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# SCREENING AND OPTIMIZATION OF POLYHYDROXY ALKANOATE (PHA) PRODUCTION FROM ISOLATED MICROBIAL STRAINS IN COIMBATORE, TAMILNADU, INDIA

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**Abstract.** Biodegradable polymers were produced instead of polythene bags by an array of microbes and plants with potential impact to the consumers against environmental pollution. The samples were collected from municipal garbage dumping yards in five localities of the study area. About 56 strains were isolated from collected samples through serial dilution and screened for polyhydroxyalkanoate (PHA) accumulation by sudan black B, nile blue A staining, and tributyrine hydrolysis tests. The PHA accumulating isolates as M12a and M16a strains were identified, characterized through morphological, cultural, biochemical, and molecular level characterization using 16S rRNA sequencing with the aid of Basic Local Alignment Search Tool (BLAST) to conclude their identity of strains up to species level. The PHA extracted was further confirmed by FTIR spectroscopy to detect the presence of functional groups, with a peak at 1516 $cm^{-1}$ and 1637 $cm^{-1}$ corresponding to (N-O) and (C=O) groups indicating the presence of PHA and bioactive compounds were further processed for Gas chromatography (GCMS) analysis. The bacterial growth conditions were optimized for physical parameters, including temperature, pH, NaCl concentration, incubation time. The effect of inexpensive carbon sources of rice, corn, potato, and tapioca skin waste decreased production costs. The results obtained for the highest PHA accumulation were observed with rice starch as 58.51% and 58.33%, in M16a and M12a, respectively.

***Keywords:*** *Bioplastics, Polyhydroxyalkanoate, Bacillus licheniformis, Fourier Transform Infrared spectroscopy, GCMS, Nile blue A, Sudan black B, Optimization of pH, Temperature, Incubation time and NaCl concentration.*

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## 1. Introduction

Indiscriminate disposal of non-biodegradable petroleum-based plastics havocked the environment and made the planet earth densely polluted. The consumption of non-renewable fuels for plastic production is directly linked with diminishing oil resources and also polluting the environment, either directly or indirectly. In response to the hazardous effect and oil scarcity, there has been expanded attention for producing biodegradable plastic from renewable sources. During last decade, the production and consumption of plastic materials were sky rocketing, which makes life on Earth alarming. PHA is the linear polyester material being synthesized by various microorganisms, serving as an intracellular reservoir for energy and carbon supply (Jendrossek, 2009). With its combined properties of biodegradability, biocompatibility, and thermoplasticity, PHA gained significant importance as a promising candidate for bio-based plastics and biomaterials (Han *et al*., 2017; Amaro *et al*., 2019). To date, about 150 different monomers have been incorporated into PHA chains under various

fermentation conditions, yielding PHA with different characteristics (Lee *et al*., 2019). Microorganisms such as bacteria, fungi, and yeast can produce biopolymers with a diverse range (Bhuwal *et al*., 2013). Bacteria can accumulate PHA as carbon energy storage material in the range of 0.2 - 0.5 µm in diameter under nutrient-depleted conditions such as excess carbon source, deficient nitrogen, phosphorous, oxygen, sulphur, and magnesium (Shrivastava *et al*., 2010; Phanse *et al*., 2011). *Bacillus megaterium., Rhizobium spp., Azotobacter spp., Pseudomonas spp., Alcaligenes latus, Alcaligenes vinelandii,* and recombinant *Escherichia coli* have the potential to accumulate biopolymers under nutrient-depleted conditions (Bhuwal *et al*., 2013). The range, size, number of granules, macromolecular structure, monomer composition and physicochemical properties vary according to the type of organism (Arshad *et al*., 2007). PHA produced from various genera of microbes has attracted attention for the production of plastics from microbes. PHAs are generally applied in drug delivery, microcarriers, medical devices and tissue engineering due to their biodegradability, immune compatibility, less toxicity and biocompatibility nature. This can simultaneously be utilized as biofuel additives, metabolic regulators, paper coating, feminine hygiene products, food packaging, marine, paint industry, printing material, and agriculture (Ataei *et al.,* 2008; Bhuwal *et al.*, 2013). The key problem associated with the commercialization of PHA is the production cost due to their highly expensed media, ingredients and recovery rate have created much interest in developing new efficient fermentation techniques and recovery processes (Singh & Parmar, 2011). In connection with the increasing demand for replacing plastic materials, PHA has tremendous market potential, but high production cost has limited its commercial applications (Albuquerque *et al*., 2018). Bacteria can utilize various substrates as media, which paved a path for reducing production cost. Hence inexpensive carbon sources were studied to reduce its production cost in large-scale commercial production. The waste products from various food industries shall be employed for PHA production due to their dual benefits of utilizing the waste and cost-effective production of eco-friendly bioplastics. There are wide range of inexpensive carbon sources namely molasses, date syrup (Ataei *et al*., 2008), edible oil (Marjadi & Dharaiya, 2011), activated sludge food waste, rice bran, mango peel, and potato starch have been used for PHA production as a good strategy. The non-halophilc and halophilic microbes can produce PHA by utilizing various inexpensive raw materials; thus, the fermentation cost shall reduced (Obruca *et al*., 2018). High contamination resistance of halophilic bacteria makes it suitable for PHA production using an unsterile seawater medium for the growth of halophilic bacteria at least for two months (Chen *et al*., 2020). Bluepha, a company in China, is producing PHB and PHBV using *Halomonas species* as a microbial cell factory/fermentation unit. There are still certain challenges faced while using halophiles for large-scale production as the treatment of saline effluent is difficult (Liu *et al*., 2019). This research is focused on non-halophilic (mesophilic) species and their PHA synthesis, along with its challenges and future applications.

The objectives of this research were to isolate, screen, and characterize prominent PHA producing bacterial isolates from municipal garbage dumping yards and enhance the PHA production by optimizing their cultural conditions such as altering pH, temperature, incubation time, NaCl concentration and to evaluate their ability to produce PHA on various cost-effective carbon sources such as corn, rice, potato, and tapioca. PHA production on different inexpensive renewable carbon sources was compared through cell dry weight, PHA content (%), and cell growth measurement.

## 2. Materials and methods

### *I. Sample collection, isolation and purification of bacteria*

Soil samples were collected from the upper layer of study area, where most of the microbial activity occurs. The soil samples of about 10 grams were collected in a clean, dry and sterile screw cap bottle along with the spatula from municipal garbage dumping yards in Coimbatore, Tamil Nadu, India, named M1, M2, M3, M4, and M5, respectively and stored at room temperature until further analysis. The carbon source such as *Zea mays* (corn), *Oryza sativa* (raw rice), *Solanum tuberosum* (potato) and *Manihot esculenta* (tapioca) was purchased from the local markets of Coimbatore. Those procured ingredients were washed thoroughly in running tap water, distilled water to remove the adhered soil, microbial agents, and dried in partial shade for 48 hours, and then it was grounded into fine powder. Bacterial isolates were obtained by serial dilution, followed by the pour plate technique. At the end of incubation, the isolates based on their distinct morphological features on the nutrient agar were selected and further purified by sub culturing on nutrient agar until they obtained pure culture for future research.

### *II. Screening for PHA producing bacteria*

The purified bacterial isolates were screened to detect PHA accumulation with Sudan black B, Nile blue A plate assay, and tributyrine hydrolysis test.

#### *II.a. Sudan black B staining (Babu et al., 2014)*

A thin bacterial smear was prepared on a sterile glass slide, and the smear was flooded with 0.3% sudan black B solution and dried for 10 minutes. The dried slide was washed with sterile distilled water and counterstained with safranin for 30 seconds. The stained slide was again washed with sterile distilled water, blot dry with tissue paper. A few drops of immersion oil were added and examined under a light microscope with 40x magnification for spotted bluish-black colonies.

#### *II.b. Nile blue A staining (Bhuwal et al., 2013).*

The sudan black B positive isolates were inoculated on PHA detecting medium, with the composition of (glucose 10 g/L, beef extract 3 g/L, peptone 5 g/L, sodium chloride 8 g/L and agar 15 g/L) supplemented with nile blue A stain at a concentration of 0.5 µg / mL. The plates were incubated at 37°C for 48 hours and observed under UV light.

#### *II.c. Tributyrine hydrolysis test (Marjadi & Dharaiya, 2011).*

Both sudan black B and nile blue A positive isolates were further confirmed by inoculating a single line streak on tributyrin agar plate (Peptic digest of animal tissue 5 g/L, yeast extract 3 g/L, agar 15/L) and incubated at 37°C for 48 hours.

### *III. Characterization of bacteria*

#### *III.a. Cultural and Morphological Characterization*

The positive colonies were inoculated and incubated on to the nutrient agar. The morphological features were analysed by observing its culture character, colony characteristics like margin, elevation, consistency, opacity, pigment on plate, and Gram's staining to differentiate the shape.

### *III.b. Biochemical characterization and Molecular identification*

Biochemical tests including indole, methyl red, voges proskauer, citrate utilization, catalase, oxidase and urease were performed in triplicates. The ability of bacterial isolates to hydrolyze starch and gelatin was also detected on the defined media and incubated at 37°C for 48 hours. The fermentative utilization of carbohydrates was observed by seeding the culture onto the defined medium containing sugar of sucrose, D-glucose, D- fructose, starch, maltose, lactose, and D- sorbitol. The molecular level of characterization was performed by 16S rRNA sequencing for the obtained bacterial isolate. The sequence analysis and alignment were carried out through NCBI, BLAST tool.

### *IV. Optimization*

About 5 variables such as incubation temperature, pH, NaCl concentration, incubation time, and carbon sources were selected to optimize cultural conditions to obtain efficient results. The positive strains were inoculated into the nutrient agar and incubated at 30°C, 37°C and 40°C for 48 hours in triplicates. The optimum pH was determined for PHA producing bacteria by inoculating the strains in nutrient medium with varying pH as 6.5, 7.5 and 8.5 were adjusted, incubated at 37°C for 48 hours. The optimum concentration of NaCl was identified by inoculating bacterial strains in the production medium employed with 1%, 5% and 10% NaCl concentration and incubated at 37°C for 48 hours of incubation. The growth was determined by measuring absorbance at 610 nm in ELICO CL 223 Colorimeter, the results were recorded and tabulated. The optimum incubation time was analysed by incubated at 37°C for incubation time optimization. The colorimetric values were observed at the regular intervals of 24 hours, 48 hours, 72 hours, and 96 hours by measuring absorbance at 610 nm, the results were recorded and tabulated. The PHA positive strains were further inoculated into 250 ml of mineral salt medium containing corn starch, potato starch, rice starch and tapioca skin powder incubated at 37°C for 72 hours in a shaking incubator with 120 rpm. The biomass concentration was measured at 610 nm, values were recorded and tabulated.

### *V. PHA production*

The positive strains were further inoculated into PHA production media in 250 ml of mineral salt medium containing $(NH_4)2SO_4$-2g/L, $KH_2PO_4$-13.3g/L, MgSO4-1.3 g/L, citric acid-1.7g/L, glucose-2g/L and 10ml/L trace element solution ($FeSO_4.7H_2O$-10g/L, $ZnSO_4.\ 7H_2O$-2.25g/L, $CuSO_4.\ 5H_2O$-1g/L, $MnSO_4.\ 5H_2O$-0.5g/L, $CaCl_2.\ 2H_2O$-2g/L, $(NH_4)_6MO_7O_{24}$-0.1g/L) and incubated at 37°C for 72 hours in shaking incubator with 120 rpm in triplicates. Bacterial growth was monitored by measuring the biomass using ELICO CL 223 Colorimeter and the values were recorded.

### *V.a. Extraction of PHA*

The PHA extraction was carried out through the sodium hypochlorite digestion method (Arshad *et al*., 2007) with required modifications in triplicates. After 72 hours of cultivation, cells were centrifuged for 10 minutes at 4000 rpm and the pellet was collected. The pellets were further dissolved in 0.4% of sodium hypochlorite, incubated at 37°C for 1 hour and centrifuged at 4000 rpm for 5 minutes. The collected pellet was washed in acetone, water and dissolved in chloroform. After chloroform evaporation, the PHA was collected, and its content (w/w) is known as percentage of PHA in dry cell mass and quantified using this formula,

**PHA accumulation (%)** = Dry weight of extracted PHA (g/L) × 100% /DCW (g/L)
**Residual biomass (g/L)** = DCW (g/L) – Dry weight of extracted PHA (g/L).

***V.b. Characterization of PHA***

To qualitatively characterize PHA monomers, the dry cell samples were first subjected to methanolysis with 15% sulphuric acid/methanol (2 mL) and chloroform (2 mL) at $100^0$ C for 140 minutes in screw capped test tubes (Kato *et al*., 1996) after cooling 1 mL of water was added to separate the organic and aqueous phase. The organic phase containing methylester derivatives was analyzed with Shimadzu 8400S Fourier transform infrared spectrometer in the mid-IR region of 500 to 4000 cm-1, with an Agilent 7820A GC System equipped with a –DB-5 column (30 m × 0.25 mm; 0.25 µm film thickness), interfaced with an Agilent mass selective detector 5977E inter MSD. Oven temperature program was from 100 to 270°C at 10°C/min; helium was used as carrier gas and the flow rate is 1.2 ml/min.

## 3. Results and discussion

***I. Sample collection, isolation and purification of bacteria***

Soil samples were collected from the upper layer of study area where most of the microbial activity occurs. About 56 bacterial isolates were obtained from the 5 different soil samples collected. The purified bacterial isolates were further subjected to identification of PHA accumulating bacteria. The isolates were streaked onto nutrient agar, subcultured and stored at $4^0$ C till further investigation.

***II. Screening for PHA producing bacteria***

***II.a. Sudan black B staining***

About 22 out of 56 bacterial strains were showed bright colour intensity with sudan black B staining and confirmed the ability to produce PHA. Sudan black B staining preliminarily confirms the accumulation of PHA granule under light microscope at 40X magnification. Fig. 1 reveals the bluish-black colony of the isolates, such as M12a and M16a. Sudan black B was considered as a preliminary test for PHA accumulation (Phanse *et al*., 2011). Black–blue coloration with sudan black B indicates the presence of lipophilic compounds, a preliminary test for detecting PHA accumulating bacteria.

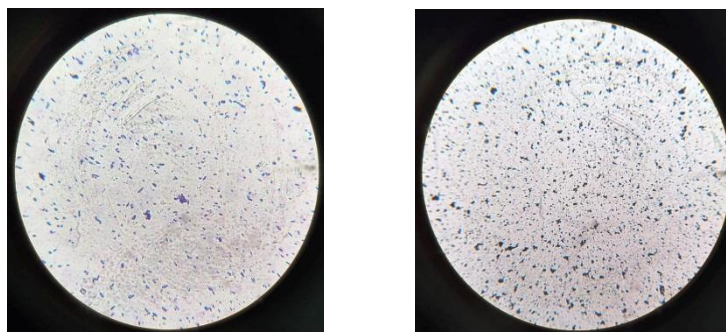


**Fig. 1.** Sudan Black B staining of M12a and M16a(a) M12a and (b) M16a

***II.b. Nile blue A staining***

About 2 isolates of 22 positive strains of sudan black were stained positively with nile blue A staining plate assay method, a specific dye for PHA detection. The two

bacterial isolates such as M12a and M16a showed bright orange to pink fluorescence on exposure to UV light. Nile blue A considered as more specific dye for PHA accumulation analysis, produce characteristic red or pink fluorescence under ultraviolet light (Maytham *et al.,* 2016). The earlier findings were in agreement with the findings of this research.

***II.c. Tributyrine hydrolysis test (Marjadi & Dharaiya, 2011).***

M12a and M16a isolates were also showed positive for tributyrin hydrolysis test depicted a clear zone around the bacterial colonies after incubation on medium shows lipolysis activity of bacteria by secreting lipase enzyme. It further indicated that both the strains are capable of producing lipase enzyme.

***III. Characterization of bacteria***

***III.a. Cultural and Morphological Characterization***

PHA accumulating bacteria were classified up to genus level using morphological, gram's staining with the help of Bergey's Manual of Determinative bacteriology. M12a and M16a were found to be *Bacillus sp*. The cultural and morphological characters of M12a and M16a were observed in Fig. 2 and presented in Table 1. Gram staining was performed for M12a and M16a observed in light microscope under 40x to identify the shape and Gram character. Both bacterial strains were identified as gram-positive rod based on staining properties.

**Fig. 2.** Colony Morphology of M16a and M12a on nutrient agar

**Table 1.** Colony morphology and Grams staining of M12a and M16a

| S. No | Characteristics | M12a | M16a |
|---|---|---|---|
| 1 | Color | White creamy | White creamy |
| 2 | Margin | Erose | Erose |
| 3 | Elevation | Flat | Flat |
| 4 | Opacity | Opaque | Opaque |
| 5 | Consistency | Rough, Wrinkled | Rough, Wrinkled |
| 6 | Gram staining | Gram positive rod | Gram positive rod |

***III.b. Biochemical properties of bacteria***

The M12a and M16a were able to utilize citrate and positive for Methyl red, Voges Proskauer, oxidase test and negative for indole test. They were unable to grow on urea agar, indicating that both cultures have no urease activity. Gelatinase activity was not observed in the M16a strain, and M12a had revealed positive for gelatinase activity. The bacterial strains could hydrolyze starch, lipid by producing amylase and lipase

enzymes, respectively. Both the strains were able to ferment sugars like sucrose, starch, glucose, fructose, and maltose. They were unable to ferment lactose and D sorbitol. Gram staining, morphological characteristics and biochemical tests revealed that the PHA producing strains belong to the genera *Bacillus.* They shall accumulate 90 % of their dry cell weight as PHA under nutrient-depleted conditions. It is also advantageous over other species due to the absence of lipopolysaccharide in the external layer, making its extraction process much easier.

### *III.c. Molecular identification*

In addition to morphological and biochemical characterization, 16S rRNA gene sequencing was performed to identify bacteria at their species level and to differentiate between closely related bacterial species at the highest level of accuracy, rapidly. The 16S rRNA sequence analysis of M12a and M16a revealed with highest similarity in the range of 99.4 % with *Bacillus licheniformis* strain with accession number NR_118996.1, and their length was observed in the range of 1500 base pairs. The results of 16S rRNA sequencing for M12a and M16a were presented in Table 2.

**Table 2**. Closets organisms and % identifies of isolated strains obtained in this study

| S. No | Strain Name | Source | Closets strain (Accession number) | % Identity | E value |
|---|---|---|---|---|---|
| 1 | M12a | Municipal garbage dump yards | *Bacillus licheniformis* NR_118996.1 | 99.54% 1500/1507(99%) | 0.0 |
| 2 | M16a | | *Bacillus licheniformis* NR_118996.1 | 99.40% 1484/1493(99%) | 0.0 |

### *III. Optimization of temperature, pH, carbon sources, salt concentration and incubation time*

The change in incubation temperature, pH, carbon sources, salt concentration, time interval affect the growth and PHA accumulation. PHA production is associated with bacterial biomass, and it enhances with maximum biomass concentration (Ataei *et al.,* 2008). Bacterial isolates were grown at 30°C, 37°C, and 40°C, after 48 hours of incubation. The optimum temperature was determined as 37°C for M16a after measuring the optical density values, growth sharply decreased at 30°C and 40°C, and the results were tabulated (Table 6). The optimum pH for bacterial cultures was determined after 48 hours of incubation with varying pH by measuring the optical density values using a spectrophotometer. The M16a showed effective growth at pH 7.5, which was lesser than M12a. From the results, the optimum NaCl concentration for PHA accumulating bacterial strains was found to be as 5%, and the results were tabulated below. Thus, the test results suggested that increasing NaCl concentration resulted in increasing biomass of PHA accumulating bacterial strains and the profiles depicted in Table 3. Various carbon sources of corn, potato, rice, and tapioca used to optimize the growth of bacteria. Among these, rice starch is considered more effective, followed by tapioca, corn, and potato starch. The results were tabulated in Table 4.

**Table 3.** Optimization of cultural parameters

| Sl No | Isolate Name | Temperature OD value at 610 nm | | | pH OD value at 610 nm | | | NaCl Concentration OD value at 610 nm | | | Incubation time (hours) OD value at 610 nm | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 30°C | 37°C | 40°C | 6.5 | 7.5 | 8.5 | 1% | 3% | 5% | 24 | 48 | 72 | 96 |
| 1 | M12a | 0.05 | 0.17 | 0.06 | 0.04 | 0.06 | 0.11 | 0.03 | 0.08 | 0.14 | 0.02 | 0.05 | 0.02 | 0.01 |
| 2 | M16a | 0.01 | 0.09 | 0.04 | 0.06 | 0.18 | 0.11 | 0.07 | 0.14 | 0.18 | 0.04 | 0.07 | 0.03 | 0.02 |

**Table 4.** Optimization of carbon sources

| Sl.No | Bacterial isolate | OD value at 610 nm | | Cell dry weight (g/10 ml) | | Extracted Polymer Weight (g/10 ml) | | Residual biomass (g/10 ml) | | PHA accumulation (%) | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | M12a | M16a | M12a | M16a | M12a | M16a | M12a | M16a | M12a | M16a |
| 1 | Corn Starch | 0.39 | 0.46 | 0.22 | 0.46 | 0.053 | 0.216 | 0.167 | 0.244 | 24.09% | 46.95% |
| 2 | Potato starch | 0.31 | 0.41 | 0.046 | 0.09 | 0.010 | 0.03 | 0.036 | 0.06 | 21.875% | 33.33% |
| 3 | Rice starch | 0.53 | 0.59 | 0.06 | 0.094 | 0.035 | 0.055 | 0.025 | 0.039 | 58.33% | 58.51% |
| 4 | Tapioca starch | 0.48 | 0.57 | 0.18 | 0.27 | 0.09 | 0.15 | 0.09 | 0.12 | 50% | 55.50% |

It has been reported that temperature plays a vital role in determining the PHA accumulation in bacteria. Desouky *et al*., (2014) optimized PHA producing *Bacillus thuringiensis* at different temperatures at 25°C, 30°C, 35°C, 37°C and 40°C, and this study reported 35°C as optimum temperature for PHA accumulation. The effect of pH is also considered an inevitable cultural parameter in determining the PHA accumulation. Desouky *et al*., (2014) optimized PHA producing *Bacillus thuringiensis* at different pH such as 3, 5.5, 7, 7.5, 8 and 10, determined 7.5 as optimum pH for the PHA accumulation. Babu et al. (2014) determined the effect of NaCl salt concentration for PHB accumulating bacterial strains isolated from sewage soil samples at 0.1% - 10% salt concentration and determined 0.1 % is best for PHB accumulation. In this 5% NaCl salt concentration is effective for bacterial growth and accumulation. The results show that optimum incubation time was determined as 48 hours for both the bacterial strains, and its growth falls after 48 hours. Desouky *et al*., (2014) obtained a maximum PHA yield 2.5g/L after 72 hours of incubation. Ataei *et al*., (2008) showed PHA accumulation of *Bacillus sp* between 36 hours after inoculation and biomass reached a maximum after 50 hours.

The study showed that bacterial strains were found to utilize rice starch, produced a higher quantity of PHA, and accumulated 58% by both strains. Results of the comparative study revealed that production rate was enhanced in M16a than M12a. Inexpensive carbon sources appear to be the best alternatives for high-priced carbon sources. Several waste products from agriculture and industrial water have been applied as carbon sources in microbial PHA accumulation to bring down the PHA production rate and waste management expense to attain a sustainable way to reach the green world (Amaro *et al*., 2019). Bhuwal et al. (2013) supplied inexpensive pulp, paper, and cardboard industry sludge and wastewater as a sole carbon source to produce PHA. Bacterial strains accumulated PHA up to 79.27% and 77.63%, respectively. Vijayendra *et al*., (2007) used corn steep liquor as nitrogen source for polyhydroxybutyrate through *Bacillus* sp CFR 256. After 72 hours of fermentation PHA obtained was 8.20 g/L and 51.20% dry cell biomass. Poomipuk *et al*., (2014) produced PHA by *Cupriavidus* sp

KKU38 using cassava starch hydrolysate and obtained PHA in yield of 61.60%. Huang *et al*., 2006 obtained PHA using an archean *Haloferax mediterranei* inexpensive extruded rice bran (ERB) and native rice bran (NRB) alone and along with extruded corn starch (ECS). But ERB gave a better yield than NRB also reduced the cost of expensive. ERB along with ECS at 1: 8 showed 77.8 g/L. This helps to diminish production costs and the environmentally polluting substance that can be converted into eco-friendly bioplastic. A combination of synthetic biology, systemic biology, morphology engineering, advanced industrial biotechnology can reduce PHA production costs and complex downstream processes (Chen *et al*., 2020).

***V.a. Production and extraction of PHA***

The strain of *Bacillus licheniformis* was screened for PHA production. It was observed in M12a and M16a while supplemented with a minimal salt medium where glucose as a carbon source for its growth. The cultivation was performed at 37°C and an agitation rate of 120 rpm. In the present study, both the bacterial isolate produced a copious amount of PHA during growth using the MSM by utilizing single carbon source glucose. The PHA produced by bacterial isolates was extracted after 48 hours of incubation using digestion with sodium hypochlorite and chloroform. The growth rate, weight of PHA obtained, PHA yield percentage, and residual biomass of bacterial strains were calculated using PHA dry cell weight. The results were tabulated in Table 5.

**Table 5.** Growth measurement of PHA accumulating bacteria

| Sl. No | Bacterial isolate | OD value at 610 nm | Cell dry weight (g/10 ml) | Extracted Polymer Weight (g/10 ml) | Residual biomass (g/10 ml) | PHA accumulation (%) |
|---|---|---|---|---|---|---|
| 1 | M12a | 0.29 | 0.077 | 0.019 | 0.018 | 24.67% |
| 2 | M16a | 0.36 | 0.068 | 0.024 | 0.014 | 35.29% |

Bacterial growth was determined by measuring the optical density at 610 nm. Among the two strains, M16a produced a better PHA yield of 35.29% than M12a at 24.67%. The residual biomass was calculated by estimating the difference between dry cell weight and the weight of extracted PHA.

***V.b. Characterization of PHA***

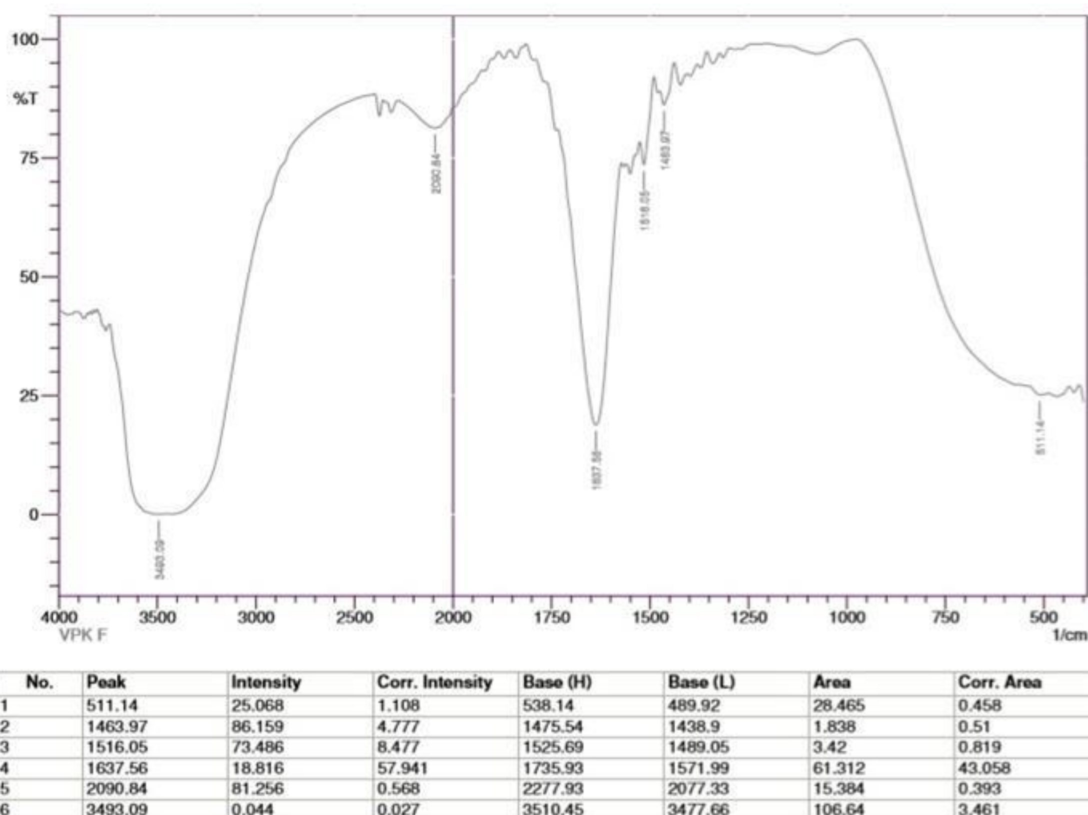
***a Fourier Transform Infrared Spectroscopy (FTIR)***

About 2 grams of PHA was dissolved in 5 ml of chloroform and allowed for evaporation to obtain the polymer. The extracted, thus obtained from M16a, was further confirmed by FTIR spectroscopy for detecting the presence of functional groups. Infrared spectra for PHA performed in the range of 4000-500 $cm^{-1}$, with multiple peaks. In the present study FTIR spectra showed absorption bands at 3493 $cm^{-}$1, 2090 $cm^{-}$1, 1637 $cm^{-1}$, 1463 $cm^{-1}$, 1516 $cm^{-1}$ and 511 $cm^{-1}$. The PHA extracted showed intense absorption at 1463 $cm^{-1}$ representing the presence of $CH_3$ group. The peak at 1516 $cm^{-1}$ and 1637 $cm^{-1}$ corresponds to (N-O) and (C=O) groups, respectively. The peak at 1637 $cm^{-1}$ indicated the presence of thioester carbonyl (C=O) stretching group. It also showed a peak at 2090 $cm^{-1}$ indicated the presence of (C=C) stretching group. The absorption at 3493 $cm^{-}$1 indicates the presence of (O-H) bending group. Also, the absorption peak recorded at 511 $cm^{-1}$ show (C-Br), (C-O) and (C-C) stretching group. The C = O, C = C and C = O at 1516 $cm^{-1}$ , 1637 $cm^{-1}$ and 2090 $cm^{-1}$ respectively indicates the presence of compounds responsible for PHA production. The extracted PHA from M16a showed a

similar absorption rate to earlier research. The absorption peak obtained at 1637 $cm^{-1}$ is exactly similar to 1639 $cm^{-1}$ (Shah, 2012) and 1634 $cm^{-1}$ (Maytham *et al.*, 2016). Similarly, the absorption peak at 1463 $cm^{-1}$ was also identical to the 1443 $cm^{-1}$ and 1453 $cm^{-1}$ peak obtained by (Maytham *et al.*, 2016). In Gumel et al. (2012) obtained peak at 3420 $cm^{-1}$ indicates the presence of (O-H) group of the polymer chain. From the results, the functional group of the PHA was identified as $CH_3$, C=C and C=O by FTIR spectroscopy, and the results were presented in Table 4 and Fig. 3. The earlier findings were in accordance with the findings of the current study.

**Table 6.** Characteristics of IR absorption frequencies of organic functional group

| S. No | Characteristics absorption ($cm^{-1}$) | Functional bonds | Functional groups |
|---|---|---|---|
| 1 | 511 | C-Br | Alkyl halides |
| 2 | 1463 | $CH_3$ | Methyl |
| 3 | 1516 | N-O | Nitro compounds |
| 4 | 1637 | C=O | Carbonyl |
| 5 | 2090 | C=C | Alkene |
| 6 | 3493 | O-H | Alcohol |




| No. | Peak | Intensity | Corr. Intensity | Base (H) | Base (L) | Area | Corr. Area |
|---|---|---|---|---|---|---|---|
| 1 | 511.14 | 25.068 | 1.108 | 538.14 | 489.92 | 28.465 | 0.458 |
| 2 | 1463.97 | 86.159 | 4.777 | 1475.54 | 1438.9 | 1.838 | 0.51 |
| 3 | 1516.05 | 73.486 | 8.477 | 1525.69 | 1489.05 | 3.42 | 0.819 |
| 4 | 1637.56 | 18.816 | 57.941 | 1735.93 | 1571.99 | 61.312 | 43.058 |
| 5 | 2090.84 | 81.256 | 0.568 | 2277.93 | 2077.33 | 15.384 | 0.393 |
| 6 | 3493.09 | 0.044 | 0.027 | 3510.45 | 3477.66 | 106.64 | 3.461 |

**Fig. 3**. FTIR graph of extracted polymer of M16a

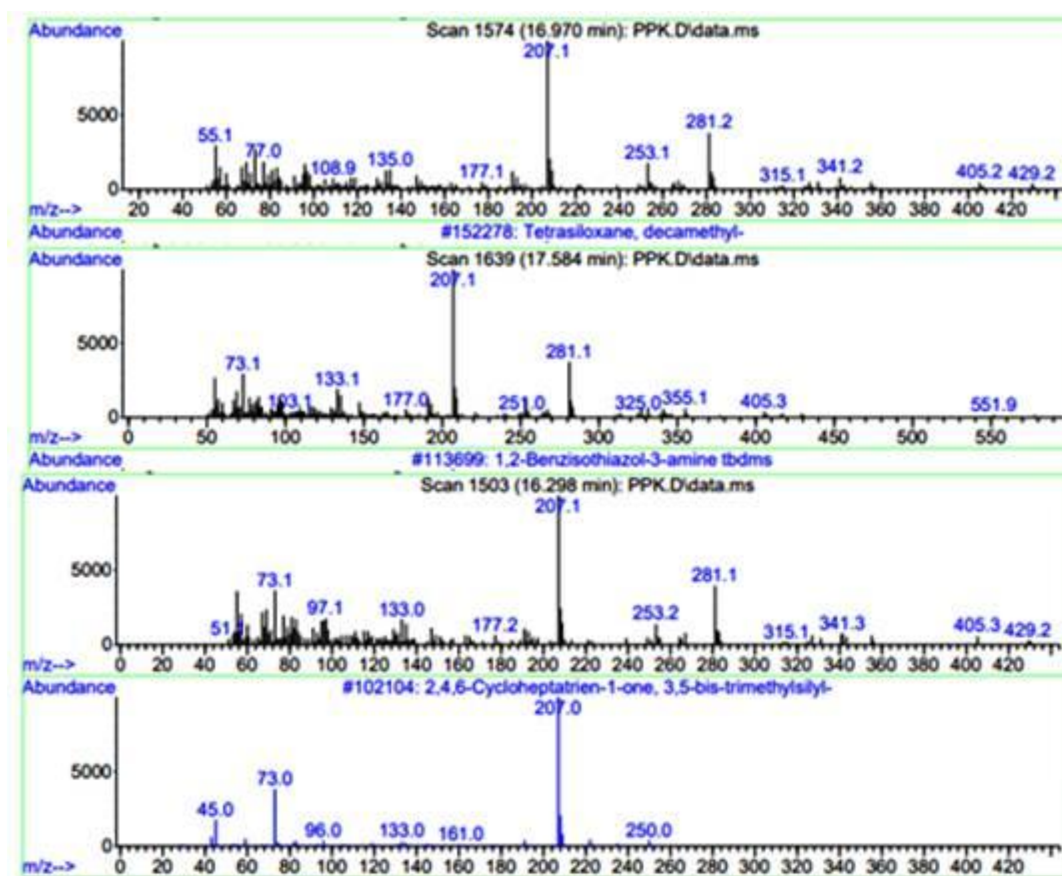



**Fig. 4** GC-MS analysis result of PHA

**Table 7.** Characteristics of GC-MS analysis of PHA extracted from M16a

| Peak No | Retention time | Area (%) | Compound name |
|---|---|---|---|
| 1 | 16.298 | 9.85 | 2,4,6-Cycloheptatrien-1-one, 3,5-bis-trimethylsilyl |
| 2 | 16.412 | 4.48 | N-Methyl-1-adamantaneacetamide |
| 3 | 16.478 | 4.65 | 1,4-Bis(trimethylsilyl)benzene |
| 4 | 16.714 | 10.00 | 1,2,4-Benzenetricarboxylic acid, 4-dodecyl dimethyl ester |
| 5 | 16.771 | 3.67 | 1,4-Bis(trimethylsilyl)benzene |
| 6 | 16.818 | 2.46 | Trimethyl[4-(1,1,3,3,-tetramethylbutyl)phenoxy]silane |
| 7 | 16.970 | 11.12 | Tetrasiloxane, decamethyl |
| 8 | 17.017 | 4.79 | Tris(tert-butyldimethylsilyloxy)arsane |
| 9 | 17.064 | 3.00 | 1,4-Bis(trimethylsilyl)benzene |
| 10 | 17.102 | 5.45 | 1,2-Bis(trimethylsilyl)benzene |

| | | | |
|---|---|---|---|
| 11 | 17.282 | 13.76 | Silicic acid, diethyl bis(trimethylsilyl) ester |
| 12 | 17.348 | 5.31 | Trimethyl[4-(1,1,3,3,-tetramethylbutyl)phenoxy]silane |
| 13 | 17.490 | 7.87 | Trimethyl[4-(2-methyl-4-oxo-2-pentyl)phenoxy]silane |
| 14 | 17.584 | 4.73 | 1,2-Benzisothiazol-3-aminetdbms |
| 15 | 17.631 | 3.07 | 1,2-Benzisothiazol-3-amine tdbms |
| 16 | 17.735 | 4.63 | Trimethyl[4-(2-methyl-4-oxo-2-pentyl)phenoxy]silane |
| 17 | 17.792 | 1.16 | Trimethyl[4-(1,1,3,3,-tetramethylbutyl)phenoxy]silane |

The present study indicated that about 17 biodegradable compounds were available in the analyzed sample and presented in Table 7. The compounds were benzene and their derivates as 1,2-Benzisothiazol-3-amine, Decamethyl and 3,5-bis-trimethylsilyl were shown in peaks and its retention time as 17.631, 16.970 and 16.298 along with the other available compounds. It positively indicated the production of PHA. The results were similar to the findings of Okwuobi & Ogunjobi, (2013). The compounds can make PHA a promising candidate to be used in medical and environmental areas.

## 5. Conclusion

This study isolated PHA accumulating bacterial strains from municipal waste dumped soil and identified as *Bacillus licheniformis* by morphological, grams staining, biochemical characterization, and 16S rRNA sequencing. The isolates were screened by Sudan black B and Nile blue A staining. Among 56 bacterial isolates, 2 strains namely M12a and M16a were isolated, purified, preserved and found to be promising for PHA accumulating bacteria. The PHA production was done in a minimal salt medium for both M12a and M16a, the maximum growth was observed in M16a. The PHA produced was extracted through sodium hypochlorite digestion method and qualitatively analysed by FTIR and GCMS analysis. Bacterial isolates M12a and M16a were optimized at different cultural parameters for maximum PHA production. The result indicates that bacterial isolate showed maximum growth at 37° C and 5% NaCl concentration, pH 8.5 was optimum for M12a and 7.5 for M16a. Bacterial growth was increased in time dependent manner, and the highest optical density value was obtained at 48 hours of incubation. There is a gradual decrease after 48 hours were observed for both strains. Optimization of carbon sources helps improve biomass production, thereby enhancing PHA production and making it more cost-effective. This study demonstrated the low-cost medium containing inexpensive agricultural waste products as a carbon source could be used for cultivating the high cell density of *Bacillus licheniformis* to produce PHA in a higher cell density. Application of engineered bacteria can reduce PHA production and extraction complexity, thus leading to a decline in production expense. The combination of mutated multiple division patterns and elongated bacterial cell size enhances the PHA accumulation.

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