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Production of Bioplastic by Polyhydroxybutyrate (PHB) accumulating *Bacillus megaterium* VAS05 and Determination of its Chemical and Mechanical Characteristics

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Abstract

The Polyhydroxybutyrate (PHB) producing *Bacillus megaterium* was isolated from organic waste dumping sites in Kannur, Thalassery, Kannur District, Kerala, India. The strain identity was confirmed through biochemical tests and molecular sequencing and was designated *Bacillus megaterium* VAS05; the nucleotide sequence was submitted in GenBank with accession no. MW193404. The PHB production maximized at an incubation period of 72 h (4.1 g/l); the optimum PHB production was noted at a temperature 37°C and pH 7.0 in which 63.71 % and 62.13% yield respectively of the biopolymer was obtained. The supplementation of the carbon sources (1% w/v), sucrose and lactose separately with the Minimal Davis medium yielded high level of PHB (5.44 and 5.57 g/l respectively). The nitrogen sources studied couldn't impact the production of PHB (ammonium sulphate, beef extract, malt extract) except for Green Gram powder (1% w/v), the supplementation of which as the sole source of nitrogen yielded 6.04 g/l of PHB. The PHB was partially purified by hot chloroform precipitation and bioplastic sheet was prepared. The FTIR analysis showed the characteristic peaks corresponding to C─H, C═O, OH functional groups and strong stretching peaks within the range of 1800 and 1600 cm^{-1}. The XRD analysis revealed intense peak at 31.83˚ indicating crystalline nature of PHB. The thermal transition of the bioplastic was noted at a temperature of 106.07°C. The tensile strength of the extracted bioplastic film was very low (1.38 MPa) which necessitates further experiments towards improving the mechanical properties of the bioplastic. Evident degradation could be observed for biopolymer after 40 days of burial in the soil while the synthetic plastic sheet remained unaltered. The study revealed the possible prospects of bioplastic from PHB produced by soil bacteria as a better alternative to the synthetic non-degradable plastics.

Key words

Bacillus megaterium VAS05, Polyhydroxybutyrate (PHB), bioplastic, carbon, nitrogen source, tensile strength

Introduction

The menace the plastic pollution has created over years has forced man to think on developing new strategies to reduce, reuse and replace them to save the environment. The approaches to replace the synthetic plastics have been centred on to developing quality bioplastics with properties comparable with synthetic counterparts in terms of strength and durability; and could be easily degraded by soil microorganisms. There has been a recent surge of interest in the research on bioplastics in the global level as one of the measures to mitigate environmental pollution [1]. The bio-based or biodegradable plastics, are commonly called the 'bioplastics', several of which are already commercialised with several different applications [1,2,3,4,5]. Bioplastics with several applications find entry into the commercial market as materials for packaging [6], compost bags [7], in biomedicine [8,9], and use in different industries [10].

Polyhydroxyalkanoic acids (PHAs) are produced in archae, bacteria and in few eukaryotes such as yeasts and fungi as natural intracellular carbon and energy reserve compounds. Several different bacteria such as *Alcaligenes latus*, *Ralstonia eutropha*, *Azotobacter beijerinckii*, *Bacillus megaterium*, and *Pseudomonas oleovorans*and some fungi and archaea accumulate the biopolymer as reserve food material[11]. The polyhydroxybutyrate (PHB) is the most common biodegradable polymer among different PHAs and has potency to replace the conventional non-degradable synthetic plastics [12,13] and show material properties that are similar to polypropylene[14]. Bacteria produce different forms of PHB [15]. As reported by Lee et al. [16],eighty different forms of PHB have been detected in bacteria.Several researchers have isolated PHB producing bacteria from different sources; Danial *et al*. [13] isolated *Bacillus wiedmannii*AS-02 OK576278from cattle manure sample which produced PHB utilizing sugar fruit peel waste as nutrient source. Studies on the PHB-producing bacteria*Erythrobacter aquimaris* isolated from the mangrove rhizosphere, Red Sea,Saudi Arabia was conducted by Mostafa*et al*. [17]. Trakunje *et al*. [18] reported PHB production bya rare actinomycete species, *Rhodococcus pyridinivorans* BSRT1-1 from wastewater treatment area of Kasetsart University, Bangkok, Thailand. This study focuses on the isolation and screening of PHB-producing soil bacteria from organic waste dumping sites in Kannur, Kerala; to optimize the biopolymer production and to extract it, as well as determining its chemical and mechanical characteristics.

Materials and Methods

Isolation and screening of PHB producing bacteria

The soil samples were collected from the organic waste dumping sites in Kannur, (Kerala) India in sterile polythene bags and transported to the laboratory. The serially diluted samples were plated on nutrient agar medium and observed for morphologically different colonies at 37°C for 7 days. Eight morphologically different colonies (VAS01 – VAS08) were cultured on nutrient and Minimal Davis Agar and screened for PHB production using standard protocol [19].

For the rapid detection and isolation of PHB producing bacteria, 0.02% alcoholic solution of Sudan black B was applied to stain bacterial colonies and the plates were kept undisturbed for 30 min. The excess dye was then decanted and plates were rinsed gently by adding 100% ethanol. Colonies unable to incorporate the Sudan black B appeared white, while PHB producers appeared bluish black [11,20]. The pure culture of the producer strain designated

VAS05 was further stained by Sudan black B and observed under the microscope for confirming. The morphology of the PHB producer was further confirmed by Scanning Electron Microscopy.

Measurement of dry biomass

For dry biomass measurement the culture was centrifuged at 10,000 rpm for 15 min, and the pellet was dried in an oven at 55 °C to constant weight [14,21]

Production and Extraction of PHB

The PHB producing isolate VAS05 was cultured in Minimal Davis Medium at 37º C for 3 days for PHB extraction. The procedure was performed using Sodium hypochlorite-chloroform method as per Nehra *et al.*[22] with minor modifications. Following incubation, 10ml of culture was centrifuged at 6000 rpm for 10 minutes and supernatant discarded. The pellet was suspended in 5 ml of 4% sodium hypochlorite and 5 ml of hot chloroform and incubated at 37˚ C for 1 hour. After incubation, the suspension was centrifuged at 3000 rpm for 10 minutes. Upper and middle phases were discarded and 5 ml of hot chloroform added to the bottom phase. 5 ml of ethanol and acetone mixture (1:1) was added to precipitate the granules. The precipitate was allowed to evaporate for dryness at 30˚C, and then the weight of PHB was measured [23].

Biochemical and molecular identification of PHB producer VAS05

The PHB producing strain VAS05 was characterized by studying their colony morphology and biochemical characteristics in accordance with Bergey's Manual of Determinative Bacteriology [24]. 16S rRNA studies were carried out to confirm the species level identification of VAS05. The DNA of VAS05 was isolated using CTAB method and amplified using universal forward and reverse primers (5'-CGC GGC CTA TCAGCT TGT TG-3') and 16R (5'-CCG TAC TCC CCA GGC GGGG-3').The PCR amplified product was sequenced using the Big Dye Terminator (BDT) v3.1 cycle sequencing kit on an ABI 3730xl Genetic Analyzer. The sequenced product was subjected to BLAST studies to identify the nearest homology and phylogenetic tree drawn using Mega 10.0 software. The evolutionary history was inferred by using the Maximum Likelihood method and Tamura-Nei model[25]. The bootstrap consensus tree inferred from 500 replicates [26] is taken to represent the evolutionary history of the taxa analyzed [27].

Effect of Culture Conditions on the production of PHB

The effect of incubation temperature, pH, carbon and nitrogen sources were determined. The growth of VAS05 strain congruent to the production of PHB in nutrient broth and minimal Davis medium under different culture conditions was also determined. The growth of the VAS05 strain was monitored by measuring the aliquots at an absorbance of 610 nm (OD_{610}) using a UV – Visible double beam (HLS1 – 19191 India) spectrophotometer for every 24 h for 6 days [28]. The cultures grown for PHB assays were evaluated by comparing the dry weight of extracted PHB.

The effect of temperature and pH on the growth of the strain was determined by culturingVAS05 at various temperatures (25, 30, 37 and 40°C) and pH adjusted to 5.0, 6.0, 7.0, 8.0 and 9.0 using 1 N HCl / 1 N NaOH in Minimal Davis broth medium and nutrient broth respectively in separate experimental setups.

Effect of Carbon and Nitrogen source on PHB production

To determine the best carbon source for PHB production, 100 mL of sterile minimal Davis medium media with 1% w/v of different carbon sources (sucrose, lactose, starch, and sago rice powder; pH 7.0) were inoculated with 10 ml inoculum and incubated for 72 h at 37 °C after sterilization. The growth, dry weight and PHB production were estimated as described previously.

The effect of nitrogen source on PHB production was determined by supplementing the 100 ml production medium with different nitrogen sources (1% w/v) *viz*., ammonium sulphate, beef extract, malt extract and green gram powder and pH adjusted to 7.0; 10% inoculum was added to the sterilized media and incubated for 72 h at 37 °C following which growth, dry weight and PHB production were estimated as previously described.

Production of Bioplastic from extracted PHB

The PHB producerVAS05 was cultured in Minimal Davis medium at 37°C for 72 h with supplemented lactose and green gram (1% w/w) as carbon and nitrogen source respectively to upsurge PHB production. The PHB produced was extracted and dry weight calculated as per the foregoing protocol. The pellet was dried at 120˚C for 30 minutes, cooled and weighed. Ten grams of extracted PHB was taken in a beaker and 3 ml of 0.5 N HCl added to it with continuous stirring. Subsequently 2 ml of Glycerol and 0.5 N NaOH and allowed to liquidate. The mixture is spread on a ceramic tile after adequate mixing and placed in oven at 120°C and baked for 3 to 4 h. The tile is allowed to cool and the film scraped off the surface.

Determination of the tensile strength and thermal behaviour of the bioplastic

The tensile strength of the prepared sample was tested using ASTM D 882 at CIPET, Kochi. The tensile strength and grip separation of the thin plastic film were based on the elongation to break of the material and were calculated from crosshead displacement. The tensile samples were cast in a collapsible aluminum mold based on ASTM standard D882 for tensile tests and the specimen was prepared as rectangular strips by compression molding based on ASTM D882 for tensile properties of thin plastic sheeting. The results were expressed in terms of tensile strength, tensile modulus, and elongation at break. The moisture and ash content of the material was calculated using ASTM6980 standards.

Differential Scanning Calorimetry (DSC) Analysis: The thermal transition of the bioplastic film was determined using (DSC) analysis ASTM D3418.

Fourier Transform Infrared Spectroscopy Analysis

FT- IR spectrum was measured using Nicolet 6700 FT-IR spectrometer (Thermo Scientific, United States). The samples were scanned between a wave number range 4000-400 cm^{-1} with a total of 64 scans per sample at a resolution of 4 cm^{-1}[29,30,31].

X-Ray Diffraction

XRD measurement of PHB was performed at room temperature on RIGAKU Smart lab X-ray diffractometer employing nickel-filterd Cu-Kα(λ = 1.5406 E; 40 kV, 30 mA)in the 2θ range of 2 – 50˚ at 25˚C, using a scan speed of 10˚ / min. The degrees of polymers crystallization were estimated from the XRD spectra [31]

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Soil Degradation Test

The degradation of the biopolymer was observed by soil burial test. The 5 x 5 cm biopolymer sheet was buried in soil (500 g) taken in a tray. Similar sized polythene sheet was buried in another tray with soil. Both the sets were observed for degradation after 40 days after which they were observed for any change in physical texture.

Statistical Analysis

All experiments were carried out in triplicates and the data analysis in terms of mean and standard deviation of the growth, dry weight and PHB production was conducted in Excel 10.0.

Results and Discussion

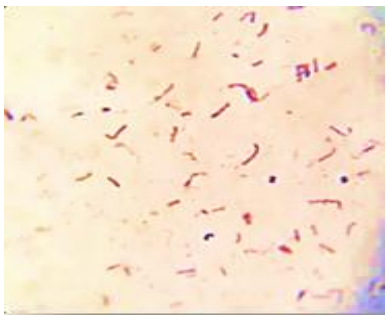
From among the eight distinct strains of bacteria isolated from the soil sample, the strain VAS05 produced Polyhydroxy Butyrate (PHB) as revealed by Sudan Black staining. The PHB producer strain VAS05 was confirmed as *Bacillus megaterium*based on their morphological, biochemical and molecular characteristics. The microscopic and SEM image of the PHB producer strain *Bacillus megaterium* is shown in Fig. 1.

The biochemical characteristics clamped the strain as Gram positive, catalase negative, positive to starch hydrolysis, negative to gelatin hydrolysis, non-spore forming and long rods. The partial 16S rRNA sequence obtained when subjected to BLAST showed highest similarity to *B. megaterium* and *Bacillus flexus* strains. The phylogenetic tree drawn using Mega 10.0 software is given in Fig. 2. The gene sequence was submitted to GenBank under the accession number **MW193404**.

Earlier reports also suggest isolation of PHB from *B. megaterium* strain collected from different vicinities [32,33,34] and the even the first known bio-based plastic, polyhydroxybutyrate (PHB), was discovered from the bacterium *Bacillus megaterium* by a French researcher, Maurice Lemoigne in 1926 [35].

Fig. 1. Microscopic (a) and SEM (b) image of PHB producing *Bacillus megaterium* VAS05

(a)



(b)

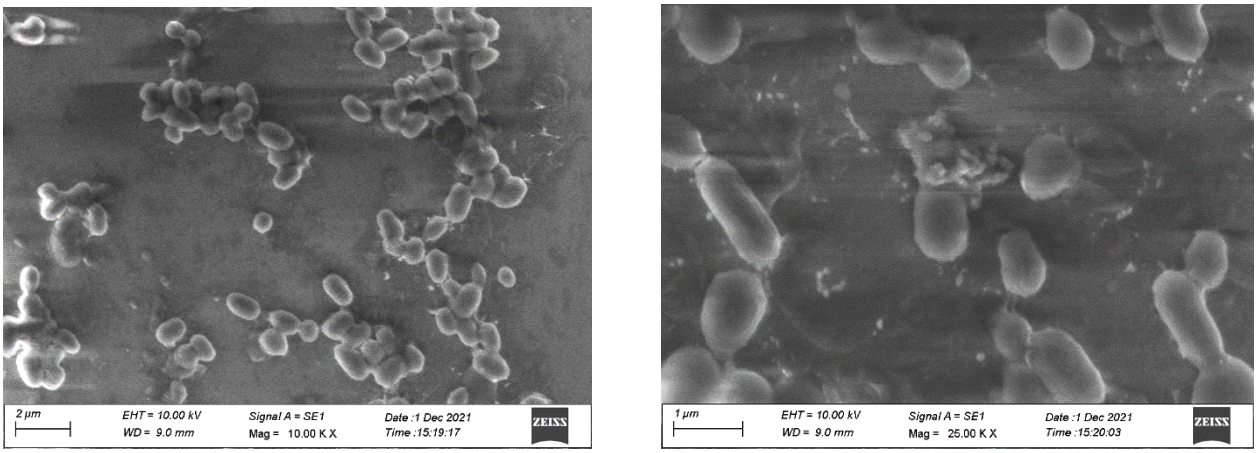
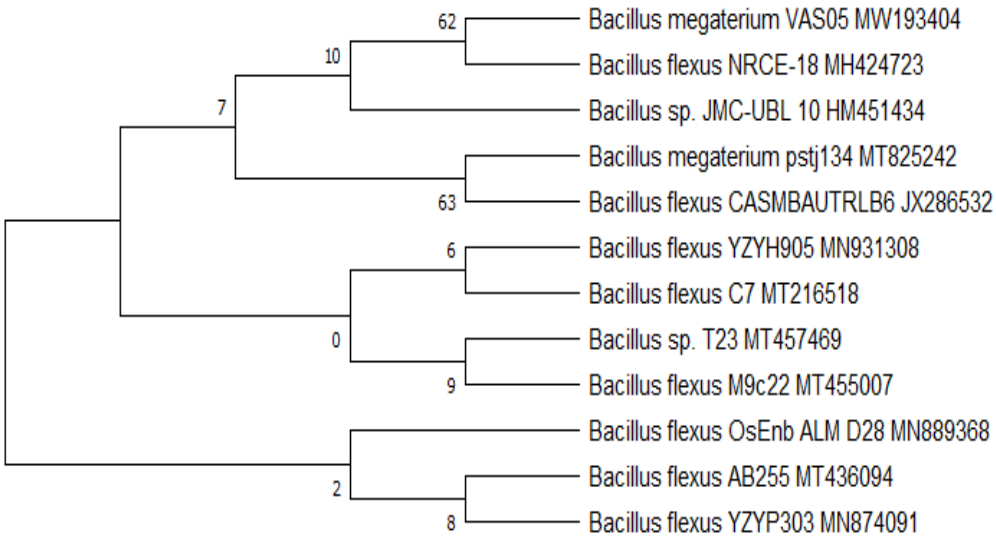


Fig. 2. The Bootstrap Consensus Tree inferred based on 16S rRNA gene sequences showing phylogenetic relationship between strain *Bacillus megaterium*VAS05 and closely related taxa of the genus Bacillus



Effect of different culture conditions on the growth and PHB production by *B. megaterium* VAS05

The growth of the PHB producer strain *B. megaterium* VAS05 in the Minimal Davis medium maximized at the 72th hour of incubation (OD_{610} = 0.92; PHB production - 4.1 g/l) (Fig. 3) and then declined. This might be due to nutrient depletion, which forces the bacteria to use the accumulated PHB as energy source similar result were reported earlier by Bhagowati *et al*. and Penkhrue *et al*. [31,36]. The growth and the PHB production were maximum at 37°C and at a pH of 7.0 (Table 1). Reports by Singh *et al*. [37] concluded 30°C as the optimum temperature for Bacillus sp. [31]. Similar observations were noticed by Singh *et al*. [38] in *Bacillus subtilis* NG220 from sugar industry waste water and by Alshehrei [39], that showed maximum yield of PHB at pH 7. The optimum temperature for the growth of the strain along with PHB production was 37°C with PHB yield of 5.18±0.07 g/l.

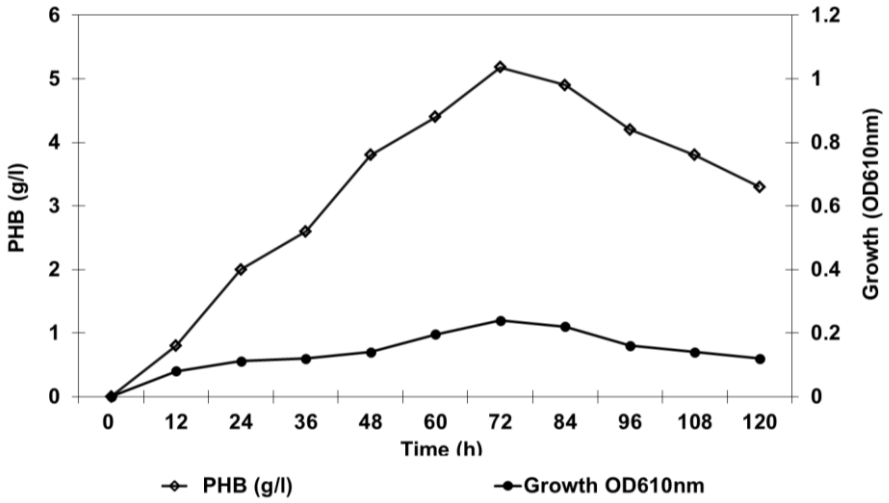


Fig. 3. Growth and PHB production of *B. megaterium* VAS05 at different incubation period with temperature maintained at 37°C and initial media pH of 7.0

Table 1. Effect of temperature, pH, carbon and nitrogen sources on growth and production of PHB by *Bacillus megaterium* VAS05

Temperature / pH / Nutrient source	Growth $OD_{610\ nm}$	Dry biomass (g/l)	PHB (g/l)	%PHB (w/w)
Temperature (°C)				
25	0.77±0.037	3.23±0.208	0.87±0.06	26.93
30	0.93±0.015	4.9±0.230	2.73±0.21	55.71
37	1.14±0.052	8.13±0.351	5.18±0.07	63.71
40	1.01±0.052	7.16±0.251	3.17±0.25	44.27
pH				
5	0.4±0.02	2.97±0.493	0.79±0.08	26.6
6	0.85±0.01	4.47±0.251	2.33±0.15	52.13
7	1.13±0.02	8.37±0.208	5.2±0.36	62.13
8	0.8±0.01	6.20±0.30	3.2±0.31	51.61
9	0.55±0.01	3.73±0.208	0.57±0.40	15.28
Carbon source (1% w/v)				
Sucrose	1.26±0.058	8.83±0.230	5.44±0.23	61.61
Lactose	1.28±0.049	8.93±0.251	5.57±0.058	62.37
Starch	0.54±0.393	4.27±0.208	2.18±0.106	51.05
Sago rice powder	0.77±0.026	6.53±0.152	3.41±0.213	52.22
Nitrogen Source (1% w/v)				
Ammonium Sulphate	0.77±0.065	7.06±0.256	3.5±0.28	49.58
Beef Extract	0.69±0.015	6.83±0.305	3.45±0.18	50.51
Malt Extract	0.80±0.035	6.86±0.14	3.29±0.18	47.96
Green Gram	1.25±0.01	9.34±0.152	6.04±0.20	64.67

Effect of Nutrient sources on PHB production

Sucrose and lactose (1% w/v) as carbon sources induced PHB production (5.44 g/l and 5.57 g/l respectively) in *Bacillus megaterium* VAS05. This result was in accordance to the research study by Thapa *et al*. [40] that showed three of their soil isolates preferring sucrose over other carbon sources. Reports by Mohanrasu *et al*. [41] showed maximum PHB production with addition of glucose as sole carbon source for *B. megaterium* isolated from marine water. Working with different carbon sources in PHB producing media, Thapa *et al*. [40]observed higher PHB yield on *Bacillus pumilus*, *Bacillus pasteurii* and *Bacillus*

sphaericus that preferred sucrose while *Bacillus megaterium* preferred glucose. The study conducted by Getachew and Woldesenbet [14]reported that *Bacillus sp.* accumulated 25%–35% (w/w) PHB during glucose fermentation.

Out of various organic and inorganic nitrogen sources studied, Green gram supported the maximum (6.04±0.20 g/l) PHB production followed by ammonium sulphate, beef extract and malt extract (Table 1). Alshehrei [39] observed ammonium sulphate to be the best nitrogen source for PHB production in different microorganism including *B. subtilis*. The growth and PHB production were high in the presence of 1% Green gram**.**Similar results were noted in earlier studies too [42,43,44].

Production of Bioplastic from extracted PHB and determination of its characteristics

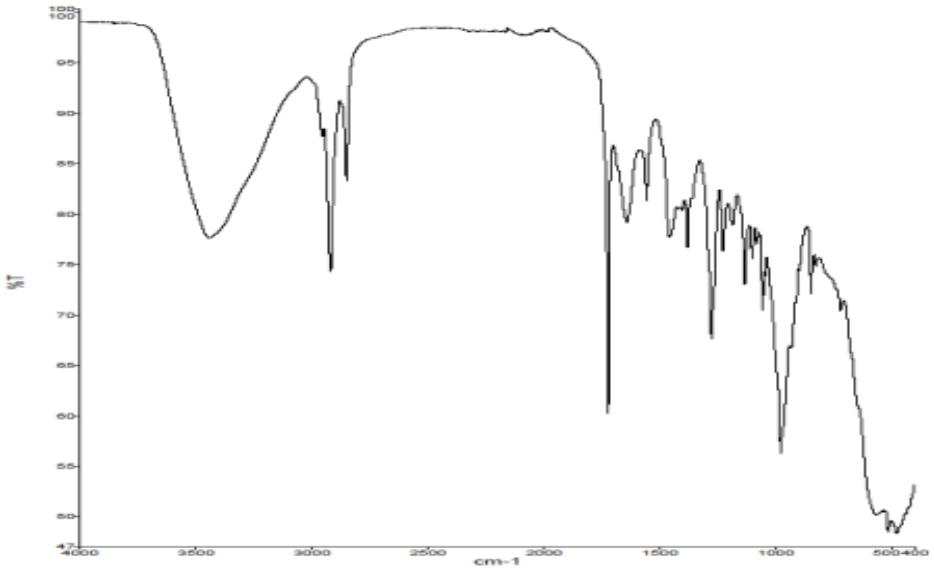
The figure 4(a & b) shows the appearance of the extracted PHB as dried powdery and bioplastic sheet. The biopolymer extracted was characterized by Fourier transform infrared (FTIR). The thermal transition was analysed by DSC analysis and the structural integrity determined by XRD analysis.

Fig. 4. Biodegradable plastic produced by *B. megaterium* VAS05

(a)  **(b)** 

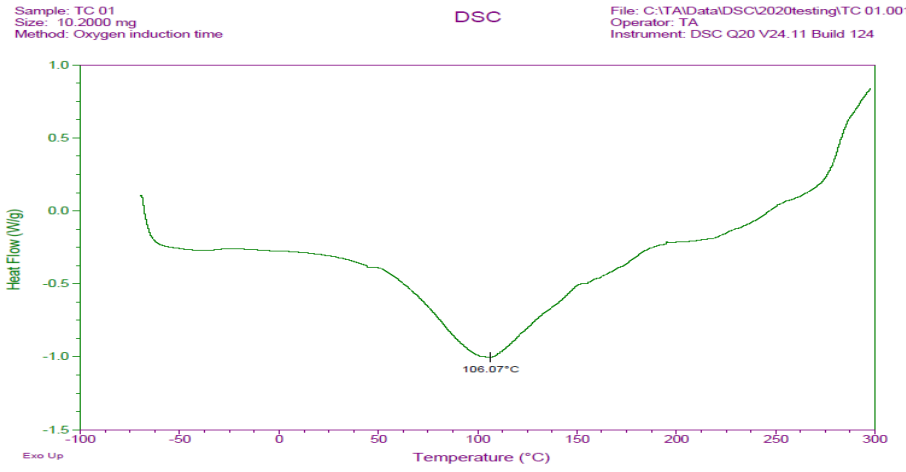
FTIR Analysis: FTIR analysis was performed to determine the functional groups present in PHB extracted from *B. megaterium*. The FTIR spectrum revealed the characteristic peaks corresponding to C─H, C═O, OH functional groups and strong stretching peaks within the range of 1800 and 1600 cm^{-1}. Intense absorption bands noticed at 1738 cm^{-1} corresponded to C=O carbonyl stretching and presence of aliphatic groups at 1272 cm^{-1} absorption band were noticed. Multiple vibrational modes between 1300 -1000 cm^{-1} confirmed presence of C-O ester groups. The bends between 2930 to 3000^{-1}cm indicated the presence of C-H stretching of methyl and methylene stretching groups while band at 3470^{-1} are related to O-H stretching. Similar reports of carbonyl and methyl groups were reported in PHB selected from different isolates [18,45,46,47] further confirming the properties of PHB extracted from VAS05 strain.

Fig. 5. Fourier transformed infrared spectroscopy (FTIR) analysis of extracted PHB



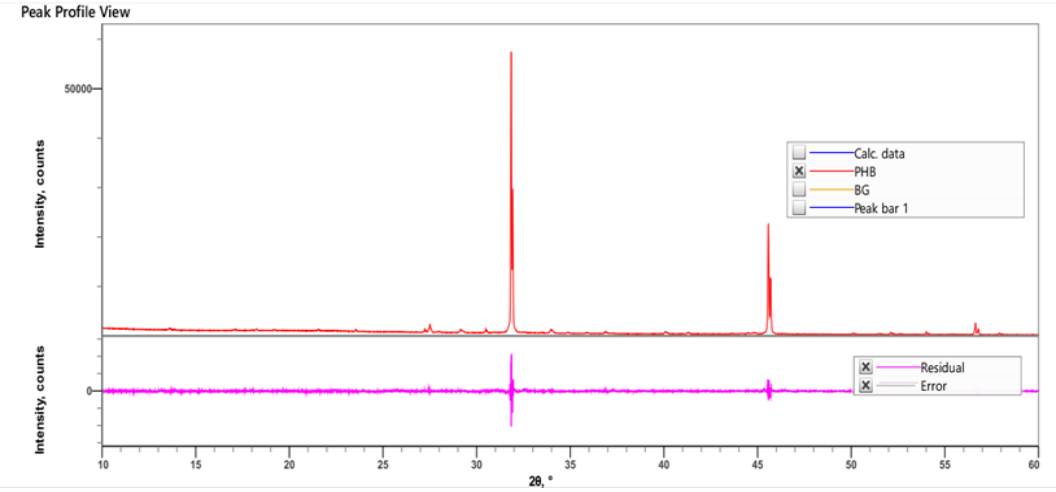
DSC Analysis: The thermal transition of the bioplastic film was determined using Differential Scanning Calorimetry (DSC) analysis (ASTM D3418).Based on the thermograms the peaks reach maximum temperature for melting at 106.07°C in studied PHB. The result correlate with studied conducted by Sultan and Johari [48]. Chaijamrus and Udpuay[49] reported T_c for PHB synthesized from *B. megaterium*to be ~113°C, whereas Oliveira *et al.*[50]reported T_c for PHB synthesized from *C. necator*to be ~86°C.

Fig. 6. DSC for PHB synthesized by *B. megaterium*VAS05



XRD Analysis**:**Structural integrity of extracted product was analyzed by X- Ray diffractometer. The product exhibited a characteristic peak at 2θ values of 31.83˚and 45.55˚. The increased intensity of peaks showed that the polymer has more organized packed crystalline structure. The highest intense peak was observed at 31.83˚ indicating crystalline nature of PHB. The diffraction patterns of PHB and blend PHB by Bhagowati *et al.,* [36] showed 2θ values at similar ranges of 26.80°, 31.74°, 45.59°, and 56.22° at varied intensities.

Fig.7. XRD patterns obtained from PHB



Determination of Tensile Strength

The tensile strength of the prepared sample was tested at standard procedure ASTM D 882 at CIPET, Kochi. The tensile strength of the extracted bioplastic film was 1.38 MPa. The tensile strength is related to the molecular weight of the polymer. The value obtained is noted very lower when compared to the standard PHB which is usually between 11-40MPa[51]. According to Balani et al. [52], the low molecular weight of the polymer ensues weak van der Waals forces developing loosely bonded polymer chains which causes lower strength although crystallinity is present. The Table 2 shows the different values in terms of tensile strength, elongation at break, moisture and ash content of the studied PHB. The lower mechanical strength of the PHB studies indicates the need for further experiments in terms of blending with other biopolymers, plasticizers, composites etc. which can improve the mechanical properties of the biopolymer[53,54].

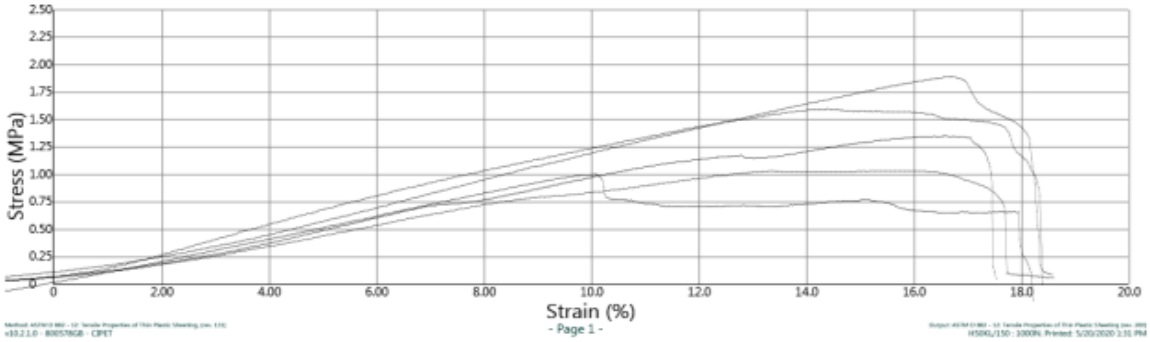
Table 2. Mechanical properties of PHB extracted from *B. megaterium* VAS05

Parameter	PHB properties
Tensile Strength	1.38 MPa
Elongation at break	17.46 %
Moisture Content	13.66 %
Ash Content	5.54 %

The stress–strain curve for PHB is shown in Fig.8; the PHB biopolymer shows brittle behavior. Similar observations were made by Mosnáckováet *al*. [54] for unfilled PLA/PHB blends. The mechanical properties of PHB can be significantly affected by the ratio of

amorphous and crystalline moieties; PHB being a semi-crysalline polymer. The high crystallinity and brittleness of PHB makes them unsuitable for products requiring high agility and flexibility [53].The secondary crystallization and physical ageing can further increase the brittleness of PHB over time. The mechanical properties of PHB can be improved by different approaches and strategies, which includedrawing and thermal treatment, blending with materials from natural sources and synthetic polymers and forming reinforced composites with natural fibers and inorganic fillers[55].

Fig. 8. Stress-strain curve for PHB



Degradation Test: Soil burial test has shown the obvious degradation of the soil buried biopolymer after 40 days while the synthetic plastic (polythene sheet) remained unaltered.The alteration in the texture of bioplastics indicated degradation and decay in the soil environment; thus, providing prospects as alternative to the synthetic non-degradable plastics by suitable modifications. This finding is in agreement with previous studies [14,56].

Conclusion

The biodegradable plastic, Polyhydroxybutyrate(PHB) was produced by *Bacillus megaterium* VAS05 isolated from organic waste dumping site in Kannur, Kerala, India. The maximum PHB production by the isolate was obtained during 72 h at 37°C and pH 7.0. The lactose or sucrose as the carbon source and the green gram powder as the nitrogen source elicited PHB production. The chemical characteristics of the extracted PHB was determined by FTIR, DSC and XRD analysis, while the mechanical properties revealed through determination of Tensile strength. The study provides prospects for application of the extracted PHB from *Bacillus megaterium* VAS05 as bioplastic. The study also points to the need for further studies on large scale PHB production and on improving the mechanical properties of the same.

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