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Isolation, Screening and Selection of Efficient Polyhydroxybutyrate (PHB) Synthesizing Bacteria

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Polyhydroxybutyrate (PHB) are polyesters of hydroxyalkanoates synthesized by various bacteria as intracellular carbon and energy storage compounds and accumulated as granules in the cytoplasm of cells. This biological macromolecule with wide range of applications in pharmaceutical, packaging, and different personal hygiene products. The present study deals with the enhanced production of PHB producing bacteria from soil were isolated, characterized and screened by Sudan black and Nile red staining methods. Screened organisms were subjected to the basis of morphological, biochemical and 16S rRNA analysis, the isolates were provisionally identified as *Bacillus safensis* EBT1 (JX679410), *Thermus thermophilus* EBT2 (KC684277), *Bacillus halodurans* EBT3 (KC008602), *Rhodocyclus purpureus* EBT5 (KC768770) and *Bacillus megaterium* EBT7 (JX870045). The strain EBT1 showed higher amount of PHB accumulation when compared to the other strains and was comparable with that of the other isolated strains. The PHB inclusions in cells were identified and affirmed using Fluorescent microscopy and FTIR analysis respectively.

Keywords: Polyhydroxybutyrate, Isolates, Sudan black, Nile red, Fluorescent microscopy.

A fully biodegradable polymer is defined as a polymer that is completely converted by living organisms, usually microorganisms, to carbon dioxide, water and humic material. Biodegradable materials under development include polylactides, polyglycolic acids, polyhydroxyalkanoates (PHAs), aliphatic polyesters, polysaccharides and their co-polymers[1-3]. Amongst these microbially-formed polyhydroxyalkanoates (PHAs) is the most suitable for significant contributions as bioplastics or biodegradable plastic. Poly-hydroxyalkanoates (PHA) have attracted a lot of attention recently as biodegradable thermoplastics, and poly-hydroxybutyrate (PHB) is one of PHA and it is the best known[4,5]. PHA can be synthesized by over 30% of soil-inhabiting bacteria. Many bacteria in activated sludge, in high seas, and in extreme environments are also capable of making PHA. In the last 10 years, PHA has been developed rapidly to find applications in various fields. In nature, prokaryotic microorganisms respond to sudden increases in essential nutrients in their usually hostile environment by storing important nutrients for survival during prolonged period of starvation[6,7]. PHAs are one such storage compound. PHAs are usually produced when carbon sources are in excess. The carbon sources are assimilated, converted into hydroxyalkanoate (HA) compounds and finally polymerized into high molecular weight PHAs and stored as water insoluble granules in the cell cytoplasm. PHAs are an excellent storage compound because their presence in the cytoplasm, even in large quantities does not disturb the osmotic pressure of the cell[8,9]. Polyhydroxybutyrate (PHB) is a polymer or family of PHAs that was first isolated and characterized

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in 1926 by French microbiologist Maurice Lemoigne as a constituent of bacterium *Bacillus megaterium*. Since then PHB has been shown to occur in a variety of taxonomically different groups. Most of the organisms are capable of accumulating PHB up to 30–80% of their cellular dry weight. PHB is produced by microorganisms apparently in response to conditions of physiological stress[10, 11]. The polymer is a primarily product of carbon assimilation (from glucose or starch) and is employed by microorganisms as a form of energy storage molecule to be metabolized when other common energy sources are not available. Microbial biosynthesis of PHB starts with the condensation of two molecules of acetyl-CoA to give acetoacetyl-CoA, which is subsequently reduced to hydroxybutyryl-CoA[12, 13]. This study aims at studying the capability of a locally isolated bacterium for high production of biopolymer such as polyhydroxybutyrate. This is an effort towards development of natural plastics from bacteria, which have potential to replace synthetic plastics and thereby in the long run eliminate the non-degradable plastics.

MATERIALS AND METHODS

Isolation of microorganism

Biomass for PHB accumulation was collected in the form of Activated sludge from sewage treatment plant (STP) in Nesapakkam, Chennai belonging to dairy and food processing industry. PHB producing bacterial biomass was subjected to selective enrichment by aeration in synthetic medium containing (g/L of distilled water) 20 glucose, 0.574 $(NH_4)_2HPO_4$, 1.5 K_2HPO_4, 0.4 $MgSO_4.7H_2O$, and trace element solution, 1ml (stock solution (mg ml^{-1}) of 25 Na_2SO_4, 25 $FeSO_4.7H_2O$, 4.40 $MnSO_4.4H2O$, 0.079 $CuSO_4.5H_2O$, 73.4 $CaCl_2.2H_2O$). The enriched bacterial biomass was subjected to serial dilutions. 10^{-5} and 10^{-6} dilutions were plated on nutrient agar medium (Peptone 5 g/L, Yeast extract 3 g/L, Sodium chloride 5 g/L, Glucose 1 g/L, Agar 18 g/L, pH 7.0 (30 °C) by spread plate method. The isolated Strains were sub cultured in nutrient broth and pure cultured on nutrient agar medium. The same nutrient broth was used as growth media for all the isolates till determination of potent strain and evaluation of optimum nutrients.

Pretreatment

The collected samples were placed in sterile container and stored at 4 °C. Each gram/ml of the sample was suspended in 9 ml of sterile distilled water and mixed well for two minutes. The samples were heated at 60 °C for 60 min in water bath and then serial dilution technique was performed for the isolation of different bacteria. Saline: Sodium Chloride- 0.85g, distilled water - 100 ml. The ingredients were dissolved in distilled water and sterilized at 121 °C at 15 lbs for 15 minutes. Nutrient agar medium: Peptone - 0.5 g, Yeast extract - 0.5 g, NaCl - 0.5 g, Agar - 2 g, distilled water - 100 ml, pH - 7.0. The ingredients were dissolved in distilled water and sterilized at 121 °C at 15 lbs for 15 minutes[14].

Sudan Black B Staining

Purified strains were screened for PHB accumulation using Sudan black B staining. Prepare a thick smear from the culture suspension in a clean glass slide. The smear was air dried and heat fixed on a glass slide and then stained with a 3 % Sudan Black B (w/v in 70 % ethanol, Sigma) solution for 10 minutes. Safranin Stain: The Safranin powder (0.5 g) was separately weighed and mixed with 100 ml of distilled water and stored in amber bottle for further use. The smear was washed with distilled water and then allowed to react with safranin solution for 10 seconds. The smear was then rinsed with distilled water and air dried. Stained samples were observed under oil immersion at 1000x magnification with direct bright-field illumination using an Olympus CX31 microscope with Lumenera infinity 2 CCD camera.

Fluorescent staining method

Nile red and Nile blue A staining were used to develop simple and highly sensitive staining method to detect PHB directly in growing colonies. These dyes were directly added to the DMSO to give a final concentration of 0.5 mg dye (ml medium)$^{-1}$. 10 ml of 48 hours old culture of the isolates was transferred to an eppendorf tube containing of Nile red (“sigma”) and incubated for 30 minutes at 30 °C. After the incubation period, the culture was centrifuged at 4000 rpm, for 5 min. The pellet was collected and resuspended in distilled water. Nile red stained smear under fluorescent microscope at 465 nm excitation[15].

Preparation of Inoculum

The primary inoculum was prepared in

Luria Bertani medium (Peptone -1g, Yeast extract - 1 g, $NaCl_3$ -1 distilled water - 100 ml, pH - 7.2 ±0.2 in 250 ml conical flask containing 50 ml of sterile medium and inoculated from the stock culture. The fresh overnight culture was used as an inoculum for production of polyhydroxybutyrate. The ingredients were dissolved in distilled water and sterilized at 121 °C at 15 lbs for 15 minutes. The positive isolates showing PHB granules were inoculated in production medium[16]. 100 ml of production medium was prepared in 250 ml conical flask and sterilized. 1 % inoculum was transferred aseptically into the production medium and incubated at 37 °C for 48 hours. The medium was agitated at 100 rpm for better aeration and growth of the organism.

Methodology for molecular work

Biochemical tests

The identification of the bacterial isolates with the ability to produce 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. Eight different tests were performed in selected PHB producing bacteria; namely catalase test, indole production test, citrate utilization test, urease, Starch hydrolysis, Gelatine - liquefaction, Casein hydrolysis, carbon source utilization tests for their biochemical characterization.

16S rRNA Sequence analysis

Sequence alignments provide a powerful way to compare novel sequences with previously characterized genes. Both functional and evolutionary information can be inferred from well-designed queries and alignments. The bacterium was characterized and identified by 1.4 kb of 16S rRNA gene sequencing using universal primers as distinguished by Solaiman *et al.*, 2005[17]. The 16S rRNA sequence was analysed using BLAST -Basic Local Alignment Search Tool sequence analysis tool available in NCBI (National centre for Biotechnology Information http:// www.ncbi.nml.nih.gov), provides a method for rapid searching of nucleotide and protein databases. Since the BLAST algorithm detects local as well as global alignments, to test the regions for similarity with other 16S rRNA sequences may provide important clues to the function of uncharacterized nucleotides and proteins.

Phylogenetic tree analysis

Phylogenetic tree analysis is the technique of methodically demonstrating an evolutionary relationship between species. This method is useful to determine whether group of genes are related through a process of divergent evolution from a common ancestor or the result of convergent evolution. In the present study a phylogenetic tree was constructed using BLAST tree tool. The sequence was analysed using Molecular Evolutionary Genetics Analysis (MEGA) version 5 and the corresponding Phylogenetic tree was constructed.

Growth media

Nutrient agar medium used for strain cultivation consisted of: Peptone -5 g/L, Yeast extract -3 g/L, Sodium chloride -5 g/L, Glucose -1 g/L, Agar -18 g/, distilled water- 1000 ml and pH was adjusted to 7.0. Each litre of nutrient broth used for PHB production has the same composition as that of nutrient agar except Agar.

PHB assay

The PHB assay was carried out using the method of Law and Slepecky (1961) [18].The pellet was lyophilized and digested with 30 % sodium hypochlorite solution at 37ÚC for 30 min. The sample was then centrifuged at 8,000 rpm for 30 min and washed sequentially with distilled water (5x), acetone (5x), and ethanol (5x) before being dissolved in chloroform (5x). The chloroform was allowed to vaporize completely at room temperature, and the sample was further treated with concentrated H_2SO_4 and incubated at 100ÚC for 30 min. Absorbance of the resultant solution was measured at 235nm using (Schimadzu UV- 1601, Japan) with crotonic acid as a standard.

Analytical methods

Cell dry weight was determined gravimetrically. Culture samples in 10 ml aliquots were centrifuged at 3000 ×g for 20 min and the pellet was washed twice with distilled water. The cells were transferred to a dry pre-weighted petridish and dried to a constant weight at 110°C.

Characterization of PHB

The presence of different functional

groups in PHB was checked by Fourier Transmission Infra-Red spectroscopy. Extracted PHB (2 mg) and standard PHB from Sigma (2 mg) were dissolved in 500 ìl of chloroform and layered on NaCl crystal. After the chloroform was evaporated, the PHB polymer film was subjected to FTIR analysis[19].

RESULTS AND DISCUSSION

Isolation of PHB accumulator

The sewage treatment plant contributes a real unbroached resource for novel bacteria and particularly the biopolymers producing strains. And this investigation was directed to isolate a diverse range of PHB producers from various samples of sewage treatment plant with the hope of identifying different classes of PHB accumulators. Totally 30 types of bacteria were isolated from activated sludge and contaminated soil were screened for PHB accumulation. In all, 30 representative microorganisms were isolated on nutrient agar amended with glucose (1 %), purified and maintained as pure cultures.

Screening of the isolates for PHB production

All the 30 isolates were subjected for microscopic screening for PHB production using Sudan black B and Nile red fluorescent staining. It was observed that out of 30 isolates as many as seven were found to accumulate PHB. It was interesting to note that all the 7 strains were isolated from activated sludge shows positive for PHB production. Based on the presence of more lipophilic inclusions observed in Sudan black B staining and the high intensity of fluorescence detected in viable Nile red staining the potential PHB producer was identified. Five strains show the deeply dark purple granules of PHB for Sudan black B staining (Figure. 1) and granules were observed as reddish orange fluorescence at an emission wavelength of 465 nm for Nile red staining (Figure. 2). Out of 7 isolates five were strong accumulators and the rest 2 were poor accumulators of PHB. The positive bacteria were assigned the code number depicting the place of the research laboratory. Legends are EBT1, EBT2, EBT3, EBT5 and EBT7.

Molecular identifiction of the isolates

The five active PHB accumulators was then identified and confirmed by morphological, biochemical characteristics was presented in Table 1. Microbiological properties were compared with

Table 1. Morphological and biochemical Characteristics of the five isolates

Characteristics	EBT1	EBT2	EBT3	EBT5	EBT7
Colony colour	White	White	White	Red	White
Pigment	Nil	Yellow	Nil	Nil	Nil
Shape	Rod	Rod	Rod	Curved rod	Rod
Gram's staining	+	-	+	-	+
Fluorescence	Nil	Nil	Nil	Nil	Nil
Motility	+	+	+	+	+
Spore formation	+	+	+	+	+
Growth at 50ÚC	+	+	+	+	+
Gelatin- liquefaction	+	+	+	+	+
Starch hydrolysis	+	-	+	-	+
Casein hydrolysis	+	+	+	+	+
Citrate utilization	-	+	-	+	-
Indole Production	-	-	-	-	-
Catalase test	-	+	-	+	-
Urease test	-	-	-	-	-
Glucose	+	+	+	+	+
Fructose	+	+	+	+	+
Sucrose	+	+	+	+	+
Maltose	+	+	+	+	+
Xylose	+	+	+	+	+
Galactose	+	+	+	+	+

Bergey's manual of determinative bacteriology[20]. All the five isolates were gram -positive except EBT2 and EBT5 which was gram negative rods.

Phylogenetic tree analysis

Analysis of the 16S rRNA gene sequences on five isolates was performed using NCBI BLAST (National centre for Biotechnology Information http://www.ncbi.nml.nih.gov). The Complete sequences were aligned to the homologous sequence available for *Bacillus* strains. The BLAST (NCBI) search using the sequences showed 99 % homology to other 16S rRNA gene sequences. The sequences of the 16S rRNA gene of the isolate strains (~1.4kb) were deposited in the GenBank sequence database and were given the following accession numbers: *Bacillus safensis* EBT1(JX679410), *Thermus thermophilus* EBT2(KC684277), *Bacillus halodurans* EBT3(KC008602), *Rhodocyclus purpureus* EBT5(KC768770) and *Bacillus megaterium* EBT7(JX870045). Phylogenetic tree analysis and sequence similarity calculations after neighbour

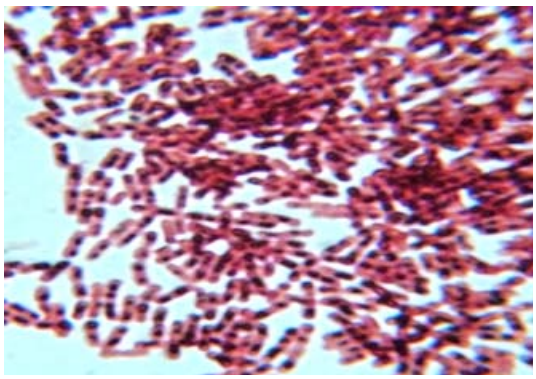

Fig. 1. Sudan black B staining of PHB granules (Black section)

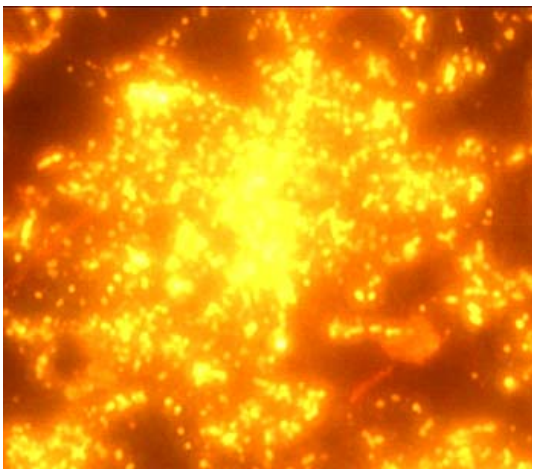

Fig. 2. Nile red stained under fluorescent microscope

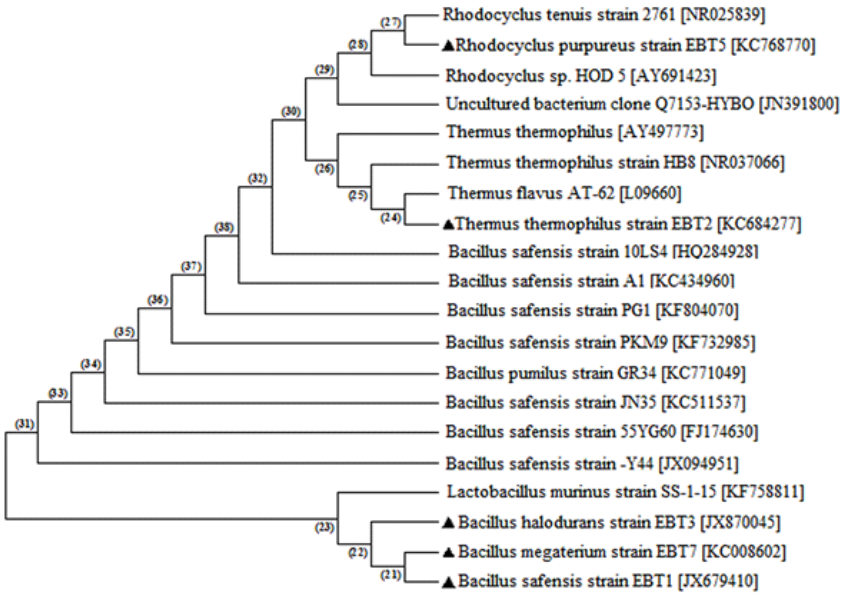

Fig. 3. Phylogenetic relationship by neighbour-joining analysis of 16S rRNA sequences; The scale represents the evolutionary branch length. GenBank accession numbers are given in parentheses

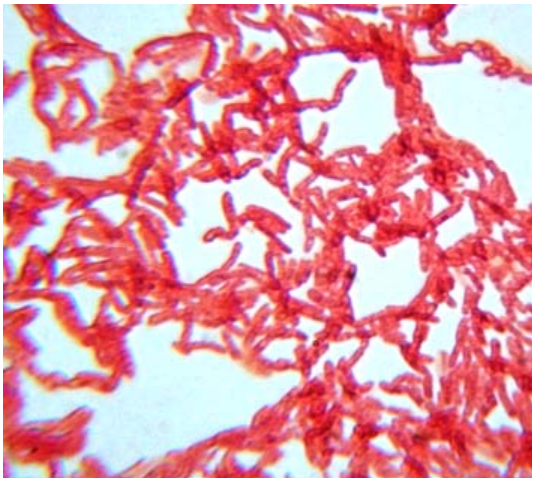

Fig. 4. Promising isolates selected based on the PHB production [conditions: pH 7, Temperature 45 °C, Incubation Time 48 h]

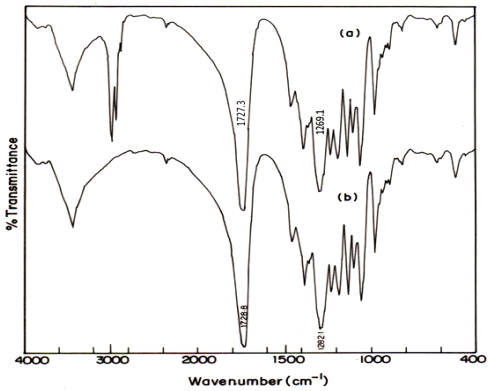

Fig. 5. PHB synthesizing positive isolate EBT1

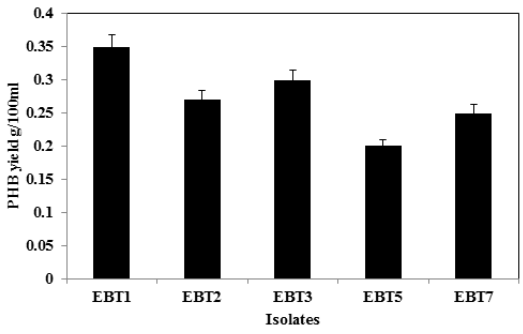

Fig. 6. FTIR spectrum of PHB produced by *Bacillus safensis* EBT1 (a) compared with standard PHB from Sigma(b).

joining analysis showed strong homology with other bacterial sp strains available in the database. The Phylogenetic tree was constructed using neighbour-joining method by MEGA 5software shown in Figure. 3.

Selection of promising bacterial isolates

Based on the PHB yields, promising isolates were selected covering all the sources of the isolates were EBT1, EBT2, EBT3, EBT5 and EBT7 which produced PHB yields of 0.35, 0.27, 0.30, 0.20 and0.25 g/100 ml, respectively. Among the 5 promising bacterial isolates, one efficient strain (*Bacillus safensis* EBT1 shown in Figure. 5) were selected based on PHB assay using Law and Splepecky method (1961) and subjected for further optimization studies. *Bacillus safensis* EBT1 accumulated higher PHB of 0.35 g/100ml which is superior to the other four strains EBT2, EBT3, EBT5 and EBT7. The result obtained from this study has confirmed the hypothesis that the sludge isolates are interesting and yet unexploited reservoir for PHB producing bacteria. This is the first report elucidating the spore-associated bacteria as a potential source of PHB production.

FTIR Spectroscopy results

FTIR spectra of the extracted polymer show peaks at 1727.3 cm^{-1} and 1269.9 cm^{-1} corresponding to specific rotations around carbon atoms specific to certain funtional groups (Fig. 6). The peak at 1727.3cm^{-1} corresponds to C=O stretch the ester group present in the molecular chain of highly ordered crystalline structure . The peak at 1269.3 cm^{-1} corresponds –CH group.These peaks are corresponding to the peaks obtained for the standard PHB (Sigma) at 1728.8 cm^{-1} and 1282.1 cm^{-1} confirming that the extracted polymer is PHB.

CONCLUSION

The outcome of this study offers greater potential in research, for further investigation with *Bacillus safensis* EBT1 on PHB production. The systematic position of Bacillus safensis strain EBT1 was determined based on 16S rRNA sequence. BLAST homology analysis revealed that the sequence of strain EBT1 showed 100% sequence identity with more than 60 Bacillus sp from the NCBI database. Finally the polymer compound was confirmed as Polyhydroxybutyrate by FTIR analysis. Based on the results of the present study,

it is concluded that Bacillus safensis isolated from sewage sludge showed better characteristic PHB producing ability. With all these advances, it is likely that PHB will become a major biodegradable plastic in a wide range of applications in the near future and will eliminate the disposal problems and environmental hazard as are prevalent with conventional plastic materials.

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