ORIGINAL ARTICLE

Potential screening of butyrate type of polymer obtained from *Alcaligenes latus*

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Abstract

Objective: There has been considerable interest in the development of biodegradable polymers such as poly-β-hydroxybutyrate (PHB) from bacterial origin which could help in solving probable problems due to the use of synthetic polymers. Many synthetic polymers are being used now-a-days in drug delivery systems. However, synthetic polymers have certain disadvantages such as their nonbiodegradability and so probability of bioaccumulation. Such accumulations for long time in body are harmful. This explains a need of easily biodegradable and a biocompatible polymer. Bacteria can synthesize a wide range of biopolymers which are biodegradable, biocompatible and have material properties suitable for medical applications. Bacterial polymers such as PHB are found to be functionally more effective and can be better for use in the pharmaceutical field. **Materials and Methods:** These studies started with screening of better producer of PHB from soil. This study represents a work on screening of bacterial isolates capable of producing PHB, and production of PHB using laboratory scale fermentation procedures. Using nutrient agar, we could screen four bacteria from soil, capable of producing PHB, using Sudan black-B staining. We also used a pure bacterial culture of *Alcaligenes latus* obtained from Microbial Type Culture Collection Chandigarh as a producer of PHB. **Results:** Out of these five bacterial isolates, *A. latus* found to produce PHB in relatively more amounts yielding about 55%. We used *A. latus* for production of PHB in large quantity. The morphological tests, physiochemical assessment, and characterizations of finished polymer were carried out. PHB shows viscosity 0.7993 (Pa.s), sharp peak arouse at 176.12°C in differential scanning calorimetry analysis, 235.8 nm λmax, successive Fourier transform infrared analysis, energy dispersive X-ray spectroscopy, and microscopic imaging analysis (scanning electron microscope [SEM]) were performed to get satisfactory reasoning for confirmation. **Conclusion:** PHB was used for formulating various formulations and was found to give good results, hence was concluded to be a better polymer in the pharmaceutical field.

Key words: *Alcaligenes latus,* bioprocessing techniques, characterization, poly-β-hydroxybutyrate production

INTRODUCTION

Bio-based polymers are materials which are produced from renewable resources. The terms bio-based polymers and biodegradable polymers are used comprehensively in the literature, but there is a key difference between the two types of polymers. Biodegradable polymers are defined as materials whose physical and chemical properties undergo decline and completely mortify when exposed to microbes, carbon dioxide (aerobic) processes, methane (anaerobic processes), and water (aerobic and anaerobic processes).[1] Bio-based polymers can be biodegradable or nondegradable. Similarly, while many bio-based polymers are biodegradable, not all biodegradable polymers are biobased. Biodegradable polymers are seen as the budding solution to manage problems concerning worldwide environmental and solid waste management. These biodegradable plastic materials can retain the desired material properties of conformist synthetic plastics and can be completely degraded without parting any undesirable rest.[1,2] With the aim of substituting the functionality of plastics of the petrochemical source, as well as mounting the range of application of bioplastics to an extensive range, polyhydroxyalkanoates (PHAs) are

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supposed as the most suitable materials because of their adaptability in terms of physical properties and chemical characteristics.[2] Polyhydroxybutyrate (PHB) is the most widely studied member of the PHA family and the first one that has been produced at industrialized scale. It has been used, among other applications, to produce bottles, films, and fibers for biodegradable wrapping materials and as mulch films for agriculture.[3] However, applications of PHB are not restricted to these areas, and they have been extensive to osteosynthetic resources, bone plates, surgical sutures, rivets, tacks, and many other materials in medicine.[4] At industrial level, PHB is commonly produced from *Cupriavidus necator* (*Alcaligenes eutrophus*) and *Alcaligenes latus* (*Azohydromonas lata*). These microbes can accrue PHB up to 80% of the dry cell weight. PHB produced from these Gram-negative organisms is employed in a wide variety of products.[5] However, for its use in the area of biomedicine, an extra separation stage should be carried out since Gram-negative organisms contain endotoxins in the outer membrane lipopolysaccharide. The viability of microbial large scale production of PHB is dependent on the development of a low-cost method that produces biodegradable plastics with properties similar or better to petrochemical plastics. The commercial production of PHB has been using relatively economical substrates such as methanol, beet molasses, ethanol, starch and whey, cane molasses as a sole carbon source. Various nitrogen-rich media, such as casein hydrolysate, yeast extract, tryptone, cas amino acids, corn steep liquor, and collagen hydrolysate, have been used in PHB bioconversions using either *C. necator* or recombinant *Escherichia coli* strains.[6] However, unrefined carbon sources such as corn syrup, cane molasses, beet molasses, or malt extract, also support PHB formation, obtaining yields of PHB comparable to, even better than the refined sugars. Beet molasses and malt extract promoted higher polymer production per liter due to a growth stimulatory effect. The metabolic pathway of PHB involved the regulation of its synthesis in the microbial cells. PHB was produced by a variety of microorganism under the environmental stresses such as nutrient limitation, i.e., nitrogen, phosphorus or oxygen limitation. The microorganism and the strategy of production were affected on the duration of fermentation, growth rate, carbon source concentration, etc.[7]

There is generous interest in the development of biodegradable polymer like PHB by environmentally efficient bacterial origin. This may help to solve trouble produced by the synthetic or semi-synthetic polymer. Synthetic polymer may produce hazardous problem during formulation that produces the bioaccumulation in the body. This accumulated trace of polymer creates health-related problems. PHB is found functionally more and can be better for use in pharmaceutical formulations. This will help to improve pharmaceutical drug delivery of different therapeutic agents. This biopolymer is an effective tool to control or extend the drug release rate of pharmaceutical formulation. PHB is the biopolymer include in the formulation because of their thermoplasticity and biodegradability features. It is considered as more environmentally friendly and sustainable method for obtaining the polymer. These improvements contribute to make medical treatment more efficient and to minimize side effects and other type inconvenience for patients.[8]

The current study was based on screening of biopolymer which would satisfy the rising demand of sustainable economic biopolymer production from bacterial genera. Screened PHB has found to be an optimistic role in biomedical applications and pharmaceutical drug delivery scenario. The variety of (semi) synthetic polymer available in market and these many synthetic polymers are being used now-a-days in drug delivery systems synthetic polymers have certain disadvantages such as their high production cost, nonbiodegradability, toxicity, and so it proven their impact on probability of bioaccumulation.[9,10] Such accumulation for long-time hampers the human health. Hence, these demerits avoided by application of screened biopolymer candidate PHB as drug release retardant tool. It is an efficient intracellular carbon energy-storage biopolymer; this is produced by number of Gram-positive and Gram-negative bacteria under nutrient limitation conditions to their growth.

A. latus grows in minimal medium without any added growth factors. Indeed, the ability of *A. latus* to accumulate PHB is so dominant that the PHB content in the cells could reach up to 48% of the cell dry weight. Majorly carbon and nitrogen sources are necessary for their intragranular polymer production, generally glucose used as carbon source and peptone as nitrogen source but the high economical investment is the main inconvenient parameter for polymer production. This problem is overcome by use of cheaply available molasses as carbon source and ammonium chloride as nitrogen source.

Variety of extraction methods are used solvent extraction, chemical digestion surfactant, sodium hypochlorite, etc. The average molecular mass of PHB is also affected on the method of extraction to cause severe damage to the granule, mostly an important loss of molecular mass of the polymer. In addition, the separation condition such as pH, temperature, duration, and biomass to aqueous phase could reduce degradation.[11] This work, in order to study the effect of the molasses in the medium on *A. latus* growth and PHB accumulation and productivity were estimated by the time during growth. The evaluation of the PHB production as a low-cost process affects the properties of the biopolymer synthesized by this bacterium, the chemical structure (Fourier transform infrared [FTIR]) and the thermal properties of PHB obtained from this fermentation were determined. PHB was effectively screened from prime PHB producer *A. latus* yielded the highest polymer production using cheap C and N sources for molasses and ammonium chloride, respectively. The morphological tests, physiochemical assessment, and characterizations, *viz,* rheology, differential

scanning calorimetry (DSC) analysis, X-ray diffractograms (XRD), and microscopic imaging analysis (scanning electron microscope [SEM]) were performed to get satisfactory reasoning for confirmation of finished polymer substance.

MATERIALS AND METHODS

Bacterial fermentation was carried out using *A. latus* strain no. 2311 Microbial Type Culture Collection, Chandigarh. A lyophilized culture was reactivated at 30°C for 24 h in a growth medium as nutrient agar (NA) medium containing NA 3.5%, peptone 2.5%, NaCl 0.5%, yeast 0.1%, beef extract 0.5%, agar powder 3%, and distilled water. The nutrient components as sugar, nitrogen, phosphate, and trace elements were sterilized separately at 121°C for 20 min.

Screening and Characterization of Biopolymer from Microbial Source

Primary screening of microbial source

Collection of soil samples

Soil samples were collected near from Biotechnology Department TKIET campus, Warananagar. It was collected apart from 3 to 4 cm deep from surface was used for isolation of the bacteria. It was kept in plastic bags and marked with collection details and remained protected from the light and used for further study.[12]

Isolation and purification of bacterial strains

The collected soil samples screening to obtain spore forming bacteria was performed by heating sample at 80°C for 10 min. Samples were prepared using sterile distilled water. Soil sample supernatants were subjected to serial diluted 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5}. 0.2 ml of diluted soil samples were plated on NA plates and incubated at 28°C for 24 h. For testing their ability of producing PHB, the isolates were manually transferred on NA plate by spot inoculation.[12]

Media preparation and sterilization

Composite media ingredients were taken in flask. The above media was sterilized for 121°C, 15 lb, for 15 min and allow to cool it; after media was kept in pre-sterilized laminar air flow in between two Bunsen burner to avoid cross contamination. Aseptically transferred the media into sterilized Petri plate and allowed to cool for 15 min, and after that, the desired culture transplant was used for further study. The stationary phase media plates were incubated at 30°C for 48 h.[12]

Effect of pH on PHB production

Different initial pH of the medium (6.0-8.0) was used to check whether pH has any noticeable effect on PHB production. The initial pH of the medium was adjusted by 1 N hydrochloric acid or Sodium hydroxide.[12]

Screening of PHB-producing bacterial strains

Sudan black B staining technique

Well-isolated, morphologically distinct colonies were subcultured on NA plates and were subsequently stained with Sudan black B (0.3% in 96% ethanol). Colonies stained with Sudan black B were selected as a positive for PHB production. The Sudan black B positive isolates were further confirmed by Nile blue viable colony staining; wherein 0.5 µg/ml Nile blue dye solution in alcohol (final concentration) was added to the sterilized media.[13,14] The selected PHA producing isolates were also analyzed for their gram nature.

Nile blue staining technique

Heat-fixed cells were treated with 1% Nile blue A for 10 min and the excitation wavelength of 460 nm selected and excitation was observed. Glycogen and polyphosphate did not stain. Nile blue A stain appeared to be a more specific stain for PHB than Sudan black B.[13,14]

Volutin granule staining technique

Every microorganism has special characteristics; these organisms store reserve food material in the form of granule. This reserve food granule is called as Volutin granule. To stain volutin granule, Albert's staining method was employed. This technique evaluated for the presence of granular lipid constituent like PHB in intracellular region of bacterial strains. In above staining methods, bacterial colonies positive for PHB production were selected by observing the granules under fluorescence microscope,[13,14] OLYMPUS Reflected Fluorescence System, (Olympus Corporation, Japan) using BXRFA fluorescence illuminator, fitted with Image Analyzer. After staining confirmation, isolates were selected further studies. Stained isolate images for Nile blue and Volutin granule staining are illustrated in Figures 1 and 2 respectively.

Morphological characterization of microbial source

Colony characterization

The colony characters, *viz*., size, shape, margin, elevation, opacity, and color were observed on agar medium.[15]

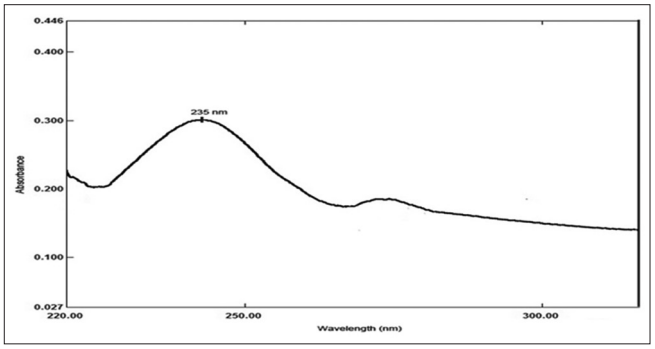

Figure 1: Ultraviolet-visible scanning spectra of polyhydroxybutyrate biopolymer solution

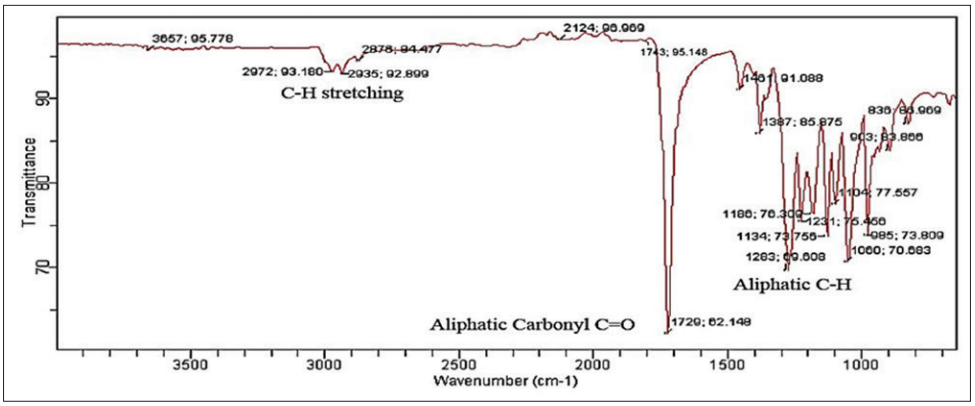

Figure 2: The Fourier transform infrared spectrum of polyhydroxybutyrate biopolymer

Cultivation of selected isolates and standard PHB producer on media

Selected six isolates and the standard organism; *A. latus* was inoculated separately onto NA plates and nutrient broth. The plates and broth were incubated at 30°C for 48 h.[15]

Harvesting of cell mass

Cell mass from agar plates was scrapped with the help of spatula and that from broth 5 ml of 48 h at 30°C individual grown isolate was centrifuged at 6000 rpm for broth culture and 4000 rpm for solid state agar medium at 10°C; centrifugation time 15 min was employed for both medium, these processes were carried out in cooling centrifuge (Remi, Mumbai). Then, obtained cell pellets were suspended in deionized water. The pellet of cell mass allows to air dry and after that placed in pre-weighed Petri plates dried to a constant weight at 60°C.[15,16] The plates were weighed again and dry cell weight of each isolate was calculated.

Production of biopolymer

PHB recovery

In the PHB, recovery process was done using two steps such as cell lysis and solvent extraction.[17]

Cell lysis

The cell mass pellet was treated with sodium hypochlorite (10 ml/50 ml culture pellets) and incubated at 30°C for 2 h. Sodium hypochlorite could not release all of the available protein, further treatment became necessary; which was subjected to homogenize by the homogenizer for 10 min, which was subjected homogenized cell mass further treated by passing the ultrasonic waves by immersing probe sonicator's rod in cell mass pellet to attain firm cell lysis process.[17]

Solvent extraction technique

PHB granules were extracted using chloroform extraction method. The above cell mass centrifuged at 6000 rpm for 15 min supernatant was collected and filtered through Whatman No. 1 filter paper and the non-PHB cell mass discarded. Obtained cell mass pellet suspension treated with chloroform at 45°C with constant shaking on rotary shaker for 24 h. PHB from chloroform phase was recovered by nonsolvent precipitation method comprising (7:3 v/v) methanol and water. Followed by chloroform saturated cell mass suspension was poured into separating funnel; allowed to stand for separation, further that saturated chloroform layer was collected and kept at room temperature until PHB crystals emerge out.[17]

Characterization of PHB biopolymer

Physiochemical characterization

The polymer samples were evaluated for its color, odor, and appearance.

Melting point determination

The melting point of PHB biopolymer was determined. Small amount of PHB sample was introduced into capillary tube attached to graduated thermometer and constant heat was supplied to Thiele's tube containing paraffin oil assembly suspended in the bath.[18] The temperature at which polymer just melted was recorded.

Solubility determination

Solubility of polymer sample was determined by adding excess amount of polymer into solvent system ranging from polar to nonpolar ones. These solvents allow to keep at room temperature for 24 h with occasional shaking on rotary shaker.[18]

Rheological characterization

Viscosity determination

The shear viscosity is defined as the ratio of shear stress to the shear rate under test conditions. Viscosity test was performed using Brookfield R/S-CPS+ Rheometer. Measurements were carried out using plate-plate type instrument and using C75-2 spindle at 32 ± 2°C. A gap of 0.5 mm was kept between two plates.[19] The viscosity of PHB sample was loaded between two plates and viscosity was recorded.

Spectral characterization

Ultraviolet (UV) spectrum

Determination of the UV absorption maxima was recorded by convenient spectrophotometric analysis of bacterial PHB has been devised. Quantitative conversion of PHB acid to crotonic acid by heating in concentrated sulfuric acid and to found out scanning range of PHB. A standard curve was

established with PHB conc. ranging from 2 to 10 µg/ml. Solution of higher concentration of the screened PHB[20] was kept as standard and read with sulfuric acid as blank in the range of wavelength from 200 to 400 nm using a UV spectrophotometer (UV 1800, Shimadzu).

FTIR spectroscopy

PHB was characterized by FTIR spectroscopy. The spectrum was recorded using FTIR spectrophotometer (Agilent ATR CARY 630). The system employs a diamond ATR element that made proper contact between sample for evaluation without scratching of the window and sample analysis was done feasibly.[21] The scanning range was 3800-800/cm.

Thermal characterization (DSC)

The DSC study was carried out on PHB to confirm its thermal properties and purity. The DSC patterns were recorded on a METTLER TOLEDO STARe System. 2 mg of polymer was heated in crimped aluminum pans at a scanning rate of 20˚C/min in an atmosphere of nitrogen gas flow 40 ml/min using the range of 40-280°C.[22]

Microscopic imaging analysis (SEM)

SEM is essentially a high magnification microscope, which uses a focused scanned electron beam to produce images of the sample, both top-down and, with the no necessary sample preparation, cross-sections. Safeguard sample authenticity; safeguard credibility maintained by no coating requirement or other preparation before analysis. A microscopic observation of PHB was performed using a SEM. Sample was sputtered with gold-palladium and then observed at different magnifications. The sample was analyzed using SEM (JEOL JSM 6360, Germany) under 500, 1000, 2000, 5000, and ×10000 magnification, operating at a voltage of 5 kV to produce the micrographs.[23]

X-ray powder diffraction

XRD of PHB was recorded for 2θ between 4° and 50° at 0.1° intervals and scanning rate of 6 s^{-1}using a BRUKER D8 Advance (BRUKER AXS UK) diffractometer equipped with copper tube operating at 40 kV and 40 mA.[24]

Nuclear magnetic resonance (NMR) spectroscopy

The polymer was suspended in spectrochem grade deuterochloroform ($CDCl_3$). The ^{1}H NMR and the ^{13}C NMR spectral analysis of sample were obtained at 300 MHz using a model VARIAN Mercury 300 MHz NMR spectrometer. The chemical shift scale was in parts per million (ppm).[25]

RESULT AND DISCUSSION

Screening and Characterization of Biopolymer from Microbial Source

Primary screening of microbial source

Soil samples were collected near from the Biotechnology Department TKIET campus, Warananagar. It was found to be loamy and blackish in color. Isolation and purification of bacterial strains from soil sample was carried out. It was reported that 25 different viable colonies are produced on NA plate with time span of 24 h at 30°C.

Screening for PHB producing bacterial strains

It was found that, out of 25 isolated colonies, four showed morphologically showed prominent black to the cell mass after flooding with Sudan black-B solution [Table 1]. The obtained cell mass from the replica plate was then observed by lipid granule staining, and the cells were confirmed to be containing lipid granules.[13,14] Thus, these four isolates were screened as producer of PHB granules. These isolates were then purified from the replica plate. The obtained purified isolates were coded as S1, S2, S3, and S4 before colony characterization.

Morphological Characterization of Microbial Source

Colony characterization

The colony characters, *viz*., size, shape, margin, elevation, opacity, and color were observed on agar medium.

Harvesting of cellular biomass

Table 2 shows the dry weight of biomass per 100 ml of nutrient media of the soil isolate and the standard organism *A. latus* obtained from agar and broth medium. The all bacterial strains were capable of PHB synthesis. Bacteria accumulate maximum PHB during the stationary phase, in nutrient broth medium, PHB yield becomes limited as compared to stationary phase due to nutrients availability becomes critical after some period that reason was took into account and NA

Table 1: Dry weight of obtained cellular biomass

Isolate code	Dry weight of biomass per 100 ml media (mg)	
	Agar	Broth
S1	110	61
S2	176	82
S3	87	41
S4	232	118
AI	370	195

Table 2: PHB yield showed by all microbes on NA medium

Isolate	PHB yield/dry cell weight (% w/w)
S1	12
S2	20
S3	8
S4	17
AI	27

PHB: Polyhydroxybutyrate

medium was selected for further studies. The lowest amounts of biomass showed by soil isolate S3 that was 87 mg and 41 mg in NA and nutrient broth, respectively.[15,16] Soil isolate S4 showed 232 mg in NA, respectively. By considered other view of production standard PHB producer *A. latus* gave yields 370 mg of biomass which was greater than biomass yielded by other soil isolates organism hence that standard organism was selected for further production.

Production of Biopolymer

Extraction of PHB

NA was selected as the medium for maximum PHB yield. When all the bacterial isolates were grown on NA medium, they produced different proportions of PHB yield.[17] The isolate S3 was yielded least amount of PHB yield 8% w/w dry cell weight while, at the same time other isolates S1, S2, and S4 yielded 12, 20, and 17% w/w of dry cell weight, respectively [Table 2]. However, among all the isolates, *A. latus* showed maximum production of PHB which was about 27% w/w of dry cell weight.

Selection of Specific Media Composition for *Bacillus megaterium*

Method-A

In Method-A, the media composition was made using commercially available carbon source Glucose with nitrogen source peptone. The prime importance was gained by this Method-A due to universal acceptance, but the high economy was spent on carbon and nitrogen sources, due to that reason Method- B was took into account [Table 3].

Method-B

In the Method-B, media composition contained carbon and nitrogen source molasses and ammonium chloride, respectively, both sources were replaced by same quantities of carbon and nitrogen source followed by Method-A. The carbon and nitrogen sources used in Method-B are available cheaply as compared to sources used in Method-A [Table 4].

As *A. latus* showed maximum production of PHB, it was decided to use this organism for further PHB production. The specific media for *A. latus* required for the optimum production of PHB was obtained from the literature. This media was optimized further for PHB production by differing the concentration of glucose in the media [Tables 3 and 4]. From Table 4, it was found that *A. latus* showed minimum production of PHB in al1 media which contained 1% glucose, whereas in media containing 2% glucose, i.e., Al2 media, *A. latus* showed maximum production of PHB about 55% w/w dry cell weight. PHB production in Al3 media containing 3% glucose was nearly close to that in Al2 media; hence, it was decided to use Al2 media composition for further production of PHB.[17]

Characterization of PHB Biopolymer

Physiochemical characterization

The PHB biopolymer samples were evaluated for its organoleptic properties.[18] The biopolymer is white, crystalline, stiff, and brittle plastic in nature, molecular weight 1.29×10^4 g/mol, as well as odorless and melting point is in the range of 171-175°C.

Solubility determination

Solubility of PHB was confined or easy to soluble in different types of polar to nonpolar solvents [Table 5].[18]

Rheological characterization

Viscosity determination

The flow property of a system is expressed in terms of viscosity. Simply, viscosity is an index of resistance of liquid to flow. The higher the viscosity of liquid, greater is the resistance to flow. When solvent added into polymer, the groups get easily hydrated in solution. When polymer molecules move, the hydrated solvent sheath moves. As a result, the size of polymer unit increases hence increases the resistance the resistance to flow. The rheological characterization of PHB was performed using Brookfield R/S-CPS+Rheometer.

Measurements were made by plate-plate assembly. It was shown that viscosity of the PHB was measured as a function

Table 3: Specific media composition contained C and N source for *A. latus*

Ingredients	Quantity (g/L)		
	Al1	Al2	Al3
Agar	25	25	25
Peptone	10	10	10
Meat extract	10	10	10
Potassium dihydrogen phosphate	1.5	1.5	1.5
Disodium hydrogen phosphate	9	9	9
Magnesium sulfate	0.2	0.2	0.2
Calcium chloride	0.01	0.01	0.01
Citric acid	0.1	0.1	0.1
Glucose	10	20	30
Trace element solution	1 ml	1 ml	1 ml
Agar	25	25	25

PHB: Polyhydroxybutyrate, *A. latus: Alcaligenes latus*

Table 4: PHB yield of *B. megaterium* in BM specific media

Media	PHB yield dry cell weight (%w/w)
Al1	31
Al2	55
Al3	52

PHB: Polyhydroxybutyrate, *B. megaterium: Bacillus megaterium*

of temperature. Increasing the temperature leads to a change in the viscosity. As the temperature increases, the system acquires thermal energy which facilitates the breaking of the cohesive forces. Increasing temperature range of the polymer substance that has impact on separation of molecule and reduces the intermolecular forces leads to reduced flow of the system. Temperature Here, the measurements were made at constant temperature 33.9°C. The viscosity of PHB was affected by thermal instability due to this reason, especially care was taken at the time of measurements; overall temperature of environ was maintained, and measurement was carried out. Viscosity measurement of PHB assignments was represented in Table 6.

Spectral characterization

UV spectrum

UV spectroscopy is very useful and feasible analytical technique to assaying the variety of compounds. In this technique, quantitative conversion of PHB acid to crotonic acid by heating in concentrated sulfuric acid and to found out scanning range of PHB (Law and Slepecky method). Sulfuric acid containing PHB solution was kept as test and read with sulfuric acid as blank in the range of wavelength from 200 to 400 nm using the UV spectrophotometer. The reported absorbance maxima (λmax) value for PHB is 235 nm.[20] UV absorption maxima was recorded, and λmax was found to be at 235.8 nm. So, it was concluded that the obtained λmax value much closer to reported value that conforms the primary authentication of PHB polymer [Figure 1].

FTIR Spectroscopy

FTIR spectra of the screened PHB polymer are shown in Figure 2. A strong and sharp absorbance band at 1729/cm could be assigned to the C=O stretching mode of the crystalline parts in PHB. The small, almost invisible shoulder positioned at 1743.cm, is also attributed to the same C=O stretching mode although it arises from the amorphous parts. In addition, the peaks at 1283 and 1231/cm are assigned to the C–O–C stretching modes of the crystalline parts. The strong peaks, detected near 1186 and 1134/cm, are also attributed to the C–O–C stretching band. Usually, the presence of the absorption bands above 3000/cm is connected with the possibility of a C–H–O hydrogen bond, but these are very negligible in FTIR spectrum of PHB. The weak shoulders, positioned at 2935 and 2972/cm, could be an evidence of

Table 5: Solvent system used in PHB solubility determination

Solubility	Solvent
Highly soluble	Chloroform
	Dichloromethane
	Acetic anhydride
	1N sodium hydroxide
	Acetic acid
Poorly soluble	Toluene
	Benzene
	Dioxane
	Pyridine
Practically insoluble	Water
	Methanol
	Ethanol
	Hexane
	Benzene
	Acetone

PHB: Polyhydroxybutyrate

Table 6: Rheological behavior of PHB biopolymer solution

Parameter	Minimum	Maximum	Average
Viscosity (Pa.s)	0.4303	2.2936	0.7993
Shear stress (Pa)	22.9754	53.1547	32.2269
Shear rate (1/s)	10.0170	100.1070	55.0323

PHB: Polyhydroxybutyrate

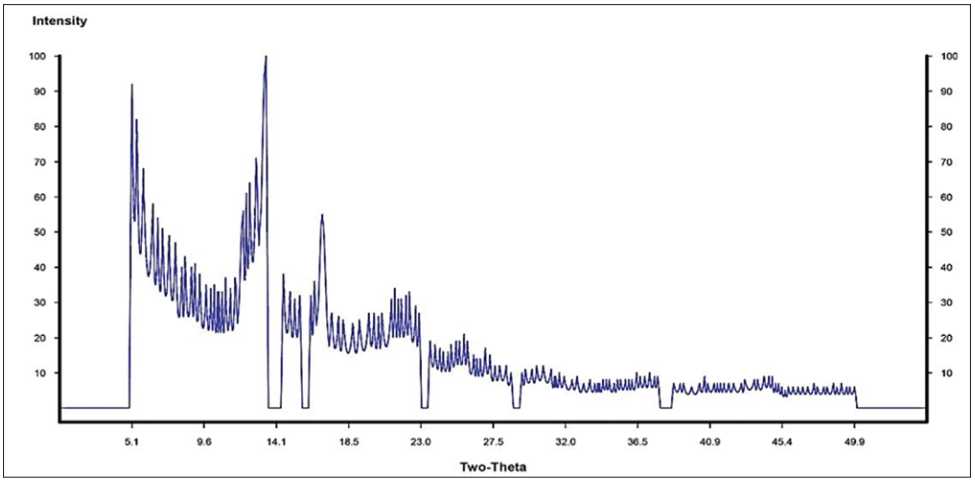

Figure 3: The X-ray diffraction pattern of polyhydroxybutyrate biopolymer

C-H stretching. The weak band at 3657/cm is attributed to its hydroxyl end-groups.[21]

X-ray spectrometry

Solid polymer can exist in a crystalline or amorphous state. The diffraction profile of PHB biopolymer exhibits peak at 13.5°.[26] The peaks observed at 13.5°, 15.5°, and a small shoulder at 20° supports the DSC data that the polymer exists in a crystalline form [Figure 3].

Thermal characterization

DSC

DSC provides a measurement of the rate, magnitude, and temperature at which chemical or physical changes occur in a polymeric substance or system during heating or cooling. The heated PHB underwent an endothermic peak at around 166.5°C indicating the melting point of the polymer. Furthermore, it can be noticed that at approximately 143°C the PHB powder begins to melt, which is completed at 180°C. The thermogram also indicates that the polymer may be undergoing rapid degradation at temperatures above 199.9°C. The higher heat of fusion/enthalpy which was about 92.14 J/g revealed the higher crystallinity of the polymer. It can also be noted that in the temperature range of 140-180°C, transitions take place in the PHB at three temperature ranges, the point at which melting starts, a characteristic melt peak of the crystalline phase at 166.5°C and the point at which PHB completely melts are observed [Figure 4]. It was also found that coalescence of the crystals in PHB starts occurring at 143°C.

Microscopic imaging analysis

Scanning electron microscopy

The sample was analyzed in SEM (JEOL JSM 6360, Germany) under ×500, ×1000, ×2000, ×5000, and ×10000 magnification. SEM was illustrated with a high degree of magnification; highlighted surface features of screened PHB polymer. PHB enabled with irregular fracture surface due to its crystalline structure and brittle surface morphology.

PHB magnified at ×500 illustrated hard stone like surface structural arrangement and at ×1000 magnification showed speckled particulate arrangement. PHB revealed irregular surface area with crest and trough at ×2000 magnifications. Polymorphic granular matrix withheld at surface was shown in ×5000 magnifications. PHB at ×10000 showed that saturated dense globular microparticles at the surface. So, it has revealed that surface morphology of PHB distinctly discerns the polymer surface between the continuous spiky topographic arrangements of the major phase with many pores and protrusions.[23] Materials have microscopically complex structure with a random distribution of dense globular pore sizes, due to its hard, brittle nature found its prime applicability in the packaging industry and pharma formulation practice. SEM micrographs at varied degree of magnifications are illustrated in Figure 5.

NMR Analysis

The ^{1}H NMR spectra obtained from extracted PHB is shown in Figure 6. The spectrum shows a doublet at 1.28 ppm which is attributed to the methyl group coupled to one proton. The doublet of quadruplet at 2.51 ppm is attributed to the methylene group adjacent to an asymmetric carbon atom bearing a single atom.[24] The multiplet at 5.25 ppm is characteristic of methylene group. Two other signals are observed, a broad one at 1.60 ppm which may be due to water and another one at 7.15 ppm attributed to the solvent used, i.e,. chloroform.

Figure 7 represents ^{13}C NMR spectra of PHB. Four narrow lines appeared at very strong intensities in addition to the weak lines associated with bacterial cellular material. These four peaks were assignable to the methyl (19.729 ppm; CH_3), methylene (40.273 ppm; CH_2); methane (67.52 ppm; CH), and carbonyl (169.09 ppm; C=O) carbon resonances of PHB.[25]

CONCLUSION

The current study was based on finding a polymer which would be helpful to pharmaceutical field. Now-a-days, a number of synthetic polymers are being used to formulate different dosage forms. However, they have certain limitations such as their nonbiodegradable nature, toxic properties, bioaccumulation, etc. Hence, it was decided to isolate a

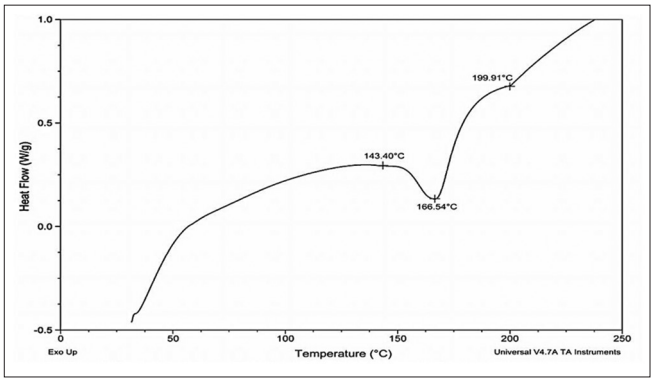

Figure 4: The differential scanning calorimetry thermogram of polyhydroxybutyrate biopolymer

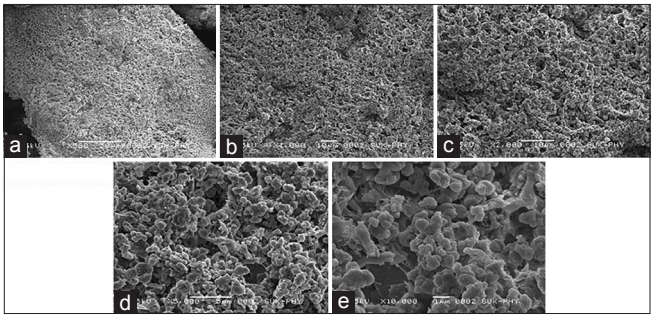

Figure 5: Scanning electron microscopic imaging analysis of polyhydroxybutyrate biopolymer magnified at (a) ×500, (b) ×1000, (c) ×2000, (d) ×5000, and (e) ×10000 magnification

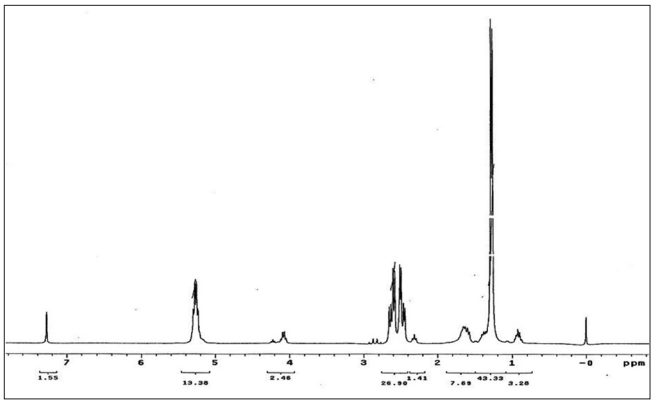

Figure 6: ^{1}H NMR spectra of polyhydroxybutyrate biopolymer

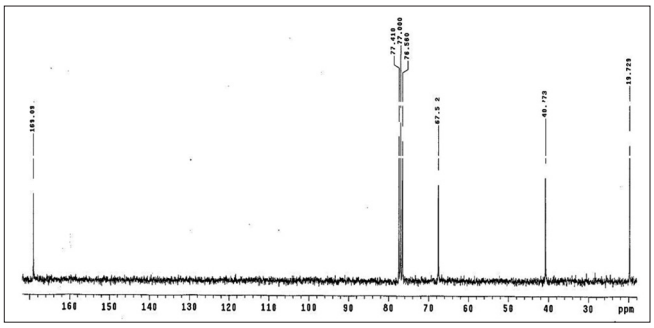

Figure 7: ^{13}C NMR spectra of polyhydroxybutyrate biopolymer

polymer from natural sources which would overcome these limitations. Bacterial source was selected as a natural source for polymers because they can synthesize a wide range of biopolymers that serve diverse biological functions and have material properties suitable for numerous industrial and medical applications. Further points that require research are the scale-up of the process, and much more effort is required in this area to increase the production of bioplastics to successfully replace the nondegradable plastics. Thus, the future of bioplastics depends on the efforts toward fulfilling requirements of price and performance. At present, PHB is the only members of PHAs that are produced on a commercial scale. Even though the price of PHAs is still too high, current advances in fermentation and purification technology as well as the development of superior bacterial strains by recombinant DNA technology are likely to lower the price of PHA so that it is close to that of other biodegradable plastic materials. The competitive advantage of the environmentally friendly and highly processive synthesis of biodegradable and, in many cases, biocompatible polymers will become increasingly attractive for industry.

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