ACCELERATED SLUDGE SOLUBILISATION UNDER SULPHATE REDUCING CONDITIONS: THE EFFECT OF HYDROLYTIC ENZYMES ON SLUDGE FLOC SIZE DISTRIBUTION AND EPS COMPOSITION

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ABSTRACT

Extracellular polymeric substances (EPS) are the construction materials for microbial aggregates such as biofilms, flocs and sludge, and greatly contribute to the structural integrity of sludge flocs in wastewater treatment processes. The loss of integrity of the sewage sludge floc is believed to be due to enhanced hydrolysis of important structural components such as lignin, protein and cellulose in the sludge floc matrix. The mechanism of enhanced sludge floc fracture, due to the action of enzymes hydrolysing these structural components, remains a key element in our understanding of how the floc integrity in systems utilising a sulphate reducing system is compromised. A range of relatively non-specific exogenous enzymes (ß-glucosidase, cellulase, proteases: trypsin, pronase E and chymotrypsin) were added to a sulphidogenic bioreactor- (containing both sulphate reducing bacteria (SRB) and a methanogenic bacterial system) and a (control) methanogenic bioreactor sample, and the effect of these enzymes on sludge floc size (diameter) distribution and EPS composition was investigated. Sludge samples from the bioreactors were examined under bright field and differential interference contrast light microscopy. Proteolytic and glucohydrolytic activity of the enzymes were monitored using standard enzyme assaying techniques, and Bradford, Somogyi-Nelson, and total carbohydrate assays were performed to establish the composition of the EPS (after extraction with 3% (v/v) glutaraldehyde and Sephacryl S-400 size exclusion chromatography).

Sludge flocs present in the sulphidogenic environment of the sulphidogenic bioreactor were found to have smaller diameters than their counterparts present in the methanogenic bioreactor. The addition of hydrolytic (i.e. proteolytic and glycohydrolytic) enzymes resulted in an increased rate of matrix hydrolysis, leading to increased rates of floc fracture and deflocculation. The presence of ß-glucosidase, cellulase, and proteases naturally residing within the sludge floc was confirmed. We propose that the addition of commercially available enzymes may be prohibitively costly on a large scale, and that the activity of the enzymes naturally residing within the floc matrix be optimised or enhanced.

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As the bulk of the EPS was shown to be composed of mainly polysaccharides, we propose that by increasing the activity of the naturally occurring ß-glucosidases residing within the floc matrix, the process of deflocculation may be enhanced. As sulphide has been shown to increase the activity of this very important key enzyme, we propose that this is one of the contributing factors why sludge solubilisation is accelerated under sulphate reducing conditions.

INTRODUCTION

Sulphate reduction by Sulphate Reducing Bacteria (SRB) has long been identified as a potential process for Acid Mine Drainage (AMD) bioremediation. The SRB (obligate anaerobes) have the ability to utilise inorganic sulphate as a terminal electron acceptor during an ATP-requiring reaction, but require the supply of a low molecular weight carbon source as an electron donor for sulphate reduction. As SRB are unable to degrade long polymeric molecules such as polysaccharides, proteins or lipids, they rely on the activity of hydrolytic and acidogenic bacteria (involved in anaerobic digestion) to supply the monomeric carbon/energy source. Primary sewage sludge has been recognised as a low cost, readily available carbon source for SRB. In addition, SRB present in sulphate-reducing systems have also been shown to dramatically accelerate the rate of primary sewage sludge hydrolysis and solubilization by methanogenic bacteria in our bioreactors.

Sludge components are cemented together by the extracellular polymeric substances (EPS) produced from bacterial cellular metabolism, cell autolysis and the wastewater itself. Significant changes in sludge structure and microbial composition can result from the hydrolysis of the EPS and degradation of the sludge matrix (Nielsen *et al*, 1996). Polysaccharides form the bulk of the extracellular material in the EPS, up to 65% of the total extracellular material (Horan and Eccles, 1986), but other organic substances, such as proteins, lipids and nucleic acids are also present (Goodwin and Foster, 1985; and Zhang *et al*., 1998).

Anaerobic storage of sludge is known to lead to rapid sludge deflocculation . Several factors are believed to play a role during this process. Starkey and Karr (1984) have proposed that the production of EPS is inhibited as a result of the anaerobic processes taking place. Nielsen *et al.* (1996) reported that a significant degradation of the sludge floc matrix occurred during anaerobic storage over a few days, and that the reduction in sludge was mainly due to degradation of the sludge protein and carbohydrates. Since it has been found that proteins are a major constituent of EPS and that they may be involved in binding the floc together (Dignac *et al.*, 1998), the large reduction in proteins and some reduction in the carbohydrate content after a few days is the most significant observation. This indicates in some way that the reduction in proteins and carbohydrates (to a lesser extent) affects the floc strength, which leads to deflocculation and reduced dewaterability (Nielsen *et al.*, 1996).

Associated with these sludge flocs is a certain amount of enzymatic activity, which is required in order to break down the complex molecules into their basic monomers. The bacteria present in the sludge convert complex particulate and dissolved organic matter into low-molecular weight compounds by extracellular hydrolytic enzymes. Proteolytic, lipolytic and cellulolytic enzymes, synthesised within bacterial cells, hydrolyse the adsorbed macromolecules into smaller subunits that can be transported across the cell membrane and then metabolized (Levine *et al*., 1985). Several different types of enzymes have been reported in sludge, such as aminopeptidases, dehydrogenases, galactosidases, lipases and phosphatases. It has also been shown that for specific

enzymes, the largest activities have been observed for leucine aminopeptidase and β-glucosidase. These hydrolytic processes require exoenzymes and can be seen as being the overall rate-limiting step for the mineralisation of organic matter in the sludge treatment process. The exoenzymes found in sludge may originate from the influent sewage or from the sludge itself or even as actively secreted exoenzymes. Chrost and co-workers (as referenced by Frølund *et al.*, 1995) found that exoenzymes are either directly associated with the cell surface of the producer, or are in the free form, dissolved in water, or adsorbed on surfaces other than that of their producer (e.g. immobilised in the sludge matrix within the EPS).

The breakdown of sewage and sludge effluents is a universal problem, and little is known about the mechanism and biochemistry/enzymology associated with the anaerobic process. A study of the various enzymes active in the process would provide invaluable information as to their role in the loss of sludge floc integrity, with particular reference to the role of β-glucosidases and proteases. The objective of this study was therefore to add a range of commercial proteases, β-glucosidase and cellulase to the sludge, and to determine which of the added enzymes would be best suited for utilisation in the accelerated sludge process, compromising the floc matrix integrity and thereby promoting the rate of enhanced sludge digestion under sulphate reducing conditions.

EXPERIMENTAL

Fresh methanogenic and sulphidogenic sludge, Olympus BX50 microscope, Olympus PM-C35DX camera, 10% Formalin, commercial β-glucosidase from Almonds (Sigma G-0395: EC 3.2.1.21), commercial cellulase from *Aspergillus niger* (Sigma C-1184: EC 3.2.1.4), ρ-nitrophenol-β-D glucoside, Alkaline copper tartrate, Nelson's arsenomolybdate, N,N-dimethyl-p-phenylene diamine dihydrochloride, 3% Glutaraldehyde, and Sephacryl S400 were used. All reagents used were of the highest analytical grade available

Reactor design and setup

Two different bioreactor systems were used during the course of this project, a 20L laboratory-scale continuously stirred tank reactor sulphidogenic reactor (SR; Fig. 1) and a 10L continuously-stirred methanogenic reactor (MR; figure not shown). The bioreactors were set up as described previously (Akhurst *et al*., 2002). The chemical oxygen demand (COD) of the primary sludge was determined as described by APHA (1985) and Akhurst *et al.,* 2002.

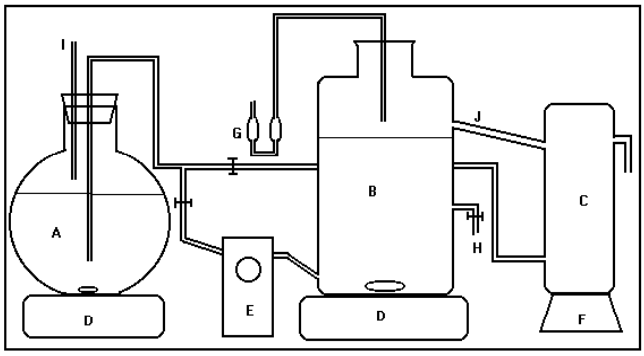

Figure 1. The sulphidogenic bioreactor (SR) under anaerobic conditions. A-Feed vessel, B-Anaerobic reactor, C-Overflow vessel, D-Stirrer, E-Peristaltic pump, F-Support base, G-Gas trapping device, H-Sampling port, I-Influent port, J-Overflow port

β-glucosidase and protease assays

This β-glucosidase assay was modified from Colowick and Kaplan (1988) and measured the amount of ρ-nitrophenol produced per minute, which provided an accurate measurement of the β-glucosidase activity within the sample. Enzyme and substrate blanks were performed by omitting the 200μl enzyme solution (sample) and the 1.0ml ρ-nitrophenol-β-D-glucoside substrate respectively, and replacing them with equal volumes of acetate buffer. The absorbance values (less the enzyme and substrate controls) were then determined at 430nm and translated to μmol of nitrophenol using a standard curve relating μmol nitrophenol to absorbance at 430nm. A modfication of the azocasein protease assay method proposed by Richards *et al.* (1984) was used to determine the preotease activity in the samples. All tests and controls were performed in duplicate.

Somogyi-Nelson, Phenol-sulphuric and Bradford protein assays

Total reducing sugars were determined according to the Somogyi-Nelson assay according to method of Colowick and Kaplan (1988). Total carbohydrate content was determined according to a modification of the phenol-sulphuric assay method of Colowick and Kaplan (1988), and adapted for use in a microtiterplate reader (PowerWave$_X$, Bio-Tek Instruments, Inc). Protein was determined according a modification of the Bradford method as outlined in the Sigma product number B6916 informational sheet and also adapted for use in a microtiterplate reader (PowerWave$_X$, Bio-Tek Instruments, Inc). All the assays above were performed in duplicate.

Floc sizing measurements

Flocs were sized using an Olympus BX50 light microscope, under both x200 and x400 magnification, using an eyepiece micrometer/graticule. One hundred flocs were sized per sample, as per Barbusinski and Koscielniak (1997). The average floc diameter (assuming a spherical shape) was calculated for each sample, using the data obtained from 100 flocs.

Assessing the effect of the commercial enzymes

The effects of both β-glucosidase and cellulase in terms of sludge floc diameter were evaluated by adding various commercially available enzymes at a concentration of 10% (w/v) to methanogenic sludge. The solution was then incubated at 37°C for 24 hours. Samples were collected at the following times: 0, 3, 6, 12 and 24 hours. Each sample was analysed using an Olympus BX50 microscope, and 100 flocs per sample were counted. The changes in average floc diameter versus time was then determined.

Extraction of the EPS

Samples (45ml) from each reactor (MR and SR) were collected and centrifuged at 9000g for 5 minutes at 4°C. The supernatant was removed and the pellet resuspended in 45 ml tap H_2O, after which it was centrifuged at 9000g for a further 5 minutes at 4°C. This step was repeated three times. After the third wash, the supernatant was discarded and the pellet was resuspended in 3% (v/v) glutaraldehyde. The sample was then incubated overnight with slow agitation at 4°C, and then subjected to Sephacryl S400 chromatography.

RESULTS AND DISCUSSION

The sludge flocs present in the sulphidogenic bioreactor have often been observed to be of a smaller size (diameter) that those found in the methanogenic bioreactor. As can be seen by a representative sample from each bioreactor in Figure 2 (a and b) (column graph of %frequency versus floc diameter in divisions of 10μm), floc diameters ranged from 10-109μm in the methanogenic bioreactor and 10-59μm in the sulphidogenic bioreactor respectively. A large fraction of the sludge flocs (52%) in the sulphidogenic bioreactor were in the range of 10-19μm. Average floc diameter for the flocs in the methanogenic and sulphidogenic bioreactors (calculated from a sample of 100 flocs) were detemined as 43μm and 23μm respectively. Parker *et al.* (1971) reported a bimodal floc size distribution

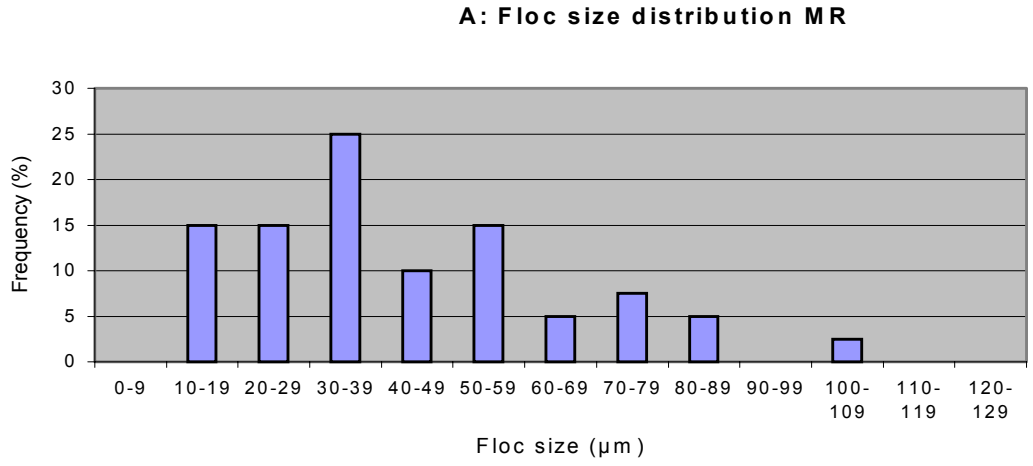

Figure 2. Floc size (diameter) distribution for sludge flocs obtained from the methanogenic (A) and sulphidogenic (B) bioreactors

from 0.5 to 5µm and 25 to 3000µm in activated sludge, while Mueller *et al.* (as quoted by Urbain *et al.,* 1993) reported a single floc size range of 20 to 200µm. Barbusinski and Koscielniak (1997) reported a significant decrease in average floc size diameter from 125µm to 65µm within the first four days during the course of aerobic digestion. The size range of the flocs in our bioreactors seem to to fall in a similar range, with a floc size (diameter) range of 10-109µm

It was decided to use the sludge obtained from the methanogenic bioreactor sample for further studies, as this sludge sample contained flocs of a larger size range and was free from any sulphate/sulphide which was subsequently shown to have a significant effect on sludge floc size and enzyme activities. The addition of commercially added enzymes such as proteases, β-glucosidase and cellulase resulted in a rapid decrease in methanogenic floc diameter over the time period studied (See Figures 3 and 4) The addition of 10% (w/v) β-glucosidase led to rapid decrease in average floc diameter from 56.96µm to 29.48µm within six hours (a reduction in floc diameter of almost 48.24%), while the reduction in floc diameter due to the addition of 10% (w/v) cellulase appeared to proceed at a much slower rate (Figure 3). A reduction in floc diameter of 56.96 to 38.1µm (i.e. a reduction of only 33.11%) was observed in the case of cellulase over a six hour incubation period. Cellulase required an incubation time of almost 12 h in order to effect a similar reduction in floc diameter as compared to β-glucosidase (6 h). A slight decrease from 56.96 to 43.36µm (23.88%) was also observed in the case of the control incubation, in which no enzyme had been added. This is believed to be due to the background rates of de/flocculation due to the conditions under which the experiment was employed e.g. a temperature of 37°C and continuous shaking.

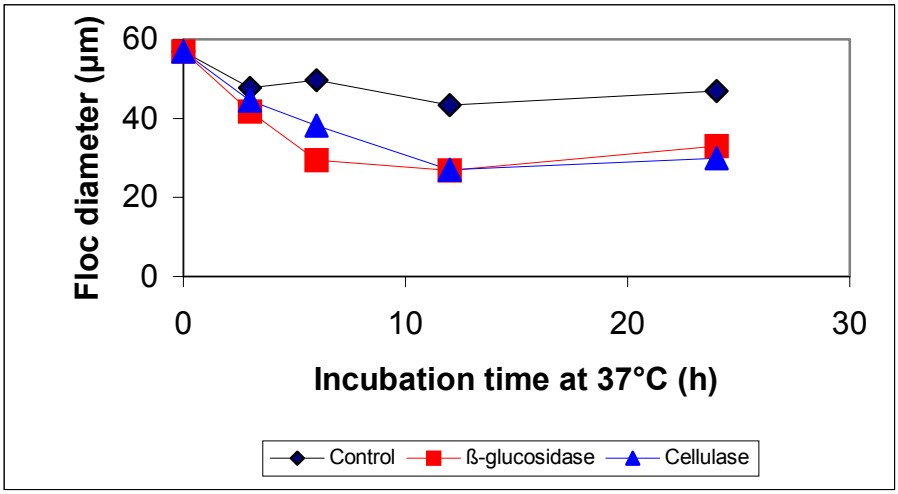

Figure 3. The effect of β-glucosidase and cellulase (10% w/v) on sludge floc diameter at 37°C.

The addition of (10% w/v) proteases also resulted in a rapid decrease in floc diameter (Figure 4). Again, a slight decrease from 56.96 to 43.36µm (23.88%) was also observed in the case of the control incubation, in which no enzyme had been added. Alpha-chymotrypsin (10% w/v) was the most efficient protease, resulting in a 54.51% decrease in floc diameter from 56.96 to 25.91µm within six hours. Pronase E and trypsin were

slightly less efficient, leading to reductions in floc diameter of 53.11% from 56.95 to 26.71 for both enzymes over an incubation interval of 12 h. All the proteases, as well as both β-glucosidase and cellulase, appeared to reach an equilibrium after a period of 12 hours (Figs 3 and 4), which may be indicative of the lack of stability of these two commercial enzymes within the sludge samples at a temperature of 37°C.

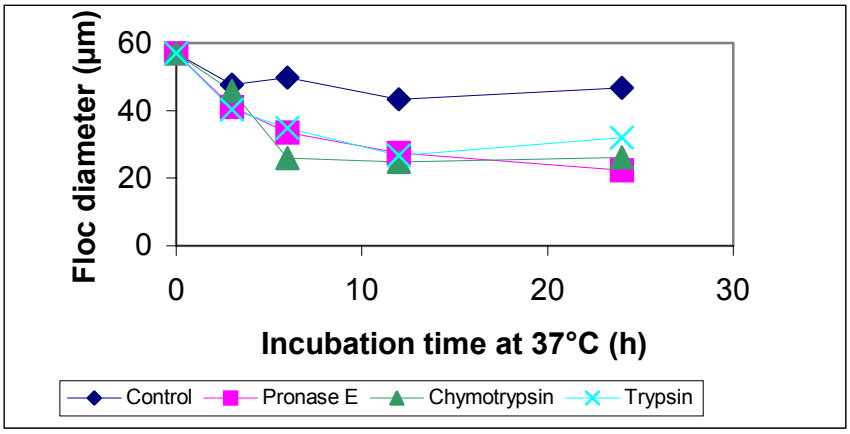

Figure 4. The effect of proteases (10% w/v) on sludge floc diameter at 37°C.

The significant differences observed in the efficiencies of β-glucosidase,cellulase and the various proteases may be due differences in their molecular masses. For the sake of comparison with literature, enzymes were added on % (w/v) basis rather than on a per molar or micromolar basis. Scheidat *et al.* (1997) reported that a production of soluble COD (an increase in soluble COD of 50-100%) was made possible by the addition of hydrolytic enzymes such as peptidases, cellulases and carbohydrolases at a temperature of 50°C, while only a small effect (15-40%) on soluble COD was observed at 38°C. The higher rate of COD production at 50°C was concluded to be due to the fact that the hydrolytic enzymes had temperature optima closer to this temperature. Enzyme concentrations of 1.2% (w/v) and 29% (w/v) were used throughout the course of their study.

As the addition of exogenous enzymes at a level of 10% (w/v) can be prohibitively expensive, optimising the activities of those enzymes (β-glucosidase, cellulase and the various proteases) already present in the methanogenic sludge appeared to be a more feasible option. In fact, work performed in our laboratory has indeed indicated that many of these enzymes are already present in sufficiently high amounts within the methanogenic and sulphidogenic bioreactor sludges. Frølund *et al.* (1995) reported that for the specific enzymes tested, the largest activities were found for β-glucosidase and leucine aminopeptidase. Nybroe *et al.* (1992) have stated that aminopeptidase activity, and related proteolytic activity, have also been used to study bacterial decomposition in aquatic ecosystems. Figures 5 shows the levels of β-glucosidase activity present in the two bioreactor sludges. Both β-glucosidase- and protease activities were mainly found in the pellet fractions, indicating that these enzymes are mainly cell-associated or located within the EPS matrix. Levels of β-glucosidase were established to be 3.062 and 4.633 µmol/min for sludge samples from the methanogenic and sulphidogenic bioreactor respectively (Figure 5). The activities of both enzymes (β-glucosidase and proteases) were higher in the sulphidogenic bioreactor sludge as compared to the methanogenic bioreactor sludge (Figures 5, data not shown for proteases).

There appears to be a definite link between the larger activities observed in the sulphidogenic bioreactor sludge, and the fact that the flocs in these samples are of smaller diameter. There is increasing evidence of a possible relationship between sulphide concentration and β-glucosidase/protease activity, and sludge floc size (diameter). Indeed, previous work in our lab has supported the fact that these enzyme activities are enhanced by increasing levels of sulphide (Akhurst *et al*. 2002).

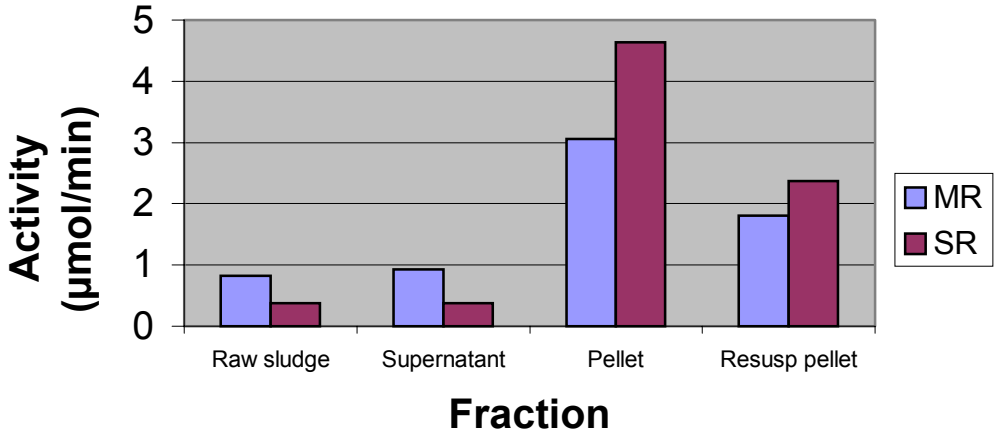

Figure 5. Fractionation studies with β-glucosidase. Resusp pellet = resuspended pellet after centrifugation

EPS was extracted from the sludge samples via treatment with 3% (v/v) glutaraldehyde and analysed using size exclusion chromatography on Sephacryl S-400. The composition of the EPS was characterised using Bradford protein, glucose and total carbohydrate assays. $A280_{nm}$ profiles obtained showed the presence of two peaks, peak 1 at tubes 6-11 and peak 2 at tubes 16-21 (results not shown) indicating the presence of protein and other aromatic compounds. We have subsequently established that peak 1 belongs to glutaraldehyde, while peak 2 is believed to contain the majority of the total carbohydrate portion (as the largest component of the EPS). The concentration of total carbohydrate was quite high (a peak height of 60.689 mg/ml) as compared to that observed for total protein (a peak at tube 43 of 0.281 mg/ml). This observation that the EPS is mainly composed of carbohydrate (hence the term “extracellular polysaccharide”) has also been reported by others in the field e.g. Horan and Eccles (1986) and Azeredo *et al.* (1998). However, Dignac *et al.* (1998) found that between 70 and 80% of the extracellular organic carbon found in the EPS could be attributed to proteins and sugars, of which proteins appeared to be the major component of the EPS.

CONCLUSIONS

The EPS matrix that surrounds the biomass greatly contributes to the structural integrity of sludge flocs in wastewater treatment processes. The loss of integrity of the sewage sludge floc is believed to be due to enhanced hydrolysis of important structural components such as lignin, protein and cellulose in the sludge floc matrix. The mechanism of enhanced sludge floc fracture, due to the action of enzymes hydrolysing the structural components of EPS, remains a key element in our understanding of how the floc integrity in systems utilising a sulphate reducing system is compromised.

It appears as if sludge flocs which are present in a sulphidogenic environment (with the production of large amounts of sulphide), are generally of smaller diameter than those found in a methanogenic environment.
The flocs which were present in the bioreactors ranged in diameter from 10-59µm and 10-109µm for the sulphidogenic and methanogenic bioreactors respectively. Sulphide is therefore believed to play a crucial role in deflocculation (as the average floc diameter was smaller in the sulphidogenic bioreactor sample), which would explain the phenomenon of accelerated sludge solubilisation under sulphate reducing conditions, which has been observed in our laboratory and by other researchers (Whiteley *et al.*, 2002).

Significant sulphate reduction resulting in sulphide has been observed during the anaerobic storage of sludge, and the presence of sulphide may have a deteriorating effect on the sludge floc by reducing Fe(III) to Fe(II) as FeS (Nielsen and Keiding, 1998). Divalent cations are known to be excellent flocculating agents, and it can be generally assumed that even at low concentrations, Fe(III) promotes flocculation and will form stronger flocs than Ca^{2+} ions. Therefore when Fe(III) is selectively removed from the sludge floc by the formation of FeS, the Ca^{2+} would partially substitute for the Fe compounds (Nielsen and Keiding, 1998). This would result in weaker floc formations, due to the presence of FeS, which is more negatively charged and therefore doesn't contribute to flocculation. However, the role of enzymes in this process cannot be underestimated, and a study of the effect of sulphide on the enzymology of this process and the sludge flocs is currently underway. This will be the subject of a subsequent publication elsewhere (Akhurst *et al.* 2002).

The effect of the addition of the various relatively non-specific exogenous enzymes (ß-glucosidase, cellulase, proteases: trypsin, pronase E and chymotrypsin) on sludge floc size (diameter) was investigated in the absence of sulphide (i.e. methanogenic sludge). It is evident that the addition of these enzymes to the sludge floc caused an increased hydrolysis of the matrix by the breaking of those peptide and sugar linkages maintaining the integrity of the floc, leading to increased floc fracture, deflocculation and consequent sludge solubilisation. The addition of exogenous enzymes to a methanogenic or sulphidogenic bioreactor on a large scale would be prohibitively costly, and it is proposed that those enzymes already present within the floc matrix, and those which are cell-surface bound, be exploited. Evidence showing that these enzymes are indeed found on the cell-surface or within the floc matrix has been put forward (Akhurst *et al.* 2002).

As the bulk of the EPS was shown to be composed mainly of polysaccharides, we propose that by increasing the activity of the naturally occurring ß-glucosidases residing within the floc matrix, the process of deflocculation may be enhanced. As sulphide has been shown to increase the activity of this very important key enzyme (Akhust *et al.*, 2002), we propose that this is one of the contributing factors why sludge solubilisation is accelerated under sulphate reducing conditions

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