Screening of Surfactant Producing Bacterial Strains Isolated from Soil Samples of an Automobile Workshop

Erum Shoeb[1,*], Uzma Badar[1], Jameela Akhter[2], Faiza A. Ansari[1], Maheen Waqar[1] and Maqsood A. Ansari[1]

[1]*Department of Genetics, University of Karachi, Karachi-75270, Pakistan and* [2]*Centre for Molecular Genetics, University of Karachi, Karachi-75270, Pakistan*

Abstract: Isolation of biosurfactant producing bacteria from our local environment was the main focus of our study. Disposal of waste of all kinds including oil in automobile workshops is a common observation in Karachi. Considering the likelihood of existence of biosurfactant producing organisms at hydrocarbon contaminated areas, we have collected soil samples from automobile repair workshop and isolated biosurfactant producing bacterial isolates. Total of eight bacterial strains (DGEF01-08) were isolated out of which DGEF03, DGEF05 and DGEF07 showed positive activity through oil spreading technique. Hemolytic activity was observed in all the eight isolated strains. DGEF01, DGEF02, DGEF03 and DGEF08 showed alpha hemolytic activity whereas the strains DGEF04, DGEF05, DGEF06 and DGEF07 showed beta hemolytic activity. All the strains had positive CTAB agar plate activity and tolerance against hydrocarbon *m*-xylene. Analysis of growth curves with and without stress of all the strains showed that hydrocarbon tolerance mechanism is inductive in case of DGEF01 and 03 whereas the rest of the strains showed constitutive (non-inducible) mechanism of tolerance. Detection of *rhlAB* gene through PCR also gave positive results for DGEF01, DGEF03, DGEF05, DGEF06 and DGEF08, which indicate rhamnolipid producing *Pseudomonas* species. These isolates are valuable source to investigate further for future industrial applications.

Key Words: Biosurfactant, oil contamination, screening, isolation, hydrocarbon, *m*-xylene.

INTRODUCTION

Automobile workshops create many different types of hazardous wastes during regular services which predominantly include used oil and fluids [1]. Such wastes are routinely disposed within the vicinity of workshops. Oil contaminated regions are reported to be a suitable place for the isolation of biosurfactant producing bacterial strains [2].

Biosurfactants are surface active agents produced by certain specialized microorganisms including bacteria, fungi and yeast [3]. These microbes produce biosurfactant, either secreted extracellularly or attached to parts of the cell membrane, predominantly during growth phase [4]. Besides being non-toxic and biodegradable, biosurfactants are amphiphilic molecules with high specificity [5, 6]. They are highly stable at extremities of temperature, pH and salt concentrations [7]. These molecules have the ability to decrease the surface and interfacial tension [8]. In addition, biosurfactants are promising natural surfactants that offer several advantages over chemically synthesized surfactants, such as lower toxicity, biodegradability and ecological acceptability [9, 10]. Biosurfactants are thus used as an alternative for chemical surfactants [11]. Biosurfactants belong to various classes including glycolipids, lipopeptides, fatty acids, phospholipids, neutral lipids and lipopoly-saccharides [12].

*Address correspondence to this author at the Department of Genetics, University of Karachi, Karachi-75270, Pakistan, Tel: +92-21-99261300-2218, Fax: +92-21-34966045, E-mail: erumsh@uok.edu.pk

The range of industrial applications of biosurfactants includes excellent detergency, emulsification, foaming, wetting, penetrating, thickening, metal sequestering and resource recovering [13]. Most crucial property of biosurfactants, which has captivated the researchers of today, is its use in bioremediation of pollutants, health care and food processing [14].

Since the last decade, increasing attention has been paid to the isolation of biosurfactant producing organisms. However, the high production costs and low strain productivities, limit its wide application [15]. With this scope, the present study proposes an effective methodology to search for biosurfactant producing bacteria from oil contaminates soils.

MATERIALS AND METHODS

Sampling Area

For the isolation of biosurfactant producing bacteria, the soil sample was collected from an automobile workshop where the soil usually remains soaked with spilled petroleum products.

Enrichment and Isolation of Bacterial Isolates

5 gram of oil spilled soil sample of automobile repair workshop was inoculated in 50 ml LB broth [16] and

0250-5363/12

incubated at 37ºC for 24 hours. 100 µl of O/N grown cultures were spreaded on R2A medium agar plates [3]. The plates were then incubated at 37ºC for 24-48 hrs. Morphologically different colonies were selected and purified [17]. The selected bacterial isolates were stored in LB agar slants and kept under refrigerated conditions for further screening.

Identification Through GSP Agar

The cultures were streaked on Glutamate Starch Phenol Red (GSP) agar plate and incubated at 37 ºC for 24-48 h.

Screening for Biosurfactant Production

The isolated strains were tested for biosurfactant production through following methods.

Oil Spreading Technique

10 µl supernatant (from culture broth) was added to the surface of oil as described by Nasr, *et al*., [18]. Occurrence of clear zone was an indication of biosurfactant production. 10 µl of culture media without any growth was taken as a control.

Hemolytic Activity

Hemolytic assay was performed in blood agar plates [19]. 50 µl broth cultures were spot-inoculated on to blood agar plates and incubated for 48 h at 37ºC. The plates were visually inspected for zone of clearance (hemolysis) around the colony. Complete and incomplete hemolysis was designated as α (alpha) and β (beta) hemolytic activity [20].

CTAB Agar Plate

Blue agar plates containing cetyltrimethylammonium bromide (CTAB) (0.2 mg ml^{-1}) and methylene blue (5 mg ml^{-1}) were used to detect extracellular glycolipid production [21]. Biosurfactants were observed by the formation of dark blue halos around the colonies.

Tolerance Against Hydrocarbon (*m*-xylene)

In order to analyze ability of isolates to grow in presence of hydrocarbon *m*-xylene, fresh overnight cultures were spreaded on solidified LB agar plates and 2 ml. *m*-xylene was poured over the spreaded plates followed by incubation at 37ºC for 24 hrs. Plates were sealed using parafilm-M to avoid evaporation of *m*-xylene from petri dishes.

Bacterial Growth Curves

The growth curves of biosurfactant producing strains were constructed for the determination of log phase and also to determine induction of tolerance mechanism against hydrocarbon *m*-xylene. Cell growth was monitored with DU 730 spectrophotometer of Beckman Coulter which was used to measure the optical density at 600 nm (OD_{600}). For log phase determination first three readings were taken at 15 min intervals and later at 30 min intervals till the culture enter the stationary phase indicated through the graphs plotted for time vs OD. For induction, overnight cultures were grown with and without stress of 1 ml. *m*-xylene. Overnight cultures were used to inoculate cultures with *m*-xylene (Test) and without *m*-xylene (Control), resulting into three combinations: control → control, control → test and test → test growth curves [17]. OD1 was taken as control (O/N) to control growth curve; OD2 was taken as control (O/N) to stress growth curve; and OD3 was taken as stress (O/N) to stress growth curve.

Detection of *rhlAB* gene

For detection of *rhlAB* gene through PCR, genomic DNA was isolated through phenol/chloroform method [16]. Primers used for *rhlAB* and PCR conditions provided were as reported byKumar *et al*. [22].

RESULTS

Isolation of Bacterial isolates

Total of eight aerobic bacterial strains were isolated, purified and screened for the biosurfactant production. The strains were coded as DGEF01-08.

Identification Through GSP Agar

All the isolated strains DGEF01-08 were found to be surrounded by red-violet zone on GSP medium, accordingly, isolates were identified as Pseudomonas spp.

Screening for Biosurfactant Production

Following results are obtained after screening tests.

Oil Spreading Technique

Overnight cultures of eight isolated strains were centrifuged and added to oil containing plates. The strain DGEF03, 05, 07 showed the clear zone by being able to displace the oil around the colony indicating biosurfactant production (Fig. **1**). No clear zone was observed with control.

Hemolytic Activity

The hemolytic activity was observed in all the eight isolated strains, results showed α (alpha) hemolytic activity of strain DGEF01, 02, 03 and 08 and the β (beta) hemolytic activity of strain DGEF04, 05, 06 and 07 (Fig. **2**).

CTAB Agar Plate

CTAB Agar Plates results showed the dark blue halos in all the isolates as shown in Fig. (**3**) showing positive activity of Biosurfactant production.

Fig. (1). Results of oil spreading technique showing oil displacement around the colonies.

Fig. (2). Results of hemolytic activity; Left side of plate showing α (alpha) hemolytic activity and right side showing β (beta) hemolytic activity.

Tolerance Against Hydrocarbon (*m*-xylene)

Heavy growth was observed after 24 hrs. of incubation in all the eight strains which showed tolerance against hydrocarbon *m*-xylene (Fig. **4**).

Growth Curves for Biosurfactant Production

Different strains exemplified a varying patterns of growth which are shown through graphs (Fig. **5**). To find out whether *m*-xylene tolerance mechanism is inducible or conserved, overnight cultures grown with and without stress were inoculated in stress and plotted graphs were compared for their initial response against *m*-xylene. Parallel growth curve was plotted for control without stress. It has been observed that response against *m*-xylene of strains DGEF01 and 03 was inducible, while that of strains DGEF02, 04, 05, 06, 07 and 08 was non inducible/ constitutive in nature.

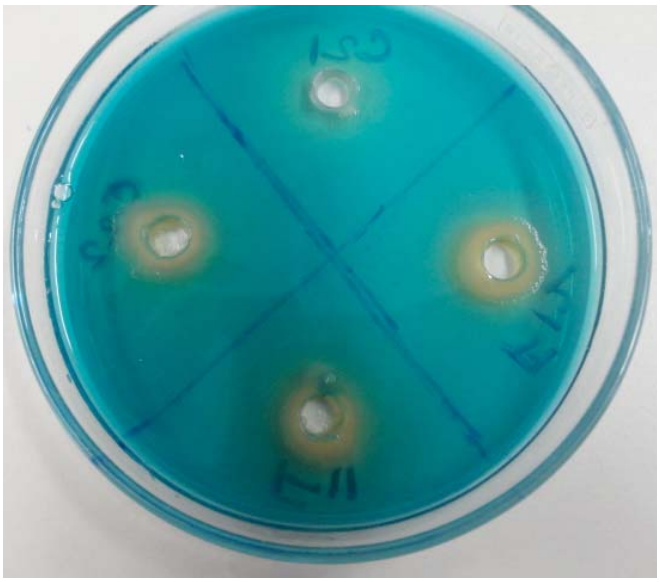

Fig. (3). Results of CTAB Agar Plates showing dark blue halos.

Fig. (4). Culture growing in presence of *m*-xylene.

Detection of *rhl* gene

For detection of *rhlAB* gene through PCR, when the primers rhlABf and rhlABr were used, a single PCR fragment of the expected size (~777 bp) was amplified as shown in Fig. (**6**), in strains DGEF01, 03, 05, 06, 08 indicating the occurrence of *rhlAB* gene.

DISCUSSION

Research in the area of biosurfactants has expanded quite a lot in recent years due to its potential use in different industries. The focus of the present study is to isolate and

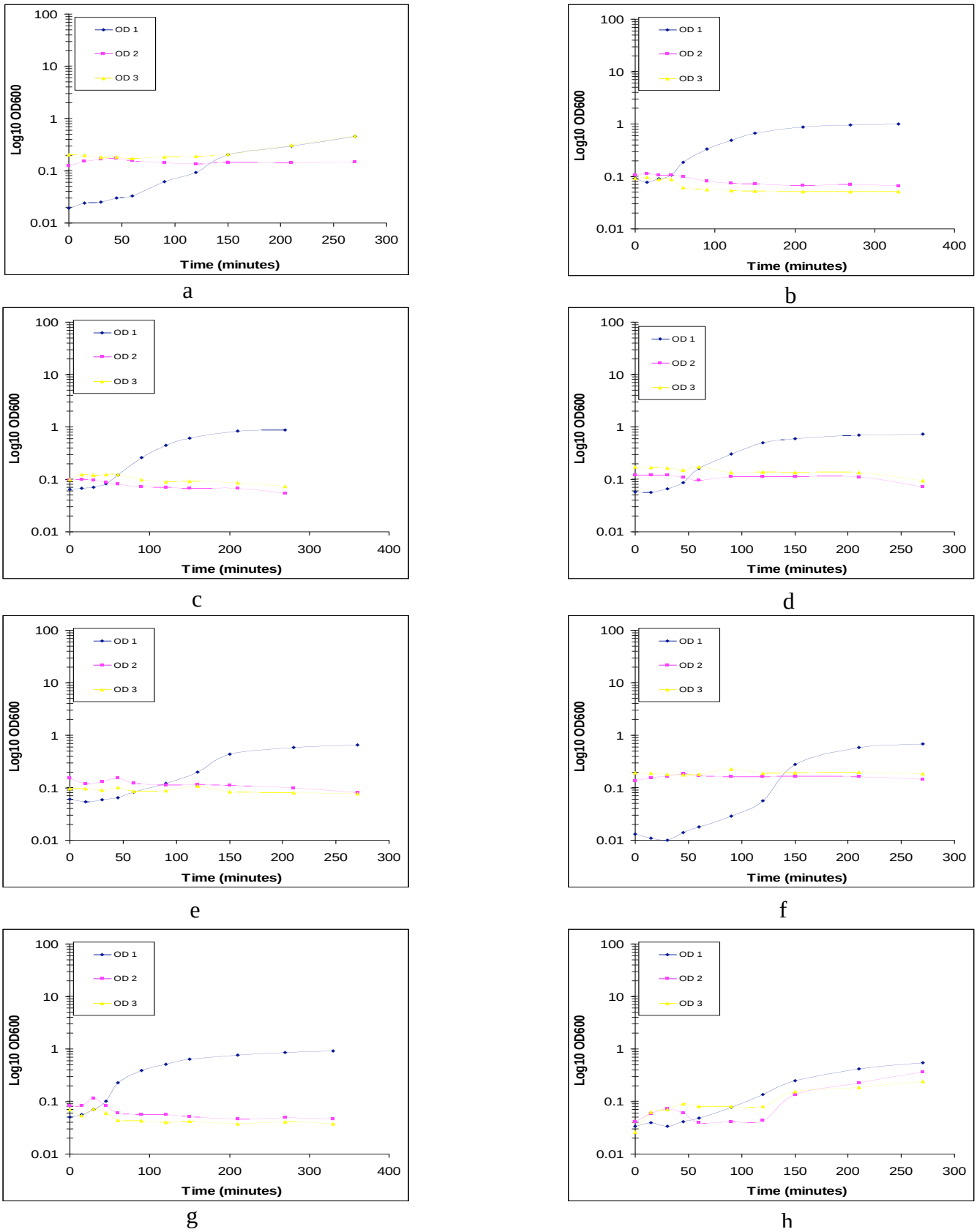

Fig. (5). Growth Curves of isolated strains. OD1 was taken as control (O/N) to control growth curve; OD2 was taken as control (O/N) to stress growth curve; and OD3 was taken as stress (O/N) to stress growth curve. DGEF01 (a), DGEF02 (b), DGEF03 (c), DGEF04 (d), DGEF05 (e), DGEF06 (f), DGEF07 (g), DGEF08 (h). Inductive tolerance mechanism in observed in DGEF01 (a) and DGEF03 (c) while rest of the strains showed non-inducible mechanism of tolerance.

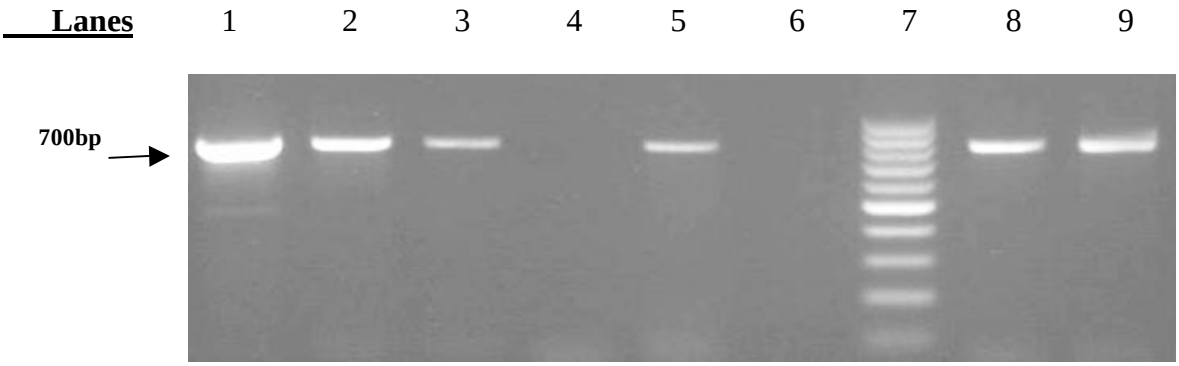

Fig. (6). Gel Photograph showing the results of PCR for the detection of *rh1ab* gene. Lane 1 & 2=DGEF01, Lane 3=DGEF03, Lane 4=DGEF02, Lane 5=DGEF05, Lane 6=DGEF04, Lane 7=Ladder (100bp: Biotools Cat#31.006), Lane 8=DGEF06, Lane 9=DGEF08. DGEF01 (Lane 1&2), DGEF03 (Lane 3), DGEF05 (Lane 5), DGEF06 (Lane 8) and DGEF08 (Lane 9) showing band of ~777 bp.

characterize indigenous biosurfactant producing bacterial isolates so as to select suitable biosurfactant producers. Automobile service and repair shops are the largest small quantity generators of hazardous waste. Auto repair shops create many different types of extremely dangerous wastes during their daily operations [23, 24, 25].

Biosurfactant producing microorganisms are naturally present in the oil and hydrocarbon contaminated soils [26]. We have been able to isolate bacteria with the ability to produce biosurfactants from the soil samples collected from automobile workshop. Biosurfactant production was confirmed by the conventional screening methods including oil spreading technique, hemolytic activity and blue agar plate method.

Oil spreading technique was used as reported by Urum and Pekdemir [27] and three of the isolates could successfully displace the oil hence indicating production of biosurfactant. Displacement of oil clearly is a sign of extra-cellular surfactants present in the supernatant of cultures.

Strains were tested for haemolytic activity, which is regarded by some authors as indicative for biosurfactant production and used as a preliminary method for bacterial screening [19, 11, 28]. Hemolytic activity observed in all the eight isolated strains, DGEF01, 02, 03 and 08 gave alpha (incomplete) hemolytic activity while the strains DGEF04, 05, 06 and 07 had beta (complete) hemolytic activity.

Blue agar plate method is a semi quantitative agar plate method that is based on the formation of an insoluble ion pair of anionic surfactants with the cationic surfactant CTAB and the basic dye methylene blue [29]. All the isolated strains showed positive CTAB agar plate activity.

All the isolates were further assessed for tolerance against hydrocarbon *m*-xylene. Through growth curves analysis with and without stress of all the strains it was found that hydrocarbon tolerance mechanism is inductive in case of DGEF01 and 03 while rest of the strains showed constitutive (non-inducible) mechanism of tolerance. Isolates with the inductive tolerance mechanisms grow slowly at the initial exposure of the stress as compare to the already exposed cultures to the stress.

Detection of *rhlAB* gene through PCR also gave positive results for the isolates DGEF01, 03, 05, 06, 08 indicating the occurrence of *rhlAB* gene. Detection of this gene in DNA extracts of the isolates has established the ability of the microbe to produce rhamolipid type of biosurfactant. Five out of eight isolates gave a single band of expected size with the primers designed based on the *rhlAb* sequences in database. *rh1ab* gene is reported to be involved in production of Rhamnolipids, which are naturally occurring biosurfactants exhibit several promising industrial applications [30]. Biosurfactant activity of rhamnolipids makes them excellent candidates for support in the breakdown and removal of oil spills. Rhamnolipids also demonstrate antibacterial and antifungal activities, suggesting possible roles in the medical and agricultural industries [4]. Furthermore, it might be expected that isolates DGEF02, 04 and 07 produce some other type of commercially important biosurfactants. As biosurfactants are derived from a natural sources, each of these typees are an attractive alternative to synthetic compounds [31].

As a result of screening tests it can obviously be noticed that all the isolates are promising and showed potential to be utilized for upcoming molecular and chemical investigations.

ACKNOWLEDGEMENT

Authors are pleased to acknowledge Dean, Faculty of Science, University of Karachi for providing the research grant and support.

REFERENCES

[1] Abidemi, O.O. Levels of Pb, Fe, Cd and Co in Soils of Automobile Workshop in Osun State, Nigeria. J. Appl. Sci. Environ. Manage., 2011, 15 (2) 279-282.

[2] Francy, D.S.; Thomas, J.M.; Raymond, R.L.; Ward, C.H. Emulsification of hydrocarbon by surface bacteria. J Industrial Microbiol, 1991, 8: 237-46.

[3] Anandaraj, B.; Thivakaran, P. Isolation and Production of Biosurfactant producing organism from oil spilled soil. J. Biosci. Tech, 2010, 1 (3) 120-126.

[4] Desai, J.D.; Banat, I. M. Microbial Production of Surfactants and their Commercial Potential. Microbiology and Molecular Biology Reviews, 1997, 47–64.

[5] Zajic, J.E.; Panchal, C.J. Bioemulsifiers. Crit. Rev. Microbiol., 1976, 5: 39-66.

[6] Cooper, D.G.; Zajic, J.E. Surface-active compounds from microorganisms. Adv. Appl.Microbiol., 1980, 26: 229-253.

[7] Desai, J.D. Microbial surfactants: Evaluation, types, production and future applications. J. Sci. Ind.Res., 1987, 46: 440-449.

[8] Banat, I.M. Characterization of biosurfactants and their use in pollution removal-state of art (review).Acta Biotechnol., 1995, 15: 251-267.

[9] Makkar, R.S.; Cameotra, S.S.; Banat, I.M. Advances in utilization of renewable substrates for biosurfactant production. AMB Express, 2011, 1:5. doi:10.1186/2191-0855-1-5

[10] Das, P.; Mukherjee, S.; Sen, R. Improved bioavailability and biodegradation of a model polyaromatic hydrocarbon by a biosurfactant producing bacterium of marine origin. Chemosphere, 2008, 72:1229–1234.

[11] Banat, I.M.; Makkar, R.S.; Cameotra, S.S. Potential commercial Application of Microbial Surfactants. Applied Microbiol. Biotechnol., 2000, 53:495-508.

[12] Rosenberg, E. Microbial biosurfactants. Critical Reviews in Biotechnology, 1986, 3:109-132.

[13] Soberón-Chávez, G.; Maier, R.M. Biosurfactants: a General Overview. In: Soberón-Chávez G, editor. Biosurfactants. Springer-Verlag; Berlin, Germany, 2011, 1–11.

[14] Banat, I.M. Biosurfactant production and possible uses in Microbial Enhanced Oil Recovery and Oil Pollution Remediation: review. Biores. Tech., 1995, 51: 1-12.

[15] Davis, D.A.; Lynch, H.C.; Varley, J. The production of surfactin in batch culture by Bacillus subtilis ATCC 21332 is strongly influenced by the conditions of nitrogen metabolism. Enz. Micro Technol., 1999, 25(3-5): 322-329.

[16] Sambrook, J.; Fritsch, E.F.; Maniatis, T. Molecular cloning: A laboratory manual, 2nd edition. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 1989.

[17] Shoeb, E. Genetic Basis of Heavy Metal Tolerance in Bacteria. Ph. D. thesis. University of Karachi, Karachi, Pakistan, 2006.

[18] Nasr, S.; Soudi, M.R.; Mehrnia, M.R.; Sarrafzadeh, M.H. Characterization of novel biosurfactant producing strains of Bacillus spp. isolated from petroleum contaminated soil. Iranian J. Microbiol., 2009, 1 (2), 54-61.

[19] Mulligan, C.N.; Cooper, D.G.; Neufeld, R.J. Selection of microbes producing biosurfactants in media without hydrocarbons. J. Ferment. Technol., 1984, 62:311–314.

[20] Misawa, N.; Hirayama, K.; Itoh, K.; Takahashi, E. Detection of alpha- and beta-hemolytic-like activity from Campylobacter jejuni. Clin. Microbiol., 1995, 33(3): 729–731.

[21] Siegmund, I.; Wagner, F. New method for detecting rhamnolipids excreted by Pseudomonas species during growth on mineral agar. Biotechnol. Tech., 1991, 5, 265-268.

[22] Kumar, M.; León, V.; De Sisto Materano, A.; Ilzins, O.A.; Luis, L. Biosurfactant production and hydrocarbon-degradation by halotolerant and thermotolerant *Pseudomonas* sp. World J. Microbiol. Biotechnol., 2008, 24: 1047-1057.

[23] Imevbore, A.A.A.; Adeyemi, S.A. Environmental monitoring in relation to pollution and control of oil pollution. In: Proc. Seminar on the Petroleum Industry and the Nigerian Environment, 1981, 135 – 142.

[24] Edebiri, R.A.O.; Nwanokwale, E. Control of pollution from internal combustion engine used lubricant. Proc. Int. Seminar Petroleum Industry and the Nigeria Environment. 1981, 9-12.

[25] USEPA. Treatment technologies for site cleanup. Annual status report, office of Solid Waste and Emergency Response (5102G), 10th ed., United States Environmental Protection Agency, 2001.

[26] Bento, F.M.; de Oliveira, C.F.A.; Okeke, B.C.; Frankenberger Jr., W.T. Diversity of biosurfactant producing microorganisms isolated from soils contaminated with diesel oil. Microbiological Research, 2005, 160: 249-255.

[27] Urum, K.; Pekdemir, T. Evaluation of biosurfactants for crude oil contaminated soil washing. Chemosphere, 2004, 57: 1139-1150.

[28] Carrillo, P.; Mardaraz, C.; Pitta-Alvarez, I.S.; Giulietti, A.M. Isolation and selection of biosurfactant-producing bacteria. World Journal of Microbiology and Biotechnology, 1996, 12:82-84.

[29] Pinzon, N.M.; Ju, L.K. Improved detection of rhamnolipid production using agar plates containing methylene blue and cetyl trimethylammonium bromide. Biotechnology Letters, 2009, 31(10), 1583-1588.

[30] Gunther, N.W.IV; Nuñez, A.; Fett, W.; Solaiman, D.K.Y. Production of Rhamnolipids by Pseudomonas chlororaphis, a Nonpathogenic Bacterium. Appl. Environ. Microbiol., 2005, 71(5): 2288–2293. doi: 10.1128/AEM.71.5.2288-2293.

[31] Youssef, N.; Simpson, D.R.; Duncan, K.E.; McInerney, M.J.; Folmsbee, M.; Fincher, T.; Knapp, R.M. In situ biosurfactant production by Bacillus strains injected into a limestone petroleum reservoir. Applied and Environmental Microbiology, 2007, 73:1239-1247.