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Biodegradation of aromatic hydrocarbons by Haloarchaea and their use for the reduction of the chemical oxygen demand of hypersaline petroleum produced water

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ABSTRACT

Ten halophilic Archaea (Haloarchaea) strains able to degrade aromatic compounds were isolated from five hypersaline locations; salt marshes in the Uyuni salt flats in Bolivia, crystallizer ponds in Chile and Cabo Rojo (Puerto Rico), and sabkhas (salt flats) in the Persian Gulf (Saudi Arabia) and the Dead Sea (Israel and Jordan). Phylogenetic identification of the isolates was determined by 16S rRNA gene sequence analysis. The isolated Haloarchaea strains were able to grow on a mixture of benzoic acid, p-hydroxybenzoic acid, and salicylic acid (1.5 mM each) and a mixture of the polycyclic aromatic hydrocarbons, naphthalene, anthracene, phenanthrene, pyrene and benzo[a]anthracene (0.3 mM each). Evaluation of the extent of degradation of the mixed aromatic hydrocarbons demonstrated that the isolates could degrade these compounds in hypersaline media containing 20% NaCl. The strains were shown to reduce the COD of hypersaline crude oil reservoir produced waters significantly beyond that achieved using standard hydrogen peroxide treatment alone.

1. Introduction

Petroleum and natural gas reservoirs frequently contain hypersaline waters. During production of crude oil and natural gas large amounts of reservoir water is typically also produced (produced water) particularly as the wells decline in oil production (Speight, 2007). Produced water is by far the largest volume byproduct or waste stream associated with oil and gas production and handling it presents challenges and costs to operators (Veil et al., 2004). According to the Produced Water Society "in 1993, 1.09 trillion gallons of produced water were generated – enough water to flow over Niagara Falls for 9 days. Sixty-five percent of the produced water generated in the US is injected back into the producing formation, 30% into deep saline formations and 5% is discharged to surface waters. Produced water salinity in the US ranges from 100 mg L^{-1} to 400 000 mg L^{-1} (seawater is 35 000 mg L^{-1})" (Produced Water Society, 2011).

Not only is the saline content of produced water typically high, the organic composition, which is a major contributor to chemical oxygen demand (COD), is complex, frequently containing aromatic compounds including phenolics and aromatic hydrocarbons. Hydrocarbon aromatics dissolved in production waters primarily include toxic mono and low to medium molecular weight polycyclic aromatic hydrocarbons (PAHs) due to their relatively high solubility in comparison to nonaromatic fractions of fossil fuels such as alkanes (Veil et al., 2004). The most soluble of these aromatic hydrocarbons are benzene, toluene, ethylbenzene and xylenes (BTEX), naphthalenes, phenanthrenes and dibenzothiophenes.

Although much of the produced water is typically reinjected into the reservoirs for enhanced recovery or disposal, large amounts are discharged into the sea and on land into evaporation pits of various designs or treated in some fashion and released into the environment. Produced water remains with the crude oil during transport from the production facilities via pipeline or tankers. As crude is offloaded to storage facilities and refineries, any produced water that comes along with the oil is separated before secondary pipelining or refining. As a result, produced water is a significant potential source of toxic aromatic contaminated hypersaline water. If discharged into the environment without prior treatment, deliberately or be accident, it can cause hazards by contamination of soil, and surface, ground and marine water.

One way or the other produced water must be disposed of in manner that complies with regulations, which includes COD and targeted chemicals, such as aromatics and hydrogen sulfide. Conventional treatment relies primarily on physico-chemical treatments include gravity separation and dissolved air flotation of suspended solids, chemical oxidation of hydrogen sulfide using hydrogen peroxide, de-emulsification, coagulation and flocculation of suspended solids that contribute to colloidal COD. However,

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these treatments are not designed to remove dissolved aromatic compounds. Organics can be removed with conventional wastewater treatment processes such as activated sludge systems, however, the saline content of these systems must be well below that frequently found in produced water for them to work efficiently (Lefebvre and Moletta, 2006).

It is now well established that aromatic compounds can be readily degraded in aerobic environments within soils, sediments and waters with salinities up to and including that of seawater (~3.5%) (Van Hamme et al., 2003). However, little is known about their metabolism in hypersaline environments. Haloarchaea are a class of the Euryarchaeota found in hypersaline environments that require salt concentrations in excess of 2 M (or about 10%) to grow, and optimal growth frequently occurs at higher concentrations, typically 20–25%, which covers a broad range of salinities frequently found in produced water.

Although few Haloarchaea strains with aromatic hydrocarbon degradation capability have been described to date (Le Borgne et al., 2008), a range of aromatics have been shown to be degraded and used as carbon sources by these microorganisms. *Haloferax volcanii* D1227 isolated from oil brine-contaminated soil was shown to degrade monoaromatic carboxylic acids, such as benzoate, cinnamate and 3-phenylpropionate (Emerson et al., 1994) and *Haloarcula* sp. D1 was shown to metabolize p-hydroxybenzoic acid (Fairley et al., 2002). The Haloarchaea strain EH4 was found to be capable of degrading a wide range of *n*-alkanes and the aromatic hydrocarbons acenaphthene, phenanthrene, anthracene, and 9-metylanthracene while growing in a medium with 310 g L^{-1} of salts (Bertrand et al., 1990). An extremely halophilic archaeon from a salt marsh was shown to degrade various saturated and aromatic hydrocarbons at salinities of up to 31% and grew optimally at 22% NaCl (Kleinsteuber et al., 2006). More recently, Tapilatu et al. (2010) isolated a *Haloferax* strain (MSNC 14) able to grow on phenanthrene from a shallow crystallizer pond in Camargue, France, with no known contamination history using *n*-alkanes as the source of carbon and energy.

The ability to degrade aromatics under hypersaline conditions suggests these microorganisms could be useful for bioremediation in hypersaline environments. Conventional microbiological treatment processes do not function well at high salt concentrations indicating that microbial bioremediation of undiluted hypersaline produced water requires halophiles (Lefebvre and Moletta, 2006; Singlandea et al., 2006). Information on the ability of halophiles to treat hypersaline environments contaminated with aromatics is limited. However, biological treatment of highly saline effluents, such as produced water from the oil industry, has received some study (Pendashteh et al., 2010).

Prior to discharge at sea, the Brazilian national oil company PetroBras treats produced water received at oil terminals with hydrogen peroxide primarily to remove hydrogen sulfide and prevents its formation during storage. However, after treatment, the organic content, particularly phenol levels is still high, and consequently, so is the COD level of the produced water, which remains of concern with regard to the impact on oxygen demand and the introduction of toxic compounds at the marine discharge site.

The goal of this work was the isolation of halophilic Haloarchaea able to degrade aromatic hydrocarbons and the evaluation of these isolates for their ability to reduce COD levels of petroleum produced water beyond that achieved with hydrogen peroxide treatment alone. Samples taken from five hypersaline sites were used as sources for the isolation of Haloarchaea able to metabolize aromatic compounds. Haloarchaea able to metabolize a range of aromatic compounds were isolated and identified. In addition, we present data on the use of these isolates for reducing the COD content of petroleum reservoir produced waters.

2. Materials and methods

2.1. Source of strains

Haloarchaea were isolated from 5 hypersaline environments designated as follows: Uyuni Salt Marsh, Bolivia (**BO**), Cabo Rojo marine salterns, Puerto Rico (**PR**), sabkhas (salt flats), Saudi Arabia (**AA**), Dead Sea, Jordan (**MM**), and Cahuil marine salterns, Chile (**CL**). *H. volcanii* (previously *Halobacterium volcanii*) DSMZ 3757 (also known as *H. volcanii DS2*), a halophilic archaeon isolated from bottom sediment from the Dead Sea (Mullakhanbhai and Larsen, 1975) was supplied by Fundação Oswaldo Cruz (FIOCRUZ, Rio de Janeiro – RJ, Brazil).

2.2. Media

A mineral high salt medium (MHSM) adapted from Mevarech and Werczberger (1985) was used for the enrichment, isolation and characterization of microorganisms. The medium composition was as follows (g L^{-1}): NaCl (200), KCl (3.75), NH_4Cl (0.267), K_2HPO_4 (0.174), $MgSO_4$ (37) and $CaCl_2$ (0.5) together with 0.1% (v/v) trace-elements solution (Widdel and Bak, 1992), pH 7.2. The autoclaved medium was then amended with 0.1% (v/v) filter-sterilized vitamin solution (Wolin et al., 1963). Aromatic compounds were added as indicated. PAH compounds were dissolved in acetone prior to addition. For solid medium, 1.5% (w/v) bacteriological agar (Difco) was added before autoclaving. It should be noted that this medium is an optimal minimal medium for the growth of *H. volcanii*, and hence it is perhaps not surprising that the organisms isolated on this medium were all members of the genus *Haloferax*.

A rich medium (MHSM-YT) was used for preparation of inoculum for use in evaluating growth on various aromatic compounds. MHSM-YT was prepared by supplementing MHSM with yeast extract (0.3% w/v) and tryptone (0.3% w/v) before autoclaving.

2.3. Enrichment and isolation of Haloarchaea

Enrichments and isolation of Haloarchaea was performed as described previously (Cuadros-Orellana et al., 2006). Enrichment cultures were prepared in 125 mL Erlenmeyer flasks containing 50 mL MHSM amended with 0.4 mM p-hydroxybenzoic acid and 100 μg mL^{-1} chloramphenicol to prevent the growth of bacteria. Water or sediment samples were added to the medium in the proportion 1:50 (v/v or w/v). Cultures were incubated aerobically at 40 °C on a rotary shaker operating at 240 rpm for 10 d or until good growth was observed. After three consecutive transfers, aliquots were serially streaked onto solid medium of the same composition for isolation of colonies.

Strains isolated on p-hydroxybenzoic acid were evaluated for their ability to degrade various aromatic compounds and reduce the COD of produced water as described.

2.4. 16S rDNA analysis of Haloarchaea isolates

Genomic DNA of the halophilic strains was obtained using the AquaPure® Genomic DNA kit (Bio-Rad Laboratories) using the manufacturer's protocols.

Phylogenetic identification of the Haloarchaea isolates was performed by sequence analysis of cloned 16S ribosomal RNA genes. Primers specific for 16S rRNA gene sequences from Archaea, 1Af (5′-TCYGKTTGATCCYGSCRGAG-3′) and 1100Ar (5′-TGGGTCTC GCTCGTTG-3′) (Embley et al., 1992), were used to amplify the genomic DNA of the isolates. The polymerase chain reaction (PCR) was performed in a volume of 50 μL containing 5 μL of 10X

buffer with $MgCl_2$ (Eppendorf), 8 mM dNTP's (deoxyribonucleotide triphosphates), 2.5 μL of each primer (diluted 1:9 in MilliQ water), 0.5 μL of Taq polymerase (Eppendorf) and 50–100 ng of DNA, using the following thermal cycling conditions: initial denaturation at 95 °C for 5 min, 25 cycles of denaturation at 94 °C for 1 min, annealing at 50 °C for 1 min, extension at 72 °C for 2 min, followed by a final extension at 72 °C for 10 min. The amplified products were analyzed by electrophoresis in 1% agarose gels.

The PCR amplified products were sequenced by NewBiotechnic, S.A., Seville, Spain, using a 3100 Genetic Analyzer (Applied Biosystems) according to the manufacturer's protocols. The sequences obtained for each isolate were assembled into contigs using the Phred/Phrap/Consed program, Linux version (Ewing et al., 1998; Gordon et al., 1998). The sequences (all between 870 and 1040 bp in length) were compared against the Nucleotide collection (nr/nt) database, which includes all GenBank, RefSeq Nucleotides, EMBL, DDBJ and PDB sequences (excluding EST, GSS, STS, PAT, WGS and HTGS phases 0, 1 and 2 databases), using the nucleotide blast (blastn) Basic Local Alignment Search Tool available through the National Center for Biotechnology Information website (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Construction of the phylogenetic tree was performed using the Fast Minimum Evolution method (FASTME program) (Desper and Gascuel, 2004) available as an option in the NCBI Blast program suite. The tree image shown in this paper was made using the Dendroscope program (Huson et al., 2007).

2.5. Growth of isolated Haloarchaea strains on monoaromatic acids and PAHs

Inoculum for each isolate was prepared by transferring colonies from MHSM agar plates, containing 0.4 mM p-hydroxybenzoic acid and 100 μg mL^{-1} chloramphenicol, to 50 mL of MHSM-YT media. Cultures were grown at 40 °C with shaking at 150 rpm for 168 h (7 d). A volume of 20 mL of the culture was centrifuged for 10 min at 5 °C and 3000*g* and the resulting cell pellet were transferred to Erlenmeyer flasks (125 mL) for growth experiments. Those strains that presented best growth were selected for growth on polyaromatic hydrocarbons.

For growth on monoaromatic acids cell pellets were resuspended in 50 mL of MHSM amended with a mixture of benzoic acid, p-hydroxybenzoic acid, and salicylic acid (1.5 mM each), with or without the addition of 0.05% yeast extract. For growth on polycyclic aromatic hydrocarbons cell pellets were resuspended in 50 mL of MHSM containing a mixture of naphthalene, anthracene, phenanthrene, pyrene and benzo[a]anthracene (0.3 mM each), with or without 0.05% yeast extract.

Inoculated flasks were incubated at 40 °C with shaking at 150 rpm for 168 h. Noninoculated controls (abiotic control) were incubated under the same conditions. Growth of the strains was determined visually by following the appearance of a red coloration characteristic of all isolates and attributed to the formation of carotenoid pigments typical of Haloarchaea (Oren, 1994).

2.6. Determination of PAH degradation by high performance liquid chromatography (HPLC)

Evaluation of the degradation potential of the isolated strains was performed in the same media as used for PAH growth experiments. This experiment was run in triplicate and results were expressed as the averages of the 3 measurements. Non-inoculated controls containing PAH's were incubated under the same conditions. Control cultures containing acetone without PAHs were also inoculated and in all cases no growth or color formation was observed.

After 168 h of incubation at 40 °C with shaking at 150 rpm the cultures were transferred to a separatory funnel and PAHs were extracted with 20 mL volumes of dichloromethane. The dichloromethane in the combined extracts was removed by rotator evaporation and the PAH's were resuspended in acetonitrile. Degradation was determined by HPLC using a ZORBAX C_{18} reverse-phase column (SUPELCO). Separation was achieved by isocratic elution in acetonitrile:water (70:30) as mobile phase, with a flow rate of 0.6 mL min^{-1} and UV absorbance detector set at 254 nm. Duplicate 20 μL injections were performed for each sample and peak areas for each compound were averaged and percent degradation was calculated by comparison to the peak areas obtained for the non-inoculated controls.

2.7. Treatment of produced water with Haloarchaea isolates

Produced water, which had been treated with hydrogen peroxide to reduce COD and hydrogen sulfide, was obtained from the Petrobras (Petróleo Brasileiro SA) refinery in São Sebastião, SP, Brazil, and used with and without sterilization (autoclaving for 15 min at 121 °C). The hydrogen peroxide treated produced water contained approximately 100 g L^{-1} of NaCl and a COD of 1345 mg L^{-1}. No other components were added. Erlenmeyer flasks (125 mL) containing 50 mL of the produced water were inoculated and incubated for 168 h as described above for the aromatic compound degradation experiments except that cell pellets from 40 mL cultures were used to inoculate 100 mL of produced water. Non-inoculated controls were also run. COD reduction was determined and degradation was expressed as % of COD reduction. Experiments were run in triplicate and results are expressed as the averages of the 3 measurements.

To determine COD the produced water cultures and raw produced water were centrifuged at 3000*g* for 15 min at 5 °C and the supernatant removed to separate screw cap tubes. The primary interference in COD measurements is chlorides. Due to the high salt content of the produced water, excess chloride was removed by addition of 2.6 g of silver nitrate to 6 mL of sample supernatant followed by vortexing for 1 min and then centrifugation for 1 min at 2000*g*. The supernatants were removed to separate screw cap tubes for subsequent analysis. COD was determined as follows: 2 mL of supernatant in a screw cap tube was amended with 0.05 g of mercury sulfate, 0.5 mL of 1.0 N potassium dichromate and 2.5 mL of concentrated sulfuric acid (containing 5.5 mg silver sulfate per gram), and refluxed for 2 h at 150 °C. After cooling, COD was determined by absorbance using a Hach model DR-2000 spectrophotometer (Hach Company, USA) according to manufactures instructions.

3. Results and discussion

3.1. Isolation and phylogenetic identification of Haloarchaea

Haloarchaea were isolated from 5 hypersaline environments. Previous analysis demonstrated that all isolates were Gram negative, pleomorphic, motile, red-pigmented, and cell extracts showed absorption peaks at 527 nm and 494 nm, characteristic of carotenoid pigments of Haloarchaea (Cuadros-Orellana et al., 2006). Lipid analysis of these strains demonstrated that they contained diether lipids characteristic of Archaea (Cuadros-Orellana et al., 2006).

After evaluation of growth on p-hydroxybenzoic acid ten isolates were chosen for further characterization. 16S rDNA phylogenetic analysis of the chosen Haloarchaea isolates revealed that all were members of the genus *Haloferax*. The isolates and the GenBank accession numbers for the 16S rDNA sequences are as follows (isolate designation (GenBank accession number)): BO3

(HQ438272), BO6 (HQ438273), BO7 (HQ438274), PR13 (HQ438275), MM17 (HQ438276), MM27 (HQ438277), AA31 (HQ438278), AA35 (HQ438279), AA41 (HQ438280) and CL47 (HQ438281). None of the isolates had identical 16S sequences. Table 1 shows the results of blast analysis of the 16S sequences obtained from the isolates and the first top ranking Blast match for each isolate. It should be noted however, that the choice of the first ranking Blast match is somewhat arbitrary because in the case of all of the cloned 16S genes there are numerous other sequences with equivalent scores and the distinction between species based on 16S often is in the 1–2% difference range.

In order to get a better understanding of the phylogenetic relationships of the isolates a 16S phylogenetic distance tree was created from multiple sequence alignments using all the isolates, the first two highest ranking matches for each isolate and additional *Haloferax* strains with more distant relation to the isolates (Fig. 1).

Using the distance tree as a guide three groupings can be inferred for the Haloarchaea isolates. The first group contains isolates BO7, AA41, MM27, MM17 and PR13, all of which appear to be most closely related to *H. alexandrinus* strains, the type strain of which was isolated from a solar saltern in Alexandria, Egypt (Asker and Ohta, 2002). The second contains isolates BO3, BO6, AA31 and AA35, which appear to share closest relation with strains of *H. alexandrinus* and *H. prahovense*, the type strain of which was isolated from a hypersaline environment in Prahova county, Romania (Enache et al., 2007). The fourth grouping contains only CL47. CL47 is the most distantly related to the other isolates and most closely related to *H. sulfurifontis* whose type strain (strain M6T) was isolated from a sulfide- and sulfur-rich spring in south-western Oklahoma, USA (Elshahed et al., 2004).

3.2. Growth on aromatic compounds

The ten strains were then grown in a mixture of p-hydroxybenzoic acid, benzoic acid and salicylic acid, (1.5 mM each) without and with 0.05% yeast extract. *H. volcanii* DSMZ 3757 was grown in the same media for comparison. Growth was evaluated on the basis of the extent of red pigment formation (Table 2). With the exception of AA35 and AA41 all isolates exhibited poor grow on this mixture of aromatic acids as the sole carbon source in the absence of 0.05% yeast extract. However, growth in the presence of these compounds was significantly higher when 0.05% yeast extract was present. Isolates BO6 and AA41 grew the best in the presence of yeast extract and overall growth of all strains grown in the presence of yeast extract was as good or better than that of strains AA35 and AA41 in the absence of yeast extract.

The isolates were then grown in a mixture of naphthalene, anthracene, phenanthrene, pyrene and benzo[a]anthracene (0.3 mM each) without and with 0.05% yeast extract (Table 3). In the absence of yeast extract all strains, with the exception of BO7, grew poorly as indicated by red pigment formation. The addition of 0.05% yeast extract produced similar results as for growth on the mixture of aromatic acids, producing a significant improvement in pigment formation for all isolates and *H. volcanii*. The isolates demonstrating the most intense pigment formation in the presence of yeast extract were BO7, PR13, MM17, MM27, along with the control strain *H. volcanii*.

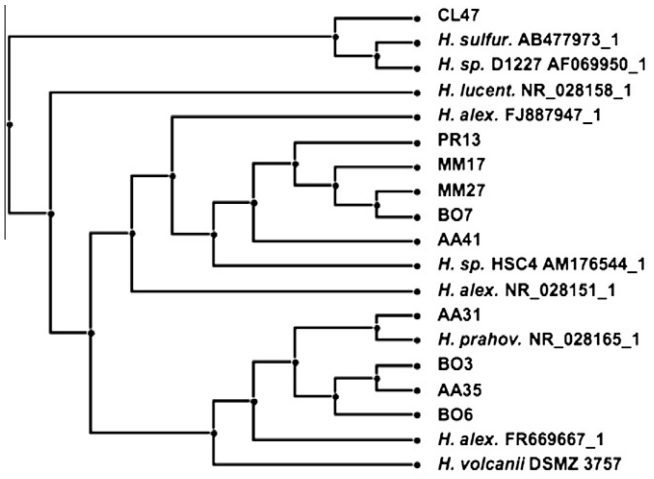

Fig. 1. 16S rDNA cladogram of the Haloarchaea isolates, their first two blast hits and additional *Haloferax* strains derived from a distance tree matrix produced using the Fast Minimum Evolution method. Abbreviations: alex. = *alexandrinus*, lucent. = *lucentense*, prahov. = *prahovense* and sulfur. = *sulfurifontis*.

Table 1
Phylogenetic identities of the haloarchaea isolates based on 16S sequences.

Query strain(s)	First top ranking blast match	GenBank accession	Query coverage	Identity
BO3, BO6, AA31, AA35	*Haloferax alexandrinus* strain KCTC 12962	FR669667.1	98%, 99%, 100%, 100%	99%, 98%, 99%, 99%
BO7, MM17, AA41, PR13	*Haloferax sp.* CS1-9	GQ478076.1	100%, 100%, 99%	99%, 99%, 99%
MM27	*Haloferax sp.* HSC4	AM176544.1	97%	97%
CL47	*Haloferax sulfurifontis* JCM 12327	AB477973.1	99%	96%

Table 2
Growth of strains in MHSM containing p-hydroxybenzoic acid, benzoic acid and salicylic acid, (1.5 mM each) without and with 0.05% yeast extract[a].

Strains	Growth without yeast extract (h)			Growth with yeast extract (h)		
	48	72	168	48	72	168
BO3	+	+	+	++	++	++
BO6	+	+	+	+++	+++	+++
BO7	+	+	+	++	++	++
PR13	+	+	+	++	++	++
MM17	+	+	+	++	++	++
MM27	+	+	+	++	++	++
AA31	+	+	+	++	+++	+++
AA35	+	+	++	++	++	++
AA41	++	++	++	+++	+++	+++
CL47	+	+	+	++	++	+++
H. volcanii	+	+	+	++	++	++

[a] Pigment formation was scored as + (slight), ++ (moderate) and +++ (intense).

Table 3
Growth of strains in MHSM containing naphthalene, anthracene, phenanthrene, pyrene plus benzanthracene (0.3 mM each) without and with 0.05% yeast extract[a].

Strains	Growth without yeast extract (h)			Growth with yeast extract (h)		
	48	72	168	48	72	168
BO3	–	+	+	–	+	++
BO6	–	+	+	–	+	++
BO7	+	++	++	+	++	+++
PR13	–	+	+	–	++	+++
MM17	–	+	+	–	++	+++
MM27	–	+	+	–	++	+++
AA31	–	+	+	–	+	++
AA35	–	+	+	–	+	++
AA41	–	+	+	–	+	++
CL47	–	+	+	–	+	++
H. volcanii	–	+	+	–	++	+++

[a] Pigment formation was scored as – (no formation), +(slight), ++ (moderate) and +++ (intense).

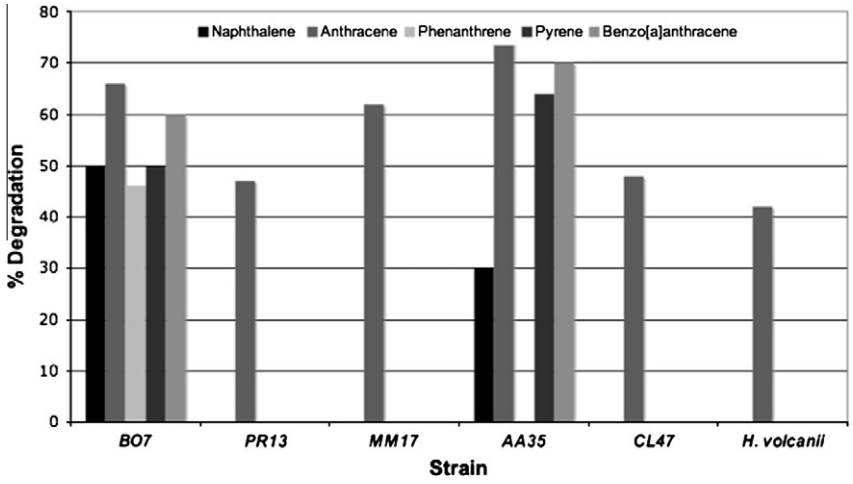

Fig. 2. Degradation of aromatic hydrocarbons by Haloarchaea isolates in the absence of 0.05% yeast extract after 168 h of growth.

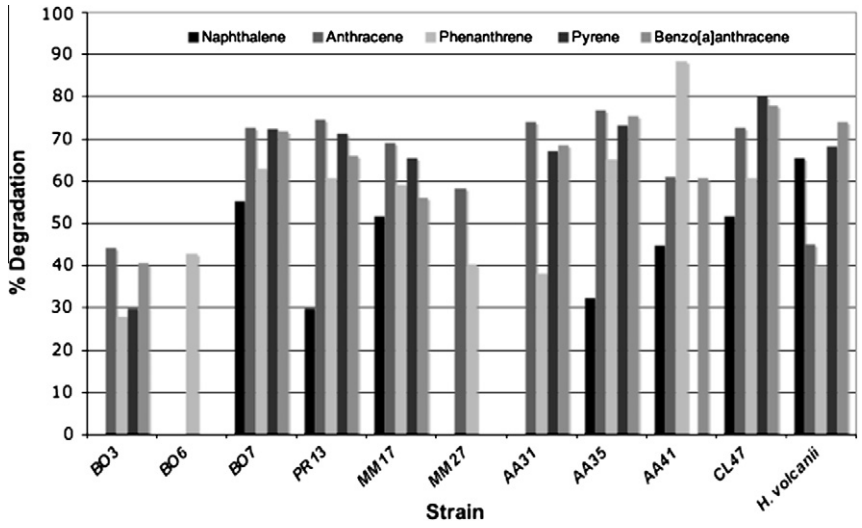

Fig. 3. Degradation of aromatic hydrocarbons by Haloarchaea isolates in the presence of 0.05% yeast extract after 168 h of growth.

To determine the extent of PAH degradation supernatants from cultures grown in MHSM for 168 h with and without 0.05% yeast extract were analyzed by HPLC. In the absence of 0.05% yeast extract only isolates BO7, PR13, MM17, AA35 and CL47 along with *H. volcanii* produced appreciable reductions in PAH content (Fig. 2). However, the extent of removal was not uniform and only isolate BO7 produced a reduction in all five compounds, all of which were reduced by greater than 45%. In addition, isolate AA35 significantly reduced the concentration of all PAHs with the exception of phenanthrene. *H. volcanii* and isolate CL47 were only able to reduce the concentration of the two ring PAH naphthalene (approximately 45%). Interestingly, isolates PR13 and MM17 only reduced the concentration of the three ring PAH phenanthrene.

Addition of 0.05% yeast extract produced a marked increase in PAH removal for all cultures, and in some cases the range of PAH degradation as well (Fig. 3). Under these conditions isolates BO7, PR13, MM17, AA35, CL47 and *H. volcanii* degraded all five PAHs with BO7, MM17 and CL47 degrading over 50% of each compound. Isolates BO6 and MM27 degraded only phenanthrene and anthracene, respectively. Isolates BO3, BO6 and MM27, which did not degrade naphthalene produced the least overall PAH reduction. Isolate AA31, which also did not degrade naphthalene, was more effective in removing the other four PAH's.

3.3. Reduction of COD in petroleum produced water by Haloarchaea isolates

Growth on aromatic acids and hydrocarbons suggested that the Haloarchaea isolates could be useful in the bioremediation of petroleum produced water, which typically is contaminated with these or related compounds. To evaluate this possibility, the isolates were grown, along with the control strain *H. volcanii*, for 168 h in produced water previously treated with hydrogen peroxide that was either non-sterile or sterilized. The effect on the COD content of the produced water is shown in Table 4. In the absence of added Haloarchaea the COD was reduced by 20.2% and 18%, for the non-sterile and sterile produced water, respectively. These data show that 18% of the COD was either volatilized or air oxidized over the 168 h period and also suggests that indigenous microorganisms present in the produced water had a limited ability to reduce COD.

Addition of individual Haloarchaea isolates produced a significant increase in COD reduction in all cases except that of the non-sterile cultures with MM17 where it actually was decreased. The greatest COD reduction was produced by isolates BO6, PR13, AA31, AA35, and CL47 all of which decreased the COD by greater than 65% in the non-sterile cultures. Sterilizing the produced water

Table 4
ACOD reduction (%) following growth of the strains in produced water for 168 h.

Strain	Reduction of COD (%) non-sterile	Reduction of COD (%) sterile
BO3	38	51
BO6	71	67
BO7	34	58
PR13	66	66
MM17	13	39
MM27	68	54
AA31	87	74
AA35	66	49
AA41	59	48
CL47	73	50
H. volcanii	34	25
H_2O_2 treatment only	20	18

had the effect of reducing the extent of COD removal for all of these isolates except PR13, which may indicate that the sterilization process produced more recalcitrant or toxic compounds. However, this was not true in the case of isolates BO3, BO7 and MM17, which saw an increase in COD removal in the sterile cultures. *H. volcanii* produced relatively little COD removal with 34.4% and 25.2% reduction in non-sterile and sterile produced water, respectively. The extent of COD reduction did not necessarily correlate with the range of aromatic hydrocarbons susceptible to degradation for each strain. Isolates PR13, AA31, AA35, and CL4 demonstrated a broad aromatic degradation range and high produced water COD reduction, *H. volcani*i, which was shown to degrade all five PAHs, did not perform well in reducing COD. Conversely, isolate BO6, which only demonstrated the ability to degrade naphthalene, reduced the COD of produced water 71.1% and 67.2%, in non-sterile and sterile produced water, respectively.

4. Conclusions

These results suggest that the ability to metabolize aromatic compounds including PAH's is common to a variety of hypersaline environments and maybe a widespread feature among the Haloarchaea genus *Haloferax*. In addition, the ability of the Haloarchaea isolates to reduce the COD of petroleum produced water suggests that the use of Haloarchaea for the bioremediation of hypersaline environments contaminated with aromatic compounds is possible and a promising area for future research.

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