ADDIS ABABA UNIVERSITY

ADDIS ABABA INSTITUTE OF TECHNOLOGY

SCHOOL OF CHEMICAL AND BIO ENGINEERING

Production of poly-hydroxybutyrate (PHB) from glucose derived from sugar cane bagasse using Bacillus subtilius

A Thesis submitted to Addis Ababa Institute of Technology, In Partial Fulfillment of the requirement of degree of Master of Science in chemical Engineering (biochemical Engineering Stream)

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ADDIS ABABA INSTITUTE OF TECHNOLOGY (AAiT)

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DECLARATION

I declare that this thesis for the M.Sc. Degree at Addis Ababa University, hereby submitted by me, is my original work and has not previously been submitted for the degree at this or any other university, and that all resources of materials used in this thesis have been duly acknowledged.

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This thesis has been submitted for examination with my approval as University Advisor.

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Abstract

Most plastic materials are produced from petrochemicals. However, due to their non-degradable nature, they are causing serious environmental problems. The objective of this study was to obtain a bio-plastic material known as poly-hydroxybutyrate (PHB) based on glucose extracted from sugarcane bagasse with the help of bacillus subtilius. under stressing media condition. The extraction process stages were pretreatment, hydrolysis, fermentation, extraction, and recovery. The first stage was pretreating sugar cane bagasse and hydrolysis using dilute sulfuric acid in 1:8 solid/liquid ratios. The three factors analyzed were temperature, pH, and fermentation time for extraction of PHB. Synthesized PHB was characterized using Sudan staining, UV spectrophotometer and FTIR. The Response Surface Methodology with Box-Behnken experimental design was used to find the optimum extraction conditions. The effects of extraction parameters (temperature, pH, and fermentation time) on the response (poly-hydroxybutyrate yield) were analyzed. The optimum PHB yield of 6.82 g/l (0.17 g/g of bagasse) of was obtained at 37.3 0c, 7.2 pH and 48.6 hours. From the statistical analysis, all the factors had significant effect on the yield. Moreover, the interaction effects of temperature and pH, temperature and fermentation time, and pH and fermentation time had also significant effects on the yield.

***Keywords**: sugarcane bagasse, bacillus subtilius, poly-hydroxybutyrate (PHB) and bio-plastic*

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List of Acronyms

ANOVA	Analysis of variance
$CuSO_4$	Copper sulfate
Cu_2O	Cuprous oxide
FTIR	Fourier Transform Spectroscopy
H_2SO_4	Sulfuric acid
Mcl-PHBs	Medium chain length Polyhydroxybutyrate
NaOH	Sodium hydroxide
PA 11.	Polyamide 11
PBAT	Polybutylene-adipate-co-terephthalate
PCL	Polycaprolactones
PE	Polyethylene
PEF	Polyethylene furanoate
PET	Polyethylene terephthalate
PHA	Poly-hydroxyalkanoate
PHB	Poly-hydroxybutyrate
PLA	Polylactic acid or polylactide
PP	Polypropylene
PS	Polystyrene
PTT	Polytrimethylene terephthalate
Scl- PHBs	Short chain length Polyhydroxybutyrate
sCO_2	supercritical carbon dioxide
UV	Ultra-Violate

Chapter one

Introduction

1.1. Background

The rapid growth of the human population increases the use of plastics and this led to a huge accumulation of non-degradable waste material (Luengo et al. 2003). Due to the desired properties of plastic like its low density, strength, durability, and resistance to degradation it uses for different purposes. However, its resistance to degrade creates high amounts of accumulation recalcitrant plastics in the environment, so it becomes dangers for the biosphere (Sukan, Roy, and Keshavarz 2014). For this reason, finding environmental friendly degradable plastics is the best alternative (Camargo et al. 2012).

Biomaterials, as the name implies, are biologically synthesized and catabolized by different organisms and have high applications in biotechnology (Luengo et al. 2003). They are biodegradable and biocompatible. They can be easily consumed by microbes and have no toxic effect on the host (Kumar and Thakur 2017).

Bio-plastic are polyesters, produced by different kinds of microbes under different nutrient and environmental conditions. These polymers, which are usually lipid in nature, are accumulated as storage materials allowing microbial survival under stress conditions (Brandl et al. 1990). The number and size of the granules, the monomer composition, macromolecular structure, and physico-chemical properties vary, depending on the producer organism (Luengo et al. 2003).

Biopolymers are an alternative to petroleum-based polymers with a wide range of environmental advantages. Biodegradable materials under development include polylactides, polyglycolic acids, polyhydroxyalkanoates (PHAs), aliphatic polyesters, polysaccharides and their co-polymers and/or blends. Amongst these, PHAs are of particular interest because they possess thermoplastic characteristics and resemble synthetic polymers to a larger extent. PHAs are degraded to carbon dioxide and water in aerobic conditions and methane in anaerobic conditions by microbes 2 found in soil, water and other various natural habitats

PHB is a special type of bio-plastics, that is synthesized and catabolized by microorganisms particular0ly bacteria. PHBs are macromolecules synthesized by bacteria and are inclusion bodies accumulated as reserve material when the bacteria grow under different stress conditions (Saito 1994)

1.2. Problem of statement

Plastic materials are mostly produced from petrochemicals. But, due to their non-degradable nature, they are causing serious environmental problems (Chee et al. 2010). They are causing problems such as affecting the appealing quality of cities, water bodies and natural areas due to their durability and resistance to degrade beside to their high usage due to their desirable properties (Kamisah et al. 2005). In addition, the huge accumulation of recalcitrant plastics in the environment becomes danger for the biosphere (Sukan, Roy, and Keshavarz 2014). For this reason, the biodegradable plastics is the best solution to protect the environment from those problems caused by non-degradable plastics.

Among the different kinds of biodegradable plastics, poly-hydroxybutyrate (PHBs) is the one which is 100% biodegradable (Getachew and Woldesenbet 2016a). From sugar production process, large amount of sugarcane bagasse is generated as by-product in Ethiopia. It is under-utilized and sometimes creates environmental problem since huge piles of the byproduct are left unattended being susceptible to fire hazard. It contains certain amount of glucose. Thus, it is possible to use glucose derived from sugarcane bagasse for production of biodegradable plastic. Also, different studies used recombinant bacteria to produce biodegradable plastics but, in this study, only one species of bacteria is used to produce it. So, in these thesis work poly-hydroxybutyrate (PHBs) is produced from sugarcane bagasse by using *bacillus subtilius.* This can offer environmentally friend solution to the non-biodegradability issues of petroleum-based plastics. Moreover, it provides some application of bioplastic according to the Ethiopian current policy.

1.3. Objectives

1.3.1. General objective

The general objective of this research is production of poly-hydroxybutyrate from glucose derived from sugarcane bagasse by using *bacillus subtilius.* under stressing media condition.

1.3.2. Specific objective

To achieve the general objective the following specific objectives are addressed

- To hydrolyze sugar cane bagasse into glucose
- To investigate effect of processing conditions (temperature, fermentation time and pH) on PHB production.
- To characterize the extracted poly-hydroxybutyrate

1.4. Significance of the study

This study is an experimental study of PHB extraction method and its significance

- ✓ Helps to reduce the imported amount of petrochemicals since the conventional petrochemical plastics will be replaced by plant-based polymers
- ✓ Since the product is biodegradable it can serve as a compost for the soil and improves the quality of soil and reduces additional fertilizer cost (Prados and Maicas 2016).
- ✓ To substitute petrochemical-based plastics with bioplastic which is biodegradable
- ✓ To minimize the environmental impact due to the huge accumulation of sugar cane bagasse.
- ✓ To minimize the ecological problem that arises from the degradation of recalcitrant synthetic polymers

Chapter Two

Literature review

2.1. Plastics

In early 1600 B.C. human started to use natural rubber and polymerized the rubber into different useful object. Diverse usage and manufacturing of plastics and plastic products began in 1839 when polystyrene (PS) and vulcanized rubber were discovered.(Kehinde 2019) After 1930, plastic was used everywhere, especially in fashion, communication and electrical and automotive industries. Since then mass production of plastic began and it has constantly expanded (Ojumu, Yu, and Solomon 2004).

Nowadays plastics are very dominant and it is utilized by manufacturing industries ranging from pharmaceutical to automobiles. Also it used in routine house hold packaging material, in bottles, cell phones, printer etc. (Muhammad Shamsuddin 2017). They are abundant and important because of their high molecular weight, low reactivity, long durability, and cost efficiency these, make them easily chemically manipulated into different strength and shape (Kumar and Thakur 2017)

Plastics have become a large environmental problem. Because of their long durability and molecular structure it takes hundred to thousand years to degrade plastic (Ojumu, Yu, and Solomon 2004). The plastic residue in landfills causes high environmental impact into the ecosystem. Researchers have conducted many researches for managing plastic waste on earth by finding eco-friendly alternative to plastics (Righi et al. 2016).

Bio-plastics are one of eco-friendly plastics, which are disposed in environment and can easily degrade through the enzymatic actions of microorganisms (Roh, Bauchan, and Huda 2012). The degradation of biodegradable plastics give rise to carbon dioxide, methane, water, biomass, hemic matter and various other natural substances which can be readily eliminated (Muhammad Shamsuddin 2017).

2.1.1. Types of plastic

According to their nature plastics can divide into different categories.

Natural plastics – these are naturally occurring materials that can be said to be plastics because they can be shaped and molded by heat. An example of this is amber, which is a form of fossilized pine tree resin and is often used in jewelry manufacture (Brydson J.A. 1999).

Semi synthetic plastics - these are made from naturally occurring materials that have been modified or changed but mixing other materials with them. An example of this is cellulose acetate, which is a reaction of cellulose fiber and acetic acid and is used to make cinema film (Goodship 2017).

Synthetic plastics - these are materials that are derived from breaking down, or 'cracking' carbon-based materials, usually crude oil, coal or gas, so that their molecular structure changes. This is generally done in petrochemical refineries under heat and pressure, and is the first of the manufacturing processes that is required to produce most of our present day, commonly occurring plastics (Shah et al. 2008).

Synthetic and semi synthetic plastics can be further divided into two other categories. These two categories are defined by the ways in which different plastics react when heated.

Thermoplastics - these are plastics that can be softened and formed using heat, and when cool, will take up the shape that they have been formed into. But if heat is reapplied they will soften again. Examples of thermoplastics are acrylic and styrene, probably the most common plastics found in school workshops (Roh, Bauchan, and Huda 2012).

Thermosetting plastics - these are plastics that soften when heated, and can be molded when soft, and when cool they will set into the molded shape. But if heat is reapplied they will not soften again, they are permanently in the shape that they have been molded into. Why this happens we will look at later (Goodship 2017)**.** Examples of thermosetting plastics are polyester resins used in glass reinforced plastics work, and melamine formaldehyde used in the manufacture of Formica for kitchen work surfaces (Shah et al. 2008).

2.1.2. Demand of plastic

The annual plastic production was increasing in these past decade; in 2008 the annual plastic production was estimated to be 245 million tons globally (Chee et al. 2010). Nowadays, almost 39.6% of overall plastic usage is for packaging, this is followed by building and construction, automotive, electrical and electronics, household materials and agriculture applications at 20.4%, 9.6%, 6.2% 4.1% and 3.4%, respectively. The other 16.7% uses for appliance, mechanical engineering, furniture and medical (Kehinde 2019).

According to data from the association Plastics Europe, world production of plastics amounted 280 million tons in 2011:

- 235 million tons of primary materials (used in the production chain);
- 45 million tons used to produce the coating, welding, spraying, painting and varnishing; this represents an increase of 3.7% of world's production of plastic from the 270 million tons in the year 2010.

Current World Production Rate of Plastics

Globally, plastic production was expected to be 380 million tons in 2018. Since 1950 to 2018, plastics of about 6.3 billion tons have been produced worldwide, 9% and 12% of which have been recovered and burned, respectively (Comăniţă et al. 2016). Plastics of about 5 million tons are yearly used up in UK alone, with only about one-quarter recycled, and the rest landfilled. Annually, approximately 500 billion plastic bags are used out of which an estimated 13 million tons ends up in the ocean, killing around 100,000 marine lives (Goodship 2017).

Future Projection of Production of Plastic

Generally, approximately 311 million tons of plastics were produced in 2014, expected to twofold in about 20-year time and possibly multiply by 2050. International Energy Agency World Energy Outlook in 2015 estimated that, the largest application, plastic packaging (26% of the total volume), is envisaged to have continuous strong growth, which might double within 15 years, with a possibility of fourfold increase by 2050 (Kehinde 2019).

Demand of plastic in Ethiopia

During the period 2009 – 2011, the local plastic manufacturing sub sector has imported on average 67,235 tons of various type plastic polymers of which the largest share (40.58%) is accounted by polyethylene and related polymers followed by polypropylene and related polymers (19.48%) and polyvinyl chloride and related polymers. In 2015, the plastic consumption volume in Ethiopia reached around 172,000 tons. This figure is expected to increase to some 308,000 tons of plastic by 2020 (Ababa 2005).

In Ethiopia waste recycling is carried out by poor and marginalized social groups who resort to scavenging/waste picking for income generation and some even for everyday survival. This is widespread throughout urban areas of the developing world and it is reported that up to 2% of the population in Asian and Latin American cities depend on waste picking to earn their livelihood (Woldegiorgis. 2017).

2.1.3. Effect of plastic on the environment

Plastics are made up of synthetic organic polymers which are widely used in different applications ranging from water bottles, clothing, food packaging, medical supplies, electronic goods, construction materials, etc. and it is vital and adaptable product with different properties, chemical composition and application. At first plastic was assumed to be harmless and inert, however, with time disposal of plastic into the environment has led to diverse associated problem (Clunies-ross 2019).

Currently, plastic wastes are widely environmental burden because of their durability. They are located everywhere and persistence in the environment especially in the aquatic environment. Plastics have several toxic constituents among which are phthalates, poly-fluorinated chemicals, bisphenol, brominated flame retardants and antimony trioxide which can reach out to have adverse effects on environmental and public health (Kehinde 2019).

The world plastic demand is dominated by the thermoplastic polypropylene, polyethylene and polyvinyl chloride. Plastic packaging is easy to carry and use, but plastic is a strong pollution factor. Moreover, a nonrenewable natural resource - oil - is needed for plastic production (almost

4% of world oil consumption is used as raw materials for plastic production), while the finished material is not biodegradable (Comăniţă et al. 2016)**.**

Plastic polymers are not considered toxic, but plastic materials contain some residual monomers. Also, many chemical compounds used in the plastics manufacturing as additives, in particular plasticizers are dangerous to human health and the environment, along with some degradation products that may be released during the plastic life cycle (Proshad et al. 2018). it also facilitate the reduction of greenhouse gas emissions and high concentration of plastic can lead to serious effects for human health and the environment (Ganguly 2019)**.**

Impact and risk caused by plastic production and consumption

Plastics production and waste continue to rise various problems and environmental threats. It is difficult to recycle plastic waste due to its heterogeneity during collection (Proshad et al. 2018). Incineration of plastic waste can be applied for energy recovery, but plastics combustion can generate emissions of toxic and hazardous and gases could be done by thermal degradation which contributes to global warming (Kehinde 2019).

Besides these practices, the recovery of chemicals such as monomers Environmental risk is the result of the interaction between human activities developed in an unsustainable way and the environment (Clunies-ross 2019). Increasing concerns over preserving environmental quality, in particular water quality have stimulated the development of a variety of technologies for reducing the environmental impact of human activities on the nonrenewable and vulnerable resource (Ganguly 2019).

Currently, the density of the plastic waste is almost 100 times higher than 40 years ago. The main impacts and risks caused by plastic waste on aquatic environment consist in: changing habitats of aquatic species change the hydrological system of water and sediments, destruction of plankton and phytoplankton (Brydson J.A. n.d.). Therefore, ocean pollution affects the ecosystem on many levels. Risks caused by discharge of uncontrolled wastes such as plastics in water are presented in the following sections (Comăniţă et al. 2016).

Human health risks due to plastics in the environment

Plastic can affect all underground and surface water bodies, with imprevisible and negatively impacts and risks on wildlife, ecological habitats, health of coastal communities. Plastic particles in surface water columns are photodegradable, becoming increasingly smaller (up to molecular level) (Bergmann, Gutow, and Klages 2015). Toxic substances resulting from plastic degradation (such as bisphenol-A, styrene, phthalates) are then consumed by plankton, thus becoming part of the food chain, reaching humans in the end, which cause are neurotoxic and carcinogenic compounds, can generate disorders to human health (Haight and Antadze 2012).

2.2. Bio-plastic

Biomaterials are like the name indicates they biologically synthesize and catabolized by different organisms and have high applications on biotechnology. They are biodegradable and biocompatible means the easily consumed by microbes and have no toxic effect on the host conferring upon them a considerable advantage with respect to other conventional synthetic products. Bio-plastics are a special type of biomaterial. They are polyesters, produced by a range of microbes, cultured under different nutrient and environmental conditions (Luengo et al. 2003).

A bio-plastic is a plastic that is made partly or wholly from polymers derived from biological sources such as sugar cane, potato starch or the cellulose from trees, straw, and cotton. They are not just one single substance, they comprise of a whole family of materials with properties and applications (Street, Lieberman, and Muhlbauer n.d.).

They are either biodegradable or biobased products or both. Biodegradable plastics are a polymer that breaks down into and converted to methane, water and carbon dioxide due to the activity of microbes. Whereas bio-based they may not biodegradable but they convert smaller fragments by the action of abiotic factors such as UV radiation, oxygen attack, and biological attack (Muhammad Shamsuddin 2017).

Biodegradation: A biological process in which, a polymer breaks into smaller particles with the help of microbial activity and converted into methane, water and carbon dioxide. The mechanism of bio degrade the polymer depends upon the thickness and composition of the material (Gill 2014).

Degradation: The process of disintegration of the polymer into smaller fragments by the action of abiotic factors such as UV radiation, oxygen attack, and biological attack. The most common degradable plastics are polyethylene (Gill 2014).

Bio-based plastics: are plastics derived from natural resources or biomass or biodegradable. They may or may not be biodegradable but recyclable. The mechanical properties are quite similar as those derived from fossil for example, Bio- PVC, bio- PE derived from sugarcane (Mayra 2017).

Bio-based polymers can be categorized in three groups:

1. Polymers extracted directly from biomass e.g. proteins (whey, casein, collagen, soy), lipids (triglycerides), and polysaccharides (cellulose, starch, chitin gums)
2. Polymers synthesized from bio-derived monomers e.g. polylactides
3. Polymers produced by natural or genetically modified organisms e.g. microalgal and bacterial cellulose and other inclusion bodies, e.g. poly(hydroxylalkanoate) (Khosravi-Darani and Bucci 2015).

Compostable plastics: A plastic that have capability to undergo biological decomposition in composite and breaks down into carbon dioxide, water, inorganic compounds and biomass without leaving toxic substances to the atmosphere. The compostable products can also degrade by the mechanism of enzymes. For example PLA is suitable for both methods to degrading completely (Shah et al. 2008).

2.2.1. Advantage and disadvantage of bio-plastics

2.2.1.1. Advantage of bio plastic

The main advantage of bio-plastic products is that they are produced from renewable resources rather than fossil resources. The usage of renewable resources would contribute to a reduction of greenhouse gases emission through the reduced carbon footprint (Shah et al. 2008). Compared to petrochemical plastics, the bio-plastics production can emit about 80% less carbon dioxide. The production of bio-plastics also consumes 65% less energy than the production of petrochemical plastics environmental problems like uncontrolled dumping on land and disposal at sea, and the related emission of toxic substances. However, effective implementations of collection, sorting

and recycling practices and public awareness are also essential to reward the benefits of bio-plastics (Selvamurugan and Sivakumar 2019).

- ✓ ***Potentially a much lower carbon footprint.*** A plastic made from a biological source sequesters the CO2 captured by the plant in the photosynthesis process. If the resulting bio-plastic degrades back into CO2 and water， this sequestration is reversed. But a permanent bio-plastic， made to be similar to polyethylene or other conventional plastics, stores the CO2 forever. Even if the plastic is recycled many times, the CO2 initially taken from the atmosphere remains sequestered (Shah et al. 2008).
- ✓ ***Lower energy costs in manufacturing***. Plastics are made from ~4% of the oil that the world uses every year. With oil scarcity the manufacture of plastics becomes increasingly exposed to fluctuating prices (Chen 2014).
- ✓ ***Do not use scarce crude oil***. In contrast, each kilogram of plastic typically requires 20 kilowatt hours of energy to manufacture, more than the amount needed to make the same weight of steel. Almost all this comes from fossil sources(Brandl et al. 1990).
- ✓ ***Reduction in litter and improved compostability from using biodegradable bioplastics.*** Plastic single use shopping bags are the most obvious example of how plastics can pollute the environment with huge and unsightly persistence. A large fraction of the litter in our oceans is of disposable plastic bags. Cities and countries around the world are taking action against the litter, sometimes by banning non-degradable plastic bags entirely (Hill n.d.).

2.2.1.2. Disadvantage of bio-plastic

Bio-plastics have many significant advantages but uncontrolled and improper disposal of the bio-plastic wastes are also contributing to the problems like littering and, soil and water pollution (Kumar and Thakur 2017). Similar to conventional plastics, the bio-plastic wastes are littering also harmful to wildlife the disposal of bio-plastic wastes into a landfill may contribute to the greenhouse gases emission. The other disadvantage of bio-plastic is their high manufacturing cost .At last, the cultivation of crops for manufacturing bio-plastics can create competition on cultivable land for food production (Selvamurugan and Sivakumar 2019).

2.2.2. Demand of bio- plastic

At present, bio-plastics represent about 1% of the more than 359 million tonnes of plastic produced annually. But the demand of bio-plastic is increasing, and with more sophisticated biopolymers, applications, and products emerging, the market for bioplastics is continuously rising and expanding. According to the latest market data compiled by European Bioplastics in cooperation with the research institute novan Institute, global bioplastics production capacity is set to increase from around 2.11 million tonnes in 2019 to approximately 2.43 million tonnes in 2024 (Bioplastics 2020).

New and innovative biopolymers, such as bio-based PP (polypropylene) and PHAs (polyhydroxyalkanoates) show the highest relative growth rates. In 2019, biobased PP entered the market on a commercial scale with a strong growth potential due to the widespread application of PP in a wide range of sectors. These polyesters are 100 percent bio-based and biodegradable, and feature a wide array of physical and mechanical properties depending on their chemical composition (Haight and Antadze 2012).

Bio-based, non-biodegradable plastics altogether, including also the drop-in solutions bio-based PE (polyethylene) and bio-based PET (polyethylene terephthalate), as well as bio-based PA (polyamides), currently make up for over 44 percent (almost 1 million tonnes) of the global bioplastics production capacities (Williams et al. 2012).

The production of bio-based PE is predicted to continue to grow, as new capacities are planned to come online in Europe in the coming years. Intentions to increase production capacities for bio-based PET, however, have not been nearly realized at the rate predicted in previous years but actually declined. Instead, the focus has shifted to the development of PEF (polyethylene furanoate), a new polymer that is expected to enter the market in 2023. PEF is comparable to PET but 100 percent bio-based and is said to feature superior barrier and thermal properties, making it an ideal material for the packaging of drinks, food and non-food products (Gill 2014).

Biodegradable plastics altogether, including PLA, PHA, starch blends and others, account for over 55.5 percent (over 1 million tonnes) of the global bio-plastics production capacities. The

production of biodegradable plastics is expected to increase to 1,33 million in 2024 especially due to PHA's significant growth rates (Bioplastics 2020).

Bioplastics are used in an increasing number of markets, from packaging, catering products, consumer electronics, automotive, agriculture/horticulture and toys to textiles and a number of other segments. Packaging remains the largest application for bio-plastics with more than 53 percent (1.14 million tonnes) of the total bioplastics market in 2019 (Gill 2014).

2.2.3. Types of bio-plastic

Bio-plastics are partly bio-based, biodegradable or both. Generally, the family of bio-plastics is approximately divided into three main groups :

1. Bio-based or partly bio-based non-biodegradable plastics are such as bio-based PE, PET and bio-based technical performance polymers such as PTT.
2. Plastics those are both bio-based and biodegradable, such as PLA and PHA.
3. Plastics that are based on fossil resources and are biodegradable, such as PBAT (Chen 2014).

Commonly used types of bioplastics are based on cellulose, starch, glucose, and oil. Specific techniques are then employed to convert these feedstock into thermoplastic starch, polylactic acid, poly-3-hydroxybutyrate, polyamide 11 and bio polyethylene (Kumar and Thakur 2017).

1. Thermoplastic Starch

Thermoplastic starch is the most significant and widely used bioplastic. Flexibility and plasticizer such as sorbitol and glycerine are added to process the starch. Thermoplastic starch generally represents just one component of which starch-based bioplastics are formed (Zaharadeen and Sirajo 2018). The second part of the blends consists of water repellent and biologically degradable polymers like polyester, polyesteramides, polyester urethanes or polyvinylalcohol. Throughout the melting process, the water-soluble, disperse starch phase and the water-insoluble, plastic are bond together to form a waterproof starch plastic. Applications of thermoplastic starch are bags, yogurt tubs, cups, plant pots, cutlery, diaper foil, coated paper, and cardboard. Most of the starch derives from crops such as potatoes or corn (Williams et al. 2012).

2. Polycaprolactones (PCL)

PCL is biodegradable thermoplastic polyester synthesized by chemical conversion of crude oil. This biopolymer has good water, oil, solvent, and chlorine resistance, with a low melting point, glass transition temperature, and low viscosity, and processable using conventional melt blending technologies. PCL is being investigated for its use in biomedical utensils, pharmaceutical controlled release systems, and in biodegradable packaging (Mayra 2017). PLA is a thermoplastic biopolyester produced from L-lactic acid, which is usually produced from the fermentation of corn starch, and can be biodegraded by some bacteria (e.g. *Alcaligenes faecalis*) and fungi (Khosravi-Darani and Bucci 2015).

3. PLA

PLA (polylactic acid or polylactide) is characteristics resemble conventional fossil fuel-based plastics such as polyethylene (PE), polypropylene (PP) and polyethylene terephthalate (PET). It can easily be processed on manufacturing facilities that already exists for the production of common petrochemical-based plastics – no further industrial investments are required. PLA is mostly produced by the fermentation of starch from crops, commonly corn, wheat or sugarcane into lactic acid followed by subsequent polymerization (Kumar and Thakur 2017).

Its blends have a wide range of applications including computer and mobile phone casings, biodegradable medical implants, foil, molds, tins, cups, bottles and packaging devices (Street, Lieberman, and Muhlbauer n.d.). PLA and PLA copolymer plastics have already been used successfully for medical and pharmaceutical purposes such as the production of screws, nails, plates, and implants that can be resorbed by the body. Also, the use of PLA – nanoparticles as drug carriers or MRI contrast agents is currently investigated (Zaharadeen and Sirajo 2018).

PLA is a very versatile bioplastic. By varying composition and quality it can be designed to biodegrade quickly or last for years. Additionally, PLA possesses extraordinary stability, as well as extremely high transparency (Ghuttora 2016).

However, PLA also has a significant disadvantage. The plastic softens at a temperature of about 60 degrees Celsius, which limits its application for the production of packages for hot drinks and

food. Copolymerization with heat resistant polymers and the addition of fillers overcome these drawbacks (Street, Lieberman, and Muhlbauer n.d.).

4. PHB

Polyesters are one of the most important classes of thermoplastic polymers. They can be formed from the reaction of a di-acid or acid anhydride and a diol with the elimination of water, or by ring-opening polymerization of cyclic (di-) esters (Griebel, Smith, and Merrick 1968). According to the composition of their main chain, polyesters are classified as aliphatic, semi-aromatic and aromatic. Aromatic reactants improve the hardness, rigidity, and heat resistance, whereas aliphatic acids and diols increase the flexibility, lower the melting or softening point and improve the processability (Fonseca, Sá, and Lima-neto 2016).

PHB is polyesters, made from diacid and diol components. Poly-3-hydroxybutyrate is generally produced by bacteria processing glucose or starch. These polyesters are known for their biocompatibility and biodegradability, and their capability to be blended with various other commercial polymers (Fonseca, Sá, and Lima-neto 2016). This polymer is often blended with other resins to improve their processing and end use properties. It can also be blended with starch to lower cost and to increase the biodegradability. Its characteristics are similar to those of the fossil crude oil-derived plastic polypropylene (Prados and Maicas 2016)

PHB is distinguished from most other currently available biodegradable plastics primarily by its physical characteristics such as the insolubility in water and its resistance to hydrolytic degradation (Asrar et al. 2002). It produces a transparent film at a melting point of 175 °C and is biodegradable without residue. PHB is probably the most common type of a substance class termed as polyhydroxyalkanoates, but also many other polymers of this polyester class are produced by a variety of organisms. The application of PHB blends varies from the fabrication of glues to hard rubber (Gu 1998).

5. PA 11

A biopolymer derived from natural oil is polyamide 11 (PA 11). This polyamide bioplastic is also known under the trade name Rilsan. Although, PA 11 derives from renewable resources (castor beans) it is not biodegradable (Chen 2014).

It is used in high-performance applications such as automotive fuel lines, pneumatic airbrake tubing, electrical anti-termite cable sheathing, oil, and gas flexible pipes and control fluid umbilical, sports shoes, electronic device components, and catheters (Brandl et al. 1990).

6. PE

Polyethylene (PE) is generally known as a fossil-based polymer. However, it can simply be converted form bioethanol (by dehydration) which is produced on a large scale by fermentation of agricultural feedstocks such as sugar cane or corn. Bio-polyethylene is chemically and physically identical to traditional polyethylene – it does not biodegrade but can be recycled (Zaharadeen and Sirajo 2018).

2.3. Poly-3-hydroxybutyrate (PHB)

PHB is natural polyester, synthesized by a wide range of organisms, particularly by some bacterial strains. They have very interesting properties such as being biocompatible and totally and rapidly biodegraded to carbon dioxide and water by a large number of microorganisms (Nonato, Mantelatto, and Rossell 2001).

PHB can be compounded to thermoplastic resins that have physicochemical and mechanical properties similar to petrochemical-based polymers, e.g., polyethylene and polypropylene, and standard plastic-engineering molding procedures can be applied to them (Sabarinathan et al. 2018).

The homopolymer PHB is itself quite brittle, a fact that reduces the range of its applications. Nevertheless, nucleating agents, plasticizers and other additives allow PHB to be used in several processes, especially injection molding for pots, caps and other single, hard pieces. Other uses, such as molds for metal casting, prosthetics, paper, and granule coating, composites (starch, cellulose) are under development (Nonato, Mantelatto, and Rossell 2001).

2.3.1. Synthesis of PHB

PHB is produced in the cells of microorganisms, as product of microbial secondary metabolism, usually in conditions when the cells are exposed to nutrient stress or in an uncomfortable environment such as carbon-excessive with limited nutrients which is possible in both gram-positive and gram-negative bacteria (Griebel, Smith, and Merrick 1968).

PHB is synthesis by different method. There are three main ways of synthesizing PHB materials, the first approach being through ring opening polymerization (ROP) of β-butyrolactone (BL). Another approach is through the use of natural/transgenic plants (Asrar et al. 2002).

The third approach of obtaining PHB materials is through bacterial fermentation. When under optimal fermentation conditions, it is possible that more than 90% of the cells dry weight may be comprised of PHB materials. This approach is the most commonly used in the synthesis of PHB (Figure 2.1). PHB synthesis relies on a central carbon metabolite from acetyl-CoA through a sequence of three enzymatic reactions: (Fournet, Mcdonald, and Mojicevic 2020)

I. The reversible condensation of two acetyl-CoA moieties forming acetoacetyl-CoA, catalyzed by β-ketothiolase (PhaA);
II. Acetoacetyl-CoA reduction to (R)-3-hydroxybutyryl-CoA by an acetoacetyl-CoA reductase (PhaB);
III. The polymerization of (R)-3-hydroxybutyryl-CoA catalyzed by the enzyme PHB synthase (PhbC gene) to produce PHB. The biosynthetic pathway of PHB from acetyl-CoA.

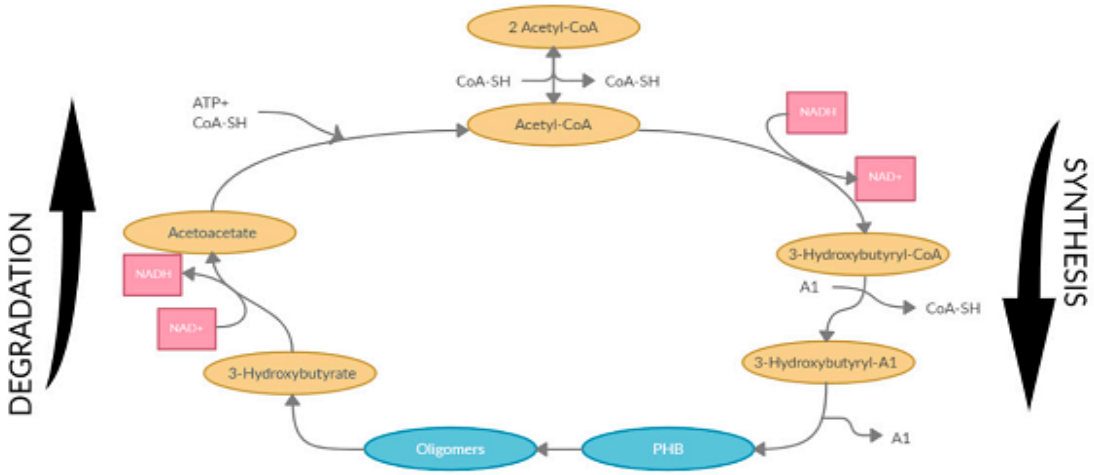


Figure 2. 1:- PHB synthesis and degradation process

Source- (Fournet, Mcdonald, and Mojicevic 2020)

2.3.2. Properties of Poly-3-hydroxybutyrate (PHB)

Poly-hydroxybutyrate (PHB) is biodegradable thermoplastic polyester produced by bacterial fermentation, which easily biodegradable. PHB is characterized by having a methyl functional group (CH_3) and an ester linkage group (−COOR), it is these functional groups that are responsible for the materials thermoplastic, hydrophobic, high crystallinity, and brittle characteristics(Fournet, Mcdonald, and Mojicevic 2020). PHB has a very high potential for manufacturing uses due to its high crystallinity (50-70%), excellent gas barrier (water vapor permeability around 560 g. µm/m^2/day) (Pachekoski et al. 2009).

Physical properties are similar to those of polypropylene, which is Semi-rigid, translucent, good chemical resistance, tough, good fatigue resistance, integral hinge property, good heat resistance. PHB has an elasticity modulus of 3 GPa and tensile strength at break of 25 MPa. However, PHB has some disadvantages, such as high fragility, showing 3-5% tensile elongation at break, and low thermal stability above its melting point, with marked degradation starting at 200 °C (Thapa et al. 2019).

PHB is a partially crystalline material with a high melting temperature, and a high degree of crystallinity. It is not water-soluble but is 100 % biodegradable and has optical activity, piezoelectricity, and good barrier properties. Young's modulus and tensile strength of PHB are comparable to those reported for PP, but the elongation at break (5±10 %) is significantly lower (Thapa et al. 2019). Thus, stiffness of PHB is attributed to cracks within the PHB spherulites that form under conditions of non-externally applied stress. Long storage at room temperature causes brittleness to increase. PHB does not have any residues of catalysts because the sources of production are microorganisms. It is isotactic and does not include chain branching. So it flows easily during processing (Khosravi-Darani and Bucci 2015).

The figure below indicates the molecular structure of PHB. There are two main groups of PHBs, namely, short chain length (scl)-PHBs and medium chain length (mcl)-PHBs. Scl-PHBs consist of 3-5 carbon atoms and mcl-PHBs contain 6-14 carbon atoms. They are also identified as homo-polymers and hetero-polymers depending on whether one or more than one type of hydroxylbutyrate units is found as monomer units in the polymer (Righi et al. 2016). Scl-PHAs have high crystallinity with a melting point of 180˚C and an elongation to break of 5%. However mcl-PHBs and their copolymers have low crystallinity (20% - 40%) and do not break easily

(extension to break of 300% - 450%). They behave as elastomers and their composition can be manipulated for a range of application. The physical and chemical properties of PHBs are depends on type of micro-organism, media ingredients, and fermentation conditions, modes of fermentation and recovery methods (Sukan, Roy, and Keshavarz 2014).

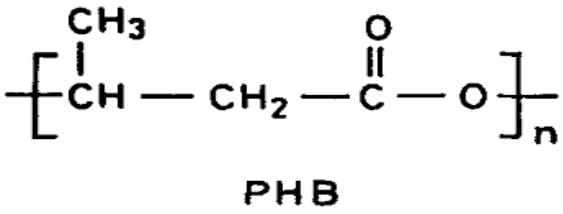


Figure 2. 2:- Molecular structure of PHB

2.4. Parameters affecting PHB Production

PHBs vary in their physical and chemical characteristics owing to their varied monomer content. Factors affecting the monomer content include:

2.4.1. Type of microorganisms

PHB production process mainly depends on the type of microorganism used for the process. There are two major groups of bacteria that have the potential to utilize raw substrates for the production of PHB. These bacteria grow based on the culture conditions required for PHB synthesis (Roh, Bauchan, and Huda 2012).

As for the first group, microbes belong to this group requires the limitation of essential nutrients such as nitrogen, magnesium, sulphur and phosphorus for the synthesizing of PHB from an excess carbon source. Bacteria that fall in this group include *bacillus spp.*, *R. eutropha*, *Protomonas oleovorans* and *Protomonas extorquens* (Petrasovits et al. 2007).

The second group of bacteria belongs to those that do not require limitation of nutrient for PHB synthesis. For these particular types of bacteria, biopolymer is accumulated during the growth phase of bacteria and microbes included *Alcaligenes latus* and recombinant *E. coli*. It is nearly 300 bacteria have been identified to have the ability of producing PHB bioplastic (Morgan and Luginbuhl 1968).

Both Gram positive and gram-negative bacteria were earlier reported to produce PHB. Gram negative bacteria are reported to produce quantity of PHB suitable for commercial production. However, these gram-negative bacterial cell walls possess lipopolysaccharides (LPS) as cell wall constituents which may limit PHB from these sources for biomedical applications. The genus *Bacillus* is gram positive and seems to be good candidate for the production of PHA/PHB for biomedical applications. Such gram positive bacteria lack the LPS and intensive investigations are required for their exploitation in PHB production (Babruwad et al. 2015).

2.4.2. Media ingredients

In fermentation process, the main carbon supply is sugar sources. But it is very costly to use raw sugar for industrial scale. Some of the available waste that has the potential for substrates replacement includes sugarcane bagasse, oil palm front juice, soya flour, molasses, rice branch, fruit peels and pulp (Petrasovits et al. 2007).

In addition to the carbon source type, concentrations of N and P will directly affect the amount and efficiency of PHB synthesis. Numerous studies have demonstrated that higher PHB levels are available at limited nitrogen or phosphorus concentrations (Science 2018).

2.4.3. Fermentation conditions

Fermentation condition are the factors the affect the fermentation process, these are temperature, pH and time.

Effect of Temperature on fermentation

The effect of different temperatures was studied for the PHB production by different strain of microorganisms. Incubation temperature plays a pivotal role in the metabolic process of an organism. The fermentation temperature in the range of 28-45ºC is superlative for the production of PHB. Optimum temperature for the production of PHB corresponds to the growth temperature of particular microorganism. Commonly, PHB is produced by Bacillus sp. in the range of 30-38°C. As the temperature increased, the rate of physiological processes also increased which enhanced the growth of microorganism to a certain limit, after that it started decreasing (Rehman and Aftab 2016).

Effect of pH on fermentation

Typically, metabolic processes are highly susceptible to mild changes in pH. The cell dry weights for each parameter were calculated using the formula corresponding to measure the accumulation of PHB from the biomass. And also, the pH change indicates on concentration substrate used for the process. The selected bacteria growth range is 7-7.6 (Yogesh et al. 2014).

Effect of Time on fermentation

The influence of incubation period was studied for the optimization of PHB production process. It has been reported that bacteria able to produce PHB are divided into two categories. The first category involves those which produce PHB during stationary phase when N, P, Mg and oxygen are limited and carbon source is in surplus while, second category involves PHB accumulation during the growth phase. The physiological features of the microorganism are imperative for the production of bioplastic (Rehman and Aftab 2016). In different studies, the fermentation medium was incubated for different time intervals. The cell mass increased steadily, leading to a maximum cell density then it followed by a gradual decline. During the decline phase when microorganisms undergo scarcity of food due to the absence of energy supply, they release definite PHB depolymerase which hydrolyze the polymer to water soluble monomers or oligomeric esters. The hydrolyzed products are taken up by the cells and metabolized. The incubation period is depending on type of microorganisms used for the process, the incubation period of *bacillus spp.* is between (12-75hr) (Sabarinathan et al. 2018).

2.4.4. Modes of fermentation

A number of different fermentation processes can be used to obtain PHBs. These include: discontinuous processes such as batch culture, fed-batch culture, and repeated fed-batch culture and continuous processes such as continuous fed-batch systems using gaseous substrates, one-stage chemostat process, two-stage chemostat process, and multi-stage chemostat process in continuously stirred tank reactor (CSTR)-bioreactor cascades (González-García et al. 2011).

In the batch fermentation, the concentration of the nitrogen and carbon sources is restricted by the physiological preconditions of the production strain. Due to the low productivity associated with

batch culture processes, a simple 'repeated batch' approach has recently been evaluated to enhance the volumetric productivity (Sabarinathan et al. 2018). The report emphasized that the volumetric productivity was successfully increased and displayed a major advantage over simple batch processes in the production of PHB materials. This new approach also eliminates the non-productive time required for the cleaning, refilling, and sterilization of the bioreactor between individual batches (Fournet, Mcdonald, and Mojicevic 2020).

In the fed-batch systems, the addition of the precursor substrate is usually feed through pulses when the concentration falls below a set value. However, without a proper feeding strategy this culture would not result in a much higher productivity than observed for batch culture processes. A number of reports have determined optimal feeding rates of carbon and nitrogen sources based on the material being processed. In the case of PHB, one example is when the nitrogen and carbon sources are refers into the process according to the consumption of the biomass up until a PHA-poor biomass is achieved] (Hamieh, Olama, and Holail 2013). One of the main challenges associated with fed-batch fermentation is the control over the feeding substrate concentration to allow an ideal range in terms of limiting and inhibiting levels. Therefore, the substrate feeding approach used for the successful production of a high percentage of accumulated PHB is essential (Joshua Dhivyan Gnanasekhar 2012).

The continuous fermentation processes are recognized to operate under steady, controlled conditions, where factors that can affect the process such as the pH, nutrient supply, and concentration of the product are kept constant. The constant conditions required to generate high active biomass, however result in only small fraction of accumulated material. This is due to the fact that PHB production depends on the physiological stress response placed on the microorganisms when essential nutrients are limited or depleted (Fournet, Mcdonald, and Mojicevic 2020). In order to produce PHB, a cell growth phase occurs first where the bacteria are fed with essential nutrients to the level needed for PHB production and then nutrients are depleted after a certain time to trigger secondary metabolism and encourage polymer biosynthesis. hence, it is impossible to complete PHB production using a continuous one-step process at sufficient productivity when conditions are kept constant (Joshua Dhivyan Gnanasekhar 2012).

Therefore, continuous two-stage and multi-stage fermentation processes are better suited for this purpose and hence, this is currently the most common method of producing PHB materials, allowing for stable processing conditions and higher productivity (Hamieh, Olama, and Holail 2013). Continuous fermentation processes can facilitate high productivity of PHB materials, including the production from cultures of high specific growth rates, however, the execution of such cultivation in industry for PHB production has been limited due to the continuous processes being prone to microbial contamination and productivity interruptions leading to financial losses (Fournet, Mcdonald, and Mojicevic 2020).

2.5. Processing methods for PHB

Biosynthetic polymers may be produced through microbial or plant route. Currently, microbes are considered to be the major source for the production of PHBs, although PHBs can also be produced in plants. Microbial biosynthesis of bioplastics requires technical viability and feasibility for a challenging production. While an extensive range of PHAs are produced with varying characterize low productivity and high costs compared to the traditional mineral-based plastics (Mikkili et al. 2014a).

2.5.1. Selection of Microorganisms

Diversities of Gram-positive and Gram-negative bacteria (over 300 species, examples of which include Pseudomonas sp., Bacillus sp. and Methylobacterium sp.) carry the metabolic ability to biosynthesis. PHBs and accumulate them in their cytoplasm as carbon and energy sources in the shape of granules. Biosynthesis of PHBs by bacteria is usually in response to stress conditions such as nutrient (e.g. nitrogen or phosphate) limitation with an excess carbon source. However, some bacteria such as A. vinelandii UWD and A. eutrophus, A. latus and a mutant Azotobacter vinelandii are able to accumulate PHAs under non-limiting conditions (Sukan, Roy, and Keshavarz 2014).

Among these bacterias, bacillus sp. is selected because it has high efficiency, high ability, and lacks toxicity to extract PHB (Hassan et al. 2016).

Bacillus subtilis is started to be paid attention as the potential producer of PHB after its performance in production of metabolites, bioremediation and generation of bioenergy. It is formerly recognised

in the industrial scale production of amino acids, recombinant proteins and fine chemicals but never been tried for the biopolymers production (Morgan and Luginbuhl 1968).

Bacillus subtilis also known as grass bacilli are Gram-positive bacteria and well-known bacteria species that are capable to grow within many environments. Their capabilities that can be isolated from many environments, making them seems like they are broadly adapted to grow in various environmental condition (Mikkili et al. 2014b).

Bacillus subtilis, like other members of bacillus species, may form a highly resistant dormant endospores when undergo nutrient deprivation and other environmental stresses. It has been reported that among potential Bacillus spp., the PHB yield vary from 11% to 69% w/w of dry cell weight (Morgan and Luginbuhl 1968).

2.5.2. Media

The choice of media is important not only to supply optimal conditions for the production of a variety of PHBs in different bacteria but also to do so with high volumetric productivity to provide a final product that is economically competitive with the traditional plastics. Different cost-effective sources used for fermentation include media containing molasses, corn steep liquor, whey, wheat, and rice bran starch and starchy wastewaters, effluents from the olive mill and palm oil mill, activated sludge and swine waste. The choice of media, partly, depends on whether the microorganism is wild type or recombinant and whether it needs nutrient limiting conditions (Keshavarz and Roy 2010).

The selection of media depends on the type of microorganisms, productivity, and economically visibility. Among those media source bagasse chosen for this research, because of different reasons like, during the hydrolysis of the bagasse, cellulose conversion is greater than 90% that is better than the others. Pretreatment for the bagasse hydrolysis is simpler than the other source since it required less energy and less cost. The availability of bagasse is high when compared to other cellulose sources (Tekle 2019).

Cellulose is a major structural component of cell walls, and it provides mechanical strength and chemical stability to plants. Hemicellulose is a copolymer of different xylose (C5) and glucose

(C6 sugars that also exist in the plant cell wall. Lignin is polymer of aromatic compounds produced through a biosynthetic process and forms a protective layer for the plant walls.

Cellulose is the β-1, 4-polyacetal of cellobiose (4-O-β-D-glucopyranosyl-D-glucose). Cellulose is more commonly considered as a polymer of glucose because cellobiose consists of two molecules of glucose. The chemical formula of cellulose is (C6H10O5) n and the structure of one chain of the polymer:

Many properties of cellulose depend on its degree of polymerization (DP), i.e. the number of glucose units that make up one polymer molecule. For instance, cellulose from wood pulp has a DP between 300 and 1700. The nature of bond between the glucose molecules (β-1,4 glucosidic) allows the polymer to be arranged in long straight chains. Cellulose is found in both crystalline and amorphous structure. The coalescence of several polymer chains leads to the formation of micro fibrils, which in turn are united to form fibrils. In this way cellulose can obtain a crystalline structure (Mikkili et al. 2014b).

2.5.3. Fermentation

Many PHB fermentations are carried out in two stages. The aim is to produce a high cell density culture in the first stage (growth) and then to increase the concentration of PHBs during the second stage that is usually a nutrient-limited fermentation. Fermentation conditions depend on the demands of the microbe and often a temperature, stirrer speeds, dissolved oxygen tension and pH (González-García et al. 2011).

Two types of fermentation processes i.e., batch and fed-batch fermentations used for production and multiplication of these bacterial strains. Fed-batch cultivation is, however, more efficient than batch cultivation as very high cell concentration can be achieved in limited time and has an additional benefit of controlling the medium composition by substrate inhibition. A two-stage cultivation process where firstly, the cells are allowed to multiply to a predetermined concentration in a medium having excess of nutrients followed by their transfer to the second stage medium with limited nutrient supply exhausting their carbon reserve to make PHB (Kumar and Thakur 2017).

Fed-batch fermentation process chooses because of it is more efficient and very high cell concentration can be achieved in limited time and has an additional benefit of controlling the medium composition by substrate inhibition (Sukan, Roy, and Keshavarz 2014)

2.5.4. Recovery

Recovery is the last process in the PHB process. Different recovery techniques are used for recovery, such as the organic solvent extraction, cell disruption followed by aqueous extraction, enzymatic digestion extraction using supercritical CO_2. None of these recovery procedures possess all the necessary requirements for an efficient and economical large-scale process. The major drawbacks are cost, safety, and scalability (Keshavarz and Roy 2010).

1. **Organic solvent extraction**

PHB recovered using organic solvent like chloroform, dichloromethane (methylene chloride), polychlorinated ethane (1,2-dichloroethane, 1,1,2-trichloroethane, 1,1,2,2-tetrachloroethane). These solvents perform well for extraction of PHB, because they only dissolve lipid part of the cell that can easily be removed prior to the intrinsic PHB extraction. The other solvents like, ether, hexane, and acetone can only be applied for precipitation of PHB (Koller and Niebelschütz 2013).

2. **Supercritical fluid extraction**

Supercritical fluid extraction is more and more implemented for efficient extraction of numerous high value products from various natural matrices; also, biochemical reactions can be accomplished in the environment of supercritical fluids. In this context, supercritical CO_2 (sCO_2) is regarded as a very convenient and safe solvent due to the fact that it has high solubility for numerous compounds with a certain hydrophobicity, and it evaporates completely and fast right after the extraction without any remaining residues, hence, no subsequent drying of the product is needed. Therefore, it was reasonable to consider sCO_2 also as a potential solvent for PHA extraction. These findings are contradictory to the conclusions made by other research groups, where sCO_2 turned out to be an efficient solvent for lipids and other hydrophobic cellular components, but not for PHB nor for poly(3-hydroxyoctanoate) (Koller and Niebelschütz 2013).

2.6. Application of PHB

These biopolymers have a Wide range of applications in fields such as

- ✓ Biomedical: - sutures, supports of tissue cultures for implants, dressing parts of bones and replanted veins, engineering of heart valves and pins
- ✓ Pharmacy: - encapsulation of medicine for controlled release of drugs
- ✓ Food packaging :- Food packaging is a main step of the food chain, the purpose of which is mechanical support, transition, extension of shelf life, and preservation of food (Khosravi-Darani and Bucci 2015).
- ✓ Agriculture: - encapsulation of fertilizer
- ✓ Veterinary: - encapsulation of veterinary medicinal product
- ✓ Environmental: - bags, bottles, disposable items, items of personal hygiene, films of involvement, degradable diapers and remediation of areas affected by oil spills
- ✓ Industrial:- recovery of oligomers and monomers for new use in the synthesis of polymers (Rahnama et al. 2012).

Chapter Three

Materials and methods

3.1. Materials

3.1.1. Raw materials

Fresh bagasse was obtained from Wonji Shewa Sugar Company in February 2020, by using local transportation. The collected bagasse was dried for 2 weeks by using sunlight.

The strain of bacillus subtilis was collected from Ethiopian Institution of Biodiversity around British council near Megenagna, Addis Ababa in September 2020.

3.1.2. Chemicals

The chemicals used were

- Mineral salts medium (MSM) [composition (g/L): Urea (1.0), Yeast extract (0.16), KH_2PO_4 (1.52), Na_2HPO_4 (4.0), $MgSO_4 \cdot 7H2O$ (0.52), $CaCl_2$ (0.02), Glucose (40), and trace element solution 0.1 ml]
- Trace element contained ($ZnSO_4 \cdot 7H_2O$ (0.13), $FeSO_4 \cdot 7H_2O$ (0.02) and H_3BO_3 (0.06)).
- Nutrient broth (1% lactose,0.02$MgSO_4$,0.01%NaCl, 0.05%KH_2PO_4, 0,25% Peptone and 0.25% yeast extract)
- Sodium hypochlorite
- Acetone
- Methanol
- Chloroform
- Hydrochloric acid (1N)
- Sodium hydroxide
- Sulfuric acid
- Distilled water

3.1.3. Equipment

The equipment used were: -

- Beaker
- Pipette
- Plates
- Loop
- Measuring cylinder
- Digital balance
- pH meter
- Incubator
- Autoclave
- Orbital shaker
- Freezer dryer
- centrifuge
- UV-Spectrophotometer
- FTIR
- Compound microscope

3.2. Method

3.2.1. Experimental flow sheet

The experimental setup was extraction of PHB from sugar cane bagasse using bacillus subtilis. The diagram indicates each chemical step in the extraction process.

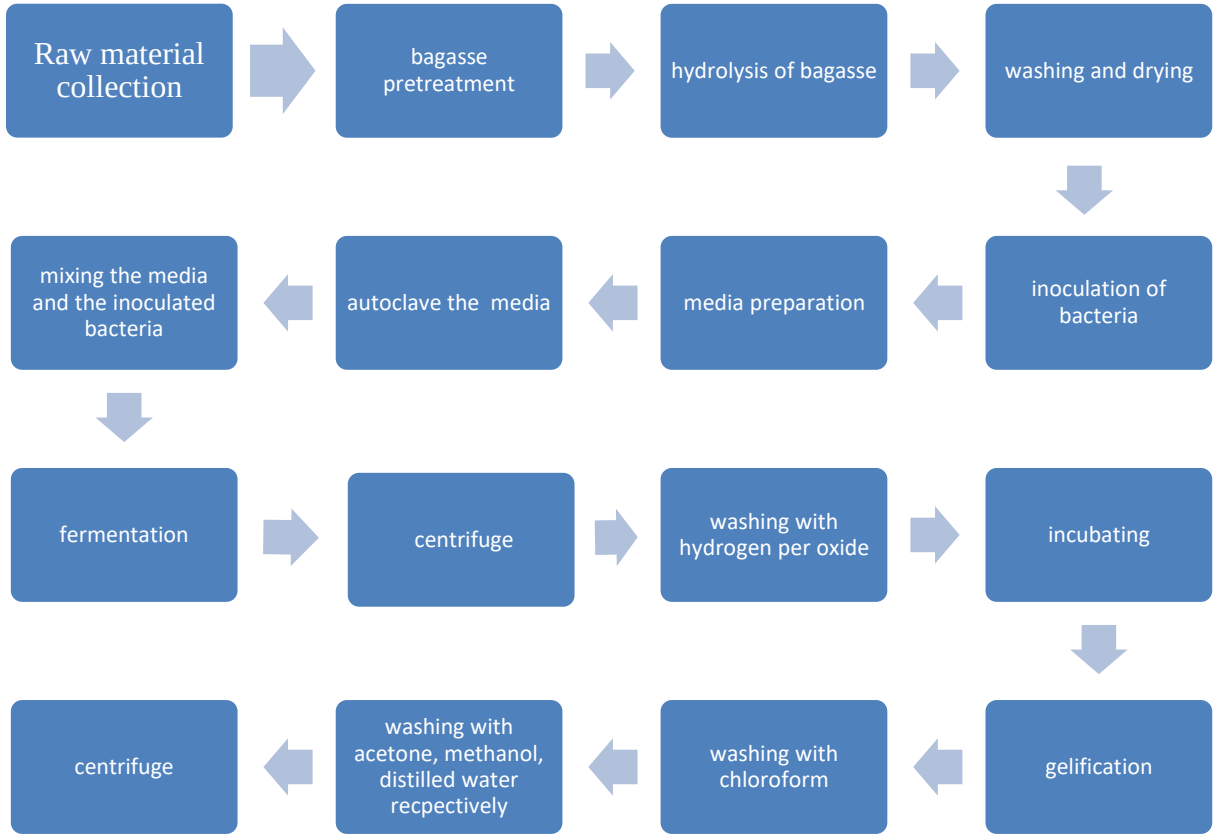


Figure 3: 1:- Experimental flow sheet

3.2.2. Treatment of bagasse

3.2.2.1. Pretreatment of sugar cane bagasse

The sugarcane bagasse collected from Wonji Shewa sugar factory was dried on open air for one week. The dried bagasse was crushed and sieved to less than 0.1mm diameter. The fine sugarcane bagasse was soaked in 1% concentration of H_2SO_4 in 1:10 w/v solid liquid ratio at 121^0C for 15min. It was then washed, filtered and dried. The purpose was to remove waste and pretreat the bagasse and these pretreatment methods are not that much efficient compared to the other techniques to extract glucose but this method is better because the hydrolyzed glucose is suitable

for bacterial growth. Using other methods may cause un suitable situation for bacterial growth (Dussán et al. 2014).

The material was further treated with 1.5% sodium hydroxide (NaOH) in 1:20 w/v solid-liquid ratio at 10^0C for 1hr. The purpose was to remove the hemicellulose and further pretreatment. It was then washed with distilled water and filtered. and dried in open air (Dussán et al. 2014).

3.2.2.2. Hydrolysis of cellulose

The pretreated cellulose–lignin hydrolyzed with 2% dilute sulfuric acid for 15 min at temperature of 145^0C. After that, it was washed with distilled water until the pH regulated then filtered and dried. In this step the dilute acid decomposes the cellulose-lignin into its monomers. The extracted sample tested by using benedict method. This test conducted to identify the amount of glucose extracted from sugar cane bagasse (Dussán et al. 2014).

3.3.2. Inoculation of bacteria

The strain of Bacillus subtilis gathered from Ethiopian Institute of Biodiversity was inactive when it was collected. So, to activate the bacteria, it was inoculated into nutrient agar (composition:0.5% peptone, 0.3% yeast extract, 0.3% beef extract, 1.5% agar, 0.5% NaCl) for 24 hrs. After 24 hours the activate bacteria transfer into nutrient broth (composition: 1% lactose, 0.02% MgSO4, 0.01% NaCl, 0.05% KH2PO4, 0.25% Peptone and 0.25% yeast extract) to create suitable environment for the bacteria duplicate and multiply then, it was incubated for 24hr (Getachew and Woldesenbet 2016b).

3.3.3. Fermentation

The fermentation process was started by preparing the mineral salt media. The media contain: (0.5g Urea, 0.08g Yeast extract, 0.76g KH_2PO_4, 2g NaOH, 0.26g $MgSO_4{\cdot}7H_2O$, 0.01g $CaCl_2$, 20g extracted Glucose from sugarcane bagasse, and 0.05ml trace element solution). The trace element solution contained (0.06g $ZnSO4{\cdot}7H_2O$, 0.01g $FeSO4{\cdot}7H_2O$ and 0.03g H_3BO_3). To prepare the media Excess amount of glucose and limited amount nutrients was used. The glucose and the trace element solution were autoclaved separately, to avoid reaction at high temperature. Then, 0.05ml trace element solution added to 500 ml glucose solution (Getachew and Woldesenbet 2016a).

From 24-hour cultured bacillus subtilis one ml was taken and added to one liter of the prepared mineral salt media and incubated in shaker incubator at 150 rpm and different temperature, time and pH. Bacillus subtilis is aerobic bacteria so the fermentation condition was aerobic fermentation.

3.3.4. Extraction of PHB

After fermentation the sample was extracted using organic solvent extraction method. Twenty ml of sample was taken from each experimental run and it was centrifuged at 5500 rpm for 25 min. Then, the supernatant and white slurry was formed. After that, the supernatant was removed and the white slurry on the pellet was mixed with sodium hypochlorite and it was incubated at 30 °C for 2 hr (Thapa et al. 2019). The mixture of the product and sodium hypochlorite was centrifuged at 5000 rpm for 15 min. Then, it washed with distilled water, acetone and methanol respectively. The formed product was treated with 5 mL boiled chloroform and kept at 4 °C for overnight. The purpose of this organic solvent extraction disrupts the cell and extracts PHB.

3.4. Characterization method

3.4.1. Benedict test

After the hydrolysis, cellulose-lignin was breakdown into reducing sugar. All monosaccharides are reducing sugars. They all have a free reactive carbonyl group. Glucose is one of the monosaccharides. Benedict test was used to check the change of the cellulose-lignin into glucose. In the benedict test the sample was mixed with benedict solution and heated for 3 minutes.

Benedict test was used to detect the presence and estimate the amount of reducing sugar (Sugar with a free aldehyde or ketone group). In benedict test copper sulfate ($CuSO_4$) react with electrons from the aldehyde or ketone group of the reducing sugar to form cuprous oxide (Cu2O) red brown precipitate (King Saud University 2015).

Figure 3: 2:- Benedict test of hydrolyze sugar cane bagasse

3.4.2. Chemical characterization of PHB

3.4.2.1. Staining Reactions and Microscopy

Microbiologists have traditionally detected the presence of PHB granules in bacterial cells by staining with Sudan black B. (1, 2, 3) Staining with Sudan black B is considered as a presumptive test for the presence of PHB under a fluorescence microscope (Godbole 2014).

Ten ml of media was centrifuged at 6000 rpm for 20 minutes and Sudan staining was done to confirm PHB production after the PHB production confirmed the extractions of PHB proceed (Thapa et al. 2019). The Sudan staining was used for qualification test of PHB.

3.4.2.2. Fourier Transform Spectroscopy (FTIR)

Fourier transform infrared (FTIR) is the one of method of infrared spectroscopy in which infrared radiation is passed through a sample some radiation is absorbed by the sample and some are passes through. The resulting signal at the detector is a spectrum representing a molecular 'fingerprint' of the sample. The usefulness of infrared spectroscopy arises because different chemical structures produce different spectral fingerprints.

The functional group of the sample is recorded by the FTIR spectra technique. This technique recorded the qualitative characterization of the surface functional group. The sample measured on Spectrum 65 FT-IR (PerkinElmer) in the range 4000-400 cm^{-1} (resolution 4 cm^{-1}, number of scans: 4) using KBr pellets technique.

3.4.2.3. UV spectrophotometer

A variety of techniques can be used to determine and estimate the presences of extracted PHB from sugarcane bagasse. UV-VIS spectroscopic is simple, cost-effective and rapid tests for detecting PHB. UV-visible spectroscopy uses light in the visible ranges or its adjacent ranges. The PHB was scanned at wave length ranging from 200 to 300 nm using UV 3200 Spectrophotometer and the characteristic peaks were detected with chloroform standard. The absorbance at those wavelengths were recorded.

3.4.3. Experimental design

Data analysis was carried out by DESIGN EXPERT version 12 software (Box-Behnken design) to evaluate the effects of the process variables; temperature (34ºC, 37ºC and 40ºC), fermentation time (24 hr., 48 hr. and 72 hr.) and pH (6.5, 7 and 7.5) on the yield of PHB. These range choose because the growth behavior of *bacillus subtilis*. The Box-Behnken experimental design prepared 17 experiments on the pattern generated through software. The response variable was PHB yield. This design of the experiment helps us to optimize the process parameters for more than three levels and minimize an experimental error that is increased experimental accuracy. Significance of the result was set from analysis of variance (ANOVA). Table below shows the preparation of experiment using design expert's software version 12 with three level and three factors.

Table 1:- Experimental design factors and levels for extraction of PHB

Run no	Factor 1 Temperature	Factor 2 pH	Factor 3 Fermentation time (hour)
1	34	7	24
2	37	6.5	24
3	34	7	72
4	37	7	48
5	37	7	48
6	34	6.5	48
7	40	6.5	48
8	40	7.5	48
9	37	7	48
10	40	7	72
11	37	7.5	72
12	37	7	48
13	37	7	48
14	34	7.5	48
15	40	7	24
16	37	6.5	72
17	37	7.5	24

Chapter four

Result and discussion

4.1. Benedict test

In the benedict test after the sample was mixed with benedict solution and heated for 3 minute the result confirms that the color of the sample was changed to brick red. This brick red color indicates that the amount of glucose in the extract was more than 2%.

The color changes green → 0.1-0.5 % sugar

The color change yellow → 0.5 – 1 % sugar

The color change orange → 1 – 1.5 % sugar

The color change to red → 1.5 - 2 % sugar

The color change to brick red → > 2% sugar (Arval 2019).

Figure 4: 1 Result of benedict test of hydrolyze sugar cane bagasse

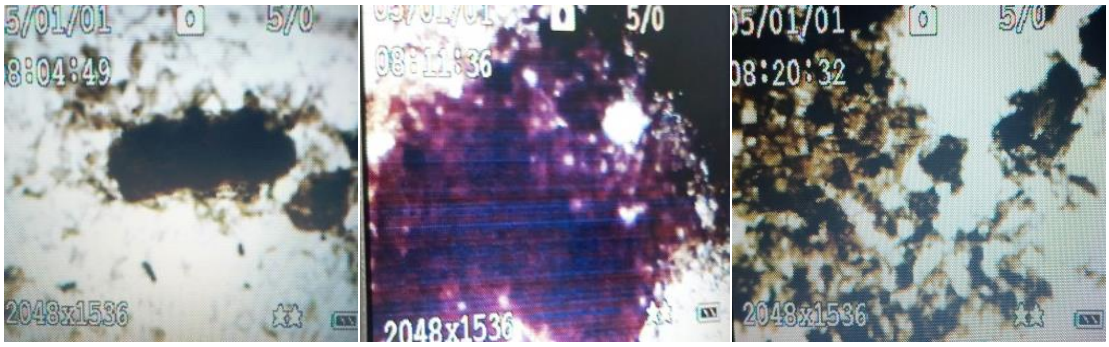
4.2. Determination of PHB production by staining

The production of PHB was determined by using Sudan staining under compound microscope. At staining, if PHB was produced, the color of the sample was changed to dark blue/black.

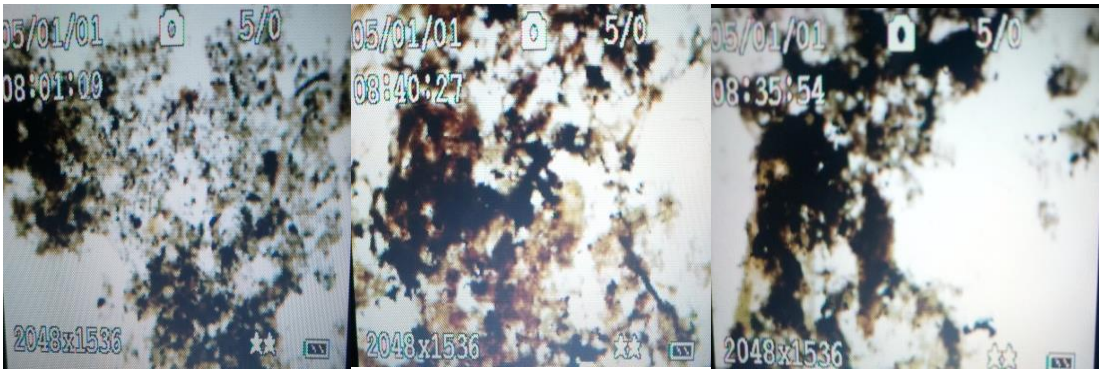
A) The first three pictures indicate Sudan Staining result at constant pH but varied temperature and time. These are at 6.5 pH, 37 ^{0}C T and 24 hr. time, at 6.5 pH, 34 ^{0}C T and48 hr. time and at 6.5 pH, 37 ^{0}C T and time 72 hr. respectively.

B) The second three pictures indicate Sudan Staining result at constant pH but varied temperature and time. These are at 7 pH, 34 ^{0}C T and 24 hr. time, at 7 pH, 37 ^{0}C T and 48 hr. time and at 7 pH, 40 ^{0}C T and time 72 hr. respectively. When compared to the result in A, the production of PHB in the parameter listed under B is comparable high. This is because of the at pH of 7 the media is very suitable for the bacteria to produce higher PHB and also higher yield than yield at parameter on C.

C) The last two pictures indicate Sudan Staining result, at 7.5 pH, 37 ^{0}C T and 24 hr. time, at 7.5 pH, 37 ^{0}C T and 72 hr. time respectively. The result is lower than both under A and C as the pH of media is close to base.



(A)



B.

C.

Figure 4: 2:- Qualitative test of PHB using Sudan stain

The result picture indicates that the PHB production was highly affected and varies at different temperature, time and pH. The Sudan staining method indicates the qualitative production of PHB.

As general the from the picture above the Sudan staining gives the best result at 7 pH, 37^0C and 48 hours respectively.

4.3. Fourier Transform Spectroscopy (FTIR) analysis of PHB

FTIR spectrum of the PHB sample was measured by dissolving 1 mg extracted sample of PHB in 5 ml chloroform. After pellet was formed by adding KBr, the spectra were recorded on Spectrum 65FT-IR (PerkinElmer) in the range 4000-400 cm^{-1} (resolution 4 cm^{-1}, number of scans: 4). In this analysis the Fourier Transform Infra-Red spectrum of the PHB sample reveled these peaks at 3385, 2931.5, 1608, 1509, 1423.5, 1168, 1081.7, 855.6, 705, 552.8 cm^{-1} whereas these peaks are lying between 3385 cm-1 and 552 cm^{-1}(Jangra, Batra, and Sikka 2016). The peak at 3385 cm^{-1} indicated stretching strong H bond created by the terminal OH groups found in sugarcane bagasse. These are comparable with results obtained by (Getachew and Woldesenbet 2016b). The peak at 2931.5 and 1423.5 cm^{-1} which represents the methane groups, followed by a peak at 1608 corresponds to C=O stretch of an ester group present in highly ordered crystalline structure. The at 1509 cm^{-1} peak indicates the presence N–H amide protein in the polymer whereas the peak at 1168 cm^{-1} corresponds to CH group. The presence of these marked peaks demonstrated the presence of PHB. The peak 1081.7 cm^{-1} represent –C–O– polymeric group in the sample sugarcane bagasse. The other peak 855.6, 705, 552.8 cm^{-1} correspond to the presence of alkyl halides. These are

comparable with results obtained by (Getachew and Woldesenbet 2016b). The ester functional group and the methane group at these peaks highly representative of the presence of PHB. These all-prominent absorption bands confirm that the polymer extracted from the sugarcane bagasse were PHB.

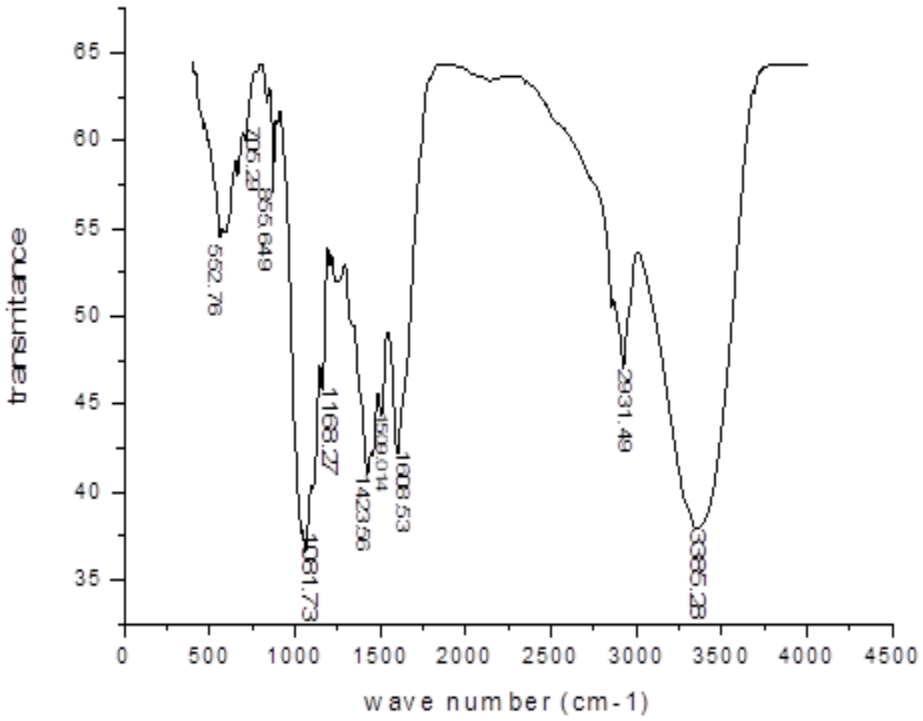


Figure 4: 3: - FTIR pattern of PHB from bagasse

4.4. UV-Vis spectrophotometer analysis of PHB

This analysis was done by using chloroform as blank sample because chloroform is one of the plastic synthesizers and it was the best indicator for the presence of PHB. The figure 4.3 indicate the graph the absorbance at the wavelength between 200-320nm range. The point indicates the absorbance of PHB at these range. Absorbance and concentration are directly proportional so the higher the absorbance indicates the higher concentration. UV–Vis scanning of the extracted polymers showed the occurrence of PHB.

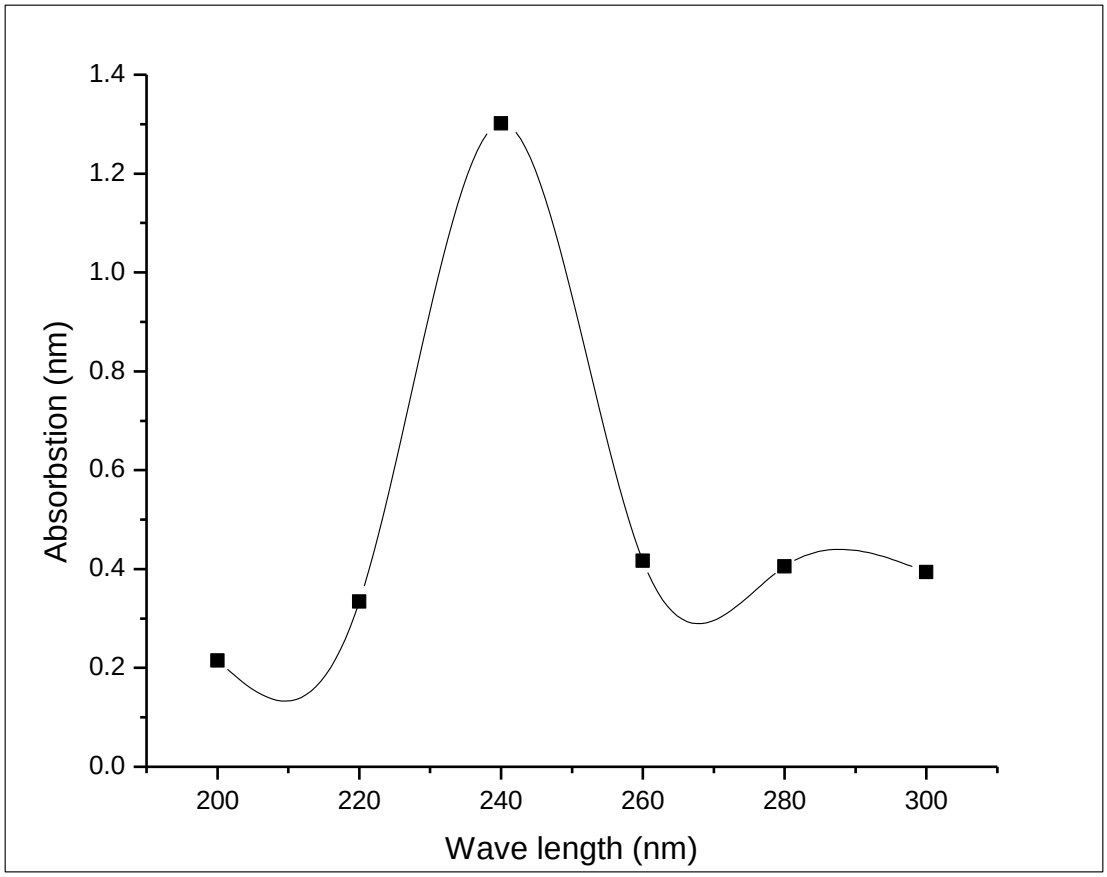


Figure 4: 4: - UV- Vis spectroscopy for PHB

The graph indicates that the quantitative result of PHB extraction. The relationship between absorbance and concentration is directly proportional. The higher peak indicates there is the high absorbance at that wave length. Higher absorption is obtained at 240nm wave length.

4.5. Effect of Process Parameters on the Extraction of PHB

4.5.1. PHB yield

The yield of PHB after final drying process was weighed and analyzed for each experimental run. From the experiment the maximum yield gained was 6.82 g/l and the minimum was 2.1 g/l.

Table 2:- Experimental PHB yield from bagasse

Std	Run	Factor 1 A: temperature degree Celsius	Factor 2 B: pH	Factor 3 C: fermentation time Hours	Response 1 Yield g/g
1	6	34	6.5	48	2.43
2	7	40	6.5	48	2.6
3	14	34	7.5	48	3.15
4	8	40	7.5	48	4.1
5	1	34	7	24	2.1
6	15	40	7	24	2.67
7	3	34	7	72	2.44
8	10	40	7	72	3.016
9	2	37	6.05	24	2.752
10	17	37	7.5	24	3.2
11	16	37	6.5	72	2.43
12	11	37	7.5	72	4.14
13	13	37	7	48	6.79
14	5	37	7	48	6.78
15	4	37	7	48	6.8
16	12	37	7	48	6.82
17	9	37	7	48	6.77

4.5.2. Statistical analysis for the yield PHB

This design of the experiment helps us to optimize the process parameters for more than two levels and minimize an experimental error that is increased experimental accuracy. The Box-Behnken experimental designs were used to obtain the model equation. The experiments were performed in

random order to avoid systematic error. Significance of the extraction parameters was analyzed using ANOVA. The coded terms were listed below

A = temperature

B = pH

C = fermentation time

AB = the interaction between temperature and pH

AC = the interaction between temperature and fermentation time

BC = the interaction between pH and fermentation time

Table 3:- ANOVA of fitted model for the yield of PHB

Source	**Sum of Squares**	**df**	**Mean Square**	**F-value**	**p-value**	
Model	57.54	9	6.39	17061.27	< 0.0001	Significant
A-temperature	0.6418	1	0.6418	1712.89	< 0.0001	
B-Ph	2.40	1	2.40	6393.83	< 0.0001	
C-fermentation time	0.2126	1	0.2126	567.24	< 0.0001	
AB	0.1521	1	0.1521	405.91	< 0.0001	
AC	9.000E-06	1	9.000E-06	0.0240	0.8812	
BC	0.3982	1	0.3982	1062.57	< 0.0001	
A^2	19.43	1	19.43	51844.74	< 0.0001	
B^2	10.43	1	10.43	27838.48	< 0.0001	
C^2	18.35	1	18.35	48965.38	< 0.0001	
Residual	0.0026	7	0.0004			
Lack of Fit	0.0011	3	0.0004	1.03	0.4689	not significant
Pure Error	0.0015	4	0.0004			
Cor Total	57.54	16				

Table 3 showed that the effect of temperature, pH and fermentation time and also the interaction effect between temperature and pH, temperature and fermentation time and pH and fermentation

time. The P values were used as a tool to check the significance of each of the coefficients, which in turn are necessary to understand the pattern of the mutual interactions between the test variables. The larger the magnitude of F-test value and the smaller the magnitude of P-values, the higher the significance of corresponding coefficient. Values of P less than 0.05 indicate that the model terms are significant. In this case the three factors which are the temperature, pH and fermentation time are significant model terms and have significant effect on the yield of the product. Values greater than 0.0500 indicate the model terms are not significant. Therefore, the interaction effects between temperature and fermentation time is not significant. To improve the model the insignificant model terms were reduced. The table below is a modified statistical analysis when the insignificant model terms are eliminated.

4.5.3. Model equation

A representative equation in which it represents the whole model with a single mathematical relation is a model equation. The model equation of extraction PHB from sugar cane bagasse that was developed by the software has been shown below in the table. Practically, from the equation it can be easily observed that, increment in individual factors also made the yield to increase parallelly. In other hand, even though the combined factors affect the yield strongly.

Equation 1: - final equation in terms of coded factors

$$Yield = 6.792 + 0.28325 * A + 0.54725 * B + 0.163 * C + 0.195 * AB + 0.0015 * AC + 0.3155 * BC - 2.148 * A^2 - 1.574 * B^2 - 2.0875 * C^2$$

In the above equation, the significant effect of each variable indicated. The pH has high effect on the yield of PHB, also the interaction between pH and fermentation time has high significant effect.

Equation 2: - final equation in terms of actual factors

$$Yield = 16.8447 * A + 83.1665 * B + 0.169896 * C + 0.13 * AB + 0.0262917 * BC - 0.238667 * A^2 - 6.296 * B^2 - 0.00362413 * C^2 - 605.737$$

The above equation indicates the final equation in terms of actual factors. The significant effect of each variable indicated. The pH has high effect on the yield of PHB compared to temperature and

fermentation time; also, the temperature has moderate effect compared to fermentation time. Moreover, the interaction between pH and temperature and the interaction between pH and fermentation time have significant effect on the yield of PHB. The interaction between temperature and fermentation time has no significant effect on PHB yield.

4.5.4. The Effect of temperature on the Extraction of PHB

It was noted from the preliminary experiments the amount of PHB was highly dependent on the temperature of fermentation. In this study, to explore the optimum conditions for maximum of PHB from sugar cane bagasse, the temperature of fermentation varies. As shown in the figure below the temperature was varied from 34^{o}C to 40^{o}C.

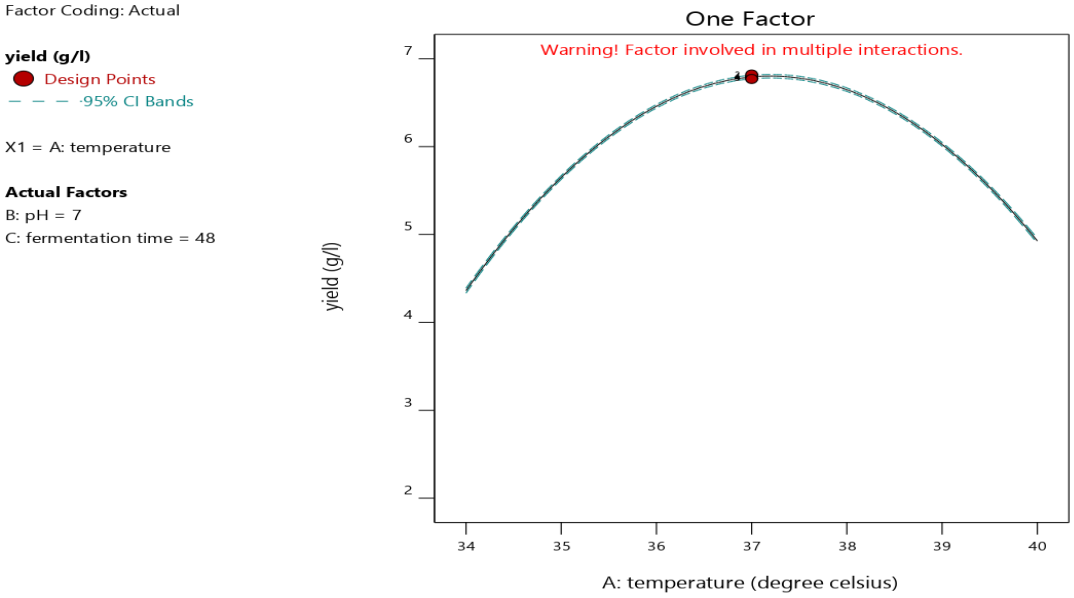


Figure 4: 5: - Effect of temperature on PHB extraction from bagasse

In this case extraction temperature was chosen 34, 37 and 40^{o}C. The yield of PHB increases with increasing temperature in small amount. From the figure above the maximum yield was found at the extraction temperature of 37^{o}C that is 6.82g. Respectively with an increase in temperature gradient, PHB yield was increased simultaneously from 2.1g to 6.82g for 34^{o}C and 40^{o}C depend on the extraction time.

4.5.5. The Effect of pH on the Extraction of PHB

It was noted from the preliminary experiments the amount of PHB was highly dependent on the pH of the media. In this study, to explore the optimum conditions for maximum of PHB from bagasse, the pH varies. As shown in the figure below the pH was varied from 6.5 to 7.5.

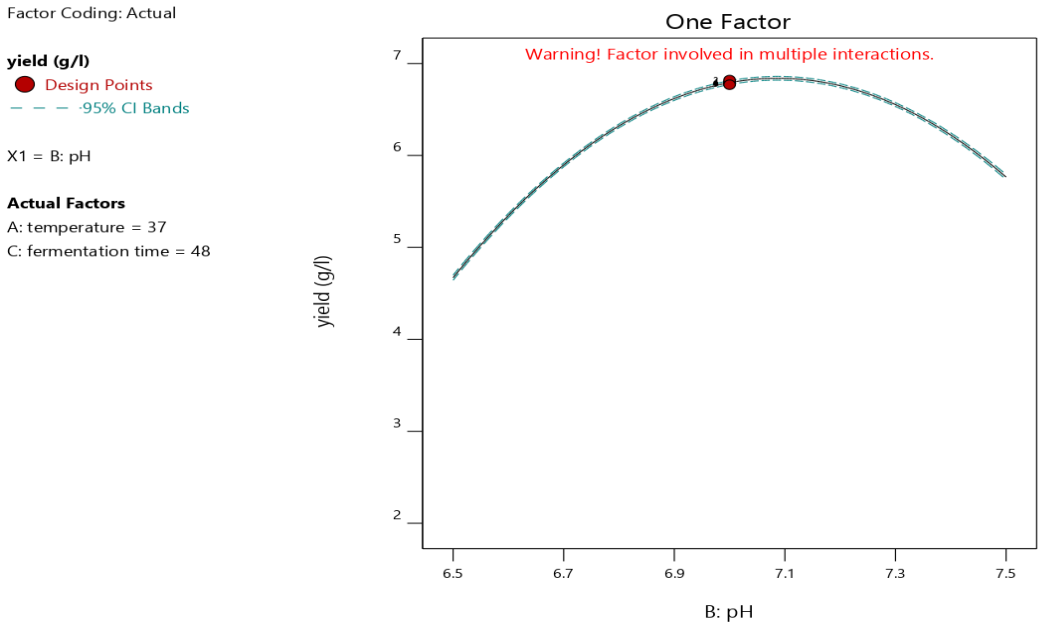


Figure 4: 6: - Effect of pH on PHB extraction from bagasse

The extraction of PHB has higher yield in a 7 pH of media than the rest. Considering pH of media, the optimum yield found was 6.82g at given time of extraction. At this pH, the value of yield is higher because the condition of media is more comfortable than others pH value.

4.5.6. The Effect of fermentation time on the Extraction of PHB

Extraction is increasing simultaneously with an increment of fermentation time mostly. But further increment of time may cause more stress to bacteria's and cause shortage of nutrient.

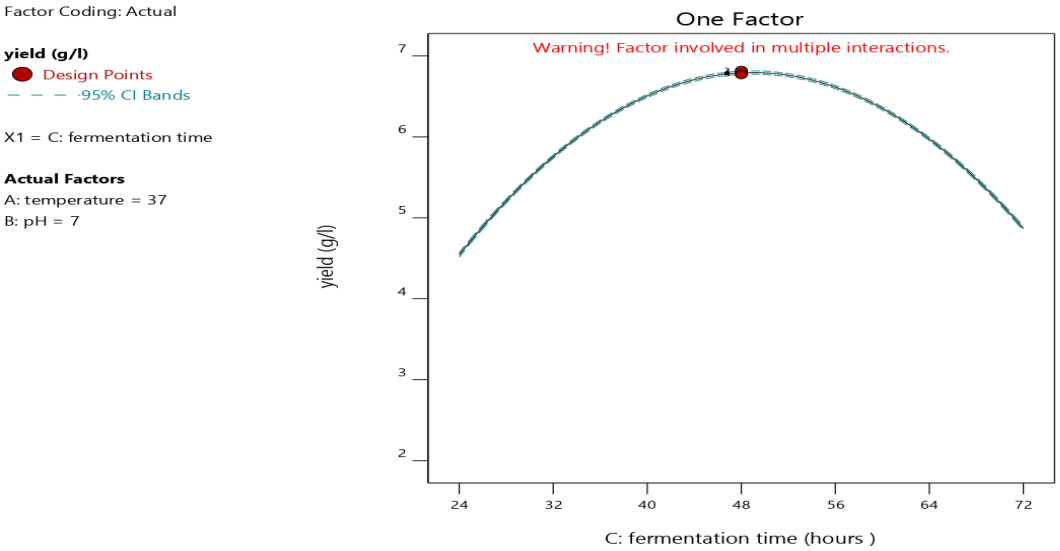


Figure 4: 7: - Effect of fermentation time on PHB extraction from bagasse

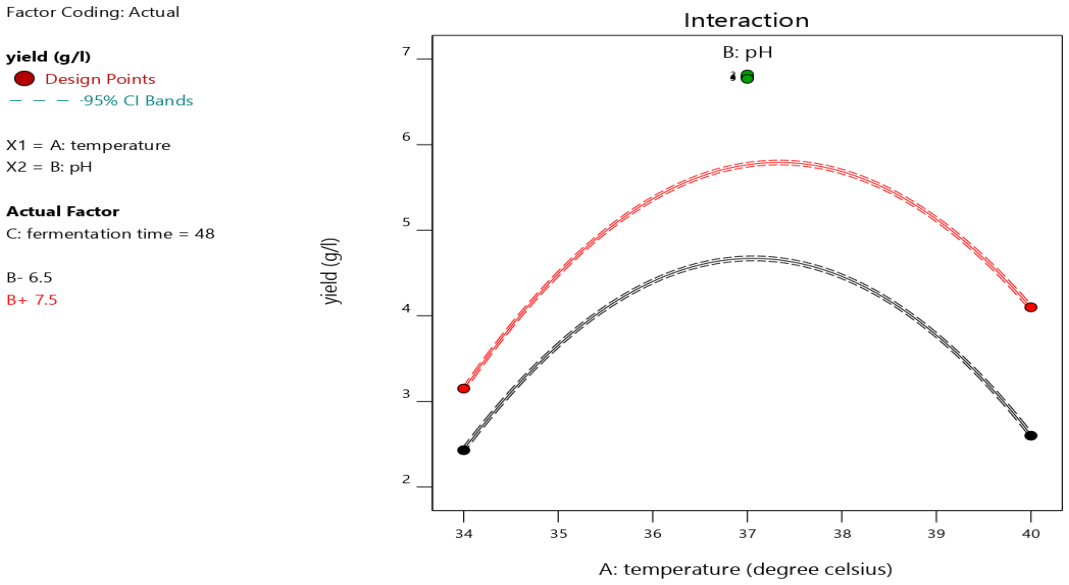
The graph above indicates that the raise of yield was found with comparative increment of fermentation time. In this study the extraction time varies from 24hours to 72 hours. Time which is below 24 hours of fermentation time yields with a negligible mass of PHB. The maximum yield was found when the extraction process was going for 48 hours and it was 6.82 at medium temperature.

4.5.7. Interaction Effect on the Yield of PHB

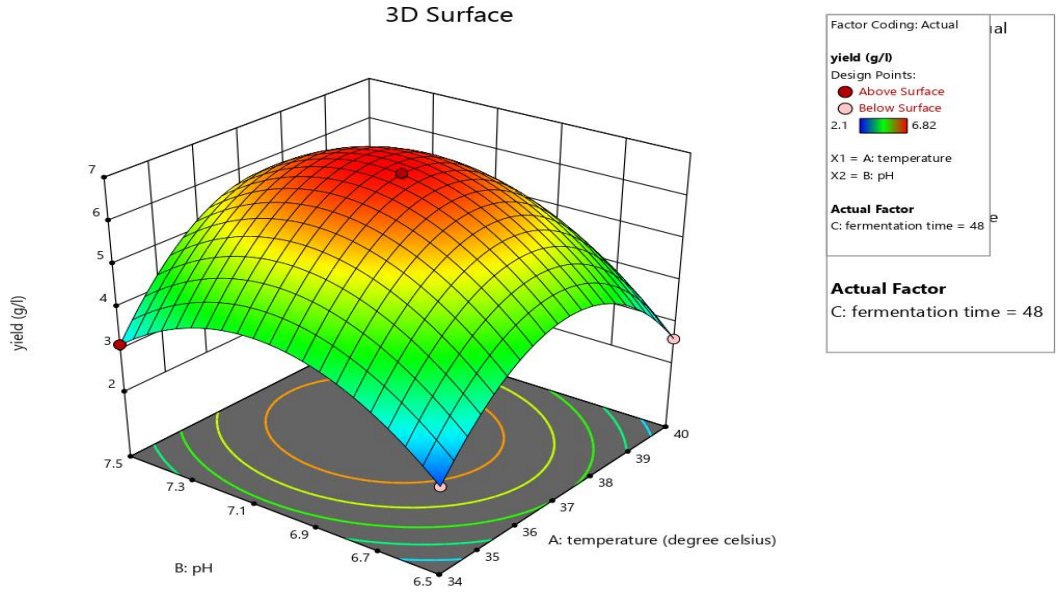
The study of the effects of the combinations of three factors were discussed and some important results described in figure below. The result shows that there is interaction effect between those factors on the PHB yield.

4.5.7.1. Interaction effect between temperature and pH on the yield of PHB

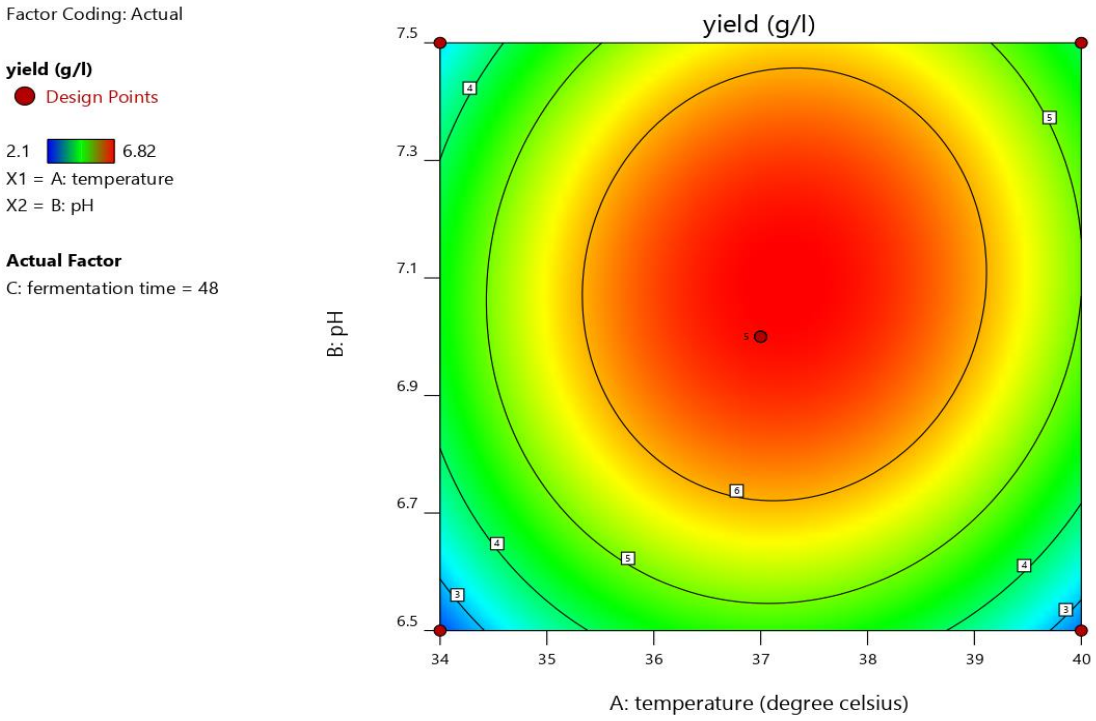
There is no point of interaction between these two factors. The interaction between pH and temperature has significant effect on the yield of PHB. As the graph indicated the slight change of this factor highly affect the yield. In figure (C) the contour indicate high interaction means high yield of PHB.


Factor Coding: Actual
yield (g/l)
Design Points
95% CI Bands
X1 = A: temperature
X2 = B: pH
Actual Factor
C: fermentation time = 48
B- 6.5
B+ 7.5
Interaction
B: pH
yield (g/l)
A: temperature (degree celsius)

(A)


3D Surface
yield (g/l)
B: pH
A: temperature (degree celsius)
Factor Coding: Actual
yield (g/l)
Design Points:
Above Surface
Below Surface
X1 = A: temperature
X2 = B: pH
Actual Factor
C: fermentation time = 48

(B)



(C)

Figure 4: 8: - The interaction effect of temperature and pH on PHB yield

The Figure above indicates that, the interaction between pH and extraction temperature was significantly affect the response. From the ANOVA Table, the p-value of pH and extraction temperature was <0.0001. This means the p-value of the interaction is less than the p-value of the model design. Generally, there was combined effect of pH and extraction temperature observed on the yield. So, the yield was dependent of pH and extraction temperature influence at the same time. The proportionate increment of pH and extraction temperature was significantly affecting the yield. When extraction temperature goes from 34 to 40^{o}C and pH goes from 6.5 to 7.5 yield is increased.

4.5.7.2. Interaction effect between temperature and fermentation time

The interaction between temperature and fermentaion time has no significant effect on the yield of PHB. They both increase simultaneously but the interaction effect is low. The graph indicates the yield was increased in the time of extraction starting from 24 to 72 hour with a maximum yield of

6.82g. So, the interactive factor of temperature and extraction time was significantly affecting the yield.

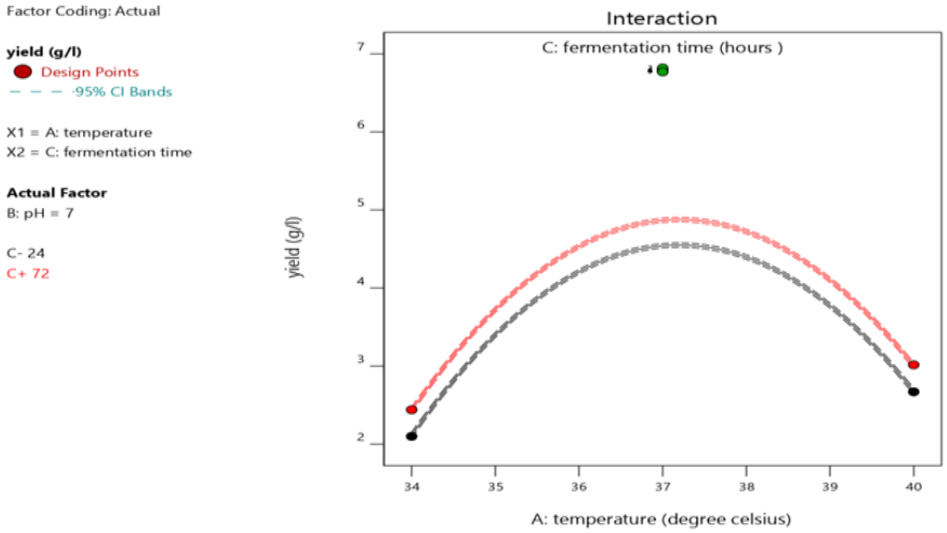
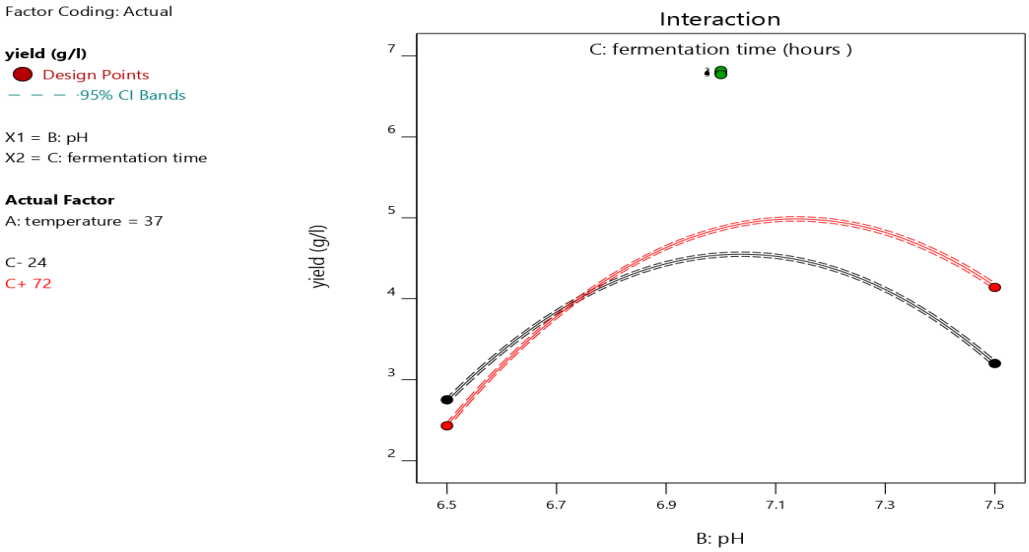


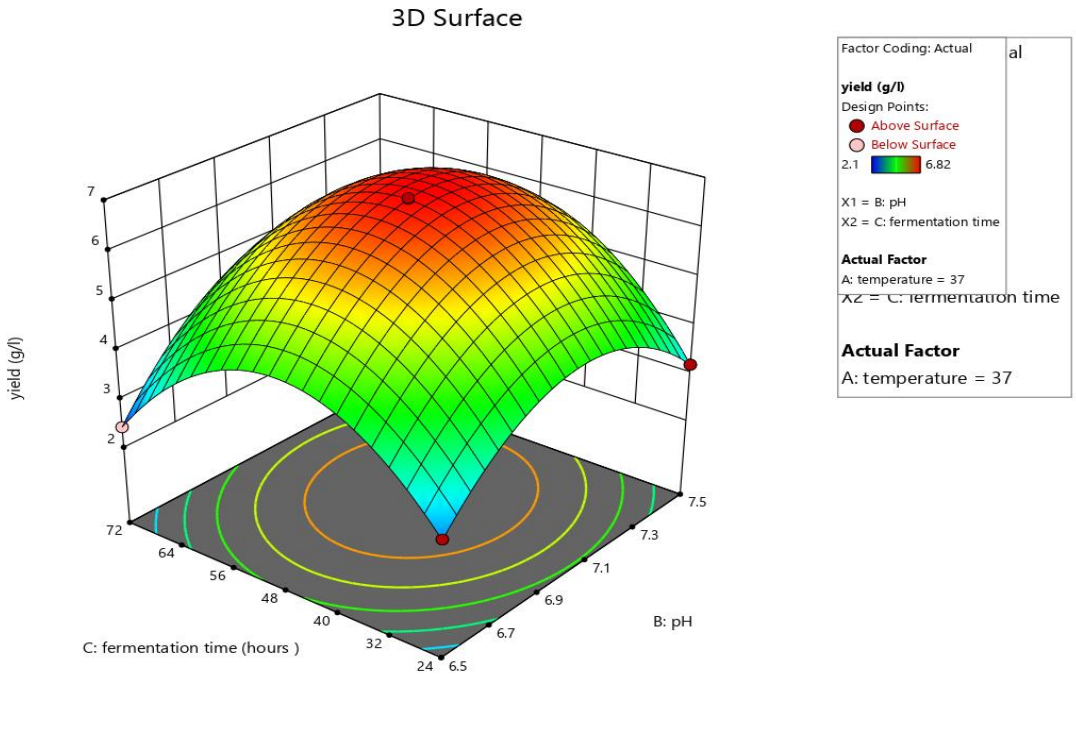
Figure 4: 9: - The interaction effect of temperature and fermentation time on PHB yield

3.5.7.3. Interaction effect between pH and fermentation time

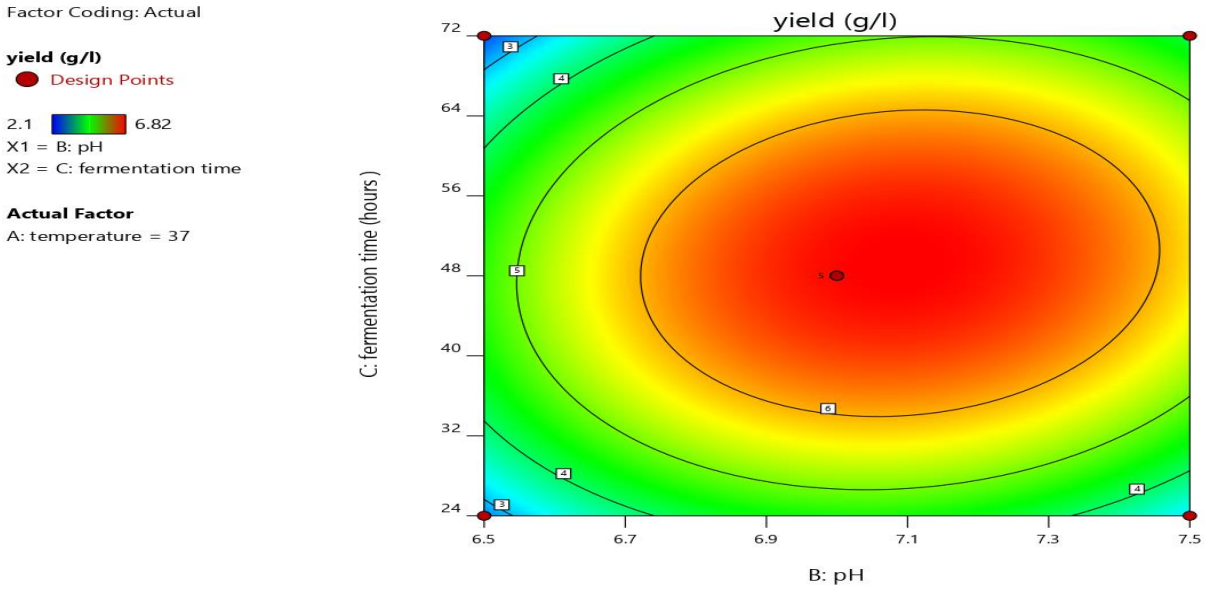
From the interaction graph (a) of below, the point of interaction is indicated well at the center of the two lines. That means at the point of interaction both pH and fermentation time have higher effect on the yield. This indicates the yield was significantly increased in the time of fermentation starting from 24 to 72 hour with a maximum yield of 6.82g. So, the interactive factor of pH and fermentation time was significantly affecting the yield.


Factor Coding: Actual
yield (g/l)
Design Points
95% CI Bands
X1 = B: pH
X2 = C: fermentation time
Actual Factor
A: temperature = 37
C- 24
C+ 72
Interaction
C: fermentation time (hours)
yield (g/l)
B: pH
2
3
4
5
6
7
6.5
6.7
6.9
7.1
7.3
7.5

A


3D Surface
yield (g/l)
C: fermentation time (hours)
B: pH
Factor Coding: Actual
yield (g/l)
Design Points:
Above Surface
Below Surface
2.1
6.82
X1 = B: pH
X2 = C: fermentation time
Actual Factor
A: temperature = 37
Actual Factor
A: temperature = 37

B.



C.

Figure 4: 10: - The interaction effect of pH and fermentation time on PHB yield

4.5.8. Optimal Process Parameters to Prepare Maximum yield PHB

Preliminary observations show that PHB yield varies significantly according to the experiment parameters, reaching values of 2.1-6.82 g/l under certain operating conditions. To find the optimum extraction process parameters for the highest PHB yield, the extraction process parameters were optimized subject to constraint shown in the figure below. Five solutions were found and the solution with the highest yield was chosen. The maximum yield was 6.82682 under the optimum process variables at 37.26 ^{0}C, 48.64 hours and 7.15 pH of extraction temperature, fermentation time and pH of media. In order to verify the optimization results, the selected parameters were validated in the laboratory with suggested process parameters. The result from the experiments confirmed the selected solutions.

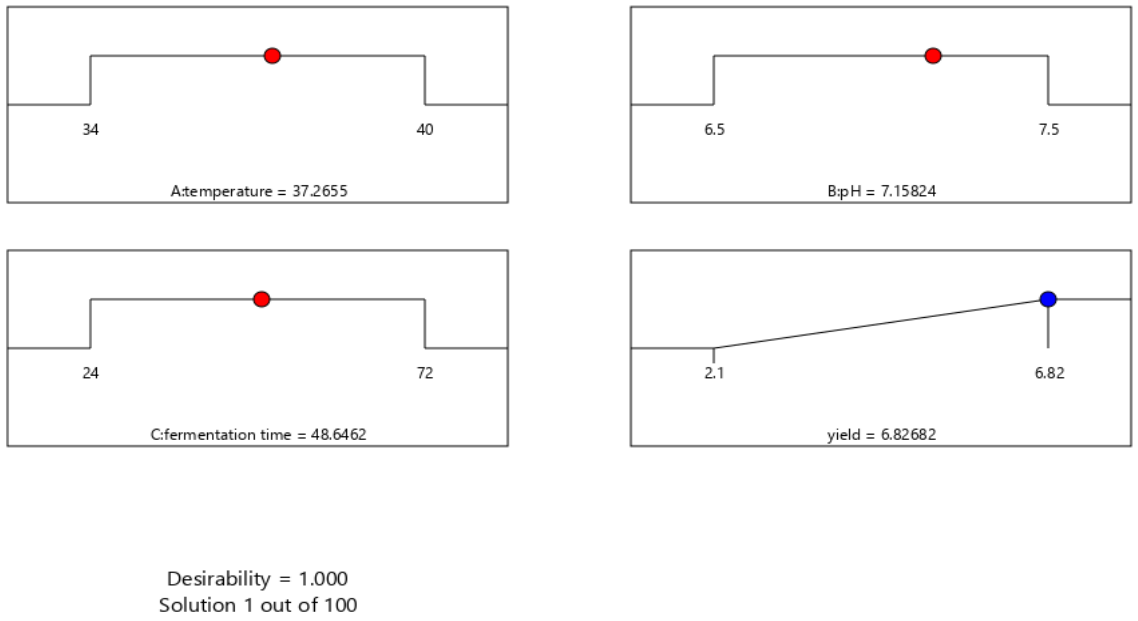


Figure 4: 11: - Optimal yield of PHB

Chapter five

Conclusion and recommendation

5.1. Conclusion

PHB is natural polyester, synthesized by a wide range of organisms, particularly by some bacterial strains. They have very interesting properties such as being biocompatible and totally and rapidly biodegraded to carbon dioxide and water by a large number of microorganisms.

Extraction of PHB from sugar cane bagasse was carried out in five main stages. These stages are pretreatment of bagasse, hydrolysis, fermentation, extraction and recovery. In this study three variables used and their effect on the yield was studied i.e., temperature (34, 37 & 40 ^{0}C), pH (6.5, 7 & 7.5) and fermentation time (24, 48, 72 hours) were used as a factor. Significance checking and yield optimization was implemented using RSM of Box-Behnken method. The effect of each variable and the interaction between the factors implied using ANOVA. The combination of temperature – pH, temperature—fermentation time, pH– fermentation time had a significant effect on the extraction yield of PHB.

Characterization of the extract indicate, the benedict test of the bagasse was brick red and it indicate the amount of glucose was more than 2 %, and the photometric result after staining indicate the presence of PHB on the cell.

An optimal yield of PHB was obtained at 37.26 ^{0}C, 48.6 hours and 7.15 pH. This means higher yield is obtained at Neutral pH, medium extraction temperature and medium extraction time. Depending, on the result of Box-Behnken method an optimized yield of 6.82 g/g was gained by hydrolyzing 40gm of sugar cane bagasse. The result obtained by using one strain Bactria (*bacillus subtilis*) is comparative with the results of others study which used recombinant strain of bacteria (Getachew and Woldesenbet 2016b) and the result gained from this work is better. Based on these facts it can be concluded that, it is evident that the chosen method of extraction was efficient, and reliable to use PHB.

The results of this study confirmed that easily available raw material like bagasse can be used for the production of PHB and it can also use for reducing the cost of biodegradable plastics, reducing environmental pollution problems caused by conventional plastics and solving disposal problem of the agricultural wastes.

5.2. Recommendation

If some further research work is carried out to explore PHB for substitute plastics , recommended studies are listed below:

- This area required further study.
- Comparative study was needed on the types of bacteria strain to select the efficient type of strain for production of PHB.
- Concentration of PHB must be analyzed using known concentration of PHB (pure) by using calibration curve method using UV Spectroscopy. The lack of pure PHB in the market limits this study from determining the exact concentration.
- The optimization of the extraction process using Box-Behnken method of experimental design was analyzed by considering a higher constraint factor value. Since, the coefficient of variance was less than 10%, optimization was representing for this design space only. If reproducibility of the model is needed a full factorial experiment with replicate should be undertake to consider as an operational design rather than a space design.
- It is recommended to perform preliminary design, economic feasibility study and establish economic scale for production of PHB.

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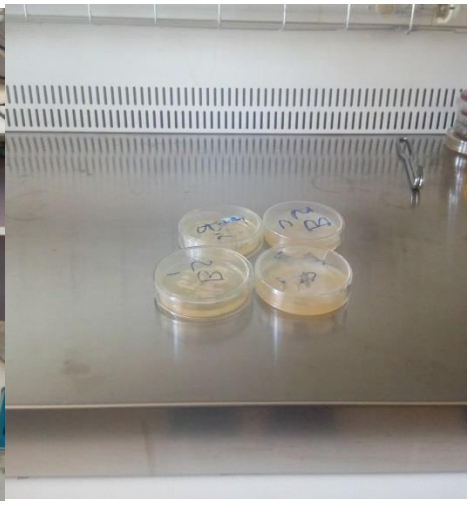
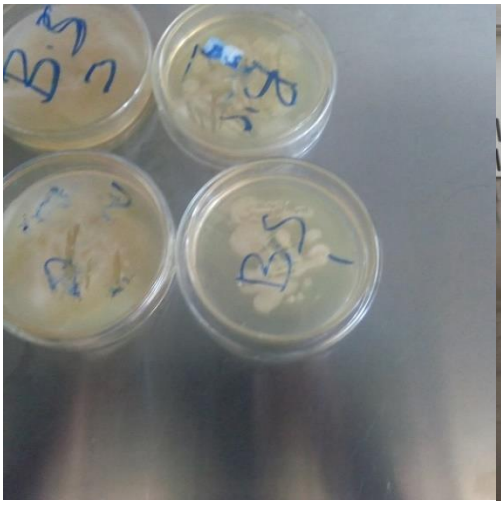
Appendix








HYBRIDIZATION INCUBAT

$CuSO4 \rightarrow Cu^{++} + SO_4^{--}$ …… 1
$2Cu^{++}$ + reducing sugar → Cu^{+}……..2
$Cu^{+} \rightarrow Cu_2O$ (precipitate)……..3

The color changes green → 0.1-0.5 % sugar
The color change yellow → 0.5 – 1 % sugar
The color change orange → 1 – 1.5 % sugar
The color change to red → 1.5 - 2 % sugar
The color change to brick red → > 2% sugar (Arval 2019).

Coefficients in Terms of Coded Factors PHB

Factor	Coefficient Estimate	df	Standard Error	95% CI Low	95% CI High	VIF
Intercept	6.79	1	0.0087	6.77	6.81	
A-temperature	0.2832	1	0.0068	0.2671	0.2994	1.0000
B-Ph	0.5473	1	0.0068	0.5311	0.5634	1.0000
C-fermentation time	0.1630	1	0.0068	0.1468	0.1792	1.0000
AB	0.1950	1	0.0097	0.1721	0.2179	1.0000
AC	0.0015	1	0.0097	-0.0214	0.0244	1.0000
BC	0.3155	1	0.0097	0.2926	0.3384	1.0000
A^2	-2.15	1	0.0094	-2.17	-2.13	1.01
B^2	-1.57	1	0.0094	-1.60	-1.55	1.01
C^2	-2.09	1	0.0094	-2.11	-2.07	1.01

Fit Statistics

Std. Dev.	0.0194	**R^2**	1.0000
Mean	4.06	**Adjusted R^2**	0.9999
C.V. %	0.4770	**Predicted R^2**	0.9996
		Adeq Precision	315.2415

The **Predicted R^2** of 0.9996 is in reasonable agreement with the **Adjusted R^2** of 0.9999; i.e. the difference is less than 0.2.