MICROBIAL PRODUCTION OF BIOPLASTIC (POLY-β-HYDROXYBUTYRATE)

SHIRLEY HEMANT BHOIR, HEMLATTA CHAKRABORTY

Abstract: Biopolymers are an alternative to petroleum-based polymers with a wide range of environmental advantages. Poly-β-hydroxybutyrate (PHB) is a biodegradable polyester, belonging to the family of polyhydroxyalkanoates (PHA). It is produced by microorganisms in the form of reserved food granules under stress conditions like excess availability of C source but limited provision of other nutrients such as N, phosphate, & sulphur. PHB has various applications in different areas like medicine, drug manufacture & agriculture. In this study, isolation & screening of PHB producing bacteria from different soil samples was carried out followed by optimization of media for maximum production of PHB by the selected isolates. The best C source was considered to be glucose. Peptone was found to be the best N source. Maintaining the C:N ratio of 15:1 & 5:1 using the best C&N source was found to be optimum for the bacterial isolates from Powai soil & Mangrove sample respectively. In order to reduce the cost of PHB production, molasses was tested as a cheaper substrate for PHB production. The advantage is that the biowastes can not only be disposed off but also value added product like PHB can be obtained. Further, FTIR analysis of the samples showed ester peaks &bio plastic formation in form of a thin film was also obtained.

Keywords: Bio-polymer, Poly-β-hydroxybutyrate (PHB), C, N.

Introduction: PHB is a natural, biodegradable polymer accumulated in the form of intracellular granules by a large variety of bacteria. From a biotechnological point of view, the ability of bio plastics to be biodegradable makes them a desirable substitute for petrochemical-based plastic, an environmental pollutant [1]. Increased production of bio plastics can significantly reduce C dioxide emissions, curtail plastic waste generation & decrease consumption of fossil fuels. The aim of this project is PHB production from microbial origin & the objectives are isolation of PHB producing bacteria from different soil samples, screening for high PHB producers from the isolated bacteria & their identification, optimization of cultural parameters for maximum PHB yields, use of cheaper substrate like molasses for production of PHB, characterization of the samples by FTIR analysis, determination of PHB accumulation & bioplastic production.

Materials & Methods:

Isolation of bacteria from different soil samples: Soil samples from gardens of Powai & Vasai, soil sample from paddy field (Shahapur), soil nearby garage (Kurla), soil nearby industrial effluent drainage (Mithi river, Kurla), mangrove soil (Thane creek) were collected. The soil samples collected were enriched in Nutrient broth supplemented with 1% glucose & further isolated on Nutrient agar supplemented with 1% glucose. Mucoid bacterial colonies were assigned names based on their source of isolation & further subjected to rapid screening for PHB production following the viable colony method of screening using Sudan Black B dye [2].

Quantification of PHB production & selection of isolates:

All the Sudan Black B positive isolates were subjected to quantification of PHB production as per the crotonic acid assay [3]. By referring to the standard curve, the quantity of PHB

produced by the isolates was determined. Based on the PHB yields, two isolates (Powai 4 & Mangrove 1) were selected for further studies.

Characterization of PHB producing isolates: The selected PHB producing isolates were identified upto the level of genus by means of morphological & biochemical tests as per the procedures outlined in Bergey's Manual of Systematic Bacteriology.

Microscopic visualization of PHB producing bacteria:

PHB producing bacteria were detected using the lipophilic stain Sudan black B. Sudan black stain was prepared as a 0.3% solution (w/v) in 70% ethanol. Smears of PHB producing bacteria were prepared on glass slides & heat fixed & subjected to Sudan black B staining.

Optimization of cultural parameters for maximum PHB production:

Different factors affecting PHB production were optimized *viz*., N (N) source, & the C:N ratio.

Effect of different N sources on PHB production:

The bacterial isolates were grown in M-9 medium with the best C source i.e glucose, & different N sources were used *viz.,* peptone, casein, ammonium sulphate, & yeast extract, all at 1.0 g/100ml concentration under shaker conditions [4]. After 48 hrs, PHB yields were quantified as per the method of John & Ralph (1961). Based on the yield data, the best N source was arrived at.

Effect of different C: N ratios on PHB production:

Effect of different concentrations of glucose as the C source on PHB production:

The bacterial isolates were grown in M-9 minimal broth having the best N source & different concentrations of glucose as the C source *viz.,* 2.5%, 5%, 10%, 15%, 20%, 25% & 30% under shaker conditions. After 48 hrs, PHB yields were quantified as per the John & Ralph method (1961). Based on the data, the optimum concentration of glucose as the C source was arrived at.

Effect of different concentrations of the N source on PHB production: The bacterial isolates were grown in M-9 minimal broth with the optimum concentration of C source but varied concentrations of N source viz., 0.1%, 0.5%, 1%, 1.5% & 2% under shaker conditions. After 48 hrs, PHB yields were quantified as per the John & Ralph method (1961). The optimum N concentration was arrived at. Based on the data, the best C: N ratio was obtained.

Use of inexpensive substrate (molasses) for PHB production: Snethil Kumar. B and Prabhakaran (2006)[5] used different bio effluent and found high production of PHB in molasses based substrate medium.

Molasses was used as a inexpensive substrate in place of glucose for PHB production [6]. The concentration of molasses added was according to the optimum concentration of C source obtained.

The total carbohydrate content of molasses was estimated by the Fehling's titration method. Accordingly, amount of molasses to be added in the medium was calculated by the formula:

The amount of molasses = Optimum concentration of glucose for PHB production X 100

to be used The total carbohydrate content of molasses: Both the selected isolates were inoculated in optimised medium & M-9 minimal broth containing molasses as the C source & incubated under shaker conditions for 48 hrs. After 48 hrs, PHB yields were quantified as per the John & Ralph method (1961).

***Fourier Transform Infra-Red Spectroscopy* (FTIR) analysis:** FTIR analysis of the sample was done at Haffkine's Institute, Parel, Mumbai. The analysis was carried out using the Shimadzu

analyser at the range of 400-4000 cm^{-1} wavelength.

PHB accumulation:

The positive isolates were inoculated in M-9 minimal broth supplemented with best C&N source at optimum concentrations & incubated under shaker conditions for 48 hrs. After 48 hrs, the broth was pelleted at 10,000 rpm for 10 min using pre-weighed centrifuge tubes & the pellet was washed with saline& the wet cell weight was calculated. The pellet was resuspended in equal volume of 4% sodium hypochlorite & incubated at room temperature for 30 min. The mixture was then centrifuged & the supernatant discarded. The cell pellet containing PHB was again washed with acetone & ethanol. Finally, the polymer granules were dissolved in chloroform. The chloroform was filtered; the filtrate was poured in a pre-weighed glass petri dish & dried in an oven. The petri dish with extracted PHB was weighed. The wet cell weight & dry wt of PHB extracted (g/100ml), & PHB accumulation (%) were determined.

Bi-Bioplastic production: The positive isolates were inoculated in M-9 minimal broth supplemented with best C source i.e. glucose (Paramjit Singh and Nitika Parmer,2011) [7] & the best N source i.e. peptone at optimum concentrations & incubated under shaker conditions for 48 hrs. The cell pellet containing PHB was again washed with acetone & ethanol. The polymer granules were then dissolved in chloroform. The chloroform was filtered & the filtrate was poured as a thin layer in a glass petri dish & dried in an oven, forming a film.

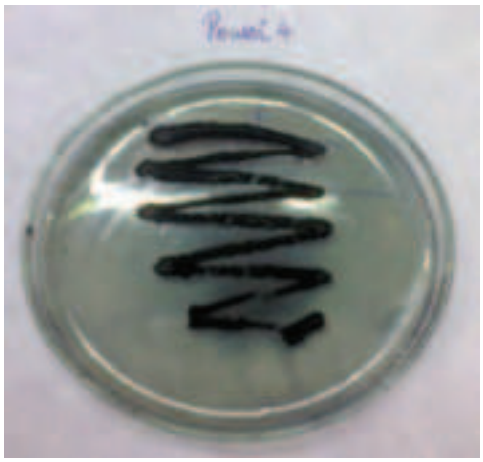


Figure 1: Sudan Black B testing of the isolates

Table 1: Viable colony staining of PHB producing bacteria & their scoring

Sr. No	1	2	3	4	5	6	7	8	9
Code Name of the isolate	Garage 1	Garage 2	Mangrove 1	Mithi 1	Paddy 1	Powai 1	Powai 2	Powai 3	Powaii 4
Scoring of black color colony	++	++	+++	+	++	+	++	+++	+++

Legend:

+++ : Strongly stained isolated colonies

++ : Medium stained isolated colonies

+ : Light black stained isolated colonies

Results & Discussion:

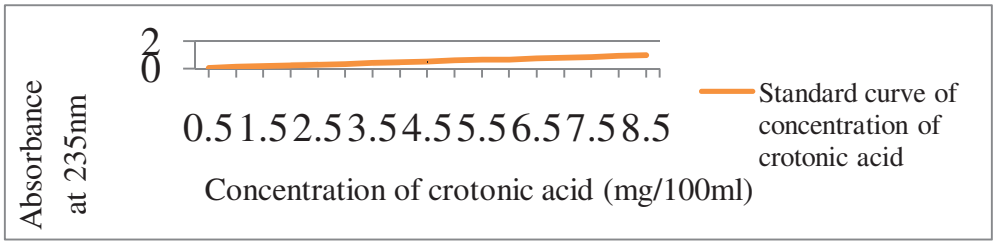
Isolation & screening of the isolates for PHB production:

12 mucoid bacterial colonies were isolated, purified & maintained as pure cultures. The screening for PHB production was done using viable colony staining by Sudan black B stain as shown in fig 1. All 12 isolates were selected based on colony characteristics and out of which 9 were PHP +ve. Out of these three were strong accumulator, four were moderate accumulator and 2 were poor accumulator as indicated in table 1. The colour of the Sudan black B colonies were visually scored. Out of 12 isolates, 9 were found to accumulate PHB.

Quantitative screening of the isolates for PHB production:

The 9 isolates obtained from the preliminary screening were subjected to quantification of PHB production following the technique of John & Ralph(1961). The organisms were grown in M-9 minimal broth supplemented with 2% glucose & were subjected to PHB quantification. The PHB yield from different isolates is tabulated in table 2. Of these the two culture Powai4 and Mangrove 1 gave best yield of PHB accumulated in the cells.

Sr. No	1	2	3	4	5	6	7	8	9
Code Name of the isolate	Garage1	Garage2	Mangrove1	Mithi1	Paddy1	Powai1	Powai2	Powai3	Powai4
PHB Yield mg/100ml	0.95	0.9	2.6	0.8	0.85	0.7	0.9	2.3	4.45



Graph 1: Standard curve of concentration of crotonic acid (mg/100ml) at 235 nm

By referring to the standard curve of crotonic acid, PHB production by the isolates was quantified. PHB production varied from 0.7 mg/100ml to 4.45 mg/100ml. Powai 4 isolate was the highest PHB producer with4.45mg/100ml(graph 1). In this method PHB is converted to crotonic acid which is brown coloured.

One gram of PHB is equivalent to 1 gram of crotonic acid (Law & Slepecky, 1969) [8]. Based on the PHB yields, 2 isolates viz., Powai 4

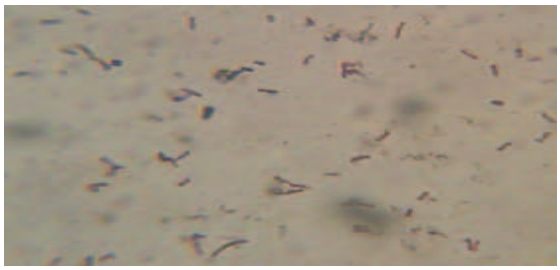
(4.45mg/100ml) & Mangrove 1 (2.6mg/100ml) were selected for further studies.

Identification of the PHB producing bacteria:

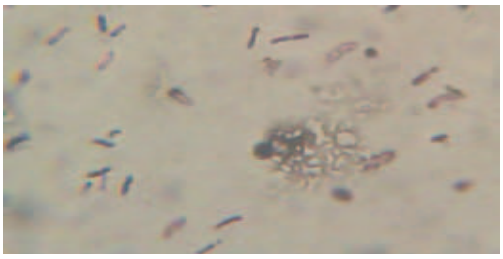
The isolates Powai 4 & Mangrove 1 were identified as *Bacillus spps*. based on the Bergey's Manual of Bacteriological Identification.

Microscopic detection of PHB:

The identified PHB producing bacteria were shown to accumulate PHB via microscopic staining techniques using Sudan black B(fig 2). Both the isolates displayed reactivity in the form of intense purple granules against a saffranin counter-stained cytoplasm.



Powai 4



Mangrove 1

Figure 2: Sudan Black B staining of the isolates

Optimization of cultural parameters for maximum PHB production by the selected isolates:

Effect of different N sources on PHB production: Amongst different N sources, peptone was found to be the best N source with PHB yield of 4.5 mg/100 ml & 2.6 mg/100ml for Powai 4 & Mangrove 1 respectively.

The PHB yield produced by selected bacterial isolates as influenced by different Nitrogen (N) source is tabulated in table 3.

Table 3: PHB yields produced by selected bacterial isolates as influenced by different Nitrogen (N) sources

Isolates	N source (1%)	PHB yield (mg/100ml)
Powai 4	Ammonium sulphate	0.95
	Casein	4.25
	Peptone	4.5
	Yeast extract	2.5
Mangrove 1	Ammonium sulphate	0.85
	Casein	2.0
	Peptone	2.6
	Yeast extract	1.6

Effect of different C:N ratios on PHB production:

Different C:N ratios were maintained using the best C&N sources in the M-9 minimal broth &

their effects on PHB production were studied.

Effect of different concentrations of glucose as the C source on PHB production:

Out of the seven concentrations, 15% was found to be optimum for Powai 4 supporting the highest PHB production of 5.15mg/100 ml whereas 5% was obtained to be the best C concentration for Mangrove 1 with PHB production of 2.95mg/100ml as indicated in table 4.

Table 4: Influence of different concentrations of glucose on PHB production by the selected isolates

Concentrations of glucose (%)	PHB yield (mg/100ml)Powai 4	PHB yield (mg/100ml) Mangrove 1
2.5	0.95	2.5
5	1.95	2.95
10	3.75	2.65
15	5.15	2.0
20	4.5	0.95
25	3.4	0.7
30	1.0	0.65

Effect of different concentrations of peptone (the best N source) on PHB production:

Out of the five concentrations of peptone, it was found that peptone at a concentration of 1.0 g/100ml supported the highest PHB production (5.2 mg/100ml & 3.0mg/100 ml for Powai 4 & Mangrove 1 respectively when compared to other levels as shown in table 5.

Table 5: Influence of different concentrations of peptone on PHB production by the selected isolates

Concentrations of peptone (g/100ml)	PHB yield (mg/100ml) Powai 4	PHB yield (mg/100ml) Mangrove 1
0.1	1.75	0.98
0.5	2.85	1.9
1	5.2	3.0
1.5	3.5	2.4
2.0	1.9	0.7

Under normal conditions, bacteria synthesize their body materials like proteins & grow. But, during nutrient limiting conditions, bacteria may shift their protein synthesis to PHB synthesis for survival. To exploit this phenomenon, experiments were carried out to study the accumulation of PHB with excess C & N limiting conditions. [9].

Sujatha et al (2003) [10] showed that higher PHB +ve strains were obtained from sewage sludge and tannery effluent as compared to garden and field soil. Ayub *et al* (2004) [11] showed PHB accumulation was maximum by stress resistant strains of bacteria.

PHB yields produced by the selected isolate using agro industrial waste.

The total carbohydrate content of molasses was estimated to be 67.23gm% using the Fehling's titration method. Molasses was used as a cheaper C
source for PHB production from agro industrial waste product [12]. Data revealed that the yields on this waste was significantly higher than those produced using the optimized medium.

Table 6: PHB yields produced by the selected bacterial isolates on cheaper substrates

Isolates	Media	PHB yield (mg/100ml)
Powai 4	Optimized medium	5.2
	Medium with molasses as C source	8.4
Mangrove 1	Optimized medium	3.0
	Medium with molasses as C source	5.6

As shown in fig 6 results of the study indicated that it is possible to convert carbonaceous material present in the molasses, into environmentally friendly PHB polymer. Both the strains showed increased PHB content when grown on molasses.

FTIR analysis:

The IR spectra showed mainly two intense absorption bands at 1710.86 cm^{-1} & 1236.37 cm^{-1} for PHB obtained from the isolate Powai 4 corresponding to

C=O & C-O stretching groups respectively. Similarly, bands from the isolate Mangrove 1 at 1593.2 cm^{-1} & in a range of 1296.16 - 1355.96 cm^{-1} were obtained corresponding to C=O & C-O stretching groups respectively. FTIR analysis of both the samples showed significant peaks that corresponds to the ester group which confirms the presence of PHB.

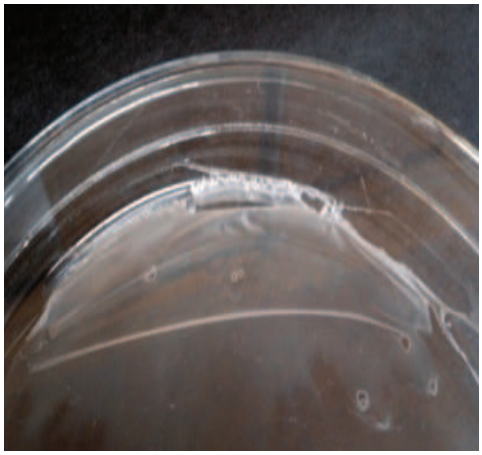
PHB accumulation:

The % of PHB accumulation was found to be 26.92% & 6.81% in Powai 4 & Mangrove 1 isolates respectively.

Bioplastic production:

PHB polymer was extracted from the M-9 minimal broth supplemented with best C source (glucose) & the best N source (peptone) at optimum concentrations in which the two bacterial isolates, Powai 4 & Mangrove 1 were inoculated as shown in fig 3. A thin film of bioplastic was also obtained as shown in fig 4.

Figure 3: Extract of PHB polymer Figure 4: Thin layer of bioplastic produced



Conclusions: PHB is a natural, biodegradable polymer accumulated in the form of intracellular granules by a large variety of bacteria [13]. In this study two isolates Powai 4 & Mangrove 1 showed best PHB prod--uction and were identified as *Bacillus spp.* The best C source was considered to be glucose & the best N source was found to be peptone. Maintaining the C:N ratio of 15:1 using the best C&N source was found to be optimum for Powai 4 whereas a C:N ratio of 5:1 was optimum for Mangrove 1. These findings were in accordance with the work done by Ackermann and Babel Wolfgang, 1995,[14]. Molasses was evaluated as an inexpensive substrate & it supported PHB production & increased PHB yield. FTIR analysis of the samples confirmed the presence of Poly-β-hydroxybutyrate (PHB). The percentage of PHB accumulation was found to be 26.92% for Powai 4 & 6.81% for Mangrove 1. A thin film of bio plastic was obtained from the extract of PHB.

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Shirley Hemant Bhoir/ Hemlatta Chakraborty

Department of Micobiology/ K. J. Somaiya College of Science & Commerce/

Vidyavihar/Mumbai-77/bhoirshirley@gmail.com