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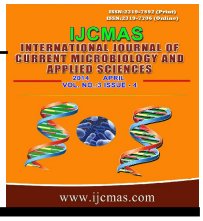

Original Research Article

Assessment Mixed Culture of *Actinomyces* and *Sacchromyces* for biodegradation of Complex Mineral Oil hydrocarbon

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K e y w o r d s

Bioremediation control; Mixed cultures; Biosurfactant; 16S-rRNA gene.

A B S T R A C T

The biodegradation of mineral oil hydrocarbon by mixed culture of hydrocarbon-degrading organisms was investigated. The mixture or consortium of bacteria, denoted as AS2 consisted of 2 microorganisms. Microorganisms were *Actinomyces octodloyts, Saccharomyces cerevieace.* Microorganisms used in this study were from the Biotechnology and Genetic Engineering Research Unit (BGERU) Microbial Bank collection strains, at Taif University, KSA and were isolated from hydrocarbon-contaminated soil samples by enrichment technique on hydrocarbons as the sole carbon and energy source. The strains of the mixture were identified as *Actinomyces octodloyts, and Saccharomyces cerevieace,* by means of 16S-rRNA genetic method. The strains were selected based on the criteria that they were able to display good growth under complex mineral oil. Each of these strains demonstrated a strong ability to grow as a single strain on a hydrocarbon as sole carbon source. Their ability to degrade complex mineral oil hydrocarbon were monitored by gas chromatography (GC) for 6 days. The biodegradation percentage of mineral oil at 3% ml/L in liquid medium was 41.8% after 4 days of growth only. Potential biosurfactant production tested using the two methods named modified drop collapse (MDC) and blue agar plate (BAP) showed that the species are biosurfactant producers. Thus, these two isolates have potential to be useful for bioremediation of sites highly contaminated with petroleum hydrocarbons.

Introduction

Hydrocarbon degradation in soil by microorganisms has undergone renewed emphasis because of the increased incidence of petroleum-based pollution. Knowledge about hydrocarbon degradation is needed to determine how microorganisms might be utilized in the removal of the pollutants from the environment. Bioremediation is a modern method in which the natural ability of microorganisms is employed for the reduction of the concentration and/or toxicity of various chemical substances, such as petroleum derivatives, aliphatic and aromatic hydrocarbons, industrial solvents, pesticides and metals. The class

of petroleum products known as mineral oils can be generally understood to include a variety of products which go by different names such as white oils, lubricating oils, light fuel oils, residual fuel oils, as well as transformer and cable oils (Gary and Handwerk, 2001). Mineral oils refer to all oils which are made from dewaxed paraffin-based crude oils which are blended with additives to particular properties for specific uses (Aluyor and Ori-jesu 2009). Mineral oils are composed of straight and branched chain paraffinic, naphthenic, and aromatic hydrocarbons with 15 or more carbons in a complex mixture (Aluyor and Ori-jesu 2009).

Biological treatment most commonly involves the breakdown of contamination into nontoxic forms using microbiological processes (Lee et al., 1998). Therefore, bioremediation may be defined as the use of living organisms to remove environmental pollutants from soil, water and gases (Collin, 2001). The advantages of employing mixed cultures as opposed to pure cultures in bioremediation have been demonstrated. It could be attributed to the effects of synergistic effects among members of the consortium. Moreover, some substances can be decomposed only by cometabolism. The mechanisms in which petroleum degraders' benefit from synergistic relationships may be complex. It is possible that one species removes the toxic metabolites of the species preceding it. It is also possible that the second species are able to degrade compounds that the first are able to only partially degrade it (Alexander, 1999). Further research should be directed towards understanding the roles of individual members in influencing the effectiveness of a microbial consortium or mixer. Rambeloarisoa et. al. (1984) demonstrated a consortium of 8 strains made up of members of 6 genera to be able to effectively degrading crude oil.

The vast range of substrates and metabolites present in hydrocarbon impacted soils surely provides an environment for the development of a quite complex microbial community (Butier and Mason, 1997). Microbial populations that consist of strains that belong to various genera have been detected in petroleum-contaminated soil or water (Sorkhoh et al., 1995, Chikere et al.,2009). This strongly suggests that each strain or genera have their roles in the hydrocarbon transformation processes. More recently, microbial degradation was found to be an available alternative method over the conventional methods. Microbial treatment can control contamination of soils or water with crude oil, used or fresh petroleum products by reducing the length of the paraffin and oil molecules and by producing by-products that act as surfactants and paraffin and oil solvents. However, information on numbers and local species of microorganisms as well as their efficiency in degradation of complex mineral oil in Saudi Arabia is scarce. However, the biodegradation of these compounds using mixed cultures or microbial consortia isolated from contaminated sites with petroleum products has not been evaluated and these microorganisms are adapted to grow and thrive under environments with high concentrations of complex mineral oils, and the effects of environmental factors on the microbial growth are not known yet.

High molecular weight hydrocarbons are highly difficult to degrade. High molecular weight biosurfactants are highly efficient emulsifiers that work at low concentrations and exhibit considerable

substrate specificity, they are produced by a large number of bacteria and they are composed of polysaccharides, proteins, lipopolysaccharides, lipoproteins etc. (Banat et al., 2000, Zhang et al., 2012). In general microorganisms produce biosurfactants to increase their interfacial area for contact to give improved uptake of hydrophobic substrates. However, it has been observed that the exopolymers synthesized by these strains in media with glucose as carbon and energy source, had a remarkable capacity of emulsifying hydrocarbon compounds (Martinez-Checa et al. 2002, Zhang et al., 2012). 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 phylogenetic diversity of microbial communities can be tested by molecular methods, like fingerprinting or cloning and sequencing of PCR-amplified rRNA genes. These techniques have been employed to isolates and consortia enriched from natural environments. The 16S-rRNAmethod was also used to monitor the microbial diversity in environments and to follow transitions in community structure upon seasonal changes or along spatial gradients in salinity, temperature, and availability of substrates and minerals (Kleinsteuber et. al. 2006). The use of 16S- rRNA gene sequencing to examine genetic 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 recorded that suitable sequence differences in the 16S- rRNA gene could be used for bacterial identification (Sacchi et al. 2002) and for subtyping and identifying hyper virulent bacterial clones (Nilsson et al. 2003).

This work represents a continuation of our research in the area of hydrocarbon biodegradation technology. The present study aims to characterize isolates using 16S-rRNA gene technique, to produce biosurfactant and to degrade complex mineral oil as sole carbon substrate source in pure culture or mixed culture efficiently.

Materials and Methods

Microbial strains

Microorganisms were *Actinomyces octodloyts* AF104, *Saccharomyces cerevieace* AF203. Microorganisms 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 from mechanic workshops and gas stations contaminated soils by enrichment culture technique in our previous work in our laboratory. The two isolates showed good growth on Bushnell- Haas enrichment mineral medium(BHM) amended with hydrocarbon and were selected based on the growth and degradation ability.

Growth potential of hydrocarbon-utilizing microorganisms

Inocula were routinely grown in Luria–Bertani (LB) broth medium (g L^{-1}): peptone, 10.0; yeast extract, 5.0; NaCl, 5.0 (Miller, 2007). 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) containing (g/l): $MgSO_4$.7H2O, 0.2; K_2HPO_4, 1.0; KH_2PO_4, 1.0; $FeCl_3$, 0.05; NH_4NO_3, 1.0; $CaCl_2$, 0.02; pH 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. Flasks were amended with mineral oil 1, 3, and 5 % (v/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 on mineral oil was monitored by measuring the optical density (O.D.) at 595 nm in 2 ml cuvettes using a spectrophotometer (Biophotometer plus, Eppendorf).

The net dry weight for the 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. Mineral 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).

Biodegradation of hydrocarbons

Strains were incubated overnight in 50 ml LB broth medium in triplicate, pH 7.5 at 30^{o}C (shaken culture: 150rpm). Cells were centrifuged and washed twice with the liquid inorganic salts BHM. The pellet was suspended in 5ml BHM and inoculated into 250 ml of BHM in 3 flasks supplemented with 3 concentrations of mineral oil: 1, 3 and 5%(v/v) as sole carbon and energy sources.

Biodegradation Efficiency

Samples taken from the flasks were mixed with equal volumes of hexane and shaken to extract mineral oil. Residual mineral oil was monitored using the method used by Martinez-Checa et. al. (2002).

Biosurfactant production screening using the modified drop collapse method (MDC)

To prepare the assay, three plates were rinsed successively with hot water, 75% ethanol, distilled water and dried with air. After preparation, plates were equilibrated and coated with a thin layer of crude oil. The preparation was left for 24 h to ensure a uniform oil coating. Bacterial or yeast suspensions of all isolated strains were prepared and OD (595 nm) was adjusted to 0.8 for each strain. A volume of 0.5 μl of each organism suspension was transferred on the thin oil layer.The shape of the drop was inspected after 1 min; if the drop remained beaded, the result was scored as negative (-). If the drop collapsed, the result was scored as positive (+) (Bodour and Miller-Maier, 1998).

Biosurfactant production screening using the blue agar plate method (BAP)

This method shows an ionic biosurfactant producing strains by color reaction. Mineral salts agar medium (MSA) (Siegmund and Wagner, 1991) supplemented with carbon sources (glycerol), 2%, cetyltrimethylammonium bromide (CTAB) 0.5 mg/ml), methylene blue (0.2 mg/ml) were prepared. Three Plates was streaked with organism of interest and incubated at 30°C for 24 h. A dark blue halo around the culture was considered as positive for biosurfactant production. Sodium dodecyl sulfate (SDS), 1 mg/l and sterile distilled water were used respectively as positive and negative controls.

DNA extraction, PCR, and sequence analysis

DNA Extraction

The genomic DNA of *Actinomyces* sp. sample was extracted using a bacteria DNA Preparation Kit and Yeast 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'-CCGAATTCGTCGACAACAGAGTTTG ATCCTGG CTCAG-3') and reverse rD1 (5'-CCCGGGATCCAAGCTGGAGGT G ATCCAG CC-3') for *Actinomycetes* and primers P1 (ATCAATAAGCG GAGGAAAAG and P2 CTCTGGCTTCACCCTATTC for yeast as described by Ren et al. (2007). The PCR reaction mixture contained 10 *P*mol of each primer and 12.5 µl of 2xSuperHot 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 0.5ug/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 microorganisms.

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 of microbial candidates

Mixtures of organisms were obtained during enrichment using 0.1% yeast extract in BHM. Screening on an agar plate containing mineral oil resulted in isolation of several candidates from the contaminated sites. Two isolates of *Actinomyces*sp. and three isolates of *Saccharomyces* sp. were identified and characterized. Strains AF104 and AF203 were local isolates isolated by enrichment technique and deposited in our microbial bank at Taif University during our previous work (Shahaby and El-Tarras, 2011) in our laboratory. The 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 Apikit profiles (2009). Phenotypic examination of the recovered microorganisms revealed that they belong to the genera of *Actinomyces,* and *Saccharomyces*. Two isolates AF104 and AF203 showed good growthon BHM amended with complex mineral oil and were selected based on the growth and degradation ability. Strains AF104 and AF203 showed optimal growth at 30^{o}C.

The results of 16S-rDNA sequence alignment analysis revealed that 16S-rDNA sequence of strain AF104 and AF204 were more than 98% identical to that of *Actinomyces octodloyts, and Saccharomyces cerevieace,* respectively. *Psedomonas aeruginosa*, *Bacillus subtilis* and *Halomonaseurihalina* (moderately halophilic bacterium) species were effective bacteria in the biodegradation of heavy hydrocarbons (mineral 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 et al., 2013).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.

Nucleotide sequence accession numbers

The partial 16S-rRNA gene sequences that were determined have been deposited in the GenBank, EMBL, and DDBJ nucleotide sequence databases under accession no. NJ700209 for *Saccharomyces cerevieceae* AF203 and NJ700210 for *Actinomyces odontolyticus* AF104.

Growth rates and biomass of the microbial candidate's mixture

Optical density and biomass were determined simultaneously. The linear relation between OD_{595} and dry mass was obtained during growth on 1% of mineral oil. The specific growth rate and net dry weight of the two isolates were determined and illustrated in Table 1. These figures indicate the effluence of the specific growth rate and biomass precipitation on a period of bacterial cultivation in BHM containing 1 % (v/v) hydrocarbon. Little adaptation occurred at 5% mineral oil, indicating that the highest hydrocarbon exceeded the strains capability to adapt.Table (1) summarize the results of growth rates, and biomass content during

growth at 1% (v/v) concentration of complex mineral oil for a week under laboratory conditions. The specific growth rates of the two isolates on mineral oil showed that strain AF203 was faster than strain AF104in growth in mineral salts medium BHM containing 1 % (v/v) hydrocarbon. Most growth occurred in the first2 days for the two strains resulting in good biomass production (Table 1). Maximum specific growth rates (μ_{max}) for AF104 and AF203 strains were occurred after two and three days of growth being 0.073 μ h^{-1} and 0.064 μ h^{-1}, respectively. Strains were also grown on 3% and 5% hydrocarbon (data not shown). However, growth on 1% hydrocarbon was better than 3 and 5 % (v/v) concentrations. Moreover, isolate AF203 produced more biomass from hydrocarbon being 3.85 g cells l^{-1}mineral oil hydrocarbon after six days of growth. No change was observed in pH during the first seven days of incubation for strains.

The reduction in heavy hydrocarbon fractions by biodegradation of paraffinic hydrocarbons using *Pseudomonas* and *Actinomyces* species was noticed (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).

Degradation capacity of hydrocarbon by isolates

The hydrocarbon degradation capacity of selected isolates in BHM supplemented with complex mineral oil are illustrated in Fig. 1-3. No evaporation of hydrocarbon from the screw-capped flasks was observed throughout the experiments. All isolates showed the ability to utilize mineral oil as sole carbon source however, the efficiency of utilization varied among strains, with AF203 being the best (40.2% removal within 5 days) at 5% hydrocarbon concentration.AF104 was able to degrade mineral oil by more than 38.3%, whereas the mixture of AF104 and AF203 named (AS2) could degrade about 41.8% after 4 days only at 3% concentration of hydrocarbon. Two reference strains *Actinomyces*, *Saccharomyces* used as negative control could only degrade approximately 9 and 7% of mineral oil, respectively under the same conditions (data not shown). The results on the assessment for degradation capacity were similar to those of growth (Fig. 1). In mineral oil 5% -supplemented BHM, a small degree of degradation was observed only in cases of using consortium of AF104 and AF203 strains (AS2) being 32.3 % after 2 days only. The consortium degrades 39.4% of hydrocarbon after 2 days only at concentration 3%. Mineral oil was utilized to the extent of 38.3% for AF104 after 6 days at 5% concentration, 40.2% after 5 days at 5% concentration and 39.4% for consortium of both AF104 and AF203 (AS2) after 2 days only of growth. Strain AF203 was more efficient than strain AF104 being 40.2% and 38.3% at 5% concentration, respectively. One might thus expect that incubation at 3% or 5% hydrocarbon beyond6 days would have resulted in even more mineral oil degradation than the 41.8% observed. Interestingly, CFU and total cell counts at 3% and 5% concentration were even higher than those in the maximally active incubation at 1% hydrocarbon concentration (data not shown). This indicates reduced hydrocarbon degradation per cell at the higher mineral oil concentration, which goes along with lower protein content per cell. The maximally active incubation at 1%

hydrocarbon preserved the highest metabolic versatility. Given that the two strains were capable of strong growth in both solid and liquid media at 30°C and 3% or 5% of hydrocarbon as sole carbon source, we tested the performance of each of the two strains separately via rotary shaker under these conditions Table (2) and Fig. 1-3.However, these percentages also demonstrated a moderate level of mineral oil biodegradability by each of these two strains performed separately at 5% concentration.

Zhao et al. (2011) selected consortium by positive end dilution method. The consortium consisted of *Rhizobiales* sp., *Pseudomonas* sp., *Brucella* sp., *Bacillus* sp., *Rhodococcus* sp., *Microbacterium* sp. and *Roseomonas* sp. and removed nearly 52.1% of crude oil at initial concentration of 10,000 mg l^{-1} at 30 °C within 7 days. The strains *Pseudomonas* sp., *Brucella* sp. *and Rhodococcus* sp. likely played a key role in the crude oil degradation(Abdel-Megeed et al., 2012).The mixed populations of *Pseudomonas*, *Rhodococcus*and *Bacillus* are capable of degrading mineral oil of up to 120 ppm. However, biodegradation ofmineral oil was fast by the mixed culture comparing to the biodegradtion of each strain separately.

Lazar et al. (1999), Pokethitiyook et al. (2002), and Sadeghazad and Ghaemi (2003) reported that, *Pseudomonas* and *Bacilli* species were the most effective microorganisms in the biodegradation of heavy hydrocarbons. Lazar et al. (1999) also, stated that microorganisms involved with the microbial treatment of crude oil, are generally live, naturally occurring, and are mainly facultative anaerobic, pathogenic, contain no sulphate-reducing bacteria or slime-forming bacteria and are environmentally safe. On the other hand, Saadoun (1997) determined ability of *Rhodococcus erythropolis* to degrade mineral oil and other hydrocarbons by using a Warburg constant volume respirometer. Results of oxygen uptake indicated that hexane and tetrade cane were more degradable than mineral oil and decane. *R. erythropolis* exhibited highest QO_2 values (2.9 and 2.8) when exposed to tetradecane and hexane, respectively. Mineral oil and decane were degraded more slowly with QO_2 values 0.87 and 0.94, respectively. Alvarez et al. (2011) evaluated the effectiveness of monitored natural attenuation, bioenrichment, and bioaugmentation using a consortium of three actinomycetes strains in remediating two distinct typical Brazilian soils that were contaminated with crude oil, with or without the addition of NaCl. Microcosms were used to simulate bioremediation treatments over a 120-day period. During this period, they monitored total petroleum hydrocarbons (TPHs) and n-alkanes degradation and changes in bacterial communities. Over time, they found the degradation rate of n-alkanes was higher than TPH in both soils, independent of the treatment used. Suggesting that the total bacterial community in the soils was mainly affected by the experimental period of time, while the type of bioremediation treatment used was the main factor influencing the actinomycetes populations in both soils.Growth on mineral oil 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.,1986,1987) or methylcyclohexane for bacteria able to grow on a wide range of alicyclic compounds (Trudgill, 1984).The reduction in heavy hydrocarbon fractions by biodegradation of paraffinic hydrocarbons using *Pseudomonas* and

Table.1 Growth rates, biomass yield and biosurfactant production (using MDC and BAP techniques) of two strains AF104and AF203grown in Bushnel-Hass medium amended with 1% of mineral oil hydrocarbon.

Strains	Biomass yield (g cells /Lmineral oil)	Growth rate (μ) (h^{-1})	Biosurfactant production
Actinomyces(AF104*)*	3.58	0.071	+
Sacchromyces(AF203)	3.85	0.062	+

Table.2 Biodegradation performance of complex mineral oil hydrocarbon by aconsortium of *Actinomyces* sp.AF104 and *Saccharomyces*sp.AF203 (AS2)

Culture Name	Hydrocarbon concentration%	Incubation time (days)	Biodegradation %
*Sacharomyces*sp.	3	6	39.5
*Actinomycets*sp.	5	6	38.3
Mixture of *Actinomyces*sp. and *Saccharomyces* sp. (AS2)	3	4	41.8

Fig.1 Performance of *Actinomyces*AF104 in various concentration of hydrocarbon and incubation time at 30 ^{o}C. M. O., mineral oil content.

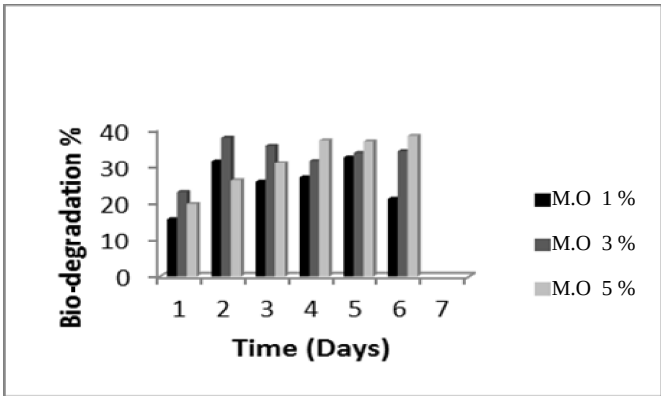


Fig.2 Performance of *Saccharomyces* AF203 in various concentration of hydrocarbon and incubation time at 30 ^{o}C. M. O., mineral oil content.

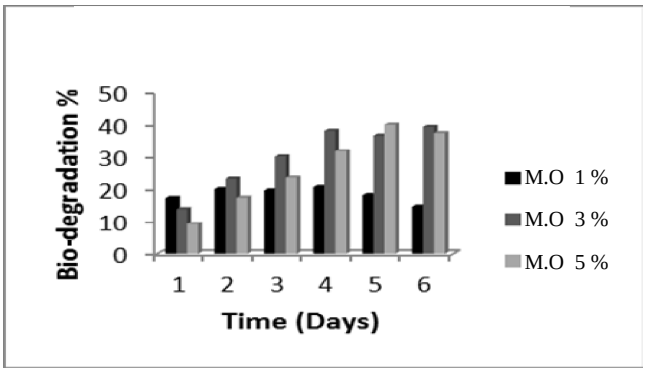
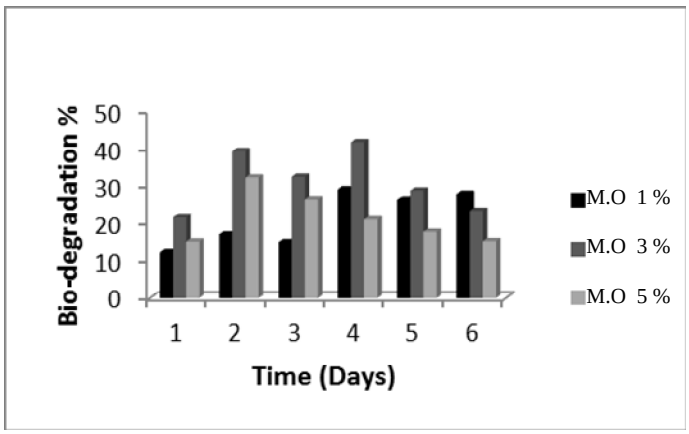


Fig.3 Performance of *Actinomyces*AF104 and *Saccharomyces* AF203 consortium (AS2) under various concentration of hydrocarbon and incubation time at 30^{o}C. M. O., mineral oil content



Actinomyces species was noticed (Etoumi, 2007). It was mentioned that the lower the concentration of hydrocarbons the higher was the utilization (El_Tarras et al., 2012, Shahaby et. al., 2013). Biodegradation would be required if the contaminated site lacked proper microorganisms (Kauppi et al., 2011). Therefore, isolation of pure microbial strains or enrichment of microbial consortium from contaminated sites for biodegradation of hydrocarbons is considered as an approach to provide inocula for bioaugmentation (Liu et al., 2011 and Kauppi et al., 2011). Due to microbial complexity and diversity, a microbial consortium could work better and more stable than pure culture for the bioremediation of crude oil-contaminated soil. Mainly because that crude oil is a complex mixture consisting of aliphatics, aromatics, resins and asphaltenes (van Hamme et al., 2003 and Malina and Zawierucha, 2007). Although, a crude oil-degrading consortium could be made up by combining a number of individual microbial strains, the bioremediation performance of the combined bacteria usually is not satisfactory (Komukai-Nakamura et al., 1996 and Ko and Lebeault, 1999). Demonstrated a consortium of 8 strains made up of members of 6 genera to be able to effectively degrading crude oil (Rambeloarisoa et al. 1984). Interestingly, only 5 of these strains were able to grow in pure cultures using a variety of hydrocarbons. However, when the other 3 strains were removed from the consortium, the effectiveness of the mixed culture was remarkably reduced. These further support the theory that each member in a microbial community has a significant role and may need to depend on the presence of other species or strains to be able to survive

Production of biosurfactant

Biosurfactant 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).Using the two qualitative methods (MDC and BAP), results demonstrated that *Actinomyces* sp. AF104 and *Saccharomyces* sp.AF203 strains used in this study were biosurfactant producers (Table 1).

This can explain the mineral oil degradation performance obtained with these two strains. Knowing that biodegradation depends strongly on hydrocarbon emulsion, the use of biosurfactant producer strains like *Actinomyces*, and *Saccharomyces* species in bioremediation technology seems to offer more potential than chemical surfactant, due to their structural diversity, biodegradability and biocompatibility relative to synthetic surfactant (Abalos et al., 2004, Amaral et al., 2010a, 2010b, Konishi, 2010, Shubhrasekhar et al., 2013). Some yeasts are preferred to bacteria as sources for biosurfactants, mainly due to their GRAS status for environmental and health safety reasons (Campos-Takaki et al., 2010).These results mean that the bacterial consortium obtained is capable of using the complex mineral oil as a carbon and energy source under these conditions.

In conclusion complex mineral oil hydrocarbons are highly difficult to degrade. A functional bacterial consortium consisting of *Actinomyces* and *Saccharomyces* was selected based on the growth and degradation ability. Strains AF104 and AF203 showed optimal growth at 30^{o}C.The results mean that the bacterial consortium obtained is capable of using the complex mineral oil as a carbon and energy source in different environmental conditions. After 4 days incubation, this consortium removed nearly 42% of 3% hydrocarbon content. The strains *Actinomyces* sp. and *Saccharomyces* sp. likely played a key role in the mineral oil degradation. The study of complex mineral oil degradation demonstrated that the selected functional consortium significantly enhanced the mineral oil removal efficiency up to 38–42% over a 6 day period in comparison with 7- 9% in the controls. The amount of mineral oil removed vs. mineral oil content suggested probable first order reaction kinetics for the mineral oil removal *in vitro* and higher rate was achieved with bioremediation.In general, microorganisms produce biosurfactants to increase their interfacial area for contact to give improved uptake of hydrophobic substrates. Both microorganisms of consortium AS2 produced biosurfactants and this improved breakdown of complex mineral oil hydrocarbon. The highest rate of hydrocarbon degradation occurred when member of consortium are biosurfecant producers. These two strains were able to produce biosurfactants. Therefore, the main phase of mineral oil removal by the two strains was achieved in the first four days of treatment. These percentages also demonstrated a moderate level of mineral oil biodegradability by each of these two strains performed separately. However, the effect of different environmental factors onthe growth of the stabilized microbial consortium might affect the performance of the consortium.The results revealed the possibility touse these microbes for the reduction of complex mineral oil in ecosystems where they accumulate and cause pollution problems. Furthermore, results indicated that the microbial consortium AS2 had a promising application in bioremediation of oil-contaminated environments and could be potentially used in microbial enhanced oil recovery (MEOR).

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