ORIGINAL RESEARCH ARTICLE

Accumulation of Poly-hydroxy-butyric Acid (PHB) by Bacillus Strain Isolated from Paddy Field of Kathmandu University Premises

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Abstract

In this study, the effect of applying nutrient limitation on the production of Poly-hydro-oxy-butyric acid (PHB) from soil bacteria was examined. PHB is a biodegradable polymer which provides a reserve of carbon and energy. PHB was extracted by chloroform dispersion method. The amount of synthesized PHB was determined as crotonic acid by spectrophotometry. We found that Nitrogen limiting condition stimulated PHB accumulation. The highest level of PHB accumulation was observed in DNB-6 strain which accumulated 31 % of the dry mass at 20 % glucose concentration. The probabilistic identification of bacteria by PIBwin software version 1.9.2 showed that the strain DNB-6 was close in nature to Bacillus cereus.

Keywords: Polyhydrooxybutyrate, Cell dry weight, Nutrient limitation, biodegradable, bioplastic.

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Introduction

Poly-hydro-oxy-butyric acid (PHB) is a member of a polymer belonging to a group polyhydroalkanoate (PHA). A wide variety of prokaryotic organisms have been shown to accumulate this polymer, including numerous heterotrophic and autotrophic aerobic bacteria, photosynthetic anaerobic bacteria, gliding bacteria, actinomycetes species, cyanobacteria [1].

PHB is a biodegradable thermoplastic which provides a reserve of carbon and energy and accumulates as intracellular granules when grown under nutrient limiting conditions [2]. Three distinct enzymatic reactions involved in the PHB biosynthetic pathway. The initial reaction involves condensation of two acetyl-COA molecules to form acetoacetyl-CoA, which is catalyzed by ß-ketothiolase encoded by *phbA*. This step is followed by the reduction of acetoacetyl-CoA by an NADPH- dependent acetoacetyl-COA dehydrogenase encoded by *phbB*. Then, the (R)-3- Hydroxybutyrl- COA monomers are polymerized into PHB by PHB synthase encoded by *phbC* (**Figure 1**) [3].

The synthetic plastics are made from petrochemicals which are not renewable. They do not readily biodegrade and often the collection and transport of this waste is difficult and expensive. Due to the non-biodegradable characteristics of petrochemicals-derived plastic materials much interest has been created in the development of biodegradable plastics. Biodegradation is chemical degradation of materials brought about by the action of naturally occurring microorganisms such as bacteria and algae [4].

Biodegradable plastics produced from renewable sources are considered a potential substitute for conventional petrochemical plastics because of their biodegradability and non-toxicity characteristics [1, 5-7]. PHB is one of the potential raw materials for producing biodegradable plastic. The PHB production capacities of bacteria have been investigated for possible application in industry. However, the use of PHB in industrial applications has been hampered mainly by their high production cost compared to petrochemical-based polymers [8,9]. The condition for bacterial PHB production can be met in soil, due to its heterogeneous nature. It may become a limiting factor for bacterial growth especially in some nitrogen poor (carbon-rich) sites. Soil *Bacillus* species have been shown to accumulate PHB during the sporulation of bacterial growth. PHB production by the isolate has been favoured by the glucose concentration [10].

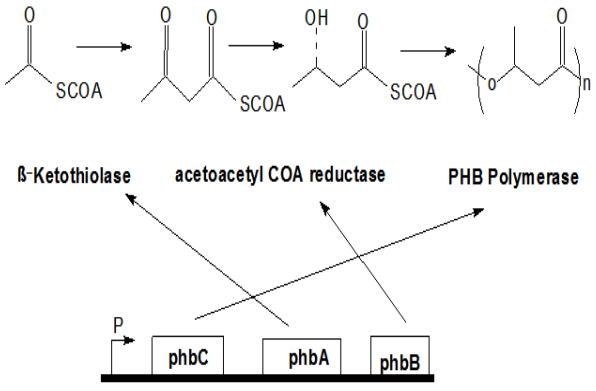

Figure 1: Biosynthetic pathway for P (3HB). P (3HB) is synthesized in a three step pathway by the successive action of B-ketoacetyl-CoA thiolase (PhbA), acetoacetyl CoA reductase (phbB), and P (3HB) polymerase (PhbC). The three enzymes are coded by the genes of the phbCAB operon. A promoter upstream of phbC transcribes the complete operon.

This study makes an effort to evaluate the PHB-producing efficiency of *Bacillus* species isolated from soil, located in the paddy field of Kathmandu University premises under nutrient limited conditions. The polymer has been extracted from a selected *Bacillus* species strain and characterized partially.

Materials and Methods

Isolation and purification of soil Bacteria

The soil sample for the isolation of bacteria was collected during the months of March to May. Soil Samples were collected from five different areas of paddy field of Kathmandu University premises. Fifteen soil isolates were named as (DNB-1 – DNB-15) and further screened randomly to check their PHB-producing efficiency. Each gram of the sample was suspended in 9 ml of sterile distilled water and shaken vigorously for 2 min. The sample was heated at 80 °C for 10 minutes in water bath. After heating, dilution of 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6} g/ml were prepared from the soil suspension for plate counts and spread on Nutrient Agar medium. After incubation at 30 °C for 24- 48 hours, serially diluted plates were picked up and examined microscopically. As standard practice, plates having 30-300 colonies were chosen for isolating the single bacterial colony.

The spore morphology, gram staining, motility and several biochemical tests were carried out to characterize the bacteria. The isolate was identified on the basis of comparison of these characters with those described in *Bergey's Manual of Determinative bacteriology* [11].

Screening for PHB production

To test the production of PHB, seed culture of isolated colonies were grown in 10 ml Nutrient broth and incubated for 24 hours at 30 °C. Later, the seed culture was transferred to 50 ml in 250 ml Erlenmeyer flasks with a 2% (v/v) inoculums and incubated at 30 °C with vigorous orbital shaking at 150 rpm. The culture was grown to a stationary phase and the cultures were examined by fluorescence microscopy after staining with nile red. It has been proved that nile red is an excellent vital stain for the detection of intracellular lipids. The cells were flame-fixed on slide glass, then a drop of 0.1 µg/ml nile red solution was added to the smear. After being heated over a flame for 1 second, the smear was covered with a cover-glass and examined under the fluorescence microscope. Nile red staining gives strong fluorescence [12].

PHB Analysis

Dry cell mass was treated with a dispersion containing chloroform and 80% Sodium hypochlorite in water at a 3:1 ratio. The mixture was then incubated at 30 °C for 1 hour and then centrifuged at 10,000 g for 10 minutes. Three different layers were formed. The upper phase was a hypochlorite solution, the middle phase contained non-PHB cell material and undisrupted cells, and the bottom phase was chloroform containing PHB. The upper phase was removed first with a pipette. The chloroform layer was also drawn using a pipette. The PHB was then extracted from the chloroform layer by slowly introducing the chloroform into ten volumes of ice cold methanol with continuous stirring.

Chemical Determination of PHB

Chloroform extract was dried at 40 °C and 10ml of concentrated sulphuric acid was added. Then they were heated at 100 °C in a water bath for 20 min. After cooling, the sample was transferred to a silica cuvette and the absorbance at wavelength of 235 nm is measured against a sulphuric acid as a blank.

Results

Of the fifteen samples evaluated for PHB production, DNB-6 isolate accumulated the maximum PHB (31.91%) of dry cell weight. The isolate was identified as a member of genus *Bacillus* when biochemical and morphological test was matched to *Bergey's Manual of Determinative Bacteriology*. Citrate utilization, growth pattern, carbohydrate utilization and number of other tests as summarized in the (**Table 1, Figure 2**). When the results of these tests were inserted in PIBwin software version 1.9.2, the strain DNB-6 was identified to close in nature to *Bacillus cereus* with ID score of 0.76.

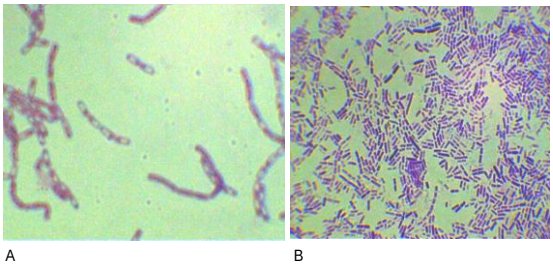

Figure 2 A, B: Gram staining and Endospore test. A: Gram staining of DNB-6, B: Endospore of strain DNB-6.

Growth curve analysis

The growth curve was plotted under nitrogen deficient media (NDM), phosphate deficient media (PHDM), and potassium deficient media (PDM), sulfur deficient media (SDM) **(Figure 3).**

Table 1: PBB production and characteristics of DNB-6

PHB production and characteristics of DNB-6 strain	
PHB % dry weight	31.96 %
Spore formation	central
Colony type	circular
Gram staining	Positive, rod
Casein hydrolysis	positive
Starch hydrolysis	positive
Motility test	positive
Nitrate reduction	positive
Catalase test	positive
Oxidative fermentation	positive

It was observed that the DNB-6 showed good growth at nitrogen deficient media **(Figure 3).** The growth curve was plotted up to 72 hours in 6 hours interval of time at 20% glucose concentration **(Figure 3).**

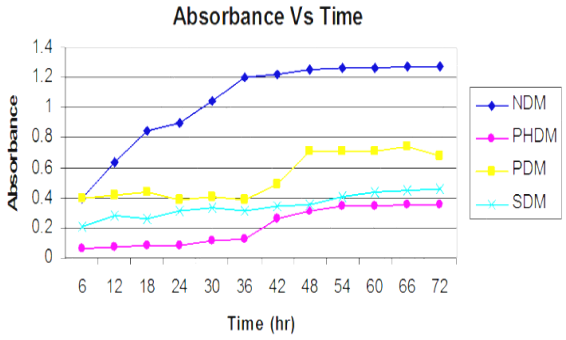

Figure 3: Comparison of Growth curve of DNB-6 under nitrogen deficient media (NDM), Phosphate deficient media (PHDM), Potassium deficient media (PDM) and sulfur deficient media (SDM) at 20% Glucose concentration.

PHB yield analysis

Production of PHB was maximum at 48 hours (**Figure 4)** when DNB-6 strain was subjected to nitrogen limiting at 20% glucose concentration.

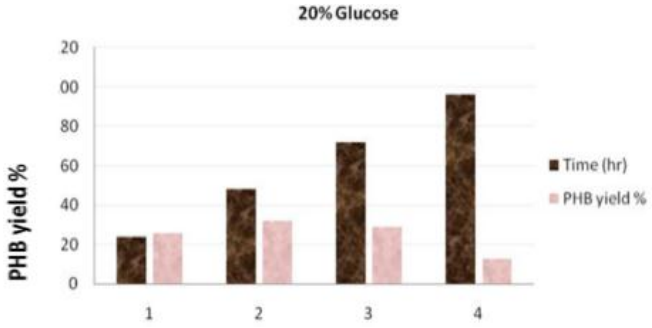

Figure 4: Time profile of PHB accumulation under nitrogen deficient media at 20 % Glucose concentration (1: 24 hr, 2: 48 hr, 3: 72hr and 4: 96hr).

The intensity of fluorescence was checked at different time interval under nitrogen limitation by fluorescence microscope (**Figure 5A, 5B, 5C, 5D).** Time profile of growth and PHB production accumulation have indicated that maximum PHB production during starting of stationary phase and degraded during late stationary phase.

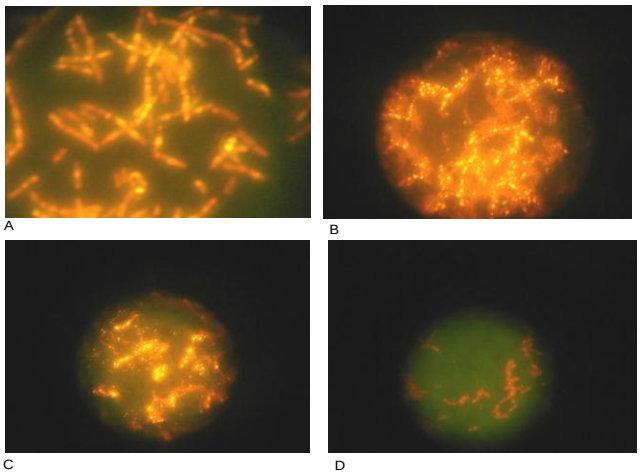

Figure 5: PHB intensity under fluorescent microscope. **A:** PHB intensity at 24 hours of strain DNB-6, **B:** PHB intensity at 48 hours of strain DNB-6, **C:** PHB intensity at 72 hours strain DNB-6 **D:** PHB intensity at 96 hours of Strain DNB-6.

Discussion

Growth curve analysis showed that PHB was a growth associated product and its accumulation is significantly increased when all cultures reached from exponential phase to till-stationary phase. Different intensity of PHB obtained at different time also coincided with the PHB yield at different time. The analysis of PHB yield was carried out in nitrogen deficient media because the growth curve as well as PHB intensity observed under this condition was better than in other conditions. The bacteria were harvested at 24 hours, 48 hours, 72 hours and 96 hours. The yield percentage of PHB was maximum at 48 hours at 20% glucose concentration. This reflected that by this time the bacteria had already entered the stationary phase. It was observed that the bacteria start accumulating PHB granules in its stationary phase (Anupam et al., 1999). This high yield of polymer could be because of using glucose as the carbon source. *Bacillus cereus* when grown on structurally unrelated carbon such as fructose, sucrose and gluconate has produced interesting other polymer beside PHB [13].

There was variability in PHB accumulation at different time profile. The highest PHB producing efficiency was found in isolate DNB-6. The maximum PHB yield was 31.91% of dry weight under nitrogen deficient media at 20% glucose concentration. The recovery process was carried by chloroform dispersion method. The PHB yield was parallel to the bacterial growth.

In conclusion, soil bacterial isolate found were capable of accumulating PHB as an energy material.

The present study showed that the maximum PHB production was under nitrogen limitation condition which agrees well with other study that suggests PHB production was maximum under nutrient limiting condition.

Acknowledgements

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Author Contributions

Assistant Prof. Sangita Shakya designed and supervised the project. Deepak Upreti prepared the manuscript. Deepak Upreti, Naresh Prasad Sapkota and Bibek Aryal share the equal amount of contributions in this work.

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