Research Article

Screening of Biodegradabale Polymer (Poly-B-Hydroxybutyric Acid) Producing Bacteria from Soil and Study on the Effect of Different Carbon Sources on Production

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ABSTRACT

Plastics play a major role in our everyday lives and can be seen everywhere, from water bottles to prosthetics. However, these polymers take many years to degrade and pose environmental problems. To overcome this, the production of environmental-friendly plastics is of great interest. Bioplastics has physical properties similar to the synthetic plastic and are of biological origin and could be used as substitutes for petroleum derived synthetic plastics because of their similar physical and chemical properties. They are completely biodegradable too. PHB, is the well-known member of the PHA series of polyesters is the reserve polymer in the form of intracellular granules found in many types of bacteria. These bacteria grow in a wide variety of natural environments e.g. in soil, sea water, sewage sludge or compost.

In the present study, an attempt was made to isolate efficient PHB producing bacteria from soil. PHB accumulators were screened based on viable colony staining method using Sudan Black B and were subjected to quantitative estimation of PHB. From the selected promising bacterial isolates, one deep PHB accumulator was selected and identified. The morphological, biochemical characteristics and colony characteristics of the organism suggested that it as a Bacillus species. The studies on the effect of different carbon sources on the PHB production by the selected bacteria, by analyzing the concentration of PHB produced indicated that maltose and sucrose favored maximum accumulation of PHB by this isolate.

Keywords: *Polyhydroxyalkanoates*, PHB, Plastics, Sudan Black

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INTRODUCTION

As Synthetic plastics are of petrochemical origin and are non-degradable, they cause waste disposal problems and leads to environmental pollution and create great havoc to environment. Although plastic have many advantages and applications, its non-biodegradability and recalcitrant nature towards microbial degradation [1] is a major drawback, which forced us to think upon a material which can replace plastic. This has directed the scientists towards alternative plastic sources that are environmentally friendly, biologically degradable under appropriate conditions, and can be obtained from cheap sources such as waste products, microorganisms, etc [2]. Bioplastics are polymers produced by microorganisms and are biodegradable [3].

Among the various biodegradable polymer materials, polyhydroxyalkanoates (PHA's) are prospective substitutes for conventional petrochemical plastics because of their similar properties to various thermoplastics and elastomers, and complete degradability upon disposal under various environments. PHA are polyesters that are synthesized by certain gram positive as well as gram negative microorganisms intracellularly as energy storage particles [4]. PHA has physical properties similar to the synthetic plastic and could be used as substitutes for petroleum derived synthetic plastics because of their similar physical and chemical properties. They are completely

biodegradable too. Polyhydroxybutyrate (PHB) is the best categorized PHAs [5].

PHB, a member of the PHA series, is a biodegradable and biocompatible thermoplastic produced by various microorganisms such as *Azotobacter, Bacillus, Archaebacteria, Methylobacteria, Pseudomonas, Haloferax, Vibrio, etc*. as a carbon or energy storage material and is readily biodegradable. It can be moulded and spun into fibers, can be polymerized into plastics [6]. It could be used in food packaging, plastic films, surgical sutures, controlled drug delivery, etc [7]. PHB has various applications in different areas like medicine, drug manufacture, agriculture and various industrial purposes. Some of the major applications of PHB are in drug delivery in developing medical sutures, bone marrow scaffold, tissue engineering devices and agriculture products [8, 9]. PHB is produced by microorganisms in the form of reserved food granules under stress condition like excess availability of carbon source but limited provision of other nutrients such as nitrogen, phosphate, oxygen and sulphur.

Several studies are reported on the screening of PHB producing bacteria and optimization of culture conditions for maximal production and isolation of PHB [10, 11, 12, 13, 14, 15]. The present study involves the screening of several soil bacterial isolates for PHB accumulation and preliminary studies for optimizing the cultural condition for PHB accumulation in a selected strain of bacteria.

MATERIALS AND METHODS

Chemicals

All the chemicals used in this study were of analytical grade procured from reputed Indian manufacturers.

Collection of samples

Soil samples of different consistency were collected from various sources and used for the isolation of bacteria. Dry sand, damp garden soil, dry garden soil, Plant organic matter rich soil and animal organic matter rich soil were taken for the isolation of PHB producing bacteria.

Isolation of bacteria from different samples

1g of soil sample collected were serially diluted in sterile distilled water and plated on nutrient agar medium supplemented with 1% glucose and incubated at room temperature for 24-48 hrs. The predominant bacterial colonies were picked up, and preserved on nutrient agar slants till further use.

Preparation of crotonic acid standard curve

Standard curve of PHB was prepared following the method of Slepecky & Law [16]. Pure PHB when dissolved in 10 ml concentrated H_2SO_4 and heated for 10 minutes it will get converted into crotonic acid. Various concentrations of crotonic acid were prepared in concentrated H_2SO_4 and absorbance was read at 235 nm against a sulfuric acid blank. Standard graph was plotted between concentration of crotonic acid and OD at 235 nm.

Rapid screening of native bacterial isolates for PHB production

For rapid screening of PHB, all the bacterial isolates were tested for PHB production by viable colony method of screening using Sudan black B stain [17]. Nutrient agar medium supplemented with 1% glucose was sterilized and cooled to 45^{o}c. The medium was poured in to sterile petriplates and allow to solidification. The plates were taken and divided in to 4 equal parts and in each part a bacterial isolated was spotted. The plates were incubated at room temperature for 24-48 hrs. After incubation, ethanolic solution of Sudan Black B solution (0.02%) 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. The dark blue colonies were taken as positive for PHB production. All the positive isolates were assigned by code numbers based on their source of isolation. The assays were done in duplicates.

Quantification of PHB production in selected isolates: by isolating and quantifying PHB

All the Sudan Black B positive isolates were subjected to quantification of PHB production as per the method of John & Ralph [18]. The representative bacterial colonies inoculated in to 5 ml of nutrient broth and incubated for 24 hr at room temperature. After incubation the bacterial cells containing the polymer were pelleted at 1500 rpm for 10 min, and the pellet washed with saline and recentrifuged to remove the unwanted materials. The pellet

was suspended in equal volume of sodium hypochlorite (2 ml) and chloroform (2 ml) and the mixture incubated at room temperature for 24 hr. The whole mixture was again centrifuged and 0.5 ml of chloroform layer separated by using micro pipette without disturbing the mixture. The polymer granules were dissolved in hot chloroform. The test tubes were incubated to evaporate the chloroform after that concentrated 10 ml H_2SO_4 was added, and boiled at100^o C for10 minutes. The addition of sulfuric acid converts the polymer into crotonic acid which is brown coloured. The solution was cooled and the absorbance read at 235 nm against a sulfuric acid blank. By referring to the standard curve, the quantity of PHB produced was determined. The assays were done in duplicates.

Quantification of PHB production in selected isolates: by Sudan black staining of broth culture

The 13 organisms isolated were inoculated in to the test tubes which containing 3 ml of nutrient broth media and incubated at room temperature for 24 hours. Each assay was done in duplicates. After incubation, Sudan black solution (0.02%) added to the tube and kept undisturbed for 30 minutes. After that the tubes were centrifuged at 1500 rpm for 10 minutes, the stained cells were washed with sterile saline and the pellet suspended in saline was read at 600 nm using cell pellet suspended in saline without the Sudan black staining as blank. During incubation, the cells having PHB granules will take up the Sudan black and the colour could be measured using a calorimeter at 600 nm.

Characterization of the selected PHB producing bacterial isolate

The selected, most efficient PHB producing bacterial isolates were subjected to a set of morphological, physiological and biochemical tests for the purpose of identification.

The thirteen potent PHB accumulating strains were examined for their colony morphology, cell shape and gram reaction as per the standard procedures given by Barthalomew & Mittewer [19]. The colony characters viz., shape, colour, elevation, surface appearance, etc were observed on Nutrient agar medium. Gram staining, Endospore Staining and Motility test were performed.

Various Biochemical tests such as Catalase test, Oxidase test, Indole production test, Methyl red test, Voges Proakauer test (VP), Citrate utilization test, Nitrate reduction test , etc were carried out as per the method given by Cappuccino & Sherman [20] with 24 hr old cultures.

Detection of PHB production in the isolates was also done following fluorescent staining method using acridine orange. The appearance of yellow coloured granules when observed under fluorescent microscope at 460nm inside the cell indicated PHB production.

Effect of different carbon source on PHB production by the Selected Bacterial isolate

The selected bacterial isolates were grown in test tubes containing 5 ml nutrient broth with different carbon sources viz., glucose, fructose, sucrose, maltose, starch, glycerol, coconut oil, sodium acetate and trisodium citrate at 1 per cent level. The tubes were incubated at room temperature for 24 hours. After incubation, PHB produced by the isolates were quantified spectrophotometerically by the method of John & Ralph [18].

Briefly, after incubation the bacterial cells containing the polymer were pelleted at 1500 rpm for 10 min, and the supernatant discarded. The pellet was suspended in equal volume of sodium hypochlorite and chloroform (2 ml) and the mixture incubated at room temperature for 24 hr. The whole mixture was again centrifuged and 0.5 ml of chloroform layer separated by using micro pipette without disturbing the mixture. The polymer granules were dissolved in hot chloroform. The test tubes were incubated 24hr to evaporate the chloroform after that concentrated 10 ml H_2SO4 was added, and boiled at 100^o C for10 minutes. The addition of sulfuric acid converts the polymer into crotonic acid which is brown colored. The solution was cooled and the absorbance read at 235 nm against a sulfuric acid blank. By referring to the standard curve, the quantity of PHB produced was determined. The assays were done in duplicates.

RESULTS AND DISCUSSION

Isolation of Bacteria from various soil samples

Five different soil samples (dry sand, dry garden soil, damp garden soil, animal organic matter rich soil and plant organic matter rich soil) were collected and about 41 bacteria were isolated on nutrient agar fortified with glucose (1%), purified and maintained as pure cultures. All the isolates were given code numbers based on the soil of origin.

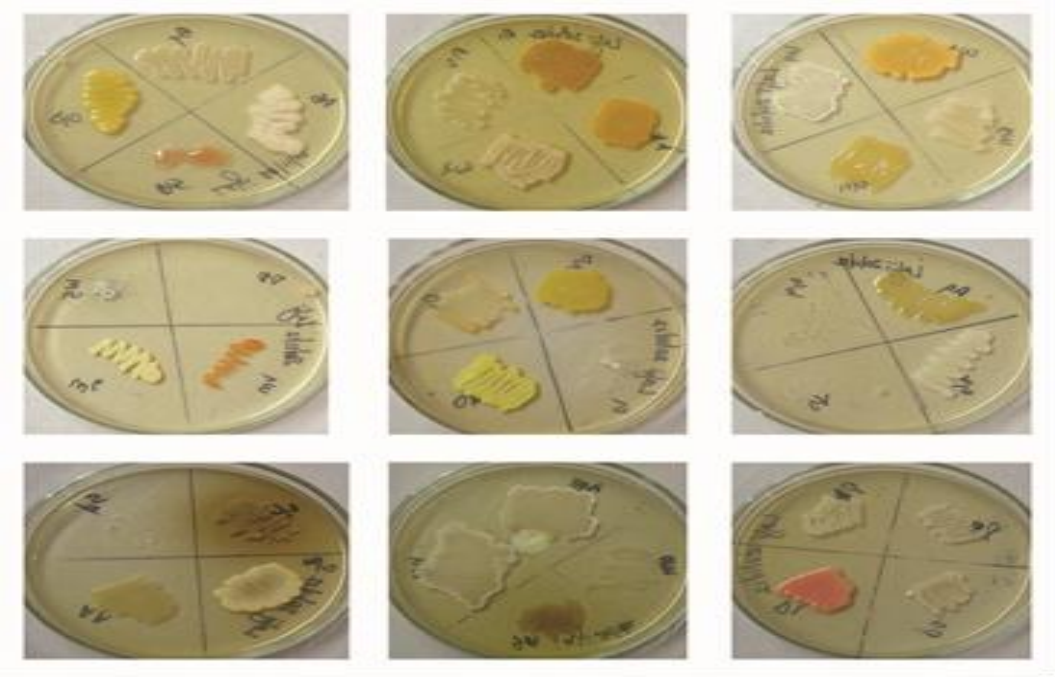

Figure 1: Colonies of isolates from various soil samples

Screening of the Isolates for PHB Production

All the 41 isolates were subjected for visual screening for PHB production using Sudan black B. It was observed that out of 41 isolates, 13 accumulate PHB (**Fig. 2**).

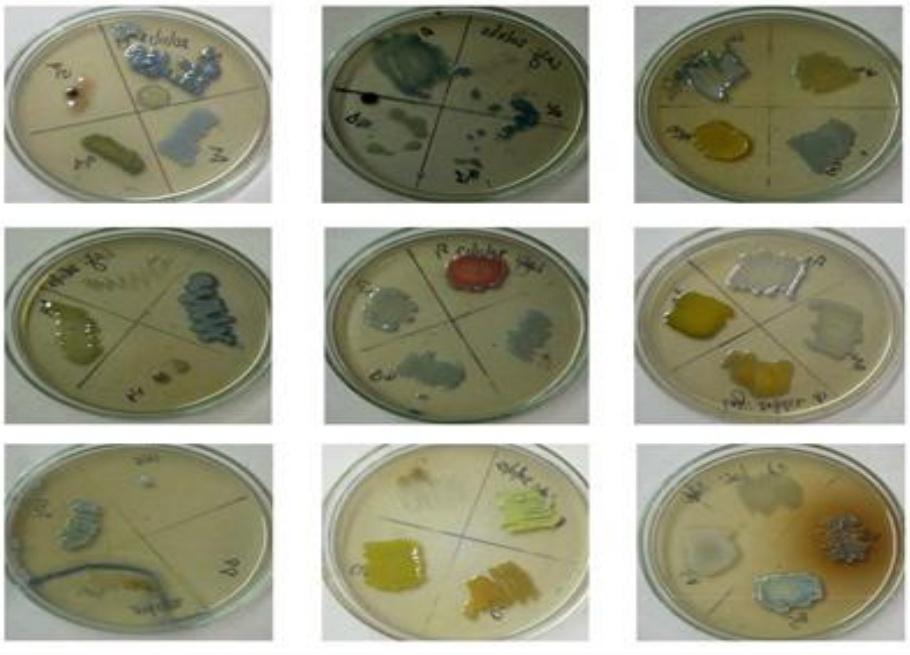

Figure 2: Screening of the Isolates for PHB Production by Sudan black staining

Selection of high level PHB accumulator from the screened isolates – Sudan Black staining of broth culture

When the 13 isolates were screened by Sudan Black staining of broth culture, a few isolates (9 Nos) showed maximum absorbance compared to others indicating maximum staining of cells which was further indicative of higher level accumulation of PHB in these isolates. The isolates that showed maximum accumulation are given in (**Table 1**).

The data given in (**Table 1**) indicated that the isolates S1, S2, S5, S6, W1, W3, W9, A8 and D6 gave maximum PHB accumulation as shown by Sudan black staining of cells.

Table 1: Selection of high level PHB accumulator from the screened isolates by *Sudan Black staining of broth culture*

Isolate	OD at 600 nm
S_1	0.99
S_2	0.62
S_5	0.86
S_6	0.80
W_1	0.71
W_3	0.68
W_9	1.23
D_6	0.79
A_8	0.59

Selection of high level PHB accumulator from the screened isolates – Quantitation of Isolated PHB using crotonic acid standard graph

The level of PHB was quantitated by isolating from broth cultures and using crotonic acid standard curve. The results showed that the isolate from S_2 contained about 0.221 µg of PHB equivalent crotonic acid /ml. S2 had produced a dry granule like growth and this might be the reason for the comparatively lesser calorimetric reading after sudan black staining of S2 grown in broth.

Selection of high level PHB accumulator from the screened isolates – Sudan Black staining of Agar culture

In (**Fig. 3**), the rightmost one is isolate S2, which was selected for further studies since it was the deepest accumulator of PHB, maintained as pure culture and used for further studies.

Figure 3: Five deep accumulators as seen after Sudan Black staining

Characterizations of the selected PHB accumulator isolate (S2)

White**,** round opaque, irregular colonies were seen on nutrient agar medium and upon staining they were found to be Gram positive motile and spore forming bacteria.

This organism was indole negative, methyl red negative, voges proskauer negative, citrate negative, nitrate negative, TSI positive, catalase positive, oxidase negative and fermented glucose, maltose, mannose, lactose and fructose with the production of acid but no gas production.

The biochemical characteristics and colony characteristics of the organism suggested that it may be a Bacillus species.

The appearance of yellow coloured granules inside the cell was seen after acridine orange staining when observed under a flourescent microscope and this indicated PHB accumulation inside the cell.

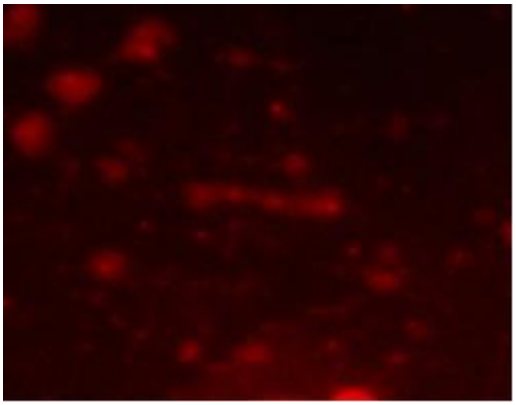

Figure 4. Bacteria showing PHB granules (Yellow coloured granules inside the cell) following acridine orange staining

Effect of different carbon sources on PHB production by isolated organism

Table 2: Effect of different carbon sources on PHB production by the selected bacteria

Carbon source	Concentration equivalent to crotonic acid (µg/ml)
Starch	1.6
Glycerol	1.48
Glucose	1.36
Coconut oil	0.70
Fructose	0.26
Sucrose	**1.75**
Trisodium citrate	1.53
Sodium acetate	1.6
Maltose	**1.75**

The studies on the effect of different carbon sources on the PHB production by the selected bacteria, by analyzing the concentration of PHB produced indicated that maltose and sucrose favored maximum accumulation of PHB (**Table 2**).

Poly-β- hydroxybutyrate (PHB) is a degradable biopolymers, produced by several bacteria under unfavorable conditions. PHB can be used instead of plastic to solve one of the greatest problems facing the environment, the persistence in the environment and non-biodegradability. PHB can be used for making biodegradable, biocompatible, and thermoprocessible plastic materials [10]. The present study aimed to isolate and characterize PHB-producing bacteria from different soil sources and optimizing the production of PHB on different carbon sources.

CONCLUSION

The present study was carried out to isolate bacterial isolates from different soil samples. Dry sand was found to contain more PHB producers compared to other soil samples. Screening was done based on viable colony staining method using Sudan Black B and quantitative estimation of PHB and one deep PHB accumulator was selected and identified to be a Bacillus species. The studies on the effect of different carbon sources on the PHB production, by analyzing the concentration of PHB produced indicated that maltose and sucrose favored maximum accumulation of PHB by this selected bacteria compared to other tested carbon sources.

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