Screening, identification and characterisation of Polyhydroxybutyrate producing bacteria from garden soil

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Research Article

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Abstract

Plastic pollution need to be resolved as it affects air, water, land. The favourable alternative for plastics would be Polyhydroxybutyrate (PHB) from bacterial origin, which are biodegradable and biocompatible biopolymers. Focus on the PHB producing bacteria is done by collecting garden soil sample. Five colonies of Sudan black blue positive isolates were chosen, extracted, and produced. One of the strains (SM1) - a potent producer as confirmed by crotonic acid assay, was further subjected to large scale production. The PHB thus produced was analysed using Fourier Transform Infra-Red spectroscopy (FTIR) and Gas Chromatography Mass Spectrometry (GCMS) to confirm the presence of functional groups. X-Ray crystallography revealed that it is of crystalline nature and are pictured by Scanning electron microscopy photography. DNA was isolated from the strain SM1, and the gene for 16S rRNA has been sequenced and submitted in GENBANK, (Accession No: MZ363886). The organism was found to be Bacillus cereus as predicted by 16S rRNA and NCBI BLAST. A phylogenetic tree was constructed using MEGA software. Bioplastic preparation was done under laboratory scale and the produced bioplastic was successfully degraded using Pseudomonas species. The prepared bioplastic from bacteria was biodegradable and eco-friendly.

1. Introduction

Plastic is affordable, flexible, robust, long-lasting, abrasion-resistant, thermal- and an electromagnetic insulator. Huge varieties of materials are made of plastics because of their durability, bringing medical and scientific advancements, energy conservation and many other socio-economic advantages. As a result, over the last 60 years, plastics demand has risen considerably from approximately 0.5 million tons in 1950 to more than 260 million tons now. [1]. Issues related to plastic waste disposal and plastic debris are not yet settled. Abandoned plastic pollutes a variety of natural land, coastal and fresh water environments, with media reports of plastic pollution also on the highest peaks and mountains [2]. The small plastic fragments contaminate the soil, and hence these are widespread in municipal and sewage sludge. Transportation of plastic to streams, rivers along with surface run-off water and inundation was also reported [3, 4]. The most frequently used plastic products found in the world are cigarettes butts, plastic water cups, bottle tops, candy wrappers, plastic grocery bags, plastic lids, straws and other plastic and foam containers [5, 6, 7]. Domestic and municipal composting are the most common disposal choices for these materials. Biodegradable polymers may also make considerable contributions to plastic recycling, waste reduction and sustainable resource use [8]. In case of polyethylene, mulching films will not favour degradation; therefore, in the first place, biodegradable films are the better choice. If buried in the soil together with the leftover plant materials, it may decay by the usual microflora [9].

Based on the application and endurance, many biodegradable bioplastic are accessible. PolyLacticAcid (PLA), Poly Hydroxy Alkanoates (PHA), cellulose and starch are among these polymer substances. Similarly, biodegradable plastics may be reused for several times and are suitable for incineration too. Depending on the type and need, the development of a circular economy through alternative edge controls could be instituted in commercial, domestic and industrial composting using anaerobic digestion; thus limiting the pollution in its panorama. [10, 11]. Out of the available bioplastic, bio-polyethylene, polyl-polyurethane, and bio-polyethylene are the most used non-biodegradable bioplastic [12].

In recent years, the highly influential type of bioplastic are Poly-Hydroxy Alkanoates [13]. PHA is a form of green plastics that lower CO₂ emissions and resilience, devoid of petroleum products and with voluminous commercial uses [14, 15]. Currently, oil-derived plastics costs less than PHAs, [16] while several experiments using agricultural
and industrial waste materials have demonstrated that the use of waste materials may reduce PHAs’ production costs by statistically optimizing them [17, 18].

In relation to the form of microbe involved, the use of low-cost substrates partakes more concern vividly because the substrate or carbon source is the costliest factor in the development of PHAs [24]. PHAs are commonly used in medicine as early as 1970s [29]. PHA-based products are widely employed by physicians’ viz., biodegradable surgical staples, bolts, sheets, clips, and cords, bioabsorbable suture substance and skin staples. There are also medical tools and usages like wound and burn bandages, fabrics for periodontal regeneration, surgical mesh, bioprostheses, patches for surgical repair, hernioplasty, coronary stents, nerve regeneration mesh tubes, artificial heart valves, and surgical equipment synthesized using PHAs [30].

Poly Hydroxy Butyrate (PHB), a well-studied form of PHA, was discovered in 1925 [19]. PHB is a plastic with biological degradability and has a Molecular Weight (MW) of 1x104–3x106 Da [20]. The glass transition temperature, crystalline density, and amorphous nature of PHB are 180°C, 1.26, and 1.18 g (cm3)−1, respectively [21]. Regardless of the fact that PHAs have similar tensile strength and Young’s modulus to Poly Propylene (PP), PHB has a lower extension to break (5%) than PP (400 percent) [22, 23]. PHBs are easily compostable and non-toxic derived from (R)-3-hydroxyalkanoic acids [25] which have thermoplastic properties identical to crude oil based plastics [26, 27, 28].

They are used in drug discovery as constituents of novel formulations, because of their knack in targeted delivery, sustained action, decreased toxicity, and improved stability [31].

They are considered promising because they are biodegradable and are formed as a secondary metabolite in the cells of microorganisms [32], usually when the cells are under nutritional stress or in an adverse situation such as carbon-excess with insufficient nutrition [33], which can occur in both gram-positive and gram-negative bacteria.

When critical nutrient sources are distorted or exhausted, bacteria clump together to retain carbon and energy [59]. It's worth noting that bacteria have been tested to produce PHBs as intercellular granules, with studies claiming that the total number of genera may be as high as 70 [34] containing nearly 300 bacterial strains [35]. *Alcaligenes, Pseudomonas, Bacillus, Micrococcus, Staphylococcus* are commonly available genera that produces PHBs. Currently, only a few bacterial strains have been effectively used to produce PHB on a commercial grade [36, 37].

The current research focuses on the isolation of potent PHB producing microbes from the garden soil of Killiyoor block of Kanyakumari District, which can produce considerable amount of the polymer under optimum conditions. Garden soil has been chosen because, for centuries, bacteria have been exploited in growing crops [69]. Soil bacteriology possess many unique microbes which have the capacity to produce a wide range of macromolecules including enzymes, antibiotics, polymeric substances etc.

The soil microbes from the collected soil are isolated through serial dilution and plating technique. Screening is done at the initial stage of isolation itself using Sudan black blue staining. Crotonic acid assay studies are helpful in the quantification of PHB and scrutinizing the potent PHB producer. The extracted PHB is analysed with Fourier Transform Infra-Red Spectroscopy (FTIR) and Gas Column and Mass Spectroscopy (GCMS) to confirm the functional groups of PHB. Further X-Ray crystallography is done to understand the three-dimensional molecular structure of the crystal and then also imaged through Scanning Electron Microscopy (SEM).

To characterize and identify the PHB producing organism, various biochemical tests are performed. 16S rRNA gene sequencing of the potent strain and BLAST analysis help in identification of the strain. The ultimate objective is the
mass production of PHB, bioplastic preparation and biodegradation of PHB using the *Pseudomonas* strains, indicating that the bioplastic produced is degradable and safe for use.

2. Methodology

2.1. Sample collection:

The humus soil sample was collected aseptically in a sterile container from the garden area, Nattalam, Kanyakumari District, Tamilnadu. Nattalam is a village located in the Killiyoor Block of Kanyakumari District. It is 28 kilometres west of Nagercoil, the district headquarters. The average temperature is between 25 & 30° C, with a humidity level of about 50 percent [38].

2.2. Serial dilution & plating technique for bacterial isolation: To carry out a series of dilutions, one gm soil sample was measured and placed in test tubes with 10 ml of pre-sterilized water. Before using, it is thoroughly combined by vortexing for 10 minutes and softly shaken. 100 ul of diluted sample was spread on the nutrient agar plates after serial dilution for up to 10⁻¹⁰ dilution. The plates were incubated at 37° C to promote bacterial growth [66].

2.3. Sudan black blue staining and screening of isolates:

2.3.1. In petriplates:

PHB-producing bacteria were screened with 0.02 gm Sudan black B stain dissolved in 100ml ethanol after incubation. The stained plates were left untouched for 20 minutes. After that, the residual dye was stripped and the plates were cleaned for 30 seconds with 80 percent ethanol. PHB producers were bluish black, signalling a positive outcome, while white colonies indicated a negative outcome. Randomly, five positive strains were selected and used for further studies. The positive isolates were sub-cultured many times to obtain pure colonies [40]. The positive strains were designated as SM1, SM2, SM3, SM4 & SM5.

2.3.2. In slides:

Staining with 0.3 percent Sudan Black B in ethanol confirmed the existence of PHB granules in the cytoplasm. To produce a clean solution and remove the sediments, it was purified. Five bacterial strains were smeared and heat fixed on a clean glass slide. It was stained for about 20 minutes with the specified dye, then counter stained for about 10 to 20 seconds with safranin. The slide was first air dried before being viewed under a microscope [40].

Under temperature controlled condition, the selective strains testing positive for Sudan black blue stain were maintained as slants in test tubes.

2.4. Colony morphology and Biochemical characterisation:

The selected cultures and their colonies were morphologically analysed and tabulated. Gram's staining was performed for identification of gram positive and gram negative bacteria. The various biochemical tests were performed, and the strains were identified by Bergy's manual of classification [41].

2.5. Production of PHB:

The five indigenous cultures were inoculated in nitrogen deficient and carbohydrate sufficient medium and incubated for about 72 hours. Extraction of PHB was carried out according to previous studies. Following incubation, the cultures were centrifuged at 10,000 rpm for about 10 minutes to obtain the culture filtrate. Supernatant was
discarded and to the pellet 10ml of sodium hypochlorite was added and mixed well. Then it was incubated for about 1 hour in a water bath maintained at 50°C. After one hour it was centrifuged at 5000 rpm for 15 minutes. Further, the pellet was washed with distilled water, acetone and ethanol. Finally, 5ml of boiling chloroform was taken, and the pellet was dissolved in it. The contents were kept for evaporation overnight in a sterile glass plate at 4°C overnight. After evaporation, the powdered PHB settled in the glass plate was collected by scratching with a sterile spatula and stored in glass bottles for further analysis [42].

2.6. Quantification of PHB by Crotonic acid assay:

2.6.1. PHB stock preparation:

The stock was prepared by dissolving 10 mg of standard bacteriological PHB in 10 ml of sulphuric acid [43].

2.6.2. Preparation of working standard solution:

Varied concentration of PHB weighing from 100 µg to 1000 µg/ml was taken in a series of test tubes. It was made up to 1ml with distilled water. To each tube, 4ml of conc. Sulphuric acid was added carefully through the sides of the test tube. The tubes were incubated in a boiling water bath for about 40 minutes [43].

2.6.3. Crotonic acid assay:

The PHB powders of all the five strains were taken in a test tube. To that, 5ml of concentrated sulphuric was poured through the sides of the test tube and kept in a boiling water bath for about 40 minutes. Now, the PHB crystals are converted to crotonic acid. The absorbance was measured at 235 nm with varied PHB concentration range from 100–1000 µg/ml PHB. The concentration of the test sample was estimated by comparing with a standard. From these results, the potent PHB strain was identified [44].

2.7. FTIR analysis:

FTIR analysis was performed to elucidate and confirm the functional groups of PHB. The PHB powder of 1 mg was dissolved in 5ml of chloroform and after evaporation FTIR analysis was done. FTIR Spectra were recorded at 400 cm$^{-1}$ to 4000 cm$^{-1}$ range using ITRACER-100, SHIZMADU instrument.

2.8. GCMS Analysis:

Following the process of methanolysis, GCMS Analysis of the extracted polymer was done. The Polymer sample was dissolved in 1ml chloroform and 1ml of 2 M sulphuric acid was added through the sides of the test tube, it was then incubated for about 1 hour in a boiling water bath. It was then cooled and 0.5 ml of demineralised water was added. The organic phase containing methyl esters of 4hydroxy alkanoic acids were analysed using GCMS [45]. Mass spectra were recorded in The Agilent portfolio GCMS instrument with column DB-5, Temperature range 100°C – 270°C (10°C/minute) with a flow rate of 1.2, carrier used helium gas and analysed by Mass hunter/library/NIST11.L – Chemstation integrator.

2.9. X-Ray Crystallography Analysis:

The Crystalline nature of PHB analysed on the basis of two-dimensional X-Ray diffraction Data in BRUKER ECO8 ADVANCE machine with scan type coupled two theta, time per step 32.0, temperature 25°C and Goniometer radius 255.0, detector name LYNXEYE < detector opening angle 2.452.

2.10. SEM Analysis:
Scanning Electron microscopy helped in studying the microstructure, surface morphology and chemical composition of the samples. For quality analysis nearly eighty percentage of the study area was scanned, the edges were left because they may have extraneous particles [46].

2.11. 16SrRNA gene sequencing and BLAST Analysis:

DNA was isolated from the organism SM1 using NucleoSpin® Tissue Kit. The presence of DNA was assessed by Agarose Gel Electrophoresis. Sequencing reaction was done in a Gene Amp PCR System 9700 (Applied Biosystems, USA) using the BigDye Terminator v3.1 Cycle sequencing Kit (Applied Biosystems, USA) following the prescribed protocol [47]. The sequence quality was checked using Sequence Scanner Software v1 (Applied Biosystems, USA). Sequence alignment and required editing of the obtained sequences were carried out using Geneious Pro v5.1 [48]. The obtained 16 S rRNA sequences were compared with all the accessible sequences found in databases using BLAST tool. (Basic Local Alignment Search tool). The phylogenetic tree was constructed using MEGA 11 software using neighbourhood joining method.

2.12. Bioplastic preparation with PHB:

The PHB powder were produced in large quantity to further facilitate bioplastic preparation. The produced PHB powder was taken in a petridish and dissolved in chloroform by gentle heating. It was then allowed to dry. After drying, a thin sheet of plastic would be developed; it was carefully removed from the petridish.

To overcome the brittle nature of PHB, it was even blended with starch and glycerol. Seventy percentage of chloroform dissolved PHB and thirty percentage starch were mixed and few drops of glycerol was added to the mixture. It was then heated gently, and the mixture was spread on aluminium foil and petriplates, it was allowed to dry for about three days and the plastic sheets were obtained [49].

2.13. Biodegradation of Bioplastic:

The developed plastic sheets were checked for biodegradation using *Pseudomonas* species. The nutrient agar plates were prepared and gel punctured at the centre to form a cavity. The PHB was dissolved and 100µl was poured in the centre cavity. Then, the entire petriplate was spread with *Pseudomonas* species. It was then incubated in an incubator at 37°C for 24 hours. After 24 hours, the petriplate was checked for the presence of hollow zone [50].

3. Result And Discussion

Soil contains different bacteria, producing various enzymes and antibiotics that are explored for years. PHB producing bacteria are found in almost all the types of soil. Speaking about the chemical nature of PHB, the ester linkages connect β-hydroxybutyrate and form PHB [49]. PHBs are made up of only R (alkyl group) side chains, and they lack S (Sulphur) side chains and hence are considered as biodegradable materials [50]. Sorting out the best PHB producers and production with cheap raw materials are necessary and thus can be an alternative to the conventional pollution causing plastics.

The bacterial isolates were screened and isolated for PHB production. Out of the numerous colonies grown under nutrient agar medium, 70–80% of the isolates showed positive result for Sudan black blue staining (Fig. 1). Out of it, only five colonies were randomly selected for further studies.

The indigenous isolates were labelled as SM1, SM2, SM3, SM4 and SM5. Colony morphology was studied by the growth of pure colonies in nutrient agar plates by spread plate technique (Fig. 4). The strains further stained with
Sudan black blue in slides were observed under microscope and photographed (Fig. 2). All strains showed PHB granules clearly under the microscope. Biochemical characterization of the isolates were checked through various tests (Fig. 5). Gram stained colonies were observed under oil immersion objective lens and photographed. Among the five colonies two of them i.e. SM1 & SM4 were found to be gram positive rods whereas SM2, SM3, SM5 were found to be gram negative rods.

PHB could be produced by growing cultures in the specialized medium by using trace element solution. The method followed for PHB production is sodium hypochlorite method as described by Gurubasappa et al., [51]. All the five strains were grown in a specialized PHB production medium for about three days. Studies stated that PHB production was better achieved at 72 hours of incubation [52]. Dry weight molecular mass of PHB were calculated. After evaporating chloroform, the petriplates showed a whitish powdery substance sticked onto it [53]. It was scratched smoothly with the help of sterile scalpel and blade. The powdered PHB (Fig. 3) was collected in a sterile container and stored at room temperature. The quantitative PHB estimation was done spectroscopically [54]. Among the five Strains SM1, SM3 and SM5 showed maximum concentration i.e. 180, 155, 105 micrograms/millilitre. After estimation, SM1 was found to be producing maximum PHB quantity, followed by SM3 and SM5. The strain SM1 was found to be a better PHB producer and used for further studies.

The FTIR spectra of strain SM1, (Fig. 6) showed a sharp absorption band at 1728.22 cm\(^{-1}\) corresponding to the characteristic carbonyl group. Similar results were observed with indicative peak of C = O at 1629.90 cm\(^{-1}\)[55]. The peak at 1282.66 cm\(^{-1}\) corresponds to –CH group, that a band at about 1,280–1,053 cm\(^{-1}\) characterizes valance vibration of the carboxyl group [55]. The band at 3441.01 cm\(^{-1}\) represents intra molecular hydroxyl group of carboxylic acid. Similar reports were found indicating carboxylic acid with peak value of 3889 cm\(^{-1}\) [56]. The band at 1055.06 cm\(^{-1}\) represents C-O stretching group. In concordance with these values, similar results were found at 1033.02 cm\(^{-1}\) [57]. The presence of these characteristic bands confirm the presence of PHB in the sample [58].

The peaks of PHB are well-defined, and sharp peaks denote the crystalline nature of PHB. The results indicate that PHB are crystallized since the water molecules were removed. Degree of crystallinity (IC) is an important factor for mechanical performance and accessibility since it affects the molecular conformation and intermolecular packaging of atoms of the material. The percentage of crystallinity was calculated from diffracted intensity measured according to Vonk's method [59, 60]

GCMS peaks indicate the presence of functional groups in the query compound. The mass spectra (Fig. 7) represents the existence of carbonyl and hydroxyl ends of the functional group. The presence of ester group is indicated with m/z value 73, methyl ester group, butenyl group is denoted with m/z value of 341. Thus, GCMS analysis indicate the characteristic fragmentation patterns indicating the presence of PHB. The elemental analysis agrees well with the molecular formula of PHB [61, 62]. SEM analysis interprets the microstructure and morphology of PHB (Fig. 8). Due to the polymeric nature of PHB, no specific patterns were individually recognized.

The obtained 16SrRNA sequence of the strain was submitted to Genbank and the accession Number is MZ363886. The obtained 16SrRNA sequence is given below:

>`SMSPAC1

GCTCTTATGAAGTTAGCGGACGGGTAGTAACACGTGGGATACCTGCCATAAGACTGGGATAACTCCGGGAAACCG
GGGCTAATACGCGATAACATTTTGAACCGCATGGTCTCGAAAATTGAAAGGCGCCTTCGCTGTCACCTTATGATGGACCCG

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BLAST analysis of SM1 showed 100 percentage identity and thus indicating the organism as Bacillus cereus. Phylogenetic tree was constructed using Mega Software Evolutionary Analysis tool (MEGA) using Neighbor Joining (NJ) method [53]. The NJ method is fast and practically useful for analysing large data sets. The scale bar corresponds to 1.00 nucleotide substitution per sequence position. Phylogram, (Fig. 9) with the Bootstrap values at the nodes [63] clearly indicate that the isolated organism is closely related to Bacillus cereus. Reports also indicated Bacillus species as one of the potent PHB producer [63, 64, 65]. Nearly 30 Bacillus strains capable of producing PHB were isolated from the intestine of various fishes [64]. Research findings include PHB producing Bacillus strains that accumulate 9–44.5% of cell dry weight [65] and PHB producing Bacillus strain with 20.63% yield [66]. These reports conclude that the Bacillus strains are yielding good amount of PHB. The isolated organism Bacillus cereus is a PHB producer as reported by the above-mentioned studies.

For development of bioplastic these polymers were produced in large quantities with repeated production cum extraction. The effectively produced polyhydroxybutyrates were made into thin polymeric sheets (Fig. 10). The biopolymer was dissolved in chloroform by heating to form paste like sticky component. It was spread evenly in the aluminium foil for drying. Since the polymer sheets are brittle in nature, the quality can be improved by blending with many ingredients like starch, agarose and glycerol. The addition of these chemicals improve the elasticity of the sheets. Fine blending is necessary to provide uniform thickness, and heating avoids residue formation.

Biodegradability analysis was carried out with the aid of Pseudomonas strains. The appearance of clear halo zones in the petriplates (Fig. 11) indicate that these bio molecules are completely degradable.

4. Conclusion

On the basis of these research findings, it is concluded that the strain Bacillus cereus (SM1) is a potent PHB producer. The produced PHB was analysed through various analytical techniques and confirmed. The PHB thus produced is biodegradable under laboratory conditions by Pseudomonas species. Furthermore, large scale production of PHB in fermenters will yield promising amounts and can be helpful for large scale bioplastic preparation.

Declarations

Statements & Declarations:

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Contribution by authors:

All the authors contributed equally to this research work, including conceptualization, design and discussion.

Data availability:

The 16SrRNA gene sequence is submitted in NCBI GENBANK database and found under the accession number MZ363886.

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Figures

Figure 1

Sudan black blue staining in Petridish
Figure 2

Sudan black blue staining in slides

Figure 3

Petriplate displaying produced PHB
Figure 4

Colony morphology of bacterial strains
Figure 5

Biochemical characterization of the isolates SM1, SM2, SM3, SM4, SM5

a: IMVIC test, gram staining, motility test
b: Carbohydrate utilization test
c: Triple sugar ion test

♣ Strains showing positive result are indicated
♣ SM1-SM5 are bacterial strains
Figure 6

FTIR Spectra of the strain SM1

Figure 7

GCMS spectrum of Polyhydroxybutyrate
Figure 8
Microstructure, Surface morphology of PHB (SEM imaging)

Figure 9
Phylogenetic Tree of the bacteria SM1 through MEGA 11 tool, Neighbor joining method
Figure 10

Bioplastic production – thin plastic sheet preparation

Figure 11

Biodegradation by Pseudomonas strain