Enhancement of Polymeric Poly-(\(\beta\))-Hydroxy Butyrate (PHB) production from \textit{Alcaligenes faecalis} through the Optimisation process

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**Article History:**
Received on: 15 Oct 2020
Revised on: 15 Nov 2020
Accepted on: 17 Nov 2020

**Keywords:**
Biopolymer, Polyhydroxybutyrate, Polyhydroxyalkanoates, FTIR, HPLC

**ABSTRACT**
Polyhydroxybutyrates (PHBs) are biodegradable polymers synthesised and stored as cytoplasmic inclusions in various bacteria. They have a wide variety of applications in various fields such as Biomedical, food, agriculture, and pharmaceutical industries and used as a vehicle for controlled-release drug delivery system. The PHB producing microorganisms were isolated from the dump soil, screened by fluorescence microscope at 490 nm and characterised by 16sRNA sequencing. Process parameters optimisation is performed for maximum PHB production by changing the parameters, viz., temperature, pH, different carbon, and nitrogen source. The isolate showed maximum PHB accumulation in the concentration of 0.07 mg/mL after 72 hours incubation at 35°C and in pH 7 showed the maximum concentration of 0.055 mg/mL. FT-IR characterised PHB shows the bands at 3426 cm\(^{-1}\) are due to the presence of C–H methylene and methyl groups and retention time of the peak at 12.39 min was determined HPLC. D-Glucose was found to be the best carbon source for the maximum production of PHB in the concentration of 0.0319 mg/mL and the media supplemented with peptone as the nitrogen source showed the 0.0723 mg/mL is the maximum accumulation of PHB in the cells; thus, the isolate shows the potential of PHB production for further exploitation.

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ISSN: 0975-7538
DOI: [https://doi.org/10.26452/ijrps.v11i4.3933](https://doi.org/10.26452/ijrps.v11i4.3933)

**INTRODUCTION**
Polyhydroxyalkanoates (PHA) are natural polyesters produced by a large number of Gram-positive and Gram-negative bacteria. They are accumulated intracellular during nutrient-stress conditions and function as energy and carbon reserves inside the cell (Verlinden \textit{et al.}, 2007). PHAs have long since been identified as potential bio-plastics since they are carbon-based polymers derived from microorganisms. Among Polyhydroxyalkanoates, Poly-3-hydroxy butyrate (PHB), made of hydroxybutyric acid monomers, are the most common and extensively used (Fukui \textit{et al.}, 1997).

Limited nitrogen and excess of carbon sources lead to the accumulation of the PHB in the prokaryotic cells (Schubert \textit{et al.}, 1988). The absence of nitrogen enhances the biosynthesis of PHB as it decreases the synthesis of amino acid but increases the acetyl CoA synthesis, which helps to activate the PHA synthase enzyme (Asada \textit{et al.}, 1999).

PHB is derived from natural renewable sources and
100% biodegradable and is; hence it is an ideal bioplastic to combat the adverse effects of synthetic plastic usage (Wei et al., 2011).

PHB differentiates itself from other biodegradable plastics, as it possesses unique properties such as highly resistant to hydrolytic degradation, UV resistance, oxygen permeability, and water insolubility when compared to the other biodegradable plastics. They are biocompatible and are suitable for medical applications. On the other hand, PHB has low resistance to acids and bases and is soluble in chloroform and other chlorinated hydrocarbons. They are entirely degradable by environmental microbes and gives carbon dioxide and water upon degradation (Kusaka et al., 1997). The usage of PHB can effectively reduce the pollution caused by greenhouse gas emissions from plastic wastes (Saito and Doi, 1994).

PHB has found a wide range of applications in various areas of biomedical, pharmaceuticals, and agriculture-related applications, such as surgical sutures, supports of tissue cultures for implants, implants for surgeries, surgical meshes, wound dressing, replanted veins, and as a vehicle for controlled-release drug delivery system.

In the food industry, as a biodegradable packaging material such as eco-friendly bags, bottles, disposable items, the PHB is used (Harding et al., 2007).

Despite the many desirable properties and widespread applications, PHB has still not been commercialised for industrial purposes due to the high cost of production, which is many times more than that of synthetic plastics. More research is to be dedicated to reducing the cost associated with scaling up PHB production.

We aim to optimise the various factors like low-cost substrate, pH, and temperature, which play an essential role in reducing the cost of PHB production and characterisation of produced PHB by FTIR and HPLC analysis.

MATERIALS AND METHODS

Isolation and Screening of PHB producing Bacterial cultures

The microorganisms were isolated from the soil samples collected from waste dumping ground and diluted and plated on Nutrient agar plates incubated at 37°C for 48 hours. The pure isolates were used for the screening of PHB granules by the Nile blue staining method. They were viewed under a fluorescence microscope at a wavelength of 490 nm (Ostle and Holt, 1982).

Molecular Characterisation of Strains by 16S rRNA sequencing

To identify the isolates at a species level, the genomic DNA was purified and amplified using the 16S rRNA gene amplifying universal primers and were sequenced with Applied Biosystems 3130xl Genetic Analyzers. The neighbour-joining method is used to construct the phylogenetic trees using the MEGA-X program (Saitou and Nei, 1987; Kumar et al., 2018).

Extraction and Quantification of produced PHB in the potent isolates

The isolates selected for the intensity of their PHB granules were refined in LB media supplemented with D-glucose as a carbon source at 150 rpm in a rotational shaker. Following two days of incubation, the extraction of PHB was performed following the sodium hypochlorite-chloroform method of Singh and Parmar with slight modifications (Singh and Parmar, 2011). In brief, 5ml of culture were centrifuged at 10000 rpm for 10 mins followed by a wash in phosphate-buffered saline (PBS). The cell pellet was then resuspended in 2.5ml of 4% sodium hypochlorite and 2.5ml of hot chloroform and incubated for 60 mins at 37°C. The tube was then centrifuged at 8000 rpm for 10 mins. The chloroform layer was transferred to the fresh glass tube, and 10 ml of concentrated sulphuric acid was added. The addition of sulphuric acid converts PHB to dark brown coloured crotonic acid. The glass tubes were cooled, and the absorbance was measured at 235 nm against sulphuric acid.

Characterisation of extracted PHB by FTIR analysis

Cells were harvested by centrifugation at 3000 rpm for 10 min, and the pellet was resuspended in the methanol and incubated overnight at 4°C to remove the pigments. After incubation, the tube centrifuged, and the pellet was dried at 60°C for 1 hour. FTIR analysis was carried out using Perkin Elmer Fourier 110 spectrophotometer. About 2mg of PHB pellet is mixed with 20 ml of KBr. The mixture was compressed into a translucent disc. The IR spectrum was documented between the ranges of 400 to 4000 cm−1 (Chandrika et al., 2015).

Characterisation of extracted PHB by HPLC analysis

The sample was centrifuged at 10,000 rpm for 10 minutes and the pellet dried at 80°C overnight. The dry pellets were boiled in concentrated. H2SO4 at 90°C for 30 minutes and were diluted with 5mM of H2SO4 before filtering. 200µl of the sample was further diluted with 1mL of
5 mM H$_2$SO$_4$ and filtered using a membrane (0.45). HPLC analysed the filtered 50 $\mu$l sample with a ROA column 78 X 300 mm (Carr, 1966).

Optimisation of process parameters for maximum PHB production

Effect of Temperature and pH

The isolate was refined using LB media as a supplement source and incubated at various temperatures and pH like 25°C, 35°C, 37°C, 40°C and pH 6, pH 7, pH 8, and pH 9, respectively. Estimation of PHB and biomass was carried out after 48 hours with the help of UV-vis spectrophotometry at 235 nm (Law and Slepecky, 1961).

Effect of Carbon and Nitrogen sources

The isolate refined in LB media was supplemented with various carbon and nitrogen sources like Glucose, Sucrose, Maltose, Cellulose, Fructose and Tryptone, Peptone, Ammonium Sulphate respectively, incubated for 48 hours. The produced PHB and the biomass were estimated using UV-Vis spectrophotometry at 235 nm (Spiekermann et al., 1999).

RESULTS AND DISCUSSION

Sample selection and isolation of bacterial strains from dumped soil

Samples collected from wasteland were subjected to serial dilutions of 10$^{-2}$, 10$^{-3}$, and 10$^{-4}$ and plated on LB agar media. These Petri plates were incubated for 24 hours at ambient temperature. Growth was observed on the plate from where individual colonies were selected and streaked again onto a new plate to gain purity of the sample.

Screening of PHB granules by Nile Blue A

Wild strains were stained with Nile Blue A for the screening of PHB granules, which was produced as an intracellular compound. These were observed under a fluorescence microscope at 490 nm. The granules showed a bright reddish-orange colour (Zhenggui et al., 2011). The isolate showed the best results out of the 12 strain was selected (Figure 1).

Molecular Characterisation of Strains by 16S rRNA sequencing

The bacteria colony, which showed maximum PHB accumulation, was selected and characterised by 16S rRNA sequencing. The 16 rRNA sequence was amplified, and the sequence was compared with sequences available in the GenBank using a BLAST search. Their sequence relationships were analysed using the software MEGA X to understand the evolutionary distance of sequenced bacteria by constructing the phylogenetic tree, and the bacterial strain was identified as *Alcaligenes faecalis* – JG9A (Figure 2).

Characterisation of PHB granules

FTIR analysis

PHB extracted from the sample was used for recording IR spectra in the range 4000–400 cm$^{-1}$. FTIR is the best technique to study the internal compound of microorganisms, especially for PHB identification (Yellore and Desai, 1998). The infrared spectrum showed three prominent absorption bands at 1131 cm$^{-1}$, at 1130 cm$^{-1}$ and at 1191 cm$^{-1}$; which is specific for C–O and C=O groups. The bands at 3426 cm$^{-1}$ are due to the presence of C–H methylene and methyl groups in PHB isolate (Figure 3).

HPLC analysis

HPLC analysis was performed to confirm the presence of crotonic acid by measuring the absorbance of 214 nm for the sample. Figure 4 shows the retention time of the peak at 12.39 min was determined for the PHB isolate. (Reema et al., 2003) also reported that PHB isolated from *Enterobacter aero-
Optimisation of different Parameters for PHB Production

The factors such as temperature, pH, Carbon source, and Nitrogen sources affect PHB production and growth of isolates.

Effect of Temperature on PHB production

A range of temperatures like 25°C, 35°C, 37°C, and 40°C was selected for the optimisation. Figure 5 shown for the same, and the ideal temperature was found to be at 35°C, where the PHB concentration is high, compared to other temperatures. The PHB concentration was found to be at a maximum value of 0.07 mg/mL when incubated at 35°C, followed by incubation at 25°C.

Effect of pH on PHB production

The results show that the production of PHB is positively affected by slight changes in pH, and it is crucial for bacterial growth. Figure 6 shows that pH affects cell dry weight and PHB concentration. pH 7 was found to support the maximum accumulation of PHB amounting to 0.055 mg/mL. The biomass concentration was also observed to be a maximum of 0.47 mg/mL for pH 7 within the pH ranges used. The optimum time of incubation was 72hr. (Myshkina et al., 2008) found a similar result for PHB production using Azotobacter chroococcum 7B2, and (Hashimoto et al., 1993) also reported high PHB yield at pH 7 using photosynthetic bacteria. The study published by Tavernier et al. (1997) on two different strains of Rhizobium meliloti cultured in different sources of nitrogen, carbon, and varying pH levels and their exopolysaccharide and PHB production is in parallel to the results obtained in this study as both the strains showed higher PHB content at pH 7 (Tavernier et al., 1997).

Figure 6: Effect of different pH values on growth and PHB production

Effect of carbon sources on PHB production

The different carbon sources decide the production of metabolites. The sources were glucose, sucrose, fructose, cellulose, and maltose, to understand the reactivity of bacteria towards the production of PHB. The media supplemented with glucose was found to support PHB production the best. The increased PHB production and biomass observed in glucose after 72 hrs is found to be 0.0319 mg/mL, and the results were depicted in Figure 7.

A study on Hydrogenophaga pseudoflava for PHB biosynthesis by Choi and Lee (1999) gave similar results in which 1% D-glucose gave the maximum PHB content of 67.30%. (Lasemi et al., 2013) reported that the production reached a maximum value as the glucose concentration was increased to 50g/L and then slightly reduced for further increase.
Effect of Nitrogen Sources on PHB production

Nitrogen sources used to affect the PHB production mechanism of the bacteria. When they are in excess, they show inhibitory effects and can lower the cultivation—the nitrogen sources like Tryptone, peptone, and ammonium sulfate for the optimisation process. The PHB concentration was found to be maximum in peptone, i.e., 0.0723 mg/mL, followed by Tryptone and Ammonium sulfate Figure 8. This result is in parallel with the result obtained by Yüksekdağ et al. (2004) in the study on Bacillus subtilises 25 and Bacillus megaterium 12 strains. The study determined that protease peptone was the best nitrogen source giving a maximum PHB yield of 78.69% and 77% in the respective strains. A study on Bacillus mycoides DFC1 by Narayanan and Ramana (2012) reported that the maximum cell growth and PHB yield were obtained when the glucose and peptone levels in the media were optimised to 17.34g/L and 7.03g/L.

CONCLUSIONS

There is a clear need to minimise the use of plastic waste and to create advances that can assume an essential part in relieving the environmental pollution and disposal problems caused by conventional plastics. The results of this study confirmed that cheaply available carbon and nitrogen sources can be used for the production of PHB, leading to reduced cost of biodegradable plastics. The capability of various bacterial species and recombinant strains is being investigated with regards to creating biodegradable plastics by expanding PHB yield and biomass. But, due to the vast effect of the high cost of carbon substrates on generation cost, a standout amongst the most imperative ways to diminish the costs is to utilise dumping soil in which bacteria are naturally present.

Although all the ten colonies isolated for the study gave positive results for PHB production, one isolate was found to have a high amount of PHB producing bacteria. The study on different media conditions through optimisation proved that the media containing glucose as the carbon source and peptone as nitrogen supplement is the most favourable for PHB production, and the production decreases in the presence of excess nitrogen sources. The optimal pH was found to be 7, and the temperature was observed to be 35°C for maximum PHB synthesis in the bacterial cell.

ACKNOWLEDGEMENT

The authors are grateful to the authorities of SRM Institute of Science and Technology, Kattankulathur, Tamil Nadu, India.

Conflict of Interest

The authors declare that they have no conflict of interest for this study.

Funding Support

The authors declare that they have no funding support for this study.

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