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Isolation, Screening and Characterization of Polyhydroxybutyrate (PHB) Producing Bacteria from Landfill Soil

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A B S T R A C T

Today, environment is polluted with waste which is not degradable. Waste placed at landfill dumping site which contain plastics account about 20% by volume of municipal solid wastes and reduce the capacity of precious landfill sites. Poly hydroxyl butyrate (PHB) which is a biodegradable and biocompatible thermoplastic compound has broadly similar physical properties to poly (propylene). It has many applications in medicine, veterinary practice, tissue engineering materials, food packaging and agriculture due to its biodegradability. In the present study, we focus on screening of bioplastics producing bacterial isolates from the soil sample from dumping site. The bacteria were characterized by their cultural characteristics, morphology and biochemical test. PHB producing bacterial isolate was further detected by using *Sudan black- B* staining reagent. Attempt was made to produce and extract PHB by using our bacterial isolate. Isolate exhibited significant PHB yields, thus showing a potential for further exploitation. Further analyses are currently ongoing to try to extract and characterize PHB granules.

Introduction

Plastic is one of the materials are relatively inert and cannot be degraded in natural environment, unlike wood, paper, natural fibres or even metal and glass. Plastics which thrown into river, ocean and other water made pollution occur. Furthermore, it is danger to environment if plastic is burned because it contains toxic chemical substance. In a survey among the developed countries, in average of 398 kg of domestic waste are generated annually by each person. But now, societal concerns and a growing awareness throughout the world have triggered a product and processes which contributed and loss of environment quality. Extensive use of petroleum-derived plastics (approximately 269 million tons used globally in 2015) increases the environmental concerns of nonbiodegradable wastes, including contamination with small fragments of toxic compounds leaching out of landfills into ground water and the emission of greenhouse gases and other organic pollutants during the degradation process (Bernard, 2014). Consequently, environmental concerns have prompted research into the development of the utilization of biodegradable polymer alternatives to petroleum based plastics. Among the biodegradable plastics, polyhydroxyalkanoates (PHAs) are highly attractive as their properties are similar to conventional plastics and include biodegradability, apparent biocompatibility, and manufacturing from renewable resources (Shah *et al.,* 2008). PHAs are lipid-like, water-insoluble, polyester molecules that are synthesized and accumulated as intracellular granules for energy reservation by a variety of microorganisms under unbalanced growth conditions, normally in the presence of excess carbon with a limitation of at least one essential nutrient such as nitrogen, phosphorus, sulfur, or oxygen (Bernard, 2014). Among the PHAs, poly(3-hydroxybutyrate) [P(3HB), PHB] is the best characterized PHA (Pena *et al.,* 2014) that can be synthesized by various bacteria, including grampositive *Bacillus megaterium, B. subtilis, B. thuringiensis*, and *Corynebacterium glutamicum* and gram-negative *Cupriavidus necator* (formerly known as *Alcaligenes eutrophus* or *Ralstonia eutropha*), *Azotobacter vinelandii,* and *Pseudomonas mendocina* (Pal *et al.,* 2009; Pena *et al.,* 2014; Chanasit *et al.,* 2016; Hassan *et al.,* 2016). The PHB polymer has very similar properties to petroleum polymer, but this polymer degrades completely into carbon dioxide and water under aerobic conditions. To date, PHB has been used in the packaging industry, agriculture, the food industry, and recently in the medical and pharmaceutical fields (Pena *et al.,* 2014). The production of PHB at an industrial scale is achieved by using gram-negative bacteria that have been reported to contain outer membrane lipopolysaccharide (LPS) endotoxins (Chen and Wu, 2005; Tan *et al.,* 2014).

Among the PHA-producing gram-positive bacteria, Bacillus spp. produce and accumulate various monomer compositions of PHAs and have been reported to be ideal hosts for PHB production (Valappil *et al.,* 2007; Moorkoth and Nampoothiri, 2016)

Materials and Methods

Sample collection and isolation of pure cultures

Soil samples were collected aseptically from Pirana Landfill dumping site at Ahmedabad, Gujarat, India. One gram of soil 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^{-5}, followed by spread plating of 100µl 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%) (Borah *et al.,* 2002).

Primary screening of PHB producing bacteria

Detection for PHB production was employed by using lipophilic stain Sudan Black B (Schlegel *et al.,* 1970). 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, ammonia production test, sugar utilization test, catalase test, oxidase test, urea hydrolysis test, starch utilization test and oxidative-fermentative test, starch hydrolysis test, geletin hydrolysis test, lipid hydrolysis test, dehydrogenase test, triple sugar iron test and litmus milk test. All tests were carried out using standard protocols proposed by (Cappuccino and Sherman, 1992).

Biopolymer production by isolated bacteria

PHB production was carried out by inoculating 1% of PHB positive isolate into production media. The composition 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 (Mikkili *et al.,* 2014). Production media was incubated at 37oC for 72 hrs in incubator shaker.

Extraction and quantification of PHB

PHB was extracted from the isolate by using the sodium hypochlorite method (Cappuccino and Sherman, 1992). All the Sudan black- B positives isolate were subjected to quantification of PHB production as per the method of John and Ralf method (John and Ralf, 1961). According to this method, 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. Quantification was done by using the following formula.

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

Results and Discussion

In this work, we investigated the potential presence of microorganisms able to synthesize the biopolymer PHB from pirana landfill dumping soil in a unique environmental area of where this species is endemic and preserved.

Isolation of PHB producing bacteria by Sudan Black -B staining

Six different colonies obtained 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 6 bacteria, 1 was found to be Sudan positive, i.e. it was capable of producing lipid granules which could have the presence of PHB. A number of *Bacillus* sp. has been reported to accumulate 9–44.5% DCW PHB (Borah *et al.*, 2002).

Characterization of PHB producing isolates

PHB producing bacterial isolate was further characterized by Gram staining, morphological and biochemical tests as shown in Table 1, 2 and 3. Isolate was Gram positive, rod shaped, spore former and identified as *Bacillus sp.* Morphology of isolate is presented in figure 1 and 2. This positive result is explained by the activity of the lipophilic endospore membranes that allow the Malachite Green to cross the membrane and to retain the green coloration (Oktari *et al.*, 2017).

Table.1 Cultural characteristics of Isolate

Characteristics	Results
Size	medium
Shape	round
Elevation	Pulvinate
Margin	Entire
Consistency	Moist
Pigmentation	Nil
Appearance	Normal
Odor	Odorless
Surface	Smooth

Table.2 Results of Gram's staining

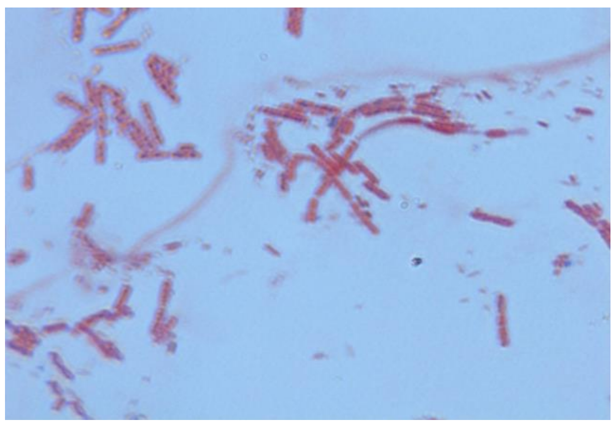
Characteristics	Results
Size	Medium
Shape	Bacilli
Arrangement	Single/chain
Gram reaction	Gram positive

Table.3 Biochemical characterization of Isolate

Sr. No.	Biochemical test	Results
1	Methyl red test	Positive
2	Voges proskauer test	Positive
3	Citrate utilization test	Positive
4	Indole production test	Negative
5	Urea hydrolysis test	Negative
6	Nitrate reduction test	Positive
7	Ammonia production test	Positive
8	Starch hydrolysis test	Positive
9	Geletin hydrolysis test	Negative
10	Lipid hydrolysis test	Negative
11	Dehydrogenase test	Negative
12	Triple sugar iron test	Positive
13	Litmus milk test	Negative

Fig.1 Morphology of Isolate

Fig.2 Black color granules of isolated bacteria were seen by sudan black staining



Extraction of PHB producing isolate

In the present study, we have noticed that the bacterial isolate was able to produce substantial amounts of PHB 21% during growth using the simplified LB media. *Bacillus*, that showed similar morphological characteristics and was able to accumulate 60% of intracellular PHB [21].

In the present study showed that isolation of Biopolymer producing bacteria *Bacillus sp.* which has been identified and characterized from the pirana dumping site. Among the three soil samples which were used gave the isolated single positive and high amount of PHB was accumulated. The production of PHB was found to increase along with the increase in the biomass. Further studies are required to optimize the growth media to improve the PHB yield and to reduce the cost of production media along with suitable PHB induction media components.

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