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Characterization of Polyhydroxybutyrate Producing Bacterium Isolated from Sewage Sample

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ABSTRACT

The increasing importance of the non-degradable plastics has emerged as one of the major concerns. For this, research is being ventured from the existing reserve to produce bioplastics on the basis of biodegradability properties. Polyhydroxybutyrate, a thermoplastic that is biodegradable as well as environmental friendly, is one such example. Present study identifies potential bacteria strains producing Polyhydroxybutyrate from sewage soil sample collected from Tenali, Guntur District. AR15, one of the derivatives from the ten bacterial isolates recognized through Sudan Black staining, was perceived to be a potential PHB producer. A preliminary identification of the isolate was achieved through biochemical characterization and was found to be Bacillus sp. Biopolymer obtained from the isolate was characterized by FTIR and TGA. Further the biopolymer compared with standard and was seen to be Polyhydroxybutyrate.

1. INTRODUCTION

In today's world, plastics are used more than any other material. Undeniably, today's society cannot function without plastics whether it is in the automobile, construction, biomedical, packaging industries and much more. Although plastic is economically and socially beneficial, it is currently damaging to the environment. The annual production of plastic has risen to more than 300 million tonnes [1]. The average amount of plastic recycled is 9% and the average amount incinerated is 12%. In addition, 79% of plastic ends up in landfills and the ocean each year, with over 10 million tons entering the ocean according to the studies of Geyer et al. [2] and Jambeck et al. [3]. Plastics' chemical inert nature and strong physical properties make them ideal materials for everyday applications but making it hard for them to break down and decompose in the environment [4]. Microplastics are formed when plastic items dissolve into smaller fragments over time, which are absorbed into the food chain by organisms at the base, thus causing plastic material to build up as the food chain moves upwards. Organic pollutants are also allowed into the food web by plastic pollution due to their adsorption on the surfaces of plastics in Ref. [5]. In addition to causing environmental damage, over 99% of our current plastics are produced from finite reserves of petroleum. Global population growth as well as expanding middle classes are expected to drive demand for plastic in many parts of the world. The number of biodegradable plastics produced currently is less than 1% as suggested by De Bartolo et al. [6]. Therefore, it is imperative to develop bioplastics which are essential to prevent the destruction of the environment, and they must be derived both from renewable materials that can be sustainably sourced and that biodegrade into harmless compounds. A bioplastic refers to a plastic material that is either biodegradable or biobased in the study of Rujnić-Sokele and Pilipović [7]. The biodegradability of plastics is affected by various factors such as properties of the polymer blend itself (like the presence of which functional group, molecular weight of polymer chains, presence of additives, tacticity and crystallinity) and environmental conditions, which may be chemical, biological or physical in nature (like microbial community, exposure to sheer forces, UV radiation and temperature). It refers to using biomass instead of petroleum reserves as a raw material source. Nevertheless, a biodegradable plastic does not always mean it is bio-based (like polycaprolactone made from fossil fuels). Likewise, there is no guarantee that bio-based plastic compounds are biodegradable (like biomass source that produces polyethylene). Nonetheless, bioplastics should be both biobased and biodegradable in order to reduce the environmental pollution created by the production of these plastics and ensure they are sustainably sourced [8]. Bioplastics of the first generation are biobased and biodegradable. Ranganadha et al. [9] proposed that the source of these first-generation bioplastics is terrestrial crop biomass. They either utilize biopolymers occurring in nature like starch or cellulose, or they undergo even more processing in order to form plastics like polylactic acid and polyhydroxyalkanoates [10]. The growing competition for arable land, fertilizer, usage of fresh water and increase in prices of food were some of the downsides of such products in Ref. [11]. Brodin et al. [12] proposed that the agricultural waste was incorporated for second generation bioplastic technology while discarding biomass for the production of bioplastics. However, global plastic demand cannot be restored using waste stream generated bioplastics alone as they are not large enough in

volume and high enough in quality and consistency in order to provide sufficient biomass [13]. The current study included screening of PHB producing bacteria and how PHB is characterized to determine its quality.

2. MATERIAL AND METHODS

2.1 Collection, isolation and preservation of bacterial culture sample

Sewage soil sample was collected aseptically from Tenali with *GPS* coordinates 16° 14' 12.1884" N and 80° 38' 50.9136" E. The samples were collected in a clean sterile bag. One gram of each soil sample with 10 ml of distilled water was serial dilution was the preparation of 10^{-1} to 10^{-7}, then 0.1 ml each dilution was spread plated. Microbes isolation was done by using this technique are sterile nutrient agar media using plates and incubated for 37℃ for 24-48 hours. The purified cultures are maintained on nutrient agar slant and incubated at 4℃.

2.2 Screening for PHB positive colonies

A staining method called Sudan Black was used for qualitatively screening PHB production of all the isolated bacterial colonies.Sudan Black stain was briefly prepared in 60% of ethanol as a 0.3% solution (w/v). Smear bacterial culture was prepared on a glass slide and fixed by heat then stained for 10 minutes with Sudan Black solution, for 1 min 0.5% of safranin rinsed with tap water and counter-stained. Stained samples were observed by using a Light microscope for 1000x magnification under oil immersion.

2.3 Extracting PHB from isolates of bacteria

Centrifugation of 10 mL of bacterial culture (1000 rpm/15 min) was used to achieve the extraction of PHB from bacterial isolates. Sodium solution was then used to digest the resultant pellet at 37℃ for 1 hour. After a further centrifugation at 5000 rpm for 15 minutes, distilled phosphate buffer saline, water, acetone and methanol were used to wash the mixture. The pellet was then dissolved in 5 mL of boiling chloroform after washing and retained to obtain crystals of PHB after the complete evaporation.

2.4 PHB sample and assay identification

Polyhydroxybutyrate assay was used to identify the sample.

2.5 Characterization of PHB

2.5.1 Analysis by FTIR

About one mg of PHB extract sample was dissolved in chloroform 5 ml. After cell pellets were formed by adding KBr. The spectra were recorded at the range 3,800-800 cm^{-1} by Spectrum 65 Fourier Transform – Infrared Spectroscopy.

2.5.2 TGA analysis

Approximately 5-10 mg of PHB was loaded in to aluminium crucible and subjected to heat in the temperature range i.e. from 35℃ to 700℃ with a heating rate of 10℃ min^{-1}. Thermo gravimetric analysis is used to evaluate the change in weight during heating and thus determine the thermal stability of the PHB.

3. RESULTS AND DISCUSSION

3.1 Isolation and screening of colonies producing PHB

A total of 30 bacterial isolates were screened to produce PHB. After the preliminary screening, 10 of these isolates were screened positive. Potential producers of PHB were chosen if the bacterial isolates appeared bluish white under UV. The 10 isolates were then subjected to secondary screening. Out of these 10 isolates, further studies have been chosen for AR15 because of its great potential for producing PHB (Table 1).

Table 1. PHB yield from isolated bacterial colonies

S No.	Bacterial isolate	PHB Yield (g/L)
1	AR01	0. 79
2	AR03	0. 71
3	AR04	0.52
4	AR06	0.49
5	AR10	0.44
6	AR13	0.38
7	AR14	0.58
8	AR15	0.88
9	AR18	0.81
10	AR26	0.30

3.2 Screening of isolate AR15 by Sudan Black B

Bacterial isolate AR15 screened positive to Sudan Black B (0.3 gm in 100 ml of 70% ethanol) as shown in Figure 1 when observed under Olympus microscope at 100X.

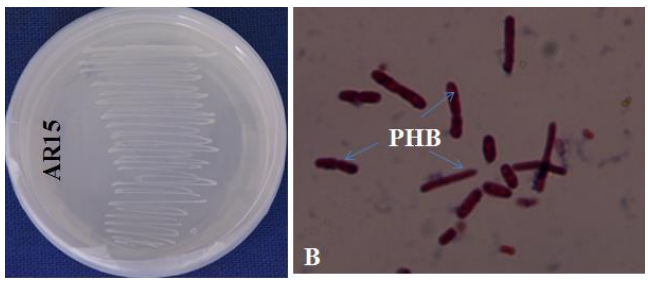

Figure 1. a) bacterial isolate b) Sudan Black B staining

3.3 Law and Slepecky method of PHB assay [14]

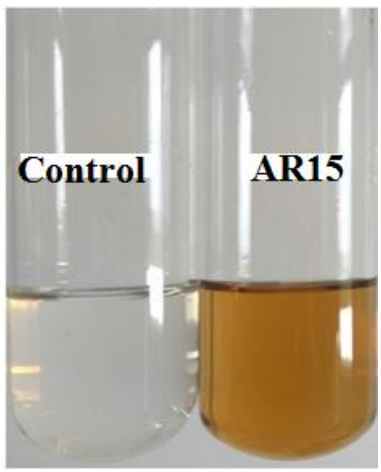

Figure 2. PHB assay by Law and Slepecky method

Considerable amount of accumulation of PHB was exhibited by isolates of bacteria viz., AR01, AR03, AR04, AR06, AR10, AR13, AR14, AR15, AR18 and AR26 which is shown in Table 1. Due to its potential to produce maximum yield of PHB, further studies have been chosen for the bacterial isolate AR15. Quantitative conversion of PHB to crotonic acid under boiling conditions (Figure 2) was used as the basis for the PHB assay using concentrated H_2SO_4.

3.4 PHB extraction

Extraction of PHB was done by the modified method of Senthil and Prabakharan [15]. Hot air oven was used to dry the crude PHB which was extracted into centrifuge tubes as pellet. A large number of gram positive and gram negative bacterial species were reported to accumulate PHB granules by early researchers.

3.5 PHB characterization

FT-IR analysis

The Fourier transform infrared spectroscopy technique is commonly used to characterize the functional groups present in a polymer. In an IR spectrum, each constituent in the extracted polymer gives a separate absorption peak. Absorption peaks of isolate AR15 at 3290 cm^{-1} were revealed by FTIR spectrum which correspond to aliphatic ester carbonyl C=O group. The peak at 2918 cm^{-1} and 2849 cm^{-1} indicate terminal hydroxyl group (-OH). The absorption peak at 1790 cm^{-1} represents O-H group related to aliphatic compounds. The peak at 1734 cm^{-1}corresponds to C=O stretch. The absorption peak at 1448cm^{-1} and 1370 cm^{-1} indicate C–O ester bond, CH vibrations of–CH_2 and–CH_3 functional groups as shown in Figure 3. The absorption peaks at 500-1000, 1220 and 1098 cm^{-1} represents C=O ester group, C–O stretch and OH group. The FT-IR spectral results of biopolymer correlated with findings of Tripathi and Srivastava [16] and Ranganadha et al. [17].

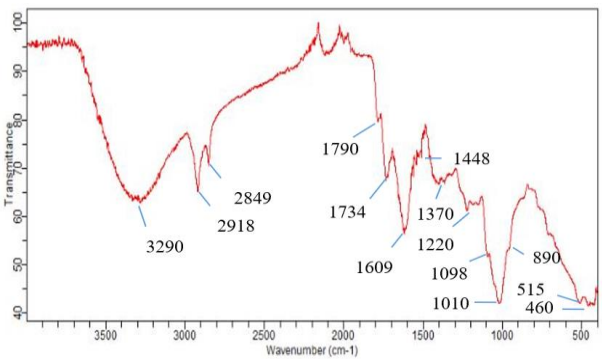

Figure 3. FT-IR analysis of Polyhydroxybutyrate from isolate AR15

TGA analysis

Thermal decomposition studies of Polyhydroxybutyrate obtained from the *Bacillus sp* AR15 was obtained in the temperature range from 30℃-400℃ using Thermal analysis. Thermal decomposition pattern of Polyhydroxybutyrate obtained from the *Bacillus sp* AR15 is shown in Figure 4 which shows the initial decomposition temperature as 208℃ and at 688℃ about 82% of the polymer was decomposed. Complete thermal decomposition of the polymer was not possible because of presence of inorganic cell dry mass.

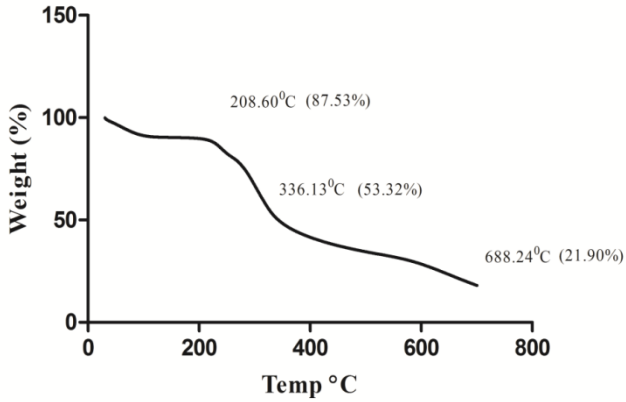

Figure 4. TGA analysis of Polyhydroxybutyrate from isolate AR15

4. CONCLUSIONS

Now a day researchers are focusing on biopolymer-producing microorganisms for developing biodegradable plastics. This reduces the biggest environmental pollution which problem is caused by conventional plastics and is solving agricultural waste disposable problems. From this study, it is concluded that isolate AR15 showed ability to accumulate PHB. Sudan Black staining method showed positive results for PHB. The confirmation analysis of PHB is done using analysis for FTIR and TGA analysis. Finally, this current research into these bacteria may indicate their unique involvement in PHB production. Bio plastics are molecules with a bright future due to their unique properties and wide range of biotechnological uses.

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