ISSN 0975-6299 Vol 2/Issue 1/ Jan-Mar 2011

International Journal of Pharma and Bio Sciences

RESEARCH ARTICLE BIOTECHNOLOGY

AMPLIFICATION OF BIOSURFACTANT PRODUCING GENE (rhlB) FROM *PSEUDOMONAS AERUGINOSA* ISOLATED FROM OIL CONTAMINATED SOIL

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ABSTRACT

Bacteria could utilize hydrocarbon as a carbon source in an oil contaminated environment. A total of thirty oil contaminated soil samples were collected from the several districts of Tamil nadu for the isolation and characterization of *Pseudomonas aeruginosa* by using gram staining, motility and biochemical tests, it was compared with standard strain of *P.aeruginosa* (ATCC 9027). All the *Pseudomonas* strains isolated from the samples have been reduced the surface tension and showed the emulsification index very effectively. The results of antagonistic test showed, the *Pseudomonas* strain was effective against *Bacillus subtilus* and *Staphylococcus aureus* due to the production of bio surfactant and the genomic DNA of *P. aeruginosa* was isolated through standard methods and amplified with specific primers (kpd 1 & kpd 2) to ensure the presence of rhl B gene (723 bp- rhamno lipid gene) involved in the production of bio surfactant and the high quality band of amplified gene (723 bp) was observed.

KEY WORDS

Contaminated soil, Bio surfactant, *P. aeruginosa,* rhlB gene and Primers

INTRODUCTION

The quality of soil is very important for the living things, when it contaminated with oils (fuel oils, hydraulic oil, automotive oils and lubricating oils) it leads to severe land pollution. Most oil contamination is the result of accidental spills and leaks. Now a day's several attentions have been paid to solve this problem. To recover the oil contaminated soils some types of amphiphilic nature surfactants are used. These compounds can reduce the free energy of the system by replacing the bulk molecules of higher energy at an interface and used for soil washing or flushing due to their ability to mobilize the contaminants. These surfactants have been used industrially as adhesive, flocculating, wetting and foaming agents [1], based on their abilities to lower surface tension, increase solubility, detergency power, wetting ability and foaming capacity. Generally the bio surfactants are grouped as glycol lipids, lipo peptides, phospho lipids, fatty acid natural lipids, etc [2]. A group of bio surfactant that has been studied extensively about rhamno lipids from *Pseudomonas aeruginosa* [3&4] and encoded by rhlB gene1 this is responsible for rhamnosyl transferase 1 and these is regulated by regulatory gene in some cases some regulatory gene affecting the rhamnolipd synthesis in *P. aeruginosa* [5]. The choice of surfactants is primarily based on product cost [1] and effective action on oil contaminants. The bio surfactants have play important roles in environmental remediation [6], so the new markets are currently being developed to use bio surfactants producing bacteria in the bioremediation of contaminated land [7]. Noordman et al., [8] studied the effects of the bio-surfactant from the *P. aeruginosa* on hexadecane degradation. These researches was mainly focused on the amplification of biosurfactant gene from P.aeruginosa isolatedom oil contaminated soils and screen the effective *P. aeruginosa* strain from the different locations for remediation purpose.

MATERIALS AND METHODS

Sample Collection and Processing of Samples

Oil contaminated soil samples (includes petrol, diesel and kerosene), were collected from the different areas of tamil nadu(Table-1) with a depth of 2-3 inches from the ground level using clean spatula. The soil was transferred into a sterile polythyne bags and stored at 4°C until using. The soil samples (10 g) were suspended in 100 ml of sterilized Saline/ double distilled water in 250ml conical flask, the samples were then kept in shaker at 200rpm for 48hrs to ensure complete mixing of soil and subsequently 1 ml of this suspension was added to 9 ml saline to obtain desired dilutions up to 10^{-6}. 0.1ml of 10^{-5} dilution of each samples were spread on nutrient agar media. The inoculated plates were incubated at 37°C for 24 hrs and observed for pigmented colonies. The pigmented colonies were then streaked onto the Cetrimide agar medium, (a selective medium for *Pseudomonas aeruginosa*) and incubated at 37°C for 24hrs. The isolated colonies were characterized by various biochemical tests.

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Table 1
The Samples Collected from Different District of Tamil Nadu, India.

S.No	Sample Name	Sample collection sites
01	PS01	Mechanic shed (Diesel) Kallakurichi, Villupuram District, Tamil Nadu.
02	PS02	Mechanic shed (Diesel) Villupuram District, Tamil Nadu
03	PS03	Mechanic shed (Petrol) Thiruvannamalai District, Tamil Nadu.
04	PS04	Mechanic shed (Petrol) Sengal pattu, Kancheepuram District, Tamil Nadu.
05	PS05	Mechanic shed (Petrol) Ashok Nagar, Chennai, Tamil Nadu.
06	PS06	Mechanic shed (Petrol) Kumba gonum District, Tamil Nadu.
07	PS07	Mechanic shed (Crude Oil) Ariyalur District, Tamil Nadu.
08	PS08	Mechanic shed (Petrol) Jeyankondam, Ariyalur District, Tamil Nadu.
09	PS09	Bus Depo (Diesel) Vadapalani, Chennai, Tamil Nadu
10	PS10	Auto Workshop (Petrol) Attur, Salem District, Tamil Nadu.
11	PS11	Oil Engineering Workshop (Diesel) Attur, Salem District, Tamil Nadu.
12	PS12	Automobile Workshop (Petrol) Adyar, Chennai, Tamil Nadu.
13	PS13	Diesel filling station (Diesel) Sangarapuram, Thivannamalai District, Tamil Nadu.
14	PS14	Diesel filling station (Diesel) Chidambaram, Kadalur District, Tamil Nadu
15	PS15	Diesel filling station (Diesel) Kallakurichi, Villupuram District, Tamil Nadu
16	PS16	Diesel filling station (Diesel) Ulundur pet, Villupuram District, Tamil Nadu.
17	PS17	Petrol filling station (Petrol) Osur, Krishnagiri District, Tamil Nadu
18	PS18	Petrol filling station (Petrol),Sangarapuram, Thiruvannamalai District, Tamil Nadu
19	PS19	Petrol filling station (Petrol) Chetpet, Chennai, Tamil Nadu.
20	PS20	Petrol filling station (Petrol) Cuddalure District, Tamil Nadu.
21	PS21	Diesel filling station (Diesel) Chinna Salem, Villupuram District, Tamil Nadu.
22	PS22	Petrol filling station (Petrol) Teynampet, Chennai, Tamil Nadu.
23	PS23	Mechanic shed (Diesel) Thindivanam, Villupuram District, Tamil Nadu.
24	PS24	Diesel filling station (Diesel) Attur, Salem District, Tamil Nadu
25	PS25	Petrol filling station (Petrol) Neyvely, Kadalur District, Tamil Nadu
26	PS26	Mechanic shed (Petrol) Villupuram District, Tamil Nadu.
27	PS27	Automobile workshop (Diesel) Thiruvannamalai District, Tamil Nadu.
28	PS28	Oil Engineering workshop (Petrol) Thirukoilur, Villupuram District, Tamil Nadu.
29	PS29	Diesel filling station (Diesel) Jeyankondam, Ariyalur District, Tamil Nadu.
30	PS30	Diesel filling station (Diesel) Kallakurichi, Villupuram District, Tamil Nadu.

Production and Screening of Bio Surfactants

The isolated culture (*P. aeruginosa*) was inoculated in MS (Mineral Salt medium) medium and kept in shaker at 200 rpm/min for 48 hours to ensure complete aeration at 35°C. The productions were conducted in 250ml of medium in1000 ml conical flasks. The screening of bio surfactants from *P. aeruginosa* were characterized by production of yellowish green pigment in cetrimide agar medium and also screened by the following three methods [9].

(i) *Surface Tension;*

Based on the surface tension reducing capacity of *P. aeruginosa* on oils were used to screen the bio surfactants and it was calculated by using standard drop weight method [10&11].

Surface tension(T) = mg /3.8r Nm^{-1}

Where,

m : mass of one drop of the liquid.
g : acceleration due to gravity.
r : radius of the capillary tube.

To determine the surface tension, mass of the medium has to be calculated and simply weighing the drop of the medium.

Mass of one drop the medium, m = W_2-W_1 / total droplet.

Where,

W_2 : weight of the sample with beaker.
W_1 : weight of the empty beaker.

(ii) Emulsification Index:

The ability of *P.aeruginosa* on the emulsification index on hydrocarbon was calculated by standard method [12].

E 24 = (height of the emulsified layer/ total height of the hydrocarbon)*100

(iii)Antagonistic Test:

The antagonistic property of *P. aeruginosa* was tested with *Bacillus subtilus, Staphylococcus aureus, Aeromonas,sp* and *Escherichia coli,* for the antimicrobial activity of the bio surfactant [13].

Isolation of Genomic DNA from P. aeruginosa and Detection of rhlB Gene:

1.5 ml of overnight broth cultures were taken in micro cetrifuge tubes to isolate the genomic DNA by using modified method of [5]. The isolated DNA samples were run in an agarose gel electrophoresis there the dark orange bands were observed under UV transilluminater and isolated samples were subjected to PCR analysis to detect the rhlB gene with the help of kpd1 (**forward primer: 5_ GCC CAC GAC CAG TTC GAC 3_**) and kpd2 (**reverse primer: 5_ CAT CCC CCT CCC TAT GAC 3_**) primers which has homologous sequence with 1030-1048 bp region of the rhlB gene. After amplification, the final PCR product was ran in 1% agarose in TE buffer and the gel was stained with EtBr. After the completion of reaction, add 5µl of loading dye, mix it and load the total 25µl into agarose gel electrophoresis.

RESULTS AND DISCUSSION

About thirty different (Petrol, Diesel and Crude oil) oil contaminated soil samples were collected from different districts of Tamil Nadu (Table 1). Total viable and non-viable populations were counted from the soil samples by using serial dilution method (Table 2). The viable cells ranging from 4.1×10^5 to 4.4×10^6 CFU/gm, were isolated from oil contaminated soil. These results were correlated with the work of [14&15] from undisturbed and contaminated arid southwestern soils.

Table 2
Number of Total Viable Bacteria from Each of the Soil Sample (Pseudomonas aeruginosa Strains)

S.No	Sample Number	Average Number of Bacteria Present
1	PSO2	5.7×10^5
2	PSO3	3.8×10^6
3	PS06	2.6×10^6
4	PS07	5.1×10^5
5	PS08	2.2×10^6
6	PS11	2.7×10^6
7	PS13	3.7×10^6
8	PS17	4.4×10^6
9	PS19	7.1×10^5
10	PS21	3.8×10^6
11	PS23	4.4×10^6
12	PS25	3.8×10^6
13	PS26	3.4×10^6
14	PS27	3.1×10^6
15	PS29	2.9×10^6
16	PS30	4.1×10^5

Qualitative measurement of surface tension

The surface tensions of the medium were reduced from the range of 0.064 Nm^{-1} to 0.028 to 0.058 Nm^{-1}. Almost the resemble results was obtained by [10&11] (Table-3). Among the 16 viable cultures (viable cells), 14 were found to be effectively reduced the surface tension which indicates the presence of surface active agents, except samples PS08 and PS21.

Table 3
The Surface Tension Reduced Values of oil contaminated soils

S.No	Sample	Surface Tension N/m^{-1}
01	CONTROL	0.064
02	STANDARD STRAIN (ATCC 9027)	0.028
03	PS02	0.030
04	PS03	0.029
05	PS06	0.058
06	PS07	0.028
07	PS11	0.046
08	PS13	0.052
09	PS17	0.031
10	PS19	0.037
11	PS23	0.032
12	PS25	0.042
13	PS26	0.033
14	PS27	0.038
15	PS29	0.040
16	PS30	0.038

Antagonist Test

Antagonistic activity of *P. aeruginosa* was tested against *Bacillus subtilus, Staphylococcus aureus, E.coli, and Aeromonas hydrophila*. It was observed that the culture spent medium had exhibited some toxic effect, and inhibits the growth of selected microorganisms. This result was correlated with *Pseudomonas aeruginosa* isolated from Soya been oil refinery wastes [13] and was tested against *Staphylococcus aureus* and *E.coli.*

Emulsification test

The isolated samples/cultures were tested for the emulsifying activity with Petrol, Kerosene (crude oil) [16] and Diesel. It was found that E_{24} was highest 75% with Petrol, 66.6% with Kerosene and 70% with Diesel in the culture samples PS07 and lowest in the sample PS13 35% with Petrol, 22% with Kerosene and 25% with Diesel (Fig.1).

Fig.1

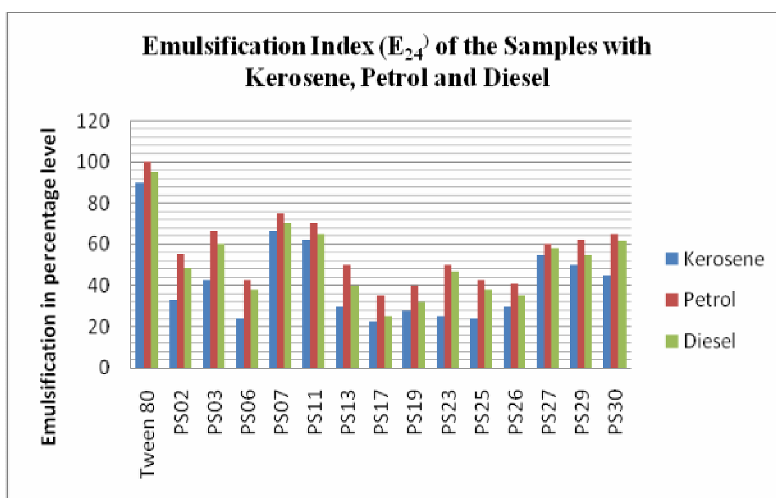


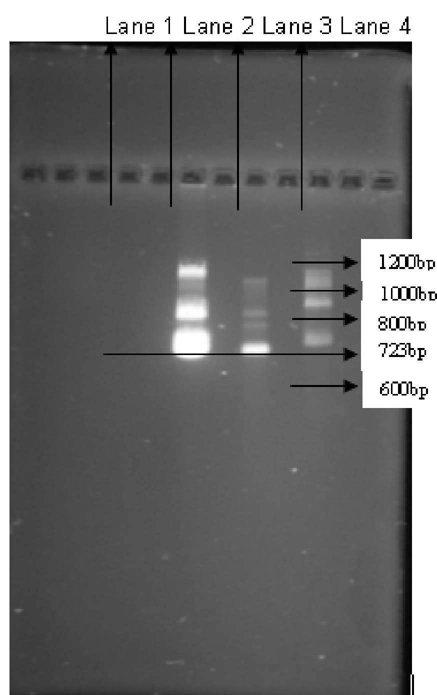
Fig. 1

Shows 14 cultures were emulsified the oils (Kerosene, Petrol and Diesel) very effectively and it were compared with Tween 80 (standard emulsifier).

Detection of rhlB Gene

The results showed that all the strains showed the amplified product of rhamnosyl transferase I (rhlB) gene. The observed bands were compared with molecular marker (800 bp) and the presence of amplified product of 723 bp level confirmed the presence of rhlB gene (Fig.2) and this study was supported by the work of [5].

Fig.2



Amplification of genomic DNA of P. aeruginosa

Lane 1= Genomic DNA (723 bp-rhlB); Lane 2 = Genomic DNA (723 bp-rhlB); Lane 3 = Marker DNA (800 bp); Lane 4 = Marker DNA (800 bp)

CONCLUSION

The investigation showed that among the 16 viable culture of *P. aeruginosa* only 14 cultures had the ability to produce the bio surfactant it were confirmed by amplification of rhlB, which is 723 bp level (analyzed with primers kpd 1 and kpd 2) and it was effectively reduced the surface tension and emulsifying the oils (Petrol, Diesel and kerosene) and it can be effective against some bacterial groups (*Bacillus subtilus, Staphylococcus aureus, Aeromonas,sp* and *Escherichia coli*). Due to effective activity of *P. aeruginosa* on surface tension reduction and emulsification activity on oils it could be very effective for the bioremediation on oil contaminated soils

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