Production, Extraction, and Characterization of Green Biodegradable Bioplastics Polyhydroxybutyrates (PHB) for Biomedical Applications

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Abstract

Production of Bioplastics from Hemp fibers
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Biodegradable plastics are that kind of plastics that will decompose naturally, when environmental microorganisms metabolize and break down the chemical bonds present in the structure of biopolymer.Bio-plastics offer an advantage to earth by reducing carbon footprint and use of fossil fuel. Bio-plastics are completely biodegradable and can be recycled.

The focus of the study was to produce green bioplastics by utilizing the sugar hydrolysate of Hemp fibers. Producing of Bioplastic was pursued in fermentation experiments, where HEMP was utilized as feedstock to produce PHB. Hemp fibers were initially pretreated, and then enzymatically hydrolyzed to produce PHB. Hemp can produce PHB (poly(3-hydroxybutyrate) when the environmental conditions are controlled. The objective of the study was to optimize simple sugar availability from hemp for *Ralstonia eutropha*. Powedered hemp with the contration of 5% NaOH, and 1.5g/ L of enzyme addition was found to yield the best restluts in the production process of Bioplastics as the fractional insoluble solids (FIS) of $\simeq 61\ \%$ was significantly better compared to other combinations of pre-treatments studied. The enzyme hydrolysis yield of 10.9 % and PHB yield of $\simeq 43\ \%$ were also found in this study.

Chapter 1

1.1. Introduction

Plastic materials have become an integral part of our lives, and are widely used in synthesizing shopping bags, garbage, water cans, industrial toys, and many other household, industrial, and construction materials. Despite the importance of plastic material in industry, it imposes serious Environmental problems. Plastic materials are not degraded in nature, which has a negative impact on human health. According to Mikkili *et al*., (2014), the most appropriate alternative to those substances are the biopolymers produced by organisms that are naturally dissolved in water and CO2.

Bio-polymers are produced from natural renewable materials as a result of microorganisms' growth such as, algae, Yeast, and bacteria. Those microorganisms are capable of producing many biodegradable polymers in nature such as, polyhydroxyalkanoate (PHA). polyhydroxyalkanoate accumulates in cells as a source of energy and carbon. The accumulation (or the synthesis) of HPA polymers increases when deficiency in nutrients and lack of nitrogen occurs during growth. Polyhydroxybutyrate (PHB) is classified as one of the most important polymers of PHA, as it has mechanical properties similar to polypropylene properties. Additionally, PHB is biodegradable and nontoxic; therefore, it can be produced from Carbon cheap sources (Aswin *et al*., 2014). PHB is a homogeneous polymer, which has a solid structure that is characterized by its 4-6 carbon atoms. High crystallization rate is another characteristic of this polymer, which reaches up to 50%. The melting point of PHB depends on its solid structure and crystallization grade, which ranges from 160 -180 m (Vooren Van, 2012).

PHB can be produced from a variety of micro-organisms that are able to assemble those polymers in their cells. Certain micro-organisms, such as *Aeromonas* bacteria, *Alcaligenes, Bacillus, Micrococcus, Pseudomonas Rhodococcus, Staphylococcus,* are capable of fermenting cheap costed carbonic sources such as, agricultural and industrial waste (e.g salsa, shark and wheat bran) and the residues of the starch industry (Al-Charen *et al*., 2014). Bacillus species such as *B. amyloliquefaciens , B. licheniformis, B. laterospous, B. firmus, B. cereus, caogulans B., B. sphaericus, B. mycoides, B. megaterium, B. macerans, B. thuringiensis, B subtilis,* have the potential to produce PHA at a rate of 11% to 70%. (Rasnani, 2014). Moreover, PHB are used in many fields including, medical Pharmaceuticals, food packaging, food packaging Utensils, spoons and dishes (Pradhan, 2014).

1.2. Biodegradable polymers

Biodegradable polymers are naturally acceptable as they do not pose any harm to the environment; and are degraded by oxidation or Biodegradation without leaving any harmful residues to the environment. Additionally, those polymers have the ability to partially or entirely decompose by enzymatic processes organized by microbiology (Poirier *et al*., 1995). Starches and its derivatives are naturally biodegradable materials, which has been an object of many important studies. In order to use the starch as a polymer, large amounts of plasticizers such as, capsol and sorbitol, should be added. Those plasticizers are water-threatening nature, with an addition ratio of 20-40% of starch weight to accomplish the change in the mechanical properties and the glass transition of the starch and its derivatives (Lourdin *et al.*,1999). The starch, such as cellulose, can be used to produce naturally degradable polymers as materials in the lab, since these materials are available in large quantities, reasonably priced, and are natural sources of

renewable. Although the material of starch has many advantages, there are some disadvantages such as the impact of harvest time in their chemical composition, their ability to absorb large amounts of water, humidity, change of their characteristics over time, and sensitivity to heat. The chains that make up starch are of the most severe defects due to the strong hydrogen bonds that bind the groups of hydroxyls. On the other hand, Cellulose is more crystallized than starch; therefore, when producing a biodegradable polymer, one of its derivatives should be used in this process (Guruprasad and Shashidhara, 2004).

There are several types of biodegradable polymers, including PHA, Polylactides, polycrystalline polymers, and polysaccharides. Additionally, there are polymers resulting from blending one polysaccharide with synthetic polymer such as mixing starch with partially dissolved Polypropylene or completely dissolved PHA (Patwardhan and Srivastava, 2004; Lee,1996). One of the most biodegradable substance and widely used one is polylactic acid (PLA), compared to PHB and polymers that are manufactured from petrochemicals. PLA is a reasonably priced polymer; however, it suffers from many disadvantages such as slow crystallization and low resistance. In addition, PLA's mechanical properties are hindered its use in many of applications, although lactic acid can be produced on an industrial scale by fermentation of sugar by Acetic acid bacteria, but polymer production needs to process chemical polymerization matter which makes production processes more complex and time-consuming (Yokohara and Yamaguchi, 2008; Datta *et al*., 1995). This has led researchers to explore new polymers that are biodegradable such as PHB. The classification of biodegradable polymers to their sources of production is shown in Figure 1.2 (Yokohara and Yamaguchi,2008; Datta *et al*.,1995).

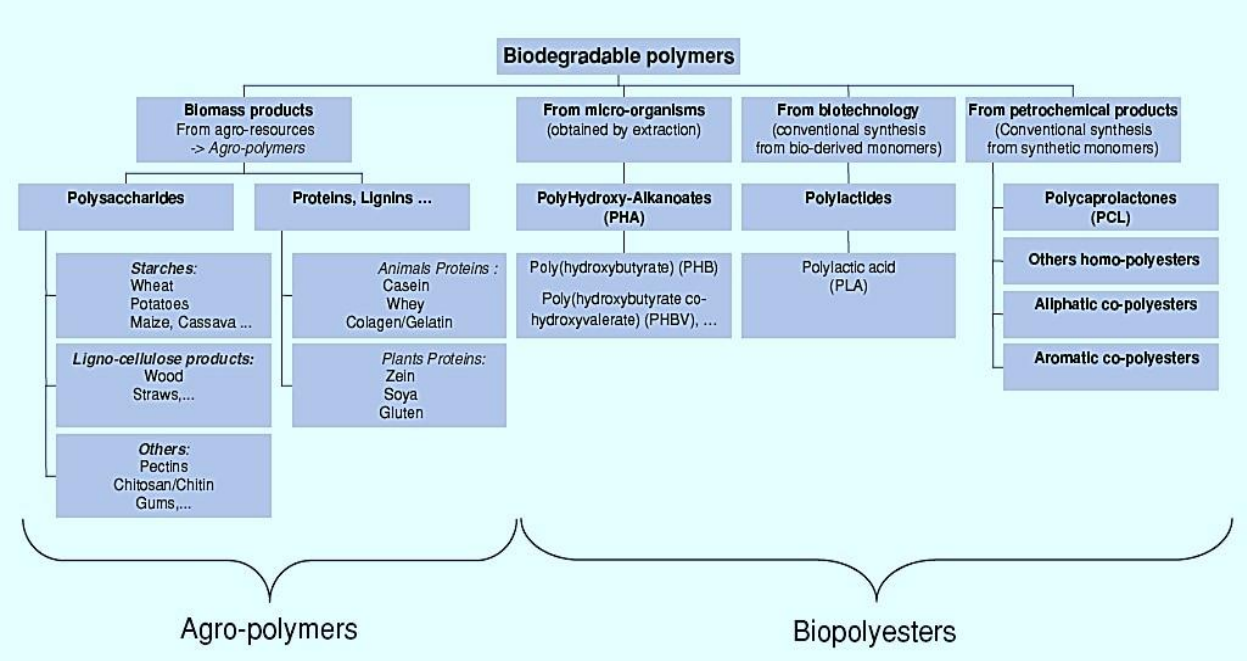


Figure 1.2: Classification of biodegradable polymers

1.3. Polyhydroxyalkanoates (PHAs)

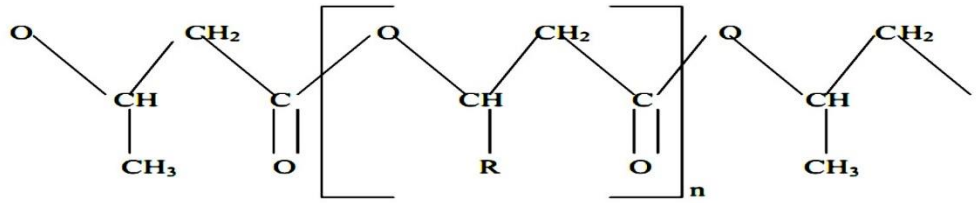
Polyhydroxyalkanoates (PHAs) is a group of natural polymers that are synthesized in bacterial cell within granules. PHAs can be divided into two groups depending on the carbon atoms in the polymer chain. The first group is short-chain, which has 3-5 carbon atoms. The second group is medium-chain, which has 6-14 carbon atoms. The functional group might contain number of unsaturated bonds containing groups of 3-hydroxyl. The chains are straight or branched and contain aliphatic or uremic aggregates (Heok 2009; Doi et al., 1992).

Madison and Huisman (1999) divided PHAs to three main groups based on the number of carbon atoms present in the core units of the first group polymer. The first group contains less than 5 Carbon atoms, which is referred to as short chain. The second group contains (6-14)

carbon atoms, called the middle of the series. Finally, the third and final group contains 14 atoms and it's referred to as a long chain. The PHAs produced are different depending on the carbon source used in the center of production. While the accumulation, size and number of granules within the cell depends on the type of microbiology used in production (Macário ,2009).

PHAs are found inside the cell and can be seen by electron microscopy in separate granules (2.0-5.0) micrometers concentrated within the cytoplasm. The molecular weight ranges between 2x105 – 3x106 Dalton. Pigmentation is used in pigments such as, Sudan black B, Nile blue A and Nile red, for the detection of granules containing PHAs. The grains are shown with bright orange color using a spectrometer, fluorescence spectroscopy (Anderson and Dawes, 1990).

Figure 1.3 shows the structural formula of Polyhydroxyalkanoates (PHAs).



n varies from 600 to 35000

R= hydrogen	Poly(3-hydroxypropionate)
R=methyl	Poly(3-Hydroxybutyrate)
R=ethyl	Poly(3-hydroxyvalerate)
R=propyl	Poly(3-hydroxyhexanoate)
R=pentyl	Poly(3-hydroxyoctanoate)
R=nonyl	Poly(3-hydroxydodecanoate)

Figure 1.3: the structure of Polyhydroxyalkanoates (PHAs) and its polymers (Lee, 1996).

PHAs in nature completely decompose to CO and H_2O due to the effectiveness of some microbes' enzymes. In addition to this important feature, PHA polymers can be produced from simple substances. The possibility of producing PHAs from simple substances such as sucrose, starch, cellulose and agricultural residues as opposed to industrial plastics that need non-renewable materials such as oil industries are the most important features of these polymers. The industrial

plastics have many problems and harmful effects on the environment such as distortion of the city, potential risks of burning waste and production of dioxin, which is a toxic substance. Due to those harmful properties, PHA is considered to be an ideal alternative to plastics Petrochemicals (Macário, 2009; Hocking and Marchessault , 1994) .

PHB is the first and best-known type of PHAs. More than 300 species of microorganisms are able to produce different types of PHAs. All types of PHAs are esters that are stored in cell cytoplasm and have certain properties such as, high molecular weights, insoluble in water and heat-fed. In addition, the PHB polymers possess physical properties similar to industrial plastics and they are naturally degradable by microorganisms (Moralejo And Garate, 2014; Macário, 2009).

1.4. *Polyhydroxybutyrate* (*PHB*)

Polyhydroxybutyrate (PHB) is the most widely produced biodegradable polymer of PHAs. It is a homogeneous polymer composed of 3-hydroxybutriate units, which is discovered by Maurice Lemoigne in 1926 in Bacillus megaterium bacteria in the study of the possibility of isolating a polymer-containing solid material Greasy are PHB (Van Vooren, 2012). The chemical structure of PHB is shown in figure 1.4. For more than 30 years, it remained classified as fat, not multi-esters and industrial production in 1982. This polymer is made up of the cellular granules in the cytoplasm. The PHB granules provide an important source of energy stored in the microorganisms. The granules are responsible for the increase of carbonic source production and the decrease in nitrogen and phosphorus production in the center of growth. Granules are an energy source that is an organization of oxidation within the cell. The absence of these granules in the affected living cells leads to degradation of other cellular components such as, RNA and protein, during starvation. The presence of the PHB granules in Bacillus is a source of energy

and carbon during spore's production by/ in the cell. (Macário, 2009; Chandrashekharaiah, 2005).

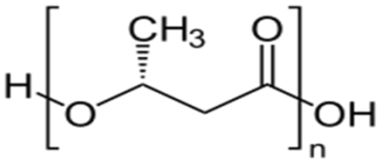


Figure 1.4: the chemical structure of the polymer Polyhydroxybutyrate, PHB (Van Vooren, 2012)

According to Lee (1996), PHB is one of several types of esters that is created by various microorganisms, which sets back-up energy when exposed to adverse environmental conditions such as, lack of essential nutrients and a significant increase in the available carbon source. The presence of PHB in bacteria plays a role similar to the role of fats in human's body and in plants.

Most PHAs of the microbiology are PHB and have a short-chain structure. The HPAs accumulate within cells and compose 90% of the weight of dry cells in some types of bacteria. Furthermore, bacteria can produce and store other polymers such as Polyhydroxyvalerate or polyhydroxyhexanoate. The presence of those compounds and their ratios depends on the type of carbon source used by Microbiology in the production of bioplastics. Studies have shown that some bacteria produce high-efficiency PHB such as E. coli, Bacillus spp., and Alcaligenes eutrophus (Wang et al., 2007; Wang and Yu, 2000). The microorganisms that can produce PHB consist of more than 90 genera, including aerobic and anaerobic ones. Some examples of those bacterial species are; primordial bacteria, light bacteria, Arachea, which are less able to produce

this polymer than were Saccharomyces cerevisiae species. The production ranged between 4-28mg\g of Dry cell weight (Kocharin, 2013; Chandrashekharaiah, 2005)

1.5. Properties of PHB

PHB is the most common type of PHAs in nature among biodegradable polymers produced from microorganisms and has characteristics similar to industrial plastics produced from petrochemicals such as thermoplasticity, melting, water insoluble, high purity, nontoxic, high temperature tolerance during molecular polymerization processes (Hocking et al.1994).

According to Lee (1996), PHB is a homogeneous polymer consisting of aliphatic fatty acids containing hydroxyl groups, whose melting point is 179˚and its crystallization rate is 80%. It begins melting at a temperature higher than 50m and resembles the properties of both polypropylene and polyethylene when being treated with heat. Moreover, its 100% (or completely) resistant to water as shown in table 1.5.

According to Hrabak (1992), PHB has distinct properties compared to plastics used in enormous quantities such as polypropylene. PHB is a natural and dynamic polymer that combines three exceptional features; high stability at elevated temperatures, 100% water and moisture resistance, 100% natural decomposition. Therefore, those three properties allow PHB to be used as a substitute for plastics manufactured from petrochemicals and makes it suitable for waste recycling. Most naturally degradable materials contain some defects such as water solubility and moisture sensitivity, while PHB overcome those problems and has little optical purity. Furthermore, it has permeability to oxygen and UV resistance compared to polypropylene (Chandrashekharaiah, 2005). Also, studies have shown that PHB's high oxygen permeability, compared to polypropylene and polyethylene, makes it one of the best materials used in food packaging and enriches the addition of antioxidants (Huey, 2006; wang *et al*., 2013).

According to Mousavioum (2001), the resistance of PHB to UV is less when acids and bases are present in the center, and when its dissolved in chloroform or any other solvent containing chlorine. the melting point between 175 (-177) m, nontoxic, tensile strength is similar to polypropylene, Sewage drainage facilitates anaerobic microorganisms from PHB degradation while polypropylene floats on the water surface. Furthermore, PHB is suitable for medical use and food preservation.

PHB has a high melting point and crystallization rate. This makes the manufacturing process difficult due to crystallization. Therefore, it can be made in the form of molds or membranes. It has a young coefficient of 3.5G pa. Furthermore, tensile strength is polypropylene uniform, but its elongation is less (Macário, 2009).

PHB is a well-known homogeneous polymer that is highly crystallized. It is made up of fine and regular particles. The polymer's hardness does not come only from the crystallized region, but in the non-crystallized region. The polymerization ratio plays an important role in the fact that PHB is a semi-amorphous compound. Its molecular weight affects many of its properties, such as decomposition, mechanical strength and hardness. It may weigh from 200-3000 kilodalton depending on the growth conditions of bacteria and the components used in production (Wang, 2011).

Table 1.5: The Physical Specifications of Polypropylene and PHB (Park et al., 2001)

Proberties	Polypropylene	PHB
Melting temperature m	**186-171**	**182-171**
Ratio of Crystallinity %	**70-65**	**80-65**
Molecular weight M.wt. (Dalton)	**^{5}10 × 7-2.2**	**^{5}10 ×8-1**

Glass transition temperature	**15-**	**15-10**
Density g/cm^3	**0.94-0.905**	**1.25-1.23**
Curvature coefficient (Young) (GPa)	**1.7**	**4-3.5**
Tensile Strength (MPa)	**38**	**40**
Oxygen permeability	**1700**	**45**
UV Resistance	**Bad**	**Good**
Solvent resistance	**Good**	**Bad**
Biodegradation	**Does not compose**	**Compose**

1.6. Synthesis of PHB in the cell

PHB polymerization can be produced through chemical polymerase reactions of β-butyrate or β-hydroxybutyric acid as primary units. Due to the difficulty of controlling the practical conditions and the high production cost, this method is not economically feasible to produce a PHB polymer. The biological synthesis of the PHB polymer inside the cell depends on the activity of three successive enzymes starting with the β-ketoacyl-CoA thiolase enzyme, which is responsible for stimulating the first step in the cell's PHB synthesis. This enzyme is part of the family of enzymes responsible for the removal of the Thiol (SH) To produce acyl-CoA and –acetylCoA. Following this reaction, two acetyl-CoA molecules bind by carbonate (CC) to produce acetoacetyl-CoA. This enzyme is found in nature in both Prokaryotic and Eukaryotic organisms. Acetoacetyl-CoA exists in two types depending on the subject matter under which it works. The first type is B-ketoacyl-CoAs and has a wide specialty. It works on compounds containing 4 -16 carbon atoms. Fatty acid is found in the mitochondria in eukaryotic organisms but in the bacteria is found in the cytoplasm. The second type is β-ketoacyl-CoA which has a

narrow range of activity and is specialized in carbon chains containing (3-5) carbon atoms. The biological synthesis of the thiolase enzyme is the beginning of a variety of vital processes including the formation and storage of ketones, isoprenoid synthesis and composition of acetoacetyl-CoA (Masamune *et al.*,1989).

Thiolase enzyme inhibits high levels of free Co-A formed after the entry of acetyl-CoA to the cycle of triscarboxyl acid. When growth stops due to nutrient depletion such as nitrogen, oxygen, phosphates, magnesium, sulfur and lack of carbon flow to the cycle of triscarboxyl acid, acetyl-CoA and reduction of Co-A level, the activity of thiolase enzyme increases and it gets directed to acetyl-CoA, leading to the production of the polymer (Genser *et al*., 1998). Acetyl-CoA is generated from several metabolic pathways, the most common ones are, sugar degradation or beta oxidation (Sudesh *et al*, 2000).

The second step in the bio-synthesis process is stimulated by the productions of acetoacetyl-CoA enzyme, which acts on the reduction of acetoacetyl-CoA to 3-hydroxybutyryl-CoA With NADPH + H + enzymatic facilities (Fukui *et al*., 1987).

The final step is based on the production of PHB polymerase, a member of the PHAs polymerase family whose molecular weight is about 63,000 Dalton and is dissolved or linked in the PHB granules found in the cytoplasm. This enzyme works on the polymerization of single units of hydroxybutyryl-3 after removal of coA and PHB polymer production (Jie, 2007).

Several studies have been conducted to produce PHB from plants directly or through genetically modified plants by transferring the genes responsible to produce PHB from bacteria to plants. The Arabidopsis plant was genetically modified to produce PHB in its green cells, as it contains the three enzymes responsible for producing the polymer. However, Verlinden *et al*., (2007) stated that the percentage of PHB is Less than 10% of the weight of dry cells, but increased

accumulation of PHB within the plant cells led to the deterioration of the cell and prevented the growth and development of the plant. Unlike bacteria, in which the accumulation of PHB within the cell is up to 90%, and it acts as a source of energy and prevents the deterioration of the cell when exposed to harsh environmental conditions (Verlinden *et al.*, 2007; Nawrath *et al*., 1994). The theoretical yield of PHB's production from various carbon sources is estimated by the pathway of the chemical reactions. The NADP + formed in the second step of the pathway is calculated for PHB synthesis. The reconstitution of NADPH + H is thought to result from Glucose Isocitrate dehydrogenase enzyme or 6-phosphate dehydrogenase. (Zakariya, 2010; Yamane,1992). The metabolic pathway of PHB is shown in figure 1.6

1.7. Production of PHB from Microorganisms

A wide spectrum of microorganisms was used in the production of PHB, which included gram positive and gram-negative bacteria, some molds and yeast. *Ralstonia eutropha* is the bacteria used in the lab, which produced glucose as a source of carbon for the production of PHB, (Kim *et al*., 1988). PHB produced from *Alcaligenes latus* and used sucrose in the center of PHB production instead of glucose reduced the cost of production compared to glucose, so sucrose is much cheaper than glucose (Wang and Lee,1997).

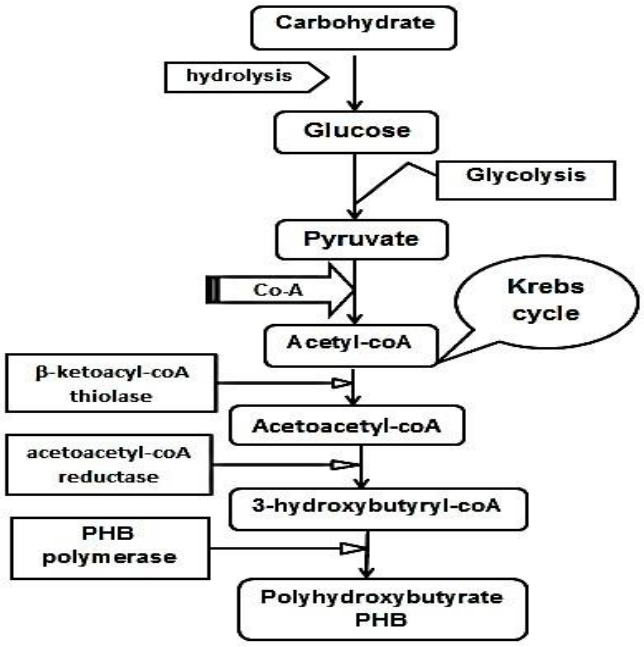


Figure 1.6: The metabolic pathway of PHB production from carbohydrates in bacteria (Supono et al.,2013)

According to Aslim et al. (1998), four species, *Lactobacillus, Lactococcus, Pediococcus* and *Streptococcus,* were used in the production of PHB. The product was extracted with chloroform using suxalite. The highest production was found in *Lactobacillus* (6.6-35.8 %). While PHB production *Lactococcus, Pediococcus and Streptococcus* species was (9-20.9 %), (8-1.1 %) and (6.8-17.2%) respectively. The broth of MRS and Elliker was used in the production.

Slater *et al*. (1994) succeeded in the transmission of genes responsible for the production of PHB from *Ralstonia eutropha* bacteria to a wild strain of *E. coli*, which resulted in an increase in production PHB from 50% to 80% of the weight of dry cells.

Pal and Paul (2000) identified 70% of *Azotobacter* isolates, which are capable of stabilizing soil nitrogen that could produce PHB in a range of 25-47% of dry cell weight. While the yield of *A.chroococcum* isolates was 70 % after studying the optimal conditions for their growth.

Also, this study isolated Fifteen types of yeast from Kombucha tea and Candida krusei, Kloeckera apiculata, Kluyveromyces africans and Saccharomyces cerevisiae. The accumulation rates of the PHB produced was measured to be 5.0- 16.6%. In addition, Pal and Paul (2000) studied the effect of carbon and nitrogen source with respect to the percentage of PHB produced from the yeast of Rhodotorula 60 glutinous Var. glutinous and yeast 27 Saccharomyces diastaticus. It was found that the use of different nitrogen sources does not affect the production of PHB from R. glutinous60Var. glutinous. On the other hand, developing on a medium containing mannitol source of carbon led to an increase in the amount of PHB by 21.95%. The use of different carbonate sources in the production of PHB from yeast 27S. diastaticus did not change the proportions of the PHB product. However, when developing on a medium containing Trypton, which had a nitrogen source, led to an increase in the PHB by 25%.

Ralstonia eutropha is classified as an example of the microorganisms that have the ability to produce PHB. In addition, *R. eutropha* has the ability to consume a wide spectrum of carbon sources such as plants and vegetable oils. The amount of PHB accumulating in bacterial cells is 90% of the weight of dry cells. Furthermore, PHB production of those bacteria with the diet system fed to the liquid plant 6100 mg/L. (Patwardham and Srivastava, 2004). Two studies carried out by Das *et al*. (2004) and (2005) isolated 15 isolates from *Listeria murrayi* and detected the presence of PHB granules within their cells. The 10 isolates were able to produce PHB after dyeing with Sudanese Black B. The highest percentage of PHB was isolated from QGR, which was 7.81% dry cells. In addition, the study analyzed the products using the technique GCMass to find several different compounds, most notably di-n-octyl phthalate, ethyl cyclopropane, and Oleic acid.

Aremu *et al*. (2010) stated that production of PHB from *Pseudomonas aeruginosa* bacteria. The development of bacteria in a medium containing the decomposed starch for

84 hours was 0.106 g / l, while the percentage of PHB was 50%. Ceyhan and Ozdemir (2011) produced PHB from sewage waste using *Enterobacter aerogenes* 12Bi and used the hypochlorite method to extract the resulting PHB. The percentage of the product ranged from 16.66 to 96.25% when five concentrations of sewage were used (10, 30, 50, 80, 100) % and showed that the best concentration is 80%.

Shaaban *et al.* (2012) obtained 180 bacterial isolates from 50 Egyptian soil samples and used them in the production of PHB after their development on a medium containing 1% glucose, 0.1% ammonium sulfate and 7 pH control. The species of *Pseudomonas* showed the highest production of PHB was 68.85% followed by the species of *Strenotrophomonas* with 64.67% of PHB production. Srilakshmi and Ramachandra Rao (2012) isolated 10 isolates Of the *Staphylococcus* species. Five of the isolates were found to have the ability to synthesize PHB within the cells after being stained with a Blue Nile dye. The five isolates were grown in a medium of Luria broth Bertani and the average production rate was (0.028-0.197) g / 100 ml. Also, the highest Production was found at isolation DYCK3.

Wang *et al.* (2013) used beet juice in the production of PHB after the development of *Alcaligenes latus* bacteria. The highest weight of the dry cells of bacteria was 10.30 g / L. While the concentration of PHB was 4.01 g / L at 38.66%. The melting temperature was 151.46 m with a crystallization rate of 43.12%. While the degree of thermal decomposition weight was 283.69 m.

Bhagowati (2013) showed that *Vibrio* spp., *Pseudomonas* spp., and *Bacillus* spp. are classified as the best bacterial species in the production of PHB because of their high adaptability in various harsh environmental conditions, resistance to environmental stresses, growth in the simplest nutrients, agricultural residues, as well as their high efficiency in assembling and accumulating PHB within its cells.

Singh *et al*. (2013) used *B.subtilis* NG220 for the extraction of PHB from a liquid residue of the sugar with the addition of 1% ammonium sulfate as a nitrogen source to the growth medium 1% ammonium sulfate as a nitrogen source. The output quantity of PHB was 5.297 g / L and the yield of PHB was 51.8%. the study was able to diagnose the polymer using GC-mass and tested it in the soil. After 30 days, the polymer lost 70% of its weight.

Nair *et al*. (2014) isolated *Vibrio* sp.MCCB237 bacteria from the degraded marine algae and studied the best sources of carbon and nitrogen to produce PHB of these bacteria The results showed that pectin and glycerol gave the highest yield of PHB at 0.289 g / L and 0.246 g / L respectively. While the best nitrogen sources were the yeast extract which gave 0.246 g PHB / L. Other conditions were the use of a vibratory incubator at 125 cycles / min at 30 ° C, pH 7.5, and 72 hours. Getachew and Woldesenbet, (2016) investigated the production of PHB among fifty bacterial strains isolated from different localities, ten PHB accumulating strains were selected and compared for their ability to accumulate PHB granules inside their cells. Isolate Arba Minch Waste Water (AWW), identified as *Bacillus* spp, was found to be the best producer when glucose was used as carbon source and peptone as nitrogen source.

1.8.1. PHB extraction from cells

One of the most important factors affecting the amount of beta-hydroxy is the process of extraction from bacteria (Poirier et al., 1995). There are several methods of extraction of beta-hydroxybutyrate from bacterial cells, including organic solvent extraction (Ramsay *et al.,* 1994; Brandl *et al.,* 1990) enzyme digestion (Holmes and Lim, 1990), mechanic methods (Yoshida et al., 1995) and many other methods. The PHB extraction and purification step is a complementary step to production. In the last two decades, several methods of extracting the substance have been

examined for the purpose of finding a viable economic method. There are several ways to extract Polyhydroxybutyrate (PHB), this paper will mostly focus on the extraction using:

1) Solvent extraction.
2) Digestion of sodium hypochlorite.
3) Enzymatic digestion.

1.8.2. Summary of these three extraction methods:

1.8. 2. 1. Solvent extraction

This method is used either on a small scale as in laboratory experiments and widely for commercial production purposes. This method is a commonly used method that can be applied to many microbiological organisms that produce Polyhydroxybutyrate. Polyhydroxybutyrate dissolves in organic solvents such as chloroform, methylene chloride and 1, 2-dichloroethane (1,2-dichloroethane). These three solvents are used to extract PHB from the biomass of the bacteria.

The method of extraction of PHB in chloroform is done by firstly, hot chloroform treatment. Then the separation of PHB from the rest of the fat by sedimentation with diethyl ether, hexane or methanol and others is the second step. Finally, re-dissolving PHB in chloroform is carried out (Doi,1990). In another study, three different chlorinated solvents: chloroform, methylene chloride and ethylene-2-dichloride were used in the extraction process of PHB. The best method of extraction was obtained when using chloroform, methyl chloride and ethylene dichloride respectively (Ramsay *et al*.1994).

1.8. 2. 2. Digestion of sodium hypochlorite (NaOCl) and Sodium hydroxide (NaOH)

Sodium hypochlorite dissolves all cellular components except for a Polyhydroxyalkanoate (PHAs), so it leaves PHB intact. The biomass digestion of sodium hypochlorite leads to a decrease in molecular weight of approximately 50% of the polymer (Berger *et al*., 1989). PHB was extracted from *R.eutropha* bacteria after the digestion with hypochlorite preceded by initial treatment with

a substance such as Sodium dodecyl sulfate (SDS). This method yielded a high molecular weight of the polymer, so it is possible to maintain the PHB granules allowing them to be used in several fields (Ramsay et al. 1990). While the effects of NaOH on Hemp fibers can be seen as the cleavage rate of the lignin bonds between cellulose and hemicellulose increases as shown in Figure 1.8.2.2 (Madadi*et al.*, 2018; Kabir *et al.* 2013)

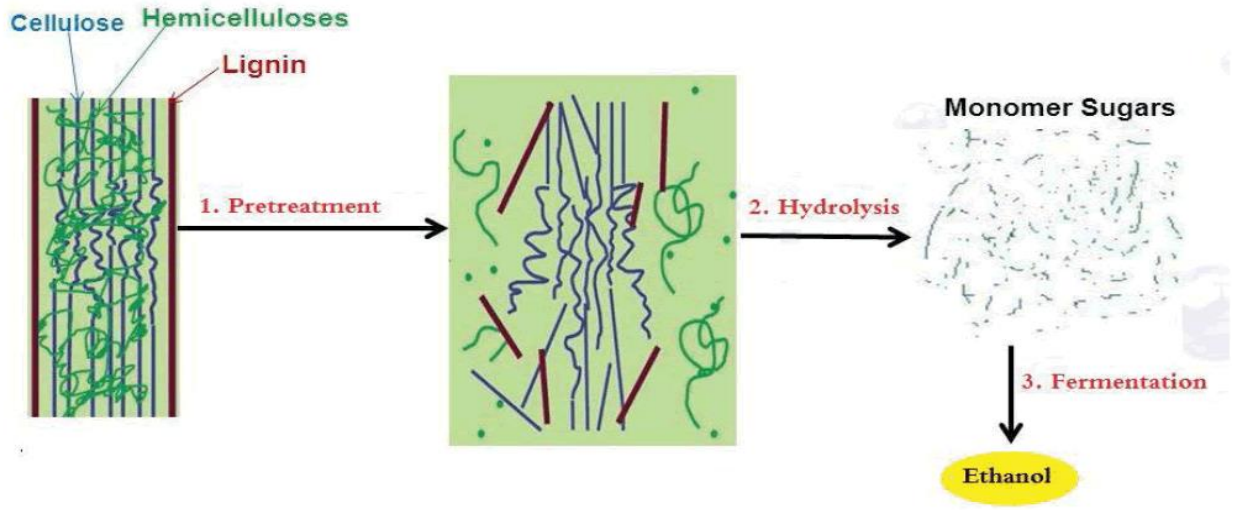


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Figure 1.8.2.2: Cleavage of lignin bonds when subjected to NaOH . Hydrolysis resulted in the production of sugar (Madadi et al., 2018)

1.8. 2. 3. Enzymatic digestion

Due to the high cost of the solvent extraction method, it was replaced by enzymatic digestion; and this replacement was developed by Imperial Chemical Industries (ICI). The method includes the following steps: Heat treatment of 100-150 ° C for cell breakage and nuclear acid checking, then digestion by enzymes followed by washing using anionic surfactant detergents to dissolve the cellular components, except for PHB. Finally, bleaching PHB with hydrogen peroxide is done.

The enzymes used in digestion are lysosome, phosphorylase, phospholipase, Licithinace, and protease. These enzymes work to digest most of the cellular components except the PHB which remains intact (Jacquel *et al*., 2008).

Chapter 2
Literature Review

2.1. PHB

Five different methods were used to compare their efficiency in extracting and learning the best method for extracting beta-hydroxybutarite from the *Bcepacia Bacteria* in table 2. The results showed that the best method of extraction is the method of cracking glass beads with SDS with sodium hypochlorite at 50 ° C as the percentage of beta-hydroxybutarate output was 57% of dry cell weight in pH 11 and 50 ° C; followed by sodium hypochlorite as the output of PHB was recorded to be 51.3% as shown in table 2. It was noted that the best concentration of sodium hypochlorite to extract PHB is 5.6%. Sodium hypochlorite works to break down cells and dissolve cellular components except PHB. Treatment with sodium hypochlorite is effective in dissolving the cellular components but it breaks the PHB when added in greater quantities (Hahn *et al.,* 1994; Ramsay *et al.,* 1990). Sodium hypochlorite is a strong oxidant and; thus, it affects the molecular weight of PHB. In addition, the extraction of sodium hypochlorite leaves chlorinated waste which contaminate the environment (Budwill *et al.,* 1992; Ramsay *et al.,* 1990). In some cases, sodium hypochlorite is used with organic solvents such as calcitone and alcohol, which are medium-polar solvents that dissolve most cellular components such as nucleic acids Phosphatase, and protein substances. The exposure of cells to 50 ° C helps to break down cells better than 37 ° C which facilitates the extraction of PHB. Glass beads method is widely used to recover internal proteins (Chisti and Moo-Young,1996;1994; Harrison,1991; Kula and Schutte, 1987; Middelberg, 1995). Then, SDS is added to help cracking by interacting with the fatty components of the cell

membrane, increasing its size and then exploding the membrane to produce micelles of SDS and lipid phosphate of the membrane (Ramsay *et al*., 1990). SDS does not have the ability to break down cells in a manner that permits the release of all its internal proteins. A study of the genetically engineered *sacchromyces cervisiae* has shown that the use of SDS with chloroform was not an effective method of cell decomposition, instead cell decomposition is more efficient when treated with glass beads (Garrido *et al*., 1994). In this study glass beads along with SDS sodium hypochlorite was found to be an efficient method of cell decomposition (Tamer *et al*., 1998). The organic solvent extraction method was not efficient in extracting as the PHB collected was 37.8%. There are several non-chloroform organic solvents used in PHB extraction: dichloroethane, 1,1,2-trichloroethane, acetic anhydride and propylene-carbonate. Then an American company called ICI used organic solvents at the beginning of the PHB extraction (Berger et al., 1989), but this method is expensive (Poirier *et al.,* 1995). In addition, the organic solvent PHB extraction process, such as chloroform, requires large quantities of chloroform because the solution is very viscous and difficult to perform extraction from it (Lee *et al.,* 1999). Also, the organic solvent extraction of PHB breaks the form of PHB granules; and this step is important in some applications such as strong fiber production (Barham, 1990; Ramsay *et al*., 1990). Moreover, the method of lysing the cell using sodium hydroxide is easy, simple, inexpensive and environmentally friendly. The extraction residues are harmless to the environment, and no degradation of PHB occurs during extraction (Ramsay *et al*., 1989).

Table 2.1: Percentages of PHB extracted using five different extraction methods (Barham, 1990; Hahn et al., 1994; Ramsay et al., 1990; Poirier et al., 1995).

Extraction method	Percentage of PHB
1)Method of sodium hypochlorite	
0.2 %	13 %
1 %	23 %
3 %	36.7 %
5.6 %	51.3%
2) ulrasound	
2 minutes interval	11 %
4 minutes interval	26.5 %
6 minutes interval	21.3 %
3) Chloroform	37.8 %
4) Method of Naoh	
30 °C degrees for 1 hour	
Starting sample with 10% of Naoh	8 %
Sample with 30% of Naoh	20 %
60 °C degrees for 1 hour	
Starting sample with 10% of Naoh	14.3 %
Sample with 30% of Naoh	33 %
5) treating the cells with glass beads + SDS + sodium hypochlorite	
SDS with pH 11 and temperature 37 ° C	41 %
SDS with pH 11 and temperature 50 ° C	57 %
SDS with pH 13 and temperature 50 ° C	32.5 %

PHB appears inside the cells as Inclusion bodies, so PHB is expected to be a high-purity polymer. The cell wall must be destroyed to the release of PHB granules, ie polymer extraction from biomass, should be destroyed. The economic cost of production is significantly correlated with the PHB extraction and purification process. Several solvents were studied for PHB extraction with the aim of finding a relatively cheaper method with a higher extraction rate. Best solvent suggested are methylene chloride, dimethylsulphoxide 1,1,2,2-Tetrachloroethane, 1,2-Dichloroethane, Di, 2,2,2-trifluoro ethanol, ethyl acetate, trichlorethylene, and methylformamide (Terada and Marchessault 1999; Choi and Lee, 1997; Braunegg *et al*., 1978).

The sodium hypochlorite solution and chloroform have advantages that make them the most practical and useable in PHB extraction. These advantages include their high solubility properties of PHB, the ease of extraction and their possible use with dry cells. The percentage to extract PHB from cells using these solvents can reach to 91% and the purity of the polymer produced to more than 97% (Hahn *et al.*, 1994). Ryu *et al.* (2000) studies a way to extract PHB from *Ralstonia eutropha* using sodium hypochlorite solution and chloroform and the addition of 1500 mg / l aluminum ions and 1000 mg / l iron ions to the growth medium and as a binding agent between cells. this study used 50% of the sodium hypochlorite and chloroform solution prepared; and it was found that the PHB purity of the product was 90-94% and 98-99%, respectively.

According to Rawte and Mavinkurve (2002) a 1: 1 mixture of ethanol and acetone can be used to wash the resulting biomass after fermentation. This solution has helped to get rid of the water interfering with the resulting PHB polymer. This mixture has limited effectiveness in the decomposition of cell walls and helps in extracting cellular lipid compounds without affecting the PHB. Jacquel *et al.,* 2008 was able to isolate PHB from the production medium that contained edible oil as the carbon source. Hexane was used as a solvent to remove oil residues from the

center. The biomass was then dried, and the sodium hypochlorite solution was added to break down cell components except for PHB, after chloroform was added to dissolve PHB.

The propylene carbonate solvent has a high boiling point and can be used several times without dehydration and low toxicity. It was used after the addition of acetone in the extraction of PHB from the bacteria. The percentage of PHB was 95% and its purity was 84% and the molecular weight of the polymer was 7.4×10^5 Dalton. While the results of chloroform use were 94%, the percentage of PHB and its purity was 98% and its molecular weight was 1×10^5 Dalton (Fiorese *et al.*, 2009).

Sayyed *et al.*, (2009) pointed out that the best pool of PHB granules within the cells of *Alcaligenes faecalis* BCCM 2374 was shown in 24 hours of incubation in a medium containing a little nitrogen and at 30 ° C temperature; and it was found a decrease of PHB output after 24 hours of incubation. This paper used the sodium hypochlorite solution to break down and digest the contents of the cell other than PHB, and then a mixture of ethanol and acetone by 1: 1 added to dissolve and precipitate the fatty substances in the cell. Then PHB was extracted by adding chloroform and the amount of PHB output was 5.6 g / l compared to other methods such as the use of chloroform only in extraction which gave an output of PHB of 0.63 g /l. PHB extraction is important in obtaining a high percentage of PHB and usually is extracted in three stages. First, we need to tear the cells by adding a chemical or using physical methods such as the use of ultrasound or the use of enzymatic methods. Then, extraction of PHB should be done with organic solvents to separate it from the rest of the cellular components. The final step is to dispose of the solvent by evaporation or sedimentation in another solvent to improve the extraction ratio (Yu, 2014).

2.2. Molecular Weight

One of the most prominent features considered for polymers is their molecular weight, because molecular weight is one of the most important factors affecting its physical properties such as tensile strength and elasticity. These qualities are significantly reduced when the molecular weight is less than 4×10^5 Dalton; and the PHB becomes a very fragile material when the molecular weight is less than 200,000 Dalton (Wong *et al.*, 2005). There are several factors that affect the molecular weight of the PHB, including the extraction method, the nature, and composition of growth medium of the product.

2.2.1. Effect of molecular weight extraction methods for PHB

The method of extracting affects the molecular weight of the PHB, so the extraction method should be efficient and appropriate to maintain the molecular weight at least 400000 Dalton to be suitable for thermal plastic applications (Luzier, 1992). The results showed in table 3 that the molecular weight of PHB extracted from *Pseudomonas cepacia* when treated by chloroform was higher than that of other PHB extracted by other methods. As the molecular weight this PHB extracted from *Pseudomonas cepacia* was 4.7×10^5 Dalton followed by the glass beads and SDS - Sodium Hypochlorite as it was recorded to be 3.8×10^5Dalton. Also, the molecular weight of PHB varies according to the microorganism producing it. The molecular weight of PHB extracted by chloroform from bacteria *Azotobacter vinelandii* UWD was 3.4×10^6 Dalton. While the molecular weight of the PHB extracted by sodium hypochlorite was 2.83×10^6 Dalton (Page & Cornish,1993). The molecular weight of PHB extracted from genetically engineered *E. coli* is 1×10^6 Dalton (Zhang *et al.*, 1994). The molecular weight of the PHB extracted from *A. eutrophus* bacteria is 3.53×10^5Dalton and *A.latus* 2.90×10^5Dalton (Turesin *et al.*, 2000).

In comparison with another strain of *Pseudomonas cepacia*, the molecular weight of PHB extracted in chloroform was estimated at 5.37×10^5 Dalton (Ramsay et al., 1989). The molecular weight of PHB derived from *Pseudomonas* sp.K was estimated at 3×10^5 Dalton (Suzuki et al., 1986). While the molecular weight of PHB derived from *Pseudomonas135* was 3.7×10^5 Dalton (Daniel et al., 1992). The molecular weight of *Methylocystis* SPP was estimated at 2×10^6 Dalton when grown in a solution of methane (Wendlandt, 2001).

Table 2.2.1: The molecular weight of PHB when being extracted using different methods (Turesin et al., 2000; Wendlandt, 2001; Zhang et al., 1994).

Methods of extraction	Molecular weight in Dalton
extraction by Chloroform	4.7×10^5
extraction by beads - SDS - Sodium Hypochlorite	3.8×10^5
Extraction of sodium hydroxide	3.5×10^5
Ultrasonic extraction	2.4×10^5
Sodium hypochlorite extraction at a concentration of 0.2%	3.7×10^5

2.3. Hemp fibers producing sugar through hydrolysis

Hemp has been utilized for the creation of fiber and paper; but lately, there has been expanding enthusiasm concerning utilizing this yield for the creation of cellulosic biofuels in biorefineries and as a source of energy. Hemp have a high cellulose content (Moxley *et al*., 2008). This is a principal reason why this feedstock is often used in fibre production. Also, hemicellulose is one of the content of Hemp; and Xylose is the major sugar, by mass, in hemp

hemicelluloses. Hemp biomass is a complex and recalcitrant matrix made of 55 % cellulose, 16 % hemicellulose and 4 % lignin (Garcia *et al.* 1998). Lignocellulosic biomass typically needs a pre-treatment in order to make cellulose more accessible to enzymes; and Alkaline treatment by NaOH is one of the most common pre-treatment used to treat Hemp (Pakarinen et al., 2012). Zhao *et al*., (2009) stated that the common class of enzymes that are used in lignocellulosic biorefineries are cellulases and xylanases. Lignin-degrading enzymes are also considered important in the biological and biochemical pre-treatment of biomass in order to avoid the formation of inhibitors. The starch content of hemp varies between the different anatomical components of the plant. Typically, it is highest in the leaves, where photosynthesis takes place, and lower in the stems. The starch content can also vary according to the maturity of the plant. Enzymes are biocatalysts which aid in the biochemical conversion of biomass to biofuels in biorefinery processes. These biofuels use the enzymes to produce monomers from the structural polymers through hydrolysis; so that these would then be able to be fermented to the ideal fuel used by yeast or microorganisms (Moxley *et al*., 2008; Pakarinen et al., 2012).

2.5. Generalities of Biomaterials

2.5.1. Definition of Biomaterials

Biomaterials can be defined as any industrial substance that can contact living tissue without causing any toxic reaction within the body (Ohgushi., 1999). The report issued by the European Scientific Forum held in 1986 defined biomaterials as non-living substance that are used for certain medical requirements as they can interact (adapt) with the biological system of the organism (Ohgushi., 1999; Ahmmed., 2010). These alternative substances are divided into two main categories according to their acceptance within the biological system of the organism.

The first is the bioactive inertial group, which contains substances that are acceptable within the organism's biological system but do not interact with it. Gold is the classic example of the use of inert substances in biology. The second is defined as a group of active substances that are well accepted within the biological system of the organism; and are integrated with the living tissue. The above classification of the material can also apply to a group of ceramics called bioceramics (Hench, 1991).

Biomedical ceramics are witnessing an industrial boom and a continuous development supported by a large amount of medical research, with the aim of developing the medical ceramics industry. Research has focused particularly on the biological field, which is the biggest obstacle to improving the properties of medical ceramics. This has led to the development of alternative materials; and thus, the possibility of improving human life (Hench, 1991; Eliaz and Metoki, 2017).

2.5.2. The Properties of Biomaterials

For Biomaterials to perform their intended purpose, they must possess some of the main properties to be safely used when communicating with living tissue. These substances should not exhibit any negative reaction; and they must have good corrosion resistance. Elements resulting from the corrosion of these substances should have the least possible risk of poisoning or injuring the adjacent tissue. Of course, the intrinsic properties of biomaterials vary to meet the different application requirements (Hench, 1991; Eliaz and Metoki, 2017).

For some tissues, it is necessary that the material be decomposable to be gradually replaced. On the one hand, in the case of osteosynthesis, we need a stable material and have good resistance, therefore, excellent mechanical properties. Generally, the requirements that must be taken into

account in the biomaterials implants are their dynamic compatibility and sterilization (Black and Hastings, 2013):

2.5.2.1 Dynamic compatibility

The common definition of biocompatibility can be that the substance does not cause any negative reaction to the host tissue. This definition is very simplistic, but if we want to be more specific. The definition of the biocompatibility can be as the material's ability to show a specific reaction with the host tissue (Black and Hastings, 2013; Boch And Niepce, 2010). As the Biomaterials should not show a negative response in the host body, especially in the formed bond between implant and tissue.

Choosing the material of the implants is a critical step as these implants will be impeded within the human bodies. Thus, studying the reaction between the implants and the living tissue; and how the living body responses to the type of the material chosen for the implants are two important steps to guarantee the success rate of the transplantation (Black and Hastings, 2013). Moreover, the time will take for the material of the implants to react with the living tissues has a direct relationship with the plasma of the body. Also, the porosity factor has a direct relationship with the plasma of the body. Thus, the response of the living tissue to the distribution and size of pores on the biomaterial implants has a direct relationship with the success rate of transplantation (Black and Hastings, 2013).

However, there is almost always an initial inflammatory reaction or infection to the implanted biomaterial. If the infection continues for a long time, it can cause necrosis of the tissue; this should be avoided. This definition implies that the reaction of the host tissues must be known when interacting with these biomaterials; four types of reaction to the biomaterial can be identified when interacting with the host tissues (Anderson *et al.*, 2008):

A. Poisoning:

The elements released from the implanted biomaterials can kill surrounding tissue cells or cause irritation to the host.

B. Biologically inactive:

The implanted biomaterial remains isolated from the original tissue. As a protective mechanism, the fibroblast cells form a fibrous capsule around the biomaterial to isolate the implant from the host tissue.

C. Biologically active:

The cultured biomaterial promotes the formation of an interface with the tissue. This mechanism allows for a tight connection between the implant and the host tissue to avoid the relative movement of the link.

D. Bio-absorbent:

The implanted biomaterial gradually disappears over time; and is replaced by the new tissue.

The process of implanting alternative metal materials in the body of an organism - titanium and steel - is temporary because of its weak adhesion to the host tissue. In addition, biologically inactive ceramics (inert) such as Si_3O_4, ZrO_2, Al_2O_3, and SiC is also not successful as these substances shows no growth on the surface of the bond between the biomaterial and the living tissues. While, actively active ceramic material, when cultured within the living tissue, produces strong layer of adhesion (Anderson *et al.*, 2008; Black and Hastings, 2013).

2.5.2.2. Sterilization

The use of biomaterials for surgical purposes as substitutes for bone parts of the human body has been known for decades (Ohgushi, 2000). However, these surgical interventions were

generally unsuccessful until 1860, when Leicester developed sterilization techniques. Only during the 20th century were biomaterial implants processes performed as important alternatives in the medical field. Biomaterial implants attracted attention to the development and improvement of their biological properties to make them more durable; as these biomaterials enable doctors to improve the lives of people suffering from accidents or congenital defects or any other type of health problems related to the disruption of any function of the body. At present there are many techniques that can be followed for sterilization, mainly ethylene oxide sterilization and gamma sterilization (Ohgushi, 2000). There is not one preferred technique instead of the other, but it varies according to the type of biomaterial being processed. For example, ethylene oxide can leave some toxic residual ingredients on the biomaterials that can be harmful to the end user or patient. Ethylene Oxide (EO) is the residue that may remain after processing has been completed. Ethylene Chlorohydrin (ECH) is the residue that may form when EO encounters free chloride ions. Ethylene Glycol (EG) – the residue that may form when EO encounters water (Boch And Niepce, 2010). According to the behavior of the biomaterial implanted within the body; we can divide the biomaterials into three different varieties according to its acceptance within the biological system of the organism. The first of which is a group of substances known as biologically inactive substances. This group includes a group of substances that are accepted within the biological system of the organism but do not interact with it, such as gold which considers to be the classic example used for Transplantation of alternative materials. The second group is known as bioactive substances; and is well accepted within the biological system of the organism as it can be integrated with the tissue of the organism. The last group is bio-absorber which will be discussed in the next section (Boch And Niepce, 2010).

2.6. Classification of biomaterials

Alternative materials suitable for transplantation within the body of the organism should be available for a variety of characteristics such as biological adaptability, providing harmony with the living tissue, as well as good mechanical properties. On this basis, the materials were classified into three sections: biologically inactive (inert), biologically active, and finally bio-resorbable substances (Amid, 1997):

2.6.1. biologically inactive Materials (inert materials)

Biologically inactive Biomaterials are one of the first substances used in the transplantation within the body of living organisms. These materials have good mechanical properties as they are solid materials, but they live after implanting adjacent to the cells of the living tissue without interacting with them positively or negatively. These biomaterial substances are biologically incompatible with the living tissue, which requires mechanically stabilizing them within the body of the organism to cease their movement. These substances almost do not cause any reaction or inflammation, and not rejected by the surrounding tissue of the body. Also, the most important of the vitally inactive ceramic materials are aluminum and zirconium (Amid, 1997; Loza et al., 2016).

2.6.2. Biologically active Materials

Active biomaterials have good biological properties as they can interact with the living tissue. Active biomaterials be classified into three sections (Hench 1991; Mas-Moruno *et al.*, 2014):

1. Ceramics: the most important types of this category are:

    1.A. Hydroxyapatite: the chemical formula is $Ca_5(PO_4)_3(OH)$ which is similar chemically the structure of bone.

    1.B. Triccalcium Phosphate: which is converted into hydroxyapatite within the living medium of organism.

2. Glass ceramics: glass ceramics are characterized by having the same chemical compositions as glasses but differ from them in that they are typically 95-98% crystalline by volume, with only a small percentage vitreous. Good resistance to thermal shock, high strength, and high impact resistance are all the characteristics of the glass ceramics. Glass ceramics can be produced by dissolving a glass mixture of primary oxides, re-shaping it and treating it thermally until the output crystallizes. Phlogopite ($(Na,K)Mg_3(AlSiO_{10})F_{2,)}$ and β-wollastonite (β-$CaO.SiO_2$) are the two common types of glass ceramic.
3. Bioglasses: 45S5 is most important type of this category.

The importance of using biologically active materials lies in its ability to stimulate bone regeneration. Also, these biomaterials promote the reactions that lead to the proliferation of the reconstructed bone; thus, renew the membrane with it. Among the biomaterials, bioactive ceramic materials have attracted attention to offer greater potential for treating osteoarthritis within the body by substituting them. Heinz and his colleagues successfully installed the first biologically active glass in 1971 to treat osteoarthritis; and it was called 45S5 which is currently known as glass porcelain. This substance when being implanted within the body starts a series of reactions which in turn leads to the formation of a link between the implant to the bone. In the last decade there has been a significant trend towards the use of biologically active substances. These materials are known to have adhesion between their surface and tissue by forming a bioactive hydroxyapatite layer (HA) on the surface as the result of their biological reaction; this biological reaction will also lead to the formation of the bond between the implant and the tissue. Generally, these

biomaterials are characterized by their positive reaction to the tissue through the formed bond. In 1994, Heinch introduced a hypothesis for measuring the biological activity of a substance known as calculating the adhesion factor for biologically active materials which led to the proposal for a new classification of bioactive substances related to the determination of what is known as the adhesion coefficient (Hench 1991).

2.6.2.1. Coefficient of adhesion

Hench, (1996) defined that the level of biological activity of the material can be determined according to a coefficient known as the adhesion coefficient which is related to time $t_{0.5bb}$. This relation expresses the rate of development of the bond between the biomaterial and the surrounding tissue. The equation of coefficient of adhesion can be expressed as below:

Bioactivity Index (I_B) = $100/t_{0.5bb}$

Where $t_{0.5bb}$ is the time taken for more than half of the interface to bond. Based on this relationship we can classify the biologically active material into two classes (Hench, 1996):

- Class A: any material with the value of I_B greater than 8, like 45S5, will bond to both soft and hard tissues. Thus, these biomaterials can stick not only to bone but even to soft tissue.
- Class B: It contains a group of substances that have an adhesion factor of less than 8 but higher than zero. For example, the industrial hydroxyapatite which binds only to the hard bone tissue.

Figure 2.6.2.A shows the bioactivity index of multiple materials; and whether or not these materials to be considered as biologically active, inactive, or absorbed materials. All of these glass materials contained a constant of 6 wt.% P_2O_5.

Figure 2.6.2.B shows the attachment of bone formation to the surface of a group of biochemicals over time.

2.6.3. Biologically Resorbed Materials

This group represents the materials that can disappear completely after implantation. For example, polypropylene or polycyclic acid-soluble mixtures (glycolic)

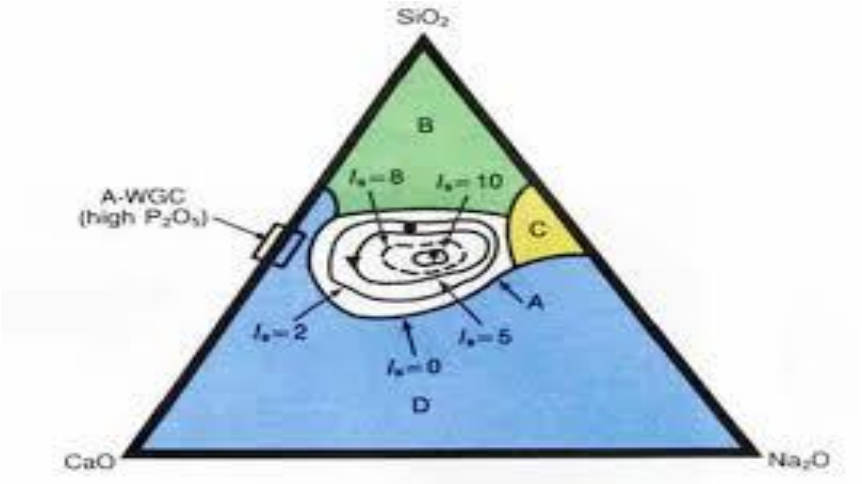


Figure 2.6.2.A: This diagram shows the rate of adhesion of bones and soft tissues to the biomaterials and glass porcelain examined. The white region in the middle of the diagram (A) represents the active biomaterials as the values of I_B are greater than zero and less than 8; these materials are bond to bone. The yellow region (C) shows these materials are resorbed within 10-30 days in tissue. The green region (B) represents inactive biomaterials (inert) as the values of I_B are greater than 8 and less than 10. D region shows that the compositions of the glass materials are not technically practical (Hench, 1991).

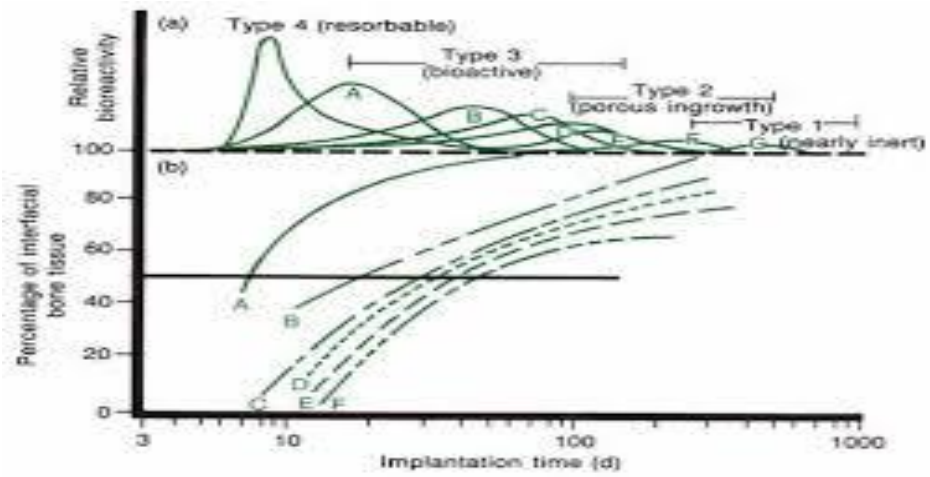


Figure 2.6.2.B: Spectrum of Bioactivity Collection of Glass Ceramics (Hench 1996).

2.7. Applications of Biomaterials

Below are some uses of biomaterials such as bioplastics and bioceramics in the medical field:

2.7.1. PHB as a biomaterial in the Medical field

PHB is a biomaterial which have been used lately in some applications of the medical field.

A) PHB as a medical scaffold:

Given the biodegradability and biocompatibility of PHA, an obvious medical application for the polymer is for scaffolding material in tissue engineering. A study published by Khorasani *et al*, (2011) found that PHB can be modified and be used as a scaffold to promote the proliferation and differentiation of neural progenitor cells derived from P19 cells. PHB was made as a porous scaffold which support the attachment and growing of neural stem cells. Also, in another study PHA was modified to be used as a bone scaffold but it was blended with natural raw materials or other biodegradable polymers, including starch, cellulose derivatives, lignin, poly (lactic acid), polycaprolactone and different PHA-type blends, before being used. Also, chemical modification to PHB was applied with respect to two important synthesis approaches: block

copolymerization and graft copolymerization in the process of creating the bone scaffold (Li, Z, 2016). Also, a copolymer of polyglycolic acid (PGA) and PHB was used to produce pulmonary valve leaflets and pulmonary artery scaffolds in sheep. This study was followed up by construction of a PHA-based heart valve scaffold, which was again surgically inserted into sheep (Brigham *et al*., 2012)

B) PHB is as drug delivery

study using PHB microspheres demonstrated that release of the anti-tumor drug rubomycin inhibited proliferative activity of Ehrlich's carcinoma in mice (Wu *et al*, 2016). Pseudo-PHA granules (i.e. nanoparticles), fabricated in vitro, have also been shown to be effective drug delivery devices. In a recent study, rhodamine B isothiocyanate (RBITC) was targeted to cancer cells and used PHB as a drug delivery (Kim *et al*., 2010).

C) In cardiovascular system

One of the most advanced applications of PHA polymers in cardiovascular products has been the development of a regenerative poly(3HB) patch that can be used to close the pericardium after heart surgery, without formation of adhesions between the heart and sternum (owald and Johans-son-Ruden, 1997)

2.7.2. Artificial heart regulators And Cardiovascular repair/ regeneration

in the United States alone, 45% of the 250,000 heart-valve replacement processes require mechanical heart transplants. The rate of these surgeries clearly demonstrates the need to intensify research in this area for broader applications in the future. Biomaterials are used in the repair and replacement of impaired heart tissues. Synthetic materials are made from polymers

and metals while natural materials are made from animal or human tissues. Both synthetic and natural materials are the two types of biomaterials used in regeneration and repair of cardiovascular tissue. Figure 2.7.1 shows an overview of how biomaterials are used in the medical field to treat cardiovascular diseases (Lam and Wu, 2012).

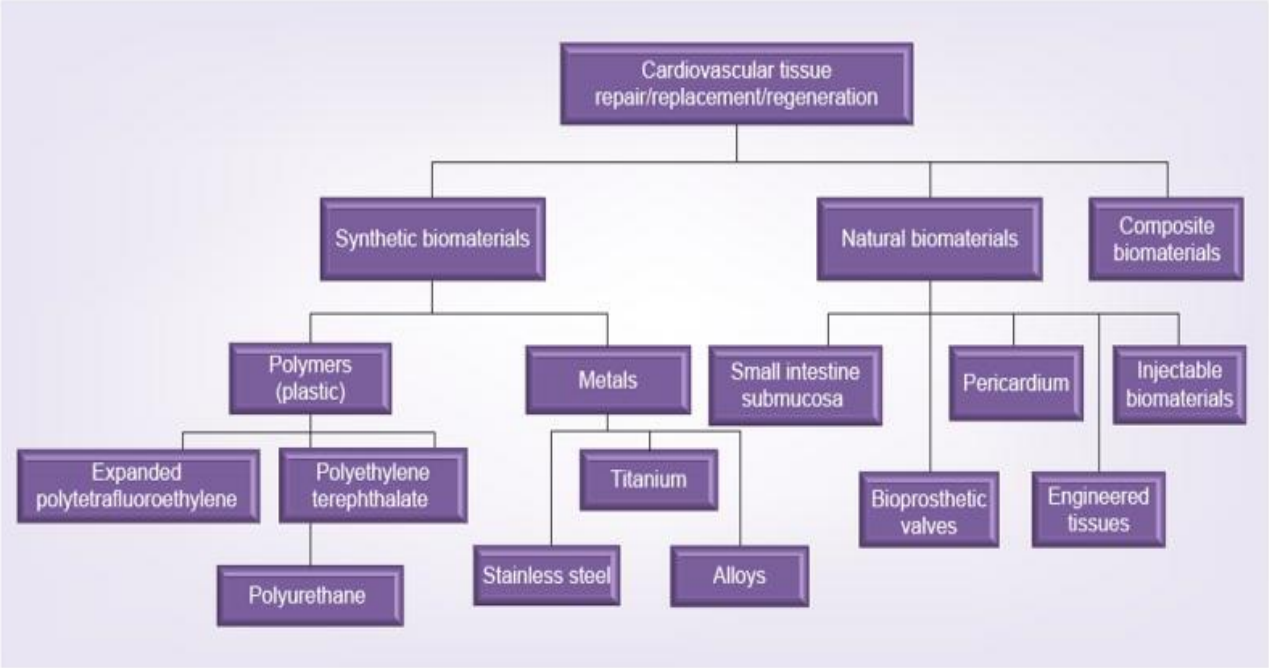


Figure 2.7.1: Synthetic and natural subdivision of the biomaterials used to treat defects and diseases of the cardiovascular system (Lam and Wu, 2012).

2.7.3. Restoration/Repairing of the skin

The damaged skin is often replaced by artificial skin produced from the patient's own cells. It is impossible to use the patient's own cells when the damage is great; thus, artificial skin cells are used to repair this significant damaged skin. Biomaterials divided into natural biopolymers and synthetic biopolymers are used as skin substitutes when there is a lack of sufficient donor tissue.

Natural biopolymers promote the improvement of cell response. While some uses of synthetic biomaterials are shown in Table 2.7.2.

Table 2.7.2: Types of synthetic biomaterials, their uses, and their brand names (Winter, 1995)

Type of polymeric dressing	**Brand name®**	**Use for**
Polymeric foam	Flexzan	Chronic wounds
	Biopatch	
	Crafoams	Burns
	Biatain	Mohs surgery and wounds
	Cutinova	Laser resurfacing wounds
Polymeric hydrogels	Cultinova Gel	Chemotherapy peels
	Biolex	
	TegaGel	
	2nd skin Flexderm	Ulcers
	Dry dressing	Laser resurfacing
Polymeric alginates	AlgiSite	Thickness burns
	AlginSan	
	Sorbsan	Surgical wounds
	Kaltostat	High exudate wounds
	Omiderm	Chronic ulcer
Polymeric hydrocolloides	Idosorb	Chronic ulcer
	Debrisan	Burns
	Sorbex	Average thickness wounds

2.7.4. Applications of Biomaterials in Dentistry and Restoration of Bones

For the last two decades, bio ceramics are used in dentistry and bone marrow surgeries. Also, artificial teeth and bones have been used in the field of medicine. Surgical cement is frequently used in the present era. Industrial joints are surrounded by bioceramic materials to reduce the potential for corrosion and inflammation of joints (Tatullo *et al*., 2015).

2.7.4.1. Bioceramics:

Bio ceramics, like bio glasses, are materials that are compatible with living tissue of organisms; and are a kind of biomaterial. Bio-ceramics vary from oxide ceramic, which is toxic as an inert substance that does not have chemical effects in the body, to the materials that are absorbed within the body after being replaced by the repaired living tissues. Bioceramics are used in many medical processes as they assist in repairing the tissues; and is used mainly as rigid implants. Bioceramics differ from porcelain ceramics as their composition is close to the composition of the living tissues of the body. Some of the bioceramics are also composed of heavy metal oxides (Hench 1991; Eliaz, And Metoki, 2017).

2.7.4.2.The History of Bioceramics:

Before 1925, medical implants were often made of relatively pure metals. Then the year 1925 saw the beginning of the use of medical alloys such as 4V-64Al-T alloy. In 1969 Heinz and others discovered that different types of glass and ceramic could be implanted in living bones and tissues (Eliaz, And Metoki, 2017). Heinz was on his way to the conference, he met a colonel returning from the Vietnam War. The colonel told Heinz how the bodies of the injured soldiers often rejected the medical implants. Heinz was interested in this issue and began to search for materials compatible with the living body, resulting in the creation of a biologically compatible material called bioglass. Following this discovery, a complete scientific research called the study of biomedical ceramics was created. On April 26, 1988, the first international symposium on medical ceramics was held in Kyoto, Japan (Hench 1991; Eliaz, And Metoki, 2017).

2.7.4.3.In Dentistry

The use of medical ceramics in the field of dentistry has been widely accepted, whether it is dental implants or restoration, using dental fillings of ceramic materials. Dental implants are

based on pins attached to the jaw to carry alternative teeth and are usually made of biologically active materials as they can adhere to the host tissue. The process of dental implants or the restoration process requires the use of solid bioceramic materials, resistant to friction, and should be the same color of the natural teeth. Thus, sintering zirconia and aluminum oxide are the two examples used in dentistry (Al-Haddad *et al.*, 2016).

A. The property of bone fusion in dentistry (Osseointegration)

The concept was proposed by professor Branemark at the beginning of the year 1979. Grafts are placed inside the bone by creating a close bond between them, without allowing them to move from its place (Al-Haddad *et al.*, 2016). Then, the bone can be cured around it. This can be compared to treating a fracture of the limbs, by preventing it from moving by placing the cast around it to allow the formation of the bone tissues. During the healing phase, the bone that is attached to the dental implant is formed.

- From 3 to 4 months in the lower jaw.
- 4 to 6 months in the upper jaw.

B. Cosmetic Dental Implants with Zirconia

These implants consist of a part that penetrates the gums and implants within them, where it is similar to the natural roots of the teeth. The upper part is a zircon (ceramic) which has the same color as the natural tooth color as shown in Figure 2.7.3.B

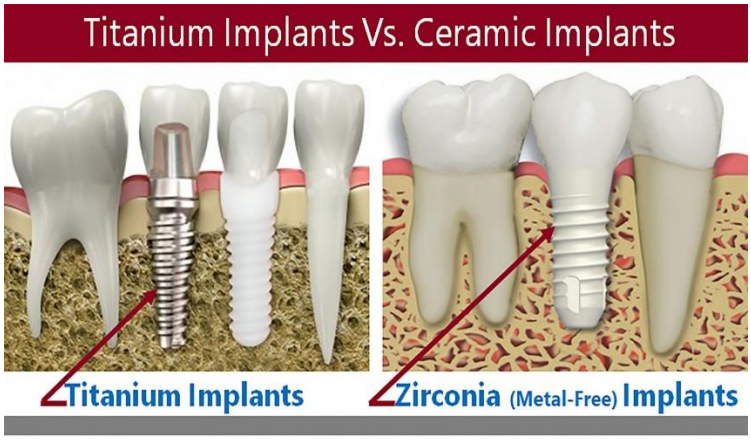


Figure 2.7.3.B: Zirconia implants which is a type of bioceramic implants

(https://www.ariadentalcare.com/metal-free-zirconium-implants)

Here are some steps to Zirconia implants (Al-Haddad *et al.*, 2016):

A) The visible part that passes through the gums like the root of the natural tooth as shown in Figure A

Figure A

http://www.mexicoimplantdentisttijuana.com/Implants.html

B) Picture B took on the day when the second upper grinding teeth are replaced by zirconia implants. The picture shows protective metal screws, and Zirconia circle is directly below them

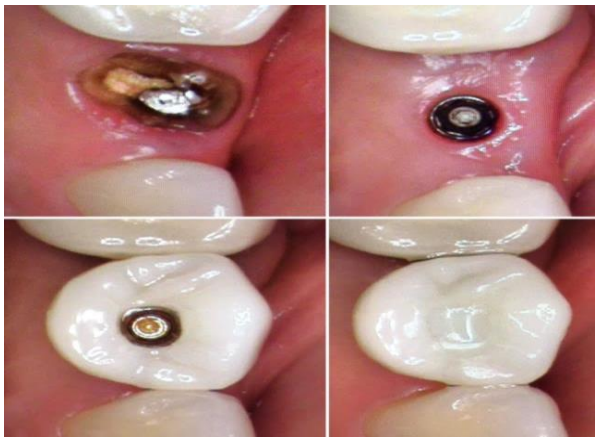


Figure B

(http://caringdentists.com/wpblog/?cat=5)

C) Figure C shows gum healing after 3 months of planting.

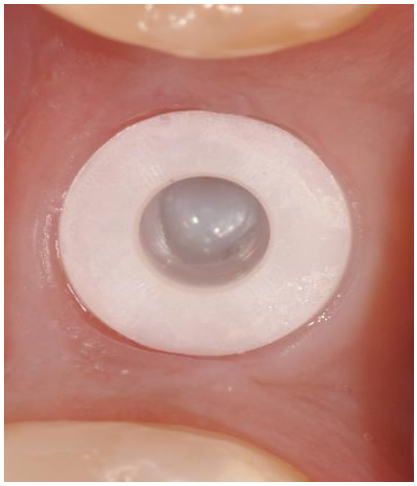


Figure C (Cionca *et al*., 2017)

D) Lastly, the ceramic crown will be placed which is shown in figure A

The advantage of using these bioceramic zirconia implants is to obtain adhesion between zircon and gum, and then the fusion of the gum to provide a protection of the rest of the implant in the bones against bacteria (Al-Haddad *et al*., 2016).

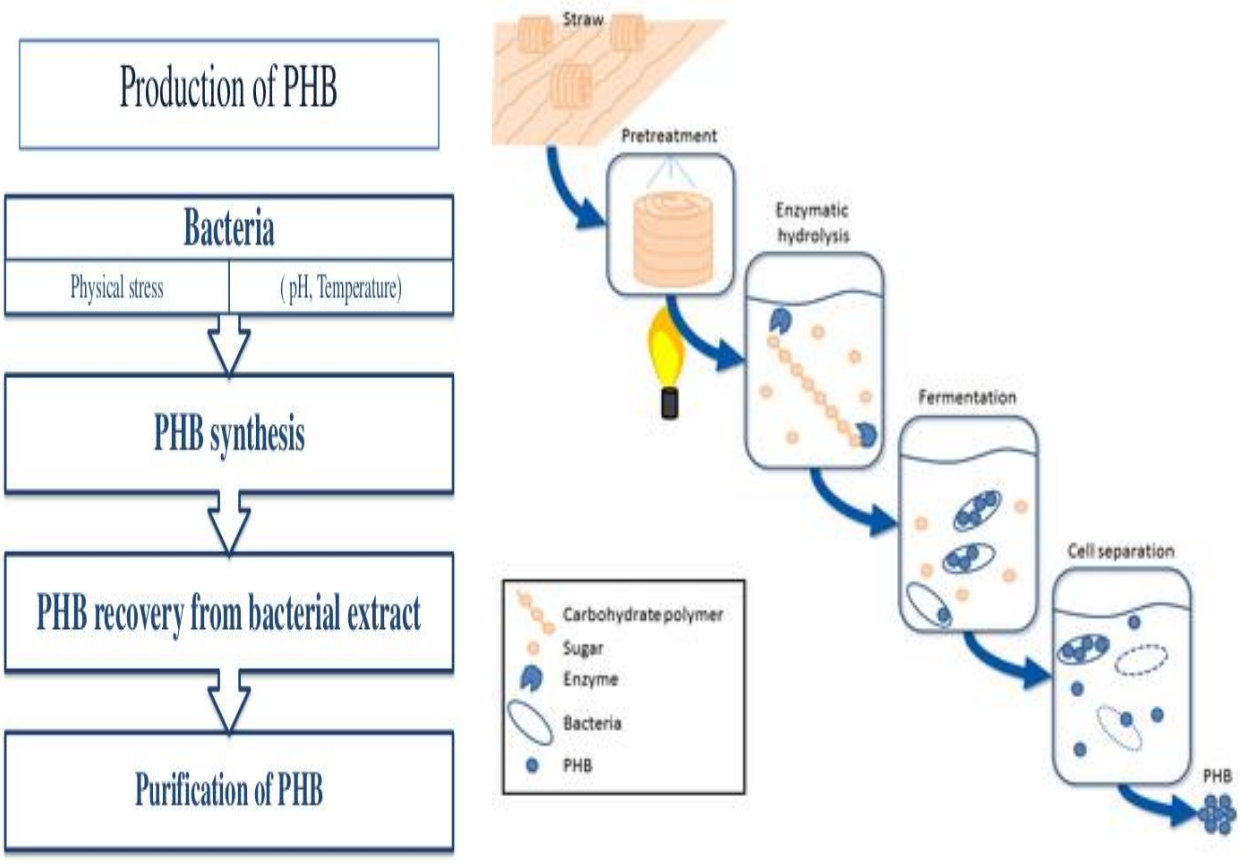
2.7.4.4. A. Bone repair

Bioceramics are utilized to repair and support the bones after being exposed to shocks or injuries. Biologically active materials such as Tricalcium phosphate (TCP), $Ca_3(PO_4)_2$, or biologically inactive materials covered by layers of TCP can be used to treat deficiency of the bones. TCP will provide a good environment for the bones to renew (Afzal 2014).

A conclusion can be reached from this chapter is that biomaterials are different when compared to other substances with a range of chemical and biological reactions. These reactions occur in the bond between the tissue and the implant which generally ends up with so-called bioactive fixation. The behavior of biomaterials varies from inactive substances to an absorbent substance; and others capable of creating an environment that can stimulate the formation of an association between living tissue and them.

2.5. Objectives of this investigation

Decades ago, the promotion of plastic bags began to replace the use of paper bags, because they are characterized by their light weight and stay for a long time without changing. But the idea of producing plastic has become an undesirable idea because of the danger it poses to the environment. Also, plastics are not ideal because they cause the distortion of nature. The objective of this study was to produce bottles of environmentally friendly bioplastics. This study aims to use sustainable green feedstock of hemp to utilize the production of PHB. The conversion of lignocelluloses to fermentable sugars was one of the underlying methods by using enzymatic hydrolysis. Hemp was chosen in the study due to its low cost and availability in Canada. Figure 5.2. summarizes the overall principle followed in this investigation.



A B

Figure 5.2.A. The main reason behind this investigation is to produce PHB from Ralstonia eutropha by using hemp as the main source of carbohydrates. 5.2.B shows that during the fermentation process the bacteria will use the glucose generated from the enzymatic hydrolysis to produce PHB

Chapter 3

Experimental Methods and Procedures

3.1. Materials and bacterial strain

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 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 **Error! No text of specified style in document.**.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

3.1.1 *Ralstonia eutropha* activation

In this investigation, *Ralstonia eutropha* (ATC 17699) was obtained from the American Culture Collection Center (Manassas, US). The bacteria obtained frozen and deactivated, so activation process was needed before using this bacteria in this study. A growth medium was utilized to activate *Ralstonia eutropha.* The growth medium was prepared by mixing up:1 gram beef extract, 1 gram tryptone, 0.2 gram yeast extract, and 100 ml distilled water in 250 Erlenmeyer flasks. Then, the mixture was mixed using the Thermo Scientific MaxQ 4450 incubator shakers for 30 minutes. Then, a sterilizing process was carried out by autoclaving the

growth medium at 121 ^{o}C for 20 minutes. After the autoclaving process, the growth medium was allowed to be cooled at room temperature within an aseptically Biological Cabinet, The Labgard Class II, Type A2 Biological Cabinet, which was cleaned by wiping the surface with ethanol; and then UV light was used for further sterilization for 10 minutes. Then under the hood of the Biological cabinet, 10 ml of *Ralstonia eutropha* was mixed with the previously prepared growth medium. Finally, *Ralstonia eutropha* was 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). All the above steps were carried out during the first day.

3.2. Hemp treatment

3.2.1. Hemp manual treatment

The hemp fibers were obtained from a ranch in Manitoba and stored at room temperature in ziplock bags. powdered hemp was the form that this study required. To acquire powdered hemp, firstly, the hemp fibers were manually separated from leaves. Secondly, hemp fibers were treated with lab scale blender to establish a powder form of the hemp fibers (Davis *et al.* 2013). The grinded hemp was then stored in ziplock bags at 27 ^{c}o.

3.2.2. Hemp treatment with alkali solution (NaOH)

Initially, 100 grams of powder hemp was added to 1-liter of alkali solution (NaOH) concentrations of 2 %, 5 %, and 10 % (w/v)). To establish 2 % NaOH, 20 grams of NaOH was placed within a 1000 ml volumetric flask; then distill water was used to make up the volume to 1000ml of the flask. The same previous procedure was used to establish 5 % and 10 % of NaOH,

whereas 50 grams and 100 grams of NaOH in 1000 ml Erlenmeyer flask of distill water were used respectively. The reaction is exothermic, once cooled, the diluted alkai solution was added to the grinded hemp. Then, the pretreated hemp was placed into a Thermo Scientific MaxQ 4450 shaker at 32^{o}C for 60 minutes; and were continuously stirred within the incubator shaker at a constant rate of 200 rpm. After 60 minutes the pretreated hemp was autoclave at 121^{o}C for another 60 minutes. After pre-treatment, the pretreated hemp mixture was filtered by an 11 cm Buchner Funnel using 0.5-micron filter paper to separate the solid hemp fibers from the liquid solution. 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). Hemp manual separation and alkali treatment were carried out during the first day.

At the end of treating the hemp with NaOH, and then treating it with heat (autoclave), a slurry was produced. One phase contains the lignin, insoluble ash, and cellulose. The second phase is made up of solubilized sugar material (Sluiter *et al.* 2016).

3.2.3. Hemp solid fibers treatment with Enzymes

The dried solid fraction of hemp which was placed in the oven during the first day was treated with enzymes to produce hydrolysis reaction. Then two 500 ml Erlenmeyer flasks were prepared by adding 50 grams of dried hemp in each flask; so, a total of 100 grams of dried hemp was used. Then, 1.5 g of Cellulase enzyme was added to one of the flask; and 1.5 g Cellulase along with 1.5 g of Xylanase enzymes were added to the second flask. Cellulase from *Trichoderma Reese,* and Xylanase from *Trichoderma longibrachiatum* enzymes were taken from the fridge of the lab without any modifications to them. Different combination of enzymes

(Cellulase from *Trichoderma Reese*, and Xylanase from *Trichoderma longibrachiatum*), were added at different concentrations (Refer to Table 3.2.3) 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 3.2.3 represents the sample of Pre-treated hemp studied followed by Enzyme Addition Studied.

Flasks Number	**Pre-treatment Conditions**		**Enzyme Addition**		
	NaOH concentration (%)	Autoclave used	Cellulase (g/l)	Novozyme (g/L)	Xylanase (g/L)
Flask 1	2	Yes	-	-	-
Flask 2	2	No	1.5	-	-
Flask 3	5	No	1.5	-	-
Flask 4	5	No	0.7	-	-
Flask 5	5	No	3.0	-	-
Flask 6	5	No	1.5	1.5	-
Flask 7	5	No	1.5	1.5	1.5
Flask 8	5	Yes	1.5	1.5	1.5
Flask 9	5	Yes	1.5	1.5	1.5
Flask 10	5	No	-	1.5	1.5
Flask 11	10	No	1.5	-	-

3.2.4. Addition of Nitrogen Source

The nitrogen-limited medium was prepared in 750 ml of distilled water by mixing of 3.3 g of $K_2H_2PO_4$, 0.45 g of Fe (III) NH_4 Citrate, 0.0075 g of $CaCl_2$, and 0.375 g of $MgSO_4$-$7H_2O$. Then, 1 mL of trace elements was added to the solution. Then this mixture was autoclave for 60 minutes at 121 ^{o}C.

3.2.5. The Trace Solution:

The trace solution was ordered by the lab coordinator and kept in the fridge; and no further modifications were applied to it. 1 ml of this trace solution was added to the nitrogen limited medium.

3.2.6. Addition of Carbon Source

A solution of 0.75 g/L of NH_4Cl in 250 ml of distilled water was prepared. The second option of carbon source was 0.50 g/L of Urea along with 0.25 g/L of NH_4Cl in 250 ml of distilled water. The solution was then autoclave for 60 minutes at 121 ^oC.

3.2.7. Adding all of it together: *Ralstonia eutropha* Addition after controlling the PH

After 72 hours of enzyme hydrolysis, hemp fibers were filtered using 11 cm Buchner funnel to separate the liquid and solid hemp; but this time the aqueous solution was kept while the solid hemp was discarded. The pH of remaining hydrolysate was then adjusted to be in a range of 7-9 (preferably 7.8), as this range was found to be the optimal PH for the bacteria to grow (Smolders *et al.*, 1994). A study conducted by Salehizadeh, and Loosdrecht (2004) found when the pH values falls in a range 7.3 to 8.7, an increase up to 20 % in the production of PHB can be established.

Then, the solution was autoclaved for 60 minutes. After autoclaving and the solution was allowed to cool down at room temperature. Then the nitrogen limiting medium was added along with the carbon limiting medium. Then, 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.

3.2.8. The collection of PHB by centrifuging

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 in the fridge.

3.2.9. Water and oil bath

the test tubes were obtained from the fridge; and 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). After, this solution was subjected to a water bath. Water bath followed the oil bath to collect the solid layers of bioplastic after the evaporation of the liquid solution.

Chapter 4
Results & Discussion

4.1. Measuring the effects of alkali and heat treatment (pre-treatment)

The Hemp was treated chemically by different concentrations of NaOH after reducing their size by using the blender. Toward the finish of the pre-treatment, a slurry solution was created. Slurries contain two phases and have historically been separated prior to characterization with wet chemical analytical methods. In this study, the overall composition of the slurry is determined by equation 1 which distinguish the dry portion of solid slurry from the liquid phase of the slurry. The solid slurry was resulted after using the filter papers to exclude the wet portion from the solid.

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

Whereas, the Numerator ($Solid\ Fraction_{Dry}$) represents the solid portion of the slurry, the denominator ($Slurry_{Dry}$) represents the wet portion of the slurry.

The solid phase contains much of the lignin, cellulose, and insoluble ash. The solubilized material, or soluble solids, is another class of material in the slurry that typically is comprised of sugars, low molecular weight lignin, products of carbohydrate depolymerization, inorganic compounds, acetic acid and extractive materials. FIS measures how much of lignin removed after the process of pre-treatment. Table 4.1 represents the results of FIS after treating the samples in the flasks with NaOH and high temperature (thermal).

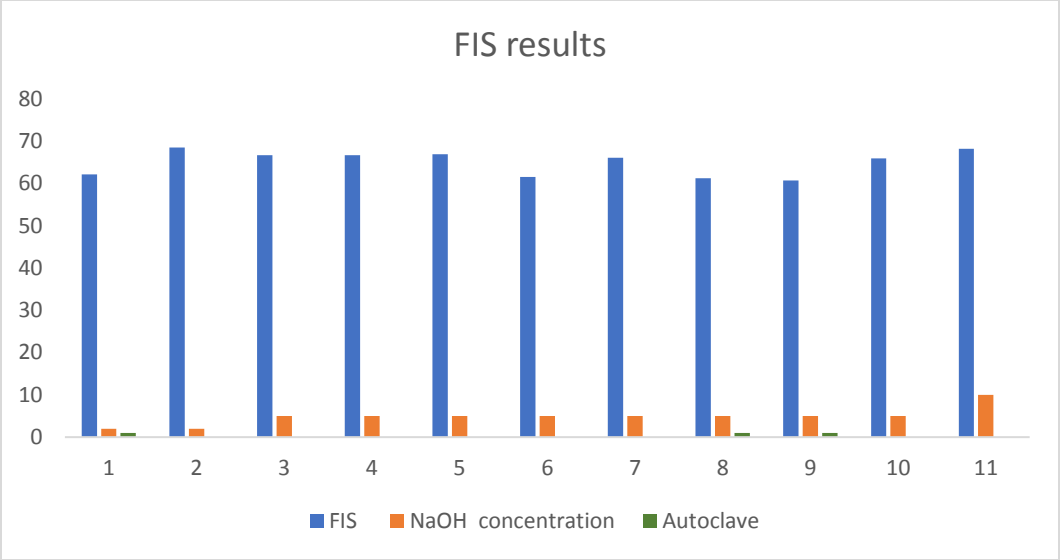


Figure 4.1 shows the numerical values of FIS where the first two flasks had 2% of NaOH, and Flask number 11 had 10% of NaOH while the rest eight flasks has 5% of NaOH. Also. Autoclaving is using a method of thermal treatment, and it applied to flask 1, 8, and 9.

A study done by Cantero-Tubilla, (2017) found that increasing the concentrations of alkali yielded lower values of FIS as NaOH and thermal treatments can disturb the lignin-hemicellulose bonds and solubilize bonds among lignin/hemicellulose. Thus, 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 (Cantero-Tubilla, 2017). Also, these treatments promote the digestion of hemicellulose by disturbing these bonds; resulting in reducing the crystallizing of hemp. Moreover, as the concentration of NaOH increases, the percentage of lignin removal increase as the cleavage rate of lignin and glycosidic bonds increases (Cantero-Tubilla, 2017; Wunna *et al.*

2017). Finally, figure 1.8.2.2 in chapter 1 explains the dynamic of these bonds when being treated with NaOH.

The results obtained from table 4.1 confirmed the conclusion made above from the studies carried by Cantero-Tubilla, (2017); Wunna *et al.* (2017). As seen from table 5.1 treating the samples with a higher concentration of NaOH reduces the recovery of insoluble materials as FIS= 68.52 % of flask 1 containing 2% NaOH had a higher FIS value compared to flask 6 with 5% NaOH (66.06%).

A second conclusion can be made as flasks 0 and 6 had lower recovery of their insoluble materials compared to flask 1 and 7, respectively (62.13% compared to 68.52 %, and 66.06 % compared to 61.26 %), table 4.1. Each of flasks 0 and 6 had been treated with NaOH only, while flasks 1 and 7 were treated with both thermally and chemically with NaOH. Thus, the conclusion from this table is that using thermal methods along with alkali treatments is not useful. Thus, treating hemp with multiple methods reduces the recovery of the insoluble materials.

4.2. Measuring PHB Recovery, HPLC, and Enzymatic Hydrolysis

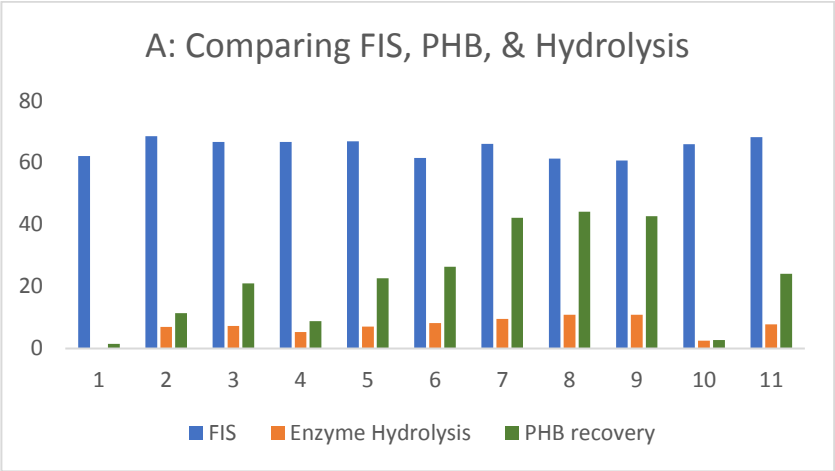
PHB cells were collected after fermentation. HPLC was accustomed investigate the sugar content after hydrolysis and fermentation. Agilent Technologies 1260 infinity series equipped with autosampler injector and a refractive index detector (serial # DEAA302993). Sampling was done at intervals the sterile clean biological cupboard. Samples diluted by an element of fifty, centrifuged at 4000 g for quarter-hour and filtered through forty-five µm filter. Samples were collected at the beginning of fermentation then intervals of twenty-four hours till finish of fermentation. Sterilized Eppendorf tubes were accustomed store a pair of milliliter samples for every interval. Samples were keep at -80 C in Revco Elite and fridge till quantified. Sugar was quantified victimization HPLC equipment. The results obtained from analyzing 3 samples.

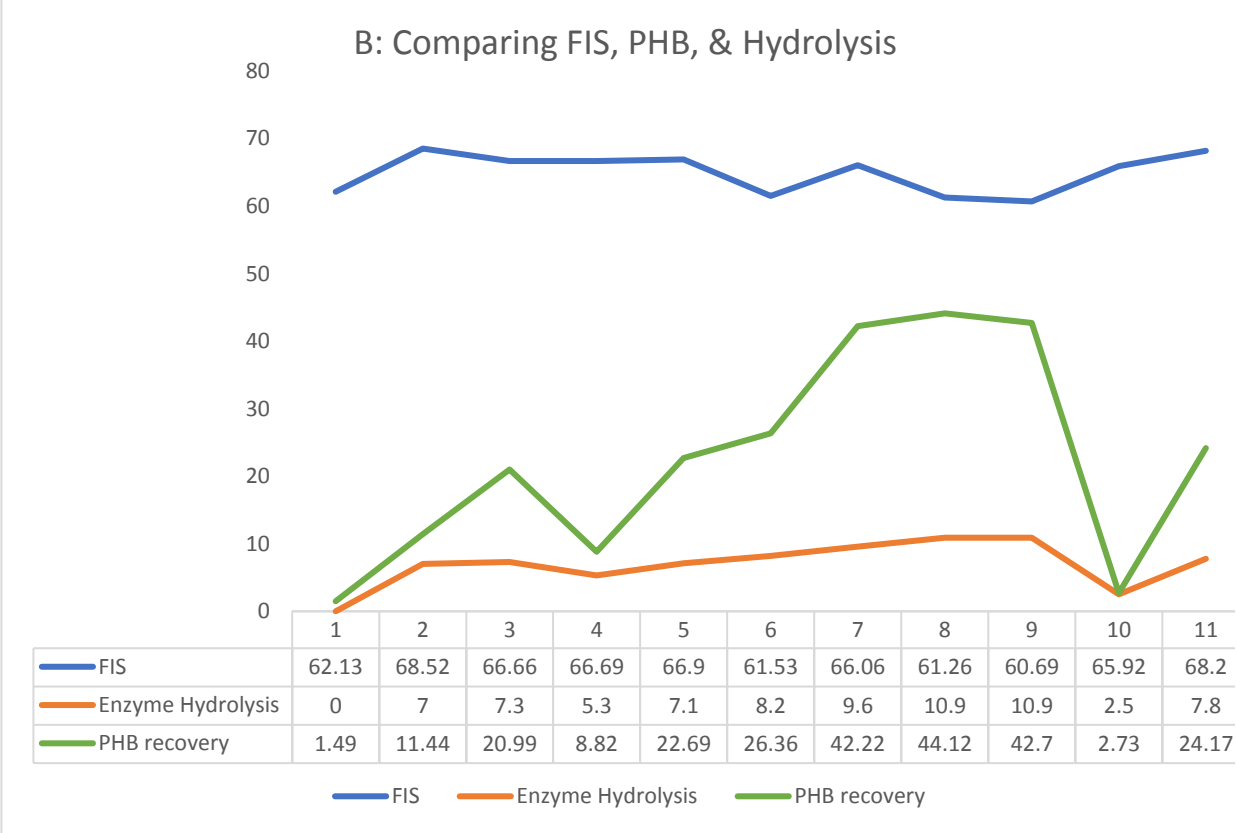
Moreoverm the Shodex SP0810 column served the purposed of measuring sugar concentrations. The mobile part of HPLC was set as water HPLC grade microfiltered was used. The mobile part was utilized to increase the rate of flow to 0.6 ml/minute for half-hour. Following this step, a constant flow resulted.

Table 4.1 shows that the all the content of sugar that was present before the fermentation process got consumed within the 72 hours following fermentation.

Table 4.1 the numerical values of FIS, Enzyme Hydrolysis, and PHB when treating the hemp with different concentrations of NaOH added to 11 flasks.

FLASK	FIS (%)	Sugar Composition		Total Sugars (g/L)	Enzyme Hydrolysis Yield (%) – Before Fermentation	Enzyme Hydrolysis Yield (%) – After Fermentation	Average Wet Mass Produced (grams)	Average Dry Mass Produced (grams)	Yield (%)
		Glucose (g/L)	Xylose (g/L)						
1	62.13	0	-	0	0	0	9.12	0.03	1.49
2	68.52	7.6	2.3	9.7	7	0	23.78	0.68	11.44
3	66.66	15.5	2.7	18.1	7.3	0	25.35	1.33	20.99
4	66.69	6.1	0.7	6.8	5.3	0	16.32	0.36	8.82
5	66.90	17.4	3	20.3	7.1	0	25.57	1.45	22.69
6	61.53	19.9	1.2	21.2	8.2	0	26.25	1.73	26.36
7	66.06	22.9	10.3	33.2	9.6	0	30.13	3.18	42.22
8	61.26	28.1	10.9	38.9	10.9	0	30.09	4.28	44.12
9	60.69	28.4	11.1	39.4	10.9	0	33.68	4.33	42.7
10	65.92	1.2	3.2	4.5	2.5	0	16.1	0.11	2.73
11	68.2	14.3	2.7	17	7.8	0	22.01	1.33	24.17





	1	2	3	4	5	6	7	8	9	10	11
FIS	62.13	68.52	66.66	66.69	66.9	61.53	66.06	61.26	60.69	65.92	68.2
Enzyme Hydrolysis	0	7	7.3	5.3	7.1	8.2	9.6	10.9	10.9	2.5	7.8
PHB recovery	1.49	11.44	20.99	8.82	22.69	26.36	42.22	44.12	42.7	2.73	24.17

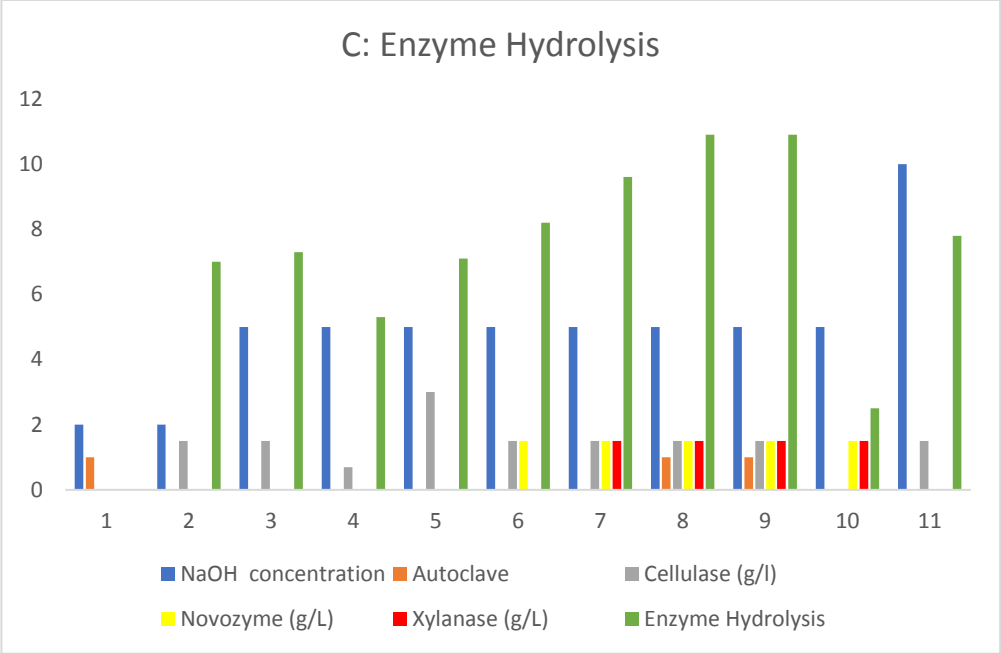


Figure 4.2 Comparing the results of FIS, Enzyme Hydrolysis, and PHB in the 11 flasks. Figure 4.2.A is a bar representation of the results while 4.2.B is a linear representation of the results. Both figure A and B represent the same data but using different visual representation. Figure C shows the effects of the enzyme hydrolysis when adding unique combinations of three different enzymes (Cellulase, Novozyme, and Xylanase), to the 11 samples.

As seen from figure 4.2.B when thermal along with alkali treatments were used in samples 1, 8, and 9, lines of FIS, enzyme hydrolysis, and PHB show the same pattern as they are all concaving downward. On the other hand, mixed patterns of lines can be witnessed in figure 4.2.B. Figure 4.2.B shows that as FIS line concave down, the two lines of PHB recovery and Enzyme hydrolysis concave upward, so the relationship is an inverse one. Also, figure 4.2.B shows that enzyme hydrolysis increases as the concentration of NaOH increases from 2% to 5%. These results are consistent with the results found in a study conducted by Mukherjee et al.,

(1993) as they found that hemicelluloses and lignin materials were removed more rabidly when increasing the concentration of an alkali treatment. Moreover, Kabir *et al*. (2013) found that the high concentration of NaOH disrupts the cellulose backbone; leading to the fast breakages of hemicelluloses and lignin; thus, better percentages of PHB recovery will follow. Moreover, the Highest PHB recovery and enzyme hydrolysis were recorded when using both thermal and alkali treatments in samples 8 and 9 (Figure 4.2). Additionally, figure 4.2.C confirms that using multiple pre-treatments (thermal and alkali) increased the yield of enzyme hydrolysis as when comparing samples 8 and 9 to the rest of samples. These results can be explained as treating these samples with heat and NaOH had affected the structure of cellulose backbone, resulting in the breakage of the bonds (Yuan *et al.,* 2012).

On the other hand, a study done by Madadi *et al.,* (2018) explains that after subjecting the hemp to alkali treatment, the hydrolysis will convert the products into mono sugars by cleaving the glycosidic bonds. Then, these resulted mono sugars can be used for fermentation process or others as seen in Figure 4 in chapter 1. figure 4.2.C shows the different effects of three enzymes on the results of hydrolysis along with applying thermal and alkali treatments to samples 8 and 9 (Figure 4.2.C). Enzyme hydrolysis yielded the highest results when using the same concentration of the three enzymes under investigation (Cellulase, Novozyme, and Xylanase). The results obtained from Figure 4.2.C is consistent with results published by Weiss *et al*., (2013), as it was found the hydrolysis of cellulose by cellulase enzymes required subjecting the cellulose to a large amount of this enzyme. Cellulase enzymes are costly which is the main problem to the industrialization of lignocellulose conversion processes. Thus, this study found that a combination of cellulase, novozyme comparing to cellulase by itself, is as effective in the hydrolysis of cellulose, and less costly (Weiss *et al.,* 2013). Sun *et al*., (2015) found that a

cellulase mixture made of cellulase, β-glucosidase, xylanase and lytic polysaccharide monooxygenases AA9 was the most successful in the process of cellulose's hydrolysis (Sun *et al*., 2015). Also, cellulase enzyme was added by itself to samples 2,3,4,5, and 11; and could elicit the hydrolysis of cellulose. Whereas, a combination of novozyme, and xylanase enzymes was added to sample 10; and a combination of novozyme and cellulase was added to sample 6. When comparing the results of sample 10 and sample 6, cellulose was the driven factor to cause greater percentages of hydrolysis. Cellulase enzyme is by far one of the most effective enzymes to be used alone in the enzymatic hydrolysis (Kim *et al.*, 2001; Hasegawa *et al*., 2016). A study conducted by Hasegawa *et al*., (2016) suggested that recycling cellulase enzymes after each enzymatic hydrolysis could solve the problem of their high cost as it was found that approximately 70% the of cellulase activity remained in the solid residue after 70% of the glucan was hydrolyzed, and 22% of cellulase activity remained in the solid residue after 99% of glucan was hydrolyzed. Multiple studies reached the fact that glucose release is controlled by removing of xylose and hemicellulose coating on cellulose. Also, these studies found that xylanase enhances the removal of xylose; and a mixture of cellulase and xylose is effective in the process of hydrloysis when compared to using each of the enzmes by themselves (Kim *et al.*, 2001; Kumar and Wyman, 2009; Yang and Wyman, 2004). These studies affirm why adding xylanase to samples 7,8,9,and 10 in this investigation icreases the products of enzymatic hydrolysis.

Conclusion

In this investigation, the sugar source in the hemp was the main source to produce PHB; roughly 5 kg from bioplastics material was accumulated by the end of this experiment. resulting in the production of bioplastics. This investigation is promising as the products of hemp can be used to produce environmentally friendly plastics which won't have any harmful effects on the lives of human beings. Also, hemp is a reliable source to produce bioplastics since its abundance in Canada; as in 2012 Canada had certified hemp as a bio-based crop and food safety accreditation was provided (Salentijn *et al.* 2015). This law of the certifying hemp increases the production of hemp in Canada. Moreover, the global markets of producing hemp are expected to double between 2016 to 2020 (Salentijn *et al.* 2015). Thus, hemp is indeed reliable, accessible source to produce bioplastics.

There are some concerns that were arise during this investigation on the possibilities to produce higher quantities of PHB. One of the concerns is on the possibility to produce bioplastics without the need of using extraction methods (water and oil bath). If bioplastics can be produced with skipping this last step, the rate of producing hemp will be faster. Enzymatic hydrolysis can take up more than 48 hours. Thus, the rate of production will be accelerated if a method of faster enzymatic hydrolysis is found. These concerns needed further investigations.

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