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The Role of Exopolysaccharide, Biosurfactant and Peroxidase Enzymes on Toluene Degradation by Bacteria Isolated From Marine and Wastewater Environments

Afrouzossadat Hosseini Abari [1], Giti Emtiazi [1*], Seyed Mahdi Ghasemi [1]

[1] Department of Biology, Faculty of Science, University of Isfahan, Isfahan, IR Iran

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ABSTRACT

Background: Toluene which widely exists in petroleum and its related products has gathered much attention due to its adverse effects on health and carcinogenic potential. Since microorganisms are able to utilize petroleum hydrocarbon as carbon and energy sources, they can be used for bioremediation applications.

Objectives: The aim of this study was to isolate toluene degrading bacteria from wastewater and seawater. The production of exopolysaccharide, biosurfactant and peroxidase enzymes such as laccase and catalase were investigated to determine the effect of them on toluene degradation.

Materials and Methods: To screen and isolate toluene degrading bacteria, contaminated seawater and wastewater samples were added to toluene containing mineral media (MM). The biochemical and molecular characteristics of the isolates were then studied.

Results: From seawater, two toluene degrading *Bacillus* and one *Sporosacina* species and from wastewater a novel high capable toluene degrading strain, Bacterium Ex-DG74 were isolated and introduced. *Bacterium Ex-DG74* showed tolerance to 15 % (v/v) toluene but the marine isolated species could tolerate only 1 % (v/v) toluene. This bacterium also showed the highest catalase and membrane-bound laccase activity. The spore-forming marine bacterium, *S. halophila* produced large amounts of exopolysaccharide, biosurfactant and extracellular laccase.

Conclusions: The results of the present research indicated that EPS, biosurfactant and peroxidase enzymes can have essential roles on toluene tolerance and biodegradation. These native microbial isolates could be considered as a powerful approach for the in situ bioremediation of hydrocarbon-contaminated sea and wastewater.

Implication for health policy/practice/research/medical education:
This research introduced toluene degrading bacteria as suitable strains for bioremediation of toluene contaminated water environments by the production of high amounts of exopolysaccharides, biosurfactants and peroxidase enzymes.

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1. Background

Aromatic hydrocarbons are co mMon groundwater and soil contaminants associated with petroleum product releases (1). Among these, toluene is one of the most important concerns because of its toxic and carcinogenic potential. Toluene which widely exists in petroleum and its related products is used in fuels, and also as a solvent and raw material for the production of plastics, synthetic

* *Corresponding author:* Giti Emtiazi, Department of Biology, Faculty of Science, University of Isfahan, Isfahan, IR Iran. Tel: + 98-3117932457, Fax: + 98-3117932456, *E-mail:* Emtiazi@yahoo.com

fibers and pesticides (2). Although degradation of aromatic compounds by physical and chemical methods is costly, the bioremediation of these compounds is a low cost option and due to the capability of complete mineralization could be a desirable method (3). Bioremediation, which is one of the most effective means of toxic material removal from contaminated water and soils, can be achieved by stimulating the local microbial population to use some of these compounds as their only source of carbon and energy (4).

The native microbial strains of each environment are more efficient than the others because of their adaptability with the environmental conditions of the area such as temperature, pH, salinity and etc. (5-7). Some important products which can help the native microbial strains to tolerate and degrade aromatic hydrocarbons are exopolysaccharides (EPS), biosurfactants and peroxidase enzymes. It seems that the production of these compounds is the bacterial response to the extreme environmental conditions (8-10). EPS are believed to protect bacterial cells from desiccation, heavy metals, organic compounds and other environmental stresses and can efficiently emulsify toluene and other aromatic hydrocarbons (8-11). Due to the very low water solubility and bioavailability of petroleum hydrocarbons, biosurfactants which are amphiphilic compounds are produced to enhance hydrophobic substrates uptake and utilization (12). After emulsification and uptake, peroxidase enzymes such as laccase and catalase play an important role in degradation and detoxification of toxic pollutants (13-15).

2. Objectives

The aim of this research was to compare production of exopolysaccharide, biosurfactant and peroxidase enzymes and to find their role in tolerance increase and bioremediation of toluene by the bacteria isolated from water.

3. Materials and Methods

3.1. Sampling

To screen and isolate toluene degrading bacteria, contaminated seawater samples were collected from a depth of 15 cm and stored in sterile 100-mL bottles from three different sites in the Persian Gulf (the coasts of Bushehr, Bandar-Abbas and Qeshm Island) and two sites in the Caspian Sea (the coasts of Bandar-Anzali and Gisoum) and wastewater samples were collected in spring from Isfahan Province, Iran, and transferred on ice to the laboratory.

3.2. Isolation and Selection of Toluene Degrading Bacteria

A liquid mineral medium (mM) was used to isolate toluene degrading bacteria. This medium contained (gL-1): KH_2PO_4, 4; Na_2HPO_4, 4; NH_4Cl, 2; $MgSO_4$, 0.2; $CaCl_2$, 0.001 and FeCl3, 0.001 in 1000 mL distillated water and pH 6.8. About 5 mL seawater and 1 mL wastewater samples were added to 50 mL of sterile mM media in 250 mL flasks. 1 % (v/v) toluene was added to sterile mM media and then the flasks were incubated at 28°C. After 1 week of enrichment, 1 mL of cultures were transferred into fresh media and incubated further. After four subcultures, an appropriate dilution of the culture was spread onto the toluene agar (mineral medium with toluene and agar) plates. Phenotypically different colonies obtained from the plates were transferred to fresh mM media with and without toluene to eliminate autotrophic and agar-digesting bacteria. The procedures were repeated and the isolates which exhibited growth on toluene were stored for further characterization (16).

3.3. Identification of the Isolates

Identification of the isolated strains was based on colony morphology, microscopic observation of the cell cycle, Gram stain, acid-fast stain, the catalase test, the oxidase test, oxygen requirements, motility and the ability to grow on different carbon sources according to the standards of microbial identification (17). To identify the best toluene utilizing strain 16S rRNA gene was amplified with DG74-AGGAGGTGATCCAACCGCA as a forward primer and RW01-AACTGGAGGAAGGTGGGGAT as a reverse (18). Sequence analysis was performed by Eurofins MWG Operon's sequencing service, Germany.

3.4. Growth Rate and Toluene Removal Assay

The growth rates of the isolates were routinely assessed indirectly by a turbidity measurement at 600 nm in a UV–visible spectrophotometer (Shimadzu UV-160, Japan). The toluene removal assay was carried out by dissolving residual toluene of medium in 3 mL n-hexane and reading the optical density of the toluene against a blank at 200 to 400 nm wavelength (16).

3.5. Laccase Activity Assay

Laccase activity was measured in 1 mL reaction measure containing 75 mM catechol as the enzyme substrate in 50 mM sodium phosphate buffer, pH 5 and 200 μL of culture fluid or bacterial biomass. The progress of the reaction was monitored at 440 nm for 10 min. One unit of laccase activity was defined as a change in A440 of 1 mL in 1 min (19).

3.6. Exopolysaccharide (EPS) Production Assay

The production of EPS was studied by Microtiter-plate test in nutrient broth medium. At first, the McFarland Standard culture of toluene degrading bacteria was prepared. Three wells of a sterile 96-well flat-bottomed plas-

Table 1. Biochemical Characteristics of the Isolated Strains

Strain	Morphology	Growth in NaCl (15 %)	Growth in 50°C	Nitrate Reduction	Urease	Catalase	Oxidase	OF/ Glucose	Anaerobic Growth	Biochemical Maximum Identity
CS	Spore-forming rod	–	–	+	+	+	–	F/O	–	*B. firmus*
PG-1	Spore-forming rod	–	+	+	+	+	–	F	–	*B. amyloliquefaciens*
PG-2	Spore-forming coccus	+	–	+	–	+	+	F/O	+	*S. halophila*
WW	Coryne form	+	+	–	–	+	+	F/O	+	*Ex-DG 74*

tic tissue culture plate with a lid were then filled with 200 μL of each bacterial suspension. Negative control wells contained broth only. The plates were covered and incubated aerobically for 24 h at 28°C. Then, the growth phase medium was carefully removed by pipette, the wells of the microtiter plates were then rinsed with 200 μL PBS (phosphate buffer saline) and the surface of the wells stained with 200 μL of 25 % aqueous crystal violet for 10 min. After rinsing and drying of the plates, 200 μL of 33 % glacial acetic acid was added to each well, and the optical density was measured at 492 nm (20).

3.7. Biosurfactant Production Assay

To determine biosurfactant production by the isolates, the oil spreading technique was used. 50 mL of distilled water was added to a large petri dish (25 cm diameter) followed by the addition of 20 μL of crude oil to the surface of the water. 10 μL of the bacterial culture was then added to the surface of the oil (21). The diameter of the clear zone on the oil surface was measured and compared to the concentration of biosurfactant ranging from '+' to '++++' corresponding from partial to complete spreading on the oil surface. The lack of biosurfactant production was scored as negative.

3.8. Catalase Assay

The same fresh biomasses of the strains were added to 1×9 cm test tube with 500 μL of 3 % H_2O_2 and were slowly shaken. Then the tubes were placed against a dark background and observed for i mMediate bubble formation (O_2 + water = bubbles). The length of produced foam in tubes was measured and reported in centimeter unit (22).

4. Results

4.1. Isolation and Characterization of Toluene Utilizing Bacteria

After sampling and enrichment procedures, twenty toluene degrading bacterial strains were isolated from wastewater and seawater of the Persian Gulf and the Caspian Sea. Among them, two Gram-positive rods, one Gram-positive cocci isolated from seawater (strains *CS, PG-1* and *PG-2)* and one Gram-positive coryneform isolated from wastewater (strain *WW*) were selected for more studies. The Gram-positive, spore-forming, oxidase-negative and catalase-positive rods which produce convex orange colonies were identified as *Bacillus amyloliquefaciens*. The Gram-positive spore-forming, oxidase-negative and catalase-positive rods which produce slime white colonies were identified as *Bacillus firmus*. The Gram-positive endospore-forming cocci, which had aerobic growth and was able to reduce nitrate, oxidase and catalase positive, was identified as *Sporosarcina halophila* (*Table 1*).

Table 2. Sequence and High Homology of the Best Toluene Utilizing Strain

Strain	Sequence of 16S rDNA	Maximum Identity	NCBI Accession Number
WW	TCATGAATCACACCGTGGTAACCGTCCCCCCGAAGGTTAGACTAGCTACTTCTGGTGCAACCCACTCCCATGGTGTGACGGGCGGTGTGTACAAGGCCCGGGAACGTATTCACCGCGACATTCTGATTCGCGATTACTAGCGATTCCGACTTCACGCAGTCGAGTTGCAGACTGCGATCCGGACTACGATCGGTTTTGTGAGATTAGCTCCACCTCGCGGCTTGGCAACCCTCTGTACCGACCATTGTAGCACGTGTGTAGCCCAGGCCGTAAGGGCCATGATGACTTGACGTCATCCCCACCTTCCTCCAGTT	Uncultured bacterium clone A1-E3_M13R (98 %)	Ex-DG74 (HQ414235.1)

The 16s rDNA sequence analysis for wastewater strain which had the highest toluene degradation activity indicated that the strain was close to Uncultured bacterium clone A1-E3_M13R (98 %) and was registered as *Bacterium Ex-DG74* (HQ414235.1) in NCBI (*Table 2*).

4.2. Growth Rate and Toluene Removal by the Isolates

The removal of toluene was observed after 24 h incubation of the isolates in the mineral medium supplemented with toluene (*Figure 1A* and *Figure 1B*). The results indicated that *Bacterium Ex-DG74* had a high ability of growth on toluene as the sole source of carbon and energy and removed toluene by 82 %. This strain had the capability of growth on the medium contained 15 % toluene, whereas the other strains grew only on 1 % toluene containing medium.

4. 3. Laccase Assay in the Vegetative Cells

The supernatant and the biomass of all strains grown on toluene containing media indicated that the vegetative cells had laccase activities. As it is shown in *Figure 2* supernatant of *Sporosarcina halophila* and *Bacillus amyloliquefaciens* had higher extracellular laccase activity than the other strains. On the other hand, the biomass of *Bacterium Ex-DG74* showed the highest membrane-bound enzyme activity (*Figure 2A* and *Figure 2B*). In this situation, induction of laccase production with toluene was more effective than without toluene.

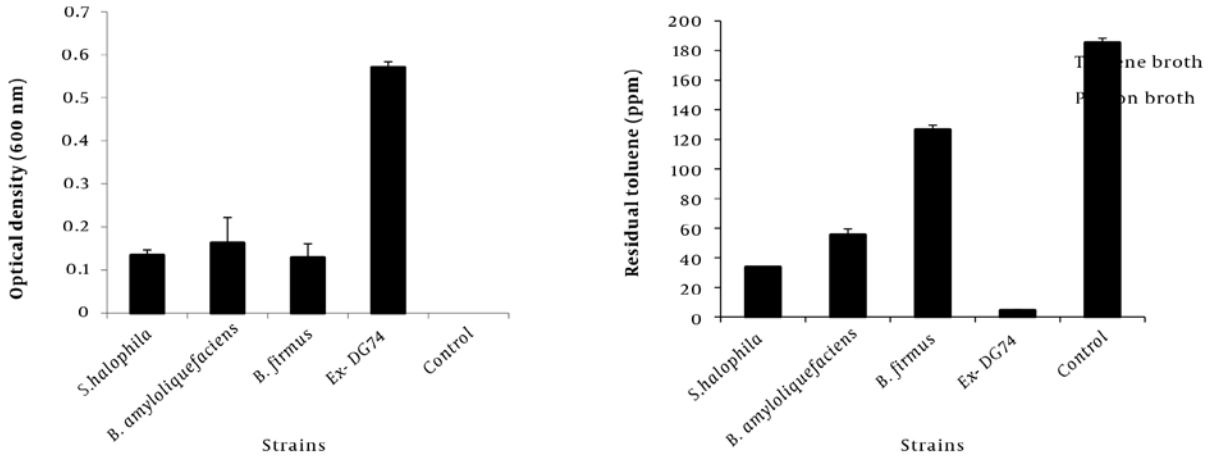
4.4. Exopolysaccharide Production Assay

According to the results, the production of EPS by the marine bacteria, *S. halophila* and *B. amyloliquefaciens* was higher than the wastewater isolate (*Table 3* , *Figure 3*). Thus, it can be concluded that EPS may be an effective factor on toluene biodegradation in marine environment. Therefore, it seems that the production of exopolysaccharide is the bacterial reaction to hard environment conditions such as presence of aromatic hydrocarbons like toluene.

4.5. Biosurfactant Production Assay

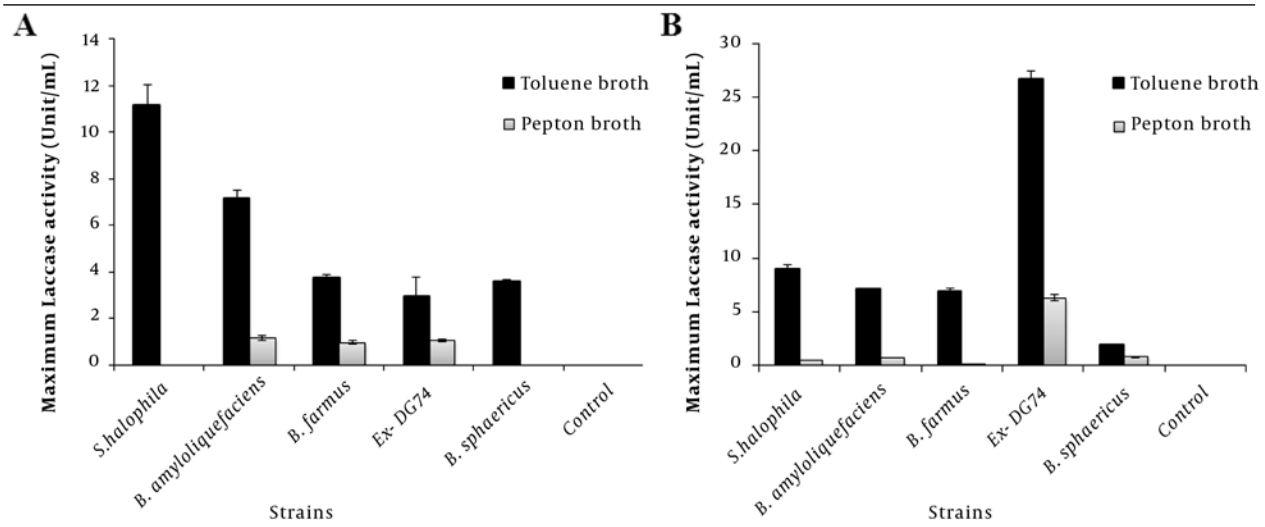
Table 4 shows the results obtained from the oil spreading method. The results indicated that *S. halophila* had the highest biosurfactant production rate. It is assumed that production of biosurfactant by this strain is a mechanism to decrease toluene toxicity and also to increase its bioavailability.

Figure 1. Comparison of growth and toluene removal rates among the isolated strains.



A, The maximum growth of the isolates in toluene containing medium after 24 h; B, Analysis of toluene consumption by the isolates, assayed by UV spectroscopy in 261 nm after 24 h (the average of three replications was shown). Control sample was the sterile medium without bacterial inoculation.

Figure 2. Comparison of Laccase Activity Among The Isolated Strains

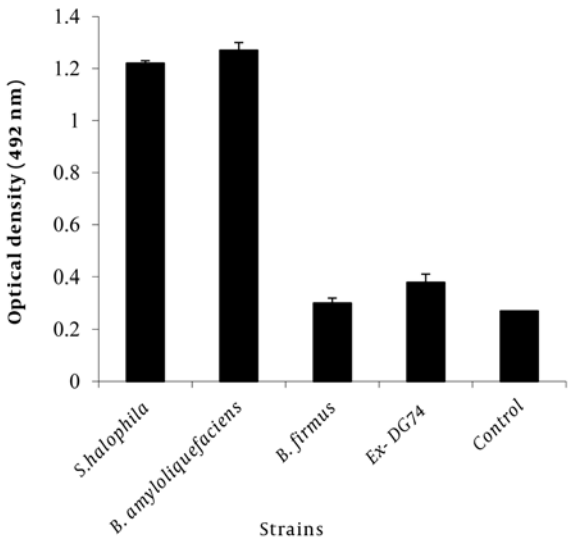


The maximum laccase activity in supernatants, A, and biomass of vegetative cells; B, grown in toluene and peptone broth (the average of three replications was shown). Control sample was the sterile medium without bacterial inoculation.

Table 3. Comparison of EPS Production in the Isolated Strains

	EPS Production
S. halophila	+++
B. amyloliquefaciens	+++
B. firmus	+
Ex-DG74	+
Control	-

Figure 3. EPS Production by the Isolated Strains (the Average of Three Replications Was Shown).



Control sample was the sterile medium without bacterial inoculation

4.6. Catalase Assay

As it is shown in *Figure 4,* qualitative assay of catalase production indicated that all strains had catalase activity. The maximum catalase activity was achieved by *Bacterium Ex-DG74* which can efficiently help it in toluene bioremediation.

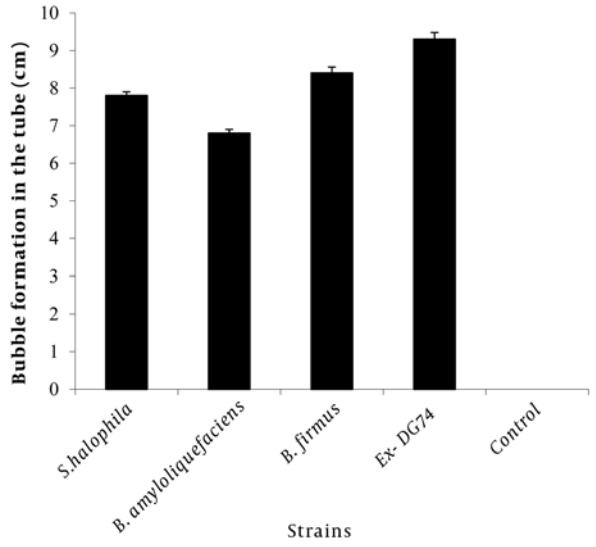
5. Discussion

It is obvious that toluene and other volatile hydrocarbons are toxic to many kinds of life. In this research, toluene degrading bacteria in sea and wastewater were isolated and investigated. The results suggested that these microorganisms might have potentials in bioremediation of toluene in water. As previously described, toluene degrading bacteria in near shore surface water and wastewater showed a relatively wide diversity, including species of *Pseudomonas, Rhodococcus*, *Exiguobacterium*, *Bacillus* and *Halomonas* (16, 23, 24). In this report, a novel

Table 4. Biosurfactant Production Assay by Oil Spreading Method

	Toluene Broth Zone Diameter, cm	Nutrient Broth Zone Diameter, cm
S. halophila	7	4.5
B. amyloliquefaciens	1	2
B. firmus	0	2.5
Ex-DG74	0	1
Control	0	1

Figure 4. Comparison of Catalase Activity Among the Isolated Strains (The Average of Three Replications Was Shown).



Control sample was the sterile medium without bacterial inoculation

toluene degrading bacterium, two *Bacillus* and one *Sporosacina* species were introduced, including *Bacterium Ex-DG74*, *Bacillus firmus*, *Bacillus amyloliquefaciens* and *Sporosarcina halophila*. Among these isolates, *Bacterium Ex-DG74* showed a great ability to remove high concentrations of toluene with maximum laccase activity rate in biomass (membrane-bound laccase).

As mentioned by Hullo *et al.* (25), toluene biodegradation in vegetative cells may be under the impression of laccase activity. Catalase is an antioxidant enzyme which is produced in almost all living organisms. Since toluene metabolites may cause oxidative DNA damage, it is assumed that this effect can be prevented by catalase action. As it is shown in *Figure 4*, *Bacterium Ex-DG74* also had higher catalase activity than the other strains. Thus, we concluded that the high ability of toluene tolerance and biodegradation by *Bacterium Ex-DG74* was related to its strong laccase and catalase activity. Thus, we suggested that this bacterium could be considered as a powerful approach for the bioremediation of toluene-contaminated wastewaters. Production of EPS and biosurfactant increases the bioavailability and biodegradation rate of petroleum hydrocarbon under extreme environmental conditions (26, 27).

Among the isolates, we also suggest the spore-forming marine bacterium, *S. halophila* as a potential tool for in situ bioremediation of hydrocarbon-contaminated seawaters due to its great ability in the production of EPS, biosurfactant and extracellular laccase. In conclusion, the production of EPS, biosurfactant and peroxidase enzymes by the isolates have essential role in tolerance and bioremediation of toluene in marine and wastewater environments and these native microbial strains of each environment are also more efficient than the others because of their adaptability with the area environmental conditions such as temperature, pH, salinity and etc.

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References

1. Keith L, Telliard W. ES&T Special Report: Priority pollutants: I-a perspective view. *Environ Sci Techol*. 1979;**13**(4):416-23.
2. Nahar N, Alauddin M, Quilty B. Toxic effects of toluene on the growth of activated sludge bacteria. *World J Microbiol Biotechnol*. 2000;**16**(3):307-11.
3. Abu Hamed T, Bayraktar E, Mehmetoğlu Ü, Mehmetoğlu T. The biodegradation of benzene, toluene and phenol in a two-phase system. *Biochem Eng J*. 2004;**19**(2):137-46.
4. Ti mMis KN, Pieper DH. Bacteria designed for bioremediation. *Trends Biotechnol*. 1999;**17**(5):200-4.
5. Chang LK, Ibrahim D, Omar IC. A laboratory scale bioremediation of Tapis crude oil contaminated soil by bioaugmentation of Acinetobacter baumannii T30C. *Afr J Microbiol Res*. 2011;**5**(18):2609-15.
6. Atlas RM, Cerniglia CE. Bioremediation of petroleum pollutants: Diversity and environmental aspects of hydrocarbon biodegradation. *Bioscience*. 1995;**45**(5):332-8.
7. Kota S, Barlaz MA, Borden RC. Spatial heterogeneity of microbial and geochemical parameters in gasoline contaminated aquifers. *Pract Period Hazard Toxic Radioact Waste Manage*. 2004;**8**(2):105-18.
8. Abbasi A, Amiri S. Emulsifying behavior of an exopolysaccharide produced by Entrobacter cloacae. *Afr J Biotechnol*. 2008;**7**(10):1574-6.
9. Banat IM. Characterization of biosurfactants and their use in pollution removal - State of the Art. (Review). *Acta Biotechnologica*. 1995;**15**(3):251-67.
10. Nichols CA, Guezennec J, Bowman JP. Bacterial exopolysaccharides from extreme marine environments with special consideration of the southern ocean, sea ice, and deep-sea hydrothermal vents: a review. *Mar Biotechnol*. 2005;**7**(4):253-71.
11. Kazy SK, Sar P, Singh SP, Sen AK, D'Souza SF. Extracellular polysaccharides of a copper-sensitive and a copper-resistant <i>Pseudomonas aeruginosa</i> strain: synthesis, chemical nature and copper binding. *World J Microbiol Biotechnol*. 2002;**18**(6):583-8.
12. Barathi S, Vasudevan N. Utilization of petroleum hydrocarbons by Pseudomonas fluorescens isolated from a petroleum-contaminated soil. *Environ Int*. 2001;**26**(5–6):413-6.
13. Boon EM, Downs A, Marcey D. Catalase: H2O2 oxidoreductase. Catalase Structural Tutorial Text. 2007 [updated 2007]; Available from: http://biology.kenyon.edu/BMB/Chime/catalase/frames/cattx.htm.
14. Kunamneni A, Camarero S, Garcia-Burgos C, Plou FJ, Ballesteros A, Alcalde M. Engineering and Applications of fungal laccases for organic synthesis. *Microb Cell Fact*. 2008;**7**:32.
15. Leahy JG, Colwell RR. Microbial degradation of hydrocarbons in the environment. *Microbiol Rev*. 1990;**54**(3):305-15.
16. Wang L, Qiao N, Sun F, Shao Z. Isolation, gene detection and solvent tolerance of benzene, toluene and xylene degrading bacte-

ria from nearshore surface water and Pacific Ocean sediment. *Extremophiles*. 2008;**12**(3):335-42.
17. Slepecky RA, Hemphill HE. The genus Bacillus. In: Dworkin MF, S., Rosenberg E, Schleifer KH, Stackbrandt E, editors. *The Prokaryotes*. 3rd ed ed. NY: Springer; 2006.
18. Bickley J, Short JK, McDowell DG, Parkes HC. Polymerase chain reaction (PCR) detection of Listeria monocytogenes in diluted milk and reversal of PCR inhibition caused by calcium ions. *Lett Appl Microbiol*. 1996;**22**(2):153-8.
19. Ruttimann C, Schweber E, Salas L, Cullen D, Vicuna R. Lygninolytic enzymes of white rot basidiomycetes Phlebia brevispora and Ceioporiopsis subvermispora,. *Biotechnol Appl Biochem*. 1992;**16**(1):64-76.
20. Stepanovic S, Vukovic D, Dakic I, Savic B, Svabic-Vlahovic M. A modified microtiter-plate test for quantification of staphylococcal biofilm formation. *J Microbiol Methods*. 2000;**40**(2):175-9.
21. Morikawa M, Hirata Y, Imanaka T. A study on the structure-function relationship of lipopeptide biosurfactants. *Biochim Biophys Acta*. 2000;**1488**(3):211-8.
22. Kubica GP, Jones WD, Jr., Abbott VD, Beam RE, Kilburn JO, Cater JC, Jr. Differential identification of mycobacteria. I. Tests on catalase activity. *Am Rev Respir Dis*. 1966;**94**(3):400-5.
23. Hinteregger C, Streichsbier F. Halomonassp., a moderately halophilic strain, for biotreatment of saline phenolic waste-water. *Biotech Lett*. 1997;**19**(11):1099-102.
24. Onysko KA, Budman HM, Robinson CW. Effect of temperature on the inhibition kinetics of phenol biodegradation by Pseudomonas putida Q5. *Biotechnol Bioeng*. 2000;**70**(3):291-9.
25. Hullo MF, Moszer I, Danchin A, Martin-Verstraete I. CotA of Bacillus subtilis is a copper-dependent laccase. *J Bacteriol*. 2001;**183**(18):5426-30.
26. Darvishi P, Ayatollahi S, Mowla D, Niazi A. Biosurfactant production under extreme environmental conditions by an efficient microbial consortium, ERCPPI-2. *Colloids Surf B Biointerfaces*. 2011;**84**(2):292-300.
27. Emtiazi G, Ethemadifar Z, Habibi MH. Production of extra-cellular polymer in Azotobacter and biosorption of metal by exopolymer. *Afr J Biotechnol*. 2004;**3**(6):330-3.