Microbial polyhydroxybutyrate production by using cheap raw materials as substrates

G. Mahitha* and R. Jaya Madhuri

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ABSTRACT
Bioplastic, Polyhydroxybutyrate (PHB) is well known for its environmental friendliness and complete decomposition into water and carbon dioxide by microorganisms. The main drawback of PHB commercialization is its high production cost which is 10 times higher than that of synthetic plastic. So, the present research work mainly focussed on the fermentative production of PHB by *Bacillus amyloliquifaciens* and *Nocardiopsis potens* using low cost raw materials like Molasses, wheat bran, rice bran, ragi bran, jambul seed powder, orange peel and whey as substrates. *Bacillus amyloliquifaciens* and *Nocardiopsis potens* gives maximum PHB 16.5 µg/ml and 26.8 µg/ml respectively in the medium containing molasses and wheat bran as a substrates. Further the functional groups of extracted PHB were confirmed by Fourier transform infrared spectroscopy.

Introduction

The low cost, stability, durability, good mechanical and thermal properties of plastic make it the best choice for wide spread applications and worldwide problem [1] because they are non-degradable. Decades have been invested on extensive research to develop bioplastic like Polyhydroxybutyrate as a substitute for recalcitrant petrochemical plastic. It is used for packaging materials, bags, containers, disposable cups, diapers and also in surgical materials [2]. Polyhydroxybutyrate is biologically synthesized polyester produced by many prokaryotes and also degraded by bacteria. Polyhydroxybutyrate is an intracellular lipid reserve granules accumulated in many bacteria when cell’s surroundings contain unbalanced growth condition [3]. The optimal conditions for PHB production by microorganisms usually include high concentration of carbon source and limited concentration of N, P, S or trace elements like Mg, Ca, Fe. During the normal growth conditions, nutrients are used for the synthesis of proteins which are essential for the growth of bacteria. The nitrogen source depletion leads to the cessation of protein synthesis which in turn inhibits citrate synthase and isocitrate dehydrogenase enzymes and consequently slows down TCA cycle. As a result acetyl-CoA routes to Polyhydroxybutyrate synthesis [4]. The PHB production by fermentation, the substrate and recovery costs are high, making their use unattractive. Thus, the use of waste materials can substantially reduce substrate cost and also downsize the production costs. Hence, the present research work has been aimed to use waste residues as a substrates for the fermentative production of PHB from marine microorganisms.

Methodology

Micro organisms used for PHB production

Polyhydroxybutyrate accumulating bacteria were isolated from marine water collected from the coastal area of Bay of Bengal and screened by Sudan Black B staining method [5]. The positive isolates KMM1 and CMM3 were subjected to 16s rRNA sequencing and the resulting nucleotide sequences are submitted to Genbank for the allotment of accession numbers.

Inoculum preparation

The flasks containing 50 ml of marine broth were inoculated with one loop full of potent isolates culture and incubated at 37° C for 24 hours. 1ml of inoculum was added to the 100ml of media in the fermentation process.

Preparation of fermentation media
The PHB producing strains, selected by Sudan Black B staining method, were quantified for the PHB production in Nitrogen limited (N) condition. The N-limited media [6] consists of (NH$_4$)$_2$SO$_4$ - 2g/L; KH$_2$PO$_4$ - 2 g/L; MgSO$_4$.7H$_2$O- 0.2 g/L; Na$_2$HPO$_4$ - 0.6 g/L and Yeast Extract - 0.2 g/L. The composition of trace element solution were FeSO$_4$.7H$_2$O - 10 g/L; ZnSO$_4$.7H$_2$O - 2.25 g/L; CuSO$_4$.5H$_2$O - 1 g/L; MnSO$_4$.5H$_2$O - 0.5 g/L; CaCl$_2$.2H$_2$O - 0.2 g/L; Na$_2$B$_4$O$_7$.7H$_2$O - 0.23 g/L; (NH$_4$)$_6$Mo$_7$O$_{24}$ - 0.1g/L and 35% HCl - 10ml/L.

**Substrates**

The N-limited media supplemented with 1% of the following cheap substrates Orange peel, molasses, wheat bran, rice bran, jambul seed powder, whey and ragi bran as a carbon source [4] and incubated in an orbital shaker at 120 rpm by maintaining their optimal conditions like pH and Temperature which are determined in the previous study [7].

**Purification and Determination PHB**

After 48hrs of incubation 10 ml of fermented broth was centrifuged at 6000 rpm for 15 min. Then, the pellets were suspended in 5 ml of sterile water and dried for 24 hrs at 100°C. To cell suspension, 5 ml of sodium hypochlorite solution was added and incubated at 60°C for 1 hour [8] and centrifuged at 6000 rpm for 15 min and the supernatant was separated. To extract cell lipids and other molecules (except PHB) from supernatant, added 5 ml of 96% (1:1 v/v) ethanol and acetone. Now, 10 ml of chloroform was added to the tube by placing it in hot water bath (60°C). Chloroform was evaporated to obtain PHB crystals. Ten ml of 98% H$_2$SO$_4$ was added at 60°C and kept for 1 hr to convert PHB crystals into crotonic acid. After cooling to 25°C, the amount of PHB was determined spectrophotometrically at 235 nm against H$_2$SO$_4$ as blank with crotonic acid as standard [9].

**FTIR Analysis**

The polymer extracted from the fermentation medium was analyzed qualitatively by Fourier transform infrared spectroscopy (FTIR) to know the presence of different functional groups. 1mg of standard PHB (sigma) and extracted PHB dissolved in 5 ml of chloroform. Chloroform was evaporated and KBr pellet was prepared with the resulting PHB [10]. Spectra were recorded in 4000 cm$^{-1}$ to 400 cm$^{-1}$ range.

**Results and Discussion**

Bioplastics are produced and also easily disposed into water and carbon dioxide [11] without causing any harm to the environment by micro organisms present in various types of environments such as soil, sea, lake water and also in sewage water. Two bacterial marine isolates KMM1 and CMM3 showed PHB granules on Sudan Black B staining method (Fig 1&2). KMM1 has 99% homology with *Bacillus amyloliquifaciens* and CMM3 has 97% homology with *Nocardiopsis potens* on 16s rRNA sequencing.

![Fig 1: PHB granules of *Bacillus amyloliquifaciens*](image1)

![Fig 2: PHB granules of *Nocardiopsis potens*](image2)

KM091730 and KU324482 are the accession numbers allotted by Genbank for the *Bacillus amyloliquifaciens* and *Nocardiopsis potens* respectively and the resulting phylogenetic trees (Fig 3&4) are given below.
Good biodegradability and biocompatibility of PHB have attracted much interest in their use but the production cost is too high when compared to that of conventional plastic. Mainly the cost of carbon feedstocks used in the fermentative production can significantly affect polyhydroxybutyrate production cost [8]. Raw materials like Molasses, wheat bran, rice bran, ragi bran, jambul seed powder, orange peel and whey are used in this study as a carbon substrates for the PHB fermentative production. The amount of PHB was estimated by sodium hypochlorite assay method. *Bacillus amyloliquifaciens* had maximum PHB production (16.5 µg/ml) in the medium containing molasses as a substrate (Fig 5). *Nocardiopsis potens* gives maximum PHB production (26.8 µg/ml) in the medium containing wheat bran as a substrate (Fig 6).

Hydrolysis of fruit wastes and other raw materials is essential for the release of simple sugars which are easily metabolized by bacteria [12]. In this study, *Bacillus amyloliquifaciens* and *Nocardiopsis potens* utilize such wastes directly for bioconversion into PHB because they produce extracellular amylase enzyme.
The extracted PHB polymer from the *Bacillus amyloliquifaciens* and *Nocardiopsis potens* were characterized by FTIR for the confirmation of functional groups and it was shown in figures 7, 8 and 9. The band found at 1639 cm\(^{-1}\) and 1611 cm\(^{-1}\) corresponds to thioester (C=O) bond which is similar to the report of Shah (2012) [13] while the one found at 1413 cm\(^{-1}\) and 1411 cm\(^{-1}\) is CH\(_3\) asymmetric deformation and the peak at 965 cm\(^{-1}\) corresponds to C-O-C bond. The peaks at 2921 cm\(^{-1}\) and 2920 cm\(^{-1}\) belongs to (CH, CH\(_2\), CH\(_3\)) and the peaks around 3275 cm\(^{-1}\) and 3287 cm\(^{-1}\) corresponds to terminal –OH group [14-15].
**Conclusion**

PHB producing marine isolates *Bacillus amyloliquifaciens* and *Nocardiopsis potens* utilized the raw materials as a carbon source effectively without any pre treatment. The polymer extracted from the fermentation medium was analysed...
qualitatively by FTIR along with standard PHB (sigma) for the confirmation of functional groups.

Conflict of interest
The authors declare that there is no conflict of interest regarding the publication of this paper.

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