CAPSULAR IMMOBILISATION OF SULPHATE-REDUCING BACTERIA AND APPLICATION IN DISARTICULATED SYSTEMS

DOUGLAS SANYAHUMBI

CAPSULAR IMMOBILISATION OF SULPHATE-REDUCING BACTERIA AND APPLICATION IN DISARTICULATED SYSTEMS

A thesis submitted in fulfilment of the
requirements for the degree of

Doctor of Philosophy
of
Rhodes University

by

DOUGLAS SANYAHUMBI

MARCH 2003

ABSTRACT

Biotechnology of sulphate reducing bacteria has developed rapidly in recent years with the recognition of their extensive and diverse biocatalytic potential. However, their application in a number of areas has been constrained due to problems including poor cell retention within the continuous bioprocess reactor environment, and contamination of the treated stream with residual organic feed components and cell biomass. These problems have so far excluded the application of biological sulphate reduction in the treatment of 'clean' inorganic waste streams where components such as sulphate, acidity and heavy metal contamination require treatment.

This study investigated the effective immobilisation of sulphate reducing bacterial cultures and proposed that the disarticulation of the electron donor and carbon source supply using such systems would create the basis for their application in the treatment of 'clean' inorganic waste streams.

A functional and stable sulphate reducing culture was selected and following evaluation using a number of techniques, was immobilised by encapsulation within a calcium-alginate-xanthum gum membrane to give robust capsules with good sulphate reduction activity. The concept of disarticulation was investigated in a swing-back cycle where the carbon source was excluded and the electron donor supplied in the form of hydrogen gas in a continuous up-flow capsule-packed column reactor. Following a period of operation in this mode (4-12 days), the system was swung back to a carbon feed to supply requirements of cell maintenance (2-3 days). Three types of synthetic 'clean' inorganic waste stream treatments were investigated, including sulphate removal, neutralisation of acidity and heavy metal (copper and lead) removal.

The results showed:

- Sulphate removal at a rate of 50 mg SO_4^{2-}/L/day/g initial wet mass of capsules during three 4-day cycles of electron donor phase. This was comparable to the performance of free cell systems;
- Neutralisation of acidity where influent pH values of 2.4 and 4.0 were elevated to above pH 7.5;

- Copper removal of 99 and 85 % was achieved with initial copper concentrations of 2 and 60 mg/L respectively;
- Percentage lead removal values of 49 and 78 % were achieved;

This first report on the application of the concept of capsular immobilisation and disarticulation in the treatment of ‘clean’ inorganic waste streams will require future studies in order to extend the development of the full potential of the concept.

TABLE OF CONTENTS

CHAPTER 6:
CONCLUSIONS AND FUTURE DEVELOPMENTS

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

$\Delta G'$	Gibbs free energy
Aam/SA	Acrylamide / sodium acrylate
AAS	Atomic absorption spectrophotometer
AIPS	Algal integrated ponding system
Alg	Alginate
AMD	Acid mine drainage
APS	Adenosine phosphosulphate
ATP	Adenosine triphosphate
BOD	Biochemical oxygen demand
CG	Cell generating
COD	Chemical oxygen demand
CSTR	Continuous stirred tank reactor
DCM	Dichloromethane
DCM	Delignified cellulosic material
dddH_2O	Triple distilled de-ionised water
ddH_2O	Double distilled de-ionised water
dH_2O	Distilled de-ionised water
DWAF	Department of Water Affairs and Forestry
EDTA	Ethylenediaminetetraacetic acid
EGSB	Expanded granular sludge blanket
EPS	Extracellular polymeric substances
ER	Epoxy resin
ETLP	Electron transport level phosphorylation
GP	Gluten pellets
HPLC	High performance liquid chromatography
HRT	Hydraulic retention time
IC	Inhibition concentration
IFBC	Immobilised fed-batch column

kPa	Kilopascals
MPB	Methane producing bacteria
PAA	Polyacrylamide
PAG	Polyacrylamide gel
PAN	Polyacrylonitrile
PCH	Petroleum hydrocarbons
PEI	Polyethyleneimine
Pg	Postgate
ppm	Parts per million
PUF	Polyurethane foam
PVA	Polyvinyl alcohol
PVF	Polyvinylformal
rpm	Revolutions per minute
SBR	Sequencing batch reactor
SEM	Scanning electron microscopy
SLP	Substrate level phosphorylation
SRB	Sulphate-reducing bacteria
SRP	Sulphate-reducing prokaryotes
SRBM	Sulphate reducing bacteria media
SRM	Sulphate reducing media
TCA	Tricarboxylic acid
UASB	Up-flow anaerobic sludge blanket
UHT	Ultra high temperature
VSS	Volatile suspended solids
RXD	hexahydro-1,3,5-trinitro-1,3,5-triazine

ACKNOWLEDGEMENTS

There are so many people whose contribution to this thesis is note-worthy in some way. It is customary to start by acknowledging one's supervisors and I will not divert from that custom as both my supervisors have afforded me so much of their time and effort and have provided me with invaluable support and guidance on this research project and more importantly on life during the most testing times. So my heartfelt thanks and acknowledgment to my supervisors Professors John Duncan and Peter Rose, for the wisdom of a lifetime that you have been happy to share and impart.

My family and I have had to deal with so much tragedy in the last two years, their support has been pivotal in my getting through the last two years and I would like to dedicate this thesis to them: to my dearest Mom (Margaret) who has remained stronger that I ever could have and has kept together a family that has faced tragedy after tragedy, for your love Mom that is unconditional, for your prayers full of tears that have meant more to me than I can put in words, for your un-swerving faith in God which you have not only shared with me, but also showed me, I will forever be grateful. To my Dad (John Douglas) and my brother (Cris) who both died in the last two years, whom I never got the chance to say goodbye to personally, but hope they knew that I love them and miss them each day that I live, for the amazing love and support for my educational endeavours for which they sacrificed so much but will not get the opportunity to see the final product, thank you, you will always be a part of me. To my twin brother Derrick who has always been there for me, who has moved the world for me at times and has given new meaning to the words "brother's keeper", thanks for the sacrifices Des, I hope you know that I can never really pay you back, but would do anything I can for you. To my sisters Cynthia and Theo and their families, who have encouraged and supported me in so many ways, for your prayers that I believe have moved the hand of God on my behalf, I love and thank you so much.

To Moira Pogrund who may as well have adopted me for the duration of my time in Grahamstown, you've seen me when I've been happy, in pain and ready to give up and have always supported me and given up so much of your time (and car) for my cause, thanks for the encouraging words or kick up the back-side as the situation demanded you will always be a special part of my life. To Bruce Smith for the encouragement (in your own special way)

that I'm sure you always knew I needed and appreciated, thanks, see you at the next farewell party. I'll miss you.

Thanks to the rest of my "mothers" in Grahamstown, Rhona Duncan, John Gillam, Desiree Wicks, Marion Penney, Ruth, who have fed me encouraged me and given sound advice (I fear not always taken) on coffee, red-bull, toast, vitamins, women and work.

Humungous thanks to the Heavy Metals Lab 129C crowd that I have seen come and go, Kate Swabey, Rose Payne, Rob van Hille (Dr), Leigh Nightingale (groovy first ever digsmate), Mamta Bhagat, Paula Antunes (Dr), Marcus Selepe, Cherie-Lynne Mack, Jo Burgess (Dr) and to the refugees in the lab Stew Clark, Nuraan Khan and Sheena Fraser who've all encouraged me and shared part of their lives with me.

Thanks to all the staff in the Department who've probably been too polite to point out that I should move on, for their support and encouragement. Special thanks to Prof Steph Burton, Prof Rosie Dorrington and Prof Greg Blatch for taking the time to take a professional and personal interest in me. Thanks to all my colleagues in the Department for the academic and social stimuli. To Louise and Joan for all you did for me so willingly, to make my life in the Department that much better. To Prof Mike Burton for quick lessons in 3rd order polynomials and your time and assistance. To Robin Cross, Shirley Pinchuck & Marvin Randall for the help with the EM work and socials on current world affairs

Huge thanks to the proofreaders, Gary Burgess, who is such a cool oke (same as 'bloke' Gary), and to Tracy Hammond, also thanks to Mike Ludewig and Sally Clark for the much needed lighter moments during writing up, I will always remember that when all else fails, I will turn the music up loud and dance. Thanks Fritha for help with checking references & Jo again for the second check. Matthew Charlseworth for a desperately needed place to crash.

For the amazing unconditional friendship and support, thanks to Lisa Gibson, Joanne Gilfillan, Abi & Arno, Ida (and Mira), Bronwyn Tweedie, Sarah Jefferson, Henry the Roman and Tania the new Roman, Marisa, Liza, Janet Tyson, Jeff G-Strachan (enjoyed the cards evenings), Carol & Kasch, Kev & Alix, Tracy Minton, Taz Davidson the list goes on.

It would be a sin to forget all my Stanley Kidd House "kids", who felt that I sometimes needed reminding that they already had Dads, thanks for the opportunities to play Dad, Mom and 'judge, jury and executioner' at times.

Financial assistance over the years from The Andrew Mellon Foundation, NRF and Canon Collins Educational Trust for Southern Africa is gratefully acknowledged.

CHAPTER 1
IMMOBILISATION OF THE SULPHATE-REDUCING BACTERIA

1.1 Biocatalytic Potential of Sulphate-Reducing Bacteria

Although there had been earlier suggestions attributing the presence of hydrogen sulphide in biological environments to sulphate reduction activity by living organisms, Beijerinck is believed to have cultured the first sulphate-reducing organism in 1895 (Salle, 1954).

1.1.1 Sulphate-reducing bacteria

Sulphate-reducing bacteria (SRB) comprise a mixed group of morphologically and nutritionally diverse, strictly anaerobic bacteria that use sulphate as the terminal electron acceptor. The still more widely used term 'SRB' has been used here instead of the more recent term sulphate-reducing prokaryotes (SRP). They oxidise a range of compounds including organic acids, fatty acids, alcohols, and H_2 as carbon and electron donor sources. A significant aspect of SRB metabolism is the production of hydrogen sulphide (H_2S/HS^-), a strong reducing agent, capable of inhibiting the growth of both anaerobic and aerobic microorganisms (Gibson, 1990). The SRB perform dissimilatory (and assimilatory) sulphate reduction, with dissimilatory far exceeding assimilatory reduction. The genera of SRB are generally defined in terms of their morphology rather than physiology. The majority of SRB are reported to stain Gram-negative with *Desulfovibrio* being the most encountered genus, but the Gram-staining behaviour of SRB is diagnostically unreliable (Boopathy *et al.,* 1998a; Zehnder, 1988).

Although morphologically diverse, SRB are considered to be physiologically unified. Currently eighteen genera of dissimilatory SRB are known, and these have been placed into two broad physiological subgroups. The first group contains genera such as *Desulfovibrio*, *Desulfomonas*, *Desulfotomaculum* and *Desulfobulbus*, they can use lactate, pyruvate, ethanol or certain fatty acids as carbon and energy sources. Group II includes genera such as *Desulfobacter*, *Desulfococcus*, *Desulfosarcina* and *Desulfonema* that specialise in the oxidation of fatty acids, particularly acetate (Madigan *et al.,* 1997).

The reduction of sulphate to hydrogen sulphide is an eight-electron reduction reaction.

$$SO_4^{2-} + 8\ e^- + 8\ H^+ <====> H_2S + 2\ H_2O + 2\ OH^- \quad (1.1)$$

The reaction proceeds through a number of intermediate stages. The stable sulphate ion is first activated by the enzyme adenosine triphosphate (ATP)-sulphurylase to give adenosine phosphosulphate (APS). In dissimilatory sulphate reduction, the sulphate in APS is then reduced to sulphite releasing adenosine monophosphate (AMP). In assimilatory reduction another phosphate is added to APS to form phosphoadenosine phosphosulphate (PAPS). Only then is the sulphate reduced. Sulphite is the first product of sulphate reduction in both cases (Madigan *et al.,* 1997).

The following equations show some of the other important reactions occurring in SRB systems, where nCH_2O represents a carbohydrate source (equation 1.2), and equation 1.3 gives the reaction that occur in SRB that are able to use hydrogen as an electron donor source:

$$2CH_2O + SO_4^{2-} \rightarrow H_2S + 2HCO_3^- \quad (\Delta G = -80\ KJ/mol) \quad (1.2)$$

$$4H_2 + SO_4^{2-} \rightarrow HS^- + OH^- + H_2O \quad (\Delta G = -38.1\ KJ/mol) \quad (1.3)$$

$$H_2S + H_2O \rightarrow H_3O+ + HS^- \quad (K_1 = 1.0 \times 10^{-7} \text{ at } 20\ °C) \quad (1.4)$$

$$HS^- + H_2O \rightarrow H_3O+ + S^{2-} \quad (K_2 = 0.8 \times 10^{-17} \text{ at } 20\ °C \quad (1.5)$$

$$2CH_3CHOHCOO^- + 3SO_4^{2-} \rightarrow 6HCO_3^- + 3HS^- + H^+ \quad (\Delta G = -253\ KJ/mol) \quad (1.6)$$

The dissociation constant K_2 of equation 1.5 means that the formation of sulphide as S^{2-} in all solutions with a pH of less than 14 is negligible. Therefore, sulphides occur, predominantly, as HS^- or H_2S as represented in equation 1.4. Equation 1.6 represents the reaction for the complete oxidation of lactate, for organisms that cannot completely oxidise lactate, the ΔG of that molar reaction is – 160 KJ/mol (Gibson, 1990).

1.2 Biotechnology of Sulphate-Reducing Bacterial Applications

As far back as the late 1940s and early 1950s several researchers had already shown interest in SRB and their potential in bioprocess applications. Zobell (1946) and Miller (1950) both investigated the deposition of copper sulphide ores and sulphide ores of other metals, and

their experimental results on sulphur isotope distribution suggested that sulphide deposits of copper, iron, lead, zinc, gold and uranium may be of biogenic origin.

Bannick and Muller (1952) passed wastewater derived from the digestion of straw with sulphite through a column with SRB and were able to remove enough sulphite to allow the wastewater to undergo methane fermentation, a process otherwise inhibited by sulphite.

Sulphate-reducing bacteria were used in the gas production industry to treat effluent wastewaters contaminated with thiosulphates and thiocyanates, both compounds toxic to fish and sewage organisms. *Thiobacillus* was the main organism believed to be responsible for the metabolism of thiocynate. *Thiobacillus thiocyanoxidans* was isolated from this environment by Happold (1957).

Interest in the biocatalytic potential of SRB has since been developed into numerous and diverse industrial applications.

1.2.1 Acid mine drainage

Earlier work on acid mine drainage (AMD) was reported by Tuttle and colleagues (1968, 1969a, b) who passed AMD solution through a porous dam of wood-dust with a consortium of cellulolytic bacteria and SRB and observed sulphate reduction and sufficient alkalinity was generated to raise the pH to acceptable levels and at the same time removed iron from solution. Olem and Unz (1980) investigated the use of a rotating-disc biological contacter for the treatment of AMD, where continuous oxidation of ferrous iron (Fe^{2+}) by thiobacilli was assumed to precede the precipitation of ferric iron (Fe^{3+}) by neutralisation.

Many researchers since then (Gyure *et al.,* 1990, Rowley *et al.,* 1994, Johnson, 1995; Elliot *et al.,* 1998, Rose *et al.,* 1998; Van Hille *et al.,* 1999; Whittington-Jones, 2000; Foucher *et al.,* 2001, Kolmert and Johnson, 2001; Kaksonen *et al.,* 2003) have reported on bioprocess application of SRB in the treatment of AMD.

The acidity and high dissolved metal composition of AMD have extensive detrimental effects on both aquatic and terrestrial environments. Addition of $Ca(OH)_2$ and CaO to the AMD has been the conventional method for treating AMD. The lime neutralises the acidity and precipitates the metals as hydroxides. However, a metal-laden solid waste results which is

difficult to dispose, and even with dewatering, the metals remain prone to remobilisation (Lyew *et al.,* 1994).

Dill *et al.* (1995) found that using producer gas as a source of electrons and carbon gave lower sulphate reduction rates and biomass production in the removal of sulphate from AMD. Addition of growth supplements was found to stimulate SRB growth and therefore increase the rate of sulphate reduction.

The formation of metal sulphides is the main mechanism used by SRB to remove toxic metals from solution. Low solubility products of most toxic metal sulphides mean that even with low sulphide generation, precipitation is good (White *et al.,* 1997). Although most SRB are mesophilic with maximum growth occurring at pH values of 6-8, some isolates have been found to be able to tolerate and grow in moderately acid conditions, pH value of 3-4. In these environments the SRB probably maintain relatively neutral microenvironments around cell flocs due to their metabolic activities (White *et al.,* 1997)

Elliot *et al.* (1998) designed an up-flow porous- medium anaerobic bioreactor, which was inoculated with SRB. Under a continuous flow of medium containing 16.1mM sodium lactate at pH values of 4.5, 4.0, 3.5, and 3.25, the SRB were able to reduce sulphate, removing 38.3 % of the influent sulphate at pH 3.25. Elliot and his colleagues' results contradicted earlier published work of Gyure *et al.* (1990) who found that at pH 3.8, organic acid concentration greater than 5 mM completely inhibited biological sulphate reduction. However, results from Elliot *et al.,* (1998) suggest that SRB may be applied in the remediation of AMD and wastewaters where the pH value is around 3.0.

The rates of sulphide generation, and therefore metal removal, by SRB from different wetlands treating AMD were found to be significantly different (Webb *et al.,* 1998). The lack of correlation between sulphide generation and metal removal suggested that there might be other factors (e.g. binding to the biomass or complexing with extracellular polymeric substances), which also contribute to metal removal. The effect of these other factors varied between different metal ions, with zinc and iron removal, and amount of sulphide generation, showing direct correlation, compared to manganese, which remained in solution (Webb *et al.,* 1998).

Glombitza (2001) used a mixed culture of SRB immobilised onto porous material for the precipitation of heavy metals and purification of mine wastewater with methanol as a cheap carbon source. Sulphate reduction rates using AMD from a lignite mine reached 132 mg/l/h. Heavy metals removal was almost 100 % and the acidic water increased in pH from 3.0 to 6.9. The excess sulphide generated was oxidised and used for the production of sulphur (Glombitza, 2001).

Similar work on the treatment of AMD using an SRB based system was carried out by García *et al.* (2001). They used SRB isolated from the bottom of a pyritic tailing pond and reported the removal of 9 000 ppm of sulphate and found the culture was able to grow in the presence of up to 100 ppm of copper and 30 ppm of iron. The system was also able to alkalise the medium provided that the initial pH value was greater than 4.0.

1.2.2 Metal-laden wastewaters

Natural immobilisation of metal ions as sulphide ores has been found to take place naturally in many environments. Sulphate-reducing bacteria are believed to be responsible for the deposition of many metal sulphide ores (Zobell, 1946; Miller, 1950).

The Biosulphide Process described by Rowley *et al.* (1994) involved the separation of chemical precipitation of sulphide metals from the biological conversion of sulphate to sulphide. Advantages of this process are that the SRB biomass is not exposed to the fluctuating conditions of the wastewater influent, which means bacterial sensitivity to toxic compounds is eliminated. Hydraulic retention times (HRT) are greatly reduced even with highly contaminated streams, as there is no need for the biomass to acclimatise. The metal sulphide sludge produced in the Biosulphide Process is excluded from the biological reactor where it can cause plugging, toxicity and abrasion (Rowley *et al.,* 1994).

Biosorption and/or bioaccumulation of metals by microorganisms is probably one of nature's safeguards for reducing toxicity of metal ions in the microorganisms' environment. The potential applications of these phenomena, however, are governed by certain criteria or characteristics of the biosorbent. These include metal affinity, rate of metal uptake, selectivity, temperature tolerance, versatility and robustness (Eccles, 1995).

Sulphate-reducing bacteria have also been used in the treatment of electroplating effluent. Song *et al.* (1998) investigated the effect of different electron donors and toxic materials on the activity of SRB in the bioremediation of electroplating effluent. The inhibition concentration (IC) of copper that reduced SRB activity by 50% (IC_{50}) was found to be 100 mg/L. On the other hand, 130 mg/L of chromium (VI) only caused about 15 % decrease in SRB activity, while 10 mg/L of cyanide caused about 30% inhibitory effect compared to the control. Little difference was observed in the affinity of the SRB for glucose as an electron donor source compared to lactate and acetate. In this application however, the maximum glucose utilisation rate was lower than the maximum rates for lactate and acetate. Taking into account the costs, handling and purchase availability involved in supplying the different carbon sources for electroplating effluent treatment, glucose was considered a reasonable carbon source for the SRB (Song *et al.,* 1998).

Jalali and Baldwin (2000) were able to grow SRB in a solution with up to 150 mg/L of copper and remove copper to below 0.1 mg/L. Better copper removal was determined in the presence of bacterial cells compared to when the cells were removed from solution by centrifugation. The authors concluded that the presence of copper in solution might play a role in stimulating the bacteria and improving metal precipitation. Increased copper precipitation may have been due to improved sulphide production and production of extracellular polymeric substances (EPS), seen as slime under electron microscopy.

Chen *et al.* (2000) investigated the adsorption of copper(II) and zinc(II) ions on stationary phase cells of *Desulfovibrio desulfuricans* and the effect of adsorption on the SRB cells. Equilibrium adsorption values for copper(II) and zinc(II) of 16.7 mg Cu/g dry cell and 49.6 mg Zn/g dry cell were achieved at pH values of 5.0 and 6.6 respectively. There was negligible comparative copper(II) and zinc(II) adsorption at pH values less than 4.0, confirming the importance of the solution pH and ionic strength. The possible role of EPS in the removal of metal ions from solution was also noted.

Mining and mineral processing of polymetalic ore in the Vromos Bay area, near the Black Sea coast of southeastern Bulgaria have resulted in the contamination of surrounding agricultural land with radioactive elements uranium, radium and thorium. Other toxic heavy metals (e.g. copper, cadmium and lead) are also present. Laboratory experiments demonstrated efficient treatment of the soils by an *in situ* treatment method, where acidified waters were used to

solubilise the metals and the SRB played a role in the immobilisation of the metals. Real field application of this process gave promising results. However, the authors noted the need for detailed characterisation of the subsurface geologic and hydrogeologic conditions of the area to prevent soil effluent migration and subsequent pollution of surface and ground waters (Groudev *et al.,* 2001a & b).

Foucher *et al.* (2001) reported on the selective precipitation of metals from real effluent collected from the disused Chessy-Les-Mines, using biogenic hydrogen sulphide (H_2S). A fixed-bed reactor was fed with an H_2/CO_2 mixture, and the gas mixture from the bioreactor was subsequently bubbled into a stirred reactor fed with the real effluent. Feeding some of the sulphide treated effluent into the SRB reactor was found to stimulate sulphate reduction. Copper and zinc could be selectively recovered from other metal ions present in the effluent under different pH conditions.

Three SRB strains isolated from saltpans were found to have optimum growth at 90 - 100 % salinity on substrates like formate, acetate, lactate, butyrate, ethanol and benzoate. Different SRB strains were found to be able to tolerate mercury chloride ($HgCl_2$) and lead nitrate ($Pb(NO_3)_2$ concentrations of up to 200 µg/mL and 500 µg/mL respectively. Therefore, the potential exists for the application of hypersaline SRB that are more tolerant to heavy metals than mesosaline SRB in the precipitation of metals (Harithsa *et al.,* 2002).

Due to the increased application of platinum group metals in industry, new methods for their recovery are constantly being investigated. Chemical extraction methods generally use toxic compounds, are expensive and difficult due to the complex aqueous chemistry involved. Several researchers have reported on the biological reduction of these metals. For example, *Desulfovibrio desulfuricans* has been reported to enzymatically reduce uranium(VI) to stable UO_2 (Lovley *et al.,* 1991; Francis *et al.,* 1994). Lloyd *et al.* (1998) used resting cells of *D. desulfuricans* which were challenged with a set concentration of $Pd(NH_3)_4Cl$, and analysis of the cells using energy-dispersive X-ray microanalysis confirmed the presence of reduced palladium. Hydrogenase and possibly cytochrome c_3 activity were reported to be involved in palladium reduction.

The sulphate-reducing bacterium, *Desulfovibrio desulfuricans* was found to be able to couple the oxidation of an organic compound to the enzymatic reduction of uranium(VI) to

uranium(IV), which precipitated out of solution from groundwater contaminated with uranium(VI) as uraninite ($UO_2 \cdot xH_2O$). The groundwater was collected from mill tailings sites with uranium(UVI) concentration of up to 50 mg/L and also contained nitrate and sulphate. Addition of nutrients in the form of ethanol and trimetaphosphate was found to stimulate the SRB (Abdelouas *et al.,* 1999 & 2000).

Lloyd *et al.,* (2001) reported on the reduction of technetium(VII), chromium(VI), selenium(IV), and tellurium(IV) by representatives of three genera of SRB. Specific electron donors were found to be able to effect reduction of specific metal ions with some of the metals being reduced to base metals. Lloyd *et al.,* (2001) also reported on the ability of a newly identified SRB strain, *Desulfotomaculum reducens*, to couple growth to metal reduction.

1.2.3 Biodegredation of aromatic/nitroaromatic and toxic organic compounds

Earlier work by McCormick *et al.* (1976 & 1981) demonstrated the sequential degradation of the aromatic compound hexahydro-1,3,5-trinitro-1,3,5-triazine (RXD) by sewage sludge under anaerobic conditions. Application of SRB in nitroaromatics degradation is dependent on the ability of some SRB to utilize nitrate in addition to sulphate, as the terminal electron acceptor (Keith and Herbert, 1983). The respiratory enzyme responsible is a nitrite reductase, which is believed to be closely associated with a hydrogenase enzyme (Steenkamp and Peck, 1981).

Sulphate-reducing bacteria are able to oxidise a wide range of substrates, more than 75 reported by Widdel and Bak, (1992). *Desulfobotulus sapovorans* (formerly *Desulfovibrio*) and *Desulfoarculus baarsii* (formerly *Desulfovibrio*) are able to utilise fatty acids up to C_{18}, (Widdel and Bak, 1992) with the latter being the only genera able to completely oxidise its substrates (Widdel, 1988). Drzyzga *et al.,* (1993) described a new SRB isolate, *Desulfoarculus* strain SAX, which is able to completely oxidise certain aromatic compounds, including benzoate and 4-hydroxybenzoate to CO_2. Strain SAX is also able to use substrates typical of the genus, *Desulfovibrio* such as H_2, pyruvate, lactate, and malate in the presence of sulphate (Drzyzga *et al.,*1993). Therefore, strain SAX has promising potential application in waters and/or soils contaminated with aromatic compounds.

Only recently has the ability of microbial degradation of explosives and nitroaromatic compounds by anaerobic bacteria been demonstrated. Nitro groups in the aromatic ring have been implicated as the cause of toxicity of most of these compounds. The use of pesticides, plastics, pharmaceutics, explosives and the landfill dumping of industrial waste, are the main contributors of nitroaromatic compounds to soil and water environments (Boopathy *et al.,* 1998a). The SRB, *Desulfovibrio* sp. (strain B) was demonstrated to be able to degrade 100 mg/L of 2,4,6-trinitrotoluene (TNT) within ten days using pyruvate as the main substrate and SO_4^{2-} as the electron acceptor. Other substrates that supported TNT degradation were lactate, ethanol, formate and a hydrogen/carbon dioxide mixture. SRB were also able to effectively degrade TNT (100 %) and related compounds from soil slurry (Boopathy and Kulpa, 1992; Boopathy *et al.,* 1993; 1998a & b).

Kuever *et al.* (1993) described the isolation and characterisation of a new spore-forming sulphate-reducing bacterium that grew by complete oxidation of catechol. Most of the SRB capable of using aromatic compounds were isolated mostly from marine environments and only a few e.g. *Desulfobacterium catecholium, Desulfotomaculum sapomandens,* and some *Desulfococcus multivorans* strains, grow in fresh water media (Tasaki *et al.,* 1991). The new SRB strain was described as of the genus *Desulfotomaculum* strain Groll. This isolate showed complete oxidation of various aromatic compounds including phenol, catechol, benzoate, *p*- and *m*-cresol, benzyl alcohol and vanillate. Hydrogen and carbon dioxide were used as substrates rather than lactate. Degradation of aromatic compounds was by o-demethylation and subsequent complete mineralisation. The versatility of strain Groll in the degradation of more than 20 different aromatic compounds makes its scope for application in wastewater treatment very promising (Kuever *et al.,* 1993).

Chloroform is suspected to be a carcinogen and as such poses a threat to public health. It is widely used in industry as a solvent and occurs in landfill leachates. Chloroform cannot be degraded under aerobic conditions except under methanotrophic conditions (Bouwer *et al.,* 1981). Although anaerobic methane archea have been shown to degrade chloroform, their activity is inhibited at chloroform concentrations above 16.74 μM (the more common term, anaerobic methane producing bacteria (MPB) has been used here). Sulphate-reducing reactors under the same conditions showed much higher chloroform transformation rates without any inhibition (Gupta *et al.,* 1996). Chloroform transformation in SRB occurred mainly by reductive dehalogenation to form dichloromethane (DCM). Dichloromethane

degradation in SRB was much slower compared to chloroform transformation, whereas in the methanogenic system the rate of DCM transformation was about the same as that for chloroform transformation (Gupta *et al.,* 1996).

An effluent high in organic load (4-6 kg /m^3 COD) and salt content (1.85 kg/m^3 SO_4^{2-} and 16.2 kg/m^3 Cl^-) is generated by the Chilean fishmeal industry during hydraulic unloading of fish from ships. Marine sediment and pig manure were used as anaerobic inocula for the treatment of the saline effluent. The marine inoclum gave a higher methanogenic:sulphate reduction ratio of 0.0025 at 37 °C. At COD:SO_4^{2-} ratios lower than 0.5, and at hydrogen sulphide concentrations greater than 0.22 kg/m^3, methane production decreased by up to 50 % (Aspé *et al.,* 1997).

Fang and Zhou (1997) were able to treat sulphate-rich wastewater containing benzoate and cresol isomers in an UASB reactor at 37 °C. Results of their experiments suggested that most of the sulphidogens in the reactor were acetogens producing acetate, which was subsequently utilised by methanogens to yield methane.

Constructed wetlands are designed for passive treatment of wastewater. Such a system was used to treat waters contaminated with crude oil and toxic heavy metals (cadmium (Cd), copper, lead, manganese and iron. Although the SRB were not reported to have played a role in the degradation of the oil portion of the wastewater, they were attributed with the efficient removal of all the metal ions to well below acceptable discharge levels for agriculture and/or industry (Groudeva *et al.,* 2001). In contrast to the wetlands data above, which found no contribution by SRB in the degradation of the oil component of wastewater, several researchers have reported on the degradation of petroleum hydrocarbons (PCH) in PCH-contaminated aquifers (Lovley, 1997; Wiedemeier *et al.,* 1999; Anderson and Lovley, 2000). Schroth *et al.* (2001) were able to detect microbial sulphate reduction in a PCH-contaminated aquifer using push-pull tests combined with stable sulphur isotope analyses.

1.2.4 Degradation of lignocellulosic materials

Sulphate-reducing bacteria were found to be able to grow in the sulphate-rich wastewater from the paper and pulp industry. Hydrogen sulphide generated by SRB was found to be a contaminant in the paper and pulp industry, where it was believed to be responsible for the break down of cellulose material (Bannick and Muller,1952).

There is little literature available on the degradation of lignocellulosic materials by SRB. Pareek *et al.* (1998) reported on the mineralisation of lignocellulosic material (office paper and newspaper) under sulphidogenic conditions. Although mineralisation of lignocellulosic material under methanogenic conditions was also reported, it was two times lower than under sulphidogenic conditions.

1.2.5 Sulphate-rich wastewaters

One of the more obvious applications of SRB is in sulphate removal from sulphate-rich wastewater. Sulphate-reducing bacteria reduce sulphate to sulphide and then photosynthetic sulphide oxidisers and thiobacilli play a role in the oxidation of the free sulphide to elemental sulphur (Postgate, 1982; Lettinga, 1985).

Maree and Strydom (1985) and Maree *et al.*(1986, 1991) have worked on the removal of sulphate from industrial wastewater and were able to show that producer gas is a viable carbon and electron donor source for biological sulphate reduction.

Maree and Hill (1989) described a method for the removal of sulphate from sulphate-rich industrial effluent by SRB. The hydrogen sulphide produced was subsequently oxidised to elemental sulphur when it was brought into contact with a ferric solution. This represents one way of dealing with the hydrogen sulphide produced in the anaerobic stage of sulphate reduction to give a valuable by-product in the form of elemental sulphur.

Due to the use of sulphuric acid in many chemical production industries, sulphate is often present in the wastewaters and therefore SRB can be used in the treatment of the wastewater. Some SRB carry out incomplete oxidation of organic matter to acetate while the rest are able to completely oxidise organic matter to hydrogen sulphide and carbon dioxide. In some industries where methane production is the desired end point, the inhibitory effect of hydrogen sulphide becomes problematic. Although SRB play a role in citric acid production wastewater treatment, completely metabolising propionate and H_2/CO_2, the methanogens were found to be able to out-compete the SRB for acetate with competition for butyrate and ethanol also taking place (O'Flaherty *et al.*, 1998).

Large amounts of sulphates and sulphides are used and discharged in the chrome tanning industry. The resultant effluent is not only high in sulphates and sulphides but has a high chemical oxygen demand (COD) loading. van Groenestijn, (1999) reported on a treatment process which employed an UASB or expanded granular sludge blanket (EGSB) reactor in which most of the COD was eliminated and converted into biogas and the sulphates were reduced to hydrogen sulphide. The hydrogen sulphide was then passed into ferric-chelate (Fe(III)-EDTA) (redox mixture) to give elemental sulphur, hydrogen gas and ferrous-chelate mixture. In this way the system was set up for sulphate removal and sulphur recovery.

Silva *et al.* (2002) were able to remove up to 97 % sulphate from wastewaters were the initial sulphate concentration ranged from 12 000 to 35 000 mg/L using a 94.2 L anaerobic fixed bed reactor. Using an EGSB reactor set-up, de Smul *et al.* (1999) achieved 80-90 % sulphate removal at 33 °C, at a sulphate loading rate of 4 g/L/day, provided the COD to sulphate ratio was at least 6.

1.3 Problems in the Development of Sulphate-Reducing Bacterial Biotechnology.

Before the 1970s, application of SRB and anaerobic digestion processes for wastewater treatment were not considered a viable alternative to aerobic treatments. The anaerobic process was restricted to sewage and manure digestion as it was thought to be too sensitive and unstable in active water treatment applications (Visser, 1995; Boopathy *et al.,* 1998a).

While numerous problems have been resolved in SRB biotechnology applications in recent years, two in particular have continued to constrain further development; poor cell retention and ‘clean’ waste stream treatment.

The slow growth rates of SRB tend to exacerbate the major problem of poor cell retention in applied continuous bioprocesses where biomass wash-out in the effluent stream reduces reactor volumetric productivity. In such cases, attempts to create high dilution rates result in significant loss of biomass in the effluent stream (Pilkington *et al.,* 1998).

Sulphate-reducing bacteria require an electron donor and a carbon source for sulphate reduction and cell growth, and in most cases one compound fills both roles (e.g. lactate, ethanol, and several other low molecular weight organic compounds) (Knobel and Lewis, 2002). Providing a combined electron donor and carbon source is relatively easily accomplished and sometimes the wastewater of interest already has a high organic load that serves this purpose.

The problem arises when the wastewater of interest contains mainly inorganic pollutants requiring treatment and has very low or zero organic content. The introduction of an organic component, as biomass or organic feed, into a 'clean' (inorganic) waste stream introduces unwanted organic pollution, which is generally not completely oxidised to carbon dioxide and water. This may then require further downstream processing for its removal. The high residual organic load that results from current SRB treatment systems excludes the SRB from 'clean' waste stream bioprocess application.

'Clean' waste streams, in the present study, have been defined as those waste streams or effluents that contain little or no organic pollution, and the only pollutants present are one or a combination of inorganic components such as sulphates, metal ions and acidity. These will include certain mine wastewaters, boiler feed waters and treated waters emerging from upstream treatment operations.

The study reported here has focused on these problems and has investigated the use of immobilised cell systems and disarticulation of carbon and electron donor supply as potential solutions. The background to the proposed hypothesis is discussed below.

1.4 Immobilisation

Bioprocess applications, for the most part, are employed in continuous reactor systems. Batch reactors were originally applied in activated sludge processes in what were known as the 'fill and draw' processes. Batch reactors involve extensive operator control and where possible have tended to be phased out in most industries. The sequencing batch reactor (SBR) is a modification of the typical batch process in that various processes are designed to be carried

out in the same reactor with a sequence of events occurring under micoprocessor control (Horan, 1990).

Completely mixed reactors have the advantage of homogenous distribution of the waste load in the reactor tank. However, bad design may result in raw sewage passing through the system untreated, and floc loading may be reduced resulting in poor settlability (Horan, 1990).

Plug-flow reactors are a type of continuous reactor process where the influent is introduced at one end of a high length to breadth ratio tank and removed at the other end with no mixing. A gradient of decreasing biochemical oxygen demand (BOD) and increasing suspended solids is established along the length of the reactor (Horan, 1990).

The UASB is now one of the more widely applied continuous reactor process in use today (Fang and Zhou, 1997; Elliot *et al*., 1998;Timur *et al.,* 2000). There may be variation of the UASB reactor set-up, e.g. the EGSB reactor (de Smul *et al.,* 1999) but the basic principles are the same. The influent is introduced from the bottom of the reactor and flows upwards through a 'sludge blanket' that is retained within the system.

Free cells in suspension provide useful information in laboratory research, but for more rigorous industrial microbial applications, they have several disadvantages that include small particle size, low mechanical strength and low density, which limit reactor design and make it difficult to separate the biomass from the effluent. In packed-bed or fluidised-bed reactors immobilised biomass has greater potential (Gadd, 1988).

1.4.1 Advantages of cell immobilisation

Due to the problem of biomass loss due to wash-out encountered in continuous bioreactor processes, immobilisation has been an option that has received considerable attention. This is of particular importance in reactor systems that apply microorganisms with very slow growth rates and is especially problematic where cells are low EPS producers and natural adhesion properties are hence also low. Loss of biomass would mean a significant decrease in reactor productivity due to lower cell load and lower cell densities.

Cell immobilisation has been defined as, 'the physical confinement or localisation of cells to a certain defined region of space with the preservation of some desired catalytic activity'. Immobilisation can, obviously, be applied to enzymes and other compounds of interest. (Nedovic *et al*., 1998).

Numerous advantages have been reported for immobilisation over free suspended cells in continuous reactor processes and these include:

- Higher cell densities and cell loads – immobilisation reduces and may, in some cases, eliminate biomass loss through wash out.
- Increased volumetric productivity – this is linked to cell densities and load. Increased biomass load results in increased reactor activity and consequent volumetric productivity.
- Shorter overall reaction time – also linked to biomass load and densities.
- Reuse of biomass with shorter reactor 'down-time' – immobilisation allows for easier handling of the biomass which is important in processes where biomass recovery for reuse and handling is routine.
- Improved substrate utilisation – due to concentrated pockets of biomass which each act as intense reaction microenvironments.
- Simplified process design – managing macro rather than micro particulates
- Protection of cells – immobilisation provides some elements of protection for the biomass from what are sometimes harsh environmental conditions of pH, temperature and toxic metabolites.
- Improved biomass separation and retention.

(Kierstan and Coughlan, 1985; Gadd, 1988; Pilkington *et al.,* 1998; Park & Chang, 2000).

1.4.2 Principle methods of immobilisation

A range of techniques and support materials have been investigated for cell immobilisation. These can be divided into four main groups according to the physical method employed for immobilisation, 1) entrapment within a porous matrix, 2) attachment or adsorption to a pre-formed carrier, 3) self aggregation by flocculation (natural) or cross-linking agents (artificial) and 4) cells contained behind a barrier (usually semi-permeable) see figure 1.1.

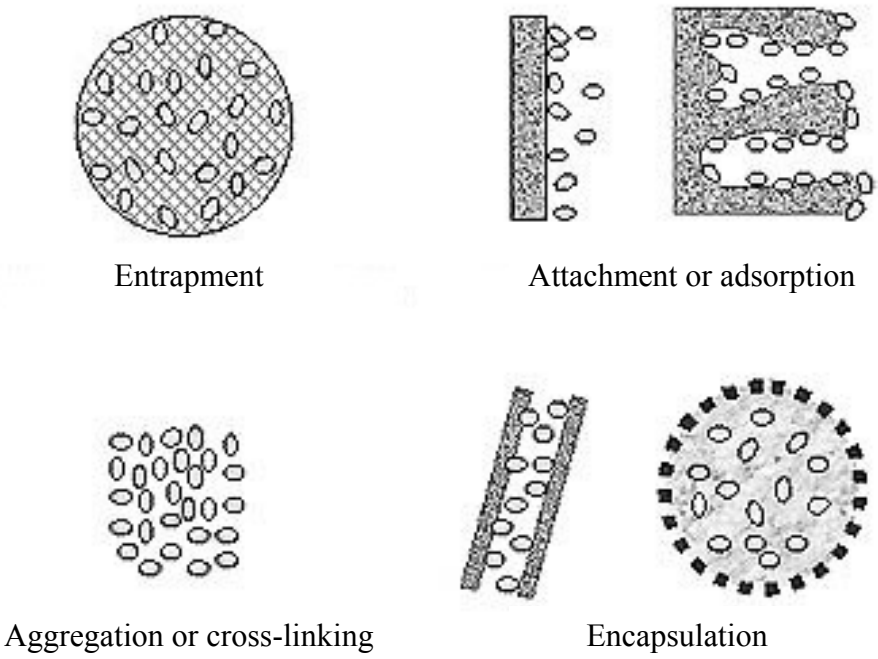

Figure 1.1: Principle methods of cell immobilisation (Pilkington *et al.,* 1998).

1.4.2.1 Entrapment

Cells are confined within a three-dimensional matrix, but are free to move within the compartments or pores in the material. Substrate and products are able to diffuse to and from the cells. Membrane matrices and several polymers are available for the entrapment of cells, including, alginate, *kappa*-carrageenan, agar/agarose, cellulose, collagen, polyacrylamide, polyurethane foam (Trevan, 1980; Mattiasson, 1983a; Brodelius, 1985; Scott, 1987).

There are limitations with the entrapment methods. Substrate and product molecular size is limited (this may not be a major problem in aqueous systems), the carrying capacity or loading of the matrix is limited and leaching of the cells into the media occurs with growth, eventually leading to loss in biomass (Kuhn, 1988, Kuhn and Pfister, 1989). The process of entrapment may also result in high cell death e.g. entrapment by acrylamide or polyurethane polymerisation may be initiated by persulphate or ultraviolet light (Mattiasson, 1983b).

1.4.2.2 Attachment or adsorption

Absorption offers a very mild method of immobilisation and is therefore widely used. It should be noted that some of the entrapment methods involve some attachment or adsorption process, e.g. entrapment in polyurethane foam.

Limitations of the adsorption method include the reversible nature of binding which means desorption of immobilised cells may occur due to changes in the aqueous environment e.g. pH; as in the entrapment method, loading capacity is limited and continued growth results in loss of the biomass to the medium; a nutrient gradient is established at the matrix/biomass boundary with the cells closer to the matrix being exposed to the least amount of nutrients; some microorganisms do not naturally attach to surfaces or they attach poorly and are, therefore, readily leached into the media (Scott, 1987; Brodelius, 1985; Trevan, 1980).

1.4.2.3 Self aggregation or cross-linking

Some microorganisms have the tendency to clump/self aggregate and form flocs. Production of extracellular polymeric substances (EPS) by some microbes is seen as a mechanism designed to allow aggregation in order to create a closely controlled microenvironment for the cells. Biomass floc formation varies between microorganisms, for example, methane producing bacteria (MPB) produce strong cell aggregates with good settlability compared to SRB which form weak aggregates, both organisms produce EPS. Covalent linking technology is used to induce aggregation of cells (Brodelius, 1985).

Self-aggregation and cross-linking have some of the limitations of the methods already discussed: the chemistry of the aqueous environment can disrupt flocs or cross linkages, thus releasing the cells into the media. Again loading capacity is limited with cross-linking and little room is available to allow continued growth (Brodelius, 1985).

1.4.2.4 Encapsulation

In contrast to other methods of immobilisation discussed so far, encapsulation maintains the solution environment around the biomass rather than maintaining physical or chemical forces required for immobilisation (Klei *et al*., 1985). Immobilisation by encapsulation overcomes most of the problems associated with other immobilisation methods. In this process the conventional method of calcium alginate beads formation (entrapment) is reversed. A mixed solution of cells and calcium chloride is dropped into a solution of sodium alginate while mixing. A semi-permeable calcium alginate membrane immediately forms on the surface of the microaqueous droplet by ionic interaction (Klein *et al*., 1983, 1986). Wall thickness, pore size, surface charge and mechanical strength of the capsule can be altered by using reagents at different concentrations (Tampion and Tampion, 1987; Park and Chang, 2000).

The liquid core capsule has several advantages for microbial immobilisation over conventional entrapment methods: the liquid core provides a microenvironment with a large area for cell containment; mass transfer resistance inside the capsule is reduced; nutrients can diffuse in and out of the capsule freely; the cells' microenvironment provides some measure of protection for the cells from the immediate environment (Park and Chang, 2000).

1.4.3 Examples in cell immobilisation

There are many examples of the application of the immobilisation techniques in cell immobilisation. The selection of an immobilisation method depends on various factors, including the type of organism, the conditions or environment of application, the availability of the matrix in convenient quantities, the mechanical and chemical stability of the matrix, the complexity of the immobilisation process and its effect on the biomass (Hermanson *et al*., 1992), bioreactor configuration, ease of operation scale-up and physiological and environmental safety of the materials used (Nedovic *et al.*, 1998). Once all these factors have been taken into account, the appropriate choice of immobilisation method is applied.

A cylindrical column with immobilised *Rhizopus arrhizus* in glass beads was prepared for the adsorption and recovery of uranium from solution in a continuous lab-scale reactor. The reactor's uptake capacity was 50 mg/g, and this could be maintained over 12 repeated cycles. All the adsorbed uranium could be desorbed from the biomass (Tsezos *et al*., 1989).

Duncan *et al.* (1992) investigated the immobilisation and application of yeast and algal cells for bioremediation of heavy metals. Yeast and algal cells were immobilised by polyacrylamide gel cross-linking, entrapment onto filter paper and immobilisation onto polypropylene hollow fibre membranes. All immobilisation methods improved metal adsorption and the biomass could be reused up to ten times after reconditioning.

A *Bacillus* species was immobilised by embedding in a water-soluble polymer, polyethyleneimine (PEI) and then cross-linked using gluteraldehyde. The immobilised product was rigid, but less stable than charcoal and seemed to lose metal binding capacity, this was probably due to the cross-linking of potential binding site. Treatment of the immobilised product with hot caustic increased its porosity and metal binding capacity (Brierley and Brierley, 1993).

Rhizopus arrhizus was immobilised onto various supports using various methods, alginate (Alg), polyacrylamide (PAA), epoxy resin (ER) and polyvinylformal (PVF) materials, all of which were tested for the removal of cadmium (Cd^{2+}) from solution. Polyvinylformal removal of Cd^{2+} removal was almost the same as that for free biomass and increased with higher cell loading. Polyvinylformal is an inert, non-inhibitory immobilisation matrix (Tobin *et al.,* 1993).

Immobilisation of yeast cells has been investigated extensively. Green *et al.* (1996) investigated the microencapsulation of yeast cells in polyamide microcapsules by interfacial polymerisation and subsequent application as biocatalysts in organic solvents. The resultant microcapsules showed good mechanical strength and rigidity with shaking over a number of days with no significant deterioration.

Sağ *et al.* (1995) demonstrated that although Ca-Alg beads were able to adsorb copper(II) ions, in an up-flow reactor, *Zoogloea ramigera* cells immobilised onto Ca-Alg beads showed higher equilibrium copper removal. The immobilised *Z. ramigera* and Ca-Alg showed copper removal of 94.3 % and 63.8 % respectively at a flow rate of 3.6 ml/min and initial copper concentrations higher that 100-150 mg/L.

Immobilisation of four mesophilic strains of lactic acid bacteria separately and/or co-entrapped in κ-carrageenan/locust bean gum gel beads allowed monitoring of their application in continuous pre-fermentation of ultra heat treated (UHT) skim milk in a stirred tank bioreactor. High and stable lactic acid and cell productivity were achieved. The immobilised cells showed very good resistance to psychotrophic contamination (Sodini *et al.,* 1997).

Aksu *et al.* (1998) immobilised *C. vulgaris* onto Ca-Alg and agarose and then compared the adsorption of copper ions (Cu(II)) by the two systems in packed-bed columns. Calcium-alginate is a good adsorbent of Cu(II) ions and only a slight increase in adsorption was observed with Ca-Alg immobilised *C. vulgaris.* On the other hand, agarose immobilised *C. vulgaris* clearly increased the Cu(II) adsorption. Over longer time periods, Ca-Alg-algae systems adsorbed more Cu(II) than the agarose system.

Ca-Alg immobilised *Candida stellata* cells were able to influence the analytical profile of wines with increased glycerol (70 %) and succinic acid content compared to the *Saccharomyces cerevisiae* fermentation control (Ferraro *et al.,* 2000).

Immobilisation of *Chlamydomonas reinhardtii* in Ca-Alg beads resulted in no nitrate consumption at concentrations below 0.14 mM nitrate, whereas complete nitrate consumption was observed with free suspended cells. Immobilisation seemed to confer some protection on the cells and allowed them to maintain high nitrate uptake viability over a wider range of pH (5.5-8.0) and temperature (25-38 °C) than free suspended cells (Garbayo *et al,* 2000).

The filamentous yeast, *Trichosporon cutaneum* R57 with phenol degrading ability was chemically immobilised onto modified polyacrylonitrile (PAN) granules and fibers. The rate of phenol biodegradation was higher with *T. cutaneum* R57 cells immobilised on PAN fibres than on PAN beds (granules), showing potential for application of the immobilised process in the purification of phenol-containing wastewater (Godjevargova *et al*., 2000).

A new cryogel immobilisation technique was used to immobilise *Aspergillus* sp. CX-1 in macroporous cryoPAG for the production of extracellular hydrolytic enzymes. The extracellular enzymes of interest, exo-1.4-β-glucanase, endo-1.4-β-glucanase, β-glucosidase and xylanase all showed higher activities and stability with long term repeated batch cultivation compared to free mycelia. The fungus immobilised in the composite cryoPAG with polypropylene non-woven fabric had the highest exo-1.4-β-glucanase activity, best mechanical strength, and the best enzyme production for up to 85 days. The macroporous structure of the cryoPAG allows efficient mass transfer and resultant improved growth conditions. The spongy cryoPAG can be used in large blocks and does not break up into granules (Lusta *et al.,* 2000).

The tendency of *Acidithiobacillus ferrooxidans*, previously named *Thiobacillus ferrooxidans*, to attach to surfaces makes it a good candidate for immobilisation (Kelly and Wood, 2000). *A. ferrooxidans* is involved in the bioleaching of sulphide ores for metal recovery. The direct mechanism involves the production of iron(III) by the bacteria for leaching of sulphide ores. Glass beads, activated carbon, ion-exchange resin, sand, polystyrene, polyurethane and many other supports have been investigated. *A. ferrooxidans* immobilisation onto glass beads occurred on the jarosite (iron(III)sulphate) formed in cultures. However, stirring prevented

the jarosite from being deposited onto the glass beads. The results suggest the importance of setting conditions for jarosite production in biofilm formation (Pogliani and Donati, 2000).

Heavy metal tolerant bacteria selected from the Rio de la Plata coasts in Argentina were immobilised in polymeric matrices prepared by gamma irradiation of 2-hydroxyethyl methacrylate and 2-hydroxyethyl acrylate at –78 °C in the presence of water, glycerol and polyvinyl alcohol. Bacteria immobilised on acrylic hydrogels could immobilise twice as many Cr(III) ions compared to polyvinyl alcohol membranes (Degiorgi *et al*., 2001).

The fungal biomass of *Rhizopus nigricans* was immobilised by entrapment in five different polymeric substrates - Ca-Alg, polyvinyl alcohol (PVA), polyacrylimide, polyisoprene and polysulfone. Polymer entrapment was more successful than immobilisation by adsorption onto polyurethane and coir fibre. After free biomass, polysulfone gave the best chromium(VI) uptake capacity of 101.5 mg Cr/g sorbent, and could be reused at least 25 times after regeneration (Bai and Abraham, 2003).

A novel denitrifying bacterium, *Pseudomonas butanovora* was immobilised in composite beads and packed into a reactor. Nitrate removal efficiency of the reactor was almost 100 % at ethanol-C : nitrate-N ratios of 3:1 and 1.5:1 with relatively short HRTs of 2.47 and 3 hours respectively in continuous systems (Kesserű *et al.,* 2003).

Saccharomyces cerevisiae, strain AXAZ-1 was immobilised on delignified cellulosic material (DCM) and gluten pellets (GP). The immobilised yeast biomass and free cells were used in separate batch fermentations at different temperatures. The volatile constituents were extracted and analysed. The combined effect of temperature and immobilisation gave wines with a more fruity character due to higher esters:alcohol ratio. Delignified cellulosic material-supported yeast offers a promising alternative to wine fermentation, giving a wine with more fruity character, lower fusel alcohol and intermediate acidity. Delignified cellulosic material is a cheap and abundant support of food grade purity for yeast immobilisation (Mallouchos *et al.,* 2003).

Yeast cells (*Saccharomyces cerevisiae*) were immobilised during multiplication, onto acrylamide/sodium acrylate (Aam/SA) hydrogels by adsorption. Ethyl alcohol production by the hydrogels was determined. The swelling of the hydrogels on immersion into the nutrient

media meant that more nutrients were available for the yeast cells. Different cross-linkers can be used to give different pore sizes. Ethyl alcohol production in the immobilised system was found to increase (Öztop *et al.,* 2003).

1.4.4 Immobilisation of sulphate-reducing bacteria

The immobilisation of SRB has been sparsely reported in the literature and most research on immobilisation of anaerobic bacteria has tended to focus on the methane-producing bacteria (MPB) (Alphenaar, 1999). There are contradictory reports on the relative abilities of MPB and SRB to be effectively immobilised. Isa *et al*. (1986a & b) reported that MPB were better able to immobilise in sludge beds and hence, out-compete SRB, resulting in wash-out of SRB from bioreactors. Several other researchers (e.g. Alphenaar, (1999); Basu & Baldwin, (2000); Finnie *et al.,* (2000); Glombitza, (2001)) have reported on the immobilisation of SRB, but none of these reports include immobilisation by encapsulation of the cells as will be described later in this chapter.

Sulphur dioxide reduction by SRB flocs in continuous stirred tank reactors (CSTR), and by SRB immobilised on potassium-carrageenan gel matrix or onto polymeric porous BIO-SEP® beads in column reactors has been demonstrated, using municipal sewage as a carbon and electron donor source. BIO-SEP® beads are commercially available porous polymeric-based resins, while κ-carrageenan is a sulphonated polysaccharide which can be cross-linked with cations (e.g. potassium). On heating, the potassium-carrageenan forms a low viscocity solution, which produces a strong gel on cooling. A reduction of sulphur dioxide to hydrogen sulphide in the CSTR of 2.1 mmol/L/h was reported compared to 16.5 mmol/L/h for the BIO-SEP® polymeric beads. However, sulphite concentrations appeared to affect sulphur dioxide reduction (Selvaraj *et al*., 1997).

Pumice, an inert solid support, and granulated sludge particles were both found to provide suitable support for SRB attachment. However, the mechanical strength of the sludge granules decreased with time. The activity of the immobilised SRB in UASB reactors was determined in terms of the rates of organic COD removal and volatile suspended solids (VSS) concentration, and was found to be 0.79 and 0.55 gCOD/gVSS/day for pumice and granular sludge –immobilised SRB respectively (Alphenaar, 1999).

Other types of supports reported for the immobilisation of sulphate-reducing bacteria include calcium-alginate beads, polyurethane foam (PUF), lava rock and Ringlace® (a synthetic lace-like material, made from polyvinylidene fibres). The first two supports gave the best results by maintaining the greatest amount of biomass attached to the support over time at 84 % and 73% respectively. However, the Ringlace® and lava rock showed poor support of the biomass at 37 % and 6% respectively. The sulphate reduction rates of 79 (±10) and 97 (±9) mg/L/day for calcium-alginate beads and PUF immobilised SRB respectively were comparable to the rate for free suspended cells of 81 (±14), all in static batch cultures (Basu & Baldwin, 2000).

Nagpal *et al.* (2000) immobilised SRB onto porous glass beads and reported sulphate reduction rate in a fluidised bed reactor to be up to 6.33 g/ L/day with a hydraulic retention time of 5.1 hours. Kolmert and Johnson (2001) reported average sulphate reduction rates of 0.25-0.30 $gSO_4^{2-}dm^{-3}day^{-1}$ with acidophilic SRB immobilised in porous glass beads.

Finnie *et al.* (2000) immobilised SRB within a hydrous, alcohol-free silica matrix with less than 0.1 % of the immobilised biomass leached into the nutrient solution over 10 days. Long-term storage of the silica gels also found to be possible. A maximum sulphate reduction rate of 264 mg/L/day was achieved in batch cultures and after 10 weeks of storage at room temperature under nitrogen, similar sulphate reduction rates were re-established after six days in new nutrient media (Finnie *et al.*, 2000).

A laboratory pilot plant with SRB immobilised on porous ceramic carriers in a 3.9 m^3 bioreactor was used in the treatment of acid mine wastewater and gave improved sulphate reduction rates compared to free suspended cells. Sulphate reduction rates by immobilised SRB were reported to be as high as 3168 mg/ L/day. Metal removal was almost 100 % with the pH of the acidic stream increased from a value of 3.0 to 6.9 (Glombitza, 2001).

No reports were found on the immobilisation of SRB by encapsulation as described in this research study.

1.5 Encapsulation of Sulphate-Reducing Bacteria: Rationale for Proposed Research Study

The application of SRB biocatalytic processes in anaerobic wastewater treatment is widespread and includes sulphate removal, sewage treatment, AMD treatment, metal removal and biodegradation of a number of toxic and recalcitrant organic compounds. Wastewater treatment/remediation is one of the biggest industries applying microbe bioprocessing.

Two related problems may be identified in the development of SRB biotechnology firstly that of biomass loss in continuous bioprocess reactors and secondly the high residual organic contamination that limits the application of SRB biotechnology in 'clean' waste stream treatment.

'Clean' waste stream treatment may include sulphate removal, pH neutralisation of acidic waters, heavy metal removal and calcium and magnesium removal in the treatment of water hardness. 'Clean' streams are differentiated by the lack of or minimal organic contamination of the waters and there is a need for a bioprocess treatment system that does not add an undesirable organic contamination.

Immobilisation of SRB by encapsulation in a liquid core within a permeable calcium-alginate-xanthan gum membrane is proposed as a method for providing effective immobilisation of the SRB to reduce or eliminate biomass loss which is experienced with all other immobilisation methods. Immobilisation is also necessary for the effective evaluation of the proposed novel concept of disarticulation of the electron donor and carbon source supply in order to eliminate the problem of residual organic contamination of treated streams and allow for the possible application of SRB bioprocess systems in 'clean' waste stream treatment.

In most systems where SRB biocatalytic processes are applied, the electron donor and carbon source is the same compound and is organic in origin e.g. lactate, ethanol, acetate, sewage feeds. In all these cases, treatment of inorganic waste streams becomes problematic as the use of an organic feed would introduce or increase the organic load of the effluent.

A schematic representation of the energy production in sulphidogens is given in figure 1.2. SRB obtain their energy through electron transport level phosphorylation (ETLP), represented in figure 1.2 by the shaded area, basically the reduction of sulphate to sulphide. When sulphate is low and an organic electron donor is present, energy is obtained through substrate level phosphorylation (SLP), un-shaded region in figure 1.2, through the hydrolysis of acetyl phosphate. However, other bacteria need to be present to utilise the hydrogen generated in SLP to prevent the increase in external hydrogen partial pressure, which would reduce SRB's ability to oxidise the organic carbon source (Holland *et al.,* 1987).

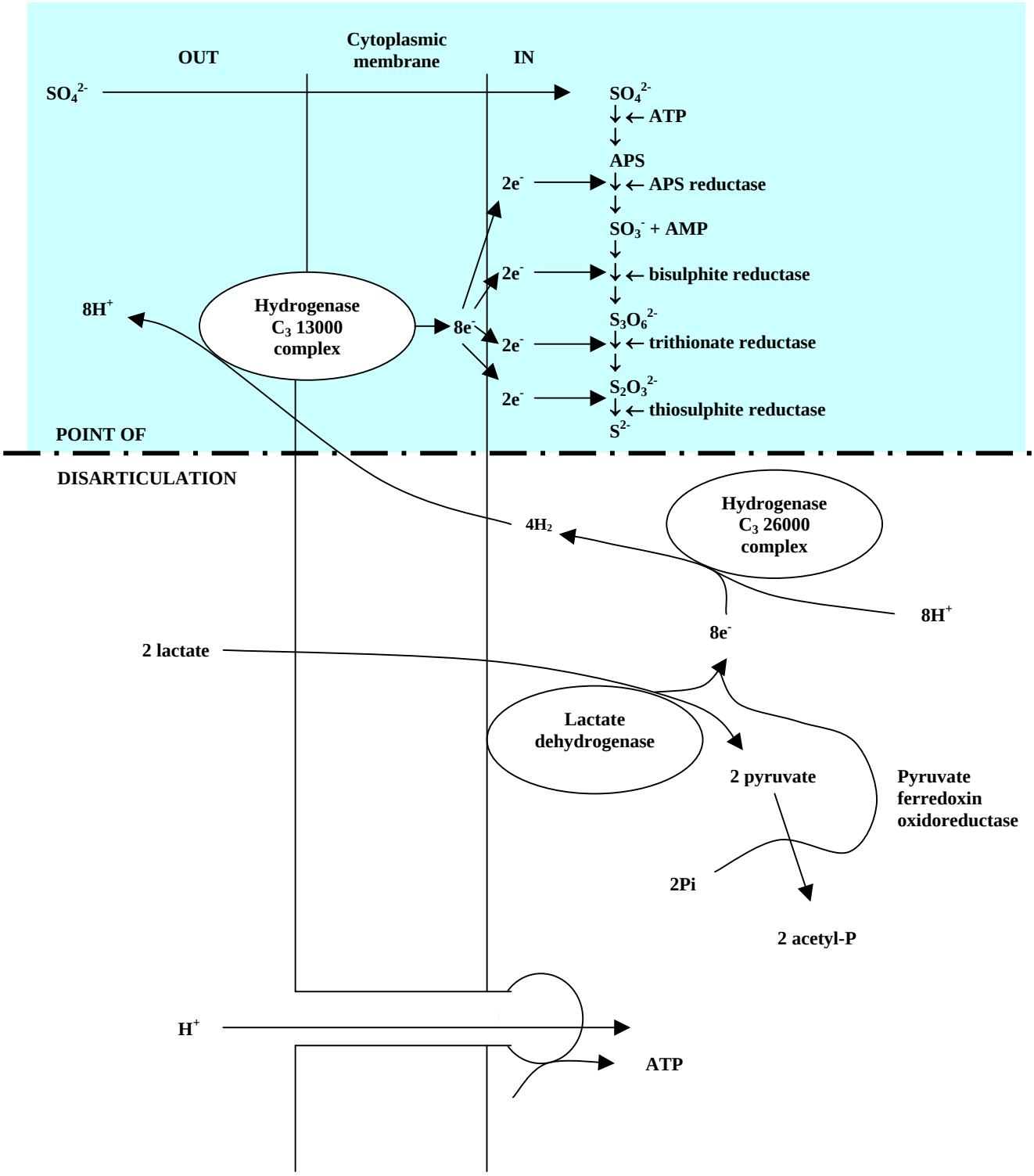

Figure 1.2: Schematic diagram for energy production in sulphidogens. Un-shaded area represents substrate level phosphorylation (SLP) and the shaded area represents electron transport level phosphorylation (ETLP) (Holland *et al.*, 1987).

Eight electrons in total are needed to reduce sulphate to sulphide and this is where ETLP is coupled to SLP, through the net movement of eight protons that leave the cytoplasm, following substrate oxidation (Holland *et al.,* 1987).

In order to effect the disarticulation of the electron donor and carbon source coupling in an SRB system, a swing back cycle would need to be applied in which the immobilised SRB may be exposed to and withdrawn from the stream being treated. In the treatment regime all sources of carbon would be excluded from the system and hydrogen gas provided as the sole electron donor source. Essentially, the external electron donor in the form of hydrogen would maintain ETLP. After a period of operation, the cells would need to be exposed again to the carbon source to provide for SLP. The desired effect would be to un-couple the sulphate reduction process from organic substrate oxidation but maintain sulphate reduction activity throughout.

Although producer gas has, in some cases, been employed to overcome the problem of high residual organic loads, it has not been applied to 'clean' waste stream treatment. In such cases, hydrogen gas served as the electron donor source and carbon dioxide gas or carbon monoxide gas served as the carbon donor source (du Preez and Maree, 1994; van Houten, 1996; Bredwell *et al*., 1999).

1.6 Research Hypothesis

Based on the rationale underpinning the argument for encapsulation and disarticulation as important prerequisites for extending SRB biotechnology in 'clean' waste stream bioprocess application, the following hypothesis has been proposed.

- The effective immobilisation of SRB will enable the disarticulation of electron donor and carbon source delivery and thus facilitate the treatment of 'clean' inorganic wastewater streams.

1.7 Research Objectives

Given the absence of previous reports or experience on encapsulation methods for SRB, it was identified at the onset that this research programme would need to establish the appropriate conditions under which encapsulation would be effected as the starting point of the study. It was identified that the following would be required:

- Selection of a competent SRB culture and its biomass production in sufficient quantities, to facilitate practical investigation of immobilisation procedures;
- Investigation of the reusability potential of SRB biomass in multiple recovery and reuse strategy applications;
- Evaluation and selection of optimum immobilisation methods;
- Investigation of the concept of electron donor and carbon source disarticulation in the application of 'clean' waste stream treatment;

CHAPTER 2
CULTURE SELECTION AND GROWTH CHARACTERISATION

2.1 Introduction

The investigation of SRB immobilisation processes and their successful application in bioprocess development depends, in the first instance, on the selection of an appropriately adapted and viable culture for the purpose.

The basic role of the SRB as terminal degraders of organic compounds means that they are most effective within a consortium of anaerobic bacteria. Most SRB use low molecular weight compounds as electron donors and carbon sources and therefore require the presence of fermentative and other anaerobic bacteria that are able to degrade more complex carbon compounds (Zehnder, 1988). They are therefore found in environments where organic substrates and dissolved sulphates are available.

One such environment is the Rhodes BioSure Process® developed by Rose *et al.* (1998) for the treatment of AMD. A pilot plant was operated at the Rhodes University Experimental Field Station in Grahamstown. Research on the sulphate-reducing population in the Rhodes BioSure Process® has demonstrated highly effective and functional mixed co-cultures in sulphate removal from mine wastewaters.

Although a pure culture would probably allow for more rigorous and detailed investigation in the laboratory, application of such a system would be difficult due to the absence of the general co-existence and co-dependence known to occur in natural SRB mixed co-cultures. Also, due to the heavy emphasis on application in the present research study, it was important to identify and select the most functional biocatalyst. It was therefore decided to work with culture isolates from the anaerobic bioreactor component of the Rhodes BioSure Process® pilot plant which had the advantages of being locally available, extensively characterised and found to be effective and functional in application for wastewater treatment (Whittington-Jones, 2000; Bowker, 2002; Chauke, 2002).

Evaluation of a range of culture isolates would allow the selection of the culture with the best characteristics to be used in further investigations.

Therefore, the objectives of the investigation outlined in this chapter were:

- To evaluate and select a number of isolates from the anaerobic bioreactor component of the Rhodes BioSure Process® pilot plant.
- To evaluate the performance of the culture isolates in sulphate-reducing activity and identify and choose the culture isolate which demonstrated the best activity.
- To characterise the chosen culture isolate by microscopic examination.
- To ascertain the optimum growth media for the chosen culture isolate from selected SRB media.
- To evaluate the performance of the chosen culture isolate in multiple cycle and reuse experiments, which mimic the expected routine handling and movement of an immobilised culture through the electron donor and carbon source stages of the swing-back cycle.

2.2 Material and Methods

2.2.1 Chemical reagents and solutions

All experimental work was carried out using doubled distilled and de-ionised water (ddH_2O) or triple distilled de-ionised water ($dddH_2O$). All reagents were of an analytical grade and purchased from Merck, South Africa. Borosilicate glassware was used for all experiments after first washing, rinsing with 15 – 20 % nitric acid (HNO_3) and then rinsing with distilled, de-ionised water (dH_2O). Any pH adjustments were carried out using 1 M sodium hydroxide (NaOH) and 1 M hydrochloric acid (HCl) solutions as required

2.2.2 Microorganisms

A mixed culture of SRB was obtained from a sulphate-reducing bioreactor, a component of the Rhodes BioSure Process® pilot plant located at the Rhodes University Experimental Field Station. Using SRB-enrichment media and plating techniques, Chauke (2002) selected 35 isolates assumed to be pure cultures. Five culture isolates denoted A27, B2, C3, E28 and F7

were randomly selected from Chauke's 35 isolates and were maintained as stock cultures and used for all future studies as required.

2.2.3 Nutrient media

Four different SRB growth media were selected from Atlas (1993) and evaluated for cultivation of SRB cultures; Postgate B (Pg-B), modified Postgate C (Pg-C), sulphate-reducing bacteria medium (SRBM) and sulphate reducing medium (SRM). Media composition is reported in appendix 1.

2.2.4 Sulphate-reducing bacteria fed-batch experiments

Shaker flask studies were carried out in 250 mL Erlenmeyer conical flasks, and the experimental set up was as shown in figure 2.1. Cell generator (CG) reactors for generating biomass were 2 L and 3 L cylindrical vessels run as fed-batch systems with a 10 % (v/v) inoculum from the appropriate stock culture. Shaker flask and cell generator reactors were all wrapped in tin-foil to prevent the growth of photosynthetic organisms and were agitated at 100 revolutions per minute (rpm) on Labcon® shakers in a 30 °C and using a 3 hours ON and 1 hour OFF regime. During sampling reactor vessel headspace was sparged with nitrogen to maintain the anoxic environment. Growth performance was measured by various means including optical density (OD) readings at 600 nm. Other methods investigated for the measurement of growth performance include; most probable number (MPN), direct counts after staining using epifluorescence microscopy, dry weight determination and fluorescence measurements using a spectrofluorometer. Sulphate (SO_4^{-2}) was measured by high performance liquid chromatography (HPLC), using a Waters 510 HPLC with a 430 conductivity detector (see appendix 1 for sample preparation), or using a Merck Spectroquant-based method (see appendix 1 for sample preparation). Sulphide off-gas was collected in a zinc acetate trap and was measured using the Merck Spectroquant methylene blue method and sample reading on the Merck NOVA 60 Spectrophotometer (see appendix 1 for sample preparation). The pH was monitored daily using a Cyberscan 2500 pH meter. Alkalinity was determined by titration with sulphuric acid as described in appendix 1.

2.2.5 Gram-staining and Scanning Electron Microscopy

Samples were Gram-stained for photomicroscopy according to the procedure described by Murray *et al.*, (1994). Sample preparation for scanning electron microscopy (SEM) is described in appendix 2.

2.2.6 Multiple cycle and reuse of sulphate-reducing bacteria culture

The use of the SRB culture in successive multiple cycle and reuse experiments was undertaken by centrifuging at 5 000 rpm for 10 minutes using a Beckman Model J2-21 centrifuge. The pellet fraction was then resuspended in new medium to start another fed-batch flask reactor study. This procedure was repeated over a number of cycles.

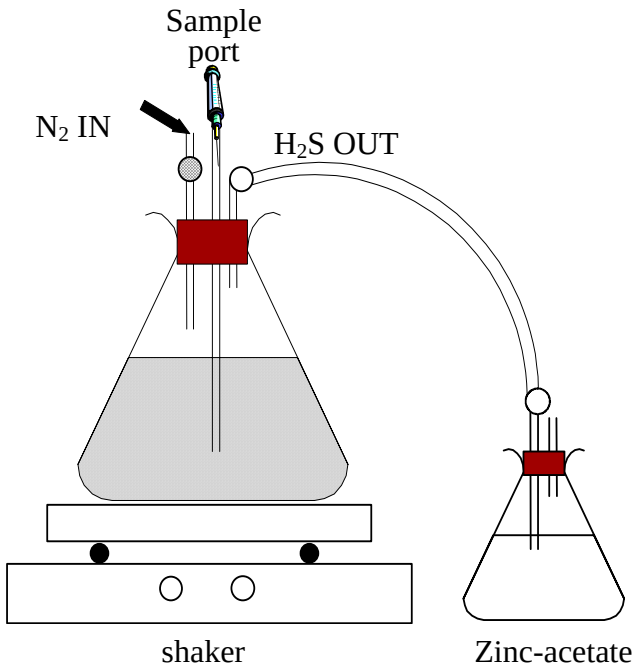

Figure 2.1: Configuration of shaker flask reactors used in the studies described.

2.3. Results and Discussion

Various methods were used to investigate and compare the growth characteristics and sulphate reduction activity of the five SRB culture isolates, A27, B2, C3, E28 and F7 in order to select the culture isolate which demonstrated the best characteristics for further study.

2.3.1 Growth and performance of sulphate-reducing bacterial culture isolates

Evaluation of the growth of the culture isolates is reported here in terms of OD readings at 600 nm. However, several other methods e.g. most probable number (MPN), direct counts after staining using epifluorescence microscopy, dry weight and protein determination and fluorescence measurements using a spectrofluorometer were attempted (results not included),

but these methods were found to be un-reliable and not reproducible. The performance of the culture isolates was evaluated as a function of their sulphate-reducing ability and subsequent sulphide generation. pH profiles were also generated as they give a good indication of the state of an SRB culture.

Results given in figure 2.2 of the change in OD readings at 600 nm for the five isolates report an average of two duplicate experiments.

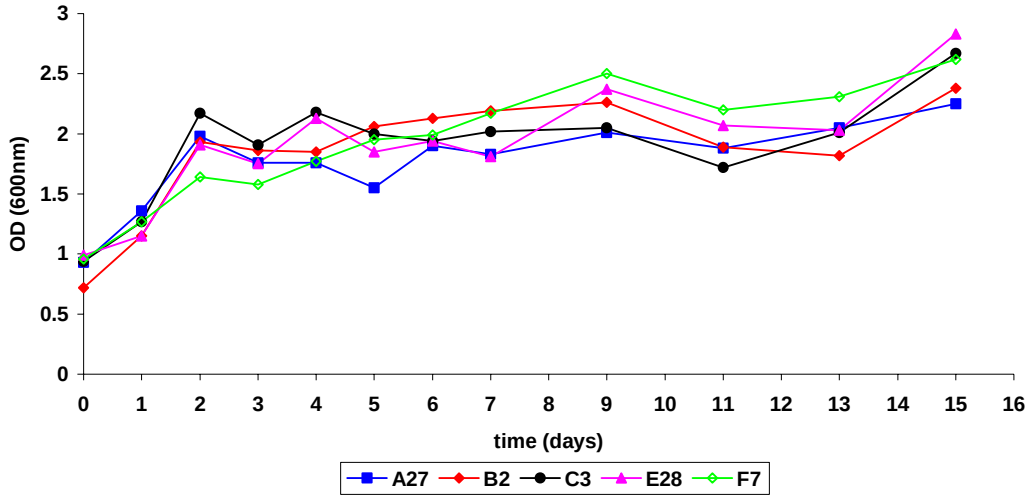

Figure 2.2: Change in optical density at 600 nm for different SRB isolates in fed-batch, 250 mL shaker flask reactors with Modified Postgate C medium.

In the presence of iron in the media, a black precipitate of iron sulphide was produced by SRB growth. Large serial dilutions were required in order to obtain a satisfactory spectrophotometer reading at 600 nm. This introduced a source of error, as did the presence of extracellular polymeric substances (EPS) produced by the SRB, which lead to aggregation and clumping of the SRB cells. The final OD reading on day 15 showed that isolate E28 had the highest OD reading at 600 nm of 2.83. The next best isolates, C3 had an OD reading of 2.67. Isolate A27 had the lowest OD reading at 600 nm of 2.25. Therefore, no major differences in the performance of the isolates were apparent from OD readings.

The results for the change in sulphate concentration and sulphide generation given in figure 2.3 and 2.4 respectively represent an average of two duplicate experiments. Rapid sulphate reduction was observed in the first 24 hours of the experiment with isolate E28 showing the

best sulphate reduction activity compared to the other isolates. Percentage sulphate reduction for isolate E28 was 15 % compared to 11 % for the next best isolate B2, and 2 % for the worst performing isolate F7, after 24 hours. Isolate E28 was found to maintain its comparatively higher sulphate reduction activity for the duration of the experiment.

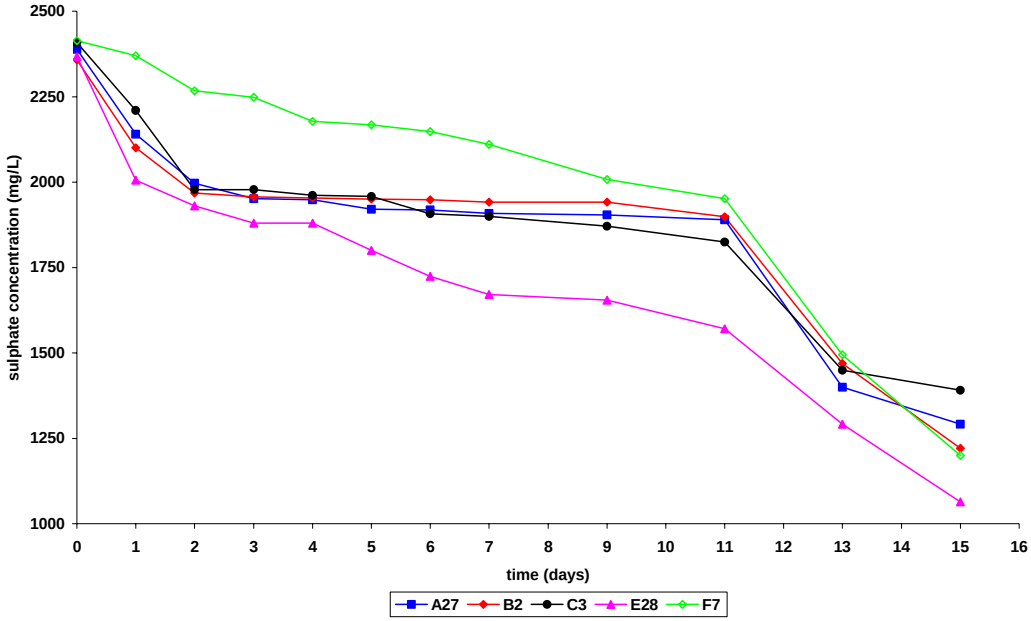

Figure 2.3: Sulphate reduction activity for different SRB isolates over time in fed-batch, 250 mL shaker flask reactors with Modified Postgate C medium.

Between day one and day 11, percentage sulphate reduction activity for isolate E28 was more than double that of the worst performing isolate (B2) at 22 and 10 % respectively and the percentage sulphate reduction of the next best isolate by day 11 was 18% for F7. At the end of the experiment, isolate E28 still showed the best sulphate reduction activity with a final average percentage removal of 55 % compared to 50 % for F7, the next best isolate, and 42 % for C3, the worst performing isolate. The biphasic trend for sulphate reduction observed for all samples may suggest a change in dominant species within the systems.

There was very little to no detectable sulphide generated in the reactors in the first seven days of the experiment (figure 2.4, sulphide detection lower limit ~ 0.02 mg/L). However, a rapid increase in sulphide generation was observed from day 11, which may correspond to the rapid increase in sulphate reduction observed over the same time period. The highest amount of sulphide generated by day 9 was found to be by isolate F7, at 6.0 mg/L compared to 2.4 mg/L for E28. However, from day 10 to the end of the experiment E28 maintained the highest amount of sulphide generation with a final sulphide concentration of 66 mg/L. This was

considerably higher than that generated by the next best sulphide generating isolate A27 of 51 mg/L, and the worst isolate B2 of 38 mg/L.

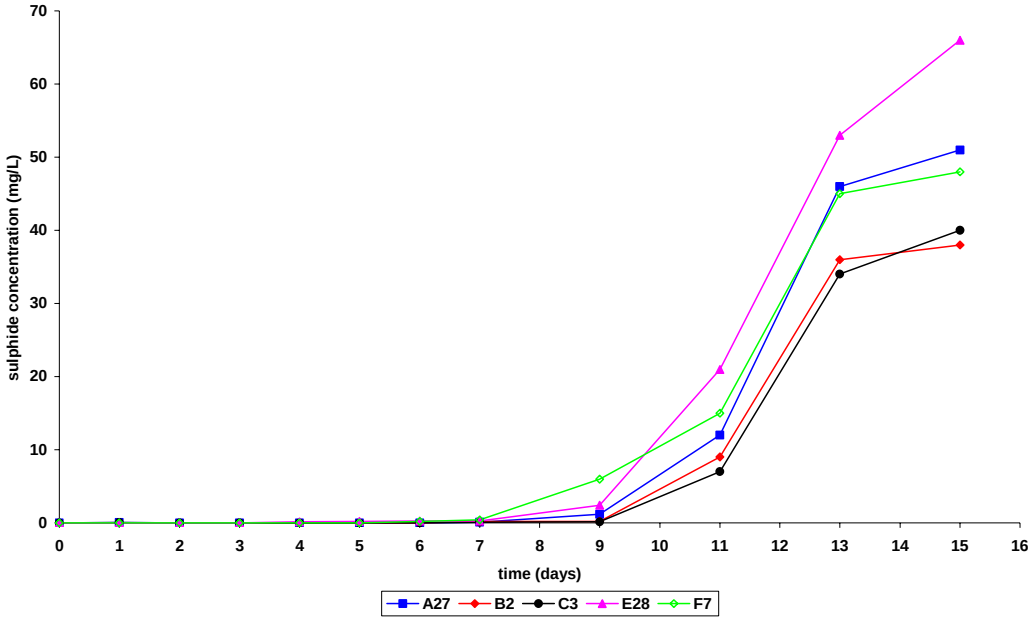

Figure 2.4: **S**ulphide generation for different SRB isolates in fed-batch, 250 mL shaker flask reactors.

The pH profiles for the SRB isolates over 15 days given in figure 2.5 are an average of two duplicate experiments. Little or no change in pH was observed over the first day, probably due to the very low initial pH, and also acclimatisation by the isolates. The pH values were then found to increase after day one for all isolates with the exception culture isolate F7, where the pH value was approximately 5.5 on day five, compared to pH values of 6.5 to 7.0 for the other isolates. There was no obvious explanation for this observation as the starting conditions for all the reactors were similar. It is worth noting that five days is generally not considered a long acclimatisation period for SRB, which are known to grow slowly (Visser, 1995). Over the next 10 days, the pH values of all isolates steadily increased to between 7.5 and 8.0. There were no large differences in the pH profiles to clearly distinguish the isolates, however, after day five, isolates A27 and E28's pH profiles were consistently higher than those for the other isolates.

The results from monitoring the pH of an SRB reactor gives a good indication of the status of the SRB culture within the reactor. Active SRB cultures are able to maintain the pH of their environment above 6.0 (Widdell, 1988).

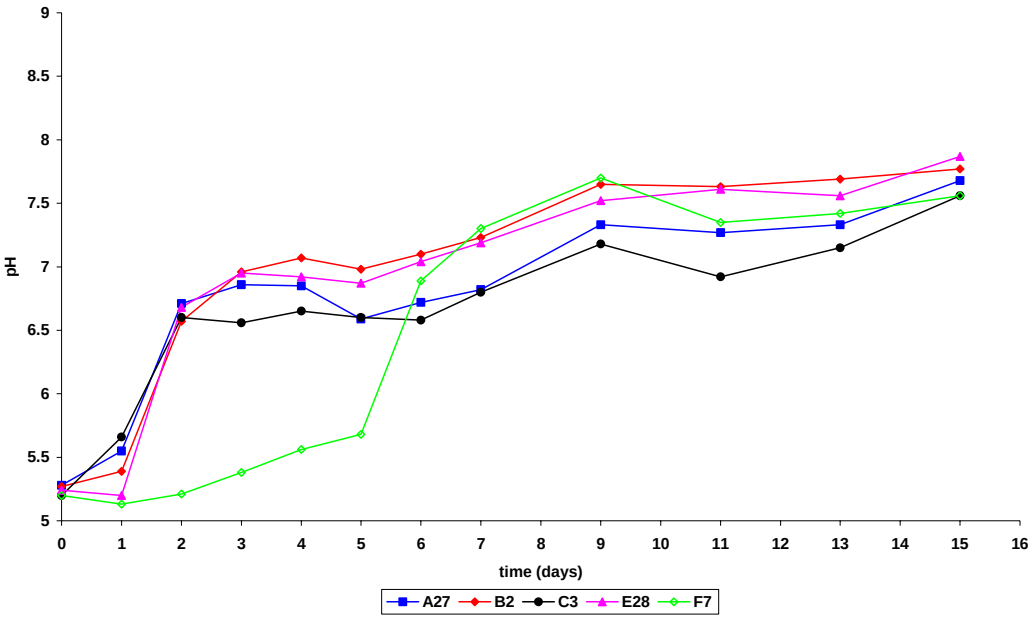

Figure 2.5: pH profile for different SRB isolates in fed-batch, 250 mL shaker flask reactors.

Table 2.1 gives the average sulphate reduction and sulphide generation rates for all the culture isolates over the duration of the experiment (15 days) and compares these to the average rates over day 11 to day 15 (4 days), where the most rapid changes in sulphate reduction and sulphide generation were observed (see figures 2.3 and 2.4)

Table 2.1: Average rates of sulphate reduction and sulphide generation for SRB isolates

	Average sulphate reduction rate (mg SO_4^{2-}/L/day)					**Average sulphide generation rate (mg HS^-/L/day)**				
Isolate	**A27**	**B2**	**C3**	**E28**	**F7**	**A27**	**B2**	**C3**	**E28**	**F7**
Day 0 to 15 (total)	73	76	68	87	81	3.4	2.5	2.7	4.4	3.2
Day 11 to 15(most rapid)	473	475	456	393	488	9.8	7.3	8.3	11.3	8.3

During days 11 to 15, the period of the most rapid activity, the sulphate reduction rate for isolate E28 was lower at 393 mg SO_4^{2-}/L/day, compared to the highest reduction rate of 488 mg SO_4^{2-}/L/day for isolate F7. However isolate E28 showed the highest sulphide generation rate during the full period of 11.3 mg HS^-/L/day compared to 9.8 mg HS^-/L/day for isolate A27 with the next highest rate of sulphide generation.

Overall, isolate E28 showed the highest average sulphate reduction and sulphide generation rates of 87 mg SO_4^{2-}/L/day and 4.4 mg HS^-/L/day compared to 81 mg SO_4^{2-}/L/day and 3.2 mg HS^-/L/day for isolate F7, the next best isolate, and compared to 76 mg SO_4^{2-}/L/day and 2.5 mg HS^-/L/day for B2, which was the worst performing isolate.

While the differences in the average sulphate reduction and sulphide generation rates between the isolates are not very large, overall, isolate E28 showed superior growth and performance characteristics compared to the other four culture isolates with 55 % sulphate reduction. Therefore, isolate E28 was selected as the culture isolate of choice for all subsequent experiments.

2.3.2 Microscopy of isolate E28

As isolate E28 was chosen for all subsequent experiments, microscopy work was undertaken to investigate the morphology and Gram-staining characteristic of the isolate. Figure 2.6 is a photograph of Gram-stained isolate E28 (a) and an SEM micrograph of isolate E28 (b).

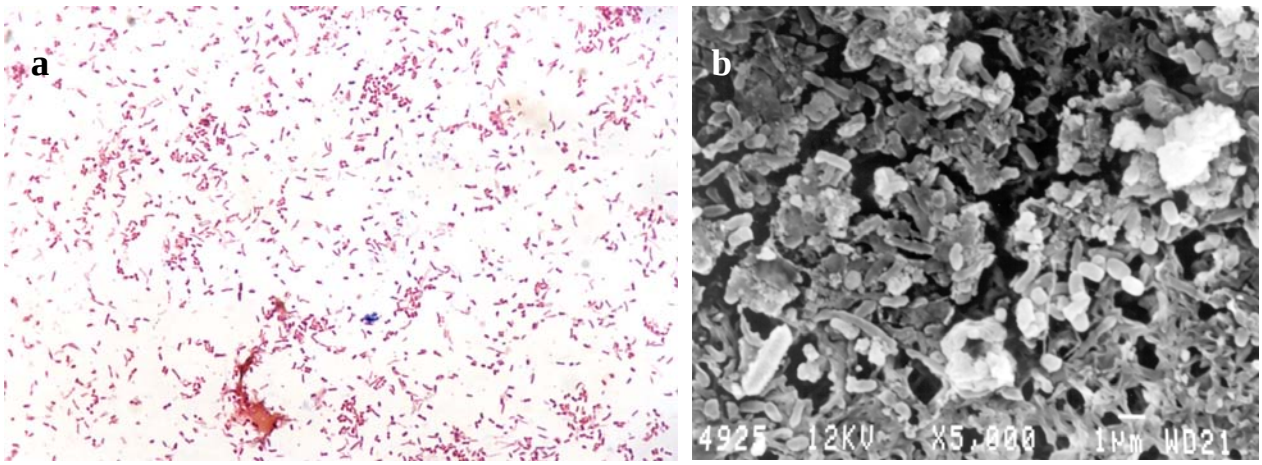

Figure 2.6: a) Photograph of Gram-stained and **b)** SEM micrograph of SRB isolate E28.

It was clear from figure 2.6 that isolate E28 was a mixed co-culture, as morphologically different bacteria were present on the photograph and micrograph. The majority of the bacteria comprising E28 were found to be Gram-negative rods, however, some gram-negative cocci and/or ovoid bacteria present these were observed more clearly on the SEM micrograph.

The culture E28 was plated onto agar plates and single colonies were picked off the plate and Gram-stained in an attempt to ascertain the different morphologies of the bacteria comprising culture E28. The 3 photographs in figure 2.7 below shows the results of the Gram-stained representative bacteria of culture E28.

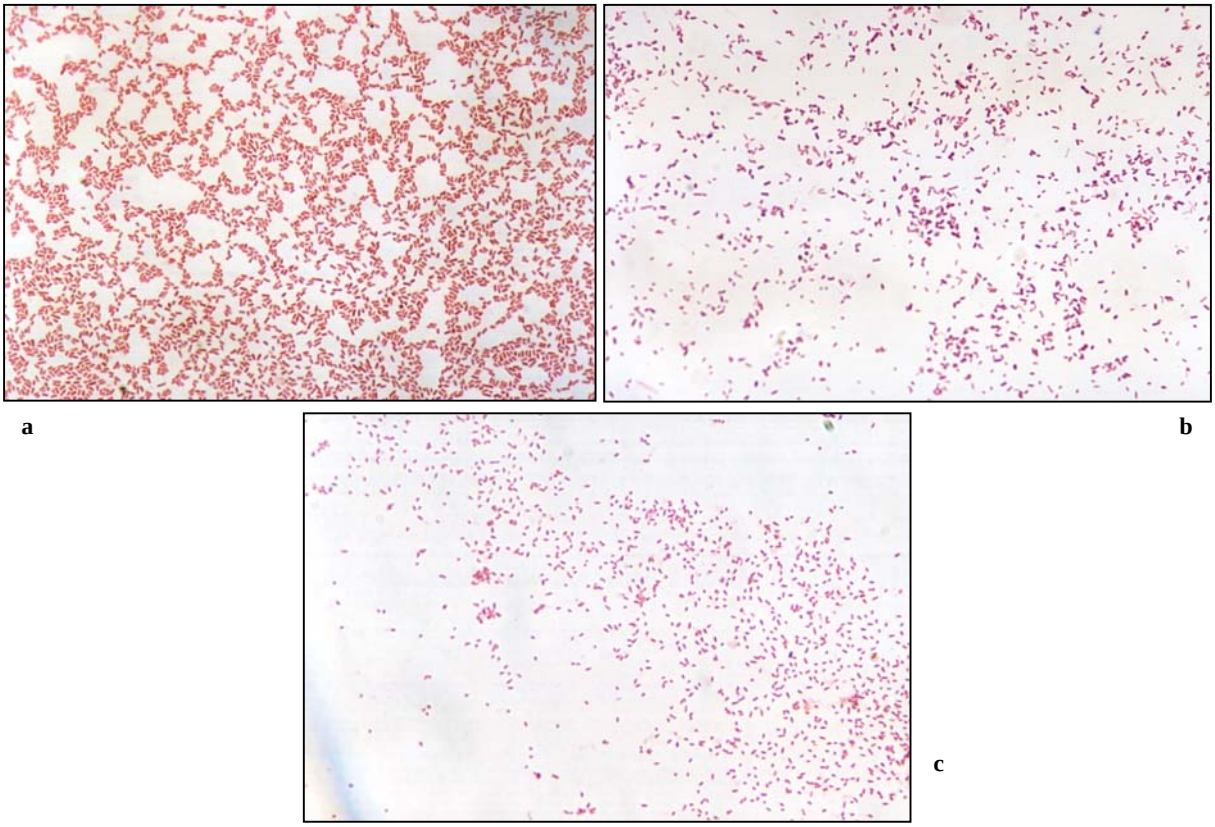

Figure 2.7: Gram-stained bacteria comprising isolate E28. **a** = large gram-negative rods, **b** = short, gram-negative ovoid bacteria and **c** = gram negative ovoid/cocci

Culture E28 was found to comprise two or three morphologically different anaerobic bacteria. Unpublished 16S RNA molecular work on culture E28 by Chauke (2002) identified the SRB component on the mixed co-culture as a *Desulfovibrio* species.

Given that culture E28 was found to be a stable and functionally superior culture in its performance compared to the other isolates that were evaluated, its mixed status was not seen to be a problem as long as it continued to function as a stable co-culture during immobilisation and subsequent manipulation. This would need to be determined during the course of subsequent studies.

2.3.3 Evaluation of optimal growth media for culture E28

Four different SRB media were selected from Atlas (1993) for evaluation in order to determine the media that provided the best support for the growth of the selected culture E28. The four media chosen were Postgate B (Pg-B), Postgate C (Pg-C), sulphate reducing medium (SRM) and sulphate reducing bacteria medium (SRBM). There are several differences in the compositions of these four media as outlined in appendix 1.

Media evaluation experiments were carried out in 250 mL flask reactors, with the set up as previously described. The results report on the sulphate reduction, sulphide and alkalinity generation and the changes in pH with the use of different media. All the results are an average of 3 separate experiments.

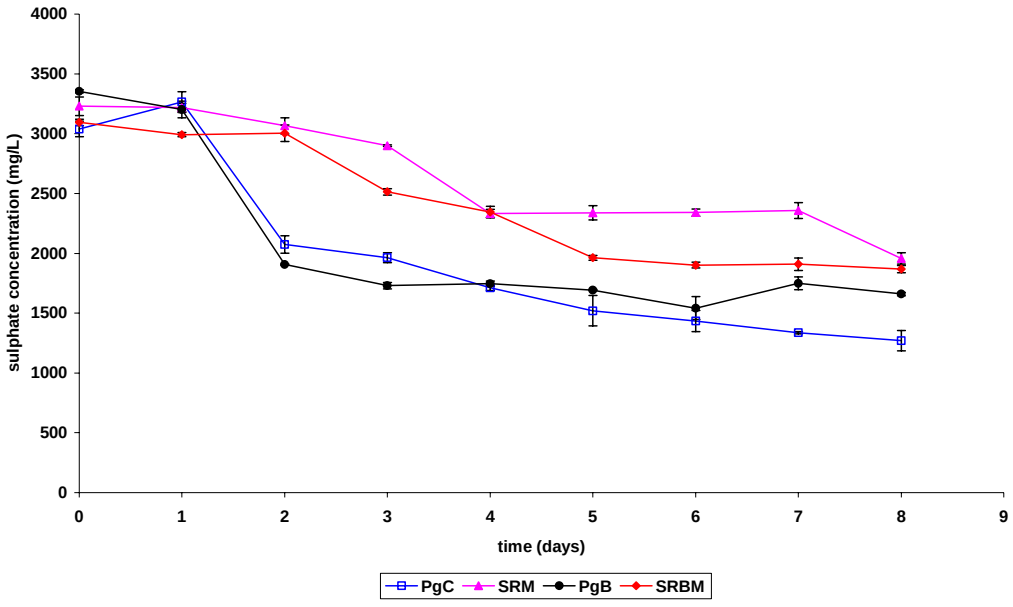

Figure 2.8: Sulphate reduction over time by SRB growing in different media. Fed-batch, 250 mL shaker flask reactors.

Figure 2.8 shows the profiles for sulphate reduction for the four different media over eight days. The sulphate reduction profiles for the reactors Pg-B and Pg-C were similar throughout the experiment. Percentage sulphate reduction after two days was greatest for Pg-B at 43 % and Pg-C had the next highest percentage sulphate reduction of 31 %. The other two media, SRM and SRBM showed considerably lower percentage sulphate reduction values of 5 and 3 % respectively after two days. After day four and for the remainder of the experiment, Pg-C showed the best sulphate reduction compared to the other three media. At the end of the experiment on day eight percentage reduction for Pg-C was 58 %, with the next best media observed to be Pg-B at 51 %. The percentage sulphate reduction for SRM and SRB were still considerably lower at 39 and 40 % respectively.

The profiles for sulphide generation for the four media are shown in figure 2.9. After day two, the sulphide generated in the Pg-C reactor of 306 mg/L was more than double that of the reactor, SRM, with the lowest sulphide generation at 147 mg/L. Sulphide generated in the Pg-B and SRBM reactors after day 2 was similar at 293 and 263 mg/L respectively. A considerable difference in the amount of sulphide generated on day eight between the media reactor with the best sulphide generation, Pg-C at 340 mg/L and that with the lowest amount of sulphide generated, SRM at 185 mg/L was observed. Although sulphide generation by the next best performer, SRBM at 330 mg/L was comparable to that of Pg-C, overall activity of the Pg-C reactor was found to be better.

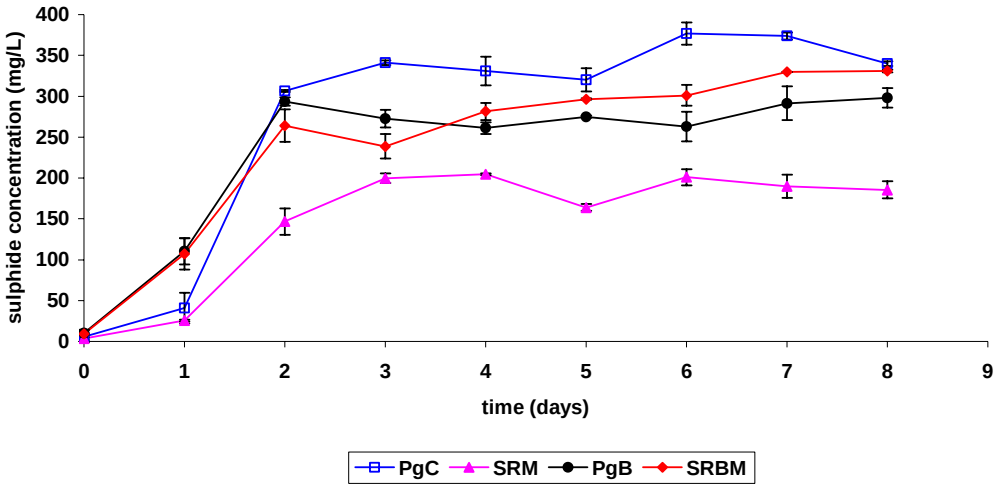

Figure 2.9: Sulphide generation for SRB growing in different media. Fed-batch, 250 mL shaker flask reactors.

The generation of alkalinity (figure 2.10) was observed to occur rapidly in all four reactor systems, reaching equilibrium after two to three days. Modified Pg-C media showed the highest alkalinity generation from day two of approximately 2690 mg $CaCO_3$/L, the next highest was that of the Pg-B reactor of approximately 2320 mg $CaCO_3$/L. In comparison, the amounts of alkalinity generated by SRBM and SRM reactors after day two were 2225 and 2160 mg $CaCO_3$/L. The average equilibrium alkalinity generated calculated from day three to day eight showed that the Pg-C reactor reached the highest equilibrium at approximately 2700 mg $CaCO_3$/L, compared to 2470, 2200 and 2370 mg $CaCO_3$/L for Pg-B, SRBM and SRM respectively.

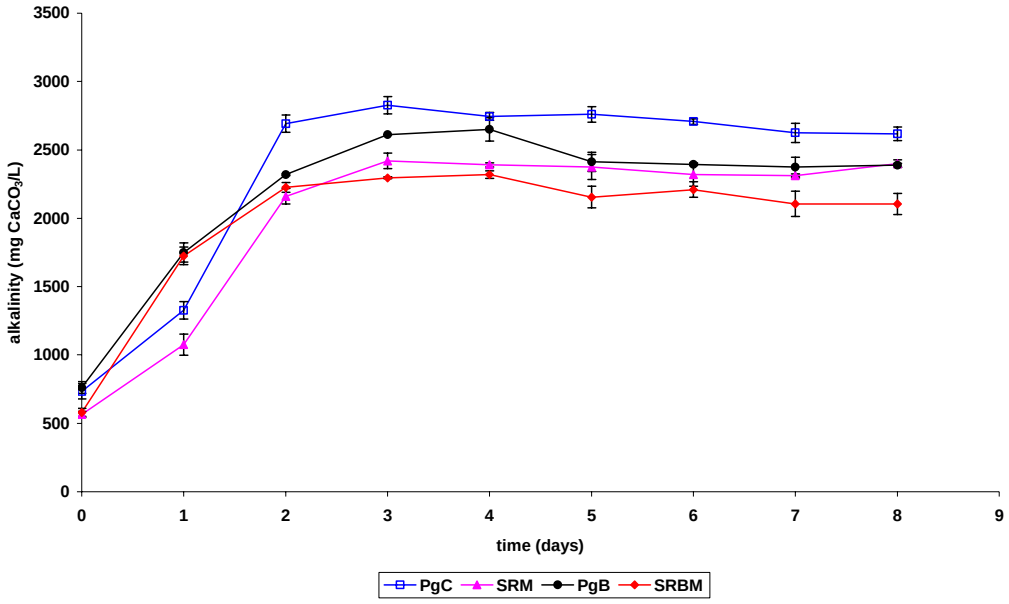

Figure 2.10: Alkalinity generation by SRB growing in different media. Fed-batch, 250 mL shaker flask reactors.

The pH profiles for the four different media are shown in figure 2.11. No large differences were observed in the pH profiles of all four reactors with the different media. The general trend for all the curves was very similar with all systems maintaining the pH between 6.5 – 8.0 throughout the experiment.

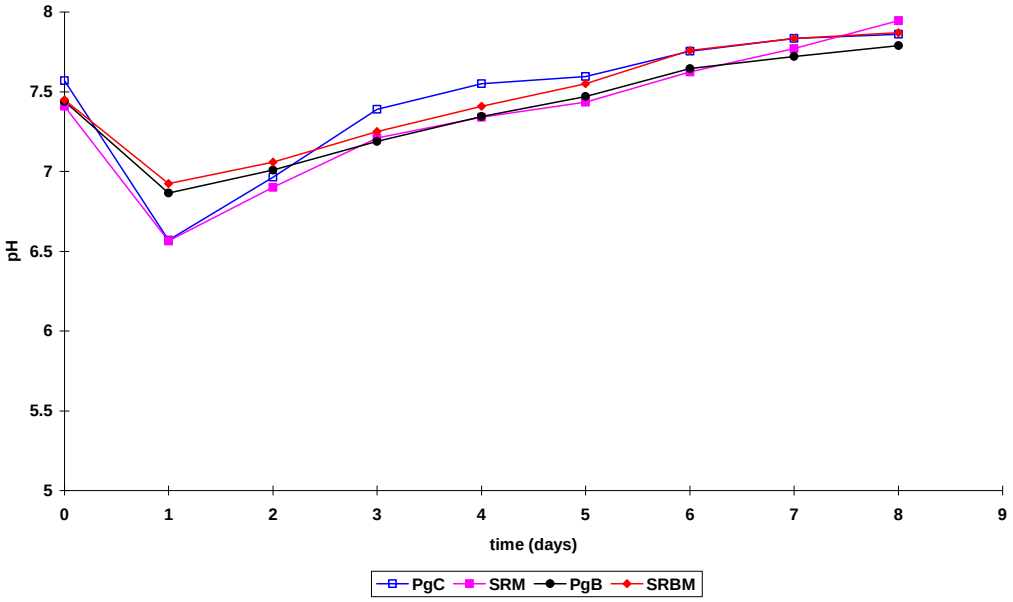

Figure 2.11: pH profiles of SRB in different media. Fed-batch, 250 mL shaker flask reactors.

A summary of the average sulphate reduction and sulphide generation rates is given in table 2.2. It was apparent from the results given in table 2.2 that Pg-C provided the best support for the cultivation of culture E28. The rate of sulphate reduction was 221 mg SO_4^{2-}/L/day compared to 211 mg SO_4^{2-}/L/day for Pg-b which gave the next best sulphate reduction rate, with SRM and SRBM giving similar but comparatively much lower sulphate reduction rates of 159 and 153 mg SO_4^{2-}/L/day respectively.

Table 2.2: Average sulphate reduction and sulphide generation rates in different media

	Sulphate reduction rate (mg SO_4^{2-}/L/day)				**Rate of sulphide generation (mg HS^-/L/day)**			
Media	**Pg-C**	**SRM**	**Pg-B**	**SRBM**	**Pg-C**	**SRM**	**Pg-B**	**SRBM**
mean over 8 days	221	159	211	153	42	23	36	40

Although the sulphide generation rate for Pg-C of 42 mg HS^-/L/day was not much higher than the next best media, SRBM at 40 mg HS^-/L/day, the results as a whole supported the

conclusion that Pg-C was the best of the four media evaluated for cultivation of culture E28. Modified Pg-C was therefore used in all subsequent experiments.

2.3.4 Multiple cycle and reuse of sulphate-reducing bacterial culture E28

Successful application of SRB in the bioprocess systems envisaged required that the culture be robust and versatile, as most systems would involve routine start-up and shut-down periods where biomass would shift from one mode of reactor operation to another. This would include the ability of the culture to survive short periods of exposure to air and when returned to an anoxic environment, quickly re-establish sulphate reduction activity.

Shaker flasks reactors with a set amount of biomass wet mass were set up in order to evaluate the performance of culture E28 in multiple cycle and reuse experiments. Initial experiments passed the biomass through three cycles of reaction, spin down, biomass washing, resuspension and reaction once again. Sulphate reduction, sulphide determination and pH were determined. A subsequent set of multiple cycle and reuse shaker flasks were set up and this time run over six cycles to evaluate the resilience of culture E28, and determine if there were any trends that were established over a longer time period of operation. Sulphate reduction, sulphide generation, alkalinity generation and pH were determined over the six cycles. Results for multiple cycle and reuse experiments over three cycles shown in figures 2.12 and 2.13 are averages of three separate experiments.

Figure 2.12 shows the sulphate reduction and sulphide generation profiles of the initial multiple cycle and reuse experiments over three cycles. All three cycles show rapid sulphate reduction in the first two to three days. The average rates of sulphate reduction during the periods of rapid sulphate reduction were 530, 256 and 341 mg SO_4^{2-}/L/day and the overall rates of sulphate reduction were 180, 54 and 125 mg SO_4^{2-}/L/day for cycle one, two and three respectively. The average percentage sulphate reduction for cycles one, two and three respectively were 67, 48 and 47 %. There was no definite overall trend observed over the three cycles for sulphate reduction over the three cycles. Although there was a decrease in the average percentage sulphate reduction from the first to the second cycle it appears to remain steady in the third cycle at approximately 47.5 %.

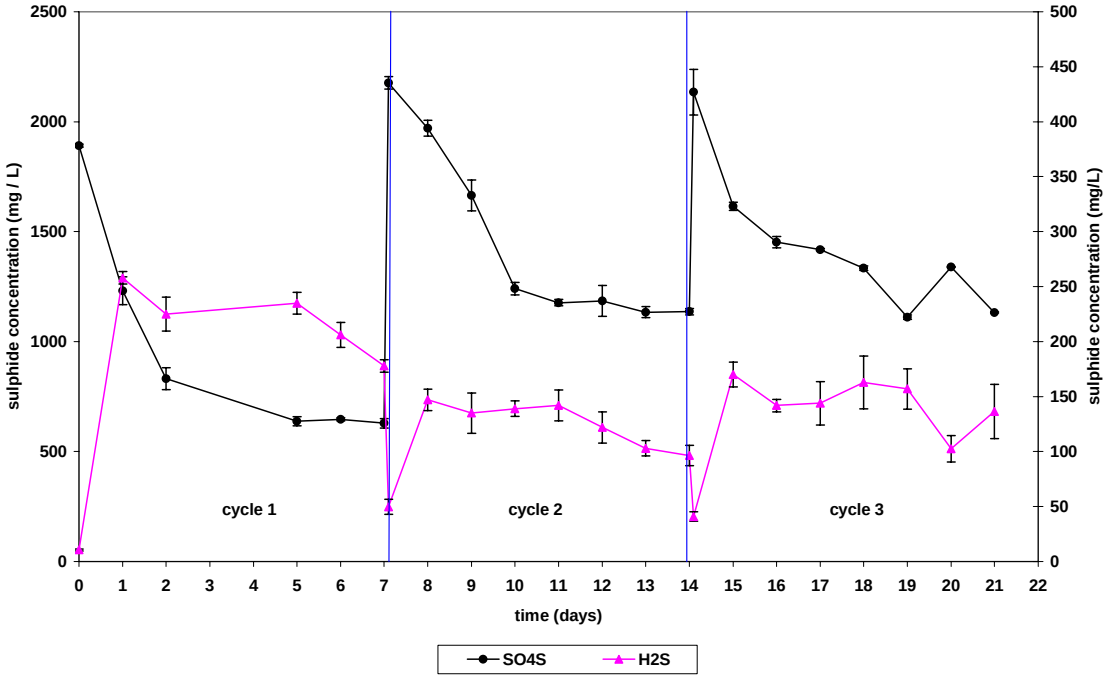

Figure 2.12: Sulphate reduction and sulphide generation for 500 mL, fed-batch shaker flask reactor experiments over 3 cycles.

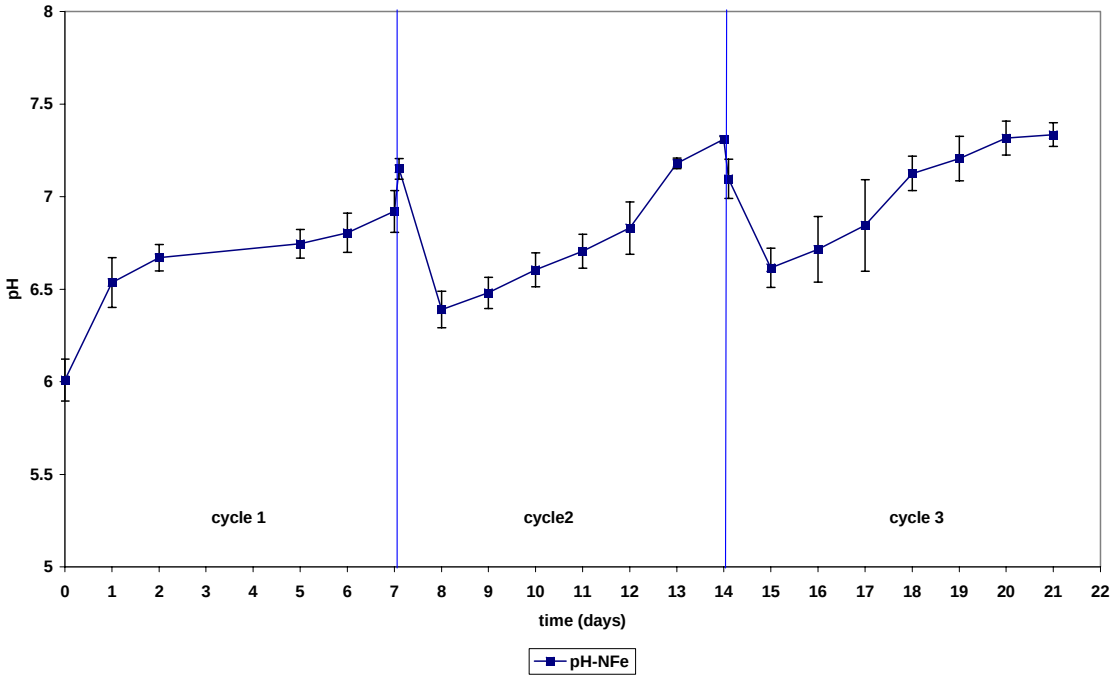

Figure 2.12: pH profile for 500 mL, fed-batch shaker flask reactor experiments over 3 cycles.

Sulphide generation over the three cycles reflected the trends observed with sulphate reduction. The maximum amount of sulphide generated over the three cycles was approximately 250, 140 and 170 mg/L for cycles one, two and three respectively, again, no obvious trend for sulphide generation over the three cycles was observed.

The pH profile for the three cycles of the first multiple cycle and reuse performance evaluation experiment is shown in figure 2.13. Each cycle showed the expected trend of increase in pH with time. In each cycle the culture was able to maintain the pH above 6.0 and at the end of each cycle, the pH was approximately 7.0.

The results for multiple cycle and reuse experiments over six cycles are shown in figures 2.14 and 2.15. These results show the averages of two duplicate experiments.

Figure 2.14 shows that the sulphate reduction capacity of the SRB culture was maintained throughout the six cycles over 42 days. The fastest rate of sulphate reduction in each cycle occurred within the first three days (results in table 2.3) followed by a steady decrease in sulphate reduction for the rest of the cycle. Average percentage sulphate reduction during the period of maximum sulphate reduction rate for cycles one to six consecutively were 53, 41, 38, 59 74 and 73 % respectively. Sulphate reduction activity appears to improve after the first three cycles. In the last two cycles, the initial sulphate concentrations were increased to approximately 2400 mg/L, (compared to approximately 1500 mg/L in the first four cycles) to determine the effect of increased initial sulphate concentration in multiple cycle and reuse of culture E28. The average percentage sulphate removal during the period of most rapid sulphate reduction rate were found to be higher, at 74 and 75 %, in the last two cycles compared to those determined for the first four cycles. The last 3 cycles showed that the sulphate concentration in solution ended up below the 500 mg/L mark, which was not the case for the first three cycles.

There was corresponding high sulphide production at the early stages of each cycle (figure 2.15). The maximum sulphide concentrations for cycles one to six consecutively were 262, 153, 161, 265, 196 and 199 mg/L. Sulphide concentrations were found to decrease rapidly following a decrease in the rate of sulphate reduction in the first one to two days.

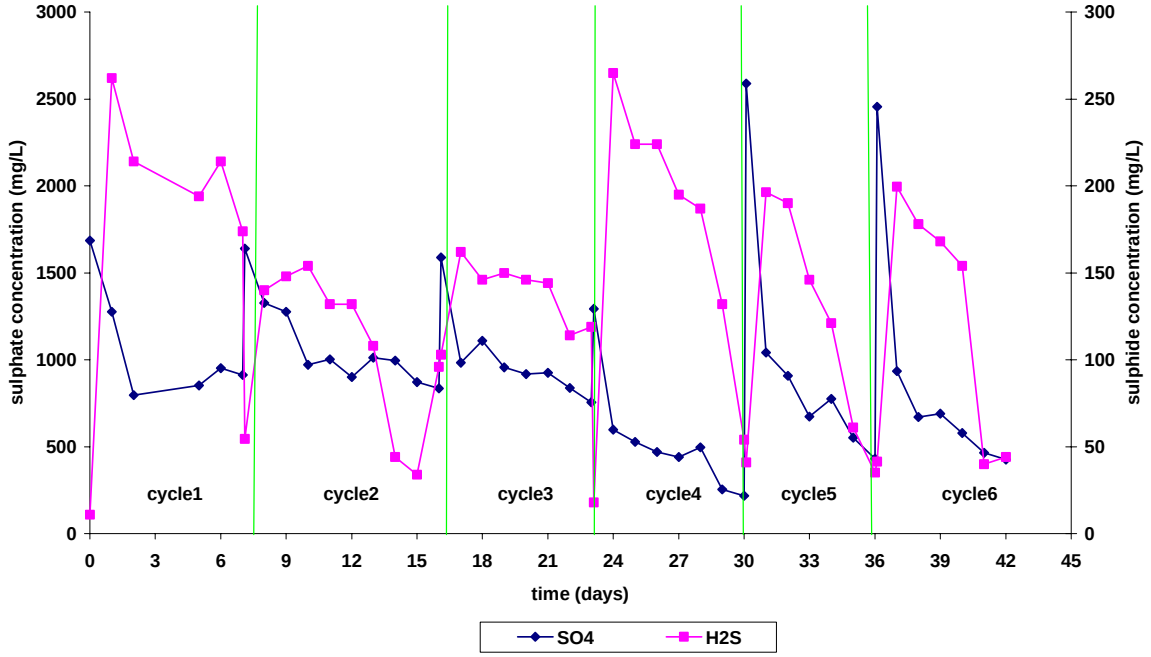

Figure 2.14: Sulphate reduction and sulphide generation in 500 mL, fed-batch shaker flask reactor experiments over 6 cycles.

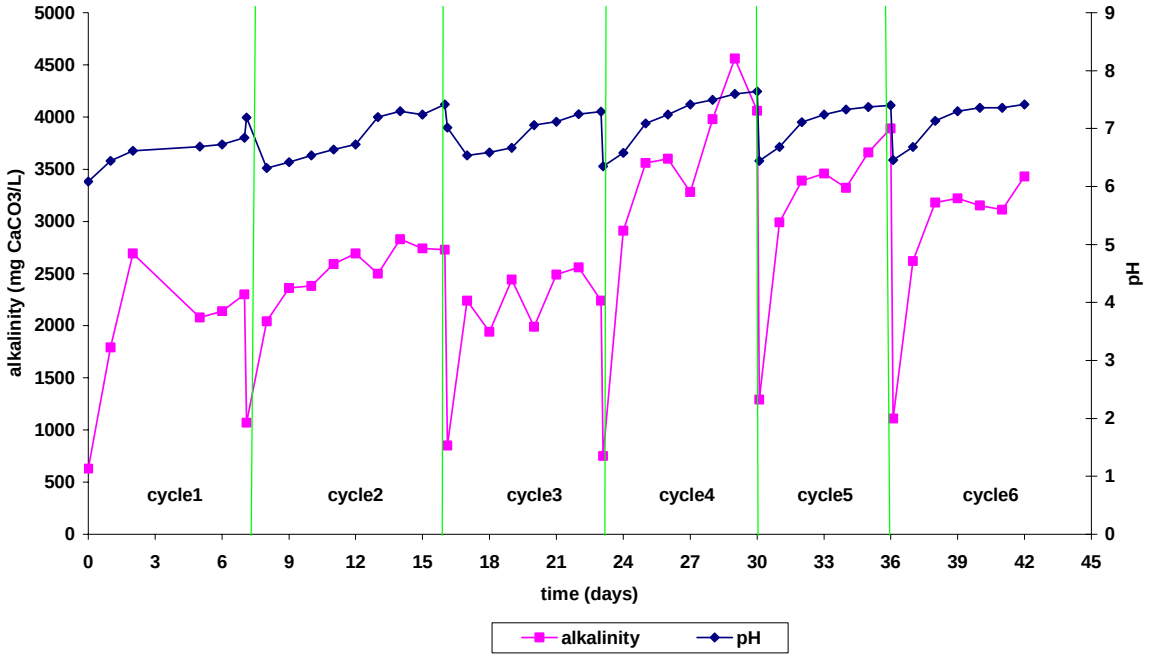

Figure 2.15: pH profile and alkalinity generated in 500 mL, fed-batch shaker flask reactor experiments over 6 cycles.

Figure 2.15 shows the pH profile and alkalinity generation profile for the multiple cycle and reuse of culture E28 over the six cycles and 42 days. In each cycle there is the gradual increase in pH and the corresponding increase in alkalinity generated. In the first three cycles, the amount of alkalinity generated reaches equilibrium of approximately 2500 mg $CaCO_3$/L, values comparable to those observed for the culture E28 in different media (see figure 2.10). Alkalinity generation for the last three cycles was found to be comparatively greater than that for the first three cycles, with an average equilibrium of approximately 3250 mg $CaCO_3$/L. The trend of gradual increase in pH and alkalinity is similar for all six cycles; increase in pH is a good indication of a healthy SRB system.

The rates of sulphate reduction in the first two or three days of each cycle where reduction was observed to be fastest (average maximum sulphate reduction rate) and the total sulphate reduction rate for each cycle over the six or seven days (average total sulphate reduction rate) are given in table 2.3. The average total percentage sulphate removal is also given in table 2.3.

Table 2.3: Rates of sulphate reduction and percentage sulphate removal over 6 cycles

	Cycle					
	1	**2**	**3**	**4**	**5**	**6**
Average total sulphate reduction rate (mg SO_4^{2-}/L/day)	110	115	104	134	360	338
Average maximum sulphate reduction rate: day 1-3 (mg SO_4^{2-}/L/day)	445	222	304	383	638	893
Percentage sulphate removal (%)	46	49	52	83	83	83

The average total sulphate reduction rate determined for cycle six was 338 mg SO_4^{2-}/L/day compared to 110 mg SO_4^{2-}/L/day for cycle one, a three fold increase in the average rate of sulphate reduction from cycle one to cycle six. The average maximum rate of sulphate reduction over the first three days in cycle six was approximately double that determined for cycle one over the same period. Percentage sulphate removal in cycles 4, 5 and 6 were all almost double the percentage determined in cycle one (see table 2.3).

The data in table 2.3 suggests that the sulphate reduction activity of the SRB culture E28 improves over the six cycles. This is could be due to an increase in biomass concentration, but may also be as a result of a more acclimatised and therefore more robust SRB culture. Although the initial sulphate concentration at the start of cycles 5 and 6 (figure 2.15) were higher that for the first 4 cycles, a larger percentage change in sulphate concentration was observed in cylces 5 and 6.

The results on the multiple cycle and reuse performance of culture E28 showed that the selected culture was both robust and versatile over at least six cycles in 42 days. The consortium reproduced reliably which indicated the stability of the co-culture and suggested that the E28 consortium was a suitable candidate for future immobilisation experiments.

2.3.5 Activity of biomass generating reactors

At this point, a suitable culture (SRB consortium E28) had been selected; the media for its optimum growth identified and its sulphate-reducing performance had been evaluated. The robust and versatile nature of the SRB consortium E28 had been investigated and ascertained in multiple cycle and reuse experiments, the next point that needed to be addressed was the production of the biomass in sufficient quantities for the manufacture of immobilisation systems. Therefore, cell-generating (CG) reactors were set up and their sulphate-reducing activity and biomass generating ability investigated.

A 10 L SRB reactor inoculated with the SRB consortium E28 was set up in a round flat-bottom flask and was used as the stock culture for the inoculation of all subsequent CG reactors. The CG reactors were set up in 2 L and 3 L cylindrical glass containers covered with tin foil to eliminate the possible growth of photosynthetic organisms. The CG reactors were incubated at 30 °C with agitation at 100 rpm using a three hours ON and one hour OFF regime.

Figures 2.16 and 2.17 show representative sulphate-reducing activity results for the 2 L and 3 L reactors respectively. The graphs generated in figure 2.16 and 2.17 are averages of three separate experiments. Sulphate reduction, sulphide generation and pH measurements were used to evaluate the activity of the cell-generating reactors. The reactors had new media added when the rate of sulphate reduction was observed to have slowed down.

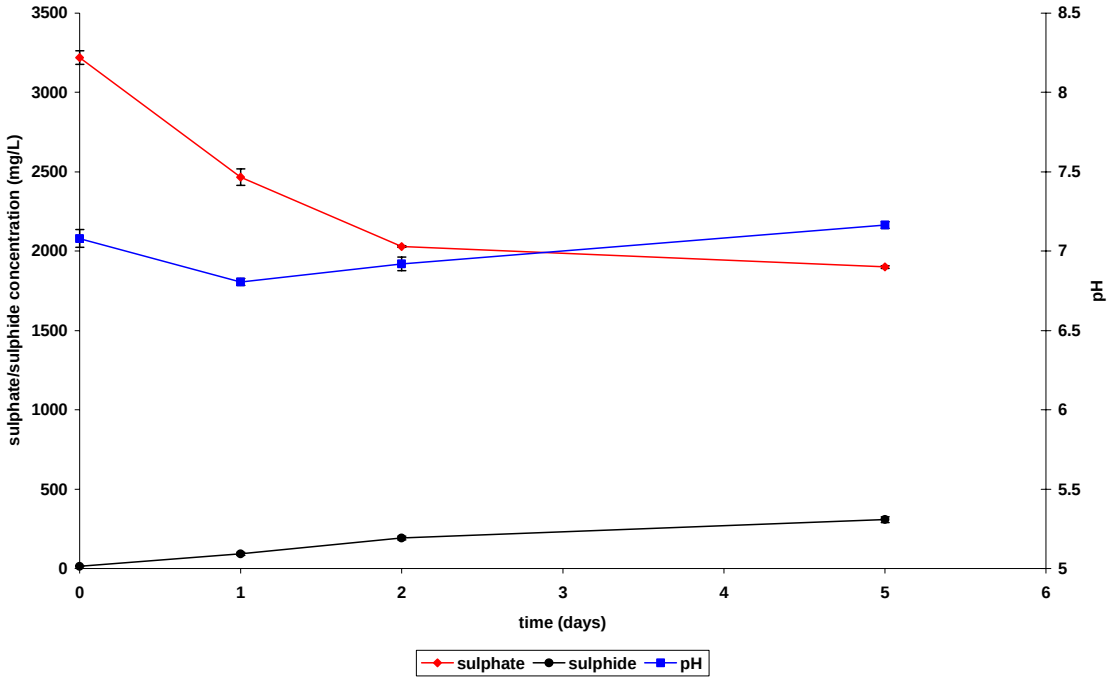

Figure 2.16: Sulphate reduction, sulphide generation and pH profile for a 2 L fed-batch, cell-generating reactor.

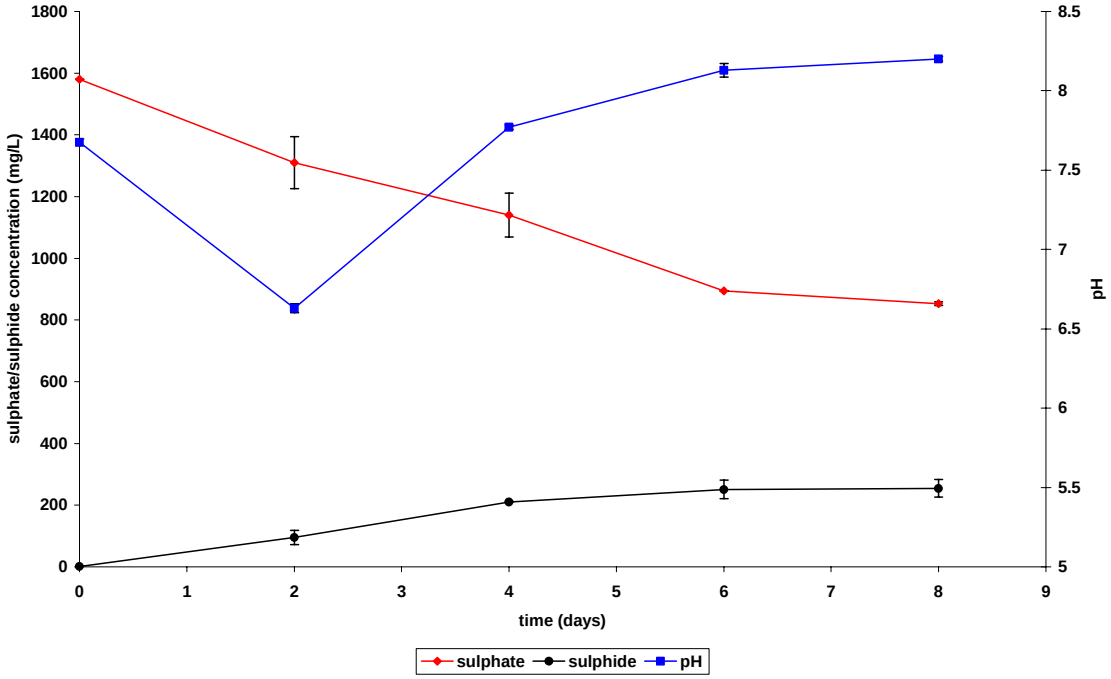

Figure 2.17: Sulphate reduction, sulphide generation and pH profile for a 3 L fed-batch, cell-generating reactor.

The sulphate reduction and sulphide generation profiles for the different reactors were found to be different despite similar start-up conditions. However, it was found that with all the reactors, activity was observed within a day of start-up, suggesting good adaptation of the SRB to the system. Maximum sulphide production in the 2 L reactors was observed to reach as high as 308 mg/L and 275 mg/L in the 3 L reactors. There was no apparent sulphide (H_2S) inhibition of the systems, probably due to the daily sparging of the reactor headspace with nitrogen during sample collection. The average maximum rates of sulphate reduction rates were 595 and 114 mg SO_4^{2-}/L/day and the average total sulphate reduction rates were 263 and76 mg SO_4^{2-}/L/day for the 2 and 3 L CG reactors respectively

Table 2.4 below provides a summary of the results for some of the cell-generating reactors. The average rate of sulphate reduction and sulphide production was found to be higher in the 2 L reactors when compared to the 3 L reactors. However, the differences in the amount of biomass produced (wet mass) were not as large as would have been expected from the more active 2 L reactors.

Table 2.4: Cell generators, rates of sulphate reduction, sulphide and biomass production

	Reactor volume size (L)	**Average rate of SO_4^{2-} reduction (mg/L/day)**	**Average rate of H_2S production (mg/L/day)**	**Wet mass (g) After (number) of days**
CG1	2	107	85	5.2 (5)
CG2	2	267	56	5.1 (5)
CG3	2	131	23	5.7 (7)
CG4	3	46	27	6.9 (8)
CG5	3	90	29	6.9 (8)
CG6	3	91	34	6.3 (8)

Results from CG reactors showed that rapid activity can be obtained and that it was possible to generate sufficient active biomass that could be used for the manufacture of immobilisation systems for further research.

2.4 Conclusion

Initially five SRB culture isolates from the anaerobic component of the Rhodes BioSure Prosess® pilot plant were evaluated in order to select the isolate with the best growth characteristics and best sulphate-reducing activity. The SRB consortium E28 was found to perform better than the other 4 culture isolates, showing the best growth as determined by OD readings at 600 nm. Sulphate reduction activity of SRB consortium E28 was also comparatively better than the other 4 isolates with the highest average sulphate reduction and sulphide generation rates of 87 mg SO_4^{2-}/L/day and 4.4 mg HS^-/L/day compared to 81 mg SO_4^{2-}/L/day and 3.2 mg HS^-/L/day for isolate F7, the next best isolate. The SRB consortium E28 was therefore chosen for all subsequent experiments.

Microscopy work showed that E28 was a mixed co-culture mostly comprising Gram-negative rods. Although the advantages of working with a single culture in laboratory experiments were noted and appreciated, a decision was made to work with the mixed culture E28 due to the emphasis of the research study on application and also due to the superior performance observed for this SRB consortium. Therefore, no attempt was made to purify SRB consortium E28 as it gave the desired characteristics, showing the highest activity. The SRB consortium E28 was found to be robust and versatile, able to withstand periods of exposure to air and centrifugation between three and six cycles for 21 and 42 days respectively and maintain good sulphate reduction activity with short start-up periods throughout multiple cycle and reuse.

In comparison to four other SRB media, Pg-C medium was found to provide the best nutritional support for the cultivation of SRB consortium E28. The best sulphate reduction activity by SRB consortium E28 was shown to occur in Pg-C medium in which the average sulphate reduction rate was 221 mg SO_4^{2-}/L/day with Pg-B medium providing the next best sulphate reduction rate of 211 mg SO_4^{2-}/L/day. Therefore Pg-C medium was used for all subsequent experiments.

Multiple cycle and reuse experiments simulated the operating regime in the proposed swing-back cycle in immobilised systems where routine handling of the biomass would move it

between electron donor and carbon source. Selection of a robust and versatile culture was therefore of great importance. The multiple cycle and reuse studies showed SRB consortium E28 to be a very robust and versatile culture, able to undergo at least six reuse cycles in 42 days with the sulphate activity showing a threefold increase in the sulphate reduction from cycle one to cycle six.

Production of cell biomass in cell generating reactors was found to be possible and that sufficient wet mass of SRB consortium E28 (2.2 – 2.6 g/L) could be generated in five to seven days.

Following the selection and performance evaluation of a suitable culture isolate and determining its robustness in multiple cycle and reuse, evaluating the immobilisation potential of the biomass for application in the proposed process was the next point to be addressed.

CHAPTER 3
IMMOBILISATION OF THE SULPHATE-REDUCING BACTERIAL CONSORTIUM E28

3.1. Introduction

The major disadvantage associated with the utilisation of slow growing, free suspended cells of SRB in continuous bioprocess reactor systems is the loss of biomass through wash-out in the effluent. The result of biomass loss in such reactor systems is decreased volumetric productivity (Kierstan and Coughlan, 1985; Boopathy *et al.*, 1998a; Park and Chang, 2000).

Immobilisation offers an appropriate solution to the many disadvantages of freely suspended cell systems. These include higher reaction rates due to increased cell densities, which results in higher specific product yields, an ability to apply continuous processes with high dilution rates without the problem of biomass wash-out, easier handling and control, cheaper reactor design and easier separation of biomass and product (Mattiasson, 1983b).

A number of considerations need to be taken into account for the efficient development of any bioreactor system employing immobilised living biomass. The choice of immobilisation matrix and method of immobilisation affects the resultant catalytic activity of the immobilised biomass, for example, highly porous supports may provide a large surface area for biomass attachment but considerable mass transfer resistance may be encountered. In order to be practically applicable, bioprocesses require high catalytic activity and operationally stable immobilised cells that are capable of periodical rejuvenation or reuse. The volumetric productivity of a bioprocess is important in determining reactor size for a given throughput level. Also, the effective product concentration in the effluent stream would determine the extent and cost of downstream processing, and include substrate and other process costs (Venkatasubramanian and Karkare, 1983).

Several encapsulation methods are available for application, and each one requires evaluation for suitability for intended use. Rha and Rodriguez-Sanchez (1988) described the

strengthening of capsules by adding an alginate solution to chitosan, poly-L-lysine, polyethylamine or polyvinylyamines. Goosen *et al.* (1989) immobilised living tissue (e.g. islets of Langerhans) in semi-permeable hydrogel membranes with a poly-L-lysine outer layer. Baker *et al.* (1992) described the encapsulation of biological material in non-ionic polymer beads for application as agricultural agents; Batich *et al.* (1994) showed that *Oxalobacter formigenes* encapsulated in alginate capsules maintained activity for several months. Treatment of the gel droplets with a polyelectrolyte was reported to strengthen the capsules. G.ang.ser.o.slahed.d *et al.* (2000) described a method for generating high strength capsules, and rather than simply using poly-L-lysine or chitosan, the authors provided a polyvalent ion (e.g. Ca^{2+}) in the polyanion-polycation membrane-forming step.

The conventional methods for cell immobilisation are based on maintaining physical or chemical forces necessary for immobilisation. This is true for attachment, adsorption and entrapment immobilisation methods (Klei *et al.,* 1985). In contrast, encapsulation methods maintain a solution environment around the cells and the calcium-alginate membrane formed around the cells allows free diffusion of substrate and product through it while containing the cells within the polymeric membrane (Chang *et al.,* 1998). Immobilisation by encapsulation offers several advantages, not only over freely suspended cells, but also over conventional immobilisation methods. Cell loading in entrapment/adsorption methods is limited due to the limited surface area available to the cells, and only the cells on the periphery of the bead or support are exposed to high concentrations of substrate, thus establishing a concentration gradient. In the case of encapsulated cells, dry cell densities reach values as high as 310 g/L, based on the inner volume of the capsule (Park and Chang, 2000).

All previous work on the immobilisation of SRB has reported on conventional attachment or entrapment methods, by contrast, this study has investigated the encapsulation of SRB in calcium-alginate capsules. The terms ‘capsule’ and ‘bead’ have in previous literature been used somewhat loosely and interchangeably, however, in this study, the working definition of capsule and encapsulation will be the immobilisation of cells in a liquid core within a semi-permeable membrane.

3.2 Materials and Methods

3.2.1 Chemical reagents and analysis

As previously described in chapter 2

3.2.2 Microorganisms

The SRB consortium E28 used was selected from a number of other culture isolates as previously described in chapter 2.

3.2.3 Immobilisation methods

3.2.3.1 Immobilisation in alginate beads

A 2.5 % solution of sodium alginate was made by slowly adding sodium alginic acid salt to 100 mL of water in a beaker with vigorous stirring. A pellet of SRB from liquid culture was obtained by centrifugation at 5000 rpm using a Beckman Model J2-21 centrifuge. The wet mass of the pellet was determined before resuspension in 2.5 % sodium alginate solution to give a 6 % (w/v) 'cell-alginate' solution and mixed to homogeneity.

The cell-alginate solution was placed in a 10 mL disposable syringe and added drop-wise to a beaker of 2 % calcium chloride and gently stirred with a magnetic stirred and follower. Altering the dripping speed and the height of the syringe above the calcium chloride solution controlled the size of the beads. The beads were then washed in distilled water and either placed in media or stored in a solution of 0.5 % (w/v) calcium chloride.

3.2.3.2. Encapsulation in Calcium-Alginate-Xanthan gum membrane

A one step immobilisation method was carried out with appropriate modifications, according to the method outlined by Chang *et al.* (1998).

After determining the wet mass, the SRB pellet was resuspended in a mixture of 1.3 % (w/v) calcium chloride and 0.26 % (w/v) xanthan gum (XG) at room temperature (~20-25 °C) to give a 35-40 % (w/v) SRB-XG- calcium chloride solution. Using a 10 mL disposable syringe, this mixture was added drop-wise to a solution (400 mL) of 0.6 % (w/v) sodium alginate (Na-alg) with 0.1 % (v/v) Tween 20 with stirring and allowed to react for 10 minutes. The

resultant capsules were then washed with distilled water for 10 minutes and were then added to 1.3 % (w/v) calcium chloride solution and allowed to harden for 20 minutes with stirring. Figure 3.1 gives a schematic representation of the capsule production method followed.

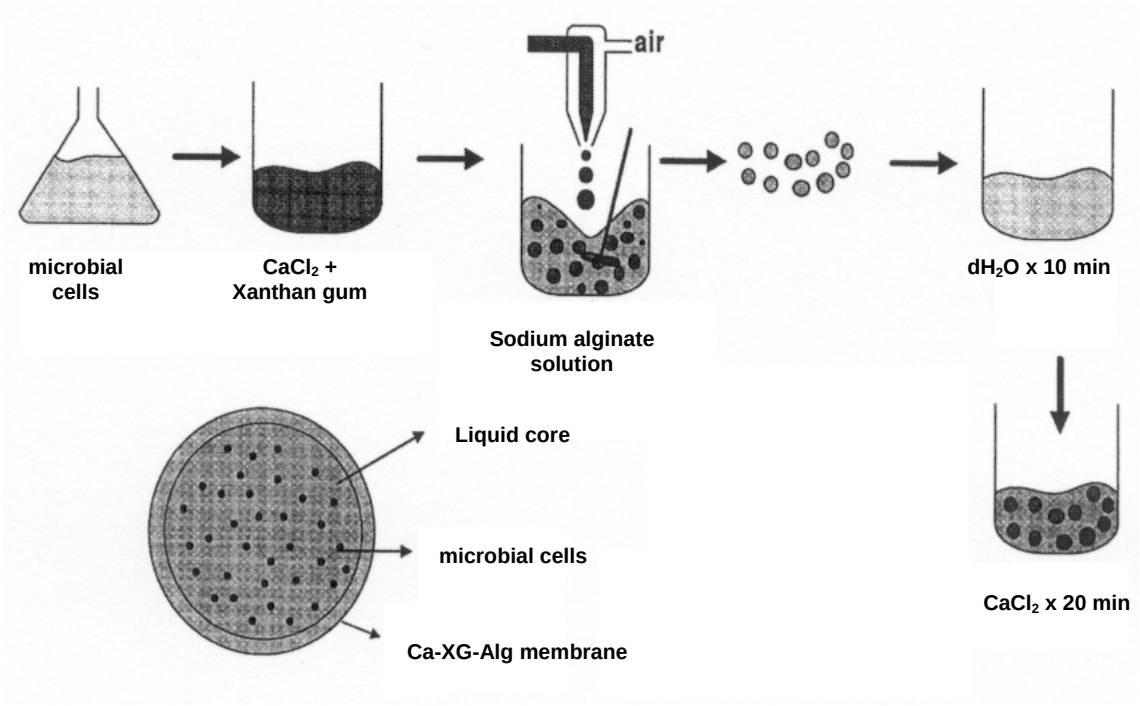

Figure 3.1: Schematic capsule preparation method by liquid droplet forming-one-step method (modified from Park and Chang, 2000)

3.2.3.3 Encapsulation in Calcium-Alginate-Poly-L-lysine membrane

A two-step pregel dissolving immobilisation method was carried out with appropriate modifications, according to the method outlined by Goosen *et al*. (1989).

After determining the wet mass, the SRB pellet was resuspended in a 2.5 % (w/v) Na-alg solution at room temperature (~20-25 °C), to give a 6 % (w/v) SRB-Na.Alg solution mixed to homogeneity. Using a 10 mL disposable syringe, this mixture was added, drop-wise, to a solution (400 mL) of 2 % (w/v) calcium chloride, which was stirred to give conventional alginate beads. Altering the speed of the drops and the distance of fall controlled bead size.

The alginate beads were then washed in a dilute 2-cyclohexylamino-ethane sulphonic acid (CHES) solution with 1.1 % (w/v) calcium chloride. The washed alginate beads were incubated for six minutes in a solution of 0.05 % (w/v) poly-L-lysine (molecular weight 24 700) and again washed in dilute CHES solution with 1.1 % (w/v) calcium chloride and physiological saline. The calcium-alginate-poly-L-lysine beads were then incubated for four minutes in a 0.03 % (w/v) sodium alginate solution to allow cross-linking of any residual poly-L-lysine on the surface of the beads. The beads were then incubated for four minutes in 0.05 M citrate buffer to re-liquify the inner calcium alginate core while maintaining a calcium-alginate-poly-L-lysine outer membrane. Physiological saline was used for the final wash. Figure 3.2 presents a schematic diagram of the procedure followed.

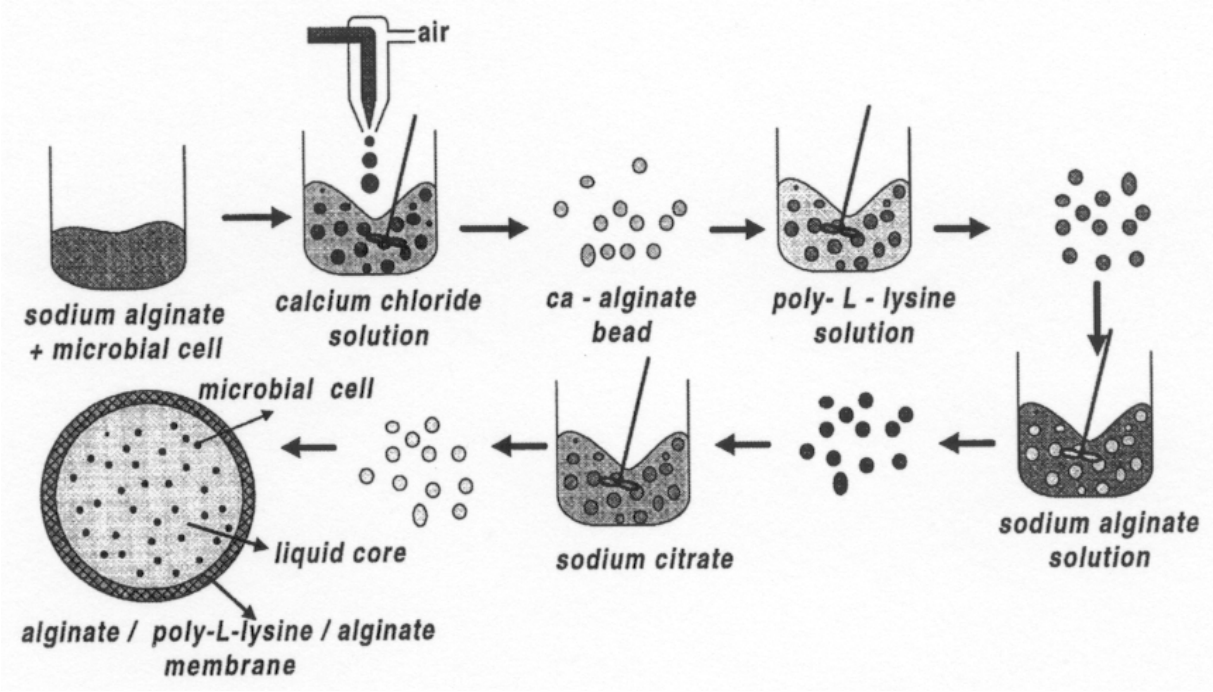

Figure 3.2: Schematic capsule preparation method by pregel dissolving-two-step method (Park and Chang, 2000)

3.2.4 Fed-batch multiple cycle and reuse experiments

Experiments were carried out in duplicate in 500mL or 1 L Erlenmeyer flasks as fed-batch systems on Labcon shakers at 100 revolutions per minute (rpm) in a 30 °C constant environment room. Samples were taken once a day while sparging the vessels with nitrogen to maintain the anoxic environment. Leaching of biomass into the medium was monitored by

direct observation of the capsules for signs of breaking up and by Gram-staining of medium samples.

For multiple cycle and reuse experiments, reactors were set up and run as for fed-batch experiments described above. The capsules were then transferred to new fed-batch shaker flask reactors with fresh media after filtering through a Buchner funnel.

3.2.5 Column studies

In fed-batch packed column studies, the capsules were place within a sealed perspex column with a sampling port, a gas outlet that was connected to the zinc acetate sulphide trap, and an inlet through which nitrogen was bubbled from the bottom of the reactor to maintain an anoxic environment during sampling. Experiments were carried out in a 30 °C constant environment room. The basic reactor design and experimental set up is shown in figure 3.3.

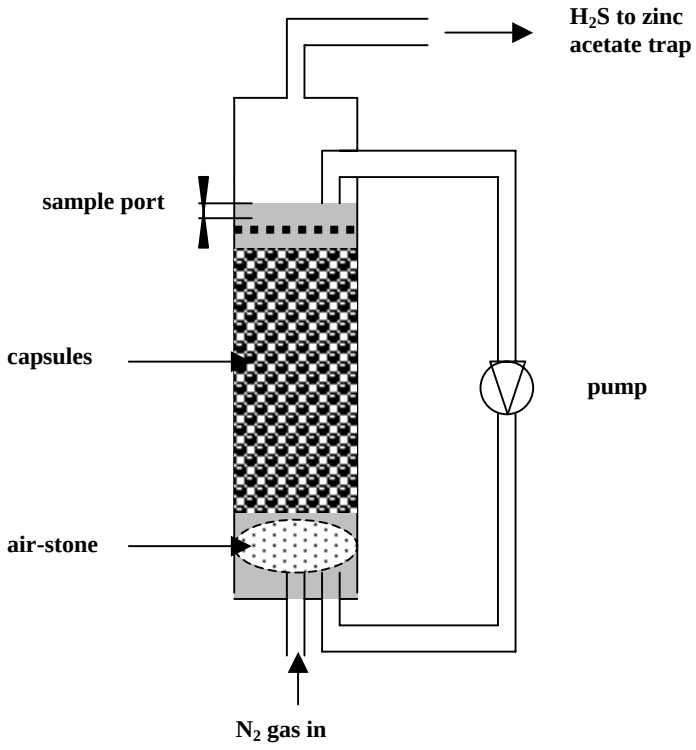

Figure 3.3: Diagrammatic representation of fed-batch packed column reactor set up.

Reactor vessels were of two volumetric sizes, 250 mL and 1.5 L. Media was continuously re-circulated at 2.5 mL/min in the smaller reactor and 15 mL/min in the large reactor to give a hydraulic retention time (HRT) of approximately 100 minutes.

In continuous packed column studies, the experimental set up was similar to that described above for fed-batch packed column studies. The reactor vessel was a glass column with a water jacket used to maintain reactor temperature at 30 °C, which allowed the experiments to be carried out on a laboratory bench top. A diagrammatic representation of the basic continuous packed column reactor system is presented in figure 3.4.

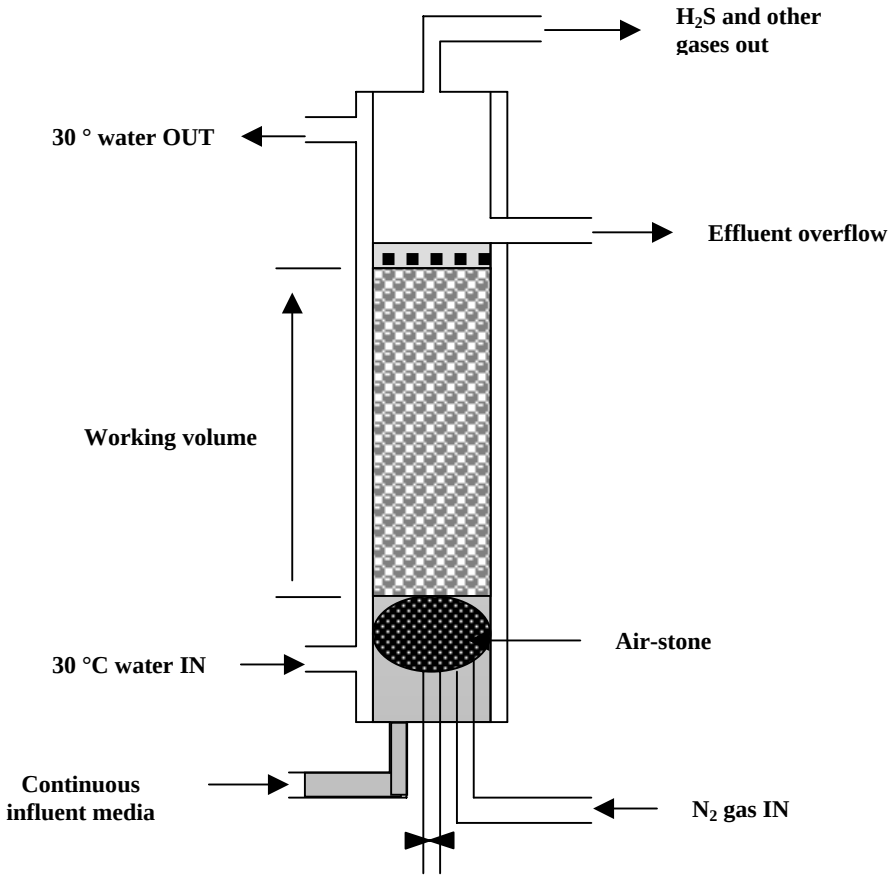

Figure 3.4: Diagrammatic representation of the configuration of a continuous up-flow packed column reactor.

The term 'working volume' was used to define the volume of the column occupied by the capsules (see figure 3.4). The working volume, the flow rate and the HRT were approximately 59 mL, 0.4 mL/min and 148 minutes respectively. Nitrogen gas was constantly bubbled into the system to maintain anoxic conditions.

In both fed-batch and continuous packed column systems, samples were collected daily for analysis. The integrity of the capsules and leaching of biomass into the medium was monitored by direct observation of the capsules for signs of breaking up and by Gram-staining of medium samples.

3.2.6 Optimisation of capsule production

Two semi-automated systems were developed for capsule production as shown in figure 3.5 A and B, in order to speed up the production of capsules and also to produce capsules of a more standard size and quality.

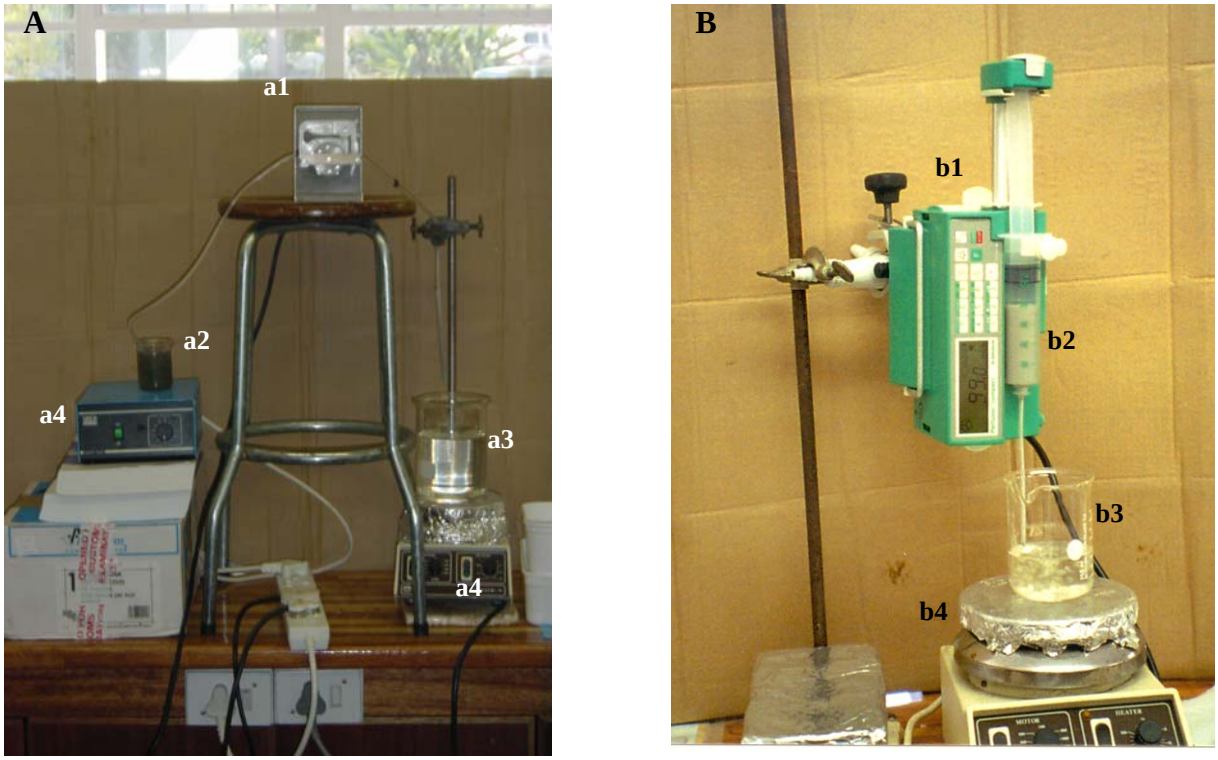

Figure 3.5 A and B: Semi-automated systems for the production of calcium-alginate capsules in the laboratory. **a1** = peristaltic pump, **b1**= Braun® medical syringe pump, **a2 & b2** = cells + calcium chloride + xanthan gum, **a3 & b3** = sodium-alginate + Tween 20, **a4 & b4** = magnetic stirrer

In figure 3.5A, the SRB-XG- calcium chloride solution in a beaker was placed on a magnetic stirrer with follower and stirred gently in order to maintain a homogeneous mixture. This mixture was added drop-wise to a stirred solution of sodium alginate with Tween 20 (made up as previously discussed) using an Ismatec® peristaltic pump. The rest of the capsule production was as previously described in section 3.2.3.2.

In figure 3.5B, a maximum of 55 mL of the SRB-XG- calcium chloride solution in 60 mL syringe was added drop-wise to a stirred solution of sodium alginate with Tween 20 (made up as previously discussed) using a Braun® medical syringe pump. When optimised, the operating parameters and output of the system are detailed in table 3.1. Completion of capsule production was as outlined previously in section 3.2.3.2.

Table 3.1: Optimised operating and output conditions for capsule production using the Braun® medical syringe pump

Operating / Output parameter	Optimum setting
Optimum pump flow rate	99 mL/min
Syringe volume	55 mL
Height of syringe above liquid level	1.5 cm (± 0.5)
Magnetic stirrer speed	500 rpm (±50)
Capsule produced per unit volume	15 capsules/mL (± 2)
Average cell content per capsule	0.035 g/capsule (± 0.005)
Average capsule size (diameter)	0.4 mm (± 0.1)

3.3 Results and Discussion

3.3.1 Immobilisation in alginate beads

Initial SRB consortium growth medium, modified Pg-C, contained iron and sulphate-reducing activity was indicated by the medium turning black as iron sulphide precipitated out of solution. Therefore, initial calcium-alginate beads of SRB consortium were black in colour due to the presence of the iron sulphide. Subsequent culture production in the absence of iron in solution produced beads grey in colour. Iron was excluded from all subsequent growth media in order to exclude any possible interference by the iron in metal precipitation studies.

Therefore, original calcium-alginate immobilised SRB consortium beads were either black or grey in colour as shown in figure 3.6. The black and grey calcium-alginate beads both had a similar diameter of 4 mm and when handled, had a solid and robust feel.

3.3.1.1 Scanning electron microscopy of calcium-alginate beads

Scanning electron microscopy micrographs (figure 3.7) provide an indication of the arrangement the SRB consortium within the calcium-alginate matrix (A and B). Figure 3.7 C and D show the surface structure of the alginate bead after preparation for SEM

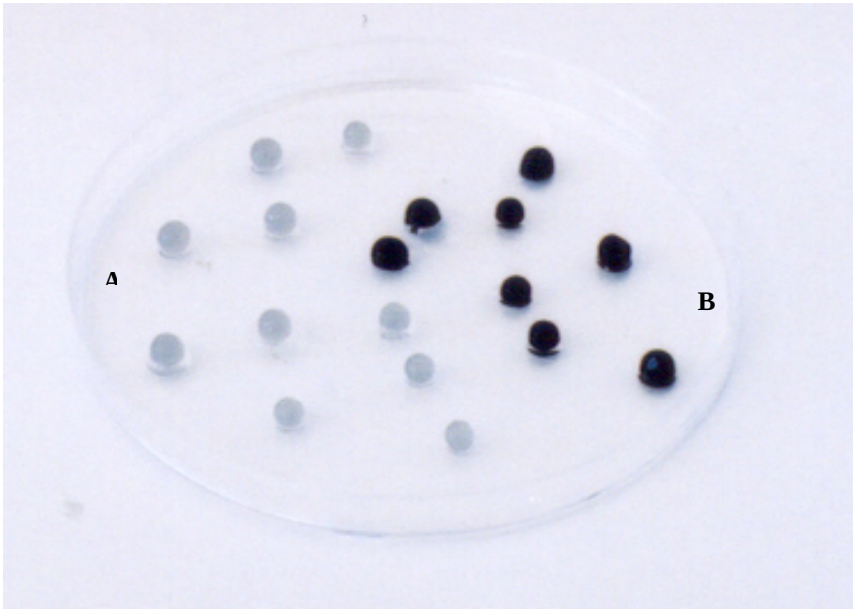

Figure 3.6: Sulphate-reducing bacterial consortium immobilised in sodium alginate beads. **A** = SRB consortium grown in the absence of iron, **B** = SRB consortium grown in the presence of iron.

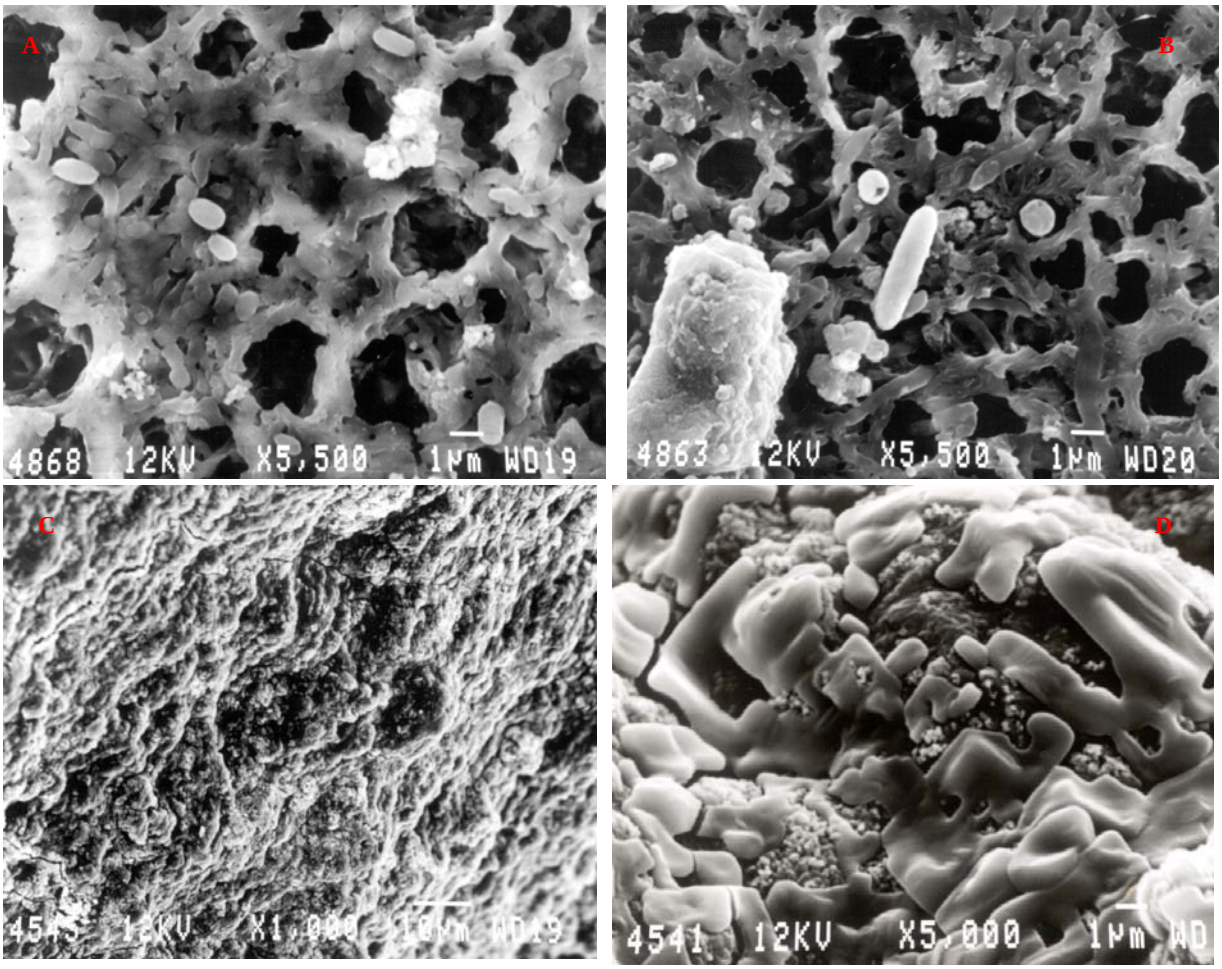

Figure 3.7: Scanning electron micrographs of calcium-alginate beads-immobilised sulphate-reducing bacteria. **A** and **B** = SRB within the calcium-alginate matrix; **C** and **D** = surface of calcium-alginate beads.

The SRB consortium cells were entrapped within a calcium-alginate matrix and figure 3.7 A and B show the cells within what appears to be an open and porous matrix structure. Some of the cells can also be seen to be trapped in the porous spaces of the matrix structure. A few cells appear to be free from the matrix structure, this may be due to some degradation of the matrix during preparation for SEM. The surface structure of the calcium-alginate beads was observed to be relatively amorphous (figure 3.7 C and D).

3.3.1.2 Performance of sulphate-reducing bacterial consortium immobilised in calcium-alginate beads in fed-batch shaker flask studies

The sulphate reduction performance of the immobilised SRB consortium in calcium-alginate beads is given in figures 3.8 and 3.9, each graph is an average of two duplicate experiments. These results were used to evaluate the effect of immobilisation of the SRB consortium in calcium-alginate beads on their sulphate reduction activity and determine the effect of the presence of iron in the beads. SRB consortium immobilised in calcium-alginate beads that were black in colour are identified with the prefix "Fe" and the calcium-alginate beads that were grey in colour due to the absence of iron in their growth media, have the prefix "NFe", while un-immobilised free suspended cells have the suffix "free".

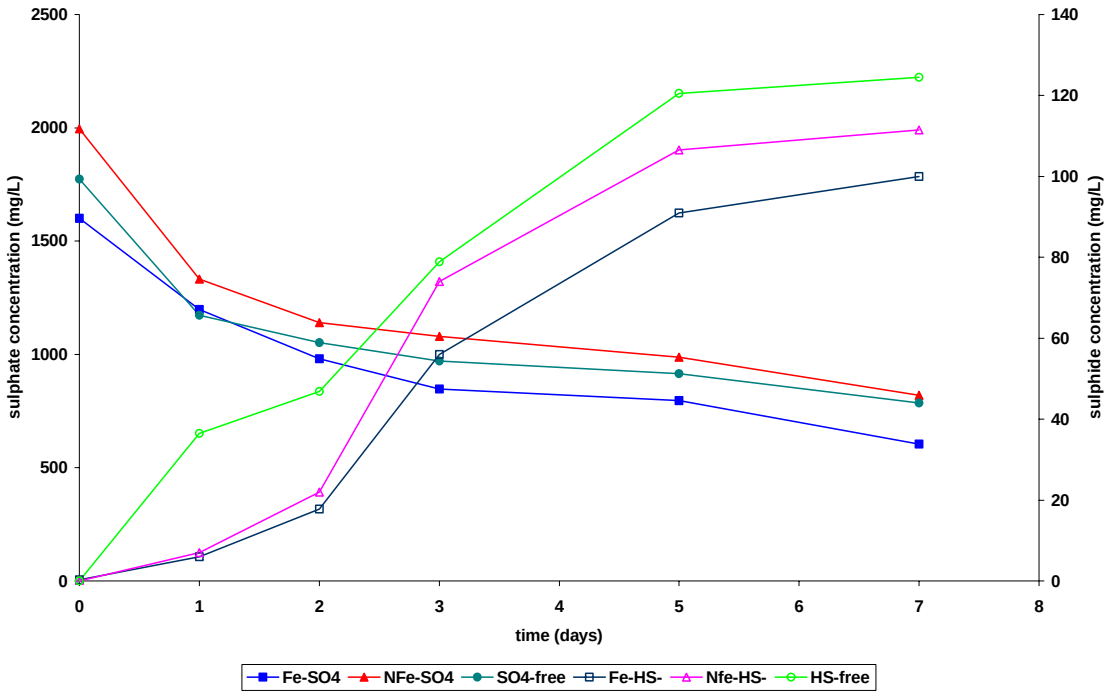

Figure 3.8: Sulphate reduction and sulphide generation profiles for un-immobilised and immobilised sulphate-reducing bacterial consortium (in medium with iron-Fe and no iron-NFe) in calcium alginate beads in fed-batch 500 mL shaker flask reactors.

The profiles for sulphate reduction and sulphide generation for the SRB consortium immobilised in calcium alginate beads were comparatively similar to the profile shown for un-immobilised free suspended cells. The average percentage sulphate removal after seven days was 62, 59 and 56 % for black and grey calcium alginate beads and the un-immobilised SRB consortium cells respectively. The sulphate reduction rates on day three for the black and grey calcium alginate beads were approximately 310 and 428 mg SO_4^{2-}/L/day compared to 361 mg SO_4^{2-}/L/day, suggesting that there is not a large difference in activity between the calcium alginate immobilised SRB consortium and un-immobilised SRB consortium in the first three days. The overall average sulphate reduction rate after 7 days showed slightly better rates for the calcium alginate immobilised SRB consortium beads compared to free un-immobilised SRB consortium cells (see table 3.2).

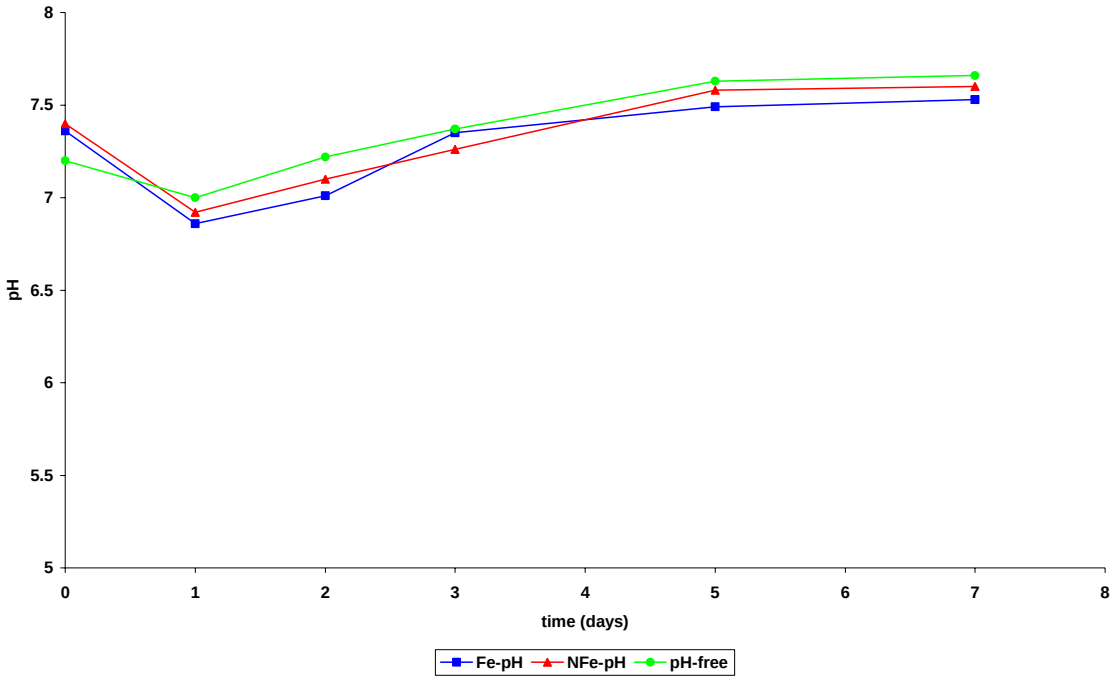

Figure 3.9: pH profiles for un-immobilised and immobilised sulphate-reducing bacterial consortium (in medium with iron-Fe and no iron-NFe) in calcium alginate beads in fed-batch 500 mL shaker flask reactors.

Figure 3.9 shows the pH profiles for the SRB consortium immobilised in calcium alginate beads and the un-immobilised SRB consortium. The pH profiles show an expected trend. Active SRB increase the pH of the aqueous environment in which they are found through the production of bicarbonate alkalinity and the generation of sulphide, which also contributes to the biogenic alkalinity. On release as hydrogen sulphide the removal of hydrogen ions from solution increases the pH.

Table 3.2: Average percentage sulphate reduction, rates of sulphate reduction and sulphide generation for un-immobilised and immobilised sulphate-reducing bacterial consortium in shaker flasks.

	Average Percentage Sulphate reduction (%)			**Average Sulphate reduction rate (mg SO_4^{2-}/L/day)**			**Average Sulphide generation rate (mg HS^- /L/day)**		
Culture	**Fe**	**NFe**	**Free**	**Fe**	**NFe**	**Free**	**Fe**	**NFe**	**Free**
Day 0 to 7 (total)	62	59	56	143	168	141	39	46	58

Slightly better average percentage sulphate reduction and average rate of sulphate reduction was achieved by SRB consortium immobilised in calcium alginate beads compared to freely suspended cells. However, average sulphide generation was found to be considerably higher in the reactor with the free un-immobilised SRB consortium. This result was unexpected, but figure 3.8 also showed that sulphide generation was higher in the un-immobilised system compared to both immobilised systems.

In subsequent experiments, both SRB consortium grown in the presence and absence of iron were used for immobilisation in calcium alginate beads, as there did not appear to be considerable differences in their sulphate reducing activity.

3.3.1.3 Sulphate-reducing bacterial consortium immobilised in calcium-alginate beads in multiple cycle and reuse studies

The advantages of moving from free suspended biomass to immobilised biomass were discussed in chapter one. In chapter two the importance of evaluating the performance of biomass in multiple cycle and reuse was also discussed. Figures 3.8 and 3.9 showed that the immobilisation process had no detrimental effect on the sulphate-reducing activity of the SRB consortium. Multiple cycle and reuse experiments were then carried out to determine the robustness of the calcium alginate beads and their sulphate reducing activity.

The immobilised SRB consortium were placed in shaker flasks and moved to fresh medium over three cycles (six to seven days) over a total of 19 days. The sulphate reduction and sulphide generation profiles are given in figure 3.10 and the pH and alkalinity profiles in figure 3.11. The results shown represent an average of two duplicate experiments.

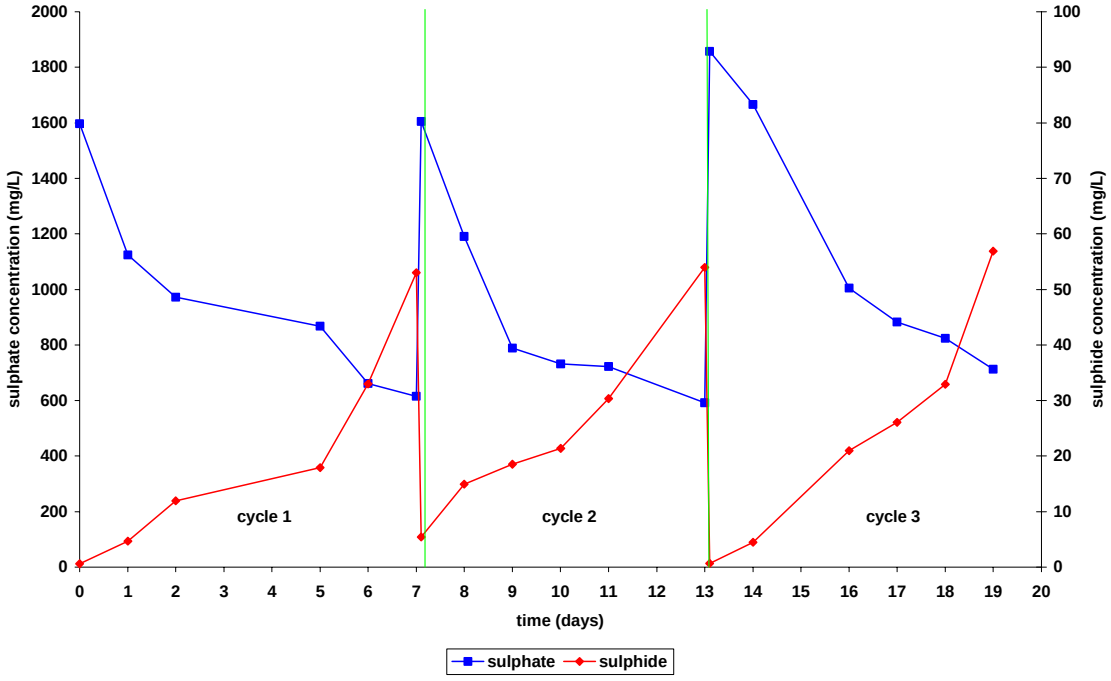

Figure 3.10: Sulphate reduction and sulphide generated over 3 cycles for sulphate-reducing bacterial consortium immobilised in calcium alginate beads in fed-batch 500 mL shaker flask reactors.

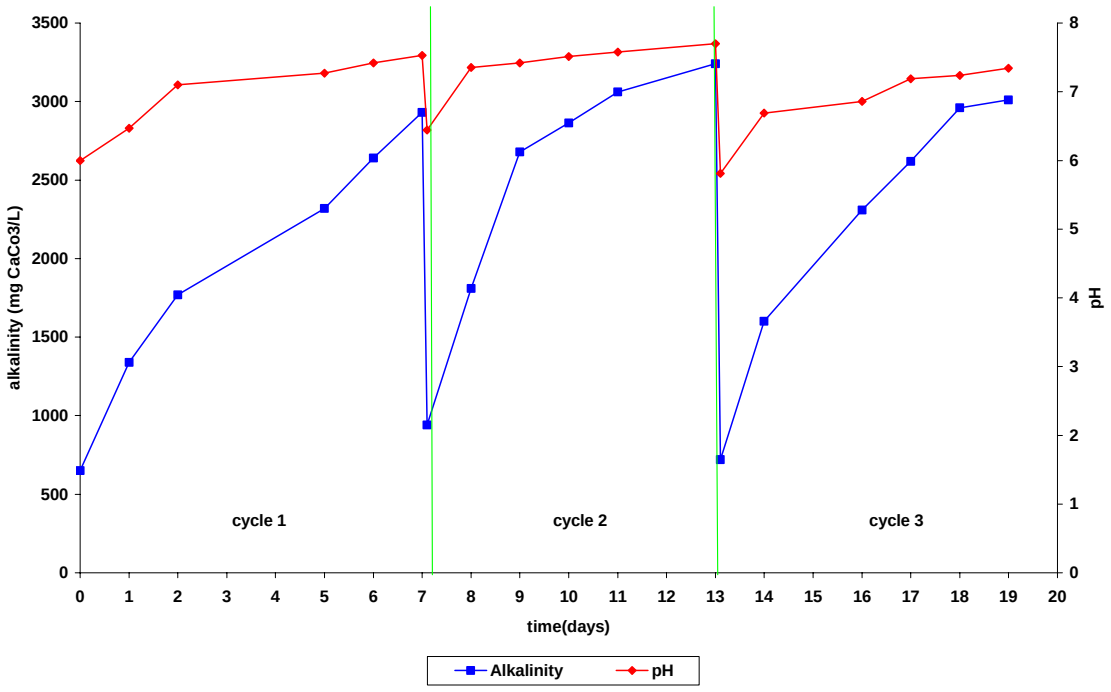

Figure 3.11: pH profile and alkalinity generated over 3 cycles for sulphate-reducing bacterial consortium immobilised in calcium alginate beads in fed-batch 500 mL shaker flask reactors.

The SRB consortium immobilised in calcium alginate beads showed consistently high sulphate reduction over the three cycles. Each cycle showed over 60 % sulphate reduction, which is higher than that for the free suspended SRB consortium (see table 3.2). The average rates of sulphate reduction for the three cycles were 140, 169 and 191 mgSO_4^{2-}/L/day respectively over the duration of each cycle, indicating an increase in performance. This could have been due to an increase in biomass within the bead, but because no suitable and reliable method for measuring any increases in biomass was found, a conclusive reason for improved performance could not be reached. The maximum rates of sulphate reduction occurred in the first two days for cycles one and two and in the first three days for cycle three and were 313, 408, and 426 mg SO_4^{2-}/L/day. These rates of sulphate reduction compare favourably with those found for un-immobilised biomass (see table 2.3). However, considering that for each mole of sulphate reduced, a mole of sulphide is generated, the amounts of detectable sulphide found in solution and in the zinc acetate traps were found to be much lower than would have been anticipated (figure 3.10). The average rates of sulphide generation were 7.5, 8.1 and 9.4 mg HS^-/L/day for cycles one, two and three respectively.

The SRB consortium immobilised in calcium alginate beads also showed consistently high alkalinity generation over the three cycles. The maximum alkalinity generated for cycles one, two and three were 2930, 3240 and 3010 mg $CaCO_3$/L respectively. The pH in each cycle had increased to above pH 6.5 by the end of day one and was maintained above 7 for the remainder of each cycle. Therefore the pH and alkalinity profiles gave the anticipated trends.

3.3.1.4 Integrity of calcium-alginate beads

At the end of the first cycle, the calcium alginate beads showed no signs of breaking up or disintegrating. Gram stained medium samples showed little to no sign of biomass leaching into solution. It appeared that the calcium alginate beads had maintained their integrity into the second cycle of the multiple cycle and reuse studies showing good sulphate-reducing activity. However, early in the second cycle the calcium alginate beads started to show signs of disintegration. Figure 3.12 shows some of the calcium alginate beads in a petri-dish at the end of the second cycle where, although most of the beads were still intact, some had disintegrated completely and pieces of the beads were clearly visible. During the third cycle, disintegration of the calcium alginate beads proceeded more rapidly and the multiple cycle and reuse experiments were discontinued on day 19.

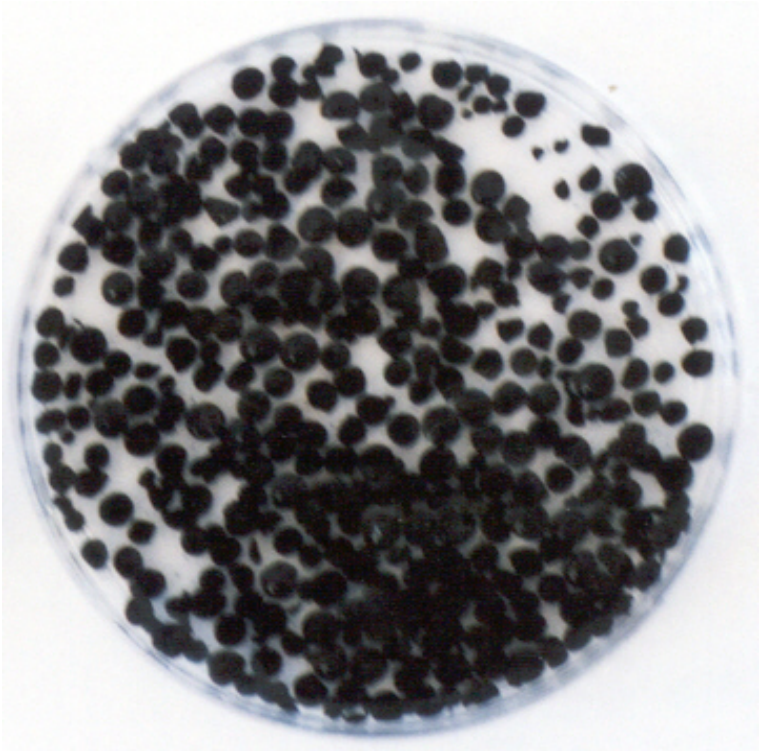

Figure 3.12: Appearance of alginate beads at the end of the second cycle of the multiple cycle and reuse experiments with broken pieces of calcium alginate beads clearly present.

Disintegration could have been due to several reasons. The shaking flasks meant constant agitation of the beads and the friction created between calcium alginate beads probably contributed to their disintegration. The presence of any metal chelating compounds in the media would also have played a role in sequestering the calcium cations involved in the cross-linking needed to maintain the structure of the beads. Compounds that would have been involved in disrupting the cross-linking structure of the beads would have included phosphates and citrate. However, considering the low concentrations of these compounds in the medium, this alone should not account for the extensive disruption of the beads that is only evident by the end of the third cycle. The sulphidogenic environment may also have contributed to some extent toward the disruption and disintegration of the calcium alginate capsules.

Figures 3.13 a and b show gram-stains of the suspension medium at the end of the second and third cycles respectively, and were used to determine the presence of leached biomass and calcium alginate matrix debris.

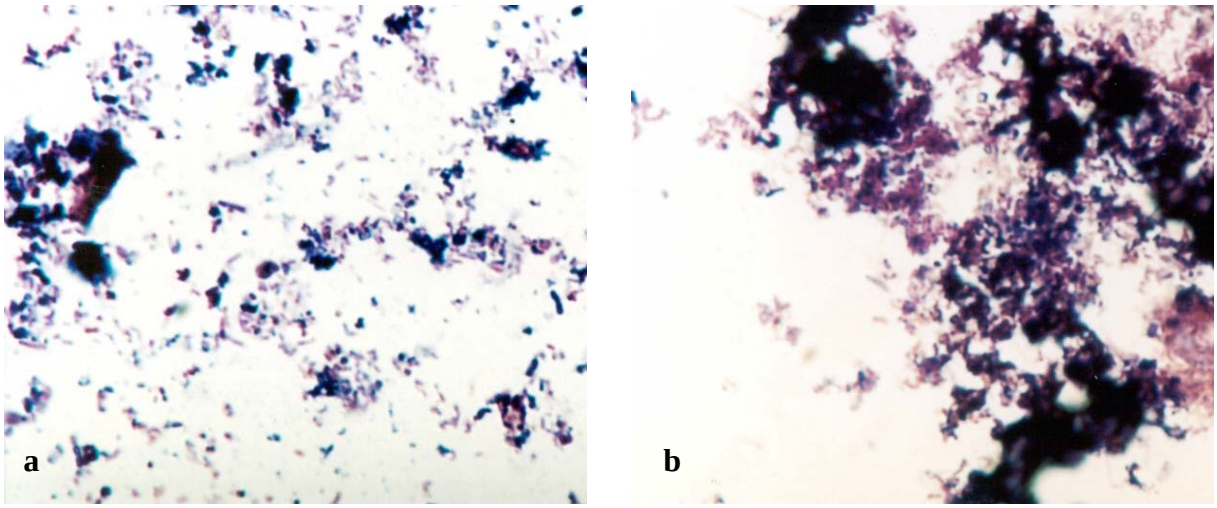

Figure 3.13: Gram-stain photographs of suspension medium samples from calcium alginate beads-immobilised SRB consortium shaker flask reactors. **a** = sample from cycle 2 and **b** = sample from cycle 3.

It was clear that as the calcium alginate beads broke up, more cells and bead matrix debris were released into the medium. Figure 3.13 showed that the suspension medium Gram stain samples at the end of the third cycle contained much more bead debris and biomass cells.

3.3.2 Immobilisation by encapsulation

As was the case with the SRB consortium immobilised in calcium alginate beads, initial SRB consortium immobilised by encapsulation within a calcium alginate xanthan gum (Ca-alg-XG) or calcium alginate poly-L-lysine (Ca-al-PLl) membrane were black in colour, simply due to the presence of iron in the original modified Pg-C medium used. Subsequent immobilisation was carried out using SRB consortium grown in the absence of iron to eliminate all possibilities of iron interference in capsule application studies.

Figure 3.14 shows SRB consortium Ca-alg-XG capsules immediately after production. A close-up view of the calcium alginate capsules (figure 3.14a) clearly showed the Ca-alg-XG membrane around the capsule. Figure 3.14b gives an indication of the grey colour of calcium alginate capsules produced from SRB consortium grown in the absence of iron in the medium. The sizes of the capsules were 0.4 to 0.5 mm in diameter (figure 3.15) and standardising the production method gave capsules of a more consistent size. The capsules were different in texture from beads, they did not have the hard, velvet feel to them but were found to be elastic and had a smooth and slippery surface which probably helped in protecting the capsules from mechanical damage.

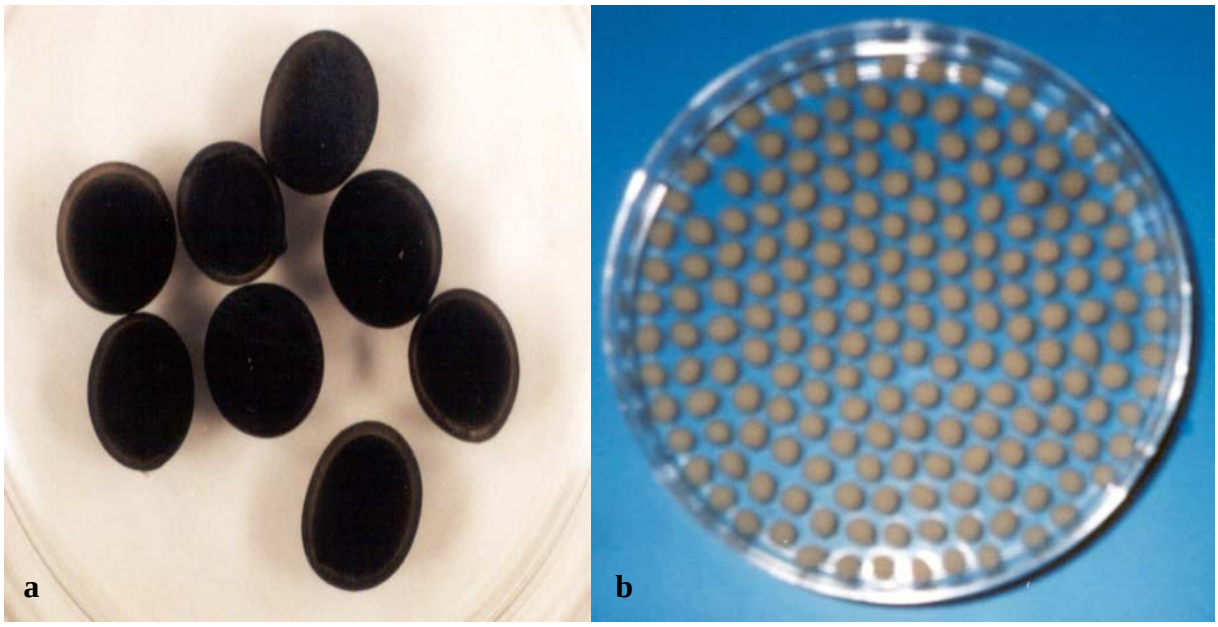

Figure 3.14: a) Close-up photograph of sulphate-reducing bacterial consortium immobilised in calcium-alginate xanthan gum capsules with the semi-permeable membrane clearly visible. **b)** representative grey calcium alginate capsules.

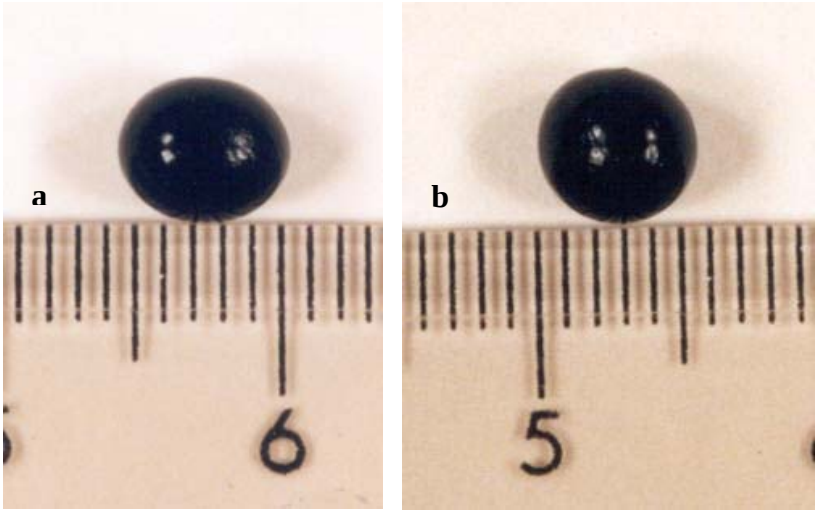

Figure 3.15: Representative sizes of capsules with sulphate-reducing bacterial consortium immobilized in **a)** Calcium –alginate-poly-L-lysine capsule; **b)** Calcium-alginate-xanthan gum capsule

3.3.2.1 Scanning electron microscopy of calcium-alginate capsules

The SEM micrographs for calcium alginate capsules are shown in figure 3.16. Due to the problems presented in sectioning the liquid core of the calcium alginate capsules for SEM, samples were prepared for SEM using cryo-freezing techniques as outlined in appendix three.

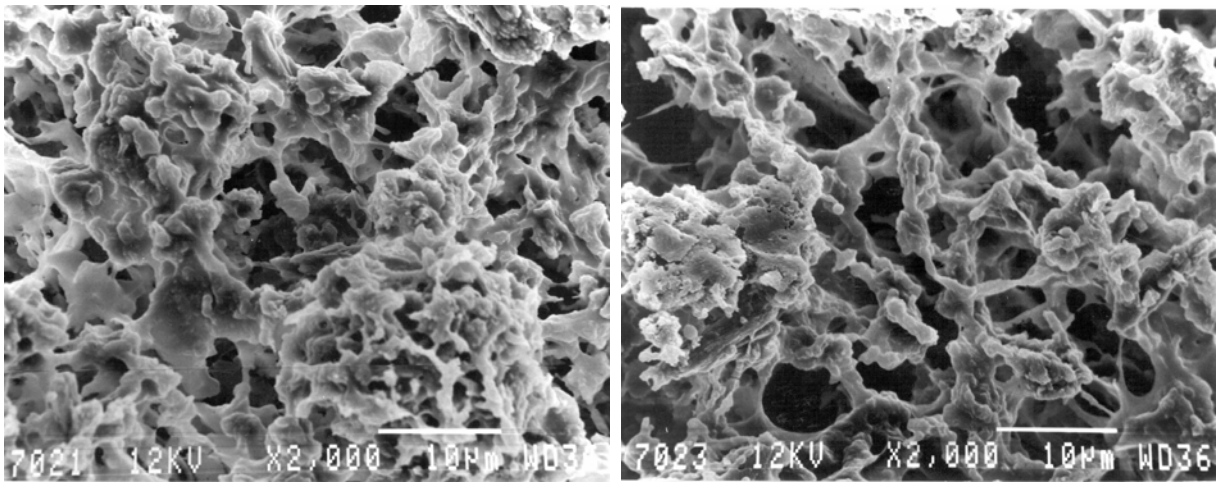

Figure 3.16: Cross-sectional cryo-scanning electron micrographs of sulphate-reducing bacterial consortium immobilised in calcium alginate capsules.

Some distinct differences were apparent when the SEM micrographs for the calcium alginate capsules were compared to the SEM micrographs of the calcium alginate beads. Whereas the calcium alginate beads were observed to have a more open porous structure, the calcium alginate capsules appeared to have a more tightly packed conformation, which is what would be expected in a liquid culture. The biomass in calcium alginate beads is therefore more visible compared to the calcium alginate capsules that are found within a liquid medium. Figure 3.17a shows a clearer view of the packing of cells within the calcium alginate capsules. The cross-sectional membrane structure shown in figure 3.17b appears to be a multi-layered structure, which probably contributed to the greater stability and strength of calcium alginate capsules compared to calcium alginate beads that have a cross-linked porous and more open structure.

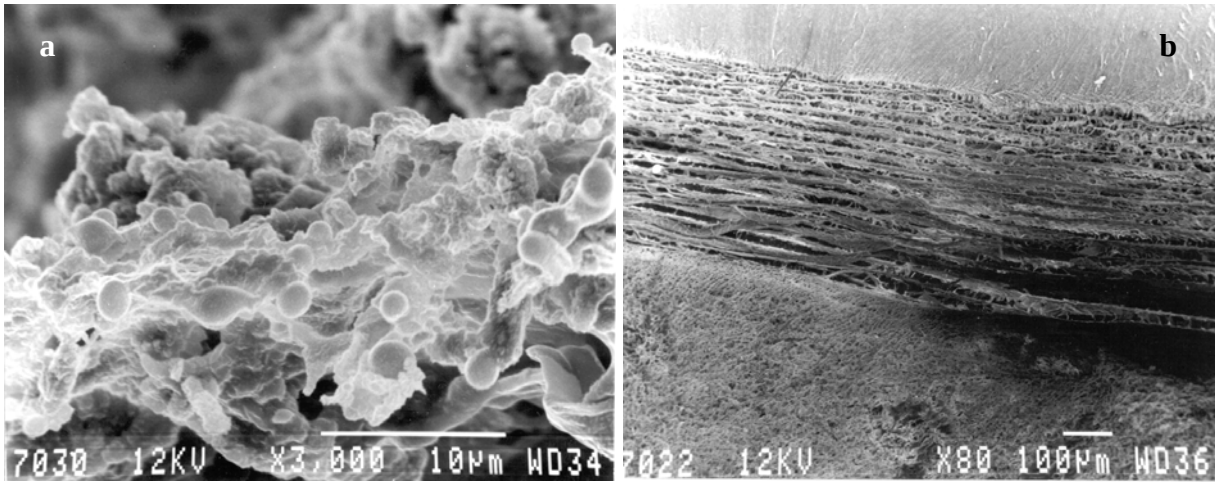

Figure 3.17: Cross-sectional cryo-scanning electron micrographs of the inside (**a**) and the membrane (**b**) of SRB consortium immobilised in calcium alginate capsules**.**

3.3.2.2 Multiple cycle and reuse studies

Fed-batch shaker flask experiments were carried out in 1 L Erlenmeyer flasks comparing the performance of un-immobilised SRB consortium biomass (UI), ca-alg-XG gum immobilised SRB consortium capsules (IX), ca-alg-PLl immobilised SRB consortium capsules (IP) and a negative control of autoclaved medium with no SRB consortium cells present. At set intervals, the biomass from all four systems were moved to shaker flasks with fresh medium after separating and washing the immobilised SRB consortium capsules and after centrifugation and re-suspension for the free suspended un-immobilised biomass. The multiple cycle and reuse study was carried out over five cycles and a 45-day period.

Certain experimental conditions e.g. initial sulphate concentrations, duration of cycles and periods of down time (when the biomass was stored in buffer in an anaerobic hood) were constantly altered for each cycle in order to determine the performance of the calcium alginate capsules under conditions that mimicked the variable conditions expected for real application or in industry.

Multiple cycle and reuse sulphate-reduction activity profiles were generated and compared for the 3 systems, UI, IP, IX. Each cycle is numerically labelled and the periods of down time are labelled "dt". The results shown represent an average of two duplicate experiments. No sulphate reduction or sulphide generation was detected for the negative control.

The sulphate reduction profiles for UI, IP and IX are shown in figure 3.18. The average initial sulphate concentration for the three SRB consortium systems was approximately 1850 mg/L. The first cycle was the longest, running for five days, and it probably represented a time of acclimatisation with the lowest percentage sulphate removal values of approximately 80, 54 and 79 % for UI, IP and IX respectively. However, the average sulphate reduction rates of 282, 214 and 283 mg SO_4^{2-}/L/day for UI, IP and IX respectively in the first cycle were comparatively similar and even early on in the experiment these values were considerably better than the rates achieved for SRB consortium immobilised in calcium alginate beads. Percentage sulphate removal in subsequent cycles was much higher than in the first cycle with values calculated for UI being very similar to those for both IP and IX, most of these were above 90%. The final sulphate concentrations in all but IP in cycle one were below 500 mg/L, an important value for most industries' discharge requirements.

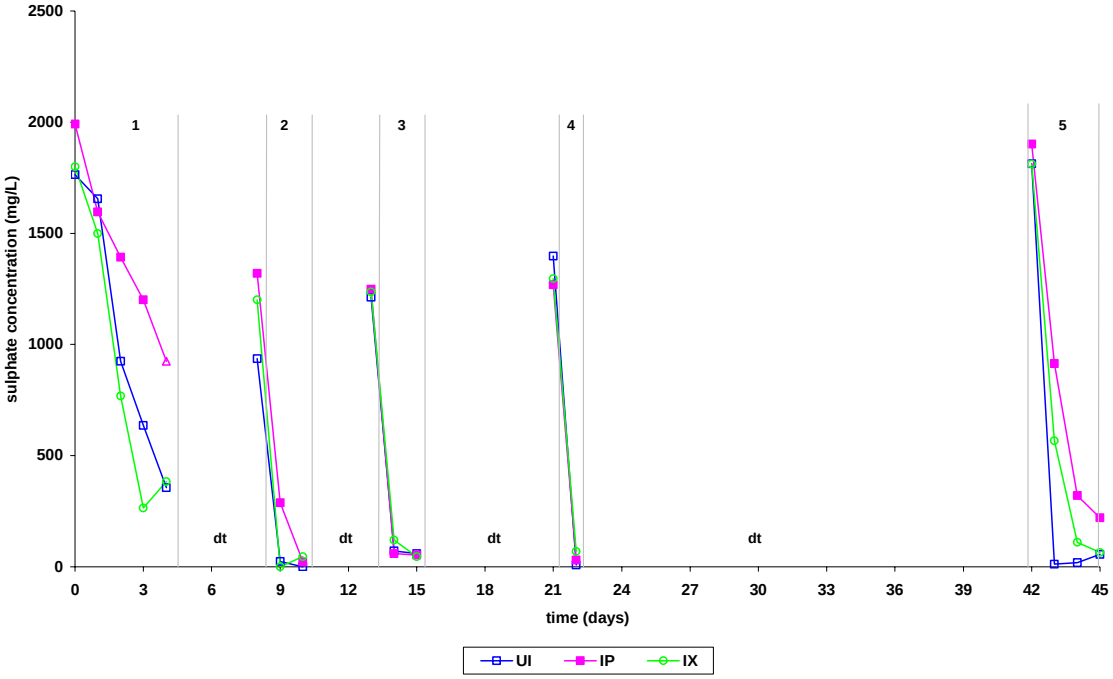

Figure 3.18: Sulphate reduction profiles in multiple cycle and reuse experiments for 5 cycles over 45 days for SRB consortium immobilised in calcium alginate capsules and un-immobilised SRB consortium, dt = down time.

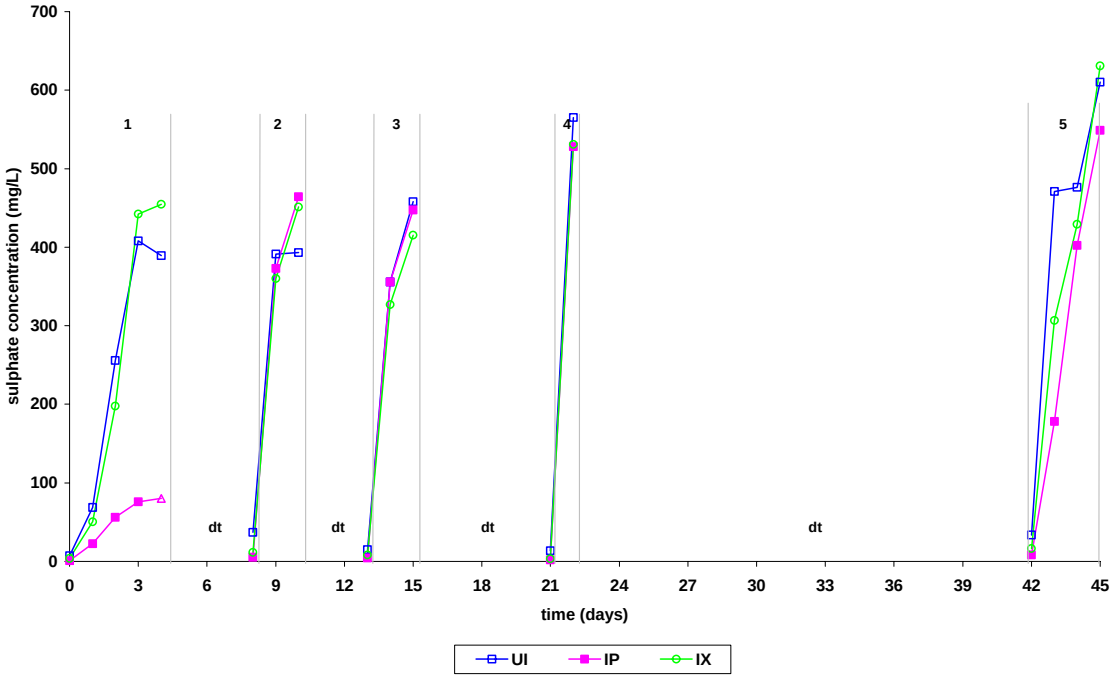

Figure 3.19: Sulphide generation profiles in multiple cycle and reuse experiments for 5 cycles over 45 days for SRB consortium immobilised in calcium alginate capsules and un-immobilised SRB consortium, dt = down time.

Sulphate reduction rates were also calculated with reference to the initial wet mass of the SRB consortium used in a reactor or for immobilisation. Although this method of calculating rates has its limitations due to the change in biomass with time, it was the only method found to be consistent as extensive attempts to use published methods for reliable and accurate enumeration of SRB consortium biomass were found to be completely ineffective. Calculated sulphate reduction rates with respect to initial biomass wet mass are given in tables 3.3 and 3.4.

Sulphide generation profiles for all three SRB consortium systems UI, IP and IX (figure 3.19) gave more anticipated results compared to those found for SRB consortium immobilised in calcium alginate beads (figure3.10). Reflecting the lowest amount of sulphate reduction found in the first cycle, IP had the lowest amount of sulphide generated of approximately 80 mg/L compared to 390 and 455 mg/L for UI and IX respectively. However, by the second and subsequent cycles, as was found with sulphate reduction, the amount of sulphide generated by IP was comparable and similar to that by UI and IX. The rates of sulphide generation in UI and both IP and IX were very similar throughout the whole experiment. The rates of sulphide generation in the fifth cycle, for example was 17.5, 16.4 and 18.6 mg HS^- /L/day/g initial biomass wet mass for UI, IP and IX respectively..

High alkalinity generation was found in all three SRB consortium systems. The average rate of alkalinity generation in the first cycle was 34, 10 and 31 mg $CaCO_3$/L/day/g of initial biomass wet mass for UI, IP and IX respectively. These rates were found to increase considerably by the second cycle and the highest rates of alkalinity generation in the fifth cycle were 109, 93 and 94 mg $CaCO_3$/L/day/g of initial biomass wet mass. The increase in alkalinity generation rates suggest an improvement in all three systems with time, which may indicate adaptation of the three SRB consortium systems.

The differences in initial sulphate concentrations and duration of down times did not appear to detrimentally affect the sulphate reducing activity of any of the SRB consortium systems. All systems showed rapid start up profiles on being re-introduced into shaker flasks (see rates in table 3.3). In cycle four following five days of down time with an average initial sulphate concentration of 1300 mg/L, the percentage sulphate reduction for all SRB consortium systems was 95 % or better in one day. The longest down time of 19 days was before cycle

five where the initial sulphate concentration was approximately 1850 mg/L, percentages sulphate reduction for the three systems were 97, 88 and 96 % for UI, IP and IX respectively.

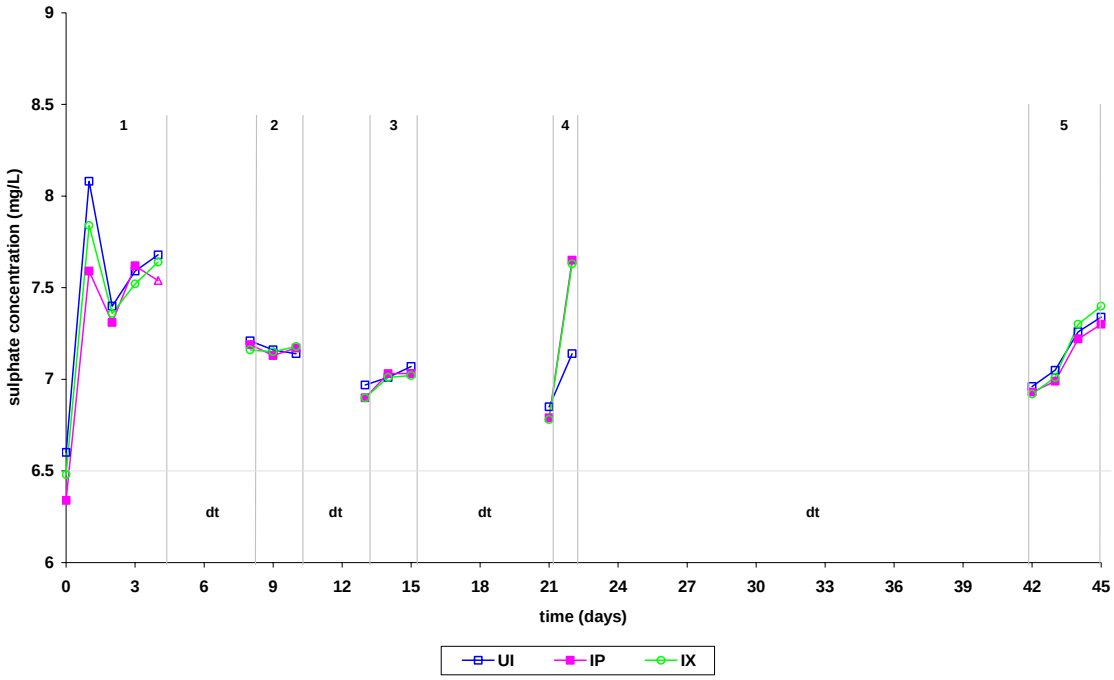

Figure 3.20: pH profiles in multiple cycle and reuse experiments for 5 cycles over 45 days for SRB consortium immobilised in calcium alginate capsules and un-immobilised SRB consortium, dt = down time.

In all the reactors, the pH was maintained above 6.5 as shown in figure 3.20. The optimum pH range for SRB growth is 6.5 to 8.5. SRB growth has been reported to be inhibited at pH values below 6 and above 9 (Widdel, 1988). The monitoring of pH, therefore provides an important indication on the state of an SRB reactor. A healthy and active SRB system will increase or maintain the pH of the aqueous environment in which it is found at above 6.5. Bicarbonate alkalinity generated by an active system helps to buffer the aqueous environment and increase the pH.

The pH profiles, sulphate reduction and sulphide generation profiles all provide an indication of satisfactory performance of the un-immobilised and capsule-immobilised SRB consortium. The rates shown in tables 3.3 and 3.4 suggest good biocatalytic activity by the immobilised systems which compares well with the un-immobilised system.

Table 3.3: Average rates of sulphate reduction for sulphate-reducing bacterial consortium immobilised in calcium alginate capsules.

Cycle	Down time before cycle (days)	Duration of cycle (days)	Average Initial sulphate concentration (mg/L)	Average rate of sulphate reduction (mg SO_4^{2-}/L/day)			Average rate of sulphate reduction (per g initial wet mass) (mg SO_4^{2-}/L/day/g)		
				UI	IP	IX	UI	IP	IX
1	0	4	1850	282	214	283	26	19	26
2	3	2	1150	468	649	577	43	58	52
3	2	2	1230	576	598	596	52	54	54
4	5	1	1320	695	620	613	63	56	56
5	19	3	1850	586	560	583	53	51	53

The trend observed for sulphide generation in figure 3.19 was one of increasing amount of sulphide generation over the five cycles for all three systems, which suggested an improvement in the SRB consortium's performance over time despite all the variable conditions of down time and initial sulphate concentrations and cycle duration that were applied to the systems. However the sulphate rates (table 3.3) and sulphide and alkalinity rates in table 3.4 seemed to reach a steady value and showed no increase after that.

Table 3.4: Percentage sulphate reduction and average rates of sulphide and alkalinity generation for sulphate-reducing bacterial consortium immobilised in calcium alginate capsules.

Cycle	Percentage sulphate reduction (%)			Average of sulphide generation (mg HS^-/L/day)			Average rate of sulphide generation (per g initial wet mass) (mg HS^-/L/day/g)			Average rate of alkalinity generation (mg $CaCO_3$/L/day)			Average rate of alkalinity generation (per g initial wet mass) (mg $CaCO_3$/L/day/g)		
	UI	IP	IX	UI	IP	IX	UI	IP	IX	UI	IP	IX	UI	IP	IX
1	80	54	79	79	16	91	7.0	1.4	8.2	288	100	350	34	10	31
2	100	98	96	196	232	225	16.2	20.9	20	1180	1115	1050	150	101	96
3	95	96	96	229	224	208	20.1	20.1	18.5	1145	1080	1080	104	98	98
4	99	98	97	283	264	266	25.1	23.9	24	2050	2970	2950	93	135	132
5	97	88	84	203	183	210	17.5	16.4	18.6	623	675	655	109	93	98

There were no major differences in activity between the free un-immobilised SRB consortium and the two immobilised SRB consortium systems when considering the rates calculated in tables 3.3 and 3.4. This suggests that no sulphate reducing activity was lost through the immobilisation process. It also suggests that there was no marked improvement in working with immobilised SRB consortium in fed-batch shaker flask systems. The crucial point that was noted in these experiments was that the immobilised SRB consortium did not lose their biocatalytic activity. The major advantage of this and the immobilisation process would be evident in column systems as the shaker flasks are not in anyway representative of the proposed methods of application of the calcium alginate capsules.

Sulphate reduction and sulphide generation rates given in tables 3.3 and 3.4 suggest good growth of the SRB consortium encapsulated in calcium alginate membranes cross-linked with either xanthan gum or poly-L-lysine. These rates are comparable and in most cases better than those for free suspended cells as determined and reported in table 2.3.

Results in tables 3.3 and 3.4 show that there was little or no increase in sulphate reduction or sulphide production from the second cycle onwards all these remained relatively steady. These results indicate that increasing cell mass either does not take place or if it does, it does not result in continuously increasing sulphate reduction. This observation was not expected and was not resolved in this study

3.3.2.3 Integrity of calcium-alginate capsules

No obvious signs of deterioration of the calcium alginate-xanthan gum capsules were observed during the five cycles and 45-day period of the study. The maintenance of sulphate reduction activity suggested that the immobilisation process did not inhibit SRB performance within the capsules. However, the Ca-alginate-poly-L-lysine capsules were not found to be as robust as the Ca-alginate-xanthan gum capsules. There were signs of deterioration of the capsules, as evidenced by the presence of support matrix debris and SRB in solution became apparent by the third cycle (figure 3.21). Figure 3.22 shows more detail of the deteriorating Ca-alginate-poly-L-lysine capsules. The cracks in the membrane suggest lack of elasticity within the semi-permeable membrane, which resulted in breakage and release of capsule contents.

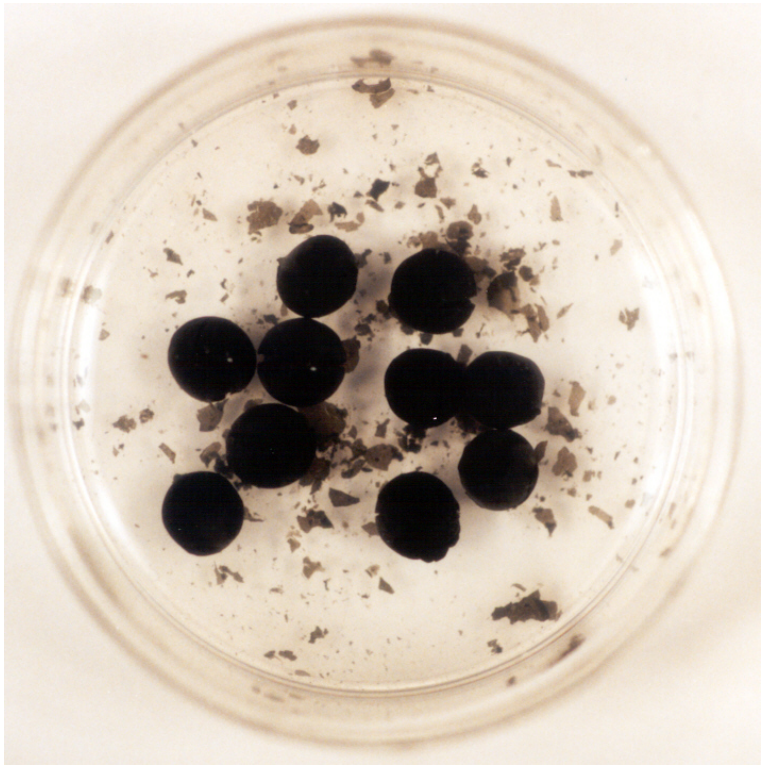

Figure 3.21: Calcium-alginate-poly-L-lysine capsules showing signs of deterioration by the third cycle in the course of the multiple cycle and reuse study.

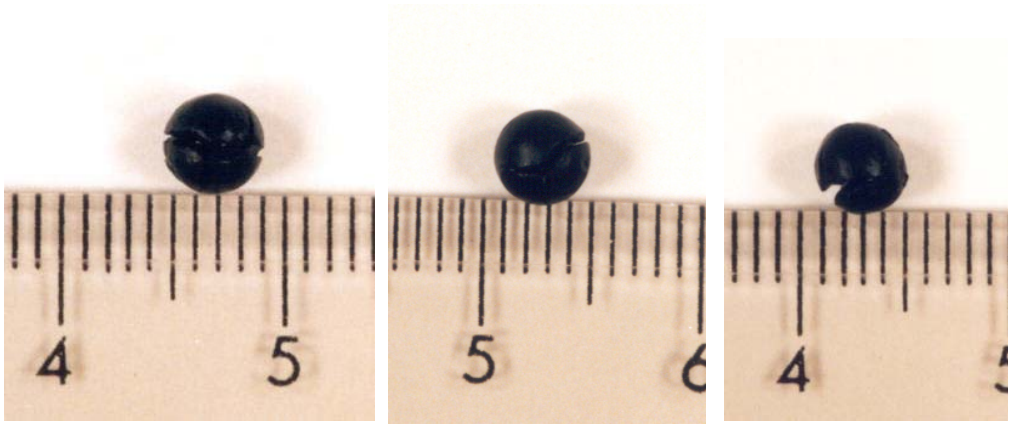

Figure 3.22: Close-up view of calcium alginate-poly-L-lysine capsules breaking up by the third cycle in the course of the multiple cycle and reuse study.

3.3.2.4 Fed-batch, 48-hour shaker flask experiments.

Previous results suggested that most of the SRB activity occurred within the first two days of start up, so a 48 hour experiment was set up using immobilised and un-immobilised SRB to monitor the progress of sulphate reduction and sulphide generation much more closely in the

initial 24 hours of the experiment, and then determine pH, sulphate and sulphide concentrations after a further 24 hours of incubation.

The sulphate reduction and sulphide generation graphs for the 48-hour experiment are shown in figure 3.23 and the pH profile is shown in figure 3.24. In the first 24 hours there was little to no differences in the rate of sulphate reduction between the un-immobilised and immobilised SRB consortium. The average sulphate reduction rates after 24 hours were 22.5, 20.4 and 21.3 mg SO_4^{2-}/L/h for UI, IP and IX respectively (table 3.5). After 48 hours, the rates of sulphate reduction for UI and IP were similar at 11.9 and 13.5 mg SO_4^{2-}/L/h respectively compared to that for IX, which was considerably higher at 21.3 mg SO_4^{2-}/L/h.

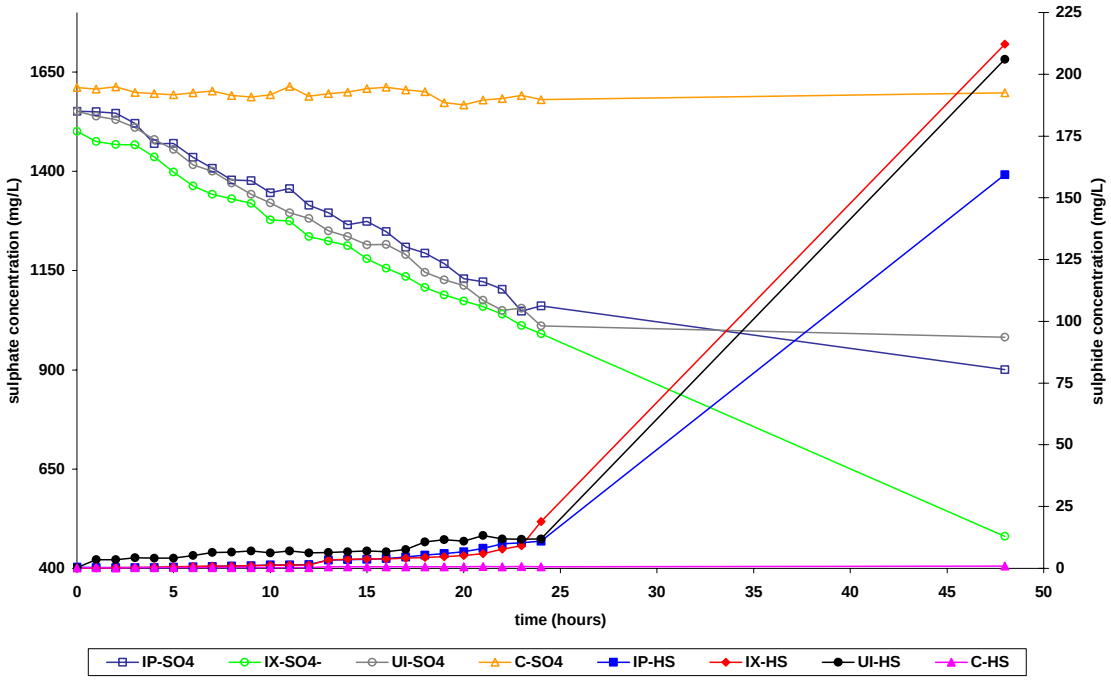

Figure 3.23: Sulphate reduction and sulphide generation profiles over 48 hours for SRB consortium immobilised in calcium alginate capsules and un-immobilised SRB consortium and a negative control (C) in 500 mL fed-batch shaker flask reactors.

Approximately 30 % sulphate reduction occurred in the first 24 hours for the three systems, however the corresponding sulphide concentrations detected were comparatively much lower and did not reflect the 30 % sulphate reduction. After 48 hours, the average percentage sulphate reduction was 42, 37 and 53% for UI, IP and IX respectively, and the sulphide concentrations determined were more reflective of the sulphate reduction at this point. Sulphide concentrations were 206, 159 and 212 for UI, IP and IX respectively.

The pH profile (figure 3.22) shows that the pH was maintained above 6.5 and steady increases in pH value only began after approximately 14 hours with the exception of the un-immobilised reactor. Gradual but consistent increases in pH value in the un-immobilised reactor started after approximately 16 hours.

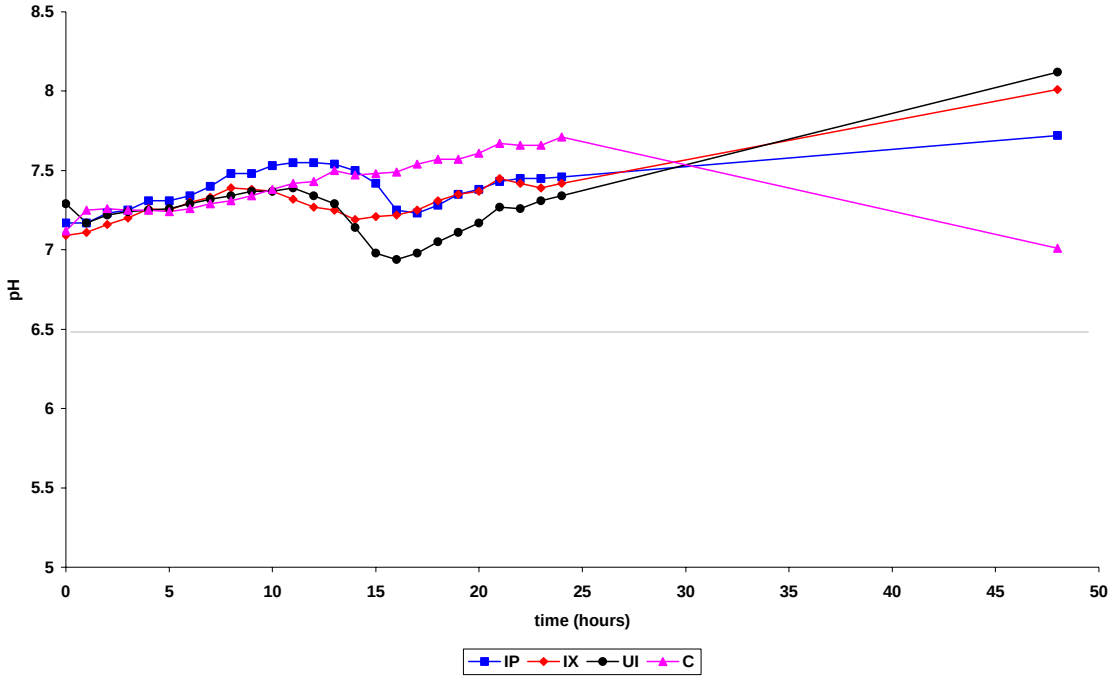

Figure 3.24: pH profiles over 48 hours for SRB consortium immobilised in calcium alginate capsules and un-immobilised SRB consortium and a negative control (C) in 500 mL fed-batch shaker flask reactors

Several factors including initial pH and anoxic conditions probably contribute to how a given reactor starts up, as does the acclimatisation of the biomass to the aqueous environment. The multiple cycle and reuse show slow start up for the immobilised and un-immobilised SRB consortium biomass in the first cycle (figure 3.18) compared to subsequent cycles where initial sulphate and sulphide rates were observed to be rapid (see table 3.3). The 48-hour experiment carried out showed that sulphate activity seemed to start almost immediately (figure 3.23) however, sulphide was only detected about 16 to 18 hours after the start of the experiment. Sulphide generation between 24 and 48 hours was found to occur rapidly for the un-immobilised and immobilised SRB consortium. As expected, the negative control showed no sulphate reduction and no sulphide generation.

The rates of sulphate reduction and sulphide generation for the 48-hour experiment are given in table 3.5.

Table 3.5: Rates of sulphate reduction, sulphide generation and percentage sulphate reduction over 48 hours for SRB consortium immobilised in calcium alginate capsules and un-immobilised SRB consortium.

Time (hrs)	Average rate of Sulphate reduction (mg SO_4^{2-}/L/h)			Average rate of Sulphide generation (mg HS^-/L/h)			Average percentage sulphate reduction (%)		
	IX	IP	UI	IX	IP	UI	IX	IP	UI
1st 24	21.3	20.4	22.5	0.8	0.5	0.5	40	32	35
2nd 24	12.1	6.7	1.2	6.2	8.0	8.1	29	15	3
48	16.7	13.6	11.9	4.8	3.6	4.5	53	42	37

The rates given in table 3.5 showed that the rate of sulphate reduction in the first 24 hours was highest for all three systems but overall, IX performed the best of the three systems, UI, IP and IX in the 48-hour experiment. IP always performed worse than both IX and UI.

Due to the fragile nature and breakage of both calcium alginate beads (after two cycles, ~ 13 days) and calcium-alginate-poly-L-lysine capsules (after two cycles, ~ 13 days), all subsequent capsule production and experiments utilised SRB consortium immobilised in calcium-alginate-xanthan gum capsules only.

3.3.2.5 Fed-batch packed column studies

The move from shaker flasks to packed column systems was a pivotal study in the evaluation of the capsule system for the proposed bioprocess application. While the fed-batch, shaker flask-type systems are generally not the form of bioprocess application to be used in industry, They provide an initial indication of success for the continuous flow, static packed bed column-type set up which does simulate the industrial scale bioprocess application. The most commonly used type of column reactor in industrial bioprocess application of SRB is the UASB reactor.

Since immobilisation of SRB was geared toward bioprocess application of the SRB in continuous column reactor systems, it was important to move from fed-batch shaker flasks to packed column studies once the suitable form of the biomass had been identified and its performance evaluated.

Sulphate reducing bacterial consortium immobilised in calcium alginate-xanthan gum capsules were found to be the most robust of the immobilised SRB consortium systems investigated in shaker flask studies. Good biocatalytic activity in both fed-batch and multiple cycle and reuse studies was also shown. Sulphate reducing bacterial consortium immobilised in calcium alginate-xanthan gum capsules were therefore, identified as the most suitable form of SRB biomass for continued performance evaluation in packed column studies.

Initial packed column reactors were set up as fed-batch column systems. There were reactors of two different volumetric sizes, approximately 250 mL (small) and 1.5 L (large), both with a HRT of approximately 1.7 hours.

Figures 3.25 and 3.26 are representative of the sulphate reduction activity profiles observed for the fed-batch column studies. The results shown in figures 3.25 and 3.26 are averages of two duplicate experiments.

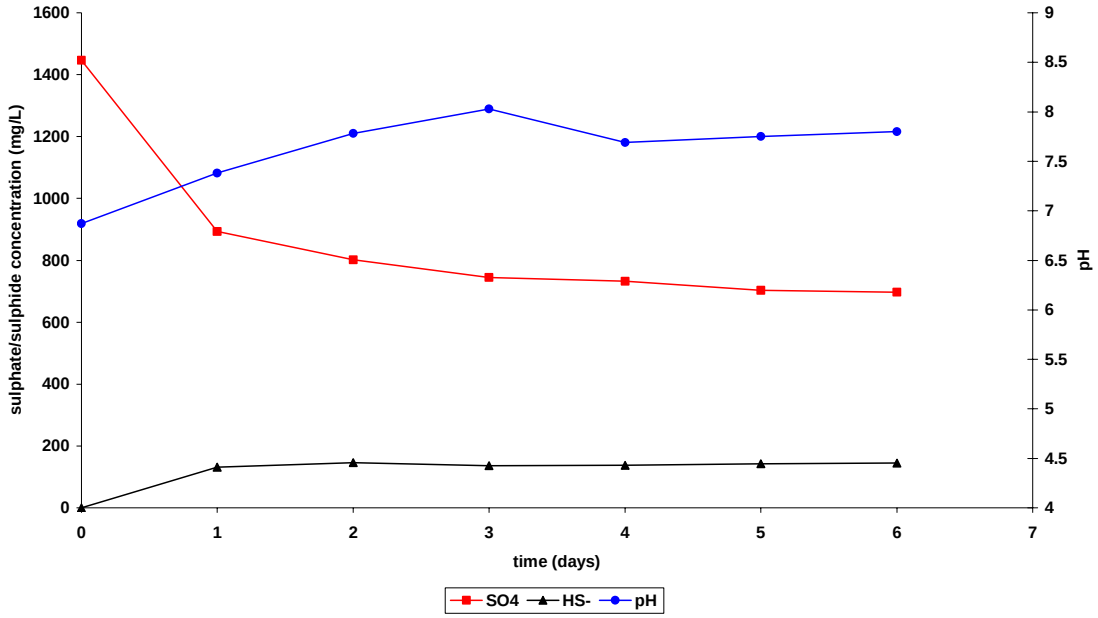

Figure 3.25: Sulphide generation, sulphate reduction and pH profile for calcium-alginate-xanthan gum immobilised SRB consortium in a 250 mL packed column reactor.

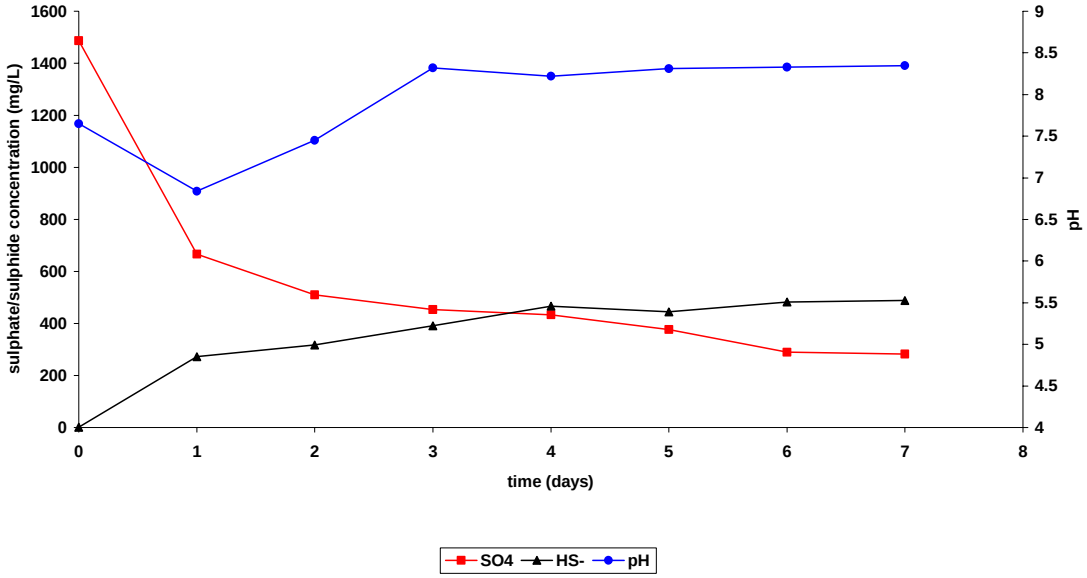

Figure 3.26: Sulphide generation, sulphate reduction and pH profile for calcium-alginate-xanthan gum immobilised SRB consortium in a 1.5 L packed column reactor.

Figure 3.25 gives the profiles for the smaller 250 mL reactor and figure 3.26 shows the profiles for the larger 1.5 L reactor. Although the trends for sulphate removal, sulphide generation and pH were similar for both figures 3.24 and 3.25, there were some differences in sulphate reduction performance. Sulphate concentration in the larger reactor was lowered from 1500 mg/L to below 600 mg/L by the second day, approximately 66 % sulphate reduction on day two. Whereas in the smaller reactor, sulphate concentration was not lowered to below 700 mg/L until day 6, and the average percentage sulphate reduction was 45 % on day two. The sulphide generation curves reflect the difference observed in sulphate reduction, sulphide generated in both reactors on day two was 146 mg/L and 318 mg/L for the small and large column reactors respectively. The pH values in both reactors were maintained between 6.5 and 8.5 with the pH values in the large reactor being maintained above 8.0 from day three.

Several fed-batch packed column reactors were run in the course of this study. Table 3.6 gives the rates of sulphate reduction and sulphide generation for figures 3.25 and 3.26 and a second set of fed-batch packed column studies for which the graphs were not shown. Maximum rates are calculated from regions on the curve of greatest slope. The calculated ratio of initial biomass wet mass to volume given in table 3.5 gives an indication of the initial biomass concentration in each packed column.

Table 3.6: Rates of sulphate-reducing activity for sulphate-reducing bacterial consortium in fed-batch packed column reactors

			Sulphate		Sulphide	
Reactor size	**Ratio of initial wet mass / volume (g/L)**	**Max. rate SO_4^{2-} reduction (mg SO_4^{2-} /L/day)**	**Max. rate SO_4^{2-} reduction (mg SO_4^{2-} /L/day/g initial wet mass)**	**Final percentage SO_4^{2-} reduction (%)**	**Max. rate H_2S generation (mg/L/day)**	**Max. rate H_2S generation (mg/L/day/g initial wet mass)**
250 mL A*	16	234	65	52	29	7.9
250 mL B	24	781	121	92	116	18
1.5 L A*	13	345	17	81	130	6.5
1.5 L B	12	122	7	44	32	1.8

* data for figures 3.25 and 3.26

The data contained in table 3.6 show that the smaller reactors had better rates of activity when compared to the large reactors. However, the large reactor 1.5 L A's maximum sulphate reduction rate per unit wet mass (345 mg SO_4^{2-}/L/day) was more than double that of the other large reactor (122 mg SO_4^{2-}/L/day) but was in turn much lower than the values determined for both small reactors. This is probably due to the fact that the larger reactors contained more un-productive reactor volume compared to the smaller reactors e.g the large reactors had about 12 or 13 g of biomass per L of media compared to 16 or 24 g of biomass per L of media for the small reactors. The use of smaller columns meant minimized un-productive reactor volume. The large differences in the activity rates for the small reactors may have been due to the differences in the initial biomass wet mass.

3.3.2.6 Continuous up-flow packed column studies

The results achieved with immobilised SRB consortium capsules in fed-batch packed column studies showed good sulphate reducing activity (table 3.6), and therefore the packed column systems were set up for continuous up-flow studies in order to simulate industrial scale process application.

The continuous up-flow packed column reactors were designed to eliminate "wasted" volume in the reactor where there were no capsules present. The reactors' volumetric size was described in terms of a "working volume" which was defined as the volume in the column

occupied by the capsules. The average working volume of the continuous up-flow packed columns was 59 mL run at a flow rate of 0.4 mL/min and a HRT of approximately 2.45 hours.

Figures 3.27 and 3.28 show the profiles for sulphate reduction and sulphide generation in continuous up-flow packed columns with immobilised SRB consortium capsules. The experiments were run with 2 L of medium and when that was exhausted, the reactor was washed with 200 mL of a 0.5 % calcium chloride solution and new 2 L of media was passed through the column. The results in figure 3.27 are the average of three separate experiments with standard deviation error bars.

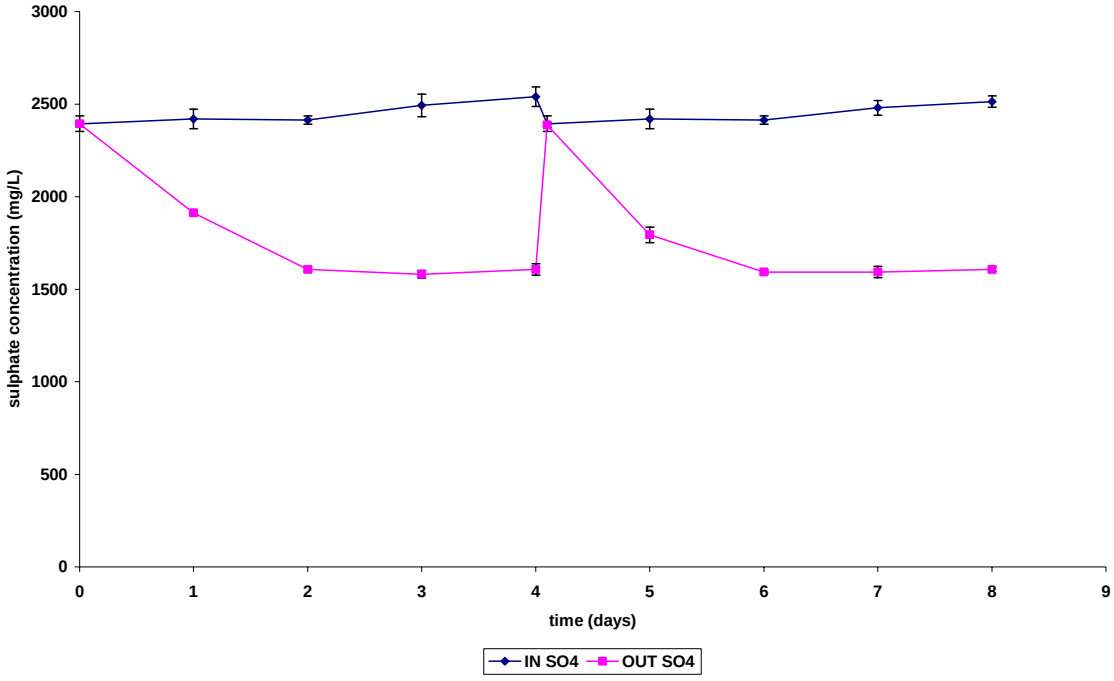

Figure 3.27: Influent and effluent sulphate concentrations for immobilised SRB consortium capsules in a continuous up-flow packed column reactor.

In the first continuous cycle, there was a gradual decrease in effluent sulphate concentration until day two after which an equilibrium effluent sulphate concentration value of about 1600 mg/L emerged (compared to the influent sulphate concentration of approximately 2500 mg/L, a difference of 900 mg/L or 36 % sulphate removal). The relatively constant difference in influent and effluent sulphate concentration from day two suggested that the system would have reached a steady state. On re-starting the continuous column with new media, the trend observed for sulphate removal was very similar to the first cycle. After two days the system again reached equilibrium at approximately 1600 mg/L effluent sulphate, which represented

36 % sulphate removal, and, again, the system had probably reached steady state sulphate removal. The average rates of sulphate removal in the two cycles from day two (steady state) are given in table 3.7 and were 884 mg SO_4^{2-}/L/day and 871 mg SO_4^{2-}/L/day for cycle one and two respectively.

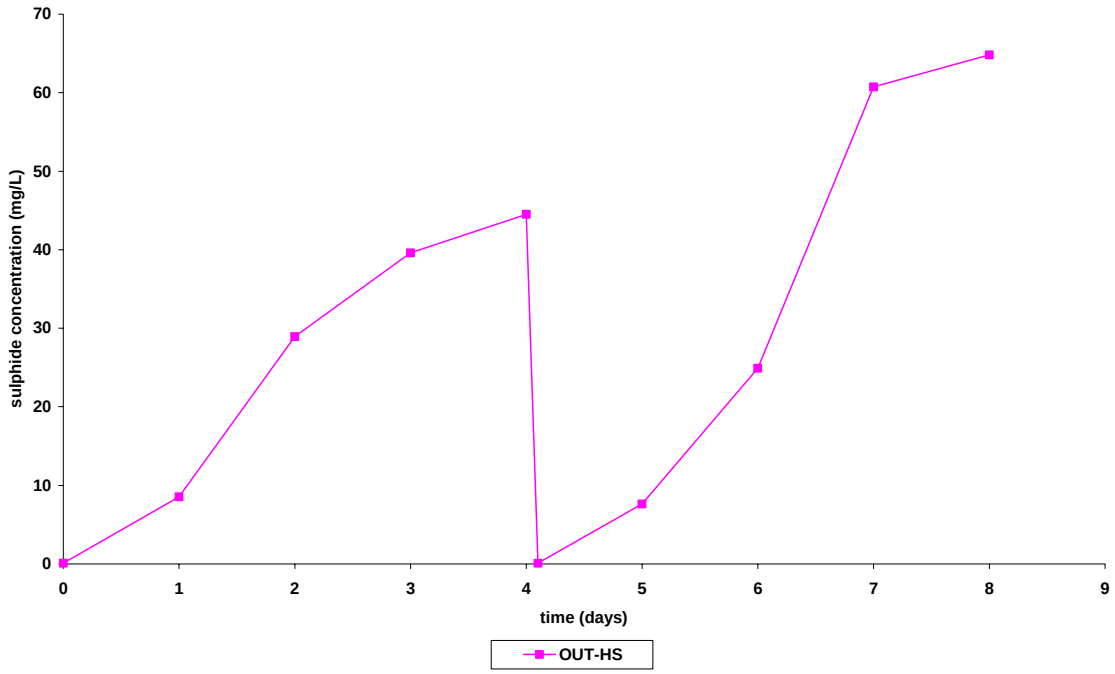

Figure 3.28: Influent and effluent sulphide concentrations for immobilised SRB consortium capsules in a continuous up-flow, packed column reactor.

As expected, the influent sulphide concentration was negligible at approximately 0.1 mg/L, which is the Spectroquant limit of detection for sulphide and was not shown in figure 3.29. The trend for sulphide concentration in the effluent complimented the trend observed for sulphate reduction. The maximum detectable sulphide concentrations observed were approximately 45 mg/L and 65 mg/L in cycle one and two respectively, which was an unexpected result considering the much higher values determined for the fed-batch column systems (figure 3.25 and table 3.5). However, the continuous reactor system set-up makes monitoring and detection of all the hydrogen sulphide generated difficult. Samples taken at the effluent point came into contact with air and some sulphide is lost. Therefore, the detected sulphide was determined from the dissolved sulphide in the effluent and the sulphide in the zinc acetate trap.

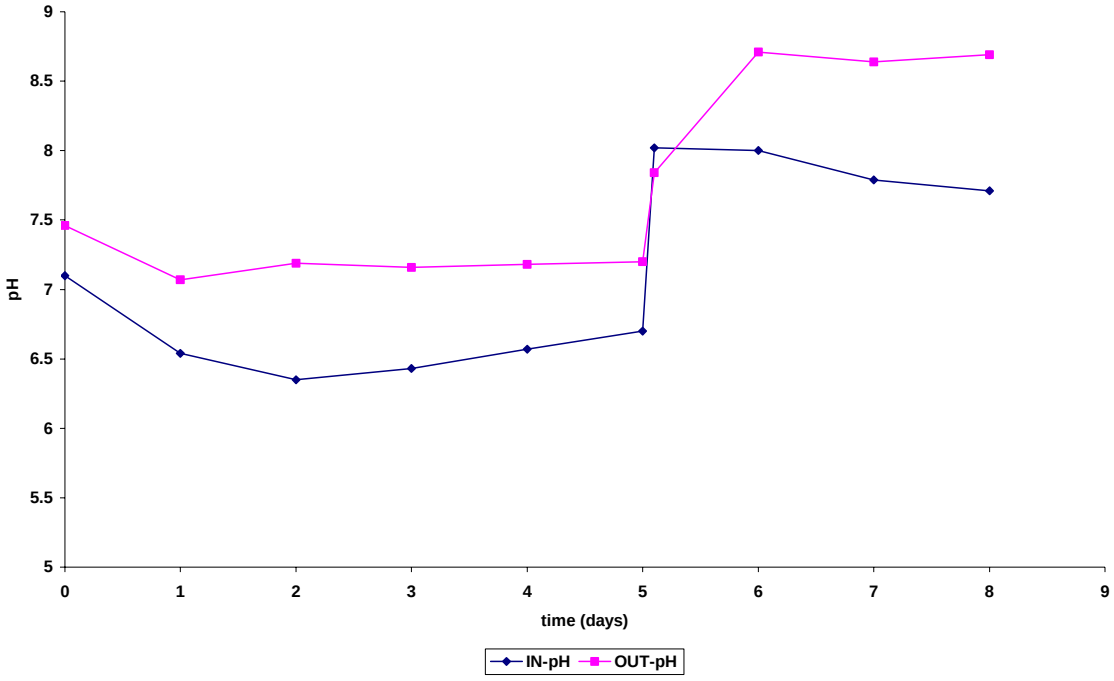

Figure 3.29: Influent and effluent pH values for immobilised SRB consortium capsules in a continuous up-flow, packed column reactor.

Figure 3.30 shows the influent and effluent pH values for the continuous up-flow packed bed reactor with immobilised SRB consortium capsules. Active SRB generate bicarbonate (and sulphide) alkalinity which contributes to the buffering of the aqueous environment and increase in pH. Therefore the observed increase in pH between the influent and effluent was expected as active SRB activity had been confirmed by the sulphate reduction and sulphide generation results. The profiles in figure 3.30 show that although the new media had a different influent pH, the system was again seen to increase the effluent pH and maintain it above the influent pH for the duration of the experiment.

Results showing the calculated changes in sulphate and sulphide concentrations between the influent and effluent streams for this experiment are given in figure 3.31. The graphs showed that sulphate reduction for the two cycles reached steady state from about day three, at approximately 900 mg/L. The change in sulphide concentration was approximately 45 mg/L in the first cycle and 65 mg/L in the second cycle, but as mentioned earlier, accounting for all the sulphide generated in the continuous set up was difficult.

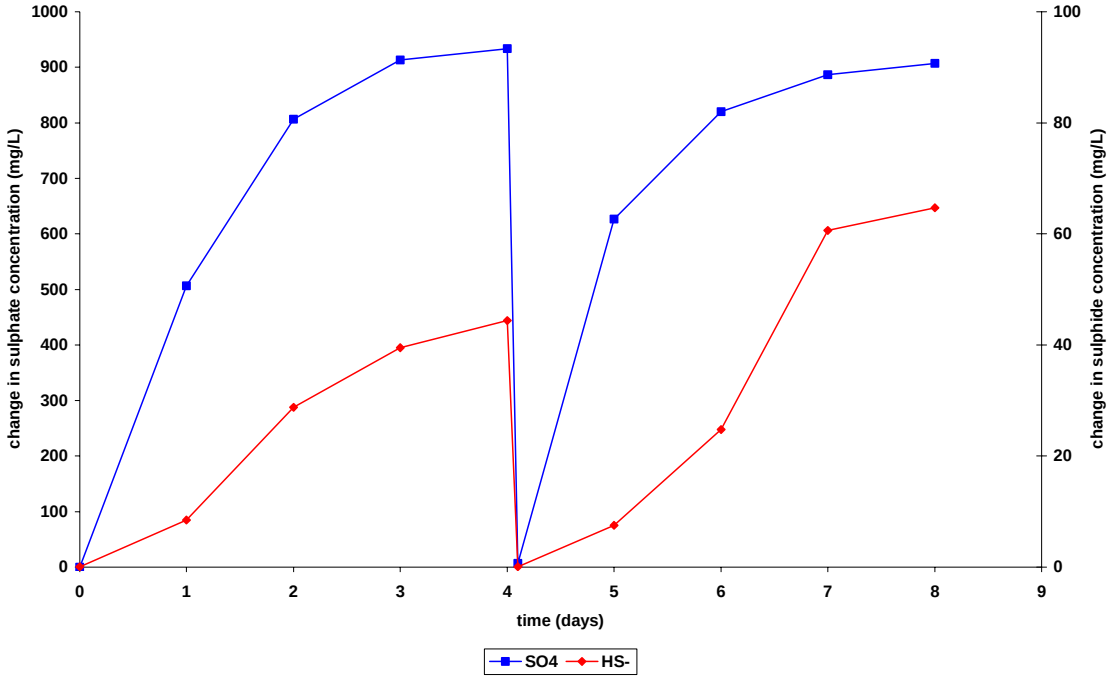

Figure 3.30: Change in influent and effluent sulphate and sulphide concentrations for immobilised SRB consortium capsules in a continuous up-flow, packed column reactor.

Table 3.7 shows that the sulphate reduction and sulphide generation rates for the immobilised SRB consortium capsules in continuous up-flow, packed column reactors was higher than rates reported for fed batch packed column systems in table 3.6 and the different immobilised fed-batch shaker flask systems in table 3.3. These rates are also much higher than those reported for free suspended cells as reported in table 2.1. The only comparable rates are those in table 2.3 calculated for free suspended SRB consortium in multiple cycle and reuse experiments.

Table 3.7: Rates of sulphate reduction and sulphide generation and percentage sulphate reduction for immobilised SRB consortium capsules in a continuous up-flow, packed column reactor.

	Ave. rate SO_4^{2-} reduction (mg SO_4^{2-} /L/day)	**Max. rate H_2S generation (mg HS^-/L/day)**	**Ave. percentage SO_4^{2-} reduction (%)**
Cycle 1	884	38	36
Cycle 2	871	76	36

3.4 Conclusions

The main bottleneck expected in the production of SRB capsules was in the generation of adequate quantities of biomass. Sulphate-reducing bacteria are slow growing (Zehnder, 1988) and it was necessary to have several cell-generating reactors running at any one time to ensure the availability of sufficient biomass.

The cross-linked matrix of calcium-alginate beads was found to be susceptible to disruption by any metal chelating groups present in solution. Sulphidogenic conditions also appeared to affect the integrity of the calcium-alginate beads over a relatively short time (less than 15 days). Therefore, although cell immobilisation by entrapment on calcium alginate beads was simple in effort and mild on the biomass, it was found to be unsuitable for the immobilisation of SRB as the matrix was not robust enough to withstand mechanical damage and the harsh sulphidogenic conditions associated with SRB. There was little to no difference in the sulphate reducing activity of calcium-alginate beads made from SRB consortium grown in the absence or presence of iron, the sulphate reduction rates for SRB consortium grown in the absence or presence of iron were 168 mg SO_4^{2-}/L/day and143 mg SO_4^{2-}/L/day respectively (table 3.2).

In immobilisation by encapsulation, a mixture of the cells, calcium chloride and xanthan gum were added drop-wise into a mixture of sodium alginate and a surfactant (Tween 20), which in effect reversed the procedure used for the production of alginate beads. The result is that on contacting the alginate solution the calcium cations on the outside of the droplet immediately cross-link to form a calcium-alginate semi-permeable membrane. The method involving poly-L-lysine is slightly different, but the xanthan gum and poly-L-lysine served similar functions in both cases. The xanthan gum and poly-L-lysine are able to interact with any exposed alginate groups on the calcium-alginate membrane matrix, which have not participated in the cross-linking process. This ensures that there are no exposed groups in the matrix that are available for attack by chelating and other groups. Xanthan gum and poly-L-lysine also added elasticity to the calcium-alginate matrix, allowing some expansion by the membrane, which is important for cell growth, hence improving the cell loading capacity of the capsules. The surfactant, Tween 20, was used to partially open up the membrane matrix and allow easier gaseous and nutrient exchange between the inside of the capsule and its immediate environment. Therefore the cross-linking groups in the matrix were never exposed

for attachment by chelating agents. No experiments were carried out to determine the diffusion limitation in the beads as no hindrance to the sulphate reduction and sulphide generation processes was observed.

Due to the more involved process used in the two-step pregel dissolving immobilisation method for the calcium-alginate-poly-L-lysine encapsulation, there is more room for error. The time given to redissolve the inner calcium-alginate layer affected the thickness of the membrane, and over-exposure resulted in weaker membranes that were fragile and therefore, broke up in the sulphidogenic systems. The two-step pregel system was therefore found to be unsuitable for SRB immobilisation.

Immobilisation of SRB by encapsulation within a calcium-alginate-xanthan gum semi-permeable membrane was found to give robust immobilisation matrices for SRB. The capsules were found to be able to last up to 45 days with regular handling and exposure to air for short periods of time. The permeability problems encountered with the xanthan gum capsules appeared to have been solved by adjusting the constituents' concentrations. Concentrations of the calcium and alginate and the Tween 20 affect the resulting membrane, cross-linking that is too tight results in an impermeable membrane and the build-up of gas within the capsules which results in the capsules floating and eventually bursting and releasing their contents.

Application of the calcium-alginate immobilised SRB capsules in continuous systems gave promising results. There was removal of 36 % of sulphate from solution in a 56 mL column at a HRT of 2.3 hours. Steady state was reached after approximately 30 HRTs on day three.

Although some reactor systems had longer lag periods at start up, none of the immobilisation methods seemed to have any detrimental effects on the activity of the biomass. Sulphate reduction and sulphide generation was observed in all the relevant studies. Studies with the immobilised SRB in columns in both fed-batch and continuous systems gave clear results of sulphate reduction and sulphide generation, and showed promise for application in continuously operated systems.

CHAPTER 4
DISARTICULATED ELECTRON DONOR AND CARBON SOURCE SUPPLY IN THE APPLICATION OF IMMOBILISED SULPHATE-REDUCING BACTERIA:
1. SULPHATE AND NEUTRALISATION TREATMENT OPERATIONS

4.1. Introduction

The results presented in chapter three determined that it was not only possible to immobilise an SRB consortium by encapsulation within a calcium-alginate-xanthan gum membrane, but that it was also possible to maintain good sulphate-reducing performance throughout. With the exception of the continuous up-flow packed column studies, the basic performance of the immobilised SRB consortium system was found to be as effective as the free suspended SRB consortium biomass.

Successful immobilisation of the SRB consortium has been shown to achieve these important objectives:

- The problem of loss of biomass through wash-out in the overflow for continuous bioprocess application, which consequently leads to reduced reactor productivity has been resolved;
- Effective handling and transfer of biomass in multiple cycle and reuse operations has been demonstrated. This has substantial advantages compared to sludge transfer mechanisms used for cell retention in wastewater treatment processes using SRB;
- A mechanism now exists by which both biomass and the organic carbon source may be separated from the treatment stream.

Current application of SRB in wastewater treatment is limited to those that already contain a reasonably high organic load to act as an organic electron donor and carbon source, or where necessary, an organic electron donor and carbon source is supplied and then applied directly to the effluent. The result is an effluent with high residual organic carbon content, which

requires further downstream treatment after the anaerobic treatment process (du Preez and Maree, 1994). The biomass itself also constitutes an organic load on the treated water. This outcome is not acceptable in the treatment of 'clean' stream inorganic wastewaters as it would remove one pollutant, sulphate, metal ions or acidity but introduce a new pollutant, as the residual organic load. du Preez and Maree (1994), Dill *et al.* (1995) and van Houten (1996) have all reported on the use of hydrogen as an electron donor and carbon dioxide or carbon monoxide as a carbon source, but not in the treatment of 'clean' waste streams.

In this chapter a possible solution to the problem of the application of SRB biotechnology to 'clean' stream treatment of aqueous solutions was examined. The concept of disarticulating the electron donor and carbon source supply by the application of the immobilised SRB as introduced in chapter 1 is evaluated.

In order to effect the disarticulation of the electron donor and carbon source coupling, this study looked at providing an organic substrate as a carbon and electron donor source (Pg-C) to a continuous up-flow packed column reactor with calcium-alginate immobilised SRB consortium capsules. Then at given time intervals, switch-back cycle was operated in which an organic medium was replaced with an inorganic medium containing sulphate but no carbon source and hydrogen as the electron donor.

Essentially, this would disarticulate ETLP and SLP but ETLP would be maintained by the external electron donor (hydrogen) provided. The desired effect would be to un-couple the sulphate reduction process from organic substrate oxidation. During this period, it is expected that in the absence of a carbon source, cell growth and cell maintenance processes would gradually slow down and eventually stop, but since sulphate reduction is independent of cell growth, it can be maintained for a period until again carbon is applied and provided that an electron donor if available.

$$4H_2 + SO_4^{2-} + H^+ \rightarrow 4H_2O + HS^- \qquad \Delta G^{\circ\prime} = -152.2 \text{ kJ} \qquad (4.1)$$

Equation 4.1 would, therefore, represent the energy generating process within the cell (Zehnder, 1988).

4.2 Materials and Methods

4.2.1 Chemical reagents and analysis

As previously described in chapter 2

4.2.2 Microorganisms

The SRB consortium E28 used was selected from a number of other culture isolates as previously described in chapter 2.

4.2.3 Experimental set-up for the disarticulation experiments

Experiments were all carried out in duplicate, in glass columns with an average maximum volumetric size of approximately 60 mL with a water jacket to keep the reactors vessels at 30 °C. Capsules of immobilised SRB consortium were placed inside the column with modified Postgate C medium pumped in from the bottom of the column. Initially full medium was pumped in at 0.10 - 0.13 mL/min for a set number of days, after which inorganic medium with a known sulphate concentration or acidified to a given pH with sulphuric acid was pumped in at the same rate. Concurrent with full medium supply, nitrogen gas was bubbled into the reactor to maintain anoxic conditions. On switching over to an inorganic influent stream, nitrogen and hydrogen gas were bubbled in at a maximum pressure of 3-5 kPa. Influent and effluent samples were taken once a day for analysis. Figures 4.1 - 4.3 illustrate the experimental set-up.

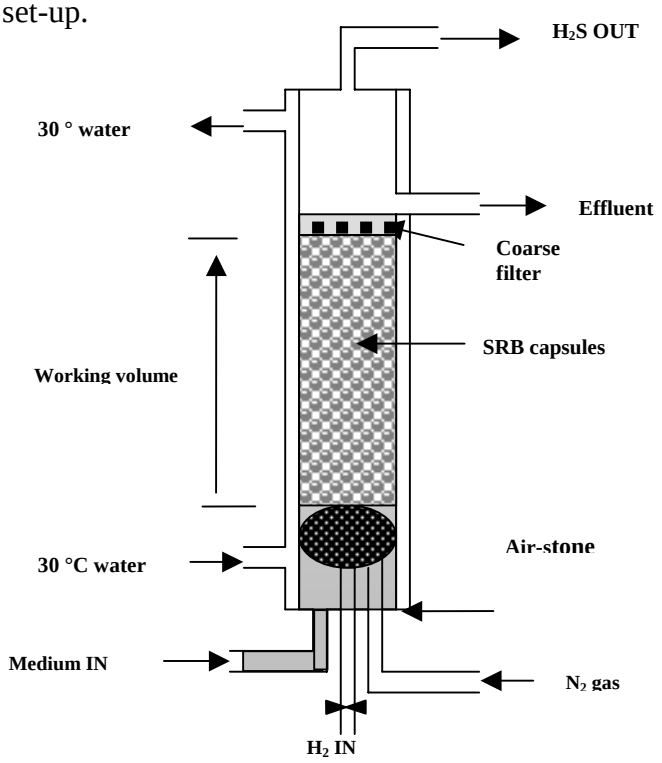

Figure 4.1: Diagrammatic representation of the column configuration for immobilised SRB in a continuous fluidised, up-flow reactor.

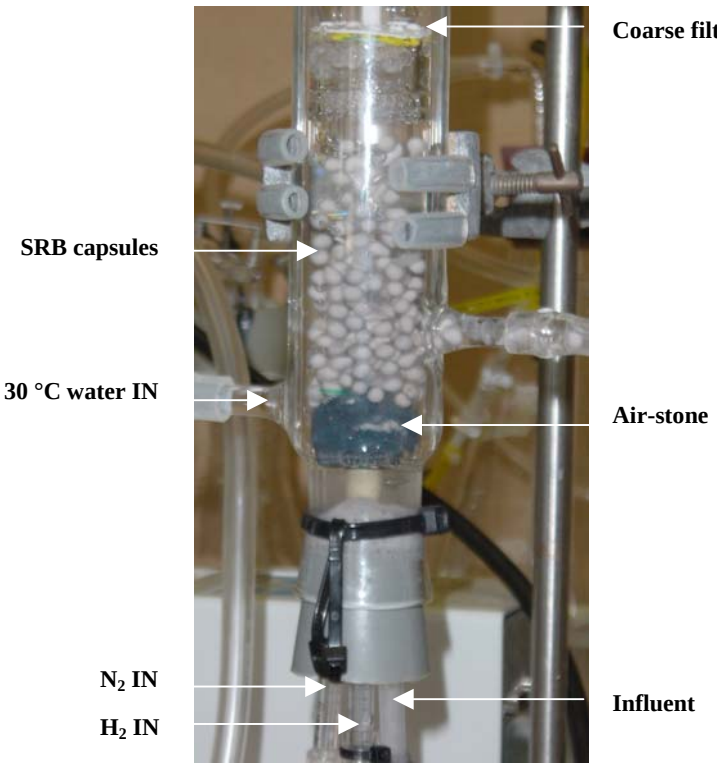

Figure 4.2: Close-up photograph of the column configuration for immobilised SRB in a continuous fluidised, up-flow reactor.

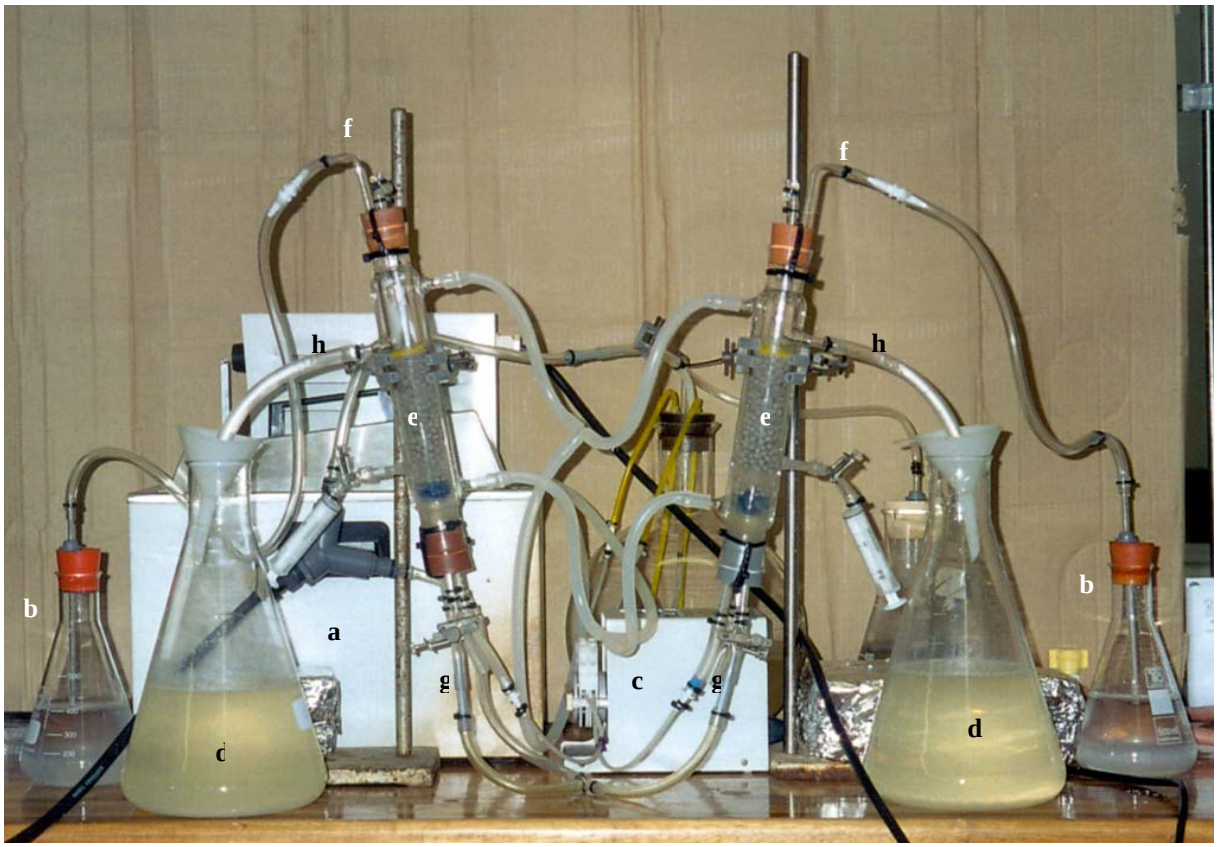

Figure 4.3: photograph showing the experimental set-up for disarticulation and application experiments in duplicate. **a** = 30 °C water bath; **b** = zinc acetate sulphide traps; **c** = peristaltic pump for influent; **d** = effluent; **e** = SRB capsules in column; **f** = sulphide-out port; **g** = medium and gases inlet pipes and port; **h** = sampling ports.

4.3 Results and Discussion

4.3.1 Sulphate treatment

Immobilised SRB consortium with disarticulated electron donor and carbon source supply were evaluated for sulphate removal from aqueous solution, in continuous up-flow packed column reactors. The sulphate reduction profile for the experiment is given in figure 4.4. The periods of carbon supply (full medium) and the periods of electron donor supply alone (hydrogen but no carbon) are labelled 'fm', and 'Hnc' respectively. IN-SO4 represents the influent sulphate concentration and OUT-SO4 represents the effluent sulphate concentration. 'IN-OUT' represents the difference calculated between the influent and effluent concentration.

Two duplicate experiments were carried out and sulphate samples where assayed in triplicate. Rapid sulphate reduction was observed on start-up and the average sulphate reduction rate in the first cycle of full medium was 417 mg SO_4^{2-}/L/day (see table 4.1). On switching to inorganic medium with hydrogen as the electron donor for the next four days, sulphate concentration decreased rapidly and by day two sulphate reduction was near equilibrium state with the average rate of sulphate reduction in the last three days at a maximum average steady state rate of 900 mg SO_4^{2-}/L/day. This steady state sulphate reduction rate was maintained throughout the first cycle of Hnc. So although the cycle was switched back to full medium on day six, the results suggest that it may be possible to disarticulate the carbon and electron donor sources and maintain sulphate reduction activity for a much longer period. After day six, full medium was reintroduced to allow cell growth and cell repair to take place for two days and the average sulphate reduction rate in that time was approximately 350 mg SO_4^{2-}/L/day (table 4.1). However the maximum average sulphate reduction rate calculated for the second full medium period was 820 mg SO_4^{2-}/L/day. The maximum average sulphate reduction rates during steady state for the next two periods of inorganic aqueous streams were 974 and 990 mg SO_4^{2-}/L/day respectively. The overall average sulphate reduction rates for each cycle of carbon supply or electron donor phase are given in table 4.1. The maximum average rate of sulphate reduction for the last cycle of full medium was 1067 mg SO_4^{2-}/L/day.

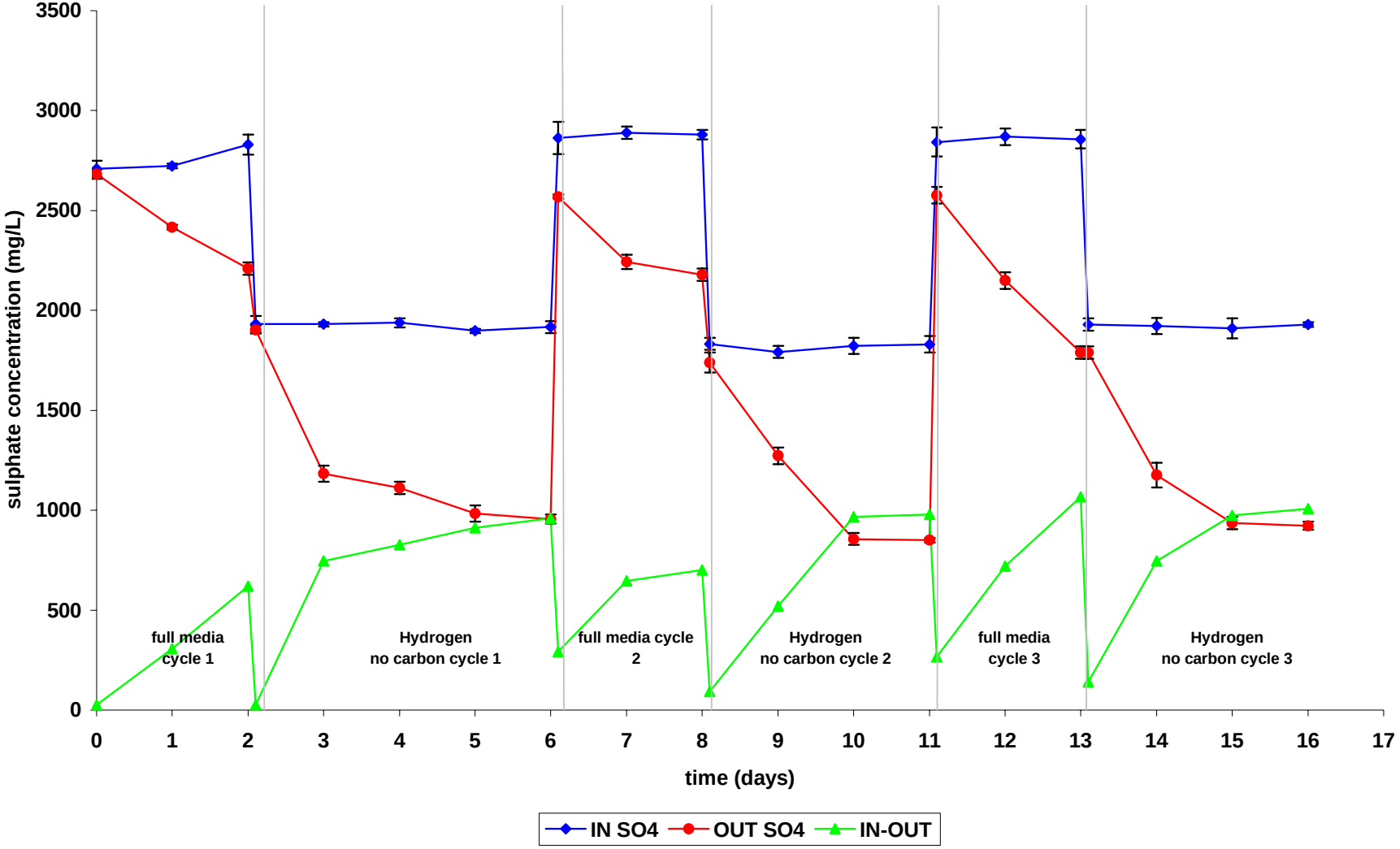

Figure 4.4: Sulphate profile in a disarticulated continuous up-flow packed column reactor with immobilised SRB consortium.

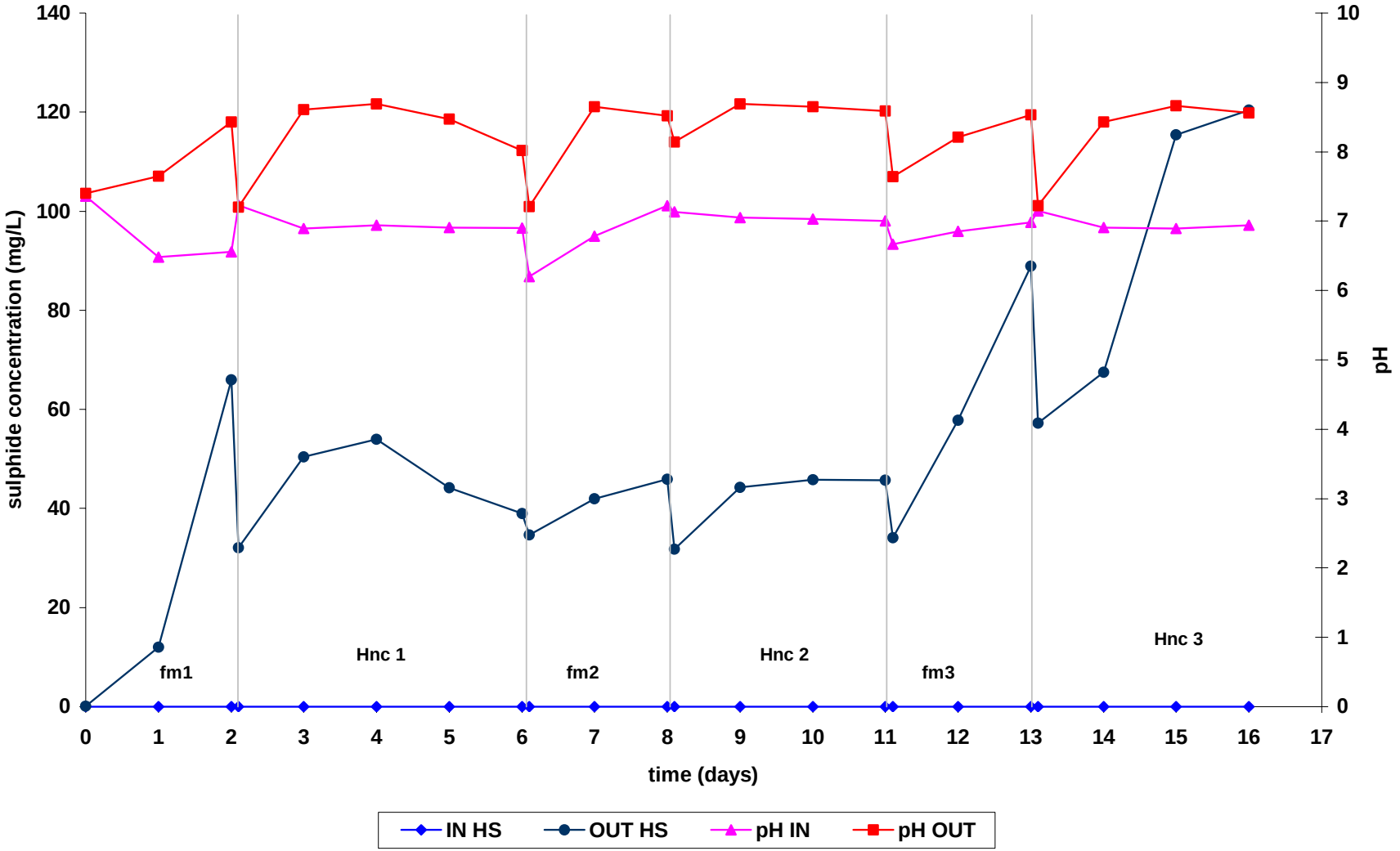

Figure 4.5: Sulphide and pH profiles in a disarticulated continuous up-flow packed column reactor with immobilised SRB consortium.

On day eight, the influent was again switched back to an inorganic stream. The switching between full medium to allow cells to recover and inorganic effluent to determine the sulphate reducing ability was repeated in the experiment and the results show reproducible evidence of the ability of SRB to maintain their sulphate-reducing capacity in the absence of an organic carbon source provided an alternative electron donor source is available, in this case hydrogen gas.

Figure 4.5 gives the corresponding sulphide generation profiles and the pH profiles for the system. The effluent pH was maintained above the influent pH, which is a good indication of an SRB system operating under sulphidogenic conditions. pH values for the effluent were between 7.0 and 9.0. Sulphide generation corresponded well with sulphate removal with higher sulphide generation taking place in the last two cycles. However, compared to flask cultures in which it is much easier to control sulphide release and trapping, in continuous systems this is much more difficult as sulphide is lost around the effluent overflow where sulphide oxidation and loss to the atmosphere may occur.

Table 4.1 shows sulphate reduction rates calculated for the continuously operated system and allowed some comparison between full medium and inorganic medium streams as influents to the reactor. The average rate of sulphate reduction per day was calculated at the end of each cycle, and the average sulphate reduction rates were calculated with respect to the initial wet mass of the capsules.

Table 4.1: Rates of sulphate reduction for disarticulated, sulphate removal experiments

	Average percentage Sulphate removal (%)		**Overall average rate of sulphate removal (mg/L/day)**		**Overall average sulpate removal rate (mg/L/day/g initial capsules wet mass)**	
Cycle	**Full medium**	**Hydrogen & no carbon source**	**Full medium**	**Hydrogen & no carbon source**	**Full medium**	**Hydrogen & no carbon sauce**
1	22	50	310	868	17	49
2	24	54	350	853	20	48
3	37	52	336	956	19	53

Overall rates of sulphate reduction were better for periods with inorganic compared to full medium. These results tended to support the hypothesis that sulphate reduction can be uncoupled from the oxidation of an organic carbon source, and although cell growth probably slows down and may even stop in the absence of a carbon source, there is a period during which the cell is able to sustain sulphate reduction. This critical point at which the change over should occur still needs to be determined, but was not reached in the course of these experiments.

4.3.2 Acidity treatment

The application of the disarticulated immobilised SRB system in the neutralisation of acidic solutions was examined at two pH values, at pH 2.4 and 4.0.

4.3.2.1 pH 2.4

The changes in influent and effluent pH and alkalinity are given in figure 4.6. In the first two days, the influent was full medium and the expected pH profile was observed where the effluent pH was higher than the influent pH and was maintained between 6.5 and 8.5, a good indication of sulphidogenic activity. On day three, the influent stream was switched to an inorganic acidic stream with a pH value of approximately 2.4 and within a day the effluent pH value was above 7.5. The immobilised SRB were able to maintain the pH above 6.5 for four days after which the pH started to decrease. The pH value had dropped to below 6.0 on day five of exposure to the acid stream. The influent was, therefore, switched back to full organic full medium for two days to allow the SRB to recover. On day 10, the influent stream was, again, switched over to the inorganic acidic stream and the trend was similar to the first inorganic stream cycle. The effluent pH value was raised to higher than 7.5 and this was maintained for approximately four days and on day five the effluent pH value dropped to below 6 and the influent stream was again switched back to the full organic medium.

It is possible to account for the observed trends in the acidic aqueous solutions, by considering the reactions occurring in SRB systems. Assuming complete oxidation of an organic substrate such as lactate, the following equations reaction would take place:

$$2CH_3CHOHCOO^- + 3SO_4^{2-} \rightarrow 6HCO_3^- + 3HS^- + H^+ \quad (4.2)$$

Where hydrogen is the sole electron donor and in the absence of a carbon source, the reaction would be:

$$4H_2 + SO_4^{2-} + H^+ \rightarrow 4H_2O + HS^- \quad (4.3)$$

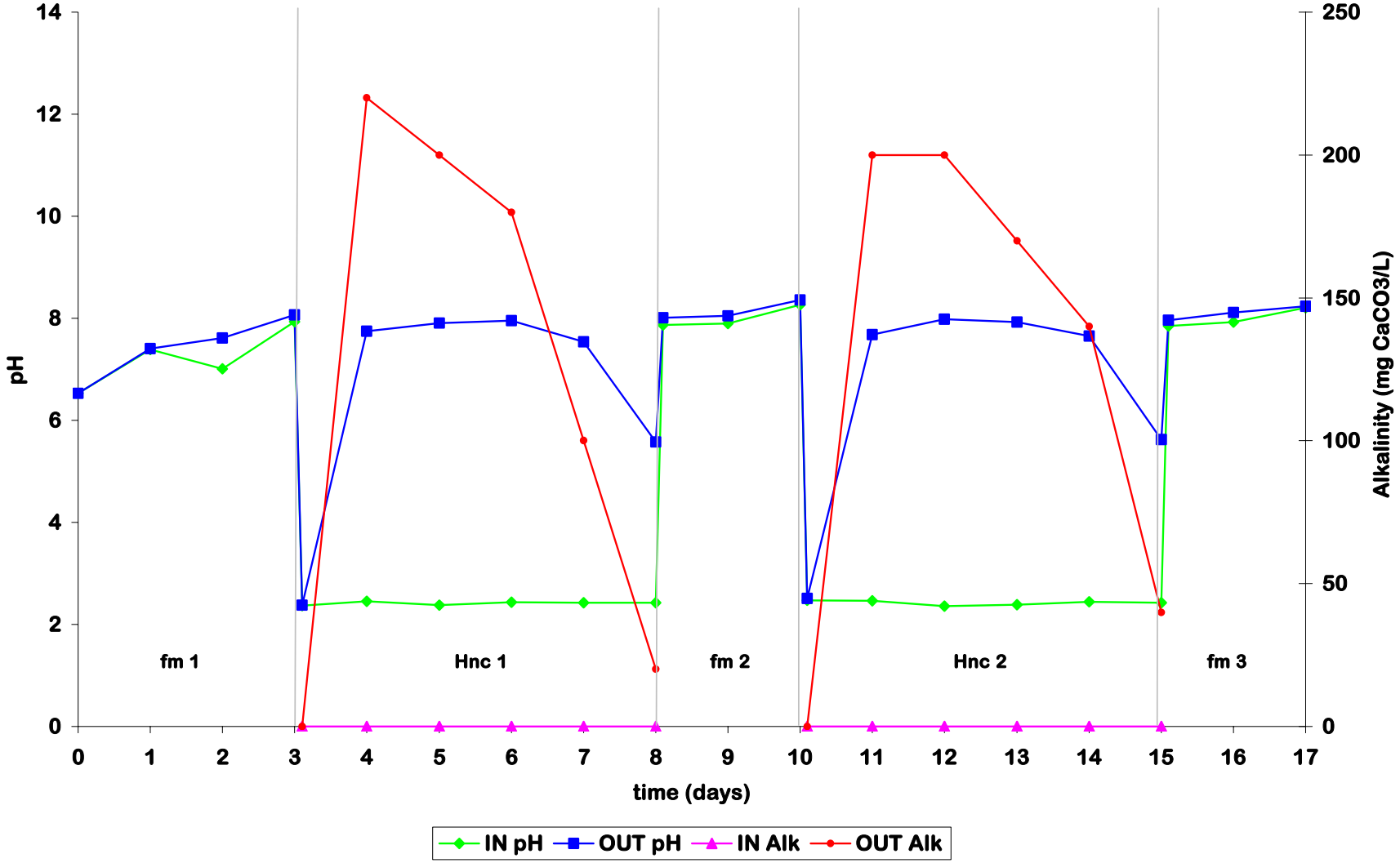

Figure 4.6: pH and alkalinity profiles in an acid neutralisation (pH 2.4) disarticulated continuous up-flow packed column reactor with immobilised SRB consortium.

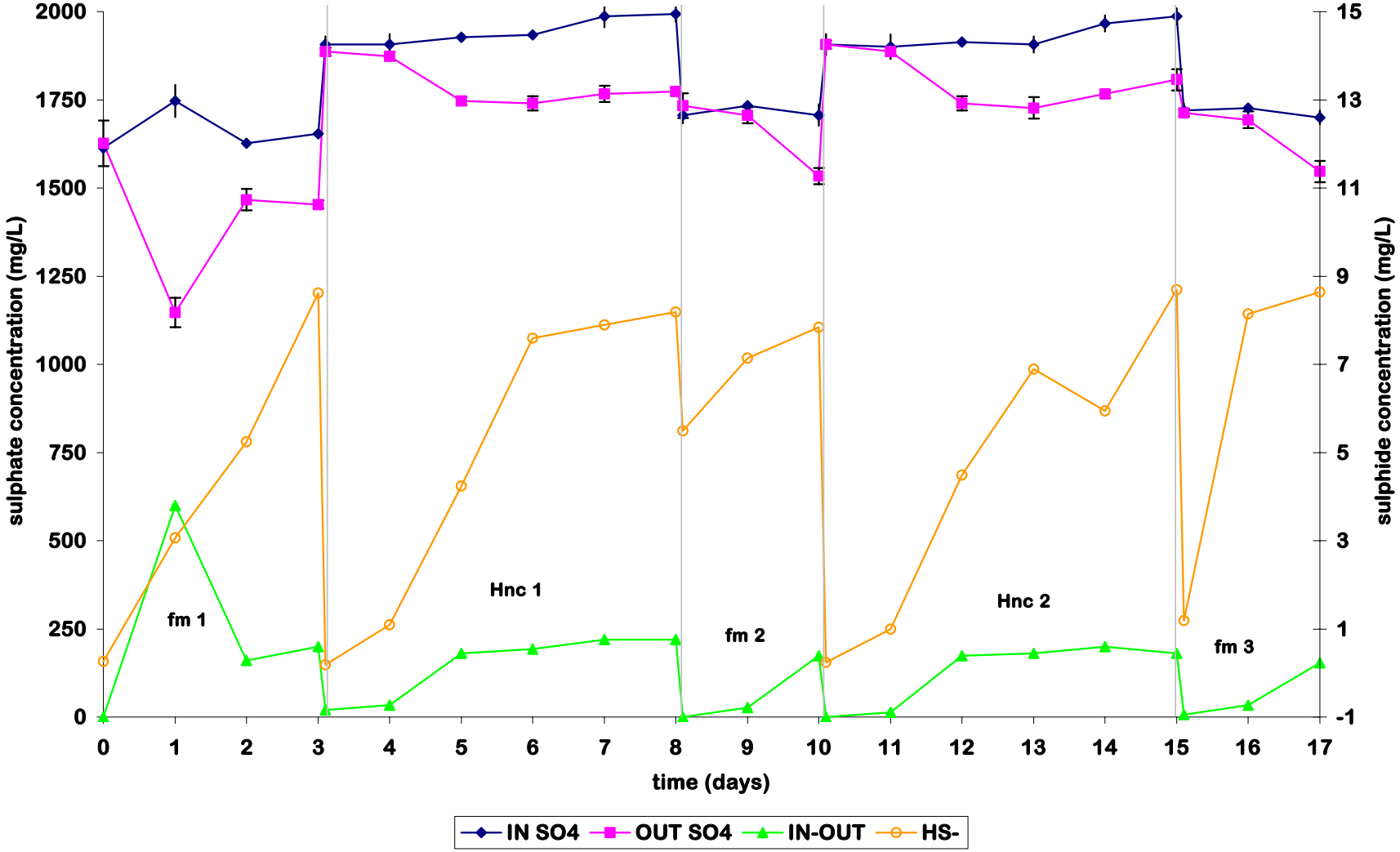

Figure 4.7: Sulphate/sulphide profiles in an acid neutralisation (pH 2.4) disarticulated continuous up-flow packed column reactor with immobilised SRB consortium.

In the first instance the bicarbonate generated provides biogenic alkalinity that acts to buffer the pH of the environment and maintain the pH above 4.5. On the other hand H_2S production results in hydrogen ions being removed from the system with subsequent increases in pH.

Sulphate reduction and sulphide generation profiles for the disarticulation and acid neutralisation experiments at pH 2.4 are given in figure 4.7. The trends for sulphate reduction were very similar to those given in figure 4.4 for the system which was used to investigate sulphate removal at a more neutral pH value. The difference, however, was in the amount of sulphate reduction and sulphide generation. The low pH value of 2.4 had a pronounced effect on the SRB system. Whereas equilibrium sulphate reduction in figure 4.4 with an inorganic stream was around 900 mg/L, in the case of the pH 2.4 experiment it was less than 250 mg/L. However, it seems that this low sulphate reduction activity was sufficient to raise the influent pH value from about 2.4 to above 7.5. Sulphide generation was also low, with the maximum detected sulphide being approximately 10 mg/L.

Table 4.2: Rates of sulphate reduction for disarticulation, pH 2.4 acid neutralisation experiments

	Average percentage Sulphate removal (%)		**Average rate of sulphate removal (mg/L/day)**		**Average sulphate removal rate (mg/L/day/g initial capsules wet mass)**	
Cycle	**Full medium**	**Hydrogen & no carbon source**	**Full medium**	**Hydrogen & no carbon source**	**Full medium**	**Hydrogen & no carbon sauce**
1	19	8.9	320	173	13	7.1
2	6.0	7.7	100	149	4.1	6.1
3	5.5	-	97	-	4.0	

Table 4.2 shows that the rates of sulphate removal were much lower for the inorganic, pH 2.4 acidic influent when compared to those in table 4.1 for sulphate removal from an inorganic influent at a neutral pH value, suggesting that pH plays an important role is SRB activity, and although SRB can adjust the pH of their environment, their activity decreases.

There have been reports of SRB activity ceasing at pH values below 3.5, however, Elliot *et al.* (1998) have shown that SRB can be acclimatised to acidic conditions and although initial

SRB activity is low, once established, the activity increases. The immobilised SRB may be able to adapt more quickly due to partial protection from the semi-permeable membrane.

4.3.2.2 pH 4.0

A similar experiment to the one above was carried out with an influent pH of approximately 4.0. The experiment was carried out over 32 days with longer (12 days) periods where the influent was the inorganic acidic stream, labelled on the graph as 'Hnc' (+hydrogen, no carbon). During the initial three days, the influent was full medium (labelled 'fm' on the graph). Figure 4.8 shows the pH values for the influent and effluent, and the alkalinity determined for the influent and effluent over the 32 days.

The system was expected to cope better with an inorganic acidic influent at pH 4.0 and the results given in figure 4.8 suggest that this was the case. Effluent pH values were maintained above a value of 8.5 for the majority of the experiment. Although there was a switch back to full medium after 12 days of inorganic acidic influent, there was no observed deterioration in sulphate reduction activity in the system (see figure 4.9) due to the inorganic acidic influent. This suggested that it may be possible for the immobilised SRB to maintain sulphate reducing activity and neutralisation of a pH 4.0 inorganic influent over a longer period of time. Sulphate reduction equilibrium of approximately 400 mg/L was reached at the end of the first inorganic acidic influent cycle, which probably represented steady state for the system. The equilibrium sulphate reduction value in the second cycle was higher, approximately 500 mg/L, which may suggest adaptation of the system to the inorganic acidic influent. These equilibrium values were higher than those observed for the pH 2.4 inorganic acidic influent, which were less than 250 mg/L (see figure 4.7). The equilibrium values of 400 and 500 mg/L were still lower than those obtained for sulphate reduction at more neutral pH values (see figure 4.4) of approximately 900-1000 mg/L.

Alkalinity generation (figure 4.8) differed considerably between periods of full medium and those of the inorganic acidic stream. These results were expected, and as equation 4.2 shows, a large amount of bicarbonate alkalinity is generated during lactate oxidation. pH adjustment during the periods of inorganic acidic influent would mostly be due to sulphide alkalinity and proton consumption. However, there was some detectable alkalinity during the 12 days of inorganic acidic influent and this reached an equilibrium value of approximately 260 mg $CaCO_3$/L (figure 4.8).

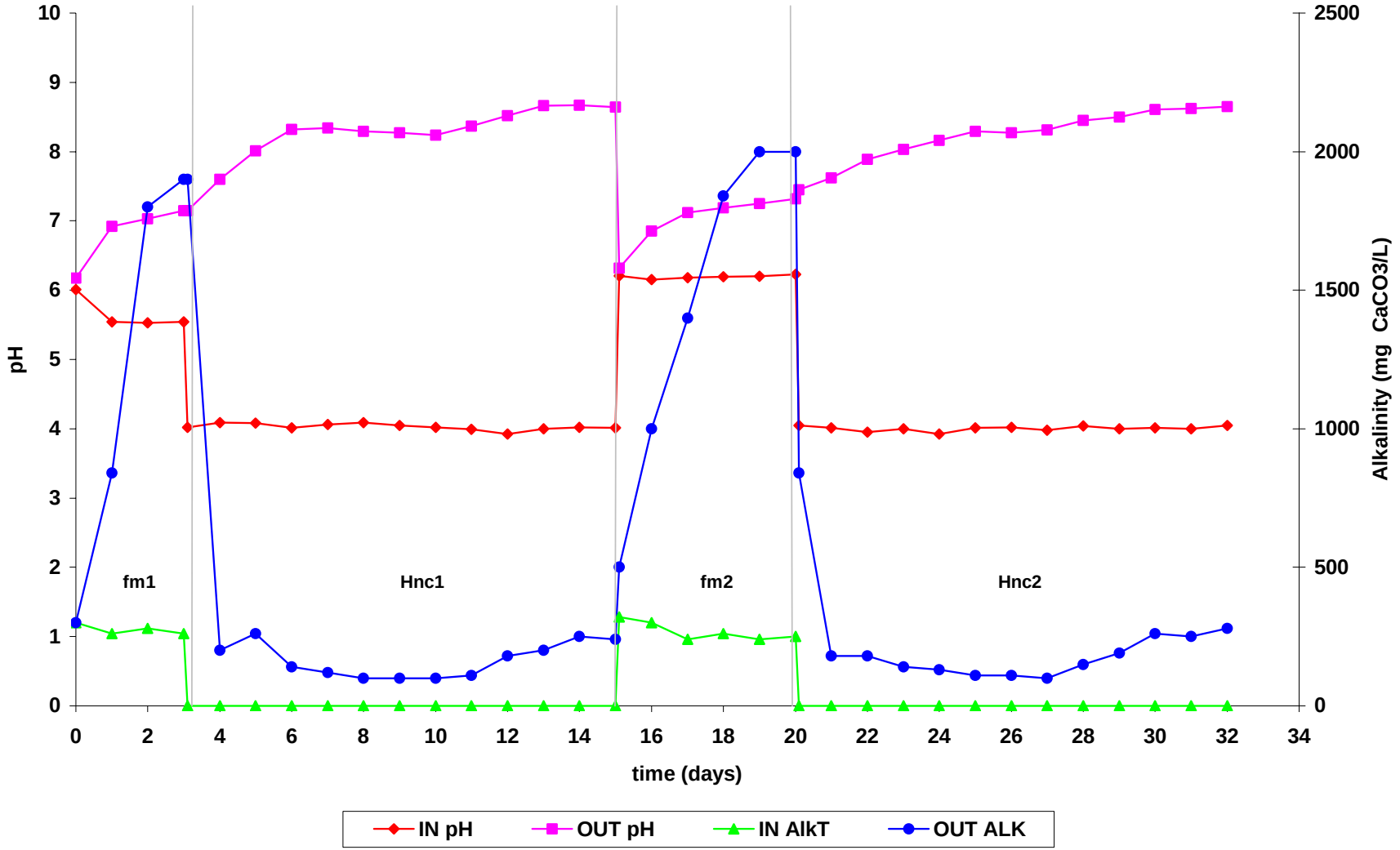

Figure 4.8: pH and alkalinity profiles in an acid neutralisation (pH 4.0) disarticulated continuous up-flow packed column reactor with immobilised SRB consortium.

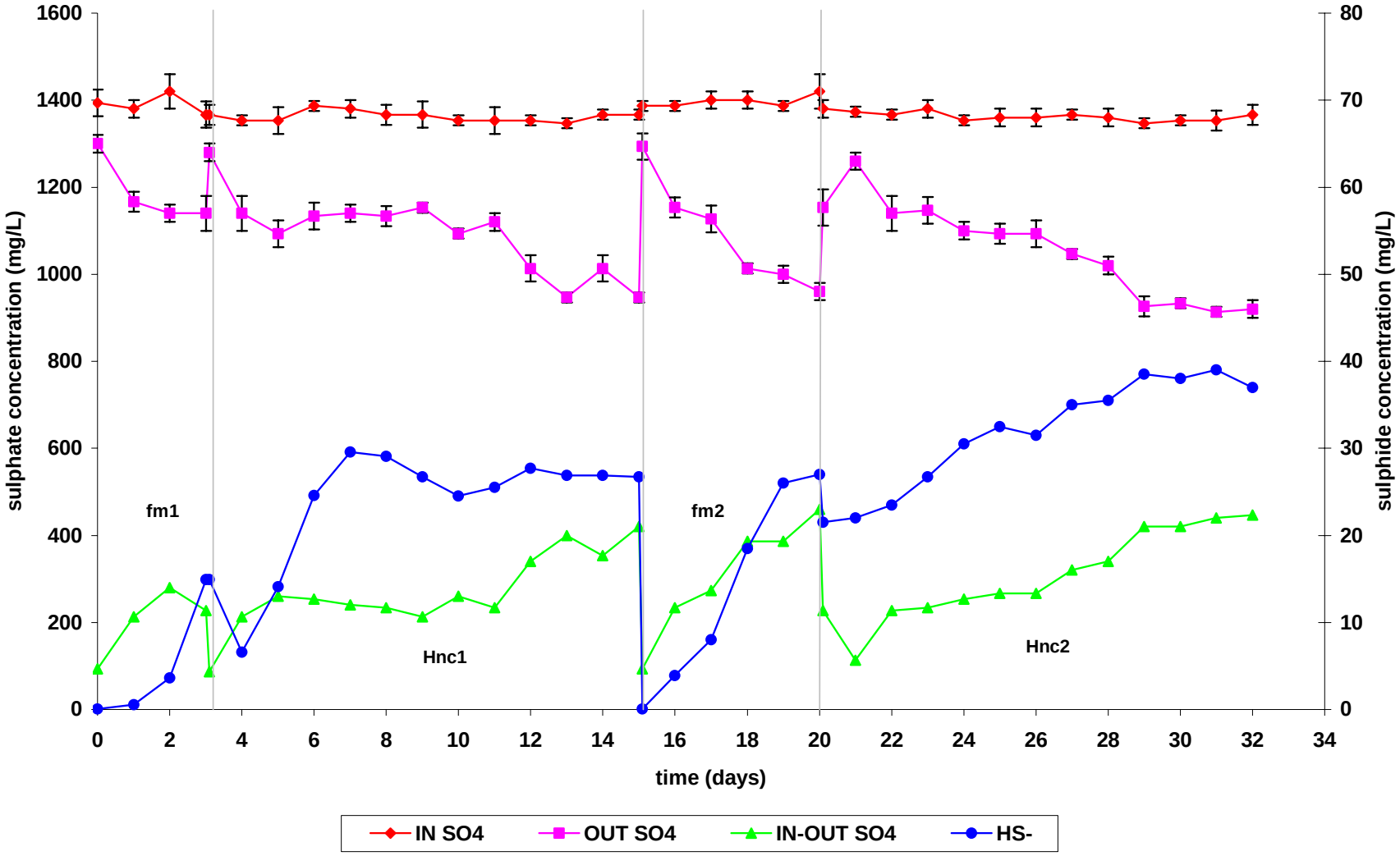

Figure 4.9: Sulphate/sulphide profiles for acid neutralisation (pH 4.0) in a disarticulated continuous up-flow packed column reactor with immobilised SRB consortium.

The amount of sulphide generated in this pH 4.0 experiment (figure 4.9) is generally higher than that generated for the pH 2.4 experiment. Sulphide concentration in the first inorganic acid influent cycle was approximately 30 mg/L and 35 mg/L in the second cycle compare to approximately 10 mg/L in the pH 2.4 experiments, another indication of increased SRB activity at pH 4.0.

The sulphate removal rates for the pH 4.0 experiments are given in Table 4.3. These rates were considerably higher than those determined for the inorganic influent at pH 2.4, see table 4.2.

Table 4.3: Rates of sulphate reduction for disarticulation, pH4.0 acid neutralisation experiments

	Average percentage Sulphate removal (%)		**Average rate of sulphate removal (mg/L/day)**		**Average sulphate removal rate (mg/L/day/g initial capsules wet mass)**	
Cycle	**Full medium**	**Hydrogen & no carbon source**	**Full medium**	**Hydrogen & no carbon source**	**Full medium**	**Hydrogen & no carbon sauce**
1	19	21	271	292	13.8	14.8
2	26	24	367	331	18.6	16.8

Sulphate removal rates were still lower than those determined for the system at a neutral pH value (table 4.1). The percentage sulphate removal values from the full medium cycles above and the neutral pH system were, however, very similar, probably due to the reduced adverse effects of a system at a pH value of 4.0 compared to 2.4.

4.4 Conclusions

Disarticulation of the electron donor and carbon source in bioprocess application of immobilised SRB was found not only to be possible, but in some cases yielded better sulphate reduction rates when compared to systems where the organic substrate, lactate, was the electron donor and carbon source.

Disarticulation was investigated in two areas of SRB bioprocess application: sulphate removal and neutralisation of acidic aqueous solutions.

In sulphate reduction systems where the aim was solely the removal of sulphate, sulphate reduction activity was observed to continue in the absence of a carbon source for 16 days when switching between full medium and the Hnc systems with no carbon source supplied. Experiments with longer periods of electron donor but no carbon supply will need to be carried out in future to define the critical time that disarticulation could be effected and without deleterious effects on sulphate reducing activity of the biocatalyst. Average rates of sulphate reduction with respect to the initial wet mass of the capsules were 43 and 50 mg SO_4^{2-}/L/day/g and average percentage sulphate removal was 28 and 52 % for the carbon supply and electron donor phases respectively.

Immobilised SRB were shown to be able to raise the pH of acidic aqueous solutions from pH values of 2.4 and 4.0 to above 7.5 during periods of disarticulated electron donor and carbon source. The average sulphate removal rates with respect on the initial capsules' wet mass were 7.0 and 6.6 mg SO_4^{2-}/L/day/g and average percentage sulphate removal was 10 and 8 % for the full medium and electron donor phase respectively, in the pH 2.4 system. Average rates of sulphate removal for the pH 4 system were 16.2 and 16.8 mg SO_4^{2-}/L/day/g and average percentage sulphate removal was 22.5 and 22.5 % for the full medium and Hnc periods respectively. The lower values determined for acid neutralisation suggested that pH had a considerable effect on the sulphidogenic systems where the system is self-buffering. However, experiments over 32 days also suggested longer term Hnc may be possible as the sulphate reduction activity was maintained over 12-day Hnc periods, even though the activity rates were considerably reduced at low pH. Kolmert and Johnson (2001) reported similar findings with immobilised acidophilic sulphate-reducers. As with the sulphate experiments, future studies may determine the end point of Hnc cycles, and their effect on the biomass.

Since disarticulated electron donor and carbon source supply in the application of the immobilised SRB consortium were successfully applied to the treatment of inorganic sulphate and acidic aqueous streams, it was decided to investigate the process in one of the largest areas of SRB bioprocess application, metal precipitation from aqueous solution.

CHAPTER 5
DISARTICULATED ELECTRON DONOR AND CARBON SOURCE SUPPLY IN THE APPLICATION OF IMMOBILISED SULPHATE-REDUCING BACTERIA:
2. HEAVY METAL-CONTAMINATED WASTEWATER TREATMENT

5.1. Introduction

The ability of SRB to remove metals from solution, by precipitation as metal sulphides, is probably a mechanism for reducing toxicity in their immediate environment (Eccles, 1995). However, SRB have found wide application in the treatment of metal-contaminated effluents (Rowley *et al*., 1994; Song *et al*., 1998; Foucher *et al*., 2001; Harithsa *et al*., 2002).

As pointed out in chapter 4, the application of SRB in wastewater treatment has in general been limited to wastewater streams with organic components that are used as both an electron donor and carbon source supply by the SRB, or to streams where organic components may be added. The residual organic content of the anaerobic treated stream usually requires further down stream treatment. The result has been the exclusion of application SRB in clean waste streams with very little or no organic load. This situation applies in numerous instances of metal-contaminated wastewaters.

In this chapter performance of immobilised SRB in the removal of metal ions from a 'clean' inorganic aqueous stream with disarticulation of the electron donor and carbon source supply is investigated.

5.2 Materials and Methods

5.2.1 Chemical reagents and analysis

As previously described in chapter 2

5.2.2 Microorganisms

The SRB consortium E28 used was selected from a number of other culture isolates as previously described in chapter 2.

5.2.3 Experimental set-up

As described in chapter 4 with the exception that the inorganic solution contained metal ions of known concentration. The experimental set-up was as illustrated in figures 4.1 - 4.3.

5.3 Results and Discussion

Copper and lead were chosen from a list in the South African Water Quality Guidelines Manual due to their different toxicity levels and represent two ends of the heavy metal toxicity spectrum. Toxic concentration level for copper is comparatively high; with acute poisoning found to occur above 30 mg/l. Lead toxic concentrations on the other hand are low; Lead above 10 mg/L may cause acute health problems (DWAF, 1996).

5.3.1 Application of immobilised SRB consortium capsules in the treatment of a copper-laden aqueous solution

Periods where full medium was supplied are labelled ‘fm’, and periods were hydrogen was supplied but no carbon source was supplied are labelled ‘Hnc’ on the graphs. IN-SO4 represents the influent sulphate concentration and OUT-SO4 represents the effluent sulphate concentration. ‘IN-OUT’ represents the difference calculated between the influent and effluent concentration.

5.3.1.1 Removal of 2 mg/L copper from aqueous solution

The first experiment investigated the removal of copper from an aqueous solution at an initial concentration of approximately 2 mg/L, with intervals of electron donor and carbon source supply disarticulation, was monitored over 32 days. A low concentration of copper was chosen in the initial experiment to evaluate the performance of the disarticulated system as a polishing step at low concentrations. The results for the changes in pH and copper and the changes in sulphate removed and the sulphide generated between the influent and effluent are given in figures 5.1 and 5.2 respectively.

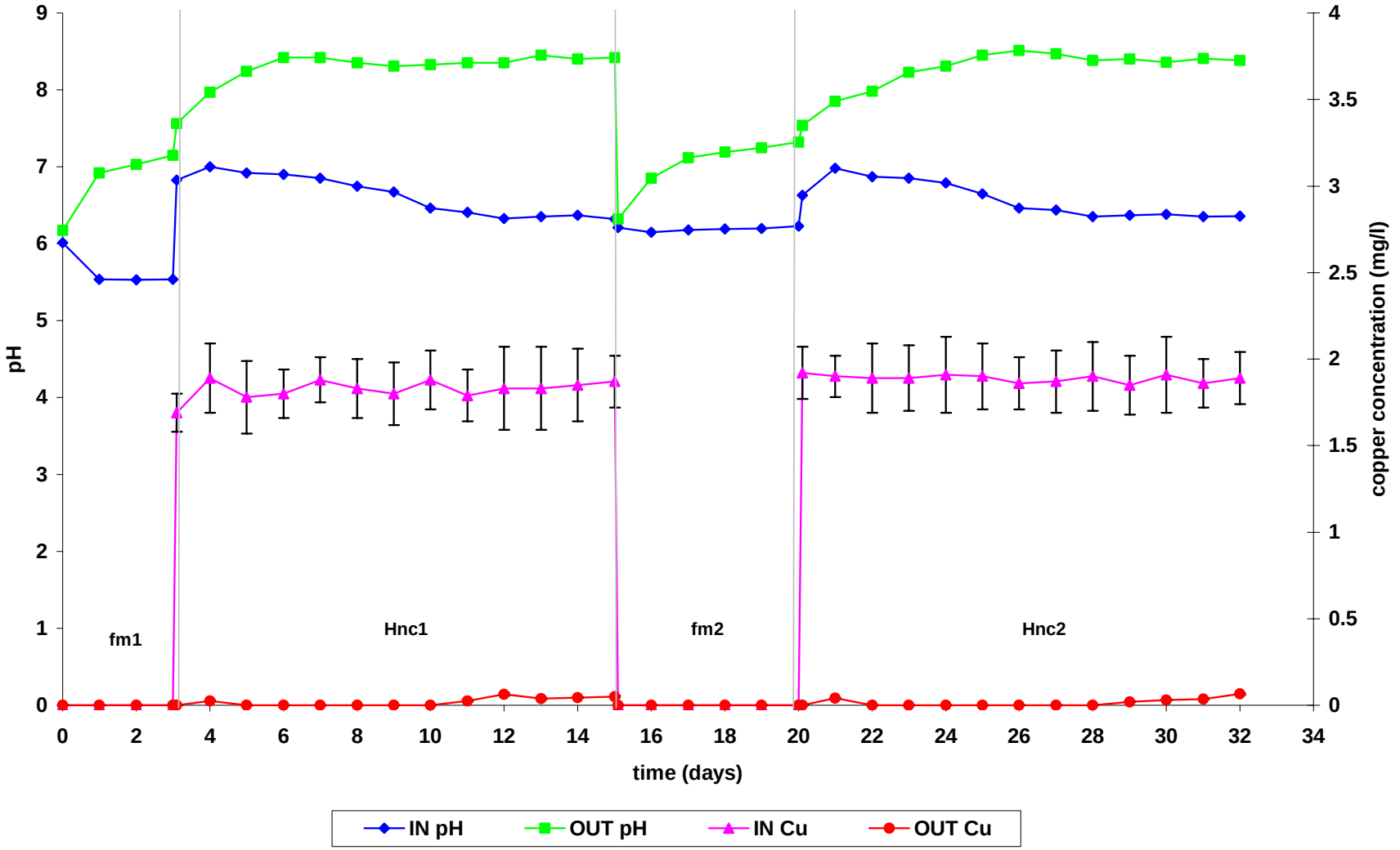

Figure 5.1: pH and copper (2mg/L) removal profiles in a disarticulated continuous up-flow packed column reactor with immobilised SRB consortium.

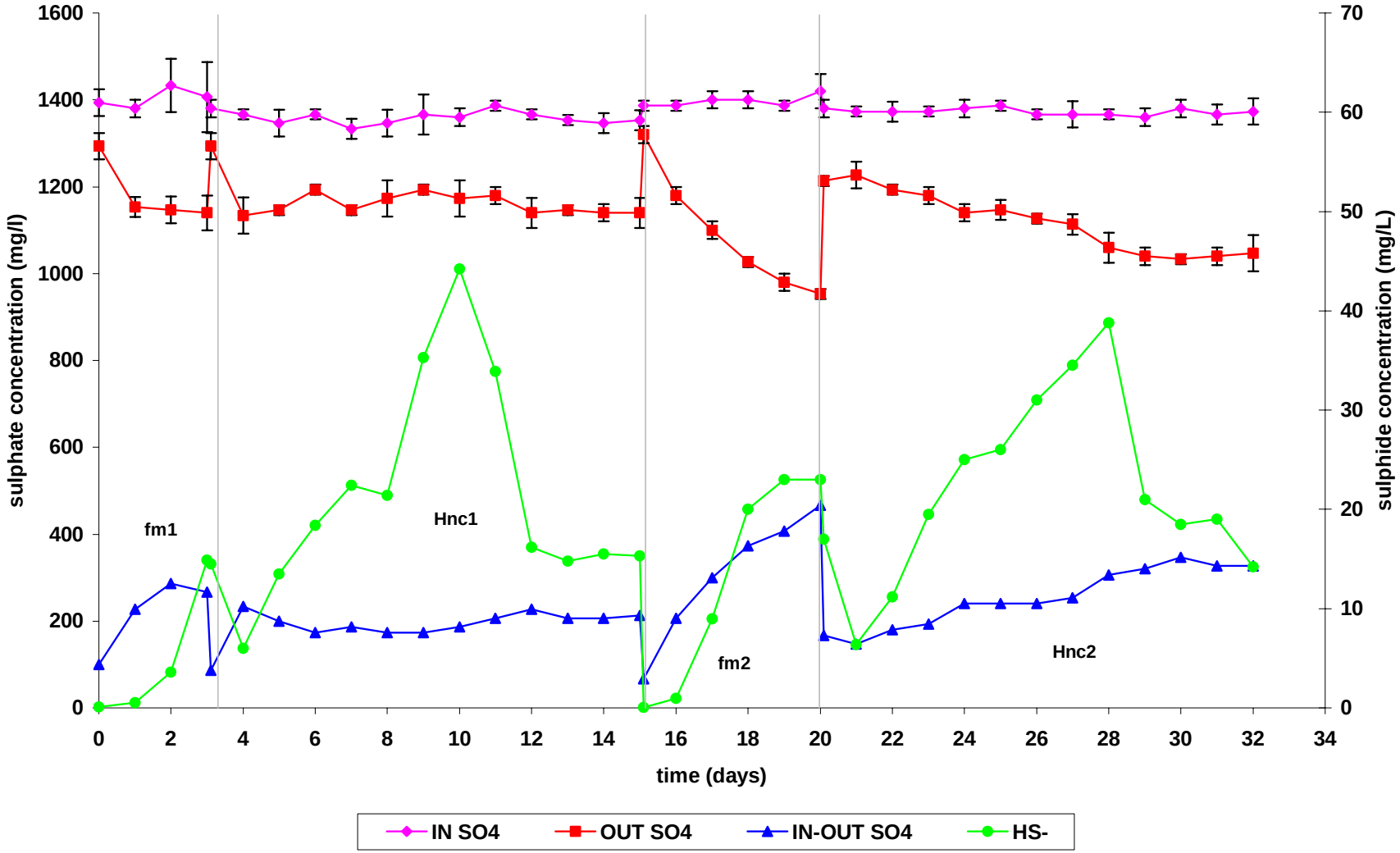

Figure 5.2: Sulphide generation and sulphate reduction profiles for a copper (2 mg/L) removal experiment in a disarticulated continuous up-flow packed column reactor with immobilised SRB consortium.

Copper removal from solution was observed immediately on switching from the full medium to the inorganic stream spiked with copper. The surfaces of the capsules were observed to go from the original grey colour to black, as a black precipitate of copper sulphide adhered to the surface of the capsules. Figure 5.3 shows two columns reflecting the observed difference in colour between a column with an inorganic influent with copper and one without.

Figure 5.3: Continuous up-flow packed columns with calcium-alginate-xanthan gum encapsulated SRB. **A** = column with an inorganic influent stream spiked with copper where the SRB capsules have gone black in colour; **B** = column where the influent does not contain copper, and the SRB capsules are grey in colour.

Black copper sulphide was also observed settling at the bottom of the column, but due to the fluidised nature of the reactors from nitrogen and hydrogen bubbling, most of the copper sulphide settled out in the effluent-collecting vessel. A control experiment was carried out to determine the copper-adsorption capacity of inactive SRB capsules and therefore determine how much of the observed copper removal was due to adsorption onto the capsule rather than sulphide precipitation. In the first cycle with the inorganic effluent, it was found that on average, the adsorption of copper onto the capsule accounted for approximately 12 % of observed copper removal. In the second cycle it only accounted for approximately 10%. This is probably due to a decrease in the amount of surface area on the capsules still available for copper adsorption. Adsorption of copper onto the surface of the capsules did not appear to have any adverse effects on the integrity of the capsules.

Table 5.1 gives the percentage sulphate and copper removal, and the rates of sulphate removal. Effluent pH was maintained well above a value of 6.5 throughout the 32-day period of this experiment. Influent copper concentration was approximately 2 mg/L, and the highest detected copper in the effluent was approximately 0.07 mg/L, suggesting almost 100 % copper removal from an initial concentration of 2 mg/L.

Sulphate removal at start-up with full medium was approximately 300 mg/L, this value stabilised at approximately 200 mg/L with the first inorganic influent cycle (Hnc1). The full medium period from day 15 saw sulphate removal and sulphur generation increase, probably an indication of a more stable and acclimatised system. Sulphate reduction reached an equilibrium value of approximately 300 mg/L (figure 5.1).

Table 5.1: Percentages and rates of sulphate and copper (2 mg/L) removal in disarticulated up-flow continuous systems (averages are calculated for the duration of the cycle).

	Average percentage Copper removal (%)		**Average percentage Sulphate reduction (%)**		**Average rate of sulphate reduction (mg/L/day)**		**Average sulphate reduction rate (mg/L/day/g initial capsules wet mass)**	
Cycle	**Full medium**	**Hydrogen & no carbon source**	**Full medium**	**Hydrogen & no carbon source**	**Full medium**	**Hydrogen & no carbon source**	**Full medium**	**Hydrogen & no carbon sauce**
1	0	99	21	15	293	206	14.9	10.5
2	0	99	26	20	364	274	18.5	13.9

Rates of sulphate reduction at equilibrium in the two inorganic stream cycles Hnc1 and 2 were 212 and 325 mg SO_4^{2-}/L/day respectively after approximately 20 HRTs in each case. Sulphate reducing activity of the immobilised SRB appeared unaffected over 12 days of an inorganic influent stream spiked with 2 mg/L of copper. Copper removal was still at approximately 100 % on day 32, with little to no sign of any decrease. The trend observed for sulphide generation was more variable.

5.3.3.2 Removal of 60 mg/L copper from aqueous solution

The South African water quality guideline for domestic use recommends that the concentration of copper should be less than 30 mg/L in potable water. Copper concentrations above 30 mg/L are believed to cause acute poisoning (DWAF, 1996). Therefore, for the next experiment an initial copper concentration of 60 mg/L was used to determine whether the system was able to levels to below 30 mg/L.

Figure 5.4 illustrates copper removal and the pH profile for an initial copper concentration of approximately 60 mg/L. In the first three days, the influent into the column was full medium without copper. The influent pH value of the full medium was above 6.5 for the duration of the experiment and the effluent pH was approximately 8. As in previous experiments, the effluent pH was maintained above the influent pH. The inorganic influent spiked with copper had a pH value of approximately 5.5 and the immobilised SRB were able to raise this to a pH value greater 8.0. Copper removal was observed from the first day of switching over to the inorganic copper influent. Copper concentration in the effluent decreased from the first day at approximately 22 mg/L to approximately 6 mg/L by day eight. A similar trend was observed in the second Hnc period from day 10 to day 15. Control experiments determined that copper removal by adsorption to the capsules accounted for approximately 15 % of the copper removed in the experiment.

Sulphate removal and sulphide generation profiles are given in figure 5.5. Sulphate removal equilibrium throughout the whole experiment was approximately 200 mg/L irrespective of the electron donor source. Although detectable sulphide was generally low, this was expected due to the precipitation of copper sulphide. Sulphide concentration in the periods of full medium (fm1, 2 and 3) influent was observed to be higher than during the periods of inorganic influent, probably because there was no copper in the full medium to react with the sulphide. The change in the colour of the capsules in the presence of copper was evident.

The average rates of sulphate reduction at equilibrium for the two inorganic cycles Hnc1 and 2 were 138 and 145 mg SO_4^{2-}/L/day respectively and were reached after nine HRTs in both cases.

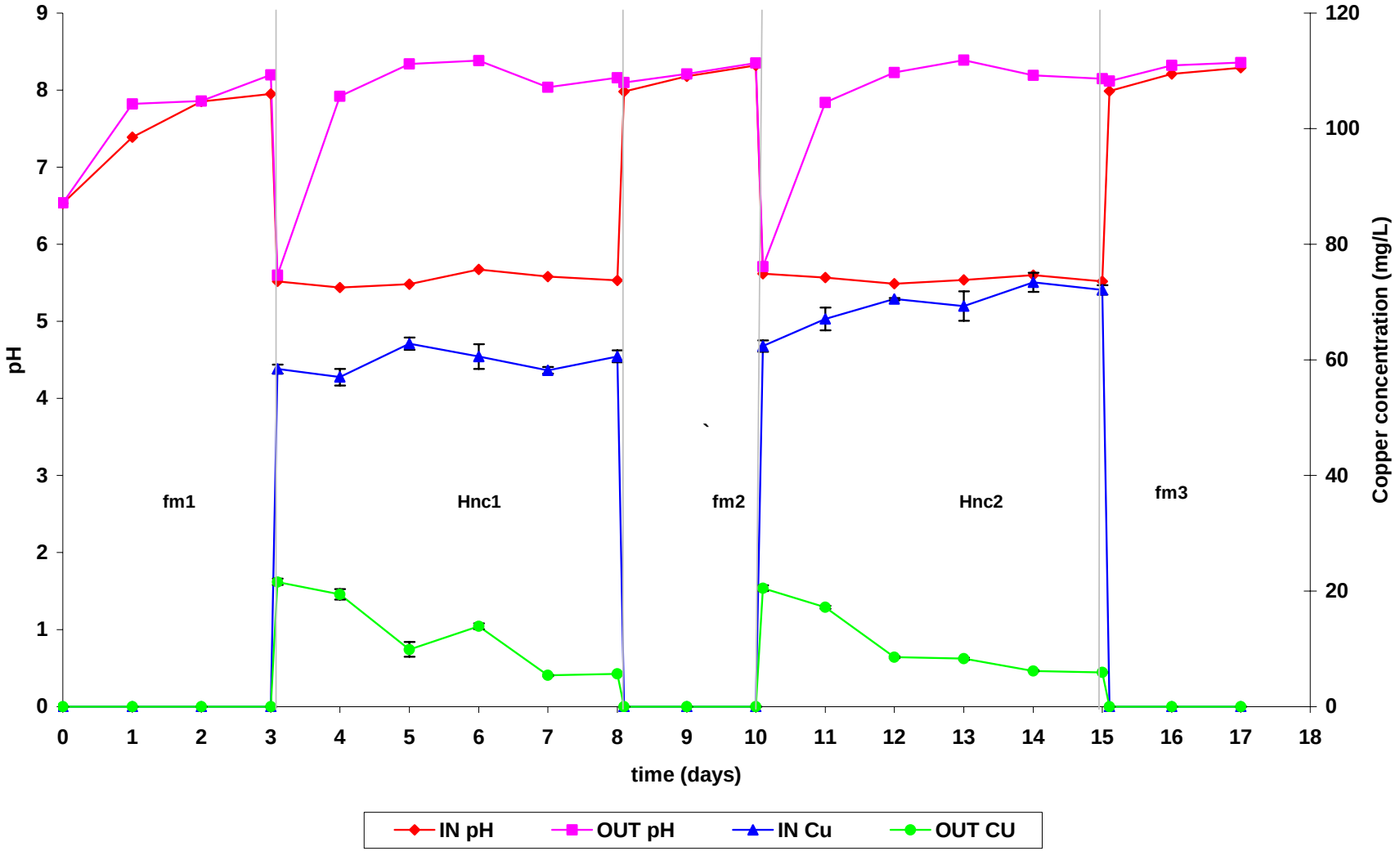

Figure 5.4: pH and copper (60mg/L) removal profiles in a disarticulated continuous up-flow packed column reactor with immobilised SRB consortium.

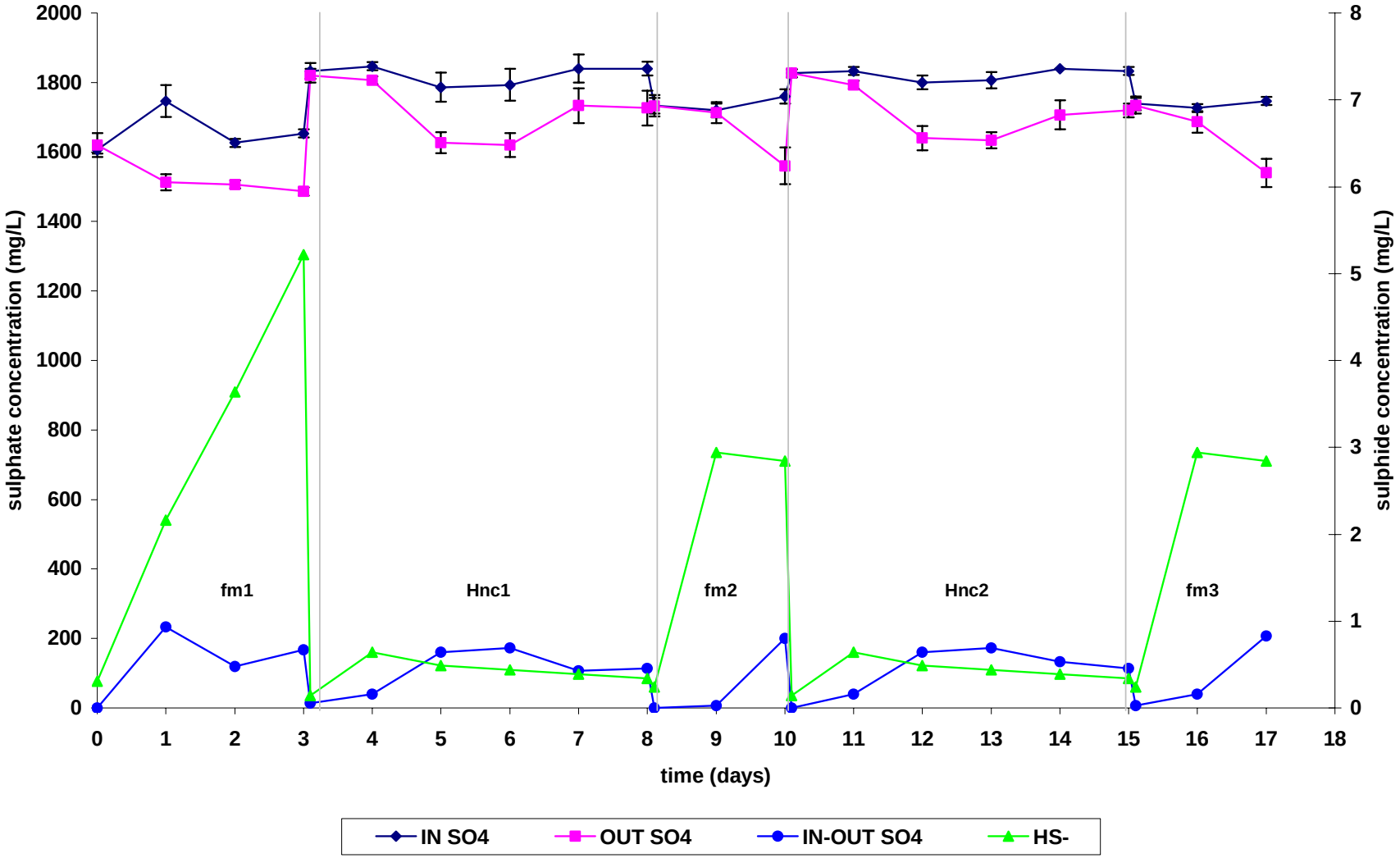

Figure 5.5: Sulphide generated and sulphate reduction profiles for a copper (60 mg/L) removal experiment in a disarticulated continuous up-flow packed column reactor with immobilised SRB consortium.

Rates and percentages of sulphate and copper removal are given in table 5.2. The average sulphate reduction rates per day for copper removal at 2 mg/L were considerably higher than those for copper removal at 60 mg/L (table 5.1). It may be possible that the higher copper concentration had an adverse effect on SRB activity. Sulphate removal per initial gram wet mass of capsules was subsequently also greater for copper removal at 2 mg/L. Compared to the 99 % copper removal observed at 2 mg/L of copper, percentage copper removal at 60 mg/L copper was approximately 83 and 87 % for the two electron donor (Hnc1 and 2) phases.

Table 5.2: Percentages and rates of sulphate and copper (60 mg/L) removal in disarticulated up-flow continuous systems.

	Average percentage copper removal (%)		**Average percentage sulphate reduction (%)**		**Average sulphate reduction rate (mg/L/day)**		**Average sulphate reduction rate (mg/L/day/g initial capsules wet mass)**	
Cycle	**Full medium**	**Hydrogen & no carbon source**	**Full medium**	**Hydrogen & no carbon source**	**Full medium**	**Hydrogen & no carbon source**	**Full medium**	**Hydrogen & no carbon sauce**
1	0	83	10.3	6.7	173	121	7.1	5.0
2	0	87	5.9	6.8	104	124	4.2	5.1
3	0	-	7.3	-	127	-	5.2	-

5.3.2 Application of immobilised SRB consortium capsules in the treatment of a lead-contaminated aqueous solution

Lead was chosen for this study due to its toxicity at low concentrations. The South African water quality guidelines manual suggests that lead concentrations in water for domestic use be kept below 10 mg/L (DWAF, 1996).

5.3.2.1 Removal of ~10 mg/L lead from aqueous solution

The graphs in figures 5.6 and 5.7 give the pH and lead removal profiles and the sulphate removal and sulphide generation profiles respectively for a continuous up-flow packed column system with intervals of disarticulated electron donor and carbon source.

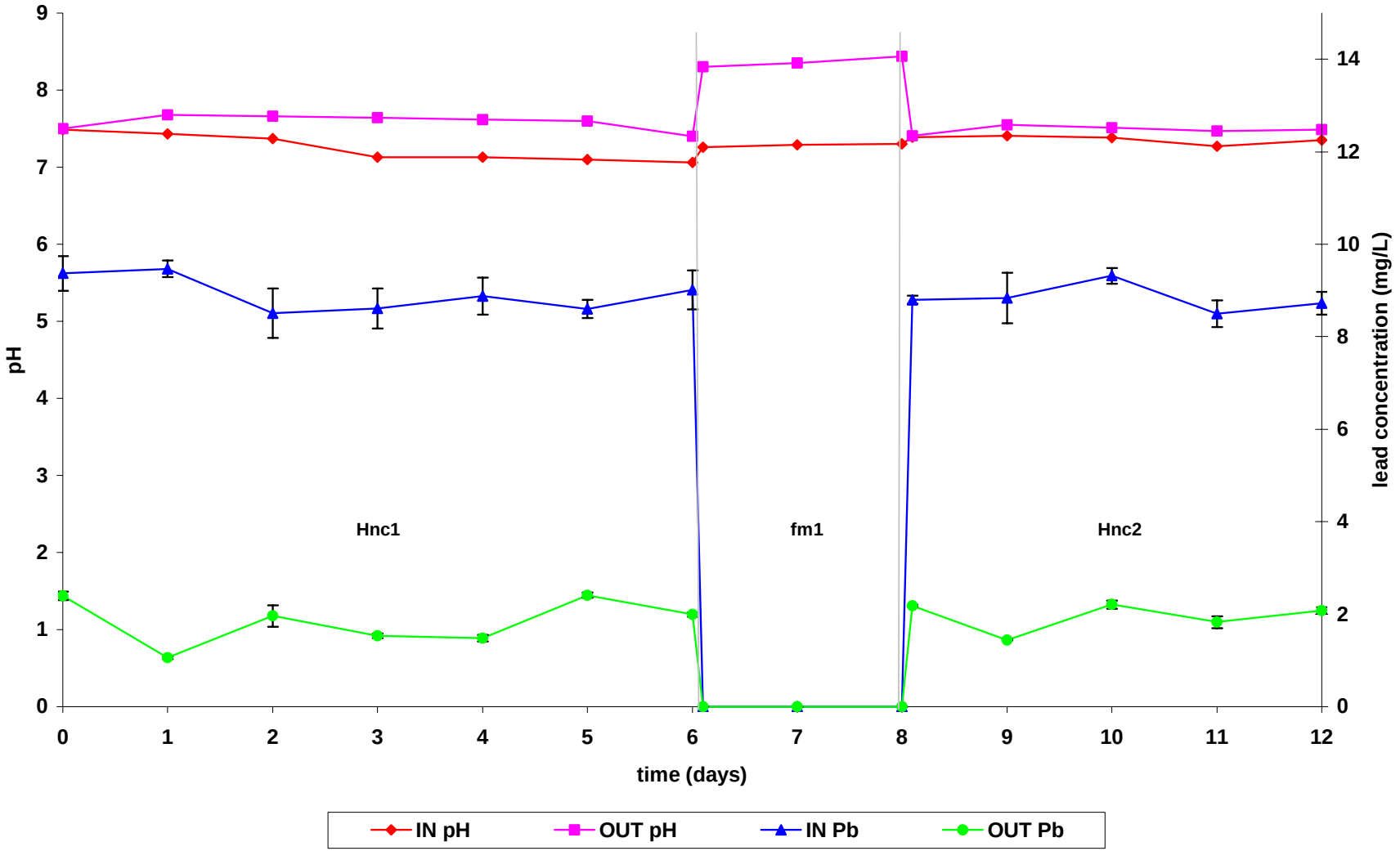

Figure 5.6: pH and lead (10 mg/L) removal profiles in a disarticulated continuous up-flow packed column reactor with immobilised SRB consortium.

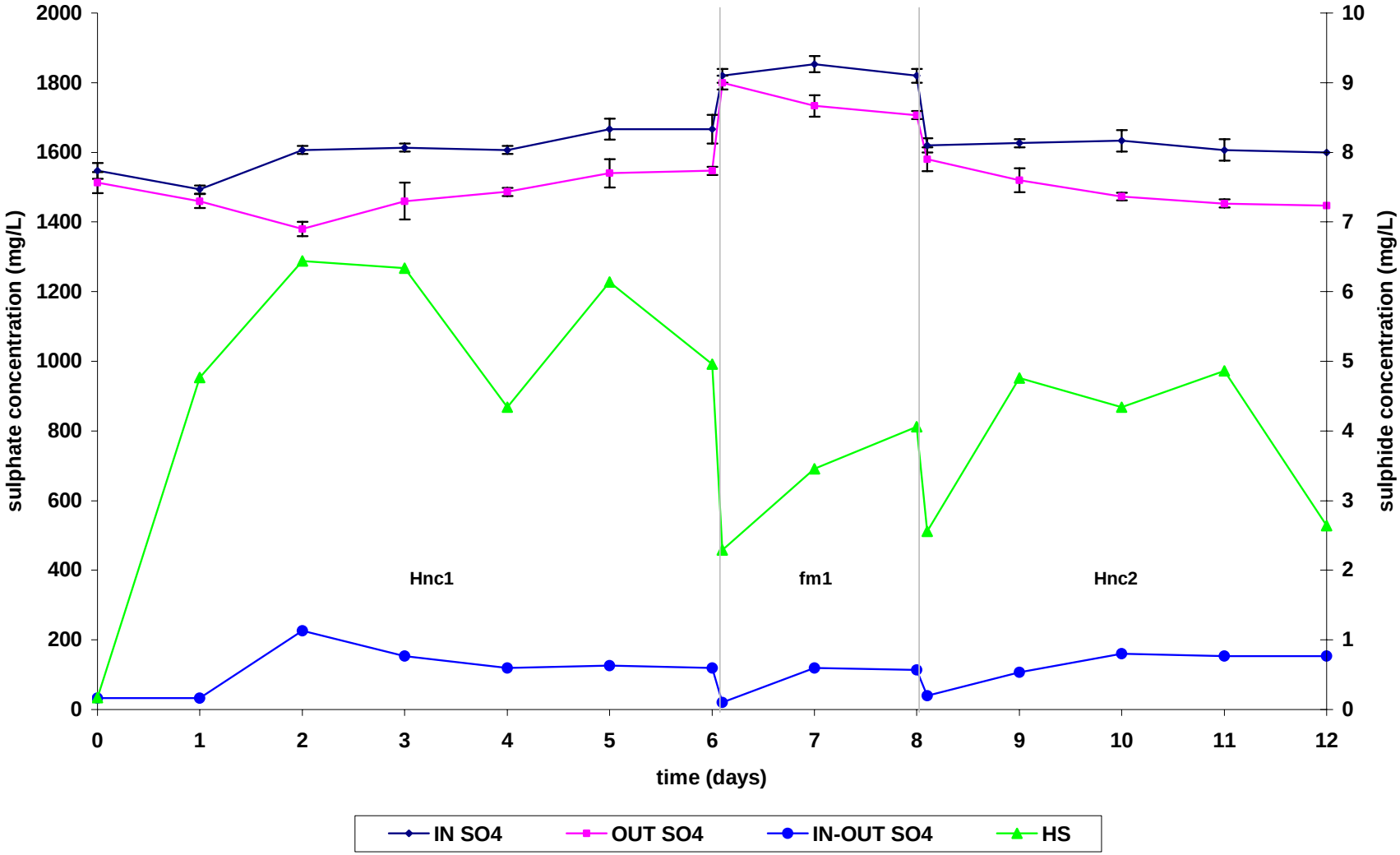

Figure 5.7: Sulphide generated and sulphate reduction profiles for a lead (10 mg/L) removal experiment in continuous disarticulated up-flow packed column reactor with immobilised SRB consortium.

The influent for the first six days of this experiment was the inorganic medium spiked with lead. The pH trend was as expected, with the effluent pH value higher than the influent pH value and was maintained this way in for the duration of the experiment (figure 5.6). Lead concentrations in the influent and effluent streams were approximately 9 and 2 mg/L respectively, an average removal of 78 %. A control experiment run concurrently to ascertain the contribution of the capsules with no sulphidogenic activity determined that in the first inorganic influent cycle, the capsules accounted for approximately 11 % of the lead removal and 7 % during the second inorganic influent cycle. Although the influent was only switched over to the full medium after day six for two days, it appeared that sulphate reduction was able to start without an initial period of full medium influent. As in previous experiments the system appears to be able to maintain metal removal for the duration of the experiment (12 days in this instance).

Although sulphate reduction was evident in figure 5.6, where sulphate removal was reflected in concurrent generation of sulphide, rate of sulphate removal equilibrium in the first inorganic cycle, Hnc1 was approximately 130 mg SO_4^{2-}/L/day and 156 mg SO_4^{2-}/L/day in Hnc2 after 13 and 5 HRTs respectively. The average detectable sulphide concentration in the first inorganic cycle (Hnc1) was approximately 6 mg/L compared to 5 mg/L in the second cycle. Sulphide generation during the full medium influent period was much lower than was expected. The trend over that time suggested that sulphide generation was progressing, although very slowly. Detectable sulphide in column systems seems to be low, but the low values may also suggest that most of the sulphide is being used in metal precipitation. A light brown to black precipitate of lead sulphide was seen on the surface of the capsules and observed settling out in the effluent collection flask.

Table 5.3 is a summary of the percentage lead and sulphate removal and sulphate reduction rates. Percentage lead removal was lower than that achieved for copper removal. Rates of sulphate reduction were also much lower for the lead system when compared to the copper systems. If there was any toxicity to the immobilised SRB system from lead, there was no observed evidence to support this. As with the other metal removal experiments, the rates of sulphate reduction determined for systems with metal ions were lower than those for systems in which metal-free inorganic sulphate-rich streams were treated.

Table 5.3: Percentages and rates of sulphate and lead (~10 mg/L) removal in disarticulated up-flow continuous systems.

	Average percentage lead removal (%)		Average percentage sulphate removal (%)		Average sulphate removal rate (mg/L/day)		Average sulpate removal rate (mg/L/day/g initial capsules wet mass)	
Cycle	**Full medium**	**Hydrogen & no carbon source**	**Full medium**	**Hydrogen & no carbon source**	**Full medium**	**Hydrogen & no carbon source**	**Full medium**	**Hydrogen & no carbon sauce**
1	0	79	6.9	8.4	127	136	5.2	5.6
2	0	77	-	9.5	-	153	-	6.3

5.3.2.2 Removal of ~3 mg/L lead from aqueous solution

In the previous experiment it was found that lead concentration from the influent to the effluent was reduced from approximately 9 to 2 mg/L. In this experiment, the influent lead concentration was introduced at approximately 3 mg/L in order to see if the system would reduce the lead concentration to below 2 mg/L and thus simulating a polishing step for lead.

The experiment was conducted for a longer period of 32 days and the periods of inorganic influent were extended in order to determine whether the lack of an organic carbon source would affect the activity of the immobilised SRB. Full medium was supplied for the first three days, and then the influent was switched over to an inorganic stream spiked with lead.

The pH profile and metal removal are given in the graph in figure 5.8. Influent pH with full medium in the first three days was less than 6.0 and was increased by the sulphidogenic system to approximately 7.0. The inorganic influent pH value was raised from approximately 7.0 to approximately 8.5. Lead removal in the system did not decrease much below the 2 mg/L, which was similar to the observation in the previous experiment, even though the initial lead concentration in this case was approximately 3 mg/L compared to 10 mg/L in the previous experiment. The average lead concentration in the effluent was approximately 1.6 mg/L. Detectable sulphide generated in the effluent of approximately 15 mg/L suggests that a considerable amount of sulphide did not react with the lead.

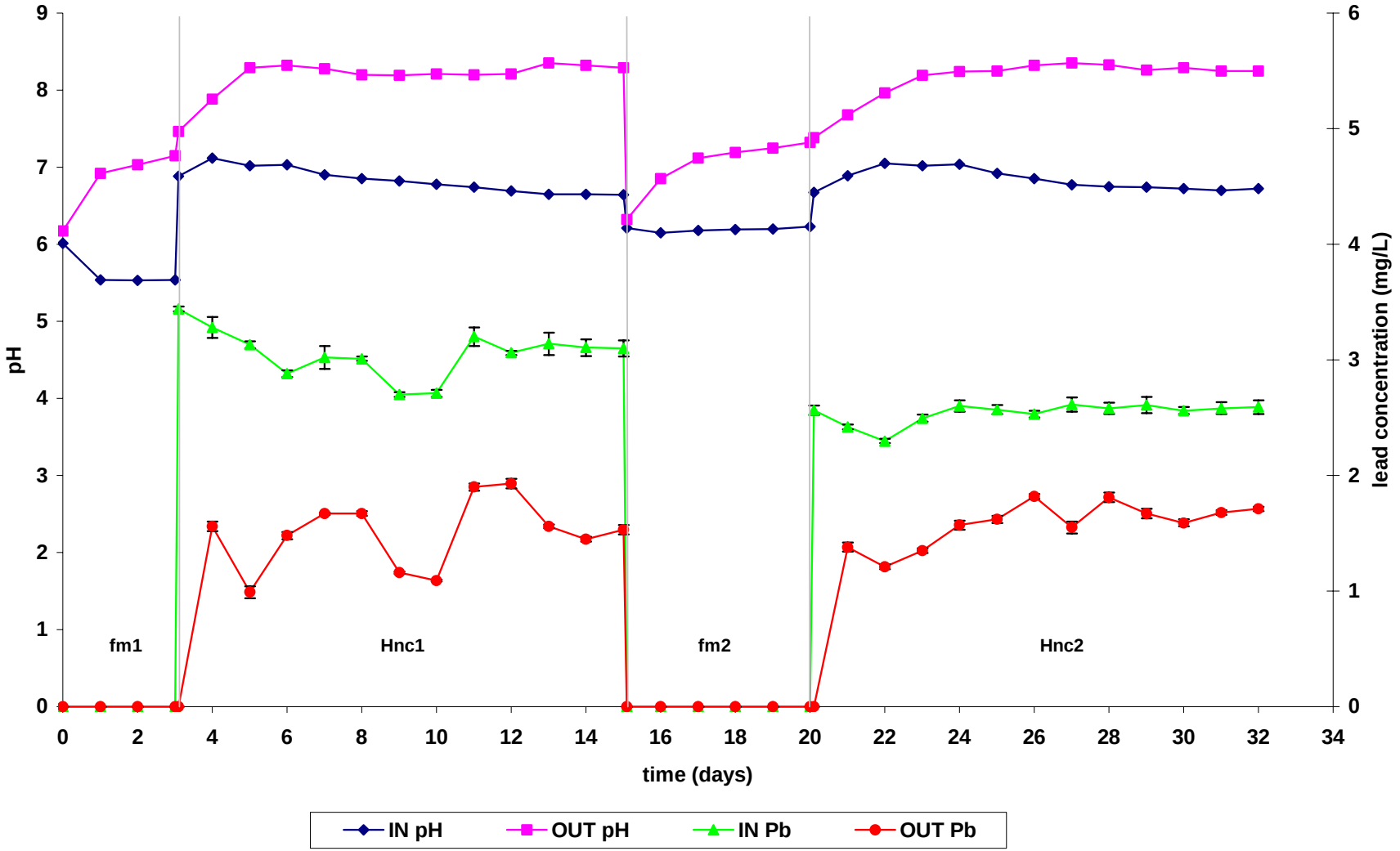

Figure 5.8: pH and lead (3 mg/L) removal profiles in a disarticulated continuous up-flow packed column reactor with immobilised SRB consortium.

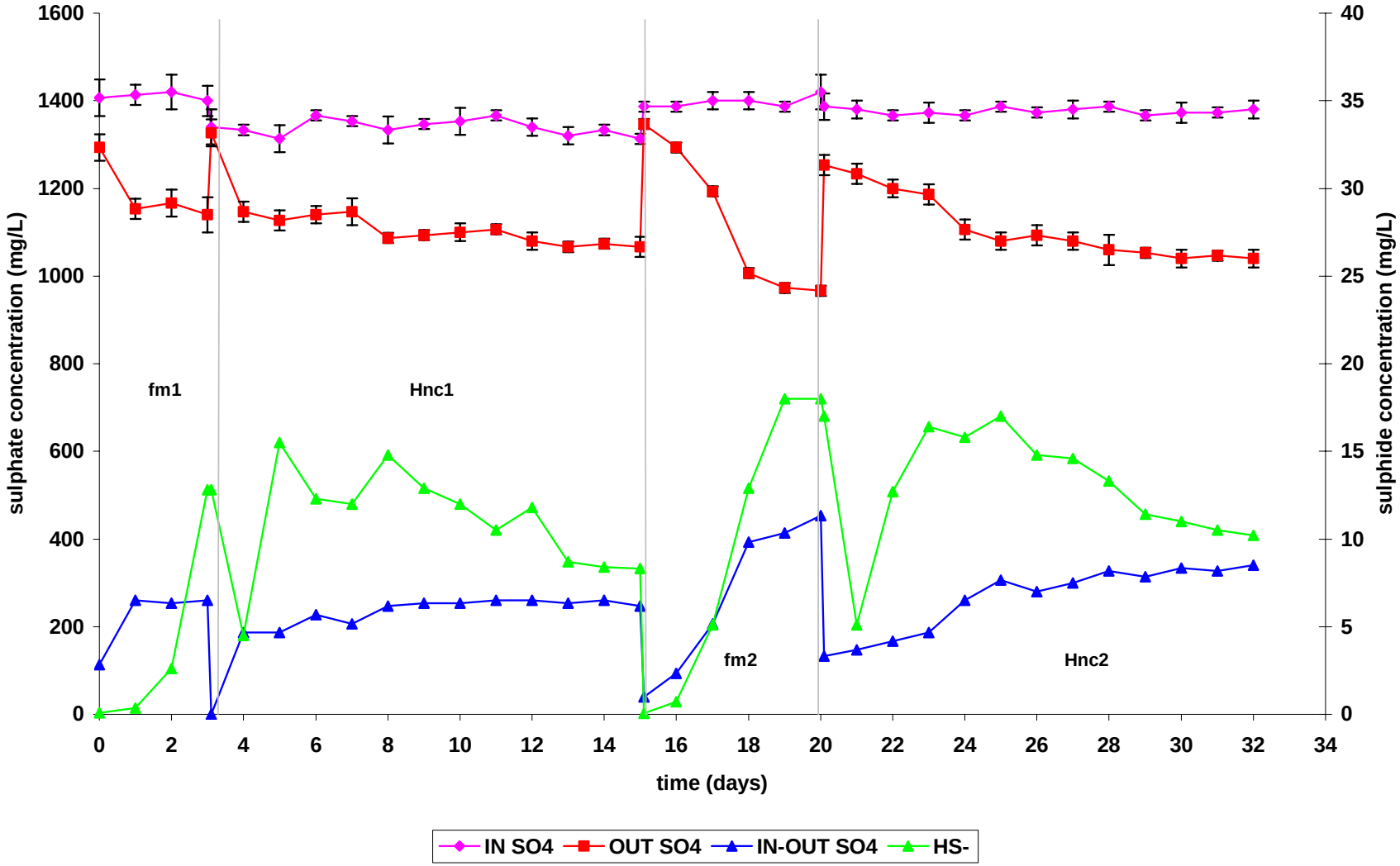

Figure 5.9: Sulphide generated and sulphate reduction profiles for a lead (3 mg/L) removal experiment in a disarticulated continuous up-flow packed column reactor with immobilised SRB consortium.

Figure 5.9 shows that during the first three days, with full medium influent, sulphate removal reached equilibrium at approximately 250 mg/L and was approximately 400 mg/L during the second full medium cycle. Sulphate removal reached the same approximate equilibrium value of 250 mg/L during the first inorganic influent cycle and about 300 mg/L in the second cycle.

The rates of sulphate reduction (table 5.4) in both the full medium and inorganic influent streams were considerably higher than those determined for the lead removal experiment at 10 mg/L (table 5.3), even though percentage lead removal was better in the 10 mg/L lead experiment. Sulphate reduction rates in this experiment are comparable to those determined for copper at 2 mg/L. Percentage lead removal in this experiment was 54 % in the first inorganic influent cycle and 43 % in the second, both of these are much lower than the 78 % achieved for the 10 mg/L lead experiment. However, the results suggested that the system was less efficient when treating inorganic streams with low concentrations of lead compared to copper, with which the percentage removal from a 2 mg/L copper solution was close to 100 %.

Table 5.4: Percentages and rates of sulphate and lead (~3 mg/L) removal in disarticulated up-flow continuous systems.

	Average percentage lead removal (%)		**Average percentage Sulphate reduction (%)**		**Average rate of sulphate reduction (mg/L/day)**		**Average sulphate reduction rate (mg/L/day/g initial capsules wet mass)**	
Cycle	**Full medium**	**Hydrogen & no carbon source**	**Full medium**	**Hydrogen & no carbon source**	**Full medium**	**Hydrogen & no carbon source**	**Full medium**	**Hydrogen & no carbon sauce**
1	0	54	21	18	296	237	15	12
2	0	43	23	21	320	285	16	15

5.4 Conclusions

Metal removal experiments investigated the ability of immobilised SRB in continuous bioprocess systems, to precipitate copper and lead from aqueous solution as metal sulphides during periods of disarticulation of the electron donor and carbon source supply. Average copper removal was approximately 99 and 85 % for copper solution at approximately 2 and 60 mg/L respectively. Average rates of sulphate reduction were 16.7 and 12.2 mg SO_4^{2-} /L/day/g initial wet mass of capsules and average percentage sulphate removal was 23.5 and 17.5 % for the full medium and Hnc periods respectively for the 2 mg/L copper solution. For the 60 mg/L copper solution, average rates of sulphate reduction were 5.5 and 5.1 mg SO_4^{2-} /L/day/g initial wet mass of capsules and average percentage sulphate removal was 7.8 and 6.8 % for the full medium and Hnc periods respectively. The lower rates for 60 mg/L copper solution may suggest toxic effects of high concentrations of copper on the immobilised SRB consortium. Song *et al*. (1998) have reported on the toxicity of copper to SRB, 100 mg/L of copper was found to reduce SRB activity by 50 %. Detection of the copper and sulphide in solution was probably due to dissociation of the copper sulphide complex on lowering the pH of the solution for sulphide determination.

Dissolved lead concentrations of approximately 3 and 10 mg/L were used and average lead removal was approximately 49 and 78 % respectively. The final aqueous lead concentration was slightly below 1.8 mg/L in both experiments. Average rates of sulphate reduction were 15.5 and 13.5 mg SO_4^{2-}/L/day/g initial wet mass of capsules and average percentage sulphate removals were 22 and 20 % for the full medium and Hnc periods respectively for the 3 mg/L lead solution. Average rates of sulphate reduction for the 10 mg/L lead solution were 5.2 and 6.0 mg SO_4^{2-}/L/day/g initial wet mass of capsules and average percentage sulphate removal was 6.9 and 9.0 % for the full medium and Hnc periods respectively. Percentage lead removal for the 3 mg/L solution was much lower than for the 10 mg/L lead solution, which was in contrast to the results observed for the copper solutions. A toxicity effect would be expected to be more pronounced at higher lead concentrations. However, the rates of sulphate activity were found to be higher at lower concentrations of lead, which is the expected trend if toxicity affects the activity of the immobilised SRB consortium.

Control experiments carried out using inactive immobilised SRB showed that the calcium-alginate-xanthan gum capsules accounted for a maximum of approximately 15 % metal removal and the black precipitates of copper and lead sulphides were evident on the surface of the capsules. The amount of metal-sulphide precipitate on the capsules obviously decreased with increasing number of cycles.

Determination of the maximum periods of Hnc fell outside the scope of the present study, which was concerned with establishing and verifying the disarticulation process. Therefore, maximum periods of Hnc were not determined. However, the results for the removal of copper suggest that the electron donor and carbon source supply can be disarticulated for much longer than the 12-day intervals investigated. Figure 5.1 to 5.4 showed no signs of deterioration in sulphate reduction and copper removal once steady state had been achieved. Steady state was determined where stable operating conditions had been established and following at least 5 reactor volume changes (approximately 2 days). The results also seem to suggest adaptation of the SRB consortium to a metal laden environment, this was more the case with lead removal where sulphate reduction was initially less compared to that for the copper solutions.

Different metal ions will probably require evaluation as their effect on the SRB may vary. However the study found that it was possible to successfully disarticulate the electron donor and carbon source supply in the application of an immobilised SRB consortium for the treatment of metal-contaminated aqueous solutions.

The results of the disarticulation of the electron donor and carbon source supply and the results of the subsequent application studies of such systems now allow further investigations into a new area of SRB bioprocess application, the utilisation of immobilised SRB in the treatment of clean stream waste waters, which has, up to this point, not been possible.

CHAPTER 6
CONCLUSIONS AND FUTURE WORK

6.1 Conclusions

The rationale for this research project derived from two constraints observed in the development of SRB biotechnology. Poor cell retention in continuous bioprocess reactors results in the loss of biomass, and consequently reduced cell-loading capacity and volumetric productivity of reactors. Also, high residual organic content, including cell biomass, in treated wastewaters has, to date, excluded the application of SRB bioprocesses in the treatment of 'clean' inorganic waste streams.

In view of the above-mentioned problems, effective immobilisation of the SRB was proposed as a pre-requisite for separating the biocatalyst from the treated stream. Effective immobilisation would also provide the basis for the swing-back cycle in which the electron donor and carbon source supply could be disarticulated to effect sulphate reduction and cell maintenance functions in two separate phases of operation.

To test the proposed hypothesis, it was necessary to select a suitable sulphate reducing culture and also evaluate, select and develop an effective immobilisation method for the culture. The immobilised SRB system would then be used to verify the proposed disarticulation concept and finally evaluate its application in the treatment of 'clean' inorganic waste streams.

An efficient sulphate reducing culture was selected from isolates collected from the Rhodes BioSure® Process pilot plant. This culture was shown to contain a *Desulfovibrio* within a stable consortium of three to four separate morphological types.

The most effective method of immobilisation of the SRB consortium was shown to be encapsulation in a liquid core within a Ca-alg-XG membrane, which resulted in robust and resilient capsules. The performance of the Ca-alg-XG capsules was found to be comparable to, and in some cases better than that found for free-suspended cells with the added advantage of easier handling and good mechanical properties.

The exclusion of all sources of carbon while at the same time supplying an external electron donor in the form of hydrogen gas effected the disarticulation of the electron donor and carbon source supply in the immobilised SRB systems. Successful sulphate reduction and sulphide generation during these periods demonstrated the concept of electron donor and carbon source disarticulation.

Swing-back cycles between full organic medium and inorganic aqueous streams (with hydrogen supplied as the sole electron donor) were used to evaluate the application of immobilised SRB in disarticulated systems for the treatment of synthetic 'clean' inorganic waste streams for sulphate removal, neutralisation of acidity and the removal of heavy metals from solution.

The average sulphate reduction rate in the electron donor phase was 50 mg SO_4^{2-}/L/day/g initial wet mass of capsules, which represented an average of 52 % sulphate removal. Sulphate reduction rates were found to be greater during the electron donor phase compared to carbon supply phase. No breakthrough points present as sulphate reduction impairment was observed during the three 4-day cycles of the electron donor phase. This indicates competent carbon storage by the culture over this period.

Reduced sulphate reduction activity in the electron donor phase was however, observed during neutralisation of acidic inorganic streams. Average sulphate reduction rates of 6.6 and 16.8 mg SO_4^{2-}/L/day/g initial wet mass of capsules at influent pH values of 2.4 and 4.0 respectively were recorded. This is 13 and 34% of neutral sulphate removal systems. Despite reduced sulphate reduction rates, continuous acidity neutralisation (effluent pH > 7.5) was maintained by the system over 12-day periods under disarticulated conditions. Neutralisation results suggest that the sulphate reduction process, and not just alkalinity generation in SLP, contributed toward pH elevation, probably through proton consumption in the generation of sulphide.

Average copper removal in the electron donor phase conditions was 99 and 85 % at initial copper concentrations of 2 and 60 mg/L respectively. No breakthrough points in sulphate reduction activity were observed during two 12-day cycles. Jalali and Baldwin (2000) had found inhibition of sulphate reduction in free cell systems by initial copper concentrations above 50 mg/L, which may explain the reduced rate of sulphate reduction.

The average sulphate reduction rates the electron donor phase for lead removal were 13 and 6 mg SO_4^{2-}/L/day/g initial wet mass of capsules, representing average percentage lead removal values of 49 and 78 % for the 3 and 10 mg/L lead solutions. These results contrast those reported by Sani *et al.*, (2001) who observed no measurable growth of *Desulfovibrio desulfuricans* at 3 mg/L lead in a free cell system. The low sulphate reduction rates suggest possible lead toxicity, but results in this study, for 3 and 10 mg/L initial lead concentrations suggest that the Ca-alg-XG membrane may confer an element of protection on the encapsulated cells. Adsorption to the capsule accounted for approximately 15 % of metal removal detected for both the copper and lead solutions. During experiments, black metal sulphide precipitates were observed to adhere to the capsules' surface.

The following broad conclusions may be drawn from the study:

- An efficient and stable SRB culture is necessary for the immobilisation process;
- Immobilisation by encapsulation in Ca-alg-XG membrane was produces robust and versatile capsules with comparatively good sulphate reduction activity;
- The use of immobilised cells facilitates the disarticulation of electron donor and carbon source supply, which enables the treated stream to be free of both biomass and organic contaminants;
- Immobilised SRB consortium in disarticulated bioprocess reactors may be successfully applied in the effective treatment of synthetic 'clean' inorganic waste streams for the removal of components such as sulphate, acidity neutralisation and copper and lead removal from solution;

The results of this study seemed to support the research hypothesis, and demonstrated the concept of disarticulation of the electron donor and carbon source supply in biological sulphate reduction systems;

6.2 Future Work

While the study on SRB immobilisation and disarticulation of the electron donor and carbon source in the treatment of 'clean' inorganic waste streams has provided an initial

demonstration of the concept, substantial further research will be required to explore the full potential of the idea. Future investigations would need to consider the following:

- Enumeration is a crucial requirement in evaluating biomass generation within the immobilised system. The problems of accurate routine biomass measurement are not trivial and will need to be resolved in order to further develop the application of the proposed system;
- The critical breakthrough points where sulphate reduction can no longer be maintained in the absence of a carbon source and the point where swing-back to full medium supply is required needs to be determined;
- Optimisation of the capsular strength requirements associated with reactor scale up development of the system also need to be determined;
- For full development of the system, a wider range of potential applications will need to be investigated;

REFERENCES

Abdelouas, A., Lutze, W., Gong, W., Nuttall, E., Strietelmeier, B.A. and Jarvis, B.J. (2000) Biological reduction of uranium in groundwater and subsurface soil. *The Science of the Total Environment,* 250: 21-35.

Abdelouas, A., Lutze, W., Nuttall, E., Gong, W., Fritz, B. and Crovisier, J-L. (1999) Enzymatic reduction of U(VI) in groundwaters. *Earth and Planetary Sciences,* 328: 321-326.

Aksu, Z., Eğretli, G. and Kutsal, T. (1998) A comparative study of copper (II) biosorption on Ca-alginate, agarose and immobilised *C.vulgaris* in a packed-bed column. *Process Biochemistry,* 33 (4): 393-400.

Alphenaar, A. (1999) Anaerobic granular sludge characterisation and factors affecting it's functioning. PhD thesis. Wageningen Agricultural University, The Netherlands. pp 147-159.

Anderson, R.T. and Lovley, D.R. (2000) Anaerobic bioremediation of benzene under sulphate-reducing conditions in a petroleum-contaminated aquifer. *Environmental Science and Technology,* 34 (11): 2261-2266.

Aspé, E., Martí, M.C. and Roeckel, M. (1997) Anaerobic treatment of fishery wastewater using a marine sediment inoculum. *Water Research,* 31 (9**):** 2147-2160.

Atlas, R.M. (1993) Handbook of microbiological media. Parks, L.C. (Ed.). CRC Inc. Florida. pp 71, 566-567, 856-858.

Bai, R.S. and Abraham, T.E. (2003) Studies on Chromium(VI) adsorption-desorption using immobilised fungal biomass. *BioresourceTechnology,* 87: 17-26.

Baker, C.A., Brooks, A.A., Greenley, R.Z. and Henis, J.M. (1992) Encapsulation of biological material in non-ionic polymer beads. United States patent number 5 089 407.

Bannick, H.F. and Muller, F.M. (1952) Utilisation of waste liquors from digestion of straw with monosulphite. *Antonie van Leeuwenhoek (International Journal of General and Molecular Microbiology),* 18. 45-54.

Basu, O. and Baldwin, S.A. (2000) Attachment and growth of sulphate-reducing bacteria on different support materials. *Environmental Technology,* (21): 1293-1300.

Batich, C. and Vaghefi, F. (1994) Process for microencapsulation. United States patent number 5 286 495.

Boopathy, R., Kulpa, C.F. and Manning, J. (1998a) Anaerobic biodegradation of explosives and related compounds by sulphate-reducing bacteria and methanogenic bacteria: A review. *Bioresource Technology,* 63: 81-89.

Boopathy, R., Manning, J. and Kulpa, C.F. (1998b) Biotransformation of explosives by anaerobic consortia in liquid culture and in soil slurry. *International Biodeterioration and Biodegradation,* 41: 67-74.

Boopathy, R. and Kulpa, C.F. (1992) Trinitrotoluene (TNT) as a sole nitrogen source for a sulphate-reducing bacterium *Desulfovibrio* sp. (B Strain) isolated from an anaerobic digester. *Current Microbiology,* 25: 235-241.

Boopathy, R., Wilson, M. and Kulpa, C.F. (1993) Metabolism of 2,4,6-trinitrotoluene (TNT) by *Desulfovibrio* sp. (Strain B). *Applied Microbiology and Biotechnology,* 39: 270-275.

Bouwer, E.J., Rittmann, B.E. and McCarty, P.L. (1981) Anaerobic degradation of halogenated 1- and 2-carbon organic compounds. *Environmental Science and Technology,* 15: 596-599.

Bowker, M.L. (2002) The biology and molecular ecology of floating sulphur biofilms. MSc thesis, Rhodes University, South Africa.

Bredwell, M.D., Srivastava, P. and Worden, R.M. (1999) Reactor design issues for synthesis-gas fermentations. *Biotechnology Progress,* (15) 5: 834-844.

Brierley, C.L. and Brierley, J.A. (1993) Immobilisation of biomass for industrial application of biosorption. *Biohydrometallurgical Technologies,* 35-44.

Brodelius, P. (1985) Immobilised plant cells: Preparation and biosynthetic capacity. In: Immobilized cells and enzymes – A practical approach. Woodward, J. (Ed.). IRL Press Ltd, Oxford, UK. pp 127-137.

Chang, H-N., Seong, G-H., Yoo, I-K., Park, J-K. and Seo, J-H. (1998) Method for immobilisation of whole microbial cells in calcium alginate capsules. United States patent number 5 766 907.

Chauke, C.G. (2002) The molecular microbial ecology of sulphate reduction in the Rhodes BioSURE process. MSc thesis, Rhodes University, South Africa.

Chen, B-Y., Utgikar, V.P., Harmon, S.M., Tabak, H.H., Bishop, D.F. and Govind, R. (2000) Studies on biosorption of zinc(II) and copper(II) on *Desulfovibrio desulfuricans*. *International Biodeterioration and Biodegradation,* 46: 11-18.

Clesceri, L.S., Greenberg. A.E. and Eaton, A.D. (eds) (1998) Standard methods for the examination of water and wastewater. 20th Edition. American Public Health Association, Washington. pp 2-27 – 2-29.

Cross, R., Botha, T. and Pinchuck, S. (2001) Preparation of biological material for electron microscopy. Rhodes University, South Africa.

Daims, H., Ramsing, N.B., Schleifer, K-H. and Wagner, M. (2001) Cultivation-independent, semiautomatic determination of absolute cell numbers in environmental samples by fluorescence *in situ* hydridisation. *Applied and Environmental Microbiology,* 67 (12**):** 5810-5818.

De Smul, A., Goethals, L. and Verstraete, W. (1999) Effect of COD to sulphate ration and temperature in expanded-granular-blanket reactors for sulphate reduction. *Process Biochemistry,* 34: 407-416.

Degiorgi, C.F., Pizarro, R.A., Smolko, E.E., Lora, S. and Carenza, M. (2001) Gamma ray preparation of hydrogels for bacteria immobilsation. *Radiation Physics and Chemistry,* 63 (1): 109-113.

DWAF (Department of Water Affairs and Forestry). (1996) South African water quality guidelines (first edition) – Volume 8: Field Guide. pp 2, 19, 28, 39, 49-50.

DWAF (Department of Water Affairs and Forestry). (1998) Minimum requirements for the handling, classification and disposal of hazardous waste. Second Edition. South Africa.

Dill, S., du Preez, L., Graaf, M. and Maree, J. (1995) Biological sulphate removal from acid mine drainage utilising producer gas as a carbon and energy source. *Water Sewage and Effluent,* 15 (1): 43-44.

Drzyzga, O., Küver, J. and Blotevogel, K-H. (1993) Complete oxidation of benzoate and 4-hydroxybenzoate by a new sulphate-reducing bacterium resembling *Desulfoarculus*. *Archives of Microbiology,* (159): 109-113.

Du Preez, L.A. and Maree, J.P. (1994) Pilot-scale biological sulphate and nitrate removal utilising producer gas as energy source. *Water Science and Technology,* 30 (12**):** 275-285.

Duncan, J.R., Brady, D. and Wilhelmi, B. (1992) Immobilisation of yeast and algal cells for bioremediation of heavy metals. In: Methods in Biotechnology. Volume 2. Bioremediation Protocols. Sheehan, D. (Ed.). Humana Press Inc., Totowa, New Jersey. pp 91-97.

Eccles, H. (1995) Removal of heavy metals from effluent streams – why select a biological process? *International Biodeterioration & Biodegradation,* 5-16.

Elliot, P., Ragusa, S. and Catcheside, D. (1998) Growth of sulphate-reducing bacteria under acidic conditions in an up-flow anaerobic bioreactor as a treatment system for acid mine drainage. *Water Research,* 32 (12): 3724-3730.

Fang, H.H.P. and Zhou, G-M. (1997) Anaerobic degradation of benzoate and cresol isomers in sulphate-rich wastewater. *Water Science and Technology,* 36 (6-7): 7-14.

Ferraro, L., Fatichenti, F. and Ciani, M. (2000) Pilot scale vinification process using immobilised *Candida stellata* and *Saccharomyces cerevisiae*. *Process Biochemistry,* 35 (10):. 1125-1129.

Finnie, K.S., Bartlett, J.R. and Woolfrey, J.L. (2000) Encapsulation of sulphate-reducing bacteria in a silica host. *Journal of Materials Chemistry,* (10) 1099-1101.

Foucher, S., Battaglia-Brunet, F., Ignatiadis, I. And Morin, D. (2001) Treatment by sulphate-reducing bacteria of Chessy acid-mine drainage and metals recovery. *Chemical Engineering Science, (*56): 1639-1645.

Francis, A.J., Dodge, C.J., Lu, F., Halada, G.P. and Clayton, C.R. (1994) XPS and XANES studies of uranium reduction by *Clostridium* sp. *Environmental Science and Technology,* (28): 636-639.

G.ang.ser.o.slashed.d, O., Skaugrud, O., Dettmar, P., Sj.ang.k-Braek, G. and Jolliffe, I. (2000) High strength capsules, process for preparing and using the same. United States patent number 6 165 503.

Gadd, G.M. (1988) Accumulation of metals by microorganisms and algae. In: Biotechnology, Volume 6b. Rehm, HJ and Reeds, G (eds). VCH, Weinheim. pp 425-427.

Garbayo, I., Vigara, A.J., Conchon, V., Martins Dos Santos, V.A.P. and Vílchez, C. (2000) Nitrate consumption alterations induced by alginate-entrapment of *Chlamydomonas reinhardtii* cells. *Process Biochemistry,* 36 (5): 459-466.

García, C., Moreno, D.A., Ballester, A., Blázquez, M.L. and González, F. (2001) Bioremediation of an industrial acid mine water by metal-tolerant sulphate-reducing bacteria. *Minerals Engineering,* 14 (9): 997-1008.

Gibson, G.R. (1990) Physiology and ecology of sulphate-reducing bacteria. *Journal of Applied Bacteriology,* 69: 769-797.

Glombitza, F. (2001) Treatment of acid lignite mine flooding water by means of microbial sulphate reduction. *Waste Management,* 21: 197-203.

Godjevargova, T., Aleksieva, Z. and Ivanova, D. (2000) Cell immobilisation of *Trichosporon cutaneum* strain with phenol degrading ability on new modified polymer carriers. *Process Biochemistry,* 35 7: 699-704.

Goosen, M.F.A., O'Shea, G.M. and Sun, A.M.F. (1989) Microencapsulation of living tissue and cells. United States patent number 4 806 355.

Green, K.D., Gill, I.S., Khan, J.A. and Vulfson, E.N. (1996) Microencapsulation of yeast cells and their use as a biocatalyst in organic solvents. *Biotechnology and Bioengineering,* 49, 5: 535-543.

Groudev, S.N., Georgiev, P.S., Spasova, I.I. and Komnitsas, K. (2001a) Bioremediation of a soil contaminated with radioactive elements, *Hydrometallurgy,* 59: 311-318.

Groudev, S.N., Spasova, I.I. and Georgiev, P.S. (2001b) *In situ* bioremediation of soils contaminated with radioactive elements and toxic heavy metals. *International Journal of Mineral processing,* 62: 301-308

Groudeva, V.I., Groudev, S.N. and Doycheva, A.S. (2001) Bioremediation of waters contaminated with crude oil and toxic heavy metals. *International Journal of Mineral Processing,* 62: 293-299.

Gupta, M., Gupta, A., Suidan, M.T. and Sayles, G.D. (1996) Biotransformation rates of chloroform under anaerobic conditions – II. Sulphate reduction. *Water Resources,* 30 (6): 1387-1394.

Gyure, R.A., Konopka, A., Brooks, A. and Doemel, W. (1990) Microbial sulphate reduction in acidic (pH 3) strip-mine lakes. *FEMS Microbiology Letters,* 73 (3): 193-201.

Happold, F.C. (1957) Biological treatment of effluents. *Research, London,* 10: 443-448.

Harithsa, S., Kerkar, S. and Bharathi, P.A.L. (2002) Mercury and lead tolerance in hypersaline sulphate-reducing bacteria. *Marine Pollution Bulletin,* 44: 726-732.

Hermanson, G.T., Mallia, A.K. and Smith, P.K. (1992) Immobilised affinity ligand techniques. Academic Press Inc., California. pp 1-53.

Holland, K.T., Knapp, J.S. and Shoesmith, J.G. (1987) Anaerobic Bacteria – Tertiary level biology. Blackie & Son Ltd, London. pp 134-197.

Horan, N.J. (1990) Biological wastewater treatment systems: Theory and operation. John Wiley & Sons, West Sussex. pp 1, 41-71.

Isa, Z., Grusnmeyer, S. and Verstraete, W. (1986a) Sulphate reduction relative to methane production in high-rate anaerobic digestion: Microbiological aspects. *Applied Environmental Microbiology*. 52 (3): 580-587.

Isa, Z., Grusnmeyer, S. and Verstraete, W. (1986b) Sulphate reduction relative to methane production in high-rate anaerobic digestion: Technical aspects. *Applied Environmental Microbiology*. 52 (3): 572-579.

Jalali, K. and Baldwin, S.A. (2000) The role of sulphate reducing bacteria in copper removal from aqueous sulphate solutions. *Water Research,* 34 (3): 797-806.

Johnson, D.B. (1995) Acidophilic microbial communities – candidates for bioremediation of acidic mine effluents. *International biodeterioration and biodegredation,*: 41-58.

Kaksonen, A.H., Riekkola-Vanhanen, M-L. and Puhakka, J.A. (2003) Optimisation of metal sulphide precipitation in fluidised-bed treatment of acid wastewater. *Water Research,* 37: 255-266.

Keith, S.M. and Herbert, R.A. (1983) Dissimilatory nitrate reduction by a strain of *Desulfovibrio desulfuricans*. *FEMS Microbiology Letters,* 18 (1-2): 55-59.

Kelly, D.P. and Wood, A.P. (2000) Reclassification of some species of *Thiobacillus* to the newly designated genera *Acidithiobacillus* gen. nov. *Halothiobacillus* gen. nov. and *Thermithiobacillus* gen. nov. *International Journal of Systematic Evolutionary Microbioog,* 50: 511–516

Kesserű, P., Kiss, I., Bihari, Z. and Polyák, B. (2003) Biological denitrification in a continuous-flow pilot bioreactor containing immobilised *Pseudomonas butanovora* cells. *Bioresource Technology,* 80: 75-80.

Kierstan, M.P.J. and Coughlan, M.P. (1985) Immobilisation of cells and enzymes by gel entrapment. In: Immobilized cells and enzymes – A practical approach. Woodward, J. (Ed.). IRL Press Ltd, Oxford, UK. pp 39-48.

Klei, H.E. Sundstrom, D.W. and Shim, D. (1985) Immobilisation of enzymes by microencapsulation. In: Immobilized cells and enzymes – A practical approach. Woodward, J. (Ed.). IRL Press Ltd, Oxford, UK. pp 49.

Klein, J., Stock, J. and Vorlop, K.D. (1983) Pore size and properties of spherical Ca-alginate biocatalysts. *European Journal of Applied Microbiology and Biotechnology,* 18: 86-91.

Klein, J., Vorlop, K.D. and Steinert, H.J. (1986) German patent DE 3432923.

Knobel, A.N. and Lewis, A.E. (2002) A mathematical model of a high sulphate wastewater anaerobic treatment system. *Water Research,* 36: 257-265.

Kolmert, A. and Johnson D.B. (2001) Remediation of acidic waste waters using immobilised, acidophilic sulphate-reducing bacteria. *Journal of Chemical Technology and Biotechnology*, 76: 836-843.

Kuever, J., Kulmer, J., Jannsen, S., Fischer, U. and Blotevogel, K-H. (1993) Isolation and characterisation of a new spore-forming sulphate-reducing bacterium growing by complete oxidation of catechol. *Archives of Microbiology,* 159: 282-288.

Kuhn, S.P. (1988). Bioaccumulation of metals using immobilised *Zoogloea ramigera*. PhD dissertation, The Ohio State University, Ohio, U.S.A.

Kuhn, S.P. and Pfister R.M. (1989) Adsorption of mixed metals and cadmium by calcium-alginate immobilised *Zoogloea ramigera*. *Applied Microbiology and Biotechnology*, 31: 613-618.

Lettinga, G. (1985) Anaerobic wastewater treatment and its potential. *European water and sewage,* 89: 22-27.

Lloyd, J.R., Mabbett, A.N., Williams, D.R. and Macaskie, L.E. (2001) Metal reduction by sulphate-reducing bacteria: physiological diversity and metal specificity. *Hydrometallurgy,* 59: 327-337.

Lloyd, J.R., Yong, P. and Macaskie, L.E. (1998) Enzymatic recovery of palladium by using sulphate-reducing bacteria. *Applied and Environmental Microbiology,* 64 (11). 4607-4609.

Lovley, D.R., Philips, E.J.P., Gorby, Y.A. and Landa, E.A. (1991) Microbial reduction of uranium. *Nature,* 350: 413-416.

Lovley, D.R. (1997) Potential for anaerobic bioremediation of BTEX in petroleum-contaminated aquifers. *Journal of Industrial Microbiology and Biotechnology,* 18 (2/3): 75-81.

Lusta, K.A., Chung, I.K., Sul, I.W., Park, H.S. and Shin, D.I. (2000) Immobilisation of fungus *Aspergillus sp.* by a novel cryogel technique for production of extracellular hydrolytic enzymes. *Process Biochemistry,* 35 (10): 1177-1182.

Lyew, D., Knowles, R. and Sheppard, J. (1994) The biological treatment of acid mine drainage under continuous flow conditions in a reactor. *Trans Ichem E.,* 72B: 42-47.

Madigan, M.T., Martinko, J.M. and Parker, J. (1997) Brock - Biology of Microorganisms. 8th edition, Prentice-Hall Inc. New Jersey. 671-674.

Mallouchos, A., Komaitis, M., Koutinas, A. and Kanellaki, M. (2003) Wine fermentation by immobilised and free cells at different temperatures. Effect of immobilisation and temperature on volatile by-products. *Food Chemistry,* 80: 109-113.

Maree, J.P. and Strydom, W.F. (1985) Biological sulphate removal from a packed bed reactor. *Water Research,* 19 (9): 1101-1106.

Maree, J.P., Gerber, A. and Strydom, W.F. (1986) A biological process for sulphate removal from industrial effluent. *Water SA,* 12 (3). 139-144.

Maree, J.P. and Hill, E. (1989) Biological removal of sulphate from industrial effluents and concomitant production of sulphur. *Water Science and Technology,* 21: 265-276.

Maree, J.P., Hulse, G., Dods, D. and Schutte, C.E. (1991) Pilot plant studies on biological sulphate removal from industrial effluent. *Water Science and Technology,* 23: 1293-1300.

Mattiasson, B. (1983a) Immobilised cells and organelles. Volume I. CRC Press Inc., Florida. pp 4-25.

Mattiasson, B. (1983b) Immobilised viable cells. In: Immobilised cells and organelles. Volume II. Mattiasson (Ed.). CRC Press Inc., Florida. pp 23-37.

McCormick, N., Feeherry, F.E. and Levinson, H.S, (1976) Microbial transformation of 2,4,6-trinitrotoluene and other nitroaromatic compounds. *Applied Environmental Microbiology,* 31: 949-958.

McCormick, N.G., Cornell, J.H. and Kaplan, A.M. (1981) Biodegradation of hexahydro-1,3,5-trinitro-1,3,5-triazinc. *Applied Environmental Microbiology,* **42**. 817-823.

Miller, L.P. (1950) Formation of metal sulphides through the activities of sulphate-reducing bacteria. *Plant Research,* 16: 85-89.

Murray R.G.E., Doetsch, R.N. and Robinow, C.F. (1994) Determinative and cytological light miscrosopy. In: Manual of methods for general and molecular bacteriology. Gerhart, P., Murray, R.G.E., Wood, W.A. and Krieg, N.E. (Eds.), ASM, Washington, DC. pp 21-41.

Nagpal, S., Chuichulcherm, S., Peeva, S. and Livingston, A. (2000) Microbial sulphate reduction in a liquid-solid fluidised bed reactor. *Biotechnology and Bioengineering,* 70 (4): 370-380.

Nedovic, V.A., Leskosek-Cukalovic, I. And Vunjak-Novakovic, G. (1998) Immobilized cell technology (ICT) in beer fermentation – a possibility for environmentally sustainable and cost-effective process. http://www.rcub.bg.ac.yu/~todorom/tutorials/rad15.html (accessed 24 October 2001).

O'Flaherty, V, Lens, P., Leahy, B. and Colleran, E. (1998) Long-term competition between sulphate-reducing and methane-producing bacteria during full scale anaerobic treatment of citric acid production wastewater. *Water Research,* 32 (3): 815-825.

Olem, H. and Unz, R.F. (1980) Rotating-disc biological treatment of acid mine drainage. *Journal of Water Pollution Control Fed,* 52: 257-269.

Öztop, H.N., Öztop, Y.A., Karadağ, E., Işikver, Y. and Saraydin, D. (2003) Immobilisation of *Saccharomyces cerevisiae* on to acrylamide-sodium acrylate hydrogels for production of ethyl alcohol. *Enzyme and Microbial Technology,* 32: 114-119.

Pareek, S., Kim, S.K., Matsui, S. and Shimizu, Y. (1998) Hydrolysis of (ligno) cellulosic materials under sulphidogenic and methanogenic conditions. *Water Science and Technology,* 38 (2): 193-200.

Park, J.K. and Chang, H.N. (2000) Microencapsulation of microbial cells. *Biotechnology Advances,* 18 (4): 303-319.

Pilkington, P.H., Margaritis, A., Mensour, N.A. and Russell, I. (1998) Fundamentals of Immobilized Yeast Cells for Continuous Beer Fermentation: A. Review. *Journal of the Institute of Brewing,* 104: 19-31.

Pogliani, C. and Donati, E. (2000) Immobilisation of *Thiobacillus ferrooxidans*: importance of jarosite precipitation. *Process Biochemistry,* 35 (9): 997-1004.

Postgate, J.R. (1982) Economic importance of sulphur bacteria. *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences,* 298 (1093): 583-600.

Rha, C. and Rodriguez-Sanchez, D. (1988) Encapsulated active material system. United States patent number 4 749 620.

Rose, P.D., Boshoff, G.A., van Hille, R.P., Wallace, L.C.M., Dunn, K.M. and Duncan, J.R. (1998) An integrated algal sulphate reducing high rate ponding process for the treatment of acid mine drainage wastewaters. *Biodegradation,* 9: 247-257.

Rothery, E. (1980) AA-1275 and AA-1475 series spectrophotometers: Operation Manual. Varian Techtron Pvt. Ltd. Mulgrave, Australia. pp 1-15.

Rowley, M.V., Warkentin, D.D., Yan, V.T. and Piroshco, B.M. (1994) The Biosulphide process: Integrated biological/chemical acid mine drainage treatment – results of laboratory piloting. Conference presentation, International Land Reclamation and Mine Drainage Conference and the Third International Conference of Acid Mine Drainage. Pittsburgh. pp 205-213.

Sağ, Y., Nourbakhsh, M., Aksu, Z. and Kutsal, T. (1995) Comparison of Ca-alginate and immobilised *Z. ramigera* as sorbents for copper(II) removal. *Process Biochemistry,* 30 (2): 175-181.

Salle, A.J. (1954) Fundamental principles of bacteriology. 4th Edition, McGraw-Hill Book Company, Inc. New York. pp 355-356.

Sani, R.K., Geesey, G. and peyton, B.M. (2001) Assessment of lead toxicity to *Desulfovibrio desulfuricans* G20: influence of components of Lactate C medium. *Advances in Environmental research,* 5: 269-276.

Schroth, M.H., Kleikemper, J., Bolliger, C., Bernasconi, S.M. and Zeyer, J. (2001) *In situ* assessment of microbial sulphate reduction in a petroleum-contaminated aquifer using push-pull tests and stable sulphur isotope analysis. *Journal of Contaminant Hydrology,* 51: 179-195.

Scott, C.D. (1987) Immobilised cells: a review of recent literature. *Enzyme Microbiology and Technology,* 9: 66-73.

Selvaraj, P.T., Little, M.H. and Kaufman, E.N. (1997) Biodesulphurisation of flue gases and other sulphate/sulphite waste streams using immobilised sulphate-reducing bacteria. *Biotechnology Progress,* 13 (5): 583-589.

Silva, A.J., Varesche, M.B., Foresti, E. and Zaiat, M. (2002) Sulphate removal from industrial wastewater using a packed-bed anaerobic reactor. *Process Biochemistry,* 37: 927-935.

Sodini, I., Boquien, C., Corrieu, G. and Lacroix, C. (1997) Microbial dynamics of co- and separately entrapped mixed cultures of mesophilic lactic acid bacteria during the continuous pre-fermentation of milk. *Enzyme and Microbial Technology,* 20: 381-388.

Song, Y-C., Piak, B-C., Shin, H-S. and La, S-J. (1998) Influence of electron donor and toxic materials on the activity of sulphate-reducing bacteria for the treatment of electroplating wastewater. *Water Science & Technology,* 38 (4-5): 187-194.

Steenkamp, D.J. and Peck, H.D. (1981) Proton translocation associated with nitrite respiration in *Desulfovibrio desulfuricans*. *Journal of Biological Chemistry.* 256: 5450-5458.

Tampion, J. and Tampion, M.D. (1987) Immobilised cells: principles and applications. Cambridge University Press, Cambridge. pp 1-19, 75-77, 97-99, 113-135.

Tasaki, M., Kamagata, Y., Nakamura, K. and Mikami, E. (1991) Isolation and characterisation of a thermophilic benzoate-degrading sulphate-reducing bacterium. *Desulfotomaculum thermobenzoicum* sp. nov. Arch Microbiology, 155: 348-352.

Timur, H., Ozturk, I., Altinbas, M., Arikan, O. and Tuyluoglu, B.S. (2000) Anaerobic treatability of leachate: comparative evaluation of three different reactor systems. *Water Science and Technology,* 42 (1-2): 287-292.

Tobin, J.M., L'homme, B. and Roux, J.C. (1993) Immobilisation protocols and effect on cadmium uptake by *Rhizopus arrhizus* biosorbents. *Biotechnology Techniques,* 7 (10): 739-744.

Trevan, M.D. (1980) Immobilized enzymes – An introduction and applications in Biotechnology. John Wiley and Sons Ltd. USA. pp 1-55.

Tsezos, M., McCready, R.G.L. and Bell, J.P. (1989) The continuous recovery of uranium from biologically leached solutions using immobilised biomass. *Biotechnology and Bioengineering,* 34: 10-17.

Tsezos, M. and Deutschmann, A.A. (1990) An investigation of engineering parameters for the use of immobilised biomass particles in biosorption. *Journal of Chemical Technology and Biotechnology,* 48: 29-39.

Tuttle, J.H., Randles, C.I. and Dugan, P.R. (1968) Activity of micro-organisms in acid mine water. I. Influence of acid mine water on aerobic heterotrophs of a normal stream. *Journal of Bacteriology,* 95: 1495-1503.

Tuttle, J.H., Dugan, P.R., MacMillan, C.B. and Randles, C.I. (1969a) Microbial dissimilatory sulphur cycle in acid mine water. *Journal of Bacteriology,* 97: 594-602.

Tuttle, J.H., Dugan, P.R., and Randles, C.I. (1969b) Microbial sulphate reduction and its potential utility as an acid mine water abatement procedure. *Journal of Applied Microbiology,* 17: 297-302.

Van Groenestijn, J.W. (1999) Sulphur recovery from tannery wastewater. *Leather,* 19-21.

van Hille, R.P., Boshoff, G.A., Rose, P.D., and Duncan, J.R. (1999) A continuous process for the biological treatment of heavy metal contaminated acid mine water. *Resources, Conservation and Recycling,* 27: 157-167.

Van Houten, R.T. (1996) Biological sulphate reduction with synthesis gas. Ph.D. thesis, Wageningen Agricultural University, Wageningen, The Netherlands. pp 9-18.

Venkatasubramanian, K. and Karkare, S.B. (1983) Process engineering considerations in the development of immobilised living cell systems. In: Immobilised cells and organelles. Volume II. Mattiasson (Ed.). CRC Press Inc., Florida. pp 134-143.

Visser, A. (1995) The anaerobic treatment of sulphate containing wastewater. PhD Thesis, Wageningen Agricultural University, Wageningen, The Netherlands. pp 1-4, 81-108.

Webb, J.J., McGinness, S and Lappin-cott, H.M. (1998) Metal removal by sulphate-reducing bacteria from natural and constructed wetlands. *Journal of Applied Microbiology,* 84: 240-248.

White, C., Sayer, J.A. and Gadd G.M. (1997) Microbial solubilisation and immobilisation of toxic metals: key biogeochemical processes for treatment of contamination. *FEMS Microbiology Reviews,* 20: 503-516.

Whittington-Jones, K. (2000) Sulphide-enhanced hydrolysis of primary sewage sludge : implications for the bioremediation of sulphate-enriched wastewaters. Ph.D. thesis, Rhodes University, South Africa.

Widdel, F. (1988) Microbiology and ecology of sulphate- and sulphur- reducing bacteria. In: Biology of anaerobic microorganisms. Zehnder, A.J.B. (Ed.). Wiley & Sons Inc. New York. pp 469-585.

Widdel, F. and Bak, F. (1992) Gram-negative mesophilic sulphate-reducing bacteria. In: The prokaryotes, 2nd Edn. Balows, A., Trüper, H.G., Dworkin, M., Harder, W. and Schleifer, K.H. (eds). Springer, New York. pp 3352-3378.

Wiedemeier, T.H., Rifai, H.S. and Newell, C.J. (1999) Natural attenuation of fuels and chlorinated solvents in the subsurface. Wiley & Sons, New York. pp 617.

Zehnder, A.J.B. (1988) Biology of anaerobic microorganisms. John Wiley & Sons Inc. New York. pp 471-476.

ZoBell, C.E. (1946) Marine Microbiology. Chronica Botanica Co. Waltham, MA.

APPENDICES

APPENDIX 1:

Alkalinity Determination

Potentiometric titration to end-point pH method:

A 10 mL (or 5 mL if reactor volume small) sample was placed in a 100 mL beaker on a stirrer bar with a pH pencil probe attached to a calibrated Cyberscan pH meter. The sample was titrated with 0.02N standard sulphuric acid to an end-point pH of 4.5 for total alkalinity determination, which was calculated using the equation below:

$$\text{Alkalinity (mg } CaCO_3\text{/L)} = \frac{A \times N \times 50\,000}{\text{mL sample}}$$

Where:

A = mL standard acid used

N = normality of standard acid (Clesceri *et al.*, 1998).

Copper Analysis - Spectroquant

Copper analysis was carried out according to the Merck Spectroquant reagent kit 1.14767 for photometric analysis based on the reaction of copper (II) with cuprizon (oxalic acid bis [cyclohexylidene hydrazide]) reagent in an alkaline medium to give a blue colour complex.

Samples were diluted using dddH_2O to fall within the detection range of 0.1 – 9.5 mg/L and read on the Merck Spectroquant NOVA 60 instrument with internal standards.

Operation	**Volume/Amount**
Sample (20-30 °C)	5 mL
Add Cu -1A and mix to dissolve	1 level spoon (green)
Add Cu -2A and mix	5 drops
Leave to stand for 5 minutes	
Measure	On Spectroquant or against a blank at 595 nm

Lead Analysis – Atomic Absorption Spectrophotometer

Atomic absorption spectrophotometer operating parameters for lead analysis

Flame	Wavelength (nm)	Lamp current (mA)	Slit width (nm)	Working range (mg/L)	Sensitivity (mg/L)
A-A*	217	5.0	1.0	0.2 - 20	± 0.06

* A-A = Air-Acetylene (Rothery, 1980).

Samples with suspended solids or particulate matter were first filtered using a millipore filter system with a 25 mm diameter, 0.45 μm pore size nylon filter.

SBB Media

	Composition per litre			
Component	**Pg-B**	**Pg-C**	**SRBM**	**SRM**
Sodium lactate (60 % soltn)	4 mL	6 mL	4 mL	4 mL
$MgSO_4.7H_2O$	2.0 g	0.1 g	2.0 g	2.0 g
Na_2SO_4	-	2.0 g	1.0 g	1.5 g
NH_4Cl	1.0 g	1.0 g	1.0 g	-
$CaSO_4$	1.0 g	-	-	-
$CaCl_2.6H_2O$ / $CaCl_2.6H_2O$	-	0.5 g	0.1 g	0.1 g
$Fe(NH_4)_2(SO_4)_2.6H_2O$	-	-	-	3.92 g#
Yeast extract	1.0 g	1.0 g	1.0 g	-
Peptone	-	-	-	2.0 g
Beef extract	-	-	-	1.0 g
KH_2PO_4	0.5 g	0.25 g	0.5 g	0.5 g
$FeSO_4.7H_2O$	0.5 g	0.004 g	0.5 g*	-
Ascorbic acid	0.1 g	0.1 g	0.1 g*	0.05 g#
Thioglycollic acid	0.1 g	0.1 g	0.1 g*	-
Sodium citrate.$2H_2O$	-	0.3 g	-	-

Pg-B = Postgate B; Pg-C = modified Postgate C; SRBM = Sulphate reducing bacteria medium; SRM = Sulphate reducing medium

* = made separately in 10 mL and added to 980 mL of media

= made separately in 100 mL and add 10 mL to 1 L of media

Autoclaved and the pH adjusted to 7.0 – 7.5 (Atlas and Parks, 1993).

Sulphate (SO_4^{2-}) Analysis - HPLC

Sample preparation:

Sample preparation was carried out immediately after sample collection to avoid prolonged exposure of samples to the atmosphere.

A sample was diluted 1 in 10 in a total volume of 1.5 mL and filtered using a millipore filter system with a 25 mm diameter, 0.22 μm pore size nylon filter. The filtrate was then filtered slowly through a Waters C-18 solid-phase organics extraction cartridge, this filtration was repeated through a regenerated Waters C-18 solid phase organics extraction cartridge. The sample was then ready for analysis by HPLC.

Waters C-18 solid phase organics extraction cartridges were regenerated twice before discarding. Regeneration was carried out by slowly eluting with 1 mL of triple distilled water ($dddH_2O$) followed by 1 mL of methanol and another 1 mL of $dddH_2O$ and allowed to dry before use.

HPLC Mobile Phase for anion sulphate (SO_4^{2-})

Stock: 5.52g of *para*-Hydroxybenzoic acid was dissolved in less than 500 mL $dddH_2O$ in a 2 L volumetric flask, and then 250 mL of Methanol was added before making up the solution to the 2 L mark with $dddH_2O$.

Running buffer: 300ml of the stock mobile phase was made up to approximately 800 mL, the pH was adjusted to 8.3 with 0.1N sodium hydroxide before making up to the 1 L mark. The running buffer was then filtered through a 7.5 cm diameter, 0.45 μm nylon filter and degassed under vacuum.

Samples were run on a Waters model 510 pump, the HPLC column used was a Hamilton PRP-X100 10 μm, 150 x 4.1 mm column, attached to a Waters 430 Conductivity Detector. The instrument had a Rheodyne injection port and used BORWIN v.1.5x. software for data and peak analysis. A standard curve was generated using sodium sulphate. 20 μL samples were injected and run at a flow-rate of 2 mL/min, the sulphate peak's retention time was approximately 10 minutes.

Sulphate (SO_4^{2-}) Analysis - Spectroquant

Sulphate analysis was carried out according to the Merck Spectroquant reagent kit 1.14791 for photometric analysis based on the reaction of barium iodate with sulphate ions to give barium sulphate and iodate ions which react with tannins in a weak acid medium to give a reddish-brown colour.

Samples were diluted using de-oxygenated dddH_2O (minimize oxidation of sulphides in solution to sulphate) to fall within the detection range of 10 – 600 mg/L and read on the Merck Spectroquant NOVA 60 instrument with internal standards.

Operation	**Volume/Amount**
Sample	2.5 mL
Add SO_4^{2-} -1A and mix	2 drops
Add SO_4^{2-} -2A and mix	1 green microscoop
Shake well repeatedly for 5 minutes while at 40 °C	
Add SO_4^{2-} -3A and mix	2.5 mL
Filter through a 0.45 μm pore-size nylon filter – repeat if still turbid	
Add SO_4^{2-} -4A and mix	4 drops
Mix and stand at 40 °C for 7 minutes	
Measure	On Spectroquant or against a blank at 515 nm

Sulphide Analysis - Spectroquant

Sulphide analysis was carried out according to the Merck Spectroquant reagent kit 1.14779 for photometric analysis based on the reaction of hydrogen sulphide with N,N'-dimethyl-1,4-phenylene-diamine dihydrochloride and oxidation with iron(III) to methylene blue.

Hydrogen sulphide was released from the zinc sulphide complex by lowering the pH of the samples from the zinc acetate gas traps.

Samples were diluted using de-oxygenated dddH_2O to fall within the detection range of 0.02 – 1.50 mg/L and read on the Merck Spectroquant NOVA 60 instrument with internal standards.

Operation	**Volume/Amount**
Sample (5-25 °C)	5 mL
Add HS-1A and mix	1 drop
Add HS-2A and mix	5drops
Add HS-3A and mix	5drops
Measure	On Spectroquant or against a blank at 665 nm*
* with concentrations greater than 3 mg/l wait 5 minutes before reading.	

APPENDIX 2:

Electron Microscopy

Phosphate buffer:	Solution A	35.8g/l	$Na_2HPO_4.2H_2O$
	Solution B	13.6g/l	KH_2PO_4

Solutions A and B were mixed in the ratio 4:1 to give a 0.1 M, pH 7.3 solution.

Buffered gluteraldehyde:	10ml	25% ultrastructure grade gluteraldehyde
	90ml	Phosphate buffer

The solution was stored in a dark bottle at 4°C.

1% Buffered Osmium Tetroxide

Solution 1	2.55% $Na_2HPO_4.2H_2O$
Solution 2	2.52% NaOH
Solution 3	5.4% glucose
Solution 4	41.5ml Solution 1
	8.5ml Solution 2

Fixative consists of 45ml Solution 4, 5ml Solution 3 and 0.5g OsO_4.
Dissolve the osmium tetroxide crystals in Solution 4 by warming in the fume hood.
Cool and decant into a clean flask, add Solution 3 and pH to 7.3 with 0.1M HCl.
Store in a dark bottle at 4°C. (Cross, 2001)

Scanning electron microscopy (SEM)

Calcium-alginate beads were dried and then fixed overnight at 4 °C in a 2.5 % buffered gluteraldehyde solution. After 2 washes with phosphate buffer, the samples were put through a series of 10-minute alcohol dehydration steps starting from 30 % through to absolute ethanol in 10 % dilution steps. The sample was then subjected to critical point drying in the appropriate instrument to eliminate all liquid within the specimen while minimising distortion.

A portion of the sample was then cut off and placed onto a clean specimen stub using double-sided conductive tape. This sample was gold coated to improve secondary electron emission and to reduce charge build-up. An electron microscope JEOL JSM 840 was used to view the sample (Cross *et al.,* 2001).

Cryo-SEM

Initial attempts to prepare calcium-alginate capsules by conventional SEM method as described above resulted in the membrane dissolving in gluteraldehyde. Therefore, a cryo-SEM technique was used.

In cryo-SEM the sample is observed in a frozen hydrated state, prepared by making use of a specialised piece of equipment called a Hexland CT 1000.

Capsules were attached to specimen holders with TissueTek and rapidly plunge-frozen in sub-cooled (slushy) nitrogen and transferred under vacuum to a Hexland Cryo-SEM unit attached to a JEOL JSM 840 scanning electron microscope. In order to expose the interior of the capsules they were fractured either under sub-cooled nitrogen following initial freezing or on the cold stage in the preparation chamber of the Hexland Cryo-SEM unit. The frozen and fractured capsules were then transferred to the cold stage in the SEM specimen chamber and heated to -80C to allow sublimation of ice, and thus "etching" of the fractured surface, to expose the cells. When adequate "etching" had taken place the capsules were transferred back to the cryo preparation chamber for sputter-coating with gold following which they were returned to the cold stage of the SEM specimen chamber for SEM examination. Micrographs were recorded digitally via a JEOL WinStor image handling system and conventionally on Agfapan APX 100 film (Cross *et al*., 2001).