Production of Polyhydroxybutyrate (PHB) from batch fermentation of hemp wastes via co-fermentation of *Cupriavidus necator* with *Paraburkholderia sacchari*

by

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Abstract

Polyhydroxybutyrate (PHB) is a biopolymer from the polyhydroxyalkanoates (PHA) family, produced as a result of secondary metabolic reactions for energy storage in cells. It has gained considerable attention due to its biodegradability, biocompatibility, mechanical properties and potential to replace petrochemical based plastics, addressing the growing environmental concerns related to plastic pollution. Moreover, PHB is a promising material for applications in packaging, agriculture, biomedical devices, and more. However, challenges in its production, including high production costs and resource demands, hinder its large-scale adoption. The price for biodegradable plastics is approximately 20-30% higher than traditional plastics and the total production cost is heavily influenced by the cost of raw materials costs such as feedstock and water resources. As a result, optimizing its production process is critical for improving its economic viability and sustainability.

In this study, hemp biomass has been explored as an alternative feedstock to produce PHB. Hemp biomass is a byproduct of the textile industries and hemp seed processing industries that is obtained as a remnant part after bast fiber and seed are removed from the shoot system of the plant. Being rich in cellulose and hemicellulose, hemp biomass can be processed into glucose and xylose rich hydrolysate that can be consumed by bacteria such as *Cupriavidus necator* and *Paraburkholderia sacchari* to produce PHB. *C. necator* can produce PHB from glucose and lignin while *P. sacchari* can coferment glucose and xylose to produce PHB.

This thesis focused on exploring various factors that affect PHB production from hemp hydrolysate using two different microorganisms. Chapter 1 entails the literature review of the relevant information regarding hemp biomass as feedstock and PHB production procedures via

C. necator and *P. sacchari*. In chapter 2, pretreatment parameters for alkaline treatment of hemp biomass were optimized and the PHB production capacity of the two bacteria, in individual and combined manner, in hemp hydrolysate were studied. Pretreatment of hemp biomass with 1% sodium hydroxide for 1 h at 130°C was observed to be significantly superior during pretreatment optimization. Similarly, hydrolysate with *C. necator* only achieved maximum PHB yield of 0.433 g/g sugars (specifically from glucose) at 48 h from the start of fermentation while combination of *C. necator* and *P. sacchari* showed significant effects on utilization of both glucose and xylose in the hydrolysate to produce maximum yield of 0.341 g/g sugars at 48 h. To reduce water demand during washing of solids prior to hydrolysis, unwashed solids were processed under different alternatives in chapter 3 where maximum PHB yield of 0.65 g/g sugars was obtained for liquid hot water pretreated hemp solids and undetoxified prehydrolysate using *C. necator*, which consumed lignin derivatives and sugars in the media. Finally, chapter 4 concluded the findings and understanding of this research study along with recommendations for future experiments.

Table of Contents

Table of Contents viii

List of Figures ix

List of Tables xi

Acknowledgements xii

Dedication xiv

1 Chapter 1: Introduction, Thesis objectives and Literature Review 1

1.1 Introduction 1

1.2 Thesis objectives 3

1.3 Literature review 4

1.3.1 Existing PHB production system in industries 4

1.3.2 Sources of substrate: hemp stalk 5

1.3.3 Pretreatment of hemp stalk 6

1.3.4 Biosynthetic pathway / metabolism 10

1.3.5 Microorganisms: *Paraburkholderia sacchari* and Cupriavidus necator 11

1.3.6 Fermentation 13

1.3.7 Characterization of PHB 16

1.3.8 Conclusion 17

1.4 References 18

2 Chapter 2: Investigation of synergistic effects of *P. sacchari* and *C. necator* for production of PHB from hemp hurds 23

2.1 Abstract 23

2.2 Flowchart 23

2.3 Introduction 24

2.4 Materials and methods 26

2.4.1 Hemp as carbon source 26

2.4.2 Compositional analysis of hemp 27

2.4.3 Pretreatment of hemp 27

2.4.4 Enzymatic hydrolysis 28

2.4.5 Optical density measurement and cell mass calculation 28

2.4.6 Microorganism and inoculum 29

2.4.7 PHB production 30

2.4.8 High-performance liquid chromatography (HPLC) sugar analysis 31

2.4.9 PHB quantification 31

2.4.10 Gas chromatography mass spectrometer (GCMS) for PHB quantification 32

2.4.11 Calculations 32

2.4.12 Statistical analysis 33

2.5 Results and discussion 33

2.5.1 Optimization of pretreatment of HS solids 33

2.5.2 Effects of bacterial combination on PHB yield 35

2.5.3 Variation between batches 47

2.6 Conclusion 49

2.7 References..50

3 Chapter 3: Investigation of alternatives against washing pretreated solids to produce PHB via *C. necator* 53

3.1 Abstract..53

3.2 Introduction...54

3.3 Materials and Methods 56

3.3.1 Hemp stalk (HS) as substrate 56

3.3.2 Compositional analysis of hemp..56

3.3.3 Cell mass measurement...57

3.3.4 Pretreatment and enzymatic hydrolysis 57

3.3.5 Optical Density measurement...58

3.3.6 Lignin measurement...59

3.3.7 PHB production 59

3.3.8 Dilution of alkaline hydrolysate 60

3.3.9 High performance liquid chromatography (HPLC) sugar analysis 60

3.3.10 Determination of PHB using gas chromatography-mass spectrometer (GCMS)....60

3.3.11 Calculations 61

3.3.12 Statistical Analysis 62

3.4 Results and Discussion: 62

3.4.1 Optimization of enzymatic hydrolysis 62

3.4.2 Dilution of alkaline hydrolysate versus growth viability of *C. necator*..................69

3.5 Conclusion 71

3.6 References...71

4 Chapter 4: Summary, Conclusion and Recommendations..73

4.1 Summary and Conclusion 73

4.2 Recommendations 74

List of Figures

Figure 1.1: PHB biosynthetic pathway adapted from Gęsicka et al. (2021) 11

Figure 2.1: Production of PHB using co-fermentation of *P. sacchari* and *C. necator* 23

Figure 2.2: Glucan and xylan content in pretreated biomass under different pretreatment conditions 34

Figure 2.3: Time series data of PHB fermentation for Control 1 and Cnec hdlz 37

Figure 2.4: Comparison of PHB yield in control 1 and Cnec hdlz 38

Figure 2.5: Time series data of PHB fermentation for Control 2 and Psac hdlz 40

Figure 2.6: Comparison of PHB yield in control 2 and Psac hdlz 41

Figure 2.7: Time series data for optical densities (solid lines) and pH (broken lines) of Control 2 and Psac hdlz 42

Figure 2.8: Time series data of PHB fermentation for Control 3 and Comb hdlz 43

Figure 2.9: Comparison of PHB yield in control 3 and Comb hdlz 44

Figure 2.10: Time series data for pH (solid lines) and OD (broken lines) for control 3 and Comb hdlz 45

Figure 2.11: Time series data of PHB production in Cnec hdlz, Comb hdlz and Psac hdlz throughout the fermentation. 46

Figure 2.12: Comparison of PHB yield in Cnec hdlz, Psac hdlz and Comb hdlz 47

Figure 2.13: Comparison between PHB yield of Comb hdlz and Psac hdlz from two batches (I and II) 48

Figure 2.14: Time series data for PHB production over time in Comb hdlz and Psac hdlz two batches (II and I) 49

Figure 3.1: Flowchart of PHB production from unwashed solids with prehydrolysate as in one pot treatment process..........54
Figure 3.2: Efficiencies of methods (a), (b), (c) and (d) used in enzymatic hydrolysis in terms of glucose yield and xylose yield..........63
Figure 3.3: Time series data of glucose and xylose consumption for control, washed LHW solids (WSH), detoxified LHW solids (DTX), undetoxified LHW solids (UNDTX) and alkaline pretreated solids throughout the fermentation..........65
Figure 3.4: Time series data of mean optical densities ± standard deviation depicting *C. necator* growth for control, washed LHW solids, detoxified LHW solids, undetoxified LHW solids and AP solids throughout the fermentation..........66
Figure 3.5: Comparison of PHB (g/l) produced in 24 h, 48 h and 72 h for control, washed LHW solids, detoxified LHW solids, undetoxified LHW solids and (AP) solids throughout the fermentation..........68
Figure 3.6: Comparison of PHB yield produced in 24 h, 48 h and 72 h for control, washed LHW solids, detoxified LHW solids, undetoxified LHW solids and (AP) solids throughout the fermentation..........68
Figure 3.7: Lignin concentration in washed LHW solids, detoxified LHW solids, undetoxified LHW solids and (AP) solids before and after the fermentation...........69
Figure 3.8: Time series for growth of *C. necator* in different dilution ratio hydrolysate media..........70

List of Tables

Table 1.1: Comparison between different pretreatment conditions used in the experiments in this thesis 7

Table 1.2: Summary of the fermentation conditions used in the literature review 14

Table 2.1: Composition of first batch of HS 33

Table 3.1: Hydrolysis conditions for solid and liquid used to produce WSH, DTX, UNDTX and APH 58

Table 3.2: Composition of second batch of HS 62

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Dedication

Dedicated to my loving husband, my whole family and my teachers whose guidance made me who I am today.

1 Chapter 1: Introduction, Thesis objectives and Literature Review

1.1 Introduction

Plastic, once hailed as a miracle, now stands as a global nuisance, its prevalent applications in day-to-day life and excessive durability wreaking havoc on a global scale that has caused environmental and ecological issues from the depths of our oceans to the peaks of our mountains. More than 400 million tons of plastic are manufactured annually across the world and following the present trend, plastic use is projected to almost triple by 2060 (OECD, 2022). However, only about 15% of gross plastic produced is recycled annually, which means continuous piling of plastic wastes at landfills, oceans, and dumpsites in gigantic amounts (Eze et al., 2021). The accumulation of plastic waste in the environment has resulted in severe ecological harm, such as detrimental effects on marine life and the emission of greenhouse gases during the manufacturing process. If the present production and disposal patterns persist, it is predicted that around 12 billion metric tons of plastic waste will accumulate in landfills or in the natural surroundings by 2050 (Geyer et al., 2017). Conventional plastic is processed out of fossil fuels sources, which are non-renewable, via highly energy intensive processes, as a result huge amounts of greenhouse gas are emitted throughout its life cycle accounting alone for approximately 1.8 gigatons of carbon dioxide equivalent (Gt CO_2e), or 3.7% of global emissions (OECD, 2022). Plastics' long-life span ranging from 20 to 500 years has caused issues such as decreasing ratio of landfill capacity to plastic waste amount and microplastic pollution in agriculture and groundwater contamination due to leachate from landfills (Wan et al., 2022). In response to this issue, there is an increasing focus on the advancement of alternatives such as

biodegradable bioplastics, which are made from renewable carbon source and degrade to CO_2 and water, thus reducing the quantity of plastic waste in the environment.

Bioplastics, such as polyhydroxyalkanoates (PHAs), are biodegradable plastics synthesized by microbial cells from renewable resources such as sugars from starch or cellulose, polylactic acids and natural fats (Ali et al., 2023). They have emerged as a promising alternative to traditional petroleum-based plastics. Having properties such as biodegradability, biocompatibility, thermoprocessibility, and flexible strengths, polyhydroxyalkanoates (PHAs) are a group of biologically produced polyesters. The most prevalent member of this family, poly-(R)-3-hydroxybutyrate (PHB), was discovered first in 1925 in *Bacillus megaterium* by Lemoigne (Chee et al. 2010) and has been extensively researched ever since. PHBs have been used in various industrial applications including packaging materials, the development of biofuels, medical implants, and drug delivery systems (Chen et al., 2011). Consequently, the bio-based polymer industry is rapidly growing, with a 2020 market size of around 2.2 million tons, making it approximately 0.5% of the fossil-based polymer industry (Garside, 2021). One of the primary constraints leading to low bioplastic production is the high cost, primarily attributed to the expenses associated with feedstock, which account for most of the production costs and additional land occupation along with long term concerns such as food security and extensive use of associated chemicals such as pesticides. Moreover, bioplastic production is expected to expand to 2.87 million metric tons by 2025, demanding innovation in the existing scenario of renewable feedstock to make it more economically viable and environmentally sustainable (Garside, 2021).

Regarding renewable resources, biomass can be typically categorized into three types – first generation such as food and cash crops, second generation such as agricultural residues, and

third generation such as algae (Ho et al., 2014). Use of first-generation biomass for commercial production of bioplastic has high efficiency in terms of energy input, however, it is associated with a food crisis, as it competes with food production for land, water, and other resources. This raised concerns about bioplastic sustainability and its potential negative impact on food security of the expanding world population. In this scenario, second generation biomass, such as switchgrass, corn fiber, hemp, sugarcane bagasse, paddy straw, sugarcane waste, molasses, glycerol, and waste oil, can be considered as a practical alternative (Sirohi et al., 2020). Among second generation biomass types, agricultural wastes have tremendous capacity to be potential feedstocks due to their abundance and cosmopolitan nature. The cellulose and hemicellulose in these agricultural wastes can be broken down into monomer sugars, such as glucose and xylose, which can serve as carbon source for bacteria like *Paraburkholderia sacchari* to produce PHB inside their cells. The primary hindrance for the chemical breakdown of lignocellulose is recalcitrance of lignin, which protects the cellulose and hemicellulose in cell walls. Various pretreatments, such as physical, chemical and biological methods, have been developed to overcome this hindrance. Hydrolysis of pretreated solids with enzyme is later performed for depolymerization of structural components into oligomers and monomers.

1.2 Thesis objectives

1. Study synergistic effects of two PHB producing bacteria (*P. sacchari* and *Cupriavidus necator*) in increasing PHB yield from hemp stalk hydrolysate as substrate.
2. Reduce water demand by exploring alternative options against washing pretreated solids prior to hydrolysis for PHB production.

1.3 Literature review

1.3.1 Existing PHB production system in industries

As per survey data from EUBIO_Admin (2023), most bioplastic products produced annually are from strong polymers like polylactic acid (PLA), polyhydroxyalkanoates (PHA) and polyamides (PA), among which only 52.1% are biodegradable. PLA has the largest share and PHA has the third largest share of the biodegradable bioplastics market. PLA and PHA are expected to be the primary components of bioplastics within five years with almost half of the production being used in the packaging industries. Boey et al. (2022) and McAdam et al. (2020) compared mechanical properties of bioplastics with conventional plastics and showed that PHA, specifically PHB, has more strength but is more brittle than polypropylene (PP). The brittleness of PHB can be improved by reinforcement with natural or inorganic fillers. To produce PHB, commonly used feedstocks are vinasse, sugarcane molasses, glucose, sucrose and methanol. Glucose sources, being widely available carbon sources with high PHB yield and low substrate processing cost, are extensively investigated (Choi & Lee, 1999). Commercially used microorganisms to produce PHB include *C. necator, Alcaligenes sp., Azotobacter sp., Bacillus sp., Nocardia sp., Pseudomonas sp., and Rhizobium sp.* (McAdam et al., 2020). Cyanobacteria, also known as blue green algae, are another attractive alternative for industrial production of PHB (Carpine et al., 2020). The life cycle assessment performed by Koch et al. (2023) demonstrated significantly reduced environmental impacts using photoautotrophs, such as cyanobacteria, in comparison to use of biomass feedstock. Nevertheless, there are major issues associated with industrial scale-up of photosynthetic cultures such as light availability, oxygen accumulation, culture contamination and nutrient supply (Yashavanth et al. 2021). Regardless of the source of carbon, the recovery of PHB produced as carbon storage in cytoplasm of bacteria is

expensive. One option to reduce the cost of PHB production is to reduce the cost of feedstock. For example, if commercial glucose costing $0.5/kg could be replaced by hydrolyzed corn starch costing $0.22/kg for PHB synthesis by recombinant *E. coli* keeping all other fermentation parameters constant, the production cost of PHB would be reduced from $ 4.91/kg to $ 3.72/kg PHB (Choi & Lee, 1999).

1.3.2 Sources of substrate: hemp stalk

Hemp is commercially produced primarily for its seeds for pharmaceutical purposes and, to a lesser extent, fibers for textiles. It can thrive in a wide range of temperatures and precipitation levels, demonstrating high resilience to adverse soil conditions like high salinity and pest infestations (Manian et al., 2021). The biomass remaining after seed harvest is usually burned, plowed under, composted, fed into anaerobic digestion, or buried. The majority of hemp biomass consists of hemp stalks where bast fibers account for about 20-30% of the stalk while the remaining portion is hemp hurds (Types of Hemp Fibre - Canadian Hemp Trade Alliance, n.d.). Plant cell walls are classified as primary and secondary based on maturity. The primary cell wall, thicker in structure, consists of cellulose, pectin, hemicellulose, and lignin. The secondary cell wall, formed next to the primary, contains cellulose, hemicellulose, and pectin, with varying proportions and compositions across different layers (Manian et al., 2021). Lignin naturally protects plants against pathogens and pests, giving them a hard structure but also making cellulose and hemicellulose difficult to release. To degrade cellulose into sugar monomers, it is necessary to break the lignin seal, hemicellulose sheath, and disrupt the crystalline structure of cellulose. During pretreatment and/or hydrolysis, lignin and hemicellulose decompose, breaking down cellulose into glucose monomers, and hemicellulose into monomers like xylose, with pectin's side-chain substitutions yielding arabinose and galactose (Petit et al., 2019). Hemp

biomass consists of cellulose content ranging 36.5-75.6% and hemicellulose content ranging 10.1-32.8%, which is quite high in comparison to other biomass types such as corn stover, corn fiber, and wheat straw. (Zhao et al., 2020), (Sipos et al., 2010). Hemp fiber alone comprises 53-91% cellulose and 4-18 % hemicellulose boosting the overall glucan and xylan content in hemp stalk (Liu et al., 2017). With potential of high hydrolysis yield greater than 90%, hemp ultimately is a promising feedstock for yielding high sugar concentration hydrolysate and higher gross profit per hectare than kenaf, switchgrass and sorghum (Das et al. 2017).

1.3.3 Pretreatment of hemp stalk

Hemp stalks are structurally built of lignin, hydrophobic in nature, and recalcitrant against any enzymes or other exterior agents. The intricate crystalline structure of cellulose and hemicellulose are enclosed by the lignin, protecting them from the direct access of treatments. Consequently, biomass must be subjected to pretreatment with the primary objective to rupture the lignin structure resulting in the release of cellulose and hemicellulose to make them readily accessible by enzymes for further hydrolysis into monomers. Pretreatment enhances the porosity and increases the surface area of the biomass (Bokhari et al. 2021). As mentioned in Mankar et al. (2021), there are several techniques of pretreatment such as chemical pretreatment (acid, alkaline, organosolv, ammonia fiber explosion), physical pretreatment (milling, extrusion, ultrasound), biological pretreatment and mechanical pretreatment. Each of the techniques has their unique specialty along with some drawbacks. Milling, acid, alkali pretreatment and liquid hot water treatment are some of the common techniques used in industries.

Table 1.1: Comparison between different pretreatment conditions used in the experiments in this thesis

Pretreatment	Condition	Advantages	Disadvantages
Physical pretreatment	Particle size reduction to 2 mm using milling equipment	• Increase biomass surface area • Aids in breaking down crystallinity of structure components	• High energy consumption • Unable to remove lignin from biomass
Alkaline pretreatment	Use of alkali such as NaOH with biomass and treat at high temperature and rotational speed	• Dissolve intermolecular ester bond and remove lignin efficiently • Less caustic than acid pretreatment and forms no inhibitory byproducts	• High capital for corrosion resistant equipment • Challenging to handle pretreatment wastewater • Inefficient reagent recovery
Hydrothermolysis	No use of chemical but treat biomass at high agitation speed and high temperature only	• No chemical used • Easier to handle pretreatment wastewater	• High energy consumption • High water demand compared to alkaline pretreatment

Physical pretreatment such as milling typically involves reducing the particle size of the biomass to enhance the destruction of structural components' crystallinity and aid in overcoming

mass and heat transfer limitations in downstream processes (Bokhari et al. 2021). Biomass is physically deconstructed usually without chemicals in dry or wet state using mechanical forces such as compression, impact, abrasion, attrition and shear caused by interaction between biomass, the surface wall of the reactor, and the impact source. Hammer mill, disk mill, ball mill, roller mill, PFI mill, and knife mill are some of the commonly used milling machines used in biomass pretreatment worldwide (Kim et al. 2016). However, high energy consumption for operation and inability to remove lignin from the biomass are some of the limitations preventing from relying entirely on milling as a pretreatment method (Mankar et al. 2021).

One of the most efficient techniques to disrupt and remove lignin from biomass is alkaline pretreatment. Alkaline chemicals, such as sodium carbonate, ammonia, and hydroxides of potassium, calcium, sodium, and ammonium, dissolved in water are used to pretreat biomass under high temperature to support saponification of intermolecular ester bonds in the lignin-carbohydrate complex and cross-linking xylan in hemicellulose followed by swelling and chemical modification of biomass eventually increasing the porosity and surface area of substrate (Xu and Sun 2016). Sebestyén et al. (2011) demonstrated the significant chemical change in composition of hemicellulose in hemp due to removal of acetyl groups by alkaline treatment along with solubilization of free small lignin units neutralizing formation of potential acidic inhibitors such as acetic acid. Milling together with moderate loading size of alkali such as 0.05 to 0.2 g NaOH per g biomass could yield recovery of more than 90% percent glucan and 80% xylan with no need of detoxification prior to downstream processes (Stoklosa et al., 2024). Moreover, in comparison to acid pretreatment, alkali reagents are less caustic in nature and do not produce as many inhibitory byproducts. Compared to organosolv pretreatment, the chemicals used in alkaline pretreatment are cheaper and it does not involve risks related to volatility and

flammability associated with the chemicals used (Mankar et al. 2021). However, high capital and processing cost due to the need for corrosion resistant equipment, inefficiency in reagent recovery, and difficult to manage pretreatment wastewater are some of the drawbacks associated with alkaline pretreatment of biomass.

Hydrothermolysis, also called liquid hot water (LHW) pretreatment, is an example of physicochemical pretreatment that enhances digestibility of lignocellulosic biomass, producing beneficial structural and chemical alternations while being environmentally friendly. LHW pretreatment uses no chemicals but water as solvent at high temperatures (usually above 160 °C) to solubilize lignin and hemicellulose (Keskin et al. 2019). Xylose recovery of 85% was achieved after treating sugarcane bagasse with LHW pretreatment. During pretreatment, pH was maintained between 4-8 at saturated vapor pressure for 180°C, however, the pH decreased upon heating beyond 200°C due to enhancement of degradation reactions, resulting in an increase in acidic inhibitory products, (Yu et al. 2013). Zhao et al. (2020) achieved 85-98% glucan and 67-71% xylan recoveries by treating industrial hemp with water at 170°C for 30 min, which resulted in less lignin content and more glucose yield after enzymatic hydrolysis than was observed for hemp that underwent dilute acid pretreatment. Formation of inhibitors, such as HMF and furfural, due to high glucan and xylan decomposition was less severe in LHW pretreatment than in dilute acid pretreatment that served as advantage during hydrolysis. However, significant energy input and water consumption are some of the associated drawbacks of LHW pretreatment. Zhuang et al. (2016) reported almost 6 times more energy demand and more than 3 times water consumption per kg sugarcane bagasse (SB) with LHW pretreatment than that with sodium hydroxide treatment. The resource demand for treating SB with LHW pretreatment

combined with aqueous ammonia was comparable to the resources demand for alkaline pretreatment of SB.

1.3.4 Biosynthetic pathway / metabolism

PHB synthesis is a secondary metabolism process that bacteria perform to store excess carbohydrate as a water insoluble accumulated structure in the form of organelles as a preparation for survival during a carbon deficient situation (McAdam et al., 2020). The main enzymes involved during the PHB synthesis process are PHB polymerase and phasin (PhaA, PhaB and PhaC). PHB synthesis starts with accumulation of PHB polymerase followed by the subsequent recruitment of phasin, hence, granule formation is controlled by the cell itself by controlling these polymerase activities. Glucose is broken down into pyruvate, which is oxidized into acetyl- CoA to enter the Kreb's cycle. As the 4-carbon chain proceeds undergoing multiple modification forming intermediates in the cycle, an electron is transported to cause phosphorylation of adenosine diphosphate (ADP) into adenosine triphosphate (ATP) as energy molecules. *C. necator* is reported to control its PHB formation by controlling this phosphorylation (Koning et al., 2023).

As depicted in Figure 1.1, PHB synthesis typically involves 3 steps: 1) condensation of two acetyl-CoA molecules to form acetoacetyl-CoA mobilized by β-ketothiolase (PhaA), 2) reduction of acetoacetyl-CoA into (R)-3-hydroxybutyryl-CoA mobilized by reductase (PhaB) and 3) finally polymerization of (R)-3-hydroxybutyryl-CoA into PHB encoded by enzyme PHB synthase or PhaC gene (McAdam et al., 2020). The polymerase directly binds phasin through its unstructured N-terminal domain during synthesis, however, under starvation, intracellular depolymerase enzyme (PhaZ) catabolizes PHB into the (R)-3-hydroxybutyryl-CoA, which is further catabolized into acetyl-CoA (Yáñez et al., 2022).

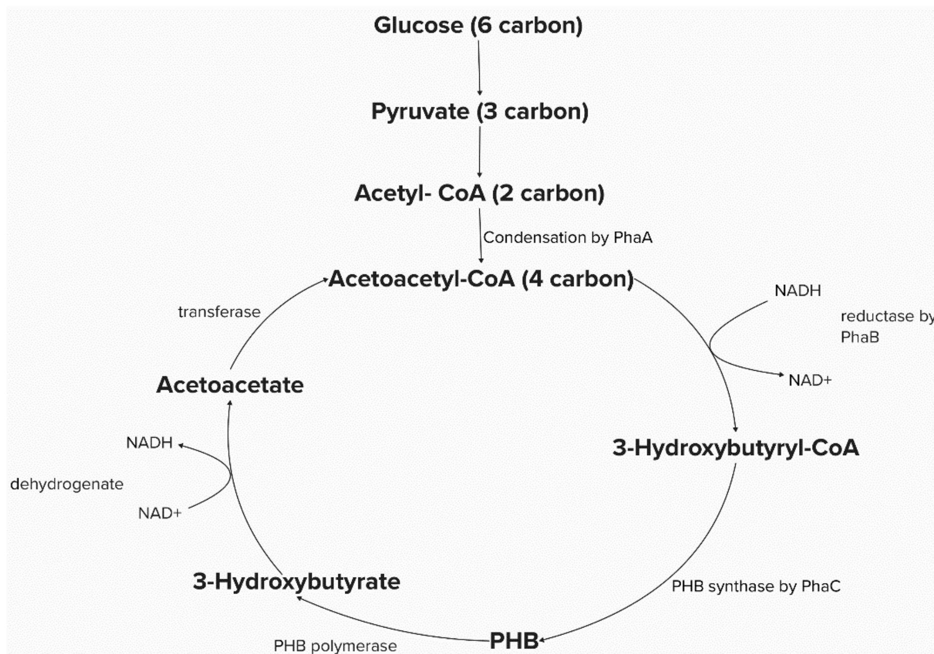

Figure 1.1: PHB biosynthetic pathway adapted from Gęsicka et al. (2021)

1.3.5 Microorganisms: *Paraburkholderia sacchari* and *Cupriavidus necator*

P. sacchari is a Gram-negative bacterium isolated in 1990 from soil of a sugar plantation in Brazil (Gomez et al. 1996). It is capable of metabolizing various inexpensive carbon sources available in biomass hydrolysate including sucrose, hexoses such as glucose, galactose, and mannose, and pentoses such as xylose and arabinose under optimized fermentation conditions (Nascimento et al. 2016, Dietrich et al. 2019, Li and Wilkins 2021). *P. sacchari* is facultative for survival, however, it demands aerobic conditions to grow extensively and accumulate PHB. Widely known for its ability to conferment multiple sugars against carbon catabolite repression, Dietrich et al. (2019) also reported *P. sacchari* to accumulate PHB up to 71% of its cell dry weight (CDW) while consuming five sugars from hardwood holocellulose hydrolysate after 48 h. Oliveira-Filho et al. (2021) emphasized *P. sacchari* as a bacterial chassis with reference to its low

biological risk nature and high PHB yields from sucrose (0.29 g/g) and xylose (0.38 g/g) along with product versatility such as xylitol and xylonic acid. However, citric acid is preferred by *P. sacchari* over sugars, hence, uptake of glucose starts only after citric acid is consumed while consumption of glucose suppresses xylose consumption rate in high glucose media (Domingues et al. 2021).

C. necator, formerly known as *Ralstonia eutropha*, is widely recognized for its robust capability to produce PHB. Being the first strain to be used for commercial PHB production, this bacterium can efficiently convert a variety of simple to complex carbon sources, including simple sugars, oils, glycerol, lignin and organic acids, into PHB, making it an attractive candidate for bioplastic production (Zhang et al. 2022). For instance, Hänisch et al. (2006) reported that *C. necator* could accumulate PHB up to 90% of its CDW with high cell density. The genetic and metabolic pathways of *C. necator* have been extensively studied, easing metabolic engineering efforts aimed at enhancing PHB yield and productivity. Genetic modifications, such as the overexpression of PHB synthase genes and the deletion of competing pathways, have led to significant improvements in PHB accumulation even in oxygen limiting conditions (Tang et al. 2020). Additionally, various fermentation strategies, including fed-batch and continuous processes, have been optimized to maximize PHB production while the ability of *C. necator* to use renewable feedstocks such as agricultural waste and industrial by-products further underscores its potential for sustainable bioplastic production. Li and Wilkins (2020) achieved up to 4.5 g/l PHB accumulation from lignin-derived compounds contained in alkaline pretreatment liquor from corn stover using a fed-batch reactor. As the metabolic engineering of recombinant *C. necator* advances to improve its inability to utilize sugars naturally, researchers have already succeeded in making a *C. necator* strain that can convert glucose primarily into

PHB (Olavarria et al. 2022). Biglari et al. (2018) produced 6.8 g/l PHB with 76 % CDW from 25 g/l glucose after 48 h. However, its ability to use sucrose and co-ferment pentoses alongside hexoses are some of the characteristics that researchers are still exploring.

1.3.6 Fermentation

PHB fermentation primarily focuses on optimization of nutrients and technical parameters such as pH, temperature, agitation speed, and oxygen availability for proper bacterial growth in media. Batch fermentation and fed-batch fermentation are some of the widely used techniques for PHB fermentation. Batch culture is known for its flexibility and low processing cost while fed batch can achieve high cell densities and high PHB yield due to its control over each technical parameter such as pH, feeding strategy and dissolved oxygen. (Lopez-Arenas et al., 2017).

Flask culture or stirred bioreactors can be used in the laboratory to represent batch fermentation. Khattab & Dahman (2019) used hemp hurds hydrolysate to produce 13.4 g/l PHB after 80 h of batch fermentation in flasks rotated at 200 rpm with a temperature of 30°C using *C. necator*. The maximum sugars obtained from the enzymatic hydrolysis with enzymes (cellulase, cellobiase and xylanase) was 53 g/l of both glucose and xylose combined. Paul et al. (2023) diluted a hydrolysate containing 56 g/l glucose obtained from 92% efficient enzymatic hydrolysis of industrial hemp to 20 g/l glucose to ferment with *E. coli* at agitation speed of 200 rpm at a temperature of 30°C. The *E. coli* strain produced1.77 g/l PHB in 48 h. Cesário et al. (2014) adopted both batch and fed batch fermentation to produce PHB via *P. sacchari* from wheat straw hydrolysates and compared it with PHB obtained by fermenting commercial sugars in the same concentration. In flask culture, maximum PHB produced using undiluted wheat straw hydrolysate (WSH) was 2.4 g/l. When WSH was diluted by two-fold, the PHB

concentration produced nearly doubled to 4.4 g/l. Apparently, undiluted WSH supported similar cell biomass growth with less sugar consumption and hence less PHB production than control. The increased amount of consumed sugars and almost doubled PHB concentration in diluted WSH accordingly supports the hypothesis that *P. sacchari* may prefer other rapidly consumable carbon sources, such as citrate that was present as a buffer from the enzymatic hydrolysis, over sugars available in undiluted hydrolysate resulting less PHB production despite similar cell growth as that in diluted hydrolysate. PHB yield of 0.19 g/g hydrolysate sugars was observed in the shake flask experiment, which was greater than that of 0.18 g/g commercial sugars. Maximum PHB yield reported was 0.22 g PHB/ g hydrolysate sugars in 2 L stirred- tank reactor with 1.6 g/l per h productivity.

Table 1.2: Summary of the fermentation conditions used in the literature review

Fermentation conditions	Substrate	Organism used	PHB production	Reference
Batch fermentation at 200 rpm, 30°C for 80 h	Hemp hurd	*C. necator*	13.4 g/l	Khattab & Dahman (2019)
Diluted 56 g/l glucose media to 20 g/l and ferment in batch mode at 200 rpm, 30°C for 48 h	Industrial hemp	*E. coli*	1.77 g/l	Paul et al. (2023)
Batch fermentation (undiluted)	Wheat straw	*P. sacchari*	2.4 g/l	Cesário et al. (2014)

Batch fermentation (two-fold diluted)			4.4 g/l	
Shake flask experiment at 32°C	Wheat straw Commercial sugars	*P. sacchari*	0.19 g PHB/ g sugars 0.18 g PHB/ g sugars	Cesário et al. (2014)
Shake flask experiment for 30 h under alternating dissolved oxygen concentration from (3 ppm, 0.5 ppm) every 60 min	Commercial glucose	*Lactobacillus delbrueckii* + *C. necator*	0.237 g PHB/ g sugars	Tohyama et al. (2002)
Shake flask experiment at 30 °C, 200 rpm for 72 h	Commercial glucose and xylose (3:1 ratio)	*Bacillus sp.* + *C. necator*	2.12 g/l (PHB production enhanced by 40%)	Lee et al. (2021)
Shake flask experiment at 30 °C, 180 rpm and pH 7	Lignin and sugar	*Pseudomonas putida*	1.38 g/l	Arreola-Vargas et al. (2021)

Coculture techniques have been adopted in several experiments showing effectiveness in co-fermentation of glucose and xylose for product enhancement. Tohyama et al. (2002) modeled the combined use of first *Lactobacillus delbrueckii* to convert glucose into lactate and then *C. necator* to eventually convert that lactate into PHB achieving maximum PHB yield of 0.237g/g in 30 h. The researchers varied the dissolved oxygen concentration at either 3 ppm or 0.5 ppm

every alternating hour. This subsequent conversion of substrate into metabolite and metabolite into final product was reported to be promising if the culture conditions were properly controlled. Lee et al. (2021) used co-culturing of *Bacillus sp*. SM01, that can utilize xylose to produce PHB, and *C. necator*, that convert glucose into PHB, to enhance the PHB production by approximately 40% than that produced by monoculture at the glucose: xylose ratio 3:1.

Similarly, co-utilizing multiple components in the substrate, such as lignin and sugars, in the hydrolysate has emerged as an attractive alternative advocating integrated biorefineries. These approaches not only alleviate the dependence on sugars as the sole feedstock but also provide new economic perspective to process unwashed solids with reduced processing steps and water resource demand. Arreola-Vargas et al. (2021) produced medium chain length PHAs (mcl-PHA) at a concentration up to 1.38 g/l via co-fermentation of lignin and sugars released in alkaline pretreated corn stover hydrolysates with an engineered strain of *Pseudomonas putida*. Sugar-rich hydrolysates were reported to promote cell biomass growth and increase in PHA titer by 71% in comparison to using lignin as the sole substrate. The increase in soluble substrate concentrations in medium increase the cell dry weight (CDW) in the media while decreasing the PHA content and PHA yield due to potential inhibition caused by increased concentration of degradation products such as weak acids, furans and phenolic compounds (Liu et al. 2019).

1.3.7 Characterization of PHB

For characterization of PHB contained in bacteria samples from fermentation, there are multiple methods to adopt including UV-visible absorption spectroscopy, Fourier transform infrared spectroscopy (FT-IR), gas spectroscopy-mass spectrometry (GC-MS), nuclear magnetic resonance (NMR), high-performance liquid chromatography (HPLC), and differential scanning calorimetry (DSC) as explained in (Xu et al. 2010). Hassan et al. (2016) explains thermal

analysis of purified PHB samples using a thermogravimetric analysis instrument as an additional method of assessing thermal stability of PHB. ATR-FTIR spectroscopy was used by (Thapa et al. (2019) to analyze the functional groups present in PHB. The prominent groups were CH_3, CH_2, C-O, C=O, OH, and CH and they played a crucial role in confirming the presence of PHB. Juengert et al. (2018) analyzed PHB granules by fluorescence microscopy qualitatively using Nile Red staining (also known as Nile blue oxazone) while Wang et al. (2014) determined PHB content of cells by measuring crotonic acid from acid-catalyzed depolymerization of PHB in acid column HPLC. Options of multiple methods that can be employed for the qualitative analysis of PHB granules as well as for the quantitative assessment of PHB content, enhances the overall understanding of PHB properties.

1.3.8 Conclusion

PHB, having comparable mechanical properties with conventional petroleum plastics and being environmentally friendly, is one of the most researched and explored microbial products from renewable feedstock. This thesis is focused on exploring possibilities of the production of PHB from hemp stalk as feedstock biomass in multiple prospective techniques. Pretreatment methods such as alkaline treatment and LHW pretreatment can be used to overcome the recalcitrant nature of lignocellulose structure with few inhibitory degradation products in hydrolysates for fermentation. In the second chapter, hemp stalk hydrolysates were subjected to fermentation in three treatment methods using individual and combined application of *C. necator* and *P. sacchari* and their potential to produce PHB was observed. The third chapter primarily addresses the co-fermentation of lignin with sugars obtained from enzymatic hydrolysis of unwashed solids simulating one pot treatment condition.

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2 Chapter 2: Investigation of synergistic effects of *P. sacchari* and *C. necator* for production of PHB from hemp hurds

2.1 Abstract

This study aimed to attain high PHB yield using alkaline pretreated hemp stalks as a feedstock for microbial fermentation, hence, we attempted to optimize the different parameters involved in producing PHB. Hemp was pretreated with 1% sodium hydroxide at 130 °C for 1 h which enhanced the hydrolysis yield up to 97.91% for glucose and 99.75% for xylose. During batch fermentation, nitrogen and phosphorus content were increased which enhanced cell growth and sugar consumption rates. 50 mM phosphate buffer was used for pH control. which reduced the rate of pH decline throughout the experiment. While *Cupriavidus necator* alone in hydrolysate produced maximum PHB yield of 0.433 g/g sugars at 48 h, combined fermentation of *Paraburkholderia sacchari* and *C. necator* showed synergistic effects in co-fermentation of sugar and xylose while also increasing PHB yield up to 0.341 g/g sugars in comparison to using *P. sacchari* alone. Higher PHB yields were achieved in hydrolysates than in control.

2.2 Flowchart

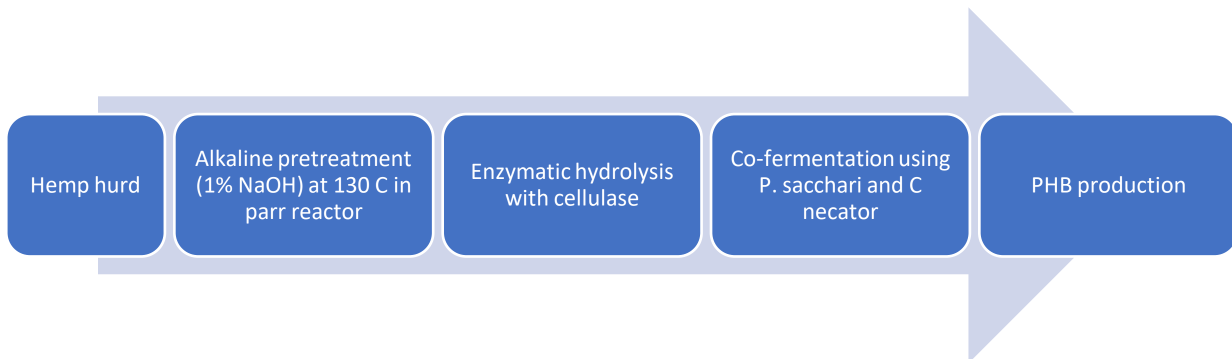


Figure 2.1: Production of PHB using co-fermentation of *P. sacchari* and *C. necator*

2.3 Introduction

The increasing demand for plastics to advance lifestyles and the resulting plastic pollution with decreasing plastic management options has been a critical global issue. Petrochemical based plastic products are also problematic due to their exceedingly long decomposition time, ranging from 20 years to 500 years, leading to serious environmental consequences. This prolonged breakdown time makes it challenging to dispose of them in an environmentally responsible manner. Bioplastics that are biodegradable, biocompatible, and produced using renewable sources of raw materials, could replace petrochemical based plastics. Polyhydroxybutyrate (PHB) is a commercially used bioplastic with expanding use in the packaging, cutlery, and pharmaceutical industries. PHB has physical and mechanical properties comparable to non-renewable plastics in terms of tensile strength, melting temperature, and transparency (McAdam et al. 2020). However, PH's higher degree of crystallinity results in a stronger, stiffer, and yet more brittle plastic than alternative conventional plastics, which is something researchers are working on to improve (McAdam et al. 2020). Similarly, the current market price of PHB is more than three times higher than that of petroleum-based plastics, with feedstock accounting for the majority of the total production cost (Manikandan et al. 2021). Lignocellulosic biomass, being low cost, renewable and cosmopolitan in nature, could serve as a sustainable feedstock to produce PHB on a commercial scale.

While sources of cheap yet feasible sources of feedstock are being extensively explored, commercially available simple sugars are too expensive for industrial PHB production. On the other hand, agricultural wastes are something that industries are investing huge amounts in to manage. Valorization of agricultural residues as feedstock for commercial PHB production could address multiple industrial issues in terms of sustainability, economy, and environmental

management. Switchgrass, sugarcane bagasse, corn stover, sawdust, and wheat straw are some of the commonly used agricultural wastes with high sugar yield. Likewise, hemp, also known as *Cannabis sativa*, having multiple benefits, can be considered as a viable alternative. Ability of hemp to grow comparatively faster, even in marginal land, not requiring uses of pesticides or fertilizers and most importantly one of the rich sources of glucan and xylan content, that later produce glucose and xylose for fermentation into bioplastic, makes it competent and viable source for bioplastic (Nath, 2022). Nevertheless, these biomasses must undergo though multiple pretreatment and hydrolysis steps in prior to subjecting for production of PHB due the recalcitrance nature of lignin, which is responsible to protect intricated structure of cellulose and hemicellulose in nature.

Hemp is commercially produced predominantly for its seeds, commonly used in pharmaceuticals and supplements, and secondarily for bast fiber used in textiles. Hemp agricultural waste consists of cellulose, hemicellulose, and lignin, which upon subjection to pretreatment and enzymatic hydrolysis, break down into glucose and xylose by disruption of lignin (Yoon et al., 2014). Microorganisms such as *Paraburkholderia sacchari* and *Cupriavidus necator* consume those sugars and store excess carbon in the form of PHB in their cells, up to 67% and 90% of their dry weight, respectively (Oliveira-Filho et al., 2021, Sen et al., 2019). Upon reaching a certain level of PHB, cells are harvested, and bioplastic is extracted from the cells. *P. sacchari* is well known for its ability to consume both glucose and xylose and converting them into PHB (Oliveira-Filho et al., 2021), however, does not have much history of producing PHB in high sugar concentrated media in batch fermentation unless diluted and performed fed-batch in bioreactors. Meanwhile, as Gang et al. (2019) and Khattab and Dahman (2019) have shown, *C. necator* can produce high PHB such as more than 10 g/l with high sugar concentration

in media during batch fermentation. *C. necator* preferably consumes hexoses to produce PHB consequently, the probability of unused sugars at the end of the fermentation is higher than *P. sacchari*. Furthermore, the ability of *C. necator* to utilize lignin derived monomers like vanillin and inhibitory aromatics in the media such as benzoic acid and p-coumaric acid, can leverage efficiency against inhibition to produce PHB from hydrolysates. Using these two bacteria in the same fermentation could unleash new possibilities such as co-fermenting two main sugars in the media as well as yielding high PHB production in batch fermentation without dilution.

This chapter focuses on production of PHB in two conditions: 1) using hydrolysate from washed solids via combination of these two bacteria emphasizing the advantage of dissimilar nature of two different bacteria, 2) from hydrolysate of unwashed solids, pretreated and hydrolyzed with different pretreatment techniques simulating one pot treatment method.

2.4 Materials and methods

2.4.1 Hemp as carbon source

Locally produced hemp stalk (HS) was obtained from the Department of Agronomy at Kansas State University and stored at room temperature in an open bag in a dry condition. HS was milled subsequently using 6 mm and 2 mm sieve in a hammer mill with speed controller (Benshaw, RSI003S42W, IP66, Pittsburgh, USA) at the rotational speed of 4500 rpm. The moisture content of 2 mm size HS was checked using a Moisture Analyzer (HE73, 115 V, Mettler-Toledo, LLC, Columbus, OH, USA). If the moisture was less than 8%, it was stored in air-tight containers; otherwise, it was dried at room temperature overnight prior to storage.

2.4.2 Compositional analysis of hemp

For preparation of HS to be analyzed for composition, ground HS was sieved according to the procedure explained in Hames et al. (2008). Ash content of sieved biomass was calculated as per Sluiter (2008a) and biomass was further processed to calculate extractives content following procedure of Sluiter (2008b). The remaining solid after subsequent sieving and extraction was subjected to compositional analysis in accordance with the procedure by (Hames et al. 2008).

2.4.3 Pretreatment of hemp

Sodium hydroxide solution (NaOH) was used for alkaline pretreatment of HS in a 1 l floor stand Parr Reactor with pneumatic lift (4577 HP/HT Pressure reactor, Parr Instrument Co., Moline, IL, USA). 50 gm dry mass of HS was used with 5 gm of NaOH in 450 ml of HPLC water to ensure a 10% solid to liquid ratio in the final mixture (i.e. 0.1 g NaOH/ g HS). The pretreatments were carried out at the varying concentration range of NaOH from 1-2 % (w/v) and temperature range between 130 °C – 170 °C for time range between 30 - 120 min until optimized at 1% NaOH and 130 °C for 60 min on the basis of the compositional analysis of pretreated solids. The stirrer speed was maintained constant at 400 rpm throughout each experiment. After completion of the pretreatment, the HS content was cooled down to less than 50 °C by continuously circulating running water at room temperature though the reactor cooling coil. Later, pretreated mixture was passed through a coffee filter using vacuum filtration to segregate solids and prehydrolysate, which were stored separately at 4 °C in closed containers. Compositional analysis was performed for both solids and prehydrolysate following procedures developed by the National Renewable Energy Laboratory (NREL) (Sluiter et al. 2008).

Pretreated solids were further hydrolyzed with cellulase enzyme to prepare hydrolysate for fermentation.

2.4.4 Enzymatic hydrolysis

Refrigerated pretreated solids were washed with very dilute sulfuric acid of (0.06 mM) until the pH was normalized from greater than 8.5 to approximately 5 in a vacuum filtration apparatus. After the liquid was drained out, the moisture content of solids was determined, and the dry weight of the solids was calculated. Approximately 10 g dry weight solids were added to each 250 ml Kimax Baffled flask along with 50 mM acetate buffer at pH 5 and cellulase enzyme (Cellic CTec3 HS, Novozymes, Bagsvaerd, Denmark) at a concentration of 20 FPU/g glucan to attain 10 % solids loading in the mixture. Pretreated solids in the flasks were autoclaved at 121 °C for 20 min. The enzyme activity of the cellulase was determined in FPU by performing Filter Paper Assay procedure (Adney and Baker 2008). After cooling down the solids, cellulase enzyme along with pH 5 acetate buffer was added to the flasks and covered with silicon sponge closures. The mixture was incubated in a shaker at agitation speed of 200 rpm for 72 h at 50 °C.

After 72 h in the shaker, the contents were filtered using a coffee filter in a vacuum filtration apparatus. The solids on the paper were discarded while the liquid hydrolysate obtained was stored in refrigerator at 4 °C for fermentation.

2.4.5 Optical density measurement and cell mass calculation

A spectrophotometer (BioMate 3, 335905P, Thermo Electron Co, Madison, WI, USA) was used to measure optical density (OD) of cultures to measure cell mass concentration. In order to find the linear relationship between optical density and cell mass in a given sample for each of the bacteria, monoculture media was diluted in 4 different proportions (0%, 25%, 50%

and 75%) with HPLC water. Each dilution was mixed by pipette and optical densities of each dilution were taken. The dilutions were then transferred into 2 ml centrifuge tubes. The tubes used were preheated for 24 h in an 80 °C oven and cooled down to room temperature in a desiccator to take their weights prior to transferal of samples into them. The tubes with samples were centrifuged at the rotational speed of 12,000 rpm for 5 min and supernatant was discarded. The tubes containing cell pellets were dried for 24 h in an 80 °C oven overnight and weights were taken after cooling down to room temperature to plot OD versus cell mass curves.

2.4.6 Microorganism and inoculum

Two bacteria were used in this experiment: *C. necator* (DSM 545) and *P. sacchari* (DSM 17165). Both bacteria were purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany) and were obtained in the form of dry pellets. The pellet of *C. necator* was regenerated in 20 ml seed medium containing meat extract (3 g/l) and meat peptone (5 g/l) as suggested by DSMZ. First-generation cells (100 µl) were sub-cultured in 20 ml seed medium containing meat extract (3 g/l), meat peptone (5 g/l) and glucose (30 g/l) for a second generation. *P. sacchari* was regenerated up to two generations in 25 ml seed medium for 22-24 h for each generation. Bacteria subcultures were grown at 30 °C for 24 h in 250 ml baffled flasks with silicon sponge closures while mixing at 250 rpm. A culture with OD 10 was stored in 50 % glycerol in 2 ml cryovials in a -80 °C freezer for future use.

Before fermentation, one cryovial of each bacterial species from the -80°C freezer was inoculated into a positive control sugar solution until the OD of 8-10 was reached in 24 h. The culture was centrifuged at the centrifugal force of 1509 × g for 10 min. The supernatant was discarded, and the cell pellet was washed with 0.89% NaCl solution twice before using as

inoculum. Inoculum was added to each flask with fermentation media to attain a starting OD of at least 1.

2.4.7 PHB production

For PHB production, hemp stalk hydrolysate was the main source of carbon while 1 g/l $(NH_4)_2SO_4$ and 1 g/l yeast extract was added as nitrogen sources. In addition, the fermentation media was supplemented with 4.5 g/l $Na_2HPO_4 \times 2H_2O$, 1.5 g/l KH_2PO_4, 0.2 g/l $MgSO_4 \times 7H_2O$, 0.01 g/l $CaCl_2 \times 2H_2O$, 0.06 g/l $Fe(NH_4)_3$ citrate and 1ml/l trace element solution, as specified by Ramsay (1990), in 50mM phosphate buffer to maintain pH at 6.8. The pH of fermentation media with hydrolysate was adjusted with 5 M H_2SO_4 and 5 M NaOH to pH 6.8 and the final mixture was passed through a 0.2 mm membrane filter for sterilization. After addition of inoculum, fermentation was carried out at continuous agitation of 250 rpm at 30 °C for 72 h in 250 ml baffled flasks with silicon sponge closures. The working volume of fermentation media was 100 ml with 10% inoculum volume and each treatment in the experiment was done in triplicate. Controls were prepared by mimicking the sugar and salts concentration in the hydrolysate for each treatment. Samples were collected at 0, 24, 48 and 72 h for analysis of PHB concentration, pH, sugar concentration and optical density.

For inoculum of both bacteria (*P. sacchari* and *C. necator*), one vial of cells for each bacterium was incubated in positive control solution at 30 °C for 24 h. The cell culture was centrifuged and washed twice with 0.89% NaCl as stated in section 2.4.6 and then, suspended in 81 ml sterile HPLC water. Consequently, 9 ml of that suspension was added to each flask (such as 3 controls and 3 hydrolysates). For combined treatment, 4.5 ml of the suspension of each bacterium was added to each flask (such as 3 combined controls and 3 combined hydrolysates).

2.4.8 High-performance liquid chromatography (HPLC) sugar analysis

For monomer sugars, each sample was analyzed using the high-performance liquid chromatography (HPLC) equipment (G1316A, Agilent Technologies 1200 Series, Santa Clara, CA, USA) after filtering though the 0.2-micron PES filter into a 2 ml HPLC vial. For viscous sample from fermentation media with bacteria, each was centrifuged at the centrifugal force of 12879 × g for 5 min and the liquid supernatant was filtered and analyzed. An Aminex HPX-87P column (300 × 7.8 mm, Bio-Rad Lab, Hercules, USA) was used to analyze the compounds.

2.4.9 PHB quantification

For PHB quantification of each sample from fermentation, the procedure explained in Dai et al. (2015) and Juengert et al. (2018) was followed. Each sample (2 ml) was centrifuged in a 15 ml polypropylene tube at the centrifugal force of 2054 × g for 10 min. The supernatant was discarded, and the centrifuged cell pellets were washed with 0.89% NaCl solution twice. The cells were dried overnight at 55 °C in oven until constant dry weight of cells was achieved. 2 ml each of methanol acidified with 3% v/v 72% H_2SO_4 and chloroform was added to dried cells in a glass tube and incubated at 105 °C in an oven for 3 h. After that, samples were cooled down in an ice water bath for 5 mins. HPLC grade water (1 ml) was added to each sample and vortexed vigorously for 30 s before letting stand until two phase separation was observed. The organic phase (liquid at bottom) was retrieved with glass pipette and dried using anhydrous sodium sulphate before putting it in a vial for analysis in GCMS. External standards were prepared using 4 mg, 8 mg, 12 mg, 16 mg, 20 mg, 24 mg of sodium DL-3-hydroxybutyrate (H0231, TCI America, Portland, OR, USA) following the same procedure.

2.4.10 Gas chromatography mass spectrometer (GCMS) for PHB quantification

PHB was quantified using gas chromatography mass spectrophotometer (GCMS- Agilent Technologies, 6890 GC, v N.86.07) using Agilent column (30 m×250 µm ×0.25 µm) ZB-1701. The injector temperature was 230 C, and the injection mode was split with 60:1 split ratio. The pressure of the carrier helium was 8.2 psi with constant flow rate of 1 ml/min through column. 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. The temperatures of MS source temperature and the MS quad temperature were 230 °C and 150 °C, respectively. Data analysis was performed using chromatography studio (MassHunter GC/MS Acquisition B.07.06.2704, Santa Clara, CA, USA).

2.4.11 Calculations

Glucose and xylose yields were calculated as follows:

$$\text{Glucose yield} = \frac{\text{glucose}\left(\frac{g}{l}\right) + \text{cellobiose} * \frac{360}{342}\left(\frac{g}{l}\right)}{\text{mass fraction of glucan} * 1.11 * \text{pretreated solids concentration in hydrolysis}\left(\frac{g}{l}\right)} \times 100\%$$

Eq 2.1

$$\text{Xylose yield} = \frac{\text{xylose}\left(\frac{g}{l}\right)}{\text{mass fraction of xylan} * \frac{150}{132} * \text{pretreated solids concentration in hydrolysis}\left(\frac{g}{l}\right)} \times 100\%$$

Eq 2.2

In order to calculate PHB yield, following formula was used:

$$\text{PHB yield} = \frac{\text{PHB concentration at t time}}{\text{Sugar concentration}(t0) - \text{Sugar concentration}(t)} \qquad \text{Eq 2.3}$$

Where PHB and sugar concentrations (g/l),

t0 = 0 h and t = 24 h, 48 h, and 72 h.

2.4.12 Statistical analysis

Data were presented as mean ± one standard deviation. Comparisons between means were conducted using analysis of variance (ANOVA) using SAS (Version 9.4 M7, SAS Inst. Inc., Cary, NC, USA). Means from different independent variables were separated using Fisher's protected least significant difference method. The statistical difference between the different groups of data was studied at a 95% confidence interval.

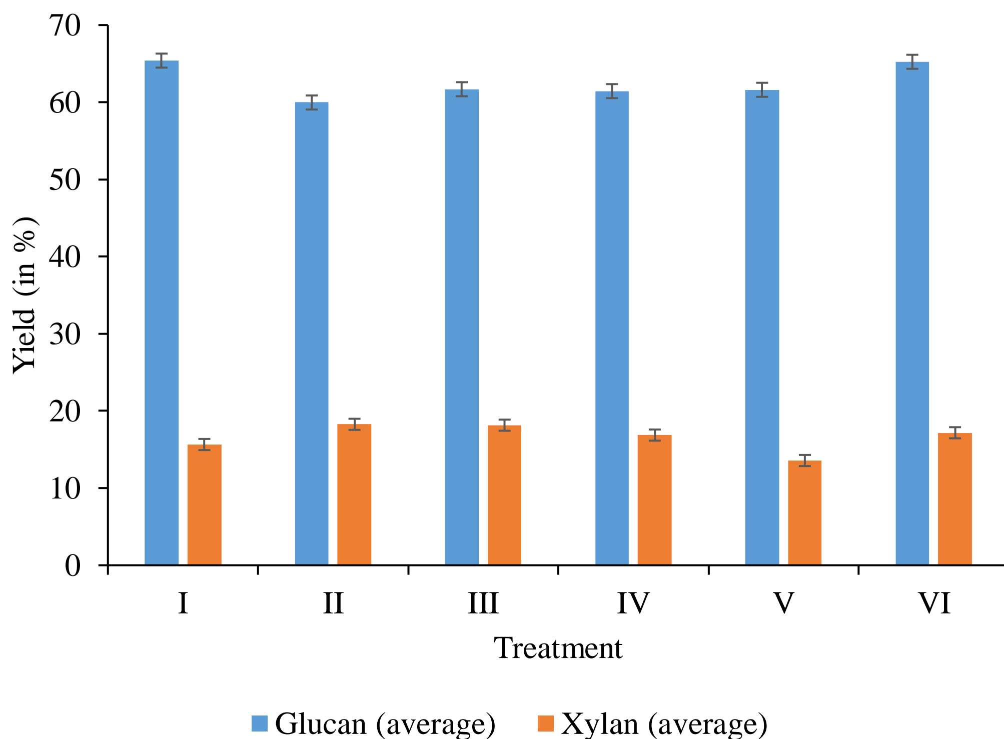
2.5 Results and discussion

2.5.1 Optimization of pretreatment of HS solids

HS was analyzed to determine the composition of structural polysaccharides, lignin, and other cell wall components. The composition is in **Error! Reference source not found.**.

Table 2.1: Composition of first batch of HS

Components	Content (%)
Glucan	46.42
Xylan	12.65
Galactan	2.45
Arabinan	1.4
Mannan	3.39
Lignin	15.14
Extractives	13.3
Ash	3.15



Treatment	I	II	III	IV	V	VI
Temperature	170°C	130°C	130°C	130°C	130°C	130°C
Pretreatment time	30 min	30 min	1 h	1 h	1 h	2 h
NaOH concentration	1%	1.5%	1.5%	2%	1%	1%

Figure 2.2: Glucan and xylan content in pretreated biomass under different pretreatment conditions

Figure 2.2 presents the average glucan and xylan content in pretreated hemp stalks subjected to various pretreatment conditions, indicating the efficiency of each condition. Under the conditions of 1% NaOH at 170°C for 0.5 h, the glucan content was the highest at 65.39%, with a xylan content of 15.65% followed by 65.23% glucan content in prolonged pretreatment condition up to 2 h at 130°C and 1%NaOH. For the condition of 1.5% NaOH at 130°C for 0.5 h, glucan content was slightly lower at 59.97%, while xylan content was 18.26%. When the duration was increased to 1 h at the same concentration and temperature, the glucan content rose

to 61.69%, and xylan content was 18.15%. With 2% NaOH at 130°C for 1 h, the glucan content was 61.44%, and xylan content was 16.87%. For the conditions of 1% NaOH at 130°C for 1 h, glucan content was 61.61%, and xylan content was 13.57%. Lastly, extending the time to 2 h at 1% NaOH and 130°C resulted in a glucan content of 61.20% and a xylan content of 16.08%. Overall, glucan content ranged from 59.97% to 65.39%, while xylan content varied from 13.57% to 18.26%, indicating some sensitivity to pretreatment conditions.

No significant difference ($p<0.05$) was found in glucan contents obtained from the treatments with varying NaOH concentration, however, treatments with 1.5% and 2% NaOH showed significantly higher xylose content than rest of the treatments. Similarly, prolonged pretreatment condition up to 2 h and increase in temperature up to 170°C resulted in significantly higher glucan and xylan contents while treatment "1%, 130°C, 1 h" achieved the least xylan content compared to the rest of the treatments. Hence, pretreating further HS at 130°C using 1% NaOH for 1 h was chosen to accumulate least xylose content possible in the hydrolysate considering that both *C. necator and P. sacchari* prefer glucose over xylose. Also, less time, energy, and NaOH are being used for treatment "1%, 130°C, 1 h" to produce glucan content comparable to that produced with increased pretreatment duration, temperature and NaOH.

2.5.2 Effects of bacterial combination on PHB yield

HS hydrolysates and a control sugar mixture mimicking the sugar concentration in hydrolysates were fermented with three treatment factors, *C. necator* only (Cnec hdlz), *P. sacchari* only (Psac hdlz), and combination of both bacteria (Comb hdlz) as an attempt to explore the feasibility of HS hydrolysates as a sustainable, cost-effective renewable PHB feedstock.

2.5.2.1 Comparison of control 1 and C. necator only hydrolysate (Cnec hdlz) - PHB conc and yield

Figure 2.3 shows the trend of glucose consumption (g/l) and PHB production (g/l) during every timepoint of the fermentation of control 1 and Cnec hdlz. The fermentation started with 52.66 g/l glucose and 14.54 g/l xylose in the media for both Control 1 and Cnec hdlz among which 84% of glucose was consumed in Cnec hdlz to produce a maximum PHB concentration of 15.53 g/l in 72 h. Xylose concentrations were consistent throughout the fermentation in both control 1 and Cnec hdlz except in the last 24 h for which the consumption was 1 g/l because *C. necator* prefers glucose over xylose. Over twofold residual glucose remained at the end of the fermentation in Control 1, compared to Cnec hdlz, reduced the PHB yield for Cnec hdlz compared to Control 1, even though approximately 2 g/l more PHB was produced in Cnec hdlz than in control 1 at 48 h and 72 h. This could be attributed to the additional lignin derivatives and helpful acids such as acetic acid in the hydrolysate under inhibiting limit such as 0.5 g/l (Marudkla et al. 2018) that favored more consumption of sugars in comparison to control (Wang et al. 2014). However, only 0.5 g/l and 1.3 g/l of xylose was consumed in Control 1 and Cnec hdlz, respectively, by *C. necator*.

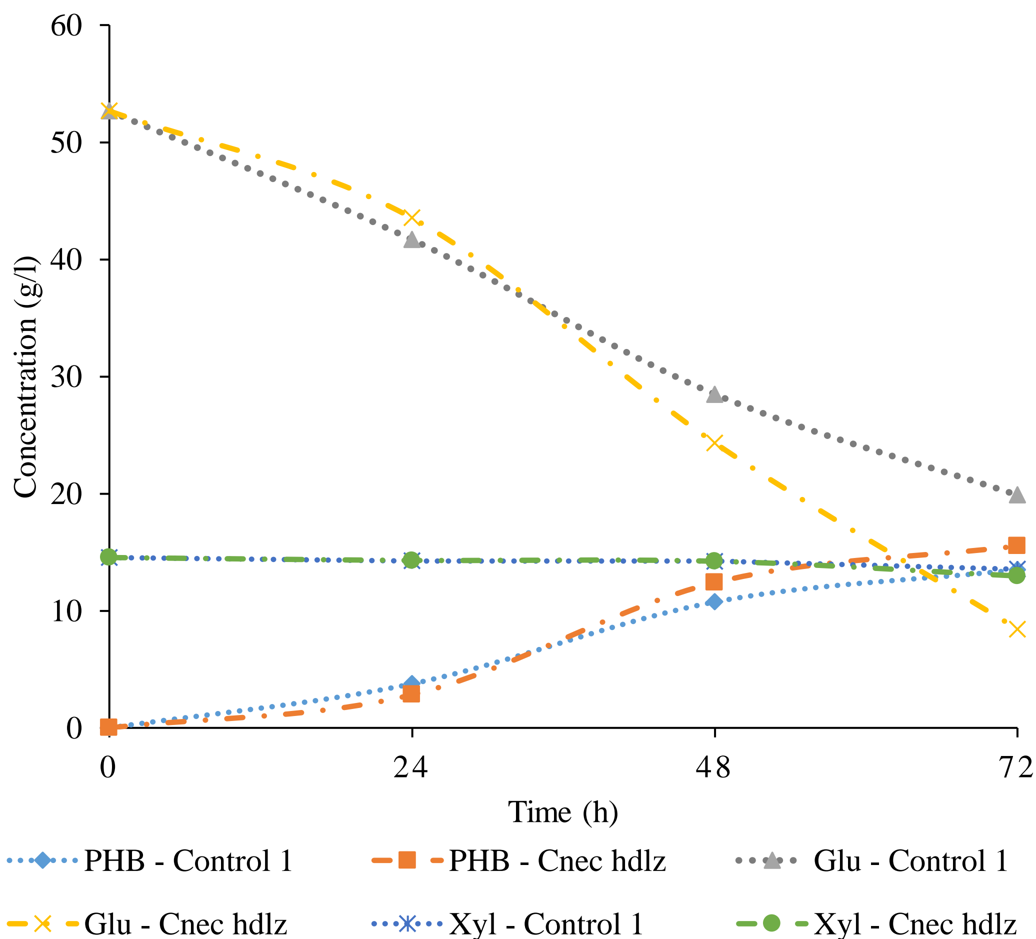


Figure 2.3: Time series data of PHB fermentation for Control 1 and Cnec hdlz

Figure 2.4 displays the PHB yields from the fermentations of Cnec hdlz and Control 1. The PHB produced in control 1 was 3.76 g/l, 10.75 g/l and 13.49 g/l with PHB yield of 0.334 g/g, 0.439 g/g and 0.399 g/g after 24, 48 and 72 h respectively as illustrated in Figure 2.3 and Figure 2.4. Comparable PHB yields were observed in Cnec hdlz which were 0.302 g/g, 0.433 g/g, and 0.339 g/g after 24 h, 48 h and 72 h, respectively. PHB concentration for Cnec hdlz was significantly greater at each time point than that for control 1 at significance level of 0.05. With respect to PHB yield, no significant difference was found between control and hydrolysate

at 24 h and 48 h however, control 1 had significantly greater PHB yield than that for Cnec hdlz at 72 h.

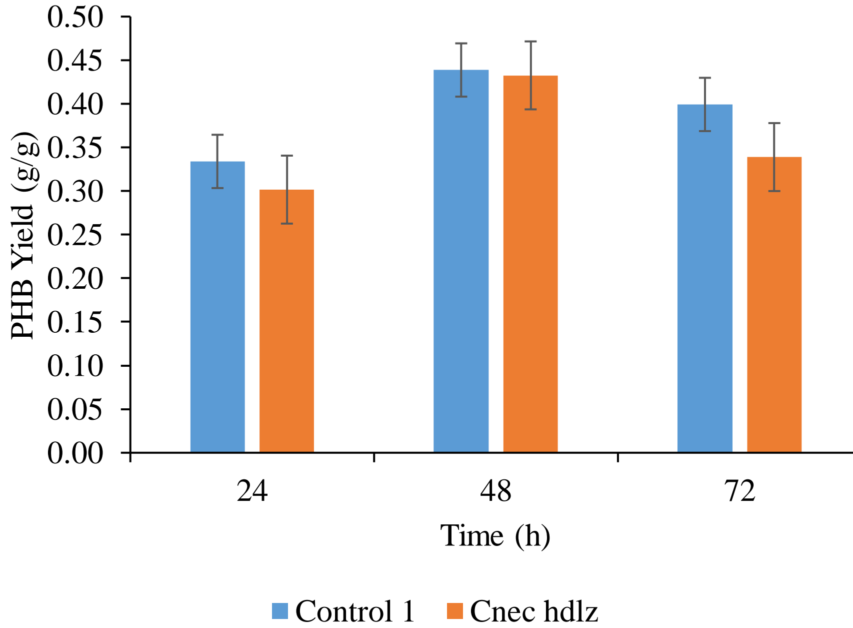


Figure 2.4: Comparison of PHB yield in control 1 and Cnec hdlz

2.5.2.2 Comparison of control 2 and P. sacchari only hydrolysate (Psac hdlz) - PHB concentration and yield

P. sacchari is well known for its ability to co-ferment sugars. As shown in Figure 2.5, *P. sacchari* consumed more glucose and less xylose in control 2 than in Psac hdlz at every time point. The initial concentrations of glucose and xylose were 51.22 g/l and 13.66 g/l, respectively, in both media. *P. sacchari* utilized 46.06% of glucose and 49.27% of xylose in Psac hdlz to produce a PHB concentration of 3.83 g/l after 72 h. *P. sacchari* utilized 40.82% of glucose and 24.90% of xylose in Control 2 to produce a PHB concentration of 2.64 g/l after 24 h. *P. sacchari* consumed 61.17% of initial glucose and 35.25% of initial xylose after 48 h, and 67.55% of initial glucose and 36.23 % of initial xylose after 72 h, but only 2.52 g/l PHB and 1.28 g/l PHB were

produced at 48 h and 72 h, respectively. As illustrated in Figure 2.6, maximum PHB yield of 0.146 g/g sugars was achieved in Psac hdlz in 48 h while control 2 produced only 0.105 g/g PHB yield in first 24 h. The PHB yield decreased with time till 0.03 g/g at the end of the fermentation at 72 h.

PHB yield was significantly greater for hydrolysate than for control at every time point throughout the fermentation ($p<0.05$). In the first 24 h, control produced significantly more PHB than Psac hdlz. However, the result was opposite in 72 h from the start of fermentation such as Psac hdlz produced more PHB in significant amount than control 2. Meanwhile, PHB produced by control 2 and Psac hdlz was not significantly different at 48 h and all analysis was carried out based on significance level of 0.05.

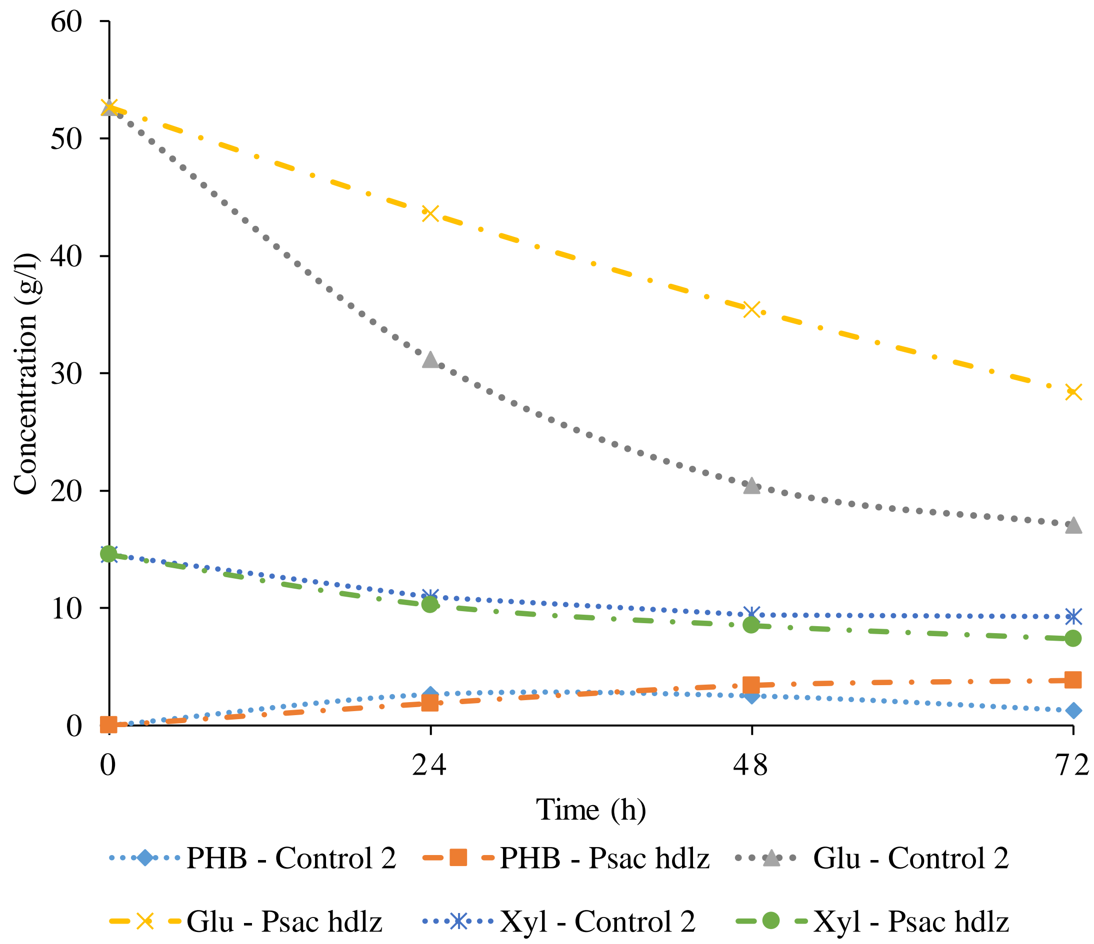


Figure 2.5: Time series data of PHB fermentation for Control 2 and Psac hdlz

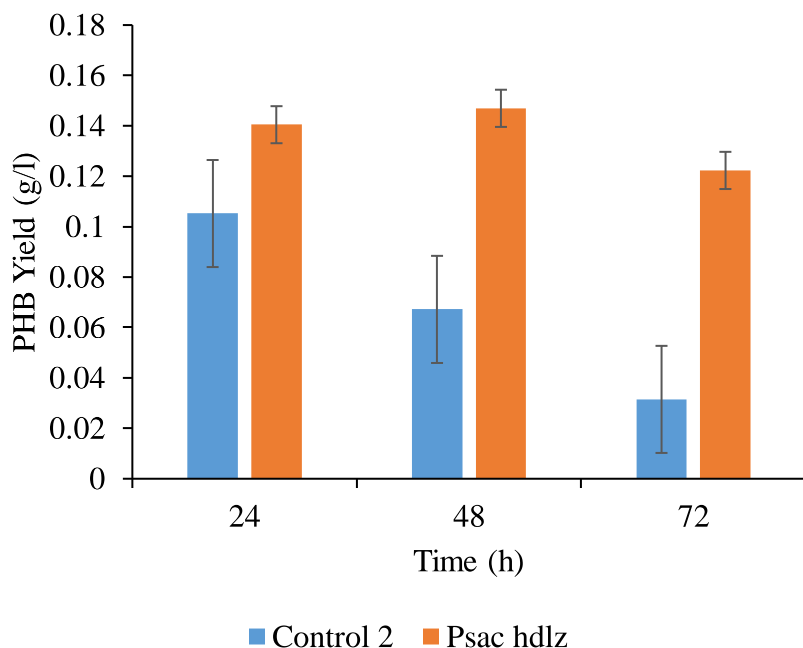


Figure 2.6: Comparison of PHB yield in control 2 and Psac hdlz

A sharp decline was observed in the first 24 h in control 2 (Figure 2.7) due to the excess release of acids such as acetic acid from the metabolic reaction during exponential growth of *P. sacchari* . The pH dropped from 6.81 to 3.99, which restricted bacterial growth and PHB production, hence, control 2 ended up with least PHB (1.29 g/l) with decreased bacterial cell concentration (13.85 g/l) at pH 3.87 at 72 h from the start of fermentation. Cell growth throughout fermentation in Psac hdlz was observed to have no substantial decrease in pH over time as shown in Figure 2.7. Consequently, PHB production kept increasing over time (24 h, 48 h and 72 h) as illustrated in Figure 2.5. This can be attributed to the resistance of Psac hdlz to pH changes with increasing bacterial growth, which is caused by the buffer strength of phosphate buffer possibly accompanied by additional acetate buffer present in Psac hdlz. The pH in Psac hdlz remained favorable throughout the fermentation yet, *P. sacchari* probably consumed easier carbon sources available in the media over the glucose and xylose leaving abundant residual

sugars such as 28.04 g/l glucose and 7.37 g/l xylose at the end of the fermentation (Cesário et al. 2014).

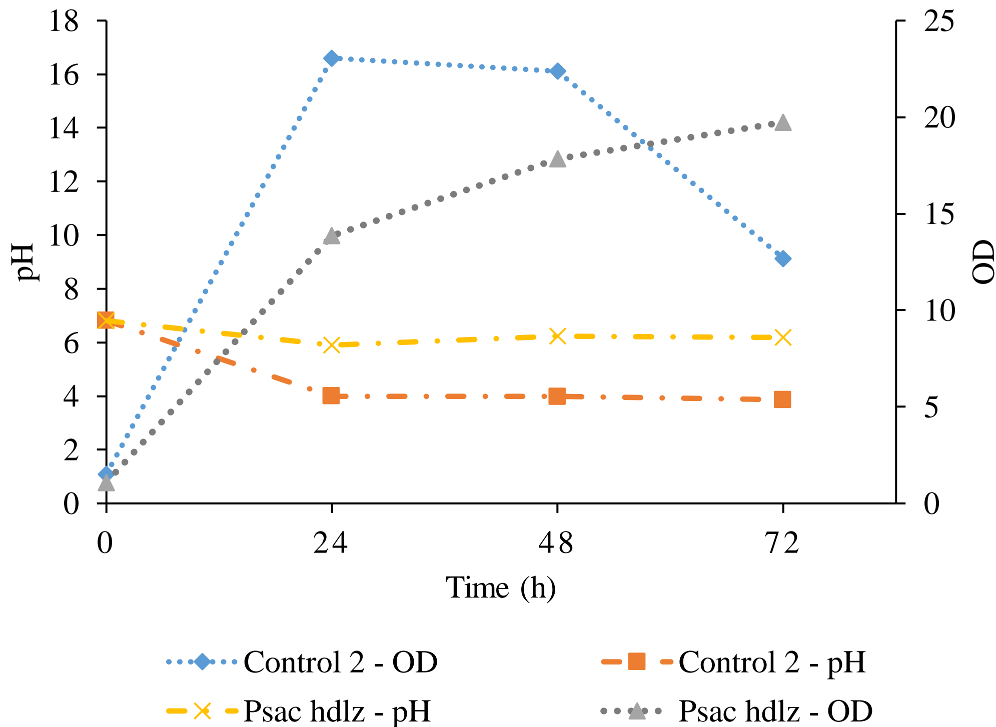


Figure 2.7: Time series data for optical densities (solid lines) and pH (broken lines) of Control 2 and Psac hdlz

2.5.2.3 Comparison of control 3 and hydrolysate with two bacteria: C. necator and P. sacchari (Comb hdlz)- PHB conc and yield

Figure 2.8 demonstrates the trend for sugar consumption over time in Control 3 and Comb hdlz throughout the fermentation and Figure 2.9 shows the PHB yields attained in at three time points during the fermentation. Both glucose and xylose were consumed in Control 3 and Comb hdlz. Based on the observation that *C. necator* did not consume substantial xylose in control 1 and Cnec hdlz throughout the fermentation, *P. sacchari* must have been responsible for

xylose utilization. The initial concentration at the beginning of the fermentation was 52.66 g/l glucose and 14.54 g/l of xylose for both Control 3 and Comb hdlz. 38.72% of initial glucose and 18.5% of initial xylose concentration were used to produce 4.13 g/l PHB in Control 3 during the first 24 h which resulted in a maximum yield of 0.18 g/g. PHB yield decreased over time due to reduced PHB synthesis despite 75.04% of total glucose and 27.06% of total xylose being utilized by the end of the fermentation in Control 3. *P. sacchari* may have dominated during the exponential growth in the first 24 h of the fermentation in the control 3, which would have resulted in sharp decline of pH from 6.81 to 4.04 as depicted in Figure 2.10. Consequently, the acidic environment did not favor further production of PHB by either of the bacteria resulting in yields decreasing to 0.083 g/g by the end of the fermentation.

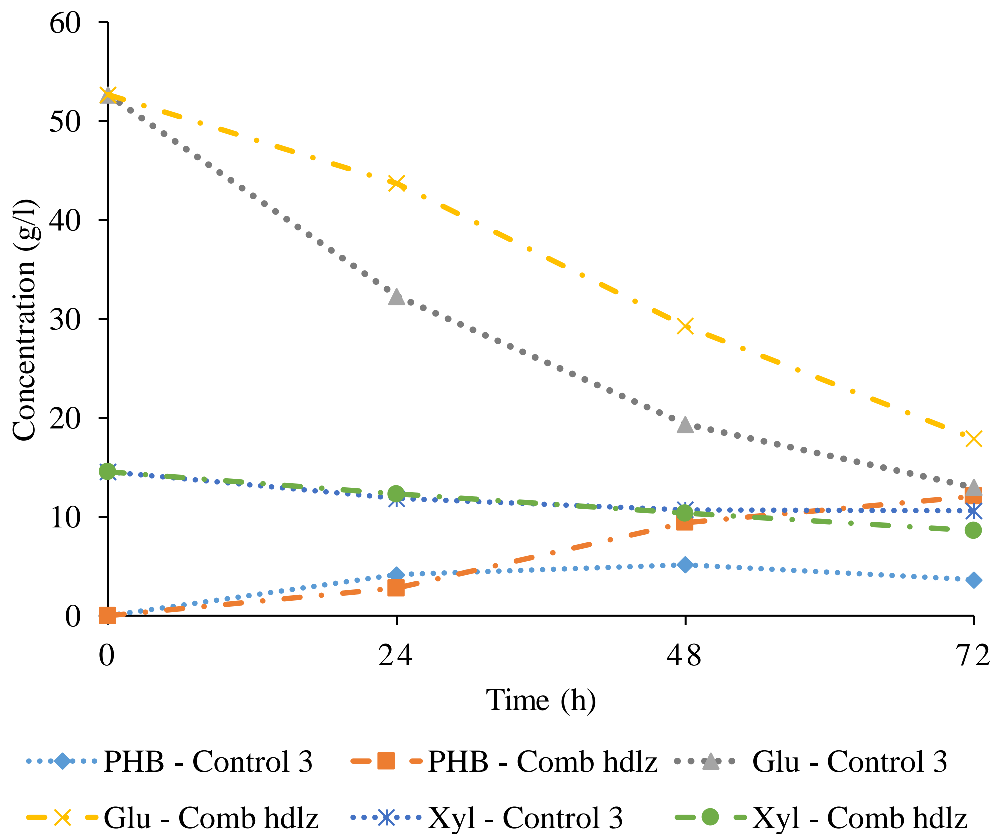


Figure 2.8: Time series data of PHB fermentation for Control 3 and Comb hdlz

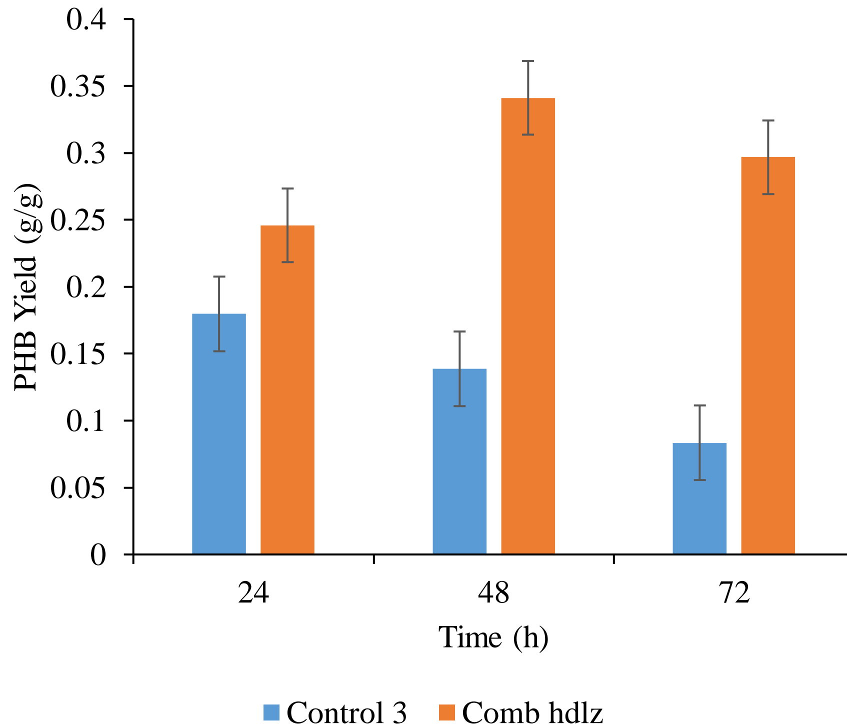


Figure 2.9: Comparison of PHB yield in control 3 and Comb hdlz

On the contrary, as shown in Figure 2.10Figure 2.10 , resistance of Comb hdlz against pH drop supported growth of bacteria with a favorable environment for PHB synthesis resulting in a maximum PHB concentration of 12 g/l PHB from utilization of 66.02% of total glucose and 40.90% of total xylose by the end of the fermentation. The maximum yield for Comb hdlz was 0.341 g/g, obtained at 48 h, and the pH value for the media was consistent throughout the fermentation.

On statistical analysis, PHB yield was found to be significantly higher for Comb hdlz than that for control 3 at every time point throughout the fermentation. In PHB production, Control 3 initially produced significantly more PHB than Comb hdlz during the first 24 h. However, by 72 h after fermentation began, the trend changed, with Comb hdlz producing a significantly higher amount of PHB. Meanwhile, no significant difference was found in PHB

produced from control and Comb hdlz at 48 h and all analysis was carried out based on significance level of 0.05.

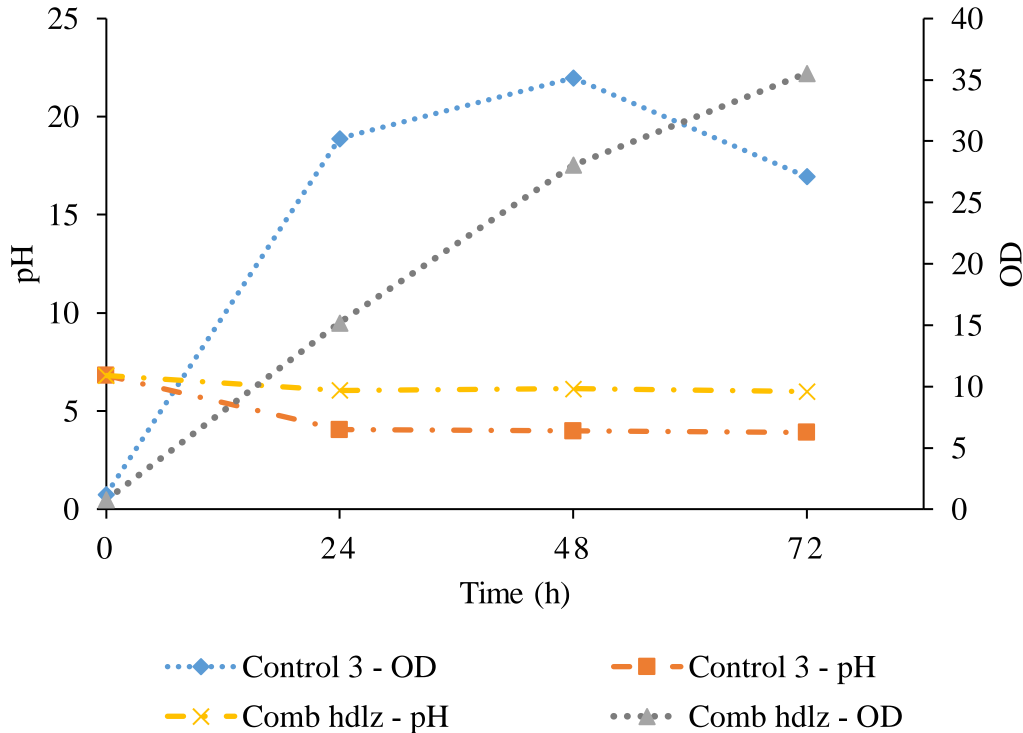


Figure 2.10: Time series data for pH (solid lines) and OD (broken lines) for control 3 and Comb hdlz

2.5.2.4 Comparison of C. necator, P. sacchari and both combined - PHB yield in hydrolysate

Figure 2.11 displays the PHB concentration observed in Cnec hdlz, Psac hdlz and Comb hdlz while Figure 2.12 illustrates the comparison between the PHB yields at 3 time points (24 h, 48 h and 72 h) during fermentation. No significant difference was found between PHB concentrations produced in Cnec hdlz and Comb hdlz during the first 24 h while P sac hdlz produced the least PHB among the three treatments. Significant differences ($p<0.05$) were found in PHB concentrations among all three bacterial treatments at 48 h and 72 h with Cnec hdlz

producing the highest concentrations and Psac hdlz producing the lowest concentrations. A similar trend of having maximum PHB yield at 48 h was observed in all three hydrolysates. The maximum PHB concentration and yield at every time point was observed in Cnec hdlz. Significant differences ($p<0.05$) were found in PHB yields among all three bacterial treatments with Cnec hdlz having the highest yield and Psac hdlz having the lowest yield at all time points.

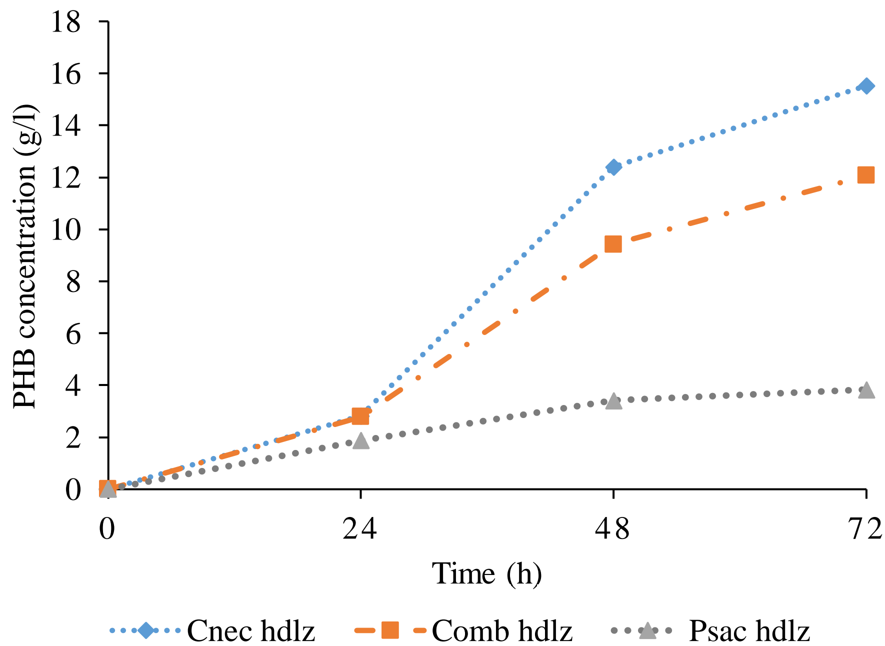


Figure 2.11: Time series data of PHB production in Cnec hdlz, Comb hdlz and Psac hdlz throughout the fermentation.

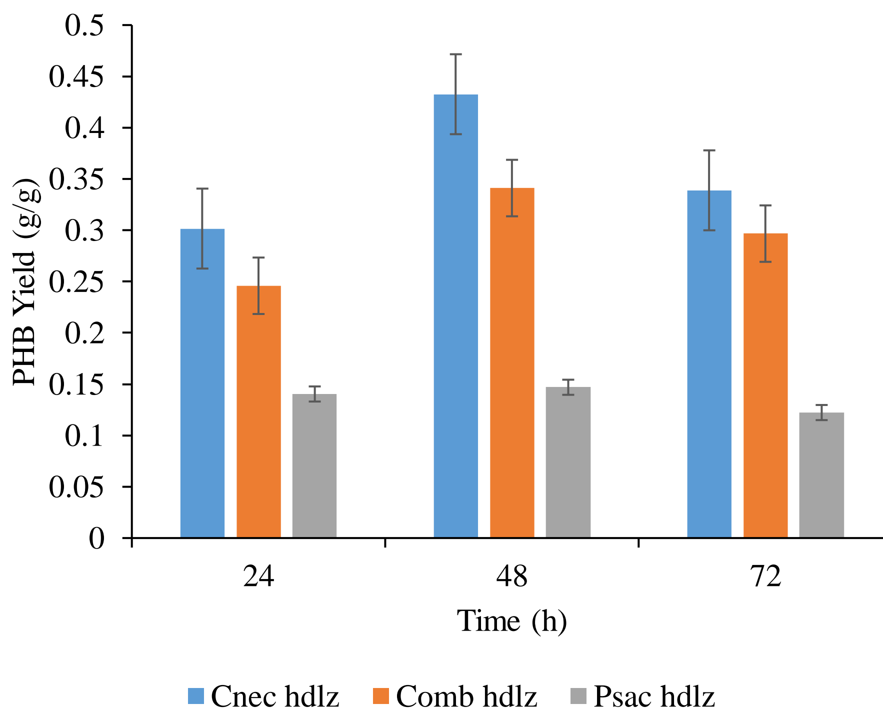


Figure 2.12: Comparison of PHB yield in Cnec hdlz, Psac hdlz and Comb hdlz

2.5.3 Variation between batches

The 3×2 factorial experiment with 3 replicates was performed twice due to unexpected modification in the *C. necator* working stock potentially induced by the change in culture media. *C. necator* did not produce any PHB with hydrolysate while it produced up to 10.44 g/l PHB with control. Similarly, this strain of *C. necator* suppressed the PHB production capacity of *P. sacchari* when applied in combination in hydrolysate, however, the same combined bacterial treatment yielded up to 5.514 g/l PHB in control. The strain of *P. sacchari* was the same throughout all the experiments.

Figure 2.13 shows the comparison between PHB yields and Figure 2.14 displays PHB production throughout fermentation of Comb hdlz and Psac hdlz using different cultures. Clearly, *P. sacchari* from culture II attained almost double the PHB concentration and yield in PHB as

compared to that from batch I at each time point of fermentation while suppressed *P. sacchari* in Comb hdlz from batch II could not produce PHB at all. Statistical analysis shows significant difference between PHB yields at all three time points and between PHB productions at 48 h and 72 h from the start of fermentation in Psac hdlz from culture I and II ($p<0.05$). Hence, we could conclude that the results in bacterial activity were affected by a culture effect.

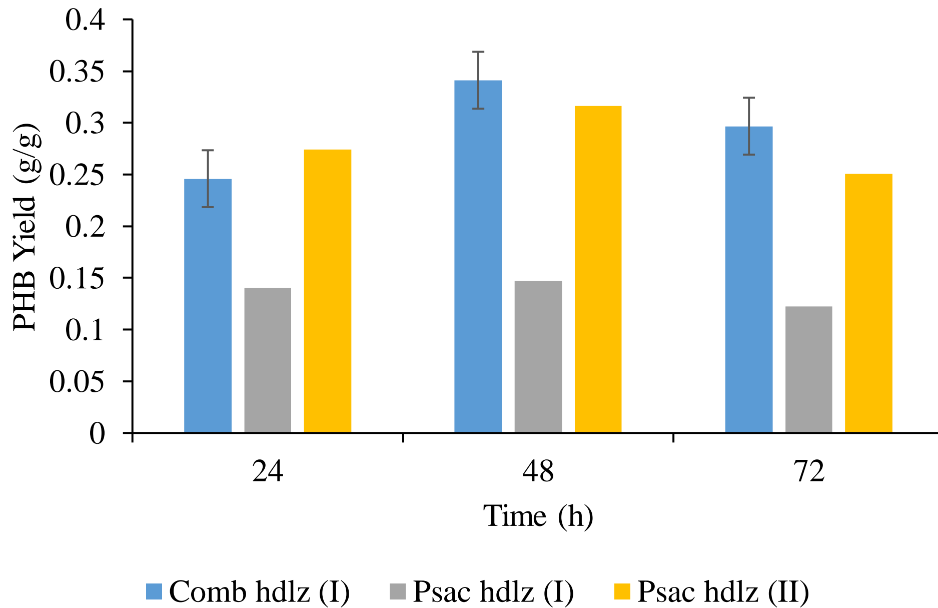


Figure 2.13: Comparison between PHB yield of Comb hdlz and Psac hdlz from two batches (I and II)

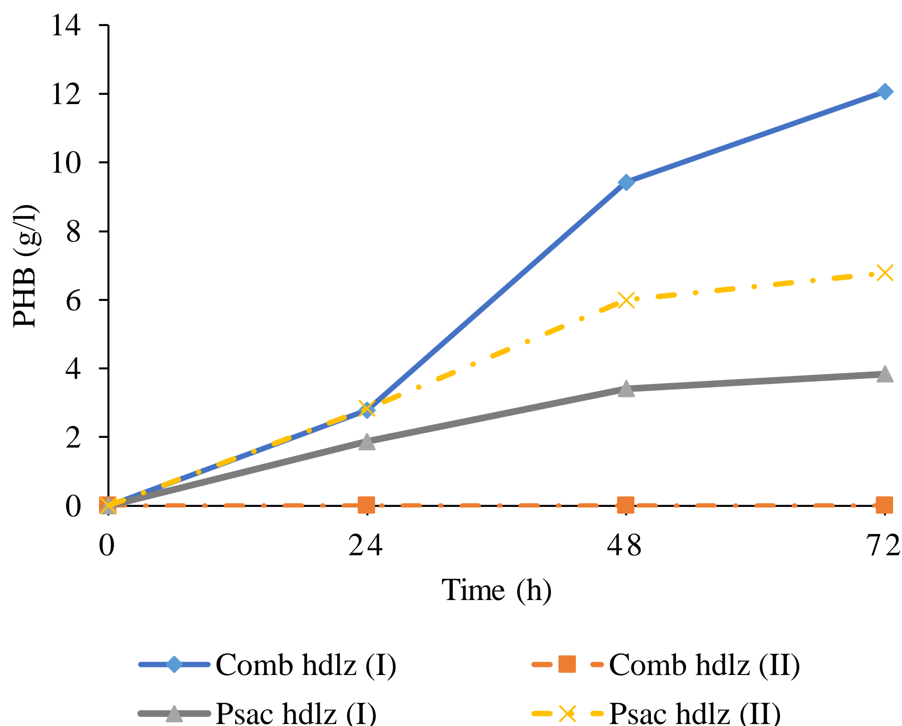


Figure 2.14: Time series data for PHB production over time in Comb hdlz and Psac hdlz two batches (II and I)

2.6 Conclusion

This study confirmed the compatibility of *C. necator* and *P. sacchari* in co-fermentation of glucose and xylose in alkaline pretreated hemp hydrolysate to produce PHB. Comparable PHB with respect to that produced by single culture was achieved via co-culture of these two bacteria however, hydrolysate with *C. necator* only produced the maximum PHB concentration. The highest PHB yield was obtained at 48 h for all hydrolysates while *C. necator* achieved the maximum PHB yield of 0.433 g/g sugars. Interestingly, more PHB was produced in HS hydrolysates than in control solutions even though both hydrolysates and control solutions had the same glucose and xylose concentrations.

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3 Chapter 3: Investigation of alternatives against washing pretreated solids to produce PHB via *C. necator*

3.1 Abstract

The escalating demand for sustainable alternatives to petroleum-based plastics has catalyzed significant interest in biopolymers, particularly polyhydroxyalkanoates (PHAs). In this study, we explored the valorization of unwashed pretreated hemp stalk (HS) for the production of polyhydroxybutyrate (PHB), a PHA variety, via microbial fermentation using *Cupriavidus necator* DSM 545. HS was pretreated using two techniques: hydrothermolysis (LHW) at 170°C for ½ h and alkaline pretreatment (AP) with 1% sodium hydroxide at 130°C for 1 h. Intermediate washing was avoided to simulate the mixture condition in a one pot treatment condition, which is a streamlined process that integrates multiple treatments and processes in a single vessel without intermediate handling. However, AP filtered unwashed solids were hydrolyzed with water to lower the inhibitor content in the media up to 28% of that hydrolyzed with prehydrolysate resulting 70 % glucan conversion efficiency. LHW filtered wet solids were hydrolyzed with detoxified and undetoxified prehydrolysate resulting in 93% and 85% glucose yield, respectively where detoxification was performed by agitating with 50 g/l charcoal for an hour. Maximum PHB of about 1.12 g/l at 48 h was produced in detoxified hydrolysate while a maximum yield of 0.65 g PHB per g sugars in 24 h was achieved in undetoxified hydrolysate by preferentially using lignin derivatives over the sugars in the media. PHB (1.08 g/l) was produced in AP hydrolysates in the first 24 h which reduced in 48 h and 72 h subsequently, implying PHB depolymerization by *C. necator* instead of further synthesis.

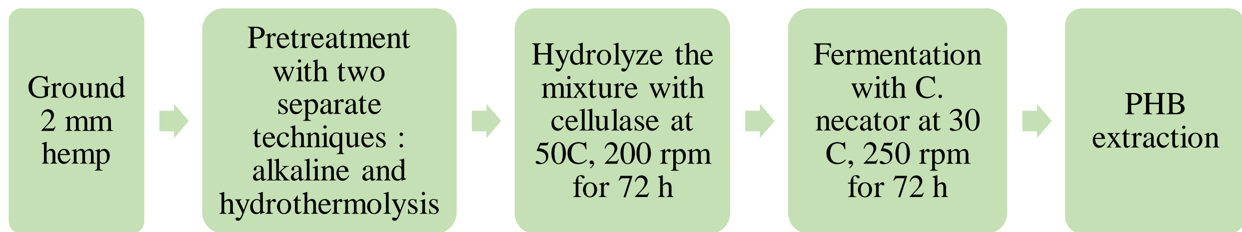

Figure 3.1: Flowchart of PHB production from unwashed solids with prehydrolysate as in one pot treatment process

3.2 Introduction

Biopolymers, particularly bioplastics, are gaining momentum as sustainable alternatives to traditional petroleum-based plastics due to their biodegradability and lower environmental impact. As global concerns over plastic waste and pollution intensify, the demand for bioplastics has surged across various industries, including packaging, agriculture, automotive, and consumer goods. The global bioplastics market is projected to grow significantly in the coming years, driven by both regulatory pressures and consumer demand for eco-friendly products. According to market reports, the global bioplastics market was valued at over USD 11 billion in 2022 and is expected to continue expanding at compound annual growth rate of 18.8% by 2030, with an increasing number of companies adopting bioplastic materials in their production processes (market report 2023). This increasing demand can also be attributed to the flexibility in the alteration of mechanical properties of bioplastics as desired via use of additives and molecular engineering. Studies like Li et al. (2016) have shown that biopolymers such as polyhydroxyalkanoate (PHA) can now be modified to achieve comparable strength and flexibility to traditional plastics though innovations like blending, plasticization, and copolymerization. This growing market presence highlights the potential of bioplastics to play a crucial role in the shift toward more sustainable materials in global industries.

The industrial production of PHAs has grown significantly in recent years due to their potential as a biodegradable alternative to conventional plastics, with applications in packaging, agriculture, and medical fields. However, large-scale production faces notable challenges, including high costs of raw materials and the need for specialized fermentation processes, which make it more expensive than petroleum-based plastics. The efficiency of microbial synthesis is often constrained by feedstock limitations. Likewise, utilization of lignocellulosic biomass as a potential feedstock is hindered due to lignin which complicates PHA production and reduces yields. Furthermore, a significant issue in using lignocellulosic biomass for bioplastics production is the massive clean water consumption during washing of pretreated solids prior to hydrolysis(Yuan et al. 2021). This washing not only depletes water resources but also generates large volumes of wastewater that need to be treated, adding both environmental and economic burdens. These challenges underscore the need for advancements in processing techniques to overcome the barriers posed by lignin and excessive water usage.

A one-pot treatment process for processing pretreated solids offers a solution to the significant water consumption associated with washing solids in PHB production. In this approach, biomass undergoes a simplified, streamlined treatment that integrates multiple steps, such as pretreatment, hydrolysis, and fermentation, eliminating the need for intermediate washing stages and significantly reducing water usage. However, a major challenge arises from the presence of inhibitors, such as lignin-derived compounds, formed during the pretreatment process. These inhibitors can hinder microbial activity, preventing bacteria from effectively consuming the sugars needed for fermentation, which can lead to reduced PHA yields.

Cupriavidus necator DSM 545 is a versatile mutant bacterium known for its ability to consume sugars like glucose to accumulate polyhydroxybutyrate (PHB), a type of PHA, as an

intracellular storage material under nutrient-limited conditions. One of its remarkable features is its ability to grow in media containing lignin derivatives, which are typically inhibitory to many microorganisms. Lignin, a complex polymer found in plant biomass, breaks down into various aromatic compounds during pretreatment, which can hinder microbial growth. However, *C. necator* has shown the ability to metabolize some of these lignin-derived compounds, such as ammonium acetate, benzoic acid and p-coumaric acid, effectively using them as carbon sources along with sugars to produce PHB (Wang et al. 2014). Weng et al. (2023) used metabolically engineered *C. necator* to produce 3.81 g/l PHB in 72 h from corn stover hydrolysates containing glucose and xylose in the presence of lignin-derived aromatics such as p-coumaric acid and ferulic acid. In this study, HS was pretreated using two different techniques, hydrothermolysis and alkaline pretreatment, and the unwashed solids were hydrolyzed with water or prehydrolysate in the presence of cellulase, along with ensuring the optimal limit of lignin derivatives for bacterial viability by diluting the media specifically in the alkaline pretreated hydrolysate to produce PHB.

3.3 Materials and Methods

3.3.1 Hemp stalk (HS) as substrate

A second batch of HS was obtained from the Department of Agronomy at Kansas State University and processed as explained in section 2.4 above2.4. Dry 2 mm size hemp was stored in an air-tight container for future use.

3.3.2 Compositional analysis of hemp

The same procedures explained in section 2.4.2 was used to analyze composition of the samples.

3.3.3 Cell mass measurement

Cell mass for a given sample was calculated by interpolating the optical density (OD) versus cell mass equation as explained in section 2.4.5 where OD was measured using the spectrometer (BioMate 3, 335905P, Thermo Electron Co, Madison, WI, USA).

3.3.4 Pretreatment and enzymatic hydrolysis

Two types of pretreatments were used for HS namely alkaline pretreatment (AP) with 1% sodium hydroxide solution as explained in section 2.4.3 and hydrothermolysis. The liquid to solid ratio and agitation speed were same in both pretreatments however, the hydrothermolysis was operated at 170°C for 0.5 h with reference to the condition used in Zhao et al. (2020). The mixture from the pretreatment was separated using a coffee filter and the liquid (prehydrolysate) to solid ratio was calculated based upon their masses and the moisture content of the filtered solids. The solid to liquid ratio during enzymatic hydrolysis was the same as in the mixture obtained after pretreatment mimicking the condition in the one pot treatment process.

Pretreated solids were hydrolyzed with two different liquids separately: water and prehydrolysate. Four types of hydrolysis were carried out as follows: (a) washed LHW solids with water, (b) unwashed LHW solids with detoxified LHW prehydrolysate, (c) unwashed LHW solids with undetoxified LHW prehydrolysate and (d) unwashed alkaline pretreated solids with water. Before hydrolysis, pH of solids was neutralized by washing with dilute 0.06 mM H_2SO_4 solution until pH of eluent was about 5 to prepare water hydrolysate only for method (a) while detoxification was performed by agitating prehydrolysate with 50 g/l activated charcoal for 1 h for method (b). Solids for methods (b), (c) and (d) were filtered to remove prehydrolysate, then they were combined with either water or prehydrolysate maintaining the solids to liquid ratio

observed after pretreatment prior to enzymatic hydrolysis. Meanwhile, the pH of prehydrolysate was neutralized with 5 M HCl until pH 5 was attained. Wet solids were autoclaved at 121°C after filtration while prehydrolysate were sterilized via 0.2-micron membrane filtration and then, added to a 250 ml Kimax Baffled flask in presence of 50 mM acetate buffer at pH 5 with cellulase enzyme (Cellic CTec3 HS, Novozymes, Bagsvaerd, Denmark). After 72 h in an incubator shaker set at 50°C and the mixture was filtered with a coffee filter by vacuum filtration. The solids retained on the filter paper were discarded, while the resulting liquid hydrolysate was stored at 4 °C in a refrigerator for fermentation.

Table 3.1: Hydrolysis conditions for solid and liquid used to produce WSH, DTX, UNDTX and APH

Hydrolysis condition	**Method A (WSH)**	**Method B (DTX)**	**Method C (UNDTX)**	**Method D (alkaline)**
Solid state	washed	Not washed	Not washed	Not washed
Liquid	water	Detoxified prehydrolysate	Undetoxified prehydrolysate	water

3.3.5 Optical Density measurement

A spectrophotometer (BioMate 3, 335905P, Thermo Electron Co, Madison, WI, USA) was used to measure optical density (OD) of cultures to measure cell mass concentration and lignin concentration in the media.

3.3.6 Lignin measurement

Three ml of samples were taken and diluted to a volume of 100 ml with HPLC water followed by shaking in a vortexer. After ensuring the homogeneity of the mixture, 50 μl of the diluted solution were further mixed with 1450 μl water to produce a final diluted solution. 100 μl of 8 mM $K_3Fe(CN)_6$ was added to each of the final diluted solutions in a 2 ml tube followed by the immediate addition of 100 μl 0.1 M $FeCl_3$ and was mixed thoroughly in a vortexer for 5 min (Xie et al. 2017). After mixing, the absorbance of the sample at a wavelength of 700 nm was measured in a spectrophotometer with reference to HPLC water as a blank control. Additionally, a standard calibration curve was prepared by treating solutions with known Klason lignin concentrations as described above and preparing a linear concentration vs. absorbance standard curve. Hence, lignin concentration in experimental samples was determined from the standard curve.

3.3.7 PHB production

For fermentation, five types of mixtures were used as media namely, (I) control, (II) water hydrolysate from hydrolysis method 'a,' (III) detoxified LHW hydrolysate from method 'b,' (IV) undetoxified LHW hydrolysate from method 'c,' and (V) alkaline hydrolysate from method 'd.' The primary carbon source was glucose and xylose for media I and II while media III, IV, and V had both sugars and lignin derivatives. One g/l $(NH_4)_2SO_4$ were added as nitrogen sources along with 4.5 g/l $Na_2HPO_4.2H_2O$, 1.5 g/l KH_2PO_4, 0.5 g/l $MgSO_4.7H_2O$, 0.1 g/l $CaCl_2.2H_2O$, 0.06 g/l $Fe(NH_4)_3$ citrate and 5 ml/l trace element as described in Ramsay (1990). Control was prepared by replicating the sugar concentration in the alkaline hydrolysates which had the highest sugar concentration among all media.

Seed medium was prepared from 30 g/l glucose, 3 g/l meat extract and 5 g/l meat peptone, and was used to incubate bacteria for 24 h until OD of 8-10 was obtained. Bacteria inoculated in 20 ml seed medium was used for each fermentation media with 40 ml working volume resulting in a starting optical density of media more than 4. Cells were washed with 0.89% NaCl solution twice before adding to the media and 20% bacterial volume was used in the entire flasks. Samples were taken every 24 h until 72 h from the start time of fermentation.

3.3.8 Dilution of alkaline hydrolysate

The sterile fermentation media prepared from the enzymatic hydrolysis of alkaline pretreated solids with prehydrolysate in the presence of cellulase (Cellic CTec3 HS, Novozymes, Bagsvaerd, Denmark), was diluted with water in 5 different ratios: 25%, 31.25%, 37.5%, 43.75%, and 50%; and then subjected to fermentation with the salts and inoculum as explained in section 2.4.7. Each treatment was performed in triplicate and the optical densities of the media were observed in spectrometer at the wavelength of 600 nm in 24 h, 48 h and 72 h from the start of the fermentation.

3.3.9 High performance liquid chromatography (HPLC) sugar analysis

Monomer sugars were analyzed using high-performance liquid chromatography (HPLC) equipment (G1316A, Agilent Technologies 1200 Series, Santa Clara, CA, USA). The sample preparation technique and the column characteristics were kept the same as mentioned in section 2.4.8.

3.3.10 Determination of PHB using gas chromatography-mass spectrometer (GCMS)

2 ml of each sample, to be tested to determine amount of PHB, was subjected to the procedure explained in the section 2.4.9and analyzed using calibrations from external standards

of 1 mg, 2 mg, 4 mg, 6 mg and 8 mg from commercial sodium DL-3-hydroxybutyrate (H0231, TCI America, Portland, OR, USA) processed in the same way to the samples. PHB was quantified by preparing the samples and standards as explained in section 2.4.9 and using gas chromatography mass spectrophotometer as per the conditions stated in section 2.4.10.

3.3.11 Calculations

In order to calculate yields, following formula were used:

a) Hydrolysis efficiency

Glucose yield (in percentage) =

$$\frac{\text{glucose}\left(\frac{g}{l}\right)+\text{cellobiose} * \frac{360}{342}\left(\frac{g}{l}\right)}{\text{mass fraction of glucan} * 1.11 * \text{preatreated solids concentration in hydrolysis}\left(\frac{g}{l}\right)+\text{glucose in prehydrolysate}\left(\frac{g}{l}\right)} \times 100\% \qquad \text{Eq 3.1}$$

and,

Xylose yield (in percentage) =

$$\frac{\text{xylose}\left(\frac{g}{l}\right)}{\text{mass fraction of xylan} * \frac{150}{132} * \text{preatreated solids concentration in hydrolysis}\left(\frac{g}{l}\right)+\text{xylose in prehydrolysate}\left(\frac{g}{l}\right)} \times 100\% \qquad \text{Eq 3.2}$$

b) PHB yield

$$\text{PHB yield} = \frac{\text{PHB concentration at t time}}{\text{Sugar concentration}(t0)-\text{Sugar concentration}(t)} \qquad \text{Eq 3.3}$$

Where, PHB concentration and sugar concentration are at g/l,

t0 = 0 h and t = 24 h, 48 h and 72 h

3.3.12 Statistical Analysis

Data was presented as mean ± one standard deviation. Comparisons among means were subjected to an analysis of variance (ANOVA) using SAS (Version 9.4 M7, SAS Inst. Inc., Cary, NC, USA). Means from different independent variables were separated using the Fisher's Protected Least Significant Difference. Significant differences among groups of data were determined at a 95% confidence interval.

3.4 Results and Discussion:

3.4.1 Optimization of enzymatic hydrolysis

HS was analyzed to determine the composition of structural polysaccharides, lignin, and other cell wall components. The composition is in **Error! Reference source not found.**2.

Table 3.2: Composition of second batch of HS

Components	Content (%)
Glucan	40.55
Xylan	7.13
Galactan	0
Arabinan	1.09
Mannan	3.28
Lignin	28.1
Extractives	10.47
Ash	3.7

Figure 3.2 shows the yields of glucose and xylose conversion on the basis of sugars attained at the end of hydrolysis. Four different treatments were subjected to HS solids and liquid as explained in section 3.3.3. The undetoxified LHW hydrolysate (from method ‘c’) was observed to have the lowest conversion yields with 62.87% glucose and 65.52% xylose while detoxification of LHW hydrolysate (from method ‘b’) increased the yields to 87.63% glucose and 86.52% xylose, respectively. Water hydrolysate from LHW (from method ‘a’) and alkaline pretreated hydrolysate (from method ‘d’) were observed to have higher xylan conversion than glucan conversion, with 92.97% and 97.09% xylose yields for method a and d, respectively, in comparison to 67.17% glucose yield for method ‘a’ and 70.33% glucose yield for method ‘d.’ Both glucan and xylan conversion yields for all 4 methods were significantly different ($p<0.05$) from each other.

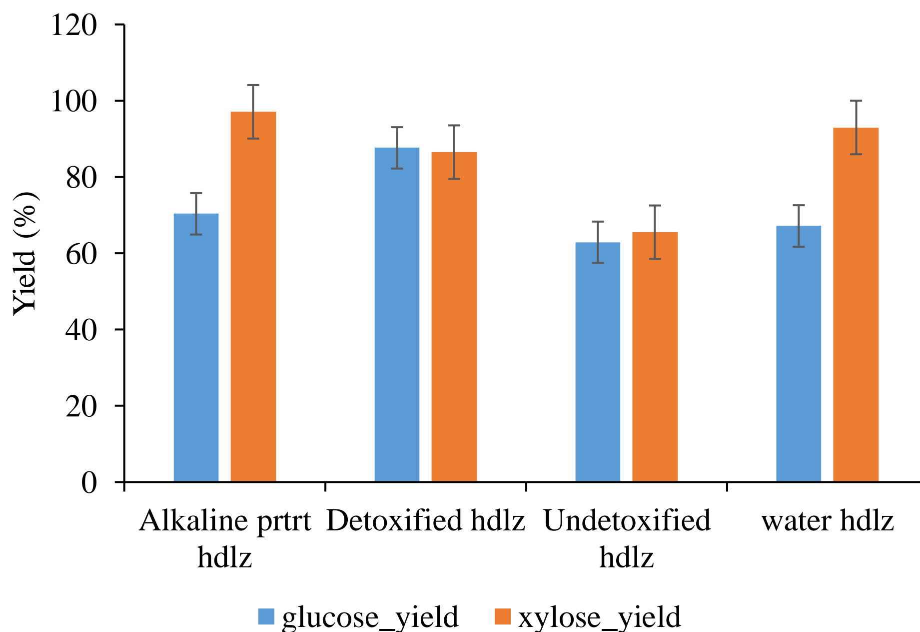

Figure 3.2: Efficiencies of methods (a), (b), (c) and (d) used in enzymatic hydrolysis in terms of glucose yield and xylose yield

Similar to the results shown in the section 2.5.2, *C. necator* could not consume xylose in the media while glucose was consumed the most in the control. The varying sugar concentrations at the start of fermentation were due to the different methods adopted to perform hydrolysis. The starting glucose concentrations were 31.66 g/l, 28.24 g/l, 28.90 g/l, 26.46 g/l and 29.59 g/l for control, washed hydrolysate (method 'a'), detoxified hydrolysate (method 'b'), undetoxified hydrolysate (method 'c') and alkaline pretreated hydrolysate (method 'd'), respectively.

As demonstrated in the Figure 3.3, sharp decline in glucose concentration can be seen in the control, due to the absence of inhibiting factors such as lignin degradation products, sugar degradation products and hemicellulose derived products inherent in hydrolysates (Tengborg et al. 2001), that resulted 1 g/l of residual glucose at 72 h and significantly different ($p<0.05$) from rest of the methods in terms of glucose consumed at every time point. Washed solids with fewer inhibitors from lignin derivatives due to use of water instead of prehydrolysate showed steady decrease of glucose to 14.29 g/l at 72 h followed by steady consumption of glucose in detoxified hydrolysate to 12.44 g/l in 72 h. On the contrary, a slower decline in glucose concentration was observed in alkaline pretreated hydrolysate and undetoxified hydrolysate at every time point of the fermentation ending up with 17.73 g/l and 18.80 g/l residual glucose at the end of the fermentation, respectively. Glucose concentrations were not significantly different for all treatments except for control during 48 h and 72 h from the start of the fermentation. Meanwhile at 24 h, glucose concentrations for WSH and control were different from the rest of the methods at the significance probability of 0.05.

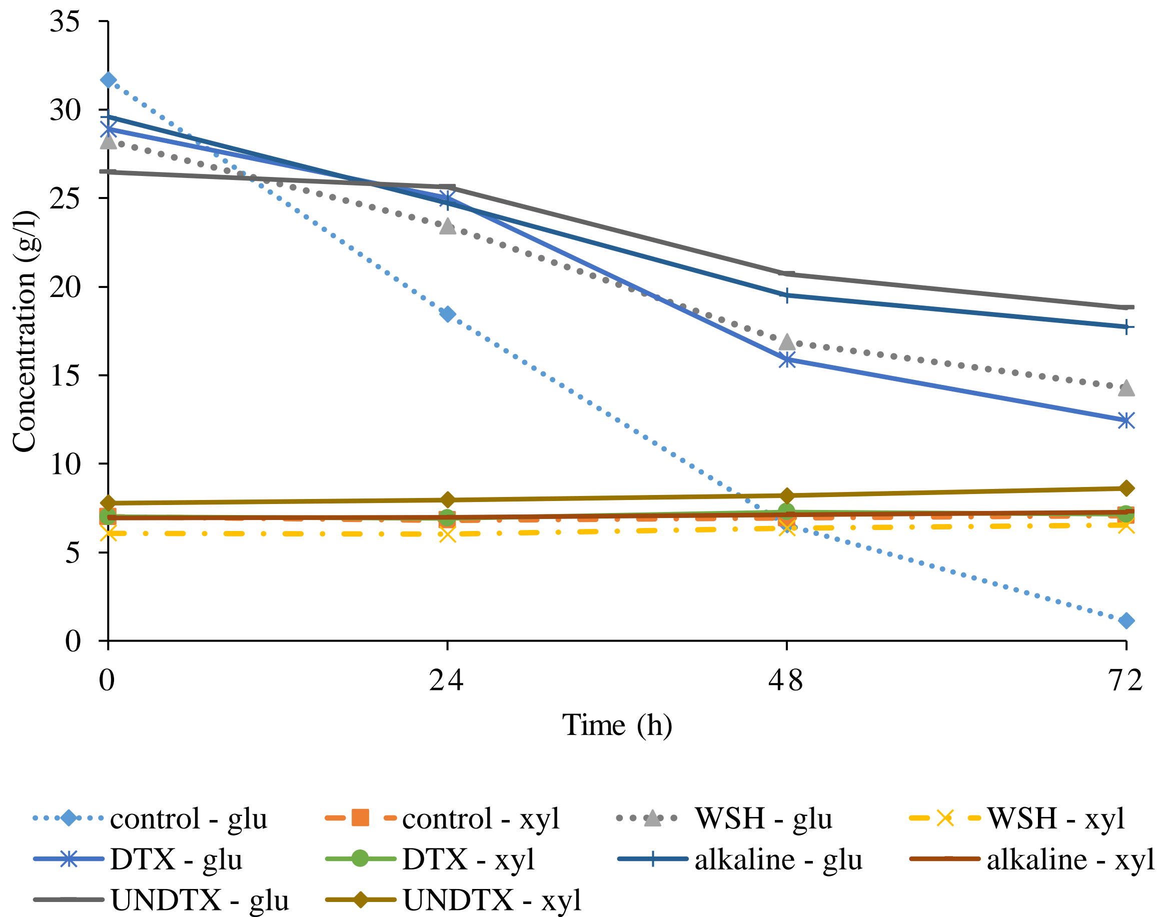

Figure 3.3: Time series data of glucose and xylose consumption for control, washed LHW solids (WSH), detoxified LHW solids (DTX), undetoxified LHW solids (UNDTX) and alkaline pretreated solids throughout the fermentation

Figure 3.4 depicts the growth of *C. necator* in the control and media obtained via methods (a), (b), (c) and (d). The initial optical densities (OD) for control, washed hydrolysate and alkaline pretreated hydrolysate were 4.46, 4.77, and 4.56, respectively, while that for DTX and UNDTX started at 5.15 and 5.41, respectively, which can be attributed to the additional darkness of prehydrolysate used when water was used as a blank. A lag phase was observed in UNDTX while a significant increase ($p<0.05$) in OD was observed in control for the first 24 h followed by WSH

and alkaline, which were significantly indifferent from each other and finally by DTX. *C. necator*: At 48 h, due to large variations in the OD of UNDTX, no significant differences in OD were observed among WSH, DTX and UNDTX ($p<0.05$) or among alkaline, WSH and UNDTX. Similarly in 72 h, overlapping could be observed in the significantly indifferent groups such as WSH and DTX, DTX and UNDTX and finally UNDTX and alkaline made three different groups whose means of OD were indifferent from each other at $p<0.05$.

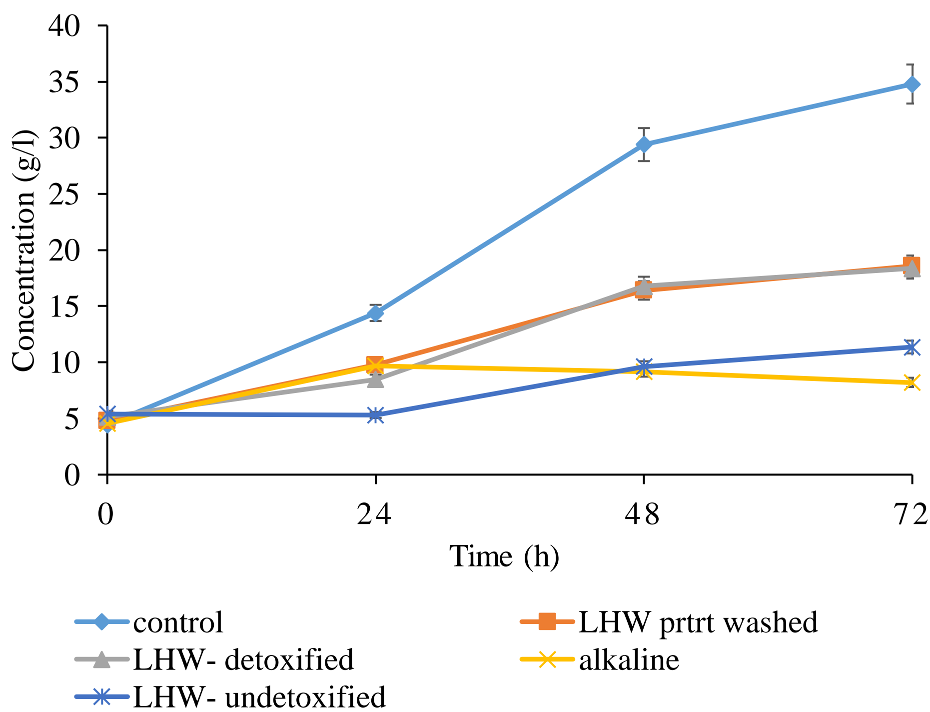

Figure 3.4: Time series data of mean optical densities ± standard deviation depicting *C. necator* growth for control, washed LHW solids, detoxified LHW solids, undetoxified LHW solids and AP solids throughout the fermentation

As illustrated in the Figure 3.5 and Figure 3.6, maximum PHB concentration of 3.80 g/l was produced in the control with a yield of 0.15 g/g sugars at 48 h, followed by the washed solids hydrolysate (WSH) which produced 2.00 g/l PHB with a yield of 0.18g /g. The PHB yield

decreased from 0.24 g/g at 24 h for control when 41.8% glucose was consumed to produce 3.28 g/l PHB, however, PHB was not produced at the same rate as it was in the first 24 h even though 79.35% and 96.47% of glucose was consumed in 48 and 72 h, resulting low yields for the control. Likewise, WSH produced 0.69 g/l and 1.37 g/l PHB by consuming 15.2 % and 44.05% glucose at 24 h and 72 h, respectively. A similar trend of maximum PHB production at 48 h can be seen in all media except in alkaline pretreated hydrolysate (APH). Conforming with the optical density trend in APH, 1.08 g/l PHB was produced in the first 24 h, after which PHB concentration declined to 0.51 g/l at 72 h. PHB concentrations up to 0.86 g/l, 1.12 g/l, 0.73 g/l were produced in DTX with yields of 0.22 g/g, 0.09 g/g, 0.04 g/g in 24 h, 48 h and 72 h, respectively. Meanwhile, the least PHB concentrations of 0.62 g/l, 0.67 g/l, 0.43 g/l was produced in UNDTX, however, with the highest PHB yields of 0.65 g/g, 0.41 g/g and 0.21 g/g at 24 h, 48 h and 72 h, respectively (Figure 3.6). This anomalous yield is a result of PHB production based on consumption of lignin derivatives rather than available sugars in the media. Figure 3.7 displays the reduced lignin concentration before and after fermentation which supports the hypothesis that lignin derivatives were used to produce PHB in the cells in UNDTX media.

Statistically, there was no difference found PHB production in different media such as WSH, DTX, UNDTX and APH used except for control at the significance level of 0.05 at 24 h and 72 h while WSH and control were found to be significantly different from rest of the media at 48 h in terms of PHB concentration. PHB yields for all media were found to be similar at 48 h and 72 h, while PHB yield for UNDTX was significantly higher than the other media at 24 h.

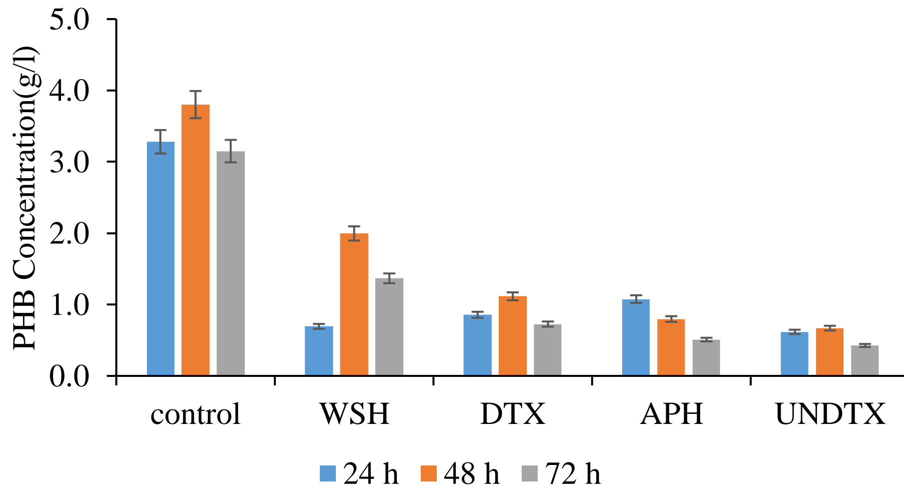

Figure 3.5: Comparison of PHB (g/l) produced in 24 h, 48 h and 72 h for control, washed LHW solids, detoxified LHW solids, undetoxified LHW solids and (AP) solids throughout the fermentation.

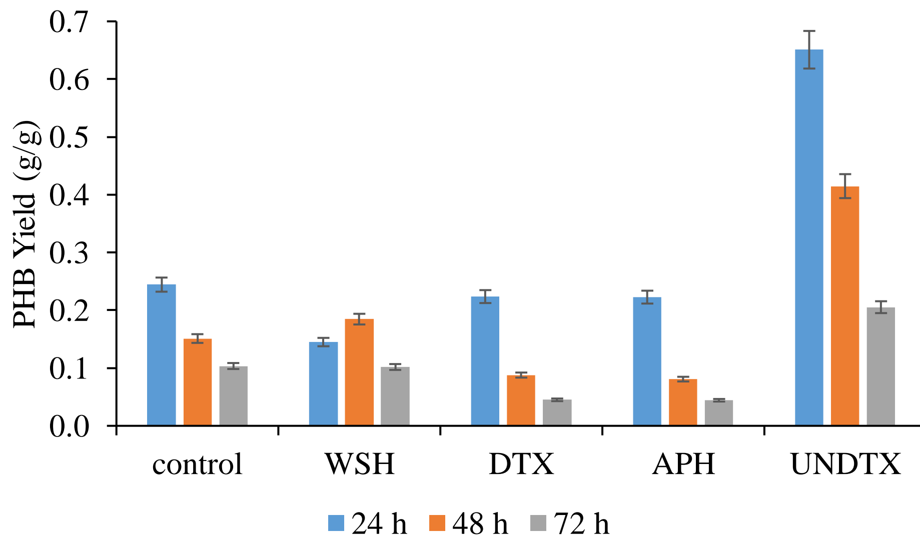

Figure 3.6: Comparison of PHB yield produced in 24 h, 48 h and 72 h for control, washed LHW solids, detoxified LHW solids, undetoxified LHW solids and (AP) solids throughout the fermentation

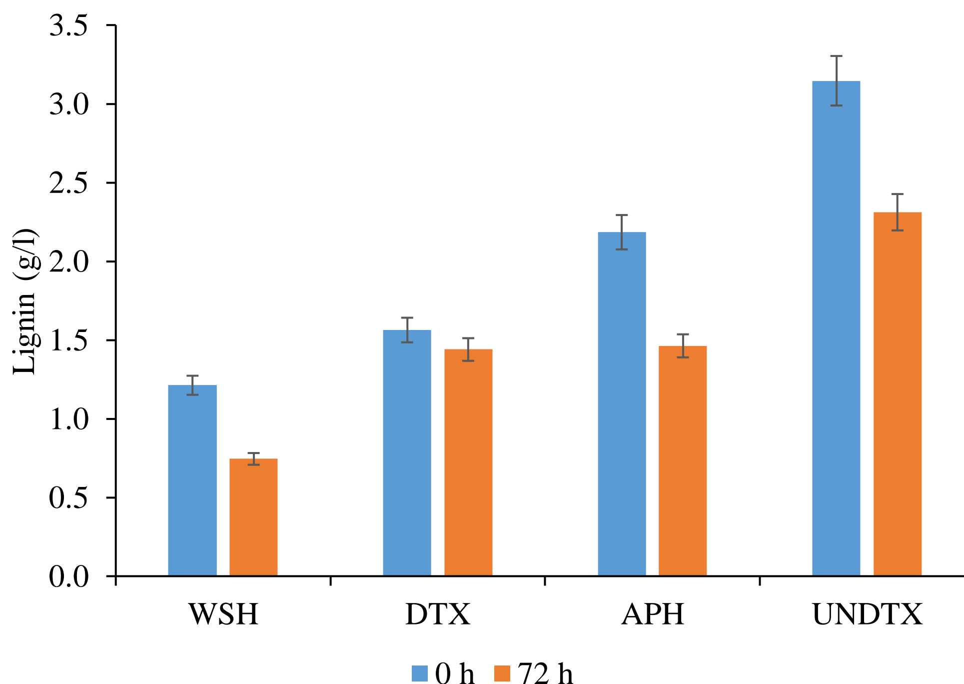

Figure 3.7: Lignin concentration in washed LHW solids, detoxified LHW solids, undetoxified LHW solids and (AP) solids before and after the fermentation.

3.4.2 Dilution of alkaline hydrolysate versus growth viability of *C. necator*

Since the lignin derivatives in the unwashed alkaline solids hydrolyzed with prehydrolysate suppressed the growth of *C. necator* in the media during fermentation, the hydrolysate media was diluted with water from 25% to 50 % As demonstrated in

Figure 3.8, 25% and 31.25% media concentrations supported bacterial growth in the first 24 h, followed by 37.5% media concentration where lag phase of 24 h could be observed. At 48 h, maximum ODs of more than 10 were observed in 25% and 31.25% diluted media, while a similar OD was observed in 37.5% diluted media at 72 h. No growth was observed in the 43.75% and 50% diluted media.

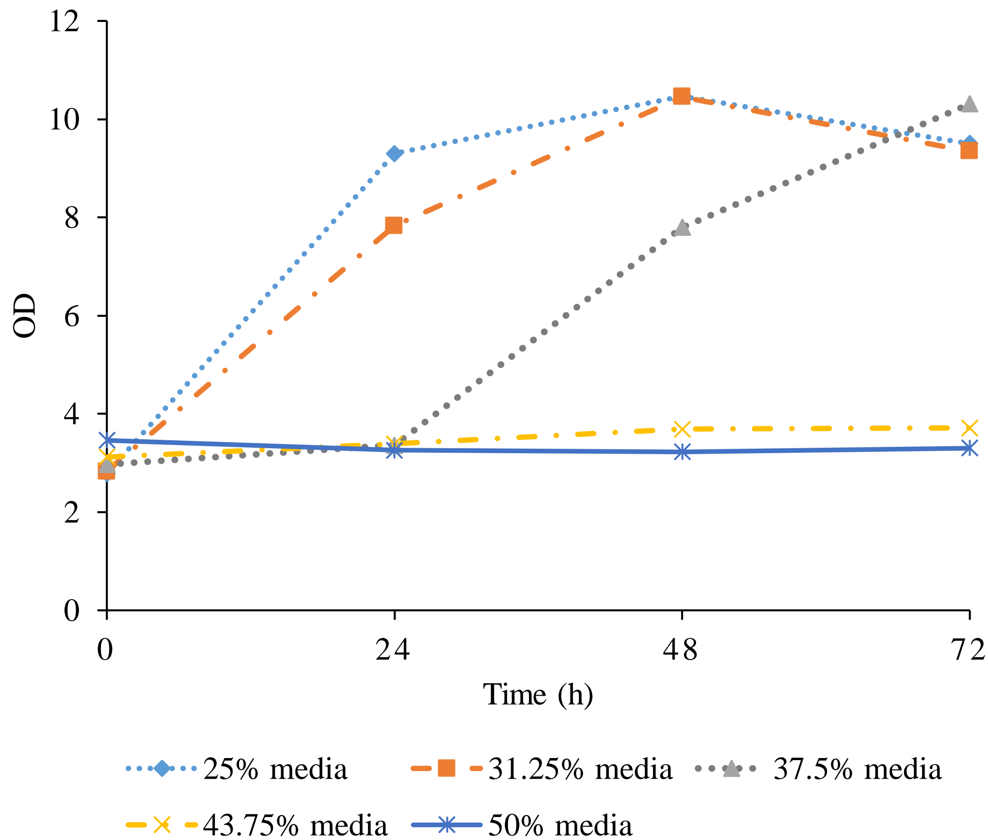

Figure 3.8: Time series for growth of *C. necator* in different dilution ratio hydrolysate media

ODs at 24 h in 25% and 31.25% diluted media were found to be significantly ($p<0.05$) different from the other media, while the OD at 48 h for 37.5% media differed significantly ($p<0.05$) with first two dilution ratios as well as with the last two dilution ratios. At 72 h, OD in the first three diluted media such as 25%, 31.25% and 37.5% differed significantly ($p<0.05$) from the rest two media (43.75% and 50%). These results concluded that the lag phase and viability of *C. necator* is inhibited with increasing lignin derivatives content and stops growing if the lignin content exceeds 37.5%.

3.5 Conclusion

This study demonstrated the valorization of hemp stalk for PHB production using *C. necator* without the intermediate washing of pretreated solids against substantial water consumption problem for washing in industries. Hydrothermolysis followed by detoxification seemed to be better pretreatment technique than alkaline method to process solids for one pot treatment however, alkaline pretreated solids must be processed with equivalent water instead of prehydrolysate during enzymatic hydrolysis for its viability for bacterial growth. PHB production was observed using the one pot treatment process, which would reduce water demand for washing, however, the low PHB concentration would not be economically feasible unless the production and yield of PHB is significantly enhanced.

3.6 References

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4 Chapter 4: Summary, Conclusion and Recommendations

4.1 Summary and Conclusion

This thesis investigated hemp stalk as a promising feedstock for sustainable production of PHB. The cellulose-rich nature and hardiness of hemp biomass yielding high sugar concentrated hydrolysate makes it viable for commercial PHB production. *Cupriavidus necator* and *Paraburkholderia sacchari*, being two different natured PHB producing microorganisms, can benefit from different factors in the fermentation media while complementing their metabolic capabilities and contributing in PHB synthesis cooperatively. Parameters of key processes used throughout the entire PHB production journey were optimized to increase the PHB yield.

In chapter 2, pretreatment was optimized using 1% sodium hydroxide solution at 130°C, which yielded hydrolysate with glucose concentration up to 61.6 g/l and xylose concentration up to 13.57 g/l. Batch fermentation was carried out in shake flasks with *C. necator* and *P. sacchari*, both separately and in combination. Nitrogen and phosphorus content had to be increased to ensure increase of bacteria and desired product in the media. Flasks with *C. necator* only obtained the best PHB yield of 0.43 g/g sugars at 48 h from the start of fermentation and a PHB yield of 0.341 g/g sugars was obtained in combined bacterial treatment. The highest PHB yield was obtained in all hydrolysates at 48 h from the start of fermentation and more PHB was produced in hydrolysates than in controls for all types of bacterial treatment due to consistent pH condition boosted by buffer strength of 50 mM phosphate buffer.

Chapter 3 focused on processing pretreated solids for further hydrolysis and fermentation without washing the associated lignin derivatives and inhibiting components, simulating the one pot treatment condition with an objective of reducing substantial water demand for the PHB

production with *C. necator*. Hemp stalks were pretreated with two types of pretreatments: hydrothermolysis and 1% sodium hydroxide treatment. Bacteria in alkaline pretreated mixture was inhibited by high concentration of lignin derivatives from producing PHB, hence, the prehydrolysate had to be below 37.5% of the original concentration to be feasible for growth of *C. necator* and PHB production. The maximum PHB yield of 0.22 g/g sugars was produced using alkaline pretreatment in the first 24 h while the maximum theoretical PHB yield that can be attained is 0.478 g PHB per g glucose, if PHB is converted from glucose and 0.573 g PHB/ g xylose if PHB is converted from xylose. Detoxified LHW hydrolysate supported the growth of *C. necator*, however, the bacteria could produce only 0.22 g/g PHB yield while bacteria in undetoxified LHW pretreated hydrolysates preferred lignin and its derivatives over sugars in the media ultimately achieving highest PHB yield of 0.65 g/g sugars. From a scaling up perspective for commercialization, PHB production with unwashed pretreated solids would be beneficial in terms of environmental sustainability and cost, however, the fact that only about 1 g/l of PHB only was produced would not make it feasible from an industrial point of view.

4.2 Recommendations

To build upon the findings of the research, future studies can be focused on optimizing the bacterial strains and conditions used for PHB production. While synergistic effects of two bacterial strains demonstrated positive effects on co-fermentation of glucose and xylose and produced comparable PHB concentration and yield, it could not produce as much PHB as obtained when *C. necator* alone was used, hence, exploring a broader range of microbial combinations, or even genetically engineered strains, could further enhance PHB yield and maximize sugar consumption at the end of the experiment. Additionally, optimizing environmental factors such as dilution rate,

nutrient composition, pH, temperature and other essential parameters throughout the fermentation in fed batch mode can be studied to further improve PHB yield.

Regarding the one pot treatment process, future research can optimize the fermentation parameters such as pH, lignin contents and fermentation salt content, to increase PHB yield from either of the pretreatments used. Regular monitoring of pH and limiting the amount of lignin content and its derivatives could be some of the considerable factors for this objective. Catalyzation by additional components such as laccase or mediator can also be explored to overcome the inhibition during fermentation to increase efficiency of the process. Finally, it is recommended to evaluate the economic feasibility of implementing these water-saving strategies at a commercial scale, ensuring that they provide both environmental and cost benefits.