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RESEARCH ARTICLE

Characterisation of PHA accumulating *Bacillus* sp associated with petroleum and diesel oil contaminated soil using traditional techniques

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Abstract

The extensive usage of petrochemical plastics, due to their versatile properties especially durability is causing severe problem in waste management affecting the aesthetic qualities of cities, waterbodies and natural areas. Environmental biotechnology has the intention of increasing sustainability of production processes by employing biological systems and thereby benefiting the environment. Microorganisms are a biological system which is generally used for the reduction of pollution from air, aquatic or terrestrial systems. Problems concerning the global environment have created much attention in developing eco-friendly products. The bacterial polymer(PHA) is a alternative for synthetic plastics. In this study, an attempt was made to isolate PHA producing microorganisms from petroleum and diesel oil contaminated sites. The bacterial consortium was screened and confirmed for *Bacillus* strains producing polyhydroxyalkanoates (PHA) by colony morphology and biochemical analysis. The PHA production was confirmed by PHA activity assay. The PHA extract was apparently confirmed by TLC.

INTRODUCTION

The development of human civilization throughout history has led to growing disruption of the natural balance and the occurrence of different types of pollution. Environmental pollution with petroleum and petrochemical products has been recognized as a significant and serious problem[1].Diesel engine oil, which is one of the major products of crude oil, constitutes a major source of pollution in our environment. Therefore diesel engine oil can enter into the environment through wrecks of oil tankers carrying diesel oil, cleaning of diesel tanks by merchants, war ships carryingdiesel oil and motor mechanics[2].

Problems concerning the global environment have created much attention in developing eco-friendly products .Biopolymers are one product that can help to overcome problems caused by petrochemical polymers. Biopolymers are generated from renewable natural sources and are often biodegradable and nontoxic[3].Hydrocarbons from oil are used as a source of nutrients and energy for microorganism growth, and at the same time, microorganisms decompose them to naphthenic acids, alcohols, phenols, hydroperoxides, carbonyl compounds, esters, and eventually to carbon dioxide and water[4].(Eglinnton, 1975; Marković et al., 1996).The ability of microorganisms to utilize hydrocarbons in oil contaminated environments has been documented[5].The presence of oil degrading microorganisms such as bacteria and fungi is not restricted to a particular ecosystem and has been found in the Arctic, Antarctic and temperate region but little work has been reported in high temperate ecosystem[6].

This is possible because microorganisms have enzyme system to degrade and utilize diesel oil as a source of carbon and energy[7]. Suggested reasons for the reduced plant growth in diesel oil contaminated soils range from direct toxic effect on plants and reduced germination to unsatisfactory soil condition due to insufficient aeration of the soil because of the displacement of air from the space between the soil particles by diesel engine oil [8,9]. Bioremediation

is considered a non-destructive, cost-effective, and sometimes logisticallyfavourablecleanup technology, which attempts to accelerate the naturally occurringbiodegradation of contaminants through the optimization of limiting conditions[10].

Therefore, the development and use of biodegradable plastics is gaining more serious attention. The most extensively studied thermoplastic biopolymers are the polyhydroxyalkanoates (PHA) and polylactic acid LA)[11].A number of bacteria producing PHAs are *Alcaligeneseutrophus, Alcaligeneslatus, Azotobactervinelandii, Rhizobium sps*6, *Bacillus sps*7, *methylotrophs, pseudomonads*, and recombinant *Escherichia coli*[12,13,14].PHAs are synthesized by numerous bacteria as intra cellular carbon or as energy storage compounds and are accumulated as granules in the cytoplasm of the cell[15].Renewable carbon sources can be used for the production of these water insoluble strong polymers. The production cost of bioplastics being the main criteria can be reduced by using cheap carbon sources and nutritional supplements with feeding strategies[16].Polyhydroxyalkanoates (PHA) are biodegradable polyesters and elastomers, which gets accumulated as cytoplasmic inclusions in certain bacteria during unbalanced growth condition as an intracellular storage material of carbon and energy, usually characterised by an excess carbon supply and lack of one or more essential nutrients[17,18]. The present work has been focused on this approach, aiming to isolate novel bacterial strains capable of producing polyhydroxyalkanoate from oil contaminated sites.

MATERIALS AND METHODS

Sample Collection and Isolation of Pure Cultures

Oil contaminated soils were collected from various sources in Trichy, and were used for isolation of pure cultures. One gram of soil sample is dispensed in 10ml of sterile distilled water and then this was subjected to serial dilution and 0.1ml from each dilution was plated on nutrient agar medium, by spread plate method for propagation of microbial growth. The isolated bacterial colonies were subcultueerd in nutrient broth and pure cultured on nutrient nutrient agar medium[19].

Characterisation of PHA producing isolates

PHA producing strains were identified and characterised by morphological and biochemical characterisation according to the Bergey's Manual of Determinative bacteriology[20].

Morphological characterization

Morphological features were identified by growing the cultures on nutrient agar media and gram staining and endospore staining was performed[21].

Biochemical characterization

Different Biochemical tests were carried out includes IMVIC tests, catalase test, oxidase test[22,23] .

Extraction of PHA

Each culture were centrifuged at 8000 rpm, 40 c for 10 minutes to harvest the cells. The culture supernatant was taken. P^H of the culture supernatant was lowered to 2 with 5 M Hcland incubating at 40 c for 24 hours. The precipitate was separated by centrifugation at 8000 rpm for 20 minutes. This white precipitate formed culture was selected[24].

PHA ACTIVITY ASSAYS

Emulsification Activity (E24):

The emulsifying capacity was evaluated by an emulsification index (E24). The emulsifying activity of the culture supernatant was estimated by adding 3ml of the supernatant and adding equal volume of petrol to the same tube. The tube was vortexed for 10 seconds to 1 minute. Then held stationary for 1 minute and then visually examined for turbidity of stable emulsion. Emulsifying power was measured by vortexing equal volumes of the centrifuged culture with petrol for 1 minute and determining the percentage of volume to settle for 24 hours and the height of the emulsion was measured. Emulsifying property of the PHA is also carried out with diesel, crude oil, gingelly oil and food oil by using following formula[25].

E24 = (Height of emulsion formed / Total height of solution) X 100

Oil Displacement Test

Twenty microliter of crude oil was put onto the surface of 50µl of distilled water in a petridish. A thin membrane of oil formed immediately. Then 10microliter of supernatant was gently dropped on the center of the oil membrane. A clear halo was visible. The area of this circle was measured and calculated for oil displacement area(ODA) using the following equation[26].

ODA= 22/7 $(radius)^2$ cm^2

Characterization of PHA crude extract

Thin Layer Chromatography (TLC):

Thin layer chromatography is one of the valuable and versatile method for the analysis of wide range of biomolecules. TLC is most widely used for the detection of the presence of compounds and for the separation of mixture of compounds. The separation in TLC is due to the differential partition of solute between the stationary and mobile phases.

A thin uniform layer of stationary phase was made on a glass plate. The plate was air dried for 15 minutes and then over-dried for 10-15 minutes and 100^0 C. 20 microliter of each sample and standard sugars were spotted on a line drawn about 1.5-2.0 cm from the bottom. The TLC plate was plated gently in a mobile phase contained in a chromatographic tank and allowed for solvent development. As the solvent front reached about 1-2 cm from the top of the plate, the plate was removed and air dried. The plate was sprayed with the spraying reagent and treated at 100^0 C for 10 minutes[27].

RESULTS AND DISCUSSION

Polyhydroxyalkanoates (PHA) are the only bioplastics fully synthesised and polymerised by microorganisms making part of a family of polyesters with several structures. Since 1926, when the first PHA was identified, the poly (3-hydroxybutyrate) (p (3HB)) , over 80 distinct monomer units have been found as constituents of PHAs in diverse bacteria[28,29]. For PHA producing microbial cells , PHAs serve as carbon and energy storage material in times of unbalanced nutrient availability. Under conditions of starvation, these reserve materials can be mobilized, giving a survival advantage to the cells[30]. For the present study of aim,11 oil contaminated soil samples which includespetrol, diesel and kerosene, were collected from different areas. (Table:1).Among the 11 samples, all the soil samples produced white colonies, out of whichonly 5 samples are found to be positive for *Bacillus* species. The colonies were subcultured for various experiments. The isolated cultures were confirmed by Gram'sstaining, spore staining and biochemical test(Table:2). The isolates were comparedwith standard strain of *Bacillus liqueniformis*ATCC 2132. After theextraction process, the extracted PHA activity was assessed by follwing tests.

EMULSIFICATION TEST:

The isolated samples were tested for the emulsifying activity with petrol,diesel, crude oil. It was found that E24 was highest 90% with crude oil in the culture sample (CF 11) , 60% with diesel in the culture sample CF 01,CF 03, CF 10(Table:3)..

OIL DISPLACEMENT TEST

The strain CF 11 exhibited the higest activity for oil displacement test andCF 06 strain exhibited lowest activity (Table:3).

DROP COLLAPSING TEST

The isolated 5 strains showed positive results for drop collapsing test. (Table:3)

TLC:

The extracted PHAs were examined by thin layer chromatography andvisualized by spraying with α- naphtol solution. The compounds were observed underUV light as pink spots which indicates the surfactin extraction from *Bacillusliqueniformis*.(fig:4).

Table - 1 Sample collection from different areas

S.No	Sample Name	Area of Collection	Gram Staining
1	CF 01	Mechanic shop (crude oil)	+ve rod
2	CF 02	Petrol Filling station (Petrol)	+vecocci
3	CF 03	Diesel Filling station (Diesel)	+ ve rod
4	CF 04	Ration shop (Kerosine)	-ve rod
5	CF 05	Bus stand (Diesel)	-ve rod
6	CF 06	Petrol Filling station (Petrol)	+ve rod
7	CF 07	Automobile workshop	+vecocci
8	CF 08	Mechanic shop (crude oil)	-ve rod
9	CF 09	Petrol Filling station (Petrol)	-ve rod
10	CF 10	Mechanic shop (crude oil)	+ve rod
11	CF 11	Petrol Filling station (Petrol)	+ve rod

Table - 2 IDENTIFICATION TEST FOR PHA PRODUCING *Bacillus Liqueniformis*

S.No	TEST	STANDARD STRAIN (*Bacillus liqueniformis* ATCC 2132)	ISOLATED SAMPLE
1.	GRAM STAINING	Gram positive rod	Gram positive rod
2.	SPORE STAINING	Green Colour spores	Green Colour spores
3.	CATALASE TEST	+ve	+ve
4.	OXIDASE TEST	+ve	+ve

Table - 3 EMULSIFICATION INDEX, OIL DISPLACEMENT AREA AND DROP COLLAPSING TESTOF EXTRACTED PHA FROM *Bacillus liqueniformis*

S.No	SAMPLE	EMULSIFICATION INDEX E24			OIL DISPLACEMENT AREA (ODA) (cm^2)	DROP COLLAPSING TEST
		PETROL	DIESEL	CRUDE OIL		
1	CF 01	60	50	60	0.196	+ve
2	CF 03	50	60	50	0.282	+ve
3	CF 06	60	50	70	0.1256	+ve
4	CF 10	50	60	50	0.282	+ve
5	CF 11	70	60	90	0.3849	+ve

Fig – 1 Emulsification of Diesel by PHA extracted from *Bacillus liqueniformis*

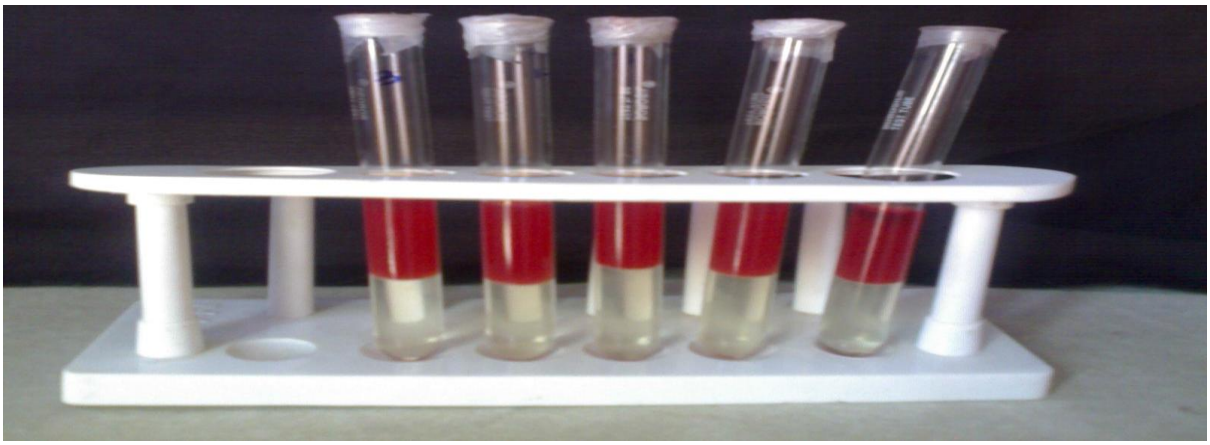


Fig –2 Illustrate the Emulsification of petrol by PHA extracted from *Bacillus liqueniformis*

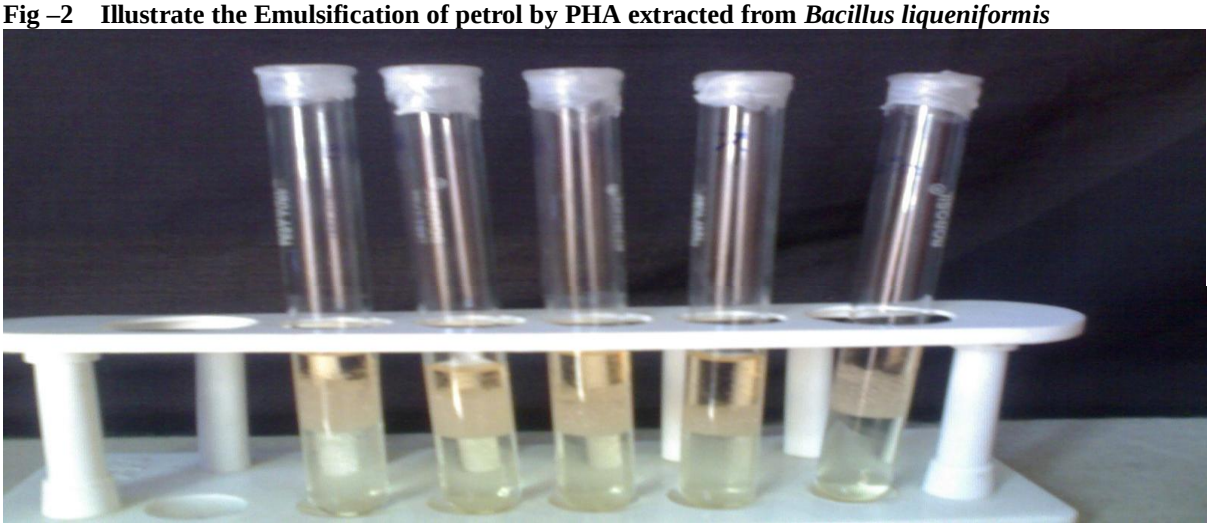
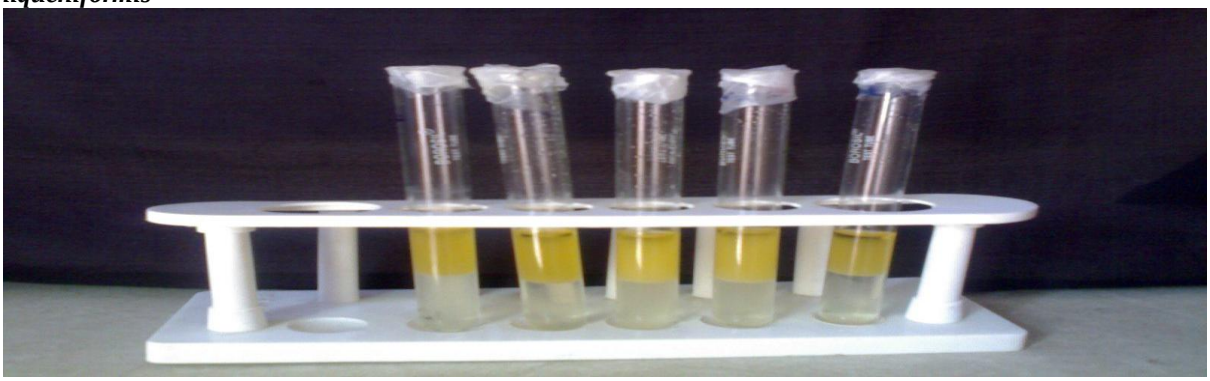
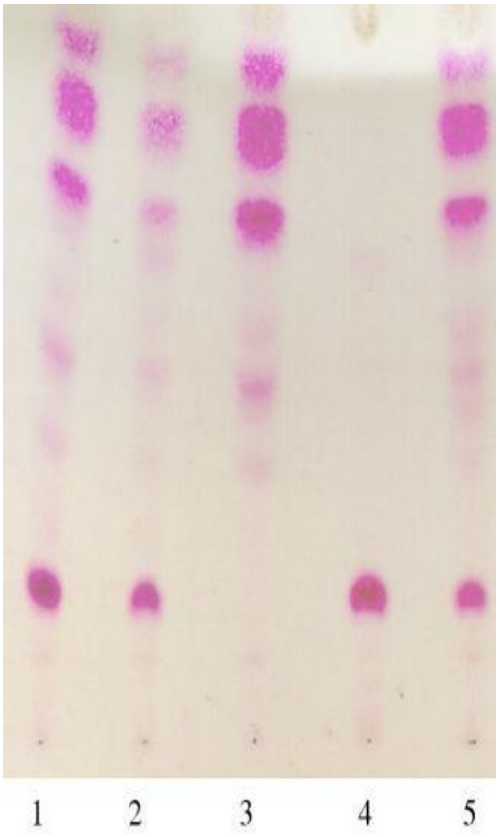


Fig : 3 - Illustrate the Emulsification of crude oil by PHA extracted from *Bacillus liqueniformis*



THIN LAYER CHROMATOGRAPHY



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