Research Article

Poly β Hydroxy Butyrate (PHB) Biosynthesis in *Bacillus*

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ABSTRACT

This study deals with the production of Poly β Hydroxy Butyrate (PHB) by microorganisms which were isolated from activated waste sludge. Six different strains were selected based on their morphological characteristics. Nitrogen limited medium was provided to organisms for influencing the production of PHB inside their cytoplasm as an energy and carbon source reservoir. The PHB screening was performed with the aid of Sudan Black staining and colony viable assay. The PHB positive culture was taken for molecular identification and the organism was identified as *Bacillus* sp. Effect of various process parameters on PHB production such as pH, temperature, incubation periods, carbon sources, nitrogen sources, agro waste products of the medium were optimized. The optimum temperature was attained at 37°C and pH was 8, Ammonium sulfate was found to be the best supporter for the growth in PHB. The optimum PHB growth was estimated at 48hrs of time interval. Finally the Biosynthetic pathway involved in PHB production was also studied.

Keywords: Biopolymer, Biosynthetic pathway, Molecular identification, Medium optimization, Poly β Hydroxy Butyrate (PHB).

INTRODUCTION

Polyhydroxybutyrate (PHB) is naturally formed macromolecules produced by many bacteria in response to various environmental conditions during their growth cycle, and therefore are referred to as natural polymers. Bacteria accumulate PHB in their cytoplasm as carbon and energy reservoir, the accumulation of PHB is increased when growth is limited by a nutrient other than carbon, such as nitrogen, oxygen, phosphorous, sulfur and trace elements like magnesium, calcium and iron deficiency.[1] Among Polyhydroxy alkanoates , PHB is the best known polyester, due to its structural properties similarity to those of polypropylene,[2] with the advantage of being biodegradable, biocompatible, non toxic and produced from renewable carbon and energy sources.[3] PHB has many applications in medicine, veterinary practice, tissue engineering materials, food packaging, drug delivery carriers, biofuels and agriculture.[5-8] Reducing the costs of PHB production by optimizing fermentation process is the basic research objective for industrial application. The main objective of this study was to isolate, characterize and identify PHB producing strain from activated waste sludge. The biopolymer was identified by chemical characterization using spectrometric chemical assay. Furthermore, PHB positive culture was taken for molecular identification of the organism and it was identified as *Bacillus* sp. Special emphasis was given to optimization of the effect of different parameters such as pH, temperature, incubation periods, carbon sources, nitrogen sources and agro waste products of the medium for PHB biopolymer production.

MATERIALS AND METHODS

Sample collection and isolation

In this study, a total of 6 bacterial strains were isolated from activated waste sludge collected in Chennai.

Screening for bacterial endospore

The isolates were screened for endospore using Schaeffer-Fulton Endospore staining technique. Among the 6 strains, green color endospore were observed in 4 strains and further screened for PHB granules using Sudan black staining technique.

Screening for PolyHydroxy Butyrate

Among the 4 strains, dark purple colour PHB granules were observed in only one strain (Figure 1). The strains gave positive results for Sudan black staining was utilized for further studies.

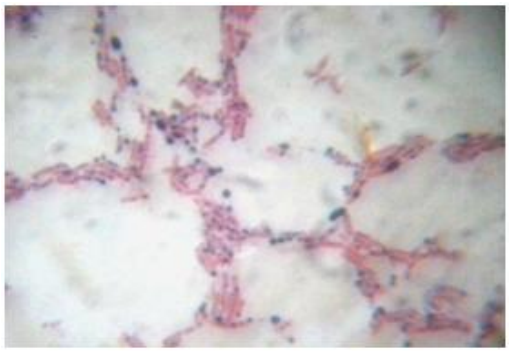

Figure 1: Strain 2 cells show clearly that they contain PHB as dark purple granules

Confirmation screening for PHB

Viable colony assay

Again the 6 isolates were taken for viable colony assay. The colony stained with Sudan black shows positive result for PHB production and colony does not stained with Sudan black shows negative result for PHB production.

Isolation of best Polyhydroxy butyrate producer

The selected isolate was inoculated in production medium[9] for screening the best PHB producer. The best PHB producer was estimated based on spectrometric chemical assay using Law and Splepecky method (1961)[10] and subjected for further optimization studies.

Molecular identification of the isolated bacteria

Molecular identification was performed by extracting the genomic DNA from isolated bacteria and PCR amplification of the 16S ribosomal RNA gene were amplified by using Primers

Forward: 5' AGAGTTTGATCCTGGCTCAG 3'

Reverse: 5' AAGGAGGTGATCCAGCCGCA 3'

and the Cycling conditions: initial denaturation at 94°C for 4 minutes, followed by 30 cycles of 90°C for 20 seconds, 45°C for 30 seconds, 68°C for 50 seconds, followed by final elongation step at 72°Cfor 7 minutes, the amplified PCR products were sequenced commercially and the 16S rRNA nucleotide Sequence was analysed by Basic Local Alignment Search Tool (BLAST) available at NCBI and also Phylogenetic tree analysis was performed.

Parameter optimisation studies for production of PHB

The time courses of PHB production during fermentation, influence of various pH levels, different carbon and nitrogen sources on their overall production were investigated using Production medium.[9]

Effect of Incubation time on PHB production

Around 500ml of sterile production medium was prepared and 1% inoculum was added aseptically. The inoculated medium was incubated at 37°C with shaking at 100 rpm. Around 10 ml of culture was aseptically drawn periodically at 6 hours intervals up to 72 hours. The PHB production was determined using alkaline digestion method.[11]

Effect of Temperature on PHB production

100 ml of sterile production medium was prepared in different conical flask and inoculated with 1% inoculum. Each flask was incubated at different temperatures such as 25°C, 30°C, 37°C, and 42°C for 48 hours. The PHB production was determined using alkaline digestion method.[11]

Effect of pH on PHB production

100 ml of sterile production medium was prepared in different conical flasks and each flask was adjusted to different pH such as 6.5, 7, 7.5, 8, 8.5 using 0.1N NaOH and 0.1N HCl. After sterilization, flasks were inoculated with 1% inoculum and incubated at 37ºC for 48hours. The PHB production was determined using alkaline digestion method. [11]

Effect of Carbon Sources on PHB production

100 ml of sterile production medium (pH – 8) was prepared in different conical flasks. In each flasks were amended with different carbon sources (2 %) such as Fructose, Glucose, Lactose, Maltose and Sucrose. Other cheap carbon sources such as bagasse, molasses, soya, wheat bran, rice bran were also used for better production of PHB. The flasks were inoculated with 1% inoculum and incubated at 37°C for 48 hours. The PHB production was determined using alkaline digestion method.[11]

Effect of Nitrogen source on PHB production

100 ml of sterile production medium (pH - 9.0) was prepared in different conical flasks with Glucose as carbon source. In each flask were amended with different nitrogen sources (0.5%) such as Peptone, Casein, Gelatin, Potassium nitrate, Ammonium sulphate, Urea. The flasks were inoculated with 1% inoculum and incubated at 37°C for 48 hours. The PHB production was determined using alkaline digestion method.[11]

Effect of different agro waste products

Various natural agro waste products like rice bran, wheat bran, sugarcane bagasse and molasses were used as (2 %) substrates (by replacing sugar components in production medium) for effective PHB production. Agro wastes were collected, dried and powdered. The substrates were sterilized and seeded with 1% inoculum. They were incubated at 37°C for 48 hours. The amount of PHB produced from different substrates was estimated.[11]

RESULTS AND DISCUSSION

The growth study of the organism is essential for the production of PHB. Growth study was performed for the selected isolate using modified nutrient medium.[9] In order to determine the optimum production time for maximum PHB production; the samples were collected at 6 hours intervals and analyzed for the estimation of PHB. In the growth study we found that up to 18th hour the production of PHB was very low and then there is a gradual increase in the production. Maximum PHB production was observed from 36th hour to 60th hour; from there onwards gradual decrease in the PHB production was observed. Based on the results analyzed at different time intervals, it was determined that the maximum production of PHB was at 48th hour. (Table 1)

Maximum PHB is produced with incubation time 48hrs and the yield was estimated to be 280µg/ml.

Different Parameters

The environmental parameters like pH, temperature shows great influence on the growth of the organisms and the production of PHB.

Effect of Temperature

In order to determine the effect of the incubation temperature for the better PHB production, different incubation temperatures were maintained for production process. Based on the readings it was observed that the selected strain have a temperature optima at 30°C. (Table 2)

Table 1: Effect of Incubation Time on PHB Production

Time of culture withdrawal	PHB (µg/ ml)
6th hour	32
12th hour	44
18th hour	88
24th hour	152
30th hour	184
36th hour	224
42th hour	242
48th hour	**280**
54th hour	262
60th hour	250
66th hour	240

Table 2: Effect of Temperature on PHB production

Temperature(°C)	PHB (µg/ ml)
25	157
30	204
37	**284**
42	232

The optimum temperature was found to be 37°C and the PHB produces was 284µg/ml.

Effect of pH

The optimal pH for PHB was determined by alkaline digestion method. Based on the readings it was observed that the selected strain shows maximum PHB production when it was maintained at pH 8. (Table 3)

Table 3: Effect of pH on PHB Production

pH	PHB (µg/ ml)
6.5	201
7	213
7.5	241
8	**272**
8.5	224

The maximum PHB is produced at pH 8 and it was found to be the optimum pH for PHB production.

Effect of Carbon Sources

Different carbon sources were screened for maximum production of PHB for the selected isolate. As it is seen from Table 4, except for lactose, the rest of the carbon sources gave satisfactory production of PHB. However if maximum productivity was considered Sucrose was taken as best carbon source. (Table 4)

Table 4: Effect of Different Carbon Sources on PHB Production

Carbon source	PHB (µg/ ml)
Glucose	240
Fructose	251
Sucrose	**294**
Maltose	168
Lactose	143

Among 5 different carbon sources given, sucrose shows the best result for maximum PHB production.

Effect of Nitrogen Sources

The nitrogen sources are of secondary energy sources for the organisms which play an important role in the growth of the organism and the production. Different nitrogen sources were screened for maximum production of PHB for the selected isolates. As it is seen from Table, the lowest PHB production was obtained with casein and gelatine. Peptone and Potassium nitrate gave much more satisfactory result among the selected nitrogenous source. Since productivity is concerned, Ammonium sulphate shows the maximum PHB production and considered as best sources in this study. (Table 5)

Table 5: Effect of Different Nitrogen Sources on PHB Production

Nitrogen sources	PHB (µg/ ml)
Peptone	252
Potassium nitrate	187
Ammonium sulphate	**296**
Urea	154
Casein	140
Gelatin	132

The maximum yield of PHB was estimated to be 296µg/ml and the optimum nitrogen source was ammonium sulphate.

Effect of different agro waste products

Different agro waste products were taken for best PHB production. As mentioned in table 6, molasses is considered to be the best and cheaper source for PHB production. (Table 6)

Molasses was found as the best medium for PHB production. The yield was 298µg/ml. Among all used

media and conditions, molasses had given the highest yield of PHB.

Table 6: Effect of different agro waste products on PHB production

Agro waste products	PHB(µg/ ml)
Rice bran	225
Wheat bran	150
Sugarcane bagasse	100
Molasses	**298**

Molecular identification of the isolated bacteria

According to Bhairavi Ghate *et al.*,[12] the viable colony screening assay had been used for final confirmation of PHB production in culture 2. According to the alkaline digestion method,[11] the concentration of PHB was estimated in µg/ml.

The isolated bacterium from activated waste sludge was identified as *Bacillus* sp. The blast hit and the Phylogenetic tree also indicates that the identified *Bacillus* sp. is 99% similar to many other species of *Bacillus* including *Bacillus amyloliquefaciens* and *Bacillus subtilis* etc. (Table 7)

Table 7: Sequences producing significant alignments

Description	Max score	Total score	Query cover	E value	Max ident	Accession
Bacillus amyloliquefaciens subsp. plantarum YAU B9601-Y2 complete genome	2732	27210	100%	0.0	99%	HE774679.1
Bacillus sp. K7SC-9A 16S ribosomal RNA gene, partial sequence	2732	2732	100%	0.0	99%	JF799962.1
Bacillus subtilis gene for 16S ribosomal RNA, partial sequence, strain: A97	2732	2732	100%	0.0	99%	AB501113.1
Bacillus amyloliquefaciens FZB42, complete genome	2726	25459	100%	0.0	99%	CP000560.1

CONCLUSION

The present study highlights the accumulation of PHB and its production in bacteria. The optimization parameters and medium that were discussed would be very useful for industrial production of PHB in future. The agro waste products would be much cheaper medium for the mass production of PHB. As said earlier, Molasses is capable of producing a large amount of PHB than using other agro industrial residues as substrates. The gene Pha A encoding the enzyme 3 ketothiolase is the precursor for PHB biosynthesis in bacteria. The specific gene sequencing would be also performed in future for deeper study of biosynthetic pathway involved in PHB production. As PHB is an alternative for plastics, it would be more useful if it is synthesized in higher concentrations. The addition of a new species to the existing list of PHB-producing microorganisms will provide new ways for the production of cost-effective biodegradable plastics.

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