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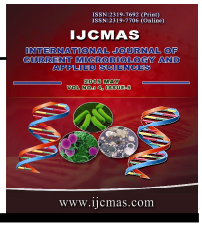

Original Research Article

Bioremediation of Petroleum Oil by Potential Biosurfactant-Producing Bacteria using Gravimetric Assay

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Keywords

Bioremedation, Biosurfacants, Oil-degrading bacteria, Gravimetry.

A B S T R A C T

Bioremediation is an efficient strategy for cleaning up sites contaminated with organic pollutants. Oil is worldwide contaminant that can reach toxic concentrations that are detrimental to the environment and human health. The present study investigated bioremediation, by gravimetric analysis, the ability of theoil-degrading bacteria to produce biosurfecants. Four bacterial strains were used in the study. Strains were local isolated strains by enrichment culture technique from polluted sites in industrial area of Taif, Saudi Arabia. These isolates were identified through morphological, biochemical and 16S-rRNA gene sequencing techniques as *Bacillus thuringiensis* AT5, *Actinomyces octodloyts* AF104, *Pseduomonas aerogenosa* AF11-GT and *Pseduomonas stutzeri* AT3.Their ability to degrade crude oil hydrocarbon were monitored by gravimetric assay for 5 days. The total viable count of isolates ranged from 1.7 $x10^4$ - 2.1$x10^6$ CFU mL^{-1} culture. The four strains showed relatively high capacity and wide spectrum to degrade the crude oil hydrocarbons. Strain AF11-GTshowed high biodegradation efficiency up to 93.41 % when grown on 3, and 5g L^{-1} oil hydrocarbon by the second day.An increase in oil degradation was correlated to an increase in cell number indicating that the bacterial isolates were responsible for the oil degradation. Potential biosurfactant production methods showed that isolates are biosurfactant producers and the superior isolate was AF11-GT. Our results obtained demonstrate the potential for biodegradation of AF11-GTisolate *in situ* and/or *ex situ* as well as in microbial enhancing oil recovery (MEOR) agent.

Introduction

Nature has been a potential source of oil-degrading agent for years. Recently, attention has been given to oil pollution remediation and microbial enhancing oil recovery processes. The risk of crude oil spillage, involved in many activities of petroleum industry, poses a serious human health and environmental problems, due to the possibility of air, water and soil contamination. Crude oil, because of its

characteristics is one of the most significant pollutants in the environment as it is capable of causing serious damages to humans and the ecosystem. Prolonged exposure and high oil concentration may cause the development of liver or kidney disease, possible damage to the bone marrow and an increased risk of cancer. Recent oil spill occurrences in soils are motivating studies in the soil bioremediation field, especially due to the lack of scientific knowledge in the bio-treatment of this type of polluted soil. Microbial bioremediation has been shown to be a potent technique for the breakdown of contaminations into less harmful form (Pratt et al., 1999). To attenuate the environmental effect caused by water pollution by hydrocarbons, the bioremediation was less aggressive and more adjusted to maintain the ecological balance (Rosato, 1997).

Microbial remediation of hydrocarbon compounds was found to be an available alternative method over the conventional methods. Microbial treatment can control hydrocarbons pollution by reducing the length of the hydrocarbon molecules and by producing by-products that act as biosurfactants and solvents (Banat 1995 and Wolicka et al., 2009). The maximum benefit of bioremediation process is the mineralization in which the pollutant is degraded to CO_2 and H_2O by the aerobic metabolic way (Banat, 1995). There are two bioremediation techniques which can be used in all the available technologies of treatment in order to try to maximize its efficiencies (Wolicka et al., 2009): the biostimulation, in which there is the increase of the indigenous populations activity by adding nutrients and/or a terminal electron acceptor, and the bio-augmentation, in which there is the increase of the pollutant degradation potential by adding exogenous degrading microbial strains. The search for effective and efficient methods of oil removal from contaminated sites has intensified in recent years, because microbial degradation that in responsible for clearing untreated oil spills is slow (Banat, 2000). Microbial remediation of a hydrocarbon–contaminated site is accomplished with the help of a diverse group of microorganisms, particularly the indigenous bacteria present in soil.

The growth and proliferation of oil utilizing microorganisms in polluted soil is greatly influenced by the availability of nutrients and their hydrocarbonoclastic property. Mechanical method to reduce hydrocarbon pollution is expensive and time consuming. Gas chromatography and mass spectrometry is usually used to provide type analysis of petroleum products, which gives the percentages of hydrocarbon types such as alkanes and cycloalkanes in the oil. The analysis type can provides the relative composition, the amounts has to be estimated by another technique, called gravimetric assay. Gravimetric would give satisfactory result in experiments that utilize large amounts of crude oil (Latha and Kalaivani, 2012).

Biosurfactants are the surface active agents that are amphipathic in nature and possess both hydrophilic and hydrophobic moieties that reduce the surface and interfacial tensions between two immiscible liquids. The polar and non-polar moieties present in the structure of biosurfactants allow them to accumulate at inter-phase between liquids of different polarities and form micelles thereby reducing surface tension and facilitating hydrocarbon uptake and emulsification. The interest in biosurfactants is taking much more attention these days due to their promising quality towards the environment. The biosurfactants are preferred over their chemically synthesised counterparts because of their higher

biodegradability and selective nature towards the environmental factors like temperature, pH, and salinity. They enhance the recovery of oil by reducing the interfacial tension (IFT) between the oil and water interfaces, or by mediating changes in the wettability index of the system. Microbial surfactants have several advantages over chemical surfactants such as lower toxicity, higher biodegradability and effectiveness at extreme temperatures or pH values (Kosaric, 1992). However, the biosurfactants are not able to compete with the chemical surfactants due to their higher production costs (Gautam and Tyagi, 2006, Pacwa-Plociniczak et al., 2011). With the emergence of worldwide energy crises, the microbial enhanced oil recovery (MEOR) was focused on in recent years. One of the principles is the production of the biosurfactants by petroleum microbes. Surfactants can decrease interfacial tension of water, oil and rock, emulsify crude oil, and change wettability, etc. (Pansiripata et al., 2010). Besides, MEOR, biosurfactants are widely used in environmental biological remediation, fermentation engineering, cosmetic industry, food industry, etc.

The increasing use of PCR, over the last few years, rapid template purification, and automated DNA sequencing has dramatically reduced the time necessary to yield a high-quality sequence. The use of 16S- rRNA gene sequencing to study the relatedness of prokaryotic species is well established and has led to increased availability of 16S- rRNA databases. The convergence of these technical and computational advances has also enhanced the application of 16S- rRNA gene sequence analysis to bacterial identification (Rantakokko-Jalava et al., 2000). It was recently reported that subtle sequence differences in the 16S rRNA gene could be used for species identification (Sacchi et al., 2002) and for subtyping and identifying hyper virulent bacterial clones (Nilsson et al., 2003).

The present work was carried out to study bioremediation, by gravimetric analysis, the ability of the organism to produce biosurfecants and to evaluate the efficiency of crude oil removal by the addition of oil-degrading microorganisms.

Materials and Methods

Microorganisms

Bacterial strains were *Bacillus thuringiensis* AT5, *Actinomyces octodloyts* AF104,*Pseduomonas stutzeri* AT3 and*P.aerogenosa* AF11-GT. The isolates used in this study were from the Biotechnology and Genetic Engineering Research Unit (BGERU) Microbial Bank collection of strains, at Taif University, KSA. Strains were local isolated strains by enrichment culture technique from polluted sites in industrial area of Taif, Saudi Arabia.The four isolates showed good growth on Bushnell- Haas enrichment mineral medium (BHM) amended with crude oil andwere selected based on the growth and degradation ability.

Growth potential of hydrocarbon-utilizing microorganisms

Inocula were routinely grown in Luria–Bertani (LB) broth medium. Media were autoclaved at 120 °C for 20 min. Cultures were grown overnight first on LB medium without hydrocarbon addition. Then, grown on Bushnell- Haas enrichment mineral medium (BHM).The pH was adjusted to 7.2 and sterilized at 121^{o}C for 15 min. Bacteria were grown in 250 ml Erlenmeyer flasks for one week in a rotary shaker. A 100 ml medium was dispensed in 250ml conical flasks.Flasks were amended with crude

oil**:**0, 3 and 5 g/L (w/v) for each organism. The pH of media was adjusted to 7. One ml was taken to measure turbidity at 595 nm with spectrophotometer. Growth oncrude oilwas monitored by measuring the optical density (O.D.) at 595 nm in 2 ml cuvettes using a spectrophotometer (SmartSpec Plus Spectrophotometer, Bio-Rad).The net dry biomass was determined simultaneously. A 1 mL of culture was centrifuged at 1500 rpm for 10 min, washed twice with distilled water, poured into a pre-weighed container, dried overnight at 90 °C to constant weight and cooled for reweighing. Crude oil adapted cells were harvested and washed twice with BHM and the pellet suspended in 0.1M phosphate buffer at pH 7.0. Cells were harvested by centrifugation for 5 min at 3,000 x g at room temperature. The growth rates of cultures in exponential phase were determined from linear regressions of log10 absorbency vs. time, calculating a least squares fit of data from the exponential growth phase, and determining the slope of this line. The instantaneous growth rate (μ) was determined from the slope of this line x ln10; μ had the dimensions h^{-1} (Koch, 1984).

Enumeration of microorganisms

Bacteria were enumerated by making tenfold serial dilution of flasks using physiological saline. From the diluted sample, using a dropper pipette, 1 ml of each dilution was dropped onto Petri dish then the plate count technique was employed; Bushnell- Haas agar media amended with crude oil as carbon source were poured (Gerdhardt et al., 1984). Duplicates of plates were used for each dilution. Plates were incubated for 48 – 72 h at 30^{o}C in an incubator. Each inoculum of microorganism developed into a discreet colony. All plates yielding 30 - 300 colonies were counted. The number of viable microorganisms in the sample was calculated from the number of colonies formed, the volume of inoculum used by dropper pipette and the dilution factor expressed in colony forming unit (CFU) (Krieg, 1984).

Biosurfactant Assay

Two methods were employed in this study modified drop collapse method (MDC) (Bodour and Miller-Maier, 1998) and Blue agar plate method (BAP) (Siegmund and Wagner, 1991).

Surface tension measurements

Surface tension(mN/m)was measured using an Auto-tensiomat (Fisher Scientific Co., Pa., USA) according to the manufacturer instructions.

Emulsification stability (E_{24}) index

Emulsification stability(E_{24}) of strains was measured by adding 2 ml of oil to the same amount of each culture. The sample was mixed with a vortex for 2 min and left for 24 hours. E_{24} index was calculated as percentage of height of emulsified layer (mm) divided by height of liquid column (mm) (Sarubbo et al., 2006).

Biodegradation of crude oil

Strainswere incubated overnight in 50 ml LB broth medium in triplicate, pH 7.5 at 30^{o}C (shaken culture: 150 rpm). Cells were centrifuged and washed twice with the liquid inorganic salts BHM. The pellet was suspended in 5ml BHM and inoculated into 100 ml of BHM in 3 flasks supplemented with 3 concentrations of crude oil: 0,3, and 5 gL^{-1} (w/v), as sole energy and carbon sources (Al-Ghawar field) in eastern of KSA. Flasks were incubated at 30°C for 5 dayson a rotary shaker at 150 rpm.

Extraction of crude oil

For estimation of oil degradation rates by gravimetric assay10ml of n-hexane was added to each flask. The contents were transferred to a separating funnel and extracted. Extraction was employed twice to ensure complete recovery of crude oil. The extract was treated with 0.4g of sodium sulphate to remove water and decanted into a beaker leaving behind sodium sulphate. The extract was evaporated to dryness in a rotary evaporator under reduced pressure.

Biodegradation efficiencyand gravimetric assays

Residual crude oil was monitored using the method used by (Saxena, 1990).The residual crude oil was measured by extraction of crude oil from the BHM medium and evaporating it to dryness in rotary evaporator at 40°C under reduced pressure. The volume of extracted crude oil was deducted from beaker weight. The degradation percentage was calculated as follows:

Residual crude oil weight = Weight of beaker containing extracted crude oil – Weight of empty beaker.

Degraded crude oil amount= Weight of crude oil added in the media – Weight of residual crude oil

Degradation % = Amount of crude oil degraded / Amount of crude oil added in the media x 100

Molecular genetics analysis

DNA extraction

The genomic DNA of *Actinomyces* sp. and the three bacteria species were extracted using bacteria DNA Preparation Kit (Jena Bioscience, Jena, Germany) according to the manufacturer's instructions (www.jenabioscience.com).

PCR amplification of 16S-rRNA gene

Primer sequences used to amplify the 16S-rRNA gene fragment were: primers forward fD1 (5'-CCGAATTCGTCGACAACAGAGTTTGA TCCTGG CTCAG-3') and reverse rD1(5'-CCCGGGATCCAAGCTGGAGGTGATCC AG CC-3') for *Actinomycetes* and U1 [5CCA GCA GCC GCG GTA ATA CG3] and U2 [5ATC GG(C/T)TAC CTT GTT ACG ACT TC3] for bacteriaas described by Ren et al. (2007). The PCR reaction mixture contained 10 *P*mol of each primer and 12.5 µl of 2x SuperHot PCR Master Mix (Bioron, Ludwigshafen, Germany) mixed with 50 to 100 ng of DNA template. Sterile d. H_2O was added to a final volume of 25 µl. Thermal cycler (Uno II, Biometra, Germany) with the following thermal profile: 94 °C for 4 min., 94 °C for 1 min., 55 °C for 1 min., 72 °C for 1.5 min, the number of cycles was 35 cycle and the post PCR reaction time was 72°C for 5 min.

Analysis of the PCR products

The PCR reaction products were electrophoresed with 100 bp ladder marker (Fermentas, Germany) on 10 x 14 cm 1.5%-agarose gel (Bioshop, Canada) for 30 min using Tris-borate- EDTA Buffer. The gels were stained with 0.5 ug /ml of ethidium bromide, visualized under the UV light (Watanabe et al., 2001) and documented using a GeneSnap 4.00- Gene Genius Bio Imaging System (Syngene, Frederick, Maryland, USA).

Sequencing of 16S-rRNA gene

The PCR-products of each organism was purified from excess primers and nucleotides by the use of AxyPrep PCR

Clean-up kit (AXYGEN Biosciences, Union City, California, USA) and directly sequenced using the same primers as described for the amplification process. The microorganisms DNA sequences were determined with the chain-termination method on an ABI 3730 DNA sequencer by a commercial service (Seoul, Korea). Sequences were aligned in the GenBank database using the BLASTN program at the National Center for Biotechnology Information (NCBI), and percent homology scores were obtained to identify bacteria.

Statistical analysis

Statistical analysis was performed using the SPSS 10.0 software. Data underwent a one-way ANOVA test, and means were compared using Duncan's multiple range tests at 5% significance level.

Results and Discussion

Identification, Morphological, Biochemical and Molecular Characterization

Table 1 show morphological, physiological and biochemical characteristics of isolates.Strains were local isolates isolated by enrichment technique and deposited in our microbial bank at Taif University, Saudi Arabia (Shahaby and El-Tarras, 2011, El-Tarras et al, 2012) in our laboratory. **T**he isolates were identified on the basis of their cultural and biochemical characteristics according to Bergey's Manual of Determinative Bacteriology (9^{th} edition) (Holt et al., 1994) and Api kit profiles (ApiBioMerieuxsa, 2009). Colony morphology on nutrient agar plate, AT5showed creamy, big spreading, finely wrinkled and slimy. In AF11-GTshowed large, opaque irregular colonies with earthy odors, AF104-MH was medium white colony with gray center, and AT11 was small yellowish colony. In Blood agar plates showed the heamolysis. Phenotypic examination of the recovered microorganisms revealed that they belong to the genera of *Actinomyces*, *Bacillus* and *Pseudomonas* (Table 1). Four isolates *Actinomyces odontolyticus* AF104-MH, *Bacillus thuringiensis* AT5,*Pseudomonas aerogenosa* AF11-GT and *P. stutzeri* AT11 showed good growth on Bushnell- Haas medium amended with crude oil as a carbon source and were selected based on the growth and degradation ability. All selected strains showed optimal growth at 30 ^{o}C.

The data of 16S-rDNA sequence analysis showed that 16S-rDNA sequence of strain AF104-MH, AT5, AF11-GT and AT11 were 98% identical to that of *Actinomyces octodloyts*, *Bacillus thuringiensis*, *Pseudomonas aerogenosa* and *P. stutzeri*, respectively.

Some of these microorganisms have earlier been isolated, identified and reported as hydrocarbon bio-degraders by Alexander (1999), Abalos (2004), Etoumi (2007), Abdel-Megeed et al (2012), Shahaby and El-Tarras (2011)and El-Tarras et al(2012). The ability of these isolates to produce a color change in the medium is presumably due to the reduction of the indicator by the oxidized products of hydrocarbon degradation. The total color change (colorless to dark blue) supports the fact that the isolates are potential hydrocarbon oxidizers**(**BAP method**)**. Among the four strains that produced total color change, *Pseudomonas aerogenosa* and *Bacillus thuringiensis* displayed the fastest onset and highest extent of biodegradation.

Psedomonas aeruginosa, *Bacillus subtilis* and *Halomonas eurihalina* species were effective bacteria in the biodegradation of

heavy hydrocarbons (petroleum oil) and n-tetradecane (Martinez-Checa et. al., 2002, Sadeghazad and Ghaemi, 2003, Shahaby and El-Tarras, 2011, and El- Tarras et al., 2012, Shahaby, 2014).Isolation of alkane degrading microorganisms from oil contaminated soil has been reported by several researchers. Nazina et al (2005) have obtained hydrocarbon oxidizing *Geobacilli* strains from formation waters of oil fields.

Accession numbers of strains

The partial 16S-rDNA gene sequences of strains that were determined have been deposited in the GenBank, EMBL, and DDBJ nucleotide sequence databases under accession nos. JQ621963 for *Bacillus thuringiensis* AT5,NJ700210 for *Actinomyces odontolyticus* AF104-MH, NJ700212 for *Pseudomonas aerogenosa*AF11-GT and JQ342094 for *P. stutzeri* AT11.

Growth kinetics and dry weight (biomass)

Table 2 show the growth pattern, dry biomass and bacterial density from petroleum oil by the four selected bacterial isolates. Optical density OD_{595} and dry weight were estimated simultaneously. The linear relation between Optical density and dry weight were obtained during growth on crude oil. These figures indicate the effluence of the specific growth rate and biomass precipitation on a period of bacterial cultivation in BHM containing crude oil hydrocarbon. Little adaptation occurred at higher oil concentration, indicating that the highest hydrocarbon concentration exceeded the strains capability to adapt.The specific growth rates of the isolates on oil showed that strain AF11-GTfollowed by isolate AT 11were faster than other strains AF104- GT and AT11in growth in mineral salts medium BHM containing 3g(w/v) oil. Most growth occurred in the first 2 days for all strains resulting in good biomass production. Maximum growth rates (μ_{max}) for AF11-GTwas 2.11 $\mu\ h^{-1}$followed by other strains. Strains were also grown on 5g L^{-1}oil (data not shown). However, growth on 3 g L^{-1} (w/v) oil hydrocarbon was better than 5 gL^{-1}(w/v) concentration. Furthermore, isolate AF11-GT produced more biomass from oil being 2.11 g cells l^{-1} after only 2 days of growth.

In total viable counts of bacteria were ranged between $1.7x10^4$ - $2.1x10^6$ CFU mL^{-1} culture. The results presented in Table 2. From this table that the number of viable bacteria especially AF11-GT followed by AT5 are greater than the other isolates.

The decrease in hydrocarbon fractions by biodegradation of hydrocarbons using *Bacillus, Pseudomonas* and *Actinomyces* species was recorded (Etoumi, 2007). It was mentioned that the lower the concentration of hydrocarbons the higher was the utilization. Similar growth rates and biomass on hydrocarbon were obtained (Etoumi, 2007, Shahaby and El-Tarras, 2011).

Biodegradation and biosurfecant production

Table 3 illustrate the biodegradation pattern, and production of biosurfactant by the four selected bacterial isolates. Biosurfactant are produced by many bacterial strains that can degrade or transform the components of petroleum hydrocarbon. They are non-toxic, non-hazardous, biodegradable and environmentally friendly compounds (Banat et al., 2000). Using the two qualitative methods (MDC and BAP), results demonstrated that allstrains used in this study were biosurfactant producers (Table 3). This explains the oil degradation performance obtained with these four strains. Knowing that biodegradation

depends strongly on hydrocarbon emulsion, the use of biosurfactant producer strains like *Bacillus, Actinomyces*, and *Pseudomonas* species in bioremediation technology seems to offer more potential than chemical surfactant, due to their structural diversity, biodegradability and biocompatibility relative to synthetic surfactant (Banat, 1995, Banat et al, 2000, Abalos et al, 2004, Bayoumi et al, 2010, Shubhrasekhar et al, 2013). These results indicate that the bacterial isolatesare capable of using the petroleum oil as a carbon and energy source under these conditions.

The activity of the four strains on 0,3, and 5 g L^{-1}oil (w/v) was monitored and the rate of biodegradation were used as indication for the ability of these isolates to grow on petroleum oil hydrocarbon. All strains showed positive response up to 93.41% (Table 3) when grown on 3g L^{-1} oil by the second day. The bacterial isolate AF11-GT was nearly two fold more biodegradation efficacy of oil than strains AT11 and AF11-GT. The strain AT5 was moderate in activity being 61.63 %. The lowest activity rates were recorded on 5g l^{-} oil (data not shown). The biodegradation of hydrocarbon was starting to decrease after the third day. The higher the concentrations of petroleum oil the lower of biodegradation capacity. Similar results were obtained by number of researchers (Kumara 2006, Etoumi 2007, EL-Tarraset al 2010, Shahaby 2014).Growth on oil derivate before exposure to specific hydrocarbon compounds was intended as a pre-enrichment step, similar to the initial enrichment of polychlorinated biphenyl (PCB) degraders on biphenyl (Bedard *et al.*, 1987) or methylcyclohexane for bacteria able to grow on a wide range of alicyclic compounds (Trudgill, 1984).Latha and Kalaivani (2012) used the method of gravimetric analysis to estimate biodegradation rates but it was over estimation and it was unrealistic.

Biosurfactants were not detected in culture broth of bacterial isolates grown in MSM containing n-hexadecane hydrocarbon (Kumara 2006). On contrast, other researcher, Margaritis et al (1979), Etoumi (2007) and Shahaby (2014) detected biosurfactants for bacterial strains of *Actinomyces* and *Pseudomonas* species when grown on n-hexadecane hydrocarbon. This contrast is might due to nature of bacteria and carbon sources present in media.

The emulsification indices and surface tensions recorded for crude oil hydrocarbon in Table 4, illustrate a difference in emulsification activity and surface tension of isolates on the oil hydrocarbon. An emulsification index and surface tension of 93% and 70.41 mN m^{-1} were achieved for AF11-GH followed by AT5, respectively. The lowest strains were AF104-MH and AT11 being 31, 29% and 55.33, 54.21 mN m^{-1},respectively. Thus, isolates AF11-GH and AT5 showed a preferential emulsification activity and surface tension when growing on crude oil as sole carbon and energy sources.

Emulsification Activity and surface tension are among the most important characteristics of biosurfactant producing microorganisms (Gautam and Tyagi 2005). These characteristics have been shown to increase the biodegradation rate of hydrocarbons, stimulate microbial growth, decrease crude oil viscosity and enhance oil recovery at oil fields (Margaritis et al.,1979, Pokethitiyook et al,2002, Sadeghazad and Ghaemi, 2003).Mandri and Lin (2007) reported that the *P. aeruginosa* had degraded 90% in 4 weeks.

The isolates produced biosurfactants with varying concentration as culture supernatants exhibited variable degree of colors (Table 3) when tested with BAP method and emulsification activities (Table 4). Cleary, strainAF11-GH showed better performance compared to the other three strains. These results are comparable with those reported by Etoumi (2007) and Margaritis et al (1979) when using *Pseudomonas* species.

Table.1 Morphology, physiology, and growth of strains on LB media

Strain	*Actinomyces odontolyticus* AF104-MH	*Bacillus thuringiensis* AT5	*Pseudomonas aeruginosa* AF11-GT	*Pseudomonas stutzeri* AT11
Colony color	White	Creamy	Greenish	Yellowish
Morphology	Long thin hyphae	Long bacilli	Short rods	Short rods
Gram Stain	+	+	-	-
Motility	+	+	+	+
Oxidase reaction	-	+	+	-
Catalase reaction	+	+	+	+

Table.2 Growth rates and Biomass yield of strains grown in Bushnell-Hass medium amended with oil as a sole carbon and energy source

Strains	Growth rate (μ)(h^{-1})	Biomass yield (g cells $Loil^{-1}$)	Number of bacteria (CFU)
AF-104-MH	0.081	1.96	$1.7x10^4$
AT5	0.062	0.821	$3.2x10^5$
AF11-GT	0.879	2.11	$2.1x10^6$
AT11	0.051	0.71	$2.7x10^5$

Table.3 Biodegradation rateand biosurfecant production of strains grown in Bushnell-Hass medium amended with crude oil hydrocarbon as sole carbon and energy source

Strains	Biodegradation rate (%)	Biosurfecant production
AF-104-MH	53.6	+
AT5	61.63	++
AF11-GT	93.41	+++
AT11	52.32	+

Table.4 Emulsification stability index (E24) andsurface tension(mN/m) of cell free supernatant of the strains grown in Bushnell-Hass medium amended with crude oil hydrocarbon as sole carbon and energy source

Strain	Emulsification Index (E_{24})	Surface Tension (mN/m)
AF-104-MH	31	54.21
AT5	87	69.63
AF11-GT	93	70.41
AT11	29	55.33

In conclusion microorganisms with high oil-degrading performance are essential for bioremediation of contaminated siteswith crude oil.It indicates the degradation, may be due to production of emulsifiers, surfactants etc. Bioremediation of soil contaminated with crude oil has been considered as a cost-effective technology (Gentilia et al., 2006).

Hydrocarbonthat is resistant to both oxidation and assimilation when present alone in the medium, may be oxidized if attacked by organisms that are simultaneously oxidizing other hydrocarbons. This process, called cooxidation, was noted by Leadbetter & Foster (1958) when investigating the oxidation of ethane, propane, and butane by *Pseudomonas mechanica* while growing on methane, the only hydrocarbon supporting growth. Growth of*R. erythropolis* on complex mineral oil was required to incite the decomposition of the tested hydrocarbons. Finally, biodegradation means complete mineralization of a compound to inorganic form.

Biodegradation has been widely received by the public. However a number of factors must be taken into consideration before *in situ* biodegradation can be applied. These includes, microorganism and capacity to produce biosurfecant, type and concentration of oil contaminated, prevalent climatic conditions, type of environment that has been contaminated and nutrient content as well as pH of the contaminated site.

Biosurfactants are produced by many bacterial strains that can degrade or transform the components of petroleum products. They are non-toxic, non-hazardous, biodegradable and environmentally friendly compounds (Banat et al 2000).

Finally, this study provides anexcellent bacterial strain with a promising role in possible uses in microbial enhanced oil recovery and oil pollution remediation. Strain AF11-GT might be a good potent strain and cheap carbon substrate e.g. milk whey for cell growth and production of surfactant may employ.The surfactant production process needs to be optimized at both laboratory and field level in order to produce high amount of surfactants.

References

Abalos A, M. Vinas, J. Sabaté, M.A Manresa, A.M Solanas, 2004. Enhanced biodegradation of Casablanca crude oil by a microbial consortium in presence of a rhamnolipid produced by *Pseudomonas aeruginosa* AT10. Biodegradation 15:249-260.

Abdel-Megeed A., I.I Abou Elseoud, A.A Mostafa, A.N Al-Rahmah, S.AEifan 2012. Biodegradation of mineral oil by bacterial strains isolated from contaminated soils. Afr J of Microbiol Research, 6(42): 6994-7002.

Alexander, M., 1999. Biodegradation and Bioremediation. 2nd Edn., Academic Press, San, Diego

Aluyor E. O, M. Ori-jesu 2009. Biodegradation of mineral oils – A review. African Journal of Biotechnology 8 (6), 915-920.

Alvarez M., J. M. Marques, E. Korenblum, L. Seldin, 2011. Comparative Bioremediation of Crude Oil-Amended Tropical Soil Microcosms by Natural Attenuation, Bioaugmentation, or Bioenrichment. Appl. Environ. Soil Science, 2011:1-10.

Amaral P.F.F, M.A.Z Coelho, I.M.J Marrucho, J.A.P Coutinho 2010b. Biosurfactants From Yeasts: Characteristics, Production and Application, in: Biosurfactants, ed. Sen R, pp:236-247. West Bengal, India

Amaral PF, MA Coelho , IM Marrucho , JA Coutinho (2010a). Biosurfactants from yeasts: characteristics, production and application. Adv Exp Med Biol.672:236-49.

APi, 2009. BioMerieuxsa 69280 Marcy I'Etoile, France. http://www. biomerieux. com.

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

Banat, I. M. 1995. Biosurfactants production and possible uses in microbial enhanced oil recovery and oil pollution remediation: a review. Biores Tech. 51: 1–12.

Bedard, D. L, R. Unterman, L. H Bopp, M. J Brennan, M. L Haberl, C. Johnson. 1986. Rapid assay for screening and characterizing microorganisms for the ability to degrade polychlorinated biphenyls. Appl. Environ. Microbiol. 51:761-768

Bedard, D.L., R.E. Wagner, M.J. Brennan, M.L. Harbel, J.F. Brown, Jr 1987. Extensive degradation of arochlors and environmentally transformed polychlorinated biphenyl's by *Alcaligenes eutrophus* H850. *Appl. Environ. Microbiol.,* 53:1094-1102

Bodour A.A, R. Miller-Maier 1998. Application of a modified drop collapse technique for surfactant quantitation and screening of biosurfactant producing microorganisms. J. Microb. Methods 32:273-280.

Butier, C.S, J.R Mason. 1997. Structure function analysis of the bacteria aromatic ring hydroxylating dioxygenase. Advanc. Microbiol. Physiol. 38: 47-84.

Campos-Takaki G.M, L.A Sarubbo , C.D Albuquerque 2010. Environmentally friendly biosurfactants produced by yeasts. Adv Exp Med Biol. 672:250-60.

Chikere C.B, G.C Okpokwasili, B.O Chikere. 2009. Bacterial diversity in a tropical crude oil-polluted soil undergoing bioremediation. A. J. Biotech. Vol. 8 (11), pp. 2535-2540.

Collin, P.H, 2001. Dictionary of Ecology and the Environment. 4th Edn., Peter Collin Publishing,

El –Tarras, A. E, A. F Shahaby, N.S Awad, 2012. *In Vitro* screening for natural oil degrading bacteria and evaluation of their biodegradation potential for hydrocarbon. African J. Microbiology Research Vol. 6(49), pp. 7534-7544

Etoumi A. 2007. Microbial treatment of waxy crude oils for mitigation of wax precipitation. J. Petrol. Scien. Engin.55 (1-2): 111-121.

Gary J.H, G.E Handwerk 2001. Petroleum Refining Technology and

Economics."4th Edition Marcel Dekker Inc. New York USA

Holt, J.G., N. R. Krieg, P. H. A Senath, J .T Staley, ST Williams, (eds.). 1994. Bergey's Manual of Determinative Bacteriology, 9th edition. Baltimore: Williams and Wilkins.

Kauppi S., A. Sinkkonen, M. Romantschuk 2011. Enhancing bioremediation of diesel-fuel-contaminated soil in a boreal climate: comparison of biostimulation and bioaugmentation. International Biodeterioration & Biodegradation, 65: 359–368

KleinsteuberS, V. Riis, I. Fetzer, H. Harms, S. Müller 2006. Population Dynamics within a Microbial Consortium during Growth on Diesel Fuel in Saline Environments. Appl Environ Microbiol. 72(5): 3531-3542.

Ko S.H., J.M. Lebeault 1999. Effect of a mixed culture on co-oxidation during the degradation of saturated hydrocarbon mixture. Journal of Applied Microbiology, 87: 72–79

Koch, A.L. 1984. Growth measurement, In: P. Gerhardt, R.G.E Murray, R.N Costllow, E.W Nester, W.A Wood (eds.), Manual of Methods for General Bacteriology. p 179-208. American Soci. Microbiol. Washington, DC, USA.

Komukai-Nakamura SK, T.H Yamauchi, Y. Inomata, K. Venkateswaran, T.H Yamamoto, S. Harayama, 1996. Construction of bacterial consortia that degrade Arabian light crude oil. J. Ferment. Bioeng. 82:570-574.

Konishi, M.A, 2010. Application of Deep Sea Yeast-Production of Biosurfactants. The Review of High Pressure Science and Technology , 20 (4): 347-353.

Latha R. , R Kalaivani , 2012. Bacterial Degradation of Crude Oil by Gravimetric nalysis. Adv. Appl. Sci. Res., 3(5):2789-2795

Lazar, I., A. Voicu, C. Nicolescu, D. Mucenica, S. Dobrota, I.G Petrisor, M. Stefanscu, L. Sandulescu, 1999. The use of naturally occurring selectively isolated bacteria for inhibiting paraffin deposition. J. Petrol. Sci. Eng. 22: 161–169.

Lee, J.K., D. Park, B.U. Kim, J.I. Dong, S. Lee, 1998. Remediation of petroleum contaminated soil by fluidized thermal desorption. Waste Manage., 18: 503-507.

Liu H.J., C.Y. Yang, Y. Tian, G.H. Lin, T.L. Zheng, 2011. Using population dynamics analysis by DGGE to design the bacterial consortium isolated from mangrove sediments for biodegradation of PAHs. International Biodeterioration & Biodegradation, 65: 269–275

Malina G., I. Zawierucha, 2007. Potential of bioaugmentation and biostimulation for enhancing intrinsic biodegradation in oil hydrocarbon- contaminated soil. Bioremediation Journal, 11:141–147.

Mandri T., J. Lin, 2007. Isolation and Characterization of Engine Oil Degrading Indigenous Microorganisms in KwaZulu-Natal, South Africa, African Journal of Biotechnology. 6(1): 23-27.

Martnez-Checa, F., F.L Toledo, R. Vilchez, E. Quesada, C. Calvo, 2002. Yield production, chemical composition, and functional properties of emulsiWer H-28 in media containing various hydrocarbons. Appl. Microbiol. Biotechnol. 58, 358–363.

Nazina, T.N, D.S Sokolova, A.A Grigoryan, A.B Poltaraus, T.P Tourova, A.M Lysenko, G.A Osipov, S.S Belyaev, 2005. *Geobacillus jurassicus* sp. nov., a new thermophilic bacterium isolated from a high-temperature petroleum reservoir, and the validation of the *Geobacillus* species. Syst. Appl. Microbiol. 28: 43–53.

Nilsson, W.B, R.N Paranjype, A. DePaola , M.S Strom. 2003. Sequence polymorphism of the 16S rRNA gene of *Vibrio vulnificus* is a possible indicator of strain virulence. J. Clin. Microbiol. 41:442-446.

Rambeloarisoa, E., J.F. Rontani, G. Giusti, Z. Duvnjak , J.C. Bertand, 1984. Degradation of crude oil by a mixed population of bacteria isolated from sea-surface foams. Mar. Biol., 83: 69-81.

Rantakokko-Jalava K, S Nikkari, J Jalava, E Eerola, M Skurnik, O. Meurman, O. Ruuskanen , A Alanen, E Kotilainen, P Toivanen, P Kotilainen. 2000. Direct amplification of rRNA genes in diagnosis of bacterial infections. J. Clin. Microbiol.38**:** 32-39.

Ren N, D Xing, BE Rittman, L Zhao, T Xie, X Zhao, 2007. Microbial community structure of ethanol type fermentation in bio-hydrogen production. Environ. Microbiol. 9: 1112–1115

Saadoun I., 1997.Degradation of complex mineral oil and other hydrocarbons by *rhodococcus erythropolis***.** Actinomycetes 8 (3): 53-57

Sacchi CT, AM Whitney, LW Mayer, R Morey, A Steigerwalt, A Boras, RS Weyant, T Popovic. 2002. Sequencing of 16S- rRNA gene: a rapid tool for identification of *Bacillus anthracis.* Emerg. Infect. Dis. 8:1117-1123.

Sadeghazad, A. , N.Ghaemi, 2003. Microbial prevention of wax precipitation in crude oil by biodegradation mechanism. Soc. Pet. Eng. SPE 80529, 1–11.

Sarubbo LA, JM Luna, GM Campos-Takaki, 2006. Production and stability studies of the bioemulsifier obtained from a new strain of Candida glabrata

Saxena, MM, 1990. Environmental analysis: Water, soil and air. 2nd ed. Bikaner, India : Agro Botanical Pub. P, 186.

Shahaby A F, A E El-Tarras, 2011. Population Dynamics, Isolation and Characterization of Potential Engine Oil Degrading Indigenous Bacteria in Contaminated Sites in Taif, KSA". Intern. J. petrol. Sci. and Technol. 5 (1): 47-62.

Shahaby A.F, N. A. Awad, A.E El-Tarras, 2013. Isolation and Molecular Characterization of Novel Bacterial Strains Possessing High Efficiency to Degrade Mineral Oil. Life Science J, 10(3): 2563-2572

Shubhrasekhar C., M. Supriya, L.Karthik, K. Gaurav and K. V. Bhaskara Rao 2013. Isolation,, Characterization and Aplication of Biosurfactant produced by Marine Actinobacteria isolated from Saltpan Soil from costal area of Andhra Pradesh, India**.** Res. J. Biotech Vol. 8(1): 18-

Siegmund I, F. Wagner 1991. New method for detecting rhamnolipids excreted by *Pseudomonas* spp. during growth on mineral agar. Biotechnol. Tech. 5:265-268.

Sorkhoh, N. A.; Al-Hasan, R.H.; Khanafer, M. and Radwan, S.S. 1995. Establishment of oil-degrading bacteria associated with cyanobacteria in oil – polluted soil. J. App. Bacteriol. 78, 194-199.

Trudgill, P.W. 1984. Microbial degradation of the alicyclic ring. In D.T. Gibson (ed.), Microbial degradation of organic compounds. Marcel, Dekker, Inc., New York

van Hamme J.D., A. Singh, O.P. Ward, 2003. Recent advances in petroleum microbiology. Microbiology and Molecular Biology Reviews, 64: 503–549

Ward D. M., T. D. Brock, 1978. Hydrocarbon Biodegradation in Hypersaline Environments. Appl. Environ. Microbiol., 35 (2): 353-359

Watanabe K, Y Kodama, S Harayama 2001. Design and evalution of PCR primers to amplify bacterial 16S ribosomal DNA fragments used for community fingerprinting. J Microbiol. Meth. 44: 253–262.

Zhang X, D Xu, C Zhu, T Lundaa and K E Scherr, 2012. Isolation and identification of biosurfactant producing and crude oil degrading *Pseudomonas aeruginosa* strains[j]. Chem. Engin. J. 209:138-146.

Zhao D, C Liu, Y Zhang, Q Liu, 2011. Statistical bioremediation condition optimization of crude oil in soil applying response surface by microbial consortium KW8-2. Journal of China University of Petroleum (Edition of Natural Science), (6): 30–33.

Pratt CJ, Newman AP and Bond PC (1999) Mineral oil bio-degredation within a permeable pavement: long term observations. Water Science and Technology, 1999, 29,(2), pp103-109.

Rosato, Y.B. 1997. Biodegradação do Petróleo. In: Microbiologia Ambiental, (Eds). I.S. Melo; J.L. de Azevedo. Embrapa-CNPMA, Jaguariúna. Pp. 307–334.

Wolicka D, Suszek A, Borkowski A, Bielecka A. Application of aerobic microorganisms in bioremediation in situ of soil contaminated by petroleum products. Biores Technol.2009;100:3221–3227. doi: 10.1016/j.biortech.2009.02.020.