

Isolation of Polyhydroxybutyrate (PHB) Producing Bacteria, Optimization of Culture Conditions for PHB production, Extraction and Characterization of PHB

Christina Thapa¹, Pallavi Shakya¹, Rabina Shrestha¹, Sushovita Pal¹, Prakash Manandhar²
Department of Biotechnology, SANN International College, Purbanchal University, Nepal¹
Department of Microbiology, St. Xavier's College, Nepal²

Abstract

Polyhydroxybutyrates (PHBs) are energy reserves synthesized by different micro-organisms such as *Alcaligenes*, *Pseudomonas*, *Staphylococcus*, Algae, in excess of carbon and limitation of nutrients like nitrogen. These biopolymers are suitable alternate to synthetic carbon-based polymers. However, the high production cost limits their commercialization. The aim of this study was thus, focused on optimization of culture condition for maximum PHB production in an attempt to reduce the production cost. The micro-organisms for this purpose were isolated from 4 different soil samples and screened for PHB production. Culture conditions for these organisms were optimized by changing the parameters, viz., incubation time, pH, carbon source and NaCl concentration. Thus, optimized culture condition was used to culture the isolates for extraction of PHB and its analysis. The extracted compounds on FTIR-analysis gave characteristic C=O peak of PHB, thus, confirming the seven isolates to be PHB producers. Results for optimized parameters for the isolated PHB positive species showed that synthesis of PHB was maximum at 48 hours i.e. during the early stages of stationary phase. However, different isolates favored different culture conditions. Highest PHB accumulation and growth of isolates were seen at pH 7 and 9. Similarly, it was observed that glucose was favored by 4 isolates and sucrose was favored by 3 isolates. Interestingly, NaCl concentration did not cause significant effect on neither the bacterial growth nor the PHB production. During the extraction of PHB from the optimized culture conditions, extraction of PHB from broth gave significant yield than that from agar. A good PHB yield from broth amounting to 36.41% and 34.59% was observed for *Bacillus pasteurii* and *Micrococcus luteus* respectively, showing a potential for their exploitation in industrial PHB production. At optimized conditions, 7 isolates exhibited significant PHB yields, thus showing a potential for further exploitation.

KEYWORDS: Bioplastics, Biopolymer, Polyhydroxybutyrates, PHB, Fourier Transform Infra-Red Spectroscopy (FTIR)

*Corresponding Author

Email: rabeenastha@gmail.com

Introduction

Traditional plastics are synthetic carbon-based polymers that are made from non-renewable source, mostly from petroleum. Due to their relatively low cost, ease of manufacture and flexibility, the demand of plastics is ever-growing. However, plastics, being man-made, are not recognized by micro-organisms.^[13] Thus, they take very long to degrade i.e. 450 years on average for degradation of a plastic bottle.^[7] Plastic debris also poses considerable threat by choking and starving wildlife, distributing non-native and potentially harmful organisms, absorbing toxic chemicals and degrading to micro-plastics that may subsequently be ingested.^[2] Also, due to high cost of recycling, plastics are rarely recycled leading to crammed

up landfills. For eradication of these and various other problems such as carbon emission during incineration, biodegradation of plastic is a must. Considerable amount of interest in the development and production of an alternative, biodegradable plastics or bioplastics is being done. Among them polyhydroxy alcanoic acids (PHAs) are drawing much attention as they have nontoxic residual products and low environmental permanence.^[9] Depending on the types of carbon sources available and the biochemical pathways operating in the cell, microorganisms are capable of synthesizing various types of PHAs. Poly [R- 3-hydroxybutyrate] (P[3HB]) is the first type of PHA identified and is the most common PHA found in nature.^[1]

PHBs are carbon and energy reserve polymers produced in bacteria, archaea, and in few eukaryotes, such as yeasts and fungi when carbon source is in plentiful and other nutrients such as nitrogen, phosphorous, oxygen or sulphur are limited. The storage molecule is then metabolized under unfavorable conditions when other common energy sources are not available. Some bacterial species which naturally produce PHB are *Ralstonia eutrophes*, *Alcaligenes*, *Pseudomonas*, *Bacillus*, *Rhodococcus*, *Staphylococcus* and *Micrococcus*. [1]

PHB is ecofriendly, biodegradable, biocompatible and is accumulated up to 90% of cell dry weight. [10] PHB based plastics made by combining PHB with other biocompatible polymers (like 3-hydroxyvalerate) [8] find many applications in agriculture, packaging, and medical field including drug delivery and tissue engineering. [10] In spite of these interesting properties, industrial production of PHB is still not well established due to its high production cost. This has made it unable to compete with conventional plastics in the commercial market. The PHB content and its composition are influenced mainly by the strain of the microorganism, the type of substrate employed and its concentration, and other growth conditions such as pH, time and temperature. [11] Therefore, much research is needed to discover and identify novel species with vastly superior production capacity and optimization of conditions for maximal synthesis of PHB.

This research focuses on isolation and characterization of PHB producing bacteria from easy and convenient sources i.e. sewage soil sample. The objective was to analyze the extracted PHB by different isolated organisms in optimized physical and chemical conditions. Biochemical and morphological tests are performed for the identification purpose. In an attempt to overcome the limitations associated with costly substrates, this research is designed to use and test the efficiency of readily available and relatively cheap carbon sources such as sucrose, glucose and fructose in PHB production. Moreover, it also focuses on optimization of various growth conditions such

as incubation time, pH and NaCl concentration for increase in the polymer production from respective PHB positive organism. Considering the optimum growth conditions and carbon source of each organism, PHB extraction and its characterization using FTIR analysis was done. The extracted PHB was calculated as percentage yield of the cell dry weight obtained.

Materials and Methods

Sample collection and isolation of pure cultures

Soil samples were collected aseptically from topsoil of four sites viz., Teku Dumping Site, Balaju Industrial Site, Banks of Dhobi Khola and Budhanilkantha Animal Waste Manure.

One gram of each sample was dispersed in 10ml of sterile distilled water and heated at 80°C for 10 minutes to isolate only endospore forming bacteria. Serial dilution of these samples was done up to 10^{-3} , followed by spread plating of 100 μ l diluted samples on nutrient agar plates. Thereafter, the plates were incubated at 30°C for 48 hours. Pure culture of morphologically distinct colonies was grown in modified agar plates. The constituents of Modified agar plates are: Beef extract (0.3%), Peptone (0.5%), Sodium Chloride (0.8%), Glucose (1%), and Agar (1.5%). [12]

Primary screening of PHB producing bacteria

Detection for PHB production was employed by using lipophilic stain Sudan Black B. [3] Stain was prepared by dissolution of 0.3 gm powdered stain in 100 ml of 70% ethanol. For microscopic studies, smears of colonies were heat-fixed on clean, grease-free glass slides, followed by staining with 0.3% solution of the Sudan Black B. After leaving the slides undisturbed for 15 minutes, immersion in xylene and counterstaining with safranin (5% w/v in sterile distilled water) was performed. Cells appearing blue-black under microscope were accredited as PHB positive strains.

PHB positive strains were preserved on two vials, viz., working and stock vials, containing agar slants with 2% glycerol for preservation.

Morphological and Biochemical Characterization of PHB positive Isolates

Distinct morphological features of the isolates were recorded on the basis of shape, color and size. Similarly, cellular morphology was studied under the microscope using Gram Staining and Endospore Staining. Standard microbiological methods were employed for identification of isolated bacteria by biochemical tests. The tests performed were IMViC test, nitrate test, sugar utilization test, catalase test, oxidase test, starch utilization test and oxidative-fermentative test.

Growth Curve Study of Isolates

PHB producing medium was used to study the growth and production of PHB. The components of the media are: Glucose - 1g, Peptone -

0.25g, Yeast extract - 0.25g, NaCl - 0.01g, KH₂PO₄ - 0.05g, MgSO₄ - 0.02g and pH at 7. [6]

One percent inoculums from activated PHB positive isolates were inoculated in conical flasks containing PHB producing media, followed by incubation of the culture for 48 hours at 37°C with occasional shaking. At an interval of every 4 hours, the samples were collected to perform Sudan staining and the biomass reading was done using spectrophotometer at 640nm.

Optimization of PHB production

Effect of pH

Every microorganism has a minimum, an optimum and a maximum pH for growth. To standardize the optimum pH for the production of PHB, the PHB positive bacterial cultures were inoculated in PHB producing media at different pH (2, 4, 7, 9 and 11) and incubated at 37°C for 48 hours with occasional shaking. The pH values were taken in order to cover different acidic, neutral and basic pH ranges. After incubation, the samples were screened using Sudan stain to confirm PHB production and turbidity of the media due to bacterial growth was measured by spectrophotometer at 640nm.

Effect of NaCl Concentration

Microorganisms vary widely in their NaCl tolerance. Thus, PHB producing media with

different NaCl concentrations (0.1%, 0.5%, 2%, 5%, and 10%) was prepared. After autoclaving, 1% of activated culture was added to each tube and incubated at 37°C for 48 hours. The samples were collected after 48 hours for Sudan Staining and for measurement of O.D. at 640nm.

Effect of Carbon Sources

2% glucose, sucrose, and fructose were added into PHB producing media as carbon sources and the selected isolates were grown in it. After incubation and screening by Sudan stain, the PHB produced by the isolates was quantified spectrophotometrically for the selection of carbon source that showed highest PHB production.

Extraction of PHB

The optimized pH and carbon source for each bacterium were used for the extraction of PHB by solvent extraction method [4] with slight modifications. Firstly, 1% of PHB positive strain was inoculated in PHB producing media of optimized pH and carbon source and it was incubated at 37°C. After each 4-hour, 1 ml of media was centrifuged at 11,800 rpm for 20 minutes and Sudan staining was done to confirm PHB production. When the PHB production was confirmed, which mostly ensued after 48 hours, 50 ml of bacterial cell culture growth was taken and pelleted at 5000 rpm for 25 minutes. The dry weight of the pellet was taken and then it was washed with acetone and ethanol successively. For the recovery of PHB, equal volume of 6% sodium hypochlorite was used to re-suspend the pellet and it was incubated at 37°C for 10 minutes. This was followed by centrifugation at 5000 rpm for 30 minutes to sediment the lipid granules. The pellet obtained was washed with acetone and ethanol followed by hot chloroform treatment. After the pellet dissolved in chloroform, Whatman filter paper was used to filter out the cell residues so that only PHB is present in the chloroform solution. Finally, the filtrate was evaporated in hot air oven at 40°C and dry weight of extracted PHB was measured. The percentage of PHB accumulation was calculated using the formulae:

$$\frac{\text{Dry weight of extracted PHB } \left(\frac{g}{ml}\right) \times 100}{\text{Dry weight of biomass}}$$

strains inoculated in broth media as well as in

This procedure was repeated for all the PHB positive strains and PHB was extracted from the was found that five of the strains belonged to

Table 1. Morphological Characteristics of Strains

Morphology	T101	B139	Y202	K302	X102	D301	L402
Gram's Test	+	+	+	+	+	+	+
Cell Size	L: 1µm B: 2 µm	L: 3µm B: 1µm	Diameter: 2 µm	Diameter: 2 µm	L: 5 µm B: 1 µm	L: 5 µm B: 1 µm	L: 5 µm B: 1 µm
Shape	Rod	Rod	Cocci	Cocci	Rod	Rod	Rod
Spore	+	+	-	-	+	+	+
Margin	Irregular	Smooth	Smooth	Smooth	Wooly	Smooth	Smooth
Color	White	Light Yellow	Yellow	Yellow	Pinkish White	White	White
Elevation	Flat	Drop-like	Convex	Drop-like	Flat	Flat	Convex
Opacity	Translucent	Opaque	Opaque	Opaque	Translucent	Opaque	Opaque

agar media.

Characterization of PHB

The presence of prominent functional groups such as CH₃, CH₂, C=O, C-O, CH, and OH is a critical decider of presence of PHB. ATR-FTIR spectroscopy (Spectrum65 FTIR spectrometer) was used for qualitative identification and for checking the presence of such groups in the extracted compounds.

Table 2. Identification of Strains

S.N.	Strain	Identification from Bergey's Manual of Bacteriology	from Bergey's Determinative
1.	T101	<i>Bacillus pumilus</i>	
2.	B139	<i>Bacillus megaterium</i>	
3.	Y202	<i>Arthrobacter</i> sp.	
4.	K302	<i>Micrococcus luteus</i>	
5.	X102	<i>Bacillus pasteurii</i>	
6.	D301	<i>Bacillus cereus</i>	
7.	L402	<i>Bacillus sphaericus</i>	

Result and Discussion

Isolation and Screening

Altogether, 23 colonies, which were distinct, were chosen based on their shapes and colors. After 24-48 hours culture period, Sudan Black B staining was done to confirm the presence of PHB granules. Among 23 bacteria, 7 were found to be Sudan positive, i.e. they were capable of producing lipid granules which could have the presence of PHB.

Morphological Characterization

The result of morphological and biochemical characterization of the 7 Sudan positive bacteria is shown in Table 1 and Table 2 respectively. It

Bacillus species and 2 strains, Y202 and K302, belonged to *Arthrobacter* species and *Micrococcus luteus* respectively.

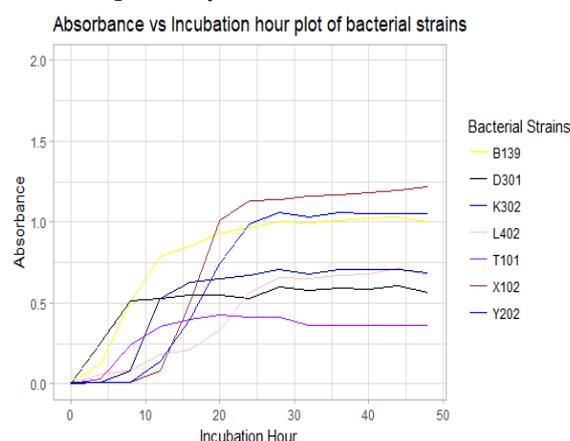


Figure 1: Graph showing growth curve of isolates. It was found that high amounts of black stained granules were obtained in the 48-hour period for all the bacteria. Therefore, 48 hours was chosen as the optimized incubation time for PHB production.

Growth Curve Analysis

The inoculated cultures were incubated at 37°C for 48 hours and readings were taken at 640 nm using spectrophotometer. 640 nm was taken as the required wavelength because the Wavelength of Measurement (WM) of OD depends on growth of the culture and here, we expect higher growth.

The Absorbance vs. Incubation hour plot of bacterial strains plotted using R-programming has been shown in the Figure 1.

Optimization of Culture Conditions

The 7 strains of Sudan positive bacteria were subjected to growth in the PHB producing media prepared with different pH, NaCl and

carbon sources. The graphs of Absorbance vs the optimized conditions of bacterial strains were plotted using R-programming (Figure 4, 5 and 6 respectively).



Figure 2: Sudan Stained black PHB granules of strain L402



Figure 3: Sudan Stained black PHB granules of strain D301

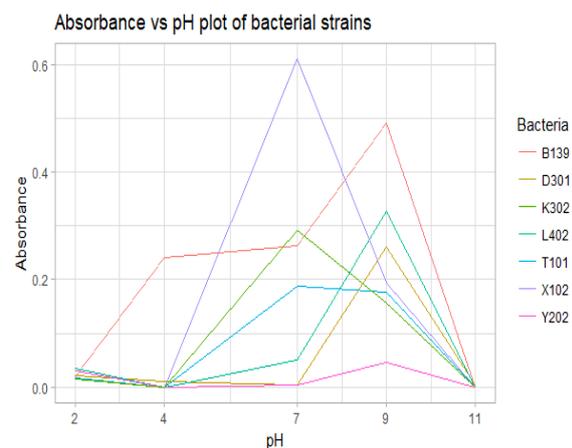


Figure 4: Graph showing absorbance vs. pH plot of bacterial strains. It was established that, for *Bacillus megaterium* (B), *Arthrobacter sp.*(Y), *Bacillus cereus* (D) and *Bacillus sphaericus* (L) the optimized pH was 9, for *Micrococcus luteus* (K) and *Bacillus pasteurii* (X), the optimized pH was 7 and for *Bacillus pumilus* (T), pH 7 and pH 9 were compatible. Thus, we can deduce that acidic pH is not suitable for PHB production.

Extraction of PHB

Extraction was performed from both PHB producing broth and agar. It was observed that extraction from broth gave much better results than that from agar from all of the species except *Arthrobacter spp.* The highest percentage of PHB accumulation from broth culture was shown by *Bacillus pasteurii* and the lowest was shown by *Arthrobacter spp.* Similarly, from agar,

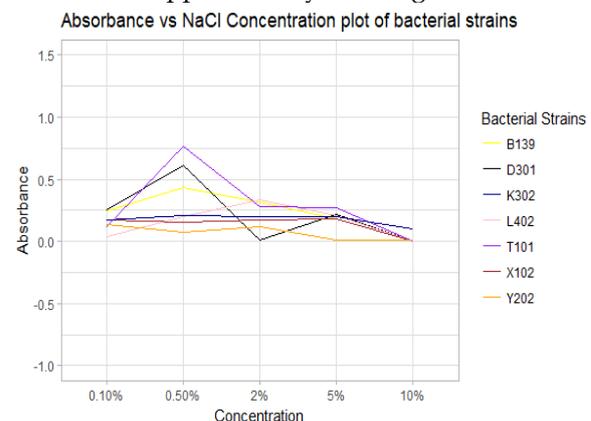


Figure 5: Graph showing absorbance vs. NaCl Concentration plot of bacterial strains. It was established that *Bacillus pumilus*(T), *Bacillus megaterium* (B) and *Bacillus cereus* (D) grew the most in the concentration of 0.5%, *Arthrobacter spp.* (Y) and *Bacillus sphaericus* (L) grew the most in the concentration of 2% while *Micrococcus luteus* (K) and *Bacillus pasteurii* (X) grew uniformly in all the concentrations except in 10%.

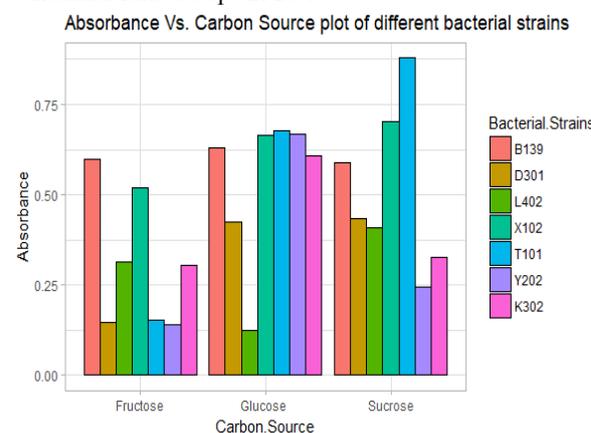


Figure 6: Graph showing effect of different carbon sources on bacterial growth. It was observed that *Bacillus* species could grow and produce PHB in both Glucose and Sucrose. *Bacillus pumilus* (T), *Bacillus pasteurii*(X) and *Bacillus sphaericus* preferred Sucrose while *Bacillus megaterium* (B) preferred glucose and *Bacillus cereus* preferred both Glucose and Sucrose. *Arthrobacter spp* (Y) and *Micrococcus luteus* (X) preferred glucose.

Arthrobacter spp. showed highest accumulation of PHB. *Bacillus pasteurii*, *Bacillus pumilus*, *Bacillus sphaericus* did not show any growth in

agar possibly due to limited availability of nutrients in their respective growth conditions.

Table 3. Comparison between percentage PHB accumulation from isolates grown in broth and

Table 3. Comparison between percentage PHB accumulation from isolates grown in broth and agar

S.N.	Bacterial Code	Bacteria	%PHB accumulation from broth	%PHB accumulation from agar
1.	X102	<i>Bacillus pasteurii</i>	36.41	-
2.	K302	<i>Micrococcus luteus</i>	34.59	17.65
3.	B139	<i>Bacillus megaterium</i>	28.63	4.12
4.	T101	<i>Bacillus pumilus</i>	21.46	-
5.	L402	<i>Bacillus sphaericus</i>	18.45	-
6.	D301	<i>Bacillus cereus</i>	14.91	9.35
7.	Y202	<i>Arthrobacter spp.</i>	8.56	20.65

agar

FTIR Analysis

The functional groups of extracted PHB were identified using FTIR Analysis. The functional groups of PHB extracted from *Bacillus pasteurii*, *Arthrobacter spp.*, *Micrococcus luteus* and *Bacillus cereus* was confirmed as C=O groups.

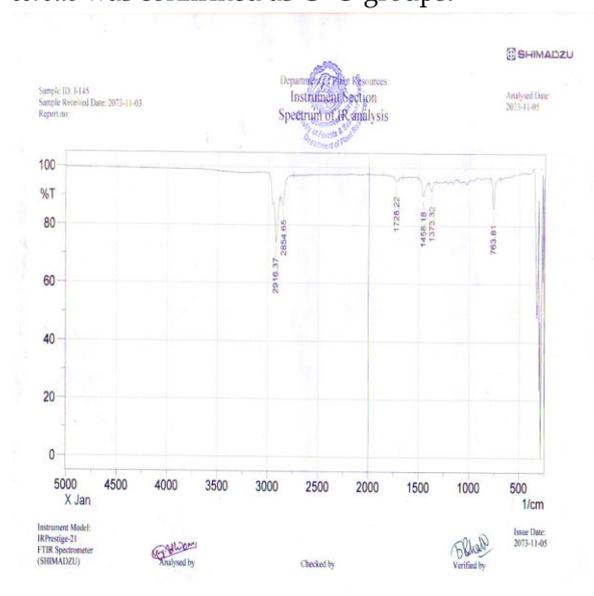


Figure 7: FTIR Analysis of extracted product from X102 (*Bacillus pasteurii*)

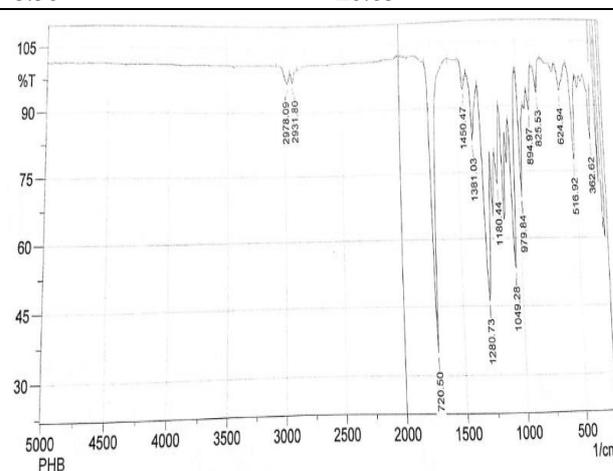


Figure 8: FTIR Analysis of Standard PHB

Conclusion

The present study was designed for the isolation of effective poly-hydroxybutyrate producing strains from soil to yield maximum PHB under optimized conditions. From our research, we found out that cosmopolitan “Everything is Everywhere” population such as *Bacillus*, *Arthrobacter* and *Micrococcus* species were able to produce PHB in considerably good quantity compared to other isolated species. Consequently, the effect of various parameters like carbon source, incubation time, pH and NaCl concentration on PHB production were seen to be species specific. Similarly, the production from broth and fermentation methods gave much better results than that from agar in all of the isolated species except *Arthrobacter*. PHB yield from broth amounting to 36.41% and 34.59% was observed in *Bacillus pasteurii* and *Micrococcus luteus* respectively, showing a potential for their exploitation in

industrial PHB production. Further characterization of extracted products with the help of ATR-FTIR analysis showed prominent functional groups CH₃, CH₂, C=O, C-O, CH and OH, which when compared with the standard PHB curve, confirms the extracts as PHB. Hence, this project focused on the isolation of microorganisms from soil samples of polluted sites and the optimization of conditions for the production of PHB effectively and frugally.

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