Bacterial Polyhydroxyalkanoate Polymer Extraction and Characterization from Solid Waste Dumping Site, Adama City, Ethiopia

Muzeyin Husen

A Thesis Submitted to The Department of Applied Biology School of Applied Natural
Presented in Partial Fulfillment of the Requirement for the Degree of master's in biology Specialization in Applied Microbiology

Office of Graduate Studies

Adama Science and Technology University

January, 2024

Adama, Ethiopia

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Advisor: Seid Mohammed

(PhD) Co-advisor: Fedlu Kedir (PhD)

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DECLARATION

I hereby declare that this Master Thesis entitled “Bacterial Polyhydroxyalkanoate Polymer Extraction and Characterization from Solid Waste Dumping Site Adama City, Ethiopia” is my original work. That is, it has not been submitted for the award of any academic degree, diploma, or certificate in any other university. All sources of materials that are used for this thesis have been duly acknowledged through citation.

Muzeyin Husen		
Name of student	Signature	Date

RECOMMENDATION

we, the advisor(s) of this thesis, hereby certify that I/we have read the revised version of the thesis entitled "**Bacterial Polyhydroxyalkanoate Polymer Extraction and Characterization from Solid Waste Dumping Site Adama City, Ethiopia**" prepared under our guidance by **Muzeyin Husen** submitted in partial fulfillment of the requirements for the degree of Master of Science in Applied Microbiology. Therefore, I/we recommend the submission of a revised version of the thesis to the department following the applicable procedures.

Seid Mohammed (PhD)		
Major Advisor	Signature	Date
Fedlu Kedir (PhD)		
Co-advisor	Signature	Date

APPROVAL PAGE

we, the advisors of the thesis entitled "**Bacterial Polyhydroxyalkanoate Polymer Extraction and Characterization from Solid Waste Dumping Site Adama City, Ethiopia**" and developed by **Muzeyin Husen**, hereby certify that the recommendation and suggestions made by the board of examiners are appropriately incorporated into the final version of the thesis.

Seid Mohammed (PhD)		
Major Advisor	Signature	Date
Fedlu Kedir (PhD)		
Co-advisor	Signature	Date

APPROVAL OF BROAD OF REVEIWERS

we, the advisors of the thesis entitled " **Bacterial Polyhydroxyalkanoate Polymer Extraction and Characterization from Solid Waste Dumping Site Adama City, Ethiopia** " and developed by **Muzeyin Husen**, hereby certify that the recommendation and suggestions made by the board of examiners are appropriately incorporated into the final version of the thesis.

Seid Mohammed (PhD)		
Major Advisor	Signature	Date
Fedlu Kedir (PhD)		
Co-advisor	Signature	Date

We, the undersigned, members of the Board of Examiners of the thesis by **Muzeyin Husen** have read and evaluated the thesis entitled " **Bacterial Polyhydroxyalkanoate Polymer Extraction and Characterization from Solid Waste Dumping Site Adama City, Ethiopia** " and examined the candidate during open defense. This is, therefore, to certify that the thesis is accepted for partial fulfilment of the requirement of the degree of Master of Science in Applied Microbiology.

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LIST OF ACRONYMS

CDW	Cell dry weight
CMC	Carboxymethyl cellulose
DIN	German Institute for Standardization
ERB	Extruded rice bran
ECS	Extruded cornstarch
FID	Flame ionization detector
FTIR	Fourier transform infrared spectrum
GC-MS	Gas chromatography-mass spectroscopy
ISO	International Organization for Standardization
MALDI TOF	Matrix-assisted laser desorption/ionization-time of flight
MDB	Minimal D avis Broth
MR-VP	Voges Proskauer test
NBA	Nile blue A
OD	Optical density
PC	PHA content
PHA	Polyhydroxyalkanoate
PHB	Polyhydroxybutyrate
PHO	Polyhydroxyoctanoate
PHV	Polyhydroxyvalerate
SBB	Sudan black B
TSI	Triple sugar iron
UV	Ultraviolet
XRD	X-ray diffraction

ABSTRACT

Conventional plastic wastes are very challenging to the environment, and their production cost also creates an economic crisis due to petrochemical-based plastics. The recent study aimed to extract and characterize bacterial polyhydroxyalkanoate (PHA) polymer collected from solid waste dumping site of Adama City. In this study, soil samples were collected, and bacterial isolates were grown on Minimal Davis Agar (MDA). Sudan Black B (SBB) and Nile Blue A (NBA) staining were carried against these isolates. Various biochemical tests were done for these SBB and NBA-positive isolates. Polyhydroxyalkanoate extraction was determined for the best fluorescence-producing isolate, Enterococcus sp. ASWI-7. Effects of carbon sources, incubation time, pH, salt concentration, and temperature were performed for the best PHA producer, Enterococcus sp. ASWI-7. In total, 95 SBB-positive isolates were obtained. From these, 65 NBA -positive isolates were screened. This result indicates that the minimum PHA content (PC) was detected from Bagasse (14.3±1.496%), whereas the maximum PHA content (PC) was detected from glucose (42.59±1.3%) at 37 °C and pH 7. The highest PHA content was obtained by the NaCl-$CHCl_3$ method (53.82±2.17%), followed by the NaCl-NaOCl method (47.92±3.125%). Based on MALDI–TOF MS analysis, these isolates were designated as Bacillus sp. ASWI-19, Enterobacter sp. ASWI-21, Enterococcus sp. ASWI-7, and Pseudomonas sp. ASWI-17. However, the 16S rRNA sequence exhibited that Enterococcus sp. ASWI-7 isolate was aligned with Faecalis with 97.3% sequence similarity, which could be a new isolate. Fourier transform infrared (FTIR) spectrum analysis indicates the presence of band at 1743 cm^{-1} due to the presence of polyhydroxyalkanoate polymer carbonyl group (C=O). Gas chromatography Mass spectrometry (GC/MS) analysis showed presence of butane dioic acid, dimethyl ester, butyl 9-tetradentate, heptadecanoic acid, 11,14-octadecadienoic acid, methyl ester, tetra decanoic acid, and tridecanoic acid after thermal degradation against Enterococcus sp. ASWI-7. The powder XRD pattern of PHA has shown peak values of $2\theta = 19.80^o$ and 44^o corresponding to (11 0) and (222) crystal planes of the PHA polymer. The average crystal size for PHA polymers was also 12.15 nm against Enterococcus sp. ASWI-7. The study concluded that Enterococcus sp. ASWI-7 was found to be the best candidate for PHA production after its growth conditions had been optimized. Thus, the isolate can be employed for Industrial applications for PHA production.

Keywords: *Characterization, Enterococcus species, Extraction, Isolates, Polyhydroxyalkanoates (PHA)*

1. INTRODUCTION

1.1. Background of the study

Plastics have been in use since the 1800s, and over the past 220 years, their demand has exponentially increased, indicating their widespread application in solving various problems (Kurian & Das, 2021). They are an integral part of our everyday life. The non- degradable nature of petroleum-based plastics contributes to environmental pollution. As they accumulate in the environment for decades, they cause environmental and health problems. They contaminate water resources and pose a threat to marine animals and birds who can become entangled in them, leading to injuries and death (Sathya et al., 2018). Since petroleum is the raw material for plastic production, the declining availability of oil leads to a decrease in plastic production. This situation highlights the importance of developing alternate methods to produce plastics. Some traditionally used polymers, like silicone, have been suspected of causing cancer. This raises concerns about the potential health risks associated with certain types of plastics (Shishatskaya & Volova, 2004).

The chemical industry has been involved in both the production of conventional plastics and the development of biodegradable alternatives for polyhydroxyalkanoates (PHA) recovery from biomass. This is driven by the need for nontoxic, biodegradable, and biocompatible polymers. Polyhydroxyalkanoate is described as biopolyesters that offer environmentally friendly advantages such as biocompatibility, biodegradability, and compostability. These properties make them an attractive alternative to petroleum-based plastics (Rodrigues et al., 2022). Biodegradable polymers, such as PHAs have gained attention as eco-friendly thermoplastics because they can be produced from renewable biomass resources. Additionally, unlike many petroleum-based plastics, PHAs are completely degraded to CO_2 and H_2O by environmental microbes. This makes them a promising alternative to conventional plastics in terms of reducing environmental pollution and promoting sustainability (Insomphun et al., 2014).

Polyhydroxyalkanoates have indeed gained significant attention from both research and industry due to their unique properties of biodegradability and biocompatibility. Polydroxyalkanoates (PHA)s are primarily produced from bacterial cells under nutrient-stress conditions, such as limited phosphorus, nitrogen, sulfur, and excess carbon sources.

There are various types of PHA, including polyhydroxybutyrate (PHB), polyhydroxyhexanoate, polyhydroxyoctanoate (PHO), and polyhydroxyvalerate (PHV). Polydroxyalkanoates (PHA) molecules are stored as carbon and energy reserves in many bacteria. When characterized, they can form into different monomers. Additionally, PHAs exhibit thermoplastic and elastomeric properties, making them versatile for various applications. These properties make PHA attractive for use as sustainable alternatives to conventional plastics, as they can be produced from renewable resources and are biodegradable (Wei et al., 2009)

Poly-3-hydroxybutyrate (PHB) is indeed the most common type of PHA that has been discovered. Polyhydroxybutyrate was the first class of PHA to be identified and studied (Sathya et al., 2018). It was isolated and characterized by Lemoigne from the bacterial strain *Bacillus megaterium* in 1926. Polyhydroxybutyrate (PHB) exhibits several characteristics that make it an attractive alternative to conventional plastics. These include thermoplastic processability, hydrophobicity, complete biodegradability, and biocompatibility (Sandhya et al., 2013). Chemically, PHAs are polyoxoesters of R-hydroxyalkanoic acid monomers. Depending on the number of carbon atoms, PHA is classified into two main types: short-chain-length PHA (PHA scl) and middle-chain-length PHA (PHA mcl). Short-chain-length PHA (PHA scl) contains 3 to 5 carbon atoms. These biopolymers are stiff, brittle, and possess a high degree of crystallinity. Middle-chain-length PHA (PHA mcl) contains 6 to 14 carbon atoms. It is flexible and possesses low crystallinity, tensile strength, and a high melting point (Sathya et al., 2018).

Polyhydroxyalkanoate granules are complex molecules and are sometimes referred to as carbonosomes. There are four different types of proteins associated with PHA granules: Polyhydroxyalkanoate synthases, PHA depolymerases, phasins, and regulatory proteins. Among them, phasins are the most common and they cover the outer layer of the granules (Maestro & Sanz, 2017). The key enzymes involved in PHA biosynthesis are polyester synthases, also known as PHA synthases. These enzymes catalyze the conversion of (R)-3-hydroxyacyl-CoA substrates to PHAs, releasing CoA in the process (Rehm, 2007). To screen microbial strains for the presence of intracellular PHB granules, staining methods can be used. The colony staining method using Nile blue and Sudan black is commonly used, with Nile blue being the most sensitive and specific stain for detecting PHB granules (Altaee et al., 2017b). Polyhydroxyalkanoate recovery has been using halogenated solvents such as chloroform, sodium hypochlorite, sodium chloride, and the dispersion of

chloroform and sodium hypochlorite. Sodium hypochlorite (NaClO) is an oxidizing chemical that dissolves the non-PHA cellular mass (NPCM), while PHA granules remain in solid form. However, chloroform can dissolve the PHA granules (Rodrigues et al., 2022).

Polyhydroxyalkanoate has indeed found applications in various industries such as medical, telecommunications, furniture, packaging materials, shopping and garbage bags, clothing, liquid containers, footwear, toys, household products, industrial products, and building materials (Sathya et al., 2018). Biodegradable plastics, including PHA, offer several advantages. They are produced from renewable sources through biological processes, which enhances environmental sustainability. Additionally, the production of bioplastics contributes to economic sustainability. However, the production cost of bioplastics using conventional technologies is often higher than that of petrochemical-based plastics. Therefore, reducing the production cost of bioplastics by utilizing inexpensive raw materials and technological innovations is crucial for their wider adoption and application (Ivanov et al., 2015).

Polyhydroxyalkanoate can be produced from various microorganisms, primarily bacteria and occasionally archaea. Among the bacteria, at least 75 different genera have been isolated. Some of these include *Bacillus megaterium, Klebsiella aerogenes, Pseudomonas. oleovorans, Pseudomonas. putida, Sphaerotilus natans,* and *Cupriavidus necator*. These bacteria have been stored around 80% of their dry biomass as PHA in the form of intracellular inclusions under stress conditions during fermentation. In addition to bacteria, PHA-producing archaeal genera have also been identified. These include *Haloferax, Halogeometricum, Natrinema, Haloarcula,* and *Halobacterium*. Researchers have reported that *Haloferax* is the dominant archaeal genus obtained from samples of marine coastal sediment (Mohammed et al., 2019b).

Widespread use of PHA is currently limited due to high production costs. For example, the cost of PHB-co-v resin, a biodegradable polymer, is approximately $30/kg, which is more expensive than conventional plastics like polypropylene, which costs around $2/kg.

1.2. Statement of the problem

Due to their durability and resistance to degradation, petroleum-based plastics are the material chosen for consumer goods. These non-degradable conventional plastic wastes are very challenging to the environment by causing environmental pollution (Sathya et al., 2018). To solve this problem biodegradable polymers are gaining attention due to increased awareness of the waste issue and adverse environmental effects. The intriguing feature of plastics is how easily bacteria can break them down. Therefore, understanding how materials and bacteria interact is necessary for this and it is crucial to consider the microbial breakdown of natural and synthesized polymers. Due to biodegradability, biocompatibility, and replacement for petrochemical-based polymers, PHA has gained significant commercial and research interest. However, the industrial production of these polymers was constrained due to the expensive carbon sources. Therefore, PHA production from renewable and cheap agro- based carbon sources are a sustainable solution for global environmental problems. To overcome this problem many studies have been done on bioplastic polymers. However, there are not any published research papers on the extraction and characterization of these PHAs polymers in Ethiopia. So, the current study was focused on the extraction and characterization of Polyhydroxyalkanoate polymer derived from PHA-producing bacteria isolated from the Adama city solid waste damping site.

1.3. Objectives of the study

1.3.1. General objective

- To extract and characterize polyhydroxyalkanoate polymer derived from bacterial isolates from Adama city solid waste dumping site soil sediment.

1.3.2. Specific objectives

- To screen polyhydroxyalkanoate polymer producing bacterial isolates using SBB and NBA from solid waste dumping sites
- To determine the suitable solvents employed for PHA polymer extraction.
- To characterize and identify the extracted PHA polymers using various analytical

instruments Fourier Transform Infrared, X-ray Diffraction nd to analyze using GC-MS).

- To evaluate the optimum culture condition for potential PHA producing bacterial isolates
- To identify the isolates using MALD-TOF MS and 16S rRNA

1.4. Scope of the study

This study was carried out at Adama Science and Technology University. It aimed to extract and characterize polyhydroxyalkanoate polymer derived from PHA-producing bacteria isolated from the Adama city solid waste dumping Site

1.5. Significance of the study

The significance of this study is to give information on the extraction and characterization of Polyhydroxyalkanoate polymer derived from PHA-producing bacteria isolated from the Adama city solid waste dumping site. Another significance is to know suitable solvents employed for PHA polymer extraction and to predict the optimum culture condition for candidate PHA producing bacteria. This is study used to produce eco-friendly and cheap bioplastics from bacteria using cheap substrates. Also helps to minimize the cost expend for the production and commercialization of petroleum-based plastics using cheap renewable carbon sources

2. LITERATURE REVIEW

2.1. History of PHA

The history of PHA research dates to the early 20th century when Maurice Lemoigne, a French microbiologist, discovered B. *megaterium* that produced poly-β-hydroxybutyric acid (PHB) under nutrient-limited conditions. This discovery laid the foundation for further exploration of PHAs as natural polyesters. In the 1950s, Karl Ziegler and Giulio Natta developed catalysts for the synthesis of polyolefins, which sparked interest in PHAs as sustainable alternatives to petroleum-based plastics. During the 1970s, researchers identified over 100 different PHA monomers and discovered copolymers of PHB with other hydroxyalkanoates, such as poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV). In the 1980s, PHAs gained recognition as biodegradable plastics that could potentially replace traditional plastics in various applications. The 1990s the application of genetic engineering and metabolic engineering techniques to improve PHA yield and diversity. In the 2000s, researchers explored new carbon sources and bacterial strains for PHA production, as well as developed new methods for PHA recovery and purification. In the 2010s, PHAs were further developed for biomedical, agricultural, and environmental applications, including tissue engineering, drug delivery, biodegradable packaging, and bioremediation (Chen & Patel, 2012). By the end of the 1950s, research on *Bacillus* species had provided evidence that poly(3-hydroxybutyrate) (P(3HB)) serves as an intracellular carbon and energy reserve in these microorganisms. It was also discovered that gram-negative bacteria frequently produce this reserve polymer. Overall, the history of PHA research has seen significant advancements in understanding PHA production, characterization, and application, making it a promising area for sustainable materials development (Sudesh et al., 2000).

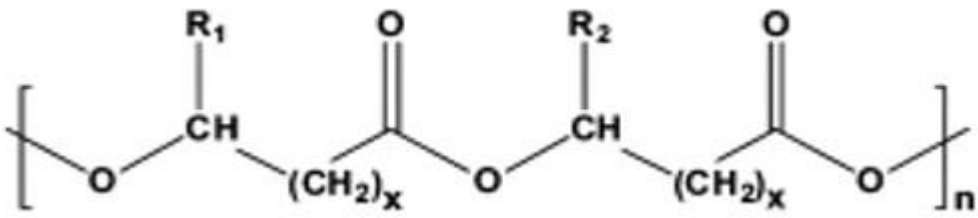


Figure 1: The structure of the PHA compound picture obtained from Madkour et al. (2013)

2.2. Biosynthesis of PHB and PHA

The Biosynthesis of PHB possesses three steps such as two acetyl-CoA molecules are condensed by the β-keto thiolase (*PhaA*), resulting in the formation of acetoacetyl-CoA. Acetoacetyl-CoA is then reduced to (R)-3-hydroxybutyryl-CoA by the (R)-specific acetoacetyl-CoA reductase (*PhaB*). The polyester synthase (*PhaC*) utilizes (R)-3-hydroxybutyryl-CoA as a substrate to produce PHB. These genes encoding the PHB biosynthesis proteins (*PhaA*, *PhaB*, and *PhaC*) are often co-localized and arranged in an operon. Polyhydroxyalkonoates are synthesized from medium-chain length (R)-3-hydroxyfatty acids. Fatty acid metabolic intermediates are directed towards PHA biosynthesis if the carbon source is converted to acetyl-CoA without using the fatty acid β-oxidation pathway. The transacylase *PhaG* mediates the synthesis of (R)-3-hydroxyacyl-CoA from the relevant acyl carrier protein (ACP) thioester. The (R)-3-hydroxyacyl moiety is then transferred to CoA by the transacylase. These processes highlight the enzymatic steps involved in the biosynthesis of PHB and PHAs (Figure 2), as well as the utilization of different carbon sources and intermediates in their production (Rehm, 2007).

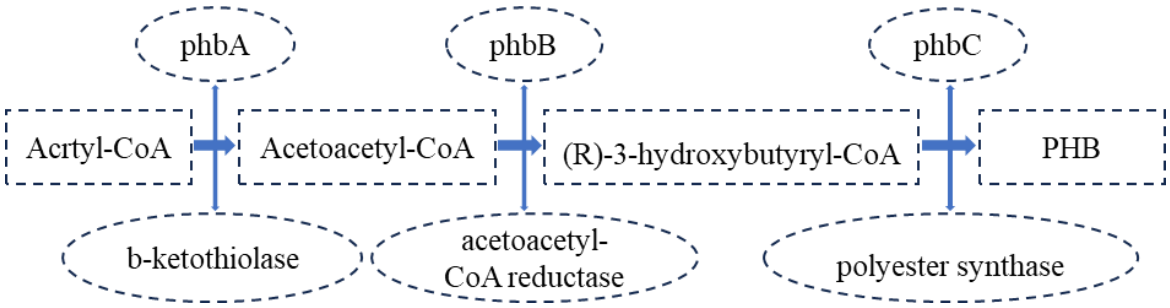


Figure 2: The biosynthesis of the PHB and PHA compounds adapted from Rehm (2007).

2.3. Properties of PHA

There are several properties exhibited by PHA, some of them are discussed below.

(1) **Semi-crystalline structure**: PHAs have a semi-crystalline structure, and their melting temperature (Tm), glass transition temperature (Tg), and thermal degradation temperature (Td) serve as indicators of their thermal and mechanical characteristics (Priyanka Kumari & Dr. Madhuri Girdhar, 2022).

(2) **Variations in Tm, Tg, and Td**: PHAs exhibit varied Tm, Tg, and Td within specific ranges due to their structural variations. The ranges reported are non-observable to 177 °C

for Tm, 52 to 4 °C for Tg, and 227 to 256 °C for Td (Tan et al., 2014).

(3) **Mechanical properties**: PHAs also exhibit varied mechanical properties within these temperature ranges. These properties include Young's modulus (0.008 to 103 MPa) elongation at break (2% to 1000%), and tensile strength (8.8 to 104 MPa).

(4) **PHB limitations and modifications**: PHB has a high degree of crystallinity due to its stereoregular structure, which limits its application as a polymeric substance. To overcome this weakness, researchers have employed chemical modifications such as copolymerization with different PHA monomers or combining PHB with other polymers. One such modification is the addition of 3-hydroxyvalerate (3HV) to PHB, creating PHBV, which exhibits more suitable physical characteristics for industrial applications, such as lower Tm, reduced stiffness, and higher toughness Click or tap here to enter text (Anderson et al., 1990).

(5) **Differences between SCL-PHAs and MCL-PHAs**: MCL- PHAs, such as 3-hydroxyoctanoate and 3-hydroxydecanoate, have lower Tm and crystallinity compared to SCL-PHAs. This higher elastomeric nature of MCL-PHAs expands their potential applications (Davis et al., 2013).

2.4. Structural Diversity of PHA

PHAs are divided into two main categories based on the length of their chains. MCL PHAs have medium-chain lengths, while SCL PHAs have short-chain lengths. The chain length of PHAs affects their crystallinity, elongation ability, and tensile strength. SCL PHAs, being crystalline polymers, are rigid and brittle, with higher melting temperatures (typically ranging from 42 to 65 °C) and greater crystallinity. On the other hand, MCL PHAs exhibit elastomeric qualities, with lower melting temperatures, increased flexibility, and lower crystallinity (around 25% less crystalline) (Grigore et al., 2019). MCL PHAs are often referred to as "real elastomers" due to their lower melting temperatures. If the temperature exceeds their melting temperature (Tm), they become sticky and amorphous. Bacteria utilize the oxidation process to synthesize MCL PHAs. This involves the conversion of various carbon sources into acetyl-CoA, which then undergoes polymerization to form MCL PHAs (Wecker et al., 2015).

The primary substrate for MCL-PHA synthesis is acyl CoA, which is released at the end of each cycle by utilizing two carbon atoms. The enzyme PhaJ converts 2-trans-enoyl CoA

to R-3-hydroxyacyl CoA, and PhaC polymerizes R-3-hydroxyacyl CoA to MCL-PHA. The final steps of the oxidation process are catalyzed by thiolases and dehydrogenases (Zhao et al., 2020). Various fermentation mechanisms can be used for MCL-PHA production using co-substrates like decanoic acid (DA) and derived sugars (Oliveira et al., 2020). *Pseudomonas* sp. is the primary producer of MCL-PHA and can metabolize all sugars except glucose. When both sugars are provided together, hexoses become available, leading to faster glucose metabolism. The structure of MCL-PHA is dependent on the type of carbon source provided. *Pseudomonas* sp. synthesizes MCL-PHA through two distinct pathways: oxidation in the presence of substrates like alkanoic acid and de novo fatty acid biosynthesis in the presence of structurally unrelated substrates like carbohydrates. All sugars can be metabolized by them, excluding glucose. As a result, when both sugars are given in combination, hexoses are made available. As a result, glucose is metabolized more quickly (Urbina et al., 2018). MCL-PHA is structurally reliant on the kind of carbon source offered. MCL-PHA is synthesized in *Pseudomonas* sp. by two distinct pathways: oxidation in the presence of substrates such as alkanoic acid and de novo fatty acid biosynthesis in the presence of structurally unrelated substrates like carbohydrates (Oliveira et al., 2020).

2.5. Biodegradability of polymers and its classification

Biopolymers can be composted and can degrade in the environment. Microorganisms convert biodegradable polymers into water and carbon dioxide. Biopolymers that can be composted can be added to a commercial composting process, where they will decompose by 90% in just six months. Biopolymers can be composted and can degrade in the environment. Biopolymers can be divided into two main categories: those made from living organisms and those made from renewable resources but still requiring polymerization (Chapuel & Reyes, 2019; Nair & Laurencin, 2007). According to how they are produced, biopolymers can be categorized into various groups (Chapuel & Reyes, 2019). From biomass, such as polysaccharides (starch, cellulose, and proteins), polymers are directly extracted or eliminated (Figure 3). Biologically or genetically engineered bacteria- produced polymers. Polyhydroxyalkanoates make up most of the biobased polymers in this category as of right now, but bacterial cellulose is being developed as well. Polymers are created using renewable biobased monomers in a traditional chemical synthesis process. Polylactic acid, a bio polyester made of lactic acid monomers, provides

a nice illustration. Polymers made from synthetic monomers using traditional synthesis. Aliphatic and aromatic polyesters are two examples.

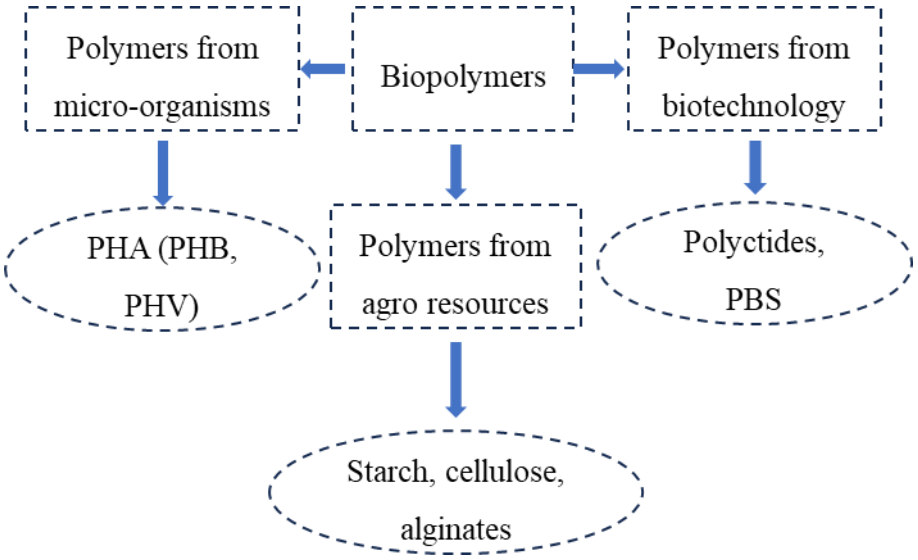


Figure 3: The classifications of the polymers as taken from Chapuel & Reyes (2019).

2.6. Bioplastics

Bioplastics are a family of polymeric materials that can be either bio-based, biodegradable, or both. The term "bioplastic" does not necessarily mean that the material is made up of biomass. (1) Fossil-based biodegradable bioplastics: Some plastics derived from fossil fuels can still be considered bioplastics if they possess biodegradable properties. These plastics may come from non-renewable sources but can break down naturally over time, reducing their environmental impact. (2) Biobased non-biodegradable bioplastics: Some plastics are derived from renewable resources (biobased) but are not biodegradable. These plastics may offer other environmental benefits, such as reduced carbon emissions during production, but do not break down easily in the environment. (3) Biobased biodegradable bioplastics: True bioplastics are those that are both biobased and biodegradable (Figure 4). These materials are not only derived from renewable resources but also can degrade naturally, minimizing their impact on the environment. They are considered both eco-friendly and sustainable (Faizan Muneer et al., 2021).

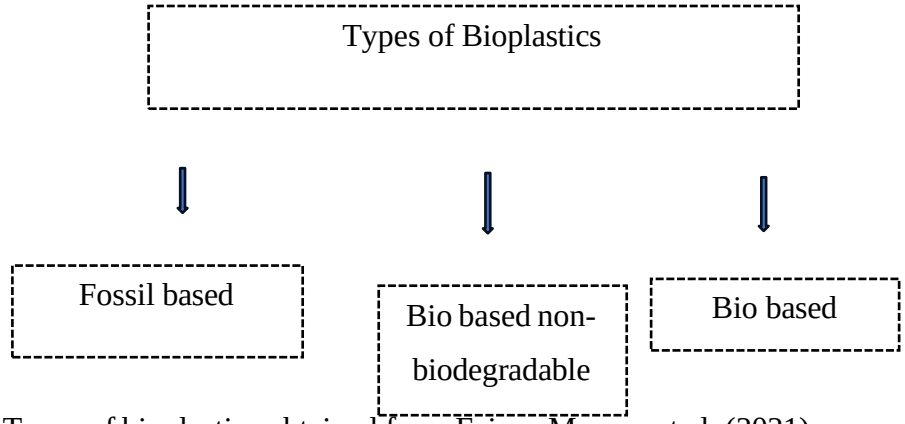


Figure 4: Types of bioplastics obtained from Faizan Muneer et al. (2021).

The most common definition of biodegradable polymers is that they degrade and decompose under normal environmental conditions (aerobic and anaerobic) within an acceptable period after their useful life. This definition aligns with the capacity of PHAs to biodegrade. Biodegradable polymers, including PHAs, can be broken down by microorganisms such as fungi, bacteria, yeasts, and actinomycetes present in the environment. These microorganisms decompose the polymers into compostable products, including water, carbon dioxide, and biomass. The ISO (International Organization for Standardization) and DIN (German Institute for Standardization) have provided criteria for defining biodegradable polymers. According to ISO standards, chemical alteration of the substance by microbes is necessary for biodegradable polymers. DIN standards require the conversion of polymers into microbial me tabolic products (Sathya et al., 2018). PHAs, such as PHB (polyhydroxybutyrate), can be degraded by natural non-specific lipases and esterases found in microorganisms. In vivo studies have shown that PHA implants and medical devices degrade where they are implanted. The presence of lipase activity in the gastrointestinal tract suggests that lipases may be involved in the digestion of PHAs in vivo (Löbler et al., 1999)

2.7. Biocompatibility

Due to their biocompatibility, they are used in medical applications. For use in medical applications, materials must be biocompatible, which means that they should not cause severe immune reactions when introduced to soft tissues or the blood of a host organism during degradation in the body to be considered biocompatible. PHAs not only appear in microorganisms as storage materials but are also ubiquitous in other natural plants as well as animals, and their metabolism and excretion are both well understood. The monomeric component of P(3HB) and R-3-hydroxybutanoic acid is a product of cell metabolism,

produced during fatty acid oxidation in the liver. This hydroxyl acid is a ketone body that is biosynthesized in the mitochondria of the liver and is used by the brain as a fuel source. 3-Hydroxybutyric acid is a normal constituent of human blood in concentrations between 0.3 and 1.3 mM (Zinn et al., 2001). An attractive progress has been made after the finding of the very widespread dispersal of PHB as a low molecular weight oligomer (120-200 monomers) in microorganisms, plants, and animals, including humans. In many cases, this form of PHB is found as a PHB calcium polyphosphate complex in membranes that seems to function as an ion channel through cell membranes. Various medical applications of PHA have been explored extensively in recent years. PHAs have been used to develop devices including nerve repair devices, repair patches, cardiovascular patches, orthopedic pins, adhesion barriers, guided tissue repair/regeneration devices, nerve guides, tendon repair devices, bone-marrow scaffolds, tissue-engineered cardiovascular devices, and wound dressings (Chen & Wu, 2005; Hazer, 2010; Valappil et al., 2006). So far, various tests on animal models have shown polymers, from the PHA family, to be compatible with a range of tissues. The surface properties of PHA films are favorable for the proliferation and attachment of tissue cells (Hazer, 2010; Wu et al., 2009), suggesting that PHA is suitable for scaffolding materials in tissue engineering. NIH 3T3 fibroblast cells have been shown to adhere and proliferate on PHA membranes (Shishatskaya & Volova, 2004). Also, mesenchymal stem cells were shown to adhere and proliferate on several PHA substrates, with a terpolymer poly(hydroxybutyrate-co-hydroxyvalerate-cohydroxyhexanoate) (P (HB- co-HV-co-HHx)) (Ji et al., 2009; Wei et al., 2009).

2.8. Methods of recovery of PHA

2.8.1. Chloroform

Since PHA is an intracellular product, the method applicable for the effective separation of PHA from other biomass components can be complex and costly. The most common method for the extraction of PHA from biomass is solvent extraction by using chloroform. Chloroform extraction is a very simple and effective method to separate PHA granules from biomass. By using this method, highly purified PHA can be obtained without the degradation of PHA molecules (Table 1). Other halogenated hydrocarbon solvents such as dichloromethane, dichloroethane, and chloropropane can be also used to extract and purify PHA from the cell biomass. However, these methods are not suitable for the mass production of PHA as the solvent is potentially hazardous to health and the environment (Chee & Lau,

2017).

2.8.2. Sodium Hypochlorite

This method of PHA extraction is based on the treatment of cells with sodium hypochlorite. This oxidizing agent degrades at appropriate concentrations selectively all polymeric non-PHA cell constituents, whereas PHA mostly resists the chemical attack of hypochlorite and remains. PHA, which does not dissolve in sodium hypochlorite and remains solid, can then easily be separated by filtration or centrifugation (Table 1). One advantage of the hypochlorite method is that the cells need not be dried before the treatment, although the method is much more effective if dried cells serve as starting material. This saves time and energy and reduces the costs for downstream processing. In general, this process is easy to handle and is with some precautions also applicable to a large scale. Heat formation during the application must be carefully taken care of because the digestion of biomass by hypochlorite is a strongly exothermic process. This was demonstrated in a recent study when PHB was isolated by a simplified method from biomass of *Rastonia. eutropha*. The simplifications of the methods consisted of the use of a much higher cell density of 3% (w/v) and the use of only a 13% (w/v) aqueous solution of sodium hypochlorite. By these measures, the total volume occurring during the application as well as the required amounts of chemicals and energy were significantly reduced. Disruption by sodium hypochlorite can result in a high purity of the polymer with a PHA content in the isolated material of up to 99% (Chee & Lau, 2017).

2.8.3. Dispersion of sodium hypochlorite and chloroform

The dispersion of hypochlorite and chloroform consisting of an organic chloroform phase and an aqueous phase with 30% hypochlorite, about 91% of the polyester could be recovered, yielding a material with 97% purity. The procedure decreased the molecular weight of the PHA only marginally, but it required a large amount of chloroform. NaOCl when combined with chloroform helps in the solubilization of PHA (Table 1). The chloride ion of the halogenated solvents solubilizes PHA (Estrela et al., 2002). NaOCl prevents contamination of PHA with proteins and other compounds by neutralizing amino acids resulting in the formation of salt and water (Aramvash et al., 2018). These chemicals can destroy the cell wall and proceed with the release of PHA (Aljuraifani et al., 2019; Estrela et al., 2002)

2.8.4. SDS treatment

By incorporating themselves into the lipid bilayer of the cytoplasmic membrane of the cell envelope, several surfactants, including sodium dodecyl sulfate (SDS) and others, break down cells (Table 1). Because the surfactant also solubilized non-PHA cellular components, for instance, P(3HB) from *R. eutropha* cells was released into the solution surrounded by the cellular debris). One benefit of SDS is that P(3HB) can be recovered directly from a high cell density culture broth without the need for pretreatments. If an SDS/biomass ratio greater than 0.4 was utilized, P(3HB) was extracted from *R. eutropha* cells with a purity of over 95% and a recovery rate of more than 90%. To get PHA with a higher recovery and with a purity exceeding 97%, other agents such as hypochlorite and sodium hydroxide must be used in addition (Samorì et al., 2015).

Table 1: Methods of PHA recovery, advantages, and disadvantages adapted from Chavan et al. (2021).

S.N.	Methods	Advantages	Disadvantages
1	Chloroform	A very simple, effective, and highly purified product can be produced, with no degradation of the PHA molecule	Not suitable for mass production of PHA
s2	Dispersion of sodium hypochlorite and chloroform	Highly purified product	Decreases molecular weight
3	SDS treatment	Highly recovering of PHA	expensive
4	Sodium Hypochlorite	Destroy cell wall and release of PHA, high purity	May digest polymer

2.9. Factors affecting PHA degradation and yield

PHA degradation is primarily influenced by two elements: environmental conditions and PHA material characteristics. Due to microbial activity that produces PHA-degrading enzymes, PHA can spontaneously break down in the environment. As a result, the environment greatly affects the development of PHA-degrading bacteria, which in turn alters

the secretion and production of PHA depolymerase as well as the enzymes' ability to degrade substances. Some of the most important factors that are essential for the development of the microbial community in the environment are humidity (moisture level), temperature, the presence or absence of oxygen, and the availability of nutrients. PHA production has been affected by different factors, including the carbon-nitrogen ratio, nutrients, method of fermentation, and fermenter operating parameters. Microorganisms can use various carbon sources, such as sugars, fatty acids, and organic acids, for their growth and PHA accumulation. Various strains can have different preferences for nitrogen, such as ammonia, urea, or nitrate (Yean et al., 2017).

2.10. Applications of Biopolymers

Biopolymers, due to their biocompatible and biodegradable nature, can be used to improve the performances of other biologically active molecules in a product. They can also be modified to suit various potential applications which include synthesis of nanomaterials, biomedical applications, the food industry, and packaging. Some of the potential application areas of biopolymers are described as follows.

2.10.1. Biomedical Applications

The biomedical applications of biopolymer materials, such as those in tissue engineering, pharmaceutical transporters, and medical devices, have generated a lot of interest recently. Gelatin, a typical biopolymer, has many uses in medicine, including as an adhesive and for treating wounds. PHAs have been widely used in biomedical engineering, including in the development of tissue engineering techniques and drug delivery systems (Figure 2). Although synthetic biopolymers, proteins, and polysaccharides are favored, they lack the mechanical qualities and stability in water settings required for medical applications (Shrivastav et al., 2013).

2.10.2. Drug Delivery

Polyhydroxyalkanoates have potential as biomaterials for use in traditional medical devices, drug delivery, and tissue engineering since they are typically biodegradable and thermos processable. PHA polymer degradation in the host organism's tissues provides the potential to couple this event with the release of bioactive substances, such as antibiotics or anticancer drugs. When utilized for localized administration of drugs and the regulated release of a drug

over several months, biodegradable polymers can be inserted into the body and contain an entrapped drug HAs can be produced into films, porous matrices, microcapsules, microspheres, and nanoparticles (Figure 2). They are hydrophobic and biocompatible. A PHA homopolymer or copolymer can capture or microencapsulate drugs. Numerous medications, including anesthetics, antibiotics, anti-inflammatory pharmaceuticals, anticancer treatments, hormones, steroids, and vaccines, have been delivered using microsphere- or microcapsule-based delivery methods (Nobes et al., 1998; Orts et al., 2008).

2.10.3. Food Industry

In addition to providing a competitive advantage due to a more sustainable and environmentally friendly image, replacing oil-based packaging materials with biobased films and containers may also result in improved technical properties. Currently, food coatings, packaging materials, and encapsulation matrices for functional meals all use biopolymers. In addition to lowering the overall carbon footprint associated with food packaging, they offer innovative methods to extend product shelf life. These biobased polymers are especially helpful in three key areas for food-related applications: food packaging, food coating, and edible films for food and encapsulation (Figure 2). Certain biodegradable polyesters and thermoplastics including starch, PLA, PHA, and other materials that can be processed using standard machinery are the most commercially viable materials for food packaging. Gingerade and Price reported using PHA microspheres as steroids (Gangrade & Price, 1991).

2.10.4. Packaging Applications

Currently, certain biodegradable polyesters that can be produced using standard machinery are the most economically viable materials for food packaging. These materials are already utilized in several monolayer and multilayer food packaging applications (Figure 2). The sustainable biopolymers used in monolayer packaging are starch, PHA, and PLA, three of the most extensively studied thermoplastics. The most crucial factor to consider for bio-based food packaging applications is its barrier qualities. Water vapor is transmitted via packaging due to hydrophilic polymers' often weak moisture resistance, which degrades the quality of food. Shorter shelf lives, higher expenses, and ultimately more waste are the results of this. The addition of various nanofillers, such as nano clays, metal oxide nanoparticles, and others, is another method for enhancing the barrier characteristics of biopolymers

(Sathya et al., 2018)

2.10.5. Applications of bioplastic in agriculture

Another huge market for PHA-containing bacterial biomass-derived nanocomposite bioplastic applications is agriculture. Dark plastic mulch, which is now utilized to control weeds, lessen soil water evaporation, and warm the soil for earlier planting, could be replaced in one possible application (Figure 2). Under plastic mulch, millions of hectares of agricultural land are farmed. However, non-biodegradable plastic mulch needs to be laboriously removed from the field each year for disposal or recycling, which uses energy and harms the environment. Utilizing film made of nanocomposite bioplastic from bacterial biomass that contains PHAs has the advantage of offering all the advantages of conventional plastic mulch while being able to be left in situ for natural biodegradation on the field. Making slow-release fertilizers with bioplastic coating or encasing nutrients in bioplastic granules, bars, or films is another agricultural use for nanocomposite bioplastic made from bacterial biomass and PHAs. For instance, as bioplastic degrades over a 0.5-2- year period, fertilizer incorporated in bioplastic bars can be utilized on oil palm plantations as time-released fertilizers (Gangrade & Price, 1991)

Table 2: The different Applications areas of PHA compounds as described in (Sathya et al., 2018)

S.N.		Application of PHA
1	Agriculture	Control releases of pesticides, herbicides, and fertilizer Control water evaporation
2	Packaging materials	Food packaging water resistance to paper
3	Food Industry	food coatings, packaging materials
4	Drug Delivery	Used to deliver numerous medications, including anesthetics, antibiotics, anti-inflammatory pharmaceuticals, anticancer treatments, hormones, steroids, and vaccines
5	Construction industry	used for insulation walls and partitions and construction of non-structural (internal) elements such as separating walls and partitions for temporary constructions
6	Hygiene products	Diapers, sanitary napkins, training pants, bandages

3. MATERIALS AND METHODS

3.1. Description of the study area

The study was carried out at Adama, Oromia region which is located around 92.2 kilometers southeast of Addis Ababa, Ethiopia. Adama is situated at an elevation of 1712 meters and coordinates are 8.54ºN 39.27°E. The city is situated between the Great Rift Valley to the east and the foot of an escarpment to the west as indicated in Figure 5. It has an annual temperature of 22°C and 400–800 mm of rainfall. The overall area of the city is 9,616,399.5 m^2 (OWWDS, 2012).

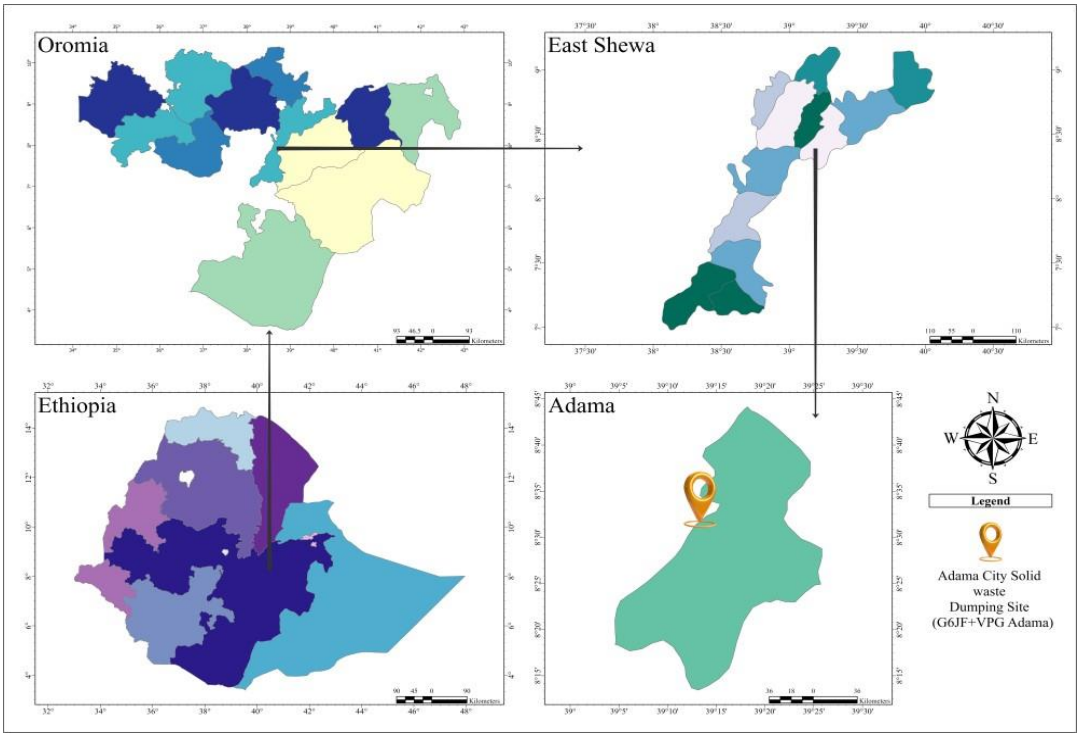


Figure 5: Location of the study area

3.2. Sample collection

A 15 Soil samples were collected from a solid waste dumping landfill in Adama City, Ethiopia. The bacterial isolates were obtained from the soil samples by pure culture by streak cultur techniques Briefly, the serial dilution of the soil samples was carried out and the isolates were obtained on Minimal Davis Agar Mohammed & Ray (2022), (per liter) (1.0 g Dextrose, 7.0 g K_2HPO_4, 1.0 g $(NH_4)_2SO_4$, 0.5 g sodium citrate, 2.0 g KH_2PO_4, 0.1 g $MgSO_4 \cdot 7H_2O$, 15 g/Agar). The pH was adjusted at 7.2 ± 0.2 and incubated at 37 °C 72 h The obtained pure culture was then maintained on a Nutrient agar plate (Peptone, 5 g/l;

sodium chloride, 5 g/l; Beef extract, 1.5 g/l; Yeast extract, 1.5 g/l; Agar, 15 g/l. The final pH was adjusted at 7.4 ± 0.2 at 37 °C for 24 h) for further use references??).

3.3. Primary detection methods for PHA-producing isolates

Detection for PHB production was employed by using lipophilic stain SBB (Thapa et al., 2019). The stain was prepared by dissolution of 0.3 gm powdered stain in 100 ml of 70% ethanol (v/v). For microscopic studies, colonies' smears were heat-fixed on clean, grease-free glass slides, followed by staining with 0.3% solution (w/v) of the SBB. After leaving the slides undisturbed for 15 min, immersion in xylene and counterstaining with safranin (5% w/v in sterile distilled water) was performed. Cells appearing blue-black under the microscope were accredited as PHB-positive strains (Desouky et al., 2014).

3.4. Secondary screening methods for PHA-producing isolates

The positive isolates obtained on SBB were subjected to NBA staining. The staining was conducted according to the methods of modification Ostle & Holt. (1982). A smear of each bacterial isolate was prepared on a microscope slide and air-dried. The smears were fixed by immersing the slides in 10% acetic acid for 10 min. The smears were stained with 0.1% NBA solution for 10 minutes. The slides were rinsed with deionized water and counterstained with 0.5% safranin O solution for 10 seconds. The slides were rinsed again with deionized water and blotted dry with a paper towel. A cover slip was placed over each smear and observed under an epifluorescence microscope with a blue filter.

3.5. Morphological characterization

Smear preparation and gram staining Gram staining was performed according to the method of Castenholz et al. (2001). Briefly, cleaned microscopic slides were taken. One drop of sterile distilled water was placed in the center of the microscopic slide using a micropipette. Using an aseptic technique, a small part of a single colony was taken and transferred from a solid medium to a distilled water drop on a microscopic slide using the sterilized loop. A thin smear was carried out and allowed to air dry at room temperature. The bacterial cells were fixed by passing the slides through the Bunsen burner flame for two-three times. The heat-fixed smears were placed on the staining rack covered with crystal violet and left for 1 min. The slide was washed gently with tap water and covered with Gram’s iodine for 1 min and the iodine was washed off by tilting the slide. The smear was decolorized with 95% (v/v)

of ethyl alcohol for about fifteen seconds and immediately washed with water. The smear was covered with safranin for one minute and washed with tap water. Finally, the smear was blotted and dried in the air to examine under the oil immersion objective (De Vos et al., 2009).

3.6. Biochemical tests

Different biochemical tests were performed to identify the isolated bacteria strains and described as follows.

3.6.1. Indole test

This test was used to identify microbes that can break down tryptophan to indole. Sterilized tubes containing tryptophan broth (4ml) were inoculated with a loopful of the isolates and incubated at 37 °C for 24-28 h. At the end of the incubation period 0.5 ml of Kovac's reagent was added. The presence/absence of a ring was used to judge a positive/negative test for indole production ability (Dawodu & Akanbi, 2021).

3.6.2. Citrate utilization test

The citrate utilization test was performed on Simmons citrate agar. Bacterial isolates were inoculated on Simmons citrate agar medium, and slants were incubated at 37°C for 48 h. A negative citrate utilization test is indicated by the lack of growth and color change in the tube. A positive citrate result is indicated by growth and a blue color change (Lemenh et al., 2021).

3.6.3. Catalase test

Three drops of 3% H_2O_2 (v/v) solution were added to bacteria cultures grown on a nutrient broth medium (how much broth?? The age of the broth??). The bubble formation was indicated as a positive reaction, while no bubble formation was considered a negative reaction for the catalase test (Lemenh et al., 2021).

3.6.4. Urease test

The urea hydrolysis test was conducted according to the method described by(Lemenh et al. (2021). A loopful of bacterial culture was inoculated into a test tube containing urea broth and then incubated for 24 h at 37 °C. The development of a bright pink color was indicative

of a positive result for urea hydrolysis.

3.6.5. Methyl Red Test

The MR-VP test was performed as prescribed by Shoaib et al. (2020). Briefly, methyl Red and Voges Proskauer test (MR-VP) broth prepared in two sets was inoculated with a loopful of the bacterial isolates and incubated for 48 h at 30 °C. Two to three drops of methyl red alcoholic solution were added to the first tube. The appearance of a distinct red color was used as indicative of a positive reaction for the MR test.

3.6.6. Voges-Proskauer Test

Voges-Proskauer Test was performed according to the method Shoaib et al. (2020). Voges Proskauer test (MR- VP) broth prepared in two sets was inoculated with a loopful of the bacterial isolates and incubated for 48 h at 30 °C. α-naphthol solution (5 % solution in 70% ethyl alcohol) (v/v) was added and shaken gently for 15 min.

3.6.7. Triple sugar iron (TSI) test

Slants of triple sugar iron medium were prepared in the test tubes by autoclaving at 121 ^{o}**C**. Using a sterile technique; a20 ml off the experimental bacterium from 24h old pure culture was inoculated into the tubes using a stab and streak inoculation method with an inoculating needle. The screw caps were not fully tightened, and the tubes were incubated for 24 h at 37 °C. Fermentation is indicated by the yellowing of the butt and the slant of the Triple Sugar Iron (TSI) agar medium. If gas was formed during the fermentation, it is shown in the butt either by the formation of bubbles or cracking of the agar (Aditi et al., 2017).

3.7. Recovery of PHA

3.7.1. Recovery of PHA polymers by NaCl treatment

Polyhydroxyalkanoates recovery was conducted according to Mohammed & Ray (2022).. A 200ml of PHA- producing potential isolates were cultured for 72 h at 35^{o}**C** and biomass was collected by centrifugation (8400 rpm, 10 min at 4 °C). The biomass dried to constant weight overnight at 50-60 °C. The dried biomass (pellet) was pretreated with 6.25M NaCl solution (w/v) at 37 °C for 1 h for PHA recovery. It was centrifuged (8400 rpm, 10 min at 25 °C).

Bacterial pellets were washed three times by dH_2O and centrifuged (8400 rpm, 5 min). The pellet was treated with acetone (100%) for 2 h. It was then air-dried. Finally, the PHA contens was recovered.

3.7.2. PHB recovery by using the dispersion of sodium hypochlorite and chloroform

It was performed following the methods of Sei Kwang Hahn et al. (1995). It was also used for analytical purposes. A 1-g portion of cell powder was treated with a dispersion containing 1 ml of chloroform and 1ml diluted sodium hypochlorite solution (4%) (1:1) (v/v). After the cell powder was treated at 37 °C for 1 h, the mixture was centrifuged (8400 rpm, 20 min, 25 °C) for 10 min, which resulted in three separate phases. The upper phase was a hypochlorite solution, the middle phase contained non-PHB cell material and undisrupted cells, and the bottom phase was chloroform containing PHB. The upper phase was removed first with a pipette, and the middle phase was separated by filtration from the chloroform phase using Whatman filter paper. PHB was recovered from the chloroform phase by non-solvent precipitation (hexane (100%) (v/v). Finally, the chloroform and hexane were removed by evaporation using the oven at 50 °C; the PHA contents were determined (Mohammed & Ray, 2022).

3.8. Growth kinetics for bacteria isolate

The bacterial isolate was tested for determination of generation time, and specific growth rate in Minimal Davis Broth medium. Briefly, a single colony of the pure bacterial isolates was inoculated into 5 ml of nutrient broth and incubated for 12 h to prepare the preculture. One ml of this overgrown culture was aseptically transferred into a flask containing 100 ml of sterile Minimal Davis Broth medium and incubated at 37 °C at 150 rpm for 54 h. Sample aliquots were taken out in four regular time intervals for turbidimetric analysis, staining, viable cell count, and extraction of PHA from the obtained biomass (Mohammed et al., 2019b).

3.9. Optimization for different parameters

3.9.1. Effect of temperature on PHA production

Minimal Davis broth was prepared and sterilized at 121 °C for 15 min. After sterilization, 100 ml of broth medium was poured into a 250 ml conical flask and supplemented with 1% glucose (w/v) as a carbon source. The bacterial isolates (2.5 ml) were inoculated into a broth medium. The cultures were incubated at different temperatures (25, 30, 35, and 37 °C) on a rotary shaker at 150 rpm for 72 h. Finally, the pellets were centrifuged (8400 rpm, 10 min at 25 °C) extracted, and quantified .

3.9.2. Effect of carbon source on PHA production

Effects of various carbon sources on PHA production by the isolate were studied. Briefly, Minimal Davis Broth (pH 7) was prepared supplemented with 1% (w/v) of Bagasse, , glucose, molasses, and sucuroses as the sole carbon source. The suitability of different carbon sources was evaluated for PHA production. A 2.5% (v/v) of overgrown preculture of the isolate was used as inoculums in 200 ml of production medium and incubated on a rotary shaker at 150 rpm and 37 °C for 72 h. After the incubation hour, the pellets were centrifuged (8400 rpm, 10 min at 25 °C), extracted, and quantified by gravimetric methods (Kemavongse et al., 2008)

3.9.3. Effect of incubation time on PHA production

Effects of different incubation times 24, 48, 72, and 96 on PHA production in isolate were also studied using Minimum Davis broth and glucose as a sole source of carbon. Samples were taken out at time intervals and PHA production was estimated using the standard protocol (Mohammed et al., 2019).

3.9.4. Effect of pH on PHA biosynthesis

Different pH values of the medium (7.0 to 11.0) were used to check whether the pH has any effect on PHA biosynthesis. Minimal Davis broth with different pH values (7.0 to 11.0) was set and sterilized at 121 °C for 15 min. The pH of the medium was adjusted by HCl or NaOH. After sterilization, a 2.5 ml (v/v) 24 h old culture was taken and transferred to 500 ml conical flasks containing 200 ml PHA biosynthesis medium and supplemented with 1% filter-sterilized glucose, fructose, molasses (w/v), and sucrose as a carbon source. The broth

culture was inoculated with bacterial isolates (2.5%) and incubated on a rotary shaker at 150 rpm and 37 °C for 72 h. Finally, the pellets were centrifuged (8400 rpm, 10 min at 25 °C), extracted, and quantified by using gravimetric methods (Mohammed & Ray, 2022).

3.9.5. Effect of salinity on PHA biosynthesis

The effect of salt was determined at various concentrations (0.1, 0.2, 0.5, and 1M). Briefly, the pure cultures obtained on SBB and NBA was inoculated into a sterilized MDB medium. The medium broth was incubated for 48 to 72 h. After incubation, the biomass was collected by centrifugation (10,000 rpm, 25 ^{o}C, and 10 min) and dried overnight at 40 ^{o}C using an oven. The CDW (g/L) was measured from dried biomass.

3.10. MALDI-TOF MS analysis

Four isolates were assessed by MALDI–TOF MS (specification). Nutrient agar was inoculated with the sample for 24 hr at 37°C. A microplate (96 MSP, Bruker®– Billerica, USA) was transferred with each colony. A lysis solution (70% formic acid; Sigma–Aldrich®) covered the bacterial sediment. A matrix solution (alpha-ciano-4- hidroxi-cinamic acid diluted in 50% acetonitrile and 2.5% trifluoroacetic acid, Sigma– Aldrich®) was added with a 1-L aliquot. A mass spectrometer (MALDI–TOF LT Micro flex, Bruker®)

3.11. Construction and analysis of the phylogenetic tree

The 16S rRNA gene sequences were extracted from the whole genome sequence of the De novo assembly of the bacterial isolate. using ContEst16S server (https://www.ezbiocloud.net/tools/contest16s). The highest sequence fragment was chosen and used for BLAST analysis. The 16S rRNA gene sequence of other taxonomically related strains was collected from the GenBank database using the EzTaxon server (https://www.ezbiocloud. net/identify for comparison. The phylogenetic tree performed following the methods of Mohammed et al. (2020)

3.12. Characterization of PHA granules

The cell biomass was collected, and the residual was removed to collect PHA granules for bacterial isolate. The collected granules were further dissolved within Chloroform. The crystal PHA was collected after evaporation of Chloroform at 50 ^{O}C. The prepared PHA was characterized using FTIR, GC-MS, and XRD according to the following methods

Mohammed et al. (2020).

3.12.1. Fourier-Transform Infrared Spectroscopy (FTIR)

The extracted PHA sample was mixed homogeneously with potassium bromide (KBr) in a 1:10 ratio and scanned using a Nicolet 6700 FT-IR spectrometer (Thermo Scientific, Waltham, MA, USA) under a spectral range of 4000–400 cm^{-1}. Each peak of determining results was shown as functional groups of polymers (Mohammed et al., 2020).

3.12.2. X-ray Diffraction (XRD)

XRD is commonly used for diffraction intensity correlated to crystallinity determination (SHIMADZU Corporation (Japan), XRD-7000 X-RAY DIFFRACTOMETER). A diffractogram of 2 g of the extracted PHA powder was performed at room temperature with nickel-filtered Cu-Kα radiation (wavelength = 0.1542 nm) in the scattering angle range of 2θ = 2–50◦ at 25 ^{O}C at a scan speed of 10◦ min^{-1}. The maximum particle size and crystallinity were determined (Shah & Kumar, 2021).

3.12.3. Analysis of PHA polymers using GC–MS

Analysis of PHA monomeric compositions before and after thermal treatment using a GC–MS (7890B GC-240 ION TRAP MS) equipped with a flame ionization detector (FID). Approximately 10 mg of pure PHA polymer obtained was subjected to methanolysis in sealed test tubes with 1 mL $CHCl_3$, 0.85 mL methanol, and 0.15 mL concentrated H_2SO_4. After the mixtures` were cooled and the seal was broken, to which 1 mL deionized water was added, the contents were homogenized, sand the bottom phase was transferred into an Eppendorf to which approximately 0.5-1 g of sodium sulfate was added to remove moisture and centrifuged at 5000 rpm for 5 min. The supernatant was used for GC analysis. The sample was analyzed in a Shimadzu 2010 Plus-gas chromatograph using an Elite-1 (fused silica gel-polymethyl siloxane) capillary column (30 m length, 0.32 mm I.D. and 0.25 μm film), nitrogen as the carrier gas with a flow rate of 1 mL/min; the fame ionization detector (FID) was operated at 250 °C with an injector port temperature of 230 °C and the column temperature at 200 °C. The oven temperature was set to 55 °C for 7 min with a ramp of 4 °C/min up to 100 °C, 10 °C up to 200 °C and held at 200 °C for 10 min (Stanley et al., 2020)

4. RESULTS AND DISCUSSION

4.1. Isolation and characterization of PHA-producing bacteria

It is interesting to note that the study focused on isolating plastic-degrading bacteria with PHA production ability from plastic waste. In this study, 98 bacterial isolates were obtained from the soil sample collected from the Adama city solid plastic waste dumping site and some of them were indicated in (Figure 6). Both gram-positive and gram-negative were screened for the PHA biosynthesis Figures a, b, c, and d after growing on MDA using the plate culturing method.

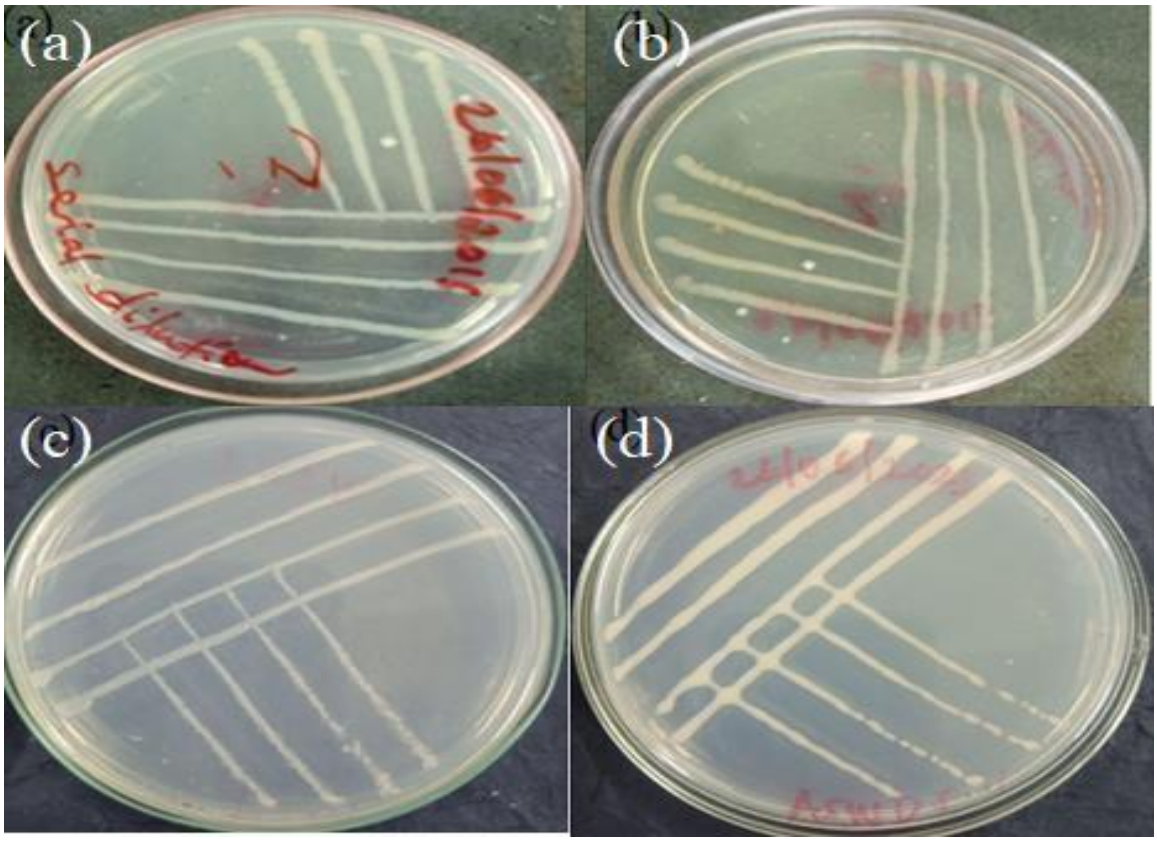


Figure 6: PHA-producing bacteria isolated from Adma City Solid waste Dumping Site (a) represents *Bacillus sp. ASWI-19*, (B) *Enterococcus sp. ASWI-7*, (C) *Pseudomonas sp. ASWI-17*, and (d) *Enterobacter sp. ASWI-21* after growing on MDA after being supplemented with 1% (w/v) of glucose.

The morphological characterization for PHA-producing bacterial isolates was performed. From Gram staining, purple bacterial isolates color was observed, Figure 7a indicating that it was gram-positive *Enterococcus sp. ASWI-7.* As illustrated in Figure 7b bacilli rod-shaped, was appeared. It indicates that *Bacillus sp. ASWI-19.* Figures 7c and d indicate pink and color from gram staining and indicate that gram-negative bacteria isolated ASWI- 17 and ASWI-21 respectively.

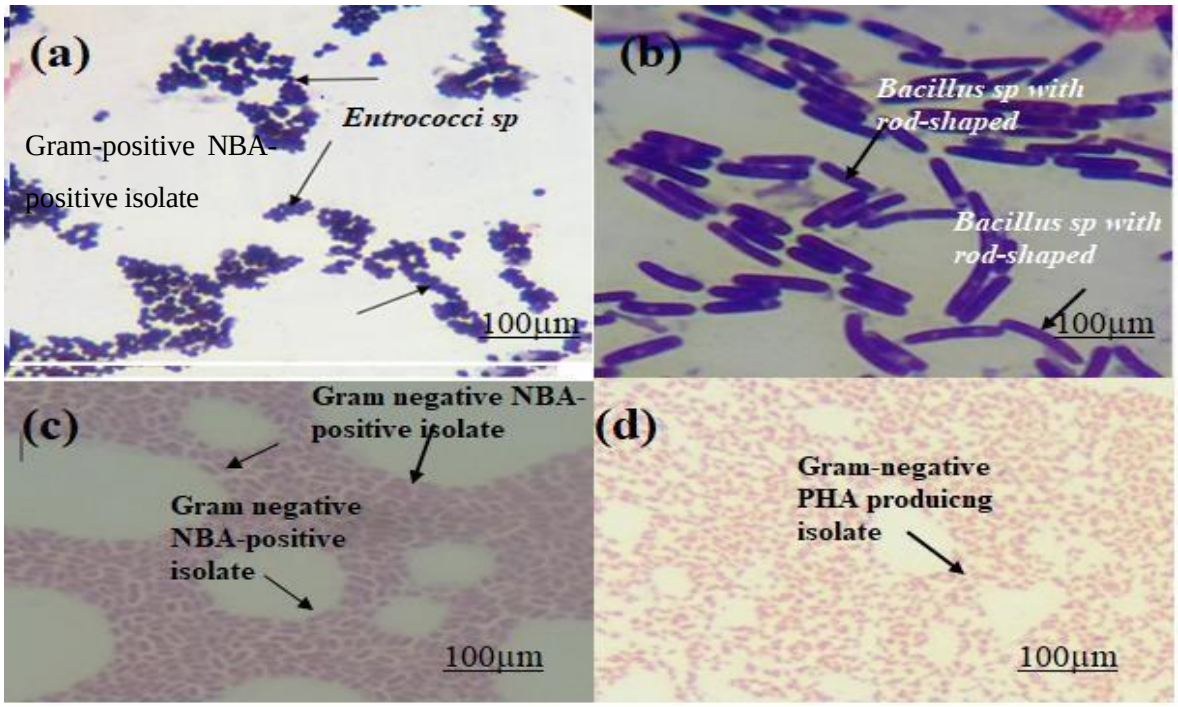


Figure 7: (a) The arrow indicates microscopic examination of the *Enterococcus sp. ASWI-7.* isolate shows that it is a cocci-shaped bacterium and appears purple after Gram-staining. (b) The arrow indicates microscopic examination of the *Bacillus sp. ASWI-19* isolate shows that it is a long rod-shaped bacterium isolated from the Adama city solid waste dumping site and appears purple after Gram-staining (c) The arrow indicates a microscopic examination of the PHA-producing *Pseudomonas sp. ASWI-17* isolate shows that it is a rod-shaped bacterium and appears pink in color after Gram-staining. (d) indicated *Enterobacter sp. ASWI-21* appeared red color appeared after gram staining**.**

4.2. Screening PHA-producing isolates by SBB

In this study, 95 isolates showed black-blue coloration when stained with Sudan black B, a preliminary screening agent for lipophilic compounds, and 65 isolates showed positive results with Nile blue A staining (Figure 8), a specific dye for the PHA granules. Both gram-positive and gram-negative bacteria showed PHA production. Similar to the current study Bhuwal et al. (2013a) reported that both gram-positive and gram-negative bacteria showed PHA production.

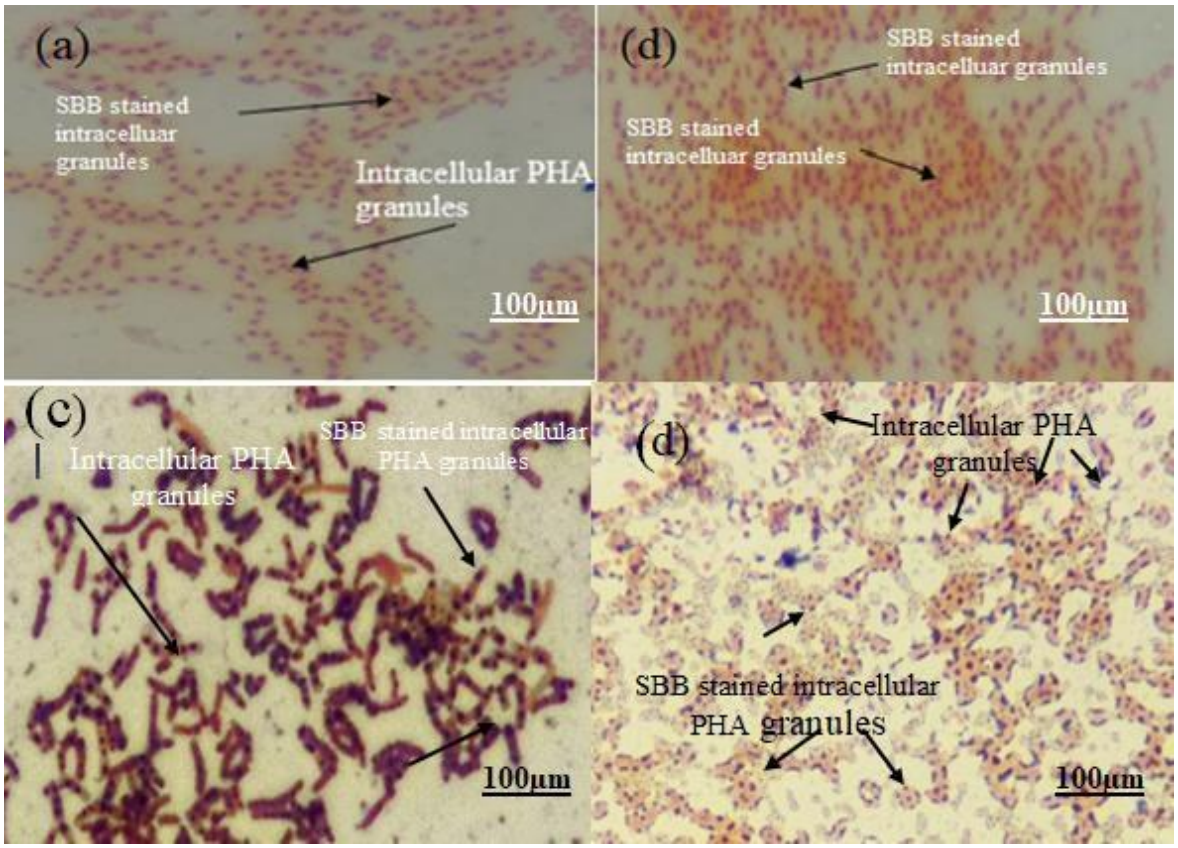


Figure 8: Indicates intracellular PHA granules after stained with NBA for bacteria isolated from Adama city solid waste dumping site. (a) The arrow indicates the SBB staining potential isolate that screened from a plastic waste dumping site area, which shows intracellular PHA granules after it has been grown on glucose as a sole carbon source and stained with SBB solution and accumulated black bioplastic intracellular granules with a red background as observed under the microscope (100x) and intracellular PHA granules that developed from 1% glucose for bacteria isolated for plate staining against *Pseudomonas sp. ASWI-17*. (b) intracellular PHA granules after *Enterobacter sp. ASWI-21* (c) Indicates

intracellular PHA granules shown after being stained with SBB against *Bacillus sp. ASWI-19.* (d) the arrow indicates the SBB staining against *Enterococcus sp. ASWI-7.*

SBB is a preliminary screening agent that gives a black-blue coloration to PHA-positive bacteria, while NBA is a more specific dye that fluoresces under UV light. as indicated in Figure 9. This study is similar to the previous report Shah & Kumar (2021) in which forty PHA-producing bacterial strains were obtained from soil samples using SBB staining. Out of these strains, six showed higher PHA accumulation when confirmed by Nile Blue A staining. The highest PHA producer was identified as *B. halotolerant* DSM8802, which accumulated 44.5% PHA of cell dry weight using domestic kitchen waste as a carbon source. Another study showed that eight PHA-producing bacterial strains were isolated from landfill and effluent treatment plant samples using SBB staining (Rayasam et al., 2020). All these strains were able to produce PHA from pomegranate peels as a novel and low-cost substrate. These studies demonstrate the diversity of Gram-positive bacteria that can make PHAs using various carbon sources and the usefulness of SBB and NBA staining methods for screening and confirming PHA producers for the diversity of bacterial species.

4.3. Screening of PHA-producing isolates using Nile blue A (NBA)

Bacterial isolates were also tested with Nile Blue A for PHA-specific screening as indicated in figure 9. It was easy, fast, and cost-effective to screen PHA-producing bacterial species from other non-producing isolates using this dye. The bacteria strain was able to produce more granules and have higher fluorescence intensity (Figure 9) when NBA dye was de-stained with 8% acetic acid (v/ v). In agreement with the current findings, Fluorescence microscopy using Nile Blue A staining for bacterial cells with high PHB levels showed bright orange fluorescence that indicated PHB accumulation inside the cells (Altaee et al., 2017).

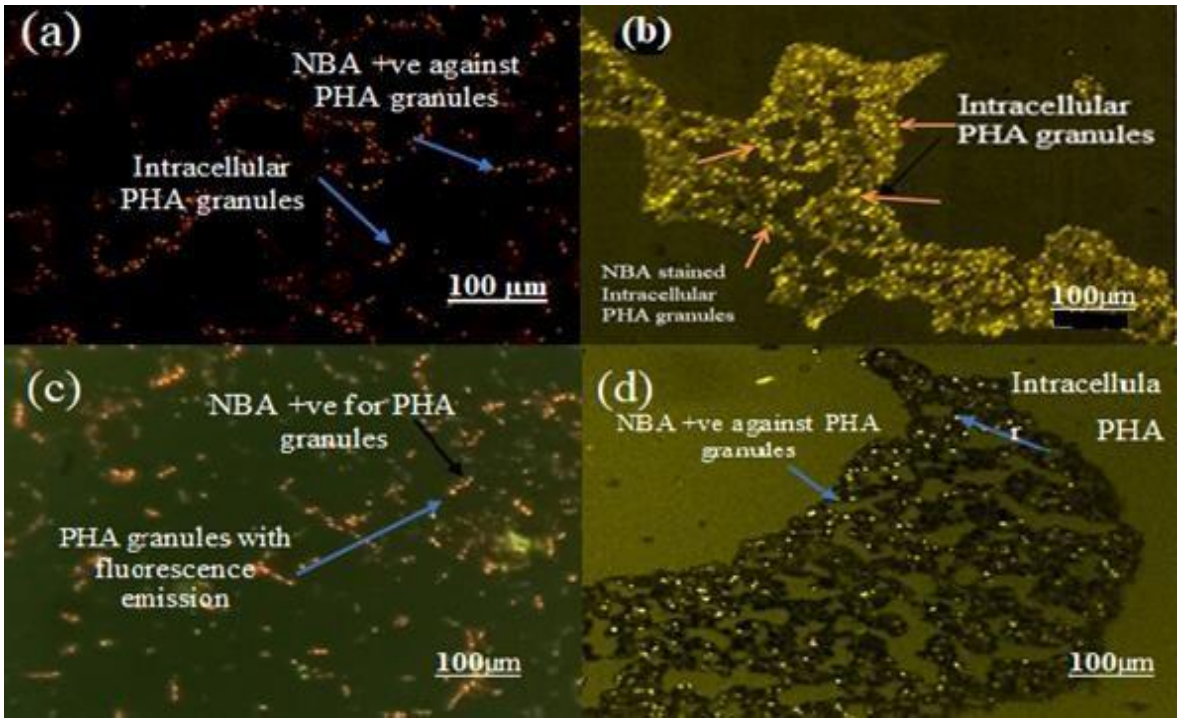


Figure 9: PHA-producing isolated slide stained with NBA examined under a fluorescence microscope 40x for bacterial isolated from Adama city solid waste damping site. (a) Indicates intracellular PHA granules after stained *Enterobacter sp. ASWI-21*, which shows intracellular PHA granules after it has been grown on glucose as a sole carbon source and stained with NBA fluoresces their intracellular granules that developed from 1% glucose. (b) indicates the intracellular PHA granules against *Pseudomonas sp. ASWI-17* showed fluoresces for their granules. (c) Intracellular PHA granules against *Bacillus sp. ASWI-19* (d) Indicates the intracellular PHA granules stained after stained with NBA against *Enterococcus sp. ASWI-7.*

Bhuwal et al. (2013b) isolated and screened 15 PHA-producing bacteria from pulp, paper, and cardboard industry wastes using NBA staining. They found that *Enterococcus* sp. NAP11 and *Brevundimonas* sp. NAC1 showed the highest PHA production among the isolates. Another study was Mohammed & Ray (2022) which screened for PHA-producing bacteria from the Loktak Lake sediment samples using NBA staining. They identified a new strain of *Bacillus* sp. LPPI-18 produced PHB when grown on glucose, molasses, or D-fructose as carbon sources. NBA staining is a simple, rapid, and reliable method for screening and characterizing PHA-producing bacteria. However, it has some limitations, such as the need for UV light equipment, the interference of other fluorescent compounds in the samples, and the possible toxicity of NBA to the bacterial cells. These same authors confirmed *Alcaligenes eutroph*us, *P. oleovoram,* and *P. putida* PHA biosynthesis using NBA

dye. 24, 48, 72, and 96 h were done for NBA staining. The binding tendency of this dye to bacteria that harbor granules has been based on carbon source, environmental condition, and period of granules to be developed.

4.4. Biochemical tests

The biochemical tests were performed for the PHA-producing isolated from the Adama City Solid Waste Dumping Site. It was performed for 4 MALDI TOF identified isolated named, *Bacillus sp. ASWI-19, Enterobacter sp. ASWI-21, E. faecalis, and Pseudomonas sp. ASWI-17* as indicated in Table 3.

Table 3: biochemical tests for the PHA producing bacterial isolates

Isolates	Catalase	Urease	Methyl red	Motility	VP	Indole	Glucose fermentation	Lactose fermentation	Sucrose fermentation	H_2S	MALDI TOF	16srRNA
ASWI-7	_	_	+	_	+	_	+	+	+	_	✓	✓
ASWI-17	+	_	_	+	_	_	+	+	+	+	✓	
ASWI-19	+	+	+	+	+	_	+	+	+	_	✓	
ASWI-21	+	_	_	+	+	_	+	+	+	_	✓	

Key: + indicates a positive result for a given biochemical test whereas – indicates a negative result

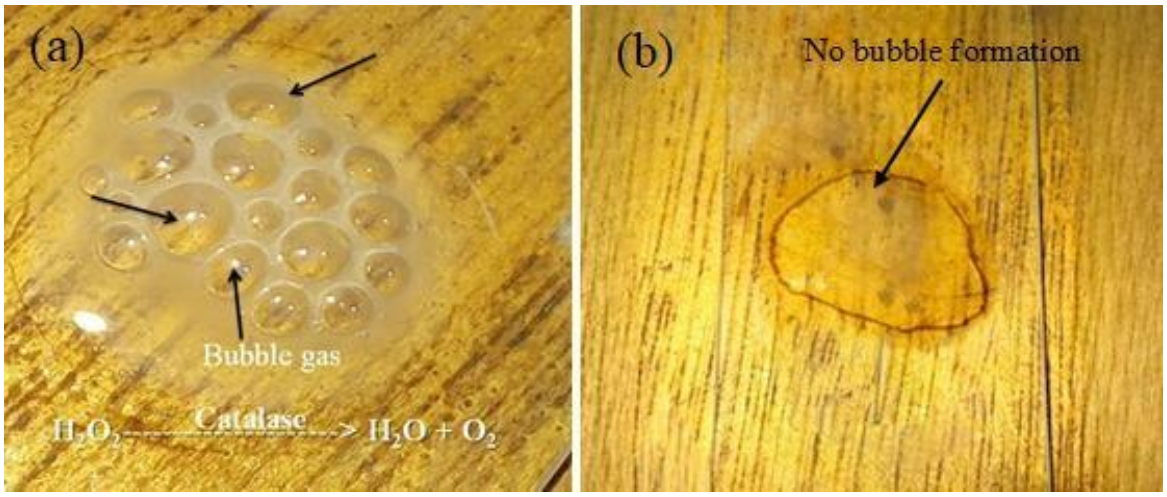


Figure 10: (a) Gram- catalase-positive bacteria isolate that obtained soil sediment and designated as from *Enterococcus sp. ASWI-7*. This isolate has a catalase enzyme that can breakdown H_2O_2 into oxygen and water which results in the formation of bubbles of gas.

In our study, the isolate showed catalase positive as indicated in Figure 10 It was also suggested that this isolate harbored a catalase enzyme that was able to break down H_2O_2 into oxygen and water which resulted in the formation of bubbles gas. In agreement with this study, it was reported that there was a formation of bubble gas when H_2O_2 was applied to *Staphylococci* species (Hafeez & Aslanzadeh, 2018). These catalase tests were thus found to be employed as a distinguishing method of *Enterococcus sp. ASWI-7* is a similar report for *Streptococci* and other anaerobic bacterial species (Hafeez & Aslanzadeh, 2018). Due to the presence of catalase enzyme against the recent results, this isolation can be employed for various industrial applications.

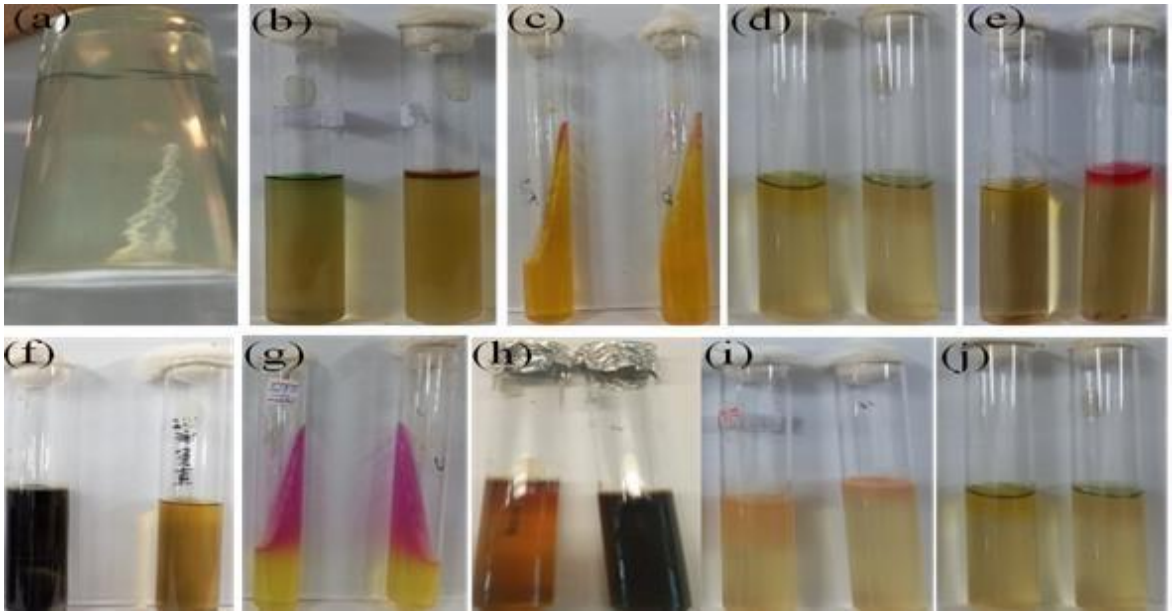


Figure 11: The biochemical tests (a, b, and c) motility test negative, methyl red positive, and triple sugar positive against *Enterococcus sp. ASWI-7* isolate. (d and e) VP test negative, motility test positive, indole negative against ASWI-17. (f and g) citrate positive, motility positive, and triple sugar positive for ASWI-19. (h, I, and j) motility test, positive, VP positive, and MR negative for bacteria isolated from the Adama city solid waste dumping site for ASWI-21**.**

4.5. Identification (MALDI–TOF MS)

Using MALDI -TOF- MS, certain *Bacillus, Enterococcus, Enterobacter*, and *Pseudomonas* species were obtained. These NBA-positive isolates were identified as *Bacillus sp. ASWI-19, Enterobacter sp. ASWI-21, Enterococcus sp. ASWI-7, and Pseudomonas sp. ASWI-17* based on protein profiles. There is charge-to-mass ratio is displayed in the Appendix. Their score for these isolates was found to be above >2.00.

4.6. Phylogenetic tree for newly isolated Enterococcus sp. ASWI-7

The 16S rRNA analysis of *Enterococcus sp. ASWI-7* using EzTaxon-e server Kim et al. (2012) species showed that the isolates were related to *Enterococcus* species and finally designed as *Enterococcus ASWI-7*. The complete 16S rDNA for *Enterococcus* spp. determined and submitted in GenBank, NCBI. A sequence comparison by BLAST

analysis confirmed the close affiliation of the strain to the genus *Enterococcus*. The strain exhibited the highest sequence similarities of (97.3%), with *E. quebecensis* CCRI-16985T (GU457262), (96.5%) with *E. rivorum* LMG 25899^{T} (MIEK01000071) and 95.6% with *E. alcedinis* L34 (Figure. 12). Another study Mohammed et al. (2019a) reported that the strain exhibited highest sequence similarities of (100%), with *B. aryabhattai* B8W22T (EF114313), 99.86%) with *B. megaterium* NBRC 15308T and 98.8% with *B. flexus* NBRC 15715T. Similarly Altaee et al. (2017a) revealed 99% identity to the complete sequence of the 16S rRNA gene of *Rhodococcus equi* strain DSM20307 (accession no. NR 041910.1), 98% identity to the complete sequence of 16S rRNA of *R. opacus* strain B4 (accession no. NR074632.1) and 98% identity to a partial sequence of 16S rRNA of *R.wratslaviensis* strain NCIMB 13082 (accession no. NR 026524.1). The fourth closest one was 97% identity to the complete sequence of 16S rRNA *Nocardia farcinica* IFM 10152 strain (accession no. NR074702.1).

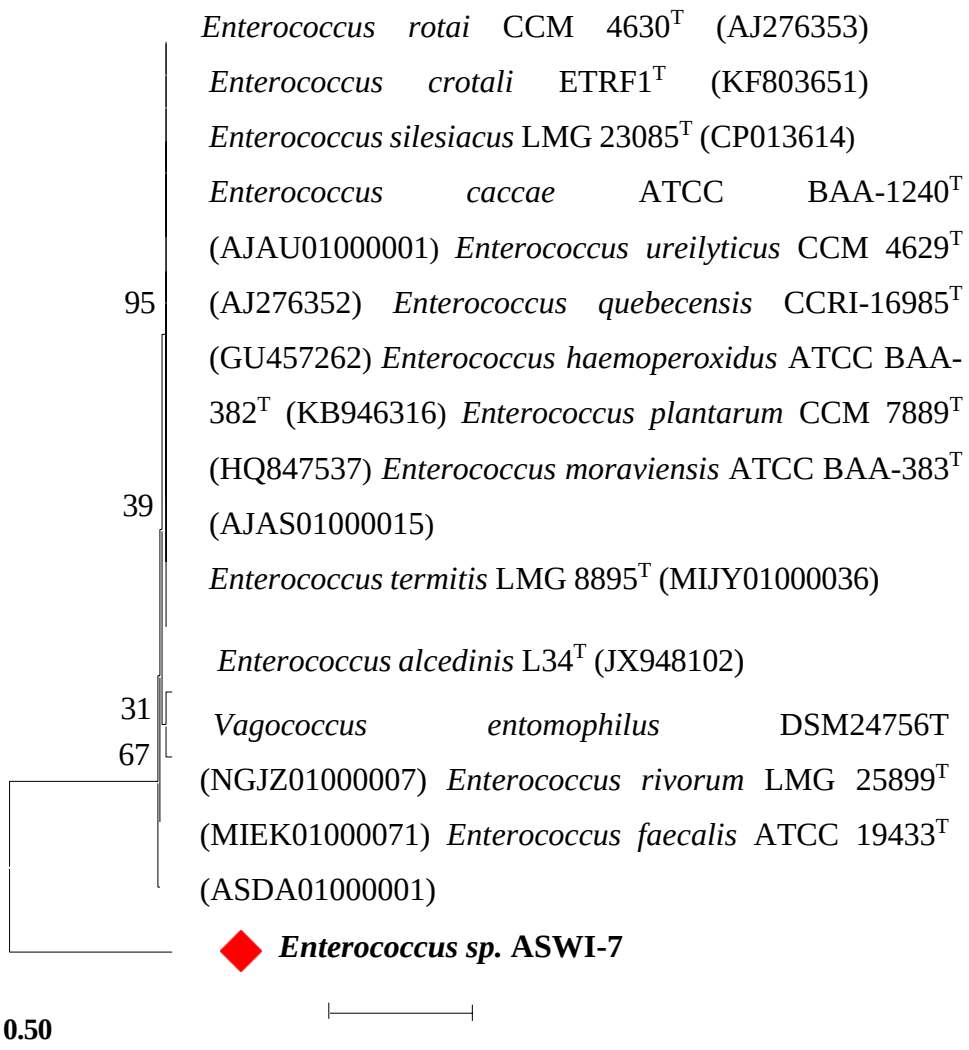


Figure 12: Evolutionary analysis by Maximum Likelihood method. The strain exhibited the highest sequence similarities of (97.3%), with *E. quebecensis* CCRI- 16985T (GU457262), (96.7%) with *E. rivorum* LMG 25899^{T} (MIEK01000071), and96.51% with *E. alcedinis* L34. The evolutionary history was inferred by using the Maximum Likelihood method and Tamura-Nei model (Tamura & Nei, 1993). The tree with the highest log likelihood (-4730.79) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Tamura-Nei model and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. This analysis involved 15 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. There were a total of 1519 positions in the final dataset. Evolutionary analyses were conducted in MEGA11 (Tamura et al., 2021a)

The evolutionary distances were computed using the p-distance method Thomas (2001), and are in the units of the number of base differences per site. The analysis involved 15 nucleotide sequences. Codon positions included were 1st + 2nd + 3rd + Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 1406 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Kumar et al., 2007). In these results, no outgroup was reported in the analyses.

Table 4: Sequence similarity between *Enterococcus* sp. ASWI-7 (our isolate) and other strains with their respective GC% and accession number

S.N.	Organism	Strain	Accession	GC%	Pairwise Similarity (%)
1	*Enterococcus sp.*	ASWI-7	Not deposited		The recent
2	*E. faecalis*	ATCC 19433^{T}	ASDA01000001	38.7	97.3
3	*E. quebecensis*	CCRI-16985^{T}	GU457262	52.9	96.7
4	*E. rivorum*	LMG 25899^{T}	MIEK01000071	51.8	96.51
5	*E. alcedinis*	L34^{T}	JX948102	52.2	95.63
6	*E. haemoperoxid us*	ATC C BAA-382^{T}	KB946316	35.8	95.52
7	*E. rotai*	CCM 4630^{T}	AJ276353	53.7	95.44
8	*E. crotali*	ETRF1^{T}	KF803651	53.7	95.44
9	*E. moraviensis*	ATCC BAA-383^{T}	AJAS01000015	36.1	95.37
10	*E. silesiacus*	LMG 23085^{T}	CP013614	36.4	95.37
11	*E. ureilyticus*	CCM 4629^{T}	AJ276352	53.7	95.37
12	*E. plantarum*	CCM 7889^{T}	HQ847537	53.3	95.37
13	*E. caccae*	ATCC BAA-1240 ^{T}	AJAU01000001	35.3	95.301
14	*E. termitis*	LMG 8895^{T}	MIJY01000036	52.8	95.30
15	*E. wangshanyuan ii*	MN05^{T}	CP021874	37.5	95.30
16	*E. ureasiticus*	DSM 23328^{T}	MIJZ01000009	51.3	95.23
17	*E. larvae*	BWM-S5^{T}	MW338870	53.8	94.73
18	*E. hirae*	ATCC 9790^{T}	CP003504	36.9	94.092

19	*E. canis*	NBRC 100695^{T}	BCQJ01000024	50.9	94.09
20	*E. durans*	NBRC 100479 ^{T}	BCQB01000108	51.8	94.02

4.7. Methods of PHA recovery

PHA-producing bacterial isolates were collected from Adama City solid waste dumping site and *Bacillus sp. ASWI-19, Enterobacter sp*. ASWI-21, *Enterococcus sp. ASWI-7,* and were screened for PHA-producing bacteria. The extraction was performed for *Enterococcus sp.* ASWI-7, because no more information was reported on the extraction and characterization of the cocci-shaped bacteria. This result showed that the highest PHA content was obtained by the NaCl-$CHCl_3$ method (53.82±2.17%), followed by the NaCl- NaOCl method (47.92±3.125%), and $CHCl_3$-NaOCl method (11.458±1.042%) as illustrated in (Figure 13). These values were consistent with the literature data on PHA recovery methods. The NaCl-$CHCl_3$ method was also the most efficient regarding PHA purity, cell disruption, and solvent recovery. Therefore, it is suggested that the NaCl-$CHCl_3$ method is a suitable technique for PHA extraction from bacterial cells.

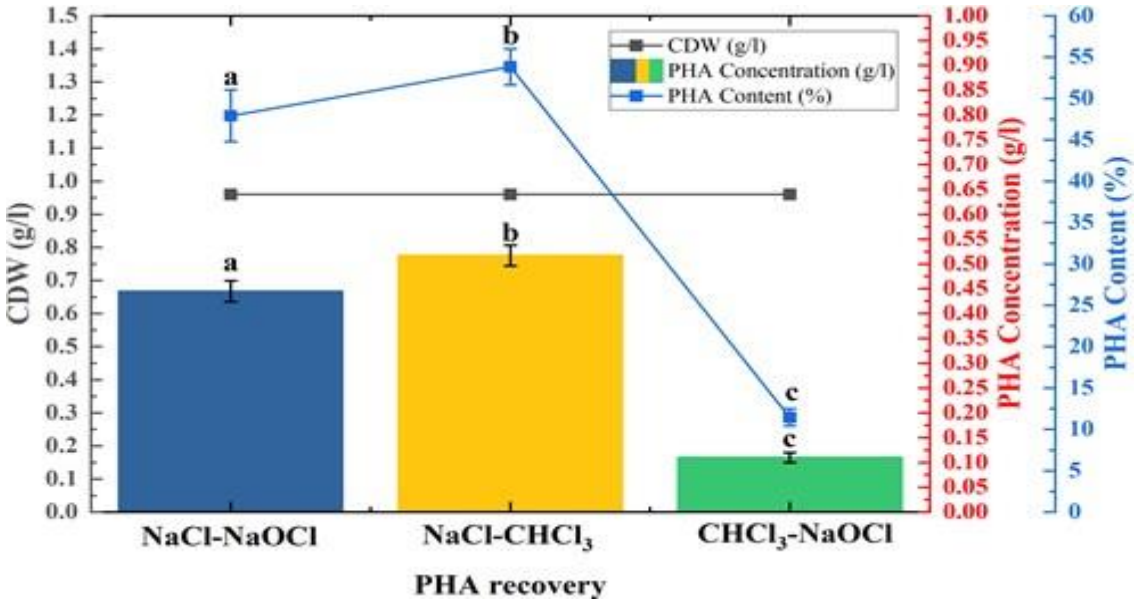


Figure 13: Methods of PHA recovery NaCl-$CHCl_3$, NaCl-NaOCl, and $CHCl_3$- NaOCl using *Enterococcus sp.* ASWI-7 from Adama city solid waste dumping site from 1% (w/v) of glucose as carbon source.

4.8. Optimization PHA PHA-producing bacteria for different parameters

4.8.1. Effect of carbon source on PHA biosynthesis

In this study, it was compared that the PHA biosynthesis from four different carbon sources: bagasse, glucose, molasses, and sucrose against *Enterococcus sp. ASWI-7*, which is an SBB and NBA-positive isolate. The cell dry weight (CDW), PHA concentration (P. conc.), and PHA content (%) were obtained for the recent solate, *Enterococcus sp. ASWI-7*, after 72 h of the incubation period. The results showed that glucose was the most suitable carbon source for PHA biosynthesis against *Enterococcus sp. ASWI-7*, followed by molasses, sucrose, and bagasse. The minimum CDW was detected for Bagasse (0.56 g/L) with 0.067 g/L P. conc. and PHA content 14.3±1.5%, whereas the maximum CDW was obtained from glucose (0.9567±0.025 g/L) with 0.41±0.02 g/L P. conc. and PHA content 42.6±1% This result was similar to the previous report Mohammed & Ray (2022) in which 37.00±1.67% PC was obtained MDA had been utilized in the presence of a medium suitable carbon source against *Bacillus* sp. LPPI-18 strain. Molasses yielded a CDW of 0.77±0.02 g/l, with PHA conc. 0.11±0.01 g/l, and PHA content of 14.30067±14.30067%, and sucrose resulted in a CDW of 0.7±0.0153 g/l, with PHA conc. 0.0967±0.035 g/l, and PHA content 13.149±4.825209%. `

The statistical analysis revealed that there was a significant difference in PHA content between glucose and bagasse (*p=0.000*) but not a significant variation between Bagasse and molasses (*p=1.000*). The results showed that both glucose & molasses and glucose & sucrose resulted in a p-value of 0.00, indicating that there was a significant difference in the PHA biosynthesis between the two carbon sources (Figure 13). . The study has shown that no significant variation (*p=0.618*) was observed between sucrose and molasses regarding PHA content. In terms of PHA concentration, there was no significant difference between Bagasse and molasses (*p=0.061*), Bagasse and sucrose (*p=0.169*), and molasses and sucrose (*p=0.521*) (Figure 13, Additional File: Table A_{15}).

This finding suggests that glucose is the most suitable carbon source for PHA production by *Enterococcus sp. ASWI-7* and bagasse, molasses, and sucrose can also be used as alternative substrates with lower efficiency. These results suggest that glucose is the most suitable carbon source for PHA biosynthesis for *Enterococcus sp. ASWI-7* isolate, which is a PHA-

producing isolate. However, bagasse is the most minor carbon source of which PHA granules were detected when it had been supplied as a sole carbon source. In the present study, bagasse is found to be a cheap agricultural carbon source employed to produce PHA polymer with less amount of PHA contents. Similarly, Huang *et al*. (2006) obtained a 140 g/L cell concentration with 77.8 g/l PHA concentration and 55.6% PHA content using extruded rice bran (ERB) and extruded cornstarch (ECS) (1:8 g/ g) as a carbon source against *H. Mediterranean*, archaeal bacteria.

This study suggested that utilization of these cheap agro-wastes, such as bagasse and sugar cane molasses, for PHA biosynthesis, is cost-effective and increases marketing potential and extensive coverage once a potential strain has been employed at the commercial level. A similar finding proposed by, Wang *et al*. (2013) indicated that, industrial-scale production of PHA has been restricted due to production costs being elevated. Thus, sugar cane molasses gave (14.301±1.496) g/l dry biomass at 72 h, which is a higher biomass reported when compared with bagasse. In this study, the maximum PC accumulation occurred from 60 to 72 h for these isolates.

All the bacterial species do not have the same ability to utilize sucrose as a carbon source. Some are utilized very efficiently, but some cannot use and produce PHA. In this study, *Enterococcus sp. ASWI-7* could not produce PHA polymers when sucrose (1% w/v) was supplied as a main carbon source. Its ability to utilize diverse carbon sources may be limited. It could be suggested that sucrose is a double sugar and, hence it is difficult for an isolate to break down its monomers. The identical isolates may also lack invertase enzymes that can degrade sucrose, which is supplied as a sole carbon source. It has also been observed that sucrose utilization can be used as an identification technique.

4.8.2. Effect of incubation time on PHA biosynthesis

The results showed that *Enterococcus sp. ASWI- 7* had the highest PHA content (PC) of 42.59 ± 1.35% at 72 h of incubation. However, further increasing the incubation period to 96h resulted in a decrease in PC of *Enterococcus sp. ASWI-7* to 32.02 ± 0.36% (Figure 13) . Statistical analysis revealed that PC was significantly affected by time across all time interval levels (p= *0.000*), indicating a temporal influence on PC. However, the comparison between 48 and 96 h showed no significant difference (*p=0.440*), implying that the material effect on PC was not linear. Furthermore, CDW and PHA conc. had significant variations across all time points (24, 48, 72, and 96 h) with (*p=0.000*) demonstrating a solid influence of time on

CDW and PHA conc. (Figure 13, Additional File: Table A_{17}).This suggests an optimal incubation time for maximizing PHA production by the bacterial isolates from *Enterococcus sp. ASWI-7*. Thereafter, production starts decreasing with time while biomass remains relatively high until 72 h. These results were comparable to (Chaudhry *et al*., 2011). The study demonstrates the potential of using waste-derived bacteria as sources of PHAs for bioplastic applications. These isolates may use this PHA intracellular granule as a carbon.

4.8.3. Effect of pH PHA biosynthesis

The results showed that the highest PHA content was obtained at pH 7 (50.145±2.3%), followed by pH 8 (32.457 ± 1.988%), pH 9 (25.6789± 1.2%), and pH 11 (19.3±2.4 %) (figure 14). This indicates that *Enterococcus sp. ASWI-7* prefers a neutral to slightly alkaline environment for PHA production and PHA synthesis is inhibited at high pH levels. The ANOVA test showed that the PHA content of the cells grown at pH 7 and pH 8 did not differ significantly ($p = 0.229$), which means $p > 0.05$. However, the PC between pH 7 and pH 9 ($p = 0.000$) and between pH 7 and pH 11 ($p = 0.000$) differed significantly, which means $p < 0.05$. The CDW and PHA concentrations of all pH values (7, 8, 9, and 11) also differed significantly with $p<0.05$. High pH may tend to inactivate enzyme activities during these bacterial growths and polymerization (Mohammed *et al*., 2019b). (Figure 14 Additional File: Table A16).

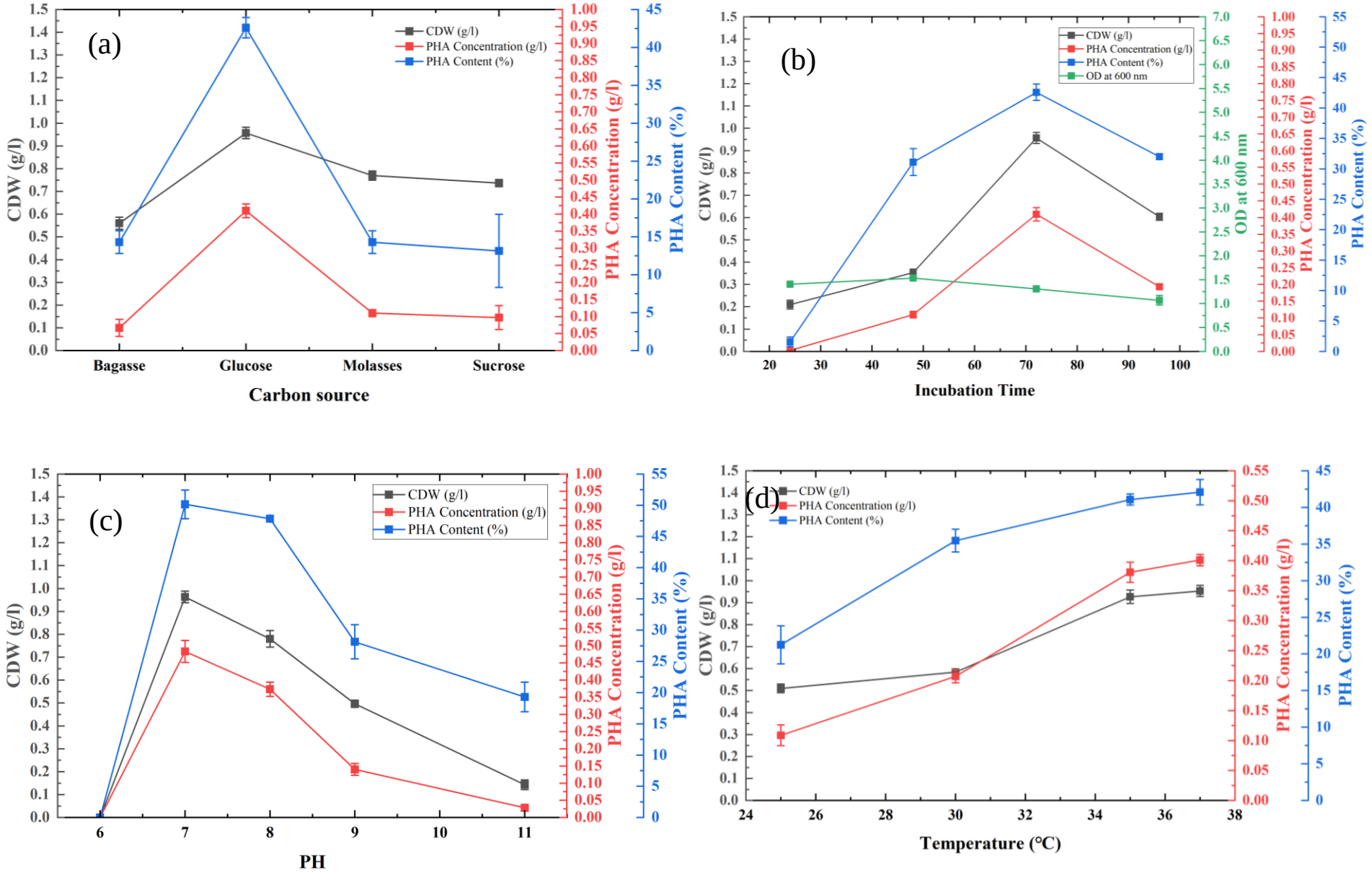
4.8.4. Effect of salt concentration against PHA

The growth of the different salt concentrations of sodium chloride in Minimal Davis broth was observed (Figure 14e). The optimum salt concentration for the growth of *Enterococcus sp. ASWI-7* in the MDB was indicated by the result to be 1.282M at the optimum growth temperature. In the present study, up to a 1M concentration of sodium chloride was grown on by the isolate *Enterococcus sp. ASWI-7*. In a flask containing a concentrated solution of sodium chloride, less prolific growth was observed than in a flask of MDB with less concentrated salt 0.5M. The isolate *Enterococcus sp. ASWI-7* can grow in a high salt concentration of sodium chloride was observed from this study. Biomass production for *Enterococcus sp. ASWI-7* isolates were also evaluated against the salt concentrations (Figure 14 Additional File: Table A_4). Statistical analysis revealed that it is not significantly different at concentrations 0.1M and 0.5M ($p=0.347$), but all other concentrations are significantly different at ($p=0.00$) (Figure 13, Additional File: Table A_4).

4.8.5. Effect of temperatures on PHA biosynthesis

The current results showed that *Enterococcus sp. ASWI-7* can produce PHA at these tested temperatures, but the PHA content (PC) and composition significantly varied. The lowest PC (21.24±2.59%) was recorded at 25 °C, while the highest PC (42.08±1.732%) was obtained at 37 °C. The PC against 30 and 35 °C were 35.467±1.55% and 41.069±0.756%, respectively. More PHA productions have been recorded for both bacterial species over a range of 25-37 °C a t pH 7. PHA was obtained at 25-37 °C even though the maximum PC have been detected at 35– 37 °C. The ANOVA test revealed that in terms of CDW, the temperature had a significant effect on the CDW of the cells, as the cells grown at 25 °C had a lower CDW than the cells grown at 30 °C ($p = 0.005$). However, the temperature did not have a significant effect on the PHA content of the cells, as there was no statistical difference between the PHA content of the cells grown at 35 and 37 °C ($p = 0.201$) (Figure 14 Additional File: Table A_7).

An increase in temperature might have been affected the PHA biosynthesis since the enzyme used for the polymerization of PHA granules inactivated. Singh *et al*. (2013) reported that the maximum PHB concentration (5.201 g/L) was obtained at 40 °C after 72 h of incubation period. In line with this study, it has been reported that as the temperature (40 °C) increases the PHB biosynthesis decreases due to the denaturation of PHA polymers (Mohammed *et al*., 2019b)


(a)
CDW (g/l)
PHA Concentration (g/l)
PHA Content (%)
Bagasse
Glucose
Molasses
Sucrose
Carbon source
(b)
OD at 600 nm
Incubation Time
(c)
PH
(d)
Temperature (°C)

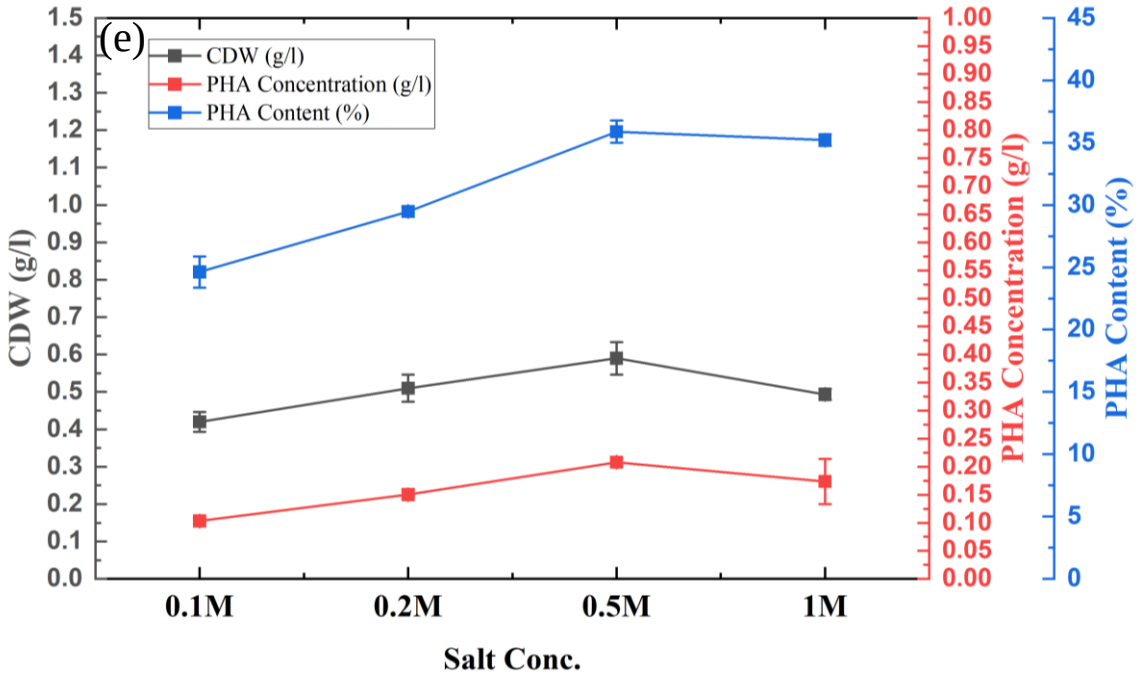


Figure 14: (a) PHA biosynthesis from four different carbon sources: bagasse, glucose, molasses, and sucrose against *Enterococcus sp. ASWI-7* which is an SBB and NBA-positive isolate. (b) Optimization of *Enterococcus sp. ASWI-7* isolate for the different incubation times (24h, 48h, 72h, and 96h). (c) Effect of Temperature on CDW, P. conc. and PC when 1% glucose was supplemented as a carbon source for *Enterococcus sp. ASWI-7*. Data represented mean ± SD of three replicates (n = 3) of Bioplastic content. (d) for the isolate *Enterococcus sp. ASWI-7* over a range of 7–11 pH value when 1% glucose was supplemented as a carbon source. Data represented mean ± SD of three replicates (n = 3) of Bioplastic contents. (e) The effect of salt concentration against PHA-producing bacteria.

4.9. Growth kinetics

The growth kinetics of a PHA-producing bacterium, was obtained from the Adama solid waste dumping site, was displayed as indicated in (Figure -15) based on the viable count, biomass, and optical density of the culture at different time intervals. The results showed that there was no growth observed from 0 to 5 h, indicating a lag phase where the bacteria were adapting to the new environment. After 5 h, the culture entered an exponential growth phase, where all the parameters increased rapidly until 44 h. This phase was characterized by a high metabolic activity and cell division rate. After 44 h, the culture reached a stationary phase, where the growth rate was balanced by the death rate. Our result agrees with (Altaee *et al*., 2017). The results show that OD increases with time, peaking at about 38 h. Starting from 40 h the cells enter the stationary phase before beginning to decline and remaining markedly high even after 50 h. The viable count, biomass, and optical density remained constant until 54 h, suggesting that the bacteria had reached their carrying capacity and were limited by nutrients or oxygen. As indicated in Figure 15 after 54 h, the culture entered a decline phase, where the death rate exceeded the growth rate. The viable count, biomass, and optical density decreased gradually, indicating that the bacteria were dying or becoming inactive due to unfavorable conditions. Our result agrees with the finding described by Monod (1949).

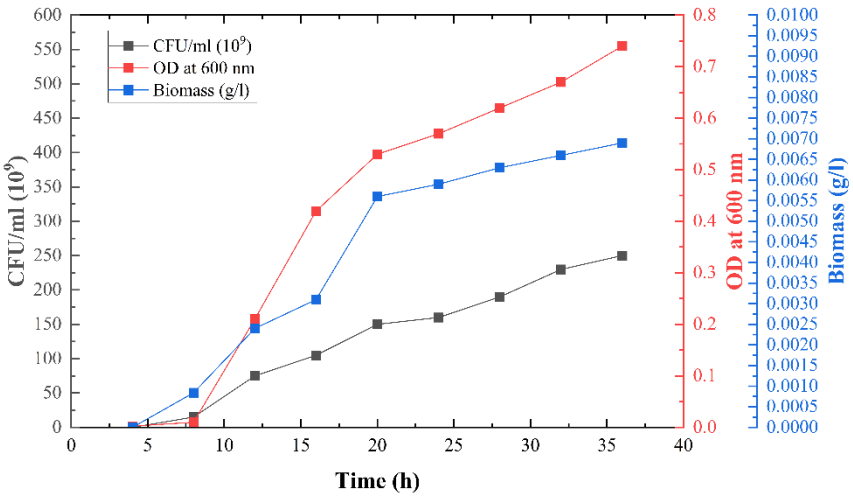


Figure 15: Growth kinetics for PHA-producing *Enterococcus sp. ASWI-7* bacteria by using 1% of glucose as a carbon source.

4.10. Characterization of PHA compounds

4.10.1. Fourier transform infrared spectrum (FTIR) Analysis

As illustrated in Figure 16, the absorption peak or band of the fingerprinting part ranged 1638.55, indicating the presence of C=O in the recent PHA polymer derived from the PHA-producing isolate, *Enterococcus sp. ASWI-7*. A similar absorption peak was predicted for the polymer derived from *Bacillus* sp. BPPI-14 and *Bacillus* sp. BPPI- 19 was indicated in Mohammed *et al*. (2019). In this study, the functional group (1743.54 cm^{-1}) for the recent PHA polymer was predicted. This is an intense peak at 1743.54 cm^{-1} wave number, which indicates the presence of the carbonyl stretching, a typical ester group (Figure 16). In line with this study, Kovalcik *et al*. (2017) estimated the occurrence of the carbonyl group (1722 cm^{-1}) against *Synechocystis* salina PHA polymer after the FTIR had been carried out. The same authors predicted the occurrence of aliphatic ester group vibrations (C-O-C) at the intense peaks of 1280, 1227, and 1181 cm^{-1}.

The methyl and methylene groups were also predicted for the recent PHA polymer at intense peaks between (Figure-16) 2922.21 and 3292.14 cm^{-1} against ASWI-7, PHA- producing isolate. In agreement with this study, the occurrence of methyl and methylene groups was estimated in the range of 3000-2800 cm^{-1} against PHA polymer, which is derived from *S. salina* isolate (Kovalcik *et al*., 2017). The presence of absorption bands between 1743.54 and 1038.14 cm^{-1} could show the availability of polymer and copolymers like Polyhydroxybutyrate (PHB) and polyhydroxybutyrate-co-hydroxyvalerate-P(HB-co- HV), which are a similar report to (Shamala *et al*., 2009) against PHB and HV-copolymer with 1723 & 1281 and 1738, 1134, 1215 cm^{-1}, respectively. As illustrated in Figure 16, the absorption band was detected near the 1743.54 cm^{-1} intense band, which is inconsistence with the previous finding Randriamanhefa *et al*. (2003), a characteristic feature of Polyhydroxyalkanoate (PHO) and identified as ester band (1742 cm^{-1}). These collective intense peaks at various points indicated the presence of PHA polymers and other copolymers. The PHA could be related to PHB, PHH, PHO, and other PHA.

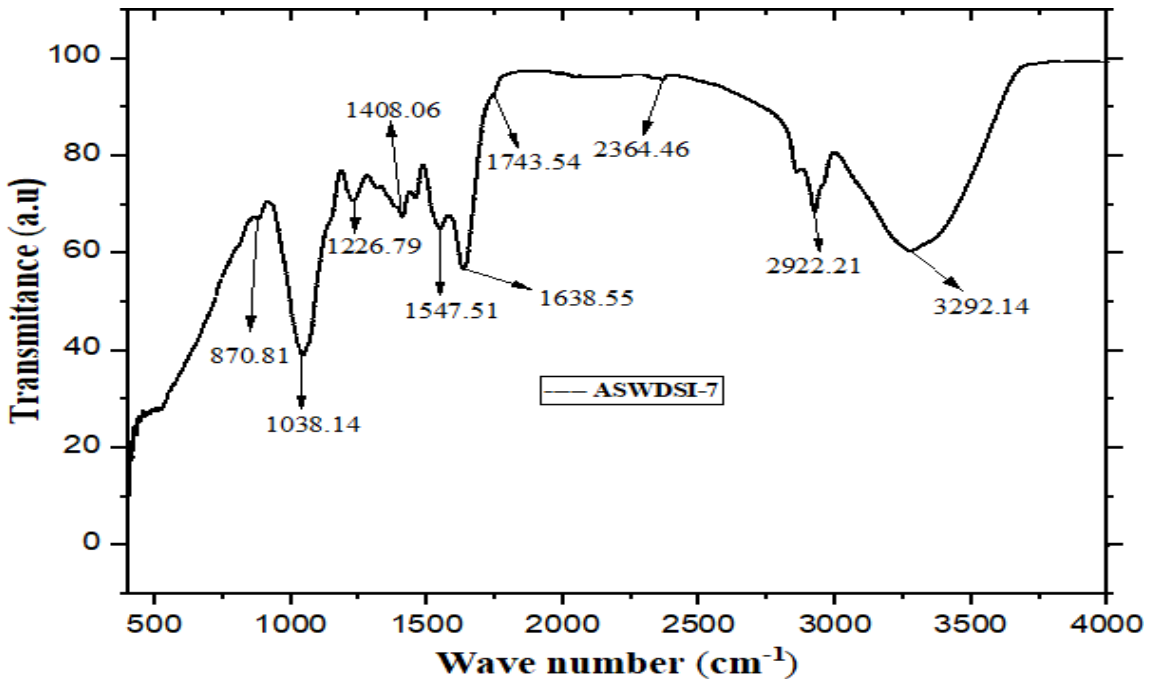


Figure 16: Fourier transform infrared spectrum (FTIR) of the *Enterococcus sp. ASWI-7* polymer that obtained from soil samples.

4.10.2. GC-MS analysis

The thermal degradations of PHAs are employed to produce specific monomers of PHAs, which belong to hydrocarbon, ester, methyl groups, and organic acid. Less percentage (2.56%) of eicosane P793, Methyl 16-acetylhydroxypalmitate, 11,14-Octadecadienoic acid, methyl ester, 13-Octadecenoic acid, methyl ester, heptadecanoic acid, 16-methyl-, methyl ester, tricosane P1059, Butyl 9-tetradecenoate, and others (Table 5) detected from the recent PHA-producing isolate after the thermal degradation of PHA polymers had been performed. These compounds belonged to hydrocarbon and ester groups following the pyrolysis products at 280 °C. In line with this study, it was shown that diethyl phthalate, eicosane, and hexadecane were reported as the possible intermediates of the biodegradation of polyethylene (Mohammadi *et al*., 2022). The maximum percentage of PHA polymers detected after thermal degradation had been performed was Benzene, 1, 2-dimethyl (7.69%). A similar study demonstrated that Benzene, 1-(1, 1-dimethyl ethyl)-3, 5-dimet (0.36%, at 8.18 min) and Ethyl cyclopropane carboxylate (49.508% at 4.974 min) reported against *Bacillus cereus* BNPI-92 (Mohammed *et al*., 2020). The thermal degradations of PHBs are predominantly including such as propene, 2- butenoic acid, propenyl-2-butenoate, and butyric-2-butenoate. The same authors reported that propene, 2-butenoic acid, 2-pentanoic

acid, propenyl-2-butenoate, propenyl-2- pentanoate, butyric-2-butenoate, and pentanoic-2-pentanoate were detected after the thermal degradation of poly (HB–HV) (Kato *et al*., 2018).

In this study, other monomers such as octadecane P630 (5.13%), 11,14-octadecadienoic acid, methyl ester (2.15%), cyclo hexadecane P489 (5.13%), 13-octadecenoic acid, methyl ester (2.56%), heptadecanoic acid, 16-methyl-, methyl ester (2.56%), Methyl 13-methyltetradecanoate (2.56%) and Tetra decanoic acid, 12-methyl, methyl ester (2.56%) were detected. These monomers were less concentrated among the detected profile of compounds after the GC-MS analysis had been carried out against PHA polymers (Table 5 Additional File: Figure 17). These compounds are mostly methyl ester groups after thermal degradations have been done. Following the study, the monomer composition of the PHA polymer such as 36 mol% of 3-hydroxy octanoate (3-HO), 34 mol % of 3-hydroxy decanoate (3-HD), 13 mol % of 3-hydroxydodecanoate (3-HDD); 15mol% of 3- hydroxy- tetra decanoate (3-HTD) and 2 mol % of 3-hydroxyhexadecanoate (3-HHD) following GC-MS analysis against *mcl* PHA (Aoyagi *et al*., 2002) that obtained from *P. putida*. Following thermal degradation (Py–GC/MS analysis) at 280 ^{o}C, only e-caprolactone was detected from poly (R)-3-hydroxybutyrate (Kathiraser *et al*., 2007).

Table 5: Compound profiles using GC-MS analysis after thermal degradation of PHA polymer derived from *Enterococcus sp. ASWI-7* isolate that grows on glucose as a sole carbon source.

S.N.	Name of compounds	MF	CAS Registry Number	RT (min)	Base Peak	Content (%)
1	Hydrazinecarboxylic acid, phenylmethyl ester	$C_8H_{10}N_2O_2$	5331-43-1	3.31	91	5.26
2	1-Decanol	$C_{10}H_{22}O$	112-30-1	3.642	85.1	5.13
3	Benzene, 1,2-dimethyl-	C_8H_{10}	95-47-6	4.443	91	7.69
4	Dodecanal	$C_{12}H_{24}O$	112-54-9	5.342	105	2.56
5	1-Dodecene	$C_{12}H_{24}$	112-41-4	5.416	57	2.56

6	1,3,5-Trimethylbenzene or 1,2,3-Trimethylbenzene	C_9H_{12}	108-67-8	5.902	105	2.56
7	Butanedioic acid, dimethyl ester	$C_6H_{10}O_4$	106-65-0	6.932	115	2.56
8	1,4-Dimethylbicyclo [2.2.0]hexane, dideutero-	$C_8H_{14}D_2$	997019-64-5	7.253	99	2.56
9	Phenol, 2,4-bis(1,1-dimethylethyl)-	$C_{14}H_{22}O$	96-76-4	13.541	191.1	5.13
10	Tridecanoic acid, 12-methyl-, methyl ester	$C_{15}H_{30}O_2$	5129-58-8	16.059	74	2.56
11	Myristic acid P503	$C_{14}H_{28}O_2$	544-63-8	16.397	57	2.56
12	Octadecane P630	$C_{18}H_{38}$	593-45-3	16.488	57	5.13
13	Cyclohexadecane P489	$C_{16}H_{32}$	295-17-0	16.54	57	5.13
14	Methyl 13-methyltetradecanoate	$C_{16}H_{32}O_2$	5129-59-9	16.746	74	2.56
15	Tetradecanoic acid, 12-methyl-, methyl ester	$C_{16}H_{32}O_2$	5129-66-8	16.837	74	2.56
16	Phthalic acid, butyl tetradecyl ester	$C_{26}H_{42}O_4$	997863-90-9	17.661	149	2.56
17	Methyl 11-hexadecenoate	$C_{17}H_{32}O_2$	997448-32-7	17.965	55	2.56

18	Palmitic acid ME P721	$C_{17}H_{34}O_2$	112-39-0	18.159	74	2.56
19	Cyclotetradecane P391	$C_{14}H_{28}$	295-17-0	18.811	57	5.13
20	Methyl 16-acetylhydroxypalmitate	$C_{19}H_{36}O_4$	0-00-0	18.874	57.1	2.56
21	11,14-Octadecadienoic acid, methyl ester	$C_{19}H_{34}O_2$	56554-61-1	19.807	67	2.56
22	13-Octadecenoic acid, methyl ester	$C_{19}H_{36}O_2$	56554-47-3	19.859	55	2.56
23	Heptadecanoic acid, 16-methyl-, methyl ester	$C_{19}H_{38}O_2$	5129-61-3	20.076	74	2.56
24	Stearyl alcohol P721	$C_{18}H_{38}O$	112-92-5	20.568	57.1	5.13
25	Butyl 9-tetradecenoate	$C_{18}H_{34}O_2$	997498-24-2	21.575	57.1	2.56
26	1,1,3,6-tetramethyl-2-(3,6,10,13,14-pentamethyl-3-ethyl-pentadecyl) cyclohexane	$C_{32}H_{64}$	997904-10-3	22.056	207	5.13
27	Tricosane P1059	$C_{23}H_{48}$	638-67-5	23.755	57.1	2.56
28	Hentriacontane	$C_{31}H_{64}$	630-04-6	24.059	57.1	2.56
29	Eicosane P793	$C_{20}H_{42}$	112-95-8	24.762	207	2.56

The total presence of compounds such as 1,3,5-trimethylbenzene or 1,2,3-trimethylbenzene, Phenol, 2,4-bis(1,1-dimethylethyl), myristic acid P503, hentriacontane, stearyl alcohol P721 and others identified by GC-MScould be due to the impurity of PHA polymers from the given of GC-MS spectral data (Additional File: Figure - 17) at various RT (Table 5). Similarly, it was estimated that the contaminant peak was detected at about 13 min after the GC analysis had been conducted against PHA polymers, which were recovered by enzymatic digestion treatments. Previous study indicates that Myristic acid is used as the primary substrate for the biosynthesis of PHA with various monomers such as C6, C8, C10, C12, and C14, in which C8 is the predominant component (Tan *et al*., 2022). However, (Li et al., 2003) reported that butyric acid, decanoic acid, heptanoic acid, myristic acid, octanoic acid,

palmitic acid, octanoic acid, stearic acid, and others are employed as the main carbon source for biosynthesis of 3-hydroxybutyric acid (3HB), 3- hydroxyhexanoic acid (3HC), 3-hydroxyheptanoic acid (3HH) and 3-hydroxyvaleric acid (3HV), against *Chromobacterium violaceum, Klebsiella aerogenes* (pCV8, pUMS) and *R. eutropha* PHB-4(pCV8) bacterial species. In this study, Butane dioic acid, tridecanoic acid, Octadecanoic acid, heptadecanoic acid, and octadecadienoic acid were detected following thermal degradation using GC-MS analysis. This could indicate that the recent isolate may produce polymers such as PHB, polyhydroxy-octadecenoic acid (Kim *et al*., 2000), poly (β-hydroxy octanoate) (PHO) (Tamura *et al*., 2021b), Polyhydroxy- 3-hydroxyvalerate (PHV) (Tilbrook *et al*., 2014) and other monomer composition (mol%) of PHAs such as Dodecanoic, Eicosanoid, Octadecanoic and Octanoic (Ballistreri *et al*., 2001).

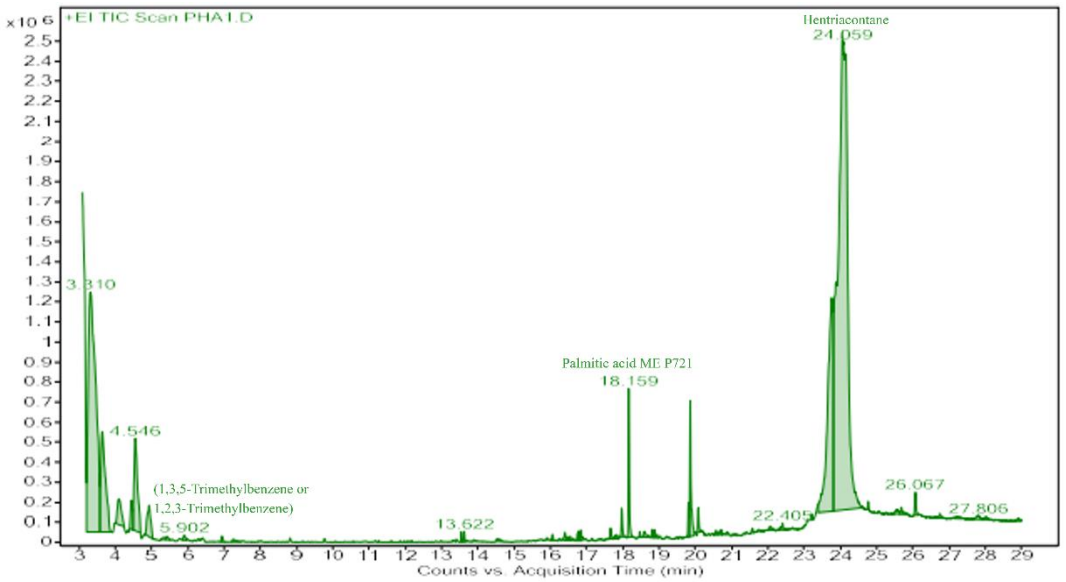


Figure 17: Gas chromatogram of PHA. The major compound of PHA polymers detected after thermal degradation ~~had been performed~~ was Benzene, 1, 2-dimethyl (7.69%).

4.10.3. X-ray Diffraction Crystallography (X-RD)

To analyze the structure of the PHA extracted from ASWI-7 through the method (Mohammed et al., 2020), XRD was employed. The powder XRD pattern of the sample was recorded on an X-ray diffractometer at the range of 2θ from 10 to 80 with a scan rate of 20/ min using Cu Kα (λ = 1.5406 A0) radiation with an accelerating voltage of 40 KV. Different phases present in the samples were evaluated (Saqib *et al*., 2018). The average particle size was calculated using Debye Scherrer's formula.

$$D = \frac{0.94}{\beta cos\theta} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots$$

Where, D is the Crystallite size, □ = 1.54 A0 is the X– ray source wavelength, β is the Full Width at Half Maximum (FWHM) of a peak, and □ is the peak position (Arokiyaraj *et al*., 2014). The X-ray diffraction pattern of PHA gives details about the purity, crystallinity, and morphology sample. The powder XRD pattern of PHA has shown peak values of 2θ = 19.80o, and 44 o corresponding to (1 1 0), and (222) crystal plane of the PHA polymer. The standard PHB profile X-ray diffraction shows prominent peaks at 2θ = 13.4°, 17.15°, 20.1°, 22.7°, 25.5°and 30.3° which correspond to (020), (110), (101), (111), (121) and (002) position by (Salgaonkar & Bragança, 2015) which strongly agreed with our finding for glucose polymer derived from *Enterococcus sp. ASWI-7*.

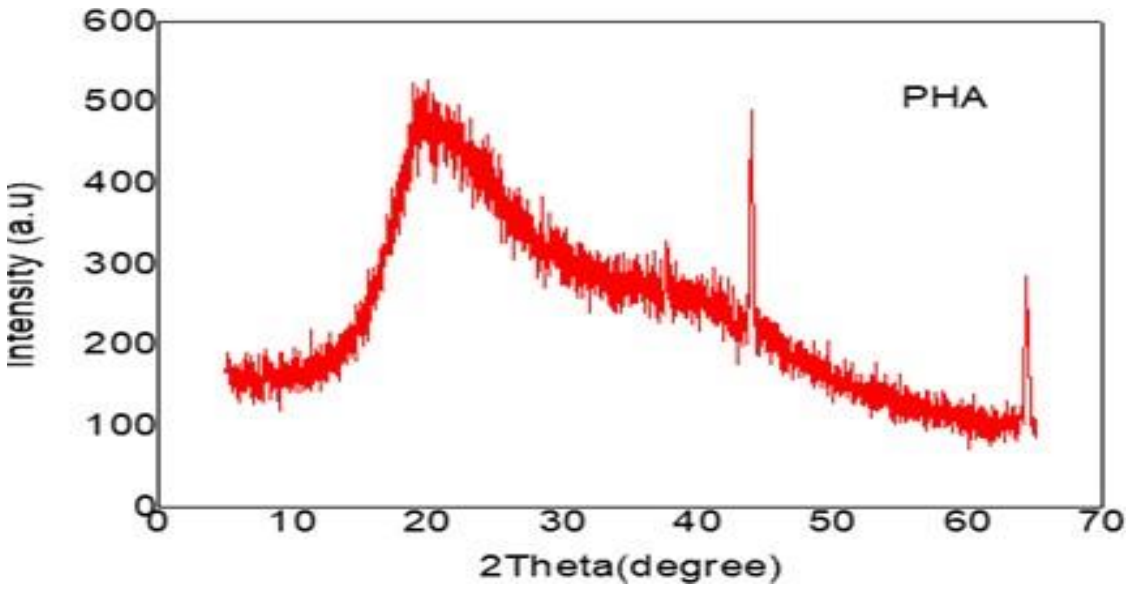


Figure 18: XRD analysis of PHA compounds

Table 6: Average crystallite size PHA polymer was calculated using the above equation. XRD structural information of PHA polymeric material was presented in the following

Peak	2θ(degree)	θ(degree)	FWHM (degree)	FWHM (radian)	Crystal size (nm)
1	19.80	9.90	13.3	0.232	0.63
2	44	22	0.38	0.0066	23.66
Average crystal size					**12.15**

5. Conclusion

Generally, the recent study was about the extraction and characterization of PHA from bacteria isolated from soil samples of the Adama city solid waste dumping Site soil sediments. The new potential bacterial species isolated from waste dumping site of Adama City were belonged to *Bacillus sp. ASWI-19, Enterobacter sp. ASWI-21, Enterococcus sp. ASWI-7, and Pseudomonas sp. ASWI-17.* Biochemical tests, MALDI–TOF MS, and 16S rRNA were performed for the *Enterococcus sp. ASWI-7 Enterococcus* sp. ASWI-7 was able to produce PHA when s provided with 1% carbon source such as bagasse, glucose, molasses, and sucrose. In this study, more PHA content was obtained from glucose, and less content was obtained from bagasse and no PHA content was obtained in carbon source free medium. . pH of 7 and temperature of 37 ◦C for PHA biosynthesis grown on MDB were found as the optimal conditions for isolates in degrading polyethylene. PHA characterizations were performed by FTIR, XRD, and GC/MS to identify the composition, degree of crystallinity, and functional group of the compound. A This study showed that waste dumping sites could be a potential source of polyethylene-degrading and producing bacterial isolates.

6. Recommendation

Based on the findings of the research the following recommendations are made:

- The potential candidate of the PHA-producing cocci-shaped bacteria *Enterococcus species* was not well studied before so, further investigation on the isolation and extraction of PHA compound from *Enterococcus species* is necessary.
- From Adama city solid waste dumping site cocci-shaped bacteria *Enterococcus sp. ASWI-7* was isolated and the PHA compound was extracted.
- The Adama city solid waste dumping site should be explored more in hopes of finding novel sources of bioplastics-producing microorganisms. Therefore, Further study should be carried out on the isolation and characterization of bioplastic-producing bacteria from the Adama city solid waste dumping site.

7. References

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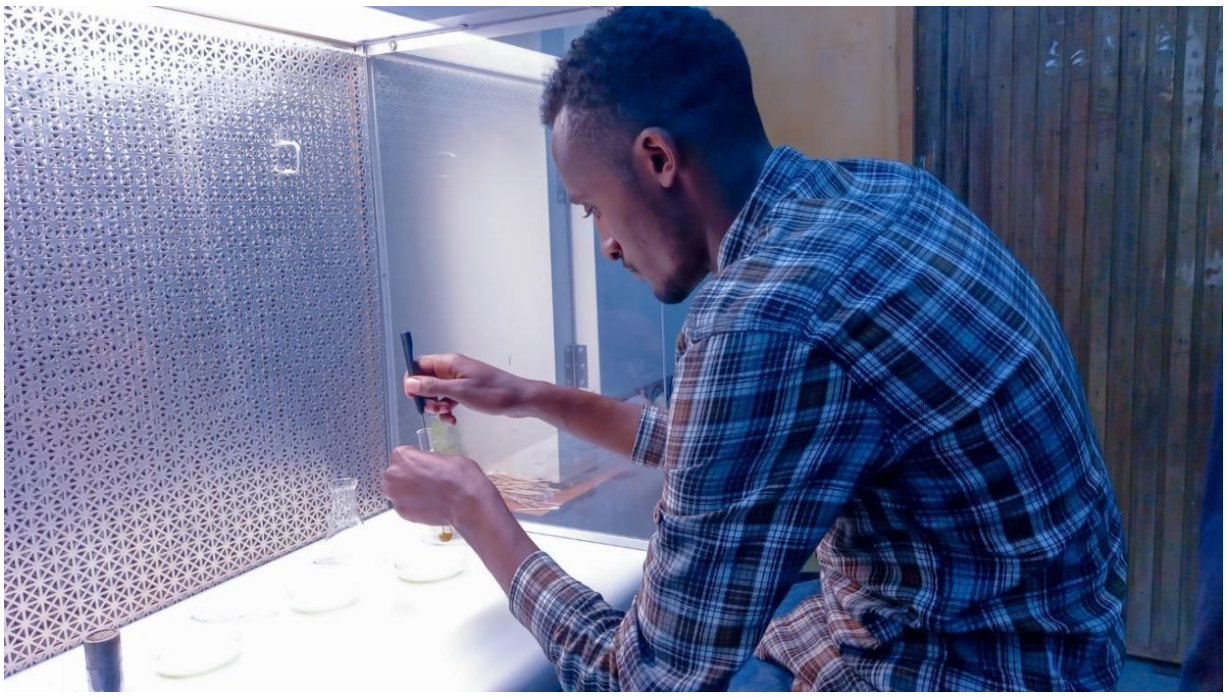
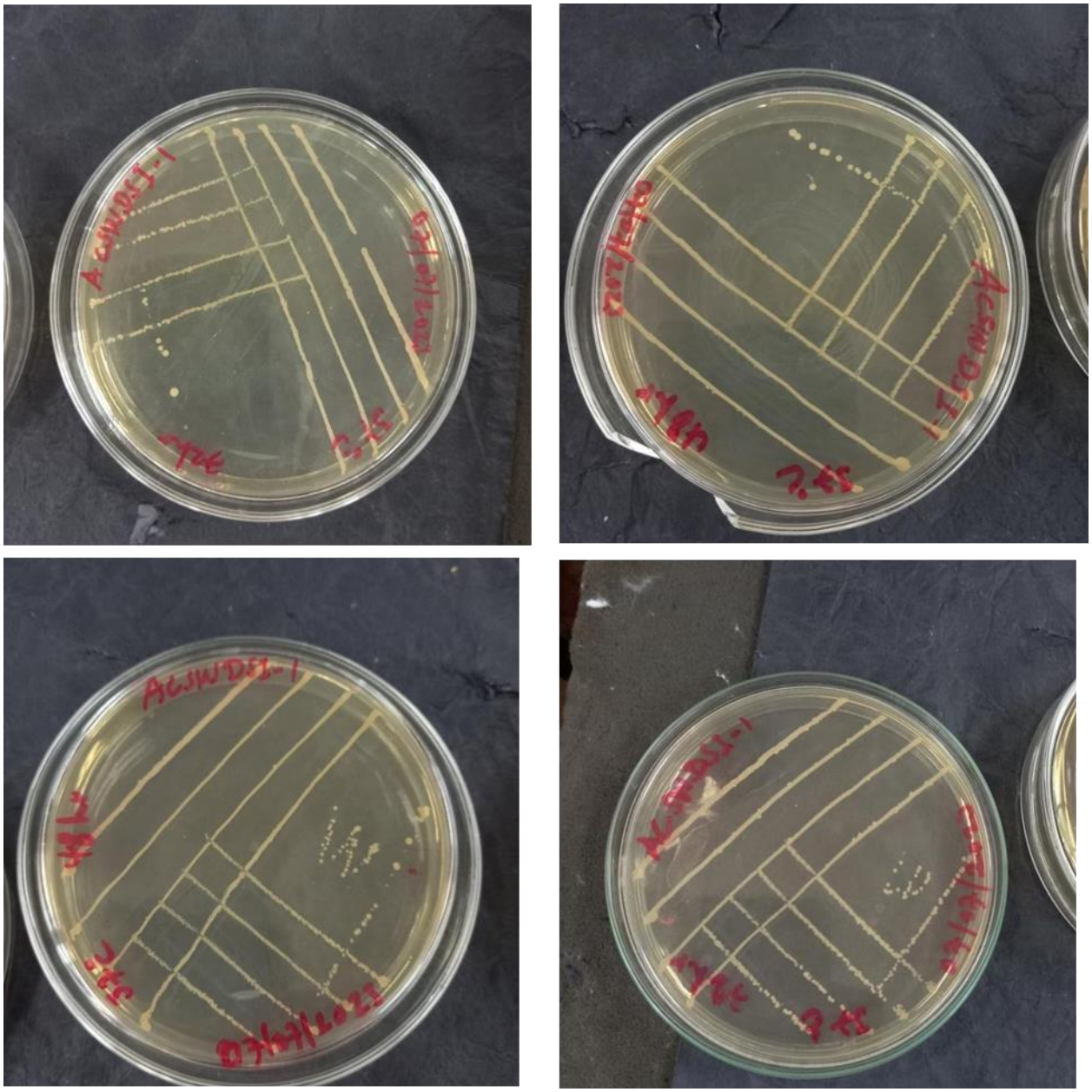
8. Appendices

Appendix 1: sampling area: Adama city solid waste dumping site

Appendix 2: Streaking PHA-producing bacteria in applied biology laboratory.



Appendix 3: Pure culture of bacterial isolated from Adama city solid waste damping



Appendix 4: Screening of PHA producing bacteria using SBB and NBA at Oromia regional laboratory

Appendix 5: Measuring UV Spectrometer for the optimization of PHA Producing Bacteria

Appendix 6: Statistical analysis

Table A_1: Mean, SD, minimum and maximum against CDW and various salt conc. Salinity

CDW against salinity								
Conc.	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
0.1 M	3	.420000	.0264575	.0152753	.354276	.485724	.3900	.4400
0.2 M	3	.510000	.0360555	.0208167	.420433	.599567	.4700	.5400
0.5 M	3	.590000	.0435890	.0251661	.481719	.698281	.5600	.6400
1 M	3	.493333	.0152753	.0088192	.455388	.531279	.4800	.5100
Total	12	.503333	.0687992	.0198606	.459620	.547046	.3900	.6400

Table A_2: Multiple Comparisons and significant variation among CDW and salt concentration for the dependent Variable: CDW against salinity LSD

(I) conc.	**(J) conc.**	**Mean Difference (I-J)**	**Std. Error**	**Sig.**	**95% Confidence Interval**	
					Lower Bound	Upper Bound
0.1 M	0.2 M	-.0900000*	.0262467	.009	-.150525	-.029475
	0.5 M	-.1700000*	.0262467	.000	-.230525	-.109475
	1 M	-.0733333*	.0262467	.023	-.133858	-.012808
0.2 M	0.1 M	.0900000*	.0262467	.009	.029475	.150525
	0.5 M	-.0800000*	.0262467	.016	-.140525	-.019475
	1 M	.0166667	.0262467	.543	-.043858	.077192
0.5 M	0.1 M	.1700000*	.0262467	.000	.109475	.230525
	0.2 M	.0800000*	.0262467	.016	.019475	.140525
	1 M	.0966667*	.0262467	.006	.036142	.157192
1 M	0.1 M	.0733333*	.0262467	.023	.012808	.133858
	0.2 M	-.0166667	.0262467	.543	-.077192	.043858

	0.5 M	-.0966667*	.0262467	.006	-.157192	-.036142

*. The mean difference is significant at the 0.05 level

Table A_3: Mean, SD, minimum and maximum for PHA contents against various salinity concentration

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
0.1 M	3	24.623333	1.2483723	.7207481	21.522205	27.724462	23.2300	25.6400
0.2 M	3	29.469667	.4136911	.2388447	28.442001	30.497332	29.0000	29.7800
0.5 M	3	35.878667	.8924670	.5152661	33.661656	38.095678	35.0800	36.8420
1 M	3	35.202667	.4720978	.2725658	34.029911	36.375423	34.6930	35.6250
Total	12	31.293583	4.8417350	1.3976885	28.217292	34.369875	23.2300	36.8420

Table A_4: Multiple Comparisons with dependent Variable: PHA contents against salinity LSD

(I) conc.	(J) conc.	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
0.1 M	0.2 M	-4.8463333*	.6768736	.000	-6.407207	-3.285460
	0.5 M	-11.2553333*	.6768736	.000	-12.816207	-9.694460
	1 M	-10.5793333*	.6768736	.000	-12.140207	-9.018460
0.2 M	0.1 M	4.8463333*	.6768736	.000	3.285460	6.407207
	0.5 M	-6.4090000*	.6768736	.000	-7.969873	-4.848127
	1 M	-5.7330000*	.6768736	.000	-7.293873	-4.172127
0.5 M	0.1 M	11.2553333*	.6768736	.000	9.694460	12.816207
	0.2 M	6.4090000*	.6768736	.000	4.848127	7.969873
	1 M	.6760000	.6768736	.347	-.884873	2.236873
1 M	0.1 M	10.5793333*	.6768736	.000	9.018460	12.140207
	0.2 M	5.7330000*	.6768736	.000	4.172127	7.293873

0.5 M	-.6760000	.6768736	.347	-2.236873	.884873

*. The mean difference is significant at the 0.05 level.

Table A_5: Mean, SD, minimum and maximum for PHA concentration against various salinity concentration

Descriptive

PHA conecentration against salinity

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
0.1 M	3	.103333	.0057735	.0033333	.088991	.117676	.1000	.1100
0.2 M	3	.150333	.0100167	.0057831	.125451	.175216	.1400	.1600
0.5 M	3	.208000	.0072111	.0041633	.190087	.225913	.2000	.2140
1 M	3	.173667	.0055076	.0031798	.159985	.187348	.1700	.1800
Total	12	.158833	.0402285	.0116130	.133273	.184393	.1000	.2140

ANOVA

PHA concentration against salinity

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.017	3	.006	107.220	.000
Within Groups	.000	8	.000		
Total	.018	11			

Post Hoc Tests

Multiple Comparisons

Table A_6: Dependent Variable: PHA concentration against salinity at LSD

(I) conc.	(J) conc.	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
0.1 M	0.2 M	-.0470000*	.0060000	.000	-.060836	-.033164
	0.5 M	-.1046667*	.0060000	.000	-.118503	-.090831
	1 M	-.0703333*	.0060000	.000	-.084169	-.056497
0.2 M	0.1 M	.0470000*	.0060000	.000	.033164	.060836
	0.5 M	-.0576667*	.0060000	.000	-.071503	-.043831
	1 M	-.0233333*	.0060000	.005	-.037169	-.009497
0.5 M	0.1 M	.1046667*	.0060000	.000	.090831	.118503
	0.2 M	.0576667*	.0060000	.000	.043831	.071503
	1 M	.0343333*	.0060000	.000	.020497	.048169
1 M	0.1 M	.0703333*	.0060000	.000	.056497	.084169
	0.2 M	.0233333*	.0060000	.005	.009497	.037169
	0.5 M	-.0343333*	.0060000	.000	-.048169	-.020497

*. The mean difference is significant at the 0.05 level.

Table A_7: Multiple Comparisons and significant variation among various Temperatures

Multiple Comparisons						
Dependent Variable: CDW(g/L) against various Temperature						
LSD						
(I) Value	(J) Value	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
25 ºC	30 ºC	-.07333*	.01915	.005	-.1175	-.0292
	35 ºC	-.41667*	.01915	.000	-.4608	-.3725
	37 ºC	-.44333*	.01915	.000	-.4875	-.3992
30 ºC	25 ºC	.07333*	.01915	.005	.0292	.1175
	35 ºC	-.34333*	.01915	.000	-.3875	-.2992
	37 ºC	-.37000*	.01915	.000	-.4142	-.3258
35 ºC	25 ºC	.41667*	.01915	.000	.3725	.4608
	30 ºC	.34333*	.01915	.000	.2992	.3875
	37 ºC	-.02667	.01915	.201	-.0708	.0175
37 ºC	25 ºC	.44333*	.01915	.000	.3992	.4875
	30 ºC	.37000*	.01915	.000	.3258	.4142
	35 ºC	.02667	.01915	.201	-.0175	.0708

***. The mean difference is significant at the 0.05 level.**

Table A_8: Mean, SD, minimum and maximum for CDW (g/l), PHA conc. (g/l) and PHA contents

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
PHA contents	24 h	3	1.572333	.7794526	.4500172	-.363934	3.508601	1.0400	2.4670
	28 h	3	31.116667	2.205840	1.273542	25.637055	36.596279	28.5700	32.4300
CDW (g/l)	72 h	3	42.590167	1.345117	.7766038	39.248710	45.931623	41.1935	43.8770
	96 h	3	32.020000	.3649658	.2107131	31.113375	32.926625	31.6000	32.2600
	Total	12	26.824792	15.982273	4.6136851	16.670139	36.979444	1.0400	43.8770
	24 h	3	.210000	.0200000	.0115470	.160317	.259683	.1900	.2300
	28 h	3	.353333	.0152753	.0088192	.315388	.391279	.3400	.3700
	72 h	3	.956667	.0251661	.0145297	.894151	1.019183	.9300	.9800
	96 h	3	.603333	.0152753	.0088192	.565388	.641279	.5900	.6200
	Total	12	.530833	.2963554	.0855504	.342538	.719129	.1900	.9800
	24 h	3	.003300	.0016462	.0009504	-.000789	.007389	.0023	.0052
	28 h	3	.110000	.0100000	.0057735	.085159	.134841	.1000	.1200
	72 h	3	.410000	.0200000	.0115470	.360317	.459683	.3900	.4300
PHA conc. (g/l)	96 h	3	.193333	.0057735	.0033333	.178991	.207676	.1900	.2000
	Total	12	.179158	.1562823	.0451148	.079861	.278455	.0023	.4300

Table A_9: Multiple Comparisons at LSD for CDW (g/l), PHA conc. (g/l) and PHA contents

Dependent Variable	(I) CODING	(J) CODING	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
PHA contents	24 h	28 h	-29.5443333*	1.1117419	.000	-32.108015	-26.980652
		72 h	-41.0178333*	1.1117419	.000	-43.581515	-38.454152
		96 h	-30.4476667*	1.1117419	.000	-33.011348	-27.883985
	28 h	24 h	29.5443333*	1.1117419	.000	26.980652	32.108015
		72 h	-11.4735000*	1.1117419	.000	-14.037181	-8.909819
		96 h	-.9033333	1.1117419	.440	-3.467015	1.660348
	72 h	24 h	41.0178333*	1.1117419	.000	38.454152	43.581515
		28 h	11.4735000*	1.1117419	.000	8.909819	14.037181
		96 h	10.5701667*	1.1117419	.000	8.006485	13.133848
	96 h	24 h	30.4476667*	1.1117419	.000	27.883985	33.011348
		28 h	.9033333	1.1117419	.440	-1.660348	3.467015
		72 h	-10.5701667*	1.1117419	.000	-13.133848	-8.006485
CDW (g/l)	24 h	28 h	-.1433333*	.0158114	.000	-.179794	-.106872
		72 h	-.7466667*	.0158114	.000	-.783128	-.710206
		96 h	-.3933333*	.0158114	.000	-.429794	-.356872
	28 h	24 h	.1433333*	.0158114	.000	.106872	.179794
		72 h	-.6033333*	.0158114	.000	-.639794	-.566872
		96 h	-.2500000*	.0158114	.000	-.286461	-.213539
	72 h	24 h	.7466667*	.0158114	.000	.710206	.783128
		48 h	.6033333*	.0158114	.000	.566872	.639794
		96 h	.3533333*	.0158114	.000	.316872	.389794
	96 h	24 h	.3933333*	.0158114	.000	.356872	.429794
		28 h	.2500000*	.0158114	.000	.213539	.286461
		72 h	-.3533333*	.0158114	.000	-.389794	-.316872
PHA conc. (g/l)	24 h	28 h	-.1067000*	.0094520	.000	-.128496	-.084904
		72 h	-.4067000*	.0094520	.000	-.428496	-.384904
		96 h	-.1900333*	.0094520	.000	-.211830	-.168237
	28 h	24 h	.1067000*	.0094520	.000	.084904	.128496
		72 h	-.3000000*	.0094520	.000	-.321796	-.278204
		96 h	-.0833333*	.0094520	.000	-.105130	-.061537

72 h	24 h	.4067000*	.0094520	.000	.384904	.428496
	28 h	.3000000*	.0094520	.000	.278204	.321796
	96 h	.2166667*	.0094520	.000	.194870	.238463
96 h	24 h	.1900333*	.0094520	.000	.168237	.211830
	28 h	.0833333*	.0094520	.000	.061537	.105130
	72 h	-.2166667*	.0094520	.000	-.238463	-.194870

***. The mean difference is significant at the 0.05 level.**

Table A_{10}: Mean, SD, minimum and maximum values against various carbon sources

Carbon source	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
Bagasses	3	.5600	.02646	.01528	.4943	.6257	.53	.58
Glucose	3	.9567	.02517	.01453	.8942	1.0192	.93	.98
Molasses	3	.7700	.02000	.01155	.7203	.8197	.75	.79
Sucrose	3	.7367	.01528	.00882	.6987	.7746	.72	.75
Total	12	.7558	.14823	.04279	.6617	.8500	.53	.98

Table A_{11}: Multiple Comparisons at LSD against various carbon sources with PHA CDW g/L) Dependent Variable:

(I) Value	(J) Value	Std. Error	Sig.	95% Confidence Interval	
				Lower Bound	Upper Bound
Bagasse	Glucose	.01810	.000	-.4384	-.3549
	Molasses	.01810	.000	-.2517	-.1683
	Sucrose	.01810	.000	-.2184	-.1349
Glucose	Bagasse	.01810	.000	.3549	.4384
	Molasses	.01810	.000	.1449	.2284
	Sucrose	.01810	.000	.1783	.2617
Molasses	Bagasse	.01810	.000	.1683	.2517
	Glucose	.01810	.000	-.2284	-.1449
	Sucrose	.01810	.103	-.0084 .1349	.0751
Sucrose	Bagasse	.01810	.000		.2184
	Glucose	.01810	.000	-.2617	-.1783
	Molasses	.01810	.103	-.0751	.0084

Table A_{12}: Mean, SD, minimum and maximum values various carbon sources with PHA CDW g/L)

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
Bagasse's	3	.0667	.02517	.01453	.0042	.1292	.04	.09
Glucose	3	.4100	.02000	.01155	.3603	.4597	.39	.43
Molasses	3	.1100	.01000	.00577	.0852	.1348	.10	.12
Sucrose	3	.0967	.03512	.02028	.0094	.1839	.06	.13
Total	12	.1708	.14663	.04233	.0777	.2640	.04	.43

Table A_{13}: Multiple Comparisons at LSD against various carbon sources for PHA

concentration

Multiple Comparisons

Dependent Variable: PHA concentration

LSD

(I) Value	(J) Value	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Bagasses	Glucose	-.34333*	.01986	.000	-.3891	-.2975
	Molasses	-.04333	.01986	.061	-.0891	.0025
	Sucrose	-.03000	.01986	.169	-.0758	.0158
Glucose	Bagasses	.34333*	.01986	.000	.2975	.3891
	Molasses	.30000*	.01986	.000	.2542	.3458
	Sucurose	.31333*	.01986	.000	.2675	.3591
Molasses	Bagasses	.04333	.01986	.061	-.0025	.0891
	Glucose	-.30000*	.01986	.000	-.3458	-.2542
	Sucrose	.01333	.01986	.521	-.0325	.0591
Sucrose	Bagasses	.03000	.01986	.169	-.0158	.0758

Glucose	-.31333*	.01986	.000	-.3591	-.2675
Molasses	-.01333	.01986	.521	-.0591	.0325

***. The mean difference is significant at the 0.05 level.**

Table A_{14}: Mean, SD, minimum and maximum values against various carbon sources for PHA contents

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
Bagasses	3	14.3007	1.49573	.86356	10.5851	18.0163	12.66	15.58
Glucose	3	42.5902	1.34512	.77660	39.2487	45.9316	41.19	43.88
Molasses	3	14.3007	1.49573	.86356	10.5851	18.0163	12.66	15.58
Sucrose	3	13.1490	4.82521	2.78584	1.1625	25.1355	8.00	17.57
Total	12	21.0851	13.18282	3.80555	12.7092	29.4611	8.00	43.88

Table A_{15}: Multiple Comparisons at LSD against various carbon sources for PHA contents

Dependent Variable: PHA contents

LSD

(I) Value	(J) Value	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Bagasses	Glucose	-28.28950*	2.21985	.000	-33.4085	-23.1705
	Molasses	.00000	2.21985	1.000	-5.1190	5.1190
	Sucrose	1.15167	2.21985	.618	-3.9673	6.2706
Glucose	Bagasses	28.28950*	2.21985	.000	23.1705	33.4085
	Molasses	28.28950*	2.21985	.000	23.1705	33.4085
	Sucrose	29.44117*	2.21985	.000	24.3222	34.5601
Molasses	Bagasses	.00000	2.21985	1.000	-5.1190	5.1190
	Glucose	-28.28950*	2.21985	.000	-33.4085	-23.1705
	Sucrose	1.15167	2.21985	.618	-3.9673	6.2706
Sucrose	Bagasses	-1.15167	2.21985	.618	-6.2706	3.9673
	Glucose	-29.44117*	2.21985	.000	-34.5601	-24.3222
	Molasses	-1.15167	2.21985	.618	-6.2706	3.9673

***. The mean difference is significant at the 0.05 level.**

Table A_{16}: Multiple Comparisons between PH against PHA contents

Dependent Variable	(I) CODING	(J) CODING	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
PHA contents	24 h	28 h	-29.5443333*	1.1117419	.000	-32.108015	-26.980652
		72 h	-41.0178333*	1.1117419	.000	-43.581515	-38.454152
		96 h	-30.4476667*	1.1117419	.000	-33.011348	-27.883985
	28 h	24 h	29.5443333*	1.1117419	.000	26.980652	32.108015
		72 h	-11.4735000*	1.1117419	.000	-14.037181	-8.909819
		96 h	-.9033333	1.1117419	.440	-3.467015	1.660348
	72 h	24 h	41.0178333*	1.1117419	.000	38.454152	43.581515
		28 h	11.4735000*	1.1117419	.000	8.909819	14.037181
		96 h	10.5701667*	1.1117419	.000	8.006485	13.133848
	96 h	24 h	30.4476667*	1.1117419	.000	27.883985	33.011348
		28 h	.9033333	1.1117419	.440	-1.660348	3.467015
		72 h	-10.5701667*	1.1117419	.000	-13.133848	-8.006485
CDW (g/l)	24 h	28 h	-.1433333*	.0158114	.000	-.179794	-.106872
		72 h	-.7466667*	.0158114	.000	-.783128	-.710206
		96 h	-.3933333*	.0158114	.000	-.429794	-.356872
	28 h	24 h	.1433333*	.0158114	.000	.106872	.179794
		72 h	-.6033333*	.0158114	.000	-.639794	-.566872
		96 h	-.2500000*	.0158114	.000	-.286461	-.213539
	72 h	24 h	.7466667*	.0158114	.000	.710206	.783128
		28 h	.6033333*	.0158114	.000	.566872	.639794
		96 h	.3533333*	.0158114	.000	.316872	.389794
	96 h	24 h	.3933333*	.0158114	.000	.356872	.429794
		28 h	.2500000*	.0158114	.000	.213539	.286461
		72 h	-.3533333*	.0158114	.000	-.389794	-.316872
PHA conc. (g/l)	24 h	28 h	-.1067000*	.0094520	.000	-.128496	-.084904
		72 h	-.4067000*	.0094520	.000	-.428496	-.384904
		96 h	-.1900333*	.0094520	.000	-.211830	-.168237
	28 h	24 h	.1067000*	.0094520	.000	.084904	.128496
		72 h	-.3000000*	.0094520	.000	-.321796	-.278204

	96 h	-.0833333*	.0094520	.000	-.105130	-.061537
72 h	24 h	.4067000*	.0094520	.000	.384904	.428496
	28 h	.3000000*	.0094520	.000	.278204	.321796
	96 h	.2166667*	.0094520	.000	.194870	.238463
96 h	24 h	.1900333*	.0094520	.000	.168237	.211830
	28 h	.0833333*	.0094520	.000	.061537	.105130
	72 h	-.2166667*	.0094520	.000	-.238463	-.194870

***. The mean difference is significant at the 0.05 level.**

Table A_{17}: Multiple Comparisons between incubation time against PHA contents

Dependent Variable	(I) CODING	(J) CODING	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
PHA contents	24 h	28 h	-29.5443333*	1.1117419	.000	-32.108015	-26.980652
		72 h	-41.0178333*	1.1117419	.000	-43.581515	-38.454152
		96 h	-30.4476667*	1.1117419	.000	-33.011348	-27.883985
	28 h	24 h	29.5443333*	1.1117419	.000	26.980652	32.108015
		72 h	-11.4735000*	1.1117419	.000	-14.037181	-8.909819
		96 h	-.9033333	1.1117419	.440	-3.467015	1.660348
	72 h	24 h	41.0178333*	1.1117419	.000	38.454152	43.581515
		28 h	11.4735000*	1.1117419	.000	8.909819	14.037181
		96 h	10.5701667*	1.1117419	.000	8.006485	13.133848
	96 h	24 h	30.4476667*	1.1117419	.000	27.883985	33.011348
		28 h	.9033333	1.1117419	.440	-1.660348	3.467015
		72 h	-10.5701667*	1.1117419	.000	-13.133848	-8.006485
CDW (g/l)	24 h	28 h	-.1433333*	.0158114	.000	-.179794	-.106872
		72 h	-.7466667*	.0158114	.000	-.783128	-.710206
		96 h	-.3933333*	.0158114	.000	-.429794	-.356872
	28 h	24 h	.1433333*	.0158114	.000	.106872	.179794
		72 h	-.6033333*	.0158114	.000	-.639794	-.566872
		96 h	-.2500000*	.0158114	.000	-.286461	-.213539
	72 h	24 h	.7466667*	.0158114	.000	.710206	.783128
		28 h	.6033333*	.0158114	.000	.566872	.639794
		96 h	.3533333*	.0158114	.000	.316872	.389794
	96 h	24 h	.3933333*	.0158114	.000	.356872	.429794
		28 h	.2500000*	.0158114	.000	.213539	.286461
		72 h	-.3533333*	.0158114	.000	-.389794	-.316872
PHA conc. (g/l)	24 h	28 h	-.1067000*	.0094520	.000	-.128496	-.084904
		72 h	-.4067000*	.0094520	.000	-.428496	-.384904
		96 h	-.1900333*	.0094520	.000	-.211830	-.168237
	28 h	24 h	.1067000*	.0094520	.000	.084904	.128496
		72 h	-.3000000*	.0094520	.000	-.321796	-.278204

	96 h	-.0833333*	.0094520	.000	-.105130	-.061537
72 h	24 h	.4067000*	.0094520	.000	.384904	.428496
	28 h	.3000000*	.0094520	.000	.278204	.321796
	96 h	.2166667*	.0094520	.000	.194870	.238463
96 h	24 h	.1900333*	.0094520	.000	.168237	.211830
	28 h	.0833333*	.0094520	.000	.061537	.105130
	72 h	-.2166667*	.0094520	.000	-.238463	-.194870

***. The mean difference is significant at the 0.05 level.**

Appendix 7: MALDI-TOF MS Identification Results

Wudassie Diagnostic Center	Wudassie Advanced Medical Laboratory	Document No. WAML/ALS/F5.8-001	
		Ser. No:	Ver. No. 1.0
	Laboratory Result Report Form	Page 1 of 1	Effective date: June 01,2023

Identification Report

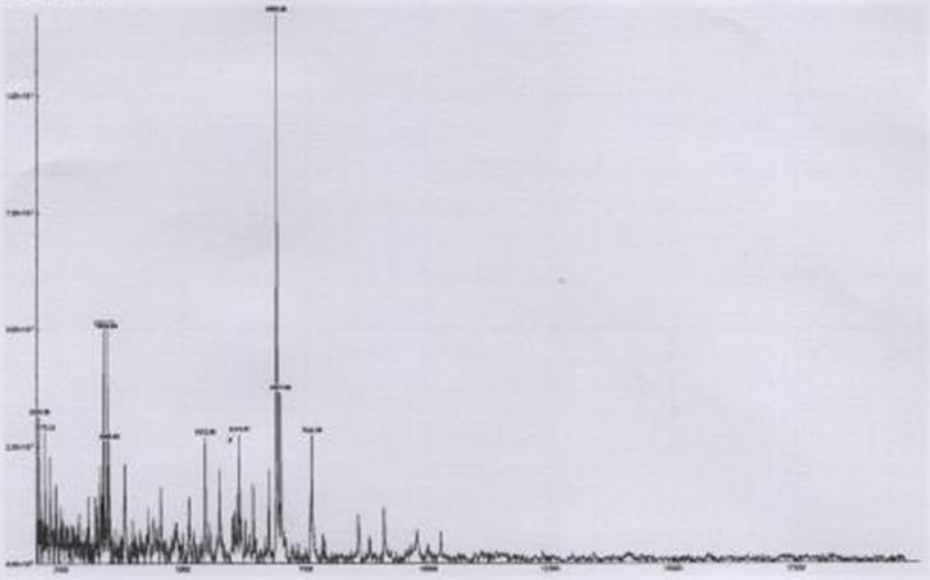
Sample Info:

Sample ID	10
Patient ID	
Spot	A2
Acquisition Time	2023-12-07 15:11:02

Identification Result:

Genus	*Pseudomonas*
Species	*fluorescens*
Score	2.27
Scoring Criteria	< 1.7 Unreliable; 1.7-2.0 Probable Genus; > 2.0 Probable Species

Spectrum :



Remarks:

Report No.:EX-Accuspec18C44335A29 Hash:74B631CAC24B85A4 Gen. Time:2023-12-07 15:14:25

Wudassie Diagnostic Center	Wudassie Advanced Medical Laboratory	Document No. WAML/ALS/F5.8-001	
		Ser. No:	Ver. No. 1.0
	Laboratory Result Report Form (Identification Report)	Page 1 of 1	Effective date: June 01,2023

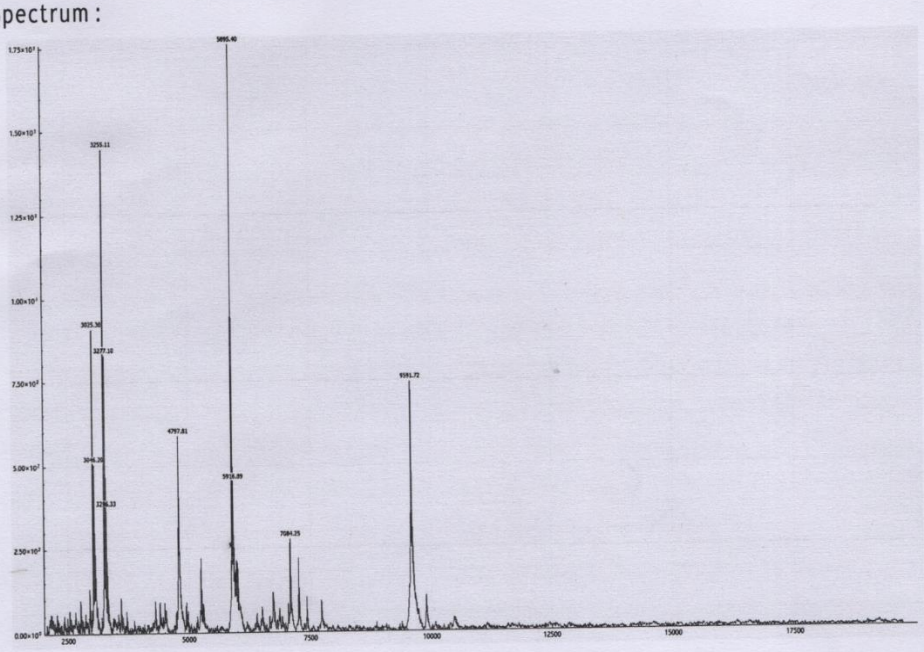
Sam ple Info:

Sam ple ID	S1
Patient ID	
Spot	A5
Acquisition Tim e	2023-12-27 09:42:37

Identification Re sult:

Genus	*Bacillus*
Species	*subtilis*
Score	2.70
Scoring Criteria	< 1.7 Unreliable; 1.7-2.0 Probable Genus; > 2.0 Probable Species

Spectrum :



Rem arks:

Wudassie Diagnostic Center	Wudassie Advanced Medical Laboratory	Document No. WAML/ALS/F5.8-001	
		Ser. No:	Ver. No. 1.0
	Laboratory Result Report Form	Page 1 of 1	Effective date: June 01,2023

Identification Report

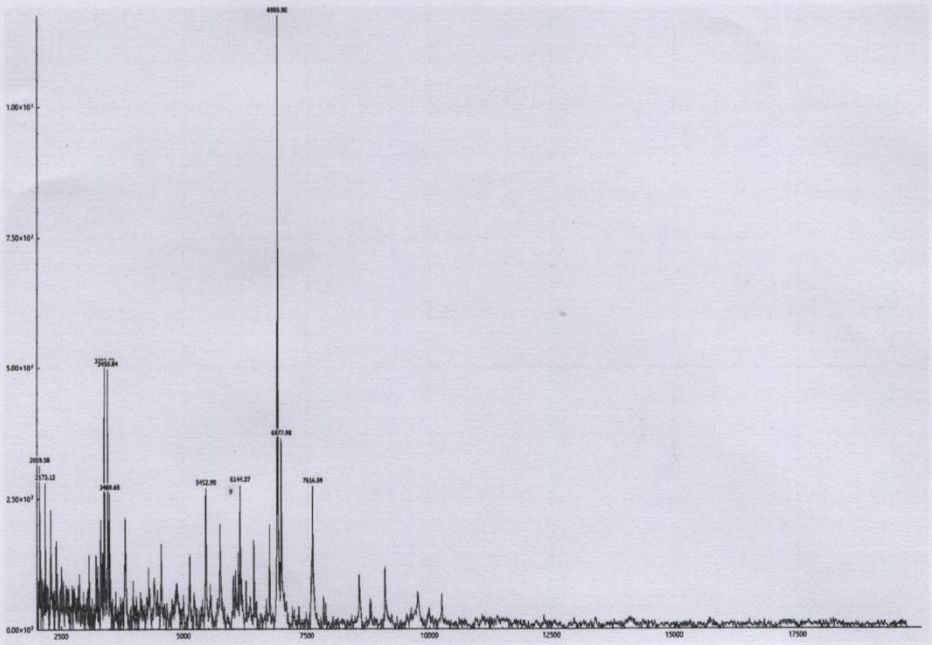
Sample Info:

Sample ID	15
Patient ID	
Spot	A7
Acquisition Time	2023-12-27 15:11:02

Identification Result:

Genus	*Enterobacter*
Species	*aerogenes*
Score	2.30
Scoring Criteria	< 1.7 Unreliable; 1.7-2.0 Probable Genus; > 2.0 Probable Species

Spectrum :



Remarks:

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