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Bioprospecting and Characterization of poly-β-hydroxyalkanoate (PHAs) producing *Pseudomonas Spp.* isolated from edible oil contaminated soil

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Plastics have battle to biological breakdown that result in its accumulation in the environment. This accentuates the need to search for biodegradable polymer, which is readily disposable and degradable. Bacteria synthesize and accumulate polyhydroxy alkanote (PHA) as carbon source under limiting conditions of nutrients. Bioprospecting for poly-β-hydroxyalkanoate (PHA)-accumulating micro-organisms was carried out in edible oil contaminated soil of north Gujarat region. They were isolated in unbalanced culture medium (high carbon/nitrogen ratio) with various edible oil used as carbon source. PHAs producing bacteria were identified by staining with Sudan black and solubilising cellular components in sodium hypochlorite. Two bacterial strains isolated, screened and characterized from edible oil contaminated soil samples were selected due to their capacity of growing in the presence of edible oil and at the same time producing PHA. The isolates were identified by PCR of the 16S rDNA gene using universal primers. The isolates were *Psudomonas aeruginosa and Pseudomonas fluorescence.*

Key words: Biopolymer, native microorganism, Sudan black, biodegradable

Improper disposal of plastics has threatened natural environment worldwide since long time ago. Conventional petrochemical plastics are recalcitrant to microbial degradation (Flechter, 1993). Excessive molecular size seems to be mainly responsible for the resistance of these chemicals to biodegradation and their persistence in soil for a long time (Atlas, 1993). To overcome this problem, the production and applications of eco-friendly products such as biodegradable plastics becomes inevitable. Polyhydroxyalkanoates (PHAs) which produced mainly by bacteria provide a degradable alternative to petrochemical plastics (Anderson and Dawes, 1990; Kumar *et al.*, 2004). PHAs are polyesters that accumulate as inclusions in a wide variety of bacteria. PHA have been attracting considerable attention as biodegradable plastics due to their similar properties to various thermoplastics and elastomers, which

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have been used in consumer products, and completely degraded to water and carbon dioxide upon disposal under various environments (Lee and Choi, 1999). PHAs have thermo mechanical properties similar to synthetic polymers such as polypropylene, but are truly biodegradable in the environment (Yu *et al*.2002). Generally, seven classes of biopolymers are distinguished, namely, polynucleotides, polyamides, polysaccharides, poly isoprenes, lignin, poly pohosphate, and PHA. PHA are produced by a variety of bacterial species (Shamala *et al*., 2003) and can be produced from renewable carbon sources (Brandl *et al*., 1990). The composition of the polymer synthesized is governed by two main factors, that is, the bacterial strain being used and the carbon source being used to grow the bacteria. Polyhydroxyl butyrate (PHB) is a biodegradable thermoplastic polymer that has many advantages similar to that of many conventional petrochemical derived plastics (Oliveira *et al*., 2004). The main advantage is that, the biodegradable polymers are completely degraded to water, carbon dioxide and methane by anaerobic microorganisms in various environments such as soil, sea, lake water and sewage and hence, is easily disposable without harm to the environment. Poly 3-hydroxy butyrate (PHB) belongs to the PHA and is used widely as a storage compound produced by the bacteria. This is observed as hydrophobic inclusions in the cytoplasm of B*acillus megaterium* and in many gram negative and gram-positive bacteria (Brandl *et al*., 1988). Degradability can be categorized as either photodegradable or biodegradable. Photo degradation leads to breakdown of the polymers into non-degradable smaller fragments leading to loss of structural integrity of the material. In contrast, biodegradable polymers are either partly or fully decomposed. PHAs are high molecular mass polymers with properties similar to conventional plastics such as polypropylene (Reddy *et al*., 2003). Therefore, they have a wide range of applications, such as in the manufacture of bottles, packaging materials, films for agriculture and also in medical applications (Oliveira *et al*., 2004). The major barrier to their wide acceptance is the high cost, particularly the costs of carbonaceous raw materials (40%) and polymer recovery (26%) (J. Yu *et al.* 2002*)*. Therefore, the identification of alternative cost-effective substrates for the production of PHA has become an important objective for the commercialization of bioplastics (Preethi *et al.* 2012).

Present study illustrates the methodology employed for isolating two *Pseudomonas* strains from edible oil contaminated soil which were able to accumulate PHAs, selecting the best isolates, their molecular characterization by partially sequencing the ribosomal 16s RNA gene, and characterizing the biopolymer so obtained by differential scanning calorimeter.

Materials and methods

Soil sample collection

Isolates were obtained from edible oil contaminated soil of various edible oil producing industries located in the north Gujarat region (23^0 35' 13" to 24^0 30' 57" N and 72^0 24' 47" to 73^0 24'47" E) and which is supposed to be rich of edible oil degrading microorganisms. The samples were collected and kept in plastic bags, marked with collection details and remained protected from the light in a refrigerated room at 4°C.

Isolation and Screening of PHA-producing bacteria

The colonies were isolated by the methods described by Marjadi and Dharaiya (2011) and Wong *et al.,* (2000) on mineral salt basal medium (MSM), which is supplemented with sesame oil (1%w/v) as a sole carbon source; pH of the medium was adjusted to 7.0, media were autoclaved at 121°C and 15 psi for 15 minutes. The

equivalent of 5 g of dry soil was taken and cultured in 100 ml of MSM medium; following 12 hrs incubation, serial dilutions were carried out from enriching culture using isotonic saline solution until a 10^{-8} dilution was reached. 100 µl of the last three dilutions were spread on Petri dish with unbalanced MSM solid medium or unbalanced C/N ratio, containing the three separate carbon sources. The plates incubated at 30°C to isolate bacterial colonies.

Each of these cultures was smeared on glass slide, heat-fixed and stained with Sudan Black B *(Loba)* to detect the presence of intracellular granules. At the same time cultures was spread on tributyrine agar plate for the detection of lipase producer. Only those isolates which could grow on tributyrine agar plate and synthesize PHA were selected as PHA producers. All the selected isolates were identified through biochemical test.

Microorganisms being able to accumulate PHAs were selected in MSM and grown in duplicate in 3 fresh MSM broth, each dish containing different edible oil as carbon source (sesame oil, palm oil, soybean oil and coconut oil) and incubated at 30°C for 72 hrs on rotary shaker at 150 rpm, at 37 ^{0}C .At the end of the cultivation (72 hrs), cells were harvested by centrifugation at 10,000g for 10 min. The cell pellet was then resuspended in 100 ml of hexane by vortexing and then centrifuged again at 10,000g for 5 min to remove the remaining oils.

Extraction of and estimation of PHB

5 ml of culture should be centrifuged at 10, 000 g for 10 minutes and supernatant should be discarded. The pellet should be suspended in 2.5 ml of 4 % sodium hypochlorite for digestion and 2.5 ml of hot chloroform and should be incubated at 37°C for 1 hour. The suspension should be centrifuged at 1500 g for 10 minutes. (The upper phase contains hypochlorite solution and the middle phase contains chloroform with cell debris).The bottom phase containing PHB with chloroform was collected and further should be followed by extraction with hot chloroform and precipitated with ethanol and acetone (1:1).The precipitate was allowed to evaporate for dryness at 30^{o}C to obtain PHB crystals and weighted. The polymer content (w/w) was defined as the percentage of PHB in dry cell mass. The amount of PHB in the extracted samples was determined spectrophotomertrically at 235 nm (Lee et al., 1995; Law and Slepecky, 1960).

Molecular characterization of Isolates

The both native PHA-accumulator isolates were first characterized by partially sequencing the ribosomal 16s RNA gene through commercial customer service, at Agharkar Research Institute, Pune, India. Briefly, Each reaction mixture contained approximately 10ng of DNA; 2.5mM MgCl2; 1xPCR buffer (Genei®, Bangalore, India); 200 µM each dCTP, dGTP, dATP and dTTP; 2pmol of each, forward and reverse primer; and 1U of Taq DNA polymerase (Genie®, Bangalore, India) in a final volume of 20 µl. FDD2 and RPP2 primers were used as universal primers to amplify almost entire 16S rRNA gene as described previously (Muyzer *et al.,* 1993 and Rawling, 1995). The PCR was performed using the Eppendroff® Gradient Master cycler system for 30 cycles. One 5-min-cycle at 94°C; 30 cycles of 1-min at 94°C, 1-min at 602°C and 1-min at 72°C and an extension cycle of 10-min at 72°C. The purified DNA amplicons (PCR products) were sequenced using the ABI PRISM® Big Dye™ Terminator Cycle sequencing Ready Reaction kit and the automatic DNA sequencer (Applied Biosystems®, U.S.A.). The sequence generated from automated sequencing of PCR amplified DNA was analyzed through DNA Sequence Analyzer computer software (Applied Biosystem®). The sequences of the partial 16S rRNA were compared with the 16S rRNA sequence

available in the public nucleotide databases at the National Center for Biotechnology Information (NCBI) by using its World Wide Web site *(http://www.ncbi.nlm.nih.gov)*, and the BLAST (Basic Local Alignment Search Tool) *(http://blast.ncbi.nlm.nih.gov/)* algorithm (Altschul *et al*., 1997) to find out possible homologous isolated strains with previous characterized strains.

Results and Discussion

Isolation and Screening of PHA-producing bacteria

Total 25 bacterial strains were isolated by enrichment cultivation using edible oil as the sole carbon source, form the samples collected from various oil industries within north Gujarat region. Out of sixteen strains, nine strains previously showing turbid colonies and now are able to secrete the lipase and detected by zone of hydrolysis on Tributyrine agar plates . PHB granules were observed in nine strains for (DSM-1, 2, 4, 5, 6, 7, 8, 9 and 14) in all cells when stained with Sudan black staining. Sudan black is a lipophilic dye (Ceyhan and Ozdemir, 2011) attached to PHB granules with pink black back ground (Figure-1).

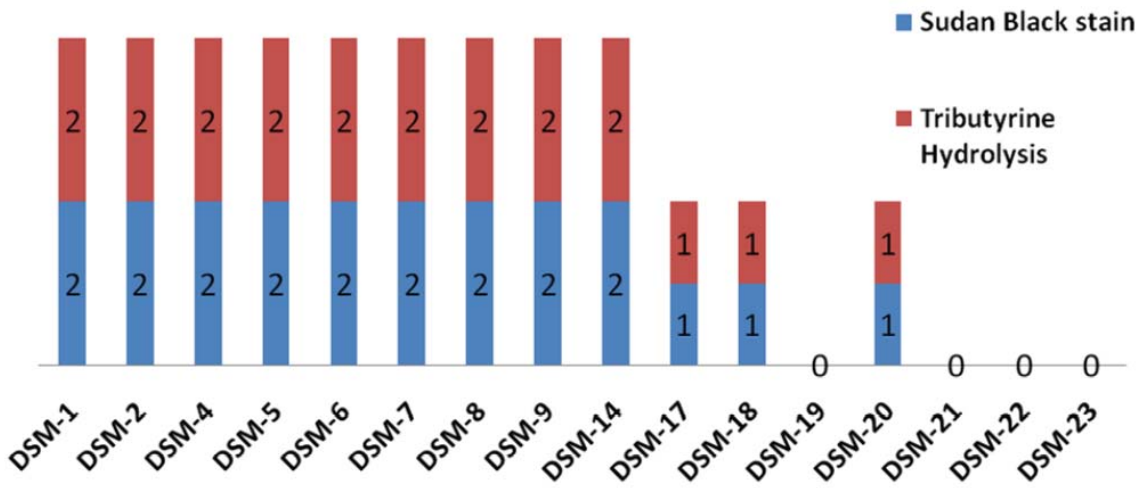

Fig. 1. Screening of Isolates. 0 – No granules observed, No zone of hydrolysis observed; 1 – Slight granules and zone of hydrolysis observed; 2 - Granules and zone fully observed

With regard to biochemical characterization, sugar utilization and enzyme secretion profile of edible oil degrading and PHB like granules producing strain showed probable resemblance to *Pseudomonas Spp.(5 and 7)* were selected as a producer of PHB like granules and ultimately for further molecular characterization.

PHB was isolated from the production medium by solvent extraction technique. The sodium hypochlorite digestion process enables in the digestion of cells and release of the PHB granules outside the cells for easy extraction of PHB. The conversion to crotonic acid by hot concentrated sulfuric acid proved to be about one-third time more sensitive than alkaline hydrolysis of PHB extraction and analysis.

Due to the α, β, unsaturated bond, crotonic acid has an absorption maximum at 235 nm in concentrated sulfuric acid and this property was used for ultraviolet (UV)

measurement. The amount of PHB in the extracted samples was determined with UV spectrophotometer at 235 nm with reference to the standard graph of 3- hydroxy butyric acid (Figure 2).

For 16S rRNA sequence analysis, the genomic DNA was extracted from the *Pseudomonas spp. (DSM-5,7).* Both PHB accumulator bacterial isolates were successfully amplified with primer RPP2. The 16S rRNA gene from these strains was amplified by polymerase chain reaction (PCR) and 500 bp of the product was sequenced and identified at genus level using 16S rRNA partial sequencing.

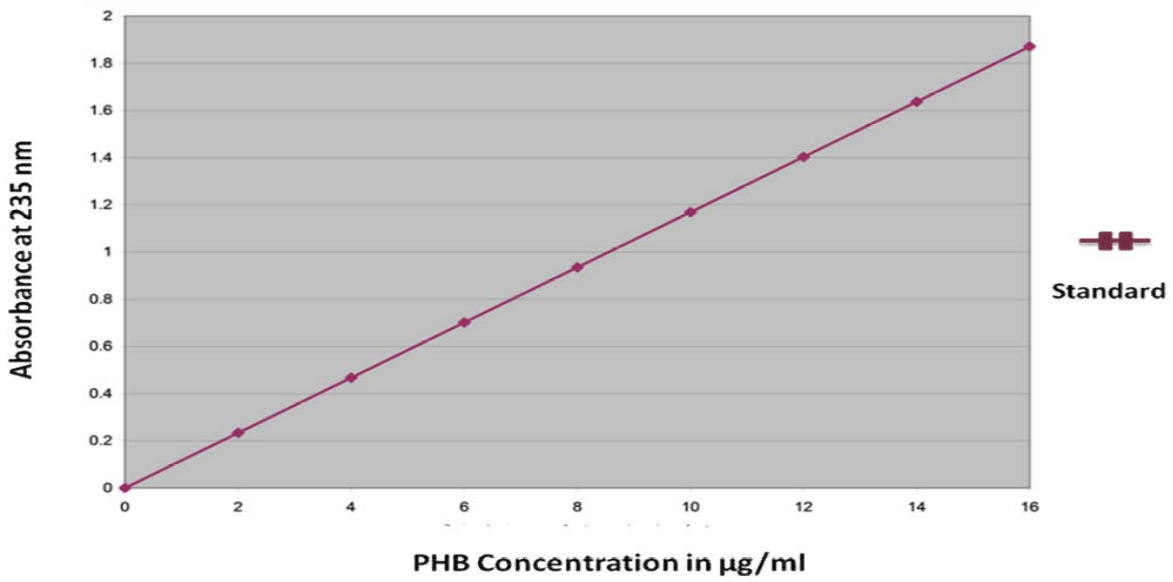

Figure 2. Standard curve of PHB

Table-1 Fermentation of PHB in Carbon rich MMSB medium after 24 hrs

Microorganisms	OD (235nm)	PHB Concentration in µg/ml
Pseudomonas (DSM-5)	0.040±0.014	10± 3.52
Pseudomonas (DSM-7)	0.038± 0.012	9.5 ± 3.01

Partial sequencing of 16S rRNA and its homology search indicated that DSM-5 and 7 were strains of *Pseudomonas.* The Partial 16S rRNA sequence of DSM-5 and 7 were queried to search for homology using nBLAST. The best hit DSM-5 was *Psudomonas aeruginosa* strain NBRAJG78 with 95% identities and an E-value = 0.0. Hence, DSM-5 belongs to the genus *Pseudomonas* and species *aeruginosa.* Similarly, for DSM-7 *Pseudomonas fluorescence* strain LMG 7220 with 98% identity.

Psudomonas aeruginosa is a gram-negative rod shaped bacterium commonly found in soil, water, skin flora and most manmade environment throughout the world (Raja *et al.,* 2009) and its Poly 3-(hydroxyalkanoates) production ability was reported from oily substrate and fatty acids (Haba *et al*., 2007 and Ballistreri *et al*., 2001).

Though *P. aeruginosa* is not normally recognized as PHB producer but currently it has been recognized to produce it (Aremu *et al*., 2010 and Mountassif *et al.,* 2010). However, in the present study it is evident that this organism is producing 10±3.52 µg/ml PHB concentration after fermentation for 24 hrs (Table-1).

Pseudomonas fluorescens is a gram-negative bacterium found in soil, water and plant surfaces and has been used for several bioremediation applications to degrade pollutants (Baggi *et al.,* 1983). It has previously been experimentally shown to produce PHB (Jiang *et al.,* 2008) and its genomic sequence indicates the presence of the Pha A/B/C/Z genes involved in PHB metabolism (Paulsen *et al.,* 2005). Recently it has been isolated from oil contaminated soil (Sarin *et al.,* 2011).

Conclusion

The finding leads to the conclusion that bacterial strains isolated from the edible oil contaminated soils were characterized by partial 16s rRNA sequencing. The sequence was deposited in Gene bank/DDBJ/EMBL up to genus and species level by using nBLAST homology searching. Further, the characterization of PHB like granules by various methodologies and their comparison with standard PHB as described earlier shows that the extracted polymer from the microbial isolate posses almost similar properties and was finally confirmed to be PHB (Poly-β-hydrxybutyrate).

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