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TREATMENT OF HYPERSALINE INDUSTRIAL WASTEWATER BY A MICROBIAL CONSORTIUM IN A SEQUENCING BATCH REACTOR

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ABSTRACT

Hypersaline effluents are produced by various industrial activities. Such wastewater, rich in both organic matter and salt (> 35 g l^{-1}), is difficult to treat by conventional wastewater treatment processes. It is necessary to use halophilic bacteria. In this study, a bench-scale sequencing batch reactor (SBR) was inoculated with halophilic sediments in order to treat an agri-food effluent containing 120 g salt l^{-1}. The micro-organisms were able to treat carbon and nitrogen, provided the pH in the reactor was neutralised with phosphoric acid. Soluble COD and Soluble TKN removal attained 83% and 72% respectively. 16S rDNA identification of the halophilic microbial community showed high diversity.

Keywords: Tartaric acid production, halophilic micro-organisms, molecular microbiology, SSCP.

INTRODUCTION

Worldwide, the pollution removal in hypersaline effluents is likely to represent up to 5% of the total global wastewater treatment requirement. Moreover, the developing countries in particular are confronted with this kind of problem since the industrial and urban wastewater in these countries is often discharged into depressions in saline zones. Thus, the removal of xenobiotics in saline ecosystems is a problem that affects the industrial sector in general and the peri-urban environment of many developing countries.

Various industrial sectors are confronted with the problem of hypersaline effluents generated by their production activity. This is particularly the case in industries that use calcium tartrate crystals as raw material sold to the winemaking and agri-food sectors. The calcium tartrate is recovered from alkaline solutions extracted from the tartar-removing process in wine tanks. These solutions are made up of water, a salt compound (tartar, sodium and potassium), residual soda and organic matter from the wine. The precipitation of calcium tartrate is obtained by acidification (HCl) and the addition of a calcium salt ($CaCl_2$). The crystals are dried and washed to eliminate excess chloride, thus generating hypersaline effluents that must be treated to conform to the French and European legislation.

The level of salt in the effluent, close to 120 g l^{-1} (mainly NaCl and KCl), makes the correct operation of a conventional wastewater treatment system impossible [1]. The main dysfunctions caused by salt have been reviewed in 1995 [2]: first of all, conventional micro-organisms are sensitive to abrupt ionic changes and, in addition, they do not tolerate salt concentrations higher than 50 g l^{-1}. Also, high salt levels disturb the microbial metabolism, with a consequent fall in the removal yields of organic matter: the nitrification reaction, *inter alias*, is particularly inhibited. Moreover, salt tends to increase the suspended solids (SS) in effluents, on account of its lysis effect on many organisms (protozoa amongst others [3]) and because of its inhibiting action on flocculation [2]. Lastly, bacterial acclimation to salt is quickly lost if salinity suddenly drops.

In the treatment of hypersaline effluents, the advantages of using halophilic bacteria have already been reported. The bacteria best adapted to the effluent considered in this study are moderate halophiles, i. e. in order to ensure

their development, the salt concentration must lie between 30 and 150 g l^{-1} [4].

Among the studies undertaken on halophilic bacteria, most have aimed to define the bacteria's taxonomic position. Such information makes it possible to define the types of carbon-containing substrates, such as sugars or protidic substances [5, 6], to be used for growing the given micro-organisms. A very small percentage of these halophilic strains have been tested on carbon-containing substrates of an aromatic type like those that are likely to be found in the agri-food effluent studied in this paper. A study was carried out on the representative of the aromatic acid monomers in C6-C1, the benzoate [5]. Later, the removal of phenol (representative of non-acid aromatic monomers) was studied in greater depth in halophilic conditions using a micro-organism identified as *Pseudomonas halodurans* [7].

In non-saline conditions, the bacteria are known for their varied metabolism, but little information exists with regard to their potential for aerobically degrading polluting molecules under saline conditions. An unidentified bacterial strain, isolated in 1978 from the Great Salt Lake (Utah, USA), was able to degrade a non-aromatic non-polar pollutant, hexadecane, under saline conditions of less than 200 g NaCl l^{-1} [8]. More recently, some recalcitrant compounds such as organophosphoric pesticides have been successfully degraded by a micro-organism identified as belonging to the *Alteromonas* genus [9]. Similarly, the database "Biodegradative Strain Database", available on the Web, refers to the removal of the 2.4-D (2.4-dichlorophenoxyacetate) by a species of the *Halomonas* genus.

The team that obtained significant results on the decomposition of phenol in a saline medium [7] described an aerobic moderate halophile taken out of the Great Salt Lake. This species, identified as belonging to the *Halomonas* genus, was able to degrade 0.1 g phenol l^{-1} as its only source of carbon and energy. This study was carried out using a synthetic saline wastewater similar to the effluents resulting from the oil industry in which phenolic compounds are commonly found. This bacterium degraded phenol in a medium containing NaCl concentrations between 10 and 140 g l^{-1}. Such maximum salt concentrations are similar to those found in some agricultural and food industries or in saline depressions which receive agricultural and urban wastewater in south Mediterranean countries. Under optimal growth conditions (between 30 and 50 g NaCl l^{-1}), the removal of 0.1 g phenol l^{-1} was complete at the end of 13 h for the only strain ever studied. For higher salt concentrations, the complete mineralisation of phenol was slower, reaching 100 h for 140 g NaCl l^{-1} [7].

In 1994, a sequencing batch biofilm reactor (SBBR) was inoculated with moderate halophiles, recovered from the Great Salt Lake, in order to treat a synthetic effluent containing 150 g salt l^{-1} [10]. The removal yields measured on phenol exceeded 99%. The experiment was renewed in 1995 [2], using a SBR with free culture, this time reaching an average yield of 99.5%. In Japan in 2000 [11], a hypersaline agri-food effluent (150 g NaCl l^{-1}) was purified by halo-tolerant bacteria. Chemical oxygen demand (COD) removal of about 90% was attained in an aerobic SBR. On the other hand, when the cultivation mode was changed to continuous culture, the COD removal was from 60 to 70%. Other potential applications do exist, e.g. recycling a micro-algae culture medium after a biological treatment [12].

All in all, in the whole corpus of scientific work published on the metabolism of halophilic micro-organisms, there is little reference to the removal by biological agents of hypersaline agri-food effluents and especially to the breaking down of aromatic compounds in saline conditions. However, according to those studies, it seems possible to treat hypersaline effluents using microbiological techniques, despite the considerable obstacles created by salt. Little research has gone beyond the pilot stage and most has used synthetic effluents.

In this paper, we treated a complex industrial hypersaline effluent in a bench-scale aerobic SBR using microbiological techniques. Furthermore, whereas most studies have adressed the removal of carbon only, we also tried to eliminate nitrogen. Lastly, the identification of the micro-organisms involved in the treatment was carried out by 16S rDNA identification.

MATERIALS AND METHODS

Influent

The influent used in this study resulted from the activity of a tartaric acid production plant. The analytical parameters of the influent appear in table 1. A glossary of these parameters and other terms used in this paper is presented at the end of the paper.

Table 1. Characteristics of the influent used for the experiment.

PH	7.8
Salt (g l^{-1})	120
SS (mg l^{-1})	1760
VSS (mg l^{-1})	460
CODt (mg l^{-1})	4340
CODs (mg l^{-1})	3770
TOCt (mg l^{-1})	1750
TOCs (mg l^{-1})	1580
TKNt (mg l^{-1})	190
TKNs (mg l^{-1})	150

Bench-scale Reactor

The bench-scale SBR (figure 1) had a volume of 5 L. The tubes inserted into the top of the reactor ensured the filling and the withdrawal of the effluent. Peristaltic pumps provided a fill flow and a draw flow of 42 and 39 ml min^{-1} respectively. An air compressor ensured bubble aeration

transferred in 2 ml tubes and stored frozen at – 20° C. Extraction and purification of total genomic DNA was carried out, using a protocol based on mechanical cell disruption by heat treatment (70 °C for 1h) in the presence of zirconium beads [17]. Nucleic acids are recovered after several washes with polyvinylpolypirolidone to remove PCR inhibitors before alcohol precipitation. Concentration and size of DNA (around 50 kb) were estimated by electrophoresis on a 0.7% agarose gel and viewed by ethidium bromide with ultraviolet emission.

Amplification, Cloning and Sequencing of the V3 Region of 16S rDNA

Highly variable V3 regions of microbial 16S rDNA genes were amplified by PCR using bacterial primers (W31 – W49) (see table 2). Samples were treated according to the protocol PCR-cloning – Amplification of 16S rDNA previously described [18]. For each reaction the solution, along with enzyme *Tampon Red Taq*® 5 U/µl in the amount of 5 µl, dNTP 2,5 mM in the amount of 4 µl and 2 - 2 µl of bacterial primers (w31 100 ng/µl – w49 100 ng/µl), was added to 1 µl of DNA diluted in water. PCR conditions were as follows: an initial denaturation step at 94°C for 2 min, followed by 30 cycles of a three-stage program with 1 min at 94°C, 1 min at 61°C and 1 min at 72°C, the final elongation step running for 10 min at 72°C. PCR products were purified with a QIAamp kit (Quiagen). Purified product sizes (~200pb) and concentrations were checked by electrophoresis on a 2% agarose gel containing ethidium bromide with ultraviolet emission. Correct PCR products were cloned and transformed into *Escherichia coli* using the pCR® 4-TOPO® vector kit according to supplier instructions (Invitrogen).

E. coli with inserts of proper size were screened by PCR on colonies with plasmid targeted primers T7 and P13. Afterwards, purified PCR products were sequenced with a dye-terminator cycle sequencing reaction kit with AmpliTaq DNA polymerase FS kit buffer (Applied Biosystems) and the T7 primer. Sequence reaction products were analysed on an ABI model 373A genotyper apparatus (Applied Biosystems).

SSCP Analysis

Single Strand Conformation Polymorphism (SSCP) analyses were performed for overall detection of microbial populations and for a study of their dynamics. SSCP analysis makes it possible to separate DNA fragments of a similar size according to their configuration (secondary structure). Targeting the 16S rDNA V3 region, which permits the phylogenetic discrimination of microbial species, enables the reactor microbial community to be monitored by one profile of peaks, where each peak corresponds to a different sequence of 16S rDNA V3 region i.e. to one bacterium. The height of the peaks corresponds to the quantity of 16S rDNA sequence after PCR amplification.

The amplification of the V3 region of 16S rDNA PCR-SSCP was carried out with specific primers w91 – w104 (see table 2) from total DNA. An initial denaturation step at 94°C for 2 min, was followed by 30 cycles of a three-stage program with 30 sec at 94°C, 30 sec at 61°C and 30 sec at 72°C, and a final elongation for 10 min at 72°C. DNA polymerase was *Pfu* turbo (Stratagene). PCR-SSCP products were purified using the kit QIAquick and were estimated by gel-electrophoresis.

For electrophoresis, PCR-SSCP products were diluted in water before mixing with 18.75 µl formamide (Genescan-Applied Biosystems) and 0.25 µl internal standard (ROX, Genescan-Applied Biosystems) [18]. The mixture was denatured by heating at 95°C for 5 min and cooled in watery ice for 10 min. Single strands of DNA molecules made stable secondary conformations which were separated by capillary electrophoresis. SSCP analyses were performed with the automatic sequencer abi310 (Applied Biosystems). DNA fragment detection was done with the fluorescent W34 primer. The results obtained were analysed by GeneScan® 3.1 (Applied Biosystems).

To identify SSCP peaks of interest, 16S rDNA V3 from reactor samples were amplified and cloned into *E. coli* as described above. Cloned inserts were amplified by PCR using the plasmid targeted primers T7 and P13. PCR-SSCP on the resulting DNA fragment produced, after SSCP analysis, single peaks which were compared with total microbial community

Table 2. Sequences and target positions of primers used in this study.

Primer	Sequence	Position in *E.coli* (Brosius *et al.*)[19]	Target
W31	TTACCGCGCTGCTGGCAC	R[b] 515-533	16S rDNA universal
W49	ACGGTCCAGACTCCTACGGG	F 329-348	16S rDNA *bacteria*
W104[a]	6-FAM-TTACCGCGCTGCTGGCAC	R 515-533	16S rDNA universal
W91	HEX-ACGGTCCAGACTCCTACGGG	F 329-348	16S rDNA *bacteria*
T07	TAATACGACTCACTATAGGG	-	plasmid
P13	GACCATGATTACGCCAA	-	plasmid

[a] The primer w104 is marked at 5′ end with fluorescent phosphoramidite - TET (Applied Biosystems).
[b] F and R correspond to forward and reverse primer.

profiles for peak assignation. Sequencing of interesting cloned V3 regions was carried out and, finally, the identification of micro-organisms corresponding to peaks was successfully established.

Sequence Analysis

16S rDNA V3 sequences (of about 200 bp) were identified by comparison with sequences available in databases using the BLAST program. The nucleotide sequence data reported in this work will appear in the GenBank nucleotide database under accession numbers AY188696 to AY188715.

RESULTS - DISCUSSION

Evolution of Reactor Performance during the Experiment

The concentrations in soluble COD (CODs) and soluble TKN (TKNs) and corresponding removal yields are shown in figures 2 and 3. The experiment can be divided into three distinct periods:

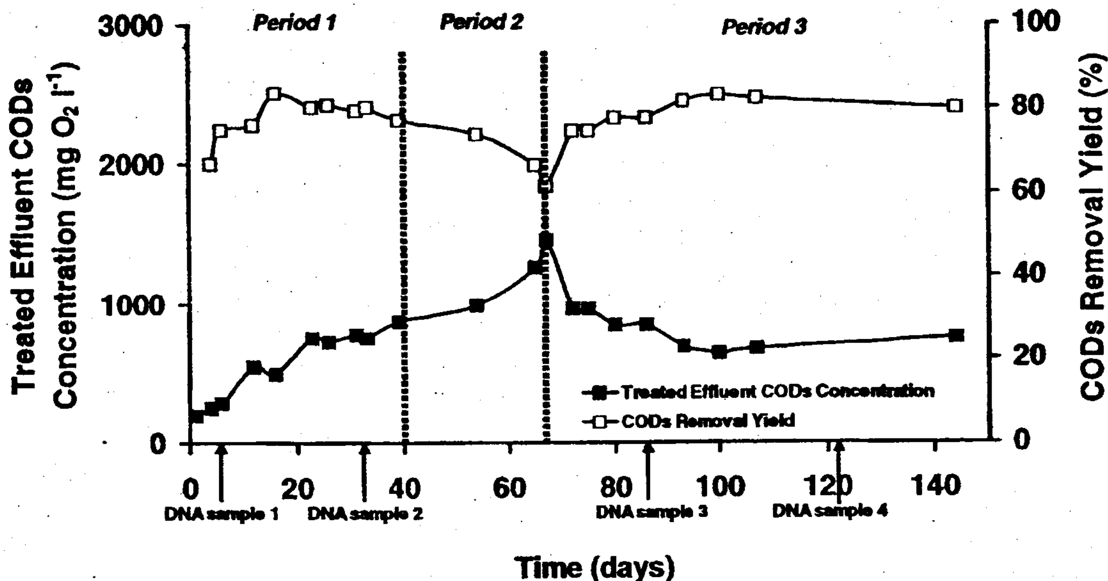

Figure 2. Evolution of treated effluent CODs concentration and CODs removal yield during the experiment. Days when DNA samples were collected from the middle of the digester are indicated by an arrow.

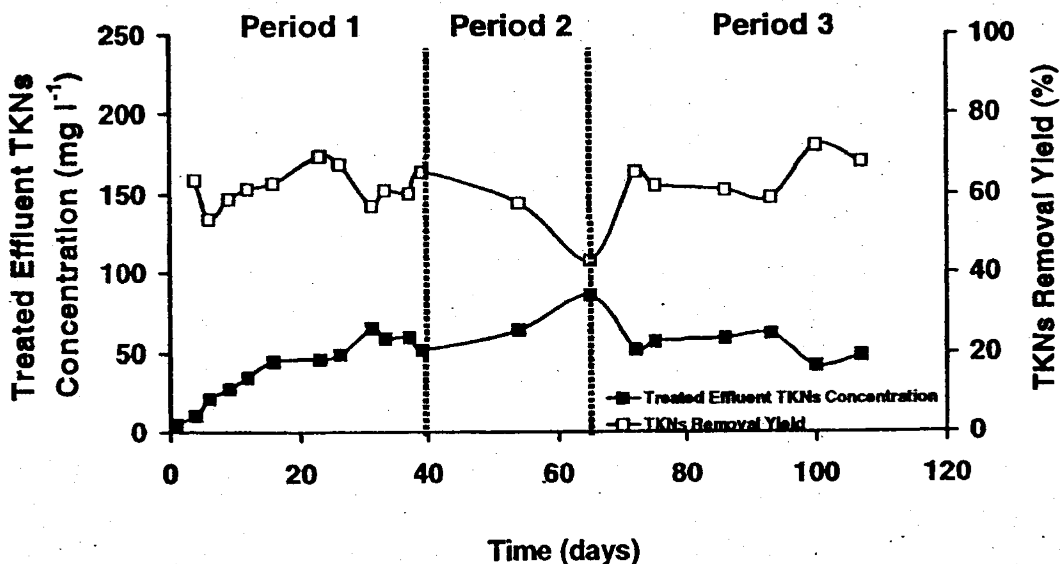

Figure 3. Evolution of treated effluent TKNs concentration and TKNs removal yield during the experiment.

- Period 1 was a starting period (approximately 40 cycles), during which salinity was increased gradually to reach that of the real effluent (120 g l^{-1}). Other operations were carried out during this phase: halophilic bacteria adjunction at cycle 17, doubling of the load at cycle 34 by doubling the flow rate. During this period, the reactor was not working under steady state conditions and its performance in removing CODs and TKNs decreased slowly, as the salt concentration increased, and in spite of the halophilic bacteria adjunction. Four millilitre samples were collected on days 6 and 33 for DNA extraction.
- Period 2 was a period of deterioration of the reactor performance (30 cycles approximately), during which salinity was stabilized near 120 g l^{-1} . This period also corresponded to a phase of microbial adaptation or selection due to the harsh conditions. The removal efficiency fell to 61% and 43% for the CODs and TKNs, respectively. At the same time, the bacterial biomass decreased considerably, from 5.1 g VSS l^{-1} at cycle 26 (the maximum value obtained during the experiment, in the days which followed the inoculation of halophilic sediment) to 1.9 g l^{-1} at cycle 65. As previously considered, the pH in the reactor increased up to 9, probably because of the consumption of residual organic acids, originating in the wine, contained in the influent. The ecosystem was destabilized by the extreme conditions of the medium (hypersalinity and alkaline pH) and this disturbance generated a considerable reduction in the removal yields, as well as in the biomass growth rate. Furthermore, a neutralisation of pH thanks to hydrochloric acid, carried out as of cycle 60, did not enhance the performance of the system.
- Period 3 was a period of enhancement of the process, followed by a stabilization phase. At cycle 67, phosphoric acid was substituted for hydrochloric acid. Within the next five days, the performance of the process was clearly enhanced. Figures 2 and 3 show that this period was characterised by a rapid improvement of the removal yields, followed by a stabilization of the system. Analysis of the biomass indicated a growth recovery up to 3.5 g l^{-1} at cycle 93, which is conventional for a low loaded plant treating an industrial effluent without VSS in the influent [20]. Thereafter the biomass remained constant, thus no sludge wastage was required. Four millilitre samples were collected on days 87 and 121 for DNA extraction.

Monitoring the performance of the SBR over time showed it is possible to treat highly saline effluents provided the pH is neutralised with phosphoric acid. The maximum removal yields reached 83% and 72% for the CODs and TKNs, respectively. These yields were obtained during period 3. As previously indicated [11], the inhibition due to alkaline pH added to the inhibition due to salt and prevented the optimal operation of the process and it was necessary to remove one of the two inhibitions in order to reach better removal yields. In addition, the positive impact of phosphoric acid indicated a phosphorus deficiency in the influent. The phosphorus requirement could be estimated to be 20 mg l^{-1}, using a conventional COD/P ratio of 200/1. After biomass acclimation, phosphorus was brought using phosphoric acid. The major problem encountered when treating the hypersaline effluent in the halophilic SBR was SS which consisted of dispersed particles that failed to form flocs and settle prior to draw. SS averaged 1800 mg l^{-1} in the treated effluent in this study, one third of this value consisting of VSS, as reported in figure 4. According to this VSS loss, the sludge age ranged from 40 to 50 days during period 3.

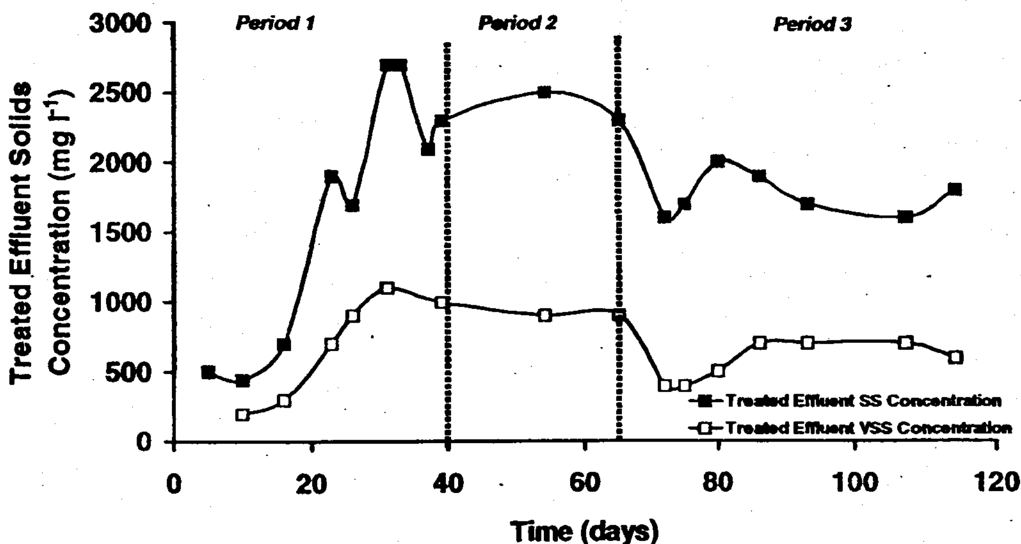

Figure 4. Evolution of treated effluent SS concentration and treated effluent VSS concentration during the experiment.

The high turbidity in the effluent explains why CODt and TKNt removal yields were much lower than those of the soluble fraction, optimal yields reaching respectively 68 and 46%, during period 3 (data not showed). These flocculation problems in hypersaline wastewater have already been reported [2]. Several factors may contribute to effluent turbidity: first of all, the lack of filamentous organisms, which were rarely observed during the study period, may contribute to this phenomenon. Filaments do indeed contribute to the mechanical integrity and the structure of the flocs [21]. In their absence, flocs are essentially made of individual particles which are easily disrupted. In addition, the protozoa, which play a role in the elimination of the micro-organisms by grazing them, were rarely observed during the study period. This may also have resulted in increasing effluent turbidity. Finally, the density of salt water is higher than that of fresh water and thus reduces the settling velocity of dispersed particles.

Study of a Single Operation Cycle of the SBR

This study was carried out during cycle 100, during the third period previously described, at a time when the reactor was stabilized. Figure 5 shows the removal of soluble TOC (TOCs) during this cycle in parallel with the dissolved oxygen concentration. It appears that carbon removal took place exclusively during the aerobic phase. This removal was very fast (4 h) and took place in two stages, according to the dissolved oxygen curve: the first stage lasted one hour and corresponded to the removal of easily biodegradable compounds. Then a second three-hour stage was probably linked to the removal of more recalcitrant molecules contained in the influent. It appears from figure 5 that the cycle duration could be halved since, after 12 hours, there was no further removal of TOCs. The purpose of the anoxic period was to allow nitrification/denitrification to take place in the reactor, but no such phenomenon could be observed during the operation cycle of the SBR (data not showed).

Dynamics of Bacterial Communities During the Experiment

Profiles of bacterial communities (figure 6) were obtained by PCR-SSCP analysis and their evolution was studied during the experiment. All profiles' areas were large and peaks were not isolated, which indicated high biodiversity. Profile 1 (obtained on day 6) was very different from profiles 2, 3 and 4 (obtained on day 33, 87 and 121): only four peaks (A, B, D and E) on profile 1 were still present later. This first profile probably corresponded to the urban wastewater treatment sludge inoculated on day 1. Then, after halophilic sediments were inoculated on day 17, the profiles changed quickly.

The area of profile 2 was less than the area of profile 1. This indicated a decrease in diversity due to the harsh conditions (high salt concentration and high pH). This period corresponded to microbial adaptation and/or selection. After the pH was neutralized and time passed, the area increased on profile 3, which meant that diversity increased. Finally, profiles 3 and 4 were similar (see peaks A to H. Only the height of the peaks, corresponding to proportions, changed).

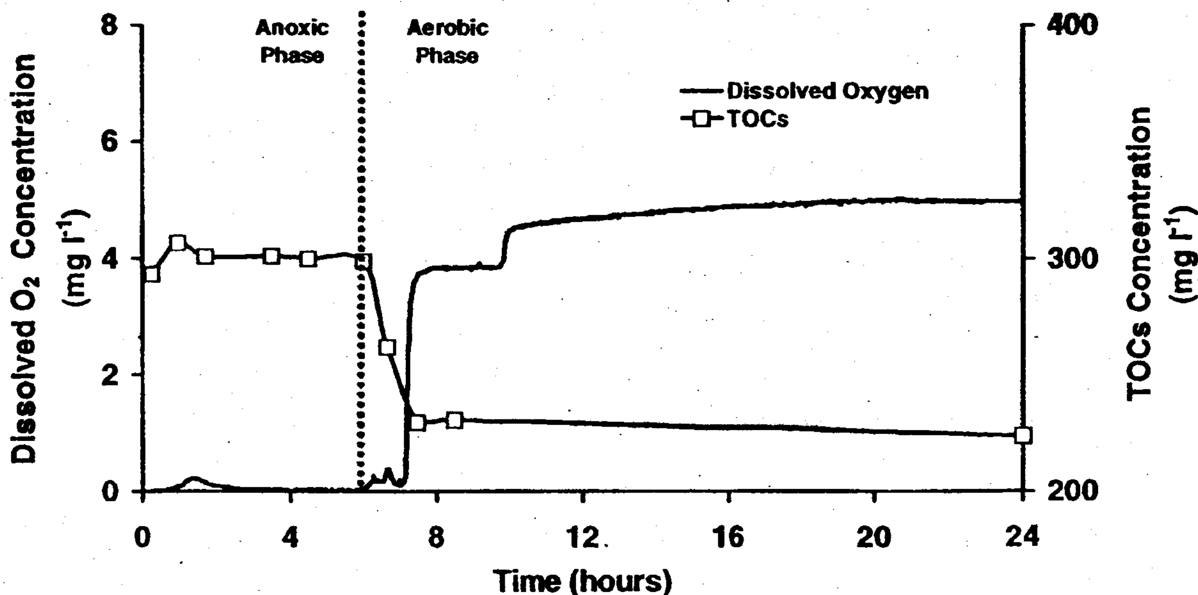

Figure 5. Variation in dissolved O_2 concentration and TOCs concentration during day 100.

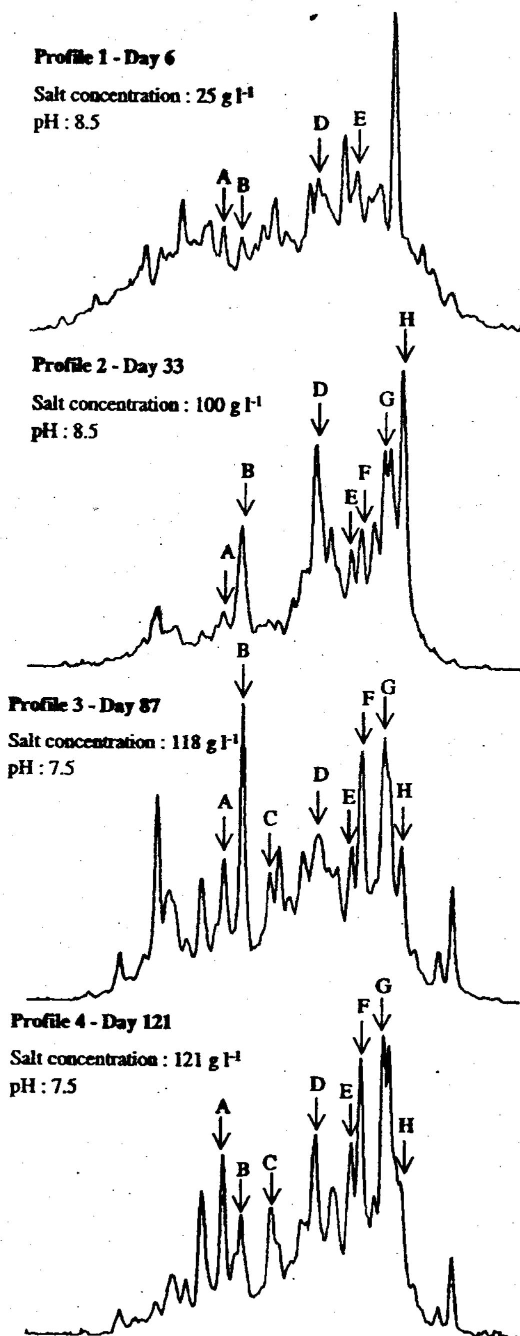

Figure 6. Dynamics of SSCP patterns of bacterial 16S rDNA region amplification products.

Thus, the dynamics of the bacterial communities were an accurate reflection of the evolution during the experiment. The addition of a halophilic biomass completely changed the bacterial community (see the difference between profile 1 and 2). But on profile 2, diversity was less than on profile 1. After acclimation was completed, diversity increased again (see profile 3).

Basically, the diversity (number of dominant 16Sr DNA) of the salt-tolerant ecosystem (profile 4) appeared to be similar to the diversity of the non-salt tolerant ecosystem (profile 1).

Microbial Community Identification on SSCP Patterns on Day 121

A 16S rDNA V3 region clone library was established from a bacterial DNA sample and called VM. PCR-SSCP analyses were conducted on 49 bacterial clones for identification. The results of 16S rDNA sequencing analysis are shown in Table 3.

According to 16S rDNA sequencing, the microbial clones belonged to five divisions of *Bacteria*: *Gamma-Proteobacteria* (27 clones), *Alpha-Proteobacteria* (3 clones), *Cytophagales* (13 clones), Gram positive low GC (5 clones), and BRC1 (1 clone). More than half of the clones belong to the division of *Gamma Proteobacteria*. The *Cytophagales* are very well represented, too. Lastly, one clone (VM51) belongs to lineage BRC1 which has been considered as a novel candidate division [22]. Up to the present, this division has included 16S rDNA genes from bulk soil and rice roots of flooded rice environments. VM51 has a sequence similarity of 89% with these 16S rDNA genes from anoxic soil.

Many of the 16S rDNA are close to **16S rDNA** of identified marine bacteria while others are common in oil fields, which are often associated with the presence of saline water. Among them, many *Halomonas sp.* are known for their capacity for biopurification (see introduction) and many *Marinobacter sp.* are known for their capacity in the biological removal of oil hydrocarbons discharged into the marine environment. It can be concluded that these particular bacteria took part in the removal of pollution in the effluent studied here.

Considering the marine habitat, it has already been reported [23] that most bacteria identified by molecular methods belong to the divisions of *Proteobacteria* (67%) and *Cytophagales* (25%).Within the framework of this study, 30 clones out of 49 (61%) belong to the division of *Proteobacteria* and 13 clones out of 49 (27%) belong to the division of *Cytophagales*. Thus, the majority of the halophilic 16Sr DNA identified in this study belongs to the same division as those of a marine ecosystem.

The microbial clones clustered within 20 Bacterial OTUs were distributed as follows in the five divisions: *Gamma Proteobacteria*, 6 OTUs – *Cytophagales*, 8 OTUs – Gram + low GC, 3 OTUs – *Alpha Proteobacteria*, 2 OTUs – BRC1, 1 OTU.

For only a few OTUs do the closest 16S rDNA correspond to uncultured or unidentified bacteria, which may seem surprising. Except for VM51, which belongs to a new division (BRC1), other OTUs belong to previously known divisions. One of the reasons explaining this fact may be the particular attraction of extreme mediums for researchers whose work has thus led them to a good understanding of divisions which include extremophilic bacteria.

Table 3. Phylogenetic affiliation of the 16S rDNA sequences.

OTU name	N°. of clones	Divergence within OTU	Division	Closest 16S rDNA	Accession number	Source	Sequence similarity
VM40	9	1.2%	Proteobacteria gamma	*Halomonas sp. BYS-1*	AY062217	Unknown	100%
VM02	1	0%	Proteobacteria gamma	Uncultured bacterium clone MN16BT2-9	AF361651	Deep-sea carbonate crusts	99%
				Halomonas sp. YIM-kkny11	AY121436		98%
VM31	1	0%	Proteobacteria gamma	Uncultured bacterium clone MN12BT3-44	AF361672	Deep-sea carbonate crusts	100%
				Halomonas sp. SYM P12	AB085660		99%
VM03	7	1.2%	Proteobacteria gamma	*Marinobacter sp.* DS40M8	AF199440	Subseafloor habitats associated with a deep-sea volcanic eruption	100%
VM14	2	0.6%	Proteobacteria gamma	Unidentified eubacterium SCB45	U64022	Marine bacterioplankton	100%
				Marinobacter sp. NK-1	AB026946		98%
VM32	7	0%	Proteobacteria gamma	Marinobacterium georgiense	AB021408	Unknown	100%
VM64	1	0%	Proteobacteria alpha	Blastochloris sulfoviridis	AY117149	Thermotolerant purple non-sulfur bacterial strain	98%
VM26	2	0%	Proteobacteria alpha	*Sinorhizobium morelense*	AY024335	Leucocephala-associated bacterium	100%
VM30	2	0.6%	Cytophagales Flavobacteria	Marinilabilia salmonicolor	M62422	Unknown	98%
VM56	1	0%	Cytophagales Flavobacteria	Marinilabilia salmonicolor	M62422	Unknown	96%
VM55	2	0%	Cytophagales Flavobacteria	Unidentified bacterium	AJ409009	Unknown	93%
				Bacteroides sp.	AB064818		93%
VM33	4	1.9%	Cytophagales Flavobacteria	*Psychroflexus torquis*	AF001365	Antarctic sea ice	94%
VM23	1	0%	Cytophagales Flavobacteria	Uncultured CFB group bacterium	AF440843	Crude oil-contaminated coastal marsh	92%
				Cytophaga sp.	AB015525		85%
VM50	1	0%	Cytophagales Flavobacteria	*Flavobacterium salegens*	M92279	Hypersaline Antarctic lake	100%
VM11	3	1.2%	Gram + Low GC	Unidentified Hailaer soda lake bacterium	AF275702	Hypersaline soda lake of Inner Mongolia	92%
				F24 *Alkalibacterium olivoapovliticus*	AF143512		90%
VM25	1	0%	Gram + Low GC	*Tetragenococcus halophilus*	AB041349	Fish sauce	98%
VM05	1	0%	Gram + Low GC	*Haloanaerobium congolense*	U76632	African oil field	98%
VM60	1	0%	Cytophagales Flavobacteria	Uncultured cytophagales bacterium clone LA7-B21N	AF513957	Hypersaline lake and brackish pond of remote Hawaiian island	93%
VM06	1	0%	Cytophagales Flavobacteria	Uncultured delta proteobacterium MT23	AF211273	Hydrogen sulfide-rich black mud from marine coastal environments	93%
				Cytophaga sp	AB015264		95%
VM51	1	0%	BRC	Uncultured soil bacterium PBS-III-16	AJ390451	Bulk soil and rice roots of flooded rice microcosms	89%

16 OTUs out of 20 correspond to halophilic genes, which indicates that no phenomenon of adaptation occurred but, rather, a selection of halophilic bacteria. This result was foreseeable given the remarks of Woolard and Irvine already described [2]: non-halophilic bacteria do not tolerate salt concentrations higher than 50 g l^{-1}.

Final identification tests using primers of *Archaea* gave negative results (data not shown), which means that Bacteria alone were active in the hypersaline reactor.

CONCLUSIONS

The data presented in this paper show that halophilic bacteria can be used to treat hypersaline wastewater. Bacteria taken from the sediments in the evaporation basins of an agri-food plant generating hypersaline effluents were maintained for more than 100 cycles in a SBR. Whereas most of the papers already published have focused on the removal of carbon compounds in synthetic effluents, the bacteria studied here were able to treat the carbon and nitrogen pollution of a complex effluent produced by the same industry from where they originated. The removal yields reached 83, 88 and 72% for the CODs, TOCs and TKNs, respectively. But high levels of SS remained in the treated effluent.

The results of this experiment have, however, proved the feasibility of treating hypersaline industrial wastewater, providing the parameters of the reactor are fully controlled. The techniques of molecular microbiology have shown the phylogenetic diversity of the halophilic species that developed in the SBR operated for this study. The diversity of a salt-tolerant ecosystem can be similar to the diversity of a non salt-tolerant one.

GLOSSARY

$CaCl_2$: Calcium chloride (mg l^{-1})
CODs: (soluble) Chemical oxygen demand (mg O_2 l^{-1})
CODt: (total) Chemical oxygen demand (mg O_2 l^{-1})
HCl: Hydrochloric acid (mg l^{-1})
KCl: Potassium chloride (mg l^{-1})
N_2: Dinitrogen (mg l^{-1})
NaCl: Sodium chloride (mg l^{-1})
NO_3^-: Nitrate (mg l^{-1})
TKNs: (soluble) Total kjedahl nitrogen (mg l^{-1})
TKNt: (total) Total kjedahl nitrogen (mg l^{-1})
O_2: Dioxygen (mg l^{-1})
OTU: Operational taxonomic unit
PCR: Polymerase chain reaction
SBBR: Sequencing batch biofilm reactor
SBR: Sequencing batch reactor
SS: Suspended solids (mg l^{-1})
SSCP: Single strand conformation polymorphism
TOCs: (soluble) Total organic carbon (mg l^{-1})
TOCt: (total) Total organic carbon (mg l^{-1})
VSS: Volatile suspended solids (mg l^{-1})

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