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A comparison of carbon/energy and complex nitrogen sources for bacterial sulphate-reduction: potential applications to bioprecipitation of toxic metals as sulphides

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Detailed nutrient requirements were determined to maximise efficacy of a sulphate-reducing bacterial mixed culture for biotechnological removal of sulphate, acidity and toxic metals from waste waters. In batch culture, lactate produced the greatest biomass, while ethanol was more effective in stimulating sulphide production and acetate was less effective. The presence of additional bicarbonate and H_2 only marginally stimulated sulphide production. The sulphide output per unit of biomass was greatest using ethanol as substrate. In continuous culture, ethanol and lactate were used directly as efficient substrates for sulphate reduction while acetate yielded only slow growth. Glucose was utilised following fermentation to organic acids and therefore had a deleterious effect on pH. Ethanol was selected as the most efficient substrate due to cost and efficient yield of sulphide. On ethanol, the presence of additional carbon sources had no effect on growth or sulphate reduction in batch culture but the presence of complex nitrogen sources (yeast extract or cornsteep) stimulated both. Cornsteep showed the strongest effect and was also preferred on cost grounds. In continuous culture, cornsteep significantly improved the yield of sulphate reduced per unit of ethanol consumed. These results suggest that the most efficient nutrient regime for bioremediation using sulphate-reducing bacteria required both ethanol as carbon source and cornsteep as a complex nitrogen source.

Keywords: sulphate-reducing bacteria; bioremediation; complex nitrogen sources; substrates

Introduction

Microbial treatment of metal/radionuclide-contaminated solid and liquid wastes is a topic of current concern, with a wide range of microbial mechanisms being employed in laboratory and field studies. These include biosorption, accumulation and precipitation as well as oxido-reductive transformations [12,18,26]. Some of the most efficient mechanisms rely on metal precipitation and immobilisation as sulphides as a result of bacterial sulphate reduction [12]. Sulphate-reducing bacteria are an important component of natural and artificial wetlands and contribute significantly to metal immobilisation by precipitation [4,13,16]. Fixed-bed bioreactor systems have also been successfully applied on a laboratory and pilot scale to remove acidity, sulphate and metals [11,14]. Where anaerobic sludge blanket reactors are used to treat the organic (BOD) component of waste waters, metal sulphide precipitation may also be a valuable secondary process [7]. In fact, a sludge-blanket reactor has also been used in a system specifically engineered to remove zinc and sulphate from contaminated ground water [2,24] which, so far, represents the best example of a microbial treatment process in commercial operation [12].

The present work was carried out as part of the development of a microbiological process for bioremediation of metal-contaminated soils. The integrated process comprises microbial leaching of metals from soils by an aerobic process using sulphur-oxidising bacteria to produce a metal-loaded, acidic liquor from which the sulphate and metals are removed and the acidity partially neutralised by reduction of the sulphate under anaerobic conditions by sulphate-reducing bacteria. Both reduction in metal concentration and rise in pH of simulated leachates during continuous-flow stirred tank culture of sulphate-reducing bacteria were a function of sulphate reduction, so that the sulphate-reducing bacteria component of the process could be optimised by maximising sulphate reduction [27]. This simultaneously removed metals, sulphate and acidity [2,12] as a result of the very low solubilities of metal sulphides [6] and the low dissociation of hydrogen sulphide in aqueous solution [23]. Sulphide precipitation resulted in the removal of metals from the liquor to potentially very low levels suitable for environmental discharge [8,25].

Sulphate-reducing bacteria are heterotrophic organisms which dissimilate carbon via respiratory mechanisms [22,23]. The range of carbon/energy sources used by sulphate-reducing bacteria as a group is very wide and includes alcohols, organic acids and hydrocarbons, although sugars are rarely utilised [15]. However, individual strains are able to metabolise only a limited range of these substrates. These substrate preferences have been used to divide sulphate-reducing bacteria into three groups based on carbon dissimilatory patterns and the preferred enrichment substrate, but which also differ in aspects which may be significant for biotechnological processes, such as growth rate [28]. The hydrogen-lactate group comprises mainly *Desulfovibrio* and *Desulfotomaculum* species which utilise the organic acids lactate, pyruvate, succinate, fumarate and malate. They metabolise ethanol with the end-pro-

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ducts being acetate and/or CO_2. Members of this group can also use hydrogen as an electron donor in the presence of CO_2, acetate or other organic carbon source. These are the fastest growing group of sulphate-reducing bacteria with doubling times, under favourable conditions, around 3–4 h [23] and, because of this, are of interest as potential components of a biotechnological process for metal decontamination. The sulphate-reducing bacterial component of the integrated treatment process was a mixed culture, enriched for sulphate reduction and other useful properties including metal and acid tolerance [27]. The use of a mixed culture, adapted to the process conditions, has several advantages over pure cultures for environmental biotechnology. It is intrinsically less liable to contamination from other microorganisms and may also be able to oxidise certain carbon sources more completely than pure cultures [9,19] or utilise sources such as sugars which are not metabolised by pure cultures of sulphate-reducing bacteria [20]. The composition of a mixed culture is also modified by selection when process conditions are altered because component organisms possess differing optima for growth and activity [5,17,21]. This provides a route for process improvement which is unavailable in pure culture. The mixed culture we used was enriched using lactate as the carbon/energy source in order to obtain fast-growing organisms, but it was clearly desirable to explore the use of other potential substrates for the sulphate-reducing bacteria component of the integrated process. The carbon/energy substrate is clearly an influential variable because of its influence on growth rate and culture composition as well as a potential effect on the economics of a commercial process. In addition, it has been observed frequently that the presence of a complex nitrogen source stimulates growth and activity of sulphate-reducing bacteria [28]. The present study was therefore carried out to determine the effect of several substrates and substrate mixtures and the effects of additional complex nitrogen nutrients on sulphate reduction by a mixed sulphate-reducing bacterial culture in order to optimise use in an integrated biological process for the treatment of metal-contaminated wastes.

Materials and methods

Organisms and culture

The organisms employed were a mixed culture of sulphate-reducing bacteria, derived from enrichment cultures from a number of sources. The sulphate-reducing mixed culture was maintained in continuous culture on SRB1 medium comprising (g L^{-1}): sodium lactate, 7.0; Na_2SO_4, 1.8; KH_2PO_4, 0.25; NH_4Cl, 1.0; yeast extract, 1.0; $CaCl_2{\cdot}2H_2O$, 0.06; $MgCl_2{\cdot}6H_2O$, 0.06; $FeCl_3$, 0.004. One millilitre per litre of a metals stock containing 50 mM each of Cd, Co, Cr, Cu, Mn, Ni, and Zn chlorides was also added. The inflow pH was 6.0 and no pH control was employed. The flow rate was 0.05 h^{-1} and the temperature and pH of the broth were 20°C and 7.4 respectively.

Comparison of carbon/energy sources

A comparison of carbon/energy sources was carried out using 20 ml of SRB1 medium in a sealed Universal bottle. This medium was made up in 11 batches and dispensed in 20-ml aliquots in Universal bottles which were sealed using butyl-rubber serum caps. O_2-free N_2 was sparged into the headspaces using hypodermic needles inserted through the caps. The bottles were then sterilised by autoclaving them (121°C, 15 min). Inoculum for experimental batch cultures was prepared by adding 2.0 ml of a chemostat culture to a Universal bottle of sterile, anaerobic medium and incubating it for 72 h. SRB1 medium was used for the experimental cultures, with the following modifications: lactate was omitted, except in controls and replaced with the carbon/energy sources under test. Yeast extract (Lab M, Bury, UK) was included as there is evidence that rapid adaptation to different carbon sources requires complex nutrients [23]. However, to minimise its effect as a carbon source, yeast extract was supplied at a concentration of 0.1 g L^{-1}. The concentration of the carbon/energy sources was also reduced to 1/10 of that used in SRB1 as previous experiments had indicated that this would favour sulphate-reducing bacteria over other organisms. Each carbon/energy source was supplied at a concentration which gave an equimolar carbon concentration (18.8 mmol C L^{-1}). The concentrations were (mM): ethanol, 9.75; sodium acetate, 9.75 or sodium lactate, 6.5. Where added, $NaHCO_3$ was initially present at 0.44 mM. The initial pH of batch cultures was 6.0; 20-ml portions of the required medium were added to 25-ml Universal bottles which were sealed, sparged with N_2 and sterilised as above. The bottles were then inoculated with 2 ml of the inoculum culture, and 2 ml of H_2 gas (at room temperature and pressure) was injected into the appropriate bottles. The cultures were incubated at 20°C for 72 h. Replication of both inoculation and assays was carried out blockwise.

Continuous culture experiments were carried out in SRB1 medium using carbon/energy sources at a concentration equivalent to 188 mmol L^{-1} carbon. The concentrations were (mM): sodium lactate, 65; ethanol and sodium acetate, 98; and glucose, 33. The initial dilution rate was 0.005 h^{-1} (the lowest obtainable) as the growth rate on some substrates was expected to drop after changing the substrate. The temperature was maintained at 20°C. A constant sparge of oxygen-free nitrogen was maintained at between 1–2 L h^{-1} to prevent ingress of atmospheric O_2. After the initial change of medium, reactors were run for 3 weeks to allow settling before samples were removed. Sampling was repeated after 1 week to verify that the reactor conditions were stable. On raising the flow-rate in the course of these experiments the reactors were allowed a 2-week settling period before sampling. To sample, 15 ml of culture was removed from the reactor. The solids and liquor of 10 ml of this sample were separated by centrifugation ($1200 \times g$, 20 min). One millilitre of whole broth was used for a protein assay and the remainder was utilised for organic assays. A separate sample was removed anaerobically for assay of soluble sulphides. Gas samples were removed from the headspace using hypodermic syringes inserted through butyl rubber seals.

Comparison of additional nutrients

Experimental cultures were grown in batch culture in a base medium comprising SRB1 medium using ethanol as the carbon/energy source made up as above but omitting the

yeast extract. Twenty-millilitre aliquots of this medium were prepared anaerobically in Universal bottles as above. The additional carbon and complex C/N sources were prepared as batches of concentrated stocks at the following concentrations (g L^{-1}): sodium lactate, 1.0; ethanol, 0.5; yeast extract (Difco, West Molesey, UK), 10.0; and cornsteep (Merck/BDH, Lutterworth, UK), 10.0 which were sterilised by autoclaving (121°C, 15 min). Two-millilitre aliquots of the additive tested were added to each bottle of base medium just prior to inoculation using a hypodermic syringe; 2 ml of inoculum was then similarly added. Cultures were incubated for 24 h and then four replicate cultures for each treatment were removed and sampled. The remainder were incubated for a further 48 h and then sampled.

Effect of cornsteep concentration

Cultures were prepared as above using SRB1 medium with ethanol as the carbon/energy source with the addition of cornsteep at concentrations between 0–20.0 g L^{-1}. Cultures were incubated and samples were removed in quadruplicate as above and analysed for sulphide and protein concentrations. Sulphide determinations were carried out immediately after opening the sealed bottles. Where protein determinations were not carried out immediately, cultures were stored frozen at −30°C.

Effect of cornsteep on culture yield parameters

The experiment to determine the effects of cornsteep was carried out in CSTR culture using ethanol as carbon substrate. The sulphate and ethanol concentrations were respectively, 20 and 40 mM. Cornsteep was added at a concentration 1/10 that of the ethanol (0.2 g L^{-1}). After equilibration, samples were taken at 48-h intervals and analysed for soluble sulphide, sulphate, ethanol and protein. The pH and E_h of the cultures were also recorded.

Analytical methods

The culture OD_{550} and protein concentration were used as an estimate of biomass present in the culture. To extract protein, 1.0 ml of whole culture was homogenised with a glass homogeniser and pipetted into a test-tube. Two hundred microlitres of 4 M NaOH were added and mixed for 10 min, shaking it 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 using the Bradford method [3] standardising with 1 mg ml^{-1} bovine albumin (Sigma Chemical Co, Poole, UK) solution.

Sulphide was assayed by DC polarimetry using a Metrohm 663 VA stand (Metrohm UK Ltd, Buckingham, UK) and Eco-Chimie μ-Autolab controller and software (Windsor Scientific Ltd, Slough, UK). To collect a sample from a CSTR, a 7-ml Bijou bottle was filled with N_2, and approximately 5 ml of culture were removed from the reactor using a hypodermic syringe and a dip-tube. The culture was then transferred to the Bijou bottle by inserting the syringe needle through a rubber serum-cap. This procedure prevented access to atmospheric oxygen. Samples between 100 and 1000 μl were pipetted from the sample bottle (or culture bottle in the case of batch cultures) into the polarograph vessel. The vessel contained 20 ml of 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. The half-wave potential for sulphide was between −0.69 and −0.72. The polarogram was plotted and the peak-height estimated using Eco-Chemie Electroanalytical system software. Calibration was by means of a calibration curve using a range of volumes of 20.2 mM Na_2S, prepared by dissolving 5.0 g of ACS grade $Na_2S{\cdot}9H_2O$ (Sigma Chemical Company) in 1.0 L of 0.1 M NaOH (Analar grade). The analyte and standard volumes were chosen to keep the peak current below 5×10^{-7} A as the response was non-linear at higher levels.

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. The eluant was comprised of: boric acid, 4.5 mM; mannitol, 13.5 mM; TRIS, 3.8 mM; and acetonitrile, 2%; 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 phase was then diluted to the concentration range 0–500 μM and filtered through a 0.45-μm mesh size PTFE syringe filter (HPLC Products Ltd, Macclesfield, UK) prior to injection.

Ethanol was assayed by gas chromatography (GC). Solids were separated by centrifugation (9000 × *g*, 5 min). One hundred microlitres of 1.0 M methanol were added per ml as internal standard and the supernatant phase was filtered through a 0.45-μm mesh size PTFE syringe filter (HPLC Products Ltd). Ten to twenty-five microlitres of filtrate were injected into a Varian gas chromatograph with Alltech Porapak-T 80–100 mesh column packing a 6 ft (180 cm), 2-mm i.d. glass column. The column and injector temperatures were 140°C and the flame ionisation detector temperature was 180°C.

Organic acids were analysed by high performance liquid chromatography (HPLC) using an ion-suppression technique. Initial sample preparation was identical to that for the ethanol assay. Twenty microlitres of sample were injected into a Waters HPLC system using a 25-cm × 4.6-mm column packed with Spherisorb S5 C8 (Phase Separations Ltd, Deeside, Clwyd, UK). The eluant used was 0.02 M phosphoric acid (pH 2.1) at a flow rate of 0.9 ml min^{-1}. A Waters 490E programmable wavelength UV detector, operating at 200 nm was used. The system was controlled using Waters Millennium software.

Solid and liquid broth components were separated by centrifugation (1100 × *g*, 10 min) of 10.0-ml aliquots of whole broth. The pellet was dissolved by incubating it 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 phase was acidified by addition of 1.0 ml of 6 M HNO_3. Both fractions were filtered through a 0.45-μm mesh size PTFE syringe filter (HPLC Products Ltd). Headspace gases were assayed by GC using a Varian model 90-P instrument with a 1.3-m Porapak-Q 80/100 column (Alltech Associates Applied Science Ltd, Carnforth, UK). Injector, column and detector temperatures were all 25°C. The mobile phase was He_2 (50 ml min^{-1}). The retention times

were CO_2 1.1 min and H_2S, 5.5 min respectively. One millilitre of headspace gas was removed using a hypodermic syringe and injected without any further treatment. E_h and pH were measured by means of platinum and glass electrodes respectively (Russell pH Ltd, Auchtermuchty, UK). 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 Ltd, Leighton Buzzard, UK). The system was operated using LabTech Notebook software (Adept Scientific Software Ltd, Letchworth, UK). For the purposes of the present study, these variables were logged but not controlled.

Results and discussion

Biomass and sulphide production on different substrates in batch culture

The duration of batch experiments was chosen so as to restrict the experiment to the exponential growth phase. The initial (24-h) sample was taken just after the end of lag phase and the doubling time was approximately 6–8 h for the control experiments. This procedure allowed the application of results more directly to continuous processes than would be possible had the batch culture proceeded to completion with significant substrate depletion [1]. The carbon/energy source was varied, using ethanol or acetate in combination with bicarbonate and H_2 either singly or together. Lactate was used as an energy source in control cultures because it is the most complete C-source for the H_2/lactate group of sulphate-reducing bacteria [28]. The OD_{550} of the various treatments after incubation is shown in Figure 1a. The lactate-grown cultures produced significantly more biomass than any of the others but addition of neither H_2 nor CO_2 led to any increase in biomass production in the presence of other substrates. There were no metals added to these cultures so that the production of sulphide precipitates was minimal and did not interfere with use of OD_{550} as an assay for biomass. The increased yield of sulphide in lactate-grown cultures compared to other carbon sources was less pronounced than biomass yield, and it appeared that the presence of bicarbonate and H_2 marginally stimulated H_2S production when ethanol or acetate was the main carbon source (Figure 1b). The ratio of sulphide to the amount of biomass produced was also greater in both acetate- and ethanol-grown cultures than in lactate-grown cultures and was stimulated by H_2 and possibly by bicarbonate (Figure 1c). This indicated that lactate was more favourable for growth of biomass than the other substrates and suggests that the presence of bicarbonate and H_2 possibly stimulated sulphide production over biomass.

Sulphate reduction on various substrates in CSTR

All of the substrates used in this experiment supported sulphate reduction although the amount of sulphate reduced varied markedly. Sulphate was supplied at an initial total concentration (from all sources) of 14.2 mM. As described previously, the amount of sulphate reduced varied with the flow-rate through the bioreactors. Comparison between cultures grown on different substrates was not straightforward as cultures grown with acetate or glucose as carbon source appeared to wash out at flow rates between 0.005 and

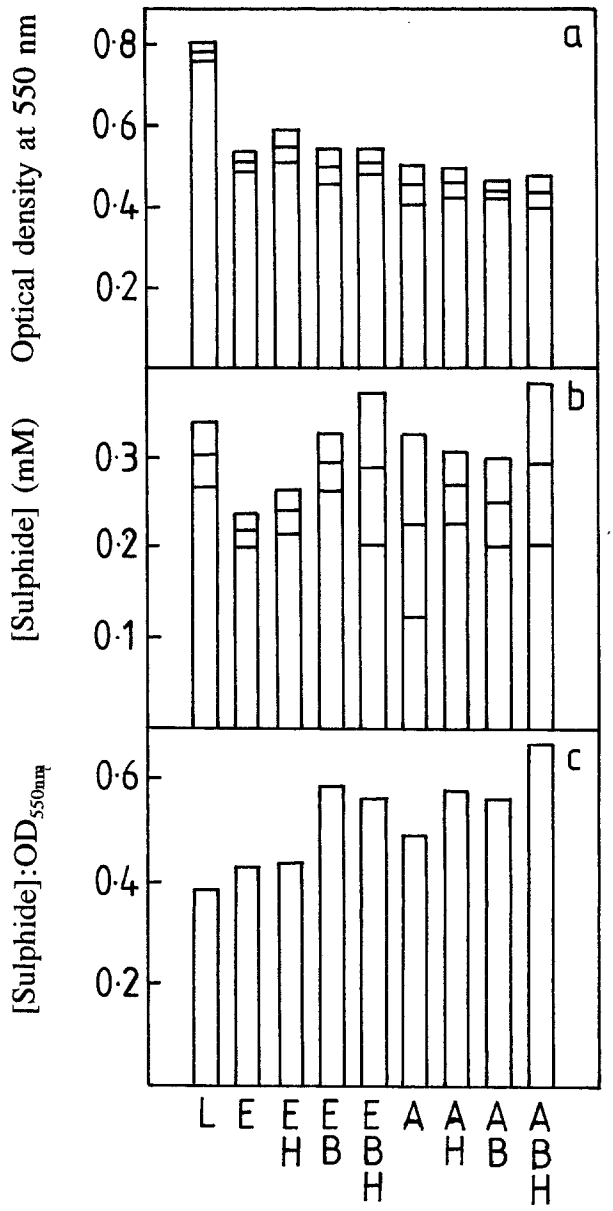

Figure 1 Growth and sulphate reduction by mixed sulphate-reducing batch cultures on various combinations of potential substrates as indicated by: (a) $OD_{550\ nm}$, (b) sulphide concentration of culture supernatants, and (c) ratio of sulphide to biomass concentration (OD_{550}). The substrates were: bicarbonate (B), H_2 (H), sodium lactate (L), ethanol (E) and sodium acetate (A). All points are derived from four separate cultures. The standard error of the mean (s.e.m.) and the mean value are indicated by the cross-bars.

0.01 h^{-1}, whereas cultures using ethanol washed out between 0.05 and 0.08 h^{-1}, and lactate cultures washed out between 0.08 and 0.1 h^{-1}. In addition, the cultures were mixed, so that both physiological state and species composition would have varied significantly between treatments. Table 1 shows the sulphate reduction at the highest dilution rate used that did not produce washout in CSTR culture. In addition, Table 1 shows data for an internal sedimentation bioreactor culture using the same medium which was grown at the same dilution rate as the corresponding CSTR and is included for comparison. An approximate maximum rate of sulphate reduction per litre of reactor working vol-

Table 1 Sulphate reduction using various carbon substrates

Substrate	Dilution rate (h^{-1})	Sulphate reduced[b]	
		mmol L^{-1}	mmol h^{-1}
Glucose	0.01	4.7 ± 2.1[c]	0.047 ± 0.021
Acetate	0.005	9.0 ± 3.4	0.045 ± 0.016
Lactate	0.08	11.4 ± 0.41	0.91 ± 0.033
Ethanol (CSTR)[a]	0.05	12.2 ± 0.19	0.61 ± 0.01
Ethanol (ISR)	0.08	19.1 ± 0.24	0.96 ± 0.015

Sulphate reduction was measured in continuous culture on SRB1 medium at the dilution rates shown. Except for the ISR, which was running at an arbitrary dilution rate, the rate was the highest used which maintained a steady state.
[a]CSTR = continuous-flow stirred tank reactor; ISR = internal sedimentation reactor.
[b]The initial sulphate concentration was 14.2 mM except in the ISR using ethanol where it was 20 mM and all substrates were supplied at a concentration of 230 mM carbon.
[c]The errors indicated are the standard error of the mean based on four separate determinations in each case.

ume is also given. It can be seen that the substrates tested fell into two distinct groups. Lactate and ethanol gave almost complete sulphate reduction while cultures using acetate reduced less than half of that present in the inflow. Glucose-grown cultures were intermediate between these groups, presumably reflecting the occurrence of both lactate and acetate as the main products of glucose fermentation by non sulphate-reducing components of the mixed culture. These acids were apparently responsible for the low pH of the glucose-grown cultures.

Utilisation of substrates and metabolic products in CSTR

Table 2 shows the organic substrates and products present in the reactor supernatant medium and off-gases. The products of ethanol, lactate and acetate oxidation were those that would be expected from established knowledge of the physiology of these organisms, the only soluble product detected from the former two substrates being acetate. The supernatant medium from glucose-utilising cultures, however, was more complex, including significant amounts of acetate and lactate as well as a number of unidentified minor components (Table 2). The main components which could be utilised by sulphate-reducing bacteria were therefore lactate and acetate [15]. Methane was not detected in any of these reactors so the only route for gaseous efflux of carbon was as CO_2 which was present in significant quantities in all of the off gases. It is apparent that the biomass protein concentration varied less between the CSTR cultures grown on different substrates than did the other variables shown in Tables 1 and 2, which also supports the view that the different substrates selected different components from the mixed culture. Both E_h and pH reflect the dominance of sulphate-reducing bacteria in the system as H_2S is a strongly reduced form and reduction of H_2SO_4 to H_2S removes a significant proportion of the acidity [27].

Effect of additional nutrients on batch growth and sulphate reduction by sulphate-reducing bacteria using ethanol as substrate

Very little growth had occurred in any of the cultures after 36 h incubation; consequently the 72-h samples were used to compare the cultures. The additional substrates could be divided into two groups, additional carbon sources (ethanol and lactate) and complex nitrogen sources (yeast extract and cornsteep). HPLC analysis of the media confirmed that no detectable extra lactate, acetate or other carbon/energy sources were added in the nitrogen sources. The addition of pure carbon substrates had no effect on the final sulphide concentration after 72 h incubation. This confirms that the ethanol concentration was not limiting to the cultures. However, both of the additional nutrients that contained complex nitrogen sources stimulated sulphide production, very strongly in the case of cornsteep (Figure 2a). The ratio of sulphide produced to ethanol utilised was calculated as an index of the yield of sulphide. Neither of the additional carbon substrates significantly affected this variable but the effect of both yeast extract and cornsteep was marked; cornsteep gave a more than 4-fold increase (Figure 2b).

The biomass, as represented by the protein concentration in cultures after 72 h incubation, was not affected by the provision of extra carbon/energy sources. However, as might be expected, biomass was increased by the provision

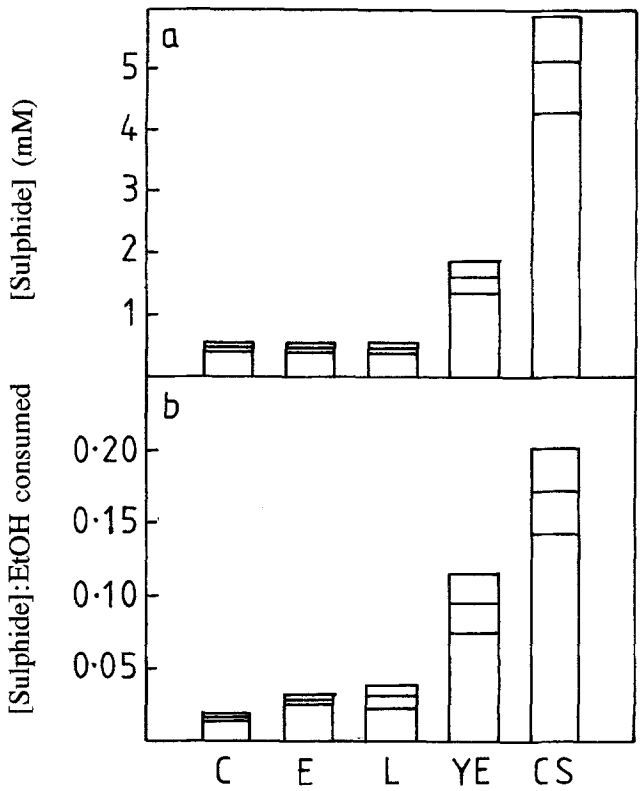

Figure 2 The effect of additional carbon and complex nitrogen nutrients on: (a) sulphide concentration, and (b) sulphide yield for ethanol consumed in batch cultures of a sulphate-reducing mixed culture using ethanol as substrate with additional carbon and complex nitrogen sources. C, control; E, additional ethanol (0.5 g L^{-1}); L, sodium lactate (1.0 g L^{-1}); YE, yeast extract (0.5 g L^{-1}); CS, cornsteep (0.5 g L^{-1}). The error range (s.e.m.) and mean are indicated by the cross-bars. Each experiment was performed in quadruplicate.

of complex nitrogen sources, with cornsteep giving the greatest yields (Figure 3a). The ratio of protein produced to ethanol consumed was used as an estimate of the protein yield. This also showed a marked increase in the presence of both yeast extract and cornsteep but none in the presence of additional carbon sources (Figure 3b). The initial concentration of ethanol in the base medium was high (108 mM) to minimise the possibility of carbon limitation affecting the outcome of the experiment. The maximum utilised was approximately 20% of the ethanol, so that ethanol depletion did not affect the course of the experiment. None of the additional substrates stimulated ethanol utilisation significantly (Figure 4). The ratio of sulphide to protein concentration was also enhanced by the addition of complex nitrogen sources (Figure 5) which implies that the activity of the biomass was also enhanced by the addition of cornsteep to the growth medium.

Effect of varying the concentration of a complex nitrogen source (cornsteep) in batch culture

Over the range tested, the protein concentration increased almost linearly with the concentration of cornsteep added (Figure 6a). The sulphide concentration reached a maximum at approximately 1.8–2.0 g L^{-1} cornsteep (Figure 6b). The maximum ratio of sulphide : protein present in the culture occurred at 1.5 g L^{-1} cornsteep (0.3 g cornsteep g^{-1} ethanol) (Figure 7). This represented an optimum concentration for batch culture.

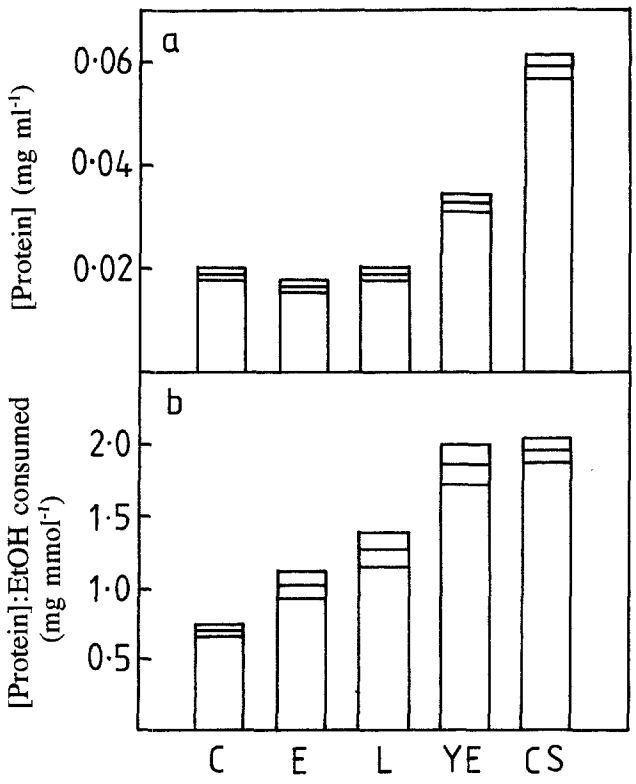

Figure 3 The effect of additional carbon and complex nitrogen nutrients on: (a) protein concentration, and (b) ratio of protein concentration to ethanol consumed in batch cultures of a sulphate-reducing mixed culture using ethanol as substrate with additional carbon and complex nitrogen sources. Other details as Figure 2.

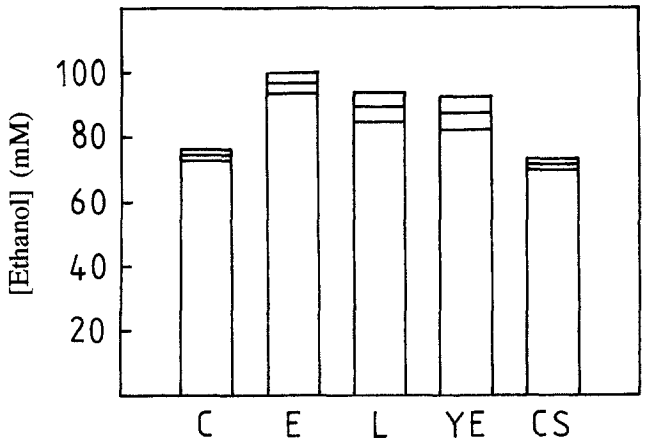

Figure 4 Ethanol concentration in batch cultures of a sulphate-reducing mixed culture using ethanol as substrate with additional carbon and complex nitrogen sources. Other details as Figure 2.

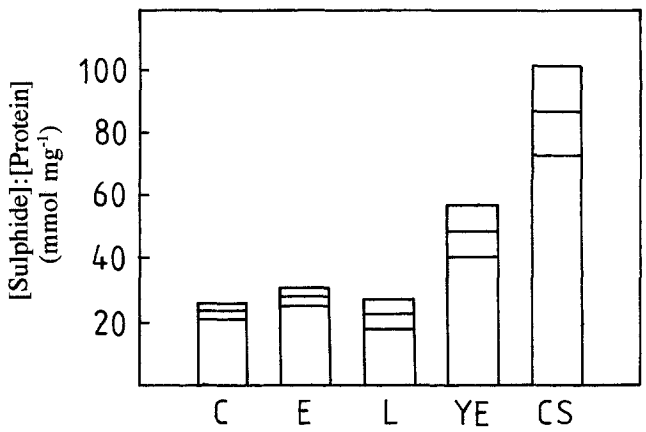

Figure 5 Effect of additional nutrients on the activity of biomass as indicated by the ratio of sulphide to protein concentration in batch cultures of a sulphate-reducing mixed culture using ethanol as substrate with additional carbon and complex nitrogen sources. Other details as Figure 2.

Apparent yield coefficients and culture parameters in continuous culture in the presence and absence of complex nitrogen (cornsteep)

The culture in the presence of cornsteep maintained a consistently lower redox potential than in the absence of cornsteep (−356 ± 9 mV and −269.3 ± 17 mV respectively) and the pH of the former trial was also consistently higher than the latter (7.9 ± 0.4 and 6.3 ± 0.1 respectively). Previous results indicated that this resulted from stimulation of sulphate reduction [27] as the mean sulphate concentrations were 7.32 ± 2.56 and 15.74 ± 1.93 mM in the presence and absence of cornsteep. The protein concentrations were 61 ± 12 and 20 ± 2 μg ml^{-1} respectively. There was no ethanol detected remaining in the medium, confirming that ethanol was the limiting nutrient. The yield of protein was 1.52 ± 0.25 μg $mmol^{-1}$ ethanol consumed in the presence and 0.5 ± 0.05 μg $mmol^{-1}$ ethanol in the absence of cornsteep, respectively. The yield of sulphate reduced was 0.82 ± 0.2 and 0.61 ± 0.19 mmol $mmol^{-1}$ ethanol respectively, in the presence and absence of cornsteep, although the improved yield of sulphate reduction was less marked than the improvement in the yield of biomass (protein).

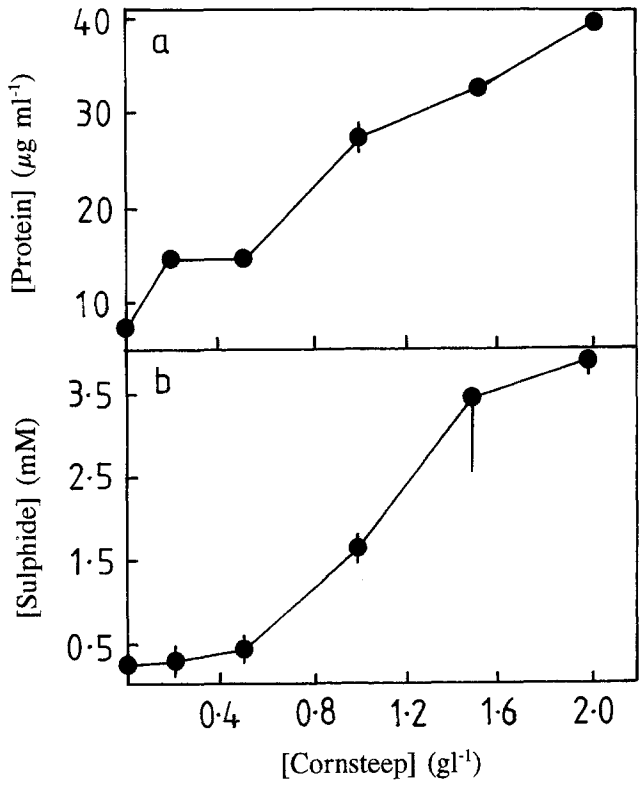

Figure 6 Effect of initial cornsteep concentration on: (a) protein production, and (b) sulphide concentration in sulphate-reducing mixed batch cultures using ethanol as substrate. Each point is the mean of four replicates and the bars indicate s.e.m.

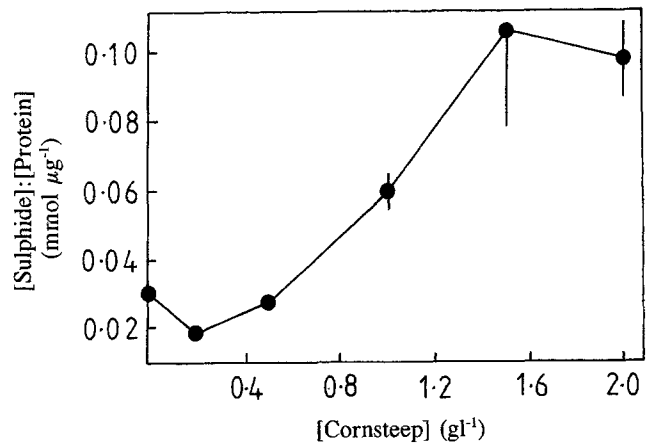

Figure 7 Effect of initial cornsteep concentration on the activity of sulphate-reducing mixed batch cultures (indicated by the ratio of sulphide to protein concentrations) using ethanol as substrate. Each point is the mean of four replicates and the bars indicate s.e.m.

From the point of view of a biotechnological process, ethanol was the most useful carbon/energy source in that it supported efficient sulphate reduction and growth. Lactate supported better growth of sulphate-reducing bacteria, but there was less sulphate reduced per unit biomass. Ethanol was also the substrate utilised in the Shell-Budelco process, but comparative studies between substrates were not reported for that system [2]. The pattern of utilisation of glucose observed here was similar to that found in a previous study [10] but the higher substrate concentrations used in the present study produced significant differences. In both studies, glucose was fermented in the mixed culture and the fermentation products, mainly acetate and some lactate, supported sulphate reduction. These products, however, were produced as the acetic and lactic acids and consequently the pH of the CSTR culture dropped below limits which permitted optimum growth of sulphate-reducing bacteria (Table 2).

In continuous culture, the slower growth rate on ethanol as substrate resulted in a lower maximum dilution rate. It was therefore possible that the addition of other nutrients as a source of carbon skeletons or complex nitrogen would improve performance. The addition of small additional amounts of pure carbon/energy substrates (lactate and ethanol) had no apparent effect on either growth or sulphate reduction. However both yeast extract and cornsteep resulted in an increased protein content and sulphate reduction and in increased yield of both per mol ethanol utilised. Of the two, cornsteep had the stronger effect on both variables. The additions appeared to result in both more active biomass as shown by the sulphide produced per μg protein and in a greater biomass concentration as measured by the protein concentration. This advantage gained from the addition of cornsteep was carried over into continuous culture and feedback bioreactors so that the increased efficiency can represent a significant economic gain for metal bioprecipitation processes.

Table 2 Carbon substrates and products

Product	Substrate supplied				
				Ethanol[c]	
	Glucose	Acetate	Lactate	CSTR	ISR
LIQUOR					
Glucose	+[a]	ND	ND	ND	ND
Lactate	+	−	+	−	−
Acetate	+	+	+	+	+
Ethanol	−	−	−	+	+
Other[b]	+	−	−	−	−
GASES					
CO_2	+	+	+	+	+
Methane	−	−	−	−	−
BIOMASS					
Protein (mg ml⁻¹)	0.022	0.012	0.026	0.018	0.063
pH	5.5	6.0	6.5	6.5	6.5
E_h (mV)	−350	−437	−501	−480	−418

[a]The presence of compounds is indicated as +; absence as −. ND indicates that the compound was not determined in the samples.
[b]Unidentified, probably organic acid, peaks in HPLC chromatograms.
[c]CSTR, continuous-flow stirred tank reactor; ISR, internal sedimentation reactor.

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