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**RESEARCH ARTICLE**

# Using of biosurfactant and bacterial enzymes for treatment of the oily polluted soils

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*Abstract*

Biosurfactants are valuable microbial amphiphilic molecules (consisting of molecules having a polar water-soluble group attached to a water-insoluble hydrocarbon chain) with effective surface-active and biological properties applicable to several industries and processes. In recent years natural biosurfactants had attracted attention because of their low toxicity, biodegradability, and ecological acceptability. This work includes eighteen spore-former isolates belong to *Bacillus* genus that were isolated from twenty five samples of oily polluted soil. Eight strains were showed the ability to produced biosurfactants by spotting over the blood agar plates, and six isolates specifically 15ASA was showed a good lipase and biosurfactant producing, when grown in mineral salt medium at 30°C, with lower surface tension of water from 72 to 32.25mN/m. The best results were obtained when using olive oil and sodium nitrate as carbon and nitrogen sources respectively. Emulsifying index E24 was done and showed that benzene and olive oil were the best substrates (59.2%), while engine oil, vegetable oil and crude oil were less good substrates for emulsification (55.5, 55.5 and 37.03)% respectively. Test of displacing the oil (engine oil, vegetable oil, benzene, olive oil and crude oil) showed that zones of displacement with (30, 40, 35, 15 and 25)mm respectively. Results showed that 70% of the initial concentration of engine oil (1gm) was degraded after 72h of incubation

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## INTRODUCTION

Produced water is an industry challenge which affects the whole production process. The produced water handling system is a key part of most production facilities. If the discharge requirements are not met, there may be a significant environmental and economic impact. Furthermore, produced water can take up valuable space in the oil transport lines, reducing the amount of oil that can be produced.

Microorganisms utilize a variety of organic compounds as the source of carbon and energy for their growth. When the carbon source is an insoluble substrate like a hydrocarbon (C**x**H**y**) microorganisms facilitate their diffusion into the cell by producing a variety of substances, like biosurfactants. Some bacteria excrete ionic surfactant, which emulsify hydrocarbon substrates in the growth medium, like rhamnolipids which produced by different *Pseudomonas* (1), or the sophorolipids produced by several *Torulopsis* sp. (2). Other microorganisms are capable of changing the structure of their cell wall, by synthesising lipopolysaccharides or non-ionic surfactants in their cell wall. Example of this group are: *Candida lipolytica* and *Candida tropicalis* which produce wall-bound lipopolysaccharides when growing on *n*-alkanes (3), and *Rhodococcus erythropolis* and many *Mycobacterium* sp., which synthesise non-ionic trehalose corynomycolates (4). There are lipopolysaccharides, such as emulsan, synthesised by *Acinetobacter* sp. (5), and lipoproteins, such as surfactin and subtilisin, produced by *Bacillus subtilis* (6).

Surfactants are surface active agents with wide ranging properties including the lowering of surface and interfacial tensions of liquids. Surface tension is defined as the free surface enthalpy per unit area and is the force acting on the surface of a liquid leading to minimization of the area of that surface. Both synthetic and natural surfactants exist capable of reducing the surface tension of water from 72mN/m to around 27mN/m (7). Microbial surfactants or biosurfactants are surface-active amphipathic molecules produced by a number of microorganisms. They occur in nature as a diverse group of molecules comprising of glycolipids, lipopeptides and lipoproteins, fatty acids, neutral lipids, phospholipids, polymeric and particulate biosurfactants (8 and 9). Beside their regular roles in enhanced oil recovery, bioremediation and industrial emulsification, in recent years, microbial surfactants have been found to possess several properties of therapeutic and biomedical importance, e.g. antibacterial, antifungal and antiviral properties. They also have anti adhesive action against several pathogenic microorganisms (10, 11 and 12).

Lipids (fats, oils and greases) are major organic matters in municipal and industrial wastewater and can cause severe environmental pollution. Wastewater produced from edible oil refinery, slaughter house, wool scouring and dairy products industry contains a high (> 100mg/L) concentration of lipids (13 and 14). High concentration of these compounds in wastewater often causes major problems in biological wastewater treatment processes. Because of their nature they form a layer on water surfaces and decrease oxygen transfer rate into the aerobic process (15).

Oils and fats are part of a group of compounds known as fatty esters or triglycerides, and their hydrolysis essentially involves reactions with water to produce valuable free fatty acids and glycerol. Enzymatic hydrolysis of triglycerides may be carried out at ambient conditions. In this technology an aqueous solution of lipase is contacted with the oil, forming a liquid – liquid dispersion. The lipase is enzyme that specifically catalyze the hydrolysis of the oil into free fatty acid and glycerol at the interface between the two liquids. The hydrolysis reaction yields 1mole of glycerol and 3moles of fatty acids per mole of triglycerides. Triglycerides here on called "lipids", do not dissolve in the water phase, so the reaction has to take place at the interface of the water and lipid phase (16).

The research goal to isolate biosurfactant and lipase producing strains from environmental pollutant oil waste, and evaluate their activity at laboratory.

## Materials and Methods

### Isolation of bacteria

Fifteen samples of oily polluted soil were heat-treated (80ºC for 10min) to kill all vegetative cells and individually placed on nutrient agar plates. After 24hr of incubation at 30ºC, colonies were recovered and purified by streaking on fresh nutrient agar.

### Hemolytic activity

For determining the hemolytic activity, the blood agar was prepared by adding 5% of human blood to blood agar base medium. Then isolates were spot inoculated and incubated at 37ºC for 3 days. The plates were observed for the zone of clearance around the colonies (17).

### Lipase detection

Plate assay for the lipase activity was determined according to the method of Samad *et al*., (1989) (18) with a slight modification. Briefly, the agar plates comprised of 2% tween 80, 2.5% agar and 0.5% methyl red, were made ready and wells were cut. Then 20µl of cell free culture supernatant was added to the well cut over the agar plate and incubated overnight at room temperature. The plates were then observed for the zone of clearance around the well. The sterilize distilled water was used as negative control.

### Screening assays for biosurfactant production

10 % of each isolates were inoculated and grew aerobically in mineral salt medium (MSM) for 7 days, at 30ºC and stirred at 150rpm, composition (gm/l) : $Na_2HPO_4$ (2.2), $KH_2PO_4$ (1.4), $MgSO_4.7H_2O$ (0.6), $FeSO_4.7H_2O$ (0.01), NaCl (0.05), $CaCl_2$ (0.02), yeast extract (0.02) and (0.1)ml of trace element solution containing (gm/1): (2.32)gm $ZnSO_4.7H_2O$, (1.78)gm $MnSO_4.4H_2O$, (0.56)gm $H_3BO_3$, (1.0)gm $CuSO_4.5H_2O$, (0.39)gm $Na_2MoO_4.2H_2O$, (0.42)gm $CoCl_2.6H_2O$, (1.0)gm EDTA, (0.004)gm $NiCl_2.6H_2O$ and (0.66)gm KI. pH of the medium was adjusted to 7.0 ± 0.2. Carbon and nitrogen sources were added separately. The carbon source used was olive oil (2% w/v), with $NaNO_3$ (1gm/l) as nitrogen source (19). Then surface tension measurement were determined on cell free supernatant obtained by centrifuging the cultures at 6000rpm for 30min.

### Oil displacement test

Petri plate (150mm diameter) was filled with 40ml of distilled water, and added 15µl of weathered hydrocarbons, such as (engine oil, vegetable oil, benzene, olive oil and crude oil). Then 10µl of cell free culture supernatant was

carefully placed on the centre of the oil film. After 30sec of incubation the diameter of clear halo zone was measured (20).

**Emulsification assay (E24)**

E24 of isolate was determined by adding 2ml of cell free culture supernatant to 2ml of hydrocarbons including (engine oil, vegetable oil, benzene, olive oil and crude oil) were mixed well with a vortex for 2min and leaving to stand for 24hr. The E24 index is given as percentage of height of emulsified layer (mm) divided by total height of the liquid column (mm) (21).

**Extraction of bacterial biosurfactants**

After 7days of cultivation, bacterial cells were removed from culture broth (in mineral salt medium) by centrifugation at 5000rpm for 10min. Supernatant of bacterial isolate was acidified to pH 2.0 with 1N HCl and placed in a refrigerator at 4ºC overnight. The pellets were obtained by centrifugation at 7000rpm for 10min, dissolved in a lot of distilled water, then the pH was adjusted to 7.0 with 2M NaOH. The crude biosurfactant was extracted as recommended (22).

**Extraction of lipase**

Bacterial culture (2%) was inoculated in medium (peptone 0.3%, yeast extract 0.1%, sodium chloride 0.05%, tween 80 0.5% and distilled water 100ml) (23). The components were adjusted to pH 7.0 before autoclaving at 121°C for 15min and incubated at 30°C for up to 72h, the culture was centrifuged at 6000rpm for 30min and the supernatant (crude enzyme ) was collected.

**Biodegradability test**

Biodegradability test performed in 500ml elementary flasks containing mineral salt medium and 1gm of engine oil, then added 50ml crude biosurfactant and 50ml crude lipase, incubated in shaker incubator at 150rpm for 72h. After end of incubation period, the media centrifuged at 10000rpm for 15min to remove the cells. The filtrate transformed into 250ml separating funnel and extracted by addition 50ml of hexane (24). The mixture (hexane and oil) was mixed thoroughly and left to dry. The final weight of flask was noted and the difference between the final and the tare weight represent the mass of weathered oil recovered in the sample.

## Results and Discussion

**Collection of samples**

Samples of oily pollutant soil were heat-treated to kill all vegetative cells and incubated on a solid medium to allow germination and growth of heat-resistant spores. Analysis of pure colonies based on morphological characteristics and checked by microscopy (gram staining and spore staining). From a total of fifteen samples of soil, eighteen spore-former isolates were isolated.

**Blood agar hemolysis**

Initially, spore-former isolates (*Bacillus* genus) were screened for its ability to produce biosurfactants by spoting over the blood agar plate (Table 1). Eight strains were showed a significant zone of clearance around the colony, confirming the production of surface active molecules (Fig 1).

Table (1) Effect of *Bacillus* strains in blood hemolysis on blood agar plates

| Strains | Blood hemolysis |
|---|---|
| 1 ASA | +++ |
| 2 ASA | - |
| 3 ASA | - |
| 4 ASA | - |
| 5 ASA | +++ |
| 6 ASA | - |
| 7 ASA | + |
| 8 ASA | - |
| 9 ASA | - |

| | |
|---|---|
| 10 ASA | ++ |
| 11 ASA | + |
| 12 ASA | - |
| 13 ASA | - |
| 14 ASA | - |
| 15 ASA | ++ |
| 16 ASA | ++ |
| 17 ASA | - |
| 18 ASA | ++ |

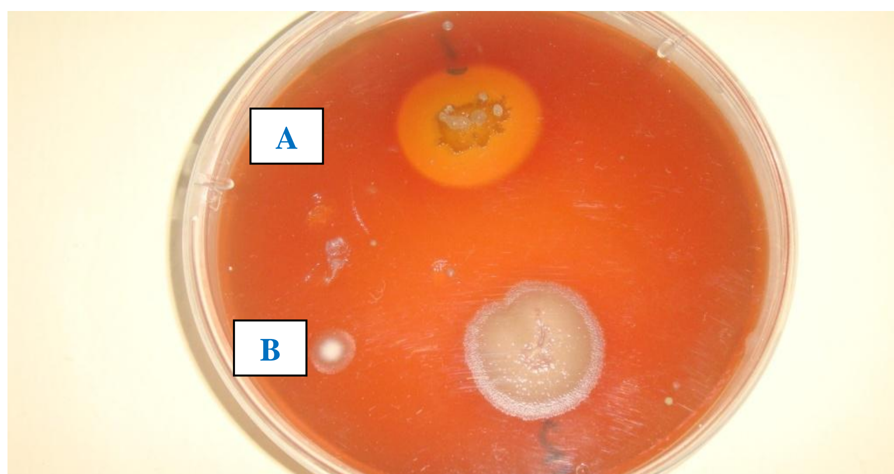

Figure (1) Blood hemolysis (A) positive result (B) negative result

Lysis of blood agar has been recommended as a method to screen for biosurfactant activity. This method is useful in predicting the promising strains regarding biosurfactant production, since, in most cases, the degree of lysis of red blood cells is directly proportional to the concentration of biosurfactant production. However, it has not been determined that all kinds of biosurfactants have a hemolytic activity and microorganisms may produce chemicals other than biosurfactants that can cause hemolysis (25).

**Lypolytic activity**

Six strains of *Bacillus* genus were produced lipase (Table 2). In fact, the lipase producing microorganisms is found and degrading fat and oil pollutant sources, such as wastewater (Fig 2). So, nutritional requirements of lipolytic bacteria could be usefully applied in developing strategies for bioremediation of fat pollutant aqueous systems (26).

Table (2) Efficiency of *Bacillus* genus in lipase production

| Strains | Lipase production |
|---|---|
| 1 ASA | + |
| 5 ASA | + |
| 7 ASA | + |
| 10 ASA | - |
| 11 ASA | - |
| 15 ASA | + |
| 16 ASA | + |
| 18 ASA | + |

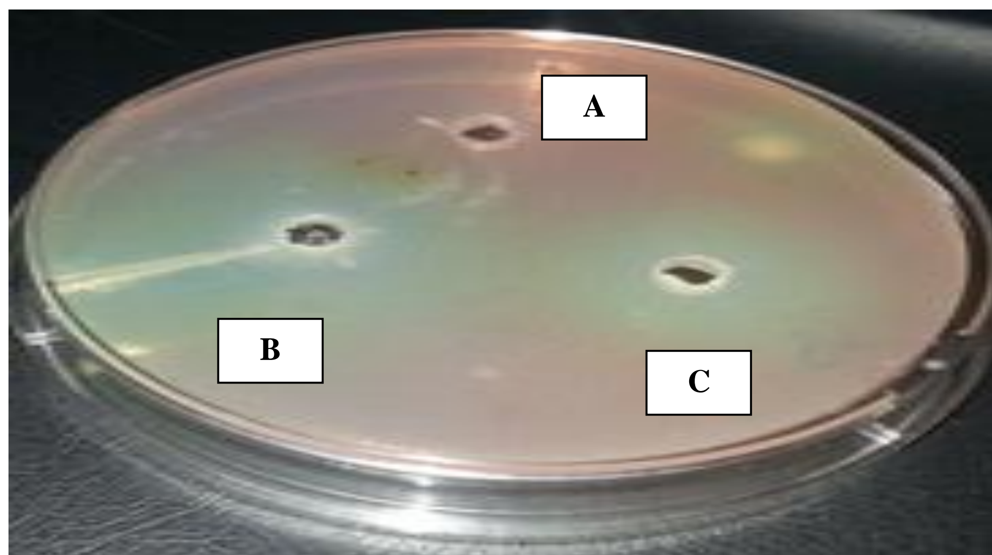

Figure (2) Screening of lipolytic activity (A) negative result (B) and (C) positive results

**Biosurfactant production**

Cell-free culture of strain 15ASA grown in liquid medium at 30ºC, showed a good biosurfactant lowering surface tension of water from 72 to 32.25mN/m compared to others (Fig 3). Surface tension measurement would be the choice of method to quantify biosurfactant production. However, it gave an indirect relationship to the concentration of biosurfactant available in the liquid. Reduction of surface tension was indicative to the production of biosurfactant by the microbe. The higher reduction of surface tension would lead to the high concentration of biosurfactant available (27).

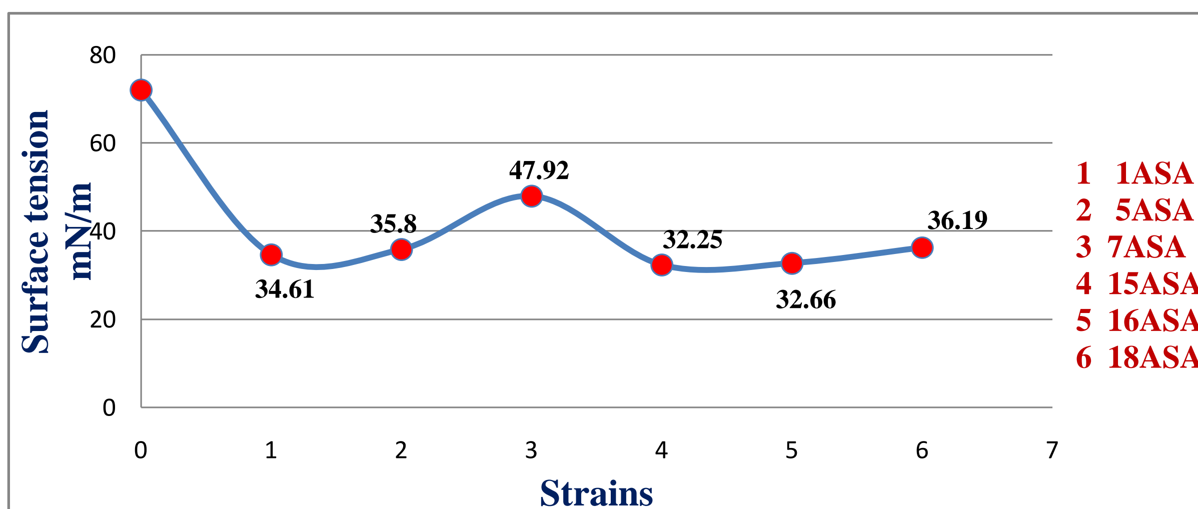

Figure (3) Surface tension of *Bacillus* strains

**Oil spreading technique**

The supernatant of 15 ASA strain was added to the plates containing water and oil (engine oil, vegetable oil, benzene, olive oil and crude oil). The strain displaced the oil showing a zone of displacement with (30, 40, 35, 15 and 25)mm. respectively. According to (20), the amount of biosurfactant necessary to obtain a clear detectable zone

over an oil layer is called as the minimum active dose (MAD) of the corresponding biosurfactant.The results obtained were noted down (Fig 4).

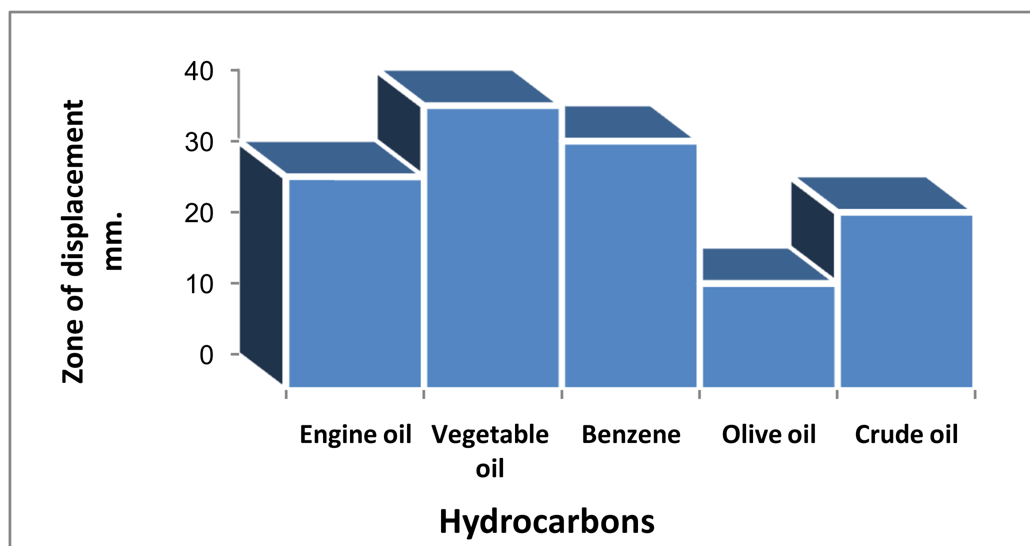

Figure (4) Oil spreading technique of biosurfactant against different hydrocarbons

**Emulsification stability (E24)**

15 ASA strain have the ability of emulsifying (E24) oil (engine oil, vegetable oil, benzene, olive oil and crude oil) were showed that stabilization of oil and water emulsion was commonly used as a surface activity indicator (28). As shown in (Fig 5), all hydrocarbons tested served as substrates for emulsification by the biosurfactant. Benzene and olive oil were the best substrates with (59.2%). Engine oil, vegetable oil and crude oil were less good substrates for emulsification with (55.5, 55.5 and 37.03)% respectively.

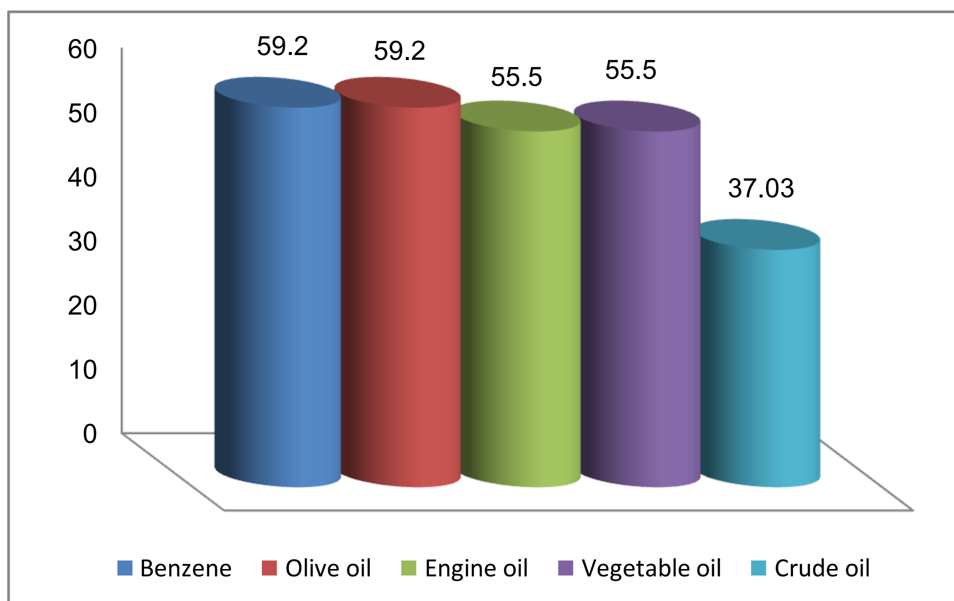

Figure (5) Emulsification activity (E24) of biosurfactant against different hydrocarbons

**Application of biosurfactant and lipase**

At first, we evaluated the degradation capabilities of the engine oil by *Bacillus* strain, by cultivating it on MSM containing crude of biosurfactant and lipase, supplemented with 1gm of engine oil as the sole carbon source. The evolution of the growth distinguished by brake down large molecules of oil into small droplets in medium culture, this indicates the production of bioemulsifier during the degradation process. After 72h of incubation the results showed that 70% of the initial concentration of engine oil (1gm) was degraded. Addition of crud biosurfactant and lipase to the production media stimulate the ability of the isolate to emulsify and degrade engine oil.

## References


1. Guerra-Santos, L.; Kappeli, O. and Fiechter, A. (1986). Dependence of *Pseudomonas aeruginosa* continuous culture biosurfactant production on nutritional and environmental factors. Applied Microbiology and Biotechnology, 24, pp: 443-448.
2. Cooper, D. G. and Paddock, D. A. (1983). *Torulopsis petrophilum* and surface activity. Applied and Environmental Microbiology, 46, pp: 1426-1429.
3. Fukui, S. and Tanaka, A. (1981). Metabolism of alkanes by yeasts. Advances in Biochemical Engineering, 19, pp: 217-237.
4. Ristau, E. and Wagner, F. (1983). Formation of novel anionic trehalosetetraesters from *Rhodococcus erythropolis* under growth limiting conditions. Biotechnology Letters, 5, pp: 95-100.
5. Rosenberg, E.; Zuckerberg, A.; Rubinovitz, C. and Gutnick, D. L. (1979). Emulsifier of *Arthrobacter* RAG-1: isolation and emulsifying properties. Applied and Environmental Microbiology, 37, pp: 402-408.
6. Cooper, D. G.; Macdonald, C. R.; Duff, J. P. and Kosaric, N. (1981). Enhanced production of surfactin from *Bacillus subtilis* by continuous product removal and metal cation additions. Applied and Environmental Microbiology, 42, pp: 408-412.
7. Christofi, N. and Ivshina, I. B. (2002). Microbial surfactants and their use in field studies of soil remediation. Journal of Applied Microbiology, 93, pp: 915–929.
8. Desai, J. D. and Banat, I. M. (1997). Microbial production of surfactants and their commercial potential. Microbiol. Mol. Biol. Rev., 61, pp: 47-64.
9. Mukherjee, S.; Das, P. and Sen, R. (2006). Towards commercial production of microbial surfactants. Trends Biotechnol., 24, pp: 509-515.
10. Cameotra, S. S. and Makkar, R. S. (2004). Recent applications of biosurfactants as biological and immunological molecules. Curr. Opin. Microbiol., 7, pp: 262-266.
11. Singh, P. and Cameotra, S. S. (2004). Potential applications of microbial surfactants in biomedical sciences. Trends Biotechnol., 22, pp: 142-146.
12. Rodrigues, L. ; Banat, I. M. ; Teixeira, J. and Oliveira, R. (2006). Biosurfactants: potential applications in medicine. J. Antimicrob. Chemother., 57, pp: 609-618.
13. Wakelin, N. G. and Forster, C. F. (1997). An investigation into microbial removal of fats, oils and grease. Bioresour. Technol., 59, pp: 37-43.
14. Lefebvre, X. ; Paul, E. ; Mauret, M. ; Baptiste, P. and Capdeville, B. (1998). Kinetic characterization of saponified domestic lipid residues by aerobic biodegradation. Water Res., 32, pp: 3031-3038.

**15.** Becker, P. ; Koster, D. ; Popov, M. N. ; Markossian, S. ; Antranikian, G. and Markl, H. (1999). The biodegradation of olive oil and treatment of lipid-rich wool wastewater under aerobic thermophilic condition. Water Res., 33, pp: 653-660.

**16.** Pronk, W. ; Kerkhof, P. J. A. ; Helden van, C. and Van't Reit, K. (1988). The hydrolysis of triglycerides by immobilized lipase in a hydrophilic membrane reactor. Biotechnol. Bioeng., 32, pp: 512-518.

**17.** Carillo, P. ; Mardarz, C. and Pitta-Alvarez, S. (1996). World J. Microbiol. Biotechnol., 12, pp: 82.

**18.** Samad, M. Y. A. ; Razak, C. N. A. ; Salleh, A. B. ; Yunus, Z. W. ; Ampon, K. and Basri, M. (1989). J. Microbiol. Methods., 9, pp: 51.

**19.** Abouseoud, M. ; Maachi, R. and Amrane, A. (2007). Biosurfactant Production from olive oil by *Pseudomonas fluorescens*. Communicating Current Research and Educational Topics and Trends in Applied Microbiology.

**20.** Sriram, M. I. ; Kalishwaralal, K. ; Deepak, V. ; Gracerosepat, R. ; Srisakthi, K. and Gurunathan, S. (2011). Biofilm inhibition and antimicrobial action of lipopeptide biosurfactant produced by heavy metal tolerant strain Bacillus cereus NK1. Colloids and Surfaces B: Biointerfaces, 85, pp: 174-181.

**21.** Sarubbo, L. A. (2006). Production and stability studies of the bioemulsifier obtained from a strain of the *Candida glabrata* UCP 1002. J. Biotechnol., 9, pp: 400-406.

**22.** Haddad, N. I. A. ; JI, W. and Bozhong, M. (2009). Identification of a biosurfactant producing strain: *Bacillus subtilis* HOB2. Protein & Peptide Letters, 16, pp: 7-13.

**23.** Rahman, R. N. Z. R. A. ; Leow, T. C. ; Salleh, A. B. and Basri, M. (2007). *Geobacillus zalihae* sp. Nov., a thermophilic lipolytic bacterium isolated from palm oil mill effluent in Malaysia. BMC Microbiology, 7, pp: 77.

**24.** Ho, J. and Rashid, M. (2008). Application of Ez-enzyme in bioremediation of oily sludge. J. of Technology, 4, pp: 21-32.

**25.** Youssef, N. ; Duncan, K. E. ; Nagle, D. P. ; Kristen, N. S. ; Roy, M. K. and Michael, J. M. (2004). Comparison of methods to detect biosurfactant production by diverse microorganisms. Journal of Microbiological Methods, 56, pp: 339-347.

**26.** Zeng, X. ; Xiang, X. ; Peng, W. and Fengping, W. (2004). Screening and characterization of psychrotrophic, lipolytic bacteria from deep-sea sediments. J. Microbiol. Biotechnol., 14, pp: 952-958.

**27.** Makkar, R. S. and Cameotra, S.S. (1997). Biosurfactant production by a thermophilic *Bacillus subtilis* strain. Journal of Industrial Microbiology and Biotechnology, 18, pp: 37-42.

**28.** Cooper, D. G. and Goldenberg, B.G. (1987). Surface-active agents from two *Bacillus* species. Appl. Environ. Microbiol., 53 (2), pp: 224–229.