PRODUCTION OF BIOPLASTICS FROM RENEWABLE AND SUSTAINABLE FEEDSTOCK RESOURCES

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Abstract

Production of Bioplastics from Renewable and Sustainable Feedstock Resources

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This study illustrates the potential opportunity for the utilization of hemp to produce PHB (poly(3-hydroxybutyrate). The objective of the study was to optimize simple sugar availability from hemp for *Ralstonia eutropha*. The use of three pre-treatment methods (grinded – 5% NaOH – Autoclave at 121 ^{o}C for 60 minutes) was able to provide a better fractional insoluble solids (FIS) of $\simeq$ 61 % that was significantly better compared to other combinations of pre-treatments studied. Optimum enzyme dosage was also determined by comparing different enzyme concentrations and found that three enzymes should contain a dose of 1.5 g /L. The optimum pre-treatment and hydrolysis conditions resulted in a better enzyme hydrolysis yield of 10.9 % and PHB yield of $\simeq$ 43 %. Results also demonstrate that sonification did not improve PHB recovery, while pH control increased PHB recovery.

Keywords: Hemp, *Ralstonia eutropha*, PHB, Pre-treatment, Enzyme Hydrolysis

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Chapter 1
Introduction

Plastics are found everywhere, in homes, industry, and in different medicinal applications. They are inexpensive, durable material suitable for a wide variety of applications used by consumers on a daily basis (Cai *et al.* 2018). Plastics dominate the packaging industry and have seen exponential growth globally by shifting the use of plastics from reusable to single-use applications (Geyer *et al.* 2017). For example, plastic bags have become known as an effective single-use product (Geyer *et al.* 2017; Alam *et al.* 2018). Among consumers and commercial retailers, plastic bags have become recognized for its functionality, lightweight, and economically low-cost method of transporting goods and materials. As a result, the global plastic production is growing on a daily basis (Alam *et al.* 2018). Plastics Europe reported that the world produced 335 million tons of plastic in 2016 (PlasticEurope, 2018).

Plastics are manufactured from fossil hydrocarbons containing ethylene and propylene, making them nondegradable (Geyer *et al.* 2017). Nondegradable plastic is defined as the ones that exhibit *"lack of ability of the material to decompose or mineralize at measurable rates"* (Leslie, 2015). At present, plastic is the most common pollutant on the planet displaying serious ecological and economic issues (Avery-Gomm *et al.* 2018). For example, plastic bags have a problematic short life cycle, and after use plastic bags are generally thrown away after 20 minutes and in certain settings can be utilized up to a maximum of one year. With large amounts of plastic products being disposed (regulated and dumped illegally), this has led to adverse environmental events (land, air, water resources) and public health issues (Alam *et al.* 2018).

Generally, increase production of plastic production and short-term use of plastics being discarded, eventually reach landfills, incinerators or in the natural environment (legally or illegally) (Breyer *et al.* 2017; Geyer *et al.* 2017; Alam *et al.* 2018). Plastics are not biodegradable resulting in growing concern over its accumulation and its effects (Geyer *et al.* 2017). Petroleum-based plastics only decompose through combustion or other forms of thermal reactions (Geyer *et al.* 2017). Within landfills, heavy metals used in plastics do not degrade. The expected residual time of heavy metals is approximately 150 years (Adelopo *et al.* 2018). As heavy metals leach out, landfill plastic pollution contribute to as sources of contamination for freshwater and air (contributing to greenhouse gases) (Beyer *et al.* 2017).

Soil is a big part of urban ecosystems. Due to industrialization, urbanization, and advancements in agriculture, toxic plastic pollutant accumulates in the soil and act as a reservoir (Wang *et al.* 2018). This pollutant can migrate to the surface to the topsoil or migrate to groundwater and precipitate, then released into the environment via volatilization affecting both the atmosphere and different water sources (Wang *et al.* 2018). Plastic waste is found throughout the world's water supply. It was reported that more than 12.7 million tons of plastic waste, have already enter the ocean (Jambeck *et al.* 2015; Bour *et al.* 2018). Plastic waste is expected to grow in the following years as shipping, fishing, tourism, and other transport activities increase over time (Colmenero *et al.* 2017). Within the ocean, the plastic waste is exposed to different physical, biological and chemical processes/reactions that result in continuous fragmentation into micro-sized particles known as microplastics (Choi *et al.* 2018). Microplastics are characterized as being less than 5 mm (Bour *et al.* 2018).

As fragmentation process continues, the concentration of microplastic is expected to increase in the environment over time (Cai *et al.* 2018). In certain parts of the aquatic

environment, maximum microplastic concentration reached up to 100,000 particles / m^3 (Choi *et al.* 2018). The high concentration of microplastics has led to several different problems in the marine ecosystem. Particular threats to the marine ecosystem include the digestion of microplastics in different marine environments (Colmenero *et al.* 2017). The small size of microplastics can simply be transported to other water sources (seas, lakes, rivers, etc), by ocean currents, wind, tides etc. (Cai *et al.* 2018). Due to the size of microplastics resembling the plankton species, microplastics can be easily confused by invertebrates and other fish species (Ding *et al.* 2017).

It was reported in 2015 by de Sá, who investigated the effect of microplastic on juvenile goby that goby had difficulty differentiated between microplastic and its prey (de Sá *et al.* 2015). Other research such as the one conducted by Ding *et al.* (2017) focused on the ingestion of microplastics with zebrafish and found microplastic accumulation in different parts of the zebrafish including gills, brain, gut, and liver (Ding *et al.* 2017). Furthermore, a study completed in 2017 by Jovanović, found that fish that accumulate microplastic are at risk of developing intestinal issues, liver metastasis, lipid metabolism, behavioral and energy disturbances (Jovanović, 2017). In certain cases, ingestion of microplastics may be fatal (digestion issues and/or locomotion issues) (Choi *et al.* 2018). Locomotion issues arise from low energy reserves and unable to effectively escape from a predator (Ding *et al.* 2017; Choi *et al.* 2018). As plastic waste fragments into microplastic. Many toxic compounds that were once used as additives are released. Toxic chemicals include polychlorinated biphenyls, and nonylphenols organic pesticides (bisphenol A) (Debroas *et al.* 2017).

Generally, the toxic chemical is not covalently bonded to the plastic polymer and can be easily separated as microplastics are formed. As a result, toxic chemicals accumulate in the local

environment which can affect the normal functioning of the marine organism (Cai *et al.* 2018). It has been reported that 60 % to 80 % of floating litter in the oceans is made up of microplastics. Marine microbes are also affected by floating microplastics. Floating microplastics layers offer microbes an alternative substrate that can be used for microbial colonization and transportation, thus affecting the natural ecosystem of the ocean (Debroas *et al.* 2017). Due to the small size of microplastics, it has the ability to easily be transported to other water sources (seas, lakes, rivers, etc), by ocean currents, wind, tides etc. (Cai *et al.* 2018).

Freshwater sources can achieve up to 4 μg / L (Chen *et al.* 2017). The microplastic ingested by marine organisms/ animals are affected by the accumulation and release of toxic chemicals which also affects the quality of the surrounding environment (Hoffman *et al.* 2017). As the smaller ill organism/ animal are preyed upon, the larger animal/human ingested the toxins absorbed. As a result, moving up the food chain, have the potential to affect the human quality of life (Hoffman *et al.* 2017). Plasticizers additives are required to make consumer products more flexible and durable (Giovanoulis *et al.* 2018). Examples of plasticizers include phthalate esters, Bisphenol A (BPA), organophosphate esters (OPEs) (Wan *et al.* 2016; Chen *et al.* 2017; Giovanoulis *et al.* 2018).

Phthalates esters are used to improve flexibility, extensibility, and ease of processing (Chen *et al.* 2013). Phthalates can be categorized into two categories based on their molecular weight (Machtinger *et al.* 2018). The first category is referred to as low molecular weight phthalates that are used in personal care products (cosmetics, deodorant, shampoos, nail polish, etc.). Examples of low molecular weight phthalates include diethyl phthalate (DEP) & di-iso-butyl phthalate (DiBP). The second category is referred to as high molecular weight phthalates, which are used for adhesives, wallpaper, polyvinyl chloride, etc. Common examples of high

molecular weight phthalates include di(2-ethylhexyl) phthalate (DEHP) & di(isononyl) phthalate (DiNP) (Machtinger *et al.* 2018). Phthalates are not covalently bonded to the plastic product, therefore can leach out into the environment leading to human exposure and adverse events (Giovanoulis *et al.* 2018; Machtinger *et al.* 2018). Phthalates can contaminant the environment through direct or indirect pathways during different stages, during product use and end of life stages (disposal) (Wang *et al.* 2018).

Humans are continuously being exposed to phthalates by either digestion or inhalation. It has been reported that human's daily exposure to DEHP is ≃ 2 mg/day (Cho *et al.* 2015). Phthalates, in particular DEHP are known to be endocrine disrupting chemicals. Endocrine disrupting chemicals can lead to the development of endometriosis in humans (Cho *et al.* 2015). Generally, DEHP can be chemically broken down in the gut via hydrolysis into a metabolite known as mono-(2-ethylhexyl) phthalate. However, in females, DEHP can have an additive effect leading to infertility and other reproductive issues (Cho *et al.* 2015). Phthalates have also been associated to trigger oxidative stress in other human cells such as umbilical cells and placental cells. Oxidative stress has also been linked to cellular damage in lipids, proteins & DNA and other anti-thyroid activities (Cho *et al.* 2015; Machtinger *et al.* 2018).

Bisphenol A (BPA) containing products are found everywhere since they are used to generate many different household items including drink bottles, paints, toys, food containers, etc. (Huang *et al.* 2017; Lin *et al.* 2017; Desai *et al.* 2018). BPA global consumption was estimated to be over 5.5 million tons in 2011 (Huang *et al.* 2017). BPA can leach out from food and drink containers and migrate into stored content (Jalal *et al.* 2018). Different factors such as heat and pH (acidic or basic) influence leaching profile from BPA containers (Jalal *et al.* 2018). A study conducted by Howe *et al.* (1998) investigated the potential exposure of BPA from

coated cans. Howe *et al.* (1998) found that 6.6 µg / person per day can be transferred from can to people, after ingestion the BPA passes into the bloodstream (Howe *et al.* 1998; Jalal *et al.* 2018). Interestingly, in 2016, dog and cat pet food will generate $ 62.75 million (US) in sales (Koestel *et al.* 2017). As in humans, the increasing amount of canned food diet as an effect on a pet's health (Koestel *et al.* 2017).

As BPA reaches the gastrointestinal system, it is metabolized by the liver & gut converting it into BPA-glucuronide (Huang *et al.* 2017). The half-life of BPA is 6 hrs, as it accumulated over time, it poses a risk to develop adverse health events in the body (Huang *et al.* 2017). BPA like phthalate is an endocrine-disrupting chemical and is linked to many child and adult adverse health events (Cho *et al.* 2015; Desai *et al.* 2018). Due to the endocrine-disrupting chemical ability to bind to stimulate endogenous hormone receptors, past literature has established BPA association to reproductive, neurogenesis, neurological effects (Koestel *et al.* 2017; Desai *et al.* 2018). Interestingly, in 2016, dog and cat pet food will generate $ 62.75 million (US) in sales (Koestel *et al.* 2017). As in humans, the increasing amount of canned food diet as an effect on a pet's health (Koestel *et al.* 2017). A study conducted by Kang *et al.* (2002) investigated the amount of BPA in pet canned food. Kang *et al.* (2002) found that in 15 different cat food brands, BPA range from 13 to 136 ng /g. Kang *et al.* (2002) also observed 11 to 206 ng /g in 11 different brands of dog food (Kang *et al.* 2002). In 2017, Koestel *et al.* (2017) determined the effects from a two-week canned food diet on dogs and found BPA levels were closely related to bicarbonate levels in the blood. As BPA increased the bicarbonate levels also increased. This relationship illustrated the potential higher risk of dogs developing renal and gastrointestinal disorders (Koestel *et al.* 2017).

Global accumulation of plastic waste is a growing problem (Changwichan *et al.* 2018). To address this growing threat to the environment along with the reduction of petroleum resources, researchers are focused on developing/improving plastic recyclability (Avery-Gomm *et al.* 2018; Choi *et al.* 2018; Landon-Lane, 2018). One area of emphasis is to produce biodegradable plastic through renewable resources (Landon-Lane, 2018). Biodegradable plastics or bioplastics are referred to a category of plastics that are made from renewable biomass feedstock (plant-based) and other organic compounds that have the ability to degrade in the environment into smaller &safer particles (Changwichan *et al.* 2018; Landon-Lane, 2018).

Bioplastics can be categorized into 4 different categories.

- Group 1: Bioplastic polymer derived directly from biomass such as starch & cellulose (Garcia-Garcia *et al.* 2016).
- Group 2: Bioplastic polymer derived from monomers retrieved from renewable biomass through a chemical reaction (Garcia-Garcia *et al.* 2016).
- Group 3: Bioplastics polymers derived from non - renewable resources. An example includes fossil fuel (Garcia-Garcia *et al.* 2016).
- Group 4: Bioplastic derived from bacterial fermentation (Garcia-Garcia *et al.* 2016).

Bioplastics such as polyhydroxyalkanoates (PHA) can be produced through a microbial fermentation process (Group 4) and under certain processing conditions can produce similar characteristic similar to petroleum-based plastics (Changwichan *et al.* 2018). As bacteria are exposed to undesirable growth conditions, they are exposed to an oversupply of carbon with a limitation of one essential nutrient such as phosphorous or nitrogen (Cesario *et al.* 2014). As a

result, during the fermentation process bacteria accumulate in the form of intracellular granules in the cytoplasm that serves as an energy source (Azizi *et al.* 2017). Past literature has established PHA benefits including being biocompatible and exhibiting thermoplastic characteristics (Zhila *et al.* 2018). Most importantly, PHA exhibits physical properties comparable to petroleum-based polymers (Yang *et al.* 2011). Table 1 compares PHB versus fossil-fuel produced plastic characteristics

Table 1.1 Compares PHA polymer characteristics with fossil-fuelled based plastics.

Properties	PHB	Fossil-Fuelled based plastics- Polypropylene
Crystallinity (%)	60	70
Tensile Strength (MPa)	40	34.5
Melting Point (^{o}C)	180	176
Glass Transitional Temperature	4	-10
Density (g/cm^{3})	1.25	0.91
UV Light Resistance	Good	Poor
Biodegradability	Good	Poor

Table 1.1 based on average values. Adapted from Akaraonye *et al.* 2010

Polyhydroxybutyrate (PHB) is a type of PHA (Azizi *et al.* 2017). Typically, PHB exhibit biocompatible, thermoplastic, nontoxic properties that can be used in the field of medicine (ex. controlled drug release applications) (Henrich *et al.* 2012; Huschner *et al.* 2015; Azizi *et al.* 2017). Figure 1 illustrates the chemical formula of PHB.

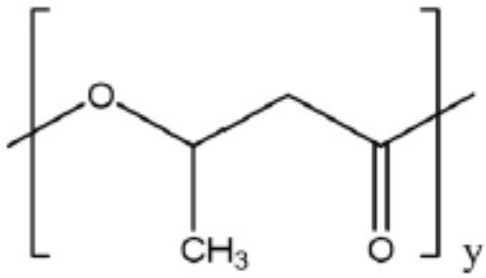

Figure 1.1 Illustrates the chemical formula for PHB. Adapted from Goonoo et al. 2017

Chapter 2
Literature Review

2.1. PHB

PHB can be made by either gram-negative or gram-positive bacteria under certain conditions (Yang *et al.* 2011). *Ralstonia eutropha* is one the most recognized bacteria among PHA-generating microorganism to effective accumulate large amounts of PHB. (Taguchi *et al.* 2003; Huschner *et al.* 2015). *Ralstonia eutropha* is also known as Cupriavidus necator is a gram-negative bacterium belonging to the of β- proteobacteria (Lee *et al.* 2016). It has the ability to consume renewable carbon sources to synthesize PHB (Taguchi *et al.* 2003). Environmental parameters such as temperature, pH, dissolved oxygen and other environmental conditions (carbon to nitrogen ratios (C: N)) all influence PHB production via *Ralstonia eutropha* (Huschner *et al.* 2015; Kosseva, *et al.* 2018). The ideal temperature for *Ralstonia eutropha*-PHA production requires a temperature between 25 ^{o}C to 40 ^{o}C, pH range between 5.5 to 7.9 (Huschner *et al.* 2015). Typical conditions of nutrient stress require an abundant source of carbon with a small (limited) amount of nitrogen, (Yang *et al.* 2011).

Two conditions to consider:

- Low C: N ratio: In this condition, more carbon is used to produce energy compounds, meaning less carbon for PHB production. The nitrogen is used for cell growth (Chakraborty *et al.* 2009).

- High C: N ratio (ideal): In this condition, more carbon is available meaning more PHB production, while limited nitrogen cannot be used for cell growth (Chakraborty *et al.* 2009).

When bacteria are subjected to unbalanced growth conditions, they have the ability to accumulate PHA and other intracellular compounds (Noda *et al.* 2005; Burniol-Figols *et al.* 2018). The PHA acts as an energy storage mechanism (Noda *et al.* 2005). PHB which is a short chain length PHA is stored in the cytoplasm as an insoluble droplet (Lin *et al.* 2017). Once the energy storages are exhausted, microorganism uses the excess PHB for growth (Lin *et al.* 2017).

2.2. Biorefinery

Biorefinery is defined as *"the sustainable processing of biomass into a spectrum of marketable products (food, feed, materials, and chemicals) and energy (fuel, power, and heat)"* (Huijgen *et al.* 2012; IEA, 2014). An example is biofuels in particular bio-ethanols. Bio-ethanol is widely used in the United States & Brazil as a good alternative for fossil fuels used for transportation (Pakarinen *et al.* 2012; Kuglarz *et al.* 2016; Lee *et al.* 2016). Another important feature of biofuel is the ability to be incorporated into existing fuel systems without difficult (Kuglarz *et al.* 2016).

Initially, bio-ethanol were produced using starched-based plants such as sugar cane. However, difficulties arise as the crop itself is not sustainable and/or crop cultivation is not possible in different parts of the world (Kuglarz *et al.* 2016). Next generation bio-ethanol shift the use of starch-based material to lignocellulosic-based that was attained from dedicated biomass or other forms of agriculture residues (Kuglarz *et al.* 2016). The goal of second-

generation bio-ethanol is to use lignocellulosic material that has the ability to reduce carbon footprint in the atmosphere (Park *et al.* 2010; Pakarinen *et al.* 2012).

2.3. Lignocellulosic Biomass

Lignocellulosic-based material such as wheat straw and rice straw & softwood are highly available inexpensive sources of carbohydrates. They are considered renewable and do not compete with global food production (Park *et al.* 2010; Cesario e*t al.* 2014). Lignocellulosic is a reliable resource for biorefinery (Huijgen *et al.* 2012). The three main components of lignocellulose are cellulose (β -1,4-linked glucose polymer) hemicellulose (polymer containing hexose & pentose) and lignin (phenol cross-linked polymer) (Galletti *et al.* 1991; Kupski *et al.* 2018). These three components make up the cell wall which acts as a physical barrier aimed to protect the inner tissues (Kupski *et al.* 2018). The cell wall physical barrier acts to support plant structural, impermeability and protector other forms of attack (microbial attack & oxidative stress) (Cesario *et al.* 2014). Efficient disassembly into individual components is a prerequisite for an economically feasible lignocellulosic biorefinery (Huijgen *et al.* 2012).

Despite the individual components chemical structure, cellulose and hemicellulose are securely held by lignin in the cell wall (Jeun *et al.* 2015). The use of these two sugars is regulated by the structural resistance of the cell wall. This structural resistance is known as biomass recalcitrance (Jeun *et al.* 2015). Parameters affecting recalcitrance include biomass structural heterogeneity, amount of lignin and cellulose crystallinity (Wang *et al.* 2017). Once liberated, cellulose and hemicellulose demonstrate a good source of carbon to be used in further biological processes (Cesario *et al.* 2014).

2.4. Other Biomass Considered

Sugars are an important source in human diet. Researchers aim to use non-food renewable resources for PHB production to prevent competition with food resources (Davis *et al.* 2013). Sources such as crude glycerol have been tested for PHA production (Burniol-Figols *et al.* 2018) Glycerol is an attractive biomass due to its high availability and low cost. In fact, glycerol is the by-product of biodiesel (Fukhi *et al.* 2014). However, there are three major challenges associated with glycerol as a resource (Burniol-Figols *et al.* 2018).

1. After the biodiesel production, glycerol along with other impurities are produced (Burniol-Figols *et al.* 2018). These impurities cannot be used in pharmaceutical industries. Further refinement is needed (Mozumder *et al.* 2014).
2. The molar mass of polymer produced at the end of production, affecting the quality of the polymer (Mozumder *et al.* 2014; Burniol-Figols *et al.* 2018)
3. The actual product produced only contains hydrobutrate which have to produce undesirable thermal characteristics when compared to PHB. Further processing is required (Burniol-Figols *et al.* 2018).

Another example is grass. Globally there are over 2.3 billion hectares of the total grass agriculture area available (Davis *et al.* 2013). In Europe alone, grass makes up approximately 240 million hectares of available grassland. The grass is an attractive biomass since it does not require fertilizer (cost) & plowing of soil (labor) (Davis *et al.* 2013). Also by 2030, Europe would gain access to 19 million hectares of grass that would not disrupt Europe's food/agriculture sector (Davis *et al.* 2013). Grass is made up of two components, with one component which makes up of cellulose, hemicellulose, and lignin. Depending on the species,

generally grass is made up of 25 – 40 % cellulose, 15- 50% hemicellulose and 10 – 30 % lignin (Davis *et al*.2013; Cerrone *et al.* 2015). However due to its high lignin content, impedes its ability to be a bioaviable source, making grass less favorable to use (Davis *et al.* 2013).

Table 2.1 Summarizes the cellulose, hemicellulose and lignin content within different biomass

Biomass	**Cellulose (%)**	**Hemicellulose (%)**	**Lignin (%)**	**Reference**
Wheat Straw	30	50	20	Dahman *et al.* 2014
Rice Straw	31	22	13	Chen *et al.* 2011
Rice Husk	31	24	14	Reddy *et al.* 2005
Barley Straw	38	30	16	Reddy *et al.* 2005
Sugar Cane	43	31	11	Martin *et al.* 2007
Banana	63	7	7	Reddy *et al.* 2005
Pineapple	76	18	9	Reddy *et al.* 2005
Grass	33	33	20	Reddy *et al.* 2005
Hemp	55	16	4	Garcia *et al.* 1998

Note: Average content is listed from Reddy *et al.* 2005. Different species of the same biomass experience different climate and environmental stress (Cerrone *et al.* 2015). Table adapted from Reddy *et al.* 2005

Table 2.2 List of agriculture waste in relation to the production of cell dry weight (CDW) and PHA produced

Source	**Microorganism**	**CDW (grams/liter)**	**PHA Type**	**PHA produced (w/w)**	**Reference**
Pea shells	*Bacillus sphaericus*	2.50	PHB	1.60	Kumar et al 2016
Low-quality waste animal fats (swine/cattle)	*Ralstonia eutropha*	4.50	PHB	-	Riedel *et al.* 2015
Pineapple	*Bacillus sphaericus*	4.20	-	0.06	Suwannasing *et al.* 2015
Glycerol	*Ralstonia eutropha*	1.81	P(3HB)	1.08	Fukui *et al.* 2014
Soybean Oil	*Ralstonia eutropha*	6.80	P(3HBco-3HHx)]	6.0	Insomphun *et al.* 2014
Rice	*Bacillus firmus*	1.68	PHB	-	Sindhu *et al.* 2013
Rice Husk	*Bacillus mycoides*	0.47	P(3HBco-3HV)	0.07	Narayanan *et al.* 2014
Wheat Straw	*Ralstonia eutropha*	15.3	PHB	-	Dahman *et al.* 2014
Sunflower Husk	*Ralstonia eutropha*	8.82	PHB	7.69	Saratale, *et al.* 2015
Rice Paddy straw	*Ralstonia eutropha*	10.87	PHB	9.88	Saratale, *et al.* 2015
Orange Juice Waste	*Ralstonia eutropha*	9.58	PHB	7.31	Lagunes *et al.* 2016
Sugar Cane Waste	*Bacillus sphaericus*	9.00	PHB	5.00	Getachew *et al.* 2016
Banana Waste	*Bacillus sphaericus*	7.80	PHB	2.10	Getachew *et al.* 2016
Corn Cob	*Bacillus sphaericus*	9.30	PHB	4.80	Getachew *et al.* 2016

2.5. Pre-treatment

Production of PHB bioplastic requires three main stages. The first is pre-treatment followed by enzyme hydrolysis and finally fermentation (Zhu *et al.* 2015). Most agriculture waste considered is made up of a high amount of lignin which affects the performance of enzyme hydrolysis. In order to achieve maximum potential from enzyme hydrolysis, the agriculture

waste must be first be pretreated (Zhu *et al.* 2015). In the cell wall, cellulose is a water-insoluble high crystalline structure made up of a long chain polymer of β-D-glucopyranose units. Hemicellulose is a heterogenous branched polymer made up of xylose & hexose, galactose (Cesario *et al.* 2014; Gao *et al.* 2017). Lignin is an amorphous complex made up of a heteropolymer of phenylpropene units (Gao *et al.* 2017). Lignin acts as a barrier of cellulose that aims to improve the overall plant structure while restricting access to microbial attacks (enzymes) (Cesario *et al.* 2014; Zhou *et al.* 2018).

The biggest challenge in pre-treatment is the destruction of the cell wall lignin component (Bule *et al.* 2016). Pre-treatment accounts for up to 40 % of the total processing cost (Zhang *et al.* 2009). The selection of a suitable pre-treatment process should consider the raw material, environmental impact, cost and further down streaming processes (Yuan *et al.* 2018). The ideal pre-treatment process should be able to provide material digestibility along with high yield of essential components (Yuan *et al.* 2018). Examples of pre-treatment processes investigated in the literature include, physical, chemical, thermal (Mohsenzudeh *et al.* 2012).

2.5.1. Physical Pre-treatment

Physical treatment is also known as mechanical pre-treatment and is used to reduce particle size and crystallinity while increasing the specific area and reduce the degree of polymerization (Gao *et al.* 2017). An example is ball milling. Ball milling is used to produce fine particles and has shown to increase enzyme digestibility (da Silva *et al.* 2010). A study conducted by da Silva *et al.* 2010 who investigated ball milling time (30 minutes to 120 minutes) effect on sugarcane bagasse and straw. da Silva *et al.* (2010) found that increasing the ball milling time for both bagasse and straw increased glucose and xylose yield. However, da Silva *et al.* (2010) found that depending on the component of sugarcane, different times were required to

achieved similar glucose and xylose yield. Bagasse took 60 minutes to produce approximately 79 % glucose and 72 % xylose. While sugarcane straw took, 90 minutes (30 minutes longer) to produce approximately 78 % glucose and 57 % xylose (da Silva *et al.* 2010).

2.5.2. Chemical Pre-treatment

Chemical pre-treatments are considered to provide faster and efficient degradation of the cell wall (Mancini *et al.* 2018). Examples of the chemical pre-treatment include acidic and alkalization (Zhu *et al.* 2015; Yuan *et al.* 2018).

2.5.2.1. Acidic Pre-treatment

One widely used form of chemical pre-treatment is known as acidic pre-treatment (Zhu *et al.* 2015). Acidic pre-treatment works by hydrolyzing the hemicellulose into its individual monosaccharide composition. The lignin is condensed and precipitated at the same time (Jaisamut *et al.* 2016). Cellulose is released from lignin and enzymes used in enzyme hydrolysis now have more accessibility to the cellulose (Jaisamut *et al.* 2016). Utilization of a dilute acidic pre-treatment is cheap and can be easily applied (Zhu *et al.* 2015). An example is acidic pre-treatment is diluted sulfuric acid (Dahman *et al.* 2014).

There are three major challenges associated with acidic pre-treatment. Firstly, not all lignin is precipitated, affecting the overall enzyme hydrolysis process (Zhu *et al.* 2015). Secondly, toxic by-products that inhibit enzyme hydrolysis are produced during the decomposition of the cell wall (Jaisamut *et al.* 2016). Common by-products include phenolics (hydroxybenzoic acid), furfural, hydroxymethylfurfural (HMF) and other organic acids (Zhu *et al.* 2015; Zhou *et al.* 2018). HMF is generated from the dehydration of hexose such as glucose while furfural is produced by the dehydration of pentose such as xylose (Jaisamut *et al.* 2016).

The final drawback is linked to the corrosion of equipment (Wu *et al.* 2018). These three challenges provide additional cost and neutralization processes (labor & cost) that negatively impact the overall bioplastic production cost (Jaisamut *et al.* 2016).

2.5.2.2 Alkalization Pre-treatment

An alternative to acidic pre-treatment is the use of a different type of chemical pre-treatment known as alkalization (Liu *et al.* 2015). For each glucose molecule within in the cellulose chain, there are three free hydroxyl groups, which makes the region polar (George *et al.* 2014). This makes bast fibers incompatible with nonpolar matrices. Furthermore, the hydroxy groups impact moisture absorption which can result in increased degradation rate (George *et al.* 2014). Alkalization can be used to prevent these issues (George *et al.* 2014). The crystalline regions are covered by the amorphous regions, making it difficult to interact with the cellulose. Alkalization can reduce the number of hydroxyl groups that exist in the amorphous region (Kabir *et al.* 2013). Examples of alkylation include sodium hydroxide and sodium carbonate (Yuan *et al.* 2018). Alkalization works by saponification and cleavage of the lignin bonds between cellulose and hemicellulose (degradation of ester and glyosidic linkages) (Brodeur *et al.* 2011; Mancini *et al.* 2018). This leads to cellulose swelling, surface area for enzyme interaction increases while decreasing crystallinity & degree of polymerization (Liu *et al.* 2015).

Typically, alkalization pre-treatment conditions are less severe compared to acidic pre-treatments. Alkaline pre-treatments can be executed under ambient environments but require a longer duration of time (Brodeur *et al.* 2011). For example, the study conducted by Sun *et al.* (1995) who studied alkaline pre-treatment effect of wheat straw cell wall found that pre-treating wheat straw with 1.5 % NaOH for 144 hours at 20 ^{o}C can result in 60 % removal of lignin (*Sun et al.* 1995; Brodeur *et al.* 2011). Alkalization involves soaking the biomass in the alkaline

solution at a certain temperature for a duration of time (Brodeur *et al.* 2011). A Study conducted by Zhao *et al.* (2007) studied alkaline pre-treatment at low temperatures effect on spruce found that using 7% NaOH pre-treatment for 24 hours at 23 ^{o}C can reduce lignin content by 18.7 % (Zhao *et al.* 2007; Brodeur et al, 2011). After alkalization, a neutralization stage is required to remove lignin before enzyme hydrolysis can begin (Brodeur *et al.* 2011).

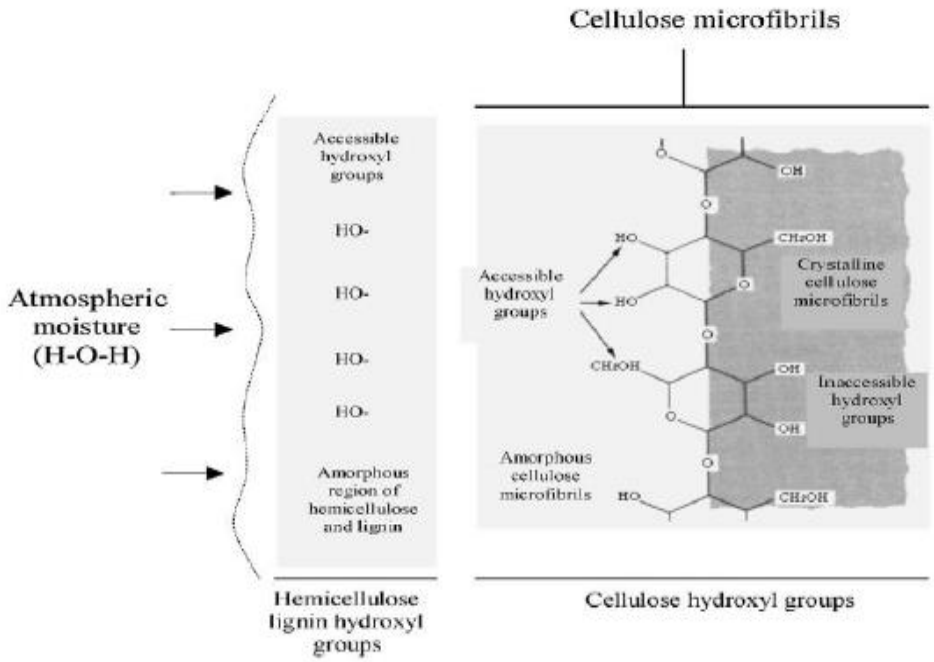

Figure 2.1Represents NaOH interaction with Hemp bast fibers. Adapted from Kabir et al. 2013

2.5.3. Thermal Pre-treatment

Chemical treatments are efficient but difficult to control (Rajput *et al.* 2018). In terms of acidic pre-treatment toxic by-products are produced (Rajput *et al.* 2018). Physical treatment, on the other hand, provides low investment and operational convenience (Rajput *et al.* 2018). Another physical treatment is known as a thermal treatment. Thermal treatment in literature is

referred to as steam explosion or hydrothermal pre-treatment (Rajput *et al.* 2018). Thermal pre-treatment is an environmentally friendly process, where the biomass is exposed to heat under a specific pressure over a specific period of time (Ferreira *et al.* 2013; Romani *et al.* 2016). Generally, the temperature used is between 50 to 240 ^{o}C (Rajput *et al.* 2018). After the duration of time, the biomass is ejected into normal pressure, resulting in an explosion of macromolecules (Ferreira *et al.* 2013). Different factors affect the performance of thermal treatment including residence time, the temperature used and moisture content (Ferreira *et al.* 2013). These parameters affect the release of toxic by-products (Furfural, HMF) and the success of pre-treatment (Ferreira *et al.* 2013). The advantage of thermal pre-treatment is the use without chemicals, less equipment erosion and limitation of concentration of toxic by-products (when parameters are controlled) (Wu *et al.* 2018). It should be noted that thermal pre-treatment alone cannot significantly affect the biomass cell wall (Wu *et al.* 2018). To further enhance the accessibility to cellulose a combination of physical/chemical pre-treatment process is required (Chen *et al.* 2018; Wu *et al.* 2018).

Table 2.3 Compares Physical, chemical, thermal pre-treatments

Pre-Treatment Process	**Influential Parameters**	**Advantages**	**Disadvantages**	**Reference**
Physical				
Ball-Milling	-Duration of time -Biomass used	-Produce Fine Particles -Low investment -Ease of operation -No chemicals	-Cleaning of Equipment	Da Silva *et al.* 2010 Gao *et al.* 2017
Thermal	-Residence time -Temperature used	-Environmentally friendly -No Chemicals	-Energy Requirements	Rajput *et al.* 2018 Ferreira *et al.* 2013 Wu *et al.* 2018

	-Moisture Content	-Less Equipment Erosion		
Chemical				
Acidic	-Concentration used	-Inexpensive -Produce High Sugar yield	-Toxic by-products (HMF, furfural) -Erosion of equipment	Zhu *et al.* 2015 Zhou *et al.* 2010 Dahman *et al.* 2014
Alkalization		-Cellulose swelling -Increase surface area -Decrease crystallinity -Decrease of polymerization	-May affect Cellulose and Hemicellulose	Liu *et al.* 2015 Mancini *et al.* 2018

2.5.4. Combinational Pre-treatment

Thermal pre-treatment has been effective in the use of hardwood, softwood, sugar cane and wheat straw (Ballesteros *et al.* 2006). To further enhance the pre-treatment stage both acidic and alkali chemical pre-treatment are used to improve productivity (Ballesteros *et al.* 2006). Past literature establishes the use of physical/chemical combination used to increase the yield of sugars and while decreasing the temperature and time requirements (Ballesteros *et al.* 2006). Hence reduce energy requirements reducing pre-treatment production cost (Wu *et al.* 2018).

A study conducted by Ballesteros *et al.* (2006) illustrated the use of 0.9 % H_2SO_4 combining with a further 10 minutes of thermal pre-treatment to achieve optimal yield with wheat straw (Ballesteros *et al.* 2006). Wu *et al.* (2018) demonstrated improved enzyme hydrolysis through the use of thermal pre-treatment and post alkylation. In this study, autohydrolysis was performed at 140 ^{o}C for 40 minutes followed by 1-hour exposure to 6 %

sodium hydroxide (Wu *et al.* 2018). As a result, enzyme hydrolysis increased from 36 % (controlled) to 83.7 % (Wu *et al.* 2018). Thus, the combination of physical-chemical pre-treatment is shown to decrease cost and minimize environmental pollution (Wu *et al.* 2018).

Table 2.4 Summarizes the different biomass and corresponding pre-treatment comparing cellulose, hemicellulose and lignin results

Biomass	**Pre-treatment**		**Composition**		**Reference**
		Cellulose / glucan	Hemicellulose/ xylan + arabinan	Lignin	
Jute Fibers	Soaked 5 % NaOH for 4 hours	72.9	12.6	11.7	Liu *et al.* 2009
	Soaked 5 % NaOH for 4 hours with post 1.5% KH-570 and for another 4 hours	77.6	11.4	9.6	
Almond Shell	2step: First step: Sieving (140 um) & Drying at 60^{o}C for 24 hours Second Step: 5% NaOH (80^{o}C for 2 hours) then 10 % H_2O_2 (80^{o}C for 2 hours). Finally drying at 60^{o}C for 2 hours.	46.25	7.56	29.85	Sánchez-Safont *et al.* 2018
Rick Husk	2step: First step: Ultracentrifugally milling (125 um) & Drying at 60^{o}C for 24 hours. Second Step: 5% NaOH (80^{o}C for 2 hours) then 10 % H_2O_2 (80^{o}C for 2 hours). Finally drying at 60^{o}C for 2 hours.	31.13	18.6	28.25	

Seagrass	2step: First step: Ultracentrifugally milling (125 um) & Drying at 60^{o}C for 24 hours. Then 3% CH_3COOH Second Step: 5% NaOH (80^{o}C for 2 hours) then 10 % H_2O_2 (80^{o}C for 2 hours). Finally drying at 60^{o}C for 2 hours	38	21	27	
Hemp	Untreated	46.1	10.1	18	Pakarinen *et al.* 2012
	Steam explosion (200 ^{o}C for 5 minutes)	69.6	5.5	16	
	1 % NaOH	83.6	8.4	7.2	
Wheat Straw	0.35 M NaOH for 6 hours	48.8	23.6	21.9	Yuan *et al.* 2018
	0.35 M NaOH for 6 hours followed by 0.35 Na_2CO_3	46.6	23.0	20.1	
	2 % NaOH with Autohydrolysis (140 ^{o}C)	93.3	80.7	33.9	Wu *et al.* 2018

2.5.5. Other Considerations

A pre-treatment of lignocellulosic biomass issue is related to cost and ability to overcome biomass recalcitrance. While researchers still focus on improving the pre-treatment stage for lignocellulosic material (Kreuger *et al.* 2011). Other research is now shifting focus towards another economically feasible crop that can provide a higher yield while providing a low environmental impact (Kreuger *et al.* 2011).

2.6. Hemp

An alternative to lignocellulosic biomass is hemp (Kreuger *et al.* 2011). Hemp is Cannabis that is mainly made up of cannabidiol (Schluttenhofer *et al.* 2017). In North America, hemp can only be considered as "hemp" when the concentration of THC (Tetrahydrocannabinol) is below 0.3% (Schluttenhofer *et al.* 2017). Hemp can produce high biomass yield while having a low susceptibility to pest (Kreuger *et al.* 2011; Kuglarz *et al.* 2016). The plant itself can adapt and grow in different geographic climates as illustrated in Figure 1 (Salentijn *et al.* 2015). Canada, China, and North Korea are among the highest largest producers of hemp (Schluttenhofer *et al.* 2017). Canada had banned production of hemp from late 1930s to 1998. From 1998, the Canadian government has provided different grants to develop/ grow new hemp cultivators and improve hemp processing technologies. In 2012, to fuel the industry, Canada had certified hemp as a bio-based crop and food safety accreditation was provided (Salentijn *et al.* 2015). As demand for renewable sustainable products increased, hemp has the potential to be used for this growing market (Schluttenhofer *et al.* 2017). Recently hemp has integrated into the automotive, textile, paper, bio-fuel, construction, cosmetic & personal care industries (Salentijn *et al.* 2015). Recently there has been an increased demand for hemp products from the United States, while many other countries around the world are now considering hemp as a highly sustainable crop. The global markets are expected to double between 2016 to 2020 (Schluttenhofer et al. 2017).

Hemp is made up a woody core and bast fibers (Schluttenhofer *et al.* 2017; Xie, 2017). In 1998, a study conducted by Garcia *et al.* (1998) investigated the individual components of hemp bast fibers and woody core. The study found that bast fibers provided a higher amount of cellulose, hemicellulose, pectin, and lignin compared to woody core (Garcia *et al.* 1998). In

order to attain the maximum cellulose, hemicellulose content, the bast fibers must be separations from the woody core. The process of separations is known as retting. Retting is responsible for the improvement the quality of hemp for a future product for use (Schluttenhofer *et al.* 2017). Typically, hemp cell wall is made up of 55 % cellulose, 16 % hemicellulose and 4 % lignin (Garcia *et al.* 1998). Hemp high cellulose content and low lignin composition have the potential to be used for different bioplastic applications (Kreuger *et al.* 2011).

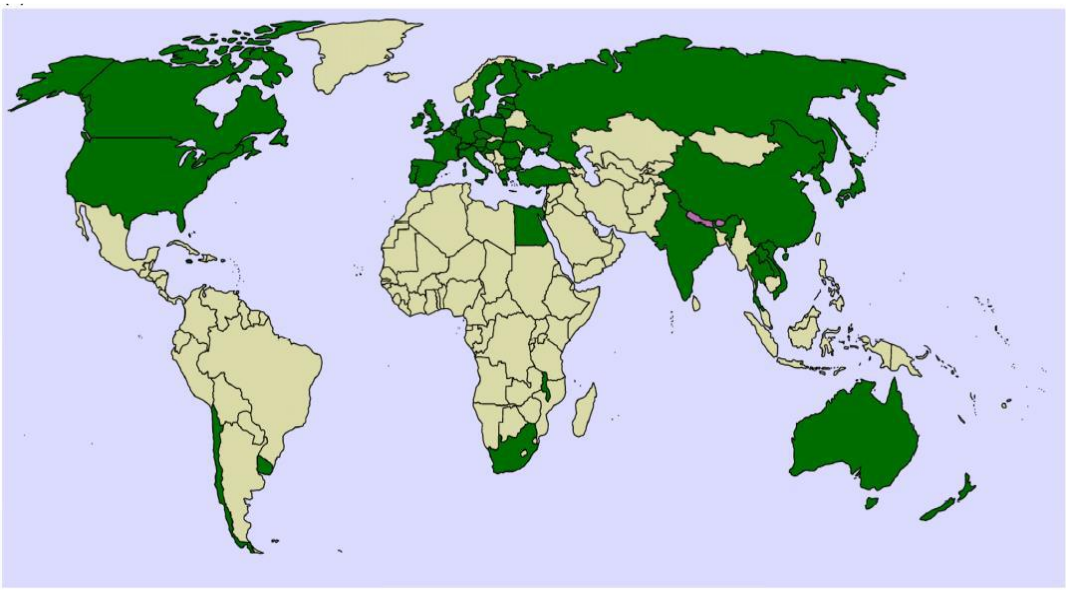

Figure 2.2 Illustrates countries that are currently producing hemp. Adapted from Schluttenhofer et al. 2017

2.7. Extraction Method

There are many different processes used to recover PHB from bacteria (Yang *et al.* 2011). Extraction processes include organic solvents, differential digestion, and detergent base (Yang *et al.* 2011). The extraction process influences the polymer quality (Macagnan *et al.*

2017). Organic solvents such as chloroform and dichloromethane are generally used to extract PHB. The disadvantage of the organic solvent is the use of large amounts required to successfully extract PHB. This, in turn, can account for 50 % or more of the total cost of polymer production (Yang *et al.* 2011). However, the use of an organic solvent is considered a simple recovery method with the ability to remove unwanted endotoxins that cause degradation to the polymer (Kunasundari *et al.* 2011).

Organic solvent extraction is made up of two stages.

- Stage 1: PHB release via cell membrane permeability changes (Kunasundari *et al.* 2011).
- Stage 2: PHB precipitation generated from non-organic solvents. Examples include methanol and ethanol (Kunasundari *et al.* 2011).

Table 2.5 Compares different PHB extraction methods

Extraction Method	**Beneficial Properties**	**Non-Beneficial Properties**
Organic Solvent Ex. Chloroform, dichloromethane	-Endotoxin Elimination -Generates High molecular Weight Product -Generate High Purity -Minor Polymer degradation	-Not Eco-friendly - A large amount of solvent used -Intensive Labour -Time Consuming
Chemical Digestion Method Ex. Sodium Hypochlorite	-Reproducible molecular weight recovered	-Low Purity
Supercritical Fluid Ex. Ammonia, Methanol	-Simple -Cheap -Eco-friendly	-Hard to recover polar compounds -Clean up issues -Compatibility issues with natural compounds

Adapted from Kunasundari *et al.* 2011

2.8. Fermentation Process

In order to move towards a large scale of production, bioreactors should be used (Kaur *et al.* 2015). There are different bioreactors available including batch, fed-batch, and continuous reactors. Bioreactors offer cost benefits such as

- Operating at required temperature to maximize production
- Reducing the potential of contamination to the product

Adapted from Kaur *et al.* 2015

Batch fermentations are considered the easiest and should be used as the initial assessment towards developing PHB (Kaur *et al.* 2015). Past literature focuses on different operating procedures, novel carbon sources conversion, along with the use of different bacteria (Kaur *et al.* 2015). It is described as a closed system, where at the start of the experiment, the substrate and other components are added, react within the reactor and once the reaction is completed, the product can be removed (Kaur *et al.* 2015). The disadvantage of this fermentation process is that it requires additional substrates or components that cannot be added during cultivation. The sample cannot be withdrawn during the reaction period, so no intermediate analysis can be conducted (Kaur *et al.* 2015).

Fed-batch fermentations avoid the issue of starvation of bacteria that usually occur in batch reactors at the end of the reaction. Fed-batch reactors also provide a solution to add addition substrates to the system during the cultivation period. This allows the substrate to be within their ideal fermentation substrate concentration. Other parameters that fed-batch fermentation reactors can control include the pH, concentration of substrate and dissolved oxygen. Controlling these parameters allow an end result of high cell density and PHB production (Kaur *et al.* 2015).

The highest fermentation performance process involves the use of continuous fermentation bioreactors. Advantages of continuous bioreactors include high dilution rates resulting in high concentrations that can provide highly stable production rates (Kaur *et al.* 2015). Disadvantages of continuous bio-reactors include the high risk of bacterial contamination, which can affect the time and cost associated with loss of production. However, it should be noted that an appropriate environmental procedure should be in place, along with more resistant bacteria can help lower the risk of contamination (Kaur *et al.* 2015).

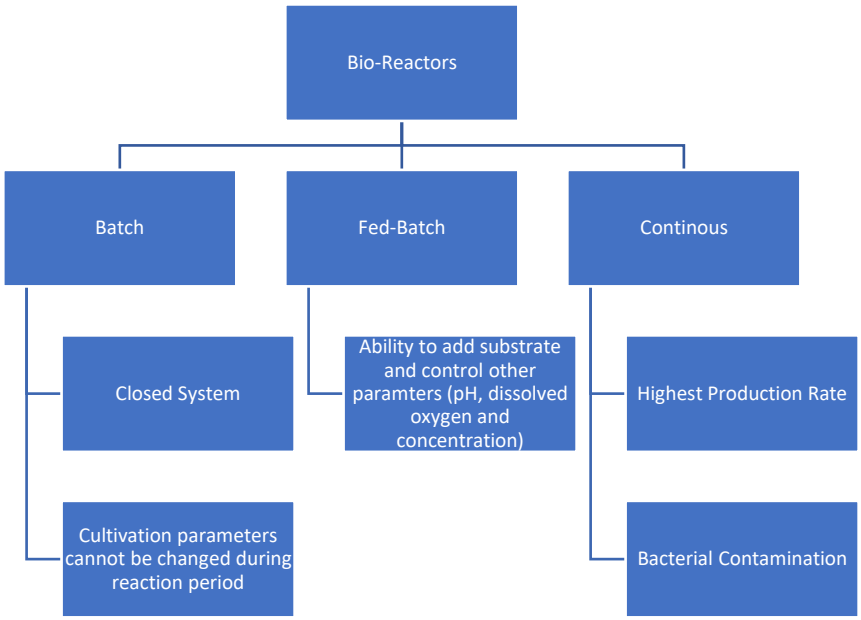

Figure 2.3 Summarizes bioreactors categories and features. Adapted from Kaur et al. 2015

2.9. Processability

The major challenge for industrial production of bioplastics is to acquire/achieve cost competitiveness against conventional fossil fuels produced plastics (Akiyaman *et al.* 2003). In

order to achieve competitiveness, researchers and industrial partners must develop cost competitive methods to increase production (Akiyaman *et al.* 2003). One strategy to overcome PHB properties is to add a copolymer. The addition of copolymers has the ability to decrease the melting point and glass transition temperature while improving internal stability (Modi *et al.* 2011). An example is a study completed by Modi *et al.* (2011) where poly-(3-hydroxy valerate) was added to PHB which helped improve processing temperature by decreasing the melting point. This study demonstrated the potential of co-polymer addition to being used in processed into polymers similar to other petroleum-based products (Modi *et al.* 2011).

An alternative to co-polymer addition is the blending method. Generally, the blending method is easier and faster way to improve PHB properties and lower processing cost (Abdelwahab *et al.* 2012; Mousavioun *et al.* 2013). Most importantly the blending method is environmentally friendly, solvent-free and can be easily executed in different designed chemical reactions under different processing conditions. The results from these designs can then be easily manipulated to meet the final desired requirements (Pryzbysz *et al.* 2018). Blending starch with PHB has gained interested in producing low-cost bioplastic (Ma *et al.* 2014). Starch is a unique resource since it's renewable and can be derived from different biomass including potatoes, wheat and maize (Ma *et al.* 2014). Interestingly, starch is the ideal candidate to fossil-based polymers since it's cheaper to produce starch compared to other oil. However, starch has a high molecular weight and exhibits severe thermal degradation, limiting its processability on a large scale (Ma *et al.* 2014). An alternative to starch is the use of thermoplastic starch (TPS) which are made from gelatinized starch and additives that help improve starches processability (Ma *et al.* 2014). Studies such as the one conducted by Ma *et al.* (2014) who investigated starch effect illustrate the use of starch with PHB blends can improve toughness (Ma *et al.* 2014).

Literature has also used PCL (Polycaprolactone) as a component in PHB blends. PCL is a hydrophobic aliphatic polyester. PCL is obtained from both non- renewable resources such as crude oil and renewable resources such as polysaccharides (Gutiérrez *et al.* 2017). PCL is a biodegradable thermoplastic that exhibits a low viscosity, low melting point ($\simeq 60\,^{o}C$), low glass transitional temperature ($\simeq -60^{o}C$), semi-crystalline structure (Gutiérrez *et al.* 2017). This allows PCL to be processed easily with typically manufacturing equipment. Other advantages of PCL include it being biocompatible, excellent stretch-ability and low water vapor permeability (Gutiérrez *et al.* 2017).

Currently, Polylactic acid (PLA) is the first choice for the commercial biodegradable polymer used to produce films in food packaging applications (Arrieta *et al.* 2015). PLA like PCL has a semi-crystalline structure, derived from lactic acid. Lactic acid can be found in different renewable resources such as corn, sugar beet and wheat (D'Amico *et al.* 2016). Lactic acid is also found in the human metabolic cycle, which indicates that lactic acid is non-toxic and biocompatible (Darie-Niţă *et al.* 2015). PLA is resistant to solvents and provides a good barrier to vapor and other gases (Darie-Niţă *et al.* 2015). Other advantages of PLA include its ability to be degraded and retuned backing into its original component (lactic acid) via ester hydrolysis. The lactic acid can then be recycled and be used for subsequent production of PLA (Darie-Niţă *et al.* 2015). For these reasons, The FDA (Food and Drug Administration) has approved it use (Darie-Niţă *et al.* 2015).

PHB and PLA share common properties. For example, PLA has a melting point of $\simeq 180\,^{o}C$, and becoming brittle as a final product (D'Amico *et al.* 2016). PLA itself has a semi-crystalline structure and a glass transitional temperature of $\simeq 80\,^{o}C$. Due to these characteristics, PLA also exhibits poor toughness and difficulty being processed at higher stress levels (Darie-

Niţă *et al.* 2015). Production cost of PLA can be high compared to petroleum-based plastics (Darie-Niţă *et al.* 2015). In order to overcome these problems, PHB blends provide a method to improve plastic and cost properties (D'Amico *et al.* 2016). Different blends provide different properties. For example, a study conducted by Zhang *et al.* (2011) who investigated the mechanical properties between different ratios of PLA-PHB blends found that PLA (75 %) / PHB (25 %) illustrated better tensile characteristics and mixing behavior compare to other blends studied (Zhang *et al.* 2011). Literature has also shown that PLA and PHB together appropriate mixing is dependent on the molecular weight of each component. For example, when PHB is mixed with a high molecular weight (MW > 18,000) demonstrated a biphasic separation, while PHB mixed with a low molecular weight (MW < 18,000) produces a well-balanced mixture (Abdelwahab *et al.* 2012). On the other hand, PLA can also be mixed with low molecular weight PHB (MW < 9400) and miscible with high molecular weight PHB (MW> 9400) (Abdelwahab *et al.* 2012). Factors affecting plasticizers performance include chemical structure of polymer and other processing parameters related to mixing (Garcia-Garcia *et al.* 2016). Other factors such as biocompatibility, compatibility with polymer, the amount of plasticizer used to achieve desired properties should all be considered (Darie-Niţă *et al.* 2015).

2.10. Objectives

Petroleum-based plastics are not biodegradable and accumulate over time, causing environmental pollution (Sun *et al.* 2018). The increasing concern over petroleum resources and environmental impact has led to extensive research focused on developing biodegradable plastics acquired from renewable resources (Scaffaro *et al.* 2018). Past literature has focused on lignocellulosic biomass to produce PHB, however there is limited information available

regarding to the use of hemp (Dahman *et al*. 2014). The purpose of this study is to examine the ability to produce PHB using renewable and sustainable green feedstock of hemp. As shown in Table 2.1, hemp has a relatively high cellulose and the lowest lignin content compared to another biomass reviewed. Hemp was also chosen due its low cost and availability in Canada (Figure 2.2) (John *et al.* 2007; Schluttenhofer *et al.* 2017)

As a result, different pre-treatments and hydrolysis methods were studied to determine optimize simple sugars availability from hemp for separate hydrolysis and fermentation process to increase PHB production. Table 2.2 summarizes agriculture waste in relation to the production of cell dry weight and PHA produced. Examining Table 2.2 reveals that *Ralstonia eutropha* are able to produce higher cell dry weight and PHB compared to other bacterial species. In this study, *Ralstonia eutropha* will be used in an attempt to increase production.

In this study, a comparison between pre-treatment effectiveness was created between different combinations of physical, chemical and thermal pre-treatments and their effect on PHB production. Enzyme production effectiveness was also established by comparing enzyme type, dosage, and combination enzyme therapy to determine optimum parameters in regards to PHB production. Extraction comparison in relation to sonification and its effect on PHB production was also determined. Samples were collected with time to determine pH control effect on PHB production.

Chapter 3
Experimental Methods and Procedures

3.1 Materials

In this study, *Ralstonia eutropha* (ATC 17699) was purchased from the American Culture Collection Center (Manassas, US). Hemp fibers obtained from a farm in Manitoba. Chemicals were purchase from Sigma Aldrich, Baker &Adamson, AnalaR analytical reagents. All materials were used as received without further purification unless mentioned. Water HPLC grade submicron filtered purchase from Fisher Chemicals. Tables 3.1and 3.2 summarizes all chemicals and enzymes that were used in the present study. Table A.1 in Appendix A summarizes equipment serial per batch number with corresponding manufacture that were used in the study.

Table 3.1 Represents the chemicals used in this study

Chemical	Batch Number	Manufacture
Ammonium Chloride	129K01881	Sigma-Aldrich
Ammonium iron (III) citrate	SLBR612V	Sigma-Aldrich
Beef Extract	SLBQ3908V	Sigma-Aldrich
Boric Acid	SLBQ5348V	Sigma-Aldrich
Calcium Chloride	070M0053V	Sigma-Aldrich
Chloroform	SHBH8512	Sigma-Aldrich
Citrate Acid Monohydrate	0901M0157V	Sigma-Aldrich
Copper Chloride, Reagent grade, 97 %	MKAA0267	Sigma-Aldrich
D-(+)-Glucose ACS reagent	SLPBP5997V	Sigma-Aldrich
D-(+)-Xylose, minimum 99 %	010M0063	Sigma-Aldrich
Hemp	N/a	Farm in Manitoba
Hydrochloric Acid	SZBE1710V	Sigma-Aldrich
Manganese (II) Sulfate Monohydrate, Minimum 99 %	126K0111	Sigma-Aldrich
Magnesium Sulfate anhydrous, reagent	208094-5006	Sigma-Aldrich

Nickel (II) Chloride hexahydrate	SLBQ6963V	Sigma-Aldrich
Potassium Citrate	2106	Baker & Adamson
Potassium dihydrogen orthophosphate	7701361	AnalaR analytical reagent
Sodium Hydroxide	MKBR2876V	Sigma-Aldrich
Sodium Molybdate Dihydrate	1441416V	Sigma-Aldrich
Sodium Phosphate Dibasic ACS reagent	096K0141	Sigma-Aldrich
Tryptone	059k0108	Sigma-Aldrich
Yeast Extract	SLBM5481V	Sigma-Aldrich
Water HPLC Grade Submicron Filtered	177528	Fisher Chemical
Zinc Sulfate Heptahydrate, 99 %, A.C.S. reagent	07128CJ	Sigma-Aldrich

Table 3.2 Represents the enzymes used in this study

Enzymes	**Batch Number**	**Manufacture**
Cellulase from *Trichoderma reesi* ATCC 26921	129K1869	Sigma-Aldrich
Cellulase from *Trichoderma reesei*	SLBS6244	Sigma-Aldrich
Novozyme 188	078K0709	Sigma-Aldrich
Novozyme 188	079K1446	Sigma-Aldrich
Xylanase from *Thermomyces lanuginosus*	018K1473	Sigma-Aldrich

3.2. Hemp Biomass Pre-treatment & Enzyme Hydrolysis

3.2.1. Hemp Physical Pre-treatment

In this study, hemp was the sole biomass used. The hemp fibers were obtained from a farm in Manitoba and stored at room temperature in airtight bags. The hemp fibers were then manually separated from leaves. After separation, hemp was subjected to particle size reduction

using a lab scale blender (Davis *et al.* 2013). The grinded hemp was then stored in airtight bags at 27 ^{o}C.

3.2.2. Hemp Alkalization and Thermal Pre-treatment

Initially, 100 grams of grinded hemp weight using balance and transferred into Erlenmeyer Flasks. Alkali pretreated hemp was obtained by suspending 100 grams grinded hemp in 1-liter alkali solution (NaOH) concentrations (2, 5, 10 % (w/v)). To produce 2 % NaOH, required 20 grams of NaOH and was placed within a 1000 ml volumetric flask. Distill water was used to make up the volume to 1000ml. For 5 % NaOH, 50 grams NaOH in 1000 ml distill water. For 10 % NaOH, 100 grams NaOH in 1000 ml distill water was used. The reaction is exothermic, once cooled, the diluted alkai solution was added to the grinded hemp.

The pretreated hemp was then placed into a preheated incubator shaker (Thermo Scientific MaxQ 4450) at 32^{o}C for 60 minutes. The Erlenmeyer flasks were continuously stirred within the incubator shaker at a constant rate of 200 rpm. After 60 minutes the pretreated hemp was autoclaved at 121^{o}C for another 60 minutes. After pre-treatment, the pretreated hemp mixture was filtered into the solid faction (including insoluble solids) and a liquid fraction using an 11 cm Buchner Funnel (0.5-micron filter paper was used). The solid fraction was washed with deionised water until pH 7 was achieved (Kulgarz *et al.* 2016). The solid hemp fraction was then placed in the oven at 80^{o}C for 24 hours to remove moisture (Sluiter *et al.* 2016).

3.2.2.1. Pre-treatment Quality Control

Accurate characterization of hemp and process intermediates is necessary for economic evaluations and quality control. Pretreated slurries are heterogenous mixtures and can affect

further intermediate steps and overall PHB production (Slutier *et al*. 2011). Pretreated slurries are made up of a solubilized and insoluble phase. The solubilize phase contains the sugars, while insolubilize phase contains lignin. The National Renewable Energy Laboratory (NREL) define the wash method for the solid fraction. This method involves exhaustive and sequential washing with distilled water to remove liquor from the solid phase (Sluiter *et al*. 2016). The solid fraction also called pre-treated slurries had to be washed with deionised water until pH 7 was achieved. Washing is conducted under a Buchner funnel, it should be noted that disproportionate vacuum should not be used as water evaporation will affect composition of pretreated biomass (Sluiter *et al*.2011). Calculating the efficiency of the pre-treatment method is known as FIS (fraction insoluble solids) which indicates the amount of lignin removed (Slutier *et al.* 2011; Slutier *et al*. 2016). It uses the solid fraction recovered after drying in relation to total wet slurry. In this study, FIS provides a comparison of samples for consisted based used for quality control. Consistency is based on pre-treatment used (Slutier *et al*. 2016).

$$Fractional\ Insoluble\ Solid(FIS) = \frac{Solid\ Fraction_{Dry}}{Slurry_{Dry}} * 100 \quad \text{Equation 1}$$

where FIS represents the fraction insoluble solids; Solid $\text{Fraction}_{\text{Dry}}$ represents the dry mass of solids after pre-treatment, and Solid $\text{Slurry}_{\text{Dry}}$ represents represent a wet slurry (Sluiter *et al.* 2016).

3.2.3. Enzyme Hydrolysis

The recovered dried solid fraction was then subjected to enzyme hydrolysis. The solid fraction acts as a feed source and was placed into 500 ml Erlenmeyer flasks. Different combination of enzymes (Cellulase from *Trichoderma Reese*, Novozyme 188 and Xylanase from *Trichoderma longibrachiatum*), were added at different concentrations (Refer to Table 6) along with 50ml – 0.5 M citrate buffer (pH ≃ 4.8). The citrate buffer was made from 8.236 g of potassium and 5.254 g of citric acid and mixed to 500 mL of de-ionized water. The hemp-enzyme mixture pH was adjusted to 7. The Thermo Scientific MaxQ 4450 was then mixed the mixture for up to 72 hours at 48 ^{o}C.

Table 3.3Represents the sample of Pre-treated hemp studied followed by Enzyme Addition Studied.

Sample Name	**Pre-treatment Conditions**		**Enzyme Addition**		
	NaOH concentration (%)	Autoclave used	Cellulase (g/l)	Novozyme (g/L)	Xylanase (g/L)
Batch 0	2	Yes	-	-	-
Batch 1	2	No	1.5	-	-
Batch 2	5	No	1.5	-	-
Batch 3	5	No	0.7	-	-
Batch 4	5	No	3.0	-	-
Batch 5	5	No	1.5	1.5	-
Batch 6	5	No	1.5	1.5	1.5
Batch 7	5	Yes	1.5	1.5	1.5
Batch 8	5	Yes	1.5	1.5	1.5
Batch 9	5	No	-	1.5	1.5
Batch 10	10	No	1.5	-	-

*Samples were Autoclave 121^{o}C for 60 minutes
* Batch 8 had an additional extraction step (Ultrasonication)

3.3. Bioplastic Production

Bioplastic production experiments were conducted in 500 ml Erlenmeyer flasks that were incubated with Thermo Scientific MaxQ 4450 at 28 ^{o}C for five days. Fermentation was always conducted using *Ralstonia eutropha*, and hydrolysate solution of hemp that contains mixture of sugars (prepared according to section 3.2.2).

3.3.1. *Ralstonia eutropha* activation

Ralstonia eutropha was activated in a basal mixture. The basal mixture was made up of 1 gram beef extract, 1 gram tryptone and 0.2 gram yeast extract. These components were mixed with 100 ml distilled water in 250 Erlenmeyer flasks. The mixture was mixed using the Thermo Scientific MaxQ 4450 incubator shakers for 30 minutes. After thorough mixing, the basal mixture was autoclaved at 121 ^{o}C for 20 minutes. The Labgard Class II, Type A2 Biological Cabinet was aseptically cleaned using ethanol (surfaces and equipment) and UV was used further sterilization for 10 minutes. After autoclave, the basal medium was allowed to be cooled to room temperature within an aseptically Biological Cabinet. After cooling, *Ralstonia eutropha* sample was then added to the basal medium under aseptic conditions. Adaptation of *Ralstonia eutropha* ATCC guidelines for activation led to *Ralstonia eutropha* being incubated for 120 hours at an initial pH of 7 at temperature of 30 ^{o}C within Thermo Scientific MaxQ 4450 (150 – 200 rpm) (Dahman *et al.* 2014).

3.3.2. *Ralstonia eutropha* addition

After 48 hours of enzyme hydrolysis, hemp fibers were filtered using 11 cm Buchner funnel. The remaining hydrolysate was then adjusted to pH of 7. The nitrogen-limited medium was produced adding to the hydrolysate a mixture of 3 g/L of Na_2HPO_4-$12H_2O$, 0.5 g/L of KH_2PO_4, 1g/L NH_4Cl, 0.1 g/L $MgSO_4$-$7H_2O$, 1.2 mg/L Fe (III) NH_4 and 10 mL of trace elements. The trace elements composed of 10 mg/L $ZnSO_4$- $7H_2O$, 3mg/L $MnCl_2$-$4H_2O$, 30 mg/L H_3BO_3, 20 mg/L $CaCl_2$-$6H_2O$, 1 mg/L $CuCl_2$- $6H_2O$, 2mg/L $NiCl_2$-$6H_2O$ and 3 mg/L $NaMoO_4$-$2H_2O$. After all, components were added, the pH was adjusted to 7. Hydrolysate and the nitrogen limiting medium was then autoclaved (Dahman *et al.* 2014). The activated Ralstonia *eutropha* (5 % w/v) was added to the hydrolysate and nitrogen limited nutrient medium at a temperature of 28 ^oC +/- 2 ^oC. The solution was sterilized and then placed in the Thermo Scientific MaxQ 4450 for a maximum of 5 days. After 5 days, PHB was produced within the flask.

3.4. Extraction

After fermentation, PHB extraction was required. Fermentation resultant broths contained PHB lyophilized cells. The broth was placed in VWR test tubes and centrifuged at 4000 g for 15 minutes using accuSpin 400 Centrifuge. After centrifuging the samples, solid formed at the bottom (contained PHB lyophilized cells). The liquid layer was discarded. The solid phase was then immersed in a chloroform. For each gram of PHB lyophilized cells, approximately 50 ml of chloroform was added. The chloroform and PHB were mixed thoroughly using Analog Vortex Mixer VM-3000. Once completed, all test tubes mixtures were placed within the round bottom flask. The round bottom flask was then attached to a reflux condenser under a heated oil bath (80

^{o}C) for 4 hours (Dahman *et al.* 2014). The PHB was recovered from the precipitating solvent. The weight of PHB was then measured (Dahman *et al.* 2014).

3.5. HPLC Analysis

HPLC was used to investigate separate hydrolysis and fermentation process sugar content. Sampling was done within the aseptic cleaned biological cabinet. All surfaces and equipment used were aseptically sterilized using ethanol and further utilization of UV for 10 minutes. Samples diluted by a factor of 50, centrifuged at 4000 g for 15 minutes and filtered through 45 µm filter. Samples were collected at the start of fermentation and then intervals of 24 hours until end of fermentation. Sterilized Eppendorf tubes were used to store 2 ml samples for each interval. Samples were stored at -80 ^{o}C in Revco Elite Plus Freezer until quantified. Sugar was quantified using HPLC apparatus. The results obtained from analyzing three samples.

Sugar concentration was determined using HPLC apparatus; Agilent Technologies 1260 infinity series equipped with autosampler injector and a refractive index detector (serial # DEAA302993). The Shodex SP0810 column was used to determine sugar concentrations. HPLC was operated using water HPLC grade microfiltered as the mobile phase. The mobile phase was used to increase the flow rate to 0.6 ml/minute for 30 minutes. A constant flow rate was then achieved. Adjusting flow rate reduces noise generated and provides a constant pressure within HPLC apparatus. The maximum allowable pressure was 300 bar. The column temperature was maintained at 80 - 85 ^{o}C. Once all parameters were achieved and maintained, the autosampler took 50 µL for each sample. Each sample was analyzed for 35 minutes. It should be noted that HPLC grade water from Fisher Scientific was purchase in an attempt to reduce noise generated from the analysis. Also using HPLC grade water improved methodology previously described by Syed *et al.* (2014). Data were processed using the Openlab software. HPLC testing vials were

filled to headspace to reduce the loss of solvent in the vapor phase (Syed *et al.* 2014). The reliability of HPLC results was confirmed by running analysis (sugars standards & samples) triplicate. Sugar concentrations were determined from calibration curves (Appendix A) from standard sugar solutions of known concentrations (10 – 1000 µg / µL)

3.6. Statistical Analysis

In this study, separate hydrolysis and fermentation analysis were repeated three times. Results are calculated average values according to Equation 1. Table 6.4 to Table 6.10 in Appendix display the raw data for individual sugar concentrations, FIS results in sugar concentrations and corresponding average, standard deviations and relative standard deviations percentage. Standard deviations were calculated according to Equation 2 and represented as error bars in Chapter 4 & 6 Figures. All results listed are displayed as average values (Equation 2) (Kulgarz *et al.* 2016).

$$\mu = \frac{1}{n} * \sum_{i=1}^{n} x_i \qquad \text{Equation 2}$$

where µ represents the arithmetic mean; n represent the total number of samples tested (n=3), and x_i represents the observed value for each sample.

$$\sigma = \sqrt{\frac{1}{n} * \sum_{i=1}^{n} (x_i - \mu)^2} \qquad \text{Equation 3}$$

Let $\sigma\ represent$ the standard deviation

$$RSD = 100 * \frac{\sigma}{\mu} \qquad \text{Equation 4}$$

Let RSD represent the relative standard deviation in (%)

Results for all replicates are listed in Table A.2 to A.8 in Appendix A. According to these results average standard deviation in Table A.2 range from 0.14 to 1.01 with an average standard deviation of 0.76. The relative standard deviation error for Table A.2 in the range of 0.2 to 1.6 %. Table A.3 and A.4 represent the sugar available after enzyme hydrolysis. The standard deviation ranges from 0.01 to 0.47 with an average standard deviation of 0.22. The relative standard deviation for A.3 & A.4 is 0.08 to 30 %. Table A.5 &A.6 represent sugar reading during fermentation. The standard deviation ranges from 0.07 to 0.31 with an average standard deviation of 0.22. The relative standard deviation for A.5 & A.6 is 0.5 to 10.8 %. Table A.7 shows the wet mass produced in the experiment. The standard deviation ranges from 0.12 to 2.68 with an average standard deviation of 1. The relative standard deviation for Table A.7 ranges from 0.5 to 11.1 %. Table A.8 & A.9 represent Batch 7 pH readings. The standard deviation ranges from 0.31 to 0.93 with an average standard deviation of 0.60. The relative standard deviation for Table A.8 &A.9 ranges from 4 to 19.4 %. According to Table A.2 to A.8 the average standard deviation of the whole study is 0.51.

Chapter 4
Results & Discussion

4.1. Pre-treatment Effectiveness

In this study, hemp was exposed to several different combinations of pre-treatments including particle size reduction, different alkali concentrations (NaOH) and thermal. At the end of the pre-treatment stage, a slurry was produced. The slurry contained two phases. One phase contains the lignin, insoluble ash, and cellulose. The second phase is made up of solubilized material (typically the sugars required) (Slutier *et al.* 2016). FIS results are based on the amount of dry slurry remaining in relation total wet slurry produced. FIS is calculated using Equation 1 in Chapter 3. Ensuring FIS reproducibility & repeatability is a key indicator for both quality controls in terms of ensuring removal of residual alkali, moisture and lignin, hence helping to control cost (Sluiter *et al.* 2016). As shown in Table 4.1, as the number of pre-treatment methods increased, the lowered amount of insoluble contents was recovered. For example, Batch 0 that was pretreated with 2% NaOH in addition to thermal pre-treatment showed a lower amount of insoluble recovery (FIS ≃ 62.13 %) compared to Batch 1 which utilized only 2 % NaOH pre-treatment (≃ FIS 68.25 %). The same relationship was demonstrated with Batch 6 (FIS ≃ 66.06 %) which had a higher FIS value compared to Batch 7 (FIS ≃61.26 %) which used 5 % NaOH and thermal pre-treatment. The results are significant since the p-value calculated was less than 0.05.

Table 4.1 Summarises results for FIS obtained for different pre-treatment followed Table 3.3

Sample Name	**FIS (%)**
Batch 0	62.13 +/- 1.00
Batch 1	68.52 +/- 0.72
Batch 2	66.66 +/- 1.75
Batch 3	66.69 +/- 0.82
Batch 4	66.90 +/- 0.96
Batch 5	61.53 +/- 1.53
Batch 6	66.06 +/- 0.19
Batch 7	61.26 +/-0.40
Batch 8	60.69 +/- 0.30
Batch 9	65.92 +/- 0.40
Batch 10	68.2 +/- 0.14

From Table 4.1, it also shows that the severity of pre-treatment also has an effect on FIS. For example, Batch1 which incorporated 2 % NaOH had a higher FIS value compared to Batch 6. Meaning that the severity of pre-treatment has an effect on insoluble fraction. These results are significant since the p-value was below 0.05. The higher concentration of pre-treatment attacks the hemp structure by decreasing the crystallinity allowing for cellulose interaction and decreasing the FIS (Liu *et al.* 2015). In the fermentation process, lignin has the ability to inhibit bacterial growth. Lignin itself is an aromatic rigid polymer making it a complex matrix composed of phenolic compounds of phenyl propionic alcohols (Wunna *et al*. 2017). The matrix contains functional groups such as hydroxyl, methoxy, and carbonyl (Wunna *et al.* 2017). The purpose of alkali treatment pre-treatment is to disturb the lignin-hemicellulose bonds and solubilize the internal surface and bonds between lignin and hemicellulose, enhancing hemicellulose digestibility (Wunna *et al* 2017).

A study conducted by Wunna *et al.* (2017) investigated alkali pre-treatment effect on lignin removal found that alkali conditions such concentration have an effect on lignin removal (Wunna *et al.* 2017). As concentration increases the percentage of lignin removal increase while

xylan production increases (Wunna *et al.* 2017). The higher concentration resulted in the ability to cleave lignin and glycosidic bonds that lead to decrease in crystallinity and increase swelling. The increasing concentration also results in saponification of acetyl and uronic esters bonds that allow enzymes access the cellulose (Chen *et al.* 2013). In a study conducted by Chen *et al.* (2013), who investigated alkali pre-treatment parameters found that cellulose has a low reactivity to alkali solutions. Since alkali solutions such as NaOH neutralize the phenolics which are actually contained with lignin (Jaisamut *et al*. 2013; Wunna *et al.* 2017).

4.1.1. PHB and Sugar Results

Figure 4.1 represent s the pre-treatment effect on hydrolysis yield, PHB recovery and FIS. Examining this figure reveals that as the concentration of NaOH increases, the amount of hydrolysis yield and PHB recovery increases from 2 to 5%. When correlating these results to FIS as shown in Figure 4.1, lower the FIS results demonstrated both a higher hydrolysis yield and PHB recovery. A study conducted by George *et al.* (2014) explained that the concentration of pre-treatment used is essential for hemp swelling which exposes the cellulose backbone, which influences the overall efficiency of enzymatic hydrolysis (George *et al.* 2014). That the pre-treatment used affects the surface adhesion forces, de-fibrillation, and smoothness of hemp (George *et al.* 2014).

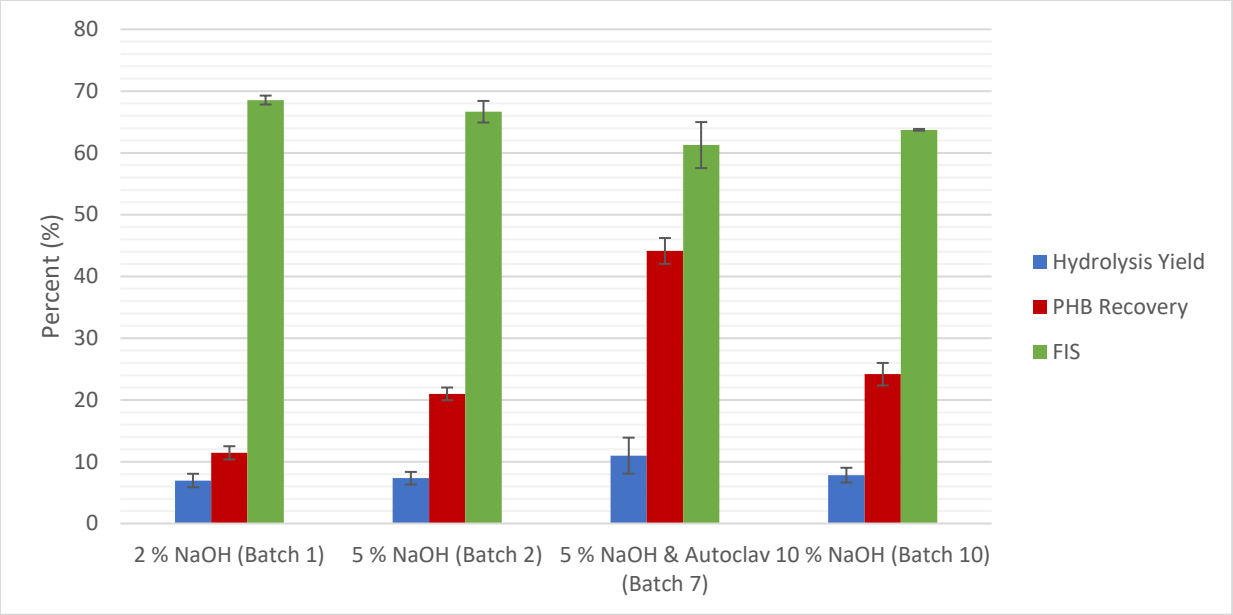

Figure 4.1Represents the pre-treatment effect on hydrolysis yield, PHB recovery and FIS. All samples were grinded as a first pre-treatment step.

Table 4.2 displays the results for the sugar concentration before and after fermentation. After examination of Table 4.2, it should be noted that after fermentation all sugars were consumed 72 hours. Batch 2 demonstrated a slightly higher hydrolysis yield compared to Batch 1. This higher hydrolysis corresponded to a higher PHB recovery. The higher concentration of NaOH used resulted in a higher removal of hemicellulose and lignin from the original pretreated hemp fibers that resulted in higher PHB recovery (Kabir *et al.* 2013).

Table 4.2 Summary of sugar concentration before and after fermentation followed Table 3.3

Sample Name	Sugar Composition		Total Sugars (g/L)	Enzyme Hydrolysis Yield (%) – Before Fermentation	Enzyme Hydrolysis Yield (%) – Before Fermentation	**Average Wet Mass Produced (grams)**	**Average Dry Mass Produced (grams)**	**Yield (%)**
	Glucose (g/L)	Xylose (g/L)						
Batch 0	0.00	-	0.0	0.0	0.0	9.12	0.03	1.49
Batch 1	7.6	2.3	9.7	7.0	0.0	23.78	0.68	11.44
Batch 2	15.5	2.7	18.1	7.3	0.0	25.35	1.33	20.99
Batch 3	6.1	0.7	6.8	5.3	0.0	16.32	0.36	8.82
Batch 4	17.4	3.0	20.3	7.1	0.0	25.57	1.45	22.69
Batch 5	19.9	1.2	21.2	8.2	0.0	26.25	1.73	26.36
Batch 6	22.9	10.3	33.2	9.6	0.0	30.13	3.18	42.22
Batch 7	28.1	10.9	38.9	10.9	0.0	30.09	4.28	44.12
Batch 8	28.4	11.1	39.4	10.9	0.0	33.68	4.33	42.7
Batch 9	1.2	3.2	4.5	2.5	0.0	16.10	0.11	2.73
Batch 10	14.3	2.7	17.0	7.8	0.0	22.01	1.33	24.17

When Batch 10was used, it demonstrated a higher hydrolysis yield and PHB recovery compared to Batch 1 and Batch 2. However, Batch 10 only produced less dry mass compared to BATCH2. When comparing FIS, Batch 10 had a higher FIS compared to Batch 2, which inversely affected the quantity of PHB produced. Between Batch 2 and Batch 10, the p-value was greater than 0.05, there was no significant difference between the two. The combination of pre-treatments used (NaOH with thermal treatments) provided a higher hydrolysis yield and PHB recovery compared to single pre-treatment use. FIS was also smaller. Demonstrating an overall better product compared to the other samples produced. These results are significant since the p-value was less than 0.05.

4.2. Enzyme Effectiveness

4.2.1. Enzyme Addition

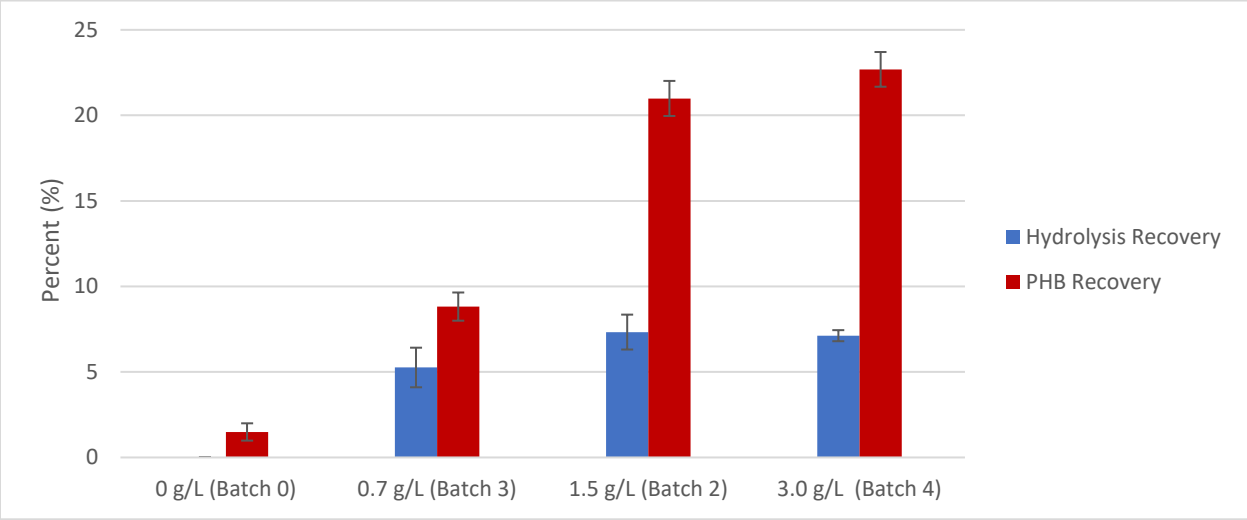

Figure 4.2 Represents the cellulase-T.ressi concentration effect in relation to hydrolysis recovery and PHB recovery

The most influential rate-limiting stage is the enzyme hydrolysis which converts the pre-treated biomass into mono- and oligomeric sugars that can be used for further processing (Martin-Sampedro *et al.* 2013). Hence there is a need to improve our understanding of the interaction between enzymes and the hemp substrate. The goal is to develop a proficient enzyme hydrolysis method that is cost effective (Martin-Sampedro *et al.* 2013).

According to Table 4.2, samples that incorporated cellulase provided a higher total sugar value compared to Batch 9 which did not use cellulase. Cellulase has the ability to convert cellulose to glucose. Cellulase works by cleaving the β – 1,4 glycosidic bonds. That is bonded to the glycosyl units. A widely accepted industrially for of cellulase is derived from *Trichoderma reesi* (T.*reesi*). The cellulose exoglycanase and endoglucanase of β – 1,4 glycanase are made from specific units of complex proteins known as domains (Martin-Sampedro *et al.* 2013). The

two main domains found within cellulase-T.*reesi* is catalytic domain and cellulase binding domain. The two forms are bonded to each other via peptide bonds (Martin-Sampedro *et al.* 2013).

The cellulase binding domain is used for interacting with insoluble cellulose surfaces, while the catalytic domain specializes in the hydrolysis reaction. Together these domains make cellulase – T.*reesi* a very reliable form of cellulase (Martin-Sampedro *et al.* 2013). A study conducted by Saratale *et al.* (2015), investigated enzyme dosage and its effect on PHB. Saratale *et al.* (2015) found that as enzyme dosage increase, enzyme hydrolysis also increased, but to a certain extent. They found from 5 to 10 FPU (Filter paperase activity) per gram, the enzyme hydrolysis increased, after 10 FPU / gram there was no significant effect in hydrolysis yield and PHB recovery (Saratale *et al.* 2015).

In this study, different concentrations of cellulase-T.*reesi* were investigated the optimized cellulase activity. Figure 4.2 Represents the cellulase-T.ressi concentration effect in relation to hydrolysis recovery and PHB recovery. Examining Figure 4.2, it was observed that 0 g /L cellulase provided smaller hydrolysis recovery and PHB recovery followed by 0.7 g/L then 1.5 g/L and 3.0 g/L. The results are significant since the p-value was less than 0.05. There was also no significant difference between 1.5 g/L and 3.0 g/L (p-Value 0.37) in relation to enzyme hydrolysis yield which reflected in the PHB yield. The severity of pre-treatment was the same for Batch 2 and Batch 4 resulting in similar FIS values. The pretreated substrate was constant, while the dose of cellulase increased. Therefore, increasing the cellulase dosage to 1.5 g/L is an effective way to produce PHB.

4.2.2. Enzyme Synergy Effect

Low enzyme hydrolysis yield from cellulase and high-cost limit its ability on a large scale (Yang *et al.* 2018). Therefore, exploring different enzyme combinations is a method used to improve hydrolysis recovery and PHB recovery (Yang *et al.* 2018). A key issue in enzyme hydrolysis hemicellulose. Hemicellulose acts as a barrier that limits access to cellulose (Yang *et al.* 2018). An enzyme known as xylanase acts to disrupt biomass xylan structure within hemp (George *et al.* 2014; Yang *et al.* 2018). In this study, single and combination of enzymes were used. Combinations included Cellulase & Novozymes 188 and Cellulase & Novozymes 188 & Xylanase. Each sample used 1.5 g /L per enzyme. In Figure 4.3 display the effect from enzyme addition in relation to hydrolysis yield and PHB recovery. Figure 4.3 displays that three enzymes provided the highest hydrolysis yield and PHB recovery followed by Batch 5. (two enzymes) and Batch 2 (cellulase only). Batch 7 provided a higher total sugar of 38.9 g/L compared to Batch 5 which was 21.2 g/L. This resulted in a higher Batch 7 average dry mass produced of ≈ 4.28 grams while Batch 5 only provided ≈ 1.73 grams. The results agree with Zhang *et al.* (2013). Zhang *et al.* (2013) investigated the number of enzyme addition during hydrolysis. They found that increasing the number of enzymes has a positive effect on enzyme hydrolysis. Increasing the enzyme hydrolysis amount, therefore improves PHB production (Zhang *et al.* 2013).

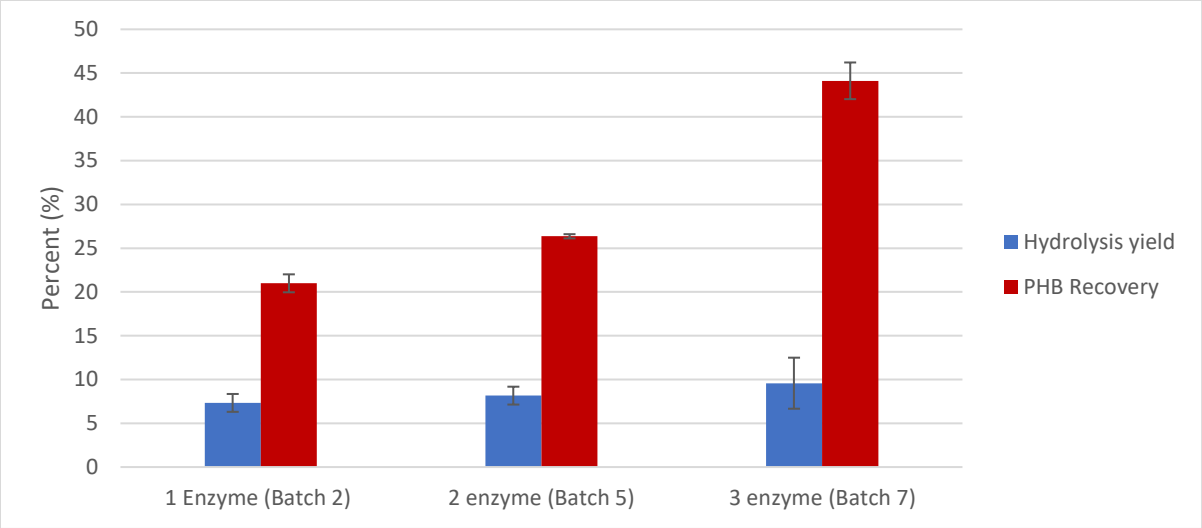

Figure 4.3 Represents the effect from enzyme addition in relation to hydrolysis yield and PHB recovery

The results are also in agreement with George *et al*. (2014) who studied the effects of enzymes on surface properties. Xylanase alone resulted in hemp surface roughness reduction, that exposed pre-treated hemp-cellulose backbone. Reducing the hemicellulose and lignin content resulted in a decrease in contact angle allowing better interaction between the enzyme and the pre-treated hemp. (Geroge *et al.* 2014).

4.3. Extraction

Figure 4.4 represents extraction with/ without sonification in relation to hydrolysis yield and PHB recovery. Batch 7 does not incorporate the sonification method while Batch 8 utilizes sonification. Examining Figure 4.4. it shows that the PHB recovery is better without sonification. The results are not significant since the p value (0.86) is higher than 0.05. These results are not in agreement with literature.

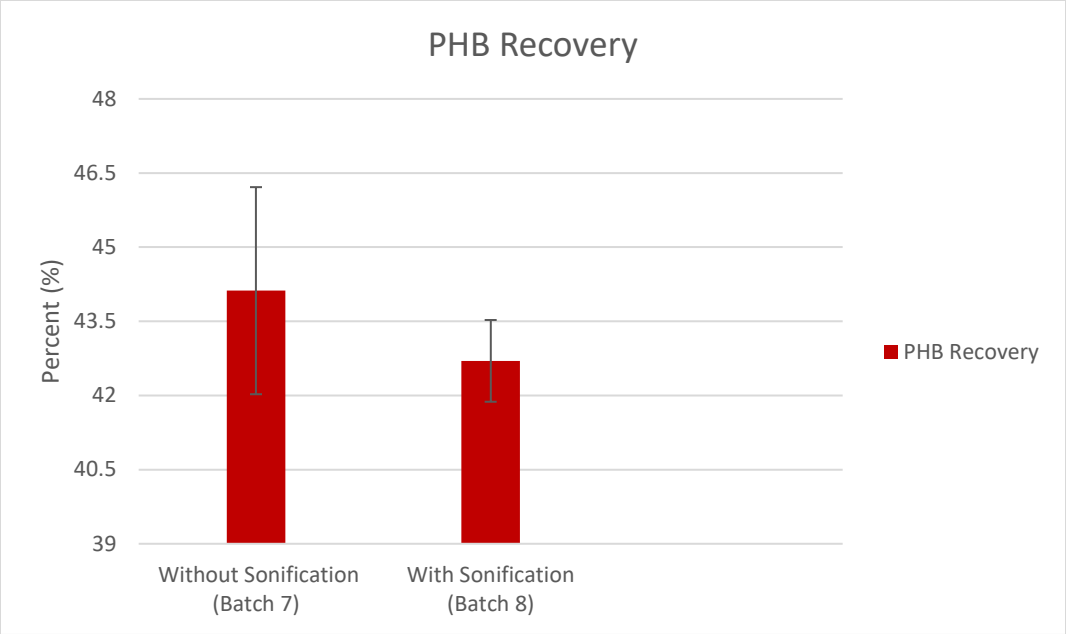

Figure 4.4 Represents extraction with/ without Sonification in relation to hydrolysis yield and PHB recovery

The results do not agree with Ishak *et al.* 2016. Ishak *et al.* (2016) investigated. sonification effect on solvent extraction on PHB. The goal in any PHB extraction is to reduce cost by reducing the time of extraction and organic solvent used. Therefore, the proficient mass transfer is needed to reach this goal (Ishak *et al.* 2016). An excellent method to enhance the mass transfer process in fluids is the use of PHB recovery is ultrasound irradiation (Ishak *et al.* 2016). Ultrasound irritation also known as sonification is used to produce rapid-recurrent pressure changes in the samples. Sonification is described as having two stages, the first being rarefaction and the other is compression stages. Rarefaction stage involves producing empty space within a solid object which produces gas and vapor microbubbles (Ishak *et al.* 2016). During the compression stage, the microbubbles will grow in size, once reaching an adequate size, the microbubbles will implode (intense turbulence) which would result in a shock wave that spreads through the polymer fluid (Ishak *et al.* 2016). These two stages overall produce effective mass

transfer. Viscosity is also a critical parameter in regards to the success of sonification (Ishak *et al.* 2016). It should be noted that the viscosity of the polymer solution influences the microbubbles within the empty space within the polymer solution (Ishak *et al.* 2016). Organic solvents are less viscous (Ishak *et al.* 2016).

Ishak *et al.* (2016) studied different sonification parameters including duration and frequency. They found that sonification works best for 5 minutes under sonication frequency of 37 kHz. Ishak *et al.* (2016). Increased the viscosity of their polymer solution by adding a non-solvent (hexane), therefore improving the polymer recovery. It should be noted the amount of nonsolvent is also critical, the too much nonorganic solvent can act as a precipitate (Ishak *et al.* 2016).

4.4. Bacteria Influence on PHB

4.4.1. Total Sugar Concentration Profile

Ralstonia eutropha is a well-known bacterium that can synthesize PHB from different renewable resources (Taguchi *et al.* 2003). Dahman *et al.* (2014) investigated production using wheat straw as the sole source using *Ralstonia* in two techniques. One known as separate hydrolysis and fermentation (SHF) and the other was glucose (as control). Dahman *et al.* (2014), study demonstrated a better production in terms of dry cell weight, hydrolysis yield and PHB with SHF compared to glucose control. For example, SHF produces 7.1 g/L while glucose as a control produced 4.6 g/L. In this study, SHF was used to produce PHB. Figure 4.5 represents the total sugar concentration profile using the separate hydrolysis and fermentation. Examining this figure reveals that most sugar produced during pre-treatment was utilized with 48 to 72 hours,

except sample Batch 7 and Batch 8. Batch 7 and Batch 8produced higher total sugar concentrations compared to other samples, therefore took, more time to consume. The higher sugar concentration of sugar had a fast consumption within the first 24 hours then slowed down after 48 hours until end of fermentation. After the fermentation process all sugar was consumed. This is in line with Dahman *et al*. (2014), where 98 % of sugar was consumed after fermentation (Dahman *et al.* 2014).

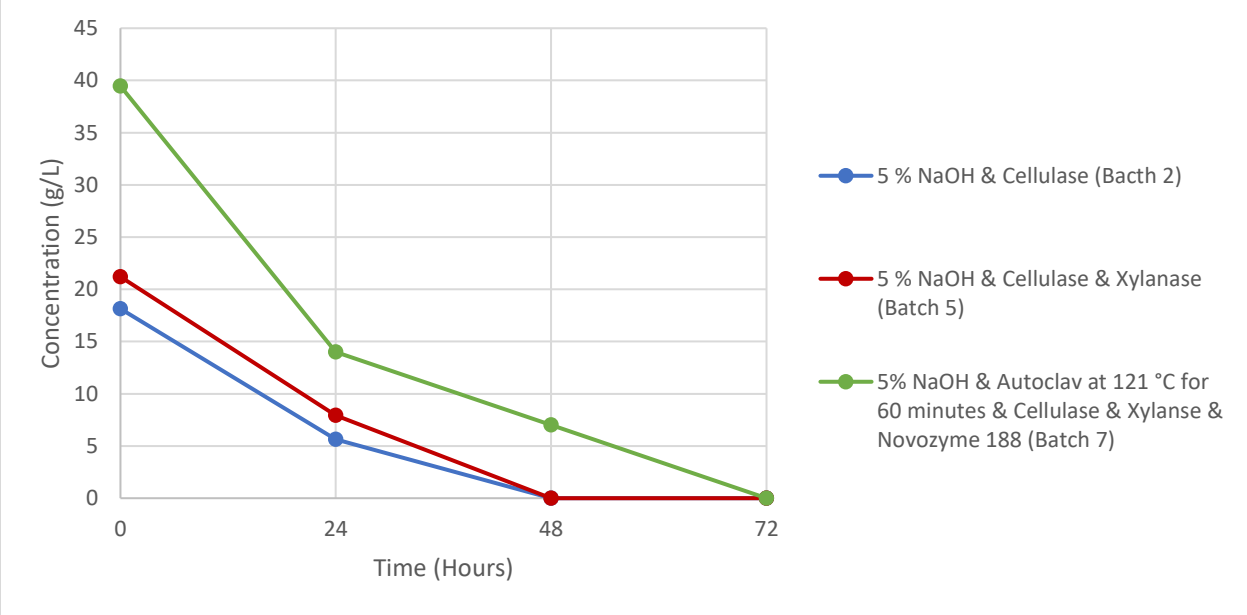

Figure 4.5 Represents the total sugar concentration profile using the separate hydrolysis and fermentation

4.4.2. pH effect on PHB

PHB accumulates with Ralstonia eutropha cytoplasm as lipophilic inclusions (Macagnan *et al.* 2017). This occurs in two stages. The first stage is referred to as the inoculum phase. This is where bacterial cell growth occurs in the basal medium. The second phase is referred to as a polymer accumulation stage under nutrient limitation and high carbon concentration resulting in

a high carbon to nutrient ratio. (Macagnan *et al.* 2017). The polymer accumulation stage works under nutritional limitation which acts as the driving force stage until a PHB saturation is achieved (Villano *et al.* 2014). Within fermentation time, *Ralstonia* is continuously exposure to both nutrients and carbon source (high substrate consumption), resulting in a bacterial growth response increasing achieving a high cell density while PHB production would decrease (Villano *et al.* 2010; Villano *et al.* 2014; Macagan *et al.* 2017). pH can manipulate PHA production. A study conducted by Oehmen *et al.* 2014 determine pH effect on biomass concentration and PHA storage response. Oehmen *et al*. (2014) pH controlled resulted in a higher PHA production rate. As pH rises, the microbial community increases along with the PHA storage yield (Oehmen *et al.* 2014). This is also in line with the work completed by Macagnan *et al.* (2017) who investigated adjusted/controlled pH in inoculum phase to optimize PHB production using Ralstonia and also found that pH has an effect in PHB production.

Literature also states that higher PHB production is linked to high pH values between 7.5 to 9 (Villano *et al.* 2010). Villano *et al.* (2010) also investigated the effect of pH on PHB production and found that PHA composition was strongly influenced by pH. That pH control can lead to 48 % increase in production. Villano *et al.* (2010) suggested that pH control can be used in different feedstock to improve PHB production (Villano *et al.* 2010). Base on literature, pH was controlled. Figure 4.6 shows the pH outcome on PHB production optimal conditions (Batch 7). Batch 1 was used as the control. As shown in Figure 4.6, after the addition of *Ralstonia*, there was no production in the first 48 hours, however, after 48 hours, granules started to form, indicating by the pH value above 7. As illustrated in Figure 4.6 where the pH rose to 8.26 +/- 0.33 after 72 hours, 9.39 +/- 0.31 for 96 hours and 8.97 +/- 0.36 for 120 hours. Increase in pH demonstrated a bioconversion during fermentation. Batch 1 on the other hand, produced after 72

hours. The pH remained under 7, indicating bacterial cell growth. This was indicated by a change in color of these samples. For example, after 48 hours Batch 7 change color from dark brown to light brown with white PHB lyophilic cells beginning to form. While Batch1 produced a darker shade of brown compared to Batch 7. Batch 1 starting producing PHB lyophilic cells after 72 hours. pH control had led to a 27 % higher PHB yield in Batch 7 compared to Batch 1.

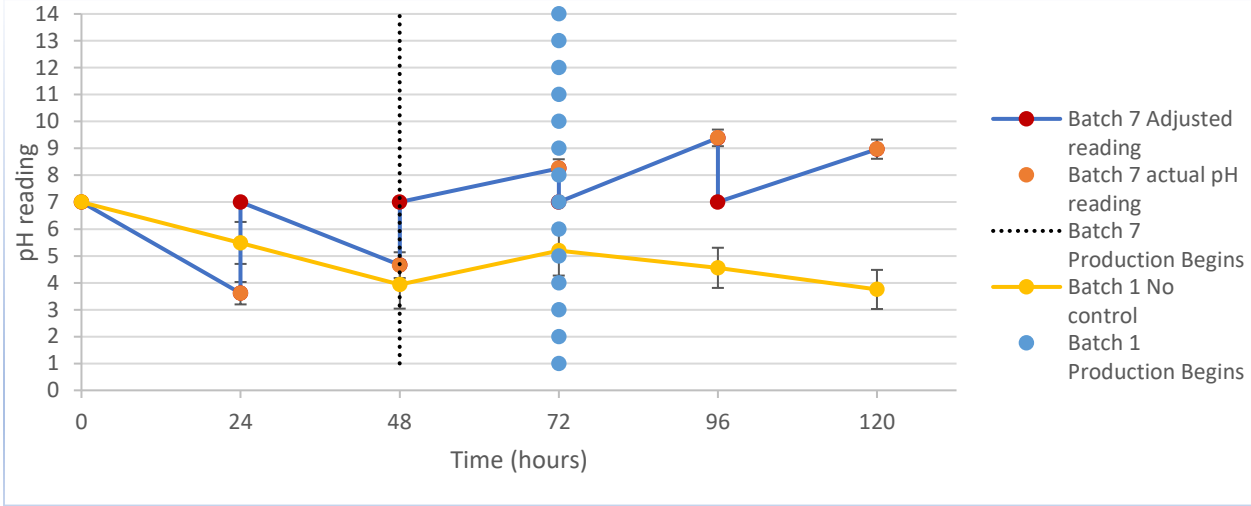

Figure 4.6 Represents the pH outcome on PHB production

Chapter 5
Outlook & Conclusion

5.1. Conclusion

In this study, different pre-treatment and enzyme hydrolysis parameters were applied to *Ralstonia eutropha* to produce large amounts of PHB. Separate hydrolysis and fermentation were used with hemp as the renewable and sustainable feedstock. Pre-treatment effectiveness involved the use of physical pre-treatment along with different combinations of chemical and thermal forms of pre-treatment. In this study, the utilization of three pre-treatment methods (grinded – 5% NaOH – autoclave at 121 ^{o}C for 60 minutes) led to higher disruption of the hemp lignin structure which led to higher removal of lignin enhancing hemicellulose digestibility and increase swelling. This led to lower FIS recovery of $\simeq$ 61 %. As a result, more cellulose was accessible for enzymes. Results were significant since the p-value was less than 0.05.

Enzyme hydrolysis effectiveness was established by comparing enzyme dosage, enzyme type and enzyme synergy effect. Enzyme hydrolysis converts the pre-treatment biomass into simple sugars used for fermentation. Enzyme dosage was compared. It was found that enzyme using 1.5 g/L is optimum for PHB production. By utilizing 1.5 g/L for each enzyme hydrolysis yield produced 7.3% and PHB recovery produced 20.99 % which represented a significantly better results compared to other dosage (0 to 3 g/L) studied. The dosage represents optimum conditions for cellulase to bind to substrate for simple sugar conversion. The combination of enzymes was also investigated. The use of three enzymes which include 1.5 g/L of each cellulase, xylanase Novozyme led to higher hydrolysis yield of 10.9 % and PHB recovery of 44.17.

pH control between Batch1 and Batch 7 was investigated. Batch 1 was studied using no pH control, while Batch 7 pH was adjusted every 24 hours. Adjusting the pH lead to overall increase in 27 % PHB yield. Finally, an extraction comparison in relation to sonification was also conducted. Sonification in this study was unable to significantly improve PHB production. Future work would require a more durable sonification apparatus that is able to work with PHB.

The combination of pre-treatment (grinded & 5 % NaOH & autoclave for 60 minutes) with the use of three enzymes (cellulase, xylanase, and Novozyme) led to higher total sugar concentration ≃ 40 g/L and higher PHB production yielding ≃ 43 %. The use of this parameters can be implemented for further commercialization in an attempt to reduce cost. Based on the literature, future work needs to incorporate a form of sonification to further improve processing capabilities.

5.2. Outlook and Future Recommendations

PHB is a conventional biodegradable plastic with a high melting point (≃ 180 ^{o}C), glass transition temperature of ≃5 ^{o}C while exhibiting a high crystalline characteristic (Garcia-Garcia *et al.* 2016) (Abdelwahab *et al.* 2012). Due to PHB high crystalline characteristic and high melting point, PHB is fragile (literature described as “brittle”) and exhibits difficult ion thermal processing that prevents its use in different applications (Anabukerasu *et al.* 2015).

- PHB Blending to improve processability results
- Investigation into low environmental impact plasticizers
- Environmentally friendly extraction methods
- Genetically modify *Ralstonia eutropha* can be used to improve PHB productivity

- FIS compositional Analysis
- Bioreactor

Appendix A

Table A.1 Represents the equipment used in this study

Equipment Model	**Serial Number**	**Manufacture**
1200 Infinity HPLC Apparatus	1260 Quad Pump (DEAB805754) TCC : DEACN12554 RID: DEAA602224	Agilent Technologies
51BL32 Commercial Blender	021115	Waring
AB104-S Balance	1129212158	Mettler-Toledo
accuSpin 400 Centrifuge	40894047	Fisher Scientific
Analog Vortex Mixer VM-3000	58916-121	VWR
Corning PC-353 Stirrer	N/a	Cole Palmer
Despatch Industrial Oven	XRCC 002991	Xerox
JMP Fumehood	001	Jamestown Metal Products
LabGard Class II, Type A2 Biological Cabinet	124021061808	NUAIRE
MaxQ 2000 CO_2 Shaker	1410080400819	Thermo Scientific
MaxQ 4450 Shaker	1413100353224	Thermo Scientific
MLS-3780 Laboratory Autoclav	731280	SANYO
PB1507–L Balance	1129231716	Mettler-Toledo
Revco Elite Plus Freezer (ULT2586-6-A42)	0125679201080623	Thermo Scientific
Seven Easy pH	1229525475	Mettler-Toledo
SP0810 Packed Column for HPLC	H912040	Shodex
UP200Ht Ultrasonic processor	35101570910	Hielscher
VMS-C7S1 Hot plate	07.162197	VMR

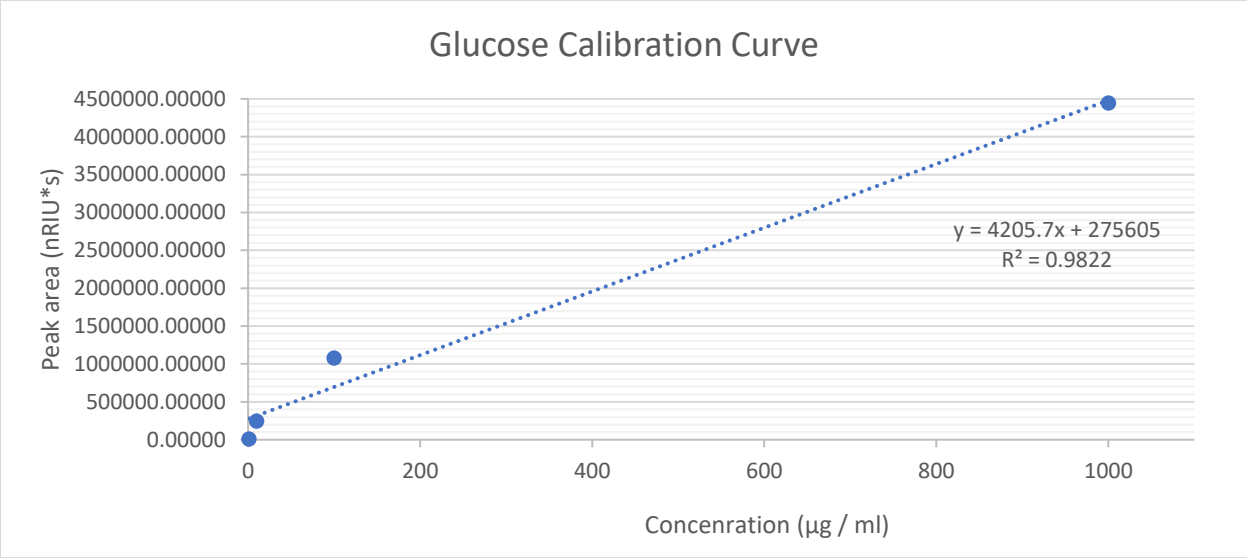

Figure A.1 Glucose Calibration Curve for known concentrations

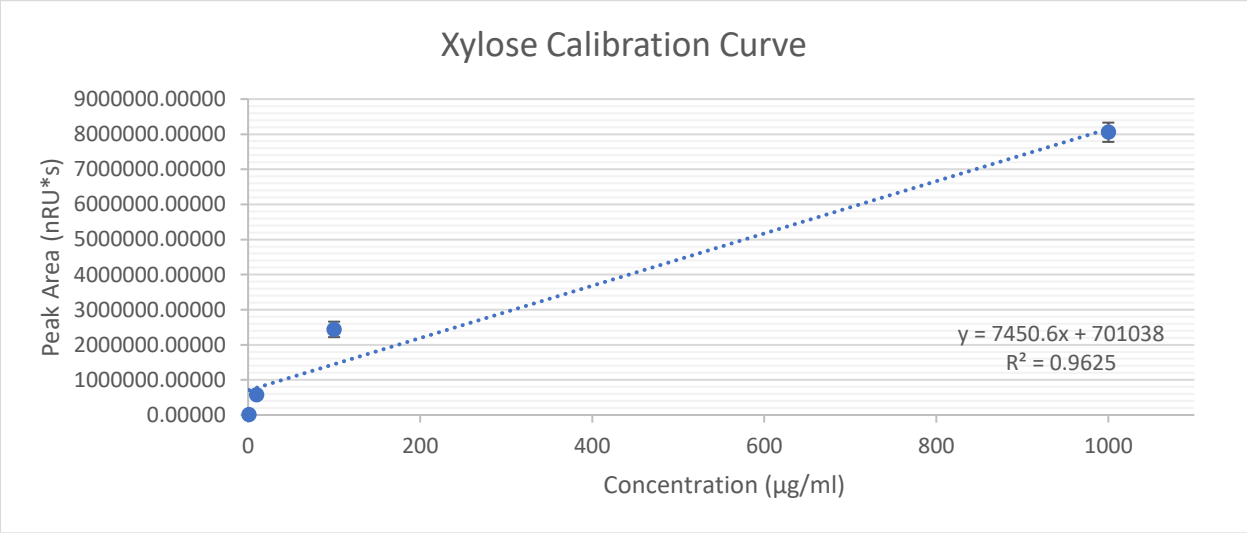

Figure A.2 Xylose Calibration Curve for known concentrations

Table A.2 Shows FIS observations and corresponding average and standard deviation

Sample Name	FIS Observation 1 (%)	FIS Observation 2 (%)	FIS Observation 3 (%)	Average FIS (%)	Standard Deviation	Relative Standard Deviation (%)
AC0	62.14	63.13	61.11	62.13	1.01	1.6
AC1	68.30	69.32	67.93	68.52	0.72	1.1
AC2	65.73	65.57	68.67	66.66	1.75	2.6
AC3	66.35	67.63	66.09	66.69	0.82	1.2
AC4	66.14	67.98	66.57	66.90	0.96	1.4
AC5	62.39	59.77	62.43	61.53	1.53	2.5
AC6	66.05	65.87	66.25	66.06	0.19	0.3
AC7	61.45	60.81	61.54	61.26	0.40	0.7
AC8	60.64	60.36	61.07	60.69	0.36	0.6
AC9	66.19	65.40	66.17	65.92	0.45	0.7
AC10	68.07	68.35	68.17	68.20	0.14	0.2

Table A.3 Represents the glucose RID reading after enzyme hydrolysis

Sample Name	Reading 1 (nRIU*s)	Reading 2 (nRIU*s)	Reading 3 (nRIU*s)	Calculated Average (g/L)	Standard Deviation	Relative Standard Deviation (%)
AC0	309250	309654	311255	8.2	0.25	3.0
AC1	308830	307273	306979	7.6	0.24	3.2
AC2	339681	341447	340666	15.5	0.21	1.4
AC3	302521	300419	301259	6.1	0.25	4.1
AC4	350802	346917	348218	17.4	0.47	2.7
AC5	358364	360429	359733	19.9	0.25	1.3
AC6	372373	372317	370938	22.9	0.19	0.8
AC7	394617	392750	393364	28.1	0.23	0.8
AC8	393737	395698	395187	28.4	0.24	0.8
AC9	280794	281913	279600	1.2	0.28	23.3
AC10	337134	335002	334909	14.3	0.30	2.1

Table A.4 Represents the xylose RID reading after enzyme hydrolysis

Sample Name	Reading 1 (nRIU*s)	Reading 2 (nRIU*s)	Reading 3 (nRIU*s)	Calculated Average (g/L)	Standard Deviation	Relative Standard Deviation (%)
AC0	-	-	-	0.00	0.0	0.0
AC1	717578.0	719441.0	717876.0	2.32	0.13	5.6
AC2	719344.0	720558.0	722875.0	2.67	0.24	9.0
AC3	707490.0	706827.0	704555.0	0.70	0.21	30.0
AC4	723296.0	723389.0	723483.0	3.00	0.01	0.3
AC5	709498.0	711391.0	710144.0	1.25	0.13	10.4
AC6	776464.0	778995.0	777977.0	10.30	0.17	1.7
AC7	783898.0	779855.0	782678.0	10.89	0.28	2.6
AC8	781069.0	786392.0	783855.0	11.10	0.36	3.2
AC9	724477.0	725312.0	725873.0	3.25	0.09	2.8
AC10	720069.0	721632.0	721741.0	2.70	0.13	4.8

Table A.5 Glucose Reading for each sample during Fermentation

AC2						
Time	Reading 1 (nRIU*s)	Reading 2 (nRIU*s)	Reading 3 (nRIU*s)	Calculated Average (g/L)	Standard Deviation	Relative Standard Deviation (%)
0	339681	341447	340666	15.5	0.21	1.4
24	299313	299178	299409	5.6	0.03	0.5
48	0	0	0	0.0	0.00	0.0
72	0	0	0	0.0	0.00	0.0
AC5						
0	358364	360429	359733	19.9	0.25	1.3
24	309478	309074	308325	7.9	0.14	1.8
48	0	0	0	0.0	0.00	0
72	0	0	0	0.0	0.00	0
AC7						
0	394617	392750	393364	28.1	0.23	0.8
24	313037	313596	313335	9.0	0.07	0.8
48	0	0	0	0.0	0.00	0
72	0	0	0	0.0	0.00	0

Table A.6 Xylose reading for each sample during fermentation

AC2						
Time	Reading 1 (nRIU*s)	Reading 2 (nRIU*s)	Reading 3 (nRIU*s)	Calculated Average (g/L)	Standard Deviation	Relative Standard Deviation (%)
0	719344	720558	722875	2.7	0.24	8.9
24	0	0	0	0.0	0.00	0.0
48	0	0	0	0.0	0.00	0.0
72	0	0	0	0.0	0.00	0.0
AC5						
0	709498	711391	710144	1.2	0.13	10.8
24	0	0	0	0.0	0.00	0.0
48	0	0	0	0.0	0.00	0.0
72	0	0	0	0.0	0.00	0.0
AC7						
0	783898	779855	782678	10.9	0.28	2.6
24	729742	734321	732827	4.2	0.31	7.4
48	0	0	0	0.0	0.00	0.0
72	0	0	0	0.0	0.00	0.0

Table A.7 Represents wet mass produced and corresponding average and standard deviation

Sample Name	Reading 1	Reading 2	Reading 3	Calculated Average	Standard Deviation	Relative Standard Deviation (%)
AC0	9.15	10.12	8.10	9.12	1.01	11.1
AC1	23.09	23.56	24.70	23.78	0.83	3.5
AC2	25.68	24.33	26.04	25.35	0.90	3.6
AC3	15.99	16.42	16.57	16.33	0.30	1.8
AC4	25.43	25.62	25.65	25.57	0.12	0.5
AC5	26.28	27.46	25.02	26.25	1.22	4.6
AC6	29.32	31.01	30.06	30.13	0.85	2.8
AC7	30.14	28.43	31.69	30.09	1.63	5.4
AC8	30.87	33.96	36.21	33.68	2.68	8.0
AC9	15.73	15.99	16.57	16.10	0.43	2.7
AC10	22.73	20.80	22.50	22.01	1.05	4.8

Table A.8 Represents AC1 pH reading and corresponding average and standard deviation

Sample Name	Reading 1	Reading 2	Reading 3	Average	Standard Deviation	Relative Standard Deviation (%)
0	7	7	7	7	0	0.0
24	5.63	4.64	6.18	5.48	0.78	14.2
48	3.72	3.17	4.93	3.94	0.90	22.9
72	5.16	6.15	4.29	5.20	0.93	17.9
96	4.5	5.33	3.84	4.56	0.75	16.4
120	4.58	3.47	3.21	3.75	0.73	19.4

Table A.9 Represents AC7 pH reading and corresponding average and standard deviation

Sample Name	Reading 1	Reading 2	Reading 3	Average	Standard Deviation	Relative Standard Deviation (%)
0	7	7	7	7.00	0.00	0
24	3.22	3.57	4.05	3.61	0.42	11.6
48	4.22	4.58	5.17	4.66	0.48	10.3
72	8.23	8.61	7.94	8.26	0.34	4.1
96	9.34	9.72	9.11	9.39	0.31	3.3
120	8.57	9.08	9.26	8.97	0.36	4.0

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