THE ROLE OF CELLULASES AND GLUCOHYDROLASES IN THE SOLUBILISATION OF PRIMARY SEWAGE SLUDGE.

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

MASTER OF SCIENCE

Of

RHODES UNIVERSITY
GRAHAMSTOWN.

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DECEMBER 2001

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LIST OF ABBREVIATIONS.

AIPS = Algal integrated ponding system
AMB = Acetophilic methane bacteria
AMD = Acid mine drainage
AMP = Adenosine monophosphate
APS = Adenosine phosphosulphate / ammonium persulphate
CMC = Carboxymethyl cellulose
COD = Chemical oxygen demand
EDTA = Ethylene diamine tetraacetate
ERB = Electrode reservoir buffer
HMB = Hydrogenophilic methane bacteria
HRT = Hydraulic retention time
MS = Methanogenic system
MUF = Methylumbelliferone
Mwt. = Molecular weight
NPF = Neutralised pooled fractions
PPA = Phenyl propanoic acid
PNPG = ρ-nitrophenylglucoside
SDS-PAGE = Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SRB = Sulphate reducing bacteria
SS = Sulphidogenic system
VFA = Volatile fatty acid
Vo = Void volume (ml)
Ve = Elution volume (ml)
YAS = Yellow affinity substance

ABSTRACT.

Biological sulphate reduction has been identified as a potentially valuable process for removing sulphate and heavy metals from industrial effluents. The role of sulphate reducing bacteria (SRB) in this process has attracted the attention of biotechnologists and recently of enzymologists due to its fundamental properties and possible role in AMD bioremediation. These obligatory anaerobic sulphate-reducing bacteria are commonly known to dissimilate sulphate for energy. Under anaerobic conditions SRB oxidize simple organic compounds such as lactic acid with the sulphate and thereby generate hydrogen sulphide (a strong reducing agent) and bicarbonate ions. The hydrogen sulphide in turn reacts with contaminant metals contained in AMD and precipitates them out of solution as metal sulphides. Bicarbonate ions neutralize AMD by reaction with protons to form carbon dioxide and water.

Organic matter in the municipal sewage sludge has been identified as a potential source of electron donors for sulphate reduction. However, this organic matter is in the polymeric form that cannot be utilised by SRB. The latter depend on the activities of other hydrolytic bacteria for the degradation of complex polymers. Hence the availability of these monomeric substrates is a major factor, which may constrain further process development and is considered a rate-limiting step.

This study is therefore undertaken to investigate the bacterial glucohydrolase enzymes involved in the digestion of the polysaccharides present in the sewage sludge with specific interest in cellulases and/or β-glucosidase enzymes. The goals of the research are to: isolate, identify, purify and quantify these enzymes; study their distribution with respect to time, pH, and temperature; maximize and quantify the hydrolysis products; study whether sulphide and sulphate have an enhancing or an inhibitory effect on the activity of enzymes; optimize the enzyme activity against substrate and/or product inhibition and soluble heavy metal salts.

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ACKNOWLEDGEMENTS.

I would like to take this opportunity to thank the following people without whom this thesis would never have materialized:

My Supervisor Prof. C. G. Whiteley, for the opportunity to become involved in a challenging area of research, Dr, B Pletschke for his encouragement and help in the lab, "Brett, I am truly grateful, thank again", fellow Postgraduates (Anu, Doug, Paula, Stewart), Prof. Blatch's lab (special thanks to Tonderai Matambo and Fretha) and everyone in lab 309.

The Water Research Commission and DAAD for financial support, and Mr. John Gillam for doing a great job as though he owed me a favour.

Special thanks to my family (especially my grandparents), and my husband Tam for their moral support and love.

Above all, "Lord I give You praise and glory for helping me finish the work that I started at Rhodes University."

CHAPTER ONE

LITERATURE REVIEW.

BACKGROUND.

1.1. Acid mine drainage (AMD) and environmental pollution.

Since the beginning of man's existence, his activities have influenced the natural bio-geochemical cycles of elements. With increasing industrial and urban development the impact of man's actions on the environment has become substantial [van Houten, 1996]. This disturbance of the natural cycles is generally caused by the discharge of industrial wastes into the environment, posing a pollution problem on the land and water systems. The impact is usually excessive growth of undesirable macro and / or microorganisms with visual and olfactory effects on the system. Some discharges containing high sulphate concentrations may change the colour of waters and cause foul smells which may be toxic to animal and plant life [Hobson and Wheatley, 1993]. The industry normally responsible for the discharge of such effluents is the mining industry. The single most important source of water contamination (from the mines) worldwide is acid mine drainage (AMD) [Easton *et al.* 1995; Younger, 1995].

1.1.1. The production of AMD.

The principles of acid mine drainage generation are fairly well understood. Pyrite normally associated with ore and coal and other sulphide minerals, on exposure to oxygen and water in the presence of oxidising bacteria such as *Thiobacillus ferrooxidans,* are oxidised into sulphate, iron and acid. The overall reaction can be summarised as follows:

$$2FeS_2 + 2H_2O + 3O_2 \rightarrow 2Fe^{++} + 4SO_4^{=} + 4H^{+}$$

The acid in turn [as it influences the solubility of many constituents] dissolves and mobilises most heavy metals into solution. AMD is thus characterised by low pH values (± 2.5), high levels of sulphate and heavy metals [Easton *et al.* 1995; Adam *et al.* 1996].

AMD can be an extensive environmental pollution problem since the oxidation processes may continue for decades or even centuries after the closure of a mine. The main sources of

AMD in abandoned mine areas are usually old waste rock dumps and rock walls in tunnels and shafts. Open pits and underground workings are often filled partly or completely with polluted water after the closure of a mine. Overflow from such systems may contribute significantly to the total transport of pollutants out of the area [Christenden, *et al,* 1996] into ground water, which may be accessed for human use. This overflow may affect the land surface contaminating receiving streams and aquifers [Easton *et al.* 1995; Johnson, 1995]. In either scenario the overall effects can be very dramatic and hostile to most forms of life [Adam *et. al,* 1996].

1.1.2 Impacts of AMD.

Acid: Hydras, sponges, flat worms and vertebrates are found rarely in AMD streams though several insect species have been found apparently thriving in some locations [Johnson, 1995]. In worst cases all aquatic life disappears, river bottoms become coated with a layer of rust-like particles and the pH decreases [Adam *et. al,* 1995]. Higher plant life also fares badly.

Metals: Aluminium, barium, cadmium, chromium, cobalt, copper, lead, mercury, nickel, selenium and zinc are heavy metals (other than iron and manganese) which may occur in South African AMD at levels which may be of concern. Some of these metals are insoluble at neutral pH, precipitate out of solution and are classically regarded as non-toxic because of this insolubility. As they go into solution, that is, in strongly alkaline or acidic conditions they are regarded as potentially toxic. Below are shown the effects of these metals on human and other macro-organisms (Table 1.1).

Sulphate: Is non-toxic to animals and humans except at very high levels when it exerts a purgative effect. Sulphate concentration must be considered where concrete is in contact with water as sulphate promotes corrosion of concrete. Sulphate is the dominant contaminant in the effluents from South African mining operations. South African rivers (unlike the other major mining countries) are heavily impounded and many of the major dams are only flushed every twenty

years. Therefore the salinity in these dams builds up, leading to problems for various end users of the water from these dams [Easton *et. al,* 1995].

Table 1.1: Typical mine drainage metal composition and their adverse effects on man and other forms of life [Easton et al. 1995].

HEAVY METAL	TOXIC EFFECTS
Aluminium	The soluble aluminium form has been implicated as a neurotoxin and with regard to irrigation water, aluminium can cause non-productivity in acid soils.
Barium	Is a muscle stimulant in its soluble form. Its fatal dose for man is 0.8 g and occurs as $BaCl_2$. It is toxic to the heart, blood vessels and nervous system and may cause kidney damage. This metal is readily precipitated from solution (and thus rendered non toxic) by sulphate.
Cadmium	Is associated with cardiovascular diseases, causes nausea and vomiting and it accumulates in the liver and kidneys. It is a recognised carcinogen and may cause the so called "Itai-Itai" disease characterised by brittle bones and intense pain. Cadmium is also highly toxic to plants and has the potential to accumulate in plants and soils to concentrations that may be harmful to humans. Cadmium is readily soluble in water and only precipitates at high pH values.
Chromium	High chromium concentrations are toxic and cause skin disorders, liver damage and ulcers. There is also reason to believe that chromium may be carcinogenic in the chromate form.
Cobalt	Though an essential element in human nutrition, cobalt is only needed in minute quantities. It exhibits a low toxic potential, except for irrigation where the toxic potential is moderate, depending on the plant species.
Copper	Affects the taste of water and is toxic to a number of plants.
Lead	Is a highly toxic and accumulative body poison which affects nerve tissues, causing anaemia, brain damage and paralysis. In children, it may result in mental retardation with convulsions in later life. May also inhibit plant cell growth at high concentrations.
Mercury	Is a highly toxic cumulative poison causing both brain and kidney damage. It is normally concentrated in the aquatic food chain and is converted to the even more toxic methyl mercury. The danger posed by mercury pollution therefore results from eating fish contaminated by mercury.
Nickel	Is also essential in small quantities for animal and plant life but can be toxic to certain plants in concentrations exceeding 0.2 mg/L. Its toxicity is reduced at neutral or alkaline pH.
Selenium	Is an essential element for normal health. If present in excess quantities, however, disease may result. It is also toxic to plants at concentrations as low as 0.025 mg/L, and toxic to livestock if forage is grown in soils with relatively high levels of added selenium.
Zinc	Although zinc is not toxic to humans, water containing high zinc concentrations should be screened for the highly toxic cadmium, which is commonly associated with zinc.

1.1.3 AMD and water balance in South Africa.

South Africa has the world's largest resources of minerals and other metals such as manganese, chromium and vanadium ores, high-grade reserves of iron ore and significant deposits of niobium. South Africa is also the world's major producer of gold and has substantial coal reserves. The mining of gold and coal currently accounts for approximately 90% of the total tonnage of metals mined in the republic of South Africa. The source of AMD pollution is therefore associated with the mining of the latter mineral resources. Mineral build-up caused by AMD presents a formidable problem in South Africa particularly in view of the fact that it imposes severe restriction on the beneficial use of the available water. The effects of AMD pollution are compounded by the fact that South Africa is an arid country with a limited water resource [Murray and Tredoux, 1999].

Water availability in South Africa is governed by the amount of rainfall reaching the rivers that is on average only 52-milliard m^3 per year (8.6%). Though improved planning for water use may be applied, only 60% of the mean annual run-off (together with ground water resources) may yield a reasonably assured supply of 31.5milliard m^3 per year [Henzen and Pieterse, 1978]. Even the available surface water resources are unevenly distributed, concentrated to a large extent in the eastern one third of the country. The remaining two thirds relies heavily on ground water. The ground water resources are also being depleted as a result of excessive abstraction due to increasing water demands with industrialization, urbanization, agricultural, recreational and domestic use as well as the growing population [Murray and Tredoux, 1999]. With just over 1200 kilolitres of available fresh water for each person each year at the present population of more than 42 million, South Africans are on the threshold of the internationally used definition of "water stress" [White paper on national water policy]. Within a few years population growth will take this country below this level. The white paper on a water policy for South Africa published in April, 1997 has reported that South Africa has less water than countries widely considered to be much drier such as Namibia and Botswana.

The above information on water balance represents the credit side in respect of distributable water [Henzen and Pieterse, 1978]. It is therefore advisable to consider the acidic mine

drainage problem in the context of the critical balance existing between the present and future demands for and the availability of water.

Because water is a precious commodity in South Africa and human life is dependent on it, there is increasingly more need for the preservation and proper management of the water resource and a rational management of wastes generated by human activities [van Houten, 1996]. Effort must be made to limit the use and wastage of water. Where possible water must be reused without sacrifice of the quality of the end product in industrial applications or health aspects for domestic purposes [Brouckaert *et. al,* 1989]. Removal of pollutants (with specific reference to AMD) from industrial effluents is one way of contributing to efforts of water conservation.

1.1.4 AMD Treatment.

Several methods exist for the treatment of mine waters, depending upon the volume of the effluent, the type and concentration of contaminants present. The approach used for controlling AMD pollution (in connection with this study) focuses on one of the existing three ways i.e. to collect and treat AMD before allowing it to flow into other water courses [Johnson, 1995]. The conventional mine drainage systems employing chemical treatment processes have been attempted and have come up with production of hazardous wastes and economic problems [Rowley *et. al*, 1994]. Details for these methods are covered in literature concerned with AMD treatment and will not be covered in this text. An effective treatment is sought to generate water of neutral pH and low acidity, and to reduce the levels of sulphates, iron and other metals present, down to the environmental limits. To be attractive, this process must be of low cost, easy to install and maintain and producing limited quantities of solid by products [Adam *et. al,* 1996]. A biological process, the anaerobic sulphate reduction by sulphate reducing bacteria, has been identified for acid mine drainage treatment [Christenden *et. al,* 1996].

1.2. Sulphate reducing bacteria (SRB).

SRB are a specialized group of anaerobes that carry out the anaerobic process of dissimilatory sulphate reduction [Gibson, 1990].

1.2.1 Habitat: These microorganisms are well recognised for their ubiquity in natural aqueous environments found in lakes, oceans estuarine sediments, saline and non-saline ponds and fresh water sediments [Barnes *et al.* 1991]. They are also found in polluted environments such as whey digesters, spoiled foods, anaerobic purification plants and deep sea vents. Other habitats in which SRB have been detected include sewage plants and more significantly they have been found in the intestinal contents of man, animals and termite gut. Over 40% of individuals tested in two different human populations have demonstrated to harbour significant members of intestinal SRB. Though the presence of SRB is associated with anaerobic micro niches some SRB have been detected in many ostensibly aerobic environments, however, with a low rate of sulphate reduction [Barnes *et al.*1991].

1.2.2 Classification: This was initially based on spore-formation with two classes identified, the non-sporing motile vibrios or rods - *Desulphovibrio* and the spore-forming straight or curved rods - *Desulphotomaculum* [Gibson, 1990; Widdel, 1988]. With further research additional genera of SRB have been discovered, several of which differed physiologically and morphologically from the known *Desulphovibrio* and *Desulphotomaculum*. Presently, their classification is based on morphology and nutrition though this classification is also complicated by the fact that morphologically similar types may differ in their nutrition whereas nutritionally similar types may have different morphology. This complexity of features led to compromises being made in classification of the SRB. The established genera and species are only an indication of a heterogeneous assemblage of microorganisms having in common merely dissimilatory sulphate metabolism and obligate anaerobiosis rather than phylogenetic relationships [Widdel, 1988]. The known SRB genera so far comprise both the Gram negative (*Desulphovibrio, Desulphotomaculum, Desulphobulbus, Desulphobacter, Desulphococcus, Desulphosarcina, Desulphuromonas)* and the Gram positive (*Desulphonema*) [Postgate, 1984; Widdel, 1988; Gibson, 1990; Radchenko and Tashirev, 1991].

Since SRB are morphologically and nutritionally diverse, they normally exist as part of a microbial consortium because of the complex mixture of naturally occurring carbon substrates

in their diverse habitats [Barnes *et. al,* 1991]. The significance of this consortium will be discussed later. All SRB share a common ability to couple the reduction of sulphate to the oxidation of carbon compounds (taking place during the anaerobic oxidation of carbon substrates), which act as electron donors. In the process, SRB obtain energy (by electron transport phosphorylation) for their growth (equation 1, section 1.3) [Reis *et. al,*1988; Gibson, 1990; van Houten, 1996].

1.2.3 Growth and nutritional requirements for SRB

(i) An electron acceptor: An inorganic electron acceptor is required by most strains and this is usually provided by the activated sulphate ion. Certain species have been shown to utilize other oxidized sulphur compounds namely: thiosulphate, tetrathionate and sulphite, whilst some strains utilize elemental sulphur [Postgate, 1984].

(ii) **The type of substrate**: Collective evidence has illustrated that SRB use low molecular weight compounds as electron donors. They depend on the activity of other hydrolytic and fermentative bacteria coexisting as integral members of the mixed microbial consortia for the initial degradation of the original polymers from biomass [Widdel, 1988; Hamilton, 1998]. Therefore, the type of substrate required by SRB, which varies according to genus, would be the principal end products of fermentation. Essentially these consist of volatile fatty acids (VFA) e.g. acetate, propionate, butyrate, C_3 and C_4 organic substrates such as lactate, pyruvate and malate; alcohols e.g. ethanol and propanol; H_2 and CO_2, and occasionally sugars and longer chain fatty acids. Although the bacteria and processes involved have not been clearly resolved, a number of sulphate reducers are also thought to metabolise certain hydrocarbons including methane [Postgate, 1984]. These fermentation products (especially the short chain fatty acids) are subsequently metabolised by hydrogen-producing acetogenic bacteria into hydrogen and acetate that function as the favoured electron acceptors during sulphate reduction [Hamilton, 1998].

(iii) Temperature: Some sulphate reducers are able to grow at temperatures below 5^0C, whilst at the opposite extreme the spore-forming thermophilic species grow comfortably at temperatures ranging form 65^0 to 80^0C [Gibson, 1990]. Hence SRB are ubiquitous.

(iv) pH: The preferred pH for their growth is around 7 and they are usually inhibited at pH values lower than 6 or higher than 9 [Widdel, 1988].

1.3. Sulphate reduction.

All plants and animals require sulphur for the synthesis of proteins. The biological transformation of sulphur in natural environments is a nutrient cycling process comprising both aerobic and anaerobic components. In its highest oxidation state sulphur exists as a sulphate ion (SO_4^{2-}) that is reduced to sulphide (S^{2-}) by most bacteria, fungi and plants before incorporation into amino acids. This process is termed assimilatory sulphate reduction and is purely a biosynthetic process. However, any sulphur compound with an oxidation state higher than that of sulphide (2-) is capable of functioning as an electron acceptor for the oxidation of carbon substrates by biological processes. For example, during dissimilatory sulphate reduction (i.e. when carbon substrates are broken down in the presence of sulphate), the sulphate ion is utilized as an oxidant for the degradation of organic material. An equivalent amount of sulphide is formed per mole of sulphate reduced [Gibson, 1990]. The overall degradation reaction taking place during dissimilatory sulphate reduction is as follows:

$$2CH_2O + SO_4^{2-} \rightarrow H_2S + 2HCO_3^- \quad \text{.....(1)}$$

Where: CH_2O represents a soluble carbon substrate.

The free sulphate anion (SO_4^{2-}) is not a suitable electron acceptor [van Houten, 1996]. The proposed pathway for the dissimilatory reduction of sulphate to sulphide by SRB involves the transportation of the sulphate ion from the environment across the bacterial cell membrane producing a highly activated adenosine phosphosulphate [APS]. APS is the actual electron acceptor that is subsequently converted to bisulphite and AMP [Widdel, 1988; van Houten, 1996; Gibson, 1990] by APS reductase. The bisulphite is then reduced to sulphide [van Houten, 1996].

The end product of sulphate reduction is sulphide, while it is extremely corrosive, binds rapidly to metals to form insoluble metal sulphides. Sulphidogenic activity of SRB therefore

offers both the potential for the removal of toxic heavy metals during downstream processing from a range of industrial plants, and in water bioremediation [Gibson, 1990].

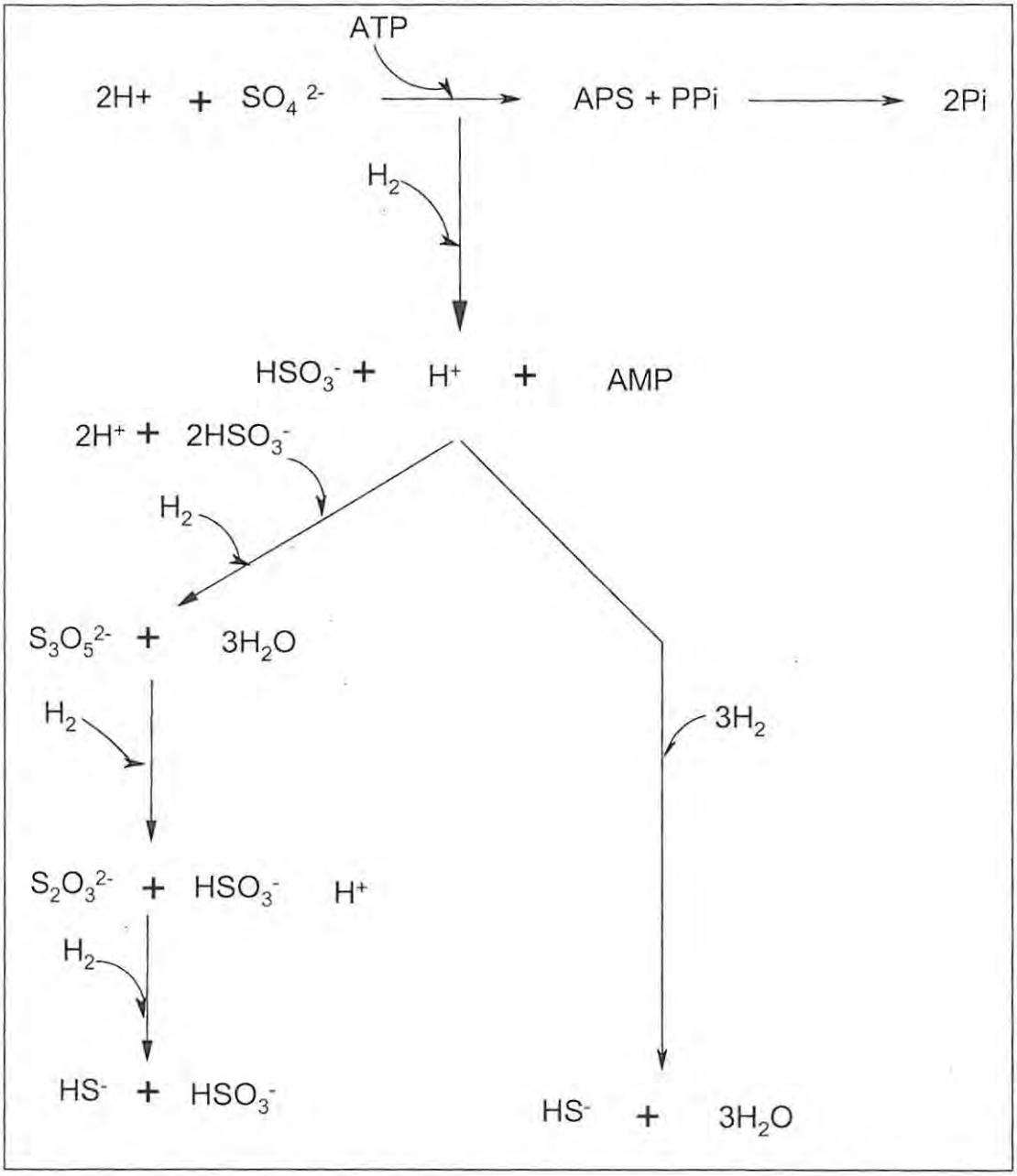


Figure 1.1: Possible pathways for dissimilatory reduction of sulphate to sulphide by SRB (adapted from van Houten 1996).

1.4. Sulphate reduction coupled to AMD bioremediation.

Sulphate reduction coupled to oxidation of organic substrates and precipitation of metal cations is illustrated by the reactions on the next page.

From reaction (1) (on page 8)

$$2CH_2O + SO_4^{=} \rightarrow H_2S + 2HCO_3^{-} \quad \text{.....(1)}$$

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

Where $2CH_2O$ represents organic substrates for growth of bacteria
M is a metal (e.g. Iron, manganese, nickel, zinc etc)
MS = Metal sulphide

The biochemical reactions taking place during sulphate reduction can be summarised as follows: under anaerobic conditions SRB reduce sulphate to form sulphide with concomitant oxidation of organic substrates (that are electron donors to bicarbonate ions). The sulphide combines with the contaminant heavy metals to form insoluble metal sulphides (which – only at low pH values - precipitate out of solution) and protons. Bicarbonate ions (which are in effect the result of excess sulphide formation) produced in reaction 1 can consume protons thus raising the pH of AMD with carbon dioxide and water as the final products.

$$HCO_3^{-} + H^{+} \rightarrow H_2O + CO_2 \quad \text{.....(3)}$$

Facts to consider: Reactions 1 and 2 will proceed under anaerobic conditions provided the pH does not inhibit biological activity. The amount of acid generated in equation 2 (from metal precipitation) equals the amount that can react with bicarbonate ions. Meaning that, if all the sulphide produced reacts with metals, no net reduction in acidity or increase in pH will occur. Should the pH of the influent AMD be less than 5, the SRB will be inhibited (as stated in section 1.2.3). In that case, incorporating lime or other alkali into the organic substrate or addition to the influent directly should raise the pH of the influent AMD. Hence the effluent discharge limitations with regards to pH are between 6 and 9 [Sing, 1992].

Since sulphate reduction cannot proceed in the absence of an electron donor (provided by the carbon substrates), the availability of the organic compounds may bring limitations to the advancement of this technology (i.e. AMD bioremediation). Furthermore, low costs are attractive in effective technological practises. Therefore, the municipal sewage sludge has

been identified as the potential cost effective, readily available feedstock of particulate organic carbon source for (SRB) sulphate reduction, but is present in a complex polymeric form. It has been mentioned that the SRB are unable to utilize polymeric molecules and depend on the activity of other fermentative bacteria for the supply of the low molecular weight carbon source. The hydrolysis of the complex particulate matter being a fermentative process involves enzymatic degradation and the whole hydrolytic process can be better defined as anaerobic degradation.

1.5. Anaerobic degradation.

Anaerobic digestion is a process in which organic matter is converted to methane and carbon dioxide in the absence of oxygen. This process is widely used for the purification of wastes containing high concentrations of organic material such as domestic sewage sludge and effluents from various industries [Hatting *et. al.*, 1969]. The primary major objectives of sludge digestion are the neutralization of odours and other offensive characteristics, the reduction of its tendency to putresce and a decrease in the number of pathogens and microbial activity. Approximately 30-40% of solid organic material is converted to methane and CO_2 and the resulting smaller amount of solid biomass can be readily dewatered (by gravity or vacuum filtration) into a more stable solid product which is easy to dispose off on land and further broken down by microorganisms [Mckiney, 1962; Forday and Greenfield, 1983; Lester and Sterrit, 1990].

1.5.1. The microbiology and biochemistry of anaerobic degradation.

There are, traditionally, four trophic groups of bacteria involved in the sequential anaerobic conversion of organic material into methane: the hydrolytic, fermentative, acetogenic and methanogenic bacteria. These groups correspond to the four respective phases of methanogenesis. Later observations have recognised a fifth group (the homoacetogenic bacteria, which will be included in this discussion) though its role in the process is not clearly understood [Forday and Greenfield, 1983]. The Schematic presentation of anaerobic degradation is illustrated in Figure 1.2.

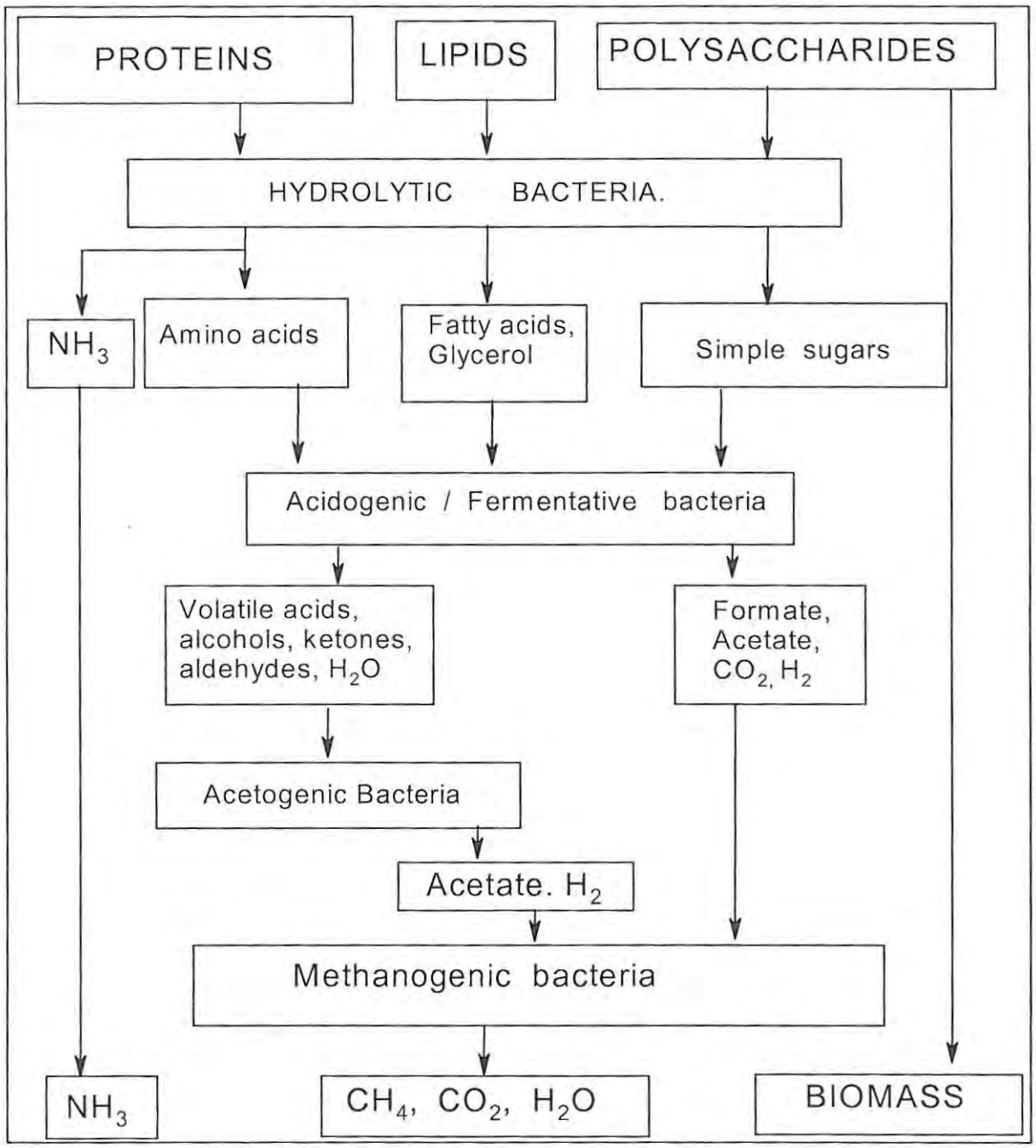


Figure 1.2: Schematic diagram showing the biochemistry of anaerobic digestion, (adapted from Lester and Sterritt, 1990).

1.5.1.1 Hydrolysis

This initial phase, hydrolysis involves dissimilation or biochemical oxidation of high molecular weight organics [Blaszyk and Korasic, 1990] such as polysaccharides, proteins and lipids into energy and simpler and soluble compounds (i.e. sugars, amino acids, fatty acids and glycerol) [Daniels, 1984]. Since the large insoluble compounds cannot be transported into

the bacterial cells [Lester and Steritt, 1990], the initial hydrolytic attack is brought about by the extracellular enzymes elaborated by the bacteria. During biochemical oxidation, electrons and protons are transferred from a reduced compound (the hydrogen electron donor) to a less reduced compound (the hydrogen electron acceptor). In the anaerobic environment the hydrogen electron acceptor is unlimited. It can either be an inorganic compound (e.g. sulphate, carbon dioxide) or simple organic compounds. Little is known about lipases and lipid hydrolysis, while various extracellular glucohydrolases and a wide range of specific and non-specific proteases are known to be present in anaerobic digesters. A large group of microbial species reported to hydrolyse polysaccharides anaerobically are listed in Table 1.2.

Table 1.2. Polysaccharide-degrading anaerobic bacteria.

Biopolymer	Bacteria
Cellulose	*Ruminococcus flavefaciens* *Ruminococcus albus* *Clostridium thermocellum* *Bacteroides succinogenes* *Acetivibrio cellulolyticus* *Clostridium longisporum* *Clostridium cellobiosporus*
Hemicellulose	*Bacteroides ruminicola* *Bacteroides fibrisolvens* *Bacteroides adolescentis* *Bacteroides eggerthii*
Pectin	*Lachnospira multiporus* *Bacteroides fibrisolvens* *Bacteroides ruminicola* *Clostridium butyricum* *Clostridium multifermentans*
Starch	*Streptococcus bovis* *Bacteroides amylophilus* *Succimonas amylolytica* *Lactobacillus* species

1.5.1.2 Fermentation.

The pattern of metabolism in the second stage involves the fermentation of the hydrolytic phase products into carboxylic or volatile fatty acids (VFA), aldehydes, ketones and methanogenic substances (formate, acetate, hydrogen, carbon dioxide and methanol). The VFA produced are lactate, propionate, butyrate, caproate, caprylate, valerate, pyruvate and heptanoate [Lester and Steritt, 1990]. As the concentration of acids produced becomes sufficiently high, the pH of the anaerobic digester decreases. As a result this phase is called *acidogenesis*. The acidogenic population is by far the largest of the trophic groups. Most of the bacteria listed in table 1.2 are part of this group [Forday and Greenfield, 1983].

1.5.1.3 Acetogenesis

The build-up of acid products that results in low pH values may retard further bacterial metabolism. Essentially the biological system approaches equilibrium by developing a third group of bacteria. This group is known as the proton reducing acetogenic bacteria and can utilize the organic acids developed [Blaszyk and Korasic, 1990]. In contrast to the hydrogen forming acidogenic bacteria, these bacteria have an obligate requirement for the disposal of electrons as hydrogen gas. Acetate, hydrogen and carbon dioxide are products of acetogenetic conversions. These conversions are an important step in the anaerobic process as the VFA produced during hydrolysis are particularly toxic to the methanogenic bacteria.

Acetogenic bacteria are the slowest growing of the trophic groups and hence represent a rate-limiting step in the anaerobic process. Inhibition of the acetogenic population that could also arise from hydrogen accumulation may result in an environment unfavourable for the (next and ultimate anerobic bacterial group) methanogenic bacteria and a further souring of the digestion [Forday and Greenfield, 1983]. However, the metabolism of amino acids results in liberation of ammonia, which in turn neutralises a portion of the remaining acids (i.e. those that are not yet converted to acetate, hydrogen and carbon dioxide). In this way, the pH rises to a more favourable level for further bacterial growth.

Homoacetogenic bacteria have been reported to contribute to the acetate pool via carbohydrate degradation. They can also donate hydrogen to the methanogenic bacteria by a

phenomenon known as interspecies hydrogen transfer. Some species in this group are able to convert H_2 and CO_2 to acetate. One species *Acetobacterium woodii* was shown to be able to degrade aromatic compounds. Their significance as hydrogen consumers under normal conditions is considered to be minor as they are unable to compete with methanogenic bacteria. They are suspected to be important for maintaining low hydrogen partial pressure perturbations in the digester, which temporarily inhibit the methanogens. Members of the acetogenic group include *Syntrophobacter, Syntrophomonas and Desulfovibrio* [Daniels, 1984].

1.5.1.4 Methanogenesis

This is the last phase of anaerobic degradation and is carried out by *methanogenic* bacteria. These bacteria are a diverse group of rod, spherical and spiral shaped organisms showing considerable intraspecies variations in cell dimension and organisation, and regularity in cell shapes. They are very strict anaerobes, requiring a lower oxidation-reduction potential for growth than most anaerobic bacteria. They are particularly sensitive to pH values above 7.5 and below 6.0 and are inhibited by unionised (protonated) volatile fatty acids. This diverse group can be divided into two categories, the hydrogenophilic methane bacteria (HMB) and the acetophilic methane bacteria (AMB - a restricted group of *Methanosarcina backeri* strains).

HMB can grow autotrophically on hydrogen and CO_2 as sole energy and carbon sources to produce methane. A few species can utilize formate. The AMB account for approximately 70% of methane generated (directly from acetate) in anaerobic digesters [Forday and Greenfield, 1983]. Methane production quickly lowers the excess acids thus permitting further degradation of the more complex organics. Methanogens also promote β–oxidation of fatty acids using CO_2 as their hydrogen acceptor and water as their electron donor. As long as the digester is able to maintain a balanced bacterial population of acid and methane formers, operating problems for anaerobic digestion can be reduced [Blaszyk and Korasic, 1990].

A large fraction of the feedstock or organic particulate matter broken down by the processes mentioned above are constituted by carbohydrates whose primary function is to provide energy in the digesters. Most carbohydrates are in the form of insoluble polysaccharides (such

as cellulosic fibrous plant material), with sometimes soluble polysaccharides and sugars from the same sources [Hobson and Wheatly, 1993]. Polysaccharides, generally more complex in structure and less biodegradable than some plant carbohydrates, are also found in the tissue residues from slaughter houses and in the intestinal secretions and bacteria in faeces. Human beings have a limited ability to digest polysaccharides and only starch can be digested by gut enzymes. The plant *cellulosic* material remains unaltered and so is void in the faeces. On the other hand, farm animals have digestive tracts that allow microbial degradation of starch and partial degradation of cellulosic material. The undigested material found in the human and animal faeces form a poorer substrate for, and require a longer time for degradation by the digester bacteria [Wang, 2000]. A general but brief discussion on the biochemical structure of polysaccharides may provide clarification on why some polysaccharides are difficult to digest.

1.6. The biochemistry of carbohydrates.

Carbohydrates contain many monosaccharides (the fundamental units of the carbohydrate class) and may have molecular weights in the millions [Clark and Switzer, 1977]. The fundamental linkage between monosaccharide units in polysaccharides is an acetal or a ketal formed by a condensation of an alcoholic hydroxyl of one monosaccharide with the hemiacetal (or hemiketal) form of the carbonyl carbon sugar, accompanied by loss of water. These linkages are called glycosidic bonds and may occur in a variety of isomeric arrangements. That is, glycosidic bonds may be formed between α or β-hydroxyls at the anomeric carbon of one sugar and alcoholic hydroxyl groups at carbons 2, 3, 4, or 6 of a second sugar. In addition, the polymer may be branched so that the two sugars have glycosidic linkages to different alcoholic hydroxyls of a third unit (e.g. the branch point in glycogen). Each glycosidic (acetal or ketal) linkage employs the reducing carbon (anomeric carbon) of one monosaccharide. Thus all oligosaccharides and polysaccharides have only a single reducing end. The α-1.4 linked glucose units in starch (Figure 1.3) allow the formation of molecules that are accessible to enzymes that can hydrolyse interglucose bonds. On the other hand cellulose has a structure that is not easily degradable by hydrolytic enzymes. Since degradation of cellulose is the subject of interest in this study an elaborate discussion on its structure is necessary.

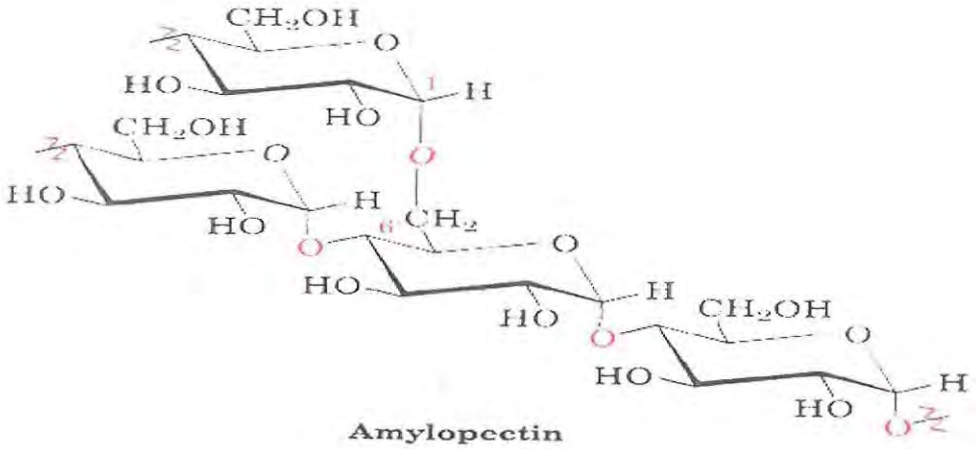


Figure 1.3: A representation of the structure of starch. The α–1.4 bonds link the glucose units and the α-1.6 acetal bonds are at the branching points.

Cellulose: is the most abundant of all naturally occurring organic compounds and probably makes up at least a third of all vegetable matter in the world. It is the main constituent of the cell walls of higher plants (Figure 1.4) where it provides the main structural feature[1].

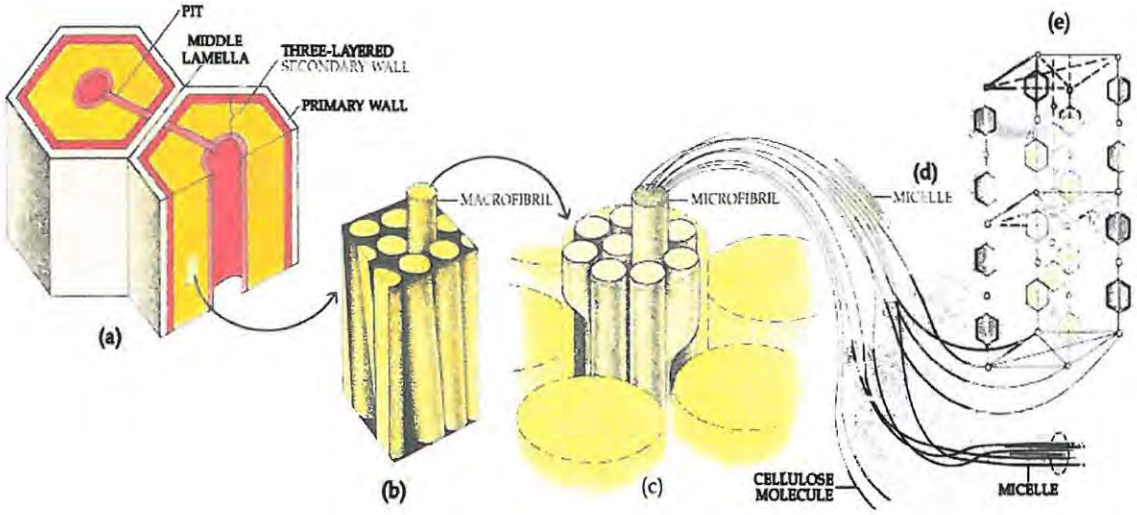
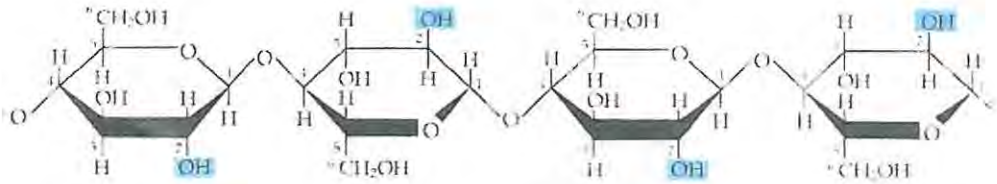


Figure 1.4: The detailed structure of a cell wall. (a) Portion of the cell wall showing the middle lamella, primary wall and three layers of the secondary wall. Cellulose, the principal component of a cell wall, exists as a system of fibrils of different sizes, (b) The largest fibrils, macrofibrils can be seen with the light microscope. (c) The macrofibrils were resolved into microfibrils about 10 nm wide. (d) Parts of the microfibrils, the micelles are arranged in an orderly fashion and impart crystalline properties to the cell wall. (e) A fragment of a micelle shows parts of the chainlike cellulose molecules in a lattice arrangement [Raven et al. 1992].

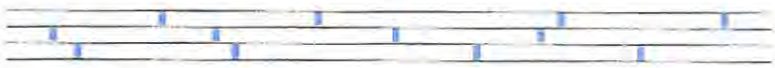
[1] http://www.biol

The word cellulose when used in a technical sense does not mean exactly the same thing as when used to denote an organic compound. Technically, "cellulose " denotes the residues obtained by subjecting vegetable materials to certain pulping processes. However in terms of biochemistry cellulose is a polysaccharide [of sufficient chain length to be insoluble in water or in dilute weak acids and alkalies] composed predominantly of repeating glucose anhydride units linked together through the 1 and 4 carbon atoms with a β-glucosidal linkage. The number of glucose anhydride units per molecule may vary over a wide range with chain lengths ranging from 500[1] to 12000 glucose residues long with no branching[1]. That is, a given sample of cellulose will thus contain many molecules of different lengths. An average value for the number of glucose anhydride units in a given sample of cellulose molecule is known as the degree of polymerization. It is the natural celluloses that have a degree of polymerization from 1 500 to higher values, but hydrolyzed material may drop into the so called 'mesocolloid' range 50-500, and still maintain the characteristics of cellulose. That is, the chain still has the connected β,1-4 linked glucose units, but at a lower number.



CELLULOSE MOLECULE

(b)



MODEL OF CROSS LINKED CELLULOSE MOLECULES

(c)

Figure 1.5: β-Glucose monomers, joined in β-1.4 linkages. (b) The structural formulae for alternating β-glucose units have been rotated 180^0 front to back to show the bonding. (c) In cellulose, the -OH groups (in colour) which project from both sides of the chain form hydrogen bonds with –OH groups on neighbouring chains, resulting in the formation of crosslinked cellulose molecules (c) [Garrett and Grisham 1999].

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[1] http://www.biol

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The glycosidic links (as opposed to the α,1-4 linkage in starch) in cellulose completely alter the properties of cellulose. The α–linkage sites of amylose are naturally bent, conferring a gradual turn to the polymer chain that results in a helical conformation. The β,1-4 linkage in cellulose involves alternating 180^0 flips of glucose units along the chain so that the chain adopts a fully extended conformation [Figure 1.5]. Juxtaposition of several such chains permits efficient interchain hydrogen bonding (Garrett and Grisham, 1999) which form between the C_3OH group and the oxygen in the pyranose ring within the same molecules (intramolecular hydrogen bonding) and those that form between the C_6OH group of one molecule and the oxygen glucosidic bond of another (Wang, 2000). This bonding results in very tightly packed cellulose molecules that form crosslinked crystal lattices or **microfibrils.** The latter are wound together and around one another to produce **macrofibrils.**

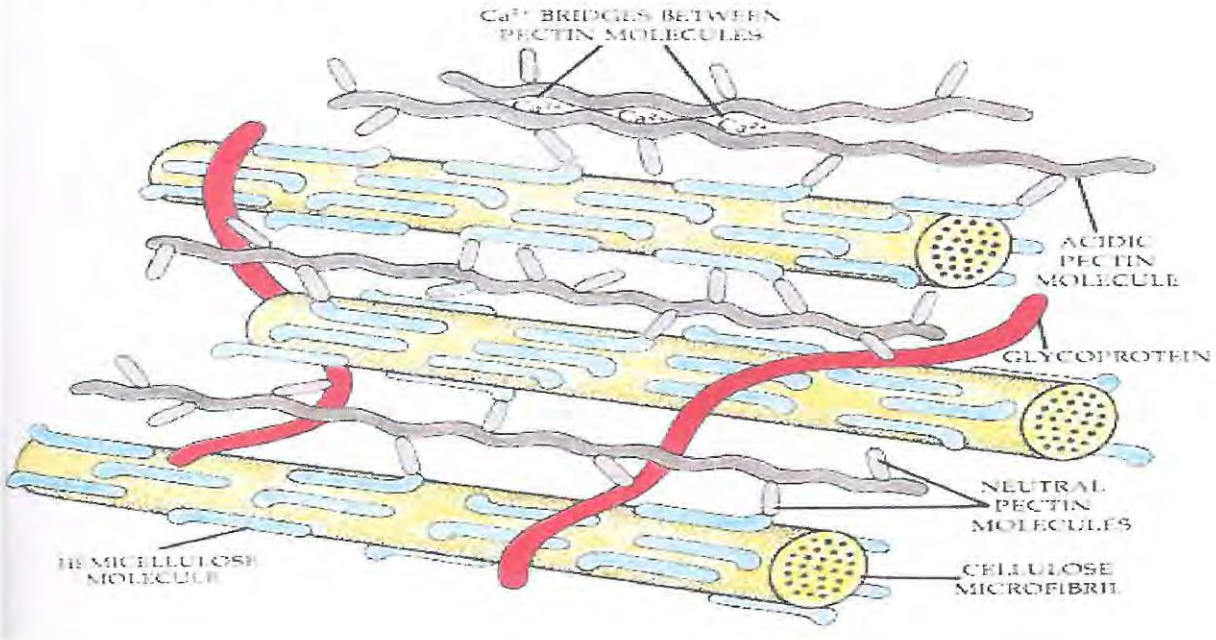

Figure 1.6: Schematic diagram showing how the cellulose microfibrils and matrix components (pectins and hemicelluloses and glycoproteins) of the primary wall may be interconnected. Hemicellulose molecules are linked to the surface of the cellulose microfibrils by hydrogen bonds. Some of these hemicellulose molecules are cross-linked by neutral pectin molecules. The latter are linked by calcium ions. The glycoproteins are attached to pectin molecules [Raven et. al. 1992].

Each macrofibril is interpenetrated by crosslinked matrix of non-cellulosic polysaccharides such as hemicellulose and pectin [Figure 1.6]. Lignin, a natural polymeric product arising from an enzyme-

initiated dehydrogenative polymerization of three primary precursors; trans-p-coumaryl alcohol, trans coniferyl-alcohol and trans-sinapyl alcohol also forms part of the cellulose framework (Bisaria and Ghose, 1981; Raven *et al.* 1992). Ordinarily, the β,1-4-glycosidic bonds themselves are not difficult to break. However, because of these hydrogen bonds and the association with non-cellulosic polymers, cellulose crystals are so tightly packed that neither water nor enzyme can penetrate them. The inability of water to penetrate cellulose also explains why crystalline cellulose is insoluble. Parts in the cellulose that have less hydrogen bonds and less association with other non-cellulosic material are termed the 'amorphous cellulose' and this type is soluble and can be easily penetrated by enzymes (Wang, 2000).

Because the cellulose structure is so complex, its degradation is also a complex process requiring the participation of many enzymes (Bisaria and Ghose, 1981), produced by a group of microorganisms. These cellulolytic microorganisms include aerobic or anaerobic and mesophilic and thermophilic fungi and bacteria which occupy a variety of habitats. Among the best characterized cellulase systems are those of the white-rot fungus, *Phanerochaete chrysosporium* and those of soft-rot fungus [Coughlan and Ljungdahl, 1988]. *Neocallimastix frontalis,* an anaerobic fungus is found in the bovine rumen may contribute to cellulose production in municipal sewage sludge. This fungus is known to produce an extracellular cellulase system that catalyses extensive hydrolysis of crystalline celullose. Among aerobic cellulolytic bacteria are *Cellulomonas, Cellovibrio, Microbispora bispora* and *Thermomonospora species.* Examples of anaerobic bacteria (predominant inhabitants of anaerobic digesters) are *Acetivibrio cellulolyticus, Bacteroides cellulosolvens, Bacteroides succinogenes, Clostridium thermocellum, Ruminococcus albus* and *Ruminococcus flavefaciens* [Coughlan and Ljungdahl, 1988].

1.7. Enzymology of cellulose

Because cellulose is formed by repeated residues of glucose - it is called a *glucan* (Matthews and van Holde, 1990) and it is for this reason that cellulolytic enzymes are called ***glucanases.*** The cellulase system is a multi-component complex of enzymes possessing different types of activities. The three main types commonly mentioned in literature are *exoglucanases, endoglucanases and cellobiase (or β–glucosidase).* All of these enzymes are required for complete hydrolysis of insoluble cellulose and they act together in a synergistic fashion (Bisaria and Ghose, 1981).

1.7.1 Endoglucanases: hydrolyse cellulose chains by cleaving β–glycosidic bonds at random producing a rapid change in the degree of polymerization. The substrate being hydrolysed in solution results in a sharp drop in viscosity relative to the rate of release of reducing sugars. Endoglucanases are generally inactive against crystalline cellulose. They attack longer chains of cello oligosaccharides (amorphous cellulose and soluble derivatives such as CM-cellulose) at a high rate and the rate of their catalytic activity increases with the degree of polymerisation [Mahalingeshwara Bhat and Wood, 1988]. It has been stated that as many as ten different polypeptide in the cellulase complexes exhibit endoglucanase activity [Coughlan and Ljungdahl, 1988].

1.7.2 Exoglucanases: found as major components in some cellulase systems [Mahalingeshwara Bhat and Wood, 1988] are present in two major forms. (a) The β,1-4 glucan cellobiohydrolase (CBH) which removes cellobiose from the non-reducing end of the cellulose chain [Mahalingeshwara Bhat and Wood, 1988]. (b) β,1-4 glucan glucohydrolases, which removes glucose units also from the non- reducing end of the cellulose chain (Bisaria and Ghose, 1981). Definitive proof of the existence of these enzymes in the cellulase complexes is still under investigation. However, the fact that cellobiose is the major (>90%) product of cellulose hydrolysis by *C. thermocellum* complexes may suggest such a possibility [Coughlan and Ljungahl, 1988].

1.7.3 Cellobiase or β-glucosidase: a very important component of the cellulase system (and the focus of this study) is responsible for the conversion of short-chain cello oligosaccharides and cellobiose (which are released by other cellulases) into glucose. The catalytic activity of β-glucosidase decreases with increasing degree of substrate polymerisation, but it does not attack cellulose. Some β-glucosidases have been reported to hydrolyse aryl-β-glucosides but not cellobiose. Other characteristics are that they are not specific for the 1.4-β-linkage only, but also possess a transferase activity that acts on glucose units to form other sugar molecules such as dimers, trimers, and higher oligosaccharides [Mahalingeshwara Bhat and Wood, 1988].

Cellulose systems containing low levels of β-glucosidase have poor saccharifying power as the cellobiose produced by exo and endoglucanases has an inhibitory effect. The synergistic contribution of β–glucosidases has therefore a vital role in the solubilization of cellulose as it attenuates the whole celluloytic process by continuous removal of cellobiose into glucose.

Extensive studies on cellobiase (under anaerobic conditions) will therefore bring some kind of advancement in the knowledge of celullose breakdown and perhaps contribute to the improvement of cellulose fermentation as cellobiose production is rate limiting (Bisaria and Ghose, 1981).

1.8 The process of cellulose degradation.

The physical properties of cellulolytic complexes of anaerobic bacteria: For effective cellulose degradation evidence obtained from extensive research on cellulolysis indicates that the anaerobic cellulolytic bacteria must attach themselves to the substrate (cellullose). The multicomponent cellulolytic complexes produced by these anaerobes termed *polycellulosomes* are located on the cell surface for this reason (Figure 1.7). The characteristic polycellulosome is extremely stable and maintains a defined supramolecular structure [Bayer and Lamed, 1988] and has a diameter of 60 nm and a calculated mass of 50 to 80 x 10^6 Da. [Coughlan and Ljungdahl, 1988].

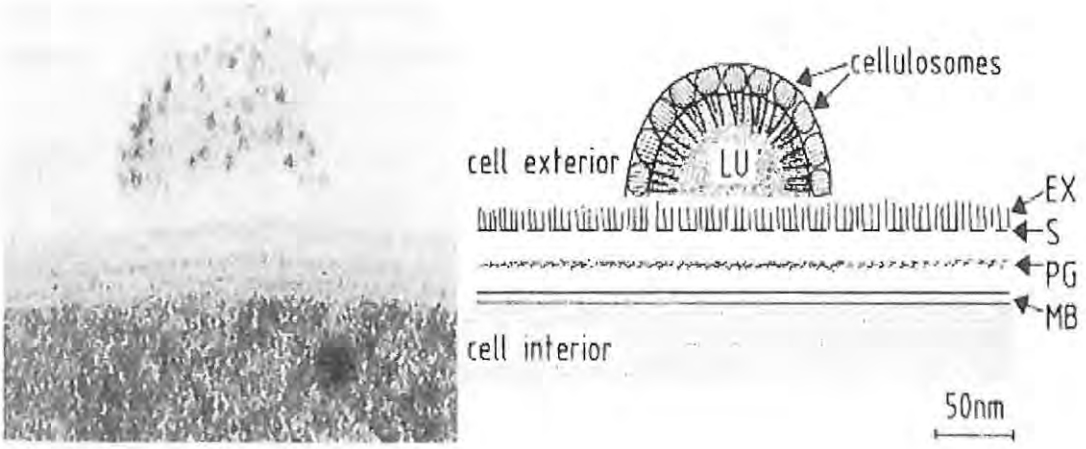

Figure 1.7: Structural details of a quiescent polycellulosomal protuberance. Left: Immunoelectron micrograph showing an anticellulosome-specific, ferritin labelled protuberance and its topographical relationship to the cell surface. Right: Diagrammatic representation of the same, based on similar micrographs (both SEM and TEM) combined with cationized ferritin labeling. The abbreviations in the Figure designate the following: MB - cytoplasmic membrane; Pg - peptidoglycan; S - "S" layer (which defines the outer dense plain of the cell surface); EX - electron transport exocellular anionic (cationised ferritin reactive) material: and LU - lumen of protuberance [Bayer and Lamed, 1988].

The polycellulosome is comprised of tightly packed spherical entities namely, cellulosomes (16 to 18 nm in diameter) with a mass of 2 – 2.5 kDa. The cellulosomes in turn are comprised of about 35 polypeptides ranging from 45 kDa to about 200 kDa [Coughlan and Ljungdahl, 1988]. Wang, [2000] states that not all of these polypeptides possess catalytic activity. Recent research has shown that one of the cellulase components is relatively inert but has the ability to recognise and attach itself to the surface of the cellulose mass [Figure 1.8] in addition to the ability of recognising and holding onto another protein component that exhibits enzymatic activities. Thus the chance of reaction is significantly enhanced by a close proximity effect [Wang, 2000]. It has been suggested that this component has a molecular weight of 200-210K and that it is not unique to just one type of bacterial species but is produced by each of the different species of anaerobic bacteria [Bayer and Lamed, 1988].

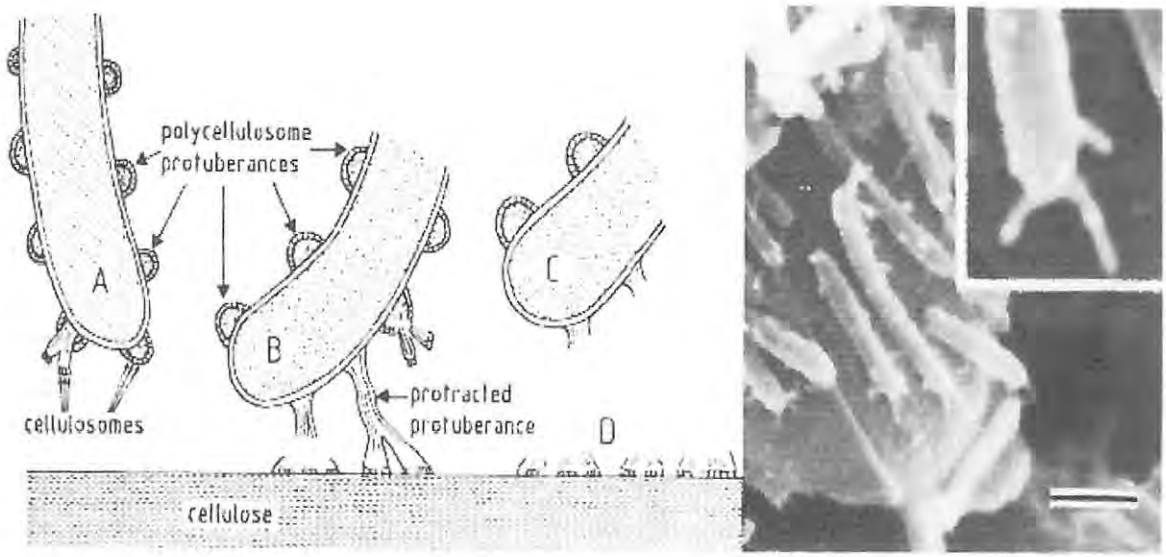

Figure 1.8: Interaction (adhesion-desorption) of C. thermocellum cells with cellulose, mediated by the protracted polycellulosomal protuberances. Left: Diagrammatic representation (based mainly on TEM studies) of major ultra structural events which, accompany the adsorption / desorption processes. Cell A represents a cell immediately prior to contact with the cellulosic substrate; cell B, a cell following contact; and cell C, a cell following detachment from the substrate leaving cellulosome clusters on the cellulose surface (D). Right: SEM of cationized ferritin-stained cells of C. thermocellum attached to microcrystalline cellulose. Insert: A higher magnification of protracted protuberances. **Bar 1μm [Bayer and Lamed, 1988].**

There are several ways by which cells may adhere to substrate surfaces. These include biospecific adhesions or relatively "nonspecific" charge-mediated or hydrophobic interactions. In many cases the initial adhesion may be followed by secondary interaction processes leading to firm colonization [Bayer and Lamed, 1988].

1.9 Factors affecting the enzyme-substrate complex formation.

Since it is essential that bacteria which attack insoluble substrates maintain physical contact with the substrate [Bayer and Lamed, 1988], factors affecting the formation and the activity of enzyme-substrate complex are critical to cellulolysis.

The Yellow Affinity Substance (YAS): During growth on cellulose some of these anaerobic bacteria e.g. *C. thermocellum* secrete a water insoluble yellow pigment that coats the substrate. The yellow pigment, carotenoid-like in nature with an elemental composition of $C_{52}H_{94}O_{19}N$ is termed the Yellow Affinity Substance (YAS). It is known to facilitate the attachment of the cellulolytic enzyme complex to cellulose, since the coated coloured cellulose had been discovered to bind greater amounts of enzyme than does ordinary cellulose. *A. cellulolyticus* is also known to secrete the water soluble YAS when growing on cellulose.

3-Phenyl propanoic acid (PPA). The enzyme complexes in other anaerobic bacteria such as *R. albus* are only formed if the growth medium contains PPA or rumen fluid, a source of PPA. PPA is a precursor of β-ketoalanine, which is a compound that is involved in the formation of a capsular structure that surrounds the cell, contains the cell complex and contains the cellulase complex. In the absence of PPA the capsular structure is not formed around the cells and around the polycellulosomes (vesicular structures). Under such conditions cellulolytic activity is still produced but is associated with low molecular weight free polypeptide and is not active against crystalline cellulose.

Ageing of bacterial culture: As the bacterial cultures age, the affinity factor (akin to the 210K polypeptide seen in all anaerobic cellulolytic complexes) is lost resulting in disaggregation of the enzyme substrate complexes. Subsequently these complexes are released from the cells into the fluid or growth medium resulting in their desorption from the substrate. The complexes may remain

for a time as functional complexes in the medium but they ultimately decompose to free polypeptide, hence the presence of the extracellular activity.

The activity of enzyme complexes against crystalline cellulose may be dependent on calcium or magnesium, dithiothreitol (or other thiols) and some additional factors mentioned below. Whether the enzyme complex is stimulated, inhibited or unaffected is dependent on (i) the nature of cellulose substrate to be hydrolysed [i.e. whether the substrate is amorphous or crystalline], (ii) bacterial species producing the enzyme and (iii) the state of the enzyme (whether the enzyme is free in solution or membrane bound). For example dependence on Ca^{++}, Mg^{++} and thiols is true for *C. thermocellum, R. flavefaciens and A. cellulolyticus*. The activity of *B. cellulosolvens* is inhibited by EDTA and stimulated by dithiothreitol or ascorbate. The activities of *R. albus* and *B. succinogenes* against crystalline cellulose are diminished under aerobic conditions and are maintained under reducing conditions. Fe^{++} has been mentioned as an essential cofactor in the *C. thermocellum* complex.

By contrast with their activity against crystalline cellulose, the complexes require neither calcium nor thiol for activity against amorphous or soluble derivatives of cellulose nor is either agent needed for the activity of the free endoglucanases against CM-cellulose [Coughlan and Ljungdahl, 1988]. Thus it is concluded that Ca^{++} and thiols could be needed for an exo-acting enzyme in the complexes even though the presence of such activity is yet to be investigated further.

1.10. The mechanism of cellulose degradation.

The unravelling of mechanisms of cellulose enzymic hydrolysis is a challenge to biochemists, geneticists, microbiologists and to those who wish to use cellulose as a source of biochemical feedstock. Although cellulose degradation is not yet completely understood, many studies on the fungal systems have provided much information for the elucidation of other cellulase systems occurring in bacteria. However these studies indicate that the mechanism of cellulose degradation in anaerobic bacteria is different from that operating in aerobic and fungal systems.

Since cellulose does not occur in a pure form, it is necessary to explain what happens to lignin and hemicelluloses for a better understanding of cellulose breakdown. Hemicellulose (a polymer composed by mannoses, xyloses, arabinoses and other sugars) is broken down by hemicellulases

which occur in two basic forms i.e. the exo and endotypes. Thus there are arabinases, galactanases, mannases and xylanases among the hemicellulose degrading enzymes (Bisaria and Ghose, 1981). Four kinds of enzymes have been implicated in lignin breakdown: ligninase, laccase, manganese peroxidase and H_2O_2 producing enzymes (Kirk, 1988). The hydrolytic activity and the synergistic interactions of these enzymes help to create more accessible cellulosic regions that can be acted upon by exo and endoglucanases. Some cellulase complexes have been found to possess xylanase activity hence the term multi- rather than unicomplex systems (Leschine, 1995). An interesting point to make is that recent work carried out on enzyme activity in sewage sludge hydrolysis, using the API-ZYM^{TN} qualitative test kit indicated the presence of galactosidases and mannosidases as well as glucosidases [Molipane, 1999] which have been mentioned to be part of hemicellulase and cellulase complexes respectively.

Because initial work on cellulolysis employed the fungal system as a model, a brief description of the enzymology of that system will be included in this text for clarity. In fungi, endoglucanases attack amorphous regions of cellulose fibres producing reducing and non-reducing ends for exoglucanases. The latter in turn hydrolyse cellobiose from more crystalline regions of the cellulose chain. Finally, β–glucosidase hydrolyse cellobiose and prevent its accumulation [Leschine, 1995].

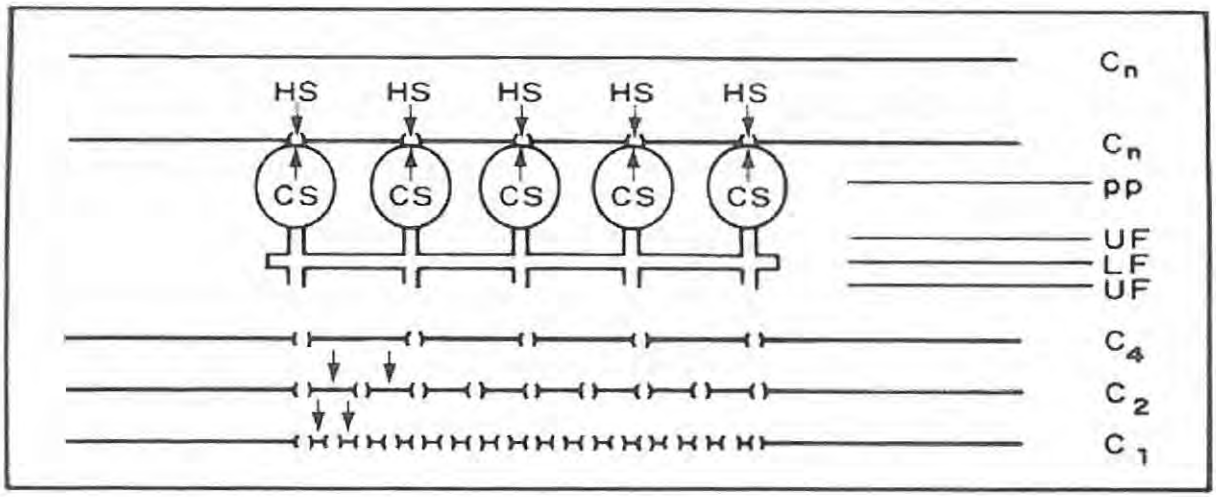

Figure 1.9: Diagrammatic view of the main aspect of the structural concept of the C. thermocellum cellulosome from isolated strains JW20 and YM4, and of the inherent structure-function relationships. (CS) - catalytic site; (HS) - hydrolysis site [i.e. the site on the cellulose chain at which a glycosidic bond is cleaved]; (UF) - ultra thin fibrils; (LF) - central string of unknown composition; (pp) - polypeptide; (C_n, C_4, C_2, C_1) - cellodextrins of various lengths, C_n being cellulose and C_1 being cellobiose [Couglan and Ljungdahl,1988].

In contrast to the cellulase system of aerobic fungi described above, the ultra structural details of cellulolytic enzyme complexes produced by anaerobic bacteria are best resolved in loose cellulosomal particles. Genes encoding several of these cellulosomal particles or polypeptides have been cloned and their nucleotide sequences determined. The cellulose integrating protein known as the CipA (i.e. 210K polypeptide) has a cellulose-binding domain [LF-central string] and nine internal sequences [ultra thin fibrils (UF)] that bind catalytic subunits. That is, the latter are attached to the central string by ultra thin fibrils (UF) and are arranged in ordered chain like arrays in a defined orientation. This orientation is such that these polypeptide chains exhibit rows of equidistantly spaced subunits. The average centre-to-centre distance between the catalytic sites of the neighbouring identical subunits has been estimated to be about four nanometres. This estimated distance is equivalent to the length of a cellooligosaccharide consisting of four cellobiose (C_4) units. It is assumed that these subunits represent individual polypeptide with enzymatic activity, implying that the lengthy cellulose fibre is aligned beside this row of subunits and that its degradation is a multi-cutting event.

This event occurs extracellularly resulting in the formation of C_4 subunits (from the cellulose chain), which in turn diffuse into the cellulosome. Inside the cellulosome the C_4 subunits are assumed to be further broken down to smaller cellodextrins (C_1, C_2), either by a similar multi cutting event mediated by chains of smaller subunits with smaller centre to centre distances also present in the same cellulosome or by single cuts [Couglhan and Ljundahl, 1988].

Such a series of proposed reactions has been reported to be consistent with the findings that many polypeptide components of cellulosomes possess enzymatic activity and that cellobiose (smaller cellodextrins) accounts for greater than 90% of the products of cellulose hydrolysis by isolated cellulosomes [Coughlan and Ljungdahl, 1988; Leschine, 1995].

1.11. Research hypothesis.

The process of sulphate reduction is driven by the availability of low molecular weight organic substrates, which act as electron donors. The occurance of primary sewage sludge organic substrates in a complex form makes these substrates unsuitable for utilization by SRB. Whittington-Jones, (1999) proposed that the greatest challenge that needs to be overcome before primary sewage can be used effectively as a source of electron donors is to increase both the rate of hydrolysis and

the yield of soluble products. It has been suggested that, two factors that have the greatest impact on the rate limiting hydrolytic step were the concentration of the hydrolytic enzymes and their substrates. To date, little or no information is available regarding the specific enzymes involved in the hydrolysis of the complex polymers and the concentration of these enzymes in the primary sewage sludge. In addition, recent studies have also shown that the rate and extent of cellulose degradation is enhanced in the presence of sulphur compounds [Coughlan and Ljungahl, 1988; Kim et. al., 1997; Pareek et. al., 1998 and Whittington-Jones, 1999]. The mechanism by which this enhancement occurs is, however, not yet understood. It has therefore been proposed that; if enhanced solubilization of primary sewage sludge in the presence of sulphur containing compounds is controlled in part by enzymes (cellulases and glucohydrolases), these enzymes have an important role in the overall process of sulphate reduction. For this reason, the behaviour of cellulases and glucohydrolases under sulphidogenic conditions needs to be addressed. Optimisation of the activity of the enzymes may improve cellulose degradation both *in situ* and in specialised bioreactors thus providing a better carbon source (glucose) for SRB in sulphate reduction.

1.12. Research aim.

These studies were therefore undertaken to investigate the role of β–glucosidase in the solubilization of primary sewage sludge.

1.13. Research objectives.

1. To identify, locate, isolate, partially purify and quantify the cellulases and glucohydrolases in sewage sludge.

2. To investigate the distribution of these enzymes with respect to time, temperature and pH.

3. To investigate the effect of sulphur compounds (sulphite and sulphide), which are produced during the process of sulphate reduction on enzyme activity.

4. To investigate the effect of heavy metals e.g. Zn^{++}, Ni^{++}, Fe^{++} and Cu^{++} on glucohydrolases.

5. To investigate the effect of volatile fatty acids [butyric, propionic and acetic] on enzyme activity.

6. To use the information obtained to improve the overall performance of sludge solubilization and design more efficient reactors.

7. To maximise and quantify the hydrolysis products from the action of the enzymes.

8. To optimise the process of sulphate reduction and AMD bioremediation.

CHAPTER TWO

THE PRODUCTION OF CELLULASES AND GLUCOHYDROLASES DURING ANAEROBIC DIGESTION OF SEWAGE SLUDGE.

2.1 INTRODUCTION.

2.1.1 Background

While chapter one deals with the microbiology and biochemical pathways of anaerobic digestion, this chapter focuses on the enzymology of primary sludge solubilization and the practical operation of anaerobic digesters. The fundamental unit of the anaerobic digestion process is a tank, usually a circular concrete tank with or without a cover. In those tanks without a cover the sludge mass at the top of the tank will dry out quite quickly forming a rather solid cover. Initially the tanks were designed to hold the sludge solids for several months, while the microorganisms slowly brought about digestion [Hobson and Wheatley, 1993].

As the volume of waste solids increased, there was a demand for a more rapid process. The addition of heat resulted in increased biological activity with a shortened digestion period. Mechanical mixing, agitation, gas mixing, low speed stirring and rapid mixing were used as a means of speeding up the process and it soon became possible to accomplish in 30 days (i.e. typical mean sludge retention period for complete digestion) [Lester and Sterrit, 1990] what used to take 4 to 6 months [Mckiney, 1962].

Decomposition of the complex organic matter present in sewage sludge is primarily a function of enzymes elaborated by anaerobic bacteria. Initially these bacteria use the organic matter as their feedstock for growth and then ultimately break it down into soluble substrates that are less hazardous and can be easily disposed off to the environment. Some of the substrates produced are those exploited by SRB as carbon source for sulphate reduction.

2.1.2 Feed source and seeding

The two basic constituents required for anaerobic digestion in tanks containing wastes are: (i) a diverse bacterial population that will carry out the different biological reactions concerned with the breakdown of different molecules and (ii) a carbon source. The seed or inoculum for digesters may be developed from the feed (in this case, the faecal waste) which is known to contain at least small numbers of an active bacterial population to initiate digestion [Hobson and Wheatley, 1993]. Another way of inoculating or seeding a digester is to use digested or digesting sludge

from another similar installation that is operating well. In order for the seeding to be effective the quantity of seed or bacterial inoculum should usually be at least 10% or more of the digester volume [Gurnham, 1955].

Along with the inoculum, many other types of bacteria such as facultative bacteria may be introduced into the digester. They may continue to grow as a minor component of the bacterial population by utilizing some of the feed or a metabolic product of the active bacterial group. On the other hand, if the digester is a continuously fed system, these bacteria may not be growing in the digester. They may be introduced continuously with the feed and will always make a certain number of the viable cells but will have no specific role in digester reactions. Special bacterial cultures and enzymes have been marketed for the purpose of starting digestion or for accelerating the activity of a digester that has been slowed by toxic materials or other causes. The effectiveness of these aids is of doubtful value [Gurnham 1955; Hobson and Wheatley, 1993]. Hence the option for studying and optimizing the enzymes already existing in the digesters will be a better option.

2.1.3 Temperature and pH control

Apart from substrates, bacteria require suitable pH and temperature conditions for optimal growth and these have been mentioned in the previous chapter. As the digestion is carried out by mixed interdependent cultures, the pH of the digester must be held at some value which allows the activity of all the bacteria without being optimum for all the reactions. Single stage reactors in which all reactions take place are therefore run at a pH of 7 to 7.5. In some cases it is possible that the pH of the feedstock may need correction if it is initially acidic or alkaline. At the Grahamstown municipal sewage digesters pH is regulated as a common practice by the addition of lime ($CaCO_3$). Similarly, temperatures at which the bacteria can grow include a wide range.

2.1.4 Mixing

Mention has been made that bacteria attacking solid substrates should maintain physical contact with the substrate. Mixing therefore ensures the dispersion of organic particulate matter to all points in the digester. In this way contact between the microorganisms and the substrate is enhanced. Digester operation, like all biological processing is subject to interference by many

other factors such as toxic materials introduced into the digester. This aspect will be dealt with, in later chapters.

Though it is a known fact that the hydrolytic reactions taking place during anaerobic digestion are a function of enzymes, only the microbiological (i.e. different bacterial species) and the biochemical (i.e. different metabolic pathways taking place) aspects of digestion have been explored. Very little is known about the actual enzymes carrying out these reactions. In this chapter, the production of cellulases and glucohydrolases during anaerobic degradation was studied. In addition, breakdown of total polymeric carbohydrate, production of reducing sugar, COD removal, pH fluctuations, removal of sulphate and production of sulphide in the bioreactors during the course of anaerobic digestion were followed.

2.2. METHODOLOGY.

2.2.1 Reactor design and operational procedures.

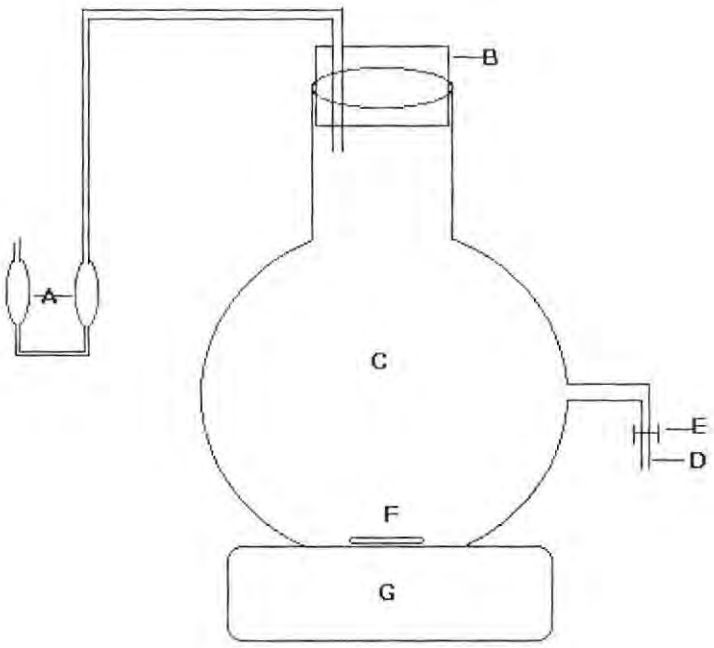

Figure 2.1: Schematic diagram of the laboratory-scale (single-stage) stirred tank reactor, used for monitoring the production of cellulases and β-glucosidases during anaerobic degradation. A - gas trap containing zinc acetate solution, B - rubber stopper, C - 10 or 20 L glass tank, D - sampling port, E - metal clamp, F - magnetic bar, G - magnetic stirrer.

Experimental setup and seeding

Lab-scale stirred-tank methanogenic (where methane was produced as one of the final products) and sulphidogenic (where sulphide was produced from sulphate reduction) reactors were set up. The schematic diagram for the setup is illustrated by Figure 2.1.

The 10 L methanogenic (control) reactor was seeded with 10% (1000ml) of a mixed culture of methanogenic bacteria collected from an old standing digester of the Grahamstown sewage plant. This reactor was used to monitor the production of cellulases and glucosidases, the breakdown of polymers and production of soluble substrates under normal anaerobic conditions (i.e. in the absence of SRB and sulphate). The sulphidogenic (experimental) reactor was used to monitor the production of cellulolytic enzymes under sulphidogenic conditions. The latter was also seeded with 10% (2000ml) inoculum of a mixed culture of SRB obtained originally from the Grootvlei dam and cultured in fermentation tanks at the AIPS (Grahamstown). Sulphate (2000 mg/L) was added to the sulphidogenic reactor to simulate the sulphate required as a substrate.

Feeding: The feed or carbon source for both reactors was fresh raw sewage sludge obtained from the underflow lines of the primary clarifiers of the Grahamstown sewage plant. The raw sludge was collected in the early morning (i.e. between 8 and 10 am.) as its nature changed during the day. The sludge was sieved through a 2 mm^2 sieve to remove particles that may cause blockage in the sampling port tube of the reactors. After sieving, the sludge was collected into 2 L plastic containers and taken to the labs for Chemical Oxygen Demand (COD) analysis. COD is a measure of oxygen equivalent of the organic matter content of a sample that is susceptible to oxidation by a strong chemical oxidant. In other words COD is an amount of oxidisable organic matter expressed in terms of the oxygen content. When the COD of the feed was obtained, the sludge was diluted to a concentration of 2000 mg/L COD as an indication of a reasonable amount of substrate. Both reactors were batch fed and operated as closed systems. The remaining raw sludge was stored at 4^0C for later use. Aluminium foil was used to cover the reactors to prevent any photosynthetic reactions that may result from the activities of facultative bacteria and exposure to light.

Mixing: Both reactors were placed on magnetic stirrers and specialized magnetic bars were placed inside. In this way, the contents of the digesters were subjected to moderate mixing to

ensure a reasonable distribution of the solid substrates and their exposure to the hydrolytic enzymes. Rubber stoppers were used to close the tanks and the latter were placed in a fume hood for extraction of any toxic gas (i.e. sulphide and methane) that may escape from the reactors. A gas trap containing zinc acetate was also used to react with H_2S escaping from the sulphidogenic reactor. Production of sulphide was indicated by the formation of a white precipitate (ZnS) in the gas trap.

2.2.2 Sampling.

Sampling for analysis was done every second day for a period of 31 days. A volume of 80 ml was sampled from each reactor and was fractionated into 20 ml plastic bottles. The samples were stored at -70^{0}C to be analyzed at the end of the digestion period, that is, on the 31st day.

2.2.3 Enzyme assays

Principle

Methylumbelliferyl derivatives, methylumeblliferyl-glucoside (MUF-Glu) and methylumbelliferyl-cellobioside (MUF-Cell) that were used as substrates, both produce the fluorescent methylumbelliferone and the attached carbohydrate, when digested by their respective enzymes [Figure 2.2]. The degree of substrate hydrolysis is determined by fluorimetric measurement of methylumbelliferone liberated in the reaction. In an alkaline solution, methylumbelliferone is excited at a wavelength of 365 nm and emits fluorescence at 455 nm.

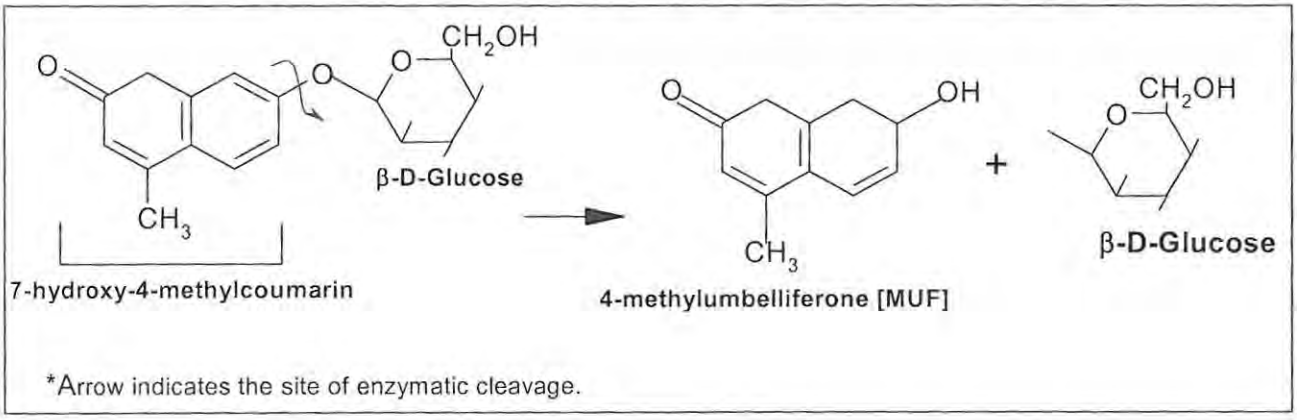

Figure 2.2: The cleavage of Methylumbelliferyl-β-D-glucopyranoside by β-Glucosidase.

Reagents.

1. All substrates and other chemicals were purchased from Sigma, except for 95% ethanol that was obtained from Rhodes University Chemistry stores.
2. 50 mM 4-methylumbelliferone in 50% ethanol.
3. (1.5 mM) 4-methylumbelliferyl-β-glucose (MUF-Glu). 1.5mM 4-methylumbelliferyl-β-cellobiose (MUF-Cell). Both substrates were dissolved in phosphate buffer (0.1M, pH 7.0).
4. Glycine buffer [60 g of glycine in 1500 ml of dH_2O and adjusted to pH 10.8 with (50% w/v NaOH solution)].
5. 10 ml plastic centrifuge tubes, 10 ml glass tubes, 3 ml glass cuvette, incubator set at 50^0C, Spectrofluorimeter [Model F-2500 Fluorescence spectrophotometer].

Procedure

All sludge samples from day 1-31, from both reactors were thawed at room temperature, samples mixed well and were ready for analysis. 0.5 ml sludge and substrate (MUF-Glu or MUF-Cell) were mixed in 10 ml plastic centrifuge tubes and incubated on a rotary shaker (300 rpm) at 50^0C for 10 and 30 min, respectively.

Reactions were stopped by addition of 2.5 ml of 95% ethanol. Tubes were thoroughly mixed and transferred to an ice bath. Suspended sludge particles (in the tubes) were removed by centrifugation at 2 460 g [JA-14 rotor, Beckman J2-21 centrifuge] for 5 min at 0^0C and the supernatant was decanted into clean glass test tubes containing 0.5 ml of glycine buffer (pH 10.8). From this point the samples were stable for at least an hour.

To measure MUF produced, 3 ml of the sample was added to a glass cuvette and fluorescence was measured on a Spectrofluorimeter at an excitation wavelength of 365 nm and an emission wavelength of 455 nm. Two types of blanks were prepared, i.e. the substrate and the enzyme blanks. For the enzyme blank, only the sludge alone was incubated at 50^0C, addition of ethanol followed, then the substrates were added, centrifuged and decanted into tubes containing glycine buffer. Enzyme blank was used to test for possible indigenous fluorescence in the sludge.

Substrate blanks were prepared in the same way. The only difference was that the substrates were incubated first at 50^0C, to test if heat had any hydrolytic effect on them.

After the blank fluorescence was read, MUF fluorescence was calibrated by adding 0.03 ml of the 50 mM MUF standard to the cuvette containing the blank, after which fluorescence was measured again.

2.2.4 Assay for total hydrolysis of polysaccharides.

The Phenol-Sulphuric method was carried out as described by Mahalingeshwara Bhat and Wood, 1988.

Principle

The method is based on colour reaction between carbohydrate and phenol reagent in concentrated sulphuric acid. The orange colour development is a result of polysaccharides acid hydrolysis to monosaccharides. Formation of an orange colour from this reaction is measured spectrophotometrically at 488 nm [Dubois et. al, 1956].

Reagents

1. 5% (w/v) aqueous solution of phenol (stored at 4^0C)
2. 98% H_2SO_4
3. Glucose stock solution [1 mg/ml]

Procedure

Sludge samples (1.0 ml) were pipetted directly into the bottom of a glass test tube (1.5 x 15 cm). Phenol reagent (1.0 ml) was added in the same way, followed by 5 ml of concentrated H_2SO_4 pipetted directly onto the mixture of phenol and sludge from a fast flow pipette or a suitable dispenser. The solutions were mixed immediately and allowed to cool before absorbance was

read at 488 nm. The absorbance values (after subtraction of reagent blanks) were then translated into glucose equivalent using a standard curve obtained by plotting glucose against absorbance. The glucose standard curve was constructed by using suitably diluted sugar solutions with a concentration range of 0-15 μg/ml, (Table 2.1).

TABLE 2.1: Preparation of glucose standard curve for total carbohydrate determination.

Glucose conc. (μg/ml)	Glucose stock solution (μl)	Distilled water (μl)	Phenol solution (ml)	Conc. H_2SO_4 (ml)
0	0	1000	1	5
1.43	10	990	1	5
2.86	20	980	1	5
4.29	30	970	1	5
5.71	40	960	1	5
7.14	50	950	1	5
8.57	60	940	1	5
10.00	70	930	1	5
11.43	80	920	1	5
12.86	90	910	1	5
14.29	100	900	1	5

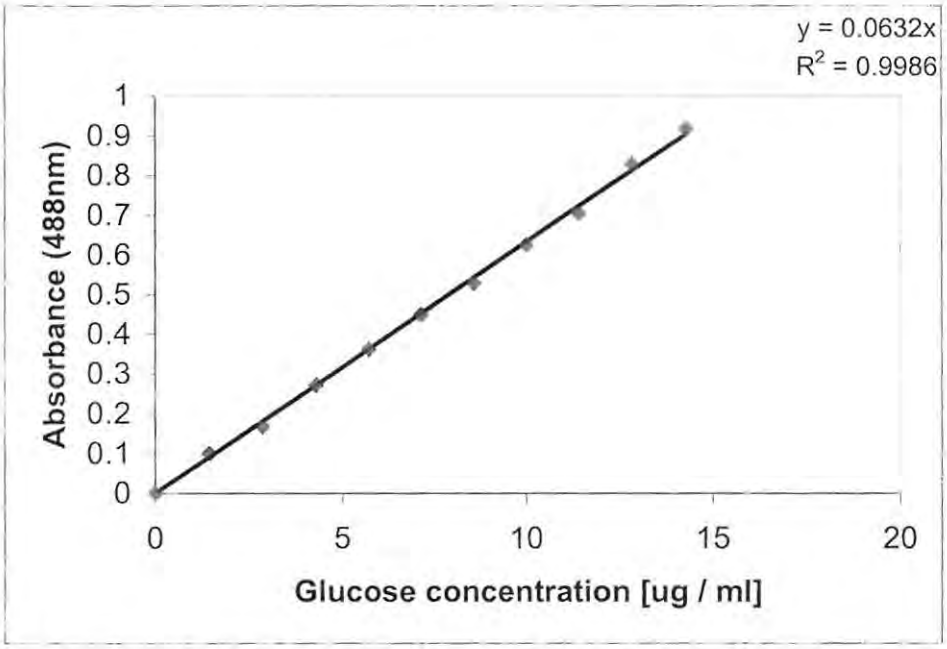

Figure 2.3: Total carbohydrate standard curve prepared with glucose for the phenol sulphuric acid method

2.2.5 Assay for estimation of glucose production.

The Somogyi-Nelson method used for determination of glucose production is a modification of the procedures described by Mahalingeshwara Bhat and Wood, (1988) and Clark and Switzer, (1977).

Principle

A sugar is heated with an alkaline solution of copper tartrate and cuprous oxide is produced. Cuprous oxide reacts with the arseno-molybdate to give a molybdenum blue color. This intense blue color is then measured spectrophotometrically at 520 nm. Sodium sulphate is included in the reaction mixture to minimize the entry of atmospheric oxygen in solution that would cause the reoxidation of cuprous oxide.

Materials

$CuSO_4.5H_2O$, $NaCO_3$, NaK tartrate, Na_2SO_4 in water, ammonium molybdate $[(NH4)_6Mo_7O_{24}.4H_2O]$, sodium arsenate $[Na_2HASO_4]$, concentrated H_2SO_4.

Reagents.

1. **Copper reagent:** Anhydrous sodium sulphate (45 g) was dissolved in 100 ml of boiled distilled water followed by 6 g of sodium carbonate, 4 g of potassium sodium tartrate and 1g of copper sulphate. The solution is made up to a volume of 250 ml and stored at 27^0 C to prevent the reagent from crystallizing when the temperature drops.

2. **Arseno-molybdate reagent:** (i) Ammonium-molybdate (25 g) was dissolved in 450 ml of distilled water followed by the addition of concentrated sulphuric acid (21 ml) to make solution A. (ii) Sodium-arsenate (3 g) is dissolved in 25 ml of distilled water to make solution B. (iii) Solution A and B are added together and incubated at 37^0C for 24-48 hours in a brown bottle then stored at room temperature. NB: the reagent is sensitive to light. The reagent must also be slightly yellow without any green tint.

Procedure.

The sludge samples (1.0 ml) from both reactors were each added to different glass test tubes (1.5 x 10 cm) containing 5.0 ml of distilled water, followed by addition of the copper reagent (1.0 ml). The mixtures were incubated for 20 min in a water bath that was set at 80^0C. The samples were then allowed to cool at room temperature for about 15 min. Arseno Molybdate reagent was added to produce a blue colour and the samples were mixed carefully on a vortex mixer. The samples were centrifuged at 2 460 g [JA-14 rotor, Beckman J2-21 centrifuge] for 10 min (at room temperature) to remove any suspended sludge particles. Absorbance was read at 520 nm and translated into glucose equivalent using a standard curve obtained by plotting micrograms of glucose added against absorbance. The concentration range for glucose standards was (0-60 μg/ml).

Table 2.2 Preparation of the glucose standard curve by the Somogyi-Nelson method

Glucose conc. (μg/ml)	Glucose stock solution (ml)	Distilled water (ml)	Copper reagent (ml)	Arseno Molybdate solution (ml)
0.00	0	6.00	1	1
6.25	0.05	5.95	1	1
12.5	0.10	5.90	1	1
18.75	0.15	5.85	1	1
25.00	0.20	5.80	1	1
31.25	0.25	5.75	1	1
37.50	0.30	5.70	1	1
43.75	0.35	5.65	1	1
50.00	0.40	5.60	1	1
56.25	0.45	5.55	1	1

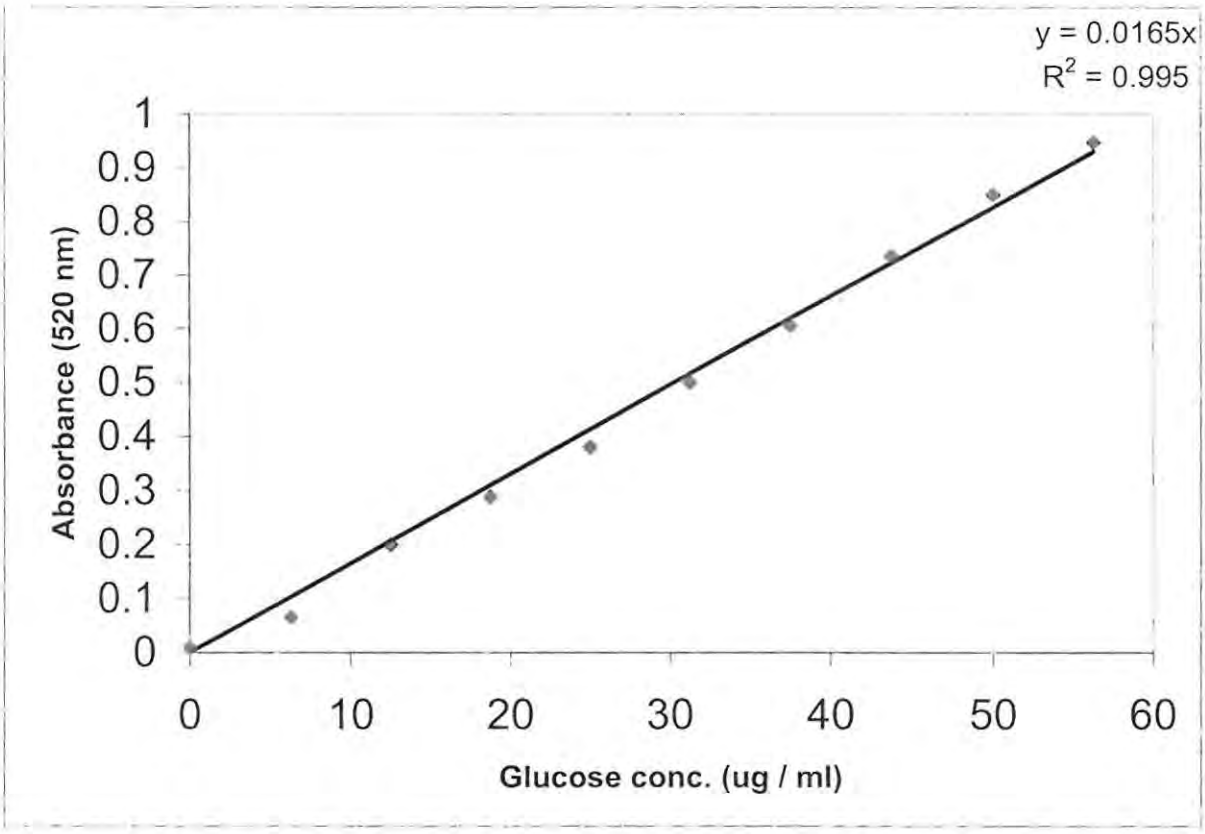

Figure 2.4: Glucose standard curve for the Somogyi-Nelson method.

2.2.6 Analytical procedures

Procedure for sulphate removal, sulphide production and COD determination were carried out as described in Standard Methods (APHA, 1985). Fluctuation of pH in both reactors for the whole course of anaerobic digestion was also monitored.

2.2.6.1 Sulphate assay.

Principle

Addition of Barium salt precipitates 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].

Reagents

1 Barium Chloride (0.205 M) solution: $BaCl_2.2H_2O$ [50 g in 1000 ml dd H_2O]

2 Buffer solution A: $MgCl_2.6H_2O$ (30 g), $CH_3COONa.3H_2O$ (5 g), KNO_3 (1 g), CH_3COOH (20 ml), dd H_2O (880 ml)]

3. Sulphate stock solution (100 µg/L): Na_2SO_4 (0.15335 g) in 250 ml of dH_2O. A range of standards between 0 and 612 mg/L were prepared as shown in Table 2.3.

Procedure

To 1 ml of each of different sludge samples 0.2 ml of buffer solution A was added, followed by addition of 0.1 ml of barium chloride solution. The tubes were mixed well. Absorbance was read at 420 nm using a Schimadzu spectrophotometer.

Table 2.3: Preparation of the sulphate standard curve.

$SO_4^=$ conc. (mg/L)	Stock solution (ml)	d H_2O (ml)
0.0 (Blank)	0.0	1.0
122	0.2	0.8
245	0.4	0.6
367	0.6	0.4
490	0.8	0.2
612	1.0	0.0

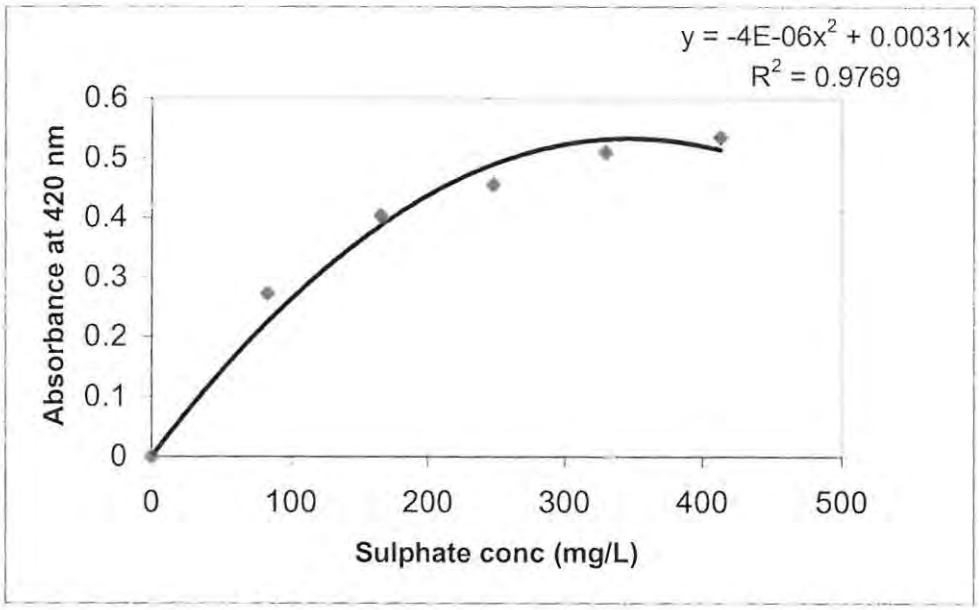

Figure 2.5: Standard curve for the turbidimetric determination of sulphate.

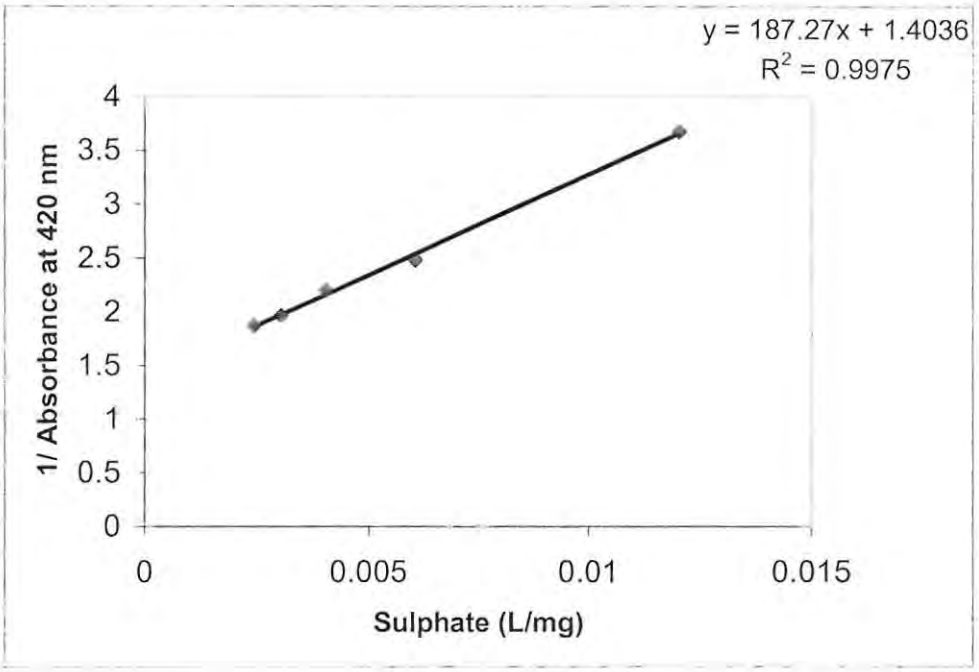

Figure 2.6: Standard curve (double inverse) for the turbidimetric determination of sulphate

2.2.6.2 Sulphide assay

Principle

The reaction of N,N-dimethyl-phenylene-diamine with hydrogen sulphide in the presence of ferric chloride results in the formation of a methylene blue thiazine dye. The concentration of sulphide is proportional to the intensity of the blue colour [Rees *et al.* 1971].

Reagents

1. Amine-Sulphuric acid stock solution (0.02 M): (2 g) N,N-dimethyl-phenylene-diamine-dihydrochloride dissolved in 500 ml of HCl.

2. Ferric Chloride solution (0.06 M): $FeCl_3.6H_2O$ (8 g) dissolved in 500 ml of HCl.

3. Sulphide stock solution: $Na_2S.9H_2O$ (0.789 g) dissolved in 500 ml of distilled water. A standard curve with concentrations ranging from 0-1 mg/L was prepared according to Table 2.4.

Procedure

Amine-Sulphuric acid solution (0.5 ml) and ferric chloride solution (0.5 ml) were added to 5 ml of the sludge samples. The mixture was allowed to stand for an hour to develop the blue colour. Absorbance was read at 670 nm using a spectrophotometer.

Table 2.4: Preparation of a the sulphide standard curve.

Conc. (mg/L)	Stock solution (ml)	dd H2O (ml)
0 (blank)	0	100
0.2	2	98
0.4	4	96
0.6	6	94
0.8	8	92
1.0	10	90

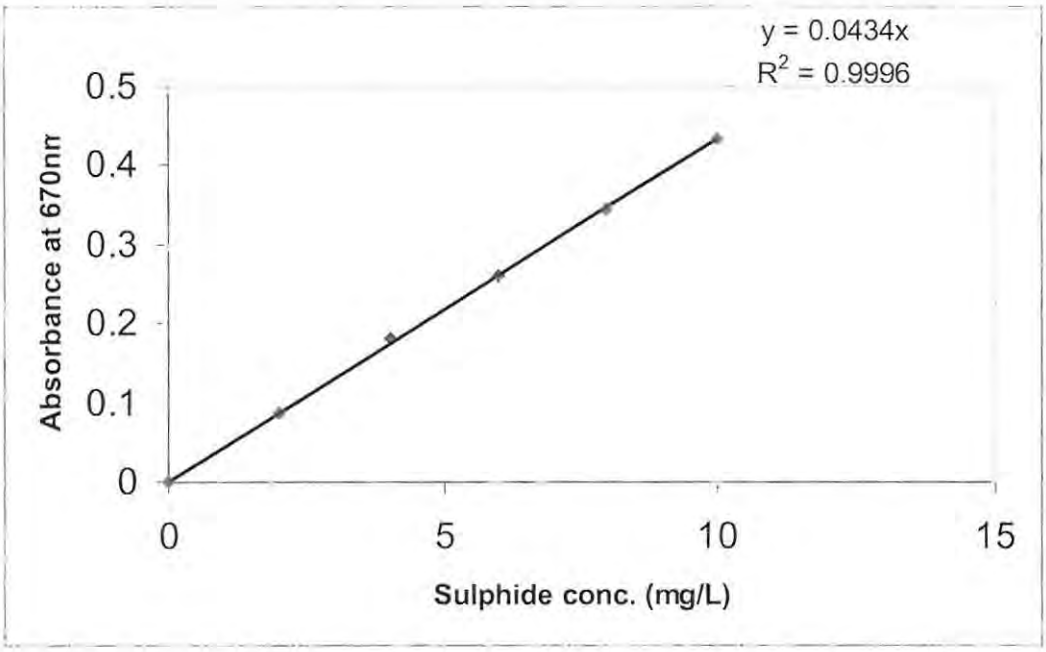

Figure 2.7: Sulphide standard curve.

2.2.6.3 COD determination: SQ 118 Method.

Principle

Most types of organic matter are oxidised by a boiling mixture of chromic and sulphuric acids. A sample is refluxed in a strongly acidic solution with a known excess of potassium dichromate ($K_2Cr_2O_7$). After digestion, the remaining $K_2Cr_2O_7$ is titrated with ferrous ammonium sulphate (FAS), the amount of $K_2Cr_2O_7$ consumed is determined and the amount of oxidisable organic matter is calculated in terms of oxygen equivalent [APHA, 1985].

Reagents.

(The reagents were supplied as part of the Photometer Spectroquant 118 kit)

1. Solution A [contains ferrous ammonium sulphate] from Merck.
2. Solution B [contains potassium dichromate and sulphuric acid], from Merck.

Procedure

Into a reaction cell specially designed for COD determination, 0.3 ml of solution A and 2.3 ml of solution B were added. Sludge samples (3.0 ml each) were added into the tubes, mixed and incubated in a thermoreactor (TR-205) at 148^0C for 2 hours. The samples were then cooled to room temperature by allowing them to stand for 5 min. For the blanks, 3.0 ml of water was added instead of the sludge samples. The COD concentration (mg/L) was measured with a Photometer Spectroquant 118 (Merck Test Kit) using the 029 method.

2.2.6.4 pH determination.

Fluctuation of pH in both reactors was also determined, using a pH meter [Orion Research - digital ionalyzer / 501].

2.3 RESULTS.

2.3.1. Enzyme production during anaerobic degradation.

In both sulphidogenic and methanogenic reactors cellulase and β-glucosidase activities were detected (Figures 2.8 and 2.9) from day 1 (i.e. first day of sampling and reactor start up) to day 31 (i.e. last day of sampling). In the crude form, the enzymes were assumed to be associated with the sludge organic particulate matter, making it difficult to determine accurate protein concentrations. As a result, activity was expressed as 1 mole of product / mg sludge biomass.

Enzyme activity was generally low in methanogenic conditions and remarkably high in sulphidogenic conditions for both enzymes. β-Glucosidase activity was 10 and 40 times higher than cellulase under both the methanogenic and sulphidogenic conditions respectively. Under sulphidogenic conditions, cellulase production was observed to be slow for the first three days, but reached its maximum from day 5 to day 13 with an average activity of 47.2 nmol/mg sludge biomass. A gradual decrease from days 15–21 leading to a sharp decline and a levelling off in activity during the last 10 days of the anaerobic process was observed. There appeared to be a similar pattern for β–Glucosidase production. However, there was a slight shift to the right in reaching maximum enzyme production. Maximum glucosidase activity was only reached from day 13–17 with an average activity of 1989 nmol/mg sludge biomass.

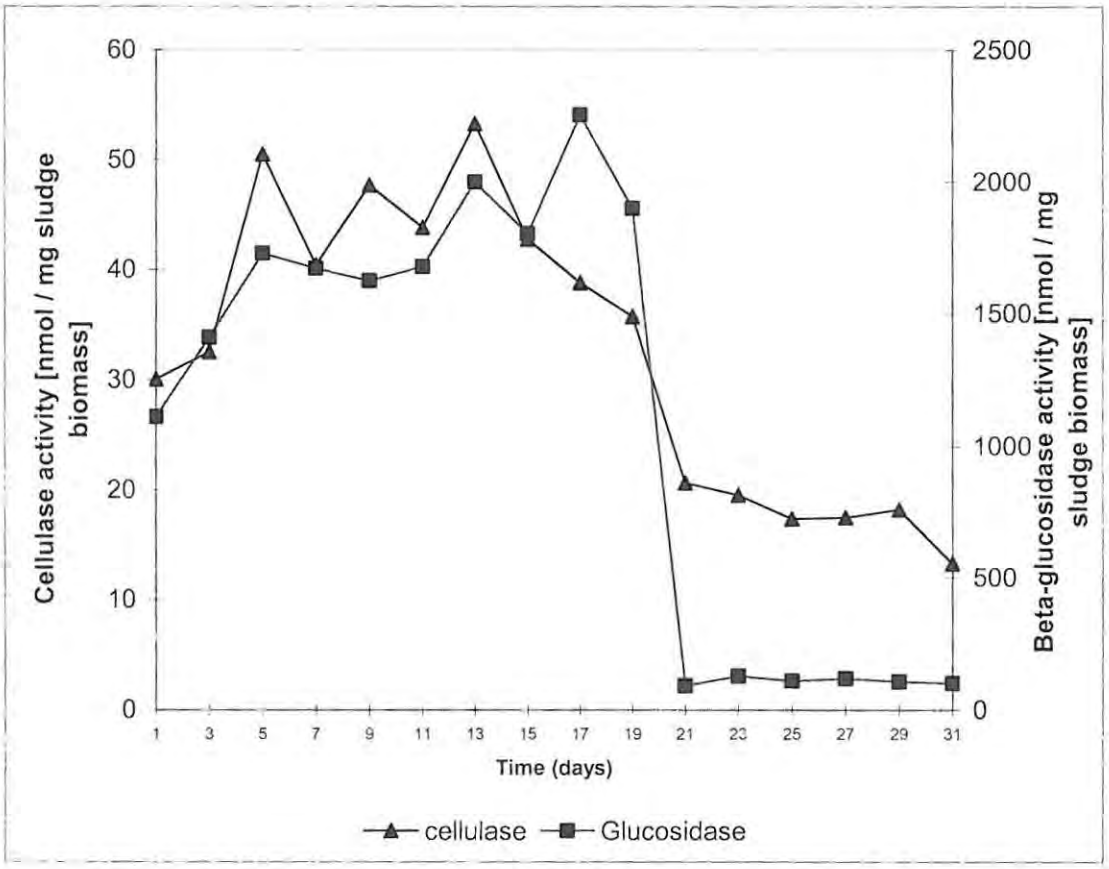

Figure 2.8: Production of cellulases and β-Glucosidases under sulphidogenic conditions.

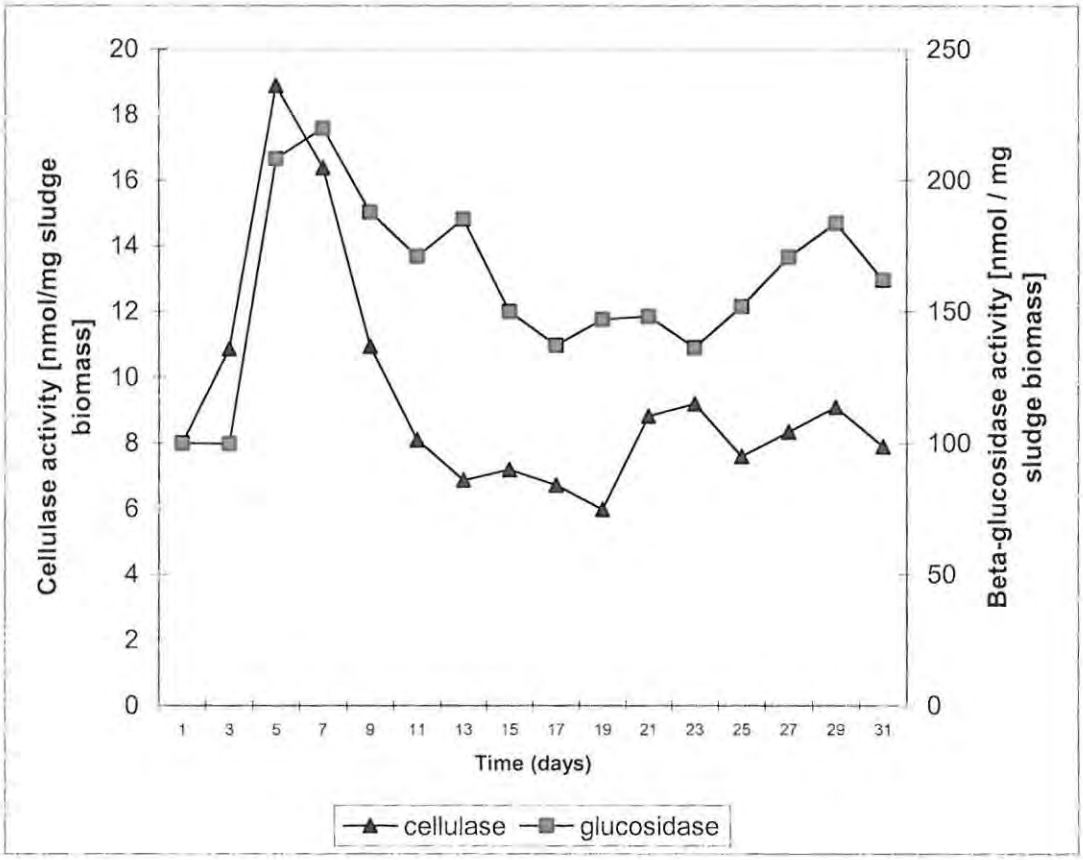

Figure 2.9: Production of cellulases and β-Glucosidases under methanogenic conditions.

Under methanogenic conditions, the production of both enzymes exhibited a similar pattern. Cellulases reached their maximum activity at day 5 while β-glucosidases reached maximum activity at day 7. A decline in activity from day 9-19, then a rise from 21--29 and another decline towards day 31 for both enzymes were observed.

Due to the fact that cellulose is a substrate for cellulases and glucose, a product of glucosidases, fluctuation in the concentrations of total polymeric carbohydrate and reducing sugar present in the bio-reactors during the course of the anaerobic process [Figures 2.10 and 2.11] were monitored.

2.3.2. Polymeric carbohydrate degradation.

Under sulphidogenic conditions, there appeared to be a 16% increase in the total carbohydrate concentration on the 13th day followed by a relatively rapid 20% decrease from day 15–31 [Figure 2.10].

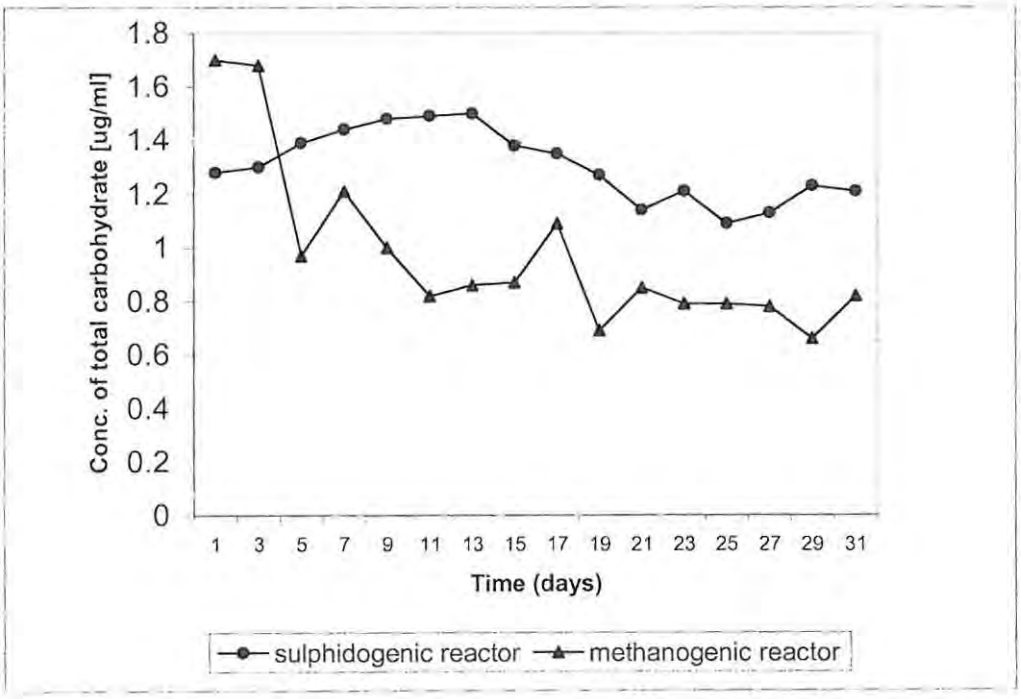

Figure 2.10: Degradation of total polymeric carbohydrate over time in sulphidogenic and methanogenic reactors.

However the net concentration of the total polymeric carbohydrate content from day 1 – 31 was negligible only 5.5 %. Under methanogenic conditions the carbohydrate content decreased from the first to the last day (1.7 μg/ml - 0.82 μg/ml) of the anaerobic process respectively, with a net decrease of 51.8 %.

2.3.3. Reducing sugar production.

In the sulphidogenic reactor, four peaks exhibiting maximum production of reducing sugar were observed on days 9, 13, 19 and 25. The highest reducing sugar production occurred at day 13. In the methanogenic reactor, there appeared to be a gradual consumption of reducing sugar during the whole course of anaerobic degradation with a net decrease of 10.8 % (Figure 2.11).

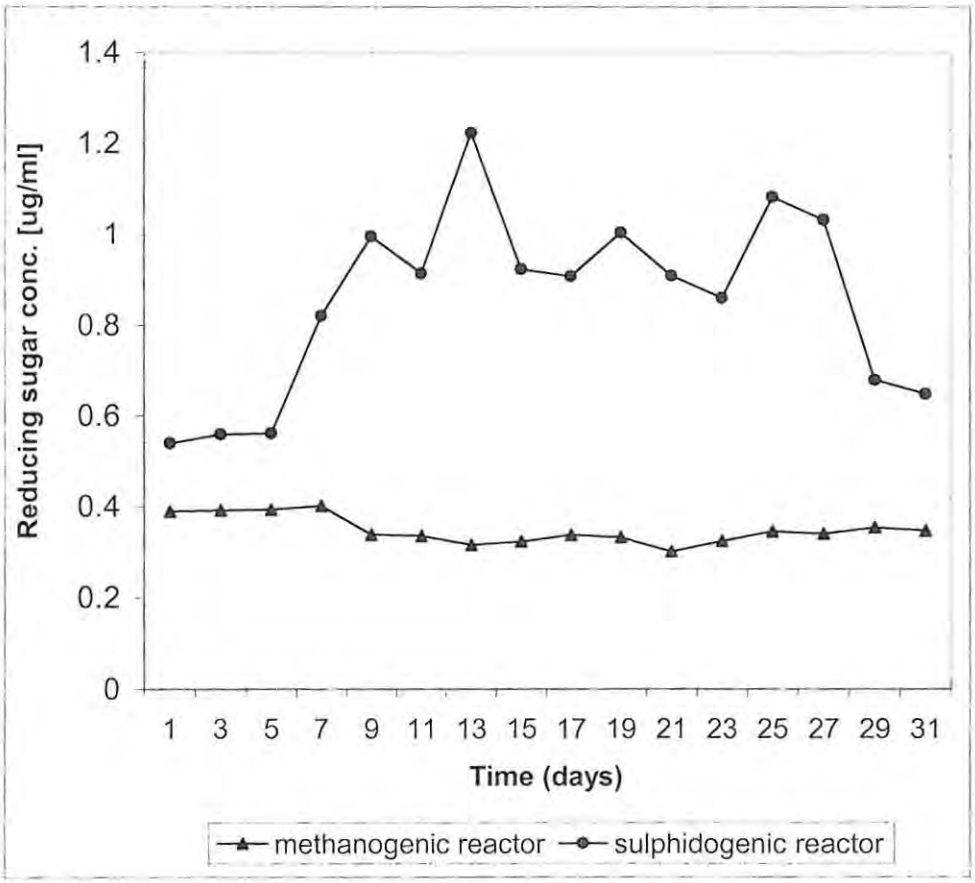

Figure 2.11: Production of reducing sugar in methanogenic and sulphidogenic anaerobic reactors.

2.3.4. Analytical procedures.

2.3.4.1. Sulphate and sulphide assays.

Standard curve plots: Due to the hyperbolic pattern displayed by the original sulphate standard curve (Figure 2.5 – p. 42), a double inverse plot was constructed for the determination of sulphate concentration (Figure 2.6 – p. 42). A linear curve with an R^2 value of 0.9996 for the sulphide standard curve was obtained from the original results (Figure 2.7 – p. 44).

Sulphate removal and sulphide production: In the sulphidogenic system, concentration of endogenous sulphate ions on the first day was 2184 mg/L while the sulphate that was added initially was only 2000 mg/L. The additional 184 mg/L of sulphate observed was ascribed to the indigenous sulphate ions present in the feed and / or SRB inoculum. Maximum sulphate removal in the sulphidogenic system seemed to occur during the first 9 days of the anaerobic process, while the trace amounts of sulphate present in the methanogenic system were completely removed by day 5 (Figure 2.12). On the other hand, maximum production of sulphide from 0-2599 mg/L in the sulphidogenic system was observed at day 13 (Figure 2.13).

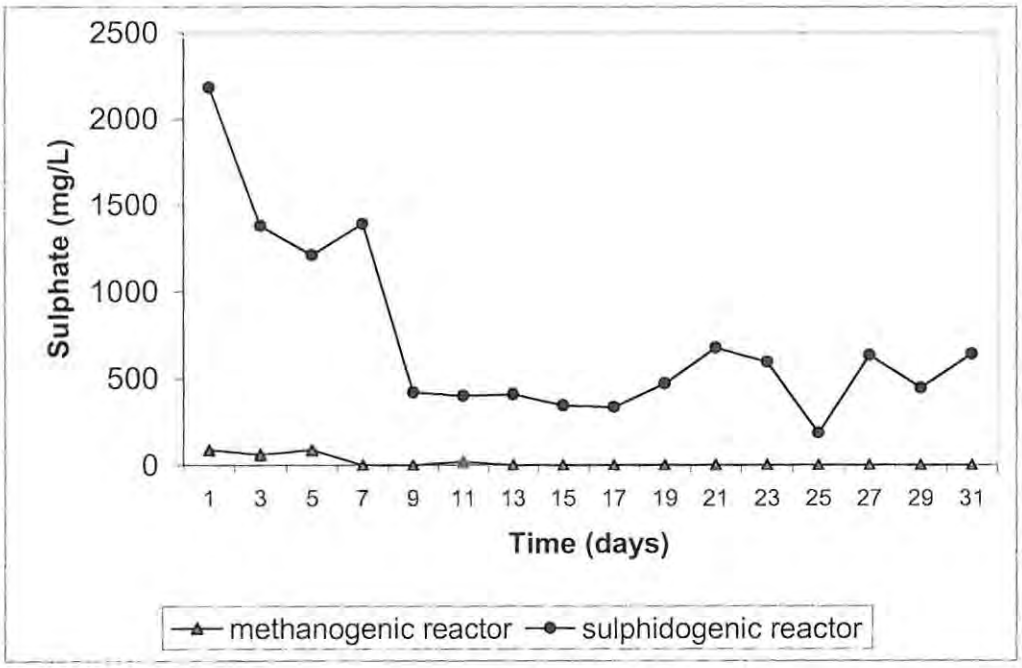

Figure 2.12: Sulphate removal from methanogenic and sulphidogenic reactors during anaerobic degradation.

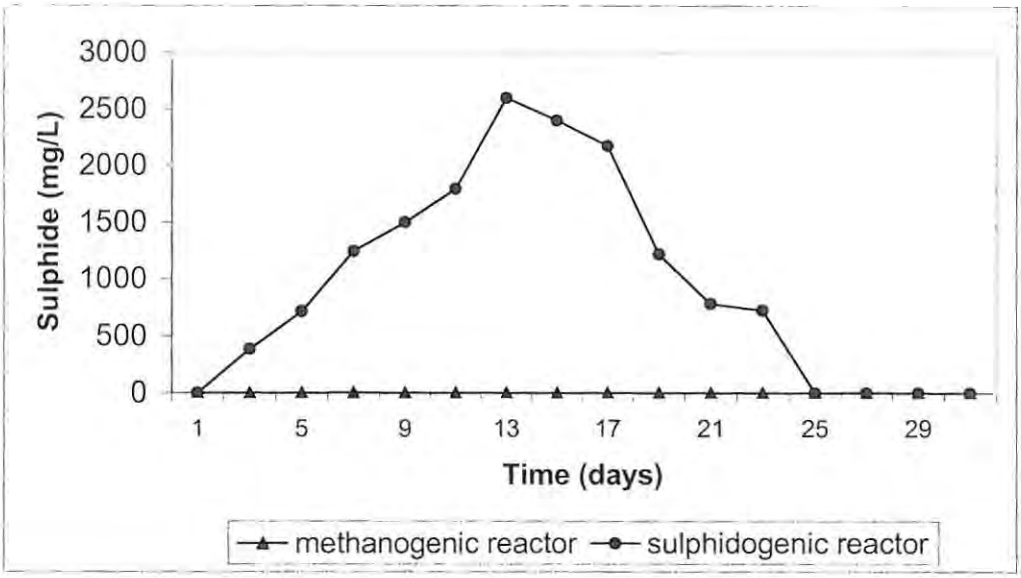

Figure 2.13: Production of sulphide in methanogenic and sulphidogenic reactors.

However, by day 25 all of the sulphide produced had escaped from the system. The time frame between maximum sulphate removal (day 9) and maximum sulphide production (day 13) was calculated to be 4 days. Only trace amounts of sulphide were observed in the methanogenic system [Figure 2.13].

2.3.4.2. COD determination.

In the methanogenic system, COD removal was gradual with a net decrease of ± 48% by day 31 [Figure 2.14]. In the sulphidogenic system, there seemed to be a rapid COD build up (of 67%) reaching its peak at day 9 followed by a rapid decrease (42.3%) from day 11-31. However, the net decrease in COD concentration for the whole process was only 3.75%.

2.3.4.3. pH Fluctuations over time.

In both systems there were no significant changes in pH, except that the pH conditions in the sulphidogenic system were slightly alkaline fluctuating between 7 and 8, while the pH in the methanogenic system was slightly acidic fluctuating between 4.9 and 6.0 [Figure 2.15].

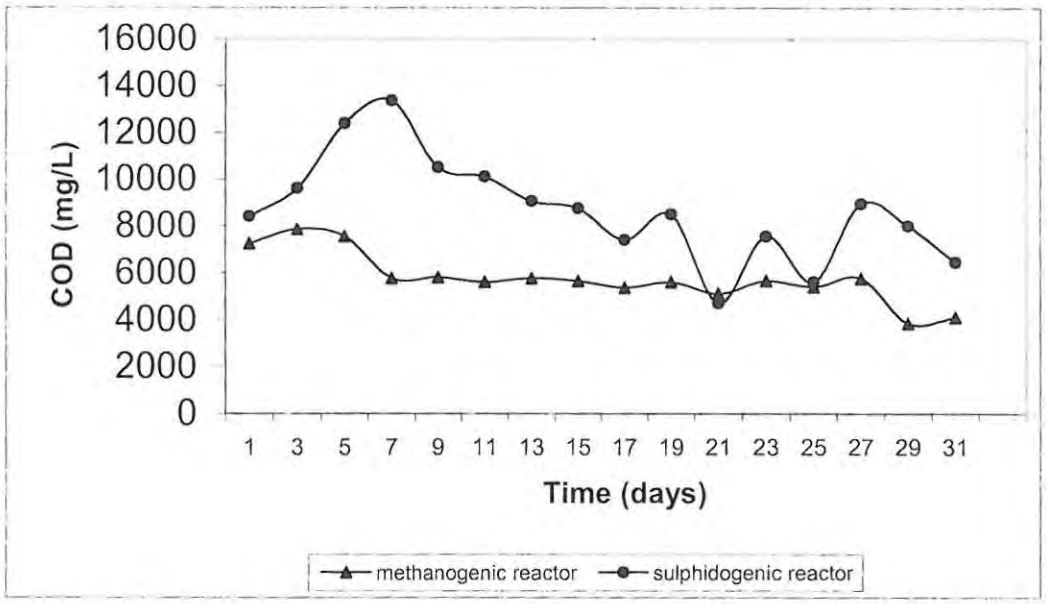

Figure 2.14: COD removal during anaerobic degradation.

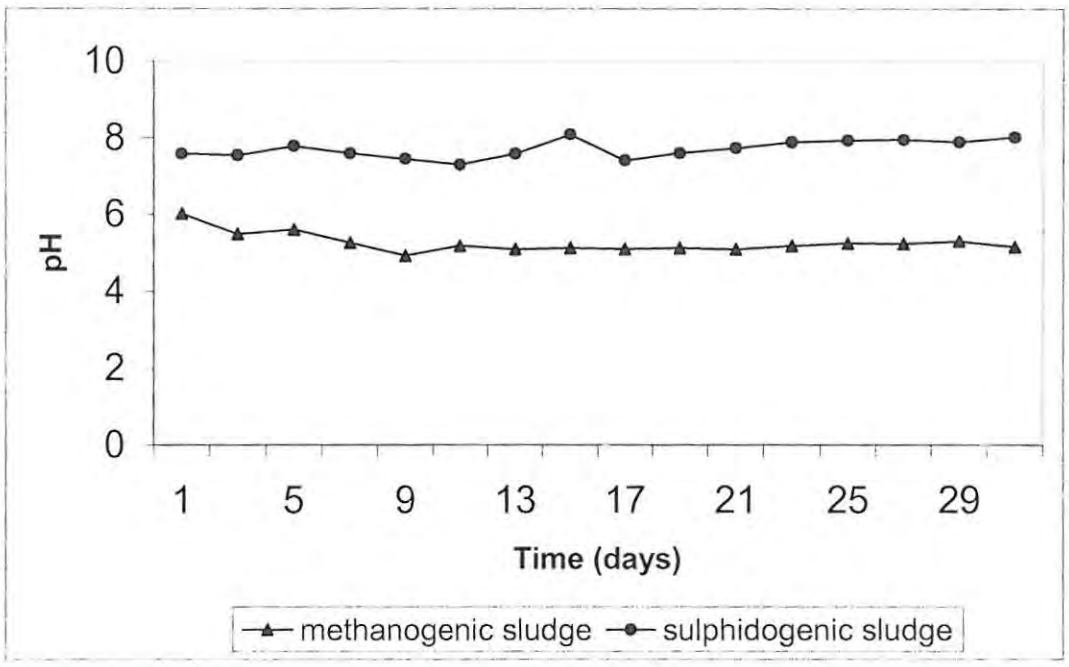

Figure 2.15: pH fluctuation during anaerobic degradation of sewage sludge.

2.4. DISCUSSION.

2.4.1. Enzyme assays.

The results of this study into the production of cellulases and glucosidases during anaerobic degradation make an important contribution towards understanding the role of these enzymes in the solubilisation of primary sewage sludge (PSS) under both methanogenic and sulphidogenic conditions.

The availability of high cellulolytic activity was indicated by high enzyme production. Since enzymes are produced by anaerobic bacteria, low enzyme activity observed during the first five days of the degradation process in both the methanogenic and the sulphidogenic systems can be attributed to a period of organic substrate uptake during the lag phase of these bacteria.

Sulphidogenic system (SS)

From day 3-5 cellulase activity increased by 10% (this could indicate bacterial growth) to reach its maximum at day 5. From days 5-13 the activity of the enzymes remained at a maximum indicating a stationary phase reached by the bacterial mixed culture. The maximum cellulase production with an average of 47.2 nmol/mg sludge biomass could have been a result of equilibrium in growth being reached leading to a consequently efficient cellulase production (Figure 2.8 – p. 47). Though maximum cellulase production was sustained for 8 days in the SS, an immediate depletion in enzyme production after the 5th day was observed in the methanogenic system (MS). Therefore, sustained activity indicated an enhanced enzyme production in the SS.

The above results correspond with work done by Kim *et al.* (1997). They demonstrated that cellulose hydrolysis under methanogenic and sulphidogenic conditions were 0.048 and 0.069 per day respectively. Within 10 days of the hydrolysis process, 48% of cellulose in the methanogenic reactor and 75% in the sulphidogenic reactor had been degraded. Similar findings were recently reported by (Pareek *et al.* 1998) who showed that the percentage and the rate of paper

mineralisation under sulphidogenic conditions was approximately twice that under methanogenic conditions.

It is well known that the susceptibility of cellulose to enzymatic hydrolysis is dependent on its accessibility to cellulases [Mahalingeshwara Bhat and Wood, 1988]. As cellulose never occurs in its pure form but is always associated with lignin and hemicellulose [Bisaria and Ghose, 1981], it can be expected that the presence of the hemicellulose and lignin will reduce the rate of cellulose hydrolysis. Thus the removal of hemicelluloses and lignin will increase the accessibility of cellulose to endo- and exoglucanases. Gharpuray *et al*, (1983), showed that factors affecting the rate of lignocellulolysis in order of importance were: specific surface area, lignin content and the crystallinity of the substrate. Recent studies have shown that the size of sludge flocs in the SS is smaller than that in the non-sulphidogenic systems. As a result, the sludge bed within the sulphidogenic system is less compact [Whittington-Jones, 1999] suggesting that a larger surface area on the substrate may be available for degradation. It may further be suggested that under sulphidogenic conditions removal of lignin and hemicellulose are enhanced giving rise to a lower density sludge.

Franklin and Mc Donald (1969), found that lignin subjected to alkali conditions formed free radicals which attack carbohydrate material either in the original fibre or in solution and thus form co-polymers of lignin and carbohydrates. The formation of such polymers result in slower delignification. Hydrosulphide ions (HS^-), one of the equilibrium products of sulphate reduction [Molipane, 1999] can act as a free radical scavenger, preventing the recondensation of lignin reactive molecules, thus preventing the repolymerization of lignin [Clayton, 1969; Franklin and Mc Donald, 1969]. In addition, the concentration of soluble phenolic compounds was observed to increase during digestion of primary sewage sludge in the presence of biological sulphate reduction or sulphide, indicating possible delignification in the presence of alkalinity [Whittington-Jones, 1999].

Furthermore, xylanases which cleave hemicellulose from cellulose function optimally in alkaline conditions (which are the existing conditions in the SS - Figure 2.15 – p. 52). These enzymes are almost always associated with cellulases, thus facilitating the accessibility of cellulose to enzymatic attack and the enhanced cellulase activity. Some SRB have been shown to have the ability to utilize phenolic compounds [Widdel, 1988; Schink et. al., 1992]. Therefore, it is

possible that SRB are directly involved with lignin degradation [Whittington-Jones, 1999]. However, this requires further investigation.

Other factors leading to enhanced enzyme production.

(i) Effects of sulphate reduction factors

Mention has been made that the activity of enzyme complexes against crystalline cellulose is dependent on a thiol-reducing agent [Coughlan and Ljungdahl, 1988; Demain and Wu, 1988]. Therefore it is possible that sulphate, sulphide and/or some of the intermediates of sulphate reduction could have a direct effect on cellulolytic activity. More details on this aspect will be discussed in later chapters.

(ii) Synergism between cellulase complex components

Cellobiose, a substrate for β-glucosidase is a product of cellulose breakdown. Therefore, the shift to the right in maximum enzyme production for β-glucosidase (Figure 2.8) between days 13 and 19 [as opposed to cellulase production between days 5-13] could suggest that glucosidases are switched on by the presence of cellobiose produced by cellulases and the converse can be true. In the presence of excess cellobiose, cellulases are inhibited. Hence the decrease in cellulase activity after the 13^{th} day.

This inhibition of cellulases coinciding with the 95% depletion of β-glucosidase activity between days 19 and 21 suggests that the release of cellobiose by cellulases is also decreasing. The breakdown of cellobiose by β-glucosidases can therefore be considered a rate-limiting step in the cellulolytic process. The overlapping sustained cellulase production with increasing glucosidase production between days 5 and 19, strongly supports the hypotheses that a synergism operates between cellulolytic enzymes.

Possible factors leading to depletion of enzyme activity after the 19th day.

(i) Substrate depletion

The substrates to be hydrolysed could be exhausted as an approximate decrease of 10% in both the polymeric carbohydrates and reducing sugar concentrations between days 19-21 is observed (Figures 2.10 and 2.11) respectively.

(ii) The adverse effects of H_2S

The adverse effects of H_2S on anaerobic degradation have been widely accepted. However, these effects have been confined to the gasification step [Malichevuru, *et. al.*, 1993] (Figure 2.13). Maximum sulphide production [2599 mg/L] on the 13th day, (which led to production of sulphide gas from HS^-, and S^{2-} intermediates) could have led to bacterial death.

(iii) Enzyme-substrate complex formation

Efficiency of enzymatic hydrolysis of solid substrates results only when the enzymes are attached to the substrate. The formation of the enzyme-substrate complex is greatly affected by aging of bacteria. Loss of the affinity factor (due to aging of bacteria) responsible for attaching cellulases to cellulose could adversely affect enzyme activity (section 1.9 – p. 24). However, the increase in enzyme activity in the MS (Figure 2.9) does not completely support the above hypothesis. Low sulphate concentration and high sulphide gas production may more adequately account for the death of bacterial culture. The death of co-existing methanogenic bacteria in the SS could have been due to competition between the SRB and methanogens for substrate as SRB are better survivors under sulphidogenic conditions.

Methanogenic system (MS)

In the MS, maximum enzyme production for cellulases and glucosidases was observed at days 5 and 7 respectively. This shift is similar to that observed in the SS. The same synergistic action between the cellulase complex components could also account for this shift. It has already been demonstrated that production of proteases associated with the sludge floc, increased during the

first 13 days of the anaerobic degradation process in the MS. This increase in enzyme activity only lasted for 7 days in the SS followed by a rapid decrease from days 9-19 [Tshivhunge, 2001]. These findings indicated enhanced protease activity with increasing sulphide concentration even at 1000 mg/L (pH 7.0), cannot predict the effect of sulphide at concentrations above this value. High sulphide concentration between days 7-19 (≥1000 mg/L) [Figure 2.13] and the decrease in protease activity within the same period could imply inhibition of proteases at higher sulphide concentrations.

Therefore, the absence of sulphide in the methanogenic system within the same time frame could account for the increased protease activity. Depletion of cellulase and glucosidase activities can be attributed to proteolytic attack. The rise in cellulolytic activity from days 19-29 accompanied the low proteolytic activity. In addition, the sustained enzyme production in the sulphidogenic system between days 5-13 and 13-19 for cellulases and glucosidases respectively [Figure 2.8] could have been due to inhibition of proteases.

Though Whittington-Jones (1999) has shown the optimum pH for carbohydrate degradation to be between 6-7 various authors [Eastman and Ferguson, 1981; Hobson and Wheatley, 1993; and Molipane, 1999] have indicated the operation of most cellulolytic enzymes to be in the acidic range i.e. between 4.8 and 7. At low pH conditions most proteases have demonstrated to have low activity [Tshivhunge, 2001]. The decrease in protease activity and the increase in cellulolytic activity could have resulted from high production of volatile fatty acids.

2.4.2. Degradation of polysaccharides.

Sulphidogenic system

A number of bacterial cellulases are glycoproteins (Knowles *et al.* 1988) and in most cases their glycoproteins have been shown to be mannose rich. The affinity of these mannose-rich polymers for cellulose crystals and their role as cellulose binding factors has been demonstrated (Demain and Wu, 1988). In addition, *Clostridium thermocellum* and at least one genus of SRB *(Desulphonema)* are gram positive bacteria [Beguin *et al.* 1988; Gibson, 1990]. Gram positive bacteria have a peptidoglycan layer which serves as their cell envelope.

The increasing polysaccharide content observed between day 1-13 in the SS could have been promoted by the increased bacterial growth [Knowles *et al.* 1988]. As the bacteria multiply exponentially, more enzymes are produced, resulting in increased production of mannan-rich polymers as the latter are part of the cellulase complex. The glycan component of the peptidoglycan layer might have contributed to the carbohydrate content as the bacteria multiply [Figure 2.10]. The decrease in polysaccharide content that is confluent with depletion in cellulase production (Figure 2.8) could be due to the disaggregation of enzyme complexes [Section 1.9 – p. 24], death of bacterial culture from H_2S intoxication, and/or further substrate hydrolysis by glucosidases.

Methanogenic system

As expected the total polysaccharide concentration in the MS decreased with increasing cellulase production (during the first 5 days of the anaerobic process). Though cellulase associated with the floc decreased after day 9, the total polysaccharide content continued to decrease as well. It has been suggested that depletion in enzyme activity could be due to disaggregation of the cellulolytic complex, where the affinity factor disassociates from the complex. As this component is mannose-rich, it could have been attacked by mannosidases that have been detected [Molipane, 1999] to be present in the sludge leading to a decrease in the carbohydrate content. However, this remains to be investigated. Other non-cellulolytic enzymes could also be playing a role in the depletion of carbohydrate present in the sludge as the assay employed includes other polysaccharides such as starch and glycogen.

2.4.3. Reducing sugar production.

Methanogenic system

Reducing sugar release (Figure 2.11) was confluent with β-glucosidase activity. From day 1-7 both reducing sugar release and β-glucosidase activity were high (Figures 2.9 and 2.11), thus the decrease in reducing sugar levels from days 9-23 coincided with the decreasing β-glucosidase activity. The slight increase in reducing sugar concentration from days 25-31 again coincided with the increase in β-glucosidase activity within the same period. These results were expected under normal conditions of the methanogenic process since some of the reducing sugar is

released from cellobiose by β-glucosidases. Fluctuation of reducing sugar concentration observed in the MS was not affected by β-glucosidase hydrolytic activity. Breakdown of other non-cellulosic carbohydrates by amylases, galactosidases, α-glucosidases etc. could also have had a significant contribution.

Sulphidogenic system

Maximum reducing sugar production between days 5-29 (Figure 2.11) coincided with high glucosidase activity observed from days 5-17 (Figure 2.8). Thus indicating the release of reducing sugar from cellobiose by β-glucosidases. Though glucosidase activity was low from days 19-29, there was a continued release of reducing sugar, ascribed to the presence of other carbohydrolases.

2.4.4. Sulphate removal and sulphide production.

Methanogenic system

The trace but negligible amounts of sulphide detected in the MS during the first five days were attributed to reduction of endogenous sulphate that could have been present in the feed. Since sulphide is a product of sulphate reduction, its absence in the MS was expected.

Sulphidogenic system

Though sulphate removal was almost complete by day 9, maximum sulphide production occurred at day 13. The period of four days between days 9-13 could be attributed to formation of intermediates (such as $S_2O_3^{=}$, HSO_3^{-}, HS^{-} and S^{2-} ions) as sulphate is not directly converted to sulphide. It was interesting to note that both β-glucosidase and cellulase activities were enhanced during the same period, suggesting that the thiol reducing agent enhancing enzyme activity could be the sulphite and the bisulphite ions rather than sulphate and sulphide. It has been mentioned that the adverse effects of sulphide on anaerobic degradation are limited to the gasification step. Therefore, if sulphide that was present in the SS was in the ionic form between days 9-13, the above hypothesis could be proven true i.e. it would not exert enzyme inhibition. This could be further supported by the fact that cellulase and β-glucosidase activities decrease only after day 13

and 17 respectively suggesting the formation of H_2S during that period. In the absence of contaminating metals, this H_2S escapes from the system to react with Zn acetate (in the gas trap) forming a white precipitate.

2.4.5. COD determination.

A decrease in COD content was an expected result for both systems. The methanogenic system had a 48% net COD decrease by day 31 reflecting the degradation in oxidisable organic matter. The increase in COD content observed in the SS was rather abnormal, since the reactors were operated as closed systems. This false increase was ascribed to the interference of the COD assay by sulphide. It was subsequently realized that in order to remove sulphide, the solution should be purged with nitrogen gas and a few drops of sulphuric acid be added.

2.4.6. pH determination.

The constant pH conditions [between 7 and 8] observed in the sulphidogenic system can be explained by the buffering system existing therein. When sulphate is reduced to sulphide, bicarbonate ions (resulting in alkalinity) are produced. On the other hand, the acidogenic phase of anaerobic degradation produces volatile fatty acids [which bring about acidic conditions]. The hydrogen ions from the acids could possibly react with the bicarbonate ions from sulphate reduction resulting in the formation of water and carbon dioxide.

Sulphate reduction: SO_4^{2-} + carbon source $\rightarrow$ HCO_3^- + H_2S

$$\text{R-COOH (VFA's)} \rightarrow \text{R-COO}^- + \text{H}^+$$

$$\text{Then :} \quad \text{HCO3}^- + \text{H}^+ \rightarrow \text{H}_2\text{O} + \text{CO}_2$$

The acidic pH conditions in the methanogenic sludge were explained by the constant production of the volatile fatty acids. However, the pH did not decrease beyond the value of 4.9 suggesting constant conversion of the produced VFA's to acetate and methane.

CHAPTER THREE

IDENTIFICATION, LOCATION AND EXTRACTION OF CELLULOLYTIC ENZYMES.

3.1 INTRODUCTION

3.1.1 Background.

It has already been suggested that though part of human diet includes fibrous plant material, human gut enzymes lack the ability to digest plant fibres such as cellulose. The fibre fraction remains unaltered and so voided in the faeces. As a result, approximately 60% of the total carbohydrate proportion (i.e. 25-40% of the overall sewage sludge) contained in settleable solids of raw sewage sludge is in the form of cellulose [Hunter and Heukelekian, 1965].

Data obtained from preliminary experiments [i.e. the 51.8% decrease in total polymeric carbohydrate content and the observed cellulase activity in the methanogenic reactor (Chapter 2)] has indicated a possible association of cellulase activity with human waste. The challenge thus faced during these studies was to investigate cellulose degrading enzymes where they were least expected to occur.

The fact that the human digestive system does not possess cellulases, and that cellulose was degraded, raised questions about the actual composition and source of the sewage contained in Grahamstown sewage digesters. Results obtained from a survey conducted by interviewing some of the operators at the Grahamstown sewage works suggested that though the sludge was mainly composed of domestic sewage (largely constituted by human waste), the slaughter house, hospitals, a fruit processing industry and materials from drainage water had a considerable contribution. No mention was made of animal farm waste.

It was also stated that approximately 90-95% of the waste entering the sewage pipes is formed by water and this fraction is filtered through to the digester tanks. The 1-5% fraction is formed by the big solid particles, rubbish, stones etc. It is the 90-95% filtrate containing polymeric organic particles, water and hydrolytic bacteria that was sampled for these studies.

3.1.2 Possible sources of cellulases found in sewage sludge.

Due to the fact that raw sludge from humans does not contain cellulolytic bacteria [Crowther and Harkness, 1975], it is necessary to account for the possible origin of the

cellulase activity detected in the sludge. It has been suggested that if a feedstock lacks a particular component, to ensure a better and more complete digestion, mixing of waste may be necessary [Hobson and Wheatley, 1993]. This could be the case with the sludge sampled from the Grahamstown digesters as the drainage water could carry environmental cellulolytic microorganism e.g. anaerobic fungi and cellulolytic bacteria contained in animal droppings. Examples of anaerobic fungi also known to form stable co cultures with methanogens, which also have a remarkable efficiency in the solubilization of crystalline cellulose, are *Neocallimastix frontalis, Piromonas communis* and *Sphaeromonas communis* [Wood *et al.* 1988].

In addition, three non-sporing gram-negative cellulolytic rods were isolated from a domestic sewage digester. No isolates were made from raw sludge, but the organisms were isolated from digesting sludge in a two stage process. More organisms were isolated from the bottom of a secondary tank than from the primary tank. Furthermore, different rods that were divided into two groups of high and low cellulolytic activities were also isolated from a sewage digester. Cellulolytic sporing rods were also obtained from samples of a digester that was started from sewage sludge and fed with powdered cellulose. A non-sporing *Acetivibrio cellulolyticus* were indirectly isolated from a sewage digester. Additional cases of cellulolytic isolates from sewage sludge are elaborately covered [Crowther and Harkness, 1975; Hobson and Wheatley, 1993].

All isolates made from sewage digesters were mainly from digesting sewage sludges and nothing was found in crude or raw sludges. The nature of these isolates varied considerably i.e. gram positive, gram negative, sporing and non-sporing. From the results of these experiments it was concluded that digesters contain a varied population of cellulolytic bacteria, which enter the digesters in minute quantities and develop during the digesting process [Hobson and Wheatley, 1993].

3.1.3 Identification

Having accounted for the origin of anaerobic cellulolytic bacteria in sewage sludge, the attempt to identify, localize and extract cellulases from digesting human waste will now make more sense. Though identification of cellulases has been briefly dealt with in the previous chapter, the initial procedures involved in the identification process were not covered. Therefore, a part of this chapter focuses on the groundwork done during the

identification process thus accounting for the selection of the assay employed for all the other studies.

3.1.4 Localization

Since the preliminary analytical procedures and enzyme assays carried out on the sludge samples employed a homogenous sludge mixture (unfractionated sludge), localization of cellulases in the sludge still remains unclear. It has been suggested that 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 the substrate [Chróst, 1991]. In the activated sludge, the exoenzymes dissolved in solution are known to be negligible. However, the association of these enzymes with the cells and their immobilization onto the sludge matrix within the extracellular polymeric substances is not clearly understood [Boczar *et al.* 1992; FrΦlund *et al.* 1995]. Localization of cellulases in the sludge is therefore a second objective of this chapter.

3.1.5 Extraction.

For the study and characterization of any enzyme, purification of that particular enzyme is necessary. Extraction is therefore the first step in the purification of an enzyme. The purpose of this step is to obtain the enzyme in a soluble form. How these enzymes are extracted will depend on their location. For extracellular enzymes such as those secreted into fermentation media or present in a cocktail medium containing cells and any other particles must be removed to prevent interference in subsequent purification steps. To achieve this, a clarification step such as centrifugation or microfiltration is required. If the extract is to be loaded onto a column, this step is also vital to prevent fouling and blocking of the column.

For an intracellular protein, disruption of cells followed by extraction may be necessary. Once disrupted, cells release enzymes readily, although in some cases the addition of detergent (e.g. SDS or triton) to release membrane-bound enzymes is advantageous [Angal and Harris, 1993]. Clarification and extraction methods used in this study were centrifugation and sonication respectively.

3.1.6. Determination of cellulose from the sludge.

It has previously been proven that the number of bacteria (per unit weight/volume) in a digester population can give an idea of the importance of the bacteria in degradation of a substrate. The number of bacteria may also reflect the concentration of a particular substrate in a digester feedstock. From these findings, it is possible that substrate concentration may reflect the quantity of bacterial population hydrolyzing that particular substrate thus giving an idea of the expected activity. For this reason, estimation of cellulose was also included in the study [Hobson and Wheatley, 1993].

3.2. METHODOLOGY.

3.2.1. Reactor design and operational procedure.

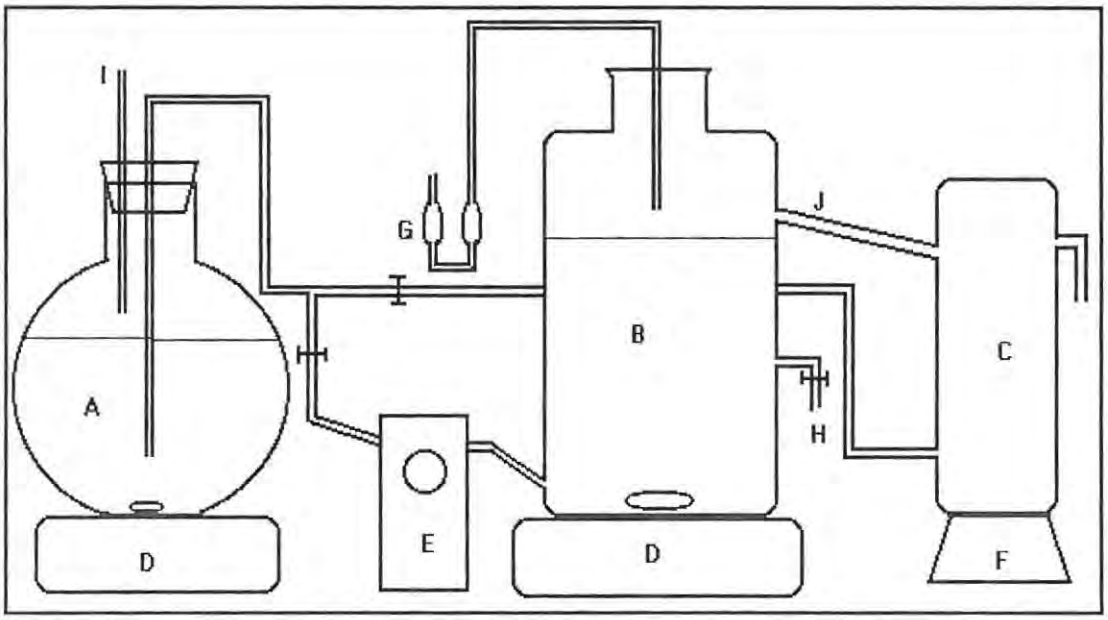

Figure 3.1: SULPHIDOGENIC CONTINUOUS SYSTEM: A-10 L tank containing primary sewage sludge (COD=2000 mg/L) and SO_4^{2-} (2000 mg/L). B – 20 L reactor containing 10% SRB mixed culture and continuously fed by A. C – 2 L plastic tank positioned on a marginally lower level to allow passive overflow of sludge effluent from B. D - Magnetic stirrers for continuous stirring of the reactor contents to prevent settling of the organic matter. E - peristaltic pump set to allow HRT of two days. F- Plastic stand. G – H_2S gas trap containing zinc acetate solution. H – Sampling port on the side of the reactor allowing samples to be drawn from the center of the sludge bed. I - Tube for air suction. J – Silicone tubing for passage of the overflow.

Two lab scale stirred glass tank reactors, the methanogenic (control) and the sulphidogenic (experimental) reactors were set up. The methanogenic reactor resembled exactly the one described in (Section 2.2.1 – p 32). Batch feeding was done on every third day. The sulphidogenic reactor was a continuous three-stage reactor illustrated by Figure 3.1.

3.2.2 Estimation of cellulose concentration in the primary sewage sludge.

Principle.

Complex carbohydrates are generally insoluble and not directly suitable for spectrophotometric assay. In general, they are first hydrolysed and the monosaccharides produced measured [Bashford and Harris 1987]. In this assay, cellulose was estimated by measuring the glucose equivalent produced from enzymatic hydrolysis of sewage sludge.

Reagents

1. Raw sludge (2000 mg/L) COD.

2. Pure cellulase (from *Trichoderma viridae*), Sigma. Enzyme was dissolved in distilled water and diluted to a concentration of 1mg/ml.

3. Acetate buffer 0.1 M, pH 5.0

4. Glass test tubes 10 ml, 50^0C water bath.

5. Centrifuge and 10 ml centrifuge tubes.

6. Somogyi-Nelson reagents and materials (see Section 2.2.5 – p. 38).

Procedure.

In a 10 ml glass test tube, 1 ml of raw sewage sludge (containing the substrate) was reacted with 1 ml of the commercial enzyme solution. 1 ml of acetate buffer (0.1M, pH 5.0) was also added. For the control, 1 ml of distilled water was used to replace the enzyme. The experiment was conducted in triplicate. The contents of the tubes were mixed using a vortex mixer and incubated at 50^0C for 30 min. Both the samples and the controls were centrifuged at 982 g [A-4-62 rotor, Eppendorf centrifuge 5810R] for 10 min. The supernatants were decanted into clean glass tubes and assayed for reducing sugar using the Somogyi-Nelson method as described in [Section 2.2.5 – p. 38].

3.2.3. Enzyme assays

3.2.3.1 Photometric assay for cellulases

NB: The term cellulase will be referring to endo- and exo-glucanases.

Pprinciple

Many methods used for assaying cellulase activity involve the measurement of either reducing sugar or total sugar released into solution. In this assay, the soluble derivative of cellulose, carboxymethyl-cellulose (CMC) was used to measure cellulase activity. The activity was determined by measuring the increase in reducing power of the solution using the Somogyi-Nelson method [Mahalingeshwara Bhat and Wood, 1988].

Reagents.

1. CMC solution 1% (w/v), prepared by dissolving 1.5 g of 0.7 degree of substitution [i.e. completely soluble in water] in 100 ml of distilled water with heating and stirring. This solution could be stored at 4^0C for a maximum of 7 days with little detectable change in the reducing power of the solution.

2. Acetate buffer (0.1M, pH 5.0).

3. Somogyi-Nelson reagents (p. 38).

Procedure.

CMC reagent (1 ml) and 1ml of acetate buffer were mixed in a 10 ml glass test tube and brought to 50^0C in a water bath. The sludge, 1 ml (expected to contain the enzyme) was then added to the tube and the mixture was incubated at 50^0C for 30 min. The tubes were then removed, centrifuged at 982 g [A-4-62 rotor, Eppendorf centrifuge 5810R] for 10 minutes. The supernatants were decanted into clean glass test tubes and assayed for reducing sugar using the Somogyi-Nelson method. There were two types of controls set up. The first (substrate) control was to determine whether CMC was hydrolysed by heat in the presence of the copper reagent. This was obtained by subjecting the substrate (1 ml) to the Somogyi-Nelson assay and the enzyme was added before the centrifugation step. The second (enzyme) control was used to measure the amount of residual sugar already present in the sludge. This was prepared by subjecting the sludge only to the Somogyi-Nelson method and CMC was added before the centrifugation step that precedes absorbance reading.

3.2.3.2 Photometric assay for β-glucosidases.

Principle.

The activity of β–glucosidase was determined by using an artificial substrate ρ-nitrophenyl-β-D-glucopyranoside. The degree of hydrolysis of the substrate was determined by photometric measurement of the ρ-nitrophenyl ion liberated in the reaction. In alkaline solution this ion forms a yellow color complex that absorbs light strongly in the 400-420 nm region [Clark and Switzer, 1977].

Reagents.

1. Sodium acetate buffer (0.1 M, pH 5.0)
2. ρ-nitrophenyl-β-D-glucopyranoside (5 mM).
3. Glycine buffer, (0.4 M, pH 10.8, i.e. 60 g of glycine dissolved in 1500 ml of

distilled water) to make solution A. 50% (w/v) NaOH solution was added drop wise to solution A until pH 10.8 was reached. The buffer was diluted by distilled water to a volume of 2 L.

4. ρ-nitrophenol standard (0.2 mg/ml).

Procedure.

In 10 ml glass test tubes, the sludge enzyme 1 ml was reacted with ρ-nitrophenol-β-D-glucopyranoside (substrate) 1 ml in acetate buffer (1 ml) pH 5.0. The tubes were incubated at 37^0C for 30 minutes. After 30 minutes the tubes were centrifuged at 982 g [A-4-62 rotor, Eppendorf centrifuge 5810R] for 10 minutes and the supernatants were decanted into clean tubes. Glycine buffer (4 ml) was added and the tubes were allowed to stand at room temperature for 15 minutes. The experiment was conducted in triplicate and the mean value was calculated. For the controls, the enzyme (sludge) was only added after the addition of glycine buffer. Absorbance was read at 405 nm. The ρ–nitrophenol standard curve was constructed by using suitably diluted ρ-nitrophenol with a concentration range of 0-42 nmol/ml [Table 3.1].

TABLE 3.1: Preparation of ρ-nitrophenol standard curve.

ρ–nitrophenol concentration. (nmol/ml)	ρ–nitrophenol volume (ml)	0.4 M glycine buffer vol. (ml)
0	0.0	7.0
4.28	0.5	6.5
8.57	1.0	6.0
12.86	1.5	5.5
17.14	2.0	5.0
21.43	2.5	4.5
25.71	3.0	4.0
30.00	3.5	3.5
34.28	4.0	3.0
38.57	4.5	2.5
42.85	5.0	2.0

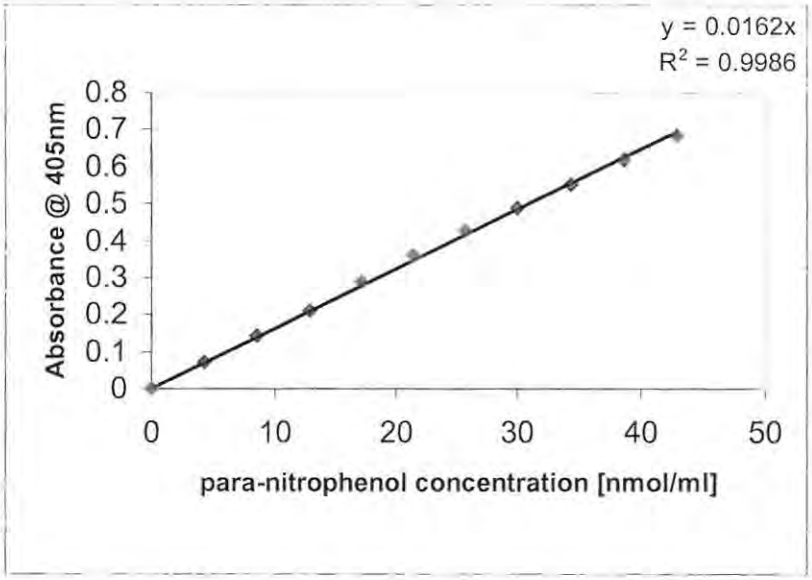

Figure 3.2: ρ-Nitrophenol standard curve for the determination of β–glucosidase activity.

3.2.4 Fluorimetric assays for cellulase and β-Glucosidase activities.

For the principle and method see Section 2.2.3 – p. 34-35.

3.2.5 Enzyme location.

To determine the location of the enzymes 50 ml of the sludge was centrifuged at 27 200 g [JA-20 rotor, J-21 Beckman centrifuge] for 10 min. The supernatant was decanted into a clean tube. The pellet was washed twice with 10 ml volumes of distilled water, centrifuged and resuspended in another 10 ml of distilled water. Both the supernatant and the resuspended pellet were assayed for enzyme activity using the fluorimetric method described above.

3.2.6 Extraction.

Enzyme preparation: Approximately 400 ml of sludge was centrifuged at 15 300 g [JA-14 rotor, J-21 Beckman centrifuge] for 30 min (4^0C). The pellet was washed 3 times with 50 ml volumes of distilled water. All supernatants were pooled together and assayed for activity. The pellet was weighed and re-suspended in 0.1 M phosphate buffer (pH 7.0) with a volume that was twice its weight.

Extraction: the re-suspended pellet was separated into 5 ml fractions and poured into 10 ml plastic centrifuge tubes. Some samples were chilled in an ice bath and kept aside for enzyme assays. These were labeled "Sonication at time 0". Other samples were sonicated at 9 W [30 s/ml]. Sonication was carried out by subjecting the samples to 30 second bursts for a total period of 5 min to optimize sonication time. All samples were centrifuged [982 g (A-4-62 rotor, Eppendorf centrifuge 5810R) x 5 min] at 4^0C and both the supernatants and the pellets assayed for enzyme activities using the fluorimetric method.

3.3 RESULTS.

3.3.1 Estimation of cellulose concentration in the primary sewage sludge

When the arseno-molybdate reagent was added, an intense molybdenum blue color was observed in the experimental tubes, suggesting the digestion of sludge cellulose by authentic cellulase and the release of reducing sugar from the cellulose chains. The amount of reducing sugar obtained from raw sludge [after subtraction of controls] was estimated to be 10 mg/ml. Upon the addition of the arseno-molybdate reagent, a blue-green color and a white precipitate were observed in the controls [containing sludge only]. No precipitate was observed in the experimental tubes.

3.3.2 Enzyme assays.

Photometric determination of Cellulase activity: No cellulase activity was detected in both the methanogenic and the sulphidogenic reactors. However, a slightly blue color was observed in the controls and the samples of the methanogenic sludge. A different result was observed in the sulphidogenic samples. Both the sulphidogenic samples and the controls had an intense blue color.

Photometric determination of β-glucosidase activity: β-glucosidase activity from the methanogenic and sulphidogenic reactors was initially studied using the synthetic chromogenic ρ-nitrophenol-β-D-glucopyranoside (PNPG). After the addition of glycine buffer (pH 10.8), a yellow color was observed in the sample tubes. Only a pale brown

color could be seen in the controls. The yellow colour resulted from the cleavage of the agluconic bond (i.e. bond between the ρ-nitrophenol and the glucose moiety) to form the yellow ρ–nitrophenolate anion, which absorbs light strongly at 405 nm in alkaline conditions [Desphande *et al.* 1983]. β–Glucosidase activity was detected in both reactors. Activity was defined as the amount of enzyme liberating 1 nmol of ρ-nitrophenol per minute. The specific activity for β-Glucosidase was estimated to be ± 0.85 nmol/min/mg of sludge biomass under methanogenic conditions and ± 35.2 nmol/min/mg of sludge biomass under sulphidogenic conditions.

3.3.3 Enzyme location.

Fluorimetric determination of Cellulase and β–Glucosidase activities: Due to the fact that cellulase activity was very low and could not be detected photometrically, a more sensitive fluorimetric method was employed. Both cellulase and glucosidase activities were detected. After the sludge was centrifuged and assayed for activity, approximately 99% of the activity for both cellulases and glucosidases was detected in the pellet. The activity in the supernatant was negligible.

A

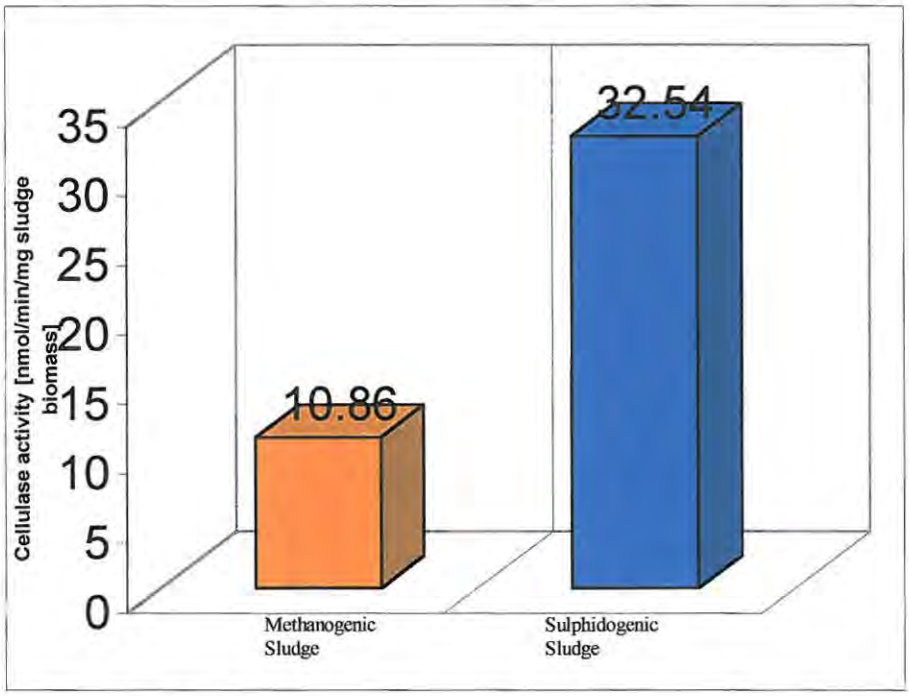

Figure 3.3A: Cellulase activity detected in the methanogenic and sulphidogenic sludge pellets.

B

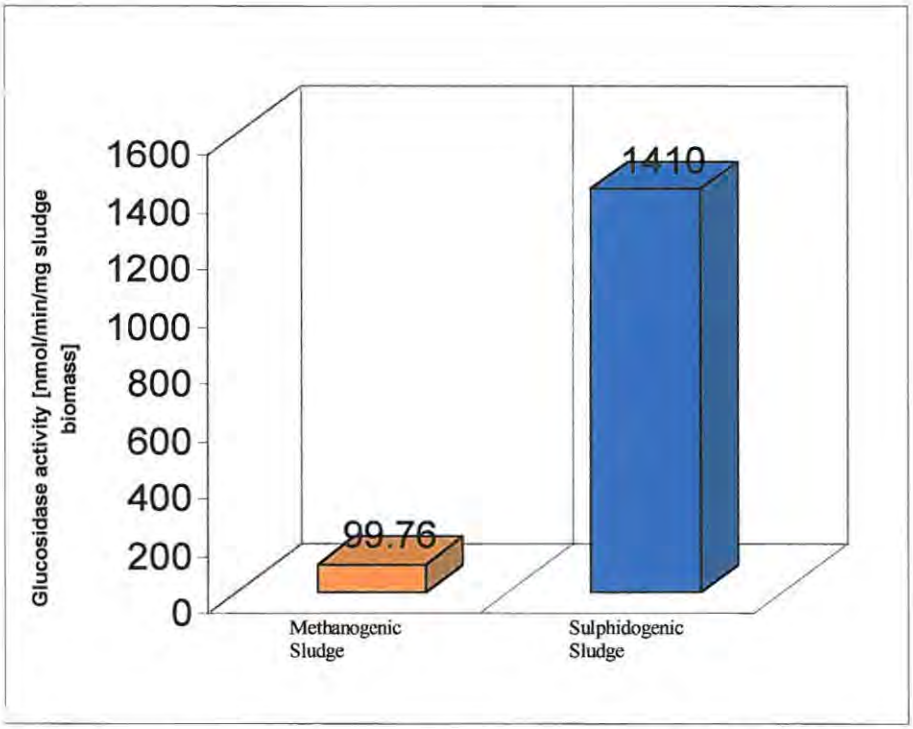

Figure 3.3B: β-Glucosidase activity detected in the methanogenic and sulphidogenic pellets.

There seemed to be a low production of enzymes under methanogenic conditions. The activity was 10.86 nmol/min/mg and 99.76 nmol/min/mg of sludge biomass (Figures 3.3A -3.3B) for cellulases and glucosidases respectively. Activity for both enzymes was generally high under sulphidogenic conditions.

3.3.4. Enzyme extraction.

Since all the activity was detected in the pellet, before sonication, the pellet was considered to have 100% activity. The time taken for both enzymes to be released from the pellet into the supernatant was approximately 4 minutes (Figure 3.4). After sonication of the pellet more than 80% of the activity was released into the supernatant. Only 8.4% for glucosidase and 22% for cellulases - of relative activity - remained in the pellet. There was no decrease in the pellet activity though sonication was prolonged up to 9 minutes suggesting that the remaining enzymes were tightly immobilized to the organic particulate matter. The percentage activity released was estimated to 91.6% for glucosidases and 78% for cellulases (Figure 3.5). However, there was an imbalance between activity released from the pellet and activity gained in the supernatant. Figure 3.6 indicates a 94% increase of glucosidase and 82% increase of cellulases in the

supernatant. Approximately 2.4% for glucosidase and 4% for cellulase activity seemed to have been lost. This loss in activity can be attributed to the denaturation of enzymes by the ultra-sonic waves of the sonicator.

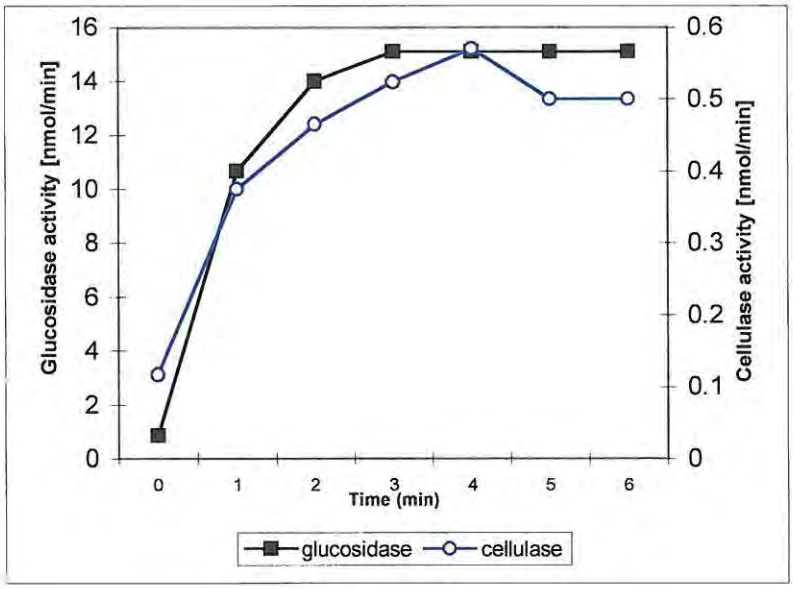

Figure 3.4: Release of enzymes from the methanogenic pellet into the supernatant vs sonication time. (Similar results were obtained for the sulphidogenic enzymes).

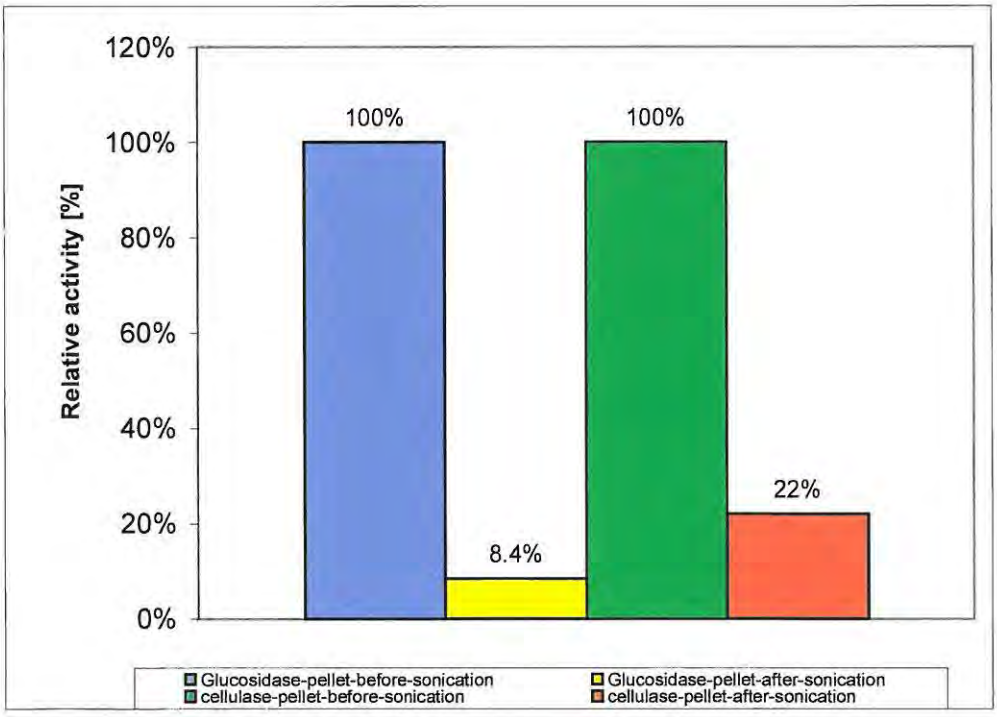

Figure 3.5: Release of enzymes by sonication from the sludge pellet.

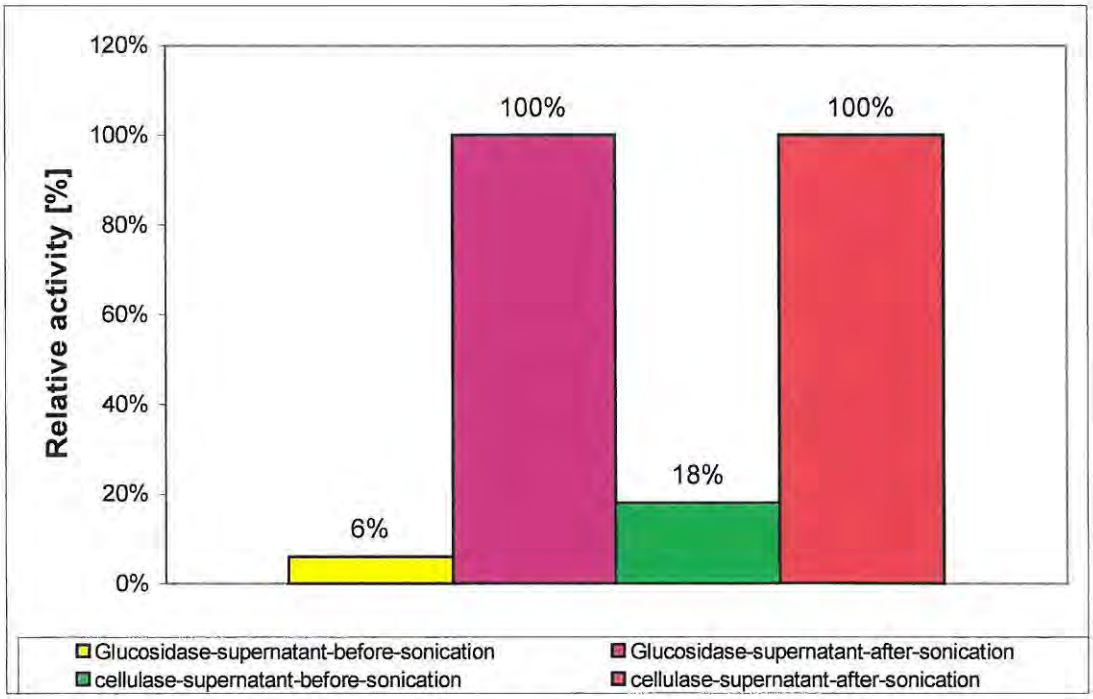

Figure 3.6 Release of enzyme activity into the supernatant after sonication.

3.4. DISCUSSION.

3.4.1 Estimation of cellulose concentration in raw sludge

From the findings of previous research studies (see Section 3.1.5), it has been hypothesized that substrate concentration may possibly reflect the quantity of bacterial population hydrolyzing that particular substrate. Therefore, the purpose of this experiment was to get an idea of the anticipated cellulase activity. Pure cellulase (1 mg/ml) produced 10 mg/ml of reducing sugar. The concentration of reducing sugar produced may give an idea of the quantity of cellulases (i.e. whether the activity is high or low) contained in the sludge, when the sludge cellulases are incubated with commercial substrate. However, two drawbacks that may arise from the hypothesis are: (i) the substrate used for sludge cellulase assay will be artificial, and (ii) the level of purity of sludge cellulase may be different from that of the pure enzyme.

Production of reducing sugar from sludge hydrolysis by pure cellulase is also evidence that cellulose forms part of the polymeric carbohydrates that was degraded during the enzyme production studies (see Chapter 2). The white precipitate (formed upon the

addition of the arseno-molybdate reagent] in the controls is undigested cellulose [Mahalingeshwara Bhat and Wood, 1988]. The absence of this precipitate in the experimental tubes was an indication of complete digestion of sludge cellulose. Therefore, it is safe to consider the concentration 10 mg/ml of reducing sugar produced, an accurate measure of degraded cellulose.

3.4.2. Enzyme assays

Photometric determination of Cellulase activity: the pale blue colour observed in the methanogenic samples as well as in the controls could be ascribed to the residual reducing sugar present in the sludge. The sulphidogenic samples and controls showed interesting results. An intense blue colour was observed in both the samples and the controls. To suggest a high cellulase activity under sulphidogenic conditions from these results would be inaccurate as the samples and controls showed no difference in the amount of reducing sugar produced. However, it is a known fact that polysaccharides are hydrolysed when subjected to heat and extreme pH conditions. The Somogyi-Nelson assay involves a step where sugar is heated with an alkaline solution of copper tartrate to produce cuprous oxide. Results from the previous chapter also showed that the pH in the sulphidogenic reactor was slightly alkaline. It is possible that the combination of sulphate and copper reagent produced high alkaline conditions that would (upon heating) hydrolyse both the CMC and sludge cellulose. Such results were not obtained in the methanogenic samples and controls. Therefore, the copper reagent alone could not be producing alkalinity.

No clear reason was found to explain why activity against CMC could not be detected in this case, since CMC is commonly used for determination of glucanases. However, two reasons suspected to have led to failure to detect cellulase were attributed to: (a) limited sensitivity of assay employed and (b) the type of substrate used.

Sensitivity of the assay: Results have shown that cellulase activity could only be detected fluorimetrically. Fluorescence methods are known to be potentially several orders of magnitude more sensitive than absorption methods [Bashford and Harris, 1987]. It is the high sensitivity of photodetectors (i.e. an element of the fluorimeter which uses diode arrays for photodetection) and the ability of monochromators (which select

appropriate excitation and emission wavelengths) to resolve incident from emitted light that makes fluorescence a particularly sensitive analytical procedure. While the absorbance of light by chromophores generally falls within the μM range, fluorimetry is capable of detecting nanomolar or even picomolar concentrations of fluorophores [Bashford and Harris, 1987]. Due to the above facts, it can be suggested that cellulase activity could have been so low that it could not be detected by absorbance spectroscopy.

The type of substrate used: Although CMC is widely used for the assay of glucanases, there are some problems attendant on its use. The degree of substitution has been shown to have a profound effect on enzyme attack. Only unsubstituted residues are attacked. It appears that at least two (or in some cases three) adjoining residues are required for enzymatic action. CMC of degree of substitution of 0.7 has been reported to have few sites that can be subject to enzymatic attack. Short incubation time and low enzyme activity could have had a negative contribution [Mahalingeshwara Bhat and Wood, 1988]. Despite the discouraging results; the production of reducing sugar in the sludge and the degradation of polymeric carbohydrates implying cellulolytic degradation motivated the use of the more sensitive technique - fluorimetry.

Photometric determination of glucosidase activity: the use of PNPG for glucosidase detection is a reliable but insensitive method as the results indicate that the assay is 10^2 times less sensitive when compared to the fluorimetric assay. In addition, the incubation period of 30 minutes for spectrophometry was 6 times longer than the 5 minutes employed in the fluorimetric assay. The two above factors have led to preference of the fluorimetric to the absorption spectroscopic technique. The 5-fold requirements of any assay are specificity, sensitivity, accuracy, precision and convenience [Bashford and Harris, 1987]. More of these requirements have been met by the fluorimetric assay.

Evidence for high affinity of MUF-Cell for endoglucanases: Chernoglazov *et al.* (1989) studied the activity of highly purified endoglucanases from *T. reseei* using MUF-Cell as a substrate. They compared the effective binding constant and k_{cat} of endoglucanase with respect to MUF-Cell to that of CMC (a polymeric substrate for the enzyme). Kinetic measurements of MUF formation by fluorimetry showed that MUF-Cell was characterized by a high affinity toward *T. reseei* endoglucanase (K_m of 1.2 ± 0.1

mM) while the maximum velocity for endoglucanase was 9 μmol/min per mg of enzyme; and k_{cat} was equal to 7.9 s^{-1} (30^0C, pH 5.0). The k_{cat} value was not much lower than that characterizing the enzyme (determined viscometrically) with respect to CMC (K_m ca.0.1 mM, kcat ca.10 s^{-1}). This allowed the use of MUF-Cell as a substrate for endoglucanases to determine fluorimetrically the activity at a level of 10^{-10} M/min, providing a 10-fold increase in sensitivity compared to CMC [Chernoglazov *et al.* 1989]. The speculation made earlier from the results obtained (Section 3.3.2 – p. 69) with regards to the type of substrate and sensitivity of the assay used, is supported by the above studies.

Despite high sensitivity, fluorimetric registration of the hydrolysis of MUF-Cell does not allow for a continuous assay of activity. The assay was carried out at pH 7.0 though the optimal catalytic activity of cellulolytic enzymes is in the pH range of 4-6. This is because MUF in the pH range 4-6 exists in a protonated form. The deprotonated form of MUF (product) has a maximal absorption at 360 nm, while the emission fluorescence has a maximum at 455nm. These values differ markedly from those of the MUF-derivatives of oligosaccharides that have their absorption maximum at 318 nm, providing a basis for highly sensitive fluorimetric determination of the reaction products.

The role of glycine buffer: Another reason pertaining to the impossibility of a continuous assay is due to the high pKa value of MUF (7.8). Therefore, the measurement of MUF in the reaction mixture is performed by titrating the reaction mixture up to pH 10 or higher. After the titration step, the MUF concentration can be determined using a calibration curve [if the fluorescence assay is being set up for the first time - to ensure that the assay is linear]. In the case of turbid solutions (e.g. sludge) containing compounds likely to quench, it is advisable to calibrate each assay by setting a zero reference point using a blank solution and a standard amount of the fluorescent compound (added when the assay is completed) [Chernoglazov *et al.* 1989]. The detection of MUF at a level of 10^{-10}-10^{-11} M due to titration to high pH values has been verified. This is the significant role played by glycine buffer (pH 10.8) in the sensitivity of the assay.

Disadvantages of using MUF-Cell as a substrate.

Extraction of MUF from the reaction mixture: a problem associated with the use of MUF-labelled substrates in sediments or in any slurry is that the produced MUF in the assay adsorbs onto the organic particulate matter. Several methods to extract MUF quantitatively from the slurry have been tested before. The extractants used were buffers of pH 2. (0.2 M glycine-NaCl-HCl) or pH 11.0 (0.2 M glycine-NaOH), 1% triton X-100 in water, 80% methanol and 80% ethanol. Extraction with ethanol resulted in a recovery of 88% of the product [Boshcker and Cappenberg, 1994]. In this study, MUF was extracted by adding 95% ethanol after the incubation period and the reaction mixture was centrifuged at [982 g, A-4-62 rotor, Eppendorf centrifuge 5810R (4^0C for 5 min).

Glucosidase interference: Though MUF-Cell is a preferable substrate for determination of endoglucanases, it does not allow for the assay of enzyme activity in the presence of other cellulase complex components. The assay is subject to interference by β-glucosidases as cellobiose is a natural substrate for these enzymes. Boschker and Cappenberg (1994), in their study of sediment glucanases, used gluconolactone (a specific inhibitor of β-glucosidase). Their findings indicated that 65% of the activity measured with MUF-cell could be attributed to the action of β-glucosidases.

An experiment comparing the activity of endoglucanases and β-glucosidases on MUF-Cell was also carried out in this study. Pure cellulase and β–glucosidase from *Trichoderma viridae* were both tested for their activity against MUF-cell under the following assay conditions: 20^0C incubation with shaking at 310 rpm for 90 minutes. Cellulase activity was found to be 0.96 nmol/ml per μg of enzyme and glucosidase activity was 0.76 nmol/min/μg of enzyme. The higher activity of cellulase suggested that though β-glucosidases hydrolyse MUF-cell, cellulase is a better substrate for endoglucanases.

Inhibition of β–glucosidase with gluconolactone for the assay of glucanases in the sludge was also attempted. These studies were unsuccessful, however, as gluconolactone introduced further solutes [even after centrifugation] to the cocktail mixture causing quenching of light and lower fluorescence readings. Therefore, the activity values

obtained from enzymatic cleavage of MUF-Cell were accepted only as an indication of endoglucanase activity, on the basis of the high affinity of MUF-cell for endoglucanases and *vice versa*. In contrast to cellulose (the natural polymeric substrate of cellulases), MUF-Cell is an oligomeric and soluble substrate. It is therefore very unlikely that the activities measured with MUF-Cell actually give a true reflection of *in situ* values of cellulose hydrolysis or exoglucanase activities. The measured activities can only be seen as potential activities that can be used to compare activities at different sampling sites and to measure changes in activity with time e.g. the study of enzyme production with time [Boshker and Cappenberg, 1994].

The determination of β-glucosidase activity using MUF-Glu was successful and accurate. Glucosidase activity detected fluorimetrically has proved the assay to be remarkably sensitive as compared to the PNPG assay. Failure to obtain the actual activity values for endoglucanases led to abandonment of studies on cellulases. Purification and characterization studies were continued with β–glucosidases only, using fluorimetry as the preferred method for assay.

3.4.3. Enzyme location.

Approximately 99% of activity was detected in the pellet and this confirmed the theory that bacteria, which attack insoluble materials, maintain physical contact with the substrate (i.e. through biospecific interactions, non-specific charge mediated or hydrophobic interactions). The location of the cellulosome (cellulase complex) on the bacterial membrane and its adhesion to cellulose has been confirmed [Bayer and Lamed, 1988]. The low cellulase activity (in the 10^{-12} M/min range) detected in the supernatants of both reactors can be ascribed to the release of the cellulase complexes from the cells and from the substrate (due to loss of the affinity factor) into the supernatant, as the bacterial cells age [Coughlan and Ljungdahl, 1988]. This theory was confirmed during the time trial studies. As enzyme activity for both was low from day 1 to day 19, some of the pellet activity seems to be released into the supernatant [Figure 3.7]. There was no definite pattern for glucosidase activity in the methanogenic sludge, but a pattern of increasing activity was observed in the sulphidogenic supernatant from day 19-27 [Figure 3.8].

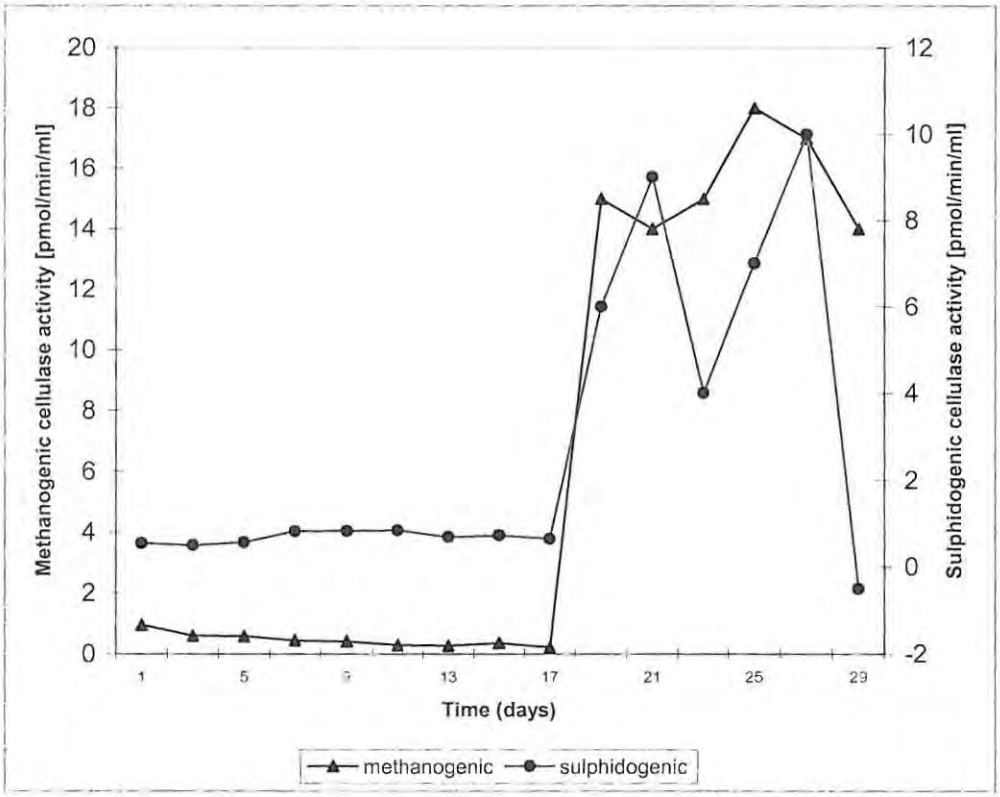

Figure 3.7: Fluctuation of cellulases in the supernatant vs time during anaerobic degradation. [from the time trial studies].

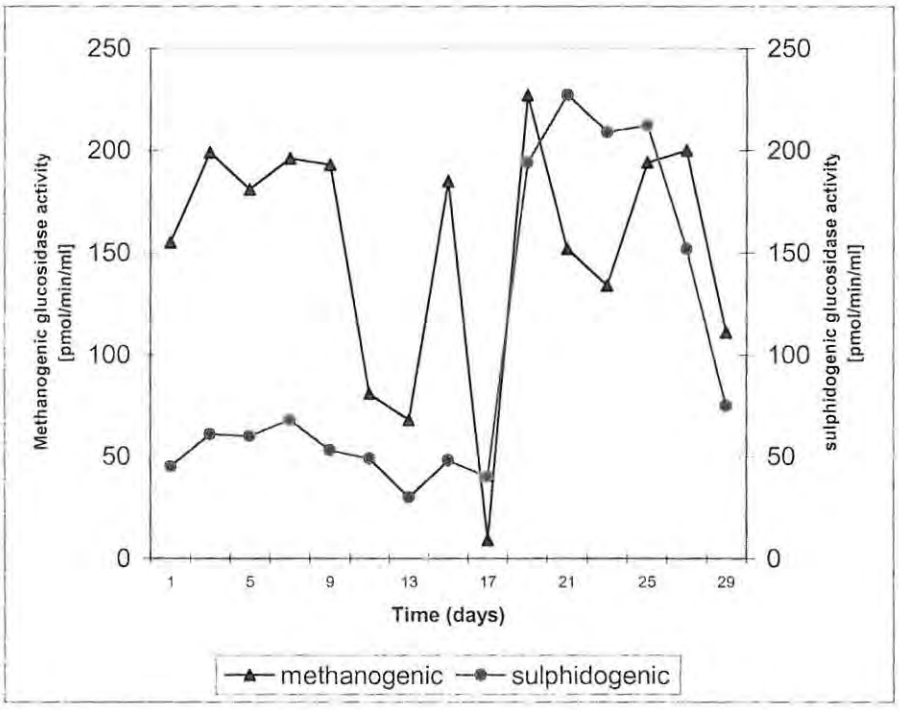

Figure 3.8: Fluctuation of the supernatant glucosidase activity vs time [from the time trial studies].

3.4.4. Enzyme extraction by sonication.

The release of the membrane bound and substrate-immobilized cellulase complex by sonication was successful. The high-frequency sound waves of the sonicator cause cell disruption by shear forces and gaseous cavitation. As the gas bubble collapse, shock waves resulting in cell disruption are formed [Angal and Harris, 1993]. Though more than 80% of activity was released into the supernatant after sonication, results have shown that some activity (i.e. 8.4% for glucosidase and 22% for cellulases – Figure 3.5 – p. 71) remained in the pellet. The relatively higher cellulase activity remaining in the pellet suggested a better resistance of these enzymes to disruption compared to that of glucosidases. After 4 min (time duration for maximal release of activity) of sonication, (Figure 3.4) there was no more release of activity from the pellet. Studies carried out on *C. thermocellum*, demonstrated that the cells of this organism adhere tightly to cellulose in a way which is resistant to vigorous stirring and harsh conditions. Therefore, the resistance of cellulases to sonication can be attributed to the tight immobilization of the cellulolytic complex onto the substrate. The immobilization of these cellulases seems to provide stability to the enzymes *in situ*, as the remaining 22% cellulase activity remained unchanged even though sonication was prolonged for nine minutes.

CHAPTER FOUR

PURIFICATION AND PARTIAL CHARACTERIZATION OF β-GLUCOSIDASE.

4.1. INTRODUCTION

4.1.1. Background

Purification is an important prerequisite for the advancement of studies on any enzyme [Janson and Ryden, 1989]. These studies can be on the activity of that enzyme, structure, structure-function relationships, as well as on characterization with regards to pH optimum, temperature optimum, temperature stability, induction and inhibition.

The requirements of these studies will determine how much protein to be purified is required; whether loss of activity can be tolerated; the degree of purity and the time taken for purification. For example, for studies of activity, relatively small amounts of active enzyme are required and high purity will not be essential provided any interfering activities are removed. If the enzyme studied is not stable, rapid isolation is important to minimize activity loss. Loss of enzyme at each purification step is inevitable. Therefore, to maximize yield, the minimum number of steps should be used. However, by minimizing the number of steps, the final purity of the enzyme is compromised [Angal and Harris, 1993]. For purification of the cellulolytic complex, the procedure only involved three steps, viz: extraction by sonication, affinity chromatography and gel filtration. Characterization of the molecular size and polypeptide composition was achieved by subjecting the enzymes to SDS-PAGE.

The procedure followed for the purification process was to isolate the whole of the cellulosome despite the fact that only glucosidase activity was determined. This approach was adopted for three reasons: the lack of stability of β-glucosidase in the isolated uncomplexed form, the ability to detect β-glucosidase specifically using the MUF-Glu substrate, despite the presence of other cellulolytic enzymes in the cellulosome, and the need for the cellulose-binding factor (which is part of the cellulosome) for the affinity chromatography step. The extraction of cellulolytic enzymes has already been dealt with in the previous chapter. When the sonic extract was obtained, the enzyme was purified from the crude mixture by affinity chromatography.

4.1.2. Affinity chromatography

Affinity chromatography is a powerful protein purification technique [Angal and Harris, 1993] that exploits the biospecific interactions existing between protein binding sites and biomolecules (also known as ligands). These ligands are immobilized on suitable particles (solid support, usually a gel matrix) that can be packed into a column. The sample containing the desired protein is then passed into this column, and the specific interaction of the protein with the ligand holds the desired protein back while others pass through (Figure 4.1) [Scopes, 1994].

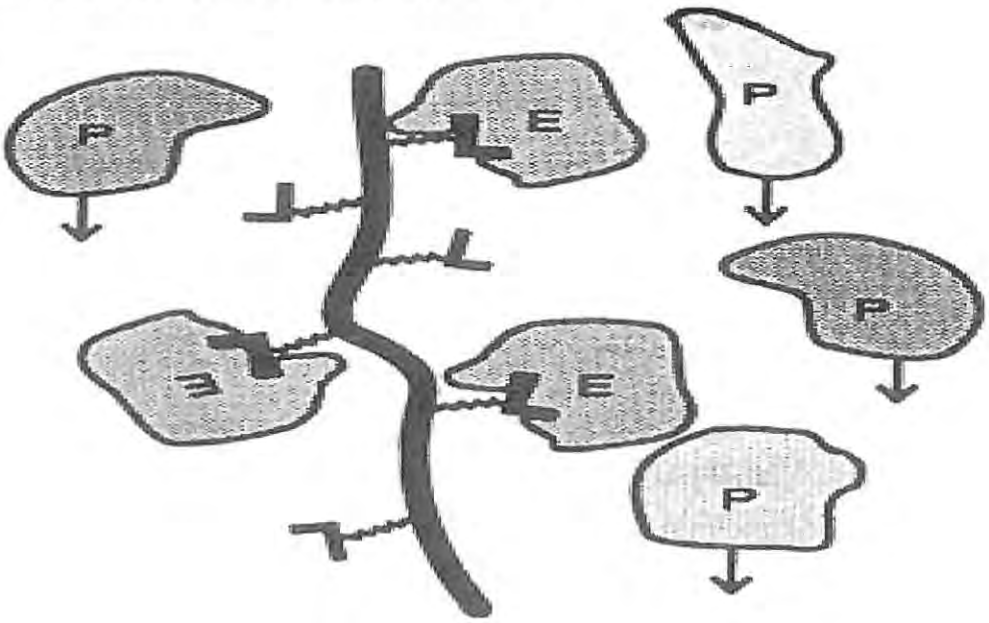

Figure 4.1: The basic principle of affinity adsorption chromatography. A ligand (L) is covalently attached to the backbone matrix e.g. cellulose. Only enzymes (E) with a specific affinity for L bind to the adsorbent. Proteins (P) pass through unaffected [Scopes, 1994].

Ligands may be small molecules such as substrates for enzymes or larger molecules such as hormones. The interaction of a binding site with a ligand is determined by the number and the distribution of complementary surfaces, as well as the overall size and shape of the ligand. The complementary surfaces involve a combination of charged and hydrophobic moieties and exhibit other short-range molecular interactions such as hydrogen bonds. The stereo-selectivity of the binding activity of an enzyme allows purification of a single enzyme available at a low concentration from a crude mixture of proteins that are present at higher concentration.

Protein-ligand interaction: For efficient binding, the adsorbent and the sample must be equilibrated with a buffer reflecting the conditions required to achieve a strong complex of the ligand with the protein to be purified [Janson and Ryden, 1989; Angal and Harris, 1993]. As indicated above, the interaction is mediated by a variety of factors. If the interaction is thought to be predominantly hydrophobic, an increase in the ionic strength or in pH may improve adsorption. Other interactions may be reinforced by addition of divalent metal ions or specific factors able to preserve a particular protein conformation. Other ways for improvement of protein-ligand interaction may be suggested if some knowledge of that particular interaction is available. Trial and error experiments may be required if nothing is known about the interaction [Angal and Harris, 1993].

Column operation: column size is usually not a critical parameter. Rather the column is governed by the capacity of the adsorbent and the amount of the substance to be purified. Capacities of affinity gels are generally high. Short, wide columns are often used to obtain rapid separations on beds of usually 1-10 ml gels. Column packing follows the usual precautions for chromatographic techniques (i.e. the recommended flow rates and packing procedures) to ensure definite bands [Janson and Ryden, 1989]. Affinity purification need not be restricted to column procedures. To achieve binding equilibrium, several approaches apply. In some cases batch-wise procedures are preferred with gentle stirring during both adsorption and desorption. This approach mainly applies when small amounts of a protein (as in the case of cellulases in the crude sludge mixture) are to be isolated from a mixture containing a large proportion of inert protein with an adsorbent of high affinity. The purification is achieved more readily by adding a slurry of the specific adsorbent to the crude mixture. The drawback in using a column for affinity chromatography is that the flow rate can deteriorate due to the application of the crude sample [Dean and Lowe, 1974; Bayer and Lamed, 1988].

Washing and elution of columns: adsorption of proteins / enzymes is followed by washing of the column with several volumes of the starting buffer to remove all unbound material. This step is monitored by UV-absorbance and is complete when the original base line is reached. Elution of the adsorbed solute is achieved by driving the adsorption equilibrium of the adsorbed solute from the stationary (solid matrix) to the mobile phase

(buffer). This can be achieved in several ways such as changing the pH, ionic strength, dielectric constant or temperature of the buffer. Ideal elution of a strongly adsorbed protein should utilize a solvent, which alters the conformation of the protein to decrease the affinity of the protein for the ligand, without being so severe as to cause partial or complete denaturation of the enzyme. In most cases, the alteration of a single physical variable suffices to effect elution, although in some cases simultaneous changes in pH and ionic strength are more effective than either alone. When elution is complete, the adsorbent has to be regenerated to remove any material still bound to the matrix. This can be done by re-equilibrating the matrix with several column volumes of the starting buffer. If necessary, detergents and denaturing agents are used depending on the stability of the adsorbent [Janson and Ryden, 1989].

4.1.3. Gel filtration

Gel filtration (basic principle) was the subsequent technique employed in the purification of the cellulosome. Gel filtration (sometimes referred to as molecular exclusion) is a form of chromatography that separates molecules on the basis of their different sizes. The basic principle is that molecules are partitioned between a mobile solvent and solvent immobilised on a solid support of defined porosity. The separation process is carried out using a gel matrix packed in a column and surrounded by solvent. The gel consists of an open, cross-linked, three-dimensional molecular network cast in bead form for easy column packing and optimum flow characteristics. The pores in the beads are of such sizes that some are not accessible to large molecules yet can be penetrated by smaller molecules.

The large molecules pass through the column and are eluted first in the void volume. If intermediate molecules are present, they will have access to some of the pore space of the beads and be withheld for a shorter time than smaller molecules. The latter may have access to all the pore space of the beads the beads and are withheld the longest; hence they migrate more slowly through the column and appear as the last components in the chromatogram. Molecules are therefore eluted in order of their decreasing size. The diagrammatic representation of the principle of gel filtration is illustrated in Figure 4.2.

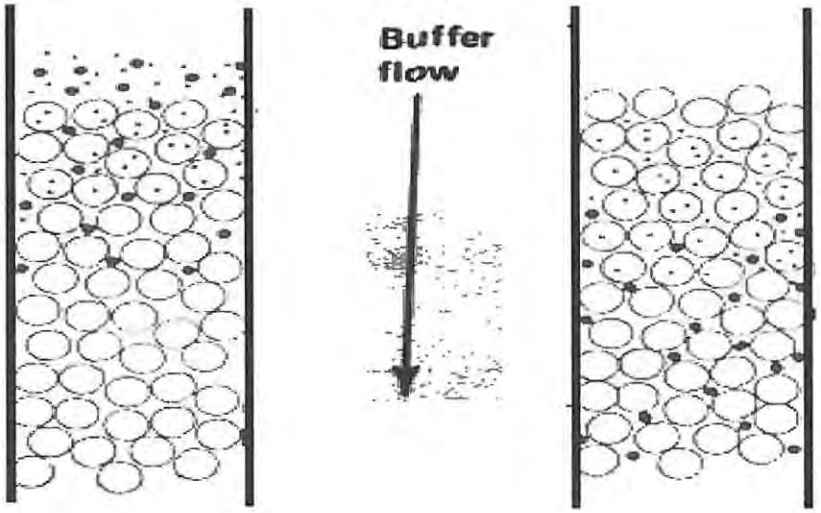

Figure 4.2: Simple diagram illustrating principles of gel filtration to separate different sized molecules in a column. Large molecules are excluded from most of the available column volume, and so move rapidly through [Scopes, 1994].

Loading of sample onto the column: to obtain a high resolution of molecules the sample must be applied in a small volume. The greater the sample volume, the greater becomes the volume in which the sample is eluted; if this volume is greater than the volume separating different components, the components will be remixed. The sample volume must therefore be lower than the separation volume between components. Typically the sample volume is kept between 1 to 5 % of the total bed volume. Sample volumes below 1% result in diffusion and natural chromatographic spreading. For sample volumes greater than 5% of the total bed volume only molecules differing in molecular size by a factor of four will be separated [Scopes, 1994].

Elution of molecules from the column: gel filtration is relatively independent of the type of eluent except in cases where possible adsorption of proteins occurs. Molecules can be eluted from the column using the appropriate buffer, i.e. one in which the protein is most stable. In most cases the elution buffer is the same as that used for equilibrating the gel beads. The volume of the eluting buffer used to elute a substance or component is known as the *elution volume* (Ve). In the case of large molecules that are excluded from the gel beads, Ve is exactly equal to the volume of buffer around the bead, which is known as the *void volume* (Vo). The samples eluted from the column can be collected at

the bottom of the column manually or using a programmed fraction collector. More information on gel exclusion (e.g. packing of columns, the different types of matrices used, etc.) is available in protein purification textbooks such as [Janson and Ryden, 1989; Angal and Harris, 1993; Scopes, 1994] as well as in parallel references. Only the basic principles can be covered in the scope of this text.

4.1.4. Concentration of enzymes

Purification steps may sometimes result in dilution of the sample. As a result, a concentration step is required to aid subsequent studies on the enzyme. For example, if the purified protein is to be used for physico-chemical characterization (such as molecular weight studies) concentration of the sample from a gel filtration column may be necessary. The purpose of the concentration step is to reduce the volume of the sample (smaller volumes are easier to handle), and to produce a higher protein concentration. Sample concentration can be done in a number of ways. Among the known different concentration methods are: precipitation by ammonium sulphate, acetone precipitation, adsorption to ion exchangers and removal of water using semi-permeable membranes – depending on the size of protein / particles that can pass through that membrane [Janson and Ryden, 1989]. In our case the concentration method employed during the purification of the cellulosome was acetone precipitation.

Acetone precipitation: The addition of acetone to an aqueous extract containing proteins has a variety of effects, which by a combined effect lead to protein aggregation. The principal effect of acetone is its reduction of the solvation power of water for a charged hydrophilic protein molecule. This can be explained in terms of the displacement of water molecules as well as their partial immobilization through the hydration of acetone. The displacement of water from around the hydrophobic areas on the proteins' surface – by acetone – results in a relatively higher solubility of these hydrophobic areas. As the protein solubility in water decreases, the electrostatic and dipolar forces cause the proteins to form aggregates that lead to precipitation [Figure 4.3]. Hydrophobic attractions are believed to have little or no influence on the formation of these aggregates because of the solubilising power of acetone on these areas [Scopes, 1994].

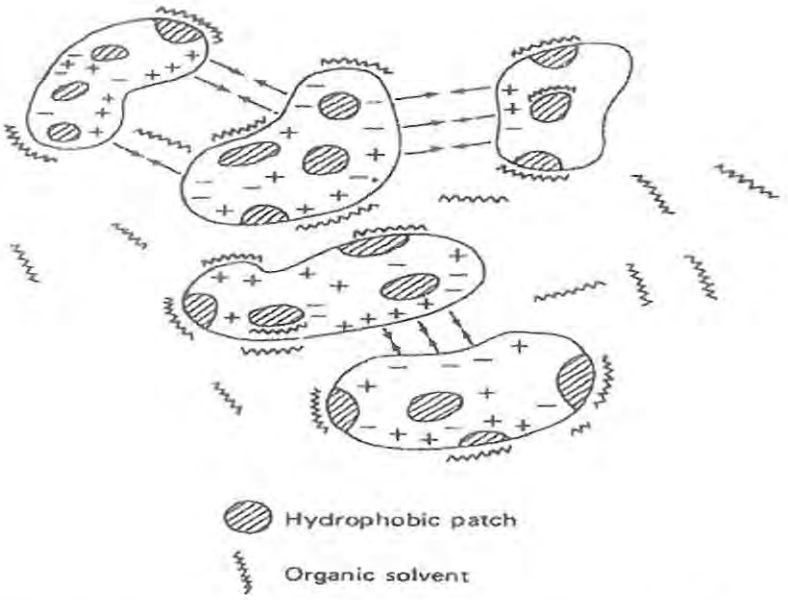

Figure 4.3: Aggregation of proteins by interactions in an aqueous - organic solvent mixture [adopted from Scopes, 1994].

Characterization of the purified protein usually follows at the completion of purification. Characterization of molecular dimensions of the protein is normally achieved by subjecting the protein to gel electrophoresis.

4.1.5. Electrophoresis

Electrophoresis refers to the migration of molecules in an electric field. Fundamental to electrophoretic separations is the fact that proteins are electrically charged particles. The charges are derived from amino acids [such as arginine, lysine, histidine, glutamic acid, aspartic acid and tyrosine] with ionogenic side groups. In addition, the proteins often have associated charged components of non-protein origin such as lipids and carbohydrates. Most of these acids and bases are relatively weak, making the overall charge of the protein strongly pH dependant.

For separation, the sample containing the proteins is placed in an electric field, which forces the electrically charged proteins to move. The separation is normally performed in a gel matrix that can either act as an inert support for the electrophoresis buffer or actively participate in the separation by interacting with the proteins. A variety of matrices including starch, agarose and cellulose acetate can be used for electrophoresis.

However, the high-resolution capacity of polyacrylamide gel electrophoresis (PAGE) makes this the method of choice for most applications. A particularly important procedure is PAGE in the presence of the anionic detergent, sodium dodecyl sulphate (SDS), by which proteins can be characterized in terms of the molecular size of their constituent polypeptides [Janson and Ryden, 1989].

The mobility or the rate of movement of molecules during electrophoresis is governed by three factors; the applied voltage, net charge on the molecule and molecular friction. Applied voltage and the net charge of the molecule are directly proportional to the mobility of the molecule. Conversely, mobility decreases with increased molecular friction caused by molecular size and shape. This set of principles can be illustrated by the following equation:

$$\text{Mobility of a molecule} = \frac{\text{(Applied voltage) (Net charge)}}{\text{Molecular friction}}$$

The voltage applied should be kept constant throughout the electrophoretic run so that the rate of movement of molecules can reflect the net charge and frictional characteristics. Practical aspects of electrophoresis will be dealt with in section 4.2 under methodology [Clark and Switzer, 1977].

4.2. METHODOLOGY

4.2.1. Extraction of enzymes

The procedure followed for extraction of the cellulosome is outlined in Section 3.2.6, p. 68. The sonic extract obtained was approximately 23 ml. A portion (3ml) was kept for assays and SDS-PAGE, and 20 ml was loaded onto a CC-31 cellulose affinity column.

4.2.2. Affinity chromatography.

Principle.

The adsorption of the cellulosome to the cellulose matrix relates to the fundamental role of cell bound cellulolytic enzymes in their adherence to their substrate.

Reagents.

1. 30 g of Whatman cellulose (CC-31) [Sigma].
2. 50 mM Tris-HCl buffer, pH 7.7.
3. 1 M NaOH
4. 10% acetic acid
5. Acetone
6. Sulphidogenic sonic extract brought to pH 7.7 with 1 M NaOH.

Procedure.

Column preparation: 30 g of dry cellulose powder (CC-31) was washed with 2 x 100 ml volumes of 50 mM Tris-HCl buffer, pH 7.7, in a glass beaker. After the second wash, the slurry was allowed to settle under gravity. The supernatant (excess buffer) was discarded and the cellulose was pre-equilibrated with the same buffer and allowed to swell, in a covered beaker overnight. After the cellulose had settled, the supernatant was discarded and the pH was adjusted once again to pH 7.7.

Batchwise adsorption: A portion of the sonic extract (20 ml) containing a crude mixture of enzymes, adjusted to pH 7.7, was added to the cellulose matrix and stirred mechanically for 1 hour at room temperature. The slurry was poured into a sintered glass funnel and filtered under suction using a Buchner flask (Figure 4.4). The slurry was sucked to damp-dryness and the filtrate was decanted into a glass bottle and stored at 4^0C for subsequent studies. The cellulose was resuspended in 100 ml of Tris-HCl buffer, pH 7.7, and then in an extra 30 ml and stirred for 10 min to remove all unbound material. The

slurry was filtered as described above. The filtrate from the Tris-buffer wash was decanted into an empty beaker and stored at 4^0C.

Batchwise desorption: The cellulosome was eluted from the cellulose matrix by resuspension of cellulose in 24 x 20 ml portions of double distilled water. The fractions (20ml) were collected after filtration into a Buchner flask and transferred to 24 x 20 ml glass test tubes. Elution was monitored by measuring the absorbance of proteins at 280 nm. All fractions were assayed for β-glucosidase activity. Fractions containing activity were pooled together and brought to pH 7.0 with 10% acetic acid. In order to concentrate the protein, the pooled fractions (NPF) were brought to 60% (v/v) acetone.

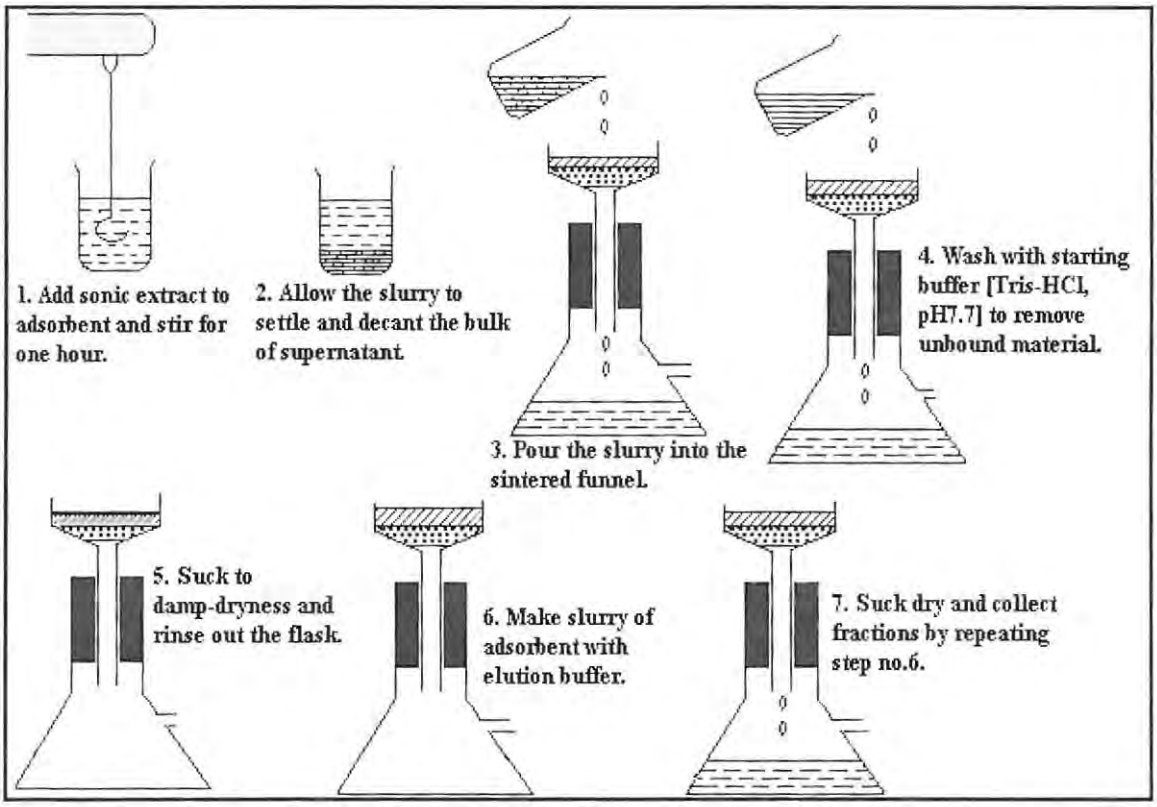

Figure 4.4: Operational procedure for batch-wise adsorption.

4.2.3. Precipitation by acetone.

Both the pooled fractions from affinity chromatography and acetone were chilled to 4^0C in an ice bath. A thermometer was placed inside the beaker containing the sample and acetone was added slowly to the sample with gentle stirring, maintaining the temperature below 10^0C until the solution reached 60% (acetone). The solution was allowed to stand in an ice bath until light brown flakes were observed. The suspension was centrifuged at 4^0C (15 300 g, JA-14 rotor, J21 Beckman centrifuge for 20 min). The supernatant was decanted into a clean beaker and returned to the ice bath for further precipitation. The combined precipitate was resuspended in Tris-buffer (10 ml). A portion was kept aside for enzyme assays and SDS-PAGE and the remainder was further purified by gel filtration.

4.2.4. Gel filtration.

Principle

The principles of gel filtration are discussed under the introduction

Reagents.

1. Sepharose 4B [Sigma].
2. Acetone
3. 50 mM Tris-HCl buffer, pH 7.7.

Procedure.

Gel filtration was carried out at room temperature on a Sepharose 4B column. The column was equilibrated and eluted with Tris-HCl buffer. The column dimensions were 0.9 x 18 cm. The gel bead volume was 25 ml. The peristaltic pump was set to a flow rate of 3 drops / second and fractions of 5 ml were collected with a fraction collector [Figure 4.5]. Absorbance at 280 nm was measured and beta-glucosidase activity was determined

in all fractions. The major peak fractions were pooled, neutralized with 10% acetic acid and brought to 60% acetone. After centrifugation, the precipitate was redissolved in 5 ml of Tris-HCl buffer. A portion of 3 ml was kept for enzyme assays and 2 ml for SDS-PAGE.

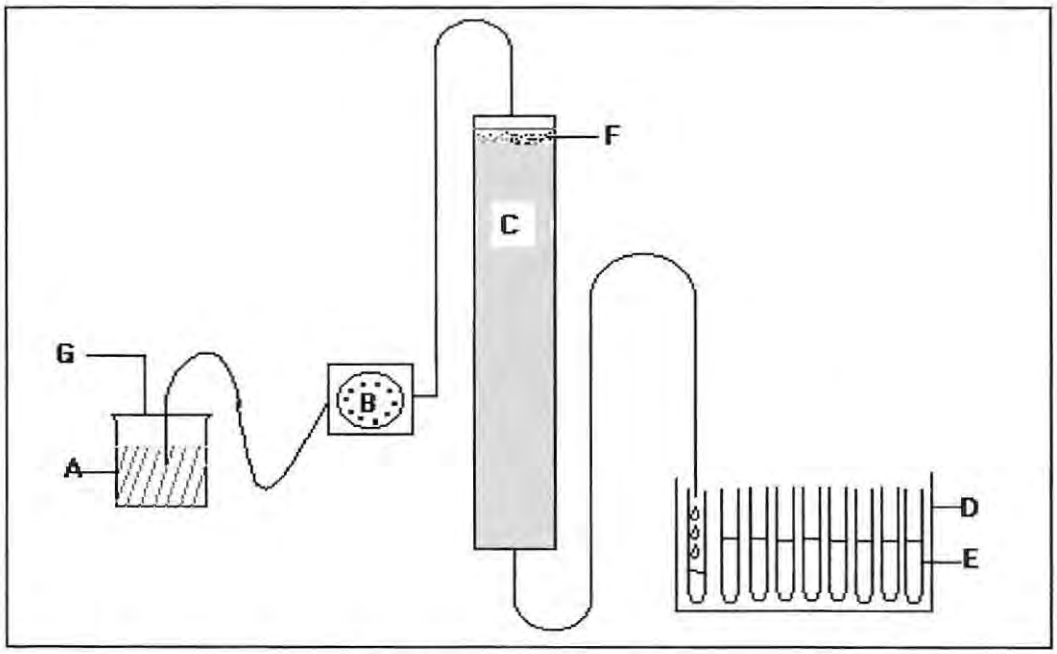

Figure 4.5: Illustrating the set up of apparatus during gel filtration. A-Beaker containing elution buffer [Tris-HCl buffer] and covered in parafilm (G). B-peristaltic pump. C - column. D – fraction collector with collecting tubes (E). F – Sample loaded on sepharose 4B.

4.2.5. SDS-Polyacrylamide gel electrophoresis.

The procedure followed was according to Laemmli (1970). Gels (12%) were used and stained with Coomassie Brilliant Blue R-250.

Reagents.

1. **Acrylamide/Bis:** (30% T, 2.67% C), prepared by dissolving acrylamide (87.6 g) and N', N' -bis-methane-acrylamide (2.4 g) in deionised water to make up a 300 ml solution. The solution was filtered and stored in the dark at 4^0C. This solution is stable for at least one month at 4^0C.

2. **SDS 10% (w/v):** SDS (10 g) was dissolved in 90 ml of water with gentle stirring. The solution was brought up to 100 ml with deionised water.

3. **Bromophenol blue 0.5 % (w/v):** Bromophenol blue (0.5 g) was dissolved in deionised water to make up a 100 ml solution.

4. **Resolving gel buffer:** [1.5 M Tris-HCl, pH 8.8], prepared by dissolving Tris base (27.23 g) in 80 ml of deionised water. The pH was adjusted to pH 8.8 with 6 M HCl. The solution was made up to 150 ml with deionised water and stored at 4^0C.

5. **Stacking gel buffer**: [0.5 M Tris-HCl, pH 6.8], Tris base (6 g) was dissolved in 60 ml of deionised water and adjusted to pH 6.8 with 6M HCl. The total volume was brought up to 100 ml with deionised water and stored at 4^0C.

6. **Sample buffer:** mix 3.55 ml of deionised water, 1.25 ml of stacking gel buffer, 2.5 ml of glycerol, 2.0 ml 10% (w/v) SDS and 0.2 ml of 0.5% (w/v) bromophenol blue to make solution A. β–Mercaptoethanol (5µl) was added to 950 µl of sample buffer to make solution B. The sample was diluted three times with solution B and heated at 95^0C for 4 minutes prior to loading.

7. **10X Electrode reservoir buffer (ERB), pH 8.3:** Tris base (30.3 g), glycine (144 g) and SDS (10 g) were dissolved in distilled water to make a 1L solution and stored at 4^0C.

8. Ammonium per sulphate (APS) 10%: 100 mg of APS was dissolved in 1 ml of deionised water. The solution was prepared fresh daily.

9. **12% Resolving gel:**

* Resolving gel buffer	3800µl
* 10% (w/v) SDS	150µl
* APS	150µl
* Acrylamide/Bis	6000µl
* Deionised water	4900µl
* TEMED	6µl

10. **5% Stacking gel:**

* Stacking gel buffer	750µl
* 10% (w/v) SDS	60µl
* APS	60µl
* Acrylamide /Bis	1000µl
* Deionised water	4000µl
* TEMED	6µl

11. **Coomasie Gel stain:** Coomassie brilliant blue (1 g) was dissolved in 450 ml of methanol, 450 ml distilled water and 100 ml of glacial acetic acid.

12 **Coomassie Gel destain:** Methanol (100 ml) was mixed with water (800 ml) and glacial acetic acid (100 ml).

Procedure.

Gel casting, loading of samples, gel staining and destaining.

1. The gel cassette was assembled vertically on a cassette stand, using clean dry glass plates and plastic clamps. The resolving gel was poured gently with a micro-pipette into the assembled gel cassette to about 1 cm below the level,

which would be occupied by the well-forming comb. Oxygen inhibits polymerization; therefore, trapping of air bubbles was avoided during casting. To avoid contact of the gel with air, a layer of distilled water was overlaid on the gel and polymerization was complete within 45 minutes.

2. After polymerization, the layer of water was removed with filter paper and the cassette was filled with the stacking gel mixture. The sample well-forming comb was inserted between the spacers and the stacking gel was allowed to polymerize for 30 minutes. After polymerization the comb and the casting frame were removed from the casting stand. The gel cassette was installed into the slot at the bottom of the electrode assembly. The electrode assembly was placed into an electrode chamber or mini tank filled with the ERB. The inner chamber of the electrode assembly was also filled with ERB.

3. The samples were loaded into the sample wells a 10 µl Hamilton syringe. The mini tank was closed with the tank lid and the electrodes were inserted accordingly.

4. The gel was run at 200 volts for 30 minutes. At the completion of electrophoresis, the ERB was poured off and the gel was removed from the gel cassette and transferred (with gloves on) to a flat glass container half-filled with the Coomassie Brilliant blue stain.

5. The container was covered with a plastic wrap and placed on a rocking shaker to agitate for 30 minutes.

6. The stain was poured out into a clean container and the gel was washed with three changes of water and the Coomassie destain solution was added. Destaining was complete after an hour and strong protein bands could be seen.

4.3. RESULTS.

4.3.1. Affinity chromatography

A volume of exactly 22.6 ml of extract was obtained from sonication of the sulphidogenic slurry. After equilibration with Tris-HCl buffer and removal of the supernatant buffer, the cellulose adsorbent slurry in a volume of 140 ml was treated with a portion of sonic extract (20 ml). After an hour of stirring and two hours to allow the slurry to settle, the cellulose adsorbent had changed from white to light brown in colour. After the removal of excess buffer the unbound material was eluted with a total of 130 ml Tris-HCl buffer (pH 7.7). Initially 100 ml was used in bulk, but the additional 30 ml (used as 10 ml portions, three times) was used for bringing the filtrate to a zero UV-absorbance value to ensure complete removal of unbound material. The elution of the cellulosome from CC-31 with distilled water produced the profile in Figure 4.6.

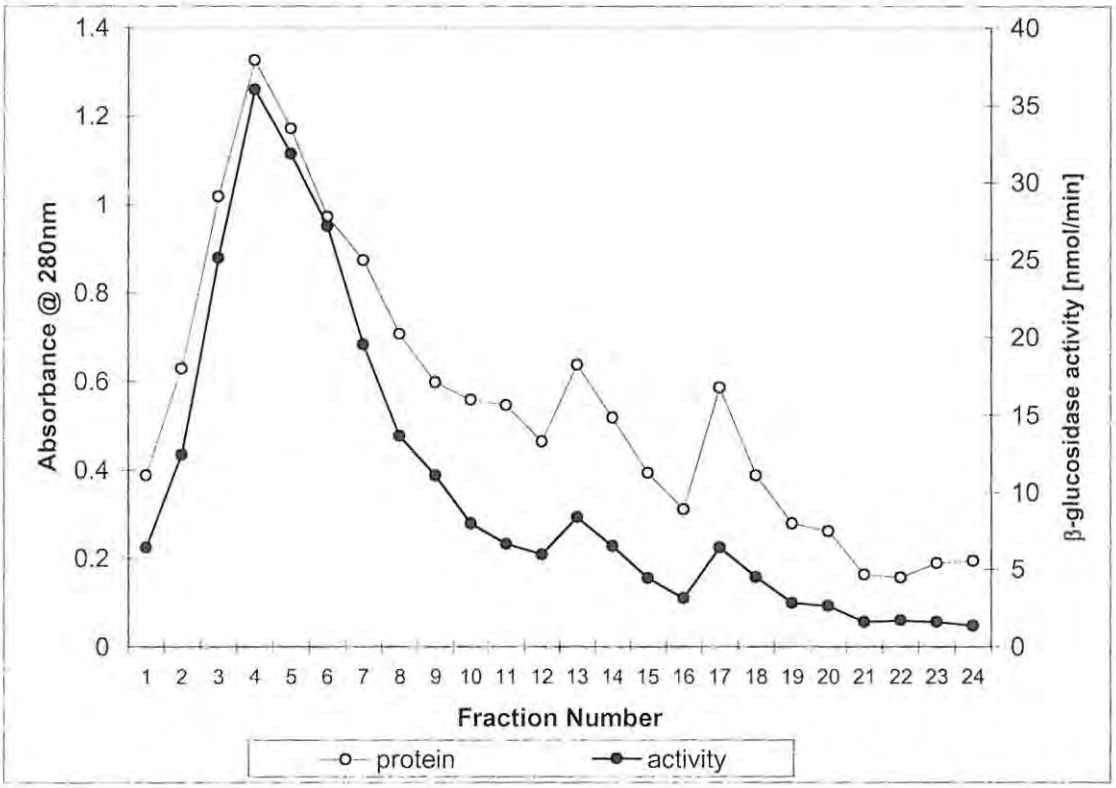

Figure 4.6: Elution profile of β-glucosidases from cellulose CC-31. Three peaks were produced; first peak from fractions 1-11, second peak from fractions 12-15 and the third peak from fractions 16-21.

The sonic extract containing the crude enzyme was resolved into three fractions, with three peaks, all having β-glucosidase activity. The bulk of activity and high protein concentration were in the first 11 fractions, while less enzyme activity was observed in fractions 12-15 and 16-21 respectively.

4.3.2. Gel filtration.

When the precipitate from CC-31 was loaded onto the Sepharose 4B only a portion of the precipitate seemed to elute through the column. Most of the protein remained at the top of the gel bed. In a similar manner to that seen in affinity chromatography, the precipitate fractionated into three protein peaks at 30 ml, 60 ml and 120 ml. Two peaks exhibiting β-glucosidase activity were observed. One of the peaks of activity corresponded to the protein peak at the void volume (i.e. 30 ml). The second activity peak was eluted with an elution volume of 95 ml.

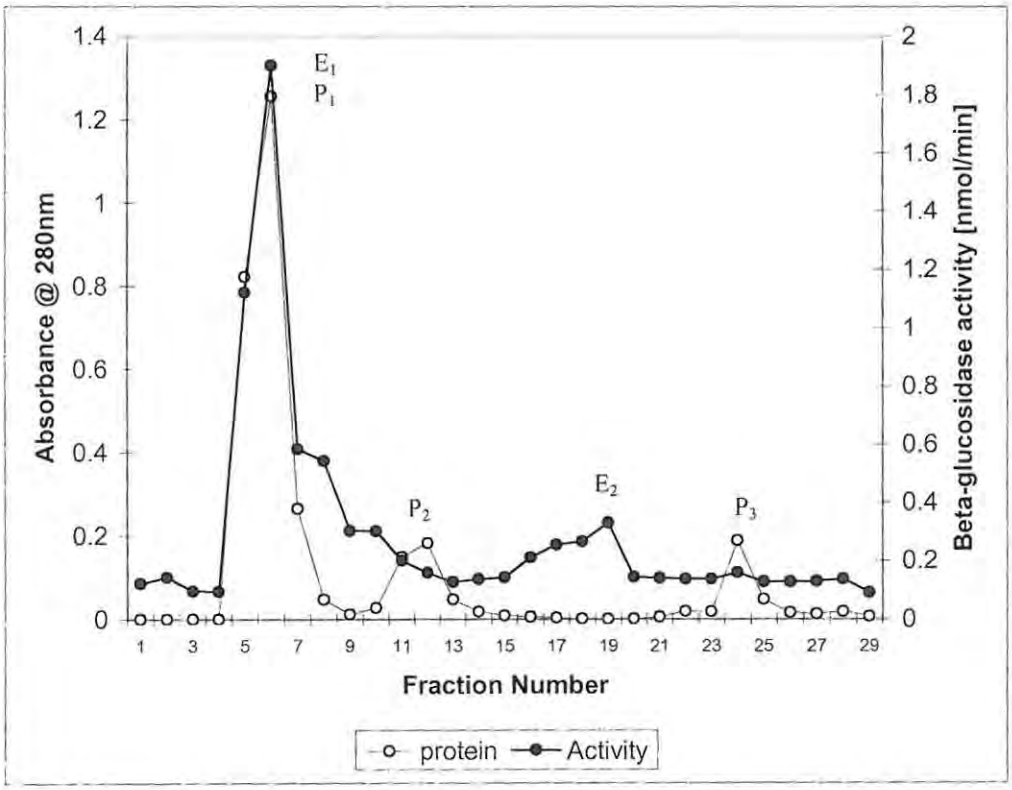

Figure 4.7: Sepharose 4B chromatography of β-glucosidases. Column dimensions 18 x 0.9 cm. Flow rate 7 ml/min. Void volume (Vo) = 30 ml. Bed volume = 25 ml. Fractionation range = 60 – 20 000 KDa. E = enzyme peak, P = protein peak.

Table 4.1 Purification table of β-glucosidases.

Preparation	Volume (ml)	Tot. Protein (mg)	Total Act. (U)	Spec. Act. (U/mg)	Enzyme Recovery (%)	Fold purification
Sonic Extract (crude)	22.6	0.68	40.68	59.82	100	1
Affinity chromat.	10	0.13	14.09	108.38	34.6	1.8
Gel Filtration.	8	0.02	5.98	299.0	14.7	5.0

4.3.3. Sodium dodecyl polyacrylamide gel electrophoresis (SDS-PAGE).

The molecular weights of the polypeptides were analysed by SDS-PAGE (12% gels). The crude extract, the affinity chromatography fraction and the Sepharose 4B fraction were loaded in lanes 1, 2 and 3 respectively [Figure 4.8].

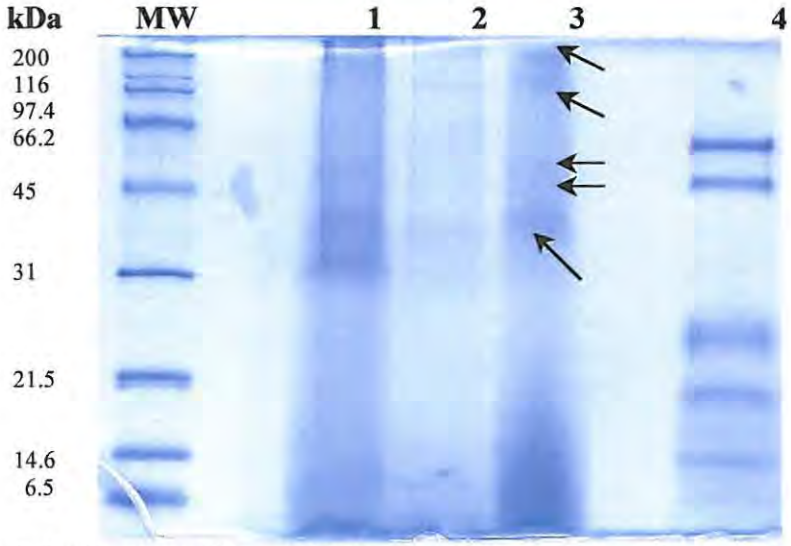

Figure 4.8: Subunit composition and biochemical characterization of the cellulosome isolated from the sewage sludge. Following SDS-PAGE (12% gels), protein content was visualised by Coomassie blue staining. Lane 1 = Crude extract, Lane 2 = affinity chromatography (precipitate), Lane 3 = Fraction from Sepharose 4B, Lane 4 = pure β-glucosidase (control) from *Trichoderma viridae*.

Protein standards (from Sigma) with molecular weights ranging from 6 500 – 200 000 Da were used. The distance migrated by standard proteins was measured and the log molecular weight vs. distance migrated (cm) plot was constructed [Figure 4.9]. In lane 1 (crude extract), dark blue bands forming a continuous shaded lane were observed. In lanes 2 and 3, five faint bands that migrated the same distances were observed. Molecular weights of the five bands from the top downwards were estimated to be 200 000, 115 000, 54 000, 46 000 and 40 000 Da using the equation: *(y = mx + c)* of the log Mwt. vs. distance migrated plot.

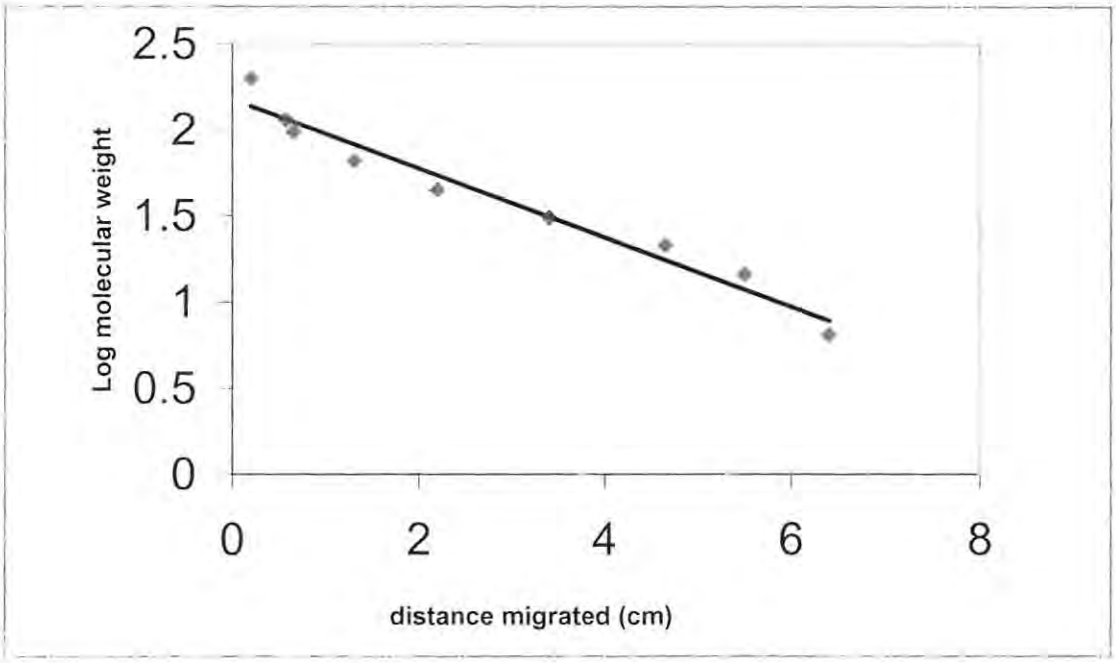

Figure 4.9: Plot of distance versus the log molecular weight of protein standards.

4.4. DISCUSSION.

Purification of cellulolytic enzymes has proven to be extremely difficult due to the complicated nature of both the enzyme system and its natural substrate. Many trial and error procedures (including ammonium sulphate precipitation, concentration of samples with PEG, desalting by dialysis) have been attempted during the purification of β-glucosidases from the sludge. Ammonium sulphate precipitation (ASP) was observed to be less effective in concentrating the enzyme when compared to precipitation by acetone.

In some cases, ASP was observed to compromise enzyme activity when compared with acetone precipitation. Dialysis (at 4^0C in 50 mM Tris-HCl buffer, pH 7.7 and / or in 0.1M phosphate buffer, pH 7.0) decreased the activity by 50%. Concentration of enzymes with PEG took long hours since the volumes from affinity chromatography were large. By the time the concentration step was finished enzyme activity was completely lost.

These results suggested the instability of glucosidases and that a faster purification procedure should be employed. Individual components may not be able to retain their enzyme activities for a long time [Coughlan and Ljungdahl, 1988], though preliminary studies have shown the cellulosome to be resistant to harsh conditions [Bayer and Lamed, 1988]. The alternative was to isolate the whole cellulosome.

The complexity and strong immobilization of the cellulosome to its natural substrate posed further difficulties during isolation. Although sonication (during extraction) could partially overcome the strong immobilization of the cellulosome on the substrate, the intactness of the cellulosome indicated that pure components of the cellulolytic complex (i.e. endo- / exo-glucanases or glucosidases) alone could not be easily obtained. Therefore, detection of fractions containing β-glucosidases relied on the specificity of MUF-GLU.

4.4.1. Affinity Chromatography.

The adsorption of the cellulosome to CC-31 after the removal of unbound material was indicated by the change of color of the adsorbent from white to light brown. The mechanism of interaction of cellulosome with CC-31 is not clearly understood, as the ligand binding the cellulosome is not known. However, the little information that is available concerning the attachment of cellulases to their natural substrate may assist in the elucidation of the interaction cellulosome with CC-31.

The interactions with cellulose are believed to be "non-specific" charge mediated or hydrophobic in nature. On the other hand, swollen beaded cellulose has a microheterogenous structure consisting of crystalline regions interconnected with

amorphous regions. Crystalline regions have hydrophobic and electrostatic sites. The amorphous regions expose the hydroxyl groups of glucose monomers on the cellulose chain making the cellulose matrix hydrophilic in nature [Dean et. al, 1985]. Pores of the cellulose beads are big enough to be penetrated by proteins of around 10^6 Da [Scopes, 1994]. From this information, it is possible that the cellulosomes enter the beads and interact with the OH groups of glucose monomers resulting in a non-specific, charge mediated, electrostatic interaction. In addition, the matrix could form salt bridges with the Cl^- ions that may be attached to the matrix.

Desorption of the enzymes from the matrix was done by decreasing the pH of the equilibrating buffer. The eluant used was distilled water (or 1% triethylamine solution) with cations as opposing ions thus reducing the strength of interaction [Dean et al., 1985]. The amount of enzyme recovered from CC-31 was 34% of the original extract (Table 4.1). This low yield was ascribed to the strong adsorption of the enzyme to the adsorbent leading to impaired desorption of significant quantities of the enzyme. This has been reported as a common observation for the interaction of cellulases with cellulose substrates despite the use of various eluants [Claydon *et al.* 1988]. It is also possible that most of the enzyme in the extract did not bind to the adsorbent due to possible interference of trace amounts of sulphate ions and other impurities since the extract was crude.

Furthermore, steric hindrance from adjacent bound proteins could also prevent more proteins to bind [Scopes, 1994] as cellulosomes are complexes that are difficult to fractionate. The ratio of the protein extract volume to the adsorbent volume was 1:7. Therefore, saturation of binding sites could not have been the case. Despite all the difficulties encountered during the affinity chromatography step, isolation of the cellulosome was successful. The purification fold increased from 1to 1.8 (Table 4.1).

4.4.2. Acetone precipitation.

Brown flakes of protein aggregates appeared after an hour after the addition of acetone. The most important practical consideration of acetone precipitation was the prevention of

protein denaturation upon addition of acetone. Hydration of acetone molecules is an exothermic reaction. When acetone was added quickly to the sample, at percentages below 20% v/v, the temperature increased. At temperatures greater than 10^0C denaturation effects are, therefore, substantial. Denaturation occurs due to disruption of intra-molecular hydrophobic interactions that maintain the protein structure. At low temperatures the protein conformation structure is less flexible. Therefore, chances of penetration of the protein structure by acetone molecules are minimized and such penetration leads to destabilization of the protein. Conversely, at high temperatures the conformational structure is more flexible. If small acetone molecules penetrate, they attach themselves through hydrophobic forces to the internal amino acid residues resulting in protein denaturation [Scopes. 1994]. Consequently, chilled acetone was added in small amounts and when 20% v/v (acetone) was reached, no further increase in temperature was observed. The remainder of the solvent was added in bulk. Protein amount in the affinity chromatography step was 0.13 mg (Table 4.1).

4.4.3. Gel filtration.

Sepharose 4B is classified under a group of agarose gels with superior resolving power [Bayer and Lamed, 1988]. It is capable of fractionating large proteins in the range of 60 – 20 000 KDa [Scopes, 1994]. Most of the proteins (Figure 4.6) with glucosidase activity were eluted at the void volume of the column, suggesting that most of these proteins have large molecular weights. Bayer and Lamed (1988) fractionated cellulosomes from *Clostridium thermocellum* using Sepharose 4B and obtained similar results. They discovered that the major form of the cellulosome comprised an apparently discrete multisubunit polypeptide of M_r = 2.1 x 10^6 Da. In addition, a vesicular form fractionating at the void volume of the Sepharose 4B column had a molecular weight of 1 x 10^6 Da.

The two other protein peaks eluted at 60 ml and 120 ml (i.e. P_2 and P_3, Figure 4.7) (with no β-glucosidase activity) could possibly be cellulolytic enzymes with endo- or exo-glucanase activities. The peak at E_2 (with glucosidase activity but a low A_{280} value) could

be comprised of proteins with little or no aromatic amino acids (tyrosine, tryptophan and phenylalanine), since it is these amino acids that absorb light strongly at 280 nm.

Since all the fractions from affinity chromatography were pooled together, precipitated and fractionated, it would be tempting to consider the three protein peaks from Sepharose the same as those from CC-31resin. This assumption may not be necessarily accurate, as the principles of fractionation of the two techniques are different. Affinity chromatography elutes proteins according to the strength of protein-ligand interaction by decreasing the pH while Sepharose 4B fractionated proteins according to their decreasing molecular weights. In addition, all the protein peaks from CC-31 corresponded to the β-glucosidase peaks, while only peaks from the Sepharose column had glucosidase activity with only the largest peak corresponding to the protein peaks.

Only 14,7% of the original extract was recovered at the end of gel filtration. This can be ascribed to the extensive protein loss between extraction and affinity chromatography, as well as the low protein recovery from affinity chromatography due to the partial elution of the CC-31 precipitate from the Sepharose column. Partial elution of the CC-31 precipitate could have arisen for two reasons. (i) the Sepharose 4B resin could have ionic charges that interact with the proteins leading to adsorption of the latter to the column. Elution of these proteins could probably be achieved by changing the ionic strength or the pH of the elution buffer. (ii) Though cellulosomes are multi-enzyme complexes they can form bigger complexes, the polycellulosomes with molecular weights ranging from 50–80 x 10^6 Da. The exclusion limit of Sepharose 4B is only 20 X 10^6 Da suggesting that the proteins adsorbed at the top of the gel bed could be polycellulosomes excluded from the column. No standards of known molecular weights were run through the Sepharose column. Therefore, the molecular weights of the fractions were not determined thus making the gel filtration only another purification step. Due a to low concentration of protein from the smaller peak at P_3 (Figure 4.7), the two activity peaks were pooled together and precipitated further with 60% acetone. The purification was 5 fold that of the crude extract with a protein concentration of 0.02 mg (Table 4.1).

4.4.4. SDS-PAGE.

The crude sample (sonic extract) contains a mixture of different proteins. When subjected to SDS-PAGE these proteins are likely to be separated into their subunits. Because the protein concentration is high, the subunits produced will be numerous such that the bands representing the polypeptide units overlap resulting in a blue shaded lane with unclear bands. In lanes 2 and 3, where isolation and purification of proteins had taken place, the lanes are clearer showing faint bands. The clarity of the lanes can be ascribed to the removal of all the unwanted proteins during isolation and the faintness of the bands, to the low concentration of protein in the loaded samples. The 14 polypeptide molecular weights of the cellulosome have been characterized [Bayer and Lamed, 1988] and their calculated masses are tabulated (Table 4.2).

Table 4.2: Polypeptide molecular weights of the cellulosome from Clostridium thermocellum.

SUBUNIT	MOLECULAR WEIGHT (Da).
S1	210 000
S2	170 000
S3	150 000
S4	115 000
S5	98 000
S6	91 000
S7	84 000
S8	75 000
S9	67 000
S10	66 000
S11	60 000
S12	57 000
S13	54 000
S14	48 000

The results of fractionation of the cellulosome from the sludge by SDS-PAGE were similar to the findings of Bayer and Lamed (1988). Four (i.e. with molecular weights = 200 000, 115 000, 54 000, and 46 000 Da) of the five bands observed in Figure 4.8 correspond closely with those in Table 4.2. The 200 000 Da subunit could possibly be the same designated the 210 kDa subunit. The latter is a glycoprotein containing about 50% carbohydrate, the majority of which is galactose. Though it does not exhibit measurable

cellulolytic activity, it is believed to have an essential role in the organization or assembly of the cellulosome complex. However, preliminary studies have shown that these multi subunits formed by these polypeptides possess cellulolytic activities. In addition, the cellulosome is remarkably stable and resistant to many procedures designed to interfere with inter-peptide bonding. Only treatment with boiling SDS effects significant dissociation of the complex into subunits [Bayer and Lamed, 1988].

CHAPTER FIVE

CHARACTERIZATION OF β-GLUCOSIDASE: THE EFFECTS OF SULPHUR-CONTAINING COMPOUNDS, HEAVY METALS AND VOLATILE FATTY ACIDS ON ENZYME ACTIVITY.

5.1. INTRODUCTION.

5.1.1 The effects of sulphur containing compounds on β-Glucosidase activity.

The connection between sulphate reduction and acid mine drainage bioremediation has been described in Chapter 1 (Section 1.4 – p. 9). The reduction of sulphate involves production of bisulphite and sulphite ions as precursors of sulphide (one of the final products of sulphate reduction). Time trial studies (Chapter 2) have shown that under sulphidogenic conditions, production of cellulases (between days 5-13) and of glucosidases (between days 5-17) increased (Figure 2.8 – p. 46) with decreasing sulphate removal (Figure 2.12 – p. 49). In the methanogenic sludge, activity of cellulases and glucosidases respectively decreased after 5 and 7 days (Figures 28. and 2.9).

Maximum sulphate removal (from a concentration of 2 184 mg/L to 420 mg/L) occurred by day 9 (Figure 2.12) while maximum sulphide production (2 599 mg/L) was at day 13 (Figure 2.13 – p. 50). After day 13 cellulolytic activity in the sulphidogenic system decreased and it was after this day that most of the sulphide in solution escaped from the system as H_2S. The activity of β-glucosidases also decreased after day 17. Furthermore, from day 1-5 at sulphate concentrations between 2 184 – 1211 mg/L and sulphide levels between 0 - 714 mg/L, enzyme activity was low but increased gradually towards day 9. The time frame between maximum sulphate removal and maximum sulphide production was 4 days. It was within this same period that maximum activity for both enzymes was observed. Since sulphite ions are intermediates of sulphate reduction to sulphide, it was assumed that these ions could possibly be maximally produced during this period. The one question that needed to be addressed was whether the sulphate, sulphite and sulphide had a stimulatory or inhibitory effect on the enzymatic activity.

5.1.2. The effects of heavy metals on β-Glucosidase activity.

AMD is characterised by a high concentration of heavy metals. Inhibition of anaerobic degradation by these heavy metals is well known [Mosey *et. al.*, 1971; Khan and Trottier, 1978]. Concentrations at which these metal effects become

apparent appear to differ widely. If it is assumed that free heavy metal ions are the direct inhibitors, reported variations in toxic concentrations have been explained in terms of the presence of harmful constituents in the sludge, which react with metal ions to form less harmful compounds. One of the constituents in the sulphidogenic sludge is H_2S, which is known to interact with metals to form insoluble metal sulphides that precipitate out of solution. The second major process capable of removing sufficient heavy metal ions from solution is their interaction with the carbon dioxide/carbonate system to form metal carbonates [Mosey *et. al.*, 1971].

Though enough information on anaerobic degradation inhibition by heavy metals is available, the effect of these metals on enzymatic activity has not been explored. Therefore the second question that needs to be addressed was the concentrations at which these heavy metals become toxic in the presence and absence of H_2S and bicarbonate ions.

5.1.2 The effects of volatile fatty acids (VFAs) on β–Glucosidase activity.

The hydrolytic stage of anaerobic degradation results in the production of VFAs, which have been pronounced suitable electron donors for sulphate reduction. Time trial studies have shown a decrease in pH in the methanogenic system from 6.02 to 4.99 from day 1 to day 9 respectively (Figure 2.15 – p. 51) and this decrease in pH seemed to be confluent with the decrease in enzymatic activity (Figure 2.9 – p. 46). The decrease in pH was associated with the production of volatile fatty acids from the hydrolytic stage of anaerobic degradation. Accumulation of VFA's is known to inhibit anaerobic degradation [Khan and Trottier, 1978].

On the other hand, the pH in the sulphidogenic system fluctuated between 7 and 8. VFA's produced in this system are neutralized by bicarbonate ions produced from sulphate reduction. Though it is known that high concentrations of VFA's inhibit anaerobic degradation, the toxic levels and their direct effects on hydrolytic enzymes have not been studied. The third objective of this chapter was therefore to investigate the effects of acetate, propionate and butyrate on enzymatic activity.

5.1.3 Determination of pH optima.

Suitable pH and temperature conditions are part of the requirements for optimal growth of bacteria. Different bacteria can grow over a wide range of pH values from quite acidic (pH ±3) to alkaline (pH ± 10 or even higher) conditions. Bacteria concerned in hydrolytic reactions of anaerobic degradation are in the middle of this pH range. The fermentation of simple sugars occurs at relatively low pH values of 5 to 6. However, degradation of polysaccharides takes place optimally at higher pH values of 6.5 to 7.0. Different research scientists have reported different pH optima ranges for cellulolytic bacteria [Hobson and Wheatley, 1993]. Such differences seemed to have been due to different sources of bacterial isolates and experimental designs. Therefore, it is important in this study to determine the pH optimum/range of β-glucosidases.

5.1.5 Optimum temperature determination for β-Glucosidases.

Hydrolytic bacteria can grow at three different temperature ranges: the psychrophilic (i.e. 0^0C to 15^0C), the mesophilic (20^0C to 45^0C) and the thermophilic (50^0C to 70^0C). The rate of polysaccharide degradation has been reported to accelerate in the thermophilic range [Hobson and Wheatley, 1993]. This accelerated hydrolysis could have been due to the combination of the heat and pH effects or due to stimulation of enzymatic activity by high temperatures. Therefore, investigation of optimum temperature of glucosidases was included as one of the objectives of this chapter.

5.1.6 Thermal stability studies for β-Glucosidases.

Finally, the stability of enzymes is advantageous for their industrial application and it is thus important to determine the feasibility of an enzyme system for a particular application. The loss of enzyme activity with time is one of the most important factors in determining the system performance and economics [Busto *et. al.*1996]. One of the causes of enzyme activity loss is inactivation resulting from thermal denaturation.

Based on the results of optimum temperature studies, thermal stability of β-glucosidases present in the sludge were also carried out. These studies were initially

part of the purification procedure since heat denaturation is sometimes used as one the purification techniques. This explains the investigation of the different preparations (i.e. crude sludge enzyme, sonic extract and ammonium precipitate fractions mentioned later in Section 5.2.4), as these fractions were part of the trial and error procedures during the purification process.

5.2 METHODOLOGY.

5.2.1 The effects of sulphur containing compounds, heavy metals and volatile fatty acids on enzyme activity.

Enzyme preparation: Due to the low recovery and instability of purified glucosidase, this study was carried out using glucosidase in its original form. Crude samples obtained from both the sulphidogenic and methanogenic reactors were centrifuged at 15 300 g for 30 minutes. The pellets obtained were washed three times with distilled water (50% of the original volume of the sample before it was centrifuged) to remove extraneous material. The mass of the final pellet was measured, re-suspended in 4x v/w of phosphate buffer (pH 7.0, 0.1M) and sonicated as described earlier. Sonic extracts were prepared according to the procedure in Chapter 3.

Reagents.

1 To yield concentrations of 4000 mg/L the following stock solutions were prepared:

Thiols.

(a) 55 mM ammonium sulphate solution: 0.552 g of $(NH_4)_2SO_4$ dissolved in 100 ml of distilled water.

(b) 33.4 mM sodium bisulphite solution: 0.528 g of $Na_2S_2O_5$ dissolved in 100 ml of distilled water.

(c) 124.5 mM sodium sulphide solution: 2.99 g of Na_2 S.$9H_2O$ dissolved in 100 ml of distilled water.

Heavy metals.

The metal ions were dissolved in water as their chloride salts.

(a) 60 mM $ZnCl_2$: 0.829 g of $ZnCl_2$ dissolved in 100 ml of distilled water

(b) 15.2 mM $FeCl_3.6H_20$ solution: 1.934 g $FeCl_3.6H_20$ dissolved in 100 ml of distilled water.

(c) 8.0 mM $CuCl_2$ solution: 1.073 g of dissolved in 100 ml of distilled water.

(d) 12.5 mM $NiCl_2$ solution: 1.62 g $NiCl_2$ dissolved in 100 ml of distilled water.

Volatile fatty acids.

(a) Butyric acid (45 mM): 42.2µl of 99% butyric acid in 10 ml of distilled water.

(b) Propionic acid (54 mM): 40.5 µl of 99% propionic acid in 10 ml of distilled water.

(c) Acetic acid (66 mM): 38.2 µl of glacial acetic acid in 10 ml of distilled water.

2. 3.0 mM stock solution: MUF-GLU substrate (0.1 g) dissolved in 100 ml of 0.1 M phosphate buffer, pH 7.0.

Procedure.

Variable volumes of the metal ions, volatile fatty acids, sulphur compounds were made up to 0.25 ml with distilled water to obtain a range of concentrations from 0 to 1000 mg/L (Table 5.1).

To 0.5 ml of the sonic extract various effectors (0.25 ml) with different concentrations were added. The mixture was incubated at room temperature for 15 min. The substrate (0.25 ml) was added to all sample mixtures and incubated at 50^0C in a rotary shaker (320 rpm x 5 minutes). The reaction was stopped by addition of 95% ethanol (2.5 ml), mixed and centrifuged at 982 g (A-4-62 rotor, Eppendorf centrifuge 5810R) for 5 min (4^0C). The samples were transferred to clean 10 ml glass test tubes containing 0.5 ml glycine buffer (50 mM, pH 10.8). Fluorescence was read as described in (Chapter 2, Section 2.2.3).

Table 5.1: Preparation of different concentrations of the various effectors (i.e. metals, volatile fatty acids and sulphur containing compounds).

Effector concentration (mg/L)	Stock solution volume. (ml)	Phosphate buffer volume. (ml)	Substrate volume (ml)	Sludge enzyme volume (ml)
0	0	0.25	0.25	0.5
200	0.05	0.20	0.25	0.5
400	0.10	0.15	0.25	0.5
600	0.15	0.10	0.25	0.5
800	0.20	0.05	0.25	0.5
1000	0.25	0.00	0.25	05

5.2.2 Determination of pH optima.

The two buffer systems used were an acetate buffer (with pH values ranging from 3.8 to 5.76) and a phosphate buffer system (with pH values from 6.0 to 8.0).

Reagents.

1. 0.1 M acetate buffer: 0.1M of acetic acid solution was titrated with 0.1 M of NaOH solution to prepare buffers of different pH values.

2. 0.1 M Phosphate buffer: prepared by titrating 0.1M NaH_2PO_4 solution against 0.1M NaOH to obtain buffers of the desired pH.

3. 0.1 mM MUF-GLU solution dissolved in distilled water.

4. 95% Ethanol.

5. 50 mM Glycine buffer, pH 10.8 (Section 2.2.3 – p. 35).

Procedure.

To 0.25 ml of substrate, 0.1 ml of enzyme was added. The mixture was made up to 1.0 ml with 0.65 ml of the appropriate buffer. The assay conditions for β-glucosidase activity were followed according to Chapter 2, Section 2.2.3 – p. 35.

5.2.3 Determination of optimum temperature for β–Glucosidase activity.

β-Glucosidase assay conditions were similar to those described in section 2.2.3 – p35, but the samples, prepared in triplicate were incubated at different temperatures ranging from 0^0C to 77^0C.

5.2.4 Thermal stability studies for β-Glucosidases.

Different enzyme fractions, the sonic extract, 10% ammonium sulphate precipitate and the crude enzyme pellet were prepared. Thermal stability of glucosidase was studied by incubating the enzyme fractions at 50^0C and withdrawing 2 ml aliquots for assay from each fraction at 15 min intervals over an incubation period of three hours.

5.3 RESULTS.

5.3.1 Effects of sulphate, sulphite and sulphide on enzyme activity.

At 200 mg/L of sulphate, activity was unaltered in the methanogenic system and was slightly increased in the sulphidogenic system. Above this concentration β-glucosidase activity was progressively slightly inhibited (Figure 5.1). In both reactors, sulphite was observed to enhance activity. At 200 mg/L of sulphite activity was increased by 192% in the methanogenic sludge and by 55% in the sulphidogenic sludge (Figure 5.2). The reduced effect of sulphite in the sulphidogenic system could be an inaccurate reflection of the actual result as the sonic extract from the sulphidogenic system already contains trace amounts of endogenous sulphite making the actual final sulphite concentration

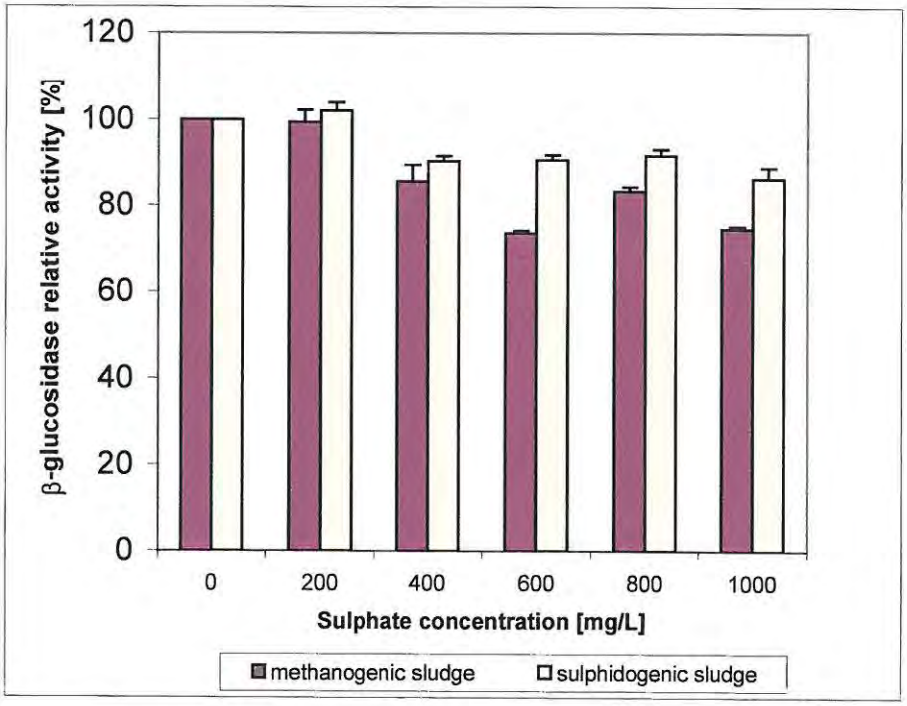

Figure 5.1:The effect of sulphate on β-glucosidase activity

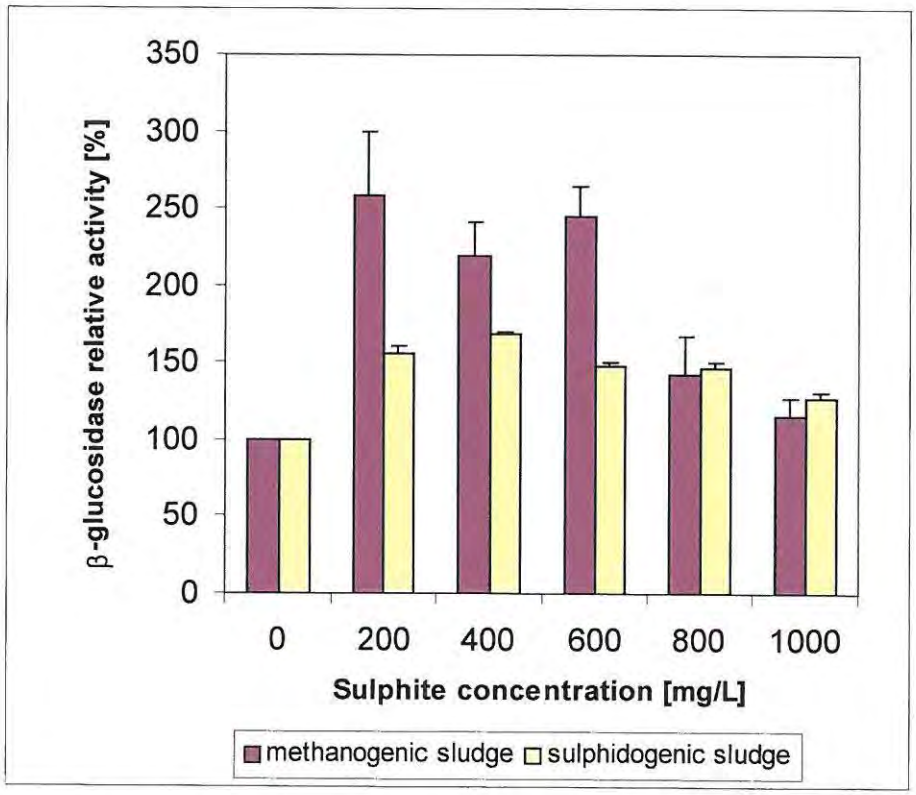

Figure 5.2: The effect of sulphite on β-glucosidase activity.

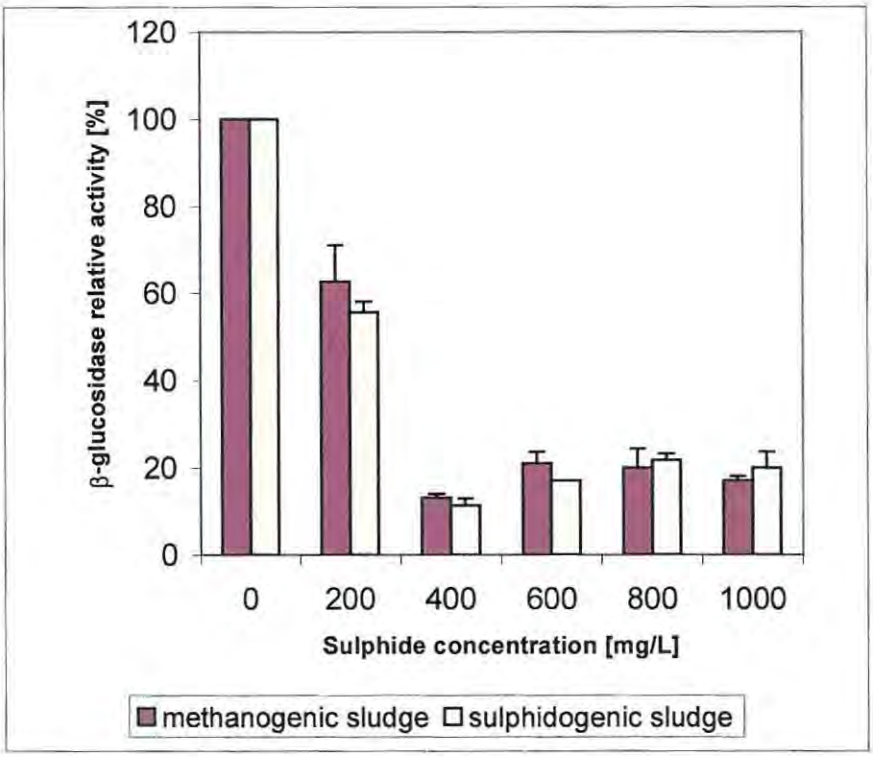

Figure 5.3: The effect of sulphide on β-glucosidase activity.

higher than the added amount. The stimulation of activity decreased with increasing sulphite concentrations. Though the decrease suggested enzyme inhibition at higher sulphite concentrations, at 1000 mg/L of sulphite activity was still enhanced by 15% in the methanogenic system and 27% in the sulphidogenic system. Though enzyme activity was enhanced more in the methanogenic system at lower concentrations, the 12% activity difference between the methanogenic and the sulphidogenic sludge (i.e. from 27% - 15%) indicated that enzymes produced in the sulphidogenic system have more tolerance for sulphite even at very high concentrations. In both systems, enzyme activity was markedly inhibited by sulphide even at low levels i.e. 200 mg/L (Figure 5.3).

5.3.2 Effects of heavy metals on enzyme activity.

Enzymatic activity was markedly inhibited by most metals, with the exception of Zn^{++} at concentrations $\leq$ 600 mg/L in the methanogenic sludge and Fe^{++} below 200 mg/L in both the methanogenic and the sulphidogenic systems (Figures 5.4 - 5.7). Fe^{++} ions showed ±9.% stimulation in the methanogenic system and 48.3% in the sulphidogenic system. The methanogenic system showed tolerance for concentrations up to

600 mg/L of Zn^{++} ions and the sulphidogenic system was inhibited. When percentage activities between both systems were compared, inhibition of enzymatic activity by copper and iron was generally lower in the sulphidogenic system (Table 5.2). For Fe^{++} 97% enzymatic inhibition was observed at 600 mg/L in the methanogenic system while the sulphidogenic system only showed ±90% inhibition.

Table 5.2: Comparing inhibition [%] of enzymatic activity by metal ions in the methanogenic and the sulphidogenic systems.

Conc.	Zinc		Iron		Nickel		Copper	
[mg/L]	Meth.	Sulph.	Meth.	Sulph.	Meth.	Sulph.	Meth.	Sulph.
200	-	67	-	-	32.7	39.7	75.0	72.7
400	-	52	95.7	66.0	30.7	46.9	87.4	77.69
600	-	56	97.0	90.0	40.7	59.0	93.7	86.6
800	35.4	60	99.0	97.4	39.0	64.7	69.0	91.4
1000	72.4	69	100	100	43.7	66.0	87.4	88.0

NB: Meth. = Methanogenic system, Sulph. = Sulphidogenic system.

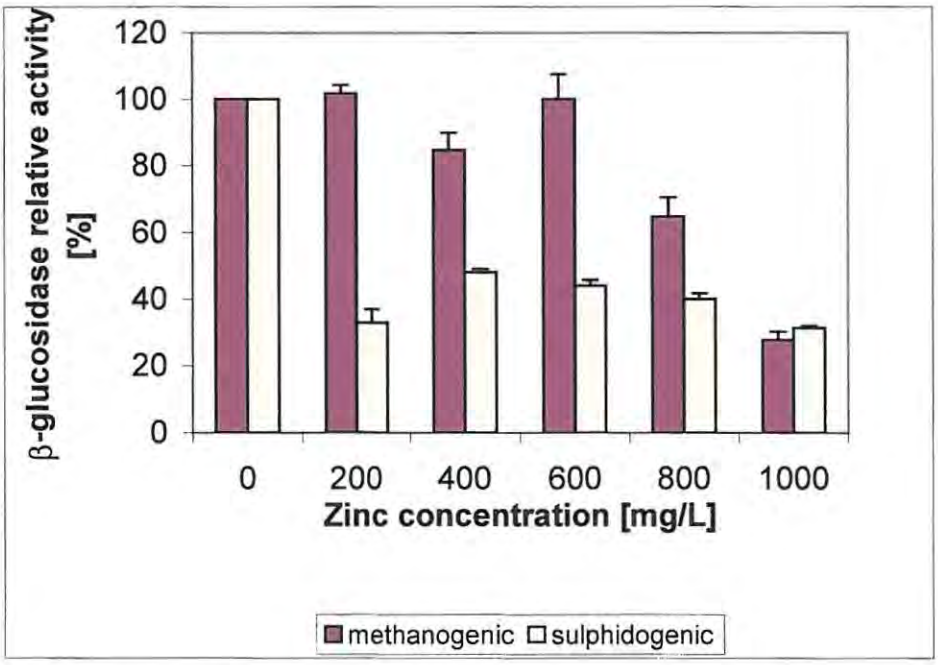

Figure 5.4:The effect of zinc on β-glucosidase activity.

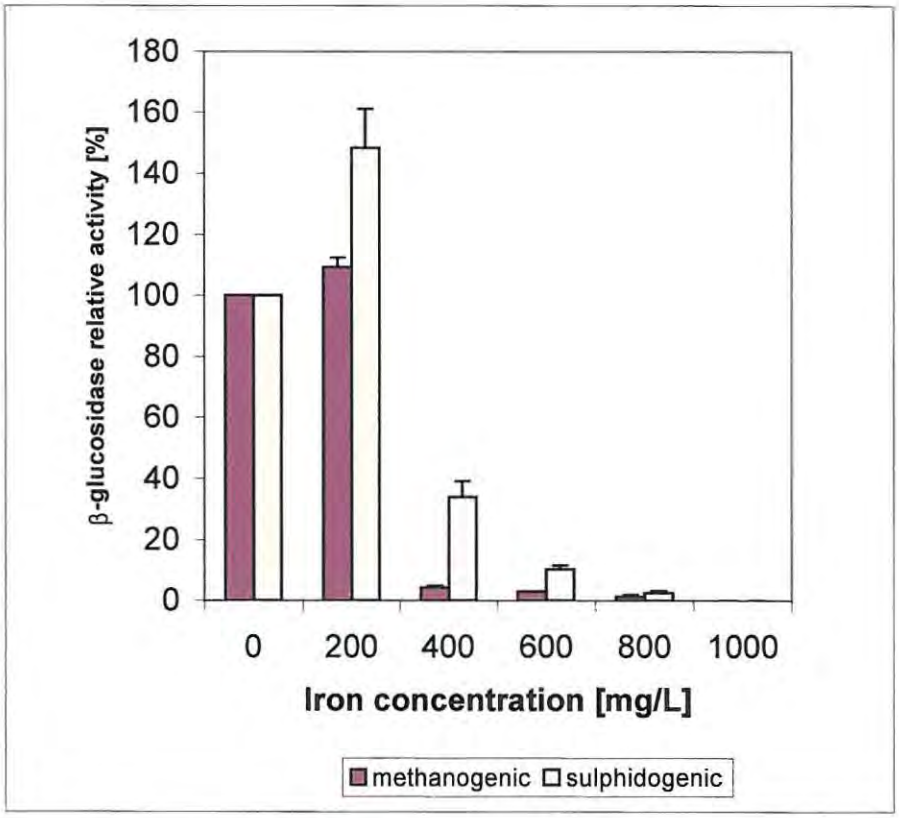

Figure 5.5: The effect of iron on β-glucosidase activity.

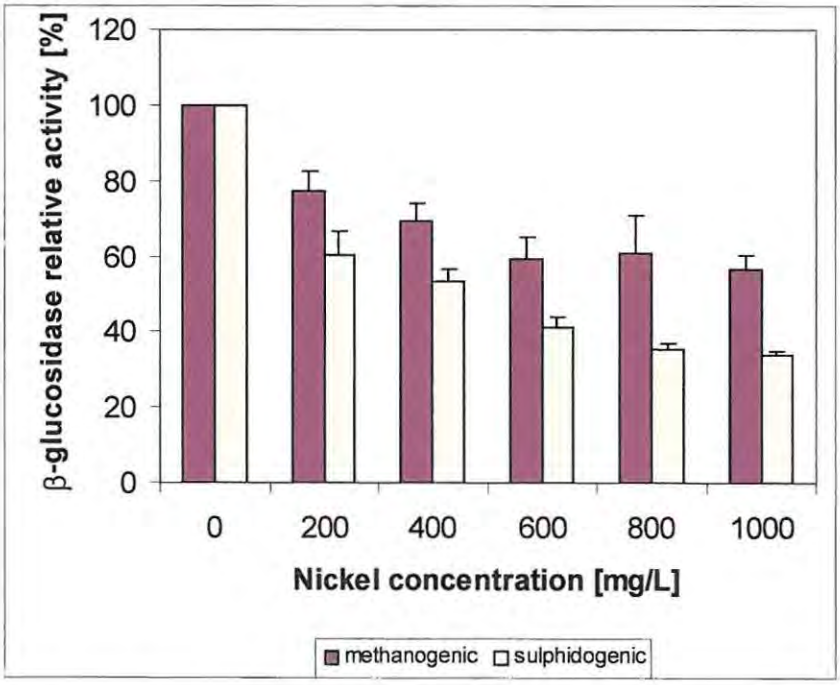

Figure 5.6: The effect of nickel on β–glucosidase activity.

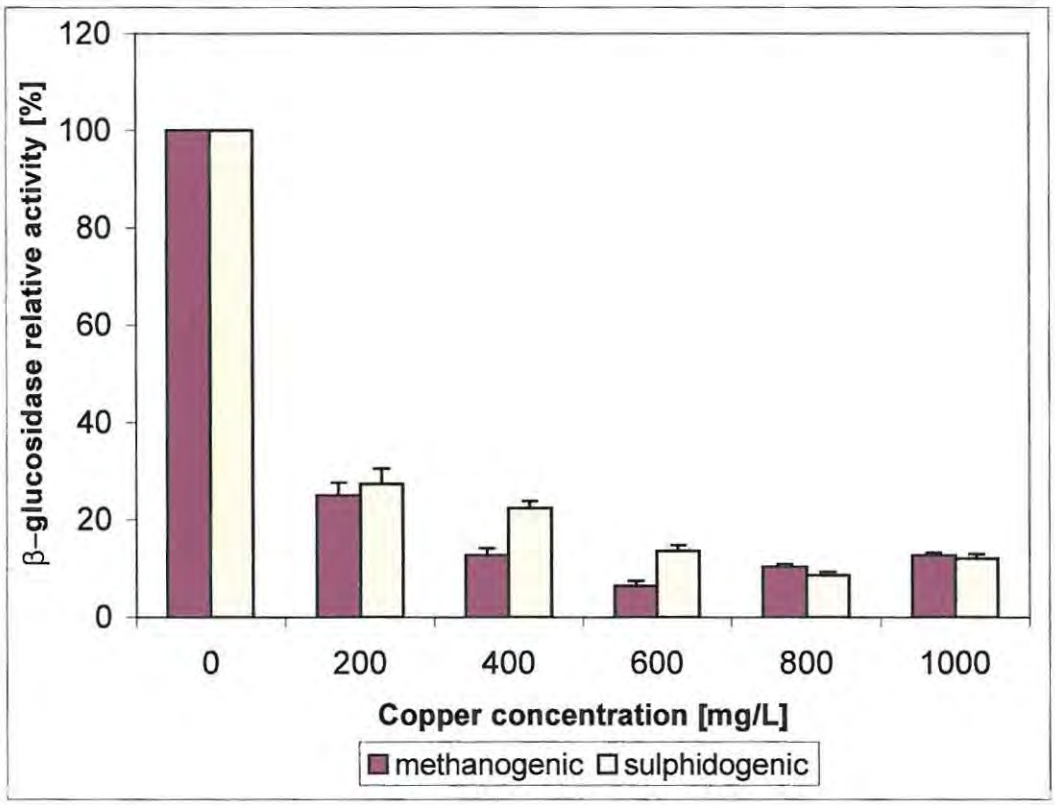

Figure 5.7: The effect of copper on β-glucosidase activity.

5.3.3 Effects of volatile fatty acids on enzyme activity.

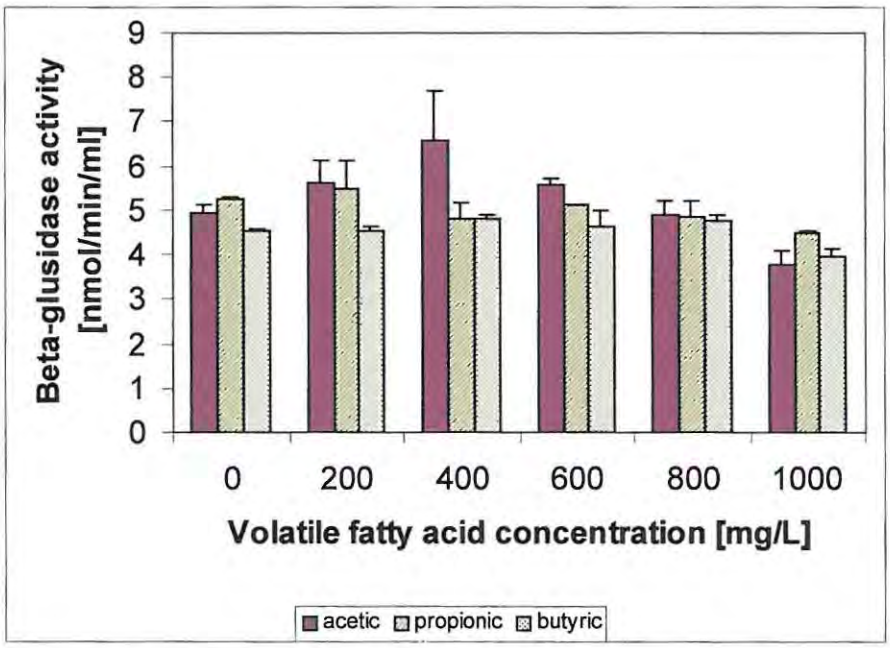

Figure 5.8: The effect of volatile fatty acids on β-glucosidase activity.

The effects of volatile fatty acids on enzymatic activity were similar in both systems. Both the propionic and the butyric acids had no significant effect on enzymatic activity except for slight inhibition observed at concentrations above 800 mg/L. Acetic acid was stimulatory up to 400 mg/L and inhibitory at concentrations above 600mg/L.

5.3.4 Optimum pH, optimum temperature and thermal stability.

The pH optima were in the range between 5.4 and 7.2 in both systems (Figure 5.9) and the temperatures at which glucosidases operated optimally were 60^0C in the methanogenic sludge and 50^0C in the sulphidogenic sludge (Figure 5.10). Though thermal stability studies on glucosidases are included as part of characterization, they were initially carried out as part of the purification procedure. The intention was to isolate glucosidases by heating them at their optimum temperature in order to denature all the undesired proteins / enzymes and interfering activities, if the sludge glucosidases are discovered to be thermostable. The three fractions (i.e. the crude pellet, sonic extract and the ammonium sulphate precipitate) that were tested all showed thermal instability when heated at 50^0C (Figure 5.11).

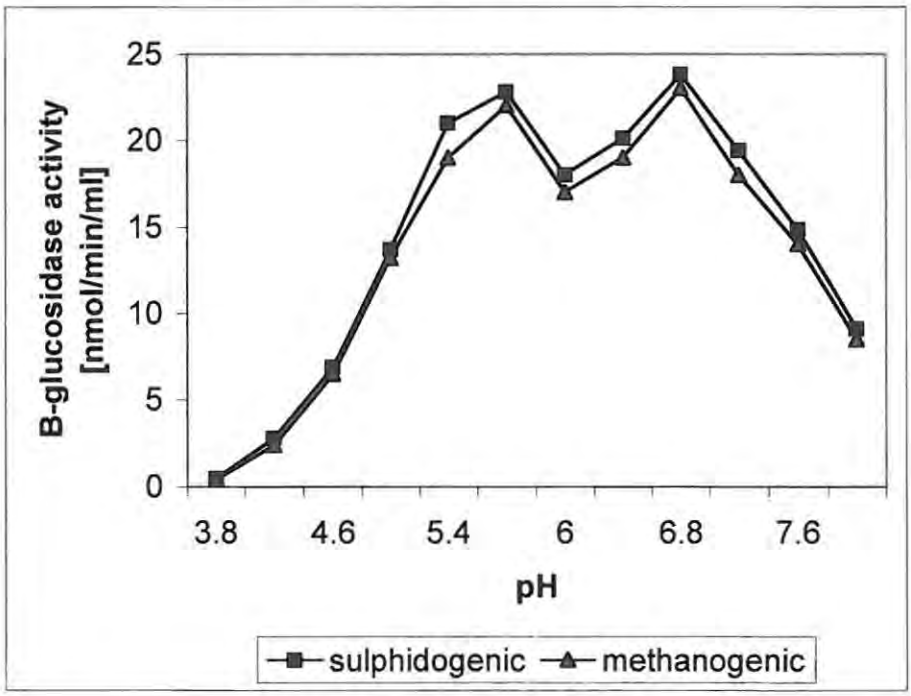

Figure 5.9: pH optimum studies on β-glucosidases.

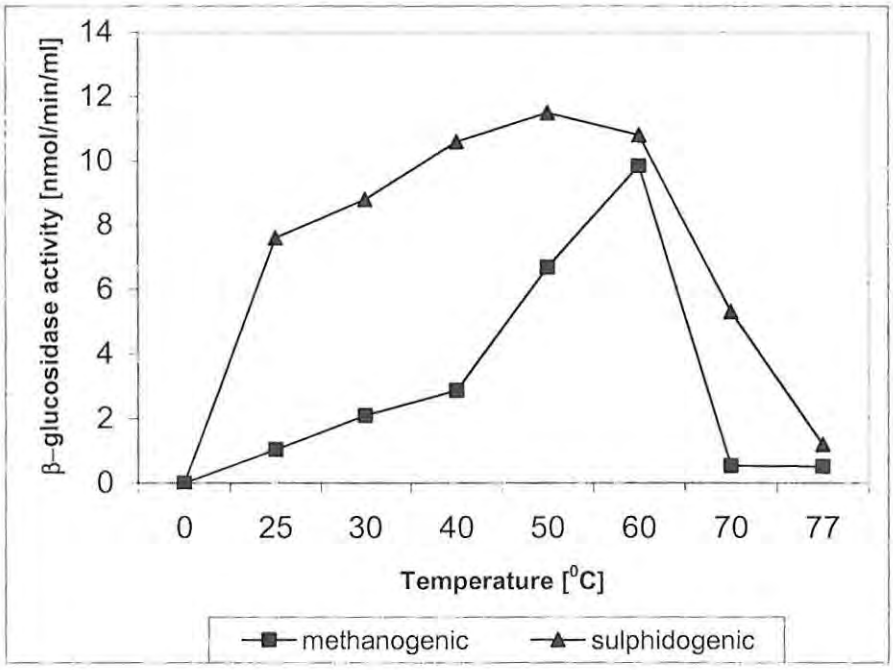

Figure 5.10: Optimum temperature for β-glucosidases in the methanogenic and the sulphidogenic systems.

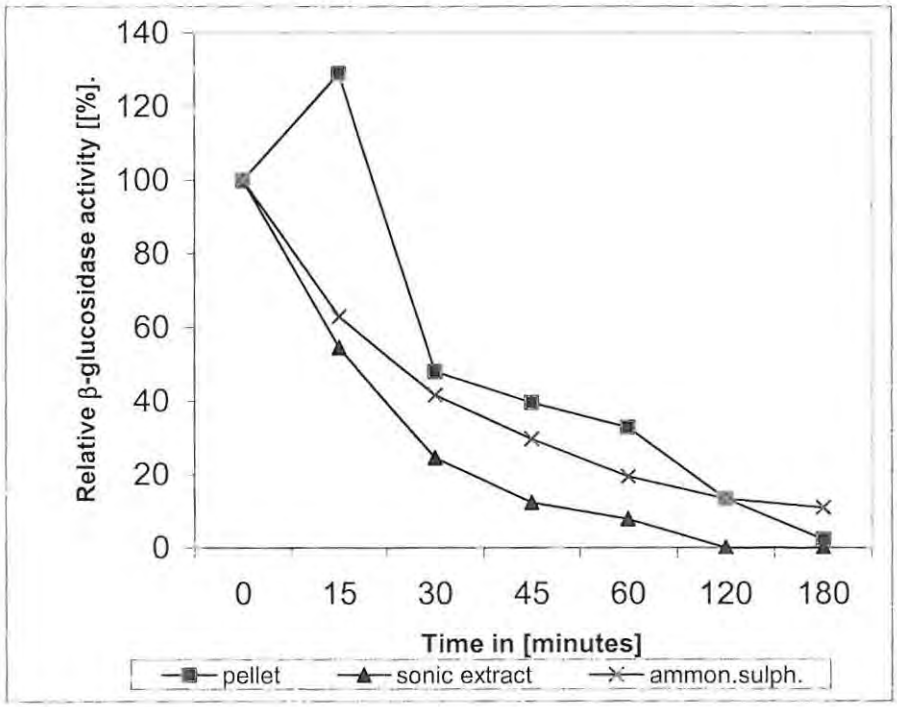

Figure 5.11: Thermal stability studies for β-glucosidase.

5.4 DISCUSSION.

5.4.1 Effects of sulphate, sulphite and sulphide on β-Glucosidase activity.

The results indicated that the presence of sulphur-containing compounds at concentrations below 200 mg/L in both the methanogenic and the sulphidogenic systems is essential for cellulose degradation by cellulolytic enzymes in the sewage sludge. With glucosidase activity, both sulphate and sulphide at low concentrations had an inhibitory effect while sulphite was stimulatory. This could be explained by the fact that sulphite is rendered a better electron acceptor compared to sulphate. In addition, stimulation of enzymatic activity could also be linked to the involvement of inorganic sulphur in the synthesis of sulphur containing amino acids, which are incorporated into microbial enzymes [Khan and Trottier, 1978].

Sulphur containing compounds have been reported to form an integral part in ferrodoxin and other compounds involved in the electron transport system as well as coenzyme M, which is involved in the methyl group transfer reactions of anaerobic degradation. Furthermore, for the cultivation of anaerobes, sulphur-containing compounds have been employed to maintain a reducing environment essential for the growth of bacteria that produce hydrolytic enzymes [Khan and Trottier, 1978]. Therefore, inorganic sulphur contained in sulphate, sulphite and sulphide appears to be an essential nutrient for the hydrolytic mixed culture present in sewage sludge. Thiols have also been reported to stimulate enzyme activity of *B. cellulosolvens* cellulolytic system as well as the activity of exo-acting enzymes of the cellulolytic complex [Coughlan and Ljungdahl, 1988].

Khan and Trottier (1978) observed that the presence of sulphate up to a concentration of 81 mg/L seemed to be necessary for anaerobic degradation and that concentrations up to 168 mg/L had little or no inhibition on cellulose degradation. They also discovered that the microbial ecosystem tolerated sulphate concentration up to 1152 mg/L without more than a 50% loss in efficiency of cellulose conversion to methane. Their findings agree with the results of this study as sulphate concentration at 1000 mg/L decreased the activity by only 20%. The sulphate tolerance by the bacterial mixed culture explains the findings that enzymatic activity, even at high

concentrations, i.e. 1000 mg/L, was only decreased by 20%. According to time trial results, sulphate concentrations from days 1-5 were between 2184 and 1112 mg/L. At such concentrations inhibition of degradation would be expected. However, the high tolerance of digester bacteria for sulphate could partly explain the observed activity during that period.

Inhibition of anaerobes by sulphides at concentrations as low as 50 mg/L (by about 50%) has been reported and complete inhibition of acetoclastic methane producing bacteria by free H_2S has been reported to occur at 200 mg/L [Isa *et. al.*, 1986]. This inhibition is suspected to be the result of intrinsic toxicity of H_2S gas to SRB and hydrolytic bacteria [Reis *ct. al.* 1992]. Isa *et al*, (1986) observed that at pH 7.5, 20% of the total sulphide present in solution existed as free sulphide. The pH of the sulphidogenic sludge fluctuated between 7 and 8 (Figure 2.14) suggesting an environment that encouraged formation of free sulphide. This could explain inhibition of enzymes at 200 mg/L (Figure 5.3) and supports the idea that the decrease in activity after day 13 (when most of the sulphide in solution was possibly converted to the non-ionized form as free H_2S) was a result of sulphide production.

5.4.2 Effects of heavy metals on β-Glucosidase activity.

Results have shown that Zn^{++}, Ni^{++}, and Cu^{++} are inhibitory to glucosidase activity. Inhibition of enzymatic activity could be explained by the fact that concentrations as high as 200 mg/L are toxic to anaerobic hydrolytic bacteria. Since these bacteria produce these enzymes, any factor affecting their viability, will have an effect on enzymes produced. Stimulation of glucosidase activity by iron in both reactors can be attributed to the fact that iron is an essential co-factor in the *C. thermocellum* complex [Coughlan and Ljungdahl, 1988]. $FeSO_4$ at $\leq$ 500 mg/L, $ZnSO_4$ at 100 mg/L, Cu^{++} ions at $\leq$ 2 mg/L and Ni^{++} ions at $\leq$ 24 mg/L have been used in the cultivation media for SRB suggesting that at low concentrations these metals are essential for growth of SRB [Hurst *et. al.*, 1997]. If SRB growth is encouraged, sulphate reduction will be accelerated, thus encouraging utilization of anaerobic degradation products such as VFA's. When accumulation of VFA's is prevented the hydrolytic enzymes of the hydrolytic stage are stimulated, accounting for the enhanced enzymatic activity.

The presence of sulphate, sulphide and carbonates has been reported to induce precipitation of metals in the digester and thus reduce their availability to the microorganisms [Isa *et al.* 1986]. If this were the case, then inhibition in the sulphidogenic system would not be expected. However, the results have shown three of the tested metals to be inhibitory. It has been reported that different valuable metals precipitate at different pH values as metal sulphides. Amongst the metals investigated, copper was discovered to precipitate at pH 1.5, zinc at pH 3-4 and nickel at pH 5-6 [Buisman, 2000].

The pH values in the methanogenic and the sulphidogenic reactors were reported to operate between 5-6 and 7-8 respectively. In addition, the bench scale assays for β-glucosidase were conducted at pH 7.0. At this pH a large fraction of the metals could be free in solution and would not have formed metal sulphides, and thus could inhibit the enzyme. The reduced inhibition by copper and iron in the sulphidogenic system, however, can be accounted for by the possible reaction of a smaller fraction of these metal ions with the sulphate and bicarbonate ions rendering the metals less toxic.

5.4.3 Effects of volatile fatty acids on β–Glucosidase activity.

The neutralising effect of VFAs on enzyme activity can be explained by the presence of bicarbonate ions, which react with the hydrogen ions that are released into solution by the acids. The reaction below illustrates the buffering activity of bicarbonate ions produced by sulphate reduction.

$$HCO_3^- + H^+ \rightleftharpoons H_2CO_3 \rightleftharpoons H_2O + CO_2$$

In addition, degradation of simple sugars occurs in acidic conditions i.e. between pH 5 and 6 [Hobson and Wheatley, 1993]. SRB and anaerobic hydrolytic bacteria are inhibited at pH values lower than 5.0 and higher than 9.0. This could account for the slight enzyme inhibition at higher concentrations of VFA's. The enhancement of glucosidase activity by acetic acid can be attributed to the fact that the acetic acid is the most favored substrate for the SRB during sulphate reduction.

5.4.4 Optimum pH, optimum temperature and thermal stability.

pH optima between 5.4 and 7.2 were expected, as carbohydrate degradation occurs between 5 and 7. The multiple peaks showing a range of pH optima can be explained by the fact the enzymes assayed are a group of glucosidases produced by different cellulolytic bacteria. The optimum temperatures between 50^0C and 60^0C suggested that glucosidases operate optimally in the thermophilic range. However, thermal stability studies have shown glucosidases not to be stable at their optimum temperature indicating that for the speeding up of hydrolysis, the reactors can't be operated at such temperatures. Thermal stability of the glucosidase fractions increased in the following order: sonic extract < ammonium sulphate precipitate < crude pellet.

The crude fraction showed a marked resistance to thermal inactivation. Approximately 34% of its initial activity remained after 1 hour (at 50^0C incubation). The residual activities for sonic extract and the ammonium sulphate precipitate were 7.8% and 19.4% respectively. The increased stability of the crude extract glucosidases is due to their immobilization to the bacterial membranes and the substrate. Free enzymes are more susceptible to denaturation by extreme heat and pH conditions [Busto *et. al.*, 1997]. This may explain the instability of the sonic extract glucosidase/(s), as sonication releases the enzymes from the substrate / organic particulate matter. In the presence of ammonium sulphate, glucosidase forms protein aggregates that lead to precipitation. It is these aggregates that enhanced enzyme stability. From the results, it can be concluded that glucosidases operate optimally in their unaltered natural state. Due to thermal instability of the sonic extract and ammonium sulphate precipitate, heat denaturation could not be included in the purification procedure for β-glucosidase.

CHAPTER SIX

CONCLUSIONS: THE ROLE OF CELLULASES AND GLUCOHYDROLASES IN THE SOLUBILIZATION OF PRIMARY SEWAGE SLUDGE.

6.1. INTRODUCTION.

Sulphate reduction, a process used for bioremediation of AMD requires electron donors usually from organic substrates of low molecular weight. Primary sewage has been identified as a rich source of these organic substrates, which are however, not readily available for utilization by SRB as they are present as polymers. The hydrolysis of complex substrates involves enzymatic degradation. Data obtained from this study was used in to explain the role of cellulases and glucohydrolases in the degradation of sewage sludge complex carbohydrates under methanogenic (control) and sulphidogenic (experimental) conditions.

6.2 The effects of sulphur containing compounds on cellulolytic enzymes during anaerobic degradation.

The time for complete methanogenic anaerobic degradation of polymers in anaerobic digesters is approximately 31 days. Preliminary studies have shown that this degradation, under sulphidogenic conditions, required a shorter period i.e. approximately 15-17 days. Therefore, production of enzymes during anaerobic degradation under methanogenic (normal) and sulphidogenic conditions over a period of 31 days was studied. Enzyme activity was generally low in the methanogenic system and high in the sulphidogenic system suggesting an enhanced hydrolysis in the sulphidogenic system.

The peak for enzyme production under methanogenic conditions was reached at day 5 for cellulases and at day 7 for glucosidases. Under sulphidogenic conditions, the highest production for cellulases and glucosidases respectively, was observed at days 13 and 17. The emerging of β-glucosidase peaks a few days later than the cellulase peaks, in both systems, supported the hypothesis that components of the cellulolytic complex operate in a synergistic fashion.

In both systems, β-glucosidase activity was higher than cellulase activity. This can be ascribed to its role in the whole process of cellulose breakdown i.e. to convert cellobiose

(a major product of cellulose breakdown and a potent inhibitor of cellulases) into glucose. If cellobiose is allowed to accumulate the rate of cellulose degradation declines significantly [Busto *et al.* 1997], therefore, abundant production of β-glucosidase enzymes is absolutely necessary. In the sulphidogenic system, enzyme production decreased drastically after day 21 while activity in the methanogenic system remained fairly high even at day 31 implying an accelerated degradation process in the sulphidogenic system.

During anaerobic degradation, enzyme activity, sulphate and COD removal, sulphide production, and pH fluctuations were monitored in both systems. Maximum sulphate removal was observed by day 9 while maximum sulphide production was at day 13 in the sulphidogenic system. Sulphite, a better electron acceptor and an intermediate for sulphate reduction was assumed to be produced between days 9 and 13. The increase in enzyme production at day 9 coincided with low sulphate concentrations (± 400 mg/L). Sustained high enzyme production was confluent with the assumed period of high sulphite production.

In the sulphidogenic system, on day 13, the peak of high sulphide production, enzyme activity was also still high, suggesting that sulphide in its ionised/soluble form (H_2S, HS^- and $S^=$) has an enhancing effect on enzyme activity. Whittington-Jones *et. al,* (2001) provided a descriptive model of this enhanced solubilization. They established that HCO_3^-, OH^- and SH^- ions produced during sulphate reduction, neutralise the acidic surface of the sludge organic particulate matter (OPM), and thereby disrupting the charges that maintain the integrity of the OPM. Whiteley *et. al.,* (2001) further explained that "the architecture of the OPM is dependent on the interaction between microbially produced polymers and the various cations present in the sludge and that slight changes in ionic composition due to removal of the sludge cations alters the conformation of the OPM".

Based on the above findings, enhanced enzyme activity can, therefore, be explained in terms of the increased rate of lignin solubilization in the presence of soluble sulphide,

which in turn, exposes cellulose to cellulase attack. The decline in enzyme activity observed after day 13 accompanied the increase of the decreasing soluble sulphide. The decrease in soluble sulphide concentration was indicative of the formation of uni-onised sulphide, which escaped from the solution as hydrogen sulphide gas. These findings supported the idea that the toxic form of sulphide is the hydrogen sulphide gas. Whittington-Jones, (1999) had observed similar results.

Since anaerobic degradation is dependent on enzymes, and since sulphate, sulphite and sulphide appear to have a multiple role on degradation, the direct effect of these sulphur-containing compounds at concentrations between 200-1000 mg/L on enzyme activity was examined. According to the findings of this study sulphate is only required at concentrations $\leq$ 200mg/L for enhanced carbohydrate degradation. Above this concentration the cellulolytic enzymes are inhibited. Sulphite was observed to have a stimulatory effect even at concentrations as high as 1000 mg/L supporting the idea that high activity observed between days 9 and 13 could have been the result of high sulphite production. These findings agree with the fact that the activity of cellulolytic complexes against crystalline cellulose is dependent on thiols, [Coughlan and Ljungdahl, 1988]. However, these thiols are required only at limiting concentrations (Chapter 5, Figures 5.1 – 5.3).

At 200 mg/L of sulphide, enzyme activity was inhibited by $\pm$ 43% in both systems. Whittington-Jones, (1999) found that enzyme activity was enhanced by sulphide at concentrations $\leq$ 100 mg/L, suggesting that 200 mg/L of sulphide was too high a concentration. A short-coming of this present study was that concentrations between 0-199 mg/L were not explored. Therefore, information on the effects of these thiols on enzyme activity, at such concentrations is not available.

6.3. The effects of heavy metals on enzyme activity.

Since sulphate reduction is employed for bioremediation of AMD (a mine effluent characterised by high heavy metal concentrations), the effects of heavy metals on enzyme

activity was also studied. The metal concentrations examined ranged between 200-1000 mg/L. In both systems copper and nickel were inhibitory, while zinc was tolerated up to 600 mg/L only under methanogenic conditions. At 200 mg/L of iron, enzyme activity was enhanced by 192% and 55% in the methanogenic and the sulphidogenic systems respectively. At 400 mg/L enzyme activity was inhibited by 96% and 66% Table 5.2 – p. 115) in the methanogenic and sulphidogenic systems respectively, suggesting that for cellulose degradation, iron present in AMD is required only at concentrations lower than 200 mg/L under sulphidogenic conditions. In addition, for the process of sulphate reduction to work, metal concentration in AMD has to be below 200 mg/L due to the fact that enzyme activity is inhibited at higher concentrations.

The presence of sulphide and carbonates in the sulphidogenic system resulted in the precipitation of heavy metals thus reducing enzyme inhibition. These findings implied the necessity for the presence of sulphides, but at controlled concentrations. Volatile fatty acids showed no significant effects on enzyme activity except for acetate that had a stimulatory effect on enzyme activity. This stimulation attributed to the fact that acetate is the most readily useable substrate (as an electron donor) for sulphate reduction. The neutralised effect of volatile fatty acids was explained in terms of the existing buffering systems of the digesters.

6.4 Enzyme distribution, optimum temperature, optimum pH and thermal stability.

Highest enzyme activity was associated with the organic particulate matter. As cellulases have to be strongly attached to cellulose for the degradation process, these findings were expected. Purification and thermal stability studies proved that these enzymes are best left immobilised to the organic particulate matter, as they are the most stable under these conditions. The temperature for their optimal hydrolytic activity was observed to be in the thermophilic range i.e. between 50^0C and 60^0C. However, to speed up cellulose degradation, operating the digesters at such temperatures is not an option since the enzymes in their crude form were stable for only 15 minutes.

The pH optima ranged from 5.4 to 7.2 suggesting that these enzymes operate in the acidic range. However, pH of acid mine drainage, which is as low as 2.0 may denature the enzymes unless, addition of the sewage sludge containing these enzymes increases the pH up to 5. The pH of primary sewage sludge is usually controlled by addition of $CaCO_3$. Therefore, neutralisation of AMD by the addition of sewage sludge could be possible.

6.5 General conclusion.

In summary, sewage sludge cellulases and glucohydrolases are produced by a group of cellulolytic bacteria existing in a consortium with other hydrolytic bacteria. Components of cellulolytic complexes depend on each other for completion of the whole process of cellulose degradation such that they operate in a synergistic and sequential fashion. At low concentrations, sulphate, sulphite and sulphide have an enhancing effect on enzymatic activity. At concentrations above 200 mg/L for sulphide and sulphate, and 600 mg/L for sulphite, these sulphur-containing compounds inhibit the enzymes. Sulphide also plays a role in precipitation of heavy metals thus reducing their availability as free ions rendering them less toxic to enzymes. Therefore, the presence of soluble sulphide at low concentrations is necessary. Trace amounts of metals are also necessary for growth of SRB and hydrolytic bacteria. However, concentrations higher than 200mg/L for zinc, nickel and copper and 400 mg/l for iron inhibit enzyme activity.

Under sulphidogenic conditions, volatile fatty acids only have a positive contribution as they provide electrons for sulphate reduction. Acidity introduced by VFA's is neutralised by HCO_3^- ions present in the sulphidogenic digester. AMD pH $\pm$ 2.0 is detrimental to the enzymes.

Future work will necessitate the setting up of a sulphidogenic reactor that includes AMD SRB, hydrolytic bacteria, sulphate and primary sewage sludge to investigate the performance of cellulases and glucohydrolases in the presence of AMD. The control reactor would be the sulphidogenic system described in Figure 3.1 (p. 63). In addition,

concentrations between 0-199 mg/L for the effects of sulphur- containing compounds, metals and volatile fatty acids should be explored.

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