Research Article (Open access)

Isolation, Screening and Characterisation of Polyhydroxyalkanoate Producing Bacteria from Oil Contaminated Site

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ABSTRACT- The development of human civilization throughout history has led to the 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. 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 the wrecks of oil tankers carrying diesel oil, cleaning of diesel tanks by merchants, warships carrying diesel oil and motor mechanics. In the present study, the microorganisms utilizing petrol and diesel oil as carbon source were isolated and investigation of their characteristics towards the production of polyhydroxyalkanoates (PHA), which is now a day well known as a biodegradable polymer.

Key-Words: Petrol and Diesel oil contamination, Bioremediation, Biodegradable bacterial polymer, Sudan Black B staining, 16sr RNA sequencing

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INTRODUCTION

Automobiles used (waste) oil contains oxidation products, sediments, water and metallic particles resulting from machinery wears, used batteries, organic and inorganic chemicals used in oil additives and metals [1]. Oil pollution occurs when oil is introduced into the environment directly or indirectly by men's impacts resulting in unfavorable change in such a way that the safety and welfare of any living organisms is endangered. Crude oil if spilled into the water spreads over a wide area forming a slick and oil in water immediately begins to undergo a variety of physical, chemical and biological changes including evaporation of high volatile fractions, dissolution of water-soluble fractions, photochemical oxidation, drill, emulsification, microbial degradation and sedimentation. The concentration of hydrocarbon and non-hydrocarbon components in crude oil from different sources differ greatly [2]. The contamination of the aquatic system with heavy metals has been on the increase since the last century due to industrial activities. Heavy metals are taken up as cations. Among the heavy metals detected in WSF are Pb, Cu, Zn, Cd, Ni, Cr, and V [3]. The extensive usage of petrochemical plastics due to their versatile properties especially durability is causing severe problem in waste management affecting the aesthetic quality of cities, water bodies and natural areas. The accumulation of plastic wastes has become a major concern in terms of the environment [4]. Biopolymers are one product that can help to overcome problems caused by petrochemical polymers are generated from renewable natural sources and are often biodegradable and nontoxic [5]. The most extensively studied thermoplastic biopolymers are the polyhydroxyalkanoates (PHA) and polylactic acid

Received: 21 Jan 2016/Revised: 13 Feb 2016/Accepted: 26 Feb 2016

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LA) [6]. In the present study, was carried out physicochemical characterization of soils from auto-mechanic workshops at different depths (0-15 cm; 15-30 cm, 30-45 cm), namely pH, clay, silt, sand, total organic carbon (TOC), exchangeable cations, and equally examined the distribution of heavy metals such as Pb, Cr, Cu, Zn, Fe, Ni, and Cd.

MATERIALS AND METHODS

Petroleum and Diesel oil contaminated water and sediment samples from various sources during post monsoon and summer season as outlined in Table 1 and used for the physicochemical, Trace metal and bacteriological analysis and also used for the isolation of bacteria. The 2000 mL of water samples were collected with a 2500 mL sterile container in each location. The sediment samples were collected by sterile spatula and stored insterile plastic bags [7].

Table 1: Collection of samples from different sources for isolation of PHA Synthesizing bacteria

S. No	Sample type	Sample code	Place of sampling	Collection time
1	Oil contaminated water sample from mechanic workshop	SW1	Woraiyur, Trichy (site 1)	Summer
2	Oil contaminated water sample from mechanic workshop	SW2	Tennur, Trichy (site 3)	Summer
3	Oil contaminated water sample from mechanic workshop	SW3	K.K.Nagar, Trichy (site 2)	Summer
4	Oil contaminated sediment sample from mechanic workshop	SS1	Woraiyur, Trichy (site 1)	Summer
5	Oil contaminated sediment sample from mechanic workshop	SS2	Tennur, Trichy (site 3)	Summer
6	Oil contaminated sediment sample from mechanic workshop	SS3	K.K.Nagar, Trichy (site 2)	Summer

Physiochemical analysis

The physiochemical parameters, i.e., pH, electrical conductivity (EC) and total dissolved solids (TDS) were measured using field kit (Thermo Orion 5-Star Ph Multi-Meter) on the site and the concentrations of soluble cations and anions (Ca_2^+, Mg_2^+, Na^+, K^+, CO_3^-, HCO_3^-, Cl^- and SO_4^{2-}) were determined according to the standard methods [8-11]. All samples were collected with precautions required for microbiological analysis, held on iceboxes and processed within 12 h of collection.

Trace Metal analysis

For heavy metal analysis, the one liter of oil polluted water was acidified immediately with concentrated nitric acid (HNO_3). For trace metal study, acidified test water samples were filtered by Whatman No. 1 filter paper and processed (APDC + MIBK) for metal analysis. The sediment samples were air-dried and smaller than (>) 63 μm in size were kept back in pre-cleaned properly. Thenceforth, the dried sediment samples were crushed by agate mortar and pestle. Both the samples were processed with an aqua-regia mixture (i.e. HCl: HNO_3= 3:1) in Teflon bomb and were incubated at 140 °C for 2-3 days after dried and sieved samples. After incubation, the reaction mixture was filtered with Whatman No.1 filter paper. The trace metals in the sea water, sea sediment and crab samples were determined by the atomic absorption spectrophotometry (GBC SensAA - AAS, Australia) in flame mode [12].

Isolation and Purification of Bacterial Strains

1 gm of oil contaminated soil sample was taken, serially diluted, plated on nutrient agar plates and incubated at 37°C for 24 h to calculate the bacterial colonies [13]. By using the above method 1 ml of sago wastewater sample was taken for analysis and to calculate the bacterial colonies. After incubation all bacterial colonies were purified on nutrient agar plates and subsequently analyzed for PHAs accumulation and confirmed by Nile blue A staining, gram reaction, motility, spores staining and biochemical tests.

Nile Blue A Staining

Heat fixed bacterial smear was stained with 1% aqueous solution of Nile blue A at 55ºC for 10 min. The slide was washed with tap water to remove the excess stain and washed with 8% aqueous acetic acid for 1 min. The stained smear was again washed with tap water and blot dried. Prior to observe, the slide was remoistened with a drop of water and coverslip was placed on the smear [14]. The slides were viewed in fluorescence microscopy at a wavelength of 480 nm.

Morphological Characterisation

The three potent PHA accumulating strains B5, B7 and B24 were examined for their colony morphology, pigmentation fluorescence, cell shape and gram reaction as per the standard procedures [15].

Biochemical Characterisation

Biochemical tests were carried out as per the method given by Cappuccino and Sherman [16] with 24 hr old cultures.

Molecular Characterisation

The selected strain was identified by ABI 3730 x l sequencer (Applied Biosystems). The 16S rRNA gene was selected with the 16S rRNA gene universal primer.

Dna Extraction, Amplification And Sequencing

Bacterial Genomic DNA was isolated using the Insta Gene™ Matrix Genomic DNA isolation kit. Extracted DNA was The 16s rRNA was amplified using bacterial universal primers for *Bacillus* sp. 27f forward primer and 1492R reverse primer. The amplified PCR product was sequenced using the 518F/800R primers. Sequencing reactions were performed using an ABI PRISM® BigDyeTM Terminator Cycle Sequencing Kits with AmpliTaq® DNA polymerase (FS enzyme) (Applied Biosystems). Amplified DNA obtained from PCR was then sequenced by using an ABI 3730xl sequencer (Applied Biosystems). The sequence of the 16s rRNA gene was compared with the 16s rRNA gene sequences available at the National Center for Biotechnology Information (NCBI) public databases by using their World Wide Web and the BLAST (Protein-Protein blast).

RESULTS AND DISCUSSION

The physicochemical properties of petrol and diesel oil contaminated sediment and water samples in mechanical workshop for the season summer is given in Table 2. The development of human civilization throughout history has led to growing disruption of the natural balance and the occurrence of different types of pollution [17]. Changes in soil properties due to contamination with petroleum-derived substances can lead to water and oxygen deficits as well as shortage of available forms of nitrogen and phosphorus [18-20]. Studies have shown that PAHs can be carcinogenic and/or mutagenic in some circumstances and have been classified as priority pollutants [21].

Table 2: Physiochemical parameters in oil polluted area water and sediment samples in Tiruchirappalli, Tamil Nadu – Summer 2015

S. No.	Parameter	SW1	SW2	SW3	SS1	SS2	SS3
1	pH	8.11	8.64	8.42	7.98	8.64	8.15
2	TDS (mg/L)	914.4	1070.6	800.8	1054.1	1255.8	1094
3	EC (µg/cm)	1451.4	1699.3	1271.1	1673.1	1993.3	1736.5
4	Salinity (ppt)	~1	~1	~1	~1	~1	~1
5	Do (mg/L)	4.6	3.5	3.1	2.8	1.8	2.2
6	BOD (mg/L)	65.3	70.1	61.2	89.4	105.7	91.5
7	TA (mg/L)	141	202	162.8	171.5	224.8	203.1
8	TH (mg/L)	174.8	156.9	148	222.7	276.4	239.1
9	$Ca2^+$ (mg/L)	83.5	72.4	68.9	101.4	124.8	108.4
10	$Mg2^+$ (mg/L)	91.3	84.5	79.1	121.3	151.6	130.7
11	Na^+ (mg/L)	120.5	104.2	93.4	108.9	129.5	115.8
12	K^+ (mg/L)	54.7	71.6	55.4	64.8	70.5	76.8
13	HCO_3^- (mg/L)	136.4	185.6	151.2	160.1	203.4	186.7
14	CO_3^- (mg/L)	4.6	16.4	11.6	11.4	21.4	16.4
15	Cl^- (mg/L)	321.5	400.8	247.8	365.2	402.6	334.5
16	SO_4^2 (mg/L)	72.8	81.3	60.5	80.5	84.7	76.5
17	$N.NO_2^-$ (mg/L)	6.4	14.5	8.9	10.5	18.9	12.4
18	OPO_4^- (mg/L)	11.4	25.9	14.6	12.4	29.8	21.3
19	Oil & Grease(mg/L)	11.3	13.4	9.4	17.6	18.6	14.5

According to Dorn *et al*., [21] hydrocarbon contains substances that are toxic to the flora and fauna found in the ecosystem. Diesel pollution is on the increase in Nigeria, as well as other developing countries. The trace metal parameters of petrol and diesel oil contaminated soil and water samples in mechanical workshop for the season post-monsoon was given in Table 3.

The presence of heavy metals in the environment and specifically in soils, industrial and domestic urban waste endangers living organisms. Once it gets into the food chain, through plants, animals and water sources lead to biomagnification and bioaccumulation in living cells and tissues [22-24].

Table 3: Trace metal parameters in oil polluted area water and sediment samples in Tiruchirappalli, Tamil Nadu-Summer 2015

S. No.	Parameter	SW1	SW2	SW3	SS1	SS2	SS3
1	Cd	2.12	4.1	2.41	3.31	4.69	2.96
2	Cr	0.68	1.12	0.57	0.95	1.21	1.03
3	Cu	2.78	4.23	3.18	4.87	5.58	4.38
4	Fe	8.92	10.56	7.45	15.65	18.94	16.54
5	Ni	0.31	0.52	0.35	0.35	0.72	0.42
6	Pb	2.14	2.64	1.87	3.12	4.12	3.64
7	Zn	4.56	5.98	4.12	7.45	9.26	7.95

Totally 31 isolates were isolated from different samples from three different sites, namely oil contaminated sites from mechanic workshop. All the strains were used for screening of PHA production.

Screening for pha producing bacterial strains

Totally five isolates from site 1 water sample (SW1) namely *Acinetobacter* sp, *Aeromonas* sp, *Aeromonas* sp, *Alcaligenes* sp. and *Bacillus* sp among the isolates only one strain *Bacillus* sp. showed PHA accumulation. In site 1 sediment sample (SS1) five bacterial strains were isolated such as *Bacillus* sp., *Bacillus* sp, *Enterobacter* sp., *Lactobacillus* sp, *Listeria* sp. From site 2 water sample (SW3) five bacterial strains were isolated namely *Paenibacillus* sp and from site 2 sediment sample (SS2) five isolates were identified. In site 3, water sample (SW3) five bacterial strains were isolated namely *Pasteurella* sp. and different types of *Pseudomonas* sp. were isolated and from sediment sample (SS3), six isolates were isolated such as *Serratia* sp., *Citrobacter* sp, *staphylococcus* sp. and different types of *Vibrio* sp. These bacterial strains were identified and confirmed by morphological and biochemical test with the help of nineth editions of Bergey's manual of determinative bacteriology. The result of biochemical test is given in Table 4. Hence out of thiry one isolate one strain are screened for high PHA producers based on nile blue A staining (Fig. 1).

Table 4: Biochemical characterizations of isolated strains from oil polluted regions in Tiruchirappalli

Culture code	Indole	Methyl Red	Voges Proskauer	Citrate	TSI				Catalase	Oxidase	Urease	Starch Hydrolysis	Carbohydrate	Nitrate	Casein Hydrolysis	Coagulase	Gelatin	ONPG	Esculin	Motility	Sporulation	L-Phenyl alanine	Gram Staining	Cocci/ rods
					Slant	But	H2S	Gas																
B1	-	-	-	-	AK	Ak	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Rods
B2	-	-	-	+	Ak	Ak	-	-	+	-	+	-	-	-	-	-	+/-	-	-	-	-	-	-	coccobacilli
B3	+	-	+	+	Ac	Ac	+	+	+	+	-	+	+	+	+	+/-	+	+	-	+	-	+/-	-	Rods
B4	-	-	+	+	Ak	Ac	-	-	+	+	-	-	-	+	-	+/-	+/-	-	-	+	-	+/-	-	Rods
B5	-	-	-	+	Ak	Ak	-	-	+	-	-	-	-	-	+	-	+	-	-	+	+	-	+	Rods
B6	-	-	-	+	Ac	Ak	-	-	+	-	-	+	+	+	+	-	+	-	+	+	+	+	+	Rods
B7	-	-	+	+	Ac	Ac	-	-	+	-	-	-	+	-	+	-	+	-	+	+	+	-	+	Rods
B8	-	+	+	-	Ac	Ac	-	+	+	-	+	+	+	+	+	-	-	+	-	+	-	-	-	Rods
B9	-	-	+	+	Ac	Ac	-	+	-	-	-	-	+	-	+/-	+/-	-	-	+	-	-	+/-	+	Rods
B10	-	+	+	-	Ac	Ac	-	-	+	+	-	-	+	-	-	+/-	-	+	+	+	-	+/-	+	coccobacilli
B11	-	-	-	-	Ak	Ac	-	-	+	-	+	+	+	+	-	-	+	-	-	-	-	+/-	-	Rods
B12	+	-	+	-	Ak	Ac	-	+/-	+	+	+	+	+	-	+	+	+	-	+	+	+	-	+	Rods
B13	+	-	-	-	Ak	Ac	-	+	+	+	+	+	+	-	+	-	-	-	+	+	+	+/-	-	Rods
B14	-	-	+	-	Ak	Ac	-	+	+	-	-	+	+	+	+	-	+	-	+	+	+	+/-	-	Rods
B15	-	-	+/-	-	Ak	Ac	-	-	+	-	+	+	+	+/-	-	-	-	-	+	+	+	+/-	+	Rods
B16	+	+	-	+	Ac	Ak	-	-	+	+	-	+	+	+	-	-	-	-	-	-	-	+/-	-	coccobacilli
B17	-	-	-	+	Ak	Ak	-	-	+	+	+	-	-	+	-	-	+	+/-	-	+	-	-	-	Rods
B18	-	-	-	+	Ak	Ak	-	-	+	-	+	-	-	+	-	-	-	-	-	+	-	+	-	Rods
B19	-	-	-	+	Ak	Ak	-	-	+	-	+	-	-	+	-	-	-	-	-	+	-	+	-	Rods
B20	-	-	-	+	Ak	Ak	-	-	+	-	-	-	-	+	-	-	-	-	-	+	-	+	-	Rods
B21	-	-	-	+	Ak	Ac	-	-	+	+	+	-	+	+	-	+/-	-	-	-	+	-	+/-	-	Rods
B22	-	-	-	+	Ak	Ac	-	+	+	+	+	-	-	+	-	-	-	-	-	+	-	-	-	Rods
B23	-	-	-	+	Ak	Ac	-	-	+	+	+	+	+	+	-	-	-	-	-	+	-	-	-	Rods
B24	-	-	-	+	Ak	Ac	-	-	+	+	+	-	-	-	-	-	-	+/-	-	+	-	-	-	Rods
B25	-	-	-	-	Ak	Ac	-	-	+	+	+	+	-	+	-	-	-	-	-	+	-	+	-	Rods
B26	-	+	+	+	Ak	Ac	-	+	+	-	+		+	+	-	-	-	+	+	+	-	-	-	Rods
B27	-	+	-	+	Ac	Ac	+	+	+	-	+/-	+	+	+	+	-	-	+	+	+	-	-	-	Rods
B28	-	-	+	-	Ak	Ac	-	-	+	+	-	+	+/-	+	+	-	-	+/-	-	-	-	-	+	Cocci
B29	+	+	+	+	Ak	Ac	-	-	+	+	-	-	-	+	-	-	-	+	-	+	-	+	-	coccobacilli
B30	+/-	+	+	+	Ak	Ac	-	-	+/-	+/-	-	+/-	-	+	-	+/-	-	+	-	+	-	+	-	Rods
B31	+	+	+	+	Ak	Ac	-	-	+	+	-	-	-	+	-	-	-	+	-	+	-	+	-	coccobacilli

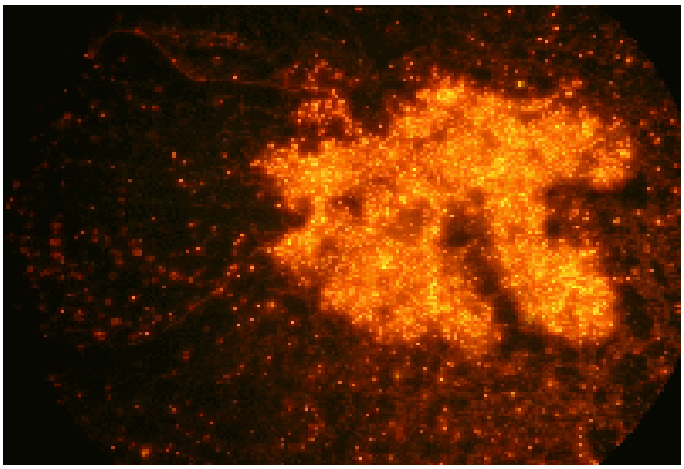

Fig. 1: Nile blue A test for *Bacillus* spp.

Molecular Identification of Pure Isolate Using 16S rRNA

The sequence of partial 16srRNA of this strain was compared against those available in the public database. The sequence is closely related to *Bacillus sp* (97% identity to 16srRNA sequence of similarity to strains). The nucleotide sequence and phylogeny based on these partial 16s rRNA sequences and related bacterial strains are shown in Fig. 2 and Fig. 3. The nucleotide sequence of 16s rRNA of the strain *Bacillus cereus* determined in this study has been deposited the in Gen Bank database under accession number KU512626.

ORIGIN
1 cgtaggatga cgctggcggc gtgcctaata catgcaagtc gagcgaactg attagaagct
61 tgcttctatg acgttagcgg cggacgggtg agtaacacgt gggcaacctg cctg-taagac
121 tgggataact tcgggaaacc gaagctaata ccggatagga tcttctcctt catgggagat
181 gattgaaaga tggtttcggc tatcacttac agatgggccc gcggtgcatt agctagttgg
241 tgaggtaacg gctcaccaag gcaacgatgc atagccgacc tgagagggtg atcggccaca
301 ctgggactga gacacggccc agactcctac gggaggcagc agtagggaat cttccgcaat
361 ggacgaaagt ctgacggagc aacgccgcgt gagtgatgaa ggctttcggg tcgtaaaact
421 ctgttgttag ggaagaacaa gtacgagagt aactgctcgt accttgacgg tac-ctaacca
481 gaaagccacg gctaactacg tgccagcagc cgcggtaata cgtaggtggc aagcgttatc
541 cggaattatt gggcgtaaag cgcgcgcagg cggtttctta agtctgatgt gaaagcccac
601 ggctcaaccg tggagggtca ttggaaactg gggaacttga gtgcagaaga gaaaagcgga
661 attccacgtg tagcggtgaa atgcgtagag atgtggagga acaccagtgg cgaaggcggc
721 tttttggtct gtaactgacg ctgaggcgcg aaagcgtggg gagcaaacag gattagatac
781 cctggtagtc cacgccgtaa acgatgagtg ctaagtgtta gagggtttcc gcccctttagt
841 gctgcagcta acgcattaag cactccgcct ggggagtacg gtcgcaagac tgaaactcaa
901 aggaattgac gggggcccgc acaagcggtg gagcatgtgg tttaattcga agcaacgcga
961 agaaccttac caggtcttga catcctctga caactctaga gatagagcgt tccccttcgg
1021 gggacagagt gacaggtggt gcatggttgt cgtcagctcg tgtcgtgaga tgttgggtta
1081 agtcccgcaa cgagcgcaac ccttgatctt agttgccagc atttagttgg gcactctaag
1141 gtgactgccg gtgacaaacc ggaggaaggt ggggatgacg tcaaatcatc atgcccctta
1201 tgacctgggc tacacacgtg ctacaatgga tggtacaaag ggctgcaaga ccgcgaggtc
1261 aagccaatcc cataaaacca ttctcagttc ggattgtagg ctgcaactcg cctacatgaa
1321 gctggaatcg ctagtaatcg cggatcagca tgccgcggtg aatacgttcc cgggccttgt
1381 acacaccgcc cgtcacacca cgagagtttg taacacccga agtcggtgga gtaaccgtaa
1441 ggagctagcc gcctaaggtg ggacagatga ttggggtgaa gtcgtacggc tacccccaaaa
1501 atgcccgcgg gagatgatcg tgatttcaag cggttctgga cactaaaacc ccctaccaga
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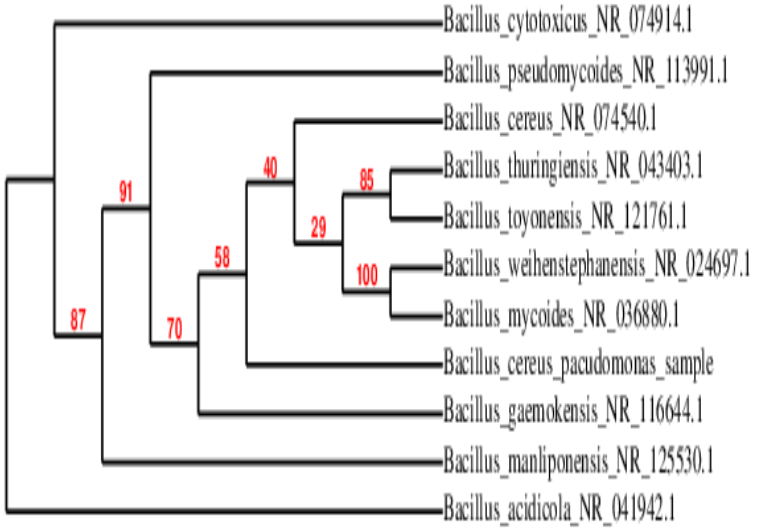

CONCLUSIONS

This study has led to the preliminary finding of bacterial sp. *Bacillus cereus* from oil contaminated mechanic workshop water sample capable of producing PHA. It was also observed that physicochemical and trace metal content of the site.

ACKNOWLEDGEMENT

One of authors (R. Rameshwari) is highly thankful to University Grants Commission (UGC), for financial support by receiving Minor Research Project (SERO/UGC, Hyderabad) No: MRP – 6112/15). The authors also thankful to Department of Civil Engineering, National Institute of Technology (NIT), Trichy, Tamilnadu, India for trace metal analysis.

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