# Enzymology of Activated Sewage Sludge during Anaerobic Treatment of Wastewaters: Identification, Characterisation, Isolation and Partial Purification of Proteases

**by**

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**ABSTRACT**

During anaerobic digestion bacteria inside the digester require a carbon source for their growth and metabolism, sewage sludge was used as a carbon source in this study. The COD content was used to measure the disappearance of the substrate. COD content was reduced by 48.3% and 49% in the methanogenic and sulphidogenic bioreactors, respectively, while sulphate concentration was reduced by 40%, producing 70mg/L of hydrogen sulphide as the end product over the first 5-7 days. Sulphate (which is used as a terminal electron acceptor of sulphur reducing bacteria) has little or no effect on the sulphidogenic and methanogenic proteases. Sulphite and sulphide (the intermediate and end product of sulphate reduction) increased protease activity by 20% and 40%-80%, respectively.

Maximum protease activity occurred on day 21 in the methanogenic reactor and on day 9 in the sulphidogenic reactor. The absorbance, which indicates the level of amino acid increased to 2 and 9 for methanogenic and sulphidogenic bioreactors, respectively. Proteases that were active during anaerobic digestion were associated with the pellet (organic particulate matter) of the sewage. These enzymes have an optimum activity at pH 10 and at temperature of 50$^{o}$C. The proteases that were active at pH 5 and 7, had optimum temperatures at 30$^{o}$C and 60$^{o}$C, respectively. Due to their association with organic particulate matter, these enzymes were stable at their optimum temperatures for at least five hours at their respective pH.

Inhibition by PMSF, TPCK and 1.10-phenanthroline suggested that proteases inside the anaerobic digester are a mixture of cysteine, serine and metalloproteases. At pH 5, however, EDTA appeared to enhance protease activity by 368% (three-fold). Acetic acid decreased protease activity by 21%, while both propionic and butyric acid at 200 mg/L cause total inhibition of protease activity while these acids at higher pH (where they exist as their

corresponding salts) exerted little effect. Copper, iron and zinc inhibited protease activity by 85% at pH 5 with concentrations ranging between 200 and 600 mg/L. On the other hand, nickel, showed an increase in protease activity of nearly 250%. At pH 7 and 10, copper had no effect on protease activity while iron, nickel and zinc inhibited these enzymes by 20-40%.

Proteases at pH 7 were extracted from the pellet by sonication, releasing 50% of the total enzymes into the solution. The enzymes were precipitated by ammonium sulphate precipitation, and further purified by ion exchange chromatography and gel filtration. Ion exchange chromatography revealed that most of the enzymes that hydrolyse proteins are negatively charged while gel filtration showed that their molecular weight is approximately 500 kDa. SDS-PAGE of sonicated sample revealed that methanogenic proteases consisted of four subunits with $M_r$ values of 66, 45, 35 and 29 kDa while sulphidogenic proteases consisted of 3 subunits with $M_r$ values of 45, 36 and 24 kDa.

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**ABBREVIATIONS**

| | |
|---|---|
| AMD | Acid mine drainage |
| ALD | Anoxic lime drains |
| APS | Adenosine phosphosulphate |
| ATP | Adenosine triphospate |
| BSA | Bovine serum albumin |
| COD | Chemical oxygen demand |
| DEAE | Diethylaminoethyl |
| dd$H_2O$ | Deionised water |
| DMSO | Dimethyl sulphoxide |
| EDTA | Ethylenediaminetetraacetic acid |
| $H_2S$ | Hydrogen sulphide |
| $M_r$ | Relative molecular weight |
| PEG | Polyethylene glycol |
| PMSF | Phenylmethyl sulfonyl flouride |
| PPi | Pyrophosphate |
| SDS | Sodium dodecyl sulphate |
| SDS-PAGE | Sodium dodecyl sulphate-polyacrylamide gel electrophoresis |
| $SO_3^-$ | Sulphite |
| SRB | Sulphur reducing bacteria |
| TCA | Trichloroacetic acid |
| TEMED | N, Nρ-Tetramethyl-ethylenediamine |
| TPCK | Tosyl-phenylalanine chloromethylketone |
| VFA | Volatile fatty acids |

# CHAPTER 1

# GENERAL INTRODUCTION

## 1.1 WATER IN SOUTH AFRICA

Water is a basic need for all living organisms, it carries the idea of baptism and new life, cleansing and healing, and the promise of growth and prosperity. Due to population growth, South Africa is approaching a crisis where water resources will no longer meet the demand. This will place great pressure on the rivers and ground waters, as more water is required for domestic, agricultural and industrial supplies.

South Africa is a semi arid country that receives a very low annual rainfall. Approximately 8.6% (on average 52.5 milliard $m^3$ per year) of South Africa's rainfall reaches the rivers. However, it was proposed that with a combination of different methods, like exploitation of groundwater sources, sea water and the river system, 60% of the annual run off can be used, giving South Africa a water supply of 31.5 milliard $m^3$ per year (Henzen and Pieterse, 1978).

It was predicated that by the year 2000 about 16.7 milliard $m^3$ of water was required for power generation, mining, industries, and domestic use, while 12.8 milliard $m^3$ was needed for agricultural use. Thus the total annual water demand to the consumer sector was 29.5 milliard $m^{3,}$ which was 2.0 milliard $m^3$ less than the total estimated distributable water of 31.5 milliard $m^3$.

It has been suggested, however, that savings can be taken to reduce water depletion by improving

utilisation of irrigation water that will save 1.5 milliard $m^3$ per annum and by water reclamation and reuse that will save 7.2 milliard $m^3$ per annum, giving the total of 8.7 milliard $m^3$. Furthermore, the water that has been saved during the last century will be depleted within the next ten years (Henzen and Pieterse, 1978; Department of Water and Forestry, 1986).

## 1.2 NATIONAL WATER ACTS AND ITS PURPOSE

Due to lack of water in South Africa, the National Government took on the responsibility to protect and manage water resources in order to achieve the sustainable use of water (Department of Water Affairs and Forestry, 1999). The first Water Act, established in 1956, (South African Union Gazette Extraordinary, 1956) was the key legislation regulating water management and its aim was to protect South Africa's limited resources from pollution. This act, however, has some limitation since it was written when there was more available water - a situation that is clearly not the case in present day.

In 1994, the Minister of Water Affairs and Forestry reviewed the Water Act by focussing on the following main areas: Water cycle; legal aspects of water; priorities for Water management; approaches to water resource management, water resource management institutions, financial assistance; Water rights (access to water supply and sanitation) and Water services (national norms and tariff standards). The new legislation has been published in 1997 under Water Act 108 (South African Acts, 1997).

## 1.3 ACID MINE DRAINAGE

### 1.3.1 Introduction

Mining remains the single most important diverse industry in mineral rich South Africa and throughout this mining sector, the use and quality of the water vary tremendously. It can be stated in general that although the mining sector is a minor user of water supplies, it is a significant contributor to water pollution through Acid Mine Drainage (AMD) (Pulles e*t al.*, 1995)

Acid mine drainage is one of the most serious environmental problems facing the coal and metal mining industry. AMD results from chemical and biological oxidation of sulphide ores, and it is characterised by low pH values, high levels of sulphate, heavy metals especially iron, copper, cobalt, nickel, zinc and magnesium (Wittman and Förstner, 1976,1977) and suspended solids. Many of these heavy metals are potentially toxic because of their ability to bind to biologically active molecules, especially proteins.

The main sources of AMD in abandoned mine areas are old waste rock dumps and rock walls which are found in tunnels and shafts, having a high concentration of sulphides and/or sulphosalts. When sulphide minerals such as pyrite and marcasite are exposed to oxygen and moisture, they form $FeS_2$ and acids that are released into the water (Funke, 1990; Johnson, 1995). AMD is a perpetuating environmental problem, since the oxidation processes may continue for decades or even centuries after the closure of a mine.

### 1.3.2 AMD Chemistry

There are four commonly accepted chemical reactions that represent the chemistry of pyrite weathering to form AMD. 1) During the oxidation of pyrites, which is the most abundant sulphide mineral, sulphur is oxidised to sulphate, ferrous iron is released and two moles of acid are produced for each mole of pyrite oxidised.

$$2\ FeS_2 + 7\ O_2 + 2\ H_2O\ !\ 2\ Fe^{2+} + 4\ SO_4^{2-} + 4\ H^+ \text{ .....(1)}$$

2) The released ferrous iron is converted to ferric iron with the consumption of acid [equation 2]. This reaction rate is pH dependent, and proceeds faster at pH values near 5 and slower under acidic conditions (pH 2-3). It is the rate-determining step in the overall acid-generating sequence. At low pH, *Thiobacillus ferrooxidans*, chemolithotrophic bacteria oxidise ferrous iron in equation 2, by increasing the overall reaction rate. These bacteria may attach to solid ore surfaces and

attack sulphide minerals directly (Lundgren *et al.*, 1986).

$$4\ Fe^{2+} + 4\ H^+ + O_2\ !\ 4\ Fe^{3+} + 2\ H_2O \text{ .....……...(2)}$$

3) The hydrolysis of iron generates acid as a byproduct. Many metals are capable of undergoing hydrolysis and the formation of their hydroxide precipitate above pH 3.5 [Equation 3].

$$4\ Fe^{2+} + 12\ H_2O\ !\ 4\ Fe(OH)_3 + 12\ H^+ \text{ .....…….(3)}$$

4) The oxidation of additional pyrite by ferric iron, generated in equation 1 and 2 releases more hydrogen ions into the aquatic environment and hence continues to reduce the pH. The $Fe^{III}(OH)_3$ formed in this reaction is called "yellow boy@ since it is a yellow-orange precipitate that turns the acidic runoff in the streams to an orange or red colour and covers the stream bed with a slimy coating. This is the cyclic and self-propagatory part of the overall reaction and takes place very rapidly and continues until either ferric iron or pyrite is depleted. The addition of equation 1 and 2 gives equation 4 (Stumm and Morgan, 1981).

$$FeS_2 + 14Fe^{3+} + 8H_2O \ !15Fe^{2+} + 2SO_4^{2-} + 16H^+ \quad .....(4)$$

## 1.4 TREATMENT PROCESSES OF ACID MINE DRAINAGE

### 1.4.1 Chemical Treatment Processes

A range of chemical processes is available for the treatment of acid mine drainage and these processes neutralise the acidity of AMD and remove metals from the solution. Water that has been subjected to these processes, however, must meet specific water quality criteria before being discharged into the streams (Todd and Reddick, 1997). Many companies use chemicals such as calcium carbonate, sodium hydroxide, sodium bicarbonate or ammonia to treat acid mine drainage, with each chemical offering the advantage of neutralising the acidity.

However, chemical treatment is an expensive process as it requires the installation of a plant with agitated reactors, precipitators, clarifiers and thickeners (Gazea *et al.,* 1996). There is the production of sludge and large volumes of unstable metal hydroxides mixed with gypsum, and these are costly to dispose. During chemical treatment the reaction time tends to be slow and there is a loss of efficiency of the system, due to coating of the limestone particles with iron

precipitates. Due to high cost of the chemicals, passive treatment offers an alternative in treating AMD. During passive treatment, which includes wetlands and anoxic limestone the natural occurring chemical and biological reactions are allowed to take place in a controlled environment.

#### 1.4.1.1 Limestone Treatment

This is an old method that has been used for the AMD neutralisation and there are three types of lime that can be used, namely, unslaked lime (CaO) with 80-90% calcium, hydrated lime ($Ca(OH)_2$) with 60-70% calcium and dolomitic lime with ($CaCO_3$ -$MgCO_3$) (Henzen and Pieterse, 1978).

The choices of a neutralising agent depend on the railage costs, economy, convenience and safety of handling. The most commonly used material is the crushed limestone $CaCO_3$ and its neutralising reaction occurs as follows:

$$CaCO_{3(s)} + H^+ \; 6 \; HCO_3^- + Ca^{2+} \text{ .....……(5)}$$

$$HCO_3^- + H^+ \; 6 \; H_2CO_3 \text{ .....……..(6)}$$

$$H_2CO_3 \; 6 \; H_2O + CO_{2(g)} \text{ .....……. (7)}$$

The limestone contains natural bicarbonate, which neutralises the hydrogen ions. Metals, however, remain in the solution and this suggests that the water from this process can have high

concentration of metals and sulphate (Hadley and Snow, 1974). Since chemical treatment is expensive, biological treatments are currently being researched and implemented.

#### 1.4.1.2 Wetland Treatment

Constructed wetlands are becoming an increasingly common method of treating water pollution, including confined animal wastewater, cropland runoff, urban stormwater, septic tank effluent, municipal wastewater effluent, acid mine drainage, industrial process water, and landfill leachate (Bastian and Hammer, 1993; Kadlec, 1995; Kadlec and Knight, 1996). This technology started in the 1950's for treatment of municipal wastewater in Germany (Brix, 1994) and it is very popular in the Eastern United States where over 400 wetlands has been constructed for AMD treatment.

In wetlands, varieties of processes are responsible for AMD treatment and these include chemical, biological and microbiological reactions, which occur in the aerobic and anaerobic zones of wetlands. Aerobic wetland consists of a large surface area pond with horizontal surface flow and the pond may be planted with cattails and other wetland species. This kind of wetland can only be effective if the pH of the influent is greater that 5.5 since aerobic wetlands can effectively treat water that is net alkaline.

Anaerobic wetland can be called compost wetland and it consists of a large pond with a lower layer of organic substrate. Spent mushroom is mostly used as the compost layer and contains 10% calcium carbonate, which is used to remove oxygen from the system. The organic substrate promotes chemical and microbial processes that generate alkalinity. Removal of oxygen by the compost leads to the reduction of sulphate and the prevention of metal oxidation as well as helping with armouring or coating of limestone by preventing its dissolution. Microbial

respiration within the organic substrate helps in sulphate reduction, producing water and hydrogen sulphide (Department of Environmental Protection, 1999).

Kuyucak and St-German (1994) investigated a number of alternative processes that combined with a wetland, for the addition of alkali to AMD. Anoxic Lime Drains (ALD) was used as a pre-treatment step, producing alkalinity at a lower cost than wetlands alone. Their use is limited, however, to mine water with $Fe^{3+}$ concentration of less than 2 mg/L, a net acidity lower than 3000 mg/L and $CaCO_3$ and dissolved oxygen less than 1 mg/L (Gazea *et al.*, 1996).

Metal removal takes place through physical/chemical cation exchange and complexation with organic matter, both of which occur, in the organic substrate. The biological and chemical processes that treat the metals and acidity are pH dependent. If pH is less than 3, the wetland will not function (Department of Interior, 1990) and this is one of the disadvantages of using wetland for the treatment of AMD.

### 1.4.2 Biological Treatment Processes

#### 1.4.2.1 Introduction

Biological processes will continue to be one of the most practical and economical means of treating large volumes of wastewaters from domestic, commercial, industrial and agricultural sources. During the past years, an aerobic process was favoured for the treatment of waste because it is easy to operate and can tolerate process fluctuations. In comparison, anaerobic

reactors have, historically, been slow to start up, less stable under fluctuation operating conditions and more expensive to install (Christensen *et al.,* 1996).

In recent years, however, power costs have risen drastically and sludge disposal has become much more difficult and costly. These changes have significantly reduced the comparative economy of aerobic treatment and led industry to re-examine other treatment methods. Among the various biological processes in use, anaerobic digestion has gained popularity due to lower production of sludge and production of useful gases (Ghosh and Pohland, 1974). New advances in treatment technology have significantly reduced the size of anaerobic reactors, costs and improved the stability of the operation.

Anaerobic digestion consists of three stages: 1) During actual digestion or liquification process, the complex organic compounds are hydrolysed by extracellular enzymes to simple soluble compounds. The cellulose and starches are hydrolysed to simple sugars, while the proteins are broken down to the amino acids and lipids to fatty acids. Facultative and anaerobic bacteria are responsible for the hydrolysis and they are also responsible for the formation of volatile acids (Perot *et al.,* 1988) (Fig. 1.1). 2) Hydrolysed substrates are converted into organic acids and alcohols. Simple sugars, amino acids, glycerol and long chain fatty acids are used as the main carbon source for growth and carbon dioxide, ammonia and saturated fatty acids are the end products. Alcohols, ketones and aldehydes are produced in small quantities while acetic, propionic, butyric and lactic acids are the most frequently produced end products during this stage (Mutkowska, 1997). 3) This step is commonly known as gasification, where the end products of acid fermentation are converted to gases mainly methane and carbon dioxide (Ghosh and Pohland, 1974). This is the reason why anaerobic decompositions are characterised by foul-

smelling and noxious gases, such as ammonia, hydrogen sulphide and by combustible gases such as methane and hydrogen. During anaerobic digestion hydrolytic reactions (stage 1) tend to limit the production of methane and it can be referred as the rate-limiting step for the overall anaerobic microbial digestion

Microorganisms capable of producing methane by the above anaerobic process are called methanogens and are found in waterlogged soils, the gut of animals, sewage sludge, manure piles, marine and freshwater sediments and hot springs. These bacteria require a strict anaerobic environment for growth with a redox potential below -300mV. They can grow over a wide range of temperature with the optimum temperature of 35$^{o}$C and they are particularly sensitive to pH values below 6 and above 7.5 (Mutkowska, 1997).

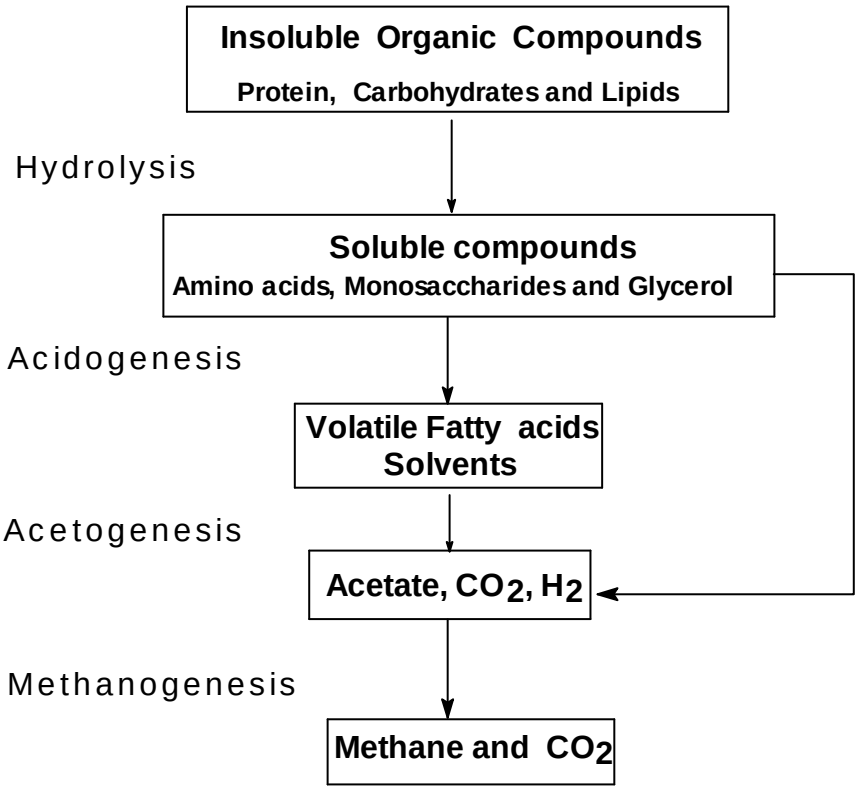


**Figure 1.1** Metabolic steps and microbial groups involved in anaerobic digestion (Perot *et al.*, 1988)

The methanogenic bacteria can use hydrogen and carbon dioxide as their carbon and energy sources and a few species have the ability to use formate as their energy and carbon sources (Forday and Greenfield, 1983). The use of acetate, however, is restricted to strains of *Methanosarcina barkeri* (Bacher and Pfenning, 1981) as it was found that 70% of methane produced was derived from acetate while only 15% was derived from formate, hydrogen and carbon dioxide (Smith and Mah, 1966).

Many industrial waste effluents contain high concentrations of sulphate and the application of an anaerobic treatment will cause the coexistence of the bacterial consortia (Colleran *et al.*, 1995). High concentration of sulphate encourages the growth of sulphate reducing bacteria (SRB) and the production of hydrogen sulphide during sulphate reduction leads to the inhibition of methanogens (Hilton and Archer, 1988). Both groups of bacteria compete for the common intermediates such as acetate, hydrogen and other short chain volatile fatty acids (VFA) in the anaerobic degradation process. Though this competition can be observed during low concentration of substrate, the SRB have the ability to use a wider range of substrate than the methanogens and hence sulphate reducing bacteria will outcompete methanogens and sulphate reduction becomes the dominant process. The biological anaerobic reactor that is dominated by the methanogens is called methanogenic reactor while the one that is dominated by the SRB is sulphidogenic reactor.

#### 1.4.2.2 Ecology and Physiology of Sulphate Reducing Bacteria

Sulphate reducing bacteria (SRB) are obligate anaerobes which can be found in anoxic estuarine sediments (Compeau and Bartha, 1987), acid mine water (Herlihy and Mills, 1985; Herlihy *et al.*, 1987), saline water (Banat *et al.*, 1981), fresh water and in soils (Watanabe and Furusaka, 1980).

These bacteria have an optimal temperature for growth around 30$^{o}$C and they prefer an environment of around pH 7.

SRB which use lactate as a carbon source and energy are divided into two groups:- the genus *Desulfotomaculum*, which are characterised as being spore-forming straight or curved rods and the genus non spore forming *Desulfovibrio*. These two genera produce different enzymes that are involved in the removal of pyrophosphate which is formed during sulphate activation by ATP sulphurylase (Liu and Peck, 1981). Extensive research of SRB over the past 25 years has led to a major reclassification with addition of numerous genera which include *Desulfobacter, Desulfobacterium, Thermodesulfobacterium, Desulfonema, Desulfomicrobim, Desulfococcus, Desulfobulbus, Desulfomonas, Desulfosarcina and Archaeoglobus* (Gibson, 1990).

The SRB have the ability to utilise inorganic sulphate, either by assimilatory or dissimilating mechanisms as one of their terminal electron acceptors in an ATP requiring reaction (Peck and Lissolo, 1988). The former sulphate reduction is a biosynthetic process in which sulphate is reduced to sulphide before incorporation into biological building blocks like amino acids (Gibson, 1990), while the latter utilise sulphate as the electron acceptor in the oxidation of energy substrate with sulphide as the end product (Hansen, 1988).

The initial step of biological sulphate reduction involves the transport of exogenous sulphate across the bacterial cell membrane into the cell (Cypionka, 1987). This proceeds by the action of ATP sulphurylase, which combines sulphate with ATP to produce adenosine phosphosulphate (APS) and pyrophosphate (PPi). APS is then cleaved to form inorganic sulphate which is converted to sulphite ($SO_3^-$) by the cytoplasmic enzyme APS reductase and eventually the

sulphite is reduced to hydrogen sulphide ($H_2S$) (Gibson, 1990).

SRB can use many substrates as their energy source (Table 1.1), and since they cannot degrade complex organic compound such as polysaccharides, proteins and lipids, they are dependent on other organisms for the supply of energy (Hansen, 1988).

**Table 1.1** Compounds that can be used as energy substrate by SRB (Hansen, 1988)

| | |
|---|---|
| Inorganic | hydrogen, carbon monoxide |
| Monocarboxylic acids | formate, acetate, propionate, butyrate, 2- and 3- methylbutyrate, fatty acids up to $C_{18}$, pyruvate, lactate |
| Dicarboxylic acids | succinate, fumarate, malate, oxalate, maleinate, glutarate, pimelate |
| Alcohols | methanol, ethanol, propanol, ethylenegycols, glycerol |
| Amino acids | glycine, serine, cysteine, threonine, valine, leucine, isoleucine, aspartate, glutamate, phenylalanine |
| Miscellaneous | fructose, choline, benzoate, nicotinic acid, phenol |

SRB also need nitrogen and the majority of these bacteria obtain their nitrogen from ammonium salts and dissimilatory reduction of nitrate and nitrite. It has been shown that nitrate can inhibit sulphide production by increasing the redox potential (Jenneman *et al.*, 1986). It was reported that some of these bacteria might be able to fix nitrogen (Widdel and Pfenning, 1984).

#### 1.4.2.3 Treatment of AMD by Sulphate Reducing Bacteria

Sulphate reduction during biological processes is based on the growth of sulphate reducing

bacteria and has been identified as a valuable process for the recovery of contaminant metals from AMD (Postgate, 1984; Widdel and Hansen, 1992; Barton, 1995) and the generation of alkalinity to neutralise the acid (Barnes *et al.*, 1991; Herrera *et al.*, 1991).

Under anaerobic conditions, SRB oxidise simple organic compounds such as lactic acid with sulphate generating hydrogen sulphide and bicarbonate ions:

$$3\ SO_4^{2-} + 2\ CH_3CH(OH)COO^- + 2\ H^+ \rightarrow 3\ H_2S + 6\ HCO_3^- \quad \text{..... (8)}$$

Hydrogen sulphide reacts with many contaminant metals to remove them from the solution as insoluble metal sulphides:

$$H_2S + M^{2+} \rightarrow MS(s) + 2H^+ \quad \text{.....……. (9)}$$

Where M includes metals such as Cd, Fe, Pb, Ni, and Zn. Bicarbonate ions react with protons to form $CO_2$ and water and remove acidity from solution as $CO_2$ gas:

$$6\ HCO_3^- + 6\ H^+ \rightarrow 6\ CO_2(g) + 6\ H_2O \quad \text{.....…… (10)}$$

The $H_2S$ and $HCO_3^-$ formed during sulphate reduction equilibrate into a mixture of $H_2S$, $HS^-$, $S^{2-}$, $HCO_3^-$, and $CO_3^{2-}$. If sufficient sulphate reduction occurs, this mixture will buffer the solution pH to a range of 6-7, though, this depends on the specific quantities and types of organic end product formed (Dvorak *et al.*, 1992).

Varieties of carbon source have been considered as energy source for biological sulphate reduction. One of the initial carbon sources to be considered was sewage sludge (Butlin *et al.*, 1956), with a system for the production of sulphur from hydrogen sulphide which was developed by Burgess and Wood (1961). Since then, many waste carbon sources have been tried for the treatment of large volumes of effluent. These include:- 1) mushroom compost - where it was found that heavy metals could be recovered separately by bubbling $H_2S$ gas through three reactors that were connected in series, at pH 1.6, 3.8 and 6.2 (Hammack and Edenborn, 1992). 2) Straw and alfalfa - though sulphate reduction was very low, unless sucrose was added to the system and there was no significant increase in the pH of water (Béchard *et al.*, 1993). 3) Whey - it was found that the pH increased from 4 to 7.6, the sulphate concentration decreased from 60mM to 10mM and sulphide concentration was found to be between 40 and 150 mg/L (Christensen *et al.*, 1996). 4) Molasses - used in a two stage reactor, though, it was expensive and unavailable in the future, due to its use in other industrial processes (Reis *et al.*, 1988, Maree and Hill, 1989). In this method, removal of biological sulphate increased with an increase in pH and sulphate reduction was more efficient in the fixed film reactor than in a stirred reactor tank. 5) Synthetic gas - (hydrogen, carbon monoxide and carbon dioxide) was used as an energy source during sulphate reduction. The study demonstrated that SRB=s are able to form biofilms under turbulent flow conditions, and a maximum conversion rate of 30g $SO_4^{2-}$/ L/d was achieved when free $H_2S$ concentrations were kept below 450 mg/L (Du Preeze *et al.*, 1992 and van Houten *et al.*, 1996).

#### 1.4.2.4 Sewage as a Carbon Source for Sulphate Reducing Bacteria

Municipal sewage treatment plants contain both a primary and a secondary sludge. Primary

sludge consists of sand, food waste, settled material of inorganic and organic compounds including influent raw sewage, whereas secondary sludge is a settling material produced at the secondary clarifiers of the sewage treatment plant after biological treatment (Choi and Rim, 1991).

There are a number of pre-treatments that have been successfully employed to produce a relatively inexpensive energy source from the sewage sludge (Bisaria, 1991) (Table 1.2). It has

**Table 1.2** Pre-treatment methods for increasing energy source during anaerobic digestion (Bisaria, 1991)

| **Pre-treatment Method** | **Underlying Principle** | **Examples** |
|---|---|---|
| Mechanical | Shearing and impacting forces yielding fine substances | Balling, hammer milling |
| Physical | Increasing pore size and partial hydrolysis of hemicellulose; depolymerisation | Steaming, wetting, freezing |
| Chemical | Removal of lignin and/or hemicellulose | Swelling agents, dilute acids, oxidising agents |
| Enzymatic | Selective removal of lignin/hemicellulose | lignin peroxidase; xylanase |
| Biological | Removal of lignin; modification of lignocellulose structure | White rot and soft-rot fungi and bacteria |

been reported that thermal pre-treatment can improve the production of biogas from waste-activated sludge (Pipyn and Verstraete, 1979). The majority of these treatments are costly, and their use in the treatment of sewage for sulphate reduction cannot be justified. This problem

could be solved by the use of a natural biodegradation of sewage sludge with bacteria that are already present in the system. These bacteria produce enzymes that are responsible for the digestion of insoluble complex compounds to soluble monomers, which are used by SRB and methanogens for their metabolism.

#### 1.4.2.5 Advantages and Disadvantages of Sulphate Reduction over Other Biological Treatment Processes

The South African economy depends on the mining of minerals. During this industrial activity, however, there is a problem of water pollution through AMD since oxidation processes may continue for decades or even centuries after the closing of the mine. AMD contains high concentrations of sulphate, solids and metals (Christensen *et al.,* 1996) and these molecules cannot be removed by the use of methanogens, leading to the use of SRB which utilise sulphate as their electron acceptor. It has been reported that sulphur reducing bacteria are able to fix free nitrogen (Widdel and Pfenning, 1984), which means that the sulphate reduction process would probably be more economical than a conventional methane digestion for organic waste high in nitrogen concentration. SRB can utilise a wide range of substrates including fatty acids which methanogens cannot use, so the sulphate reducing process should be less sensitive to pH changes and accumulation of volatile acids should be less of a problem (Hilton and Archer, 1988).

During the sulphate reduction there is production of hydrogen sulphide where the sulphide ions interact with heavy metals and the complex precipitates out of the solution (Peters and Ku, 1985). It has been discovered that metal separation and settling rates obtained with sulphide

precipitation is higher than those obtained with hydroxide precipitation (Singh, 1992; Hammack *et al.,* 1994). The other attractive feature for this process is the recovery of sulphur as a byproduct from the effluent.

A major disadvantage of this process is the high cost of organic substrate and the downstream treatment of residual organic carbon content after anaerobic treatment. This problem was overcome by replacing organic carbon source with readily available gas (Du Preeze and Maree, 1994), however, this method has a problem of costs supporting the reason for choosing sewage sludge as a cheaper and abundant carbon source. The sewage sludge consists of complex organic compounds, which needs to be broken down to simpler molecules that can be used by the bacteria as an energy source. There are many methods that can be used for the degradation of these compounds but these are expensive and they introduce foreign molecules into the system. The use of enzymes, however, is the best option, since bacteria, which are found in the original environment, produce them.

**1.5 ENZYMES**

Enzymes are biological catalysts that determine the pattern of chemical transformations and accelerate reactions by factors of at least a million. Most biological reactions do not occur at perceptible rates in the absence of enzymes. They are highly specific in the reaction catalysed and in their choice of reactants or substrates and they usually catalyse a single reaction or a set of closely related reactions. (Stryer, 1988).

Nearly all known enzymes are proteins except the catalytic active RNA molecules. Proteins are polymers, which are composed of amino acids that are covalently linked together. Their length and amino acids sequence, can differentiate them from one another leading to different structures

and functions (Creighton, 1993).

The first step during catalysis is the formation of an enzyme-substrate complex (Fig. 1.2), where the substrates are bound to enzymes at the active site cleft from which water is largely excluded when the substrate is bound. The specificity of enzyme–substrate interaction depend on the shape of the active site, which can reject a molecule that does not have a complementary shape (Stryer, 1988).

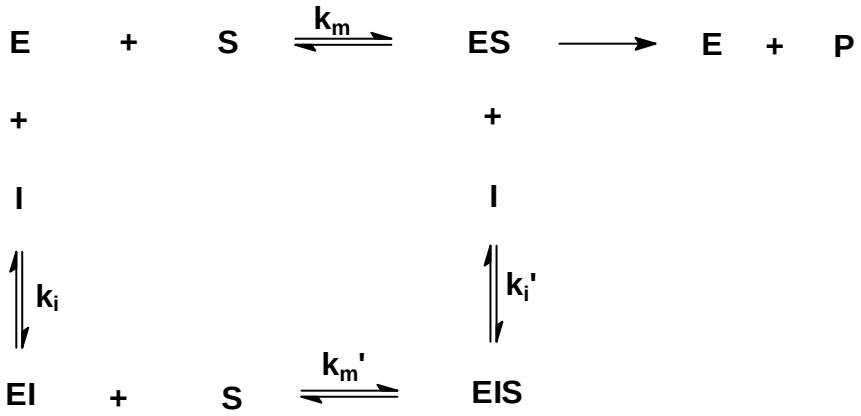


**Figure 1.2** Interaction between the enzyme (E) and substrate (S) or inhibitor (I). Competitive inhibition: I binds only to E, not to ES; Noncompetitive inhibition: I binds either to E and/or ES and Uncompetitive inhibition: I binds to ES, not to E.

Specific small molecules or ions can inhibit enzymes and their inhibition can either be reversible or irreversible. Irreversible inhibitors dissociate very slowly from its target enzyme because it becomes very tightly bound to the enzyme by either covalent or non-covalent bonds. During reversible inhibition, however, there is a rapid dissociation of enzyme-inhibitor complex. In

competitive inhibition, the substrate or inhibitor binds to the active site of an enzyme, not both. Most of competitive inhibitors are similar in shape and structure to the substrate and they bind at the active site of the enzyme preventing the interaction between enzyme and the substrate. Competitive inhibition can be overcome by increasing the concentration of substrate (Fig. 1.3) (Stryer, 1988).

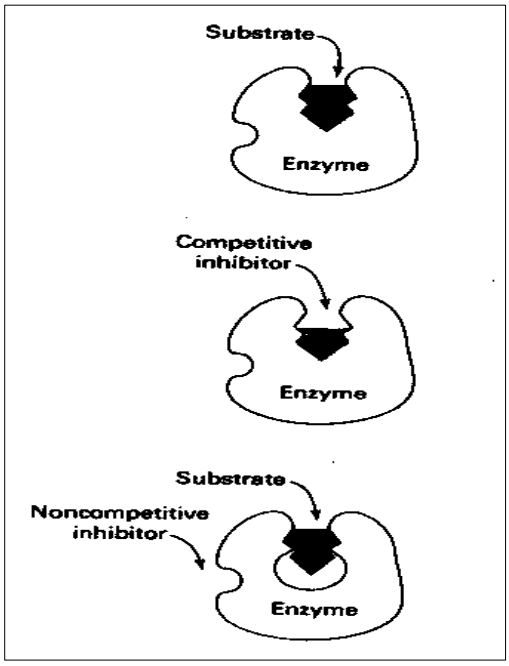


**Figure 1.3** Distinction between a competitive inhibitor and non-competitive inhibitor: (top), enzyme-substrate complex (middle), a competitive inhibitor prevents the substrate from binding (bottom), a non-competitive inhibitor does not prevent the substrate from binding (Stryer, 1988).

Non-competitive inhibition is also part of reversible inhibition, but it is different from competitive inhibition since here the inhibitor and substrate can bind simultaneously to the enzyme molecule. This means that their binding sites do not overlap. This kind of inhibition causes a decrease in turnover number of an enzyme rather than diminishing the proportion of

enzyme molecules to which the substrate binds. Unlike competitive inhibition, non-competitive inhibition cannot be overcome by raising the substrate concentration (Stryer, 1988). Uncompetitive inhibitors have the ability to bind to ES complexes, but not to the enzyme alone.

## 1.6 PROTEASES

### 1.6.1 Introduction

Proteases were among the first enzymes to be purified and crystallised, and they have triumphed in some of the successes of biochemistry. Modern concepts of enzyme specificity were established when Bergman and Fruton (1941) developed the use of synthetic protease substrates, leading to the determination of amino acids sequence of enzymes.

The second era in protease research started five years later when it was reported that trypsin catalysed activation of chymotrypsinogen without detectable proteolytic degradation. This was extended when Lorand (1950) subsequently discovered that the catalytic effect of thrombin in blood clotting was due to limited proteolysis of its substrate, fibrinogen. Since then, it has been established that specific limited proteolysis is a general mechanism displayed in many important physiological functions. The proteases are stable under normal conditions, which is accomplished by regulation that permits rapid activation and yet confine enzymes function in time and space (Reich *et al.*, 1975).

Since these enzymes are responsible for protein degradation they have become an area of interest during biological treatment of wastewaters, though progress in this field is somewhat limited. Sewage sludge, that is used as a carbon source, consists of between 19% to 28% proteinaceous materials and during anaerobic digestion, this protein material is hydrolysed into peptides and

amino acids by proteases. These smaller molecules are then available as an energy supply to the SRB and methanogens (Kotzα *et al.*, 1969).

### 1.6.2 Classification of Proteases

The nomenclature of enzymes that hydrolyse peptide bonds is very complex and it is different from the nomenclature of other enzymes. Generally, the name of an enzyme is specific to the name of its substrate and the particular reaction catalysed. The term protease was used initially to indicate enzymes which hydrolyse peptide bonds (Fig 1.4) (Mathews and van Holde, 1996). After discovering that the efficiency of these enzymes was dependent on the length of peptide chain, the terms "proteinase" and "peptidase" were introduced to indicate proteases acting on the proteins and peptides, respectively.

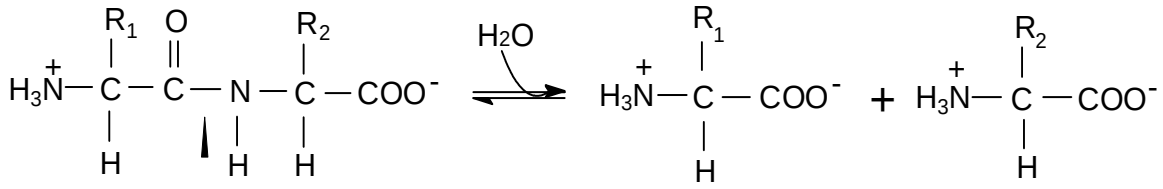


**Figure 1.4** The protease reaction which specifically cleaves the peptide bond (Mathews and van Holde, 1996)

Proteases are classified according to their mechanism of action (Neurath *et al.*, 1967; Stroud *et al.*, 1971; Titani *et al.*, 1972b). The International Union of Biochemistry recognises four mechanistic classes with each family having a characteristic set of functional amino acid residues arranged in a particular configuration to form the active site (Table 1.3). Members of each family are believed to have descended from a common ancestor by divergence (Neurath *et al.*, 1967).

**Table 1.3** Examples of proteases, subdivided into mechanistic sets (Reich *et al.*, 1975)

| **Set** | **Feature** | **Inhibitor** | **Examples** |
|---|---|---|---|
| Serine protease | active serine | fluorophosphates | chymotrypsin,<br>trypsin,<br>elastase,<br>subtilisin |
| Metalloprotease | $Zn^{++}$ | o-phenanthroline<br>EDTA | carboxypeptidases<br>thermolysin |
| Aspartic protease | acidic pH optimum | diazoketones | pepsin<br>rennin |
| cysteine protease | cysteine | iodoacetate | papain<br>cathepsin B |

The best distinction between proteases is to subdivide them into endopeptidases (acting in the interior of the polypeptide) and exopeptidases (acting at the NB and C- terminal ends). Endopeptidases are usually rate limiting and are responsible in the early stage of protein cellular degradation while exopeptidases complete the degradation of peptides to free amino acids. Once the degradation is started it proceeds rapidly and there is little or no accumulation of intermediate

products. Exopeptidases are generally monomeric proteins of small molecular weight whereas endopeptidases are multimeric proteins of high molecular weight. Many exopeptidases are metallo enzymes[1].

### 1.6.2.1 Serine Proteases

This class, characterised by the presence of a unique "Serine" residue, which forms a covalent bond with some substrates and inhibitors, is subdivided into two distinct families. One family is represented by the bacterial protease subtilisin. The other family is the trypsin family which includes chymotrypsin, trypsin, elastase and kallikrein (Creighton, 1993). Each serine protease preferentially cuts a polypeptide chain at the carboxyl side of specific kinds of amino acids. For example, trypsin cuts preferentially to the carboxylate side of basic amino acid residues like lysine or arginine, while chymotrypsin acts if a hydrophobic residue like phenylalanine is in this position. The general three-dimensional structure is different in the two families but they have the same active site geometry and catalysis proceeds via the same mechanism[2].

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1 http://crisceb.na.cnr.it/angelo/petrili/proteacl/exoendo.htm.

2 http://delphi.phys.univ-tours.fr/Prolysis/introprotease.html

The active site regions of all the serine proteases have a number of common factors of having a catalytic triad of Serine, Histidine, and Aspartic Acid. A fourth feature of the active site, however, differs from one serine protease to another and this "pocket" is always located close to the active site serine (Mathews and Holde, 1996). Catalysis of peptide bond hydrolysis by serine protease (Chymotrypsin, as an example) proceeds as shown in Fig. 1.5. The first step in the

catalysis is the formation of an acyl enzyme intermediate between the substrate and the essential serine (Fig. 1.5 a-c). Formation of the covalent intermediate proceeds through a negatively charged tetrahedral transition state intermediate and then the peptide is cleaved (Fig. 1.5 d-f).

During the second step (Fig. 1.5 g-h), which is deacylation, the acyl-enzyme intermediate is hydrolysed by a water molecule to release the peptide and to restore the serine-OH of the enzyme. The deacylation that involves the formation of a tetrahedral transition state intermediate, proceeds through a reverse reaction of acylation (A water molecule attacks the nucleophile instead of Ser residue). The Histidine residue provides a general base and accepts the OH group of the reactive Ser (Garrett and Grisham, 1999).

Serine proteases like chymotrypsin are susceptible to inhibition by organic fluorophosphates, such as tosyl phenylalanyl chloromethylketone (TPCK) and phenylmethylsulfonyl fluoride (PMSF) which reacts with the active site serine residue, but not with any of the other serines in the enzyme. The covalent bond of the enzyme-inhibitor complex is extremely stable (Garrett and Grisham, 1999).

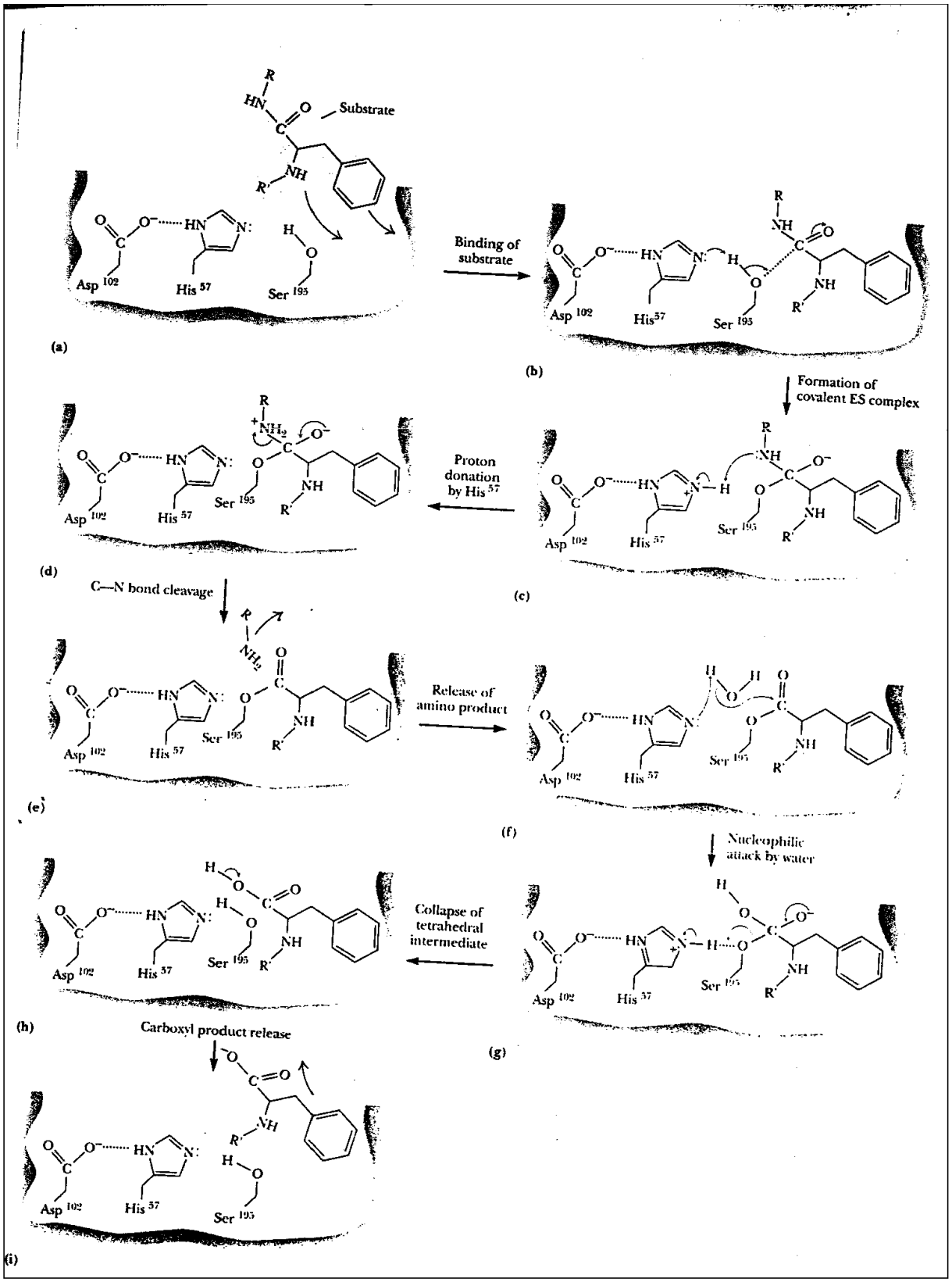



**Figure 1.5** A detailed mechanism for the chymotrypsin reaction (Garret and Grisham, 1999).

### 1.6.2.2 Cysteine Proteases

Cysteine or thiol proteases are characterised by cysteine-histidine within their catalytic site and they are found in many species, which include plants, animals and bacteria (Brocklehurst, 1985). They have a molecular weight in the range of 20 kDa to 35 kDa. These enzymes consist of single peptide chains, folded to form two domains with a deep cleft between them. The active cysteine and histidine residues occupy opposite sides of the cleft, histidine residue is partly attached to a hydrophobic region. Any interactions that affect the movement of the walls of the cleft may reorganise the catalytic site geometry (Page and Williams, 1987).

Like the serine proteinases (Fig. 1.5), catalysis proceeds through the formation of a covalent intermediate and it involves the cysteine and histidine residues (Fig. 1.6), however, the nucleophile is a thiolate ion which has a greater tendency of ionisation compared to the hydroxyl group of the serine proteases. The thiolate ion is stabilised through the formation of an ion pair

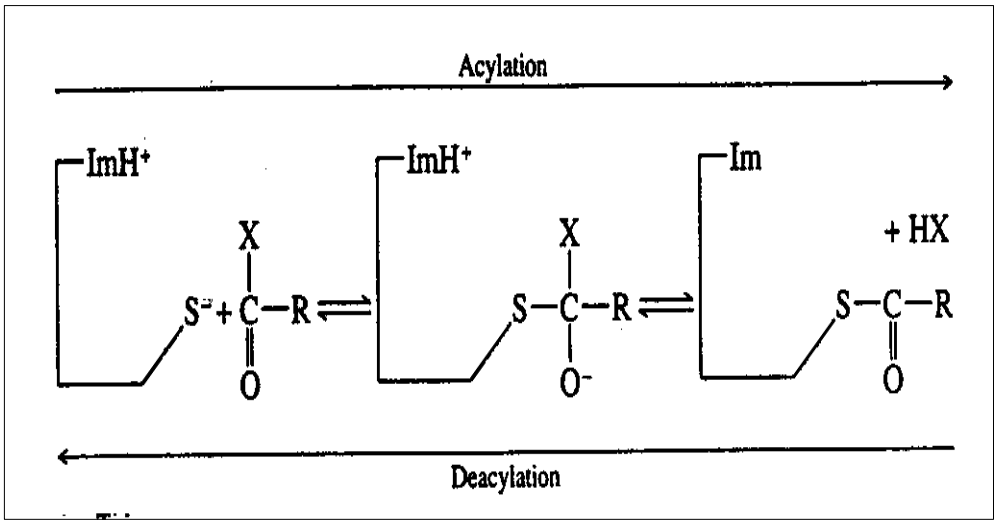



**Figure 1.6** Formation of thiolate ion during the reaction that is catalysed by thiol protease (Creighton, 1993)

with the neighbouring imidazolium group of Histidine. The attacking nucleophile is the thiolate-imidazolium ion pair and no water molecule is required. However, thiol proteases are extremely

susceptible to inhibition by many reagents such as iodoacetate, idoacetamide and methyl iodide (Page and Williams, 1987) and can be inhibited by serine inhibitors such as PMSF and TPCK.

### 1.6.2.3 Aspartic Proteases

Carboxyl proteinases are conventionally described as aspartic acid proteinases which form an interesting and mechanistically well defined class of hydrolytic enzymes (Tang, 1977). Most of aspartic proteinases belong to the family of pepsins and include digestive enzymes such as pepsin, chymosin and lysosomal cathepsin D, processing enzymes such as renin, certain fungal proteases like penicillopepsin, rhizopuspepsin and endothiapepsin. Second families comprise viral proteinases such as that from the AIDS virus (HIV) which are called retropepsins. Most of these enzymes are single chain proteins with a molecular weight of 30-40 kDa (Page and Williams, 1987).

These enzymes are predominately active at acidic pH and in many cases each posses two aspartic acid residues at the active site. They have similar three-dimensional structures, but their rate of peptide bond cleavage is different. Crystallographic studies show that these enzymes are bilobed molecules, with the active site located between the two homologous lobes. The two important catalytic aspartic residues are located at the base of the cleft in an extraordinarily polar environment and it is postulated that the deep groove is the substrate-binding site (Page and Williams, 1987).

In contrast to serine and cysteine proteases, catalysis by aspartic proteinases do not involve a covalent intermediate though a tetrahedral intermediate does exist. Two simultaneous proton transfers achieve the nucleophilic attack, one from a water molecule to the diad of the two-

carboxyl groups (Fig. 1.7 a-b) and a second one from the diad to the carbonyl oxygen of the substrate with the current CO-NH bond cleaved (Fig. 1.7 c-d). This general acid-base catalysis, which may be called “push-pull@ mechanism leads to the formation of a non-covalent neutral tetrahedral intermediate (Fig. 1.7) (Garrett and Grisham, 1999).

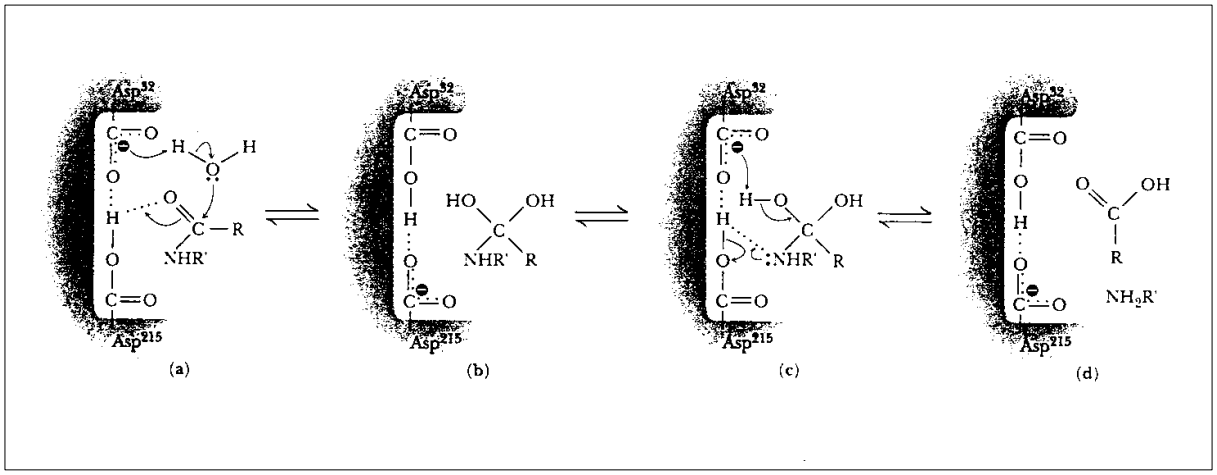



**Figure 1.7** A mechanism for the aspartic proteases (Garrett and Grisham, 1999).

### 1.6.2.4 Metallo Proteases

The metallo proteinase may be one of the older classes of proteinases found in bacteria, fungi and higher organisms. They differ widely in their sequences (Titani *et al.*, 1972a) and their structure but most of these enzymes contain a catalytically active zinc (Zn) atom which may serve different functions in different metalloenzymes. It may be directly involved in the catalytic process either in the binding step, the rate determination step or both and regulates catalytic activity by affecting the active site conformation or it may help stabilise protein structure. In some cases other metals such as cobalt, cadmium, copper and nickel can replace zinc. So far cobalt has proved to be the most valuable because it leads to a highly active metalloproteinase, while cadium and copper enzymes are generally much less active (Page and Williams, 1987).

The catalytic mechanism leads to the formation of a non-covalent tetrahedral intermediate after attack of a zinc-bound water molecule on the carbonyl group of the scissile bond. Transfer of the glutamic acid proton to the leaving group further decomposes the intermediate (Fig1.8) (Creighton, 1993).

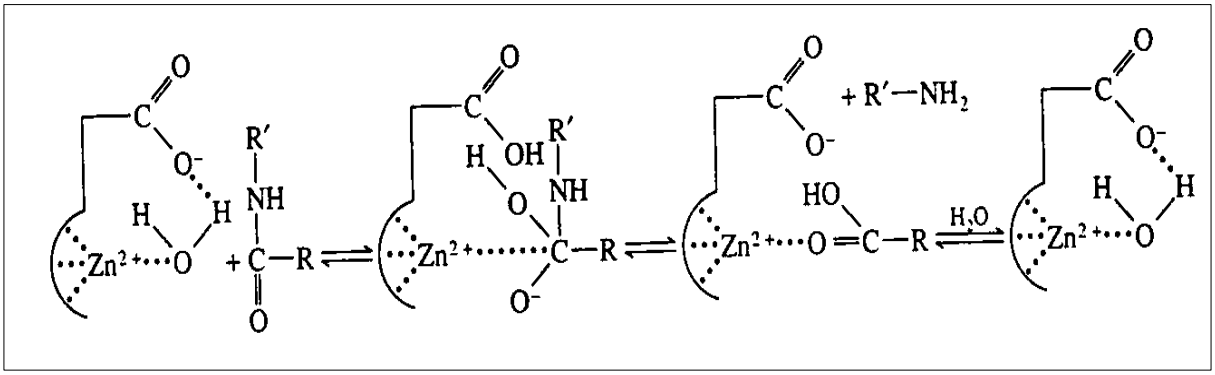



**Figure 1.8** A mechanism for the metalloproteases, in this case containing a $Zn^{2+}$ ion (Creighton, 1993).

## 1.7 RESEARCH HYPOTHESIS AND OBJECTIVES

Primary sedimentation is a widely used process for wastewater treatment. The removal of the significant fraction of the incoming organic and solids-load during primary sedimentation results in the reduction of subsequent conventional biological treatment requirements and allows the

overall optimisation of a treatment plant in terms of organic removal. Application of anaerobic treatment to the primary sludge is necessary in order to produce, through hydrolysis, the necessary carbon for the subsequent bacterial processes. Metabolism of macromolecules is different than small molecular weight compounds as a consequence of physical and biological factors, since diffusion coefficients of macromolecules are low. Complex organic compounds such as proteins, lipids and polysaccharides must be hydrolysed (by proteases, lipases and cellulases respectively) into smaller units before they can be transported across the bacterial cell wall. The Michaelis Menten enzyme kinetic model assumes that the rate of substrate utilisation is a function of enzyme and substrate concentration and rate coefficients.

The physics and biology of macromolecule metabolism is not fully understood and answers are needed to address whether the enzymes are extracellular and whether the smaller hydrolysed fragments are assimilated directly or released into the bulk medium. The Bioremediation Group at Rhodes have developed and patented novel processes for the recovery of heavy metals from acid mine drainage enhancing the yields and consequently reducing overall costs. The enzymology of the initial hydrolysis processes, the impact or influence of subsequent product metabolites of sludge and sewage digestion remain viable research objectives. The breakdown of sewage and sludge effluent is a universal problem and little is known about the mechanisms and chemistry associated with the processes. Rhodes University Bioremediation Group has, for many years been studying the biotechnology of sulphate reducing bacteria involved with removal of heavy metals from acid mine drainage. Nothing is known, however, with regards the enzymology of the hydrolysis process of the sludge and sewage The aims of this study are the following:

1. To identify the presence and the distribution of proteases in the methanogenic and

sulphidogenic anaerobic bioreactors.

2. To monitor the enzyme activity and to measure the quantity of the protein hydrolysis over a period of time in the methanogenic and sulphidogenic anaerobic bioreactors.

3. To investigate the distribution of proteases with respect to pH, temperature and thermal stability.

4. To study the effect of sulphur containing compounds such as sulphate, sulphite and sulphide; volatile acids such as acetic, butyric and propionic acid, and heavy metals such as Fe, Zn, Ni and Cu and proteases inhibitors such as 1,10-phenanthroline, EDTA, PMSF and TPCK on protease activity.

5. To isolate and purify proteases from the anaerobic bioreactors

# CHAPTER 2

# PRODUCTION OF PROTEASES DURING ANAEROBIC DIGESTION USING SEWAGE SLUDGE AS A CARBON SOURCE

## 2.1 INTRODUCTION

The activated sewage sludge has large fractions of incoming organic particulate, which mainly consists of proteins, carbohydrates and lipids (Nielsen *et al.*, 1992; Raunkjaer *et al.*, 1994). Henze (1992) showed that the particulate organics in the raw and settled wastewater varies from 40-60% and during anaerobic digestion, are broken down into simple soluble monomers which can be used by microorganisms. The cellulose and starches are hydrolysed to simple sugars by hydrolases; proteins are digested into amino acids by proteases and lipids are broken down to fatty acids and glycerol by lipases (McKinney, 1962). This hydrolysis involves the use of exoenzymes, and it is found to be the overall rate-limiting step for the mineralisation of organic matter.

Enzymatic hydrolysis of protein is an attractive means of obtaining hydrolysates with improved nutritional and functional properties. These enzymes have a wide variety of applications in food and other biotechnological industries, accounting for nearly 60% of the total worldwide enzyme sales. Proteases are categorised into alkaline, neutral or acid proteases based on their catalytic mechanism and pH optimum for activity (Ikasari and Mitchell, 1996).

Exoenzymes can originate from the sewage influent or from the activated sludge itself and can be produced either from cell autolysis or actively excreted by bacteria that are found in the sludge. These enzymes are either associated with the cell surface of the producer or are in free form, in solution or adsorbed on the surfaces of other material matter (Chr $\bar{s}$t, 1991). In the activated

sludge the exoenzymes dissolved in solution are negligible (Boczar *et al.,* 1992), and it is not clearly understood how these enzymes are associated with cells or immobilised in the sludge matrix within the extracellular polymeric substances (EPS) (FrNlund *et al,* 1995). Proteases have been immobilised on natural (organic and inorganic) and synthetic supports with inorganic support being used due to their good flow through properties, mechanical strength, regeneration and resistance to microbial attack (Chellapandian, 1998).

From all the products produced by the action of exoenzymes, amino acids and sugars are the most readily fermentable substrates, with the former being converted into pyruvate and the latter into alcohols. Pyruvate is an intermediate product that can be converted to lactate, propionate, butyrate, formate and acetate (Sterrit and Lester, 1988). The longer chain of fatty acids remain untouched until later in the overall process when methanogenic bacteria have been produced (McKinney, 1962). SRB and methanogens then use these monomers as their energy supply.

## 2.2 MATERIALS AND METHODS

### 2.2.1 Materials

Bovine serum albumin (BSA), Folin-Ciocalteau reagent, Ninhydrin reagent, Solution A and B for COD determination and other biochemicals were purchased from Sigma. Casein was obtained

from Merck. Ultra-Violet spectroscopy was performed on a UV-160A Shimadzu spectrometer and a Power Wave from Bio-Tek Instruments, Inc.

### 2.2.2 Reactor Design and Operational Procedures

A laboratory-scale stirred tank reactor which was used for these studies is shown in Fig 2.1. The

bioreactor has a working volume of 20L, sealed with a rubber stopper to avoid air from entering into the system. A gas trap containing zinc acetate was used to collect $H_2S$ escaping from the sulphate reducing anaerobic reactor.

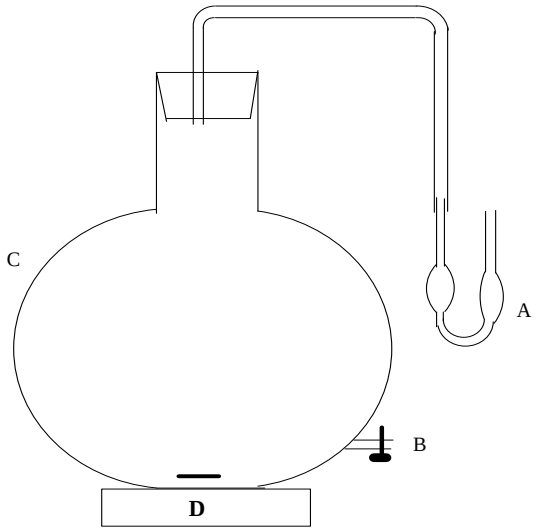



**Figure 2.1** Schematic diagram of the laboratory scale reactor used for monitoring protease production during anaerobic digestion. A: Gas Trap Device, B: Sampling Port, C: Anaerobic Reactor and D: Magnetic stirrer

### 2.2.3 Feed Source and Seeding

Sewage sludge, obtained from Grahamstown Municipal Works, was used as a carbon source in this study. The sludge was collected from the underflow lines of the primary clarifiers and macerated to break down any solids that may cause obstructions and/or blockage. The sulphate reducing anaerobic reactor was inoculated with 10% of SRB, 2000mg/L of sulphate was added to the sewage to simulate the sulphate reduction process and the chemical oxygen demand (COD)

content was made to 2000 mg/L. The methanogenic control anaerobic reactor was inoculated with 10% methanogens from Grahamstown Municipal anaerobic digester and the COD content was also made to 2000 mg/L.

### 2.2.4 Analytical Procedures

Sewage sludge samples, collected every two days (48hrs) from both reactors were centrifuged (3000 x g, 15min) and the supernatants were used for analysis of sulphate, sulphide and COD concentration. Determination of sulphate, sulphide and COD were carried out according to the analytical procedures described in Standard Methods (APHA, 1985), colorimetric method using N, Nt-diethyl-Δ-phenylenediamine (Rees *et al.,* 1971), and Merck Spectroquant$^{TN}$ system respectively (Appendix I and II).

### 2.2.5 Assay of Protease Activity

To casein (1.0 ml, 2% (w/v) in sodium phosphate buffer (0.1 M, pH 7.0) was added enzyme extract (1 ml) and the mixture was incubated ($37^{o}$C for 10min). The reaction was stopped by addition of TCA (2 ml, 5% w/v), centrifuged (3000 x g, 10min) and absorbance of the supernatant measured at 578 nm. One unit of protease activity was defined as the amount of the enzyme that generates 1:mol of tyrosine per minute under the assay conditions (Appendix II). Proteases were assayed in the supernatant after centrifugation (3 000 x g, 15min) of the sewage sludge. Any protease enzymes associated with the activated sludge matrix were assayed by re-suspending the sewage sludge in sodium phosphate buffer (0.1 M, pH 7) and performing the above assay *in situ*.

### 2.2.6 Amino Acid Determination

The total amino acid concentration in the sewage sludge was determined with the ninhydrin colour reaction (Plummer, 1978). Sewage extract (0.2 ml) and ninhydrin reagent (0.2 ml) were heated in a boiling water bath (15min). The reaction mixture was cooled to room temperature, ethanol (50% v/v, 0.3 ml) was added and the absorbance of the solution was measured at 570nm. The level of extracellular amino acids was determined from the supernatant after centrifugation (3 000 x g, 15min) of the sewage sludge.

### 2.2.7 Protein Determination

Extracellular proteins were assayed in the supernatant after centrifugation (3 000 x g, 15min) of the sewage sludge by Lowry (1951) method, using BSA as a standard (Appendix I and II). Protein reacts with the Folin-Ciocalteau reagent to give a coloured complex, due to the reaction between protein and alkaline copper and the reduction of phosphomolybdate by tyrosine and tryptophan present in the protein. The intensity of the colour depends on the amount of these aromatic amino acids present.

## 2.3 RESULTS

The sludge samples were withdrawn from the laboratory scale sulphidogenic and methanogenic reactor after 48hrs interval during anaerobic digestion. The rate at which the substrate is used in the system was determined by measuring the COD content and the result is shown in Fig.2.2. Both reactors showed an improved performance. In the sulphidogenic anaerobic system, the COD concentration increased from an initial value of 2000 mg/L to 13350 mg/L at day 5 before dropping to 7700 mg/L by day 31. The COD concentration from the methanogenic anaerobic

system fell from 8000 mg/L at day 5 to 4100 mg/L at day 31.

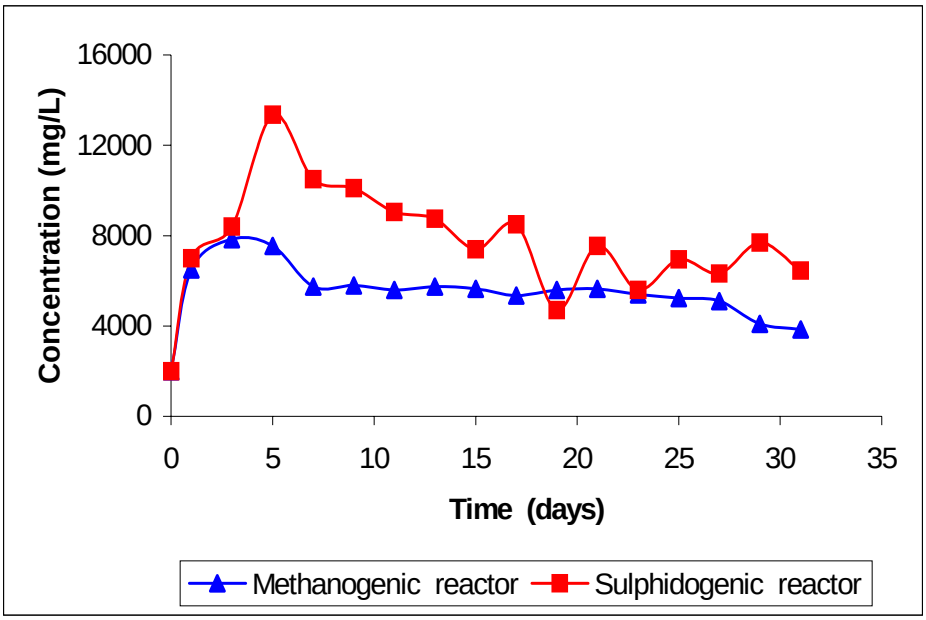



**Figure 2.2** COD concentration during anaerobic digestion

It was noticed that sulphate concentration in the sulphidogenic bioreactor was reduced from 2300 mg/L to 600mg/L during the first 5 days of anaerobic digestion producing hydrogen sulphide as an end product. This value dropped further to 500 mg/L by day 31 (Fig. 2.3). There was no sulphate reduction under the methanogenic system because of the absence of sulphate (Fig. 2.3).

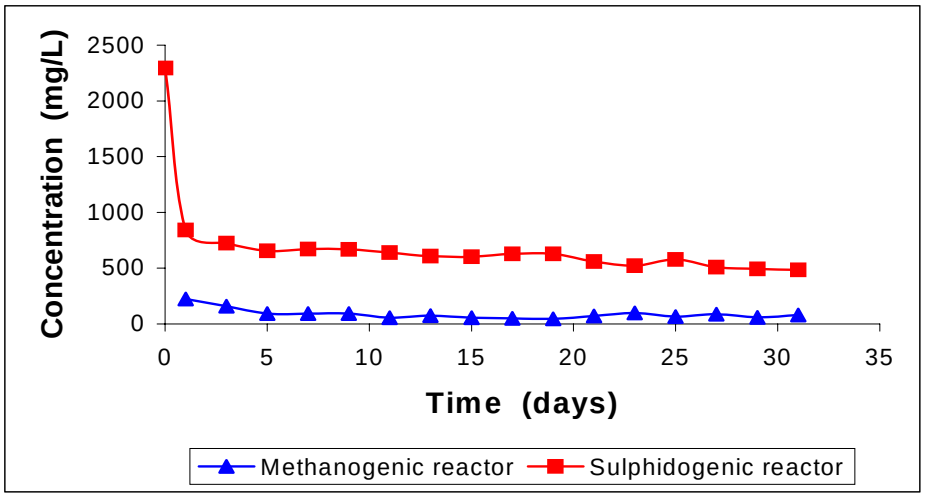



**Figure 2.3** Sulphate removal during anaerobic digestion.

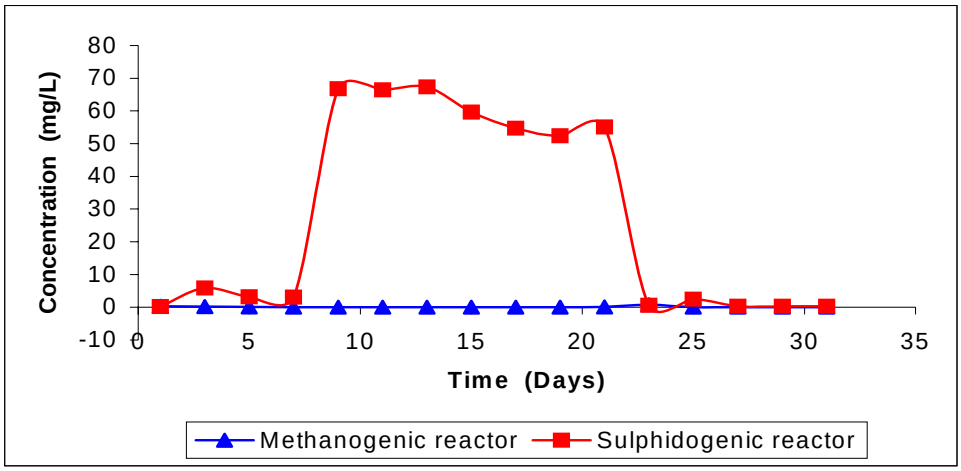
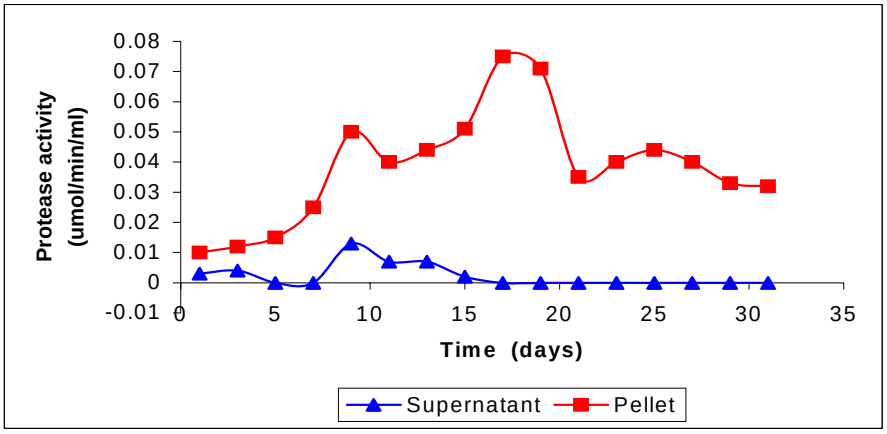



**Figure 2.4** Sulphide production during anaerobic digestion.

The production of hydrogen sulphide is shown in Fig. 2.4. A sharp increase in hydrogen sulphide of approximately 70 mg/L was seen at day 7 from the sulphidogenic bioreactor and after fluctuating between 70 and 55 mg/L for the next few days this dropped to basal levels by day 23. Again no hydrogen sulphide was detected from the methanogenic control bioreactor.

A

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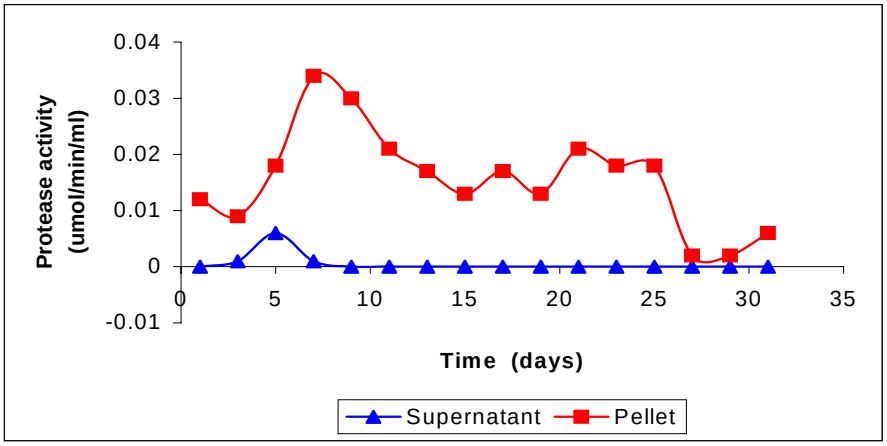



**Figure 2.5** Time course of the production of proteases in A) methanogenic and B) sulphidogenic anaerobic digester.

In order to localise the protease activity, each sample of sewage sludge was divided into two: 1) supernatant samples (after centrifugation of the sludge) were used to determine the presence of extracellular proteases and 2) re-suspended sludge pellet was used to assay the particulate or matrix bound enzymes.

From the time-course study it was found that there was little protease activity in the supernatant. The maximum activity was found to be to 0.01 μmol/min/ml at day 9 in the methanogenic reactor and 0.005 μmol/min/ml in the sulphidogenic reactor at day 5, after which the activity decreased to zero (Fig 2.5 A and B). On the other hand, the maximum protease activity was found to be in the re-suspended pellet matrix. Comparison of activities in fraction 1 and 2, indicates that the major fraction of total enzyme activity is associated within the organic floc matrix. Their peak activity occurred during day 7-9 in the sulphidogenic system and during day 17-19 in the methanogenic system, with activities of 0.035 and 0.08 μmol/min/ml, respectively (Fig. 2.5 A and B).

In this study, the concentration of extracellular protein has been monitored and the result is shown in Fig 2.6. Protein concentration increases up to 2.76 mg/ml at day 11 in the sulphidogenic reactor and up to 0.7 mg/ml at day 7 in the methanogenic reactor, after which there was a gradual decrease in protein concentration up to the end of the time course study. Since it was apparent that proteases were strongly associated with the organic floc, it would be obvious that protein itself could be adsorbed. This would lead to difficulties in measuring protein concentration within the organic floc.

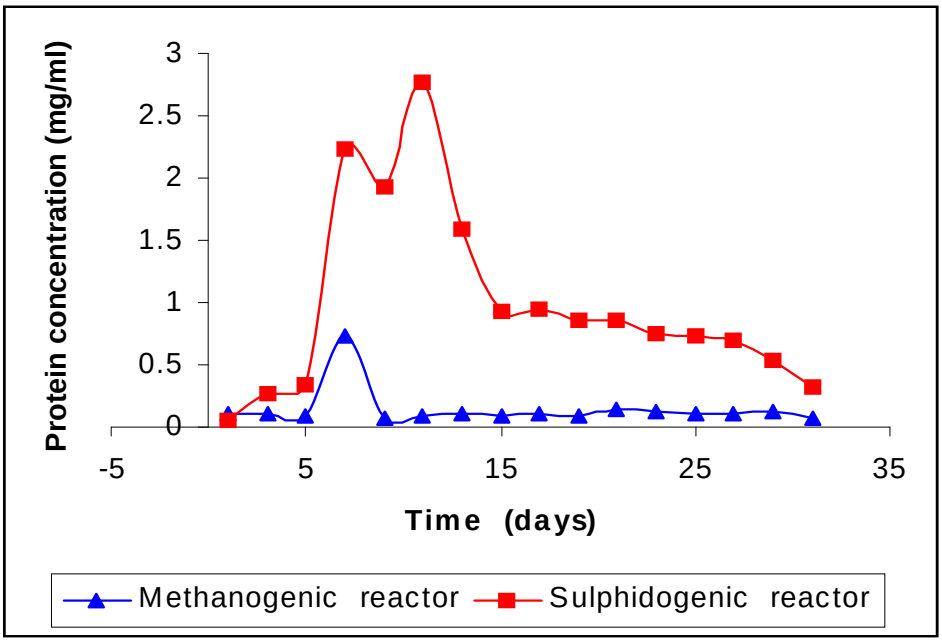



**Figure 2.6** Time course of the production of proteins from the anaerobic bioreactors.

Hydrolysis of protein material by proteases led to the production of amino acids, and their levels from both methanogenic and sulphidogenic bioreactors are shown in Fig 2.7. Initially there was a slight decrease in amino acid content until day 5 and 3, as the absorbance indicating the amino acid content decreased from 1.55 to 0.68 and from 1.35 to 1.2 for the methanogenic and sulphidogenic samples, respectively. The absorbance increased to 9.22 for the sulphidogenic sample during day 11, fluctuates between 6 and 9 until day 25 and then decreased to 1. The absorbance of methanogenic sample, however, increased to 1.7, stays constant until day 29 after which it drops to 0 at day 31.

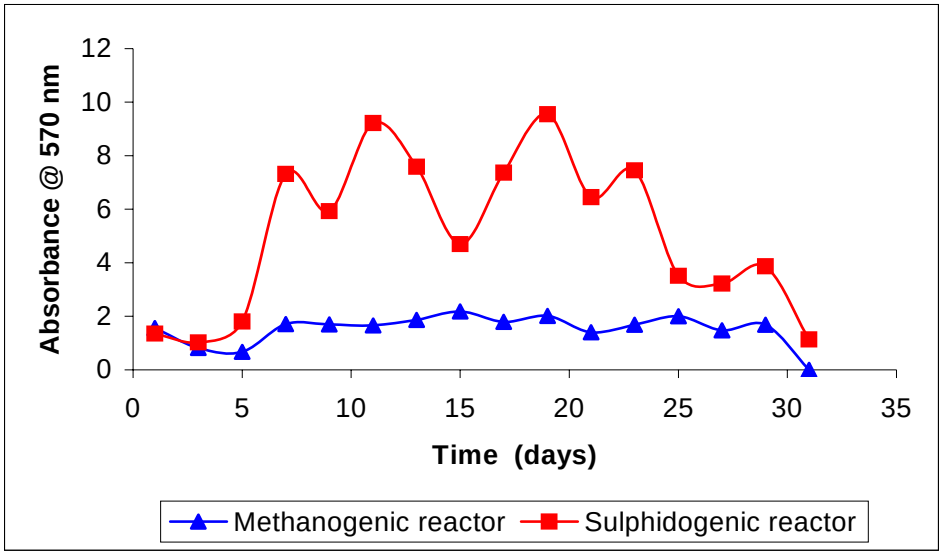



**Figure 2.7** Time course of the production of amino acids from anaerobic bioreactors.

## 2.4 DISCUSSION

During anaerobic digestion the microorganisms need an energy source for their growth and metabolism and sewage sludge was used as the particular source in this study. COD content in the system was measured in order to follow the disappearance of substrate during anaerobic digestion. It was found that the COD increased up to day 3 in the methanogenic reactor and to day 5 in the sulphidogenic reactor and after that it decreased. From this study it can be suggested that the increase in COD content could be due to the growth of bacteria inside the reactors until they stabilise and a decrease could be due to the use of the substrate by bacteria for their own metabolism. Hatziconstantinou *et al.*, (1996) suggested that it could be due to a uncontrolled hydrolysis/fermentation process that is taking place at the bottom of the tank. This was doubtful in our small reactors since they were continuously being stirred.

In the sulphidogenic reactor, SRB utilised sulphate as their electron acceptor during the

anaerobic digestion process. The production of hydrogen sulphide in the system indicated that there was sulphate reduction. In this study hydrogen sulphide concentration increased to 70 mg/L and stayed constant until day 21 after which started to decrease (Fig. 2.4). A decrease could be due to the fact that hydrogen sulphide was escaping during sampling and this was confirmed by the smell of a gas coming out of a reactor. During sulphate reduction there was formation of a white layer on top of the reactor, suggesting that there was formation of sulphur by sulphur oxidising bacteria, which occur in the presence of light.

From both reactors there was little protease activity in the supernatant compared to the activity that was found in the re-suspended pellet (which shows that there was extremely low concentration of soluble protein in the sludge supernatant). In the sulphidogenic system, the supernatant showed maximum activity of 0.005 :mol/min/ml at day 7 (Fig. 2.5) while re-suspended pellet matrix has 0.035 :mol/min/ml at day 9. In the methanogenic system, the supernatant showed maximum activity of 0.01 :mol/min/ml at day 9 while re-suspended pellet matrix has 0.08 :mol/min/ml at day 19. These results showed that proteases were either attached to the cell membrane of the producer or immobilised in the organic matter. Boczar *et al.,* (1992) showed the same results and FrNlund *et al.,* (1995) suggested that the extracellular polymers of the flocs contain a large pool of extra-cellular enzymes.

Due to their association with the pellets proteases can be reused during anaerobic treatment of wastewaters without affecting their activity, since other molecules tend to offer a protective film about them. As seen later (Chapter 4 and 5) this is a problem during enzyme extraction and purification. Another advantage of this protective film is that microorganisms do not need to waste energy in replacing the enzyme pool continuously (Goel *et al.,* 1998). Comparing the two

systems, it was found that the methanogenic reactor showed a higher protease activity than the sulphidogenic reactor and enzyme production occurred at a later stage in the methanogenic reactor during the time study (day 19 for methanogenic and day 9 for sulphidogenic reactors, Fig. 2.5, page 40). This supports the results that a non-sulphate system contained a higher enzyme activity than the sulphate containing system which were found by Whittington-Jones (1999). High enzyme activity could imply that proteases are digesting other enzymes like lipases and cellulases and are destroying themselves in the process (autolysis). If this happens in a reactor, there won't be any energy supply to the SRB and methanogens leading to a digester failure and thus would explain why a non-sulphate system takes longer to treat wastewaters.

Analysis for amino acids, which are produced during protein hydrolysis by proteases, showed that in both reactors protein degradation was taking place and high levels of these monomers were found in the sulphidogenic reactor (Fig. 2.7). On the other hand, in the methanogenic reactor low levels of amino acids were found which contradicted our beliefs that wherever there is high protease activity there should be high levels of amino acids. This could be due to the production of hydrogen sulphide in the sulphidogenic reactor, which might influence the interaction between proteases and the substrates (Chapter 3). The increase in protein concentrations in the two reactors during the time course is due to the production of other enzymes such as lipases, cellulases and glucosidases in the reactor, which are responsible for degradation of other complex organic compounds such as lipids and carbohydrates. FrNlund *et al.*, (1995) showed that there was no change in carbohydrate and protein content over time in the sludge, suggesting that protease activity could be low or rather, that protein degradation and protein production were balanced. However, in this study there was an increase in amino acid concentration with time, showing there are enough proteases for protein degradation. The little

difference in protein concentration between the influent and effluent could be due to a balance between protein degradation and production.

# CHAPTER 3

# CHARACTERISATION AND PROPERTIES OF PROTEASES FROM

# THE ACTIVATED-SEWAGE SLUDGE MATRIX

## 3.1 INTRODUCTION

To study an enzyme, it is necessary to develop a rapid assay to measure its catalytic activity. Assays are designed to measure the rate of product formation or the rate of substrate disappearance. In this study the assay that measured the rate of product formation was used due to the fact that it involved a direct measurement of enzyme activity. Frequently, the amount of product produced in a given length of time is measured in a fixed-time assay (Armstrong, 1989).

Enzyme activity is affected by the concentrations of the substrate(s), activators, and inhibitors specific for the enzyme, nonspecific effects of compounds such as salts and buffers, pH, ionic strength, temperature, even interaction with other proteins, or membranous material that might be present. In order to establish maximum activity ($V_{max}$) of an enzyme, optimal conditions should be established. Unfortunately, this is not always possible due to the cost of the substrate or its solubility (Scopes, 1982).

For the majority of enzymes, the initial rate of reaction varies hyperbolically with substrate concentration. The velocity is usually expressed as units, e.g., :moles of product produced or substrate transformed per minute, or specific activity, which is units of enzyme activity per milligram of protein. At low substrate concentrations, first order kinetics is observed but at high substrate concentrations, zero order kinetics exists. The initial rate is proportional to the substrate concentration and this relationship can be expressed mathematically by the Michaelis-Menten equation:

$$v_O = \frac{V_{max}[S]}{K_m + [S]} \qquad \text{----------} \quad 1$$

where [S] is the substrate concentration, $V_{max}$ is the limiting value of $v_o$ and $K_m$ is the Michaelis constant. However, oligomeric enzymes that contain multiple substrate-binding sites do not display this kind of kinetics as they give a sigmoidal relationship between initial rate and substrate concentration. These kinds of enzymes change their molecular conformation as a result of substrate binding, and this process is called homotropic effect (Wilson and Walker, 1994).

After establishing the $V_{max}$ and $K_m$ of the enzyme, it is clear that these values are dependent on other influences such as ionic strength, pH and temperature. Ionic strength and pH specify the state of ionisation of amino acid residues in the active site of the enzyme and catalytic activity relies on a specific state of ionisation of these residues, so enzyme activity is pH dependent. As a result, the pH profile of an enzyme is either bell-shaped giving a narrow pH optimum or it has a plateau (Wilson and Walker, 1994). During determination of enzyme activity one must consider the temperature at which the assay is carried out. There is no optimum temperature of an enzyme, but there is only optimum temperature for a given assay. Normally, enzyme assays are carried out at 30 or 37$^{o}$C due to protein denaturation at higher temperatures. However, the shorter the incubation time, the higher the apparent optimum temperature and typical apparent optima for assays taking 5-10 min to complete are in the range of 40-60$^{o}$C (Scopes, 1982).
In enzymology, inhibition studies are important and addition of enzyme inhibitors will cause a decrease in the rate of a reaction leading to the accumulation of the intermediate products. Inhibition studies are essential for the classification of enzymes and the four classes of proteases, aspartic, serine, cysteine and metalloproteases can be distinguished on the basis of their

sensitivity to various inhibitors (Abraham and Breuil, 1996). During anaerobic digestion, proteases operate over a wide range of pH values (5-11) and those that are active between pH 7-8 are usually the zinc-containing metalloproteases, which are inhibited by powerful chelating agents such as EDTA. Other proteases are sensitive to organophosphorus compounds (Sterrit and Lester, 1988).

### 3.1.1 Inhibition of digester bacterial reactions

There are a number of possible inhibitors of the reactions of anaerobic digestion and the increases in their concentration can cause the reactor to run with suboptimal efficiency or complete digestion failure. Inhibitors can be grouped into two classes: 1) end products from microbial reactions normally part of the digestion process and 2) organic or inorganic substances introduced into the digester in the feedstock (Hobson and Wheatley, 1993).

The volatile fatty acids (VFA) such as acetic, propionic and butyric acids can be inhibitors to an anaerobic digester as well as being the substrates for the microbial reaction. Accumulation of these acids inhibits the fermentation reaction producing them by an increase in hydrogen ions in the system, causing a drop of pH in the digester. It was suggested that this kind of inhibition occurs when the concentration of volatile acids is higher than 2000 mg/L (Hobson and Wheatley, 1993).

Wastewaters from industries contain high concentration of heavy metals and sulphate, which inhibit the rate of digestion by affecting bacteria inside the system. These bacteria use acetate while SRB have the ability to use some of the other volatile fatty acids such as propionic and butyric acid as their energy source (Omil *et al.*, 1996). During AMD treatment, the SRB utilise sulphate as their electron acceptor forming sulphite as a intermediate compound and eventually

producing sulphide. If the levels of sulphate become limiting during the AMD treatment, the SRB are able to use the intermediary sulphite as their electron acceptor. From these findings, it was decided to investigate the effect of these compounds on protease activity

Production of hydrogen sulphide during sulphate reduction has another advantage of removing heavy metals in wastewaters. Some of these metals such as copper and zinc are essential trace elements to living organisms, even though at higher concentration they can be toxic. Other metals such as lead and cadium do not have any known biological function. Heavy metals that have been identified to be a concern are chromium, manganese, iron, cobalt, copper, zinc, molybdenum, silver, mercury, cadium and nickel (Hickey *et al.*, 1989), and the most quoted metals that cause inhibition of sewage sludge digesters are chromium, copper, nickel, cadium and zinc. The toxicity is due to the binding of heavy metals to the residues in the active site of an enzyme leading to inactivation. It is also due to the replacement of naturally occurring metals that are found in the enzyme by metals that are in the solution. It is known that $Co^{2+}$, $Hg^{2+}$, $Fe^{2+}$and $Fe^{3+}$ form chelates with cysteine residues, while $Zn^{2+}$, $Cu^{2+}$ and $Ni^{2+}$ interact with histidine residues (Malmström and Rosenburg, 1960; Jiang *et al.*, 1994). $Zn^{2+}$ can help in the stabilisation of protein structure or regulation of catalytic activity of metalloproteases (Page and Williams, 1987).

## 3.2 MATERIALS AND METHODS

### 3.2.1 Materials

The most common chemicals used were from Sigma and were of highest purity available. Acetic

acid, Butyric acid, DMSO, EDTA, 1,10-Phenanthroline, PMSF, Propionic acid, and TPCK were obtained from Sigma, Casein was obtained from Merck and Spectroscopy was performed in a Shimadzu UV-160A spectrophotometer.

### 3.2.2 Reactor Design and Operational Procedure

The laboratory-scale continuous stirred tank reactor (CSTR) used for these studies of anaerobic digestion is shown in Fig. 3.1. The same feed source used in Chapter 2 (section 2.2.3) was used in this study.

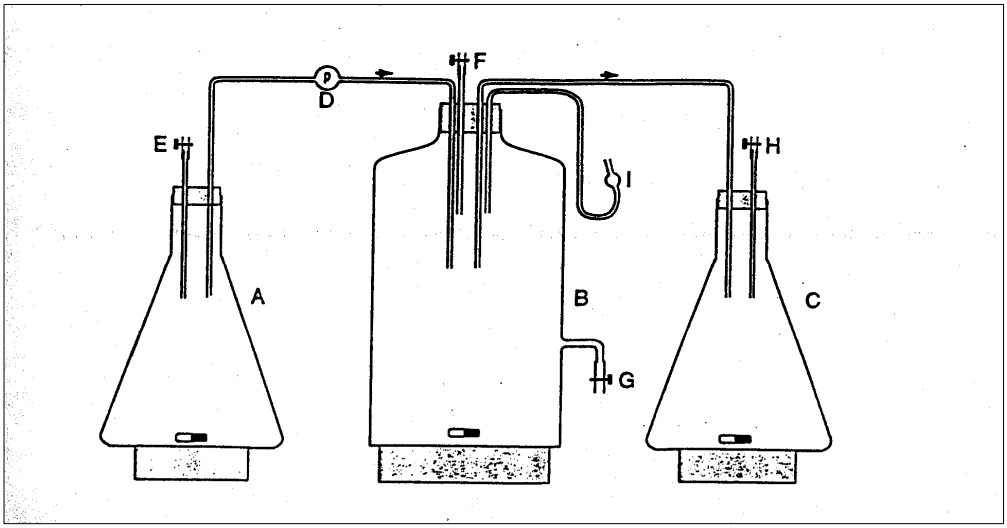



**Figure 3.1** Schematic diagram of the laboratory-scale reactor used for monitoring protease activity in the anaerobic digester. A: Feed vessel, B: Anaerobic reactor, C: Overflow vessel, D: Peristaltic pump, E: Influent Port, F: Port 1, G: Sampling port 2, H: Over flow and I: Gas trap.

### 3.2.3 Influence of pH, Temperature and Stability on Enzyme Activity

#### 3.2.3.1 pH

The effect of pH on protease activity was determined using the standard assay conditions

described in section 2.2.5 (page 36), except that the following buffers were used to dissolve casein: 0.1 M sodium acetate (pH 4-6), 0.1 M sodium phosphate (pH 7-8) and 0.1 M carbonate/bicarbonate (pH 9-11). The sample that was used as an enzyme extract was the re-suspended pellet through out the study.

#### 3.2.3.2 Temperature

To enzyme extract (1 ml) was added casein (1 ml) in buffer reflecting the particular pH of interest and the mixture was incubated at temperatures ranging between 20$^{O}$C and 70$^{O}$C for 10 min. The reaction was stopped by adding TCA (2 ml, 5% w/v) unhydrolysed protein was precipitated by centrifugation (3000 x g, 10min) and the absorbance of the supernatant was measured at 578 nm.

#### 3.2.3.3 Thermal Stability

Enzyme extract was heated at optimum temperature for 0-5hrs and the samples were collected in an hourly interval. To enzyme extract (1 ml) was added casein (1 ml) in sodium acetate buffer (0.1 M, pH 5), in sodium phosphate (0.1 M, pH 7) and in carbonate/bicarbonate buffer (0.1 M, pH 10) and the mixture was incubated (37$^{o}$C, 10 min). The reaction was stopped by adding TCA (2 ml, 5% w/v), the precipitated protein centrifuged (3000 x g, 10min) and the absorbance of the supernatant was measured at 578 nm.

### 3.2.4 Effect of Sulphate, Sulphite and Sulphide on Enzyme Activity

These compounds were dissolved as sodium salts. It should be noted, however, that sulphite was tested using sodium bisulphite, which dissociates into sodium sulphite in solution. Their

concentration was varied from 0 to 1000 mg/L. To enzyme extract (1ml) was added sulphur containing compound (0-1ml) and made up to 2 ml with water. The mixture was incubated (room temperature, 30min). Reaction was initiated by the addition of casein (1ml, 2% w/v), the mixture was incubated (37$^{o}$C, 10 min), centrifuged (3000 x g, 10 min), and the absorbance of the supernatant was measured at 578 nm. The results were expressed as a percentage of relative activity, taking into account the activity determined with a non treated enzyme sample.

### 3.2.5 Effect of Volatile Acids on Enzyme Activity

The volatile acids investigated in this study were acetic acid, butyric acid and propionic acid. To enzyme extract (1ml) was added volatile acids (0-1ml) and the reaction mixture was made up to 2 ml with water to give a final concentration of acid of 0-1000 mg/L. The mixture was incubated (room temperature, 30min) and the reaction was initiated by addition of casein (1ml, 2% w/v). To measure the residual protease activity the reaction mixture was incubated (37$^{o}$C, 10 min), centrifuged (3000 x g, 10 min), and the absorbance of the supernatant was measured at 578 nm. The results were expressed as percentage of relative activity, compared to the activity of a non treated enzyme sample.

### 3.2.6 Effect of Metal Ions on Enzyme Activity

The following metals: $Fe^{3+}$, $Cu^{2+}$, $Ni^{2+}$ and $Zn^{2+}$ were investigated and these metal ions were dissolved in water as their sulphate salts. In order to have the final concentration, which ranges from 0-1000 mg/L, metal ion solution (0-1 ml) was added to enzyme extract (1 ml) and the solution was made up to 2 ml with water. The mixture was incubated (room temperature, 30 min), the reaction started by the addition of casein (1 ml, 2% w/v) and to measure the residual protease activity the mixture was incubated (37$^{o}$C, 10 min). TCA (2 ml, 5% w/v) was used to

stop the reaction, the unhydrolysed protein were removed by centrifugation (3000 x g, 10 min) and the absorbance of the supernatant was measured at 578 nm.

### 3.2.7 Effect of known Protease Inhibitors

The effect of a number of potential inhibitors on protease activity was also investigated: a serine inhibitor, phenylmethylsulphonylfluoride (PMSF) and tosyl-L-phenyl-alanylchloromethylketone (TPCK); metalloprotease inhibitors, EDTA and 1,10-phenanthroline. EDTA was dissolved in water, while 1,10-phenanthroline, PMSF and TPCK were dissolved in dimethyl sulphoxide (DMSO) solution. To enzyme extract (1 ml) was added inhibitor (1 ml) to give final concentrations of 2.5 mM for PMSF, 100 :M for TPCK, 10 mM for EDTA and 1,10-phenanthroline. The mixture was incubated (room temperature, 30min), and a reaction was started by addition of casein (1ml, 2% w/v). The reaction mixture was incubated (37$^{o}$C, 10 min), centrifuged (3000 x g, 10 min), and the absorbance of the supernatant was measured at 578 nm. The protease activity was expressed as a percentage relative to the activity of the enzyme without inhibitors.

## 3.3 RESULTS

### 3.3.1 pH

Fig 3.2 shows the optimum pH of proteases from sulphidogenic and methanogenic bioreactors, with an optimum at pH 10 for both. At pH 11, which was higher than their optimum, these enzymes showed a 10% decrease in their activity.

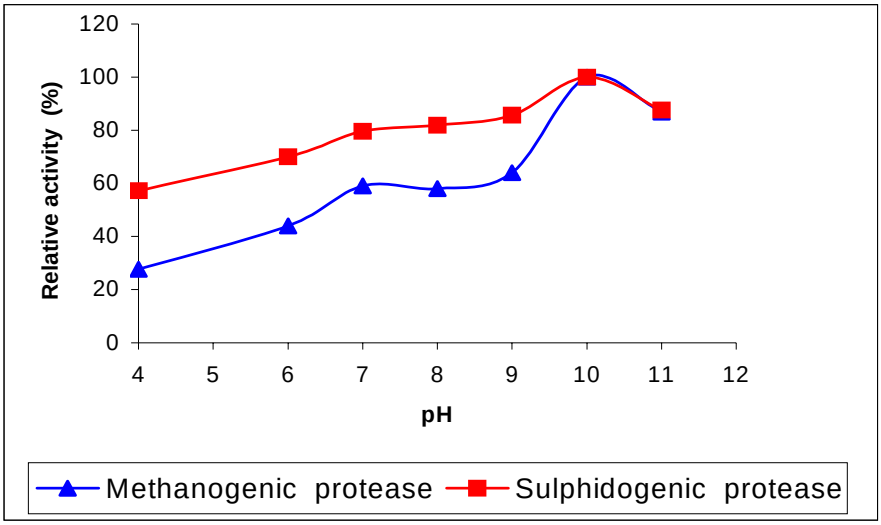
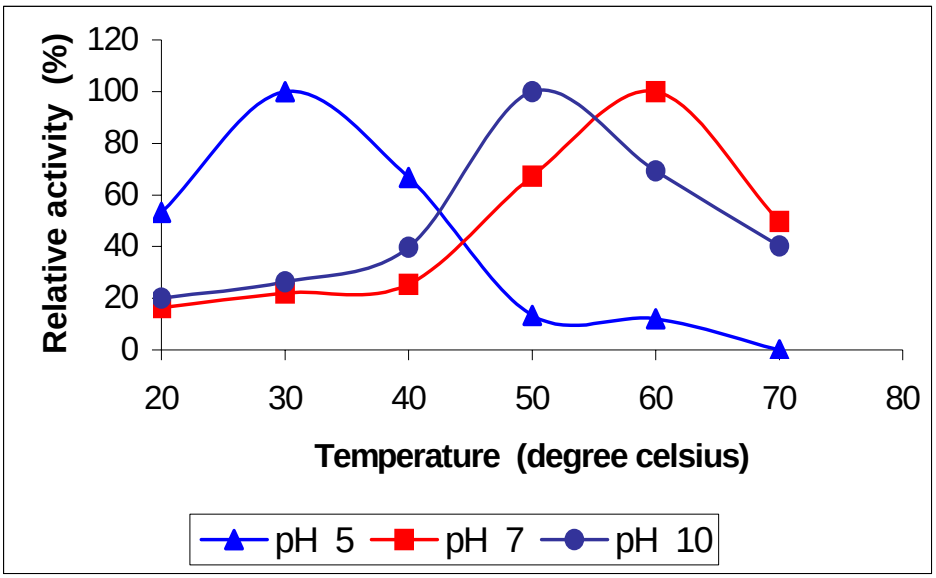



**Figure 3.2** Effect of pH on protease activity from anaerobic digesters.

### 3.3.2 Temperature

The optimal temperature for methanogenic and sulphidogenic proteases was determined at pH 5, 7 and 10. At the different pH values the proteases from both the methanogenic and sulphidogenic bioreactors seemed to have similar temperature optima (see Fig 3.3). Proteases at pH 5 have an optimum operating temperature of 30$^{o}$C, those at pH 7 have an optimum temperature of 60$^{o}$C and those at pH 10 have an optimum temperature of 50$^{o}$C.

A




B

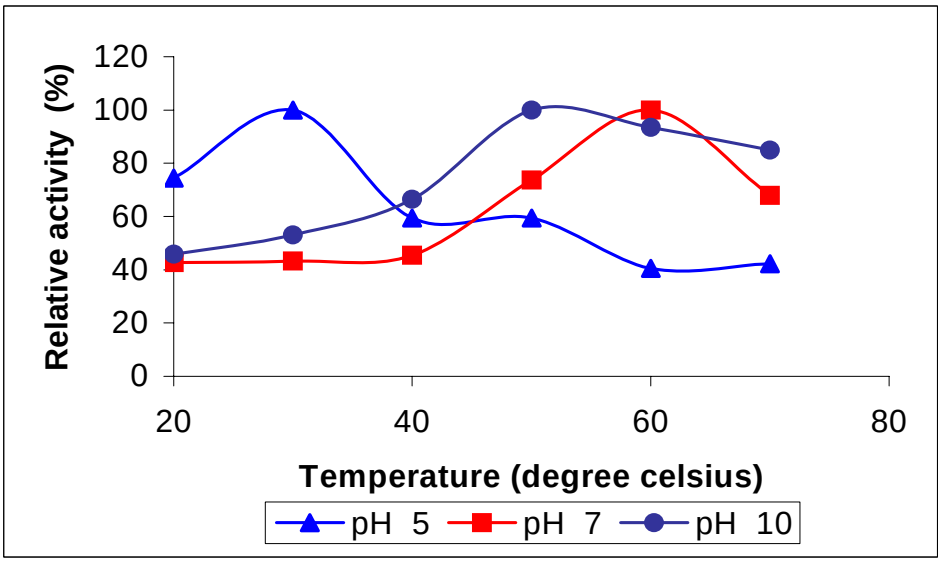
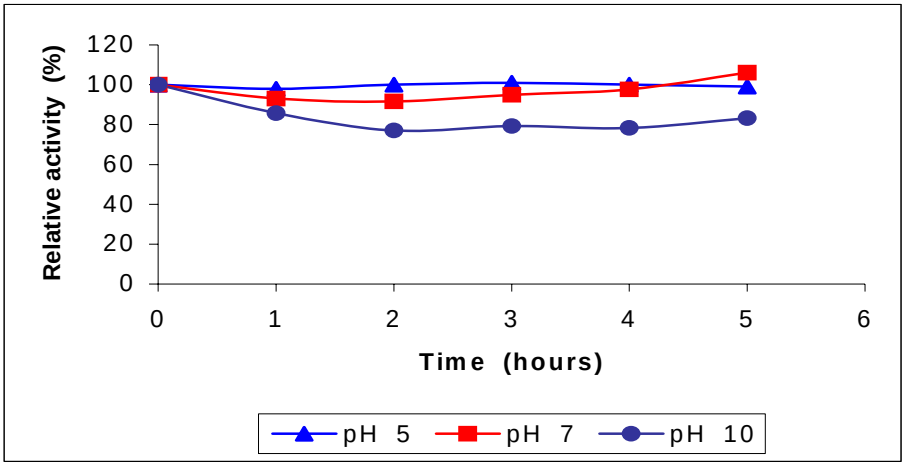



**Figure 3.3** Temperature optima of A) methanogenic and B) sulphidogenic proteases.

### 3.3.3 Thermal Stability

The enzymes from methanogenic and sulphidogenic bioreactors were stable at their optimum temperatures (pH 5, 30$^{o}$C; pH 7, 60$^{o}$C and pH 10, 50$^{o}$C) over a period of at least 5hrs (Fig 3.4).

A




B

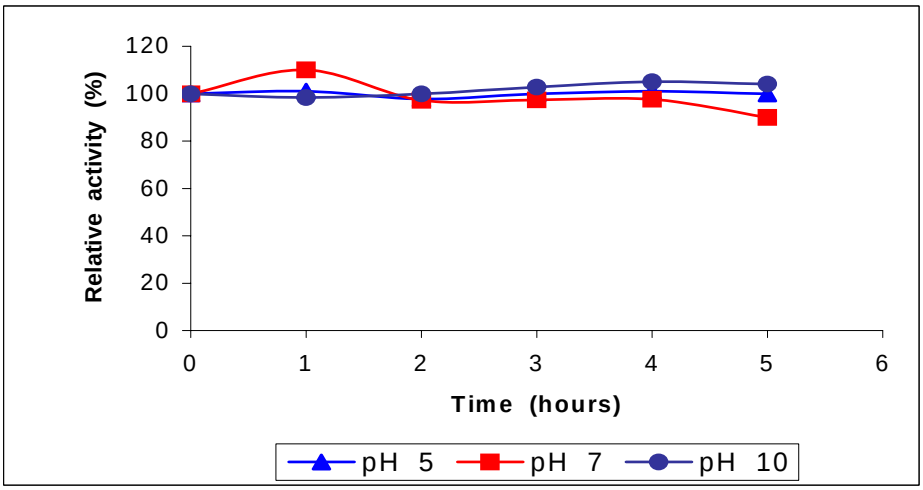



**Figure 3.4** Temperature stability of A) methanogenic and B) sulphidogenic proteases under varying pH conditions.

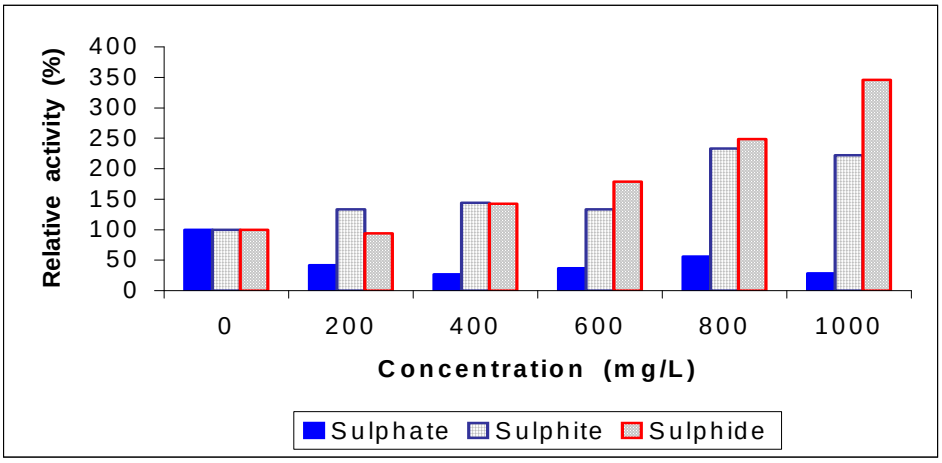
### 3.3.4 Effect of Sulphate, Sulphite and Sulphide

At pH 5 (Fig. 3.5), proteases from both anaerobic reactors were strongly inhibited by sulphate ions. The methanogenic proteases retain 28% of their activity at a concentration of 1000mg/L, while sulphidogenic enzymes retain 46% of their activity at the same concentration. The sulphite and sulphide at pH 5, however, seem to increase protease activity from both anaerobic reactors with sulphide causing the greatest change. At 1000 mg/L sulphide increased enzyme activity by 245.5% and 125% with methanogenic and sulphidogenic proteases, respectively. In the sulphidogenic reactor, however, sulphite causes a slight increase of protease activity up to a concentration of 400mg/L after which there was a decrease in protease activity.

Results of Fig 3.6 showed the effect of sulphate, sulphite and sulphide on the methanogenic and sulphidogenic proteases at pH 7. Sulphate seems to have little or no effect on protease activity from both reactors, while sulphite cause a slight increase of 26% on the methanogenic proteases at 1000mg/L. Surprisingly, sulphite seems to have no effect on proteases from the sulphidogenic reactor. Proteases from sulphidogenic and methanogenic reactors were activated by sulphide at 1000 mg/L with an increase of activity by 72.7% and 91.3%, respectively.

At pH 10 (Fig 3.7), sulphate has little effect on the methanogenic proteases while it inactivates proteases from the sulphidogenic reactor retaining 44.3% of their activity at 1000 mg/L. Sulphite seems to have little or no effect on the methanogenic proteases and it seems to increase sulphidogenic protease activity by 33.3% at a concentration of 400mg/L and at higher concentration there is inactivation of these enzymes. On the other hand, sulphide at 1000 mg/L increased enzyme activity by a further 25 and 42% on the methanogenic and sulphidogenic proteases, respectively.

A

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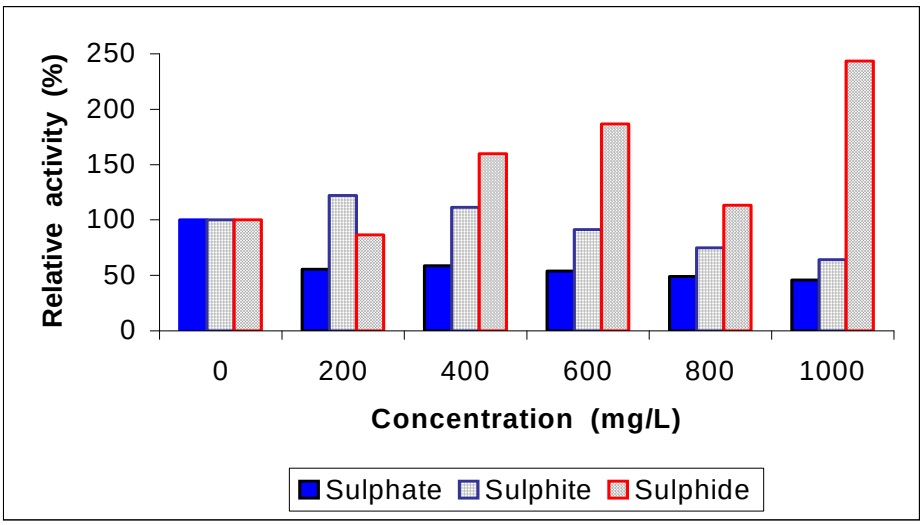
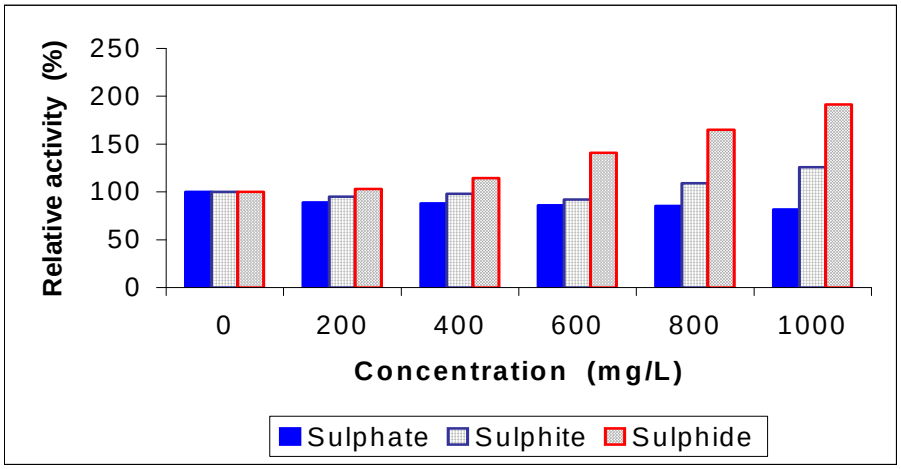



**Figure 3.5** Effect of sulphur containing compounds on the A) methanogenic and B) sulphidogenic protease activity at pH 5.

A

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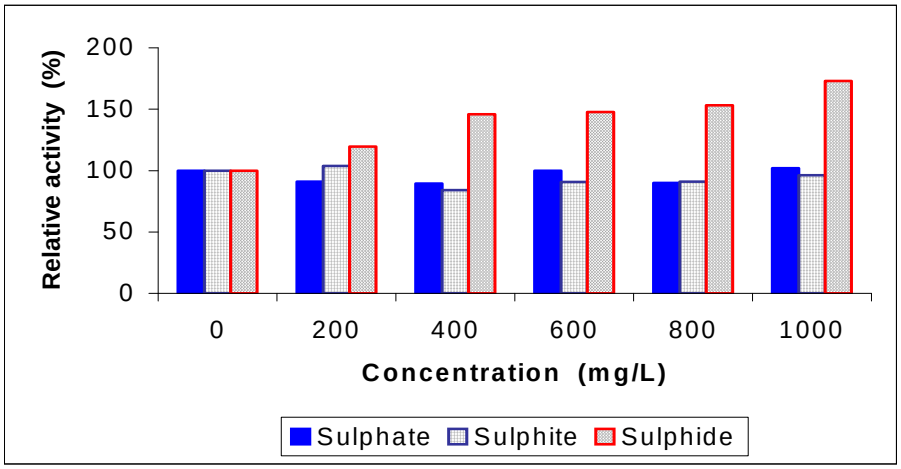
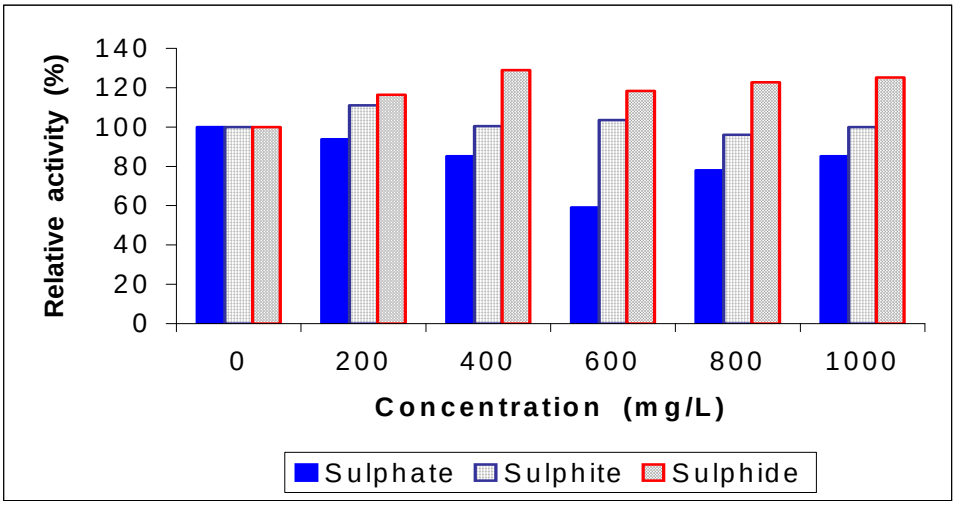



**Figure 3.6** Effect of sulphur containing compounds on the A) methanogenic and B) sulphidogenic protease activity at pH 7.

A



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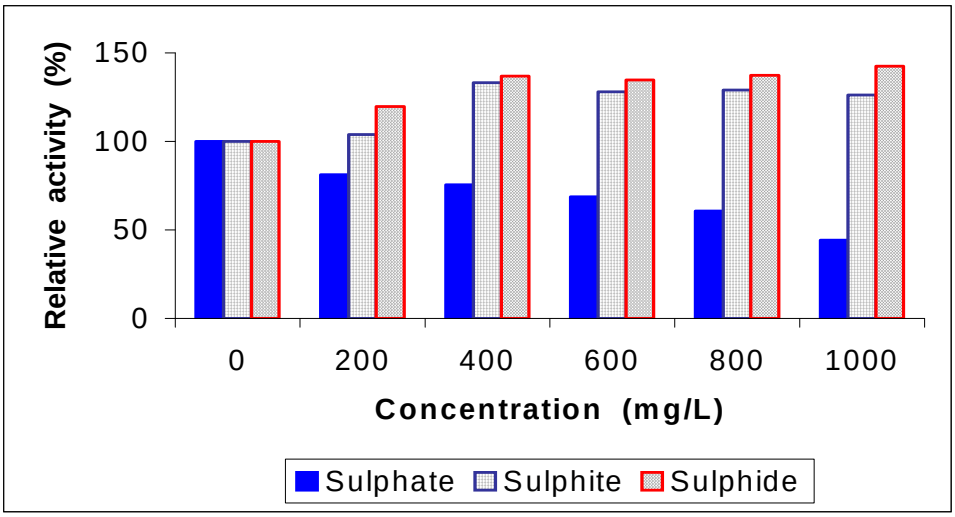



**Figure 3.7** Effect of sulphur containing compounds on the A) methanogenic and B) sulphidogenic protease activity at pH 10.

### 3.3.5 Effect of Volatile Acids

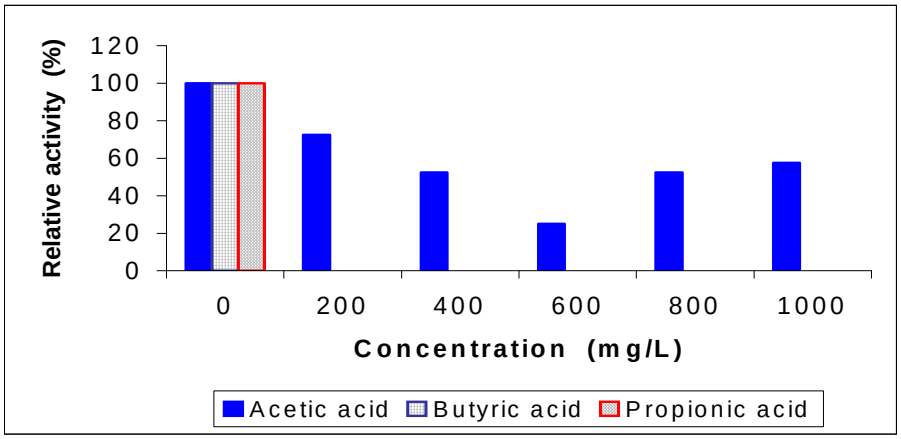
In Fig. 3.8, acetic acid causes a decrease in the activity of protease enzyme from the methanogenic reactor by 43.5 % and a slight decrease on those from the sulphidogenic reactor by 25% at pH 5. Butyric acid causes a complete inhibition of proteases from the methanogenic and sulphidogenic reactors. On the other hand, propionic acid causes a complete inhibition of proteases from the methanogenic reactor and total inhibition of sulphidogenic proteases was found after 200mg/L.

At pH 7, (Fig 3.9) acetic acid and butyric acid cause a decrease of approximately 30% in protease

activity from methanogenic reactor, while they have little or no effect on sulphidogenic proteases. At 1000 mg/L the activity decreases by 30%. Propionic acid causes a slight decrease of 16.7% of protease activity from the methanogenic sample, while it inactivate proteases from the sulphidogenic reactor causing a loss of 50% activity at a concentration of 1000mg/L.

Results in Fig 3.10 shows the effect of volatile acids at pH 10. Acetic acid causes a slight decrease of 36% and 29.1% on methanogenic and sulphidogenic protease activity, respectively, at 1000 mg/L. Butyric and propionic acid have little effects on the methanogenic proteases, causing proteases to loose activity of 26.9 and 16.5% respectively. However these acids cause a decrease in the sulphidogenic proteases, with the loss of 48.9 and 44.2% of enzyme activity, respectively, at concentration of 1000 mg/L. It should be noted that at pH 7 and 10 the volatile acids were in solution as their respective salts.

A



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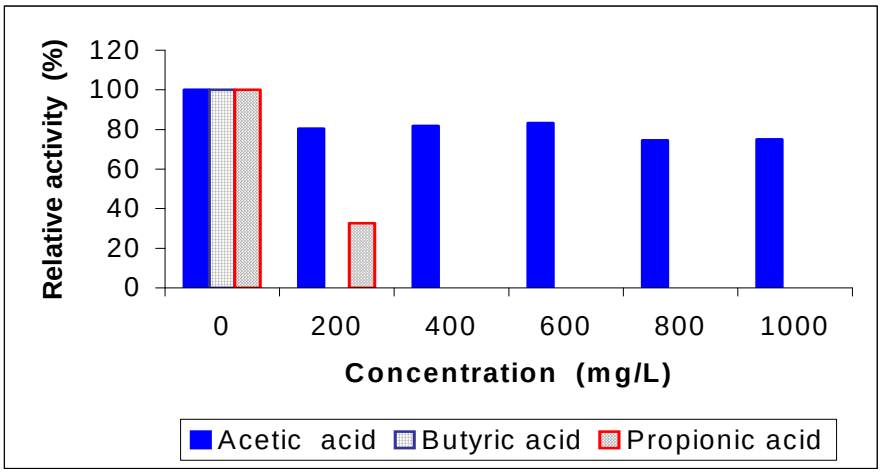
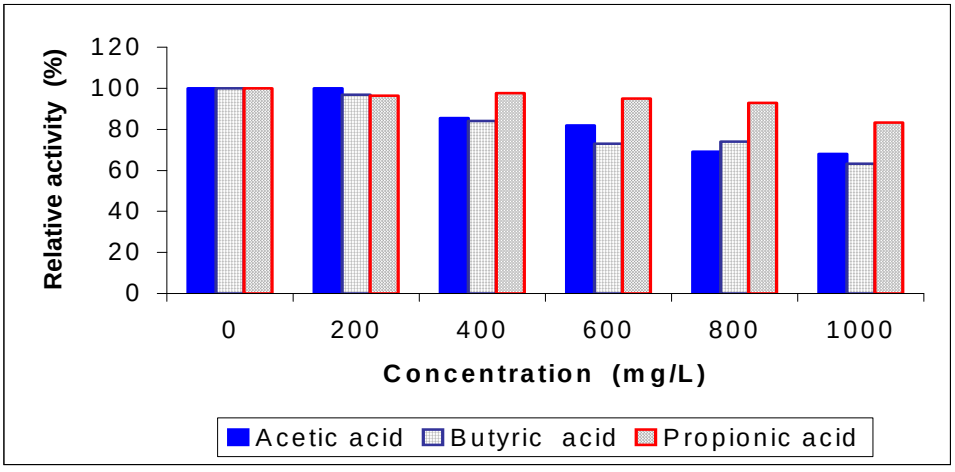



**Figure 3.8** Effect of volatile acids on the A) methanogenic and B) sulphidogenic protease activity at pH 5.

A



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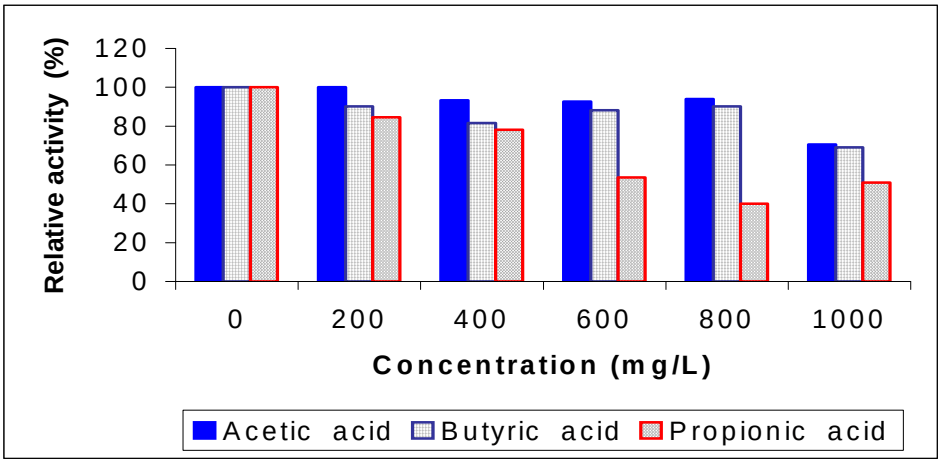
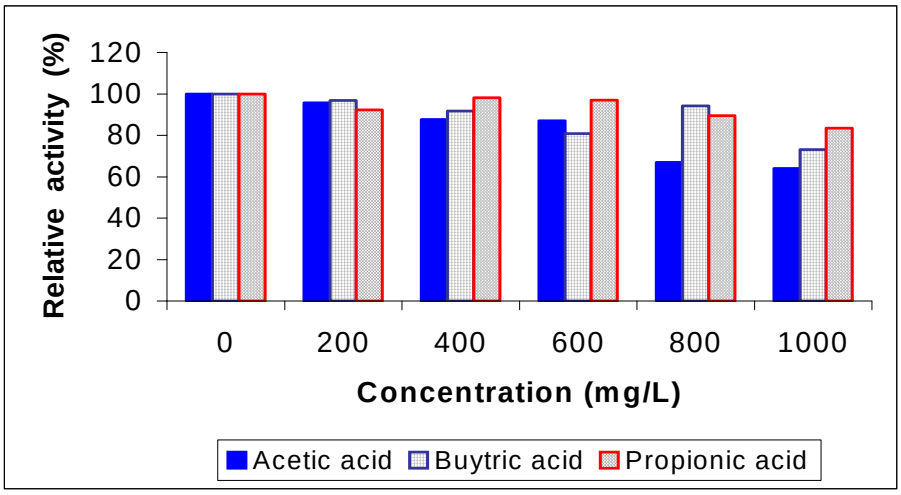



**Figure 3.9** Effect of volatile acids on the A) methanogenic and B) sulphidogenic protease activity at pH 7.

A

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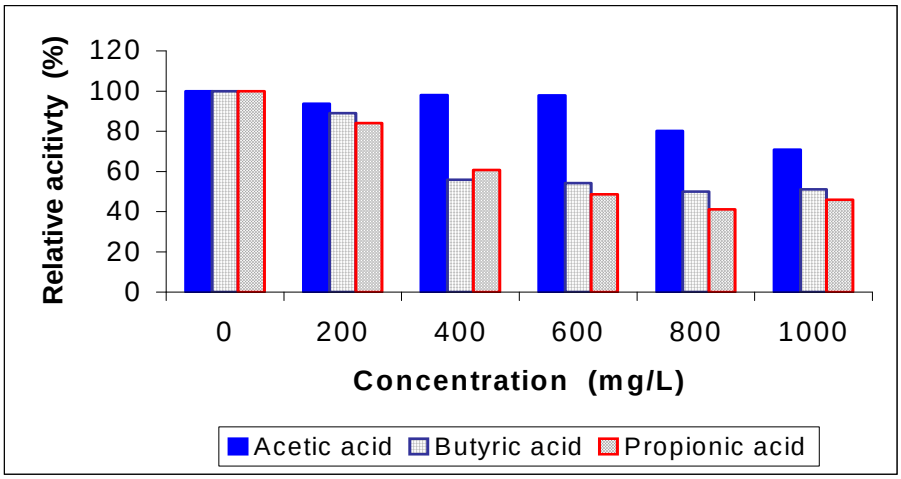



**Figure 3.10** Effect of volatile acids on the A) methanogenic and B) sulphidogenic protease activity at pH 10.

### 3.3.6 Effect of Metal Ions.

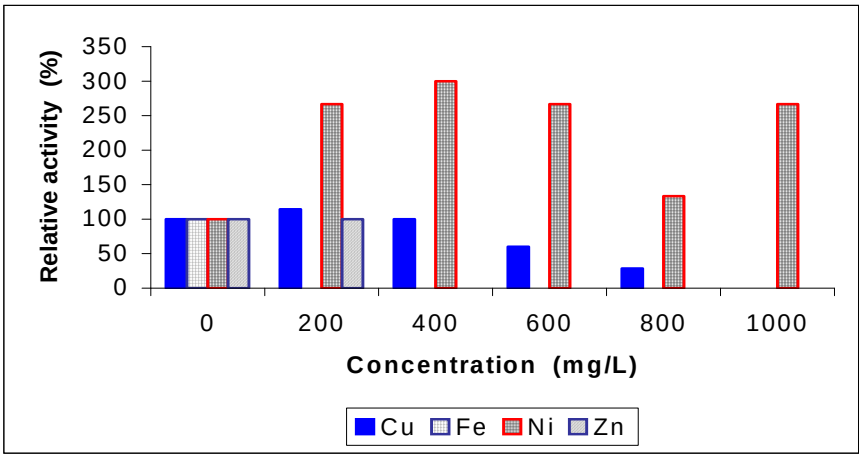
At pH 5 (Fig. 3.11), $Cu^{2+}$ has no effect on the methanogenic proteases up to 400mg/L, yet at

higher concentration of 1000 mg/L there is total inactivation. These enzymes were totally inhibited by $Fe^{3+}$ while $Zn^{2+}$ inhibited these enzymes from 400 mg/L. The sulphidogenic proteases were totally inhibited by $Cu^{2+}$ and $Zn^{2+}$ at as low concentration as 400mg/L, while $Fe^{3+}$ produces 75% inhibition at concentration of 200 mg/L. $Ni^{2+}$, on the other hand more than doubled the protease activity between a concentration of 200 and 1000 mg/L for the methanogenic sample and at 1000 mg/L for the sulphidogenic sample.

At pH 7 (Fig. 3.12), $Cu^{2+}$ has little or no effect on the sulphidogenic proteases while it caused an increase in the methanogenic protease activity. At 400 mg/L the sulphidogenic protease activity increased by two folds and at higher concentration this metal ion causes a decrease in activity. There was ~20% decrease in sulphidogenic protease activity and ~30% decrease in the methanogenic protease activity which was caused by the presence of $Zn^{2+}$. $Fe^{3+}$ at 400 mg/L caused a decrease of 45 % and 33.2 % in the methanogenic protease and sulphidogenic protease activity, respectively. On the other hand $Ni^{2+}$, activates methanogenic protease activity by 26.4% at concentration of 200mg/L, yet when its concentration is higher it caused a decrease in enzyme activity. In the sulphidogenic proteases, this metal causes a slight decrease of 10 % in their activity.

At pH 10 (Fig. 3.13), $Cu^{2+}$ activated proteases from the methanogenic reactor up to a concentration of 600 mg/L and at higher concentration there is a loss of 20% activity at 1000 mg/L. At low concentrations of this metal there was little effect on the sulphidogenic proteases. When its concentration reaches 1000 mg/L, however, protease activity decreased by 50%. $Zn^{2+}$ and $Fe^{3+}$ inactivated methanogenic and sulphidogenic protease, both metals causing a loss of activity of approximately 40% at a concentration of 1000 mg/L. There was a slight decrease in

A

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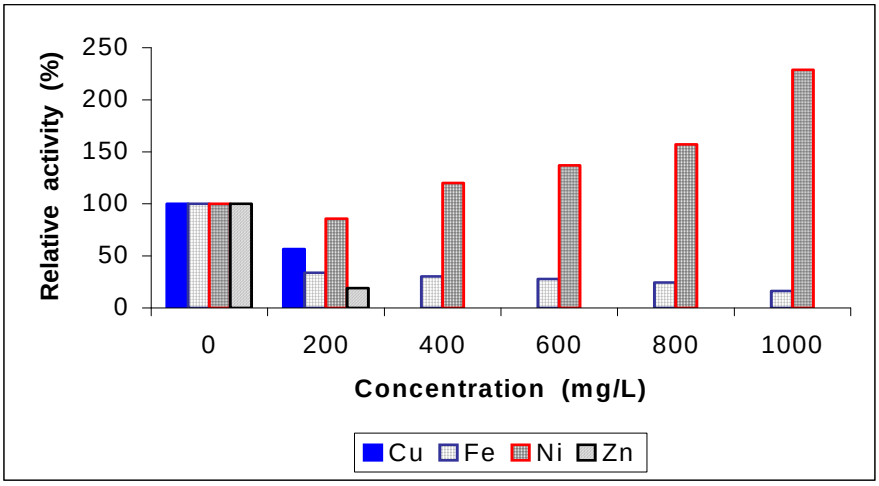
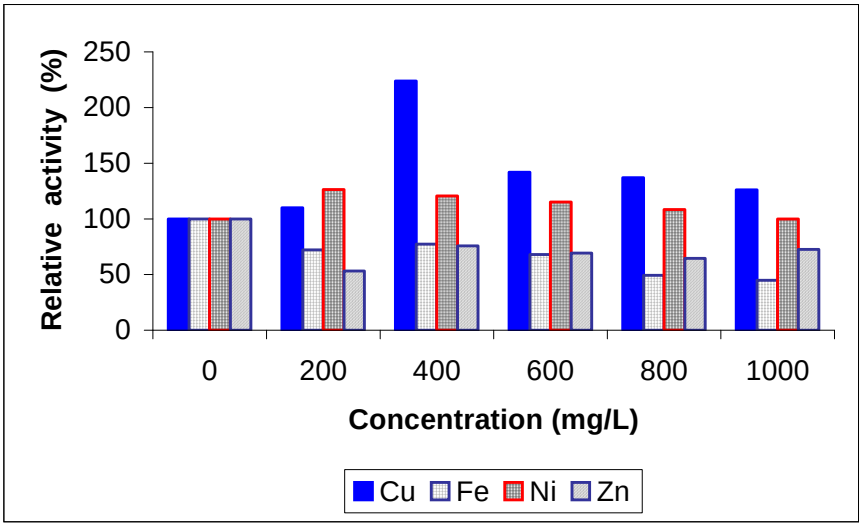



**Figure 3.11** Effect of metal ions on the A) methanogenic and B) sulphidogenic protease activity at pH 5.

A

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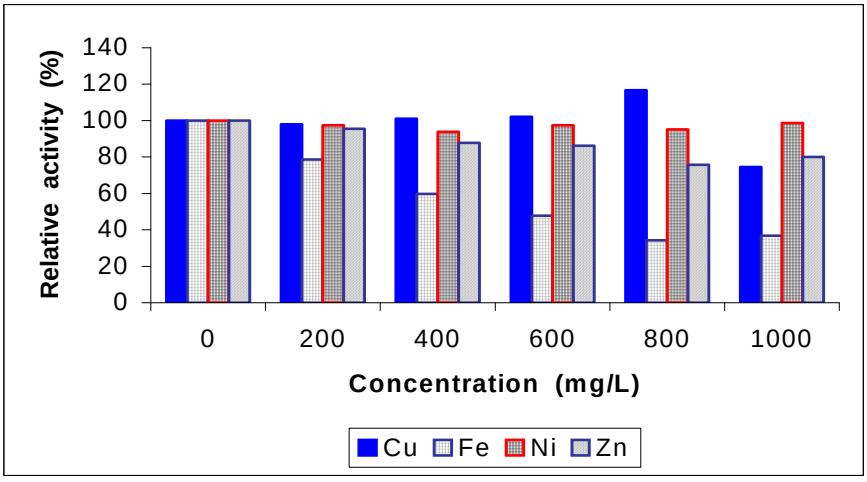


**Figure 3.12** Effect of metal ions on the A) methanogenic and B) sulphidogenic protease activity at pH 7.

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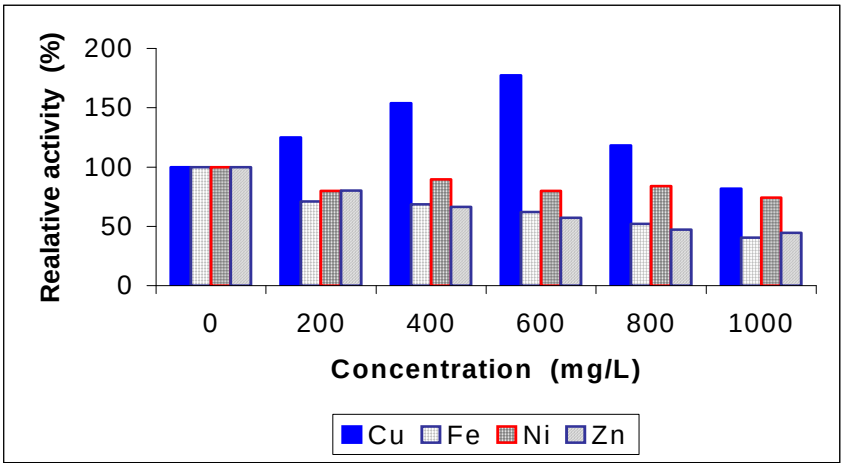


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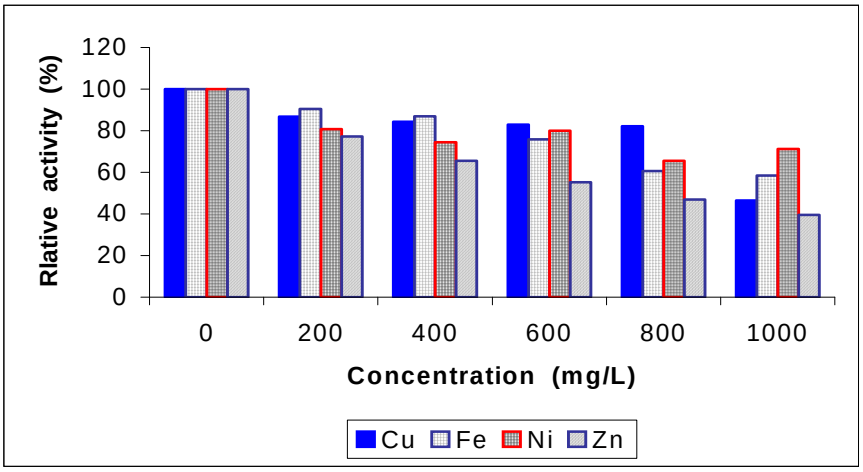



**Figure 3.13** Effect of metal ions on the A) methanogenic and B) sulphidogenic protease activity at pH 10.

protease activity when methanogenic and sulphidogenic proteases were exposed to $Ni^{2+}$,

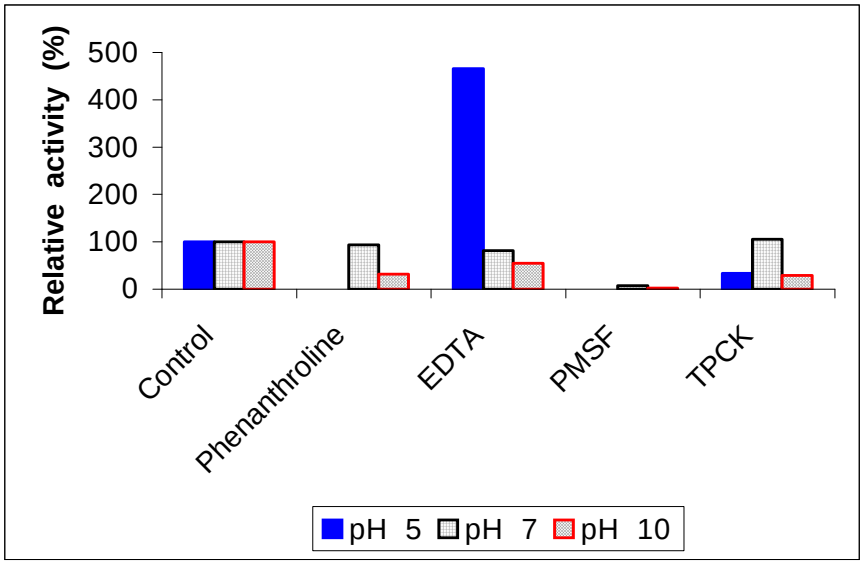
methanogenic proteases lost 20% of their activity while sulphidogenic proteases lost 30%.

### 3.3.7 Effect of known Protease Inhibitors

The metalloprotease inhibitor 1,10-phenanthroline and serine protease inhibitor PMSF caused a complete inhibition of methanogenic proteases at pH 5 while the other serine protease inhibitor TPCK caused a loss of 65.7% of activity. However, another metalloprotease inhibitor, EDTA appeared to activate protease activity by 366.7% (Fig 3.14 A). Methanogenic proteases at pH 7 were inhibited by PMSF causing an overall loss of 92.5% of enzyme activity, and yet only a slight inactivation by EDTA and 1,10-phenanthroline, with a loss of 18.7 and 6.7% activity, respectively. However, TPCK caused a slight (5%) increase in protease activity. These proteases at pH 10 were inhibited by PMSF, TPCK, 1,10-phenanthroline and EDTA leaving the protease activity of 2.1%, 29.0%, 32.2% and 54.9%, respectively (Fig 3.14 A).

Results in Fig. 3.14 B shows the effect of different protease inhibitors at different pHs on the sulphidogenic proteases. At pH 5, the metalloprotease inhibitor 1,10-phenanthroline caused a complete inhibition of these enzymes while EDTA caused a loss of their activity by 88.1%. There was a slight inactivation of protease activity by a serine protease inhibitor PMSF, causing a loss of about 30% of enzyme activity. With TPCK, however, there seems to be an activation of these enzymes by 57.5%. PMSF, 1,10-phenanthroline and EDTA inactivate these proteases at pH 7, causing a loss of activity by 60%, 45.8% and 39%, respectively; again TPCK seems to activate these enzymes by 21.2%. At pH 10, sulphidogenic proteases lost 94.9%, 79.5% and 85.4 % of activity due to the presence of TPCK, PMSF and 1,10-phenanthroline, respectively; EDTA caused a slight decrease in activity by 21.4%.

A

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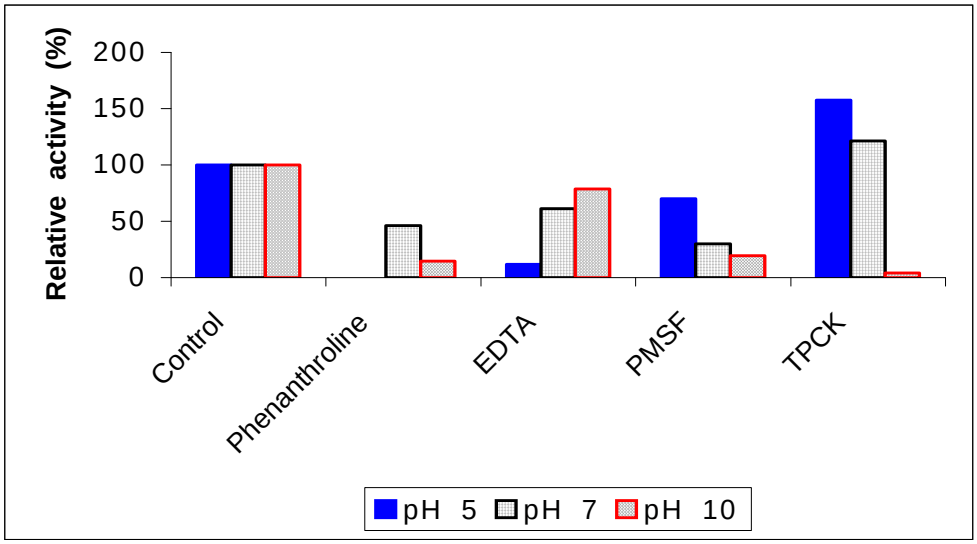



**Figure 3.14** Effect of protease inhibitors on the A) methanogenic and B) sulphidogenic protease.

## 3.4 DISCUSSION

Since it was realised that there was little or no extracellular protease activity from the methanogenic and sulphidogenic anaerobic digestion of sewage sludge attention was switched to a study and characterisation of proteases found attached or associated with the sewage sludge.

The majority of proteases from the sulphidogenic and methanogenic anaerobic bioreactor showed optimum activity at pH 10 (Fig. 3.2), which is higher than operational pH for anaerobic digestion and optimum temperature at 50$^{o}$C (Fig3.3), hinting that these enzymes are alkaline proteases. This result can be expected, since anaerobic treatment is characterised by high concentration of bacterial mass, which is present as a biofilms and/or aggregates. The suggestion that these enzyme are alkaline is in agreement with other findings that pH optima found for alkaline proteases occurs at pH 10-12 and temperature of 60-70$^{o}$C (Chu *et al.*, 1992). The other reason for having a high pH optimum could be due to the fact that these enzymes are associated with the organic particulate matter, which offers a protective film from changes of pH in the environment.

Anaerobic digesters, however, normally operate at neutral rather than alkaline conditions. Proteases are categorised as alkaline, neutral or acid proteases based on their mechanism and pH optimum for the activity and during anaerobic digestion these enzymes are active at pH between 5-7. In 1971,Markland and Smith showed that alkaline proteases were co-produced with neutral proteases in many *Bacillus* strains. The information from above led to the characterisation of enzymes that are active at neutral and acidic conditions. It was found that there are proteases that

are active at pH 5 and 7 with optimum temperatures of 30 and 60$^{o}$C, respectively (Fig. 3.3). The alkaline and neutral proteases could be thermophilic enzymes since their optimum pH was between 55 and 70$^{o}$C, while the acidic proteases seem to be mesophilic. It was suggested that the bacteria operating during thermophilic digestion are not different species from the ones that are active in a mesophilic digester and their reactions could be similar (Hobson and Wheatley, 1993). High temperature and pH offer an advantage, since it has been shown that the enzymes that have high pH and temperature optima can catalyse a reaction at low temperature and pH at their suboptimal activity (Manonmani and Joseph, 1993). From these findings, we can say that proteases at pH 10 are also involved during anaerobic digestion even though they are operating at their suboptimal activity. If the pH and temperature could be raised, high rates of protein degradation in the system could be achieved.

Methanogenic and sulphidogenic proteases at pH 5, 7 and 10 showed extensive stability (up to 5hrs) at their optimum temperature of 30, 60 and 50$^{o}$C, respectively. Puvanakrishnan and Bose, (1980) observed an increase in thermal stability of trypsin, which was immobilised on sand. This led to the suggestion that protease stability could be due to their multiattchment to the humus or soil particles that are found on the sewage sludge and which form approximately 10-20% part of the sludge (Riffaldi *et al.,* 1982). The other reason for high stability could be due to the position of the sulfide bonds of enzymes inside the humus or soil particle, which are broken down during protein denaturation.

It was found that sulphate inhibit methanogenic and sulphidogenic proteases at different pHs, while sulphite caused a slight increase in their activities, except with sulphidogenic proteases at pH 5, where there was a slight decrease (Fig 3.5-3.7). This can be expected, since sulphate and

sulphite have a similar function of diverting electrons from methanogenesis during methane production and they can be used by SRB as their electron acceptor (Frostell, 1985). However, sulphide ions were increasing the activity of the proteases from methanogenic and sulphidogenic bioreactors. If it were in the bioreactor, it could be said that the presence of these ions increases the alkalinity inside the reactor, leading to an increase in pH and higher activity of proteases. The pH inside the tube stayed the same throughout this study and it was suggested that this maybe due to the fact that sulphide has the ability to destroy the integrity of the floc (Whittington-Jones 1999) exposing more enzymes to the substrate leading to an increase in their activity.

With volatile acids, it was found that butyric acid and propionic acid totally inactivated proteases at pH 5, while acetic acid has little effect. These two volatile acids, and acetic acid seem to have little or no effect on proteases at higher pH, though there was a slight decrease in activity of pH 10 proteases with higher concentration of propionic acid (1000 mg/L) (Fig. 3.8-3.10). It should be noted, for obvious reasons that these acids would exits as their respective salts at pH 7 and/ or 10. When these acids are added into a solution they dissociate producing hydrogen ions as one of their products and the increase in hydrogen ion concentration can cause the pH to fall in a digester. The pH stayed relatively constant throughout the study, which could be due to neutralisation of volatile acids by the buffers used to resuspend the pellet. This could prove that a buildup in volatile acids would not be harmful to the bioreactor provided they are neutralised with alkalis that contain nontoxic cations. Since pH of the solution was not affected, it made us believe in what Schlenz (1947) and Buswell (1957) said “that the volatile acids are inhibitory molecules whether the pH is maintained or not”.

In this study, $Fe^{3+}$ decreases protease activity at pH 5, 7 and 10 suggesting that the proteases that

are present in both reactors contain a cysteine residue at the active site. At pH 7 and 10, $Zn^{2+}$ caused a decrease in protease activity, however, this metal inactivates methanogenic and sulphidogenic proteases at pH 5 completely. This result suggests that these enzymes may not be metalloproteases, since zinc has the ability to stabilise or activate them (Page and Williams, 1987). $Ni^{2+}$ increased protease activity at pH 5 and methanogenic protease activity at pH 7, while at the same time causing a slight decrease in methanogenic and sulphidogenic protease activity at pH 7 and pH 10 for sulphidogenic protease activity. $Cu^{2+}$caused a decrease in protease activity at pH 5, however, it has little or no effect on protease at higher values (7 and 10). This could be advantageous during anaerobic digestion, since copper has been identified as one of the most toxic metals in biological treatment (Lin, 1993). From these results we cannot really classify proteases, since their effect could be due to the solubility of metals (since most of heavy metals are soluble at acidic pH) and not the interaction between the heavy metals and the residues that are found on the active site of proteins. It was noted that at low pH most of the metals except nickel inhibited these enzymes, while at high pH they have only a slight effect (at high pH metals precipitates out of the solution). The inactivation of proteases by these metals at low pH could be due the ability of metal ions to bind with certain groups of protein molecules, especially the amino acid residues that are found at the active site of enzymes, which lead to enzyme inhibition. Sometimes it could be due to the fact that these metals are replacing natural metals that are found in the enzymes (for an example, copper can replace zinc in a metalloprotease, producing a less active enzyme) (Omil *et al.*, 1996).

At pH 5, methanogenic proteases are a mixture of metallo and serine proteases since they were completely inhibited by the serine protease inhibitor PMSF and TPCK, and metalloprotease inhibitor 1,10-phenanthroline. However, EDTA appears to activate these proteases at this pH.

This could be due to the fact that this inhibitor is a chelating agent that forms a complex with divalent ions on the floc surface resulting in the collapse of the floc infrastructure. The sulphidogenic reactor at this pH consisted of metalloproteases since both metalloprotease inhibitors EDTA and 1,10-phenanthroline had inhibited these enzymes.

A serine inhibitor PMSF inhibited methanogenic proteases at pH 7, while metalloprotease inhibitor EDTA and 1,10-phenanthroline caused a slight inactivation of these enzymes; TPCK activated them. PMSF, EDTA and 1,10-phenanthroline inhibited sulphidogenic proteases at this pH while TCPK activated them. These results suggest that proteases in both reactors at this pH are a mixture of metallo and serine proteases.

At pH 10 proteases from both reactors are a mixture of serine and metallo proteases. This is because a serine inhibitor PMSF and TPCK, and a metalloprotease inhibitor 1,10-phenthroline and EDTA have inhibited them. However, EDTA is a partial inhibitor in the sulphidogenic proteases. The author also suspects the presence of cysteine proteases, due to inhibition by a serine and cysteine inhibitor TPCK and $Fe^{2+}$. These molecules bind to the cysteine residue at the active site of cysteine proteases. From the inhibition studies we could say that serine and metallo protease are the dominant enzymes during anaerobic digestion.

# CHAPTER 4

# ENZYME LOCALISATION AND EXTRACTION

## 4.1 INTRODUCTION

As bacteria are a good source of enzymes, it would be better to consider a strain that may have high levels of a particular enzyme activity. This can either be done by conventional mutant selection or by more sophisticated cloning techniques where multiple copies of the gene encoding for the given enzyme are inserted into a specific host organism (bacterium). Fusing a gene to a strong promoter can enhance the expression of a gene, even though, this method may take time and require special expertise and handling (Scopes, 1982).

Enzymes can be dived into two classes: extracellular and intracellular, with the former tending to be more stable than the latter since they are used to the harsher outer environment to the cell. Intracellular enzymes can be found in one of three forms: free in the cytosol, fixed into multienzyme complexes or bound to membranes (Royer, 1982).

Membrane bound enzymes are found in or on the following membranes: plasma membrane, mitochondrium membranes, endoplasmic reticulum, chloroplast membrane, and erythrocyte membrane and bacterium cell wall. In many cases these enzymes are related metabolically. The intrinsic reason for enzymes to be bound to membranes is to reduce any lag period for the appearance of final products since there is a decreased diffusional path and metabolic control for channeling the metabolites from one set of fixed enzymes to another (Royer, 1982).

Multienzyme complexes can form soluble aggregates of two or more enzymes held together by numerous inter chain cross-links, ionic charges, hydrophobic interactions and covalent forces (Harris and Angal, 1994). There are two advantages for this kind of aggregation. First, the product of the enzyme that catalyses the first reaction of the pathway, does not have to go far in order to reach the enzymes that catalyses the second reaction. Secondly, one coenzyme molecule can be attached to the complex in such a way that it can conveniently transfer electrons or other chemical species from one enzyme to another. Both characteristics can be demonstrated in any artificially immobilised system (Royer, 1982).

In order to be able to isolate, purify and characterise intracellular proteins or enzymes, an efficient method of cell disruption, which is not harmful to the protein of interest, must be developed. There are standard procedures for the lysis of certain types of cells or tissues, but in some cases it is necessary to explore alternatives in order to get more enzyme into the solution (Bollag and Edelstein, 1991).

Solubilization of particulate-associated or membrane bound enzymes can be achieved by a number of different techniques. In most cases a detergent is required to release enzymes from membranes and they are divided into nonionic detergent such as Triton X-100 that tends to be milder than the ionic detergents like sodium dodecyl sulphate (SDS) (Royer, 1982). Detergents displace proteins, which are tightly bound to membrane by dissolving the membrane, and by replacing the membrane with aliphatic or aromatic chains which forms the lipophilic part of detergents. Once the protein is solubilised and its integrity confirmed, fractionation procedures could be employed, however, excess detergents can interfere with fractionation (Fig 4.1) (Scope,

1982).

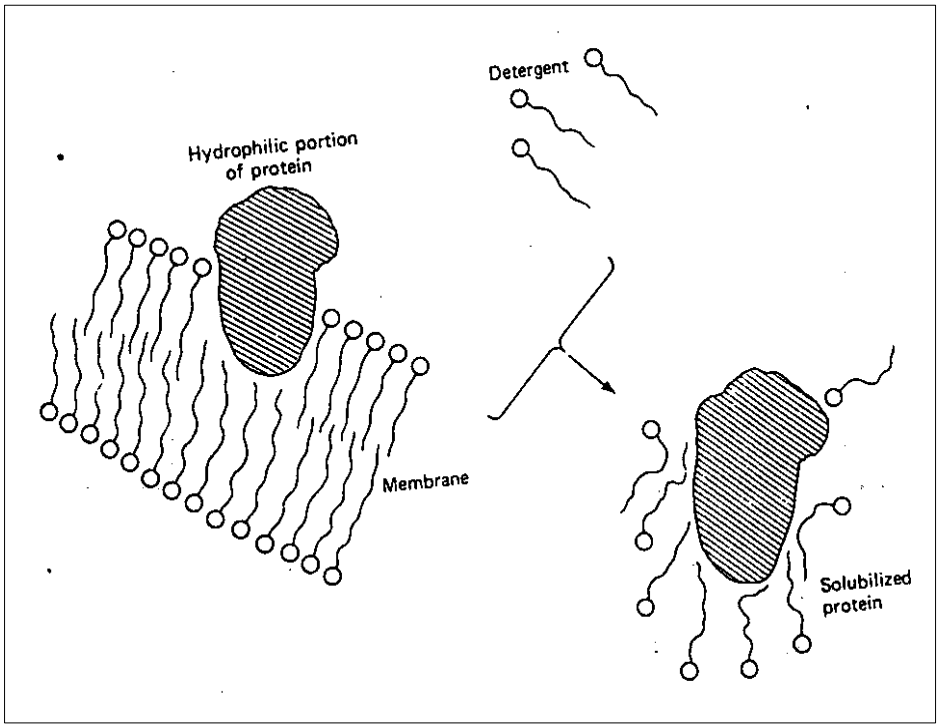



**Figure 4.1** Action of detergent in solubilising the membrane bound proteins (Scope,1982).

There are other methods that can be used to solubilise enzymes, including ultrasonication; the use of organic solvents like acetone, butanol and alcohol; pH (with or without addition of metal chelators); temperature; enzymes like proteases and lipases (Scopes, 1982) and high concentrations of urea (8 M) or guanidine (Royer, 1982).

During sonication, cells and tissues are disrupted by the creation of vibrations, which lead to mechanical shearing of the cell wall. Maximal shearing is needed to achieve maximal disruption, however, it should be below the level where there is formation of foam in the solution, since the solution will aerate, causing protein denaturation (Bollag and Edelstein, 1991). The main disadvantage of this method is generation of heat, free radicals and ions, which may cause denaturation, as the time of contact increases. At high cell concentration there is insufficient

mixing and disruption of particulate or cell membrane. To minimise protein denaturation cell paste should be kept on ice and sonication should be carried out in bursts of 30 sec or less. Successful disruption also depends on the correct choice of pH, temperature and ionic strength, and addition of surfactants could aid in protein release. This method, however, uses trial and error in order to find the best frequency and time for a particular cell disruption (Harris and Angal, 1994).

The use of organic solvents has been used since the early days of protein purification, even though, it was mostly used in the industrial scale. When organic solvents are added into the solution they lower the dielectric constant of the solution due to their solvating power (ability to reduce water activity). Thus the solubility of protein is decreased leading to the formation of protein aggregation through electrostatic attraction (Fig. 4.2).

It has been found that protein precipitation occurs at low concentration of organic solvent near its isoelectric point. The size of protein also has an influence during precipitation; large proteins will precipitate at lower concentrations of organic solvent than smaller proteins (Scopes, 1989). To minimise protein denaturation, precipitation should be carried out at $0^{o}C$ or below since high temperatures changes protein conformation enabling the molecules of solvent to gain access to the interior of the protein where they can disrupt the hydrophobic interactions. There are many other sophisticated methods that can be used for enzyme extraction but they fall outside the scope of this presentation.

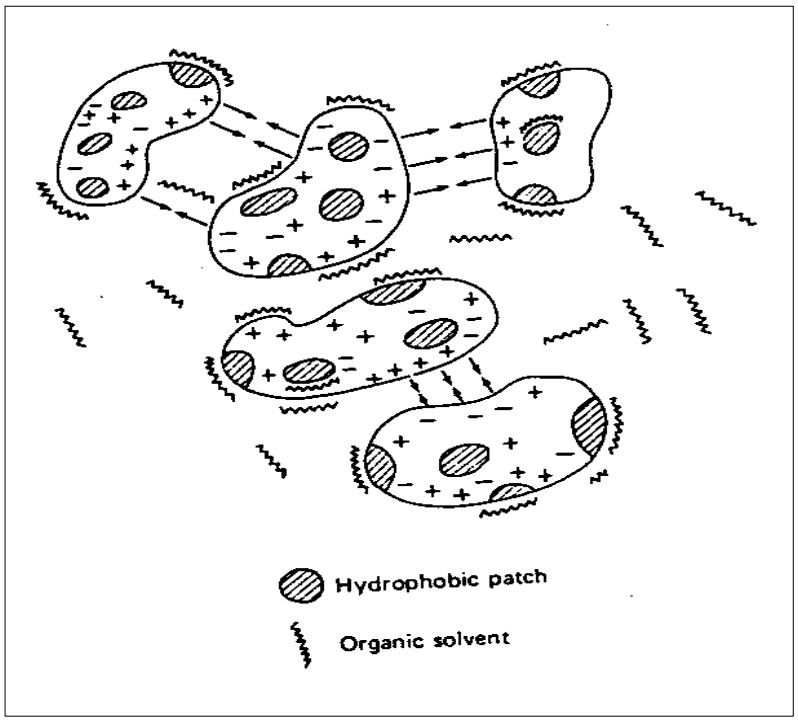



**Figure 4.2** Aggregation of proteins by interactions in an aqueous-organic solvent mixture (Scopes, 1982)

## 4.2 MATERIALS AND METHODS

### 4.2.1 Materials

TCA, Triton X-100, n-Butanol and other biochemicals were purchased from Sigma. Centrifugation was performed with Beckman J2-21 centrifuge and sonication with a Virtis Virsonic 100. Hotplate magnetic stirrer from Snijders was used to stir samples.

### 4.2.2 Sample Preparation and Fractionation

To localise the position of the enzymes, the sludge samples were divided into three fractions: 1) Raw sludge as is, 2) centrifuged sludge pellet and 3) supernatant after centrifugation. The sludge samples were collected from a continuous methanogenic and sulphidogenic anaerobic digester (Fig 3.1, page 51). Fraction 2 and 3 were obtained by centrifugation (10 000 x g, 10min), F2 referred to the resuspended pellets and F3 to the supernatant. Protease activity was assayed using casein as a substrate (section 2.2.5, page 36)

### 4.2.3 Extraction of Enzyme from the Floc Matrix of Sewage Sludge

#### 4.2.3.1 Detergent

The sludge samples were centrifuged (10 000 x g, 10min). The supernatant was discarded due to low protease activity (Fig 2.5, page 40). The sludge was resuspended in sodium phosphate buffer (0.1M, pH 7), containing Triton X-100 to have a final concentration of 0.1%. The mixture was vortexed, incubated (60 min) and centrifuged (10 000 x g, 10min). The pellets were resuspended as before and protease activity was measured in the supernatant and resuspended pellets using the standard method described earlier in section 2.2.5 (page 36).

#### 4.2.3.2 Organic Solvent

The sludge samples collected from continuous methanogenic and sulphidogenic reactors were centrifuged (10 000 x g, 10min) and the pellets were resuspended in sodium phosphate buffer (0.1 M, pH 7). n-Butanol (20% v/v) was added to the resuspended pellet solution, the mixture was stirred (30 min) and then centrifuged (10 000 x g, 10 min). The excess butanol was removed by suction and a clear solution containing proteins was filtered to obtain a clear extract. The protease activity was measured on this extract and on the residual material sediments that were

obtained after centrifugation (section 2.2.5, page 36).

#### 4.2.3.3 Sonication

Sludge samples collected from continuous methanogenic and sulphidogenic reactors were centrifuged (10 000 x g, 10min) and pellets resuspended in sodium phosphate buffer (0.1 M, pH 7) (2 volumes). The samples were sonicated (0-10min, 30 sec bursts) to optimise sonication time. The resulting sonicate was centrifuged (10 000 x g, 10 min) and protease activity was determined in supernatant and pellet using the procedure that was described in section 2.2.5.

## 4.3 RESULTS

### 4.3.1 Localisation

The results of the enzyme localisation are presented in Fig. 4.3. The crude showed the activity of the enzymes that was found inside the reactor, the resuspended pellet shows the activity of the enzyme entrapped or immobilised in the floc matrix (F2), while supernatant shows the activity of the extracellular enzymes (F3). It was found that methanogenic proteases have activities of 0.079, 0.366 and 0.033 :mol/min/ml and sulphidogenic proteases have activities of 0.246, 0.396 and 0.234 :mol/min/ml for crude, resuspended pellet and supernatant, respectively. From these results, it was found that there was high protease activity associated with the pellets with 0.366 :mol/min/ml for methanogenic and 0.396 :mol/min/ml for sulphidogenic enzyme, while the supernatant of methanogenic sample contained low activity of proteases. The supernatant of sulphidogenic samples, however, have high activity of 0.246 :mol/min/ml compared to 0.033 :mol/min/ml of methanogenic supernatant.

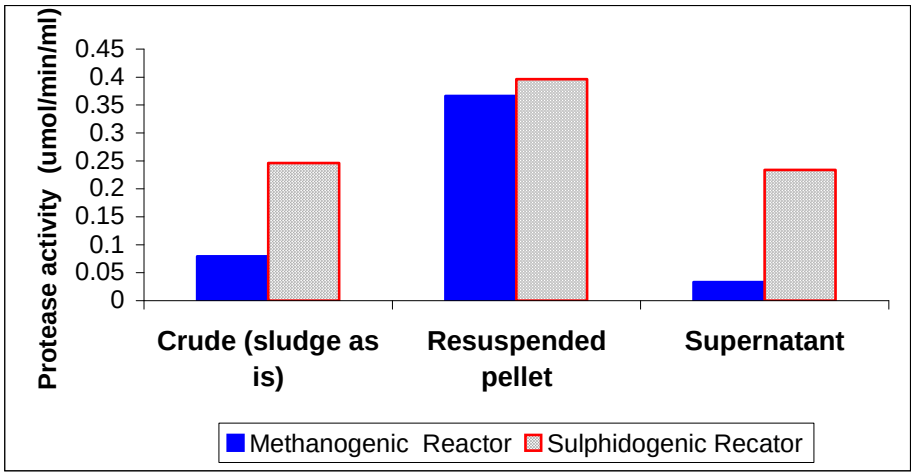



**Figure 4.3** Enzyme activity associated with different fractions in the activated sludge.

### 4.3.2 Detergent

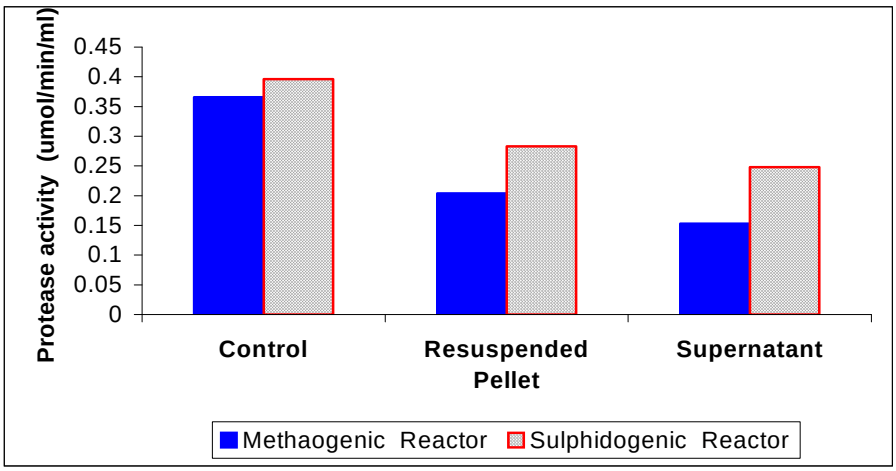



**Figure 4.4** Protease activity that was found when methanogenic and sulphidogenic proteases were extracted with the use of Triton X-100.

### 4.3.3 Solvent

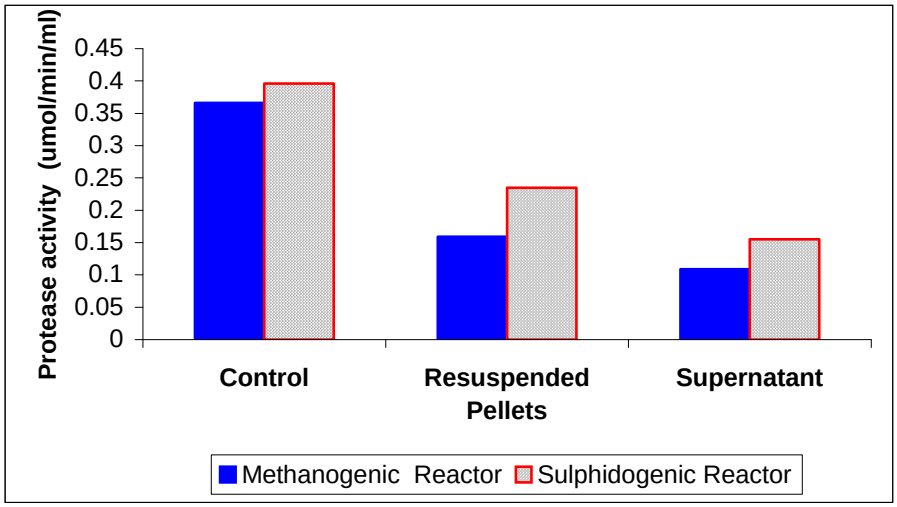


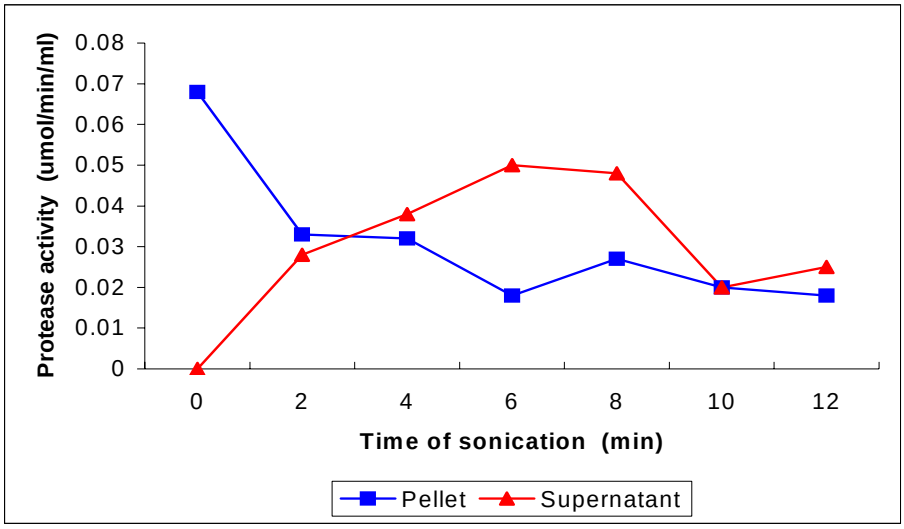

**Figure 4.5** Extraction of proteases from the anaerobic reactors using n-butanol as an organic solvent.

The other method that has been investigated to extract proteases from the matrix of sludge in this study was with the use of n-butanol as organic solvent. This method has managed to release some of the enzyme to the solution, however, there was higher enzyme activity associated with the resuspended pellet (Fig. 4.5). In the methanogenic samples, 0.11 **:** mol/min/ml of enzyme activity have been released into the solution leaving 0.16 **:** mol/min/ml in the pellet and in the sulphidogenic samples 0.16 **:** mol/min/ml activity was found in the solution while 0.24 left in the resuspended pellet after the treatment. Comparing the activity that has been determined in the pellet and supernatant with the pellet before treatment (control), some of the activities were lost during this process of enzyme extraction. Methanogenic samples had an activity of 0.366 **:** mol/min/ml while sulphidogenic samples had an activity of 0.396 **:** mol/min/ml before the start in using this technique.

### 4.3.4 Sonication

The two methods that have been studied so far have the ability to release proteases into the solution, even though there was still considerable protease activity in the resuspended pellet after the treatment. It was decided to investigate the effect of sonication on release of enzyme from the immobilised floc into the supernatant. At zero time the supernatant of both samples contained zero activity while methanogenic pellet samples contained 0.068 :mol/min/ml and sulphidogenic pellet samples have 0.065 :mol/min/ml. After 6min of sonication the methanogenic sample has released a maximum activity of 0.05 :mol/min/ml leaving 0.018:mol/min/ml in the pellet, while sulphidogenic proteases released enzyme activity up to 0.112 :mol/min/ml into the solution after 8min, leaving 0.039 :mol/min/ml in the pellet (Fig. 4.6).

A

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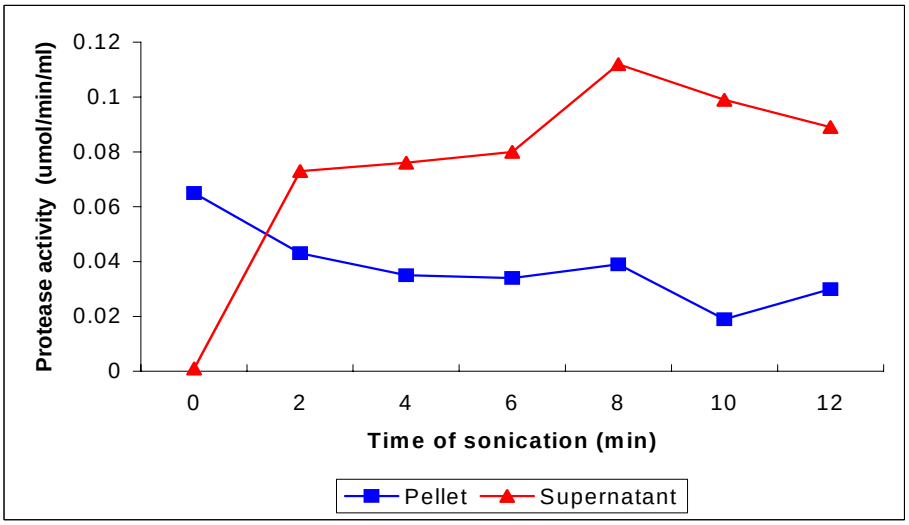



**Figure 4.6** Protease activity which was determined after sonication. A) methanogenic and B) sulphidogenic samples.

## 4.4 DISCUSSION

The observed small protease activity in the cell free fraction as compared to the fraction within the cell, indicate the major activity of proteases is associated with cells or lies within the extra cellular polymers of the floc. The lack of extracellular activity was not due to enzyme deactivation, but reflected the extremely low concentration of soluble protein present in the sludge supernatant (Boczar *et al.*, 1992).

Frrlund *et al.*, (1995) have indicated that extra cellular polymers could indeed hold large pools of extracellular enzymes. It is necessary for these enzymes to be extracted from their original source into the free solution, so that purification of these enzymes could be achieved. In this study three methods have been investigated:- the use of detergent, organic solvent and sonication.

All of these methods were able to release proteases into the solution. Butanol, however, denatured some of the enzymes during the process, in both methanogenic and sulphidogenic samples. It has been stated that alcohols with long chains such as butanol can cause a higher degree of denaturation than ethanol. However, acetone is preferred since lower concentrations are required and therefore less denaturation (Harris and Angal, 1994). The reason why n-butanol was chosen and used as organic solvent was due to its ability to precipitate proteins that are bound to lipids, which are found on the cell membrane.

With the use of Triton X-100, enzymes were partially extracted into the solution. With the methanogenic samples, high protease activity remained in the pellet after the treatment, while with the sulphidogenic samples only 50% activity remained. This detergent has been chosen because it is non-ionic and is mild in its action, and most proteins can tolerate high levels of

Triton (1-3% w/v). The use of this detergent, however, will be a problem to the enzymes that are precipitated with ammonium sulphate since Triton X-100 forms a floating layer which may frequently contain the enzyme of interest (Scopes, 1982). Due to the fact that Triton X-100 can interfere with salting out with ammonium sulphate, sonication has been adopted as a method of enzyme extraction from the floc matrix. The sonicated sample was then subjected to ammonium sulphate precipitation, ion exchange chromatography or gel filtration. This method released most of the enzyme (50%) into the solution due to disruption of flocular matrices, cell-cell interactions and cellular membrane (King and Forster, 1990).

# CHAPTER 5

# PARTIAL PURIFICATION OF PROTEASES ASSOCIATED WITH THE FLOC OF SEWAGE SLUDGE

## 5.1 INTRODUCTION

Purification of proteins is one of the most common procedures in biochemistry and it is usually the first step in the study of the physical and biological properties of proteins. To obtain a pure protein, the protein of interest should be removed from any biological matrix and contaminating proteins by an appropriate fractionation procedure. The protein purification depends on three factors, namely: quality, quantity and cost (Wilson and Walker, 1994).

Proteins differ in their sensitivity to denaturation, elevated temperatures, pH, the presence of detergents, organic solvents, salts and inhibitors during extraction and purification process. These differences are commonly used in the purification of a protein of interest and these techniques should feature during the early stages of purification. Addition of thiol-containing compounds such as mercaptoethanol, glutathione and dithiothreitol to crude enzyme preparations may prevent oxidation of sulphydryl groups, which can proceed rapidly after cell disruption (Wilson and Walker, 1994).

It should immediately be noted that, for this current investigation, certain problems and difficulties maybe encountered in attempting to purify the proteases of interest that are associated with the sewage sludge in the presence of the other ubiquitous superfluous proteases.

### 5.1.1 Protein Purification Methods

#### 5.1.1.1 Salt Fractionation

Salt precipitation is the most commonly used method for protein fractionation and it involves dissolving of the salt, into the solution that contains proteins. Addition of these salts can stabilize proteins against denaturation, proteolysis or bacterial contamination. This process depends on the hydrophobic nature of the surface of proteins and hydrophobic patches that predominate in the interior of protein (Fig. 5.1) (Harris and Angal, 1994).

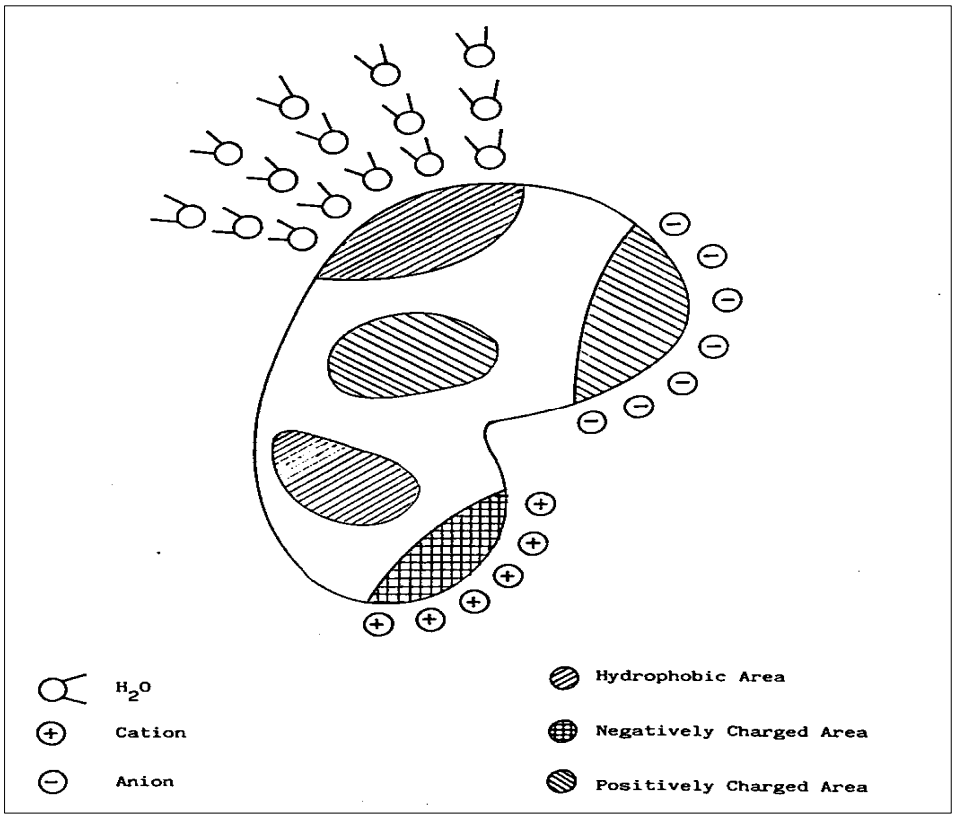



**Figure 5.1** Schematic representation of a protein showing negatively and positively charged areas on the protein interacting with ions in the solution. The hydrophobic areas on the protein interact with water molecules causing an ordered matrix of water molecules to form over these areas (Harris and Angal, 1994).

When salts are added into the system, usually at 4$^{o}$C water solvates the salt ions. As the salt

concentration increases, water is removed from around the protein, exposing the hydrophobic patches. Hydrophobic patches, which are found in one protein, interact with other patches from other proteins, resulting in aggregation. Proteins with many or large, hydrophobic patches aggregate and precipitate faster than the one with fewer, or smaller, patches and the process is referred to as salting out. The aggregates that are formed consist of several proteins and the nature of the extract will affect the concentration of salt required to precipitate the protein of interest.

There are several factors such as the nature, effectiveness and physical characteristics of salt that should be considered during the "salting out" process. The effectiveness of the salt is determined by the nature of the anion, and the most effective salts are those with multiple-charged ions such as sulphate, phosphate, acetate, and chloride. Phosphate appears to be more effective than sulphate, yet in practice, phosphate consists of $HPO_4^{2-}$ and $H_2PO_4^-$ ions at neutral pH, rather than a more effective $PO_4^{3-}$. (Scopes, 1982).

Ammonium sulphate is the most commonly used salt, due to cost, its high solubility and it can be obtained in a high degree of purity (Wilson and Goulding, 1989). Another advantage of ammonium sulphate fractionation is the stability of the sample.

#### 5.1.1.2 Column Chromatography

A chromatographic technique that is one of the most convenient methods for achieving separation of biological compounds is by partitioning them between two different media. One medium is a stationary phase, which maybe solid, gel/liquid or a solid/liquid mixture while the other one is a mobile phase, which may be a liquid or a gas. The mobile phase flows over or

through the stationary phase and the choice of these media is made in such a way that the compounds, which are going to be separated, should have different distribution coefficients (Wilson and Goulding, 1989).

There are three main principle types of chromatography: adsorption, partition and gel permeation. Other types of chromatography such as ion exchange, paper, thin layer, gas liquid, high performance liquid, affinity and gel filtration or exclusion are special adaptations of one or more of the three main types (Robyt and White, 1987).

#### 5.1.1.2.1 Ion Exchange Chromatography

Separation of proteins using ion exchange chromatography continues to be an important and popular technique for purifying proteins. The principle feature underlying this form of chromatography is the attraction between oppositely charged particles. Proteins carry both negative charge due to the presence of aspartic and glutamic acids, C-terminal carboxyl groups, and to a lesser extent, cysteine residues, and positive charge contributed by histidine, lysine, arginine and, to a lesser extent by N-terminal amines on their surface (Harris and Angal, 1994). The net charge of proteins depends on the $pK_a$ values of their amino acids and the pH of the solution.

Ion-exchange chromatography is mainly carried out in a packed column filled with an ion-exchange matrix of polystyrene, cellulose or agarose. There are two types of ion-exchanger available, those with chemically bound negative charges are called cation exchangers and those with positive charges are called anion exchangers. Proteins bind to the exchangers by electrostatic forces between the protein's surface charges and the dense clusters of charged

groups on the exchangers (Robyt and White, 1987). The choice of ion-exchanger depends on the stability of the sample components, their relative molecular mass and the specific requirements of separation.

The binding of protein to the exchangers is reversible, so that these molecules can be eluted from the column. Proteins that are bound on the exchangers can be eluted from the column by changing the pH or ionic strength, which is achieved by 0.1 to 1M concentrations of salts (Scopes, 1982)

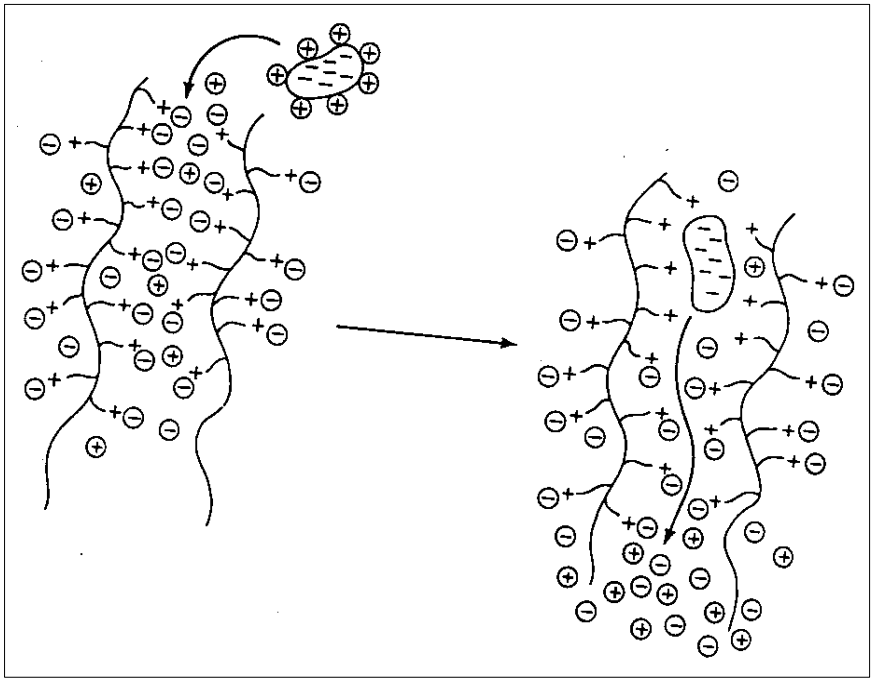


**Figure 5.2** A simple diagram illustrating the principle of ion exchange chromatography to separate different charged molecules (Scopes, 1982).

**5.1.1.2.2 Gel Filtration Chromatography**

Gel filtration has several other names including gel permeation and molecular sieving, however, the term gel filtration is the most used. The material that is used during this method is not necessarily a true gel and the procedure is not really filtration. Separation of molecules by this procedure is based on their molecular size and shape (Scopes, 1982).

The main principle of this method is that the molecules are partitioned between the solvent and a stationary phase of defined porosity. Gel particles, which are the stationary phase, are packed into a column that is equilibrated with a suitable mobile phase for the molecules to be separated. When the sample is applied onto the column, the larger molecules never enter the gel pores and they pass through the interstitial spaces and appear first in the eluate. Smaller molecules can enter the gel pores and so they will be distributed between the mobile phase outside and inside the molecular sieve. They will then pass through the column at a slower rate (Wilson and Walker, 1994).

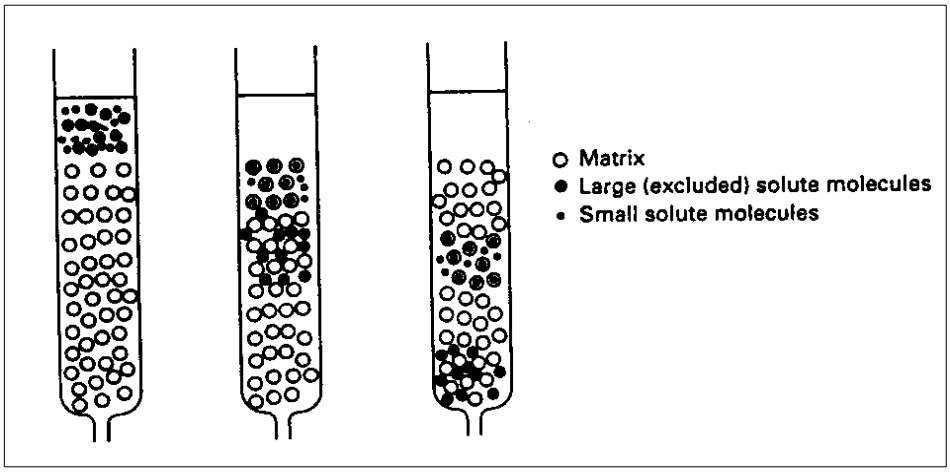



**Figure 5.3** A simple diagram illustrating the principle of exclusion chromatography to separate different sized molecules in a column (Wilson and Walker, 1994).

In a column of gel filtration medium, the behavior of a particular size molecule can be related to the total column volume $V_t$ and the void (outside the bead) volume $V_o$ by expression: $K_{av} = (V_e -$

$V_o$) / ($V_t$ – $V_o$), where $V_e$ is the elution volume of the molecule being eluted and $K_{av}$ is a coefficient, which defines the proportion of pores occupied by that molecule. Alternatively, the elution volume can be related to the elution volumes of a number of other molecules of known size, from which the molecular size of the unknown can be estimated by simple extrapolation.

#### 5.1.1.3 Gel Electrophoresis

Electrophoresis describes the migration of charged particles under the presence of an electric field and it is an ideal technique to resolve and separate the individual components of protein mixtures. This process is carried out in a support medium to counteract the effects of convection and diffusion that occur during electrophoresis, and also to facilitate the immobilisation of the separated proteins. A variety of matrices such as starch, agarose and cellulose acetate can be used for electrophoresis, however, the high resolution capacity of polyacrylamide gel electrophoresis (PAGE) makes this method the most widely used (Wilson and Walker, 1994). This method can be done under different conditions. Electrophoresis that is carried out under native conditions is often used to analyse soluble proteins with the advantage of retention of their biological and enzymatic activity. The second method is PAGE which combines with the presence of an anionic detergent, sodium dodecyl sulphate (SDS). This process is more vigorous and takes place under denaturation conditions. Here proteins are characterised in terms of the molecular size of their constituent polypeptides and can be used for monitoring protein purification (Harris and Angal, 1994).

## 5.2 MATERIALS AND METHODS

### 5.2.1 Materials

Ammonium sulphate, BSA, Coomasie Brilliant Blue R-250, DEAE Cellulose, Dialysis tubing and Low Molecular Marker and other common biochemicals were obtained from Sigma. Bio-Gel A-0.5m, Mini-PROTEAN 3 Cell, Model 2110 Fraction Collector and Power Pac 300 were obtained from Bio Rad. Casein and Polyethyleneglycol (PEG) 20 000 were purchased from Merck and spectroscopy was performed with U-V160A Shimadzu spectrophotometer.

### 5.2.2 Enzyme Extraction

The samples of sewage sludge were collected from the sulphidogenic and methanogenic anaerobic digester (Fig 3.1, page 51). All the subsequent steps were performed at 4$^{o}$C unless otherwise stated. These samples were centrifuged (10 000 x g, 10 min), the supernatant discarded and pellets resuspended in sodium phosphate buffer (0.1 M, pH 7). The suspensions were sonicated (8 W, 1.2 and 1.6 min/ml methanogenic and sulphidogenic samples, respectively, 30 sec bursts), centrifuged (10 000 x g, 10 min) and the supernatants were analysed for protease activity (section 2.2.5, page 36) and subjected to sodium dodecyl sulphate- polyacrylamide gel electrophoresis (SDS-PAGE) (10%) [Diagram 5.1].

In order to verify if proteolytic digestion is taking place after sonication, the pellets were resuspended in sodium phosphate buffer (0.1 M, pH 7) with a serine inhibitor PMSF in a final concentration of 1mM.

The sewage sludge collected from the sulphidogenic and methanogenic bioreactors were centrifuged (10 000 x g, 10 min), pellets resuspended in sodium phosphate buffer (0.1 M, pH7) were sonicated (1.2 min/ml for the methanogenic sample and 1.6 min/ml for sulphidogenic sample) and centrifuged (10 000 x g, 10 min.

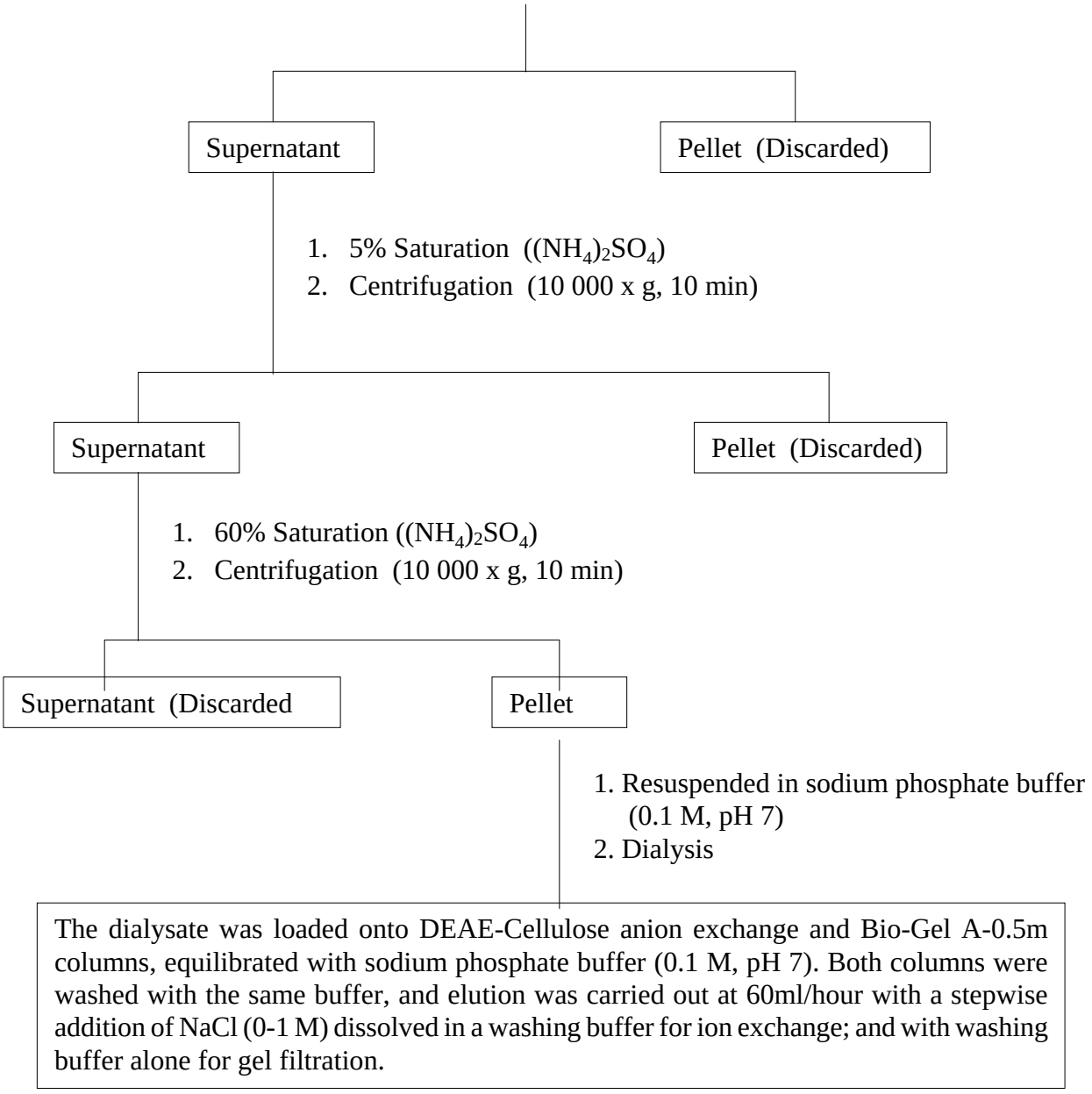



**Diagram 5.1** A flow chart showing purification of proteases from the methanogenic and sulphidogenic bioreactors

### 5.2.3 Ammonium Sulphate Precipitation

Solid ammonium sulphate was added slowly with continuous agitation over a period of 30 min to the supernatant from section 5.2.2 to give 5% saturation. The mixture was stirred (30min), centrifuged (10 000 x g, 30 min) and solid ammonium sulphate was added to the supernatant to

give 60% saturation. The suspension was centrifuged (10, 000 x g, 30 min) and precipitated proteins were dissolved in sodium phosphate buffer (0.1 M, pH 7). Redissolved proteins were dialysed against 3 x the dissolving buffer and the dialysate was analysed for protease activity (section 2.2.5, page 36) and protein concentration (Appendix I and II)

### 5.2.4 DEAE-Cellulose Ion Exchange Chromatography

The dialysate from section 5.2.3 was centrifuged (10 000 x g, 10 min) and the sample (4ml) was added to a column of DEAE-Cellulose (80 ml) equilibrated with sodium phosphate buffer (0.1 M, pH 7). The column was washed with the same buffer until the absorbance at 280 nm ($A_{280nm}$) of eluate had reached base line. The samples were eluted from the column with a stepwise addition of salt (0-1 M NaCl) dissolved in sodium phosphate buffer (0.1 M, pH 7) and 8 ml fractions collected, each being monitored for $A_{280nm}$ and potential proteolytic activity (section 2.2.5, page 36). The fractions from different peaks were collected and analysed for protein concentration, proteolytic activity and subjected to SDS-PAGE (10%). These samples were then placed in a membrane tubing and were concentrated using polyethylenegylcol (PEG) 20 000.

### 5.2.5 Gel Filtration with Bio-Gel A-0.5m

Another sample from the dialysate (5ml, section 5.2.3) was loaded on to a Bio-Gel A 0.5m column (80 ml) which was equilibrated with sodium phosphate buffer (0.1M, pH 7). This column was washed with the same buffer until $A_{280nm}$ of eluate had reached baseline. The samples

were eluted from the column with sodium phosphate buffer (0.1 M, pH 7) and 8 ml fractions collected, monitored for $A_{280nm}$ and potential proteolytic activity (section 2.2.5, page 36). The fractions from different peaks were collected, analysed for protein concentration, proteolytic activity and subjected to electrophoresis on SDS-PAGE (10%). These samples were then placed in membrane tubing and concentrated with PEG 20 000.

### 5.2.6 SDS-PAGE

The molecular weight of purified proteases was determined by 10% SDS-PAGE according to the method of Laemmli (1970) with a wide range of molecular weight markers and 5 μg BSA (66 200 Da). The gels were stained with coomassie brilliant blue R-250 in methanol-acetic acid-water and decolourised in methanol-acetic acid-water (Appendix II). Then, from a graphical plot of log molecular weights versus migrated distance, the molecular weight of purified protease can be correlated (Appendix II, Fig. 5A).

## 5.3 RESULTS

### 5.3.1 Enzyme Extraction

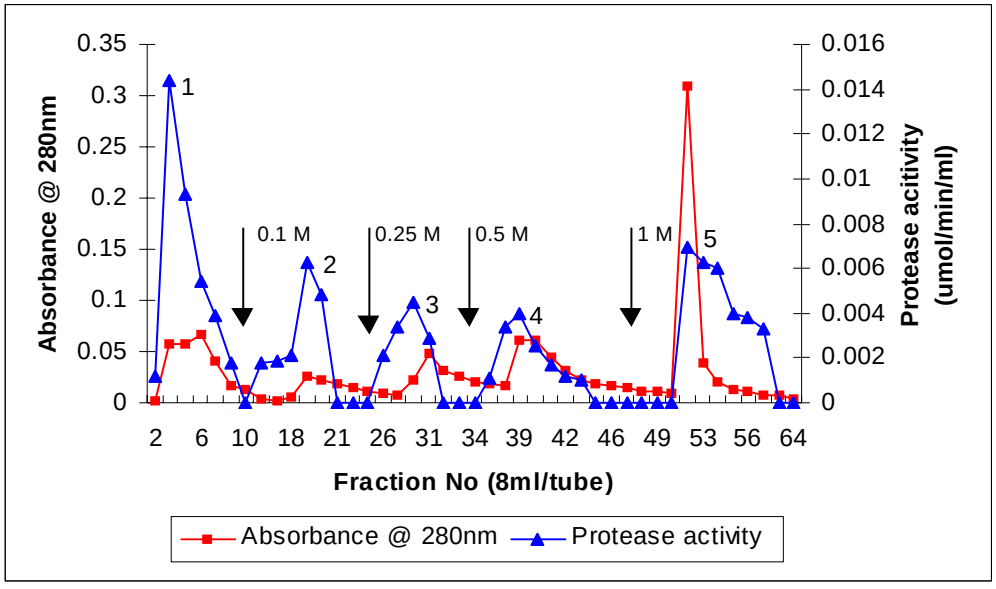
The method used for enzyme extraction was sonication releasing 50% of protease into the supernatant. Specific activity of the methanogenic samples that were purified further by ion exchange chromatography stayed the same as the one that was found for the pellets, 0.01 U/mg protein for methanogenic while their purification fold decreased from 1 to 0.92. The specific activity of sulphidogenic sample increased from 0.01 to 0.02 U/mg protein and the purification fold increased from 1 to 1.3. The protein content of both samples, however, decreased from 860.1 to 599.14 mg for methanogenic sample and for sulphidogenic sample it decreased from 432.94 to 217.79 mg (Table 5.1). However, for the methanogenic samples that were purified further by gel

filtration their specific activity and purification fold is similar to the one that were purified by ion exchange chromatography. Specific activity for the sulphidogenic sample increased from 0.01 to 0.03 U/mg protein and the purification fold increased from 1 to 2.35 (Table 5.2).

### 5.3.2 Ammonium Sulphate Precipitation

When the samples that were obtained from sonication (without PMSF) were subjected to ammonium sulphate their specific activity increased. For the samples that were purified further by ion exchange chromatography, specific activity increased from 0.01 to 0.02 U/mg protein for methanogenic sample, and from 0.02 to 0.04 U/mg protein for the sulphidogenic sample. For the sample that had undergone gel filtration, the specific activity for methanogenic sample is similar as above while for the sulphidogenic sample increased from 0.03 to 0.04 U/mg protein. Their purification factor increased from 0.922 to 3.22 for the methanogenic sample that was purified further by ion exchange chromatography and gel filtration. For the sulphidogenic sample the fold of purification increased from 1.3 to 3.25, however, for the ones that were purified by gel filtration, the purification fold did not change significantly (Table 5.1 and 5.2)

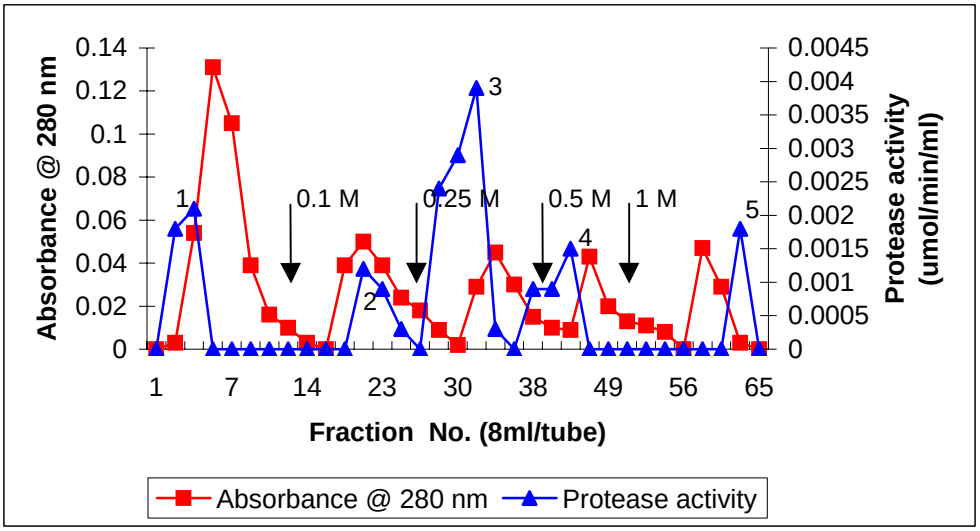
A



B

**Figure 5.4** DEAE-Cellulose ion exchange chromatography of A) methanogenic and B) sulphidogenic proteases. Column dimension: 1.5cm x 17cm. Flow rate: 60ml/hr. The proteases were eluted with stepwise addition of 0-1 M NaCl in sodium phosphate buffer (0.1 M, pH 7). ⌋ Indicate concentration of added NaCl and numbers refer to peaks that were pooled.

**Table 5.1** Purification table of the A) methanogenic and B) sulphidogenic proteases that were purified using DEAE-cellulose ion exchange chromatography




| Purification Step | Volume (ml) | Total protein (mg) | Total activity (U) | Specific activity (U/mg) | Yield (%) | Purification (fold) |
|---|---|---|---|---|---|---|
| **A. Methanogenic Sample** | | | | | | |
| Pellet | 94 | 860.1 | 4.61 | 0.01 | 100 | 1 |
| Sonication supernatant | 116 | 599.14 | 2.96 | 0.01 | 64.2 | 0.92 |
| 5-60% Ammonium sulphate | 45 | 34.79 | 0.55 | 0.02 | 11.9 | 3.22 |
| DEAE-cellulose | | | | | | |
| 1 0 M NaCl | 48 | 3.072 | 1.73 | 0.56 | 37.5 | 56 |
| 2 0.1 M NaCl | 40 | 0.80 | 0.64 | 0.80 | 13.9 | 80 |

| | | | | | | |
|---|---|---|---|---|---|---|
| 3 0.25 M NaCl | 32 | 0.58 | 0.42 | 0.72 | 9 | 72 |
| 4 0.5 M NaCl | 48 | 0.43 | 1 | 2.33 | 21.7 | 233 |
| 5 1 M NaCl | 48 | 1.25 | 1.44 | 1.15 | 31.3 | 115 |
| **B. Sulphidogenic sample** | | | | | | |
| Pellet | 30 | 432.94 | 4.92 | 0.01 | 100 | 1 |
| Sonication | 47 | 217.79 | 3.2 | 0.02 | 64.96 | 1.3 |
| 5-60% Ammonium sulphate | 22 | 26.36 | 0.97 | 0.04 | 30.29 | 3.25 |
| DEAE-Cellulose | | | | | | |
| 1 0 M NaCl | 16 | 5.04 | 0.19 | 0.04 | 3.9 | 4 |
| 2 0.1 M NaCl | 24 | 4.61 | 0.05 | 0.01 | 0.98 | 1 |
| 3 0.25 M NaCl | 24 | 3.34 | 0.17 | 0.05 | 3.42 | 5 |
| 4 0.5 M NaCl | 24 | 2.74 | 0.07 | 0.03 | 1.46 | 3 |
| 5 1 M NaCl | 8 | 1.06 | 0.01 | 0.01 | 0.29 | 1 |

### 5.3.3 DEAE-Cellulose chromatography

The ammonium sulphate dialysate was loaded onto DEAE-Cellulose column chromatography for further purification and the purification table is shown in Table 5.1. As shown in Fig. 5.4 A, five fractions showing proteolytic activity were obtained from the methanogenic sample. The samples corresponding to the peaks were pooled and showed specific activities of 0.56, 0.8, 0.72, 2.33 and 1.15 U/mg protein, and the purification fold increased from 3.22 to 56, 80, 72, 233 and 115 for peaks 1-5, respectively.

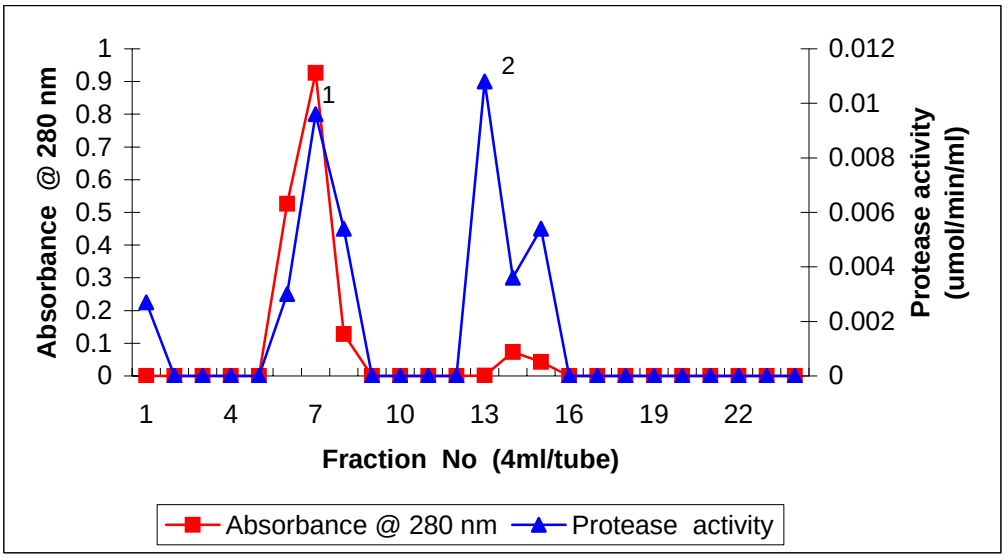
When the sulphidogenic sample was loaded onto DEAE-cellulose column chromatography, it

separates into five peaks which had potential proteolytic activity (Fig. 5.4 B). The samples that corresponded with the peaks were pooled and all of them showed protease activity. Specific activity of samples from peak 1-5 were 0.04, 0.01, 0.05, 0.03 and 0.01 U/mg protein, and their purification factors were 4, 1, 5, 3 and 1, respectively (Table 5.1). However, protein concentration decreased from 26.36 to 5.04, 4.61, 3.34, 2.74 and 1.06 mg for samples from peak 1 to 5.

### 5.3.4 Bio-gel A 0.5m Filtration

As seen in Fig 5.5 A, the sample from methanogenic reactor showed two peaks that have protease activity. The samples that correspond to each peak were pooled and recovered with the specific activity of 0.08 and 0.56 U/mg protein, and the purification fold increases from 3.22 to 8 and 56, respectively. Total protein concentration dropped from 34.79 to 2.81 and 0.43 mg (Table 5.2).

A




B

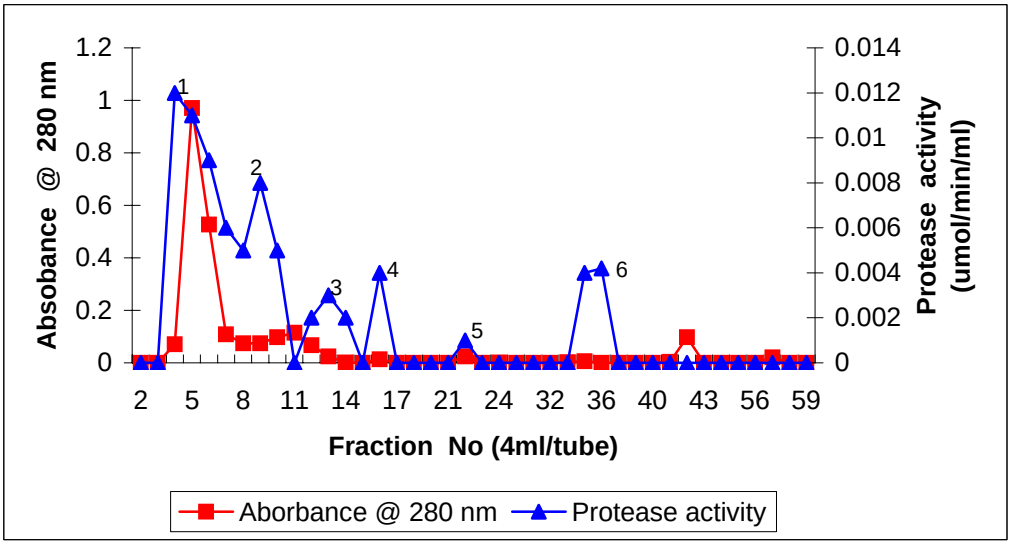



**Figure 5.5** Bio-Gel A-0.5m filtration of proteases from a) methanogenic and b) sulphidogenic bioreactor. Column dimension: 1.5cm x 17cm. Flow rate: 60ml/hr. The proteases were eluted with 0.1 M phosphate buffer (pH 7).

**Table 5.2** Purification table of the A) methanogenic and B) sulphidogenic proteases that were purified using Bio-Gel A-0.5m filtration

| Purification Step | Volume (ml) | Total protein (mg) | Total activity (U) | Specific activity (U/mg) | Yield (%) | Purification (fold) |
|---|---|---|---|---|---|---|
| **A. Methanogenic Sample** | | | | | | |
| Pellet | 94 | 860.1 | 4.61 | 0.01 | 100 | 1 |
| Sonication | 116 | 599.14 | 2.96 | 0.01 | 64.2 | 0.92 |
| 5-60% Ammonium sulphate | 45 | 34.79 | 0.55 | 0.02 | 18.7 | 3.22 |
| Bio-Gel A-0.5m | | | | | | |
| 1 | 12 | 2.81 | 0.22 | 0.08 | 4.8 | 8 |
| 2 | 12 | 0.43 | 0.24 | 0.56 | 5.2 | 56 |
| **B. Sulphidogenic sample** | | | | | | |
| Pellet | 100 | 2147 | 26.7 | 0.01 | 100 | 1 |
| Sonication | 130 | 447 | 13.07 | 0.03 | 48.93 | 2.35 |
| 5-60% Ammonium sulphate | 45 | 44 | 1.61 | 0.04 | 6.017 | 2.93 |
| Bio-Gel A-0.5m | | | | | | |
| 1 | 16 | 13.56 | 0.61 | 0.04 | 2.3 | 4 |
| 2 | 12 | 5.95 | 0.22 | 0.04 | 0.8 | 4 |
| 3 | 12 | 2.27 | 0.08 | 0.04 | 0.2 | 4 |
| 4 | 12 | 1.69 | 0.05 | 0.03 | 0.2 | 3 |
| 5 | 20 | 8.7 | 0.1 | 0.01 | 0.4 | 1 |
| 6 | 16 | 4.16 | 0.13 | 0.06 | 0.5 | 6 |

When sulphidogenic sample was subjected to gel filtration, six peaks were observed and all of them showing protease activity (Fig. 5.5 B). After pooling, samples from each peak (1 to 6) showed specific activities of 0.04, 0.04, 0.04, 0.03, 0.01 and 0.06 U/mg protein, respectively.

The purification fold of these enzymes changes from 2.93 to 4, 4, 4, 3, 1 and 6 for peaks 1, 2, 3, 4 and 5, and 6 respectively. In this case protein concentration of this sample dropped from 44 to 13.56, 5.95, 2.27, 1.69, 8.7 and 4.16 mg (Table 5.2).

### 5.3.5 SDS-PAGE

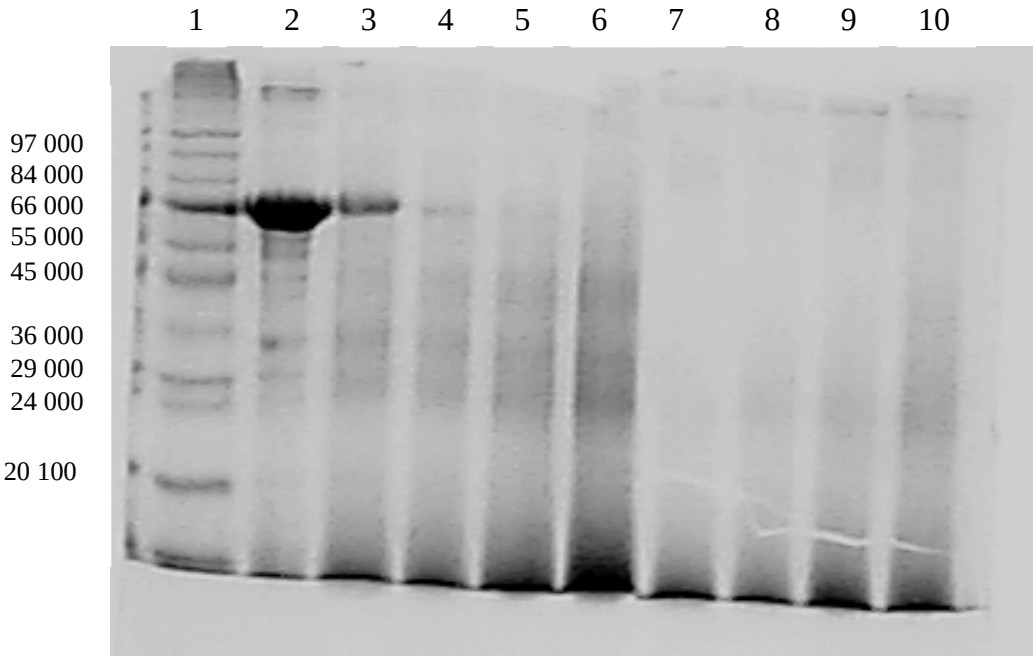



**Figure 5.6** SDS-PAGE pattern of proteases after sonication. Lane 1, molecular weight markers (Da); Lane 2, BSA; Lane3, methanogenic sample; Lane4, methanogenic sample with PMSF; Lane5, sulphidogenic sample and Lane6, sulphidogenic sample with PMSF. Lanes 7-10 contained untreated samples (Appendix I). Lane7, methanogenic sample; Lane8, methanogenic sample with PMSF; Lane9, sulphidogenic sample and Lane10, sulphidogenic sample with PMSF.

A

B

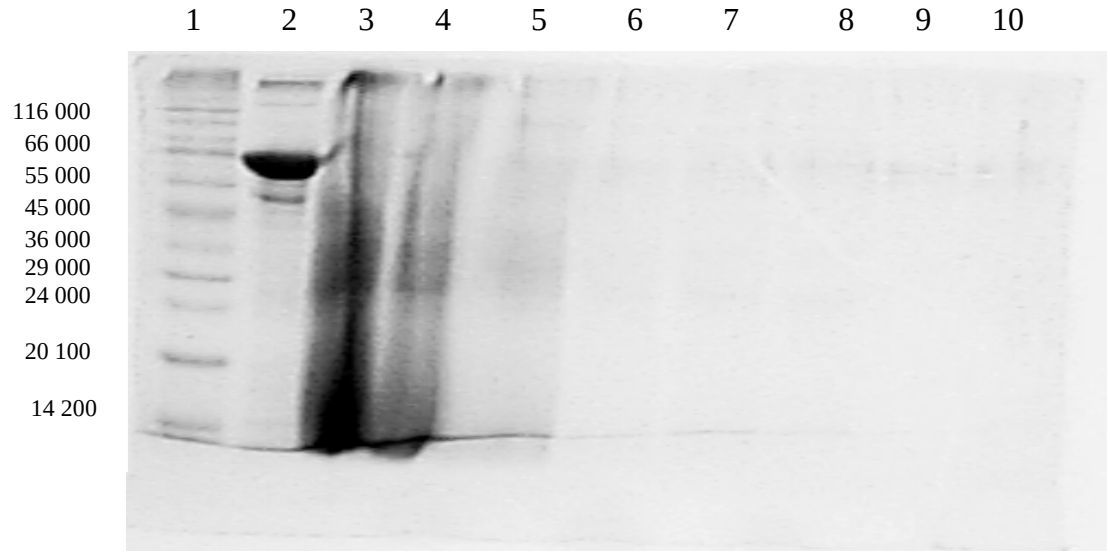



**Figure 5.7** SDS-PAGE patterns of sewage proteases obtained from A) methanogenic and B) sulphidogenic reactors. Lane1, molecular weight markers; Lane2, BSA; Lane3, resuspended pellet, Lane 4, ammonium sulphate (60%) treated sample and Lanes 5 –10, Peaks 1-6 from DEAE-Cellulose column.

The samples that were obtained from sonication with or without PMSF and those from ion exchange chromatography and gel filtration columns were subjected to SDS-PAGE (10% gel). Fig 5.6 shows the samples that were obtained from sonication, samples in lanes 3-6 contained a reducing agent and were subjected to heat except those shown in lanes 7-10. It was found that denatured samples have sharp bands on the gel and addition of serine protease inhibitor, PMSF did not affect the appearance of bands, showing that proteolytic degradation starts after sonication when most of contaminating proteins are removed from the protein of interest.

The methanogenic sample that was released from the sewage sludge floc matrix by sonication have four bands with the following $M_r$ values of 66, 45, 35 and 29 kDa, while sulphidogenic

sample has three bands with the following $M_r$ values of 45, 36 and 24 kDa (Fig. 5.6). Addition of PMSF did not have any effect in the position of bands in the gel. Results in Fig. 5.7 shows the samples that were purified further by DEAE-Cellulose anion exchange chromatography, unfortunately, no bands were detected when these samples were run on SDS-PAGE (10% gel). This is the reason why the gels of the samples that were purified by Bio-Gel A-0.5m filtration were not shown here.

## 5.4 DISCUSSION

It had been found that the enzymes produced during anaerobic digestion are associated predominately with the matrix floc of the sludge and with sonication ~ 50% of these enzymes can be released into the solution (section 2.3.4). The protein concentrations of the pellets (Table 5.1 and 5.2) are apparent protein values, since the method that was used to measure the amount of

protein is more sensitive towards the dissolved proteins and may not take into account any protein adsorbed onto the matrix.

In order to purify these enzymes, 5-60% ammonium sulphate fractionation was used as the first step in the enzyme precipitation. Enzyme purification folds increased from 0.92 to 3.22 for the methanogenic sample and 1.297 to 3.247 for the sulphidogenic sample, for the enzymes that were further purified by ion exchange chromatography. This method managed to remove some of the contaminating proteins since protein concentration decreased from 599.14 to 34.79 mg and from 217.79 to 26.36 mg for methanogenic and sulphidogenic sample, respectively. The purification fold and protein concentration for methanogenic sample that was purified further by gel filtration followed the same pattern as above. For the sulphidogenic sample, protein concentrations

decreased from 447 mg to 44 mg showing the removal of unwanted protein.

To remove unwanted protein from ammonium sulphate dialysate the samples were loaded onto DEAE-Cellulose ion exchange chromatography column that separates molecules according to their charges. Peak 1 (Fig. 5.4) contains enzyme which are positively charged as they were eluted by sodium phosphate buffer (0.1 M, pH 7) which was used as a washing buffer. Peaks 2-5 were progressively more negatively charged and were eluted with a stepwise addition of NaCl (0.1-1 M) in sodium phosphate buffer (0.1 M, pH 7) (Fig. 5.4). From this result it can be suggested that most of the proteases within the two bioreactors that are responsible for protein degradation are negatively charged. Another ammonium sulphate dialysate was loaded onto Bio-Gel A-0.5m filtration column, which separates molecules according to their sizes and shapes. This gel has an operating range of 10 000-500 000 Da meaning enzymes that have a molecular weight greater than 500 000 Da will be eluted in the void volume. Protein will be eluted in order of decreasing molecular weight. Methanogenic and sulphidogenic samples seem to contain proteases which co-elute with proteins with a molecular weight greater than 500 000 Da, since all the peaks were obtained at an early stage of elution (Fig. 5.5). From these studies it can be suggested that this kind of agarose bead in Bio-Gel A-0.5m are not good for separating proteases and a gel that has higher exclusion limit would be preferred. The other problem that was encountered in trying to purify this enzyme was a formation of a “brown coating” that was adsorbed to the exchanger/gel and remained there even after washing the resin with NaCl (2M) in sodium phosphate buffer (0.1 M, pH 7). Again, from this study it can be suggested that maybe these enzymes are lipoproteins or glycoproteins as FrNlund *et al.,* (1995) suggested that large exoenzymes were immobilised in the sludge by adsorption to extracellular polymeric substances. The methanogenic sample seems to be purified better by DEAE-Cellulose ion exchange

chromatography, than Bio-Gel A-0.5m filtration by comparing the purification fold from each method (Table 5.1 and 5.2). For the methanogenic sample that was purified by ion exchange chromatography purification fold increased from 3.22 to 56, 80, 72, 233 and 115 for peaks 1-5, respectively, and for the sample that was purified by gel filtration purification fold increased from 3.22 to 8 and 56 for peaks 1 and 2, respectively; however, for the sulphidogenic sample, the purification fold seems to be more or less the same.

To determine the molecular weight and the subunits of proteases from anaerobic reactors the samples were subjected to SDS-PAGE (10% gel). Samples that were obtained from sonication revealed that methanogenic sample consists of four subunits with the $M_r$ values of 66, 45, 35 and 29 kDa and sulphidogenic sample had 3 subunits with the Mr values of 45, 36 and 24 kDa. Unfortunately, there were no band detected when samples were purified with ion exchange chromatography and gel filtration were subjected to SDS-PAGE (10% gel). Due to the detection of molecular weight from sonicated sample, it is suggested that when samples were purified further proteolytic degradation was taking place. This can be observed during chromatography or filtration step. At first there was proteolytic activity, but after pooling and concentrating of the sample with PEG 20 000, enzyme activity was lost. From this study protease purification was successful even though this method needs to be optimised in order to get higher purification folds, especially for the sulphidogenic sample. To increase the purification fold of sulphidogenic proteases it would be advisable to increase anions inside the solution during extraction, which will compete with the negatively charged proteases in the floc matrix.

# CHAPTER 6

# GENERAL DISCUSSION AND CONCLUSION

Biological anaerobic treatment utilising sulphate-reducing bacteria has become a major topic of interest in treatment of industrial wastewaters and AMD. Application of anaerobic treatment to wastewater that has high concentration of sulphate will cause the coexistence of the bacterial consortia involved in the production of methane and reduction of sulphate (Colleran *et al.,* 1995). Both groups will compete for the common intermediates such as acetate, hydrogen and other short chain fatty acids in the anaerobic degradation process. The outcome of the competition will determine the amount of substrate removed by either SRB or methane producing bacteria (Omil *et al.,* 1996).

These bacteria need substrate to perform their reaction, and so many substrates have been used. In this project, primary municipal sewage which consisted of sand, food waste, settled material of inorganic and organics including effluent of raw sludge was the substrate because of its availability and costs. High levels of solids that may block the tubing were removed from the primary sludge by sieving. Enzymatic investigation of the hydrolysis of complex polymeric compounds in the sewage sludge showed that proteases predominate. These enzymes digest protein materials to produce amino acids which are used by the SRB and methanogens as their energy source. The disappearance of these substrates was measured by the COD content (Fig. 2.2) inside the bioreactor.

The highest protease activity was associated with the pellets, reflecting that the proteases which degrade proteins during anaerobic digestion, are found on the sewage sludge matrix. The amount of enzymes that were dissolved in solution was negligible (Boczar *et al.*, 1992 arrived at the same conclusion). The advantage of having enzymes attached to the floc matrix is that the bacteria do not need to waste energy replacing enzymes (Goel *et al.*, 1998) since they have higher operational stability. The peak of protease activity was found on day 9 for sulphidogenic reactor and on day 21 for the methanogenic reactor supporting the findings that time for sulphidogenic digestion is shorter (9-15 days) than the time required for methanogenic digestion (27-30 days).

The structure of the sludge matrix (pellet) is dependent on the interaction between the microbially produced polymers and the various cations present in the sludge. Any changes that can occur in the ionic strength and ionic composition can therefore alter the structural properties

of the sludge floc (Kieding and Nielsen, 1997). During sulphate reduction sulphide disrupts the integrity of the sewage sludge matrix, influencing the interaction between the enzymes and the substrates (Fig. 3.5-3.7) to yield high enzyme activity. Sulphide concentration, which is higher than100 mg/L, can be toxic during anaerobic digestion (McCarty, 1964) and in our study the maximum hydrogen sulphide that was found in the sulphidogenic reactor was 70mg/L, which occurred at day 7 (Fig. 2.4). The level of hydrogen sulphide dropped to zero on day 23 due to the fact that hydrogen sulphide was escaping during the sampling process. In the methane producing reactor there was no sulphide production, due to the absent of a sulphate.

Production of sulphide elevates the pH of the solution to alkaline conditions and proteases, which are closely associated with the organic particulate matter of the sewage sludge, have an optimum activity at pH 10 and at temperature of 50$^{o}$C. High protease activity at higher pH could be due to the replacement of ions that are found on the enzymes attached to the sewage floc and the ones that are generated during dissociation of buffer, leading to the release of free enzyme. High temperature denatures other enzymes leaving the protein of interest since proteases are more active at high temperature (Scopes, 1982). These proteases were stable at their optimum temperatures for about five hours due to the multipoint attachment of enzymes to the humus particle which forms a protective film around proteases, preventing autolysis (Chellapandian, 1998). The inhibition studies revealed that proteases inside the anaerobic digester are a mixture of serine and metalloproteases since they were susceptible to inhibition by PMSF, TPCK and 1,10-phenanthroline. The fact that EDTA appeared to activate proteases at pH 5 may support this

hypothesis, as the inhibitor would chelate with divalent ions removing then from the surface of the floc resulting in the disruption of the floc (Fig. 3. 14).

Heavy metals such as copper, iron, nickel and zinc can affect the digester bacteria. At low concentrations, however, these heavy metals are essential for growth and metabolism (Mutkowska, 1997). The concentration of dissolved heavy metal ions is generally kept below toxic levels by precipitating them as insoluble sulphides and/ or carbonates. In our studies, iron and zinc seem to decrease protease activity, especially at pH 5, due to the ability of these metals to form Lewis Acids and reinforce the ionic interactions to maintain the floc integrity. On the other hand, nickel activated protease activity at pH 5 and copper at pH 7 and 10 (Fig3.11-3.13). Protease inactivation may not be a result between the enzyme and metal ions but the ability of

these metal ions to interact with the surface of the floc and neutralise anionic charges that exist. This will disrupt the integrity of the floc, leading to a collapse of the floc infrastructure.

These enzymes were inhibited by the presence of volatile acids such as acetic acid, butyric acid and propionic acids (Fig. 3.8-3.10), which can be used by SRB and methanogens as an energy source. On the other hand, these volatile acids, however, can act as inhibitors of methanogenic and sulphidogenic reactors (Hobson and Wheately, 1993). If these acids inhibit proteases, then there will be no supply of soluble monomers (amino acids) available to the bacteria, leading to starvation and eventually to the death of the bacteria. Consequently the destruction of the SRB and methanogens will cause an increase in volatile acid concentration, followed by a decrease in pH, leading to a digester failure.

The majority of the proteases that were associated with the pellet were released into the solution by sonication. The shear force that is caused by sonication breaks down the bonds that are used to maintain the infrastructure of the floc, leading to the releases of these enzymes. Some of the proteases remained in the pellet suggesting that these enzymes are immobilised on the particulate matter. In order to facilitate the release of these enzymes, it would prove useful to study the interaction between the floc and the proteases by electron micrography. Most of the proteases that were released into the solution were negatively charged. It is quite possible that these proteases could interact with positively charged species on the floc surface destroying the floc integrity. A similar effect was discussed previously with the role of SH, $OH^-$, $HCO_3^-$ and heavy metals. Proteases from the anaerobic digester have a molecular weight which is approximately 500 000

Da and SDS-PAGE for sonicated samples revealed that methanogenic protease consists of four subunits while and sulphidogenic protease had 3 subunits.

Further studies should include assays for specific proteases using specific substrates. This will reduce the problem of non-specific proteolytic action, and it should be easier to determine the molecular weight of a specific enzyme rather than working with a wide range of different proteases. Specific inhibitors then could be used to specifically target the enzyme of interest.

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# APPENDICES

## APPENDIX I: PREPARATION OF ANALYTICAL REAGENTS

### 1.0 COD DETERMINATION

COD Solution A and B

### 2.0 SULPHATE DETERMINATION

#### 2.1 Buffer Solution A

| Reagent | Quantity |
|---|---|
| $MgCl_2.6H_2O$ | 30g |
| $CH_3COONa.3H_2O$ | 5g |
| $KNO_3$ | 1g |

| | |
|---|---|
| $CH_3COOH$ | 20ml |
| $ddH_2O$ | 880ml |

**2.2 0.205 M Barium Chloride**

| **Reagent** | **Quantity** |
|---|---|
| $BaCl_2.2H_2O$ | 50g |
| $ddH_2O$ | 1000ml |

**3.0 SULPHIDE DETERMINATION**

**3.1 0.02 M Amine-Sulphuric Acid Stock Solution**

| **Reagent** | **Quantity** |
|---|---|
| NN-Dimethyl-Δ-phenyldiamine dihydrochloride | 2g |
| HCl | 500ml |

**3.2 0.06 M Ferric Chloride**

| **Reagent** | **Quantity** |
|---|---|
| $FeCl_3.6H_2O$ | 8g |
| HCl | 500ml |

**4.0 PROTEIN DETERMINATION**

**4.1 Solution A:** 2% Copper sulphate - stored at $4^oC$

| Reagent | Quantity |
|---|---|
| $CuSO_4$ | 2g |
| ddH2O | 100ml |

**4.2 Solution B:** 2% Sodium tartrate -stored at 4$^{o}$C

| Reagent | Quantity |
|---|---|
| Sodium tatrate | 2g |
| ddH2O | 100ml |

**4.3 Solution C:** 2% $Na_2CO_3$ in 0.1 M NaOH - Stored at 4$^{o}$C

| Reagent | Quantity |
|---|---|
| $Na_2CO_3$ | 20g |
| NaOH | 4g |
| dd $H_2O$ | 1000ml |

**4.4 Solution D:** This reagent is prepared fresh on the day of the experiments

| **1ml Solution A + 1ml Solution B + 98ml Solution C** |
|---|

**4.5 Solution E:** Folin-Ciocalteu Phenol

**5.0 SDS-PAGE (Laemmli) RECIPES**

**5.1 Acrylamide/Bis:** (30% T, 2.67% C)

| Reagent | Quantity |
|---|---|

| | |
|---|---|
| Acrylamide | 87.6 g |
| N′ N′-bis-methane-acrylamide | 2.4 g |

Make up to 300ml with deionised water. Filter and store at 4$^{o}$C in the dark (30 days maximum)

**5.2 10% (w/v) SDS:** Dissolve 10 g SDS in 90 ml water with gentle stirring and bring to 100 ml with deionised water.

**5.3 0.5% (w/v) Bromophenol blue:** Dissolve 10 g bromophenol blue in 90 ml water with gentle stirring and bring to 100 ml with deionised water.

**5.4 Resolving Gel Buffer: (1.5 M Tris-HCl, pH 8.8)**

| **Reagent** | **Quantiy** |
|---|---|
| Tris base | 27.23 g |
| dd $H_2O$ | 80 ml |

Adjust to pH 8.8 with 6M HCl. Bring the total volume to 150 ml with deionised water and store at 4$^{o}$C.

**5.5 Stacking Gel Buffer: (0.5 M Tris-HCl, pH 6.8)**

| **Reagent** | **Quantiy** |
|---|---|
| Tris base | 6 g |
| dd $H_2O$ | 60 ml |

Adjust to pH 6.8 with 6 M HCl. Bring total volume to 100 ml with deionised water and store at 4$^{o}$C.

**5.6 Sample Buffer:** Store at room temperature

| **Reagent** | **Quantity** |
|---|---|
| Deionised water | 3.55 ml |
| 0.5 M Tris-HCl, pH 6,8 | 1.25 ml |

| | |
|---|---|
| Glycerol | 2.5 ml |
| 10% (w/v) SDS | 2 ml |
| 0.5% (w/v) Bromophenol Blue | 0.2 ml |

Add 5 µl of $-mercaptoethanol to 950 µl sample buffer prior to use. Dilute the sample at least 1:2 with sample buffer and heat at 95°C for 4 min.

**5.7 10X Electrode (Running) Buffer, pH 8.3:** Make up to 1 L and store at store at 4°C

| **Reagent** | **Quantity** |
|---|---|
| Tris base | 30.3 g |
| Glycine | 144 g |
| SDS | 10 g |

**5.8 10% Ammonium per sulphate (APS):** Prepare it daily

| **Reagent** | **Quantity** |
|---|---|
| APS | 100 mg |
| Deionised water | 1 ml |

**5.9 Making Up Gels**

**5.9.1 10% Resolving Gel**

| **Reagent** | **Quantity** |
|---|---|
| Deionised water | 4.1 ml |
| Acrylamide/Bis | 3.3 ml |
| Resolving gel buffer | 2.5 ml |
| 10% (w/v) SDS | 0.1 ml |
| 10% APS | 50 µl |
| TEMED | 5 µl |

**5.9.2 4% Stacking Gel**

| Reagent | Quantity |
|---|---|
| Deionised water | 6.1 ml |
| Acrylamide/Bis | 1.3 ml |
| Resolving gel buffer | 2.5 ml |
| 10% (w/v) SDS | 0.1 ml |
| 10% APS | 50 µl |
| TEMED | 10 µl |

### 5.9 Coomassie Gel stain

| Reagent | Quantity |
|---|---|
| Coomassie brilliant blue | 1 g |
| Methanol | 450 ml |
| dd $H_2O$ | 450 ml |
| Glacial acetic acid | 100 ml |

### 5.10 Coomassie Gel Destain

| Reagent | Quantity |
|---|---|
| Methanol | 100 ml |
| Glacial acetic acid | 100 ml |
| dd $H_2O$ | 800 ml |

## APPENDIX II: ANALYTICAL PROCEDURES

### 1. COD DETERMINATION: SQ 118 Method

**Analysis**

Measure 3ml of the sample into COD reaction cell and add 0.3mlof solution A and 2.3ml of solution B which are commercially available. Insert the reaction cell into the TR 205 and heat for 2hours at 148$^{o}$C. Remove the cell reaction and cool it at room temperature for 5minutes. Measure the COD concentration (mg/L) using a photometer SQ 118. Water is used as a blank.

### 2. SULPHATE ASSAY: Turbidimetric Method

**Principle:** Addition of Barium salt precipitate sulphate ion in acetic acid to form crystals of uniform size. A spectrophotometer is used to measure light absorption of barium sulphate suspension and sulphate concentration is determined from a standard curve (APHA, 1985).

### 2.1 Sulphate standard curve

Sulphate stock solution is prepared by dissolving 0.15335g in 250ml of distilled water and the standard ranges from (0-612mg/L)

**Table 1A Preparation of sulphate standard**

| Conc (mg/l) | Stock Solution (ml) | dd $H_2O$ (ml) |
|---|---|---|
| 0 | 0 | 1 |
| 122 | 0.2 | 0.8 |
| 245 | 0.4 | 0.6 |
| 367 | 0.6 | 0.4 |
| 490 | 0.8 | 0.2 |
| 612 | 1 | 0 |

## 2.2 Analysis

Measure 1 ml of a sample and add 0.2ml of buffer solution A. Add 0.1ml of barium chloride and vortex the mixture. Read the absorbance at 420nm using a spectrophotometer.

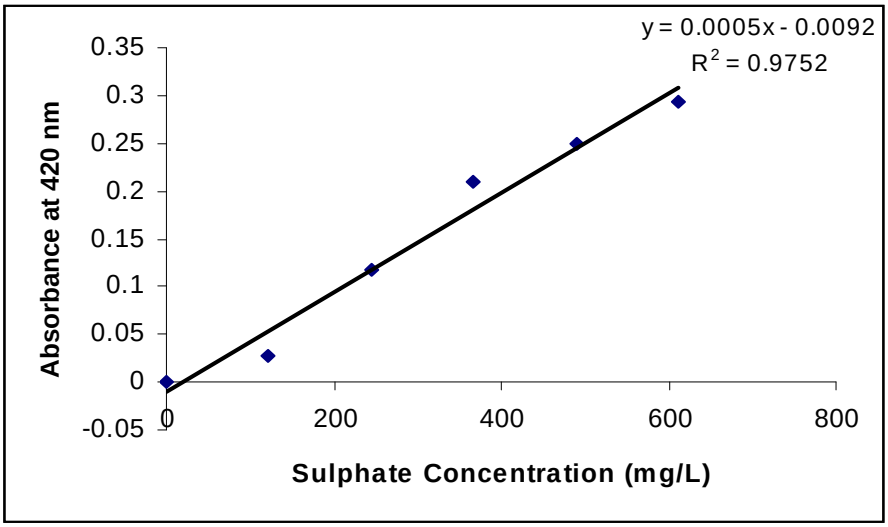


**Fig. 1A** Sulphate standard curve

## 3. SULPHIDE ASSAY

**Principle:** In this assay NN-Diethyl-Δ-phenylenediamine forms a blue colour in the presence of iron (III) ions and the intensity of the colour is proportional to the concentration of sulphide (Rees *et al.,* 1971).

### 3.1 Preparation of sulphide stock solution

The stock solution is prepared by dissolving 0.789g of $Na_2S.9H_2O$ in 500 ml of distilled water.

**Table 2A Preparation of sulphide standard curve**

| **Conc (mg/L)** | **Stock Solution (ml)** | **dd$H_2O$ (ml)** |
|---|---|---|
| 0 | 0 | 100 |
| 0.2 | 2 | 98 |
| 0.4 | 4 | 96 |
| 0.6 | 6 | 94 |
| 0.8 | 8 | 92 |
| 1 | 10 | 90 |

### 3.2 Analysis

Measure 5ml of the sample. Add 0.5ml of each amine-sulphuric acid and ferric chloride reagents. The colour is allowed to develop for an hour and the absorbance read at 670nm using a spectrophotometer.

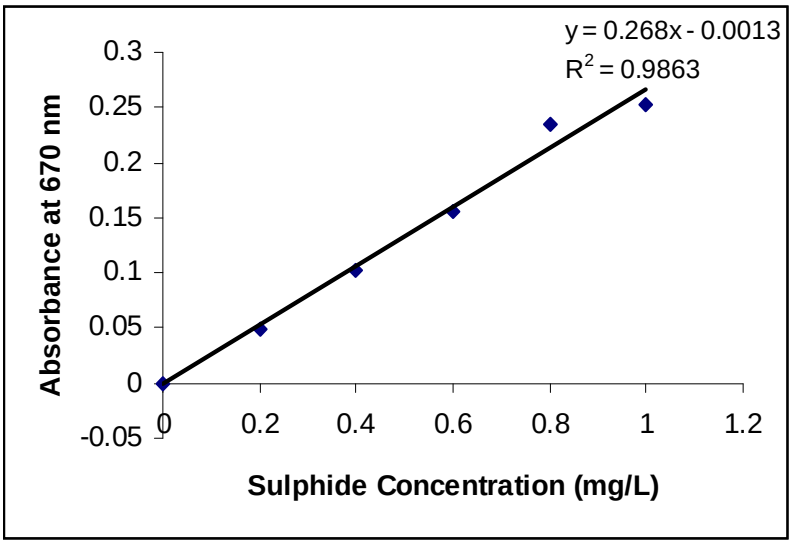


**Fig. 2A** Sulphide standard curve

## 4. PROTEIN ASSAY: Folin-Lowry Protein Method

**Principle:** This method involves two colour forming reaction. The first one involves the reaction between copper ions with peptides bonds to form a deep blue colour and this happens in the presence of alkaline copper reagent. The second one is caused by the complex inorganic salt mixture of the Folin-Ciocalteu reagent that is responsible for further colour development. This reagent is made up of sodium tungstate and sodium molybdate dissolved in phosphoric and hydrochloric acid. Its yellow colour is caused by the presence of phosphomolybdate and it is reduced by the copper-treated free or bound peptide of tyrosine and tryptophan residues, which are present in solution. The intensity of blue-green colour produced by this reaction is proportional to the protein concentration (Lowry *et al.*, 1951).

### 4.1 Preparation of Protein Stock Solution

Dissolve 0.09g of bovine albumin stock solution in 100ml of distilled water and prepare the standards ranging from 0- 0.9mg/ml (see Table 3A).

**Table 3A Preparation of Protein Standard**

| Conc (mg/ml) | Stock Solution (ml) | dd$H_2O$ (ml) |
|---|---|---|
| 0 | 0 | 1 |
| 0.18 | 0.2 | 0.8 |
| 0.36 | 0.4 | 0.6 |
| 0. 54 | 0.6 | 0.4 |
| 0.72 | 0.8 | 0.2 |
| 0.9 | 1 | 0 |

## 4.2 Analysis

Add 1ml of a sample to a test tube and add 5ml of alkaline copper reagent. Allow to stand for 10min and add 0.3ml of Folin-Ciocalteu reagent. Allow to stand for 30min and read the absorbance at 500nm using spectrophotometer.

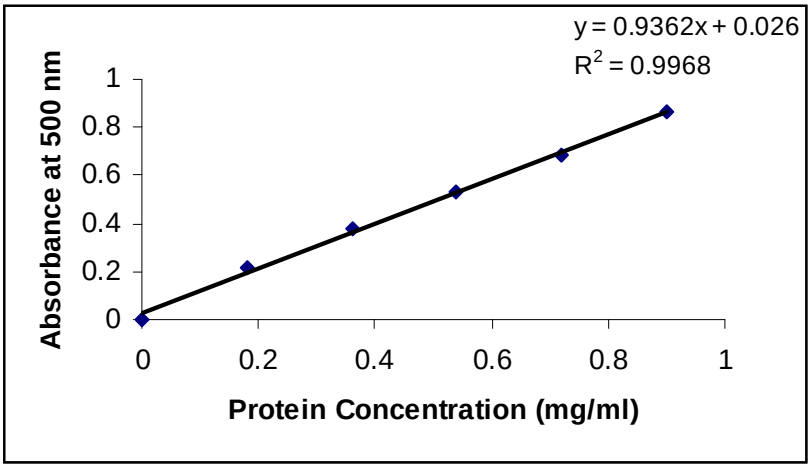


**Fig. 3A** Protein standard curve

## 5 TYROSINE STANDARD CURVE

**Principle: T**he complex inorganic salt mixture of the Folin-Ciocalteu reagent causes the development of the blue colour. This reagent is made up of sodium tungstate and sodium molybdate dissolved in phosphoric and hydrochloric acid. Its yellow colour is caused by the

presence of phosphomolybdate and it is reduced by the copper-treated free or peptide bound of tyrosine and tryptophan residues, which are present in a solution. The intensity of blue-green colour produced by this reaction is proportional to the tyrosine concentration.

### 5.1 Preparation of the Stock Solution

Dissolve 18.1 mg of L-tyrosine in 0.2 M HCl and the solution is diluted to 100 ml with water.

**Table 4A Preparation of Tyrosine Standard**

| **Conc (μmol/ml)** | **Stock Solution (ml)** | **0.2 M HCl (ml)** |
|---|---|---|
| 0 | 0 | 5 |
| 0.1 | 0.1 | 4.9 |
| 0.2 | 0.2 | 4.8 |
| 0.3 | 0.3 | 4.7 |
| 0.4 | 0.4 | 4.6 |
| 0.5 | 0.5 | 4.5 |

### 5.2 Analysis

Into the test tube add 5 ml of the sample, 10 ml of 0.5 M NaOH and then with continuous swirling 3 ml of FC reagent, which is diluted with water (1: 2). Allow to stand at room temperature for 10 min and measure the absorbance against water as a blank at 578 nm. Then construct a graph of $A_{578}$ against μmol tyrosine.

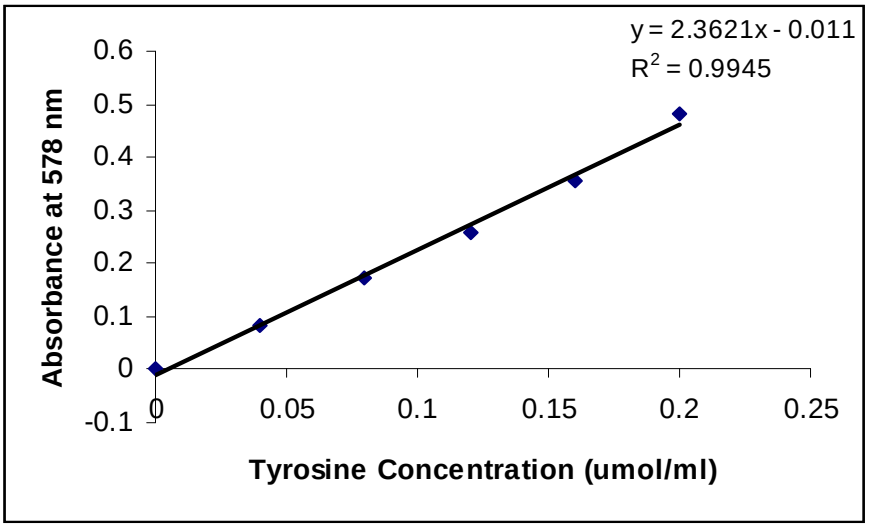


**Fig. 4A** Tyrosine standard curve

## 6 SDS-PAGE (Laemmli Method)

### 6.1 Gel Casting

Carefully add the resolving gel smoothly into the assembled gel cassette to prevent it from mixing with air and immediately overlay the monomer solution with water. Allow the gel to polymerise for 45 min. After polymerisation, remove the overlaying solution and if necessary, store at room temperature overnight, covered with 5 ml of 1.5 M Tris-HCl, pH 8.8 buffer (1:4dilution). Dry the top of the resolving gel with a filter paper, then pour the stacking gel into the cassette. Insert the desired comb between the spacers starting from the top of the spacer, then allow the stacking gel to polymerise for 30min. Gently remove the comb and the casting frame from the casting stand. Place the gel cassette into the slot at the bottom of the electrode assembly and lower the inner chamber assembly into the mini tank. Fill the inner and the lower buffer chamber with the running buffer. Load the samples into the wells with Hamilton syringe or a pipette using gel loading tips. Place the lid on the mini tank and insert the electrical leads into suitable power supply with proper polarity. Apply power to the Mini-PROTEAN 3-cell and beginning electrophoresis (200 volts) for 35 min. After electrophoresis, pour off the running buffer and remove the gels from the gel cassette.

## 6.2 Staining and Destaining Procedure

1. Gloves should be worn to prevent transfer of fingerprints to the gel. Transfer the gel into a small container containing a small amount of coomassie stain.
2. Agitate for 20min on a slow rotary or rocking shaker. Cover the container with a lid or plastic wrap during staining and destaining.
3. Pour out the stain and rinse the gel with few changes of water.
4. Add Coomassie destain. Strong bands are visible immediately on a light box and the gel is destained within an hour.
5. To destain completely, change the destain solution and agitate overnight.

## 6.3 Molecular Weight Determination

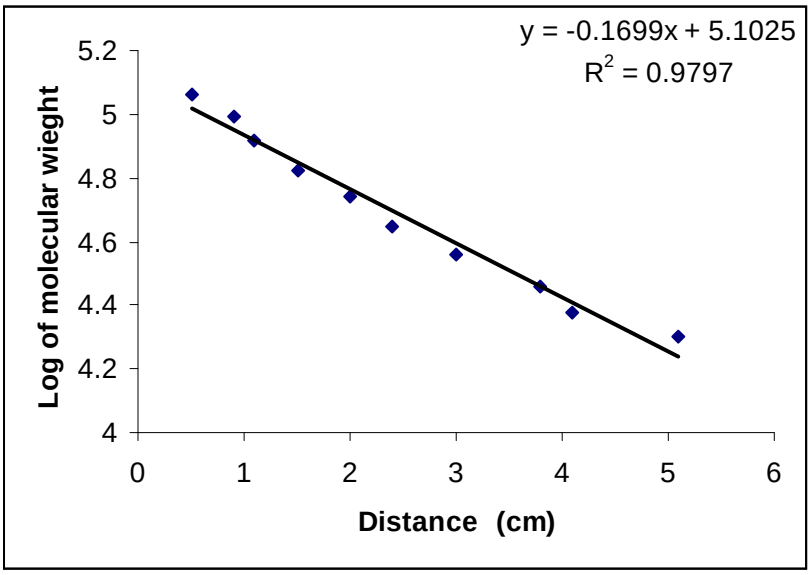


**Fig. 5A** The plot of distance versus the log molecular weight of standard.