IIETA International Information and Engineering Technology Association
Advancing the World of Information and Engineering

Environmental and Earth Sciences Research Journal
Vol. 7, No. 3, September, 2020, pp. 116-120
Journal homepage: http://iieta.org/journals/eesrj

Screening and Biochemical Characterization of PHB Producing Bacterium Isolated from Costal Region of Andhra Pradesh

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https://doi.org/10.18280/eesrj.070304

Received: 20 June 2020
Accepted: 25 August 2020

Keywords:
biopolymer, Bacillus sp, Polyhydroxtbutyratye, FT-IR, DSC

ABSTRACT

Biopolymers and their composites have been massively investigated in recent years for multiple applications especially in environmental medical and pharmaceutical fields. A thermoplastic that is both biodegradable and environmental friendly, as well as biocompatible is PHB. PHB is an intracellular granule, which is a carbon and energy reservoir for the bacteria under starvation or stress conditions. In the present work, we focused on identification of potential PHB (Polyhydroxybutyrate) producing bacterial strains from sewage soil of coastal region of Andhra Pradesh. Five bacterial isolates were identified through Sudan Black staining, out of which RR02 was observed to be potential for PHB production. Further, PHB extraction was performed by solvent extraction method. The extracted sample was characterized by FTIR, melting temperature Tm, was determined by DSC and then, compared with standard PHB for confirmation of quality. Biochemical characterization was also performed for preliminary identification of the bacterial isolate. Based on the study it was found to be *Bacillus sp*.

1. INTRODUCTION

Industrial growth and anthropogenic development over the past century have continuously increased the dependency of humans on plastics. Packaging material, building and construction, transportation, medical and health, electrical and electronics, agriculture, sports and leisure products are some of the many ways in which human population utilize plastics [1-4]. Enormous amounts of waste, which is difficult to decompose, and green house gases are generated by both the production and after life remediation of plastics, which cause various kinds of health hazard problems in humans [5]. Plastic is naturally resistant to degradation due to the molecular size which is up to 1, 50,000 Dalton. Diminishing oil resources is caused directly by the consumption of petrochemical based plastics. In order to displace this ever increasing use of plastic, a potential material could be bioplastic, which can be equally utilized in all applications [6-8]. It's degradability with time is the major merit of bioplastic over synthetic plastic. Bioplastics, in some cases are non immunogenic in nature and this can be utilized for designing medical implants and devices [9]. They are an important class of advanced biomaterial and consist of thermostable polyesters. The physico-chemical properties of bioplastics are similar to that of synthetic polymers, with an added advantage of being degradable under normal environmental conditions [10, 11]. A lot of interest for scientists and researchers has been created by biodegradable polymers such as PLA (Polyactic acid), PHA (Polyhyrdoxy alkanoates), PHB and Cellophane. Polyhydroxy alkanoates (PHAs) can be purified from bacteria, which have the ability to accumulate bioplastics under stress conditions in these forms [12, 13]. PHAs are 3-ydroxy fatty acid monomers that form linear, head-to—tail polyester. PHA is normally produced as a polymer containing 103-104 monomers. This accumulates in the bacterial inclusion size of 0.2-0.5 µm in diameter. In 1925, Lemoigne first discovered Poly-3-hydroxybutyrate (PHB) in bacteria. PHB is a linear polyester of D(-)-3 hydroxybutyric acid. PHB which is a member of the PHA family is the most widely used at industrial level for production [14, 15]. Various micro organisms such as Pseudomonas putida, Alcaligenes latus, Aeromonas hydrophila, Bacillus sps., and Ralstonia eutropha undergo nutrient imbalance conditions, like higher carbon ratio with reduced phosphorus, nitrogen and oxygen to produce the polyesters polyhydroxyalkanoates (PHAs) [16-19]. Depending upon the various groups and their position on the main chain, PHA consists of different classes. PHAs can be found as homopolymers or as co-polymer and have approximately 150 different constituents [20]. The current study involved screening of bacteria that produce PHB and the characterization of PHB which is produced for the quality.

2. MATERIALS AND METHODS

2.1 Sample collection

Soil samples were collected from different sites of sea coast situated at costal area of Guntur (Costal Region of Andhrapradesh). The samples were stored in sterile plastic bags at 4°C and were transferred to laboratory.

2.2 Isolation of PHB producing bacteria

1 gram of costal soil sample was collected from sea coast of Guntur, Andhrapradesh. Pure colonies of bacterial isolates were obtained by serial dilution pour plate technique on nutrient agar medium using L shaped spreader. All the

petriplates were incubated at 37℃ for 24-48 hours and bacterial colonies with unique characteristic features were maintained as pure cultures on agar slants and preserved at 4℃.

2.3 Morphological analysis of bacterial isolates

Pure bacterial colonies were characterized for colour, form, elevation, margin etc. Bacterial isolates were morphologically analyzed on the basis of staining and cell morphology using microscope.

2.3.1 Simple staining

The simple staining of isolate was done and analyzed for the cell size, arrangement, form, shape and pattern of the isolated strain. Methylene blue was used as simple staining dye. Smear was prepared on glass slides, heat fixed and were stained with 2-3 drops of methylene blue. Dye was washed with distilled water. The slide was placed under 100X microscope for the visualization of cell shape and cell arrangement [21, 22].

2.4 Screening for PHB positive colonies

The isolates were further screened for PHB accumulation by Sudan Black B staining.

2.4.1 Sudan Black B staining

Isolated strains were smeared on glass slide, heat fixed. Later, the smear was stained with Sudan Black B solution (Sudan Black B 0.3 gm in 70% ethyl alcohol 100 ml) and air dried, washed with alcohol, further safranin was added, 2-3 drops on the smear for 20 seconds and washed with distilled water. The slide was placed under 100x microscope (fluorescence microscope) for visualization. The bacterial cytoplasm was stained light pink and lipid granules were stained black [23].

2.5 PHB extraction from bacterial isolates

PHB extraction from bacterial isolates was carried by centrifugation of 10 mL of bacterial culture (10000 rpm/15 min.) and pellet was digested with sodium solution at 37℃ for 1 hrs. The mixture was further centrifuged at 5000 rpm for 15 min and washed with distilled phosphate buffer saline, water, acetone and methanol respectively. After washing, the pellet was dissolved in 5 ml of boiling chloroform and kept for complete evaporation to obtain PHB crystal [24].

2.6 Identification of PHB sample and PHB assay

The sample was identified by Polyhydroxybutyrate assay. Polyhydroxybutyrate assay was performed as per the standard method of Law and Slepecky [25].

2.7 Characterization of PHB

The extracted PHB was characterized by FTIR and DSC analysis.

2.7.1 FTIR analysis

FTIR is a powerful approach that is used to identify organic, polymer and inorganic materials. Infrared light for scanning samples and for observing chemical properties are used by the FTIR analytical technique. FTIR transmits infrared radiation between 10,000 and 100cm^{-1}, which receive and pass through such radiation. The radiation absorbed is converted into rotating or vibrational energy by the sample. The received image on the sensor generally shows a profile of the sample, from 4000 cm^{-1} to 400 cm^{-1}. 1mg of the PHB sample and 10mg of spectral pure anhydrous potassium bromide crystal was formed into pellet for IR analysis.

2.7.2 DSC analysis

The crystallization temperature (Tc), melting temperature (Tm), and glass transition temperature (Tg) were determined by using Differential Scanning Calorimeter. Extracted Biopolymer sample was encapsulated in aluminium crucible and heated in a temperature range from -50℃ to 400℃ at the rate of 10 ℃/min. Melting temperature was recorded at the peak of the melting endotherm [26].

3. RESULTS AND DISCUSSION

3.1 Isolation and screening of PHB producing colonies

Out of 30 bacterial isolates screened for PHB production, 05 isolates screened positive after preliminary screening. Bacterial isolates that appeared bluish white under UV were selected as potential PHB producers. Among the 05 isolates which were subjected to secondary screening isolate RR02 was highly potential for PHB production as indicated in Table 1 and hence isolate RR02 was selected for further studies. Andhrapradesh has a vast coastal region of 1000kms, Guntur region was explored for collecting soil sample. In this scenario in our study Bacillus sp. RR02 strain which was isolated successfully from coastal region of Guntur is highly potential for PHB production as shown in Figure 1.

Table 1. PHB yield from isolated bacterial colonies

S No.	Bacterial isolate	PHB Yield (g/L)
1	RR02	0.88
2	RR10	0.79
3	RR17	0.72
4	RR23	0.69
5	RR27	0.43

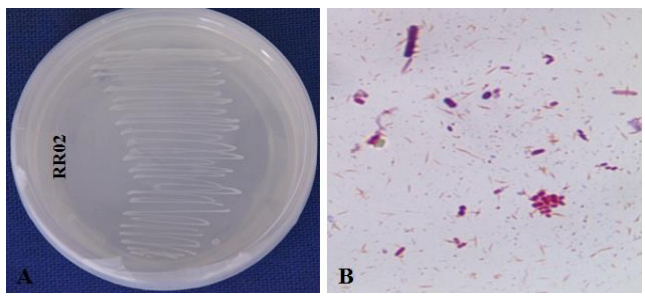

Figure 1. PHB producing positive colony a) pure colony of RR02 b) Staining

3.2 Screening of isolate RR02 by Sudan Black B

Smear of isolate RR02 was prepared on glass slide and stained with Sudan Black B (0.3 gm in 100 ml of 70% ethanol). Results were observed under Olympus microscope at 100X as shown in Figure 2.

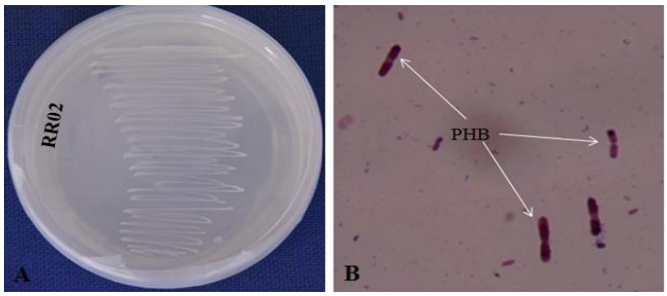

Figure 2. PHB positive Colony a) bacterial isolate RR02 b) Sudan Black B staining

3.3 PHB assay by Law and Slepecky method

PHB assay for the bacterial isolates was performed by using the standard Law and Slepecky method. The bacterial isolates viz., RR02, RR10, RR17, RR23 and RR27 showed considerable amount of PHB accumulation as shown in Table-1 Whereas, other isolates comparably yielded less amount of PHB. The bacterial isolate RR02 was choosen for further studies as it produced maximum yeild of PHB as compared to other bacterial isolates. PHB assay using concentrated H_2SO_4 is based upon quantitative conversion of PHB to crotonic acid under boiling conditions (Figure 3). Crotonic acid has got lambda max at 235 nm when H_2SO_4 is used as a solvent and it permits an accurate quantification of PHB. Biochemical characterization studies of the bacterial isolate revealed positive for PHB production.

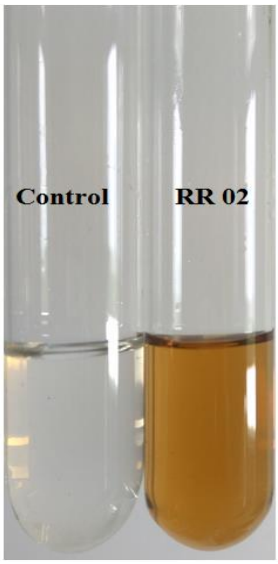

Figure 3. PHB assay of bacterial isolate RR02 by Law and Slepecky method

3.4 Extraction of PHB

The bacterial isolate RR02 was incubated in rotary shaker for 3-7 days for mass growth at 37℃. After 7 days of incubation the medium appeared turbid. Then the sample was extracted from the turbid medium using soxhlet apparatus. Crude PHB was extracted in centrifuge tubes as a pellet and was dried using hot air woven. Earlier researchers reported the accumulation of PHB granules by a large number of gram positive and gram negative bacterial species [27-32].

3.5 PHB characterization

3.5.1 FT-IR Analysis

FT-IR is used for characterization of isolated Poly-3-hydroxybutyrate from bacterial isolate [33]. FTIR spectrum of *Bacillus sp* RR02 revealed the absorption peaks at 3416 cm^{-1} and 3333cm^{-1} (Figure 4) denote terminal hydroxyl group (-OH). The absorption band at 3020 cm^{-1}represents methylene group. The absorption bands at 3020, 1609 and 1510 cm^{-1} denote the –C≡C–, –C=C–and N–O asymmetric stretch respectively. The absorption bands at 1441cm^{-1} and 1397cm^{-1} denote C–O ester bond, CH vibrations of $–CH_2$ and $–CH_3$ functional groups. The absorption bands at 1219, 1098 and 500-1000 cm^{-1} represents C=O ester group, C–O stretch and OH group. The FT-IR spectral results of biopolymer isolated from *Bacillus sp.* RR02 correlated with the earlier findings [34-36] indicating the isolated compound as PHB.

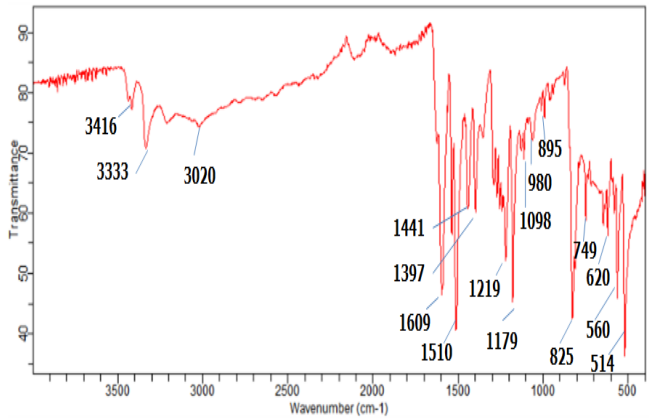

Figure 4. FTIR spectrum of powered PHB from *Bacillus sp.* RR02

3.5.2 DSC Analysis

The Differential Scanning Calorimetry thermograms (Figure 5) revealed the characteristic or DSC as shown in Figure 5 which indicated that the melting temperature Tm of PHB biopolymer is 169℃ which was very close to melting temperature Tm (175℃) of the standard PHB [37-41] with an increasing temperature range from 50℃ to 450℃.

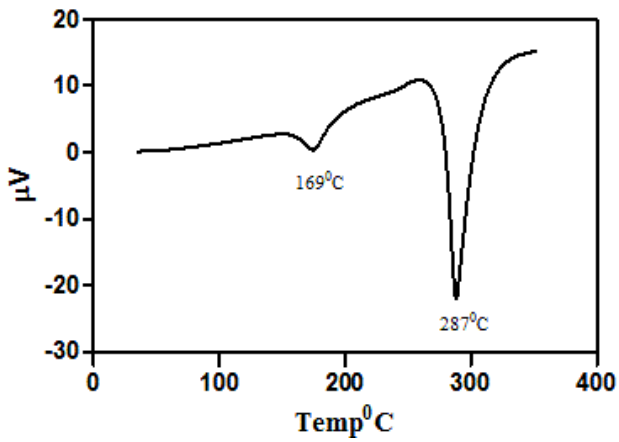

Figure 5. DSC analysis of powered PHB from *Bacillus sp* RR02

4. CONCLUSION

In the current study, different bacterial colonies were isolated and screened from soil for the production of Polyhydroxybutyrate. The isolate *Bacillus sp.* RR02 producing greater amount of PHB was studied. The biopolymer produced by *Bacillus sp. RR02* was characterized by FTIR. DSC studies revealed the melting temperature Tm of biopolymer as 169°C which was almost similar to standard PHB. The above studies reveal the potential of *Bacillus sp.* RR02 as promising alternative for commercial production of Polyhyhydroxybutyrate.

ACKNOWLEDGMENT

The author acknowledges VFSTR (Deemed to be university) and DST-FIST networking facility to carry out this work.

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