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IDENTIFICATION AND CHARACTERIZATION OF SULFUR-OXIDIZING BACTERIA IN AN ARTIFICIAL WETLAND THAT TREATS WASTEWATER FROM A TANNERY

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Wastewater from tanneries contains high concentrations of organic matter, chromium, nitrogen, and sulfur compounds. In this study, an artificial wetland is is used as the tertiary treatment in a tannery in León Gto., México. It consists of three subplots with an area of about 450 m^2. Two subplots were planted with* Typha *sp. and the third with* Scirpus americanus*. Geochemical analyses along the flowpath of the wetland show that contaminants were effectively attenuated. The most probable number technique was used to determine rhizospheric microbial populations involved in the sulfur cycle and suggested that there were 10^4–10^6 cells g^{-1} sediment of sulfate-reducing bacteria and 10^2–10^5 of sulfur-oxidizing bacteria (SOB). Representatives of SOB were isolated on media containing thiosulfate. Phylogenetic analysis of 16S rRNA of SOB isolates shows that they belong to the genera* Acinetobacter, Alcaligenes, Ochrobactrum, *and* Pseudomonas*. Most of the isolates are organotrophic and can oxidize reduced sulfur compounds such as elemental sulfur or thiosulfate, accumulating thiosulfate, or tetrathionate during growth. All isolates can use reduced-sulfur compounds as their sole sulfur source and some can use nitrate as an electron acceptor to grow anaerobically. Our results illustrate the relevance of SOB in the functioning of the wetland constructed for tannery wastewater remediation.

KEY WORDS: rhizosphere, *Scirpus americanus*, *Alcaligenes*, *Pseudomonas*, sulfides, tetrathionate

INTRODUCTION

Tanneries involved in leather production in León Gto., México, consume 1,254,750 L of water year^{-1}. There are 650 tanneries between León and San Francisco (a total distance of 20 km) and most do not recycle their wastewater because it contains high concentrations of pollutants. The wastewater cannot be discharged directly into lagoons or rivers (Alvarez *et al*., 2004).

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Nearly 80% of the tanneries use chromium salts and other chemicals such as bicarbonates, biocides, sulfates, and sulfides. In addition, organic matter is generated from skin residues. As a result, wastewater contains high chromium concentrations (3–10 g L^{-1}), biological oxygen demand (BOD) (1600 mg L^{-1} O_2), chemical oxygen demand (COD) (32000 mg L^{-1} O_2), sulfides (54 mg L^{-1}), and other nutrients (Alvarez *et al*., 2004). In attempts to remove these contaminants, wastewater from tanneries is subjected to chemical and physical purification processes, but artificial wetlands are also employed to improve treatment systems' efficiencies. Thus, tertiary methods have been proposed for removal of contaminants not only in tanneries, but also in agricultural, dairy, mine, and municipal waste (Rivera *et al*., 1997; Lloyd *et al*., 2004). The biological treatment of waste is a relatively low-input operation, maintenance costs are low, and they are relatively simple to operate (Kuehn and Moore, 1995).

Aquatic plants are essential components of wetlands, where they remove some contaminants and are able to mobilize oxygen into the rhizosphere, favoring aerobic microbial processes (Stubner, Wind, and Conrad, 1998; Gopal, 1999). Obviously, the microbial composition of wetland communities influences the quality of the effluent (Ibekwe, Grieve, and Lyon, 2003). In sediments where oxygen tension is low, fermentative and anaerobic respiration processes occur. The mineralization of organic matter may involve alternative electron acceptors such as Fe (III), nitrate, Mn (IV), or sulfate (Stubner *et al*., 1998; Holmer and Storkholm, 2001). The depletion of nitrate in wastewater that is rich in organic matter and sulfates leads to a dominance of sulfate-reducing bacteria (SRB) (Nedwell, 1982; Yang, Vollertsen, and Hvitved, 2005). Sulfide produced by SRB may be toxic to aquatic life at levels higher than 1 ppm (Bagarinao and Vetter, 1989).

Microbial oxidation of reduced sulfur compounds such as sulfide, elemental sulfur, thiosulfate, and polythionates is usually an aerobic process. Few bacteria are able to oxidize sulfur-using nitrate as the electron acceptor under anaerobic conditions (Stubner *et al*., 1998). Metabolically, sulfur-oxidizing bacteria are very diverse and include chemolithotrophs that synthesize organic carbon from CO_2 and obtain energy from sulfur oxidation. (*Thiobacillus* and *Thiomicrospira* are examples.) Heterotrophic sulfur oxidizing bacteria (SOB), on the other hand, can use organic substrates as both the carbon and energy source as well as sulfur compounds as an energy source (mixotrophy) (Jorgensen, 1982).

The relevance of the physiologically and phylogenetically diversity of SOB present in rice paddy fields has been clearly illustrated (Stubner *et al*., 1998). In contrast, little is known about their presence and role in artificial wetlands. In the present study, we examined the rhizosphere of wetland plants, for the identification and characterization of bacterial isolates and their ability to oxidize sulfur-reduced compounds.

MATERIAL AND METHODS

Wetland Operation and Sampling

Prior to discharge, wastewater from the tannery was treated with alkali. After sediment removal, the water had chemical characteristics listed in Table 1. The artificial wetland was a pilot subplot initially planted with *Scirpus americanus* (subplot III). One year later, two subplots (I and II), each planted with *Typha* sp., were added in series. Each subplot was 15 m long, 10 m wide, and 0.9 m deep, with a total surface area of 405 m^2 (Figure l). Wastewater effluent from the sedimentation pond arrived at a rate of 20 L min^{-1}. Influent totalled 80 m^3 and the average hydraulic retention time was approximately 2 d. *Typha* sp.

Table 1 Quality of biologicallytreated wastewater as measured at the different wells (E1, S1, S2, and S3)

Parameter	E1	S1	S2	S3	Removal efficiency
PH	6.0–8.7	7.9–8.1	7.5–8.1	6.9–8.7	—
Conductivity dS m^{-1}	5674–9640	7943–8030	6855–6962	2240–2721	52–72
COD mg L^{-1}	12,340–17,520	2672–3896	654–928	68–598	96–98
BOD mg L^{-1}	675–1320	324–978	190–394	39–86	93–95
Cr (total) mg L^{-1}	22–31	8–12	3–7	0.07–0.08	99
TKN mg L^{-1}	117–267	86–93	52–62	9–17	92–94
Sulfide mg L^{-1}	24–54	3–67	0.09–25	0.01–0.03	99
Sulfate mg L^{-1}	1597–2518	129–1407	734–930	169–189	88–92

Values correspond to the maximum and minimum recorded during a year. Samples were taken every 4 mo.

and *Scirpus americanus* were used for their known tolerance to and accumulation of heavy metals such zinc, lead, cadmium, and chromium (Ye *et al*., 1997; Suseela *et al*., 2002), as well as their high salinity tolerance.

The wetland started operation in 2004 and, 2 mo later, the plants had adapted to their new environment. The quality of the wastewater was measured every 4 mo. Wastewater samples were taken at entry into the wetland (well E1), between each subplot (wells S1 and S2), and at the exit of the system (well S3). Conductivity, pH, COD, BOD, total Kjeldahl nitrogen (TKN), sulfate (SO_4^{2-}), and sulfide (HS^-) analyses were performed according to Rodier (1981a, 1981b), whereas total chromium was determined by the Environmental Protection Agency (EPA) 6010 method.

Samples were taken from the sediments in the rhizosphere of plants growing at the entry (a), middle (b), and exit (c) points of each subplot, and were used to characterize the microbial populations of bacteria involved in sulfur cycling (SRB and SOB).

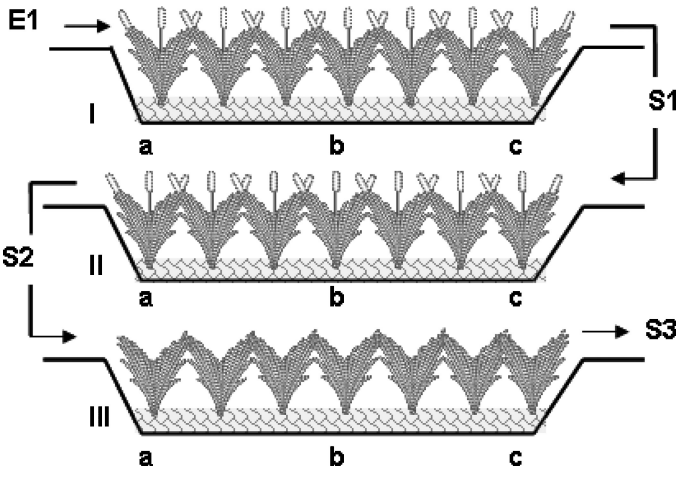

Figure 1 Diagram showing the artificial wetland showing flow of water between three subplots serially connected to one another. E1, S1, S2, and S3 are wells and the sampling points.

Most Probable Number Technique Counts of SRB and SOB

Bacterial groups were quantified using the most probable number (MPN) technique. Modified sPGC media (saline Postgate's medium) was used to grow SRB and contained (g L^{-1}) 4.0 sodium lactate, 1.0 yeast extract, 0.3 sodium citrate, 0.5 K_2HPO_4, 2.0 $MgSO_4$, 1.0 NH_4Cl, 0.2 $Fe(NH_4)(SO_4)_2.6H_20$, and 7.0 NaCl (pH 7.2). Tubes were incubated under anaerobic conditions at 28°C for 4 wk. Formation of black precipitates of ferrous sulfide was taken as a positive indication of bacterial activity (Telang, Jenneman, and Voordouw, 1999).

SOB were enumerated in modified *Thiobacillus* media B containing (g L^{-1}) 5.0 $Na_2S_2O_3.5H_20$, 3.0 KH_2PO_4, 2.0 KNO_3, 0.1 NH_4Cl, 0.7 $NaHCO_3$, 0.1 $MgCl_2$, 0.1 $CaCl_2$, 0.2 $Fe(NH_4)(SO_4)_2.6H_20$, 0.05 $MgSO_4.7H_2O$, and 0.004 Bromthymol (pH 7.0). Thiosulfate was used because sulfide is an unstable compound under aerobic conditions and is difficult to discriminate between its chemical and biological oxidation. The tubes were incubated at room temperature for 3 wk. Changes in color and turbidity indicated growth (Benner, Gould, and Blowes, 2000).

SOB Isolation and Characterization

Isolates were obtained from the MPN series and were plated onto the modified *Thiobacillu*s medium B as well as in Luria Bertani agar. Capacities of the isolates to oxidize sulfur-reduced compounds under heterotrophic conditions were tested in liquid *Thiobacillu*s medium B supplemented with citrate (10 mM) and one of the following sulfur compounds: 20 mM thiosulfate, 1% elemental sulfur or 2 mM sulfide, inoculated to 1×10^6 cells mL^{-1}, and incubated at 28°C with continuous shaking (180 rpm) for 72 h. Finally, optical density was measured and thiosulfate, tetrathionate, sulfur, and sulfite were assayed according to Masau, Key, and Suzuki (2001). Fermentation was carried out using Kliger medium and nitrate respiration was tested by gas formation and measuring the presence of nitrite (Ruby, Wirsen, and Jannasch, 1981). Chemolitotrophic assays were performed using *Thiobacillus* medium B with 20 mM thiosulfate and detecting oxidized species of sulfur. Tests of growth with a reduced-sulfur compound as the sole S source were performed using *Thiobacillus* medium B without sulphate and supplemented with 20 mM thiosulfate, 1% elemental sulfur, or 2 mM sulfide.

rRNA Gene Analysis

Phylogenetic analyses were based on polymerase chain reaction (PCR) amplification of the 16S rRNA gene using forward-primer fD1 (5′-CCGAATTCGTCGACAACAGAGTT TGATCCTGGCTCAG-3′) and reverse-primer rD1 (5′-CCCGGGATCCAAGCTTAAGGA GGTGATCCAGCC-3′). The amplification product (1500 bp) was cloned into the pCR2.1 TOPO-Cloning vector (Invitrogen, Carlsbad, CA) and sequenced. Identities were determined using the EMBL/GenBank database with the BLAST alignment tool (NCBI; www.ncbi.nlm.nih. gov/). Sequence comparisons and phylogenetic trees were made using the Mega 3.1 software package, with maximum parsimony, neighbor joining (bootstrap values of 1000) with the Jukes Cantor correction, and maximum likelihood algorithms. All the characterized isolates were deposited at the CINVESTAV (Irapuato Gto. México) culture collection.

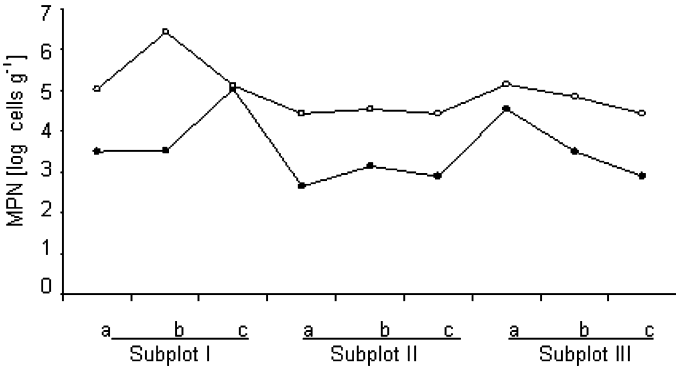

Figure 2 Abundances of SRB and SOB populations in the sediments of subplots of the artificial wetland (-○-) SRB and (-●-) SOB. MPN is reported as log cells g^{-1}.

Accession Numbers

Ten 16S rRNA gene sequences obtained from the isolates under study were sent to the NCBI GenBank (http://www.ncbi.nlm.nih.gov) and received the accession numbers from EF599307 to EF599316.

RESULTS

After 1 yr of operation, the wetlands caused the gradient of geochemical characteristics (from influent to effluent) shown in Table 1. As a result of soaking, dehairing, tanning, and finishing, input water from the tannery is high in BOD, COD, and TKN, all of which were substantially reduced during flow through the wetland as a result of biological and chemical oxidation. Remarkably, the chromium content of wastewater was reduced by 99%, probably due to the presence of *Scirpus americanus*, a known accumulator for this metal. Aqueous sulfide and sulfate concentration were diminished up to 99% and 92%, respectively.

Microbial Population of the Sulfur Cycle

The abundance of SRB and SOB populations in each subplot is shown in Figure 2. The increase in sulfide concentrations at the exit of the first subplot is correlated to an increase in the SRB populations, which declined in the last subplot (10^4 cells), probably due to decreasing organic matter in the wastewater as indicated by BOD.

Although the SOB populations in the wetland remained approximately 10^2–10^5 cells, sulfide concentrations declined markedly in the second and third subplots, likely as a result of oxidation by the SOB. In the same manner, sulfate concentrations also diminished. The largest numbers of SOB were found at the exit of the first subplot, coinciding with a high sulfide concentration in the same subplot.

Isolate Characterizations

Ten bacterial isolates that grew on *Thiobacillus* medium B were obtained from the highest dilutions (Table 2). The colony morphologies of these isolates were distinctive

and each isolate grew rapidly in rich medium (LB). All isolates were gram-negative and non-fermentative. Some, such as A11, A12, and A14, were abundant in the second and third subplots, whereas D1 was present in higher numbers in the first and third subplots. Other isolates (C11, E11, and E12) were found only in a particular subplot. Nitrate reduction and gas production under anaerobic conditions indicate that isolates A11, A12, A14, B12, C14, and D11 are denitrifying bacteria. In contrast, the oxidation of ammonium was observed in isolates C11 and C15.

Six strains showed autotrophic growth with thiosulfate as a possible electron donor and tetrathionate was detected as the oxidized sulfur compound. In the heterotrophic test, A1, A2, A4, and B2 can use sulfide (2 mM) under anaerobic conditions, with the detection of sulfide diminishing but no observed oxidized sulfur compound increasing in concentration (Table 2). Only isolates E11 and E12 oxidized elemental sulfur to thiosulfate, whereas thiosulfate was the compound oxidized by most isolates, showing tetrathionate formation. Sulfate production and changes toward acidic pHs were nt detected on any growing test, as the final pH was between 7.2 and 8.8, always more alkaline than the initial pH. Additionally, the consumption of elemental sulfur and thiosulfate as S source was detected for almost all isolates.

16. rRNA Gene Sequences and Phylogenetic Tree

16S rRNA gene sequences from our 10 isolates were included in a phylogenetic tree along with sequences from relevant reference strains. With the exception of A11, all isolates showed similarities to previously cultured species and comprised alpha, beta, and gamma-proteobacteria subgroups. A11, A12, A14, and B12 were related to *Pseudomonas* cluster (99%), with A12 and A14 corresponding particularly closely with *Pseudomonas stutzeri*. Isolates E11 and E12 were affiliated with the *Acinetobacter* sp. with both clusters within the gamma-Proteobacteria. Strains C11, C14, and C15 showed high similarity to *Alcaligenes* (98%–99%), whereas D11 was closely related to *Ochrobactrum*. These genera fall within the beta-Proteobacteria and alpha-Proteobacteria, respectively (Figure 3). *Alcaligenes* was the most widely distributed bacterial group in the three subplots, whereas the *Pseudomonas* group was only found in the second and third subplots. *Acinetobacter* was abundant only in the first subplot. In bacteriological terms, subplot III was the most diverse, perhaps because it was the first constructed subplot.

DISCUSSION

Reports in the literature about toxicity assays using *Daphnia* spp. show that the effluents from the tannery industry are considered to be toxic due to their high concentrations of organic and inorganic compounds, sulfides, and chromium salts (Cooman *et al*., 2003). Therefore, there is a need for treatments to reduce toxicants. In this regard, Alvarez *et al*. (2004) developed a biological treatment using *Eichhornia crassipes* to remove the chromium present in effluents; however, the high concentrations of salts and chromium prevented the establishment of the system.

The wetland studied here, which features *Scirpus* and *Typha*, clearly attenuates chemical constituents toxic to *Eichhornia crassipes* (COD and Cr; Table 1). The high reductions observed for COD and chromium concentrations show that the system is working properly; it is likely that the adapted plants play an important role in removing contaminants.

Table 2 Physiological characterization of S-oxidizing isolates

Characteristic	Isolate code									
	A11	A12	A14	B12	C11	C14	C15	D11	E11	E12
Origin (subplot in wetland)	II, III	II, III	II, III	III	I	II	III	I, III	I	I
Dilution factor	10^3, 10^3	10^3, 10^4	10^3, 10^3	10^4	10^4	10^2	10^3	10^4, 10^3	10^3	10^4
Gram reaction	−	−	−	−	−	−	−	−	−	−
Fermentative Kliger	−	−	−	−	−	−	−	−	−	−
Denitrification	+	+	+	+	−	+	−	+	−	−
Autotrophic growth with thiosulfate	+	+	+	+	−	−	−	−	+	+
Heterotrophy	+	+	+	+	+	+	+	+	+	+
Heterotrophic oxidizing*:										
Sulfide HS (2 mM)	−	−	−	−	−	−	−	−	−	−
Elemental sulfur 1%	−	−	−	−	−	−	−	−	+	+
Thiosulfate (20 mM)	+	+	+	+	+	+	+	+	−	−
Use of S sources**:										
Sulfide HS (2 mM)	+	+	+	+	−	−	−	−	−	−
Elemental sulfur 1%	+	+	+	+	+	+	+	+	+	+
Thiosulfate (20 mM)	+	+	+	+	+	+	+	+	+	−

*Test of oxidizing of sulfur reduced compounds.

**Test of use of sulfur sources. Gram reaction: positive (+) or negative (−), oxidizing or use of sulfur compunds: positive (+) or negative (−).

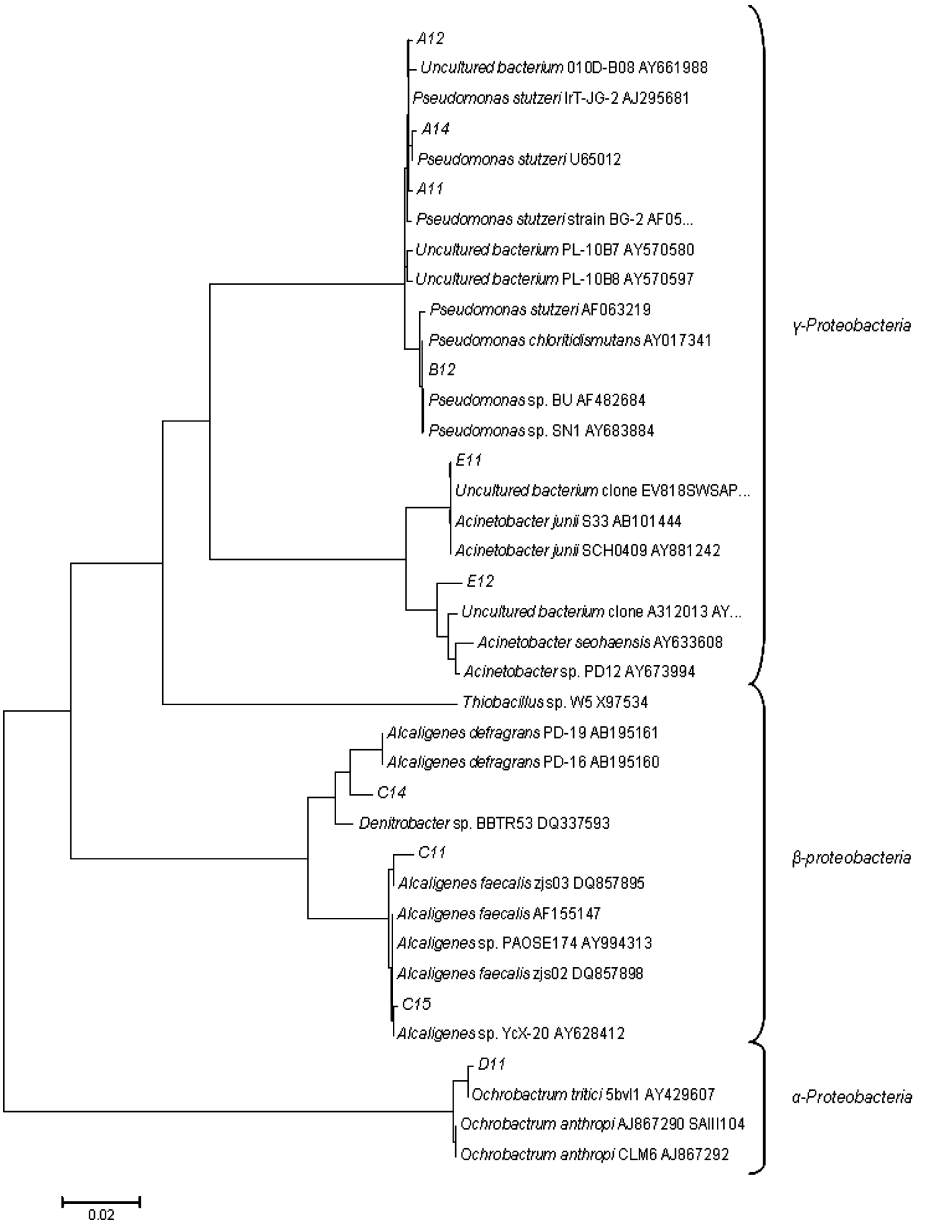

Figure 3 Phylogenetic tree showing the affiliation of ten bacterial isolates retrieved from the artificial wetland in relation to reference strains. Alphanumeric designations of new isolates are those used in Tables 1 and 2.

Microbial participation in nutrient cycles, particularly the sulfur cycle, has been studied in wetlands that receive effluents from mining in which sulfate reduction by bacteria has been recommended for removing metals by forming insoluble sulfides as final products (Webb, McGinness, and Lappin, 1998; Lloyd *et al*., 2004). In contrast, in effluents with high contents of sulfates and organic matter, as occurs in tannery effluents, the formation of sulfides is undesirable because it results in a high COD. The number of SRB present in

the wetland under study fluctuated between 10^4 and 10^6 cells per gram of sediment. Similar values have been reported for sediments in lakes and natural wetlands (Wielinga *et al*., 1999; Meier, Babenzien, and Wendt, 2004). In our test plots (Table 1), the concentration of sulfides diminished in the second and third subplots of the wetland, correlating with a decrease in the organic matter content (BOD). Lloyd *et al*. (2004) also found that organic matter regulated sulfate reduction; the addition of sucrose resulted in a larger production of sulfide. Reports in the literature indicate that in sediments and anoxic zones where the reduced sulfur compounds have accumulated, sulfide-oxidizing bacteria are present. Some aerobic or facultative anaerobes depend on final electron acceptors such as nitrate (Ruby *et al*., 1981; Eckord and Fedorak, 2002; Leduc, Leduc, and Ferroni, 2002). In the present study, the abundance of SOB was similar to the values reported for the rice paddy rhizosphere (Stubner *et al*., 1998).

Previous studies on the SOB isolated from environmental sites indicate diverse physiological processes, with regard to utilization of both sulfur and carbon compounds (Ruby *et al*., 1981; Stubner *et al*., 1998). In this study, 10 bacterial isolates were obtained and differences in the abundance of the groups depending on the subplot were observed. *Pseudomonas* was particularly predominant in the second and third subplots. The presence of this bacterial group in sulfide-rich environments has also been reported by Ruby *et al*. (1981). Sorokin *et al*. (1999) evaluated the capacity of isolates belonging to the *Pseudomonas stutzeri* group to oxidize thiosulfate under denitrifying conditions. These bacterial genera have been under intense study because they also show the capacity to degrade aromatic compounds (Lalucat *et al*., 2006). In the present work, the consumption of sulfide, elemental sulfur and thiosulfate was noticed in the culture media when incubated with *Pseudomonas* isolates, and tetrathionate was detected only in cultures amended with thiosulfate. The consumption of sulfide under denitrifying conditions may be advantageous to species oxidizing sulfur compounds under anaerobic conditions, with nitrate as the electron acceptor.

Reports on the molecular characterizations of the bacterial isolates from a wetland involved in the removal of NH_4 indicate the presence of the genus *Alcaligenes* with the capacity to nitrify (Walsh, Hill, and Moffett, 2002). This genus is also common in treatments plants of industrial effluents (Wagner *et al*., 2002) and has been identified as a participant in the desulfonation process of organosulfonate compounds (Cook, Laue, and Junker, 1999). In our study, *Alcaligenes* was present throughout the three subplots showing activities (*in vitro*) of nitrifying, denitrifying, sulfur and thiosulfate oxidizing. The participation in degradation processes of sulfur organic compounds indicates that this organism is versatile and, similar to *Pseudomonas*, likely plays an important role in the S as well as N cycles. In both genera the denitrifying capacity has been identified (Wagner *et al*., 2002), a process required in the N removal in wastewaters with heavy organic matter content.

Ochrobactrum, a genus with the capacity to oxidize thiosulfate to tetrathionate and isolated and identified from the first and third subplot, has been observed in several environments. These bacterial genera have also been isolated from the rhizoplane of wheat, showing a high dependence for the root system because it was not detected in the soil distant from the root (Lebuhn *et al*., 2000). Similar to *Rhizobium*, this genus has the capacity to establish a symbiotic association with a plant host and form nodules, as has been observed in *Lupinus albus* (Trujillo *et al*., 2005). Besides the high adsorption of metals, related to the polysaccharide production in this genus (Faisal and Hasnain, 2005), it could play an important role in toxicant sequestration considering that a combination of these bacteria with *Eichornia crassipes* resulted in a large removal of chromium from a solution. This

genus has also been identified in a denitrifying reactor for effluent treatments (Hwang *et al*., 2006) but its role in the sulfur cycle in effluents still is not clear.

The use of compounds such as dimethylsulfide as a sole sulfur source has been reported in the bacterial genus *Acinetobacter*, and cloning and identification of the hydrolase gene has led to the elucidation of an oxidation pathway (Fuse *et al*., 2000). Thus, the use of elemental sulfur as the sulfur source and oxidation to thiosulfate in laboratory assays represents a new proposed function in the sulfur cycle in the wetland under study.

In general terms, information about microbial populations present in tannery effluents is scarce, yet the treatment of these wastewaters by microorganisms is very important. In this research, it was found that the abundance of microbial populations participating in sulfur-cycle processes varied along the geochemical gradients of the wetland site.

All isolates tested were able to use a multiple reduced sulfur compound (sulfide, elemental sulfur, or thiosulfate) as a sulfur source and to incorporate it into their metabolism while avoiding the oxidation to sulfate. The isolates seem to be alkali-producing thiosulfate-oxidizing bacteria considering that the pH was increased (0.1–1.5 units) during growth through sodium hydroxide generated from the conversion of thiosulfate to tetrathionate: $4\ Na_2S_2O_3 + 2\ H_2O + O_2 \rightarrow 2Na_2S_4O_6 + 4NaOH$ (Mason and Kelly, 1988). The SOB that form tetrathionate are present in diverse environments such as soils, soda lakes, oxidizing bioreactors, and in the redox layer of the Black Sea marine sediments. All of these sites are characterized by the presence of sulfur-reduced compounds (sulfide and/or elemental sulfur) and large amounts of organic matter (Wainwright, Nevell, and Grayston, 1986; Podgorsek and Imhoff, 1999; Sorokin *et al*., 1999; Sorokin, 2003).

The role of tetrathionate in the sulfur cycle within *Catenococcus thiocyclus* has been described elsewhere (Sorokin, Robertson, and Kuenen, 1996). Tetrathionate is formed as a product of thiosulfate oxidation in culture media, promoting the chemical oxidation of sulfide (to elemental sulfur) when it is added to the medium. The strain recycles the thiosulfate to tetrathionate and more sulfide is oxidized. This mechanism couples chemical and biological reactions as they would occur in polluted environments.

Our results indicate that proper functioning of the wetland depends on the interaction of its components: plants, soil, wastewater characteristics, microorganisms, and operational conditions. The microbial activity (SOB and SRB) plays an important role on the C, N and sulfur cycles A better understanding of this mechanism could be useful in maintaining environmental quality.

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