Mixed sulphate-reducing bacterial cultures for bioprecipitation of toxic metals: factorial and response-surface analysis of the effects of dilution rate, sulphate and substrate concentration

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The effect of process variables on alkalization and removal of typical contaminating toxic metals from a simulated acid leachate by continuous mixed cultures of sulphate-reducing bacteria was studied. It was shown that the amount of metal removed and rise in pH both varied with the amount of sulphate reduction occurring, the residual sulphate concentration being the main determinant of final pH. Factorial experiments showed that sulphate reduction was enhanced by increasing the substrate concentration and inhibited by the initial sulphate concentration. The dilution rate did not exert a primary effect, but the existence of a significant interactive effect between the substrate and sulphate concentrations and the flow rate was indicative of a quantitative modification of the effect of the former two variables by the latter. The biomass concentration in the cultures was only affected by the substrate concentration indicating that the other variables acted by selection for or against sulphate-reducing components of the mixed culture. A response-surface analysis of the yield of sulphate reduction and alkalization against substrate concentration and dilution rate indicated that sulphate reduction (and alkalization) was sensitive to both of these variables where the substrate:sulphate stoichiometry was in the range 1:1–3:1. At lower sulphate concentrations complete reduction occurred at all levels while at higher sulphate concentrations washout occurred in all runs, which indicated that the key variable was the substrate:sulphate stoichiometry and its interaction with the dilution rate. Attention is drawn to the efficiency of the experimental designs employed for elucidating these factors.

Keywords: sulphate-reducing bacteria, bioremediation, biotechnology, toxic metals, process optimization

INTRODUCTION

There is increasing interest in the potential biotechnological applications of bacterial sulphate reduction for bioprecipitation of toxic metals from aqueous wastes. Sulphate is a frequently-occurring counterion to toxic metals in environmental contamination such as that arising from metallurgical applications or acid mine drainage, where it also occurs as sulphuric acid. Bacterial

sulphate reduction, by converting sulphate to sulphide, is able to simultaneously remove the toxic metal and sulphate burdens and the acidity (see Gadd & White, 1993; White & Gadd, 1996). The main mechanism whereby sulphate-reducing bacteria remove toxic metals from solution is via the formation of metal sulphide precipitates (White & Gadd, 1996; White *et al.*, 1995). The solubility products of most heavy metal sulphides are very low, in the range 1.4×10^{-15} for manganese to 2×10^{-49} for mercury (Chang, 1993). These values imply that even a moderate output of sulphide can effectively remove metals almost stoichiometrically, potentially re-

Abbreviation: CSTR, continuous-flow stirred-tank reactor.

ducing concentrations to levels well below those permitted in the environment (Crathorne & Dobbs, 1990; Taylor & McLean, 1992). Sulphate reduction also raises the liquor pH, which permits mesophilic isolates to grow in sediments associated with acidic mine and surface waters where the bulk phase pH is in the range 3–4 (Hedin & Nairn, 1993).

Systems which have previously been employed using sulphate-reducing bacteria for biological metal-removal are of three types: artificial wetlands, fixed-bed anaerobic filters, and sludge reactors. The most extensive use to date of a process utilizing sulphate-reducing bacteria is the treatment, using a sludge-blanket reactor, of contaminated ground water at the Budelco zinc-smelting works at Budel-Dorplein in the Netherlands. The pilot plant comprised a purpose-designed 9 m³ stainless steel sludge-blanket reactor using sulphate-reducing bacteria and was developed by Shell Research Ltd and Budelco BV. This plant successfully removed toxic metals (primarily zinc) and sulphate from contaminated groundwater at the long-standing smelter site by precipitation as metal sulphides and yielded outflow metal concentrations below the p.p.b. range (Barnes *et al.*, 1991; Scheeren *et al.*, 1991). The process has since been expanded to commercial scale using an 1800 m³ concrete reactor.

The present work was carried out as part of the development of an integrated process for bioremediation of metal-contaminated soils. The overall process comprises microbial leaching of metals from soils using sulphur-oxidizing bacteria to produce a metal-loaded, acid liquor and subsequently using bacterial sulphate reduction to effect precipitation of the metals and partial neutralization of the liquor. The sulphate-reducing component was carried out using an enriched and selected mixed culture derived from material collected at several sites, enriched for sulphate-reducing activity and then selected for several useful properties including maximized sulphate reduction, metal- and acid-tolerance. The use of a mixed culture, which is maintained by selective conditions, has several advantages over pure cultures for environmental biotechnology. Such a culture is intrinsically less liable to contamination from other organisms and is also able to adapt to minor changes in conditions because it comprises a number of populations with varying optima for culture variables, e.g. concentration or pH of the leachate, nutrient concentrations, temperature and redox potential. It is also potentially able to make more complete use of organic substrates through consortium growth than would be possible by a single strain (Madsen & Aamand, 1992; Hansen, 1993; Davison *et al.*, 1994). It was therefore advisable to utilize this fact as far as possible and also to minimize the impact of such contaminants by maintaining a mixed culture stabilized by the selective pressure of process conditions. The composition of a mixed culture is itself modified by selection in the course of optimizing the process conditions, which provides a further route for process improvement.

The aim of the present study was to provide a basis for optimization of the laboratory and subsequent studies of the bioprecipitation component of the process. It was

therefore designed to provide information concerning the effects of the significant nutrients and the liquor flow rate on reactor operation in a continuous system. This was carried out in simple continuous-flow stirred-tank reactor (CSTR) systems as they constitute a simple model for more complex engineered systems such as feedback reactors (Pirt, 1985) or sludge blanket reactors (Barnes *et al.*, 1991). The programme adopted utilized a factorial experiment to determine the significant variables and whether they acted to increase or decrease the yield of sulphate reduced or biomass produced and to what extent. This experimental design was also able to indicate whether there were any interactive effects between variables, i.e. alterations in the effect of one or more variables due to the level of others. The experiment was then expanded to allow the calculation of a response-surface to further characterize the interaction of the main controllable variables.

METHODS

Organisms and enrichment culture. Soil and sediment samples were obtained from a number of sites including landfill sites, aquatic sediments and industrial and mining waste tips. Some material from environmental samples in collection at the University of Dundee was also utilized. All environmental samples were first inoculated into batch culture. Where appropriate, batch enrichment cultures were further used to inoculate continuous stirred-tank or fixed-bed reactors for further enrichment and selection. Batch enrichment cultures were inoculated with 5 ml of sample sediment directly into 120 ml Postgate medium B (Postgate, 1984) in a 125 ml medical flat bottle. The medium utilized either sodium lactate or a mixture of organic acids (supplied as sodium salts) as carbon source. The bottle was tightly sealed and incubated at 25 °C for 14 d or until visible growth occurred. Then 25 ml was transferred to a further 100 ml medium in a similar bottle for a further 14 d. A portion (25 ml) of this culture was inoculated into a further culture containing 25 µM each of CuSO₄ and CdSO₄ added to the medium. This was incubated under the same conditions until growth was indicated by the formation of a dense black metal sulphide precipitate. This culture was then subcultured through a series of media containing 50, 75 and 100 µM Cu and Cd.

Continuous-flow stirred-tank reactors (CSTRs) were inoculated by adding 125 ml of culture from the third or later transfer of batch enrichment culture aseptically into an anaerobic glass chemostat containing 700 ml Postgate medium C at 25 °C. The anaerobic state was maintained by continuous sparging of the culture vessel with O₂-free N₂. The culture was grown in batch mode for 72 h, and then the flow of sterile, nitrogen-sparged medium was started at a rate of 0.01 h⁻¹. The culture was then allowed to reach a steady state, indicated by a constant sulphide content of the effluent. As the ultimate aim of the study was to develop a process for metal removal, the enrichment procedure also included a component of selection for metal tolerance. This was carried out using the same medium with addition of 25 µM each of CuSO₄ and CdSO₄ and the culture was allowed to reach constant effluent metal and sulphide concentrations. This procedure was repeated using medium containing 50, 75 and 100 µM metal salts.

Culture maintenance. The culture was maintained at 20 °C in CSTRs of working volume 700 ml and headspace 300 ml in a medium comprising (g l⁻¹): sodium lactate, 7.0; Na₂SO₄, 1.8;

KH_2PO_4 , 0.25; NH_4Cl , 1.0; yeast extract, 1.0; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.06; $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 0.06; FeCl_3 , 0.004 (SRB1 medium). One millilitre of a toxic metals stock solution comprising 50 mM each of the chlorides of Cd, Co, Cr, Cu, Mn, Ni and Zn was also added per litre. The concentrations of metals added were lower than those used for selection, reflecting the reduced amount of metal bound by the medium, but the exact amount added varied between experiments. The inflow pH was 6.0 and no pH control was employed. Anaerobic conditions were maintained by sparging with O_2 -free N_2 . E_h and pH were monitored by means of a computerized data-logging system.

Experimental cultures. Experimental cultures were maintained under the same conditions as above except that the substrate concentration and dilution rate were altered between runs. The growth medium comprised SRB1 medium with the metals omitted (factorial experiment) or a simulated leachate (response-surface analysis) containing 14.2 mM sodium sulphate to which was added an appropriate concentration of sodium lactate for each run. Each 5 litre batch of medium was sterilized by autoclaving at 121 °C for 15 min. All the experiments were carried out at 20 °C and a dilution rate of 0.05 h⁻¹ unless otherwise stated.

Sampling. Two sets of samples were removed. The first set was used to test the culture OD_{550} and pH; approximately 10 ml volumes were removed using a standard pattern fermenter-sampling device, withdrawing culture by indirect suction supplied by a 50 ml syringe. The culture samples were collected in sterile glass Universal bottles and stored at 4 °C if necessary. Since it was necessary to exclude oxygen from samples collected for sulphide assays, a glass trap, sealed with an airtight rubber seal, was permanently inserted into the outflow from the vessel. Samples (5 ml) were removed from the trap using a hypodermic syringe inserted through the seal. Bijou bottles [5 ml (nominal) volume] were equipped with serum caps and the contained air was displaced with N_2 injected through the cap. The sample was then injected into the bottle, allowing gas to escape via a hypodermic needle. In cultures where resazurin was added it remained colourless throughout this procedure, indicating that there was no significant oxidation. Each run was carried out in a fixed time schedule rather than the previously-followed procedure of monitoring each run and allowing it to reach steady state before sampling. Each was allowed 4 d settling time and then samples were removed daily over the subsequent 4 d. To aid this process, runs were carried out in such an order as to minimize the differences in operating parameters between subsequent runs. The effectiveness of this procedure was checked by monitoring the results of sampling to ensure that there were no noticeable changes over time. Samples consisting of 20 ml of the contents of each reactor were removed and the solids were separated by centrifugation (1200 g, 20 min). Supernatants and pellets were then frozen and stored at -18 °C until analysed. The supernatants were analysed for sulphate and metal content. The pellets were analysed for sulphide and metal content. Samples of broth (5 ml) were separately removed without contact with atmospheric oxygen and placed in N_2 -filled 5 ml bottles for measurement of dissolved sulphide concentration. Separate samples were also taken for protein assays.

Other analytical methods. Because of the low amount of sulphide precipitation which occurred in SRB1 medium in the absence of added metals, biomass was estimated by measuring OD_{550} using a Pye-Unicam SP600 spectrophotometer. Protein concentration was used as an estimate of biomass present in culture where this was not possible due to precipitation. A portion (1.0 ml) of whole culture was homogenized with a glass homogenizer and pipetted into a test-tube, 200 µl 4 M NaOH

was added and mixed and extracted for 10 min, shaking to resuspend at 5 and 10 min incubation. The solids were then separated by centrifugation (9000 g, 5 min) and 100 µl aliquots were assayed for protein using the method of Bradford (1976) with 5 ml of reagent standardizing with 1 mg bovine serum albumin (Sigma) ml⁻¹. Sulphide was assayed by DC polarography using a Metrohm 663 VA stand (Metrohm) and Eco-Chemie µ-Autolab controller and software (Windsor Scientific). Samples between 100 and 1000 µl in volume were pipetted from the sample bottle (or culture-bottle in the case of batch cultures) into the polarograph vessel. The vessel contained 20 ml 0.1 M NaOH which had been deaerated for 5 min using N_2 . The polarograph sweep was run between -0.4 and -0.9 V with the half-wave potential for sulphides being between -0.69 and -0.72 V. The polarogram was plotted and the peak-height estimated using Eco-Chemie electroanalytical system software. The analyte volume was chosen to keep the peak current below 5×10^{-7} A as the response was strongly non-linear at higher levels. Calibration was by means of a calibration curve using 20.2 or 2.02 mM ACS-grade $\text{Na}_2\text{S} \cdot 9\text{H}_2\text{O}$ (Sigma) dissolved in 0.1 M NaOH. The calibration curve using varying volumes of these standards was prepared within 24 h of making up the solutions to prevent inaccuracies due to volatilization of H_2S . Sulphate was assayed by ion chromatography (IC) with a Metrohm 690 ion chromatograph using an electrical conductivity detector with a Metrohm 'Supersep' IC anion column using an eluant comprising: 4.5 mM boric acid; 13.5 mM mannitol; 3.8 mM Tris; and 2% (v/v) acetonitrile; pH 7.85. The eluant was prepared in a concentrated stock which was stored under nitrogen to prevent reaction with atmospheric CO_2 . The injection volume was 100 µl, eluant flow rate was 1.5 ml min⁻¹ and full-scale detector conductance 5 µS cm⁻¹. Solids were separated from the sample by centrifugation (9000 g, 5 min), the supernatant was then diluted to the concentration range 0–500 µM and filtered through a 0.45 µm mesh PTFE filter (HPLC Technology) prior to injection. Metals were analysed by atomic absorption spectrophotometry (AAS). Solid and liquid components were separated by centrifugation (1100 g, 10 min). The pellet was dissolved by incubating in 1.0 ml 6 M HNO_3 at 80 °C for 1 h. The undigested solids were then removed by centrifugation (1100 g, 10 min) and the liquor volume was made up to 10.0 ml. The supernatant was acidified by addition of 1.0 ml 6 M HNO_3 . Both fractions were filtered through a 0.45 µm mesh PTFE filter. Metals were assayed using a Pye-Unicam SP9 atomic absorption spectrophotometer and computer using appropriate lamps and standards. Standards were made up in 0.5 M HNO_3 (see White & Gadd, 1995). E_h and pH were measured by means of platinum and glass electrodes respectively (Russell pH). The signal was read directly by a PC equipped with an Analogue Devices RTI-820 A/D conversion board with suitable interface panels (Calex Instrumentation). The system was operated using LabTech Notebook software (Adept Scientific Software). For the purposes of the present study, these variables were logged but not controlled.

Experimental design. Factorial experimental designs are used to determine the effects of a number of variables on a resultant 'yield' variable. The design used here utilized two levels of each variable. In a factorial design, each experiment comprises all possible combinations of the high and low level of each variable; the number of runs per experiment for k variables is therefore 2^k . Experimental designs of this type are generally known as 2^k factorial designs (Montgomery, 1991). Although several algorithms are available for analysing these designs the basic analysis is similar in all cases. The effect of an individual variable (main effect) is the mean difference in yield between runs where

Fig. 1. Graphical representation of the experimental design used in the response-surface experiment. In this diagram, the axes are labelled to show the actual values of the variables at each sample point in the sample-space and the coordinate-pairs show the values in normalized terms.

the variable is high and those where it is low. The mean difference between the main effects of any given variable between runs where a second variable is at a high or low level is the estimate of the interaction between those two variables. The interactions between three variables can be estimated similarly from the values of interaction of two variables at two levels of the third (Montgomery, 1991). It is important to realize that the calculated interactions are those in which the main effects are non-additive. Our analysis was implemented using Yates' algorithm (Montgomery, 1991). While a 2^k factorial design provides considerable information about the system under investigation, the number of runs required becomes unwieldy where there are more than approximately four or five variables and fractional factorial experimental designs can be utilized to reduce the number of runs although information on the interactions between variables is not available from these designs. The Plackett-Burman design (Plackett & Burman, 1946; Montgomery, 1991) is a widely used design of this type.

A response-surface is an equation which relates a number of continuous variables to the 'yield'. The relationship can be shown graphically where there are only two variables; or two-dimensional 'slices' can be calculated and plotted by keeping other variables at a constant value. The equation for the response-surface was calculated using a generalized linear regression methodology. Since the aim was to visualize the behaviour of the reactor under the expected range of operating conditions, a lowest-possible order polynomial was fitted rather than attempting to construct a 'naturalistic' model. The experimental design chosen for response-surface analysis was a central composite design using values for the flow rate and lactate concentration which gave rotatability to the design, thus simplifying calculation and error estimates with an efficient use of experimental time (Montgomery, 1991). The values are shown graphically in Fig. 1 and corresponded to normalized values of 0, +1, -1, +1.41 and -1.41 for each variable where the mid-point of the range tested was normalized to '0'. The data available from the experimental design above yielded nine sets of x_1 (lactate concentration), x_2 (dilution rate) and Y ('yield' expressed as either amount of sulphate reduced or the rise in pH). This allowed an equation in up to eight terms to be calculated. The calculation was carried out using a matrix

method (Montgomery, 1991) which was implemented using a commercial spreadsheet. The resulting equations were tested against the data using a chi-squared test for goodness of fit (Sokal & Rohlf, 1981). Where required, the equations were used to generate sets of x_1 , x_2 , and Y points which were displayed as a contour plot.

Factorial analysis of variables in stirred-tank reactors. This experiment examined the effects and interactions of three variables: sodium lactate concentration, sodium sulphate concentration and the dilution rate. The metal concentration was retained at a constant value and the variables measured were OD_{550} and H_2S concentration, which were both indicators of the activity of the culture and, in the case of H_2S , the metabolic product of greatest practical interest. The factorial design used in this experiment was a two-level design for three variables; sulphate and substrate concentrations (both varied over a tenfold range) and dilution rate (varied over a twofold range) (Montgomery, 1991). Since the number of runs was relatively small, error estimates were achieved by replication ($4 \times$) which allowed estimation of the errors by variance analysis (Sokal & Rohlf, 1981).

Response-surface analysis of the effects of substrate concentration and dilution rate on sulphate reduction and alkalization in a CSTR. This experiment had the aim of fitting a response-surface to the relationship of flow rate and carbon source concentration to growth, sulphate reduction and pH in a CSTR. Factorial experiments on sulphate-reducing CSTR cultures had indicated that significant interactions between these variables occurred. The experiment was therefore designed to derive a response-surface to visualize the interactive effects (Montgomery, 1991). As the sulphate concentration in any real situation can be expected to be unamenable to manipulation at the bioprecipitation stage, a two-dimensional response-surface, flow rate and carbon source concentration, was fitted at distinct sulphate concentrations rather than incorporating sulphate concentration as a third dimension. This had the additional advantage of greatly reducing the number of runs in the experiment and allowing a graphical presentation of the individual response-surfaces. The ranges of actual values were chosen on the basis of previous results and preliminary trials.

RESULTS AND DISCUSSION

Equilibrium pH and sulphate reduction

There was a strong correlation between the residual sulphate concentration and the pH of the reactor. The greater the residual sulphate concentration, the lower the operating pH (Fig. 2a). The relationship was not linear but was linearized by plotting $\log[\text{sulphate}]$ vs pH (Fig. 2b) which was consistent with the general power law form of dissociation kinetics. Where the sulphate was completely utilized, the pH was generally well above 7. There are several possible mechanisms whereby conversion of sulphate to sulphide in solution can raise the pH. Volatilizing H_2S drives the following reaction to the right (Postgate, 1984):

This is undoubtedly a factor and strong sparging of CSTR cultures can be seen to raise the pH by up to approximately half of one pH unit when operating around pH 7. However, this is clearly insufficient to raise the pH from

Fig. 2. Relationship of pH with (a) final sulphate concentration and (b) log of the final sulphate concentration obtained in CSTR in the response-surface experiment. All points are the mean of four separate samples.

4.0 to 6.5 or 7.0 as observed in the present study. An increase in pH of these proportions must result from the basic chemical and metabolic processes involved in sulphate reduction and there are two main potential sources. Firstly, H^+ may be involved directly in the reactions, which therefore mop up protons. For example, when using H_2 as terminal electron donor:

However, this does not occur in all bacterial sulphate reduction and is not available when growing on lactate unless other processes occurring in mixed culture produce H_2 . The main mechanism of pH increase is probably the conversion of a strong acid, H_2SO_4 (which is effectively completely dissociated in dilute solutions), to a weak acid, H_2S . H_2S dissociates in two stages:

The dissociation constants are (3) 9.1×10^{-3} and (4) 1.1×10^{-12} (Lide, 1990). This is supported by the strong positive effect of the carbon substrate concentration on the pH, which remained low in runs of the response-surface experiment where the substrate concentration was too low to allow the complete reduction of sulphate. This result also underlined the importance of operating the bacterial sulphate reduction for bioprecipitation using a high proportion of sulphate utilization, both because of

Fig. 3. Removal of (a) Co (○), Cr (●), Cu (□), Cd (■), and (b) Ni (△), Zn (▲), Mn (▽), by CSTR cultures at various levels of sulphate reduction obtained in the response-surface experiment. The initial sulphate concentration was 14.2 mM in all cases. The initial concentration of each metal was 100 μM, with the exception of Cd (30 μM) and Zn (300 μM). Each point is the mean of four separate samples.

the pH effect discussed here and also because sulphate exerts a positive effect on the E_h of the reactor contents. Since both a low pH and E_h above approximately -200 mV exert an inhibitory effect on sulphate reduction, a significant excess of sulphate may lead to the inhibition or cessation of sulphate reduction.

Metal removal and sulphate reduction

The concentration of each of the metals tested was assayed both in the supernatant and in the pelleted solids. The total amount of metals present in the samples was somewhat variable. This resulted from the strongly flocculated state of the sludge in some of the runs which led to a non-homogeneous distribution, so that the amount of sludge withdrawn in a 10 ml sample was variable. The initial concentrations of each metal were (μM): Co, 100; Cu, 100; Mn, 100; Ni, 100; Cr, 100; Cd, 30; and Zn, 300 (supplied as chlorides). The amount of metal removed was dependent on the amount of sulphate reduced in all cases except for Cu, where almost all was removed even at low rates of sulphate reduction. In all of the other metals, the amount of metal removed from solution increased with the amount of sulphate reduction until they reached a minimum level (Fig. 3). Cd, Co, Cr and Zn reached a minimum of residual metal at 3–4 mmol reduced sulphate l^{-1} and this remained almost constant at increasing sulphide concentrations (Fig. 3a, b). The percentage removal of Mn and Ni was generally lower than that of the other metals and appeared to increase more slowly with increasing sulphate reduction (Fig. 3b). The residual metal concentrations in solution are generally higher than would be expected from the solubility

Fig. 4. Culture OD_{550} (○) and sulphide concentration (●) during the factorial experiment. The arrows indicate the points at which the regime was changed. The runs are indicated by the 'high variables': 1, all variables low; S, sodium sulphate concentration high; L, sodium lactate concentration high; F, flow rate (dilution rate) high. Representative data from one bioreactor are shown.

products of individual metal sulphides, which is probably due to competitive interactions between the metals or the formation of soluble metal complexes. However, these results, taken with those above, clearly demonstrate that the three aims of the process, namely removal of metals, acidity and sulphate, are obtained via the single process of sulphate reduction. This does not, however, imply that there is only one mechanism of metal removal. The formation of insoluble metal sulphides is clearly important but the solubility of metals such as copper and nickel is also influenced by pH in the range pH 4–7 as a result of the low solubility products of their hydroxides. This therefore provided an additional mechanism for metal removal as a secondary consequence of the rise in pH resulting from sulphate reduction. The increased pH can also be expected to aid precipitation of metal sulphides since a number, such as Zn, Co, Ni and Mn sulphides, are more soluble at low pH than at neutrality.

Factorial effects

The actual concentrations of sodium lactate and sodium sulphate used were 5–30 mM and 2–20 mM, respectively, corresponding to the expected leachate ranges, and the dilution rate was varied between 0.05 and 0.1 h^{-1} . The distinct differences between the cultures grown under different conditions can be seen in Fig. 4, which is an overview of the culture OD_{550} and sulphide concentrations throughout the factorial experiment. All of the cultures settled down as expected in the 4 d period and subsequent daily sampling showed no variation with time except where washout occurred. This only occurred in the runs which had a combination of high sulphate and low lactate concentrations, and the values of sulphide and biomass production recorded are probably overestimates due to curtailment of these runs before complete washout occurred. The culture pH was also measured but did not appear to vary between the treatments, having an overall mean of 7.51 ± 0.35 . The raw data, calculated effects and the significance of the variables and their interactions on

Table 1. Sulphide and biomass concentrations in an experiment utilizing a full-factorial design with three variables at two levels (2^3 factorial design) to assess the effects and interactions of sulphate and substrate concentrations and dilution rate on bacterial growth and sulphate reduction in chemostat culture

OD_{550} was used as an estimate of biomass concentration in the cultures (see Methods). All values are derived from four determinations $\pm$ standard error of the mean (SEM).

'High' variable	Sulphide concn (mM) (mean $\pm$ SEM)	OD_{550} (mean $\pm$ SEM)
All variables low	1.9 ± 0.20	1.0 ± 0.05
[Sulphate]	0.13 ± 0.040	0.70 ± 0.11
[Lactate]	6.4 ± 0.63	1.0 ± 0.12
Flow rate	1.8 ± 0.070	0.50 ± 0.030
[Sulphate] + [Lactate]	6.5 ± 0.66	0.90 ± 0.090
Flow rate + [Sulphate]	0.39 ± 0.14	0.50 ± 0.00
Flow rate + [Lactate]	7.5 ± 0.62	1.1 ± 0.080
All variables high	5.3 ± 0.73	1.4 ± 0.030

both OD_{550} and sulphide concentration are shown in Tables 1 and 2. The effects calculated are relative measures of the sign and magnitude of the impact of the process variable on biomass and sulphide production (Montgomery, 1991). Because of its direct association with the activity of sulphate-reducing bacteria the sulphide concentration is the more useful basis for comparison. As seen in Table 2, the lactate and sulphate concentrations showed strong positive and negative effects respectively. The enhancement of sulphate reduction due to lactate was predictable and undoubtedly the result of its role as carbon/energy source, the lower concentration resulting in carbon limitation. The mechanism of the negative effect of sulphate was not obvious but was probably attributable to its effect on the redox

Table 2. Effects of main variables and interactions on sulphide and biomass concentrations

The variables and interactions considered are shown in the left-hand column. Their effects shown are measures of the enhancement (positive effects) or diminution (negative effects) at the 'high' values of each variable compared to the 'low' values of each variable in the experiment. The significance level is the probability assigned to the result in a random system (Montgomery, 1991). OD_{550} was used as an estimate of biomass concentration (see Methods). All values are derived from four determinations.

Variable	Effect			
	Sulphate concn (mM)		OD_{550}	
	Effect	Significance level (<i>P</i>)	Effect	Significance level (<i>P</i>)
Main effects				
[Sulphate]	-21	<0.001	0.15	0.01-0.05
[Lactate]	86	<0.001	6.9	<0.001
Flow rate	0.63	>0.05	0.05	>0.05
Interactive effects				
[Sulphate] + [Lactate]	4.2	>0.05	1.8	0.01-0.05
Flow rate + [Sulphate]	-8.4	>0.05	2.5	<0.001
Flow rate + [Lactate]	-1.2	>0.05	5.7	<0.001
All variables	10.6	0.05-0.01	0.45	>0.05

Table 3. Coefficients of a response-surface

The table shows the coefficients of the various terms in a cubic response-surface fitted to data on sulphate concentration and pH of a sulphate-reducing mixed culture (*Y*) against the inflow sodium lactate concentration (x_1) and dilution rate (x_2) in an equation of the form: $b_0 + b_1x_1 + b_2x_2 + b_3x_1^2 + b_4x_2^2 + b_5x_1x_2 + b_6x_1^3 + b_7x_2^3 = Y$. These coefficients were used to calculate the response-surface shown in Fig. 5. All the coefficients are those of the normalized variables shown in Fig. 1 (see text for explanation). The χ^2 parameters estimated for the fit of these models to the experimental data for sulphate reduced and pH were respectively 3.52 and 0.20. The corresponding probability levels were 0.5-0.9 and >0.9.

Term	Coefficient (b_i)		
		$Y = [\text{Sulphate}]$	$Y = \text{pH}$
x_0 (const.)	b_0	9.70	4.80
x_1	b_1	-1.56	0.19
x_2	b_2	-0.21	0.17
x_1^2	b_3	-2.35	0.56
x_2^2	b_4	-0.70	0.11
x_1x_2	b_5	-1.85	-0.15
x_1^3	b_6	-1.39	0.56
x_2^3	b_7	1.12	-0.42

balance when present in excess which raised the redox potential and thereby partially inhibited sulphate reduction. The flow rate was not significant within the range tested and none of the first-order interactions were significant but the second-order interaction between all of the variables was significant. Interactions above first

order are notoriously difficult to interpret in physical terms but this interaction can probably be best regarded as reflecting an enhanced effect of high sulphate concentration at high flow rates where the compensatory effect of greater lactate concentration was reduced due to saturation of the culture metabolic capacity. The most notable difference between the effects of these variables on sulphide and biomass production was that the sulphate concentration exerted no main effect on the latter, although the lactate concentration was still stimulatory. This reflected the behaviour of the mixed culture, which was undefined but undoubtedly contained a non-sulphate-reducing component which would have been selected for by the higher redox potential and lower pH in these runs. This underlines the importance of process conditions in maintaining the microbial composition where a mixed culture is used.

Response-surface analysis

The method of response-surface analysis employed gives an equation which relates the reactor pH or sulphate concentration to the two variables, lactate concentration and dilution rate. Although the multiple linear regression methodology employed can be used to fit data to linear equations of specific form to produce a naturalistic model, such a model was not available and the lowest order of polynomial which would fit the data was calculated. This therefore should be regarded as an interpolation polynomial giving information about the behaviour of the variables within the range covered by the experiment. The quality of the data was estimated from the central point of the experimental design which yielded *Y* values of 9.7 ± 0.65 (6.7% relative error) for sulphate reduced and

Fig. 5. Contour plot of the response-surface calculated for (a) sulphate reduction (mmol sulphate reduced l^{-1}) and (b) reactor pH, against lactate concentration and dilution rate. The data matrix was generated using the coefficients in Table 3. The standard error of the sulphate concentration at the central point of the experimental design (Montgomery, 1991) was ± 1.76 mmol l^{-1} and that of the pH was ± 0.66 units. Both estimates are based on six samples.

4.8 ± 0.14 (2.9 % relative error) for pH. The coefficients of the various terms of the cubic response-surfaces for sulphate concentration and medium pH against the lactate concentration (x_1) and the dilution rate (x_2) are shown in Table 3. These values are given for the normalized form of the equation where the experimental values of the concentration and flow-rate are transformed to lie in the range minus to plus 1.412. This corresponded to real ranges of 0–7.0 g lactate l^{-1} and dilution rate 0.01–0.08 h^{-1} (Fig. 1). These polynomials were used to generate matrices of data for contour plots of the response-surfaces which are shown in Fig. 5. Goodness of fit was estimated using a χ^2 parameter (Bailey, 1959) for the fit between the model and the original data (Table 3). This indicated that the model derived was a good fit for the data set. The response-surface for both sulphate concentration and pH confirms the importance of the lactate concentration which was by far the dominant effect (Fig. 5a, b). The plot also illustrates the more complex nature of the effect of dilution rate on sulphate reduction and pH. This was small for both variables at substrate concentrations above 45 mM but showed a strong influence at lower substrate concentrations (Fig. 5a, b), which supplies an interpretation of the interactive higher-order effects seen in the factorial study.

To incorporate the sulphate concentration, the same set of experimental runs was carried out at higher and lower sulphate concentrations, keeping all of the other variables the same. At a sulphate concentration of 5 mM, it was found that sulphate reduction was effectively complete in

all runs; at 25 mM sulphate, all runs showed progressive reduction in pH and washout occurred rapidly (data not shown). This suggests that the most important aspect of the substrate concentration was the stoichiometry between the substrate and the sulphate present. In the simple CSTR system used for these experiments, the stoichiometry necessary to maintain an adequate working pH (> 6.0) was between 3 and 4.5 mol lactate (mol sulphate) $^{-1}$ (Fig. 5b).

The rise of pH due to sulphate reduction, from 4.0 to the range 6.5–7.0, which was shown in these experiments would allow the neutralization of most acid mine waters without any additional alkali. In the case of more acidic sources, this process would probably not achieve complete neutralization and additional alkali would need to be added (as occurred in other experiments utilizing soil leachates at pH 2.0; data not shown). Sulphide precipitation using biogenic sulphide was an efficient process for removal of toxic metals but the factorial and response-surface analyses both underlined the necessity of providing an adequate substrate input to maintain sulphate reduction. The stoichiometry is greater than the 2 mol of lactate for each mol of sulphate required for incomplete lactate oxidation (Postgate, 1984), which suggests that kinetic limitations play a significant role. The observation that the biomass concentration was relatively insensitive to conditions which inhibited sulphate reduction also underlines the importance of selective pressure in such a mixed culture system to maintain the sulphate-reducing component for metal removal. Paradoxically, an excess of sulphate would appear likely to select against sulphate-reducing bacteria, presumably due to their preference for a low E_h and high pH. Although the basic operation of the system proved to be relatively simple in that both metal and acidity were removed as a result of sulphate reduction, the rate of sulphate reduction depended on a more complex mix of direct effects of the substrate and sulphate concentrations and interactive effects of these together with the dilution rate of the reactor. The experimental approach taken, using factorial methods to elucidate the significant variables followed by a response-surface analysis to provide greater detail proved to be both effective in providing information relevant to process optimization and efficient in use of experimental effort.

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