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Production of Bio-Plastics (Polyhydroxy Butyrate) from Industrial Effluent using Batch and Two Stage Batch Culture Studies

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Abstract

The present study focuses on the improvement of production of Poly Hydroxy Butyrate (PHB) using *Bacillus megaterium* MTCC 8075. The culture media and conditions were studied critically and was found that the maximum PHB production was attained in the presence of glucose as carbon source at 6% concentration with a yield of 55.0 mg/g cell weight and peptone as nitrogen source with a yield of 17.2mg/g cell weight. The optimum pH was found to be 7(22.8mg/g) and it was also investigated that PHB production was maximum corresponding to a temperature of 35°C (35.46mg/g) and at an incubation time of 72 hours (52.2mg/g). PHB was qualitatively analysed using Sudan Black staining method, extracted using sodium hypochlorite, and quantified using crotonic acid assay in UV spectrophotometer at a wavelength of 235nm. PHB production was carried out in various selected effluents like corn stalk waste and petroleum refinery effluent and corn corb yielded the maximum PHB production of 3.1mg/L. The produced PHB was confirmed using Fourier Transform Infrared (FTIR) analysis and NMR spectroscopic studies. PHB production was further improved using two-stage batch culture studies in which PHB production was compared in various nutrient limited conditions of nitrogen and phosphorous. The thermal properties of PHB was studied using Differential Scanning Calorimetry(DSC) and Thermogravimetric Analysis (TGA).

Keywords: Sudan Black, *Bacillus megaterium*, Crotonic Acid, Sodium Hypochlorite, FTIR, NMR, TGA, DSC

1. Introduction

Plastic was first coined in the history by Alexander Parks in the year 1862. The present human generation was incredibly dependent on plastics for their daily life. (Sharmila et al. 2009) But since are highly nondegradable in nature, the concept of biodegradable plastic came into existence which will be very well degraded by microorganism using sunlight, moisture, oxygen etc (Abe and Doi 2002) Polyhydroxy Alkanoates (PHA) is an intracellular microbial thermoplastic which has received much attention due to its biodegradable properties and biocompatibility. (Doi.Y. Steinbuchel et al. 2002). They are accumulated intracellularly as carbon and energy reserves under nutrient limited conditions[1–3].

The polymer family is made up of 2 major groups-aliphatic and aromatic. PHAs are aliphatic polymer naturally produced in a sugary medium like glucose or any other carbon source. The main members of PHA are Poly Hydroxy Butyrate (PHB) and Poly Hydroxy Valerate (PHV). PHB is the copolymer of 3-hydroxybutyrate which is accumulated under nutrient starving condition. It is a highly crystalline polymer with relatively high melting temperature and a glass transition temperature very close to the ambient conditions. It shows a chemical structure similar to conventional plastics Isopolypropylene (IPP). The main advantage of PHB is that it is found to be highly resistant to hydrolytic degradation[4].

PHB is largely produced in a variety of micro-organisms. About 75 genera of bacteria is found to produce

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PHB. Gram positive bacteria is found to be the better producer of PHB because the outer membrane present in gram negative bacteria (Cupriavidas necator, Alcaligenes latus) contains LPS endotoxins that may induce a strong immunogenic reaction which limits its biomedical application. On the other, Gram positive bacteria lack LPS and they secrete protein at higher concentration and the potential use of cheaper raw materials made it a better source of PHA (Choi et al. 2005). PHB was first reported in Bacillus megaterium by Leimogne. A number of micro-organism like Alcaligenes eutrophus, Azotobacter beijerinckia, Pseudomonas oleovorans, etc produces PHB extensively. Apart from bacteria, PHB is also found to be produced from green plants (Kannan et al. 1970), algae, fungi, yeast etc[5]. Among bacteria, photosynthetic bacteria, gliding bacteria, actinomycetes, cyanobacteria, even recombinant E.coli were used as a PHB producer. Among the studied microorganisms, Bacillus sp proved to be one of the most widely used PHB producers. Bhairavighate et al.[2] showed that Bacillus megaterium was able to produce 60-70% PHB of the total cell weight. Valappil et al. identified and characterized Bacillus cereus that produces large amount of PHB[6].

Under the normal growth conditions, nutrient sources are used for the synthesis of protein essential for the growth in bacteria. The Nitrogen source depletion leads to the cessation of protein synthesis which in turn leads to the inhibition of TCA cycle enzymes (citrate synthase and isocitrate dehydrogenase) and consequently slows down the TCA cycle. As a result, the acetyl coA routes to PHB synthesis. (Krithika et al. 2011). The main limitation in the commercial production of PHB as biodegradable plastic is its high production costs compared to the production of petrochemical based synthetic plastics. The situation can be compromised by replacing the carbon sources (glucose, sucrose etc) which constitutes about half of the total investment costs with cheaper inexpensive substrates like agro-residues (Kim et al. 2000, Monate 2001), sugar industry effluent, biodiesel, activated sludge from anaerobic digestor etc. (Apostotes et al. 2006).

The present study focuses on the production of PHB in Bacillus megaterium MTCC 8075. This work encompasses the optimization of media condition for the maximization of PHB synthesis and hence to utilize this optimized condition for the production in the various effluents which is chosen as the sole carbon source[8]. It also includes the comparative study of PHB production using single stage and two stage fermentation strategies. The mechanical and thermal properties are also extensively studied.

2. Materials and Methods

2.1 Microorganisms and Culture Conditions

Bacillus megaterium MTCC 8075 was obtained from Microbial Type Culture Collection (MTCC) and grown in production medium containing 1g Beef extract, 2g yeast extract, 5g peptone, 5g NaCl. The media is incubated at 37°C at 120rpm for 48 hours. Nutrient agar slants were prepared and the culture was stored at 4°C for further use[9–13].

2.2 Qualitative Analysis of PHB: Microbial Staining

PHB was identified using the Sudan Black staining procedure. Sudan Black B is a basic dye and will combine with acidic group in compound lipids thus staining phospholipids also.

Sudan black solution is prepared by dissolving 0.03g sudan black powder in 7.5mL ethanol and made up to 10mL by adding distilled water. The culture sample was heat fixed and stained with sudan black stain for 1 min. The excess stain was washed off and after drying, it was counter stained with safranin solution for 5 seconds and observed under optical microscope[14].

3. Media Optimization using Various Parameters like Incubation Time, Ph, Carbon Source and Nitrogen Source

About 2ml inoculums of Bacillus megaterium MTCC 8075 was added to 250 mL conical flask containing 100mL of sterilized media for PHB production and incubated in a rotary shaker at 120 rpm. Samples were collected at different time intervals 0, 24, 48, 72, 96 (0-96 hours at an interval of 24 hours) to find out the effect of incubation time on PHB production[15]. After the definite time of interval, PHB was extracted from the culture and quantified to find out the optimum incubation time that gives maximum PHB production.

Different pH levels (4.0-8.0) were maintained in the production medium to check pH changes have noticeable effect on PHB production. Initial pH of the medium was adjusted by either 1N HCl or NaOH[16].

Four carbon sources were selected (glucose, sucrose, lactose, maltose) to find out the effect of various carbon sources on PHB production. The strain Bacillus megaterium was inoculated in the production medium containing 5%(w/v) of these sugars for an incubation period of 72 hours at 35°C.

The effect of various concentration levels of carbon source (glucose) on PHB yield was studied by varying the glucose concentration (1-6%) in each shake flask, inoculated with Bacillus megaterium and incubated at 150 rpm for a period of 72 hours followed by extraction and quantification of PHB[17].

In order to investigate the effect of Nitrogen sources on the PHB production, Bacillus megaterium was grown in the production medium containing different nitrogen sources viz peptone, ammonium sulphate, urea, glycine, nitrates of sodium, potassium, ammonium. After incubation, the PHB was extracted and quantified from the culture broth[18].

3.1 Comparison of PHB Yield in Various Effluents

Petroleum refinery effluent which was obtained from (CPCL) Chennai Petroleum Corporation Limited Chennai and corn corb were used as the carbon source for PHB production. Defined quantity of effluent was sterilized and inoculated using Bacillus megaterium MTCC8075 After the incubation time of 48 hours, PHB is extracted and quantified using the under defined procedure[19]. The PHB produced were used for further confirmation studies hence to compare the yield obtained from that of both the effluents.

4. Quantitative Analysis of PHB

4.1 Extraction and Quantification of PHB

PHB extraction was carried out by sodium hypochlorite-chloroform method. After 48 hours of incubation at 37°C, 10ml of the culture was taken and centrifuged at 8000rpm for 15min. The supernatant was discarded and the pellet was treated with 5 mL of 6% Sodium hypochlorite solution (Loba) and the mixture was incubated at 30°C for 2 hours. After incubation, the mixture was centrifuged at 5000rpm for 15 min and then washed with distilled water, acetone, methanol respectively for washing and extraction. After washing, the pellets were dissolved in 5mL boiling chloroform (Loba) and then the chloroform was evaporated by pouring the solution on sterile glass tray and kept at 4°C[20]. After evaporation, the powder was collected for further analysis (Senthil Kumar et al. 2006).

Crotonic acid assay was the most prominent and feasible quantification technique for the production of PHB. The principle behind this assay is that the PHB crystals undergo degradation on treatment with sulphuric acid. The extracted PHB was converted to crotonic acid by adding 98%sulphuric acid and heated to 60°C for 1 hour. Crotonic acid shows maximum absorption at 235nm.The absorbance of the solution as measured at 235nm in a UV Spectrophotometer (Hitachi, USA) (Slepecky R.A. and law J.H)[21].

4.2 Confirmation Studies using NMR Analysis

The PHB produced from the industrial effluent was confirmed using NMR spectroscopy (300 MHz H-NMR). NMR spectroscopy is used to identify the carbon hydrogen framework of an organic compound. It is a non destructive, rapid analysis technique which can be done in a short duration of time[22].

5. Production of PHB using Two-Stage Batch Culture Studies

According to Doi (1990), Alcaligenes latus could store PHA up to 80% under normal growth condition. Therefore, one-step PHA production process could be used with this organism (Hrabak,1992). Literature studies reveal that two stage fed batch culture is the most widely used technique to maximize high concentrations of both cell and PHB. In first stage of growth phase, optimum nutritional conditions were provided to develop a high biomass concentration. Then, a selected nutrient was limited to stimulate PHA production in the second stage or accumulation phase. Thus second stage fermentation proved to be a better tool to improve PHB production.

5.1 Preparation of Standard Inoculums for First Stage Fermentation

Standard inoculum was prepared by inoculation of 250 ml conical flasks containing 100 ml of nutrient broth medium (medium composition given in the appendix) with a loop of tested Bacillus megaterium MTCC 8075. The inoculated flasks were incubated on rotary shaker (150 rpm) for 24 hours at 30°C. The content of these flasks were used as standard inoculums for further shake flasks experiments[23].

5.2 Second Stage Fermentation

Samples (10 ml) after 24 h were taken from the grown cultures to estimate the cell dry weight and polymer content. The remaining content of these flasks (90 ml) was centrifuged and used as crude cells or washed cells to be inoculated the second-step flasks which containing 100 ml productive media (media composition of which changes with flasks given in the appendix). Different nutrient limitation conditions were applied by varying the concentration of nitrogen source (peptone) or/and phosphorus source (Potassium di Hydrogen Orthophosphate KH_2PO_4) into the subsequent experiments as follows- (Azhar A. El-sayed et al., 2009)

Normal concentration is measured as such given in the media composition and low concentration is taken by measuring 10% of the normal concentration. All the 8 flasks are kept for incubation of 72 hours and the sample (10mL) of the culture is used for estimating the polymer content hence to obtain the optimum nitrogen and phosphorous concentration.

6. Results and Discussion

6.1 Identification of PHB

From the Figure 1 the inclusion bodies were visualized as black intracellular granules.

It was observed that those cells unable to absorb the sudan black B stain appeared pink (because of safranine counter staining), while those cells which produces PHB appeared bluish black. In the Figure 1 PHB granules are seen in bluish black against the pink coloured safranine stained cells.

6.2 Media Optimization

Age of the culture plays an important role in the PHB production because PHB might be utilized by the organism itself in the later phase of the growth. Hence, the comparison of PHB yield at various time intervals was indeed essential.

The maximum yield of PHB was obtained at 72 h after that there was a sharp decrease in the yield (Figure 2). The decrease in PHB accumulation observed after 72h is attributed due to the intracellular consumption of PHB as energy and carbon source. As reported in the literature, PHB production as all other secondary metabolite was high during the stationery phase of growth. Once the organism reached its death/decline phase, the production ceases as the organism growth rate decreases.

It has been shown in the graph that maximum PHB production was obtained for a pH of 7 and as acidity and basicity increases on either side, the production decreases.

Table 1.

Experiment trial no	Nitrogen concentration	Phosphorous concentration
1	Nil	Normal
2	Normal	Nil
3	Low	Normal
4	Normal	Low
5	Low	Low
6	Nil	Nil
7	Low	Nil
8	Nil	Low

This may be due to the adverse culture condition to the bacteria for its survival. Senthil Kumar et al. (2006).

Result showed that the PHB yield was the highest when glucose was used as carbon source, followed by sucrose and lactose, and maltose was the least (Figure 3). Glucose was the preferred carbon source and was taken for other studies. The positive effect of glucose on PHB production could be attributed due to the increased supply of the reduced cofactor, i.e., NADPH which in turn leads to the inhibition of TCA cycle enzymes. The PHB production of Alcaligenes eutrophus was more in the medium using 1% glucose as carbon source than in the medium using 1% maltose or sucrose as reported by Azhar et al., 2009

It was found that as the glucose concentration increases, PHB production was also increased. At 6% concentration, PHB yield was the highest since maximal utilization of carbon source was observed in higher concentration

It was found out that among all the nitrogen source selected, peptone was the most effective nitrogen source. This might be due to the reason that Peptone positively influenced growth and PHB production as it is needed by micro-organisms to synthesize all the enzymes that are directly involved and to induce metabolic processes in cell (Choi et al., 1999).

6.3 Comparison of PHB Yield in Various Industrial Effluent

Figure showed the comparison of PHB production on various industrial carbon source like petroleum refinery effluent, corn corb. Maximum PHB production was obtained for dairy effluent used as the sole carbon source and least production was obtained for refinery effluent. This may be due to the reason that dairy effluent contains more complex nutrient source when compared to corn waste.

6.4 Confirmation Analysis: NMR

The results of NMR studies revealed the following data which confirms that the obtained sample is PHB on comparing the peaks with that of standard. Nuclear Magnetic Resonance is a rapid and successful method for detection and quantification of PHB.

7. Conclusion

The optimal culture and media conditions for maximum production of PHB by B. megaterium was studied critically and it was inferred that out of the several carbon and nitrogen sources chosen for PHB production in B. megaterium, the most preferred carbon source and nitrogen source were found to be glucose at a concentration of 6% and peptone respectively. Also the optimum pH for PHB production was found to be 7 and the maximum production was obtained at a dilution of 50% of the raw dairy effluent. PHB yield was found to be the highest at an incubation time of 72 hours. Sudan Black staining method showed positive results for PHB. PHB was quantified using crotonic acid assay. PHB was extracted using Sodium hypochlorite method and stored for future use. The confirmation analysis of PHB is done using NMR, FTIR analysis. Also the thermal properties of PHB was extensively studied using DSC and TGA. Also the second stage fermentation using corn starch as carbon source and optimization of nitrogen, phosphorous source reveals the increased productivity of PHB.

PHB may find possible application as a polymer for tissue engineering processes and biodegradable polyester. Further studies on its culture process are in progress in order to enhance the PHB production and realise its commercial utilisation.

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