Biosynthesis and Potential Screening of Butyrate Type of Polymer Obtained from Bacillus Megaterium

Akshay Jirage¹, Khyaam Shaikh¹, Kate Vaishali¹ and Payghan Santosh¹,²*

¹Department of Pharmaceutics, Tatyasaheb Kore College of Pharmacy, India
²Shivaji University, India

*Corresponding author: Santosh Ambadas Payghan, Department of Pharmaceutics, Tatyasaheb Kore college of Pharmacy, Warananagar, Panhala, Kolhapur-416113, MS, India, Tel: +919096202858; Email: sapayghan.tkcp@gmail.com

Abstract

Diverse Bacillus strains are known as producers of polyhydroxyalkanoates (PHAs) under nutrient-limiting conditions. However, these limiting conditions have the same nutritional characteristics that stimulate spore generation in Gram-positive microorganisms. In the present work a new isolated Bacillus megaterium strain was characterized and studied in terms of its ability for producing polyhydroxybutyrate (PHB) by implementing different fermentation configurations on formulated media. The isolated strain was able to produce PHB up to 41 and 55% of its dry cell weight during bioreactor experiments employing glucose and glycerol as carbon source, respectively. The produced biopolymer was characterized and identified by using Fourier transform infrared (FTIR) techniques. PHB was effectively screened from prime PHB producer Bacillus megaterium yielded highest polymer production 41% w/w by using cheap C and N sources at 35 and 4g/L employed concentration for molasses and ammonium chloride respectively. The morphological tests, physio-chemical assessment and characterizations of finished polymer substance were carried out. PHB shows viscosity 0.7993 (Pas), sharp peak arouse at 176.12 °C in DSC analysis, 235.8nm λmax, Successive FTIR analysis, EDS and Microscopic imaging analysis (SEM) were performed to get satisfactory reasoning for confirmation

Keywords: Poly-β-hydroxybutyrate (PHB) production and characterization; Bacillus megaterium; Bioprocessing techniques

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 [1]. 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 petrochemical source, as well as mounting the range of application of bioplastics to a extensive range, polyhydroxyalkanoates (PHAs) are 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 its dry cell weight during bioreactor experiments employing glucose and glycerol as carbon source, respectively. The produced biopolymer was characterized and identified by using Fourier transform infrared (FTIR) techniques. PHB was effectively screened from prime PHB producer Bacillus megaterium yielded highest polymer production 41% w/w by using cheap C and N sources at 35 and 4g/L employed concentration for molasses and ammonium chloride respectively. The morphological tests, physio-chemical assessment and characterizations of finished polymer substance were carried out. PHB shows viscosity 0.7993 (Pas), sharp peak arouse at 176.12 °C in DSC analysis, 235.8nm λmax, Successive FTIR analysis, EDS and Microscopic imaging analysis (SEM) were performed to get satisfactory reasoning for confirmation

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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. The microorganism and the strategy of production were affected on duration of fermentation, growth rate, carbon source concentration, etc [7].

Synthetic Polymer may produce hazardous problem during formulation that produce the bioaccumulation in the body. This accumulated trace of polymer creates health related problems. PHB are 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 effective tool to control or extend the drug release rate of pharmaceutical formulation [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 Poly-β-hydroxybutyrate (PHB) has been 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, non-biodegradability, 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. Bacillus megaterium grows in minimal medium without any added growth factors. Indeed, the ability of B. megaterium to accumulate PHB is so dominant that the PHB content in the cells could reach up to 42% of the cell dry weight [11].

This work, in order to study the effect of the molasses in the medium on Bacillus megaterium 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) and the thermal properties of polyhydroxybutyrate (PHB) obtained from this fermentation were determined. PHB was effectively screened from prime PHB producer Bacillus megaterium yielded highest polymer production using cheap C and N sources for molasses and ammonium chloride respectively. The morphological tests, phisio-chemical assessment and characterizations viz; Rheology, DSC analysis, EDS and Microscopic imaging analysis (SEM) were performed to get satisfactory reasoning for confirmation of finished polymer substance.

Materials and Methods

Bacterial fermentation was carried out using Bacillus megaterium ATCC 6748. A lyophilized culture was reactivated at 30 °C for 24h in a growth medium as Nutrient agar medium containing Nutrient Agar(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 20min.

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 were collected apart from 3-4 cm deep from surface was used for isolation of the bacteria. It were 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 10min. Samples were prepared by using sterile distilled water. Soil sample supernatants were subjected to serial diluted 10⁻¹, 10⁻², 10⁻³, 10⁻⁴ and 10⁻⁵. 0.2ml of diluted soil samples were plated on nutrient agar (NA) plates [12].

Media preparation and sterilization. Composite media ingredients were taken in flask. The above media was sterilize for 121 °C, 15lb, for 15 minutes and allow cooling 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 15min and after that a desired culture transplant was used for further study. The stationary phase media plates were incubated at 30 °C for 48 hours [12]. Effect of pH on PHB production.

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

Screening of PHB producing bacterial strains: Sudan Black B Staining technique. Well isolated, morphologically distinct colonies were sub cultured on nutrient agar 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; where in 0.5μg/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 10min and the excitation wavelength of 460nm selected and excitation was observed. Glycogen and polyphosphate did not stain. Nile blue A stain appeared to be a more specific stain for poly-β-hydroxybutyrate than Sudan black B.
Volutin Granule staining technique. Every Micro-organism 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 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 BXRXA 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 Figure 1 & 2 respectively.

**Figure 1:** Nile blue staining microscopic view of Bacillus megaterium.

**Figure 2:** Volutin granule staining microscopic view.

**Morphological characterization of microbial source:** Colony characterization. The colony characters viz, Size, shape, Margin, Elevation, opacity and colour were observed on agar medium [15].

Cultivation of selected isolates and Standard PHB producer on media. Selected six isolates and the standard organism; Bacillus megaterium were inoculated separately onto nutrient agar plates and nutrient broth. The plates and broth were incubated at 30 °C for 48 hours [15].

Harvesting of cell mass. Cell mass from agar plates was scrapped with the help of spatula and that from broth 5ml of 48h at 30 °C individual grown isolate was centrifuged at 6000rpm for broth culture and 4000rpm for solid state agar medium at 10 °C; centrifugation time 15min was employed for both medium, these process was carried out in cooling centrifuge (Remi, Mumbai). Then obtained cell pellets was suspended in deionised water. The pellet of cell mass allow to air dry and after that placed in pre-weighted 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 by using two steps such as cell lysis and solvent extraction [17].

Cell lysis: The cell mass pellet were treated with Sodium hypochlorite (10mL/ 50mL culture pellets) and incubated at 30 °C for 2h. Sodium hypochlorite could not release all of the available protein, further treatment became necessary; which was subjected to homogenize by the homogenizer for 10min. 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 6000rpm for 15 min supernatant was collected and filtered through whatman No. 1 filter paper and the non-PHB cell mass (NPCM) discarded. Obtained cell mass pellet suspension treated with chloroform at 45 °C with constant shaking on rotary shaker for 24 hours. PHB from chloroform phase was recovered by non solvent precipitation method comprising (7:3v/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 colour, odour 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 Thile’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 non-polar ones. These solvents allow to keep at room temperature for 24hrs 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 by using Brookfield R/S-CPS+ Rheometer. Measurements were carried out by using plate-plate type instrument and by using C75-2 spindle at 32±2 °C. A gap of 0.5mm was kept between two plates [19]. The viscosity of PHB sample was loaded between two plates and viscosity was recorded.
Biosynthesis and Potential Screening of Butyrate Type of Polymer Obtained from Convex 2-3Mm Rod Opaque >1Mm Regular Off white Rhizoidal Circular >1Mm >1Mm Concave Soil samples were Shape Colour Opaque >1Mm Flat 1-2Mm It was Circular Pale yellow Convex Pale yellow Opaque Concave Opacity Circular Pale yellow Flat Branched Pale White Opaque Elevation Rod Light pink Regular Margin

Table 1: Colony characterization of bacterial isolates.

| Isolate Code | Colony Characteristics Parameters |
|--------------|----------------------------------|
|              | Size | Shape | Margin | Elevation | Opacity | Colour         |
| SS1          | >1Mm | Rod   | Regular | Flat     | Opaque  | Pale yellow   |
| SS2          | >1Mm | Circular | Regular | Convex   | Opaque  | Pale White    |
| SS3          | 1-2Mm | Rod     | Rhizoidal | Flat   | Opaque  | Pale yellow   |
| SS4          | >1Mm | Circular | Branched | Concave | Opaque  | Pale yellow   |
| SS5          | 2-3Mm | Concave | Regular | Convex  | Opaque  | Off white     |
| SS6          | >1Mm | Circular | Irregular | Convex  | Opaque  | Light pink    |

SS: Soil sample

Result and Discussion

Screening and characterization of biopolymer from microbial source

Primary screening of microbial source: Soil samples were collected near from 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 were reported that 19 different viable colonies are produced on Nutrient agar plate with time span of 24 hours at 30 °C.

Screening for PHB producing bacterial strains: It was found that, out of 19 isolated colonies, 6 showed morphologically distinct characteristics were flooded with Sudan black-B solution (Table 1). The obtained cell mass from plates was then confirmed by Nile blue staining technique (Figure 1) and Volutin Granule staining (Figure 2) method. These both staining technique showed positive feedback for intra-granular PHB production [13,14]. Thus, these six isolates selected as prompt producer of PHB granules before that these isolates were verified by morphological tests like colony characterization. These purified isolate were coded 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 (Table 1).
Table 2: Colony characterization of bacterial isolates.

| Isolate Code | Dry Weight of Biomass Per 100ml Media (Mg) | Agar | Broth |
|--------------|------------------------------------------|------|-------|
| SS1          | 119                                      | 73   |       |
| SS2          | 36                                       | 21   |       |
| SS3          | 52                                       | 23   |       |
| SS4          | 187                                      | 93   |       |
| SS5          | 136                                      | 88   |       |
| SS6          | 88                                       | 45   |       |
| BM           | 253                                      | 148  |       |

BM : Bacillus megaterium

Harvesting of cellular biomass. Table 2 shows the dry weight of biomass per 100ml of nutrient media of the soil isolate and the standard organism Bacillus megaterium 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 become limited as compare to stationary phase due to nutrients availability becomes critical after some time period that reason was took into account and nutrient agar medium was selected for further studies. The lowest amounts of biomass showed by soil isolate SS2 that was 36mg and 21mg in nutrient agar and nutrient broth respectively [15,16]. Soil isolate SS5 showed 136mg and 88mg in nutrient agar and nutrient broth respectively. By considered other view of production standard PHB producer Bacillus megaterium yields 250mg 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. Nutrient agar was selected as the medium for maximum PHB yield. When all the bacterial isolates were grown on nutrient agar medium they produced various amount of PHB yield [17]. The isolate SS4 was yielded least amount of PHB yield 6%w/w dry cell weight while, at the same time other isolates SS1, SS2, SS3, SS5, SS6, yielded 12, 9, 11,15,14%w/w of dry cell weight respectively (Table 3). On other hand selected standard bacterial producer Bacillus megaterium yielded 18%w/w of dry cell weight.

Table 3: PHB Yield showed by all Microbes on nutrient agar medium

| Isolate | PHB Yield/ Dry Cell Weight (% W/W) |
|---------|-----------------------------------|
| SS1     | 12                                |
| SS2     | 9                                 |
| SS3     | 11                                |
| SS4     | 6                                 |
| SS5     | 15                                |
| SS6     | 14                                |
| BM      | 18                                |

Selection of specific media composition for bacillus megaterium

Method-A: In method- A the media composition was made by using commercially available carbon source Glucose with nitrogen source Peptone. The prime importance was gained by this method-A due to universal acceptance but high economy was spent on carbon and nitrogen sources, due to that reason method - B was took into account (Table 4).

Table 4: Specific media composition contained commercial Carbon and Nitrogen Source

| Ingredients                          | Quantity (G/L) |
|--------------------------------------|----------------|
| BM1                                  | 25            |
| BM2                                  | 25            |
| BM3                                  | 25            |
| Nutrient agar                        | 15            |
| Glucose                              | 25            |
| Peptone                              | 1             |
| Beef extract                         | 2             |
| Potassium dihydrogen Phosphate       | 2             |
| Disodium hydrogen Phosphate          | 0.5           |
| Calcium chloride                     | 0.5           |
| Magnesium sulphate                   | 0.5           |
| Ferric Citrate                       | 0.5           |
| Yeast                                | 10            |
| Trace element solution               | 3mL           |

Method-B: In the method-B media composition contained Carbon and Nitrogen source Molasses and Ammonium chloride respectively both Sources was 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 compare to sources used in Method-A (Table 5).

Table 5: Specific media composition contained cheaply available carbon and nitrogen source.

| Ingredients                          | Quantity (G/L) |
|--------------------------------------|----------------|
| BM4                                  | 25            |
| BM5                                  | 25            |
| BM6                                  | 25            |
| Nutrient agar                        | 15            |
| Molasses                             | 25            |
| Ammonium Chloride                    | 3             |
| Beef extract                         | 5             |
| Potassium dihydrogen Phosphate       | 2             |
| Disodium hydrogen Phosphate          | 0.5           |

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Calcium Chloride | 0.5 | 0.5 | 0.5
Magnesium sulphate | 0.5 | 0.5 | 0.5
Ferric Citrate | 0.5 | 0.5 | 0.5
Yeast | 10 | 10 | 10
Trace element solution | 3mL | 3mL | 3mL

| Media | PHB Yield Dry Cell Weight (%W/W) |
|-------|----------------------------------|
| BM1   | 22                               |
| BM2   | 28                               |
| BM3   | 36                               |
| BM4   | 27                               |
| BM5   | 32                               |
| BM6   | 41                               |

The specific media for Bacillus megaterium required for optimum production of PHB were attained from literature. So yield of PHB in both methods was compared with each other (Table 4-6) it was found that standard Bacillus megaterium strains grown on BM specific medium they produced various extent of PHB yield. The least PHB yield showed by BM1 that was found to be 22%w/w dry cell weight which consisted 1.5% of glucose and 0.1% of peptone concentration. While maximum PHB yield showed by BM3 that was found to be 36%w/w dry cell weight at 3.5% glucose and 0.3% of peptone in media composition contained commercial carbon and nitrogen Source [17]. At same time PHB yield produced by media composition contained cheaply available carbon and nitrogen source. The yield of BM6 was found to be 27%w/w dry cell weight obtained from media containing 1.5% of molasses and 0.1% of ammonium chloride that was least yield, while 41%w/w dry cell weight of maximum yield obtained from BM6 that was found to be maximum yield as compare to yield obtained from all other media compositions. So BM6 was selected as potential PHB producer on large scale production.

Characterization of PGB Biopolymer

Physiochemical characterization

The PHB biopolymer samples were evaluated for its organoleptic properties [18]. The biopolymer are white in colour, Crystalline, stiff and brittle plastic in nature, as well as odourless and melting point is in the range of 175-177 °C.

Solubility determination

Table 7: Solvent system used in PHB solubility determination

| Solubility   | Solvent          |
|--------------|------------------|
| Highly Soluble | Chloroform       |
|               | Dichloromethane  |
|               | Acetic anhydride |

Solubility of PHB was confined or easy to soluble in different type of polar to non polar solvents [18] (Table 7).

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 in to 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 characterisation of PHB was performed using Brookfield R/S-CPS+ Remoter.

Measurements were made by plate assembly. It was shown that viscosity of the PHB was measured as a function 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 [19,20]. 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; over all temperature of environ was maintained and measurement were carried out. Viscosity measurement of PHB shown in Figure 3 and the assignments were represented in Table 8.
Table 8: Rheological behavior of PHB biopolymer solution.

| Parameter      | Minimum | Maximum | Average |
|----------------|---------|---------|---------|
| Viscosity (Pas)| 0.4303  | 2.2936  | 0.7993  |
| Shear Stress (Pa)| 22.9754 | 53.1547 | 32.2269 |
| Shear Rate (1/s) | 10.017  | 100.107 | 55.0323 |

**Spectral characterization:** Ultraviolet (UV) spectrum. Ultraviolet spectroscopy is very useful and feasible analytical technique to assaying the variety of compounds. In this technique quantitative conversion of poly-β-hydroxybutyric acid to crotonic acid by heating in concentrated sulphuric acid and to found out scanning range of PHB (Law and Sleekly method). Sulphuric acid containing PHB solution was kept as test and read with sulphuric acid as blank in the range of wavelength from 200-400nm using UV spectrophotometer. The reported absorbance maxima (λmax) value for PHB is 235nm [21,22]. Ultraviolet absorption maxima were recorded and λmax was found to be at 235.8nm. So, it was concluded that the obtained λmax value much closer to report value that conforms the primary authentication of PHB polymer (Figure 4).

Figure 4: UV-Visible scanning spectra of PHB biopolymer solution.

**Fourier transforms infrared (FT-IR) spectroscopy**

FTIR spectra of the screened PHB polymer are shown in Figure 5. PHB show peaks at 1720.56cm-1, 1279.46cm-1 and 2982.87cm-1 corresponding to specific rotations around carbon atoms specific to certain functional groups. The peak at 1720.29cm-1 corresponds to C=O stretch of the ester group present in the molecular chain of highly ordered crystalline structure. The peak at 1279.46cm-1 corresponds to -CH group modes of the crystalline parts. The Peak at 2982.87cm-1 corresponds to presence C-O-C stretching. Usually presence of absorption bands above 3000cm-1 are connected with possibility of a C-H-O hydrogen bond but these are very negligibly present in FTIR. The weak shoulders positioned at 2982cm-1 could be evidence for presence of C-H stretching. The weak band arisen at 3659 is attributed to its hydroxyl end groups [22-24].

Energy dispersive X-ray spectrometry (EDS)/ elemental analysis

In EDS Multi-Point Analysis provides integrated imaging with automated analysis including line scan and multi-point analysis features (Figure 6), whether the quantification involves the use of a standard less analysis, the peak intensities were calculated apart from the background (EDS with SEM); the peak intensities were compared to intensities of the pure elements to calculate the k-ratio; The ratio of the element’s peak height in a sample to the pure element collected under the same conditions was actually used in the quantitative calculation as the k-ratio. Corrections were made for atomic number (Z), absorption (A) and fluorescence (F) and values for above parameters are shown in Table 9. The ZAF corrections assume that the sample/detector geometry is well known, that the sample is smooth, and that it is homogeneous [25-28].

**Table 9: EDS Multi-Component Quantification for Four PHB crystals.**

| Polymer Crystal | Wt % | At % | K-Ratio | Z | A | F |
|-----------------|------|------|---------|---|---|---|
| CK              | 53.14| 69.87| 0.0763  | 1.0359| 0.1386| 1.0001|
| OK              | 11.13| 11.13| 0.0181  | 1.0186| 0.1571| 1.0004|
| NaK             | 12.14| 8.34 | 0.0589  | 0.9535| 0.5079| 1.0014|
| MgK             | 0.89 | 0.58 | 0.0046  | 0.9775| 0.5443| 1.0024|
| PK              | 0.62 | 0.31 | 0.0052  | 0.9388| 0.8822| 1.0166|
EDS is the qualitative and quantitative method of analysis. In this technique the Scanning Electron Microscopic (SEM) imaging analysis was carried out to obtain the image. The refocused images with objects were highlighted by selecting individual peak ID. In which qualitative and quantitative analysis was carried out followed by the obtained peaks. These peaks represent the series of elements presents with quantitative figures of separated element in individual object. This technique offers the analytical measurement parameters such as detected element, At%, Wt%, K ratio, Z, A and F By observing the results values The results PHB crystals comprised varied concentration of C, O, P, K, Cl, Na and Mg elements. The actual weight % was calculated by multiplying the k-ratio by 100% and dividing by the product of the Z, A and F factors. The obtained values for the entire individual particle were not same because the measured particles were not with same dimension. So obtained results are represents qualitative and quantitative analysis by showing separated peaks with varied concentration of elements [29]. The standard less results will always be normalized to 100%. The peak for detected element concentration depicted in Figure 7-10. The obtained results for A t% and Wt% for PHB crystal 1, 2, 3, and 4 from EDS are illustrated in Figure 11-14 respectively.

| Element | Wt % | At % | K-Ratio | Z | A | F |
|---------|------|------|---------|---|---|---|
| Polymer Crystal 2 | | | | | | |
| CIK | 21.94 | 9.77 | 0.196 | 0.9167 | 0.9748 | 1 |
| Total | 100 | 100 | | | | |
| Element | Wt % | At % | K-Ratio | Z | A | F |
|---------|------|------|---------|---|---|---|
| Polymer Crystal 3 | | | | | | |
| CIK | 33.93 | 45.82 | 0.0643 | 1.0304 | 0.1838 | 1.0003 |
| Total | 100 | 100 | | | | |
| Element | Wt % | At % | K-Ratio | Z | A | F |
|---------|------|------|---------|---|---|---|
| Polymer Crystal 4 | | | | | | |
| CIK | 24.18 | 41.86 | 0.021 | 1.0707 | 0.0809 | 1.0001 |
| Total | 100 | 100 | | | | |
| Element | Wt % | At % | K-Ratio | Z | A | F |
|---------|------|------|---------|---|---|---|
| CIK | 21.94 | 9.77 | 0.196 | 0.9167 | 0.9748 | 1 |
| Total | 100 | 100 | | | | |

Figure 7: EDS spectrum of PHB crystal-1.

Figure 8: EDS spectrum of PHB crystal-2.
Thermal characterization

Differential scanning calorimetric: Differential scanning calorimetric 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. This PHB polymer was composed of single endothermic components with peak temperatures about 176.12 °C. The formation of melting endothermic peak generally narrow can be due to the presence of crystalline structures [29]. PHB aroused sharp peak at 176.12 °C that analogous to the observed melting point temperature range (175-177 °C) of PHB by capillary method (Figure 15).

Microscopic imaging analysis

6.7.1. Scanning electron microscopy: The sample was analyzed in QUANTA 200 Scanning Electron Microscope under 100x, 500x, 1000x, 2000x, 5000x and 10000x magnification. SEM was illustrated with a high degree of magnification; highlighted surface features of screened PHB polymer. PHB enabled with crystalline, brittle surface morphology. PHB at 100x magnifications showed that spiky topographic arrangement. PHB magnified at 500x illustrated hard stone like surface structural arrangement and at 1000x magnification showed speckled particulate arrangement. PHB revealed irregular surface area with crest and trough at 2000x magnifications. Polymorphic granular matrix witheld at surface was shown in 5000x magnifications. PHP at 10000x showed that saturated dense globular micro particles at the surface. So it has revels that surface morphology of PHB distinctly discerns the polymer surface between the continuous spiky topographic arrangements of the major phase [29]. 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 packaging industry and pharmacy formulation practice. SEM micrographs at varied degree of magnifications are illustrated in Figure 16.

**Figure 16:** Scanning Electron Microscopic Imaging Analysis of PHB magnified at (a) 100x, (b) 500x, (C) 1000x, (d) 2000x, (e) 5000, (f) 10000x magnification.

**Conclusion**

The current study was based on screening of cost effective screening of Polyhydroxybutyrate (PHB) from bacterial genera biopolymer which would satisfy the growing demand of sustainable economic biopolymer production from bacterial genera. The cost effective polymer production with high yield and purity was the prime goal of study, so the soil isolates SS1, SS2, SS3, SS4, SS5, and SS6 were screened from soil samples. The solid state agar medium and nutrient broth were used as substrate for PHB production. Soil isolates had found to be ability to yield varying amount of PHB production, but polymer yielded by this isolates were lesser in quantity. Due to this reason Bacillus megaterium was used as prime PHB producer to yield maximum PHB production. But it was observed that polymer yield obtained from solid state substrate was higher as compare to the nutrient agar medium. The individual analysis viz; Physicochemical characterization Eg (Colour, odour, appearance melting point determination), Rheological characterization Eg (Viscosity), Spectral characterization Eg (FTIR, UV spectrum, EDS), Thermal characterization Eg (DSC), Microscopic Imaging Analysis Eg (SEM) stands for giving assurance for its purity, conformity by explaining result value.

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