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Annals of Biological Research, 2015, 6 (3):30-35
(http://scholarsresearchlibrary.com/archive.html)

Annals of Biological Research
Scholars Research Library
ISSN 0976-1233
CODEN (USA): ABRNBW

Analysis of genetic diversity among naphthalene degrading *Pseudomonas aeruginosa* strains from petroleum contaminated sites by RAPD-PCR

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ABSTRACT

The genus pseudomonas spp., are able to metabolise the petrochemical pollutants in the environment, and as a result can be used for bioremediation. Naphthalene is a major component in the petroleum that can cause damages the surrounding ecosystems. The pollutant may inhibit some microbial communities that are important in some biogeochemical cycles of that ecosystem and this affects the productivity of such ecosystems. The aim of present study was to investigate the genetic diversity of naphthalene degrading Pseudomonas aeruginosa by using random amplified polymorphic DNA-polymerase chain reaction (RAPD-PCR) method. The petroleum contaminated three different kinds of soil sample like A. petrol, B. diesel, and C. both (A&B) were collected from petrol spilling, diesel spilling and petrol+diesel spilling sites respectively in Tamil Nadu. The pure culture of P.aeruginosa isolated from cetrimide agar and this species conformed through gram staining, and various biochemical analyses. The naphthalene degrading P.aeruginosa conformed by catechol as metabolic intermediates in the substrate of naphthalene. The species of interest genetic diversity was done by RAPD-PCR method and gel image creates dendogram analysis. RAPD-PCR was carried out to determine the genetic diversity of P.aeruginosa in the petroleum by-products contaminate soil samples. Totally 39 bands was amplified by using five random (OPW-6 to 10) short singe primers. In the present study, we found that RAPD-PCR technique as a useful tool for investigation of the genetic diversity among P.aeruginosa and the number of bands and banding pattern were variable depending upon the primer and the type of species tested. This also study showed that a relative high polymorphism among P.aeruginosa isolates from sample A and C than B.

Keywords: Petroleum, RAPD-PCR, *Pseudomonas aeruginosa*, Naphthalene and Dendrogram.

INTRODUCTION

Oil spills are major hazards to the environment as they damage the surrounding ecosystems [1]. Petroleum fuel spills as a result of pipeline ruptures, tank failure, various production storage and transportation accidents are considered as the most frequent organic pollutant of soil and aquatic environment and are classified as hazardous wastes due to their cytotoxic, mutagenic and carcinogenic effects on human [2]. An ability to isolate high numbers of certain oiled grading microorganisms from an environment is commonly taken as evidence that those microorganisms are the active degraders of that environment. A number of well-known microorganisms are responsible for the biodegradation of oil hydrocarbons. Bacteria have evolved regulatory systems that ensure the synthesis of enzymes so that the initial attack on these compounds is induced only when required. Thus, for an organism with the genetic information for utilizing benzene as carbon source, the enzyme for degrading benzene is induced when benzene reaches the bacterial environment. Some of these organisms have evolved an additional and highly effective system for responding to a variety of potential growth substrate (3). The environmental isolates from soil, an oil-contaminated aquifer, and mushroom compost did not cluster away from clinical isolates(4). Most recent research has been focused on the degradation activity of oil compounds by microorganisms. Naphthalene has higher activity

over other polycyclic aromatic hydrocarbons, and the fact that naphthalene degrader enzymes are encoded by plasmid has facilitated the research about naphthalene's biological degradation. In the past, the physicochemical methods were used to degrade aromatic compounds and their derivatives, but today the priority is bioremediation. Thus, oil compounds-contaminated soils can be cleaned up by isolation, purification, and reproduction of the species with higher potency to remove these compounds (5). *P.aeruginosa* is a Gram-negative, motile, extra cellular, aerobic, and rod-shaped bacterium that belongs to the α-*proteobacteria* and it can able to consume a broad range of organic compounds. It is ubiquitous in the natural environmental settings for it can be isolated from animate and inanimate objective (6).It is also used as a model organism for a number research effort has gone into studying its ability to form biofilms (7). *P.aeruginosa* has a large genome of around 6.5 million base pairs encoding some 5270 predicted open reading frames on its single chromosome. Its chromosome possesses significantly more distinct gene families (paralogues group) than Escherichia coli, Bacillus subtilis and Mycobacterium, a factor which may contribute to its broad environmental range (8). It is also notable that 9% of the assigned open reading frames of *P.aeruginosa* encode known or putative transcriptional regulators, which have been hypothesised to enable the bacterium to adapt to a wide range of environment (9).A number of molecular tools and procedures are being employed to establish DNA fingerprinting profiles and each of these procedures has its strengths and weaknesses. The use of molecular markers to study genetic diversity will help in characterizing the *Pseudomonas sp*. isolated from milk samples (10).Polymorphism in RAPD may be due to deletion, addition or substitution of base. High diversity might be one of the possible reasons for a strain to adapt to the new environment, which is useful for its propagation and domestication of wild species. Geographically, isolated individuals tend to accumulate genetic variations during the course of environmental adaptations (11).The aim of this study was then to determine the RAPD genotyping of *P.aeruginosa* strains isolated from different petroleum contaminated soil samples to characterize their genetic diversity.

MATERIALS AND METHODS

The sampling procedure and sites was selected, three different soil samples were collected from oil spilled three locations in Tamil Nadu. We considered as1.sample-A (petrol), 2.sample-B (Diesel) and sample-C (Petrol and Diesel), this name given by based on petroleum by-products and collected site. All samples were collected under sterile manner and were immediately transferred to the laboratory. Screening methods was performed for *P.aeruginosa* from all samples as described previously (12).

2.1. Isolation and metabolically identification of naphthalene degrading bacterial species:

The all three samples (A, B, and C) were serially diluted (10^{-3}-10^{-4}) and were 1 ml of each sample transferred into 0.001 gram of naphthalene containing corresponding M9 media and kept in the shaker incubator for 8-10 days at 37°C. OD values were taken at each and every day, until it reaches stationary phase. Enriched 0.1 ml of sample was transferred into respective sterile naphthalene and salicylic acid containing M9 agar plates and incubates for 7-10 days at 37°C to obtain naphthalene degrading bacterial species isolated colonies. After incubation, naphthalene degraded colonies were identified due to yellow colour development.

2.2. Isolation and identification of *P.aeruginosa*:

The yellow colour colonies were selected and streaked onto cetrimide agar plates for selective isolation of *P.aeruginosa* and this media act as a deterrent to inhibit other bacteria and enhance the production of pyocyanin and pyoverdine pigments. All three different samples of isolate were screened by gram staining method. After that, the isolates were also identified as *P.aeruginosa* by the application of biochemical analysis including IMViC, starch hydrolysis, catalase, oxidase, Urease, nitrate production and casein test. Each isolate was obtained from different oil contaminated soil samples. The selected isolates were preserved at -70°C in TSB medium (Hi-media, India) supplemented with15% glycerol until further processing.

2.3. Genomic DNA extracted from *P.aeruginosa* isolates:

Bacterial genomic DNA was extracted from 200µl of suspension culture cells (10^4 -10^8) prepared from pure culture of pseudomonas spp., by using phenol-chloroform DNA extraction method. The DNA pellet was dissolved in 200µl of TE buffer and stored at 4°C for further use. The extracted DNA was then quantified and purity checked by using the nanodrop spectrophotometer (ND-1000). Pure DNA obtained and was with 0.8% agarose gel to check the quality of DNA.

2.4.RAPD-PCR analysis:

RAPD-PCR was performed as described previously (13), for our DNA amplification on thermocycler (Bio-Rad, USA) in a final volume of 25µl that containing 1µl of DNA (equivalent to 40ng) and master mix[10x PCR buffer (2.5µl), 2.5mM of Mgcl2 (1µl), 10mMdNTP mix (1µl), 50µM primers (1µl) for each reaction, 0.5U/reaction of Taq polymerase(0.5µl), and finally volume adjusted by sterile distilled water (17.5µl)]. We were used five different

indiscriminate primer sequences (OPW-6, OPW-7,OPW-8, OPW-9, OPW-10,) which is given in table:**01**. Optimized cycling conditions, we were followed that are given in the table:**02.**

Table:01. Primer profile and primer sequences of OPW6-10 for RAPD-PCR

Serial no	Operon code	Primer sequence (5'-3")	Annealing temperature	G+C (%)
1	OPW-1	ACGCCCGATG	34	70
2	OPW-2	CTGGACGTCA	32	60
3	OPW-3	GACTGCCTCT	32	60
4	OPW-4	GTGACCGAGT	32	60
5	OPW-5	TCGCATCCCT	32	60

Table: 02.Temperature profile and cycling condition for PCR analysis

Step	Stage	temperature	Period of Time	Cycle
1	Initial Denaturation	95°C	4 minutes	1
2	Denaturation	94°C	1 minute	30
	Annealing	32/34°C	1 minute	
	Extension	72°C	2minutes	
3	Final Extension	72°C	10minutes	1

The RAPD-PCR products were loaded on 1.5% (w/v)agarose gel with 0.5mg/ml ethidium bromide and run the gel in electrophoresis at 150v for 45 minutes. Then the banding pattern was observed and analysed in the gel documentation system (Bio-Rad, USA). RAPD fingerprints were analysed and genotyped to assign, on the basis of number and weight of band differences. Each reaction was repeated at least two times for reproducibility. At each band position, two possible alleles were considered either present (a score of 1) or absent (a score of 0). Different RAPD profiles were designed by different scores and classified as different genotypes. Phylogenetic variation was determined by converting RAPD data into frequency similarity that analysed by Unweighted Pair Group Method with Arithmetic mean (UPGMA)cluster analysis to produce phylogenetic tree.

RESULTS AND DISCUSSION

In the current study, we aimed to isolate and identify naphthalene degrading *P.aeruginosa* and its genetic diversity from different petroleum oil contaminated soil samples. The oil contaminated soil samples A,B and C were collected from different sites of oil spilling in Tamil Nadu, India.

Naphthalene degrading *P.aeruginosa* colonies were isolated from sample A, B and C in M9 agar medium. All samples were in M9 agar media, naphthalene degrading bacterial colonies turns yellow in colour indicates positive results, due to the accumulation of catechol 2,3 dioxygenase by this species which in turn converts the catechol (a colourless substrate)into 2-hydroxylmuconate semi-aldehyde (yellow in colour) in the presence of salicylic acid as intermediate. The gram staining characteristics were identified with in the mixed culture as gram negative short rods.

Table: 03: Metabolic, Gram characteristic and biochemical analysis for *P.aeruginosa*

Serial no	SAMPLES	SUBSTRATE	RESULTS
1	Petrol	Catechol in M9 with NPN&SA	Yellow
2	Diesel	Catechol in M9 with NPN&SA	Yellow
3	Petrol & Diesel	Catechol in M9 with NPN&SA	Yellow
	ORGANISMS	**SHAPES**	**GRAM CHARACTERS**
1	*P. aeruginosa*.	Short rods	Negative
	BIOCHEMICAL TEST	**INDICATION**	**RESULTS**
1	Indole production test	cherry red coloured ring	Positive
2	Methyl Red Test	No red coloured solution	Negative
3	V-P Test	No Cherry red colour	Negative
4	Citrate Utilization test	Change in blue colour slant	Positive
5	Starch Agar Test	No zone upon Iodine addition	Negative
6	Catalase Test	The colonies turned blue	Positive
7	Oxidase test	Effervescence is observed	Positive
8	Urease test	No colour change	Negative
9	Nitrate reduction test	No red coloured solution	Positive
10	Casein test	Casein is hydrolysis	Positive

The isolated *P.aeruginosa* strains were characterized by biochemical analysis. The results were observed and tabulated on the **table:03**. All of the three isolates were showed positive result to citrate utilization test, catalase test,

Indole test, oxidase test, Nitrate reduction test, and casein test and negative to MR-VP test, starch hydrolysis and Urease.

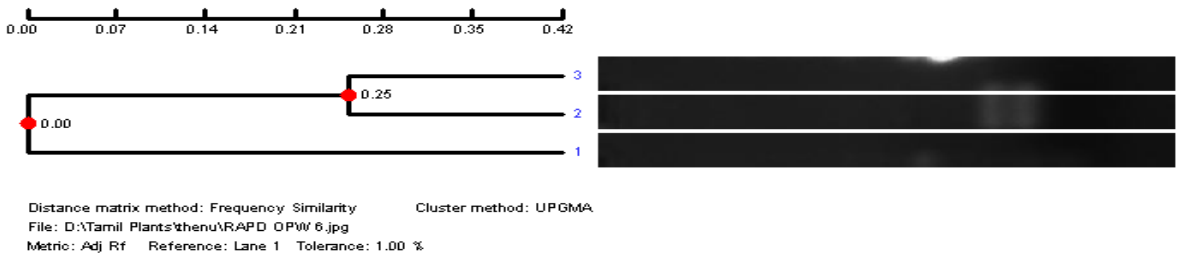
The quality of DNA was estimated by nano-drop spectrophotometer at 260/280nm, if the OD is between 1.8-2.0, then the DNA considered as pure in form.

The genetic diversity among 3 different samples of *P.aeruginosa* was determined by RAPD technique using 10 arbitrary primers from OPW-6 to OPW-10.

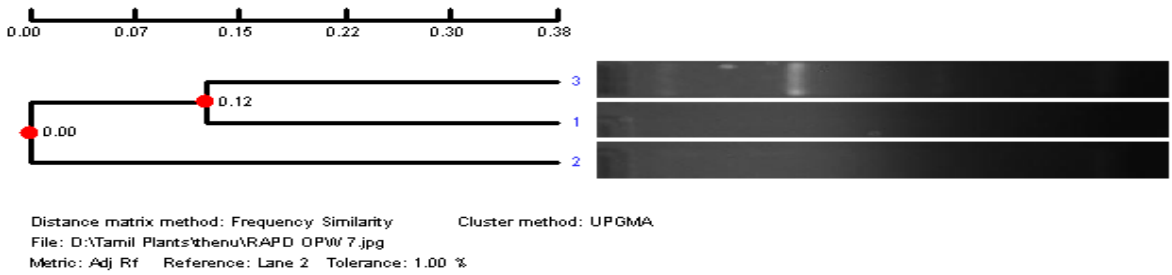
In this study, we could reveal that genetic diversity among *P.aeruginosa* was analysed by RAPD technique by using random primers (OPW 6-10). The amplified product was run on 1% agarose gel. UPGMA (Unweight Pair Group Method with Arithmetic mean) was initially designed for use in protein electrophoresis studies, but is currently most often used to produce genetic diversity among this species or with in the species for more sophisticated phylogenetic reconstruction algorithms to construct dendrogram. Cluster analysis was performed on the basis of similarity co-efficient generated from RAPD banding profiles. The cluster analysis was done separately for each primer against all three samples. Totally 39 bands were amplified in the three samples against five different primers, therefore gene diversity among *P.aeruginosa* was identified from primers (OPW6-10)**.** Phylogenetic diversity between *P.aeruginosa* was determined by using dendrogram (**fig:01.a,b,c,d,&e).**

Fig 1: Dendrogram analysis for OPW-6 to OPW-10 against three different sample on the basis of RAPD similarity matrix data by UPGMA cluster analysis

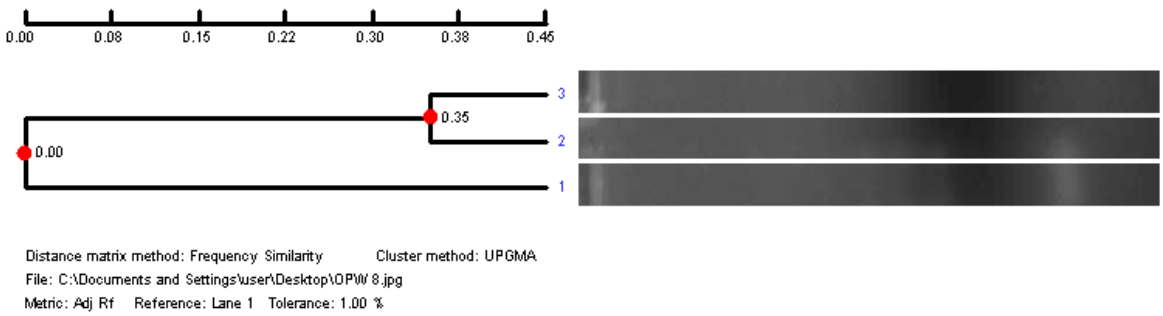
A)OPW-6



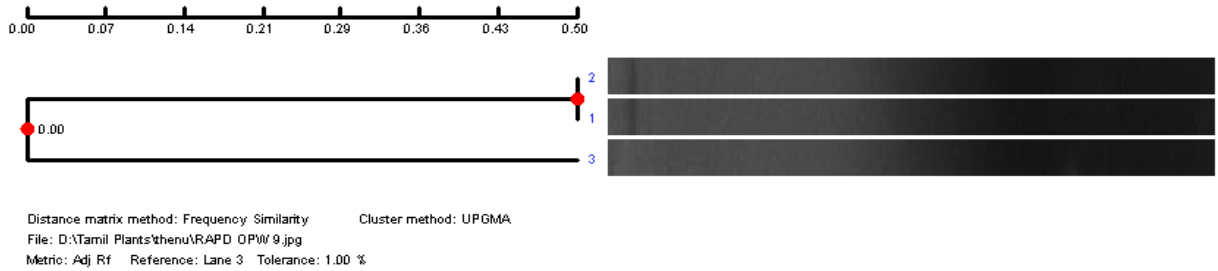
b)OPW-7



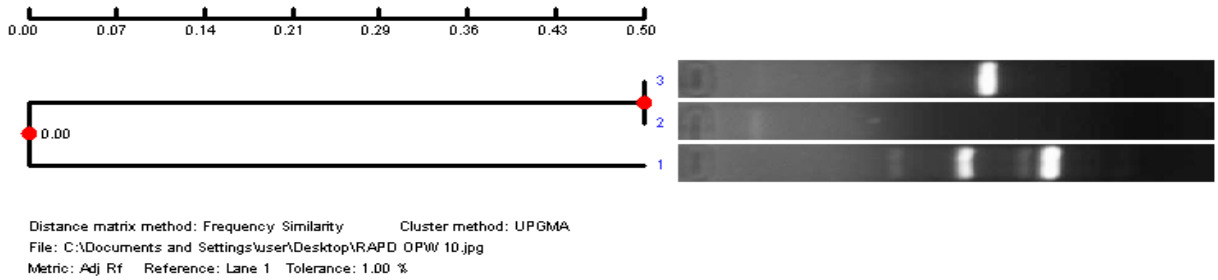
c)OPW-8



d)OPW-9



e)OPW-10



In order to conduct genetic diversity studies regarding the distribution of *P.aeruginosa* in certain setting, rapid molecular methods such as RAPD-PCR have been shown to be useful for genetic system that has shown great specificity and sensitivity to define the bacterial isolate (14). This technique utilized for genotyping among *P.aeruginosa* strains in the milk samples (10). It also used for genetic diversity in *P.aeruginosa* and they concluded that RAPD-PCR method can be used as a first screening technique in epidemiological characterization of these strains (15).The mutation by induced by UV irradiation, more effective oil degrading bacterial isolates, through increasing the activity of hydrocarbon-degradation key enzymes (Catechol 2, 3dioxygenase and Alkane monooxygenase) and also testing their adaptability to salinity, so they could be applied in oil spilled brine soil and oil polluted sea water (16). The active role of cyanobacteria isolated from textile industry effluent in the reduction of pollution level may be due to the acclimatization to its source of isolation. Biological treatment by activated sludge is better to reduce population from textile industry effluent because of its natural system of purification (17).

CONCLUSION

In the present study, we found that RAPD-PCR technique as a useful tool for investigation of the genetic diversity among *P.aeruginosa* and the number of bands and banding pattern were variable depending upon the primer and the type of species tested. This also study showed that a relative high polymorphism among *P.aeruginosa* isolates from sample A and C than B. Both petrol and diesel contaminated soils sample C isolate having higher polymorphisms against OPW6-10 primers. Our study confirmed that further analysis with more number of samples and those samples further continue to work for sequencing and also quantify the enzyme which is used to degrade petroleum by-product naphthalene.

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