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Screening Of Potential Soil Bacteria For Detection Of Polyhydroxyalkanoate (PHA) Accumulation

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Abstract: Polyhydroxyalkanoate (PHAs) have focused much attention because of their complete biodegradability and their material resemblance with synthetic plastics. For this purpose, the present study aims on isolation of those bacteria that have the potential to synthesize intracellular PHA and characterisation of PHA producing bacterial isolates so as to obtain the greatest PHA yield. 36 bacterial isolates were isolated and screened by Sudan black staining, Nile blue staining and Tributyrine hydrolysis. Out of 36 bacterial isolates, 8 isolates were screened as effective PHA producers. The selected isolates were identified phenotypically as *Pseudomonas* sp. EO1, *Aneurinibacillus* sp. EO8, *Brevibacterium* sp. EO9, *Bacillus* sp. EO11, *Pseudomonas* sp. EO18, *Bacillus* sp. LO1, *Achromobacter* sp. LO8, *Pseudomonas* sp. LO10.

Keywords: Polyhydroxyalkanoate; plastics; Sudan Black; Nile blue; Tributyrine hydrolysis

1. INTRODUCTION

Traditional petrochemical derived plastics are resistant to microbial attack and deposit in environment at a rate of 28 million tons per year [1]. In 2011, almost 280 million tons of petrochemicals-based polymers were manufactured with assumed increased in 4% per annum to 2016. Their utility are almost general as parts in automobiles, home appliances, computer equipments, packages and even medical applications are areas, where plastics clearly have become indispensable. However, its qualities of durability have caused significant problems since plastic waste gather in the environment. The accumulation of excessive plastics have arisen a global environmental problem. Plastic wastes are not generally organized by nature, as the most of plastics are not degraded by microorganisms. With the impending fossil fuel crisis, the alarming prices of petroleum and environmental impact associated with the products, the search for substitutes is necessary in reducing mankind's dependencies in non-renewable resources [2]. Biodegradable plastics provide the best replacement to prevent the environment from hazards caused by conventional petroleum based plastics, as they are 'eco-friendly' in nature. Biodegradable plastics are of many kinds with different levels of biodegradability. As biodegradable plastic, PHA was the first chief kinds of biomaterial discovered in *Bacillus megaterium* and characterized by French microbiologist Lemoeigne in 1926 [3]. Much attention has focused to PHAs because of their entire biodegradability and the affinity of their material characteristics to synthetic plastics [4]. Polyhydroxyalkanoates (PHAs) is biological polyesteric macromolecules that is naturally produced and accumulated as an intracellular granule in bacteria in response to nutritional deficiency or the existence of high carbon concentration in the growth environment [5]. Polyhydroxyalkanoate (PHA) family includes one of its candidates Polyhydroxybutyrate (PHB) that is the most usual biodegradable polymer and promising substitute to nondegradable traditional plastics. These polymers are intracellularly accumulated as inclusion bodies up to cell dry weight of 90% under nutrient stress conditions and act as reserve material for energy. As the candidates of biodegradable plastics, it shows similar physical characteristics as those of the oil-derived conventional plastics like polypropylene or polyethylene which can be molded, built into films, spun into monofilaments, and utilised to make heteropolymers with different synthetic polymers and many more scope in the field of agriculture, packaging, and medical, being biodegradable and also favourable immunologically with human tissue [6]. Recently, PHA has been establish beneficial as a new category of biofuel [7] and [8]. Except all these properties and applications, vast use of PHAs is prohibited mainly due to their high production cost compared with the oil-derived plastics [9]. High production cost of PHA is chiefly committed to the expensive carbon substrates and tedious production procedures [10]. Many efforts has been tried to minimize the PHA cost by the isolation of suitable bacterial strains to produce PHAs from a cheap carbon source such as agriculture waste, dairy waste, paper effluent, lubricating and edible oil waste. PHA accumulating bacteria can be obtained from different environmental sources for instance water and soil by using a suitable method that can monitor the PHAs

accumulation. Keeping this point in view, the present study was focused on isolation of PHA-producing bacteria from oil enriched soils to produce sufficient amount of polymer as a microbial novel end product PHA which is cost effective and ecofriendly.

2. MATERIALS AND METHODS

2.1. Sample collection

Soil samples were collected from edible oil and lubricating oil enriched soil from expeller and garage workshop located in the Haridwar (India) and its adjoining places. The samples were collected and kept in sterilized plastic bags, marked with collection details and stored at 4°C temperature.

2.2. *Bacterial isolation*

For isolation of PHA producing bacteria, six oil contaminated soil samples were screened from different regions of Haridwar. The isolation of bacteria was carried out by serial dilution, spread plate and enrichment technique. For this, sterile nutrient broth containing a gram of soil sample was incubated at 30° for 24 h. Then different dilutions (10^{-4}, 10^{-5}, 10^{-6} and 10^{-7}) were prepared by diluting broth culture in 0.9% normal saline. After this, plate of nutrient agar containing different dilutions was incubated at 30 $\pm$ 2°C for 24 h [11].

2.3. *Analysis of lipase enzyme activity in bacterial isolates*

Tributyrine agar plate method (Hi media) was used to check the oil utilising capability of oil enriched soil isolated bacteria. Isolates were also screened to examine the activity of lipase enzyme by growing them on Tributyrine agar plate [12].

2.4. *Primary screening of bacteria for the detection of PHA granules by Sudan Black B staining*

To check PHA accumulating ability in bacteria, Sudan Black B stain was used as a primary screening stain. Briefly smear of colonies taken from nutrient agar medium were stained for 15 min with 0.3% (w/v) Sudan Black B dissolved in 70% ethanol then de-colourised the stain with 50% (v/v) alcohol and counterstained with 0.5% (w/v) safranin solution. To select potential PHA producing bacteria, Sudan Black B stain was used for screening. Then safranin was washed off with distilled water and observed the slide under 100x of compound microscope [13].

2.5. *Secondary screening by Fluorescence screening*

Sudan Black B positive colonies were purified and the isolates were further confirmed by Nile blue A staining. In short, Nile blue A stain (0.5 µg/ml) suspended in carbon rich nutrient agar media containing were used for the streaking of isolated colonies. Stained plates were examined for development of fluorescence under UV-transilluminator (Biogen) at 312 nm after incubation of 24h at 30 $\pm$ 2°C [14].

2.6. Isolates Identification and characterization by phenotypic characters

Characterization and identification of PHA producing bacterial isolates were based on morphological as well as biochemical test according to Bergey's manual [15]. Based on the screening results, two potential PHA-producing strains were selected for further studies. The cultural characteristics including structure, shape, colour, cell's arrangement and Gram staining of the bacterial colonies were observed to characterize the bacterial colonies. Bacterial morphology was observed under 1000x resolution of Microscope (Olympus). Different biochemical tests such as methyl red and Voges-Proskauer, H_2S production, amylase, citrate, sucrose, dextrose, fructose, mannitol and lactose utilisation test were performed on selected bacterial isolates for 48 h at 37°C.

3. RESULT AND DISCUSSION

3.1. Isolation of PHA Producing bacteria

There are broad varieties of taxonomically and physiologically different natural bacteria and some archaea that have the capability to accumulate PHAs as bacterial storage reserve materials and accumulate them in the cytoplasm as insoluble granules. For the selection of potential PHA producers, bacterial colonies were isolated from different oil enriched soil as it was assumed to be rich in carbon source that was necessary component for the accumulation of PHA in their surrounding growth environment. A total of 36 different types of colonies (21 from edible oil and 15 from lubricating oil) were picked up from six different soil samples for further evaluation of PHA production using Sudan Black dye, Nile Blue dye and Tributyrine agar plate method.

3.2. Screening of PHA Producing bacteria

Among the 36 isolates, 24 isolates showed clear halos on tributyrine agar plate as it secretes lipase and hence are able to utilize oil present in soil sample, 14 isolates showed positive result for Sudan Black B a primary screening agent attached to accumulated PHA and observed as black blue granules in bacterial cell under light microscope (Fig.1), and 08 isolates fluoresce under UV light when grew on medium containing Nile blue A, a specific dye for the detection of PHA granules (Table 1 and Fig. 2). Eight isolates EO1, EO8, EO9, EO11, EO18 and LO1, LO8, LO10 showed positive results for all the three screening procedure via Nile blue, Sudan black and Tributyrine hydrolysis. Only those isolates which gave positive result for all the three screening procedure were selected as PHA producers. Screening of PHA accumulation through Nile blue stain at concentration of (0.5 µg/ml) has already been reported previously by Spiekermann et al. [16]. Goh

et al. screened the presence of PHA inducing bacteria by observing the fluorescent colonies flourish on nutrient rich medium under ultraviolet (UV) [17]**.** Sasidharan et al. worked on screening of polyhydroxyalkanoate (PHA) synthesizing bacteria by spot inoculation of cell culture on complex nitrogen limiting agar plates containing Nile Blue A (1 μg/ml) and agar (2%) in PHA production medium [18]. Munir and Jamil, confirmed the presence of PHA accumulating bacteria isolated from contaminated soil nearby industrial wastewater drain by using Sudan Black B method [19]. Bacteria were isolated to check the oil utilizing ability and lipase enzyme activity over tributyrine agar by Tufail et al. [20].

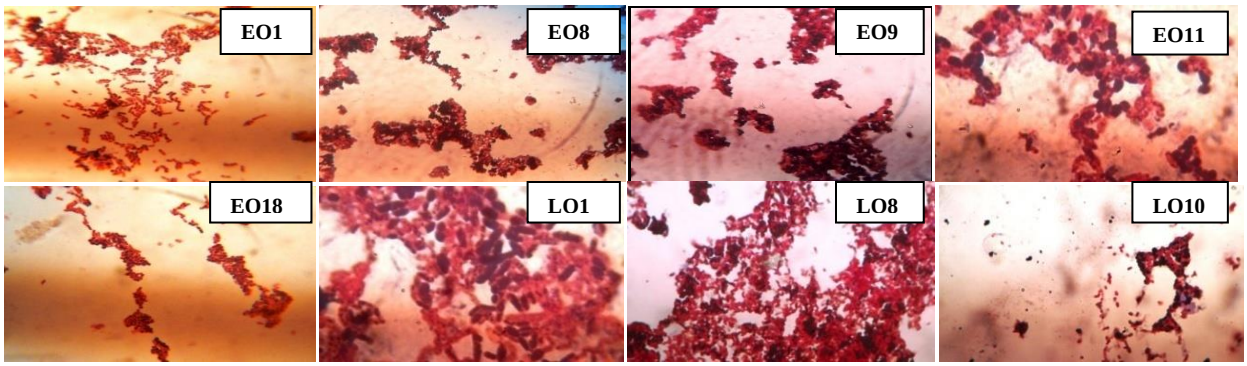

Fig.1. Sudan black B staining containing PHA inclusions in selected bacterial cells

Table 1 Screening for potential PHA producers*

Isolates	Tributyrine hydrolysis	Sudan Black stain	Nile Blue stain
Haridwar Edible Oil Soil			
E O 1	++	++	++
E O 2	+	-	-
E O 3	+	+	-
E O 4	+	-	-
E O 5	-	-	-
E O 6	+	+	-
E O 7	+	-	-
E O 8	++	+	++
Roorkee Edible Oil Soil			
E O 9	++	++	++
E O 10	+	+	-
E O 11	++	+	++
EO 12	-	-	-
EO 13	+	+	-
EO 14	-	-	-
EO 15	+	-	-
Laksar Edible Oil Soil			
EO 16	+	-	-
EO 17	-	-	-
EO 18	++	++	++
EO 19	+	-	-
EO 20	-	-	-
EO 21	+	+	-
Haridwar Lubricating Oil Soil			
L O 1	++	++	++
L O 2	+	-	-
L O 3	+	-	-
L O 4	-	-	-
L O 5	+	+	-

Roorkee Lubricating Oil Soil			
L O 6	-	-	-
L O 7	-	-	-
L O 8	++	++	++
L O 9	-	-	-
Laksar Lubricating Oil Soil			
L O 10	++	++	++
L O 11	-	-	-
L O 12	+	-	-
L O 13	-	-	-
L O 14	++	+	-
L O 15	+	-	-

*Legend: ++ = granules fully observed, ++ = granules slightly observed, - = no granules observed, ++ = fully florescence, + = slightly fluorescence, - = no fluorescence, ++ = zone fully observed, + = Zone slightly observed, - = no zone observed

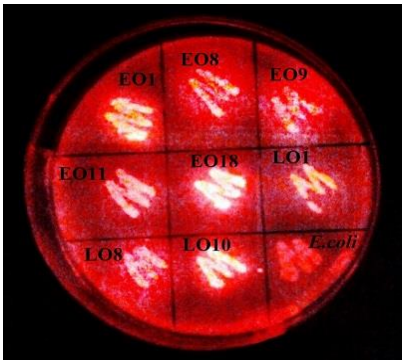

Fig.2. PHA inclusions in eight bacterial cells illuminated through Nile Blue staining under UV light and *E.coli* kept as negative control

3.3. Identification of Polyhydroxyalkanoate (PHA) producing bacteria

Out of 21 isolates from edible oil soil, 9 Gram negative & 12 Gram positive rods whereas 15 isolates from lubricating oil soil were isolated, out of which 3 Gram positive cocci, 7 Gram positive rods & 5 Gram negative rods were observed (Table 4 a and b). Classification of selected isolates up to genus level was according to morphological and biochemical characteristics based on bergey's manual of determinative bacteriology and were identified as *Pseudomonas* sp. EO1, *Aneurinibacillus* sp. EO8, *Brevibacterium* sp. EO9, *Bacillus* sp. EO11, *Pseudomonas* sp. EO18, *Bacillus* sp. LO1, *Achromobacter* sp. LO8, *Pseudomonas* sp. LO10 (Fig. 2). Many of the isolates were *Bacillus* sp. and *Pseudomonas* sp. Both the isolates are reported to be ideal PHA producers in many previous studies by Tufail et al., [20]. Potential strains were selected for later studies. Hwan et al., [21] isolated and observed greater amount of PHA in *Pseudomonas* sp. strain DR2 accumulation than other well-studied strains. Chaudhary et al., [22] selected *Pseudomonas* sp. due to its high production ability. Mainly *Pseudomonas* spp. was isolated from Antarctic soil by Goh et al., [17]. Industrial production of PHA was achieved via *Bacillus* sp. and selected as a good candidate for PHA production by Bhuwal et al., [23].

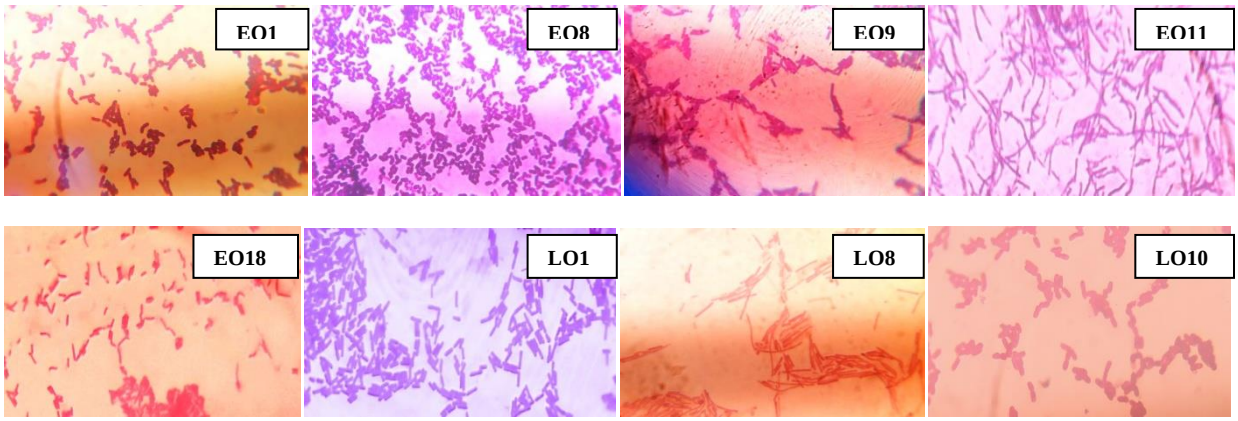

Fig.2. Gram staining of selected bacterial cells.

Table 4 a Morphological & Biochemical characteristics of selected isolates from edible oil contaminated soil

Characteristics	**EO1**	**EO8**	**EO9**	**EO11**	**EO18**
Morphological Characteristics					
Colony color	Blue-Green	Creamy greyish	Creamish white	Light Yellow	Blue-Green
Gram reaction	-	+	+	+	-
Cell shape	Rod	Rod	Rod	Rod	Rod
Cell arrangement	Single	Single	Chain	Chain	Single
Spore formation	-	+	-	+	-
Biochemical characteristics					
Citrate	+	-	+	+	+
MR	-	-	+	+	-
VP	-	-	-	-	-
Amylase	-	-	-	+	-
H_2S	-	-	+	-	-
Indole	-	-	-	-	-
Oxidase	+	+	+	+	+
Catalase	+	-	+	+	+
Sugar fermentation					
Sucrose	-	+	+	+	-
Dextrose	+	-	+	+	+
Lactose	-	+	-	+	-
Fructose	+	+	+	+	+
Mannitol	+	-	+	+	+
Probable genus	*Pseudomonas* sp.	*Aneurinibacillus* sp.	*Brevibacterium* sp.	*Bacillus* sp.	*Pseudomonas* sp.

Table 4 b Morphological & Biochemical characteristics of selected isolates from lubricating oil contaminated soil

Characteristics	**LO1**	**LO8**	**LO10**
Morphological Characteristics			
Colony color	Creamy White	White	Fluorescent yellow
Gram reaction	+	-	-
Cell shape	Rod	Rod	Rod
Cell arrangement	Short chain	Pairs	Single
Spore formation	+	-	-
Biochemical characteristics			
Citrate	+	+	+
MR	-	-	-

VP	+	-	-
Amylase	+	-	-
H_2S	-	-	-
Indole	-	-	-
Oxidase	-	+	+
Catalase	+	+	+
Sugar fermentation			
Sucrose	+	-	+
Dextrose	+	+	+
Lactose	-	-	-
Fructose	+	-	+
Mannitol	+	-	+
Probable genus	*Bacillus* sp.	*Achromobacter* sp.	*Pseudomonas* sp.

4. CONCLUSION

The objective of the current study was to isolate and characterise efficient PHA inducing bacteria so as to obtain the maximum PHA yield. According to present findings, 36 bacteria were isolated from different oil contaminated soil and the best eight screened isolates were identified at phenotypic level. All the eight isolates EO1, EO8, EO9, EO11, EO18 and LO1, LO8, LO10 can be regarded as potential PHA producing bacteria.

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