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Production of Poly (3-hydroxybutyrates) by Bacillus species isolated form Soil

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Abstract: Aliphatic polyester, PHB [poly3-hydroxybutyrate] was discovered and identified as a granular component in bacterial cells. PHB can grow in a wide variety of natural environments and is the reserve polymer (intracellular granules) found in many types of bacteria found in nature, e.g. in soil, sea water, sewage sludge or compost. In the present study, *Bacillus megaterium* and soil isolates were used to study the production of PHB granules under varied conditions of nutrients and cultural characteristics. The reference organism produced 83.78% of PHB when cultured for 24hrs. The effect of light and dark phases on growth was studied and it was evidenced that the light phase reported maximum growth of bacterium at a pH 6.0. The influence of nutrient sources on growth resulted in enhanced the growth of *B. megaterium* when supplemented with 4ml of 5% glucose. Further, screening of soil resulted in a Gram negative and positive bacillus capable of storing nearly 75.22 and 79.59% PHB when compared to *B. megaterium* (83.78%). Finally, from the work it can be emphasized that the efficient way of producing PHB was found to be pH 6.0 and exposing the medium to continuous light phase. However, supplemental addition of nutritional sources implicates that the addition of glucose enhances PHB production than the nitrogen or phosphate sources.

Key Words: PHB, *Bacillus megaterium*, polyhydroxyalkanoate, bacterial growth, optimization

Introduction

Plastics play a major role in our everyday lives. From water bottles to prosthetics, plastics can be seen everywhere. However, these oil-based polymers take many years to degrade, which poses an environmental problem in some areas; to overcome this, production of environmental-friendly plastics are been discovered [1].

In response to the problems and harmful effects of plastic wastes on the environment there has been considerable development of bio-related plastic materials. Among the various biodegradable polymer materials, polyhydroxyalkanoates (PHA's) are attractive substitutes for conventional petrochemical plastics because of their similar properties to various thermoplastics and elastomers, and complete degradability upon disposal under various environments. PHA is a biodegradable, biocompatible which is regarded as potentially useful polyester replacing petroleum-derived thermoplastics [1, 2]. PHB, is the well known member of the PHA series of polyesters, accumulates in many bacterial species as a carbon or energy storage material similar to sugar [2].

PHB is a commonly found substance and readily biodegradable aerobically and anaerobically. Microbes can use. PHB exists in the cytoplasmic fluid in the form of crystalline granule about 0.5 μm in diameter. β-hydroxybutyrate is connected by ester linkage and form PHB [3]. This can be extracted from the cells as native granule or by solvent extraction [4-6]; and processed in the same way as polypropylene. PHB is used in food packaging, plastic films, surgical sutures, controlled drug

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deliver, etc [7-8]. This bioplastic has many applications in bone plates, nails, screws (Azher et al; 2003) and in the treatment of Osteomyelitis [8].

These biopolymers were also found to increase the resistance of bacteria [9]. Lemoigne, Grelet & Croson (1950) drew attention to the different amounts of poly-beta-hydroxybutyrate obtained by *B. megaterium* on different media, and Marace & Wilkinson (1958) showed that more of this substance was formed as the glucose concentration of the growth medium was increased; the subsequent depletion of the product during the later stages of growth suggested a storage function. They also showed that organisms rich in poly-beta-hyroxybutyrate had a slower rate of autolysis than organisms poor in poly-beta-hydroxybutyrate. It is probable; therefore, that poly-beta-hydroxybutyrate acts as a reserve carbon and energy source. Tinelli (1955) found that the major part of the material was metabolized at sporulation and deduced that the two processes were intimately connected [10].

The bacterium capable of producing PHB has been identified in more than 20 bacterial genera, including Azatobacter, Bacillus, Beijernickia, Alcaligenes, Pseudomonas, Rhizobium and Rhodospirillum [11]. Many researchers have explained that soil bacteria generally produce PHB. PHB production increases if ambient conditions (pH, temperature, nutrients) are made available [8].

Materials and Methods

Organisms

Standard organism *Bacillus megaterium* MTCC 453 were obtained from the Microbial type culture collection, Chandigarh, India. The PHB producing capability of the organism was confirmed by Sudan black staining method [12]. The identification of the bacterial isolates with the ability to degrade PHB was performed on the basis of macroscopic and microscopic examination and biochemical tests. The isolates were identified macroscopically by examining colony morphology; surface pigment, shape, size, margin, surface on nutrient agar plates and microscopic examination including; Grams staining to study the staining behaviour and cell arrangement and granulation. Spore staining was also performed.

Biochemical tests

The biochemical characterizations of the isolates were performed by employing:

Carbohydrate fermentation, Catalase and Oxidase tests.

Table 1: Preliminary identification of isolates

Characteristics	Bacilli	Cocci
Gram staining	+	+
Spore staining	+	+
Oxidase	+	+
Citrate	+	-
Catalase	+	+
Starch hydrolysis	+	-
Formation of PHB	+	+
Motility	Motile	Motile
Glucose utilization	+	+
Fructose utilization	-	-
Citrate utilization	+	-
Nitrate reduction	+	-

Media

Nutrient agar medium used for strain cultivation consisted of : Peptone -5 g/L, Meat extract -1 g/L, Yeast extract – 2 g/L, Sodium chloride - 5 g/L, Agar -15 g/L and pH was adjusted to - 7.0 . Each liter of nutrient broth used for PHB production has the same composition as that of nutrient agar medium except Agar.

Optimization Tests

PHB production was monitored using different sources rich in respective components which might enhance gain in PHB dry weight. The various test employed in optimization method are:

Determination of Dry Cell Mass (DCM):

This was performed by growing bacterial isolates from soil in nutrient broth under the tested conditions. After 24 hours of incubation at 37°C, 10 mL of culture was taken from the conical flask into centrifuge tube separately. The cell pellet was washed with phosphate buffer and re centrifuged.

Supernatant was discarded and pellet was washed twice with sterile deionised water and then dried at 100°C for 24hrs. The dried material was incubated at 60 °C for 1hr with 5% (v/v) sodium hypochlorite and centrifuged at done at 6000rpm, 28-31 °C for 15 minutes. And PHB was extracted using acetone-alcohol method.

Optimization of nutritional conditions:

The isolates were incubated at 37^0C for 48 hrs with varying concentrations of Carbon (Glucose), Nitrogen (Ammonium sulphate) and Phosphorus source (Phosphoric acid) and the % production of PHB was tested at varying p^H (6, 6.5, 7and 7.5), physical conditions such as Light, Dark, Light and Dark phases to investigate their effect on dry cell weight (DCW) and PHB accumulation.

Staining methods

For the identification of soil organisms Grams staining was performed. Sudan black stain (Sudan black B powder 0.3g, 70% ethyl alcohol 100 mL) was used to show microbial intracellular lipid. In this staining, lipid inclusion granules are stained blue-black or blue-grey, whilst the bacterial cytoplasm is stained light pink and spores are stained by malachite green method [13].

Screening for PHB accumulation

Samples were collected from domestic area, Vishakhapatnam, India. Samples were screened for PHB producing bacteria from this natural carbon rich location. Initially, bacteria were isolated on Nutrient agar (NA) and the resulting bacterial colonies were used to screen poly-β-hydroxybutyrate (PHB) accumulating strains for PHB production, and staining with Sudan black B. PHB producing bacterial strains were selected based on morphological characters such as form, size, color and texture following the methods as described elsewhere [14]. Strains were routinely maintained on nutrient agar slant at 4^{o} C.

Preliminary selection of efficient PHB producer was made in our laboratory from among the different isolates obtained. The selection was based on the abundance of PHB granules in microscopic observation and PHB yield.

Extraction of PHB

After 24 h incubation at 37^{o} C, 10 mL of culture was taken and centrifuged at 10000 rpm for 15 min. the supernatant was discarded and the pellet was treated with 10mL of sodium hypochlorite and the mixture was incubated at 30^{o} C for 2hrs. After incubation, the mixture was centrifuged at 5000rpm for 15 min and then washed with distilled water, acetone, methanol respectively for washing and extraction.
After washing, the pellet was dissolved in 5mL of boiling chloroform and was evaporated by pouring the solution on sterile glass tray kept at 4^{o} C. After evaporation, the powder was collected for further analysis [15].

Recovery of PHB

Extraction by acetone-alcohol

Cell mass (g/L) obtained after 48 h growth in Nutrient agar was harvested by centrifugation at 10,000 rpm for 10 min and lysed by sodium hypochlorite at 37^{o}C for 1 h. contents were re-centrifuged at (10,000 rpm for 10 min) and the lysed cell mass was sequentially washed with distilled water, acetone: alochol (1:1) followed by precipitation in boiling chloroform (10ml). The precipitate was allowed to evaporate at room temperature to drive PHB in powder form. Percent production of PHB was calculated by using the formula

% of PHB = Total weight of PHB / Total weight of Pellet X 100

Extraction by dispersion and non-solvent precipitation

Harvested biomass (1g) was treated with a dispersion solution containing 50ml of chloroform and 50 ml of 5% (V/V) sodium hypochlorite solution. Mixture was incubated at 4,000 rpm for 10 min. Three separate phases namely, the upper phase [hypochlorite solution]; the middle phase [non-PHB cell material and undisrupted cells]; and the bottom phase [chloroform containing PHB] were obtained. PHB from the chloroform phase was recovered by filtration and non-solvent precipitation method comparing (7:3 V/V) of methanol and water.

Results and Discussion

Light-dark phases

Continuous light phase study was carried out under room temperature and the flasks were kept under fluorescent tube lights at a height of 2 meters. The flasks for dark cycle were incubated at 37^{0} C in an incubator.

Light-dark cycles are maintained as follows. The flasks were exposed to light under room temperature for a period of 4hrs and kept in dark for the next 4hrs in incubator at 37^{0}C. Optical density was measured for each of the conical flasks after 24, 48 and 72 hours respectively (Table-2).

Table 2: OD values showing effect of Light and Dark phases on growth of bacteria

	OD at 570nm (24 hr culture)	OD at 570nm (48 hr culture)
Light	0.52	**1.13**
Dark	0.35	1.08
Light & Dark	0.48	1.02

Response to p^{H}

Different pH concentrations such as 6.0, 6.5, 7.0 and 7.5 were maintained under continuous light phase to test the effect of pH on growth of bacillus. From Table-3, given below, it was evidenced that pH 6.0 favors better growth of *B. megaterium*

Effect of nutrient sources on growth

Effect of different concentrations of glucose as carbon, ammonium sulphate as nitrogen and phosphoric acid as phosphate sources were tested on the growth of bacterium.

Table 3: OD values showing effect of various p^H concentrations on growth of bacteria

p^H range	OD at 570 nm	
	24 hrs	48 hrs
Control	0.348	1.126
6.0	0.482	**1.159**
6.5	0.500	1.025
7.0	0.473	0.975
7.5	0.288	1.106

Table 4: OD values showing effect of carbon, nitrogen and phosphorous on growth of bacteria

Source	Hrs	2%		5%	
		2ml	4ml	2ml	4ml
Carbon	24	0.532	0.417	0.642	0.504
(glucose)	48	1.164	0.960	1.219	**1.362**
Nitrogen	24	0.364	0.274	0.195	0.104
(NH4)2SO4	48	0.876	0.680	0.803	0.596
Phosphorous	24	0.012	0.012	0.010	0.009
(H3PO4)	48	0.310	0.281	0.250	0.278

Form Table 4, it was found that the glucose as carbon source showed maximum O.D 1.362 at 570 nm and hence further experiments were carried out under continuous light phase at pH 6.0 with 4ml of 5% glucose as additional carbon source with 48hr incubation.

Production of PHB by soil isolates

In our study, *Bacillus megaterium* produced maximum PHB percentage and three isolates reportedly accumulated PHB granules as reserve material (Table 5). A gram -ve bacilli produced 75.22% PHB whereas two gram +ve bacilli produced 68.81 and 79.59% under optimized conditions. After 48 hrs, there was a decrease in PHB yield and increase in viscosity of the medium which might be due to the unfavorable conditions such as production of extracellular metabolites, depletion of essential nutrients in the medium or self utilization of PHB by bacteria due to inadequate nitrogen and carbon sources in the medium.

Table 5: PHB content of some bacillus strains from soil

Strains	Dry Cell Weight (g/l)	PHB (g/l)	Yield of PHB (%)
B. megaterium	1.11	0.93	83.78
Bacillus spp. +ve	2.35	0.51	21.70
Bacillus spp. -ve	2.99	0.24	8.03
Bacillus spp. +ve	0.95	0.47	49.47
Bacillus spp. -ve	2.08	0.77	37.02
Bacillus spp. -ve	1.13	0.85	75.22
Bacillus spp. -ve	2.22	0.12	5.41
Bacillus spp. +ve	1.09	0.75	68.81
Bacillus spp. -ve	1.11	0.34	30.63
Bacillus spp. +ve	3.02	0.12	3.97
Bacillus spp. +ve	0.98	0.78	79.59
Bacillus spp. -ve	3.13	0.56	17.89
Bacillus spp. +ve	2.24	0.12	5.36

Conclusion

The study presented here and the data obtained suggest that the gram +ve *Bacillus* spp. is capable of accumulating PHB up to 79% of dry cell weight which may be of interest as potential industrial organism under optimized conditions such as pH 6.0, continuous light phase with 5% glucose as additional carbon source and incubation for 48hrs.

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