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Letter to Editor

Growth and Activity of Sulphate-Reducing Bacteria in Media Containing Phosphogypsum and Different Sources of Carbon

M. Rzeczycka*, M. Błaszczyk

Department of General Microbiology, Institute of Microbiology, Warsaw University, 02-096 Warsaw, Miecznikowa 1, Poland

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Abstract

The possibility of using a mixed community of sulphate-reducing bacteria (SRB) for the biotransformation of phosphogypsum was examined. The greatest reduction of phosphogypsum (g/l) was determined in cultures with lactate (3.3), ethanol or casein (2.7), almost two-fold less in media with glucose or lactose and three-fold less in medium with acetate. In media with lactate or ethanol growth inhibition ($I = \mu / \mu_{max}$) was slight ($I = 0.80$ or 0.79) but much higher in the case of cultures with lactose ($I=0.41$), glucose ($I=0.54$), acetate or casein ($I=0.62$). In those cultures in which the concentration of sulphides was very high (about 600 mg HS^-/l) and the concentration of acetic acid did not exceed 10 mg/l (e. g. in cultures with glucose), inhibition of SRB was mainly caused by H_2S. In cultures with transient low pH value (e. g. containing lactose or acetate) the factor causing stronger growth inhibition was acetic acid. A condition for obtaining high SRB activity in media with phosphogypsum and fermentable carbon sources is constant monitoring of the reaction of the medium and/or counteracting the accumulation of toxic concentrations of hydrogen sulphide and acetic acid.

Keywords: sulphate-reducing bacteria, carbon sources, biotransformation of phosphogypsum, COD reduction

Introduction

Sulphate-reducing bacteria are obligatory anaerobes that use sulphates, thiosulphates and sulphites as final electron acceptors. The energy needed for growth is obtained through the oxidation of organic compounds:

$$SO_4^{2-} + \text{organic matter} = HS^- + H_2S + HCO_3^-$$

These bacteria usually prefer low molecular weight organic compounds, such as organic acids (lactic, pyruvic, formic, acetic) or alcohols (ethanol, propanol) as a carbon source. It is presently known that the bacteria of this group are capable of utilizing several score different organic compounds [1, 2, 3]. The division of sulphate-reducing bacteria based on: (1) type of organic substrates utilized and (2) mode of their degradation (complete or incomplete), has been commonly accepted. The ability to completely degrade organic compounds (e. g. acetate) is demonstrated by representatives of the genera *Desulfomonas*, *Desulfococcus*, *Desulfobacter, Desulfosarcina* and *Desulfotomaculum*. *Desulfobulbus* and *Desulfovibrio* break down organic compounds incompletely [1-5].

In recent years several papers have focused on the possibility of using sulphate-reducing bacteria for the purification of electroplating wastewater, drainage waters from the metallurgic industry and mining waters [6, 7]. Studies are also under way [8-11] on the utilization of sulphate-reducing bacteria for the simultaneous biodegradation of organic matter and phosphogypsum,

*Corresponding author; e-mail: m.rzeczycka@biol.uw.edu.pl

which is a waste product arising from the production of phosphoric acid from apatite concentrates and (or phosphorites.

The pathway of the degradation of carbon compounds depends on the interaction between sulphate-reducing bacteria and other fermenting bacteria (e. g. the activity of sulphate-reducing bacteria to a large degree depends on the availability of sulphates). The addition of sulphates in the form of phosphogypsum (appropriate choice of COD/SO_4^{2-} ratio) favours domination of the system by sulphate-reducing bacteria. The activity of these bacteria results not only in the breakdown of organic matter but also in the removal of sulphates (by their conversion to sulphides), reduced extent of acidification of the medium and removal of toxic concentrations of heavy metals.

The effective use of bacteria to remove various contaminants requires meeting a number of conditions. The microorganisms should be characterized by high stability, the ability to use a broad spectrum of substrates, resistance to the toxic action of such compounds as sulphates, hydrogen sulphide, heavy metals in high concentrations, high throughout and high rate of the conversion of substrates. Of key importance is the choice of proper carbon source and electron donor, as well as its cost and availability. The carbon sources used most frequently for biotechnological purposes can be divided into two major groups: simple compounds (methane, ethanol, acetic acid, sodium lactate, glucose, lactose) and complex organic materials (usually waste materials from the food industry, agriculture, forestry, excess sediments from wastewater purification plants).

The aim of the current study was to investigate the possibility of using a community of sulphate-reducing bacteria for the simultaneous biotransformation of phosphogypsum and certain organic compounds, such as: 1-sodium lactate, 2 – ethanol; 3 – casein, 4 – glucose, 5 – lactose or 6 – sodium acetate.

Materials and Methods

Microorganisms

A mixed community of sulphate-reducing bacteria isolated from various environments [9].

Media

Sulphate-reducing bacteria were cultured in minimal medium containing (in g/l distilled water): 1.0 NH_4Cl; 5.0 phosphogypsum (in the form of a deposit). The source of carbon used was (g/l): sodium lactate (4.7), ethanol (2.4), casein (5.0), glucose (3.8), lactose (6.0) or sodium acetate (5.1). Phosphogypsum was from a waste dump located in Wizów near Bolesławiec. At the time when the culture was set up the pH of the medium was 7.4. The solubility of phosphogypsum in the above medium was approx. 36% and the concentration of phosphogypsum in the solution was approx. 1.8 g/l (930 mg SO_4^{2-}/l).

Culture Conditions

The cultures were set up and maintained in 0.33 dm^3 bottles with rubber stoppers. Thirty ml of mother culture grown for about 10 days in medium were supplemented with different carbon sources (g/l): sodium lactate (1.1), ethanol (1.1), glucose (1.1), lactose (2.0), casein (2.0) and sodium acetate (1.5). These were introduced through the rubber stopper using a syringe into 270 ml medium. The cultures were incubated in thermostat for 8-15 days at 30°C.

Determinations

1 – sulphides were determined using the iodometric method with Lugol's solution (0.05M) and sodium thiosulphate (0.05M) against starch (0.5%) as an indicator.
2 – the optical density of the culture was measured in spectrophotometer at wavelength = 420 nm.
3 – chemical oxygen demand (COD) was determined as described by [12].

Calculations

1 – the specific growth rate (μ x day^{-1}) was calculated on the basis of the amount of sulphates reduced,
2 – the coefficient of inhibition was $I = \mu/\mu_{max}$;
3 – dry weight of bacteria was calculated using the formula dry wt. (g/l) = $-0.02 + 0.5 \times A_{420}$ [13] modified for determinations made at wavelength 420 nm;
4 – the COD/SO_4^{2-} ratio was calculated as the quotient of COD and concentration of sulphates in the medium;
5 – the coefficient of carbon oxidized was calculated from the proportion between COD reduction and amount of reduced sulphates;
6 – the coefficient of substrate transformation efficiency Y_s/SO_4^{2-} (g dry weight (g reduced SO_4^{2-});
7 – the maximal rate of the reaction ($V_{max}HS^-$) was calculated on the basis of maximal rate of increase in sulphides (mg HS^-/l x day);
8 – the maximal rate of sulphate reduction (V_{max} SO_4^{2-}) was calculated on the basis of maximum rate of sulphate reduction (mg SO_4^{2-}/l x day);
9 – the concentration of undissociated hydrogen sulphide [H_2S] was calculated from:

$$[H_2S] = \sum H_2S \times \frac{10^{pK-pH}}{1+10^{pK-pH}}$$

where: [H_2S] – concentration of undissociated H_2S,
H_2S – summary concentration of sulphides in the form of

soluble and insoluble sulphides in the solution and hydrogen sulphide
pK – dissociation constant of H_2S/HS^-, which at 35°C equals 6.83,
pH – reaction of the culture

10. – The concentration of acetic acid in the culture was calculated from the formula [13]:

$$\frac{\mu}{\mu_{max}} = \left(1 - \frac{[H_2S]}{547}\right)^{0.401} \times \left(1 + \left(\frac{[ac.acid]}{54}\right)^{1.08}\right)^{-1}$$

where: μ/μ_{max} – coefficient of inhibition,
$[H_2S]$ – concentration undissociated hydrogen sulphide,
[ac. Acid] – concentration undissociated acetic acid
547 – concentration hydrogen sulphide inhibiting the growth of sulphate-reducing bacteria to 100%,
54 – concentration of acetic acid inhibiting the growth of sulphate-reducing bacteria to 100%.

Results and Discussion

Studies on the use of phosphogypsum for the biodegradation of various carbon sources by a community of sulphate-reducing bacteria were conducted in media containing (g/l): sodium lactate (4.68), ethanol (2.40), casein (5.00), glucose (3.80), lactose (6.00) or sodium acetate (5.10). Three consecutive passages of the bacterial community were made, transferring them to fresh medium when a culture reached maximum activity. Growth of sulphate-reducing bacteria was observed in cultures containing phosphogypsum and all the studied carbon sources. Sulphate-reducing bacteria are able to utilize alcohols, organic acids, sugars and many other organic compounds [1, 2].

The characteristic parameters of the development of a culture in the passage in which the activity of sulphate-reducing bacteria was highest (in passage III in medium supplemented with lactate or ethanol and in passage I in media containing acetate, glucose, lactose or casein) are compared in Table 1. The greatest biotransformation of phosphogypsum was observed in media containing lactate (3.3 g/l), ethanol or casein (2.7 g/l). The biotransformation of phosphogypsum in media with glucose, lactose or acetate, was visibly lower and averaged: 1.8; 1.2 or 1.1 g phosphogypsum/l, respectively. In the case of these cultures strong acidification of the medium occurred in the first passage at the very beginning of incubation, and then receded after a few days as a result of the reduction of sulphates. In consecutive passages the drop in the reaction of the medium was even greater (as far down as pH 4.0) and the activity of the bacteria showed a gradual decrease**.** The range of pH changes in this case was much broader than, for instance, in medium with lactate (Table 1).

Similarly as in the case of phosphogypsum utilized, also the rate of sulphate reduction ($V_{max}SO_4^{2-}$) was the greatest when lactate was used (310 mg/l x day) and the lowest with lactose or acetate as the carbon source (170 mg/l x day) (Table 1).

The utilization of organic carbon accompanying the reduction of phosphogypsum was the greatest in medium with glucose (2500 mg O_2/l), acetate (2000 mg O_2/l) or ethanol (1800 mg O_2/l) and the lowest in media supplemented with lactate, lactose or casein (1300 mg O_2/l).

The COD/SO_4^{2-} ratio in the medium changed in the course of the cultivation of the community of sulphate-reducing bacteria (Table 1). It is known that sulphate-reducing bacteria dominate at a COD/SO_4^{2-} ratio lower than 1.7, whereas the higher values of the ratio favour the

Table 1. Growth of the SRB community in the medium with phosphogypsum and different carbon sources.

Parameter / carbon source	lactate	ethanol	casein	glucose	lactose	acetate
passage number	III	III	I	I	I	I
reduction of phosphogypsum (g/l)	3.3	2.7	2.7	1.8	1.2	1.1
reduction of SO_4^{2-} (mg/l)	1690	1380	1380	920	610	560
pH changes during t_{max} - t_o	7.0-7.4	5.6-7.4	5.4-7.4	4.0-7.4	4.0-7.4	6.4-7.4
$V_{max}SO_4^{2-}$ (HS^-) (mg/l x day)	310(110)	250(90)	280(100)	250(90)	170(60)	170(60)
COD reduction (mg O_2/l)	1300	1800	1300	2500	1300	2000
COD/SO_4^{2-} in the medium (t_o-t_{max})	1.4-2.6	1.7-2.2	2.6-4.6	2.2-1.9	3.0-3.3	1.5-0.9
coefficient COD/SO_4^{2-}	0.77	1.30	0.94	2.71	2.13	3.57
dry weight increase (g/l)	0.20	0.19	0.15	0.13	0.06	0.05
Y/SO_4^{2-}	0.12	0.14	0.10	0.14	0.10	0.08
$\mu/\mu_{max} = I_c$	0.28/0.35= 0.80	0.24/ 0.32=0.75	0.20/ 0.37=0.54	0.20/ 0.36=0.55	0.13/ 0.32=0.41	0.15/ 0.24=0.64

growth, for instance, of methanogenic archeons [3]. At the beginning of their incubation the described cultures contained an identical amount of phosphogypsum and organic carbon but considerably differed with respect to COD. In the course of cultivation both the COD value and concentration of phosphogypsum in the medium changed. Consequently, in cultures supplemented with lactate, ethanol, casein or lactose the conditions became gradually less favourable for the growth of sulphate-reducing bacteria and they probably began to be dominated by other groups of bacteria that are responsible for further stages of the degradation of organic compounds.

The COD/SO_4^{2-} ratio approximated the value 0.67 given by [3] only in medium with lactate and averaged 0.77. The COD/SO_4^{2-} value in media with casein and ethanol was relatively low and amounted to 0.94 and 1.30, respectively, whereas in cultures with glucose, lactose or acetate its value exceeded the theoretical value 3- to 5-fold.

According to [13] the coefficient of substrate transformation, the efficiency Y_s/SO_4^{2-} in cultures of sulphate-reducing bacteria should be 0.15. The values in the cultures supplemented with different carbon sources used in this study were from 0.08 to 0.14, with the value for cultures with lactate, ethanol and glucose being but slightly lower than the above-mentioned theoretical value (Table 1).

The specific growth rate in media with ethanol, casein or glucose was comparable to that in medium containing sodium lactate ($\mu = 0.28$, $\mu_{max} = 0.35$ day^{-1}). In the case of the remaining (with lactose or acetate) the specific growth rate was visibly lower (Table 1).

It is worth mention that in all the media containing the studied carbon sources inhibition of the specific growth rate was noted. It was the lowest in medium with lactate (I=0.80) and the highest in medium with lactose (I=0.41). It is well known [3, 13-15] that the factors affecting the growth of sulphate-reducing bacteria, besides incomplete utilization of carbon substrates, including hydrogen sulphide or acetic acid produced by the cultures. A 50% inhibition of sulphate reduction by sulphate-reducing bacteria is observed already at the concentration of 400 mg H_2S/l, whereas at 500 mg H_2S/l the process is fully halted. Reis et al. [13] observed 50% inhibition of reduction of sulphates at a hydrogen sulphide concentration of 480 mg/l and full inhibition at 547 mg H_2S/l. Sulphate-reducing bacteria are also very sensitive to the presence of even low concentrations of acetic acid: their growth is fully inhibited already at the concentration of 54 mg/l [13]. The degree of growth inhibition is therefore proportional to the concentration of hydrogen sulphide as well as the concentration of acetic acid: $I = \mu/\mu_{max} = f[H_2S] \times f[AcH]$. In turn, the action of both these inhibitors depends on the reaction of the medium: at an alkaline pH the effect of acetic acid is negligible but dramatically increases with a drop in the reaction of the medium. The concentration of hydrogen sulphide, the sulphur compound that is the most toxic for organisms, also increases at lower pH.

Reis et al. [13] drew up a mathematical model that allows studying the effect of the individual inhibitors on the growth of sulphate-reducing bacteria. This model was used in the current study to estimate the degree of inhibition of sulphate-reducing bacteria in media supplemented with different carbon sources (Table 2). Taking into account the maximal concentration of sulphides and the lower pH values of the medium occurring in consecutive passages, the concentrations of hydrogen sulphide [H_2S] and acetic acid [AcH] were calculated. The inhibition coefficients are presented in the form $I = f[H_2S] \times f[AcH]$ as well as $I = f[H_2S]$ and $I = f[AcH]$. Of the six studied carbon sources, the lowest inhibition of specific growth rate of sulphate-reducing bacteria (expressed as $I = f[H_2S] \times f[AcH]$) was observed in the cultures with lactate (in the first passage I= 0.65 and in passages II and III, I=0.80), and the greatest in medium with lactose (I=0.41 in passage I and 0.17 in passage III). In cultures containing lactate the reaction of the medium

Table 2. Concentration of hydrogen sulphide [H_2S] and acetic acid [AcH] and coefficients of inhibition ($I = \mu/\mu_{max}$) in cultures of a community of sulphate-reducing bacteria (passages I-III) set up in media with phosphogypsum and different carbon sources (calculated acc. to [13] after taking into account maximum concentration of sulphides and lowest pH value).

Parameter	Carbon source																	
	lactate			ethanol			casein			glucose			lactose			acetate		
	number of passage																	
	I	II	III	I	II	III	I	II	III	I	II	III	I	II	III	I	II	III
maximal HS^-conc. (mg/l)	550	500	680	350	500	590	600	480	510	440	350	410	340	250	140	290	220	120
minimal pH	6.8	7.0	7.2	4.8	5.4	7.0	6.8	6.0	6.0	5.2	4.4	4.8	4.0	4.0	4.0	6.4	6.8	7.0
[H_2S] conc .(mg/l)	264	200	270	350	480	240	312	418	372	430	349	405	329	249	135	230	105	50
[AcH] conc. (mg/l)	20	3	2	34	5	1	9	9	2	1	7	5	37	30	54	12	25	41
Ic=f[H_2S] x f[AcH]	0.65	0.80	0.80	0.41	0.50	0.75	0.54	0.49	0.51	0.55	0.54	0.54	0.41	0.50	0.17	0.64	0.65	0.55
I= f[H_2S]	0.88	0.84	0.82	0.66	0.53	0.75	0.71	0.56	0.57	0.55	0.60	0.58	0.70	0.78	0.89	0.80	0.95	0.97
I= f[AcH]	0.74	0.96	0.98	0.63	0.94	0.99	0.77	0.87	0.90	0.99	0.90	0.93	0.60	0.65	0.20	0.80	0.69	0.57

never dropped below the pH value of 6.8 and the concentration of hydrogen sulphide and acetate did not exceed 270 and 20 mg/l, respectively. In the case of lactate, the slight inhibition of specific growth rate in consecutive passages was brought about by both hydrogen sulphide (I= *f* [H_2S]) and acetic acid (I=*f* [AcH]) and amounted to 0.82-0.88 and 0.74-0.98, respectively. With lactose as the carbon source, when the reaction of the medium in three consecutive passages achieved a very low value (pH 4.0), this being accompanied at the same time by high concentrations of sodium acetate (54 mg/l in the last passage), a decisive effect on the growth of sulphate-reducing bacteria was the inhibitory activity of acetic acid. The coefficient of inhibition, calculated as *f*=[AcH], in the last passage attained the very low value of I=0.20 (Table 2).

To sum up the results of this study, it seems that it is possible to use a mixed population of sulphate-reducing bacteria (including species of bacteria with broad nutrient spectrum) for the anaerobic degradation of phosphogypsum and organic matter. In the studied cultures the greatest amount of dissolved phosphogypsum was removed from medium containing lactate, ethanol or casein as a source of carbon and the largest amounts of organic matter removal were observed when glucose, lactose or acetate were used. In the three last cultures a simultaneous considerable increase of the COD/SO_4^{2-} quotient, being the consequence of the presence of other, besides SRB, groups of microorganisms competing for carbon source, was observed. The removal of larger amounts of carbon compounds is, of course, favourable from the wastewater purification viewpoint, but it should be kept in mind that the growth medium can be more subject to transient acidification in the case of a microbial community, which in turn can result in the toxic activity of hydrogen sulphide and/or acetic acid (in cultures with casein or lactose, hydrogen sulphide and acetic acid, respectively, were responsible for inhibition of growth rate). It seems that a condition for the use of a community of sulphate-reducing bacteria for the simultaneous utilization of waste gypsum and organic wastewaters carrying a source of carbon degraded by fermentation, is constant monitoring of the reaction of the medium and counteracting the accumulation of compounds inhibiting the course of the process. The method described above allows precise determination of which of the factors mentioned above (hydrogen sulphide or acetic acid) is responsible for inhibiting the growth of sulphate-reducing bacteria.

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