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Screening of Petroleum Hydrocarbon Degrading Bacteria from Wastewater in Sri Lanka

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ABSTRACT

Petroleum hydrocarbon contaminated wastewater plays a major role worldwide promising a need for effective treatment strategies before discharging into natural water sources as they possess a number of negative effects on ecosystems. Biological treatment using microbial biodegradation of contaminants have been used to treat oil contaminated wastes, due to its efficient degradation and cost effectiveness. In view of that, hydrocarbon-degrading bacteria were isolated in Nutrient agar medium which were grown in a minimal medium containing oil as the sole source of carbon. Initially, identification of bacteria was done by biochemical characterization and gram reaction. Further, microbial DNA was extracted from twelve colonies of gram negative rod shaped bacteria as this study was mainly focused on *Pseudomonas aeruginosa which is* a gram negative rod and a potential petroleum hydrocarbon degrading bacterium. Microbial DNA, extracted directly from wastewater samples, were subjected to polymerase chain reaction with specific primers for *Pseudomonas aeruginosa* in order to confirm the presence of *Pseudomonas aeruginosa* in every wastewater sample as *Pseudomonas* species are renowned to be in a variety of habitats. Twelve selected colonies were also screened to identify *Pseudomonas aeruginosa* by using the same primer set. Nine colonies gave positive results for polymerase chain reaction which can be further confirmed by sequence analysis of amplified products of *Pseudomonas aeruginosa* for future bioremediation processes.

KEYWORDS: Bioremediation, Petroleum Hydrocarbon Contamination, *Pseudomonas aeruginosa*, Wastewater

INTRODUCTION

Petroleum hydrocarbon contaminated wastewater is of great concern in today's world as it has become a major source of environmental pollution. It is recorded that 1.7-8.8 million metric tons of oil is released into the world's natural water reservoirs every year (NAS, 1985; Abu and Dike, 2008) contaminating the natural systems.

In Sri Lanka, petroleum hydrocarbon contaminated wastewaters are generated mainly through accumulation of various petroleum based products in natural water bodies due to the runoff of leaks and accidental spills during transportation and, storage (Vieira *et al.*, 2006) and direct discharge of the oily wastes generated from petrochemical industry, metal processing, compressor condensates, lubricant and cooling agents, car washing, and restaurants (Lan *et al.*, 2009).

Adverse environmental and health effects caused by toxic petroleum products have increased the attention towards cleaning up strategies of contaminated sites as oil contaminated effluent generally consists of toxic substances including aliphatic hydrocarbons or paraffins (olefins), aromatic hydrocarbons (benzene, toluene, ethyl benzene and xylene (BTEX), and phenols) (Vieira *et al.*, 2006), polycyclic aromatic hydrocarbons [PAHs] (Neff, 1979) and in smaller amounts, molecules containing sulfur, nitrogen, oxygen, and metals etc. (Vieira *et al.*, 2006), which are inhibitory to plant and animal growth, equally mutagenic and carcinogenic to human being. (Alade *et al.*, 2011).

Bioremediation of oil contaminated wastewater is widely used nowadays (Raghavan and Vivekanandan, 1999; Atlas and Bartha, 1998) as it is cost-effective and will lead to complete mineralization (Das and Chandran, 2010).

A diverse group of microorganisms capable of degrading petroleum hydrocarbons including bacteria and fungi (ZoBell, 1946) are widely distributed in marine, soil and in freshwater ecosystems (Atlas and Bartha, 1973). *Pseudomonas, Achromobacter, Vibrio, Arthrobacter, Acinetobacter, Brevibacterium, , Corynebacterium, Flavobacterium,* and *Micrococcus* are recorded as the most important bacterial genera of hydrocarbon utilizers in aquatic environments where as *Pseudomonas* is the most extensively studied and isolated organism from a variety of habitats (Walker *et al.*, 1975; Mulkins-Phillips and Stewart, 1974; Jensen, 1975) which can be used as an effective bioremediation agent.

In Sri Lanka, Beira Lake, Parliament Lake, Kelani river, Bolgoda Lake and Lunawa lagoon, which are located in main industrial zones in Western Province, can be identified as major water bodies affected by continuous and

prolonged discharge of wastes (Anon, 2001; National Report Sri Lanka).

Therefore, this study was conducted as a preliminary attempt to isolate and identify potential petroleum hydrocarbon degrading bacteria present in water bodies in major industrial zones in Sri Lanka.

MATERIALS AND METHODS

Experimental Location

The study was carried out at the Department of Biotechnology, Faculty of Agriculture and Plantation Management, Wayamba University of Sri Lanka from January to April 2013.

Sample Collection

Wastewater samples were collected from selected locations; Beira Lake, Parliament Lake, Kelani River, Bolgoda Lake, and wastewater canal of Pannala Industrial Zone. Samples were taken into bottles which were washed three times with the same wastewater and stored at 4 ^{0}C for future use.

Isolation of Bacteria

Bacteria were isolated from wastewater samples of Beira Lake and Parliament Lake to identify potential petroleum degrading bacteria. The isolation was carried out in a minimal medium containing NH_4Cl (2.5 g/L), KH_2PO_4 (5.4 g/L), Na_2HPO_4 (4.7 g/L), $MnSO_4$ (0.2 g/L), NaCl (0.1 g/L) and burnt oil (2 ml/L) at pH 7 (Emtiazi *et al.*, 2004). Ten percent of the inoculum was incubated in the minimal medium for overnight at 120 rpm under room temperature. Ten micro liter of liquid culture was spread on the nutrient agar medium and incubated overnight at 37 ^{0}C. Ten colonies from each sample were selected to prepare master plates.

Identification of Bacteria

Biochemical tests and Gram's staining were performed for selected colonies.

Biochemical Tests

A drop of hydrogen peroxide was placed on a clean glass slide for catalase test and a loopful of bacteria was placed on top of it and mixed. Rate of bubble formation was recorded. Further, inoculation loop containing bacteria from selected colonies was mixed with a drop of 3% KOH for 15 sec. Development of slimy thread was observed and recorded.

Gram's Staining

A thin bacterial smear was prepared, air dried and heat fixed on to the clean glass slide. Crystal violet was added and kept for 1 min followed by addition of Iodine solution that kept for 1 min. The slide was then decolorized with 95% ethanol. Few drops of safranin was added and kept for 30 - 40 sec and rinsed slowly with distilled water. The slide was completely air dried and the smear was observed under the oil emersion.

DNA Extraction

DNA was extracted in two approaches, namely, directly from wastewater and from colonies isolated from wastewater.

Direct DNA Extraction from Wastewater Samples

Wastewater samples (treated with glucose (10 g/l) and 12 hr aeration) were centrifuged to pellet down the cells and washed twice with buffer (50 mM TrisHCl & 5 mM EDTA of pH 8.0) followed by lysis with lysis buffer (100 mM TrisHCl, 100 mM EDTA of pH 8 & 1.5 M NaCl). NaOAc and ice-cold isopropanol were added to the separated supernatant and centrifuged to obtain the DNA pellet. (Chaudhuri *et al.*, 2006; Rajapakse *et al.*, 2011). The isolated DNA was analyzed on 0.8% agarose gel.

DNA Extraction from Bacterial Colonies

Five milliliter of liquid culture was inoculated with selected bacterial strains and incubated overnight at 120 rpm. It was spun down and the pellet was re-suspended in TE buffer. Ten percent SDS and 20 mg/ml proteinase K were added to give a final concentration of 100 µg/ml proteinase K in 0.5% SDS. After 1 hr incubation at 37 °C, proteins were precipitated with 5 M NaCl and CTAB / NaCl at 65 °C for 10 min. Then DNA was purified by extraction with Phenol / Chloroform / Isoamyl-alcohol (25:24:1) and Chloroform / Isoamyl-alcohol (1:1) and precipitated with isopropanol. The pellet was washed with 70% ethanol and dissolved in TE buffer (Wilson, 1997). DNA was analyzed on 0.8% agarose gel.

PCR Amplification of DNA with Pseudomonas aeruginosa Primers

PCR amplification was done with a final reaction volume of 20 µl with 40 ng of extracted DNA, 200 µM of each dNTPs, 2 u Taq polymerase (5 u / µl), 10X Taq buffer and 0.4 µM of primer forward 5' GAACGTGCTGGTCTACGACA 3' and reverse 5' GGATACATGCTGCGGTAGT 3'. (Ekanayake *et al.*, 2010).

The PCR program consisted of an initial denauration of 5 min at 94°C followed by 35 cycles of 1 min at 94 °C, 1 min at 62 °C and 1

min at 72 °C with a final extension of 10 min at 72 °C. Final holding temperature was 4°C. PCR poducts were electrophoresesed on 1% agarose gel for 45 min at 50 V.

RESULTS AND DISCUSSION

Isolation of Bacteria

In the present study, petroleum degrading bacteria isolated from different wastewater samples were identified and characterized.

Bacterial strains which were able to grow on minimal medium with petroleum hydrocarbon have shown to posses the ability to utilize the petroleum hydrocarbon as the sole source of carbon. This confirms the ability to survive in petroleum hydrocarbon contaminated environments. These colonies were isolated on to nutrient agar medium in order to isolate the different strains of oil degrading bacteria. Twenty colonies were selected from both Beira Lake and Parliament Lake wastewater samples to prepare master plates to conduct further studies.

This study was mainly focused on *Pseudomonas aeruginosa,* a Gram-negative, aerobic, rod shaped bacterium which is a well-known petroleum hydrocarbon degrader present in every type of waste (Manukulasuriya *et al.*, 2010). Biochemical characterization was done using Catalase test, KOH test, and Gram's staining to narrow the selection for aerobic Gram negative rod shaped bacteria. Aerobic microorganisms posses the tremendous versatility in degrading complex organic compounds (Atlas and Bartha, 1998).

Identification of Bacteria

Catalase test results showed the presence of aerobic bacterial colonies among the 20 isolates except for colonies PL 05, PL 06, and PL 10 (Table 1). Most of the colonies gave positive results for KOH test except for BL 02, BL 05, BL 06, BL 08, and PL 03. The result of KOH test was further confirmed by Gram's staining (Table 1). Based on the results of biochemical tests and Gram's staining, 6 bacterial colonies BL 01, BL 03, BL 04, BL 07, BL 09, BL 10, were selected from Beira Lake (BL) and 6 bacterial colonies PL 01, PL 02, PL 04, PL 07, PL 08, and PL 09 from Parliament Lake (PL) for molecular confirmation.

DNA Extraction

DNA isolation directly from wastewater was carried out by direct lysis method. DNA obtained was good in quality in the concentration of 50 ng/µl (Figure 1).

DNA isolated from selected colonies by SDS lysis method also gave a sufficient

Table 1. Results of Biochemical tests on colonies isolated from wastewater

Col. #	KOH test	H_2O_2 test	Gram +/-	Shape of the cells	Cell aggregation
BL 01	+ >15 sec	+ (B)	-	Coccus	Single
BL 02	-	+ (QB)	+	Rod	aggregates of 3 cells
BL 03	+ <13 sec	+ (B)	-	Coccus	aggregates of 4/5 cells
BL 04	+ < 10 sec	+ (QB)	+	Rod	Single
BL 05	-	+ (QB)	+	Rod	Single
BL 06	-	+ (SB)	+	Rod	aggregates of 4/5 cells
BL 07	+ < 45 sec	+ (QB)	-	Rod	Large cell aggregates
BL 08	-	+ (QB)	-	Coccus	aggregates of 4/5 cells
BL 09	+ 15 sec	+ (QB)	-	Rod	Large cell aggregates
BL 10	+ 15 sec	+ (SB)	-	Rod	Single
PL 01	+ >15 sec	+ (QB)	-	Rod	Large cell aggregates
PL 02	+ 15 sec	+ (QB)	-	Rod	Chain like aggregates
PL 03	-	+ (QB)	+	Coccus	aggregates of 4/5 cells
PL 04	+ >15 sec	+ (QB)	+	Rod	aggregates of 4/5 cells
PL 05	+ 8 sec	-	-	Rod	Single
PL 06	+ 14 sec	-	-	Rod	Single
PL 07	+ 12 sec	+ (B)	-	Rod	Single
PL 08	+ 05 sec	+ (VSB)	-	Rod	2 cells
PL 09	+ 39 sec	+ (VSB)	-	Rod	Large cell aggregates
PL 10	+ 13sec	-	-	Rod	2 cells

BL - Beira Lake, PL - Parliament Lake, QB - Quickly Bubbled, B - bubbled, SB - Slightly Bubbled, VSB - Very Slightly bubbled

amount of good quality DNA (Figure 2 and Figure 3) which were subsequently used for PCR, for amplification of 162 bp DNA fragment of genome *of P. aeruginosa*.

PCR Amplification using Pseudomonas aeruginosa Specific Primers

PCR amplification was optimized with optimum primer concentration of 0.4 µM and by increasing the annealing temperature from 59 °C to 62 °C to obtain a better amplification of the expected product and to avoid the nonspecific amplifications at the annealing temperature of 59 °C.

Detection of Pseudomonas aeruginosa in Different Wastewater Samples

Amplification of specific fragment of 162 bp of genome of *P. aeruginosa* was detected in wastewater samples of Beira Lake, Kelani River, Bolgoda Lake, and wastewater canal of Pannala Industrial Zone confirming the presence of *P. aeruginosa* in various wastewater samples in Sri Lanka (Figure 4). In previous studies *P. aeruginosa* has identified to be in different climatic zones in Sri Lanka in different waste types (Ekanayake *et al*, 2010) confirming it is well established under the local environmental conditions.

Screening of Pseudomonas aeruginosa from Isolated Colonies

PCR amplification was done to screen the isolated colonies where, specific region of 162 bp of *P. aeruginosa* genome was amplified and it confirmed the presence of *P. aeruginosa* in selected colonies. Amplified products were observed for nine colonies, BL 01, BL 03, BL 07, BL 09, BL 10, PL 01, PL 04, PL 08, and PL 09 in between 100 bp and 200 bp region of the 100 bp ladder, confirming the detection of *P. aeruginosa* in the water sources (Figure 5). Three colonies BL 09, BL 10, and PL 09 were randomly selected and again amplified (Figure 4) for further confirmation by sequence analysis.

In the present study the identification of *P. aeruginosa*, a common Petroleum degrading bacterium, from two selected locations was successfully done, allowing opportunity for further studies in using the bacterium as a potential bioremediation agent.

Figure 1. Agarose gel electrophoresis of direct DNA of Bolgoda Lake wastewater

Figure 2. Agarose gel electrophoresis of DNA of colonies isolated from Beira Lake

Lane 1, 2: 01, Lane 3, 4: 03, Lane 5, 6: 04, Lane 7, 8: 07, Lane 9, 10: 09, Lane 11: 10

Figure 3. Agarose gel electrophoresis of DNA of colonies isolated from Parliament Lake

Lane 12, 13: 01, Lane 14, 15: 02, Lane 16, 17: 04, Lane 18, 19: 07, Lane 20, 21: 08 Lane 22, 23: 09

Figure 4. Agarose gel electrophoresis of PCR products of direct DNA

Lane 1: 100bp Ladder, Lane 2: Negative control, Lane 3, 4: BL 09, 10, Lane 5: PL 09, Lane 6: Beira Lake (direct), Lane 7: Kelani river (direct) Lane 8: BolgodamLake (direct), Lane 9: wastewater canal of Pannala Industrial Zone (direct)

Figure 5. Agarose gel electrophoresis of PCR products of colony DNA

Lane 1: 100bp Ladder, Lane 2-6: BL 01, 03, 07, 09, 10, Lane 7-10: PL 01, 04, 08 09, Lane 11: Negative control

CONCLUSIONS

The isolation of bacteria in the petroleum medium confirmed the availability of petroleum degrading bacteria. Most of the bacterial isolates were gram negative and aerobic. PCR amplification with specific primer of *Pseudomonas aeruginosa* resulted expected fragment of 162 bp confirming the presence of the bacterium in every wastewater sample. Nine bacterial isolates were identified from both Beira Lake and Parliament Lake which can be confirmed by sequence analysis.

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