Biodegradation (2011) 22:83–93
DOI 10.1007/s10532-010-9378-4

ORIGINAL PAPER

Evaluation of haloalkaliphilic sulfur-oxidizing microorganisms with potential application in the effluent treatment of the petroleum industry

P. Olguín-Lora · S. Le Borgne · G. Castorena-Cortés · T. Roldán-Carrillo · I. Zapata-Peñasco · J. Reyes-Avila · S. Alcántara-Pérez

Received: 18 December 2009 / Accepted: 15 June 2010 / Published online: 26 June 2010

Abstract Haloalkaliphilic sulfur-oxidizing mixed cultures for the treatment of alkaline–saline effluents containing sulfide were characterized and evaluated. The mixed cultures (IMP-PB, IMP-XO and IMP-TL) were obtained from Mexican alkaline soils collected in Puebla (PB), Xochimilco (XO) and Tlahuac (TL), respectively. The Ribosomal Intergenic Spacer Analysis (RISA) revealed bacteria related to *Thioalkalibacterium* and *Thioalkalivibrio* in IMP-XO and IMP-PB mixed cultures. *Halomonas* strains were detected in IMP-XO and IMP-TL. In addition, an uncultured *Bacteroides* bacterium was present in IMP-TL. Mixed cultures were evaluated at different pH and NaCl concentrations at 30°C. IMP-PB and IMP-TL expressed thiosulfate-oxidizing activity in the 7.5–10.5 pH range, whereas IMP-XO presented its maximal activity with 19.0 mg O_2 $g^{-1}_{protein}$ min^{-1}, at pH 10.6; it was not affected by NaCl concentrations up to 1.7 M. In continuous culture, IMP-XO showed a growth rate of 15 day^{-1}, productivity of 433.4 $mg_{protein}$ l^{-1} day^{-1} and haloalkaliphilic sulfur-oxidizing activity was also detected up to 170 mM by means of *N*-methyl-diethanolamine (MDEA). Saline–alkaline soil samples are potential sources of haloalkaliphilic sulfur-oxidizing bacteria and the mixed cultures could be applied in the treatment of inorganic sulfur compounds in petroleum industry effluents under alkaline–saline conditions.

Keywords Haloalkaliphilic bacteria · Sulfur-oxidizing · RISA · *Thioalkalibacterium* · *Thioalkalivibrio*

P. Olguín-Lora (✉) · G. Castorena-Cortés · T. Roldán-Carrillo · I. Zapata-Peñasco · J. Reyes-Avila · S. Alcántara-Pérez
Instituto Mexicano del Petróleo, Eje Central Lázaro Cárdenas 152, 07730 Mexico, DF, Mexico
e-mail: polguin@imp.mx

S. Le Borgne
Universidad Autónoma Metropolitana, Unidad Cuajimalpa, Sede Artificios: Artificios 40, 01120 Mexico, DF, Mexico

Introduction

Sulfur compounds are released from various industrial processes such as water treatment plants, food preparation, petroleum plants, paper industry and farming (Ramírez et al. 2009). These compounds are considered as toxic, irritating and corrosive. Many processes have been proposed to remove them such as incineration, adsorption, chemical oxidation and biological oxidation (Lens and Hulshoff 2000; Janssen et al. 2001). In the oil and gas industry, highly caustic amine solutions such as *N*-methyl-diethanolamine (MDEA) are used to remove sulfide and organic sulfur compounds from refinery hydrocarbon

streams, the sulfidic spent caustic effluents generated, are then submitted to expensive physicochemical treatments requiring high pressure and temperature to regenerate the amine solutions and eliminate sulfur compounds (Kohl and Nielsen 1997). Developing less expensive and more environmentally friendly alternatives to treat such effluents would be advantageous.

The advantage of the biotechnological process is that it is safe and affordable as it takes place at ambient temperature and normal atmospheric pressure (Cline et al. 2003). For example, the first reports of biological H_2S removal systems were performed at neutral or acid conditions (Gabriel and Deshusses 2003), but some authors suggest that under haloalkaline conditions the bioprocess is highly improved because sulfides are found in a caustic aqueous stream (pH up to 10) and a high content of sodium (up to 27.2 g l^{-1}) in some industries as in a petroleum refinery (Olmos et al. 2004; Bosch et al. 2007; Gonzalez-Sanchez and Revah 2009).

There has been more interest, in recent years, in studying soda soils and lakes since they are alkaline–hypersaline environments, and therefore a potential source of diverse microbiota. The alkaliphilic sulfur-oxidizing bacteria (ASB) are chemolithoautotrophic microorganisms able to grow at pH 9–11, and oxidize reduced sulfur compounds such as sulfide and thiosulfate (Sorokin and Kuenen 2005; Sorokin et al. 2007). *Thioalkalivibrio* are used in H_2S removal studies. These can also grow in saline environments (Bosch et al. 2007; Sorokin et al. 2008) and thus are classified as haloalkaliphilic sulfur-oxidizing (HAS) bacterium.

The main biological reactions are H_2S conversions to elemental sulfur or sulfate carried out by autotrophic sulfide-oxidizing microorganisms. These are seen in Eqs. 1 and 2, equilibrium depends on the availability of dissolved oxygen (Janssen et al. 1995; Gonzalez-Sanchez et al. 2009).

$$H_2S + \frac{1}{2}O_2 \rightarrow S^0 + H_2O \quad (1)$$

$$H_2S + 2O_2 \rightarrow {SO_4}^{2-} + 2H^+ \quad (2)$$

The aim of this research was to evaluate sulfur-oxidizing microbial mixed cultures, potentially applicable to the biological treatment of alkaline–saline effluents containing sulfur compounds.

Materials and methods

Sampling sites

Ten surface soil samples were collected from Mexican saline alkaline sites: Tehuacán, Puebla (PB), from Xochimilco (XO) and Tlahuac (TL). Temperature and pH were measured in situ during the sampling. The samples were collected, transported and stored at 4°C until analyzed.

Enrichment cultures of haloalkaliphilic sulfur-oxidizing microorganisms

The mineral medium used for the enrichment cultures of HAS bacteria was previously described by Sorokin et al. (2001) with 40 mM thiosulfate as the only energy source. This substrate was selected as a model molecule due to its stability at aerobic conditions, since sulfide is difficult to use because of its volatility and toxicity (Sorokin et al. 2008). Mineral media pH was adjusted to 10 by using 1 M NaOH. A soil mixture was prepared from each ten samples of three sites to obtain a composite sample. Five grams of each ten soil samples were inoculated into Erlenmeyer flasks containing 30 ml of mineral medium with thiosulfate, incubated at 30°C and at 180 rpm agitation. Thiosulfate concentration were monitored daily to detect the presence of sulfur-oxidizing activity. IMP-PB, IMP-XO, and IMP-TL mixed cultures were transferred three times until the substrate was completely oxidized up to the 24 h time. Biomass was centrifuged at 9,000×*g* for 15 min and re-inoculated into fresh medium. Active enriched cultures were transferred to the bioreactors.

Respirometric studies

Mixed culture sulfur-oxidizing activity was measured by means of the oxygen uptake rate $-q(O_2)$, corrected for sulfur chemical oxidation and endogenous respiration (Buisman et al. 1989; Gonzalez-Sanchez et al. 2008). Respirometric studies were performed in batch cultures in 60 ml serum bottles containing 30 ml of mineral medium (Sorokin et al. 2001) at pH 10. Inocula were grown up to 24 h and the initial protein concentration was 10 mg l^{-1}. Mineral medium was saturated with a filtered oxygen

air stream before being added to the serum bottles. The respirometric studies included the evaluation of thiosulfate and sulfide, as energy source and electron acceptor. Initial thiosulfate concentrations were adjusted from 0 to 5 mM. The effect of NaCl, MDEA and pH on the thiosulfate-oxidizing activity was also evaluated. NaCl and MDEA concentrations were adjusted from 0 to 1.7 M and 0 to 130 mM, respectively. The pH was adjusted from 6.8 to 11.0 by using a phosphate buffer, Na_2CO_3 and $NaHCO_3$. Assays were incubated at 30°C. When sulfide was used instead of thiosulfate, the initial sulfide concentrations were adjusted from 0 to 2 mM, using concentrated sodium sulfide stock solution.

Continuous cultures

Three 1.5 l continuous bioreactors were implemented to determine HAS microbial culture kinetic parameters. Bioreactors were fitted with pH and oxygen controllers (ADI 1030 biocontrollers, Applikon). Culture conditions were: pH 10 ± 0.2, temperature 30°C, 200 rpm agitation and airflow controlled to maintain an average dissolved oxygen concentration of 5 mg l^{-1} (Alcántara et al. 2004). Reactors were operated as chemostats with different dilution rates. Aliquots (10 ml) were collected to determine protein, thiosulfate and sulfate concentrations.

Molecular analyses

Microbial DNA was extracted from mixed cultures which were applied as inocula in the respirometric studies by using the Ultra Clean Soil DNA kit (MoBio). The Ribosomal Intergenic Spacer Analysis (RISA) plus a 500 bp stretch of the 16S rDNA gene were amplified with the B1055 y 23SOR primer, as previously described (Acinas et al. 1999). Polymerase chain reaction (PCR) amplifications were carried out in a T-personal thermocycler (Biometra), using the Hot Star *Taq* DNA polymerase (Qiagen). Amplification conditions were as follows: an initial denaturation at 95°C for 15 min; 35 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min; and a final extension step of 10 min at 72°C. RISA-PCR products were analyzed by electrophoresis in 1% agarose gels. The major emerging bands were cut out from the gel and purified with an UltraClean GelSpin kit (MoBio). Sequencing of the RISA fragments was performed with an ABI PRISM 310 sequencer and the B1055 primer, to obtain the sequence of the 16S rDNA gene. The obtained sequences were compared to public database sequences available at the National Center for Biotechnology Information (NCBI) by using the BLAST program (Karlin and Altschul 1990).

Analytical methods

Protein was quantified by the Lowry method (1951). The oxygen concentration in the liquid phase was measured with an oxymeter YSI (5300 Biological Oxygen Monitor). Thiosulfate and sulfate were analyzed by capillary electrophoresis using a capillary ion analyzer (Waters) equipped with a micro-capillary silica-fused column and an UV detector at 254 nm. Thiosulfate and sulfate standard solution at concentrations ranging from 5 to 100 mg l^{-1} were used. Total organic carbon (TOC) and inorganic carbon were determined with a total organic carbon analyzer (Shimadzu TOC analyzer TOC-5000A). Electrical conductivity as described by Fernández et al. (2006), was also measured.

Analysis of results (experimental data)

The effect of thiosulfate concentration was measured according to the Monod equation (Eq. 3).

$$-q(O_2) = -q_{max}(O_2) \cdot C_{S_2O_3^{2-}} / \left(K_s + C_{S_2O_3^{2-}}\right) \quad (3)$$

where $-q(O_2)$ is the specific oxygen uptake rate (O_2 $g_{protein}^{-1}$ min^{-1}); $-q_{max}(O_2)$ the maximal oxygen uptake rate (O_2 $g_{protein}^{-1}$ min^{-1}); $C_{S_2O_3^{2-}}$ the substrate concentration (mM); and K_s is the apparent affinity constant (mM).

A modified Monod model was implemented, as described by Han and Levenspiel (1988) introducing as a new parameter, the sulfide concentration; inhibiting completely the maximal sulfide-oxidizing activity: IC_{100} (Eqs. 4, 5) or the sulfide concentration; inhibiting half the maximal sulfide-oxidizing activity: IC_{50}

$$-q_{max}(O_2)_{apparent} = -q_{max}(O_2) \cdot (1 - C_{S^{2-}}/IC_{100})^n \quad (4)$$

$$K_{S\,apparent} = K_S \cdot (1 - C_{S^{2-}}/IC_{100})^m \quad (5)$$

where $-q_{max}(O_2)_{apparent}$ is the specific oxygen uptake rate (O_2 $g_{protein}^{-1}$ min^{-1}); $C_{S^{2-}}$ the substrate concentrations

(mM); IC_{100} the substrate concentration that completely inhibits the sulfide-oxidizing activity (mM); $K_{S\ \text{apparent}}$ the affinity constant (mM); and m, n is the model adjustment constants.

The culture behaviors in a chemostat bioreactor were studied by using the model described by Pirt (1975), (Eqs. 6–10) to determine different parameters.

$$C_X = D \cdot Y_{X/S} \cdot \left(C_{S_2O_3^{2-}(\text{initial})} - C_{S_2O_3^{2-}}\right) \Big/ \left(D + m \cdot Y_{X/S}\right) \quad (6)$$

$$C_{S_2O_3^{2-}} = \left(D + m \cdot Y_{X/S}\right) \cdot K_S \Big/ \left(\mu_{\max} - \left(D + Y_{X/S} \cdot m\right)\right) \quad (7)$$

$$D_{\max} = \mu_{\max} \cdot \left(1 - \sqrt{K_S / K_S + C_{S_2O_3^{2-}}}\right) \quad (8)$$

$$X_{\max} = Y_{X/S} \cdot \left(C_{S_2O_3^{2-}} + K_S - \sqrt{K_S \cdot \left(K_S + C_{S_2O_3^{2-}}\right)}\right) \quad (9)$$

$$P_{\max} = D_{\max} \cdot X_{\max} \quad (10)$$

where C_X is the biomass concentration ($mg_{protein}\ l^{-1}$); D the dilution rate (day^{-1}); $Y_{X/S}$ the substrate-to-biomass yield ($mg_{protein}\ mM^{-1}$); $C_{S_2O_3^{2-}(\text{initial})}$ the initial concentration of thiosulfate at the entrance of the chemostat (mM); $C_{S_2O_3^{2-}}$ the substrate concentration within the chemostat (mM); m the maintenance coefficient; $\mu_{\max}$ the maximal growth rate (day^{-1}); K_s the half-saturation constant (mM); $D_{\max}$ the maximum dilution rate (day^{-1}); $X_{\max}$ the maximum biomass concentration ($mg_{protein}\ l^{-1}$); and $P_{\max}$ is the maximum productivity ($mg_{protein}\ l^{-1}\ day^{-1}$).

Results

Physicochemical characterization of the samples

Soil samples (PB, XO and TL) physicochemical analyses are shown in Table 1. XO was characterized by a higher conductivity and organic carbon content, nevertheless its pH was relatively lower. PB showed the highest pH and inorganic carbon content. TL samples had a pH range from 8.1 to 10 and presented intermediate organic carbon contents compared to PB and XO. According to the Rodriguez-Valera (1993) report, soils containing >0.2% soluble salts are considered saline. PB, XO and TL samples salinity were 0.23, 0.44 and 0.18% respectively, PB and XO are therefore considered saline by means of electrical conductivity data and salinity values (NaCl).

Enrichment cultures of the haloalkaliphilic sulfur-oxidizing microorganisms

In the thiosulfate enriched cultures employed as an energy source, and electron donor, the HAS microbial cultures were obtained and designated as IMP-PB, IMP-XO and IMP-TL.

Few studies have been reported concerning the microbial community in soil and water of alkaline and saline environments in Mexico (Jan-Roblero et al. 2004; Alazard et al. 2007; Valenzuela-Encinas et al. 2009) and especially concerning HAS microorganisms (Gonzalez-Sanchez and Revah. 2007).

Thiosulfate and sulfide-oxidizing activity

The thiosulfate oxidation rate was evaluated on the basis of the oxygen uptake rate for each mixed culture. This technique has found to be a very efficient tool for kinetic parameter estimation, as the specific oxygen uptake rate and apparent affinity constant (Gonzalez-Sanchez and Revah 2009). Table 2 shows the kinetic parameters evaluated in this study. The similar values of apparent affinity constant, K_s (0.21, 0.23, and 0.22 mM) were obtained for IMP-PB, IMP-XO and IMP-TL mixed cultures, respectively. However, IMP-XO mixed culture presented the highest $-q_{\max}(O_2)$ compared to the IMP-PB and IMP-TL mixed cultures. No thiosulfate toxic effect was observed in the tested experimental range (data not shown). Thiosulfate concentrations varied from 0.1 to 10 mM and the concentration allowing a maximal oxygen uptake was established as 1 mM.

Figure 1 shows the behavior of specific oxygen uptake rate $-q_{\max}(O_2)$ as a function of different sulfide concentrations. The experimental data were fitted to the model of Han and Levenspiel (1988), obtaining determination coefficients (R^2) between 0.93 and 0.99.

The kinetic data obtained for the IMP-PB, IMP-XO and IMP-TL mixed cultures were very different from those obtained with thiosulfate. Sulfide appeared to be an inhibitory substrate according to the IC_{50} and IC_{100} values (Table 2). The IMP-XO mixed culture

Table 1 Physicochemical characterization of soil samples from Mexican saline and alkaline environments

Sample	PB	XO	TL
Origin	Tehuacan, Puebla	Xochimilco, Mexico City	Tlahuac, Mexico City
pH	9.4–10.1	8.0–9.0	8.1–10.1
Temperature (sampling) (°C)	17	28	23
Temperature (main annual temperature of the site) (°C)	21.9	16	16
Electric conductivity (mS cm^{-1})	3.28–3.85	4.25–9.04	2.28–3.15
Organic carbon (g kg^{-1})	15.3–20.6	75.4–90.4	33.1–47.1
Inorganic carbon (g kg^{-1})	8.3–13.2	1.3–1.3	6.1–11.4
Textural classification	Sandy clay loam	Sandy clay loam	Sandy loam

Table 2 Batch kinetic parameters of the IMP-PB, IMP-XO and IMP-TL mixed cultures using sulfur compounds as energy source

Parameter	Cultures		
	IMP-PB	IMP-XO	IMP-TL
Thiosulfate-oxidizing activity			
q_{max} (mg O_2 $g^{-1}_{protein}$ min^{-1})	15.6	23.6	17.0
Ks (mM)	0.21	0.23	0.22
pH			
Range	7–10.8	8–11	7–11
Optimum	9–10	10.5	9.8
NaCl			
IC_{50} (M)	1.7	ND	0.6
MDEA			
IC_{50} (mM)	96	79	27
IC_{100} (mM)	197	175	214
Sulfide-oxidizing activity			
q_{max} (mg O_2 $g^{-1}_{protein}$ min^{-1})	8.8	19.0	11.2
K_s apparent (mM)	0.16	0.30	0.06
IC_{50} (mM)	0.8	0.6	0.6
IC_{100} (mM)	1.6	1.4	0.9

ND not determined

presented the major sulfide-oxidizing activity with 19.0 mg O_2 $g^{-1}_{protein}$ min^{-1} and a K_s 0.30 mM.

Influence of NaCl and pH on thiosulfate-oxidizing activity

IMP-PB and IMP-TL thiosulfate-oxidizing activity were dependent on the NaCl concentrations (Fig. 2A). This activity decreases with increasing concentration of NaCl in the systems. The kinetic behavior at different concentrations of NaCl permitted estimation

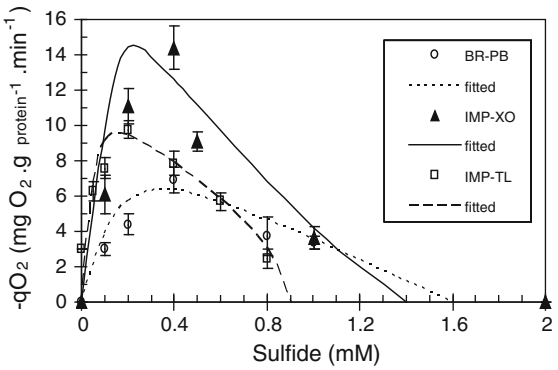

Fig. 1 Sulfide concentration effect on sulfide-oxidizing activity based on the oxygen uptake rate in IMP-XO, IMP-PB and IMP-TL mixed cultures, respectively. The *error bars* correspond to standard deviations

of the concentration of NaCl that reduced the thiosulfate-oxidizing activity by 50% (IC_{50}) (Table 2). While IMP-XO culture behavior was not affected by NaCl concentrations tested.

The test result showed that the three mixed cultures were obligate alkaliphiles with an optimum pH around 10. IMP-TL thiosulfate-oxidizing activity was 40% of its maximal activity at pH 7, while IMP-PB, at this pH was completely inactive. IMP-XO expressed an activity which is clearly alkaliphile since its maximum activity was registered at pH 10.6 and 80% of its activity was still maintained at pH 11.0 (Fig. 2B).

Effect of the MDEA in the thiosulfate oxidizing activity

In oil industrial processes amine solutions containing *N*-methyl-diethanolamine (MDEA) are used to remove

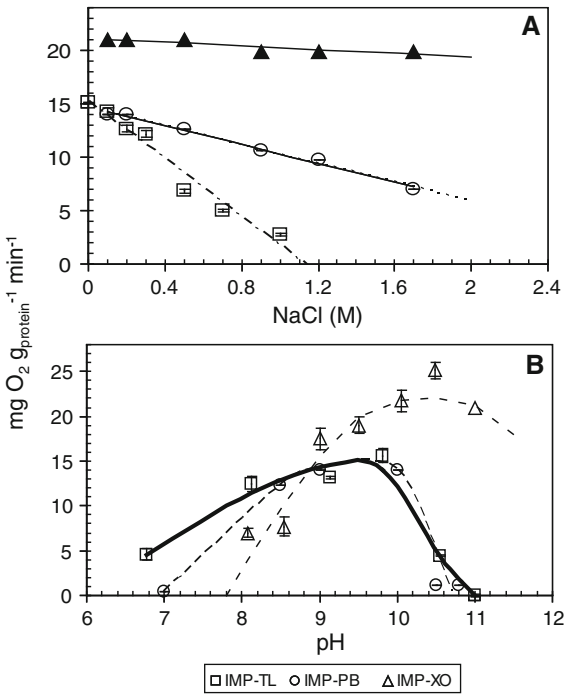

Fig. 2 **A** NaCl concentration effect, and **B** pH effect on thiosulfate oxidation rate, both in IMP-XO, IMP-PB and IMP-TL mixed cultures, respectively. The *error bars* correspond to standard deviations

sulfur compounds from refinery gaseous effluents and could therefore be found in the caustic waters flowing downstream (Carey et al. 1991). The biological treatment for these effluents must consider the effect of MDEA in the HAS microbial cultures. Even though no reports about the relationship between the thiosulfate oxidizing activity and the MDEA have been described. The three mixed cultures showed tolerance to MDEA, which could be grown on, and had thiosulfate oxidizing activity up to 170 mM of this compound. The IC_{50} and IC_{100} for MDEA are shown in Table 2, and IMP-TL mixed culture presented the highest tolerance (210 mM) to this compound. This result demonstrates that IMP mixed cultures could be used in the treatment of downstream water and the gas sweetening process.

Continuous systems

The three mixed culture behavior in a chemostat bioreactor was studied by using the model described by Pirt (1975) to determine the kinetic parameters of the biomass growth (Table 3). The results clearly highlighted the differences between the cultures. The IMP-TL system achieved biomass productivity (P_{max}) of 166 $mg_{protein}$ l^{-1} day^{-1} with a reactor dilution rate of 4.22 day^{-1}; the maximal biomass concentration within the reactor was 39.4 mg l^{-1}. The IMP-XO mixed culture had the highest maximal growth rate (15 day^{-1}) and allowed dilution rates of up to 7.14 day^{-1} and a productivity of 433.4 $mg_{protein}$ l^{-1} day^{-1}. Reactor evaluation shows that IMP-XO results are optimum to be applied within the industrial sulfur stream treatment.

Molecular characterization of the enriched haloalkaliphilic sulfur-oxidizing cultures

Molecular identification in the three mixed cultures showed a low species diversity, due to extreme growth conditions used for the enrichment cultures. Figure 3 shows the bands obtained by means of RISA analysis from IMP-PB, IMP-XO and IMP-TL mixed cultures. Five sequences obtained in this study were analyzed and these determined the closest relative taxa and were deposited into the GenBank under the accession numbers DQ154901–DQ154905.

Bands 1 and 2 were obtained in the IMP-XO mixed culture. The sequence derived from band 1 was closer to the sulfur-oxidizing bacterium *Thioalkalimicrobium cyclum* with 99% identity. Band 2 matched with *Halomonas variabilis* strain DSM 3051 (98% identity). Only one band was detected in the IMP-PB culture (band 3) and it was related (97% identity) to an uncultured Gammaproteobacterium. Bands 4 and 5 were obtained from the IMP-TL mixed culture, the first band corresponded to *Halomonas* sp. with 99% identity and the second one had 99% identity with a uncultured *Bacteroides* bacterium.

Table 3 Chemostat bioreactor kinetic parameters of the IMP-PB, IMP-XO and IMP-TL mixed cultures

Parameter	Cultures		
	IMP-PB	IMP-XO	IMP-TL
μ_{max} (day^{-1})	1.5	15	6.0
K_s (mM)	4.8	14	3.72
$Y_{X/S}$ ($mg_{protein}$ mM^{-1})	2.8	2.5	1.33
D_{max} (day^{-1})	0.98	7.14	4.22
X_{max} ($mg_{protein}$ l^{-1})	74.39	60.70	39.38
P_{max} ($mg_{protein}$ l^{-1} day^{-1})	73.18	433.4	166.09

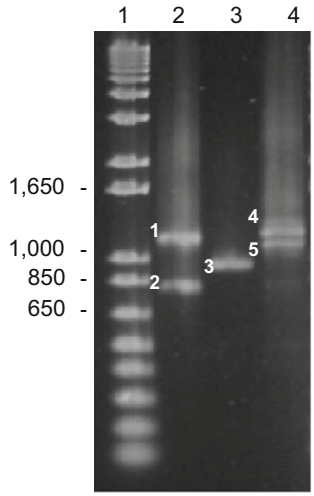

Fig. 3 Ribosomal Intergenic Spacer Analysis (RISA) in haloalkaliphilic sulfur-oxidizing cultures. *Band 1* IMP-XO *Thioalkalimicrobium cyclum* Identity (ID) 99%; *band 2*: IMP-XO *Halomonas variabilis* ID 98%; *band 3* IMP-PB uncultured *Gamma proteobacterium* ID 97%; *band 4* IMP-TL *Halomonas* sp. ID 99%; *band 5* IMP-TL uncultured *Bacteroidetes* bacterium ID 99%

Discussion

The sampling areas for this research were reported as saline alkaline environments, since their dissolved salt content were sodium-chloride, carbonates, sulfate, and in a lesser proportion salts from calcium, potassium, magnesium, lithium and boron. Some of these ecosystems are also rich in sulfur (Alcocer and Hammer 1999) and could therefore be considered as a possible source of HAS microorganisms.

The content of organic carbon in XO and TL samples indicated high organic matter content and the PB sample had medium organic matter content according to the soil classification (Fernández et al. 2006). The high inorganic carbon content may indicate the presence of carbonates as has been reported in highly alkaline environments similar to soda lakes characterized by the presence of high levels of sodium carbonate and their derivatives (Alazard et al. 2007). Total dissolved solid in PB, XO and TL were 346.5, 813.6 and 283.5 mg l^{-1} respectively. These results were similar to those described by Bárcenas et al. (2002) for soil samples from Tehuacán, Puebla and Ramos-Bello et al. (2001) for Xochimilco and Tlahuac. The authors reported a high concentration of carbonates, thus explaining the alkalinity of the samples (pH 8–10). The main temperature of sample sites were 21.9°C for PB and 16°C for XO and TL so the microorganisms found were mesophiles.

During the enrichment process one HAS mixed culture was obtained for each place. In the respirometric assays the maximal specific oxygen uptake rate $-q_{max}(O_2)$ achieved on thiosulfate by IMP-PB, IMP-XO, and IMP-TL mixed cultures were consistent with the results obtained by Sorokin and Kuenen (2005) with a pure culture of genus *Thioalkalivibrio* (4.8–35.2 mg O_2 $g_{protein}^{-1}$ min^{-1}) incubated at 30°C.

The three mixed cultures sulfide-oxidizing activity evaluation was higher than a value of 3.8 mg O_2 $g_{protein}^{-1}$ min^{-1} reported by Gonzalez-Sanchez et al. (2009) using a sulfo-oxidant consortium. The IMP-XO mixed culture presented major sulfide oxidizing activity (Table 2); suggesting that it could have a potential application for treatment streams sulfur compounds.

IMP-TL and IMP-PB were moderately salt-tolerant microorganisms capable of growing in 1.2–1.5 M Na^+. Nevertheless sulfur-oxidizing activity decreased, with respect to the Na^+ increase. IMP-XO mixed culture presented a different behavior from the other two cultures and its oxygen uptake rate was not affected by NaCl concentrations up till 1.7 M (Fig. 2A) in the measured range. IMP-XO culture was obtained from the sample with the highest salt concentration hence it is better adapted to Na^+. Besides, a microorganism related to *Halomonas*, and considered as a high salt tolerant bacterium (Shapovalova et al. 2008) was detected in the IMP-XO mixed culture.

IMP-PB and IMP-TL mixed cultures showed a similar thiosulfate-oxidizing activity (Table 2) compared to the value reported by Sorokin et al. (2003) for different *Thioalkalivibrio* strains: 17.6 mg O_2 $g_{protein}^{-1}$ min^{-1} with thiosulfate, at pH 10 and 0.5 M total Na^+. *Thioalkalivibrio* species are reported to be extremely haloalkaliphilic with an upper NaCl concentration of 4.0–4.3 M and an optimum pH of 10.5 (Sorokin and Kuenen 2005; Sorokin et al. 2008). Our results showed that IMP-PB has less NaCl tolerance.

The results obtained in continuous systems of IMP-PB and IMP-TL mixed cultures with thiosulfate only as an energy source, achieved comparable values of the specific growth rate, as those reported by Sorokin and Kuenen (2005) for *Thioalkalimicrobium* (7.92 day^{-1}),

and *Thioalkalivibrio* (2.88 day^{-1}). The substrate-to-biomass yield of IMP-XO (2.5 $mg_{protein}$ mM^{-1}) was similar to that described for *Thioalkalimicrobium* (2.5–3.7 $mg_{protein}$ mM^{-1}) obtained in a continuous culture at controlled pH and limited conditions of thiosulfate (Table 4). The chemostat results highlighted the differences between the *Thioalkalimicrobium/Halomonas* (IMP-XO) and the *Thioalkalivibrio* (IMP-PB) cultures. The first one had a 10 fold higher growth rate (15 day^{-1}) corresponding to its' higher productivity. Nevertheless the substrate to biomass yield and the maximum biomass concentration gave similar results for both cultures. IMP-TL mixed culture with no oxygen limitation and at pH 10.0 presented a complete thiosulfate oxidation with 0.5–1.5 day^{-1} dilution rates, according to the reaction described by Velasco et al. (2004) where 1 mol of thiosulfate oxidizing produced 2 mol of sulfate. At higher dilution rates (>1.5 day^{-1}) a partial thiosulfate oxidation occurred.

This result demonstrates that IMP mixed cultures could be utilized in sulfur inorganic compounds treatment, present in oil industrial streams as natural gas, spent caustic and sour waters under haloalkaline conditions.

A chemolithoautotrophic haloalkaliphilic sulfur-oxidizing bacterium *Thioalkalimicrobium cyclum* was detected in IMP-XO mixed culture. *T. cyclum* has been isolated from the stratified alkaline and saline Mono Lake, California (Sorokin and Kuenen 2005). Some authors suggest that this particular species of HAS is endemic to North America since there are no reports of it in Central Asia or Africa (Sorokin et al. 2007). *Halomonas variabilis* was found in IMP-XO mixed culture, its sequence matched with an *H. variabilis* DSM 3051 strain isolated from Great Salt Lake (Utah, USA) (Arahal et al. 2002). *Halomonas* species have been found in a variety of habitats such as saline ponds as well as deep-sea sediments and waters, they are euryhaline moderate halophiles, and some isolates are alkalitolerant (de Souza et al. 2001; Okamoto et al. 2004). Reports concerning the microbial diversity in Mexico soda lakes, suggest the presence of several members of the Halomonadaceae family such as *Halomonas magadiensis* and *Halomonas eurihalina*, as well as halophilic and alkaliphilic Gram-positive bacteria (Jan-Roblero et al. 2004).

IMP-PB mixed culture showed an uncultured *Gammaproteobacterium*, detected in the alkaline hypersaline Mono Lake, California. The closest cultivable relative was *Thioalkalivibrio* sp. strain $ALMg_2$ from north-eastern Mongolian Lake (Sorokin and Kuenen 2005). *Gammaproteobacteria* class is formed by the known groups of haloalkaliphilic sulfur bacteria, including chemolithotrophic and phototrophic groups (Banciu et al. 2008; Sorokin et al. 2006a, b). *Halomonas* sp. was detected in IMP-TL mixed culture followed by *Halomonas desiderata*, a denitrifying bacterium first described by Berendes et al. (1996) and isolated from municipal sewage. Other *H. desiderata* have been isolated from saline soils in Thailand (AY647309 GenBank accession). Sorokin and Kuenen (2005) isolated bacteria in soda lakes of the genus *Halomonas* which are heterotrophic haloalkaliphilic bacteria capable of sulfur compounds oxidation (thiosulfate and sulfide) to tetrathionate in the presence of acetate. One band was detected from IMP-TL mixed culture corresponding to a uncultured *Bacteroidetes* bacterium phylotype (DQ028407 Gen Bank accession) detected in alkaline (pH 10.4) sites from Greenland (Schmidt et al. 2006).

Conclusion

The results obtained using samples of saline alkaline Mexican soils are potential sources of haloalkaliphilic sulfur-oxidizing bacteria. The IMP-XO, IMP-PB and IMP-TL mixed cultures obtained could be applied in the treatment of inorganic sulfur compounds both at high pH and salinity. *Thioalkalimicrobium/Halomonas* (IMP-XO) culture performed well at high dilution rates and in halotolerance conditions even at 1.7 M Na^+ and pH 10.

The capability of these bacteria to grow on sulfur compounds, under highly alkaline and saline conditions, can be potentially used to develop new biotechnological processes for petroleum industrial application related to harmful sulfur compounds in natural gas treatment. Additionally, mixed cultures have the capability to grow in the presence of MDEA as found in gas sweetening processes.

Table 4 Comparison of the characteristics of haloalkaliphilic sulfur-oxidizing bacteria on thiosulfate

Culture	pH limit sulfur-oxidizing activity (optimum)	Upper salt limits M total Na^+	Maximum growth rate thiosulfate (μ_{max}, day^{-1})	Yield ($g_{protein}$/M thiosulfate)	Respiration rate with thiosulfate (qO_{2max}, $\mu M\ O_2\ min^{-1}\ mg^{-1}_{protein}$)	Source	References
Thioalkalibacter halophilus ALCO-1[a]	7.5–10 (9.5–10)	3.8	5.28[a]	1.58	1.6[a]		Banciu et al. (2008)
Thioalkalimicrobium sp.	7–10.6 (9.5–10)	2	7.92[b]	1.5–3.5	3.75[c]	Sediments soda lakes (Mongolia)	Sorokin et al. (2003); Sorokin and Kuenen (2005)
Thioalkalivibrio	7.5–10 (10)	4	2.88[b]	3.5	0.55[c]	Sediments soda lakes (Mongolia)	Sorokin et al. (2003)
Halothiobacillus Kellyi	3.5–8 (6.5)	2	10.8[d]	n.d.	n.d.	Sediment Aegean Sea	Sievert et al. (2000)
Thiomicrospira halophila	6.5–8.5 (7.8)	3.5	6.0[e]	3.5	0.44[e]	Sediments from lakes of Kulunda Steppe (Russia)	Sorokin et al. (2006b)
Thioalkalivibrio halophilus	7.5– 9.8 (8–9)	5	0.84[f]	2.6	0.25[f]	Sediments from lakes of Kulunda Steppe (Russia)	Banciu et al. (2004)
IMP-TL	7–11 (9.8)	1.1	6.0[g]	1.33	0.53[h]	Soil Tlahuac (Mexico)	This research
IMP-PB	7–10.8 (9–10)	3.0	1.5[g]	2.8	0.49[h]	Soil Tehuacan, Puebla (Mexico)	This research
IMP-XO	8–11 (10.6)	>1.7	15.6[g]	2.5	0.73[h]	Soil Xochimilco (Mexico)	This research

[a] Continuous culture pH 10, 2 M Na^+

[b] Continuous culture pH 10, 0.6 M Na^+

[c] Batch pH 10, 0.6 M Na^+

[d] Continuous culture pH 6.5, 0.5 M Na^+

[e] Continuous culture pH 7.5, 1.5 M Na^+

[f] Continuous culture, pH 9.6 4 M Na^+

[g] Continuous culture pH 10, 0.56 M Na^+

[h] Batch pH 10, 0.56 M Na

Acknowledgements The Instituto Mexicano del Petróleo supported this research with D.00011 and D.00344 projects.

References

Acinas SG, Anto NJ, Rodriguez-Valera F (1999) Diversity of free-living and attached bacteria in offshore Western Mediterranean waters as depicted by analysis of genes encoding 16S rRNA. Appl Environ Microbiol 65:514–522

Alazard D, Badillo C, Fardeau M, Cayol J, Thomas P, Roldan T, Tholozan J, Ollivier B (2007) *Tindallia texcoconensis* sp. nov., a new haloalkaliphilic bacterium isolated from lake Texcoco, Mexico. Extremophiles 11:33–39

Alcántara S, Velasco A, Muñoz A, Cid J, Revah S, Razo-Flores E (2004) Hydrogen sulfide oxidation by a microbial consortium in recirculation reactor system: sulfur formation under oxygen limitation and removal of phenols. Environ Sci Technol 38:918–923

Alcocer J, Hammer UT (1999) Saline lake ecosystems of Mexico. Aquat Ecosyst Health Manag 1:291–315

Arahal DR, Ludwig W, Schleifer KH, Ventosa A (2002) Phylogeny of the family Halomonadaceae based on 23S and 16S rDNA sequence analyses. Int J Syst Evol Microbiol 52:241–249

Banciu H, Sorokin D, Galinski E, Muyzer G, Kleerebezem R, Kuenen G (2004) *Thialkalivibrio halophilus* sp. nov., a novel obligately chemolithoautotrophic, facultatively alkaliphilic, and extremely salt-tolerant, sulfur-oxidizing bacterium from a hypersaline alkaline lake. Extremophiles 8:325–334

Banciu H, Sorokin D, Tourova T, Galinski E, Muntyan M, Kuenen J, Muyzer G (2008) Influence of salts and pH on growth and activity of a novel facultatively alkaliphilic, extremely salt-tolerant, obligately chemolithoautotrophic sulfur-oxidizing Gammaproteobacterium *Thioalkalibacter halophilus* gen. nov., sp. nov. from South-Western Siberian soda lakes. Extremophiles 12:391–404

Bárcenas AP, Tijerina ChL, Martínez GA, Becerril RE, Laqué SA, Colinas LMT (2002) Respuesta de tres materiales del género hylocereus a la salinidad sulfático-clorhídrica. Terra Latinoam 20:123–127

Berendes F, Gottschalk G, Heine-Dobbernack E, Moore ERB, Tindall BJ (1996) *Halomonas desiderata* sp. nov., a new alkaliphilic, halotolerant and denitrifying bacterium isolated from a municipal sewage works. Syst Appl Microbiol 19:158–167

Bosch P, Beusekom O, Buisman C, Janssen A (2007) Sulfide oxidation at halo-alkaline conditions in a fed-batch bioreactor. Biotechnol Bioeng 97:1053–1063

Buisman C, Post R, Ijspeert P, Geraats G, Lettinga G (1989) Biotechnological process for sulphide removal with sulphur reclamation. Acta Biotechnol 9:255–267

Carey TR, Hermes JE, Rochelle GT (1991) A model of acid gas absorption/stripping using methyldiethanolamine with added acid. Gas Sep Purif 5:95–109

Cline C, Hoksberg R, Abry R, Janssen A (2003) Biological process for H_2S removal from gas streams. The Shell-Paques/Thiopaq gas desulfurization process. Norman, Oklahoma

de Souza MP, Amini A, Dojka MA, Pickering IJ, Dawson SC, Pace NR, Terry N (2001) Identification and characterization of bacteria in a selenium-contaminated hypersaline evaporation pond. Appl Environ Microbiol 67:3785–3794

Fernández LC, Rojas ANG, Roldán CTG, Ramírez IME, Zegarra MHG, Uribe HR, Reyes, ARJ, Flores HD, Arce OJM (2006) Manual de técnicas de análisis de suelos aplicadas a la remediación de sitios contaminados. SEMARNAT, INE, IMP, México. ISBN 968-489-039-7

Gabriel D, Deshusses MA (2003) Retrofitting existing chemical scrubbers to biotrickling filters for H_2S emission control. Proc Natl Acad Sci USA 100:6308–6312

Gonzalez-Sanchez A, Revah S (2007) The effect of chemical oxidation on the biological sulfide oxidation by an alkaliphilic sulfoxidizing bacterial consortium. Enzym Microb Technol 40:292–298

Gonzalez-Sanchez A, Revah S (2009) Biological sulfide removal under alkaline and aerobic conditions in a packed recycling reactor. Water Sci Technol 59:1415–1421

Gonzalez-Sanchez A, Revah S, Deshusses A (2008) Alkaline biofiltration of H_2S odors. Environ Sci Technol 42:7398–7404

Gonzalez-Sanchez A, Tomas A, Dorado A, Gamisans X, Guisasola A, Lafuente J, Gabriel D (2009) Development of kinetic model for elemental sulfur and sulfate formation from the autotrophic sulfide oxidation using respirometric techniques. Water Sci Technol 59:1323–1329

Han K, Levenspiel O (1988) Extended monod kinetics for substrate product and cell inhibition. Biotechnol Bioeng 32:430–437

Jan-Roblero J, Magos X, Fernández L, Hernández-Rodríguez C, Le Borgne S (2004) Phylogenetic analysis of bacterial populations in waters of the former Texcoco Lake, Mexico. Can J Microbiol 50:1049–1059

Janssen A, Sleyster R, Van der Kaa C, Jochemsen A, Bontsema J, Lettinga G (1995) Biological sulphide oxidation in a fed-batch reactor. Biotechnol Bioeng 47:327–333

Janssen A, Ruitenberg R, Buisman C (2001) Industrial applications of new sulfur biotechnology. Water Sci Technol 44:85–90

Karlin S, Altschul SF (1990) Methods for assessing the statistical significance of molecular sequence features by using general scoring schemes. Proc Natl Acad Sci USA 87:2264–2268

Kohl A, Nielsen RB (1997) Gas purification. Gulf Pub. Co., Houston

Lens P, Hulshoff P (2000) Environmental technologies to treat sulfur pollution: principes and engineering. International Water Association, London

Lowry OH, Rosebrough NJ, Farr AL (1951) Protein measurement with the folin phenol reagent. J Biol Chem 193:265–275

Okamoto T, Maruyama A, Imura S, Takeyama H, Naganuma T (2004) Comparative phylogenetic analyses of *Halomonas variabilis* and related organisms based on 16S rRNA, *gyrB* and *ectBC* gene sequences. Syst Appl Microbiol 27:323–333

Olmos A, Olguín P, Fajardo C, Razo E, Monroy O (2004) Physicochemical characterization of spent caustic from the OXIMER process and sour waters from Mexican oil refineries. Energy Fuels 18:302–304

Pirt JS (1975) Principles of microbe and cell cultivation. Blackwell Scientific, Oxford

Ramírez M, Gómez J, Cantero D (2009) Removal of hydrogen sulfide and ammonia from gas mixtures by co-immobilized cell using a new configuration of two biotrickling filters. Water Sci Technol 59:1353–1359

Ramos-Bello R, Cajuste LJ, Flores-Román D, García-Calderón NE (2001) Metales pesados, sales y sodio de chinampa en México. Agrociencia 35(4):385–395

Rodriguez-Valera F (1993) Introduction to saline environments. In: Vreeland RH, Hochtein LI (eds) The biology of halophilic bacteria. CRC Press, Boca Raton, pp 1–24

Schmidt M, Priemé A, Stougaard P (2006) Bacterial diversity in permanently cold and alkaline ikaite columns from Greenland. Extremophiles 10(6):551–562

Shapovalova A, Khijniak T, Tourova T, Muyzer G, Sorokin D (2008) Heterotrophic denitrification at extremely high salt and pH by haloalkaliphilic Gammaproteobacteria from hypersaline soda lakes. Extremophiles 12:619–625

Sievert SM, Heidorn T, Kuever J (2000) *Halothiobacillus kellyi* sp. nov., a mesophilic, obligately chemolithoautotrophic, sulfur-oxidizing bacterium isolated from a shallow-water hydrothermal vent in the Aegean Sea, and emended description of the genus Halothiobacillus. Int J Syst Evol Microbiol 50:1229–1237

Sorokin D, Kuenen G (2005) Haloalkaliphilic sulfur-oxidizing bacteria in soda lakes. FEMS Microbiol Rev 29:685–702

Sorokin DY, Lysenko AM, Mityushina LL, Tourova TP, Jones BE, Rainey FA, Robertson LA, Kuenen GJ (2001) *Thioalkalimicrobium aerophilum* gen. nov., sp. nov. and *Thioalkalimicrobium sibericum* sp. nov., and *Thioalkalivibrio versutus* gen. nov., sp. nov., *Thioalkalivibrio nitratis* sp. nov., novel and *Thioalkalivibrio denitrificans* sp. nov., novel obligately alkaliphilic and obligately chemolithoautotrophic sulfur-oxidizing bacteria from soda lakes. Int J Syst Evol Microbiol 51:565–580

Sorokin D, Banciu H, van Loosdrecht M, Kuenen J (2003) Growth physiology and competitive interaction of obligately chemolithoautotrophic, haloalkaliphilic sulfur-oxidizing bacteria from soda lakes. Extremophiles 7:195–203

Sorokin D, Tourova T, Lysenko A, Muyzer G (2006a) Diversity of culturable halophilic sulfur-oxidizing bacteria in hypersaline habitats. Microbiology 152:3013–3023

Sorokin D, Tourova T, Tatjana V, Kolganova T, Spiridonova E, Berg I, Muyzer G (2006b) *Thiomicrospira halophila* sp. nov., a moderately halophilic, obligately chemolithoautotrophic, sulfur-oxidizing bacterium from hypersaline lakes. Int J Syst Evol Microbiol 56:2375–2380

Sorokin Y, Foti M, Pinkart H, Muyzer G (2007) Sulfur-oxidizing bacteria in Soap Lake (Washington State), a meromictic, haloalkaline lake with an unprecedented high sulfide content. Appl Environ Microbiol 73:451–455

Sorokin D, van den Bosch P, Abbas B, Janssen A, Muyzer G (2008) Microbiological analysis of the population of extremely haloalkaliphilic sulfur-oxidizing bacteria dominating in lab-scale sulfide-removing bioreactors. Appl Microbiol Biotechnol 80:965–975

Valenzuela-Encinas C, Neria-González I, Alcantara-Hernández RJ, Estrada-Alvarado I, Zavala-Díaz J, Dendooven L, Marsch R (2009) Changes in the bacterial populations of the highly alkaline saline soil of the former lake Texcoco (Mexico) following flooding. Extremophiles 13:609–621

Velasco A, Alcántara S, Razo-Flores E, Revah S (2004) Partial thiosulfate oxidation by steady-state continuos culture in a bioreactor-settler system. J Chem Technol Biotechnol 79:132–139