Isolation and Characterization of Halophilic Aromatic and Chloroaromatic Degrader from Wadi El-Natrun Soda lakes

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Abstract: Extremophiles are microorganisms able to survive in extreme conditions. They are good candidates for the bioremediation of hypersaline environments and also for the treatment of saline effluents. A novel aerobic bacterial strain has been isolated on the basis of its ability to utilize benzene, and toluene from the extremely saline and alkaline lakes (Soda lakes) of Wadi El Natrun. The growth rates and biodegradation ability were investigated in mineral basic media supplemented with benzene, toluene, and chlorobenzene under various growth conditions including degradation rate in the presence of different salt concentrations. The new isolate was identified by 16S rRNA as *Alcanivorax* sp.HA03. Phylogenetic analysis indicated that our isolate has 99% sequence similarity with most closely related organism *Alcanivorax* sp. TE-9 in the GenBank, which is a hydrocarbon degrading bacterium, *Alcanivorax* sp.HA03 considered as the only reported halophilic alkalophilic strain from genus *Alcanivorax* that can degrade benzene and toluene, This finding may be necessary in order to estimate the true potential of this strain to be applied in the remediation of monoaromatic and chloroaromatic compounds.
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1. Introduction

Wadi El Natrun and its alkaline inland saline lakes is an elongated depression, about 90 km northwest of Cairo. All lakes had pH values of 8.5-11 and salinity ranging from 283 to 540 g/L (Amany, 1999). Many hypersaline environments such as natural saline lakes, salt flats, solar salterns, industrial effluents, oil fields, coastal ecosystems, are often contaminated with high levels of petroleum hydrocarbons. Crude oil constituted from thousands of components which is separated into saturates, aromatics, resins and asphaltenes.

Among aerobic organisms, there is only a 1-10% occurrence of isolating alkaliphiles. While the two main physiological groups are general alkaliphiles and haloalkaliphiles, all alkaliphiles require some sodium ions for growth and maintenance, making all general alkaliphiles halotolerant to a certain degree. Some of the most highly stable alkaline environments include soda deserts like Wadi El Natrun in Egypt and soda lakes like Kenya's Lake Magadi and Soap Lake, WA (Poolman, 1988; Horikoshi, 1999; Padan *et al*., 2000; Pinkart and Kraft, 2001). Alkaliphiles and haloalkaliphiles have the potential advantage of being able to degrade aromatic and chlorinated hydrocarbons in high pH industrial wastewaters, oil-polluted salt marshes, and other contaminated environments (Margesin and Schinner, 2001).

Many Bacterial strains can degrade BTEX either aerobically or anaerobically but few have been isolated from hypersaline environments. Halophiles with biodegradative potential can be used in the bioremediation of saline environments contaminated with organic pollutants (Margesin and Schinner, 2001, Bastos *et al.*, 2000: Anton *et al.*, 2002). Isolation of halophilic and halotolerant organisms and characterization of their phylogenetic affiliation and metabolic capabilities are important for elucidating degradation pathways and developing bioremediation technologies (Garcia *et al.*, 2004). Halophiles are classified into three groups according to their optimal salt concentration for growth: slightly halophilic (1-3% w/v); moderately halophilic (3-15% w/v); and extremely halophilic (>15 % w/v) (Ventosa and Nieto, 1995). Bacteria prefer lower salinity for optimal growth (Oren, 2002).

Monocyclic aromatic compounds such as benzene, tolune, ethylebenzene, and xylene (BTEX) are common soil and groundwater contaminants and are classified as priority pollutants by the U.S Environmental Protection Agency. Benzene is a category A carcinogen. They are highly water soluble, hence can contaminate a large volume of groundwater. Leakage from underground storage tanks, pipelines, spills, and seepage from surface contaminated sites can cause major BTEX contamination (Philip *et al.*, 2005). BTEX are included in the crude oil, which contains thousands of components which is separated into saturates , aromatics, resins and asphaltenes.

Many microorganisms capable of degrading crude oil components have been isolated. However few of them seem to be important for crude oil biodegradation in natural environments. One group of bacteria belonging to the genus *Alcanivorax* does become predominant in an oil contaminated marine environment (Harayama *et al., 1*999). *Alcanivorax* plays a critical role in the natural cleaning of oil-polluted in halophilic environments. *Alcanivorax* is found in low numbers in unpolluted waters, but in high abundances in oil-polluted waters and coastlines, where it may comprise 80-90% of the oil -degrading microbial community (Harayama *et al*., 1999; Kasai *et al*., 2001; Syutsubo *et al*., 2001).

There are only very few published reports about BTEX degradation under hypersaline conditions Nicholson and Fathepure (2004 and 2005) have reported for the first time that BTEX can be degraded by microorganisms present in hypersaline environments. They were able to develop BTEX-degrading enrichment cultures using soil samples collected from oil-production facilities in Oklahoma as well as from sediment collected from the Great Salt Plain, OK. These studies have demonstrated complete mineralization of 14C-benzene to $14CO_2$ by the enrichment cultures in a medium supplemented with 2.5 M NaCl by microbial community dominant members of the enrichment community, later a pure culture of halophile, *Arhodomonas* sp. strain Seminole was isolated from the enrichment cultures. This organism degraded benzene or toluene as the sole carbon source in the presence of 15 to18 % NaCl(Nicholson and Fathepure, 2005). Li *et al.* (2006) have isolated a *Planococcus* sp. using a contaminated soil obtained from a petroleum refinery effluent in China. The organism is a moderate haloalkaliphilic and able to degrade BTEX at 20 % salt. Overall, there is a severe lack of pure cultures of halophiles that degrade petroleum hydrocarbons under hypersaline conditions. Such studies are necessary to discover novel degradation pathways of hydrocarbon degradation. Isolation of novel microbes with superior degradation potential in the presence of various salt concentrations is important for developing biological catalysts and for developing molecular tools for monitoring the presence of such microbes at contaminated hypersaline environments.

In the present study, We have successfully isolated a halophilic bacterial strain that degrades benzene and toluene as the sole source of carbon. We have studied the isolate's phylogeny, physiology, and capacity to degrade benzene and toluene at various salt concentrations.

2. Material and Methods

Soil samples and chemicals

Samples were collected from Wadi El Natrun soda lakes in Egypt at the time of sampling, the water pH and total salt content of the lakes were within the ranges 9.1–10.4 and 201–360 g l^{-1} ·Soil samples and chemicals, respectively. The dominant cation in the lakes is Na+ (2.1–4.5 M), with traces of Mg^{2+}, K^{+}, and Ca^{2+} (each less than 0.05 M in any of the lakes). The main anion was Cl^{-} (2.1–4.5 M), with lesser amounts of HCO_3^{-} ,CO_3^{-2}, and SO_4^{-2} . The carbonate/bicarbonate alkalinity varied from 0.1–0.8 M, and sulfate was less than 0.3 M. With concentrations of cations other than sodium being very low, virtually all the chloride is presumed to be present as sodium chloride.

Isolation of Benzene and Toluene utilizing Bacterium

Initially, evidence for the degradation of benzene at the soda lakes was obtained using 1 g soil from soda lakes in 50 ml mineral medium (MM) containing 1.5 M NaCl. The enrichment was initiated by adding 1 g of soil (wet weight) to duplicate 100 ml of mineral salts medium (MM). MM contained (in grams/liter): NaCl, 145; $MgCl_2$, 0.5; KH_2PO_4, 0.45; K_2HPO_4, 0.9; NH_4Cl, 0.3; KCl, 0.3. to duplicate one liter-capacity bottles, the air in the headspace served as the source of oxygen. The bottles were closed with a black rubber stopper with a hole in the middle that fit a cut 3-in. tube. The tubes were closed with Teflon-coated septa and aluminum caps. A 100 µl gas-tight glass syringe was used to introduce 22 µl of undiluted benzene and Toluene (~245 µmol) to each bottle. The bottles were incubated static in the dark at room temperature. After one month of cultivation at 30°C, 10 % of the culture was transferred to fresh medium and cultured for one more month. Benzene-degrading halphilic bacterium was isolated on agar plates prepared with MM containing 1 M NaCl with 1.5 % agar. First, a 0.1 ml of 10-fold serial dilution of the enrichment was plated onto agar plates prepared with MM containing 1 M NaCl. The plates were incubated at 30 ºC. Tiny colonies first appeared after a month. The colonies appeared smooth, round, and measured less than 1 mm in diameter. Subsequently, single colonies were aseptically transferred to liquid culture 50 ml of sterile MM-supplemented with 2 M NaCl and 25 µmoles of benzene. Then, benzene degradation was monitored using HPLC.

Phylogenetic analysis

Phylogenetic identification of the isolate was enabled by means of sequence analysis of the 16S rRNA gene. A 3 ml sample of the pure culture was centrifuged at 12,000 rpm for 15 minutes. The supernatant was decanted and the cells were washed twice with sterilized water. The cells were resuspended in 0.5 ml of sterilized water. Then,

genomic DNA was extracted from the pure culture using a MoBio Ultra Clean Soil DNA Kit (MO BIO, Carlsbad, CA). Three primers were used in the amplification of 16S rRNA. These include: Bact 27f (5'- AGAGTTTGATC (A/C)- TGGCTCAG-3'), Bact 1492r(5'-TACGG(C/T)TACCTTGTTACGACTT-3'), and Bact1098r (5'-AAGGGTTGCGCTCGTTGCG-3') (Chang *et al.*,2000). Theoretically, amplification with Bact 27f -1492r should yield 1505 bp and amplification with Bact27f -1098r should yield 1108 bp from the 16S rRNA. Amplifications with these two primer sets were used to obtain the nearly full-length sequence (1453 bp) of the 16S rRNA of the isolate. PCR amplification was performed in a total volume of 50 µl in model T Personal thermocycler (Biometra). Each PCR mixture contained 25 ng of template DNA, 0.6 µM of each primer, 1.75 mM $MgCl_2$, 200 µM of dNTPs, 1.25 U of *Taq* polymerase in Buffer A (M1865, Promega Chemicals, Madison, WI). Amplification of 16S rRNA using both primer sets consisted of an initial denaturation of the genomic DNA at 94 ºC for 3 minutes, followed by 30 cycles of denaturation at 94 ºC for 1 minute, annealing at 53ºC for 1 minute, and extension at 72 ºC for 2 minutes, and a final extension at 72 ºC for 8 minutes. PCR products were checked for expected size on 1.5 % agarose gels. The PCR product was purified by Gene JET^{TM} Gel Extraction kit (#K0691) (Fermentas). After purification, a sample of the PCR product was sequenced in both directions. The determined 16S rRNA gene nucleotide sequences were entered for BLAST searching into the Web site of NCBI (http://www.ncbi.nlm.nih.gov/blast/), and aligned using ClustalW implemented in MEGA software version 3,1(Kumar *et al*., 2004) The phylogenetic tree was constructed using TreeView version 1.6.6.

Growth condition and Growth curve

To quantify growth rate and substrate disappearance, *Alcanivorax* sp. HA03 was grown as described above and cultures harvested during late exponential growth phase by centrifugation at 7000 rpm for 10 min. Cells were washed twice with 50 mM phosphate buffer (pH 7) and resuspended in liquid mineral medium to give an OD600 nm of 0.1, degradation of benzene and toluene were tested in sterilized glass tubes containing 2 ml cell suspension (OD600nm = 0.1) and 2 mM of benzene and toluene as sole carbon source. The test tubes were incubated at 150 rpm and 30°C. At each time point, 2 test tubes were analyzed. For the estimation of the colony forming units (CFU) aliquots were serially diluted, 100 µl aliquots were plated on solid LB medium and the CFUs counted after 2 days incubation at 30°C. Uninoculated tubes and tubes without substrate (with DMSO only) served as controls.

Optimum salt concentration for benzene degradation

The ability of the new isolate to degrade benzene, toluene and chlorobenzen compounds was assessed in flasks containing 60 ml of MM-NaCl and inoculated with 3 ml liquid culture from *Alcanivorax* sp. HA03 and spiked with 1 µl (~25 µmole/Flask) of undiluted benzene, toluene, or chlorobenzene as the sole carbon and energy source. Flasks containing 60 ml MM-NaCl were incubated static at 30 °C in the dark in the presence of different concentrations of NaCl ranging from 3% - 15%.

Effect of other carbon sources on BTEX degradation

The isolate's ability to degrade BTEX and chlorobenzene in the presence of other carbon sources was studied. Flasks were setup with minimal media supplemented with 3 M NaCl and 5 mM of glucose, lactic acid, pyruvate, or Salicylate to determine the effects of these carbon substrates on benzene, toluene, or chlorobenzene degradation.

Data deposition

The 16S rRNA sequence reported in this study has been deposited in the GenBank database under accession numbers JQ015308.

3. Results

Identification of the isolate from soda lakes in Wadi El Natrun

A halophilic bacterium that degrades benzene or toluene as the sole source of carbon was isolated from the soda lakes in Wadi El Natrun. The isolate is a gram-negative, rod-shape halophilic aerobic bacterium. The isolate grew on minimal medium agar plates on benzene vapor within 2-4 weeks. Comparison of the 16S rRNA gene sequence (> 1400 bp) of the isolate with the sequences in GenBank showed 99 % sequence similarity with *Alcanivorax* sp. TE-9 the most closely related organism which is hydrocarbon-degrading *Alcanivorax* strain (Syutsubo *et al.*, 2001), 99% similarity with *Alcanivorax* sp. Qphe 3, which is polycyclic aromatic hydrocarbons, and 98% similarity with the alkane degrading strains *Alcanivorax* sp. 2B5 (liu *et al.*, 2010), *Alcanivorax dieselolei* sp. Nov (Liu and Shao, 2005), and *Alcanivorax dieselolei* strain ANT-2400 S6 (Tapilatu *et al.*, 2010).

Growth of *Alcanivorax* sp. HA03 on benzene and toluene

Alcanivorax sp. HA03 can utilize benzene (Fig. 2) and toluene (Fig. 3) as a sole source of carbon and energy. Growth was accompanied by an increase in colony forming units (CFU) and concomitant decrease in the concentration of benzene and toluene

were verified by HPLC analysis. No growth was observed in the control test tubes containing benzene only. *Alcanivorax* sp. HA03 grew rapidly at 30°C, with a doubling time of 4 hours.

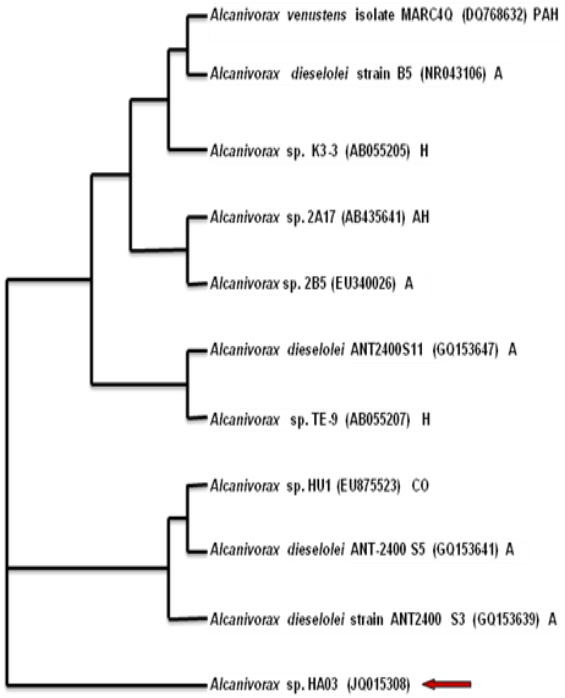

Figure 1 Phylogenetic tree based on 16S rRNA gene sequences showing the relationship of *Alcanivorax* sp. HA03 strain to other reported halophilic *Alcanivorax* strains. Accession numbers of the sequences are shown in parentheses after the strain designation. "A" aromatic degrader, "PAH" poly aromatic hydrocarbon degrader, "H" aliphatic hydrocarbon, and "CO" crude oil degrader.

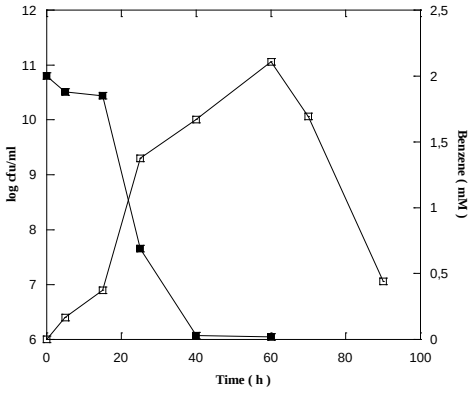

Figure 2 Growth of *Alcanivorax* sp. HA03 on 2 mM benzene as a carbon source. Growth was monitored by following colony-forming units (CFU) and substrate depletion was assessed by HPLC.

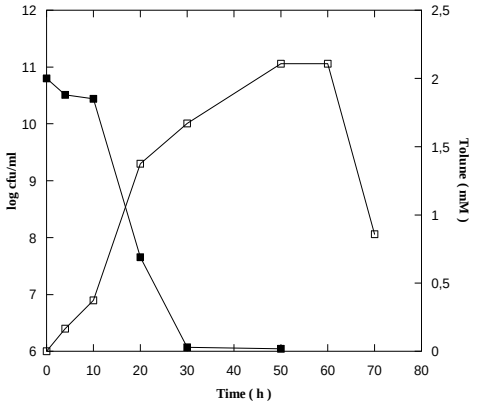

Figure.3 Growth of *Alcanivorax* sp. HA03 on 2 mM toluene as a carbon source. Growth was monitored by following colony-forming units (CFU) and substrate depletion was assessed by HPLC.

Benzene Toluene, and chlorobenzene degradation in the presence of other carbon substrates.

To study the ability of *Alcanivorax* sp. HA03 to degrade hydrocarbons in the presence of other organic substrates, we tested benzene, toluene, and chlorobenzene degradation in the presence of easily metabolizable and common substrates such as glucose, salicylate, lactate, and pyruvate. The results in Table 1 showed that degradation proceeded in the presence of all the tested substrates, except lactate. Results also showed that turbidity was much higher in flasks containing glucose, salicylate and pyruvate than in flasks with benzene, toluene, or chlorobenzene alone. No turbidity was observed in lactate bottles suggesting inhibition of growth and degradation. Data also showed that the rate of benzene degradation alone was more than toluene or chlorobenzene, the degradation also increased in the presence of glucose compared to salicylate or pyruvate. These results clearly showed that the *Alcanivorax* sp. HA03 was able to utilize benzene, toluene, or chlorobenzene alone and with other substrates simultaneously suggesting that, perhaps degradation was catalyzed by constitutive enzymes. This ability of the organism is important for field application.

Benzene, toluene, chlorobenzene degradation rate at different salt concentration

To assess the capability of *Alcanivorax* sp. HA03 to degrade monocyclic aromatic compounds and chloroaromatic compounds in flasks contain different salt concentration ranging from 3% to 15% (0.5 mol l^{-1} to 3 mol l^{-1}) (table 2), 20 µmol of benzene, toluene and chlorobenzene were added as the sole source of carbon for two months. Benzene,

toluene, and chlorobenzene were degraded in 2 weeks at a rate of 2.85, 2.15 and 0.75 µmol/flask/day respectively at salt concentration 3%, while the degradation rate of both benzene and toluene decreased to 1.45 and 1.25 µmol/flask/day respectively by increasing the salt concentration to 7% and needed almost 4 weeks at this concentration. Benzene was degraded at slower rates by increasing salt concentration from 10% to 15% (1 to 0.75 µmol/flask/day), while toluene showed no degradation at those two degradation rates (10% and 15%). No degradation for chlorobenzene occurred at the salt concentration from 7% to 15% when added as a sole carbon source even after incubating for two months.

Table 1 Benzene, toluene, and chlorobenzene degradation in the absence and presence of other carbon substrates by *Alcanivorax* sp. HA03.

Substrate	Degradation rate µmol/flask/day
Benzene	2.85 ± 0.25
Toluene	2.15 ± 0.15
Chlorobenzene	1.25 ± 0.25
Benzene + Salicylate	1.75 ± 0.14
Toluene + Salicylate	1.10 ± 0.23
Chlorobenzene + Salicylate	0.85 ± 0.15
Benzene + Pyruvate	1.60 ± 0.20
Toluene + Pyruvate	0.65 ± 0.10
Chlorobenzene + Pyruvate	ND
Benzene + Lactic Acid	ND
Toluene + Lactic Acid	ND
Chlorobenzene + Lactic Acid	ND
Benzene + Glucose	3.40 ± 0.45
Toluene + Glucose	2.90 ± 0.25
Chlorobenzene + Glucose	1.45 ± 0.10

Flasks were amended with benzene or toluene or chlorobenzene alone or with 5 mM other substrate. The Flasks were inoculated with 5 ml of benzene or toluene or chlorobenzene grown culture. The bottles were inoculated with 5 ml of benzene-grown culture. ND means no degradation

4. Discussion

In spite of high salinity and neumerous hydrocarbon contamination in Wadi El Natrun, surprisingly very few studies have been carried out to understand the microbial communities and their capacity to degrade hydrocarbon contamination either aliphatic or aromatic. In this study, we focused on the biodegradation of benzene, toluene, and chlorobenzene at high salinity from halophilic isolated strain from Wadi El Natrun.

Table 2 Benzene, toluene, chlorobenzene degradation rate at different salt concentration.

	Degradation rate µmol/flask/day		
Salt concentration	Benzene	Toluene	Chlorobenzene
3% NaCl	2.85 ± 0.25	2.15 ± 0.15	0.75 ± 0.54
7% NaCl	1.45 ± 0.72	1.25 ± 0.45	ND
10% Nacl	1.00 ± 0.4	ND	ND
15% NaCl	0.74± 0.9	ND	ND

Several studies showed the ability of bacterial populations associated with the early stages of contamination reflecting the initial utilization of various highly degradable hydrocarbon compound classes aliphatic, aromatic, and polyaromatics (Head *et al.*, 2006). The *Alcanivorax* genus has been associated with the early stages of hydrocarbon degradation and has been shown to utilize saturated hydrocarbons such as straight-chain and branched alkanes (Martins *et al*., 2010).

Alcanivorax sp. HA03 has the ability to grow on benzene, toluene and chlorobenzene as the sole carbon source. From the phylogenetic tree (Fig 1). Based on the 16S rRNA sequence data, it appeared that the isolated strain *Alcanivorax* sp. HA03 may represent a different *Alcanivorax* strain more similar to aromatic degraders than alkane degraders, Figure 1 showed the phylogenetic relationship of the *Alcanivorax* sp. HA03 with other *Alcanivorax* strains that degrade aliphatic and aromatic hydrocarbons that have been isolated from various saline environments. The phylogenetic tree clearly showed that *Alcanivorax* sp. HA03 is different than the reported aliphatic and aromatic strains, formed distinct clusters this phylogenetic clustering is to some extent reflected in their physiology and ecology.

The growth curve for *Alcanivorax* sp. HA03 on benzene and toluene showed the capability of this strain to degade benzene rather than toluene either in the presence carbon source or without carbon source, and even with increasing of salt concentrations. Analysis of the growing *Alcanivorax* sp. HA03 with other carbon source showed that this strain was able to degrade benzene or toluene or chlorobenzene in the presence of easily utilizable compounds such as glucose, pyruvate, and salicylate. However, growth and degradation of benzene was inhibited in the presence of lactic acid. The reason for this inhibition is not known. These observations are important since contaminated sites often contain a variety of easily utilizable substrates.

Our results clearly demonstrated the ability of the isolated halophilic strain from Wadi E1Natrun to metabolize benzene, toluene, or chlorobenzene as the sole carbon source at high salinity. The isolation of benzene degraders over a broad range of salinities (0% - 20%) clearly revealing the natural attenuation

potential of hydrocarbon in the Wadi E1 Natrun lakes. Such ability can be attributed to the adaptation of benzene degrader to temporal and spatial salt concentration in the site.

Alcanivorax not only degrades hydrocarbons in the laboratory, but also seems to be globally very important in the removal of hydrocarbon from salty hydrocarbon contaminated sites. It therefore has obvious potential for bioremediative interventions in polluted salty lakes, marine and coastal systems.

Overall, our study has demonstrated for the first time the ability of genus *Alcanivorax* represented by *Alcanivorax* sp. HA03 isolated from soda lakes in Wadi E1Natrun to degrade low molecular weight hydrocarbons over a broad range of salinity, suggesting the bioremediation potential of hydrocarbons at Wadi E1 Natrun.

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References

1. Amany G. Taher. Inland saline lakes of Wadi El Natrun depression, Egypt. *International Journal of Salt Lake Research.* 1999. 8: 149-169.
2. Harayama, S., Kishira, H., Kasai, Y. and Shutsubo, K. Petroleum biodegradation in mrine environments. J. Molec. Microbiol. Biotechnol. 1999. 1:63-70.
3. Head IM, Jones DM, Roling WFM. Marine microorganisms make a meal of oil. Nat. Rev. Microbiol. 2006. 4: 173-182.
4. Horikoshi, Koki. Alkaliphiles: Some Application of Their Products for Biotechnology. *Microbiol. Mol. Biol. Rev.* **1999**. *63* (4): 735-750.
5. Kasai, Y.; Kishira, H.; Syutsubo, K.; Harayama, S. Molecular detection of marine bacterial populations on beaches contaminated by the Nakhodka tanker oil-spill accident. Environ. Microbiol. 2001. 3 (4): 246-255
6. Kasai Y, Kishira H, Harayama S. Bacteria belonging to the genus *Cycloclasticus* play a primary role in the degradation ofaromatic hydrocarbons released in a marine environment. Appl. Environ. Microbiol. 2002. 68: 5625-5633.
7. Li, H., Liu,Y.H., Luo, N. Zhang, X.Y., Luan, T.G., Hu, J.M., Wang, Z.Y., Wu, P.C., Chen, M.J. and Lu, J.Q. Biodegradation of benzene and its derivatives by a psychrotolerant and moderately haloalkaliphilic *Planococcus* sp. strain ZD22. Res. Microbiol. 2006. 157: 629-636.
8. Liu, C. & Shao, Z. *Alcanivorax dieselolei* sp. nov., a novel alkane-degrading bacterium isolated from sea water and deep-sea sediment. Int J Syst Evol Microbiol. 2005. 55: 1181–1186.
9. Margesin, R.; Schinner, F. Biodegradation and bioremediation of hydrocarbons in extreme environments. *Appl. Microbiol. Biotechnol*. 2001. *56*: 650-663.
10. Martins dos Santos V., Sabirova J., Timmis K. N., Yakimov M. M *Alcanivorax borkumensis*, p. 1266–1285 *In* Timmis K. N., editor. (ed.), Handbook of hydrocarbon and lipid microbiology. 2010. Springer-Verlag, Berlin, Germany
11. Nicholson, C.A. and Fathepure, B.Z. Aerobic biodegradation of benzene and toluene under hypersaline conditions at the Great Salt Plains, Oklahoma. FEMS Microbiol. Lett. 2005. 245: 257-262.
12. Nicholson, C.A. and Fathepure, B.Z. Biodegradation of benzene by halophilic and halotolerant bacteria under aerobic conditions. Appl. Environ. Microbiol. 2004. 70: 1222-1225.
13. Oren, A. Diversity of halophilic microorganisms: Environments, phylogeny, physiology, and applications. *J. Ind. Microbiol. Biotechnol.* 2002. *28*: 56-63.
14. Padan, Etana; Krulwich, Terry Ann. Sodium Stress. In *Bacterial Stress Responses*; Storz, G., Hengge-Aronis, R., Eds.; ASM Press: Washington, D.C. 2000. 117-130.
15. Philip, J.C., Bamforth, S.M., Singleton, I. and Atlas, R.M. Environmental pollution and restoration: A role of bioremediation. In Bioremediation: Applied Microbial Solutions for Real-World Environmental Cleanup., R.M.a.P. Atlas, J.,ed. 2005. (Washington: ASM Press).
16. Pinkart, H.C.; Kraft, Z. *Anaerobic bacterial diversity in an alkaline, meromictic lake*. 101st General Meeting of the American Society for Microbiology; Orlando, Florida; 2001.
17. Poolman, B.; Driessen, A.J.M.; Konings, W.N. Effects of the Cytoplasmic pH on Solute Transport in Bacteria. In *Homeostatic Mechanisms in Micro-organisms*; Whittenbury, R., Ed.; Bath University Press: Bath, United Kingdom, 1988. 117-129
18. yutsubo, K., Kishira, H., and Harayama, S. Development of specific oliogonucleotide probes for the identification and in situ defection of hydrocarbon – degrading Alcanivorax strains. *Environ Microbiol*. 2001. 3**:** 371–379.
19. Tapilatu Y, Aquaviva M, Guigue C, Miralles G, Bertrand J-C, Cuny P. Isolation of alkane-degrading bacteria from deep-sea Mediterranean sediments. Letters in Applied Microbiology. 2010. 50:234-236
20. Ventosa, A. & J. Nieto. Biotechnological applications and potentialities of halophilic microorganism. World J. Microbiol. Biotechnol. 1995. 11: 85-94.