OPTIMIZATION OF PRODUCTION CONDITIONS OF POLYHYDROXYALKANOATES PRODUCED BY SOIL EXTRACTED BACTERIAL STRAINS ISOLATED FROM DUMPING SITE

By

Mir Khizar Rafique

Asad Hussain

DEPARTMENT OF EARTH AND ENVIRONMENTAL SCIENCES

BAHRIA UNIVERSITY, ISLAMABAD PAKISTAN

2024

OPTIMIZATION OF PRODUCTION CONDITIONS OF POLYHYDROXYALKANOATES PRODUCED BY SOIL EXTRACTED BACTERIAL STRAINS ISOLATED FROM DUMPING SITE

Mir Khizar Rafique

Asad Hussain

A thesis submitted to Bahria University, Islamabad in partial fulfillment of the requirement for the degree of B.S in Environmental Sciences

DEPARTMENT OF EARTH AND ENVIRONMENTAL SCIENCES

BAHRIA UNIVERSITY, ISLAMABAD

2024

DEDICATION

We, with immense pleasure, dedicate this research to our beautiful homeland Jammu and Kashmir. This research is also dedicated to our respected parents

ABSTRACT

Plastic waste has become the major contributor in solid waste across the world. The eye catching problem in plastic waste management is its toxicity and centuries of time for even its partial degradation. Bioplastics that are naturally biodegradable have turned into a need of the sustainable future. This study aimed to isolate and authenticate bacterial strains capable of producing Polyhydroxyalkanoate (PHA), a bioplastic from soil samples collected at a dumping site and also to assess how the concentration of carbon sources and incubation duration impact the production of (PHA). For five soil samples, preliminary selection and confirmation of PHA producing bacteria was obtained by Nile blue A and Sudan Black B dyes respectively. Effect of incubation period as 24, 48, 72(hrs.) and sugarcane bagasse as carbon source concentration of 1% and 10% was observed and PHA production was optimized at 7 pH. FTIR analysis of each of six treatments revealed characteristic peaks of PHA between 1720-1740 (cm^{-1}), the presence of ester attached carbonyl group. Besides that, the peaks for methylene band and ethyl were observed near 2924 (cm^{-1}) and 2855 (cm^{-1}) in succession that is a property of medium chain PHA. Significant peaks at 1454 (cm^{-1}) exhibited the stretching vibration of carbon oxygen bonds. The conclusion drawn from this research indicated that the maximum biomass produced from PHA producing bacteria was from treatment with 10% carbon source incubated for 72 hours.

ACKNOWLEDGMENT

Above all, we extend our sincerest gratitude to the Almighty Allah for granting us the strength and fortitude to navigate through this extensive subject, as well as for surrounding us with supportive instructors, family, friends, and exceptional academic capabilities. Our heartfelt appreciation goes to our supervisor, Dr. Umairullah Jamil (Senior Assistant Professor of Earth and Environmental Sciences) at Bahria University Islamabad, whose unwavering encouragement and guidance have been instrumental in shaping this thesis to its fullest potential. We are equally thankful to Mr. Imtiaz (Lab technician at BUIC) for his invaluable cooperation and assistance during our practical work, providing us with essential guidance and advice to ensure a conducive working environment. Finally, we are deeply indebted to the prayers and unwavering support of our parents, whose encouragement sustained us through the challenges of our research journey.

TABLE OF CONTENT

CHAPTER 1

INTRODUCTION

CHAPTER 2

MATERIALS AND METHODS

CHAPTER 3

RESULTS AND DISCUSSION

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

°C	Degrees Celsius
µl	Microliter
ABS	Acrylonitrile butadiene styrene
C	Celsius
CDA	Capital Development Authority
cm	Centimeter
CO2	Carbon dioxide
CO2 eqCO2	Carbon dioxide equivalent
e.g.	Exempli gratia (for example)
etc.	Et cetera
FTIR	Fourier Transform Infrared
g	Grams
GHG	Greenhouse gas
h	Hours
H2O	Water
HDPE	High-density polyethylene
HHW	Household Waste
i.e.	Id Est (that is)

Kg	Kilogram
LDPE	Low-density polyethylene
mg	Milligram
min	Minutes
ml	Milliliter
MP	Micro plastics
MSWM	Municipal solid waste management
Mt	Million tons
NBT	Nile Blue A dye
PC	Polycarbonate
PCB	Polychlorinated biphenyls
PE	Polyethylene
PET	Polyethylene Terephthalate
PHA	Polyhydroxyalkanoates
PHA	Polyhydroxyalkanoate
PP	Polypropylene
PP	Polypropylene
PS	Polystyrene
PS	Polystyrene
PU	Polyurethane

PVC	Polyvinyl chloride
Rpm	Revolutions per minute
sec	Seconds
SWM	Solid Waste Management
t	Tons
t/kg	Tons per kilogram

CHAPTER 1

INTRODUCTION

1.1 Solid waste management

In countries that are still growing, like ours, there are big issues with how we deal with our trash. One problem is that we don't spend enough money or build enough facilities to handle all the trash properly. This means the service we get for managing our waste isn't very good (Jagaba et al., 2021), as more people live in cities and towns, we produce more trash. This indicates that there is a greater need for services to gather all of this garbage (Sha'Ato et al., 2007). Many developing countries lack reliable data for proper waste management (Buenrostro et al., 2001). Even where data exists, it's often inconsistent, making it difficult to evaluate waste management strategies effectively (Jagaba et al.,2021). The main goals of solid waste management (SWM) strategies are to deal with health, environmental, aesthetic, land-use, resource, and economic concerns linked with improper waste disposal (Henry et al., 2006). These issues are a continuous worry globally for nations, cities, businesses, and individuals (Nemerow et al., 2009) and the global community in general. In developing countries, the amount of waste produced by growing cities overwhelms local authorities and national governments (Tacoli et al., 2012). Limited resources worsen existing inequalities experienced by vulnerable populations (Mcsweeney et al 2010).

1.2 Plastic waste

The issue of plastic contamination in natural environments has garnered considerable interest from researchers and the general public. Organisms can consume plastic or become ensnared by it, posing a hazard to the entire ecosystem (Sutherland et al., 2010). Plastic waste stands as a prime example of waste stemming from industrial advancement, and its introduction into the environment leads to serious environmental challenges. Presently, plastic goods are essential components of daily life for many, finding use across a spectrum of industries including construction, healthcare, electronics, agriculture, automotive, and packaging. (Anuar Sharuddin et al., 2016). Plastic packaging holds significant importance, constituting 26% of all plastics used, despite its shorter lifespan compared to plastics utilized in sectors like construction and automotive. Manufacturers and processors of plastic emphasize the advantages of plastic packaging, as it not only brings economic gains but also aids in reducing food spoilage and contamination. While this is valuable, even if these plastics are recycled, they eventually reach the end of their usefulness. To achieve a fully circular economy, this waste must be viewed as a resource to be reintegrated into the plastic lifecycle (PlasticsEurope, 2018).

1.2.1 Types of Plastic and wastes

1.2.1.1 Polyethylene Terephthalate (PET)

Polyethylene terephthalate (PET) is a type of plastic known for its smooth, transparent, and relatively thin qualities. It's often referred to as "stomach plastics." PET is widely used in making disposable salad dressing, margarine , mouthwash, vegetable oil, soft drink, , cosmetics, juice, and water bottles because it's resistant to inflammation and completely liquid-tight. Additionally, PET is oxygen-resistant, which helps prevent the entry of oxygen into the bottles (Proshad et al., 2018).

1.2.1.2 High-density polyethylene

Globally, the most utilized plastic is polyethylene, with high-density polyethylene being a notable variant derived from petroleum, known for its heat-resistant properties. It serves as a key component in detergent bottles, milk containers, various types of plastic grocery bags, toys, refrigerators, and more. High-density polyethylene containers are generally regarded as safe for storing food and beverages, as they pose no reported health risks, although prolonged exposure to sunlight may render them harmful, as indicated by some studies (Proshad et al., 2018).

1.2.1.3 Polyvinyl chloride (PVC)

Its attributes comprise: i. Versatility, energy conservation, adaptability to evolving circumstances, long-lastingness, and resistance to fire. ii. It serves diverse sectors like building and construction, medical, agriculture, packaging, and transportation. Furthermore, it's employed in manufacturing wires and cables, footwear, household appliances, films and sheets, furniture, bottles, and other products. (Siddiqui et al., 2013).

1.2.1.4 Low density polyethylene (LDPE)

Characteristics of LDPE: i. LDPE is known for its ease of processing, low density, broad melting range, semi-crystalline nature, low softening point, good resistance to chemicals limited moisture barrier, outstanding dielectric properties, and relatively poor resistance to abrasion and stretching. ii. Its applications range from manufacturing carrier bags, nursery bags, heavy-duty bags, and small squeeze bottles to its use in milk packaging, wire and cable insulation, and various other purposes. (Siddiqui et al., 2013).

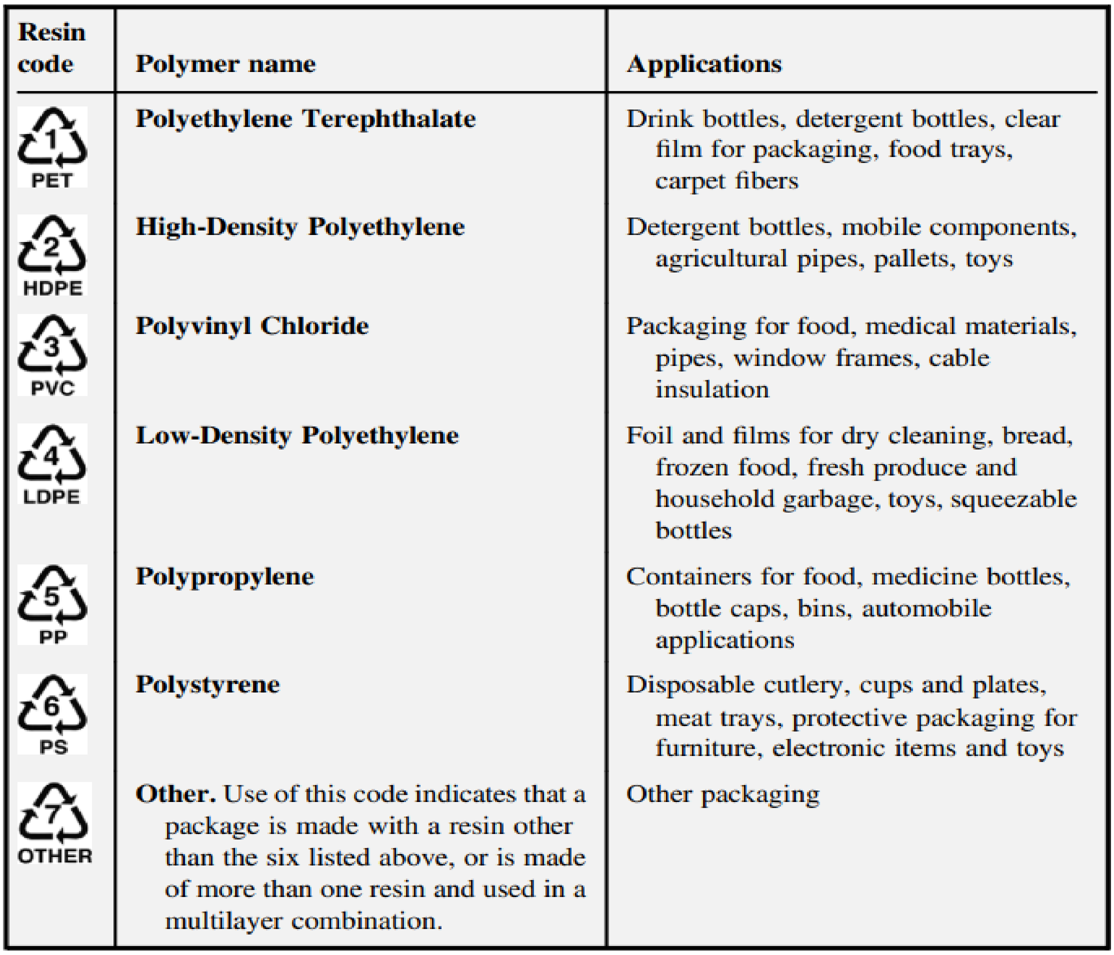
1.2.1.5 Polypropylene

Polypropylene, a robust and semi-transparent plastic, stands out for its strength and weight compared to polyethylene. It is commonly used in medications, ketchup, packaging yogurt, beverages, and more. Similar to polyethylene, polypropylene plastics are free from harmful substances. As containers for food and beverages, they are considered safe for human use (Alabi et al., 2019).

1.2.1.6 Polystyrene

Polystyrene, a material derived from petroleum, contains benzene, which is recognized as a carcinogen. It's extensively employed in making packing materials and insulating products. Items containing styrene can be hazardous to health, as continuous exposure to low levels may result in neurological issues and adverse effects on cells, including the potential for cancer. The International Agency for Research on Cancer has categorized styrene as a human carcinogen (Proshad et al., 2018).

Table 1 Main plastic types and typical applications



Resin code	Polymer name	Applications
1 PET	**Polyethylene Terephthalate**	Drink bottles, detergent bottles, clear film for packaging, food trays, carpet fibers
2 HDPE	**High-Density Polyethylene**	Detergent bottles, mobile components, agricultural pipes, pallets, toys
3 PVC	**Polyvinyl Chloride**	Packaging for food, medical materials, pipes, window frames, cable insulation
4 LDPE	**Low-Density Polyethylene**	Foil and films for dry cleaning, bread, frozen food, fresh produce and household garbage, toys, squeezable bottles
5 PP	**Polypropylene**	Containers for food, medicine bottles, bottle caps, bins, automobile applications
6 PS	**Polystyrene**	Disposable cutlery, cups and plates, meat trays, protective packaging for furniture, electronic items and toys
7 OTHER	**Other.** Use of this code indicates that a package is made with a resin other than the six listed above, or is made of more than one resin and used in a multilayer combination.	Other packaging

1.2.2 Management Problems

Presently, the global community is confronted with the pressing issue of appropriately managing and reclaiming resources from the immense volume of plastic waste. Challenges such as a shortage of technical know-how in handling hazardous waste, limited infrastructure for recycling and resource recovery, and a general unawareness of regulations are the main drivers behind the significant buildup of plastic waste (Kibria et al., 2023). The burning of plastic waste in household settings occurs through different means, such as outdoor waste fires and indoor kitchen fires fueled by solid materials. A recent research study on emissions from cooking stoves found that although plastic bags produced fewer emissions than materials like newspapers, kerosene, cloth, and wood shavings , their combustion for starting fires still resulted in increased emissions from cooking stoves (Cruz et al., 2023).

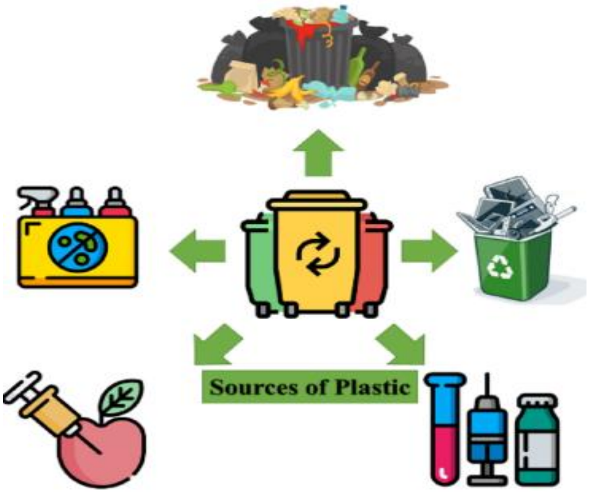


Figure 1 (a) Sources of plastic

The widespread adoption of plastics is driven by their advantageous characteristics, including lightness, durability against impacts, malleability, and resistance to bacteria. Nevertheless, this extensive utilization results in a significant volume of waste. Roughly a quarter of produced plastics are employed for long-lasting applications such as pipelines, while the majority are intended for short-term use and end up as single-use items (Dai et al., 2023). The management of municipal solid waste (MSW) encompasses multiple stages, including waste generation, collection, transportation, and disposal. To ensure effective MSW management, it's essential to have adequate infrastructure in place, along with regular maintenance and upgrades for all these processes. However, as cities experience continuous and unplanned growth, managing MSW becomes more expensive and intricate. Notably, the collection and transportation phases often consume approximately 50–60% of the overall budget allocated for MSW management, highlighting their critical role in shaping the economic feasibility of the entire MSW management system (Khan et al., 2018).

Establishing a circular plastic economy hinges on the effective recycling of plastic sourced from household waste (HHW). HHW plastic consists of recycled plastic of inferior quality alongside a range of incompatible polymers, diverse product types, and various product designs (including color and polymer reparability), which frequently result in significant material losses during sorting processes. In response, recycling programs have been introduced to bolster both the volume and standard of plastic recycling derived from household waste. (Eriksen et al., 2019). Coping with the technical and environmental complexities of waste accumulation is just part of the equation; there are also political, administrative, economic, and societal factors to consider. Waste management encompasses the use of scientific methods to tackle these challenges comprehensively. It requires careful planning, precise design, and efficient operational tools for activities such as waste collection, transportation, processing, recovery, and ultimately, disposal (Agamuthu, 2008). Waste is characterized by its loss of primary economic value yet retains inherent secondary value. Addressing the reduction and effective treatment of waste presents considerable challenges within the realm of environmental sustainability. Diverse types of waste originate from different sectors, driven by continuous technological progress in processing industries (Moya et al., 2017).

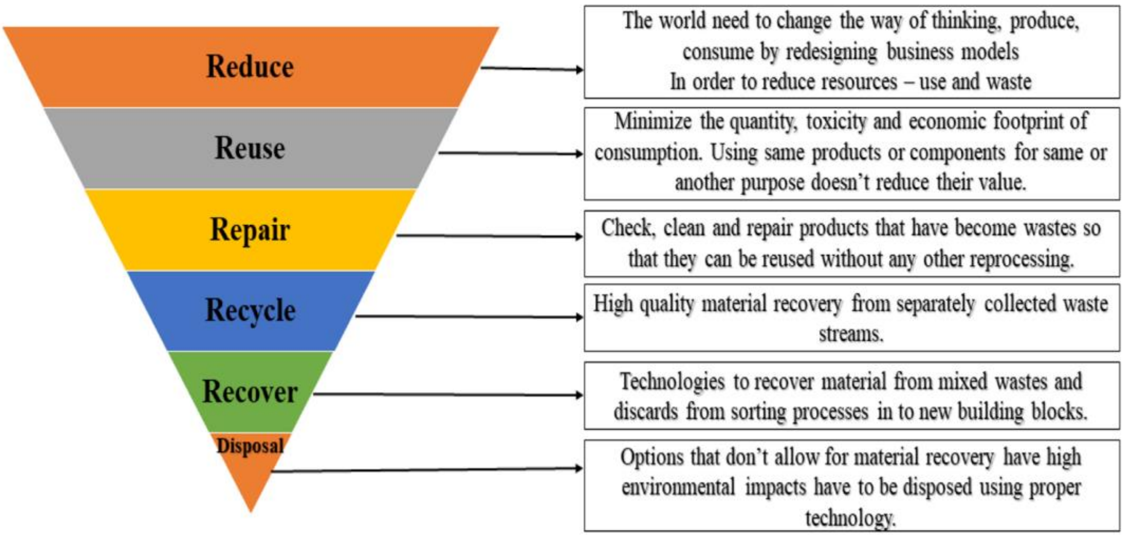


Figure 1.2 (b) Six level pyramid to reduce plastic waste

It's essential to understand that traditional waste challenges can be mitigated by reducing the accumulation of waste, alongside initiatives such as product substitution, waste recovery, and recycling. The volume of waste accumulation plays a significant role in waste management strategies. Consequently, waste materials impact the implementation of crucial precautionary measures and interventions to prevent waste accumulation, as well as essential administrative procedures related to exportation, transportation, reuse and processing, sale (Miandad et al., 2017). The recyclability of a polymer depends on its specific characteristics. Yet, recycling plastics poses technological hurdles, notably due to the risk of contamination, necessitating specialized attention. Therefore, grasping essential processes such as waste reduction, reuse, and recycling is paramount for promoting sustainable development objectives. Considering their potential to undermine environmentally friendly waste management practices, methods such as resource recovery, incineration and landfilling are viewed less favorably in the waste management hierarchy (Owusu et al., 2018).

1.2.3 Environmental Problems

The decomposition of plastic waste in landfills results in the release of carbon dioxide and methane into the air. In 2008, the decomposition of solid waste in landfills emitted an estimated 20 million tons of carbon dioxide equivalent (eqCO2) into the atmosphere. Furthermore, burning plastics and plastic products releases carbon dioxide, which contributes to global warming by trapping radiant heat and impeding its escape from the Earth's atmosphere (Chandegara et al., 2015). Burning plastics and plastic products openly releases harmful pollutants such as dioxins, heavy metals, furans and PCBs into the atmosphere. Exposure to these pollutants through inhalation can lead to serious health issues, especially respiratory disorders. The role of plastics in contributing to air pollution is particularly significant in developing and underprivileged countries, with potentially far-reaching consequences for future generations (Hamlet et al., 2018).

Plastics are currently employed in various applications, including appliances, furniture, lead-acid battery casings, and numerous other products, playing a vital role in our everyday lives. However, the widespread use of plastics, driven by their versatile nature, lightweight, affordability, and contribution to economic development, has led to their increased demand and dependence. Consequently, plastics accumulate in landfills, posing significant risks to human and animal health, and contributing to environmental pollution issues such as groundwater contamination and sanitation concerns. (Gu et al., 2017). As per the latest convention on biological diversity report, there has been a disturbing rise: incidents of fatal entanglement and ingestion of marine debris by marine animals have increased by 40% in the last decade. (Kibria et al., 2023).

Plastic waste has been discovered in numerous marine and terrestrial animals, including turtles, seabirds, and fish (Dar et al., 2022). In recent times, several studies have highlighted the prevalence of micro plastics (MP) in a range of ecosystems, with a significant focus on aquatic settings such as rivers, oceans, estuaries, Arctic waters, and estuaries, among other environments (Bellasi et al., 2020). Plastic products like nets can lead to harm, injury, and fatalities in marine animals through entanglement. Research indicates that over 260 species of vertebrates and invertebrates ingest plastics or get entangled by plastic items, resulting in over 400,000 deaths among marine mammals (Daniel, 2004). The entanglement of marine animals in plastic debris has led to the deaths of many turtles, birds, fish, and other species, primarily due to drowning or suffocation. Studies indicate that entanglement poses health risks to an estimated 243 species of marine life, often resulting in fatalities. Additionally, entanglement by plastic debris renders animals more susceptible to predation, as they are unable to untangle themselves and escape from predators (Hammer et al., 2012). Although plastic polymers are often perceived as inert and posing minimal risk to public health, concerns arise from the presence of various additives and residual monomers that may be retained within these polymers. These substances are thought to contribute to the suspected health risks associated with plastics (Araujo et al., 2002). The frequent use of personal care items can lead to heightened exposure to low molecular weight phthalates. Research suggests that men who regularly use aftershave and cologne experience elevated levels of phthalate exposure. Similarly, infants using certain infant-care products such as shampoos, lotions, and powders also demonstrate increased exposure to phthalates (Matsumoto et al., 2008).

1.3 Alternative of Plastics

The primary monomers used in plastic manufacturing, such as ethylene and propylene, are sourced from fossil hydrocarbons. Furthermore, none of the commonly used plastics have biodegradable properties. As a result, instead of breaking down, these plastics accumulate in landfills or the natural environment (Barnes et al., 2009). Mechanical recycling is essential for recovering plastic waste, utilizing physical processes to reintegrate it into the value chain. This involves a series of steps, including screening, collection, manual or automated sorting based on material type, and size reduction, thorough cleaning, and conversion into usable feedstock through extrusion and granulation. The specific order and combination of these steps are tailored to the individual characteristics of the feedstock, considering factors like its size, origin, composition and shape, and to ensure efficient and customized recovery. (Hopewell et al., 2009).

Figure 1.3 (a) Mechanical recycling loop of plastic

The excessive disposal of disposable low-density plastic waste, particularly from packaging and polymers like disposable diapers, has resulted in the overloading of landfill sites. This has negatively affected both the quantity and capacity of landfills available for disposing of various grades of trash. Additionally, the inclusion of plastics and polymers in landfills poses challenges to their long-term mechanical stability, as they have the potential to degrade and break down, leading to the production of toxic, flammable, and environmentally harmful gases (Swift et al., 2002). The practice of open-field incineration of plastic waste emerges as a significant source of air pollution. When municipal solid waste, containing approximately 12% plastics, is burned, it releases harmful substances like dioxins, furans, mercury, (PCBs) into the atmosphere. Furthermore, burning polyvinyl chloride (PVC) produces dangerous halogens, contributing to air contamination and climate change. These pollutants endanger the vegetation, environment, and the health of humans and animals. Specifically, exposure to polystyrene can result in central nervous system damage, while brominated chemicals present in plastics possess mutagenic and carcinogenic properties (Verma et al., 2016).

Biopolymers have the capability to fully and safely degrade within composting environments, making them a promising substitute for conventional plastic materials (Rapa et al., 2011). Bio-plastics, sourced from plants such as soybean oil, sweet potatoes, sugarcane, corn starch, hemp oil, naturally decompose through the activity of microorganisms like fungi, the bacteria, and algae. This characteristic makes them a promising solution for addressing both the energy crisis and reducing society's dependency on fossil fuels (Reddy et al., 2013). Bioplastics are composed of lengthy chains of monomers linked by ester bonds, thus qualifying them as polyesters. They are sorted into several types, with PHA (Polyhydroxyalkanoate) being among the most prevalent. PHA acts as a storage material for carbon and/or energy in various microorganisms when essential nutrients are scarce (Gill et al., 2014).

PHAs are synthesized by living organisms, rendering them biodegradable materials. Unlike fossil resources, PHAs are sourced from renewable raw materials, originating from natural substrates. Notably, PHAs demonstrate biocompatibility with humans and other life forms, and upon ingestion by living organisms, they are easily metabolized into non-toxic compounds (Sharma et al., 2021). Increased adoption of biodegradable plastics holds promise for reducing carbon footprints, pollution risks, and greenhouse gas emissions stemming from polymer usage. Nevertheless, this positive impact hinges on the condition that these alternatives are crafted from non-fossil resources with the aid of renewable energy (North et al., 2013). The production of PHA comes with significantly higher costs—four to nine times more expensive—than traditional plastics, mainly due to the costly production process. This encompasses expenses related to substrates, culture procurement, and sterilization. PHA is commonly produced industrially using pure cultures fed with sugars such as sucrose, glucose, and corn-derived compounds. To address cost challenges, alternative approaches involve utilizing mixed cultures as biocatalysts in conjunction with wastewater as a substrate (Bengtsson et al., 2008).

Despite the limited commercial utilization of bioplastics across different sectors, they are heavily integrated into notably disposable items like bowls, cutlery, pots, straws, crockery, packaging and consumer goods, (Chen et al., 2012).

1.4 Bioplastic (PHA) as an alternative

Across all modern societies, synthetic plastics hold immense importance due to their outstanding attributes that develop over time. These attributes, such as affordability, stability, and durability derived from their polymeric composition, render plastics indispensable components of various facets of our daily routines (Ali et al., 2021). The consumption of bioplastics globally is experiencing remarkable growth, with yearly estimates of expansion ranging from 20% to 25%. This significant uptick can be attributed to various benefits associated with bioplastics, including, increased resource independence, decreased carbon footprints, better energy efficiency, and eco-friendly properties. However, despite these promising aspects, several challenges remain unaddressed. These include high production costs, insufficient recycling infrastructure, potential raw material depletion, misinterpretation of bioplastic terminology, and the absence of comprehensive legislation, all serving as obstacles to wider adoption. (Arikan et al., 2015).

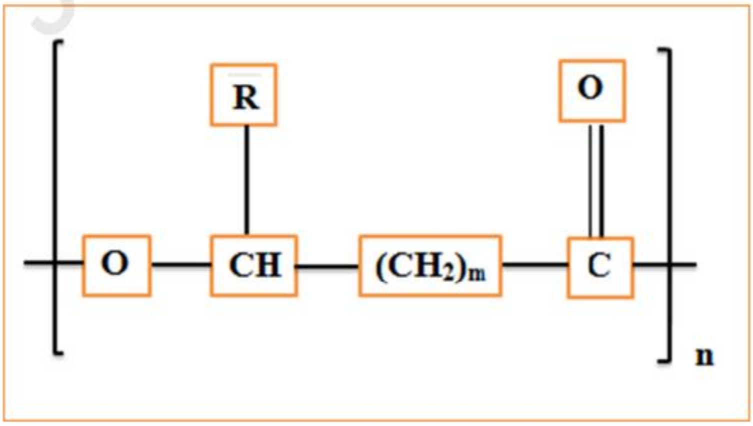


Figure 1.4 The basic structure of PHA: R- acyl group that can contain 1-13 carbons; m-1, 2 or 3; n-100 to many thousands

There is a growing recognition of biopolymers with plastic-like characteristics as viable alternatives to traditional petrochemical plastics in the market. Specifically, thermoplastic starch, poly(lactic acid), and microbial Polyhydroxyalkanoates (PHA) are currently under the spotlight in research and development endeavors (Zhu et al.,2016) . Above all, PHA represents the only category of "green plastics" due to their exceptional attributes. They are sourced from renewable resources and synthesized through biosynthesis, occurring within prokaryotic microbial cells. Moreover, PHA are biodegradable, compostable, and can be fermented into harmless byproducts such as water, CO2, or CH4. Their biocompatibility also makes them highly desirable for biomedical applications. Notably, the production of PHA does not deplete fossil resources, as they are derived from renewable sources. Their entire lifecycle aligns with the closed carbon cycle of nature, ensuring that their degradation does not contribute to increased atmospheric CO2 levels, thereby mitigating climate change (Akiyama et al.,2003).

Plastic recycling serves as a means to reduce overall plastic waste production; however, recycled plastics commonly experience a decline in mechanical performance. To counteract this issue, several approaches can be employed, including physical treatment like annealing to improve modulus and strength, incorporation of chemical stabilizers, blending with other recycled plastics, or combining recycled plastics with alternative polymers as a means of valorization (Badia et al.,2017). However, recycling inevitably produces waste either during the recycling process or at the end of the multiple recycling cycles, rendering the material too degraded for continued use. Despite numerous years of recycling endeavors, only a small proportion of plastics in the USA—less than 10%—is successfully recycled, a sharp disparity compared to the recycling rates of non-plastic materials, which ranged from 25% to 65% in 2017 (Kawai et al.,2020).

Landfilling waste plastics represents the least preferred choice in the UK waste hierarchy. Historically, it was an appealing option due to its simplicity and low cost, requiring minimal separation, cleaning, or treatment. In 1999, Western Europe disposed of 65% of the total recoverable plastics in household waste—equivalent to 8.4 million tons annually—through landfilling (APMA et al., 2002). Nevertheless, suitable landfill sites throughout Europe are becoming scarce, and there is a growing public apprehension regarding the environmental and health ramifications of landfilling. Concerns are mounting over the presence of toxic materials in municipal waste deposited in landfills, and the potential for these substances to leach into the surrounding environment (Miller et al.,2005). The explicit government policy to reduce landfill waste, exemplified by initiatives like the Landfill Directive (European Commission 1999/31/EC), poses a formidable challenge in the UK. Despite efforts, around 60% of municipal waste in England continues to be deposited in landfills, a notably higher percentage compared to roughly 37% in France and approximately 20% in Germany (EEA et al., 2007). The disposal of biodegradable materials, such as garden, bioplastic polymers, also kitchen waste, in landfills presents a notable concern, as it may lead to the production of methane, a greenhouse gas that is 25 times more effective at trapping heat compared to CO2, particularly under anaerobic conditions (Hudgins et al.,1999).

1.5 Types of Biodegradable plastics

Increased public awareness regarding the environmental implications of plastics has spurred numerous countries to adopt waste management measures, including the quest for biodegradable alternatives. As a result, there has been a marked escalation in research endeavors aimed at exploring potential solutions in this realm. This section underscores key materials presently undergoing scrutiny:

1.5.1 Polyhydroxyalkanoates (PHAs)

With their inherent biodegradability and versatile properties, these naturally sourced biopolymers stand out as highly desirable options for a diverse array of applications.

1.5.2 Polylactides

Polylactides, derived from renewable sources like corn starch, exhibit favorable biocompatibility and are experiencing growing adoption in biomedical and packaging industries.

1.5.3 Aliphatic polyesters

Aliphatic polyesters, characterized by their diverse physical and chemical properties, demonstrate potential for a wide range of applications, spanning from textiles to food packaging.

1.5.4 Polysaccharides

Polysaccharides like cellulose and starch possess distinctive biodegradability and functionalities, offering opportunities for the development of sustainable materials.

1.5.5 Co-polymers and Blends

The goal of researchers is to blend different biopolymers with traditional plastics to achieve customized properties while maximizing biodegradability (Jogdand, 1998).

The addition of materials like starch to conventional plastics, as demonstrated by products like "Bio-D" bags, can enhance biodegradation by microorganisms. However, challenges arise from limited compatibility and inadequate quantities, limiting the effectiveness of this technique. Another approach involves adopting a biological method, which focuses on creating bioplastics exclusively from renewable resources, offering a potentially more sustainable solution in the long run. While obstacles persist in achieving desired performance levels and cost-effectiveness, research efforts in this field show significant potential (Jawadekar,1992).

1.6 Sources of PHA (Polyhydroxyalkanoate)

In the production of PHA, a variety of materials have been utilized as carbon sources, including molasses sourced from sugar cane molasses, paper mill waste water, activated sludge, food waste, also plant oils such as palm oil and olive oil effluent. Additionally, agricultural wastes from the sugar industry, such fermented mash and spent wash have demonstrated significant potential for serving as carbon sources (Chaudhry et al., 2009). There's a growing understanding of how production costs can be reduced by utilizing inexpensive or nearly zero-cost carbon sources (Saratale et al., 2021). The properties of PHA biopolymers are determined by the type and arrangement of various monomeric building blocks. PHAs represent a versatile class of biomaterials, exhibiting traits ranging from elastomeric to semi-crystalline thermoplastic-like polymers (Koller et al., 2018). Poly(3-hydroxybutyrate) is the most thoroughly researched member of the PHA family. It is also the primary type of PHA bio polyester synthesized by numerous microorganisms in nature, utilizing basic feedstock like carbohydrates, alcohols such as glycerol, or fatty acids with an even number of carbon atoms. (Koller et al., 2017). The production of Polyhydroxyalkanoates (PHA) using mixed cultures has been a subject of significant study in recent years. PHA storage by mixed microbial cultures occurs under transient conditions of carbon or oxygen availability, referred to as aerobic dynamic feeding and anaerobic/aerobic process, respectively. In these processes, organisms capable of accumulating PHA, which display a range of phenotypes, are selected due to the dynamic operating conditions imposed on the reactor (Serafim et al., 2008).

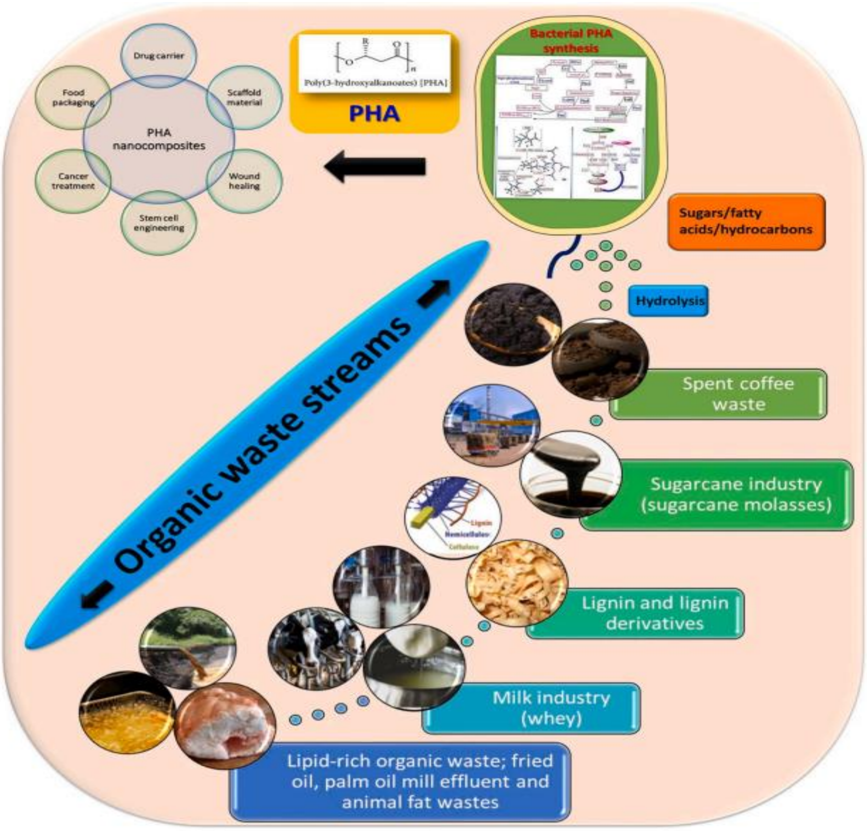


Figure 1.5 Schematic view of various organic waste streams for PHA production

1.7 Production conditions and optimization

Polyhydroxyalkanoates (PHA) are biopolymers stored as granules in various organisms under conditions of excess carbon sources and limited nutrients, serving as a carbon and reducing equivalent sink. To reduce production costs, it's crucial to identify microorganisms capable of utilizing inexpensive carbon sources and producing PHA with high yields. Numerous microorganisms have been identified for PHA production using low-cost agricultural feedstock and surplus materials (Kulkarni et al., 2015). PHA production presents economic and environmental benefits owing to its utilization of renewable resources and complete biodegradability. (Braunegg et al., 1998).

The initial phase focuses on maximizing biomass production, with PHA production commencing only in the subsequent phase once suitable conditions are established (Rondʼošová et al., 2022). Their accumulation functions as a natural mechanism for storing carbon and energy during imbalanced nutrient supply. Furthermore, it may occur when growth is constrained by the depletion of nitrogen, phosphorus, or oxygen sources alongside an increased concentration of the carbon source (Możejko-Ciesielska & Kiewisz, 2016). While nitrogen is typically the primary limiting factor, certain bacterial species exhibit increased PHA production under conditions of oxygen stress (Rond’ošová et al., 2022). Researchers employed a groundbreaking strategy by utilizing a novel medium containing rubber seed oil, an otherwise commercially insignificant substrate, for PHA production in wild Bacillus cereus (Kynadi et al., 2017). pH, substrate concentration, microenvironment, phosphorus, iron concentrations, nitrogen, are among the operating parameters that significantly affect PHA synthesis. Determining the optimal conditions involves conducting multiple experiments with different combinations of parameters, which can be logistically challenging. Traditional experimental approaches involve modifying one factor at a time while maintaining all others constant, enabling the evaluation of each factor's specific impact (Mohan et al., 2013).

Extraction of PHAs, which accumulate intracellularly, requires breaking down and removing the cell membrane. Traditional extraction techniques rely on halogenated solvents, with chloroform being the predominant choice (Mannina et al., 2019). Gas chromatography coupled with mass spectrometry (GC-MS) is a highly accurate method that permits the analysis of monomer concentration, composition, and the purity of the product (Lo et al., 2009).

1.8 Bioplastic Market

Researchers have developed several tools, such as the widely recognized "plastic spectrum," to facilitate informed decision-making in plastics selection. This framework classifies plastics according to their environmental and performance attributes, assisting stakeholders in selecting the most suitable option for particular applications (Kaith et al., 2010). The "plastic spectrum" offers a visual representation of various plastics, organizing them based on their environmental and performance qualities. Bio-based bioplastics are commonly positioned at the "right" end of this spectrum, indicating a more favorable status. This reflects their positive attributes, including their origin from renewable sources and the potential for biodegradation and composting (Alvarez-Chavez et al., 2011).

Figure 1.6 Plastic spectrum on basis of preference of use of plastic

(PU; polyurethane, PS; polystyrene, ABS; acrylonitrile butadiene styrene, PC; polycarbonate, PE; polyethylene, PP; polypropylene)

Bioplastics have made significant strides in commercialization since the inception of the plastic pyramid, positioning them ahead of conventional plastics within this conceptual framework. This progress is particularly evident in the domain of packaging films and containers, driven by their short lifespans and prevalent disposal in landfills. Notably, biodegradable bioplastics have exhibited a diverse range of properties, facilitating their application in various biomedical contexts. These applications span from the manufacturing of bone plates and screws to drug delivery systems and tissue engineering scaffolds (Sivan, 2011).

Bioplastics are gaining momentum worldwide, finding their way into a wide range of technological applications. In the United States, well-known brands like McDonald's are setting the pace by transitioning to biodegradable food containers. This movement transcends individual companies, as industry giants such as DuPont, Bayer, Dow Cargill, Danone, Nike and others are actively participating in the bioplastics revolution through the creation of biodegradable packaging solutions (Krzan et al., 2006). The global bioplastics market is experiencing remarkable growth, driving forward at a rate of 20% to 25% annually. Forecasts indicate that bioplastics' market share, currently comprising 10% to 15%, is on track for significant expansion, expected to reach 25% to 30% by 2020. This rapid expansion is underscored by the substantial increase in the market value of bioplastics, which has surged from over $1 billion in 2007 to an estimated $10 billion by 2020. Moreover, this growth trajectory is further fueled by the continuous entry of new companies into the bioplastics sector, signaling a promising future for the industry (Arikan et al., 2015).

1.9 Advantages of Bioplastics

The potential for a reduced carbon footprint with bioplastics depends on achieving permanent carbon sequestration. Unlike traditional plastics, which release captured carbon upon degradation, "permanent" bioplastics behave like conventional plastics by storing sequestered CO2 indefinitely. This characteristic persists even through recycling, making permanent bioplastics a compelling sustainable option. However, a thorough examination of specific bioplastic varieties and potential limitations like land use impact is necessary for a comprehensive understanding of their effects (Chen, 2014).

Bioplastics are notable for their utilization of renewable biomass sources like sugarcane, corn, and soy, in contrast to finite fossil fuels like petroleum. This shift holds promise for improved resource management and decreased reliance on the unpredictable geopolitical factors often linked with the crude oil market (Yu & Chen, 2008). Bioplastics offer the potential for greater energy efficiency during manufacturing in contrast to conventional plastics. This becomes particularly notable as traditional plastics consume around 4% of the global yearly oil consumption for their production. Given the diminishing oil reserves, bioplastics emerge as a valuable alternative, potentially lessening the impact of volatile oil prices on plastic manufacturing (Chen,2014).

Bioplastics present a possibly reduced environmental footprint compared to standard plastics due to their decreased greenhouse gas (GHG) emissions and lack of harmful substances. Research conducted by Yu and Chen (2008) indicates that bioplastic manufacturing emits substantially less CO2, estimated at 0.49 kg per 1 kg of resin. This represents approximately an 80% decrease compared to conventional petrochemical-based plastics, which typically release 2-3 kg of CO2 per 1 kg of resin. Such a significant reduction underscores the potential role of bioplastics in mitigating global warming through reduced emissions.

1.10 Limitations of Bioplastics

Despite their potential environmental advantages, bioplastics encounter several challenging limitations. Their current higher cost, estimated to be double that of conventional plastics, represents a significant barrier to widespread adoption. However, with anticipated larger-scale industrial production, strategies to reduce costs are expected to enhance affordability in the future. Another critical challenge is their potential to disrupt the existing plastic recycling infrastructure. If not carefully separated from conventional plastics, bioplastics can contaminate recycling streams, especially during separation processes like infrared sorting. This contamination renders the entire batch unusable, posing additional hurdles for the recycling industry (Lagaron & Lopez-Rubio, 2011).

Furthermore, sustainability concerns persist regarding bioplastic feedstock. Despite being derived from renewable sources, their production may compete with food production for land and resources, posing sustainability challenges. To tackle this issue, research is exploring the use of food by-products as alternative feedstock, aiming to reduce both energy consumption and potential competition for agricultural resources. This approach offers the potential for additional raw material sources and the advancement of a more sustainable bioplastics industry (Arikan et al., 2015).

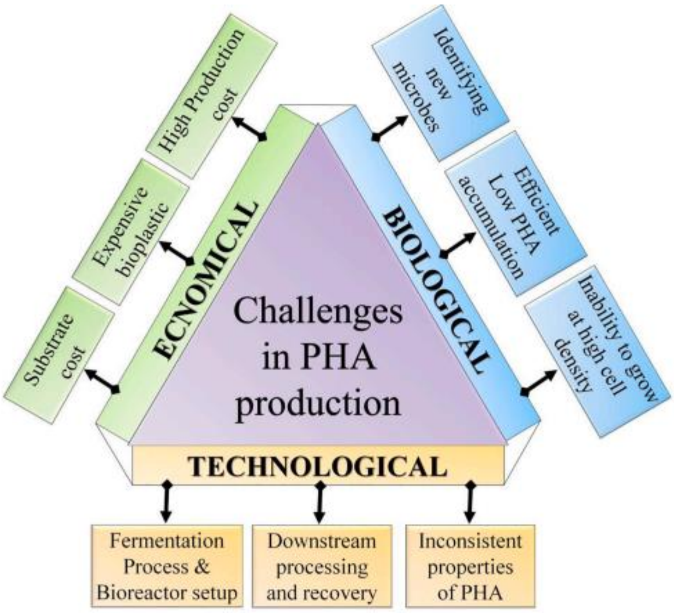


Figure 1.7 Technological, economical and biological challenges in bioplastic production

The utilization of terms like "compostable" may be misconstrued as universally applicable, despite many bioplastics requiring specific industrial composting facilities that are not universally available. This disparity fosters ambiguity and has the potential to mislead consumers. Additionally, the excessive use of terms such as "non-toxic," "eco-friendly," and "degradable" by manufacturers, lacking precise definitions, exploits consumer awareness and raises ethical concerns. Despite the anticipated growth in bioplastic production surpassing 6.7 million tons by 2018, many countries lack comprehensive legislation governing their usage, production, and waste management. This gap impedes responsible development and presents challenges in infrastructure, labeling, and consumer education (Barker & Safford, 2009).

Numerous tests are utilized to evaluate the degradation of bioplastics, highlighting the importance of standardized international methods. However, current standards lack alignment and are predominantly utilized within their country of origin. There is an immediate need to synchronize all aspects and develop a new comprehensive guide and standard exclusively for bioplastics, covering utilization, production, and waste management on a global scale. Furthermore, improvements in labeling legislation could consider factors like energy consumption, raw material utilization, and emissions during both manufacturing and usage phases (Akiran et al., 2015)

Research objectives

The current study was performed to isolate and optimize production of Polyhydroxyalkanoate. Objectives of the research are as under.

1. To isolate and confirm Polyhydroxyalkanoate producing bacterial strains from soil samples collected from dumping site.
2. To evaluate the effect of carbon source concentration and time of incubation on production of Polyhydroxyalkanoate.

CHAPTER 2

MATERIAL AND METHODS

2.1 Sampling site

For sample collection, a dumping site under capital development authority CDA located in sector I-11/4 Islamabad Pakistan had been selected. Categorically it's an open dumping site used to dump solid waste including plastic waste, paper waste, construction waste containing soil, mud and cement, organic waste containing food waste, residues of trees and plants and kitchen waste. Waste transferred on the landfill came from nearby areas of Rawalpindi and Islamabad collected by municipal authority with collaboration of CDA. Approximately one hundred garbage trucks, each containing 27-30 Tons of waste, dumped at the site on daily bases.

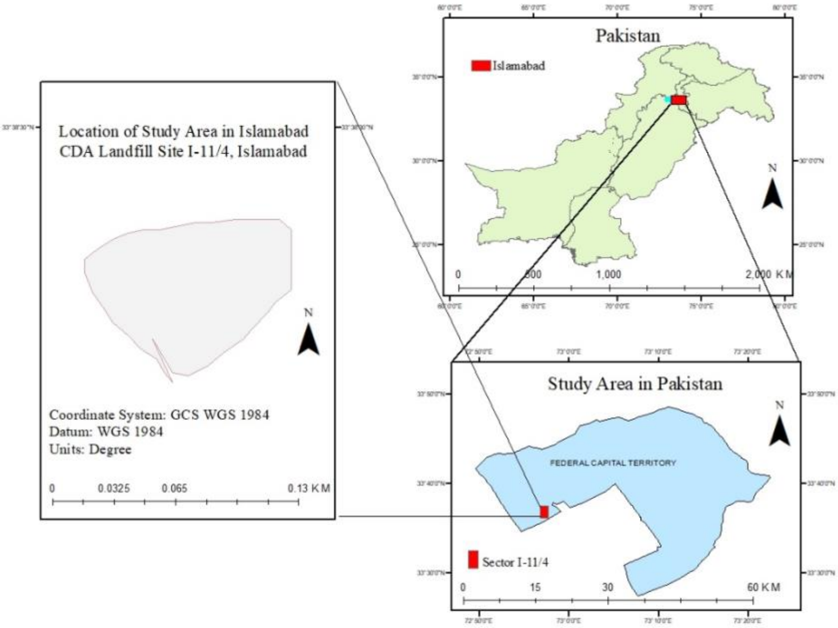


Figure 2.1 (a) Map of sampling area

2.2 Sample collection

Five different spot/locations was selected for sample collection from landfill. With the help of 50 cm long and 5 cm wide spatula, soil samples had been collected. Each soil sample reached up to 20cm depth. Ensuring that samples contained maximum amount of soil without plastic or wood waste. Immediately after collection, soil was packed in air tight bag to avoid aerial impurity (bacteria from air or any other source).

Figure 2.1 (b) Soil sample collection at CDA landfill site (I-11/4)

2.3 Isolation of bacteria

To obtained bacteria from soil samples serial dilution method was done. Each soil sample was dissolved up to seven dilutions in distilled water. In the first tube, 2g of soil was mixed with 10 ml of distilled water. Then 1ml of mixture from first tube was mixed in 9 ml of distilled water in second tube and so on up to seven tubes. Same procedure was followed by all five soil samples collected. With the help of micropipette 100 µl inoculum from seventh tube was dispersed and spreaded over nutrient agar in petri dish. Each Petri dish contained 20 ml of nutrient agar. Same procedure is followed and repeated for each diluted sample.

2.4 Preliminary selection

In order of identification and selection of most productive bacteria 2mls of Nile Blue-A dye were added and spread over the Petri dishes which were incubated for 24-48 hours at 36 C° for bacterial growth. Bacteria selection was carried out on the bases of color absorption. Bacterial colony which absorbed maximum concentration of dye appeared the darkest shade of blue was selected. Further bacterial growth streaking was performed. For streaking, inoculum loop was used. With the help of inoculum loop selective bacterial colony was carried out, dispersed over a petri dish containing nutrient agar. Then incubated up to 24-48 hours at 36 C°.

2.5 Screening

Screening is defined as a technique to determine the presence of specific type of bacteria in a sample. In order to determine bacteria production and growth, selected bacteria from preliminary selection process went under screening process. For detection two different treatments were run under two different controlled conditions in a nutrient media (1% glucose as carbon source & 5% glucose as carbon source). A standard nutrient media was selected as nutrient compound. Nutrient media contained ``4 g NaHPO4, 4g NA2HPO4, 0.01M CaCl2, 0.1M MgSO4`` mixed in 1 letter distilled water as required volume.

In that nutrient media, sodium dihydrogen phosphate ``NaHPO4`` act as weak acid to maintain acidity. ``NA2HPO4`` disodium hydrogen phosphate act as weak base. Calcium chloride ``CaCl2`` provide calcium and calcium ion to build cell structure enzymes activity and cell signalizing to bacterial body. Magnesium sulfate ``MgSO4`` provide magnesium and sulfate ions, which play role in DNA replication, energy metabolism, protein synthesis for bacterial body. All that minerals were dissolved in distilled water. Distilled water provided the purest solvent as there is no impurity in it.

Required salts were weighed in electric balance machine over a paper and then dissolved into 1 letter of distil water in a 1000 ml of flask.

In order to determine bacterial production and growth 1% and 5% of sugar as carbon source was given to bacteria explained below. (1% & 5%)

For 1% carbon source, 0.2 g of sugar was weighed and added in 20 ml of nutrient media in a test tube and for 5% carbon source, 1g of sugar in 20 ml of nutrient media was dissolved similarly. 20 ml nutrient media were measured in flask than added in a test tube and shook well with hand separately of each percentage. Selective bacteria then picked up with the help of inoculum loop and added into the same test tube.

Figure 2.3 (a) Heat drying microscope slide (left) and inoculum loop (right)

Compound was mixed well in test tube and covered with aluminum foil to avoid aerial contamination. Then incubated bacterial compound at 36 C° up to 24-48 hours. To check bacterial growth and production, 1 ml of bacterial compound was picked and spreaded over microscopic slide. Slide was heat fixed over heat flam to remove moisture content of the bacterial compound. after moisture removal 5 ml of Sudan Black-B dye was dropped over heat fixed bacteria on slide for 10 min (As Sudan Black-B dye got attached and absorbed in lipid body). Decolorizer was filled in a dropper and then used to washed the dye from slide for 10 seconds (For removal of extra dye from slide which was not attached to lipid). 5 ml of safranin was filled in a dropper and spreaded over slide for 10 seconds (safranin attached and absorbed into the cell wall of bacteria and appeared as background color). Slide was than air dried for 10-15 min to remove moisture content. For Identification of PHA slide was placed in microscope. Black colored dots represented PHA production in bacterial membrane.

Figure 2.3 (b) Observation of bacterial slides on microscope for PHA confirmation

Difference between the production and growth of bacterial body shown below; (left) bacteria with 1% of sugar appeared lighter in color as shown in fig: 2.3 (c)

(right) bacteria with 5% of sugar are greater in quantity and appeared darker as shown in fig: 2.3 (c)

1% glucose 5% glucose

2.3 (c) Growth comparison of PHA producing bacteria

2.6 Effect of temperature at 1% biomass

In order to determined PHA production on sugarcane bagasse as carbon source on different concentrations at different time intervals. Glucose was replaced with sugarcane bagasse as carbon source as per concentration of 1% and 10% in nutrient media for 24 hours, 48 hours and 72 hours at pH 7 incubated at 36 C°. pH was determined by pH meter. following treatments was made.

2.6.1 Effect of temperature at 1% biomass for 24 hr.

For 1% of Sugarcane bagasse at 24 h interval, Sugarcane bagasse was grinded in a grinder and converted into powdered, 2 g of sugarcane bagasse was weighted and mixed in 200 ml of nutrient media in 500 ml flask. After that 5 ml of bacterial compound was picked with dropper and mixed in the flask containing sugarcane bagasse and nutrient media. That compound was mixed well with hand and flash was covered with aluminum foil. Flask was incubated then for 24h at 36C° temp.

After 24 h, flask was carried out. With the help of syringe 48 ml of liquid was pumped out. Liquid was filled in Eppendorf tube, collected liquid was placed inside freezer to stop bacterial growth to determine difference in production at different time.

2.6.2 Effect of temperature at 1% biomass for 48 hr.

For 1% of Sugarcane bagasse at 48 h interval, Sugarcane bagasse was grinded in a grinder and converted into powdered 2 g of sugarcane bagasse was weighted and mixed in 200 ml of nutrient media in 500 ml flask. After that 5 ml of bacterial compound was picked with dropper and mixed in the flask containing sugarcane bagasse and nutrient media. That compound was mixed well with hand and flash was covered with aluminum foil. Flask was incubated then for 48h at 36C° temp.

After 48 h, flask was carried out. With the help of syringe 48 ml of liquid was pumped out. Liquid was filled in Eppendorf tube. collected liquid was placed inside freezer to stop bacterial growth to determine difference in production at different time.

2.6.3 Effect of temperature at 1% biomass for 72 hr.

For 1% of Sugarcane bagasse at 72 h interval, Sugarcane bagasse was grinded in a grinder and converted into powdered 2 g of sugarcane bagasse was weighted and mixed in 200 ml of nutrient media in 500 ml flask. After that 5 ml of bacterial compound was picked with dropper and mixed in the flask containing sugarcane bagasse and nutrient media. That compound was mixed well with hand and flash was covered with aluminum foil. Flask was incubated then for 72h at 36C° temp.

After 72 h, flask was carried out. With the help of syringe 48 ml of liquid was pumped out. Liquid was filled in Eppendorf tube. collected liquid was placed inside freezer to stop bacterial growth to determine difference in production at different time.

2.7 Effect of temperature at 10% biomass

In order to determined PHA production on sugarcane bagasse as carbon source on different concentrations at different time intervals. Glucose was replaced with sugarcane bagasse as carbon source as per concentration of 10% in nutrient media for 24 hours, 48 hours and 72 hours at pH 7 incubated at 36 C°. pH was determined by pH meter. following treatments was made.

2.7.1 Effect of temperature at 10% biomass for 24 hr.

For 10% of Sugarcane bagasse at 24 h interval, Sugarcane bagasse was grinded in a grinder and converted into powdered 20 g of sugarcane bagasse was weighted and mixed in 200 ml of nutrient media in 500 ml flask. After that 5 ml of bacterial compound was picked with dropper and mixed in the flask containing sugarcane bagasse and nutrient media. That compound was mixed well with hand and flash was covered with aluminum foil. Flask was incubated then for 24h at 36C° temp.

After 24 h, flask was carried out. With the help of syringe 48 ml of liquid was pumped out. Liquid was filled in Eppendorf tube. collected liquid was placed inside freezer to stop bacterial growth to determine difference in production at different time.

2.7.2 Effect of temperature at 10% biomass for 48 hr.

For 10% of Sugarcane bagasse at 48 h interval, Sugarcane bagasse was grinded in a grinder and converted into powdered 20 g of sugarcane bagasse was weighted and mixed in 200 ml of nutrient media in 500 ml flask. After that 5 ml of bacterial compound was picked with dropper and mixed in the flask containing sugarcane bagasse and nutrient media. That compound was mixed well with hand and flash was covered with aluminum foil. Flask was incubated then for 48h at 36C° temp.

After 48 h, flask was carried out. With the help of syringe 48 ml of liquid was pumped out. Liquid was filled in Eppendorf tube. collected liquid was placed inside freezer to stop bacterial growth to determine difference in production at different time.

2.7.3 Effect of temperature at 10% biomass for 72 hr.

For 10% of Sugarcane bagasse at 72 h interval, Sugarcane bagasse was grinded in a grinder and converted into powdered 20 g of sugarcane bagasse was weighted and mixed in 200 ml of nutrient media in 500 ml flask. After that 5 ml of bacterial compound was picked with dropper and mixed in the flask containing sugarcane bagasse and nutrient media. That compound was mixed well with hand and flash was covered with aluminum foil. Flask was incubated then for 72h at 36C° temp.

After 72 h, flask was carried out. With the help of syringe 48 ml of liquid was pumped out. Liquid was filled in Eppendorf tube. collected liquid was placed inside freezer to stop bacterial growth to determine difference in production at different time.

Figure 2.4 Treatments for incubation and Eppendorf tubes frozen at different time intervals (24,48 &72 hrs.)

2.7 Extraction and Analysis

In order to carry out final analysis of the product, liquid-liquid extraction of PHA from bacterial biomass was carried out. For this purpose, 48 ml volume from each prepared treatment were centrifuged at 1350 rpm in Eppendorf tube. with the help of syringe liquid was transferred into Eppendorf tube. After centrifuge biomass left at bottom of Eppendorf tube was collected with the help of spatula and filtered out at filter paper for each sample treatment separately. Each filtered biomass was transferred into a beaker and stirred up to 24 hours in chloroform (30ml of chloroform for 1 gram of each biomass). Stirred samples were filtered by filter paper to remove impurities. Filtered samples were collected in a flask and transferred into Eppendorf tube with the help of syringe. FTIR (Fourier Transform Infrared) analysis were performed for each sample to determine the detection of PHA production.

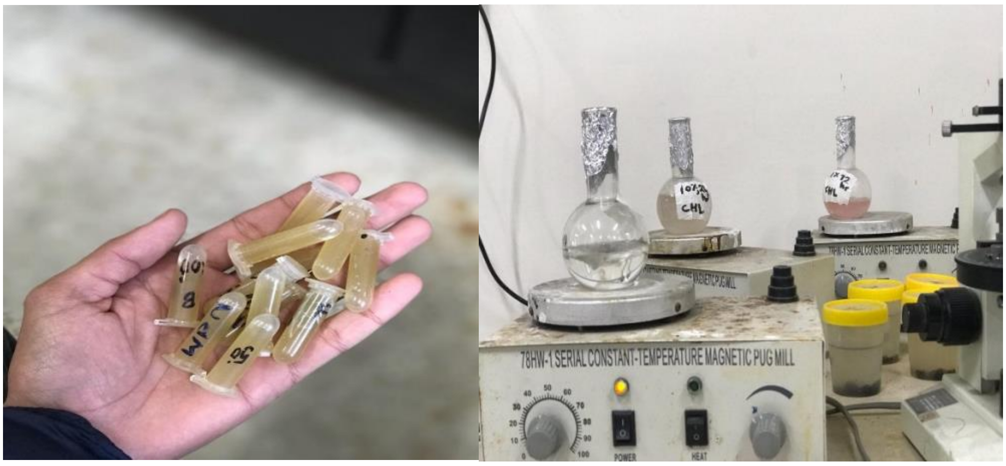


Figure 2.5 Eppendorf tubes for centrifugation (left) CHL-biomass solutions on magnetic stirrer (right)

CHAPTER 3

RESULTS AND DISCUSSION

3.1 Primary screening

Out of five soil samples taken from solid waste dumping site, three were found positive for PHA producing bacteria on the basis of absorption of Nile blue dye in the bacterial colonies as the dye binds with intracellular granules of PHA. Bacterial colony that absorbed the maximum amount of die had the darkest shade of blue and was the most productive colony. The maximum growth was observed in media containing 5% glucose as carbon source than the media containing 1% glucose as the carbon source.

Figure 3.1 PHA positive samples in primary screening

3.2 Confirmation of PHA

For the confirmation of PHA production, confirmation test was carried out using Sudan Black B dye. The bacteria from both the groups (1% glucose and 5% glucose) confirmed the PHA production when observed under compound microscope as black dots. These black dots presented the dye bindings with the lipid contents of bacterial body which also contain PHA.

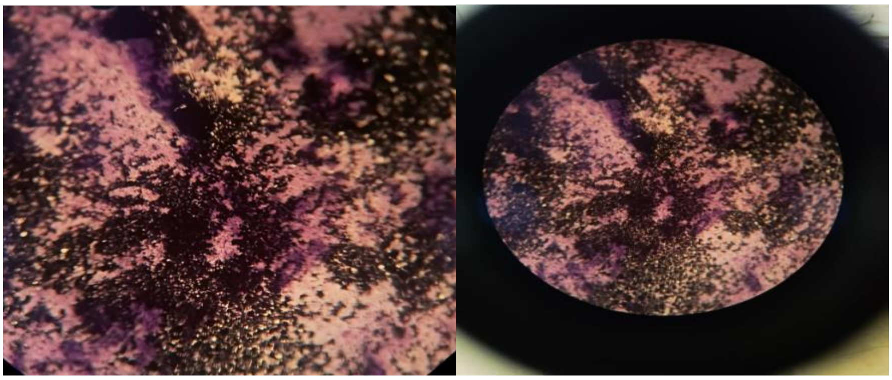


Figure 3.2 Microscopic view of Black Sudan B attachment with PHA as black dots

3.3 Post centrifugation biomass

When the carbon source was changed to sugarcane bagasse, media having 10% sugarcane bagasse exhibited more production than the media with 1% sugarcane bagasse in general but as there were six treatments the production varied. When biomass was extracted after centrifugation (at 1350 rpm) of 48 ml of each incubated sample, treatments 10% -72 hours, 1% -72 hours, 10% -48 hours, 1% -48 hours, 10% -24 hours and 1%s -24 hours extracted 3.3 gm, 2.84 gm, 2.51 gm, 2.02 gm, 1.79 gm and 1.13 gm of dry biomass respectively. The change in biomass production was expected due to change in incubation time of bacteria and carbon source concentration variations in samples. 1% -72 hours' treatment produced more biomass than 10% -48 hours indicated that time factor could overcome the carbon concentration factor if the incubation time is more than 48 hours. The sudden drop in the production between 1.79 gm and 1.13 gm indicated that bacterial growth is significant after 24 hours and in initial 24 hours' production is not that appreciable than the later.

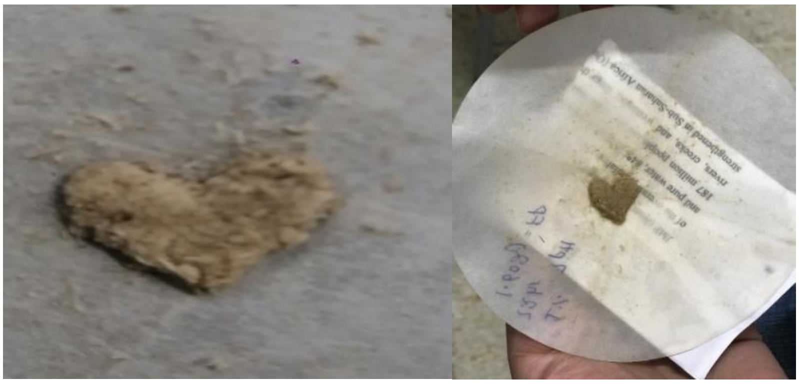


Figure 3.3 Biomass samples extracted from centrifugation of treatments

Table 2 Biomass production data (after centrifugation of samples)

Serial no.	Treatments (sugarcane bagasse conc.-incubation time)	Biomass produced after centrifugation (grams)
1	1%-24hr	1.13
2	1%-48hr	2.02
3	1%-72hr	2.84
4	10%-24hr	1.79
5	10%-48hr	2.51
6	10%-72hr	3.3

3.4 FTIR analysis

3.4.1 FTIR of treatment 1%-24hr

Treatment 1%-24 hr. was found with peaks between 1720 to 1740 (cm^{-1}) as 1730.21 cm^{-1} representing the carboxylic ester group that is an indication of PHA. Peaks at 2924.18 cm^{-1}, corresponding to 1%-24 hrs. revealed the presence of methylene band (Sharma et al., 2017).

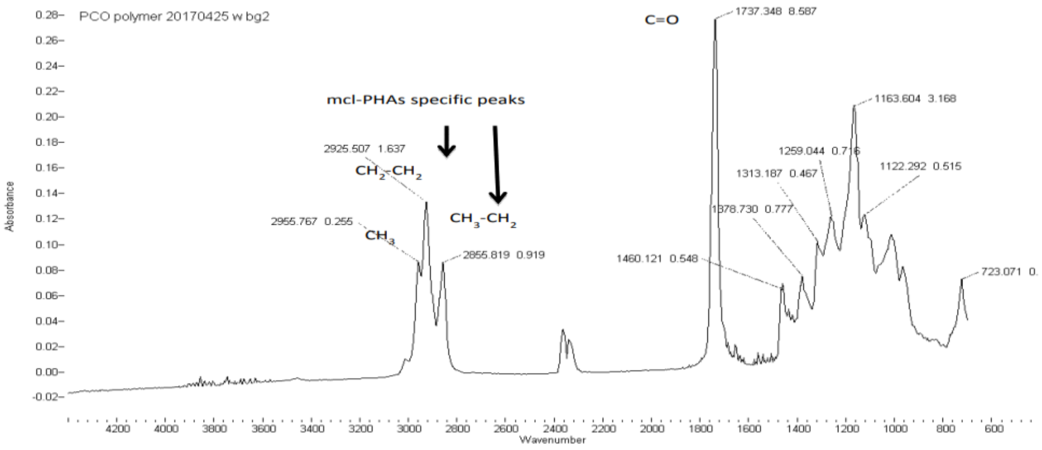


Figure 3.4 (a) Reference FTIR spectra of PHA

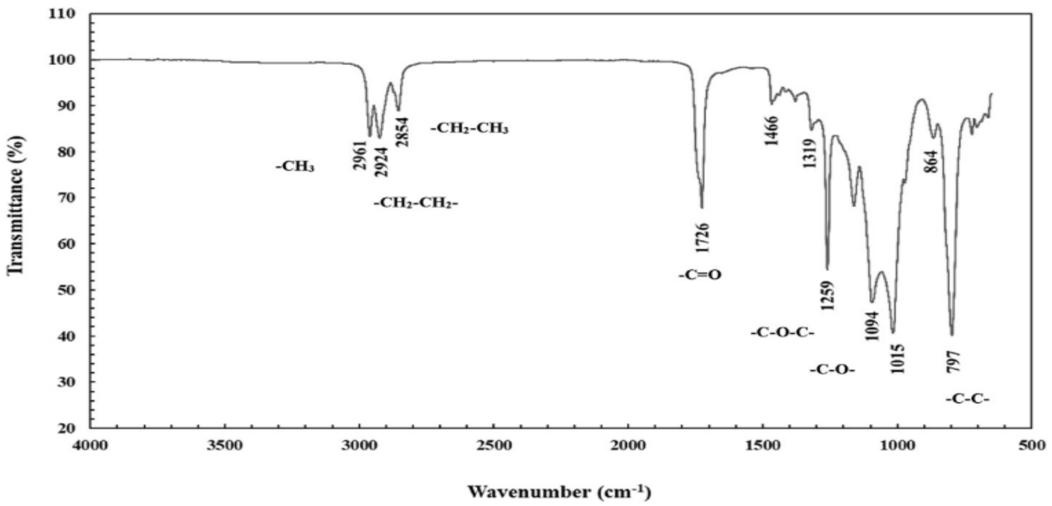


Figure 3.5 (b) Reference FTIR spectra of PHA

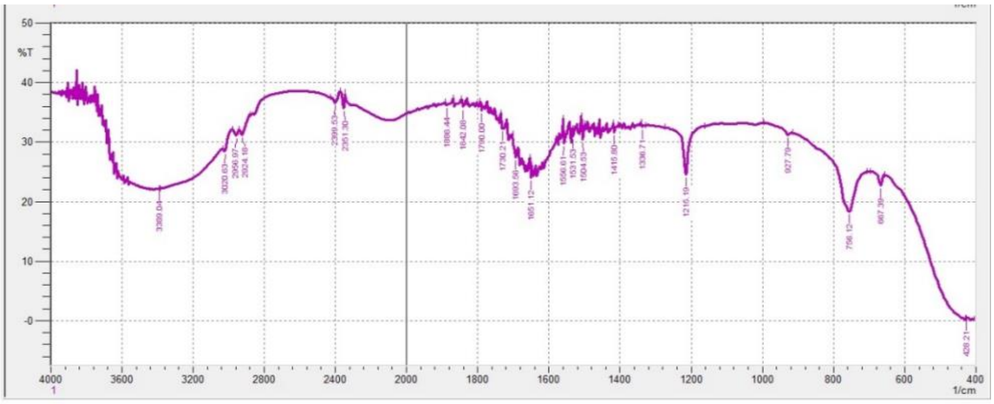


Figure 3.6 (c) FTIR spectra of treatment with 1% sugarcane bagasse and 24hr incubation time at pH-7

3.4.2 FTIR of treatment 1%-48hr

The presence of the ethyl group, confirmed by the peak at 2854.72 cm^ (-1), in this treatment, provides evidence for the formation of medium-chain-length PHA (mcl-PHA). The treatment with a concentration of 1% for a duration of 48 hours displayed a prominent peak at 1454 cm^ (-1), which corresponds to the stretching vibration of C-O groups (Tanikkul et al., 2020). The presence of the methylene band was indicated by peaks at 2926.11 cm^ (s-1), corresponding to a 1% carbon source concentration for 48 hours' incubation time (Sharma et al., 2017).

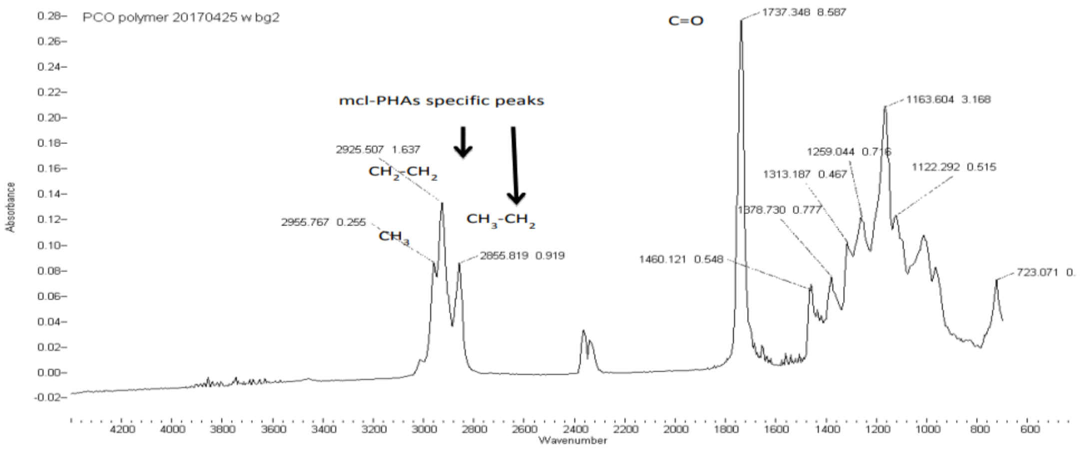


Figure 3.7 (a) Reference FTIR spectra of PHA

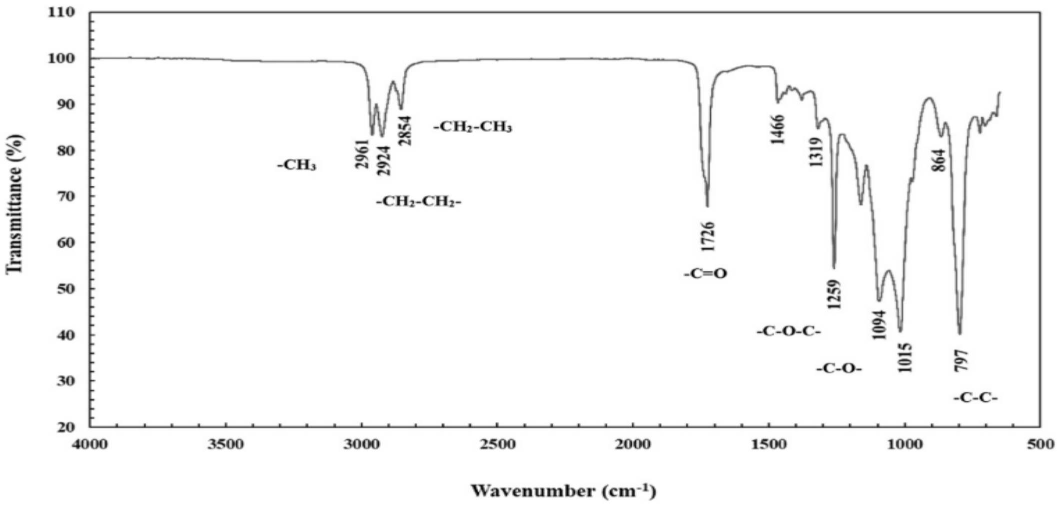


Figure 3.8 (b) Reference FTIR spectra of PHA

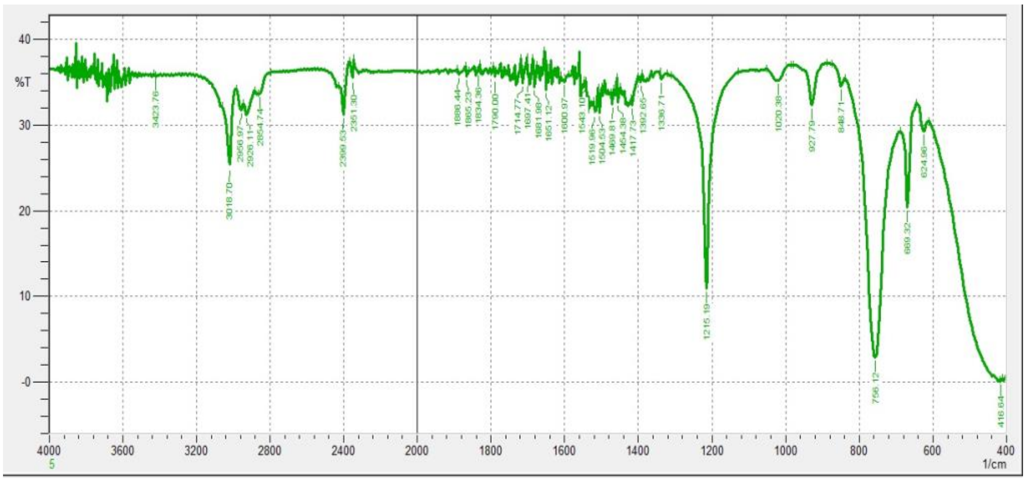


Figure 3.9 (c) FTIR spectra of treatment with 1% sugarcane bagasse and 48hr incubation time at pH-7

3.4.3 FTIR of treatment 10%-24hr

FTIR analysis of treatment 10%-24 hrs. showed peak of 1732.13 cm^{-1} that is a characteristic band of hydroxyoctanoate (HO) and hydroxydecanoate (HD) monomers of Polyhydroxy-butyrate. This treatment exhibited the peak 2854.72 cm^{-1} indicating the ethyl group confirming the formation of medium chain length PHA (mcl-PHA). The treatment at 10% for 24 hours exhibited a notable peak at 1454 cm^(-1), indicating the stretching vibration within C-O groups (Tanikkul et al., 2020). The presence of the methylene band was revealed by peaks at 2926.11 cm^ (-1), corresponding to 10% concentration for 24 hours.

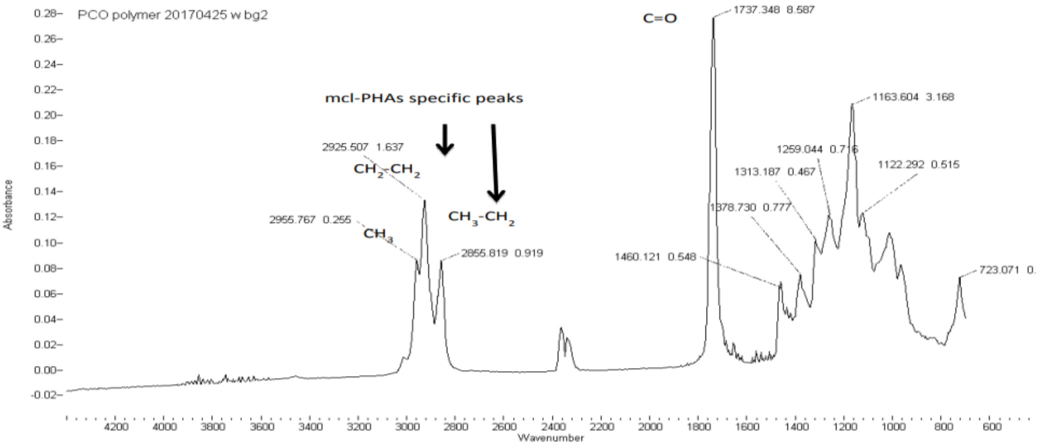


Figure 3.10 (a) Reference FTIR spectra of PHA

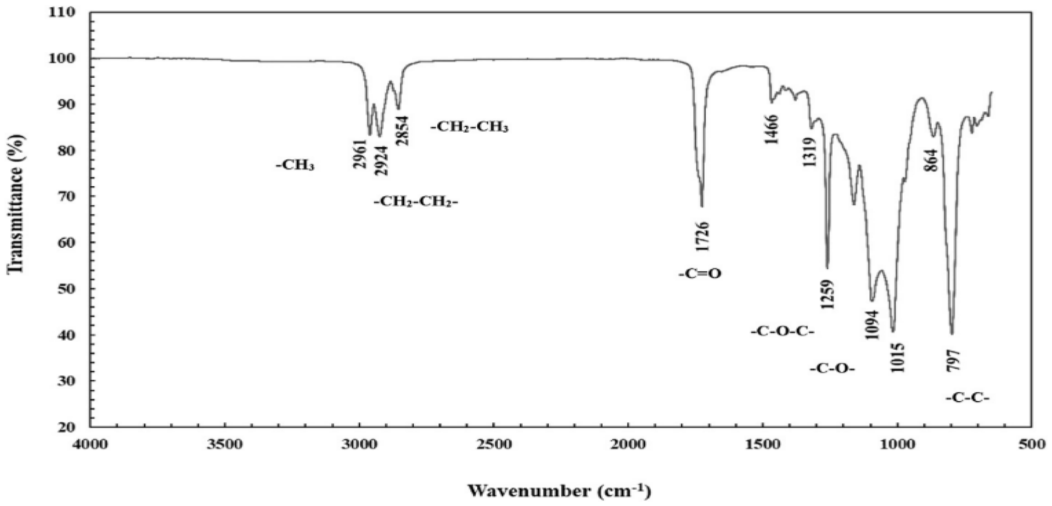


Figure 3.11 (b) Reference FTIR spectra of PHA

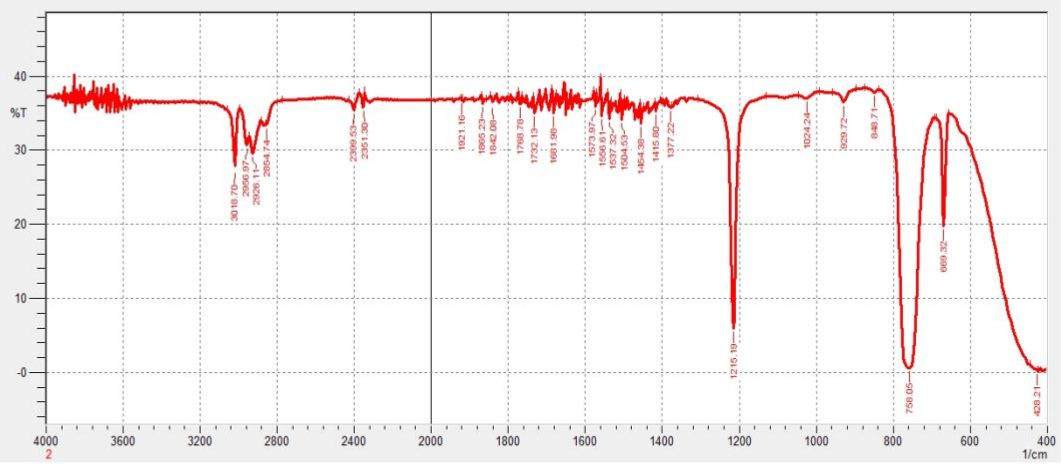


Figure 3.12 (c) FTIR spectra of treatment with 10% sugarcane bagasse and 24hr incubation time at pH-7

3.4.4 FTIR of treatment 10%-48hr

Treatment 10%-48 hr. was also found with peak between 1720 to 1740 (cm^{-1}) as 1730.21 cm^{-} representing the carboxylic ester group that is an indication of PHA. The peak observed at 2854.72 cm^ (-1) in this treatment confirms the presence of the ethyl group, thereby confirming the formation of medium-chain-length PHA (mcl-PHA). 10%-48hr gave the strong value of 1454 cm^{-1} represented the stretching vibration in C-O groups (Tanikkul et al., 2020). Peak at 2926.11 cm^ (-1), corresponding to a concentration of 10% for 48 hours, indicated the presence of the methylene band.

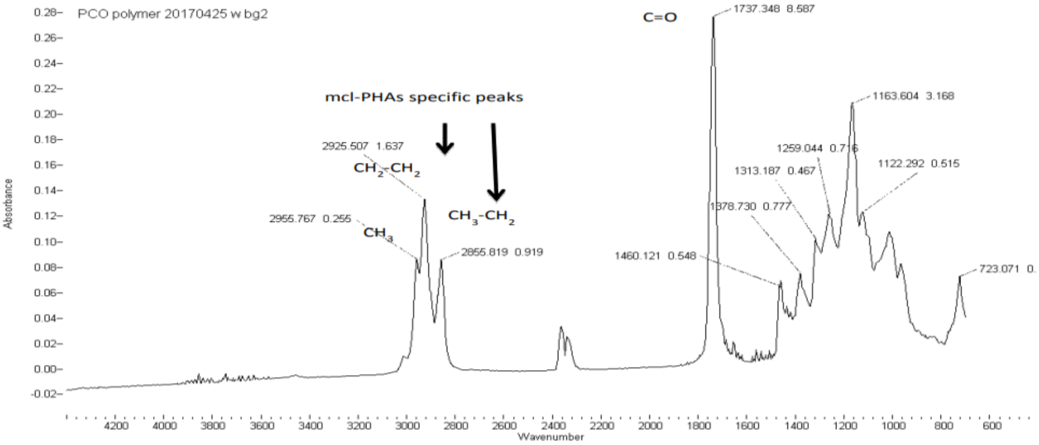


Figure 3.13 (a) Reference FTIR spectra of PHA

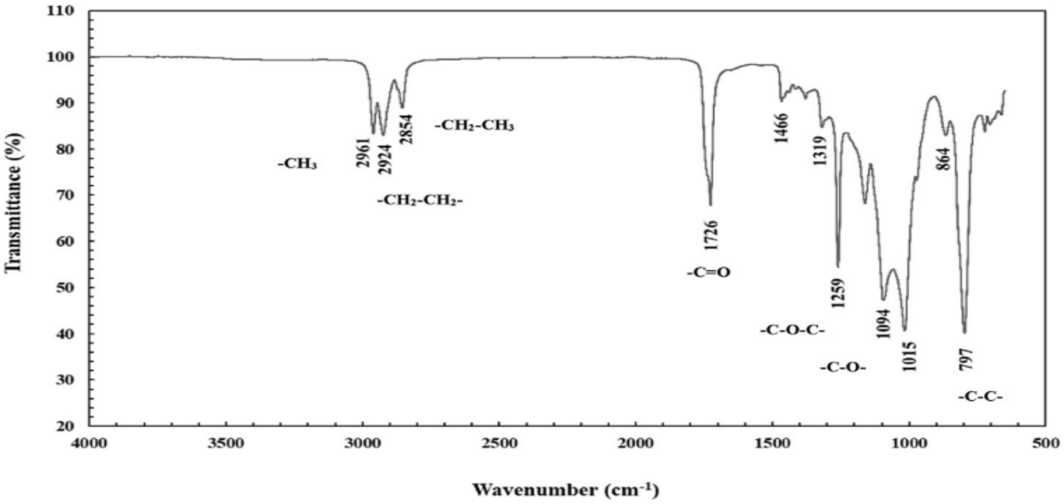


Figure 3.14 (b) Reference FTIR spectra of PHA

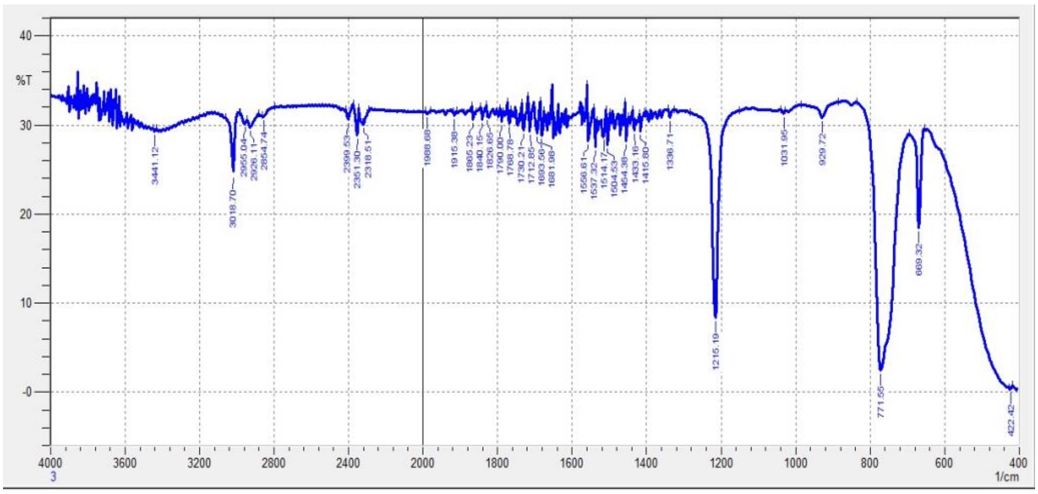


Figure 3.15 (c) FTIR spectra of treatment with 10% sugarcane bagasse and 48hr incubation time at pH-7

3.4.5 FTIR of treatment 1%-72hr

The treatment at 1%-72 hours also exhibited a peak ranging from 1720 to 1740 cm^ (-1), specifically at 1730.21 cm^ (-1), indicating the presence of the carboxylic ester group, which serves as an indicator of PHA. This treatment demonstrated a peak at 2854.72 cm^ (-1), indicating the presence of the ethyl group and confirming the formation of medium-chain-length PHA (mcl-PHA). At 1% concentration for 72 hours, a distinct peak at 1454 cm^ (-1) was observed, indicative of the stretching vibration within C-O groups. Peaks at 2924.18 cm^ (-1), corresponding to a concentration of 1% for 72 hours, indicated the presence of the methylene band.

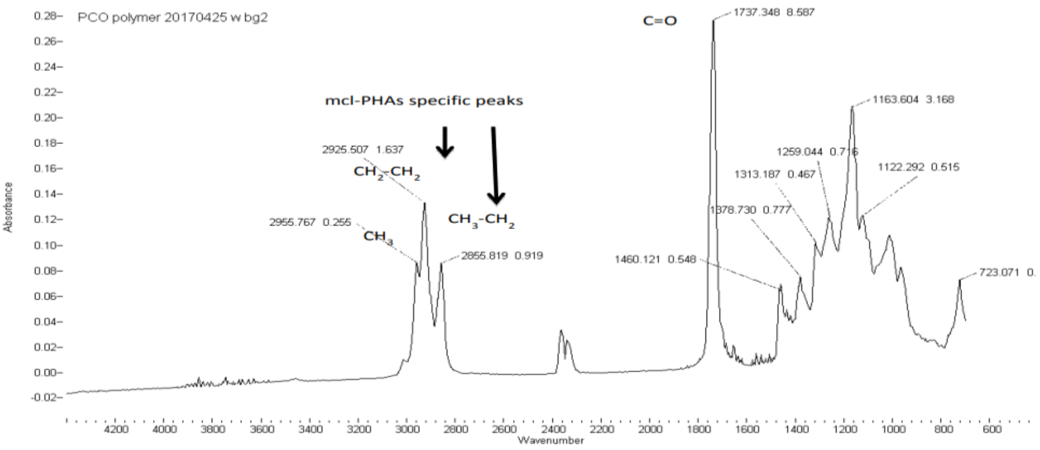


Figure 3.16 (a) Reference FTIR spectra of PHA

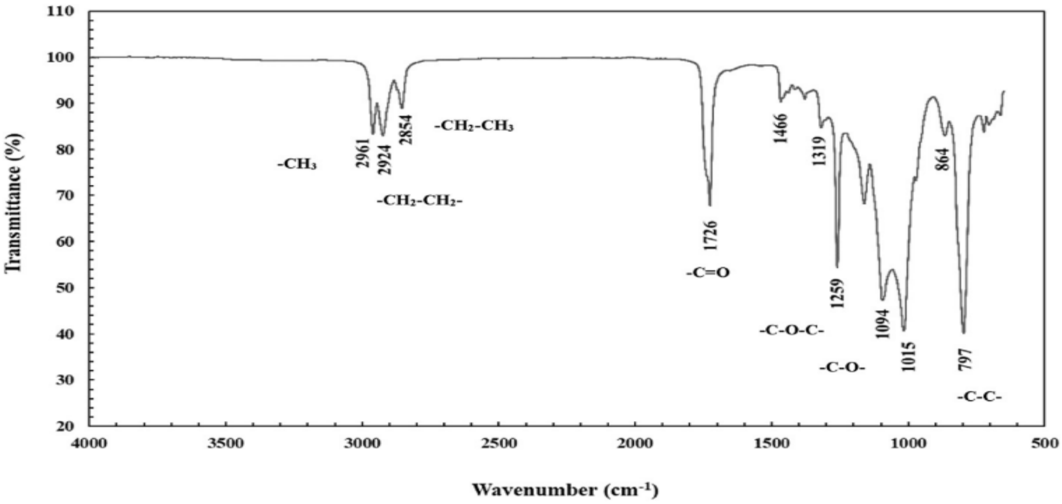


Figure 3.17 (b) Reference FTIR spectra of PHA

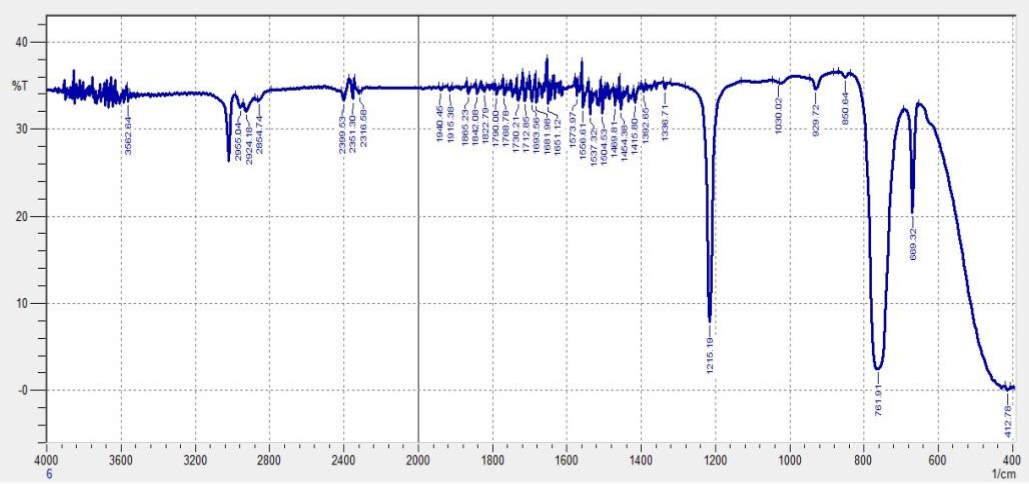


Figure 3.18 (c) FTIR spectra of treatment with 1% sugarcane bagasse and 72hr incubation time at pH-7

3.4.6 FTIR of treatment 10%-72hr

The treatment at 10% for 72 hours also displayed a peak between 1720 and 1740 cm^(-1), precisely at 1730.21 cm^(-1), signifying the presence of the carboxylic ester group—a characteristic indication of PHA (Hong et al., 1999). In this treatment, the peak detected at 2854.72 cm^ (-1) signifies the presence of the ethyl group, providing confirmation of the formation of medium-chain-length PHA (mcl-PHA). At 10% concentration for 72 hours, a significant peak at 1454 cm^ (-1) was observed, corresponding to the stretching vibration of C-O groups. The peaks observed at 2926.11 cm^ (-1), associated with a concentration of 10% for 72 hours, signify the presence of the methylene band.

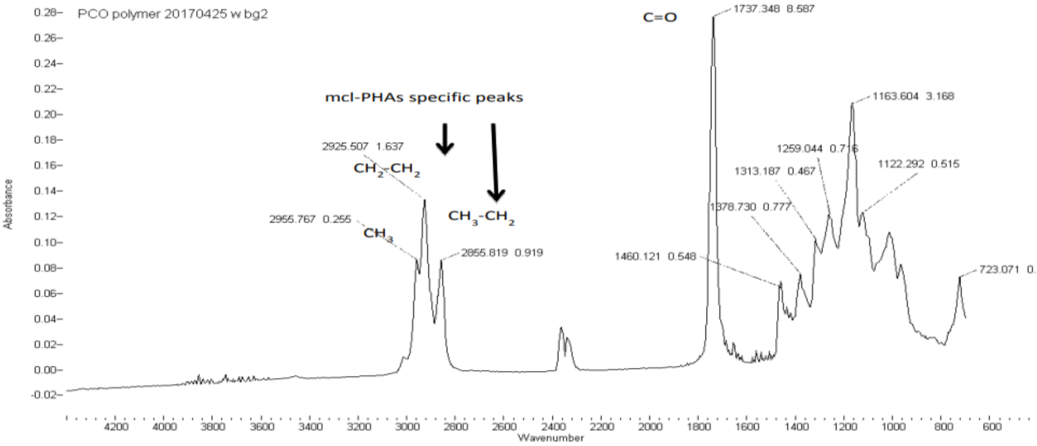


Figure 3.19 (a) Reference FTIR spectra of PHA

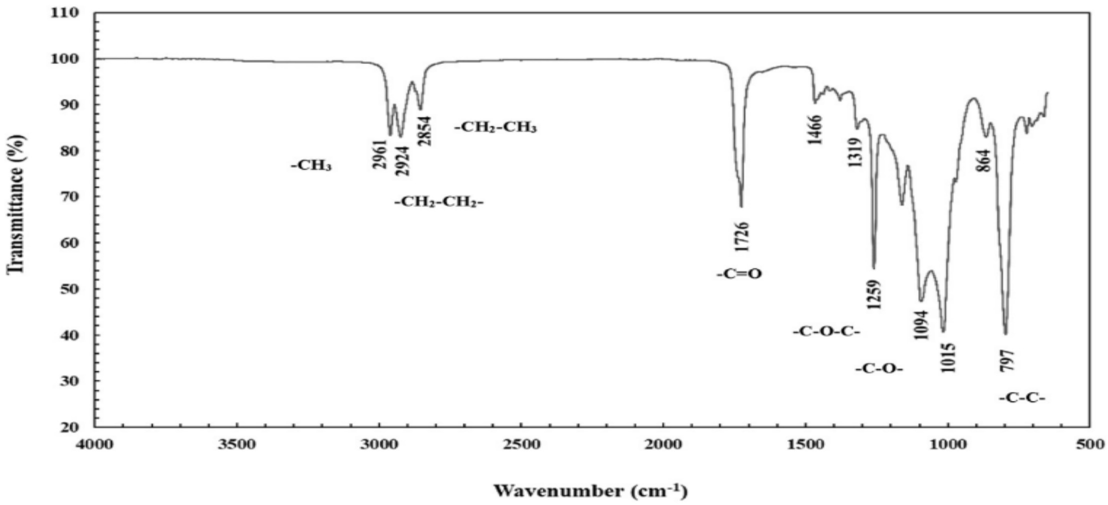


Figure 3.20 (b) Reference FTIR spectra of PHA

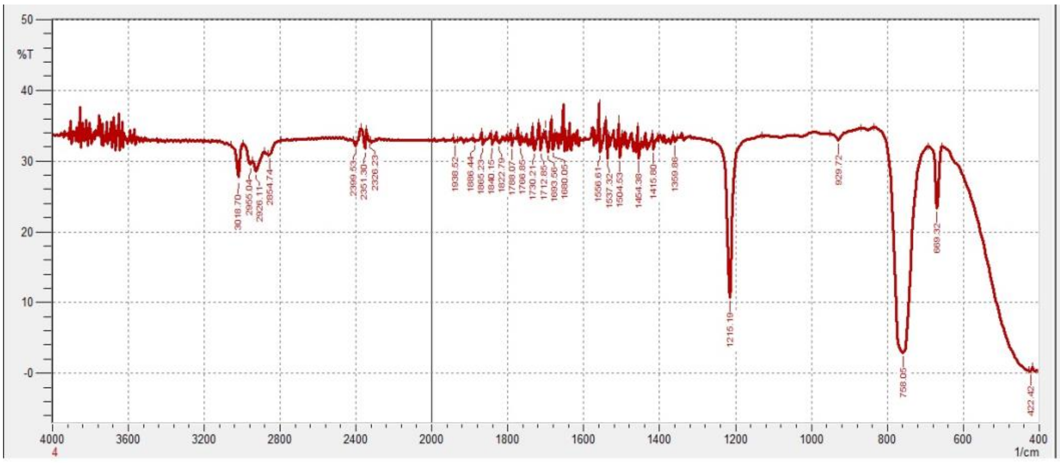


Figure 3.21 (c) FTIR spectra of treatment with 10% sugarcane bagasse and 72hr incubation time at pH-7

All six treatments exposed the medium chain length PHA formation. The imbalance between alkyl groups and in C-C or C-O bond stretching was most possibly due to different incubation time that led to formation of longer or shorter alkyl monomers.

CONCLUSIONS

1. Sufficient amount of bacterial strains capable of producing PHA were isolated and confirmed using Sudan Black-B and Nile Blue-A.
2. Maximum PHA production was observed using 10% carbon source after incubation period of 72 h.

RECOMMENDATIONS

1. PHA production should be evaluated using other low cost organic feeds
2. Physical parameters like pH and temp should be evaluated for PHA production
3. Potential for PHA's production of other organisms like fungi and actinomycetes should be evaluated
4. Molecular studies on bacterial strains capable of caring out PHA production should be carried out
5. Quantification techniques such as GCMS should also be used for PHA quantification

REFERENCES

Agamuthu, P. (2008). Challenges in sustainable management of construction and demolition waste. Waste Management & Research, 26(6), 491-492.

Akiyama, M., Tsuge, T., & Doi, Y. (2003). Environmental life cycle comparison of polyhydroxyalkanoates produced from renewable carbon resources by bacterial fermentation. Polymer Degradation and Stability, 80(1), 183-194.

Alabi, O. A., Ologbonjaye, K. I., Awosolu, O., & Alalade, O. E. (2019). Public and environmental health effects of plastic wastes disposal: a review. *J Toxicol Risk Assess*, *5*(021), 1-13.

Ali, S. S., Elsamahy, T., Al-Tohamy, R., Zhu, D., Mahmoud, Y. A. G., Koutra, E., ... & Sun, J. (2021). Plastic wastes biodegradation: Mechanisms, challenges and future prospects. *Science of the Total Environment*, *780*, 146590.

Alvarez-Chavez, C. R., Edwards, S., Moure-Eraso, R. L., and Geiser, K. 2011. "Sustainability of Bio-based Plastics: General Comparative Analysis and Recommendations for Improvement." Journal of Cleaner Production 23 (1): 46-7.

APME, M. (2002). Using waste plastic as a substitute for coal. Warmer Bulletin, (83), 20-21. APME, M. (2002). Using waste plastic as a substitute for coal. Warmer Bulletin, (83), 20-21.

Araujo, P. H. H. D., Sayer, C., Giudici, R., & Poço, J. G. R. (2002). Techniques for reducing residual monomer content in polymers: a review. Polymer Engineering & Science, 42(7), 1442-1468.

Arikan, E. B., & Ozsoy, H. D. (2015). A review: investigation of bioplastics. *J. Civ. Eng. Arch*, *9*(1), 188-192.

Badia, J. D., Gil-Castell, O., & Ribes-Greus, A. (2017). Long-term properties and end-of-life of polymers from renewable resources. Polymer Degradation and Stability, 137, 35-57.

Barker, M., and Safford, R. 2009. “Industrial Uses for Crops: Markets for Bioplastics.” Accessed January 1, 2015. http://archive.hgca.com/publications/documents/Bioplastics_web28409.pdf.

Barnes, D. K., Galgani, F., Thompson, R. C., & Barlaz, M. (2009). Accumulation and fragmentation of plastic debris in global environments. Philosophical transactions of the royal society B: biological sciences, 364(1526), 1985-1998.

Bellasi, A., Binda, G., Pozzi, A., Galafassi, S., Volta, P., & Bettinetti, R. (2020). Microplastic contamination in freshwater environments: A review, focusing on interactions with sediments and benthic organisms. Environments, 7(4), 30.

Bengtsson, S., Werker, A., Christensson, M., & Welander, T. (2008). Production of polyhydroxyalkanoates by activated sludge treating a paper mill wastewater. Bioresource technology, 99(3), 509-516.

Braunegg, G., Lefebvre, G., & Genser, K. F. (1998). Polyhydroxyalkanoates, biopolyesters from renewable resources: physiological and engineering aspects. Journal of biotechnology, 65(2-3), 127-161.

Buenrostro, O., Bocco, G., & Vence, J. (2001). Forecasting generation of urban solid waste in developing countries—A case study in Mexico. Journal of the Air & Waste Management Association, 51(1), 86-93

Chandegara, V. K., Cholera, S. P., Nandasana, J. N., Kumpavat, M. T., & Patel, K. C. (2015). Plastic packaging waste impact on climate change and its mitigation. Subbaiah R, Prajapati GV, Water management and climate smart agriculture. Adaptation of Climatic Resilient Water Management and Agriculture, Gyan Publishing House, New Delhi, India, 3, 404-15.

Chaudhry, W. N., Jamil, N., Ali, I., Ayaz, M. H., & Hasnain, S. (2011). Screening for polyhydroxyalkanoate (PHA)-producing bacterial strains and comparison of PHA production from various inexpensive carbon sources. Annals of microbiology, 61(3), 623-629.

Chen, G. Q., & Patel, M. K. (2012). Plastics derived from biological sources: present and future: a technical and environmental review. Chemical reviews, 112(4), 2082-2099.

Chen, Y. J. 2014. “Bioplastics and Their Role in Achieving Global Sustainability.” Journal of Chemical and Pharmaceutical Research 6 (1): 226-31.

Cruz, M. B., Saikawa, E., Hengstermann, M., Ramirez, A., McCracken, J. P., & Thompson, L. M. (2023). Plastic waste generation and emissions from the domestic open burning of plastic waste in Guatemala. *Environmental Science: Atmospheres*, *3*(1), 156-167.

Dai, L., Karakas, O., Cheng, Y., Cobb, K., Chen, P., & Ruan, R. (2023). A review on carbon materials production from plastic wastes. *Chemical Engineering Journal*, *453*, 139725.

Daniel, D. C. (2004). Creating a sustainable future. Jones and Bartlett Learning. Environ Sci, 8, 517-518.

Dar, M. A., Dhole, N. P., Pawar, K. D., Xie, R., Shahnawaz, M., Pandit, R. S., & Sun, J. (2022). Ecotoxic Effects of the plastic waste on marine fauna: An overview. Impact of Plastic Waste on the Marine Biota, 287-300.

Eriksen, M. K., & Astrup, T. F. (2019). Characterisation of source-separated, rigid plastic waste and evaluation of recycling initiatives: Effects of product design and source-separation system. *Waste Management*, *87*, 161-172.

European Environment Agency. (2007). The road from landfilling to recycling: common destination, different routes.

Gill, M. (2014). Bioplastic: a better alternative to plastics. Int. J. Res. Appl. Nat. Soc. Sci, 2, 115-120.

Gu, F., Guo, J., Zhang, W., Summers, P. A., & Hall, P. (2017). From waste plastics to industrial raw materials: A life cycle assessment of mechanical plastic recycling practice based on a real-world case study. Science of the total environment, 601, 1192-1207.

Hamlet, C., Matte, T., & Mehta, S. (2018). Combating plastic air pollution on earth’s day. Vital strategies environmental health division.

Hammer, J., Kraak, M. H., & Parsons, J. R. (2012). Plastics in the marine environment: the dark side of a modern gift. Reviews of environmental contamination and toxicology, 1-44.

Henry, R. K., Yongsheng, Z., & Jun, D. (2006). Municipal solid waste management challenges in developing countries–Kenyan case study. Waste management, 26(1), 92-100.

Hong, K., Sun, S., Tian, W., Chen, G. Q., & Huang, W. (1999). A rapid method for detecting bacterial polyhydroxyalkanoates in intact cells by Fourier transform infrared spectroscopy. *Applied Microbiology and Biotechnology, 51*, 523-526.

Hopewell, J., Dvorak, R., Kosior, E., 2009. Plastics recycling: challenges and opportunities. Philosophical Transactions of the Royal Society of London. Series B, Biological 364, 2115e2126.

Hudgins, M. (1999, April). Aerobic landfill studies from the USA. In 1st Int. Conf. Solid Waste.

Jagaba, A. H., Kutty, S. R. M., Baloo, L., Hayder, G., Birniwa, A. H., Taha, A. T. B., ... & Lawal, I. M. (2022). Waste derived biocomposite for simultaneous biosorption of organic matter and nutrients from green straw biorefinery effluent in continuous mode activated sludge systems. Processes, 10(11), 2262.

Jagaba, A. H., Kutty, S. R. M., Lawal, I. M., Abubakar, S., Hassan, I., Zubairu, I., ... & Noor, A. (2021). Sequencing batch reactor technology for landfill leachate treatment: A state-of-the-art review. Journal of environmental management, 282, 111946.

Jawadekar S, Plastic Feasts (Csir Golden Jubliee Series. Publi- cation & Information Directorate, New Delhi) 1992.

Jogdand S N, Chem Week, (26 May 1998) 153

Kaith, B. S., Jindal, R., Jana, A. K., and Maiti, M. 2010. “Development of Corn Starch Based Green Composites Reinforced with Saccharum Spontaneum L Fiber and Graft Copolymers—Evaluation of Thermal, Physico-chemical and Mechanical Properties.” Bioresource Technology 101: 6843-5.

Kawai, F., Kawabata, T., & Oda, M. (2020). Current state and perspectives related to the polyethylene terephthalate hydrolases available for biorecycling. ACS Sustainable Chemistry & Engineering, 8(24), 8894-8908.

Khan, M. N. H., Miah, M. A., Islam, N., Akter, M., & Ferdous, T. (2018). Profitable Opportunities for Municipal Solid Waste Management of Mymensigh City. Bangladesh J. Environ. Sci, 35, 31-38.

Kibria, M. G., Masuk, N. I., Safayet, R., Nguyen, H. Q., & Mourshed, M. (2023). Plastic waste: challenges and opportunities to mitigate pollution and effective management. International Journal of Environmental Research, 17(1), 20.

Koller, M. (2018). Chemical and biochemical engineering approaches in manufacturing polyhydroxyalkanoate (PHA) biopolyesters of tailored structure with focus on the diversity of building blocks. Chemical and Biochemical Engineering Quarterly, 32(4), 413-438.

Koller, M., Maršálek, L., de Sousa Dias, M. M., & Braunegg, G. (2017). Producing microbial polyhydroxyalkanoate (PHA) biopolyesters in a sustainable manner. New biotechnology, 37, 24-38.

Krzan, A., Hemjinda, S., Miertus, S., Corti, A., and Chiellini, E. 2006. “Standardization and Certification in the Area of Environmentally Degradable Plastics.” Polymer Degradation and Stability 91: 2819-33

Kulkarni, S. O., Kanekar, P. P., Jog, J. P., Sarnaik, S. S., & Nilegaonkar, S. S. (2015). Production of copolymer, poly (hydroxybutyrate-co-hydroxyvalerate) by Halomonas campisalis MCM B-1027 using agro-wastes. International journal of biological macromolecules, 72, 784-789.

Kynadi, A. S., & Suchithra, T. V. (2017). Formulation and optimization of a novel media comprising rubber seed oil for PHA production. Industrial Crops and Products, 105, 156-163.

Lagaron, J. M., and Lopez-Rubio, A. 2011. “Nanotechnology for Bioplastics: Opportunities, Challenges and Strategies.” Trends in Food Science and Technology 22 (11): 611-7.

Lo, C. W., Wu, H. S., & Wei, Y. H. (2009). Optimizing acidic methanolysis of poly (3-hydroxyalkanoates) in gas chromatography analysis. Asia-Pacific Journal of Chemical Engineering, 4(4), 487-494.

Mannina, G., Presti, D., Montiel-Jarillo, G., & Suárez-Ojeda, M. E. (2019). Bioplastic recovery from wastewater: A new protocol for polyhydroxyalkanoates (PHA) extraction from mixed microbial cultures. Bioresource technology, 282, 361-369.

Matsumoto, M., Hirata-Koizumi, M., & Ema, M. (2008). Potential adverse effects of phthalic acid esters on human health: a review of recent studies on reproduction. Regulatory Toxicology and Pharmacology, 50(1), 37-49.

Mcsweeney, C., New, M., Lizcano, G., & Lu, X. (2010). The UNDP Climate Change Country Profiles: Improving the accessibility of observed and projected climate information for studies of climate change in developing countries. Bulletin of the American Meteorological society, 91(2), 157-166.

Miandad, R., Barakat, M. A., Aburiazaiza, A. S., Rehan, M., Ismail, I. M. I., & Nizami, A. S. (2017). Effect of plastic waste types on pyrolysis liquid oil. International biodeterioration & biodegradation, 119, 239-252.

Miller, R. (2005). The landscape for biopolymers in packaging. Miller-Klein Associates report. Summary and Full Report available from The National Non-Food Crops Centre, Heslington, York, UK www. nnfcc. co. uk.

Mohan, S. V., & Reddy, M. V. (2013). Optimization of critical factors to enhance polyhydroxyalkanoates (PHA) synthesis by mixed culture using Taguchi design of experimental methodology. Bioresource technology, 128, 409-416.

Moya, D., Aldás, C., López, G., & Kaparaju, P. (2017). Municipal solid waste as a valuable renewable energy resource: a worldwide opportunity of energy recovery by using Waste-To-Energy Technologies. Energy Procedia, 134, 286-295.

Możejko-Ciesielska, J., & Kiewisz, R. (2016). Bacterial polyhydroxyalkanoates: Still fabulous Microbiological research, 192, 271-282.

Nemerow, N. L., Agardy, F. J., Sullivan, P. J., & Salvato, J. A. (2009). Environmental engineering: environmental health and safety for municipal infrastructure, land use and planning, and industry. John Wiley & Sons.

North, E. J., & Halden, R. U. (2013). Plastics and environmental health: the road ahead. Reviews on environmental health, 28(1), 1-8.

Owusu, P. A., Banadda, N., Zziwa, A., Seay, J., & Kiggundu, N. (2018). Reverse engineering of plastic waste into useful fuel products. Journal of Analytical and Applied Pyrolysis, 130, 285-293.

PlasticsEurope (2018) Annual review 2017–2018.

Porras, M. A., Cubitto, M. A., & Villar, M. A. (2014). Quantitative determination of intracellular PHA in Bacillus megaterium BBST4 strain using Mid FTIR spectroscopy. In *XIV Latin American Symposium on Polymers and XII Ibero American Congress on Polymers*.

Proshad, R., Kormoker, T., Islam, M. S., Haque, M. A., Rahman, M. M., & Mithu, M. M. R. (2018). Toxic effects of plastic on human health and environment: A consequences of health risk assessment in Bangladesh. International Journal of Health, 6(1), 1-5.

Râpă, M. A. R. I. A., Popa, M. E., Cinelli, P., Lazzeri, A., Burnichi, R., Mitelut, A., & Grosu, E. (2011). Biodegradable alternative to plastics for agriculture application. Romanian Biotechnological Letters, 16(6), 59-64.

Reddy, R. L., Reddy, V. S., & Gupta, G. A. (2013). Study of bio-plastics as green and sustainable alternative to plastics. International Journal of Emerging Technology and Advanced Engineering, 3(5), 76-81.

Ronďošová, S., Legerská, B., Chmelová, D., Ondrejovič, M., & Miertuš, S. (2022). Optimization of growth conditions to enhance PHA production by Cupriavidus necator. Fermentation, 8(9), 451.

Saratale, R. G., Cho, S. K., Saratale, G. D., Kadam, A. A., Ghodake, G. S., Kumar, M., ... & Shin, H. S. (2021). A comprehensive overview and recent advances on polyhydroxyalkanoates (PHA) production using various organic waste streams. Bioresource technology, 325, 124685.

Serafim, L. S., Lemos, P. C., Albuquerque, M. G., & Reis, M. A. (2008). Strategies for PHA production by mixed cultures and renewable waste materials. Applied microbiology and biotechnology, 81, 615-628.

Sha’Ato, R., Aboho, S. Y., Oketunde, F. O., Eneji, I. S., Unazi, G., & Agwa, S. J. W. M. (2007). Survey of solid waste generation and composition in a rapidly growing urban area in Central Nigeria. Waste management, 27(3), 352-358

Sharma, P. K., Munir, R. I., de Kievit, T., & Levin, D. B. (2017). Synthesis of polyhydroxyalkanoates (PHAs) from vegetable oils and free fatty acids by wild-type and mutant strains of Pseudomonas chlororaphis. *Canadian journal of microbiology*, *63*(12), 1009-1024.

Sharma, V., Sehgal, R., & Gupta, R. (2021). Polyhydroxyalkanoate (PHA): Properties and modifications. Polymer, 212, 123161.

Sharuddin, S. D. A., Abnisa, F., Daud, W. M. A. W., & Aroua, M. K. (2016). A review on pyrolysis of plastic wastes. Energy conversion and management, 115, 308-326.

Siddiqui, J., & Pandey, G. (2013). A review of plastic waste management strategies. Int. Res. J. Environ. Sci, 2(12), 84.

Sivan, A. 2011. “New Perspectives in Plastic Biodegradation.” Current Opinion in Biotechnology 22: 422-6.

Sutherland, W.J., Clout, M., Côté, I.M., Daszak, P., Depledge, M.H., Fellman, L., Fleishman, E., Garthwaite, R., Gibbons, D.W., De Lurio, J., Impey, A.J., Lickorish, F., Lindenmayer, D., Madgwick, J., Margerison, C., Maynard, T., Peck, L.S., Pretty, J., Prior, S., Redford, K.H., Scharlemann, J.P.W., Spalding, M., Watkinson, A.R., 2010. A horizon scan of global conservation issues for 2010. Trends Ecol. Evol. 25, 1–7.

Swift, G. (2002). Degradable polymers and plastics in landfill sites. *Encyclopedia of Polymer Science and Technology*, 1-13.

Tacoli, C. (2012). Urbanization, gender and urban poverty: paid work and unpaid carework in the city (p. 48). UK: Human Settlements Group, International Institute for Environment and Development.

Tanikkul, P., Sullivan, G. L., Sarp, S., & Pisutpaisal, N. (2020). Biosynthesis of medium chain length polyhydroxyalkanoates (mcl-PHAs) from palm oil. *Case Studies in Chemical and Environmental Engineering, 2*, 100045.

Verma, R., Vinoda, K. S., Papireddy, M., & Gowda, A. N. S. (2016). Toxic pollutants from plastic waste-a review. *Procedia Environmental Sciences*, *35*, 701-708.

Yu, J., and Chen, L. X. L. 2008. "The Greenhouse Gas Emissions and Fossil Energy Requirement of Bioplastics from Cradle to Gate of a Biomass Refinery." Environmental Science and Technology 42: 6961-6.

Zhu, Y., Romain, C., & Williams, C. K. (2016). Sustainable polymers from renewable resources. Nature, 540(7633), 354-362.

OPTIMIZATION OF PRODUCTION CONDITIONS OF POLYHYDROXYALKANOATES PRODUCED BY SOIL EXTRACTED BACTERIAL STRAINS ISOLATED FROM LANDFILL SITE

ORIGINALITY REPORT

SIMILARITY INDEX	INTERNET SOURCES	PUBLICATIONS	STUDENT PAPERS
8%	5%	4%	4%

PRIMARY SOURCES

1	clinmedjournals.org Internet Source	1%
2	link.springer.com Internet Source	1%
3	www.mdpi.com Internet Source	1%
4	J. H. Song. "Biodegradable and compostable alternatives to conventional plastics", Philosophical Transactions of The Royal Society B Biological Sciences, 07/27/2009 Publication	1%
5	pdffox.com Internet Source	<1%
6	Submitted to Higher Education Commission Pakistan Student Paper	<1%
7	Submitted to Ghana Technology University College	<1%