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Full Length Article

Isolation, Screening and Functional Characterization of Biosurfactant Producing Bacteria Isolated from Crude Oil Contaminated Site

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Abstract

Biosurfactants are amphiphilic compounds produced by microorganisms and have multifarious uses in industry and agriculture. The biosurfactant producing microbes are helpful in bioremediation of heavy metal, pesticides and hydrocarbon contaminated sites. They are also used as bio-control agent to protect plant against various diseases, resulting in higher crop yields. The present study was aimed to isolate potential biosurfactant producing bacteria from oil contaminated sites. Initially 37 bacteria were isolated among them nine biosurfactant produces were screened based on surface tension reduction, emulsification index, modified drop collapse test and oil displacement activity. The isolate FKOD36 was the most effective biosurfactant producer as it showed the maximum reduction in surface tension (35.15 dyne/cm) with an emulsification index of 66.7% and oil displacement activity of 3.7 mm. The bacterium was identified by using partial 16S rRNA sequencing and it belonged to genus *Klebseilla*, and was designated as *Klebseilla* sp. FKOD36. The strain was also capable of utilizing phenanthrene as the highest bacterial biomass was produced by this strain in phenanthrene amended MS media compared to other isolates. This suggests that selected strain *Klebseilla* sp. FKOD36 could have potential to degrade the hydrocarbon contaminated sites that could be helpful in conservation of natural resources.

Keywords: Biosurfactants; Isolation; Qualitative; Quantitative; *Klebseilla* sp

Introduction

Biosurfactants are very fascinating molecules having pronounced surface and emulsification activities (Van Hamme *et al.*, 2006; Singh *et al.*, 2007). They comprised a large group of various surface activities which can be classified either on the basis of their molecular weights or their origin. They comprised lipopeptides, glycolipids, phospholipids, lipopolysacchrides, neutral lipids and polysaccharides-protein complex (Desai and Banat, 1997; Christofi and Ivshina, 2002; Muthusamy *et al.*, 2008). They are produced by yeast, filamentous fungi but the most commonly reported biosurfactants produced belongs to bacteria. They can be produced by utilizing both water immiscible and water soluble substrates. Previously reported efficient biosurfactants producers belongs to genera *Pseudomonas*, *Bacillus*, *Corynebacterium* sp, *Acinetobacter* sp. *Archromobacter* sp, *Falvobacterium* sp. and Proteobacteria (Abraham *et al.*, 1998; Das *et al.*, 2008a, b; Maneerat *et al.*, 2006; Perfumo *et al.*, 2006).

Relative significance of surfactant can be indicated by the size of market for these green molecules materials and their annual growth rate. As of 2011, surfactant production increased to 15 million tons, with a worth of 25 billion USD (Transparency Market Research, 2012). Currently, biosurfactant capture a huge volume of market and is comprised 476,000 tons with a value of 1.7 billion USD. Previous reports and analysis shows that biosurfactant market will continue to grow at a rate of 3.5% annual growth rate to 2.2 billion USD (Kosaric, 2000). This is all because of their environmental friendly nature, as they are biodegradable, non toxic, ability to produce from inexpensive raw materials and stable over a wide range of harsh environments.

Now a days surfactants are reported for application in many fields of industry - pharmacy, food industry, design of washing agents, petroleum industry, environmental protection and agriculture (Rostas and Blassmann, 2009; Deleu and Paquot, 2004; Singh *et al.*, 2007; Moldes *et al.*, 2011). The biosurfactant produced by microbes are helpful in bioremediation of heavy metal, pesticides and hydrocarbon contaminated sites (Juwarkar *et al.*, 2008). They are also used as biocontrol agent to protect plant against various diseases resulting in higher crop yields (Asaka and Shoda, 1996; Krishnayya and Grewal, 2002; Gravel *et al.*, 2005; Snook *et al.*, 2009; Hultberg *et al.*, 2010). Based on their functional characteristics they have much more potential to be used in agriculture i.e. in soil

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management and plant protection strategies to ensure better place for sustainable food production. Given their properties and applications, studies on the properties of surfactants the search for biosurfactants producing bacteria is yet an interesting area of research because of the diversity of these fascinating molecules and their application in broad-spectrum application in agriculture.

The present study was conducted to isolate biosurfactant producing bacteria from oil contaminated sites. It was predicted that the crude oil contaminated sites may provide dwelling for biosurfactant producing bacteria, which not only may lower the surface tension and proved helpful in remediation of these contaminated sites. The objective of the study were the (a) isolation of potential biosurfactant producing bacteria based on their capability of reduction in surface tension and emulsification index and (2) determination of growth of selected isolate on phenanthrene amended media as possible indicator for bioremediation of hydrocarbon contaminated sites.

Materials and Methods

Collection of Soil Samples

The samples were collected near PSO oil Depot in Farid Kot (30^0 10′ 22.28″ N, 70^0 58′ 35.7″ E), Muzaffar Garh (Punjab) using the procedure described by Bodour and Miller-Maier (2000). A total of 12 soil samples were drawn. Based on the sampling location (PSO Oil Depot, Farid Kot), these samples were designated as FKOD. Each sample was put into 250 mL sterile Erlenmeyer flasks. The flasks were covered with sterile cotton wool in order to prevent any contamination. The samples were transported to the laboratory in an ice bucket for isolation of bacteria having capability of producing biosurfactants.

Isolation of Biosurfactant-producing Bacteria

A 5 g crude oil contaminated soil sample was suspended in 95 mL sterile phosphate buffer solution (PBS) in a 250 mL conical flask and placed on shaker at 200 rpm at 20ºC for 12 h. A 5 mL suspension was then inoculate to 50 mL mineral salt media (MSM) cultured amended with 1% (v/v) crude oil as carbon source. The composition (g/L) of the MS media was (g L^{-1}) Na_2HPO_4, 0.28; KH_2PO_4, 1.14, NH_4NO_3, 3.00; K_2SO_4, 0.36; $MgSO_4.7H_2O$, 0.154; $CaCl_2.2H_2O$, 0.023; $FeSO_4,7H_2O$, 0.038; NaCl, 0.10; yeast extract, 0.5. One mL trace element solution was also added to MSM. A 5 µL subculture was subsequently inoculated into fresh culture of the flasks and was incubated at the same condition. The procedure was repeated thrice to enrich microbial cultures and increase population density. Then, 50 µL of each resulting culture was spread onto MS Bacto Agar plate media and incubated for 7 days. The strains were purified by streaking agar plates as described by Oyarzabal (1999). Morphologically distinct colonies were streaked three times on fresh agar plates to obtain pure cultures. The pure cultures were transferred to Tryptic soy agar (TSA) plates and stored at 4°C in a refrigerator.

Screening of Biosurfactants and Bioemulsifier-producing Strain

Qualitative evaluation of biosurfactants: Cell-free filtrate prepared by centrifugation is necessary for biosurfactant purification and recovery. For this purpose, centrifugation of culture media was done at 12000 g for 30 min and cell free supernatant was prepared.

For primary screening of biosurfactant producing bacteria, qualitative tests were performed using drop collapse test as described by Jain *et al.* (1991). This test was carried out in the polystyrene lid of a 96-microwell (12.7 cm×8.5 cm) plate (Biolog Inc., Harward, CA, USA). Before performing test, each lid was rinsed with hot water, ethanol, distilled water three times and then dried. Then, each micro well was coated with slight 2 µL layer of oil. The wells were covered with 96-well microplate cover. These apparatuses were left for 24 h to ensure a uniform oil coating. Five µL of aliquot with seven-day enriched cultures were transferred to the prepared oil-well coated regions using a microsyringe. Each time the syringe was rinsed three times with water and then with acetone before the addition of each sample. The drop size was observed 1 min later with the aid of magnifying glass. The result was considered to be positive for biosurfactant production when the drop diameter was at least 1 mm larger than that produced with deionized water (Batista *et al.*, 2006). Each test was performed in five replications.

Quantitative measurement of biosurfactant production: Reduction in surface tension was used as one method. For this, the cell free supernatant after centrifugation at 8500 rpm was used further for measuring surface tension using du Nouy Ring type method on a Model 10 tensiomat (Fisher Scientific USA). Control was set where no inoculation was done. All measurements were done at room tempreture after carefully dipping the platinum ring in the culture solution for few second in order to attain equilibrium. Initially, for calibration of instrument, the surface tension of pure water was done. The procedure was repeated thrice and surface tension of the sample was expressed as average of three replications. The following formula was used to determine the reduction in surface tension (dyne/cm):

Surface tension reduction = Recorded surface tension in the media (before inoculation) – Recorded surface tension after inoculation (culture supernatant).

The emulsification index of the selected isolates showing positive qualitative results was screened for emulsification activity (E24%) according to the procedure of Bento *et al*. (2005). Two mL each of kerosene oil was, added to an identical amount of cell-free filtrate of different

isolates in a glass test tube with 15 mm diameter, and mixed for 2 min by a vortex. The emulsion formed in the tube was covered with parafilm and then kept under room tempreture. Height of the emulsion was calculate after 1 min and then after 24 h to determine the stability of the emulsion. Each experiment was repeated thrice to achieve accuracy of the measurement. Emulsification index (E 24%) was calculated according to formula:

$$E_{24} = \frac{\text{Height of emulsion layer}}{\text{Height of total solution}} \times 100 \qquad (1)$$

An emulsion is considered as stable if E24 is equal or more than 50% (Bosch *et al.*, 1988).

Oil spreading techniques are among the third methods. Ability of the isolates to produce biosurfactant were also be tested by their capability of oil displacement. For this purpose oil spreading techniques were performed according to the procedure as described by Morikawa *et al.* (2000) and Youssef *et al.* (2004). Briefly, 50 mL distill water was taken in a petri plate followed by 100 µL of crude oil to the water surface. Then, 10 µL of culture of each isolate tested was put on the crude oil surface. The diameter of clear zone on the oil surface was observed.

Hydrocarbon Degrading Capability: Biomass Estimation

In order to determine hydrocarbon degrading capability, isolates were grown on MSM amended with phenanthrene (1%). Biomass estimation was done by taking sample from the culture broth after 7 days. The sample was centrifuged at 10,000 rpm for 20 min at 4ºC. The cell pellets were formed which were then washed with 0.9% (w/v) NaCl solution. The dry weight was estimated (g/L) by drying the cell pellets in a hot air oven at 60-70ºC. The drying was continued until a constant dry cell weight was achieved.

Identification of Microorganism through 16S rRNA Gene

Bacterial isolate exhibiting high biosurfactant producing capability was selected and identified by partial 16S r RNA sequencing. Partial sequencing of the 16 S r RNA gens was done by the Institute for Integrative Genome Biology (University of California Riverside, USA). Sequence similarities to known bacteria were determined using the Greengenes database (DeSantis *et al.*, 2006) and submitted to GenBank for accession numbers. A neighbor joining phylogenetic tree was constructed with aligned 16S rRNA gene sequences using 1000 bootstrap replications on Mega5 (Tamura *et al*., 2011).

Statistical Analysis

All screening and biosurfactant characterization studies were performed using CRD design at triplicate under similar environmental conditions. Means and standard error were calculated for the pooled results of all experiments for each isolate. On way analysis of variance (F-test with $p<0.05$) was performed on surface tension and emulsification index data to determine significant difference potential among the isolates. All statistical analysis was performed using statistix (version 8.1) software package.

Results

Isolation and Screening of Biosurfactant Producing Bacteria

The drop collapse test was conducted for the primary qualitative screening of biosurfactant producing bacteria was highly positive for FKOD36 followed by FKOD33, FKOD31and FKOD28, while other isolates FKOD27, FKOD29, FKOD30, FKOD35 and FKOD35 also showed positive drop collapse test. These isolates were further tested for their biosurfactant production quantitatively. The results for cell free supernatant of isolates that showed positive drop collapse test indicated that all the tested isolates showed reasonably good oil displacement activity (Fig. 1). However, maximum oil displacement activity (3.6 mm) was observed in the case of isolate FKOD36, which was 18 times higher than the control in which no cell free supernatant was used. A minimum activity (1.93 mm) was recorded with isolate FKOD33 (Table 1), although it showed 9 times higher oil displacement than the control. Isolates FKOD29 and FKOD30 showed almost similar pattern in oil displacing activity and differed non-significantly from each other. They showed maximum oil displacement activity (2.1 mm) and (1.93 mm) for FKOD29 and FKOD30, respectively which is 10 times higher than the control treatment. Likewise, three other isolates FKOD31 and FKOD33 showed 13 times higher while FKOD32 showed 11 times higher oil displacement than the control treatment where no inoculation was done. Results for FKOD31, FKOD32 and FKOD33 showed similar type of results regarding oil displacement activity and had non-significant difference from each other. Additionally, it was noted that the isolate FKOD36 showed maximum oil displacement activity after 72 h of incubation period (Fig. 2). Further quantitative testing of isolates for their biosurfactant production was estimated by measuring their surface tension reduction capability. The data regarding surface tension reduction using du Nouy ring method revealed that maximum reduction in surface tension (9%) compared to control was exhibited by isolate FKOD36, followed by isolate FKOD28 (44%), FKOD33 (44%), FKOD31 (42%), FKOD32 (27%), FKOD35 (24%), FKOD29 (23%), FKOD27 (22%), while minimum was observed for FKOD30 (19%) compared to control where no inoculation was done (70 dyne/cm), which differed each other significantly at $p<0.05$ (Table 1).

Table 1: Surface tension, emulsification index (E24%) and oil displacement capability of bacterial strain isolated from crude oil contaminated site

Isolate #	Measured surface tension (dyne/cm)	E_{24} (%)	Oil displacement test (mm)	Modified drop collapse test
Control	$70.02(0)^{a}\pm0.06$	$4.63^{f}\pm0$	$0.2^{d}\pm0.1$	_
FKOD27	$57.02(18)^{bc}\pm0.80$	$33.28^{e}\pm2.0$	$2.0^{c}\pm0.11$	+
FKOD28	$48.29(31)^{e}\pm0.67$	$60.69^{b}\pm1.0$	$2.8^{b}\pm0.11$	++
FKOD29	$56.49(19)^{bc}\pm0.57$	$44.44^{d}\pm3.0$	$2.1^{c}\pm0.13$	+
FKOD30	$58.49(16)^{b}\pm0.78$	$32.80^{e}\pm2.0$	$1.93^{c}\pm0.06$	+
FKOD31	$49.15(29)^{e}\pm0.76$	$54.76^{c}\pm2.0$	$2.7^{b}\pm0.17$	++
FKOD32	$54.89(21)^{d}\pm0.85$	$52.77^{c}\pm1.0$	$2.2^{c}\pm0.11$	+
FKOD33	$48.35(30)^{e}\pm0.54$	$56.91^{bc}\pm1.0$	$2.6^{b}\pm0.06$	++
FKOD35	$56.29(19)^{cd}\pm0.48$	$42.74^{d}\pm1.0$	$2.06^{c}\pm0.06$	+
FKOD36	$35.15(49)^{f}\pm0.86$	$67.33^{a}\pm1.0$	$3.6^{a}\pm0.24$	+++

± denotes S.D
() indicates reduction in surface tension values
+++ Highly efficient; ++ Efficient; + Low efficient
LSD value (for surface tension) = 3.5610; 7.7494 (for emulsification index); 0.6606 (for oil displacement)
Significant level= 0.05

Emulsification index is considered reliable accurate method to screen isolates having capability to produce biosurfactant. It was observed that all the nine isolates (that showed positive qualitative test) showed excellent capability to emulsify hydrocarbon (Fig. 3). Among all the isolates maximum emulsification index was found for FKOD36 being 14.5 times higher than the control followed by FKOD28 (13 times), FKOD33 (12 times), FKOD31 (11.8 times), FKOD29 (9.5 times), FKOD35 (9.2 times), FKOD27 (7 times), while lowest emulsification index was found for FKOD30 (7 times) as shown in Table 1. Statistical difference among the average values of nine isolates for emulsification index was observed at $p < 0.05$. Additionally it has been observed that a good correlation occurred between emulsification index and oil displacement activity (R^2=0.92) and similarly for surface tension reduction and emulsification activity (R^2=083) (Fig. 4).

Further quantitative testing of isolates for their biosurfactant production was estimated by measuring their surface tension reduction capability. The data regarding surface tension reduction using du Nouy ring method revealed that maximum reduction in surface tension (99%) compared to control was exhibited by isolate FKOD36, followed by isolate FKOD28 (44%), FKOD33 (44%), FKOD31 (42%), FKOD32 (27%), FKOD35 (24%), FKOD29 (23%), FKOD27 (22%), while minimum was observed for FKOD30 (19%) compared to control where no inoculation was done (70 dyne/cm) as shown in Table 1 and differed statistically each other significantly at $p < 0.05$.

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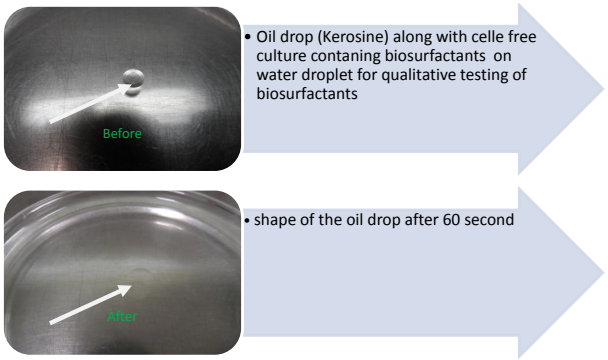

Fig. 1: Oil displacment test

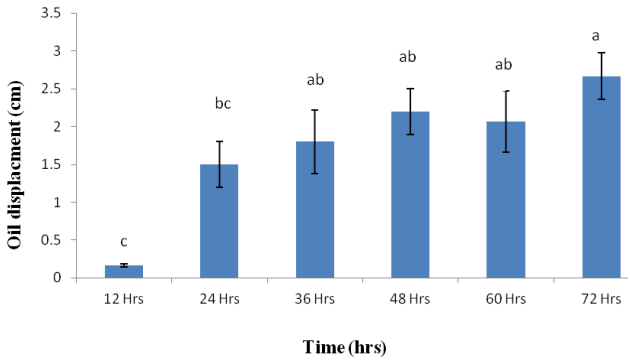

Fig. 2: Time variation in oil displacment of the selected strain *Klebseilla sp.* FKOD36

a good correlation occurred between emulsification index and oil displacement activity (R^2=0.92) and similarly for surface tension reduction and emulsification activity (R^2=083) (Fig. 4).

Bacterial Cell Biomass Production on Phenanthrene (PHE) amended Mineral Salt Medium

This assay was performed to test ability of bacterial isolates to grow on PHE amended MSM. Biomass concentration in

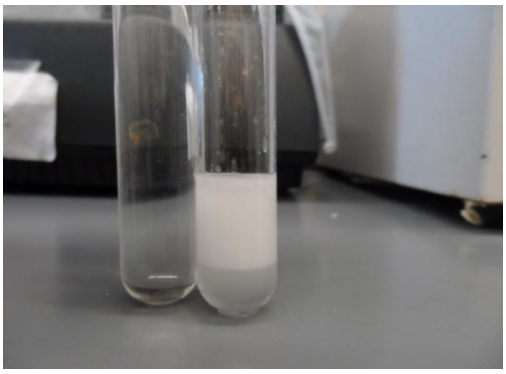

Fig. 3: Determination of emulsification index

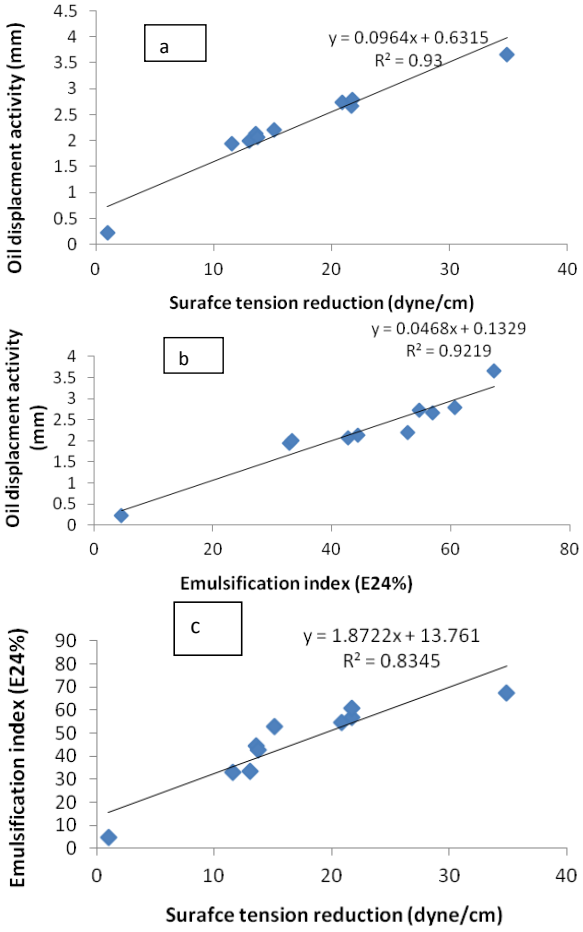

Fig. 4: Correlation between (a) the Surface tension reduction (ST) and oil displacement activity (OD), (b) Emulsification index (E24%) and displacement activity (OD), (c) Surface tension reduction (ST) and Emulsification index (E24%)

the range of 0.4-3.7 g/L was observed for the selected isolates. Maximum biomass (3.7 g/L) was observed in the case of bacterial isolate FKOD36 after 7 days incubation at 30ºC (Fig. 5), while minimum (0.4 g/L) was observed for bacterial isolate FKOD35.

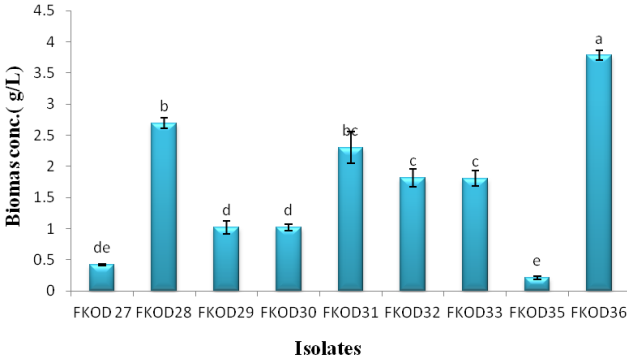

Fig. 5: Biomass estimation of bacterial isolates cultivated in phenanthrene amended MS media

Identification of Biosurfactant Producing Bacteria

Among the nine isolates, the best isolate FKOD36, which showed higher emulsification index, capability to reduce surface tension and oil displacement activity was identified as *Klebseilla sp.* FKOD36 which belonged to the gamma-proteobacteria (Fig. 6), which suggests limited distribution of these genes among bacteria. This suggests that certain taxa of surfactant-producing species may play unique roles in determining the composition and population sizes of petroleum-degrading species in oil-degrading communities as well as the rate of petroleum degradation.

Discussion

Biosurfactants play a key role in emulsification of petroleum hydrocarbons. Biosurfactants and bioemulsifiers are believed to be alternative to chemical surfactant because of their properties as eco-friendly, least toxicity, biodegradability and high specificity (Banat *et al.*, 2000). Functional properties of biosurfactants such as oil displacement, emulsification, surface activity and reduction in viscosity of crude oil for transportation are of interest. Therefore, finding biosurfactant producing microorganisms to enhance bioremediation is an important area of research.

The findings of present study revealed that nine bacterial isolates (out of 37) had a positive drop collapse (qualitative) test. This implies that these isolates had ability to produce surface active compounds, which caused reduction in surface tension. However, there are reports that qualitative test may not be sufficient to confirm the ability of bacteria to produce biosurfactants, which required to be supported by other techniques based on surface activity measurements (Mulligan *et al.*, 1984; Makkar and Cameotra, 1997). To confirm the ability of bacteria to produce biosurfactants, a quantitative analysis was performed to demonstrate the surface tension activity. All the nine isolates that had positive drop test also showed higher surface tension reduction activity, while *Klebseilla sp.* FKOD36 amongst these was excellent.

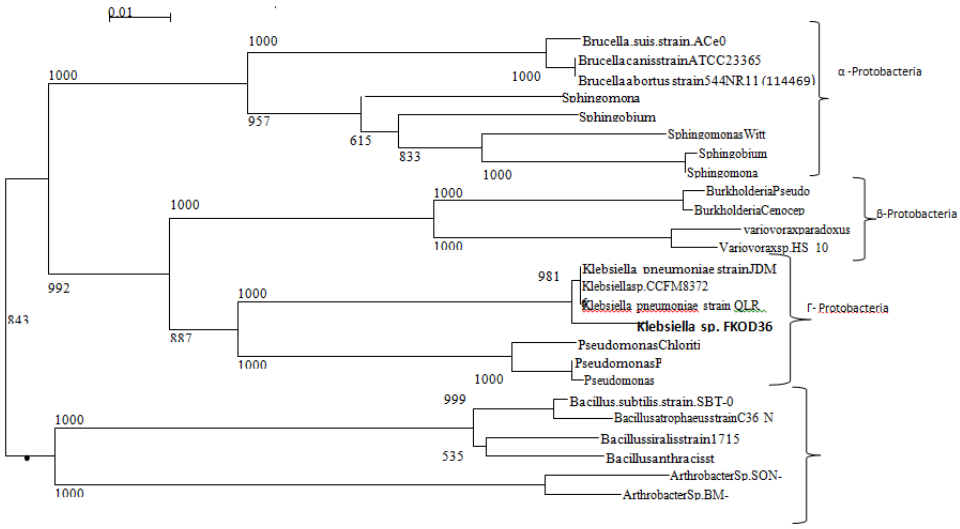

Fig. 6: Phylogenetic tree obtained by neighbor-joining analysis of 16S rRNA gene sequences, showing the position of the biosurfactant producing isolate among neighboring species of the genus *Klebsiella*

Thus, a positive correlation was observed between these two properties. It is very likely that those isolates that have ability to produce biosurfactants into the culture medium could be potential candidate for bioremediation of xenobiotic compounds because of their ability to lower surface tension efficiently, leading to more solubilization and biodegradation (Fiebig *et al.*, 1997; Kuyukina *et al.*, 2001). Since cell biomass of the selected bacterium was high on PHE amended medium, it can be suggested that this bacterial strain (*Klebseilla sp.* FKOD36) could be very effective for biodegradation of petroleum hydrocarbons.

In the present study, several bacterial isolates were obtained from soil and sludge samples contaminated with crude oil. These bacterial isolates had a variable potential to produce biosurfactant and degrade phenanthrene. One of the bacterial isolate FKOD36 showed high potential to produce biosurfactant as well as to degrade PHE. These results implied that crude oil contaminated sites contain bacteria capable of producing biosurfactant that could effectively lower the surface tension and enhance emulsification index. Thus, biosurfactants facilitate biodegradation process. That is why in this study biodegradation of PHE was substantially greater in the presence of biosurfactant than that observed in the absence of biosurfactant. Moreover, cell biomass was also greater in the case of bacterial isolate that had higher potential for producing biosurfactant. This bacterial isolate (FKOD36) was identified as *Klebseilla sp*. Previously, none of the studies reported the potential of *Klebsiella* sp. to produce biosurfactant and degrade PHE; however, *Pseudomonas and Bacillus* spp., which showed closed relatives with *Klebsiella sp*. are capable of producing biosurfactants that have been reported by researchers (Desai and Banat, 1997; Rosenberg and Ron, 1999; Maier and Soberón-Chávez, 2000). The closeness of *Klebsiella sp*. with *P*seudomonas *and Bacillus* species indicates that the selected isolate has significant genetic potential to produce biosurfactant and could be employed for bioremediation.

Conclusion

Nine out of the 37 isolates showed positive qualitative drop collapse test. However, best isolate based on surface tension reduction capability and emulsification index was found to be FKOD36 which was identified as *Klebseilla sp.* FKOD36. The selected strain showed more than 90% similarity index with *Klebseilla sp*. The biosurfactant producing strain not only exhibited excellent capability of reduction in surface tension (35 dyne/cm) but also showed higher emulsification index (66%). The results obtained suggest the possible use of *Klebseilla sp*. FKOD36 in bioremediation of crude oil and many other hydrocarbon contaminated sites.

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References

Abraham, W.R., H. Meyer and M. Yakimov, 1998. Novel glycine containing glucolipids from the alkane using bacterium *Alcanivorax borkumensis*. *Biochim. Biophys. Acta*, 1393: 57–62

Asaka, O. and M. Shoda, 1996. Biocontrol of *Rhizoctonia solani* damping off of tomato with *Bacillus subtilis* RB14. *Appl. Environ. Microbiol.*, 62: 4081–4085

Banat, I.M., R.S. Makkar and S.S. Cameotra, 2000. Potential commercial applications of microbial surfactants. *Appl. Microbiol. Biotechnol.*, 53: 495–508

Batista, S.B., A.H. Mounteer, F.R. Amorim and M.R. Totola, 2006. Isolation and characterization of biosurfactant/bioemulsifier-producing bacteria from petroleum contaminated sites. *Biores. Technol.*, 97: 868–875

Bento, F.M., F.A. de Oliveira Camargo, B.C. Okeke and W.T. Frankenberger, 2005. Diversity of biosurfactant producing microorganisms isolated from soils contaminated with diesel oil. *Microbiol. Res*., 160: 249–255

Bodour, A.A. and R. Miller-Maier, 2000. In: *Biosurfactants: types, Screening Methods and Applications: Encyclopedia of Environmental Microbiology*, pp: 750–770. Bitton, G. (ed.). John Wiley and Sons, Inc., Hoboken, New Jersey, USA

Bosch, M.P., M. Robert, M.E. Mercade, M.J. Espuny, J.L. Parra and J. Guinea, 1988. Surface-active compounds on microbial cultures. *Tenside Surfactants Detergent*, 25: 208–211

Christofi, N. and I.B. Ivshina, 2002. Microbial surfactants and their use in field studies of soil remediation: A review. *J. Appl. Microbiol.*, 93: 915–929

Das, P., S. Mukherjee and R. Sen, 2008a. Antimicrobial potentials of a lipopeptides biosurfactant derived from a marine *Bacillus circulans*. *J. Appl. Microbiol*., 104: 1675–1684

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

Deleu, M. and M. Paquot, 2004. From renewable vegetables resources to microorganisms: new trends in surfactant. *C.R. Chimie*, 7: 641–646

Desai, J.D. and I.M. Banat, 1997. Microbial production of surfactants and their commercial potential. *Microbiol. Mol. Biol.*, 61: 47–64

DeSantis, T.Z., P. Hugenholtz, N. Larsen, M. Rojas, E.L. Brodie, K. Keller, T. Huber, D. Dalevi, P. Hu and G.L. Andersen, 2006. Green genes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. *Appl. Environ. Microbiol.*, 72: 5069–5072

Fiebig, R., D. Schulze, J.C. Chung and S.T. Lee, 1997. Biodegradation of biphenyls (PCBs) in the presence of a bioemulsifier produced on sunflower oil. *Biodegradation*, 8: 67–75

Gravel, V., C. Martinez, H. Antoun and R.J. Tweddel, 2005. Antagonistic microorganisms with the ability to control Pythium damping-off of tomato seeds in rockwool. *BioControl*, 50: 771–786

Hultberg, M., T. Bengtsson and E. Liljeroth, 2010. Late blight on potato is suppressed by the biosurfactant-producing strain *Pseudomonas koreensis* 2.74 and its biosurfactant. *BioControl*, 55: 543–550

Jain, D.K., D.L. Collins-Thompson, H. Lee and J.T. Trevors, 1991. A drop-collapsing test for screening surfactant-producing microorganisms. *J. Microbiol. Methods*, 13: 271–279

Juwarkar, A.A., K.V. Dubey, A. Nair and S.K. Singh, 2008. Bioremediation of multi-metal contaminated soil using biosurfactant—a novel approach. *Ind. J. Microbiol.*, 48: 142–146

Krishnayya, P.V. and P.S. Grewal, 2002. Effect of neem and selected fungicides on viability and virulence of the entomopathogenic nematode *steinernema feltiae*. *Biocontrol Sci. Technol*., 12: 259–266

Kuyukina, M.S., I.B. Ivshina, J.C. Philip, N. Christofi, S.A.E. Dunbar and M.I. Ritchkova, 2001. Recovery of *Rhodococcus* biosurfactants using methyl tertiary-butyl ether extraction. *J. Microbiol. Methods*, 46: 149–156

Maier, R.M. and G. Soberón-Chávez, 2000. *Pseudomonas aeruginosa* rhamnolipids: biosynthesis and potential applications. *Appl. Microbiol. Biotechnol*., 54: 625–633

Makkar, R. and S. Cameotra, 1997. Biosurfactant production by a thermophilic *Bacillus subtilis* strain. *J. Ind. Microbiol. Biotechnol*., 18: 37-42

Maneerat, S., T. Bamba, K. Harada, A. Kobayashi, H. Yamada and F. Kawai, 2006. A novel crude oil emulsifier secreted in the culture supernatant of a marine bacterium, *Myroides* sp. strain SM1. *Appl. Microbiol. Biotechnol.*, 70: 254–259

Moldes, A.B., R. Paradelo, D. Rubinos, R. Devesa-Rey, J.M. Cruz and M.T. Barral, 2011. Ex situ treatment of hydrocarbon-contaminated soil using biosurfactants from *Lactobacillus pentosus*. *J. Agric. Food Chem.*, 59: 9443–9447

Morikawa, M., Y. Hirata and T. Imanaka, 2000. A study on the structure-function relationship of lipopeptide biosurfactants. *Biochim. Biophys. Acta*, 1488: 211–218

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

Muthusamy, K., S. Gopalakrishnan, T.K. Ravi and P. Sivachidambaram, 2008. Biosurfactants: Properties, commercial production and application. *Curr. Sci.*, 94: 736–747

Oyarzabal, O.A., 1999. Methods for detection, isolation and identification of *Campylobacter*. *Poult. Sci.*, 78: 4–5

Perfumo, A., I.M. Banat, F. Canganella and R. Marchant, 2006. Rhamnolipid production by anovel thermotolerant hydrocarbon-degrading *Pseudomonas aeruginosa* AP02-1. *J. Appl. Microbiol*., 75: 132–138

Rosenberg, E. and E.Z. Ron, 1999. High-and low-molecular-mass microbial surfactants. *Appl. Microbiol. Biotechnol.*, 52: 154–162

Rostas, M. and K. Blassmann, 2009. Insects had it first: surfactants as a defense against predators. *Proc. R. Soc. B.*, 276: 633–638

Singh, A., J.D. Hamme and O.P. Ward, 2007. Surfactants in microbiology and biotechnology: part 2: application aspects. *Biotechnol. Adv.*, 25: 99–121

Snook, M.E., T. Mitchell, D.M. Hinton and C.W. Bacon, 2009. Isolation and characterization of Leu7-surfactin from the endophytic bacterium *Bacillus mojavensis* RRC 101, a biocontrol agent for *Fusarium verticillioides*. *J. Agric. Food Chem.*, 57: 4287–4292

Tamura, K., D. Peterson, N. Peterson, G. Stecher, M. Nei and S. Kumar, 2011. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol. Biol. Evol*., 28: 2731–2739

Transparency Market Research, 2012. Biosurfactants Market - Global Scenario, Raw Material and Consumption Trends, Industry Analysis, Size, Share and Forecasts, 2011-2018. 121 pages. http://www.transparencymarketresearch.com/biosurfactants-market.html

Van Hamme, J.D., A. Singh and O.P. Ward, 2006. Physiological aspects. Part 1 in a series of papers devoted to surfactants in microbiology and biotechnology. *Biotechnol. Adv.*, 24: 604–620

Youssef, N.H., K.E. Duncan, D.P. Nagle, K.N. Savage, R.M. Knapp and M.J. McInerney, 2004. Comparison of methods to detect biosurfactant production by diverse microorganisms. *J. Microbiol. Methods*, 56: 339–347

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