Identification and Molecular Characterization of Polyhydroxyalkanoate Producing Bacterial Isolates from Dambal Lake Soil Sediment

Beshir Feyiso Dima

A Thesis Submitted to the department of Applied Biology, School of Applied Natural Science

Presented in Partial Fulfillment of the Requirement for the Degree of Master's inApplied Biology, (Specialization in Applied Micro Biology)

Office of Graduate Studies
Adama Science and Technology University

November ,2022
Adama, Ethiopia

Identification and Molecular Characterization of Polyhydroxyalkonoate Producing Bacterial Isolates from Dambal Lake Soil Sediment

Beshir Feyiso Dima

Advisor: Seid Mohammad (PhD)
Co-advisor: Melaku Sombo (MSc)

A Thesis Submitted to the department of Applied Biology, School of Applied Natural Science

Presented in Partial Fulfillment of the Requirement for the Degree of Master's in Applied Biology, (Specialization in Applied Micro Biology)

Office of Graduate Studies
Adama Science and Technology University

November , 2022
Adama, Ethiopia

I. Declaration

I hereby declare that this Master Thesis entitled : **Identification And Molecular Characterization of Polyhydroxyalkanoate Producing Bacterial Isolates From Dambal Lake Soil Sediment** 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.

_____ _____ _____

Name of Student Signature Date

II. Recommendation

We the advisors of this thesis hereby certify that we have read the revised version of this thesis entitled- **Identification And Molecular Characterization of Polyhydroxyalkanoate Producing Bacterial Isolates From Dambal Lake Soil Sediment** prepared under our guidance by BeshirFeyisoDima submitted in partial fulfillment of the requirements for the degree of Masters of Science in Applied Micro Biology , Therefore we recommend the submission of the revised version of the thesis to the department following the applicable procedures.

Major advisor	Signature	Date
Co-advisor	Signature	Date

III. Approval Page

I/we, the advisors of the thesis entitled "**-Identification And Molecular Characterization of Polyhydroxyalkanoate Producing Bacterial Isolates From Dambal Lake Soil Sediment** " and developed by Beshir Feyiso Dima here by certify that the recommendation and suggestions made by the board of examiners are appropriately incorporated into the final version of the thesis.

Major Advisor	Signature	Date
Co-advisor	Signature	Date

We, the undersigned, members of the Board of Examiners of the thesis by Beshir Feyiso Dima have read and evaluated the thesis entitled "**Identification And Molecular Characterization of Polyhydroxyalkanoate Producing Bacterial Isolates From Dambal Lake Soil Sediment** and examined the candidate during open defense. This is, therefore, to certify that the thesis is accepted for partial fulfillment of the requirement of the degree of Master of Science in Applied Micro Biology-.

Chair person	Signature	Date
Internal Examiner	Signature	Date
External Examiner	Signature	Date

Final approval and acceptance of the thesis is contingent upon submission of its final copy to the Office of Postgraduate Studies (OPGS) through the Department Graduate Council (DGC) and School Graduate Committee (SGC).

Department Head	Signature	Date
School Dean	Signature	Date
Office of Postgraduate Studies, Dean	Signature	Date

IV. Acknowledgement

First of all I would like to thank my advisor Seid Mohammed (PhD) for suggesting this research title and introducing me to the amazing world of the Polyhydroxyalkanotesproducing bacteria. I express my deepest appreciation to my advisor for his relentless and continuous guidance and mentorship right from the start of the proposal writing, through my laboratory work till the writing of my thesis. And so I would like to gratefully acknowledge the persistent effort of Seid Mohammed (PhD).

I would also like to extend my deepest gratitude to Mr. MelakuSombo and Mr. AbebeOlani for his kind and humble reception to his laboratory and for extending a great amount of assistance in identifying my polyhydroxyalkanoetes producing bacterial isolates and introducing me to the wonderful instrument of PCR and MALDI-TOF MS respectively.

I would like to acknowledge Adama Science and Technology University and Adama city Administration for granting me the opportunity to learn my Master‘s degree.

I‘m extremely grateful to LetaGuta who has been by my side since day one of my lab work and assisting me in every way possible, you‘ve been a great lab-mate and a very good friend.

Special thanks goes to my friend for going out of their way to help me in finding samples for my research and encouraging me to keep on going and never give up. I gratefully acknowledge the assistance of Abdurrhman Negesh who helped me in preparation of sample site map. Also I would like to recognize the help I received from Mr. Mangistu Nagesso and Mr.Mohammed Guye. The completion of my thesis paper would not have been possible without the support and nurturing of my best family Demine Tura, Mariyama Feyiso and my sons Abdi Beshir. Senyi Beshir , I am and will always be be holden to you.

V. Table of Contents

VI. List of Tables

VII. **List of Figures**

VIII. Abbreviation and acronyms

BLAST:Basic Local Alignment Search Tools

CDW cell Dry weight

DLSPI- Dambal lake sediment PHA producing isolate

LCL:PHAs: Long chain length Polyhydroxyalkanoates

MALDI TOF MS:.Matrix-assisted laser desorption/ionization-time of flight mass spectrometry

MDA: Minnimal Davis Agar

MDB: Minimal Davis broth

MSA: Multiple Sequence Alignment

MCL-PHAs: Medium chain length polyhydroxyalkanoates

NCBI: National Center for Biotechnology Information

NBA: Nile Blue A

PCR: Polymerase Chain Reaction

PHA: Polyhyroxyalkonates

PHB: polyhydroxybutyrate

PHH:polyhydroxyhexanoate

P(3HB): poly(3-hydroxybutyrate)

SBB: Sudan Black B

SCL:-PHAs Short chain length Polyhydroxyalkanoates

TBE: Tris-Borate Ethylene Diamine Tetra Acetic Acid

TE:Tris-Ethylene Diamine Tetra Acetic Acid

IX. Abstract

Plastics are conventional products with molding features . These plastic wastes accumulation are used to affect both domestic animals and wild life. *Polyhydroxyalkonoate (PHA) is a natural bioplastic that can be produced by various bacterial cells during non-carbon sources are available. The present study was aimed at identification and molecular characterization of PHA producing bacterial isolates from Lake Dambel Oromia region, Ethiopia. PHA producing bacterial isolates were screened using SBB and NBAdyes. These NBA positive isolates were identified by using Matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI TOF- MS). PCR amplification was carried out for certain NBA positive isolates. Optimization for culture conditions were conducted for frequently isolated and with stronger fluorescent intensity were conducted. The 16SrRNA genes sequence for isolate with more fluorescent intensity was perfomed. Phylogentic tree was constructed after other related bacterial strain were collected from Gene Bank database using EzTaxon server. In this study, total of 40 bacterial isolate were collected. Out of 40 isolates, 32 and 26 of them were found to be positive for SBB and NBA staining respectively. These NBA positive isolates were identified and found to be Pseudomonas spp., Bacillus spp., Klebsella sp and Staphlococcus sp. The majority of the PHA granules positive isolates were Identified as Psedomonas spp. using MALDI TOF MS techniqhes. PHA gene amipilification were checked and 540bp amplicons were obatined for various species of Pseudomonas isolates and one single Bacillus sp. The present PHA producing Psedomonas sp. DLSPI-12 isolate was found to produce more PHA granules with higher CDW and OD600nm using Glucose as a carbon source at 25^{o}C and pH 7. It is possible to conclude that Pesudomonas aeuruginosa DLSPI-12 can be employed for degrading wastes in enrich with cellulosic materials, for production of bioplastics (PHA) which is biological degradable . It will take further research to address obstacles in the way of determining the productivity of bioplastic production from micro organisms .*

***Key words**: Isolates, NBA,PCR , PHA, and SBB*

1. Introduction

1.1. Background of the Study

Plastics are conventional products with molding features. It is an organic polymer used for various industrial and domestic applications in the world for the past seventy years due to their versatility and durability (Muhammadi et al., 2015). These organic polymers are industrial derived from crude oils. The global conventional plastic products are increasing at alarming state. For instance, for the last 13 years alone, the accumulated plastic production is doubled (Selvamurugan & Sivakumar, 2019). These conventional plastics are considered as an ideal material that shown resistance for microbial biodegradation. These plastic annual productions are likely to exceed 300 million tonnes by 2010. Due to this reasoning these plastics accumulations are the main source for environmental pollution (Thompson et al., 2009). These conventional plastic pollution have been reported as a pollutant source for water bodies such as lakes, rivers, seas, oceans and the terrestrial environment (Thompson et al., 2009). Hence, these plastic wastes accumulation are used to affect both domestic animals and wild life. They are doing every thing they can to fill up land fills ,which causes waste management issues for societies all over the world. However, recent accumulations of non-biodegradable plastics in avarous parts of our planet have rapidly increased from time to time. This used to enhance an interest to ward production of various biologically degradable plastics using some potential microbial candidate using certain cost effective agro-wastes (Mohammed et al., 2019).Some of Polyhyroxyalkonates producing candidates are emerging as a solution for the problems associated with conventional plastic waste accumulation (Koller, 2017).

Various PHAs polymers which are biodegradable found to be derived from plant and diversity of bacterial isolates instead of fossil fuels (Reddy et al., 2003). These biopolymers are used for different application similar to conventional plastics. Upon degradation these bio-plastic are converted into carbon dioxide and water by the act of aerobic microorganisms within a short period of time which indicates that these bio-based materials are unlikely to persist in the environment. However, when anaerobic microorganisms are involved in biodegradation, the end product has been methane instead CO_2 and water (Selvamurugan &

Sivakumar, 2019). It is most likely that the physical and chemical properties of PHA undergo biological decline and completely degraded when exposed to bacterial spcies such as *Bacillus* sp. IBP-V002, *Enterobacter cloacae* sp. IBP-V001, and *Gracili bacillus* sp. IBP-V003 (Sandoval et al., 2021). The time required to decompose these PHAs materials are completely depends on various environmental factors (Munir et al., 2015).

PHAs are biopolyesters that numerous bacteria store as carbon and energy reserves. Because of their biomass-based origin and biodegradability, PHAs have gotten a lot of attention(Verlinden et al., 2007). PHAs compounds are intracellular PHA granules formed in the presence of available carbon and others sources. PHA has been broadly clasfied as short-chain-length (scl), medium-chain-length (mcl), and long-chain length (lcl) PHA, based on the total number of carbon atoms existing in the side chains of the PHA monomer units (Yasin & Al-mayaly, 2019). PHA synthases are the most important enzyme for PHA biosyntheis. PHA syntheses is divided into four classes based on substrate specificity, amino acid sequence, and structure (Classes I, II, III, and IV) (Sehgal & Gupta, 2020) . Class I and II PHA syntheses with only one component (*PhaC*) (61–73 kDa) could create scl PHA (e.g. in *Ralstonia eutropha*) and mclPHA (e.g. in *Pseudomonas aeruginosa*) with 3-5 and 6-14 carbon atoms, respectively (Koller & Braunegg, 2015). Class III has been reported for *Allochromatium vinosum* with *Pha C* and *PhaE* genes which is *scl* PHA. However, class IV has been documented for *Bacillus* spp.with *phaC and phaR genes* (Berekaa, 2012).

Certain bacterial isolates were employed for PHA biosynthesis. It has been estimated that these bacterial species are Gram-negatives and Gram-positives which mainly belonging to *Bacillus* spp. and *Pseudomonas* spp (Ciesielski et al., 2013). It has also been reported that *R. eutropha* which is a Gram negative bacterium has been reported for sclPHA (PHB) production at a commercial level. It has been revealed that Bacterial cells such as *Bacillus spp*.(Luengo , 2003), *Clostridium spp*.(Jaber, 2019), *Corynebacterium spp*.(Chaitanya et al., 2014), *Nocardia spp*.(Muhammadi et al., 2015), *Rhodococcus spp*(Chaitanya et al., 2014). are also well known to produce PHB. The Gram-positive genera are particularly fascinating for biopoler biosynthesis. The only wild-type bacteria that natively synthesis the commercially important co-polymer P(3HB-co-3HV) from simple carbon sources are *Corynebacterium, Nocardia,* and *Rhodococcus* (Chaitanya et al., 2014).

There are now a number of approaches for identifying bacteria that accumulate PHA (Muhammadi et al., 2015). Both phenotypic and genotypic detection approaches are included. SBB staining (Muhammadi et al., 2015);(Chaitanya et al., 2014) and NBA staining (Ostle & Holt, 1982) are two phenotypic detection methods for intracellular PHA granules that are used in the screening of PHA producers and result in dark blue or fluorescent granules. Although these approaches are quite sensitive, screening a large number of environmental isolates is a time-consuming and labor-intensive task (Sathiyanarayanan et al., 2013). Alternative staining methods for directly staining colonies and cultivating bacteria on plates containing NBA (Spiekermann et al., 1999) have recently been devised, resulting in fluorescent colonies visible under UV illumination. These colony-staining techniques can be used to screen a large number of strains. However, suitable carbon sources must be used, and PHA granule growth necessitates a long culture time (3 days). Further more, these approaches are unable to distinguish between bacteria that accumulate and those that do not accumulated Biodegradable PHA polymers. Genotypic detection approaches, on the other hand, rely on the use of molecular techniques (PCR) for identification of PHA producing bacterial isolates(Shamala et al., 2003; Sheu et al., 2000) . In fact, genotypic approaches have evolved into a highly sensitive and precise instrument for detecting and amplifying the PHA syntheses gene(Solaiman et al., 2000).

1.2. Statement of the problem

Plastics are organic polymeric materials consisting of a large collection of synthetic organic compounds that are produced by polymerization of many repeating units (monomers) that come together to build copolymers (Mulu & Abraha, 2012). Plastics enjoy a number of enormously desirable characteristics: inexpensive, lightweight, strong, durable, high strength-to-weight ratio, excellent thermal properties, electrical insulation, and resistance to acids, alkalis and solvents (Thompson et al., 2009). Over the last decades, world wide production of plastics have been increased significantly due to increasing demand in developing country as in Asia, Africa and South America (Ibrahim et al., 2021). Since 1950 to 2018, The major Environmental problem is plastics that are discarded into landfills (about 40%) and accumulation of plastics in the ocean (Hussein et al., 2021).

Therefore a sustainable plastic pollution control is necessary for the protection of human, animals and environment in our country. Most plastic industries are used additive materials like bisphenol A, phthalates, poly-fluorinated, dioxins, brominated flame retardant and antimony trioxide during manufacturing and these materials have unfavorable effects on environmental and human health (Hussein et al., 2021). Moreover, little efforts were involved in some parts of the world associated to plastic waste collection, recycling and reu(Bedade et al., 2021). Recently in Ethiopia many researches were indicated that plastics brought miscellaneous effects on environment Comăniţă et al., (2016), human health Thompson et al., (2009), Fishes Misgana & Tucho,(2022), soil, cattle and national parks (Hussein et al., 2021). Now a day in our country the Government and people are more attentive about the harmful effects of petrochemical derived plastic material in the environment. So Biodegradable plastics will be convenient alternative to conventional plastics which produced from microbial polyhydroxyalkanaotes.

Despite its ecological and economic importance, both locally and globally, Lake Dambal (Ziway) has been facing alarming environmental degradation and loss of biodiversity due to the pressure of human land-use and climate change (Tibebe et al., 2022). Substantial increases in water pollution, largely from the discharge of untreated municipal and industrial waste and high sediment load from agricultural fields caused by unchecked erosion in upper catchments, are the major causes pollution (Tibebe et al., 2022). Microbial pollution of lakes and rivers severely affects their use for domestic, agricultural, and industrial activities. Rivers and lakes can easily get pollution with pathogenic microbial agents from animal feces and sewerage systems discharged such water bodies. Several microbial studies conducted in the lakes and rivers of Ethiopia clearly showed the water bodies are contaminated with pathogens of public health concern. This is because in recent times Lake Dambal (Ziway) is exposed to anthropogenic activities due to over usage of agrochemicals like fertilizers, pesticides in which organic and inorganic pollutants releases and discharge of water from domestic sources, agricultural runoff, and horticulture including floriculture activities around the lake(Tibebe et al., 2022). The current study aim was to identify and molecular characterization of PHA producing bacterial isolates from their natural ecosystems (Dambal Lake Soil Sediment).

1.3. Objective of the study

1.3.1. General objectives

- To Identify and molecular characterization of PHA producing Bacterial isolates from Lake Dambal Soil Sediment

1.3.2. Specific objectives

- To isolate and screen PHA producing bacterial isolates from Dambal lake soil sediment
- To identify and molecular characterization PHA producing bacterial isolates
- To carried out PCR amplification for analysis of PHA producing genes
- To perform 16SrRNA gene sequence for identification for potential PHA producing isolates
- To evaluate the cultural condition for potential PHA producing isolates for suitable carbon source, pH, and temperature.

1.4. Significance of the study

Bioplastic is an important innovation and it would offer sustainable and eco-friendly alternatives to avoid the plastic pollution. Further, the intensification of this research through industrial tie-ups and promotion of the large-scale production and commercialization of the bioplastic products are also inevitable to solve the plastic pollution in our environment. The bioplastics are still in its infancy, and hence a great innovation would be developed by the intensification of research through the government and industrial funding. The continuing intense fied this research in this field would facilitate further break throughs and improvements. However, bioplastics are not the only solution to the problem of plastic pollution. The changes in consumer behaviour in buying, consuming and disposing of plastics, and wide spread public awareness of the bioplastics are also essential in effecting the control of plastic pollution.This study will provide information on bacteria that produce microbial bioplastic production from soil sediment.This paper give information of polyhydroxyalkoanates produced from different bacteria. It give awerness to the society microbial bioplasics were used as conventional plastic. This research is may be use as a reference to the other studies on microbial bioplastic production from Soil Sediment Dambal Lake**.** Moreover, this paper gives a wareness to the wider community to focus on the

environmental impacts of plastic and the importance of replacing them with other alternatives such as bioplasicts. This approach may also help integrate income generation activities while solving plastic-related environmental problems.

.

2. Literature review

2.1. Plastics and its Histocial development

Plastic is a moldable organic polymer. They are made from a variety of synthetic or semi-synthetic organic solids (Anjum et al., 2016). Almost every aspect of everyday life is associated with plastic. These plastics play important roles in transportation, telecommunications, shoes and packaging. It is also used to transport a variety of foods, beverages and other commodities (Melchor-Martínez et al., 2022). These plastics are also important raw materials for extracting crude oil and natural gas, so prices have risen since traditional plastics were extracted from crude oil. Recently, there has been a significant increase in global demand for alternatives to these non-degradable petroleum-based plastics. Expansion of conventional petrochemical plastics production and consumption are having a significant impact both visibly and invisibly on the environment and society. Improper disposal of plastics has threatened natural environment world wide since long time ago (Comăniţă et al., 2016).

Conventional petrochemical plastics are recalcitrant to microbial degradation and accumulate in environment at a rate of 28 million tons per year (Muhammadi et al., 2015). In 2011, nearly 280 million tons of petrochemicals-based polymers were produced with expected increased in 4% per annum to 2016. Production of synthetic polymers is expected to increase to around 810 million tons by 2050 (Comăniţă et al., 2016). The challenging problems associated with petroleum-based plastic accumulation in the environment and rapid depletion of natural resources being used in their production are motivating factors for research into sources and tools for alternatives to petroleum based polymers (Selvamurugan & Sivakumar, 2019).

Considering current advances in biopolymers research,bacterial polyhydroxyalkanoates (PHAs) which are biodegradable thermoplastics have shown great potential as a replacement for petroleum-based plastics.Therefore, to overcome these problems, the production and applications of eco-friendly PHAs become inevitable . Bio-based plastics are growing rapidly in recent years due to rising oil prices and the potential for depletion of oil supplies in the coming decades (Muhammadi et al., 2015).

2.2. Type Of Conventional Plastics And Their Economic Role

All plastics can be divided into two major groups based on how they respond to heat: thermosetting and thermoplastic (Plastics Europe & Conversio Market & Strategy GmbH, 2019). Plastics that can be heated, melted, and molded into the desired shape before cooling are known as thermoplastics. When heated, the synthetic thermoplastic softens and remelts. Typical thermoplastics include polyacrylates, polyesters, polyolefins, polyamides, etc. These polymer materials are also used to make packaging, disposable utensils, carpets, lab equipment, clothing, and other products (Selvamurugan& Sivakumar, 2019). In contrast to thermoplastics, thermosetting plastics are permanently hardened by curing soft liquid or solid resins. Catalysts can speed up curing, which is brought on by heat or radiation (hardener). External application of heat is not required because the reaction between the hardener and resin usually generates heat. Curing occurs in chemical reactions that cross-link polymer chains, resulting in a structure that is flammable and insoluble. Cross-linked thermosetting polymers don't melt or soften when heated; they keep their shape. Common examples of thermosetting resins include liquid polyesters used in fiber glass products and melamine-formaldehyde resins used in Formica-based kitchen worktops (Comăniţă et al., 2016).

Plastics are a wide family of polymer composites which mostly contain polymers. The class of synthetic polymers includes polyethylene (PE), that is used to make plastic bags, polystyrene (PS), that is used to make foam cups, polypropylene (PP), that is used to make fiber and bottles, polyvinyl chloride (PVC), and poly tetra fluoro ethylene (PET), which is used to make drain pipes, bottles, and food packaging (Ibrahim et al , 2021). Natural materials that have been changed or combined with other materials can also be used to produce semi-synthetic polymers by introducing them to a chemical process. Cellulose acetate, a product of the reaction between cellulose and acetic anhydride used to create films, is an illustration of this (Sandoval et al., 2021). Carbon and hydrogen are the essential components in polymers. The plastics may also include other elements including phosphorus, silicon, fluorine, chloride, sulfur, and chloride.

Polymers that are bio-based, biodegradable, or both are referred to as bioplastics. Since CO_2 absorbed during the plant growth cycle balances the CO_2 emitted during the manufacture, use, and recycling of plastics, this strategy significantly decreases the overall carbon dioxide

balance. Additionally, it is extremely cost-effective to substitute petroleum, whose price is consistently increasing, with renewable natural materials from agriculture (Ibrahim et al ., 2021). Additionally, bacterial microorganisms and frequently nanometer-sized particles, particularly carbohydrate chains (polysaccharides), can be used to produce bioplastics(Jabeen et al., 2015). In many industrial applications, such as food processing, agriculture, compost bags, and sanitation, bioplastics are currently a crucial necessity. However, relative to their synthetic equivalents, bioplastics are lower in quality. In order to meet the increasing need for bio-based and biodegradable polymers, significant research is being done on discovering new green polymer materials (Ibrahim et al., 2021) .

2.3. Production of conventional Plastics.

Plastic is produced in a number of ways and can be used in a wide range of applications (Acquavia et al., 2021). This suitability, in addition to a number of advantageous characteristics such lightness, durability, and flexibility, has led to wide spread use in today's society. As far as product holdings and food safety are concerned, packaging has an influence on both in the food industry. The majority of modern plastics are manufactured using fossil petrochemicals like natural gas or oil. However, because all these plastics are not biodegradable, their wide spread use results in a huge amount of plastic garbage. A 2016 research on the plastics industry Jankowska et al., (2022) indicates that since 1950, the plastic production has increased by 8.6 percent annually, from 1.5 million tonnes to more than 330 million tonnes. Non-biodegradable plastic enters our ecosystem, polluting it, reducing soil fertility and killing millions of living things (Acquavia et al., 2021). In order to produce bioplastic, the industry has recently begun using natural and renewable resources such fats and vegetable oils, gluten Idris et al., (2022), egg white protein Gupta et al., (2020), and sago starch (Muhammadi et al., 2015).

2.4. Biodegradable plastic polymers and their history

Bioplastics are plastics that can be decomposed naturally over time and/or are produced from substances other than fossil fuels,such as plants and microorganisms (Selvamurugan & Sivakumar, 2019). Similar to conventional plastics, bioplastics have a variety of applications in everyday situations. The bioplastics' only difference is that they are composed of biodegradable or biobased polymers. The words "bio" are used in both biodegradable and biobased polymers,

however they are different from each other (Selvamurugan & Sivakumar, 2019). The biodegradable polymers can be converted into water and carbon dioxide by the activity of microorganisms in an acceptable period of time. They can be synthesized from either natural or fossil resources. The characteristics of a compound that can be microbiologically decomposed into final products of carbon dioxide and water and is therefore unlikely to remain in the environment were referred to as "biodegradability." The term "biodegradable plastics" refers to polymers that, when exposed to microorganisms, totally decompose into carbon dioxide in aerobic processes and methane in anaerobic processes within a predetermined period of time (Jankova, 2018).

The time it takes for a material to completely decompose depends on the material, the environment (such as temperature and moisture), and the location of the decomposition (Selvamurugan & Sivakumar, 2019). Plastics that can be decomposed are those that contain hazardous metals and can be broken down by bacteria into humus. The biodegradable polymers must adhere to the specified criteria. The standards for compostable polymers are specified in three international standards: EN 13432:2000, ISO 17088:2012, and ASTM D6400-12. At least 90% of the biodegradable polymers must be converted into carbon dioxide in industrial composting facilities during a six-month period in accordance with European Standard EN 13432:2000. Additionally, during this time, particles must break down into residues with dimensions smaller than 2 mm (Jankowska et al., 2022). Not all biodegradable polymers are biodegradable plastics. The second class of bioplastics, known as biobased plastics, are made from a variety of plant-based raw materials that aren't always biodegradable. Plastics synthesized from microorganisms are called microbial bioplastics. PHA is the only microbial bioplastic that is completely synthesized from a wide variety of microorganisms. PHA biosynthesis is a microorganism that grows in an aqueous production medium at 30-37°C under atmospheric pressure with abundant carbon sources such as starch, glucose, sucrose, fatty acids and other restricted nutrients (nitrogen and mineral salts) (Koller & Koller, 2017).

Generally, the natural polymers present in microorganisms, plants, animals, etc. can be used to produce bioplastics. Additionally, monomers including sugar, disaccharides, and fatty acids are also used as the primary raw materials in the manufacture of bioplastics, where renewable resources are modified and converted into biobased polymers (Chen, 2010). The bioplastics are a

variety of products, each with special characteristics and applications that vary based on the materials used and the production methods.The following categories generally apply to bioplastics. Polyhydroxyalkanoates,Cellulose-Based Bioplastics, Polylactic Acid- Based Bioplastics and Starch-Based Bioplastic (Selvamurugan & Sivakumar, 2019).

2.5. Polyhydroxyalkanoates (PHA)

Microorganisms, plants, and animals make bio-plastics, which are chemically manufactured from biological starting ingredients such as starch, cellulose, and carboxylic acid (Chaitanya et al., 2014). The large-scale manufacture and use of bio-plastics would help to maintain non renewable fuel supplies while simultaneously addressing environmental issues. Furthermore, it may provide benefits such as a lower carbon footprint and additional waste management choices through chemical and organic recycling (Rehman et al., 2007). Bio-plastics are generally compostable, so they can be applied to the soil without causing any harm, and they will easily degrade and decompose. Unfortunately, some bio-plastics can leave toxic residues in the form of plastic fragments in soil. For example, some bio-plastics can only be degraded at high temperatures in a specialize composter. Because the marine environment does not provide an adequate environment for biodegradation, indiscriminate disposal of biodegradable plastics in the ocean may result in the death of several marine creatures (Jabeen et al., 2015). The effective adoption of collecting, sorting, and recycling techniques, on the other hand, would provide the benefit of better resource recovery during Bio-plastics disposal (Getachew & Woldesenbet, 2016).

Furthermore, bioplastics products have superior mechanical strength and thermal stability than ordinary virgin plastics. Bioplastics are also available in a variety of grades with a variety of properties (Kumar et al., 2018). Bioplastics are used in a variety of applications, including carrying bags, super-absorbent for diapers, waste water treatment, various packaging applications, medical and dental implants, catering, and hygiene products, and agricultural mulching (Sayeski, 2016)). Bioplastics produce emits roughly 80% less carbon dioxide than petrochemical plastics (Bhuwal et al., 2013). Bioplastics assembly also requires 65 percent less energy than petrochemical plastics assembly (Kumar et al., 2018). Following all potential reuse and recycling alternatives, the renewable biomass of bioplastics would be recycled and used for

energy recovery via cascades recycling Marjadi & Dharaiya, (2018), resulting in better resource recovery. Furthermore, the same authors stated that bioplastics can avoid a variety of environmental issues such as uncontrolled dumping on land and muddled disposal, as well as the hazardous compounds released as a result. However, to harvest the benefits of bioplastics, proper collection, sorting, and recycling processes, as well as public awareness, are required.

Polyhydroxyalkanoates (PHAs) are biopolyesters that numerous bacteria store as carbon and energy internal reserves (Keshavarz & Roy, 2010). Because of their biomass-based origin and biodegradability, PHAs have gotten a lot of attention (Keshavarz & Roy, 2010). PHAs compounds are intracellular PHA granules formed in the presence of an abundant carbon supply and a nitrogen, sulfur, phosphorus, and oxygen deficit. PHAs are created intracellularly by most bacteria, including gram-positive and gram-negative bacteria, when they are stressed and have a restricted nutritional supply. When bacteria have used up all of their nutrients, they must switch to self-produced PHA as a carbon source (Aljuraifani et al., 2019).

Under such unfavorable growth conditions, intracellular hydrophobic PHA granules of carbon and energy are produced as by products, instead of major ones. Accumulated PHA does not play a vital role in the cell growth of the PHA-producing microorganisms under limiting nutrient conditions. varieties of microorganisms such as recombinant and wilds trains of *Aeromonas* sp.(Chen, 2010) *Azotobacters*p. (Kourmentza et al., 2017), *Bacillus* sp. (Samrot et al., 2021); (Singh et al., 2009) *Haloferax*sp.(Bhattacharyya et al., 2015) ,*Halomonas*sp (Baptista, 2013);(Chen et al., 2012), and *Wautersia eutropha &Ralstonia eutropha* (Trakunjae et al., 2021), have been found to accumulated PHA granules.

It was also reported that micorbial cells such as geneticaly engineered *E. coli* (Hokamura et al., 2015),*Chromobacterium*sp.(Chaudhry et al., 2011)*,Methylobacterium* sp.(Chen et al., 2012)*,Sacchrophagus degradans* (El-malek et al., 2020a)*, and Thermusthermophilus*(Sohail et al., 2020), and used to accumulated PHA granules. Further more, bacterial cells such as *Cupriavidus sp*. (Idris et al., 2022) and *Pseudomonas* sp. (Licciardello et al., 2019) have been documented for PHA polymer accumulators.

Polyhyroxyalkonates can be categorized into three types of PHA. These are including such as short-chain-length (scl), mediumchain-length (mcl), and long-chain length (lcl) PHA based on the total number of carbon atoms existing in the side chains of the PHA monomer units (Samrot et al., 2021). PHA synthases is the most important enzyme for PHA biosynthesis. PHA biosyntheses is divided into four classes based on substrate specificity, amino acid sequence, and structure (Classes I, II, III, and IV) (Rehman et al., 2007).Class I and II PHA syntheses with only one component (PhaC) (61–73 kDa) could create scl PHA (e.g. in *Ralstonia eutropha*) and mcl PHA (e.g. in *Pseudomonas aeruginosa),* respectively (Behera et al., 2022). CoA thio-esters of various (R)-3-hydroxy fatty acids with 3–5 carbon atoms are preferred by class I PHA syntheses, whereas CoA thio-esters of various (R)-3-hydroxy fatty acids with 6–14 carbon atoms are preferred by class II PHA synthases. *Allochromatium vinosum* PHA syntheses feature two types of subunits: (1) PhaC and (2) PhaE (c. 40 kDa each), which preferentially produce scl PHA. Only *Bacillus* sp. has class IV PHA syntheses, which are similar to class III PHA syntheses (*PhaE* is substituted by a 20-kDa *PhaR*) (Berekaa, 2012). The commonalities of the *phaC* genes in these PHA syntheses are limited, and no standard PCR primer set covering all of these classes has been presented yet.

2.5.1. Structure of Polyhydroxyalkanoates

PHAs are polyesters with the general structural formula as indicated in Figure 1. In 1888, Beijerinck discovered PHA granules in bacterial cells for the first time (Melchor-Martínez et al., 2022). The PHA is a natural product that discovered for the first time in gram positive bacterial cells. The structure of PHAs was also characterized in the meantime (Philip et al., 2007). Poly (3HB),is also a type of PHA with a low molecular weight that can be accumulated within *Escherichia coli* cytoplasmic membrane and cytoplasm. It's also found in the membranes of yeasts, plants, and mammals. PHA is normally made up of roughly 104 monomers that clump together to form 0.2–0.5m diameter inclusions. Both gram-positive and gram-negative bacteria are able to store granules, with no harmful effects on the bacterial cells or other hosts (Berekaa, 2012). Depending on the microbe and the growth conditions, the molecular weight of these molecules ranges from $2x10^2$ to $3x10^3$kDa (Muhammadi et al., 2015). PHA builds up in cells when there is a nutritional imbalance, such as too much carbon and not enough nitrogen, phosphorus, or oxygen (Sakthiselvan & Madhumathi, 2019). Excess nutrients are stored

intracellularly by bacteria by creating insoluble biopolymers from soluble molecules. When favorable conditions for normal growth resume, the biopolymers become mobilized. Depending on the organism, the shape, physio-chemical characteristics, monomer makeup, and number and size of the granules differ (Ha and Cho, 2002).

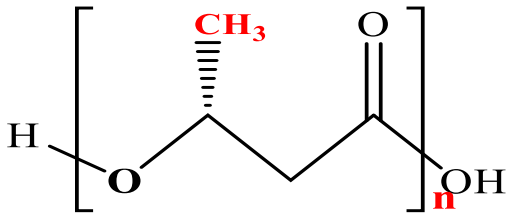

Figure 1. Structure of Polyhydroxyalkanoate (PHA) that can be produced by bacterial cells with little modification (. Reddy et al., 2003)

Table 1. Chemical structure of Polyhydroxyalkanoate (PHA)

S.N.	R-group	Full name	Short
1	CH3	Poly(3-hydroxybutyrate	PHB
2	CH2CH3	Poly(3-hydroxyvalterate	PHV
3	CH2CH2CH3	Poly (3-hydroxyhexanoate	PHH

2.5.2. PHA classification and its producers

According to Madison and Huisman (1999) PHAs are frequently grouped into three major classes based on the size of the monomers they contain;i) (scl-PHA): PHAs containing up to C_5 monomers are classified as short chain length PHAs. ii) mcl-PHA: PHAs with a chain length of C_6–C_{14} are known as medium chain length PHAs. Mostly mechanically, structurally, and thermally diverse than SCL-PHA type of polymers. However, due to the lack of efficient MCL-PHA-producing microorganisms and the necessity of costly fermentation strategies for commercial PHA production, investigation on MCL-PHA is limited (Narayanan et al., 2020). iii) lcl-PHA: PHAs having more than C_{14} monomers are referred to as long chain length PHAs. This difference is due to the substrate specificity of PHA syntheses, which can only accept 3HAs in a certain range of carbon lengths (Muhammadi et al., 2015). *Alcaligenes eutrophus* PHA synthase can polymerize 3HAs with 3–5 carbon atoms, but *Pseudomonas oleovorans* PHA syntheses can only take 3HAs with 6-14 carbon atoms (Dartiailh et al., 2021).

Short chain length PHAs (PHASCL) have less than or equal to five carbon atoms in their HA monomer unit (Sehgal & Gupta, 2020). They are the most common, highly crystallinity (55-80%) which makes them brittle and possess low melting temperature (173 – 1800C) (Ciesielski et al., 2013). Examples include: poly (3-hydroxybutyrate) P(3HB), poly(4-hydroxybutyrate) , P(4HB) and poly(3-hydroxyvalerate) P(3HV) or the copolymer P(3HB-co-3HV) as seen in Figure 1 (Verlinden et al., 2007).

Medium chain length (MCL) monomers have up to six to 14 carbon atoms present in their HA monomer unit and are called Medium-chain lengths PHAs (PHAMCL) (Muhammadi et al., 2015). Their structure could also include functional groups belonging to the halogens, olefins, cyano as well as alkyl groups Philip et al., (2007); (Muhammadi et al., 2015). Examples include poly (3-hydroxyhexanoate) P(3HHx), poly(3hydroxyoctanoate) P(3HO) and copolymers such as P(3HHx-co-3HO). According to the reports of Ciesielski*et al* .,(2013), PHAMCL are most desirable because they possess superior thermo mechanical properties. PHAMCL have lower melting temperature (39- 61^0C), are more flexible and have more elasticity than PHA SCL(Ciesielski et al., 2013).

Long chain length PHAs (PHALCL) are uncommon and have more than 14 carbon atoms present in their HA monomers ((El-malek et al., 2020a). Examples include Poly (3-hydroxypentadecanoate), Poly (3hydroxhexadecanoate.

2.5.3. Physical properties of Polyhydroxyalkanoates

These microbial based polymers can be obtained from renewable resources. This makes these polymers environmentally sustainable. As a result they're a great alternative to petrochemical thermoplastics that derived from crude oils which is non-renewable energy sources (Saharan & Sharma, 2015). PHA, which is derived from bacterial cells, has characteristics similar to traditional polymers such as polypropylene (Saharan & Sharma, 2015). Many bacteria can breakdown PHA into CO_2 and water at a high rate (3-9 months) utilizing their own secreted PHA depolymerises (Chee et al., 2010). PHB and PHV have been used in the majority of studies and reported from bacterial cells with thermal properties. The presence of functional groups such as aromatic-side chains,epoxy-, and hydroxy-, opens up a wide range of options for further

chemical modification of these microbial based polymer structure. This has an impact on the physical properties of these polymers (Chaitanya et al., 2014). PHB has a melt temperature of 1750 degrees Celsius and a glass transition temperature (Tg) of 40 degrees Celsius (Chaitanya et al., 2014). PHB, or polymer with a short chain length, is optically pure, meaning it is 100 percent pure (Chaitanya et al., 2014).

Properties of PHAs one of the most significant characteristics for processing and producing polymers'end use is their thermal and physical qualities. The molecular structure of polymers is largely responsible for their physical and thermal characteristics. The physicochemical features of scl/mcl PHAs are distinct (Chou et al., 2021). Because PHAs are susceptible to increasing in temperature and breaking down during hemelt process, their heat stability isessential. Plasticizer scan help to prevent PHAs from degrading too much during the melting process (Kumar et al., 2018a). Both P3HO and P3HO-P3HD have higher thermal degradation kinetic parameters than PHB and PHBV due to complexation on the development of six-membered transitional configuration. The thermal breakdown temperatures of P3HO with epoxy and hydroxy groups are varied. The P3HO with epoxy pendant groups might have a higher heat breakdown temperature after raising the temperature due to the bridging potential of epoxy groups. Melting Point, Crystallization, Gas Barrier, and Solubility Polymer performance is influenced by their crystallization kinetics (Kumar et al., 2020) .

In PHAs, the length of the side chains can have a big impact on how they crystallize; for example, mcl-PHAs can form a layered crystal structure, increasing the side chain length of mcl PHAs (e.g., C7 or more) and resulting in the formation of a new smectic liquid crystalline phase (Tripathi et al., 2013). Both the main and side chains of mcl-PHAs with smectic crystals can cause cold crystallization during heating (Philip et al., 2007) therefore, two melting points in mcl-PHAs have been found,which can beas cribbed to the evelopment of two distinct crystal phases (phase I and phase II) (Chen et al., 2013). Crystallization rates have been shown to be improved by reactive chemical modification of PHAs. When compared to un modified PHAs, modified PHAs with lengthy chain branching and low cross-linking densities demonstrated enhanced crystallization rates (Yang et al., 2013). These chemical alterations also enhanced the theological characteristics and processability of the material.The low crystallinity of mcl-PHAs permits them to be dissolved in non-halogenated solvents.

Mcl-PHAs with slow crystallization rate and low melting point have limited melt process ability; reactive modifications including cross linking, mixing with other polymers, and grafting techniques can be used to solve these problems in the PHAs (Muthuraj et al.,2018). The oxygen and water vapor barrier qualities of polymers are importantas their physical and thermal properties when it come stousing them for effective packaging applications such as food packing. When compared to traditional polymers, such as polypropylene and polyethylene, PHAs have better gas barrier qualities (Sandoval et al., 2021). In particular, PHA copolymers have significantly better oxygen, carbon dioxide, and odor barrier characteristics than polyethylene and polypropylene (Sandoval et al., 2021). PHBV has the best resistance to oxygen and water vapor penetration among biodegradable polymers (Marjadi & Dharaiya, 2018). When compared to PLA and PBS, the PHBV offers good potential for usage in fruit, and vegetable packaging as it has an appropriate oxygen and water vapor transfer rate (Marjadi & Dharaiya, 2018). However, the PHBV's gas barrier qualities are insufficient for the packaging of meat, cheese, and coffee. Graphene oxide and clay supplementation to PHAs has been found to increase barrier characteristics by creating a convoluted route for gas molecules (Samrot et al., 2021).

2.6. PHA Biosynthesis and their producers

2.6.1. Diversity of PHA-producing bacteria

Wide varieties of microorganisms suchas recombinant and wilds trains of *Aeromonas* sp.(Chen, 2010)*Azotobacters*p.(Kourmentza et al., 2017), *Bacillus* sp. (Samrot et al., 2021);(Singh et al., 2009),*Haloferax* sp.(Bhattacharyya et al., 2015),*Halomonas*sp (Baptista, 2013); (Chen et al., 2012), and *Wautersia eutropha & Ralstonia eutropha* (Trakunjae et al., 2021), have been found to accumulated PHA granules. It was also reported that micorbialcells such as geneticaly engineered *E. coli* (Hokamura et al.,2015),*Chromo bacterium*sp.(Chaudhry et al., 2011)*, Methylobacterium* sp. (Chen et al., 2012)*,Sacchrophagu sdegradans* (Malek et al., 2020)*, and Thermusther mophilus* (Sohail et al., 2020), and used to accumulated PHA granules. Further more, bacterial cells such as *Cupriavidus* sp (Idris et al., 2022) and *Pseudomonas* sp. (Licciardello et al., 2019) have been documented for PHA polymer accumulators. Sunlight is the source of energy to produce PHB by *cyanobacteria*, which resulted in the reduction of CO_2 (a significant green house gas) (Trakunjae et al., 2021).

Cyanobacteria possess PHA storage capacity and could produce PHA by oxygenic photosynthesis (Hokamura et al., 2015). Under nitrogen and phosphate limiting conditions, *Synehcysti*s sp. or *Synechocystis* sp. could accumulate PHB (Marjadi & Dharaiya, 2018).

Microorganisms which are capable of accumulating PHAs can be categorized into two main groups on the basis of growth parameters needed for the biosynthesis of PHA granules. The first group of microorganisms requires limited essential nutrients such as magnesium, phosphorus, nitrogen, and sulfur for PHA biosynthesis. However, they are able to accumulate high PHA granules when more carbon substrate were provided (Muhammadi et al., 2015). Some of the examples *Protomonas extorquens Ralstonia eutropha* and *P. oleovorans. Ralstonia eutropha* produce scl PHAs and are included in this group (Reddy et al., 2022). Fatty acids or hydrocarbons were fermented by most of the *Pseudomonads* spp. like *P. oleovorans* using 3-hydroxyacyl-CoA intermediates of the β-oxidation pathway to synthesis mcl PHAs. There are reports where, *Pseudomonas* strain GP4BH1has synthesized mcl-PHAs comprising 3HB and 3HO monomer units using octanoate and mcl PHAs containing 3HB, 3HO, and 3HD monomers using gluconate as carbon sources (Ciesielski et al., 2013). In another report, PHB homopolymer blended with a random copolymer poly (3-hydroxybutyrate-co-3hydroxyalkanoates) P(3HBco-3HA) containing C_4–C_{12} HA monomer units was synthesized by *Pseudomonas* sp. strain 61-3 using alkanoic acids and sugars (Guo et al., 2013). The hydrocarbon degraders are also reported as good PHA accumulators. The sites contaminated with oil rich in carbon and poor in nitrogen content create a favorable condition for microbial cells to accumulate PHA garnules(Yang et al., 2013).Many bacterial genera such as *Acinetobacte*r,*Brochothrix, Burkholderia, Caulobacter, Ralstonia, , Pseudomonas, Sphingo bacterium, a*nd *Yokenella* are included in this category (Vijay & Tarika, 2018). Halophiles require salts to grow and Archaea residing in the oceans, salt lakes, and hot springs have been reported to synthesize PHAs (Legat et al., 2010).

The marine habitats are abundant in organic matter but habitually limited in nitrogen, oxygen, and phosphorus that create a favorable environment for the marine microorganisms to produce PHA (López-Cortés et al., 2008). There are reports where highly halophilic bacteria under nutrient-deficiency conditions could accumulate polyhydroxybutyrate (PHB) (Vigneshwari et al., 2022).There are reports, where *Haloferax mediterranei* and *H. boliviensis*

were investigated to produce 65% and 56% of PHA of its total cell dry weight (CDW) using glucose and starch hydrolysate respectively (Vijay & Tarika, 2018). Oxygen limitations can enhance the production of PHAs (Quillaguaman et al., 2005). Several other sources such as anaerobic or aerobic system, activated sludge, and aerobic dynamic feeding system have been reported for PHA production.

The second group of microorganisms does not involve nutrient limitation for PHA production, in this case the PHA polyester is accumulated during the growth phase. Some of the producers are recombinant *Escherichia coli*, a mutant *Azotobacter vinelandii*, and *Alcaligenes latus.* These features are important to be reflected during the PHA synthesis (Muhammadi et al., 2015). Some of the anti biotic producing aerobic gram-positive bacterium *Streptomyces* is also investigated for PHA production with some vital metabolites (Valappil et al., 2006). *Alcaligenes eutrophus* was reported to produce copolymers of PHA suchas P(3HB-co-3HV)and P(3HB-co-4HB) (Santhanam & Sasidharan, 2010). Some of the bacterial species of *Rhodospirillum rubrum, Rhodococcus* sp. and *Rhodocyclus gelatinosus* were investigated to produce ter polymer of PHA containing 3HA monomers units of C_4,C_5, and C_6 using hexanoate as major carbon substrate (Montenegro et al., 2017) .

Besides, a random copolymer of HB and 3-hydroxyhexanoate (HH) was investigated in *Aeromonas caviae* (Santhanam & Sasidharan, 2010). In addition, LCL-PHAs were also investigated in *Fluorescent pseudomonads* spp.(Vuong et al., 2021). Many *Pseudomonas* strains (*P. fluorescens)* were reported to synthesize a P(3HB-co-3HA) copolymer comprising HA monomer units of C_4–C_{12} from hydroxybutyric acid (HB) and 1,3-butanediol (Correa-Galeote et al., 2022). A report says that *Thiocapsa p fennigii* was capable to synthesize only a PHB homopolymer using different carbon sources. However, a genetically engineered *P. putida* strain harboring the *phaC* genes of *T. pfennigii* synthesized a terpolymer of PHA, P(HB-HHx-HO) using octanoate as a carbon source (H. Wu et al., 2012).

2.7. Methods of Screening for PHA Producing Bacterial Isolates

The two most common techniques for identifying naturally occurring bacteria that make PHA from their surroundings are phenotypic-based screening and genotypic-based screening, as according research published to date. Various phenotypic detection techniques, such as Sudan black B staining (Porras et al., 2017), Nile blue A staining (Ostle & Holt, 1982) and Nile red (Kumar et al., 2018), which produce dark blue or fluorescent granules, are used for the screening of PHA producers. Additionally, it is essential to provide the appropriate carbon sources, nutrient limitation conditions, and a sufficient culture time for PHA granule accumulation in the bacterial cells before identifying and isolating PHA-producing bacteria by phenotypic methods (Sheu et al., 2000).

Sudan black B is not PHA-specific because it also stains other lipid structures in addition to PHA. According to studies, Nile blue A and Nile red are more specific to detection than Sudan black B (Shamala et al., 2003). Additionally, these methods are still unable to distinguish between bacteria that accumulate PHA granules and bacteria that accumulate lipid polymers (Sheu et al., 2000). According to studies, NB A and Nile red are more specific for detection than Sudan black B(Shamala et al., 2003). Furthermore, these methods are still unable to identify between bacteria that synthesize lipid molecules and bacteria that synthesize PHA granules (Berekaa, 2012).

Genotypic-based screening method is specific, reliable and quick which circumvents the major drawbacks inherent in phenotypic detection methods (Sheu et al., 2000). For genotypic-based screening, the degenerate or and specific oligo nucleotide primers are designed based on multiple sequence alignment results and are used as PCR primers to detect PHA synthase genes (Wu et al.,2003).The major limitations of phenotypic detection methods are solved by the rapid, accurate, and precise genotypic-based screening method (Sheu et al., 2019). Degenerate and particular oligo nucleotide primers are designed for genotypic-based screening based on the results of multiple sequence alignment and are applied as PCR primers to identify PHA synthase genes (Sheu et al., 2000).The polymerase chain reaction (PCR) provides a rapid detection of specific genes in organisms (Berekaa, 2012). The primers were designed on the basis of the highly conserved nucleic acid sequences in regions of the class II pha loci of various

Pseudomonas. Because of the unusually high consensus in these regions, the design of non degenerate PCR primers (I-179L and I-179R) was possible (Solaiman et al., 2000). Screening of various *Pseudomonas* using this method not only verified its general applicability by correctly identifying known mcl-PHA-producing strains but also led to the confirmation of species previously not characterized as accumulating mcl-PHA (Solaimanet al., 2002).Using the individual PCR fragments as a template in the second-round (semi-nested) PCR with I-179L/I-179R primers would then lead to the specific amplification of an about 0.54-kb segment of the phaC1 and phaC2 genes, respectively (Solaiman et al., 2000).Yang et al., 2013 reported PCR cloning of class II pha genes on the basis of the non degenerate primers derived from the consensus sequences of ORF1, phaZ and phaD genes of *Pseudomonas* pha loci.

MALDI-TOF-MS stands for Matrix-Assisted Laser Desorption Ionization – Time of Flight – Mass Spectrometry has been invented a long time before, but spectral fingerprints were obtained from whole bacterial cells for the first time in 1996 (Holland et al., 1996). During the same year, the spectral finger prints of different *Bacillus* species such as *Bacillus anthracis, Bacillus melitensis*, were acquired by employing the MALDI-TOF technique (Neelja et al., 2015). Since then, much attention was directed towards the identification of not only bacteria but also yeast and mold using MALDI–TOF (Koubek et al., 2012). However, MALDI-TOF was started to be routinely utilized as a first-line identification method in Microbiology labs during the last 12–15 years. The technology offers many advantages over conventional microbiological and molecular techniques, which include reliability and rapidness as it takes only a few minutes for identification of microbes simplicity, cost-effectiveness, and non-requirement of highly skilled people (Neelja et al., 2015). Moreover, the technology has the ability to identify many different types of microorganisms and thus possesses the potential to replace other identification methods in Microbiology labs (Neelja et al., 2015).

perspective and have discussed briefly its application in both clinical and environmental samples. In comparison, the more focused and comprehensive discussion of MALDI-TOF MS for its application in environmental microbiology was given by (Neelja et al., 2015). Nevertheless, in addition to the bacterial identification by MALDI-TOF, other areas of environmental microbiology such as protein profiling, metabolomics, and others were also dicribed .

2.8. Gene associated with PHA biosynthesis

2.8.1. Genes and enzymes involved in PHA synthesis

The central PHA biosynthesis pathway consists of the three basic enzymatic steps which convert acetyl coenzyme A (acetyl-CoA) intermediate to PHB . PHAs have a vast chemical diversity, with PHB being the most well-known and extensively manufactured type (Rehm, 2006). The single biosynthetic pathway for PHB is taken into consideration. These enzymes and their corresponding genes are used in the approach (Reddy et al., 2003). These genes are grouped and organized in the*phbCAB* operon. The *phaA* gene produces -ketothiolase, which catalyzes the condensation of two acetyl-CoA molecules to produce acetoacetyl-CoA, which is then converted to (R)-3-hydroxybutyrylCoA by the acetoacetyl-CoA reductase (malek et al., 2020). The enzyme is NADPH-dependent and is encoded by the *phaB* gene. The polymerization of (R)-3-hydroxybutyryl-CoA monomers mediated by PHA synthase, which is encoded by the *phaC*gene, is the last process (Shamala et al., 2003)

In microorganisms, substrates or monomers for the PHA synthase could be supplied from various metabolic pathways such as fatty acid β-oxidation, fatty acid de novo biosynthesis and citrate acid cycle (Reddy et al., 2003) . Monomers of MCL-PHA such as 3-hydroxyhexanoate (3HHx) and 3-hydroxyheptanoate (3HHp) can be channeled from the fatty acid β-oxidation pathway to PHA synthase via the catalysis reaction of R-specific enoyl-CoA hydratase (PhaJ), which convert enoyl CoA intermediates to (R)-3-hydroxyacyl-CoA. In the same pathway, epimerase and 3-ketoacyl-CoA reductase (FabG) can convert (S)-3-hydroxyacyl-CoA and 3 ketoacyl-CoAintermediates to(R)-3-hydroxyacyl-CoA, respectively (Madison and Huisman, 1999).

2.8.2.Mehanism and gene involved in the synthesis of bacterial PHA produced

Biosynthesis of PHA takes place in the cytosol of the microbial cell by different enzymatic reactions initiated from acetyl-CoAs catalyzed by substrate specific PHA synthases . Most of the bacterial strains can produce SCL-PHAs and MCL-PHAs. So far, eight pathways/mechanisms have been investigated that are involved in the synthesis of microbial bioplastics (PHAs) (Chen, 2010). The three key enzymes, like β-ketothiolase (*phaA*), NADPH dependent aceto acetyl-CoA reductase (phaB), and PHA synthase (*phaC*) have been involved in pathway I for the synthesis of

PHA. The first enzymatic reaction was catalyzed by β-ketoacyl CoA thiolase (*phbA*) that condensed two molecules of acetyl-coenzyme A (acetyl-CoA) into acetoacetylCoA. In the second reaction, the NADPH-dependent (R)-specific acetoacetyl-CoA dehydrogenase/reductase (*phbB*) enzyme reduces the acetoacetyl-CoA molecule into (R)-3-hydroxybutyryl-CoA. Lastly, the enzyme PHA polymerase/synthase (*phbC*) polymerizes the (R)-3hydroxybutyryl-CoA monomers using PHB precursor into PHB polymer (Muhammadi et al., 2015). The representative of this pathway is *Ralstonia eutropha*. A related pathway involving the degradation of PHA catalyzed by acetoacetyl-CoA synthase, 3hydroxybutyrate dehydrogenase, PHA depolymerase, and dimer hydrolase that helps to regulate PHA synthesis and its degradation. Bacterial strains of *R. eutropha,P. oleovorans,P. stutzeri, and Aeromonashydrophila* are the well-known examples of this associated pathway (Sheu et al., 2000).

Recently, Reddy et al., (2003) reported that fatty acids such as acetic acid, butyric acid, caproic acid, and propionic acid had been utilized as carbon sourcefor the synthesis of P(3HB-co-3HV) that have a higher portion of HB content using *Bacillus* sp. CYR1. Besides*, phaA, phaB, phaC*, and *phaJ* have been reported to involve for the production of P (3HB-co-3 HV) (Reddy et al., 2003). In another study,*phaC* genes and the PHA-associated gene clusters have been reported in four Antarctic isolates such as *Pseudomonas* spp. UMAB-08, *Pseudomonas*. spp. UMAB-40, *Janthino bacterium*spp. UMAB-56 and J*anthino bacterium* spp. UMAB-60 using whole-genome sequence. *Pseudomonas* strains were found to comprise Class I and Class II PHA synthase gene clusters,which are mostly involved in the biosynthesis of scl PHA and mcl PHA respectively (Reddy et al., 2022). In contrast, the *Janthino bacterium* strains contain only an uncharacterized putative *phbC* and Class I *phbC* genes. On further investigation via multiple sequence alignment, the detected genes of uncharacterized putative *phbC* are found to contain residues of highly conserved amino acid sequence and revealed catalytic *phbC* gene which are quite distinct from normal Class I, II, III, and IV *phaC* as shoen in the table 2. The findings of in vitro *phaC* enzymatic assay and PHA biosynthesis revealed that this uncharacterized putative *phaC* gene that predicted for *Janthino bacterium* sp. UMAB-60 is found to be active. This report added up new knowledge to the *phaC* database of Antarctic bacterial strains isolated from such geographically isolated and extreme environments with the other normal non-Antarctic PHA-producing microorganisms (Yang et al., 2013).

Table 2: Classes of PHA biosynthesis genes and examples of organisms that bear these genes (Muhammadi et al., 2015)

S.N.	Gene class	Type of PHA Genes involved for	Organism	Type of bioplastic produced
1	Class I	*phaC*	*Cupriavidusnecator*	sclPHA
2	Class II	*phaC1* and *phaC2*	*Pseudomonas aeruginosa*	mclPHA
3	Class III	*phaC* and *phaE*	*Allochromatiumvinosum*	sclPHA
4	Class IV	*phaC* and *phaR*	*Bacillus megaterium*	sclPHA

2.9. Main source of bioplastics

2.9.1. Individual Bacterial system for biosynthesis of Bioplastics

Bacteria that produce PHAs are frequently split into two classes based on the growth conditions necessary for PHA production. For the assembly of PHAs, the main group requires the restriction of a necessary nutrient(s). *Cupriavidus necator*, *Rhodo pseudomonas palustris*, and *Methylo bacteriumorganophilum* are among the bacteria found in this category (Kumar et al., 2020). The second group produces PHAs in cycle with growth in the growing medium. *Alcaligenes latus* and recombinant *E. coli* with PHA biosynthesis genes are among the bacteria in this group (Lin et al., 2017). *Cupriavidus necator* has received a lot of attention because of its ability to produce a lot of P(3HB) from simple carbon substrates including glucose, carboxylic acid, and ethanoic acid (Wei et al., 2011). Even olive oil, oil, and vegetable oil have been known to create around 80% Dry cell weight (dcw) P(3HB) of dry cell mass from the organism (Gupta et al., 2020). To provide PHAs, *Methylobacterium organophilum* employs simply methanol as a cheap carbon source, where as *Rhodo pseudomonas palustris* uses a combination of carbon sources. A non-sulphur photosynthetic bacterium, has potential to supply P(3HB) and P(3HB-co-3HV) using different carbon and nitrogen sources. *Rhodobacter* and *Rhodospirilium* are two other non-sulphur photosynthetic bacterial genera that have been discovered to be very resourceful in the formation of the

copolymer P(3HB-co-3HV) using a variety of carbon sources such as malate, carboxylic acid, and n-alkanoic acid (Porras et al., 2016).

Alcaligenes latus is another organism that produces PHAs using carbon sources such as glucose, molasses and sucrose with high-quality yields (Chen et al., 2013). The studys with different nitrogen sources have proven that *A. latus* is able to grow and produce PHAs with ammonium chloride and ammonium sulphate as nitrogen sources. Ammonium nitrate and urea could not support PHA production in the organism (Saharan et al., 2014). It requires a temperature of 35 ^{o}C, which makes P(3HB) production from the organism economical by lowering the demand for cooling during fermentation. Optimum biomass and PHA accumulation can be further enhanced by feeding the organism with enriched medium (Gupta et al., 2020).

2.10. Major Applicationof PHA(Polyhydroxyalkanoates)

PHAs are thermoplastics that are non-toxic, biocompatible, and biodegradable and are made from renewable resources. They must be highly polymerized, crystalline, optically active, isotactic (stereo-chemical regularity in repeating units), peizoelectric, and water insoluble. These characteristics set them apart from polypropylene. As a result of their innovative properties, they have a wide range of applications. Packaging, cosmetic containers, shampoo bottles, cardboards and papers, milk cartons and films, moisture, barriers in diapers and sanitary napkins and other personal hygiene materials, pens, combs, bullets, flavor delivery agents in foods, dairy cream substitutes, and bulk chemical production using depolymerized PHA are just a few of the applications for PHAs (Balakrishna Pillai et al., 2017).

PHAs have recently gotten a lot of press for their medical applications. Cardiovascular goods, prodrugs, nerve and soft tissue repair efficacy, dental and maxillofacial treatment, drug administration, nutrition, orthopedic and urological operations, and wound management are among them (Wu et al., 2009). These are useful as chiral precursors for the chemical synthesis of optically active chemicals because they are stereo regular (Wu et al., 2009). These chemicals are particularly useful as biodegradable carriers for long-term administration of drugs, hormones, insecticides, and herbicides. Because of their piezoelectric capabilities, they're also used as osteo synthetic materials in bone plates, surgical sutures, and vascular

replacements to stimulate bone formation. However, because to the delayed biodegradation and great hydraulic stability in sterile tissues, medical and pharmaceutical applications are limited (Valappil et al., 2006).

Medical and pharmaceutical fields gained much attention among the different PHA applications. However, agricultural applications of PHA are rather limited. Several agricultural usages are previously investigated such as agricultural nets, grow bags and mulch films. Various agriculture sectors utilize plastic materials such as greenhouse films and protection nets (El-malek et al., 2020). The usage of nets is very essential in agriculture to increase the crops quality and yield and protect it from hazards, such as birds, insects and winds (El-malek et al., 2020a). High - density polyethylene (HDPE) is the commonly used raw material for commercial agricultural nets . However, this petroleum -based plastic is harmful to animals and environment due to its non biodegradability. The usage of PHA agricultural grow bags has many advantages. Firstly, this biodegradable polymer does not exhibit any toxic residue in soil as opposed to plastic bags release harmful organic matter and toxic emission. Secondly, it can positively remove nitrogen from water. Thus, the application of PHA grow bags do not contaminate the surrounding water bodies (Jabeen et al., 2015). These bags are root -friendly as they do not cause any root deformity compared to PE bags. Subsequently, grow bags may affect the crops' growth, pathogenic immunity and the plant's ability to anchor quickly into the ground after transplanting. Furthermore, PHA grow bags eliminate double handling and recycling of bags after use (Boey et al., 2021)

3. Materials and Methods

3.1.Description of study area and sample collection

The study was carried out on soil sediment collected from Dambal Lake.The depth soil sediments were collected from Dambal Lake was 5cm and aseptically transported to Department of Biology laboratory. Lake Dambal and its feeder rivers located in the Ethiopian Rift Valley (Latitude: 7°52′–8°8′N; Longitude: 38°04′–38°56′E). Lake Dambal is a freshwater wetland found at a distance of about 160 km south of Addis Ababa. The lake is 31 km long and 20 km wide, with a surface area of 440 km^2. It has a maximum depth of 8.9 meters. Apart from seasonal runoffs and groundwater recharge, the lake is fed by two rivers, namely, Meki from the west and Qatar from the east, and is drained by River Bulbula which feeds Lake Abijata to the south. The catchment area of this lake is estimated to be 7025 km^2 and the lake lies at an altitude of 1,636 meters above sea-level. Lake Dambal water is the main valuable source for commercial fishing, irrigation and horticulture farming (Tibebe et al., 2022).

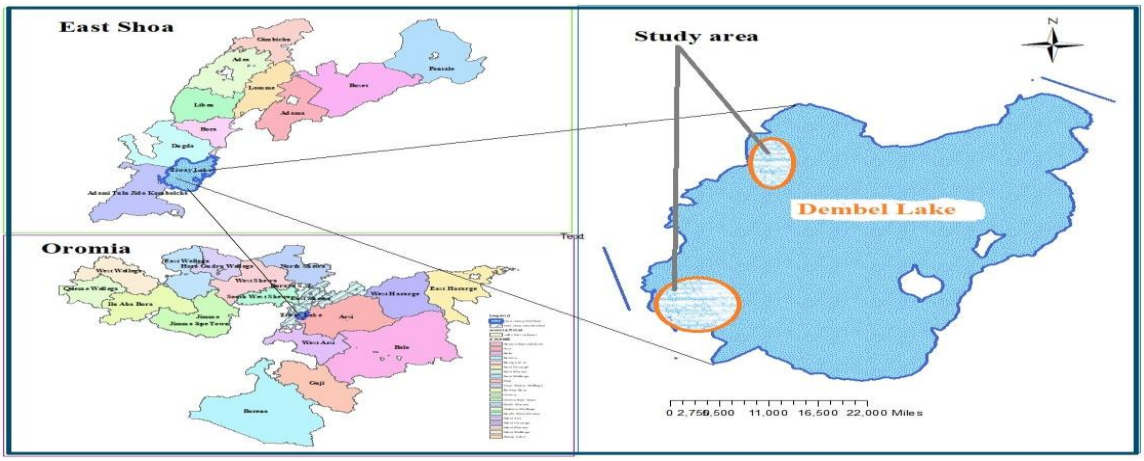

Figure. 2. Study area lake Dambal, East Shoa,Oromia , Ethiopia

3.2. Source of chemicals, reagents and instruments

All culture media and reagent such as MDA, MDB, Sudan Black B, Nile blue A reagents, and other chemicals used forscreening PHA producing bacterial isolates were purchasedfrom market stock.

3.3. Isolation of PHA producing isolates

The soil sediment sample was aseptically collected 30g of soil sediment using sterilized plastic bags from lake Dambal ,Oromia regional state ,East Shoa Zone,Batu city , Ethiopia. The samples were transported to Department of Biology laboratory Adama Science and technology Univarsity and kept at 4ºC until processing in Refregetor. The serial dilution of the soil sediment samples was carried out in test tube of 1oo ml of distilled water of 1% w/v of sediment and transfer to Minimal Davis Agar, (per liter) (1.0g Dextrose, 7.0g K_2HPO_4, 1.0g $(NH_4)_2SO_4$, 0.5g sodium citrate, 2.0g KH_2PO_4, 0.1g $MgSO4.7H_2O$, 15g/Agar and pH 7.2 ± 0.2 at 37ºC) (Himedia, Mumbai, India). The obtained pure cultures was then maintained on 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 Final pH 7.4±0.2 at 37ºC for 24hrs) for further purification.

3.4. Culture maintenance,preservation and Growth Conditions

Pure PHA producing new isolate cultures was inoculated into (Luria-Bertani broth)LB broth (Tryptone, 10g/l; yeast extract powder, 5g/l; Sodium chloride, 10 g/l pH 7.2 ± 0.2) (Himedia, Mumbai, India) and incubated at 37º C for 24 hrs. Finally, 24hrs old cultures were transferred to 50 % (v/v) glycerol solution and kept at -20 º C for next use.

3.5. PHA production medium

Production of PHA depends on the available carbon source in the media. Minimal Davis broth (per liter) (1.0g Dextrose, 7.0g K_2HPO_4, 1.0g$(NH_4)_2SO_4$, 0.5 g sodium citrate, 2.0g KH_2PO_4, 0.1 g $MgSO_4.7H_2O$ and pH 7.2 ± 0.2 at 25 ºC) was used as PHA production. It was prepared and autoclaved at 121 ºC. A 2.5% of 24h old culture were taken and transferred to 500 ml conical flasks containing 200 ml PHA production medium. The medium were supplied with 1% carbon sources (D-fructose, glucose, molasses, bagasse, D-ribose, and glycerol) (w/v) and incubated at 37 º C for 72 h on a rotary shaker at 150 rpm.

3.6. Primary screening PHAs producing isolates (Phenotypic detection methods)

3.6.1. Sudan Black B staining methods (SBB); Slide staining methods

SBB is a preliminary screening PHA producing new isolates .SBB dye solution was prepared as 0.3% in 75 ml of 95% ethanol (Marjadi & Dharaiya, 2018). A pure isolates were taken and activated infresh nutrient broth using a test tube. The cultures were inoculated at 37 ° C for 24h. Now the PHAs producing isolates of bacteria was confirmed using SBB staining. Briefly the smear was stained for 10-15 min with SBB solution ,rinsed with running water and the slide was air dried. The excess amount of strain was destained (decolorized) using xylene (100%) which is for the first time only for few several time and washed with running water. The slide smer was dried and then countered stained using safranin 0.5% (w/v) for 30s. The smear was washed with tap water and air dried. Finally the smear was observed under a microscope at 100* oil magnification power. in this staining , lipid inclusion granules . was stained as blue black ,whilst the bacteria cytoplasm was stained as red color . These SBB positive isolates of bacteria was selected and streaked onto LB agar slants and then incubated at 37ºC. These slant isolates was stored at 4 ºC for future studies. These cultures were also sub-culture every two weeks.-

3.7. Secondary screening PHA producing bacterial cells

3.7.1. Nile blue A staining; Slide staining methods

The staining was conducted following the methods of (Ostle and Holt,1982) with little modification .A single colony of bacterial was suspended in a drop of water on a slide and heat-fixed. The smear was flooded with 1% NBA stain solutionand stained at 55 ºC for 15 min in a Coplin staining Jar. But the aqueous solution of NBA was filtered beforeuse. After slide staining, excess dye was removed bywashing with distilled water. The smear were de-stained withaqueous acetic acid (8%v/v) for 1min to remove all the none bounding stains. The stained slide wasv washed with deionized water and air-dried. Finally , the smear was mounted and observed using a fluorescence microscope using oil immersion at different filter at Adama public health research center and Diagnostic regional laboratory. The images was recorded. The PHA granules inside the cells of bacteria showed orange fluorescence when the orange filter

was used and green fluorescence when green filter was used. However, when the red filter was used, the PHA granules fluoresce as deep red color.

3.8. MALDI-TOF MS Identification of Isolated PHA Producing Bacteria

MALDI-TOF MS has confirmed its potential as a rapid screening method for determining similarities between bacterial isolates, especially in such a diverse field as environmental microbiology. The diversity of the PHA producing bacteria that found in siol sediment that were isolated from Dambal lake soil sediment was conducted at laboratory of National Animal health Diagnostic and Investigation center Sebbata ,Oromia Regional State.Pure isolates of PHA producing bacterial sp. were identified following the methods of (Toubal et al.,2018) . Briefly, all isolates were initially purified and screened based on their ability to producing PHA granules prior to identification using Matrix-assisted laser desorption ionization time flight mass-spectroscopy (MALDI-TOF MS). These isolates classifications were carried out using the direct transfer method (MALDI Biotyper 3.1. User Manual, Bruker Daltonics Inc.). The representative single colonies of the possible PHA producing isolates (DLSPI) were smeared as a thin film directly into a spot on MALDI targeted plate using tooth applicator. The MALDI-TOF MS target plate was overlaid with 1µl of 70% (v/v) of formic acid and allowed to dry at a room temperature. Immediately the spot was overlaid with 1µl of matrix solution α-cyano-4-hydroxycinnamic acid in 50% (v/v) acetonitrile (HCCA) solution and allowed to dry at room temperature. The resulting spectra were compared with reference spectra by using the Biotyper 3.1 software (Bruker MALDI Biotyper, UK). The identification score cutoff values were applied to each measurement according to the manufacturer's instructions. Isolates with a score of ≥2.0 for a given species were considered adequately identified to the species level as prescribed by Tomazi et al., (2015). *E. coli* ATCC 25922^{T} was used as a standard for calibration and quality control.

3.9. Characterization Of PHA Producing Isolates

3.9.1. Morphological Characterization

Morphological characterization was performed for PHA producing isolates. Colony feature such as colony color,elevation, margin, and surface and spore formation were checked for these PHA producing morphological characterization. Microscopic examinations were performed to identify for their spore formation, morphological type as rod, streptococci and staphylococci in shape.

3.9.2. Biochemical characterization and enzyme assay

PHA positive bacterial isolates were cultured in Luria Bertani Broth (LB) ((Tryptone, 10g/l; yeast extract powder, 5g/l; Sodium chloride, 10 g/l pH 7.2 ± 0.2) Himedia, Mumbai, India at 37^{o}C for 48 h. The pellet were collected and used for biochemical tests. The PHA positive isolates were morphologically observed under compound microscope. Biochemical characterizations such as catalase test, Citrate test, gram staining, were conducted following methods of (Boone *et al*., 2001). Ability to produce little extracellular hydrolytic enzyme production were also performed as following

Extracellular amylase test: Bacterial new spp cultures was screened for amylolytic activity following methods of Vashist et al., (2013) with little modification . The pure isolate colonies were streaked on Nutrient agar (NaCl 5 g/L; peptone 10 g/L; yeast extract 10 g/L; Agar 18 g/L at pH 7.0-7.2) supplemented with soluble starch (0.1%). After incubation at 37 ^{o}C for 24-48h , the individual plates were flooded with gram's iodine to produce a deep blue colored . The soluble starch was autoclaved separately using filter sterilization .Zones of utilization were recorded for potential amylotic isolates . Finally, data were computed and analyzed.

Catalase Test: For catalase test fresh 18-24 hours old pure bacterial colonies were used. Slide method was also used for catalase test. Briefly a loop full of the isolated bacteria was smeared on a clean microscope slide and 2-3 drops of 3% (v/v) H_2O_2 was added directly on the smear, the formation of visible bubbles indicates positive result (Elmanama, 2009).

Cellulase test: Cellulase producing isolates were screened oncarboxyl methylcellulose (0.5%)(w/v) agar plate. To visualize the clearing zone , the plates was flooded with an aqueous solution of congo red 1ml/ml for 15 min.The cellu-lolytic organism were produce a clearing

zone surrounding the colony due to the digestion of carboxy -methylcellulose (Apun*et al*.,2000).

Pectinase test: Mineral salts medium (MSM) (K_2HPO_4), 1.8g; NH_4Cl, 4.0g; $MgSO_4.7H_2O$, 0.2g;NaCl, 0.1g; $FeSO_4.7H_2O$ 0.01g in 1L of dH_2O water) were prepared .A1% pectin were added to MSM medium . A loop full of pure inoculum were Streaked on to the agar plates and incubated at 37ºC for 72 h finally , Zones of utilization for pectin were recorded for respective isolates (Vijayaraghavan et al.,2013).

Protease tests: protease test were conducted according to themethods of (Ribitsch*et al*., 2012) with modification. Nutrient agar medium was prepared and supplemented with 1% skim milk. A loopful pure isolates were taken and streaked onto medium andincubated at37ºC for 25 h. Finally, the zones of inhabition were recorded for respective proteolytic isolates.

3.10. Molecular characterization for identification of obtained PHA producing isolates.

3.10.1.Genomic DNA isolation for PHA producing isolates:

The extraction of DNA for the isolated bacterial sample was carried out using the QIAGEN DNA and RNA isolation kit following the procedures of the manufacturer. Briefly pure isolates of Polyhydroxyalkanoates (PHA) producer bacteria were taken by plastic loops and added into properly labeled 2ml eppendorf tubes containing about 0.6ml of Phosphate Buffered Saline (PBS). Then the tubes were spun 20,000 × g for 5 min in a centrifuge. After the liquid was decanted 180 µl of enzymatic lysis buffer was added to the tubes and vortexed 10-20 sec. After that the tubes were incubated at 37ºC for 30 min. After the incubation, consecutively 25 µl of proteinase K was added to the tube and then 200 µl of buffer AL was added to the tubes and vortexed briefly. After vortexing the tubes were incubated at 56ºC for 30min. After incubation 200 µl of 100% ethanol was added to the tubes and vortexed briefly. Using a micropipette the entire contents of the tube were transferred into labeled spin column. Then the columns were centrifuged at 10,000 × g for 1min. After centrifugation the columns were removed from the collection tube and were placed into new collection tubes in addition with 500 µl of buffer AW1 and centrifuged again at 10,000 × g for 1 min. Then the columns were removed from the

collection tubes and placed in a new collection tube in addition with AW2 and centrifuged at 20,000 × g for 3 min. Finally the tubes were carefully removed from the centrifuge and the columns were transferred to a 1.5 ml tube and 200 µl of buffer AE was added to the column. Then the column was let to stand at room temperature for 1 min prior to centrifugation at 10,000 × g for 1min. The column was discarded and the DNA was stored appropriately at -20ºC. (QIAgenDNeasy hand book, 2006). After the genetic material samples were received they were double extracted following the manufacturers instruction using Quick-DNA™ Fungal/Bacterial Miniprep Kit Catalog No. D6005 from Zymo Research. Its quality was evaluated on 1.5% (w/v) agarose gel, a single band of high-molecular weight DNA has been observed.

3.10.2.PCR analysis for PHA producing genes

The PCR amplification of PHA producing bacteria isolates were performed with I-179-L(5′-ACAGATCAACAAGTTCTACATCTTCGAC-3′&I-179-R (5′GGTGTTGTCGTT GTTCCAGT AGAGGATGTC-3′) primers that already designed for *Pseudomonas species* Solaiman et al., (2000) which is medium chain length polymers. The expected PCR products were 540bp. The PCR mixture were consisted of 0.5ul template DNA (l00ng/ml), 2µl of 2.5mM of dNTP, 1U units of Taq DNA polymerase (Sigma), 2 µl of 10X Taq buffer, 5 µl of each designed primers. Finally, the sterile distilled water was added to a final volume of 25µl (Solaiman et al., 2000). The program was used for the amplification of gene fragments with all primer combinations were cycle of 95 °C for 5 min, followed by 35 cycles of denaturation at 94°C for 20s, annealing at 57°C for 45 s, extension at 72°C for 1min and a final extension of 72°C for 5min and incubated at 4°C. PCR was performed in a thermal cycler (Flexcycler, Germany) extracted DNA were used for amplification. All reactions were held in the thermo cycler consisting of 25 µL containing 5× PCR buffer, 0.8 mM of each dNTP, 0.5µM of each primer, 1 Taq DNA polymerase, 1.25mM $MgCl_2$, and 5 the template DNA, 10.95 distilled water. PCR amplicons and 100bp molecular marker/DNA ladder was observed by electrophoresis in 1.5% (v/v) agarose gel further stained with ethidium bromide, and viewed under a Gel documentation device.

3.10.3.PCR Amplification for 16 DNA sequence Analysis

PCR was performed in a total volume of 25 µl containing 10 pmol each of forward and reverse primers, 2.5mM of $MgCl_2$, 200 µM each of the four deoxyribonucleotide

triphosphates (dNTPs), 0.5 U of Taq DNA polymerase, 1x concentration of PCR buffer (Invitrogen, Life Technologies, Brazil) and 50 to 100 ng of isolated bacterial genomic DNA. The template was denatured by heating at pre-denaturation of 95 °C for 5 min. This was followed by 39 cycles of denaturation 30 sec at 95 °C, 45 sec annealing and 1 min elongation at 72°C, with a final extension of 7 min at 72°C. The amplicons were resolved in 1.5% agarosegel using 0.5xtrisacetate-EDTA (TAE) buffer. Bi-directional DNA sequencing reaction of PCR amplicon was carried out with 8F& 1492R primers using BDT v3.1 Cycle sequencing kit on ABI 3500Dx Genetic AnalyzPrimers Used:

3.10.4.Sequence and Phylogenetic analysis

The 16SrRNA gene sequences were analyzed with Basic Local Alignment Search Tool (BLAST) program available on the National Center for Biotechnology Information (NCBI). The 16SrRNA genes sequence of newly isolated bacterial species and other taxonomically related strains was collected from Gen Bank database using EzTaxon server (https://www.ezbiocloud. net/identify. These strains were aligned by multiple sequence alignment program (Clustal W) using MEGA version 11.0.21software package. Phylo-genetic tree was performed with the MEGA version 11.0.21 software package. Phylo-genetic tree was performed for aligned sequence and evolutionary distances was done by neighbour joining method and Kimura two-parameter distance measure.. Confidence values was estimated from bootstrap analyses of 1,000 times. *Baccilus subtilis* $CS1^T$ (KC336487.1) was designated as out-group for phylogenetic tree and other related sequences of genes were acquired from EzTaxon server (https://www.ezbiocloud.net/identify). .

3.11. Optimization of pH, temperature and carbon source

3.11.1.Optimization of pH on growth of newly isolated PHA producer.

Different pH value of the medium was used to check whether the pH hasany effect on PHA production. Minimal Davis broth ((1.0g Dextrose, 7.0g K_2HPO_4, 1.0g $(NH_4)_2SO_4$, 0.5 g sodium citrate, 2.0g KH_2PO_4, 0.1 g $MgSO_4.7H_2O$ and pH 7.2 ± 0.2 at 25 ºC) with different pH value (6.0 ,7.2 ,10)was set and sterilized at 121ºC for 15min. The pH of the medium was adjusted by HCl orNaOH. After sterilization, a 2.5ml (v/v) of 24 h old cultures were taken and transferred to

250ml conical flasks containing 50 ml PHA production medium and supplemented with 1%filter-sterilized glucose (w/v) as a carbon source. The broth culturewas inoculated with bacterial isolates (2.5%, v/v) and incubated on a rotary shaker at 150 rpm and at 37ºC for 72 h. Finally, the pellets was centrifuged (10000 rpm, 10min at 25 ºC), extracted and quantified by data analysis

3.11.2.Optimization of temperature on growth of newly Isolated PHA producer

A Minimal Davis broth (1.0g Dextrose, 7.0g K_2HPO_4, 1.0g $(NH_4)_2SO_4$, 0.5 g sodium citrate, 2.0g KH_2PO_4, 0.1 g $MgSO_4.7H_2O$ and pH 7.2 ± 0.2 at 25 ºC) was prepared and sterilized at for 15 min. After sterilization, 50 ml of broth medium was poured into 250 ml conical flask and supplemented with 1% glucose (w/v) as carbon source. The bacterial isolates (2.5 %, v/v) were inoculated into broth medium. The cultures were incubated at various temperature (25, 30, and 37 ºC) ona rotary shaker at 150 rpm for 72 h. Finally, the pellets were centrifuged (10000 rpm, 10minat 25 ºC), extracted cell dry weight (CDW) and computed by Microsoft excel.

3.11.3.Optimization Of Carbon Source On Growth Newly Isolated PHA Producer

Optimization of various carbon source on PHA producer Isolate was studied. Briefly ,Minimal Davis Broth per liter (1.0g Dextrose, 7.0g K_2HPO_4, 1.0g $(NH_4)_2SO_4$, 0.5 g sodium citrate, 2.0g KH_2PO_4, 0.1 g $MgSO_4.7H_2O$ and pH 7.2 ± 0.2 at 25 ºC (pH7) was prepared and supplemented with 1% (w/v) of D-Fructose ,D- Glucose and Glactose as sole carbon source .The solubility of different carbon source were evaluated for growth of PHA producing isolate . A 2.5% (v/v) of over night grown pure culture of the isolate was used as in oculum in 100ml of production medium and incubated on a rotary shaker at 150 rpm and at 37 ^{0}C for 72 h. After the incubation hour the pellets was centrifuged (10000 rpm ,10min at 25ºC) extracted and quantified .The Data was computed by Microsoft-ecxel.

3.11.4.Optimization of Agro-waste as carbon source

To find out the preference of carbon sources and the ability of the isolated bacteria to utilize cheap agro-wastes as non-commercial carbon sources molasses and Bagass were used from wanji sugar factory as substitutes of commercial carbon sources. The carbon source from the two materials was prepared by drying and grinding the materials into fine powder using a

grinder. Then the ground waste materials were sieved by 355µm size sieves. Then 100ml of the extract was added as a carbon source with different consentration 1%,1.5% and 2% of both agrowest carbon soucrce onto a carbon free liquid media (7.0g K_2HPO_4, 1.0g $(NH_4)_2SO_4$, 0.5 g sodium citrate, 2.0g KH_2PO_4, 0.1 g $MgSO_4.7H_2O$ and pH 7.2 ± 0.2 at 25 ^oC (pH7)at pH 7.00). After autoclaving the media were transferred into 100ml Erlenmeyer flasks and inoculated of 24 hours old fresh inoculum of the isolated bacteria was inuclated. Then the inoculums were incubated for 72 hours with agitation of 150 rpm. After 72 hours the optical density (600nm) and the biomass of the cultured media were taken in triplicates and recorded.

3.12. Data management and analysis

All of the experiments done and measurements taken were in triplicates . Data analysis such as averages, standard deviations and standard errors were computed using Microsoft excel 2010.SPSS 20 and Origin Pro8.5 statistical soft-wares were used for data analysis and for plotting graphs. The quantitative data were summarized as descriptive statistics

4. Results

4.1. Isolationof PHA producing isolate

In the present study, a total of 40 bacterial isolates were isolated and purified from Lake Dambal Soil Sediment. These 40 isolates were found to be shown variation in terms of colonies color, elevation, size and odour. They grow better when the Minimal Davis agar (MDA) medium was supplied with 1% (w/v) carbon sourse (glucose).

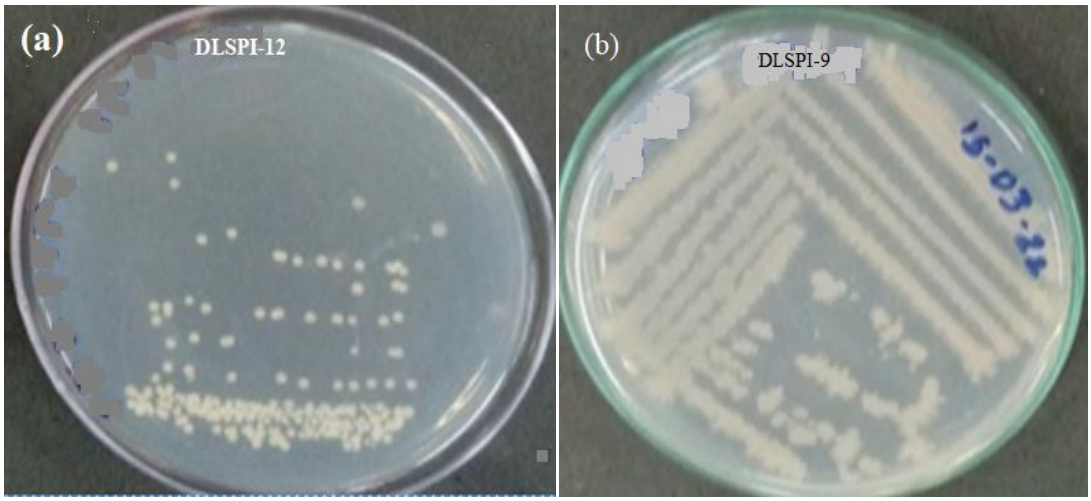

Figure.3.(a) Pure isolates these grown on MDB. (a) Indicates DLSPI-12 that isolated from soil Dambal lake soil sediment. (b) Indicates DLSPI-9 isolate shown spread growth on MDB that supplied with 1% glucose as a main carbon source.

4.2. Screening of PHA Producing Bacterial Isolated

4.2.1. Preliminary screening using SBB Staining

Out of these 40 isolates, 32 of them were shown positive results for SBB tests (Appendix 1;Table 1). In the present study, the PHA granules were shown as a distinct blue black after these isolates had been stained with SBB dye. However, the non-PHA granules parts observed as red color when these isolates were observed and stained with SBB reagents uder microscope (100x) as indicated (Figure 4a).

4.2.2. Secondary Screening Using NBA Staining

The SBB positive isolates (Figure 4b, and 4c) were characterized with NBA and found to be positive for PHA granules. As indicated in Figure 4b and 4c, distinict PHA granules appeared to have fluoresced as a bright orange color after these isolates had been stained with NBA dye against 72h old culture. For instance, the PHA granules of DLSPI-12 were observed as uniform pattern after this isolate had been supplied with glucose (1%, w/v) as a main carbon source (Figure 4b). In the present study, we realized that PHA granules appeared to have more affinity for NBA dyes than SBB dyes. As indicated in Table 3, the majority of the PHA producing isolates were found to be gram negative long rod-shaped. In the recent study, 26 isolates out of 32 isolates these shown positive results for NBA test. Cerain of the NBA positive isolates with a higher flurescence intensities were including such as DLSPI-2, DLSPI -7, DLSPI -8, DLSPI -24, DLSPI-14, DLSPI -18, and DLSPI 34. However, DLSPI-12 and DLSPI -24 were shown the highest fluerescence intensity with uniform pattern of granules when these isolates had been observed using flurescence microscope (100x) as shown figure 4b and c respectively . In the recent study, DLSPI -28 and DLSPI -32 were not shown positive results for NBA tests and hence no bright orange fluorescence emission.

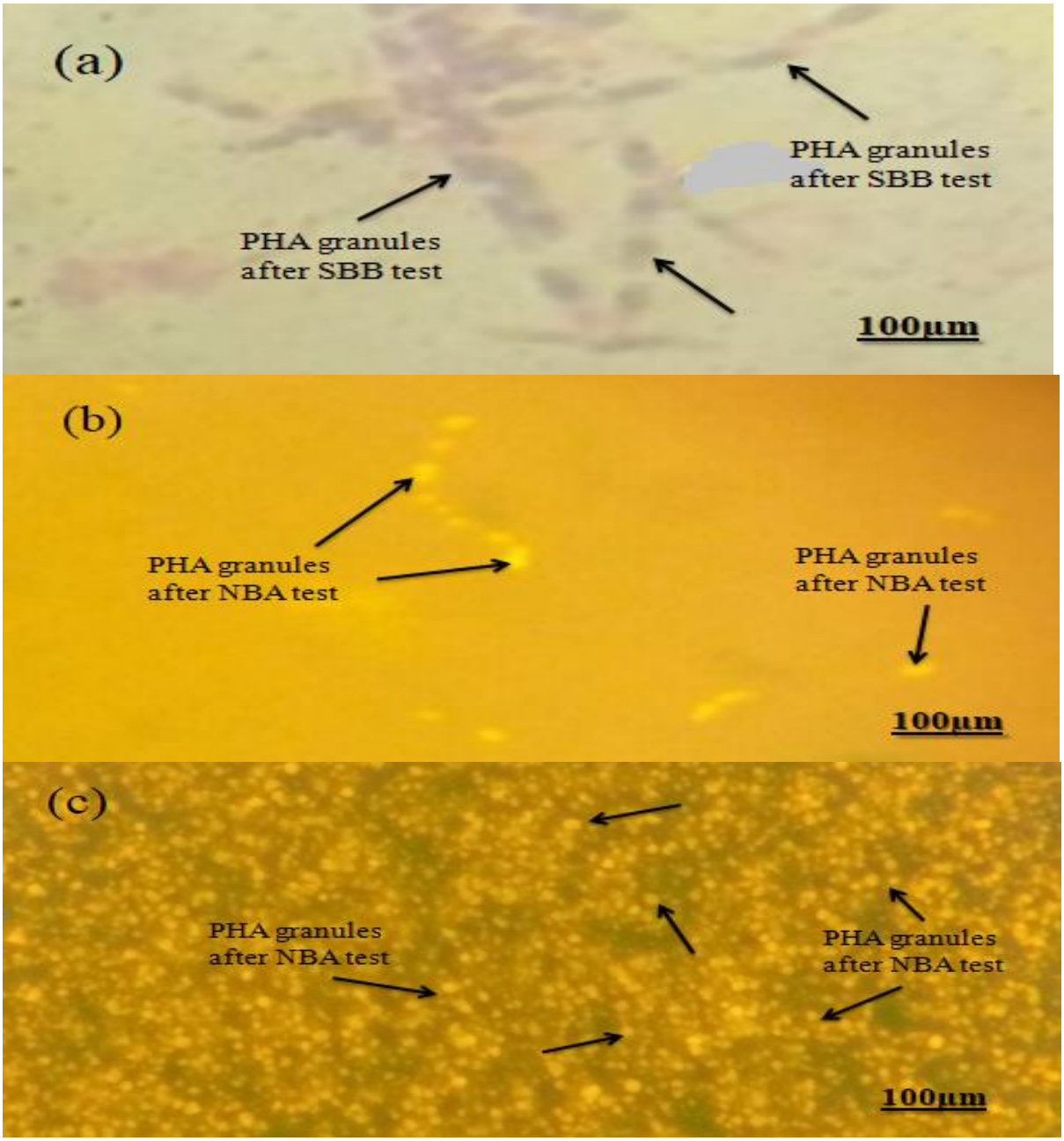

Figure .4 PHA producing isolate obtained from Dambal lake soil sediment (DLSPI-12). (a) Indicates PHA granules for DLSPI-12 isolate after staining with SBB which is Preliminary screening from 72h old broth culture. (b) Indicates PHA granules for DLSPI-12 isolate after staining with NBA which is secondary screening from 72h old culture. (c) NBA staining DLSPI -24 isolate using florensence microscope (100x) from 72h old culture that grown on MDB.

4.3. MALDI-TOF MS analysis for PHA producing bacteria identification

Diversity of these NBA positive bacteria isolates were identified by using MALD-TOF MS from soil sediments. Twenty six (65%) PHA producing potential isolates were identified using the extended direct transfer (eDT) method. In this study, these NBA positive isolates were identified as *Baccillu*s spp.*klebsiells* spp, *Psedomonas* spp, and *Staphlococcus* sp, However, the majority 14 (53.8%) of these isolates were derived to *Psedomonas* spp.(Table 3) using MALD-TOF MS. Of the 26 isolates that were correctly identified, 8 (30.7%) isolates were derived to the *Baccillu*s spp,, 1(3.8%) isolate to the *klebsiella pneumonia* and 1 (3.8%) isolate to the *Staphlococcus*sp.(Table 3). However, 2 (7.7%) isolates were unidentified (Table 3) in this study. Only two of the isolates could not be identified by the MALDI-TOF MS after two rounds of analysis.

Table 3: MALDI-TOF MS analysis for isolated PHA producing bacterial species obtained from Dambal soli sediment at sebbeta Animal Health diagnoostic insitute laboratory

S.N	Name of isolates	Possible identified isolates	Score value	S.N.	Name of isolates	Possible identified isolates	Score value
1	DLSPI -2	*P. aeruginosa*	2.46	14	DLSPI -18	*P. aeruginosa*	2.37
2	DLSPI -4	*B. cereus*	2.35	15	DLSPI -19	*P. aeruginosa*	2.23
3	DLSPI -6	*Staphylococcus sp.*	1.71	16	DLSPI -20	*B. subtilis*	2.05
4	DLSPI -7	*B. cereus*	2.3	17	DLSPI -21	*P. aeruginosa*	2.35
5	DLSPI -8	*B. cereus*	2.28	18	DLSPI -24	*B. cereus*	2.29
6	DLSPI -9	*B. subtilis*	2.26	19	DLSPI -25	*P. aeruginosa*	2.33
7	DLSPI-10	*P. aeruginosa*	2.32	20	DLSPI -23	*No identification*	0
8	DLSPI -11	*P. aeruginosa*	2.25	21	DLSPI -26	*P. aeruginosa*	2.41
9	DLSPI -12	*P. aeruginosa*	2.03	22	DLSPI -27	*No.identification*	0
10	DLSPI -13	*B. subtilis*	2.22	16	DLSPI-33	*P. aeruginosa*	2.31
11	DLSPI -14	*P. aeruginosa*	2.31	24	DLSPI-34	*P. aeruginosa*	2.36
12	DLSPI -16	*P. aeruginosa*	2	25	DLSPI-35	*B. subtilis*	1.96
13	DLSPI -17	*Klebsella pneumonia*	2.39	26	DLSPI-36	*P. aeruginosa*	1.98

4.4. Biochemical and Morphological characterization

The SBB and NBA positive more PHA affinity DLSPI-12 isolate showed various biochemical tests as indicated in (Table 4). The isolate is Gram negative and long rod-shaped. This isolate is also able to utilize various carbon souce for their growth (Table 4). The same isolate found to secret various enzymes. In this study, the DLSPI-12 isolate is posive for catalase test positive. Its optimum growth condition was in the range of pH6-10.

Table 4.Various biochemical test aginst newly isolated DLSPI-12 that isolated from soil sediment

S.N.	Various biochemical test	Result obtained against DLSPI-12 isolate from biochemical tests
	Morhological	
1	Shape	Long Rod
2	Colony color	Light yellow colony onMDA
3	Odour	+ve
4	Gram-staining	-ve
5	SBB staining	+ve
6	NBAstaining	+ve
7	Catalase	+ve
8	Extracellur amylase	-ve
9	Pectinase	-ve
10	Cellulase	+ve
11	Protease	+ve
12	D- glucose	+ve
13	D-Galactose	+ve
14	D-fructose	+ve
15	pH range	6.5-10

4.5. Enzyme activities for newly isolated *P. aeruginosa*DLSPI -12

P. aeruginosa DLSPI -12 was evalaued for certain biochemical tests and found to positive for enzymes such as cellulase, catalase, oxidase, and protease (Table4). However, this isolate was found to be negative for Extracellur amylase and Pectinase. According to biochemical resulst, *P. aeruginosa* DLSPI -12 was Gram positive, and positive for fructose, galactose and glucose frementation in the range of pH 6-10.

4.6. Molecular characterization for newly isolated pha producing bacterial spp

4.6.1. PCR detection of *phaC* sequences identification of newly isolated bacteria

Screening PHA producing bacterial isolates were confrimed using PCR which is quickly, and precisely technques for identification based on PHA producing gene ampilification. According to our findings *Psedomonas* spp with the highest MALDI TOF MS score values are the most abundant in terms of their ablity to produce PHA. Nine species of *Pseudomonas* were found to be positive for NBA test and PCR-based PHAgene ampilification. As shown in Figure 5, 540-bp PCR products were observed for these *Psedudomonas* spp. Using the I-179L/I-179R primer-pair. The PCR amplification for SBB and NBA positive *Bacillus* isolate was also observed for these with the maximum score value for MALDI TOF MS analysis. As illustrated in Figure 5, PHA producing *Bacillus* sp. DSLPI-9 isolate was meerly appeared to have shown PCR ampilification with 540bp product size.

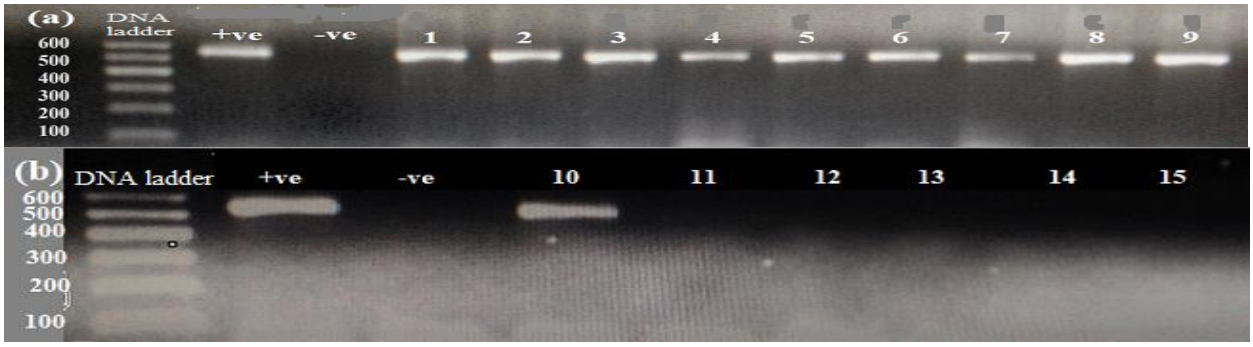

Fig. 5 (a) Amplification of PHA polymerase gene fragment for various *Psedomonas* strains (Lanes 1 -10) Lane indicated DNA marker with100bp, *psedomonas auroginosa* and E.coli were used as positive and negative control for PHA gene amplification. Lane-1 indcates P*sedomonas* sp. DLSPI-2, Lane 2 indcates P*sedomonas* sp. DLSPI-4, Lane 3 indicates *Psedomonas* sp. DLSPI-12, Lane 4 indicates P*sedomonas* sp. DLSPI-14, Lane 5 inidcates P*sedomonas* sp. DLSPI-16, Lane 6 indicates P*sedomonas* sp. DLSPI-26, , Lane 7 indicates P*sedomonas* sp. DLSPI-33, Lane 8 indicates P*sedomonas* sp. DLSPI-34 and Lane 9 indicates *Psedomonas* sp. DLSPI-36. (b) PCR amplification for PHA producing *Bacillus* spp. these isolated from soil sediment. Lane 10 indicates *Bacillus sp.*DLSPI -9, Lane 11 indicates *Bacillus* sp. DLSPI-20, Lane 12 indicates *Bacillus* sp. DLSPI-35, Lane 13 indicates *Bacillus* sp. DLSPI-8, Lane 14, *Bacillus* sp. DLSPI-24, and Lane 15 indicates *Bacillus* sp. DLSPI-7.

4.6.2. PCR amplification for the 16SrDNA for newly isolated PHA producing isolate

In this study, the newly isolated NBA positive (*Pseudomonas*sp.DLSPI-12) isolate shown sequence similarity with *Pseudomonas aeruginosa* JCM 5962^{T}(BAMA01000316 (96.88%) similarty as shown (Table 5). As indicated in Figure 6, the present NBA positive *Pseudomonas* sp. DLSPI-12 isolate is the clade with *Pseudomonas aeruginosa* JCM 5962^{T}(BAMA01000316) with different GC composition and total length (Table 5) of 16SrRNA gene sequence. The present isolate was closest to *Pseudomonas campi*S1-A32-2^{T}(MT415401) *Pseudomonas otitidis* MCC10330^{T}(AY953147),*Pseudomonas neuropathica* P155^{T}(LR797591), *Pseudomonas lalkuanensis* PE08^{T} (MF943158), *Pseudomonas mangiferae* DMKU BBB3-04^{T}(KY290607), *Pseudomonas oleovoranssubs p.Lubricantis*RS1^{T}(DQ842018),*Pseudomonas furukawaii* KF707^{T} (AJMR01000229) ,*Pseudomonas alcaligenes*NBRC 14159^{T} (BATI01000076), (Figure 6 and Table 5) which is not the same genus. *Pseudomonas humi*CCA1 ^{T}(LC145037) interms of sequence similartyas indicated in the Table 5.

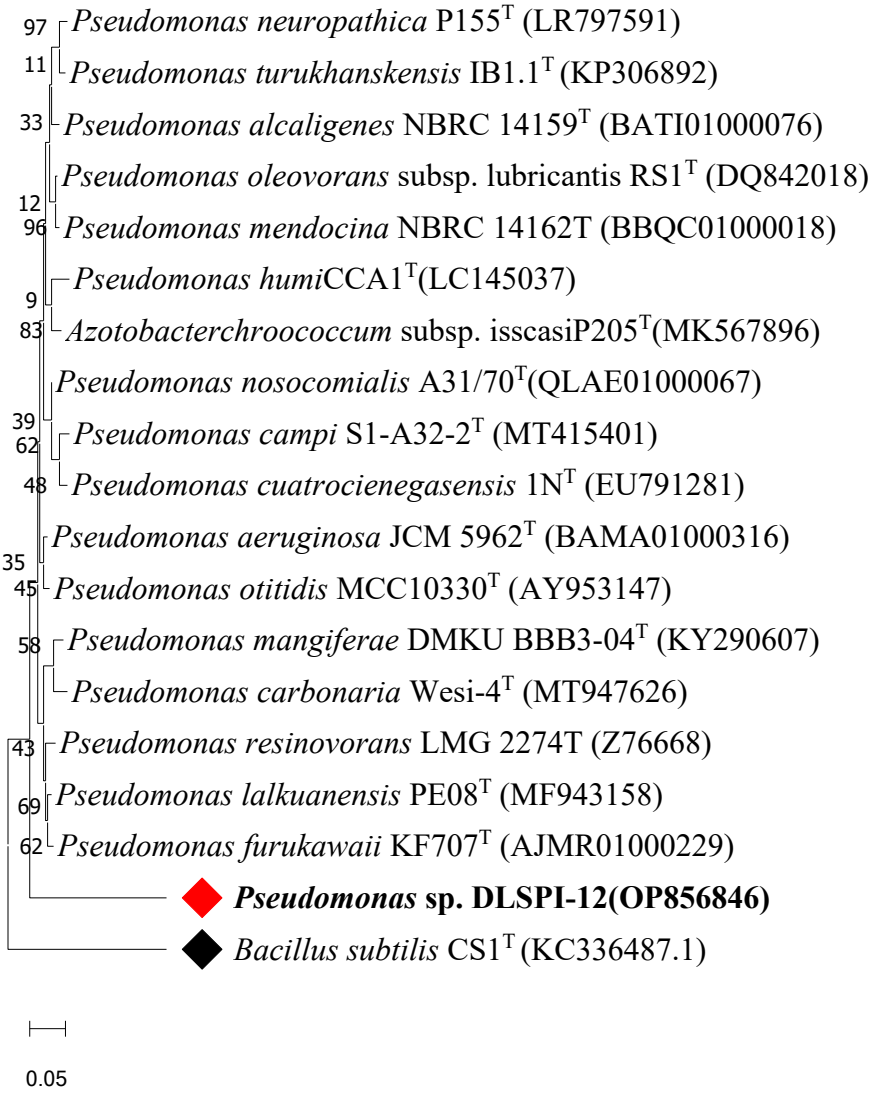

Figure 6.Evolutionary analysis by Maximum Likelihood method. The evolutionary history was inferred by using the Maximum Likelihood method and Kimura 2-parameter model. The tree with the highest log likelihood (-7403.49) is shown. The percentage of trees in which the associated taxa clustered together 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 Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree is drawn to scale (0.05), with branch lengths measured in the number of substitutions per site. This analysis involved 19 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. There were a total of 2044 positions in the final dataset with 500 bootstap of nucleotide. Evolutionary analyses were conducted in MEGA11

Table 5: Sequence similarity between our isolate and other strains with their respective GC% and accession numbers

S.N.	Sequence	Strain	Accession number	Total length (bp)	GC%	Sequence similarity with newly isolates strain (%)
1	*Pseudomonas* sp. DLSPI-12	DLSPI-12	**OP856846**	1387	53.39	Recent isolate
2	*Pseudomonas aeruginosa*	JCM 5962^T	BAMA01000316	1,458	54.32	96.90
3	*Pseudomonas campi*	S1-A32-2^T	MT415401	1,420	53.66	96.90
4	*Pseudomonas otitidis*	MCC10330^T	AY953147	1,458	53.91	96.44
5	*Pseudomonas neuropathica*	P155^T	LR797591	1,232	52.92	96.39
6	*Pseudomonas lalkuanensis*	PE08^T	MF943158	1,458	54.80	96.34
7	*Pseudomonas mangiferae*	DMKU BBB3-04^T	KY290607	1,438	54.94	96.27
8	*Pseudomonas oleovorans subsp. Lubricanti387s*	RS1^T	DQ842018	1,437	53.79	96.17
9	*Pseudomonas furukawaii*	KF707^T	AJMR01000229	1,459	54.42	96.17
10	*Pseudomonas alcaligenes*	NBRC 14159^T	BATI01000076	1,459	53.87	96.06
11	*Pseudomonas humi*	CCA1 ^{T}	LC145037	1,349	54.34	96.04
12	*Azotobacterchroococcum subsp. Isscasi*	P205^T	MK567896	1,410	55.25	95.88
13	*Pseudomonas carbonaria*	Wesi-4^T	MT947626	1,440	54.86	95.50
14	*Pseudomonas oleovorans subsp. Oleovorans*	DSM 1045^T	NIUB01000072	1,459	54.01	95.31
15	*Pseudomonas turukhanskensis*	IB1.1^T	KP306892	1,412	53.26	95.30
16	*Pseudomonas cuatrocienegasensis*	1N ^{T}	EU791281	1,430	54.06	95.30
17	*Pseudomonas resinovorans*	LMG 2274^T	Z76668	1,459	54.31	95.27
18	*Pseudomonas mendocina*	NBRC 14162^T	BBQC01000018	1,458	53.77	96.75
19	*Bacillus* subtilis	CS1^T	KC336487.1	1,458	55.08	Out group

4.7. Optimization of Growth Conditions for PHA positive Bactrial Isolates

Cultural conditions were optimized for the selected PHA producing bacterial isolatesDLSPI -12). Temperature, pH, and Carbon source preferences of selected PHA producing bacterial isolateswere tested.. All of the results are presented as Mean ± Standard Deviation (mean ± SD) of triplicates of OD_{600nm} and Biomass (BM) as cell dry weight (CDW g/l) measurements. Graph was done by SPS .

4.7.1. Optimization for pH and Temperature

In the present study, various pH values were found to affect the growth of SBB and NBA positive *Pseudomonas* sp. DLSPI-12 isolate. The minimum (0.00323±0.00024g/mL) and maximum (0.007013±0.0041g/L) CDW (g/mL) were recoded for *Pseudomonas* sp.DLSPI-12 isolate as indicated in Figure 7 at pH 10 and pH7, respectively. The *Pseudomonas* sp.DLSPI-12 isolate appeared to shown 0.003309±0.00104g/mL CDW at pH10. No significant variations were observed interms of CDW (g/mL) at 95 confidenec interval between pH6 & pH7(*P=0.317*), pH6 & pH10 (*P=0.983*), pH7 & pH10 (*P=0.326*) against *Pseudomonas* sp.DLSPI-12 isolate that isolated from soil sediment. As illustarted in Figure 7a, a SBB and NBA positive *Pseudomonas* sp.DLSPI-12 isolate appeared to give the highest OD_{600nm} (1.4276±0.0054) at pH7 and 25ºC when glucose was provided as the main carbon source. However, minimum OD_{600nm}(1.348±0.015, Figure 7a and Appendix file Table 6a) was detcetd for *Pseudomonas* sp.DLSPI-12 at pH6.

The optimum growth temperature (0.00345±4.41*10^{-5}g/L, (Figure 6b and Appendix file Table 6a) for *Pseudomonas* sp.DLSPI-12 was detected at 25ºC. However, the lowest cell Biomass (CDW, 0.0021±6.33*10^{-5} g/mL) was obatined at 37ºC after 48h of incubation period (Figure 7b and Appendix File Table 6a). As shown in Figure 7b, *Pseudomonas* sp.DLSPI-12 showesa highercell growth after the CDW (0.00323±3.84*10^{-5} g/mL) and OD_{600nm} (1.8507±0.0029) have been checked at 30ºC with optimum pH value. One Way anova at LSDtest, indicated that very significant variations were deteceted interms of cell biomass (CDW, g/mL) between 25&37ºC (*P=0.000*) and 30&37ºC (*P=0.000*) for SBB and NBA positive*Pseudomonas* sp.DLSPI-12. However, slight variations were observed between 25&30ºC (*p=0.018*) interms of CDW (g/mL) as indicated in Multiple Comparisons of Post Hoc Tests at LSD (Appendix File: Table 7b**).**

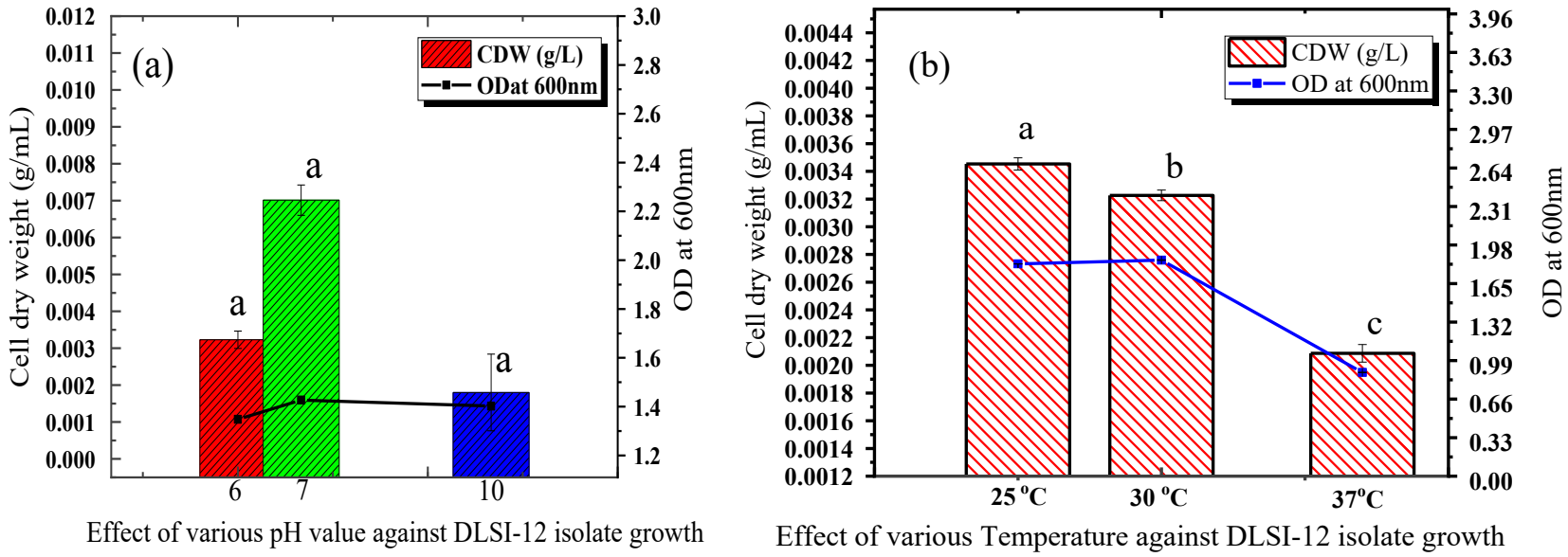

Fig. 7 Effect of various pH and temeprtaure against PHA producing isolate obtained from Dambal lake sediments (*Pseudomona* sp. DLSPI-12). a) Cell dry weight (CDW) (g/mL) obtained from DLSPI-12 isolate against various pH-values at 72h of incubation time. (b) CDW (g/L) obtained from DLSPI-12 isolate against various temperatures at 72h of incubation time. Data represented mean ± SEM of three replicate (n=3) of CDW (g/mL) against pH and temeprature. According to one way ANOVA, the CDW (g/mL) has no significant variation at 95% confidence interval by using LSD test (P> 0.05) for this isolates during growth on Minimal Devise broth (MDB) medium with the same latter on theirrespective bar.

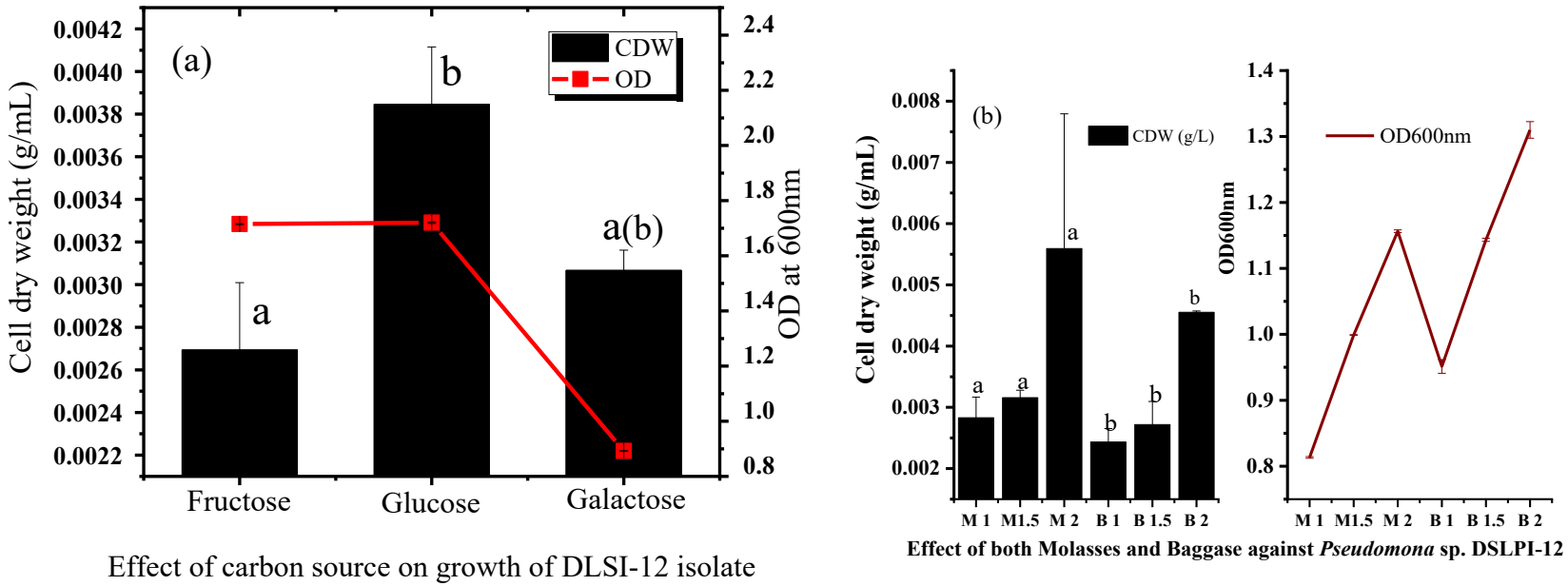

Figure 8. (a) Effect of various commercial carbon sources on (*Pseudomona* sp. DLSPI-12) isolate that obtained from Dambal lake sediments at optimum culture condition for 72h old cultures. Data represented mean ± SEM of three replicate (n = 3) of CDW (g/L) against pH and temeprature. (b) Effect of cost effective carbon source (molasses and Baggas) on (*Pseudomona* sp. DLSPI-12) isolate that obtained from Dambal lake sediments at optimum culture condition for 72h old cultures. Data represented mean ± SEM of three replicate (n = 3) of CDW (g/L) against Mollasses and Baggses. According to one way ANOVA, the CDW (g/L) has no significant variation at 95% confidence interval by using LSD test (P>0.05) for this isolates during growth on Minimal Devise broth (MDB) with the same latter on their respective bars.

The present potential PHA producing isolate (*Pseudomonas* sp. DSLPI-12) is able to utilize carbon source such as Fructose, glucose and Galactose as the main carbon source (1%, w/v) for its growth. The minimum (0.002693±0.00045g/mL) and maximum (0.003847±0.00038g/mL) CDW (g/mL) were obtained from *Pseudomonas* sp. DSLPI-12 with 1.715±0.003 and 1.705667±0.000577OD_{600nm}, for Fructose and Glucose, respectively as indicated in Figure 8a. The CDW (g/mL) and OD600nm were also documented for the present PHA granules (Figure 4a,b&c) producing isolate (Figure 8a &Appendex File: Table 2a). As indicated OneWayAnova analysis, no significant variation (*p=0.324*) was abosrved between Fructose and Galactose. However, great significant variation ($p<0.05$) (Appendex File: Table 9b) was detected between Fructose and Glucose as indicated in Figure 8a.

The optimum growth mollases and bagasses concentration (Figure 8b and Appendix File Table; 3b) for *Pseudomonas* sp.DLSPI-12 was detected at for concentration of mollases and Bagass at different percent such as 1%,1.5% and 2% respectively.However, the minimum cell Biomass (CDW, 0.002831±$5.839*10^{-5}$ g/L) was obatined at 1% (w/v) after 72h of incubation period (Figure 8b and Appendix File Table 3b). As shown in Figure 9a, *Pseudomonas* sp.DLSPI-12 showes a maximum cell growth after the CDW (0.005592±$3.84*10^{-4}$ g/L) and OD_{600nm} (1.156333±0.611258) have been checked at 2% mollases with optimum pH value. OneWay anova at LSD test, indicated that very significant variations were deteceted interms of cell biomass (CDW, g/mL) between 2% mollases &1%bagasse (*P=0.033*) and 2% mollasse &1.5% bagasse concentration(*P=0.049*) for SBBand NBApositive *Pseudomonas* sp.DLSPI-12 (Appendix file table 11 b). However, slight variations were observed between Molasse (2%, v/v)& Bagasse (1.5%w/v)(*p=0.018*) interms of CDW (g/mL) as indicated in Multiple Comparisons of Post Hoc Tests at LSD (Appendix file table 11 b).

4.8. Discussion

PHA producing isolates were the recent active research area to produce biological degradable and eco-friendly biopolymers. Screening and characterization of these isolates were also drawn considerable interest worldwide. To this reasoning, in the present study, certain SBB and NBA positive isolates which suggested for PHA biosynthesis were obtained from Lake Dambal (Ziway)soil sediment.

Certain collected bacterial isolates appeared to have granules when these isolates had been stained with SBB (Figure 4a &Appendix File: Table 1). These particular PHA granules appeared to have observed as blue black when they had been observed by microscope (100x) indicating that these isolates can be employed for PHA production at small scale level. These PHA granules were also found to be shown the highest affinity to SBB dye which is a presumptive test for identification of PHA producing isolates. This SBB tests for PHA producing isolate characterization might be employed as ataxonomic criterion for these bacterial classification. From the previous study, certain *Bacillus* spp Mohammed et al., (2019; Mohammed & Ray, (2022) were confirmed to be positive for SBB test. It has also reported that the SBB dye is shown the strongest affinity toword PHB granules and hence used to distinguish between PHA producers and non-producers (Legat et al., 2010). Similar results which obtained by other authors Murray et al. (1994) further demonstrated certain PHA and non-PHA producing potential species of bacteria can be discriminated from each other using SBB, a lipophilic dyes.

In the present study, more PHA producing isolates were confirmed by using NBA test. It was found that the NBA dye speceifically binding to the distinic part of PHA granules for 72h old pure cultures. In line with this study, certain PHA producing *Bacillus* spp were screened from soil sediment area from where plastic wastes are accumulated Mohammed et al., (2019); Mohammed & Ray,(2022) as reported for our previous work. Further study reported that Bhuwal et al.,(2013) NBA and Nile Red (NR) were the staining dyes most widly used to screen isolates that produced PHA in their natural ecosystems. According to earlier research,Ostle & Holt,(1982) reported the PHA producing bacterial isolates for the first time using NBA and NR which are the specific dyes these particulary binding to PHA granules. It has been recognized that all SBB positive isolates did not show positive for NBA test which is the secondary screening methods for PHA producing isolates. This indicates that the SBB dye might be stained

other than non-PHA granules part of bacterial cellular component which in agreement with Spiekermann et al., (1999) suggestion. According to study performed previously Kumar et al., (2018) NBA apparently has however, a greater affinity for PHA than SBB and results has indicated superior images with high contrast. Despite the fact that pathogenic bacterial isolate, *Klebsella pneumonia (*DLSPI-17) was found to positive for PHA granules after the isolate had been stained with NBA dyes which is in agreement with finding of (Valdez-Calderón1 et al., 2020).

In the present study, certain *Pseudomonas* spp, *Bacillus* spp*., klebciella* sp and *Staphylococcus alatea* were identified directly from pure isolate and isolated colony for these SBB and NBA positive bacterial isolates which were suggested for PHA biosyhthesis using protein spot. *Pseudomonas aeruginosa* is the most widly obtained isolates from soil sediment based on protein profiles using MALDI TOF MS analysis. In the previous study, The MALDI-TOF MS method discriminates bacteria on the basis of screening of characteristic peaks observed as biomarkers for nine (9) bacterial identification MALDI-TOF Koubek et al., (2012) which is less than the recent our identified isolates (26 isolates) to the species levels (Table 3). However, two of them out of the recent isolates were not identified. This could be rather than being the result of a methodological error or the failure of MALDI to detect these NBA positive bacterial isolates, it may be due to the fact that these isolates (DLSPI-23 and DLSPI-27) were not previously included in the databases or these isolates might be noble PHA isolates (DLSPI-23 & 27) producing. As indicated in MALDI TOF identification, A SBB and NBA positive *Staphylococcus* sp. DLSPI-6 was also isolated as apotential PHA granules formation. This indicated that the *Staphylococcus* sp. DLSPI-6 could employed for PHA biosynthesis which is a similar result to Melchor-Martínez et al.,(2022) reportes. They further identified isolates such as *Bacillus*, *Curtobacterium*, *Exiguobacterium, Halomonas* sp.*Paracoccus* sp. and *Planomicrobium* sp. as potential PHA producers. In contrary to our study, the previous study identified the PHA granule-associated proteins (PGAPs) (phaP) from *Haloferax mediterranei* Cai et al.,(2012*)* which is haloarchaeon using MALDI-TOF/TOF MS.

Certain bacterial cells are playing significant role for enzyme seceration. *Pesudomonas aeruginosa* DLSPI-12 able to produce cellulase, suggesting that this isolate can be employed to degrade cellulose which are constitutent various agro-wastes and hence applied for industrial application. *Pesudomonas aeruginosa* DLSPI-12 shown the highest clear zone suggesting this

isolate may produce tremandious amount of cellulase enzyme. This enzyme might be employed for various agricultural and industrial applications to degrade certain wastes these enriched with cellulosic materials. As indicated in the previous study, cellulase, protease and pectinase enzymes were produced by *Pesudomonas aeuroginosa* SD12 that isolated from Tannery waste polluted soil (Ray et al., 2010). However, our recent isolate did not produce pectinase and indicating that these *Pesudomonas aeuroginosa* SD12 and *Pesudomonas aeuroginosa* DLSPI-12 (present isolate) strains are varied.

The PHA producing isolates were characterized using PCR ampilification techniques. In this study, for certain long rod Gram negative *Pseudomona* spp, 540bp PCR products were obtained. The presence of this PHA gene product is employed to further validate the present PHA granules in these PHA producing potentail isolates these have laready confirmed by SBB and NBA tests. In line with this study, reported *phaC1/C2* gene ampilification for *P. citronellolis* NRRL B-2504, *Pseudomonas corrugata* 388, *P. fluorescens* ATCC 17824, *P. resinovorans* NRRL B-2649, and *P. putida* ATCC 17391, *P. viridiflava* ATCC 13223, using primer-pair, *I-179L* and *I-179R*. Similarly, the previous study Solaiman et al., (2000) confirmed the presence of 540 bp PHA gene amplicon for certain *Pseudomonas* spp. using the same primer-pair. The same authors screened 41 PHA producing bacteria out of 70 isolates and confirmed the presence of class I and II *phaC* genes amplification with around 550 bp and 540 bpPCR products.

Solaiman et al., (2000) finds the presence of *phaC1/C2* gene ampilifications were used to typify the isolates as medium chain length PHA (*mcl*PHA) producers microorganisms. Hence it is possible to suggested that the presence *phaC1/C2* gene our isolates which is positive for primer-pair, *I-179L* and *I-179R* positive PCR products might be *mcl*PHA producers. However, most SBB and NBA positive *Bacillus* spp. did not show for the presence of PHA gene ampilification except for *Bacillus subtilis* DLSPI-9 that have 540bp gene amplicon. This could be due to using irrelevant primer which is not designed for *Bacciluss* spp sets for PHA gene ampilifcation for these SBB and NBA positive bacterial isolates. From our study, we can realized that the presence of these genes are the most important molecular signatures for PHA synthetic ability of these isolates PHA producing isolates.

Our study indicates that this PHA-producing isolate has also shown more sequence similar to that of *Pseudomonas campi* S1-A32-2^{T} MT415401 and *Pseudomonas otitidis* MCC10330^{T}

AY953147 strains (Table 5). However, the GC composition and sequence between *Pseudomonas aeruginosa DLSPI-12* and other closely related have shown variation. This indicates that the recent isolate shows to be various when it was compared to the closest strain, *Pseudomonas aeruginosa* JCM 5962^{T} (BAMA01000316). In opposite to this study, it is revealed that López-Cortés et al.,(2008) the 16SrDNA sequences of E13 and C18 Mat obtained from Polluted Marine Microbial showed a 100% similarity with *Bacillus thuringiensis* DiSz8 sequence which is screened from a polluted sample of soil. The same authors reported that weathered granite have been recognized to be able to use as a source and habitat to harbor for PHA-producing C19 isolates which have shown a sequence similarity (99%) with endolithic *Bacillus megaterium* WN603. When *Paracoccus homiensis* DD-R11 grow on sandy beach sample, they are able to develop PHA granules and their sequence similarity (100%) were affiliated with E33, E45, and E46 strains. The sequence of strain C20R and E63 have been also shown 100% sequence similarity with gram-positive *Staphylococcus cohnii* (GTC) 728 strains. A 100% sequence similarity have been also observed between E4 and *Staphylococcus arlettae* (ATCC) (43957) sequences (López-Cortés et al.,2008).

In the present study, a SBB and NBA positive *Pseudomonas* sp. DLSPI -12 isolate gave significant amount of CDW (g/mL) and OD_{600nm} at various carbons ource, pH, and temparature. For instance, considerable amount of CDW (g/mL) and OD_{600nm} were obatined at various pH values (6-10pH) in the range of 0.0032±0.0002 to 0.007±0.0041 g/mL and 1.348±0.015 to 1.4267±0.0054, respectively (Figure7 and Appendix 2 File Table 4a). Habal et al.,(2007) finds 5.8 -9.7 g/l CDW from *Pseudomonas aeruginosa* 47T2 using various carbon sources (oleic acid, cooking oil and free fatty acid) and sodium nitrate as nitrogen source these included into mineral medium at 30ºC. However, ceratian *Bacillus* spp. Valappil et al.,(2006) were able to produce P(3HB) at pH4.5 which is contarary to the present our results. This could be due to type of strains involved in PHA biosynthesis and other culture condiction Chanasit et al.,(2016) has also previously reported that 3.65 g/L biomass is obtained from *Pseudomonas mendocina PSU* at pH 7.3 using a biodiesel liquid waste as the main carbon source which is greater biomass than the present isolate (0.007±0.0041 g/mL).

The CDW (g/mL) increased at 25 and 30ºC for *Pseudomonas* sp. DLSPI-12 indicating that this isolates prefered the mesophilic environment for more biomass and cellular formation. However, as the temperature rised, the CDW (g/mL) obtained for the present isolate is decreasing from

0.00345±4.41x10^{-5} to 0.0021±6.33 x10^{-5}g/mL at 37ºC as indicated in Figure 8. In agreement with the present study Maalej et al.,(2014), it has been reported that the exo-polysaccharide (EPS) biosyhthesis and cellular growth for *Pseudomonas stutzeri* AS22 were reduced by almost half at 30 and 37°C, respectively.

Pseudomonas sp. DLSPI-12 utilize fructose, glucose, and glactose as the main carbon sources (1%, w/v) for their growth. Various CDW (g/mL, Figure 8 and Appendix 2 File Table 2a) and OD_{600nm} were obtained with a significant variation ($p<0.05$) (Appendix File: Table 8_b) when these carbon source had been supplied in MDB medium. When glucose was employed as the only carbon source, more CDW (0.003847± 0.00027g/ml) and OD_{600nm}(1.72±0.0027) were obtained for *Pseudomona* sp. DLSPPI-12 (Figure 8 and Appendix File Table 8a) suggesting that this isolate might be produced more PHA content and productivity. In line with the present study, higher CDW (1.52g/L) was recorded for *Pseudomonas corrugata* Solaiman et al., (2000) that grown on E medium which supplemented with glucose as the main carbon source. Previous study have showed that *P. putida* $KT442^T$is used to produce high PHAwhen glycerol and glucose substrates are empoyed as the main carbon source(Dartiailh et al., 2021). when compared to the recent of our results, the highest CDW (1.62g/L) was recorded from *Pseudomonas corrugata* grown on E medium supplimentaed with oleic acid (Dartiailh et al., 2021). However, the lower CDW (0.00269±0.00031g/mL) is obatined from *Pseudomona* sp. DLSPPI-12 when this isolate was grown on MDB medium that supplemented with fructose (1%, w/v) which is a similar report to the previous study for *Bacillus* sp. BPPI-14 and BPPI-19(Mohammed et al., 2019). Previuos study revealed that the greatest growth of *Pseudomonas aeruginosa* MTCC 5210 were recorded when glucose had been employed as the main carbon sources. The same authors found the least OD_{600nm} for *Pseudomonas aeruginosa* MTCC 5210 employing the fructose as the only main carbon source.

In present study Cost effective carbon sources such as molasses and baggases had been found to employ for PHA granules biosynthesis. These cost effective carbon source such as Baggase and molasses were produce considerable amount of PHA granules and CDW (g/mL) from newly obtained *Pseudomonas* sp. DSLPI-12. The CDW (g/mL) increased when the concentration of molasses and bagasses was increased as shown (figure 8b and Appendix File Table 3b). One Way anova at LSD test, indicated that very significant variations were deteceted interms of cell

biomass (CDW, g/mL) between 2% mollases &1% bagasse (*P=0.033*) and 2% mollasse &1.5% bagasse concentration (*P=0.049*) for SBB and NBA positive *Pseudomonas* sp.DLSPI-12(Appendix File Table (10a and 11b)).When similar PHA synthesis and polymer yields were obtained utilizing sugarcane molasses as the only carbon source or with the addition of 2% (v/v) sugarcane mollases , the result obtained are both economical and environmental acceptable.

In line with this Mohammed & Ray,(2022) study newly isolated *Bacillus sp.* LPPI-18 has been found to able to accumulate PHA using different carbon sources. In this study, the maximum PC accumulation occurred from 60 to 72 h for these isolates. Molasses was found to be used as a carbon source for bacterial growth and PC accumulation. PC obtained from molasses is 28.53±3.96% with 0.48± 0.055 g/l CDW from *Bacillus sp.* LPPI-18. The study was revealed that molasses are cheap carbon sources that are able to give PHA yield at low concentration of molasses (10 g/L) at 37 °C (pH 7).

According to Naitam et al.,2022 , Bagasse is rich cellulosic waste generated from the sugarcane industry. A country like India, which is one of the leading countries in sugarcane production, also generates tonnes of bagasse as waste. After proper pretreatment, these bagasse hydrolysates can also be used for PHB production by employing various strains of PHA producing micro-organisms. Hydrolysis of bagasse released prominently glucose, xylose, arabinose, and other reducing sugars, xylose being produced at the highest concentration, 12 to 18 g L-1 under different severity conditions (Niatam et al .,2022)*R. eutropha* grown in media composed of bagasse hydrolysates 50-75% v/v and mineral medium 25-50% v/v, accumulated 55.6 to 60.2% PHB in respective media with cell dry mass production of 10-11.5 g L-1 cell dry mass production and PHB accumulation in media containing 100% bagasse hydrolysates were statistically at par with the results above mentioned.

5. Conclusionand Recommendation

5.1. Conclusion

In this study, the following points were derived with certain attributes: Various Bacteril cells were able to produce PHA granules. In this study, the PHA positive isolates were categorized and mainly beloing to *Pseudomonas* spp, *Bacillus* spp.,*Klebsella pneumonia,* and *Staphylococcus* sp. using MALDI TOF MS with various score value. *Pseudomonas aeruginosa* is the most frequently screened SBB and NBA positive isolate suggested for more PHA producing isolates in the present study.Based fluorescence intensity, out of 26 NBA positive isolates, *Pesudomonas aeruginosa* DLSPI-12 which is a Gram negative isolates was considred for optimization and found to be positive for fructose, galactose and glucose ferementation at optimum pH (7.0) and temperature (25ºC). PCR products for mclPHA gene amplification were also obtained for certain *Pseudomonas* spp and *Bacillus* sp. with 540bp amplicon size. Based on the biochemical results, and other experiments, it is possible to conclude that *Pesudomonas aeuruginosa* DLSPI-12 can be employed for degrading wastes in enrich with cellulosic materials, for production of bioplastics (PHA) which is biological degradable and eco-frendly from cheap agrowastes and other agricultural and industrial application with its limitted pathogenic incidence.

5.2. Recommendation

- Because of different reasons the full potential of the polyhydroxyalkanoates producing bactria of these bioplastic production was not studied leaving a gap in the knowledge of the PHAs producing bacteria of soil sediments. , so further investigation of the ability of the PHAs producing bacteria of these isolate is necessary.
- The PHAs producing bacteria abilities of the isolates soil sediment bacteria attention and this indicates there is the need to examine the ability of the isolates to be used as bio-plastic production ..
- The PHAs producing bacteria should be explored more in hopes of finding novel sources of bio-plastic producing microorganisms
- an enabling policy is essential for trying to cut products that can assist in substituting plastics.

6. Refrence

Acquavia, M. A., Pascale, R., Martelli, G., Bondoni, M., & Bianco, G. (2021). Natural polymeric materials: A solution to plastic pollution from the agro-food sector. *Polymers*, *13*(1), 1–39. https://doi.org/10.3390/polym13010158

Aljuraifani, A. A., Berekaa, M. M., & Ghazwani, A. A. (2019). Bacterial biopolymer (polyhydroxyalkanoate) production from low-cost sustainable sources. *MicrobiologyOpen*, *8*(6), 1–7. https://doi.org/10.1002/mbo3.755

Anjum, A., Zuber, M., Zia, K. M., Noreen, A., Anjum, M. N., & Tabasum, S. (2016). International Journal of Biological Macromolecules Microbial production of polyhydroxyalkanoates (PHAs) and its copolymers : A review of recent advancements. *International Journal of Biological Macromolecules*, *89*, 161–174. https://doi.org/10.1016/j.ijbiomac.2016.04.069

Balakrishna Pillai, A., Jaya Kumar, A., Thulasi, K., & Kumarapillai, H. (2017). Evaluation of short-chain-length polyhydroxyalkanoate accumulation in Bacillus aryabhattai. *Brazilian Journal of Microbiology*, *48*(3), 451–460. https://doi.org/10.1016/j.bjm.2017.01.005

Baptista, S. D. A. (2013). *Screening of polyhydroxyalkanoates producing bacteria isolated from marine ecosystems*.

Bedade, D. K., Edson, C. B., & Gross, R. A. (2021). Emergent approaches to efficient and sustainable polyhydroxyalkanoate production. *Molecules*, *26*(11), 1–55. https://doi.org/10.3390/molecules26113463

Behera, S., Priyadarshanee, M., & Das, S. (2022). Polyhydroxyalkanoates , the bioplastics of microbial origin : Properties , biochemical synthesis , and their applications Chemosphere Polyhydroxyalkanoates , the bioplastics of microbial origin : Properties , biochemical synthesis , and their applications. *Chemosphere*, *294*(February), 133723. https://doi.org/10.1016/j.chemosphere.2022.133723

Berekaa, M. M. (2012). Genotypic detection of polyhydroxyalkanoate-producing bacilli and characterization of phaC synthase of Bacillus sp. SW1-2. *Life Science Journal*, *9*(4), 518–529.

Bhattacharyya, A., Jana, K., & Haldar, S. (2015). *Integration of poly-3- (hydroxybutyrate-co-hydroxyvalerate) production by Haloferax mediterranei through utilization of stillage from rice-based ethanol manufacture in India and its techno-economic analysis*. https://doi.org/10.1007/s11274-015-1823-4

Bhuwal, A. K., Singh, G., Aggarwal, N. K., Goyal, V., & Yadav, A. (2013). *Isolation and Screening of Polyhydroxyalkanoates Producing Bacteria from Pulp , Paper , and Cardboard Industry Wastes*. *2013*.

Boey, J. Y., Mohamad, L., Khok, Y. Sen, Tay, G. S., & Baidurah, S. (2021). A review of the applications and biodegradation of polyhydroxyalkanoates and poly(Lactic acid) and its composites. *Polymers*, *13*(10). https://doi.org/10.3390/polym13101544

Chaitanya, K., Mahmood, S. K., Kausar, R., & Sunilkumar, N. (2014). *Biotechnological Production of Polyhydroxyalkonates by Various Isolates : A Review*. *3*(9), 1–11.

Chanasit, W., Hodgson, B., Sudesh, K., & Umsakul, K. (2016). Efficient production of polyhydroxyalkanoates (PHAs) from Pseudomonas mendocina PSU using a biodiesel liquid waste (BLW) as the sole carbon source. *Bioscience, Biotechnology, and Biochemistry*, *80*(7), 1444–1454. https://doi.org/10.1080/09168451.2016.1158628

Chaudhry, W. N., Jamil, N., Ali, I., Ayaz, M. H., & Hasnain, S. (2011). *Screening for polyhydroxyalkanoate (PHA) -producing bacterial strains and comparison of PHA production from various inexpensive carbon sources*. 623–629. https://doi.org/10.1007/s13213-010-0181-6

Chee, J., Yoga, S., Lau, N., Ling, S., & Abed, R. M. M. (2010). *Bacterially Produced Polyhydroxyalkanoate (PHA): Converting Renewable Resources into Bioplastics*. *May 2014*, 1395–1404.

Chen, G.-Q. (2010). *Plastics Completely Synthesized by Bacteria: Polyhydroxyalkanoates*. *14*, 17–37. https://doi.org/10.1007/978-3-642-03287-5_2

Chen, P., Semblante, G. U., & You, S. (2012). *Detection of Polyhydroxyalkanoate-Accumulating Bacteria from Domestic Wastewater Treatment Plant Using Highly Sensitive PCR Primers This study*. *22*, 1141–1147.

Chen, Z. Q., Deng, Y., Huang, L., Wen, Q. X., & Guo, Z. R. (2013). Influence of substrate

concentration on PHA production using fermented sugar cane as substrate. *Huanjing Kexue/Environmental Science*, *34*(6), 2295–2301. https://doi.org/10.13227/j.hjkx.2013.06.017

Chou, H., Chen, C., & Wang, H. (2021). *Connecting Three Detection Methods To Screen Polyhydroxyalkanoicates Producing Bacteria In General Nature Region Experimental*. 1–16.

Ciesielski, S., Pokoj, T., Mozejko, J., & Klimiuk, E. (2013). Molecular identification of polyhydroxyalkanoates-producing bacteria isolated from enriched microbial community. *Polish Journal of Microbiology*, *62*(1), 45–50. https://doi.org/10.33073/pjm-2013-005

Comăniţă, E. D., Hlihor, R. M., Ghinea, C., & Gavrilescu, M. (2016). Occurrence of plastic waste in the environment: Ecological and health risks. *Environmental Engineering and Management Journal*, *15*(3), 675–685. https://doi.org/10.30638/eemj.2016.073

Correa-Galeote, D., Argiz, L., Val Del Rio, A., Mosquera-Corral, A., Juarez-Jimenez, B., Gonzalez-Lopez, J., & Rodelas, B. (2022). Dynamics of PHA-Accumulating Bacterial Communities Fed with Lipid-Rich Liquid Effluents from Fish-Canning Industries. *Polymers*, *14*(7), 1–22. https://doi.org/10.3390/polym14071396

Dartiailh, C., Blunt, W., Sharma, P. K., Liu, S., & Cicek, N. (2021). *The Thermal and Mechanical Properties of Medium Chain-Length Polyhydroxyalkanoates Produced by Pseudomonas putida LS46 on Various Substrates*. *8*(January), 1–9. https://doi.org/10.3389/fbioe.2020.617489

El-malek, F. A., Khairy, H., Farag, A., & Omar, S. (2020a). International Journal of Biological Macromolecules The sustainability of microbial bioplastics , production and applications. *International Journal of Biological Macromolecules*, *157*, 319–328. https://doi.org/10.1016/j.ijbiomac.2020.04.076

El-malek, F. A., Khairy, H., Farag, A., & Omar, S. (2020b). Jo ur l P re of. *International Journal of Biological Macromolecules*. https://doi.org/10.1016/j.ijbiomac.2020.04.076

Getachew, A., & Woldesenbet, F. (2016). Production of biodegradable plastic by polyhydroxybutyrate (PHB) accumulating bacteria using low cost agricultural waste material. *BMC Research Notes*, 1–9. https://doi.org/10.1186/s13104-016-2321-y

Guo, W., Duan, J., Geng, W., Feng, J., Wang, S., & Song, C. (2013). Comparison of medium-chain-length polyhydroxyalkanoates synthases from Pseudomonas mendocina NK-01 with the same substrate specificity. *Microbiological Research*, *168*(4), 231–237. https://doi.org/10.1016/j.micres.2012.11.003

Gupta, J., Rathour, R., Medhi, K., Tyagi, B., & Thakur, I. S. (2020). 3. Microbial-derived natural bioproducts for a sustainable environment: a bioprospective for waste to wealth. In *Refining Biomass Residues for Sustainable Energy and Bioproducts*. Elsevier Inc. https://doi.org/10.1016/B978-0-12-818996-2.00003-X

Hokamura, A., Wakida, I., Miyahara, Y., Tsuge, T., Shiratsuchi, H., Tanaka, K., & Matsusaki, H. (2015). Biosynthesis of poly (3-hydroxybutyrate- co -3-hydroxyalkanoates) by recombinant Escherichia coli from glucose. *Journal of Bioscience and Bioengineering*, *xx*(xx), 6–11. https://doi.org/10.1016/j.jbiosc.2015.01.022

Hussein, B. A., Tsegaye, A. A., & Abdulahi, A. (2021). *Assessment of the Environmental and Health Impacts of disposal Plastics in Gode town , Somali regional state , Eastern Ethiopia*. *2508*(3), 455–471.

Ibrahim , Farah Syazwani Shahar , Mohamed Thariq Hameed Sultan , Ain Umaira Md Shah, S. N. A. S. 1 and M. H. M. Y. 2. (2021). Product Packaging. *The Ellis Island Snow Globe*, 207–238. https://doi.org/10.2307/j.ctv11cw45p.12

Idris, S., Rahim, R. A., & Amirul, A. A. A. (2022). Bioprospecting and Molecular Identification of Used Transformer Oil-Degrading Bacteria for Bioplastics Production. *Microorganisms*, *10*(3), 1–12. https://doi.org/10.3390/microorganisms10030583

Jabeen, N., Majid, I., & Nayik, G. A. (2015). *Bioplastics and food packaging : A review*. 1–6. https://doi.org/10.1080/23311932.2015.1117749

Jaber, N. N. (2019). *Isolation and Identification of Polyhydroxyalkanoates from two Strains of Clostridium Bifermentans Isolated from the Soil Near the Gas Station in Basrah City*. *5*(4), 9888–9892. https://doi.org/10.26717/BJSTR.2019.13.002382

Jankova, V. (2018). *Bioplastics: Opportunities and Challenges*.

Jankowska, E., Gorman, M. R., & Frischmann, C. J. (2022). Transforming the Plastic Production System Presents Opportunities to Tackle the Climate Crisis. *Sustainability (Switzerland)*,

14(11). https://doi.org/10.3390/su14116539

Keshavarz, T., & Roy, I. (2010). Polyhydroxyalkanoates : bioplastics with a green agenda. *Current Opinion in Microbiology*, *13*(3), 321–326. https://doi.org/10.1016/j.mib.2010.02.006

Koller, M. (2017). *Production of Poly Hydroxyalkanoate (PHA) biopolyesters by extremophiles ? 1*(2), 69–85. https://doi.org/10.15406/mojps.2017.01.00011

Koller, M., & Braunegg, G. (2015). *Potential and Prospects of Continuous Polyhydroxyalkanoate (PHA) Production*. 94–121. https://doi.org/10.3390/bioengineering2020094

Koller, M., & Koller, M. (2017). Advances in alkanoate (PHA) Production Edited by Special Issue Editor. *Bioengineering*, 20.

Koubek, J., Uhlik, O., Jecna, K., Junkova, P., Vrkoslavova, J., Lipov, J., Kurzawova, V., Macek, T., & Mackova, M. (2012). Whole-cell MALDI-TOF: Rapid screening method in environmental microbiology. *International Biodeterioration and Biodegradation*, *69*, 82–86. https://doi.org/10.1016/j.ibiod.2011.12.007

Kourmentza, C., Plácido, J., Venetsaneas, N., Burniol-Figols, A., Varrone, C., Gavala, H. N., & Reis, M. A. M. (2017). Recent advances and challenges towards sustainable polyhydroxyalkanoate (PHA) production. *Bioengineering*, *4*(2), 1–43. https://doi.org/10.3390/bioengineering4020055

Kumar, M., Rathour, R., Singh, R., Sun, Y., Pandey, A., Gnansounou, E., Lin, K. A., Tsang, D. C. W., & Shekhar, I. (2020). Bacterial polyhydroxyalkanoates : Opportunities , challenges , and prospects. *Journal of Cleaner Production*, *263*, 121500. https://doi.org/10.1016/j.jclepro.2020.121500

Kumar, V., Thakur, V., Kumar, S., & Singh, D. (2018a). *Bioplastic reservoir of diverse bacterial communities revealed along altitude gradient of Pangi-Chamba trans-Himalayan region*. *October 2017*, 1–9. https://doi.org/10.1093/femsle/fny144

Kumar, V., Thakur, V., Kumar, S., & Singh, D. (2018b). *Bioplastic reservoir of diverse bacterial communities revealed along altitude gradient of Pangi-Chamba trans-Himalayan region*. *June*, 1–9. https://doi.org/10.1093/femsle/fny144

Legat, A., Gruber, C., Zangger, K., Wanner, G., & Stan-lotter, H. (2010). *Identification of polyhydroxyalkanoates in Halococcus and other haloarchaeal species*. 1119–1127. https://doi.org/10.1007/s00253-010-2611-6

Licciardello, G., Catara, A. F., & Catara, V. (2019). Production of polyhydroxyalkanoates and extracellular products using Pseudomonas corrugata and P. mediterranea: A review. *Bioengineering*, *lcl*. https://doi.org/10.3390/bioengineering6040105

Lin, J., Lee, M., Sue, Y., Liu, Y., & Li, S. (2017). *Cloning of phaCAB genes from thermophilic Caldimonas manganoxidans in Escherichia coli for poly (3-hydroxybutyrate) (PHB) production*. https://doi.org/10.1007/s00253-017-8386-2

López-Cortés, A., Lanz-Landázuri, A., & García-Maldonado, J. Q. (2008). Screening and isolation of PHB-producing bacteria in a polluted marine microbial mat. *Microbial Ecology*, *56*(1), 112–120. https://doi.org/10.1007/s00248-007-9329-8

Luengo, M. Ã., & Ã, A. S. (2003). *Bioplastics from microorganisms Bioplastics from microorganisms ´ n Garcı*. *5274*(July). https://doi.org/10.1016/S1369-5274(03)00040-7

Marjadi, D., & Dharaiya, N. A. (2018). *Isolating Potential Microorganisms for Production of Poly-a- Hydroxybutyrate: A Better Option for Biodegradable Plastic*. *October*.

Melchor-Martínez, E. M., Macías-Garbett, R., Alvarado-Ramírez, L., Araújo, R. G., Sosa-Hernández, J. E., Ramírez-Gamboa, D., Parra-Arroyo, L., Alvarez, A. G., Monteverde, R. P. B., Cazares, K. A. S., Reyes-Mayer, A., Lino, M. Y., Iqbal, H. M. N., & Parra-Saldívar, R. (2022). Towards a Circular Economy of Plastics: An Evaluation of the Systematic Transition to a New Generation of Bioplastics. *Polymers*, *14*(6), 1–32. https://doi.org/10.3390/polym14061203

Misgana, B., & Tucho, G. T. (2022). *Assessment of Community ' s Perception Toward Single-Use Plastic Shopping Bags and Use of Alternative Bags in Jimma Town , Ethiopia*. https://doi.org/10.1177/11786302221085047

Mohammed, S., Panda, A. N., & Ray, L. (2019a). International Journal of Biological Macromolecules An investigation for recovery of polyhydroxyalkanoates (PHA) from Bacillus sp . BPPI-14 and Bacillus sp . BPPI-19 isolated from plastic waste land fi ll. *International Journal of Biological Macromolecules*, *134*, 1085–1096.

https://doi.org/10.1016/j.ijbiomac.2019.05.155

Mohammed, S., Panda, A. N., & Ray, L. (2019b). PT SC. In *International Journal of Biological Macromolecules*. Elsevier B.V. https://doi.org/10.1016/j.ijbiomac.2019.05.155

Mohammed, S., & Ray, L. (2022). Polyhydroxyalkanoate recovery from newly screened Bacillus sp . LPPI - 18 using various methods of extraction from Loktak Lake sediment sample. *Journal of Genetic Engineering and Biotechnology*, *7*. https://doi.org/10.1186/s43141-022-00392-7

Montenegro, E. M. da S., Delabary, G. S., da Silva, M. A. C., Andreote, F. D., & Lima, A. O. de S. (2017). Molecular diagnostic for prospecting polyhydroxyalkanoate-producing bacteria. *Bioengineering*, *4*(2), 1–10. https://doi.org/10.3390/bioengineering4020052

Muhammadi, Shabina, Afzal, M., & Hameed, S. (2015). Bacterial polyhydroxyalkanoates-eco-friendly next generation plastic: Production, biocompatibility, biodegradation, physical properties and applications. *Green Chemistry Letters and Reviews*, *8*(3–4), 56–77. https://doi.org/10.1080/17518253.2015.1109715

Mulu, B. D., & Abraha, G. (2012). The Environmental Impacts of the Disposal of Plastic Bags and Water Bottles. *Sacha Journal of Environmental Studies*, *2*(1), 81–94.

Munir, S., Iqbal, S., & Jamil, N. (2015). Polyhydroxyalkanoates (PHA) production using paper mill wastewater as carbon source in comparison with glucose. *Journal of Pure and Applied Microbiology*, *9*(Special Edition 1), 453–460.

Narayanan, M., Kandasamy, S., Kumarasamy, S., Gnanavel, K., Ranganathan, M., & Kandasamy, G. (2020). Screening of polyhydroxybutyrate producing indigenous bacteria from polluted lake soil. *Heliyon*, *6*(10), e05381. https://doi.org/10.1016/j.heliyon.2020.e05381

Ostle, A. G., & Holt, J. G. (1982). Fluorescent Stain for Poly-3- Hydroxybutyrate. *Applied and Environmental Microbiology*, *44*(1), 238–241.

Philip, S., Keshavarz, T., & Roy, I. (2007). *Polyhydroxyalkanoates : biodegradable polymers with a range of applications*. *247*(July 2006), 233–247. https://doi.org/10.1002/jctb

Plastics Europe, G. M. R., & Conversio Market & Strategy GmbH. (2019). *Plastics - the Facts 2019*. 14, 35. https://www.plasticseurope.org/en/resources/market-data

Porras, M. A., Villar, M. A., & Cubitto, M. A. (2017). Novel spectrophotometric technique for rapid determination of extractable PHA using Sudan black dye. *Journal of Biotechnology*, *255*(April), 28–32. https://doi.org/10.1016/j.jbiotec.2017.06.012

Porras, M. A., Vitale, C., Villar, M. A., & Cubitto, M. A. (2016). Bioconversion of glycerol to poly(HB-co-HV) copolymer in an inexpensive medium by a Bacillus megaterium strain isolated from marine sediments. *Biochemical Pharmacology*. https://doi.org/10.1016/j.jece.2016.11.012

Ray, A. K., Roy, T., Mondal, S., & Ringö, E. (2010). *Identification of gut-associated amylase , cellulase and protease-producing bacteria in three species of Indian major carps*. 1462–1469. https://doi.org/10.1111/j.1365-2109.2009.02437.x

Reddy, C. S. K., Ghai, R., & Kalia, V. C. (2003). *Polyhydroxyalkanoates : an overview*. *87*, 137–146.

Reddy, V. U. N., Ramanaiah, S. V., Reddy, M. V., & Chang, Y.-C. (2022). Review of the Developments of Bacterial Medium-Chain-Length Polyhydroxyalkanoates (mcl-PHAs). *Bioengineering*, *9*(5), 225. https://doi.org/10.3390/bioengineering9050225

Rehm, B. H. A. (2006). *Genetics and biochemistry of polyhydroxyalkanoate granule self-assembly : The key role of polyester synthases*. 207–213. https://doi.org/10.1007/s10529-005-5521-4

Rehman, S., Jamil, N., & Husnain, S. (2007). *Screening of different contaminated environments for polyhydroxyalkanoates-producing bacterial strains*. *1999*, 650–656. https://doi.org/10.2478/s11756-007-0144-y

Saharan, B., & Sharma, D. (2015). Bioplastics-For Sustainable Development : A Review Bioplastics-For Sustainable Development : A Review. *International Journal of Microbial Resource Technology*, *1*(April), 10–23.

Sakthiselvan, P., & Madhumathi, R. (2019). Optimization on Microbial Production of Polyhydroxybutyrate (PHB): A Review. *International Journal of Research and Analytical Reviews*, *6*(1), 243–251.

Samrot, A. V., Samanvitha, S. K., Shobana, N., Renitta, E. R., Kumar, P. S., Kumar, S. S., Abirami, S., Dhiva, S., Bavanilatha, M., Kumar, P. P., Saigeetha, S., Shree, K. S., &

Thirumurugan, R. (2021). The synthesis, characterization and applications of polyhydroxyalkanoates (Phas) and pha-based nanoparticles. *Polymers*, *13*(19), 1–29. https://doi.org/10.3390/polym13193302

Sandoval, Cho, S., Saratale, G. D., Kumar, M., Bharagava, R. N., Varjani, S., Kadam, A. A., Ghodake, G. S., Palem, R. R., Mulla, S. I., Kim, D., & Shin, H. (2021). *An Overview of Recent Advancements in Microbial Polyhydroxyalkanoates (PHA) Production from Dark Fermentation Acidogenic Effluents : A Path to an*. 1–20.

Santhanam, A., & Sasidharan, S. (2010). Microbial production of polyhydroxy alkanotes (PHA) from Alcaligens spp. and Pseudomonas oleovorans using different carbon sources. *African Journal of Biotechnology*, *9*(21), 3144–3150. https://doi.org/10.4314/ajb.v9i21.

Sathiyanarayanan, G., Saibaba, G., Kiran, G. S., & Selvin, J. (2013). International Journal of Biological Macromolecules A statistical approach for optimization of polyhydroxybutyrate production by marine Bacillus subtilis MSBN17. *International Journal of Biological Macromolecules*, *59*, 170–177. https://doi.org/10.1016/j.ijbiomac.2013.04.040

Sayeski, K. L. (2016). Challenges and Opportunities. *Teaching Exceptional Children*, *48*(3), 126–127. https://doi.org/10.1177/0040059915619928

Sehgal, R., & Gupta, R. (2020). Polyhydroxyalkanoate and its efficient production : an eco - friendly approach towards development. *3 Biotech*, 1–14. https://doi.org/10.1007/s13205-020-02550-5

Selvamurugan, M., & Sivakumar, P. (2019). *Bioplastics – An Eco-Friendly Alternative to Petrochemical Plastics*. *14*(1).

Shamala, T. R., Chandrashekar, A., Vijayendra, S. V. N., & Kshama, L. (2003a). *Identification of polyhydroxyalkanoate (PHA) -producing Bacillus spp . using the polymerase chain reaction (PCR). 1991*, 369–374.

Shamala, T. R., Chandrashekar, A., Vijayendra, S. V. N., & Kshama, L. (2003b). Identification of polyhydroxyalkanoate (PHA)-producing Bacillus spp. using the polymerase chain reaction (PCR). *Journal of Applied Microbiology*, *94*(3), 369–374. https://doi.org/10.1046/j.1365-2672.2003.01838.x

Sheu, D. S., Wang, Y. T., & Lee, C. Y. (2000). Rapid detection of polyhydroxyalkanoate-

accumulating bacteria isolated from the environment by colony PCR. *Microbiology*, *146*(8), 2019–2025. https://doi.org/10.1099/00221287-146-8-2019

Sheu, D., Wang, Y., & Lee, C. (2019). *Rapid detection of polyhydroxyalkanoate- accumulating bacteria isolated from the environment by colony PCR*. *2000*, 2019–2025.

Singh, M., Patel, S. K. S., & Kalia, V. C. (2009). Bacillus subtilis as potential producer for polyhydroxyalkanoates. *Microbial Cell Factories*, *9*(1), 1–11. https://doi.org/10.1186/1475-2859-8-38

Singh Saharan, B., Grewal, A., & Kumar, P. (2014). Biotechnological Production of Polyhydroxyalkanoates: A Review on Trends and Latest Developments. *Chinese Journal of Biology*, *2014*, 1–18. https://doi.org/10.1155/2014/802984

Sohail, R., Jamil, N., Ali, I., & Munir, S. (2020). Animal fat and glycerol to a pair A , A of bioconversion by produced water bacteria. *E-Polymers*, *20*, 92–102.

Solaiman, D. K. Y., Ashby, R. D., & Foglia, T. A. (2000). Rapid and specific identification of medium-chain-length polyhydroxyalkanoate synthase gene by polymerase chain reaction. *Applied Microbiology and Biotechnology*, *53*(6), 690–694. https://doi.org/10.1007/s002530000332

Spiekermann, P., Rehm, B. H. A., Kalscheuer, R., Baumeister, D., & Steinbüchel, A. (1999). *A sensitive , viable-colony staining method using Nile red for direct screening of bacteria that accumulate polyhydroxyalkanoic acids and other lipid storage compounds*. 73–80.

Thompson, R. C., Moore, C. J., Saal, F. S. V., & Swan, S. H. (2009). Plastics, the environment and human health: Current consensus and future trends. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *364*(1526), 2153–2166. https://doi.org/10.1098/rstb.2009.0053

Tibebe, D., Zewge, F., Lemma, B., & Kassa, Y. (2022). Assessment of spatio - temporal variations of selected water quality parameters of Lake Ziway , Ethiopia using multivariate techniques. *BMC Chemistry*, 1–18. https://doi.org/10.1186/s13065-022-00806-0

Tomazi, T., Gonçalves, J. L., Barreiro, J. R., Arcari, M. A., & Dos Santos, M. V. (2015). Bovine subclinical intramammary infection caused by coagulase-negative staphylococci increases somatic cell count but has no effect on milk yield or composition. *Journal of Dairy Science*, *98*(5), 3071–3078.

Toubal, S., Bouchenak, O., Elhaddad, D., Yahiaoui, K., Boumaza, S., & Arab, K. (2018). MALDI-TOF MS detection of endophytic bacteria associated with great nettle (urtica dioica l.), grown in Algeria. *Polish Journal of Microbiology*, *67*(1), 67–72. https://doi.org/10.5604/01.3001.0011.6145

Trakunjae, C., Boondaeng, A., Apiwatanapiwat, W., & Kosugi, A. (2021). Enhanced polyhydroxybutyrate (PHB) production by newly isolated rare actinomycetes Rhodococcus sp . strain BSRT1 - 1 using response surface methodology. *Scientific Reports*, 1–14. https://doi.org/10.1038/s41598-021-81386-2

Tripathi, L., Wu, L., Dechuan, M., Chen, J., Wu, Q., & Chen, G. (2013). Bioresource Technology Pseudomonas putida KT2442 as a platform for the biosynthesis of polyhydroxyalkanoates with adjustable monomer contents and compositions. *Bioresource Technology*, *142*, 225–231. https://doi.org/10.1016/j.biortech.2013.05.027

Ur Rehman, S., Jamil, N., & Husnain, S. (2007). Screening of different contaminated environments for polyhydroxyalkanoates- producing bacterial strains. *Biologia - Section Botany*, *62*(6), 650–656. https://doi.org/10.2478/s11756-007-0144-y

Valappil, S. P., Misra, S. K., Boccaccini, A. R., & Roy, I. (2006). *Biomedical applications of polyhydroxyalkanoates , an overview of animal testing and in vivo responses*. 853–868.

Valdez-Calderón1, A., Quezada-Cruz3, & M. B.-S. & M., Carrillo-Ibarra4, S., & M. Rodríguez5 & N. G. Rojas-Avelizapa6 & M. A. Islas-Ponce1, 2 & A. F. Angeles-Padilla1 & & A. Garrido-Hernández3 & A. M. Rivas-Castillo1, & Received: (2020). *Production of polyhydroxybutyrate (PHB) by a novel Klebsiella pneumoniae strain using low-cost media from fruit peel residues. May 2021*. https://doi.org/10.1007/s13399-020-01147-5

Vashist, H., Sharma, D., & Gupta, A. (2013). A REVIEW ON COMMONLY USED BIOCHEMICAL TEST FOR BACTERIA. Innovare Journal of Life Science, 1(1), 1–7.

https://doi.org/10.1109/ICDM.2013.109

Verlinden, R. A. J., Hill, D. J., Kenward, M. A., Williams, C. D., & Radecka, I. (2007). *Bacterial synthesis of biodegradable polyhydroxyalkanoates*. *102*, 1437–1449. https://doi.org/10.1111/j.1365-2672.2007.03335.x

Vigneshwari, J., Diagnostics, O. A., & Natesan, V. (2022). *Review on halophilic microbes and their applications REVIEW ARTICLE*. *February*.

Vijay, R., & Tarika, K. (2018). *Production of Polyhydroxyalkanoates (PHAs) using Synthetic Biology and Metabolic Engineering Approaches*. *13*(1), 99–109.

Vijayaraghavan, P., Gnana, S., & Vincent, P. (2013). A simple method for the detection of protease activity on agar plates using bromocresolgreen dye. 4, 628–630.

Vuong, P., Lim, D. J., Murphy, D. V, Wise, M. J., & Whiteley, A. S. (2021). *Developing Bioprospecting Strategies for Bioplastics Through the Large-Scale Mining of Microbial Genomes*. *12*(July), 1–11. https://doi.org/10.3389/fmicb.2021.697309

Wei, Y. H., Chen, W. C., Huang, C. K., Wu, H. S., Sun, Y. M., Lo, C. W., & Janarthanan, O. M. (2011). Screening and evaluation of polyhydroxybutyrate-producing strains from indigenous isolate Cupriavidus taiwanensis strains. *International Journal of Molecular Sciences*, *12*(1), 252–265. https://doi.org/10.3390/ijms12010252

Wu, H. A., Sheu, D. S., & Lee, C. Y. (2003). Rapid differentiation between short-chain-length and medium-chain-length polyhydroxyalkanoate-accumulating bacteria with spectrofluorometry. *Journal of Microbiological Methods*, *53*(1), 131–135. https://doi.org/10.1016/S0167-7012(02)00232-4

Wu, H., Jiang, D., & Cai, P. (2012). *Adsorption of Pseudomonas putida on soil particle size fractions : effects of solution chemistry and organic matter*. 143–149. https://doi.org/10.1007/s11368-011-0441-5

Wu, Q., Wang, Y., & Chen, G. (2009). *Medical Application of Microbial Biopolyesters Polyhydroxyalkanoates*. *973*(2006), 1–12. https://doi.org/10.1080/10731190802664429

Yang, C., Zhang, W., Liu, R., Zhang, C., Gong, T., Li, Q., Wang, S., & Song, C. (2013). Analysis of polyhydroxyalkanoate (PHA) synthase gene and PHA-producing bacteria in activated sludge that produces PHA containing 3-hydroxydodecanoate. *FEMS Microbiology*

Letters, *346*(1), 56–64. https://doi.org/10.1111/1574-6968.12201

Yasin, A. R., & Al-mayaly, I. K. (2019). *Polyhydroxyalkanoate : a biodegradable polymer (a mini review) Polyhydroxyalkanoate : a biodegradable polymer (a mini review)*. https://doi.org/10.1088/1742-6596/1378/4/042007

7. Appendix

Appendix 1 Table 1. Isolation and charactirization of bacterial isolates

S.N.	sample name	SBB	NBA	MALDTOF- MS analysis result	score value	*PHC1* and PHC2 (540bp) pirimer
1	DLSPPI -1	+ve	-ve	NP	NP	NP
2	DLSPPI -2	+ve	+ve	*P. eroginosa*	2.46	+ve
3	DLSPPI -3	+ve	-ve	NP	NP	NP
4	DLSPPI -4	+ve	+ve	*bacciluscerus*	2.35	-ve
5	DLSPPI -5	+ve	-ve	NP	NP	NP
6	DLSPPI -6	+ve	+ve	*B. cerus*	2.3	+ve
7	DLSPPI -7	+ve	+ve	*St. arlettae*	1.71	NP
8	DLSPPI -8	+ve	+ve	*B. cerus*	2.28	-ve
9	DLSPPI -9	+ve	+ve	*baccilus subtilis*	2.25	-ve
10	DLSPPI -10	+ve	+ve	*P. eroginosa*	2.31	+ve
11	DLSPPI -11	+ve	+ve	*P. eroginosa*	2.36	NP
12	DLSPPI -12	+ve	+ve	*P. eroginosa*	2.26	+ve
13	DLSPPI -13	+ve	+ve	*P. eroginosa*	2.03	NP
14	DLSPPI -14	+ve	+ve	*P. eroginosa*	2.32	+ve
15	DLSPPI -15	+ve	-ve	NP	NP	NP
16	DLSPPI -16	+ve	+ve	*P. eroginosa*	2	+ve
17	DLSPPI -17	+ve	+ve	*P. eroginosa*	2.39	-
18	DLSPPI -18	+ve	+ve	*P. eroginosa*	2.37	-
19	DLSPPI -19	+ve	+ve	*P. eroginosa*	2.23	-
20	DLSPPI -20	+ve	+ve	*B. subtilis*	2.05	-ve
21	DLSPPI -21	+ve	+ve	*P. eroginosa*	2.35	-
22	DLSPPI -22	+ve	-ve	NP	0	-
23	DLSPPI -23	+ve	+ve	no identification p	0	NP
24	DLSPPI -24	+ve	+ve	*bacciluscerus*	2.29	NP
25	DLSPPI -25	+ve	+ve	*P. eroginosa*	2.33	NP
26	DLSPPI -26	+ve	+ve	*P. eroginosa*	2.41	+ve

27	DLSPPI -27	+ve	+ve	no identification p	0		NP
28	DLSPPI -28	+Ve	-ve	NP	NP	NP	
29	DLSPPI -29	-Ve	NP	NP	NP	NP	
30	DLSPPI -30	-Ve	NP	NP	NP	NP	
31	DLSPPI -31	-Ve	NP	NP	NP	NP	
32	DLSPPI -32	+Ve	-ve	NP	NP	NP	
33	DLSPPI -33	+ve	+ve	*P. eroginosa*	2.31	+ve	
34	DLSPPI -34	+ve	+ve	*B. subtilis*	2.22	-ve	
35	DLSPPI -35	+ve	+ve	*B. subtilis*	1.96	-ve	
36	DLSPPI -36	+ve	+ve	*P. eroginosa*	1.98	+ve	
37	DLSPPI -37	-Ve	NP	NP	NP	NP	
38	DLSPPI -38	-Ve	NP	NP	NP	NP	
39	DLSPPI -39	-Ve	NP	NP	NP	NP	
40	DLSPPI -40	-Ve	NP	NP	NP	NP	

Key note: NP=Not performed

Appendix 2: Optimization of temperature,PH and various carbon source

Table S_{2a}: Effect temperature on Growth PHA producing *P. aeuroginosa* DSlPI-12 isolate

	Biomass g/ml					OD at 600nm				
Temprature	Isolatees	R1	R2	R3	Av	sd	R1	R2	R3	Av	Sd
25	DLSI-12	0.00347	0.00352	0.00337	0.00345	6.23832E-05	1.817	1.818	1.82	1.818333	0.956884
30	DLSI-12	0.000367	0.0033	0.00371	0.00356	0.00158723	1.856	1.85	1.846	1.850667	0.974071
37	DLSI-12	0.002	0.00205	0.00221	0.00208	8.96289E-05	0.89	0.892	0.885	0.889	0.467659
A) Effect of PH on growth of isolated PHA producing Bacteria *pseudomonas aeuroginosa* DSlPI-12											
	Biomass g/ml						OD at 600nm				
PH	isolated	R1	R2	R3	Av	sd	R1	R2	R3	av	Sd
6	DLSI-12	0.00298	0.0037	0.00301	0.00323	0.01292	1.345	1.375	1.324	1.348	0.020928
7	DLSI-12	0.0152	0.003519	0.00232	0.007013	0.028052	1.419	1.437	1.424	1.426667	0.007587
10	DLSI-12	0.00235	0.005387	0.00219	0.003309	0.013236	1.4	1.399	1.407	1.402	0.003559
C) Effect of Carbon source(*pseudomonas aeuroginosa* DSlPI-12) Odat 600nm											
	Biomass g/ml						Odat 600nm				
Carbon source	isolated	R1	R2	R3	Av	sd	R1	R2	R3	Av	SD
Glucose 1%	DLSI-12	0.00352	0.00364	0.00438	0.003847	0.000380292	1.723	1.715	1.723	1.720333	0.003771
Galactose1%	DLSI-12	0.00507	0.00223	0.0023	0.0032	0.001322598	0.891	0.893	0.893	0.892333	0.000943
Fructose	DLSI-12	0.00245	0.00231	0.00332	0.002693	0.000446791	1.712	1.715	1.718	1.715	0.002449

Table S_{3b}Cost effective carbon source against *P. aeuroginosa* DSlPI-12 isolate growth

Cost effective carbon source							OD_{600nm}					
Cost effective CS (%, v/v)	R1	R2	R3	Mean	SD	SEM	R1	R2	R3	Mean	SD	SEM
Molasses 1	0.002157	0.003172	0.003165	0.002831	0.000584	0.000337	0.815	0.814	0.811	0.813333	0.002082	0.001202
Molasses 1.5	0.003401	0.003038	0.003037	0.003159	0.00021	0.000121	1	0.999	0.998	0.999	0.001	0.000577
Molasses 2	0.00895	0.00638	0.001447	0.005592	0.003813	0.002201	1.16	1.156	1.153	1.156333	0.003512	0.002028
Cost effective CS (%, w/v)	R1	R2	R3	Mean	SD	SEM	R1	R2	R3	Mean	SD	SEM
Bagasse 1	0.002553	0.002012	0.00274	0.002435	0.000378	0.000218	0.972	0.941	0.941	0.951333	0.017898	0.010333
Bagasse 1.5	0.003343	0.00277	0.002042	0.002718	0.000652	0.000376	1.147	1.144	1.139	1.143333	0.004041	0.002333
Bagasse 2	0.00455	0.00452	0.00459	0.004553	3.51E-05	2.03E-05	1.322	1.323	1.285	1.31	0.021656	0.012503

Key note: CS indicates Carbon source

Table S_{4a}: Descriptive test using OneWay ANOVAs for various pH-values against SBB and NBA positive *Pseudomonas* sp.DLSPI-12

Descriptives								
Cell Dry Weight(CDW, g/L)								
	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
CDW (g/L) at pH6	3	.0032300	.00040731	.00023516	.0022182	.0042418	.00298	.00370
CDW (g/L) at pH7	3	.0070130	.00711545	.00410811	-.0106628	.0246888	.00232	.01520
CDW (g/L) at pH10	3	.0033090	.00180138	.00104003	-.0011659	.0077839	.00219	.00539
Total	9	.0045173	.00412489	.00137496	.0013467	.0076880	.00219	.01520

Table S_{5b}: Multiple Comparisons of Post Hoc Tests using OneWay ANOVAs for various pH-values against SBB and NBA positive *Pseudomonas* sp.DLSPI-12 at LSD test (0.05)

Multiple Comparisons						
Dependent Variable: Cell Dry Weight (CDW, g/L) LSD						
(I) pH	(J) pH	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
CDW (g/L) at pH6	CDW (g/L) at pH7	-.00378300	.00346540	.317	-.0122625	0.0046965
	CDW (g/L) at pH10	-.00007900	.00346540	.983	-.0085585	0.0084005
CDW (g/L) at pH7	CDW (g/L) at pH6	.00378300	.00346540	.317	-.0046965	0.0122625
	CDW (g/L) at pH10	.00370400	.00346540	.326	-.0047755	0.0121835
CDW (g/L) at pH10	CDW (g/L) at pH6	.00007900	.00346540	.983	-.0084005	0.0085585
	CDW (g/L) at pH7	-.00370400	.00346540	.326	-.0121835	0.0047755

Table S_{6a}: Descriptive test using OneWay ANOVAs for various Temperatures against SBB and NBA positive *Pseudomonas* sp.DLSPI-12

Descriptives								
Cell Dry Weight (CDW, g/L)								
	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
CDW (g/L) at 25°C	3	.00345333	.000076376	.000044096	.00326360	.00364306	.003370	.003520
CDW (g/L) at 30°C	3	.00322667	.000066583	.000038442	.00306126	.00339207	.003170	.003300
CDW (g/L) at 37°C	3	.00208667	.000109697	.000063333	.00181417	.00235917	.002000	.002210
Total	9	.00292222	.000638686	.000212895	.00243128	.00341316	.002000	.003520

Table S_{7b}: Multiple Comparisons of Post Hoc Tests using OneWay ANOVAs for various Temperatures (°C) against SBB and NBA positive *Pseudomonas* sp.DLSPI-12 at LSD test

Multiple Comparisons						
Dependent Variable: Cell Dry Weight (CDW, g/L) LSD						
(I) Temperature	(J) Temperature	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
CDW (g/L) at 25°C	CDW (g/L) at 30°C	.000226667*	.000070396	.018	.00005441	.00039892
	CDW (g/L) at 37°C	.001366667*	.000070396	.000	.00119441	.00153892
CDW (g/L) at 30°C	CDW (g/L) at 25°C	-.000226667*	.000070396	.018	-.00039892	-.00005441
	CDW (g/L) at 37°C	.001140000*	.000070396	.000	.00096775	.00131225
CDW (g/L) at 37°C	CDW (g/L) at 25°C	-.001366667*	.000070396	.000	-.00153892	-.00119441
	CDW (g/L) at 30°C	-.001140000*	.000070396	.000	-.00131225	-.00096775
*. The mean difference is significant at the 0.05 level.						

Table S_{8a}: Descriptive test using OneWay ANOVAs for various Carbon sources (1%, w/v) against SBB and NBA positive *Pseudomonas* sp.DLSPI-12

Descriptives								
Cell dry weight (CDW, g/L)								
	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
CDW (g/L) for Fructose	3	.00269333	.000547205	.000315929	.00133400	.00405267	.002310	.003320
CDW (g/L) for Glucose	3	.00384667	.000465761	.000268907	.00268965	.00500368	.003520	.004380
CDW (g/L) for Galactose	3	.00306667	.000165025	.000095277	.00265672	.00347661	.002900	.003230
Total	9	.00320222	.000629003	.000209668	.00271873	.00368572	.002310	.004380

Table S_{9b}: Multiple Comparisons of Post Hoc Tests using OneWay ANOVAs for various Carbon sources (1%) against SBB and NBA positive *Pseudomonas* sp.DLSPI-12 at LSD test

Multiple Comparisons						
Dependent Variable: Cell dry weight (CDW, g/L) LSD						
Carbon source	(J) carbon source	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
CDW (g/L) for Fructose	CDW (g/L) for Glucose	-.001153333*	.000347563	.016	-.00200379	-.00030288
	CDW (g/L) for Galactose	-.000373333	.000347563	.324	-.00122379	.00047712
CDW (g/L) for Glucose	CDW (g/L) for Fructose	.001153333*	.000347563	.016	.00030288	.00200379
	CDW (g/L) for Galactose	.000780000	.000347563	.066	-.00007046	.00163046
CDW (g/L) for Galactose	CDW (g/L) for Fructose	.000373333	.000347563	.324	-.00047712	.00122379
	CDW (g/L) for Glucose	-.000780000	.000347563	.066	-.00163046	.00007046
*. The mean difference is significant at the 0.05 level.						

Table S_{10a}: Descriptive test using OneWay ANOVAs for various Carbon sources (1%, w/v) against SBB and NBA positive *Pseudomonas* sp.DLSPI-12

Descriptive								
CDW								
	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
Molasses at 1% conc.	3	.002831	.0005839	.0003371	.001381	.004282	.0022	.0032
Molasses at 1.5% conc.	3	.003159	.0002099	.0001212	.002637	.003680	.0030	.0034
Molasses at 2 % conc.	3	.005592	.0038130	.0022014	-.003880	.015064	.0014	.0090
Bagasse at 1% conc.	3	.002435	.0003781	.0002183	.001496	.003374	.0020	.0027
Bagasse at 1.5% conc.	3	.002718	.0006520	.0003765	.001099	.004338	.0020	.0033
Bagasse at 2% conc.	3	.004553	.0000351	.0000203	.004466	.004641	.0045	.0046
Total	18	.003548	.0017878	.0004214	.002659	.004437	.0014	.0090

Table S_{11b}: Multiple Comparisons of Post Hoc Tests using OneWay ANOVAs for various Carbon sources (1%) against SBB and NBA positive *Pseudomonas* sp.DLSPI-12 at LSD test

Multiple Comparisons						
Dependent Variable: CDW LSD						
(I) Coding	(J) Coding	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Molasses at 1% conc.	Molasses at 1.5% conc.	-.0003275	.0013121	.807	-.003186	.002531
	Molasses at 2 % conc.	-.0027611	.0013121	.057	-.005620	.000098
	Bagasse at 1% conc.	.0003962	.0013121	.768	-.002463	.003255
	Bagasse at 1.5% conc.	.0001129	.0013121	.933	-.002746	.002972
	Bagasse at 2% conc.	-.0017221	.0013121	.214	-.004581	.001137
Molasses at 1.5% conc.	Molasses at 1% conc.	.0003275	.0013121	.807	-.002531	.003186
	Molasses at 2 % conc.	-.0024337	.0013121	.088	-.005292	.000425
	Bagasse at 1% conc.	.0007237	.0013121	.591	-.002135	.003582
	Bagasse at 1.5% conc.	.0004403	.0013121	.743	-.002418	.003299
	Bagasse at 2% conc.	-.0013947	.0013121	.309	-.004253	.001464
Molasses at 2 % conc.	Molasses at 1% conc.	.0027611	.0013121	.057	-.000098	.005620
	Molasses at 1.5% conc.	.0024337	.0013121	.088	-.000425	.005292
	Bagasse at 1% conc.	.0031573*	.0013121	.033	.000299	.006016
	Bagasse at 1.5% conc.	.0028740*	.0013121	.049	.000015	.005733
	Bagasse at 2% conc.	.0010390	.0013121	.444	-.001820	.003898
Bagasse at 1% conc.	Molasses at 1% conc.	-.0003962	.0013121	.768	-.003255	.002463
	Molasses at 1.5% conc.	-.0007237	.0013121	.591	-.003582	.002135
	Molasses at 2 % conc.	-.0031573*	.0013121	.033	-.006016	-.000299
	Bagasse at 1.5% conc.	-.0002833	.0013121	.833	-.003142	.002575
	Bagasse at 2% conc.	-.0021183	.0013121	.132	-.004977	.000740

Bagasse at 1.5% conc.	Molasses at 1% conc.	-.0001129	.0013121	.933	-.002972	.002746
	Molasses at 1.5% conc.	-.0004403	.0013121	.743	-.003299	.002418
	Molasses at 2 % conc.	-.0028740*	.0013121	.049	-.005733	-.000015
	Bagasse at 1% conc.	.0002833	.0013121	.833	-.002575	.003142
	Bagasse at 2% conc.	-.0018350	.0013121	.187	-.004694	.001024
Bagasse at 2% conc.	Molasses at 1% conc.	.0017221	.0013121	.214	-.001137	.004581
	Molasses at 1.5% conc.	.0013947	.0013121	.309	-.001464	.004253
	Molasses at 2 % conc.	-.0010390	.0013121	.444	-.003898	.001820
	Bagasse at 1% conc.	.0021183	.0013121	.132	-.000740	.004977
	Bagasse at 1.5% conc.	.0018350	.0013121	.187	-.001024	.004694
*. The mean difference is significant at the 0.05 level.						

```
LOCUS       OP856846                  1387 bp    DNA     linear   BCT 23-
NOV-2022
DEFINITION  Pseudomonas sp. strain DLSPI-12 16S ribosomal RNA gene,
partial
            sequence.
ACCESSION   OP856846
VERSION     OP856846.1
KEYWORDS    .
SOURCE      Pseudomonas sp.
  ORGANISM  Pseudomonas sp.
            Bacteria; Proteobacteria; Gammaproteobacteria;
Pseudomonadales;
            Pseudomonadaceae; Pseudomonas.
REFERENCE   1  (bases 1 to 1387)
  AUTHORS   Beshir Feyiso,D. and Seid Mohammed,E.
  TITLE     Identification And and Molecular Characterization of
            Polyhydroxyalkonoate Producing Bacterial Isolates from Dambal
Lake
            Soil Sediment
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 1387)
  AUTHORS   Beshir Feyiso,D. and Seid Mohammed,E.
  TITLE     Direct Submission
  JOURNAL   Submitted (18-NOV-2022) Department of Applied Biology, Adama
            Science and Technology University, Adama Gara Lugo, Adama
City,
            Oromia +251, Ethiopia
COMMENT     ##Assembly-Data-START##
            Sequencing Technology :: Sanger dideoxy sequencing
            ##Assembly-Data-END##
FEATURES             Location/Qualifiers
     source          1..1387
                     /organism="Pseudomonas sp."
                     /mol_type="genomic DNA"
                     /strain="DLSPI-12"
                     /db_xref="taxon:306"
     rRNA            <1..>1387
                     /product="16S ribosomal RNA"
```

```
ORIGIN
        1 gtcgagcgga tgaaggggag cttgctcctg gattcagcgg cggacgggtg
agtaatgcct
       61 aggaatctgc ctggtagtgg gggataacgt ccggaaacgg gcgctaatac
cgcatacgtc
      121 ctgagggaga aagtgggggа tcttcggacc tcacgctatc agatgagcct
aggtcggatt
      181 agctagttgg tggggtaaag gcctaccaag gcgacgatcc gtaactggtc
tgagaggatg
      241 atcagtcaca ctggaactga gacacggtcc agactcctac gggaggcagc
agtggggaat
      301 attggacaat gggcgaaagc ctgatccagc catgccgcgt gtgtgaagaa
ggtcttcgga
      361 ttgtaaagca ctttaagttg ggaggaaggg cagtaagtta ataccttgct
gttttgacgt


      421 taccaacaga ataagcaccg gctaacttcg tgccagcagc cgcggtaata
cgaagggtgc
      481 aagcgttaat cggaattact gggcgtaaag cgcgcgtagg tggttcagca
agttggatgt
      541 gaaatccccg ggctcaacct gggaactgca tccaaaacta ctgagctaga
gtacggtaga
      601 gggtggtgga atttcctgtg tagcggtgaa atgcgtagat ataggaagga
acaccagtgg
      661 cgaaggcgac cacctggact gatactgaca ctgaggtgcg aaagcgtggg
gagcaaacag
      721 gattagatac cctggtagtc cacgccgtaa acgatgtcga ctagccgttg
ggatccttga
      781 gatcttagtg gcgcagctaa cgcgataagt cgaccgcctg gggagtacgg
ccgcaaggtt
      841 aaaactcaaa tgaattgacg gggcccgca caagcggtgg agcatgtggt
ttaattcgaa
      901 gcaacgcgaa gaaccttacc tggccttgac atgctgagaa ctttccagag
atggattggt
      961 gccttcggga actcagacac aggtgctgca tggctgtcgt cagctcgtgt
cgtgagatgt
     1021 tgggttaagt cccgtaacga gcgcaaccct tgtccttagt taccagcacc
tcgggtgggc
     1081 actctaagga gactgccggt gacaaaccgg aggaaggtgg ggatgacgtc
aagtcatcat
     1141 ggcccttacg gccagggcta cacacgtgct acaatggtcg gtacaaaggg
ttgccaagcc
     1201 gcgaggtgga gctaatccca taaaaccgat cgtagtccgg atcgcagtct
gcaactcgac
     1261 tgcgtgaagt cggaatcgct agtaatcgtg aatcagaatg tcacggtgaa
tacgttcccg
     1321 ggccttgtac acaccgcccg tcacaccatg ggagtgggtt gctccagaag
tagctagtct
     1381 aacgcgc
//
```