ISOLATION OF HIGHLY PERFORMANT SULFATE REDUCERS FROM SULFATE-RICH ENVIRONMENTS

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INDEX ENTRIES : biodegradation, hydrogen, inhibition, isolation, sulfate-reducing bacteria, sulfide.

ABSTRACT

Eleven pure strains of sulfate-reducing bacteria have been isolated from lab-scale bioreactors or disposal sites, all featuring relatively high concentrations of sulfate, and from natural environments in order to produce sulfide from gypsum using hydrogen as energy source. The properties of the eleven strains have been investigated and compared to these of three collection strains *i.e*. *Desulfovibrio desulfuricans* and *vulgaris* and *Desulfotomaculum orientis*. Particular attention was paid to the absolute and relative sulfide production rate and to the hydrogen sulfide inhibition level. By comparison to the collection strains, a 75 % higher production rate and a hydrogen sulfide inhibition level about twice higher *i.e.* 25.1 mM have been achieved with strains isolated from sulfate-rich environments. The strain selection, particularly from sulfate-rich environments, should be considered as an optimization factor for the sulfate reduction processes.

INTRODUCTION

Many biotechnological processes of sulfate reduction have been investigated throughout the world. In the past decades, the basic aims of these investigations was often to provide sulfur from gypsum or sulfate wastes when the sulfur supplies were running low (Butlin et al. 1956; Burgess & Wood 1961; Sadana & Motey 1962; Maree 1988; Maree et al. 1991). More recently, the objective of the study of the biological sulfate

reduction was the protection of the environment through the removal of heavy metals (Cork & Cusanovitch 1979 ; Salmon et al . 1990 ; Barnes et al. 1992 ; Dvorak et al. 1992 ; Stucki et al. 1993) and/or sulfate (Somlev & Tishkov 1992 ; Van Houten et al. 1994-1996 ; Hiligsmann et al. 1996 ; Deswaef et al. 1996 ; Kaufman et al. 1997) from waste water or solid wastes.

The fundamental approach of most of these investigations was to optimize the sulfate reduction by comparing different kind of reactor design, biomass carrier, organic substrate, electron donor or other nutrients and physical conditions without paying real attention to the strain selection.

The biotechnological process recently investigated (Hiligsmann et al. 1996) involved dissimilatory sulfate-reducing bacteria (SRB) in order to produce sulfide from gypsum wastes and oxidize a cheap residual organic substrate as an electron donor. Four strains of incompletely lactate-oxidizing (into acetate) SRB and one strain of completely acetate-oxidizing SRB (Widdel & Bak 1992 ; Widdel & Hansen 1992) had been isolated (method described by Postgate 1984) from several samples of natural environments and had been studied in bench-scale bioreactors. The results clearly showed the feasibility of the biotechnological process which took advantage of the two types of SRB, in order to oxidize completely the organic substrate used as an electron donor. However, the acetate oxidation by SRB was to be further improved.

According to Badziong et al. (1978) and Widdel & Hansen (1992), acetate may be metabolized by SRB through two pathways. In the first one, acetate acts both as electron donor and carbon source for cell biosynthesis. In the second metabolic pathway, acetate is used as a carbon source jointly with carbon dioxide when hydrogen is used as the sole electRon donor. Except for halophilic strains such as *Desulfobacter postgatei*, which are not

very interesting for a waste treatment process, the catabolism of acetate as electron donor is very slow (Postgate 1984 ; Widdel & Hansen 1992). In contrast, the hydrogen metabolism is known to be as competitive as the lactate metabolism for sulfate reduction biotechnological processes (Du Preez et al. 1992 ; Van Houten et al. 1994-1996).

The purpose of the present study was to isolate new strains of SRB using hydrogen as energy source. Therefore, the sulfate reduction process that we have already investigated with lactate as the first electron donor could be followed by an acetate degradation performed in a larger reactor with hydrogen, from chemical or biotechnological production, as an electron donor. The new strains isolated from lab-scale bioreactors or disposal sites, all featuring relatively high concentrations of sulfate, and from natural environments were investigated and compared with three SRB strains from international collections. Particular attention was paid to the absolute and relative (per cell) sulfide production rate and to the hydrogen sulfide inhibition level.

MATERIALS AND METHODS

Collection strains

Desulfovibrio desulfuricans (subsp. Desulfuricans Essex6 ATCC 29577) and *Desulfovibrio vulgaris* (subsp. Vulgaris Hildenborough ATCC 29579) were obtained from the Laboratorium voor Microbiologie (University of Gent, Belgium). *Desulfotomaculum orientis* (ATCC 19365) was supplied by the Deutsche Sammlung von Mikroorganismen und Zellkulturen.

Media

The culture media described by Postgate (1984) were slightly modified. Medium B (in grams per liter of demineralized water) : KH_2PO_4, 0.3; $MgSO_4 \cdot 7H_2O$, 0.5; $FeSO_4 \cdot 7H_2O$, 0.5; $CaSO_4 \cdot 2H_2O$, 1 (or more when indicated); NH_4Cl, 0.5; yeast extract, 1; ascorbic acid, 0.2; sodium thioglycolate, 0.1; EDTA, 15 µM; resazurin (redox indicator), 0.001; sodium lactate, 4.7 (for cultures with lactate as electron donor) or sodium acetate trihydrate, 4 (for cultures with hydrogen as electron donor). Medium E : K_2HPO_4, 0.2; $MgSO_4 \cdot 7H_2O$, 0.2; $(SO_4)_2Fe(NH_4)_2$, 0.2; Na_2SO_4, 0.5; $CaCl_2 \cdot 2H_2O$, 0.5; yeast extract, 1; ascorbic acid, 0.2; sodium thioglycolate, 0.1; sodium lactate, 3.5; resazurin (redox indicator), 0.001; agar, 15. Hydrochloric acid and sodium hydroxide were used to adjust pH in media B and E to 8.5 before being autoclaved (after sterilisation, pH is near 7.1).

Medium PCA for aerobic purity check (in grams per liter of demineralized water) : casein peptone, 5; yeast extract, 2.5; glucose, 1; agar, 15. Medium PCAan for anaerobic purity check (in grams per liter of demineralized water) : casein peptone, 5; yeast extract, 2.5; glucose, 1; agar, 15; $(NH_4)_2 Fe(SO_4)_2 \cdot 6H_2O$, 0.2.

All chemicals were of analytical or extra pure quality and supplied by Merck, Union Chimique Belge, Sigma or Organotechnie (La Courneuve, France; for casein peptone and yeast extract).

Strains isolation procedure

The isolation techniques were applied on samples of natural (poorly salted or unsalted) environments such as manures (pig, bovine and rabbit) or mud taken from a pond, on samples of lab-scale biomethanisation and sulfate-reduction bioreactors and on samples of gypsum taken from disposal sites (about 10 and 2 years old). The isolation procedure has followed a

relatively particular pathway already described (Hiligsmann et al. 1995) and summarized below. At the beginning, the aim of the experiments was to isolate SRB strains oxidizing acetate as electron donor. Therefore, Postgate's (1984) general technique for strain isolation, involving enrichment cultures in medium B and colony growth in medium E (both media containing 4 g/l of sodium acetate trihydrate and incubated at 30 °c), was applied three times successively to each sample. Because this procedure did not generate pure strains according to purity checks for both aerobic and anaerobic contaminants, more selective media have been used for SRB isolation from the contaminated strains.

Widdel & Bak's (1992) isolation method was carried out in different way : with sodium dithionite as the sole redox-poising agent, with dithionite plus sulfide and subsequently with the same redox-poising agent but with 2-deoxy-D-glucose. The 2-deoxy-D-glucose is analogous to glucose and may inhibit microorganisms growing on it, without affecting SRB which are, apart from some rare strains, unable to use glucose as electron donor or organic substance for biosynthesis (Widdel & Hansen, 1992). Media containing an autoclaved culture of the facultative anaerobic contaminant were also used as mentioned by Sato (1988). However, only one pure strain of SRB was obtained but it showed a very slow metabolism in medium containing the sole acetate as electron donor. Other experiments demonstrated that the presence of a particularly predominant contaminant (defined as Z and present in about all the samples) was of great importance for SRB because it provided them another electron donor that is hydrogen. In fact, attempts to isolate acetate-oxidizing SRB strains would have lead to hydrogen-utilizing strains.

Eventually, a rapid isolation of the SRB from the contaminated cultures was obtained by using a selective antibiotic against the Z

contaminant. The antibiotic and contaminant names will not be divulged because of industrial interest.

According to Widdel & Bak (1992), most of the SRB strains that are able to use hydrogen as electron donor are also lactate-oxidizing strains. Therefore, isolation techniques with antibiotics have been applied, with greater ease, in media containing lactate instead of a gas mixture. Furthermore, the isolated strains did not contradict the former statements of Widdel & Bak. The contaminated strains containing only facultative anaerobic contaminants have been enriched by successive cultures incubated at 30 °c. These cultures were realized in 25 ml hermetically stoppered tubes completely filled with a 200 µl inoculum and medium B already inoculated (24 h ago) with a strong culture of the Z contaminant. A subculture without pre-inoculation and containing the selective antibiotic was checked for both aerobic and anaerobic purity after SRB growth.

When both facultative and strict anaerobic contaminants were present in the contaminated cultures, the successive cultures had to be followed by strain isolation procedure carried out in stoppered tubes (25 ml) completely filled with medium E. One mL of inoculum was successively diluted in 24 ml of sterile medium E held molten at 43 °c. Each culture tube was supplemented with 1 ml of a strong culture of the Z contaminant, mixed and incubated at 30 °c once the agar had set. Tubes were broken at convenient points and samples of black colonies (due to precipitation of iron sulfide) were withdrawn with a straight platinum wire. The wire was dipped in hermeticallly stoppered tubes which had been complelely filled with sterile medium B containing the selective antibiotic.

This method allowed to obtain other pure SRB stains according to purity checks for both aerobic and anaerobic contaminants.

Culture in serum bottles

Hermetically stoppered tubes were completely filled (25 ml) with medium B containing 5 g/l of gypsum and 4 g/l of sodium acetate trihydrate and adjusted to pH 8.75. After sterilization and cooling, the content of two tubes was transfered in a 100 ml sterile glass bottle. The medium was inoculated with 200 µl of cell culture (maintained at high growth level by three successive cultures in medium B containing sodium lactate) and supplemented with 250 µl of a 1M Na_2CO_3 solution (added from an autoclaved stock solution). A sterile butyl stopper was inserted in each bottle using a flamed tool as described by Hermann et al. (1986). A perforated metal cap was then screwed to seal the stopper to the bottle.

A gassing probe equipped with a sterile hypodermic needle was placed through the butyl stopper in order to fill the bottle with a filter-sterilized (0.2 µm pore size) gas mixture of hydrogen and carbon dioxide (80 % and 20 %, respectively; containing less than 3 ppm O_2 and supplied by l'Air Liquide). A second sterile needle was also placed through the stopper for 4 min (gassing phase) and then withdrawn in order to overpressurize the bottle (0.5 bar measured with a 3 bar full scale manometer) with the gas mixture.

The pH after gassing was near 6.9. The bottles were incubated at 30°C on an orbital shaker at 180 RPM. Since the hydrogen and CO_2 were consumed by the bacteria, the bottles were daily overpressurized, just before sampling by means of sterile syringes and needles.

The cultures realized with an initial gypsum concentration of 20 g/l, in either 100 ml or 500 ml serum bottles, were performed in similar conditions. However, 95 % of the gypsum was sterilized directly in the bottles. The 500 ml serum bottles contained 100 ml of medium B supplemented with 720 µl of a 1M Na_2CO_3 solution and inoculated with

500 µl of cell culture. The gassing time was raised to 15 min.

Cell cultures used as inoculum in the batch bioreactors were realized according to this method in 500 ml serum bottles with 11 g/l of gypsum (initial concentration).

Batch bioreactor

A 2.5 l glass vessel (2 l BIBBY culture vessel) with a stainless steel lid equipped with septum, shaft with 2 Rushton turbines (4 blades, height 10 mm , diameter 45 mm), 0.2 µm gas filters and tubings for sampling, gas inlet, gas outlet and temperature regulation was used as the batch bioreactor (constructed at University of Liege). Medium B (950 ml; gypsum, 1g/l; sodium sulfate, 8.3 g/l; without ascorbic acid and sodium thioglycolate) was autoclaved at 120 °C for 20 min, cooled under nitrogen and inoculated with 45 ml of cell culture in 500 ml serum bottle. After inoculation, the medium was gassed with a gas mixture of H_2 and CO_2 for 5 min (gas bubbling in the medium).

Needles placed through the septum were used to maintain pH (405-DPAS-SC-K8S/225 INGOLD combined probe) in the range 7.2 - 7.4 (the probe deviation due to sulfide was taken into account) by means of sterile H_2SO_4 (0.5 N) or NaOH (0.5 N) addition. Redox-poising agents like thioglycolate or ascorbic acid were added during inoculation to prevent their degradation by oxygen during cooling. The stirring speed and temperature were kept constant at about 120 RPM and 30 °C, respectively. The redox potential of samples was determined with an INGOLD Platinum combined probe. During culture, the bioreactor was regularly overpressurized by 0.5 bar with the H_2-CO_2 gas mixture.

Analytical methods

Sulfate was determined by the turbidimetric method : sulfate ions are precipitated by barium and the solution turbidity was analyzed by a spectrophotometer at 450 nm (Greenberg et al. 1985). Sulfide was determined photometrically using the methylene blue method applied to liquid samples (ranging from 20 µl to 1 ml) which had been collected in 5 mL of a 2 % (w/v in water) zinc acetate solution (Fogo & Popowsky 1949; Florin 1991). It should be noted that the sulfide contained in the gaseous compartment was not taken into account but, since the physical conditions (pH and temperature) were identical for each strain, the sole soluble sulfide might be considered in the strain comparisons.

Cell concentration was determined by microscopic observations on Bürker counting chamber (mean of 10 countings) after dilution in a 0.4 % formic aldehyd fresh solution and settling in the counting chamber for about 10 min. Formic aldehyd prevents the SRB motility and then allows the bacteria to settle.

Cell dry weight was determined from a 1 l cell culture in medium B (containing sodium lactate, 2.3 g/l, yeast extract 0.2 g/l, $FeSO_4 \cdot 7H_2O$, 0.05 g/l; $CaSO_4 \cdot 2H_2O$, 1 g/l; Na_2SO_4, 5 g/l and $CaCl_2 \cdot 2H_2O$, 0.2 g/l). The culture was centrifuged at 100 g for 15 min in order to remove the precipitated molecules (i.e., iron sulfide and residual gypsum) from the culture. The cell suspension was centrifuged at 17,700 g for 30 min and the supernatant was replaced by a 5 % NaCl solution supplemented with EDTA (15 mM). The centrifuged cells were suspended and washed 4 times. Eventually, the centrifuged cells were mixed in 100 ml of NaCl-EDTA solution. A 1 ml aliquote was sampled for cell counting and the rest was filtered free of bacteria on cellulose acetate membrane filter with a pore size of 0.2 µm. The filter was dried at 105 °c until the weight was constant.

Determination of sulfide production parameters

In the experiments realized in serum bottles, the absolute H_2S production rate (mM H_2S /d) was calculated as the ratio between the relative increasing of the total sulfide concentration (between two consecutive measured values) and the time period. The maximum H_2S production rate per cell (mol H_2S / d. cell) was calculated as the ratio between the maximum absolute H_2S production rate and the mean cell concentration observed during the related time period.

In the experiments carried out in batch bioreactors, the measured values of sulfide and cell concentration versus time were best fitted by third order polynomial equations. The maximum absolute H_2S production rate was determined as the slope calculated at the curve inflection point (second derivative being nil). The maximum H_2S production rate per cell was determined as the ratio between the maximum absolute H_2S production rate and the cell concentration calculated at the related culture time.

RESULTS AND DISCUSSION

Comparison of the 14 SRB strains

The isolation procedure using selective antibiotics generated eleven pure strains according to purity checks for both aerobic and anaerobic contaminants. These strains and the three others from collection were compared on the basis of three criteria : their maximum absolute production rate (mM H_2S /d), the production rate per cell observed at the maximum absolute production rate (mol H_2S /d.cell) and the sulfide concentration that inhibits their growth. These results are presented in figure 1. The production rates were determined from four cultures in 100 ml serum bottles with 5 g/l of gypsum in the culture medium. This gypsum was completely reduced after 200 h of culture. The gypsum initial concentration was changed to 20

g/l during the study of the sulfide inhibition level. Figure 2 illustrates the sulfide inhibition study concerning the F and G strains.

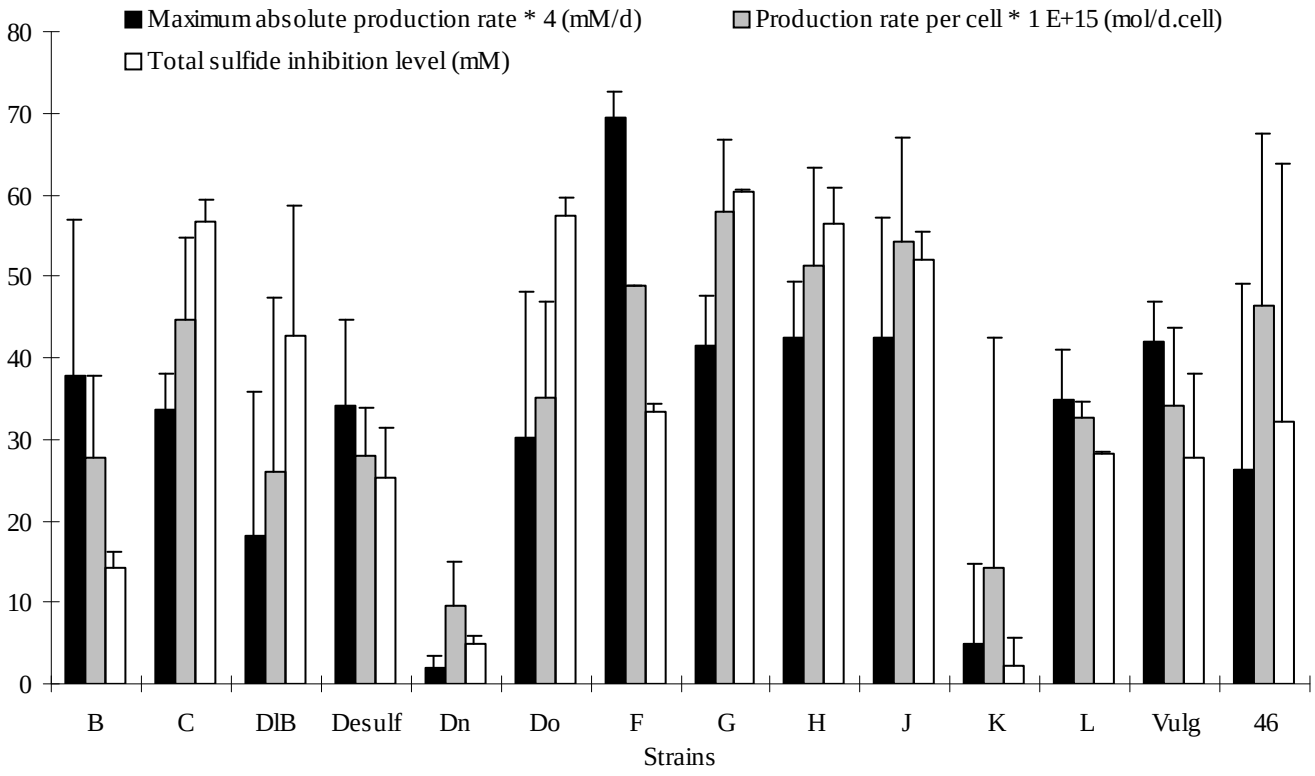

Fig. 1. Results of the comparisons of the 11 isolated SRB strains and the 3 collection SRB strains. Maximum absolute H_2S production rate [µM/d]; H_2S production rate per cell [mol/d.cell] calculated at the maximum absolute H_2S production rate and total sulfide inhibition level [µM] (including related standard deviation).

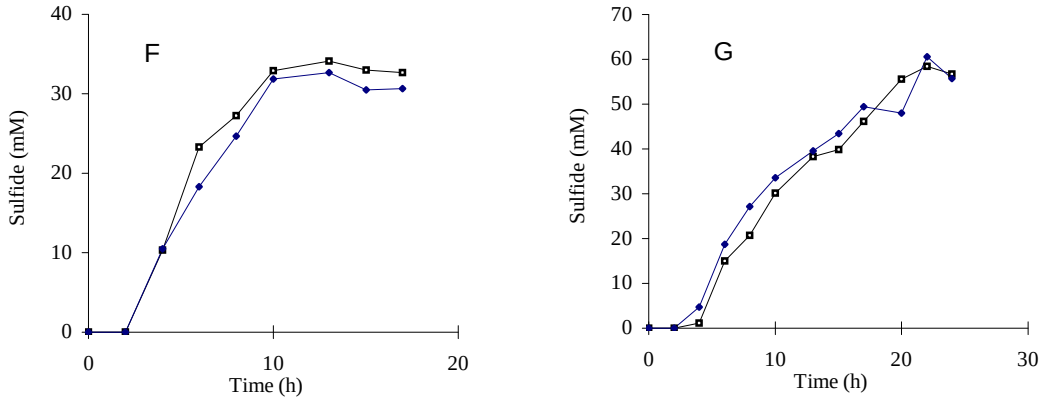

Fig. 2. Total sulfide concentration [µM] in the culture medium B (gypsum, 20 g/l) during

cultures in 100 ml serum bottles with the F and G strains (repeated twice).

The strains defined as DlB, Dn, K and 46 showed a relatively long lag phase (more than four days) usually followed by a slow growth. After 15 days of culture, some samples had not grown yet despite right and similar initial conditions in regard to the pH and redox potential. Nevertheless, these results were considered in the calculation of the standard deviation leading to large values. The lag phase of the other strains was shorter than two days and the maximum production rate was reached after about three days of culture. The Dn strain achieved relatively slight results compared to the others. This would be a consequence of its capability to form spores. However, this strain type could not be excluded from the strain selection procedure but it seems not adapted for this industrial process.

The F, G, H and J strains are the most interesting one according to the three selection criteria. Among them, F, G and H were isolated from sulfate-rich environments. It should be noted that the bacteria defined as Desulf, F and Vulg are smaller (two to three times shorter) than the bacteria of the other strains. By contrast, they lead to a higher cell number in the cultures.

According to Reis et al. (1992), hydrogen sulfide is the inhibitory form for SRB. Figure 1 shows that the Desulf and Vulg strains, *i.e. Desulfovibrio desulfuricans* and *vulgaris*, have achieved a total sulfide inhibition level of 25.2 mM and 27.7 mM, respectively. The relative distribution of H_2S and HS^- at pH 7.15 (measured at the end of cultures) is about 1:1.41. Therefore, the total sulfide concentrations could be converted in free H_2S concentrations of about 10.5 mM and 11.5 mM, respectively. By comparison, Okabe et al. (1992) mentioned a H_2S inhibition level for *Dv. desulfuricans* of 14.5 mM and other authors reported total inhibition of

growth at free H_2S concentrations of about 16 mM (Reis et al. 1992; Stucki et al. 1993). No higher levels have been mentioned except by Isa et al. (1986) but in mixed culture of SRB and methanogenic bacteria. Our experiments would confirm these results since the pure G strain reached a free H_2S concentration of 25.1 mM.

According to the morphological observations and the sulfide inhibition investigations, the F strain (13.9 mM free H_2S inhibition level) could be considered as similar to the *Dv. desulfuricans* and *Vulgaris* strains.

Other experiments with the F, G, H and J strains in 500 ml serum bottles containing 100 ml of culture medium have led to similar results of sulfide inhibition levels. It should be noted that, at the end of bioconversion (i.e., after about 15 days for the F strain and 30 days for the others), a residual gypsum concentration of about 6 g/l was observed for the F strain and a concentration ranging from 0.5 to 1 g/l was observed for the G, F and J strains. Therefore, the gypsum was not in limiting concentration.

Selection of the most efficient strain

In order to select the most efficient strain for the sulfate reduction biotechnological process, the four strains defined as F, G, H and J had to be investigated with better accuracy. Therefore, each strain was cultivated in 2.5 l bioreactors equipped with pH regulation (conditions described in Materials and Methods). The results of these experiments are presented in figures 3 and 4 and in table 1.

The investigations carried out in serum bottles and in 2.5 l bioreactors have demonstrated that the F strain was the most efficient one because it had led to the highest absolute production rate. However, it was inhibited by a H_2S concentration about twice lower than that observed with the G strain

which is the most resistant strain to H_2S toxicity. Furthermore, among the strains which are constituted of morphologically similar bacteria (i.e., G, H and J), the strain defined as G showed greater absolute and relative production rates.

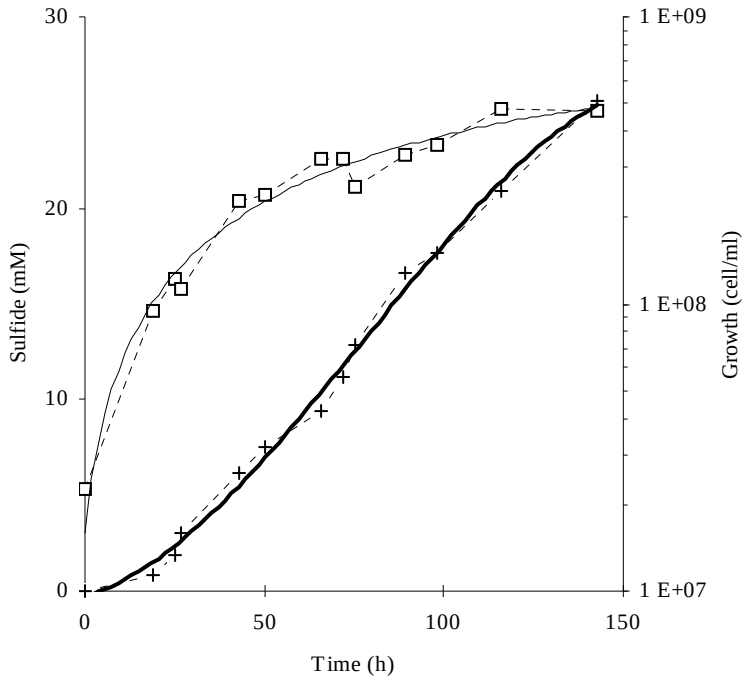

Fig. 3. (Î □) Growth of the G strain and (+) total sulfide concentration [μM] in the culture medium B (gypsum, 1 g/l and sodium sulfate, 8.3 g/l) during 2.5 l batch culture (including polynomial fit curves).

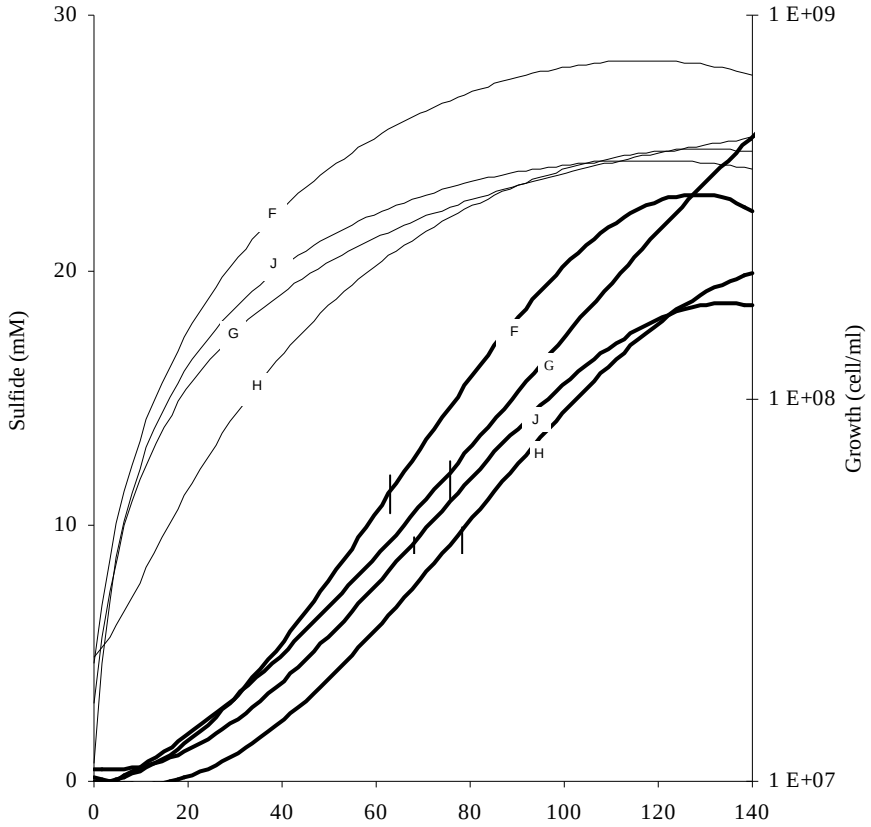

Fig. 4. (-) Growth of the F, G, H and J strains and (-) total sulfide concentration [µM] in the culture medium B (gypsum, 1 g/l and sodium sulfate, 8.3 g/l) during 2.5 l batch cultures. Polynomial fit curves. The marks indicate curve inflection point.

Table 1. Results of the investigations in 2.5 l bioreactors with the most efficient SRB strains. Maximum absolute H_2S production rate and H_2S production rate per cell calculated at the maximum absolute production rate

	Strains			
	F	G	H	J
Maximum absolute H_2S production rate (mM/d)	6.4	5.5	5.3	5
H_2S production rate per cell calculated at the maximum absolute H_2S production rate (mol/d.cell)	$1.4\ 10^{-14}$	$1.7\ 10^{-14}$	$1.7\ 10^{-14}$	$1.5\ 10^{-14}$

It should be noted that, although they have relatively different sizes, the F and G strains have similar cell dry weight, i.e. $2.2\ 10^{-13}$ g/cell and 2.8

10^{-13} g/cell, respectively. It should also be noted that their maximum production rate per gram of cell dry weight are very close (i.e., difference not exceeding 0.1 %). Therefore, since it is assumed that cell dry weight is closely related to the assimilated carbon for biosynthesis, it can be concluded that, per unit of assimilated carbon, the F and G strains have similar H_2S production rates.

Conclusions

The main purpose of the experiments presented in this study was to select efficient SRB strains using hydrogen as an electron donor in order to improve the biotechnological process of producing sulfide from the industrial by-product gypsum. Therefore, SRB strains were isolated from natural environments and samples of industrial origin featuring relatively high concentrations of sulfate.

The investigations carried out in serum bottles and bioreactors with the 11 isolated strains and the three collection strains (i.e. *Desulfovibrio desulfuricans* and *vulgaris* and *Desulfotomaculum orientis*) emphasized that the strain selection, particularly from sulfate-rich environments, should be considered as an optimization factor for the sulfate reduction processes. Indeed, the most efficient strains defined as F and G were isolated from gypsum and gypsum plus phosphate disposal sites, respectively.

Although the F strain achieved a higher H_2S production rate, the G strain seems more adapted for the biological sulfate reduction processes. In fact, per unit of assimilated carbon, the production rates of both strains would be similar and, furthermore, the sulfide inhibition level of the G strain is about twice higher (i.e., 25.1 mM free H_2S concentration) than that observed with the F strain. Therefore, in an industrial process, these results which confirm those mentioned by Isa et al. (1986), would allow to increase

the H_2S concentration in the gaseous effluent going out of the bioreactor. This opportunity is positive for the chemical process that would oxidize the H_2S into sulfur for recycling in the manufacture of sulfuric acid (Winter et al. 1989).

Further experiments will be carried out in order to identify the G strain and to investigate its overall H_2S production rate through continuous bioconversion.

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