COMPARATIVE STUDY FOR EFFECTIVE EXTRACTION METHOD FOR POLYHYDROXYBUTYRATE (PHB) FROM *BACILLUS SPPS*

SAGAR PARMAR, NAGESH MALIK

Abstract: Plastics have been an integral part of our life. However, disposal of these non-biodegradable (petrochemical derived) plastics poses a threat to our environment. Thus, much interest has been gained in developing biodegradable plastics. Polyhydroxybutyrates (PHBs) are polymers that accumulate as carbon energy in microbial cells and provide an alternative to petrochemical plastic because of their biodegradability properties. However, one of the major problems in commercializing PHB is the high production cost due to ineffective extraction methods. The aim of this study was to do a comparative study to find the most effective method for extraction of PHB from *Bacillus spps.* Out of the two methods compared the diethyl ether method was found to be more effective over chloroform method for all the Bacillus spps. isolated.

Keywords: Polyhydroxybutyrates (PHBs), petrochemical plastic, *Bacillus spps.*

Introduction: Industrialization, urbanization, improper agricultural practices and various anthropogenic activities are responsible for pollution and loss of environmental quality. Problems concerning the global environment have created much attention in developing eco-friendly products. Biopolymers are one product that can help to overcome problems caused by petrochemical polymers. Biopolymers are generated from renewable natural sources and are often biodegradable and nontoxic. They can be produced by biological systems (microorganisms, plants and animals), or chemically synthesized from biological materials (sugars, starch, natural fats and oils, etc.). [1]

In order to find alternative materials, researchers have developed fully biodegradable plastics. A fully biodegradable polymer is defined as a polymer that is completely converted by living organisms, usually microorganisms, to carbon dioxide, water and humic material. Other advantages of these materials over petrochemical plastics are that they are natural, renewable and biocompatible. [1] Biodegradable materials under development include polylactides, polyglycolic acids, Poly hydroxyalkanoates (PHAs), aliphatic polyesters, polysaccharides and their co-polymers and blends. Polyhydroxybutyrate or PHB are of particular interest because they possess thermoplastic characteristics and resemble synthetic polymers to a larger extent. They can be produced by biological systems (microorganisms, plants and animals), or chemically synthesized from biological materials (sugars, starch, natural fats and oils, etc.). PHB extracted from bacterial cells show material properties that are similar to polypropylene. [1] Polyhydroxybutyrate or PHB are linear polyesters produced in nature by bacterial fermentation of sugar or lipids. They are produced by the bacteria to store carbon and energy. Polyhydroxybutyrate or PHB is accumulated by numerous microorganisms and is the best characterized PHA. PHB was first discovered in bacteria. It is a unique intracellular polymer accumulated under stress conditions but with excess carbon source. During starvation, PHB serves as carbon and energy source and is rapidly oxidized thereby retarding the degradation of cellular components, combating the adverse conditions as in

rhizosphere.[2] As PHB are the reserve food material almost all microorganism produces PHA as intracellular inclusions under nutrient limiting conditions. Following are the genus of organisms which are involved in PHA production. *Alcaligenes, Axobacter, Bacillus, Beijerinckia, Chromobacterium, Clostridium, Defluviicoccus, Klebsiella, Micrococcus, Photobacterium, Ralstonia, Rhodobacter, Rhodopseudomonas, Rhodospirillum, Staphylococcus.* [3]

Materials and methods:

Isolation of Bacillus spps. :

Soil sample was collected from garden of Vivekenand Education Society's College of Arts, Science and Commerce, Chembur. 1 gram of soil was transferred to 100 millilitre (ml) of sterile saline aseptically. The mixture was then vortexed for even dispersion of microorganisms and was then pasteurized at 60° C for 60 minutes, was cooled and diluted and were isolated on sterile Nutrient Agar plates. The plates were then incubated at 30°C for 24 hours. [4]

Screening of the isolates for PHB production: All the bacterial isolates were qualitatively tested for PHB production following the viable colony method of screening using Sudan Black B dye. [5] All the five isolates were sub-cultured on Nutrient Agar slants with 1 % Glycerol and incubated at 30°C for 48 hours. Saline suspension of all the isolates were spot inoculated onto Nutrient Agar plates with 1 % glucose. *Escherichia coli* was used as negative control an *Bacillus subtilis* as a positive control. Ethanolic solution of (0.02%) Sudan Black B was spread over the colonies and the plates kept undisturbed for 30 minutes. They are washed with ethanol (96%) to remove the excess stain from the colonies.

Production of PHB:

A biphasic growth conditions were used for production of PHB. [6] 2 ml of 0.1 adjusted O.D. culture suspensions was aseptically transferred to a flask containing 25 ml of sterile Nutrient Broth and incubated at R.T. on Shaker for 24 hours. 10 ml of medium was centrifuged and the pellet obtained was aseptically transferred to a flask containing 25 ml of sterile Nitrogen deficient medium after once washing with sterile saline to remove any traces of the medium. The flasks were then incubated at R.T. on Shaker for 48 hours.

Extraction of PHB:

As PHB is produced intracellular it was extracted from the cell using two different extraction methods to select a method which supports highest PHB production.

Chloroform method: the cell biomass was separated from the culture medium by centrifuging the culture broth at 3000 rpm for 25 min. The washed cell biomass was then treated with sodium hypochlorite for 2 hours. This mixture was then centrifuged and the pellet that was obtained was washed with D/W, acetone and methanol. PHB was extracted from this by dissolving it in boiling chloroform and allowing the chloroform to evaporate. [7]

Diethyl ether method: the cell biomass was extracted by centrifugation at 3000 rpm for 20 min. The washed cell biomass was then treated with sodium hypochlorite for 15 min. The mixture was then centrifuged and PHB was extracted by treating the pellet that with di-ethyl ether and incubation for 15 min.

As the extracted PHB is converted to Crotonic acid by treating it with conc. Sulphuric acid, quantification of PHB was carried out using standard plot of Crotonic acid.

Result and discussion:

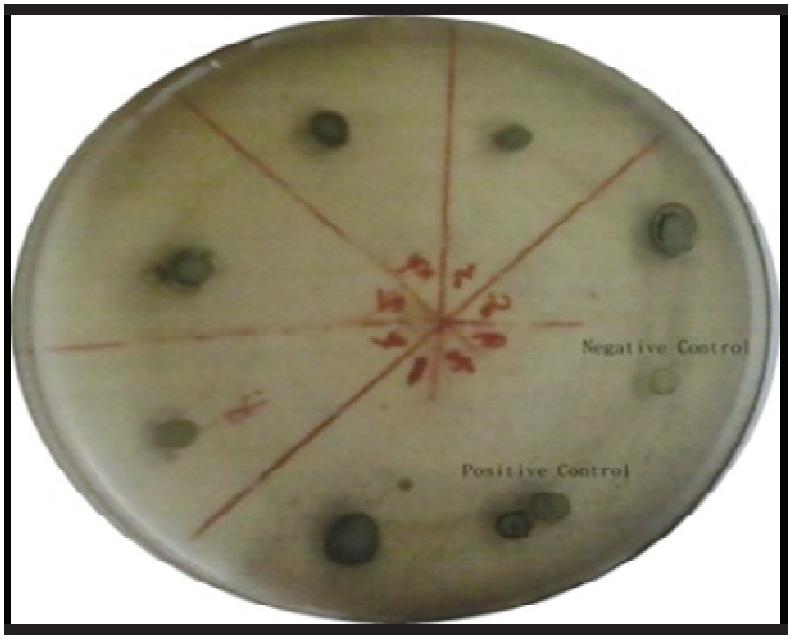
Isolation of bacillus spps. :

As the aim was to isolate only *Bacillus spps*. soil sample was pasteurized at 60°C for 60 minutes. Five different isolates were obtained from the soil sample and were coded as GS1, GS2, GS3, GS4, and GS5. The following images give the details of the growth obtained.

Screening of the isolates for PHB production:

A viable colony staining method was used to screen all the five isolates for their PHB producing abilities. All the five isolates were subjected for visual screening for PHB production using Sudan Black B (Fig1). The colour of the Sudan black B colonies were visually scored in comparison with that of the reference strain *Bacillus subtilis*. It was interesting to note that all the five isolates were found to accumulate PHB.

Fig. 1 Visual screening for PHB production using Sudan Black B



Quantification of PHB produced:

All the Sudan Black B positive isolates were subjected to quantification of PHB production. [8] The extracted PHB was dissolved in 10 millilitres of concentrated sulphuric acid (H_2SO_4). The addition of sulphuric acid converts the polymer into Crotonic acid which is brown in colour. The solution was cooled and absorbance was recorded at 235 nm using a U.V.-Visible Spectrophotometer against a sulphuric acid blank. 1 gram of PHB is equivalent to 1 gram of Crotonic acid. [3] So by referring to the standard plot, the quantity of PHB produce was determined. Both the extraction protocols were used for PHB extraction to select the method which is more efficient in PHB extraction. Di-ethyl ether method was found to be more efficient than Chloroform method in PHB extraction as all the five isolates gave a higher PHB yield using Di-ethyl ether method (Fig 2).

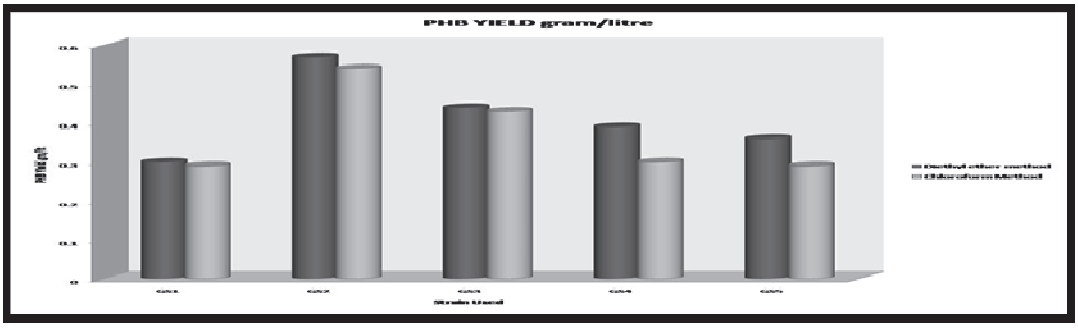


Fig 2. Comparative analysis of PHB production by diethyl ether method and Chloroform method.

A longer exposure to Sodium hypochlorite in Chloroform method might be the reason for poor extraction. Study conducted by Xuan et al. established that although sodium hydroxide (NaOH) was used in scl-PHA recovery from Gram negative bacterium, this treatment induced cell lysis leading to an increase in viscosity and loss of PHA. Heat treatment to coagulate the macromolecules is responsible for the increased viscosity, may aid recovery but a significant loss of polymer can still be expected. Hence Di-ethyl ether method is found to be effective in PHB extraction because neither is NaOH used for cell lysis nor is heat treatment for extraction and hence less loss of the polymer is expected. The results are as shown in table 1.

Table 1: Comparative analysis of PHB production by di-ethylether method and Chloroform method.

Strain Used	Di-ethylether method	Chloroform Method
GS_1	0.33	0.29
GS_2	0.57	0.54
GS_3	0.44	0.43
GS_4	0.39	0.30
GS_5	0.36	0.29

Summary: In present study, an attempt was made to do a comparative analysis of the most effective method for extraction of PHB from Bacillus spps. The salient features of the findings are outlined below

Soil sample was collected from VES college garden and five different isolates were obtained. All these were subjected for the detection of PHB accumulation by employing viable colony staining method using Sudan Black B stain.

All the five isolates were found to be PHB positive and then were subjected to quantitative estimation of PHB production. PHB yield varied from 0.29 gram/litre to 0.57 gram/litre.

Diethyl-ether method was found to be more effective for extraction as compared to chloroform method.

Conclusion: The accumulation of petrochemical plastic waste in the environment is an increasing problem. So with an interest to help mankind in an eco-friendly way, the present study was directed towards the production of Bioplastic from Bacillus *spps.* The main aim of the study was to do a comparative analysis for the most effective method for the extraction of PHB. The results obtained showed that Diethyl-ether method was found to be more effective for extraction as compared to chloroform method.

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Sagar Parmar/ Nagesh Malik/ Department of Microbiology/

V. E. S. College of Arts Science and Commerce/ Chembur/ Mumbai-400071/

India/drnageshmalik@yahoo.co.in