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Production of poly- β-hydroxybutyrate (phb) from Bacillus cereus

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Research Article

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INTRODUCTION

Plastics and polymers are an integral part of our daily existence. However, because of their properties of durability and resistance to degradation, they accumulate in the environment at the rate of about 25 million tons per year[1,2]. These form 8% by weight and 20% by volume of the landfills [3]. They inhibit enzyme activities due to highly hydrophobic in nature. The low surface area of plastics with inherent high molecular weight further compounds the problem[4]. Environmental problems associated with usage and especially dispersal of plastics has stimulated the formulation of legislations regulating polymer use. With increasing public and political awareness, and to satisfy the environmental imperatives vast research has been directed towards finding suitable substitutes that are biodegradable besides retaining the desired properties of the conventional plastics. Biodegradable polymers from renewable point of sources are of particular interest, which fit into the ecological cycle[5]. Since the potential for utilizing biological systems as a source of biodegradable materials is becoming increasingly attractive, polyhydroxyalkanoates have gained importance, as they possess properties close to polypropylene.

The chemical nature of these biopolymers is first studied in Bacillus megaterium that exist as inclusion bodies[6,7] and named them as poly-3-hydroxy-butyricacid P (3HB). This compound became more popular due to studies on several Bacillus strains[8] and phototropic bacteria[9]. The identification of polyhydroxyalkanoates other than P (3HB) especially poly-3-hydroxyvalerate P (3HV) and poly-3-hydroxyhexanoate P (3HHx)[10]. Accumulation of 95% P(3HB), 3% poly-3-hydroxyheptanoate P(3HHp), 2% of an 8-carbon hydroxyalkanoate (HA) and trace amounts of 3-other HA compounds by batch grown Bacillus megaterium using capillary gas chromatography was reported[11].

Poly-3-hydroxy-butyric acid has limited applications due to its brittle nature. Incorporation of other 3HA significantly enhanced the biopolymer properties which signified the beginning of the second stage of research on PHAs. Production of P (3HB-co-3HV), a co-polymer of industrial importance, took place during this stage. The emphasis now shifted to identification of all the 3HAs that could be associated with the bacterial polyesters. During this period it became clear that not only Gram negative but also a wide range of Gram positive bacteria, cyanobacteria (aerobic photosynthetic), non-sulfur and sulfur purple bacteria (anaerobic photosynthetic), archaea can synthesize and accumulate these 3HAs.

The present study reports on isolation and identification of an efficient biopolymer producing bacterium.

MATERIALS AND METHODS

Screening for biopolymer producing bacteria

1 gm of soil samples, collected from starch industrial effluent sites around Hyderabad, India, were suspended in different 100 ml sterile distilled water flasks and serially diluted up to 10-8. The samples were agitated for 10 minutes on a shaker and 0.2 ml of samples were spread on mineral salts medium plates containing (g/l) Sucrose 20.0; KH2PO41.0; (NH4)2SO4 2.0; MgSO4 7H2O 0.2; CaCl2 2H2O 0.02; FeSO4 7H2O 0.01. To the above solution, 1 ml of trace solution containing (g/l) ZnSo4 7H2O 0.2; H3BO3 0.6; MnCl2 4H2O 0.06; CoCl2 6H2O 0.4; CuSO4 4H2O 0.02; NaMoO4 2H2O 0.06 was added. The pH of the

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medium was adjusted to 6.5 ± 0.2. The plates were incubated at 350C for 48 hours. The isolated colonies with different colony morphologies were picked and the cultures were purified by repeated streaking on mineral salts medium plates and finally pure cultures of isolates were maintained on nutrient agar slants at 40C.

Table 1: Preliminary characterization of bacterial isolates obtained during this study.

S.No	Grams Nature	PHB production
1	Gram positive *Bacilli*	7.69%
2	Gram negative *Bacilli*	5.0%
3	Gram positive *Bacilli* with endospore	20.28%
4	Gram positive *Cocci*	Not detected
5	Gram negative *Cocci*	Not detected
6	Gram negative short *Bacilli*	6.0%

Table 2: Morphological and some physiological studies of efficient PHB producing Bacteria isolated during this study.

S.No	Morphological characters	Results
1	Cell shape	Rod
2	Motility	Random Motility
3	Endospore	Positive, subterminal and oval
4	Colony shape	Minute, mucoid, round and raised.
5	Optimum temperature	350–370 C
6	Optimum pH	6.5–7.0

Analytical methods

Bacteria obtained from the above study were screened for PHB accumulation capability. The PHB accumulated in all isolates were assayed using spectroscopic method[12].The isolates were separately inoculated in to 50 ml mineral salts medium broth in 250 ml conical flasks and were incubated on rotary shaker at 350C with 225 rpm /min for 48 hours. 2 ml of culture was collected at intervals of 24 and 48 hours and centrifuged at 10000 rpm for 15 minutes. The supernatant was discarded; the pellet was washed twice with distilled water and centrifuged at 10000 rpm for 15 minutes. The final pellet thus obtained was dried in hot air oven at 600C for 24 hours. After drying, the total bacterial dry cell weight was determined. The dried cells were lysed by incubating with sodium hypochlorite for one hour at 600 C. Lysed samples were then finally subjected to centrifugation at 10000 rpm for 15 minutes and supernatant was collected in a sterile test tube. To the collected supernatant, 5 ml of 96% ethanol and 5 ml of acetone (1:1) was added. 10 ml Chloroform was added in to each tube to extract PHB. The chloroform was then evaporated at room temperature so that, the PHB granules appear as crystals. Finally, 10 ml of Conc. H2S04 was added and heated for one hour at 600 C to convert PHB into crotonic acid. Crotonic acid formed was measured at 235 nm against sulphuric acid blank using UV- visible spectrophotometer (UV-160A Shimadzu). The amount of PHB per gram dry weight of bacterial cells was determined using a standard curve of PHB (Sigma).

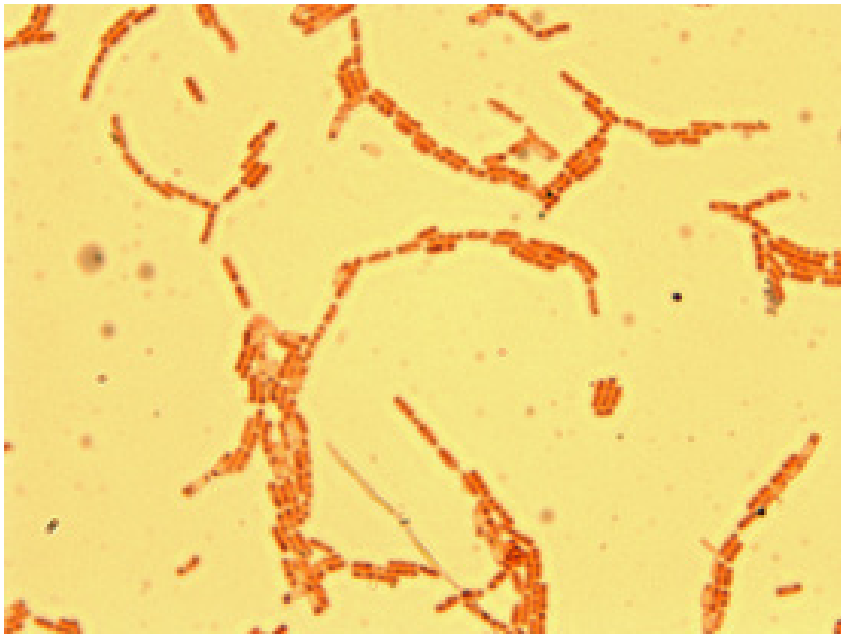

Figure 1: Sudan black staining of PHB accumulated in *Bacillus cereus* in 24 hours.

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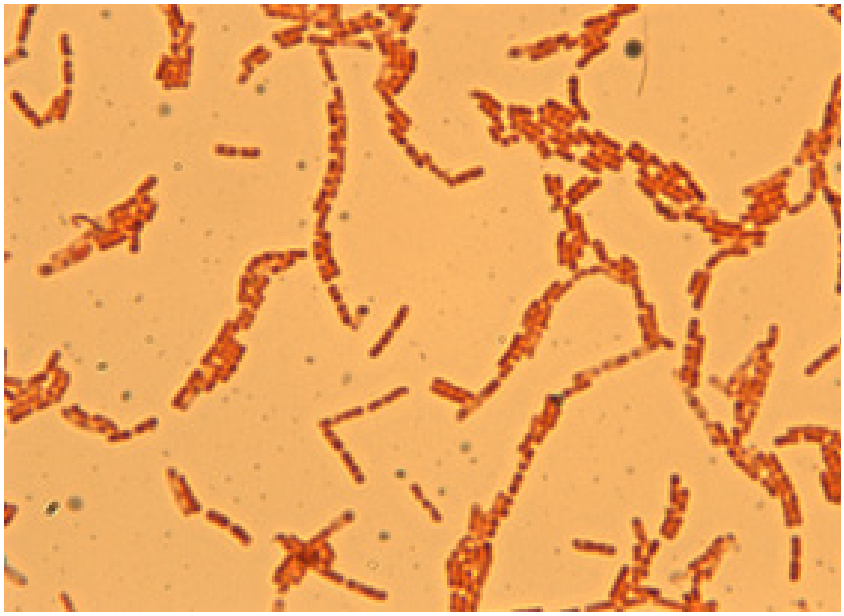

Figure 2: Sudan black staining of PHB accumulated in *Bacillus cereus* in 48 hours.

Morphological and biochemical characterization

Preliminary characteristics of the bacteria capable of accumulating PHB were studied by observing morphological characteristics and the efficient bacterium among them was further identified by studying biochemical characteristics as per Bergey's manual of determinative bacteriology[13].PHB granules accumulated in the bacterium were observed by sudan black –B staining[14].

Figure 3: Transmission electron micrograph of PHB accumulated in *Bacillus cereus*.

RESULTS AND DISCUSSION

Screening of soil samples resulted in obtaining 18 colonies, of which, six colonies showed different colony morphologies and were selected for further characterization. Preliminary morphological characterization and the PHB accumulation efficiency of these six bacterial isolates is given in Table 1. PHB accumulation of a Gram positive endospore forming Bacillus was observed to be more than the other five isolates in Table 1. Cocci isolated during this study, failed to accumulate PHB under the tested conditions. The morphological and some other growth characteristics of efficient PHB producing isolate is given in Table 2. The isolate gave positive result for methyl red, citrate, lysine decarboxylase and ornithine decarboxylase and negative result for urease, nitrate reduction, tryptophanase and Voges-proskauer test.

Based on the morphological and biochemical characterizations, the efficient PHB producing bacterial Isolate was identified as *Bacillus cereus*.

Sudan black –B staining of PHB accumulated in Bacillus cereus was observed as round stained granules, considered to be fat bodies prominently in almost all the rods from the samples collected at 24 hours (Figure 1). However, samples collected at 48th hour showed more prominent PHB granules (Figure 2). Transmission electron micrograph of the PHB accumulated in the

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bacterium is shown in Figure 3.

Polyhydroxyalkanoates (PHAs) are aliphatic polyesters naturally produced via microbial process on Carbohydrates based medium, where they act as carbon and energy storage material in bacteria. They were the first biodegradable polyesters to be utilized in plastics. The two main members of the PHA family are polyhydroxybutyrate (PHB) and polyhydroxyvalerate (PHV). Poly β-hydroxybutyrate (PHB) accumulates as energy reserve material in many micro-organisms like Alcaligenes, Azotobacter, Bacillus, Nocardia, Pseudomonas, Rhizobium, etc[15]. PHB is accumulated during the stationary phase of growth by these organisms and used later as an internal reserve of carbon and energy[16,17]. PHB is an energy storage material produced by a variety of bacteria in response to environmental stress and nutrient imbalance[18]. PHB exists as discrete granules in the cell cytoplasm with an average number of 8-12 granules per cell and are typically of 0.2-0.5μm in diameter and possess a membrane coat of 2-3nm thick[19]. Because PHB is biodegradable, there is considerable interest in using PHB for packaging purposes as opposed to other plastic materials in order to reduce the environmental impact of human garbage [20-22].

CONCLUSION

Many Bacillus species have been reported to accumulate PHB at 9%–44.5 % dry cell weight similarly; in the present study we have isolated *Bacillus cereus* that has the capacity of accumulating 20.28% PHB. The isolate PHB accumulation efficiency can be enhanced by optimizing physiological conditions. The PHB yield can also enhanced by subjecting the isolate to various mutagens and selecting a mutant with higher PHB accumulation than the wild type. B. cereus is ubiquitous in nature and an opportunistic pathogen, often associated with two forms of human food poisoning, characterized by either diarrhoea and abdominal distress or nausea and vomiting. The organism produces an emetic toxin and entero toxin responsible for these symptoms. To the best of our knowledge, we are first to report a novel strain of Bacillus cereus with considerable efficiency in accumulating PHB. Further optimization studies are understudy which can promise better yields of PHB from this bacterium.

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