ADDIS ABABA INSTITUE OF TECHNOLOGY

SCHOOL OF CHEMICAL AND BIO ENGINEERING

BIOCHEMICAL ENGINEERING STREAM

Investigating the possibility of producing Polyhydroxyalkanoates (PHAs) Using Hydrolysates of Napier grass by *Burkholderia Sacchari* strain

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

By

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I hereby declare that the thesis named **"Investigating the possibility of producing Polyhydroxyalkanoates (PHAs) Using Hydrolysates of Napier grass by *Burkholderia Sacchari* strain"** is entirely my own work, with the exception of quotations and citations, which have been properly acknowledged. I further declare that it has not previously or currently been submitted for any other degree at Addis Ababa University or other educational institutions.

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DECLARATION

I hereby declare that the thesis named "**Investigating the possibility of producing Polyhydroxyalkanoates (PHAs) Using Hydrolysates of Napier grass by *Burkholderia Sacchari* strain**" is entirely my own work, with the exception of quotations and citations, which have been properly acknowledged under the supervision of Dr. Sintayehu Nibret. I further declare that it has not previously or currently been submitted for any other degree at Addis Ababa University or other educational institutions.

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Abstract

Bacteria produces Polyhydroxyalkanoates (PHAs), which are macromolecules. They are inclusion bodies that build up as reserve resources while bacteria grow under various stress situations. PHAs have been chosen as alternatives for the development of biodegradable polymers due to their rapid degradability under natural environmental circumstances. The purpose of this study was to produce reducing sugars from lignocellulosic Napier grass and examine PHA production by the impact of temperature, pH, and culture time on growth yields by employing the produced reducing sugars as carbon sources using response surface methodology via Box- Behnken design (BBD).

Burkholderia Sacchari strain was chosen from more than 300 bacterial strains capable of PHA accumulating strains based on its ability to consume both hexose and pentose sugars and has a capacity of accumulating high mass PHA granules inside its cells. At 200 rpm, optimal pH, fermentation temperature, and incubation time for the isolate to produce the highest PHA were 7, 35 °C, and 48 hour, respectively.

When hydrolysates of Napier grass was employed as carbon sources and ammonium sulphate as nitrogen sources, the strain was able to collect 62.1% PHA from its total biomass. The extracted PHAs' FTIR spectra show strong peaks at wavenumbers that are unique to PHAs as C–H, CH2, O-H, C=O, and C–O groups. The extract's resemblance appropriateness for bioplastic production were validated by UV–Vis spectrophotometric analysis.

***Keywords**: Polyhydroxyalkanoate, Napier grass, Glucose-rich hydrolysate, Box Behnken design, PHA productivity, Burkholderia Sacchari, FTIR*

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Acronyms

ANOVA	Analysis of variance
ASTM	American Society for Testing and Materials
CSTR	Continuous Stirred Tank Reactor
FTIR	Fourier transform infrared spectroscopy
HPLC	High Performance Liquid Chromatography
Mcl-PHAs	Medium chain length Polyhydroxyalkanoates
PA	Polyamide
PE	Polyethylene
PET	Polyethylene terephthalate
PHA	Poly-hydroxyalkanoate
phaA	β-Ketothiolase
phaB	Acetoacetyl-CoA reductase
phaC	PHA synthase
PHB	Poly-hydroxybutyrate
PLA	Polylactic acid
PP	Polypropylene
PS	Polystyrene
PVC	Polyvinylchloride
Scl- PHAs	Short chain length Polyhydroxyalkanoates
UV	Ultraviolet

1. Introduction

1.1. Background

Durability and versatile mechanical and thermal properties make conventional plastics useful for many applications. As durability of plastics offers great performance for a long period without significant deterioration by resisting the effects of use and ageing. However, they are not readily biodegradable and thus accumulate in the environment and directly lead to waste disposal problems (George et al., 2020).

In view of rising energy demand and the decrease of fossil resources, the global economy is currently trying to replace recognized energy sources with greener, bio-based and renewable alternatives (Bhatia et al., 2019; Venkata Mohan et al., 2016).

Environmental, health, and political concerns, as well as the aforementioned problems, motivated the development of bioplastics such as Polyhydroxyalkanoates (PHAs); polyesters produced during the secondary metabolism of bacteria and archaea in the presence of excess carbon sources and insufficiency of nutrients inside the cell's cytoplasm, allowing for a lower environmental impact (Colombo et al., 2020; Laycock et al., 2014). In the PHAs group, polyhydroxybutyrate (PHB) is gaining enormous consideration due to its extraordinary characteristics, such as a higher degree of crystallinity, tensile strength, and melting temperature (175 ^{o}C) with low permeability for H_2O,O_2, and CO_2 (Ganesh Dattatraya Saratale et al., 2020).

According to Bugnicourt *et al*. biodegradable bio plastics are basically divided in to thee groups according to their sources: biomass based (Proteins, Lipids, and Polysaccharide), microorganism based (PHA) and biotechnology based (Polyactides and Polylacticacid) (Bugnicourt et al., 2014). In sustainable PHA production using non-edible, industrial and agricultural wastes, abundant and renewable carbon sources such as waste biomass are used. This will not only ensure agreement with zero waste policies but also enable upcycling of the generated wastes within bio refineries to high value products (Radhika & Murugesan, 2012; Sayyed et al., 2021). Cost optimization production process will be done by using waste materials (cheap carbon sources), recombinant strains techniques and use of mixed microbial culture (MMC) with open systems that do not

require sterile conditions, coupled to that of wastes or surplus based feeds (Jong Il Choi & Lee, 1997; Ienczak et al., 2013).

Conditions of external substrate excess and limitation were shown to select for microbial populations with enhanced capacity to store PHAs (Serafim et al., 2008). Bacterial PHA synthesis is known to occur under conditions in which growth is restricted by either an external (lack of nutrient or electron acceptor) or an internal factor. It was proposed that MMC operated under excess and limited conditions were subjected to an internal growth limitation arising from the alternate substrate availability, which compelled the organisms to a physiological adaptation (Beccari et al., 1998).

Methylobacterium sp., Cupriavidus necator, Ralstonia eutropha, Bacillus sp., Pseudomonas sp., Enterobacter sp., Citrobacter sp., macrocytogenes, vinelandii, A, C. Oleovorans, P. necator, Protomonas extorquens Escherichia sp., Klebsiella sp., Azotobacter beijerinckii, and *Rhizobium sp., A. A.* are some examples from over 300 heterotrophic gram-negative and gram-positive bacterial species capable of synthesizing PHAs have been isolated and defined well(Bartels et al., 2020; Bhatia et al., 2019; Jong Il Choi & Lee, 1997; Hindi et al., 2017; Muneer et al., 2020; Purushothaman et al., 2001; Santhanam & Sasidharan, 2010; Wong et al., 2012; Zain et al., 2016; Y. H. P. Zhang et al., 2009). *Burkholderia Sacchari* was chosen for this investigation because it can convert both glucose and xylose, the primary sugars in lignocellulosic hydrolysates, into PHA in greater quantities.

Because of its high availability and low cost worldwide, lignocellulosic biomass, which mainly consists of cellulose, hemicellulose, and lignin as main component, has recently received increased attention as a promising sustainable and carbon-neutral feedstock for the development of biofuels and platform chemicals (Bhatia et al., 2019). For instance, polysaccharides can be hydrolyzed by using different chemical, physical, enzymatic or combination of those methods from cellulose and hemicellulose, followed by biotransformation into different biofuels such as bioethanol, hydrogen, biodiesel, paper, lumber and bioplastics.

Napier grass is one type of lignocellulosic material that contains cellulose, hemicellulose and lignin as main alignment and has C_4 metabolism (Basso et al., 2014; Lu et al., 2019). It is a potential

feedstock for production of biofuel like biogas (Rodriguez et al., 2017), bioethanol (Ohimain, 2014) and bio butanol (He et al., 2017) as it has strong adaptableness, high growth rate, high biomass yield, drought resistance and wide availability properties. In order to extract monosaccharides from Napier grass, different pretreatment methods are being investigated by different researchers. Cellulose ethanol derived from Napier grass has been extensively studied by Yasuda et al by using Low-Moisture Anhydrous Ammonia (LMAA) Pretreatment combined with enzymatic hydrolysis of Napier grass to obtain a total sugar yield of 0.35 g- sugar per g-biomass, ethanol yield of 74.1% by using *Escherichia coli* (Yasuda et al., 2014). Napier grass has been also converted to butanol by pretreatment, enzymatic hydrolysis and ABE fermentation (He et al., 2017).

In this study, Napier grass was chosen for the carbon source and undergoes with NaOH pretreatment and enzyme hydrolysis to extract monosaccharides from it, which in turn can be used to produce PHA. To the best of the author's knowledge, this is the first study to explore the use of Napier grass as a feedstock to produce PHA.

1.2. Statement of the Problem

With an ever-growing world population, climate change, globalization and industrialization; have intensified the uncontrollable and rapid use of non-renewable energy sources such as fossil fuels and minerals makes planet earth losing its natural resources (Muhammadi et al., 2015). While the growing environmental problem has many sources, the most common organizations directly or indirectly involved are synthetic polymers, in particular plastics. Petrochemical plastics such as polyethylene terephthalate (PET), polyvinylchloride (PVC), polyethylene (PE), polypropylene (PP), polystyrene (PS) and polyamide (PA) have been commonly used as packaging materials. However, due to the high demand for their use for many purposes (low cost, durability and functional advantages), improper discarding, and environmental persistence, their use has actually been cut due to their low biodegradability, which causes significant environmental problems (López-Rubio et al., 2004; Marsh & Bugusu, 2007). Any chemical from these packaging materials can be migrated into food and contaminate the foodstuffs. The contamination at certain level would be poisoning and risking human health.

In 2019, global plastics production reached 368 million metric tons, which accounts around 621.9 billion US dollar. It is projected that the COVID-19's effect on the industry will cause a 0.3% decline in 2020. It is estimated that there would be more plastic particles in the ocean than fish by 2050 (Zhao et al., 2021).

In this regard, there is a societal and environmental need for replacing these materials with alternatives that are more environmentally friendly and biodegradable. To overcome these drawbacks, bioplastics based on renewable resources that are biodegradable, does not harm environment, and non-toxic would be needed as a replacement for synthetic plastics. PHAs, which are fully biodegradable and biocompatible nature; makes them an important substitute for synthetic plastics. They are microbially synthesized (Ganesh Saratale et al., 2021; Neetu Israni & Shivakumar, 2020).

The major challenge for industrial production of PHA is the reliable and scalable supply of carbon sources, as it makes up to 48-50% of PHA production costs (George et al., 2020; Pradhan et al., 2017). So producing PHAs using cheap carbon source would reduce the production cost. Napier grass was largely used for animal feeds, phytoremediation and feedstock for biofuel production (Ayotamuno et al., 2006; Rengsirikul et al., 2013; Yasuda et al., 2014). Stems of Napier grass which are not food competent, agricultural waste, abundant and renewable carbon sources are available in the country can be used for the production of PHAs as a feedstock. In Ethiopia, leaf part of Napier grass is used for animal feedstocks. Hence, it is possible to use the stem part of Napier grass as carbon source for the production of PHAs as it is usually discarded as waste. By using NaOH and enzymatic hydrolysis, high amount of glucose rich hydrolysates can be extracted. In addition, production of PHAs from the cheap, waste, and sustainable substrate Napier grass by *Burkholderia sacchari* strain can solve the aforementioned problems.

1.3. Objective

1.3.1. General objective

The general objective of this research is to investigate the possibility of producing PHAs from glucose rich hydrolysates of Napier grass by *Burkholderia Sacchari* strain.

1.3.2. Specific objectives

In order to attain the general objectives, the following specific objectives were addressed:

- Investigating the yield of glucose from Napier grass by using alkaline and enzyme hydrolysis.
- Investigating the effect of PHAs production process parameters (incubation temperature, fermentation time and pH of the media) on the quality and quantity of PHAs.
- Analyze the characteristics of the extracted biopolymer.

1.4. Significance of the research

This study is an experimental research on PHA production and has the following significances:

- This study will pave the way for further researches on the production of PHAs from Napier grass or other cheap locally available precursors using similar or different methods.
- The study also helps to reduce the amount of imported plastics by replacing them with locally produced bioplastics.
- It increases feedstock for animals and soil fertility by intentionally planting more Napier grass.
- Contributing to carbon credit strategy by increasing cultivation of Napier grass all over the country.

Reducing greenhouse gas (GHG) emission and carbon footprint from production of plastic and plastic waste if it is commercialized.

1.5. Scope of the study

This study explores production of PHAs from glucose rich hydrolysates of Napier grass under unbalanced growth conditions. The study also focuses on effects of incubation temperature, fermentation duration, and pH of the medium on the generation of PHA and characterization of the final product.

2. Literature review

2.1. Plastics

Plastics are man synthesized polymers that are able to withstand microbial attack, sturdiness, light weight, and resistance to water (Shah et al., 2008). The term "plastic" is derived from the Greek word "plastikos," which means "able to be molded into various shapes"(Fried, 1995). Plastics are used all over the world, from drinking cups and disposable silverware to various parts of automobiles and motorcycles. Plastics production has increased dramatically over the last 60 years global plastic output totaled, around 0.5 million tons in 1950 to 368 million tons in 2019 (Lomwongsopon & Varrone, 2022). They are critical to the global trade market as well as material packaging. However, because of the slow rate of degradation, they have caused environmental concern.

Plastics are classified into two types based on their degradability: biodegradable plastic and non-biodegradable plastic. Thermoset and thermoplastic are also the two other types of plastic materials. When heated, thermoset materials harden and become difficult to mold, polymerize and crosslink upon heating. The majority of phenolic plastics are thermosets (phenol-formaldehyde, polyurethane (PUR) and urea-formaldehyde). The application of heat to thermoplastics can soften them repeatedly. This is the most common type of plastic, and its production is growing faster than thermoset materials. Polyethylene and polypropylene (polyolefins), polyvinylchloride (PVC), polyethylene terephthalate (PET), polycarbonate and polystyrene are the most important thermoplastics (Brodin et al., 2017).

2.2. Plastic Waste

The production rate of synthetic plastics are very high as the number of populations around the globe and their demand increases with high consumption; however recycling and non-standard sorting processes of waste plastics is very low. Plastic waste is human made or recovered plastic solid waste that exists in the natural environment and causes disturbing effect towards humans and the environment. Plastics and pollution are two terms that are indistinguishably linked (Lebreton & Andrady, 2019). The global plastic industry is growing promptly; expected to be worth nearly 1700.8 billion USD in 2025 (The Business Research Company, 2021). Synthetic plastics are fossil

fuel based petroleum products that are limited in nature as they need large amount of energy for its extraction and processing that contributes to current problems like human health risks or biome noxiousness, “greenhouse effect” and “global warming”. These effects are caused because of synthetic plastics have short time span and are often incinerated, elevating the atmospheric CO_2 concentration. Afterwards the CO_2 can accumulate in the atmosphere, contributing to the stated climatic effects. Even though incineration of solid wastes can be utilized for energy recovery systems, it has serious health effects to human beings (Braunegg et al., 2007).

2.3. Bioplastics

Polymers are natural or synthetic compounds structured as chains of repeating basic molecular units (monomers). They are macromolecules due to their high molecular weight. Polymeric macromolecules can be natural polymers like; proteins, cellulose, starch and chitin, peptides, DNA, and RNA that are produced by microorganisms. Synthetic polymers such as traditional plastics like PE and PVC and biopolymers such as PHA are also polymeric macromolecules.

Bioplastics are categorized as thermoplastics, thermosets, or elastomers based on how they respond to heat, and they can be biodegradable (such as PLA, PHAs, PHB, PHV& PHBV) or non-biodegradable (like bio-PVC & nylon) (de Vargas Mores et al., 2018; M. H. Rahman & Bhoi, 2021). Bioplastics are polymers typically made from renewable resources such as plants or crops (corn, sugars, potatoes, etc.) and by a variety of microorganisms (bacteria and archaea). Such bioplastics can solve the problem of plastic waste if they are biodegradable within short time range. Bioplastics are formed by polymerizing sugars found in plants or animals. Bioplastics can be cellulose (e.g. cellulose acetate and cellophane), organic polyethylene, starch (thermoplastic starch), aliphatic polyesters, protein etc. based according to their sources (Brodin et al., 2017; Sarkingobir et al., 2021).

In general, bioplastics are plastics that are biodegradable (chemically synthesized polymers, starch-based biodegradable plastics, and PHAs (the only 100% biodegradable polymer)), completely biological in origin or partially biologically derived (Khanna & Srivastava, 2005; A. Rahman, 2014).

Bioplastics are grouped in to three main types based on their monomeric units as (Polylactic Acid PLA), Bio Polyethylene (BioPE) and PHA by using raw materials after pretreatment to change the polysaccharide to monomeric units (Brodin et al., 2017).

2.3.1. Polylactic Acid (PLA)

PLA is aliphatic polyester derived from **α**-hydroxyl acids produced from lactic acid (commonly produced by carbohydrate fermentation of corn Dextrose) obtained from renewable resources like maize, corn, starch or sugarcane followed by polymerization and are biodegradable. It can be synthesized by the condensation and polymerization of lactic acid or through the ring-opening polymerization of lactide (M. Kumar et al., 2017; Lim et al., 2008). For example, in order to produce PLA, corn is milled and dextrose is removed. Dextrose is fermented in large vats by bacteria or yeast to produce lactic acid, which acts as a monomer in the synthesis of Polylactic acid. Lactic acid is transformed into oligomers known as lactides throughout this process. PLA is formed by the joining of the lactic acids (2-hydroxy propionic acid) (Philp et al., 2013). Polysaccharides, lipids (including poly-p-hydroxybutyrate), and polyphosphates are the three primary groups of chemicals that have traditionally been regarded as potential storage molecules in microorganisms.

When compared to conventional petroleum-based polymers like PS and PET, PLA has relatively good optical, physical, mechanical, low processing temperature and barrier characteristics. Hydrolysable property in human body make PLA for biomedical and pharmaceutical application as well as for packaging purposes (Auras et al., 2003).

2.3.2. Bio Polyethylene (BioPE)

BioPE is formed by catalytic polymerization of ethylene grouped under a family of aliphatic polyolefin with a market share of 30% global plastic market. BioPE might replace petroleum-based PE in a wide range of uses, including containers, shampoo bottles, pipes, and kitchenware. BioPE is made from ethylene, which is obtained through catalytic dehydration of bioethanol (European Bioplastics, 2017). Bio ethanol is produced by fermentation of sugarcane feedstock that

in turn used for production of bio ethylene as replacement of petrochemical feedstock (Brodin et al., 2017).

2.3.3. Polyhydroxyalkanoate (PHA)

PHAs are microbial bio polyesters mainly produced by microorganisms through fermentation of renewable resources like carbohydrates, lipids, alcohols or organic acids and exist inside the cell or cytoplasm of the microbe (Jong Il Choi & Lee, 1997).The finding of short chain length PHA was the first stage of PHAs development (scl-PHA). It was discovered by Beijerinck as cellular inclusions of bacteria and other microorganisms in 1888 (Khanna & Srivastava, 2005). From 1926 to 1944, Lemoigne established the presence of the biopolymer (PHB, polyhydroxybutyrate) in the genus *Bacillus*, and in 1942, PHB granules were separated from protoplasm (Lemoigne, 1926).

Different hydroxylalkanoic acids; homo or copolysters depending on monomeric configuration are formed based on the type of strains, carbon source and culture methods used during the production process under osmotic stress conditions and nutrient imbalance makes variation in structure and physiochemical properties (Jong-il Choi & Lee, 1999) . Poly-3-hydroxybutyrate (PHB)), poly-3-hydroxybutyrate-co- 4-hydroxybutyrate (P(3-HB-co-4-HB)), poly 3-hydroxybutyrate-co-valerate (PHBV) and polyhydroxybutyrate-co-hexanoate (PHBH) are some of PHAs that are commercially available differed by their monomeric units. During production of PHAs, fermentation and down-streaming process are required (Mohapatra et al., 2020).

Different strains produce PHAs as energy and carbon source via excess carbon source and limiting nutrients like nitrogen, phosphorus, Sulphur, Oxygen, or magnesium. After these process occurred, the energy stored in the cell degraded and used (Khanna & Srivastava, 2005).

2.4. Biodegradability

The action of living microorganisms or their enzymes using sustainable raw materials (biomass) as carbon source produce biodegradable plastics. Most biodegradable plastics are used for packaging materials and special applications in the medical field because they can be degraded in a variety of conditions by bacteria that produce PHA depolymerases. They produce small amount of greenhouse gases, toxic pollutants and energy compared to petroleum based plastics. The degree

of breakdown and the time required for complete mineralization (oxidation to CO_2 and H_2O) when degradation is aerobic, and methane production in addition to CO_2 and H_2O when degradation is anaerobic, are the major parameters for the biodegradation process (Muhammadi et al., 2015).

According to the International Standard Organization (ISO) a polymer is classifiable as biodegradable if its chemical structure can be modified when subjected to the action of organisms such as bacteria, fungi, and algae giving rise to the loss of some typical properties of the starting material. The biodegradability shall be demonstrated for each of its significant organic constituent present in greater than 1% with respect to the dry mass. ISO allowed organic constituents whose non-biodegradable component appears in a quantity not exceeding 1%.

	Petrochemical	Partly bio-based	Bio-based
Non-biodegradable	PE, PP, PET, PS, PVC	Bio-PET, PTT	Bio-PE
Biodegradable	PBAT, PBS(A), PCL	Starch blends	PLA, PHA, Cellophane

Figure 2.1: Comparison of Biodegradability of Bio-based and Petrochemical Plastics (van den Oever et al., 2017)

2.5. Polyhydroxyalkanoates

PHAs are microbial bio polyesters largely produced by bacteria inside cells that are water insoluble in the range of 0.2-0.5 micrometer as a source of energy to overcome unfavorable conditions (Dawes & Senior, 1974; Singh Saharan et al., 2014).*Acinetobacter*,*Pseudomonas*, *Sphingobacterium, Brochothrix, Caulobacter, Ralstonia, Burkholderia,* and *Yokenella* are bacterial strains capable of producing PHA while degrading oil and have been isolated from oil contaminated sites. *Cyanobacteria* as photosynthetic prokaryotes, *Burkholderia terricola, Lysobacter gummosus, Pseudomonas extremaustralis, Pseudomonas brassicacearum,* and *Pseudomonas orientalis* as plant growth promoting rhizobia, *Archaea* as Halophiles, *Streptomyces* as antibiotic producers are other types of strains capable of producing PHA (Bilia et al., 2021; Ganesh D. Saratale & Oh, 2015; Sayyed et al., 2021; Singh Saharan et al., 2014).

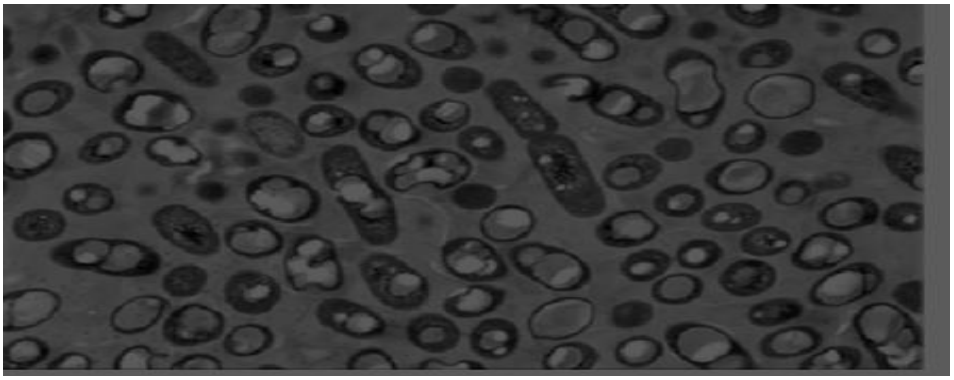

Figure 2.2: Microscopic image of PHA granules(Sharma et al., 2021)

2.6. Structure and Classification of PHAs

PHAs are stored inside the cells of strains from a few granules up to 90% of the dry weight of the cell. In reality, PHAs are a wide set of polymers that are very diverse in terms of the amount of CH_2 groups in the main chain and the alkyl group in the (R) position, with over 150 distinct PHA monomers (Solaiman et al., 2000). From PHAs family, Poly (3-hydroxybutyrate) (PHB), a homo polymer of 3-hydroxybutyrate, is the best prevalent and characterized member of them.

On the basis of chemical structure of monomers, PHAs can be homopolymers having only one type of monomer such as poly (3-hydroxybutyrate) and poly (3-hydroxyhexanoate) or copolymers having more than one type of monomer such as (3-hydroxybutyrate-co-hydroxyhexanoate). Hence, PHAs properties depend on its monomer composition.

PHAs are classified based on number of carbon monomer units as Short chain length PHA (scl-PHA) consist of monomeric building blocks of 3-5 carbons e.g. P(3HB), P(4HB),& P(3HV) like from *Alcaligenes eutrophus*, Medium chain length PHA (mcl-PHA) consist of monomeric units of 6-14 carbons e.g. P(3HHx) & P(3HO) like from *Pseudomonas* and Long chain length PHA (lcl-PHA) contain monomer building blocks of 15 carbons or more than 15 carbons with the same general structure (Z. Li et al., 2016; Sharma et al., 2021). Monomer chain length influences melting point, hydrophobicity, degree of crystallinity and glass transition capacity. The type of microbe and the growth conditions employed determine the molecular weight of the PHAs generated varies from $2*10^5$ to $3*10^6$ Da (S. Y. Lee et al., 1997).

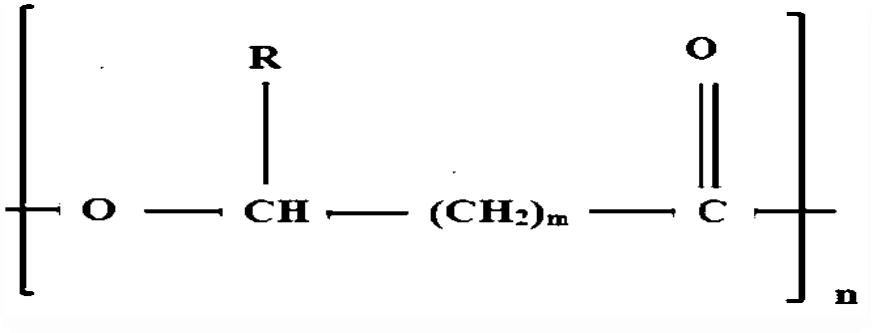

Figure 2.3: General structure of PHA

Where: R (alkyl side chain)- contains 1 up to 13 carbons; m=1, 2 or 3; n=100 to 35,000 of monomeric repeat (degree of polymerization) (Sharma et al., 2021).

2.6.1. General properties of PHAs

PHAs mostly show similar properties as petroleum-based polymers. Melting temperature, glass transition temperature (Tg), crystallinity, tensile strength, and extension to break percentage are some of the physical properties that depend on their monomer composition and purity. They are thermoplastic biopolymers that differ in chemical composition and by the length of the side chain like melting temperatures ranging from 40 to 180 °C. Differential Scanning Calorimetry (DSC) is commonly used to characterize the melting endotherms of crystallized PHA samples. DSC is also used to measure Tg of PHA in melt-quenched amorphous materials with P(3HB) homopolymer is about 4 °C (S. Kumar & Abe, 2010).

PHAs are water insoluble and resistant to hydrolytic degradation, nontoxic and even upon degradation, PHAs does not release any toxic compounds and are biocompatible. They are soluble in chloroform and many other chlorinated solvents. They have high degree of polymerization, high degree of biodegradability, and thermoplasticity. They usually sink in water allowing them for anaerobic degradation. In addition, they are good UV resistant and stiff while they have poor resistance towards acids and bases (Bugnicourt et al., 2014; S. Kumar & Abe, 2010; Sarkingobir, Sarkingobir, & Lawal, 2021; Sarkingobir, Sarkingobir, Lawal, et al., 2021).

Table 2.1: Properties of PHA's summary(Bugnicourt et al., 2014; Padermshoke et al., 2005)

Property [units]	Values
Glass transition temperature, Tg	−50 to 4 °C
Melting temperature, Tm	40 -180 °C
Crystallinity degree, Xcr	40-60 %
Young's modulus, E	1-2 GPa
Tensile strength, δ	15-40 MPa
Oxygen transmission rate, OTR	55.12 cc mm m^{-2}·day^{-1}
Water vapour transmission rate, WVTR	2.36 g mm m^{-2}·day^{-1}
Elongation at break, Ɛ	1-15 %

Table 2.2: Different types of PHAs with their properties (Bugnicourt et al., 2014; Koller, Salerno, et al., 2013; Luangthongkam et al., 2019; van der Walle et al., 2001; Volova et al., 2013)

PHA Polymer	Melting temp. (°C)	Glass transition temp. (°C)	Crystallinity (%)	Tensile strength (MPa)	Extension to break (%)
P(3HB)	177	2	60	43	5
PHBV	145	− 1	56	20	50
P(3HB-*co*-4HA	146	NR	26.7	389	N.R
P(4HB)	60	− 50	N.R	104	1000
P(3HB-co-3HV)	150	− 7.25	55–70	25	20
PHV	106.2	− 15.7	N.R	6.6	3.5
P(3HB-*co*-4HB)	166	N.R	N.R	28	45
P(3HO-co-3HH	61	− 35	N.R	9	380

PHA	173. 8	23. 39	N.R	15.9	3.6
P(3HB-co-3HO)	133	− 8	N.R	17	680
PHBHx	127	− 1	34	21	400
PHB4B	150	− 7	45	26	444
P(3HB-co-3HHx)	122	N.R	N.R	10.3	1.1

2.7. Lignocellulosic Materials

Lignocellulosic materials are the most abundant raw materials on earth amounting around 200 billion tons annually, which include lignin (complex polyphenolic structure), cellulosic (b-D-1, 4-glucan), and hemicellulosic (D-arabinose, D-xylose, D-mannose, D-glucose, D-galactose, and sugar alcohols) fibers. Lignocellulose accounts for 60% of total plant biomass. The proportions of lignin (5–25%), cellulose (30–80%), and hemicellulose (10–40%) in Lignocellulosic biomass vary depending on type of biomass used (Chandel & Singh, 2011; M. Kumar et al., 2017).

Cellulose having the molecular formula $(C_6H_{10}O_5)n$ is an organic chemical molecule that has long-chain polymer of β-(1→4)-D-glucopyranoside units that are crystalline linear structure with rigid component of cell wall. Hemicellulose mainly classified based on the residue sugar on the backbone as pentose like xylose and arabinose, and hexoses such as glucose, galactose and mannose that is a branched polysaccharide unit. Lignin is a complex organic polymer that embedded cellulose and hemicellulose, which acts as a binder, conferring on plants structural support, impermeability and resistance against microbial attack and oxidative stress (Cesário, Raposo, de Almeida, et al., 2014).

Agricultural and forestry residues, municipal solid waste, and herbaceous and woody crops are all sources of lignocellulose biomass. The major challenges to use lignocellulosic materials for biotechnological conversion is the high stability due to a high density of ester and ether bonds between lignin and carbohydrate part of the biomass. In order to convert such materials in to useful products, several pretreatment methods were developed. Among different methods, enzymatic

hydrolysis and chemical hydrolysis are the main methods to convert these materials to smaller units, monosaccharides.

Lignocellulosic materials like rice straw, corn straw and bagasse are among those materials used in several end products as they contain high percentage of carbohydrates and low lignin (Obruca et al., 2015). Lignocellulosic materials have significant potential for the production of a wide range of industrial and commodity products such as paper, lumber, bioethanol, biodegradable polymers such as PHAs, and a variety of fine chemicals.

2.8. Pretreatment Methods

The internal structure of lignocellulosic biomass is complex and it is made of cellulose, hemicellulose, and lignin. Increasing surface area by reducing cellulose crystallinity improves and accelerates degradation process by microorganisms. Hence, lignocellulosic materials should be conditioned suitably to undergo fermentation process (S. Kumar & Abe, 2010; Obruca et al., 2015). In general, proper pretreatment of lignocellulosic materials in addition to enzymatic hydrolysis is needed to convert such materials to glucose units. Cellulase is a type of enzyme that have a potential of degrading cellulose components in to glucose yielding variety of products. There are different pretreatment methods for lignocellulosic materials such as physical (chipping, grinding and milling, extrusion, ultrasound, microwave, etc.), thermal (steam explosion 160-220 °C, oil bath and autoclave), chemical (acids and alkalis), biological (bacterial, fungi and enzymatic activity) (Koller, Niebelschütz, et al., 2013; Obruca et al., 2015).

2.9. Demand of Bioplastics

Bioplastics are becoming more popular, with the global bioplastics market expected to grow by 36 % over the next five years (European Bioplastics, 2017). In 2015, bio-based plastics accounted for more than 75% of global bioplastics manufacturing capacity. In 2010, the proportion of durable bioplastics in worldwide production capacity was only around 45% of the entire share of 0.8 million metric tons of bioplastics. Biodegradable plastics market size is anticipated to reach USD 6.12 billion by 2023, up from USD 3.02 billion in 2018, at a compound annual growth rate (CAGR) of 15.1%. Rising incomes in emerging nations, as well as rapid expansion in the agricultural &

horticulture, packaging & bags, and textile industries, are boosting the biodegradable plastics market (Research and Markets, 2022).

The worldwide PHA market is expected to reach USD 98 million by 2024, increasing at a CAGR of 11.2 percent from USD 57 million in 2019. Globally, Europe is the most important market for PHA, followed by North America and Asia-Pacific region. The existence of many PHA companies, including Bio-on (Italy), Biomer (Germany), Natureplast (France), Total Corbion PLA (Netherlands), Mitsubishi Chemical Corporation (Japan), and EarthBi (Germany), has a beneficial influence on the market. Furthermore, the region's rising usage of biodegradable plastic in packaging and food service, bio-medical, and agricultural applications is increasing demand for PHAs (Escobar & Britz, 2021).

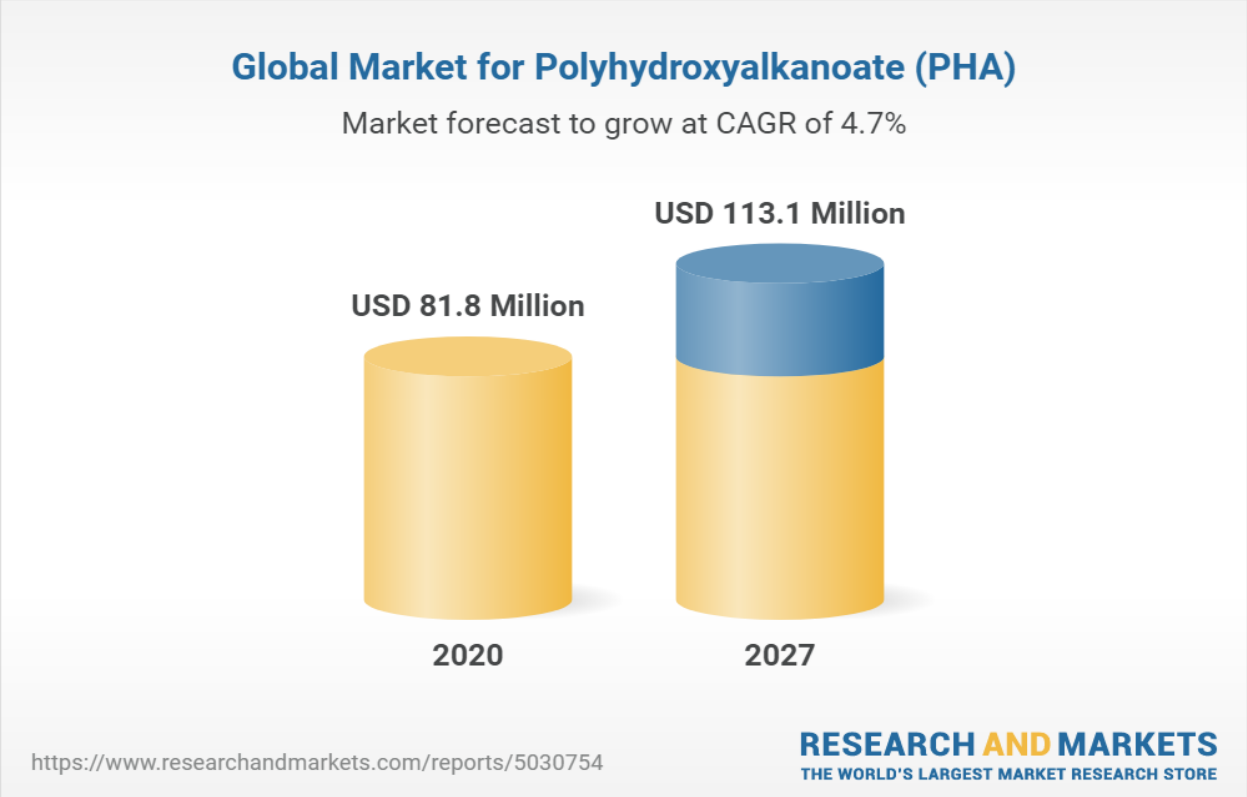

Figure 2.4: Global PHAs Market (Research and Markets, 2022)

Table 2.3: Durable Bioplastic Market Growth *(Verma, 2020)*

Year	Total capacity (million tons)	Durable (%)
2010	0.7	42.3
2013	1.58	62.4
2014	1.697	60.9
2015	3.952	75
2016	4.16	76.8
2020 (Estimated)	5.95	79

2.10. Biosynthesis of Polyhydroxyalkanoates

PHA can be produced in three ways: enzymatic catalysis, synthesis in genetically modified plants, and microbial fermentation. Among these, microbial fermentation was discovered to be an efficient method (Ahn et al., 2001; Park et al., 2001). The catalytic mechanism for PHA synthesis by bacteria incorporates many routes that are connected to microbe metabolic processes such as glycolysis, β -oxidation, and so on. There are three PHA biosynthesis routes that occur spontaneously in bacteria.

Pathway I

With the aid of the β -ketothiolase enzyme, two molecules of acetyl-CoA (from the Tricarboxylic Acid Cycle) are condensed into a molecule of acetoacetyl-CoA in this route. An NADPH acetoacetyl-CoA reductase (phaB) converts acetoacetyl-CoA into 3-hydroxybutyryl-CoA and finally PHA synthase (phaC) forms ester bonds and polymerizes the monomers into P(3HB). A related PHA degrading pathway consists of a PHA depolymerase, a hydrolase, a dehydrogenase and an acetoacetyl-CoA synthase (Laycock et al., 2013; Yogesh et al., 2012).

Pathway II

It is used by some species of *Pseudomonads* for mcl PHAs, or PHA copolymer synthesis of (R)-3-hydroxybutyrate and (R)-3-hydro- xyhexanoate. The substrates for this process come from the -

oxidation of fatty acids. A 3-ketoacyl-CoA reductase, an epimerase, a (R)-enoyl-CoA hydratase, an acyl-CoA oxidase, and an enoyl-CoA reductase are all required for 3-hydroxy acyl-CoA production. PHA synthase then polymerizes the resulting monomers. Simple carbon sources are used in the third route (III) to generate (R)-hydroxy acyl-CoA monomers with the help of acyl-ACP-CoA transacylase (Yogesh et al., 2012).

Pathway III

The primary precursor molecule of 3-hydroxy acyl-CoA that leads to PHA production is (R)-3-hydroxy acyl-ACP. Simple, low-cost carbon sources such as glucose, sucrose, lactose, and others obtained by microorganisms from environmental sources such as wastewater, activated sludge, animal fats, and hydrocarbons are used to convert (R)-hydroxy acyl intermediates from their acyl carrier protein form to Co-A form via the action of acyl-ACP-CoA transacetylase, and then hydroxyalkanoate monomers are eventually polymerized (Castilho et al., 2009; Laycock et al., 2014).

PHAs are produced by certain bacteria via several routes. *Aeromonas caviae*, for example, lacks a thiolase and a reductase, instead relying on an enoyl-CoA hydratase to manufacture (R)-3-hydroxy monomers from fatty acid oxidation. Depending on the fatty acids utilized, this bacterium may produce a variety of copolymers.

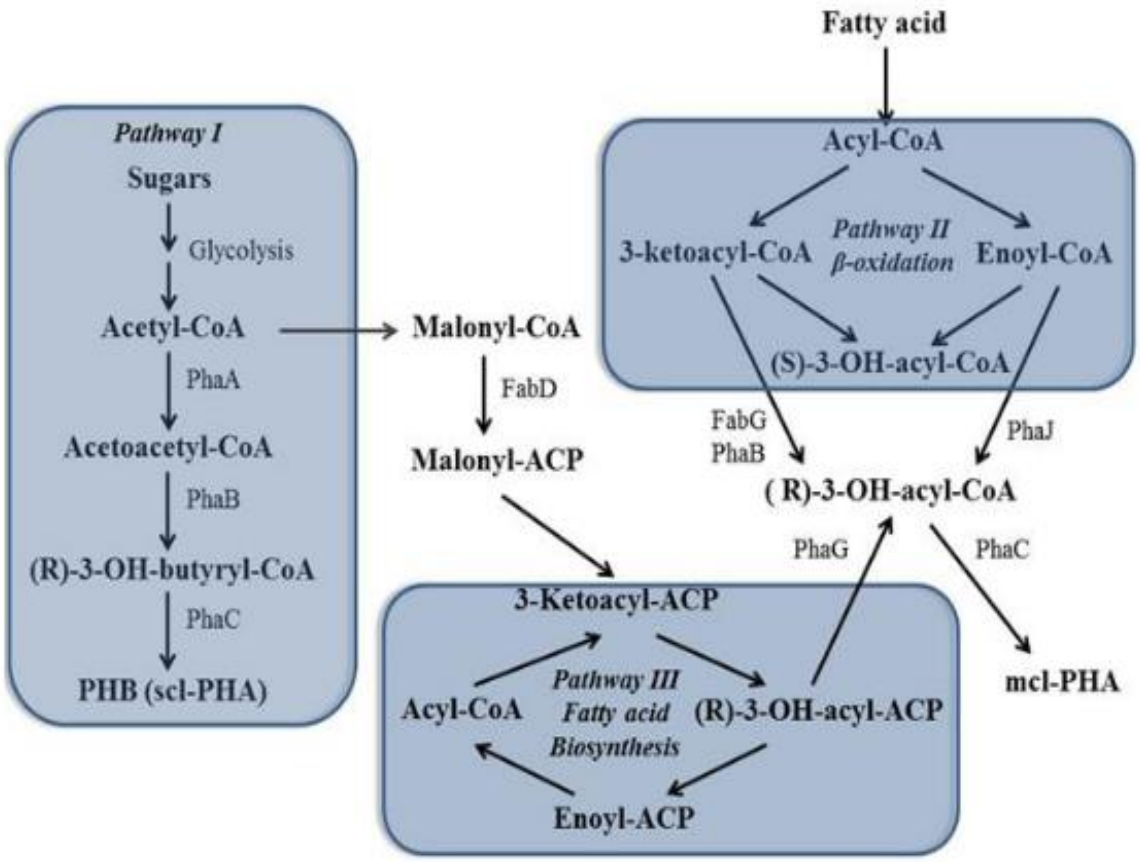

Figure 2.5: Biosynthetic Pathways of PHAs (Kalia et al., 2019)

In general, the choice of operation methods is influenced by several factors during the PHA production process, such as microbial selection, substrate or raw material (carbon source i.e. glucose or complex waste material) for the fermentation process, bioreactor types, culture (pure or mixed), fermentation modes (batch, fed-batch, continuous), and PHA extraction methods.

2.11. Fermentation Methods

Many PHA 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 PHAs 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. Batch, fed-batch and continuous fermentations are the three types of fermentation processes that

are commonly used for production and multiplication of these bacterial strains. Fermentation can take place in a single stage or many stages in a sequencing batch reactor (SBR).

Batch Fermentation

Batch fermentation mode is when the substrate and producing microorganism are added to the reactor before the fermentation starts, there is no addition or withdrawal of nutrients or products during the process and it is a closed system. Because of its versatility and cheap operating costs, batch fermentation for PHA synthesis is a common technique. However, it is linked to low PHA productivity because after using the carbon source, bacterial cells destroy the stored PHA, resulting in lower PHA content.

Fed-batch Fermentation

Fed-batch fermentation is 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 PHA. Kulpreecha *et al*., (2009) employed fed-batch cultivation and obtained cell mass of (72.6 g L^{-1} DW) and PHB content (42 percent of cell dry wt.) in a short culture time (24 h), resulting in increased PHB productivity (1.27 g L^{-1}) by using *Bacillus megaterium*.

Continuous Fermentation

Another alternative for the third operation method for PHA generation is continuous culture, also known as chemostat. The culture broth is continually changed with sterile media in this approach. The carbon source is continually supplied in excess in Chemostat culture, limiting one or more nutrients (e.g., phosphorous or nitrogen). Chemostat is extremely controlled since the specific growth rate may be kept constant by changing the dilution rate. As a result, given the right conditions, continuous fermentation has the potential to provide the greatest levels of PHA production (Zinn et al., 2001).

In this study, fed-batch fermentation process was chosen because it is 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 (Singh, 2017). Substrates, both carbon and nitrogen, sources are added to avoid any depletion during the production process until the preferred cell biomass yield is achieved in fed batch fermentation process. Continuous fermentation method was not selected because of resource limitation.

2.12. Extraction and Purification of PHAs (Downstream processing)

Extraction and purification of PHA macromolecules produced by microorganisms is a critical step in further processing and determining products quality and quantity. Different methods are used to ensure a polymer of high quality and purity, with varying purification outcomes and recovery from non-PHA cell mass. Residual biomass is primarily composed of polypeptides phospho lipids, DNA, RNA, and peptidoglycans and separated by using sedimentation, filtration, centrifugation methods (Koller, Niebelschütz, et al., 2013). Solvent extraction, flotation extraction, digestion extraction, supercritical fluid extraction, and aqueous two-phase extraction are some of PHAs extraction methods. Cell lysis and PHA recovery are steps that are almost universally used in all of these extraction and purification methods. To separate these polymeric macromolecules from all other cell debris mater, different extraction solvents or surfactants are used (Muneer et al., 2020).

The recovery system can influence the amount of product recovered, the ease of subsequent purification steps, and the final product quality. The first step in the recovery method is to separate the cells from the fermentation broth. To recover the PHA granules, the bacterial cell must be ruptured and the protein layer that coats the PHA granules must be removed. PHA is extracted directly from ground biomass by dissolving it in an organic solvent, typically propylene carbonate, chloroform, chlorolimited methylene chloride, and dichloroethane.

Solvent Extraction Method

Solvent extraction method involves three steps. It starts with pretreatment of cells to rupture and expose PHA granules. After that, the granules are made soluble in a suitable halogenated solvent

(chloroform, dichloromethane, polychlorinated ethane, etc.) and non-halogenated solvent (acetic acid, acetic acid anhydride, n-methl-pyrrolidone, and tetrahydrofuran, ethyl- and methyl lactate) followed by non-solvent solution for precipitation (waraich, 2009). The solvent extraction method of PHAs offers the advantages of eliminating endotoxins, high polymer purity, easy operation, and undamaged polymer, making it the most widely used extraction approach (Raza et al., 2019). The hazardous nature of chloroform (high environmental and health risk), in particular, is a key disadvantage of this approach.

Digestion Method

Because equipment is the main cost in PHA production, the digestion method using low-cost chemicals or enzymes provides an efficient and cost-effective recovery process. In chemical digestion, sodium hypochlorite and chloroform are used, as well as surfactants such as anionic sodium dodecyl sulfate (SDS), which disrupts cells by integrating itself into the lipid bilayer membrane, whereas in enzymatic digestion, a series of steps such as heat treatment, enzymatic hydrolysis, and surfactant washing are used, as well as proteolytic enzymes (Alcalase, Neutrase, Lecitas and Lysozyme) are target specific and can recover PHAs efficiently (Jacquel et al., 2008; Koller, Niebelschütz, et al., 2013; Koning & Witholt, 1997).

Flotation Method

Flotation method is solvent extraction in a modified form. When cell debris is left in chloroform for 72 hours at 30 °C, it floats to the surface of the extraction solvent. High purity (98%) with an 85 percent recovery efficiency, as well as a reduction in polymer waste during extraction can be achieved using this method. However, this method is slow and time consuming process (Ibrahim & Steinbüchel, 2010).

Supercritical Fluid Extraction

Supercritical fluid extraction is highly applicable for extraction of high value products and uses gaseous CO_2 (because of its high solubility for numerous compounds and fast right after the extraction), ammonia, and methanol (Hejazi et al., 2003). Temperature and operating pressure are important factors in changing the behavior of the cell membrane to extract PHA. Despite its great

complexity, the supercritical fluid extraction approach yielded PHAs of the highest purity (99.99 percent) for medical applications while also being more environmentally friendly (Hejazi et al., 2003; Williams & Martin, 2005).

Biological Extraction Method

Insects such as black soldier fly can feed on lyophilized cells of *Cupriavidus necator* (grown on a high carbon source for intracellular PHB synthesis) to enhance their protein content during the early stages of development. When the feeding is over, PHB can be removed from the fecal pellets using the black soldier fly larvae's gut. This biological technique extracts 82.95 percent pure PHB. High purity (82.95%) of PHB granules can be extracted using this method (Mohamad et al., 2020).

In general, the application of a PHA recovery approach is multidimensional and, among other things, is determined by different factors such as the microbial production strain (various strains have varied cell envelop fragilities), type of PHA in terms of molecular makeup (scl-PHA, mcl-PHA), PHA's intracellular load, needed product purity as established by the biopolymer's eventual application, chemicals for PHA recovery such as extraction solvents, may eventually be disposed of in-house, and allowable effect of the isolation process on the final molar mass of PHA (Koller, Niebelschütz, et al., 2013).

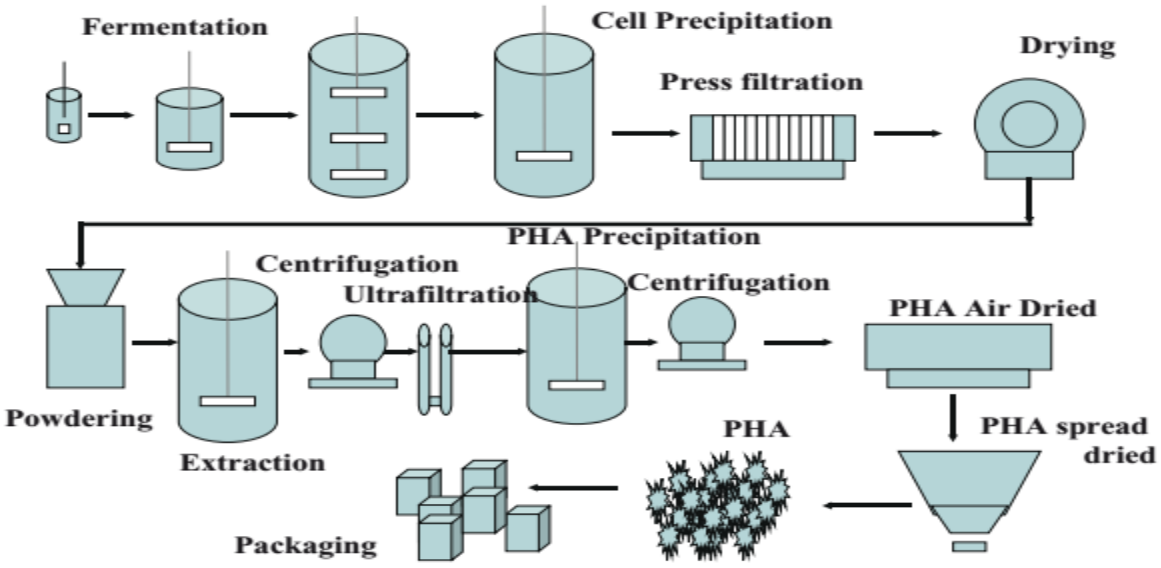

Figure 2.6: General PHAs Industrial Production and Extraction Process

2.13. Parameters Affecting PHA Production

Effect of Fermentation Period on PHA Production

The percentage of yield will be used to monitor PHA production from the first 24 hours of incubation to the start of the logarithmic phase at 120 hours. A previous report showed that there is a link between bacterial growth and PHA accumulation, and 96 hours of cultivation was an ideal period for maximal PHA synthesis by Bacillus subtilis and E. coli (Zakaria Gomaa, 2014). PHA buildup by a given bacterial species is directly related to its biomass, which eventually decreased as carbon was depleted, resulting in PHA consumption.

Effect of Initial Temperature on PHA Production

Changing the incubation temperature will reveal a significant connection between PHA production and incubation temperature. Most of the time, the greatest PHA synthesis and bacterial biomass occurs at 35 °C. However, it will vary when we employ different microbial strains, for example, *Nacardiopsis potens, Rhizobium elti, Pseudomonas stutzeri*, and *Vibrio harveyi* may generate maximal PHA at 30 °C while 50 °C was the optimum for marine *Saccharococcus* thermophiles. (Belal, 2013; Kalaivani & Sukumaran, 2013).

Effect of NaCl Concentration on PHA Production

Controlling the salinity of the medium within a reasonable range is required to prevent excessive osmotic stress from affecting PHA synthesis by changing NaCl content. In general, stress circumstances are thought to modify polymer synthesis and boost degradation potential in order to promote bacterial survival. The intracellular level of PHAs can reach 71.1 % when NaCl concentration was 250 g L^{-1} using *Haloferax mediterranei* (Cui et al., 2017). Passanha *et al*. showed that the addition of 9 g L^{-1} NaCl can result in the greatest PHA generation, yielding 30 % more PHA than the control. It was shown that increasing the concentration of NaCl to 15 g L^{-1} inhibits the formation of PHA. As a result, NaCl addition can be utilized as a straightforward, low-cost, long-term, non-toxic, and non-reactive external stress approach for boosting PHA production by using *Cupriavidus necator* (Passanha et al., 2014).

Effect of Carbon Sources on PHA Production

Carbon is the foundation of nearly all biomass sources. Carbon is used as an energy source by living organisms, whether multicellular or unicellular, for regular metabolic functions. Carbon is clearly the most important component for the synthesis of PHAs by bacteria and all other microorganisms. There is a vast amount of carbon feed stock accessible in nature that may be utilized as a sustainable and low-cost source of carbon to feed microorganisms that produce PHAs. Glucose is the most prevalent and well-studied source of energy for microorganisms. Glucose, sucrose, lactose, and a variety of other polysaccharides can be used as carbon sources for bacteria, and a significant amount of PHA can be extracted (Favaro et al., 2019; Hawas et al., 2016).

The carbon supply is a significant nutritional element that plays an important role in PHA synthesis because the bacterium stores it in the form of PHA granules. Carbon is employed as a fundamental dietary component to meet energy needs while also acting as a biogenetic substrate for microbial growth and PHA synthesis. As a result, selecting the appropriate carbon source will enhance the yield of PHA synthesis. Carbon's three key roles for microorganisms are cell maintenance, cell biomass production, and polymerization of PHAs molecules (N. Israni & Shivakumar, 2015).

One of the primary drawbacks of total PHA production costs is the high cost of the carbon substrates chosen as feed stocks. As a result, selecting an inexpensive and frugal carbon substrate is critical in order to allow a market total cost for the end product. Up to 50% of the prices are determined by the precursor substrate materials, primarily the carbon supply. The selectivity of carbon sources is determined by their cost, practicality, and the microorganisms employed in the PHA manufacturing process, as various bacteria require different carbon sources for growth and energy. Carbon sources as growth substrates are classified to three main groups' triglycerol, sugars and hydrocarbons (sucrose, lactose, starch and lignocellulose) (Fournet et al., 2020; Santhanam & Sasidharan, 2010; Urtuvia et al., 2014).

Effect of Nitrogen Sources on PHA Production

The nitrogen supply is the main limiting element for PHA synthesis, since bacterial strains require a high concentration of nitrogen at the start of the fermentation process in order to build high biomass, and then begin producing the polymer as a survival mechanism when nitrogen levels fall

(Hungund et al., 2013). A variety of organic and inorganic nitrogen sources are being studied by various researchers. Inorganic nitrogen sources such as sodium nitrate, potassium nitrate, diammonium hydrogen orthophosphate, ammonium chloride, ammonium sulphate, ammonium nitrate, ammonium acetate and organic nitrogen sources like glycine, peptone, beef extract, yeast extract, tryptone, urea. (Hungund et al., 2013; Kulpreecha et al., 2009; Tripathi et al., 2012; Zakaria Gomaa, 2014).

Effect of C:N Ratio on the PHA Production

PHA production and bacterial growth were dramatically reduced at a high C:N ratio due to substrate inhibition, where glucose has an inhibitory impact at a high level, influencing the specific growth rate and PHA production. This discovery highlights the importance of nitrogen limitation, which is inversely proportional to PHA production.(Patel et al., 2017). Mostafa *et al* utilized *Erythrobacter aquimaris* and varied the C:N ratio in the medium at 4.7:1, 9.2:1, 18.4:1, and 36.8:1 and discovered that the greatest PHB production occurred when the C:N ratio in the medium was 9.2:1 and reached 7.3 g/L.(Mostafa et al., 2020).

2.14. Applications of PHAs

Petroleum crisis in 1970's was the major turning point for companies to produce bio-based products. PHA is one of the bio-based products that gains much attention for a potential substitute for petroleum-based plastics. Because PHA is biocompatible , nontoxic and degradable, it is expected to replace synthetic non-degradable polymers in industrial applications such as packaging, agriculture, leisure, fast food, hygiene, medicine, and biomedical applications (Muhammadi et al., 2015).

Biomedical applications of PHAs

PHAs have received a lot of interest as materials for biocompatibility, biodegradability and green credentials implants in biomedical and tissue engineering applications (Chen, 2010). The key benefit in the medical industry is that a biodegradable plastic may be introduced into the human body and then removed. PHA has excellent biocompatibility since it is a byproduct of cell metabolism, and 3-hydroxy butyric acid (a byproduct of breakdown) is routinely present in blood

at quantities ranging from 0.3 to 1.3 mmolL^{-1} (Zinn et al., 2001). PHB, in particular, has been shown to be biocompatible with a variety of cell types that makes them used as drug delivery systems. Some of the applications are to deliver antineoplastic agents to cancer cells, targeting certain breast cancer cells nanoparticle of PHAs, for releasing the antibiotics gentamycin and tetracycline, bone tissue engineering applications (produce scaffolds), in prolonging stability of silver nanoparticles, for inhibiting pathogenic bacterial growth in aqua system (Gumel et al., 2013; Kılıçay et al., 2011; J. Lee et al., 2011; Puppi et al., 2010; Wei et al., 2009; X. Zhang et al., 2009).

Agricultural Applications of PHAs

PHA offers a wide range of agricultural uses. Under harsh conditions, PHA improves the nutritional qualities and shelf life of plants. Controlled release of pesticides and insecticides is one of the specific uses of P(3HB) in agriculture. PHA has the potential to be used in agriculture in a variety of ways, including biodegradable plastic sheets for crop protection, seed encapsulation, fertilizer encapsulation, and so on (González et al., 2020).

Industrial Applications of PHAs

PHAs have the potential to replace petrochemical-based polymers in a variety of other applications. PHAs are hydrolyzed and then methyl esterified to produce 3-hydroxyalkanoates methyl esters with energy content equivalent to bioethanol as biofuel from renewable biomass (Gao et al., 2011;Z. J. Li et al., 2009)). In the paper industry, PHA latexes have been utilized for surface coating and as sizing agents by utilizing paper drying temperature, drying duration, pressure, and polymer composition (Bourbonnais & Marchessault, 2010). PHAs have been used for packaging materials with a small amount due to its high glass transition temperature, which causes brittleness under normal usage circumstances. They have also been used in food industry(Castro Mayorga, 2017; Pérez-Arauz et al., 2019).

3. Material and Method

3.1. Chemical and Equipment

All chemicals were analytical grades and purchased from various suppliers. Napier grass were obtained from Menagesha mushroom farm in Ethiopia. Napier grass was chopped with scissors, crushed using a cutting mill machine, and pretreated for glucose production with NaOH and a multicomponent Cellulase Enzyme. The concentration of synthesized reducible sugars was determined using the Dinitrosalicylic Acid reagent via UV/VIS spectroscopy. Luria Bertani broth was utilized for growth media with Na_2HPO_4, K_2HPO_4,$(NH_4)_5Fe(C_6H_4O_7)_2$,$CaCl_2{\cdot}2H_2O$,$MgSO_4$ and $(NH_4)2SO_4$ as minimal salt media for propagation and fermentation by *Burkholderia sacchari* strain that produced PHA. Chloroform, sodium hypochlorite, methanol and distilled water were used for the extraction of PHA from the dried cell biomass and Sudan B. Black and Safranin were also utilized for staining purposes.

3.2. Methodology

Figure 3.1 illustrates the methodology used to conduct the experiment. This study has four major parts: glucose production, inoculation, propagation and fermentation, extraction and purification and characterization of the produced PHA. Initially, pretreatment of Napier grass by using NaOH and enzymes and characterization of the produced reducible sugars were determined. Afterwards, reducible sugar was mixed with others for propagation and fermentation purposes by varying fermentation temperature, pH and time. The PHAs were then separated and purified from the cell debris. Finally, characterization of the purified PHA were investigated.

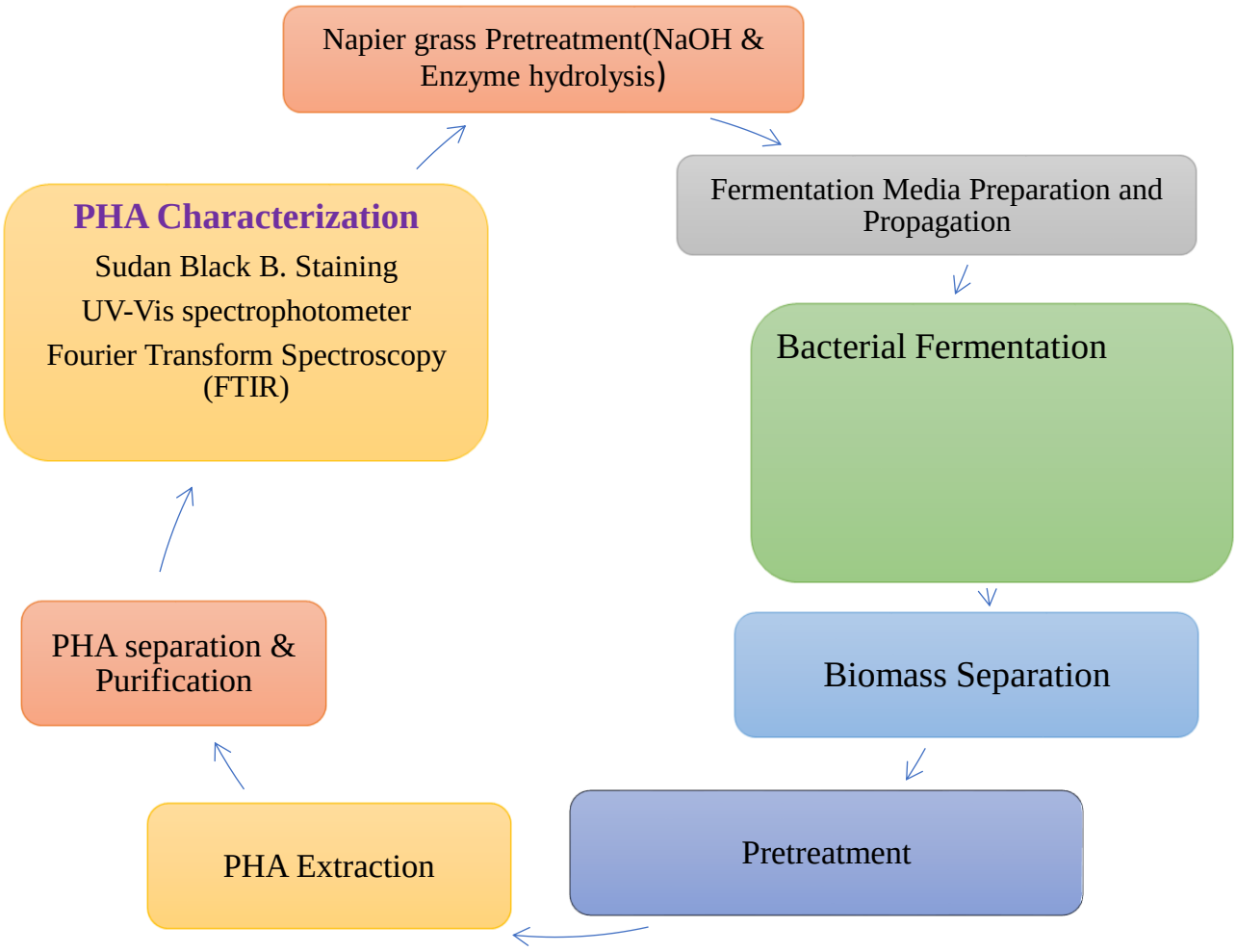

Figure 3.1: Major Steps in PHA Production Process

3.2.1. Material

Stems of Napier grass were obtained in Menagesha mushroom Farm, Ethiopia. The Napier grass was milled; sun dried for two weeks, and then chopped into small (1–2 cm) pieces with scissors and cutters before use. The samples are next crushed using a cutting mill machine and sieved with a 150mm sieve.

Figure 3.2: Napier grass Processing

3.2.2. Alkaline Hydrolysis

To remove lignin from the Napier grass, the most frequently utilized alkaline pretreatment techniques (NaOH) were used in this study (Phitsuwan et al., 2016). NaOH was chosen because it has lower reaction temperature and pressure, employs less corrosive chemicals than acids, and uses simpler reactors. Furthermore, alkaline reagents selectively interact with lignin by cleaving the

hydrolysable links in the lignin-carbohydrate complex and altering lignin structures (Kim et al., 2016) .The NaOH pretreatments listed below were carried out. In a sealed Erlenmeyer flask, 50 g of the Napier grass sample was submerged in 300 mL of 2% (w/v) NaOH (a solid to liquid ratio of 1:6). The NaOH pretreatment bottles were autoclaved at 121 ^{o}C for 1 hour. After that, they were allowed to cool to ambient temperature.

Following the pretreatments, each pretreated solid was separated from the liquid fraction using a filter. The solid portions were rinsed in distilled water until their pH was neutral. Following filtering, the solid residue was dried for at least 48 hours in a hot-air oven at 80 ^{o}C before being kept in a sealed plastic bag at room temperature for later enzymatic hydrolysis. The pretreatment's objective is to break down the lignin component and disturb the crystalline structure of the cellulose in order to enhance the accessibility of the hydrolyzing enzyme according to (J. W. Lee et al., 2015).

3.2.3. Enzymatic Hydrolysis

Enzyme hydrolysis was done according to Kongkeitkajorn *et al* (Kongkeitkajorn et al., 2020; Pensri et al., 2016) methods. Multi component Cellulase (Novozymes, Denmark) was used to hydrolyze pretreated grasses. In a 500-mL laboratory container, 100 mL of 50 mM citrate buffer solution (pH 5.0) was mixed with the pretreated grass with loading of 15 %(W/V). After that, the enzyme was added at a loading volume of 2 ml/g substrate and combined, and the mixture was incubated in a water bath at 50 ^{o}C with continual mixing for 72 hour with shaking at 150 rpm. 0.5 g/L sodium azide was added to the process to avoid microbiological contamination. By boiling the contents for 5 minutes, the hydrolysis reaction was halted. After all samples are withdrawn, the hydrolysate was centrifuged for 15 minutes at 4 ^{o}C and 4000 rpm, and the supernatant was tested for total reducing sugar using the DNS method.

3.2.4. Inoculation

In this investigation, *Burkholderia sacchari,* which was recently reclassified as *Paraburkholderia sacchari*, brought from Switzerland, was utilized. To grow the organism, Luria Bertani broth (NaCl, 5 g/L; Tryptone, 10 g/L; and yeast extract, 5 g/L) was employed. In a 500 mL shake flask, 100 mL of media was inoculated with one overnight culture from an agar slant. The bacterial

isolates were incubated for 24 hours at 30 degrees Celsius and 150 revolutions per minute (Oliveira-Filho et al., 2020). After 24 hours, the entire 50-mL culture was transferred aseptically to a fresh 250-mL LB broth culture (total 300 mL; enclosed in a 500-mL Erlenmeyer flask) and cultured as previously described.

3.2.5. Fermentation Media Preparation, Propagation and Fermentation

The following mineral salts media (MM) composition was modified to restrict nitrogen availability (in g/L): KH_2PO_4, 9.0; Na_2HPO_4 $2H_2O$, 3.0; $(NH_4)2SO_4$, 2.0; $MgSO_4 \cdot 7H_2O$, 0.2 g; $CaCl_2 \cdot 2H_2O$, 0.02; $(NH_4)_5Fe(C_6H_4O_7)_2$, 0.03 according to (de Sousa Dias et al., 2017). Trace elements solution (2 mL/L), prepared using H_3BO_3 (0.30 g/L); $CoCl_26H_2O$ (0.20 g/L); $ZnSO_47H_2O$ (0.10 g/L); $MnCl_24H_2O$ (0.03 g/L); $NaMoO_42H_2O$ (0.03 g/L); $NiCl_26H2O$ (0.02 g/L); $CuSO_4$ $5H_2O$ (0.01 g/L). The media was made in bulk and then distributed evenly across the test flasks in accordance with the protocols devised for maximum growth. This prior culture was applied as an inoculum to mineral salts media (10% v/v) (Penkhrue et al., 2020). Separate autoclaves were used for the $MgSO_47H_2O$, $CaCl_2$, 2H2O, Hydrolysates and $(NH_4)_5Fe(C_6H_4O_7)_2$ solutions were added to the medium aseptically. Glucose rich hydrolysates were added 5(%W/V). pH was adjusted to the experimental run by adding NaOH (5M) or H_2SO_4 (5M) and 200 rpm in a shake incubator.

3.2.6. Downstream Process

3.2.6.1. Measurement of Dry Biomass

Dry biomass is measured by taking samples (10 mL) from the culture media at set intervals and centrifuging them at 8000 rpm for 15 minutes. Remove supernatant and collect pellet at the bottom by rinsing with distilled water. The pellets were dried at 70°C, and weighed in an analytical scale to constant weight. The pellet samples are kept at room temperature in a dry place until PHA extraction is performed (Acosta-Cárdenas et al., 2018; Getachew & Woldesenbet, 2016).

3.2.6.2. PHA Extraction

Solvent extraction followed by non-solvent precipitation was employed to recover (Kunasundari & Sudesh, 2011). Following that, the lyophilized cells were placed in a conical flask with 100 mL

sodium hypochlorite (5%) and 100 mL chloroform. For 12 hours, the mixture was stirred in a rotary shaker at 200 rpm and 30 °C. The suspension was placed in a separating funnel and allowed to stand for 30 minutes to allow the three phases to separate: an upper phase containing hypochlorite solution, a middle phase containing non-PHA material (cell debris and undisturbed cells), and a bottom phase containing PHA solubilized in chloroform. To precipitate the PHAs, the bottom phase was decanted into a beaker and nine parts methanol were added. Finally, the PHA-precipitate was dried at 30 °C by evaporation, yielding white flakes or powder (Singh et al., 2021).

3.3. Characterization of Napier grass

Moisture content

Determination of % Moisture content was done according to ASTM D 4442 – 92 (Reapproved 2003) with a little modification. A 1 g sample of the laboratory-prepared material was put in a clean and dry crucible and baked at 105 ^{o}C for 2 hours to measure the moisture content and the % moisture was determined using the method below:

$$(\%)Moisture = \frac{(W1-W2)}{W1}\ X\ 100 \quad \text{.....3.1.}$$

Where: W1= original mass, g (gram).

W2= oven-dry mass, g (gram).

Ash content

Estimation of the percent Ash content was done according to ASTM D 1102 - 84 (Certified in 2001) with a little modification. A 2 gram sample of oven-dried (105 ^{o}c) laboratory produced material and that has been ground so finely that it can pass through a 425-µm sieve was placed in the crucible, and the combined weight of the crucible and specimen was measured. The crucible and its contents were put in the muffle furnace and fired until the carbon was completely eliminated. The sample was maintained for four hours with the final ignition temperature set at 600^{o}C. The crucible and its contents were removed four hours later and placed in a desiccator to

cool. The weight of the crucible and its contents was carefully calculated. The following calculation was used to compute the ash.

$$(\%)Ash\ content = \frac{W1}{W2}\ X\ 100 \quad \text{.....3.2.}$$

Where, W1=weight of ash (grams).

W2=weight of oven-dried sample (grams).

3.4. Determination of Total Reducing Sugar using Dinitro Salicylic Acid (DNS) Method

In the manual approach, 30 ml of modified DNS reagent was added to 10 ml of sample plus 10 ml of distilled water in a test tube, which was then heated in a boiling water bath for 15 minutes and chilled for at least 15 minutes. The absorbance was then measured at 540 nm. Several modifications in sample volume and wavelengths were investigated. Glucose standard curves were developed (Marsden et al., 1982; Miller, 1959).

3.5. Screening by Sudan Black B. Stain

After PHA production was established, ten milliliters of media were centrifuged at 6000 rpm for 20 minutes and Sudan staining was performed to confirm PHA production. A smear of 72 hour old strains was stained for 15 minutes with 0.3 percent (w/v) Sudan Black B. solution, then destaining with alcohol (50 percent v/v) and counterstaining with safranin solution (0.5 percent, w/v) (Sayyed et al., 2021) with Digital compound light microscope, Optika Italy.

3.6. UV–Vis Spectrophotometer Analysis of PHA

The extracted PHA was dissolved in chloroform and scanned in the range of 200–320 nm against a chloroform blank a Perkin-Elmer Lambda 950 UV/VIS spectrometer, with the spectrum evaluated for a strong peak at 240 nm (Selvakumar et al., 2011).

3.7. FTIR Analysis

PHA was validated using FTIR spectroscopy after 1 mg of sample and 10 mg of spectral pure anhydrous potassium bromide crystals were crushed thoroughly and formed into a pellet. Using FTIR (Thermo SCIENTIFIC iS50 ABX, spectrometer), the relative intensity of transmitting light energy was evaluated against the wavelength of absorption 4000–400 cm^{-1} (Radhika & Murugesan, 2012).During FTIR analysis, bands will indicate the carbonyl group (C=O), OH groups, C–H stretching methyl and methylene groups, and C–O group.

3.8. Experimental Design and Statistical Analysis

Box-Behnken designs were chosen for this study as they offer higher order response surfaces with fewer runs than a traditional factorial approach and since the extreme points are unknown and the effect of curvature must be considered. A Box–Behnken design with three parameters and repeated center points was utilized to statistically optimize the fermentation medium for high PHA yield. The factors examined were the fermentation incubation temperature (A), pH (B), and incubation time (C). This model was used to assess the impact of individual and combination interactions between the three independent variables on PHA production. The design generated a total of 17 experimental runs as it was shown in Table 3.1 which were determined using the "Design Expert" software.

Design-Expert software Version 12 was used to analyze and interpret the outcomes of the trial runs (StatEase).

Table 3.1: Experimental Design Factors and Levels for Extraction of PHA Run

		Factor 1	Factor 2	Factor 3	Response 1
Std	Run	A:Temperature	B:pH	C:Time	PHA yield
		°C		hr.	g/L
5	1	33	7	24	
15	2	35	7	48	
1	3	33	6.5	48	
16	4	35	7	48	
12	5	35	7.5	72	
4	6	37	7.5	48	
13	7	35	7	48	
3	8	33	7.5	48	
11	9	35	6.5	72	
6	10	37	7	24	
10	11	35	7.5	24	
9	12	35	6.5	24	
7	13	33	7	72	
2	14	37	6.5	48	
17	15	35	7	48	
8	16	37	7	72	
14	17	35	7	48	

4. Results and Discussion

4.1. Proximate Analysis of Napier grass

Moisture content

The prepared Napier grass moisture content was determined using ASTM D 4442 – 92. The experiment was carried out in triplicate and yielded a result of 6.47 ±0.09 % moisture content. Moisture content indicates that how much water is present in the biomass. High amount of moisture content affects the storage life of biomass by developing fungus and other microorganisms, which inhibits the fermentation process. The lower levels of moisture 6.47 ±0.09 % from this study indicates lower effect on the biomass used as feedstock for the fermentation.

Ash content

The calculated result was 15.8±0.5 %, similar with (Wilsie et al., 1940), the ash percentage ranging from 15.4 to 19.2 %. The ash content is an approximate measure of the mineral content and inorganic elements in biomass, and it is the amount of residue left after dry oxidation. This inorganic materials have a negative effect on microbial growth for fermentation. The Variation for different ash content may be differences in harvesting time and age of the material.

4.2. Benedicts' Test

The benedict test indicates that the color of the hydrolysate was changed to brick red after it was mixed with benedict solution and heated for 5 minutes and the color changes to brick red as it was shown from figure 4.1, signifies the solution has more than 2% of reducible sugar (Aryal, 2019.) Benedict's reagent comprises blue copper (II) sulfate ($CuSO_4$)$5H_2O$, which is reduced to red copper (I) oxide by aldehydes, resulting in carboxylic acids being formed. Copper oxide is water insoluble and thus precipitates. Depending on how many copper (II) ions are present, the color of the final solution can range from green to brick red (Benedict, 1911; Pataca et al., 2007). Color change to bricks red indicates the pretreated Napier grass using NaOH and Cellulase enzyme hydrolysis produces significant amount of reducible sugars that can be used as a feedstock for fermentation process.

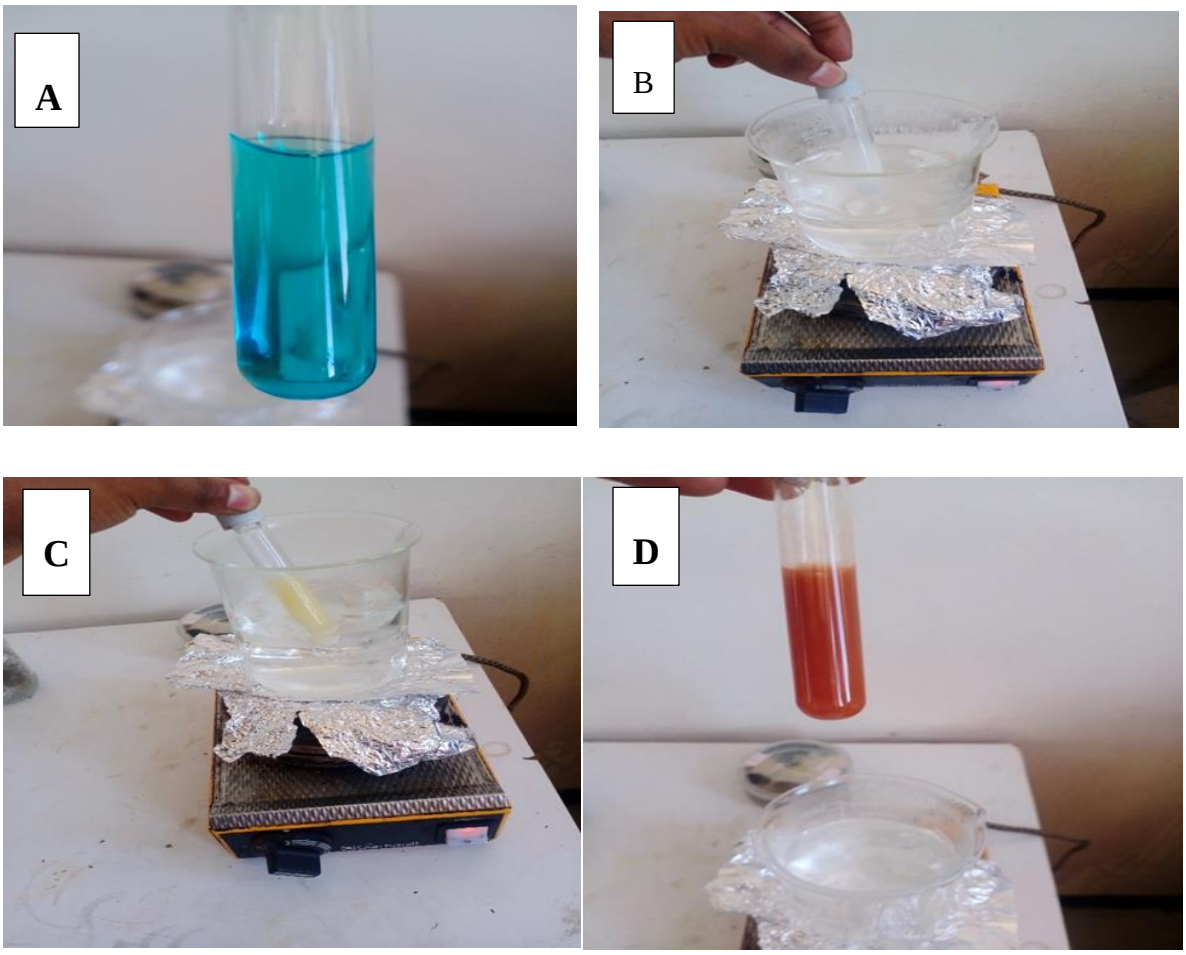

Figure 4.1: The Benedict Test of the Produced Reducible Sugar.

From figure 4.1, A. shows sample and Benedict reagent solution; B. solution changes its color after one minutes; C. color change to slightly to red; whereas D. shows the color changes to Bricks Red after 5 minutes.

4.3. Total Reducing Sugar Analysis of the Hydrolysates

The DNS approach was used to estimate sugar reduction (Miller, 1959) . The samples absorbance was measured at 540 nm, and DNS containing distilled water was used as a blank in a Perkin-Elmer Lambda 950 UV/VIS spectrometer (Shelton, CT 06484 USA). To estimate the total reducing sugar after alkaline pretreatment and enzymatic hydrolysis, a standard curve was

performed by the DNS technique, and the generated calibration data and graph is shown figure 4.2.

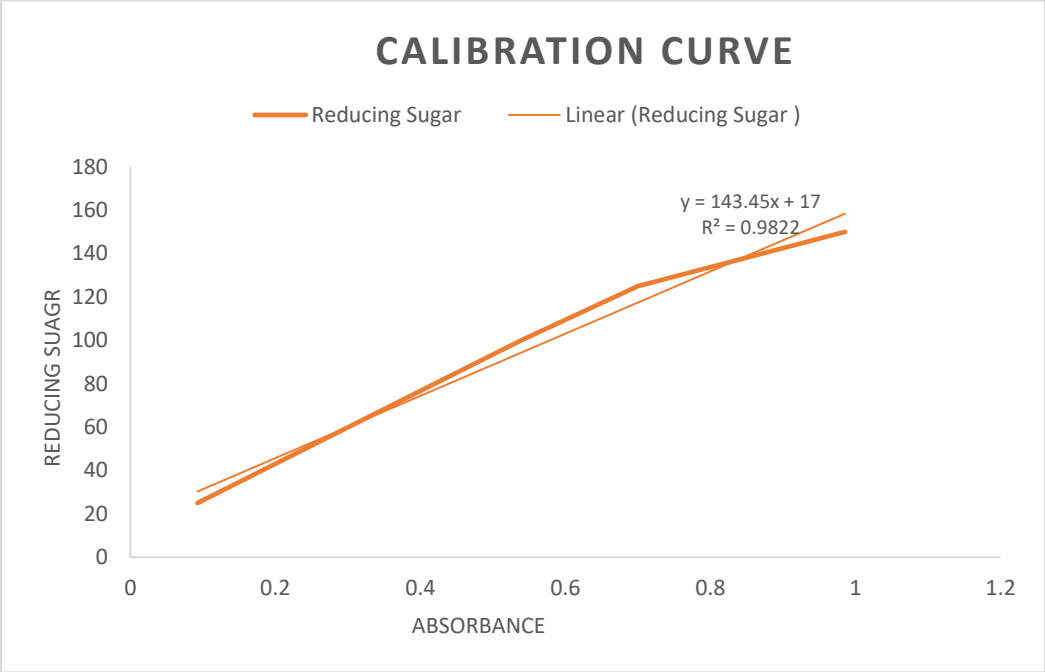

Figure 4.2: Reducing Sugar Calibration Graph

Absorbance of the standard sample and main sample were calibrated using the Lambda 950 Uv-Vis spectrophotometer and the equation developed is shown in the inset of figure 4.2.

The sample was diluted 200 times following hydrolysis and DNS treatment procedures for the reduction of noise in the spectrometer. The sample absorbance at 540nm was computed using the aforementioned equation, and the total reducing sugar content was determined to be 28.74 g L^{-1}. In this study, 3.2 g of sugar was recovered, which is less than a previous study that yielded 3.9 g of sugar from a 5 g sample load (He et al., 2017). This indicates from Napier grass, high amount of reducible sugars were produced using NaOH and Cellulase enzyme, is a potential candidate for PHA production. Higher quantities of fermentable sugars in the hydrolysate are preferred for fermentation to generate higher PHA concentrations. The major cause of this disparity is the age during cultivation and kind of Napier grass used for this investigation.

4.4. Sudan Black B. Staining

Sudan Black B. is a frequently used dye that may be applied directly to cell cultures, solid media plates, or bacterial cells mounted on slides to give the cells a colored appearance, allowing PHA producers to be distinguished from non-PHA producers (Chavan et al., 2021) except for cell membranes and other lipid-containing cell components, this is due to their capacity to absorb enough dye and give noticeable fluorescence.

PHA accumulators are blue black. According to Sudan Black B. staining test, the isolate was positive PHA producer as shown in Figure 4.3. In addition, Sudan staining demonstrates that PHA is produced in a high-quality manner and around two-thirds of cells show positive result on producing PHAs (Jong-il Choi & Lee, 1999).

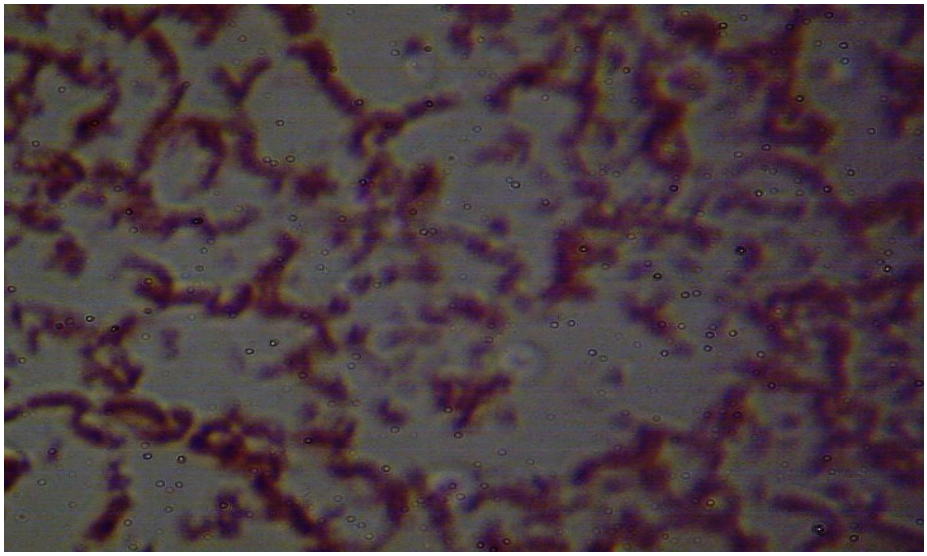

Figure 4.3: Microscopic Image of PHA Granules by Sudan Black B. Staining

4.5. UV-Vis spectrophotometer Analysis of PHA

Chloroform was used as the blank sample in this investigation since it is one of the best solvent for plastics and the best indicator for the presence of PHA. Figure 4.4 depicts the PHA absorbance graph at wavelengths ranging from 200 to 320 nm. Because absorbance and concentration are proportional, the greater the absorbance, the greater the PHA concentration. The larger the peak,

the greater the absorption at that specific wavelength. At 240 nm wavelength, absorption is greater and sharp peak, confirming the production and existence of PHA granules (Selvakumar et al., 2011).

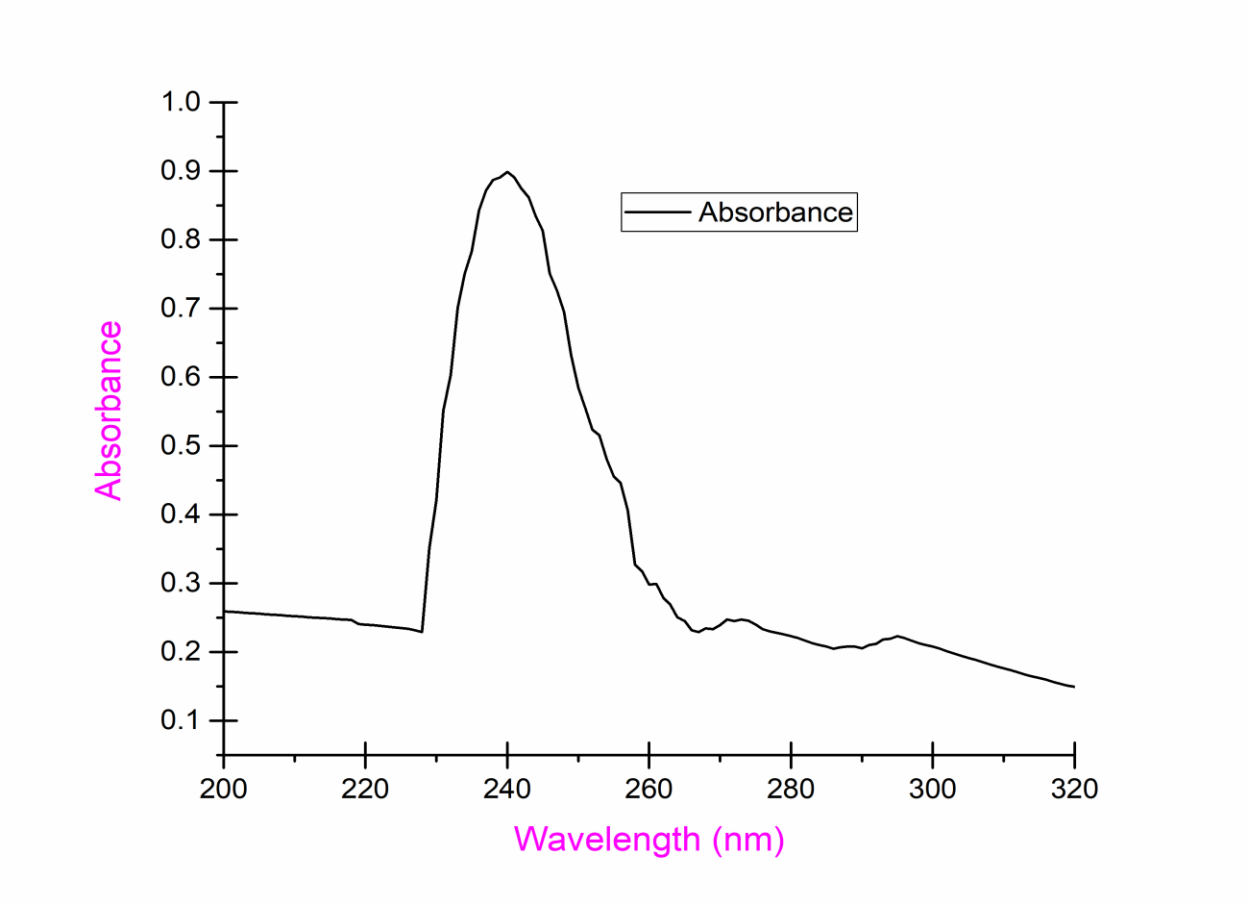

Figure 4.4: UV- Vis Spectroscopy of PHA

4.6. FTIR Spectrophotometer Analysis of PHA

To determine the functional groups present in the PHA isolated from *Burkholderia Sacchari,* an FTIR analysis was performed and the FTIR spectra is shown in Figure 4.5. Peaks in the range 2800–3000 cm^{-1} are associated with asymmetric stretching of CH_3, antisymmetric stretching of CH_2, and symmetric stretching of CH_3. C–H stretching of methyl and methylene groups were attributed to the peaks at 2930 and 2958 cm^{-1}, respectively (Atifah et al., 2001).

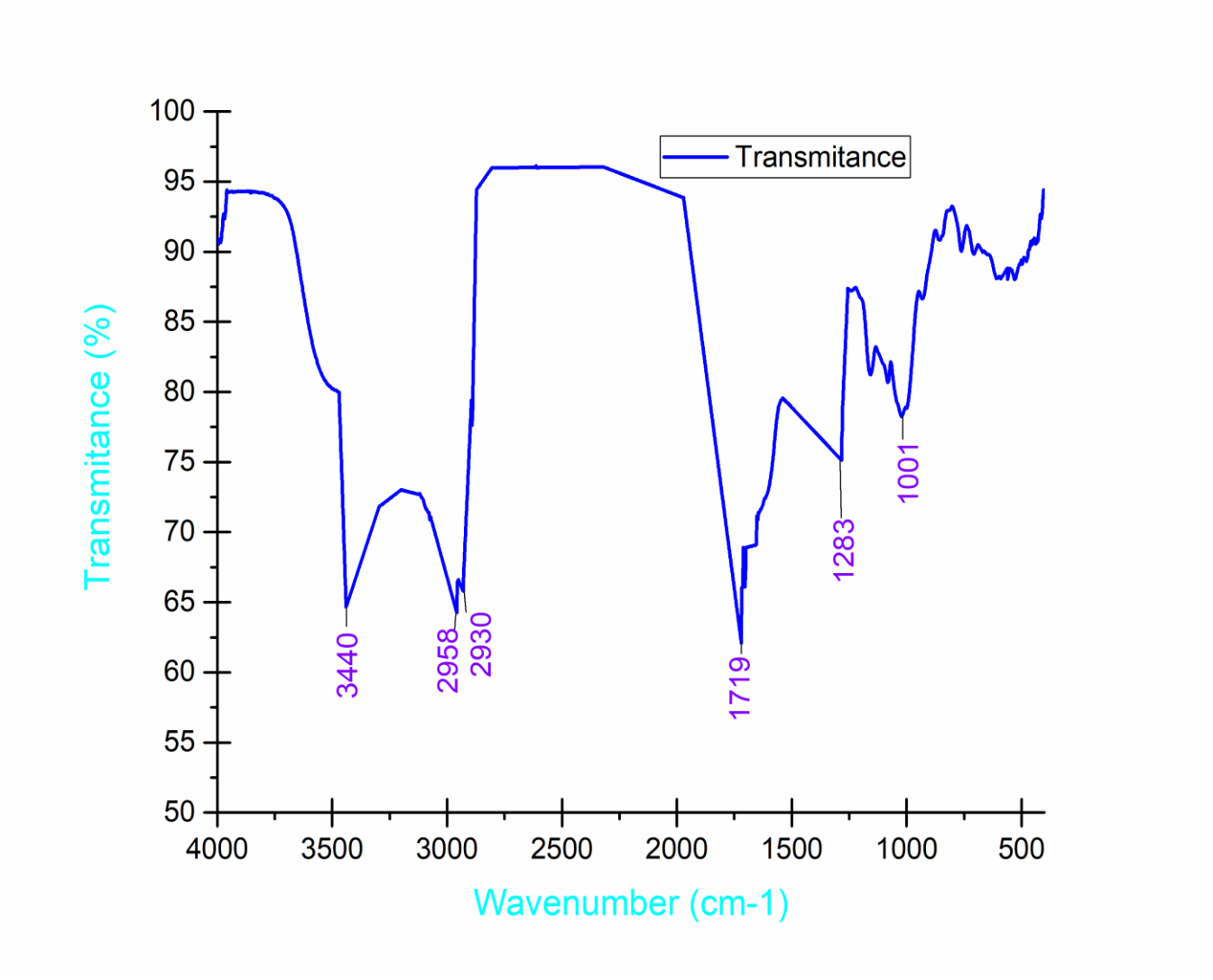

Figure 4.5: FTIR Spectra of the Extracted PHA

The peaks at 3440 cm^{-1} suggested the formation of a strong stretching H bond by the terminal OH groups (Geethu et al., 2019). The C=O and C-O stretching groups represented by absorption bands at 1719 cm^{-1} and 1283 cm^{-1}, respectively (Geethu et al., 2019; Odeniyi & Adeola, 2017), are the functional groups in the monomeric PHA. The bands at 1184 cm^{-1} are C-O-C (Bhatia et al., 2019; Mostafa et al., 2020). A material's infrared spectrum indicates its chemical components by producing a typical absorbance spectrum.

4.7. Experimental Results and Statistical Analysis on PHA Production

4.7.1. Experimental Results on PHA Production

A Box–Behnken design (BBD) with three parameters and repeated center points was utilized to statistically optimize the fermentation medium for high PHA yield. The parameters examined were incubation temperature (A), pH (B), and incubation time (C) of the fermentation medium. The 17 experiments carried out by varying the three parameters using the BBD design with their respective response, PHA yield, is shown in Table 4.1.

The yield of PHA after the final drying process was monitored and assessed for each experimental run. Trials 1 and 17 gave the minimum and maximum PHA yield of 3.15 and 7.42 g L^{-1}, respectively, for varied combinations of temperature (33 and 35 °C), pH (7) and time (24 and 48 hour). The main technique used to enable bacterial cells to build a substantial quantity of intracellular PHA is the limitation of a nutrient needed for growth and an excess of the carbon source (Doi et al., 1990).

Table 4.1: Experimental PHA yield from Hydrolysates of Napier Grass

		Factor 1	**Factor 2**	**Factor 3**	**Response 1**
Std	Run	A:Temperature(^{o}c)	B:pH	C: Time (hr.)	PHA yield(g/L)
5	1	33	7	24	3.15
15	2	35	7	48	7.4
1	3	33	6.5	48	3.5
16	4	35	7	48	7.41
12	5	35	7.5	72	5.2
4	6	37	7.5	48	5.14
13	7	35	7	48	7.38
3	8	33	7.5	48	4.2
11	9	35	6.5	72	3.42
6	10	37	7	24	3.7
10	11	35	7.5	24	4.3
9	12	35	6.5	24	3.74
7	13	33	7	72	3.49
2	14	37	6.5	48	3.58
17	15	35	7	48	7.4
8	16	37	7	72	4.016
14	17	35	7	48	7.42

Burkholderia sacchari growth and PHA production were studied at various temperatures, pH levels, and incubation periods. Figure 4.6 depicts the results of *Burkholderia sacchari* growth and PHA production from various cultivation period. In this study, cell growth continued until the nitrogen in the medium was depleted and polymer began to build within the cells at a cell dry weight (CDW) of around 5 g L^{-1} and reached maximum at a CDW of around 12 g L^{-1}. When

cultivated on the optimized medium, the strain produced the highest production of PHAs, with maximum accumulation at 48 hour (7.42 gL^{-1}) extracted from its CDW reach's a maximum 59% and a minimum of 3.15 gL^{-1} PHA. Better cell growth can lead to increased PHA production.

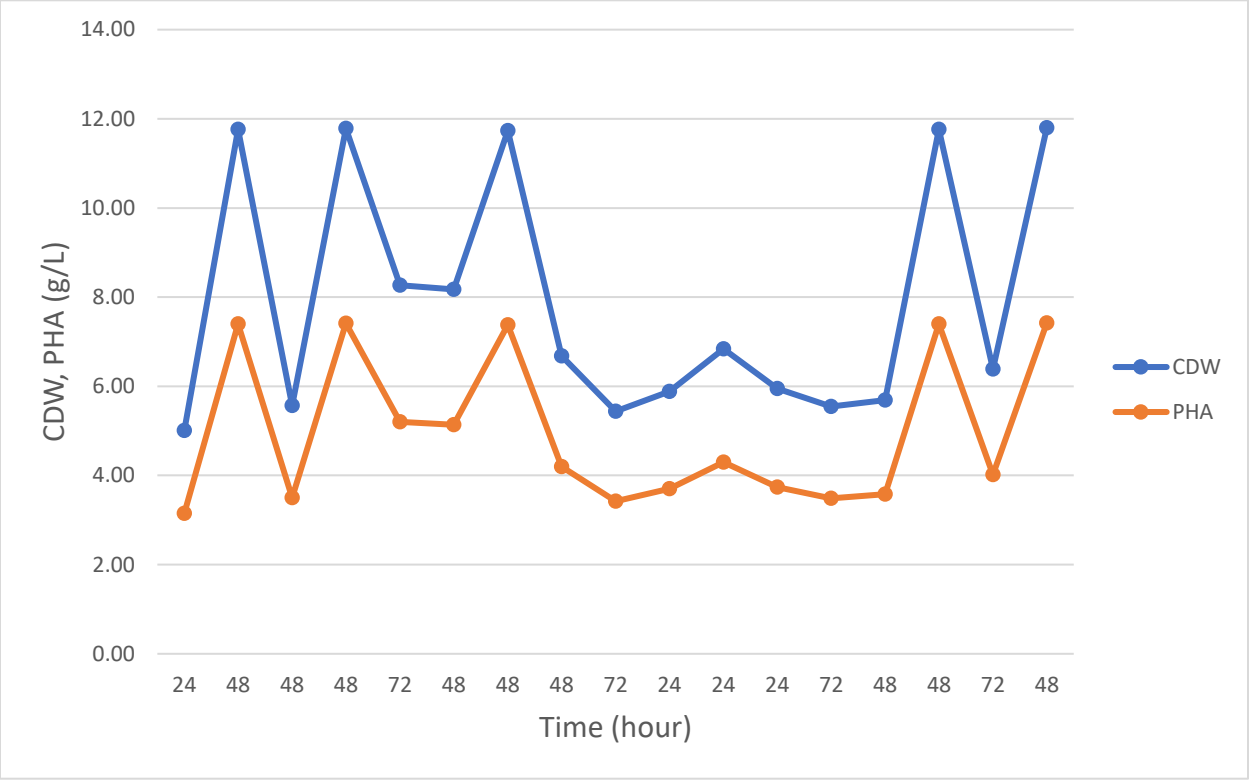

Figure 4.6: *Burkholderia sacchari* Growth and PHA Production

4.7.2. Statistical Analysis on PHA Production

ANOVA was used to test the capacity of polynomial expression to predict the answers statistically. Table 4.2 shows the ANOVA for the quadratic model of PHA yield. The processed data from the experimental design allowed for the computation of the regression equation coefficients.

Table 4.2: ANOVA for the Response Surface Quadratic Model of PHA Production

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	**46.73**	**9**	**5.19**	**13008.13**	**< 0.0001**	**Significant**
A-Temperature	0.5492	1	0.5492	1375.83	< 0.0001	
B-pH	2.65	1	2.65	6626.70	< 0.0001	
C-Time	0.1910	1	0.1910	478.43	< 0.0001	
AB	0.1849	1	0.1849	463.24	< 0.0001	
AC	0.0001	1	0.0001	0.3608	0.5670	
BC	0.3721	1	0.3721	932.25	< 0.0001	
A^2	15.79	1	15.79	39558.79	< 0.0001	
B^2	7.79	1	7.79	19525.63	< 0.0001	
C^2	14.83	1	14.83	37145.41	< 0.0001	
Residual	0.0028	7	0.0004			
Lack of Fit	0.0019	3	0.0006	2.90	0.1651	not significant
Pure Error	0.0009	4	0.0002			
Cor Total	46.73	16				

The resulting second-order polynomial equation is given in equation 4.1

$$Y = \beta 0 + \beta iXi + \beta ijXiXj + \beta iiXi^2 \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \quad (4.1)$$

Where Y represents the anticipated response, β0 represents the model constant, βi represents the linear coefficient, βii represents the quadratic coefficient, βij represents the interaction coefficient, and Xi represents the independent variable. The parameters that were statistically not significant ($P > 0.05$) and their interactions were removed from the equation.

Figure 4.7 shows graphical illustration of the smallest discrepancy between the experimental (straight line) and projected (squares) responses for the BBD to achieve a high PHA yield. The expected and actual experimental responses of biomass were found to be comparable, with only minor differences between the results.

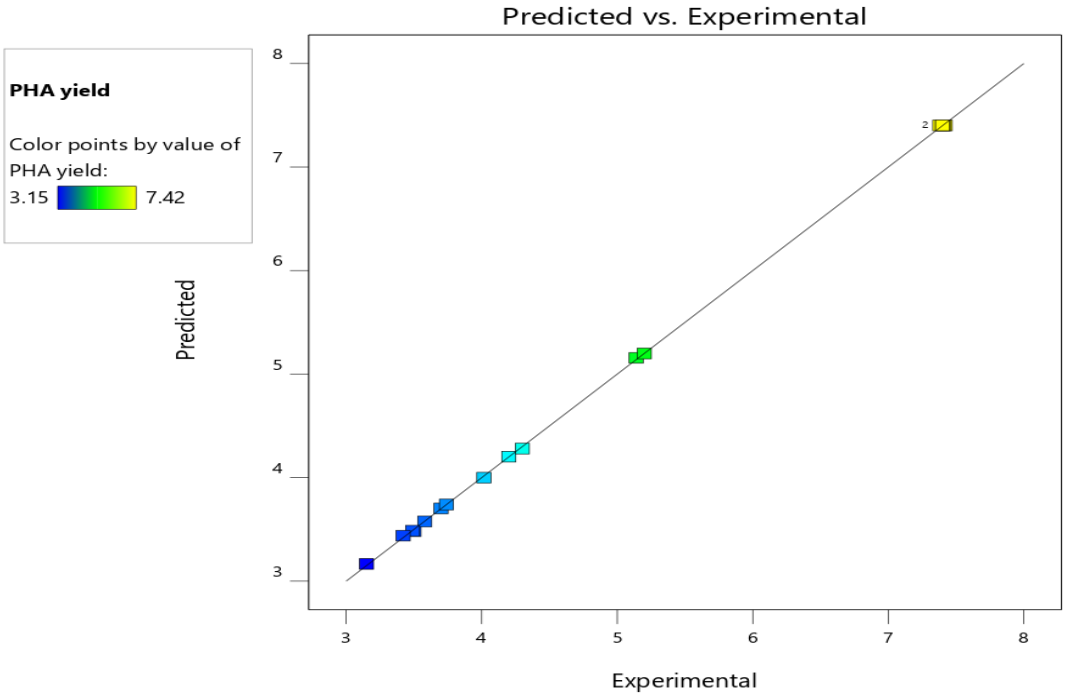

Figure 4.7: Graphical Illustration of Experimental and Predicted Responses

The lowest departure from the straight line indicates less variance from the expected value and a reasonable correlation between experimental and predictive data, demonstrating the BBD's strong prognostic capacity (Tesfaye et al., 2018).

The second-order polynomial equation with final equation in terms of coded factors and actual factors is given by equation 4.2 and 4.3, respectively.

PHA yield(Y) =7.402 + 0.262 * A + 0.575 * B + 0.1545 * C + 0.215 * AB + -0.006 * AC + 0.305 * BC -1.9365 * A^2 + -1.3605 * B^2+ -1.8765 * C^2…… (**4.2)**

PHA yield(Y) = -811.754 + 32.5208 * A + 68.593 * B + 0.145646 * C+ 0.215 * AB -0.000125 * AC + 0.0254167 * BC -0.484125 * A^2 -5.442 * B^2-0.00325781 * C^2…… (**4.3)**

P-values less than 0.0500 indicate model terms are significant. In this case A, B, C, AB, BC, A^2, B^2 and C^2 were statistically significant model terms suggesting the selected operation parameters fermentation temperature (A), pH (B), and incubation period (C) influencing PHA production. The model F-value of 13008.13 and $p<0.0001$ indicate that the model is significant, and there is a 0.01 % probability that an F-value this big is due to noise. All three parameters have a clear quadratic impact, which was consistent with the substantial effects ($p <0.0001$) of quadratic terms in Table 4.2. The linear effects of the three variables were all highly significant ($p< 0.0001$), however, the interaction effects of temperature with cultivation time was not significant ($p > 0.05$).

The F-value of 2.90 for the lack of fit indicates that the lack of fit is not significant in comparison to the pure error. There is a 16.51 % possibility that a significant lack of fit F-value is caused by noise. We want the model to fit, thus a minor lack of fit is acceptable. A non-significant lack of fit suggests that the model equation was adequate to forecast PHA production under any combination of variable values.

A satisfactory R^2 value of 0.9999 supported the model's applicability, suggesting that the model could explain 99.99 percent of the variability in the response and that less than 1% of the variations occur during the trials, indicating a convincing fit of the model to the experimental data (Table 4.3). Coefficient of determination (R^2), is the percentage of variation in the answer that can be attributed to the model rather than random error, and R^2 should be at least 80% for a successful model fit (Cao Y *et al* 2008). The Adjusted R^2 of 0.9999 is reasonably close to the Predicted R^2 of 0.9993, with a difference of less than 0.2 indicating appropriate signal, and this high number further confirms the high significance of the model.

Table 4.3: Fit Statistics for PHA yield

Std. Dev.	0.0200	**R^2**	0.9999
Mean	4.97	**Adjusted R^2**	0.9999
C.V. %	0.4022	**Predicted R^2**	0.9993
		Adeq Precision	276.4171

The coefficient of variation (CV) is the percentage ratio of the standard error of the estimate to the mean value of the observed response, and a model with a CV of less than 15% can be considered reasonably reproducible because it indicates that the experiment is more reliable and the trials are more reliable (Tesfaye et al., 2018). The CV of 0.4022 %in this investigation suggests a high level of precision in the experiment. The signal-to-noise ratio is measured by Adequate Precision. It is preferable to have a ratio of more than four. For the present BBD, signal-to-noise ratio of 276.4171 suggests a good signal. This model can be used to find the way through the design world.

4.7.3. Interaction Effects on PHA yield

4.7.3.1. Interaction Effect Between Temperature and pH

As it can be seen from figure 4.8 and from the ANOVA Table p-value of interaction between temperature and pH ($p < 0.0001$), there is a significant interaction between these two independent variables on the yield of PHA. In 3D plots, the elliptical form of contours aids in predicting the significant interactions between variables (Tesfaye et al., 2018).

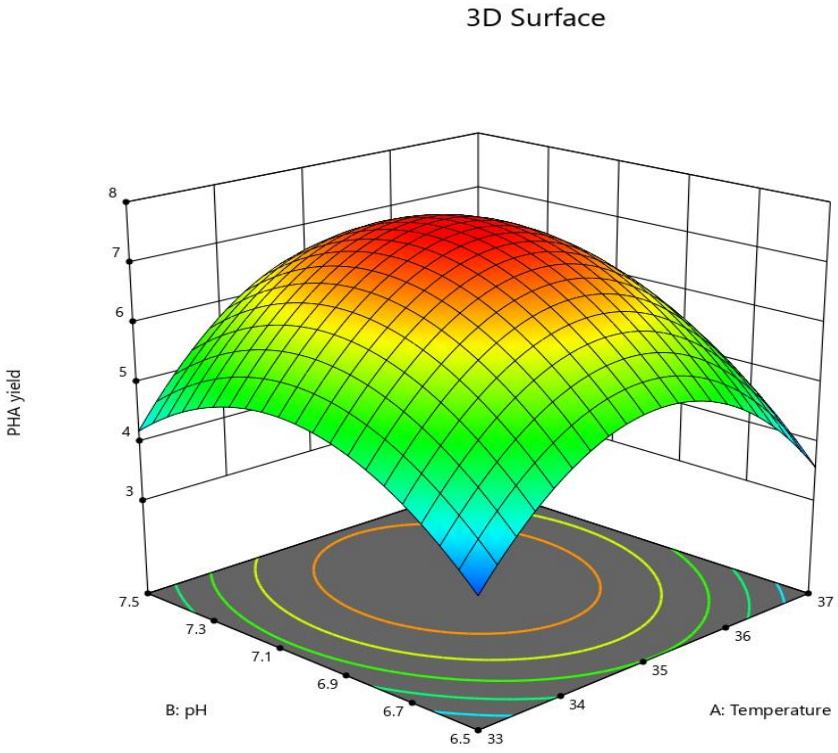

Figure 4.8: 3D plot of Interaction Effect Between Temperature and pH on the Yield of PHA

When the temperature is increased from 34 to 37 ^{o}C and the pH is increased from 6.5 to 7.5, the yield of PHA varies from 3.15 to 7.42 gL^{-1}. Figure 4.11 shows that when pH increasing from 6.5 to 7 and temperature from 33 to 35 ^{o}c maximum yield of 7.42 gL^{-1} of PHA is obtained and PHA yield is starts to decrease after pH 7 and temperature of to 35 ^{o}c. However, productivity couldn't exceed more than 7.42 gL^{-1} as high temperature slows down the metabolic activity (enzyme activity) of bacteria, reducing their ability to produce PHA (Getachew & Woldesenbet, 2016) and at high and low pH because of its effect on the bioavailability of trace elements and the regulatory enzymes responsible for PHA synthesis (Bhagowati et al., 2015). Temperature is a critical factor in bioprocesses. Temperature changes can cause a variety of microbiological reactions (Fonseca et al., 2013). pH may have effect on the bioavailability of trace elements and the regulatory enzymes responsible for PHA synthesis, ketothiolase, acetoacetyl-CoA reductase, and PHA polymerase, pH had a strong influence on PHA accumulation (Bhagowati et al., 2015).As a result, the yield was influenced by both pH and temperature at the same time. This finding is comparable to that of de Andrade *et al*, who produced PHA from *Burkholderia sacchari* using Cheese whey as a carbon source and co substrate and obtained a higher product at a temperature of 35^{o}C and a pH of neutral (de Andrade et al., 2019) and (Nascimento et al., 2016) *B. sacchari* grown with glucose at 35 °C demonstrated increased productivity and polymer output.

4.7.3.2. Interaction Effect Between Temperature and Fermentation Time

The yield of PHA is unaffected by the interplay between temperature and fermentation time. They both rise simultaneously and the interaction impact is minimal. P-value for the interaction effect of incubation temperature with time from the ANOVA table is 0.5670, indicates there is no significant interaction between these two independent variables on the yield of PHA. Figure 4.9 shows the contour plot of interaction effect between temperature and pH was circular which aids interaction between variables are not significant (Muralidhar et al., 2001).

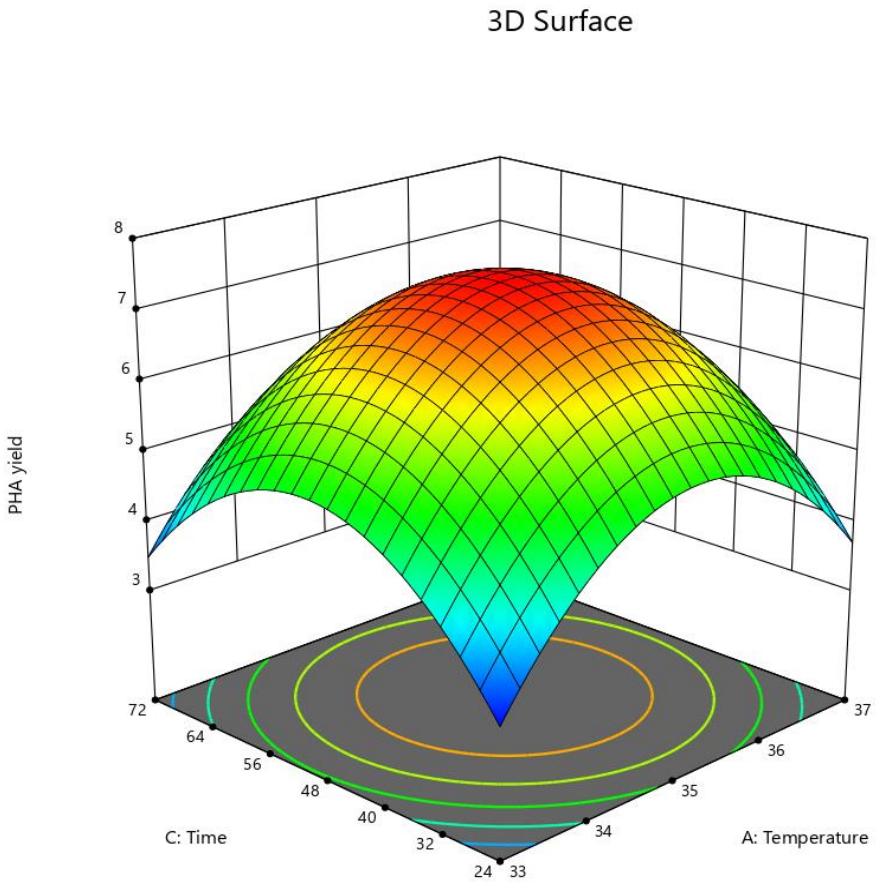

Figure 4.9: 3D Plot of Interaction Effect Between Temperature And Fermentation Time On The Yield of PHA

4.7.3.3. Interaction Effect Between pH and Fermentation Time

The site of interaction is well indicated at the center of the two lines in the interaction graph Figure 4.10 and from the ANOVA Table, the p-value of pH and fermentation time was <0.0001, indicates both pH and fermentation time have a greater impact on PHA yield. This demonstrates that the yield grew dramatically from 24 to 72 hours of fermentation, with a maximum yield of 7.42 g L^{-1}. As a result, the yield was strongly influenced by the interaction between pH and fermentation time.

In 3D plots, the elliptical form of contours aids in predicting the significant interactions between variables (Tesfaye et al., 2018). When the pH is increased from 6.5 to 7.5 and the fermentation time is increased from 24 to 72 hour, the yield of PHA rises from 3.15 to 7.42 $gL^{-1.}$ At 35 ^{o}c.

Figure 4.10 shows that at pH 7-7.1 and 48-50 hours fermentation time, a maximum yield of 7.42 g/L of PHA is obtained and PHA yield is decreased at high pH and low. After 48 hours, PHA levels start to decrease because of the polymer inside the cell being used for energy production that is in consistent with the previous studies (Zakaria Gomaa, 2014). PHA buildup by a given bacterial species is directly related to its biomass, which eventually decreased as carbon source was depleted, resulting in PHA consumption. This definitely indicates that PHA syntheses decrease at alkaline and acidic pH and at temperature higher than 35 ^{o}c because of the polymer inside the cell being used for energy production. This result is consistent with prior research, which concluded that a pH at optimal provides favorable conditions for microbial growth and the synthesis of PHA granules (Suryawanshi et al., 2020).

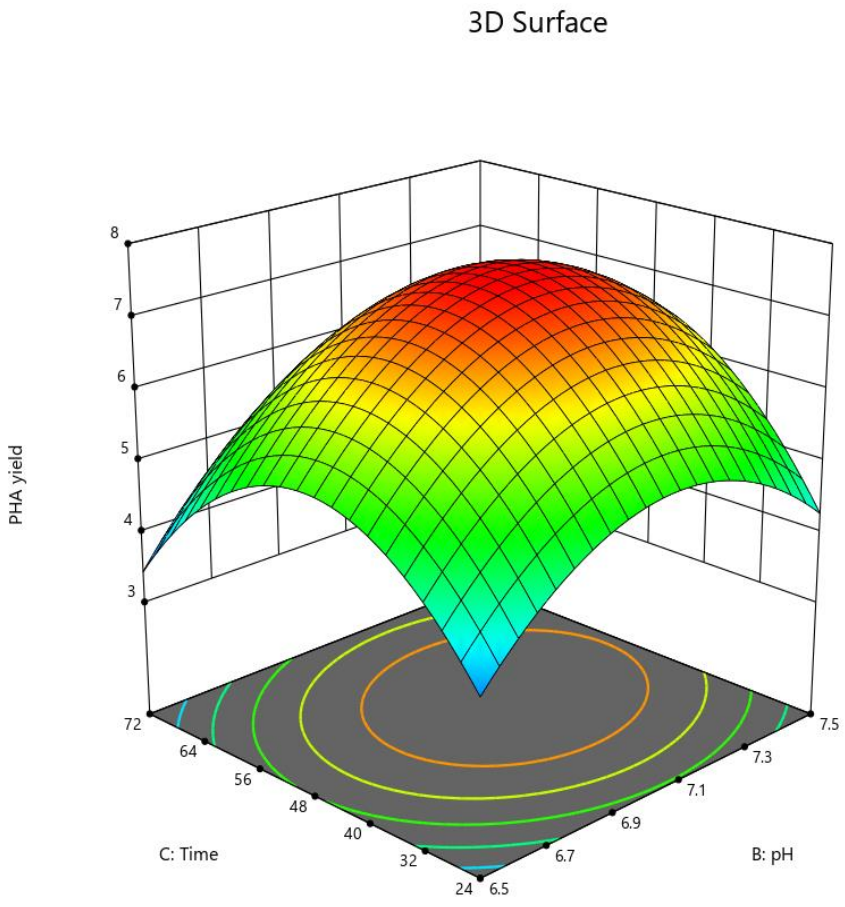

Figure 4.10: 3D Plot of Interaction Effect Between pH And Fermentation Time on the Yield of PHA

Table 4.4: Comparison of Production of PHAs using Lignocellulosic Materials

Strain	Substrate	Operation mode	Yield of PHA(g/L)	References
Burkholderia sacchari	Napier grass hydrolysates	Flask	7.42	This study
Burkholderia sacchari	Glucose + Fructose	Rotative shaker	4.1	(Gomez et al., 1996)
Burkholderia sacchari	Wheat straw hydrolysates	Rotative shaker	4.4	(Cesário, Raposo, Almeida, et al., 2014)
Burkholderia sacchari IPT189	Sucrose	Airlift Bioreactor	63	(Pradella et al., 2010)
Burkholderia sacchari	Xylose	Bioreactor	8.1	(Oliveira-Filho et al., 2020)
B.Sacchari pF5k::xylR (IPTG induced)	Xylose	Rotative shaker	5.7	(Guamán et al., 2018)

4.8. Model Validation

Experiments were conducted under the expected conditions to validate the model in the current study. The optimum PHA production was predicted at temperature of 35.388 °C, pH of 7.05145 and time of 48.1804 hour for the yield 7.4301 g L^{-1} PHA selected with high desirability of 1.0 as shown in figure 4.14. To validate the optimization findings, the chosen parameters were evaluated in the laboratory using the indicated process parameters in triplicate way. The production of PHA under the predicted optimum conditions was 7.425 g L^{-1} which was nearly the same as the predicted result. The results of the trials validated the solutions that were chosen.

The difference between experimental and projected values demonstrated a strong connection between observed and expected outcomes, confirming the validity of the response model as well as the actuality and accuracy of ideal circumstances for the variables.

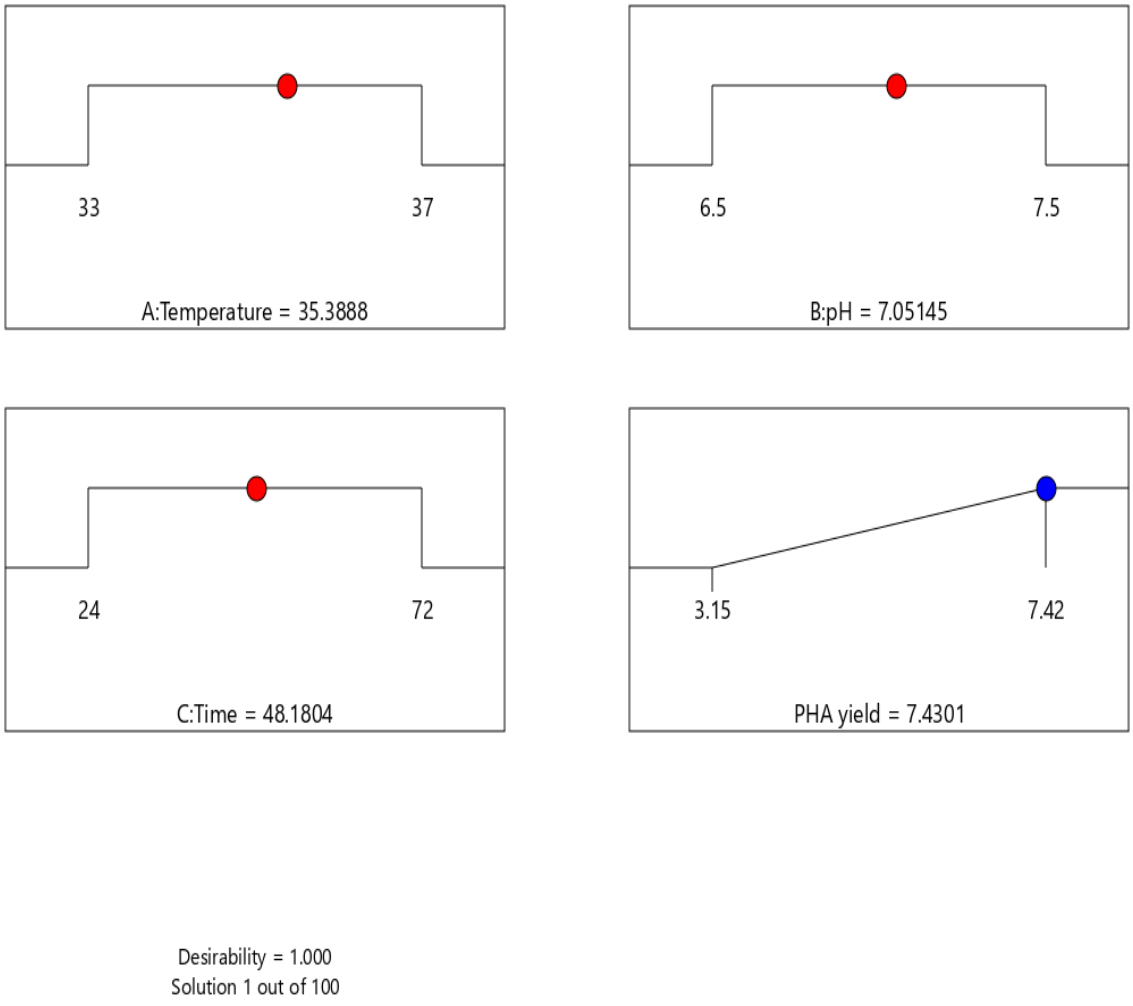

Figure 4.11: Ramps for the Selected Optimum Point Solution

5. Conclusion and Recommendation

5.1. Conclusion

It may be concluded that *B. sacchari* grew better in cultivations using hydrolysates of Napier grass as the sole carbon source at 35 °C, and neutral pH than in other examined scenarios as the strain can consume both hexose and pentose sugars that can be extracted from lignocellulosic materials.

In this study, alkaline hydrolysis of Napier grass was done first, followed by enzymatic hydrolysis and glucose rich hydrolysates were obtained. After evaluating the recovered hydrolysates, PHA, biocompatible bioplastic, was synthesized by fermentation and employing the *Burkholderia Sacchari* strain.

The extraction of PHA from hydrolysates of Napier grass took place in pretreatment of Napier grass, alkaline and enzyme hydrolysis, fermentation, extraction, and recovery stages. The process parameters; incubation temperature (33, 35, and 37 ^{o}c), pH of the media (6.5, 7 and 7.5), and fermentation time (24, 48 and 72 hours) that influenced the yield of the manufactured PHA were investigated. For optimization and to see the effect of parameters on the response, a three-variable, three-level Box–Behnken design using Response Surface Methodology was utilized. PHA bioplastic were synthesized at the optimum levels of variables.

The produced PHA with the variation of temperature, pH and fermentation time has shown statistically significant quadratic models for response of PHA yield with $p<0.0001$ and all determination coefficients R^2, nearly equal to one. The optimum PHA production was predicted at temperature of 35.38 °C, pH of 7.05 and time of 48.18 hour for the yield 7.43 g L^{-1} PHA and it was validated by doing it in triplicate using the predicted optimum conditions results 7.425 g L^{-1} PHA. The results of the trials confirming the validity of the response model.

5.2. Recommendation

If more study is conducted to investigate PHA as a replacement plastic, the following investigations are recommended:

- Further study into investigation of use different concentration of NaOH and Cellulase for production of hydrolysates of Napier grass.
- Further study into recombinant microbial strains, mixed cultures, efficient fermentations, recovery purification, and the utilization of low-cost substrates can significantly lower manufacturing costs.

It is advised that preliminary design, economic feasibility studies, and the establishment of an economic scale for PHA manufacturing be carried out.

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Appendix

Figure A1: NaOH hydrolysis Using Autoclave

Figure A2: Enzyme Hydrolysis

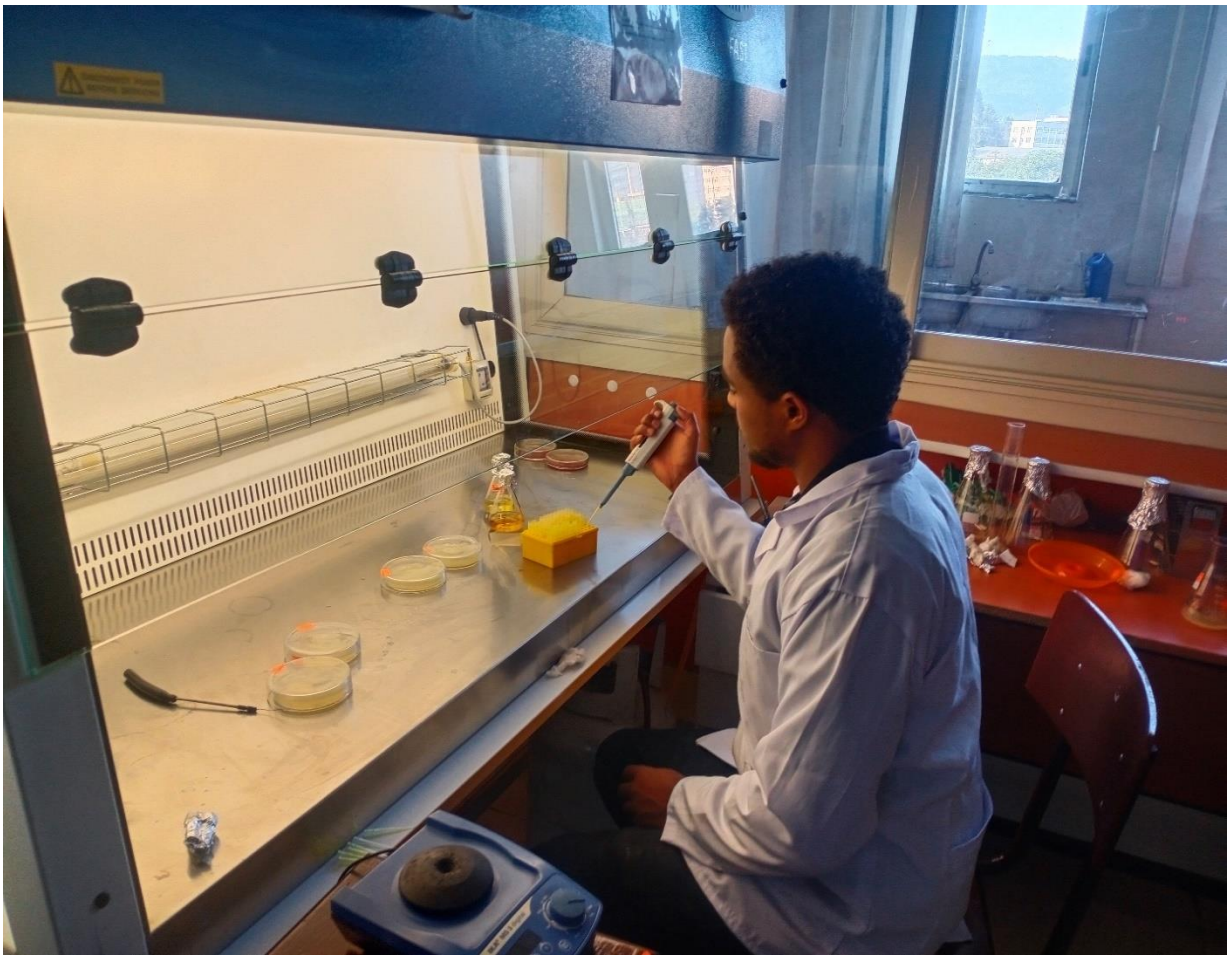

Figure A3: Inoculation of *Burkholderia Sacchari*

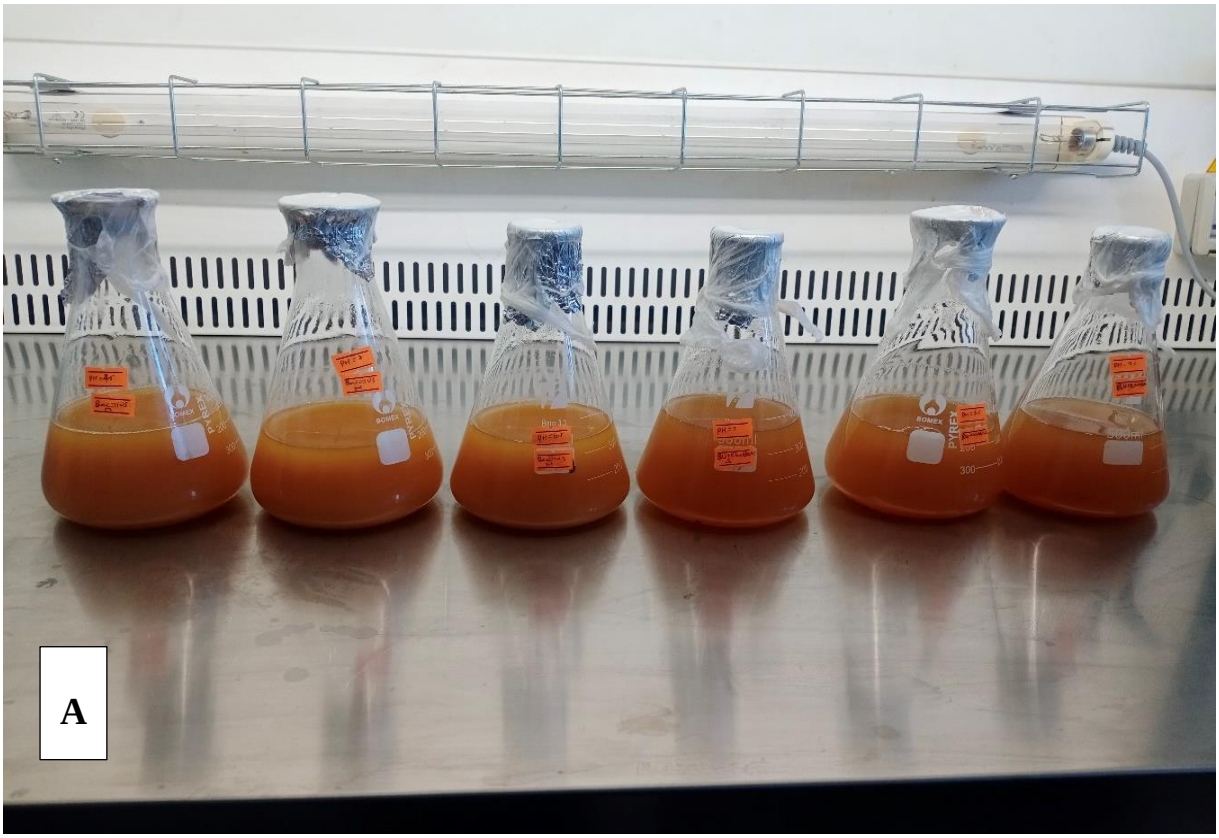

Figure A4: Propagation (A) and Fermentation (B)

Figure A5: Biomass Separation from Broth

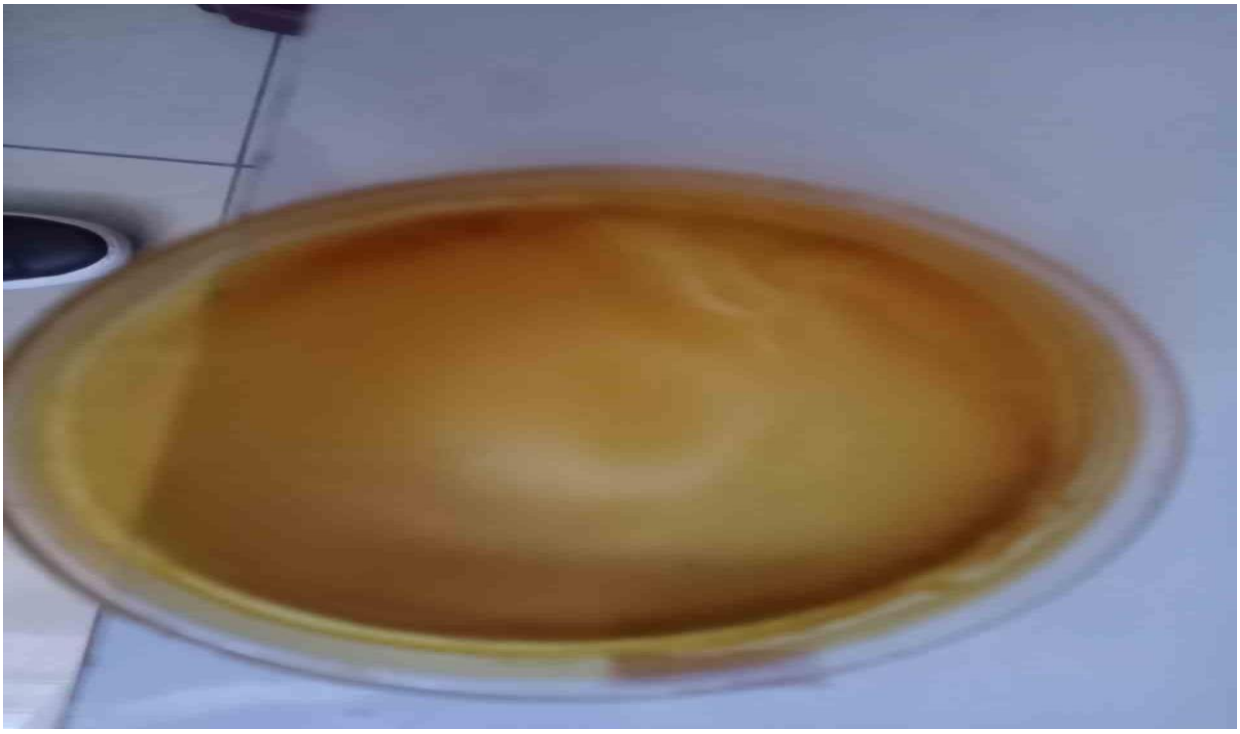

Figure A6: Separated Bacterial Biomass

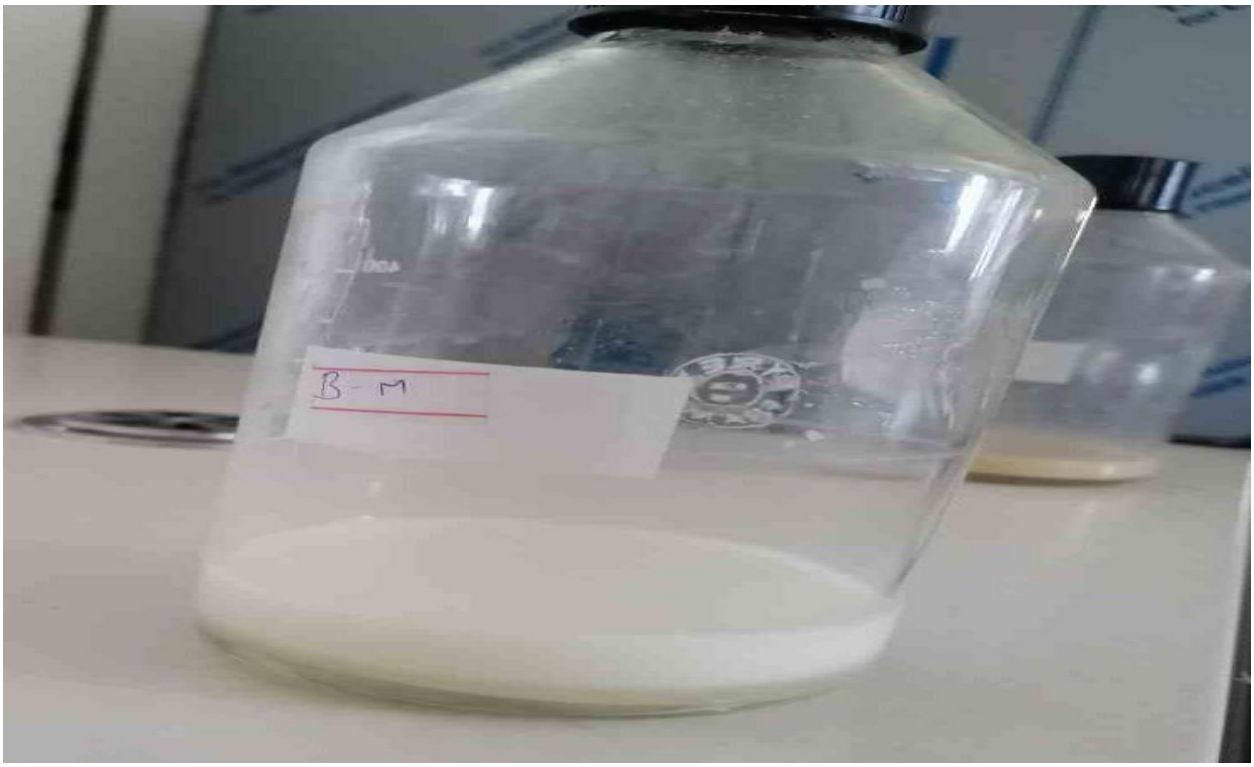

Figure A7: PHA Extraction

Table A1: Standard and Absorbance Calibration Data

Absorbance	Reducing Sugar(µg/L)
0.092568	25
0.24144	50
0.3895	75
0.53944	100
0.70014	125
0.985623	150

Table A2: Sequential Model Sum of Squares [Type I]

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Mean vs Total	419.48	1	419.48			
Linear vs Mean	3.39	3	1.13	0.3384	0.7979	
2FI vs Linear	0.5571	3	0.1857	0.0434	0.9873	
Quadratic vs 2FI	**42.79**	**3**	**14.26**	**35732.11**	**< 0.0001**	**Suggested**
Cubic vs Quadratic	0.0019	3	0.0006	2.90	0.1651	Aliased
Residual	0.0009	4	0.0002			
Total	466.21	17	27.42			

Table A3: Model Summary Statistics

Source	Std. Dev.	R^2	Adjusted R^2	Predicted R^2	PRESS	
Linear	1.83	0.0724	-0.1416	-0.3300	62.16	
2FI	2.07	0.0844	-0.4650	-1.1620	101.03	
Quadratic	**0.0200**	**0.9999**	**0.9999**	**0.9993**	**0.0320**	**Suggested**
Cubic	0.0148	1.0000	0.9999		*	Aliased

Table A4: Coefficients in Terms of Coded Factors

Factor	Coefficient Estimate	df	Standard Error	95% CI Low	95% CI High	VIF
Intercept	7.40	1	0.0089	7.38	7.42	
A-Temperature	0.2620	1	0.0071	0.2453	0.2787	1.0000
B-pH	0.5750	1	0.0071	0.5583	0.5917	1.0000
C-Time	0.1545	1	0.0071	0.1378	0.1712	1.0000
AB	0.2150	1	0.0100	0.1914	0.2386	1.0000
AC	-0.0060	1	0.0100	-0.0296	0.0176	1.0000
BC	0.3050	1	0.0100	0.2814	0.3286	1.0000
A^2	-1.94	1	0.0097	-1.96	-1.91	1.01
B^2	-1.36	1	0.0097	-1.38	-1.34	1.01
C^2	-1.88	1	0.0097	-1.90	-1.85	1.01

Table A5: Generated Solution for Optimization

Number	Temperature	pH	Time	PHA yield	Desirability	
1	**35.179**	**7.046**	**48.642**	**7.457**	**1.000**	**Selected**
2	35.185	7.091	49.867	7.478	1.000	
3	35.413	7.110	51.785	7.432	1.000	
4	35.020	7.075	45.788	7.426	1.000	
5	35.040	7.151	46.591	7.437	1.000	
6	35.265	7.028	47.479	7.428	1.000	
7	35.116	7.154	51.747	7.456	1.000	
8	35.325	7.061	48.015	7.448	1.000	
9	35.190	7.144	48.431	7.472	1.000	
10	35.101	7.109	45.886	7.439	1.000	
11	35.319	7.162	49.002	7.456	1.000	
12	35.150	7.161	47.400	7.453	1.000	
13	35.336	7.175	49.915	7.447	1.000	
14	35.083	7.112	46.866	7.457	1.000	
15	35.319	7.091	52.503	7.434	1.000	
16	35.059	7.148	46.460	7.438	1.000	
17	34.976	7.085	52.339	7.432	1.000	
18	35.017	7.104	49.277	7.471	1.000	
19	35.055	7.191	50.587	7.439	1.000	
20	34.965	7.089	47.467	7.450	1.000	
21	35.282	7.173	50.171	7.455	1.000	
22	35.155	7.031	48.824	7.446	1.000	
23	35.181	7.141	52.072	7.456	1.000	
24	35.233	7.080	52.678	7.436	1.000	
25	35.373	7.114	52.192	7.435	1.000	
26	35.236	7.181	52.441	7.430	1.000	
27	35.255	7.142	48.231	7.467	1.000	
28	35.368	7.130	47.141	7.442	1.000	
29	35.267	7.175	46.933	7.432	1.000	
30	35.356	7.128	50.644	7.458	1.000	
31	34.946	7.123	50.057	7.457	1.000	
32	35.339	7.142	49.239	7.462	1.000	
33	35.187	7.055	45.600	7.421	1.000	
34	35.251	7.082	47.963	7.466	1.000	
35	35.420	7.151	48.402	7.438	1.000	
36	35.245	7.103	51.500	7.463	1.000	
37	35.195	7.088	48.088	7.473	1.000	
38	35.434	7.154	48.593	7.435	1.000	
39	34.937	7.140	49.640	7.452	1.000	
40	34.900	7.105	51.079	7.440	1.000	
41	35.230	7.115	47.850	7.471	1.000	

42	35.350	7.060	48.497	7.446	1.000	
43	35.151	7.138	51.351	7.467	1.000	
44	35.133	7.131	48.215	7.474	1.000	
45	35.253	7.134	47.295	7.459	1.000	
46	35.141	7.119	47.855	7.473	1.000	
47	35.058	7.093	48.174	7.470	1.000	
48	35.357	7.099	52.782	7.423	1.000	
49	35.438	7.147	51.148	7.431	1.000	
50	35.327	7.067	50.141	7.453	1.000	
51	35.108	7.097	45.643	7.434	1.000	
52	35.121	7.080	48.840	7.475	1.000	
53	35.109	7.061	49.718	7.466	1.000	
54	35.187	7.066	52.709	7.430	1.000	
55	35.109	7.179	52.038	7.437	1.000	
56	35.326	7.108	48.317	7.464	1.000	
57	35.332	7.148	49.762	7.462	1.000	
58	35.092	7.147	47.843	7.463	1.000	
59	35.266	7.155	49.111	7.466	1.000	
60	35.010	7.078	46.014	7.430	1.000	
61	35.132	7.160	46.813	7.443	1.000	
62	35.146	7.150	49.865	7.473	1.000	
63	35.038	7.091	49.787	7.472	1.000	
64	35.238	7.064	49.106	7.465	1.000	
65	35.068	7.083	46.701	7.451	1.000	
66	35.041	7.151	49.216	7.465	1.000	
67	35.153	7.066	47.960	7.465	1.000	
68	35.011	7.109	50.526	7.467	1.000	
69	34.967	7.155	50.097	7.451	1.000	
70	35.311	7.040	50.524	7.434	1.000	
71	35.062	7.109	50.956	7.469	1.000	
72	35.427	7.109	47.074	7.429	1.000	
73	35.047	7.155	50.089	7.464	1.000	
74	35.213	7.150	48.082	7.466	1.000	
75	35.225	7.108	51.488	7.465	1.000	
76	35.401	7.127	51.202	7.443	1.000	
77	35.222	7.110	48.149	7.474	1.000	
78	35.130	7.194	49.798	7.445	1.000	
79	35.151	7.065	48.701	7.469	1.000	
80	35.271	7.084	47.217	7.456	1.000	
81	35.072	7.100	51.012	7.468	1.000	
82	35.172	7.124	53.260	7.434	1.000	
83	35.079	7.038	50.724	7.442	1.000	
84	35.138	7.185	47.195	7.432	1.000	
85	35.404	7.076	49.156	7.444	1.000	

86	35.044	7.102	52.378	7.446	1.000	
87	35.091	7.048	49.753	7.457	1.000	
88	35.269	7.112	49.321	7.476	1.000	
89	35.287	7.087	48.079	7.465	1.000	
90	35.009	7.165	50.455	7.451	1.000	
91	34.986	7.065	51.599	7.439	1.000	
92	35.360	7.110	47.533	7.451	1.000	
93	35.158	7.147	51.035	7.468	1.000	
94	34.832	7.113	48.343	7.425	1.000	
95	35.259	7.048	52.146	7.425	1.000	
96	35.023	7.066	49.196	7.462	1.000	
97	35.318	7.124	50.868	7.463	1.000	
98	35.108	7.057	49.361	7.464	1.000	
99	35.197	7.138	49.242	7.477	1.000	
100	35.297	7.062	50.437	7.452	1.000	