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

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Abstract:

Plastic pollution should be resolved as it affects air, land and water. 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 PHB was 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. Scanning electron microscopy helps in picturised the microstructure and surface morphology of PHB. 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 BLAST (Basic Local Alignment Search tool). 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. The bacterial based bioplastics can be produced easily under laboratory conditions and to be practically applicable, it has to be produced in large scale in industries. While compared with the conventional plastics it is costly, but while considering the biodegradability property it has to be implemented. Large scale implementation of bioplastics will protect our environment from xenobiotic polyethylene-based plastics.

Keywords: Polyhydroxybutyrate, Fourier transform infrared spectroscopy, Gas chromatography mass spectrometry, Scanning Electron Microscopy, 16 S rRNA gene sequencing, Xenobiotics.

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 [5-7]. 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 bioplastics 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, polyol-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 CO_2 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 testified 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. 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. Scanning Electron Microscopy (SEM) is helpful in knowing the microstructure and surface morphology. To characterize and identify the PHB producing organism, various biochemical tests are performed. 16S rRNA gene sequencing of the potent strain and BLAST analysis helps 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.

Methods:

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 Kanniyakumari 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].

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.

Sudan black blue staining and screening of isolates:

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.

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.

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].

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^{o}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].

Quantification of PHB by Crotonic acid assay:

PHB stock preparation:

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

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].

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].

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.

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.

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].

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.

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].

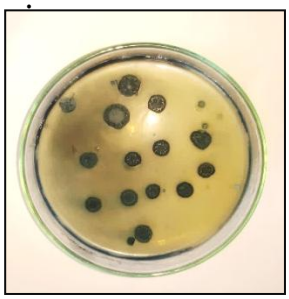
Biodegradation of Bioplastics:

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 in chloroform through gentle heating 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^{o}C for 24 hours. After 24 hours, the petriplate was checked for the presence of hollow zone [50].

Results and Discussion:

Soil contains many different bacteria, producing many useful substances that are explored for years. PHB producing bacteria are found in almost all the types of soil. Garden soil is chosen because, for centuries, bacteria have been exploited in growing crops. Soil bacteriology possess many unique microbes which have the capacity to produce a wide range of macromolecules including enzymes, antibiotics, polymeric substances etc. 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.

In this study, bacterial isolates were screened and isolated for PHB production using sudan black blue stain. Sudan black blue is a fat-soluble dye and it has high affinity for fats and lipids. Sudan black is a specific stain for colouring various lipids, such as neutral lipids, phospholipids, and sterols, and can thus be used to identify bacteria with PHB compounds as a sudanophilic character (can be stained using a lipid staining). Out of the numerous colonies grown under nutrient agar medium, 70-80% of the isolates showed positive result for Sudan black blue staining. Out of it, only five colonies were randomly selected for further studies. Figure 1 illustrates sudan black blue staining done in petriplates.



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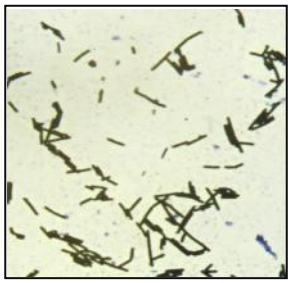

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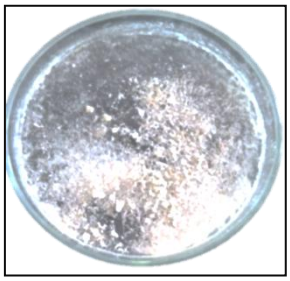

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Figure 1. Sudan black blue staining in Petridish (Strain SM1)
Figure 2. Sudan black blue staining – Microscopic observation (Strain SM1)
Figure 3. Petriplate displaying produced PHB (Strain SM1)

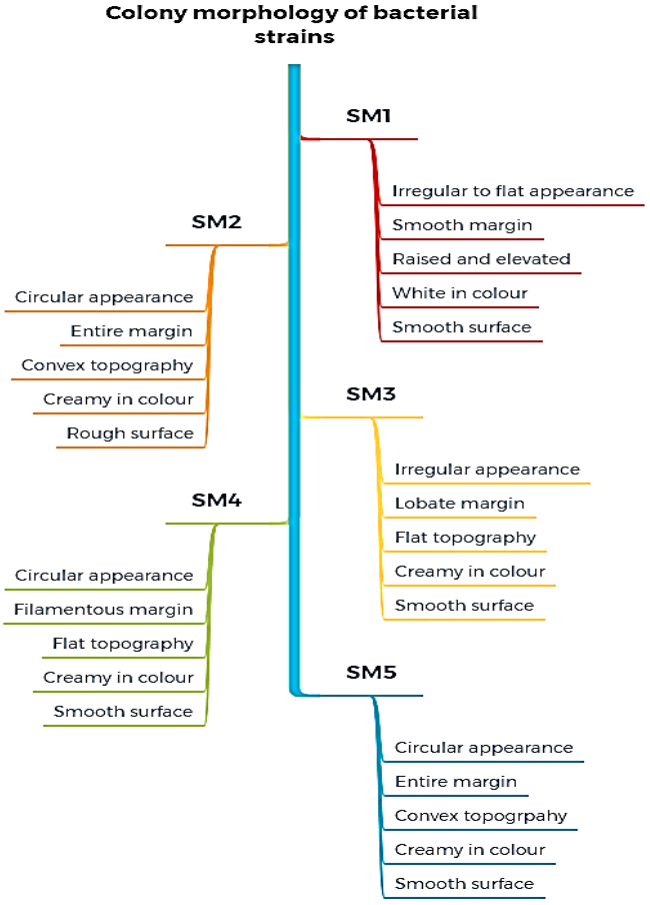


Figure 4. Colony morphology of bacterial strains

Table 1. Biochemical characterisation of the PHB positive isolates(SM1, SM2, SM3, SM4, SM5)

Tests performed	SM 1	SM 2	SM 3	SM4	SM 5
Gram staining	+	-	-	+	-
Motility	+	+	+	+	+
Spore	+	-	-	+	-
Indole test	-	-	-	-	+
Methyl red test	-	-	+	-	-
Voges proskaur test	+	-	-	+	-
Citrate utilization test	+	+	+	+	+
Catalase test	+	+	+	+	-
Oxidase test	-	+	+	-	-
Gelatin hydrolysis test	-	+	+	-	+
Casein hydrolysis test	+	+	+	+	+
Triple sugar iron test	Pink slant, yellow butt	Pink slant, yellow butt	Entire Yellow	Entire Pink	Entire Pink
Carbohydrate utilization test					
Glucose	+	+	+	+	+
Fructose	+	-	+	+	-
Galactose	-	+	+	-	+
Sucrose	+	-	-	+	-
starch	+	-	-	+	-

The indigenous Phb positive 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. The results of the colony morphology of these strains are explained in Figure 4. Colony morphology is helpful in predicting the preliminary identification of strains. The strains are streaked at the centre of the petridish and incubated

for about 24 hours which helps for the growth of a predominant colony of bacteria. The bacterial isolate SM1 had an irregular to flat appearance, with a smooth margin. The colonies of it were raised and elevated but the surface was smooth. The colonies appeared white in colour. The strain SM2 had a circular colony appearance and was creamy in colour. The margin was found to be entire, had a convex topography with rough surface. The strain SM3 had an irregular appearance and the colonies were creamy in colour. The margin edges were lobate, with a flat topography and smooth surface. The strain SM4 had a circular appearance with filamentous margin. It had a flat topography, was creamy in colour and had a smooth surface. In the strain SM5 the colonies were circular in shape and appeared creamy in colour. The topography was convex and had a smooth surface with an entire margin.

The strains further stained with Sudan black blue in slides were observed under microscope and photographed as in Figure 2. All the strains showed PHB granules clearly under the microscope. In reaction to nutritional imbalance, PHB aggregate in the cytoplasm as distinct granules to levels as high as ninety percent of the cell dry weight without disrupting the cell's osmotic pressure. The amount and size of PHA granules varies between species and among cells of the same species. It is determined by the organism's developmental stage and its adverse environment. 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. Although selected randomly, rod shaped bacteria were preferred for PHB production. It was because the bacteria will accumulate more amount of Phb than cocci shaped bacteria. Biochemical characterisation was performed and the results are tabulated as in Table 1. Bio chemical test results are helpful in preliminary identification of the organism. The biochemical test results are corelated with Bergy's manual of classification of bacteria and the strains were identified.

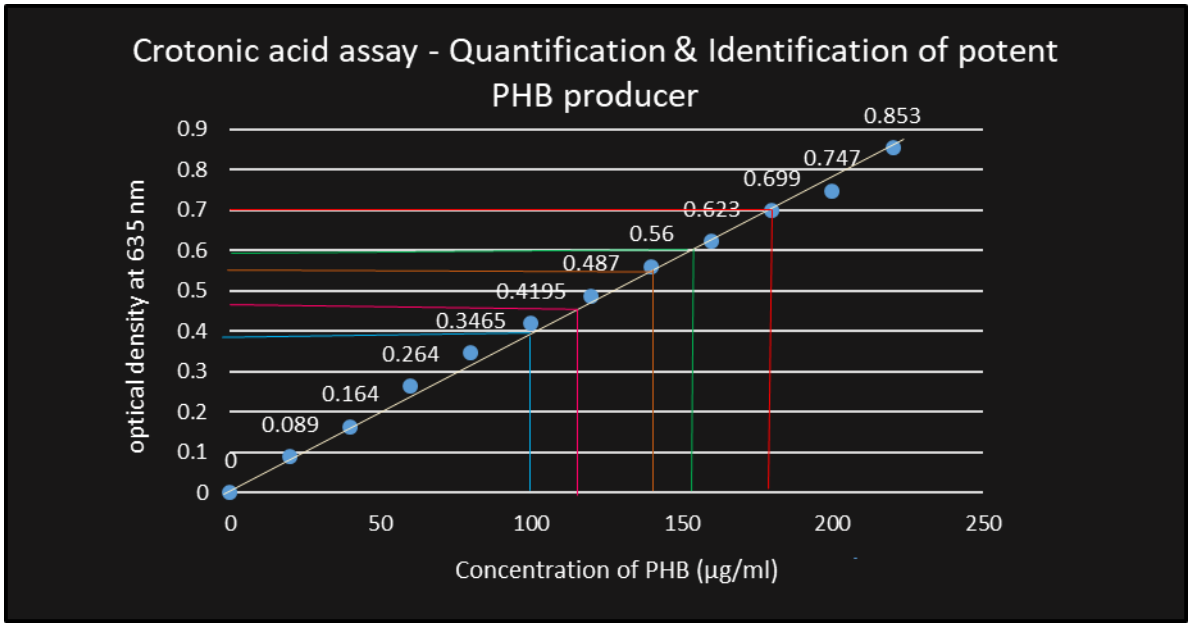


Figure 5. Crotonic acid assay – Quantification of PHB
{Concentration of strains indicated in different colours - SM1 (180 µg/ml), SM3 (155 µg/ml), SM5 (142 µg/ml), SM2 (123 µg/ml), SM4 (101 µg/ml)}

PHB could be produced by growing cultures in the specialized medium containing trace element solution. The method followed for PHB production is sodium hypochlorite method as described by Gurubasappa [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]. 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 (Figure 3.) was collected in a sterile container and stored at room temperature. The quantitative PHB estimation was done spectroscopically [54]. Crotonic acid assay was helpful in quantifying the amount of PHB produced and thus identifying the potent producer. Among the five Strains SM1, SM3 and SM5 showed maximum concentration i.e. 180, 155, 142 micrograms/millilitre through crotonic acid assay which was interpreted in Figure 5. When sulphuric acid was added to Phb and heated, it was converted to a brownish precipitate called crotonic acid. After estimation, SM1 was found to be producing maximum PHB quantity, followed by SM3 and SM5. The strain SM2 produced moderate quantity i.e 123 µg/ml and the strain SM 4 produced least quantity of 101 µg/ml. Thus, the strain SM1 was found to be a better PHB producer and used for further studies.

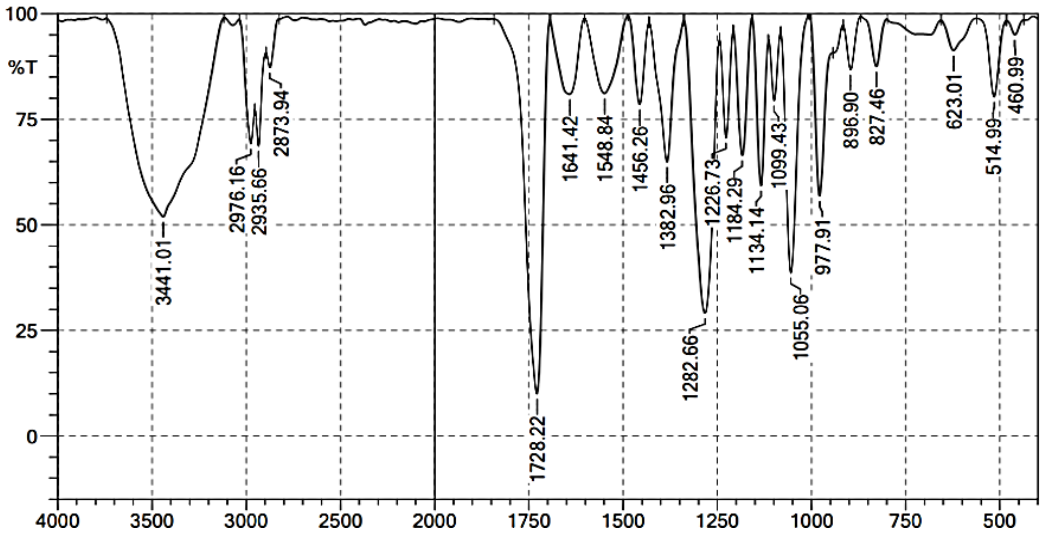


Figure 6. FTIR Spectra of the strain SM1

The FTIR spectra of strain SM1, (Figure 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 confirmed the presence of PHB in the sample [58].

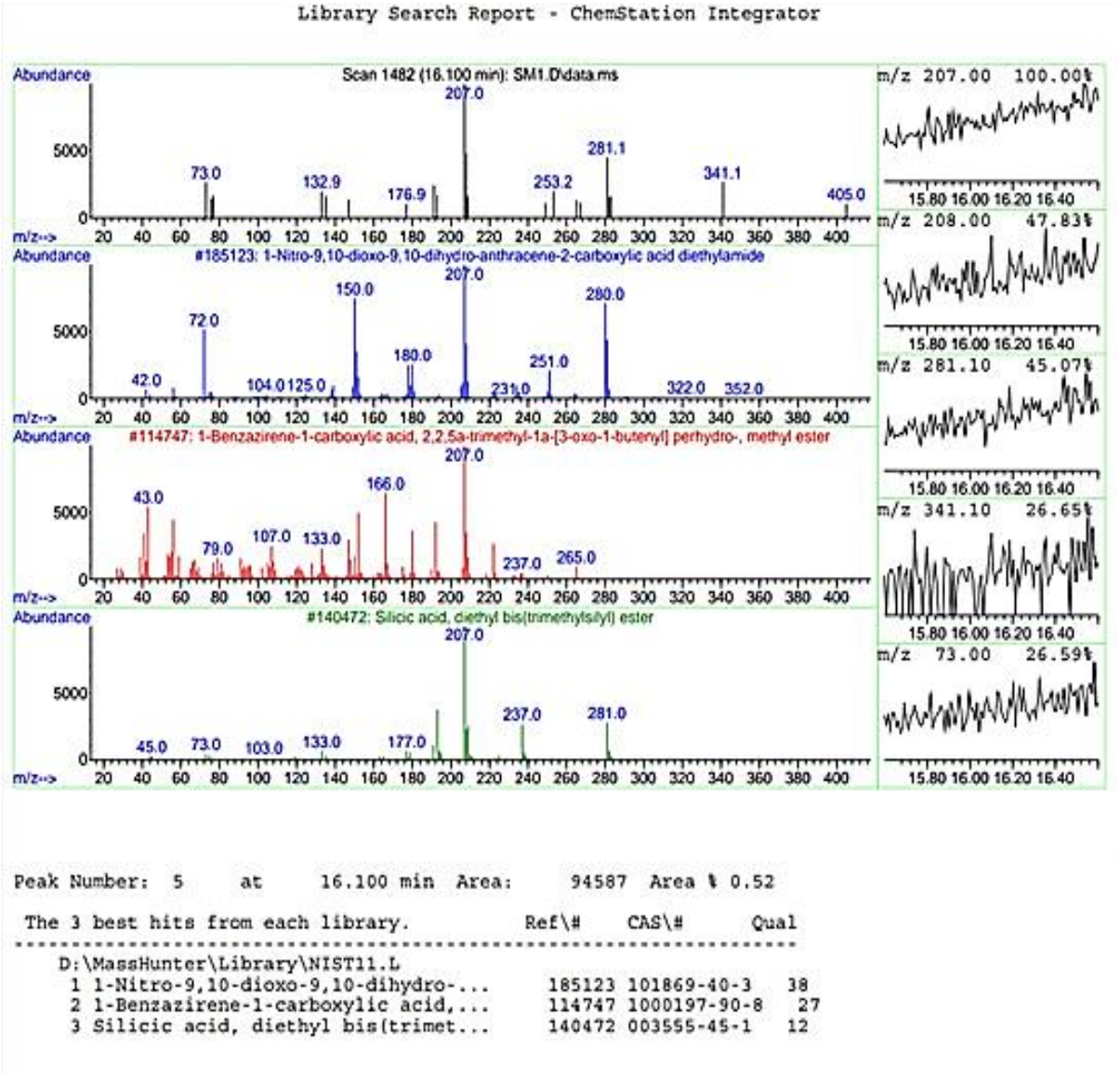


Figure 7. GCMS spectrum of Polyhydroxybutyrate

GCMS peaks indicate the presence of PHB in the query compound. The mass spectra Figure 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 (Figure 8). Due to the polymeric nature of PHB, no specific patterns were individually recognized.

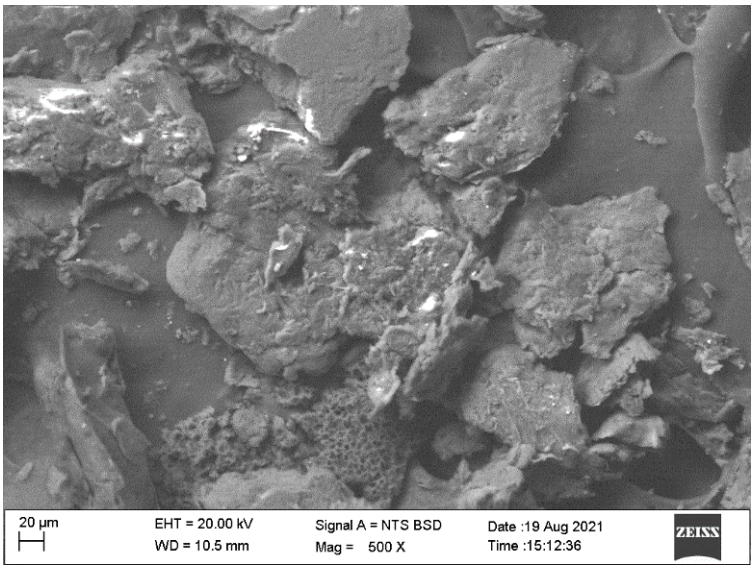


Figure 8. Microstructure, Surface morphology of PHB (SEM imaging)

DNA was isolated from the strain SM1, mass produced in PCR and it was sequenced. 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

GCTCTTATGAAGTTAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTGCCCATAAGACTGGGATAACTCCGGGAAACCG
GGGCTAATACCGGATAACATTTTGAACCGCATGGTTCGAAATTGAAAGGCGGCTTCGGCTGTCACTTATGGATGGACCCG
CGTCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCAACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACAC
TGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCA
ACGCCGCGTGAGTGATGAAGGCTTTCGGGTCGTAAAACTCTGTTGTTAGGGAAGAACAAGTGCTAGTTGAATAAGCTGGC
ACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTATC
CGGAATTATTGGGCGTAAAGCGCGCGCAGGTGGTTTCTTAAGTCTGATGTGAAAGCCCACGGCTCAACCGTGGAGGGTCA
TTGGAAACTGG

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, (Figure 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.

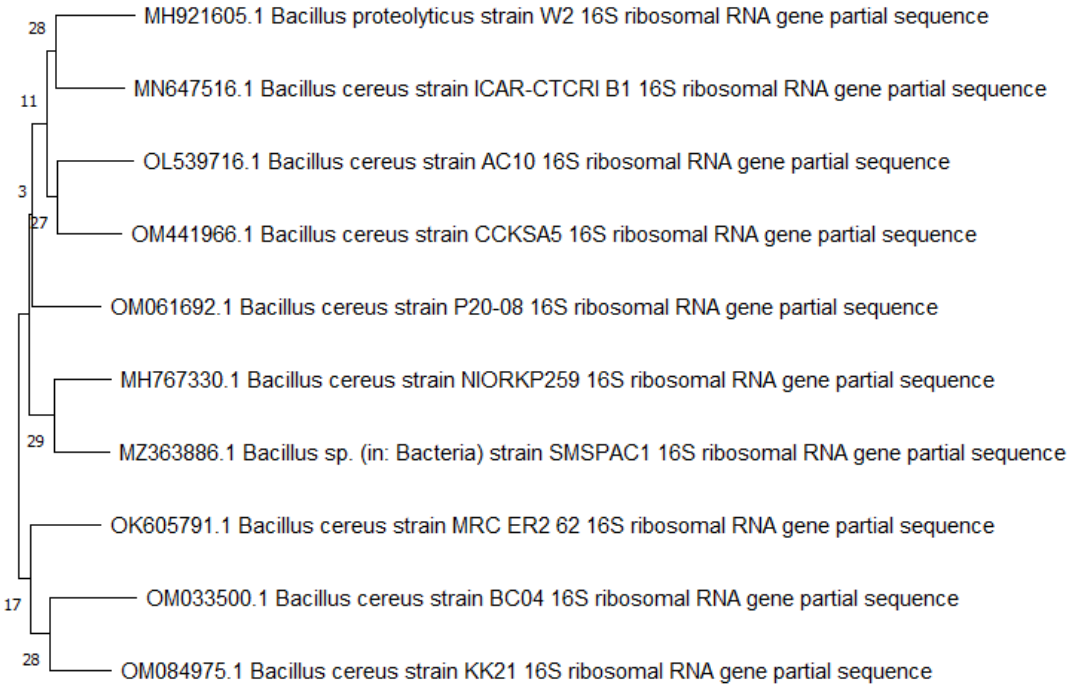
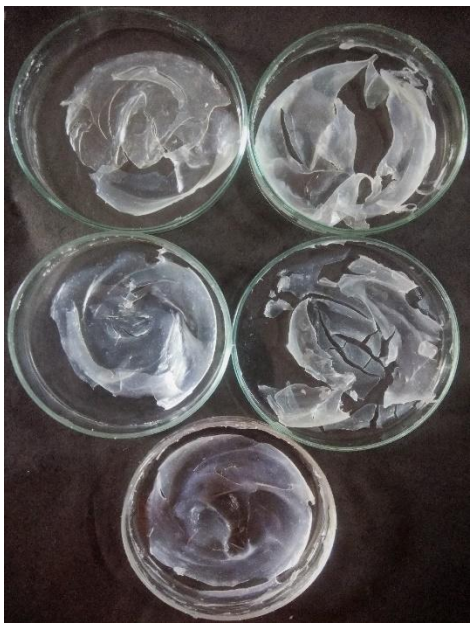


Figure 9. Phylogenetic tree of the bacteria SM1 through MEGA 11 tool, Neighbor joining method



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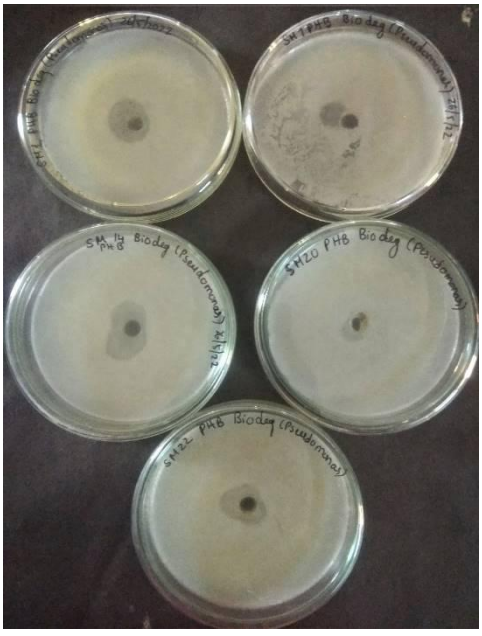

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Figure 10. Bioplastic production – thin plastic sheet preparation

Figure 11. Biodegradation by *Pseudomonas* strain

For development of bioplastics these polymer was produced in large quantities with repeated production cum extraction. The effectively produced polyhydroxybutyrates were made into thin polymeric sheets as in Figure 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 will improve the elasticity of the sheets. Fine blending is necessary to provide uniform thickness and heating avoids residue formation.

Biodegradablility analysis was carried out with the aid of *Pseudomonas* sp. The appearance of clear halozones in the petriplates (Figure 11) indicate that these bio molecules are completely degradable. The Phb degrading bacteria are able to utilise phb present in medium. As a result, halozonees are formed at the places of bacterial inoculation.

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 the presence of PHB 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 of phb and can be helpful for large scale bioplastic preparation. The usage of phb based plastics would defenitely be a solution to the global plastic pollution because of their biodegradability.

Statements & Declarations:

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All the authors contributed equally to this research work, including conceptualization, design and discussion. Data collection and experimental procedures were carried out by Ms.S.Mahitha. Data analysis, interpretation of results, discussion and conclusion was collectively done by all the authors.

Data availability:

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

References:

1. Andrady AL, Neal MA. Applications and societal benefits of plastics. Philos Trans R Soc Lond B Biol Sci. (2009); 364(1526):1977-84. https://doi: 10.1098/rstb.2008.0304.
2. Thompson R, Moore C, Andrady A, Gregory M, Takada H, Weisberg S. New directions in plastic debris. Science. (2005); 310(5751):1117. https://doi: 10.1126/science.310.5751.1117b
3. Zubris KA, Richards BK. Synthetic fibers as an indicator of land application of sludge. Environ Pollut. (2005); 138(2):201-11. https://doi: 10.1016/j.envpol.2005.04.013.
4. Brinton W. F.2005Characterization of man-made foreign matter and its presence in multiple size fractions from mixed waste composting. Compost Sci. Utilizat. 13, 274–280 http://www.socialresearchfoundation.com/upoadreserchpapers/3/45/150713122910
5. UNEP (United Nations Environment Programme), Single-Use Plastic: A Roadmap for Sustainability. 2018. (Accessed on April 2022)
6. Mattsson, K., Hansson, L.A., and Cedervall, T.,Environ. Nanoplastics in the aquatic environment Sci. Process, (2015); 1712–1721. https://doi.org/10.1039/C5EM002271
7. Chelsea M. Rochman, Sara M. Kross, Jonathan B. Armstrong, Michael T. Bogan, Emily S. Darling, Stephanie J. Green, Ashley R. Smyth, and Diogo Veríssimo Environmental Science & Technology 49 (18), (2015); 10759-10761 https://doi: 10.1021/acs.est.5b03909
8. Davis, G. Song, J. H. Biodegradable packaging based on raw materials from crops and their impact on waste management. Ind. Crops Prod., 23 (2) (2006), pp. 147-161 https://www.infona.pl/resource/bwmeta1.element.elsevier-f1106d5f-ccfe-33a7-8912-ecb5418b47b6
9. B. Demetres, B. Epifania, H. Miltiadis, S. Giacomo, P. Pietro, G. Dorleta, D. Cyril. Review, mapping and analysis of the agricultural plastic waste generation and consolidation in Europe. Waste Manage. Res., 31 (12) (2013), pp. 1262-1278 https://doi: 10.1177/0734242X13507968

10. Narancic T, Verstichel S, Reddy Chaganti S, Morales-Gamez L, Kenny ST, De Wilde B, Babu Padamati R, O'Connor KE. Biodegradable Plastic Blends Create New Possibilities for End-of-Life Management of Plastics but They Are Not a Panacea for Plastic Pollution. Environ Sci Technol. (2018); 52(18):10441-10452. https://doi: 10.1021/acs.est.8b02963.
11. Emadian SM, Onay TT, Demirel B. Biodegradation of bioplastics in natural environments. Waste Manag. (2017); 526-536. https://doi.org/10.1016/j.wasman.2016.10.006
12. Petrova, M. Garner, J. Scientists Use ACS Sci-Mind: Case Study to Examine Biopolymers Industry Posted by a viverito in Career Development. American Chemical Society (2014); (Accessed on june 2022)
13. Ying, W. Jin, Y. Guo-Qiang, C. Polyhydroxyalkanoates, challenges and opportunities. Curr. Opinion Biotechnol., 30 (2014); 59-65. https://doi: 10.1016/j.copbio.2014.06.001.
14. Chen, G. Patel, M. Plastics derived from biodegradable sources: present and future: a technical and environmental review. Chem. Rev., 112 (2012), pp. 2082-2099 https://DOI: 10.1021/cr200162
15. Viviana, V. Pamela, M. González, M. Seeger. Bacterial production of the biodegradable plastics polyhydroxyalkanoates. Int. J. Biol. Macromol., 70 (2014);208-213 https:// doi: 10.1016/j.ijbiomac.2014.06.001.
16. Bora L. Polyhydroxybutyrate accumulation in *Bacillus megaterium* and optimization of process parameters using response surface methodology. J Polym Environ. (2013); 21(2):415–420. https://doi.org/10.1007/s10924-012-0529-z
17. Getachew A, Woldesenbet F. Production of biodegradable plastic by polyhydroxybutyrate (PHB) accumulating bacteria using low cost agricultural waste material. BMC Res Notes. (2016); 9(1):509–517. https://doi.org/10.1186/s13104-016-2321-y pmid:27955705
18. Gurieff N, Lant P. Comparative life cycle assessment and financial analysis of mixed culture polyhydroxyalkanoate production. Bioresour Technol. (2007); 98(17):3393–3403. https://doi.org/10.1016/j.biortech.2006.10.046 pmid:17632000
19. Lemoigne Maurice, biodegradable plastic: its promises and consequences, Darmouth undergraduate journal of science, (2013); (Accessed on april 2022)

20. Macrae RM, Wilkinson JF. Poly-β-hyroxybutyrate metabolism in washed suspensions of *Bacillus cereus* and *Bacillus megaterium*. J Gen Microbiol. (1958); 19(1):210–222. https://doi: 10.1099/00221287-19-1-210.
21. Ray D. Biocomposites for high-performance applications: Current barriers and future needs towards industrial development, (2017), First edition, Elsevier science
22. Khanna S, Srivastava AK. A simple structured mathematical model for biopolymer (PHB) production. Biotechnol Prog. (2005); 21(3):830–838. https://doi.org/10.1021/bp0495769 pmid:15932263
23. De Koning GJM, Lemstra PJ, Hill DJT, Carswell TG, O'Donnell JH. Ageing phenomena in bacterial poly [(R)-3-hydroxybutyrate]. Polymer. (1992); 33(15):3295–3297. https://doi.org/10.1016/0032-3861(92)90250-Z
24. Nath A, Dixit M, Bandiya A, Chavda S, Desai AJ. Enhanced PHB production and scale up studies using cheese whey in fed batch culture of *Methylobacterium* sp. ZP24. Bioresour Technol. (2008); 99(13):5749–5755. https://doi.org/10.1016/j.biortech.2007.10.017 pmid:18032031
25. Sudesh, Kumar & Abe, H & Doi, Y, Synthesis, structure and properties of polyhydroxyalkanoates: Biological polyesters. Progress in Polymer Science. 25. (2000); 1503-1555. https://doi.org/10.1016/S0079-6700(00)00035-6.
26. Penkhrue W, Jendrossek D, Khanongnuch C, Pathom-aree W, Aizawa T, et al. (2020). Response surface method for polyhydroxybutyrate (PHB) bioplastic accumulation in Bacillus drentensis BP17 using pineapple peel. PLOS ONE 15(3): e0230443. https://doi.org/10.1371/journal.pone.0230443
27. Kritika Sinha, Pragya Rathore. Study of Polyhydroxybutyrate producing *Bacillus sp*. isolated from Soil. Res. J. Recent Sci., Volume 4, Issue (IYSC-2015), (2015); 61-69. http://www.isca.in/rjrs/archive/v4/iIYSC-2015/12.ISCA-IYSC-2015-3BS-09.php
28. Bonartsev, A. P., Bonartseva, G. A., Reshetov, I. V., Kirpichnikov, M. P., & Shaitan, K. V. (2019). Application of Polyhydroxyalkanoates in Medicine and the Biological Activity of Natural Poly(3-Hydroxybutyrate). Actanaturae, (2019) 11(2), 4–16. https://doi.org/10.32607/20758251-2019-11-2-4-16
29. Mokhtarzadeh A., Alibakhshi A., Hejazi M., Omidi Y., Dolatabadi J.E.N., Trends Analyt. Chem. (2016); 82:367–384. https://doi: 10.32607/20758251-2019-11-2-4-16

30. Bonartsev, A.P., Bonartseva, G.A., Shaitan, K.V. *et al.* Poly (3-hydroxybutyrate) and poly (3-hydroxybutyrate)-based biopolymer systems. Biochem. Moscow Suppl. Ser. B 5, (2011); 10–21 (2011). https://doi.org/10.1134/S1990750811010045
31. Farah S., Anderson D.G., Langer R., Adv. Drug Deliv. Rev. (2016); 107:367–392. https://doi: 10.3390/ma12213586
32. Rao U., Ravichandran S., Sehgal P.K. Biosynthesis and Biocompatibility of P (3HB-Co-4HB) Produced by Cupriavidus Necator from Spent Palm Oil. Biochem. Eng. J. 200; 49:13–20. https://doi: 10.1016/j.bej.2009.11.005.
33. Bharti S.N., Swetha G. Need for Bioplastics and Role of Biopolymer PHB: A Short Review. J. Pet. Environ. Biotechnol. (2016); 7:7. https:// doi: 10.4172/2157-7463.1000272
34. Sindhu R., Ammu B., Binod P., Deepthi S.K., Ramachandran K.B., Soccol C.R., Pandey A. Production and Characterization of Poly-3-Hydroxybutyrate from Crude Glycerol by Bacillus Sphaericus NII 0838 and Improving Its Thermal Properties by Blending with Other Polymers. Brazilian Arch. Biol. Technol. (2011); 783–794. https://doi: 10.1590/S1516-89132011000400019.
35. Grigore ME, Grigorescu RM, Iancu L, Ion RM, Zaharia C, Andrei ER. Methods of synthesis, properties and biomedical applications of polyhydroxyalkanoates: a review. J Biomater Sci Polym Ed. (2019); 30(9):695-712. https://doi: 10.1080/09205063.2019.1605866.
36. Mozejko-Ciesielska J, Kiewisz R. Bacterial polyhydroxyalkanoates: Still fabulous? Microbiol Res. (2016); 192:271-282. https://doi: 10.1016/j.micres.2016.07.010.
37. Dos Santos A.J., Oliviera Dalla Valentina L.V., Hidalgo Shulz A.A., Tomaz Duarte M.A. From Obtaining to Degradation of PHB: Material Properties. Part I. Ing. Cienc. (2017); 26:269–298. https://doi: 10.17230/ingciencia.13.26.10.
38. http://www.onefivenine.com/india/villages/Kanniyakumari/Killiyoor/Nattalam (Accessed on March 2022)
39. Sanders E. R. (2012). Aseptic laboratory techniques: plating methods. Journal of visualized experiments: JoVE, (63), e3064.

https://doi.org/10.3791/3064

40. Kitamura, S., Doi, Y. Staining method of poly (3-hydroxyalkanoic acids) producing bacteria by nile blue. Biotechnol Tech 8, (1994) 345–350 (1994). https://doi.org/10.1007/BF02428979
41. Holt J.G, krieg N.R, (1994), Bergey,s manual of determinative bacteriology, second edition, Springer, Newyork.
42. Ramsay, J.A., Berger, E., Voyer, R. et al. Extraction of poly-3-hydroxybutyrate using chlorinated solvents. Biotechnol Tech 8, (1994); 589–594 (1994). https://doi.org/10.1007/BF00152152
43. Susithra K,Ramesh U, Kannan M,Ganesan R, Rajarathinam K, Screening and characterisation of bioplastics producing bacteria isolated from oil contaminated soils of Virudhunagar, Tamil Nadu, India.Pharmaceutical and biological evaluations;Vol. 4 (Issue 1): (2017);21-27
44. Atakav Y, Pinar O, Kazan D, Investigation of the Physiology of the Obligate Alkaliphilic *Bacillus marmarensis* GMBE 72^{T} Considering Its Alkaline Adaptation Mechanism for Poly (3-hydroxybutyrate) Synthesis. Microorganisms. (2021); 9(2):462. https://doi: 10.3390/microorganisms9020462. PMID: 33672214; PMCID: PMC7926669.
45. Wang F, Lee SY. Poly (3-Hydroxybutyrate) Production with High Productivity and High Polymer Content by a Fed-Batch Culture of Alcaligenes latus under Nitrogen Limitation. Appl Environ Microbiol. (1997); 63(9):3703-6. https://doi: 10.1128/aem.63.9.3703-3706.1997.
46. Obinna C Nwinyi, Tolulope A. owolabi SEM and FTIR analysis of polyhydroxyalkanoates isolated from bacteria species Fro abattoir, Nigeria, Journal of KingSaud University Science, (2019); 285-298.
47. Sambrook, J, Fritsch E, maniatis T 2001, Molecular cloning:A laboratory Manual. Cold Spring harbour laboratory, Cold Spring harbour, New York
48. Kearse M, Moir R, Wilson A, Stones-Havas S, Cheung M, Sturrock S, Buxton S, Cooper A, Markowitz S, Duran C, Thierer T, Ashton B, Meintjes P, Drummond A. Geneious Basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. Bioinformatics. (2012); 28(12):1647-9. https://doi: 10.1093/bioinformatics/bts199.
49. Minzhang Noreen L.Thomas, preparation and properties of polyhydroxybutyrate blended with different types of starch, Journal of applied polymer science, (2009); 688-694

https://doi.org/10.1002/app.30991

50. Lai. S.M, Huang. C.K, Shen. H.F, Preparation and properties of biodegradable poly (butylene succinate)/ starch blends, Journal of applied polymer science, (2005); 257-264. https://doi.org/10.1002/aa.21679
51. Kritika Sinha, Pragya Rathore, Study of Polyhydroxybutyrate producing Bacillussp. isolated from Soil, (2015), Research Journal of Recent Sciences, ISSN 2277-2502 Vol. 4(2015), 61-69
www.isca.in, www.isca.me
52. Anish Kumari Bhuwal, Gulab Singh, Neeraj Kumar Aggarwal, Varsha Goyal, Anita Yadav, (2013); "Isolation and Screening of Polyhydroxyalkanoates Producing Bacteria from Pulp, Paper, and Cardboard Industry Wastes", International Journal of Biomaterials, Article ID 752821,
https://doi.org/10.1155/2013/752821
53. Gurubasappa. G.Biradar, Shivasharana C. T. and Basappa. B. Kaliwal, Isolation and characterization of polyhydroxybutyrate (PHB) producing Bacillus species from agricultural soil. European Journal of Experimental Biology, (2015)5(3):58-65.
https://www.semanticscholar.org/paper/Isolation-and-characterization-of-(PHB)-19efbc4970f3db5e109d26af0ef79fb15f8eaba
54. Trakunjae, C., Boondaeng, A., Apiwatanapiwat, W. et al. Enhanced polyhydroxybutyrate (PHB) production by newly isolated rare actinomycetes Rhodococcus sp. strain BSRT1 using response surface methodology. Sci Rep 11, 1 (2021);
https://doi.org/10.1038/s41598-021-81386-2
55. Mostafa, Y.S., Alrumman, S.A., Alamri, S.A. et al. Bioplastic (poly-3-hydroxybutyrate) production by the marine bacterium Pseudodonghicola xiamenensis through date syrup valorization and structural assessment of the biopolymer. Sci Rep 10, 8815 (2020); https://doi.org/10.1038/s41598-020-65858-5
56. McAdam B, Brennan Fournet M, McDonald P, Mojicevic M. Production of Polyhydroxybutyrate (PHB) and Factors Impacting Its Chemical and Mechanical Characteristics. Polymers. (2020); 12(12):2908.
https://doi.org/10.3390/polym 12122908
57. Sayyed, R Z et al. Production, purification and evaluation of biodegradation potential of PHB depolymerase of Stenotrophomonas sp. RZS7." (2020), PloS one vol. 15, 1 e0220095.,

https://doi:10.1371/journal.pone.0220095

58. Gumel, A. M., Annuar, M. S. M. & Heidelberg, T. (2012) Biosynthesis and characterization of polyhydroxyalkanoates copolymers produced by Pseudomonas putida Bet001 isolated from Palm Oil mill effluent. PLoS ONE 7, e45214. https://doi.org/10.1371/journal.pone.0045214
59. Ramezani, M., Amoozegar, M. & Ventosa, A. Screening and comparative assay of poly-hydroxyalkanoates produced by bacteria isolated from the Gavkhooni Wetland in Iran and evaluation of poly-β-hydroxybutyrate production by halotolerant bacterium Oceanimonas sp. GK1. Ann. Microbiol. 65, (2015); 517–526. https://doi.org/10.1007/s13213-014-0887-y
60. Randriamahefa S, Renard E, Guérin P, Langlois V. Fourier transform infrared spectroscopy for screening and quantifying production of PHAs by Pseudomonas grown on sodium octanoate. Biomacromolecules. (2003); 4(4):1092-7. https://doi: 10.1021/bm0341040.
61. Vonk, C.G Computerisation of Ruland's X-Ray method for determination of crystallinity in polymers. Journal of Applied crysatallography, 6, (1973);148-152 https://doi.org/10.1167/50021889873008332
62. Pradhan, Sushobhan, Dikshit, Pritam Kumar, Production, Ultrasonic Extraction, and Characterization of Poly(3-hydroxybutyrate) (PHB) using Bacillus megaterium and Cupriavidus necator, Polymers for Advanced Technologies, vol 29 (2018); 68-98. https://doi.org/10.1002/pat.4351
63. Venkata Mohan, M. Venkateswar Reddy, G. Venkata Subhash, P.N. Sarma, Fermentative effluents from hydrogen producing bioreactor as substrate for poly(β-OH) butyrate production with simultaneous treatment: An integrated approach, Bioresource Technology,Volume 101, Issue 23, (2010); 9382-9386 https://doi.org/10.1016/j.biortech.2010.06.109
64. Bharti S.N., Swetha G. Need for Bioplastics and Role of Biopolymer PHB: A Short Review. J. Pet. Environ. Biotechnol. (2016); 7:7. https:// doi: 10.4172/2157-7463.1000272
65. Ganapathy, Kavitha & Kurinjimalar, Chidambaram & Krishnan, Sivakumar & Kaarthik, Muthukumar & Aravind, Rajamani & Palani, Perumal & Rengasamy, Ramasamy. (2016). Optimization of polyhydroxybutyrate production utilizing waste water as nutrient source by Botryococcus braunii Kütz using response surface methodology. International journal of biological macromolecules. 93.

https://10.1016/j.ijbiomac.2016.09.019.

66. Bora L. Polyhydroxybutyrate accumulation in *Bacillus megaterium* and optimization of process parameters using response surface methodology. J Polym Environ. 2013; 21(2):415–420. https://doi.org/10.1007/s10924-012-0529-z
67. Pal, Adwitiya & Prabhu, Ashwini & Kumar, Avinash & Rajagopal, Badri & Dadhe, Kajal & Ponnamma, Vomsi & Shivakumar, Srividya. (2009). Optimization of Process Parameters for Maximum Poly (-beta-) hydroxybutyrate (PHB) Production by Bacillus thuringiensis IAM 12077. Polish journal of microbiology, (2009); 149-54.