface models for the optimal manufacturing conditions of dairy tofu. J. Food Eng., 68(4), 471-480.

Choi, J., Lee, S.Y. 1999. Factors affecting the economics of polyhydroxyalkanoate production by bacterial fermentation. Appl. Microbiol. Biotechnol., 51(1), 13-21.

Cornell, J.A. 2011. The inclusion of process variables in mixture experiments. in: *Experiments with mixtures: designs, models, and the analysis of mixture data*, (Eds.) P. Bloomfield, N.A.C. Cressie, N.I. Fisher, l.M. Johnstone, J.B. Kadane , L.M. Ryan, D.W. Scott, B.W. Silverman, A.F. Smith, J. Teugels, Vol. 403, John Wiley & Sons. New York, pp. 354-437.

Dai, J., Gliniewicz, K., Settles, M.L., Coats, E.R., McDonald, A.G. 2015. Influence of organic loading rate and solid retention time on polyhydroxybutyrate production from hybrid poplar hydrolysates using mixed microbial cultures. Bioresour. Technol., 175, 23-33.

Garcia-Torreiro, M., Lu-Chau, T.A., Lema, J.M. 2016. Effect of nitrogen and/or oxygen concentration on poly (3-hydroxybutyrate) accumulation by *Halomonas boliviensis*. Bioprocess Biosystems Eng., 39(9), 1365-1374.

Gaspar, M., Kalman, G., Reczey, K. 2007. Corn fiber as a raw material for hemicellulose and ethanol production. Process Biochem., 42(7), 1135-1139.

Gorenflo, V., Schmack, G., Vogel, R., Steinbuchel, A. 2001. Development of a process for the biotechnological large-scale production of 4-hydroxyvalerate-containing polyesters and characterization of their physical and mechanical properties. Biomacromolecules, 2(1), 45-57.

Gupta, V., Ratha, S.K., Sood, A., Chaudhary, V., Prasanna, R. 2013. New insights into the biodiversity and applications of cyanobacteria (blue-green algae)-Prospects and challenges. Algal Res., 2(2), 79-97.

Hamalainen, J., Granstrom, T., Mollerup, F., Wang, Y., Xiong, H., Turunen, O. 2016a. Effect of enzymatic high temperature prehydrolysis on the subsequent cellulose hydrolysis of steam-pretreated spruce in high solids concentration. J. Chem. Technol. Biotechnol., 91(6), 1844-1852.

Hamalainen, J., Granstrom, T., Mollerup, F., Wang, Y., Xiong, H., Turunen, O. 2016b. Effect of enzymatic high temperature prehydrolysis on the subsequent cellulose hydrolysis of steam ' pretreated spruce in high solids concentration. Journal of Chemical Technology and Biotechnology, 91(6), 1844-1852.

He, Y.-C., Jiang, C.-X., Chong, G.-G., Di, J.-H., Wu, Y.-F., Wang, B.-Q., Xue, X.-X., Ma, C.-L. 2017. Chemical-enzymatic conversion of corncob-derived xylose to furfuralcohol by the tandem catalysis with SO42−/SnO2-kaoline and *E. coli* CCZU-T15 cells in toluene–water media. Bioresour. Technol., 245, 841-849.

Heng, K.S., Hatti-Kaul, R., Adam, F., Fukui, T., Sudesh, K. 2017. Conversion of rice husks to polyhydroxyalkanoates (PHA) via a three-step process: optimized alkaline pretreatment, enzymatic hydrolysis, and biosynthesis by *Burkholderia cepacia* USM (JCM 15050). J. Chem. Technol. Biotechnol., 92(1), 100-108.

Kalaiyezhini, D., Ramachandran, K. 2015. Biosynthesis of poly-3-hydroxybutyrate (PHB) from glycerol by *Paracoccus denitrificans* in a batch bioreactor: effect of process variables. Prep. Biochem. Biotechnol., 45(1), 69-83.

Kessler, B., Witholt, B. 2001. Factors involved in the regulatory network of polyhydroxyalkanoate metabolism. J. Biotechnol., 86(2), 97-104.

Khanna, S., Srivastava, A.K. 2005. Recent advances in microbial polyhydroxyalkanoates. Process Biochem., 40(2), 607-619.

Koller, M. 2018. Biodegradable and biocompatible polyhydroxy-alkanoates (PHA): Auspicious microbial macromolecules for pharmaceutical and therapeutic applications. Molecules, 23(2), 362.

Liou, J.-Y., Ting, C.-K., Teng, W.-N., Mandell, M., Tsou, M.-Y. 2018. Adaptation of non-linear mixed amount with zero amount response surface model for analysis of concentration-dependent synergism and safety with midazolam, alfentanil, and propofol sedation. Br. J. Anaesth., 120(6), 1209-1218.

Liu, K., Atiyeh, H.K., Pardo-Planas, O., Ezeji, T.C., Ujor, V., Overton, J.C., Berning, K., Wilkins, M.R., Tanner, R.S. 2015. Butanol production from hydrothermolysis-pretreated switchgrass: quantification of inhibitors and detoxification of hydrolyzate. Bioresour. Technol., 189, 292-301.

Liu, Z., Ying, Y., Li, F., Ma, C., Xu, P. 2010. Butanol production by *Clostridium beijerinckii* ATCC 55025 from wheat bran. J. Ind. Microbiol. Biotechnol., 37(5), 495-501.

Lopes, M.S.G., Gosset, G., Rocha, R.C.S., Gomez, J.G.C., Da Silva, L.F. 2011. PHB biosynthesis in catabolite repression mutant of *Burkholderia sacchari*. Curr. Microbiol., 63(4), 319.

Mozumder, M.S.I., Garcia-Gonzalez, L., De Wever, H., Volcke, E.I.P. 2015. Effect of sodium accumulation on heterotrophic growth and polyhydroxybutyrate (PHB) production by *Cupriavidus necator*. Bioresour. Technol., 191, 213-218.

Neter, J., Kutner, M.H., Nachtsheim, C.J., Wasserman, W. 1996. *Applied linear statistical models*. Irwin Chicago, New York, NY.

Nocedal, J., Wright, S.J. 2006. Sequential quadratic programming. 2 ed. in: *Numerical Optimization*, (Eds.) T.V. Mikosch, S.I. Resnick, S.M. Robinson, Springer-Verlag New York. New York.

Noureddini, H., Byun, J. 2010. Dilute-acid pretreatment of distillers' grains and corn fiber. Bioresour. Technol., 101(3), 1060-1067.

Obruca, S., Benesova, P., Petrik, S., Oborna, J., Prikryl, R., Marova, I. 2014a. Production of polyhydroxyalkanoates using hydrolysate of spent coffee grounds. Process Biochem., 49(9), 1409-1414.

Pan, W., Nomura, C.T., Nakas, J.P. 2012a. Estimation of inhibitory effects of hemicellulosic wood hydrolysate inhibitors on PHA production by *Burkholderia cepacia* ATCC 17759 using response surface methodology. Bioresour. Technol., 125, 275-282.

Platts, S.P.G. 2019. S&P Global Platts Petrochemical Index (PGPI), Vol. 2019.

Ramsay, B., Lomaliza, K., Chavarie, C., Dube, B., Bataille, P., Ramsay, J. 1990. Production of poly-(beta-hydroxybutyric-co-beta-hydroxyvaleric) acids. Appl. Environ. Microbiol., 56(7), 2093-2098.

Raposo, R.S., De Almeida, M.C.M., De Oliveira, M.d.C.M., Da Fonseca, M.M., Cesario, M.T. 2017. A *Burkholderia sacchari* cell factory: production of poly-3-hydroxybutyrate, xylitol and xylonic acid from xylose-rich sugar mixtures. New Biotechnol., 34, 12-22.

Rentizelas, A.A., Tatsiopoulos, I.P. 2010. Locating a bioenergy facility using a hybrid optimization method. Int. J. Prod. Econ., 123(1), 196-209.

Roberto, I.C., Felipe, M.G., de Mancilha, I.M., Vitolo, M., Sato, S., Da Silva, S.S. 1995. Xylitol production by *Candida guillermondii* as an approach for the utilization of agroindustrial residues. Bioresour. Technol., 51(2-3), 255-257.

Rodriguez-Contreras, A., Koller, M., Miranda-de Sousa Dias, M., Calafell-Monfort, M., Braunegg, G., Marques-Calvo, M.S. 2015. Influence of glycerol on poly(3-hydroxybutyrate) production by *Cupriavidus necator* and *Burkholderia sacchari*. Biochem. Eng. J., 94, 50-57.

Saha, B.C., Iten, L.B., Cotta, M.A., Wu, Y.V. 2005. Dilute acid pretreatment, enzymatic saccharification and fermentation of wheat straw to ethanol. Process Biochem., 40(12), 3693-3700.

Saha, B.C., Qureshi, N., Kennedy, G.J., Cotta, M.A. 2016. Biological pretreatment of corn stover with white-rot fungus for improved enzymatic hydrolysis. Int. Biodeterior. Biodegrad., 109, 29-35.

Saha, B.C., Qureshi, N., Kennedy, G.J., Cotta, M.A. 2015. Enhancement of xylose utilization from corn stover by a recombinant Escherichia coli strain for ethanol production. Bioresource Technology, 190, 182-188.

Samala, A., Srinivasan, R., Yadav, M.P., Kim, T.-J., Prewitt, L. 2012. Xylo-oligosaccharides production by autohydrolysis of corn fiber separated from DDGS. BioResources, 7(3), 3038-3050.

Saratale, G.D., Oh, M.-K. 2015. Characterization of poly-3-hydroxybutyrate (PHB) produced from *Ralstonia eutropha* using an alkali-pretreated biomass feedstock. Int. J. Biol. Macromol., 80, 627-635.

Saratale, G.D., Saratale, R.G., Chang, J.-S. 2013. Biohydrogen from Renewable Resources. in: *Biohydrogen*, (Eds.) A. Pandey, J.-S. Chang, P.C. Hallenbecka, C. Larroche, Elsevier. Amsterdam, pp. 185-221.

Saratale, G.D., Saratale, R.G., Kim, S.H., Kumar, G. 2018a. Screening and optimization of pretreatments in the preparation of sugarcane bagasse feedstock for biohydrogen production and process optimization. IJHE, 43(25), 11470-11483.

Saratale, R.G., Saratale, G.D., Cho, S.K., Kim, D.S., Ghodake, G.S., Kadam, A., Kumar, G., Bharagava, R.N., Banu, R., Shin, H.S. 2019. Pretreatment of kenaf (*Hibiscus cannabinus* L.) biomass feedstock for polyhydroxybutyrate (PHB) production and characterization. Bioresour. Technol., 282, 75-80.

Saratale, R.G., Shin, H.S., Ghodake, G.S., Kumar, G., Oh, M.K., Saratale, G.D. 2018b. Combined effect of inorganic salts with calcium peroxide pretreatment for kenaf core biomass and their utilization for 2,3-butanediol production. Bioresour. Technol., 258, 26-32.

Scheffe, H. 1958. Experiments with mixtures. J. Roy. Stat. Soc. Ser. B. (Stat. Method.), 344-360.

Silva, L., Taciro, M., Ramos, M.M., Carter, J., Pradella, J., Gomez, J. 2004. Poly-3-hydroxybutyrate (P3HB) production by bacteria from xylose, glucose and sugarcane bagasse hydrolysate. J. Ind. Microbiol. Biotechnol., 31(6), 245-254.

Silva, L.F., Taciro, M.K., Raicher, G., Piccoli, R.A.M., Mendonca, T.T., Lopes, M.S.G., Gomez, J.G.C. 2014. Perspectives on the production of polyhydroxyalkanoates in biorefineries associated with the production of sugar and ethanol. Int. J. Biol. Macromol., 71, 2-7.

Singh, A.K., Sharma, L., Mallick, N., Mala, J. 2017. Progress and challenges in producing polyhydroxyalkanoate biopolymers from cyanobacteria. J. Appl. Phycol., 29(3), 1213-1232.

Sreeraj, P., Kannan, T., Maji, S. 2013. Prediction and optimization of weld bead geometry in gas metal arc welding process using RSM and fmincon. J. Mech. Eng. Res,, 5(8), 154-165.

Tokiwa, Y., Calabia, B.P. 2004. Review degradation of microbial polyesters. BiotL, 26(15), 1181-1189.

Ugray, Z., Lasdon, L., Plummer, J., Glover, F., Kelly, J., Marti, R. 2007. Scatter search and local NLP solvers: A multistart framework for global optimization. INFORMS J. Comput., 19(3), 328-340.

Wisselink, H.W., Toirkens, M.J., Wu, Q., Pronk, J.T., van Maris, A.J. 2009. Novel evolutionary engineering approach for accelerated utilization of glucose, xylose, and arabinose mixtures by engineered *Saccharomyces cerevisiae* strains. Appl. Environ. Microbiol., 75(4), 907-914.

Xiao, H., Gu, Y., Ning, Y., Yang, Y., Mitchell, W.J., Jiang, W., Yang, S. 2011. Confirmation and elimination of xylose metabolism bottlenecks in glucose phosphoenolpyruvate-dependent phosphotransferase system-deficient *Clostridium acetobutylicum* for simultaneous utilization of glucose, xylose, and arabinose. Appl. Environ. Microbiol., 77(22), 7886-7895.

CHAPTER IV

4. Enhancement of polyhydroxybutyrate (PHB) production by 10-fold from alkaline pretreatment liquor with an oxidative enzyme-mediator-surfactant system under Plackett–Burman and central composite designs

Abstract

In this study, Plackett–Burman and central composite designs were applied to improve polyhydroxybutyrate (PHB) production from alkaline pretreatment liquor (APL) by *Cupriavidus necator* DSM 545 using a supplement system consisting of oxidative enzymes (laccase, aryl alcohol oxidase (AAO)), mediators (ABTS, HOBT), DMSO, silica nanoparticle Aerosol R816 and surfactant Tween 80. First, screening experiments under Plackett-Burman design showed R816, ABTS and Tween 80 could significantly enhance PHB production. Additional experiments showed that HOBT and DMSO could be removed, and laccase and AAO were needed to remain in the system. Second, a central composite design was applied to obtain the optimum supplemental levels of R816, ABTS and Tween 80. Under optimum conditions, theoretical maximum PHB production (1.9 g/L) was close to experimental PHB production (2.1 g/L). With the supplement system, a 10-fold increase was achieved compared to PHB production (0.2 g/L) without any supplements.

4.1. Introduction

Lignin is an energy-dense, heterogeneous polymer comprising 15-30% of lignocellulosic biomass, and it is the second most abundant biopolymer on Earth after cellulose (Linger et al., 2014). However, the recalcitrance and heterogeneity of oligomeric and polymeric lignins from lignin-rich streams make lignin valorization a challenge (Salvachua et al., 2015). Therefore, lignocellulosic feedstock biorefineries produce renewable fuels, chemicals and polymers mostly from polymeric sugars (Cesario et al., 2014; Frederick et al., 2016; Li et al., 2018a; Liu et al., 2015; Wang et al., 2014) and leave huge amounts of waste lignin. In fact, lignin is generally burned to produce heat and power for the facilities of lignocellulosic biorefineries. Using a lignocellulosic ethanol process as an example, it has been estimated that only 40% of the lignin obtained from biomass is needed to meet process power requirements; therefore, 60% of the lignin could be used for producing higher value products without affecting the existing uses of lignin (Sannigrahi et al., 2010). By 2030, with 25% of transportation fuels being replaced by biofuels in the US, around 50 billion of cellulosic ethanol from about 1 billion tons of biomass will be produced in the biorefineries, therefore producing 0.3 billion tons of lignin, substantially more than necessary to power these biorefineries (Shen et al., 2018). Besides, conversion of lignin to value-added products is likely to compensate for the extra costs caused by upstream pretreatment processes that have high capital costs in lignocellulosic biorefineries (Ragauskas et al., 2014).

Lignin-enriched streams are typically accessible from lignocellulosic feedstock pretreatment or as residual solids after enzymatic hydrolysis (Salvachua et al., 2015). Alkaline pretreatment can typically preserve over 90% of sugars in solids and dissolve over 50% lignin in alkaline pretreatment liquor (APL) (Kim and Holtzapple, 2005; Xu and Cheng, 2011). Aromatic compounds in APL can be ring-opened in bacteria via the β-ketoadipate pathway and converted

further to carbon and energy storage compounds such as polyhydroxyalkanoates (PHA) from acetyl-CoA through fatty acid synthesis. PHAs, of which polyhydroxybutyrate (PHB) is the most abundant, are polyesters of hydroxyalkanoates (3-hydroxybutyrate in the case of PHB) synthesized by bacteria and accumulated as granules in the cytoplasm of cells (Lee, 1996). The degree of polymerization of 3-hydroxybutyrate in PHB can reach 2,000, which makes PHB comparable to many petroleum based plastics in terms of mechanical properties. PHB is also widely applied in the pharmaceutical industry due to its biocompatibility. In the context of a lignocellulose biorefinery, conversion of lignocellulosic feedstocks into PHB can add profit and increase sustainability. Most previous studies on lignocellulosic conversion to PHB/PHA have focused on sugars (Brodin et al., 2017; Cesario et al., 2014; Dai et al., 2015; Pan et al., 2012a; Raposo et al., 2017). There are only limited studies on lignin conversion into PHB/PHA with production ranging from 0.13 to 1 g/L (Berezina et al., 2015; Linger et al., 2014; Liu et al., 2017; Shi et al., 2017; Si et al., 2018; Wang et al., 2018). The greatest PHA concentration observed in these studies was 1.0 g/L using *Pseudomonas putida* (Liu et al., 2017). The substrate for that study was alkaline pretreatment liquor (APL) that was produced from a combination of H_2SO_4 pretreatment followed by NaOH pretreatment (Liu et al., 2017). Such PHA concentrations are too low to be economical.

Cupriavidus necator DSM 545, was found to convert benzoic acid into PHB, but PHB production was only 0.4 g/L (2 g/L cell dry weight with 20% PHB content inside cells) under a fed-batch mode (Berezina et al., 2015). Because microbial assimilation of aromatic compound conversion is feasible if the aromatic rings of the compounds are less than five (Bandounas et al., 2011; Taylor et al., 2012). In previous studies, multiple enzymes and chemicals have been widely studied in terms of their roles in lignin structural and molecular changes and low molecular

aromatic compound conversion (Beckham et al., 2016; Chen and Wan, 2017a; Linger et al., 2014; Paliwal et al., 2012). Oxidative enzymes (laccase and aryl alcohol oxidase (AAO)), mediators (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS) and hydroxybenzotriazole (HOBT)) were found to enhance lignin depolymerization by laccase (Brenelli et al., 2018; Christopher et al., 2014; Xie et al., 2017; Zhao et al., 2016). These enzymes and mediators were tested as supplements in this study. Generally, surfactants were found to decrease surface tension, increase oxygen and mass transfer, and improve availability of substrates to microorganisms in fermentation broth (Pulido-mayoral and Galindo, 2004). Aerosil R816 silica nanoparticle (hereafter referred to as R816), which is slightly hydrophobic, but is dispersible in water, was hypothesized in this study to enhance PHB production based on the aforementioned mechanisms. A nonionic surfactant, such as Tween 80, was hypothesized in this study to enhance APL conversion into PHB because previously it was found to enhance lignin oxidation and depolymerization by manganese peroxidase (Hofrichter, 2002). Lignin solubility was increased by up to 70% by addition of 2% dimethyl sulfoxide (DMSO) into aqueous media (Brzonova et al., 2017). R816, Tween 80 and DMSO were also tested as supplements in this study. These enzymes and chemicals have the potential to enhance lignin conversion to PHB, but their effects on enhancing PHB production from APL have not been studied much.

In this study, a supplement system consisting of these enzymes and chemicals was applied to *C. necator* DSM 545 to enhance PHB production from APL. The objective of this study was 1) to screen the supplements that can enhance PHB production and 2) to obtain the optimum conditions (optimum levels for supplements) that lead to maximal PHB production. In the view of process control, knowing the effects of these supplements on PHB production enhancement can help to optimize PHB production.

4.2. Materials and methods

4.2.1. Microorganisms, inoculum and AAO production

C. necator DSM 545, a Gram-negative Proteobacteria and the model organism for PHB production was used to produce PHB from APL. *C. necator* DSM 545 in the form of freeze-dried pellets was purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). *C. necator* DSM 545 was revitalized and subcultured with seed medium (meat extract 3 g/L, meat peptone 5 g/L, glucose 30 g/L) for at least two generations until the OD reached 10 at 24 h. *C. necator* culture was conducted throughout the study at 33 °C at 250 rpm in 25 mL working medium in 250 mL baffled flasks with silicone sponge closures unless specified. *C. necator* culture of optical density (OD) 10 at the wavelength of 600 nm and the path length of 1 cm was preserved at -80 ℃ in 30% glycerol in 2 mL aliquots and used for inoculum preparation. One aliquot of *C. necator* culture was inoculated to seed medium and cultured for around 24 h to obtain late exponential phase culture with an OD of 8-10. Then the culture was washed with sterile 0.89% sodium chloride solution twice and used as inoculum. Inoculum was added to each experimental flask to achieve an initial OD of 5.

Aspergillus nidulans, a filamentous fungus, was used to produce AAO. *A. nidulans*, a mutant strain with the identifier A773, was obtained from the Functional Genetics Stock Culture (FGSC, Manhattan, Kansas, U.S.). The *A. nidulans* strain was further genetically modified as described elsewhere (Pardo-Planas et al., 2017a; Segato et al., 2012). *Myceliophthora thermophila* AAO was overexpressed and secreted by the resulting genetically engineered *A. nidulans*. AAO was produced by growing *A. nidulans* in a medium containing 40 g/L maltose, 10 g /L glucose, 6 g/L $NaNO_3$, 1.52 g/L KH_2PO_4, 1.52 g/L KCl, 0.52 g/L $MgSO_4 \cdot 7H_2O$, 0.001 g/L pyridoxine, and

trace of $FeSO_4 \cdot 7H_2O$ and $ZnSO_4 \cdot 7H_2O$. The medium was adjusted to pH 6.5 using 6 M NaOH solution and autoclaved at 121 °C for 20 min. Enzyme production was carried out at 37 °C and agitated at 250 rpm for 72 h. The AAO activity was determined by spectrophotometric assay, which was based on the oxidation of veratryl alcohol and the reduction of 2, 6-dichlorophenol indophenol (DCPIP) (Pardo-Planas et al., 2017b). The absorbance of the reaction at 600 nm (ε_{600}= 21,000 $M^{-1} \cdot cm^{-1}$) was monitored at 1 cm path length and 50 °C for 2 min. Each reaction contained 0.1 ml of 2 mM DCPIP, 0.1 ml of 250 mM veratryl alcohol, 0.1 ml of 50 mM phosphate buffer (pH 7.0), and 0.7 ml of enzyme sample. One unit of AAO activity was defined as the reduction of one µmol of DCPIP per min. The AAO used in this study had an activity of 535 U/L.

4.2.2 APL preparation

Corn stover was used as the lignocellulosic material for making APL. Corn stover was collected from Rogers Memorial Farm (Lincoln, NE, USA) in 2017, and it was air dried and ground in a hammer mill (Schutte Buffalo, NY, USA) equipped with a 2.36 mm sieve. To obtain lignin-enriched liquor, alkaline pretreatment on corn stover was carried out in a 1 L bench top pressure reactor (Parr Reactor Model 4848, Parr Instrument Co., Moline, IL, USA). The reactor was loaded with 60 g corn stover (dry basis) and 540 mL alkaline solution to achieve a liquid-to solid mass ratio of 10:1. The alkaline solution was prepared by dissolving 4.2 g NaOH in 540 mL deionized water to achieve a NaOH loading of 0.07g NaOH/g corn stover. The operating conditions were agitating at 500 rpm, heating to 140 °C and holding for 30 min. The pretreatment conditions were near-optimum in terms of preserving cellulose and hemicellulose in the solids while preserving lignin in the liquid according to previous studies (Chen et al., 2013; Karp et al., 2014; Linger et al., 2014). At the end of the reaction, the heating mantle was removed, and the reactor was cooled using an internal cooling coil. Liquid fractions of the slurries were separated

from the solids under vacuum filtration using Whatman #4 filter paper. APL was prepared by neutralizing the liquid fractions to pH 6.8 with 5 M HCl and autoclaving at 121 °C for 20 min.

4.2.3 PHB production

PHB production base medium contained APL (5 mL) as the carbon source, $(NH_4)_2SO_4$ (0.1 g/L) as the nitrogen source, salts ($Na_2HPO_4 \cdot 2H_2O$, 2.9 g/L; KH_2PO_4, 1.5 g/L; $MgSO_4 \cdot 7H_2O$, 0.2 g/L; $CaCl_2 \cdot 2\ H_2O$: 0.01 g/L; Fe(NH_4)citrate: 0.06 g/L) and trace elements (1 mL/L) as described previously (Ramsay et al., 1990). The $MgSO_4 \cdot 7H_2O$, $CaCl_2 \cdot 2\ H_2O$ and Fe(NH_4)citrate solutions were autoclaved separately. PHB production was conducted at 32.5 °C at 250 rpm as described in Section 4.2.1, but with a 20 mL working volume and cultivation was stopped at 7 days (168 h) for PHB quantification as described in Section 4.2.4.

To enhance PHB production, an oxidative enzyme-mediator-surfactant system was applied under Plackett-Burman and central composite designs. The experiments were carried out in three stages. First, seven supplements (laccase, AAO, DMSO, R816, ABTS, HOBT and Tween-80) were considered as seven independent variables in a Plackett-Burman design to screen the variables that could significantly enhance PHB production. Some follow up experiments were performed to see if the non-significant variables can be removed from the system. Laccase (L2157-10KU, 50 units/mg solids activity), ABTS and HOBT in the form of 1-hydroxybenzotriazole hydrate were purchased from Sigma-Aldrich (St. Louis, MO, USA). AAO was prepared as described in Section 4.2.1. DMSO was purchased from Fisher Scientific (Waltham, MA, USA). R816 (Aerosil® R816, 12 nm average primary particle size) was donated by Evonik (Essen, Germany). Tween-80 was purchased from Acros Organics (Geel, Belgium). Second, a central composite design was employed to build a polynomial equation based on results from the Plackett-

Burman Design. Third, the optimum conditions were derived from the polynomial equation by ridge analysis and validated by running an additional set of experiments. Details of the three-stage statistical design, modeling and experimental data analyses are shown in Section 4.2.5.

4.2.4 PHB quantification by gas chromatography-mass spectrometer (GCMS)

PHB concentration was determined according to the methods described by Braunegg et al. (1978). Dried cell biomass was methanolyzed at 105 °C in acidified methanol (5% H_2SO_4, v/v, 2 mL) and chloroform (2 mL) mixture for 3 h. Sodium hydroxybutyrate (Sigma-Aldrich, MO, USA) was used as an external standard. After 3 h, 1 mL deionized water (Milli-Q, 18.2 MΩ cm) was added to the cooled mixture and vortexed. The methyl-ester derivatives were extracted from the lower chloroform layer and passed through a column containing anhydrous sodium sulfate before being injected into a GC-MS.

The GC-MS was equipped with a Trace 1310 Gas Chromatograph, ISQ QD single quadrupole mass spectrometer (ThermoScientific, Waltham, MA, USA) and TG-5MS (30 m x 0.25 mm ID x 0.25 film) capillary column (ThermoFisher Scientific, Waltham, MA, USA). The temperature profile was held at 60 °C for 5 min, increase to 220 °C at a rate of 30 °C/min, then hold at 220 °C for 5 min. The injector temperature was 230 °C, and the injection mode was splitless (1 µL). The pressure of the carrier gas (helium) was 142 kPa (18 psi). The MS transfer line temperature was 250 °C, ion source temperature was 220 °C and ionization mode was electron ionization. The ionization energy was 70 eV and selective ion monitoring (SIM) mode was used. Methylated methyl-ester derivative was confirmed with SIM at 43, 71, and 74. Solvent delay was 3 min. Data were analyzed with Chromeleon 7 chromatography studio (ThermoFisher Scientific, Waltham, MA, USA).

4.2.5 Statistical design, modeling and analysis

4.2.5.1 Plackett–Burman Design

A 7-factor-8-run Plackett-Burman design experiment was conducted (Table 4.1). The selection of low (-1) and high (1) levels for each factor (Table 4.1) was based on previous literature. Two replicates per run were performed when conducting the experiments. A total of sixteen flasks were randomly assigned to 8 runs. For statistical modeling, the first-order polynomial linear model shown in Equation 4.1 was employed.

$$Y = \beta_0 + \sum_{n=1}^{7} \beta_n x_n \quad (4.1)$$

where Y is the predicted response (PHB concentration (g/L), further called PHB production throughout the study), β_0 is the model intercept, β_n is the linear coefficient and x_n is the coded level of the independent variable. The model for Equation 4.1 was implemented using 'lm' function in the "FrF2" package using "*R*-language" software/environment (Gronmping, 2014). The main effects plot for Plackett–Burman Design was obtained using 'MEPlot' function. Significant level was set as p-value < 0.05. The significant independent variables were considered for further optimization using a central composite design. Because supplements were not essential nutrients for fermentation, non-significant supplements may be removed. Follow up experiments were carried out to see if the non-significant variables could be removed.

4.2.5.2 Central composite design

The central composite design, one of the most commonly used designs with response surface methodology, is usually used to evaluate how independent variables affect the measured response with fewer experimental run requirements than equivalent factorial designs. This procedure was used to understand the overall (linear, interaction, and quadratic) effects of

independent variables. Independent variable levels and design structure were shown in Table 4. 2. The selection of five levels of three independent variables (Table 4.2) is explained in Section 4.3.1 based on results from Plackett-Burman design. When performing the experiments, three flasks per run were applied to run # 1 through 14 as three replicates and six flasks were applied to run 15 as six replicates. A total of 48 flasks were randomly placed in two identical incubators (Model I26, New Brunswick Scientific, Edison, NJ, USA) with 24 flasks in each incubator. The effect of incubator was found to be non-significant (p-value < 0.05) and removed from the model. The polynomial quadratic model shown in Equation 4.2 was employed to fit the experimental data.

$$Y = b_0 + \sum_{n=1}^{3} b_n X_n + \sum_{n=1}^{4} b_{nn} X_n^2 + \sum_{n=\mathrm{m}-1}^{2} \sum_{m=2}^{3} b_{nm} X_n X_m \quad (4.2)$$

where Y is the predicted PHB production (g/L); b_0 is the intercept; b_n is the linear coefficient; b_{nn} is the quadratic coefficients and b_{nm} is the cross product coefficients. X_n and X_m are independent variables.

The model in Equation 4.2 was fit to experimental data generated from the central composite design using RSREG procedures in SAS 9.3 (SAS Institute Inc., Cary, NC). The fitted model was evaluated based upon the model sufficiency (R^2), regression parameter significance (p-value) and test for lack of fit. P-values < 0.05 was set as statistically significant. Response surface and contours graphs for fitted Equation 4.2 were used to illustrate the surface near the optimum conditions and were plotted using MATLAB R2014a (MathWorks, Inc., Natick, MA, USA).

4.2.5.3 Optimum condition and validation

Ridge analysis was conducted using the ridge max option of the same RSREG procedure in Section 4.2.5.2 to obtain the optimum condition that led to maximum PHB production. Ridge analysis can evaluate the nature of the stationary point from the output of REREG and find the

maximum response value along the path of steepest ascent. Validation experiments were performed by running an additional set of experiments under the optimum conditions.

4.3 Results and discussion

4.3.1 PHB production-screening

Without any supplements added, PHB production was found to be 0.21 g/L using the base medium as described in Section 4.2.3. Using the supplement system under the Plackett-Burman design, PHB production is shown in Table 4.1. After fitting a first order polynomial model, the coefficient of determination (R^2) was 0.98 (Table 4.3), which indicated a good fit. Three supplements R816, ABTS and Tween 80 were found to have significant effects on PHB production (Table 4.3). R816 is a silica nanoparticle that can lower interfacial surface tension (Zargartalebi et al., 2015). The fact that it enhanced PHB production may be related to decreased surface tension because decreased surface tension was found to increase oxygen and mass transfer and improve availability of substrates to microorganisms in fermentation broth (Pulido-mayoral and Galindo, 2004). ABTS has been a successful mediator of laccase to produce low molecular weight lignin (Brenelli et al., 2018; Christopher et al., 2014). Nonionic surfactants, such as Tween-80, have been found reducing the amount of lignin remaining in the pretreated material and improve cellulolytic enzyme performance (Borjesson et al., 2007; Cao and Aita, 2013). These facts indicate that enhanced PHB production by these three supplements may be caused by lower molecular lignin availability to *C. necator*.

Because supplements are not essential nutrients, non-significant supplements identified from Plackett-Burman design may be removed from the system. Follow up experiments were conducted to see how PHB production was affected by removing non-significant factors. These

experiments were conducted based on the conditions of run #2 in Table 4.1 because PHB production of run #2 was the highest. First, both DMSO and HOBT were removed from the supplement system and PHB production was 1.95±0.21 g/L, which was not significantly different from 2.05±0.37 g/L (PHB production of run #2 in Table 4.1). Supplementing DMSO was expected to increase lignin solubility (Brzonova et al., 2017) and conversion into PHB; however, DMSO at the levels in Table 4.1 did not enhance APL conversion to PHB in this study. The fact that DMSO could be removed was important because DMSO is expensive. Mediators (ABTS and HOBT) are low molecular weight oxidants that act as electron carriers between laccase and lignin substrates (Raj and Krishnan, 2018). HOBT was one of the most successful mediators of laccase that has been used in lignin degradation and biobleaching of kraft pulps (Christopher et al., 2014). Laccase-HOBT system was applied to treat lignin first and increased lipid yield by 10 times during subsequent *Rhodococcus opacus* fermentation (Xie et al., 2017). However, HOBT did not enhance APL conversion to PHB at the levels in Table 4.1, either. Therefore, both DMSO and HOBT were removed from the supplement system.

AAO and laccase were also found to be non-significant factors from Plackett-Burman design. However, after removing DMSO and HOBT, PHB production decreased to 1.57±0.10 g/L when AAO was also removed; and PHB production further dropped to 0.71±0.18 g/L when removing laccase. Because removing laccase and AAO caused significant drop in PHB production, it was necessary to keep laccase and AAO in the supplement system. Previous studies have shown that laccase enhanced lignin conversion to PHB-like energy storage materials, such as lipids, by *Rhodococcus opacus* (Zhao et al., 2016). For example, laccase supplementation on *R. opacus* cell culture led to an exponential increase of cell growth and 17-fold increase of lipid production from kraft lignin (Zhao et al., 2016). Zhao et al. (2016) found that laccase improved the uptake of the

lower molecular weight lignin molecules by *R. opacus*. This may also explain how laccase enhanced lignin conversion to PHB in this study. The role of AAO may lie in lignin degradation by supplying hydrogen peroxide for the oxidation of aromatic substrates (Ferreira et al., 2015).

The strategy for the central composite design was to vary three significant supplements with laccase and AAO being kept at the levels in run #2 (laccase 2 U/mL and AAO 20 U/mL). The overall effects of these three supplements were evaluated and the optimum conditions leading to maximum PHB production were derived. To determine the levels of the three significant supplements used for central composite design, it was necessary to look at the main effects of the 7 factors in the Plackett-Burman design (Fig. 4.1). From low to high levels of R816, PHB production decreased. Increases in ABTS and Tween 80 resulted in increased PHB production. Therefore, R816 at low level (0.02%, m/w) and ABTS and Tween 80 at high levels (5 mM and 2.5%, respectively) were used as the center point (run # 15 in Table 4.2) in central composite design. The experimental levels for cube (-1 and 1) and star (-1.682 and 1.682) points were set around the experimental levels of the center points. The differences between the start and center points were set to be less than the differences between the two levels in Plackett-Burman design in Table 4.1. Use Tween 80 as an example, the difference (0.841) between level -1.682 (1.159) and level 0 (2) in central composite design is less than the difference (1.5) between level -1 (0.5) and level 1 (2). With the levels of three significant factors varying in Table 4.2, laccase and AAO were kept in the supplement system with the same levels of run #2 in Table 4.1 (laccase 2 U/mL and AAO 20 U/mL), while DMSO and HOBT were removed.

4.3.2 PHB production-central composite design

Experimental PHB production from the central composite design is shown in Table 4.2. PHB production increased from 0.48 to 1.28 g/L by increasing Tween 80 from 1.5 (run #3) to 2.5% (run #7). PHB production increased from 0.55 to 1.28 g/L by increasing ABTS from 4 mM (run #5) to 6 mM (run #7). The highest PHB production was obtained at the center point (run #15). The parameter estimates for the model and model fitting are shown in Table 4.4 after fitting the experimental data using RSREG procedure with ridge max option. A significant model ($p < 0.0001$) with non-significant lack of fit ($p = 0.07$) and a R^2 of 0.90 was obtained (Table 4.4). The analyses results indicated that the model (Equation 4.3) was adequate to fit the experimental data.

$$\text{PHB concentration} = -24.11 + 783.33 * \text{R816} + 4.36 * \text{ABTS} + 6.46 * \text{Tween80} + 2.88 * \text{R816} * \text{ABTS} - 23.89 * \text{R816} * \text{Tween80} + 0.25 * \text{ABTS} * \text{Tween80} - 19050 * \text{R816}^2 - 0.47 * \text{ABTS}^2 - 1.71 * \text{Tween80}^2 \quad (4.3)$$

The overall effects were exhibited by plotting two factors as the independent variables and PHB production as the dependent variable with the third factor fixed at its optimum level (Fig. 4.2). After plotting the surfaces, predicted PHB production values at the levels of -1.682 and 1.68 (Table 4.2) for three independent variables were found to be negative. Because the edges of a predicted surface are suspect, the surfaces near the boundaries (levels of -1.682 and 1.68) were cut off and only the surface near the optimum conditions are shown in Fig. 4.2. The response surface plots were consistent with the maximum PHB production at the optimum condition. All three factors (R816, ABTS and Tween 80) showed a clear quadratic effect, which was consistent with the significant effects ($p < 0.0001$) of quadratic terms in Table 4.4. All linear effects of three factors were highly significant ($p < 0.0001$) while the interaction effects of R816 with ABTS, and with Tween 80 were not significant ($p > 0.05$).

4.3.3 Optimum condition and validation

According to ridge analysis (Table 4.5), maximum PHB production was predicted to be 1.94 ± 0.08 g/L under the optimum condition (R816, 0.019%, ABTS 5.23 mM, Tween 2.12%, as highlighted in Table 4.5). As can be noticed, this condition is close to the center point condition in the central composite design. The experimental result under the optimum condition showed a PHB concentration of 2.12 ± 0.30 g/L, which was close to and validated the predicted result from ridge analysis. It is extraordinary that the supplement system (R816, 0.0196%, ABTS 5.23 mM, Tween 80 2.12%, laccase 2 U/mL and AAO 20 U/mL) improved PHB production by 10-fold from 0.21 to 2.12 g/L. To the best of the authors' knowledge, such a PHB production was higher than any previously reported PHB/PHA production (Berezina et al., 2015; Linger et al., 2014; Liu et al., 2017; Shi et al., 2017; Si et al., 2018; Wang et al., 2018). If APL is prepared more efficiently as the approach in Liu et al. (2017) and fed-batch mode is applied to optimize PHB production, PHB production is expected to be higher than 2.12 g/L.

The trend of estimated PHB production changing with three variables in Table 4.5 is consistent with the graphs in Fig. 4.2. Table 4.5 also provides us guidance for optimizing PHB production by manipulating the levels of three supplements. For example, because ABTS is an expensive supplement, it is practically meaningful at large scale if its usage can be reduced while high PHB production is maintained. This can be realized by reducing ABTS usage from 5.2288 to 5.1172 mM and Tween 80 usage from 2.1188 to 2.058%, while increasing R816 from 0.01967 to 0.01983%. As a result, PHB production is predicted to be 1.93 g/L, which is only slightly lower than 1.94 g/L. In this way, the production cost can be lowered. As many research groups have been working on screening new strains or engineering existing strains for lignin conversion to PHB (Lin et al., 2016; Numata and Morisaki, 2015; Si et al., 2018; Tomizawa et al., 2014), the supplement

system in this study can be used by geneticists to study gene expression involved in lignin conversion to PHB and metabolic pathways modulation to enhance lignin conversion into PHB. In terms of lignin structural changes, structural changes of lignin triggered by this supplement system should be studied in future studies to understand the mechanism during lignin depolymerization and conversion.

4.4 Conclusion

In this study, PHB production was improved by 10 fold from 0.21 to 2.12 g/L with a supplement system (R816 0.019%, ABTS 5.23 mM, Tween 80 2.12%, laccase 2 U/mL and AAO 20 U/mL) by *C. necator* DSM 545. Plackett-Burman and subsequent central composite designs proved to be an effective approach to screen significant factors and to optimize PHB production. Polynomial models fit experimental data well and the optimum conditions were validated. The models can be used to manipulate supplement usage to optimize PHB production. With a higher PHB production than any previous reported, this study will be the first step for efficient lignin valorization into valuable products such as PHB. The supplement system described in this study can be used to study the metabolic pathways and lignin structural changes to help understand the mechanisms. Future work will focus on scaling up the PHB production under the optimum conditions and improve PHB productivity. With more efficient pretreatment technologies and applying fermentation strategies such as fed-batch strategy, PHB production is expected to be higher.

Table 4.1. PHB production for 8 runs with 7 factors in Plackett-Burman design

Run #	Laccase (U/mL)	AAO (U/mL)	DMSO (v/v, %)	R816 (m/v, %)	ABTS (mM)	HOBT (mM)	Tween 80 (v/v, %)	PHB (g/L)
1	2 (1)	5 (-1)	0.5 (-1)	0.1 (1)	1 (-1)	5 (1)	2 (1)	0.61 (0.01)
2	2 (1)	20(1)	0.5 (-1)	0.02 (-1)	5 (1)	1 (-1)	2 (1)	2.05 (0.37)
3	2 (1)	20(1)	2 (2)	0.02 (-1)	1 (-1)	5 (1)	0.5 (-1)	0.50 (0.13)
4	0.5 (-1)	20(1)	2 (2)	0.1 (1)	1 (-1)	1 (-1)	2 (1)	0.45 (0.22)
5	2 (1)	5 (-1)	2 (2)	0.1 (1)	5 (1)	1 (-1)	0.5 (-1)	0.21 (0.03)
6	0.5 (-1)	20(1)	0.5 (-1)	0.1 (1)	5 (1)	5 (1)	0.5 (-1)	0.38 (0.01)
7	0.5 (-1)	5 (-1)	2 (2)	0.02 (-1)	5 (1)	5 (1)	2 (1)	1.70 (0.11)
8	0.5 (-1)	5 (-1)	0.5 (-1)	0.02 (-1)	1 (-1)	1 (-1)	0.5 (-1)	0.29 (0.01)

Note: 7 factors were shown as real values (coded levels). PHB values are shown as mean (standard deviation) (n=2). DMSO denotes dimethyl sulfoxide, R816 denotes Aerosil R816 hydrophobic silica, ABTS denotes 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid), HOBT denotes 1-hydroxybenzotriazole hydrate, v/v denotes volume/volume, m/v denotes mass/volume. The selection of low (-1) and high (1) levels for each factor was based on previous literature.

Table 4.2. The central composite design strucutre and experimental PHB production

Run #	Factors			Response
	R816 (m/v, %)	ABTS (mM)	Tween 80 (v/v, %)	PHB (g/L)
1	0.015 (-1)	4 (-1)	1.5 (-1)	0.27 (0.05)
2	0.025 (1)	4 (-1)	1.5 (-1)	0.22 (0.07)
3	0.015 (-1)	6 (1)	1.5 (-1)	0.48 (0.16)
4	0.025 (1)	6 (1)	1.5 (-1)	0.52 (0.19)
5	0.015 (-1)	4 (-1)	2.5 (1)	0.55 (0.22)
6	0.025 (1)	4 (-1)	2.5 (1)	0.28 (0.06)
7	0.015 (-1)	6 (1)	2.5 (1)	1.28 (0.33)
8	0.025 (1)	6 (1)	2.5 (1)	1.06 (0.29)
9	0.0116 (-1.682)	5 (0)	2 (0)	0.54 (0.24)
10	0.0284 (1.682)	5 (0)	2 (0)	0.35 (0.12)
11	0.02 (0)	3.318 (-1.682)	2 (0)	0.20 (0.06)
12	0.02 (0)	6.682 (1.682)	2 (0)	0.72 (0.25)
13	0.02 (0)	5 (0)	1.159 (-1.682)	0.28 (0.07)
14	0.02 (0)	5 (0)	2.841 (1.682)	0.88 (0.18)
15	0.02 (0)	5 (0)	2 (0)	1.91 (0.20)

Note: 7 factors were shown as real values (coded levels). PHB production values are shown as mean (standard deviation) of 3 replicates for run #1-14 and of 6 replicates for run#15. For experimental levels for cube (-1 and 1) and star (-1.682 and 1.682) points were set around the experimental levels of center points. The differences between the start and center points were set to be less than the differences between the two levels in Plackett-Burman design in Table 2. Use Tween 80 as an example, the difference (0.841) between level -1.682 (1.159) and level 0 (2) in central composite design is less than the difference (1.5) between level -1 (0.5) and level 1 (2).

Table 4.3. ANOVA for 8-run Plackett-Burman design and mode fitting

Source	DF	SS	MSE	F value	*P*-value
Model	7	6.78	0.97	47.03	< 0.0001
Laccase	1	0.072	0.072	3.49	0.099
AAO	1	0.084	0.084	4.06	0.079
DMSO	1	0.056	0.056	2.72	0.138
R816	1	2.093	2.093	101.58	< 0.0001
ABTS	1	1.543	1.543	74.93	< 0.0001
HOBT	1	0.009	0.009	0.46	0.518
Tween 80	1	2.925	2.925	141.97	< 0.0001
Pure error	8	0.165	0.021		
Total	15	6.947			
R^2	0.98				

Note: the 8 degrees of freedom of pure error are from the duplicate of 8 runs (1*8=8).

Table 4.4. Parameter estimates and model fitting for polynomial model

Parameter	DF	Estimate (standard error)	t Value	Pr > |t|
Model	9			<.0001
Intercept	1	-24.11 (2.15)	-11.23	<.0001
R816	1	783.33 (81.50)	9.61	<.0001
ABTS	1	4.34 (0.45)	9.65	<.0001
Tween 80	1	6.46 (0.81)	7.93	<.0001
R816* R816	1	-19050 (1539)	-12.38	<.0001
R816* ABTS	1	2.88 (8.28)	0.35	0.7303
ABTS * ABTS	1	-0.47 (0.04)	-12.23	<.0001
R816* Tween 80	1	-23.89 (16.56)	-1.44	0.1575
ABTS * Tween 80	1	0.25 (0.08)	3.03	0.0044
Tween 80 * Tween 80	1	-1.71 (0.15)	-11.12	<.0001
Residual		Mean square	F value	Pr > F
Lack of fit	5	0.0798	2.26	0.0709
Pure error	33	0.0352		
Total error	38	0.0411		
Fit diagnostics				
R^2		0.90		

Note: the 33 degrees of freedom of pure error are pooled from the triplicates of run # 1-14 and six replicates for run # 15 in Table 5 (14*2+5=33).

Table 4.5 Ridge of steepset ascent for R816, ABTS, Tween 80, and esimated PHB production and standard error

R816	ABTS	Tween 80	Estimated PHB production	Standard error
0.02	5	2	1.88844	0.08258
0.01983	5.11719	2.05791	1.92716	0.08196
0.01967	**5.22879**	**2.11867**	**1.94418**	**0.08015**
0.0195	5.33637	2.1813	1.93957	0.07736
0.01934	5.44099	2.24521	1.91341	0.07396
0.01917	5.54335	2.31004	1.86572	0.07055
0.019	5.64397	2.37554	1.79652	0.06809
0.01884	5.74321	2.44155	1.70584	0.06784
0.01867	5.84135	2.50796	1.59368	0.07114
0.0185	5.93858	2.57467	1.46005	0.0789
0.01833	6.03506	2.64164	1.30496	0.09131

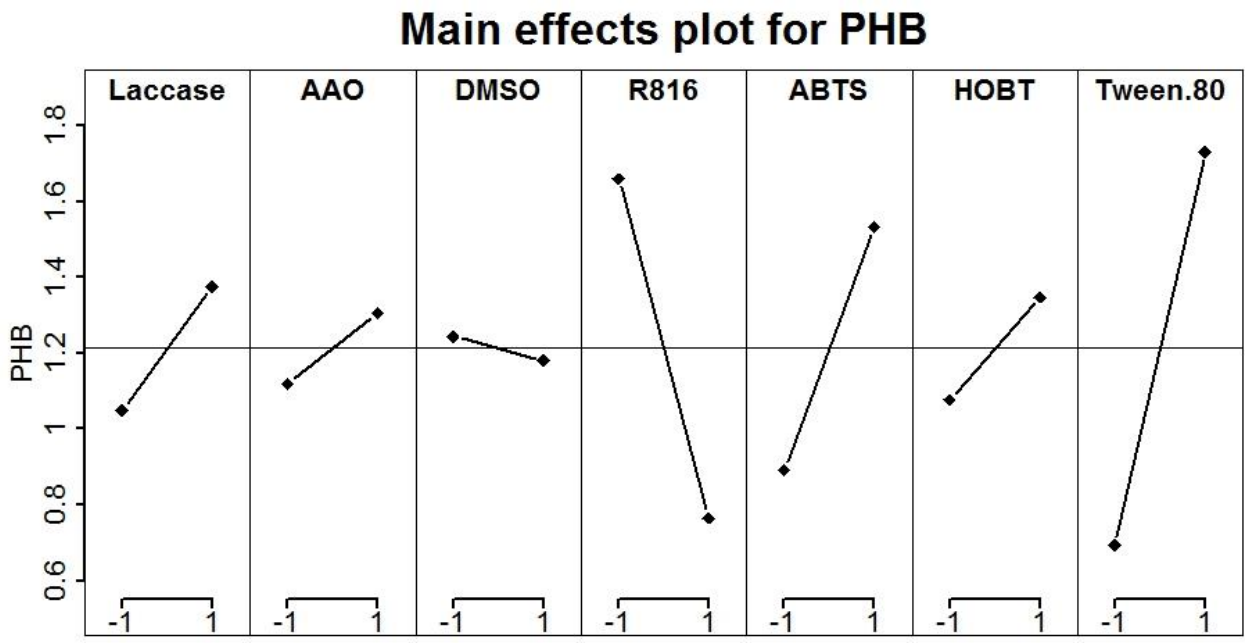

Fig. 4.1. Main effect of 7 factors from Plackett-Burman design. The squares below and above the middle horizontal line correspond to PHB production of the low (-1) and high (1) levels, respectively in Table 4.2.

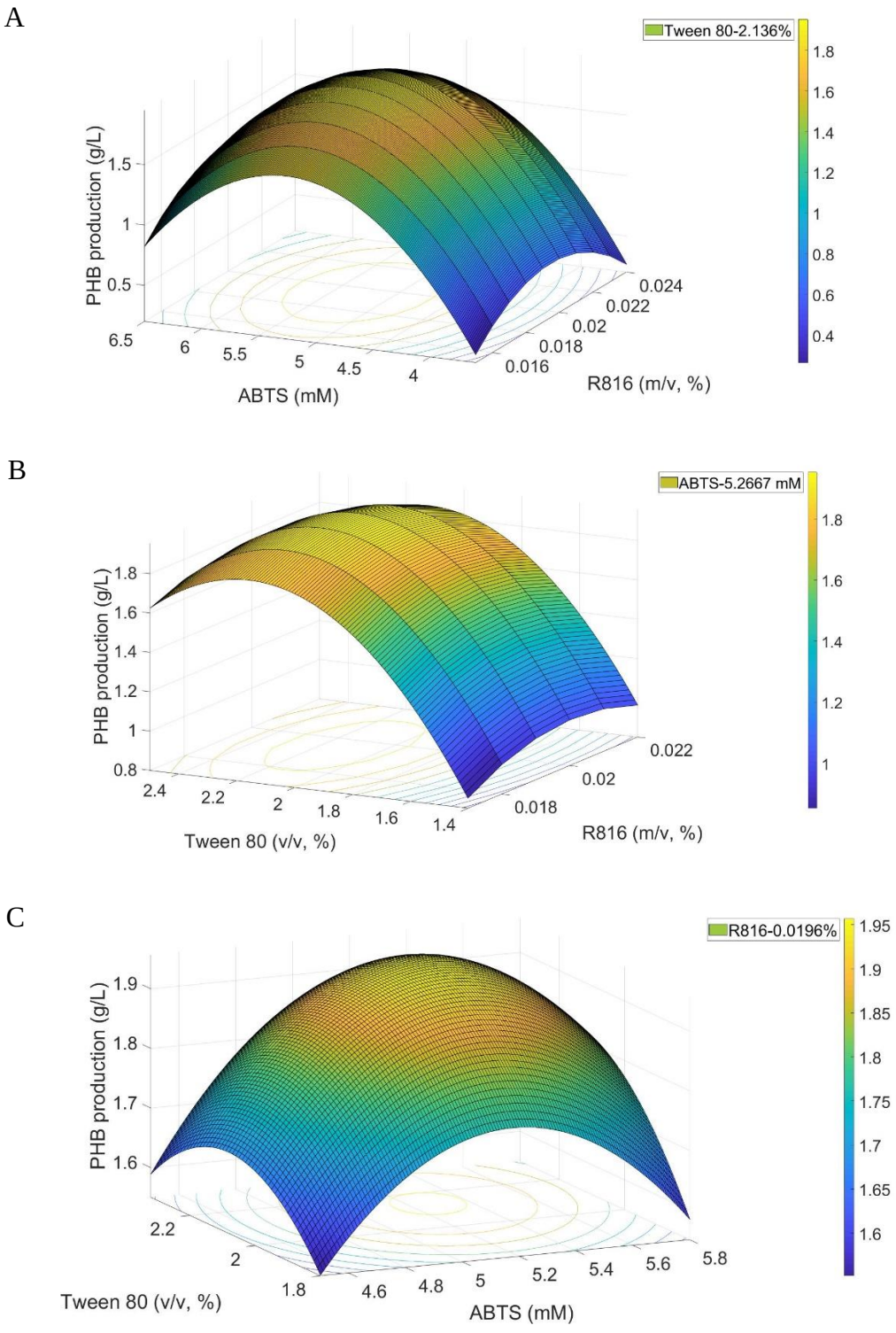

Fig. 4.2. Response surface and contour plot exhibiting overall effects of ABTS and R816 (A), Tween 80 and R816 (B), Tween 80 and ABTS (C) on PHB production.

4.5. References

Bandounas, L., Wierckx, N.J., de Winde, J.H., Ruijssenaars, H.J. 2011. Isolation and characterization of novel bacterial strains exhibiting ligninolytic potential. BMC Biotechnol., 11(1), 94.

Beckham, G.T., Johnson, C.W., Karp, E.M., Salvachua, D., Vardon, D.R. 2016. Opportunities and challenges in biological lignin valorization. Curr. Opin. Biotechnol., 42, 40-53.

Berezina, N., Yada, B., Lefebvre, R. 2015. From organic pollutants to bioplastics: insights into the bioremediation of aromatic compounds by *Cupriavidus necator*. New Biotechnol., 32(1), 47-53.

Borjesson, J., Engqvist, M., Sipos, B., Tjerneld, F. 2007. Effect of poly (ethylene glycol) on enzymatic hydrolysis and adsorption of cellulase enzymes to pretreated lignocellulose. Enzyme Microb. Technol., 41(1-2), 186-195.

Braunegg, G., Sonnleitner, B., Lafferty, R. 1978. A rapid gas chromatographic method for the determination of poly-β-hydroxybutyric acid in microbial biomass. Eur. J. Appl. Microbiol. Biotechnol., 6(1), 29-37.

Brenelli, L., Squina, F.M., Felby, C., Cannella, D. 2018. Laccase-derived lignin compounds boost cellulose oxidative enzymes AA9. Biotechnol. Biofuels, 11(1), 10.

Brodin, M., Vallejos, M., Opedal, M.T., Area, M.C., Chinga-Carrasco, G. 2017. Lignocellulosics as sustainable resources for production of bioplastics–A review. J. Clean. Prod., 162, 646-664.

Brzonova, I., Asina, F., Andrianova, A.A., Kubatova, A., Smoliakova, I.P., Kozliak, E.I., Ji, Y. 2017. Fungal biotransformation of insoluble kraft lignin into a water soluble polymer. Ind. Eng. Chem. Res., 56(21), 6103-6113.

Cao, S., Aita, G.M. 2013. Enzymatic hydrolysis and ethanol yields of combined surfactant and dilute ammonia treated sugarcane bagasse. Bioresour. Technol., 131, 357-364.

Cesario, M.T., Raposo, R.S., De Almeida, M.C.M., Van Keulen, F., Ferreira, B.S., Da Fonseca, M.M.R. 2014. Enhanced bioproduction of poly-3-hydroxybutyrate from wheat straw lignocellulosic hydrolysates. New Biotechnol., 31(1), 104-113.

Chen, Y., Stevens, M.A., Zhu, Y., Holmes, J., Xu, H. 2013. Understanding of alkaline pretreatment parameters for corn stover enzymatic saccharification. Biotechnol. Biofuels, 6(1), 8.

Chen, Z., Wan, C. 2017a. Biological valorization strategies for converting lignin into fuels and chemicals. Renew. Sust. Energ. Rev., 73, 610-621.

Christopher, L.P., Yao, B., Ji, Y. 2014. Lignin biodegradation with laccase-mediator Systems. Front. Energ. Res., 2(12).

Dai, J., Gliniewicz, K., Settles, M.L., Coats, E.R., McDonald, A.G. 2015. Influence of organic loading rate and solid retention time on polyhydroxybutyrate production from hybrid poplar hydrolysates using mixed microbial cultures. Bioresour. Technol., 175, 23-33.

Ferreira, P., Hernandez-Ortega, A., Lucas, F., Carro, J., Herguedas, B., Borrelli, K.W., Guallar, V., Martinez, A.T., Medina, M. 2015. Aromatic stacking interactions govern catalysis in aryl-alcohol oxidase. FEBS J., 282(16), 3091-3106.

Frederick, N., Li, M., Carrier, D.J., Buser, M.D., Wilkins, M.R. 2016. Switchgrass storage effects on the recovery of carbohydrates after liquid hot water pretreatment and enzymatic hydrolysis. AIMS Bioeng., 3(3), 389-399.

Gronmping, U. 2014. R package FrF2 for creating and analyzing fractional factorial 2-level designs. J. Stat. Softw., 56(1), 1-56.

Hofrichter, M. 2002. Review: lignin conversion by manganese peroxidase (MnP). Enzyme Microb. Technol., 30(4), 454-466.

Karp, E.M., Donohoe, B.S., O'Brien, M.H., Ciesielski, P.N., Mittal, A., Biddy, M.J., Beckham, G.T. 2014. Alkaline pretreatment of corn stover: bench-scale fractionation and stream characterization. ACS Sustain. Chem. Eng., 2(6), 1481-1491.

Kim, S., Holtzapple, M.T. 2005. Lime pretreatment and enzymatic hydrolysis of corn stover. Bioresour. Technol., 96(18), 1994-2006.

Lee, S.Y. 1996. Bacterial polyhydroxyalkanoates. Biotechnol. Bioeng., 49(1), 1-14.

Li, M., Marek, S.M., Peng, J., Liu, Z., Wilkins, M.R. 2018a. Effect of moisture content and inoculum size on cell wall composition and ethanol yield from switchgrass after solid-state *Pleurotus ostreatus* treatment. Trans. ASABE, 61(6), 1997-2006.

Lin, L., Cheng, Y., Pu, Y., Sun, S., Li, X., Jin, M., Pierson, E.A., Gross, D.C., Dale, B.E., Dai, S.Y. 2016. Systems biology-guided biodesign of consolidated lignin conversion. Green Chem., 18(20), 5536-5547.

Linger, J.G., Vardon, D.R., Guarnieri, M.T., Karp, E.M., Hunsinger, G.B., Franden, M.A., Johnson, C.W., Chupka, G., Strathmann, T.J., Pienkos, P.T. 2014. Lignin valorization through integrated biological funneling and chemical catalysis. Proc. Natl. Acad. Sci., 111(33), 12013-12018.

Liu, K., Atiyeh, H.K., Pardo-Planas, O., Ezeji, T.C., Ujor, V., Overton, J.C., Berning, K., Wilkins, M.R., Tanner, R.S. 2015. Butanol production from hydrothermolysis-pretreated

switchgrass: quantification of inhibitors and detoxification of hydrolyzate. Bioresour. Technol., 189, 292-301.

Liu, Z.-H., Olson, M.L., Shinde, S., Wang, X., Hao, N., Yoo, C.G., Bhagia, S., Dunlap, J.R., Pu, Y., Kao, K.C. 2017. Synergistic maximization of the carbohydrate output and lignin processability by combinatorial pretreatment. Green Chem., 19(20), 4939-4955.

Numata, K., Morisaki, K. 2015. Screening of marine bacteria to synthesize polyhydroxyalkanoate from lignin: contribution of lignin derivatives to biosynthesis by *Oceanimonas doudoroffii*. ACS Sustain. Chem. Eng., 3(4), 569-573.

Paliwal, R., Rawat, A.P., Rawat, M., Rai, J.P.N. 2012. Bioligninolysis: Recent updates for biotechnological solution. Appl. Biochem. Biotechnol., 167(7), 1865-1889.

Pan, W., Nomura, C.T., Nakas, J.P. 2012a. Estimation of inhibitory effects of hemicellulosic wood hydrolysate inhibitors on PHA production by *Burkholderia cepacia* ATCC 17759 using response surface methodology. Bioresour. Technol., 125, 275-282.

Pardo-Planas, O., Prade, R.A., Muller, M., Atiyeh, H.K., Wilkins, M.R. 2017a. Prevention of melanin formation during aryl alcohol oxidase production under growth-limited conditions using an *Aspergillus nidulans* cell factory. Bioresour. Technol., 243, 874-882.

Pardo-Planas, O., Prade, R.A., Wilkins, M.R. 2017b. High-yield production of aryl alcohol oxidase under limited growth conditions in small-scale systems using a mutant *Aspergillus nidulans* strain. J. Ind. Microbiol. Biotechnol., 44(2), 247-257.

Pulido-mayoral, N., Galindo, E. 2004. Phases dispersion and oxygen transfer in a simulated fermentation broth containing castor oil and proteins. Biotechnol. Prog., 20(5), 1608-1613.

Ragauskas, A.J., Beckham, G.T., Biddy, M.J., Chandra, R., Chen, F., Davis, M.F., Davison, B.H., Dixon, R.A., Gilna, P., Keller, M. 2014. Lignin valorization: improving lignin processing in the biorefinery. Science, 344(6185), 1246843.

Raj, K., Krishnan, C. 2018. High sugar yields from sugarcane (*Saccharum officinarum*) bagasse using low-temperature aqueous ammonia pretreatment and laccase-mediator assisted enzymatic hydrolysis. Ind. Crops Prod., 111, 673-683.

Ramsay, B., Lomaliza, K., Chavarie, C., Dube, B., Bataille, P., Ramsay, J. 1990. Production of poly-(beta-hydroxybutyric-co-beta-hydroxyvaleric) acids. Appl. Environ. Microbiol., 56(7), 2093-2098.

Raposo, R.S., De Almeida, M.C.M., De Oliveira, M.d.C.M., Da Fonseca, M.M., Cesario, M.T. 2017. A *Burkholderia sacchari* cell factory: production of poly-3-hydroxybutyrate, xylitol and xylonic acid from xylose-rich sugar mixtures. New Biotechnol., 34, 12-22.

Salvachua, D., Karp, E.M., Nimlos, C.T., Vardon, D.R., Beckham, G.T. 2015. Towards lignin consolidated bioprocessing: simultaneous lignin depolymerization and product generation by bacteria. Green Chem., 17(11), 4951-4967.

Sannigrahi, P., Pu, Y., Ragauskas, A. 2010. Cellulosic biorefineries-unleashing lignin opportunities. Curr. Opin. Environ. Sustain., 2(5-6), 383-393.

Segato, F., Damasio, A.R., Goncalves, T.A., De Lucas, R.C., Squina, F.M., Decker, S.R., Prade, R.A. 2012. High-yield secretion of multiple client proteins in *Aspergillus*. Enzyme Microb. Technol., 51(2), 100-106.

Shen, R., Tao, L., Yang, B. 2018. Techno-economic analysis of jet-fuel production from biorefinery waste lignin. Biofuels Bioprod. Bioref.

Shi, Y., Yan, X., Li, Q., Wang, X., Xie, S., Chai, L., Yuan, J. 2017. Directed bioconversion of kraft lignin to polyhydroxyalkanoate by *Cupriavidus basilensis* B-8 without any pretreatment. Process Biochem., 52, 238-242.

Si, M., Yan, X., Liu, M., Shi, M., Wang, Z., Wang, S., Zhang, J., Gao, C., Chai, L., Shi, Y. 2018. In situ lignin bioconversion promotes complete carbohydrate conversion of rice straw by *Cupriavidus basilensis* B-8. ACS Sustain. Chem. Eng., 6(6), 7969-7978.

Taylor, C., Hardiman, E., Ahmad, M., Sainsbury, P., Norris, P., Bugg, T. 2012. Isolation of bacterial strains able to metabolize lignin from screening of environmental samples. J. Appl. Microbiol., 113(3), 521-530.

Tomizawa, S., Chuah, J.-A., Matsumoto, K., Doi, Y., Numata, K. 2014. Understanding the limitations in the biosynthesis of polyhydroxyalkanoate (PHA) from lignin derivatives. ACS Sustain. Chem. Eng., 2(5), 1106-1113.

Wang, C., Yan, D., Li, Q., Sun, W., Xing, J. 2014. Ionic liquid pretreatment to increase succinic acid production from lignocellulosic biomass. Bioresour. Technol., 172, 283-289.

Wang, X., Lin, L., Dong, J., Ling, J., Wang, W., Wang, H., Zhang, Z., Yu, X. 2018. Simultaneous improvements of cell growth and polyhydroxyalkanoate production in *Pseudomonas* from a lignin derivative for lignin consolidated bioprocessing. Appl. Environ. Microbiol., AEM. 01469-18.

Xie, S., Sun, Q., Pu, Y., Lin, F., Sun, S., Wang, X., Ragauskas, A.J., Yuan, J.S. 2017. Advanced chemical design for efficient lignin bioconversion. ACS Sustain. Chem. Eng., 5(3), 2215-2223.

Xu, J., Cheng, J.J. 2011. Pretreatment of switchgrass for sugar production with the combination of sodium hydroxide and lime. Bioresour. Technol., 102(4), 3861-3868.

Zargartalebi, M., Kharrat, R., Barati, N. 2015. Enhancement of surfactant flooding performance by the use of silica nanoparticles. Fuel, 143, 21-27.

Zhao, C., Xie, S., Pu, Y., Zhang, R., Huang, F., Ragauskas, A.J., Yuan, J.S. 2016. Synergistic enzymatic and microbial lignin conversion. Green Chem., 18(5), 1306-1312.

CHAPTER V

5. Flow cytometry for quantitation of polyhydroxybutyrate production by *Cupriavidus necator* using alkaline pretreated liquor from corn stover

Abstract

Alkaline pretreated liquor (APL) from lignocellulosic feedstock pretreatment is a lignin-rich stream. Polyhydroxybutyrate (PHB), a type of biodegradable polymer was synthesized from APL by *Cupriavidus necator* DSM 545 and a high yield (2.1 g/L) was obtained in an earlier study (Chapter 4). It is of interest to monitor PHB production and cell enumeration from APL rapidly in the view of process control. However, APL has insoluble substances and is dark, which makes quantitation of cells through visible light absorbance difficult. Flow cytometry can count cells and quantitate PHB content through fluorescence intensity after fluorescence staining. However, the insoluble particles in APL may interfere with bacterial cells. A sample preparation method was developed using Nile Red staining and flow cytometry to quantify bacterial cells and PHB concentration. A linear relationship with good fitness (R^2=0.9939) was constructed to predict PHB concentration (0.2-2.1 g/L) based on fluorescence intensity acquired from a flow cytometer. A linear relationship (R^2=0.8614) between cell number and fluorescence intensity was also established. The good correlation between PHB concentration and fluorescence intensity indicates

the potential of applying flow cytometry for quantitation of PHB from APL and other media that is dark and contains insoluble particles.

5.1. Introduction

Lignocellulosic biomass biorefinery adds profit and promotes sustainability to the agricultural industry and rural economy. Multiple bioproducts (ethanol, butanol, succinic acid, lipids, enzymes) and their corresponding process design were studied for the past decades (Chen and Wan, 2017b; Ding et al., 2018; Ding et al., 2019; Faga et al., 2010; Frederick et al., 2016; Li et al., 2019a; Li et al., 2019b; Li et al., 2018a; Liu et al., 2015; Ramachandriya et al., 2013). Lignocellulosic feedstocks have a high carbon to nitrogen ratio (47-62:1) (Al-Kaisi and Guzman, 2013; Slininger et al., 2016), which makes them suitable substrates for unbalanced bacterial growth and accumulation of polyhydroxyalkonoate (PHA), which is a class of polymers bacteria produce for energy storage. One of the most common PHAs, poly-D-3-hydroxybutryate (PHB), is a biodegradable polymer that has mechanical properties comparable to many petroleum-based plastics. PHB is also widely applied in the pharmaceutical industry due to its biocompatibility. Commercial PHB produced by microbes is still 3 times more expensive than petroleum-based plastics (Aramvash et al., 2018). Raw materials make up about 50% of the production cost of PHB (Choi and Lee, 1999; Khanna and Srivastava, 2005; Mozumder et al., 2015). Conversion of inexpensive lignocellulosic feedstocks into PHB could decrease costs and increase sustainability of lignocellulosic bio-refineries.

Most research on lignocellulosic conversion into PHB has focused on sugars (Brodin et al., 2017; Cesario et al., 2014; Dai et al., 2015; Pan et al., 2012a; Raposo et al., 2017). Lignin comprises 15-30% of lignocellulosic biomass (Linger et al., 2014), but there are only a few studies

on lignin conversion into PHAs (Berezina et al., 2015; Li et al., 2019a; Linger et al., 2014; Liu et al., 2017; Si et al., 2018; Wang et al., 2018). The greatest PHA concentration observed in these studies was 2.1 g/L of PHB using *Cupriavidus necator* growing on alkaline pretreated liquor (APL) (Chapter 4). APL is a lignin-enriched streams accessible from lignocellulosic feedstock alkaline pretreatment (Salvachua et al., 2015). Other lignin sources for PHB production used in previous studies include residual lignin resulting from enzymatic hydrolysis of lignocellulosic biomass and Kraft lignin generated from the pulping and paper industry (Liu et al., 2017; Salvachua et al., 2015; Zakzeski et al., 2010). During lignin conversion into PHB by microbes, it is important to monitor rapidly bacterial cell accumulation and PHB production. Monitoring microbial cell growth through visible light absorbance is difficult since lignin is insoluble in water and their lignin suspensions are dark. For PHB production monitoring, the conventional method to quantify PHB is chromatographic analysis, which is time consuming and requires use of organic solvent to extract PHB from microbial cells. Fluorescence staining followed by flow cytometry has been studied for rapidly quantifying PHB production (Garcia-Torreiro et al., 2017; Kacmar et al., 2006; Linger et al., 2014; Zuriani et al., 2013). Flow cytometry can also be used for cell counting. Fluorescence increase has been used to confirm accumulation of PHA grown in APL (Ragauskas et al., 2014; Salvachua et al., 2015), but the APL fed to bacteria in these two studies were filtered either through 0.2 or 3 µm filters, which makes media much more clear than unfiltered APL and flow cytometry much easier. In commercial scale industry, it is highly likely that APL will be directly fed to bacteria without filtration for PHA/PHB production. To the best of the authors' knowledge, flow cytometry has not been applied to quantitate PHB produced from lignin or dark cultures with insoluble particles. Challenges are present because lignin suspensions contain insoluble substances, which may block the system of flow cytometry and interfere with bacterial cells.

Sample preparation to deal with the inference of large insoluble particles in APL has not been studied.

The objective of this study was to develop a sample preparation method and subsequent flow cytometry setup for quantitation of cell number and PHB produced from lignin. Cell culture containing PHB produced from APL by *C. necator* was used to develop the method. This study proposes the potential of applying flow cytometry to quantify *C. necator* cells and PHB concentration on bacteria growing on APL.

5.2 Materials and methods

5.2.1 Microorganism, inoculum and PHB production

C. necator DSM 545 in the form of dried pellets was purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). *C. necator* was revitalized and subcultured with seed medium (meat extract 3 g/L, meat peptone 5 g/L, glucose 30 g/L) for at least two generations until the OD reached 10 at 24 h. *C. necator* culture was conducted throughout the study at 32.5 °C at 250 rpm in 25 mL working medium in 250 mL Kimax baffled flasks with silicone sponge closure (Chemglass Life Sciences, New Jersey, USA) unless specified. *C. necator* culture of OD 10 was preserved at -80 °C with 30% glycerol in 2 mL aliquots and used for preculture preparation. One aliquot of *C. necator* culture was inoculated to seed medium and cultured for around 24 h to obtain late exponential phase culture with an optical density (OD) of 8-10. Then the culture was washed with sterile 0.89% sodium chloride solution twice and used as inoculum. Inoculum was added to each experimental flask to achieve an initial OD of 5.

5.2.2 PHB production from APL with supplements

APL was prepared as described in a previous study (chapter IV). Briefly, corn stover was pretreated with 0.07 g NaOH/g corn stover and a 10% (dry basis corn stover/alkaline solution) solid loading in a 1L bench top pressure reactor (Parr Reactor Model 4848, Parr Instrument Co., Moline, IL, USA). The operating conditions were agitating at 500 rpm agitation, heating to 140 °C and holding for 30 min. After the reaction, the reactor was cooled using an internal cooling coil. The liquid fractions of the slurries were separated from the solids under vacuum filtration using Whatman #4 filter paper. APL was prepared by neutralizing the liquid fractions to pH 6.8 with 5 M HCl and autoclaving at 121 °C for 20 min.

PHB production medium (20 mL) contained APL (5 mL) as the carbon source, $(NH_4)_2SO_4$ (0.1 g/L) as the nitrogen source, salt ($Na_2HPO_4 \cdot 2H_2O$, 2.9 g/L; KH_2PO_4, 1.5 g/L; $MgSO_4 \cdot 7H_2O$, 0.2 g/L; $CaCl_2 \cdot 2\ H_2O$: 0.01 g/L; $Fe(NH_4)$ citrate: 0.06 g/L) and trace elements (1 mL/L) as described previously in Ramsay et al. (1990) and and supplements. The $MgSO_4 \cdot 7H_2O$, $CaCl_2 \cdot 2\ H_2O$ and $Fe(NH_4)$ citrate solutions were autoclaved separately. PHB production was conducted at 32.5 °C at 250 rpm as described in Section 5.2.1, but with 20 mL working volume and cultivation was stopped at 7 days (168 h) for PHB quantification as described in Section 5.2.3. Five supplements included silica nanoparticle R816 (hereafter referred to as R816), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS), Tween 80, laccase and acryl alchohol oxidase (AAO) were added to the medium to enhance PHB production. For these supplements, R816 (Aerosil® R816, 12 nm average primary particle size) was donated by Evonik (Essen, Germany). Tween-80 was purchased from Acros Organics (Geel, Belgium). Laccase (L2157-10KU, 50 units/mg solids activity), ABTS were purchased from Sigma-Aldrich (St. Louis, MO, USA). AAO (535 U/L) was prepared as described in a previous study (chapter IV). PHB production was

conducted with varied supplements levels under a central composite design shown in Table 1 (chapter IV) and corresponding PHB production ranged from 0.22 to 2.12 g/L for 15 different samples.

For their usage, laccase and AAO were kept at 2 and 20 U/mL, respectively. Other three supplements were applied under a central composite design (Table 5.1). One sample from each run # in Table 5.1 were used for flow cytometry. These 15 samples covered a wide range of PHB concentrations. PHB quantification can be found in Section 5.2.3. Out of these 15 samples, 10 samples were chosen as training samples to build the calibration curve. For these 10 samples, their PHB concentrations have the following summary statistics (in g/L): mean 0.76, standard deviation of 0.58, median 0.53, maximum 2.12 and minimum 0.21. The other 5 samples were used as validation samples. Medium without inoculation was used as APL control samples.

5.2.3 PHB quantification by gas chromatography-mass spectrometer (GCMS)

PHB concentration was determined according to the methods described by Braunegg et al. (1978). Dried cell biomass was methanolyzed at 105 °C in acidified methanol (5% H_2SO_4, v/v, 2 mL) and chloroform (2 mL) mixture for 3 h. Sodium hydroxybutyrate (Sigma-Aldrich, MO, USA) was used as an external standard. After 3 h, 1 mL deionized water (Milli-Q, 18.2 MΩ cm) was added to the mixture and vortexed. The methyl-ester derivatives were extracted from the lower chloroform layer and passed through a column containing anhydrous sodium sulfate before being injected into a GC-MS.

The GC-MS was equipped with a Trace 1310 Gas Chromatograph, ISQ QD single quadrupole mass spectrometer (ThermoScientific, Waltham, MA, USA) and TG-5MS (30 m x 0.25 mm ID x 0.25 film) capillary column (ThermoFisher Scientific, Waltham, MA, USA). The

temperature profile was held at 60 °C for 5 min, increased to 220 °C at a rate of 30 °C/min and held at 220 °C for 5 min. The injector temperature was 230 °C, and the injection mode was splitless (1 µL). The pressure of the carrier gas (helium) was 142 kPa (18 psi). MS transfer line temperature was 250 °C; ion source temperature was 220 °C with electron ionization as ionization mode. The ionization energy was 70 eV and selective ion monitoring (SIM) mode was used. Methylated methyl-ester derivative was confirmed with SIM at 43, 71, and 74. Solvent delay was 3 min. Data were analyzed with Chromeleon 7 chromatography studio (ThermoFisher Scientific, Waltham, MA, USA).

5.2.4 Fluorescence microscopy

Before flow cytometry, fluorescent microscopy (EVOS™ FL Auto Imaging System, ThermoFisher) was used to confirm PHB accumulation in cells. The procedure for sample preparation was as follows. The cell culture having 2.12 g PHB/L was used. One mL cell culture was subjected to filtration using a 3 µm PTFE syringe filter (Tisch Scientific, Ohio, USA) to remove large insoluble particles of APL in cell culture. Filtered cell culture was centrifuged at 17,000 rpm for 3 min and washed with 10 mM phosphate buffered saline (PBS) twice. Washed cell pellets were dispersed in 100 µL PBS and 3 µL Nile Red stock solution (1 mg Nile Red/mL dimethyl sulfoxide (DMSO)) was added to the cell suspension for staining. The mixture was vortexed and incubated in the dark at room temperature for 30 min. After incubation, Nile Red stained cells were washed twice with PBS. Washed cells were dispersed in 100 µL PBS and subjected to image acquisition with a red fluorescent protein (RFP) light cube and 100× oil immersion objective. Red was chosen to be the pseudo color.

5.2.5 Flow cytometry

A flow cytometer (FACS Aria II, BD Biosciences, San Jose, CA) with a 488 nm wavelength laser coupled with 610/20 nm detection was utilized to measure the fluorescence intensity after staining. The flow cytometry signals (side scatter vs. forward scatter and side scatter vs. fluoresence intensity) were collcted. Cell cultures were subjected to the same procedure (filtration, washing, Nile Red staining and subsequent washing) as described in Section 5.2.4. To study the effect of Nile Red loading on fluorescence intensity, cell culture containing 2.1 g/L PHB was used. Instead of using only 3 µL Nile Red stock solution in Section 5.2.4, different loadings (1, 3, 5, 7 and 10 µL) of Nile Red stock solutions were added to the 100 µL cell suspension. The 100 µL cell suspension was added to 1 mL PBS and the mixture was loaded into the flow cytometer. The Nile Red loading leading to the highest fluorescence intensity was used to stain all 15 samples in Section 5.2.2 for building the calibration curve between PHB concentration and fluorescence intensity and testing the model performance.

When performing flow cytometry, data recording was stopped at 1 min for each sample and the flow rate was selected at the level “medium”. Collected signals were processed by FACSDiva software (BD Biosciences, San Jose, CA) to generate forward and side scatter and mean of fluorescence in arbitrary units (reported as fluorescence intensity). The fluorescence intensity is the mean of all of the cells in a sample. The regions of bacterial cells and Nile Red stained cells were determined by removing the signals of all control samples (PBS, Nile Red stained APL control samples, Nile Red non-stained cells + APL samples). Nile Red stained APL control samples were prepared with the same staining procedure as the samples applied on APL control samples. Nile Red non-stained cells+APL samples are the cell culture without Nile Red staining.

The signals showing fluorescence intensity were also reported as Nile Red^{+} events. Beads of size 5 µm (ACFP-50-5, Spherotech Inc, IL, USA) with known number (10^6 particles/mL) were added with the cell culture and cell number (denoted as Nile Red^{+} Cell) was calculated based on Equation 5.1.

Nile Red^{+} Cell # = (Nile Red^{+} events #/beads #) * (beads concentration/beads solution volume) (5.1)

where Nile Red^{+} events and beads # were acquired from flow cytometry, beads concentration=10^6 particles/mL, beads solution volume=25 µL.

5.2.6 Statistical analyses

All experiments were run in duplicate. Analysis of variance (ANOVA) was implemented using GLM procedure in SAS 9.3 (SAS Institute Inc., Cary, NC). Multiple mean comparisons were performed using Tukey's test using *p*-values <0.05 as statistically significant.

5.3 Results and discussion

5.3.1 PHB production

To the best of the authors' knowledge, PHB production of 2.1 g/L was the highest among all studies producing PHB/PHA from APL (Li et al., 2019a; Linger et al., 2014; Liu et al., 2017; Si et al., 2018). PHB production ranging from 0.2 to 2.1 is a wide range based on previous research (Li et al., 2019a; Linger et al., 2014; Liu et al., 2017; Si et al., 2018). The proposed process for APL conversion to PHB from corn stover is illustrated in Fig. 5.1. After 3 µm filtration and centrifugation, white cell pellets can be clearly seen while there were still some lignin particles

(black substances) in the bottom of the right centrifuge tube (Fig. 5.1). The washed pellets were used for Nile Red staining and flow cytometry.

5.3.2 Fluorescence microscopy

C. necator cells stained with Nile Red are shown in Fig. 5.2. Cells showed rod shapes. Transmitted light image overlaying with fluorescent image showed how PHB granules distributed inside bacterial cells. It was noticeable that *C. necator* cells formed different clusters (green circles in Fig. 5.2A) and different brightness (Fig. 5.2B). Red spots of different sizes and shapes inside one bacterial cell were believed to be caused by different sizes of PHB granules. The larger PHB granules are, the more likely that granules picked up more Nile Red and showed a brighter red color.

5.3.3 Flow cytometry

Flouresence intensity changing with different Nile Red loading (1, 3, 5, 7, and 10 µL stocking solution) is shown in Fig. 5.3. Nile Red loading of 3 µL showed much higher fluorescence intensity than Nile Red loading of 1 µL (p-value < 0.05). When increasing Nile Red loading to 5, 7 and 10 µL, the fluorescence intensities among these loadings were not significantly different from the intensity from 3 µL (p-value > 0.05). Therefore, 3 µL Nile Red loading was chosen for cell staining and subsequent flow cytometry.

The flow cytometry signals (side scatter vs. forward scatter and side scatter vs. fluoresence intensity) of different samples are shown in Fig. 5.4. In Fig. 5.4, side scatter signals, forward scatter signals and fluoresence intensity are denoted as SSC-A, FSC-A and NR-A, respectively. Side scatter signal reflect the morphology of cells. The more irregular the cells are, the greater the side scatter signal will be. For example, irregular cells could refer to the clusters of multiple cells in

Fig. 5.2. Forward scatter signals reflect the size of the cells. The larger the cells are, the higher forward scatter signal will be. Fluoresence intensity was used to correlate with the Nile Red stained PHB in the cell culture. Higher fluoresence intensity means more Nile Red was detected and more PHB absorbed Nile Red. However, particles other than Nile Red stained cells may also exhibit fluoresence. Therefore, it is important to select regions of interest to exclude the signals of paticles in PBS and APL.

Flow cytometry was applied to control samples including PBS, Nile Red non-stained cells+APL, and Nile Red stained APL control samples and as described in Section 5.2.5 to exclude the signals of non Nile Red stained cells. Sample having 2.12 g/L PHB was used as the example shown in Fig. 5.4. Based on the side and forward scatter signals (SSC-A and FSC-A), the selected region for all particles excluding particles in deionized water was shown in the oval shape in the "1" plots in Fig. 5.4. Based on side scatter signals (SSC-A) and fluoresence intensity (NR-A), the selected region for Nile Red stained cells was shown in the trapezoid shape in the "2" plots in Fig. 5.4.

In Fig. 5.4A, salt particles in PBS solution produced some signals in both side scatter vs. forward scatter and side scatter vs. fluorescence intensity plots. Most signals (99.2%) appear outside the region in Fig. 5.4A-2, meaning that 99.2% particles in PBS were excluded in the selected region. Fig. 5.4B shows the signals of Nile Red non-stained cells + APL samples. In Fig. 5.4B-1, Nile Red non-stained cells and APL showed a wide range of sizes and morphology. In Fig. 5.4B-2, there were only 0.01% events in the selected region. Therefore, in Figs. 5.4A-2 and 5.4B-2, particles in the region can be neglected. In Fig. 5.4B, beads of size 5 μm were shown on the right top corner of the plot and they were separated well from the cells.

As mentioned in Section 5.3.1, there were still some lignin particles in the samples after 3 μm filtration. It was important to see how these lignin particles interfered with Nile Red stained cells. In Fig. 5.4C-1, lignin particles in APL showed a wide range of sizes and morphology. There were fluorescence intensity signals for Nile Red stained APL (Fig. 5.4C-2). This was expected because lignin was previously reported to exhibit fluorescence (De Micco and Aronne, 2007; Kaliyan and Morey, 2010). In Fig. 5.4C-2, there were some lignin particles in the selected region for Nile Red stained APL control samples, but the number of events only account for 5.6% of the total 2780 events, meaning 94.4% of the lignin particles are outside the region. In Fig. 5.4D, signals of Nile Red stained cells were shown. Nile Red^+ cells and other particles overlapped in Fig. 5.4D-1, but separated well in Fig. 5.4D-2 by the selected region. There is a clear boundary between Nile Red^+ cells and other particles in Fig. 5.4D-2. Inside the regions, there are some lignin particles based on Fig. 5.4C-2. However, Nile Red^+ cells account for 43.3% of a total of 21, 244 events. After calculation, there is only around 2% ((2780 × 5.65%) / (21,244 × 43.3%) = 1.7%) lignin particles in the selected region in Fig. 5.4D-2. Therefore, we believe the region in Fig. 5.4D-2 separates Nile Red^+ cells well from other particles.

Two distinct clusters of Nile Red^+ signals were exhibited for some cultures. One of the examples is shown in Fig. 5.5B of which the PHB concentration is 0.7 g/L. These two regions indicate two different fluoresence intensities. These two distinct clusters can be caused by the different sizes of PHB granules inside cells. The presence of cell clusters (circle in Fig. 5.2A) and single cells in Fig. 5.2 could also be the reason of two distict regions of Nile Red^+ signals in Fig. 5.5.

The linear relationship between fluorescence intensity and PHB concentration showed a R^2 of 0.9939 for the calibration data (Fig. 5.6A). This R^2 value indicated good correlation between

experimental and predicted values of the response (Guan and Yao, 2008; Joglekar and May, 1987). The mean square error (MSE) for validation dataset is 0.0273. Low MSE implies the linear equation can predict PHB concentration accurately from fluorescense intensity. There is also a linear relationship between fluorescence intensity and cell number (Fig. 5.6B); however, the R^2 value of 0.8614 was lower than for the relationship between fluorescence intensity and PHB concentration (Fig. 5.6B). This probably caused by cells having varying PHB content. Therefore, monitoring fluorescence intensity to predict PHB concentration is more accurate than using it to predict cell number.

5.4 Conclusion

In this study, fluorescence microscopy using Nile Red staining confirmed PHB accumulation inside *C. necator* cells. Cell number was counted using 5 µm beads and PHB concentration was calculated based on fluorescence intensity. The developed sample preparation method worked well and a linear relationship with good fitness (R^2=0.9939) was constructed to predict PHB production from fluorescence intensity. The good correlation for a wide range of PHB concentrations indicates the potential of applying flow cytometry for PHB concentration measurement. For next-step research, the approach described in this study should be tested for monitoring real time PHB production from inoculation to the end of fermentation from APL culture by *C. necator*, and adapted to test PHB or other copolymers ([P(3HB-co-4HB), [P(3HB-co-3HV)], and [P(3HB-co-3HV-co-4HB)]), etc.) production from other media that are dark and contain insolubles.

Table 5.1. Supplements (R816, ABTS and Tween 80) formula under a central composite design structure for PHB production

Run #	Factors			Response
	R816 (m/v, %)	ABTS (mM)	Tween 80 (v/v, %)	PHB (g/L)
1	0.015 (-1)	4 (-1)	1.5 (-1)	**0.2605**
2	0.025 (1)	4 (-1)	1.5 (-1)	0.2215
3	0.015 (-1)	6 (1)	1.5 (-1)	**0.5587**
4	0.025 (1)	6 (1)	1.5 (-1)	0.5092
5	0.015 (-1)	4 (-1)	2.5 (1)	0.4292
6	0.025 (1)	4 (-1)	2.5 (1)	**0.281**
7	0.015 (-1)	6 (1)	2.5 (1)	**1.1081**
8	0.025 (1)	6 (1)	2.5 (1)	1.3961
9	0.0116 (-1.682)	5 (0)	2 (0)	0.66
10	0.0284 (1.682)	5 (0)	2 (0)	**0.4905**
11	0.02 (0)	3.318 (-1.682)	2 (0)	**0.2408**
12	0.02 (0)	6.682 (1.682)	2 (0)	0.7392
13	0.02 (0)	5 (0)	1.159 (-1.682)	0.2866
14	0.02 (0)	5 (0)	2.841 (1.682)	0.9852
15	0.02 (0)	5 (0)	2 (0)	2.12

Note: 3 factors were shown as real values (coded levels). PHB production values are one sample randomly selected from three replicates in each run. The values in bold are randomly chosen as validation samples and other 10 samples were training samples to build calibration curves. Laccase and AAO were kept at laccase 2 U/mL, AAO 20 U/mL, respectively.

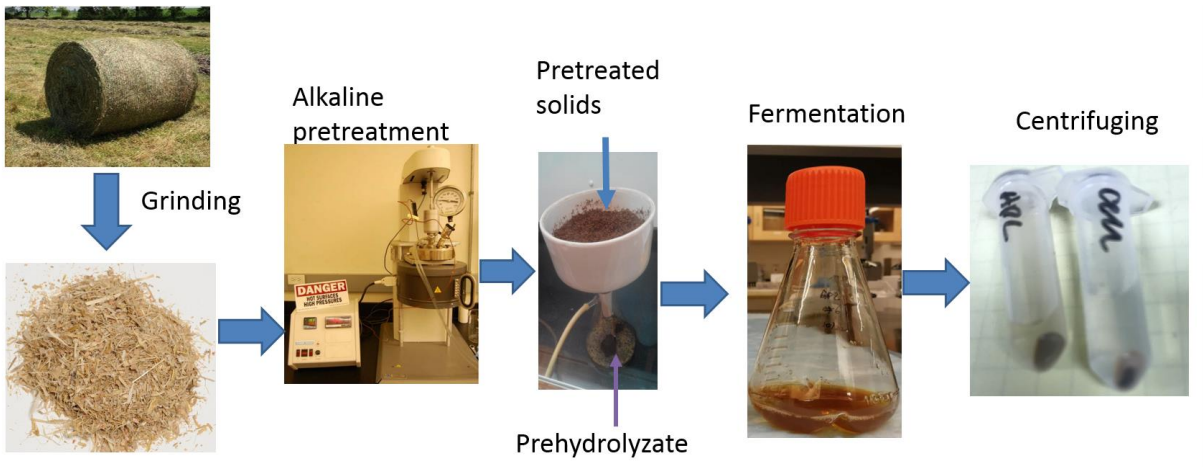

Fig. 5.1 Process of APL conversion to PHB from corn stover.

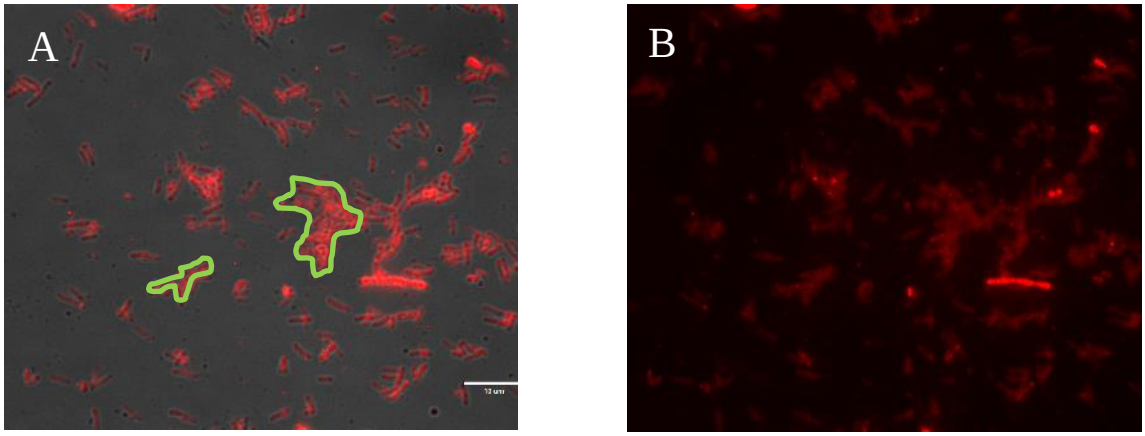

Fig. 5.2 Nile Red stained *C. necator* cells containing PHB under fluorescence microscopy. A: transmitted light image overlaying with fluorescent image, circle in green are clusters of multiple cells; B, fluorescent image.

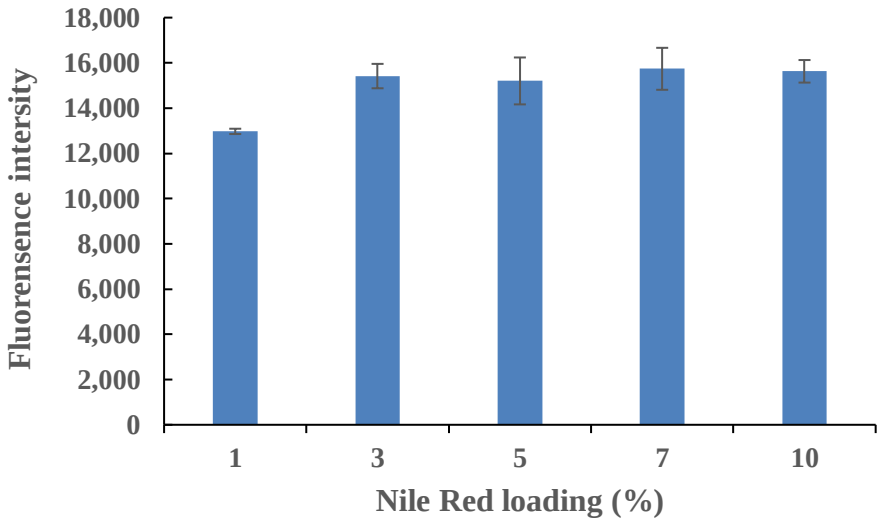

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Fig. 5.3 The effect of Nile Red loading on fluorescence intensity of the stained cell culture containing 2.1 g/L PHB. Data expressed were the mean values and standard deviations of two replicates.

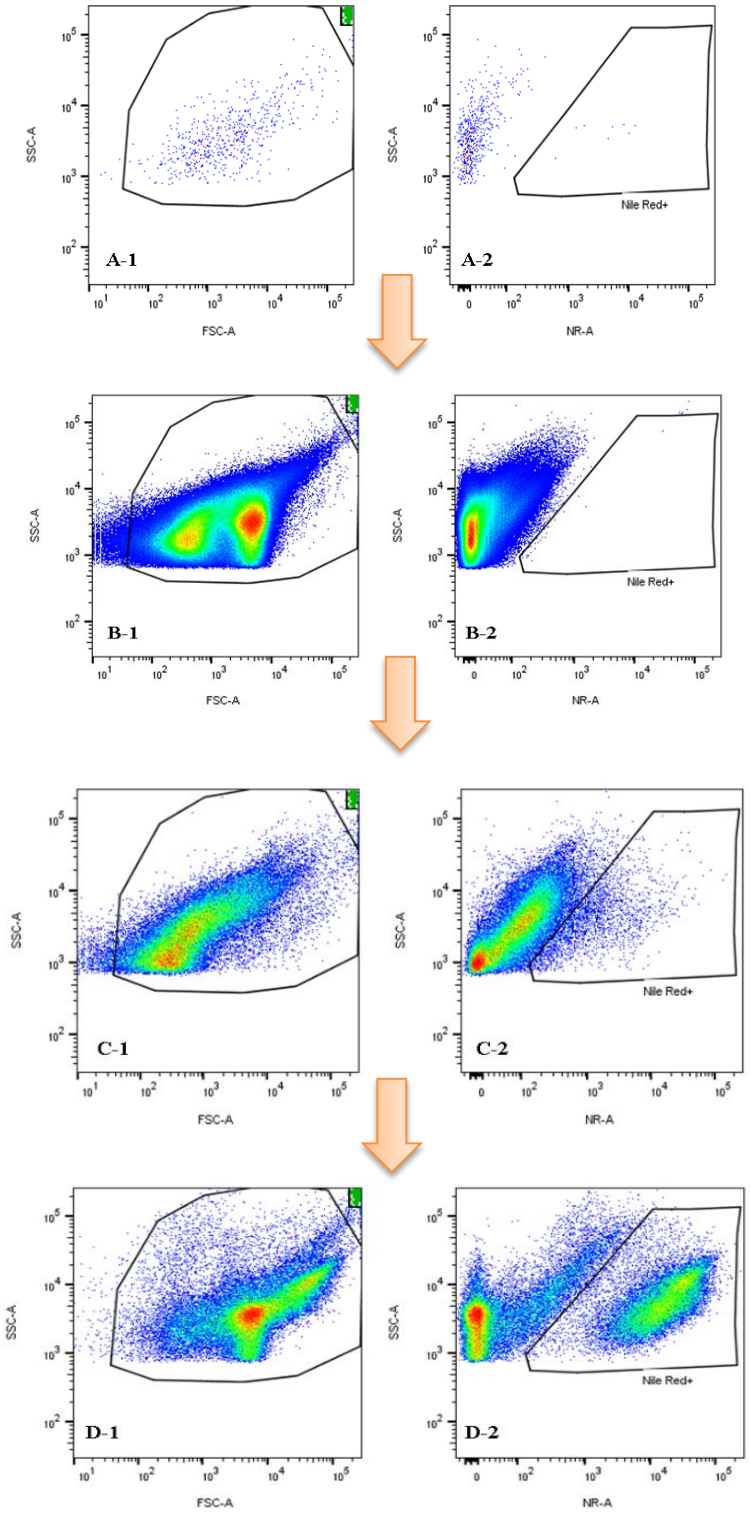

Fig. 5.4 An example to show how to select the region for Nile Red stained bacterial cells. A: PBS, B: cells, C: Nile Red stained APL, D: Nile Red stained cells. In each subplot (A-D), 1 represents side scatter vs. forward scatter, 2 represents side scatter vs. fluorescence intensity.

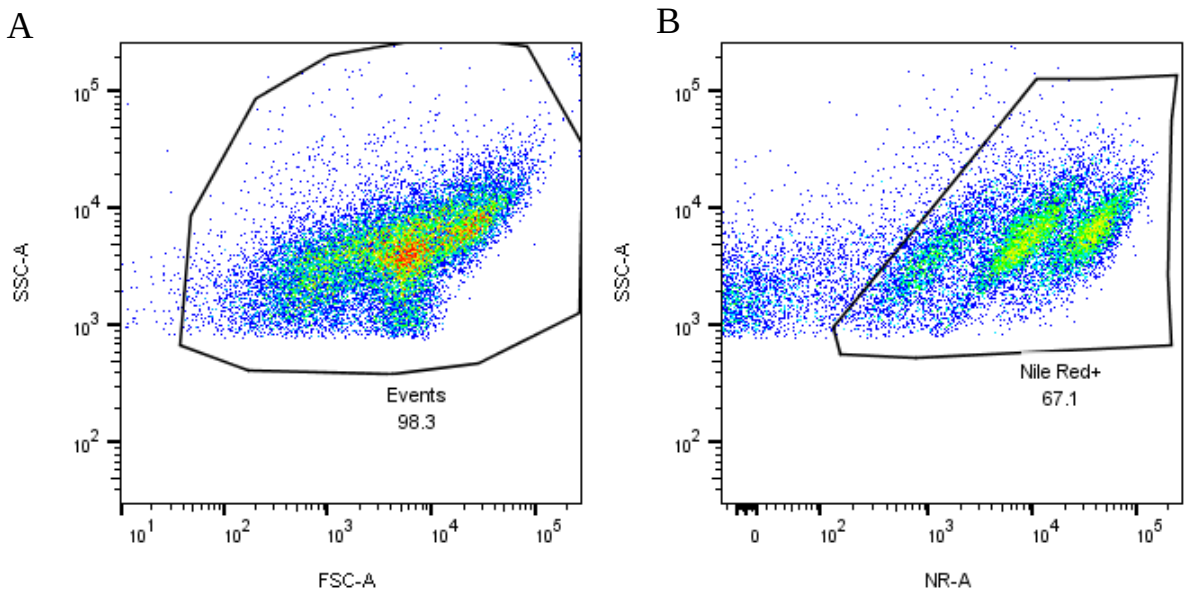

Fig. 5.5 Two portions of Nile Red indicating different sizes of PHB granules inside bacterial cell. A, side scatter vs. forward scatter and B, side scatter vs. fluorescence intensity.

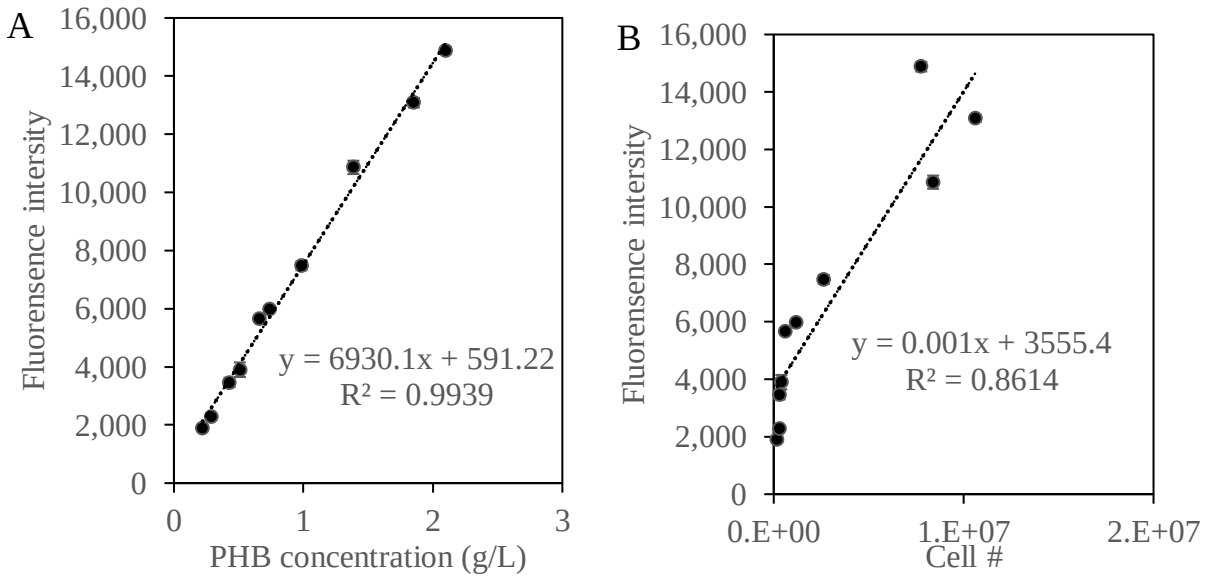

Fig. 5.6 Linear correlation between fluorescence intensity and PHB concentration (A) and between fluorescence intensity and cell number (B). Data expressed were the mean values and standard deviations of two replicates.

5.5 References

Al-Kaisi, M.M., Guzman, J.G. 2013. Effects of tillage and nitrogen rate on decomposition of transgenic Bt and near-isogenic non-Bt maize residue. Soil Tillage Res., 129, 32-39.

Aramvash, A., Moazzeni Zavareh, F., Gholami Banadkuki, N. 2018. Comparison of different solvents for extraction of polyhydroxybutyrate from *Cupriavidus necator*. Eng. Life Sci., 18(1), 20-28.

Berezina, N., Yada, B., Lefebvre, R. 2015. From organic pollutants to bioplastics: insights into the bioremediation of aromatic compounds by *Cupriavidus necator*. New Biotechnol., 32(1), 47-53.

Braunegg, G., Sonnleitner, B., Lafferty, R. 1978. A rapid gas chromatographic method for the determination of poly-β-hydroxybutyric acid in microbial biomass. Eur. J. Appl. Microbiol. Biotechnol., 6(1), 29-37.

Brodin, M., Vallejos, M., Opedal, M.T., Area, M.C., Chinga-Carrasco, G. 2017. Lignocellulosics as sustainable resources for production of bioplastics–A review. J. Clean. Prod., 162, 646-664.

Cesario, M.T., Raposo, R.S., De Almeida, M.C.M., Van Keulen, F., Ferreira, B.S., Da Fonseca, M.M.R. 2014. Enhanced bioproduction of poly-3-hydroxybutyrate from wheat straw lignocellulosic hydrolysates. New Biotechnol., 31(1), 104-113.

Chen, Z., Wan, C. 2017b. Co-fermentation of lignocellulose-based glucose and inhibitory compounds for lipid synthesis by *Rhodococcus jostii* RHA1. Process Biochem., 57, 159-166.

Choi, J., Lee, S.Y. 1999. Factors affecting the economics of polyhydroxyalkanoate production by bacterial fermentation. Appl. Microbiol. Biotechnol., 51(1), 13-21.

Dai, J., Gliniewicz, K., Settles, M.L., Coats, E.R., McDonald, A.G. 2015. Influence of organic loading rate and solid retention time on polyhydroxybutyrate production from hybrid poplar hydrolysates using mixed microbial cultures. Bioresour. Technol., 175, 23-33.

De Micco, V., Aronne, G. 2007. Combined histochemistry and autofluorescence for identifying lignin distribution in cell walls. Biotech Histochem, 82(4-5), 209-216.

Ding, C., Li, M., Hu, Y. 2018. High-activity production of xylanase by *Pichia stipitis*: Purification, characterization, kinetic evaluation and xylooligosaccharides production. Int. J. Biol. Macromol., 117, 72-77.

Ding, C., Wang, X., Li, M. 2019. Evaluation of six white-rot fungal pretreatments on corn stover for the production of cellulolytic and ligninolytic enzymes, reducing sugars, and ethanol. Appl. Microbiol. Biotechnol., 1-12.

Faga, B.A., Wilkins, M.R., Banat, I.M. 2010. Ethanol production through simultaneous saccharification and fermentation of switchgrass using *Saccharomyces cerevisiae* D5A and thermotolerant *Kluyveromyces marxianus* IMB strains. Bioresour. Technol., 101(7), 2273-2279.

Frederick, N., Li, M., Carrier, D.J., Buser, M.D., Wilkins, M.R. 2016. Switchgrass storage effects on the recovery of carbohydrates after liquid hot water pretreatment and enzymatic hydrolysis. AIMS Bioeng., 3(3), 389-399.

Garcia-Torreiro, M., Lopez-Abelairas, M., Lu-Chau, T.A., Lema, J.M. 2017. Application of flow cytometry for monitoring the production of poly (3-hydroxybutyrate) by *Halomonas boliviensis*. Biotechnol. Prog., 33(2), 276-284.

Guan, X., Yao, H. 2008. Optimization of Viscozyme L-assisted extraction of oat bran protein using response surface methodology. Food Chem., 106(1), 345-351.

Joglekar, A., May, A. 1987. Product excellence through design of experiments. Cereal Food World, 32(12), 857.

Kacmar, J., Carlson, R., Balogh, S.J., Srienc, F. 2006. Staining and quantification of poly‐3‐hydroxybutyrate in *Saccharomyces cerevisiae* and *Cupriavidus necator* cell populations using automated flow cytometry. Cytometry A, 69(1), 27-35.

Kaliyan, N., Morey, R.V. 2010. Natural binders and solid bridge type binding mechanisms in briquettes and pellets made from corn stover and switchgrass. Bioresour. Technol., 101(3), 1082-1090.

Khanna, S., Srivastava, A.K. 2005. Recent advances in microbial polyhydroxyalkanoates. Process Biochem., 40(2), 607-619.

Li, M., Eskridge, K., Liu, E., Wilkins, M. 2019a. Enhancement of polyhydroxybutyrate (PHB) production by 10-fold from alkaline pretreatment liquor with an oxidative enzyme-mediator-surfactant system under Plackett-Burman and central composite designs. Bioresour. Technol., 281, 99-106.

Li, M., Eskridge, K.M., Wilkins, M.R. 2019b. Optimization of polyhydroxybutyrate production by experimental design of combined ternary mixture (glucose, xylose and arabinose) and process variables (sugar concentration, molar C: N ratio). Bioprocess Biosystems Eng., 1-12.

Li, M., Marek, S.M., Peng, J., Liu, Z., Wilkins, M.R. 2018a. Effect of moisture content and inoculum size on cell wall composition and ethanol yield from switchgrass after solid-state *Pleurotus ostreatus* treatment. Trans. ASABE, 61(6), 1997-2006.

Linger, J.G., Vardon, D.R., Guarnieri, M.T., Karp, E.M., Hunsinger, G.B., Franden, M.A., Johnson, C.W., Chupka, G., Strathmann, T.J., Pienkos, P.T. 2014. Lignin valorization through integrated biological funneling and chemical catalysis. Proc. Natl. Acad. Sci., 111(33), 12013-12018.

Liu, K., Atiyeh, H.K., Pardo-Planas, O., Ezeji, T.C., Ujor, V., Overton, J.C., Berning, K., Wilkins, M.R., Tanner, R.S. 2015. Butanol production from hydrothermolysis-pretreated switchgrass: quantification of inhibitors and detoxification of hydrolyzate. Bioresour. Technol., 189, 292-301.

Liu, Z.-H., Olson, M.L., Shinde, S., Wang, X., Hao, N., Yoo, C.G., Bhagia, S., Dunlap, J.R., Pu, Y., Kao, K.C. 2017. Synergistic maximization of the carbohydrate output and lignin processability by combinatorial pretreatment. Green Chem., 19(20), 4939-4955.

Mozumder, M.S.I., Garcia-Gonzalez, L., De Wever, H., Volcke, E.I.P. 2015. Effect of sodium accumulation on heterotrophic growth and polyhydroxybutyrate (PHB) production by *Cupriavidus necator*. Bioresour. Technol., 191, 213-218.

Pan, W., Nomura, C.T., Nakas, J.P. 2012a. Estimation of inhibitory effects of hemicellulosic wood hydrolysate inhibitors on PHA production by *Burkholderia cepacia* ATCC 17759 using response surface methodology. Bioresour. Technol., 125, 275-282.

Ragauskas, A.J., Beckham, G.T., Biddy, M.J., Chandra, R., Chen, F., Davis, M.F., Davison, B.H., Dixon, R.A., Gilna, P., Keller, M. 2014. Lignin valorization: improving lignin processing in the biorefinery. Science, 344(6185), 1246843.

Ramachandriya, K.D., Wilkins, M., Atiyeh, H.K., Dunford, N.T., Hiziroglu, S. 2013. Effect of high dry solids loading on enzymatic hydrolysis of acid bisulfite pretreated Eastern redcedar. Bioresour. Technol., 147, 168-176.

Ramsay, B., Lomaliza, K., Chavarie, C., Dube, B., Bataille, P., Ramsay, J. 1990. Production of poly-(beta-hydroxybutyric-co-beta-hydroxyvaleric) acids. Appl. Environ. Microbiol., 56(7), 2093-2098.

Raposo, R.S., De Almeida, M.C.M., De Oliveira, M.d.C.M., Da Fonseca, M.M., Cesario, M.T. 2017. A *Burkholderia sacchari* cell factory: production of poly-3-hydroxybutyrate, xylitol and xylonic acid from xylose-rich sugar mixtures. New Biotechnol., 34, 12-22.

Salvachua, D., Karp, E.M., Nimlos, C.T., Vardon, D.R., Beckham, G.T. 2015. Towards lignin consolidated bioprocessing: simultaneous lignin depolymerization and product generation by bacteria. Green Chem., 17(11), 4951-4967.

Si, M., Yan, X., Liu, M., Shi, M., Wang, Z., Wang, S., Zhang, J., Gao, C., Chai, L., Shi, Y. 2018. In situ lignin bioconversion promotes complete carbohydrate conversion of rice straw by *Cupriavidus basilensis* B-8. ACS Sustain. Chem. Eng., 6(6), 7969-7978.

Slininger, P.J., Dien, B.S., Kurtzman, C.P., Moser, B.R., Bakota, E.L., Thompson, S.R., O'Bryan, P.J., Cotta, M.A., Balan, V., Jin, M. 2016. Comparative lipid production by oleaginous yeasts in hydrolyzates of lignocellulosic biomass and process strategy for high titers. Biotechnol. Bioeng., 113(8), 1676-1690.

Wang, X., Lin, L., Dong, J., Ling, J., Wang, W., Wang, H., Zhang, Z., Yu, X. 2018. Simultaneous improvements of cell growth and polyhydroxyalkanoate production in *Pseudomonas* from a lignin derivative for lignin consolidated bioprocessing. Appl. Environ. Microbiol., AEM. 01469-18.

Zakzeski, J., Bruijnincx, P.C., Jongerius, A.L., Weckhuysen, B.M. 2010. The catalytic valorization of lignin for the production of renewable chemicals. Chem. Rev., 110(6), 3552-3599.

Zuriani, R., Vigneswari, S., Azizan, M., Majid, M., Amirul, A. 2013. A high throughput Nile red fluorescence method for rapid quantification of intracellular bacterial polyhydroxyalkanoates. Biotechnol. Bioproc. Eng., 18(3), 472-478.

CHAPTER VI

6. Fed-batch polyhydroxybutyrate production from simulated corn fiber sugar mixtures

Abstract

Corn fiber, one of the main byproducts from the corn processing industry, is a potential raw material for production of valuable products. Polyhydroxybutyrate (PHB) is a biodegradable bioplastic that is comparable with many petroleum based plastics in terms of mechanical properties and is highly biocompatible. Corn fiber conversion into PHB could make corn biorefineries more profitable and sustainable. Glucose, xylose and arabinose are the main monomer sugars derived from upstream corn fiber processing. *Burkholderia sacchari* DSM 17165 is a bacterium strain that can convert all three sugars into PHB. Sugar streams from upstream corn fiber processing can have various sugar mixture ratios depending on pretreatment and enzymatic hydrolysis conditions. In this study, fed-batch mode was applied to produce PHB on three simulated corn fiber sugar mixtures (glucose:xylose:arabinose=4:2:1, 2:2:1, 1:2:1). The highest PHB concentration produced was 67 g/L for 4:2:1 mixture at 41 h corresponding to an accumulation of 77% of cell dry weight as PHB. Corresponding sugar conversion efficiency and productivity were 0.33 g PHB/g sugar consumed and 1.6 g/L/h, respectively. Results provide references for process control to maximize PHB production from real sugar streams derived from corn fiber.

6.1. Introduction

Multiple valuable bioproducts (ethanol, butanol, succinic acid, lipids, enzymes and polyhydroxybutyrate) and their corresponding process designs using various cellulosic feedstocks have been studied for the past decade (Chen and Wan, 2017b; Ding et al., 2018; Ding et al., 2019; Faga et al., 2010; Frederick et al., 2016; Li et al., 2019a; Li et al., 2019b; Li et al., 2018a; Liu et al., 2015; Ramachandriya et al., 2013). These bioproducts have potential to add profit and promote sustainability in the agricultural industry and rural economy. One potential cellulosic feedstock that can be used to produce the products mentioned above is corn fiber. Corn fiber constitutes ~10% of the corn (Van Eylen et al., 2011) and has low value, as its use is limited to animal feeds, primarily for ruminant animals (Rausch and Belyea, 2006). Polyhydroxybutyrate (PHB) is a polymer of particular interest due to its comparable mechanical properties to many petroleum-based plastics and its high biocompatibility. Commercial PHB produced by microbes is still 3 times more expensive than petroleum-based plastics (Aramvash et al., 2018). Raw materials make up about 50% of the production cost of PHB (Choi and Lee, 1999; Khanna and Srivastava, 2005; Mozumder et al., 2015). Corn fibers as raw materials for PHB production exhibit a few advantages. Corn fibers are inexpensive cellulosic feedstocks, and contain a small amount of lignin (<10%) (Schell et al., 2004). Corn fiber conversion into PHB can be incorporated into corn biorefinery without considering the logistic costs of raw materials supply. Therefore, corn fiber conversion into PHB could increase the value of corn crops, decrease costs of PHB production, and promote sustainability of corn biorefineries.

Typical corn fiber contains 30-50% arabinoxylan and 15-20% cellulose (Gaspar et al., 2005). After upstream processing of corn fiber, a sugar stream consisting of monomer sugars including glucose, xylose, galactose, arabinose and mannose is derived, with glucose, xylose and

arabinose being the main sugars present (Bischoff et al., 2010; Gaspar et al., 2007; Yoo et al., 2016). The ratios of glucose, xylose and arabinose vary depending on upstream pretreatment conditions. Three representative sugar mixture ratios are shown in Table 6.1. *Burkholderida sacchari* DSM 17165 is capable of converting glucose, xylose and arabinose into PHB (Bramer et al., 2001; Cesario et al., 2014). An earlier study (Chapter 3) has optimized the sugar mixture ratio for maximizing PHB production by *B. sacchari* DSM 17165 at shake flask scale. Previous studies have studied PHB production from wheat straw sugar mixture and xylose rich sugar mixtures at bioreactor scale (Cesario et al., 2014; Raposo et al., 2017). However, bioreactor scale production of PHB from corn fiber derived sugar streams was not studied before.

In this study, PHB production from three types of simulated sugar mixture (glucose:xylose:arabinose=4:2:1, 2:2:1 and 1:2:1) were demonstrated at bioreactor scale (3 L) under fed-batch mode. The ratio 4:2:1 mimics the sugar mixture from 2% NaOH pretreated corn fiber (Table 6.1), representing glucose-rich sugar mixture. The ratio 2:2:1 mimics the sugar mixture from untreated corn fiber (Table 6.1), representing sugar mixture that has similar concentrations of glucose and xylose. The ratio 1:2:1 mimics the sugar mixture from 1% H_2SO_4 (Table 6.1), representing xylose-rich sugar mixture. For these three sugar mixtures, arabinose concentration is half of xylose concentration. Cell and PHB production, cell and PHB productivity, and sugar conversion efficiency into cells and PHB were evaluated. To the author's best knowledge, this study is the first to conduct fed-batch PHB cultivation from simulated corn fiber sugar mixtures at bioreactor scale. The approach in this study may be extended for process control to maximize PHB production from real sugar streams derived from corn fiber.

6.2 Materials and methods

6.2.1 Microorganism and inoculum

B. sacchari DSM 17165 in the form of dried pellets was purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). *B. sacchari* DSM 17165 was revitalized and sub-cultured with seed medium (meat extract 3 g/L, meat peptone 5 g/L, glucose 30 g/L) for at least two generations until the OD reached 10 at 24 h. *B. sacchari* culture was conducted at 29 °C at 250 rpm in 20 mL working medium in 250 mL Kimax baffled flasks with silicone sponge closure throughout the study unless specified. *B. sacchari* culture of OD 10 was preserved at -80 °C with 30% glycerol in 2 mL aliquots and used for pre-culture preparation. One aliquot of *B. sacchari* culture was inoculated to seed medium and *B. sacchari* was cultured to obtain late exponential phase culture with an optical density (OD) of 8-10. Then, *B. sacchari* culture was washed with sterile 0.89% NaCl solution twice and used as inoculum. Four flasks of seed culture were combined in 30 mL sterile 0.89% NaCl solution as the inoculum to initiate bioreactor production of PHB. The initial OD was around 0.5 in the bioreactor.

6.2.2 PHB production under fed-batch mode

The initial medium composition for the fed-batch culture was (per L): sugar mixture, 40 g; yeast extract: 1.2 g; $(NH_4)_2SO_4$, 4.0 g; KH_2PO_4, 3.0 g; $CaCl_2 \cdot 2H_2O$: 0.03; EDTA, 0.04 mg; $MgSO_4 \cdot 7H_2O$, 1.25 g, Fe(NH_4)citrate: 0.36 g and trace elements solution, 20 mL, antifoam: 0.1 mL. The trace elements solution has the following composition (per L) (Kim et al., 1994): H_3BO_3, 0.3 g; $Na_2MO_4 \cdot 2H_2O$, 2.25 g; $MnCl_2 \cdot 4H_2O$, 2.25 g; $MnSO_4 \cdot 5H_2O$, 0.5 g; $CaC1_2$. $2H_2O$, 2 g; $Na_2B_4O_7 \cdot 10H_2O$, 0.23 g; $(NH_4)_6Mo_7O_{24}$, 0.1 g; 35% HC1 10 mL. The initial sugar mixtures contained glucose, xylose and arabinose in the ratio of 4:2:1, 2:2:1 and 1:2:1, respectively for three

types of simulated corn fiber sugar mixtures. Sugar mixture was sterilized with 0.2 μm Nalgene® Rapid-Flow™ filter units (Rochester, NY, USA). $CaCl_2$, $MgSO_4$, and trace elements are autoclaved separately. The pH was adjusted to 6.8 with NH_4OH (5 N). 30 mL inoculum described in Section 6.2.1 was added into the bioreactor to start the cultivation.

Commercial glucose in the form of dextrose monohydrate was kindly donated by Roquette Americas Inc. (Geneva, IL, USA). Xylose was donated by DuPont Nutrition & Biosciences (New Century, KS, USA). Arabinose were purchased from Hofman International Inc. (Calgary, Alberta, Canada). Yeast extract and antifoam 204 were purchased from Sigma-Aldrich (St Louis, MO, USA).

Fed-batch cultivation was carried out at 29 °C with a working volume of 1.7 L in a 3-L bioreactor (BioFlo 115, New Brunswick Scientific Co., NJ, USA) equipped with a 6-Blade Rushton impeller and temperature, pH and dissolved oxygen (DO) control. The reactor was sterilized in situ at 121 °C for 20 min, cooled and then inoculated with the inoculum described in Section 6.2.1. Culture pH was monitored by a pH-mV controller (Metter Toledo 405-DPAS-SC-K8S/225) and maintained at 6.8 by automatic addition of 5 N NH_4OH through the control unit in the bioreactor. The temperature was controlled using a heating jacket covering the reactor from outside. The mixing was carried out by a mechanical stirrer connected to the control device to adjust the mixing speed according to the DO level during the reaction phase. DO level was maintained at 20% saturation by adjusting the speed of the agitator as well as the flow rate of sterile air with cascade control. DO level was measured using an in situ DO probe (O_2 Sensors Series 6800 InPro®; Mettler Toledo). The minimum stirring speed and aeration rate was set as 400 rpm and 3.6 L_{air}/min. Profiles of sugar consumption, PHB production and cell biomass production was monitored. For fed-batch feeding, four pulse feedings were applied to add sugar mixture into the

medium at around 15, 23, 29 and 35 h for three sugar mixtures. Two pulse feeding regimes, namely low and high feeding regimes, were applied. For low feeding regime, sugar mixture of 40 g/L based on the medium volume was added at each pulse feeding. For high feeding regime, sugar mixture of 80 g/L based on the medium volume was added at the first pulse feeding and 40 g/L was added for each of the other three pulse feedings. To compare cell and PHB productivities with previous studies (Cesario et al., 2014; Raposo et al., 2017), Fed-batch cultivation was stopped at 41 h. Sampling was performed every 4-6 h to determine residual sugar, cell and PHB concentrations in the media.

6.2.3 Analytical methods

6.2.3.1 Sugar and cell biomass measurement

Sugar (glucose, xylose and arabinose) concentrations at different sampling times were determined using a high performance liquid chromatograph (HPLC, Model 1100, Agilent Technologies, Santa Clara, CA, USA). The HPLC was equipped with a Bio-Rad Aminex HPX-87H column (Bio-Rad, Sunnyvale, CA, USA) at 65 °C and a refractive index detector (RID). The mobile phase was 12 mM $NaHCO_3$ running at a flow rate of 0.6 mL/min. The mobile phase 12 mM $NaHCO_3$ was used to eliminate interference from the peak of xylonic acid at the similar relative retention time to xylose (Raposo et al., 2017; Zhang et al., 2017). The total run time for each sample was 30 min. Cell biomass was determined gravimetrically after centrifugation (13,000 rpm (12054 rcf), 5 min) and drying (105 h, 24 h) 2 mL culture samples.

6.2.3.2 PHB quantification by gas chromatography-mass spectrometer (GCMS)

PHB concentration was determined according to methods described previously (Braunegg et al., 1978; Li et al., 2019a). Dried cell biomass was methanolyzed at 105 °C in acidified methanol (5% H_2SO_4, v/v, 2 mL) and chloroform (2 mL) mixture for 3 h. Sodium hydroxybutyrate (Sigma-Aldrich, MO, USA) was used as an external standard. After 3 h, 1 mL deionized water (18.2 MΩ-cm) was added to the mixture and vortexed. The methyl-ester derivatives were extracted from the lower chloroform layer and passed through a column containing anhydrous sodium sulfate before being injected into a gas chromatograph with a mass spectrometer (GC-MS) (Trace 1310 Gas Chromatograph, ISQ QD single quadrupole mass spectrometer, ThermoScientific, Waltham, MA, USA) and a TG-5MS (30 m x 0.25 mm ID x 0.25 film) capillary column (ThermoFisher Scientific, Waltham, MA, USA). The temperature profile was held at 60 °C for 5 min, increased to 220 °C at a rate of 30 °C/min and held at 220 °C for 5 min. The injector temperature was 230 °C, and the injection mode was splitless (1 µL). The pressure of the carrier gas (helium) was 142 kPa (18 psi). MS transfer line temperature was 250 °C, ion source temperature was 220 °C and ionization mode was electron ionization. The ionization energy was 70 eV and selective ion monitoring (SIM) mode was used. Methyl-ester derivative was confirmed with SIM at 43, 71, and 74. Solvent delay was 3 min. Each chromatogram was analyzed with Chromeleon 7 chromatography studio (ThermoFisher Scientific, Waltham, MA, USA).

6.3 Results and discussion

6.3.1 Fed-batch PHB cultivation on sugar mixture (glucose: xylose: arabinose=4:2:1)

Residual sugars, cell and PHB production profiles of fed-batch PHB cultivation on sugar mixture (glucose: xylose: arabinose=4:2:1) are shown in Figs. 6.1 (low feeding regime) and 6.2

(high feeding regime). Under both feeding regimes, after each pulse feeding, three sugars were consumed simultaneously but at different rates (Figs. 6.1A and 6.2A). Glucose was consumed the fastest (2.5 and 3.0 g/h), followed by xylose (1.3 and 1.3 g/h) and arabinose was consumed the slowest (0.6 and 0.8 g/h) under low and high feeding regimes, respectively. This is consistent with previous findings showing that *B. sacchari* DSM 17165 metabolized xylose slowly compared to glucose (Guaman et al., 2018) and it preferentially consumed xylose over arabinose (Kang et al., 1998; Lopes et al., 2011). Similarly, for real sugar streams from softwood hemicellulose hydrolyzate fermented by *B. sacchari* to produce PHB, sugars were depleted at different rates in the following order: glucose, mannose, xylose, galactose, and arabinose (Dietrich et al., 2019). Under both feeding regimes, PHB production kept increasing (Figs. 6.1B and 6.2B). Cell and PHB production, productivity, and sugar conversion efficiency at the end of the cultivation (41 h) can be found in Table 6.2. The high feeding regime led to higher PHB production, similar cell PHB content (%) and productivity, and slightly higher sugar conversion efficiency compare to the low feeding regime. This was due to more carbon source being fed to the bacteria, which led to higher cell growth and PHB production under the high feeding regime.

6.3.2 Fed-batch strategy on sugar mixture (glucose: xylose: arabinose=2:2:1)

Fed-batch PHB cultivation on sugar mixture (glucose: xylose: arabinose=2:2:1) is shown in Figs. 6.3 (low feeding regime) and 6.4 (high feeding regime). Similarly, three sugars consumption rates differed in the same order: glucose, xylose and arabinose (Figs. 6.3A and 6.4A). Xylose concentration was observed to accumulate over time for both feeding regimes. For the low feeding regime, cell and PHB production increased quickly and did not increase much after 26 h (Fig. 6.3B). For the high feeding regime, cell and PHB production kept increasing through the cultivation time (Fig. 6.4B). For PHB production, productivity and sugar conversion efficiency at

41 h, low and high feeding regimes showed similar performances (Table 6.2). The high feeding regime did not enhance PHB final concentration and productivity compared to the low feeding regime as was observed for sugar mixture 4:2:1. It indicates there is no need to apply the high feeding regime for sugar mixture 2:2:1.

6.3.3 Fed-batch strategy on sugar mixture (glucose: xylose: arabinose=1:2:1)

Fed-batch PHB cultivation on sugar mixture (glucose: xylose: arabinose=1:2:1) is shown in Figs. 6.5 and 6.6. Similar to other two mixtures, three sugars were consumed at the same time but at different rates (Figs. 6.5A and 6.6A). The first pulse feeding occurred at 17 h, PHB concentration under the high feeding regime was 8 g/L (Fig. 6.6B), which was only half of 16 g/L PHB observed under the low feeding regime (Figs. 6.5B). Overall, the high feeding regime exhibited lower PHB production, productivity and sugar conversion efficiency. The lower PHB production and productivity, and sugar conversion efficiency could by caused by xylose accumulation (Fig. 6.6B).

6.3.4 Summary of cell and PHB production, productivity and sugar conversion efficiency

Fed-batch strategy by pulse feeding the carbon source is one of the most commonly applied fed-batch strategies to enhance PHB production, productivity and sugar conversion efficiency (Norhafini et al., 2019; Quillaguaman et al., 2008; Stanley et al., 2018). A summary of cell and PHB production, productivity and sugar conversion efficiency is shown in Table 6.2. Overall, the highest PHB concentration, conversion rate (PHB, g/g sugar) and productivity (PHB, g/L/h) was observed with sugar mixture 4:2:1 under the high feeding regime. The lowest PHB concentration, conversion rate and productivity were observed with sugar mixture 1:2:1 under the high feeding regime. Similarly, in Cesario et al. (2014), sugar mixture having glucose/xylose ≈ 2 showed higher

PHB production, conversion rate and productivity than sugar mixtures having glucose/xylose between 1.2 and 1.4. These results indicate that glucose-rich sugar mixture exhibited higher PHB production and productivity, and sugar conversion rate, compared to other two sugar mixtures. For sugar mixture 4:2:1 under the high feeding regime, PHB content of 77% and PHB conversion rate of 0.33 g/g sugar were achieved, which were comparable or higher than most of the previously reported studies using *Burkholderia* species to produce PHB from sugar mixtures (Cesario et al., 2014; Keenan et al., 2006; Obruca et al., 2014a; Pan et al., 2012b; Silva et al., 2004; Silva et al., 2014). Under fed-batch strategies, alternative feeding strategies, such as feeding nitrogen at certain time points (Quillaguaman et al., 2008), feeding carbon and nitrogen at constant ratio (Norhafini et al., 2019), and other control strategies, such as pH-based (Stanley et al., 2018), and DO-stat feedback control (Raposo et al., 2017) could be tested in the future to determine if PHB production and productivity, and sugar conversion efficiency can be further enhanced.

Due to slow metabolism rates of xylose and arabinose by *B. sacchari*, carbon catabolite repression is always a challenge for sugar mixture conversion into PHB (Guaman et al., 2018; Kang et al., 1998; Lopes et al., 2011). In this study, for three sugar mixtures, glucose and arabinose were not accumulated in high concentrations, but for mixtures 2:2:1 and 1:2:1, xylose accumulation was observed in concentrations > 30 g/L. Cesario et al. (2014) reported that xylose concentration above 30 g/L were inhibitory to *B. sacchari* growth. For mixture 2:2:1, this may explain that why high feeding regime did not enhance PHB production and productivity compare to low feeding regime. It should be noted that xylose concentrations were higher than 30 g/L starting form 15 h under the high feeding regime (Fig. 6.4A). By looking at the curves in Figs. 6.3B and 6.4B, after first pulse feeding at 21 h, lower cell and PHB concentrations were observed with the high feeding regime (32 and 16 g/L) than with the low feeding regime (40 and 25 g/L).

For mixture 1:2:1, xylose accumulation may also explain the lower PHB production and productivity, and sugar conversion efficiency compared to other two mixtures, and the lower PHB production and productivity under high feeding regime compared to low feeding regime. Under both feeding regimes, there were certain time windows during cultivation that xylose concentration was greater than 30 g/L (Figs. 6.5A and 6.6A). Similar to sugar mixture 2:2:1 under the high feeding regime, xylose concentrations were higher than 40 g/L for most of the time starting from 15 h.

To avoid xylose accumulation, Raposo et al. (2017) applied a higher feeding rate by using a stirring speed trigger of 900 rpm for glucose-rich mixture (glucose/xylose=1.5) and lower feeding rate by using a stirring speed trigger of 700 rpm for xylose-rich mixture (glucose/xylose=0.8). Similarly, for wheat straw hydrolyzates, when stirring speed was kept high at 1200 rpm, only glucose was consumed; when stirring speed was kept low at 900 rpm, xylose and arabinose consumption was triggered (Cesario et al., 2014). Another study used a novel glucose/xylose switching strategy (glucose was used for cell growth (at the initial stage) and xylose was used for polymer production (at the feeding stage)) to enhance poly(lactate-*co*-3-hydroxybutyrate) production using engineered *Escherichia coli* (Hori et al., 2019). The enhanced polymer production may be caused by channeling of the metabolic flux from acetyl-CoA towards polymer production over into the tricarboxylic acid cycle in xylose-fed cultures (Hori et al., 2019). These process control strategies should be tested on the ternary sugar mixtures in future studies.

In addition to process control, strain development has been active to overcome catabolite repression by isolating new strains or engineering *B. sacchari* and other bacteria. A *Bacillus* sp. isolate was found to preferentially utilize glucose and arabinose, delaying the use of xylose (Lopes et al., 2009). A few studies used engineered mutant forms of *B. sacchari* or *E. coli* to avoid xylose

accumulation (Guaman et al., 2018; Jarmander et al., 2015; Lopes et al., 2011; Silva et al., 2004). *Pseudomonas* species such as *Pseudomonas pseudoflava* was also found to metabolize all three sugars (Dietrich et al., 2013). There is a lack of molecular tools to engineer *B. sacchari* DSM 17165 (Guaman et al., 2018). Development of molecular tools to engineer *B. sacchari* DSM 17165 will be helpful to enhance PHB production, productivity and sugar conversion efficiency of this strain.

6.4 Conclusion

In this study, fed-batch strategy with pulse carbon source feeding was applied to produce PHB from three different kinds of sugar mixtures under two feeding regimes. The highest PHB concentration 66 g/L, conversion rate 0.33 g PHB g/g sugar and productivity 1.65 g/L/h was observed with sugar mixture 4:2:1 under the high feeding regime. For sugar mixture 2:2:1, there was not much difference between high and low feeding regimes. For sugar mixture 1:2:1, the low feeding regime exhibited higher PHB production, productivity and sugar conversion efficiency than the high feeding regime. High feeding regime works for glucose-rich sugar mixtures and low feeding regime is preferred for xylose and arabinose rich sugar mixture. Alternative strategies should be explored to avoid xylose accumulation if the high feeding regime is applied to sugar mixtures 2:2:1 and 1:2:1.

Table 6.1. Previous reported sugar mixture (glucose, xylose and arabinose) proportions from upstream corn fiber processing

Types	Glucose, %	Xylose, %	Arabinose, %	Simulated ratio in this study (glucose:xylose:arabinose)	References
2% NaOH treated	50.2	26.7	13.1	4:2:1	Gaspar et al. (2007)
Untreated	24.3	28.4	18.3	2:2:1	Yoo et al. (2016)
*H_2SO_4 (1%) at 121 °C for 1 h	32	44	24	1:2:1	Bischoff et al. (2010)

Note: for type*, the sugar proportions are converted from sugar concentration in g/L after hydrolysis. Other two types of sugar mixtures are converted from their polymer composition by multiplying corresponding conversion factor (1.11 for glucose, 1.136 for xylose and arabinose).

Table 6.2. PHB production, productivity and sugar conversion effieciency from three different sugar mixtures under two feeding regimes at 41 h

Sugar mixture	Feeding regime	PHB production			Sugar conversion efficiency		Productivity	
		PHB, g/L	CDW, g/L	PHB (%)	PHB, g/g sugar	CDW, g/g sugar	PHB, g/L/h	CDW, g/L/h
4:2:1	Low	54.2	68.2	79.5	0.30	0.38	1.32	1.66
	High	67.5	87.2	77.3	0.33	0.42	1.65	2.13
2:2:1	Low	46.4	67.2	69.1	0.26	0.38	1.13	1.64
	High	49.3	66.4	74.2	0.25	0.34	1.20	1.62
1:2:1	Low	44.0	77.6	56.7	0.24	0.42	1.07	1.89
	High	36.9	72.2	51.2	0.22	0.43	0.90	1.76

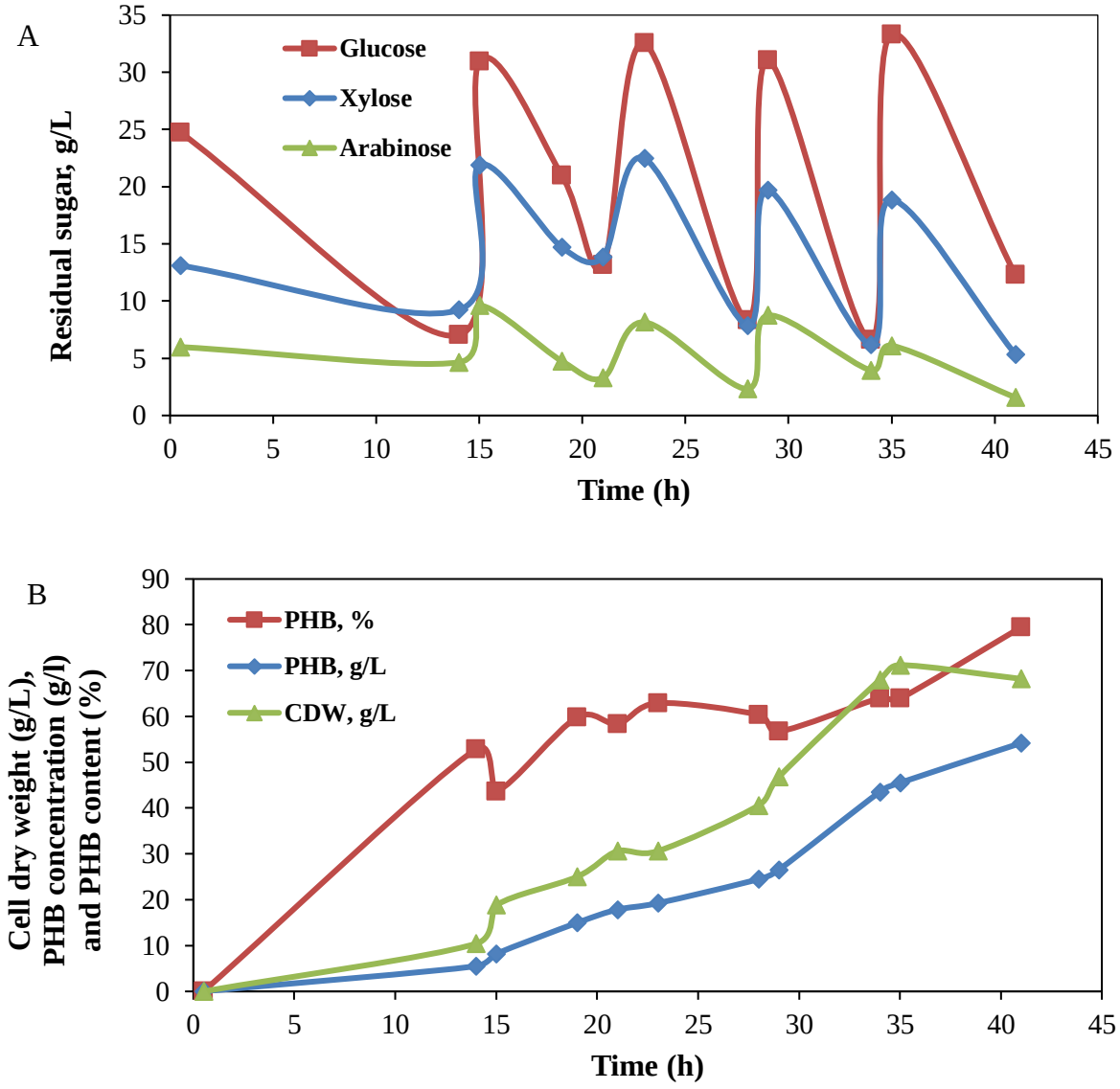

Fig. 6.1. Residual sugars (A), cell and PHB production (B) profiles for sugar mixture 4:2:1 under low feeding regime.

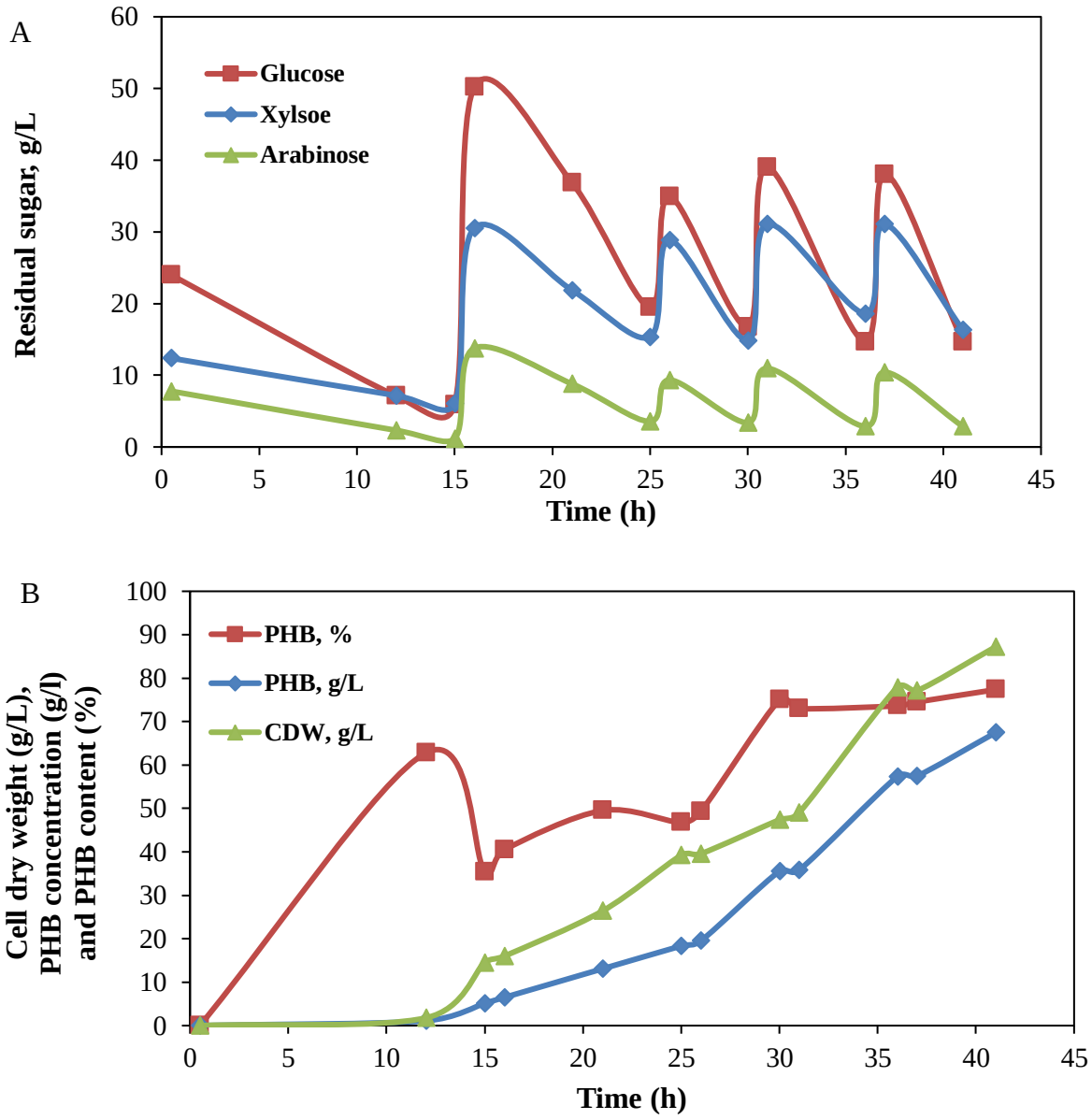

Fig. 6.2. Residual sugars (A), cell and PHB production (B) profiles for sugar mixture 4:2:1 under high feeding regime.

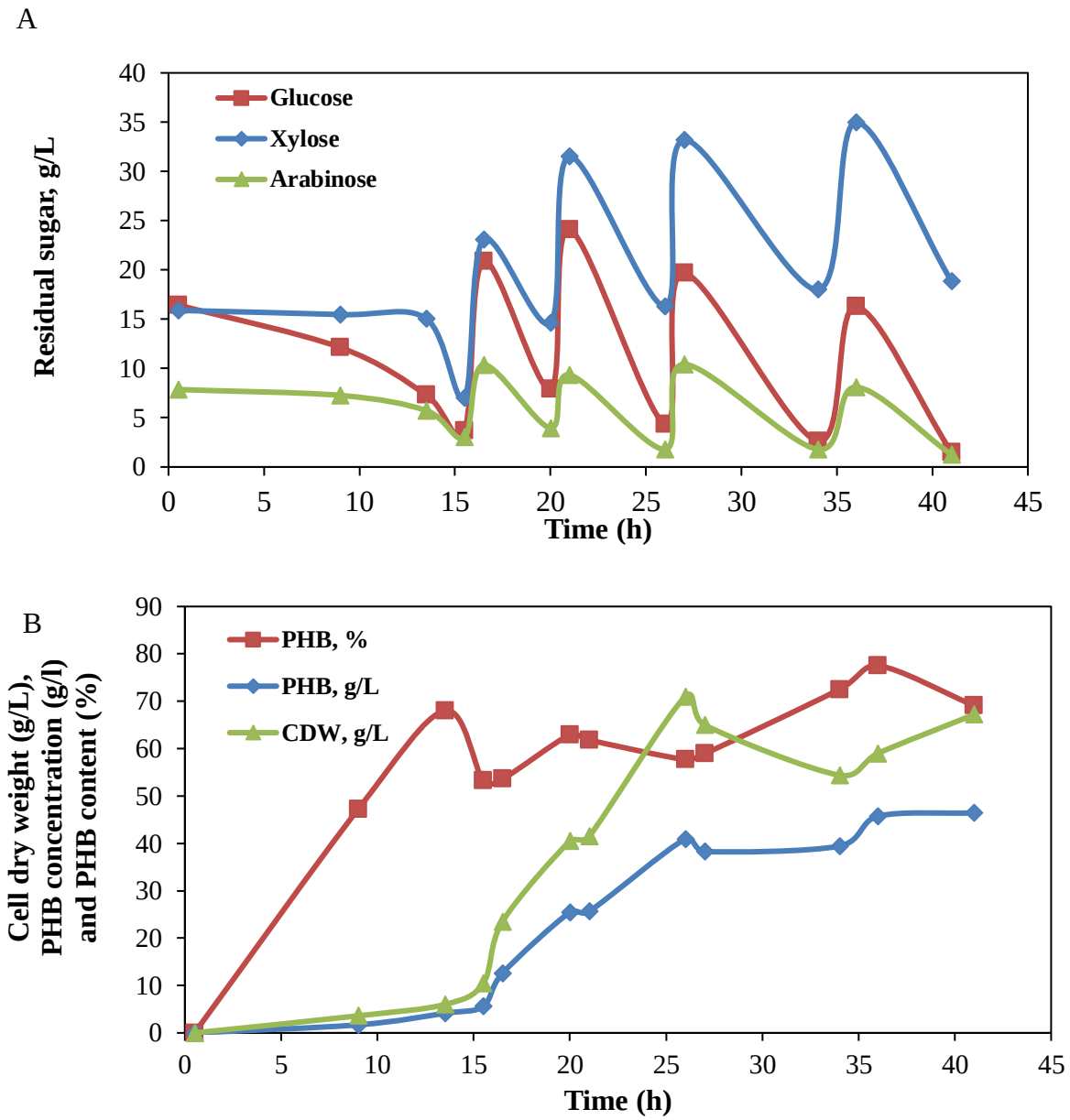

Fig. 6.3. Residual sugars (A), cell and PHB production (B) profiles for sugar mixture 2:2:1 under low feeding regime.

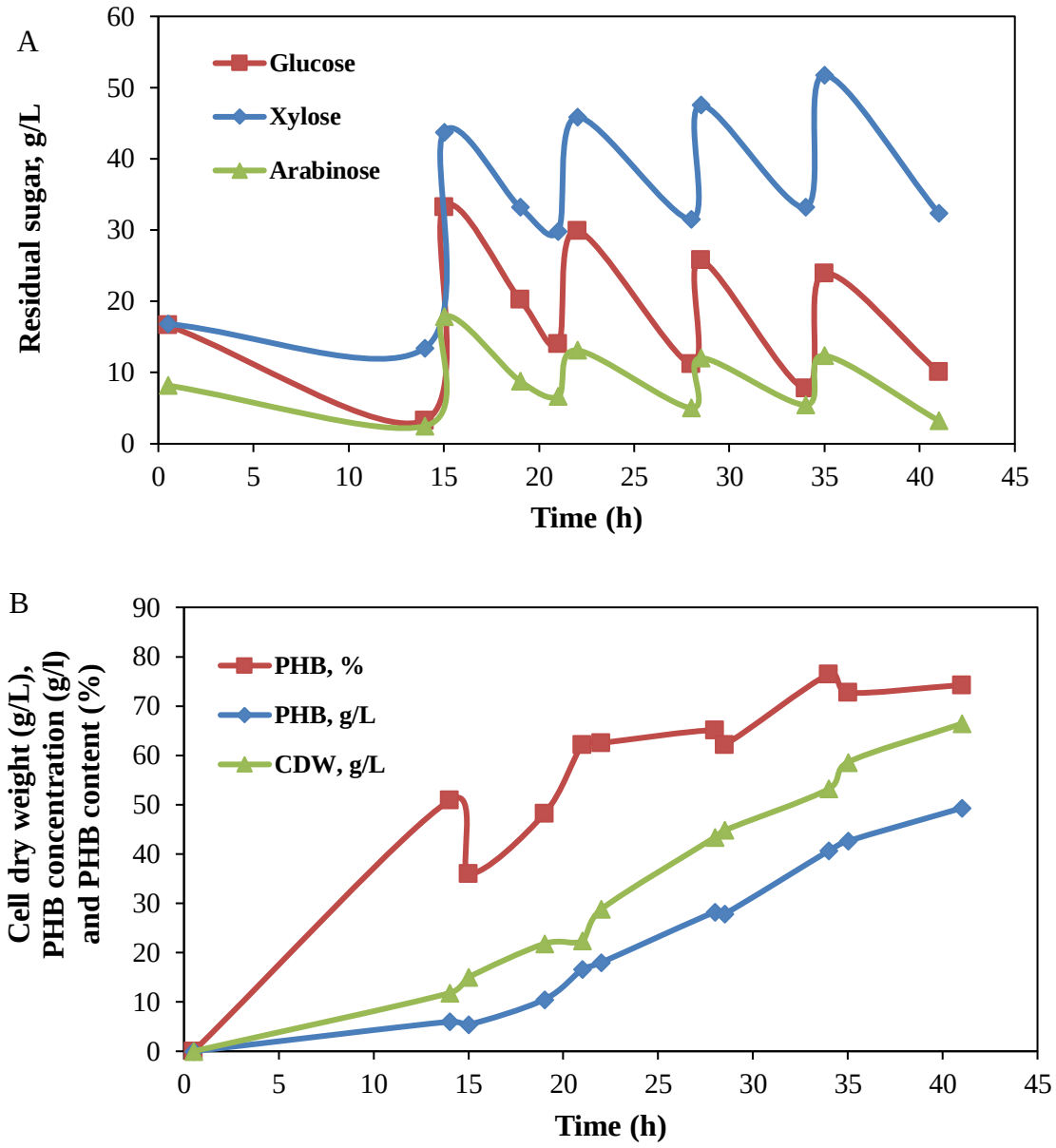

Fig. 6.4. Residual sugars (A), cell and PHB production (B) profiles for sugar mixture 2:2:1 under high feeding regime.

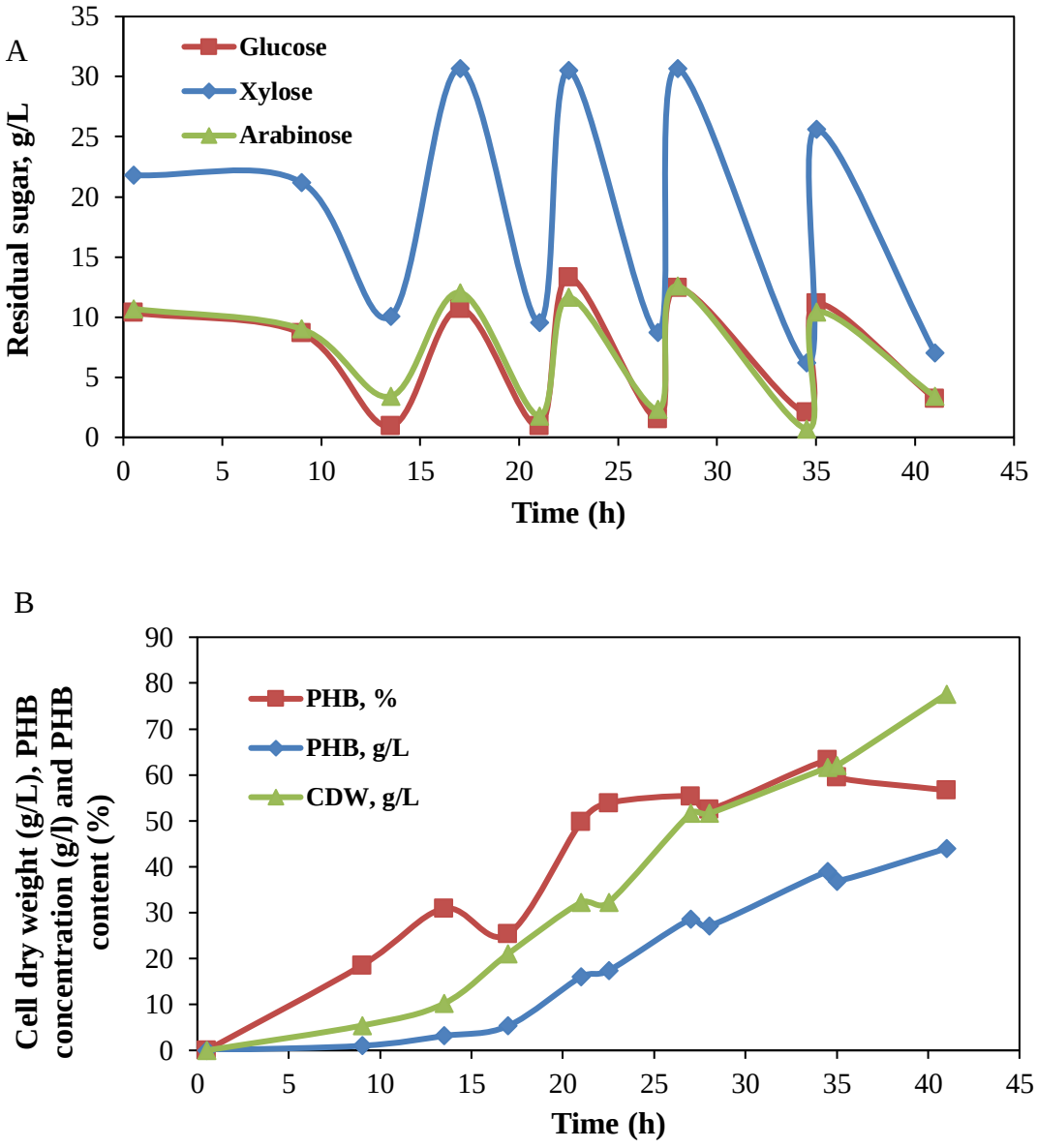

Fig. 6.5. Residual sugars (A), cell and PHB production (B) profiles for sugar mixture 1:2:1 under low feeding regime.

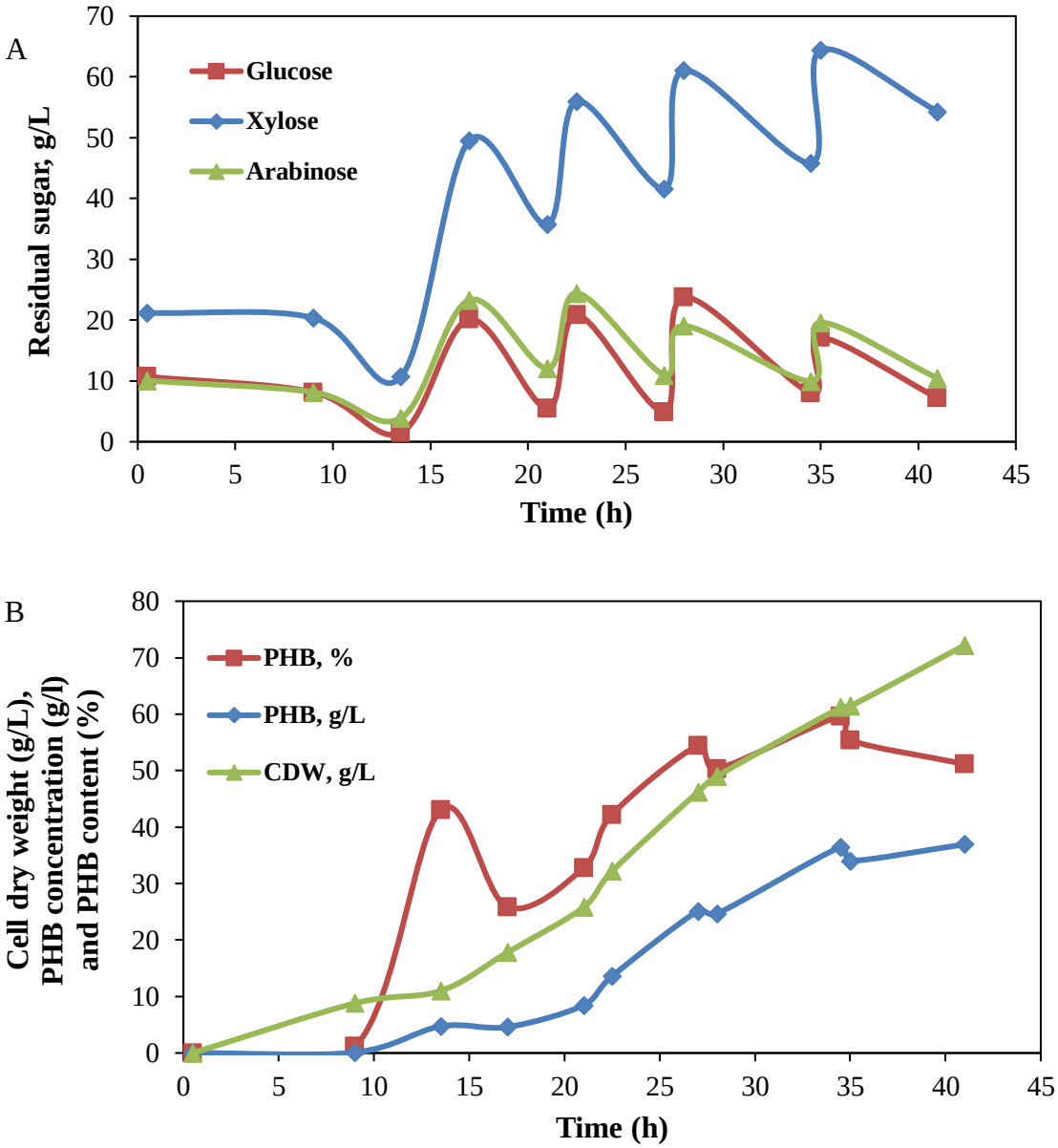

Fig. 6.6. Residual sugars (A), cell and PHB production (B) profiles for sugar mixture 1:2:1 under high feeding regime.

6.5 References

Aramvash, A., Moazzeni Zavareh, F., Gholami Banadkuki, N. 2018. Comparison of different solvents for extraction of polyhydroxybutyrate from *Cupriavidus necator*. Eng. Life Sci., 18(1), 20-28.

Bischoff, K.M., Liu, S., Hughes, S.R., Rich, J.O. 2010. Fermentation of corn fiber hydrolysate to lactic acid by the moderate thermophile *Bacillus coagulans*. BiotL, 32(6), 823-828.

Bramer, C.O., Vandamme, P., Da Silva, L.F., Gomez, J., Steinbuchel, A. 2001. Polyhydroxyalkanoate-accumulating bacterium isolated from soil of a sugar-cane plantation in Brazil. Int. J. Syst. Evol. Microbiol., 51(5), 1709-1713.

Braunegg, G., Sonnleitner, B., Lafferty, R. 1978. A rapid gas chromatographic method for the determination of poly-β-hydroxybutyric acid in microbial biomass. Eur. J. Appl. Microbiol. Biotechnol., 6(1), 29-37.

Cesario, M.T., Raposo, R.S., De Almeida, M.C.M., Van Keulen, F., Ferreira, B.S., Da Fonseca, M.M.R. 2014. Enhanced bioproduction of poly-3-hydroxybutyrate from wheat straw lignocellulosic hydrolysates. New Biotechnol., 31(1), 104-113.

Chen, Z., Wan, C. 2017b. Co-fermentation of lignocellulose-based glucose and inhibitory compounds for lipid synthesis by *Rhodococcus jostii* RHA1. Process Biochem., 57, 159-166.

Choi, J., Lee, S.Y. 1999. Factors affecting the economics of polyhydroxyalkanoate production by bacterial fermentation. Appl. Microbiol. Biotechnol., 51(1), 13-21.

Dietrich, D., Illman, B., Crooks, C. 2013. Differential sensitivity of polyhydroxyalkanoate producing bacteria to fermentation inhibitors and comparison of polyhydroxybutyrate production from *Burkholderia cepacia* and *Pseudomonas pseudoflava*. BMC Res. Notes, 6(1), 219.

Dietrich, K., Dumont, M.-J., Orsat, V., Del Rio, L.F. 2019. Consumption of sugars and inhibitors of softwood hemicellulose hydrolysates as carbon sources for polyhydroxybutyrate (PHB) production with *Paraburkholderia sacchari* IPT 101. Cellulose, 1-14.

Ding, C., Li, M., Hu, Y. 2018. High-activity production of xylanase by *Pichia stipitis*: Purification, characterization, kinetic evaluation and xylooligosaccharides production. Int. J. Biol. Macromol., 117, 72-77.

Ding, C., Wang, X., Li, M. 2019. Evaluation of six white-rot fungal pretreatments on corn stover for the production of cellulolytic and ligninolytic enzymes, reducing sugars, and ethanol. Appl. Microbiol. Biotechnol., 1-12.

Faga, B.A., Wilkins, M.R., Banat, I.M. 2010. Ethanol production through simultaneous saccharification and fermentation of switchgrass using *Saccharomyces cerevisiae* D5A and thermotolerant *Kluyveromyces marxianus* IMB strains. Bioresour. Technol., 101(7), 2273-2279.

Frederick, N., Li, M., Carrier, D.J., Buser, M.D., Wilkins, M.R. 2016. Switchgrass storage effects on the recovery of carbohydrates after liquid hot water pretreatment and enzymatic hydrolysis. AIMS Bioeng., 3(3), 389-399.

Gaspar, M., Juhasz, T., Szengyel, Z., Reczey, K. 2005. Fractionation and utilisation of corn fibre carbohydrates. Process Biochem., 40(3-4), 1183-1188.

Gaspar, M., Kalman, G., Reczey, K. 2007. Corn fiber as a raw material for hemicellulose and ethanol production. Process Biochem., 42(7), 1135-1139.

Guaman, L.P., Barba-Ostria, C., Zhang, F., Oliveira-Filho, E.R., Gomez, J.G.C., Silva, L.F. 2018. Engineering xylose metabolism for production of polyhydroxybutyrate in the non-model bacterium *Burkholderia sacchari*. Micro. Cell Fact., 17(1), 74.

Hori, C., Yamazaki, T., Ribordy, G., Takisawa, K., Matsumoto, K.i., Ooi, T., Zinn, M., Taguchi, S. 2019. High-cell density culture of poly(lactate-co-3-hydroxybutyrate)-producing Escherichia coli by using glucose/xylose-switching fed-batch jar fermentation. J. Biosci. Bioeng., 127(6), 721-725.

Jarmander, J., Belotserkovsky, J., Sjoberg, G., Guevara-Martínez, M., Perez-Zabaleta, M., Quillaguaman, J., Larsson, G. 2015. Cultivation strategies for production of (R)-3-hydroxybutyric acid from simultaneous consumption of glucose, xylose and arabinose by *Escherichia coli*. Micro. Cell Fact., 14(1), 51.

Kang, H.-Y., Song, S., Park, C. 1998. Priority of pentose utilization at the level of transcription: arabinose, xylose, and ribose operons. Mol. Cell, 8(3).

Keenan, T.M., Nakas, J.P., Tanenbaum, S.W. 2006. Polyhydroxyalkanoate copolymers from forest biomass. J. Ind. Microbiol. Biotechnol., 33(7), 616.

Khanna, S., Srivastava, A.K. 2005. Recent advances in microbial polyhydroxyalkanoates. Process Biochem., 40(2), 607-619.

Kim, B.S., Lee, S.C., Lee, S.Y., Chang, H.N., Chang, Y.K., Woo, S.I. 1994. Production of poly (3 ‐ hydroxybutyric acid) by fed ‐ batch culture of *Alcaligenes eutrophus* with glucose concentration control. Biotechnol. Bioeng., 43(9), 892-898.

Li, M., Eskridge, K., Liu, E., Wilkins, M. 2019a. Enhancement of polyhydroxybutyrate (PHB) production by 10-fold from alkaline pretreatment liquor with an oxidative enzyme-mediator-surfactant system under Plackett-Burman and central composite designs. Bioresour. Technol., 281, 99-106.

Li, M., Eskridge, K.M., Wilkins, M.R. 2019b. Optimization of polyhydroxybutyrate production by experimental design of combined ternary mixture (glucose, xylose and arabinose) and process variables (sugar concentration, molar C: N ratio). Bioprocess Biosystems Eng., 1-12.

Li, M., Marek, S.M., Peng, J., Liu, Z., Wilkins, M.R. 2018a. Effect of moisture content and inoculum size on cell wall composition and ethanol yield from switchgrass after solid-state *Pleurotus ostreatus* treatment. Trans. ASABE, 61(6), 1997-2006.

Liu, K., Atiyeh, H.K., Pardo-Planas, O., Ezeji, T.C., Ujor, V., Overton, J.C., Berning, K., Wilkins, M.R., Tanner, R.S. 2015. Butanol production from hydrothermolysis-pretreated switchgrass: quantification of inhibitors and detoxification of hydrolyzate. Bioresour. Technol., 189, 292-301.

Lopes, M.S.G., Gosset, G., Rocha, R.C.S., Gomez, J.G.C., Da Silva, L.F. 2011. PHB biosynthesis in catabolite repression mutant of *Burkholderia sacchari*. Curr. Microbiol., 63(4), 319.

Lopes, M.S.G., Rocha, R.C.S., Zanotto, S.P., Gomez, J.G.C., Silva, L.F.d. 2009. Screening of bacteria to produce polyhydroxyalkanoates from xylose. World J. Microbiol. Biotechnol., 25(10), 1751-1756.

Mozumder, M.S.I., Garcia-Gonzalez, L., De Wever, H., Volcke, E.I.P. 2015. Effect of sodium accumulation on heterotrophic growth and polyhydroxybutyrate (PHB) production by *Cupriavidus necator*. Bioresour. Technol., 191, 213-218.

Norhafini, H., Huong, K.-H., Amirul, A.A. 2019. High PHA density fed-batch cultivation strategies for 4HB-rich P(3HB-co-4HB) copolymer production by transformant *Cupriavidus malaysiensis* USMAA1020. Int. J. Biol. Macromol., 125, 1024-1032.

Obruca, S., Benesova, P., Petrik, S., Oborna, J., Prikryl, R., Marova, I. 2014a. Production of polyhydroxyalkanoates using hydrolysate of spent coffee grounds. Process Biochem., 49(9), 1409-1414.

Pan, W., Perrotta, J.A., Stipanovic, A.J., Nomura, C.T., Nakas, J.P. 2012b. Production of polyhydroxyalkanoates by *Burkholderia cepacia* ATCC 17759 using a detoxified sugar maple hemicellulosic hydrolysate. J. Ind. Microbiol. Biotechnol., 39(3), 459-469.

Quillaguaman, J., Doan-Van, T., Guzman, H., Guzman, D., Martin, J., Everest, A., Hatti-Kaul, R. 2008. Poly(3-hydroxybutyrate) production by *Halomonas boliviensis* in fed-batch culture. Appl. Microbiol. Biotechnol., 78(2), 227-232.

Ramachandriya, K.D., Wilkins, M., Atiyeh, H.K., Dunford, N.T., Hiziroglu, S. 2013. Effect of high dry solids loading on enzymatic hydrolysis of acid bisulfite pretreated Eastern redcedar. Bioresour. Technol., 147, 168-176.

Raposo, R.S., De Almeida, M.C.M., De Oliveira, M.d.C.M., Da Fonseca, M.M., Cesario, M.T. 2017. A *Burkholderia sacchari* cell factory: production of poly-3-hydroxybutyrate, xylitol and xylonic acid from xylose-rich sugar mixtures. New Biotechnol., 34, 12-22.

Rausch, K.D., Belyea, R.L. 2006. The future of coproducts from corn processing. Appl. Biochem. Biotechnol., 128(1), 47-86.

Schell, D.J., Riley, C.J., Dowe, N., Farmer, J., Ibsen, K.N., Ruth, M.F., Toon, S.T., Lumpkin, R.E. 2004. A bioethanol process development unit: initial operating experiences and results with a corn fiber feedstock. Bioresour. Technol., 91(2), 179-188.

Silva, L., Taciro, M., Ramos, M.M., Carter, J., Pradella, J., Gomez, J. 2004. Poly-3-hydroxybutyrate (P3HB) production by bacteria from xylose, glucose and sugarcane bagasse hydrolysate. J. Ind. Microbiol. Biotechnol., 31(6), 245-254.

Silva, L.F., Taciro, M.K., Raicher, G., Piccoli, R.A.M., Mendonca, T.T., Lopes, M.S.G., Gomez, J.G.C. 2014. Perspectives on the production of polyhydroxyalkanoates in biorefineries associated with the production of sugar and ethanol. Int. J. Biol. Macromol., 71, 2-7.

Stanley, A., Kumar, H.P., Mutturi, S., Vijayendra, S.N. 2018. Fed-batch strategies for production of PHA using a native isolate of *Halomonas venusta* KT832796 strain. Appl. Biochem. Biotechnol., 184(3), 935-952.

Van Eylen, D., van Dongen, F., Kabel, M., de Bont, J. 2011. Corn fiber, cobs and stover: Enzyme-aided saccharification and co-fermentation after dilute acid pretreatment. Bioresour. Technol., 102(10), 5995-6004.

Yoo, C.G., Nghiem, N.P., Kim, T.H. 2016. Production of fermentable sugars from corn fiber using soaking in aqueous ammonia (SAA) pretreatment and fermentation to succinic acid using *Escherichia coli* AFP184. Korean J. Chem. Eng., 33(10), 2863-2868.

Zhang, H., Han, X., Wei, C., Bao, J. 2017. Oxidative production of xylonic acid using xylose in distillation stillage of cellulosic ethanol fermentation broth by *Gluconobacter oxydans*. Bioresour. Technol., 224, 573-580.

CHAPTER VII

7. Fed-batch cultivation and adding supplements to increase yields of polyhydroxybutyrate production by *Cupriavidus necator* from corn stover alkaline pretreatment liquor

Abstract

In a lignocellulosic biorefinery, lignin is a waste material that is mostly burned to generate heat and power. Valorization of lignin into valuable products such as polyhydroxybutyrate increase profit and make lignocellulosic biorefineries more economically viable. Alkaline pretreatment liquor from corn stover is an example of a lignin-enriched stream. An earlier study at 250 mL shake flask scale in our lab reported PHB production from APL by *C. necator* 545 reached 2.1 g/L with supplements (laccase 2 U/mL, AAO 20 U/mL, silica nanoparticle Aerosol R816 0.02%, ABTS 5 mM and Tween 80 2% (Chapter 4). In this study, APL conversion into PHB under the same conditions was performed at 1.3 L bioreactor scale and PHB production reached 3.3 g/L. To further enhance PHB production, fed-batch cultivation with two different feeding strategies were applied. Under single pulse feeding of 300 mL medium, PHB production reached 4.0 g/L. Under 4 pulses feeding of 75 mL medium each time, PHB production reached 4.5 g/L. This is the highest PHB production from lignin that the authors are aware of in literature.

7.1. Introduction

Lignocellulosic biomass biorefineries add profit and promote sustainability in agriculture and rural economies. Multiple bioproducts (ethanol, butanol, succinic acid, lipids, enzymes, polyhydroxybutyrate) and their corresponding process designs were extensively studied over the past decade (Chen and Wan, 2017b; Ding et al., 2018; Ding et al., 2019; Frederick et al., 2016; Li et al., 2019a; Li et al., 2019b; Li et al., 2018a; Liu et al., 2015). However, most of these bioproducts were produced from polymeric sugars of lignocellulosic biomass because of the recalcitrance and heterogeneity of lignin (Salvachua et al., 2015). As an energy-dense polymer comprising 15-30% of lignocellulosic biomass and the second most abundant biopolymer on Earth after cellulose (Linger et al., 2014), lignin resulting from lignocellulosic biorefineries can be used as a feedstock for value-added products to improve the sustainability and economic feasibility of the biorefinery (Xie et al., 2016).

For the process of lignin conversion into bioproducts, generally it consists of two steps: 1) lignin polymer deconstructed and depolymerized into low molecular weight lignin; 2) low molecular weight lignin can be ring-opened in microorganisms via the β-ketoadipate pathway and converted further to bioproducts. Only low molecular weight lignins (aromatic ring # less than five) can pass the cellular membranes of microbes and enter cellular metabolism (Bandounas et al., 2011; Taylor et al., 2012). There are several previous studies converting lignin into different kinds of bioproducts (lipids, fatty acids, polyhydroxybutyrate, muconic acid and vanillin) (Da Silva et al., 2009; Kohlstedt et al., 2018; Li et al., 2018b; Linger et al., 2014; Shi et al., 2017; Wei et al., 2015). Most of these studies directly converted Kraft lignin or alkaline pretreatment liquor (APL) into bioproducts, and therefore, obtained low bioproduct yield and the bioconversion process required a long time. An early study at 250 mL shake flask scale in our lab applied a

supplement system consisting of oxidative enzymes (laccase, aryl alcohol oxidase), mediator (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid. ABTS), surfactant (Tween-80) and silicon nanoparticle (Aerosil R816) enhanced polyhydroxybutyrate (PHB) production by 10 fold (0.2 to 2 g/L) from APL (Chapter 4), given the assumption that these supplements could enhance lignin depolymerization and lignin availability to microorganisms. It is important to test PHB production with the supplement system under a larger scale and to obtain the productivity of lignin conversion into PHB.

The objectives of this study were 1) to evaluate PHB production at bioreactor scale (1.3 L) with the same supplement system described in Chapter 4 and 2) to enhance PHB production under a fed-batch mode. In this study, bioreactor (1.3 L) scale PHB production with the same supplement system in previous shake flask scale PHB production (Chapter 4) was conducted. After that, fed-batch mode was applied to enhance PHB production by two different feeding strategies: single pulse feeding and 4 pulses feeding. The parameters obtained in batch and fed-batch modes can be used to perform process control for PHB production at bioreactor scale.

7.2 Materials and methods

7.2.1 Microorganism, media and bioreactor

C. necator DSM 545, a Gram-negative Proteobacteria that can convert aromatic compounds and lignin into PHB (Berezina et al., 2015; Li et al., 2019a), was used in this study. For inoculum preparation, one aliquot of -80 °C glycerol preserved *C. necator* culture was inoculated to 25 mL seed medium (meat extract 3 g/L, meat peptone 5 g/L, glucose 30 g/L) and cultured for around 24 h to obtain late exponential phase culture with an OD of 8-10. Four flasks

of 25 mL seed culture were combined and washed with sterile 0.89% sodium chloride solution twice and used as inoculum.

For medium composition, APL prepared from NaOH pretreatment (Chapter 4) was used as the carbon source. Other components in the media included $(NH_4)_2SO_4$ (0.1 g/L) as the nitrogen source, salts ($Na_2HPO_4 \cdot 2H_2O$, 2.9 g/L; KH_2PO_4, 1.5 g/L; $MgSO_4 \cdot 7H_2O$, 0.2 g/L; $CaCl_2 \cdot 2\ H_2O$: 0.01 g/L; Fe(NH_4)citrate: 0.06 g/L) and trace elements (1 mL/L) as described previously (Ramsay et al., 1990). The supplement system contained the following five supplements: silica nanoparticle R816 0.019% (hereafter referred to as R816), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS) 5.23 mM, Tween 80 2.12%, laccase 2 U/mL and aryl alchohol oxidase (AAO) 20 U/mL (Chapter 4). For these supplements, R816 (Aerosil® R816, 12 nm average primary particle size) was donated by Evonik (Essen, Germany). Tween-80 was purchased from Acros Organics (Geel, Belgium). Laccase from *Rhus vernicifera* (L2157-10KU, 50 units/mg solids activity), and ABTS (A1888) were purchased from Sigma-Aldrich (St. Louis, MO, USA). AAO (535 U/L) was prepared as described in a previous study (Chapter 4).

Batch and fed-batch cultivation was conducted in a 0.7 L working volume in a 1.3 L bioreactor. The bioreactor (Model BioFlo115, New Brunswick Scientific, NJ, USA) was equipped with a 6-Blade Rushton Impeller and temperature and pH control, dissolved oxygen monitoring and constant aeration. Process control is described in Section 7.2.2.

7.2.2 Culture conditions

7.2.2.1. Batch cultivation

The reactor containing APL was sterilized in situ at 121 °C for 20 min, cooled and then inoculated with the inoculum described in Section 7.2.1. The initial OD for the batch culture was

around 0.5. Culture pH was monitored by a pH-mV probe (Metter Toledo 405-DPAS-SC-K8S/225) and the initial pH was adjusted to 6.8 by automatic addition of 5 N H_2SO_4 or NaOH through the control unit in the bioreactor. Under controlled pH condition, pH was maintained by automatic addition of 5N H_2SO_4. Under free pH condition, pH control was turned off. The temperature was controlled using a heating jacket covering the reactor from outside. The mixing was carried out by a mechanical stirrer connected to the control device to adjust the mixing speed according to the DO concentration during the reaction phase. DO concentration was maintained at 20% air saturation by cascade controlling the speed of the agitator as well as the flow rate of sterile air. The minimum values for speed of the agitator and airflow were 400 rpm and 0.7 L/min, respectively. The dissolved oxygen concentration in the fermenter was measured using an in situ DO probe (O_2 Sensors Series 6800 InPro®; Mettler Toledo). Sampling was conducted at certain time points and PHB was quantified as described in Section 7.2.3.

7.2.2.2. Fed-Batch cultivation

Fed-batch cultivation was conducted with two different feeding strategies: single pulse feeding of 300 mL medium and 4 pulses feeding of 75 mL medium each time. The medium is composed of APL, $(NH_4)_2SO_4$, salts and trace elements as described in Section 7.2.1 without supplements. The single pulse feeding time was at 28 h. The 4 pulses feeding time was at 28, 36, 45, 56 h. Culture pH and DO control was the same as described in Section 7.2.2.1. Sampling was conducted at certain times and PHB was quantified as described in Section 7.2.3.

7.2.3 PHB quantification by gas chromatography-mass spectrometer (GCMS)

PHB concentration was determined according to the methods described by Braunegg et al. (1978). Dried cell biomass was methanolyzed at 105 °C in acidified methanol (5% H_2SO_4,

v/v, 2 mL) and chloroform (2 mL) mixture for 3 h. Sodium hydroxybutyrate (Sigma-Aldrich, MO, USA) was used as an external standard. After 3 h, 1 mL deionized water (Milli-Q, 18.2 MΩ cm) was added to the cooled mixture and vortexed. The methyl-ester derivatives were extracted from the lower chloroform layer and passed through a column containing anhydrous sodium sulfate before being injected into a GC-MS.

The GC-MS was equipped with a Trace 1310 Gas Chromatograph, ISQ QD single quadrupole mass spectrometer (ThermoScientific, Waltham, MA, USA) and TG-5MS (30 m x 0.25 mm ID x 0.25 film) capillary column (ThermoFisher Scientific, Waltham, MA, USA). The temperature profile was held at 60 °C for 5 min, increased to 220 °C at a rate of 30 °C/min, then held at 220 °C for 5 min. The injector temperature was 230 °C, and the injection mode was splitless (1 µL). The pressure of the carrier gas (helium) was 142 kPa (18 psi). The MS transfer line temperature was 250 °C, ion source temperature was 220 °C and ionization mode was electron ionization. The ionization energy was 70 eV and selective ion monitoring (SIM) mode was used. Methylated methyl-ester derivative was confirmed with SIM at molecular weight of 43, 71, and 74. Solvent delay was 3 min. Data were analyzed with Chromeleon 7 chromatography studio (ThermoFisher Scientific, Waltham, MA, USA).

7.3 Results and discussion

7.3.1 Batch cultivation

In the previous shake flask scale study during which PHB production increased from 0.2 to 2.1 g/L with supplements (chapter 4), pH was not controlled. In this study, first, batch cultivation under the condition of free pH (pH not controlled) and without supplements was conducted. DO, stirring speed, pH and PHB production profiles were shown in Fig. 7.1. PHB production reached

0.33 g/L at 12 h (Fig. 7.1B), which is 0.6 times higher than 0.21 g/L at 168 h obtained in shake flask scale (Chapter 4). PHB production became stable at around 0.2 g/L after 36 h (Fig. 7.1B). The pH kept increasing from 6.8 to 8.8 at 56 h (Fig. 7.1B). The pH kept increasing to a maximum of 9.2 at 84 h and decreased to 9.0 at 144 h. DO dropped to 20% at around 5 h and stirring speed was increased to maintain 20% DO (Fig. 7.1A). After PHB production decreased and became stable at around 0.22 g/L, DO became stable at around 40% and stirring speed dropped back to 400 rpm (Fig. 7.1A).

Second, PHB production were conducted under the condition of controlled pH and without supplements (Fig. 7.2). PHB production reached 0.32 g/L at 12 h (Fig. 7.2B), which is similar to the PHB production under the condition of free pH and no supplements (Fig. 7.1B). This indicates that pH had no effect on PHB production under the condition of no supplements. The patterns of DO and stirring speed changing with time were similar between free pH (Fig, 7.1A) and controlled pH (Fig. 7.2A). DO dropped to 20% at around 5 h and stirring speed was increased to maintain 20% DO (Fig. 7.2A). DO became stable at around 40% and stirring speed dropped back to 400 rpm after PHB production became stable (around 0.22 g/L). It was significant that PHB production reached the maximum value of 0.3 g/L at 12 h. In other studies, for PHA/PHB production from lignin using other strains, it took several days for PHB production to reach the maximum value of 1.0 g/L (Linger et al., 2014; Shi et al., 2017). It indicated that *C. necator* DSM 545 is an efficient strain to produce PHB from lignin compared to other strains.

Previous studies showed that the optimal pH for AAO is around 5-6 (Carro et al., 2015; Ferreira et al., 2005). Laccase relative activity dropped to 40% of the activity at pH 7 (Wan et al., 2010). Although the high pH (around 9) resulting from free pH condition showed no effect on PHB production without supplements (Fig. 7.2B), high pH may affect enzyme activity when

supplements are added. Therefore, supplements were added by controlling pH at 6.8. Batch PHB production with supplements is shown in Fig. 7.3. DO dropped to 20% at around 9 h and became stable at around 34% from 19 h (Fig. 7.3A). PHB production reached a maximum of 3.34 g/L at 30 h, decreased to 3.0 g/L at 44 h and became stable (Fig. 7.3B). Similar to the 10-fold yield increase in the shake flask study (Chapter 4), the addition of supplements increased PHB production 10 fold from 0.33 to 3.34 g/L under batch fermentation mode.

7.3.2 Fed-batch cultivation

Fed-batch mode is a widely applied strategy to increase product concentration during cell cultivation. It is useful for lignin conversion because aromatic compounds in lignin are inhibitory to many enzymes and microorganisms. Previous studies have shown fed-batch cultivation increased PHA/PHB production by 0.52-1.08 fold (Liu et al., 2017; Shi et al., 2017). Two feeding strategies were applied with a total feeding volume of 300 mL medium: single pulse feeding and 4 pulses feeding. Single pulse feeding of 300 mL was initiated at 28 h, which is around the time point that highest PHB production was achieved in batch mode. After the feeding, DO dropped to 20% quickly and stirring speed was increased to maintain 20% DO (Fig. 7.4A). In around one hour, DO became stable again at around 34%. PHB production increased from 3.56 g/L at 28 h to 4.02 g/L at 42 h (Fig. 7.4B). A 13% increase was achieved with single pulse feeding of 300 mL in 14 h.

To further decrease the inhibitory effect of lignin on enzymes and microorganisms, 4 pulses feeding with a smaller volume (75 mL) each was applied in another fed-batch study. It can be seen that DO dropped to 20% and correspondingly stirring speed was increased after each pulse feeding (Fig. 7.5A). PHB production kept increasing after the first 3 pulse feedings (28, 36, 45 h) and PHB

concentration reached 4.47 g/L at 56 h (Fig. 7.5B). However, PHB production decreased after the fourth pulse feeding (4.06 g/L at 63 h). This can be explained by accumulated aromatic compounds that are not metabolized by the microorganism reaching inhibitory levels from the increasing APL being fed. The highest PHB production 4.47 g/L was 34% higher than that observed in batch fermentation with supplements and 12.55 times higher than that observed without supplements.

An overview of PHB production, time to reach maximum concentration and productivity under different strategies in this study is presented in Table 7.1. Fed-batch strategies achieved the higher PHB production (4.0 and 4.5 g/L) that that of batch cultivation (3.0 g/L) but they took longer time (42 and 56h). Therefore, batch fermentation with supplements showed the highest productivity of 0.111 g/L/h. With supplements, both fed-batch fermentation strategies showed lower productivities than batch fermentation. Single pulse feeding exhibited slightly higher productivity (0.096 g/L/h) than 4 times pulse feeding (0.08 g/L/h). Further study can be conducted to optimize the feeding time under fed-batch condition to increase PHB productivity. A comparison of PHA/PHB production between this study and previous studies are summarized in Table 7.2. This study achieved the highest PHB production (4.5 g/L) and productivity (0.080 g/L/h). This is the highest PHB production and productivity from lignin that the authors are aware of in literature.

7.4 Conclusion

In this study, at bioreactor scale (1.3 L), fed-batch fermentation strategies were conducted to enhance PHB production with a supplement system developed in a previous shake flask scale study (Chapter 4). Under batch fermentation, the addition of supplements increased PHB production from 0.33 g/L to 3.3 g/L and PHB productivity of 0.111 g/L/h was achieved. Both fed-

batch strategies increased PHB production and lowered PHB productivity compared to batch cultivation. The highest PHB production of 4.5 g/L was obtained under fed-batch strategy with 4 times pulse feeding, and the corresponding PHB productivity was 0.08 g/L/h. The supplements and fermentation strategies in this study can be used as references to enhance other bioproducts production from lignin.

Table 7.1. PHB production from APL by *C. necator* DSM 545 cultivated in bioreactor

Mode	Strategy	PHB (g/L)	Time to reach maximum concentration (h)	Productivity (g/L/h)
Batch	Free pH w/o supplements	0.33	12	0.028
	Controlled pH w/o supplements	0.32	12	0.027
	Controlled pH w supplements	3.34	30	0.111
Fed-batch	Single pulse (300 mL) w supplements	4.02	42	0.096
	4 times pulse (each 75 mL) w supplements	4.47	56	0.080

Note: w/o denotes without, w denotes with.

Table 7.2. A comparison of PHA/PHB production with previous studies

Lignin (derivatives) form	Organisms	Scale and strategy	Cell (g/L)	PHB/PHA (g/L)	Productivity (g/L/h)	References
APL	*C. necator* DSM 545	Bioreactor (1.3 L) and fed-batch	NA	4.5	0.080	This study
APL	*C. necator* DSM 545	Shake flask (250 mL) and batch	NA	2.1	NA	(Chapter 4)
APL*	*Pseudomonas. putida* KT2440	Shake flask (250 mL) and fed-batch	5.3	1.0	NA	Liu et al. (2017)
APL	*P. putida* KT2440	Bioreactor (14 L) and fed-batch	0.79	0.25	0.005	Linger et al. (2014)
APL	*C. basilensis* CGMCC 4240	Shake flask (100 mL working volume) and fed-batch**	3.99	0.8	NA	Si et al. (2018)
Kraft lignin	*C. basilensis* CGMCC 4240	Shake flask (100 mL working volume) and fed-batch**	NA	0.32	NA	Shi et al. (2017)
Vanillic acid	Recombinant *P. putida* A514	Shake flask (100 mL working volume) and batch**	0.71	0.25	NA	Wang et al. (2018)
Benzoic acid	*C. necator* DSM 545	Shake flask (300 mL) and repeated fed-batch with partial withdrawal	2	0.4	0.0018	Berezina et al. (2015)

*Note: in the study of Liu et al. (2017), APL was obtained from combinatorial H_2SO_4 pretreatment followed by NaOH pretreatment of corn stover, which was more efficient than other alkaline pretreatment processes in Linger et al. (2014), Chapter 4 and Si et al. (2018).

**Note: the paper did not mention the flask volume; 100 mL is the working volume.

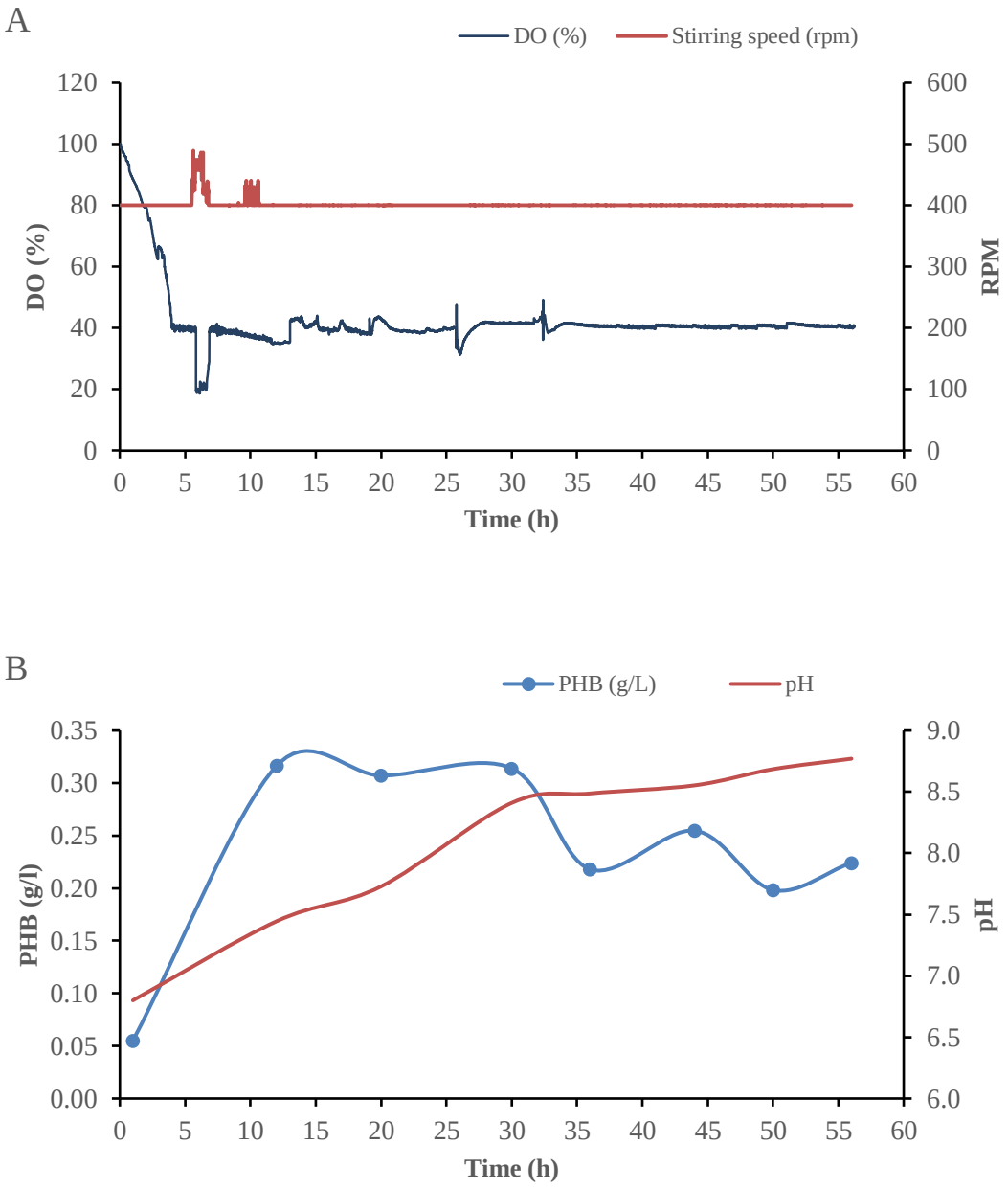

Fig. 7.1. Time course of PHB production by *C. necator* DSM 545 under the condition of free pH and without supplements.

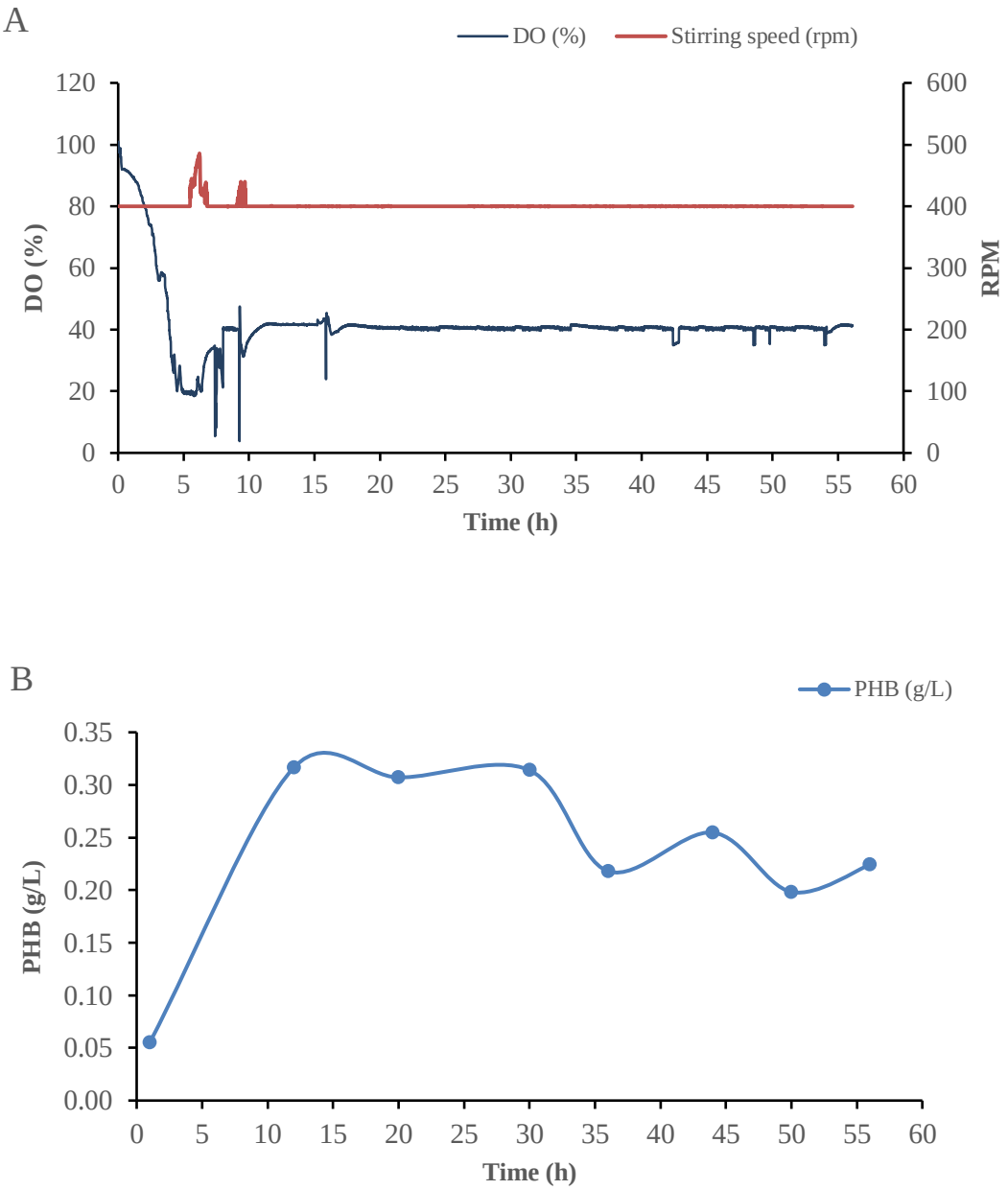

Fig. 7.2. Time course of PHB batch production by *C. necator* DSM 545 under the conditions of controlled pH (6.8) and without supplements.

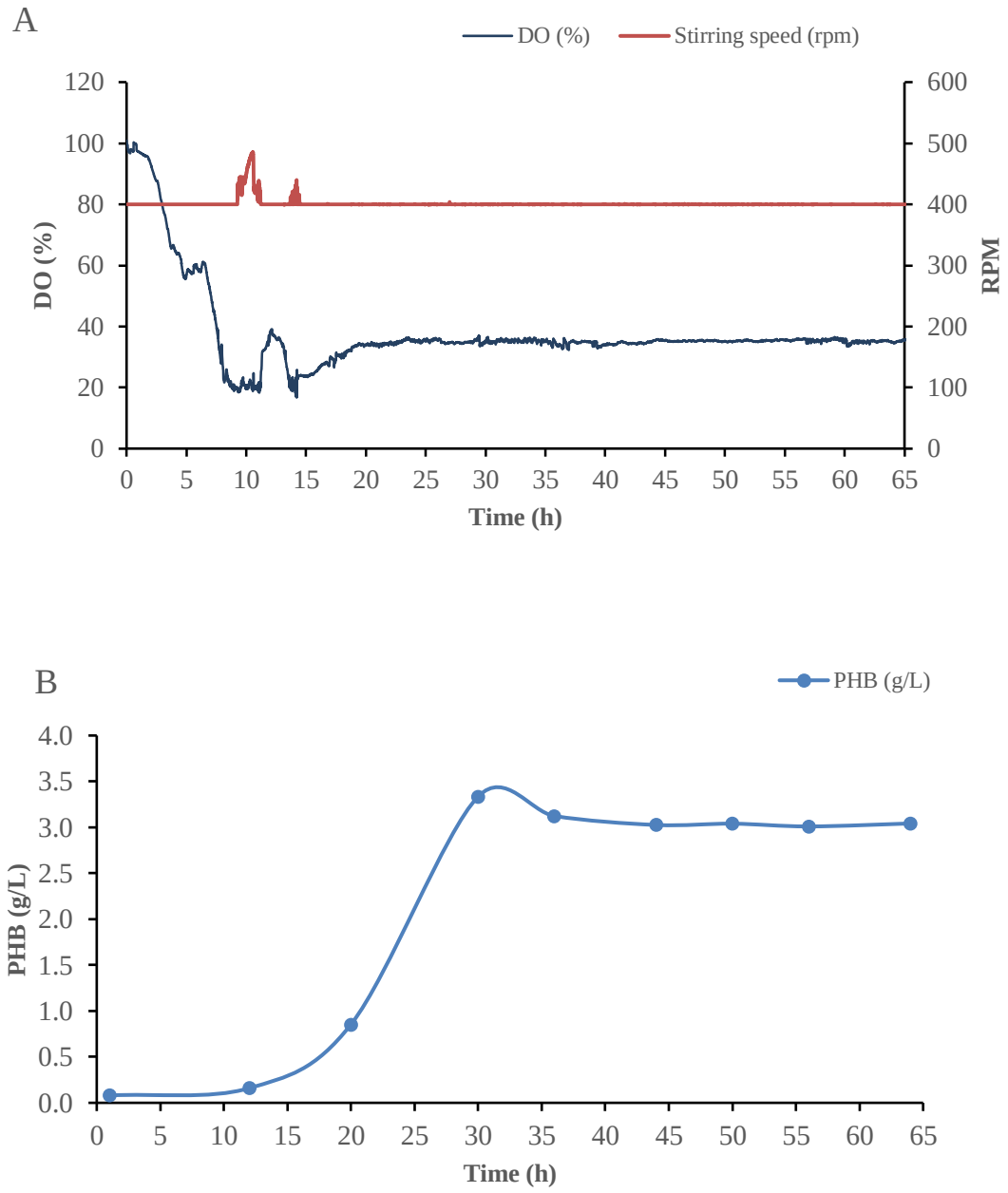

Fig. 7.3. Time course of PHB batch production by *C. necator* DSM 545 under the conditions of controlled pH (6.8) and with supplements.

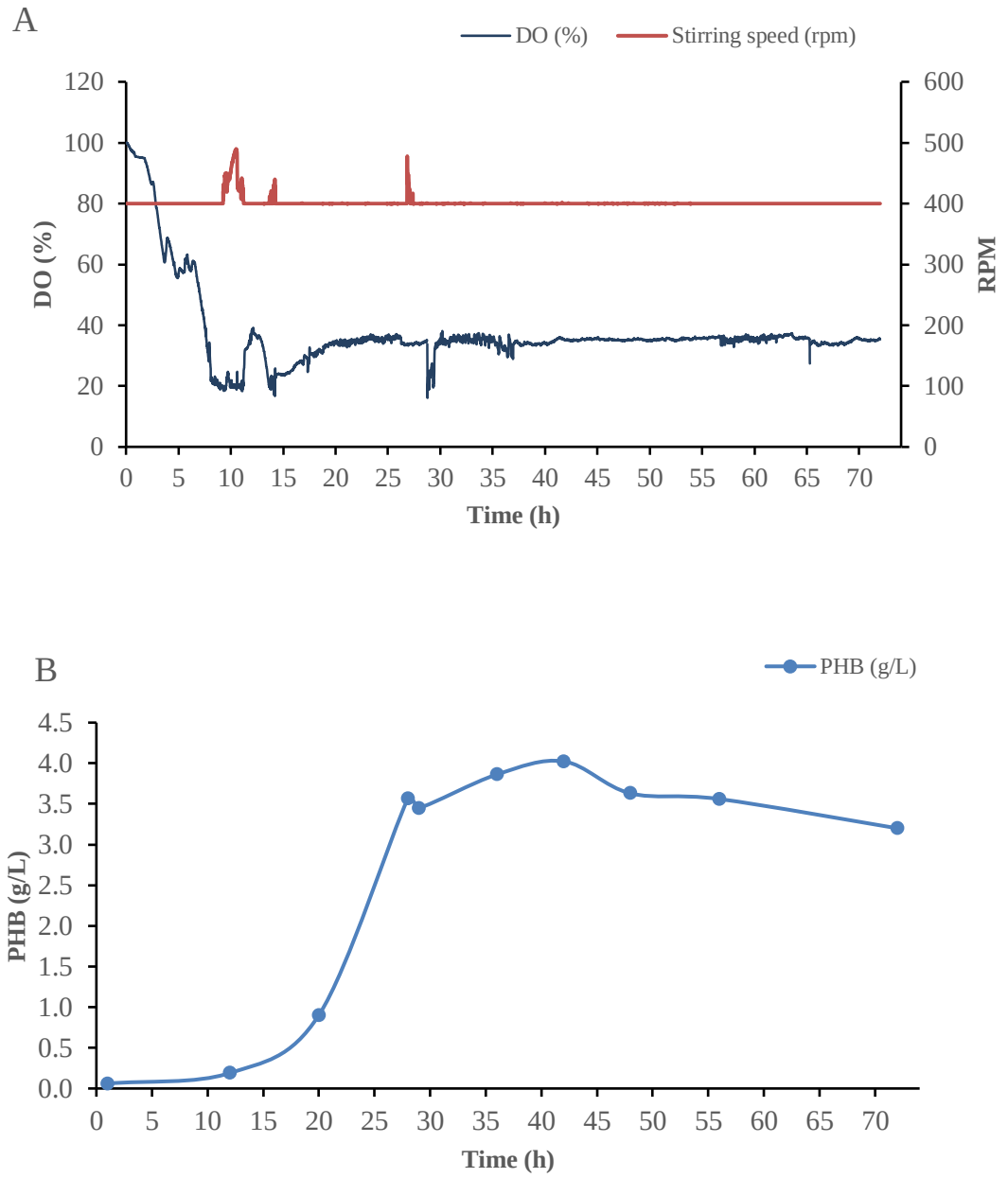

Fig. 7.4. Time course of PHB fed-batch production (single pulse feeding) by C. necator DSM 545 under the conditions of controlled pH (6.8) and with supplements. Single pulse feeding of 300 mL was initiated at 28 h.

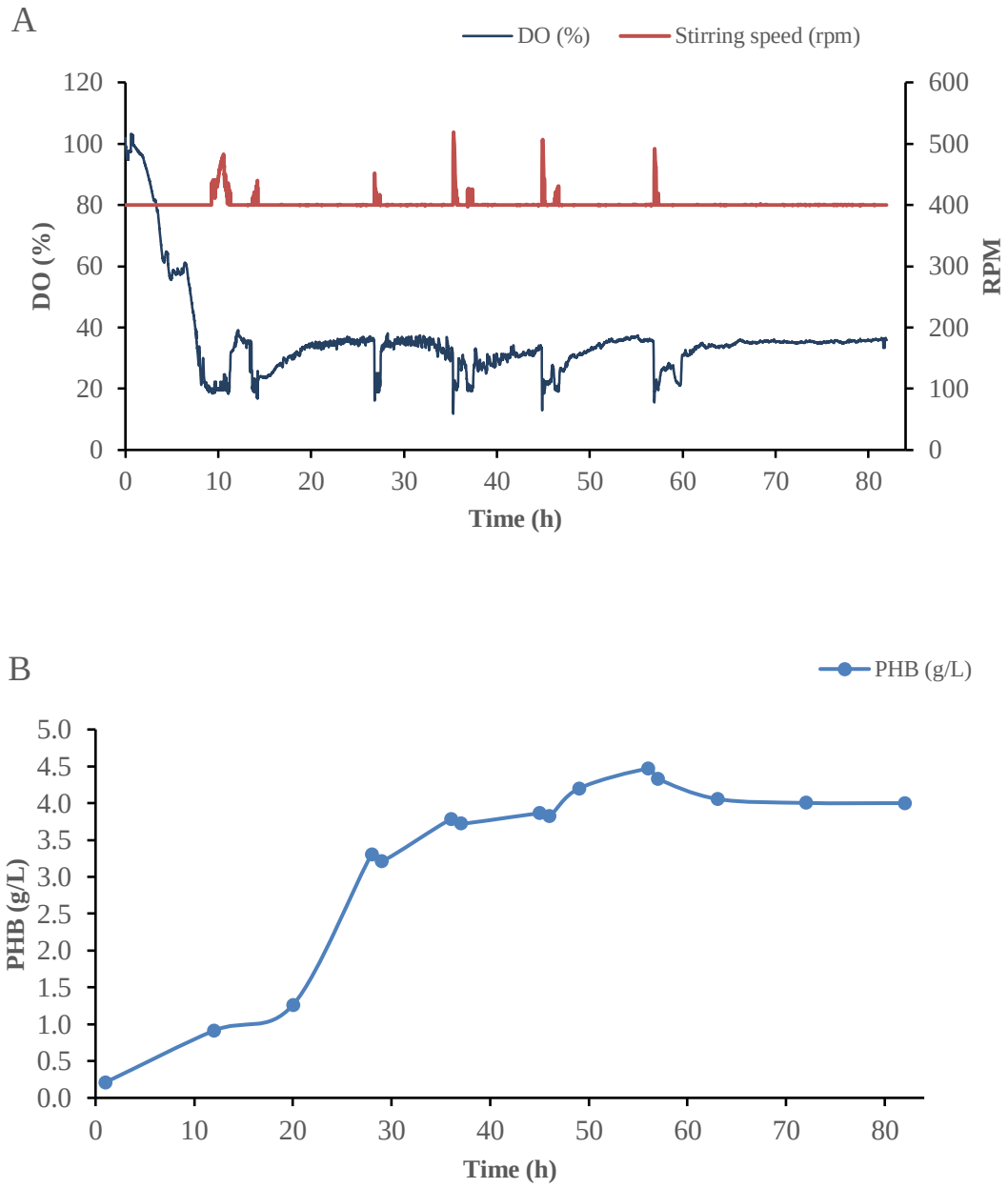

Fig. 7.5. Time course of PHB fed-batch production (4 times pulse feeding) by *C. necator* DSM 545 under the conditions of controlled pH (6.8) and with supplements. The 4 pulses feeding times are at 28, 36, 45, 56 h.

7.5 References

Bandounas, L., Wierckx, N.J., de Winde, J.H., Ruijssenaars, H.J. 2011. Isolation and characterization of novel bacterial strains exhibiting ligninolytic potential. BMC Biotechnol., 11(1), 94.

Berezina, N., Yada, B., Lefebvre, R. 2015. From organic pollutants to bioplastics: insights into the bioremediation of aromatic compounds by *Cupriavidus necator*. New Biotechnol., 32(1), 47-53.

Braunegg, G., Sonnleitner, B., Lafferty, R. 1978. A rapid gas chromatographic method for the determination of poly-β-hydroxybutyric acid in microbial biomass. Eur. J. Appl. Microbiol. Biotechnol., 6(1), 29-37.

Carro, J., Ferreira, P., Rodriguez, L., Prieto, A., Serrano, A., Balcells, B., Arda, A., Jimenez-Barbero, J., Gutierrez, A., Ullrich, R. 2015. 5-hydroxymethylfurfural conversion by fungal aryl-alcohol oxidase and unspecific peroxygenase. FEBS J., 282(16), 3218-3229.

Chen, Z., Wan, C. 2017b. Co-fermentation of lignocellulose-based glucose and inhibitory compounds for lipid synthesis by *Rhodococcus jostii* RHA1. Process Biochem., 57, 159-166.

Da Silva, E.B., Zabkova, M., Araujo, J., Cateto, C., Barreiro, M., Belgacem, M., Rodrigues, A. 2009. An integrated process to produce vanillin and lignin-based polyurethanes from Kraft lignin. Chem. Eng. Res. Des., 87(9), 1276-1292.

Ding, C., Li, M., Hu, Y. 2018. High-activity production of xylanase by *Pichia stipitis*: Purification, characterization, kinetic evaluation and xylooligosaccharides production. Int. J. Biol. Macromol., 117, 72-77.

Ding, C., Wang, X., Li, M. 2019. Evaluation of six white-rot fungal pretreatments on corn stover for the production of cellulolytic and ligninolytic enzymes, reducing sugars, and ethanol. Appl. Microbiol. Biotechnol., 1-12.

Ferreira, P., Medina, M., Guillen, F., Martinez, M.J., Van Berkel, W.J., Martinez, A.T. 2005. Spectral and catalytic properties of aryl-alcohol oxidase, a fungal flavoenzyme acting on polyunsaturated alcohols. Biochem. J., 389(3), 731-738.

Frederick, N., Li, M., Carrier, D.J., Buser, M.D., Wilkins, M.R. 2016. Switchgrass storage effects on the recovery of carbohydrates after liquid hot water pretreatment and enzymatic hydrolysis. AIMS Bioeng., 3(3), 389-399.

Kohlstedt, M., Starck, S., Barton, N., Stolzenberger, J., Selzer, M., Mehlmann, K., Schneider, R., Pleissner, D., Rinkel, J., Dickschat, J.S., Venus, J., B.J.H. van Duuren, J., Wittmann, C.

2018. From lignin to nylon: Cascaded chemical and biochemical conversion using metabolically engineered *Pseudomonas putida*. Metab. Eng., 47, 279-293.

Li, M., Eskridge, K., Liu, E., Wilkins, M. 2019a. Enhancement of polyhydroxybutyrate (PHB) production by 10-fold from alkaline pretreatment liquor with an oxidative enzyme-mediator-surfactant system under Plackett-Burman and central composite designs. Bioresour. Technol., 281, 99-106.

Li, M., Eskridge, K.M., Wilkins, M.R. 2019b. Optimization of polyhydroxybutyrate production by experimental design of combined ternary mixture (glucose, xylose and arabinose) and process variables (sugar concentration, molar C: N ratio). Bioprocess Biosystems Eng., 1-12.

Li, M., Marek, S.M., Peng, J., Liu, Z., Wilkins, M.R. 2018a. Effect of moisture content and inoculum size on cell wall composition and ethanol yield from switchgrass after solid-state *Pleurotus ostreatus* treatment. Trans. ASABE, 61(6), 1997-2006.

Li, X., Shi, J., Das, L., Tharayil, N., Zheng, Y. 2018b. A novel platform for bioupgrading of lignin to valuable nutraceuticals and pharmaceuticals. in: *2018 ASABE Annual International Meeting*, ASABE. St. Joseph, MI, pp. 1.

Linger, J.G., Vardon, D.R., Guarnieri, M.T., Karp, E.M., Hunsinger, G.B., Franden, M.A., Johnson, C.W., Chupka, G., Strathmann, T.J., Pienkos, P.T. 2014. Lignin valorization through integrated biological funneling and chemical catalysis. Proc. Natl. Acad. Sci., 111(33), 12013-12018.

Liu, K., Atiyeh, H.K., Pardo-Planas, O., Ezeji, T.C., Ujor, V., Overton, J.C., Berning, K., Wilkins, M.R., Tanner, R.S. 2015. Butanol production from hydrothermolysis-pretreated switchgrass: quantification of inhibitors and detoxification of hydrolyzate. Bioresour. Technol., 189, 292-301.

Liu, Z.-H., Olson, M.L., Shinde, S., Wang, X., Hao, N., Yoo, C.G., Bhagia, S., Dunlap, J.R., Pu, Y., Kao, K.C. 2017. Synergistic maximization of the carbohydrate output and lignin processability by combinatorial pretreatment. Green Chem., 19(20), 4939-4955.

Ramsay, B., Lomaliza, K., Chavarie, C., Dube, B., Bataille, P., Ramsay, J. 1990. Production of poly-(beta-hydroxybutyric-co-beta-hydroxyvaleric) acids. Appl. Environ. Microbiol., 56(7), 2093-2098.

Salvachua, D., Karp, E.M., Nimlos, C.T., Vardon, D.R., Beckham, G.T. 2015. Towards lignin consolidated bioprocessing: simultaneous lignin depolymerization and product generation by bacteria. Green Chem., 17(11), 4951-4967.

Shi, Y., Yan, X., Li, Q., Wang, X., Xie, S., Chai, L., Yuan, J. 2017. Directed bioconversion of kraft lignin to polyhydroxyalkanoate by *Cupriavidus basilensis* B-8 without any pretreatment. Process Biochem., 52, 238-242.

Si, M., Yan, X., Liu, M., Shi, M., Wang, Z., Wang, S., Zhang, J., Gao, C., Chai, L., Shi, Y. 2018. In situ lignin bioconversion promotes complete carbohydrate conversion of rice straw by *Cupriavidus basilensis* B-8. ACS Sustain. Chem. Eng., 6(6), 7969-7978.

Taylor, C., Hardiman, E., Ahmad, M., Sainsbury, P., Norris, P., Bugg, T. 2012. Isolation of bacterial strains able to metabolize lignin from screening of environmental samples. J. Appl. Microbiol., 113(3), 521-530.

Wan, Y.-Y., Lu, R., Xiao, L., Du, Y.-M., Miyakoshi, T., Chen, C.-L., Knill, C.J., Kennedy, J.F. 2010. Effects of organic solvents on the activity of free and immobilised laccase from *Rhus vernicifera*. Int. J. Biol. Macromol., 47(4), 488-495.

Wang, X., Lin, L., Dong, J., Ling, J., Wang, W., Wang, H., Zhang, Z., Yu, X. 2018. Simultaneous improvements of cell growth and polyhydroxyalkanoate production in *Pseudomonas* from a lignin derivative for lignin consolidated bioprocessing. Appl. Environ. Microbiol., AEM. 01469-18.

Wei, Z., Zeng, G., Huang, F., Kosa, M., Huang, D., Ragauskas, A.J. 2015. Bioconversion of oxygen-pretreated Kraft lignin to microbial lipid with oleaginous *Rhodococcus opacus* DSM 1069. Green Chem., 17(5), 2784-2789.

Xie, S., Ragauskas, A.J., Yuan, J.S. 2016. Lignin conversion: opportunities and challenges for the integrated biorefinery. Ind. Biotechnol., 12(3), 161-167.

CHAPTER VIII

8. Conclusion and Future work

The research work presented in this dissertation developed efficient processes for sugar mixture and lignin conversion into PHB. Shake flask and bioreactor scale studies were conducted for both carbon sources. At shake flask scale, advanced experimental designs including combined mixture-process, Plackett-Burman and central composite deigns were employed. At bioreactor scale, fed-batch cultivation strategy with different carbon source feeding regimes were tested. The major findings are listed in the summary of results. Recommendations for future work are proposed.

8.1. Summary of results

(1) For sugar mixture conversion into PHB, at shake flask scale (250 mL), results from combined mixture-process design showed that interactions existed between sugar mixture ratios and process variables in terms of affecting PHB production. Optimal conditions were derived from the statistical models when dealing with different sugar streams derived from upstream lignocellulosic pretreatment and hydrolysis. At bioreactor scale (3L), the highest PHB production reached 67 g/L for 4:2:1 (glucose:xylose:arabinose) mixture at 41 h corresponding to an accumulation of 77% of cell dry weight. Corresponding sugar conversion efficiency and productivity were 0.33 g PHB/g sugar consumed and 1.6 g/L/h, respectively, which are comparable to or higher than most previous studies.

(2) For lignin conversion into PHB, first, at shake flask scale (250 mL), the developed supplement system enhanced lignin conversion into PHB by 10-fold from 0.2 to 2 g/L. At

bioreactor scale (1.7 L), fed-batch cultivation further enhanced PHB production to 4.5 g/L. This is the highest PHB production from lignin that the author has been aware of in the literature.

(3) A sample preparation method was developed to predict PHB concentration using fluorescence intensity from flow cytometry. The linear relationship with good fitness (R^2=0.9939) between PHB concentration and fluorescence intensity indicates the potential of applying flow cytometry for quantitation of PHB from APL and other media that are dark and contain insoluble particles.

8.2. Recommendation for future work

(1) For sugar mixture conversion, the optimal conditions derived from the statistical model at shake flask scale and fed-batch PHB production at bioreactor scale are based on pure ternary sugar mixtures. Real sugar mixture streams contain other sugars in small amounts and other non-sugar compounds such as organic acids. The optimal conditions at shake flask scale and fed-batch PHB production at bioreactor scale should be tested on real sugar mixture streams.

(2) Supplements used in lignin conversion into PHB are expensive. For example, laccase is extracted from plant. Future work should be conducted to produce the supplement in a cheap way. In terms of genetics, supplements used in this study can be used by geneticists to study gene expression involved in lignin conversion to PHB and metabolic pathways modulation to enhance lignin conversion into PHB. For lignin structural changes, structural changes of lignin triggered by the supplement system should be studied to understand the mechanism during lignin depolymerization and conversion.