ISOLATION AND IDENTIFICATION OF PHB PRODUCING MICROORGANISMS FROM DIFFERENT SOIL SAMPLES

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***Abstract*- PHB is a unique intracellular polymer that accumulates under stress conditions and in the presence of an increased carbon source. Synthetic plastics, which bring many disadvantages, especially the environmental damage, are being considered and replaced by bioplastics. Bioplastics like PHB are considered to be one of the best alternatives due to their efficient biodegradability and several other effective properties. PHB-producing microorganisms were isolated by collecting soils from three different areas to compare the production of PHB in these soil samples. The isolates obtained were cultured on Minimal Davis Agar and then primary screening was performed using by Sudan Black staining. PHB production was confirmed by performing FTIR and GCMS analysis. The FTIR result showed that only organisms collected from organic soil samples showed the presence of PHB exhibiting the bands representing C=O, C-O, CH3, CH2, C-H and C-C stretches. The GC-MS spectrum of methanolized PHB was confirmed by a distinct peak corresponding to 2-oxetanone, 4-methylene, also known as 2-butynoic acid**

***Index Terms*- Biodegradable PHB, Sudan Black Staining, FTIR, GC-MS Analysis**

1. Introduction

PHB was first discovered by Lemigne in 1926 as a unique intracellular polymer that accumulates under conditions of stress and in the presence of an increased carbon source. It is a short chain PHA and a polymer of D-(-)-B-hydroxybutyric acid. It is an important intracellular storage compound for many bacteria, especially prokaryotic organisms. PHB can also act as a carbon and energy source. PHBs are organic macromolecules produced by bacteria and when they grow under various stress conditions such as B. a lack of nitrogen, phosphorus, sulfur, magnesium, iron, potassium or oxygen, they act as inclusion bodies that accumulate as reserve materials (Galia MB et al 2010). They are sold in markets under the Biopol brand, which are made from renewable raw materials (Byrom D. 1987; Volva TG et al., 2000). PHB properties include high crystallinity, which provides good gas transport activity. These properties make PHB better than polypropylene and polyethylene terephthalate and can also be used for food packaging applications. They also possess properties such as high melting temperature and high tensile strength, which are similar to several other synthetic thermoplastics such as polypropylene (Bucci et al. 2007).

These properties make them very useful for mass production of bioplastics that could replace petroleum-based plastics and can be a promising material for the production and development of eco-friendly, biodegradable plastics (Verlinden Raj et al. 2007).

Other properties of PHB are its biocompatibility and its low oxygen permeability. These properties are exploited for applications such as film manufacturing and blowing, injection molding, extrusion, thermoforming, coatings and biomedical products such as patching materials, stents, bone implants, drug delivery systems, tissue engineering scaffolds, etc. (Holmes, PA et al. 1985). The unique properties that distinguish PHB from other biodegradable plastics are that it is insoluble in water and has high resistance to hydrolytic degradation and UV resistance. Another use for biodegradable plastics is to use them for composting purposes, which is an alternative to landfill disposal. The reasons that contribute to its use in the medical field are its low resistance to acids and bases, its solubility in chloroform and other chlorinated hydrocarbons, and its biocompatibility. The use of these bioplastics offers potential applications in medicine, agriculture and several other industries (Ramsay et al. 1990).

The main reason to look for an alternative to petrochemical-based plastics is their environmental impact. Plastics have become an integral part of our lives and are a basic need, however, the non-degradability of these plastics causes several environmental problems. (Lee SY. 1996). Because these materials are resistant to degradation, they remain on the ground as waste in this state for several years after disposal. These plastics are inexpensive, so they are often used, but the impact

of these synthetic plastics on the environment is too bad, leading to serious ecological damage caused by the unbalanced medium. It can also lead to dangerous air, water and soil pollution leading to various human and animal health issues. Therefore, the use of an alternative such as bioplastics has recently been promoted (Brandl H et al. 1990). Since bioplastics are biodegradable, they offer the best solution to protect the environment from these dangers as they are known to be environmentally friendly. These can be converted by microorganisms into carbon dioxide, water and humic substances (Steinbach A. 1991). Plastics derived from PHB are the best among bioplastics because they are 100% biodegradable (Galia MB et al. 2010).

Some of the bacteria that produce PHA and PHB are: *Alcaligeneseutrophus*, Alcaligenes latus, *Azotobactervinelandii*, *Rhizobium sp, Bacillus sp, Methylotrophs, Pseudomonas, Recombinant Escherichia coli, Halomonascampaniensis, Alteromonaslipolytica, Aeromonas, Herbaspirillumseropedicae, etc.*

PHB and other PHAs are synthesized and deposited in the form of granules inside microorganisms as a product of microbial secondary metabolism, which can represent up to 90% of the dry weight of cells (Schlegel et al., 1961). Accumulation of PHB material is a natural technique used by microorganisms to store carbon and energy, as their synthesis is favored by environmental conditions or stress leading to a limitation of nitrogen, phosphate or oxygen (Doi 1990; Steinbach 1991; Lee 1996).

2. MATERIALS AND METHODS

2.1. SAMPLE COLLECTION

Three types of soil samples were taken from sterile containers from three different areas. These clarified soils, organic soils and agricultural

Sample-1: Sewage soil was collected from a ditch near the Krishbrindhavan Ladies Hostel and designated “Soil Sample S.

Sample-2: Organic soil taken from a home garden plant near GV Residency and was labeled "Soil Sample O"

Sample-3: Agricultural soil taken from farmland near Mettupalayam and labeled as "Soil Sample A" (Mikkili I et al. 2014)

2.2. ISOLATION OF PURE CULTURE

2.2.1. SERIAL DILUTION:

Each soil sample was serially diluted. One gram of each soil sample was placed in ten ml of sterile distilled water and mixed thoroughly. Serial tenfold dilutions from 10-1 to 10-8 were made for each soil sample. From each sample, 0.1 ml of the aliquot dilution was removed and streaked onto a nutrient agar plate using the spread plate technique. After seeding the plates, the plates were incubated at 37°C for 24 hours.

2.2.2. SUB CULTURING:

Each soil sample was serially diluted. One gram of each soil sample was placed in ten ml of sterile distilled water and mixed thoroughly. Serial 1/10 dilutions from 10-1 to 10-8 were made for each soil sample. From each sample, 0.1 ml of the aliquot dilution was removed and streaked onto a nutrient agar plate using the scatter plate technique. After seeding the plates, the plates were incubated at 37°C for 24 hours.

2.2.3. STORING: The pure cultures at 4°C inside refrigerator for future uses. Sub culturing of the stored cultures are done in an interval of 4 to 6 weeks

2.3. SCREENING OF BACTERIA FOR THE PRODUCING PHB:

- Isolated bacterial cultures were removed from the nutrient agar plates of all three specimens and streaked on Minimal Davis Agar.
- These plates were then incubated at 37°C for 2-3 days.
- After incubation, primary screening for PHB-producing organisms was performed.

2.3.1. SUDAN BLACK STAINING METHOD.

- A loopful of culture was removed from each sample onto a clean, sterile glass slide and swabbed.
- This swab is not heat set.
- After the smear was air-dried, it was stained with Sudan Black B solution and left undisturbed for 10-15 minutes
- It was washed with distilled water and then left to dry.
- It was then counterstained with safranin for 10 seconds and then washed and dried.
- The slide was observed under a 100X oil immersion microscope to screen for PHB granules.
  - **Positive Result** - Organisms seen in blue-black color under 100X oil immersion
  - **Negative result** - organisms are observed in yellow color under the 100X oil immersion.
- Once the presence of PHB-producing organisms was confirmed by primary screening using Sudan black staining, isolates from each sample plate were selected and used for further screening techniques.

2.4.PHB EXTRACTION

PHB is extracted from selected bacterial isolates using the sodium hypochlorite-chloroform method.

2.4.1. CHLOROFORM PROCESS WITH SODIUM HYPOCHLORITE

To carry out this method, the selected bacterial isolates are first cultured in Minimal Davis Medium. It was incubated at 37°C for 2-3 days. After incubation, 10 ml of broth culture from each sample was placed in a centrifuge tube and centrifuged at 6000 rpm for 10 minutes. After the initial centrifugation, pellets formed at the bottom of the tube. The supernatant was discarded and the pellets removed. The pellets were then suspended or mixed in 5 ml of 4% sodium hypochlorite and 5 ml of hot chloroform. This suspension was then incubated at 37°C for 1 hour. After incubation, the mixture was again placed in a centrifuge tube and centrifuged at 3000 rpm for 10 minutes. After centrifugation, three phases were observed, the upper, middle and lower phase. The top and middle phases were discarded and the bottom phase was removed. (Sei Kwang Hahn et al. 1993)

EXTRACTION

5 ml of hot chloroform was first added to the lower phase. A 1:1 mixture of acetone and ethanol was prepared and then 5 ml of this acetone-ethanol mixture was added. This step is performed to precipitate the granules. This mixture was then transferred to a beaker or petri dish to facilitate extraction and kept at room temperature to evaporate. The next day, the granules are scraped with a spatula. In this method, pellets from each sample were extracted. The weight of the granules obtained was measured and then collected in an Eppendorf tube. (Basavaraj H et al. 2013)

2.5. POLYMER ANALYSIS

To confirm the presence of PHB in the collected pellets, two analytical techniques were used.

2.5.1. FTIR ANALYSIS

FTIR analysis is also called FTIR spectrometry. It is carried out to identify the functional groups present in the PHB extract obtained. These are generally carried out in order to know the organic, polymeric and rarely inorganic components. This test relies on infrared light scanning the sample to observe binding properties.

TEST PROCEDURE

- 1 mg of the extracted sample was taken and dissolved in 5 ml of chloroform.
- A film of PHB polymer was obtained by evaporation of chloroform.
- This PHB polymer film was then subjected to FTIR analysis with an FTIR spectrophotometer (8400S-SHIMADZU).
- The range in which the spectra were recorded is between 4000 and 6000 cm−1. (Likitha R.V. et al. 2018)

2.5.2. GC-MS ANALYSIS

GC-MS analysis serves as a tool for qualitative and quantitative analysis. The main purpose of GC-MS analysis is the molecular analysis of the purified polymer. Centrifuged and extracted PHB cells or pellets were used for sample preparation. A defined amount of the extracted granules was added to 2 ml of chloroform in a chloroform-resistant screw-capped glass tube. If the PHB is insoluble, the sample was heated at 100°C for 3 hours. Once the PHB had dissolved, 2 mL of acidified methanol containing 10% sulfuric acid and 8 mg/mL of benzoic acid was added. The sample was then heated to 100°C for 3 hours and then cooled to room temperature. 1ml of distilled water was added to the glass tube, stirred for 30 seconds to ensure optimal mixing, then allowed to separate for approximately 1-2 minutes.

TEST PROCEDURE

- GC-MS analysis was performed using a GC-MS-QP 2010 Plus model with Rtx-5 MS capillary column (30 m x 0.25 mm x 0.25 µm film thickness).
- 3 µl of the samples were injected in splitless mode.
- The injection temperature was 260°C and the column oven temperature was 100°C.
- The mass spectrum obtained was compared with the library of mass spectra of Nist-08 and Willey-08 (Bhuwal AK et al. 2013).

2.6. IDENTIFICATION OF PHB PRODUCING ORGANISMS

Identification of organisms that produce PHB can be done by various methods, including morphological characteristics, cultural characteristics, and biochemical tests.

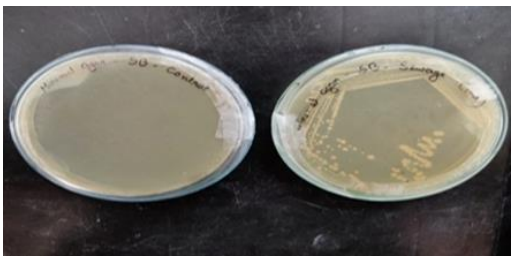
- **Morphological characteristics** - this includes microscopic observation of Gram stain, motility test and staining of endospores.
- **Cultural characteristics** - this includes macroscopic observation of colonies on solid media, including nutrient agar, mannitol salts agar and MacConkey agar.
- **Biochemical tests –** these include catalase test, oxidase test, indole test, methyl red test, Vogues-Proskauer test, citrate utilization test, etc. (Cappuccino G, Netalia Sherman, 1998).

3. RESULTS

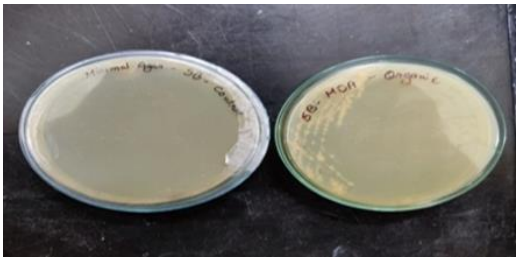
3.1. ISOLATION AND SCREENING OF PHB PRODUCING BACTERIA

To obtain more isolated colonies, the isolates were grown from feeder plates in Davis' minimal agar. Primary screening was performed by Sudan black stain, in which isolates from organic soil samples showed a blue-black color under a 100x microscope, indicating a positive result for PHB.

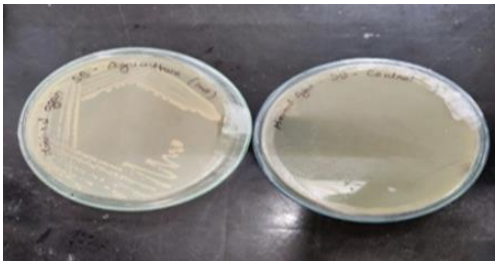
"Fig. 1" Sample S



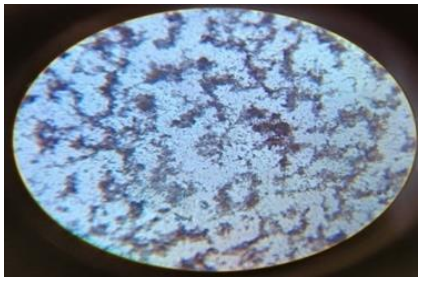
" Fig. 2" Sample O



"Fig. 3" Sample A



"Fig. 4" Sudan black staining



3.2. PHB EXTRACTION

The pellets obtained from the evaporated mixture were scraped from the beaker and the weight of the pellets obtained from each sample was measured.

- Granules obtained from a sewage soil sample - 3.8 mg
- Granules obtained from an organic soil sample - 3.1 mg
- Agricultural Soil Sample Granules - 0.2mg. These were then transferred to an Eppendorf tube and sent for FTIR and GC-MS analysis.

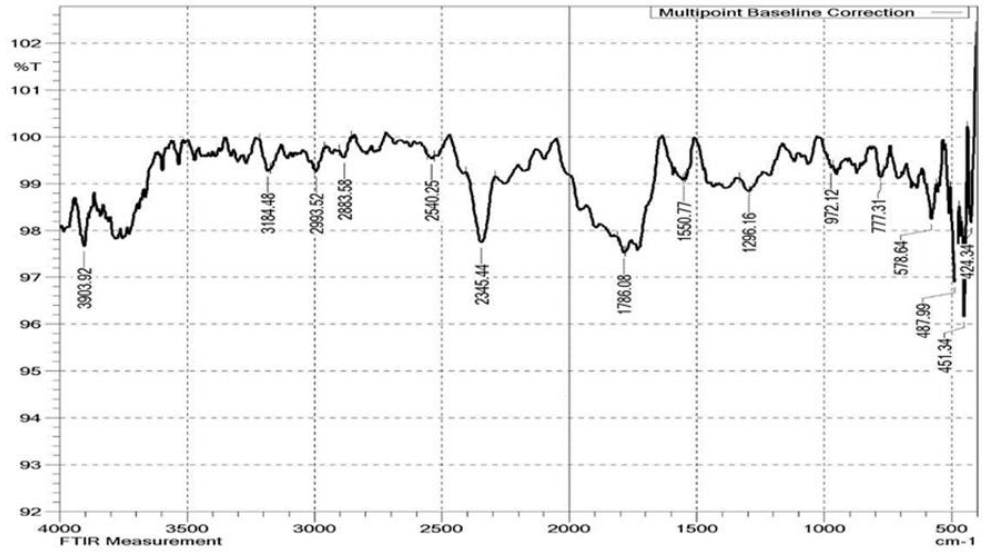
Table 1: Dry weight of extracted PHB

S.No	Sample Name	Weight of the granules obtained
1	Sample S	3.8 mg
2.	Sample O	3.1 mg
3.	Sample A	0.2 mg

3.3. FTIR ANALYSIS

The FTIR spectrum shows an adsorption band at 1786.08 cm-1 which corresponds to the ester carbonyl group representing C=O since it is between 1800 and 1680 cm-1. The series of bands between 1000 and 1300 cm-1 indicates the stretching of the C-O bond of the ester group, and the curvature at 1296.16 cm-1 in the spectrum of the sample obtained confirms the presence of a C-O bond. The region of the band from 2885 to 2845 cm-1 indicates the stretching of the CH3 and CH2 bonds and the curvature at 2883.58 indicates the presence of a CH3 or CH2 alkyl group. Elbows at 3184.48 cm-1, 2993.52 cm-1 and 2883.58 cm-1 correspond to sections C-H. The band at 177.31 cm-1 indicates the presence of a C-C band.

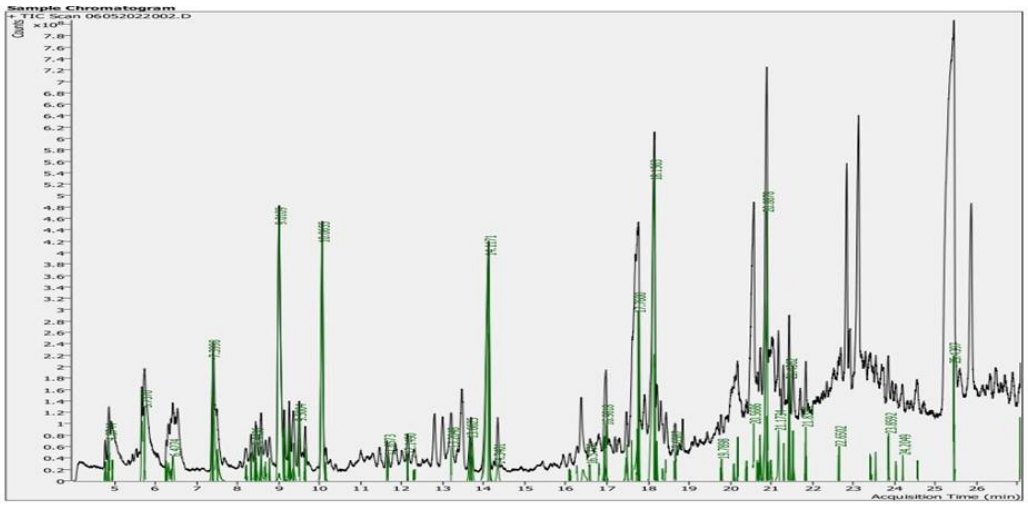
"Fig. 5" FTIR analysis of sample O



3.4. GC-MS ANALYSIS

GC-MS analysis was performed to determine the composition of PHB after the metanalysis process. GC-MS spectrum of methanolized PHB was confirmed by a certain peak corresponding to 2 oxetanone, 4-methylene also known as 2-Butynoic acid (RT 21.1734). Other surrounding elbows suggesting the presence of PHB were alanine, N-(2,2,3,3,3-pentafluoropropoxyl)-1-methylethyl ester, also known as L-2 acid -Aminobutyric (RT 16.9816), Fumaric Acid, Dicyclobutyl Ester, also called 2,6-dihydroxy-4-methoxy-3-methylphenyl-1-butanone (RT 20.8821), 3,5-dimethyldihydropyran-2,6-din , also called 3-oxo-butyric acid allyl ester (RT 19.7740) and formyl salicylic acid, also called 2-hydroxy-5- formyl benzoic acid (RT 24.5597). These peaks were the analogies with the original result. Thus, the presence of 3-hydroxybutyric components was confirmed. The initial synthesis of PHB involves the conversion of acetate to acetyl-CoA. Thus, the acetate regions of 5-methyl-4-hexen-1-yl, also known as cyclohexane acetic acid, methyl ester(RT 20.0929) and ethoxy acetylene (RT 9.2623), indicate the presence of an acetate compound, suggesting the chances of PHB synthesis This indirectly confirms the presence of PHB.

"Fig. 6" GC-MS analysis of sample O



3.5. IDENTIFICATION OF PHB PRODUCING BACTERIA

Morphological examination of the organisms was first performed by performing a Gram stain and the organisms were found to be Gram positive rods appearing in concatenated clusters. These organisms have been observed to be motile and exhibit swimming motility. It also showed the presence of spores after staining the endospores. The culture characteristics of these organisms were checked by culturing them on nutrient agar, mannitol salt agar and MacConkey agar plates. On nutrient agar, organisms grew well and colonies were raised, smooth, irregular and off-white in color, whereas on mannitol salts agar and MacConkey agar, organisms were stunted. The battery of biochemical tests revealed the organism to be catalase positive, methyl red positive and citrate positive, while the oxidase test, indole test and Voges-

Proskauer test showed negative results. When all of these results are combined, one would suspect that the organism is Bacillus sp.

Table 2: Identification of PHB producing bacteria

S. No	Identification tests	Result
1.	**Gram Staining**	Gram positive rods in chained cluster
2.	**Motility Test**	Motile
3.	**Endospore staining**	Spore forming
4.	**Nutrient agar**	Raised, smooth, irregular and creamy white in colour
5.	**Manitol Salt Agar**	Rare growth or no growth
6.	**MacConkey Agar**	No growth
7.	**Catalyse Test**	Positive
8.	**Oxidase Test**	Negative
9.	**Indole Test**	Negative
10.	**Methyl Red Test**	Positive
11	**Voges Proskauer Test**	Negative
12.	**Citrate Utilization Test**	Positive

4. CONCLUSION:

This study was conducted to isolate PHB-producing bacteria from three different soil samples and to identify the bacteria responsible for PHB production. It was also carried out to compare the production of PHB in different types of soils. From this study, it was concluded that organisms isolated from soil samples from the organic waste disposal area produced PHB. The isolated organisms turned out to be Bacillus sp. With these findings, PHB could be easily manufactured and could be used to replace synthetic plastics and prevent environmental damage, indirectly contributing to maintaining ecological balance.

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