

THE BIO-DISPOSAL OF LIGNOCELLULOSE SUBSTANCES WITH ACTIVATED SLUDGE

BY

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Declaration

I hereby certify that this dissertation is my own original work, except where specifically acknowledged in the text. Neither the present dissertation nor any part thereof, has previously been submitted at any other university.

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Abstract

Lignocellulose is the principal form of biomass in the biosphere and therefore the predominant renewable source in the environment. However, owing to the chemical and structural complexity of lignocellulose substrates, the effective and sustainable utilization of lignocellulose wastes is limited.

Many environments where lignocellulose residues are ordinarily stored can be highly acidic (e.g. landfills), and under these circumstances biodegradation of the lignocellulose is slow and unhygienic. Owing to the metabolic activities of the micro-organisms, the initially acidified habitats rapidly undergoes self-neutralization. A number of pathogenic bacteria (coliforms and *Salmonella sp.*) are present during this slow degradation process and it is therefore imperative to improve the efficiency and hygienic effects of the biodegradation of the lignocellulose.

Although the fundamentals of biodegradation of lignocellulose have been widely investigated, many issues still need to be resolved in order to develop commercially viable technology for the exploitation of these waste products. For example, owing to the complex, heterogeneous structure of lignocellulose, the degree of solubilization, modification and conversion of the different components are not clear. Likewise, the overall anaerobic degradation of lignocellulose is not understood well as yet.

In this study, the emphasis was on the promotion of solid anaerobic digestion of lignocellulose wastes for environmental beneficiation and waste reutilization. The degradation of lignocellulose in landfill environments was first simulated experimentally. Once the microbial populations and the degradation products of the system were characterized, the promotion of anaerobic digestion by use of activated sludge was studied. This included acidogenic fermentation, as well as recovery of the methanogenic phase. Moreover, special attention was given to the further disposal of humic acids or humic acid bearing leachates formed in the digestive system, since these acids pose a major problem in the digestion of the lignocellulose.

With ultrasonication, approximately 50% of the lower molecular weight fraction of humic acids could be decomposed into volatile forms, but the higher molecular weight fraction tended to aggregate into a colloidal form, which could only be removed from the system by making use of ultrasonically assisted adsorption on preformed aluminium hydroxide flocs.

This was followed by an investigation of the microbial degradation of humic acids and the toxicity of these acids to anaerobic consortia. Further experimental work was conducted to optimize the biological and abiological treatment of lignocellulose in an upflow anaerobic sludge blanket (UASB) reactor fed with glucose substrate. The humic acids could be partially hydrolysed and decomposed by the acid fermentative consortia of the granules in the UASB reactor.

Finally, solid mesothermophilic lignocellulose anaerobic digestive sludge can be viewed as a humus-rich hygienic product that can improve the fertility and water-holding capacity of agricultural soil, nourish plants and immobilize heavy metals in the environment as a bio-absorbent.

Abstrak

Lignosellulose is die hoofbron van biomassa in die biosfeer en is daarom ook die belangrikste hernubare bron in die omgewing. As gevolg van die chemiese en strukturele kompleksiteit van lignosellulose substrate, is die doeltreffende en volhoubare benutting van lignosellulose afval egter beperk. Die suurgehalte van die omgewings waar lignosellulose reste gewoonlik gestoor word, soos opvullingsterreine, kan hoog wees en onder hierdie omstandighede is die biodegradasie van die lignosellulose stadig en onhigiënies. As gevolg van die metaboliese aktiwiteite van die mikro-organismes ondergaan die aanvanklik aangesuurde habitate vinnig self-neutralisasie. 'n Aantal patogeniese bakterieë (koliforme en *Salmonella sp.*) is deurgaans gedurende dié stadige natuurlike proses teenwoordig en dit is dus van die grootste belang om die effektiwiteit en die higiëne van die bioafbakening van die lignosellulose-substraat te verhoog.

Alhoewel die grondbeginsels van die bioafbakening van lignosellulose reeds wyd ondersoek is, moet verskeie probleme nog opgelos word ten einde kommersieel haalbare tegnologie te ontwikkel vir die ontginning van afvalprodukte. Byvoorbeeld, as gevolg van die komplekse, heterogene struktuur van lignosellulose, is die graad van solubilisering en die modifikasie en omskakeling van verskillende komponente nog onduidelik. Net so word die algehele anaerobiese afbakening van lignosellulose ook nog nie ten volle verstaan nie.

In hierdie ondersoek het die klem geval op die bevordering van soliede anaerobiese digestie van lignosellulose afval vir omgewingsverbetering en die benutting van die afval. Die afbakening van lignosellulose in opvullingsterreine is eers eksperimenteel gesimuleer. Nadat die mikrobiële populasies en die afbakeingsprodukte gekarakteriseer is, is die bevordering van anaerobiese digestie deur die gebruik van geaktiveerde slyk bestudeer. Dit het asidogeniese fermentasie ingesluit, sowel as herwinning van die metanogeniese fase. Spesiale aandag is gegee aan die verdere verwerking van humussure en humussuurbevattende legates wat in die digestiewe stelsel gegenereer is, aangesien die sure probleme veroorsaak het met die vertering van die lignosellulose.

Met ultrasoniese straling is nagenoeg 50% van die lae-molekulêre massafraksie van die humussure ontbind in vlugtige vorm, maar die hoë-molekulêre massafraksie het geneig om in

‘n kolloïdale vorm te aggregeer, wat slegs uit die stelsel verwyder kon word deur middel van ultrasonies ondersteunde adsorpsie op voorafgevormde aluminiumhidroksiedvlokkies.

Dit is gevolg deur ‘n ondersoek na die mikrobiële afbreking van humussure en die toksisiteit van die sure ten opsigte van anaerobiese konsortia. Verdere eksperimentele werk is gedoen ten opsigte van die biologiese en abiologiese behandeling van lignosellulose in ‘n opwaartsvloeiende anaerobiese slikkombersreaktor (OASK) gevoer met glukosesubstrate. Die humussure kon gedeeltelik gehidroliseer en ontbind word deur die suurgistende konsortia van die granules in die OASK reaktor.

Ten slotte kan die vaste termofiliese-mesofiliese anaerobiese lignosellulose verteringslik ook gesien word as ‘n humusryke higiëniese produk wat die vrugbaarheid en die waterhoudende vermoë van landbougrond kan verhoog, plante kan voed en kan funksioneer as bio-absorbeerder van swaarmetale in die omgewing.

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List of Abbreviations

Acid Precipitable Polymeric Lignin— APPL
 Cation Exchange Capacity — CEC
 Chemical Oxygen Demand — COD
 Cezapek-Dox medium plus Humic Acid — Cezapek-Dox-HA
 Dimethylterephthalate — DMT
 Extracellular Polymers — ECP
 Fast Blue B Salt — FBB
 Fulvic Acid — FA
 Gas Chromatography — GC
 Gel Permeate Chromatography — GPC
¹H-Nuclear Magnetic Resonance Spectra — ¹HNMR
 Retention Time — RT
 Hydraulic Retention Time — HRT
 Humic acid — HA
 Humic acids — HAs
 Humic Substances — HS
 Inorganic Carbon — IC
 Liquid to Solid ratio — L/S
 Methane-Producing Bacteria — MPB
 Weight-Average Molecular Weight — Mw
 Number-Average Molecular Weight — Mn
 Z-Average Molecular Weight — Mz
 Molecular Weight — MW
 Most Probable Number— MPN
 Malt Extract Agar — MEA
 Municipal Solid Waste — MSW
 Nuclear Magnetic Resonance — NMR
 Natural Organic Matter — NOM
 Oxidation-Reduction Potential — ORP
 Organic Fraction of Municipal Solid Waste — OFMSW
 Purified Terephthalic Acid— PTA
 Raw Primary Sludge/Thickened Sludge Mixture — RAW/TWAS
 Sulphate Reduction Bacteria — SRB

Scanning Electron Microscope — SEM
Special Methanogens Activity — SMA
Total Solids — TS
Typtose Soy Agar — TSA
Typtose Soy Plus Humic Acid —TS-HA
Total Volatile Solids — TVS
Total Volatile Fatty Acid — TVFA
Tetrahydrofuran — THF
THF soluble HA— THFS-HA
Upflow Anaerobic Sludge Blanket — UASB
Unionized Volatile Acids — UVA
Volatile Acids — VA
Volatile Fatty Acid — VFA
Volatile Fatty Acids — VFAs
Volatile Solid Destruction — VSD
Volatile Suspension Solid — VSS
Yeast Nitrogen Base — YNB
Yeast Nitrogen Base plus Humic Acid — YNB-HA

Chapter 1 Introduction

Newly heightened concerns about or awareness of climatic changes, the depletion of stratospheric ozone, environmental pollution, wildlife, shrinking biodiversity, health, food safety, land degradation and pressures on non-renewable resources have been called upon the world to embrace the sustainable development ethos ⁽¹⁶³⁾. In nature, agriculture is sustainable only where the organic wastes are recycled to maintain soil humus and fertility. In practice, on the one hand, high human activity promotes intense development of soil cultivation, but the extensive extracting of the existent nutrients of the soil unavoidably brings the soil into a seriously nutrient-budged status. On the other hand, vast quantities of agricultural and agro-industrial residues generated as a result of diverse agriculture and industrial practices and municipal solid wastes/sewage sludge generated in our daily life circle have been seen to accumulate considerably. Some organic wastes are incinerated, but this practice has been criticized because of the resulting air pollution and the danger of soil erosion. Another approach has been the use of chemicals to enhance the digestibility of organic wastes, but their use could be tedious and costly, and could require further treatment to eliminate side effects. Alternatively, microbiological treatments were found to be an economical and environmentally-attractive option for the disposal of organic wastes.

Generally, most agricultural residues and organic fractions of the municipal solids and sewage sludge can be returned to the soil as nutrients through micro-organism transformation/decomposition-digestion (including hydrolysis, aerobic, anoxic and anaerobic) processes. However, in nature, these microbial processes are very slow and, ideally, the organic wastes must not be returned to the cultivated land in the raw state because that would be unhygienic for both plants and man. It would also overload the system's capacity to recycle the carbon in the wastes in the near-absence of on-site herbivores present in a balanced ecosystem. Every year, the accumulation of the biomass and organic wastes in large quantities not only results in the deterioration of the environment, but also in the loss of potentially valuable material which could be processed to yield a number of value-added products. A sustainable development ethos can be explored by developing a "Four-R strategy": reduce, re-use, recover, and recycle wastes to conserve our resources and reduce pollution by emulating, adopting and maximizing the beneficial processes of an ecosystem. Furthermore, as a countermeasure against the potential post-contamination of "artificial

manure” or chemical inorganic fertilizers in the environment, the promotion of the hygienic and value-adding disposal of organic wastes has aroused intensive interest and has been widely investigated in recent years.

The use of human wastes as a resource in agriculture has a long history. The development of water-born sewage systems in the beginning of the nineteenth century established crop irrigation as a preferred means of disposal. Sewage sludge has been, for many years, a valuable resource material when it is used as a fertilizer and a soil conditioner after composting. Processing of the raw wastes in composite mixtures in some ancient cultures reduced bulk, dissipated part of the carbon, sanitized the wastes and conserved nutrients⁽¹²¹⁾. The main benefits of sludge application in agriculture in the form of compost are: provision of major plant nutrients (in particular P and N), increased supply of some of the essential micronutrients (in particular Zn, Cu Mn and Mo), improvement in the soil structure, and increased soil water-holding capacity. Sludge can generally be considered as a slow-release fertilizer material, which can be used as a maintenance fertilizer on perennial crops, such as sugar-cane, fruit trees and grasses. It can be of particular benefit in the cases where the availability of nutrients from commercial inorganic fertilizers is low owing to factors like high leaching losses (in the case of NO_3^-) or high soil-P fixing capacity⁽¹³⁹⁾.

Activated sludge from the biological municipal waste water treatment plant which is free from heavy-metal and other toxic chemicals could be the best candidate for organic fertilizer. However, strictly speaking, it is still an intermediate product, which is rich in organics-digestion micro-organisms and should be stabilized and composted to remove the pathogen bacteria before application on the land.

On the other hand, because of the abundance of plant lignocellulose in the biosphere and the importance of different components of plant lignocellulose in industrial processes and products, it is important to be knowledgeable about its metabolism in various biological environments. In addition, to make use of the plant lignocellulose wastes before mixing them with other municipal solid wastes before dumping, can be a commendable waste management.

Since Omelianske described the degradation of cellulose with methane formation as a specific

bacterial action in 1906 ⁽¹⁸⁵⁾, the anaerobic digestion has historically been used for the stabilization of raw domestic sludge typically removed by primary sedimentation. More recently, anaerobic digestion has also been applied to chemically-treated primary sludge which contains the chemicals added for phosphorus removal and to biological sludge mixtures containing significant industrial waste contributions and exhibiting significantly different chemical and physical characteristics. Many efforts to understand the anaerobic digestion (including the microbiology, the population dynamics, the effects of environmental and nutritional conditions, feeding characteristics, operational parameters etc.) have been reviewed by Pavlostathis et al. ⁽¹⁹⁶⁾. The anaerobic fermentation of high-solid organic wastes (such as municipal solid waste and de-watered sewage sludge) has been attempted as an attractive approach for developing a recycle and or energy producing type of solid waste management system. The raw primary sludge/thickened sludge mixture, (RAW/TWAS), fed to the anaerobic digesters at the waste water treatment plant in combination with the organic fraction of municipal solid waste (OFMSW) could be a richer source of organic materials and hence lead to more effective anaerobic digestion. Anaerobic co-digestion of a RAW/TWAS sewage sludge and OFMSW mixture would take advantage of the inherent microbial population in sewage sludge to digest the OFMSW and the sludge itself. Ultimately, this would allow for resource recovery through the production of a nutrient-rich compost and energy recovery through methane bio-gas production. However, complete knowledge and efficient technology regarding the anaerobic digestion of plant lignocellulose solid wastes with activated sludge are still scarce.

Therefore, taking the abundance and the accumulation of plant lignocellulose wastes into account, the accumulation of activated sludge and its potential nutrient capacity for fertilizing land, and the advantages of anaerobic digestion, makes an effective biological utilization of plant lignocellulose waste using an anaerobic bio-degradation process a desirable option for the future. A better understanding of this complex process is of compelling interest due to the complex and refractory nature of the plant lignocellulose substrate ⁽⁷⁴⁾. Consequently, the objectives of this study were as follows:

- ◆ To simulate the microbiological decomposition of plant lignocellulose waste in landfill situations and to investigate the plant lignocellulose decomposition in sulphate acidified (landfill) conditions comparable with the controlled anaerobic digestion

process.

- ◆ To promote the anaerobic degradation of plant lignocellulose wastes with activated sludge using abiological-pretreatment and an integrated two-phase high-solid anaerobic digestion system.
- ◆ To investigate the microbial community involved in the digestion process.
- ◆ To investigate the disposal of some complex intermediate products, especially the humic acids, because of their refractivity to further bio-degradation and their potential to cause environmental problems.
- ◆ To evaluate the hygienic effects of the anaerobic digestion process and the fertility of the digested solid products as value-added soil conditioner (fertilizer).
- ◆ To establish an effective technology to utilize plant lignocellulose waste for environmental and economical benefits.

Chapter 2

Background and Theoretical Considerations

In section 1 below, the macro- and micro-structure of lignocellulose is described with respect to the relationship between the substrate structure and its decomposition process. In section 2, the fundamentals of anaerobic digestion is extensively discussed; the whole biological decomposition and anaerobic conversion process of lignocellulose is presented and the special considerations regarding anaerobic digestion of lignocellulose are discussed. In section 3, the decomposition of lignocellulose in landfill is described and the investigation's emphasis is towards the decomposition of lignocellulose in sulphate acidified landfill conditions. In section 4, the following subjects are addressed: the formation of humic acids (HAs) in the biological decomposition of lignocellulose; the characteristics and the inhibitory effects of HAs on the anaerobic digestion of lignocellulose; the biological decomposition and the non-biological alternative treatment of HAs to reduce its negative influence on the digestion process and the aqueous environment.

2.1 Lignocellulose structure

The biodegradability of the substances is dependent on its composition and chemical structure features. Knowledge of the lignocellulose structure is basic to design of the degradation process.

Lignocellulose is the term used to describe the composite of the predominant polymers of vascular biomass. It is the principal source of biomass and therefore a renewable resource. A large proportion of agroindustrial plant residues (e.g. tobacco dust) and urban waste (e.g. yard waste) is present in the form of lignocellulose. It is a complex of three classes of polymer: polysaccharides cellulose, hemicellulose and the phenolic polymer of lignin.

Cellulose is a linear polymer of D-glucose residues (building blocks) held together by β 1-4-glucosidic linkages; it makes up 50% of the mass of vascular plants and is mainly used in chemical pulping processes. The β 1-4 linkage causes the polyglucose molecules to adopt a fully-extended linear configuration, which is attained by flipping each glucose unit 180° relative to the preceding one. The glucose linkage acts as a functional group and this, with the

hydroxyl groups, mainly determines the chemical properties of cellulose. The intermolecular hydrogen bonds also stabilize each cellulose chain. The linear cellulose molecules pack together to form rod-like structures known as elementary fibrils, protofibrils or micelles, which are stabilized by intermolecular hydrogen bonds between adjacent cellulose chains. The micelles, the smallest structural units, are packed into microfibrils, which are each about 10 nm in diameter and up to several micrometres in length.

Hemicellulose is a matrix polysaccharide of the plant cell wall and is made up of xylose, galactose, mannose, arabinose, glucose and their uronic acids ^(56, 249). It includes all of the plant cell polysaccharides excluding pectin and cellulose ⁽²⁵⁸⁾. Hemicellulose is frequently branched, and sometimes hydroxyl groups of the main chain are acetylated. These two polymers are physically encrusted with lignin, to which they are covalently linked through lignin-saccharide bonds that provide mechanical strength and rigidity to plant tissues.

In contrast, lignin is an amorphous three-dimensional aromatic polymer composed of three phenylpropanoid units: coniferyl alcohol, sinapyl alcohol and p-coumaryl alcohol ⁽⁵²⁾. The lignin presents in the cell walls and middle lamellae of vascular plants and surrounding cellulose microfibrils, gives rigidity to cell walls and makes plants resistant to pathogen attack and mechanical stresses. It makes up 15%-30% of the mass of vascular plants. Lignin is synthesized in plants from coniferyl and coumaryl alcohol through radical coupling mediated by plant peroxidases. This random chemical coupling results in a complex three-dimensional phenolic polymer with a variety of bonding arrangements giving aryl glycerol- β -aryl ether, biphenyl, diphenylether, phenylcoumaran, 1,2-diaryl-propane and other such structures ⁽⁵²⁾.

Hemicellulose appears to be associated with both cellulose and lignin ⁽⁸³⁾. While cellulose and hemicellulose fractions are degradable, some of the lignin components are known to be resistant to degradation under anaerobic conditions and may hinder degradation of other components ⁽²⁴⁴⁾.

2.2 Anaerobic digestion of lignocellulose

Traditionally, anaerobic digestion technology was traditionally employed in waste water treatment to reduce the volume and weight of sludge and to produce corresponding amounts

of bio-gas. It is gaining environmental and economical importance in organic solid waste digestion due to the benefits of resource recovery and environmental impacts from the alternative treatments (e.g. incineration and landfill).

2.2.1 Anaerobic digestion process

2.2.1.1 Microbiology and biochemistry

Anaerobic (methanogenic) fermentation of organic matter is a multistage process ⁽¹⁸⁵⁾. In the first stage, the complex substrates are hydrolyzed into oligosaccharides and monosaccharides, peptides and amino acids, fatty acids and glycerol, heterocyclic nitrogen compounds, ribose and inorganic phosphate. In the second stage, acidogenesis occurs with the formation of H_2 , CO_2 , acetate and other higher organic acids through the activities of the fermentative bacteria. During the third stage, acetogenesis occurs and the organic acids that are produced in the previous stage are converted into H_2 and acetate by acetogenic bacteria. In addition, a fraction of the available H_2 and CO_2 is converted into acetate by homoacetogenic bacteria. During the last (fourth) stage, a group of methanogenic bacteria both reduces the CO_2 and decarboxylates the acetate to form methane.

Correspondingly, a complete anaerobic digestion process involves a sequence of fermentation reactions resulting in the stabilization of a complex biomass with methane and carbon dioxide as the end-products. The overall conversion process is dependent on the activities of four metabolic groups of bacteria: the hydrolytic, the obligate proton-reducing, the homo-acetogenic and the methanogenic bacteria. These microbial groups have different physiologies, growth and metabolic characteristics, and need different environmental conditions for optimal metabolic activity. The dominant digester organisms can be divided into two broad functional groups: volatile fatty acid (VFA) producers (acidogens) and VFA converters (acetogens and methanogens).

Hydrolysis and liquefaction of complex and/or insoluble organics are necessary to convert these substances into a size and form that can pass through bacterial cell walls for use as energy or nutrient sources. This is accomplished by extracellular hydrolytic enzymes, which are produced and excreted by the micro-organisms for this specific liquefaction purpose ⁽¹⁴⁰⁾.

The stabilization of bio-polymers will not occur without this initial hydrolysis step. In an acid fermentation process, the hydrolyzed bio-polymers are fermented to long chain organic acids, sugars, amino acids, and eventually to small organic acids, such as volatile fatty acids.

The overall rate of stabilization and methane fermentation can be limited by the hydrolysis step or the methanogenesis step. The rate limiting step in solid digestion is generally believed to be solubilization (hydrolysis) of particulate organic matter to soluble substrate. A successful anaerobic digestion of solid waste is firstly dependent on the hydrolysis of the bio-polymers into the monomers and the acidogenic production of VFA. It was considered that the rate-limiting step of the overall methane fermentation for sludge is hydrolysis, the methanogenesis is the rate-limiting step only in the case of soluble sugars^(181, 118). As far as the chemical and structural complexity of the lignocellulose substrate is concerned, the efficiency of anaerobic digestion of lignocellulose substrate is firstly dependent on the hydrolysis or breakdown of its large molecular (crystalline) structure. The bio-conversion, degradation and hydrolysis of crystalline lignin and cellulose is the rate limiting step in anaerobic digestion of lignocellulose.

Similarly, the overall anaerobic digestion process of the lignocellulose substrate consists of the four intimately correlated steps:

- 1) Hydrolysis, biotransformation and degradation process: convert lignocellulose polymers (lignin, cellulose and hemicellulose) into lignin moieties, reducing sugars (such as oligosaccharides and monosaccharides), phenolic aldehydes and acids, polyphenols, peptides and amino acids, fatty acids and glycerol, heterocyclic nitrogen compounds, ribose and inorganic phosphate; form humic substances. Essentially, no stabilization of lignocellulose waste occurs during the hydrolysis process; the degradable organic matter is simply converted into a soluble form that can be utilized by the bacteria. The stabilization of lignocellulose wastes cannot occur unless this initial hydrolysis step is conducted properly. The extracellular, hydrolytic enzymes must be produced in sufficient quantities and make intimate contact with the organics, in order to effect hydrolysis without limiting the overall stabilization reaction. This emphasizes the requirement of a large, active population of micro-organisms, a concentrated organic substrate and uniform mixing and temperature conditions within the reactor. It implies that the pre-treatment of

lignocellulose substrate is critical to the degradation process. Furthermore, it should be recognized that not all of the organic components of the lignocellulose are hydrolyzed to simple compounds that can be assimilated by bacteria because of the structure, inaccessibility and complex non-hydrolyzable linkages, among other factors, or humification reactions.

- 2) Acidogenic fermentation process: the hydrolyzed reducing sugars, phenolic aldehydes and acids, polyphenols, peptides and amino acids, fatty acids and glycerol are eventually fermented to smaller organic acids (volatile fatty acids) such as propionic, butyric, and valeric acid. At this stage, most of the organic material of the lignocellulose is simply changed in form and a relatively small fraction is actually consumed as energy source (i.e. stabilized). The population of bacteria responsible for acid production includes facultative anaerobes, strict or obligate anaerobes or a combination of both. Acetic acid, hydrogen, and carbon dioxide are also formed during the production of organic acids. Hydrogen is inhibitory for many of the acid-forming bacteria and must be removed from the system if acid production is to continue. At the same time, hydrogen is an energy source for some methanogenic bacteria and is rapidly consumed in the reduction of carbon dioxide to methane.
- 3) Hydrogen and acetic acid formation: at this stage, acetogenic bacteria convert propionic, butyric, and valeric acid to acetate, hydrogen and carbon dioxide. Hydrogen has been recently shown to play a key role in regulating organic acid production and consumption. If the partial pressure of hydrogen exceeds about 10^{-4} atm ^(106, 65), methane production is inhibited and the concentration of organic acids (e.g. propionic and butyric) will increase. A syntrophic association with a large, stable population of CO₂-reducing methanogens will ensure a low concentration of hydrogen.
- 4) Methane formation: Waste stabilization occurs during the methanogenic phase by conversion of the acetic acid into methane, which is separated from the system in gas form. Carbon dioxide is also produced and either escapes as gas or is converted to bicarbonate alkalinity. One of the most important characteristics of the methanogenic phase is that very few substrates can act as an energy source for the methane bacteria. Presently, it is believed that only formic acid, acetic acid, methanol, and hydrogen can be

used as energy sources by the various methanogens. Among these, acetic acid and hydrogen serve as the major substrates for methane formation in the anaerobic digestion.

The anaerobic digestion path of lignocellulose substrate to methane and carbon dioxide is summarized in Fig.2.1.

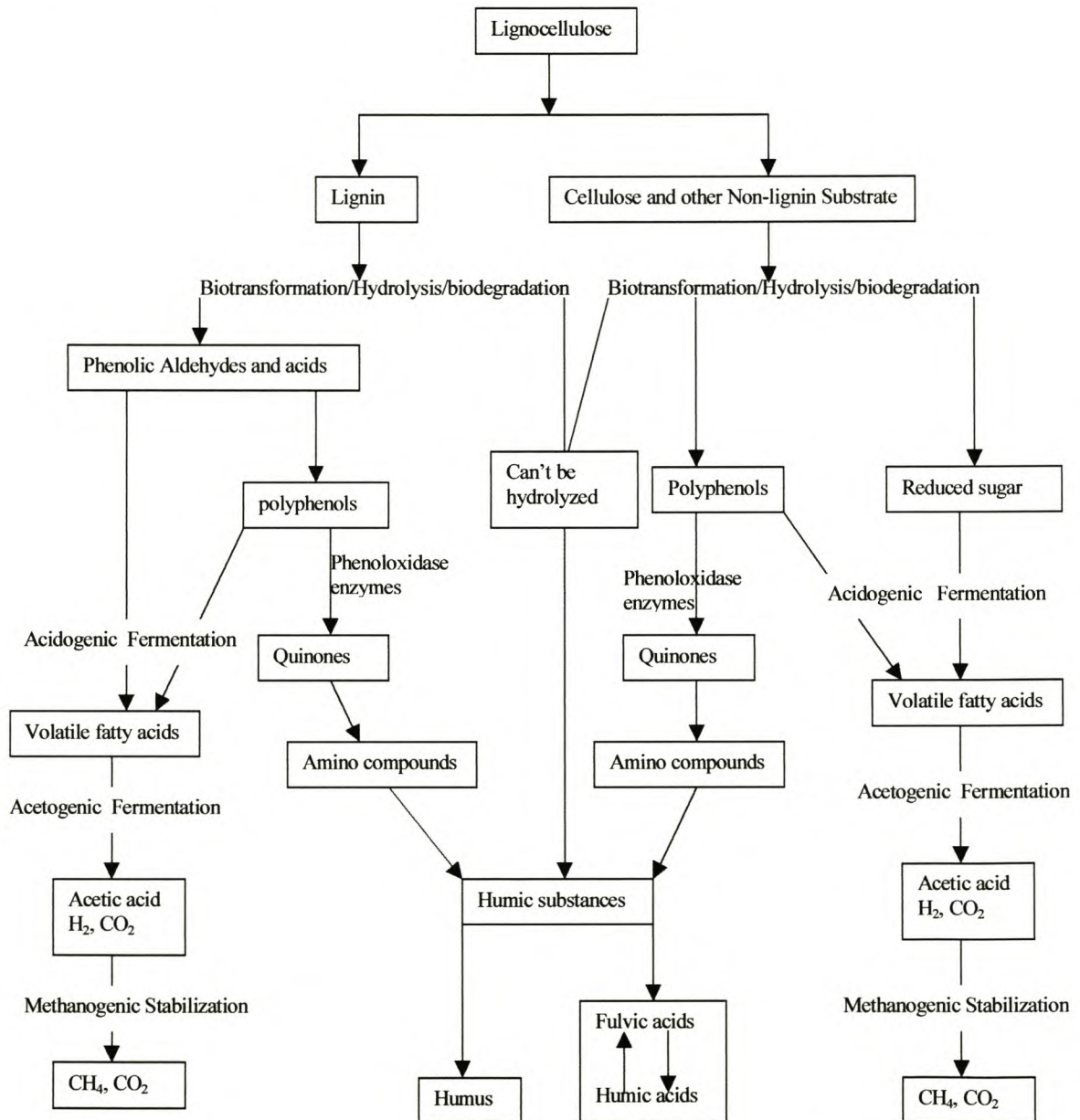


Fig 2.1 The anaerobic digestion path of lignocellulose substrate to methane and carbon dioxide⁽⁵⁵⁾

As can be seen from Fig.2.1, the biological stabilization of the lignocellulose substrate to methane is a long and complicated process. To ensure a complete stabilization of lignocellulose substrate, the following points should be taken into account:

- 1) Because of the complicated macro-and microstructure of lignocellulose, it is difficult to break it down into simple and soluble substances which can feasibly be utilized by the anaerobic consortium. Thus, the initial biotransformation/hydrolysis/bio-degradation of lignocellulose into soluble forms is critical to its further bio-degradation/utilization by an anaerobic population. This step can be enhanced by pre-treating lignocellulose physically or chemically, which, to some extent, leads to the rupturing of the rigid structure of lignocellulose.
- 2) The lignocellulose cannot be completely hydrolyzed, and a wide variety of intermediary products are formed. Some of which (e.g. phenolic aldehydes and acids and polyphenols) are still refractory to anaerobic digestion and some may re-polymerize into polydispersed polymers of aromatic and aliphatic units (humic substance), which are more complex and more highly condensed than the lignin polymer. The further utilization/conversion of the hydrolyzed products by anaerobes may thus be seriously inhibited by these intermediary products. A special treatment or further degradation of the humic substances formed in situ, especially of the HAs, is necessary because of the solubility of the HAs in normal aqueous and terrestrial habitats, and their potential toxicity to the growth of anaerobes.
- 3) Successful degradation and stabilization of lignocellulose substrate towards methane production is a consecutive biological decomposition process which involves the comprehensive activities of different functional micro-organism groups, with each stage having its specific functional micro-organisms. The presence and the growth of the particular function micro-organism can be used as an indicator by which the degradation of lignocellulose can be evaluated.
- 4) Care must be taken to reduce the pathogenic bacteria to a satisfactory level by designing and operating the anaerobic digestion process in a hygienic way so that the humus, the

final solid products of the digestion, can be used in the sustainable development of agricultural industry.

- 5) In addition, since landfill is normally used for the disposal of municipal solid wastes and will be a significant repository of the future, the decomposition of lignocellulose in landfill should be investigated to obtain the primary knowledge about the decomposition of lignocelulose in normal waste-management practice.

2.2.1.2 Biotransformation/degradation of organic fatty acids and aromatic compounds in an anaerobic process.

Predictably, organic fatty acids and aromatic compounds can be the main products in the lignocellulose hydrolysis process and can become the principal sources for the anaerobic digestion. The biotransformation/degradation of these two groups of organics has been of interest to many investigators.

Organic fatty acids:

Propionate metabolism by a mesophilic acetogenic bacterium was studied in pure cultures as well as in co-cultures and tri-cultures with methanogens. Products and inhibitory conditions observed under various substrate and consortia combinations were explained⁽⁶⁶⁾. Intermediates and products resulting from the syntrophic degradation of normal and branched C₄ to C₉ fatty acids in a methanogenic triculture were presented by Wu et al.⁽²⁶²⁾. Liu and Suflita⁽¹⁵³⁾ examined the ability of *Acetobacterium woodii* and *Eubacterium limosum* to degrade methyl esters of acetate, propionate, butyrate, and isobutyrate under growing and resting cell conditions. At relatively low concentrations, lactate was rapidly oxidized to acetate, CO₂ and H₂ in a fluidized-bed reactor inoculated first with a methanogenic consortium of three species and subsequently with a *Desulfovrio sp. Strain L1*. However, under non-sterile conditions and at high lactate concentrations, a *Clostridium sp.* culture that converted lactate to propionate was developed⁽²⁷¹⁾.

Wu et al.⁽²⁶³⁾ reported that the syntrophic fatty acid-degrading granules can be produced with a limited number of key microbial species which possess essential aggregate-forming ability,

and their association can completely mineralize the major intermediates of anaerobic digestion, i.e. acetate, propionate and butyrate.

Aromatic compounds:

Aromatic compounds generally undergo a ring reduction step, where the microbes are mostly produced. This is a result of the induction of a whole sequence of enzymes that convert an aromatic substrate into either an 'ortho' or a 'para' cyclohexane carboxylic acid derivative, followed by cleavage of the ring to the end-products CH_4 , CO_2 and aliphatic acids⁽⁸⁴⁾. These ring fission products are then funneled into the Krebs cycle through a variety of pathways depending on the organism and culture conditions^(231, 268, 20). This has been illustrated with the degradation of benzoate and phenol, two of the most studied aromatic compounds, because they are the intermediate products of numerous other aromatic compounds in the anaerobic catabolism process.

The anaerobic utilization of benzoic acid by methanogenic bacteria was first reported by Tarvin and Buswell⁽³⁶⁾, and this was later confirmed by Clark and Fina⁽⁴⁷⁾. Other aromatic compounds, which have been degraded by similar mechanisms, include catechol, pyrogallol, hydroquinone, caffeic acid, pyrrole and pyridine. The first observation of the anaerobic degradation of catechol was by Healy and Young⁽¹¹⁷⁾, and their results showed a ring cleavage step during the fermentation, with half or more of the substrate carbon being converted to methane. Hydroquinone was first reported to be degraded anaerobically by Szewzyk et al.⁽²³⁸⁾. They also investigated the degradation of catechol and again found phenol to be an intermediate, indicating that reductive dehydroxylation was the primary event during the degradative pathway.

The key steps of anaerobic phenol metabolism were studied with the help of a denitrifying *Pseudomonas* strain, and the rate-limiting intermediates were identified⁽¹⁴⁷⁾. An anaerobic consortium carboxylated phenol to benzoate at the para-positions and further transformed benzoate to 1-cyclohexene carboxylate and heptanoate. The same consortium carboxylated the ortho-, but not the meta- and para-, isomers of cresol, fluorophenol and chlorophenol⁽²²⁾. The biotransformation of benzoate, 4-hydroxybenzoate, and aniline were studied in sediment slurries amended with multiple electron acceptors, including manganese (IV) and iron (III)

oxide. Addition of molybdate inhibited the degradation of benzoate, but not that of 4-hydroxybenzoate⁽¹⁸¹⁾. Gallert and Winter⁽⁸⁹⁾ found that 4-hydroxybenzoate was activated with coenzyme A (CoA) and then reductively dehydroxylated to benzoyl-CoA in a strictly anaerobic phenol-degrading mixed culture. The generation of a proton gradient during the decarboxylation of 4-hydroxybenzoate and ATP formation by H^+ -ATPase was concluded to be a source of the observed rapid increase in cellular ATP concentrations and an increased growth yield of 1.8 g/mol after the addition of 4-hydroxybenzoate to the culture. The anaerobic benzene degradation was found in microcosms prepared with material from a contaminated aquifer⁽⁶¹⁾. Complete stoichiometric mineralization of toluene and o-xylene to CO_2 , CH_4 , and biomass under anaerobic conditions by a methanogenic consortium derived from a creosote-contaminated sediment after long lag periods (100-255 days) was reported by Edwards and Grbić-Galić⁽⁷²⁾. Seyfried et al.⁽²²⁷⁾ demonstrated that the anaerobic degradation of toluene by denitrifiers (*Pseudomonas sp. Strain T* and *strain K172*) was initiated by a direct oxidation of methyl group, with benzaldehyde and benzoate as intermediates.

The enzymatic activities involved in a complete anaerobic oxidation of catechol and protocatechuate by the sulphate-reducing bacteria *Desulfobacterium sp. Strain Cat2* were determined⁽¹⁰¹⁾. The anaerobic oxidation of catechol was found to be a CO_2 -dependent process. Kasami et al.⁽¹³⁰⁾ used ^{13}C -NMR spectroscopy to identify the anaerobic pathway for the conversion of the methyl group syringate and other phenyl methyl ethers to acetic acid by *Clostridium thermoaceticum*.

The sulphate reducing strain *Desulfococcus sp. Strain Hy5* was able to grow with hydroquinone as the sole source of carbon and energy⁽¹⁰²⁾ with gentisate the first intermediate in this degradation process. Biotransformation studies of both quinoline and methylquinoline isomers conducted with sediment slurries under methanogenic conditions at $25^{\circ}C$ showed the production of hydroxylated and methylated intermediates. Comparative metabolic studies with *Clostridium foricoaceticum* and *Clostridium aceticum* revealed acetate to be the major product of benzaldehyde degradation, with a doubling time of five hours for these species⁽¹⁰³⁾.

In addition, it was found that under denitrifying conditions, aniline was mineralized completely to N_2 and CO_2 , but two methanogenic cultures, established with anaerobic waste water digester sludge and estuarine sediment respectively, did not use aniline⁽⁶²⁾.

2.2.1.3 Environmental factors and process modifications

(1) Environmental factors

Environmental factors of concern in anaerobic digestion include nutrients (macro and micro), pH, temperature and toxic materials. For the most part, the methane-forming bacteria are thought to be the micro-organism most sensitive to environmental changes. If the hydrolysis of organic polymers is successfully reached, further acidogenic fermentation is a rapid process. However, methanogens have a slower growth rate than the acidogens upstream in the food chain, and therefore becomes the rate-limiting step in the overall conversion process. The environmental factors discussed here therefore mainly take the methanogens into consideration.

Nutrients: Nutrients must be present in sufficient quantities to ensure an efficient digestion. The nutrients required in highest concentration are nitrogen and phosphate. A commonly-used empirical formula for bacteria is $C_5H_7O_2N$ ⁽¹⁹⁴⁾. Nitrogen is used in the synthesis of proteins, enzymes, ribonucleic acid (RNA) and deoxyribonucleic acid (DNA). The phosphorus requirement for bacteria growth is about 1/7-1/5 of the nitrogen requirement. Phosphorus is required for the synthesis of the energy-storage compounds (adenosine triphosphate-ATP) and RNA and DNA. Domestic sludge usually contains sufficient quantities of nitrogen (in the form of protein, urea, ammonia) and phosphorus for efficient digestion. However, the treatment of industrial wastes or municipal wastes containing high percentages of industrial wastes may require the addition of supplementary nitrogen and/or phosphorus. Waste high in protein may be of special concern due to the potentially inhibiting levels of ammonia that may be released during protein degradation.

Other nutrients are required in lower concentrations than nitrogen and phosphorus. These include iron, nickel, cobalt, sulphur, calcium and some trace organics. The trace metals must be in solution to be available for bacterial growth. If high concentrations of sulphide are present, or are produced from sulphate, metal concentrations will decrease because of precipitation.

Activated sludge is a mixed culture full of organic - nutrient - removal micro-organisms well acclimated to the natural or chemical organic waste during the waste water treatment process. It includes the micro- and macronutrients (6-8% N and 2% P related to dry matter) necessary for biological growth ⁽¹²⁶⁾. In this regard, it can be considered as a nutrient provider for micro-organism growth.

pH: Maintenance of system pH in the proper range is required for an efficient anaerobic digestion. The general accepted range for good process efficiency is 6.5-7.6. Anaerobic conversion of organics to methane is a complex process involving many species of bacteria, with the methanogens being the most sensitive to pH changes. During system imbalance, the volatile acids produced by the acetogenic bacteria typically increase at a faster rate than can be decomposed by the methanogenic bacteria. Unless the system contains sufficient buffer capacity, the pH will drop to unacceptably low levels, and methane production will decrease and may eventually cease if the pH drop is of sufficient magnitude or duration. The dominant buffering system in anaerobic digestion is the bicarbonate system. Maintenance of sufficient alkalinity guards against failure from pH drops ⁽¹⁹⁴⁾.

Temperature: Generally, there are two optimum temperature ranges in anaerobic digestion, at 30-38°C and 50-56°C in the mesophilic and thermophilic zones respectively. Most municipal solid waste (MSW) digesters operate in the mesophilic range. However, most reports confirm that thermophilic digestion can ⁽¹⁹⁴⁾: (1) increase organic matter destruction rates; (2) increase the degree of organic matter destruction; (3) improved dewatering characteristics; and (4) increase destruction of pathogenic bacteria.

The sludge in the thermophilic reactor contains a stable population of acidic and methane forming bacteria ⁽⁶³⁾. Research on laboratory and pilot scale has shown that a first - high loading thermophilic (50-55°C) stage followed by a second mesophilic (35-37°C) stage with sufficient retention time can improve the performance of the anaerobic digestion ⁽¹²⁹⁾. There was major doubts though concerning the stability of thermophilic digestion, such as the higher accumulation of volatile acids, increased sensitivity to temperature fluctuations and possible ammonia toxicity. As far as the acidification fermentation process was concerned, the thermophilic digestion was preferable to mesophilic digestion because the ammonia toxicity could be diminished by a high organic loading rate permitted by the high digestion rate at

thermophilic temperature. Therefore, it is desirable to promote the lignocellulose hydrolysis process with less ammonia toxicity under thermophilic treatment conditions.

Toxicity and inhibition: Whether a substrate is toxic to a biological system or not depends on the nature of the substance, its concentration, and its acclimation. Many substances will stimulate the reaction in low concentration, but as the concentration increases, the effect becomes inhibiting. It should be noted that substances must be soluble to be toxic - a property that may be used to reduce the toxicity of materials such as heavy metals. Substances most commonly reported as inhibiting to anaerobic digestion are inorganics such as the alkali and alkaline-earth metals, heavy metals, ammonia-nitrogen, sulphide and a wide variety of organic compounds.

1) Ammonia-nitrogen and nitrate: Ammonia-nitrogen and bicarbonate alkalinity is produced during the digestion of organics containing nitrogen (predominately protein). In the case of sludge containing high levels of protein like activated sludge, enough ammonia-nitrogen may be released to cause inhibition. It is therefore better to mix the sludge with high carbohydrate-content organic matter (e.g. lignocellulose residues) to obtain the ideal C/N ratio for anaerobic digestion. McCarty ⁽¹⁷³⁾ reported that concentrations between 50-200 mg/l are beneficial, mainly because ammonia-nitrogen is an essential nutrient. However, ammonia-nitrogen is also thought to be toxic in two ways depending on its pH. It may be present in the form of the ammonium ion NH_4^+ , or as dissolved ammonia gas NH_3 . It was found that pH and temperature dramatically affect the concentration of $\text{NH}_3\text{-N}$ in solution. The implication that $\text{NH}_3\text{-N}$ toxicity may be more of a problem in thermophilic digestion is apparent. The unionized form of ammonia is the toxic species and the equilibrium shifts toward that species as the temperature and pH rises. The level of ammonia is believed to become inhibiting in the dry anaerobic digestion above 4 g $\text{NH}_4\text{-N/kg DS}$. The total ammonia was measured and the unionized component was calculated on the basis of the predominant pH in the digester. Kayhanian and Tchobanoglous ⁽¹³¹⁾ have suggested that the inhibiting total ammonia concentration is at the 100 mg/l level and recommended maintenance of the process below 600 mg/l. In slurry digestion at TS level below 10%, the toxicity of ammonia was very small. There are even documented cases of successful acclimatization to much larger levels than 5000 mg/l ⁽¹⁹⁹⁾. However, there is no escaping the unionized ammonia that is formed at high pH when the equilibrium shifts toward the unionized species. The unionized ammonia could

cause inhibition at levels of total ammonia well below the threshold value of 4 g/kg cited previously.

The presence of nitrate is likely to stimulate the denitrification process, and thus inhibits the methanogenesis. The interactions of methanogens and denitrifiers in the treatment of phenol containing waste waters have been investigated in an upflow anaerobic sludge blanket (UASB) reactor using a continuous experiment ⁽⁸⁰⁾. It was found that methanogenesis occurred only at chemical oxygen demand of NO_3^- -N ratios greater than 3.34. At ratios less than 3.34, methanogenesis ceased to take place and denitrification became incomplete because of an insufficient supply of substrate. When Oh et al.⁽¹⁸⁶⁾ studied the acetate limitation and nitrate accumulation during denitrification, they found that if acetate was added to the denitrifying activated sludge mixture to obtain a carbon to nitrogen (C/N) ratio in the range of 2:1 to 3:1, nitrate was completely consumed at the same rate with no nitrite accumulation, indicating that the nitrate concentration controlled the respiration rate as long as sufficient substrate was present.

2) Sulphides and sulphate -- Lawrence et al. ⁽¹⁴⁹⁾ reported that soluble sulphides in excess of 200 mg/l caused a significant decrease in methane production. It is important to note that under anaerobic conditions in a mixed culture of bacteria, sulphate is reduced to sulphide. Therefore, sulphate (in excess of 200 mg/l), represents potential sulphide toxicity.

In anaerobic ecosystems containing sulphate, sulphate reduction bacteria (SRB) and methane-producing bacteria (MPB) are competitors for fermentation intermediates such as hydrogen and acetate ^(156, 157, 260). When the sulphate concentration is high, most of the electrons could be consumed in sulphate reduction instead of in methane production. It was found that the competition between sulphate reduction and methanogenesis is governed by the rate of hydrogen and acetate production and sulphate availability ⁽¹⁵⁶⁾. When the sulphate concentrations are non-limited, but acetate and hydrogen concentrations are limited, methane production will be inhibited as the SRB lower the hydrogen partial pressure below the threshold concentration necessary for use by the MPB ⁽¹⁵⁶⁾. Additionally, based on the kinetics of substrate utilization, SRB are predicted to out-compete MPB for hydrogen. The interaction between sulphate-reduction and methanogenesis was investigated for acetate, methanol and

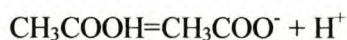
formate in six chemostats-containing mixed cultures. The kinetic parameters for the degradation of these substrates were evaluated ⁽¹⁰⁷⁾.

The degradation of cellulose and lignocellulose material was investigated in simulated landfill column reactors under sulphate reducing and methanogenesis conditions ⁽¹³⁷⁾. In the sulphate reducing reactors, methane gas production was effectively suppressed. The carbon dioxide produced during the sulphate reduction was dissolved in leachate as inorganic carbon (IC), leading to lower carbon dioxide emission from sulphate reducing reactors. Higher COD in the leachate from sulphate reducing reactors was probably due to a higher solubilization rate of cellulose and lignocellulose material. In a batch experiment, the hydrolysis of avicel-cellulose, filter paper and newspaper was faster under sulphate reducing conditions. The percentage of organic carbon bio-degradation in sulphate reducing conditions was higher than that in methanogenic conditions.

3) Heavy metals: It has been realized for a long time that the anaerobic system is particularly vulnerable to the high loading of heavy metals. The toxicity of heavy metals depends on the various chemical forms which the metal may assume under anaerobic conditions and at near neutral pH levels. Work performed by Ghosh ⁽⁹²⁾ showed that although low concentrations of some heavy metals are extremely toxic, high concentrations could be tolerated if sufficient sulphide was available for precipitation.

4) Organics: Most of the research on surface-active materials focused on anionic detergents. The ability of synthetic detergents, especially alkylbenzene sulfonates (ABS), to inhibit methane production is well known. The adsorption of the detergents on sewage solids prior to anaerobic digestion of those solids is thought to be the mechanism involved. Chlorinated hydrocarbons are extremely toxic to anaerobic digestion and have caused inhibition in a number of treatment plants in England. Hovious et al. ⁽¹²⁰⁾ examined the inhibitory characteristics of many petrochemicals. For example, ethyl benzene was found to cause little or no inhibition at concentrations from 150-1000 mg/l. However, Chou et al. ⁽⁴⁶⁾ found that an ethyl benzene concentration of 200 mg/l reduced activity by 25% with a reduction of 60% observed at 1000 mg/l. The solvent ethylene dichloride has been reported to severely inhibit methane fermentation, with inhibition starting at concentrations as low as 5 mg/l.

For a number of years there have been considerable controversy over whether volatile acids (VA) were toxic to the methane bacteria. One school of thought was that a VA concentration above 2000 mg/l was toxic ^(36, 214). However, extensive research ^(144, 170, 171, 172) conclusively showed that VA concentrations of up to 6000 mg/l can be tolerated with no loss in methane production, provided the pH is maintained in the optimal range. These researchers did show that VA concentrations of up to 6000 mg/l inhibited the acetogenic bacteria. Thus, pH control using alkaline substances is a valid procedure in maintaining methane production during imbalance digestion until the cause of imbalance is found. Andrews and co-workers ^(2, 3) and Kroeker et al. ⁽¹⁴²⁾ have postulated that it was the unionized volatile acids, UVA, that are toxic to the methane bacteria. Inhibition was reported to occur at UVA levels of 30- 60 mg/l. By using acetic acid, which is by far the predominant intermediate volatile acid as an example, the following equilibrium holds:



The concentration of UVA is thus dependent on digester pH. Even though there is still some controversy as to whether it is the UFA or low pH which inhibits the methane bacteria, it is clear that high concentrations of volatile acids can be tolerated as long as the pH stays in the optimum range of 6.5-7.6.

The inhibition of anaerobic digestion by terephthalic acid and its aromatic by-products has been studied ⁽⁷⁶⁾. It was found that the easily biodegradable compounds of acetic, benzoic and formic acid found in PTA and DMT waste water can be methanized without problem during anaerobic treatment since the degradation is not significantly inhibited by 4 carboxylbenzaldehyde as well as terephthalic and p-toluic acid. For PTA wastewater, COD removals of 40-50% may then be readily obtained at full scale. Higher COD removals would imply terephthalic acid degradation which can be obtained only after prior removal of acetic and benzoic acid from the effluent. A two- stage reactor may then be recommended in order to attain such objectives.

The toxic effect of long chain fatty acids on the acetogenic and methanogenic activity of granular methanogenic sludge was studied. Capric acid concentrations of between 6.7 and 9 mol/m³ were lethal to both acetogenic and methanogenic bacteria. The H₂-producing acetogenic and methanogenic bacteria recovered faster than the acetotrophic methanogens ⁽²⁰⁶⁾. The toxicity effects of benzene, chloroform, 1,2-dichloroethane, pentachlorophenol,

mercury, and zinc on the anaerobic mineralization of acetate in methanogenic sediments from the Rhine River in the Netherlands, were investigated ⁽²⁵¹⁾. The anaerobic biodegradability as well as the potential inhibiting effect of photo-processing effluents was tested using a batch reactor. Initially, a partial inhibition of methanogenesis was observed, mainly owing to competition between sulphate reducing and methane producing bacteria. However, with prolonged incubation, methanogenesis recovered.

(2) Two-phase anaerobic digestion process

As discussed before, the anaerobic digestion consists of a sequence of fermentation reactions that results in the stabilization and gasification of complex organic materials to methane and carbon dioxide. The overall process can generally be divided into two steps: acidification fermentation (hydrolysis and acidogenesis) and methanogenesis. Accordingly, the dominant digester organisms can be divided into two broad functional groups: VFA producers (acidogens) and VFA converters (acetogens and methanogens). When the complex organic materials such as lignocellulosic residues are to be digested, the rate-limiting step is normally the hydrolysis of particulate organic matter in the lignocellulosic residues into soluble substances. Thus, the anaerobic digestion of complex organic materials could be greatly enhanced by improving the hydrolysis (acidification fermentation stage) rate.

On the other hand, since the anaerobic digestion process is essentially diphasic, the microbial groups necessary to facilitate the overall digestion have different physiologies and growth and metabolic characteristics, and need different environmental conditions for optimal metabolic activities. For example, in the case of temperature, anaerobic digestion is generally operated in the mesophilic (30-38 °C) or thermophilic (50-60 °C) range ⁽¹⁹⁴⁾. Although the same anaerobic microbial process can take place both at the mesophilic and thermophilic temperatures, the thermophilic process has been found to be superior (especially during anaerobic digestion of solid waste and slurries) in improving sludge hydrolysis efficiency and in increasing the disinfecting effect. However, as long as hydrolysis is successful, the fermentative acidogens normally have higher growing rates than the acetoclastics and the methanogens. This may lead to a subsequent accumulation of intermediary VFA products resulting in a fall in pH value, thus deteriorating the stability of the process. In conventional one-phase thermophilic digesters, a long hydraulic retention time (HRT) is usually used to

prevent the high production rates of VFA which is caused by fast-growing acidogens and to create a balance between the VFA conversion rates and the acetogens and methanogens growing rates. Vice versa, high HRT will suppress hydrolysis and the production of VFA, thereby affecting the overall efficiency of the high-rate digester. In addition, compared with the hydrolysis-acidification fermentation micro-organisms, the acetogenic and methanogenic fermentation bacteria are more sensitive to the environmental changes. Therefore, if the hydrolysis-acidification fermentation is separated from the coupled acetogenic-methanogenic fermentation into the different optimal growing environments for each micro-organism group could substantially enhance the associated biological reactions and provide higher organics stabilization and gasification rates and greater pathogen kills ^(96, 97, 98, 48, 150) in comparison to the conventional one-phase high-rate digestion.

The two-phase anaerobic digestion processes have been extensively studied and have been used to treat industrial waste water ⁽³⁷⁾, dairy industry wastes ⁽¹⁰⁰⁾ and municipal solid wastes ⁽⁹⁷⁾. Gosh et al. ⁽⁹⁹⁾ reported that the performance of a full-scale two-phase digestion system charged with municipal sludge was considerably better than that of a one-phase high-rate digester.

The one-phase and two-phase anaerobic digestion processes were compared on the basis of the characteristics of substrate degradation and the bacterial population levels. The chemostat-type reactors, to which the starch was fed as substrate was used and both processes were operated under the same experimental conditions ⁽²⁷²⁾. It was found that the two-phase system was more stable when exposed to the changes in pH than the one-phase system. At lower SRT, the CH₄ recovery and COD removal rates in the two-phase system were higher than the one-phase system. The concentration of propionate in the effluent of the one-phase system was 30-50% higher than that in the two-phase system. The concentrations of acetate and butyrate in the one-phase system were slightly lower than those in the two-phase system. The population levels of acidogenic bacteria in both systems were in the same order (10⁸ to 10¹⁰ MPN/ml). The population levels of hydrogenotrophs were also in the same order as the acidogenic bacteria in the two-phase system, while the population levels of hydrogenotrophs were 10 to 100 times less than those of the acidogenic bacteria in the one-phase system. The number of acetic acid utilizing methanogens in the methanogenic phase of the two-phase

system were 2-10 times higher than those in the one-phase system. It is suggested that the one-phase system cannot simply be regarded as the sum of acidogenesis and methanogenesis.

Fongastitkul et al.⁽⁸⁷⁾ demonstrated the feasibility of a two-phase anaerobic sludge digestion process. Process failure and maximum system loading capacity were also investigated. The performance of a two-phase system composed of a well-mixed continuous-flow acidogenic reactor and a fluidized sand-bed methanogenic reactor was evaluated as a function of recycling of the methanogenic reactor effluent to the acidogenic reactor. This system configuration minimized the overall operational treatment cost ⁽²⁰⁹⁾. At 30 °C, a laboratory-scale anaerobic expanded granular sludge bed reactor was able to achieve more than 80% COD removal efficiency at organic loading rates up to 12 g COD /l.d for influents prepared with ethanol as model substrate at concentrations as low as 100 to 200 g COD/l ⁽¹³²⁾.

Rich and Kayhanian ⁽²⁰⁷⁾ introduced a two-stage co-composting process using the biodegradable fraction of municipal solid waste and waste water treatment plant slugs. The product (humus) met the U.S. EPA Part 503 rule for land application.

However, there is little information on the application of the two-phase anaerobic digestion in stabilizing lignocellulose residues with activated sludge.

Furthermore, improving the acidification fermentation efficiency of lignocellulose was possible by combined pre-treatment, by supplying nutrients with activated sludge and by high-rate thermophilic digestion. The restoration or development of the methanogenic phase from the high-rate acidogenic fermentation phase is expected to be the second critical step in anaerobic stabilizing of lignocellulose waste and this can be made with a two-phase digestion configuration by adjusting the optimal metabolic environments for the growth of mesophilic methanogens after the acidogenic phase (process).

(3) UASB configuration and process

Among the anaerobic treatment processes, the upflow anaerobic sludge blanket (UASB) ⁽¹⁵¹⁾ reactor has become very popular in the anaerobic biological treatment of waste water. The

implementation of the UASB reactor has been applied to a wide range of industrial and municipal waste water, as well as landfill leachate.

The advantage of the UASB reactor compared to traditional anaerobic treatment, e.g. the contact process, is the ability to retain high biomass concentrations despite the upflow velocity of the waste water and the production of bio-gas. Consequently, the reactor can operate at short HRT since the sludge retention time is almost independent of the hydraulic retention time. It can also operate at high organic loading rates because its loading rate is dependent on the amount of active biomass present in the reactor. Successful operation under these conditions requires a highly active biomass with good settling abilities. The granules can be interpreted as a spherical biofilm, and many similarities exist between biofilm development and granulation. The initial adhesion of granules begins with the initial adsorption of bacteria or adsorption to inert material such as precipitates. Once the bacterium is adhered, colonization has started. The granulation process depends on cell divisions and recruitment of new bacteria from the liquid phase. In UASB reactors, the biomass is retained as aggregates, called granules, formed by the natural self-immobilization of the bacteria, which do not employ any supporting materials such as Rasching rings or clay⁽²²¹⁾.

The metabolic reactions that occur during anaerobic digestion also suggest why the aggregation of micro-organism into granules would be advantageous. As stated before, the degradation of complex substrates into methane and carbon dioxide during anaerobic digestion involves the interaction of at least three metabolic groups. The first group of fermentative bacteria, the acidogens, conducts initial degradation of biopolymers. The acids and alcohol thus produced are utilized by a second group of bacteria, namely the acetogens. The third group of bacteria is the methanogens. Located at the end of the nutrient cascade, methanogens convert CO_2 and H_2 , acetate and a few other simple compounds into methane. Because of unfavorable thermodynamics, oxidation of propionate and butyrate is only possible if H_2 is removed efficiently, i.e. a very low hydrogen partial pressure is necessary. Propionate degradation is only possible below a partial pressure of 10^{-4} atm H_2 ^(106, 65). When a mixture of acetate, propionate and butyrate is degraded, a clear correlation exists between the degradation rate of propionate or butyrate and the hydrogen partial pressure. A slight increase in the partial pressure of hydrogen results in an immediate decrease in the degradation rate of the two volatile fatty acids^(216, 217, 218, 220). Clearly, the close association of

members of these three groups in a layered granular structure would ensure a high metabolic activity.

In recent years, a more pronounced understanding of the microbiology of immobilized anaerobic bacteria and the mechanism of granule formation has been accomplished. Extracellular polymers (ECP) in the granular sludge are important for the structure and maintenance of the granules, but the inorganic composition seems to be of less importance.

Bacterial ECP is defined as polysaccharide containing structures of bacterial origin lying outside the integral elements of the outer membrane of Gram-negative cells and the peptidoglycan of Gram-positive cells. ECP is made of organic residues, phages, lysed cells and other organic matter excreted by the microbial cells ^(49, 50). It contains polymers of saccharides, proteins, lipids, phenols and other nucleic acids. ECP can have different functions depending on the micro-organism. It can trap soluble nutrients, increase pathogenicity, or decrease the susceptibility to phagocytosis. ECP also mediates the adhesion of bacteria in natural ecosystems ⁽²³⁵⁾.

The composition of ECP affects the surface properties of the bacterial flocs and the physical properties of the granular sludge ^(88, 178). Dispersed bacteria are negatively charged and there is electrostatic repulsion between the cells. The production of ECP can change the surface charge of the bacteria, resulting in aggregation. The adsorption of bacteria depends on the surfaces of both the cells and the support to which these cells adhere. The composition of ECP is of importance for adsorption owing to ECP's influence on the surface charge and energy. Too much ECP can cause the deterioration in floc formation and, therefore, repulsion can occur.

The amount of ECP is affected by the conditions under which the granules are grown. The concentration of ECP is lower in thermophilically grown granules than in those grown mesophilically ⁽²¹⁹⁾. The amount of ECP is also influenced by the waste water composition. It was found that an increase in C/N ratio stimulated the production of extracellular polysaccharide, resulting in the improved bacterial attachment to solid surfaces ⁽³⁷⁾. A decrease in both the protein and polysaccharide content in extracellular material was seen when the feed of a UASB reactor was changed from sugar-containing waste water to a

synthetic waste water containing acetate, propionate and butyrate, with the highest lipid content in the granules in the latter reactor. Shen et al.⁽²³⁰⁾ showed that the addition of iron and yeast extract to the feed increased the amount of carbohydrates extracted from the granules; the opposite effect was seen when the iron was absent. Bull et al.⁽³⁸⁾ showed that addition of methanol to the feed improved the start-up performance of a fluid-bed reactor treating synthetic, meat wastewater.

It is not clear whether specific species produce ECP or if several or all species in the granular sludge can do so. However, it is certain that the acidogenic populations have a greater influence on the production of ECP.

It was generally agreed that the aceticlastic methanogen, *Methanosaeta*, was critical for the structure and maintenance of the granules. However, several investigators have found that *Methanosarcina spp.* can have the same important role in granules. Other bacteria are also important for the granulation process, especially ECP-producing bacteria. Hydrogen-utilizing bacteria, together with H₂-producing syntrophic bacteria, are observed in microcolonies⁽²²¹⁾. Microcolonies of syntrophic acetogens in granular structures have been observed within anaerobic granules⁽⁶⁸⁾. Syntrophic microcolonies consisting of acetogens and methanogens were major structural components of granules developed on brewery waste water⁽²⁶¹⁾.

The electron transfer in the microcolonies occurs via interspecies hydrogen transfer, while interspecies formate transfer may not be essential for interspecies electron transfer. The microcolonies give an optimal arrangement of the bacteria within the granules for effective degradation of syntrophic substances such as propionate or butyrate.

The formation and stability of the granules are essential for successful operation⁽²²¹⁾. When using the UASB reactor for waste water treatment, it is important to make a preliminary examination of the seed material and waste water both microbiologically and chemically. The microbiological analysis should give information about the microbial composition of the granules or sludge used and the ability to degrade the wastewater. The chemical analysis can give information about the waste water composition, for instance, if the necessary nutrients are present.

The discovery of new abilities for the granular sludge gives new possibilities for the design of better granules, e.g. for the bioremediation of contaminated groundwater containing xenobiotics. Also, engineered granules could be created with a higher resistance to the normal variations seen during treatment of waste water.

Bioavailability is another important factor to be considered when evaluating the anaerobic degradation process of lignocellulose. Bioavailability refers to the accessibility of a substrate for the requisite micro-organisms. In general, water-soluble substrates are more bioavailable and the likelihood of their being biodegradable is higher. Adsorption of substrate to solid matrices can also affect the susceptibility of the substrate to bio-degradation. It is known that adsorption of compounds to inert surfaces, such as granular activated carbon, may actually increase bio-degradation, covalent binding of substrates to humic materials may render the substrates more recalcitrant. Therefore, to separate the soluble intermediate degradation products from the insoluble humus can be beneficial to enhancing the process efficiency of bio-disposal of lignocellulose residues.

In addition to the many successful treatments of food/beverage waste water and even high-strength industrial waste water, the UASB was recently found to be effective in the removal of simple aromatic chemicals, such as benzoate⁽¹¹¹⁾ and phenol⁽⁷⁹⁾.

As can be predicted, the chemical structure of some components (e.g. soluble humic substances like HAs) of the leachate from the methanogenic conversion lignocellulose process are far more complicated than that of fatty acids and simple aromatic compounds. Given the uncertainty about the anaerobic biodegradability of the HA-bearing leachate in the UASB reactor, little work was done to treat this particularly complicated leachate with granules previously developed with none-HA substrate in the UASB reactor. The results thus obtained can be supplementary to the process designing of a complete anaerobic digestion of lignocellulose wastes.

2.2.2 Pre-treatment of lignocellulose

As discussed in 2.2.1, the rate-limiting step in lignocellulose digestion is firstly the hydrolysis of particulate organic matter (cellulose, hemicellulose and lignin) to soluble substrate since

the structural features of potential substrates affect their hydrolysis efficiency, thereby affecting their susceptibility to anaerobic digestion. The more complex (e.g. the more branched) the structure, the more difficult it is for micro-organisms to attack it. Thus the pre-treatment of lignocellulose is an essential prerequisite to promote the hydrolysis process and ultimately improves the overall digestion efficiency.

The pre-treatment may include mechanical, chemical, enzymatic and biological methods. Chemical, enzymatic or microbiological conversion of lignocellulose residues is affected mainly by lignin and cellulose crystallinity, leading to an ineffective degradation. Lignin impedes enzymatic and microbiological access to the cellulose, and cellulose crystallinity affects the attack rate of the mechanism on cellulose.

Numerous pre-treatment methods capable of significantly enhancing the rate and extent of hydrolysis of pure cellulose found in biomass materials have been described in literature ⁽¹²²⁾. However, most pre-treatment processes are costly when used separately and have substantial energy requirements due to the severity of the process. For example, while the hemicellulose can be readily depolymerized by being heated in the presence of dilute acids ⁽¹⁰⁾, the depolymerization of cellulose into monomeric sugars by strong acids or by commercial enzymes remains a major economic dilemma because the cellulose is often intimately associated with lignin which prevents the access to the cellulose ⁽⁸²⁾. Thus, a combined pre-treatment process could be more economical in the biotreatment of lignocellulose residues into value-added products such as fuel (energy).

Mechanical pretreatment: Utilize shearing and impacting forces to yield a fine substrate of low crystallinity index and high specific surface. Ball-milling, two-roll milling, hammer-milling, colloid-milling, vibrato energy-milling and extrusion are the representative methods ^(91, 161, 225, 177, 81). In this study, the lignocellulose material was treated in laboratory porcelain ball milling to reduce the sizes to below 2 mm.

Physical pre-treatment: Increase in pore size and partial hydrolysis of hemicelluloses (steaming), extensive depolymerization (irradiation), depolymerization and oxidation or dehydration (pyrolysis). The commonly used methods are steaming, wetting, pulping, freezing/thawing, irradiation and pyrolysis ^(184, 159, 114).

Chemical pre-treatment: Modification of the structure of the lignocellulose, reduction in crystallinity and increase in surface area. The representative agents are swelling agents (NaOH, NH₃), dilute acids (HCl, H₂SO₄, H₃PO₄), organosolve (such as methanol, ethanol, butanol, phenol); cellulose-dissolving solvents, aprotic solvents and metal complexes ^(184, 17, 211, 264, 253).

2.2.3 Lignocellulose biotransformation/degradation/hydrolysis

Actinomycetes and fungi are known to be able to degrade cellulose and other organic plant constituents, besides lignin, and to form numerous phenolic and hydroxyl aromatic acids ^(212, 165). Hemicellulose degradation involves enzymes for both main-chain and side-chain degradation, though little is known of the details, largely because the hemicelluloses themselves are so poorly understood and model compounds are generally not yet available. The degradation of each of these classes of substrate can be better understood with the availability of individual enzymes, in a pure form, made by genetic engineering methods.

As a natural substance, lignocellulose is even more complex than lignin. Nevertheless, lignocellulose degradation is a unitary problem because the materials involved are as a first approximation similar to each other. It is the interactions between degradation organisms and the substrate that must be studied - analogous to the cases of microbe-plant and microbe-animal host interactions. This involves studying the synergy between different enzymes attacking different components, and indeed between those attacking individual components. Therefore, it is useful to understand the individual degradation process of the particular constituent before the overall degradation fundamentals about lignocellulose can be established.

2.2.3.1 Cellulose enzymatic hydrolysis/bio-degradation:

The cellulose in lignocellulose is a much larger resource for the breakdown to sugars than starch. It also represents a much more complex problem because of its crystallinity and its mixture with hemicellulose and lignin. The molecular structural features of cellulose, the elementary fibril and the microfibril are important for the enzymatic degradation of cellulose. It is well known that both prokaryotic and eukaryotic systems show that the degradation of

cellulose involves a number of enzymes; in some organisms a large number are involved ⁽¹⁵⁾. Generally, the amorphous regions in the fringe micelles are attacked first in a partially crystalline cellulose fibril, leading to an enrichment of crystalline regions, which are, in turn, gradually solubilized after the loosening of their peripheral parts. Most of the work on its bio-degradation has been done with the filamentous fungus *Trichoderma reesei* (*T. reesei*)⁽⁵¹⁾ in which the cellulase is a complex of at least eight proteins that act synergistically and are subject to complex controls ⁽⁷⁵⁾. *T. reesei* produces five or more endo β -glucanases which convert the crystalline cellulose to oligocellodextrins that amount to 20-35% of the protein by weight. In addition, there are at least two cellobiohydrolases components (65-80% of total protein) which convert these to cellodextrins and cellobiose. Both of these two enzymes are subject to end product inhibition by cellobiose. Finally, there is a cellobiose that converts the cellodextrins and cellobiose to glucose and its actions are inhibited by glucose. The whole complex is also subject to catabolic repression by glucose.

2.2.3.2 Lignin bio-degradation

Fungi are considered to be the most important group of micro-organisms responsible for the cleavage of lignin. The initial step in lignin degradation by fungi involves the release of the dilignol components guaiacylglycerol- β -coniferyl ether, pinorexinol, and dehydrodiconyferyl alcohol, and the formation of primary phenylpropane (C_6-C_3) units. Among phenolic aldehydes and acids released by further decomposition of dilignols are, for example, guaiacylglycerol, coniferyl alcohol, coniferaldehyde and ferulic acid. The C_6-C_3 units also undergo oxidation in the side chains to yield a variety of low-MW aromatic acids and aldehydes, including vanillin, vanillic acid, syringaldehyde, syringic acid, p-hydroxybenzoic acid, protocatechuic acid and gallic acid. Additional OH groups may be introduced and decarboxylation may also occur during this stage, whereas the fungi involved appear to have a limited capacity for the aromatic ring cleavage of lignin.

Phenolic products are not stable, and may be subject to oxidative conversion to quinones, which may occur either chemically, e.g. in alkaline media, or more likely operated by polyphenoloxidase enzymes. The consequent, prevalently enzymatic, oxidative polymerization of mono-, di- and trihydroxyphenols, quinones and aromatic acids may occur in the absence or presence of amino acids, peptides, proteins, and amino sugars, which may

undergo condensation reactions with quinones, and thus be covalently incorporated into humic macromolecules. Oxidative polymerization also involves several other side reactions, including demethylation, oxidation of aldehyde components to carboxylic acids, decarboxylation, hydroxylation and coupling of various intermediates.

In natural terrestrial habitats (e.g. soils and compost), lignin is degraded by a complex microflora which includes both eukaryotic and prokaryotic organisms, such as white-rot fungus, *Phanerochaete chrysosporium* (*P. chrysosporium*), because of the high lignolytic activities which it exhibits. Mineralization of lignin by white-rot fungi is an established enzymatic process which occurs during the secondary phase of growth under conditions of nitrogen or carbon starvation and is mediated by a number of different enzymes, of which lignin peroxidases and Mn^{2+} dependent peroxidases are the most important ⁽¹³⁶⁾.

The following are major facts known about the degradation of lignin by white-rot fungus *P. chrysosporium*⁽¹³⁶⁾:

- 1) Overall, it is an oxidative rather than a hydrolytic process. Since no enzyme mechanism has as yet been identified, one hypothesis was that lignin degradation depended upon the production of free radicals (e.g. single oxygen or hydroxyl radicals) that would effect a chemical degradation ⁽¹¹²⁾.
- 2) Lignin degradation is not inducible by lignin itself but is triggered by severe carbon or fixed nitrogen limitation ⁽¹³⁵⁾. The biological rationale is that lignin is degraded as a stress response, so that the organism can obtain access to further sources of nutrients and energy previously made inaccessible by the presence of the lignin. Note that lignin does not contain nitrogen.
- 3) The discovery of lignin peroxidases and extracellular haem-containing enzymes, which can cleave model dimers representing some of the different bond types present in lignin (*P. chrysosporium* strain BKM-F-1767 produces a family of such enzymes ⁽¹³⁶⁾, immediately raised the issue of how the H_2O_2 that they require is generated. This could be derived from the breakdown of carbohydrates and/or of the products of lignin degradation and are likely to be coupled to the degradation of cellulose and/or hemicellulose.
- 4) As a development of the discovery of lignin peroxidases, a mechanism involving the formation of substrate radical intermediates was proposed ⁽¹³⁶⁾; such radicals might invade

the lignin molecule and be the immediate effects of its degradation. A mechanism of this type would account for the range of bonds cleaved, and makes these enzymes of particular interest.

- 5) A second class of enzymes, the manganese peroxidases, oxidizes Mn(II) to Mn(III) and it is proposed that Mn(III), chelated to organic acids, functions as an active radical that can mediate the oxidative depolymerisation of lignin in wood ⁽⁹³⁾.
- 6) Since peroxidases are implicated in the polymerisation as well as the depolymerisation of lignin, it has been particularly important to establish whether lignin peroxidases and Mn peroxidases do indeed have any in vitro depolymerising activity ^(113, 257).
- 7) *P. chrysosporium* and *T. reesei* clearly have different strategies of action on lignocellulose since *T. reesei* does not attack lignin. The present model for lignin degradation in *P. chrysosporium* is that the system consists of manganese and lignin peroxidase enzymes together with the H₂O₂ generation system that they require, and that this is coupled to carbohydrate breakdown.

Actinomycetes are another group of organisms that can degrade lignin materials, although little is known about their lignocellulose-degrading mechanism ⁽²⁵²⁾. Actinomycetes are Gram-positive bacteria which can be found on almost every natural substrate including soil and composts, freshwater basins, foodstuffs and the atmosphere ⁽²⁵⁵⁾. Their hyphal growth is well suited for the colonization of plant biomass and they secrete a range of enzymes active against lignocellulose ⁽¹⁶⁷⁾. Even so, bacterial rates of lignin degradation compare poorly with the white-rot fungus *P. chrysosporium*. This difference in lignin degradation rates between fungi and bacteria is probably due to different strategies of lignin degradation developed by these two groups of micro-organisms. Early studies ⁽¹⁹⁷⁾ indicated that streptomyces strains, in addition to being able to degrade the cellulose from lignocellulose and thereby contributing to a substantial weight loss of the substrate, is also able to degrade lignin, although to a much lesser extent. Organisms of several genera, *Streptomyces*, *Micromonospora*, *Thermomonospora* and *Actinomadura*, were identified as being able to grow on grass lignocellulose ⁽¹⁶⁸⁾. The soluble product of lignocellulose has been termed as acid precipitable polymeric lignin or APPL ^(53, 198) because it is insoluble when the pH of the medium is lowered. It has been particularly characterized using high performance liquid chromatography (HPLC) ^(26,164) and nuclear magnetic resonance (NMR) spectroscopy ⁽¹⁶⁹⁾.

Element analysis confirmed that pronounced chemical and structural changes in the lignin-carbohydrate polymer had occurred during degradation; these included oxidative reactions and the removal of methoxyl groups from lignin. Gel chromatography of the lignin-carbohydrate components showed solubilisation of lower molecular size components, particularly by *S.cyaneus* and *T. mesophila*.

It is known that many actinomycetes can utilize aromatic substrates, including lignin-related phenols. Some can perform transformation that includes hydroxylations, demethylations and aromatic ring cleavages. Lignin degradation by actinomycete is not as well studied as lignin degradation by fungi, mainly because lignin solubilization and degradation have been more difficult to demonstrate with the available techniques than with the fungal system. However, it is a promising system, which is significantly different from the *P. chrysosporium* system, and where different strains have different characteristics. Parallel studies of lignin and actinomycetes might provide interesting comparisons and suggest avenues of exploitation utilizing these differences. If the aim is the complete destruction of the lignin component of lignocellulose, then the choice of a fungal system would seem obvious. However, such degradation of lignin only occurs during the idiophase, following severe nitrogen and carbon depletion. In contrast, with actinomycetes, lignin solubilization occurs during primary growth and has no requirement for the presence of H₂O₂. Thus although lignin degradation may have more limitations, it may have the advantage of making the soluble products of lignin degradation available rather than totally decompose it to CO₂.

Although an arylglycerol- β -phenol ether was readily degraded by various actinomycetes, the presence of a substitute on the phenyl ether moiety inhibited or completely prevented its breakdown.

An important step towards elucidating the system in actinomycetes was to develop a cell-free system ⁽¹⁶⁹⁾. It was shown that supernatants from a *T. mesophila* strain yielded a soluble product that was a complex of lignin, carbohydrates (especially pentoses derived from the hemicellulose) and protein. Chemical analysis and solid-state-¹³CNMR spectroscopy both indicated similarities with HA, suggesting that actinomycetes might play an important role in the process of humification. The NMR spectra revealed an increase in the number of α -aryl ether linkages, one of the most common linkages in lignin, but there was no evidence of

extensive demethoxylation. It was found that *T. mesophila*'s solubilizing activity can be induced by either xylan or ball-milled straw, but not to a significant extent by indulin AT (an industrial lignin). An obvious possibility is that solubilizing activity is primarily xylanolytic in nature.

2.2.3.3 Lignocellulose degradation

Because of the chemical and structural complexity of the lignocellulose substrate, a description of the overall process of lignocellulose degradation doesn't yet exist for any substrate or organism. Thus, the degree of solubilisation and modification of the different components, the further fate of material that is solubilized and to what extent particular fungi or actinomycetes have similar effects on the lignocellulose substrate, are not known at the chemical level.

Lignocellulolytic enzymes comprise families that fall into two categories: hydrolytic and oxidative. Enzymes of the former category degrade cellulose and hemicellulose and each has a narrow range of substrate species. In contrast, the oxidative enzymes are non-specific and act via non-protein mediators. Cellulose degradation occurs during primary metabolism and, under laboratory conditions lignin mineralization has been characterized as a secondary metabolic (idiophasic) event, triggered by starvation for C, N or S ⁽¹³⁶⁾. The crystallinity and the mixture of cellulose with hemicellulose and lignin make the enzymatic attack on the lignocellulose more difficult than that on other glucose-based polymers such as starch since lignin impedes enzymatic and microbiological access to the cellulose.

Three actinomycetes (*Streptomyces* sp. EC22, *Streptomyces viridosporus* T7A and *Thermomonospora fusca* BD25) were assessed for their ability to degrade ball-milled wheat straw ⁽²⁴⁶⁾. All gave maximum levels of solubilized lignocellulose products at the beginning of the stationary phase of growth (72-96h). Low molecular-mass aromatic compounds extracted from the acid precipitable polymeric lignin were analyzed by reverse-phase and gas chromatography. P-coumaric acid (4-hydroxycinnamic acid), protocatechuic acid (3,4-dihydroxybenzoic acid), gallic acid (3,4,5-trihydroxybenzoic acid), gallic acid methyl ester (methyl-3,4,5-trihydroxybenzoate) and 4-methoxyphenol were recognized. The infrared spectra of the three strains were similar to the spectra of HAs, with all APPL extracts showing

carbonyl, amino, carboxyl, aliphatic and aromatic group vibrations. Peptide linkages of proteins were also detected. The results suggest a role for actinomycetes in the formation of humic substances in soils and composts.

In principle, there are at least three routes to improve the degradation performance of lignocellulose, which can be used separately or in unison. Firstly, growth conditions can be optimized, whether for individual activities, e.g. extracellular lignin peroxidase production, or for the overall process of mineralization of lignin to CO₂. For instance, the conditions under which lignin, cellulose and hemicellulose are degraded simultaneously were identified by using ball-milled straw as substrate and sodium acetate as buffer, pH 6 ⁽²³⁾. Secondly, mutations can be introduced. This has been done for cellulase-deficiency ^(73, 74). Cellulase-deficient mutants that are still able to degrade lignin (perhaps obtaining the required H₂O₂ from hemicellulose degradation) might give a nutritionally super feed for cattle since the cellulose is preserved. Another class of mutant (that would require the development of transformation methods) would be to couple lignin peroxidase expression to an inducible promoter such as that for a cellobiohydrolase I (CBH I) gene. Thirdly, one can exploit the genetic diversity of *P. chrysosporium* strains and use of their basidiospore-derived progeny in a programme of crosses. The latter approaches are mainly made at molecular level.

It was found that optimal lignin mineralisation and optimal levels of extracellular lignin peroxidase are produced under different growth conditions. Indeed, under the conditions used for optimal lignin degradation, when measuring ¹⁴CO₂ release from ¹⁴C-labelled DHP or (¹⁴C-lignin) lignocellulose (dimethyl succinate as buffer, pH 4.5, and 2% glucose), no extracellular lignin peroxidase activity is found ⁽³³⁾. However, this result may arise from the entrapment of lignin peroxidase in polysaccharide slime, which is abundantly produced in the high glucose media found to be optimal for lignin mineralisation. Thus, the measurement of extracellular enzyme levels may not be a good measure of the amount of enzyme available for lignin degradation and it may actually be misleading.

Lignin breakdown and utilization are still primarily based on chemical engineering approaches ⁽²⁰³⁾ with the lignin being recovered in polymeric form. The achievement of this step by biological methods would require the efficient release of the cellulose and hemicelluloses, and then the lignin might in principle be recovered either in polymeric form

or as monomers that could be converted into useful chemicals. In nature, the function of lignin solubilization may be primarily to allow the degrading organism to obtain access to the carbohydrates since the energetic investment in lignin solubilization is rather substantial. Thus, the basidiomycete white-rot fungus, *P. chrysosporium*, will only degrade lignin if it is depleted for carbon or nitrogen sources⁽¹³⁵⁾.

2.3 Lignocellulose decomposition in landfill

A landfill is a disposal alternative for municipal solid waste (MSW), which typically comprises 45% to 60% cellulose and hemicellulose in dry weight⁽¹¹⁾, as well as certain industrial wastes, water and waste water treatment sludge, and agriculture residues. Cellulose and hemicellulose are the major biodegradable constituents of MSW. A complex series of biological and chemical reactions begin with the burial of refuse in a landfill, and landfills represent an active anaerobic ecosystem. The decomposition of lignocellulose to methane in sanitary landfills is a microbial mediated process that requires the coordinated activity of several trophic groups of bacteria in a similar pathway that has been documented to occur in other anaerobic ecosystems.

However, when lignocellulose refuse is placed in a landfill, biological decomposition resulting in methane formation as described above does not occur immediately. A period ranging from months to years is necessary for the proper growth conditions and the required microbiological system to be established. A number of factors, including moisture content and moisture flow, pH, particle size, inoculation, nutrient concentrations and temperature have been shown to influence the onset and rate of methane production, and the two variables that appear to be most critical in controlling refuse methanogenesis are moisture content and pH. Adequate moisture and a pH around neutral are required for refuse methanogenesis. While mixed refuse contains all of the microbes required for refuse decomposition, they are undoubtedly not well distributed among all of the degradable components of refuse. Leachate recycling and neutralization have been shown to enhance the onset and rate of methane production in laboratory-scale tests.

There are five distinct but closely related phases (phase1-5) in the degradation of municipal solid waste^(200, 201). Accordingly, landfill activity may be conceived to commence with an

initial lag or adjustment phase (phase 1) which endures until sufficient moisture has accumulated to stimulate reaction opportunity and produce measurable quantities of gas and leachate. Thereafter, microbial mediation encourages further waste conversion and stabilization through several more or less discrete and sequential phases, each varying in intensity and longevity according to prevailing operational circumstances. Hence, the initial adjustment phase is followed by: a transition phase (phase 2) when the indicated field capacity is reached; the leachate and gas formation phase (phase 3) when the volatile organic acids (TVFA) become predominant with a decreased pH, elevated leachate strength (COD), and high mobility of ionic species; a methane fermentation phase (phase 4) accompanied by further production and conversion of intermediates to methane and excess carbon dioxide, reduced leachate strength, a rise in pH, low ORP, accelerated complexation and reduction of ionic species; and a final maturation phase (phase 5) when nutrients often become limited, less of the available substrate is degraded, gas production decreases, and both organic and inorganic constituents of the leachate are characteristic of post-stabilization conditions. From phase 1 to phase 2, both oxygen and nitrate are consumed, with soluble sugars serving as the carbon source for microbial activity. All of the trophic groups required for refuse methanogenesis (cellulolytic, acetogens, and methanogens) are present in fresh refuse, though there is little change in the populations. From phase 3, carboxylic acids accumulated and the pH decreases as a result of an imbalance between fermentative activity and acetogenic and methanogenic activity. There is some cellulose and hemicellulose decomposition in phase 3. The methanogen population begins to increase, and methane is detected. In phase 4, there is a rapid increase in the rate of methane production to a maximum value, a decrease in carboxylic acid concentration, an increase in pH, little hydrolysis of solids and increases in populations of cellulolytic, acetogenic and methanogenic bacteria. The accumulated carboxylic acid is the principal substrate supporting methane production in this phase. In phase 5, the methane concentration, pH, and cellulolytic and methanogenic populations remain at values similar to those in phase 4. Concurrently, the methane production rate decreases with the depletion of carboxylic acids. In addition, as carboxylic acid concentration decreases, there is an increase in the rate of cellulose plus hemicellulose hydrolysis. While acid utilization limits methane production in phases 3 and 4, solid hydrolysis limits methane production in phase 5.

In conventional landfill sites, the organic matter is degraded to methane and carbon dioxides as the final end products by methane production bacteria (MPB). In the proposed gas control

strategy, sulfate is added as an electron acceptor and the degradation of organic matter is carried out under sulfate reducing conditions by sulfate reducing bacteria (SRB). Theoretically the major end products of sulfate reduction are carbon dioxide and sulfide. The leachate along with the produced sulfide, which will remain in the leachate if a high pH is maintained, is collected and treated to convert to sulfate and then recycled back to the top of a landfill site. In addition to the control of methane gas production, sulfate reduction can also enhance the stabilization of waste, which has been suggested by recent research considering the wide range of organic substrates used by SRB along with the thermodynamic and kinetic aspects of sulfate reduction ⁽²²⁸⁾. It could be postulated that the freshly added lignocellulose residues in the landfill also undergo decomposition in the sulfate conditioned inside system of the landfill.

On the other hand, because sulfur-bearing fuels are still used in many industries, large volumes of sulfur are emitted into the atmosphere and "acid rain" is formed. Underground, a large amount of acid drainage from sulfide-ore-mines usually contains high concentration of sulfate ⁽¹⁰⁸⁾. Therefore, it can be deduced that a significant part of the bio-degradation of lignocellulosic biomass in the earth's biosphere is carried out in sulfate-dominant habitats.

Most studies on landfill bio-degradation concentrated on phase 4 where the typical anaerobic ecosystems are populated. However, little is known about the microbial composition and the degradation of lignocellulose in landfill before phase 4 under the sulfate leaching conditions. To be aware of the bio-degradation processes of lignocellulose in these sulfate acidified habitats is thus desirable.

2.4 HA formation, decomposition and further treatment

2.4.1 HA formation and decomposition

When lignocellulose undergoes biotransformation/bio-degradation by micro-organisms, humic substances (HS) will be formed as a result of re-polymerization of intermediary degradation products.

Nowadays, it is generally accepted that the major building blocks of HS originate from polyphenols of lignin or are synthesized by micro-organisms. HS are believed to form through a pathway in which the first step consists of the breakdown of all plant biopolymers, including lignin, into their monomeric structural units. Possible sources of phenols utilized in the HS formation include plant lignin, glycosides and tannins, and microbial synthesis. According to the hypothesis of Flaig et al. ⁽⁸⁶⁾, after lignin is freed from its linkage with cellulose during the decomposition of plant residues, the side chains of its building units are oxidized and demethylated, yielding polyphenols that are converted to quinones by polyphenoloxidase enzymes. Quinones that originate from lignin, and possibly from other sources, then react with N-containing compounds and polymerize to produce humic macromolecules of increasing complexity.

Based on their solubility in acids and alkalis, HS can normally be divided into four fractions: 1) humins, the portion insoluble in both alkalis and acids, consisting of lipid polymers ⁽⁵⁾, paraffinic substances, fungal melanins, and humic matter insoluble due to its high carbon content; 2) humic acid (HA), the portion that is soluble in dilute alkaline solution and is precipitated upon acidification to pH 2; 3) fulvic acid (FA), the portion that is soluble at any pH value, even below pH 2; 4) hymatomelanin acid, the alcohol-soluble portion of HA. Except for its highly complex structure against further bio-degradation, HS is also stabilized to degradation by covalent binding of its reactive sites to metals ions and clay minerals when it enters the soil.

The HS contributes substantially to improving the global soil fertility functions, including those common to other soil organic matter and soil components and those specific and typical functions which includes slow release of nutrients (such as N, P, S), high CEC, pH buffer capacity, specific physiological effects on plant growth and an extended capacity of interactions with micronutritive and/or microtoxic metal ions and xenobiotic organic molecules such as pesticides. For example, the extracellular oxido-reductases by some wood-degrading basidiomycetes and soil microfungi convert aromatic molecules in aromatic environmental toxicants to action radicals ⁽²²⁶⁾, which spontaneously polymerize or covalently bind to reactive sites of the humus molecule. They become bio-unavailable and thus ecologically detoxified ⁽²⁷⁾.

However, HA does not only have a negative influence on the efficiency of the anaerobic digestion process by rebinding some small readily digestible molecules, thus making them less accessible to the bacteria attack through its complicated aromatic-condensed macromolecular structure; it also presents environmental problems by spreading the ecotoxic compounds such as heavy metal ions and xenobiotics⁽²¹³⁾ because of its solubility in common aquatic environments, its amphipathic characteristics and its highly chemical chelating reactivity. Therefore, further treatment of HA to improve the anaerobic digestion of lignocellulose and to relieve the potential environmental problems from the digestion system is required.

Fakoussa⁽⁷⁷⁾ first demonstrated that microbes, above all filamentous fungi, could solubilize solid particles of low rank hard coal (with similar components as HA). Recently, there were reports of extracellular oxidation and the transformation of solubilized low-rank coal by wood-rot fungi⁽²⁰⁴⁾ and the depolymerization of low-rank coal by extracellular fungal enzyme systems. It was found that the extracellular oxides of white-rot fungi can transform low-rank coal macromolecules; this increased oxygen availability in the shallower 10-ml cultures favors catabolism over polymerization. The wood-decaying fungus *Nematoloma frowardii* b19, which de-polymerizes the high molecular-mass fraction of coal HAs by forming fuvic-acid-like compounds, most effectively bleached the medium supplied with the low-rank coal. Extracellular enzyme activities of oxidases and peroxidases towards 2,2'-azinobis (3-ethylbenzthiazolinesulphonate) were extractable from the agar medium. Presently, Gramss et al.⁽¹⁰⁴⁾ found that fungi, some of which are propagated in contaminated soils to control xenobiotics, metabolize humic extract compounds enzymatically.

In summary, HA can be degraded by some fungi and bacteria, while the fungi decomposition of HAs was mostly carried out by extracellular enzyme activities. Most research work has focused on the degradation of humic-like substances by pure microbial cultures, especially by white-rot fungi such as *Phanerochaete chrysosporium*^(39, 204, 205, 119, 259, 104). However, the degradation characteristics of HAs by the indigenous mixture culture, and to what extent the HAs influence the digestion activities of the indigenous anaerobic consortium, remain unknown.

2.4.2 Ultrasonic treatment of HA

Ultraviolet irradiation has been found to bring about photo-oxidation of humic materials in dilute solution ^(45, 143). Comparatively, ultrasonic irradiation is gaining particular interest because contrary to other chemical (such as acid hydrolysis, oxidation), thermal or physical (such as pyrolysis, x-ray or γ -ray irradiation) decomposition reactions, ultrasonic irradiation treatment is a non-random process where chain scissions near the center of the largest molecules are favoured. It is commonly used to break up macromolecules in solution and has been applied to a wide variety of polymers including both water-soluble and non-water soluble compounds. Cleavage takes place at certain preferential positions close to the middle of the chain, giving rise to polymer fractions of fairly definite molecular size distribution.

The first reported investigation of the degradation of polymers by high-power ultrasound was carried out by Schmid and Rommel ⁽²¹⁴⁾. The ultrasonic degradation of polymers was observed in the frequency range of 20 kHz to 1 MHz in various solvents. In general, the ultrasonic degradation process is a non-random process and the scission of polymer chains in solution occurs at a preferential position. However, details of the degradation process are still obscure. Glynn et al. ^(94, 95) have investigated the ultrasonic degradation mechanism of polystyrene in solution and proposed a general model for the prediction of the molecular weight distribution of degraded polymers.

The ultrasonic degradation of water-soluble polymers has been studied by several workers. Basedow and co-workers ^(12,14,13) have investigated dextran and poly (ethylene glycol), Ohta et al. ⁽¹⁸⁷⁾ dextran and pullulan, whilst Keqiang et al. ⁽¹³⁴⁾ have studied hydroxyethylcellulose and pol(ethylene oxide).

Sonicification (20kHz) behaviour of waxy rice starch in water has been investigated in terms of number average molecular weight (Mn) and molecular weight (MW) distribution evaluated by gel permeation chromatography. The rate of degradation was accelerated at or above the temperature where gelatinization started. High ultrasonic power also accelerated the degradation rate. After long sonication, the average molecular weight becomes the limiting constant value, and the molecular weight distribution tended to be fairly narrow. While observing the degradation curves of other water soluble α -glucans, dextran and pullulan, it

was found that branched α -glucans have larger limiting molecular size than unbranched ones⁽²⁶⁹⁾.

In addition, ultrasonic irradiation has been applied as an advanced oxidation technology for water treatment. The uses of ultrasound for the destruction of dilute aqueous solutions of some organic compounds (alcohols, ketones, aldehydes and phenol) at ambient temperatures were reported⁽²⁴⁵⁾. Some studies have been done on the application of ultrasound to the degradation of contaminated substances in water. Polycyclic aromatic hydrocarbons, parathion, geosmin, diverse phenols, hydrogen sulphide, chlorinated hydrocarbons and chlorofluorocarbons have for example been investigated.

Sonochemical decomposition of a series of hydroxybenzoic acids such as monohydroxy-, 3,4-dihydroxy-, 3,4,5-trihydroxybenzoic acid, tannic acid and reagent and prepared HAs in water under argon or air atmosphere was investigated⁽²⁷⁰⁾. It is suggested that, in the sonolysis under argon, the main sonochemical decomposition of the substances proceeds via reactions with $\text{OH}\cdot$ radicals in the bulk solution and that the contribution of thermal decomposition in cavitation bubbles or the interfacial region (between the bubbles and bulk solution) is small. In the sonolysis under air conditions, the role of oxygen was small in monohydroxybenzoic acids but increased with increasing numbers of OH groups substituted on the aromatic ring, suggesting the occurrence of decomposition of polyhydroxybenzoic acids induced by oxygen molecules at the interface. The chloroform formation potentials of 3-hydroxybenzoic acid and HA decreased due to the sonication, but the reduction in the potential was less than the corresponding amounts of decomposition of the initial substances.

(Poly)aspartate(s) and (poly)glutamate(s) are degraded by ultrasonic waves following the general pattern described for vinyl polymers and biopolymers. Cleavage takes place exclusively on the backbone, preferentially on the relatively weaker C-N bonds. As a consequence, the degradation rates increase with the increase in concentration of amide groups in the polymer. Whereas the limiting molecular weight is found to be scarcely dependent on either the irradiation conditions or the chemical structure of the polymer, the degradation rate is significantly affected by the size and shape of the side chain. Specifically, long linear alkyl side chains appear to be highly effective in accelerating the breaking process. Degradation is also affected by the presence of hydrogen and disrupting compounds, particularly when concentrations reach the values at which the helix-to-random coil transition

takes place. Ultrasonic degradation has been proved to form (poly) β -L-aspartate(s) and (poly) γ -L-glutamate(s) without altering the chemical constitution of the polymer⁽¹⁷⁹⁾.

Upon ultrasonic irradiation, organic compounds in water are degraded via several mechanisms. Three main pathways, which involve hydroxyl radical oxidation, pyrolytic degradation and supercritical water reactions, have been proposed. In the case of aqueous solution, water vapour present in the bubble is homolytically split to yield $H\bullet$ and $OH\bullet$ radicals. Chemical substrates present within the vapor phase or in the nearby liquid of the collapsing bubbles are subject to direct attack by the $OH\bullet$ radical. Volatile compounds go into the gas phase (i.e, into the gaseous bubble within the aqueous solution) and undergo direct pyrolysis. Furthermore, it has been found that hydrolysis reactions are accelerated by several orders of magnitude in the presence of ultrasound. These accelerated reaction rates have been attributed to the existence of transient supercritical water during ultrasonic irradiation. The chemical effects of ultrasound are due to the phenomenon of acoustic cavitation. Sound travels through a liquid as a wave consisting of alternating compression and rarefaction circles. If the sound wave has a sufficiently high pressure amplitude, it can overcome the intermolecular forces bonding the fluid. As a result, the liquid will break down and voids will be created, i.e. a cavitation bubble will be formed. In most liquids, cavitation is initiated at pre-existing microbubbles or weak spots where there are any type of inhomogeneity in the fluid. The inhomogeneity can be anything from particles to gas nuclei. These microbubbles grow sequentially during the compression and rarefaction cycles due to the phenomenon of rectified diffusion until they reach a critical size; in subsequent compression cycles, these cavities can collapse violently, releasing a large amount of energy. This rapid implosion is accompanied by an adiabatic heating of the vapour phase of the bubbles, which yields localized but transient high temperatures and pressures.

In aqueous sonochemistry, three different reaction sites have been postulated: (1) Interiors of collapsing cavities where temperatures of several thousand degrees and pressures of hundreds of atmospheres have been reported to exist. Water vapour is pyrolyzed to $OH\bullet$ radicals and hydrogen atoms, and gas-phase pyrolysis and/or combustion reactions of volatile substances dissolved in water take place. (2) Interfacial regions between the cavitation bubbles and the bulk solution. Although the temperature is lower than in the bubbles, a high temperature with a high gradient is still present in this region. Locally condensed $OH\bullet$ radicals in this region have been reported. (3) Bulk solution at ambient temperature where reactions of $OH\bullet$ radicals

or hydrogen atoms that survive migration from interface may occur. Recently, the role of supercritical water during cavitation has been reported. Using free radicals formed due to chain scission, the end-functional groups can be introduced; they are very useful for the more reaction sites of the HA molecule.

2.4.3 Adsorption of HAs on preformed aluminium hydroxide flocs with the aid of ultrasound

As an alternative to lessen the negative influences of HAs on the environment, removing HAs from the aquatic environment by using common water treatment methods (by absorption on preformed aluminium hydroxide flocs) was promising in practice.

In water treatment, flocculation and/or coagulation with aluminium and ferric salts have been widely used for many years to remove natural organic matter (NOM) including HAs^(234, 125, 166). However, extensive knowledge of this in situ water treatment process was limited by the fact that the adsorption of NOM to aluminium hydroxide flocs during a conventional coagulation/flocculation process is very complex, with flocculation of aluminium hydroxide particles leading to the formation of larger flocs and the adsorption of NOM molecules on flocs going on simultaneously. Therefore, the efforts to optimize this aspect by performing the adsorption of NOM on preformed aluminium hydroxide flocs⁽²⁸⁾ were necessary. It was suggested that NOM properties such as hydrophobicity, size and charge density all affect the adsorption to aluminium hydroxide floc and the extent of solubilization of aluminum; the strong hydrophobic acids (i.e., humic and fulvic acids) had greater adsorption affinities towards the aluminium hydroxide floc; the larger molecules had greater adsorption affinity on aluminium hydroxide than the smaller molecules.

With the art of aluminium slat-flocculation and/or coagulation for NOM removal, it is widely accepted that adsorption of NOM to crystalline aluminium hydroxide solids occurs through surface complexation or ligand exchange⁽⁵⁹⁾. Studies with freshly formed aluminium hydroxide precipitate suggested that surface complexation also plays a major role in aluminium coagulation⁽¹²⁵⁾. Freshly precipitated aluminium hydroxide has a high density of amphoteric surface hydroxyl groups. The reactions between NOM and the surface hydroxyl groups on aluminium hydroxide can be described by either ligand exchange reactions where

the surface hydroxyl is displaced or by ionpair formation with protonated surface sites or by a multistep process combining elements of both ^(193, 145, 60). In humic matter containing raw waters, efficient removal of organic colour and organic carbon is accomplished by coagulation with these metal ions at slightly acidic conditions, e.g. pH 5-5.5 for Al^{3+} ^(70, 69, 162). The mechanism involved is considered to be the stoichiometric reaction between the poly-anionic humate colloids and dissolved polycationic hydrolysis species of the metal ions.

Humic macromolecules are subject to complex aggregation and dispersion phenomena in aqueous media, which are of relevance to their physical behavior and chemical reactivity. The degree of irregularity and roughness of exposed surfaces of humic macromolecules is expected to have an important influence on the number, type, and availability of chemically and physically reactive sites of the HA molecule; on the adsorption capacity of HA; and on the extent of interactions between HA and metal ions on or in the aluminium hydroxide structure ⁽²⁷⁹⁾.

Much of the reactivity of the HAs in solutions is attributed to their functional groups that contain oxygen, including carboxyl, carbonyl, phenolic and methoxyl, carboxyl and phenolic groups. These groups provide most of the negative charge that adds to the mobility of humic substances in the environment, and are believed to react with metals ^(71, 9).

In HAs containing waters of high buffer capacity, the flocculation pH is very often close to the neutral point, where the predominant formation of insoluble hydroxide is expected, including the "sweep flocculation" due to the enmeshment of particulate matter by the voluminous hydroxide precipitate ⁽⁷⁾. Dissolved organic compounds are removed primarily by sorption onto the hydroxide, based upon several possible mechanisms of adsorption, such as weak physical sorption, ion exchange or surface complexation. Humic substances are well-known natural acids, able to form multi-dental chalet complexes with inorganic cations thus influencing the molecular specification of trace metals significantly ⁽²¹⁰⁾.

Therefore, the adsorption of HAs by aluminium hydroxide depends on the physical and chemical surface characteristics and micro-structures of HA and aluminium hydroxide formed in-situ. Any effects which physically or chemically affect the surface characteristics and micro-structures of HA and aluminium hydroxide could influence the adsorption

efficiency. The ultrasonic irradiation, which was known to promote microstreaming (creating turbulent flow and aiding mass transportation) and acoustic cavitation (producing local high temperatures and pressures as a result of bubble collapse) in heterogeneous systems ⁽¹⁵²⁾, may enhance the flocculation/aggregation efficiency by a variety of physical (erosion, emulsification, aggregation/flocculation) or chemical (the production of radical and excited species; single electrontransfer) ultrasound actions on the properties of HA and aluminium hydroxide flocs or the interactions at the solid liquid interface. So far, the idea to explore the effect of ultrasonic irradiation on HAs removal by aluminium hydroxide is relatively new.

The chemistry of aluminium salts and HAs in an aqueous environment and the effects that ultrasonic irradiation may contribute to the flocculation/aggregation system are complicated. The physical and chemical characteristics and structure of HA are dependent on its origin. Therefore, the removal of HA produced from the acidification fermentation of lignocellulose residues by preformed aluminium hydroxide with introduction of ultrasound irradiation into the adsorption processes needs to be evaluated. The comprehensive surface complexation of HA on preformed aluminium hydroxide applied with ultrasonic irradiation needs to be further investigated.

2.5 Summary

The lignocellulose is a complex of three classes of polymers, namely cellulose, hemicellulose and lignin. Because of the chemical and structural complexity and the intimate association of these components, biological stabilization of lignocellulose substrate to methane and carbon dioxide is naturally a slow and complicated route. The studies on microbial lignocellulose degradation from different laboratories have allowed the formulation of specific biotechnological goals, each adopting its distinctive approach.

A description of the overall process of lignocellulose degradation does not yet exist for any substrate and organism. The degradation pathway and products can be quite different with different micro-organisms and environmental conditions. The general purpose of anaerobic digestion of lignocellulose is to convert complex organic material to methane, thereby reducing the organic solids content of the sludge, its putrefaction, and its pathogen content. Considering the complexity of the chemical characteristics and structure of lignocellulose, the

following aspects of the overall digestion and utilization of lignocellulose are to be emphasized.

- 1) It can be deduced that a significant bio-degradation of lignocellulose biomass in the earth's biosphere is carried out in sulfate-dominant habitats. To enhance the knowledge of the degradation nature of lignocellulose residues in normal solid wastes management practice and to compare it with the controlled anaerobic digestion process, the biological decomposition of lignocellulose in sulfate acidified habitats is to be simulated and evaluated in a simulated landfill reactor.
- 2) The initial hydrolysis step is kinetically critical to start the anaerobic digestion. This step can be promoted by pre-treating which, to some extent, leads to the rupture of the rigid structure of lignocellulose. There are a variety of pre-treatment methods available, the comprehensive treatment methods are economically acceptable.
- 3) A wide variety of intermediary products are formed. Some of these products may polymerize into polydispersed polymers of aromatic and aliphatic units (humic substance), which are more complex and highly condensed than the lignin polymer, thus seriously inhibiting the further utilization/conversion of the hydrolyzed products by anaerobes. The further treatment and investigation of the degradation and/or removal of humic substances, especially for HAs, formed in situ of anaerobic digestion process, are necessary.
- 4) Ultrasonic irradiation is gaining particular interest as a method to decompose macromolecules in solution and has been applied to a wide variety of polymers including both water-soluble and non-water soluble compounds. Since the structure of the HAs is heavily dependent on its origin, it is difficult to compare the results obtained by different investigators. An investigation of the effects of ultrasonic irradiation on the physical and chemical characteristics of HAs extracted from the acidification fermentation (anaerobic composting) of lignocellulose residues is to be conducted. The further bio-degradation behaviour of HAs, after ultrasonic irradiation treatment, by indigenous micro-organism from the lignocellulose digester are to be investigated.

- 5) Successful degradation and stabilization of lignocellulose substrate towards methane production is a consecutive microbial process, which involves groups of comprehensive metabolic activities of different functional micro-organisms. In the initial hydrolysis stage, fungi, the particularly efficient degraders of major plant polymers, including actinomycetes, play important roles in solubilizing, transforming and decomposing the large polymers such as cellulose and lignin enzymatically. However, to what extent these initially developed fungi or actinomycetes can utilize the humic substances (e.g. HAs) or affect the growth of the subsequent obligatory anaerobic bacterium is not clear.
- 6) Since the methanogens is highly sensitive to the environmental conditions (such as pH, temperature, toxic components), even when hydrolyzed, efficient conversion of the hydrolyzed substances all the way to methane and carbon dioxide is still hard to achieve in the case of lignocellulose residues, and special consideration must be taken in the configuration design of the digestion process.
- 7) Not the whole lignocellulose can be hydrolyzed and digested. Humus, the indigestible part of the lignocellulose, is considered to have promising applications in soil conditioning after reducing the pathogen content to the satisfactory level.

Chapter 3 Experimental Work

3.1 Lignocellulose substrates and activated sludge

Lignocellulose substrates: Clean grass was collected from fresh turf cuttings on the campus of the University of Stellenbosch in November 1998. It was immediately solar dried, and then sealed in clean plastic bags before the experiment. The tobacco dust, a mixture of tobacco leaves and stalks, was collected from a farm in Paarl in October 1998. It was already solar dried, ground below 2 mm and sealed in thick plastic bags.

Table 3.1 Characteristics of the tested samples

Characteristics	Activated Sludge	Grass	Tobacco Dust
Total Solids (% TS)	24.6	91.2	94.2
Volatile Solids (% of TS)	50.71	80.11	71.97
Ash (% of TS)	49.29	19.89	28.03
Element Composition of Dry Mass			
C (%)	29.3	54.9	43.0
N (%)	3.22	2.44	2.37
NO ₃ -N (mg/kg)	41	10	308
NH ₄ -N (mg/kg)	1245	1221	761
K (%)	1.13	1.51	1.70
Ca (%)	1.78	1.19	4.29
Mg (%)	0.22	0.26	0.70
B (mg/kg)	28	23	26
Cu (mg/kg)	754	13	57
Fe (mg/kg)	1.42	0.29	0.57
Zn (mg/kg)	1068	72	761
Na (mg/kg)	1216	915	288

Activated sludge sample: The activated sludge was obtained from the Stellenbosch sewage treatment plant. This plant is operating on a mixture of domestic and industrial effluent from the town. As there are no heavy industries present in Stellenbosch, the effluent is mostly organic in nature. Bacteriological breakdown of the organic matter in raw sewage is mainly carried out by two aeration reactors. The activated sludge used in this experiment was sub-sampled from the concentrated sludge of the plant. Once sampled, it was sealed and stored under anoxic conditions at room temperature for about three months to allow some anaerobic bacteria to grow before it was combined with the lignocellulose substance as the

microorganism seed and nutrients. Characteristics of the lignocellulose substrates and activated sludge are shown in table 3.1.

3.2 Pre-treatment of lignocellulose substrate

Solubilization tests: The effects of pre-treatment methods were assayed using a modified solubilization test ⁽¹⁶⁾. The tests were performed in 500 ml gas-tight bottles positioned in the water bath at 25°C and 55°C for 5 days and 6 hours, respectively. Every 25 g of pretreated sample was mixed with 250 ml distilled water for five minutes by continuously flushing with oxygen free gas (Helium92%, Nitrogen Balance) ; the bottles were then tightly sealed and put into the temperature-controlled water bath. Tests in triplicate were used for each sample. At the end of testing, 50ml suspensions were collected and centrifuged for 15 min at 3000 rpm. The supernatant was filtered through 0.45 µm (cellulose acetate, Schleicher & Schuell). The total solid (TS) and total volatile solid (TVS) of the filtrates were determined according to Standard Methods ⁽¹⁾. Because the soluble mineral salts in grass and tobacco are marginal, the TVS of the filtrate can be considered to be the same as that of soluble organic matter.

Mechanical pretreatment: In this study, the two-roll milling method ⁽¹⁶¹⁾ was used in a laboratory porcelain milling bottle. The volume ratio of dried grass/tobacco dust and porcelain balls is about 3:2. All the samples were ground below 2 mm (100%) and the fraction between 0.2 mm-0.5 mm (about 40%) was screened for a solubilization test to evaluate the pre-treatment effects.

Physical pre-treatment: In this study, the freezing/thawing method was employed. The ground grass/tobacco dust (-2 mm) with a moisture content of around 50% (by adding distilled water) was frozen overnight in sealed plastic bags. It was then thawed at room temperature and dried in a temperature-controlled oven at 100-103°C for about 2.5 hours. Three freezing-thawing cycles were conducted before the solubilization test was performed to evaluate the pre-treatment effects. At the same time, the identical sample, which had not been frozen, was also dried in the oven under the same conditions. This parent sample was used as the control in the solubilization test to compare the pre-treatment results.

Chemical pre-treatment: In this study, the swelling agent (0.5 M NaOH) was used. This pre-treatment was realized by substituting distilled water with a 0.5 M NaOH solution in the solubilization test.

3.3 Acidogenic fermentation test and reactor

Feedstock: The feedstock was prepared by mixing activated sludge and grass/tobacco dust (2:1 w/w) in a porcelain roller for 2 hours to (1) provide sufficient contact between the fermentation culture (activated sludge) and the substrate (grass or tobacco dust); (2) get homogeneous feed for the reactor. The mixture was then sealed in plastic bags and stored in the freezer as the feedstock.

Laboratory digesters: A batch digester and a two-stage semi-continuous system with leachate recycling were employed in this study. The batch digester, without solid/leachate separation unit, was designed to investigate the effects of hydraulic retention time (HRT) and start-up pH on the fermentation performance, such as oxidation-reduction potential (ORP), pH and volatile fatty acid (VFA) content. The two-stage semi-continuous system with solid/leachate separation unit, was designed to study the effects of mass retention time and leachate recycling on the fermentation performance (ORP, pH, VFA content). Duplicated reactors were used.

The batch reactor tests were carried out in 1-liter gas-tight pressure-tolerant glass bottles. A rough sketch of the reactor is presented in Fig.3.1(a). The reactor temperature was thermostatically controlled by regulating the temperature of the water bath. The experimental procedure is as follows:

- 1) 150 g feedstock was introduced into the reactor
- 2) The proper volume of feeding medium (tap water, radical water, $\text{Ca}(\text{OH})_2$ or NaOH solution) was supplied to achieve a desired initial TS content of the reactor, with the headspace volume of the loaded reactor kept at 500-600 ml.
- 3) The reactor was vacuumed for two minuets before the reactor was flushed and gassed with oxygen-free gas.
- 4) The bottles were submerged in the water bath at a temperature between 50 and 55°C.

Gas production was measured with a wet-gas volume meter. The gas collection apparatus was assembled according to Standard Methods ⁽¹⁾. The composition of the gas, the pH and the ORP of the solution in the reactor were measured daily after the first period of incubation (at least 5 days) or every two to four days depending on the operation status of the reactor. When sampling, the reactor was flushed with oxygen-free gas (such as helium and nitrogen mixture) to prevent the oxygen contamination. The gas composition and VFA content were analyzed by GC. The TS and TVS of the samples were determined according to Standard Methods ⁽¹⁾. The pH and ORP were measured using a PHM 82 Standard pH Meter.

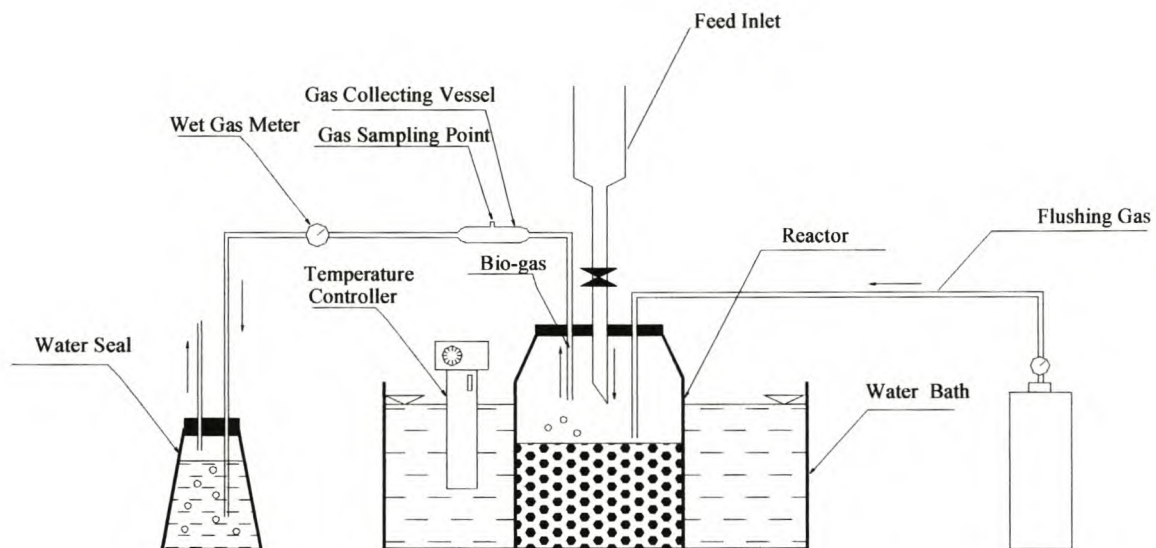


Fig.3.1 (a) Schematic diagram of experimental apparatus (batch reactor)

The two-stage semi-continuous system consists of two identical glass columns (about 1.5 liter) with a water jacket outside and a fine glass sinter at the bottom. The operation temperature of the reactor was controlled by the temperature of the water, which was pumped from the water bath, in the water jacket. This water was recycled continuously. The whole column was wrapped in thermo-insulating cloth to prevent heat from escaping. The set-up of this system is presented in Fig.3.1(b). Each column was started up with 300 g feedstock. Feeding medium of 300 ml was initially introduced through the first column. The leachate from the first column was automatically used as the inoculation medium for the second column. After one week, 10 ml leachate from the second column was sampled and analyzed

for pH, ORP, TS, TVS and VFA content. Then another 150 g of new feedstock were added to each column with the addition of 150 ml feeding medium through the first column. During sampling and loading, the same flushing procedure with oxygen-free gas was conducted to prevent oxygen contamination.

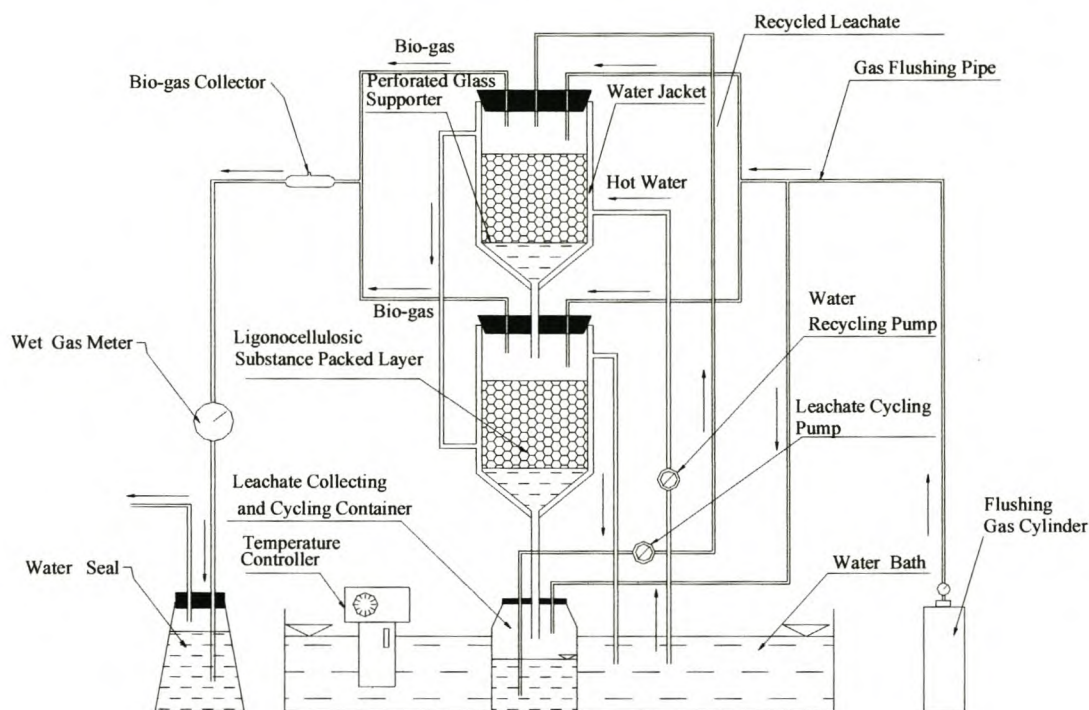


Fig.3.1 (b) Schematic diagram of experimental apparatus (semi-continuous module)

3.4 Two-phase digestion experiment and set-up

The same digestion apparatus was used as in the two-stage semi-continuous acidogenic fermentation system. The collection, pre-conditioning (including pH adjustment, dilution and reduction with reducing agent) of the leachate from the acidification digester was performed in a 1-liter gas-tight glass pressure vessel. The elution of the methanogenesis reactor using the pre-conditioned leachate was realized with a micro-tube pump. The set-up of this system is presented in Fig. 3.2

Experimental Work

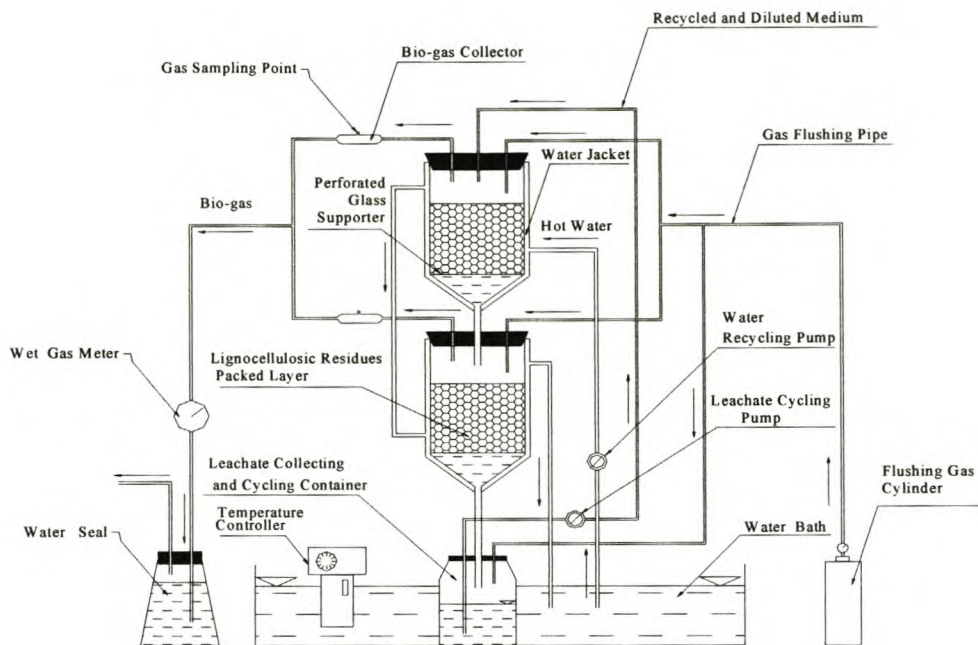


Fig.3.2 Schematic diagram of laboratory setup for two-phase digestion

Developing the acidogenic phase: At the beginning of the acidification digestion, two columns were packed with the same weight (200 g) feedstock. On the third day of digestion, another 120 g and 80 g fresh feedstocks were added into the top and bottom columns, respectively. The digesters were then left running without interruption for 2-3 weeks until the start-up of the methanogenic phase.

Starting the methanogenic process: The methanogenic process started after 2-3 weeks SRT of acidification digestion, operating under mesophilic (35°C - 38°C) condition, semi-continuously eluted with pH/ORP controlled leachate containing 0.25 g/l reducing agent (Cysteine-HCl).

Leachate preconditioning and dilution: The leachate (about 100 ml) from the acidification digester was diluted with tap water to bring the final volume to 400 ml. A certain amount of Cysteine-HCl (0.25 g/l) was added to this diluted solution before performing the pH adjustment using concentrated NaOH (1%) or HCl (1%). Then the solution was purged with an oxygen-free gas mixture (about 80% CO₂ and 20% H₂) and recycled through the digestion

system by the micro-tube pump. Since the leachate producing speeds were generally slow, the micro recycling pump only worked every other hour and was switched off overnight.

3.5 UASB reactor

The UASB reactor is typically divided into four compartments: (1) the granular sludge bed, (2) the fluidized zone, (3) the gas-solids separator, and (4) the settling compartment. The granular sludge bed is located at the bottom of the reactor. The waste water is pumped into the bottom of the reactor and passes upwards through the granular sludge bed. Here the organic compounds are biologically degraded and bio-gas is produced. In the top of and just above the granular sludge bed, a fluidized zone develops because of the production of the bio-gas. In this zone, further biological degradation can take place. The bio-gas is separated from the liquid in the gas-liquid separator. Granules with good settling abilities settle back through the fluidized zone to the granular sludge bed, while flocculated and dispersed bacteria are washed out of the reactor with the overflow; the treated water is discharged out of the reactor as qualified effluent. The thermo-wire or water-jacket is employed to control the digestion temperature of the reactor.

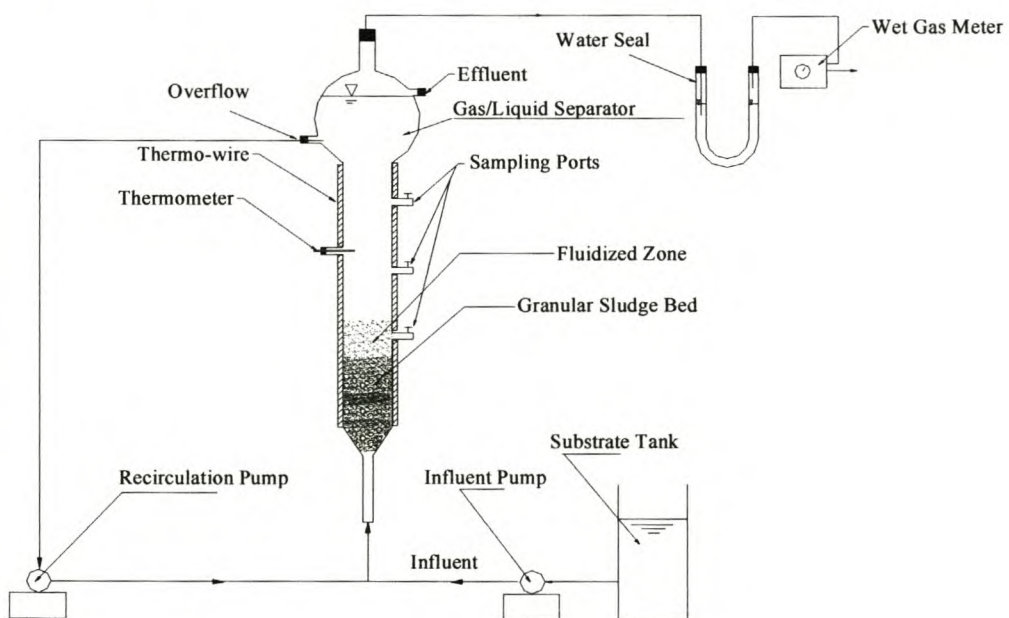


Fig.3.3 Schematic diagram of UASB reactor

A schematic diagram of the laboratory scale UASB reactor is given in Fig.3.3. The reactor (according to the design of the Food Science Department of the University of Stellenbosch) consists of a glass column portion (1.210 liter, 50 mm ID) and a 1.0 liter gas-solid/liquid separator. The total volume of 2.210 liter was used for calculation of the volumetric loading and hydraulic retention time. The reactor was operated mesophilically at about 37°C by a thermo-wire heating system. Bio-gas produced was introduced to a “U”-shaped tube water seal (16mm ID, 380 mm long) and was then measured by a wet-gas meter at room temperature. The gas sample for CH₄ and CO₂ analysis was withdrawn using a “T”-shaped gas-collecting vessel.

3.6 Lignocellulose decomposition in simulated acidified leaching solution (sulphate acidified landfill reactor)

Simulated acidified leaching solution: A sulphate solution of 0.005 M H₂SO₄ (pH around 1.80) was used as an acidified leaching solution. It was prepared from 98% H₂SO₄ (AR) and stored in a 10-liter graded plastic vessel at room temperature.

Because of the long-term degradation process of landfill, the solar dried turf grass was used as the lignocellulose residues throughout this study. Its physical and chemical characteristics are shown in Table 3.1. The seeding agent was collected from the overnight rain-soaked turf grass and was incubated in the simulated landfill tank for three months. A 2.17 kg mixture (3:1 weight base) of solar dried grass (moisture content 8.8%) and seeding grass (moisture content 82.5%) was used to load the simulated landfill tank throughout the research.

Simulated landfill reactor: A 18-liter capacity tank was used as the landfill reactor for this research. A schematic diagram of this reactor is presented in Fig.3.4. The tank was operated at room temperature. A water distribution system was attached to the inside surface of the lid to simulate the acidified leaching.

The acidified leaching solution flowed at a rate of 2.0 l/d from the reservoir into the reactor tank 500 mm below through the distribution system. After five days of simulation, another 10.0 litre simulated acidified leaching solution was added to the reservoir and the leachate collected was continuously recycled back to the reservoir at a rate 2.0 l/d. The first leachate

and gas sample were taken on the 16th day of digestion. Samples were subsequently collected every three weeks. From the second month of decomposition, 15 g of digested sample was collected from different positions of the reactor. One gram of sub-sample from the 15 g sample was used for bacterial enumeration.

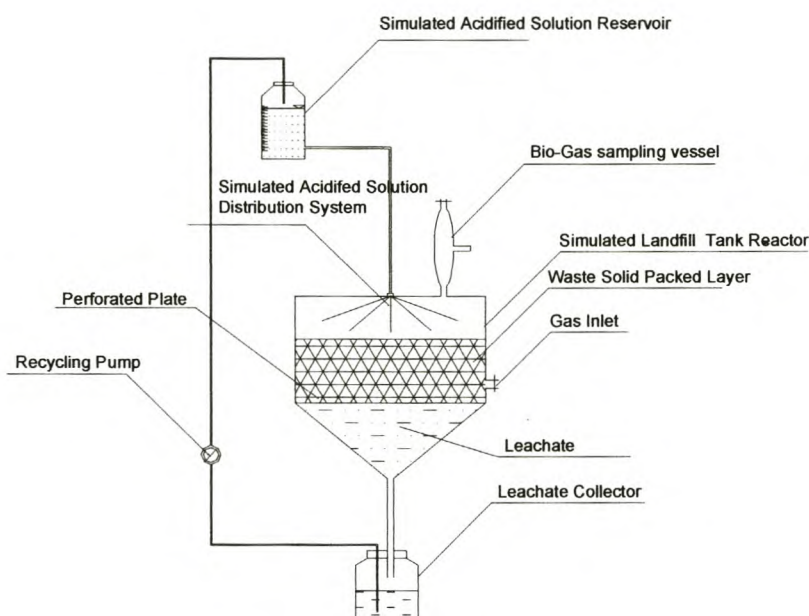


Fig.3.4 Schematic diagram of simulated landfill set-up

3.7 Ultrasonic treatment of humic acids

Humic acid extraction: The humic acids were extracted from the 4-week-anaerobic-thermophilically (50⁰C - 55⁰C)-digested humus from the laboratory digester fed with activated sludge and tobacco dust (TS₀ 21-23%, VTS₀/TS₀ 57.80%). The following extraction and purification steps were used:

- 1) The extraction was performed twice with 0.5 M NaOH under N₂ at a ratio of 10:1.5 (NaOH/Solid) in the 2.5l dark colored rolling glass bottles for 24 h at room temperature.
- 2) The 24 hour-NaOH extracting slurry (pH 11.93 to 12.36, ORP -631 mv to -676 mv) was centrifuged at 5X1000 rpm for 15 min. The supernatant was filtrated by vacuum (Whatman Filtrate Paper No. 542).

- 3) The filtrate was precipitated with 5.85 M HCl at pH 1.07-1.53 and left overnight without any stirring.
- 4) The acidified overnight standing precipitate suspension was centrifuged at 5X1000 rpm for 15 min. The precipitates were washed with 0.1 M HCl and distilled water at a ratio of 2:1(L/S) for 7 times respectively.
- 5) The supernatant and washing solutions (pH 1.20-1.83) were combined and evaporated (around 100 °C) in the dark for 4 days to recover the fulvic acid fraction. The precipitates (humic acids) were immediately freeze-dried (24-36 hours) and stored in the dark at -16 °C.

Ultrasonic irradiation of humic acids (HA): Freeze-dried HA was dissolved in 0.5 M NaOH solution to the concentration of 1.1×10^3 mg/l and the pH was adjusted to 7.33-7.34 with 1 M HCl or 1 M NaOH. Each 100 ml of this neutralized humic acid solution was then diluted by 50% and was treated with ultrasonic irradiation in a 125 ml round bottom vessel (ID 50 mm) using the U50 sonification probe (U50 control KIKA Labortechnik) operating at a frequency of 30 kHz with cycling set at 1.0 and amplitude set at 100%. The ultrasonic irradiation set-up is presented in Fig.3.5. The pH and ORP of the solution were measured immediately after irradiation using a PHM 82 Standard pH/MV Meter).

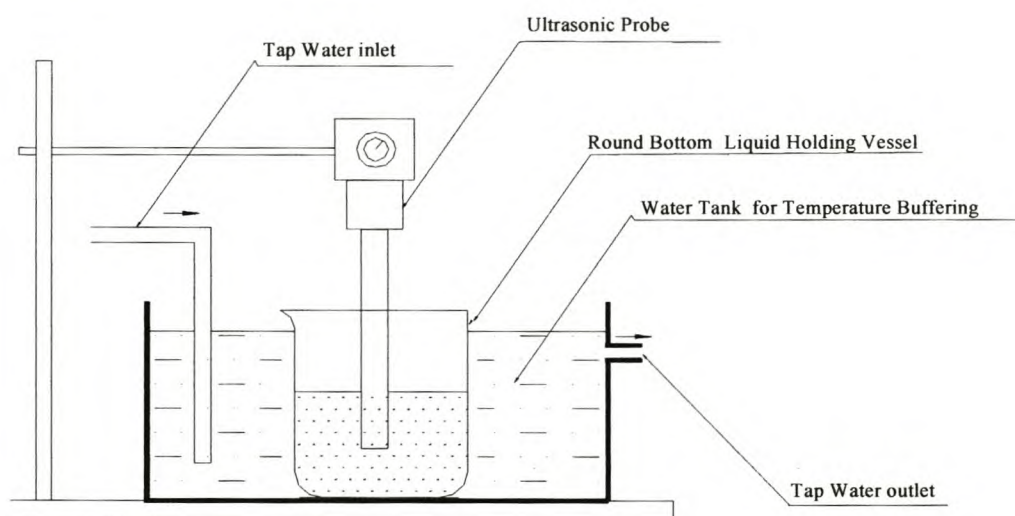


Fig.3.5 Ultrasonic irradiation apparatus

Each 20 ml of treated or untreated solution was filtrated (0.45 μ m cellulose acetate, Schleicher & Schuell) and analyzed for UV-visible absorption within a few hours. The rest of

the treated or untreated slurries were immediately freeze-dried and stored in the dark at -16°C before further analysis.

Organisms selected by using HA as single carbon source: Fungal isolates associated with lignocellulose degradation, bacteria and yeasts from the 10-month simulated lignocellulose residues landfill reactor were first screened on a 0.67% yeast nitrogen base (YNB) agar supplied with 0.25 g/l, 0.5 g/l, 1.0 g/l and 2.0 g/l of HA respectively. The HA was not pre-treated with ultrasonic irradiation and was the single carbon source (YNB-HA agar). The dominant colonies that developed after 3 weeks of incubation were sub-cultured on Malt Extract agar (MEA), which includes malt extract 7.00 g/l, Peptone 1.00 g/l, yeast extract 0.50 g/l and agar 16.0 g/l. Pure cultures were obtained by dilution plating of the dominated colonies on MEA. The bacterial strains were then sub-cultured on tryptone soy agar (TSA), with the fungi strains maintained on MEA.

Organisms selected by ultrasonic irradiation: The ultrasonic treatment of microorganisms was conducted on 25 ml of a 10-fold diluted biomass from the above- mentioned landfill reactor using a U50 sonification probe (U50 control KIKA Labortechnik) operating at a frequency of 30KHz with cycling set at 1.0 and amplitude set at 100%. The serially diluted culture suspensions were then plated on the rose bengal chloramphenicol agar; the predominant fungi and yeast colonies were sub-cultured and purified on malt extract agar plates on which bacteria growth was not detected.

All the fungi and yeast colonies for strain identification were maintained on 2% MEA at room temperature (around 20°C).

Fungal culturing and degradation: Two kinds of medium, 6.7g/l YNB medium plus 1.0 g/l HA as carbon source (YNB-HA) and modified Czapek-Dox medium (Czapek-Dox-HA) (containing 1.0 g/l HA, 0.05 g/l yeast extract, 0.5 g/l NaNO_3 , 1.0 g/l K_2HPO_4 , 0.5 g/l $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 g/l KCl, 0.01g $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ g/l) were used. Both HA with ultrasonic irradiation and HA without ultrasonic irradiation were added to the medium and autoclaved. The pH of the medium was adjusted to 6.5-7.0 to ensure the HA added in the medium was soluble. Then a loopful (3.0 mm ID) of screened fungal spores was aseptically inoculated into a 100ml volume of culture medium described above and stored in 1000 ml conical flasks. Another set

of flasks with the same medium were left un-inoculated to serve as sterile controls. The inoculated flasks (duplicates) and sterile controls were placed in a box with beakers of water to ensure a proper humidity of the air in the container and was incubated at 28 °C in the dark. At weekly intervals, 10 ml of the mycelia-free culture fluid was aseptically taken from each flask with a sterilized pipette. By using membrane filtration (pore size 0.45 µm, acetate cellulose, Schleicher and Schuell) to remove the fine mycelia (hyphae) in the filtrate, changes in the mycelia-free filtrate in absorbance at 340 nm, the FBB reactivity and the presence of reducing sugars were determined.

Bacteria, yeast culturing and degradation: A 6.7g/l YNB medium plus 1.0 g/l HA as carbon source, modified TS medium (TS-HA) (containing tryptone 17 g/l; soy peptone 3.0 g/l; sodium chloride 5.0 g/l; di-potassium hydrogen phosphate 2.5 g/l, HA 1.0 g/l) were used to monitor the degradation of HA by yeast and bacteria isolates individually. Ten milliliter of medium in 100 ml conical flasks were each aseptically inoculated with a loopful (3.0 mm ID) of bacteria or yeast colonies. Another set of flasks with the same medium was left uninoculated to serve as the blank controls. The cultures (duplicates) and sterile controls were placed in a container with beakers of water inside to maintain a proper humidity of the air in the container, the container was incubated at 28 °C in the dark for 3 weeks. At the end of 3-week-incubation, changes in absorbance at 340 nm, the FBB reactivity and the presence of reducing sugars were determined on the cell-free culture fluid (0.22 µm membrane filtration, acetate cellulose, Schleicher and Schuell).

HA toxicity on anaerobic consortia: A HA stock solution (1.0 g/l, pH 7.3-7.5) was aseptically prepared in N₂-phase. The stock solution was aseptically mixed with the modified TYEG medium, which contained 0.1 g/l tryptone, 0.1 g/l yeast extract, 0.1 g/l glucose, 0.3 g/l cysteine HCl, 10.0 ml/l trace elements solution, 10.0 ml/l vitamin solution, 5.0 g/l NaCO₃ and 2.0 ml/l phosphate solution buffer, to give final HA concentrations of 0.1, 0.05, 0.02, 0.01 and 0.00 mg/l and a final volume of 50 ml in 100 ml serum bottles. The bottles were then inoculated with 1.0 ml of anaerobic cell enrichment culture. This enriched culture was obtained from the 1.0-month-old TYEG culture, which contained 1.0 g/l tryptone, 1.0 g/l yeast extract, 1.0 g/l glucose, 0.3g/l cysteine HCl, 10.0ml/l trace elements solution, 10.0ml vitamin solution, 5.0 g/l NaCO₃ and 2.0 ml phosphate solution buffer and was inoculated with 0.1% anaerobic lignocellulose digested sludge from the reactor with 38.5% CO₂ and 49.1%

CH₄ in the headspace. All medium preparations and transfers were carried out under pure N₂ conditions. Duplicate culturing bottles were used, maintained in anaerobic atmosphere with pure N₂ and incubated in the dark at 37 °C for 3 months. The final anaerobic consortium growth in 3-month old liquid culture was monitored by measuring the biomass and by detecting the CO₂ and CH₄ contents in the gas phase of the serum bottles with a GC system.

3.8 Removal of humic acids by preformed aluminium hydroxide flocs with the aid of ultrasonic irradiation

Preparation of preformed aluminium hydroxide flocs. The method to prepare the preformed aluminium hydroxide flocs was the modified methods used by Bose et al.⁽²⁸⁾. 500 ml of standard 200 mg/l aluminium solution made from aluminium sulphate (AR, Merk) using super-Q water, 100 ml of 10 mM sodium bicarbonate solution (AR, Merk, super-Q water), and 350 ml of super-Q water were added into a 1000 ml beaker with the pH of the solution being adjusted to around 7.0 using 0.4 N NaOH or 1.0 N H₂SO₄, and the final volume was then brought to 1000 ml with super-Q water. This solution was rapidly mixed for 4 min at a speed set at 6.0 and then slowly mixed for 30 min at a speed set at 1.0 using a IKA®-WERK RH magnetic stirrer. This gave a preformed aluminium hydroxide floc solution with 100 mg Al/l.

Preparation of humic acid solution: 0.45 g freeze-dried humic acids (HA) were firstly dissolved in 450 ml of 0.5 M NaOH solution, the pH of the solution was adjusted to around 7.0 with 1 M HCl or 1 M NaOH, and the final volume was then brought to 500 ml with 1Mm sodium bicarbonate solution (pH 7). This produced a 900mg/l humic acid stock solution with a pH of around 7.0.

Ultrasonic irradiation treatment:

a) Treatment for preformed aluminium hydroxide floc suspension only: 50 ml evenly mixed preformed aluminium hydroxide floc suspension at slow stirring was transferred into a 100ml glass beaker, on which an immediate ultrasonic irradiation was performed using the U50 sonification probe (U50 control KIKA Labortechnik) operating at a frequency of 30 KHz with cycling set at 1.0 and amplitude set at 100% for 30 to 120 minutes. Ten milliliter of 900 mg/l humic acid stock solution was then immediately introduced to produce a preformed-

aluminium-hydroxide-humic acid suspension, (marked as suspension A), with a humic acid content of 150 mg/l. The control suspension, (suspension A₀), was prepared by using the same amount of untreated preformed aluminium hydroxide floc suspension and humic acid solution.

b) Treatment for preformed aluminium hydroxide floc and humic acid suspension mixture: The required volume of 900 mg/l humic acid stock solution was supplied with proper volumes of evenly mixed preformed-aluminium-hydroxide-floc suspension to bring the total volume of the mixed suspension to 60 ml (marked as suspension B₁-B₄), so that the desired concentrations of humic acid (75, 150, 300 and 450 mg/l) were obtained. The same ultrasonic irradiation procedure as that performed on the preformed aluminium hydroxide suspension was then conducted on these suspensions. The blank controls with the same concentration of humic acids were made by supplying preformed-aluminium-hydroxide-floc suspension with proper volumes of 1 mM NaHCO₃ at pH 7 (marked as solution C₁-C₄).

Adsorption experiments: After ultrasonic irradiation treatment, suspensions A₀-A₄, B₁-B₄, C₁-C₄ were respectively transferred to 125ml screw capped plastic bottles and were continuously mixed on a laboratory roller for 24 hour. As the control experiments, the same procedure was also performed on suspension A₀ and solution C₁-C₄.

3.9 Chemical or physical analytical methods

The TS and TVS of the samples were determined according to Standard Methods⁽¹⁾. The pH and ORP of the leachate was analyzed every other day using a PHM82 STANDARD pH/ORP METER.

VFA analysis: The VFA was measured by gas chromatography (GC); the sample was prepared as follows:

- 1) Centrifuge the sample at 3000 rpm for 15 minutes.
- 2) Filtrate the supernatant using 0.45 µm membrane filtrate paper.
- 3) Dilute one part 35% formic acid solution with three parts of the filtrate sample.

The preferred sample size is 4 ml - dilute 1 ml of 35% formic acid with 3 ml aqueous sample. This sample can be frozen until needed for analysis. The internal standard, hexanol, is added just before analysis.

The analyses were done on a Varian 3700 gas chromatograph, equipped with a flame ionization detector (FID) and a Nukol™ fused silica capillary column (30 x 0.53 mm i.d. and 0.5 µm film thickness) (Supelco, Inc., Bellefonte). The column temperature was initially held at 105°C for 2 min, then increased at a rate of 8°C.min⁻¹ to 190°C. The injector and detector temperatures were set at 130°C and 300°C respectively and nitrogen gas was used as carrier gas at a flow rate of 6.1 ml.min⁻¹. The results were recorded and integrated on Borwin™ (JMBS Developpements, Grenoble) integration software. All results are expressed in mg.l⁻¹ and are the average of duplicate analyses. Only a 10% variation in data was allowed, otherwise the analysis was repeated.

The bio-gas yield was measured using a wet-gas meter; the contents of CO₂, CH₄ and N₂ (during denitrification) of the bio-gas were analyzed using GC (GC 580 with TCD detector, 60/80 carboxen-1000 packing, 15 feet x 1/8 inch stainless steel columns; 45/60 carboxen-1000 packing, 2 feet x 1/8 inch stainless steel column, helium gas was used as carrier gas and the flow rate was set at 30 ml/min at 300 kPa. The column and detector temperatures were set at 100°C and 200°C respectively). With injection, the same volume (0.4 ml) of air, pure CO₂ and CH₄ respectively were injected each time before injection of the sample gas in order to: (1) check air contamination in the sampling vessel; (2) calculate the absolute percentage of the CO₂ and/or CH₄ in the bio-gas of the reactor.

UV-visible absorbances: The changes of HA, resulting from the ultrasonic irradiation, in UV-Visible absorbency at 230 nm⁽¹⁵⁴⁾ and 254 nm⁽⁵⁹⁾ and the changes of cell or mycelia-free HA degradation solutions in UV-visible absorbency at 340 nm were determined with a Cary 1E UV-visible spectrophotometer (series 94041466).

The removal efficiency of HA by preformed aluminium hydroxide flocs was also determined via UV-visible absorption at 230 and 254 nm. Five milliliter of each suspension after 24 hour adsorption experiments was filtrated through a 0.45 µm membrane filter (acetate cellulose, Schleicher and Schuell). Measured adsorption was then converted to residual concentrations

according to standard curves obtained from humic acid stock solution at pH 7, and the removal efficiency was calculated by dividing the decreased amount of humic acid concentration in liquid phase (filtrate) by the total humic acid concentration in the bulk system. Triplicate samples were prepared for analysis. The final results used to evaluate the removal efficiency were mean values of the results determined at 230 nm and those determined at 254 nm.

The untreated HA was used to make the standard solution for absorbency calibration at 230 nm, 254 nm and 340 nm. The UV-absorption of HA solution was found linear with respect to the HA concentrations in solution.

Reactivity to Fast Blue Salt (FBB): The ultrasonic irradiation effects on the HA reactivity with the FBB or the reactivity of cell or mycelia-free culturing medium with FBB were performed using the single cell kinetics method on the Cary 1E UV-visible spectrophotometer (series 94041466) according to the procedure of Gramss et al.⁽¹⁰⁴⁾. Per 1.0 ml of HA solution or cell or mycelia-free culture medium was mixed with 0.1 ml of an aqueous solution of 4.21 mM FBB. Increases in absorbency were recorded in duplicate from 0 second to 30 second at 530 nm. The reactivity was determined as the increases of absorbency over the initial 12 seconds.

Detection of the presence of reduced sugars in degradation culturing medium: The presence of sugars as the possible intermediate degradation products in the fungal culture medium was tested using the phenol-sulfuric acid methods^(67, 104). In brief: a mixture of 0.5 ml cell or mycelia-free culturing medium, 0.5 ml of a 5% (w/v) phenol solution and 2.5 ml of concentrated H₂SO₄ was left reacting for 10 min. Absorbency was determined at 490 nm, (hexoses and their methylated derivatives) and compared to blank samples in which the phenol solution had been replaced by water and the blank samples where the test solution was replaced by water.

E₄/E₆ ratios: The E₄/E₆ ratios of HA were also determined from the absorbency of aqueous solution exposed to ultrasonic irradiation with a Cary 1E UV-visible spectrophotometer (series 94041466) according to the method of Chen et al⁽⁴⁴⁾.

FT-IR-Spectroscopy: Spectra of KBr pellets, (1 mg freeze-dried HA sample in 300 mg KBr), were recorded on a Mattson GALAXY 2020 FT-IR spectrometer (Resolution 8.000, Scans 50, Signal Gain 1, Forward velocity 0.6 cm/s, Reverse Velocity 0.6 cm/s, 600-4000 nm).

¹H-Nuclear Magnetic Resonance Spectra (¹HNMR): Solutions for ¹HNMR analysis were obtained by dissolving ~20 mg of the freeze-dried HA sample in 0.5ml deuterium oxide. The spectra were determined on a Varian VXR 300 NMR Spectrometer.

Gel Permeate Chromatography (GPC) analysis: The ultrasonic irradiation effects on the molecular size (weight) distribution property of HAs were chromatographically examined by a HPLC system (Linear UV detector at 240 nm, Phenomenex – Phenogel 100 Å and 500 Å 300*7.8 mm columns, 20 µl injection volume, the freeze-dried HAs were dissolved in THF and were pumped into the system at 1.25 ml/min). Polystyrene with molecular weights of 580, 1060, 3250, 5050, and 10100, respectively, were used to calibrate the device.

Determination of residual Al concentrations: 10ml preformed aluminium hydroxide floc suspension without addition of humic acids and 24 hour-adsorption suspension mixture of aluminium hydroxide floc and humic acids were both filtrated through a 0.45 µm membrane filter (acetate cellulose, Schleicher and Schuell). The residual Al concentrations of the filtrate were analyzed by atomic absorption spectrophotometer (AA 250 plus). Duplicate samples were prepared and analyzed. The dilution factors on the residual Al concentrations in different tested suspensions were considered and the final data used were comparable results under a same initial Al concentration (Total Al concentration) base.

Microscopy: The ultrasonic irradiation effects on the physical-microstructure and morphological conformation of HA, the ultrasonic irradiation effects on the microstructure and the morphology of preformed aluminium hydroxide floc and the humic acids-aluminium hydroxide floc-adsorption-system were carried out using SEM technology. The samples were quickly freeze-dried and then attached to Al-stubs and coated with a few atoms thick layer of gold. The observation was conducted using a Topcon ABT-60 scanning electron microscope.

The morphologies of some of the filamentous fungi and bacterial isolates growing on 2% MEA and in YNB-HA liquid culture were obtained on the Topcon ABT-60 scanning electron

microscope (SEM). The samples for SEM observation were plugs of 2% MEA containing the fruiting structures and a few drops of washed biomass from YNB-HA liquid culture, which were quickly freeze-dried and sputtered with a gold coat.

The morphology of the Gram-negative bacteria consortium and the filamentous structures of some fungi grown as pure culture were also observed with an epifluorescence microscope on acridine orange stained samples using a Nikon ECLIPSE E400 microscope, a COHU CCD camera, and Scion software.

3.10 Microbiological analysis

Fungal biomass assay: The mycelia biomass was obtained by ultra-centrifuging the total volume of a 3-week culture suspension at 12400 g for 30-45 min using a BECKMAN Model Jz-21 centrifuge. The mycelia pellets, washed with double distilled water under the same centrifugation conditions 2-3 times, were freeze-dried. The final dried mass of mycelia pellets was recorded as the biomass of the fungus.

Bacteria or yeast biomass assay: A membrane filtration method was used to harvest the bacteria or yeast biomass. The procedure was as follows:

- 1) Dry the filter paper (47 mm, 0.45 μ m, acetate cellulose, Schleicher and Schuell) in a PYREX glass dish in the microwave oven at medium power for 10 minutes. Put tissue paper between the glass dish and the filter paper to avoid sticking.
- 2) Immediately put the filter paper and the glass dish in a desiccator and let it cool; weigh the filter paper just before use on an analytical balance.
- 3) Accurately record the volume of the cell suspensions; vortex the suspension for 2-3 min; filter the cell suspension through the filter (exact 5 ml) with the use of a vacuum pump.
- 4) Wash the cells three times with the same amount of distilled water.
- 5) Dry the filter paper with cells in a PYREX glass dish in the microwave oven for 15 mins. on the same power as before.
- 6) Cool the filter paper with cells in the desiccator; measure the mass using an analytical balance.

Microbial enumeration:

The serial dilution-agar spread plating method ^(90, 35) was used to quantify the viable cells in the sample. Briefly, this method involves the serial dilution of a bacterial suspension (sample) in sterile water blanks, which serve as a diluent of known volume. Once diluted, the suspensions are plated on suitable nutrient media (molten agar). Colonies between 30-300 were counted after 3-4 days incubation at 37°C for aerobic counts.

Anaerobic jars (80% N₂ and 20% CO₂ as gas phase) were used for the incubation of (facultative) anaerobic bacteria. The reductant solution (Cysteine-HCl 2.5 g/110 ml, prepared under anaerobic conditions and filter sterilized) and anaerobic nutrient medium were prepared under 100% N₂ condition and autoclaved in O₂-free gas mixtures (80% N₂ and 20% CO₂) separately. The reductant solution was added to the culture medium (2ml/1000ml) just before pouring the medium into the plates, which were inoculated with 0.1 ml of diluted sample. The inoculation and plate pouring were carried out in an O₂-free atmosphere (80% N₂ and 20% CO₂). The plates were then immediately incubated in anaerobic jars at 37 °C and colonies were counted after 2-3 weeks. These colonies were considered to be able to grow anaerobically.

The total bacterial count and acidogens counts were done aerobically, facultatively and anaerobically. For the anaerobic count, 2 ml of reductant solution, (Cysteine-HCl 2.5 g/110 ml, prepared and autoclaved under anaerobic conditions) was added. For the facultative count, the medium and the plates were prepared under aerobic conditions but the inoculated plates were incubated in the anaerobic jars (80% N₂ and 20% CO₂ as gas phase) and the colonies were calculated after 1 week.

A most probable number (MPN) enumeration ⁽¹¹⁶⁾ was performed for the quantification of methanogens and denitrifiers. A three-tube MPN analysis using 4-fold serial dilutions of the digested granule sludge was used. The medium used for the dilutions and MPN incubation of methanogens were performed as described elsewhere ⁽²⁷²⁾. The medium included (per liter): sodium formate 5.0 g; sodium acetate 5.0 g; methanol (100%) 10.0 mmol; trace elements ^{a)}10.0 ml, vitamin solution ^{b)}10.0 ml; NaHCO₃ 5.0 g; L-cysteine HCl H₂O 0.5 g; Na₂S•9H₂O 0.5 g; resazurine 0.002 g; phosphate solution ^{c)} (20.0 g K₂HPO₄ in 100.0 ml distilled water)

Experimental Work

2.0 ml . The medium was prepared and autoclaved (except vitamin solution) under 80% N₂ and 20% CO₂ conditions. Culture tubes, which were screw-capped and sealed with butyl rubber stoppers, were incubated in the anaerobic jars (80% CO₂ and 20% H₂ as gas phase) at 37 °C in the dark for 6 weeks and one dilution series per MPN analysis was autoclaved as a sterile control. Tubes was scored as positive if the methane or N₂ (analyzed by GC) in the tube was detected and the medium in the tube had become more turbid than the control tube.

Trace elements ^{a)} include (per liter): Nitrilotriacetic acid 1.5 g; Fe(NH₄)₂(SO₄)₂•6H₂O 0.2 g; Na₂SeO₃ 0.2 g; CoCl₂•6H₂O 0.1 g; MnSO₄•2H₂O 0.1 g; Na₂WO₄•2H₂O 0.1 g; ZnSO₄•7H₂O 0.1 g; AlCl₃•6H₂O 0.04 g; NiCl₂•6H₂O 0.025 g; H₃BO₃ 0.01 g; CuSO₄•5H₂O 0.01 g. Vitamin solution ^{b)} include (per liter): p-Aminobenzoic acid 10 mg; Nicotinic acid 10 mg; Calcium pantothenate 10mg; Pyridoxine hydrochloride 10 mg; Riboflavin 10 mg; Thiamine hydrochloried 10 mg; Biotin 5 mg; Folic acid 5 mg; a-Lipoic acid 5 mg; Vitamin B₁₂ 5 mg. There is no need to sterilize this solution, but it would be better to store it at 4 °C in the dark immediately after use.

Twelve groups of microbial media were included ^(8, 1):

- 1) Total anaerobic and aerobic count using TSA which included (g/l): Tryptone 17 g; Soy peptone 3.0 g; Sodium Chloride 5.0 g; di-Potassium hydrogen phosphate 2.5 g, Dextrose 2.5 g; Agar 15.0 g.
- 2) Fecal coliforms count using M-7 h FC Agar which included (g/l): Proteose pepton No.3 or Polypeptone 5.0 g; Yeast extract 3.0 g; Lactose 10.0 g; d-Mannitol 5.0 g; Sodium Chloride 7.5 g; Sodium Laural Sulphate 0.2 g; Sodium desoxycholate 0.1 g; Bromocresol purple 0.35 g; Phenol red 0.3 g; Agar 15.0 g.
- 3) Total coliforms count using Macconkey Agar which included (g/l): Peptone 20.0g; Lactose 10.0g; Bile Salts 5.0g; Sodium Chloride 5.0g; Bromocresol purple 0.01g; Agar 15.0g.
- 4) Feccal streptococci count using Streptococcus selective agar which included (g/l): Tryptone 15.0 g; Soy peptone 5.0 g; Sodium Chloride 4.0 g; Sodium Citrate 1.0 g; L-crystine 0.2 g; Sodium Sulphite 0.2 g; Dextrose 5.0 g; Sodium Azide 0.2 g; Crystal Violet 0.0002 g; Agar 13.0 g.

Experimental Work

- 5) Salmonella sp count using Brilliant Green Agar which included (g/l): Peptone 10.0 g; Yeast Extract 3.0 g; Lactose 10.0 g; Sucrose 10.0 g; Sodium Chloride 5.0 g; Phenol Red 0.08 g; Brilliant Green 0.0125 g; Agar 10.0 g.
- 6) Soil actinomycetes count using the medium which included (g/l): Glycerol 20.0 g; L-Arginine 2.5g; NaCl 1.0 g; CaCO₃ 0.1 g; FeSO₄•7H₂O 0.1 g; MgSO₄•7H₂O 0.1 g; Agar 20.0g.
- 7) Filamentous fungi count using the medium which included (g/l): Malt extract (difco) 7.00 g; Peptone (Oxoid) 1.00 g; Yeast extract (Difco) 0.50 g; Penicilling 0.50 g; Streptomycin sulphate 0.50 g; Agar 16.00 g.
- 8) Yeast count using Rose Bengal Chloramphenicol Agar which included (g/l): Peptone 3.0 g; Soy Peptone 2.0 g; Dextrose 12.85 g; di-Potassium phosphate 0.65 g; Magnesium sulphate 0.50 g; Rose Bengal 0.05 g; Chloramphenicol 0.1 g; Agar 13.0 g.
- 9) Sulphate reducer count using sulphate -reducing medium (per liter): Sodium Lactate 3.5 g; MgSO₄•7H₂O 2.0 g; Peptone 2.0 g; Na₂SO₄ 1.5 g; Beef Extract 1.0 g; K₂HPO₄ 0.5 g; CaCl₂ 0.10 g; Reductant solution (Cysteine-HCl 2.5 g/110 ml, prepare and autoclave separately under anaerobic condition) 2.0 ml; Fe(NH₄)₂(SO₄)₂•6H₂O solution (3.92 g/100 ml, filter sterilized) 10.0 ml.
- 10) Denitrifier count using denitrifier medium which included (g/l): Nutrient Broth 8.0 g, KNO₃ 0.5 g.
- 11) Acidogens count using HYA Agar which included (g/l): Proteose Peptone No.3 10.0 g; Beef Extract 1.0 g; Lactose Solution (5.0 g/10 ml, filter sterilized) 10.0 ml; Galactose Solution (2.5 g/10 ml, filter sterilized) 10.0ml; Glucose Solution (2.5 g/10 ml, filter sterilized) 10.0 ml, Agar 15.0 g.

Chapter 4

Simulation of Lignocellulose Degradation in Acidified Landfill

Municipal solid wastes are usually disposed of as landfill and therefore landfill will be a significant repository for the municipal wastes of the future. A significant bio-degradation of lignocellulose biomass in the earth's biosphere is supposed to occur in the sulfurate-dominant habitats. However, most investigations of the bio-degradation of lignocellulose have been focused on the microbial species that grow in a neutral pH. Therefore, in order to enhance our knowledge of the lignocellulose decomposition under sulphate acidified landfill conditions and to compare the effects of lignocellulose decomposition with an improved anaerobic digestion process, the bio-decomposition of lignocellulose in sulphate acidified habitats needs to be evaluated initially under landfill conditions. In this chapter, the degradation of lignocellulose residues in "acid rain" was investigated in a simulated landfill tank reactor leached with a sulphate solution under ambient temperature.

4.1 The changes in pH and ORP during simulation time course

The changes in pH of the leachate produced from the simulated landfill reactor during the time course are presented in Fig.4.1. It can be seen that the pH of the leachate rapidly increased from 1.8 to 5.65, then stabilized around 7.0 throughout the rest of the simulation time. It shows that in the sulphate-acidified environment, the microbial decomposition of lignocellulose was accompanied by an environment self-neutralization process. This neutralization effect could be attributed to the microbial activities taking place in the landfill reactor. This result confirms that those micro-organisms which can tolerate and grow in highly acidified habitats can be used as the biological alternatives to chemical neutralization agents for the treatment of sulphate-bearing acidic effluents such as the acidic discharges from coal refuse piles ⁽¹⁰⁸⁾. In this case, microbial activity could present a catalyzing effect on the increase in pH and the neutralization process. The relatively stabilized pH value at a later stage indicated that this microbial digestion system was either of a high pH buffering capacity or that the other metabolic activities which influence the pH of the system were absent or weak during this simulation period.

The changes in ORP of the leachate produced from the simulated landfill reactor are presented in Fig.4.2. The ORP of the leachate decreased from 460 mV (0 day) to -27 mV (16 day), then remained stable between -153 mV and -242 mV during the rest of the simulation period. The lower ORP at the later stage indicates that the system has been acclimatized to the development of the anaerobic consortium. It is suggested that, similar to the normal landfill process, the aerobic metabolic activities were dominant at the initial stage of this acidified landfill process. However, it can be postulated that besides the oxygen consumption the sulphate was also consumed at the initial stage according to the higher negative ORP values of the leachate.

4.2 The changes of VFA, TVS and bio-gas composition during simulation

The changes in the concentrations of total volatile fatty acids (TVFA) and various VFA of the leachate produced from the simulated landfill reactor are presented in Fig.4.3. The changes in TVS of the leachate produced from the simulated landfill reactor are presented in Fig.4.4.

The results show that the TVS of the leachate increased rapidly in the first ten weeks of decomposition. From week 10, the TVS decreased slowly and remained relatively stable. Parallel to the changes in TVS, the TVFA increased rapidly in the first ten weeks of decomposition. After week 10, the TVFA decreased at a higher rate than TVS, and the acetic, straight propionic and butyric acids were present as the major components of the VFA in the leachate.

The changes in the bio-gas composition during the simulated time course are presented in Fig. 4.5. The results show that the N₂ proportions of the headspace in the reactor remained stable throughout the simulation period; the O₂ content in the headspace was steadily consumed and slowly leveled off after week 18; the CO₂ proportions increased steadily throughout the simulation time; no methane was detected during the simulation time period.

From Fig.4.3, Fig.4.4 and Fig.4.5, it can be seen that the increase of CO₂ in the bio-gas corresponds with the decrease of TVFA in the leachate. This might be ascribed to the activities of sulphate-reducing bacteria, which produce CO₂ and H₂S using the short chain organic acids as carbon sources, because at this stage, very little or no methanogens were

likely to be growing in the system since no methane was found in the bio-gas. Thus other anaerobic VFA consumers than methanogens might be responsible for the decrease of TVFA in the leachate.

Considering of the changes in TVFA and TVS, it is suggested that the TVS did not change as much as the TVFA did, e.g. at a later stage, the TVFA content decreased much more than the TVS content. This suggests that apart from the short chain fatty acids, a number of complex organic acids (such as humic acids) were also produced and dissolved into the leachate during the decomposition process. The complex acids can not easily be used by the micro-organisms, thus they remain the principal contributors to the TVS of the leachate.

In addition, from Fig.4.3 and Fig.4.5, it can be seen that the rapid decrease of TVFA and acetic acids did not bring about a rapid rise of CO₂ in the bio-gas; this is out of what the fermentation reactions had predicted. One reason could be the occurrence of the sulphate reduction, which increased the pH of the system by the production of sulfide and OH⁻. The increased pH provides a favourable condition for dissolving the produced CO₂ in the bio-gas into the leachate as bicarbonate ions.

4.3 The distribution and compositions of microbial populations during simulated landfill process.

The total numbers of anaerobic, aerobic and the dominant groups of micro-organisms in the decomposition system after 8 weeks of landfill were examined. The results are shown in table 4.1.

The results in table 4.1 show that the total aerobic bacterial count remained higher than that of the total anaerobic bacteria throughout the simulation time, although it declined to some extent at the later stage of decomposition. Among the total aerobic, anaerobic and facultative bacterial counts, the total facultative bacterial count stayed the highest and kept increasing during the simulated process. The increasing growth of the total anaerobic bacteria was observed with the landfill process over time, although it remained lower than that of the aerobic and facultative bacteria throughout the process.

**Table 4.1 The counts and distribution of micro-organisms
during the simulated landfill time course**

Colony Forming Units g ⁻¹ solid		8 weeks	12 weeks	20 weeks
Total aerobic (10 ⁶)		12.0	11.2	8.4
Total anaerobic (10 ³)		0.0	0.2	16.4
Total facultative (10 ⁶)		28.0	108.4	136.0
Soil actinomycetes (10 ⁵)		10.6	8.8	4.6
Filamentous fungi (10 ⁶)		14.6	10.8	6.8
Yeast (10 ⁵)		78.0	26.0	10.2
Acidogenic	Aerobic 10 ⁵	62.0	18.6	12.4
	Facultative 10 ⁶	64.0	130.0	102.0
	Anaerobic 10 ²	0.0	0.2	1.14
Denitrifier (10 ²)		0.0	0.2	0.4
Sulphate-reducer (10 ³)		0.28	0.86	10.2
Total coliforms (10 ⁶)		5.64	6.82	7.46
Fecal coliforms (10 ⁵)		9.0	12.0	10.0
Fecal streptococci (10 ³)		7.4	5.0	7.0
Salmonella sp (10 ⁵)		8.2	5.26	4.88

The following results can be deduced from table 4.1:

- ◆ Noticeable growth of sulphate reducing bacteria was observed after 14 weeks of decomposition, although they were already observed at week 8.
- ◆ The growing of acidogenic bacteria during the aerobic and facultative phase was found to be much higher than that during the anaerobic phase.
- ◆ Yeast, actinomycetes and filamentous fungi were relatively stable from week 8 to week 16. After week 16, the counts of these three microbial groups declined slowly.
- ◆ The count of denitrifying bacteria was relatively lower than that of other micro-organisms analyzed so far. This implies that the inorganic nitrate content in this system might be low.
- ◆ The numbers of pathogenic bacteria (total coliforms, fecal coliforms, fecal coliforms and salmonella sp) were relatively higher from week 8 to week 20, ranging from 10³ to 10⁶. This suggests that a large number of pathogenic bacteria developed and grew

in this sulphate acidified landfill habitat. A sterilization procedure would therefore be required before the further utilization of this landfill sludge is acceptable.

The results presented in table 4.1 indicate that aerobic and facultative microorganisms were the principal microbial metabolic groups to decompose lignocellulose substance during the initial landfill process (prior to the development of phase 4 of the landfill). Yeast, filamentous fungi and facultative acidogens were the dominant microbial consortia growing in this young acidified landfill environment. The consortium of the pathogenic bacteria was also much higher in this habitat. Further sterilization treatment is compulsory before this digested sludge can be applied to agriculture land.

4.4 Growth of the microbial population in the simulated landfill system

Each organism has a pH range within which growth is possible, and usually each organism also has a well-defined pH optimum. Most natural environments have pH values between 5 and 9, and organisms with their optimum pH in this range are very common. Only a few species can grow at pH values of less than 2 or greater than 10. Especially, fungi as a group tend to be more acid-tolerant than bacteria. Many fungi grow optimally at pH 5 or below, and a few grow very well at pH values as low as 2, although their interior pH is close to neutral⁽³⁴⁾.

Fungi, including yeasts and filamentous species or moulds, are ubiquitously distributed achlophyllous, heterotrophic organisms with organized nuclei and usually with rigid walls. Because fungi possess broad enzymatic capabilities, they can actively degrade most complex natural substances and certain synthetic compounds, including some pesticides. Most fungi are aerobic or micro-aerophilic, although a few species show a limited anaerobic metabolism and a few are capable of strict anaerobic growth⁽²³⁹⁾.

A major ecological activity of many fungi, especially members of the basidiomycetes, is the decomposition of wood, paper, cloth, and other products derived from natural sources. Basidiomycetes that attack these products are able to utilize cellulose or lignin as carbon and energy sources. As one component of lignocellulose, lignin is a complex polymer in which the building blocks are phenolic compounds. The decomposition of lignin in nature occurs almost exclusively through the degradative activity of certain basidiomycetes called wood-

rotting fungi. Two types of wood rots are known: brown rot, in which the cellulose is attacked preferentially and the lignin left unchanged, and white rot, in which both cellulose and lignin are decomposed ⁽¹³⁴⁾. The secretion of ligninolytic enzymes by white rot fungi has been demonstrated in liquid cultures using defined media, complex broth, or submerged lignocellulose substrates ^(64, 190) and solid-state cultivation of lignocellulose ^(25, 208, 189).

It was found that filamentous fungi was one of the dominant microbial consortia growing in this young acidified landfill environment.

The actinomycetes are a large group of filamentous bacteria, usually gram-positive, highly diverse, aerobic, facultatively aerobic to anaerobic; many of them are hydrocarbon utilizers and common soil organisms ⁽³⁴⁾. Results showed that the presence of soil organisms might influence the production of ligninolytic enzymes by white rot fungi ⁽¹¹⁰⁾. The results obtained so far (shown in table 4.1) indicate that the population of soil actinomycetes was slightly less than that of filamentous fungi. Actinomycetes can be found in almost every natural substrate including soil and composts, freshwater basins, foodstuffs and the atmosphere ⁽²⁵⁵⁾. Their hyphal growth is well suited to the colonization of plant biomass and they secrete a range of enzymes active against lignocellulose ⁽¹⁶⁷⁾. The growth of actinomycetes with filamentous fungi confirms that besides the filamentous fungi, actinomycetes are another group of organisms that can degrade lignocellulose materials ⁽²⁵²⁾ in this highly acidified environmental milieu.

The yeasts are unicellular fungi and many of them are classified with the ascomycetes which are commonly found in soil and decaying plant materials. Yeasts usually flourish in habitats where sugars are present, such as fruits, flowers, and the bark of trees ⁽³⁴⁾. It can be seen that since yeasts generally grow on sugar-containing natural substances, the evidence of yeast being detected or not could be used to indicate whether the sugar is formed from the ligno-polymers or is degraded into VFA during the lignocellulose decomposition process.

Many organisms, including higher plants, algae, fungi, and most bacteria use sulphate as a sulfur source for biosynthesis. The ability to utilize sulphate as an electron acceptor for energy-generating processes is, however, restricted to a very special group of obligatory anaerobic bacteria, namely the sulphate-reducing bacteria. As the required ORP to conduct an

anaerobic digestion so far concerned ⁽⁵⁴⁾, sulphate is a much less favourable electron receptor than O_2 and NO_3^- . Sulphate is used as a terminal electron acceptor under anaerobic conditions by a heterogeneous assemblage of bacteria which utilize organic acids, fatty acids, alcohol and H_2 as electron donors. Certain sulphate reducers will grow completely autotrophically with CO_2 as sole carbon source, H_2 as electron-donor, and sulphate as electron acceptor. In addition to using sulphate as electron acceptor, many sulphate-reducing bacteria grow using nitrate (NO_3^-) as electron acceptor, reducing NO_3^- to ammonia, NH_3 , or can use certain organic compounds for energy generation by fermentative pathways in the complete absence of sulphate or other terminal electron acceptors. The most common fermentable organic compound is pyruvate, which is converted via the phosphoroclastic reaction to acetate, CO_2 , and H_2 . Many sulphate-reducing bacteria are known that are capable of growth on acetate as sole energy source; they oxidize acetate completely to CO_2 and reduce sulphate to sulfide ⁽³⁴⁾. Sulphate-reducing bacteria also play an important role in the breakdown of propionate ⁽¹¹⁵⁾. From table 3.1, it can be seen that the NO_3^- level in the lignocellulose sample used was relatively low. Thus, in our digestion system, the short volatile fatty acids, such as propionic acid, acetate and CO_2 were likely the main substrates for the metabolism of sulphate-reducing bacteria metabolism.

Many fermentative bacteria found during the degradation of complex substance are enteric bacteria, which include coliform bacteria ⁽⁵⁴⁾, thus it is suggested that a number of pathogenic bacteria (especially the coliforms bacteria) that were presented in this decomposition system were also responsible for the fermentation of the lignocellulose substances.

Finally, since the fungi generally grow at a slower speed and produce fewer propagules than the bacteria do, the fungi are easily outgrown by bacteria ⁽¹⁸⁾. Thus, it can be seen that the acidogenic bacteria count was found to be much higher than the filamentous fungal count, although it is suggested that the filamentous fungi played a very important role in hydrolyzing and decomposing lignin and lignocellulose substances.

Given the results and discussions above, it could be postulated that fungi, soil actinomycetes and facultative acidogenic bacteria played an important role in this acidific habitat to decompose the lignocellulose residues. The micro-organisms that most possibly dominated in this very acidic habitat might be the acidophilic bacteria, the filamentous fungi and the soil

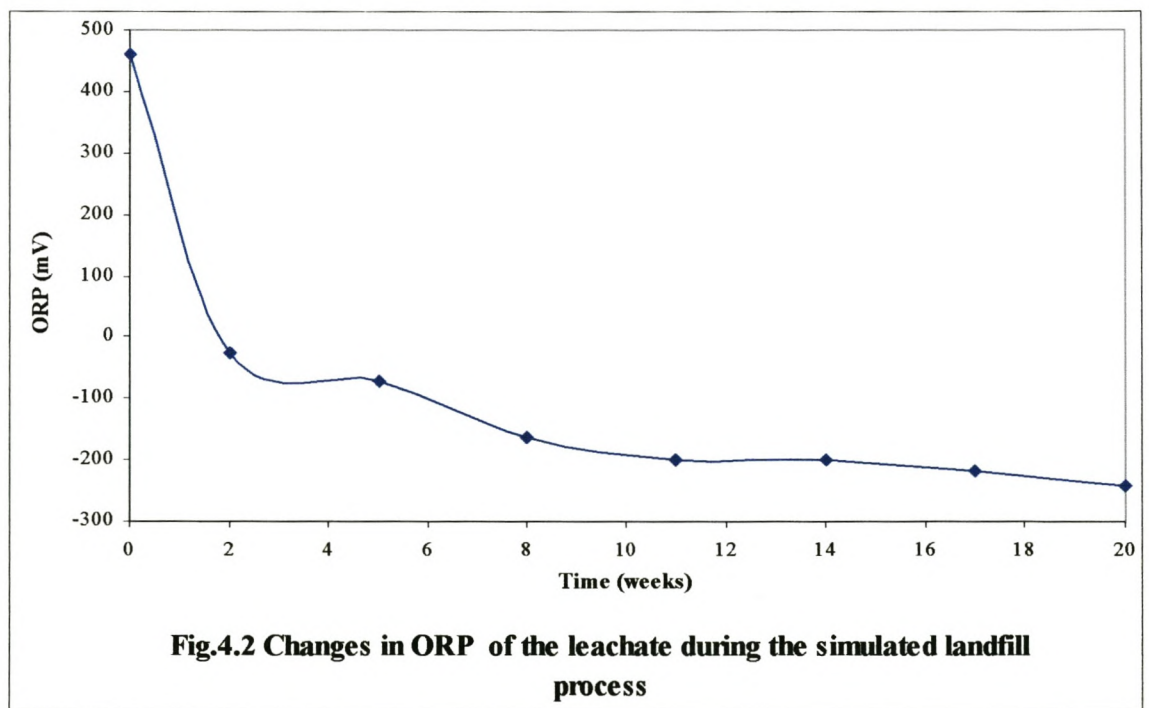
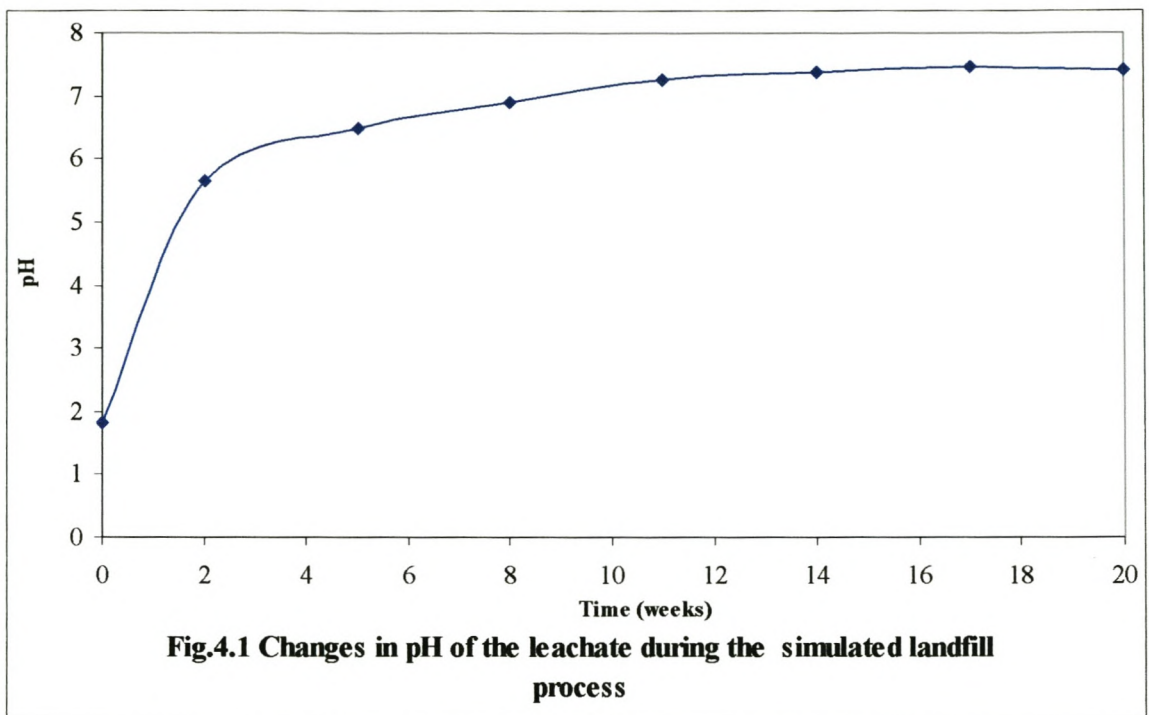
actinomycetes. Under the metabolic activities of these microorganisms, the habitats were self-neutralized, the soluble organic acids were produced and the oxygen or other potential electron-donors were consumed. Thus, the system ORP in the habitats was lowered enough to stimulate the development of anaerobic bacteria (such as sulphate-reducing bacteria).

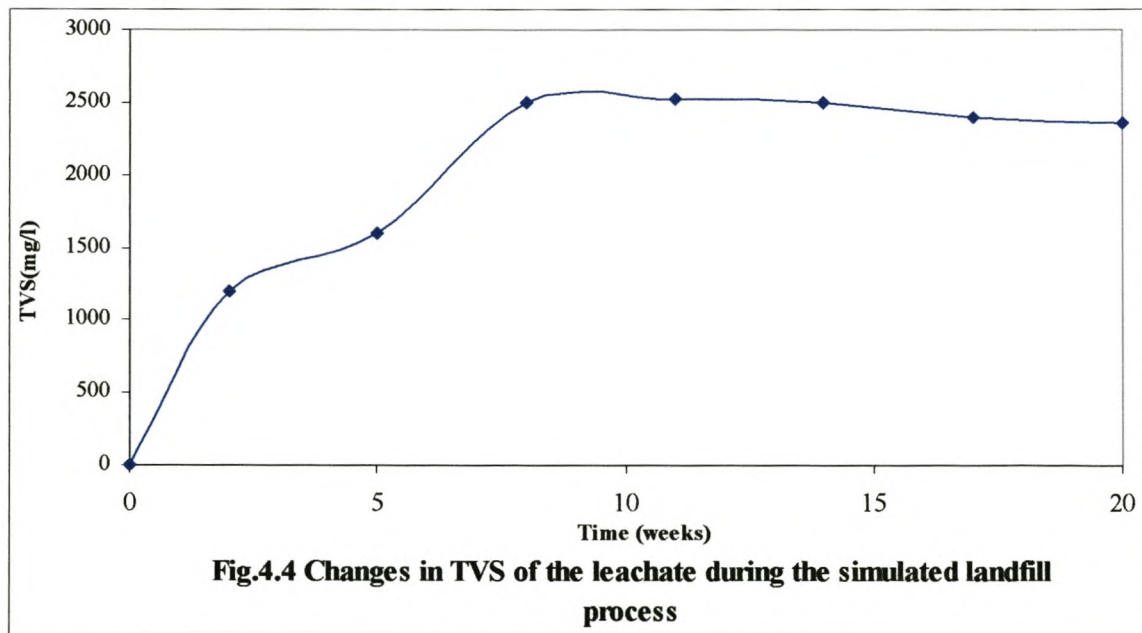
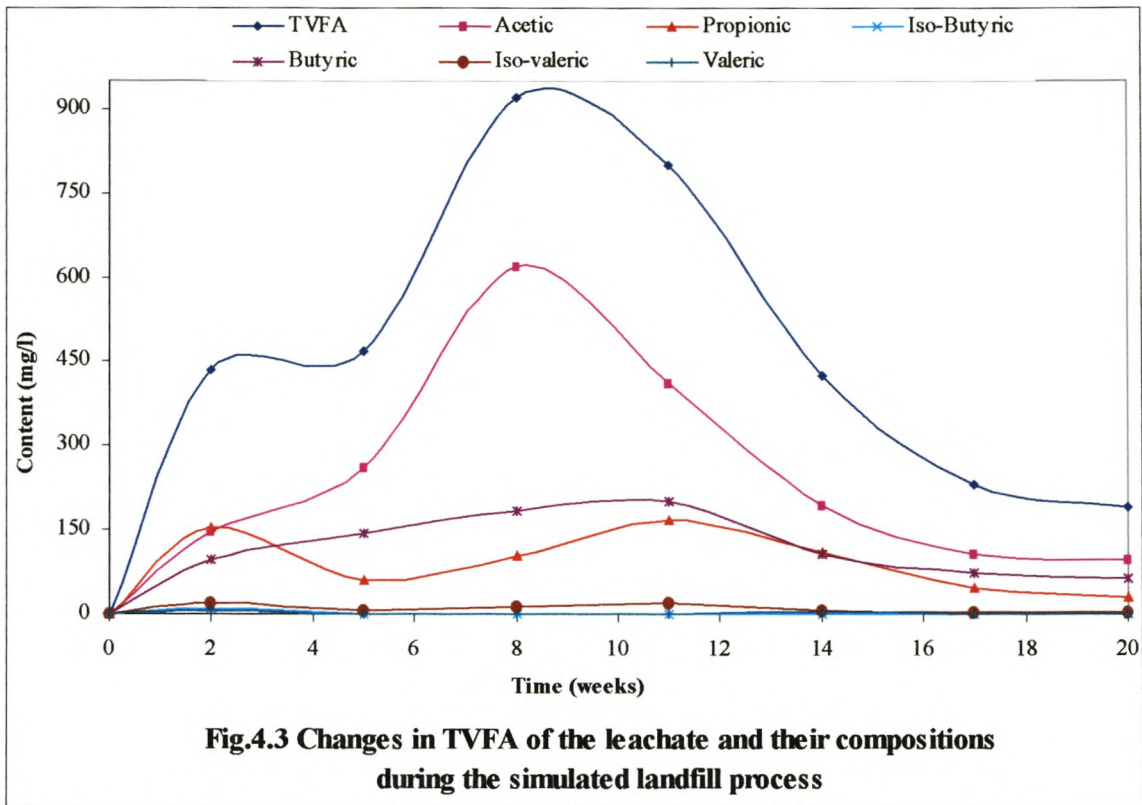
4.5 Summary

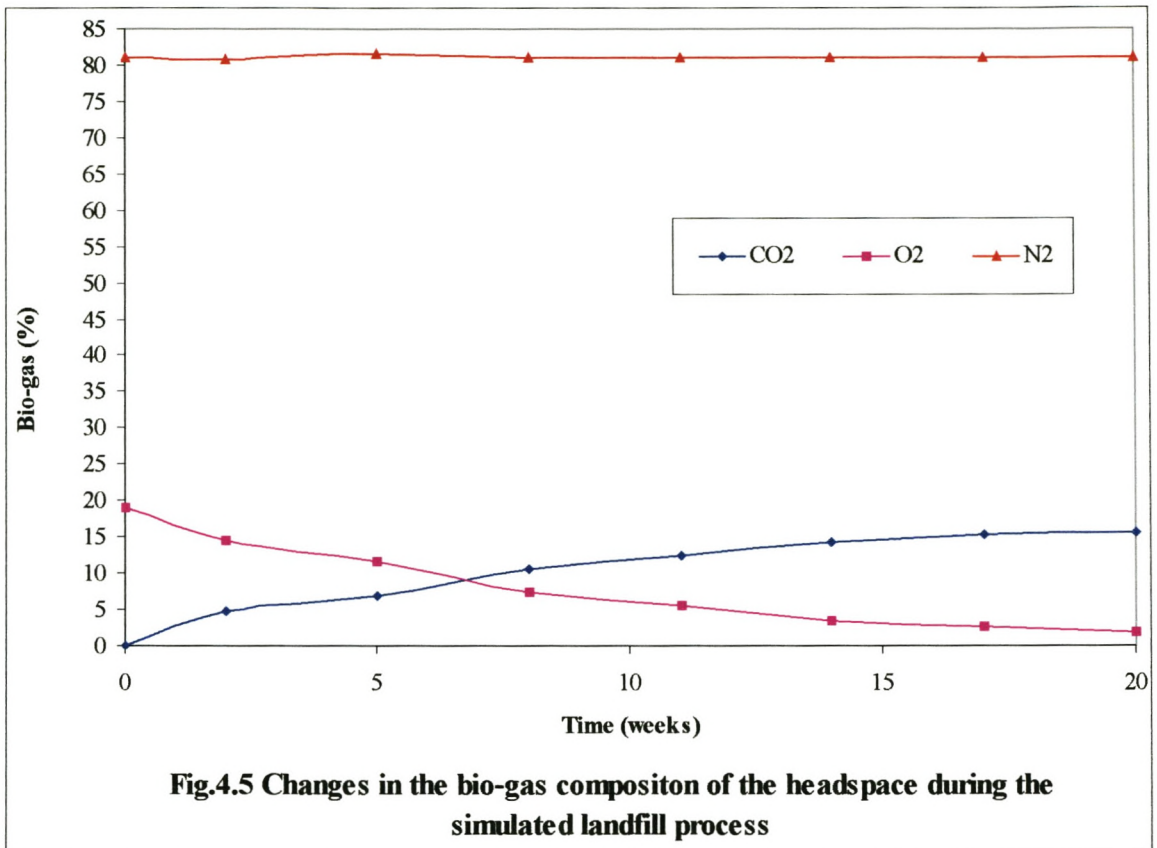
It was found that there was a number of micro-organisms in this highly acidified environment that grew on lignocellulose substrate. As a result of the metabolic activities of these microorganisms, the initially sulphate acidified habitats quickly underwent self-neutralization. The VFA and bio-gas were analyzed to evaluate the acidification fermentation process of the lignocellulose substrate with continuous sulphate leaching. Filamentous fungi, soil actinomycetes and facultative acidogenic bacteria were found to play important roles in this special acidific decomposition process. The most possible microbial consortia populating this highly acidic habitats were acidophilic bacteria, filamentous fungi and soil actinomycetes. The evidence of the yeast being detected or not could indicate whether sugar is formed from the ligno-polymers or is degraded into VFA during the lignocellulose decomposition process. After 8-10 weeks of landfill biodegradation, with the production of soluble organic acids and the consumption of oxygen and other potential oxide, the habitats becomes suitable to the growth and development of anaerobic bacteria (such as sulphate-reducing bacteria). It is suggested that a quantity of complex organic acids (such as humic acids) were generated and dissolved during the decomposition of lignocellulose substrates. These complex organic acids can not easily be used by the micro-organisms and thus remained the main components of the TVS of the leachate. The numbers of pathogenic bacteria (total coliforms, fecal coliforms, fecal coliforms and *salmonella sp.*) were relatively stable from week 8 to week 20 in the simulated landfill, ranging from 10^3 to 10^6 .

These results lead to the conclusion that the degradation of lignocellulose substances in this highly acidified habitat is possible and the acidified ecosystem can be neutralized by the metabolic activities of these micro-organisms. However, the slow decomposition process (over 20 weeks) and the presence of a large number of pathogenic bacteria in the landfill digestion suggest that it is necessary to enhance the efficiency of the lignocellulose degradation process in an environmentally and economically beneficial way.

Simulation of Lignocellulose Degradation in Acidified Landfill







Chapter 5

Promotion of Anaerobic Digestion of Lignocellulose with Activated Sludge (The Acidogenic Fermentation Process)

The complete anaerobic digestion process, mediated by a consortium of anaerobic bacteria, involves a sequence of fermentation reactions resulting in the stabilization (gasification) of a complex organic substrate with methane and carbon dioxide as the end-products. The overall conversion process is firstly dependent on the hydrolysis and liquidation of complex and/or insoluble organics to convert these materials to a size and form that can pass through bacterial cell walls for use as energy or nutrient sources. As stated in Chapter 2, due to the complicated macrostructure and microstructure of lignocellulose, it was difficult to break them down into simple and soluble substances. Thus the initial biotransformation (hydrolysis) or biodegradation of lignocellulose into soluble forms is critical for its further biodegradation/utilization by an anaerobic population.

Secondly, after hydrolysis, the ultimate feasibility of the anaerobic stabilization process strongly depends on the effectiveness of the acidogenic fermentation stage (the volatile fatty acids produced in this stage provide the substrate for methane generation or could be reclaimed as the by-products of the liquid fuel), particularly in the case of the digestion of lignocellulose residues.

However, as can be seen in Chapter 4, the decomposition of lignocellulose in landfill was noticeably slow and the higher pathogenic bacteria population in the landfill limited the utilization of the residual sludge for the fertilizing of land.

In this chapter, therefore, the promotion of acidogenic fermentation (including the hydrolysis stage) of lignocellulose substrate by combined pre-treatments and thermophilic co-digestion with activated sludge was studied.

5.1 The effects of pre-treatment on the solubility of lignocellulose

The effects of mechanical (roll-milling), physical (freezing/thawing) and chemical (0.5 M NaOH swelling) pre-treatment at 25°C and 55°C, respectively, were first evaluated by a solubilization test. The results are reported in Table 5.1. The experimental details can be referenced in Chapter 3.

Table 5.1 shows that with all the pre-treatment methods higher yields of solubility were obtained than without pre-treatment. The swelling and the size reduction (roll-milling) were more effective than the freezing/thawing method. The digestion temperature also had noticeable effects on the solubility. The solubility increased at higher temperatures.

Generally, roll-milling methods utilize shearing and impacting forces to yield a fine substrate with a low crystallinity index and a large surface area. Physical pre-treatment increases the pore size and chemical pre-treatment modifies the structure of the lignocellulose, leading to reduction in crystallinity and increase in surface area. However, most pre-treatment processes are costly when used singularly and have substantial energy requirements due to the severity of the process. Thus, combined pre-treatment processes are possibly more economically suitable. The best results could be obtained by using these three methods comprehensively when preparing the feedstock for the acidogenic fermentation tests. In addition to improving the water solubility of the substrate, these pre-treatment methods would optimize the microorganism degradation activity in the fermentation process. For example, as the microorganism often attaches and grows at interfaces, the finer the substrate, the more surface areas are available for the microorganism to attack. Degrading insoluble biopolymers by microorganisms is also expected to exhibit some interactions with the particulate substances such as attachment of the cells or adsorption of the extracellular hydrolytic enzymes to the fibres. It has been found that during growth in the presence of fibres composed of cellulose or hemicellulose, various strains of the thermophilic soil bacterium *Clostridium thermocellum* and several newly isolated thermophilic anaerobic soils adhered to the fibers⁽¹²⁸⁾. Therefore, in this study, pre-treatment (roll-milling, freezing/thawing and alkali agent) and thermophilic digestion were preferably adopted to promote the solubility of lignocellulose and thereby improving the efficiency of hydrolysis of lignocellulose in the biodegradation process.

5.2 Batch fermentation experiment

5.2.1 Effect of retention time on the process performance

One of the important factors for bacterial growth in a digester is retention time. To ensure efficient conversion of complex organic matter to their metabolites, the micro-organisms in the digester must be of a sufficient quantity, and they must have an adequate retention time to allow substrate metabolism. The retention time (RT) is an important parameter for system design and operation because it directly affects the system costs and more accurately defines the relationship between the microbial system and the digester operating conditions ⁽²⁰²⁾.

The batch system was carried out without a leachate separation unit. The first run was operated in a simple way with tap water as the seeding medium, no pH control before and during the fermentation process and with the initial total solid content (TS₀) and total volatile solid content (TVS₀) as 21-23% and 60.51% (for grass + sludge), 57.80% (for tobacco dust + sludge), respectively. Because the fluidity of leachate in the reactor is very low, the measurements of pH and ORP were performed on the slurry mixture of the 5 g sample from the digester and 5 ml distilled water. This mixture was then centrifuged for 15 min at 3000 rpm. The solid residues were washed three times with 10 ml of distilled water by centrifugation before it underwent TVS analysis. All of these supernatants were collected and filtered through 0.45 µm membrane filtrate paper. The filtrate was sampled for VAF analysis.

The degree of acidification, measured as pH and ORP changes in the digester, are presented in Fig.5.1 and Fig.5.2. The changes in volatile solid destruction (VSD) (which was calculated as a percentage of the difference between the TVS of the loaded feedstock and that of the digested solid residues divided by the TVS of the loaded feedstock) and the CO₂ content in the bio-gas are presented in Fig.5.3 and Fig.5.4. The concentrations of VFA in liquid phase are presented in Fig.5.5(1) and Fig.5.5(2).

The pH of the system quickly decreased from 6.5 during the first 5 days and then stabilized around 6.30. Parallel to the pH “plateau”, the concentrations of total VFAs changed little over time. Correspondingly, the VSD index increased during the first 15 days and then remained relatively stable.

The integrated results in pH, VFAs and VSD index above show that: (1) quick decreases in pH could result from the spontaneous acidification of dissolved substances. With the quick consumption of easily-digestible fractions of the substrate (the increased VSD index in Fig.5.3), the short chain fatty acids (volatile fatty acids, VFAs) were produced and were accumulated (the stable concentration level of total VFAs in Fig.5.5(1) and Fig.5.5(2)). A pH buffer subsystem, mainly governed by the VFAs in the bio-reactor system, was formed, thus the pH appeared stable at later RT. (2) The acidogenic fermentation process appeared to end after approximately 15 days of RT; after that no notable changes in pH, VFAs and VSD index were detected.

Although the RT was prolonged to 31 days, no methane was detected in the head-gas of the reactor. This showed that the methanogenesis was inhibited. However, the CO₂ contents in the bio-gas increased to some extent during the digestion time. Under anaerobic conditions, the high CO₂ content detected in the head-space of the reactor was largely from the acidification fermentation process.

The ORP is a measure of the general oxidation state of the system and therefore mainly depends on the H⁺ concentration, the valence status of the metal ions and the dissolved oxygen concentration. Fermentation, in principle, does not change the ORP of the environment in which it takes place because this type of energy generation for micro-organism metabolism is based only on a dismutation of organic molecules without external electron acceptors being involved. However, the direct measurements of the electrode potentials in the digester system indicate that the fermentation reduces the environment. This is probably due to the fact that the substrates of fermentors do not usually show electrochemical activity (e.g. cellulose), whereas some of the metabolites (H₂) will lower measured electrode potentials⁽⁸²⁾. Therefore, the negatively-reduced ORP in the reactor was observed during the process. Therefore, in Fig.5.2, it can be seen that the ORP of the system tended to be stable or negatively reduced to some extent during the fermentation process.

The individual cultivation conditions can be distinguished easily and exactly by measuring the oxidation – reduction potential (ORP). The values of ORP in oxic conditions are positive (higher than 50 mV as standard ORP), whereas ORP values in anaerobic conditions are negative (less than -50 mV). An ORP around 0 mV are typical of anoxic conditions⁽¹²⁶⁾. The

low ORP (below -50 mV) detected in the reactor confirmed that the process was successfully carried out under anaerobic conditions.

The results in Fig.5.5(1) and Fig.5.5(2) show that butyric acids were the dominant components of the young digestion products (early period of the digestion). When fermentation proceeded, the total VFAs (TVFAs) increased noticeably with the increases of the individual VFA, although there were some differences in the changes of VFA composition between the grass-sludge system and tobacco-sludge system in the digestion process. For example, in the grass-sludge system, during the later stage, the increase of acetic acid was accompanied by a decrease of the propionic acid - this indicated that some increase of acetic acid was probably due to the reduction of propionic acid; in the tobacco-sludge dust system, the acetic production line is above the propionic acid production line throughout the testing period - this implies that most of the augment of the acetic acid was probably from the direct fermentation products of the lignocellulose digestion. Another difference found in the tobacco-sludge system (in Fig.5.5(2)) was that the butyric concentration was much higher than the other VFA components, even higher than the acetic acid concentration in the later digestion stage. This indicates that the reduction of butyric acid to acetic acid was probably inhibited at a later digestion stage or that some acetic acid produced in the early digestion stage was re-converted into butyric acid by reverse reactions. In addition, this could be explained by the fact that the growth of the acetogenic bacteria which convert butyric acid into acetic acid was suppressed by the lower pH (seen from Fig.5.1). It was also found that the branched and longer VFA content (such as iso-butyric, iso-valeric, valeric acids) were generally much lower in these acidification products. Since the unpleasant smell from the fermentation process was mainly due to the branched and/or longer VFA intermediate products, the fermentation process with less branched VFA production would be favourable to operate in practice.

5.2.2 Effects of the pH (ORP) conditions of start-up medium

The previous solubilization test demonstrated that alkaline pre-treatment can improve the solubility of the lignocellulosic organic matter. In addition, because the reduced electro-chemical conditions were more favorable for the growth of anaerobic micro-organism than the oxidized conditions, in the second run, the tap water seeding medium was substituted by

0.5 M $\text{Ca}(\text{OH})_2$ (pH 8.02, ORP -15 mV), 0.5 M NaOH (pH 11.80, ORP -146 mV) and catholic radical water (pH 11.58, ORP -565 mV); the other conditions were kept the same as for the first run. With tobacco dust as the fed-stock, the profiles of pH, ORP, VSD and CO_2 content in the bio-gas and the VFA content against the RT are presented in Fig.5.6, Fig.5.7, Fig.5.8, Fig. 5.9 and Fig 5.10.

Fig.5.6 shows that the high initial pH of these three seeding mediums did not induce the expected increase in the pH of the reactor system. The opposite occurred with the pH “plateau” appearing earlier when compared to the first run (Fig.5.1). Furthermore, the pH values were generally lower than those in the first run. It is likely that more VFA were produced in a shorter fermentation time with the addition of these alkaline media.

Fig.5.7 shows that the ORP in the reactors started with the radical water (pH 11.58, ORP -565 mV) feeding medium was more stable and more reduced at a later stage than in the reactor started with the tap water feeding medium (Fig.5.1), although the initial greatly reduced values of the ORP in the radical water did not reduce the ORP of the digestion system immediately. The reduced ORP in the reactors starting with 0.5 M $\text{Ca}(\text{OH})_2$ (pH 8.02, ORP -15 mV) and 0.5 M NaOH (pH 11.80, ORP -146 mV) appeared relatively stable throughout the digestion time. It is suggested that the effect of the ORP status of the start-up medium on the fermentation process was dependent on the initial value of the ORP of the medium. The radical water, which had a higher reduced ORP value, presented more marked effects on the fermentation process than 0.5 M $\text{Ca}(\text{OH})_2$ (pH 8.02, ORP -15 mV) and 0.5 M NaOH (pH 11.80, ORP -146 mV) from the ORP point of view.

At this stage, methane was not yet detected in the bio-gas of the reactors. However, compared to the results in Fig.5.1, a higher CO_2 content (shown in Fig.5.8) was found in the reactors starting with NaOH and catholic radical water mediums. It quickly increased to 62% (NaOH medium) and 67% (catholic radical water medium) and stabilized around 70% (NaOH medium, catholic radical water medium), whereas less than 30% CO_2 was initially detected in the reactor starting with $\text{Ca}(\text{OH})_2$ medium. It is suggested that the addition of $\text{Ca}(\text{OH})_2$ could lead to some CO_2 mineralization in the form of CaCO_3 precipitate on the solid substance surfaces.

Fig.5.9(1) and Fig.5.9(2) show that higher VSD values were obtained in the reactor starting with the NaOH, cathodic radical water medium and $\text{Ca}(\text{OH})_2$ mediums than with the tap water medium (Fig.5.3) during the early stage of the fermentation process.

The results in Fig. 5.10(1) to Fig.5.10(6) show that the concentration of individual components of VFA increased greatly when compared to the results in Fig. 5.5(1) and Fig.5.5(2). It was found that the proportion of acetic acid was greater than the other components from the start and remained dominant throughout the fermentation process although the butyric acid is also one of the major products of the digestion. It is suggested that, besides the improvement in the solubility of the bio-polymers, the initial higher pH and more reduced ORP conditions in these feeding mediums also greatly promoted the production of acetic acid (including the butyric acid production) in the acidification process. This point is further discussed in a separate section (**Biochemical Pathways in Acidification Fermentation**).

The acid accumulation and low pH values in the batch reactor in a short RT time (2-3 days) would inhibit the growth of methanogenic bacteria. However, as far as the VSD index and VFA production are concerned, the acceleration of the acidification stage was successful.

5. 3. Two-stage semi-continuous system reactor

The low pH value in the batch reactor (without leachate stripping operation, shock loading) could be partly attributed to the inhibition of acetogenesis by the accumulation of undissociated VFA. So that the leachate stripping process and stepwise loading effects on the fermentation process was tested in the two-stage semi-continuous system reactor. a feeding medium of $\text{Ca}(\text{OH})_2$ and NaOH were tested as the initial feeding medium at three different RT times with or without leachate circulation. The operation parameters and the results (mean values at steady state of the process) are given in table 5.2.

The results indicate that the leachate circulation improved the volatile solid destruction (VSD) in 2 weeks of RT , but there was no significant improvement in VSD when RT was extended to 3-4 weeks. A significant influence of RT on VSD was observed in the fermentation process without leachate circulation from 2 weeks of RT to 3 weeks of RT; with the RT increasing

from 2 weeks to 3 weeks, VSD increased from 20% to 48% (in NaOH medium) and from 16% to 43% (in $\text{Ca}(\text{OH})_2$ medium), respectively. Three weeks of RT for the system with leachate circulation and four weeks of RT for the system without leachate circulation could be optimal. Compared with the batch reactor system with no leachate circulation, less differences in VSD were observed between the systems with the $\text{Ca}(\text{OH})_2$ medium and the NaOH medium in this semi-continuous system reactor.

In spite of the high pH values of the feeding mediums, the pH of the leachate declined below 6.0 after 2 weeks of RT and slightly increased to around 6.50 after 4 weeks of RT, but more reduced ORP and lower pH values were found in the case of leachate circulation.

When the leachate was circulated, the TVFA concentration increased steadily with increases of retention time(RT); higher TVFA and more acetic acid were formed at shorter RT. At the same time, the ratio of acetic acid to butyric acid was much higher when the leachate was circulated. This showed that more butyric acid might be converted into acetic acid when the leachate was in contact with the bio-solids of the reactor.

Compared to the results from the batch reactor, about 10%-46% CO_2 was detected in the bio-gas phase of the reactor with 0.5 M NaOH feeding medium; this was lower than the batch reactor with the same feeding medium. At the same time, small amounts of CH_4 (1%-2%) was found at a longer RT stage with or without leachate circulation. This indicated that some acid build-up inhibition was relieved with the leachate/solid separation operation.

In summary, with the leachate/solid separation operation, the digestion RT could be reduced by leachate circulation without weakening the performance of the degradation process. Some acid build-up toxicity for methanogens could be partially relieved. In terms of VFA yield and volatile solid destruction efficiency, the two-stage semi-continuous system reactor with leachate recirculation by using solid/leachate separation unit is preferable.

In addition, it was probably due to some leachate being held in the solid matrix that VFA content sampled from the leachate collector in semi-continuous system reactor were generally lower than that from the batch reactor.

5.4. Discussion of biochemical pathways in acidification fermentation.

When the bio-polymers were successfully decomposed to reduced sugars like free glucose by the microbial ecosystem, the reactions listed in Table 5.3 are considered to be possible. In order to explain their predominance in the selected biological reactions, their Gibbs Free Energy Changes are listed as well ⁽⁵⁴⁾.

In a given bio-chemical environment, excluding the kinetic factors from the outer environment, the preference for and the dominance of a specific biochemical can be predicted by the Gibbs free energy changes in that reaction and the population of the micro-organisms which function as the catalysts for the reaction. The more negative the Gibbs free energy changes and the larger the microbial population, the easier it is for that reaction to take place and to be dominant under the given circumstance.

The following information can be obtained from Table 5.3:

- Both conversion of glucose (from the biodegradation of lignocellulose) to butyric acid (and/or propionic acid), catalyzed by the fermentative bacteria, and its direct conversion to acetic acid catalyzed by the acetogenic bacteria are thermodynamically favourable because these reactions both have the strongest negative free energy changes (-264 kJ and -311 kJ). The butyric acid (and/or propionic acid) and acetic acid are therefore the two dominant components of the VFA during the acidification process.
- The conversion of butyric and/or propionic acid to acetic acid by acetogenic bacteria is thermodynamically unfavourable because it has a positive free energy change. This accounts for the build-up of butyric acid (and/or propionic acid) in the process. The butyric acid and/or propionic acid proportion in the total production of VFA was therefore relatively higher than the acetic acid at the early stage of the fermentation. This remained the case throughout the whole process when the growth of the acetogenic bacteria was too low to consume the extra butyric acid and/or propionic acid.
- In addition to the short-chain carbonoxylated products, a quantity of CO₂ and H₂ were produced in the gaseous phase. Consequently, a relatively large proportion of CO₂ was

detected in the bio-gas. Its presence and quantity in the gas phase of the anaerobic digester can be used, to some extent, as an indicator of the fermentation process and to evaluate the efficiency of the acidification process before the methanogenic process takes place. The decline in CO₂ proportion and the incline in the acetic concentration at the later stage of the fermentation might be attributed partially to the formation of acetate by H₂-cleavage bacteria when H₂ accumulation in gaseous products is high in the gaseous products at this stage. The negative free energy changes of this reaction (-95 kJ) confirm this possibility.

- While the fermentation process can be performed in anoxic circumstance, most acidogenic bacteria ⁽³²⁾ and all acetogenic bacteria ⁽¹²⁷⁾ are anaerobic. The initially reduced ORP conditions of the feeding medium with higher oxygen-toxicity-buffering capacity were beneficial to the growth of acetogenic bacteria. Therefore, when the tap water (ORP +282 mV) was substituted with radical water (ORP -565 mV) or NaOH (ORP -149 mV) as the feeding medium, the acetic acid content increased markedly and surpassed the butyric acid level significantly, along with the notable increase in the total volatile fatty acid production.
- Further, so far as the results obtained are concerned, the initial high pH of the medium did not show any inhibitory effect on the biological fermentation process. In addition to improving the solubility of the complex bio-polymers, another reason could be that the added OH⁻ radical species consumed the existing H⁺ in the system at the start of a formal fermentation process where a rapid growth of the fermentation bacteria is required. (It was observed that the pH of the tap water declined from 9.46 to 7.21 or 6.49 when 1.0 g grass or 1.0 g tobacco dust was added to 200 ml tap water; this implied that there was already a quantity of radical H⁺ in the fermentation system). This produced an initially-serious H⁺-scarce state of the system, which acted as the driving force to induce the fermentation reactions to take place in a short time. Thereafter, the pH below 6.0 in the digester was generally detected at the second day of the fermentation, in spite of the higher pH values at the start of the process. Another possibility would be that the high pH medium added initially precipitated the existing heavy metals (which was considered toxic to the microbial growth) from the sludge.

5.5 Summary

Based on the experimental results obtained the following conclusions are made:

- The combined pre-treatment method was efficient to assist the anaerobic co-digestion of lignocellulose residues with activated sludge. The mechanical (roll-milling); physical (freezing/thawing); chemical (alkali wetting agent) pre-treatment procedure increased the solubility of the lignocellulose residues, thus improving the efficiency of the acidification fermentation process.
- The influence of retention time (RT) on the destruction of lignocellulose to VFA was evident. At least 2-3 weeks of RT was required with batch reactor or semi-continuous system reactor. Generally, 4 weeks of RT was enough. The leachate circulation was necessary when short retention time was used.
- The highly reduced ORP status of the initial feeding medium (such as NaOH solution or cathodic radical waters) significantly improved the acetic acid and the total VFA in the fermentation process.
- From the point of view of the VFA production and the destruction of volatile solids, the acidification process was successful under the experimental conditions.
- Partially because of the high concentration of VFA produced in this acidification fermentation process, the methanogenesis was inhibited at this stage.
- The alleviating of the methanogenic inhibition needed further study, as discussed in Chapter 6.

Promotion of Anaerobic Digestion of Lignocellulose with Activated Sludge
(The Acidogenic Fermentation Process)

Table 5.1. Pre-treatment Effects

Feeding	Methods	Solubilization Yield(TVS/TS %)	
		25°C	55°C
Grass (-2 mm)	Distilled Water	12.4	18.2
Grass (-2 mm)	Roll-milling(0.2-0.5 mm)	28.3	33.8
Grass (-2 mm)	Freezing/thawing	17.4	22.6
Grass (-2 mm)	0.5 M NaOH	32.7	38.6
Tobacco Dust (-2 mm)	Distilled Water	13.5	20.1
Tobacco Dust (-2 mm)	Roll-milling(0.2-0.5mm)	32.5	38.2
Tobacco Dust (-2 mm)	Freezing/thawing	19.2	23.5
Tobacco Dust (-2 mm)	0.5 M NaOH	36.3	42.6

**Table 5.3 Biochemical Reaction and its Gibbs Free Energy changes in Acidification
Fermentation**

Reactions	$\nabla G_0(\text{kJ})$
Reactions Catalyzed by Fermentative Bacteria	
$(\text{C}_6\text{H}_{10}\text{O}_5) + \text{H}_2\text{O} \rightarrow \text{C}_6\text{H}_{12}\text{O}_6$	-18
$\text{C}_6\text{H}_{12}\text{O}_6 + 6\text{H}_2\text{O} \rightarrow 6\text{CO}_2 + 12\text{H}_2$	-26
$\text{C}_6\text{H}_{12}\text{O}_6 \rightarrow 2\text{CH}_3\text{COCO}_2^- + 2\text{H}^+ + 2\text{H}_2$	-112
$\text{C}_6\text{H}_{12}\text{O}_6 + 2\text{H}_2\text{O} \rightarrow \text{CH}_3\text{CH}_2\text{CO}_2^- + \text{H}^+ + 3\text{CO}_2 + 5\text{H}_2$	-192
$\text{C}_6\text{H}_{12}\text{O}_6 \rightarrow 2\text{CH}_3\text{CH}_2\text{CH}_2\text{CO}_2^- + \text{H}^+ + 2\text{CO}_2 + 2\text{H}_2$	-264
Reactions Catalyzed by Acetogenic Bacteria	
$\text{CH}_3\text{CH}_2\text{CO}_2^- + \text{H}^+ + 2\text{H}_2\text{O} \rightarrow \text{CH}_3\text{CO}_2^- + \text{H}^+ + \text{CO}_2 + 3\text{H}_2$	+72
$\text{CH}_3\text{CH}_2\text{CH}_2\text{CO}_2^- + \text{H}^+ + 2\text{H}_2\text{O} \rightarrow 2\text{CH}_3\text{CO}_2^- + 2\text{H}^+ + 2\text{H}_2$	+48
$\text{CH}_3\text{CH}_2\text{OH} + \text{H}_2\text{O} \rightarrow \text{CH}_3\text{CO}_2^- + \text{H}^+ + 2\text{H}_2\text{O}$	+10
$2\text{CO}_2 + 4\text{H}_2 \rightarrow \text{CH}_3\text{CO}_2^- + \text{H}^+ + 2\text{H}_2\text{O}$	-95
$2\text{HCO}_3^- + 4\text{H}_2 + \text{H}^+ \rightarrow \text{CH}_3\text{CO}_2^- + 4\text{H}_2\text{O}$	-105
$\text{C}_6\text{H}_{12}\text{O}_6 + 4\text{H}_2\text{O} \rightarrow 2\text{CH}_3\text{CO}_2^- + 2\text{HCO}_3^- + 4\text{H}^+ + 4\text{H}_2$	-206
$\text{C}_6\text{H}_{12}\text{O}_6 + 2\text{H}_2\text{O} \rightarrow 2\text{CH}_3\text{CO}_2^- + 2\text{H}^+ + 2\text{CO}_2 + 4\text{H}_2$	-216
$\text{C}_6\text{H}_{12}\text{O}_6 \rightarrow 3\text{CH}_3\text{CO}_2^- + 3\text{H}^+$	-311

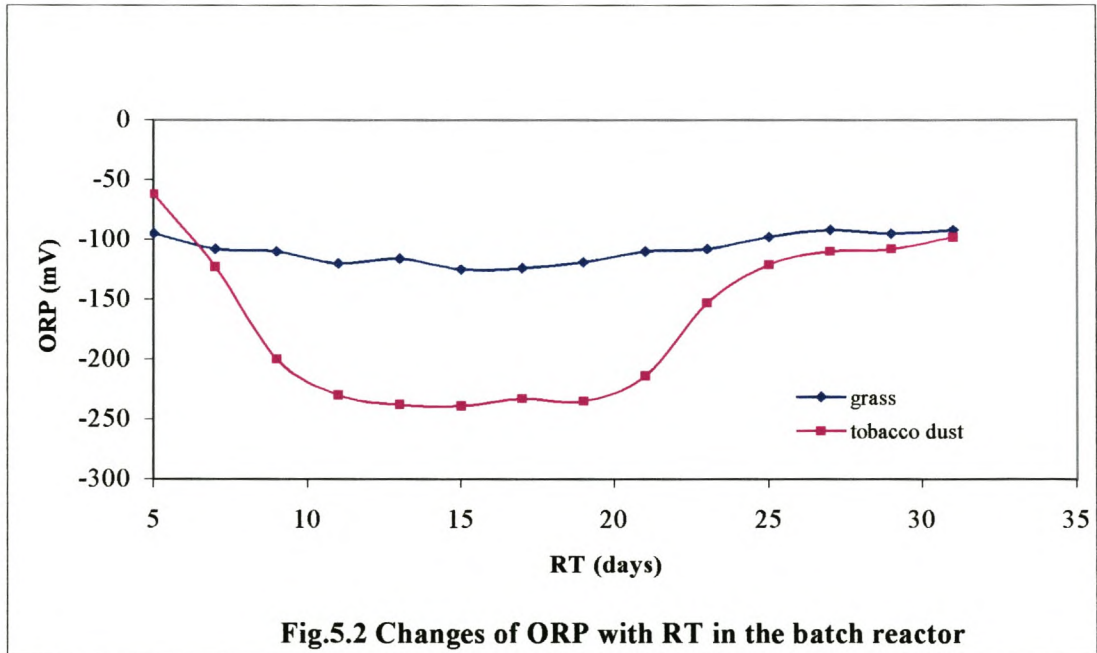
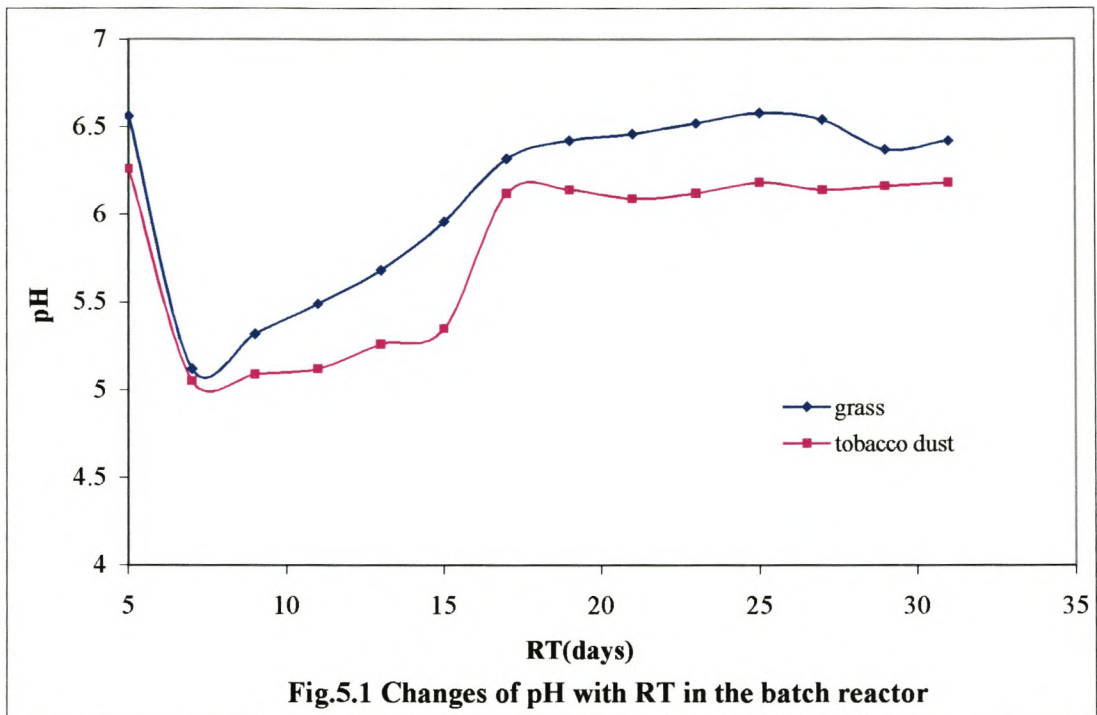
Table 5.2.
Operation conditions and digestion results of two-stage semi-continuous reactor

Runs		RT=2 weeks	RT=3 weeks	RT=4 weeks
OLR(kgVS m ⁻³ d ⁻¹)		16.22(T), 18.18(G)	11.57(T), 12.12(G)	8.68(T), 9.09(G)
Feed	TS(%)	21-23	21-23	21-23
	TVS(%TS)	57.8(T), 60.51(G)	57.8(T), 60.51(G)	57.8(T), 60.51(G)
Feeding Medium:		Ca(OH) ₂ , NaOH	Ca(OH) ₂ , NaOH	Ca(OH) ₂ , NaOH
Concentration(mol/l)		0.5	0.5	0.5
PH		8.02, 11.80	8.02, 11.80	8.02, 11.80
ORP(mV)		-15, -146	-15, -146	-15, -146
Acidification Results(1):	VSD(%)	23, 26	48, 56	56, 67
	Leachate	pH	5.4, 5.2	6.0-6.7, 6.0-6.9
		ORP	-129, -136	-176, -202
		TVFA	3904.34, 4577.32	6046.77, 7074.67
		Acetic	2780, 3208	4033.67, 5040
		Propionic	325.67, 356	713.45, 736.67
		Iso-Butyric	38.67, 53.33	52.67, 82
		Butyric	614.67, 797.33	949.65, 1073.33
		Iso-Valeric	145.33, 157.33	157.33, 142.67
		Valeric	0, 5.33	0, 0
	Bio-gas	CO ₂ %	38, 42	8, 11
		CH ₄ %	0.0	0.6, 1.4
Acidification Results(0):	VSD(%)	16, 20	43, 48	52, 60
	Leachate	pH	5.6, 5.3	6.4-7.1, 6.2-7.3
		ORP	-126, -132	-168, -195
		TVFA	3018.67, 4089.34	5194.865, 6902.66
		Acetic	1074.67, 1484	1942, 3168
		Propionic	384, 506.670	443.6, 410
		Iso-Butyric	25.335, 220	82.67, 329.33
		Butyric	1438.67, 1496	2470, 2932
		Iso-Valeric	121.33.33, 182.67	156.665, 153.33
		Valeric	0, 0	0, 0
	Bio-gas	CO ₂ %	32, 46	10, 13
		CH ₄ %	0,0	0.4,0.8

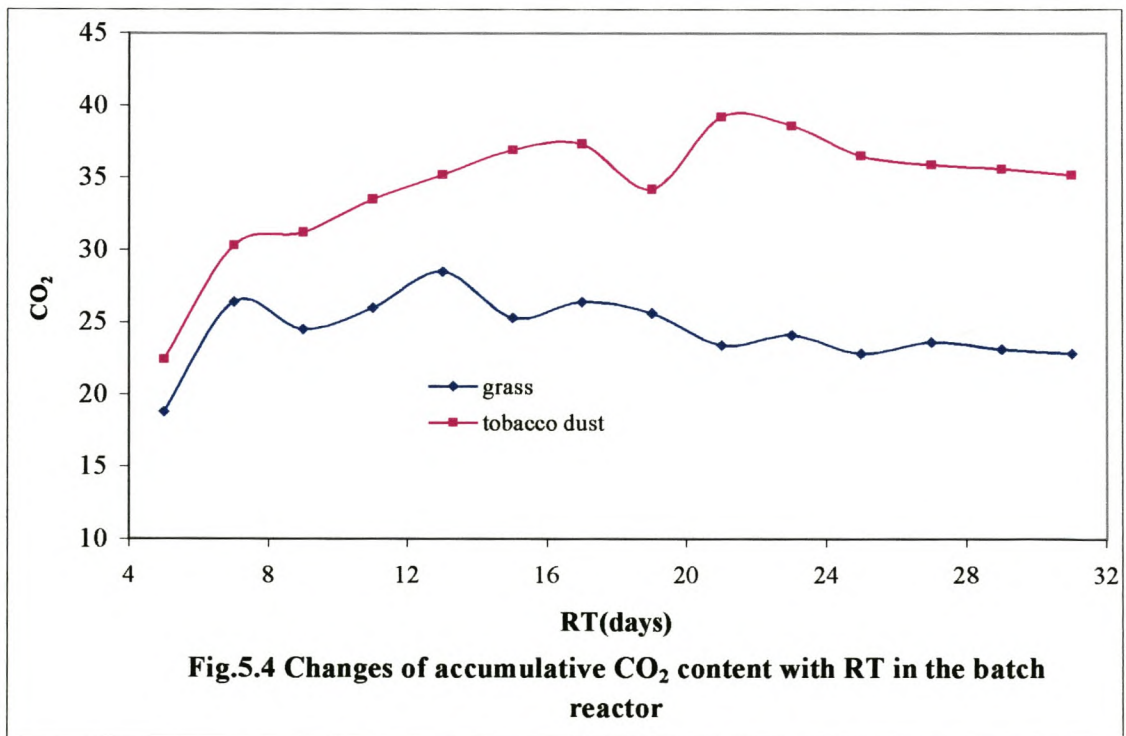
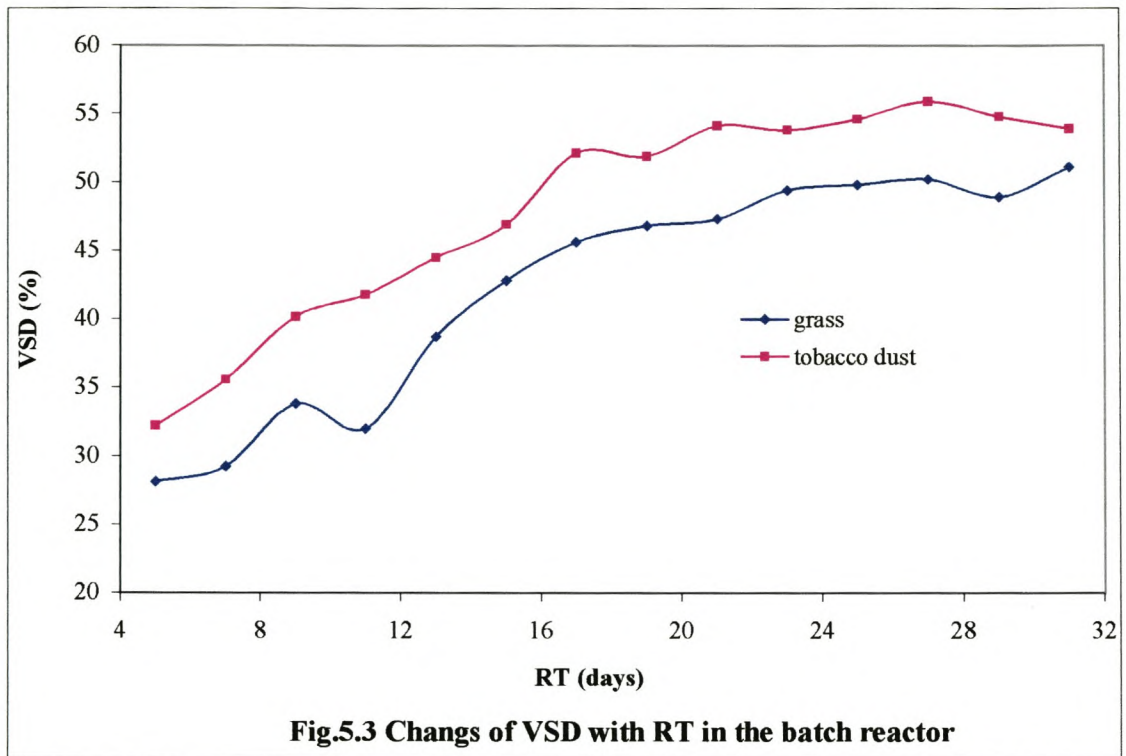
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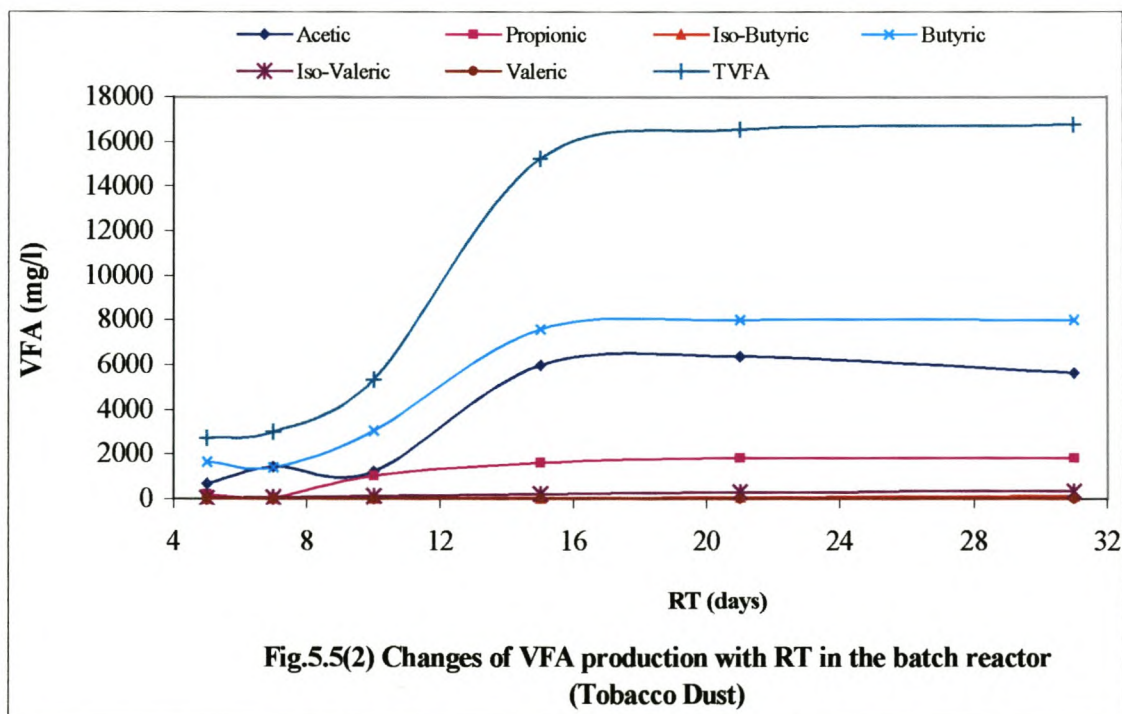
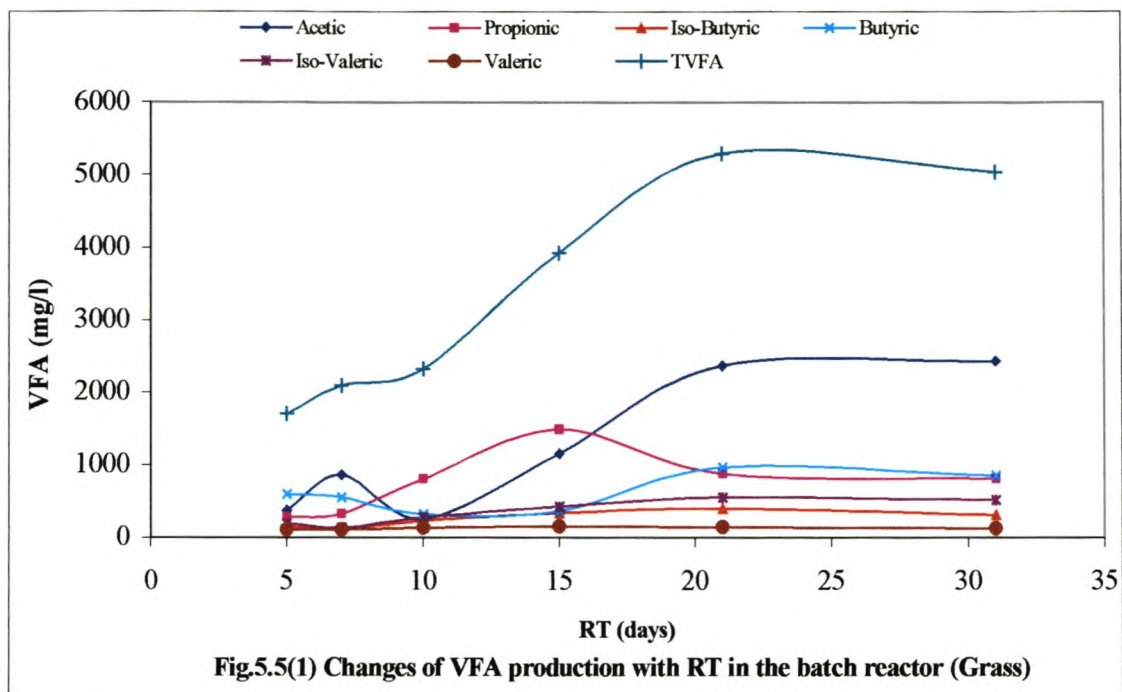
- 1.(0)-no leachate circulation; (1)-leachate circulation(every other day)
- 2.(T)-Sludge +Tobacco Dust ; (G)-Sludge+Grass
- 3.The acidification results are the mean values of the four identical reactors (two of which was fed with sludge + tobacco dust, another two was fed with sludge + grass) under the steady running conditions.

Promotion of Anaerobic Digestion of Lignocellulose with Activated Sludge
(The Acidogenic Fermentation Process)

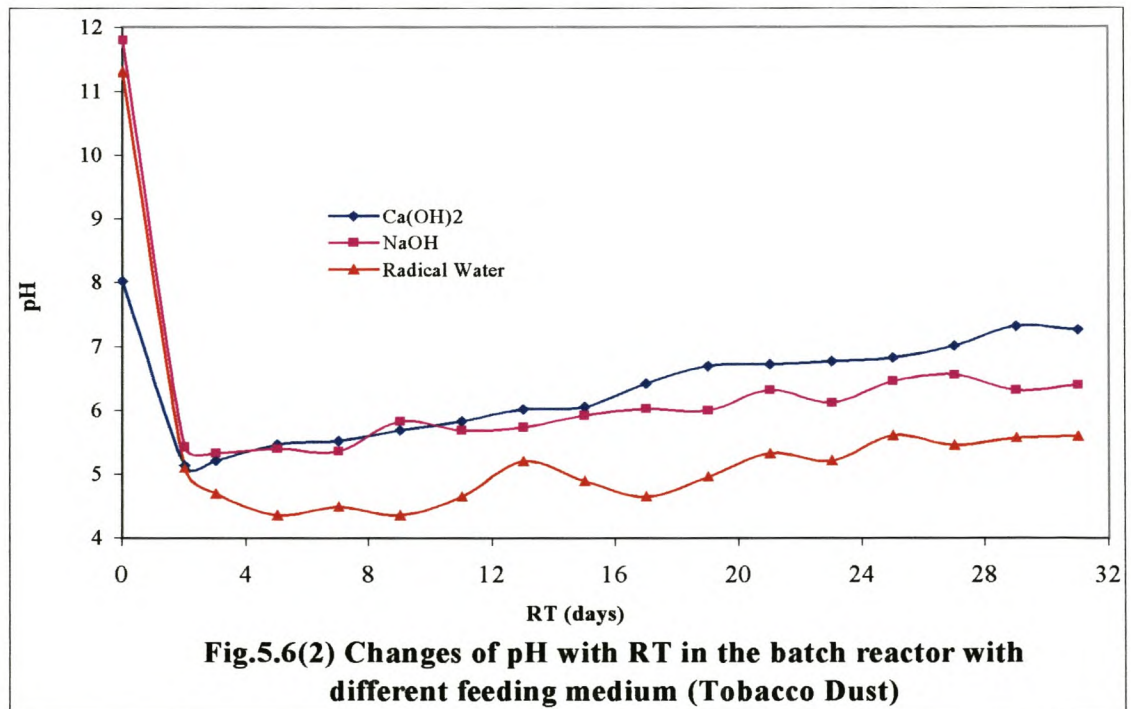
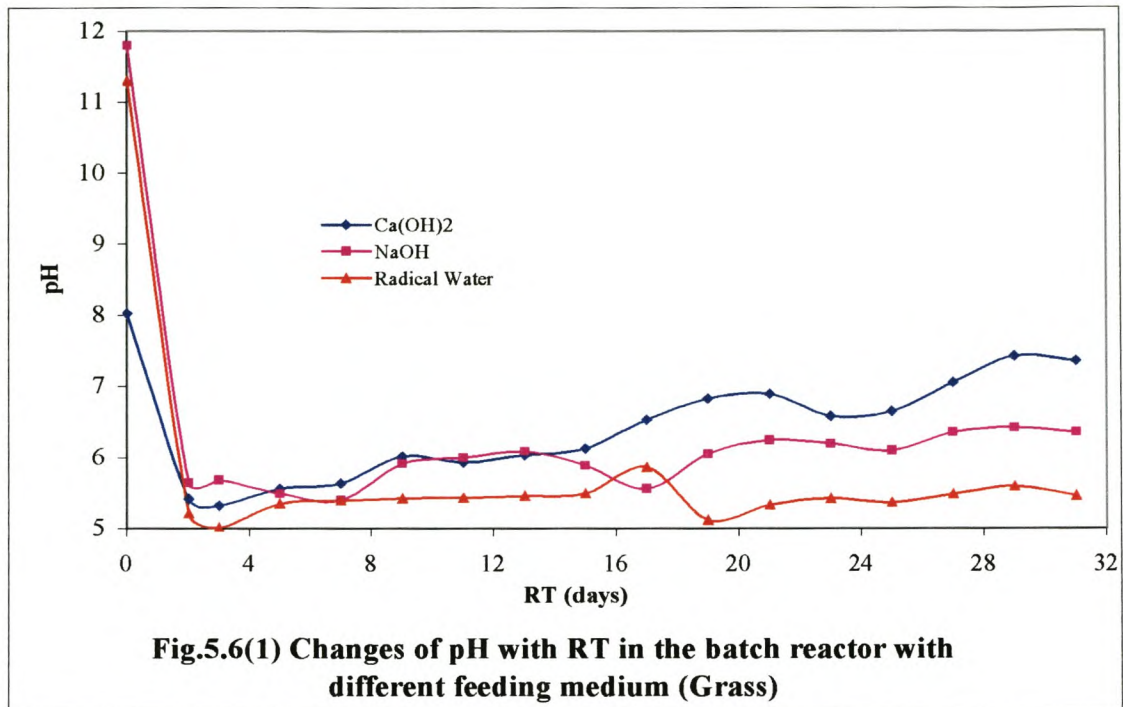


Promotion of Anaerobic Digestion of Lignocellulose with Activated Sludge
(The Acidogenic Fermentation Process)

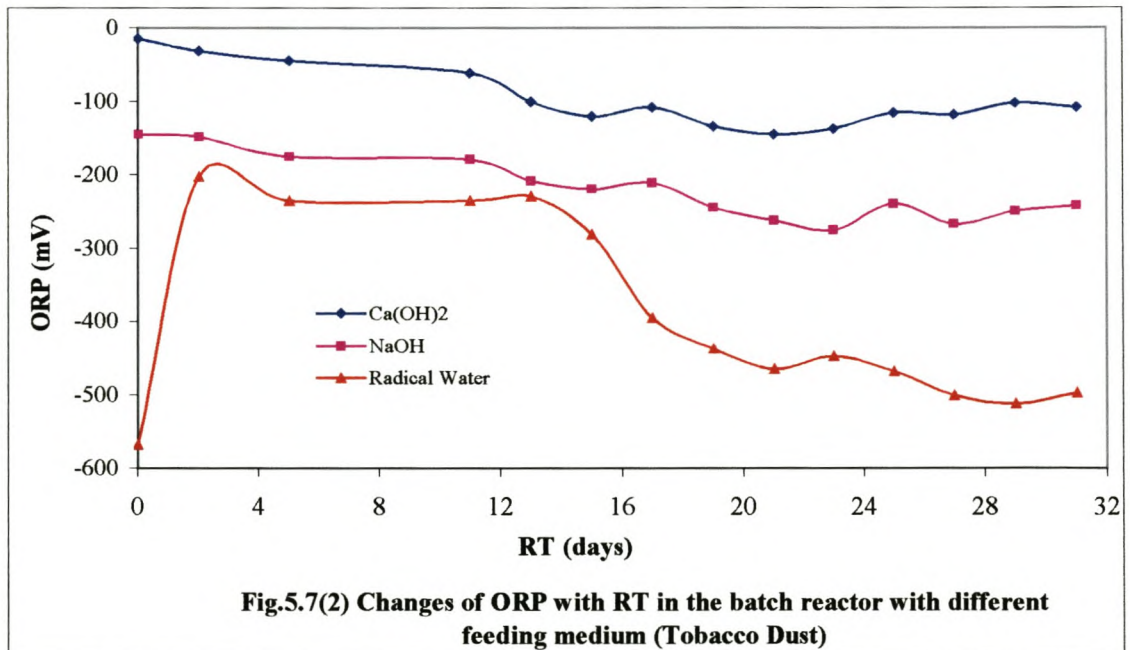
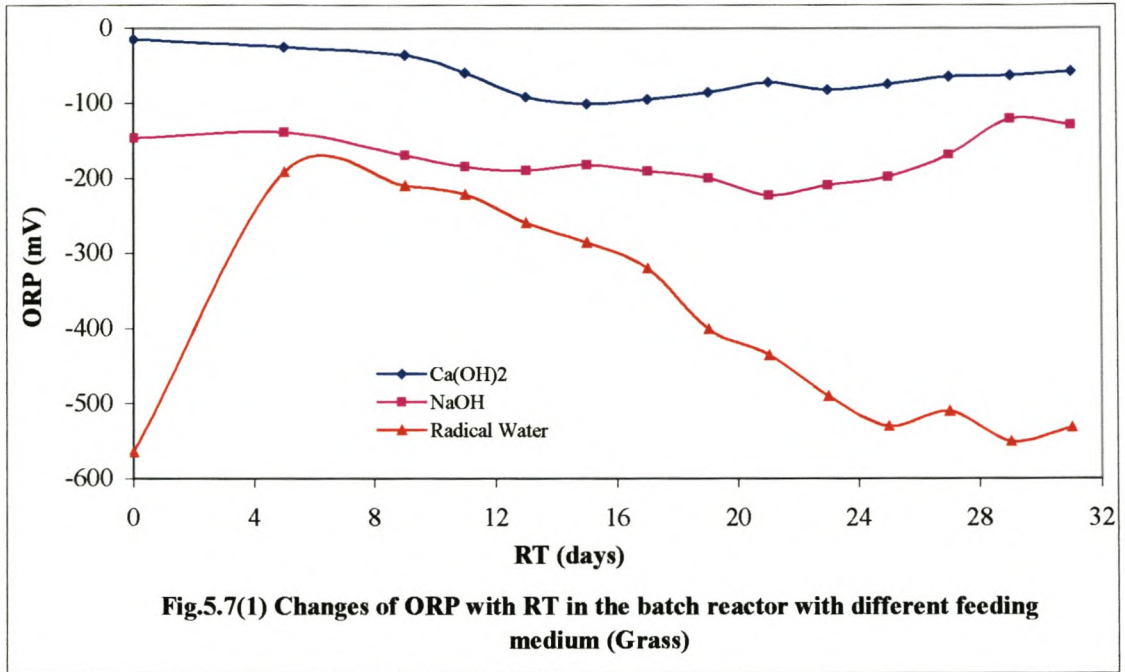




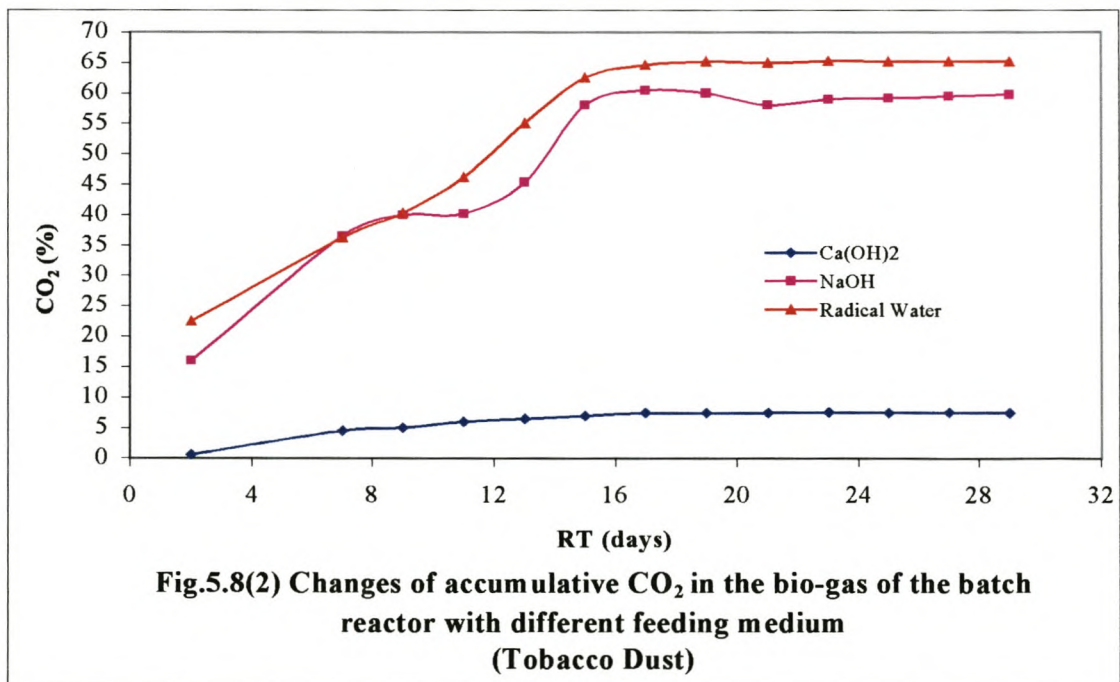
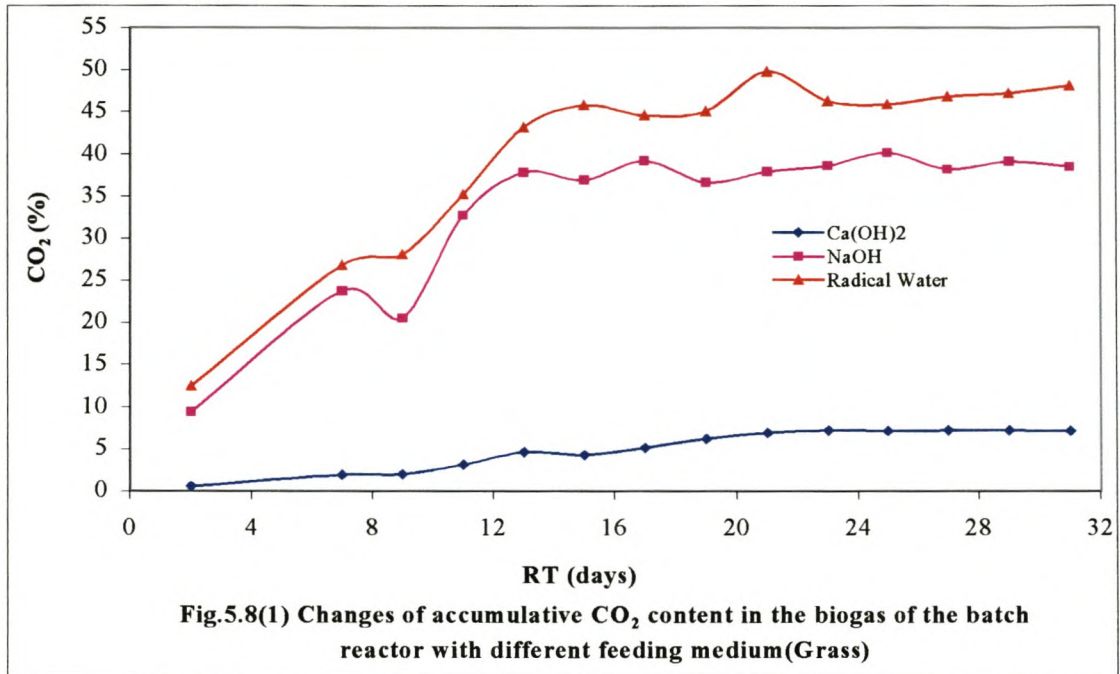
Promotion of Anaerobic Digestion of Lignocellulose with Activated Sludge
(The Acidogenic Fermentation Process)



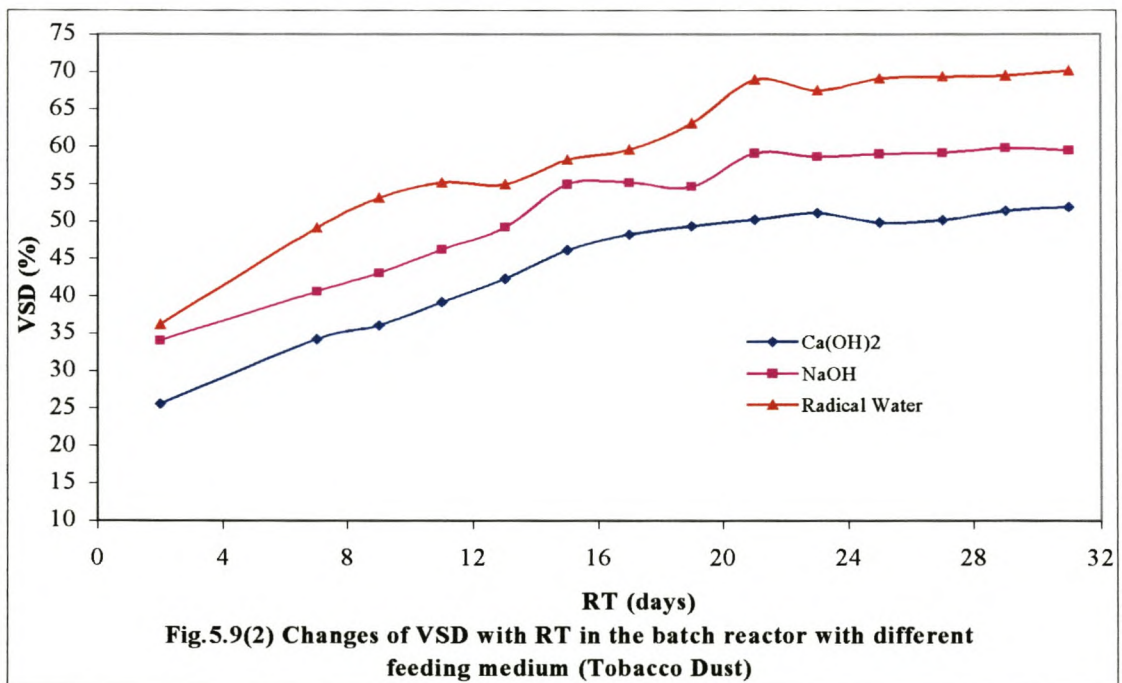
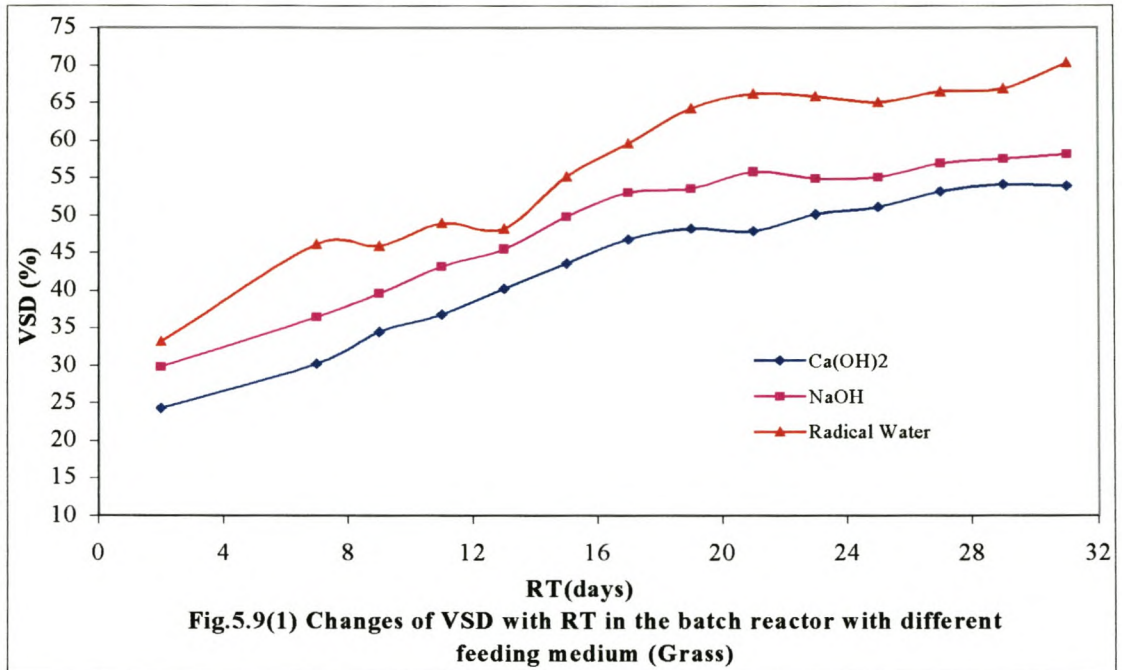
Promotion of Anaerobic Digestion of Lignocellulose with Activated Sludge
(The Acidogenic Fermentation Process)



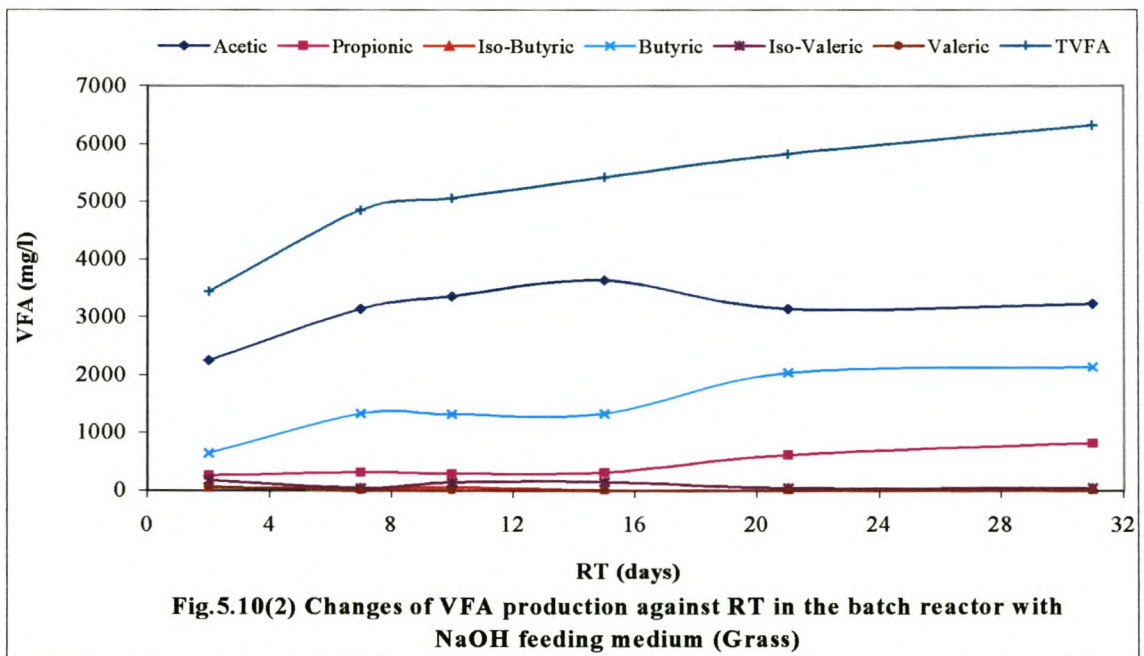
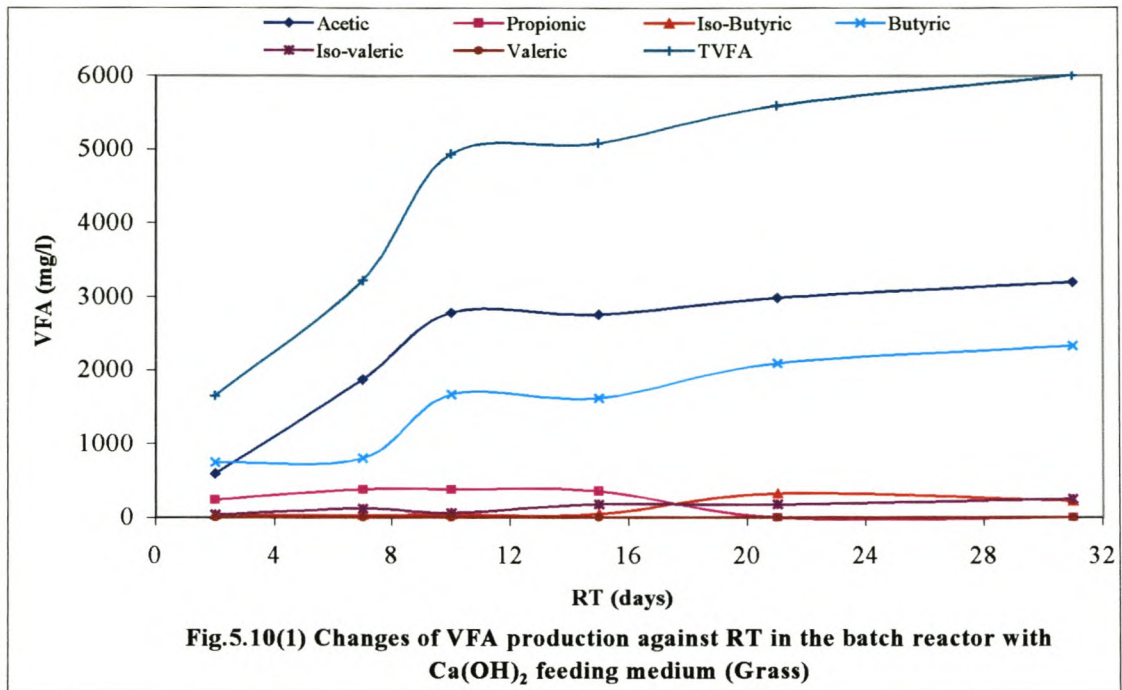
Promotion of Anaerobic Digestion of Lignocellulose with Activated Sludge
(The Acidogenic Fermentation Process)



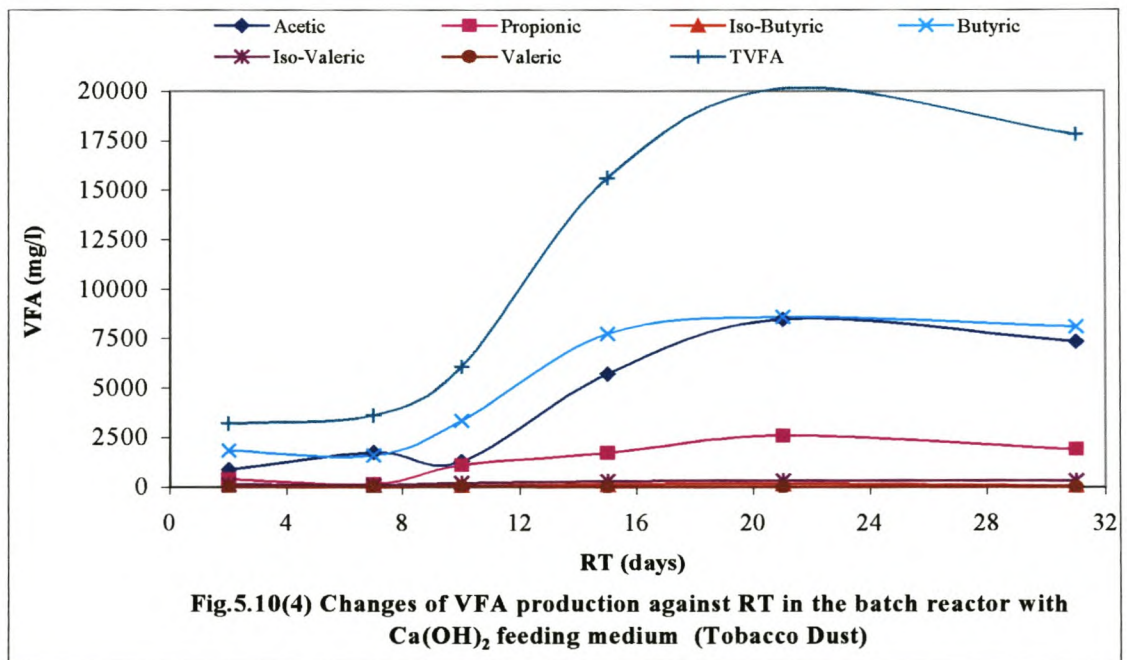
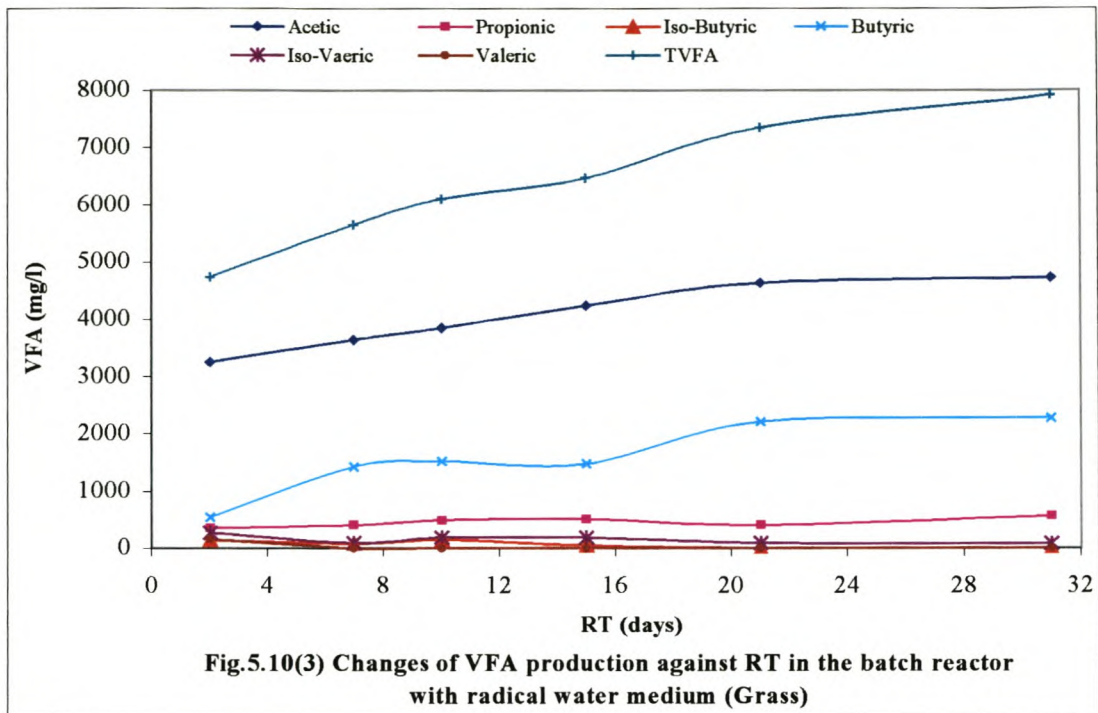
Promotion of Anaerobic Digestion of Lignocellulose with Activated Sludge
(The Acidogenic Fermentation Process)



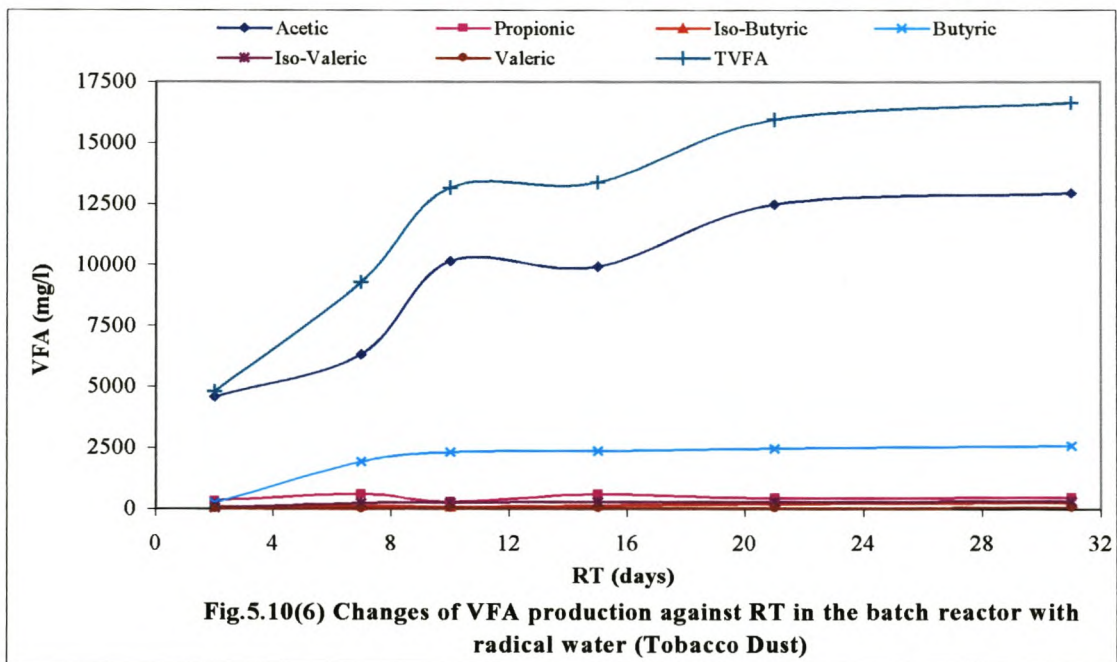
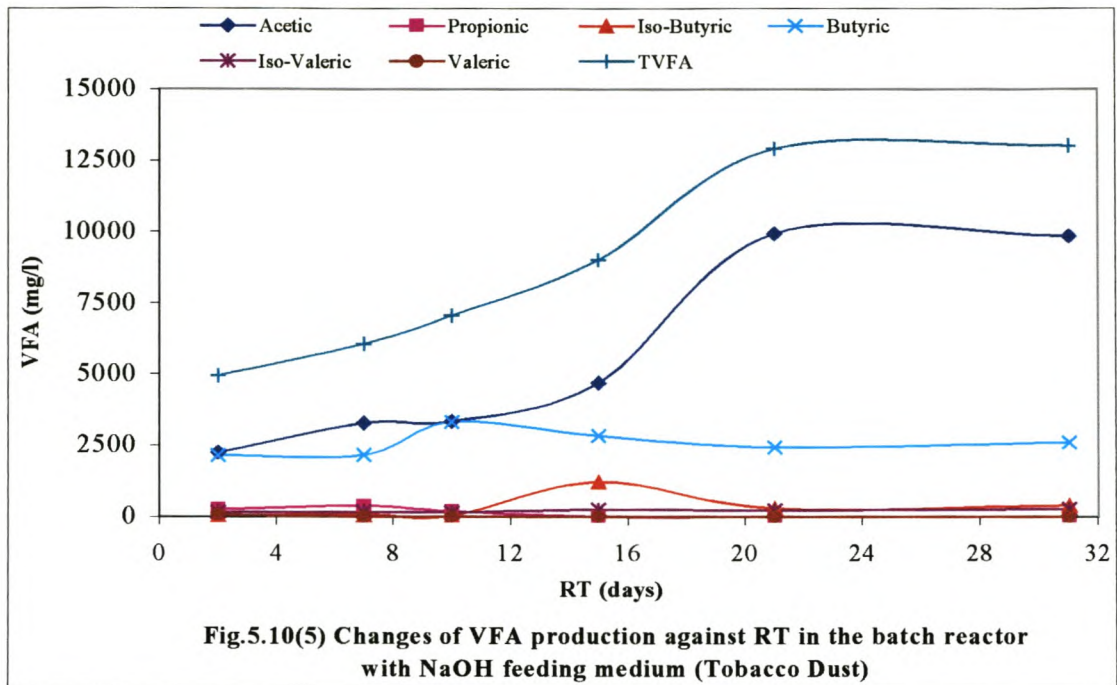
Promotion of Anaerobic Digestion of Lignocellulose with Activated Sludge
(The Acidogenic Fermentation Process)



Promotion of Anaerobic Digestion of Lignocellulose with Activated Sludge
(The Acidogenic Fermentation Process)



Promotion of Anaerobic Digestion of Lignocellulose with Activated Sludge
(The Acidogenic Fermentation Process)



Chapter 6

Anaerobic Digestion of Lignocellulose Substrate with Activated Sludge (Recovery of Methanogenic Phase from High-rate Acidification Fermentation Phase)

In Chapter 5, the combined pre-treatment method was found to be efficient in increasing the solubility of the lignocellulose residues, thus improving the efficiency of the acidification fermentation process. From the VFA production and the VSD point of view, the acidification process was successful under the experimental conditions. However, partially due to the low pH value and the high concentration of VFA produced in the high-rate acidification fermentation process, the methanogenesis was obviously inhibited at this stage. Thus, in this chapter, the recovery of a methanogenic phase from a high-rate acidification fermentation phase was studied in a semi-continuous module reactor under methophilic conditions. The experimental details can be seen in Chapter 3. The effects of the C/N ratio of the substrates in head loading, the pH and the dilution rates of the recycling leachate on the recovering process were extensively evaluated under methophilic conditions in order to relieve inhibition effects.

6.1 Effects of C/N ratio of the head feeding substrate on the denitrification process in the acidogenic fermentation stage before the development of the methanogenic stage

During the acidification fermentation process some components of the hydrolyzed-carbons in the substrate are oxidized to carbon dioxide and others are reduced to short-chain VFA during the process of releasing electrons or forming hydrogen. From the complete sludge stabilization and energy recycling points of view, further methanogenic fermentation is without a doubt preferable to any other anaerobic processes. However, in the presence of nitrate, denitrifying bacteria will reduce nitrate to nitrogen using a wide variety of substrates for supplies of carbon and electrons. Furthermore, referring to the data of Gibbs free energy changes in the bio-chemistry process ⁽⁵⁴⁾, the denitrification process, with a free energy change of -1121kJ , is thermodynamically much more favourable than the methanogenic process with a free energy change of -113 kJ . Thus denitrifiers compete with methanogens for the carbon substrates (acetate) and hydrogen from the acidification process, especially at the “Bottle Neck” acidification stage featuring the low pH and high H_2 content (H^+ and H_2

accumulation). It can be seen that the denitrification process was unavoidable during the anaerobic digestion of nitrate-bearing organic substances. However, it is possible to curtail the nitrification process to favour the development of the methanogens in the anaerobic digestion process by providing a proper C/N ratio for the feeding substrate. The interactions of methanogens and denitrifiers in the treatment of phenol-containing waste waters were investigated in a UASB reactor using a continuous experimental module⁽⁸⁰⁾. It was found that methanogens only occurred at chemical oxygen demand (COD) /NO₃⁻ -N ratios greater than 3.34. At ratios less than 3.34, methanogenesis ceased to take place and denitrification became incomplete because of an insufficient supply of substrate. Oh et al.⁽¹⁸⁶⁾ studied the acetate limitation and nitrate accumulation during the denitrification. They found that if acetate was added to the denitrifying activated sludge mixture to obtain a carbon-to nitrogen (C/N) ratio in the range of 2:1 to 3:1, nitrate was completely consumed at the same rate with no nitrite accumulation, indicating that the nitrate concentration controlled the respiration rate as long as sufficient substrate was present. The results also suggested the higher toxicity of the nitrite on the methanogenesis process when acetate was scarce. Therefore an optimum C/N ratio of the feeding substrate is also an important factor to secure the success of methanogenesis process.

Most of the investigations were carried out on waste waters and simple and pure carbon sources (such as acetate, ethanol, sucrose and phenol) were used. It can be however postulated that the competition between denitrifiers and methanogens for the carbon source and electrons also existed during the high solid anaerobic digestion of lignocellulose residues, which would contain high proportions of nitrate, (e.g. the original component or the bio-oxidation of the ammonia component of the bio-polymers before anaerobic digestion). As it is difficult to calculate the available carbon source (such as acetate, ethanol, butyrate and phenol) in lignocellulose and activated sludge for an accurate determination of C/N ratio of the feeding materials at the beginning of the digestion, it could be more practical to use the total carbon source instead of the available carbon in the case of high-solid anaerobic digestion of lignocellulose residues.

Therefore, the C/N ratio in this study was expressed as the total carbon-C/N-(NO₃) ratio by mixing the high nitrate content lignocellulose residues (tobacco dust, 308 mg/kg dry base) with the low nitrate lignocellulose residues (dry grass, 10 mg/kg dry base). This C/N ratio and

the RT were simultaneously considered to investigate the denitrification process using a series of laboratory-batch-acidification reactors (seen in Chapter 3) by monitoring the developed N_2 (one of the endproducts) proportion in the bio-gas of the reactor which was flushed with oxygen free gas, (92% Helium and 8% Nitrogen gas mixture), at the beginning of the digestion. The optimum C/N (NO_3) ratio obtained from the batch reactors was then applied to the primary feeding substrates for the two-phase methanogenic stabilization digester. The accumulative N_2 and CO_2 contents in the bio-gas composition are presented in Fig.6.1 and Fig.6.2 respectively. The methane content was found to be very low throughout the experimental time and methane detected in the bio-gas after 5 weeks of digestion was less than 2%. This implies that the methanogens were still inhibited at this stage. The pH, ORP and TVFA contents at the end of the digestion period (on the 70th day of RT) are presented in Table 6.1.

The results in Fig.6.1 show that both the C/N ratio of the substrate in the head feeding and the RT of the acidification process influenced the occurrence and the extent of the denitrification. The denitrification normally occurred after two weeks of digestion and the extent of the denitrification (N_2 content in the bio-gas) increased with increases of the RT depending on the C/N ratio. The lower the C/N ratios, the longer RT were needed to complete the denitrification (N_2 content in the bio-gas remained stable).

The results in Fig.6.2 and Table 6.1 show that the C/N ratio also influenced the performance of the acidification process. The patterns of VFA composition and the changing trends of VFA fractions appeared similar with the C/N ratio of the primary feed increasing from 31×10^2 to 91×10^2 , although the iso-butyric acid content was notably higher than the propionic acid content when the C/N ratio was 16×10^2 . This difference might be ascribed to some differences in the acidification properties between the two kinds of lignocellulose materials used (grass/tobacco dust). At higher C/N ratios, the lignocellulose was more homogeneous in composition, therefore the patterns of VFA composition and the changing tendency of VFA fractions appeared similar at higher C/N ratios of the head feeding. In addition, there was a steady build-up of the acetic acid with the increases of the C/N ratio. Since all the other VFAs, such as propionic, butyric and valeric acids, should have been converted into acetic acids before they can be used by denitrifiers or methanogens as carbon sources, the

accumulation of acetic acid at higher C/N ratios could be attributed to the lack of methanogenic metabolic activity.

At the same RT, higher TVFA and CO₂ contents were observed in the reactor with higher C/N ratio. The relatively higher ORP (-186 mV in reactor 1, compared to -297 mV in reactor 4) in the reactor with a lower C/N ratio was probably due to nitrite accumulation, thus implying an incomplete denitrification. However, the higher accumulation of propionic and butyric acids was encountered in reactor 3 and reactor 4 with higher C/N ratios, which indicates the inhibition of acetogens by the acidification products accumulated in these reactors. It could be postulated that both incomplete denitrification and severe acidification inhibit the methanogenesis process; the quicker and the more complete the denitrification and the less rigorous the acidification are, the easier and the faster the turnover of acidification phase to methanogenic phase. At the lower C/N ratio, the incomplete denitrification was the dominant inhibitor; at the higher C/N ratio, the accumulation of intermediary acid products was the dominant inhibitor. In another words, a mild denitrification was beneficial to the acidification process in that denitrification could “clean up” some acidification products, thus promoting the acidification process. Therefore, in order to develop a “healthy” integrated acidogenic and methanogenic digestion process, the C/N ratio in the head feeding substrate should preferentially be taken into consideration. Under the given experimental conditions of this study, the total Carbon-C/N-(NO₃) ratio in the range of 31×10^2 to 63×10^2 was recommended. The C/N-(NO₃) ratio of 63×10^2 was used in the rest of the experiments in this study. At this C/N-(NO₃) ratio, 18 days of RT was chosen for the acidification stage.

6.2 Recovery of the methanogenic stage by adjusting the pH and dilution rates

The anaerobic conversion of organics to methane is a complex process involving many species of bacteria. The methanogens are the most sensitive to environmental changes such as pH, and the inhibitory factors such as the build-up of highly concentrated volatile fatty acids formed from the faster acidification under thermophilic condition; denitrification; the low pH and high content of unionized acids or other concentrated toxicants that are often encountered in a high-rate acidification digester. As a result, the growth of methanogens was seriously

restrained in the high-rate acidification digesters. It can be seen that besides the C/N-(NO₃) ratios of the head feedings, the pH and dilution rates must to be carefully adjusted to prevent inhibition of the methanogenic process by the low pH, a high content of unionized acids or other concentrated toxicants.

The experiments were carried out in a semi-continuous methophilic digestion module. Under the given initially-controlled pH and the dilution conditions, the changes in pH, ORP of the digester leachate and bio-gas composition were determined in the course of the digestion time. The results are given in Fig.6.3 to Fig.6.7. The bio-gas yields (m³/kg VS added) on the 25th day of methophilic digestion are given in Table 6.2.

As expected, the methanogens were recovered to some extent under the pH adjustment and leachate dilution conditions (Fig.6.5). Compared to the results from the control reactor and so far as the methane yields in the bio-gas product were concerned, the recovery of the methanogenic phase from the acidification phase was satisfactorily achieved by adjusting the pH and diluting the inhibitory components.

The results in Table 6.2 show that the dilution rate had a greater influence on the bio-gas yield than the pH for the same digestion time. It is suggested that when the pH was controlled in the range from 7.2 to 8.2, the dilution rate had a stronger influence on the development and growth of methanogens. In addition, from Fig.6.3 and Fig.6.4, it can be seen that there were no marketable changes in pH and ORP values during the digestion time for different dilution rates. This suggests that a high pH buffering capacity exist in the digestion system. The digestion system has the ability to resist the pH vibration resulting from dilution or from microbial activities. This indicates that the inhibitory effect from concentrated unionized volatile acids or other more complicated organic toxicants were more detrimental to the growth of methanogens.

According to the results from the control reactor, adjusting the pH of the leachate from the acidification phase above 7.0 was essential in developing the methanogenic phase in the

digester; this pH value could go up to 8.0. This is confirmed by the results in Fig.6.5 to Fig.6.7. It is shown that there was no great difference in the composition of the bio-gas between these two pH value conditions, although the bio-gas product was slightly lower under pH 8.2 than under pH 7.2.

Diluting the intermediary acidification products through recycling showed positive effects on both the acidogenic and the methanogenic phases. It was found that the CO₂ content of the bio-gas was much higher during the initial stage of the methanogens digestion with the dilution process than that without the dilution process. The CH₄ content generally increased with increases in the dilution rates and the RT. However, when the dilution rate increased from 1:5 to 1:7, very little increase in CH₄ production was obtained. This suggested that the dilution process could alleviate the acids-accumulation inhibition and reduce the concentrations of the intermediary acidification products (such as dissolved phenolic and humic acids related to lignin) which presented toxicity to the methanogens. Simultaneously, the dilution process decreased the availability of the nutrients for the growth of the micro-organisms in the given digestion time. The inhibition of acid accumulation and the toxicity of intermediary acidification products normally occurred shortly after the high-rate thermophilic acidogenic digestion. After the methanogens had established, a sufficient quantity of the substrate (such as acetic acid, CO₂) needed to be efficiently provided. Thus, there is an optimum dilution rate and a high dilution rate would not always lead to the improvement of the methanogenic phase by prolonging RT.

The denitrification was not completely washed out by a high-rate acidification digestion. It was still encountered at this stage. However, with the RT prolonged, the denitrification process quickly leveled off after 10 days of methanogens acclimation (Fig.6.7).

6.3 Summary

- The thermophilic high-rate digestion with high solids loading may accelerate the hydrolysis and acidification digestion process of the lignocellulose bio-polymers.

However, thermophilic anaerobic micro-organisms are sensitive to environmental changes and it is easy to accumulate the intermediate acidification products and concentrate other toxicants in the high-rate digestion process. The preliminary studies in this chapter demonstrated that diluting the high-rate acidification system and adjusting the pH of the recycled leachate could recover the methanogenic phase from the high-rate acidogenic phase.

- The variance in the pH of the recycling leachate between 7 and 8 did not present a marked influence on the establishment of the methanogens. This implied a pH buffering capacity in the acidification digestion sludge system.
- Dilution could alleviate the acid-built up inhibition and reduce the concentrations of the toxic compounds such as lignin related phenolic and humic acids, which were produced in the process of digestion. Simultaneously, dilution decreased the availability of the nutrients to the growth of the micro-organisms in the given digestion time; thus a higher dilution rate did not always generate the positive effects on the methanogenic phase under a longer RT. An optimum dilution rate exists.
- The integrated digestion configuration composed of a high-rate thermophilic acidogenic stage followed by a dilution-pH/ORP-controlled-mesophilic stage could improve the biotransformation of lignocellulose residues to the methanogenic phase and thus ultimately stabilize the digestion system.
- The results also show that the denitrification during the anaerobic digestion of nitrate-containing lignocellulose residues was unavoidable; there was competition between the denitrifiers and methanogens for the carbon sources. Increasing the C/N ratio of the substrate in the head loading could promote the accomplishment of the denitrification process and shorten the methanogens developing process. In the case of high solid anaerobic digestion of lignocellulose residues, the bio-available carbon C in the lignocellulose was difficult to calculate precisely. The C/N ratio in this case was termed as

total carbon-C/N-(NO₃) for practical purposes. A suitable total carbon-C/N-(NO₃) ratio for the head feedings was another important factor to be adjusted for the development of the methanogenic phase.

In addition to the environmental factors, the optimum digestion is also dependent on the microbial populations functioning in the system. Examinations of the distribution and diversity of microbial populations are essential to understand the microbial reactions behind the bio-conversion process under the given experimental circumstance and this may contribute to the effective digestion of the target substances. The microbial investigation of the microorganisms in the anaerobic digestion system described in this chapter and in Chapter 5 will be discussed in Chapter 7.

Table 6.1 The acidification digestion results, (pH, ORP and TVFA), with different C/N $-(NO_3)$ ratios on the 70th day of RT

Reactor	1	2	3	4
C/N (NO_3)	$16 \cdot 10^2$	$31 \cdot 10^2$	$63 \cdot 10^2$	$91 \cdot 10^2$
pH	6.1	5.8	5.1	4.9
ORP(mV)	-186	-209	-291	-297
TVFA(mg/l)	8986.33	10364	17106.01	24132
Acetic	4680	7226.67	9930.67	11230.33
Propionic	0	610.67	2090	3265.67
Iso-Butyric	1209.67	137.33	150.67	166
Butyric	2846	2088	4608	9102.33
Iso-Valeric	251.33	301.33	326.67	368.33

Table 6.2 Bio-gas yield (m^3/kg VS added) and CH_4 content on the 25th day of methophilic digestion

pH controlled	7.2	7.2	7.2	8.2	8.2	8.2
Dilution Rate	1:3	1:5	1:7	1:3	1:5	1:7
Gas Yield (m^3/kg VS added)	0.084	0.113	0.102	0.072	0.107	0.098
CH_4 Content (%)	37.2	51.9	44.2	35.1	48.5	39.4

Anaerobic Digestion of Lignocellulose Substrate with Activated sludge
(Recovery of Methanogens Phase from High-rate Acidification Fermentation Phase)

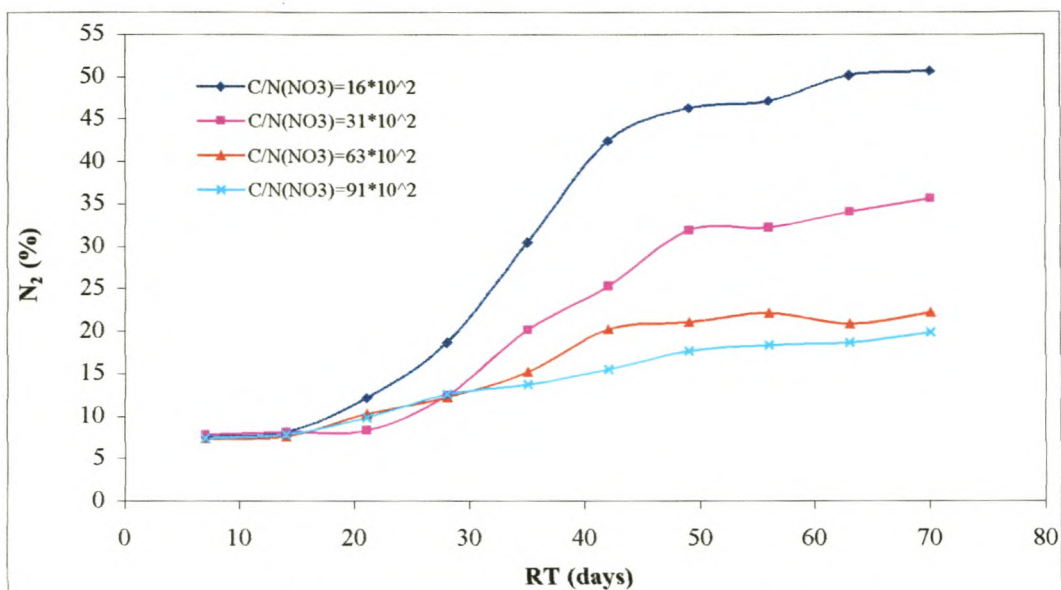


Fig.6.1 Effects of C/N(NO₃) ratios on the accumulative N₂% of the bio-gas in the acidification batch reactors

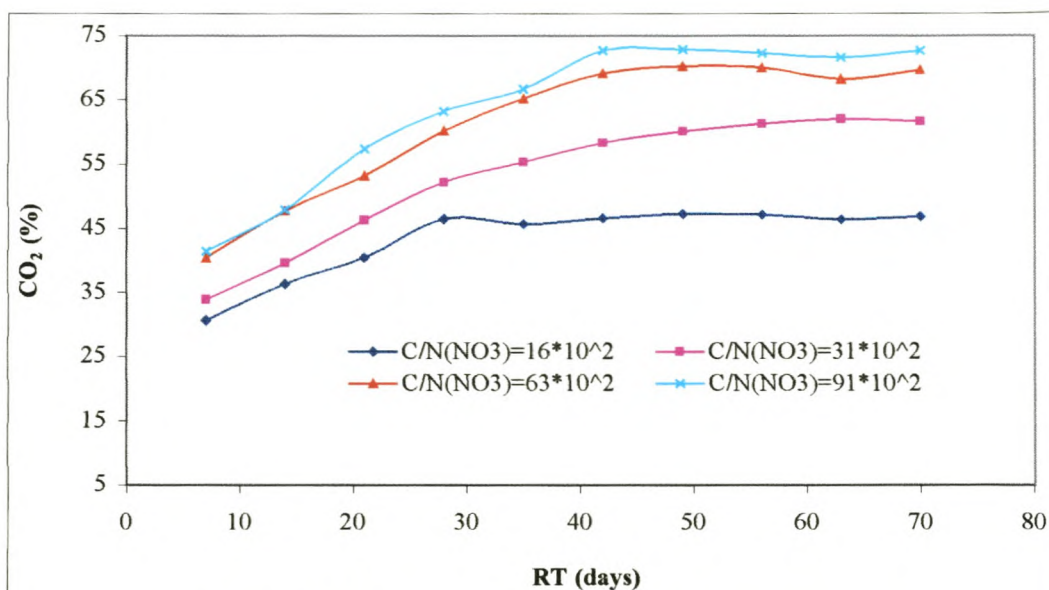
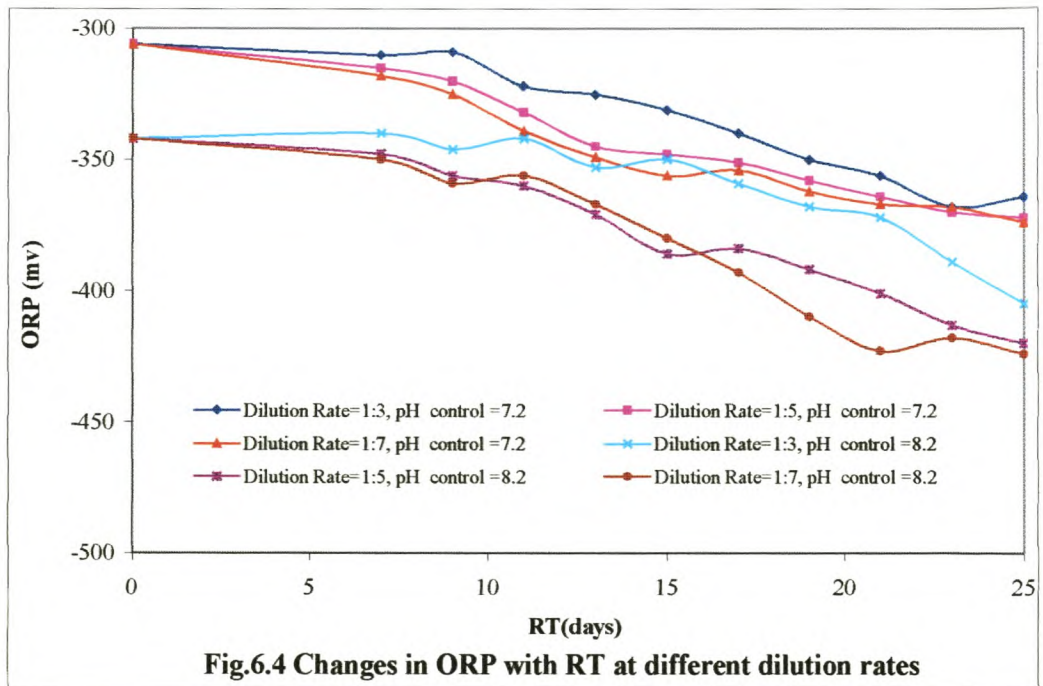
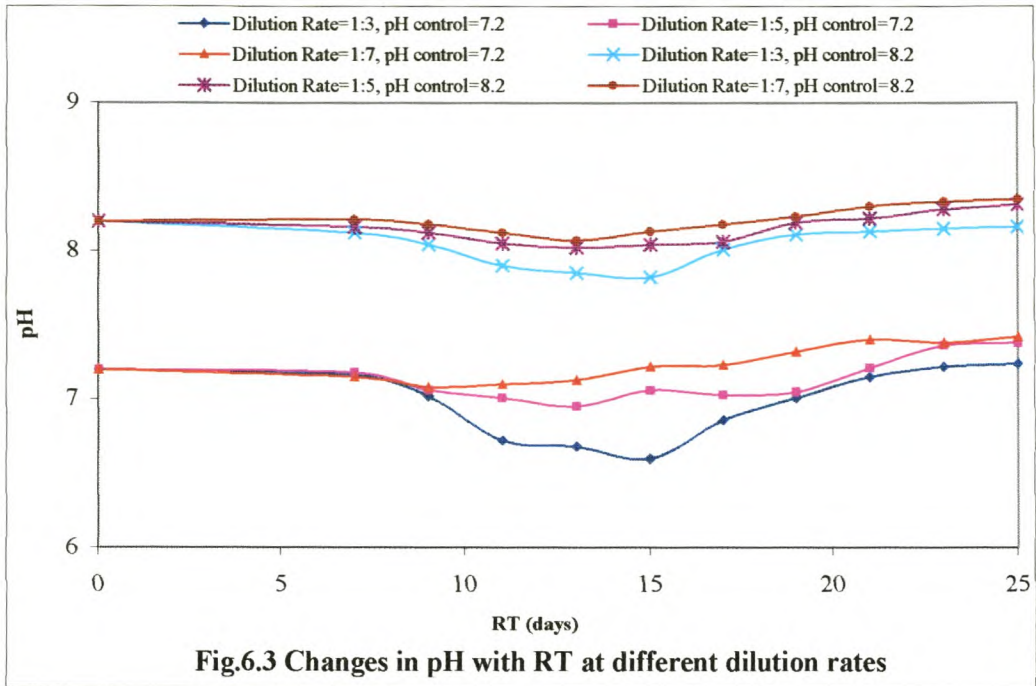
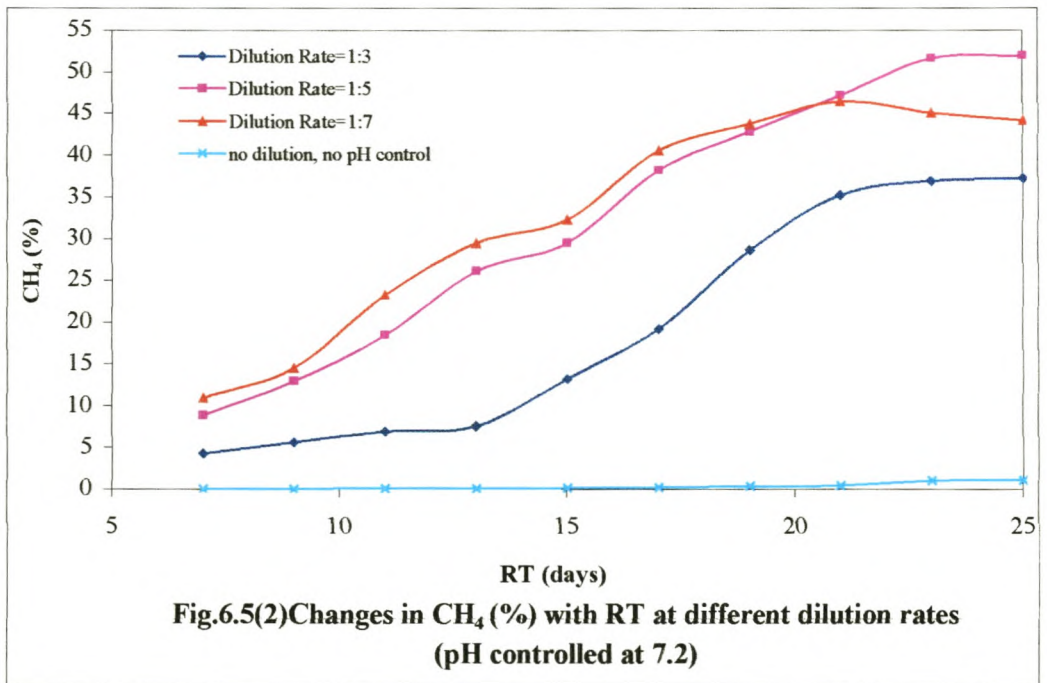
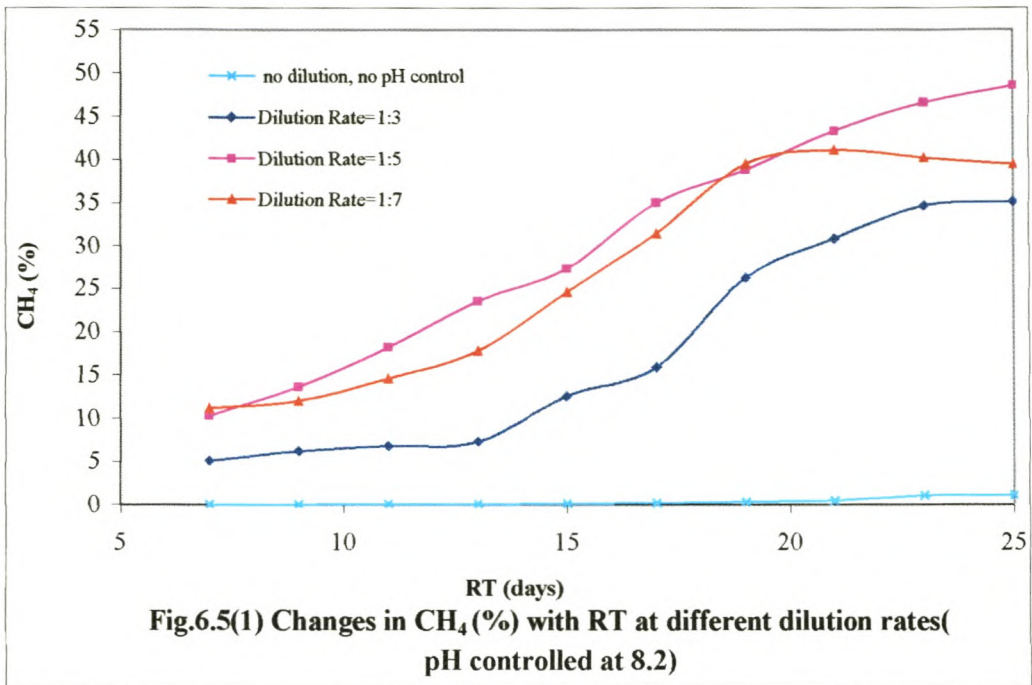


Fig.6.2 Effects of C/N(NO₃) ratios on the accumulative CO₂% of the bio-gas in the acidification batch reactors

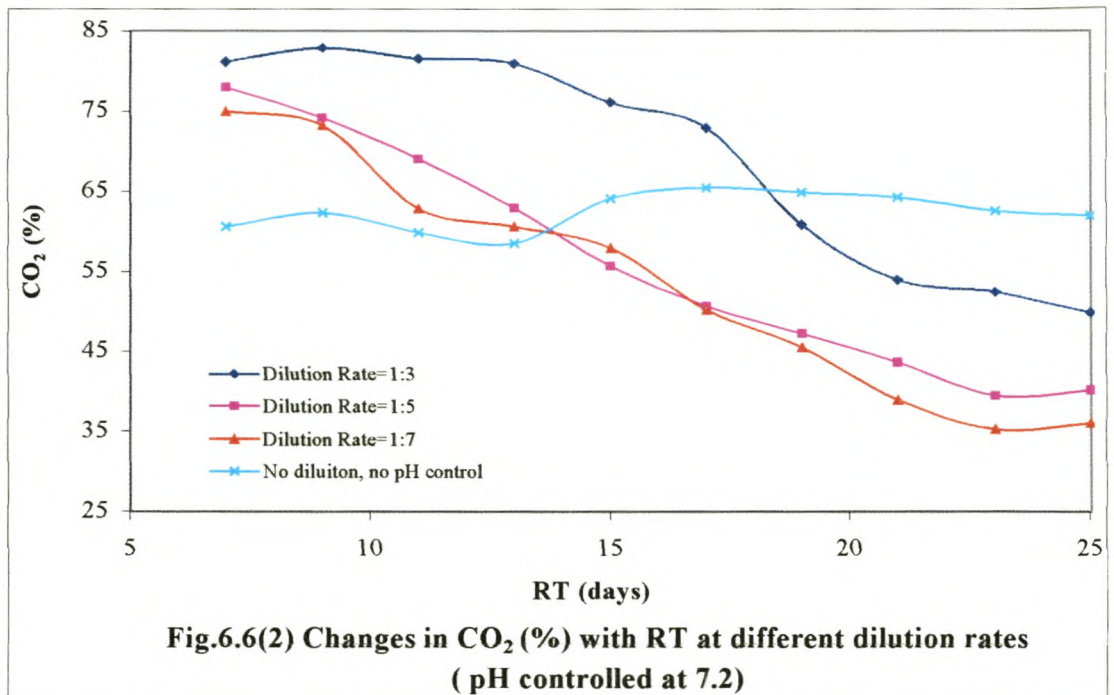
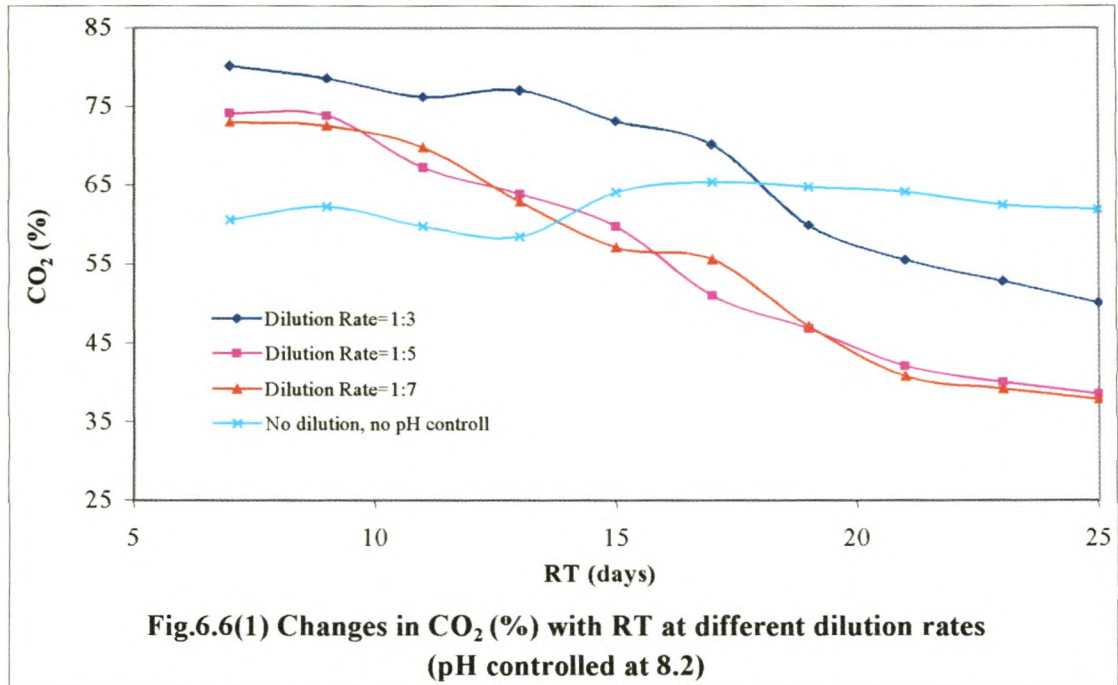
Anaerobic Digestion of Lignocellulose Substrate with Activated sludge
(Recovery of Methanogens Phase from High-rate Acidification Fermentation Phase)



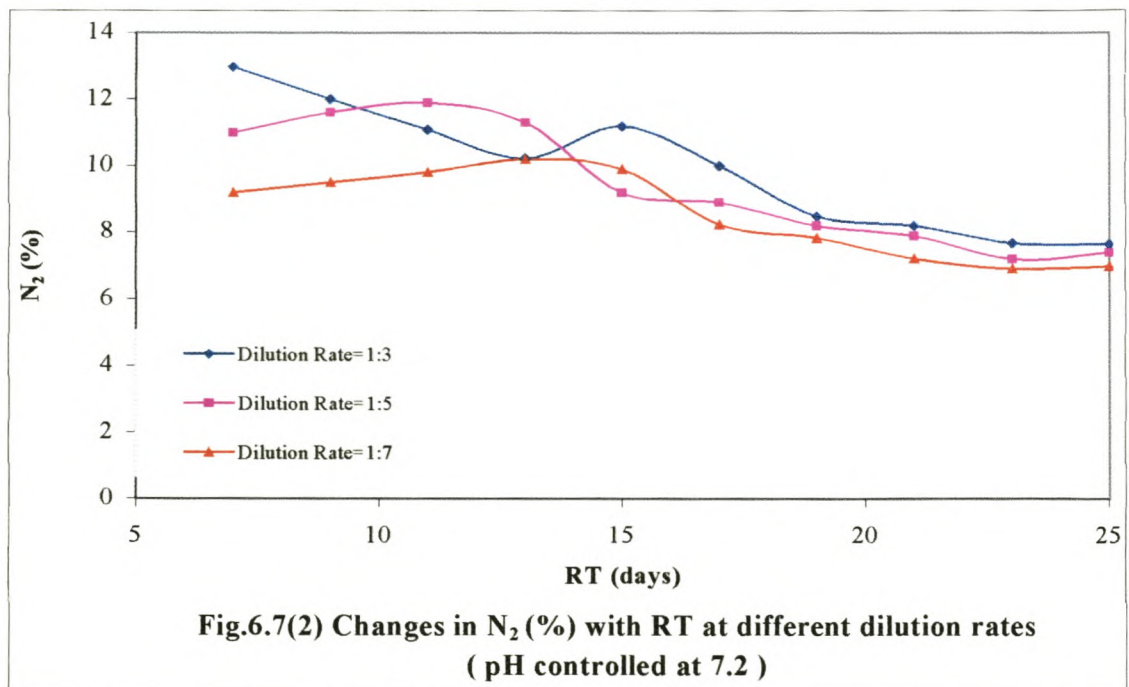
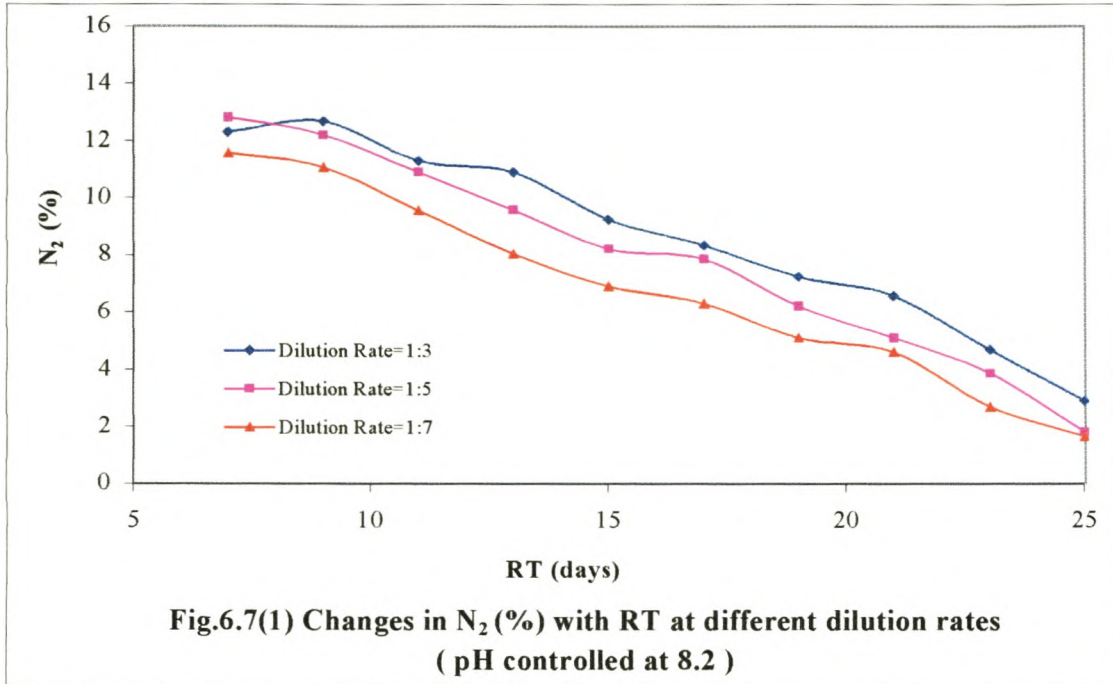
Anaerobic Digestion of Lignocellulose Substrate with Activated sludge
(Recovery of Methanogens Phase from High-rate Acidification Fermentation Phase)



Anaerobic Digestion of Lignocellulose Substrate with Activated sludge
(Recovery of Methanogens Phase from High-rate Acidification Fermentation Phase)



Anaerobic Digestion of Lignocellulose Substrate with Activated sludge
(Recovery of Methanogens Phase from High-rate Acidification Fermentation Phase)



Chapter 7

The Microbial ecosystem in Anaerobic Digestion of Lignocellulose Residues

As stated in Chapter 2, the anaerobic ecosystem is the result of complex interactions among micro-organisms of several species ⁽¹⁹⁵⁾, which are: 1) hydrolytic and acidogenic bacteria; 2) syntrophic acetogenic and homoacetogenic bacteria; 3) hydrogenotrophic bacteria; and 4) acetoclastic methanogenic bacteria. Generally, the dominant digester organisms can be functionally grouped into VFA producers (acidogenic fermentative bacteria) and VFA converters (acetogenic and methanogenic bacteria).

Methanogenic fermentation is a multistage process ^(185, 54). Because of its critical role in the gasification and stabilization of bio-solids, the methanogens in anaerobic digesters have been extensively studied in the past years ^(274, 124, 272, 155, 275). At the same time, the investigations directed at the acidogens were also reported ^(32, 41). However, in addition to the obligatory anaerobes, other microbial consortia may also be present in the digestion system, especially in the first acidification fermentation phase, and some other micro-organisms may have additional important roles in the initial stage of fermentation. According to Hungate ⁽¹²³⁾ and Toerien and Hattingh ⁽²⁴³⁾, for example, there are a small number of protozoa, fungi and yeast in fermentation bio-digesters. One recent study ^(30, 31) also reported that anaerobes could survive in planktonic and biofilm communities in aerated conditions when part of the communities included facultative or aerobic species, and the numbers and proportions of anaerobic species increased as the biofilm aged; the oral anaerobes cannot survive oxygen stress without interacting with facultative/aerobic species as a microbial community.

Further, in the digestion of complex bio-polymers (such as lignocellulose residues) all the way to the end-product methane, the rate-limiting process is the hydrolysis of the complex bio-polymers. The pre-treatment of lignocellulose residues (usually includes thermal, thermochemical, alkaline and ultrasonic methods) was necessary to enhance the hydrolysis efficiency and thus ultimately improve the methane fermentation efficiency. However the influence of the initial non-biological conditions resulting from the pre-treatment procedure, such as the initial pH/ORP and the temperature, on these microbial populations was not very clear. Therefore, in this chapter, an investigation of the growth of microbial populations was conducted to evaluate the effects of the initial pH/ORP of the feeding medium, the digestion

RT and the temperature on the composition of the microbial ecosystems developed in acidification and two-phase digesters treating lignocellulose residues with activated sludge.

The microbial samples from three kinds of laboratory reactors were analyzed: 1) An anaerobic acidification fermentation batch reactor treating the mixture of tobacco dust and activated sludge (1:2 wet weight base) at the TS_0 and VTS_0 (% TS_0) of 21-23% and 57.80% , the operation TS of 11-12%, the maximum RT of 31 days, operating at thermophilic (50-55 °C) and mesophilic (30-37 °C) conditions, respectively. 2) Anaerobic two-stage semi-continuous acidification reactors treating mixture of Tobacco Dust and Activated Sludge (1:2 wet weight base) at the TS_0 and VTS_0 (% TS_0) of 21-23% and 57.80%, the operation TS of 11-12%, the maximum RT of 28 days, operating at thermophilic (50-55 °C) and mesophilic (30-37 °C) conditions respectively, with leachate re-circulating. 3) Anaerobic semi-continuous two-phase integrated reactors for recovering the methanogenic phase from the high-rate acidification stage, treating a mixture of tobacco dust, grass and activated sludge (1:5:2 on the wet weight base) at the TS_0 and VTS_0 (% TS_0) of 21-23% and 71.74%, the maximum RT of 43 days, operating at mesophilic (30-37 °C) conditions, with leachate re-circulating and a dilution rate of 1:5. The digestion experimental set –up and microbiological analysis methods can be found in chapter 3.

The viable microbial organism counts in three types of laboratory digesters under different digestion conditions are given in table 7.1. The production and composition of VFA in digester 1 can be seen in Fig.5.5(2) and Fig.5.10(5); the production and composition of VFA in digester 2-1 under thermophilic conditions can be referenced in table 5.2. The bio-gas yield (m^3/kg VS added) and methane content in digester 2-2 were 0.113-0.139 and 48.5%-52.5%, respectively.

7.1 The effects of RT on the growth of complex microbial populations in the digester.

The results in table 7.1 generally indicate that over the digestion time, the number of aerobic microbes decreased in all the systems while the aerobic colonies were washed out of the system and the anaerobic counts increased and became dominant in the microbial community of the digestion system. The facultative counts were relatively stable throughout 2-4 weeks of

digestion, although some increase in the methanogenic phase of the digester 2-2 was witnessed.

The population of the acidogens changed in the same manner as the population of the total bacteria over the RT of the digester. This suggests that the acidogens were the principal microbes in the ecosystem of the digester.

It was found that the facultative counts were higher than the anaerobic counts during the first two weeks of digestion, which indicates that the facultative acidogens were mainly responsible for the acidification fermentation at the early stage of the digestion.

A quantity of soil actinomycetes (10^4 - 10^5), filamentous fungi (10^4 - 10^5) and yeast (0 - 10^3) were also detected in the microflora of the digesters. Higher counts were found at the early stage of the digestion. With the RT increasing, the digester became more anaerobic and these microbes gradually leveled off. This suggests that these microbes might perform the important roles in the initial digestion/hydrolysis of lignocellulose residues.

Typically, the digester community is dependent on the substrate with which it is fed and a characteristic community is likely to develop for each different substrate with which each digester is fed. Because the seeding source is from activated sludge (actinomycetes were found to be one of the most prevalent consortia in the activated sludge process) and the biomass to be digested is plant lignocellulose, it is reasonable to expect higher populations of soil actinomycetes, filamentous fungi and yeast in the younger microflora of the digesters.

In nature, bacteria rarely exists as mono-cultures. In the vast majority of environments, populations of bacteria coexist as communities, efficiently occupying all available ecological niches ⁽⁴⁾. These communities can only degrade complex substrates by acting in consortia. The results suggest that the mechanism of degradation of complex substrates such as lignocellulose is mediated by physical and/or metabolic interactions (e.g. cross-feeding) between anaerobic and facultative bacteria. Such interactions enable the survival of the degradative anaerobic species in aerated conditions, especially during the early stage of the fermentation. Furthermore, many micro-organisms can grow on solid substrates but only filamentous fungi can grow to a significant extent in the absence of free water ^(180, 232, 191). The

cultivation of filamentous fungi on solid substrates has been widely used at laboratory scale for lignocellulose fermentation ⁽²¹⁾. Bacteria and yeast can grow on solid substrates at moisture levels of 40-70%, such as in composting and in anaerobic and aerobic ensiling, but the growth and the propagation of single cell organisms always require free water. Yeast always grows on solid substrates in symbiosis with other micro-organism in composting, ensiling and some industrial solid state fermentation processes involving fruit wastes and other sugar-containing natural substances ^(163,34). The TS₀ content of our digesters was 21%-23% and much less free water was available at the start of the digestion. The relatively higher populations of soil actinomycetes and filamentous fungi detected could be attributed to their original growth from the solid states of the lignocellulose residues before the digestion. When the lignocellulose residues were hydrolysed, some yeasts were developed with the sugar-containing intermediate products. Since the mineralization (decomposition) of organic matter to non-organic molecules is essentially the degradation of particulate, soluble polymers, whatever further degradation pathway is taken to mineralize the organic matter, the first step is to hydrolyse the complex polymers to soluble molecules, often to the component monomeric units. It is after this rate-limiting step that the soluble molecules can be taken up by bacterial cells and processed further ⁽⁸²⁾. The hydrolysis of polymers takes place outside the cell membrane without any exception. The unique nutritional status of fungi is that their cells secrete extracellular enzymes which break down potential food sources which are then absorbed back into the fungal cells. This suggests that the fungi in our solid state digesters played a very important hydrolysing role as the primary decomposers in the degradation of lignocellulose.

Since yeast generally grows on sugar-containing natural substances, the evidence that the yeast was detected or not could be the signal which indicates whether sugar was produced from the ligno-polymers or was degraded into VFA during the lignocellulose digestion process.

7.2 The growth of microbial populations in the digester with the initial feeding media having different pH/ORP.

There were some differences among the digesters with different initial seeding media (tap water and 0.5 M NaOH), e.g. a higher ratio of acetic acids to butyric acids was observed in

the digester with 0.5 M NaOH as initial seeding medium than in the digester with tap water as initial seeding medium. However, there was no marked difference detected in the acidogenic populations in the microflora growing in these digesters. This suggests that either the difference in the VFA production between the digesters with different initial feeding media could be attributed to non-biological effects other than the biological effects of the feeding medium, or the total cell numbers of the microbial species are not sensitive enough to exhibit this difference. However, slightly smaller counts of total aerobic soil actinomycetes and filamentous fungi were detected in the digester with 0.5 M NaOH as initial seeding medium than with the tap water as medium. This might be explained by the fact that most aerobic microflora, including soil actinomycetes and filamentous fungi, are considered to be the indigenous micro-organisms for the lignocellulose residues fed to the digesters. They grow better in the tap water conditioned environment which is closer to their natural habitat than in the high alkaline conditioned environment with 0.5 M NaOH.

7.3 The effects of the digestion temperature on the growth of complex microbial populations in the digester.

The digestion temperature demonstrated an important effect on the growth of pathogenic microbiological populations. A number of pathogenic bacteria were washed out of the digesters under thermophilic temperatures; this confirmed that the thermophilic conditions have better sterilization effects than the mesophilic conditions. Although the other consortia appeared similar under the thermophilic and mesophilic conditions, from the point of view of sterilization benefits, the thermophilic digestion was preferable in the first acidification fermentation stage.

7.4 The effects of the phase-separation configuration on the growth of complex microbial populations in the digester.

Of all the microbial consortia detected in the digesters, the acidogens are the largest populations growing in all the digesters. However, very few or no methanogens were found in most digesters, except for the digester 2-2. This indicates that the methanogens were severely inhibited under these given digestion conditions. While comparatively higher anaerobic and facultative colonies were found in digester 2-2, the number of methane-producing micro-

organisms were found to be much less than the anaerobic count in the recovery phase of digester 2-2. This confirms that not all of the anaerobic consortia can produce methane. Additionally, this suggests that the phase separation did not lead to a total separation of methane-producing bacteria from other microflora. After the acidogenic consortia, denitrifiers were found to be the second highest dominant population in these digesters. They may be considered as some of the strong consumers of the VFA substrate in digester 1 and digester 2-1 when the methanogens were under suppression. The growth of the acidogens with prolonged RT was accompanied by increases in the production of TVFA in the digesters (see Fig.5.5(2) and Fig.5.10(5) and Table 5.2).

It was thought that the high dilution rate or the phase separation might lead to important alterations with respect to the distributions of bacteria and the intermediate routes of substrate degradation. The results in table 7.1 indicate that the phase separation did not lead to a complete separation of the acid and methane formers. It is suggested that the principle of phase separation might be that : (1) by shortening the RT to the best time for phase separation, much larger quantities of fermentative bacteria can be obtained than those in the conventional digester, so that the potential ability of fermentative bacteria, which is suppressed in the conventional digester can be brought into full play; (2) by shortening RT to the optimum time for phase separation, the same large quantities of H_2 -utilization acetogens and hydrogenotrophs can be developed with reasonable proportions; this not only intensifies the interspecies hydrogen transfer but also promotes the fermentation of substrate directly to acetic acids instead of shifting to the accumulation of propionic acids and other VFA, so that the reasonable and constant production of major intermediates such as acetic acids, propionic acids and butyric acids can be maintained in the acidogenesis; (3) when these constant productions of major intermediates enter into the second phase, it is easy for the bacteria in the second phase, especially the populations of syntrophic acetogens and hydrogenotrophs, to establish a harmonious ecosystem⁽²⁷²⁾. Therefore, the efficiency of the second phase can be enhanced.

7.5 Discussion and summary

- Usually, viable counts give an under-estimate of the true population of the micro-organisms in the samples because the culture media used can normally only recover small

or variable parts of the consortia present, especially the obligatory anaerobic methanogenic bacteria which is more sensitive to the fluctuation of the associated environment. However, results so far obtained were still meaningful for comparing the microbial community occurring in the digester system under the given digestion conditions.

- Microbial flora respond to their abiotic environment and, as a result, establish a favorable community structure. Because of the complexities of the substrate fed to the digester, many different microbiological species are necessary to facilitate a complete methane fermentation of lignocellulose residues. The first consortium of the young microbial community might be the lignocellulose naturally-indigenous consortium (such as filamentous fungi) which previously attacked these complex polymers and secreted extracellular hydrolysis enzymes into the digestion systems. Most of these are aerobic genera that rapidly exhaust the trace oxygen left in the digester system and generate a proper anoxic environment for the later development of the anaerobic acidogenic genera. The acidogens can be considered as the second consortium of the digestion microbial community which provides the methanogens (the third consortium of the community) with the suitable carbon source (VFA, CO₂) and reductants (H₂). Not all the acidogens produce methane, and a densely acidogen-populated community does not always lead to a higher methanogen population. The two-phase digestion system is more favourable than the single-phase digestion system. The microbial consortia appeared similar in thermophilic and mesophilic circumstances, but from the point of view of sterilization benefits, the thermophilic digestion was preferable to mesophilic digestion.
- Although methane fermentation appears to be limited to a few specific anaerobic bacterial genera, and because of the wide variety and the complexities of the substrate fed to the digester, many different microbial species are necessary to facilitate the complete degradation. In the case of lignocellulose residue digestion, it is likely that the first consortium of the microbial community was the indigenous lignocellulose decomposition microorganisms such as filamentous fungi and/or soil actinomycetes which initially attacked these complex polymers and secreted extracellular hydrolysis enzymes into the digestion systems. Most of them are aerobic genera which rapidly exhausted the trace oxygen left in the digester system and generated a suitable anoxic environment for the

development of the anaerobic acidogenic genera. The acidogenic bacteria can be seen as the second member of the digestion microbial community which provide the methanogens (the third part of the community) with the suitable carbon source (volatile fatty acid, CO₂) and reductants (H₂).

- A complex microbial community predicted its wide degradation functions and high adaptability to the fluctuations from the environment and the substrate. Similar to a diverse microbial consortia coexisting in majority of environments as communities that degrade complex substrates by acting in concert, these three functional microbial groups coexisted in the young microbial community of the digestion system to perform the complete degradation and stabilization of lignocellulose solid residues. As the anoxic digestion proceeded, the conditions became more favorable for the anaerobic bacteria.
- Not all the acidogens produce methane. A densely-populated acidogens community did not always lead to a higher methanogens population. It is therefore necessary to adjust the acidogenic fermentation pathway to methanogenic fermentation. From this point onwards, the two-phase digestion system is more favourable than the single-phase digestion system.
- Alkaline pre-treatment indicated some effect on the composition of the VFA in the acidification digester, but its influence on the microbial population communities was not as evident as expected.

As it was found in Chapter 6, using the dilution process could solve the inhibiting effect of the concentrated complicated organic intermediate products formed at the high-rate acidification stage. However, this will not reduce these refractory complicated intermediate products fundamentally, the special treatment for these compounds (especially the humic acids) needs further investigation to obtain a complete solution to the inhibitory and potential pollution problems, and to improve the ultimate digestion efficiency. This will be the subject of the following chapters.

**Table 7.1. Viable micro-organism counts under different digestion condition
(cfu/g digested solid mixture)**

		Digester 1				Digester 2-1				Digester 2-2	
Seeding Medium		Tap Water		0.5M NaOH		0.5M NaOH		0.5M NaOH		0.5M NaOH	L-1
Initial pH of the Medium		9.46-9.52		11.8		11.8		11.8		11.8	5.68
Initial ORP of the Medium		282-290 mv		-146 mv		-146 mv		-146 mv		-146 mv	-220mv
Digestion pH		5.26-6.16		5.73-6.56		5.20-6.90		5.68-6.78		4.80-5.68	6.95-7.42
Digestion ORP (mv)		-108~ -238		-209~ -250		-136~ -205		-112~ -220		-160~ -220	-348~ -374
Digestion Temperature		50-55 °C		50-55 °C		50-55 °C		35-37 °C		50-55 °C	35-37 °C
Digestion RT(weeks)		2	4	2	4	2	4	2	4	2	4
Total Aerobic (10 ⁶)		50.0	3.5	15.0	2.6	20.5	1.80	76.0	16.5	19.6	2.32
Total Anaerobic(10 ⁶)		2.2	24.0	4.1	39.0	6.9	34.0	1.82	26.4	17.8	29.1
Total Facultative(10 ⁶)		2.9	4.7	2.2	3.34	1.30	3.50	1.50	2.90	34.0	25.0
Soil Actinomycetes(10 ⁴)		16.0	4.5	10.0	2.4	7.60	3.2	8.5	2.4	5.5	0.70
Filamentous Fungi(10 ⁴)		30.0	9.60	12.9	2.0	14.0	2.60	15.5	1.2	21.6	3.10
Yeast(10 ³)		3.3	n.d	4.2	n.d	1.9	n.d	2.1	0.32	0.65	n.d
Acidogenic	Aerobic 10 ⁶	0.40	0.06	3.3	1.05	2.3	0.13	10.6	2.40	12.8	0.142
	Anaerobic 10 ⁶	0.56	10.4	0.82	24.3	0.52	22.4	0.31	19.6	2.3	15.4
	Facultative 10 ⁶	1.04	0.79	1.26	0.95	0.84	0.41	1.08	1.26	8.60	1.32
Total Coliforms (10 ⁴)		0.11	0.04	0.15	0.01	0.08	0.02	11.0	3.9	0.4	0.016
Fecal Coliforms (10 ³)		n.d	n.d	n.d	n.d	0.2	n.d	6.7	1.0	0.1	n.d
Fecal Streptococci (10 ³)		0.74	n.d	0.30	n.d	0.1	n.d	2.6	1.2	n.d	n.d
Salmonella sp (10 ³)		n.d	n.d	n.d	n.d	n.d	n.d	7.0	n.d	0.3	0.11
Denitrifier (10 ⁴)		16.0	43.0	30.0	90.0	24.0	72.0	28.0	65.0	9.4	11.8
Methanogens (10 ⁴)		n.d	n.d	n.d	n.d	n.d	0.02	n.d	0.03	0.01	17.6

n.d: not detected

cfu: Colony forming units

Digester 1: Acidification Batch Reactor

Digester 2-1: Semi-continuous two-stage acidification reactors

Digester 2-2: Semi-continuous two-phase methanogens recovery reactors

L-1: Leachate from phase 1 of Digester 2-2

Chapter 8

Treatment of Humic Acids Extracted from the Acidification Fermentation of Lignocellulose Residues Using Ultrasonic Irradiation

The anaerobic digestion of high-solid organic wastes (such as municipal solid waste and dewatered sewage sludge) has been observed as an attractive approach for developing a recycle or energy-producing type of solid waste management system. However, as far as the lignocellulose substances are concerned, the formation of humic substances, especially the water-soluble HAs in the anaerobic digestion process, their complete anaerobic digestion for energy production and the re-utilization of waste remain intractable problems.

The HAs, the water soluble fraction of humic substances, are not only responsible for the toxicity in the metabolic activities of the anaerobic consortium, but also present environmental problems in aqueous and terrestrial environments by spreading the ecotoxic compounds such as heavy metal ions and xenobiotics because of its amphipathic characteristics and highly chemical chelating reactivity.

Thus, special care must be taken in the treatment of HAs bearing leachate from the high-solid anaerobic digestion process of lignocellulose residues

As stated in chapter 6, using the dilution process could solve the inhibiting effect of the concentrated water-soluble fraction of humic substances formed at the high-rate acidification stage. However, the dilution process alone could not be used to degrade or decompose these humic substances. The special treatment and possible degradation fate of these complicated organic by-products needs further investigation to solve the fundamental inhibitory and potential pollution problems, thus ultimately improving the proficiency of lignocellulose digestion.

In this chapter, the effects of ultrasonic irradiation on the chemical and physical characteristics of HAs extracted from the acidification fermentation process of lignocellulose residues are investigated.

8.1 The effects of ultrasonic irradiation on the pH and ORP of the humic acid (HA) solution

The pH and ORP changes of a HA solution irradiated by ultrasonic irradiation are presented in Fig.8.1 and Fig.8.2. The results show that by increasing the ultrasonic irradiation time, the pH of the solution increased slightly from 7.30 to 7.42 and then dropped to 7.18; there was little change in the ORP of the solution (less than 10 mv) as a result of the ultrasonic irradiation. In the case of the aqueous solution irradiated by ultrasound, water vapour present in the ultrasound bubble is homogeneously split to yield H and OH radicals. HA molecules present in the vapour phase or in the surrounding liquid of the collapsing bubbles are subject to direct attack by OH radicals, thus accelerating the hydrolysis reactions by several magnitudes in the presence of ultrasound. Since the HAs behave like weak-acid poly-electrolytes with a relatively high pH buffering capacity, the changes in pH of the HA solution could be considered to be outstanding although the apparent changes were slight. The acidic nature of HA is usually attributed to the ionization behaviors of -COOH and phenolic OH groups. The changes of the HA in the pH of the solution by ultrasonic irradiation implies that the ionization behaviour of the acidic functional groups was changed partially by the reactions between OH and/or H radicals formed in the ultrasonic fields and the acidic functions of the HA molecules. Oxidation, pyrolysis and/or the mechanical degradation of HA molecules could be expected in an ultrasonic field. The changes in ORP of the aqueous HA solution was primarily attributed to the oxidation effects of the ultrasonification. The oxidation effects were possibly suppressed by the buffering capacity of the HA, and consequently, little change in the ORP of the aqueous HA solution was detected.

8.2 The effects of ultrasonic irradiation on the UV absorbance of the humic acid (HA) solution

The standard UV absorption curves at 230 nm and 254 nm are presented in Fig. 8.3. The changes of UV absorption at 230 nm and 254 nm are given in Fig.8.4. Fig.8.3 demonstrates that the UV absorbency of the HA solution at 230 nm and 254 nm has a positive linear relationship with its concentration. Fig.8.4 shows that the UV absorption of the HA solution at 230 nm and 254 nm firstly increased with the ultrasonic irradiation, then decreased under longer ultrasonic irradiation time.

The absorption of radiation in the UV-vis region of the electromagnetic spectrum arises from electron transition from bound states (outer valence orbitals) to excited electron states. In HA molecules, these exceptionally-low energy transitions are associated with the presence of chromophores, i.e. functional groups containing conjugated double bonds and sulphur, nitrogen or oxygen atoms with delocalized electronic orbitals. Electron transitions can occur within the molecular orbitals of chromophores, or the nonchromophore transfer of an electron from one chromophore to another chromophore, or to a nonchromophore (electron or charge-transfer excitation). Groups that are not chromophores but affect absorption of chromophores are called auxochromes, which typically include hydroxyl and amine groups. Despite the fact that the UV-vis spectra of HA are generally featureless owing to the extended overlap of absorption of a wide variety of chromophores affected by various substitutions, UV-vis light absorption of HA appears to increase with an increase in: (1) the degree of condensation of the aromatic ring ⁽¹³⁸⁾; (2) the total C content; (3) the molecular weight; (4) the ratio of C in aromatic rings to C in aliphatic side chains ^(224, 222); (5) the pH of the solution ^(248, 24). The changes of UV absorption in the HA at 230 nm and 254 nm as a result of ultrasonic irradiation suggest that the structure and/or contents of chromophores, such as OH-, COOH-substituted benzene rings, the intramolecular electron donor-acceptor complexes and complex highly unsaturated conjugated chromophores, had been changed by the ultrasonic irradiation. So far, by comparing the changes in the UV-absorption at 230nm and 254nm (Fig.8.4), with the changes in pH (Fig.8.1), the ultrasonic irradiation effects in this respect could be ascribed to the inducing of changes: 1) in the degree of disassociation or protonation of carboxyl and phenolic hydroxyl groups of HA; 2) in the macromolecular structure of HA resulting in greater or lesser exposure of chromophores to the light; or the introduction of an auxochrome to various positions of an aromatic ring.

8.3 The effects of ultrasonic irradiation on the reactivity of the humic acid (HA) solution with Fast Blue B Salt

Fast Blue B salt reacts with 1-naphthol and other hydroxylated aromatic compounds to form colored products which have absorption in the range of 530 nm to 618 nm ⁽²⁵⁶⁾. The changes of reactivity of the HA solution with FBB salt are presented in Fig.8.5. This indicates that the reactivity of the HA solution with FBB salt initially increased with the increase of irradiation time, then declined after 1.0 hour of irradiation. This suggests that the ultrasonic irradiation

probably increased the 1-naphthol (or other hydra-oxylated aromatic-like compounds) content in the HA solution initially and when the irradiation time was prolonged partially decomposed it.

8.4 The effects of ultrasonic irradiation on the IR spectra of the humic acid (HA) solution

The changes of IR spectra are presented in Fig.8.7 (a: 0 h ultrasonic irradiation; b: 0.5 h ultrasonic irradiation; c: 1.0 h ultrasonic irradiation; d: 1.5 h ultrasonic irradiation; e: 3.0 h ultrasonic irradiation). The associated dominant peak assignment ^(236, 223, 222, 40) and the integrated areas are listed in table 8.1.

Table 8.1. Effect of ultrasonic irradiation on the IR absorption of HA (integrated peak areas)

Function Assignment		-OH-NH, Aromatic CH Stretch	-C=OOH, C=O of Ketonic Carbon, Aromatic C=C, COO-, Hydrogen-bonded C=O stretch	C-O or OH Stretch	O substitution Arene
Wave Number (cm ⁻¹)		3002-3820	1771-1500	1200-956	900-775
Irridiation Time (Min)	0.0	177.969	16.831	7.177	0.794
	30.0	107.834	9.542	3.437	0.891
	60.0	177.548	27.052	6.277	2.601
	90.0	128.867	17.575	3.659	1.708
	120.0	141.351	12.177	3.423	0.980

The FT-IR spectra of 5 HA samples predominantly exhibited the presence of oxygen-containing functional groups: broad bands around 3002 to 3820 cm⁻¹, which could be attributed to the valence vibrations of hydroxyl groups (3400 cm⁻¹) and aromatic C-H bonds (3295-3006 cm⁻¹); shoulders at 1771-1500 cm⁻¹, which could be attributed to the C-O stretch of C=OOH and C=O of ketonic carbon, aromatic C=C, C-O stretch of COO- and hydrogen-bonded C=O); peak region in 1200-956 cm⁻¹ which can be attributed to C-O, C-N and C-C stretch; peak region in 900-775 cm⁻¹ representing the O substitution arene groups were characteristic for all samples. The absorption bands in the 2900 cm⁻¹ region were usually

superimposed on the shoulder of the broad O-H stretching band. It can be seen from the spectra in Fig.8.7 that the remarkable shifts in these main bands were not found with the ultrasonic irradiation; however, some small peaks (comparing the detail spectra a, b and c) such as the peaks around 2900 cm^{-1} (the asymmetric and symmetric stretching vibrations of aliphatic C-H bonds in CH_3 and CH_2 groups), 2400 cm^{-1} (after 1 hour ultrasonic irradiation), 1520 cm^{-1} (Amide II or Aromatic C=C stretching), 1200 cm^{-1} (aliphatic C, OH or C-O stretch of various groups) appeared in the spectra of the samples with ultrasonic irradiation. It can be seen from the table 8.1 that the intensity (integrated absorbency peak areas) of some absorption bands exhibited detectable differences due to the ultrasonic irradiation. It suggested that a greater diversity of chemical components, some weak chemical modifications in the molecular structure and slight quantity changes in functional groups of HA were probably induced by the ultrasonic irradiation.

8.5 The effects of ultrasonic irradiation on the ^1H NMR spectra of the humic acid (HA) solution

The ^1H NMR spectra of the initial HA(0) and the 2 hour-irradiated HA(1) are presented in Fig 8.8(a) and Fig 8.8(b). The chemical shifts and corresponding assignments ^(265, 266) of the absorbency peaks are summarized in table 8.2.

From the spectra in Fig.8.8, it can be seen that four sharp resonance at 0.88, 1.27, 6.71 and 8.43 ppm were observed in the initial HA sample. Three sharp resonances at 0.85, 1.25 and 8.42 ppm were observed in the HA sample with ultrasonic irradiation. Clearly the sharp signal at 6.71 ppm disappeared after irradiation. This implies that some special aromatic group (e.g. phenol) was decomposed by ultrasonic irradiation.

The signal at 2.431-3.135 ppm (protons α to carboxylic acid groups or protons of methyl and methylene groups α to aromatic rings), appeared after ultrasonic irradiation. In addition, the results in table 8.2 and Fig.8.8 show that the spectrum at 1.4-1.8 ppm, which arises from a variety of aliphatic carbons (mainly polymethylene protons and protons attached to aliphatic carbon β to aromatic rings⁽¹⁸³⁾), and the spectrum at 3.0- 4.4 ppm, which largely arises from the protons attached to carbon α to oxygen, had been changed to some extent by ultrasonic irradiation. This indicates that the main changes in the chemical structure of HA induced by

the ultrasonic irradiation took place in the carboxylic acid groups or methylene groups α to aromatic rings, a variety of aliphatic carbons (mainly polymethylene), aliphatic carbon β to aromatic rings and carbon α to oxygen.

Table 8.2. Chemical shift ranges and tentative assignments of major ^1H resonances observed from HA ^1H NMR spectra

Chemical Shift Range(ppm)		Integrated Area		Tentative Assignments
HA(0)	HA(1)	HA(0)	HA(1)	
0.559-1.062	0.393-1.022	24.661	40.298	Terminal methyl groups of methylene chains
1.062-1.439	1.022-1.743	24.761	51.709	Methylene of methylene chains; CH_2CH at least two carbons or further from aromatic rings or polar functional groups
1.439-1.758		11.806		Methylene of alicyclic compounds
1.758-2.429	1.744-2.430	17.851	23.117	Protons of methyl and methylene groups α to aromatic rings
	2.431-3.135		3.01	Protons of methyl and methylene groups α to aromatic rings; protons α to carboxylic acid groups
3.007-4.030	3.136-4.419	98.632	63.493	Protons α to carbon attached to oxygen groups; carbohydrates
6.152-7.578	6.138-7.672	33.886	28.647	Aromatic protons including phenols
8.341-8.508	8.318-8.511	0.725	0.629	Sterically hindered protons of aromatics

The spectrum at 8.3-8.5 ppm (sterically-hindered protons of aromatics) showed no significant difference in the results presented in Fig.8.8 (a) and Fig.8.8 (b). However, the resonance situated at 0.89 ppm (which indicates the methyl groups of alkyl chains at least three carbons removed from de-shielding groups, e.g. aromatic rings) in Fig.8.8 (a) shifted to 0.39 ppm in Fig.8.8 (b) after ultrasonic irradiation. This confirmed that the terminal methyl groups of the methylene chains had been changed, especially in the carboxylic acid groups, methylene groups α to aromatic rings and some aliphatic carbons (mainly polymethylene), although there were little changes taking place on the aromatic kernel structure of the HAs.

For complex macromolecules such as HA, the observed broadness of the bands in the IR spectrum was probably as a result of the extended overlapping of very similar absorption arising from individual functional groups of the same type, with different chemical environments. Although most groups of atoms vibrate with almost the same frequency irrespective of the molecule to which they are attached, this fact does not mean that the HAs displaying similar IR spectra, must have similar overall structures, but only that the net functional group and structure entities may be similar ⁽⁵⁴⁾. Therefore, it could only be concluded that the ultrasonic irradiation on the HA might induce the changes in the chemical environments of the net functional groups and structure entities of the HA macromolecule from the IR spectra. However, from the ¹HNMR spectra of the HA samples, it could be confirmed that the chemical structure of HA was changed by ultrasonic irradiation.

8.6 The effects of ultrasonic irradiation on the molecular weight and distribution of HA expressed in gel permeation chromatography (GPC)

Since not all of the HA sample were dissolved in THF, the fraction detected by gel permeation chromatography (GPC) are hereby referred to as THFS-HA. A fully acceptable molecular structure of HA could as yet not be obtained and the whole range of known molecular-weight standards suitable to the HA extracted from the fermentation stage are not available. Therefore the relative changes in retention time (RT) and absorbency of HA samples were considered to examine the effects of ultrasonic irradiation on the molecular weight (MW) of HA.

The GPC spectra of four THFS-HA samples are plotted in Fig.8.9. The statistically calculated Weight-Average Molecular Weight (Mw), Number-Average Molecular Weight (Mn), Z-Average Molecular Weight (Mz) and Polydisp Index (Polydispersity) are described in table.8.3.

The spectra show that one dominant (at a retention time of 13.62min) and four minor peaks (at RT=11.62, with MW around 983; RT=12.78-12.86, with MW around 500; RT=13.14-13.18 with MW around 430 and RT=14.46 min with MW around 292) of 240 nm absorbing fractions were detected in all the samples. In order to demonstrate the substantial changes in the spectra, the detail spectra with dominant absorbing area is given in Fig.8.10. Clearly, the

evident decrease of peak areas (reduced quantity of THFS-HA molecules) was observed in all the THFS-HA samples with ultrasonic irradiation and a shift of the peak to a higher retention time (reduced molecular weight) was observed in the THFS-HA sample exposed to 1.0 hour of ultrasonic irradiation. It is suggested that the dominant molecular weight fractions of THFS-HA were reduced effectively in quantity by ultrasonic irradiation, depending on the period of irradiation time. The longer irradiation period did not result in an exceptionally higher reduction effect. Additionally, the results in Fig.8.9 showed that the ultrasonic irradiation primarily took effect on the dominant molecular-weight fraction (mild molecular weight) of the HA. The relatively higher absorption intensity of spectra “b”, “c” and “d” at a shorter retention time than that of spectra “a” implied that some mild molecular weight fraction might aggregate into larger colloidal particles in the ultrasonic irradiation field.

Table 8.3 The statistical molecular weight and distribution of HAs in GPC Data

Irradiation Time (h)	Mw	Mn	Mz	Polydispersity	10% at Mw	90% at Mw
0	465	408	593	1.1386	754	338
1.0	601	468	847	1.2860	1142	331
1.5	607	483	829	1.2561	1116	347
2.0	629	491	858	1.2809	1203	349

In the polydisperse system like HA, the $M_w > M_n > M_z$ and $\text{polydispersity} > 1$. The M_n tends to be strongly influenced by lower molecular weight components, whereas the M_w and the M_z tend to emphasize the contribution of the heavier molecules of the mixture. The greater the value of polydispersity, the wider the range of molecular weight (MW) of the HA. From Table 8.3, it can be seen that the values of M_w and M_z in the HA system with ultrasonic irradiation were noticeably higher than those without ultrasonic irradiation. The polydispersity values of HA also increased after ultrasonic irradiation, although smaller changes in the M_n and 90% of the M_w fraction were detected after ultrasonification. These results further indicated that the wider MW composition range and some large colloidal particles of HA were formed in the ultrasonic field. This could be explained by the fact that when the ultrasonic irradiation was applied on the polydispersed system of HA, the lower MW (also the dominant) fraction decomposed first, some fractions (around 50%, from the spectra in Fig.8.10) became volatile and then disappeared from the system. The larger and heavier MW component (minor fraction

of the HA system) was difficult to be break down. Vice versa, these large molecules are easy to flocculate into colloidal particles through inter-molecular absorption, which was possibly enhanced with the increased quantity of absorbing active sites on the large molecular surface of HA by ultrasonification. Because of its amphipathic character (hydrophobic and hydrophilic) this kind of inter-molecular absorption could be ascribed to hydrophobic absorption, H-bonding absorption, and chemical-bonding absorption.

8.7 The effects of ultrasonic irradiation on the morphology of humic acid (HA)

The effects of ultrasonic irradiation on the shapes, dimensions and aggregation degree of HA can be investigated using a SEM because the fine structure is frequently revealed with unusual clarity, and particle boundary relationships can be examined in great detail under a SEM. As shown in Fig.8.11-a and 8.11-b, the HA consists of curved, closely-knit bundles of fibres and an irregular sheet (flake)-like structure of varying thickness, with the fibres often ending and starting in protrusions extending from the plane of the sheet (flake)-like structures. As can be seen from Fig.8.11-c to Fig.8.11-j, the fibres quickly diminished in number and completely disappeared after 2.0 hour of ultrasonic irradiation. However, the sheet-like structure became prominent and there were signs of increasing thickness and orientation of the multi-layered structures with fine finger-like protrusions radiating from the surface in Fig.8.11-i and Fig.8.11-j after 2.0 hours of ultrasonic irradiation. As seen from Fig.8.11-c to Fig.8.11-h, the flakes appeared to curl together at some points, forming multi-orientation and dimension structures with some fragmented open spaces and corrugated surfaces. Therefore, from the micrographs obtained on the SEM, we observed that the overall view of the initial HA was fibres and bundles of fibres that closely knit together or unite with some sheet (flake)-like structure. After ultrasonification, the fibre-like structures diminished quickly and the aggregation of closely-woven flake network structure resembling sponge and the thickened sheet structure with finger-like protrusions were frequently observed in the HA with ultrasonic irradiation. These results apparently confirmed the flocculation trend in the morphological features of HA by ultrasonic irradiation as it was proposed by the GPC monitoring results.

8.8 The effects of ultrasonic irradiation on the E_4/E_6 ratios of HA and its relationships with the UV-absorbency characteristics of HA

The effects of ultrasonic irradiation on the E_4/E_6 ratios of HA are presented in Fig.8.6. The changes of E_4/E_6 ratios in the ultrasonic irradiation time courses were similar to the changes in UV-absorbency at 230 nm and 254 nm and fast blue B salt (FBB) reactivity, increasing initially and then decreasing with prolonged ultrasonic irradiation.

As stated in Chapter 2, with ultrasonic irradiation, it is proposed that organic compounds in water mainly degrade via hydroxyl radical oxidation, pyrolytic degradation, and supercritical water reactions. In the case of a aqueous solution, water vapour present in the bubble is homolytically split to yield H and OH radicals. Chemical substrates present within the vapor phase or in the surrounding liquid of the collapsing bubbles are subjected to direct attack by OH radicals. Volatile compounds break up into the gas phase (i.e into the gaseous bubble within the aqueous solution) and undergo direct pyrolysis.

On the one hand, the UV-absorbency at 230 nm and 254 nm was mainly the measurement of aromatic content in the HA molecules. Under ultrasonic irradiation, the relatively weaker bonded alkyl side chains, long linear aliphatic side chains and C-O linked aromatic groups would be preferentially attacked and broken down into the volatile compounds and then completely escape from the solution in the gas phases with prolonged irradiation time. Thus, the ratio of aliphatic carbon/aromatic carbon could be altered by the ultrasonic irradiation. There is a good correlation between the changes in the UV-absorbency at 230 nm and 254 nm and the FBB reactivity. This indicated that the increase of UV-absorbency at 230 nm and 254 nm could initially result from the proportion of 1-naphthol or other hydra-oxylated aromatic-like compounds, which are higher than aliphatic compounds in the HA solution. The decrease in UV-absorbency at 230 nm and 254 nm could result from the decomposition and escalation of 1-naphthol or other hydra-oxylated aromatic-like compounds during the longer irradiation period, since the aliphatic compounds were easier to decompose than the aromatic-like compounds in the ultrasonic field.

On the other hand, the UV-absorbency was also affected by the size and shape of the HA molecules in the solution. In addition, it has been found in the SEM graphs that both the size and shape of the HA molecules were affected by the ultrasonic irradiation.

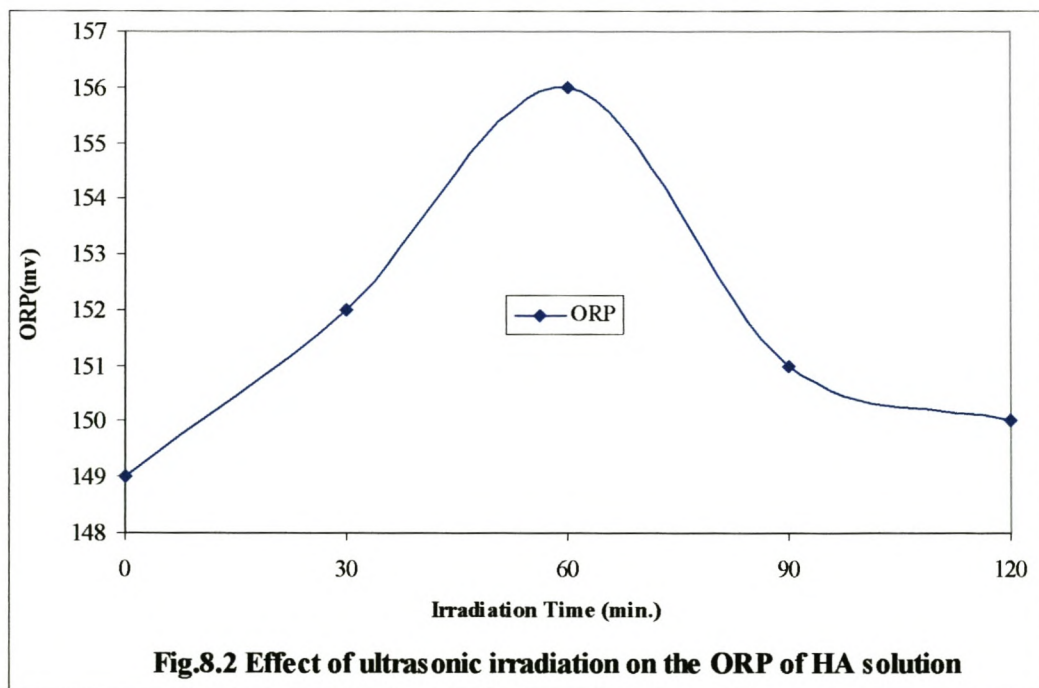
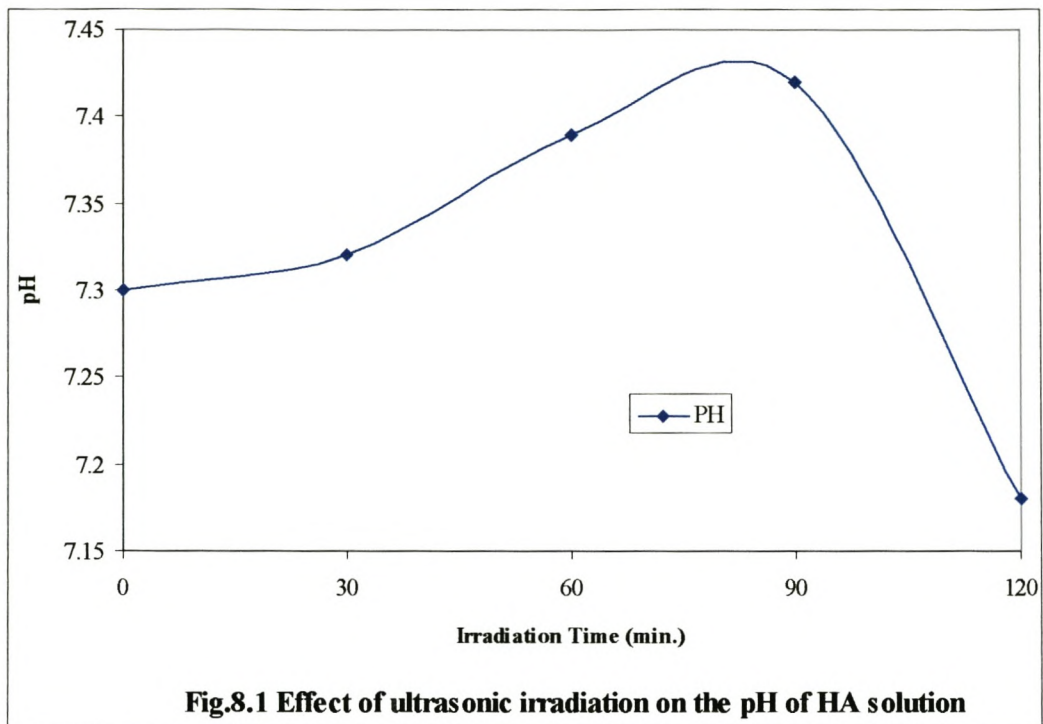
Therefore, the increasing or decreasing of UV-absorbency could not be ascribed solely to the chemical changes in the aliphatic and aromatic structures of the HA molecules.

According to Kononova ⁽¹³⁸⁾, the E_4/E_6 ratio is inversely related to the degree of condensation of the aromatic network in HA. A low E_4/E_6 ratio would be indicative of a relatively high degree of condensation of aromatic constituents in HA, whereas a high ratio would reflect a low degree of aromatic condensation and the presence of a relatively large proportion of the aliphatic structure. Schnitzer and Khan ⁽²²³⁾ suggested that light absorption of aqueous HA solutions in the visible region of the electromagnetic spectrum increased with : (1) the ratio of carbon in aromatic nuclei to C in aliphatic side chains; (2) the total C content; and (3) the molecular weight. Chen et al ⁽⁴⁴⁾ suggested that : (1) much of the observed visible absorption by HA may be due to light scattering, which thus may contribute to the lowering of the E_4/E_6 ratio in the high MW fraction of HA; (2) the E_4/E_6 ratio of HA is primarily governed by particle sizes and weights, and is not apparently related to the amount of aromatic condensed rings in the structure; and (3) the effect of pH on absorption and E_4/E_6 ratio is due to the changes in particle size possibly caused by folding/unfolding, or aggregation/dispersion of the HA macromolecules. From the results so far obtained in this study, it was found that the average molecular weight of HA increased with the aggregation/folding of the HA fractions after ultrasonic irradiation; chemical changes were also detected in a variety of aliphatic carbons (mainly polymethylene) aliphatic carbon β to aromatic rings and carbon α to oxygen and carboxylic acid groups or methylene groups α to aromatic rings. However, the chemical changes and molecular weight changes of HA did not well correlated with the changes of the E_4/E_6 ratios of HA. Therefore, the changes in E_4/E_6 of the HA solution under the experimental conditions could be the overall consequences from the changes in the aromatic constitutes/aliphatic constitute ratios of HA and the folding/unfolding, or aggregation/dispersion of the HA macromolecules caused by ultrasonic irradiation.

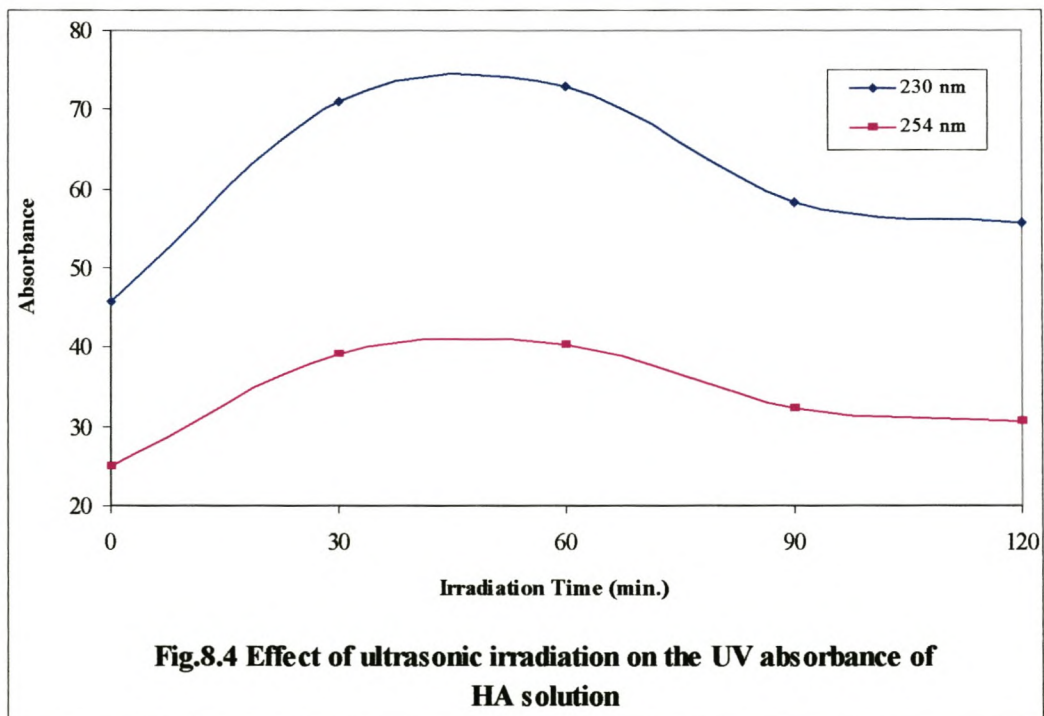
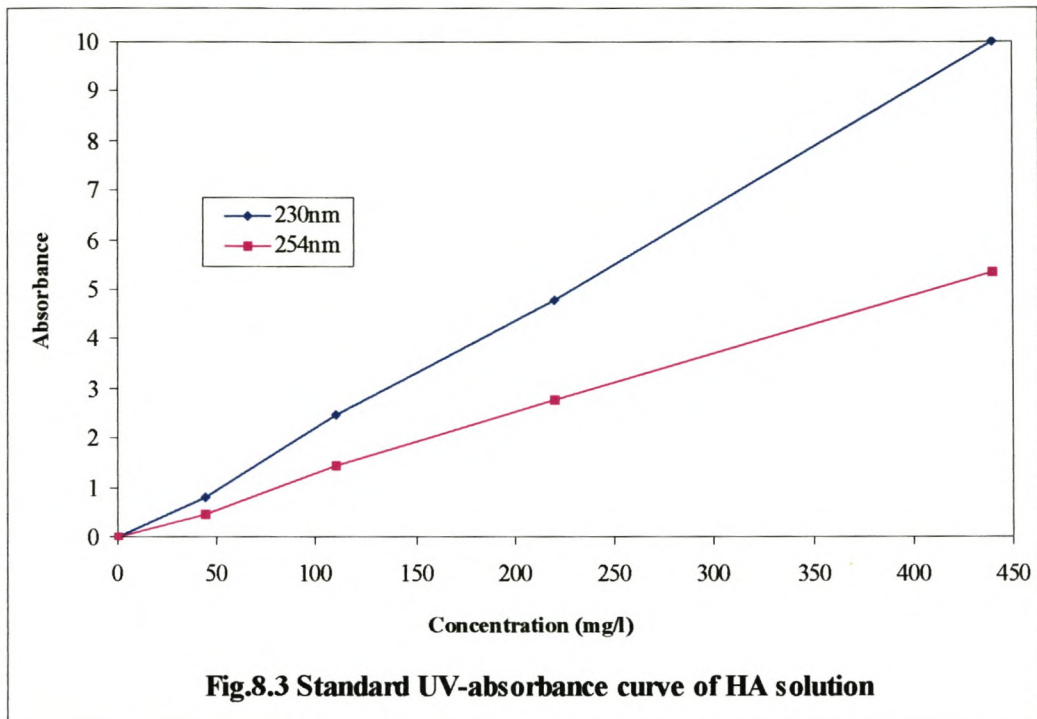
8.9 Summary

- Both the chemical and the physical characteristics of HA were changed to some extent by ultrasonic irradiation, depending on the irradiation time.
- In the polydispersed HA system, the ultrasonic irradiation affected the lower molecular fraction (dominant fraction) of the HA first. Around 50% of this fraction decomposed into a volatile form and disappeared from the system. Then, the larger molecular fraction (minor fraction) tended to aggregated into colloidal form and remained in the system.
- The chemical modifications induced by ultrasonic irradiation mainly occurred in the carboxylic acid groups, the methylene groups α to aromatic rings, a variety of aliphatic carbons (mainly polymethylene), aliphatic carbon β to aromatic rings and carbon α to oxygen.
- The changes of E_4/E_6 in the HA solutions could be the overall consequence of the changes in the aromatic constitutes/aliphatic constitute ratios of HA and the folding/unfolding, or aggregation/dispersion of the HA macromolecules caused by ultrasonic irradiation.
- The SEM micrographs showed that: the overall view of the initial HA was fibres and bundles of fibres that closely knit together or unite with some sheet (flake)-like structure; after ultrasonication, the fibre structures diminished quickly, and the aggregation of a closely-woven flake network structure resembling sponge and a thickened sheet structure with finger-like protrusions was frequently observed.
- There was good correlation between the changes in the UV-absorbency at 230 nm and 254 nm and the FBB reactivity. The effects of ultrasonic irradiation on the HA characteristics were physically, chemically and time-dependent.
- Ultrasonic irradiation can be employed to treat a HA-containing solution. However, as far as the treatment efficiency obtained are concerned, the destruction of the HAs solely by ultrasonic irradiation was not complete. Thus, it is necessary to investigate the co-operative treating effects of ultrasonic irradiation with other treatment methods, as discussed in the following chapters.

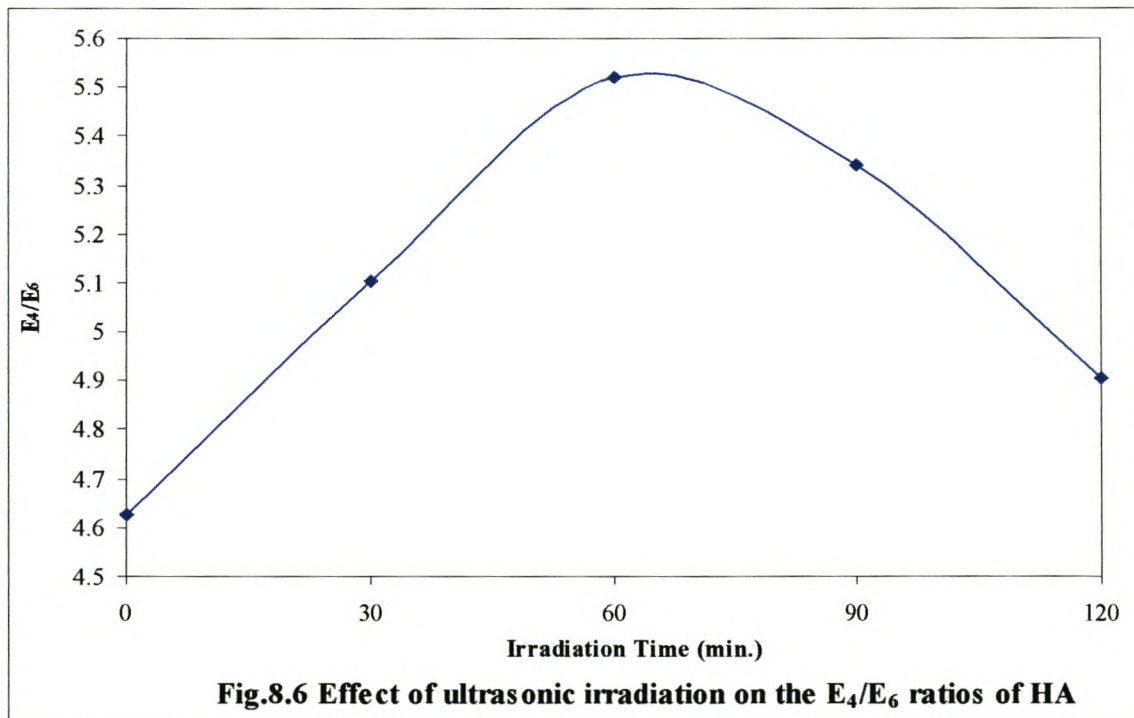
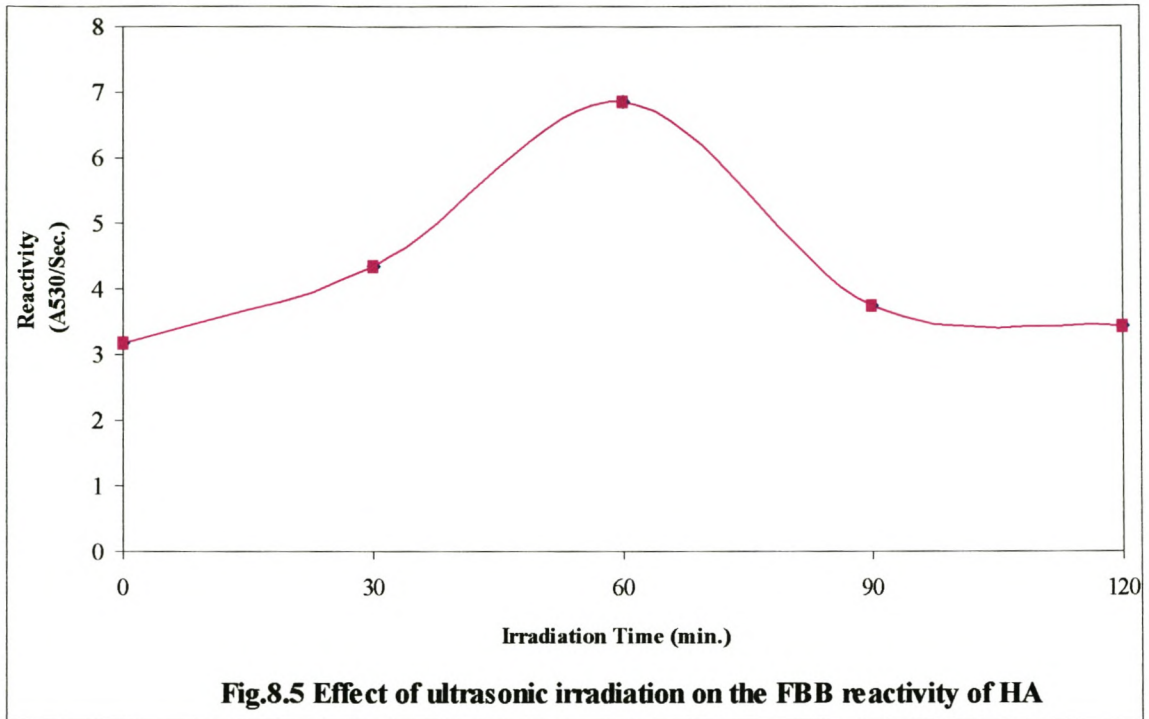
Treatment of Humic Acids Extracted from the Acidification Fermentation of Lignocellulosic Residues Using Ultrasonic Irradiation



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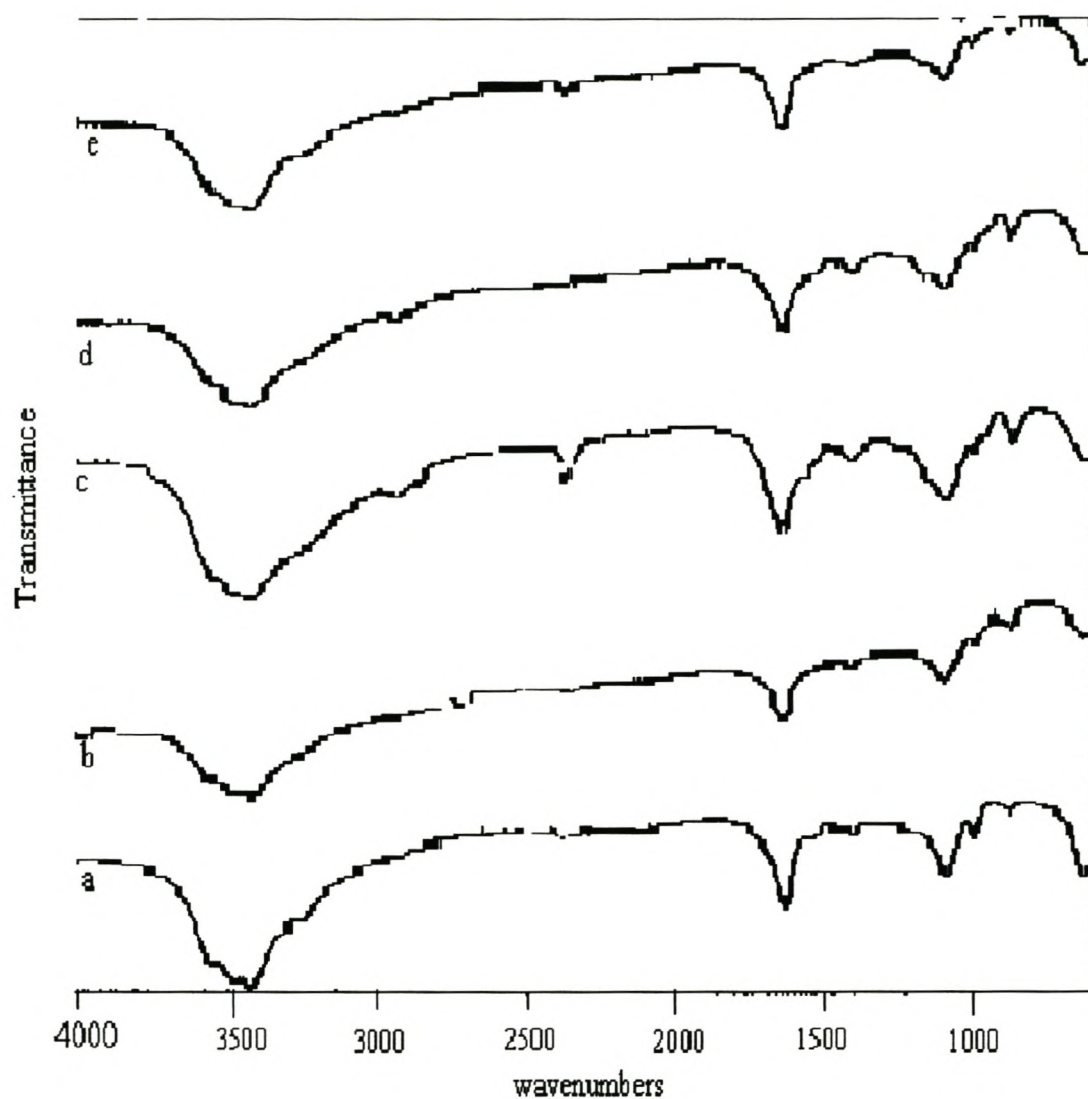


Fig.8.7(1) The FT-IR spectra of HA under different ultrasonic irradiation periods of time (a=0.0 hour; b=0.5 hour; c=1.0 hour; d=1.5 hour; e=2.0 hour)

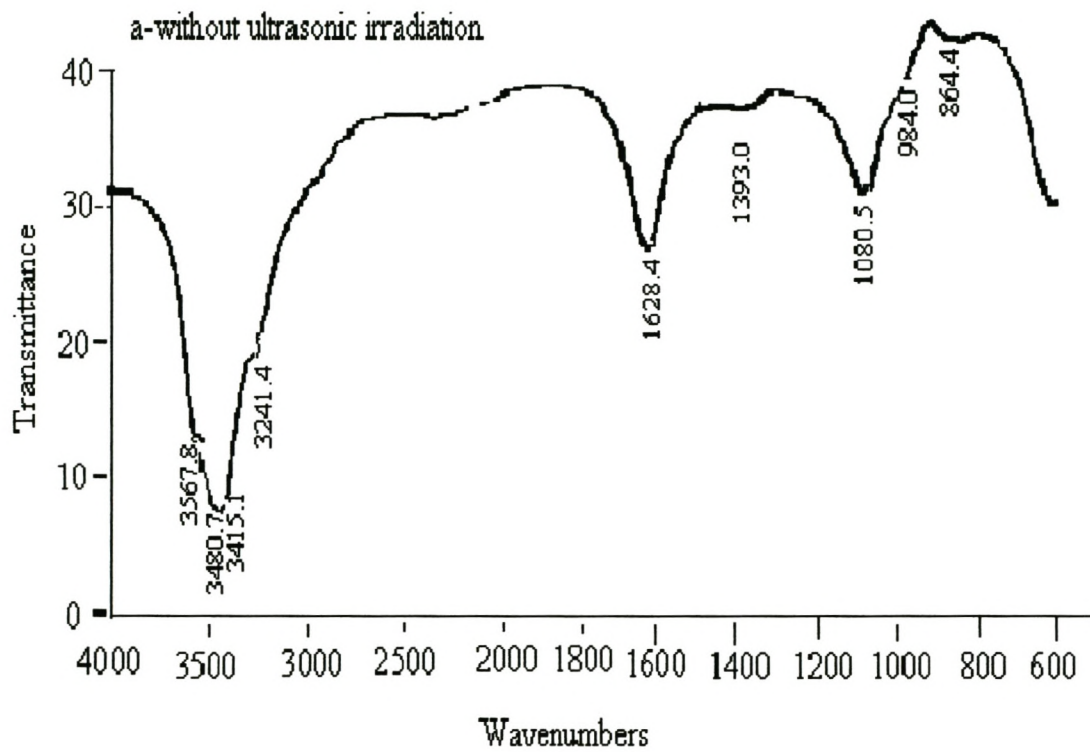
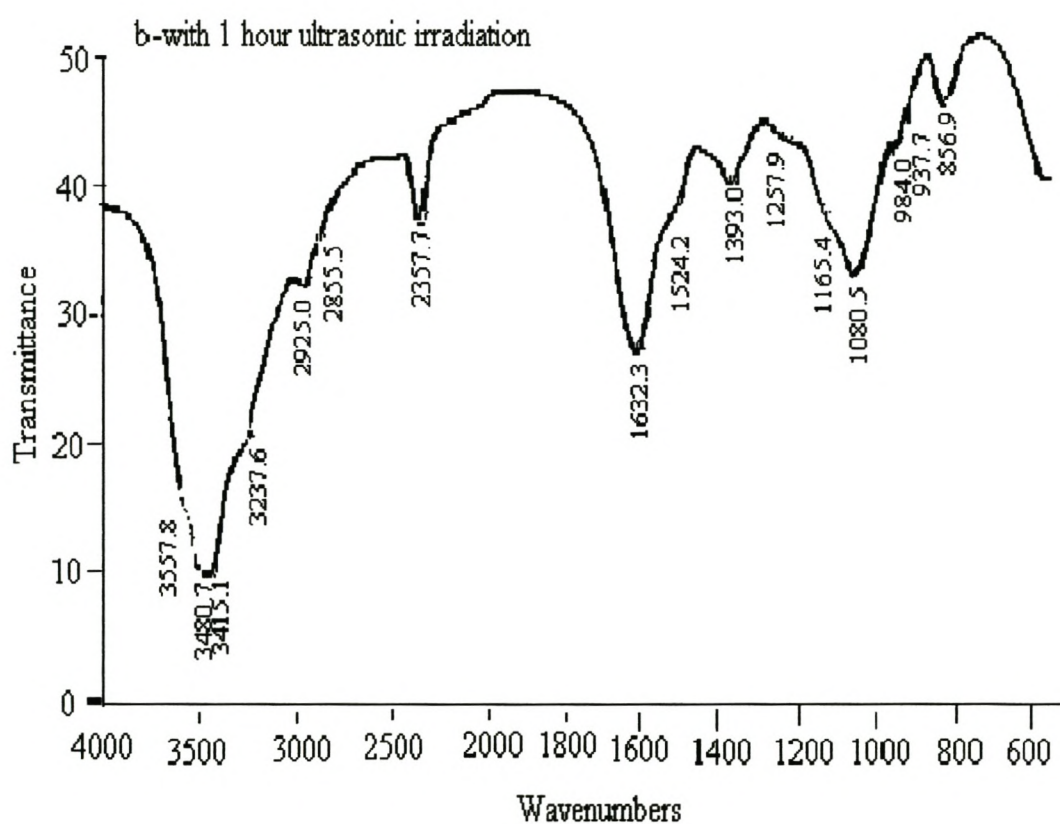


Fig.8.7 (2) FT-IR spectra of HA
(without being treated with ultrasonic irradiation)



**Fig.8.7(3) Effects of ultrasonic irradiation on the FT-IR spectra of HA
(Treated with 1.0 hour ultrasonic irradiation)**

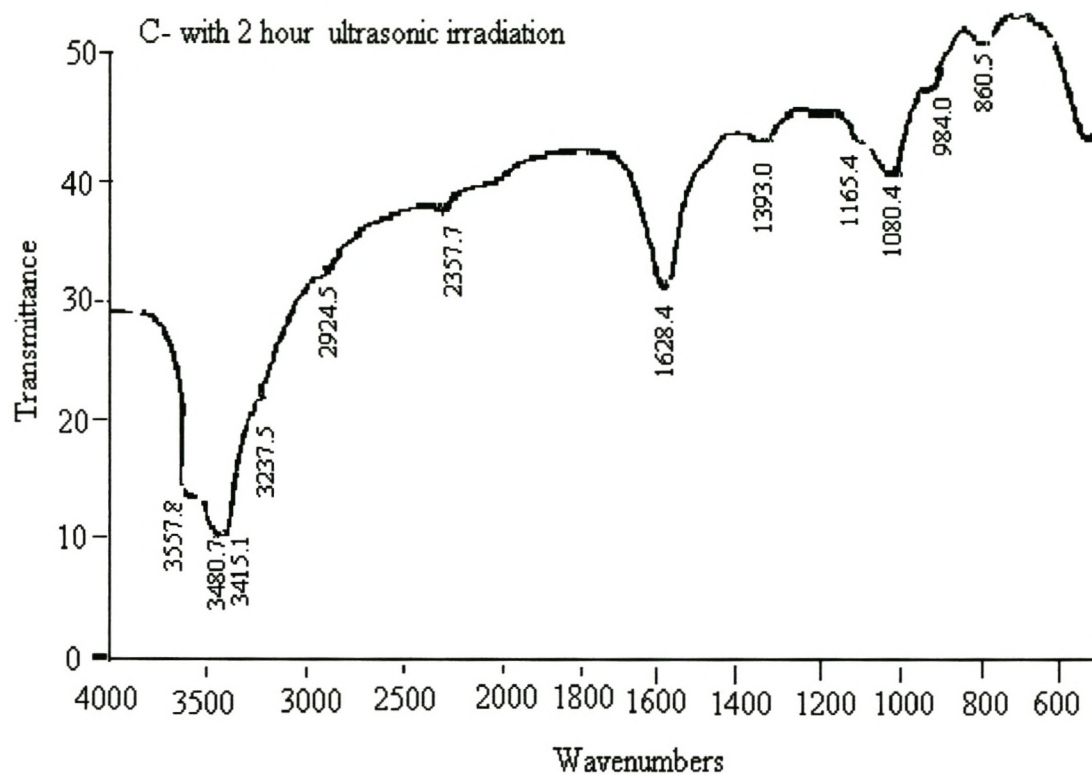


Fig.8.7(4) Effects of ultrasonic irradiation on the FT-IR spectra of HA
(Treated with 2.0 hour ultrasonic irradiation)

Treatment of Humic Acids Extracted from the Acidification Fermentation of Lignocellulosic Residues Using Ultrasonic Irradiation

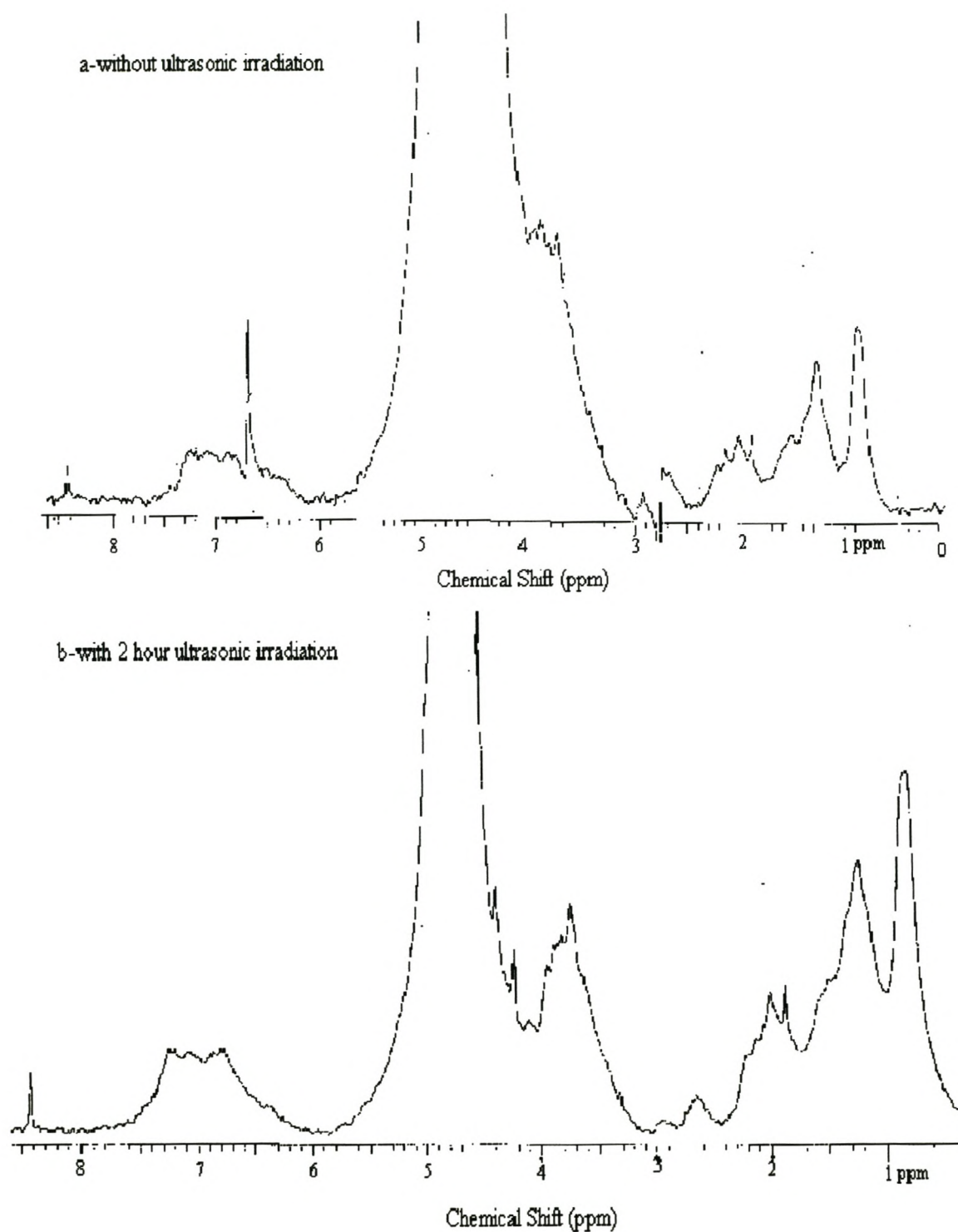
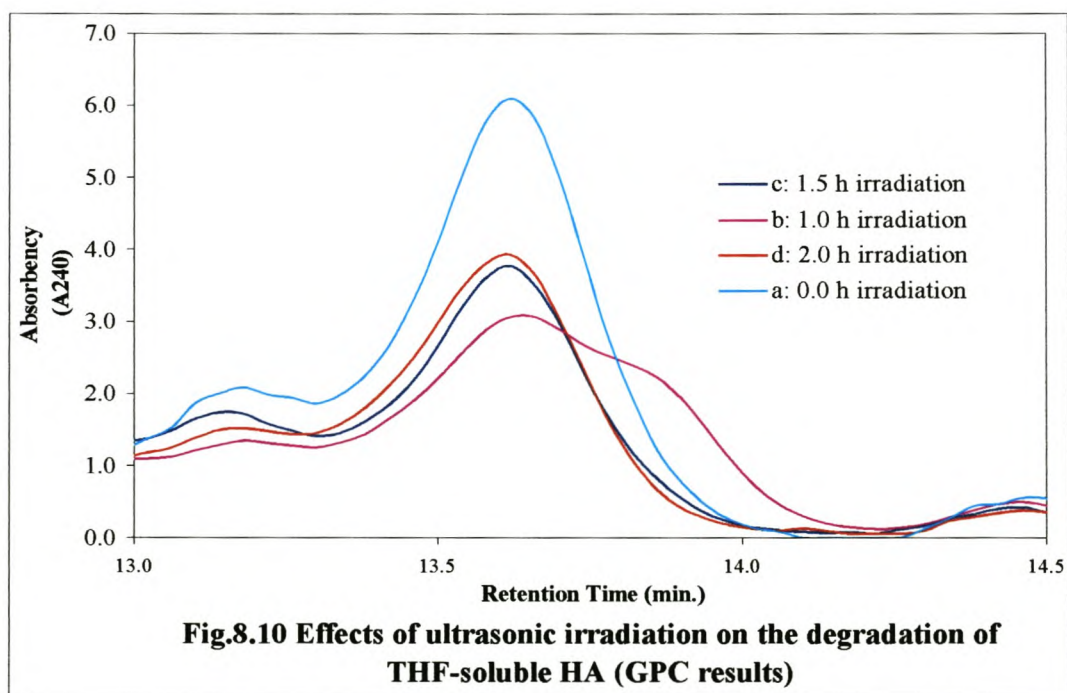
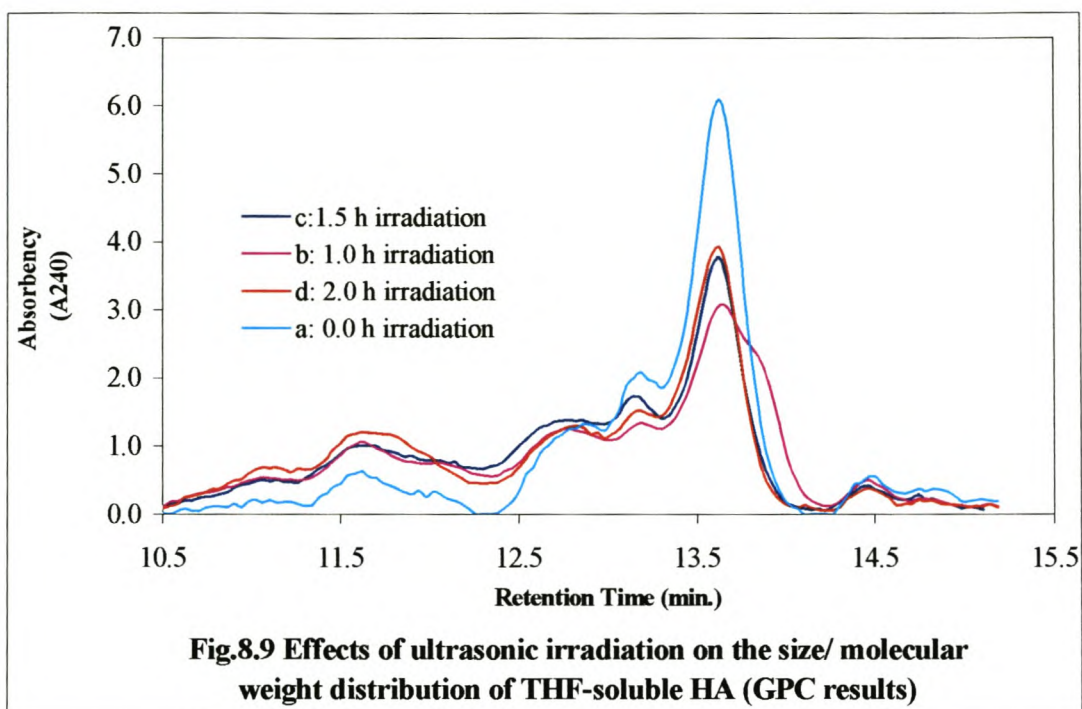


Fig.8.8 Effects of ultrasonic irradiation on the ^1H NMR spectra of HA



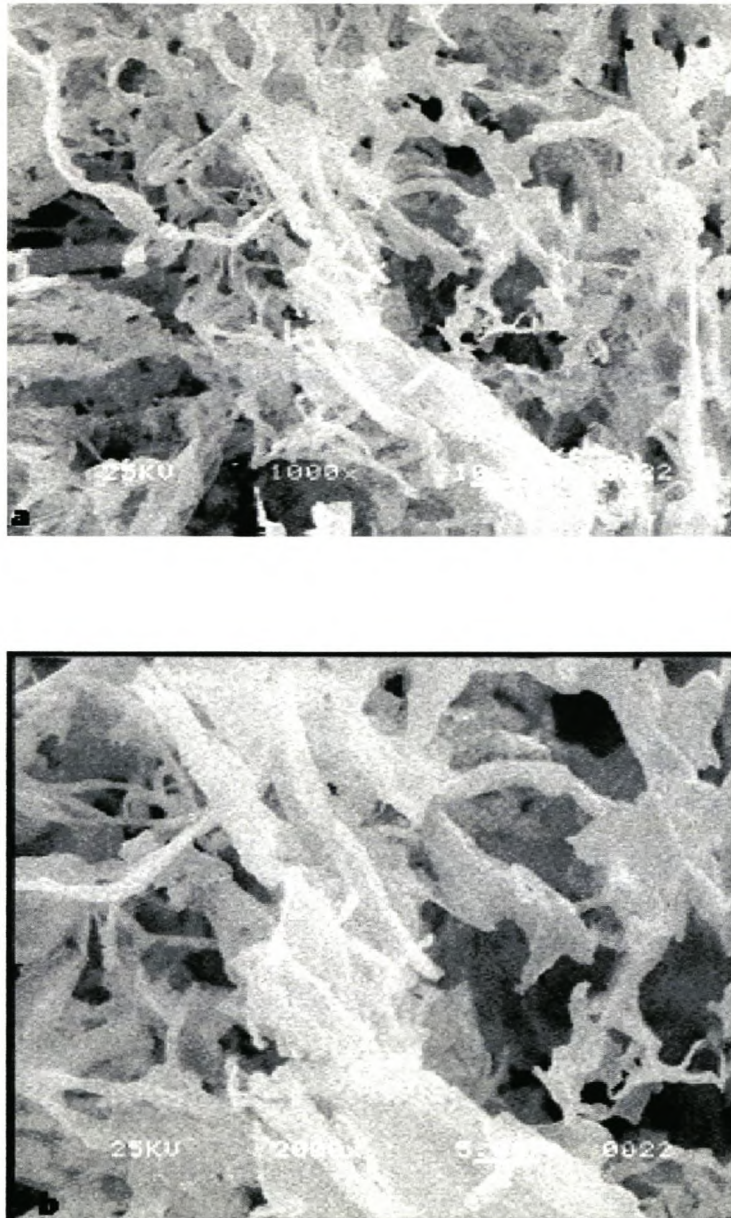


Fig.8.11 Effects of ultrasonic irradiation on the SEM micrographs of HA

(a: 0 hour ultrasonic irradiation, 1000x; b: 0 hour ultrasonic irradiation, 2000x)

Treatment of Humic Acids Extracted from the Acidification Fermentation of Lignocellulosic Residues Using Ultrasonic Irradiation

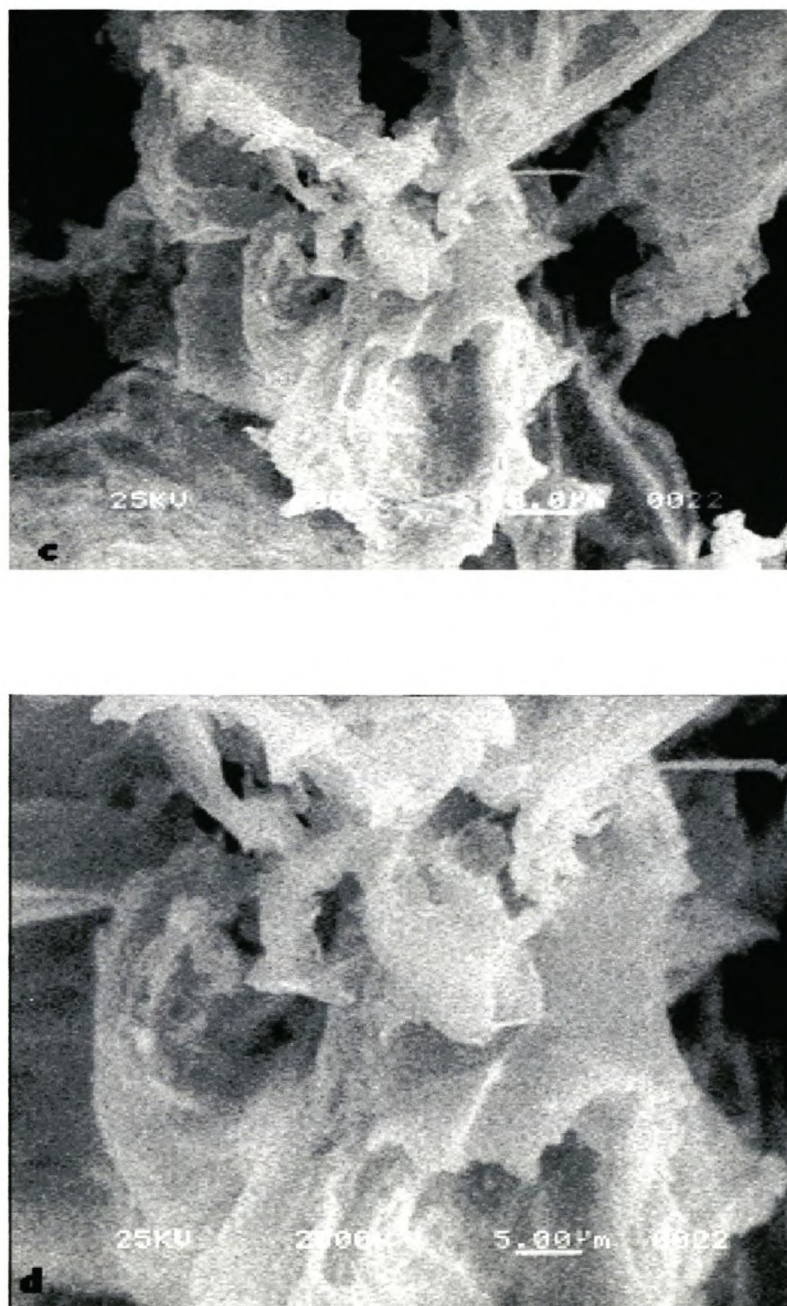


Fig.8.11 Effects of ultrasonic irradiation on the SEM micrographs of HA
(c: 0.5 hour ultrasonic irradiation, 1000x; d: 0.5 hour ultrasonic irradiation, 2000x)

Treatment of Humic Acids Extracted from the Acidification Fermentation of Lignocellulosic Residues Using Ultrasonic Irradiation

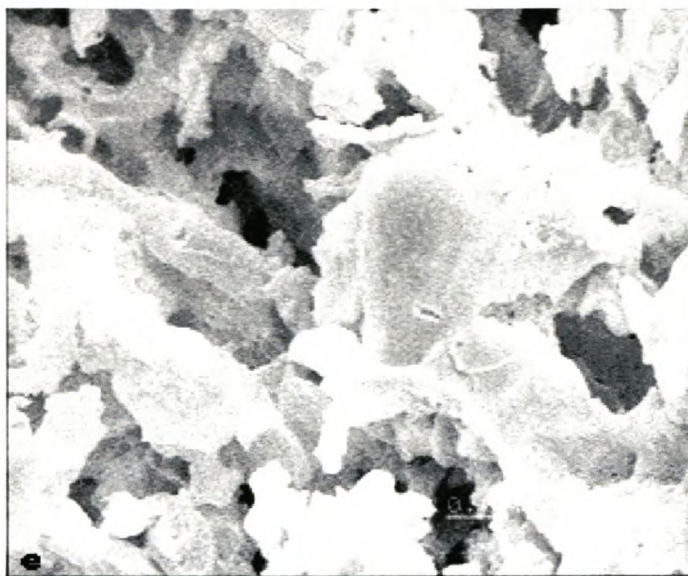


Fig.8.11 Effects of ultrasonic irradiation on the SEM micrographs of HA
(e: 1.0 hour ultrasonic irradiation, 1000x; f: 1.0 hour ultrasonic irradiation, 5000x)

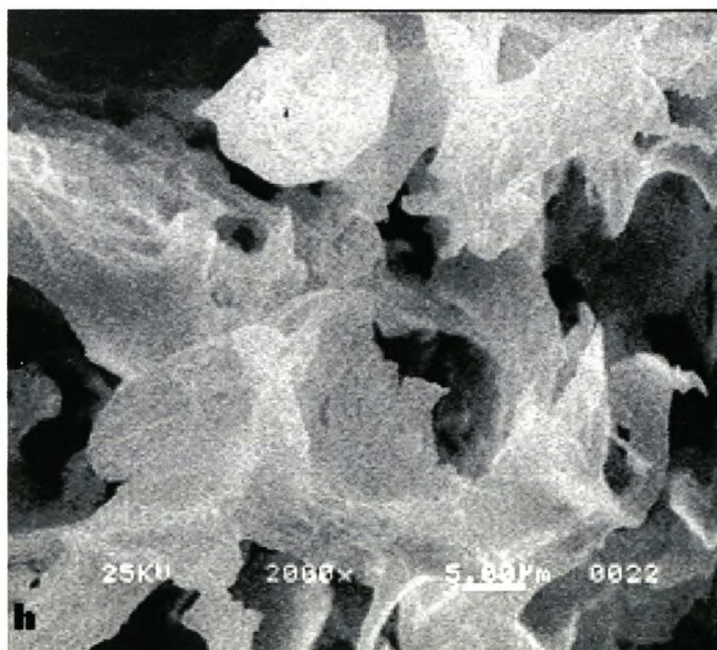
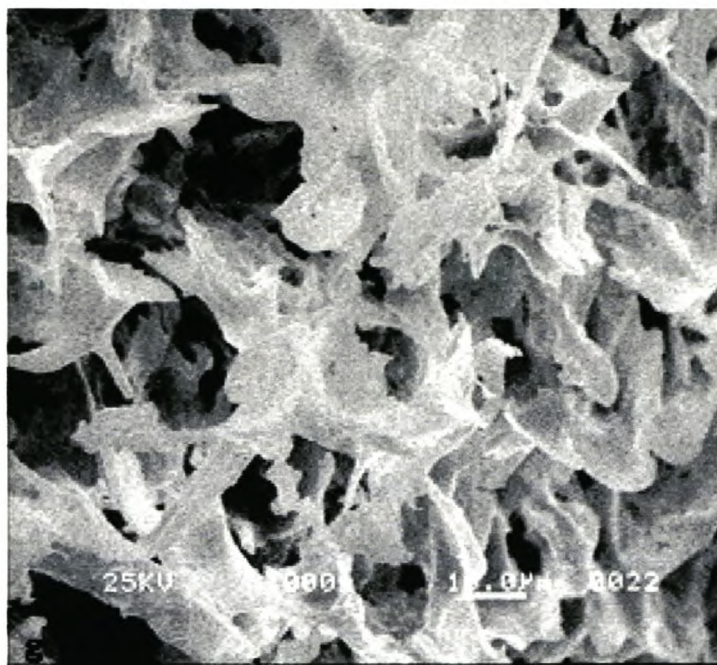


Fig.8.11 Effects of ultrasonic irradiation on the SEM micrographs of HA
(g: 1.5 hour ultrasonic irradiation, 1000x; h: 1.5 hour ultrasonic irradiation, 2000x)

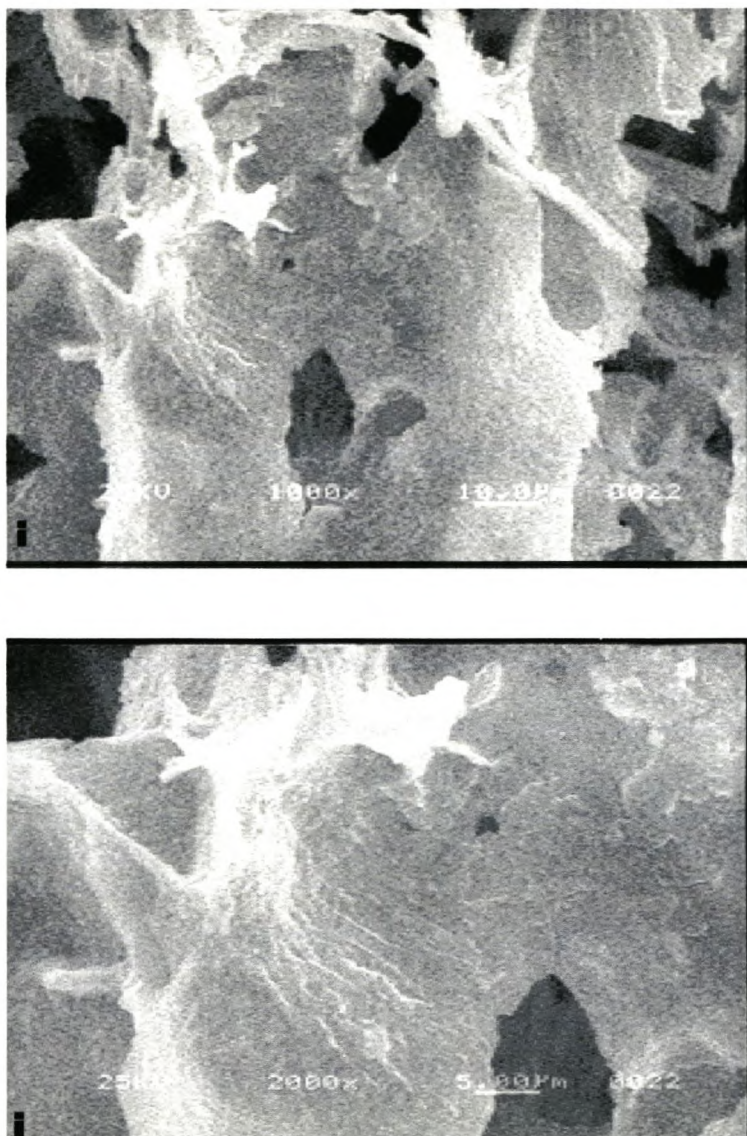


Fig.8.11 Effects of ultrasonic irradiation on the SEM micrographs of HA
(i: 2.0 hour ultrasonic irradiation, 1000x; j: 2.0 hour ultrasonic irradiation, 2000x)

Chapter 9

Effects of Ultrasound on the Adsorption of Humic Acids on the Preformed Aluminium Hydroxide Flocs

In water treatment, flocculation or coagulation with aluminium or ferric salts has been widely used for many years to remove natural organic matter (NOM), including HAs^(234, 125, 166). However, a good knowledge of this in-situ water treatment process was limited by the fact that the adsorption of NOM to aluminium hydroxide flocs during conventional coagulation/flocculation processes is very complex, with the flocculation of aluminium hydroxide particles leading to the formation of larger flocs and the adsorption of NOM molecules on flocs occurring simultaneously. Therefore, efforts to optimize this aspect by performing the adsorption of NOM on preformed aluminium hydroxide flocs⁽²⁸⁾ were necessary. It was suggested that NOM properties such as hydrophobicity, size, and charge density all affect the adsorption to the aluminium hydroxide flocs, and the solubility of the aluminium salt. The strong hydrophobic acids (i.e., humic and fulvic acids) had greater adsorption affinities towards the aluminium hydroxide flocs and the larger molecules had greater adsorption affinity on aluminium hydroxide than the smaller molecules.

The adsorption of HAs by aluminium hydroxide depends on the physical and chemical surface characteristics and the micro-structures of the HA and the aluminium hydroxide formed in-situ. Any effort that has an effect on the surface characteristics and micro-structures of HA and aluminium hydroxide could influence the adsorption efficiency.

As found in Chapter 8, the chemical and physical characteristics were both changed to some extent by ultrasonic irradiation, depending on the irradiation time. In the polydispersed HA system, the ultrasonic irradiation affected the lower molecular fraction (dominant fraction) of HA first with around 50% of this fraction being decomposed into volatile form and disappearing from the system. Then the larger molecular fraction (minor fraction) tended to aggregated into colloidal form and remained in the system. Thus, the ultrasonic irradiation is supposed to enhance the flocculation/aggregation properties of HA and aluminium hydroxide flocs by a variety of physical (erosion, emulsification, aggregation/flocculation) or chemical (the production of radical and excited species; single electron transfer) ultrasound actions. The

idea to explore the ultrasonic irradiation in the removal of HAs by aluminium hydroxide is relatively new.

The chemistry of aluminium salts and HA in an aqueous environment and the effects that ultrasonic irradiation may have on the flocculation/aggregation system are complicated, and the physical and chemical characteristics and structure of HA are dependent on the origin of HA. Therefore the objectives in this chapter were to evaluate the removal of HA, which was freshly extracted from the acidification fermentation of lignocellulose residues, by preformed aluminium hydroxide with the introduction of ultrasound irradiation into the adsorption processes and to investigate the comprehensive surface complexation process of HA on the preformed aluminium hydroxide applied with ultrasonic irradiation.

9.1 Effects of ultrasonic irradiation pre-treating on the efficiency of HA removal by preformed aluminium hydroxide flocs

The effects of ultrasonic-irradiation-pre-treating suspension A₁-A₄ and B₂ on the HAs removal efficiency by preformed aluminium hydroxide flocs are presented in Fig.9.1.

According to the results given in Fig.9.1, the removal efficiency of HAs was evidently enhanced by ultrasonic pre-treatment and was irradiation time dependent. The irradiation time of 90-120 min. was suitable because greater removal efficiency was not gained after 100 min of irradiation. The performance of preformed aluminium hydroxide flocs in the removal of HAs was also improved to some extent by ultrasonic irradiation. The reason could be that the fresh surface produced by ultrasonic irradiation on the preformed aluminium hydroxide flocs had a higher absorbing ability than the aged surface. However, the removal efficiency was comparatively higher with ultrasonic irradiation acting on HAs and the preformed aluminium hydroxide flocs simultaneously. This suggested that the effects that ultrasonic irradiation had on the preformed aluminium hydroxide and HAs, improved the absorbing or flocculating behaviours between them, thus enhancing the removal efficiency of the HAs. However, the ultrasonic irradiation effects that acted on the HA macromolecules, which are physically and chemically more complicated, were relatively more influential on the absorbing or flocculating process. In other words, the ultrasonic irradiation improved the HA removal efficiency by acting, not only on the surface characteristics of the preformed aluminium

hydroxide and the HAs, but also on the complexation reactivity between them during the aggregation or flocculation process. Therefore, the improvement in the removal efficiency of HAs was not so evident when the ultrasonic irradiation was conducted on the preformed aluminium hydroxide flocs alone.

9.2 The effects of HA concentration on the removal efficiency

The effects of HA concentration on the removal efficiency are presented in Fig.9.2. The influences of HA concentration on the removal efficiency agreed, to some extent, with the known coagulation theory. The removal efficiency increased steadily to the utmost with the portions of preformed aluminium hydroxide increasing (the portions of HAs decreasing), then decreased correspondingly to the phase destabilization caused by the over-dosage of preformed aluminium hydroxide. Under suitable concentrations of HA, the removal efficiency was relatively higher when the HA was pre-treated with ultrasonic irradiation, however, this effect levered off in the flocculating system with a higher HA concentration. This indicated that the content of HA in waste water was the basic element that affected the removal efficiency by preformed aluminium hydroxide flocs, while pre-treating with ultrasonic irradiation could enhance the removal efficiency.

9.3 The effects of ultrasonic irradiation and the HA contents on the residual Al concentrations in the adsorption system

The residual Al concentrations under different ultrasonic irradiation pre-treatment conditions and HAs contents in the adsorption system are presented in Fig.9.3 and Fig.9.4. The results show that there was no strong correlation between the residual Al concentration and the ultrasonic irradiation time, although some higher residual Al concentrations were observed with longer ultrasonic irradiation, where some ion-exchange process or re-dissolution of aluminium hydroxide might take place. However, the presence of the HAs exhibited obvious effects on the residual Al concentrations in the flocculation system. The residual Al concentration firstly increased slowly with the dosage of HA to a certain value, then decreased gradually when the HA dosage was continuously increased. It is suggested that on the one hand HA had metal dissolution capacity at low concentrations and on the other hand the multi-valence Al cations might act as bridging agents for the formation of a three-

dimensional HA matrix or gel through the charged sites (such as COO⁻ and O⁻) in the molecular structures of HA. It is these charged sites that account for the ability of HA to retain cations in non-leachable forms prevalently by coulombic and electrostatic forces. This rendered the HAs, at higher concentration, capable of sweeping more free Al species into the flocculate phase from the bulk solution.

9.4 The effects of ultrasonic irradiation on the morphology of preformed aluminium hydroxide flocs and the HAs-aluminium hydroxide floc-adsorption system

The effects of ultrasonic irradiation on the particle arrangement and morphology of preformed aluminium hydroxide flocs and on the microstructure and the surface complex morphology of the HAs-aluminium hydroxide-floc adsorption system are shown in the SEM photos (Fig.9.5(1) to 9.8(2)). As can be seen from Fig.9.5(1) to Fig.9.5(2), there was no evident morphology change in aluminium hydroxide flocs before and after 60 min of ultrasonic irradiation. However, more packed connections between the thin and lateral flake-like flocs was observed in Fig.9.5(2) under 60 min. of ultrasonic irradiation conditions. That is to say, the aluminium hydroxide flocs tend to be more flocculated after ultrasonic irradiation. From Fig.9.6(1) and Fig.9.6(2) an obvious change occurred in the morphology of aluminium hydroxide flocs; the thin lateral flakes condensed into more dimensional arrangements with short thick rod-like prominence with vertical orientation (Fig.9.6(2)). This demonstrated that an aggregation or condensation effect was introduced into the preformed aluminium hydroxide flocs system by ultrasonic irradiation. From the surface absorbing point of view, the dimensional and rough morphological structure of aluminium hydroxide should be more beneficial to the physical attachment of HA macromolecules. However, as the results in Fig.9.1 indicate, there was no evident improvement in HA removal efficiency even under the condition where the preformed aluminium hydroxide was ultrasonically irradiated for as long as 120 min. This suggests that the chemical (not physical) surface complexation played a major role in the aggregation or flocculation process. The microstructure and the surface complex morphology of HAs-aluminium hydroxide floc adsorption system are shown in Fig.9.7(1) to Fig.9.8(2), as can be seen under microscope: the HAs were found to be removed by absorbing on to the surface of the preformed aluminium hydroxide flocs in the form of small fragments or aggregated groups. The aggregates of preformed aluminium hydroxide and HAs tended to be more regular and are closely associated with longer ultrasonic irradiation

time. This further confirmed the aggregation or condensation effects that ultrasonic irradiation have on the arrangement of the flocculation system. Accordingly, it might be proposed that the aggregation or condensation of the flocculation system was one of the reasons that ultrasonic irradiation enhanced the HAs removal efficiency via preformed aluminium hydroxide flocs.

9.5 Discussion

It has been stated that the removal of HA from the soluble phase to the aluminium hydroxide surface was expected to occur mainly due to the surface complexation of the electron-donating functional groups on the HA molecules with the acceptor sites on the aluminium hydroxide surface. Many of the potential complexing groups on HA molecules are also those groups that have acidic protons⁽²⁸⁾.

The surface complexation of three HAs on in-situ formed aluminium hydroxide at a pH of around 7.0, by pH-titration methods, indicated that the adsorption results can be described by a Langmuir-equation, including the ligand content of the HAs and an affinity constant for the hydroxide surface. About 50% of the acidic functions in the HAs are complexing groups, forming 1:1-compounds. The density of complexing groups increases with molecular weight. The salicylic acid groups are involved in surface complexation⁽¹²⁵⁾.

The general effects of HAs on aluminium flocculation result in a proportional demand of Al-salts to saturate the complexing sites on the organic molecules⁽¹²⁵⁾.

As Chapter 8 indicates, the chemical modifications induced by ultrasonic irradiation mainly took place in the carboxylic acid groups, the methylene groups α to aromatic rings, a variety of aliphatic carbons (mainly polymethylene), aliphatic carbon β to aromatic rings, and carbon α to oxygen. It could be postulated that these chemical modifications induced by ultrasonic irradiation on the HA molecular resulted in more complexing sites, thus strengthening the association of HA molecules with preformed aluminium hydroxide flocs, improving the removal efficiency of HAs by preformed aluminium hydroxide flocs.

The adsorption sites for hydrophilic organics can be generally ascribed to the positively charged Al sites of the preformed aluminium hydroxide flocs. Three adsorption mechanisms

⁽²²³⁾ between preformed aluminium hydroxide flocs and humic substances could occur in the absence of aqueous metals or if the concentration of humic substances is high enough to consume the residual free metal species: (1) non-specific anion exchange, (2) specific ligand exchange, and (3) H bonding. The terms non-specific and specific (also called outer- and inner-sphere) adsorption merely describe the degree of hydration of the adsorbed molecule (e.g. HAs). Both inner- and outer- sphere adsorption require anion exchange and are therefore similar reactions. Hydrogen bonding is a sphere case of hydrophobic adsorption, where interaction with the solid phase (a relatively immobile surface-OH or other attached surface molecule) is preferred over the aqueous phase (a rapidly moving H-OH). Ion exchange (also known as ligand and anion exchange) mechanisms should not require that the exchanging compounds carry a charge. Because of its amphipathic character (hydrophobic and hydrophilic) and a wide range of group functions on its molecules, the HA can also be an exchanging neutral hydrophilic compound acting as an exchanging counter-ion (typically the proton). The proton as an exchanging counter-ion would be neutralized by surface-OH on the preformed aluminium hydroxide flocs, either by releasing H₂O into the bulk solution following the organic ligand exchange, or by forming a H bond with the surface of preformed aluminium hydroxide flocs. If the binding to the surface is through a H bonding, then the exchange is not strictly with the surface OH- groups, but rather with the H₂O compounds that are also held by electrostatic forces near the surface. It is known that one important aspect of ultrasound in heterogeneous systems is microstreaming, which creates turbulent flow and aids mass transport. Therefore, one reason that ultrasonic irradiation improved the HA removal efficiency could be ascribed to its enhancing the activity of H₂O compounds that are held by electrostatic forces near the surface of preformed aluminium hydroxide flocs and thus increasing the mass transportation efficiency of this ion exchanging process.

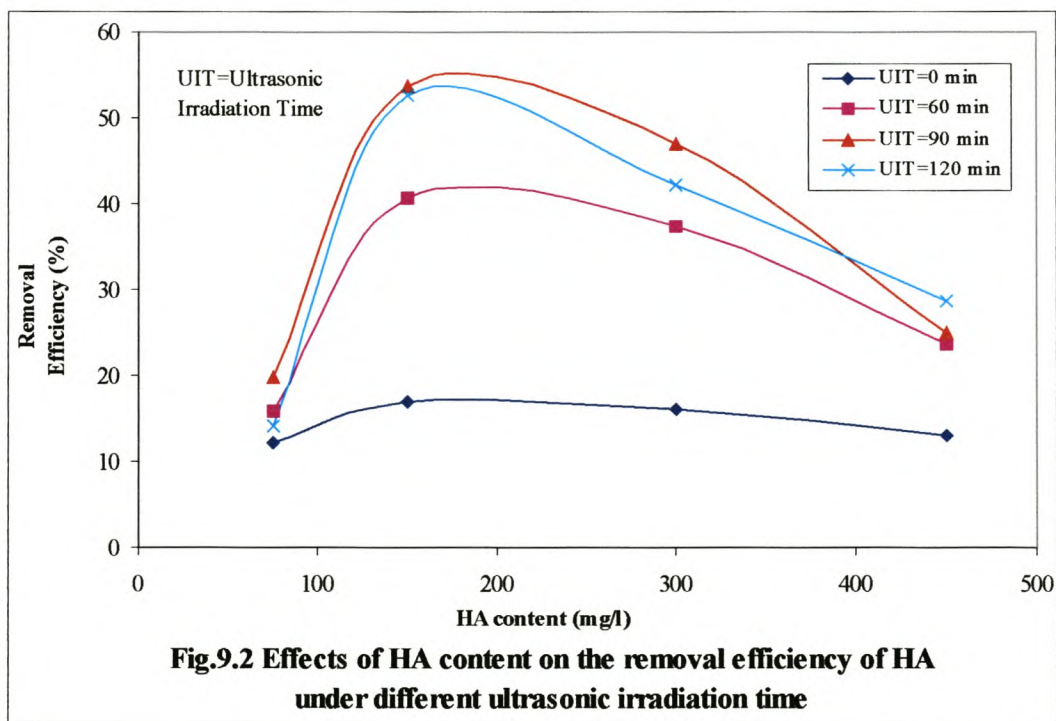
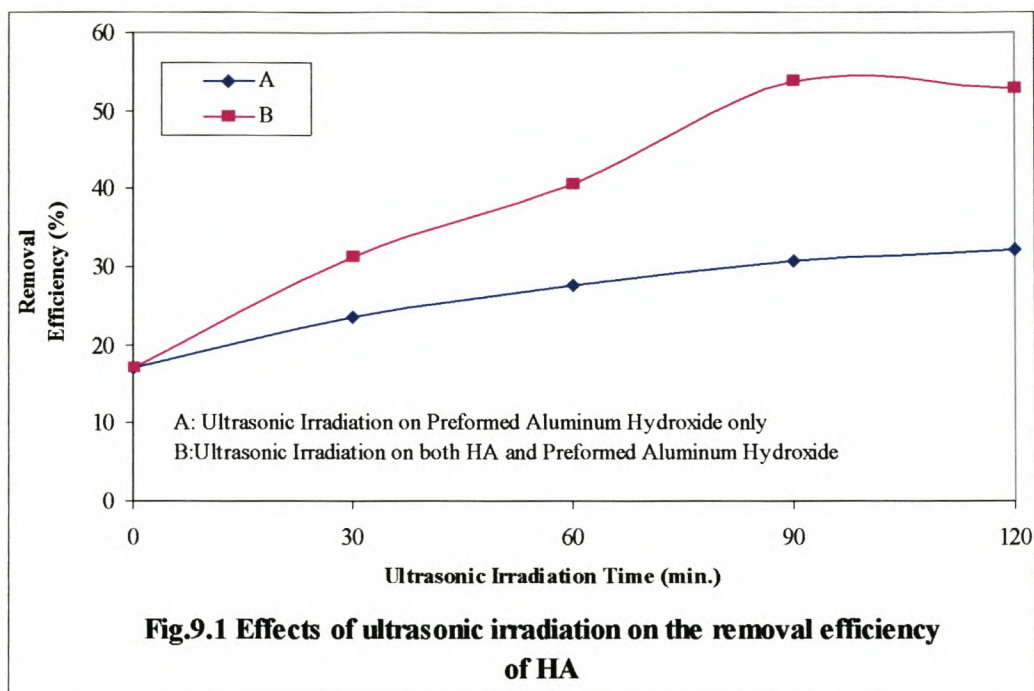
Accordingly, at least two mechanisms took place in the adsorption process. One mechanism was rapid, which involved the interaction between the positive charges of flocs and the negative charges of the polyanionic HAs. The second mechanism was much slower and might have involved an exchange process consisting of the flocs giving up OH- for the anionic sites of the HAs. The one mechanism of an electrostatic nature led to the rapid neutralization of the positive charge of the flocs; the other mechanism involved an exchange of the hydroxide groups of the flocs for the anionic groups of the humic substances. The ultrasonic irradiation did not only provide more complexing sites for the neutralization of the positive charge of the

flocs but also increased the activities of exchanging species of the flocs or enhanced their transportation efficiency for anionic groups of the HAs during the exchanging process.

9.6 Summary

Flocculation and/or coagulation with aluminium and ferric salts are widely used in the removal of natural organic matter (NOM), including HAs, in water treatment. Introduction of the ultrasonic irradiation into the process of flocculation and/or coagulation of HA by preformed aluminium hydroxide obviously improved the removal efficiency of HA. The effect was more evident when the ultrasonic irradiation was applied on the HA and the preformed aluminium hydroxide flocs simultaneously. Aggregation or condensation of the flocculation system was one of the reasons that ultrasonic irradiation enhanced the HAs' removal efficiency via preformed aluminium hydroxide flocs. The ultrasonic irradiation could not only provide more complexing sites for the neutralization of the positive charge of the flocs but could also increase the activities of exchanging species of the flocs or enhance their transportation efficiency for anionic groups of the HAs during the ion-exchanging process.

The results obtained in this chapter indicate that the direct removal of HAs from the aqueous environment by preformed aluminium hydroxide flocs was improved by ultrasonic irradiation pre-treatment. In addition to the abiological methods to remove HAs from the environment, further investigation of the effects of ultrasonic irradiation on the biodegradation characteristics of HAs are needed in order to finalize the biological stabilization of the lignocellulose waste digestion.



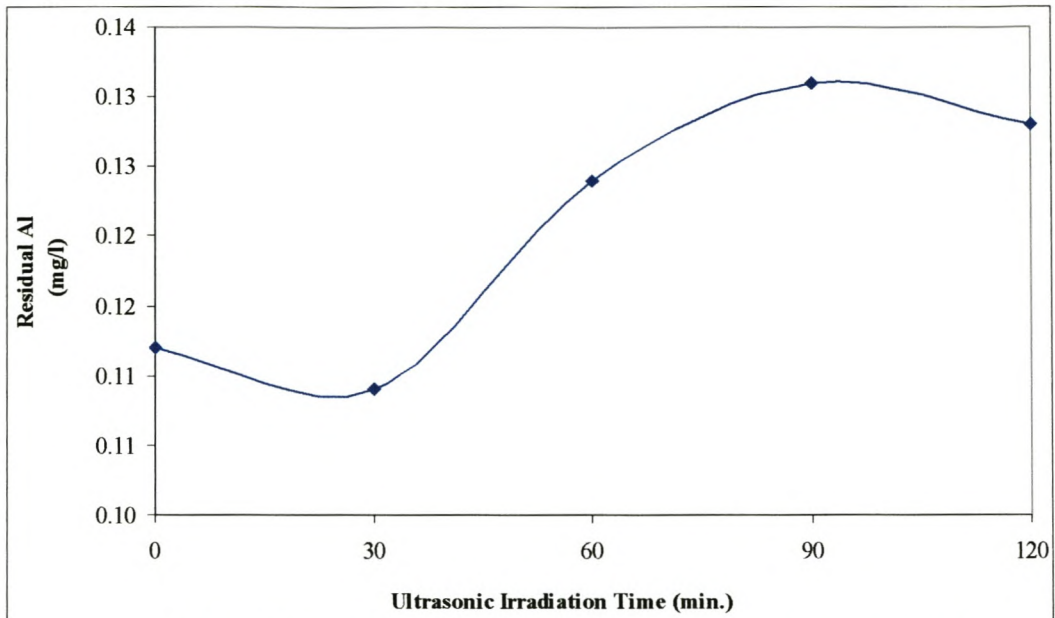


Fig.9.3 Effect of ultrasonic irradiation on residual Al concentraion in preformed aluminium hydroxide suspension

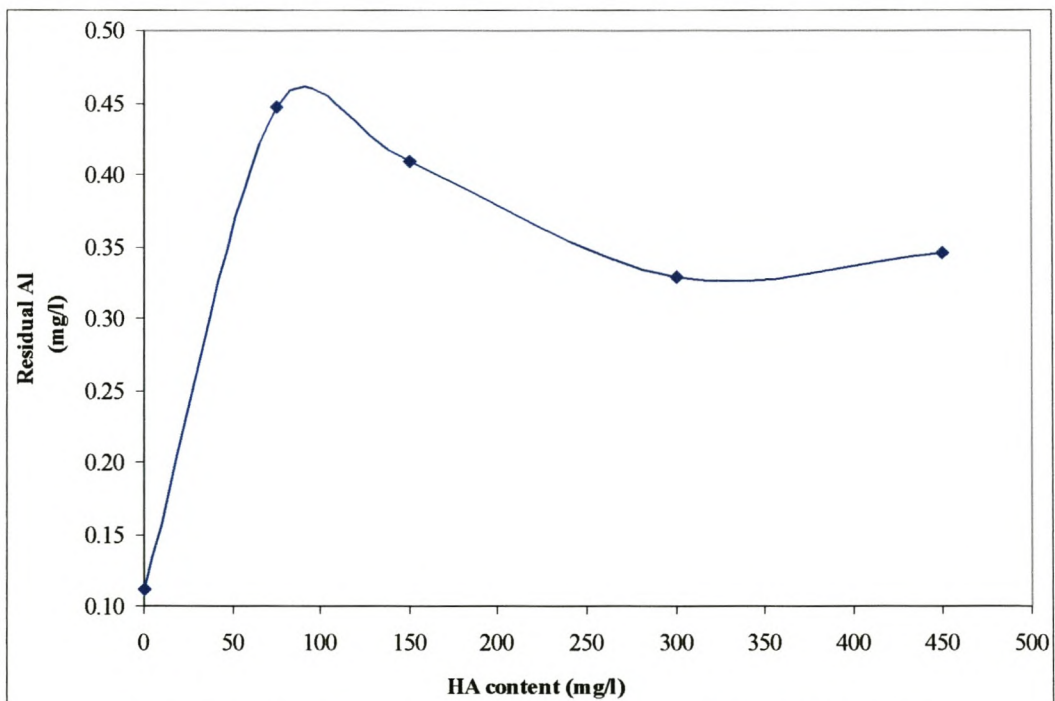


Fig.9.4 Effects of HA content on the residual concentration of Al

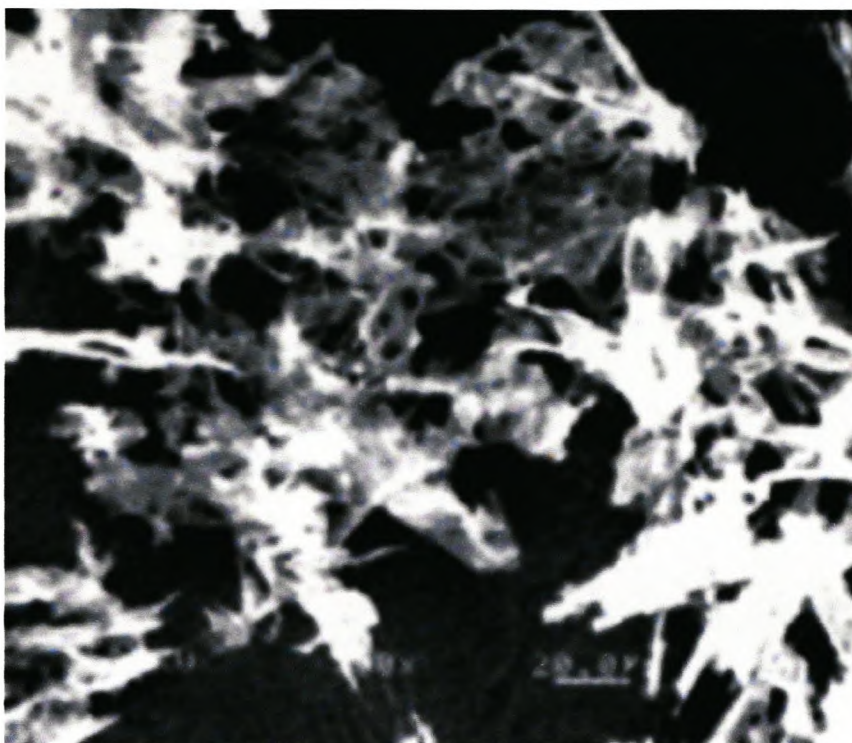


Fig.9.5(1) Preformed aluminium hydroxide flocs
(500x, no ultrasonic irradiation)

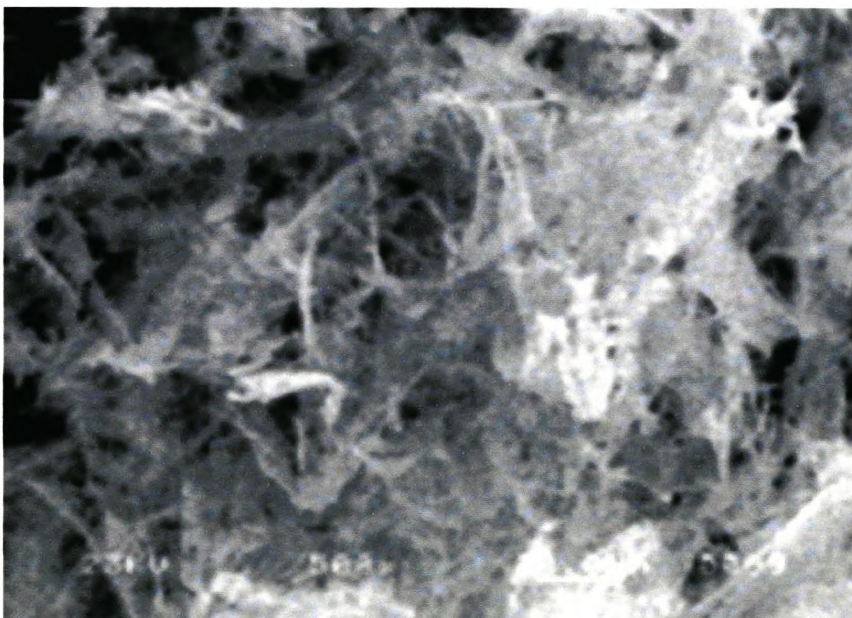


Fig.9.5(2) Preformed aluminium hydroxide flocs
(500x, ultrasonic irradiation, 60 min.)

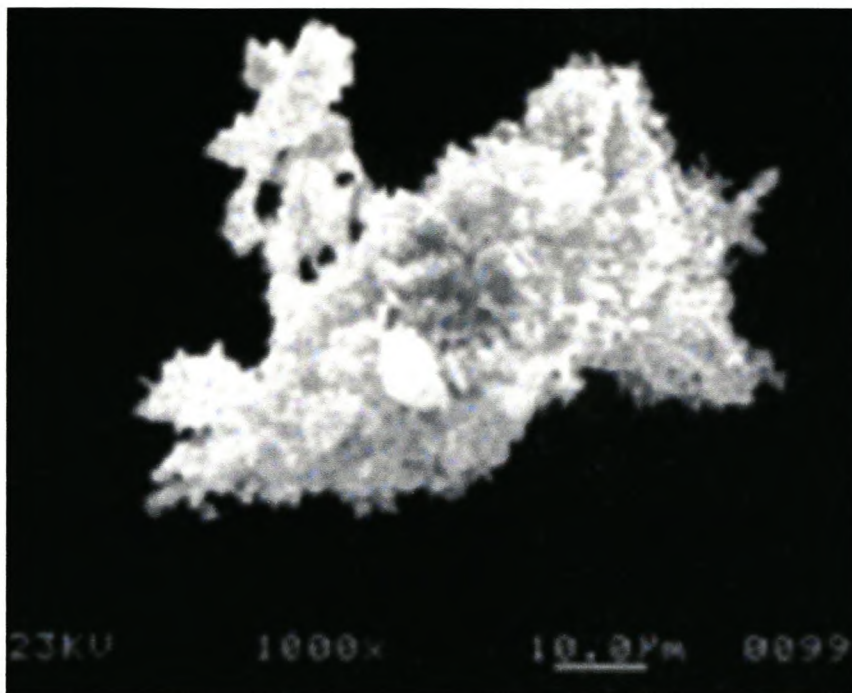


Fig.9.6(1) Preformed aluminium hydroxide flocs
(1000x, ultrasonic irradiation, 90 min.)

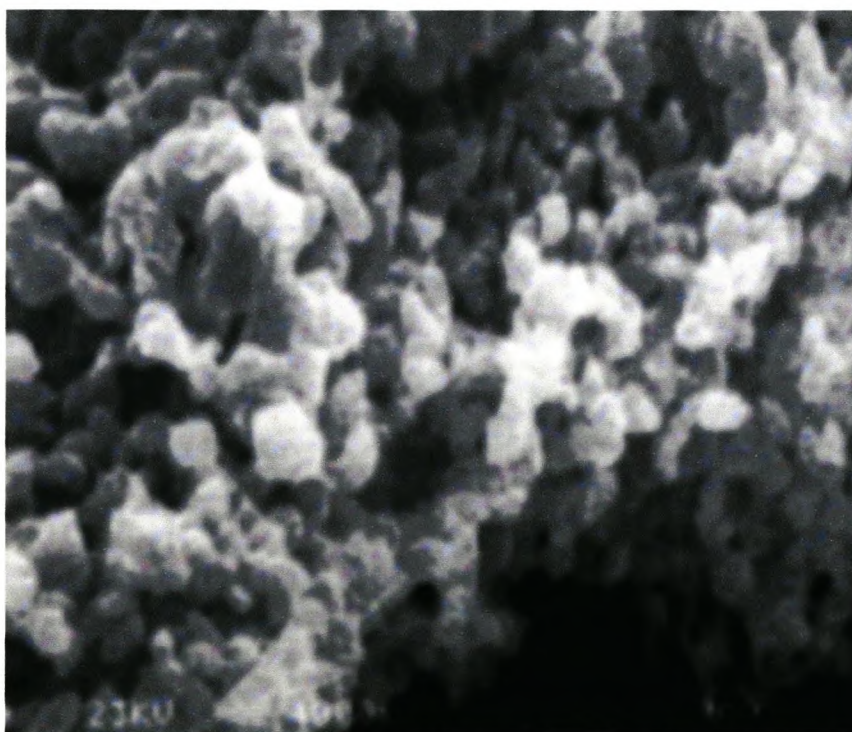


Fig.9.6(2) Preformed aluminium hydroxide flocs
(4000x, ultrasonic irradiation, 90 min.)

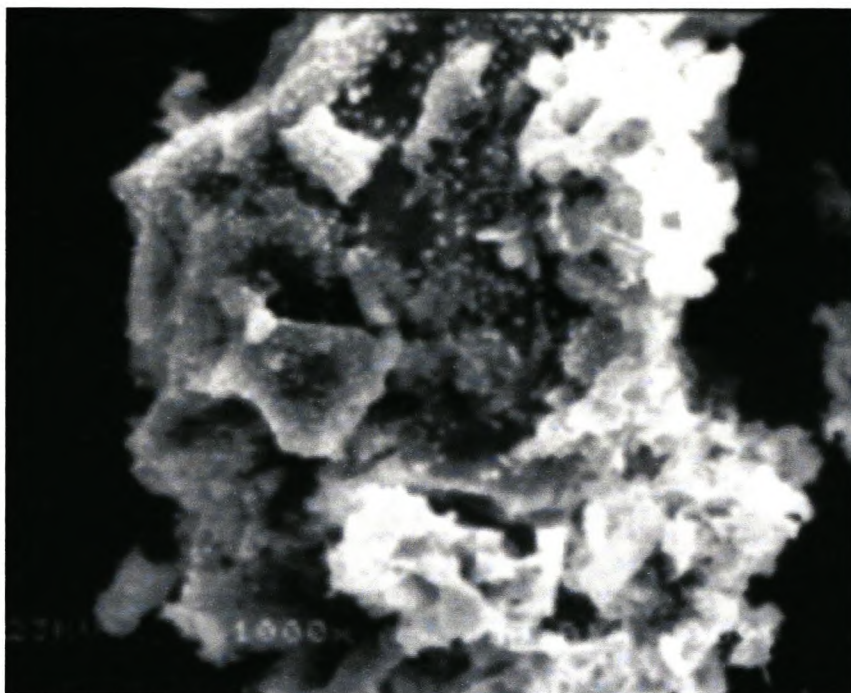


Fig.9.7(1) HAs and preformed aluminium hydroxide flocs
(1000x, ultrasonic irradiation, 60 min.)

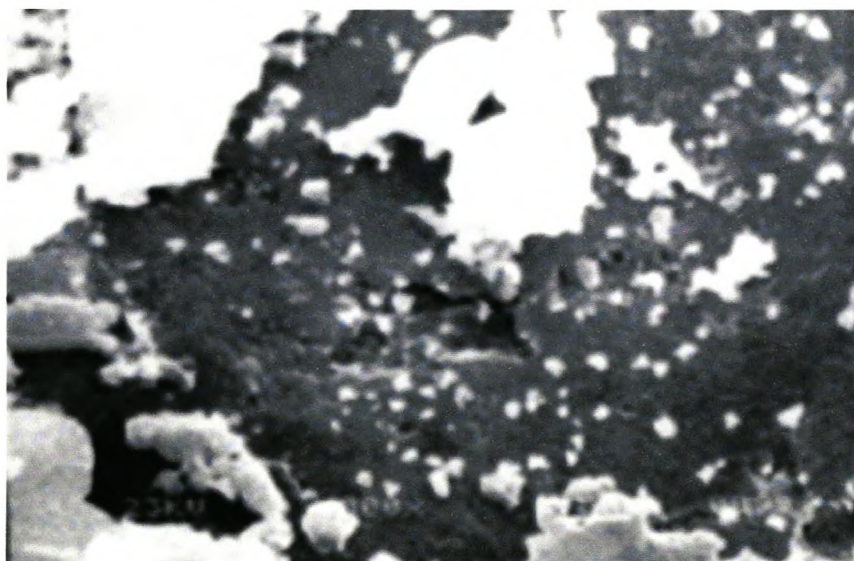


Fig.9.7(2) HAs and preformed aluminium hydroxide flocs
(4000x, ultrasonic irradiation, 60 min.)

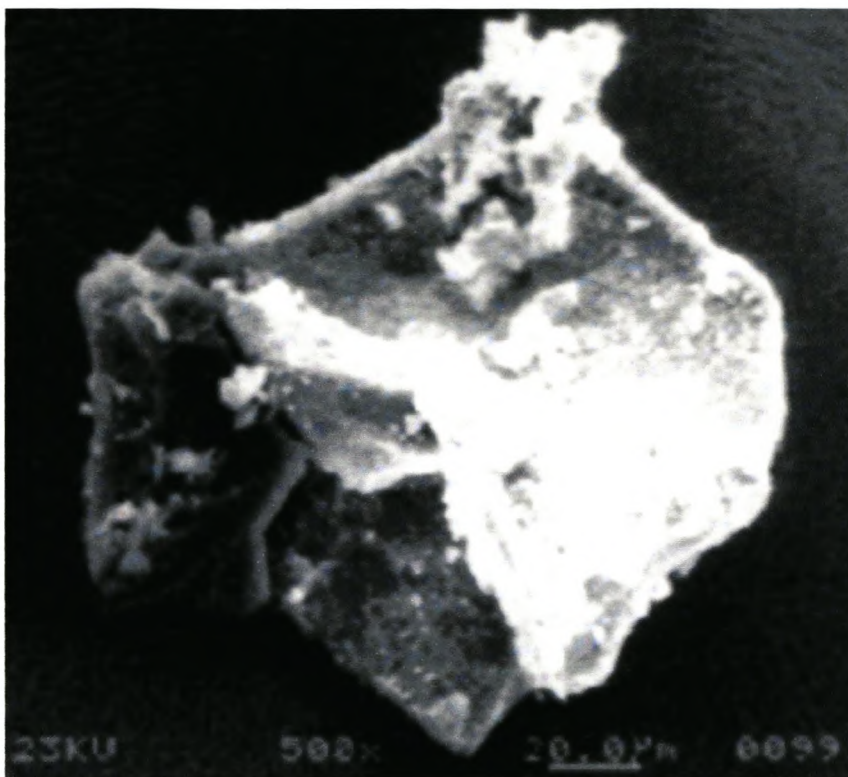


Fig.9.8(1) HAs and preformed aluminium hydroxide floes
(500x, ultrasonic irradiation, 90 min.)

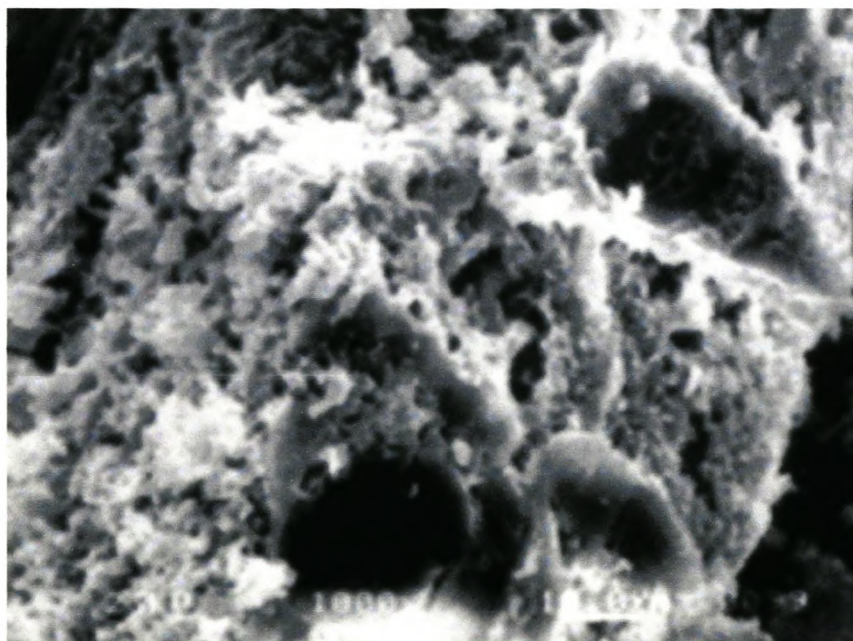


Fig.9.8(2) HAs and preformed aluminium hydroxide floes
(1000x, ultrasonic irradiation, 90 min.)

Chapter 10

Humic Acid Degradation by Lignocellulose-degradating Micro-organisms and Its Toxicity to Anaerobic Microbial Consortia

It was found that the humic acids (HAs) could be degraded by some fungi and bacteria, especially the white-rot fungi such as *Phanerochaete chrysosporium* ^(39, 204, 205, 119, 259, 104). As was stated in Chapter 7, many micro-organisms such as filamentous fungi, yeast and facultative and anaerobic fermentative bacteria were able to survive and grow in environments containing HAs. However, the possibility of HA degradation by these indigenous micro-organisms in the lignocellulose digester and its influence on the activities of the anaerobic microbial remained unknown.

In addition, the fungal decomposition of HAs was mostly carried out through extracellular enzyme activities. The ultrasonic irradiation of aqueous solutions results in the formation of free radicals (OH• and H•) due to the homogenous sonolysis of water. The OH• radical is extremely reactive and is capable of oxidizing most chemical compounds dissolved in water. This oxidizing effect rising from the ultrasonic irradiation was similar to the extracellular enzyme oxidation activity of the micro-organisms. Besides being used as a pre-treatment method to enhance the anaerobic digestion of waste activated sludge (by increasing the hydrolysis rate of waste activated sludge) ⁽²⁵⁴⁾, ultrasonic irradiation showed ⁽²⁴⁰⁾ the possibility of enhancing the growth and enzymatic activities of the micro-organisms in activated sludge. Therefore, the HA degradation process by micro-organism could be enhanced by the application of ultrasonic irradiation both on the HA and on the target degradation micro-organisms. However, little work has been found on this subject, and as was discussed in Chapter 8, so far as the treatment effects obtained were concerned, the destruction of HAs solely by ultrasonic irradiation was not considered complete. Thus, the combined effects of ultrasonic irradiation and bio-degradation of HAs need further investigation.

This chapter reports attempts that were made to investigate (1) the degradability of HAs by the indigenous microbial isolates from the anaerobic lignocellulose digestion reactor from which the HAs were extracted and (2) the HA toxicity on the anaerobic lignocellulose

digestion micro-organisms. The results obtained should provide some information about the extent to which these indigenously-produced HAs could be degraded by the indigenous lignocellulose degradation micro-organisms and of the extent to which the efficiency of the anaerobic lignocellulose digestion process could be inhibited by the HAs.

At the same time, the potential of ultrasonic irradiation on the degradation of HA by lignocellulose-degradation-associated fungi, bacteria and yeast were explored.

10.1 Screening the HAs degradative micro-organism by HAs and ultrasonic irradiation

YNB-HA agar and ultrasonic irradiation were used to screen the micro-organisms that can degrade the HAs and can tolerate the stress of the ultrasonic irradiation. It was found that the mycelial growth of fungi on YNB-HA agar was much less and weaker than those growing on a comparable malt extract agar. Therefore, it was easy to count the colonies growing on YNB-HA agar. The 3-week colonies developing on YNB-HA agar under stepwise-increasing HA concentrations are shown in Fig.10.1. The results indicate a relatively small difference (although almost an order of magnitude) in the number of colonies developing on YNB-HA agar (14×10^5 to 3.8×10^5) with increasing HA concentration from 0.25 to 2.0 g/l. Although the carbohydrate resources present in the inoculum allowed for some limited growth of the micro-organisms without using HAs, the clear zones in the agar where the colonies grew indicated that some HA had been utilized as the growing carbon source by the micro-organisms. In contrast, the mycelia growth was much faster, more dense and larger on rose bengal chloramphenicol agar inoculated with 0.1 ml diluted culture suspension, the same as the inoculation of YNB-HA agar but being applied with the ultrasonic irradiation. The 2-day colonies developing on this agar at different ultrasonic irradiation time are shown in Fig.10.2. It was found that the fungi and the yeast growing in the simulated landfill reactor were not totally killed by 30 kHz ultrasound irradiation as expected. In fact, the growth of the fungi and yeast was enhanced to some extent with ultrasound irradiation duration from 0-40 min., although it showed some decrease in growth under 60 minutes ultrasound irradiation conditions. It is possible to postulate that some fungi or yeast could tolerate the stress of exposure to ultrasound to some extent and some enzyme activities or metabolism functions in this mixed culture system could be improved by this mid-intensity ultrasound irradiation.

10.2 Microscopy morphology

Ten soil-born filamentous fungi and one gram-negative bacterial isolate, which can grow under the HA-present conditions, were included in this study. The typical morphology of the bacteria cell and the filamentous structure of some fungi were observed by an epifluorescence microscope of acridine orange stained samples using a Nikon ECLIPSE E400 microscope. The morphologies, under electric microscopy, of some filamentous fungal and bacterial isolates growing on 2% malt extract agar (2% MEA) and in YNB-HA liquid culture were obtained on a Topcon ABT-60 scanning electron microscope (SEM). The samples for SEM study were a plug of 2% MEA containing the fruiting structures and a few drops of washed bio-mass from the YNB-HA liquid culture. The samples were freeze-dried and sputtered with a gold coat. The morphologies of these micro-organisms are shown in appendix 10.1.

The dominant morphologies of bacteria (growing on TSA) and filamentous fungi growing on 2% MEA and in YNB-HA liquid culture were well observed under a SEM. As can be seen from appendix 10.1, photo 5, on 2% MEA, the conidiophore of *Aspergillus parasitics* (*A. parasitics*) is roughened, its conidia is prominently echinulated and its hyaline conidiophore is well demonstrated under the epifluorescence microscope (appendix 10.1, photo 3). However, the conidiophores and conidia of *A. parasitics* growing in YNB-HA liquid culture look smooth probably because of the HA macromolecular absorbing layer (appendix 10.1, photo 4 and photo 6) which covers the surface of the hyphae, conidiophores and conidia of the fungi. Similarly, most hyphae surfaces of the *Acremonium* gene and the *Trichoderma* gene growing in YNB-HA liquid culture (appendix 10.1, photo 16 and photo 14) did not appear as clean as those growing on 2% MEA (appendix 10.1 photo 15 and photo 13). This demonstrated that “bio-absorbing” was likely one of the mechanisms by which the filamentous fungi de-coloured the HA containing liquid.

In contrast, although the bacteria in the YNB-HA liquid culture (likely growing inside the HA macromolecular structures) appeared much smaller than those growing on TSA (appendix 10.1, photo 2). The obvious morphological differences between the bacteria and the fungi indicated their different growing model in the HA solution. The formation of compact conidia clusters on the ends of the phialide bundle of *Trichoderma* gene were frequently viewed under the SEM (appendix 10.1, photo 13), with the cluster of phialides and single terminal conidia

being observed under an epifluorescence microscope (appendix 10.1, photo 11). The structures of terminally solitary aleurioconidia and conidia chain of *Humicola fuscoatra* var. *fuscoatra* were normally observed under the SEM (appendix 10.1, photo 9.10) with its hyaline mycelium being detected under an epifluorescence microscope (appendix 10.1, photo 7). The multi-septate macro-conidia of the *Fusarium* gene was clearly seen under the epifluorescence microscope (appendix 10.1, photo 8). The awl-shaped, erect, slender phialides from the substratum, smooth-walled, guttuliform conidia of *Acremonium* gene were well observed under the SEM (appendix 10.1, photo 15 and photo 17).

It can be seen that the morphology and surface details of the bacterial cells and filamentous fungi fruiting structures on the HA free medium (2% MEA and TSA) and HA mended medium (YNB-HA) were well illustrated under the microscopes. Simultaneously, a “vivid” structural difference between the bacteria and filamentous fungi during their degradation or de-colourization of macromolecular HA was observed under the SEM and the epifluorescence microscope.

10.3 Micro-organism growth and HA decoloration

All the tested fungal and bacterial isolates demonstrated growth in HA-containing medium, except the yeast isolate. The biomass and the pH developed by fungal and bacterial isolates in the 3-week culture medium are presented in Table 10.1. Firstly, the results show that the fungal and bacterial isolates could not only grow in HA containing environment but could also utilize HA as their carbon and energy source. However, in spite of the fact that the yeast isolate could sustain the stresses of the ultrasonic vibration and exhibited some larger growth by mid-intensity ultrasonic irradiation on the environment containing HA and other carbon source, it could not survive in the environment with no other carbon source other than the HA. Secondly, no substantial differences in biomass growth were detected among the tested fungal isolates after 3 weeks of incubation. However, relatively higher values of bio-mass (46.31-58.4 mg/g HA) were found in the medium with the ultrasound treated HA as the carbon and energy source. Thirdly, the biomass yield of the bacterial culture was unexpectedly higher than the biomass of most fungi. Fourthly, the pH of the culture medium did not notably shift after 3 weeks of incubation by the fungal or bacterial isolates and this indicated that there was a buffering capacity in these microbial systems.

Compared with the control (without inoculation) sample, the spectrophotometric absorbance of cell- or mycelia-free HA medium at 340 nm was reduced to some extent by both the fungal and bacterial cultures. The reduced percentages based on the absorbance of the control sample are presented in table 10.2. All fungal isolates reduced absorbance at 340 nm gradually through the incubation time course. After 3 weeks of incubation, fungal strains and the gram-negative bacteria consortium reduced the absorbance (340 nm) of cell- or mycelia-free HA medium by 10.27-16.31 % and 8.96-10.88 % (average value). It can be seen that the difference in absorbance reduction was not as big as the difference in biomass between the ultrasonically-treated HA medium and the un-treated HA medium. This was probably due to the formation of some other optical absorbing substances, which impaired the absorbance measurement, during the process in which fungi or bacteria metabolically attacked the HA molecules.

10.4 Reactivity to Fast Blue B Salt (FBB)

In both culture fluids (YNB and modified Czapek-Dox medium) of the four fungal isolates, the reactivity of mycelia-free HA medium with FBB was reduced to some extent (69.2%-57.2%) according to table 10.3. Unexpectedly, in the culture fluids of the gram-negative bacterial consortium, the reactivity of cell-free HA medium with FBB increased exceptionally (116%-124%) compared with the control sample. According to Gramss et al. ⁽¹⁰⁴⁾, this increase was associated with the rise in alkalinity resulting from the conversion processes of the bacterial nutrient broth. However, the further noticeable difference in our study was that the reaction of the bacterial fluid-HA medium with FBB was found to be quite unstable during the course of the reaction. The reactivity changed from 10.31-8.26 in the first 0.1 min. to 0.73-1.97 in the later 0.9 min., compared with that of the control sample, which changed from 3.29-2.91 in the first 0.1 min to 1.52-1.08 in the later 0.9 min. In the bacterial culture medium where the HA was not previously treated with ultrasonic irradiation, even several big sharp absorbing peaks were observed during one minute of dynamically spectrophotometrical monitoring of FBB reactivity process. This implied that a big variety of the FBB reactive compounds were formed in the bacterial isolate culture medium as a result of the metabolic activity of the bacteria.

10.5 The presence of hexoses determined by colorimetric methods

The net absorbances (490 nm) of the fungal or bacterial solution plus 0.5% phenol and concentrated H_2SO_4 , compared to the absorbances (490 nm) of the control solution (0.5 % phenol and concentrated H_2SO_4), the absorbances of which were assumed to be 0, are presented in table 10.4. A positive value indicates the production or accumulation of hexoses and their methylated derivatives in the culture medium; a negative value indicates the consumption of hexoses and their methylated derivatives in the tested culture medium. As can be seen from table 10.4, the presence or accumulation of hexoses and their methylated derivatives were detected in most fungal culture fluids, with a relatively higher content in the HA medium which was pre-treated by ultrasonic irradiation. In contrast, the relatively higher negative value of the net absorbance was found in the bacterial culture fluids. This implied that most hexoses and their methylated derivatives were consumed by bacterial metabolic activity.

10.6 HA toxicity on the anaerobic consortium

At the end of the 3-month anaerobic incubation, the cell masses and the CO_2 and CH_4 contents in the headspace of the 50 ml anaerobic cultures, supplied with different concentrations of HAs were measured. The results (in Fig.10.3 and Fig.10.4) indicate that the cell masses decreased dramatically when the HA concentration in the culture was above 0.1 g/l. The production of CH_4 totally levelled off when the HA concentration in the culture reached 0.05 g/l, although the production of CO_2 could still be detected in the headspace throughout the incubation period even when the HA concentration in the culture was as high as 0.1 g/l.

The anaerobic bio-degradation of organic polymers, provided it can be digested, is carried out by the three groups of bacteria: the hydrolytic and fermentative bacteria; the obligate proton-reducing acetogens and the methanogens. Purging the cultures with pure nitrogen allows the culture to begin in an oxygen and CO_2 free atmosphere and it will remain this way if the organism cannot initiate growth and produce CO_2 anaerobically. Therefore, the CO_2 concentration in the closed culture vessels can be considered as one of the growth indexes of

the anaerobic organism provided the culture medium is well buffered with HCO_3 and phosphate solution buffer.

The results obtained so far imply that among the three groups of bacteria consortiums, the methanogens are the most sensitive to the toxicity of HAs. However, the fermentative consortiums can survive and grow properly on the readily obtainable carbon source (such as glucose) in dilute HA solutions, and can even survive in the environment where the concentration of HA was as high as 0.1 g/l. This could be one of the reasons why the methanogenic stage was difficult to reach, with the fermentation stage proceeding without difficulty during the anaerobic degradation of lignocellulose substances.

Since organic substrates cannot be fermented further than to acetate and H_2 , the complete mineralisation of organic matter under anaerobic conditions depends on other physiological bacterial consortia. Nevertheless, fermenting bacteria play a central role in the anaerobic community since they are the important organisms that can hydrolyse and utilize polymers (polysaccharides, proteins, etc.) in this system. All of the other important microbial players in anaerobic communities (sulphate reducers, methanogens) are capable of using only a limited number of low molecular weight substrate⁽⁵⁴⁾. The terminal mineralization depends on the activity of fermenting bacteria for substrate supply. Therefore, a complete anaerobic digestion of lignocellulose can be obtained by dilution or cleavage of the refractory humic substances, such as HA, when the fermentation is successful. This subject has been also discussed in Chapter 6.

10.7 Discussion

A careful comparison of the reduction percentages in absorbance at 340 nm, the FBB reactivity and the biomass yields of the individual fungal cultures and bacteria isolates failed to give a good correlation between these three indexes. This means that the slow, but progressive, degradation of HA by these micro-organisms was the result of the biochemical activities provided by both the isolated fungi and bacteria. Provided that the changes arose from abiotic conditions such as pH, incubation temperature and oxygen availability and that the humidity was normalized, the reductions in FBB reactivity were attributed to the aromatic-ring cleavage of the HAs; the reductions in optical absorbance at 340 nm were

associated with the qualitative and/or quantitative break-down of the HA macromolecules; the increase of bio-mass indicated the extent to which the HA was involved or consumed in the growth processes of the fungi or bacteria; the changes in concentrations of hexoses and their methylated derivatives implied the production, accumulation or consumption of the intermediate products in the culture fluids. Since the abiotic conditions for the same batch experiments were closely controlled during the investigation, the comparisons made between these indexes were able to provide an outline of the degrading abilities of the fungal and bacterial consortia.

Theoretically, in a medium with the target compound as the only carbon source, only the organisms with the corresponding degrading ability will grow. In general, fungi grow more slowly and produce fewer propagates than bacteria. Even though they are the major degraders in nature, they do not use the degraded products such as reduced sugars as a carbon source for growth. That is to say, the fungi may produce many potential biodegradation enzymes capable of degrading complex pollutants such as HAs, yet they do not use these break-down products for their sustained growth. Therefore, a relatively higher presence and accumulation of hexoses and their methylated derivatives were detected in most fungal cultures than in the bacterial cultures.

The bio-degradation strategies can be divided into three general categories: the target compound is used as carbon source; the target compound is enzymatically attacked but not used as carbon source (co-metabolism); and the target compound is taken up and concentrated within the organism (bioaccumulation). Although fungi participate in all three strategies, they are often more proficient at cometabolism and bioaccumulation than at using complex substances, such as xenobiotics, as sole carbon sources ⁽¹⁸⁾. Therefore, many fungi need a supplemented carbon source for sustained growth. As a result, the decolouration rate was slower at later incubation stages in the batch experiments.

However, owing to the following reasons, the fungi remain best biodegraders in nature. Firstly, the mycelial growth pattern gives a competitive advantage over single cells such as bacteria and yeasts, especially with respect to the colonization of insoluble substrates: fungi can rapidly ramify through substrates, literally digesting their way along by secreting a battery of extracellular degrading enzymes; hyphal penetration provides a mechanical adjunct to the

chemical breakdown effected by the secreted enzymes; and the branch-like and network structures of the filamentous fungi maximize both mechanical and enzymatic contact with the environment. Secondly, the extracellular nature of the degrading enzymes enables fungi to tolerate higher concentrations of toxic chemicals than would be possible if these compounds had to be brought into the cell. Finally, since the relevant enzymes are induced by nutritional signals independent of the target compound during secondary metabolism, they can act independently of the concentration of the substrate, and their frequently non-specific nature means that they can act on chemically diverse substrates⁽¹⁸⁾. It was therefore noticed that the absorbance reduction percentages were higher in the fungal cultures than those in the bacterial cultures while the bacterial biomass was found to be comparable to or even a little higher than most of the fungal biomass. This was possible because some parts of the HAs, which were not degraded by the fungi but were physically absorbed in the mycelia structure, were washed out of the biomass but were not included in the filtrate of the culture fluids that was measured for absorbance.

Bacteria can directly assimilate low molecular weight organics such as hexose sugars. Polymers and organic particles are hydrolyzed by membrane-bound hydrolytic enzymes. Similarly, when only polymers are available, bacteria must have exoenzymatic activity⁽⁸²⁾. In other words, quite different from fungi which can have three ways to “degrade” the polymer-like HAs from an aquatic environment by three different ways, the bacteria have only one way of degrading the polymer-like HAs, i.e. exoenzymatically hydrolyze-metabolize, (catabolic and/or anabolic), with the exoenzymatical hydrolysis being the most important step.

Little is known about the types of enzymes that slowly degrade humic material, but it can be surmised that a variety of enzymes must be involved and that they may be relatively non-specific. Oxidative enzymes play a major role in the degradation of bio-polymers, but in some cases organic acids and chelators excreted by the fungus also contribute to the process. The reduction of FBB reactivity of cell or mycelia-free culture fluids could be ascribed to the oxidative-enzymatical breaking down of the aromatic structures of the original HA and/or the further digestion of the hydroxylated aromatic compounds from the degradation of HAs.

In the batch culture, the synthesis of many fungal products and enzymes is not correlated with growth but is triggered by the limitation of an essential nutrient. For example, both the

secondary metabolite production and the production of lignolytic enzymes are correlated with the idiophase of the growth ⁽¹⁸⁾. Similar cases could exist in the fungal degradation of HA. Accordingly, it was found that the HA was slowly decolourized by the in the growing fungi.

The application of ultrasonic irradiation on the bio-degradation of HA has a twofold effect. Firstly, the cavitation and micro-streaming will bring about the physical breakdown of the polymer molecular structure, the colloidal fractions of polydispersed molecular system; The production of radical and excited species and the single electrontransfer in the homogeneous phase or at the solid liquid interface will induce chemical oxidation of some functional groups of bio-polymers. Secondly, the cavitation and micro-streaming effects on the polymer structure will simultaneously act on the micro-organism system, which will cause the weakening or enhancing of the growth of the micro-organism in the system. The efficient physically break-down of the polymer molecular structure was not well confirmed by the previous work under the sonification conditions used in Chapter 8, but a certain growth enhancement of some fungi and yeast under the same ultrasonic conditions was confirmed in this chapter. It can be postulated that the benefits of ultrasonic irradiation to improve the bio-degradation of HA by fungi and bacteria, although not highly evident, mainly resulted from the chemical oxidation of HA functional groups or from the colliding of the polydispersed HA molecular fractions. As most exoenzymes have oxidation functions, the chemical oxidation effects of ultrasonic irradiation are beneficial to the exoenzymatical hydrolysis of HA by bacteria or fungi. As far as the mycelial structure of filamentous fungi is concerned, the colloidal effects of ultrasound on the flocculation of polydispersed HA molecules also make them easier to be absorbed in the mycelial net structure of the filamentous fungi. In addition, it was reported that the larger HA molecules tended to be preferentially degraded in favour of a relative enrichment of smaller molecules ⁽²⁷³⁾.

10.8 Summary

The HA extracts from the acidification fermentation of lignocellulose residues were exposed to fungal, bacterial and yeast isolates, which were screened by a HA-carbon source and ultrasonic irradiation for three weeks in static aerobic culturing conditions at 28 °C in the dark. Its toxicity on an anaerobic consortium was tested. The degradation process of the HAs

was investigated by monitoring the absorbance at 340 nm, the FBB reactivity, the reducing sugar contents in the culturing solution and the biomass yields.

The following conclusion can be made:

- 1) The fungal and bacterial isolates (except the yeasts) from the lignocellulose waste degradation process could grow in the medium with HAs as the sole carbon source and degrade the HAs to some extent.
- 2) The enhancement of the biological decomposition of HAs by ultrasonic irradiation could be attributed to the chemical oxidation effects, which are beneficial to the exoenzymatical hydrolysis of HA by bacteria or fungi, and the colloidal effects of ultrasound on the polydispersed HA macromolecules, which made the absorption of HAs into mycelial net structures of the filamentous fungi easier.
- 3) Some fungi or yeast could sustain the stress of the ultrasound vibration to some extent with their growths being enhanced in less than 40 minutes of ultrasonic irradiation.
- 4) Among the three functional groups of anaerobic bacterial consortia, the methanogens are the most sensitive to the toxicity of HA. However, the fermentative consortium can survive and grow on the readily obtainable carbon source (such as glucose) in HA solutions even when the concentration of HA was as high as 0.1 g/l.

10.9 Further research work on HA degradation

Among the anaerobic treatment processes, the upflow anaerobic sludge blanket (UASB) ⁽¹⁵¹⁾ has become very popular for the anaerobic biological treatment of waste water because of the beneficial energy and substance flowing channel in the granules for the mutual growth of the anaerobic methane-producing consortium. Thus, the UASB reactor is another promising technological development for the treatment of HA-bearing leachate. Therefore further attention will be drawn to the investigation of the degradation efficiency of the HAs bearing leachate from the high-solid anaerobic digestion process of lignocellulose residues in the UASB reactor.

Table.10.1 The developed biomass and pH of the medium by fungi and bacteria isolates after 3 weeks of incubation

Microbial Isolate	Cezapek-Dox-HA Medium				YNB-HA Medium				TS-HA Medium			
	HA (0)		HA (1)		HA (0)		HA (1)		HA (0)		HA (1)	
	pH	Biomass mg/g HA	pH	Biomass mg/g HA	pH	Biomass (mg/g HA)	pH	Biomass (mg/g HA)	pH	Biomass (mg/g HA)	pH	Biomass (mg/g HA)
Control	6.83	0.0	7.07	0.0	6.12	0.0	6.26	0.0	6.76	0.0	6.67	0.0
Trichoderma 1	7.19	22.3	7.26	45.6	6.23	18.5	6.29	50.2				
Trichoderma 2	7.08	15.0	7.16	36.6	6.35	18.1	6.27	60.9				
Trichoderma 3	7.08	16.4	7.19	40.0	6.45	14.8	6.33	56.3				
Trichoderma 4	7.19	17.8	7.26	44.3	6.30	19.5	6.24	62.5				
Trichoderma 5	7.07	10.6	7.14	43.0	6.11	12.0	6.20	59.0				
Trichoderma 6	7.13	18.3	7.15	46.7	6.33	12.5	6.24	63.5				
Aspergillus Parasitics	7.16	14.2	7.15	54.9	6.26	23.2	6.32	54.1				
Fusarium Oxysporum	7.31	9.8	7.40	51.1	6.38	11.3	6.35	64.1				
Acrimonium	7.16	8.9	7.21	43.0	6.28	16.1	6.19	51.3				
Humicola Fuscoatra var Fuscoatra	7.25	10.8	7.32	57.9	6.35	14.9	6.41	62.1				
Gram-negative Bacteria									8.44	65.5	8.60	83.3
Average	7.162	14.41	7.224	46.31	6.304	16.09	6.284	58.4	8.44	65.5	8.60	83.3

Note: HA(0)—The HA without pretreated by ultrasonic irradiation

HA(1)—The HA pretreated by ultrasonic irradiation (at 30 kHz for 2 hours)

Table 10. 2. The reductions in absorbance (340 nm) of cell or mycelia free culture fluids during incubation

Microbial Isolate	Cezapek-Dox-HA Medium						YNB-HA Medium						TS-HA Medium	
	HA (0)			HA (1)			HA (0)			HA (1)			HA (0)	HA (1)
	Time(week)			Time(week)			Time(week)			Time(week)			Time(week)	Time(week)
	1.0	2.0	3.0	1.0	2.0	3.0	1.0	2.0	3.0	1.0	2.0	3.0	3.0	3.0
Trichoderma 1	2.70	6.91	9.31	3.40	9.65	16.2	3.31	6.02	15.8	5.61	9.36	18.3		
Trichoderma 2	2.54	6.72	10.5	4.82	10.01	15.8	4.20	6.18	17.3	5.82	10.4	16.9		
Trichoderma 3	3.11	6.04	9.34	4.09	9.26	14.1	4.09	7.24	14.6	5.12	9.22	15.5		
Trichoderma 4	1.80	6.25	11.95	4.01	10.96	17.3	5.10	7.89	15.9	6.38	9.62	18.9		
Trichoderma 5	2.63	6.10	11.04	3.86	8.21	14.8	4.26	8.65	14.2	5.69	10.22	15.2		
Trichoderma 6	2.04	6.13	11.25	4.10	9.63	14.2	5.10	7.88	13.8	6.21	9.95	16.3		
Aspergillus Parasitics	5.41	10.26	12.30	6.89	9.53	15.69	4.98	8.26	16.76	8.21	12.64	18.36		
Fusarium Oxysporum	4.01	8.35	9.68	5.33	8.26	14.68	6.12	9.10	14.08	7.68	9.23	15.83		
Acrimonium	1.35	5.59	7.65	3.21	8.22	10.35	3.68	6.02	12.64	4.22	9.64	14.11		
Humicola Fuscoatra var Fuscoatra	3.64	5.38	9.68	4.85	7.04	14.25	5.01	7.25	10.38	5.98	7.49	13.66		
Gram-negative Bacteria													8.96	10.88
Average	2.92	6.77	10.27	4.46	9.08	14.74	4.59	7.45	14.55	6.09	9.78	16.31	8.96	10.88

Note: HA(0)—The HA without pretreated by ultrasonic irradiation

HA(1)—The HA pretreated by ultrasonic irradiation (at 30 kHz for 2 hours)

Table 10.3. The changes in FBB reactivity of cell or mycelia free culture fluids in during incubation

Microbial Isolate	Cezapek-Dox-HA Medium						YNB-HA Medium						TS-HA Medium	
	HA (0)			HA (1)			HA (0)			HA (1)			HA (0)	HA (1)
	Time (week)			Time (week)			Time (week)			Time (week)			Time (week)	Time (week)
	1.0	2.0	3.0	1.0	2.0	3.0	1.0	2.0	3.0	1.0	2.0	3.0	3.0	3.0
Control with HA	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00		
Trichoderma 1	0.91	0.74	0.59	0.84	0.65	0.52	0.93	0.85	0.64	0.89	0.68	0.54		
Trichoderma 2	0.95	0.78	0.65	0.82	0.69	0.58	0.94	0.78	0.67	0.82	0.71	0.56		
Trichoderma 3	0.91	0.80	0.71	0.84	0.72	0.60	0.93	0.74	0.62	0.90	0.69	0.58		
Trichoderma 4	0.88	0.75	0.68	0.79	0.66	0.53	0.91	0.69	0.57	0.86	0.62	0.54		
Trichoderma 5	0.93	0.82	0.75	0.86	0.72	0.64	0.89	0.71	0.62	0.83	0.64	0.52		
Trichoderma 6	0.90	0.84	0.72	0.88	0.76	0.61	0.92	0.78	0.65	0.86	0.70	0.56		
Aspergillus Parasitics	0.86	0.80	0.69	0.82	0.73	0.59	0.89	0.76	0.63	0.82	0.64	0.53		
Fusarium Oxysporum	0.90	0.83	0.71	0.85	0.68	0.62	0.91	0.89	0.68	0.88	0.79	0.60		
Acrimonium	0.94	0.86	0.75	0.89	0.72	0.65	0.92	0.83	0.74	0.90	0.74	0.63		
Humicola Fuscoatra var Fuscoatra	0.89	0.80	0.69	0.84	0.75	0.60	0.91	0.87	0.76	0.85	0.71	0.66		
Gram-negative Bacteria													1.16	1.24
Average	0.91	0.80	0.69	0.84	0.71	0.59	0.92	0.79	0.66	0.86	0.69	0.57	1.16	1.24

Note: HA(0)—The HA without pretreated by ultrasonic irradiation

HA(1)—The HA pretreated by ultrasonic irradiation (at 30 kHz for 2 hours)

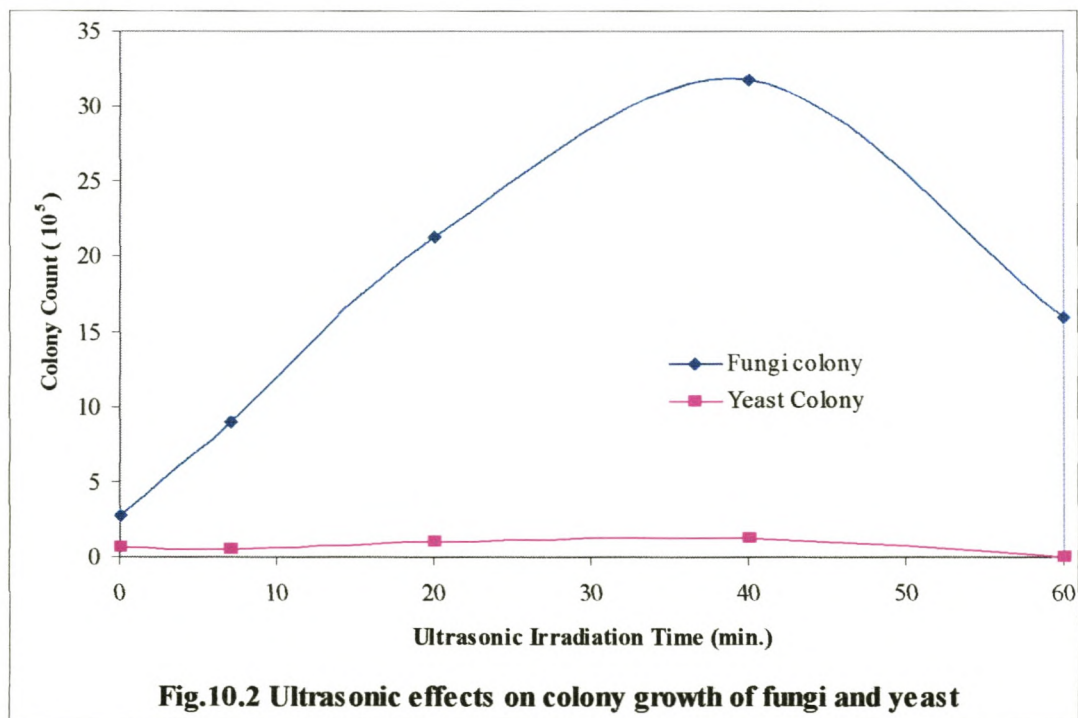
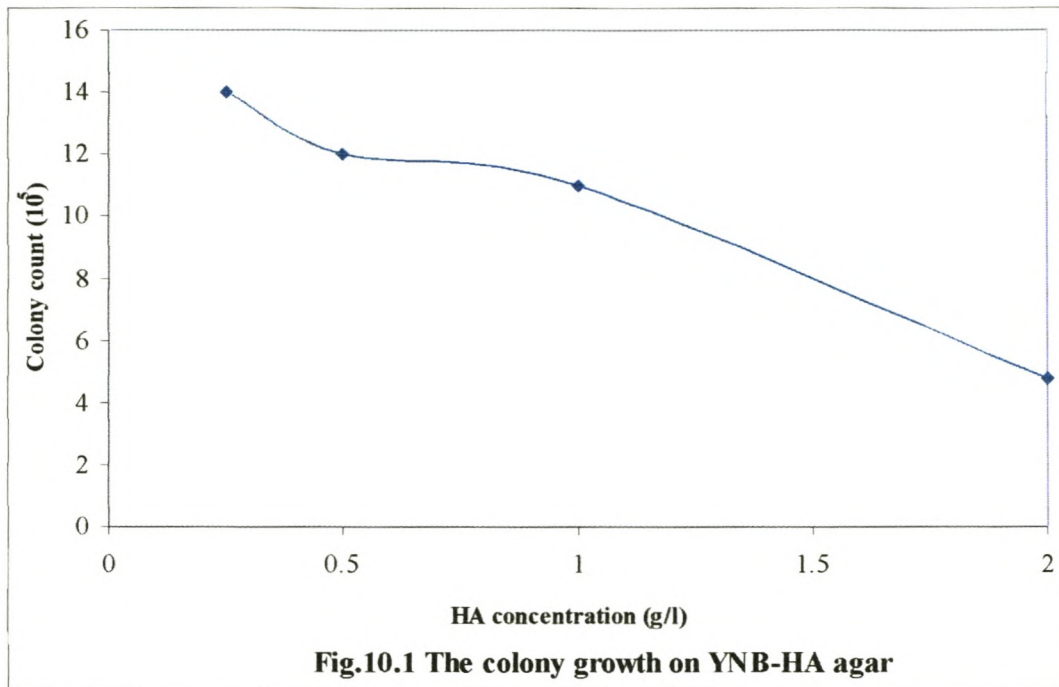
Table 10.4 Colorimetric determination of hexoses (net changes of absorbances (490nm) based on the control) in cell or mycelia free culture fluids after 3-week of incubation

Microbial Isolate	Cezapek-Dox-HA Medium		YNB-HA Medium		TS-HA Medium	
	HA (0)	HA (1)	HA (0)	HA (1)	HA (0)	HA (1)
Control with HA	0.0	0.0	0.0	0.0	0.0	0.0
Trichoderma 1	-0.09296	0.08575	0.11965	0.2212		
Trichoderma 2	0.01595	0.34335	-0.03195	0.1605		
Trichoderma 3	0.52395	1.04325	-0.2152	0.12005		
Trichoderma 4	0.28055	0.3368	0.120365	0.26403		
Trichoderma 5	0.4135	0.5095	0.03905	0.1643		
Trichoderma 6	-0.13325	0.64517	0.024006	0.51365		
Aspergillus Parasitics	0.13835	1.0419	-0.04355	0.001		
Fusarium Oxysporum	-0.039	0.4737	-0.02205	0.05815		
Acrimonium	-0.03655	0.13835	0.01024	0.02138		
Humicola Fuscoatra var Fuscoatra	0.01683	0.41537	0.00983	0.01352		
Gram-negative Bacteria					0.0883	-2.3556
Average	0.108737	0.503314	0.001039	0.153778	0.0883	-2.3556

Note: HA(0)—The HA without pretreated by ultrasonic irradiation

HA(1)—The HA pretreated by ultrasonic irradiation (at 30 kHz for 2 hours)

HA Degradation by Lignocellulose-degradating Micro-organisms and Its Toxicity to Anaerobic microbial Consortia



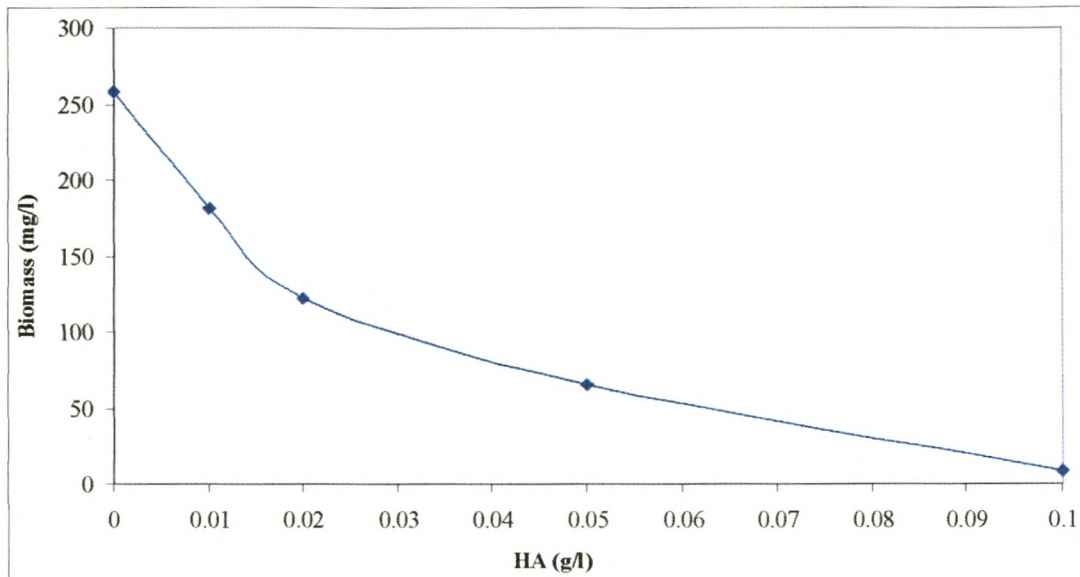


Fig.10.3 Anaerobic biomass yield under different HA concentrations

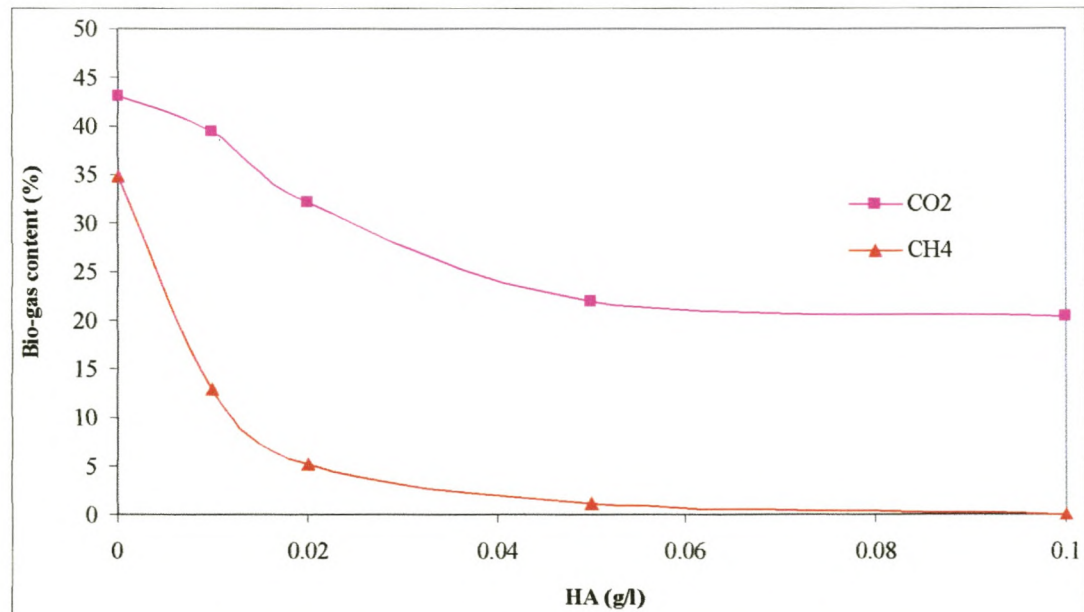
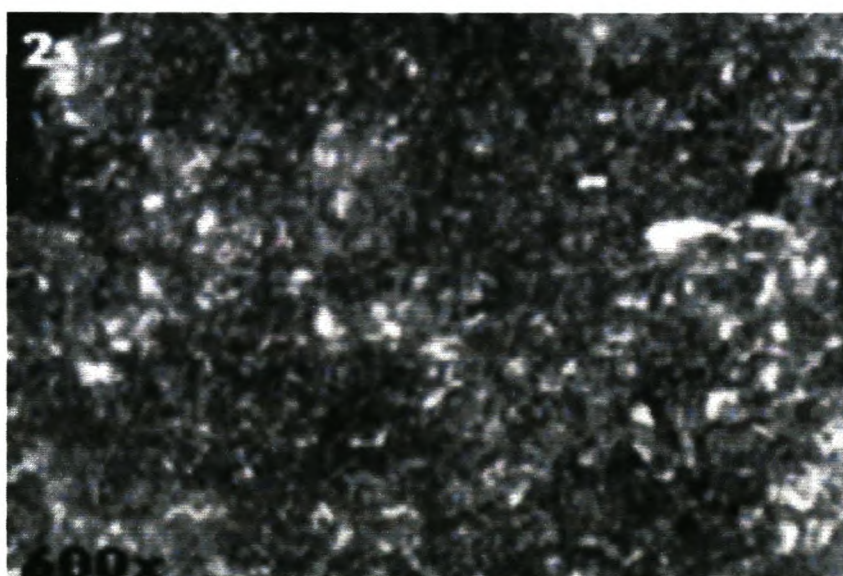
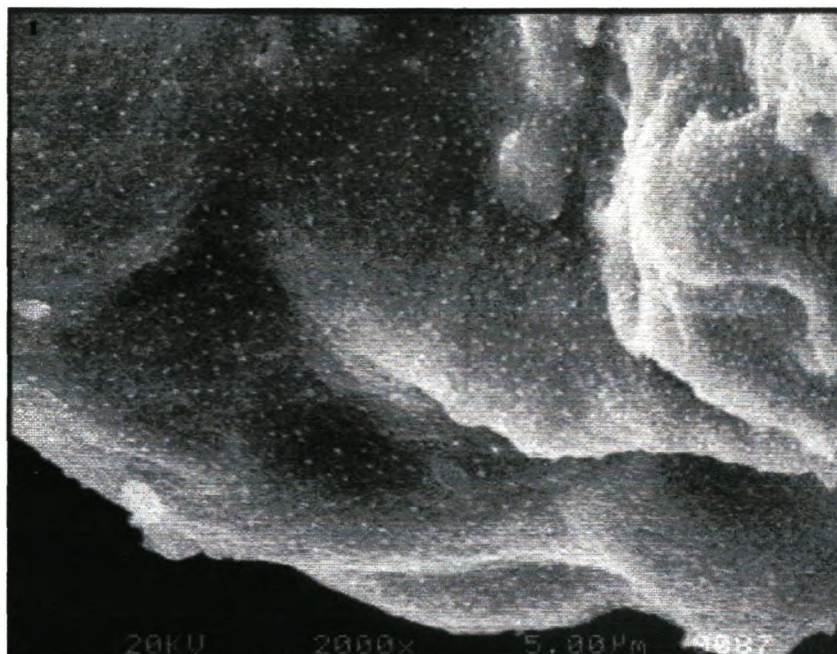
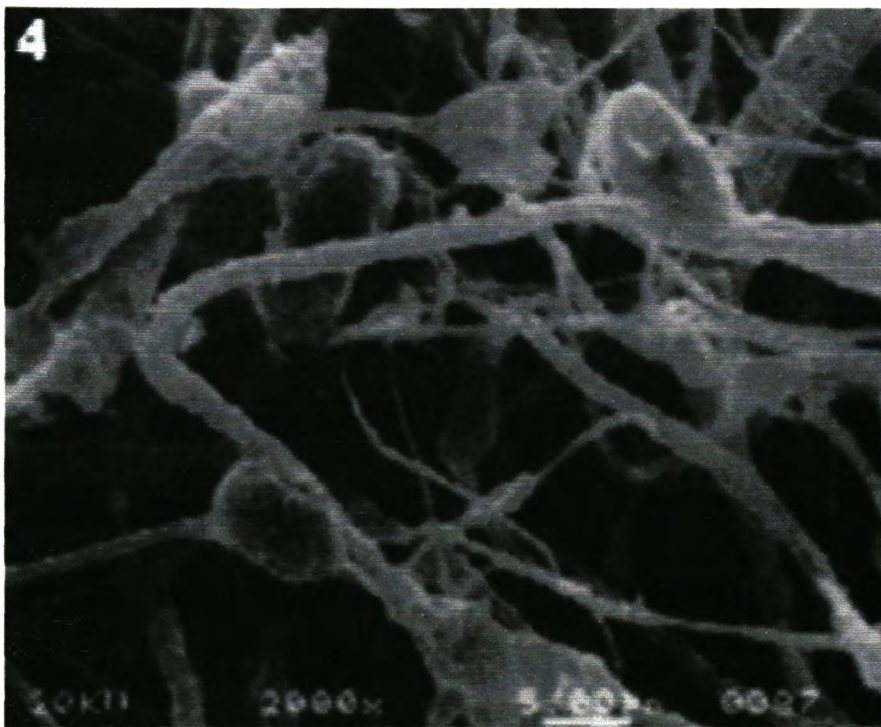
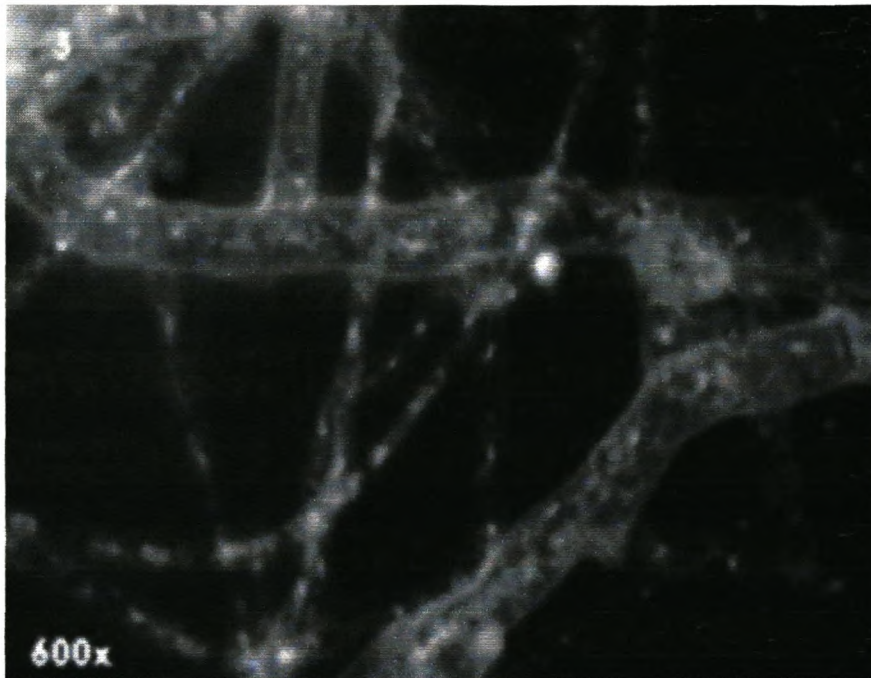
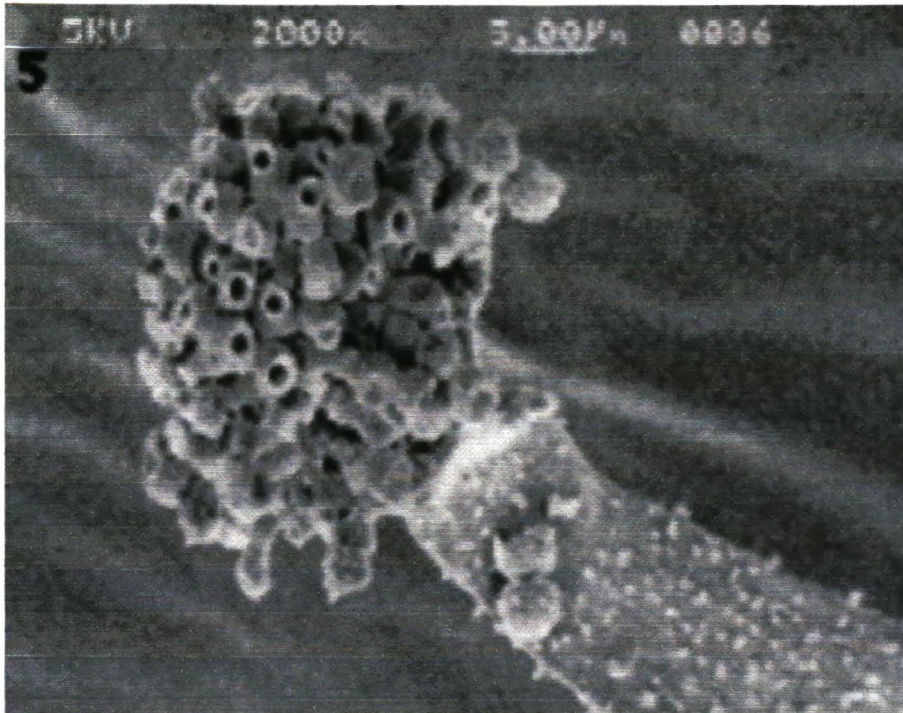


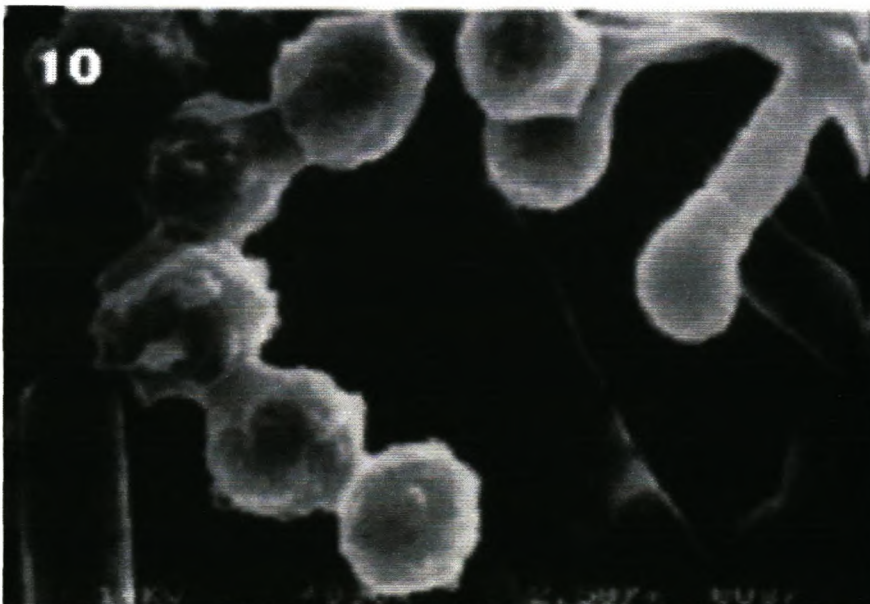
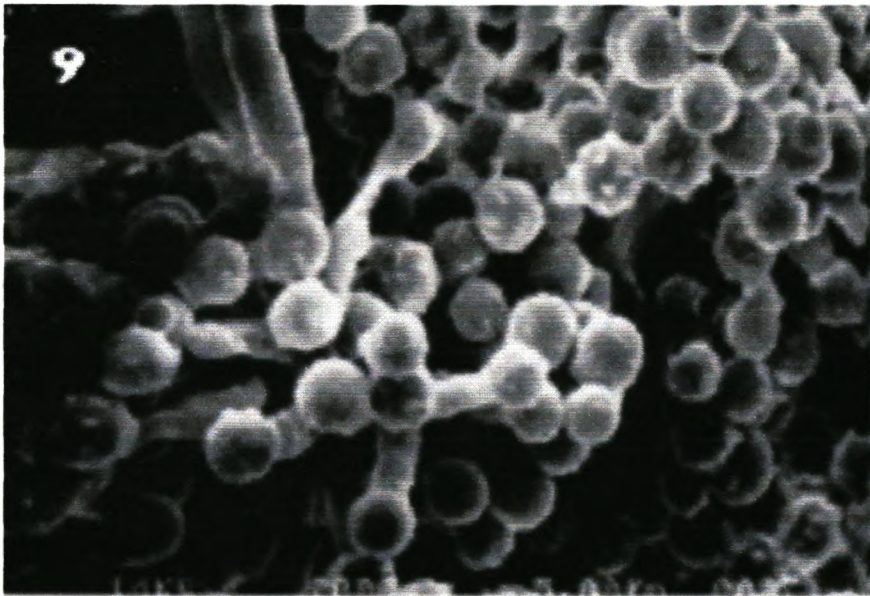
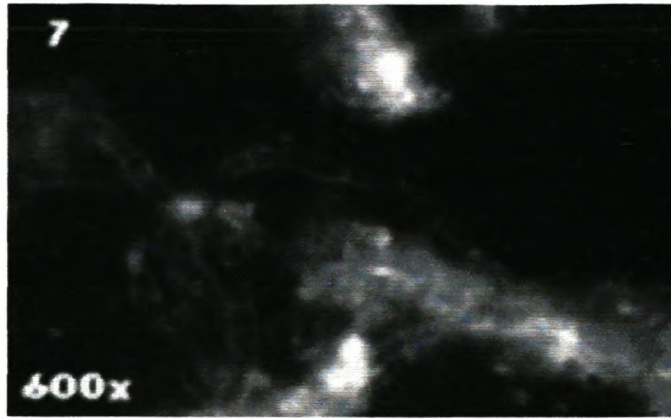
Fig.10.4 Anaerobic bio-gas composition under different HA concentrations

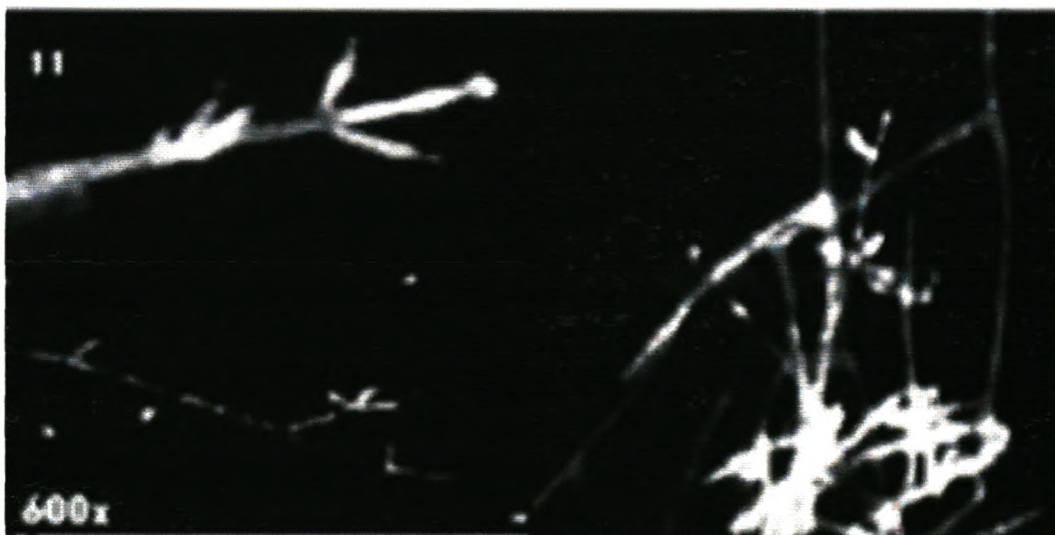
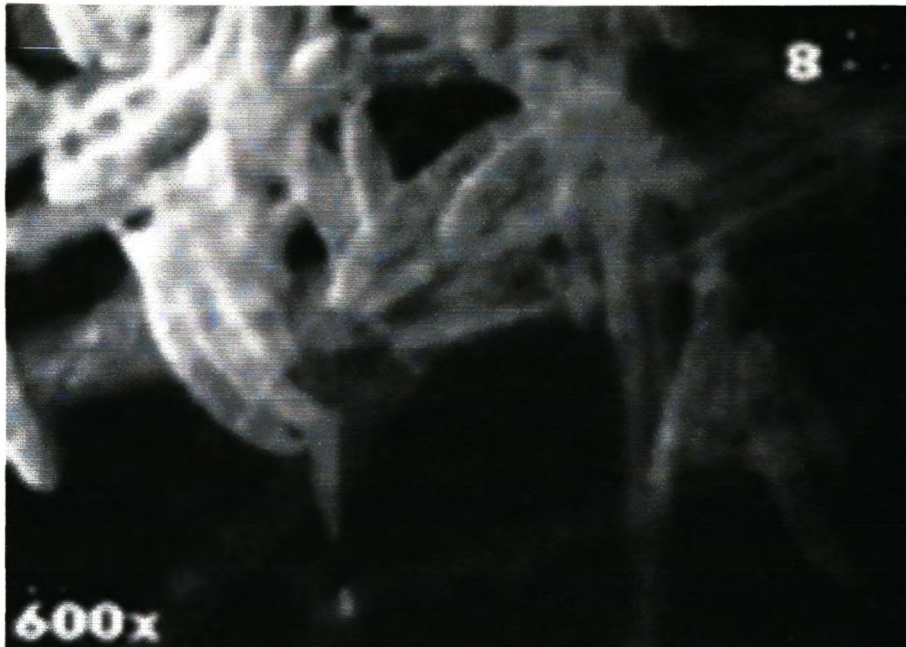
Appendix 10.1 Microscopy morphology of bacteria and fungi isolates



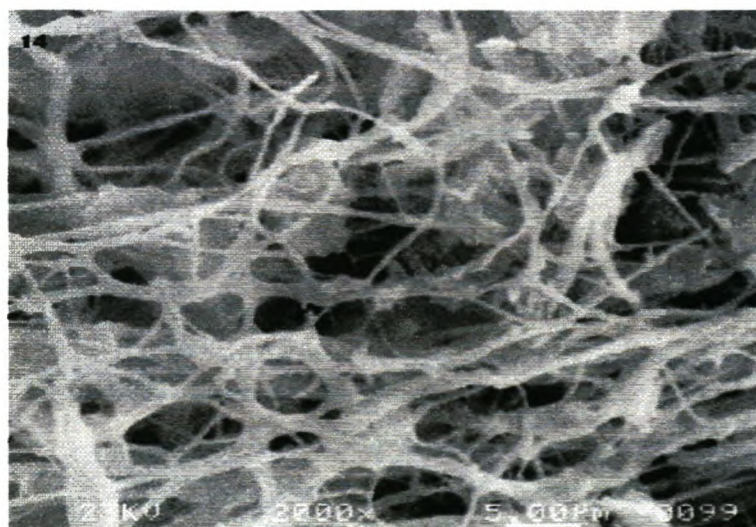
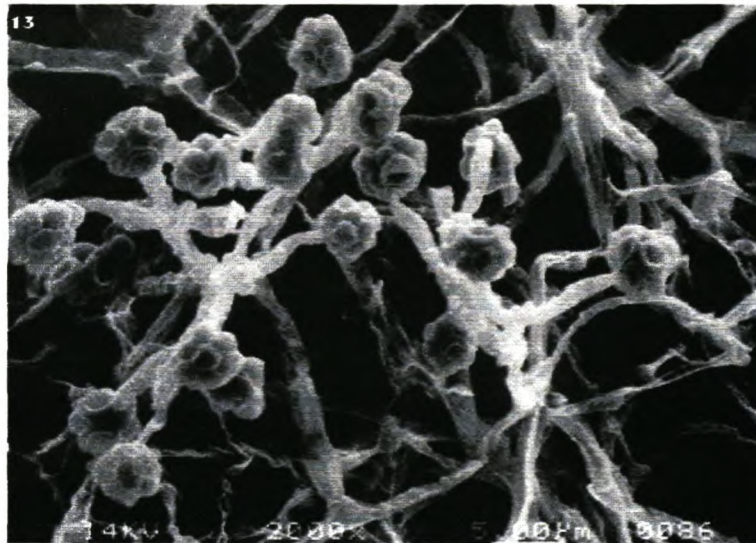
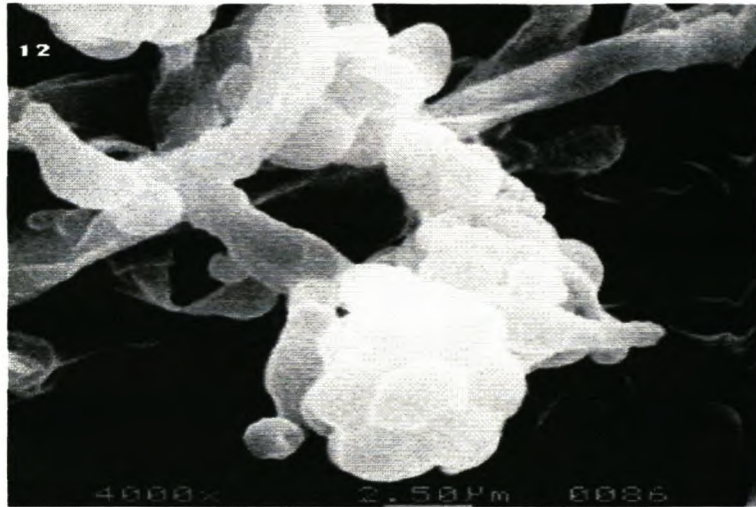


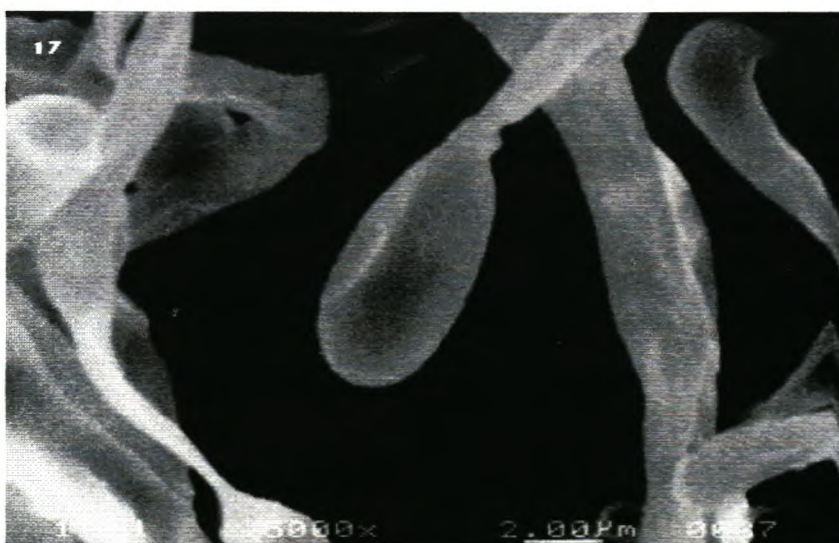
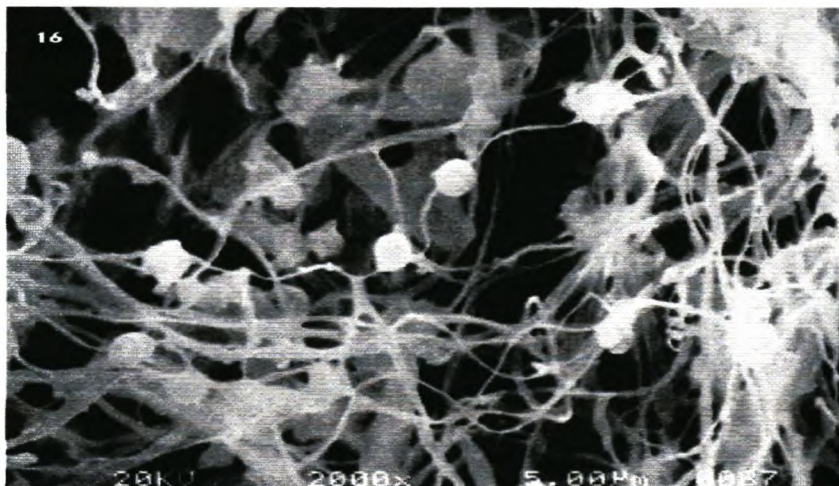
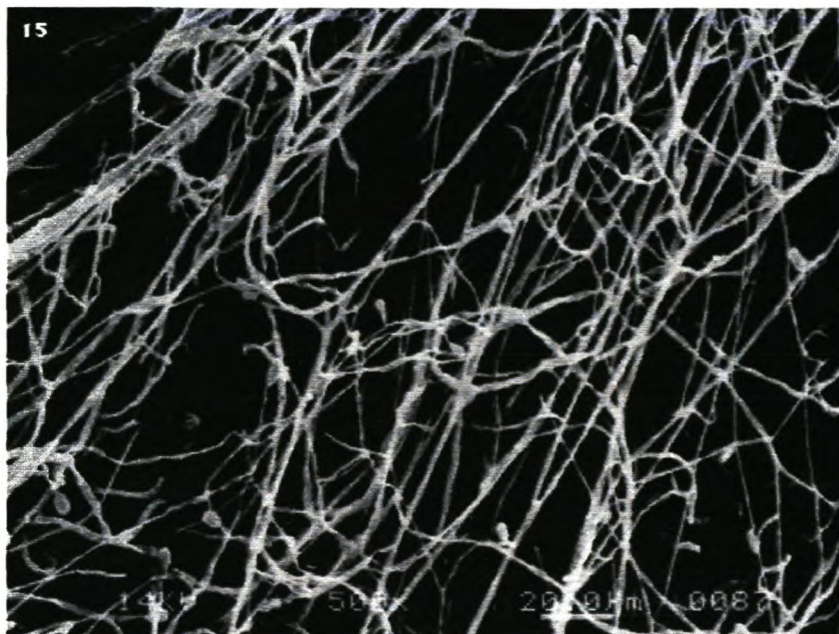






HA Degradation by Lignocellulose-degradating Micro-organisms and Its Toxicity to Anaerobic microbial Consortia





Notes:

- ◆ Photo 1: Rod-shape gram-negative bacteria consortium (SEM, 2000x, TS-HA medium)
- ◆ Photo 2: Rod-shape gram-negative bacteria consortium (epifluorescence microscope, 600x, TSA agar)
- ◆ Photo 3: Hyphae structure of *Aspergillus* gene (epifluorescence microscope, 600x, 2% MEA)
- ◆ Photo 4: Hyphae structure of *Aspergillus* gene in HA degradation culture (SEM 2000x, YNB-HA medium)
- ◆ Photo 5: Conidia heads of *Aspergillus* gene (SEM 2000x, 2% MEA)
- ◆ Photo 6: Hyphae structure and conidia heads of *Aspergillus* gene in HA degradation culture (SEM 5900x, YNB-HA medium)
- ◆ Photo 7: Hyphae structure and aleurioconidia of *Humicola* gene (epifluorescence microscope, 600x, 2% MEA)
- ◆ Photo 8: Hyphae and multi-septate macro-conidia of *Fusarium* gene (epifluorescence microscope, 600x, 2% MEA)
- ◆ Photo 9: Hyphae structure and aleurioconidia of *Humicola* gene (SEM 2000x, 2% MEA)
- ◆ Photo 10: Terminally solitary aleurioconidia and conidia chain of *Humicola* gene (SEM 4000x, 2% MEA)
- ◆ Photo 11: Hyphae structure, conidiophore and conidia of *Trichoderma* gene (epifluorescence microscope, 600x, 2% MEA)
- ◆ Photo 12: Conidiophore, conidia phialides and conidia of *Trichoderma* gene (SEM 4000x, 2% MEA)
- ◆ Photo 13: Conidiophore, bundles of phialides and conida of *Trichoderma* gene (SEM 2000x, 2% MEA)
- ◆ Photo 14: Hyphae structure of *Trichoderma* gene in HA degradation culture (SEM 2000x, YNB-HA medium)
- ◆ Photo 15: Hyphae structure, conidiophore, awl shaped, erect slender phialides of *Acremonium* gene (SEM 500x, 2% MEA).
- ◆ Photo 16: Hyphae structure, conidiophore, slender phialides of *Acremonium* gene in HA degradation culture (SEM 2000x, YNB-HA medium).
- ◆ Photo 17: Smooth-walled, guttuliform conidia of *Acremonium* gene (SEM 5000x, 2% MEA).

Chapter 11

A Primary Study on the Co-degradation of Humic Acids in the UASB Reactor Fed with Glucose Substrate

The upflow anaerobic sludge blanket (UASB) ⁽¹⁵¹⁾ has become very popular in the anaerobic biological treatment of waste-water. The UASB reactor has been implemented to treat a wide range of industrial and municipal waste-water, as well as landfill leachate.

Due to the ubiquity of HAs in lignocellulose decomposition environment and the effective usage of UASB in treating high-strength organic effluents, expanding the use of the UASB reactor to the disposal of HAs-bearing effluents from the lignocellulose decomposition process is drawing researching attention. It is necessary to evaluate the potential of the bio-degradation of HAs in a UASB reactor.

Furthermore, it is known that the development of granular sludge is the key factor for the successful operation of UASB reactors, and the characteristics of the substrate on which the bacteria grow substantially affects the granulation process. Without any doubt, the effects of HAs on the granulation process of the UASB reactor cannot be ignored. However, because some of the chemical structures of the HAs are as yet undetermined and complex, it will be a challenge to investigate the influence of the HA on the UASB process thoroughly in theory and in technology for the application of UASB reactor to HA-bearing waste waters.

To investigate all the above-mentioned subjects would be time-consuming. In this chapter, the investigation on the potential of co-degradation of HAs with glucose in UASB reactor is presented.

A schematic diagram of the laboratory scale UASB reactor is given in Chapter 3.

The seeding granules were provided by the Department Food Science of the University of Stellenbosch. The continuous experiment initially started with the feeding solution which was composed of 1.0 g/l glucose as carbon source, 0.5 g/l urea as nitrogen source, 0.5 g/l K₂HPO₄ as phosphorus source, 1.0 ml/l trace element solution, 0.25 g/l cysteine-HCl, 0.25 g/l Na₂S

• 9H₂O and 2.5 g/l NaHCO₃. After 14 days of operation and with COD loading gradually increasing from 0.67 g/l.h⁻¹ to 1.37 g/l.h⁻¹ by reducing HRT, the reactor was switched to the HAs medium which consisted of a glucose-HAs solution plus 0.5 g/l urea, 0.5 g/l and 1.0 ml/l trace element solution. The COD strength of the feeding influent was kept at 2.0 g/l, with the HA content being increased gradually by adding the concentrated HA solution, which had the COD strength of 2x10⁴ mg/l, from 0.0 ml/l to 50 ml/l over a operation period of 125 days.

The reactor was operated at a steady state for 25 days after each stepwise increase of the HA concentration, and the COD loading increased from 1.37 g/l.h⁻¹ to 3.28 g/l h⁻¹ by reducing the HRT. Samples of granules were then taken from the reactor for bio-activity analysis. The COD, VSS, pH and alkalinity of the effluent were measured by procedures described in Standard Methods ⁽¹⁾.

The measurement of the reactivity of the digestion solution with the Fast Blue B Salt (FBB), and the detection of the presence of the reduced sugars in the digestion solution in the reactor, were made using methods described in Chapter 3.

The VFA and bio-gas analyses were done following the GC methods presented in Chapter 3.

The bio-activity analysis and the specific methanogenic activity (SMA) tests were performed using a 120 ml vial. All procedures, other than those described here, are based on the Hungate technique ⁽¹⁷⁶⁾. The medium used for the incubation of the methanogens include (per liter): carbon source (glucose and sodium acetate respectively) with equivalent COD strength of 2.0 g, NH₄Cl 1.0 g, trace elements solution 10.0 ml, vitamin solution 10.0 ml; NaHCO₃ 5.0 g; L-cysteine-HCl•H₂O 0.5g; Na₂S•9H₂O 0.5 g; resazurine 0.002 g; phosphate solution (pH 7) 2.0 ml. Each sludge sample taken from the UASB reactor was washed with phosphate buffer (4%) to remove extra substrate originated from the reactor medium. All vials were incubated at 37°C. The headspace volumes of the vials were 50 ml. Except for the vials for the H₂-utilizing methanogenic activity test (in this case, no other carbon source except the carbon source of H₂ 20% and CO₂ 80% was supplied), 80% N₂ and 20% CO₂ were used as the headspace gas. Bio-gas volume production in each vial was monitored with glass syringes, and the CH₄ content in the headspace of the vials was measured using GC over 25 days of

incubation. The specific methanogenic activity (SMA) in each vial was calculated by dividing the accumulative methane production by the VSS amount of the tested granule sludge sample.

11.1 The effects of HAs on the pH and alkalinity of the UASB digestion system

The changes in the pH and alkalinity with gradually increasing HAs content during the reactor operation time course are presented in Fig.11.1 and Fig.11.2.

The results in Fig.11.1 indicate that the presence of HAs did not noticeably affect the pH of the digestion solution in the UASB reactor throughout the operation period. In fact, it shows a buffering capacity for the optimum anaerobic digestion pH range of 6.5-7.4 in the reactor. As can be seen in Fig.11.1, noticeable variations in the operation pH of the reactor were usually observed when HA was absent in the reactor.

The results in Fig.11.2 indicate that the alkalinity of the reactor increased steadily from 3000 mg/l to 5130 mg/l with the increase of HA concentration in the reactor. With the presence of HAs in the reactor, the alkalinity of the digestion solution in the reactor appeared stable when compared to the reactor in which no HAs was supplied.

Generally, the bicarbonate ion, HCO_3^- , is the major source of alkalinity in the anaerobic digesters for the neutral pH range. It is produced during digestion by the breakdown of nitrogenous compounds (such as urea) to NH_3 and then by the reaction of NH_3 with CO_2 developed from the fermentation or the methanogenesis process in the reactor. Besides HCO_3^- , volatile acid can be another important contributor to the alkalinity of the system. Under stable operation conditions, the volatile acids produced can be neutralized by the bicarbonate alkalinity. Thus the digestion system can be maintained in a neutral pH range to prevent the inhibitory effect of acidification and the total alkalinity of the system is determined mainly by the concentration of HCO_3^- formed during the digestion process. Since alkalinity is measured by titration with acid to pH 4.0-4.3, any compound that reacts with H^+ will be measured as part of the alkalinity. HAs, which are supposed to be the weak acids, may without doubt contribute to the alkalinity of the tested sample. The fact that the HA structures can be chemically altered and that the new intermediate compounds may be formed during the digestion process, make it difficult to specify the main causes of alkalinity inclination by

HAs. However, the stable pH and the increases in the alkalinity of the digestion solution in the UASB reactor supplied with HAs suggest that the presence of HAs in the UASB reactor improves the pH buffering capacity of the digestion system. This is essential to develop a well-balanced anaerobic digestion process in the UASB reactor. Based on this point, the HA can be considered not very detrimental to the operation of the UASB reactor.

11.2 The bio-modifications of the HAs in the UASB reactor

It has been found that the HAs may improve the pH buffering capacity of the UASB digestion system. For the purpose of assaying metabolic modifications of the HAs in the UASB reactor, the hydrolysis/degradation of HAs in the UASB reactor, the CH₄ production in the bio-gas of the UASB reactor, were assayed under stable conditions.

The reactivity of the UASB digestion solution (effluent) with Fast Blue B salt (FBB) under different HA concentrations during the digestion time course is presented in Fig.11.3. The presence of the reduced sugars in the effluent of the reactor under different HA contents was also monitored during the digestion process. The results are shown in Fig.11.4.

Fast Blue B salt (FBB) can react with 1-naphthol and other hydroxylated aromatic compounds to form coloured products of which the absorbance is in the range of 530-618 nm. The HA needs to be hydrolyzed/decomposed by the micro-consortium of the granule sludge in the UASB reactor before it can be used as a carbon source by the other three groups of bacteria (acidogens, acetogens and methanogens) in the UASB reactor. Therefore, the data of the FBB reactivity of the digestion solution, and the presence of reduced sugar in the reactor solution are useful indicators of the hydrolyzation/decomposition process of HAs.

The results in Fig.11.3 indicate that the reactivity of FBB with the digestion solution in the UASB reactor inclines gradually with the increase of HA concentration during the operation time course. This suggests that some proportion of the HAs were decomposed into naphthol or other hydroxylated aromatic compounds in the UASB reactor.

As can be seen in Fig.11.4, reduced sugars are also found in the digestion solution of the UASB reactor and they tend to escalate with the increase of HA concentration. This indicates

that besides the naphthol or other hydroxylated aromatic compounds, reduced sugar was also produced in the partial degradation of HAs in the UASB reactor.

From results presented in Fig.11.3 and Fig.11.4, it can be postulated that some hydrolyzation or decomposition of HAs was taking place in the UASB reactor. This implies a potential application of the UASB gradual sludge to the degradation of HA compounds.

11.3 The influences of HAs on the acidogenic fermentation process of the UASB reactor

In order to investigate the influence of HAs on the acidogenic fermentation process of the UASB reactor, the production and composition of the volatile fatty acids (VFA) of the digestion solution in the reactor fed with different concentrations of HAs, in the form of COD, were monitored on the 25th day of stable operation. The results are presented in Fig.11.5.

As shown in Fig. 11.5, the characteristics of VFA composition profiles are very similar under different HA concentrations. Acetate, butyrate and propionate are the main constituents of the VFA in the digestion solution. However, the accumulation of acetate was more evident when the HA concentration in the reactor was high. A significant accumulation of more oxidized fermentative products, the acetic acids, was detected with the increases of HA concentration. This suggests that the presence of HAs might induce the release of a large amount of electrons during the fermentation process and thus catalyze the oxidation of glucose and other larger VFA to acetic acids.

11.4 The influences of HAs on the methanogenesis process of the UASB reactor

The influence of HAs on the methanogenesis process of the UASB reactor was investigated by monitoring the methane production of the bio-gas and the special methanogenic activities of the granular sludge which was previously fed with HA bearing solutions in the UASB reactor.

The methane productions of the bio-gas under different HA feeding concentrations are presented in Fig.11.6. The results show that the methane yields of the UASB reactor decline

noticeably with the increases of the HA concentration. When the HA concentration was high, up to 50% COD, the methanogens were greatly inhibited; little methane was detected during 25 days of operation. This confirms that the HAs may inhibit the activity of the methanogens in the UASB reactor.

The specific methanogen activities of the UASB granules growing in the UASB reactor fed with different concentrations of HA are shown in Fig.11.7 to Fig.11.9. It includes fermentative methanogen activity (glucose-fed), acetic-utilization methanogen (acetate-fed) activity and H₂-utilization methanogen (H₂ 20% and CO₂ 80% - fed) activity.

As shown in Fig.11.7 to Fig.11.9, the glucose fermentation methanogen activity and H₂-utilization methanogen activity are found to be higher than the acetate-utilization activity. The interesting result is that the concentration of HA at which the UASB granule was grown did not exhibit noticeable deteriorating effects on the specific methanogen activity of the granule as had been encountered in the UASB reactor. This indicates that the inhibiting effects of the HAs on the UASB granules are reversible. The methanogenesis is released when the granules are fed with easy-digestible carbon sources which are free of HAs. As it was reported, the H₂-producing acetogenic and methanogenic bacteria recovered faster than the acetotrophic methanogens ⁽²⁰⁶⁾.

11.5 The effects of the HAs on the COD removal efficiency of the UASB reactor

In order to investigate the influence of the HAs on the COD removal efficiency of the UASB reactor, the COD of the effluent of the reactor was assayed under stable operational conditions. The COD removal efficiency against the HA concentration is presented in Fig.11.10. It can be seen that the COD removal efficiency of the reactor appeared to be slightly reduced by HAs. The COD removal efficiency of the reactor gradually decreased with the increases of HA concentration in the feeding solution, while the CH₄ production decreased remarkably under the same conditions. The fact that the low COD removal efficiency did not correlated well with the poor CH₄ production could suggest that an amount of COD in the reactor can be considered to be removed in the form of CH₄ and CO₂. Some of the HAs, which might be abiotically absorbed in the granule structure, were then calculated as the removed COD. Therefore, it was found that a large amount of removed COD did not confirm a

corresponding amount of CH₄ production of the UASB reactor. Presumably, most of the removed COD, associated with the HAs, is rather considered as the absorbed COD by the granular sludge rather than as the bio-digested COD in the reactor.

Furthermore, although the detection of naphthol or other hydroxylated aromatic compounds and reduced sugars in the UASB reactor implies that the HAs may, to some extent, undergo hydrolysis or decomposition by the granular consortium in the reactor. The cleavage of the aromatic ring of the HA molecular structure is theoretically difficult for the anaerobic bacteria; only the side chains of the molecule can possibly be split off by the microbiological activities and reduced to methane by the granules in the reactor. Therefore, most of the removed COD associated with HAs is possibly mainly the absorbed COD by the granular sludge rather than the digested COD.

11.6 Discussion

The biological information of the seeding material (granular sludge) and chemical characteristics of the feeding substances (HAs) are both essential to understand the influences of HAs on the UASB process and their potential decomposition in the UASB reactor.

11.6.1 Chemical molecular structure of HAs

The structure of the HAs is heavily dependent on their origin. The HAs were normally considered to be formed as the biologically-inert intermediate products of lignocellulose residues by an anaerobic or aerobic digestion process. Although there might be some quantity differences in the molecular functional groups of the HAs derived from different origins, the HAs generally consist of a number of aromatic, phenolic, quinonic, and heterocyclic “building blocks” that are randomly condensed or linked by aliphatic, oxygen, nitrogen, and sulphur bridges. The macromolecular structure of the HA usually bears aliphatic, glucidic, amino acidic, and lipidic surface chains as well as chemically active functional groups of various natures (mainly carboxylic, but also phenolic and alcoholic hydroxyls, carbonyls, etc.) which render the humic polymer acidic ^(236; 148).

Oxidative, reductive, and hydrolytic cleavage by inorganic chemicals, as well as by zinc dust distillation, also reveals that humic substances consist of polycyclic aromatics to which saccharides, peptides and phenols are attached ⁽²³⁷⁾. Cleavage products include phenols, phenolic acids, benzene carboxylic acids and aliphatic carboxylic acids ^(233,78). The chemical reactivity of HA is linked to its high content of oxygen-containing functional groups, including COOH, phenolic and/or enolic OH, alcoholic OH, and the C=O double bonds of quinones, hydroquinone, and α,β -unsaturated ketones ⁽²³⁷⁾. The acidity and cation exchange capacity of HA has mainly been attributed to the presence of dissociable H^+ ions in aromatic and aliphatic COOH and phenolic OH groups ⁽²²³⁾.

11.6.2 Macro- and micro-biological structure of the UASB granule

In UASB reactors, the biomass is retained as aggregates, called granules, formed by the natural self-immobilization of the bacteria. The formation and stability of the granules are essential for successful operation.

The metabolic reactions that occur during anaerobic digestion suggest that there are advantages in the aggregation of micro-organism into granules. As stated previously, the degradation of complex substrates into methane and carbon dioxide during anaerobic digestion involves the interaction of at least three metabolic groups. The first group of fermentative bacteria, namely the acidogens, conducts initial degradation of biopolymers. The acids (and alcohol) so produced are utilized by a second group of bacteria, the acetogens. The third group of bacteria is the methanogens. Located at the end of the nutrient cascade, methanogens convert CO_2 and H_2 , acetate, and a few other simple compounds into methane. Because of unfavourable thermodynamics, oxidation of propionate and butyrate is only possible if H_2 is removed efficiently, i.e. a very low hydrogen partial pressure is necessary. Propionate degradation is only possible below a partial pressure of 10^{-4} atm H_2 ^(106, 57). In granules degrading a mixture of acetate, propionate, and butyrate, a clear correlation exists between the degradation rate of propionate or butyrate and the hydrogen partial pressure. A slight increase in the partial pressure of hydrogen results in an immediate decrease in the degradation rate of the two volatile fatty acids ^(216, 217, 218, 220). Clearly, the close association of members of these three groups in a layered granular structure would ensure a high metabolic

activity. The influence of HAs on the activities of UASB granules could involve these three layer groups of bacterial consortia.

A fairly distinct localization of bacteria within granules grown on carbohydrate-containing waste-water was found. The hydrolytic and/or acidogenic bacteria were predominant in the outer part of the granules, whereas methanosaeta-like bacteria dominated in the inner part of granules⁽¹¹⁵⁾. An even more structured pattern was observed in granules grown on glucose or sucrose. It was found that the syntrophic bacterial consortia were located between an external, predominantly acidogenic layer, also including hydrogen-consuming bacteria and a core of acetate utilizers, creating optimal nutritional conditions for all its members^(158, 160). In granules grown on complex waste waters, an internal organization may be beneficial for an optimum degradation of substrate and intermediates. An aggregate granular is a stable metabolic arrangement that creates optimal conditions for all its members. It can be deduced that the more complex the substrate to be degraded, the more highly-structured the internal organization of the granules required for a successful internal energy and substrate transportation. The presence of HAs in the previously formed granules which was fed with a simple carbon source such as glucose may not greatly affect the basic structures of the well-formed granules; however, it may interfere with the energy and substrate transmission in the granule metabolic process.

11.6.3 Implications from co-consideration of structure properties of HAs and the UASB granule

The condensed aromatic, phenolic, quinonic, and heterocyclic structure of the HA makes it highly resistant to biodegradation, so that the HAs are essentially refractory to digestion and are likely to be toxic to the anaerobic consortium in the UASB reactor. However, owing to the granule's layered microstructure, the most toxicity-sensitive methanogens populated in the interior of the granule can be shielded from being exposed to the toxic pollutants (e.g.HAs) by the outer layer of the granules which are mostly composed of fermentative (hydrolysis) and acidogenic bacteria. These bacteria, which are capable of converting glucose and some other complex substrates into fatty acids, exhibited mild resistance to toxicity of HAs, as was found in Chapter 10: the fermentative consortia could survive and grow properly even when the HA concentration was as high as 0.1 g/l.

The decreases in the reactivity of the digestion solution of the UASB reactor with FBB as well as the presence and the accumulation of reduced sugar with the increases in HA concentration, furthermore confirmed that the outer layer fermentative (hydrolysis) and acidogenic bacteria remained active and responsible for the partial degradation of HA even when being exposed to a high concentration level of HAs.

The surface chains structure, such as aliphatic, glucidic, amino acidic, and lipidic chains, predicted the possibility that the HAs are partially hydrolyzed or decomposed by some special enzymes secreted by the fermentation or by the acidogenic consortia at the outer layers of the granules. A certain amount of reduced sugar was found in the digestion solution.

The chemically active oxygen-containing functional groups, such as the carboxylic, phenolic and alcoholic hydroxyl functions of the macromolecule of HAs, determine the high chemical reactivity, weak acidity and strong ion cation exchange (chelating) capacity of HAs. Thus the chemical properties of the digestion system, such as pH buffering capacity, alkalinity, and the reactivity of the fermentation products (volatile fatty acids) in the UASB reactor, could obviously be influenced by the presence of chemically active HAs. For example, the increase of alkalinity of the digestion solution in the UASB reactor by HA may be a result of the dissolving of CO₂ induced by the weak acidity of HA.

It was reported that fermenting bacteria were capable of channeling electrons from anaerobic oxidations via HAs towards iron reduction ⁽¹⁹⁾. The oxidation of propionic acid to acetic acids was observed with the presence of HAs. A similar phenomenon noticed in this study was that the accumulation of acetic acids under the same HRT and COD loading conditions became more evident when the HA concentration in the UASB reactor was increasing (Fig.11.5). The HAs were likely to act as readily accessible electron acceptors during the glucose fermentation process in that the decreased propionate (butyrate) to acetate ratio in the acidogen fermentation products of UASB reactor was observed with the presence of HAs throughout the digestion period. This can be confirmed by the fact that the increased formation of acetic acids is energetically most favourable for the fermenting bacteria, and the reduction of HAs is in accordance with the general concept that the energetically most favourable electron acceptors (such as HAs) are utilized first. In addition, anaerobic oxidation

of [1,2- ^{14}C] vinyl chloride and [1,2- ^{14}C] dichloroethene to $^{14}\text{CO}_2$ under HA reducing conditions demonstrated that waterborne contaminants can be oxidized by using HA compounds as electron acceptors⁽²⁹⁾. This suggested that natural aquatic systems have a much larger capacity for contaminant oxidation, due to the readily electron accepting property of HAs, than previously thought. In substances, the anaerobic digestion of organic substrate to methane is composed of a series of oxidation-reduction processes; any substance that contributes to the electron provision or depletion may exert regulative effects on the efficiency and products profile of this methanogenic process. Since the quinoid moieties of HAs and low-molecular-weight quinones are considered redox mediators in the chemical reduction of iron or manganese species, or of organic pollutants, the mechanisms of HAs as a redox mediator may be related to its abundantly quinoid aromatic structure. However, biologically, the mechanism of the HAs as active electron (e.g. H_2) acceptors during the methanogenic process (e.g., the potentials of HAs in regulating the catabolism and anabolism processes of methanogens) needs to be explored in detail in the near future by using pure methanogen cultures.

The toxicity of aromatic pollutants to the methanogens was dependent on the nature of the chemically functional group of the pollutants; the more hydrophobic the functional group of an aromatic chemical, the higher the toxicity to the methanogenic activity. The large amount of polycyclic aromatic chain structure of HA renders it hydrophobic, while the high content of oxygen-containing functional groups, including COOH , phenolic and/or enolic OH , alcoholic OH , and the C=O double bonds of quinons, hydroquinone, and α,β -unsaturated ketones render it hydrophilic. Thus, HA can be regarded, to some extent, as an aromatic-structured hydrophobic polymer with a large variety of hydrophilic oxygen-containing functions on the surface chains. At this point, it can be postulated that the toxicity of HA is not as severe as that of other strictly hydrophobic aromatic pollutants such as the cresols and phenols. Therefore, as found in the special methanogens activity assays, the methanogenic activity inhibited by the HA can easily be recovered by feeding the system with appropriate substrate (such as glucose, acetate, H_2/CO_2).

It is known that substrate depletion in the centre of a microbial aggregate can be caused by reduced diffusional fluxes and that this may impair the overall metabolic rates of the involved micro-organisms⁽²⁴⁷⁾. This problem is dealt with in nature by the development of syntrophic

relationships by the forming of mixed microbial consortia, in which the involved microorganisms share a common spatial microniche⁽⁴³⁾. Among these, the anaerobic granule is a typical example of a microbial aggregate. It is widely accepted that the metabolic efficiency and the growth yield of the syntrophic acetogenic bacteria, which produce hydrogen, strongly depend on the rate of H₂ removal by a consuming species such as a methanogen; this process is called interspecies hydrogen transfer. In anaerobic systems, low hydrogen partial pressure can only be achieved by interspecies transferring of molecular hydrogen from hydrogen-producing bacteria to hydrogen-oxidizing methanogens. Thus, in granule sludge, microcolonies consisting of syntrophic, propionate, or butyrate degraders and hydrogen-utilizing methanogens have frequently been observed.

Owing to their hydrophobic and their hydrophilic properties, HAs may influence the interspecies electron and substance transfer among the anaerobic bacterial functional groups by absorbing at the inter-surfaces of the microniches located in the juxtapositioning structure of the UASB granule. At these interfaces, the transportation of bio-gas (CO₂ and H₂) and substrate (VFAs) often takes place. As a readily accessible electron acceptor, HA may reduce H₂ by absorption, which was confirmed by chemical reduction of HAs with H₂, thus accelerating the formation of acetate and catalyzing the oxidation of propionate to acetate. The accumulation of acetic acid may also be intensified at the interfaces between syntrophic acid fermentation consortia and methanogenic consortia because of the absorption of HAs at these interfaces. This may lead to serious substrate (including H₂) depletion in the centre of the granule. Therefore, it was found that the methane production decreased, whereas the acetate content increased with the presence and increase of HA. From this point, the inhibitory effect of HA on the methanogenic activity can therefore be probably attributed to the accumulation of acetate, the fermentation product in the vicinity of acidogen and acetogen consortia and the substrate and H₂ depletion in the centre of the granule where the methanogen was mostly growing.

Furthermore, as it is suggested by Fick's first law of diffusion [i.e. $J = -D(dc/dx)$] that either a short distance x or a large X-H₂ (X-VFAs) concentration difference between the acetogen and the methanogen cell surface improves the interspecies metabolite transport step of the syntrophic reaction⁽⁴³⁾. However, pushing the diffusion metabolite transport by large values for this concentration difference would result in a product inhibition of the acetogenic partial

reactions by high XH_2 (X-VFAs) and, thus, limit the overall carbon and electron flux. Therefore, it was found that the accumulation of acetate with the presence of HAs did not lead to an appropriate methanogen production.

It is also possible that the HAs may be absorbed on the surface of the extracellular polymer (ECP) constitute of the granules, affecting the substrate and bio-gas transferring process between the granule and its outside nutrient-providing milieu in the UASB reactor. For example, it was reported that the sorption of phenanthrene to the soil humic fractions may result in a higher concentration of substrate in the vicinity of the bacterial cells and, therefore, may increase its bioavailability ⁽¹⁸⁷⁾. Thus, it can be assumed that the removed COD associated with the HA absorbing on the ECP can rather be considered to be the COD physically trapped in the granular matrix rather than the biological digested COD.

The pore size and porosity of the ECP matrix affects cell activity through its regulation of substrate and bio-gas transport. Substrate transporting limitations owing to the high absorbing capacity of HAs on the surface of ECP may result in autolysis of the cores of the granules, producing hollow granules. The porosity of the granules, possibly as an effect of bacterial lysis, gives an inactive core of rather large granules, and thus decreases the interior methanogenic activity ⁽⁶⁾.

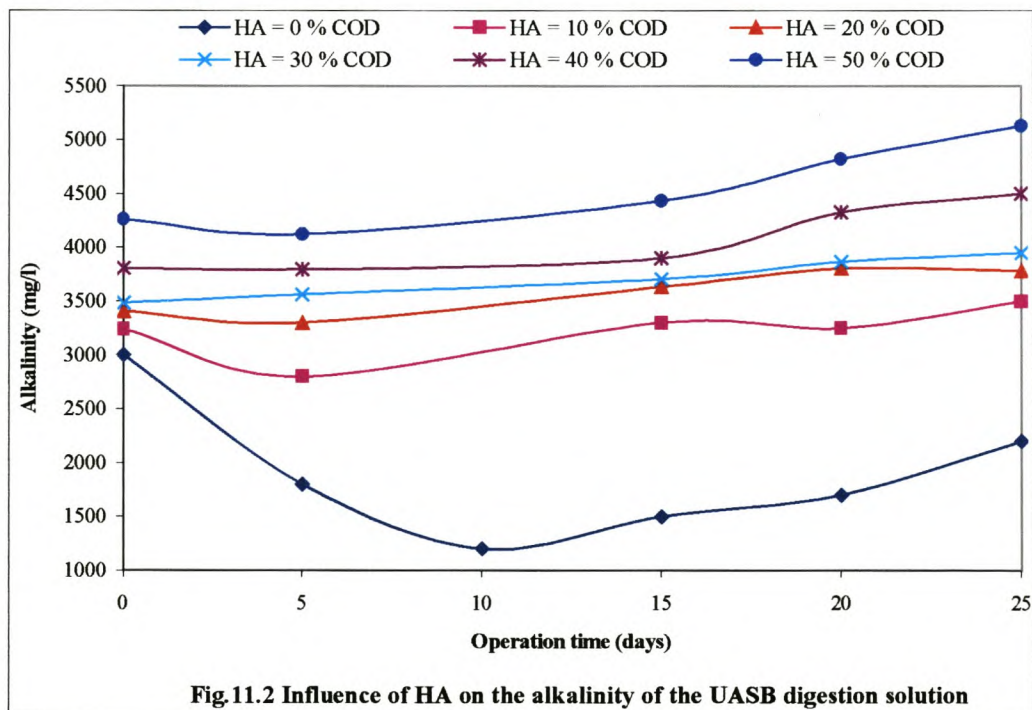
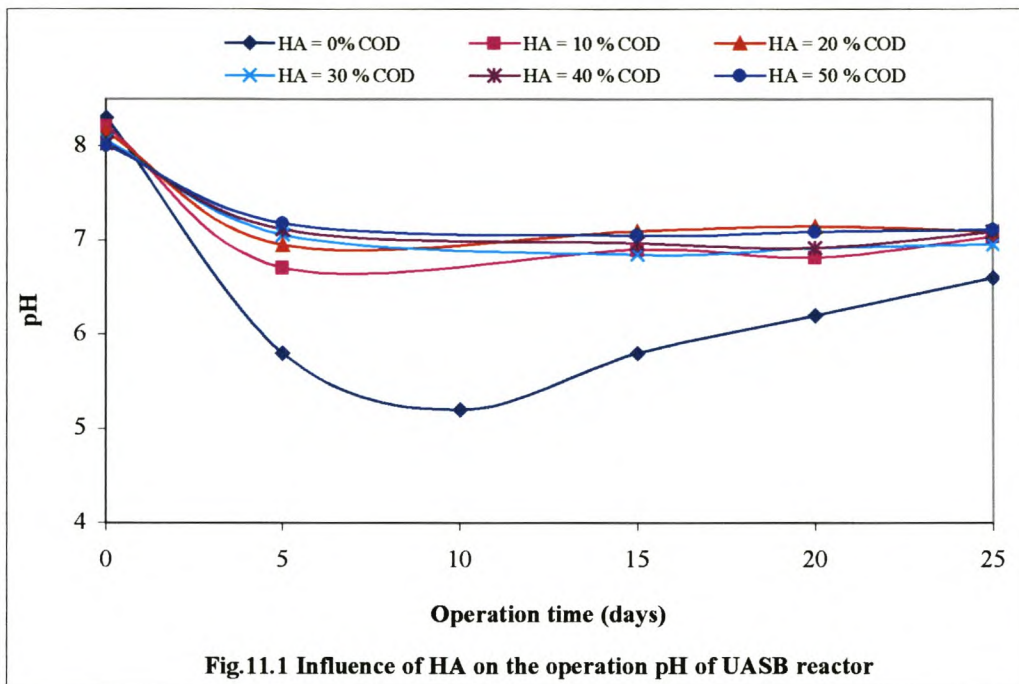
11.7 Summary

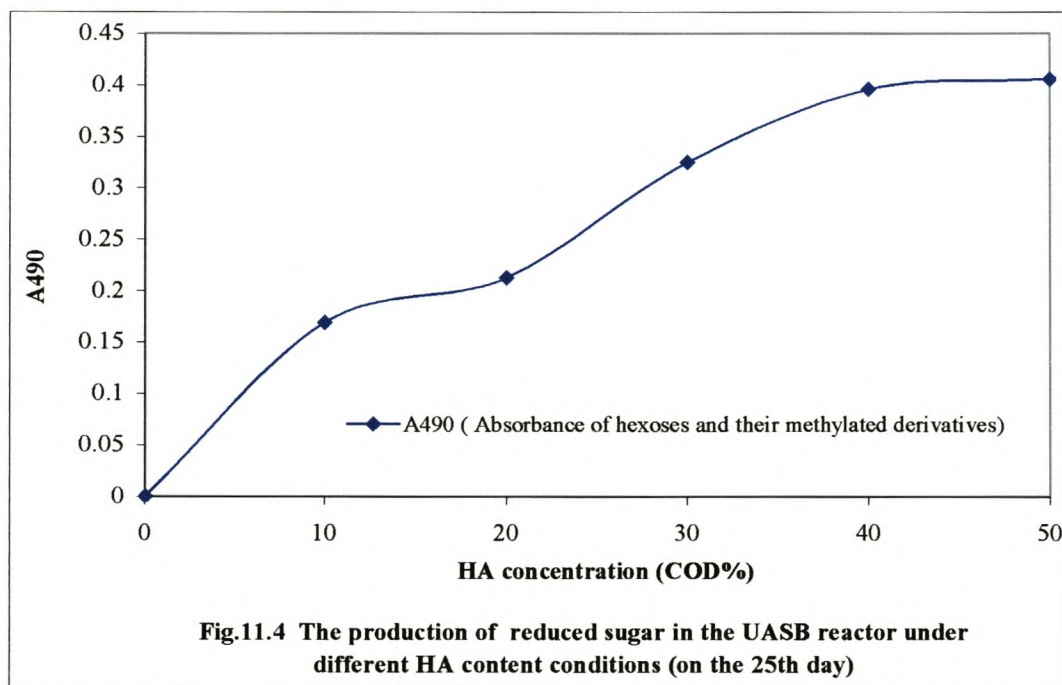
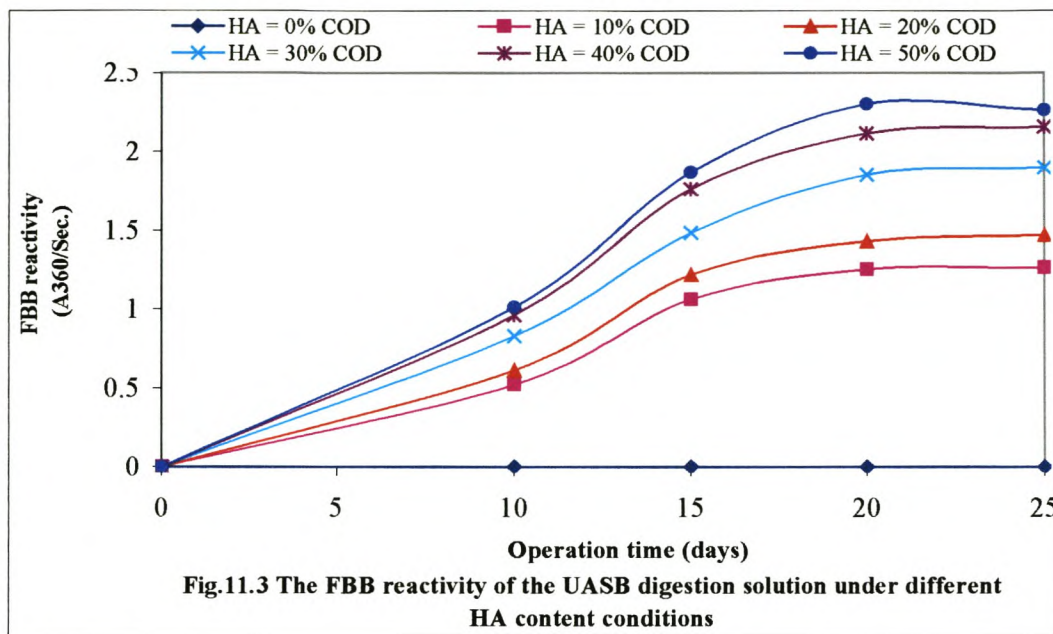
According to the results presented in this chapter, the following conclusions can be drawn:

- 1) Based on the highly chemically-heterogeneous structures of the HA macromolecule, HAs can be partially hydrolyzed and decomposed by the acid fermentative consortium in the UASB granules.
- 2) Instead of inhibiting the activities of the fermentative consortium of the UASB granule, HA probably plays a mediating role in the electron and substrate interspecies transfer among a variety of juxtaposed microniches located along the structure of UASB granule.

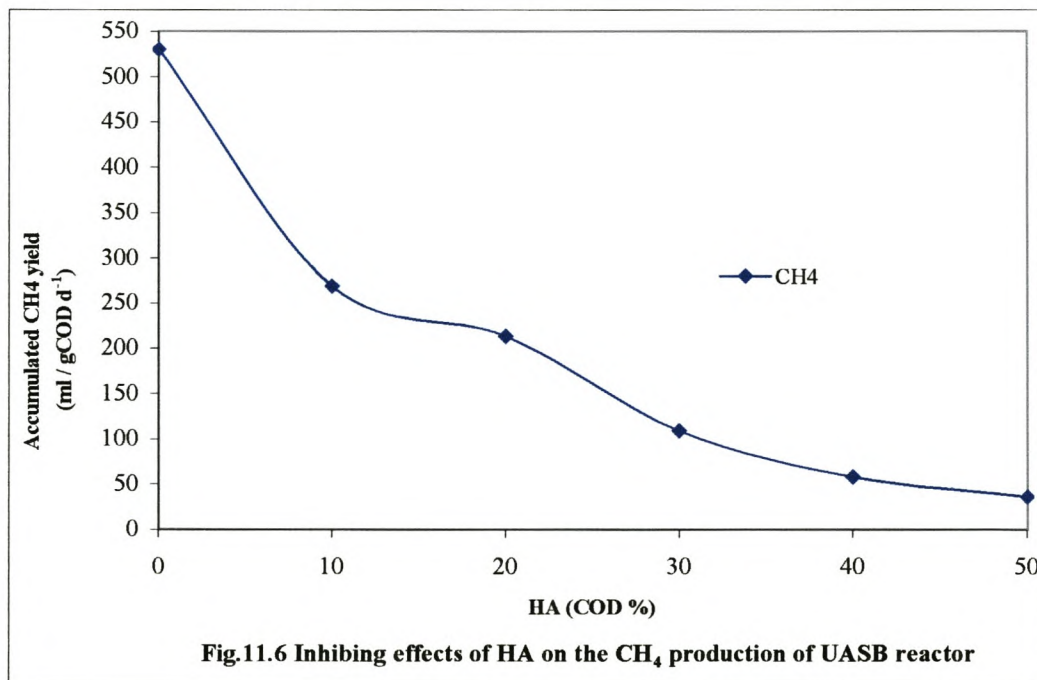
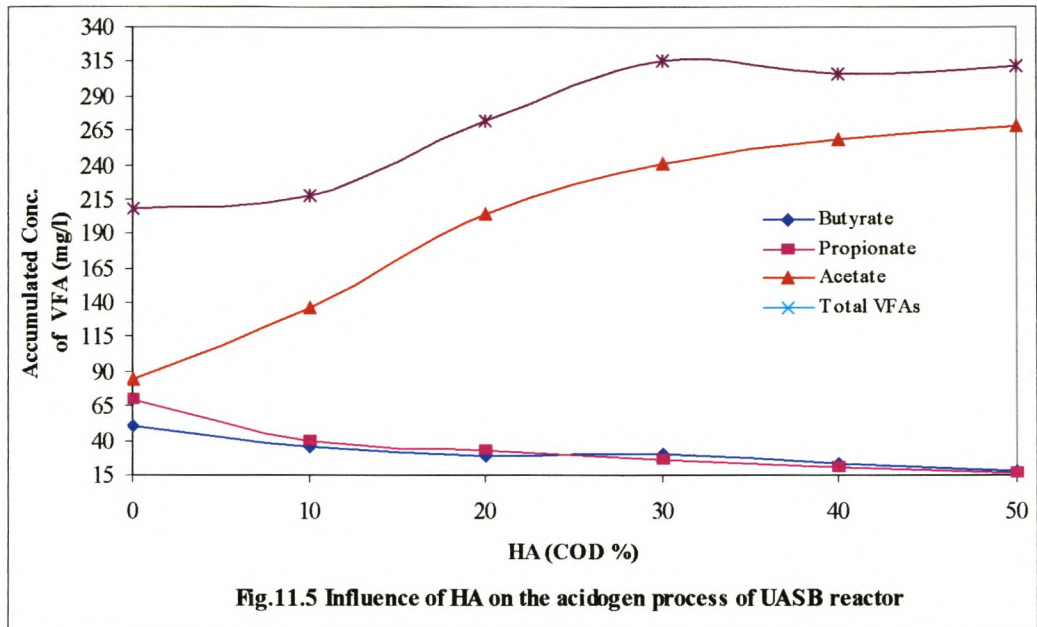
- 3) Owing to the granules layered microstructure, the most toxicity-sensitive methanogens grew in the interior of the granule can be shielded from being completely exposed to the HAs by the outer layer hydrolytic and acidogenic fermentative bacteria. The methanogenic activity inhibited by the HAs can easily be recovered by feeding with a proper substrate (such as glucose, acetate, H_2/CO_2).
- 4) The inhibiting effects of HAs on the methanogenic activity of the granule probably resulted from the accumulation of fermentative products (such as acetate) at the interfaces between the fermentative acidogen consortium and the methanogen consortium owing to the high absorbing capacity of HAs at these interfaces or on the surface of the ECP constitutes of the granules.
- 5) The treatment of HA bearing waste water is possible from this primary study. However, the influences of HAs on the granulation process, granule microstructure and interspecies transfer which are closely associated with the core methanogenic process in the UASB granule needs to be extensively investigated before a robust UASB granule which can function effectively for the HA-containing waste water.

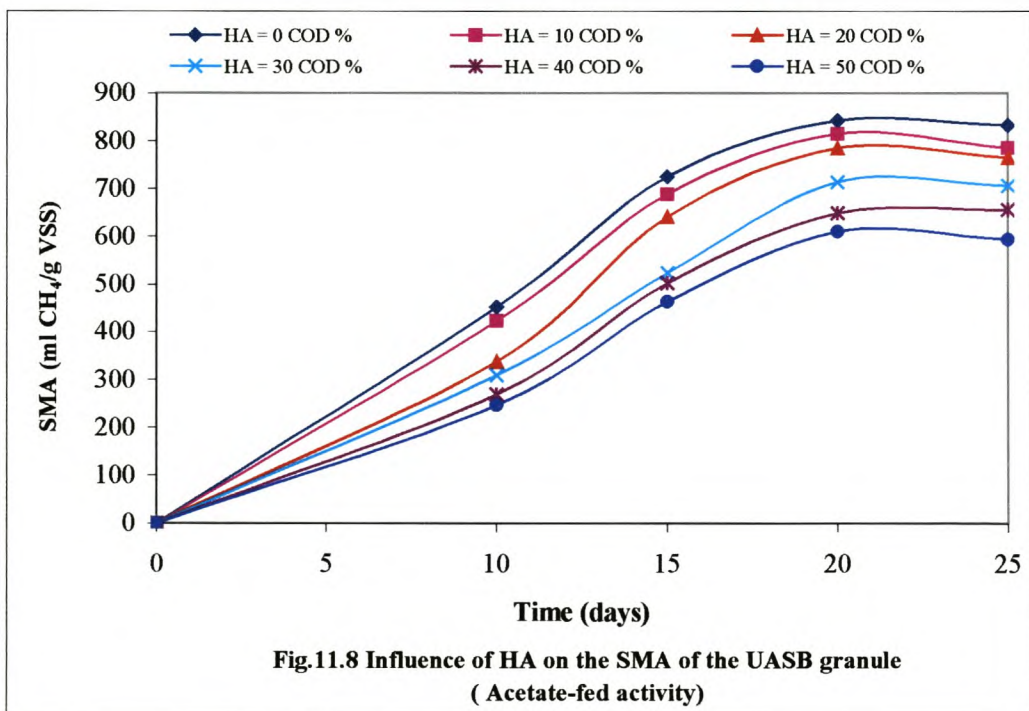
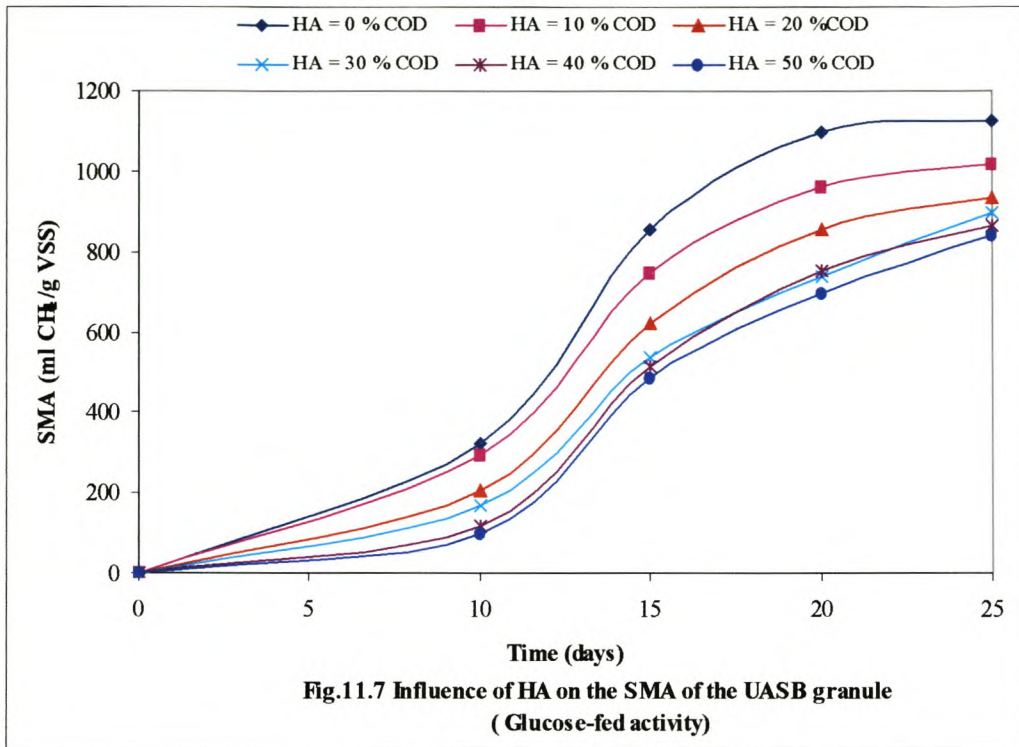
Further work on these aspects was beyond the scope of this investigation and in the penultimate chapter, the important issue of exploiting the lignocellulose solid anaerobic digestion sludge for the agricultural purpose is discussed.

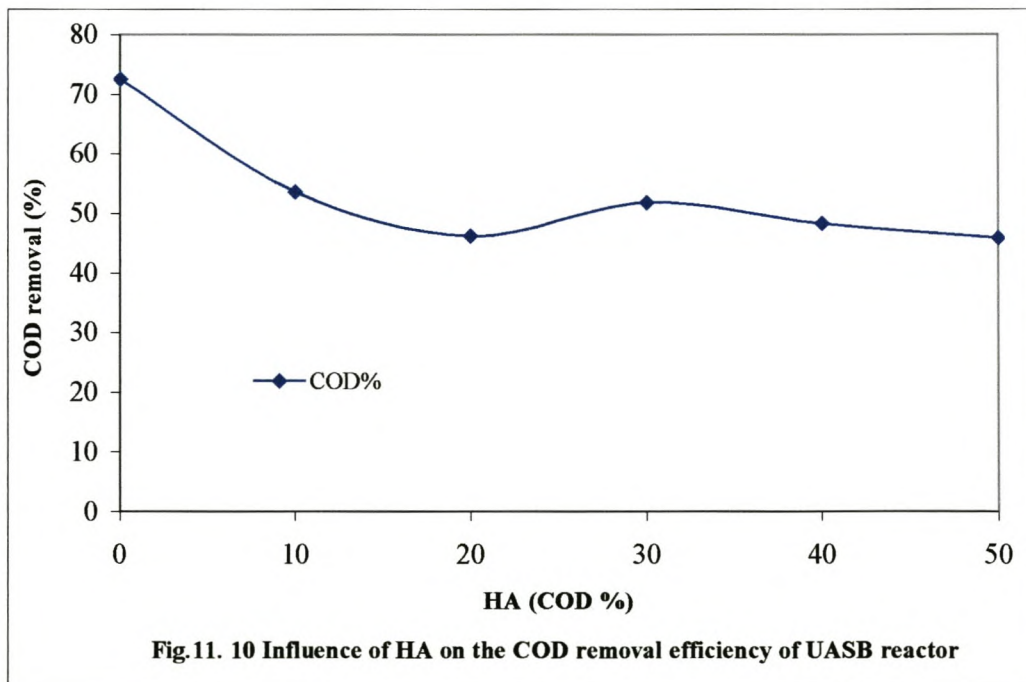
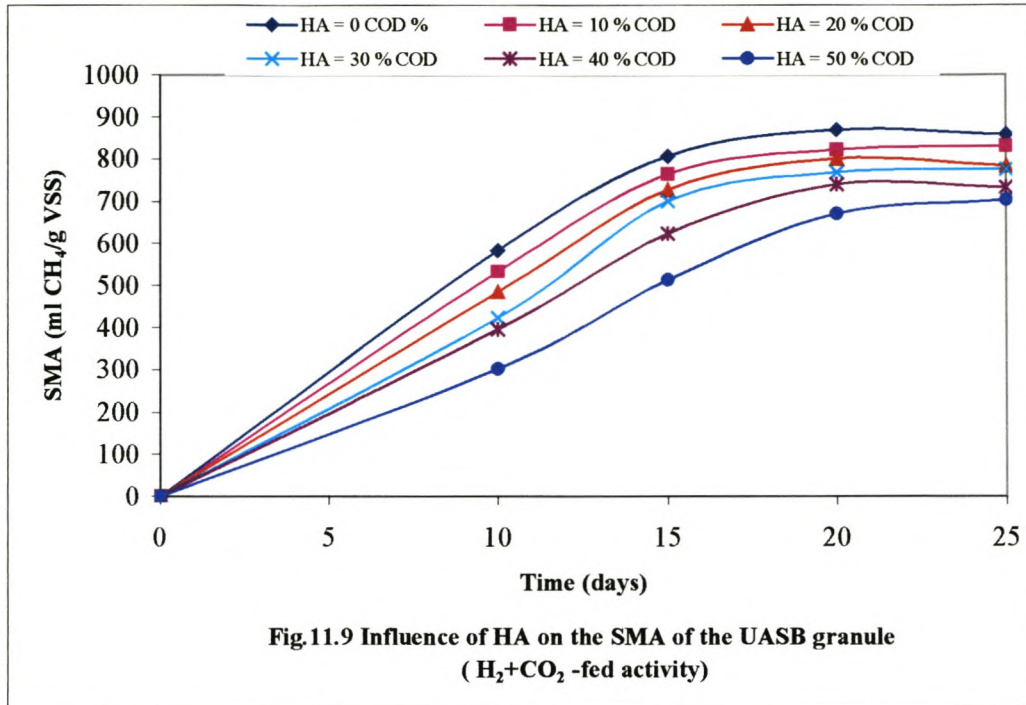




A Primary Study on the Co-degradation of Humic Acids in the UASB Reactor Fed with Glucose Substrate







Chapter 12

Applicability of Solid Anaerobic Lignocellulose Digestion Sludge to Soil Conditioning

Ideally, natural systems are typically in a steady state as determined by environmental and geological factors. In reality, agricultural systems, with crop and plant residue removal and intensive soil tillage, hardly ever reach this state. As a result of environmental concern over the use of synthetic fertilizers (e.g. nitrate leaching) and the rediscovery that, “there is but one manure which permanently keeps up the fertility of land, and that is farmyard manure”⁽¹⁴¹⁾, application of bio-treated sludge has been re-awakened.

In principle, sludge is a valuable product that should be reused if possible. Sludge may be applied to agricultural lands for further sludge treatment and improvement of the characteristics of the land. Sludge acts as a soil conditioner to facilitate nutrient transfer, increase water retention and improve soil properties. It can also serve as a partial substitute for chemical fertilizers⁽⁴²⁾.

As one of the important products from the solid anaerobic digestion process, the anaerobic digestion sludge can be considered as a relative bio-stable soil humus - an amphoteric heteropolycondensate of carbon, nitrogen, sulphur and phosphorus compounds⁽¹⁹³⁾. It shelters and nourishes soil organisms while it buffers and moderates physical, chemical and biological processes and attributes of soil.

The characteristics of sludge that affect its stability for land application include moisture content, pathogenic bacterial content, nutrients and metals. In this chapter, the hygienic effects of the anaerobic digestion process and the applicability of this anaerobic digestion sludge as a soil conditioner were investigated and evaluated.

12.1 The nutrient characteristics of anaerobic digestion sludge

As already mentioned, the digestion sludge can be used as a valuable soil conditioner and soil fertilizer for many crops because it may contain considerable amounts of nitrogen (N), phosphorus (P) and trace elements. To evaluate the nutrient value of the sludge, the nitrogen

(N), phosphorus (P), Potassium (K) and some trace element contents of dried anaerobic digestion sludge were examined . The results are presented in table 12.1. The major nutrient value comparison of this anaerobic sludge with the stabilized activated sludge and fertilizer is presented in table 12.2.

Table 12.1
Nutrient composition of dried anaerobic sludge

Parameter	Amount (g/kg)
Ash	476.9
Volatile Solid	523.1
Total Nitrogen (N-Tot.)	27.2
Water-Soluble Nitrogen (N-Sol.)	0.7
Total Phosphorus (P-Tot.)	4.3
Water-Soluble Phosphorus (P- Sol.)	0.00143
Total Potassium (K-Tot.)	14.6
Water-Soluble Potassium (K- Sol)	0.001
Ca	37.3
Mg	4.1
Na	9.831
Mn	0.312
Fe	5.757
Cu	0.342
Zn	0.261
B	0.03

As can be seen from table 12.1, the anaerobic digestion sludge contained considerable macro-nutrients (N, P, K) and a series of micro-nutrients (Ca, Mg, Mn, Na, Fe, Cu, Zn, B). The water-soluble fractions of macro-nutrients in the sludge were found to be substantially low. This implies that the release of these macro-nutrients from the anaerobic sludge into the soil will be slow. The quick loss of the nutrients because of leaching can be prevented if the sludge is applied to agricultural land.

Table 12.2**Comparisons of nutrient contents of anaerobic digestion sludge with other dried sludge**

	Nitrogen (N.%)	Phosphorus (P.%)	Potassium (K.%)
Anaerobic sludge	2.72	0.43	1.02
UASB sludge #	0.9	2.1	0.4
Activated sludge *	3.22	2.3	0.3
Fertilizer*	5	10	10

Mehmet Ali Y. Kselen⁽¹⁷⁵⁾, 1998.

* Tchobanoglous⁽²⁴²⁾, 1991.

From table 12.2, it can be seen that the nitrogen content and potassium content of anaerobic sludge are comparable with or higher than activated sludge and UASB sludge. However, it is not recommended that commercial fertilizer be completely substituted with anaerobic sludge owing to its relatively lower macro-nutrient content. Therefore, it can be suggested that the anaerobic sludge be applied together with commercial fertilizer to agriculture land.

12.2 The hygienic characteristics of anaerobic digestion sludge

In our investigation, the anaerobic digestion of lignocellulose residues was carried out under thermophilic conditions followed by mesophilic conditions with an alkaline solution as the initial feeding medium. The hygienic status of anaerobic digestion sludge was evaluated by counting the pathogenic bacteria in the sludge. For the purpose of comparison, the pathogenic bacteria counting was simultaneously carried out on the acidified landfill sludge (room temperature). The bacteria counted included total coliforms (Macconkey agar, Biolab.), fecal coliforms groups (M-FC agar, Biolab.), fecal streptococci groups (Streptococcus selective agar, Biolab) and *Salomonella sp.* groups (Brilliant Green Agar, Biolab). The results are shown in table 12.3.

From the results in table 12.3, it can be seen that the disinfecting effects of anaerobic digestion under given experimental circumstances were generally good when compared with acidified landfill sludge. The total number of coliforms in the anaerobic digestion sludge was in the range of 10^2 cfu/g, while in acidified landfill sludge it was in the range of 10^6 - 10^7

cfu/g. No fecal coliforms were detected in anaerobic digestion sludge, while they were found in the range of 10^6 cfu/g in acidified landfill sludge. *Fecal streptococci* were not found in anaerobic digestion sludge, but about 10^3 cfu/g were discovered in acidified landfill sludge at an early stage (20 weeks). *Salomonella sp.* was in the range of 10^1 cfu/g for anaerobic digested sludge, whereas about $10^5 - 10^6$ cfu/g was detected in acidified landfill sludge. These results confirm that the anaerobic digestion process under thermophilic conditions has promising disinfecting effects in contrast with a normal acidified landfill process. From a hygienic point of view, the anaerobic digestion of sludge is suitable for agricultural lands without extra disinfecting treatment, while further disinfecting treatment, such as composting, is definitely required for acidified landfill sludge before it can be applied to agricultural lands.

Table 12.3 Pathogenic bacterial count of anaerobic sludge and acidified landfill sludge (cfu/g digested sludge)

Pathogenic bacteria	Anaerobic digestion sludge		Acid landfill sludge	
	4 weeks	6 weeks	20 weeks	44 weeks
Total coliforms	1.6×10^2	1.2×10^2	7.46×10^6	8.63×10^7
Fecal coliforms	nd	nd	1.0×10^6	31×10^6
Fecal streptococci	nd	nd	7.0×10^3	nd
Salomonella sp.	0.11×10^2	0.32×10^2	4.88×10^5	35.2×10^5

cfu: Colony forming units

12.3 The water-content or water-holding characteristics of solid anaerobic digestion sludge

As water use restrictions become tighter and more of a superintendent's operating budget is used for water, water content and water-holding capacity of the sludge becomes increasingly important in its application to agricultural land. The water contained in the sludge can be classified as free water, immobilized water and bound water. Therefore, in order to evaluate the free and/or immobilized and bound water content of the anaerobic digestion sludge under our experimental conditions, four portions (20 g – 25 g) of 2 week - thermophilic - 4 week - mesophilic anaerobic digestion sludge were consistently dried in an oven at $103-105^\circ\text{C}$ for 68 hours. The cumulative water loss and evaporation rate were monitored during the drying period and the results are presented in Fig.12.1.and Fig.12.2. The relationship between evaporation rate and moisture content is presented in Fig.12.3.

From the results in Fig.12.1, it can be seen that the water loss through evaporation was less than 10% after one hour of drying. This indicated that the largest fraction of the water in the anaerobic digestion sludge is strongly held in the structure of the sludge, and it does not dewater easily. Little water was evaporated after 24 hours of drying at a temperature of 103-105 °C, more than 65% of the water can be evaporated during the first 20 hours of drying. This suggested that a large part of the water in the sludge could be utilized by plants if it is applied to the soil in the arid area.

The results in Fig.12.2 show that the water evaporation rate of the sludge increased greatly during the first 4-hour drying process; it then decreased rapidly and appeared stable with the drying process longer than 42 hours. The critical conversion point on the curve suggests that most of the free and immobilized water evaporated during the first 4 hours of drying at a temperature of 103-105 °C.

Furthermore, the types of water in the anaerobic digestion sludge can be determined from the evaporation rate versus moisture content curve in Fig.12.3. It can be seen that the critical moisture value is around 0.320 g/g sludge. The difference between the x-coordinate at the point of 0.683g/g sludge, which is the moisture content of the sludge at the start of the drying test, and the x-coordinate at the critical moisture point of 0.363 g/g sludge, can be considered as the free and immobilized water content of the sludge, which is about 0.304 g/g of sludge. The bounded water content is between x-coordinate point, at which the water evaporation rate is close to 0.0 g/h, and the x-coordinate at the critical moisture point of 0.320 g/g sludge, where the water evaporation rate reaches the maximum. Therefore, it can be calculated that the anaerobic digestion sludge has about 44.5% free water plus immobilized water and 46.9% bound water that is difficult to drain out of the sludge structure. Thus, the water-holding capacity of this sludge is relatively high and will be beneficial to agricultural application in dry-climate areas.

12.4 The metal desorption and absorption characteristics of anaerobic digestion sludge

The anaerobically digested lignocellulose sludge is essentially composed of humus and microbial debris. Thus, similar to soil humic substances, it has considerable potential to

accumulate and transport heavy metals in soil and aquatic environment through physical absorption and/or chemical chelating. In the meantime, the metals retained in the sludge could pose a potential risk to plants, animals and humans. It is important to have knowledge of the metal absorption/desorption properties of anaerobic digestion sludge for agriculture land use or soil conditioning.

The sample used was wet sludge with a moisture content of about 68%. Metal desorption experiments were performed in the pH range of 1-7 using HCl for pH adjustment. The wet sludge sample with a moisture content of about 68% was used. Under each given pH, 50 g of wet sludge was supplied with 100 ml distilled water. The mixture was kept in suspension for 12 hours. The suspensions were then filtrated (Schleicher & Schuell) and the metal contents of the filtrate were analyzed by inductively coupled plasma (ICP) spectrometry (Varian Liberty Series II Sequential ICP AES). The results are illustrated in Fig.12.4.

The results in Fig.12.4 indicate that desorption of metals such as Fe, Al, Cu, Pb, Si and Mn did not occur under normal environmental conditions (pH 6-7). Desorption of these metals only occurred at pH 1.0 or less, although a considerable desorption of Ca can take place at pH 5. The concentration of K and Na in the suspension remained relatively stable throughout the pH range from 1.0 to 8.0; there is no significant desorption of these metals under conditions with pH at 1.0 or lower than 1.0. This suggested, on the one hand, that desorption / absorption of K and Na from the sludge is pH independent, and on the other hand, it pointed out that the bindings of heavy meals on the anaerobic sludge are normally stronger than the binding of non-heavy metals such as Ca, Mg, K and Na. The interactions between these non-heavy metals and the anaerobic sludge are likely through ion-exchanging without the involvement of H^+ or OH^- .

Heavy metal incorporation properties of anaerobic sludge were investigated by measuring absorption properties of Pb, Zn, Cu and Fe on the sludge. The experiments were carried out in 600 ml beakers with 20 g wet anaerobic sludge added to a series of 100 ml stock metal solutions having concentrations of 0-300 mg/l. The mixed solutions were kept in suspension by magnetic stirring for 2.5 hours. The pH of the solutions was initially adjusted to around 7.0 by 1 M NaOH. During the absorption process, each 15 ml sub-suspension solution was taken during the absorption process for metal analysis at 0.5 hour intervals. The residual metal

content in the suspension under different metal concentrations and absorption times was analyzed by ICP spectrometry. The amount of each metal to be absorbed by the anaerobic sludge was determined. The changes in absorption amount and equilibrium concentrations of heavy metals during the time course were monitored and the results are presented in Fig.12.5 – Fig.12.8. The absorption curves under different heavy metal concentrations are given in Fig.12.9 – Fig.12.12.

As can be seen in Fig.12.5-12.8, the absorption of Pb, Zn, Cu, Fe by the anaerobic sludge rose steadily in time course and tended to stabilize after 120 minutes. This suggests that the absorption of heavy metals such as Pb, Zn, Cu, Fe is not a slow process as the absorption equilibrium can easily be reached in 2 hours. The absorption process was found to be faster and more effective under higher concentrations of heavy metals. For example, the absorption of Pb, Zn and Cu immediately (about 0.5 hour) reached equilibrium when their concentrations were as high as 300 mg/l.

The effects of heavy metal concentrations on the absorption processes can be deduced from Fig.12.9-12.12. The results indicated that the absorption of heavy metals on the anaerobic sludge increased exceptionally with the increases in the addition of heavy metals. In particular, a positive linear relationship was found between the absorption of Fe on the anaerobic sludge and the rise in the concentration of additional Fe. Compared to the increases in the amount of absorbed heavy metals on the anaerobic sludge, the residual (equilibrium) concentrations of these heavy metals in the suspensions increased less or tended to stabilize with the increasing addition of each heavy metal. This suggested that the absorption of heavy metals on the anaerobic sludge was strongly dependent on the available concentrations of the heavy metals. In another words, it can be assumed that the anaerobic sludge suspension had a high heavy metal-ions buffering capacity. Therefore, the residual concentrations of these heavy metal ions remained stable in spite of the remarkable external contributions of the heavy metal ions to the suspension system.

As was discovered, the heavy metal desorption capacity of anaerobic sludge under the environmental conditions where the pH is higher than 1.0 was extremely weak. The anaerobic sludge can therefore be considered to be an effective heavy metal absorbent rather than a heavy metal spreader in the environment. The heavy metal absorptive property of the

anaerobic sludge should not be a big obstacle for its application on agriculture land because its weak and slow heavy-metal-releasing characteristics can prevent, to some extent, the risk of intake of heavy metals by plants.

In order to investigate the effects of the additional heavy metals on the absorption/desorption properties of the originally-contaminated metals such as K, Ca, Mg, Si, Fe in the anaerobic digestion, the residual concentrations of K, Ca, Mg, Si, Fe in the suspensions under different concentrations of additional heavy metals were measured by ICP. The absorption/desorption time was 120 min. The pH of the suspensions was maintained in the range 7.0-7.6. The results were presented in Fig.12.13- Fig.12.17.

The results in Fig.12.13-Fig.12.17 indicate that the additional heavy metals such as Pb, Zn, Cu and Fe enhanced the desorption of Ca, Mg, K and Si from the anaerobic sludge. The residual concentrations of Ca, Mg, K and Si in the anaerobic sludge solutions generally showed augmentation with the increases of heavy metal addition. This suggests that the heavy metals (Pb, Zn, Cu, Fe) added into the suspensions were exchanged for some of the Ca, Mg, K and Si from the surface structures of the anaerobic sludge. Interestingly, the addition of Pb, Zn, Cu and Fe did not incur any desorption of Fe from the anaerobic sludge. Vice versa, the absorption of Fe on the anaerobic sludge was strengthened by the addition of these heavy metals. These findings further pointed towards the fact that the anaerobic sludge has a stronger affinity for heavy metals than for non-heavy metals. The absorption of these heavy metals on the anaerobic sludge in some ways resulted from the desorption of non-heavy metals such as Ca, Mg, K and Si from the anaerobic sludge. This indicated that anaerobic sludge has a selective absorption affinity for heavy metals. Since the metal toxicity to the environment comes mainly from the mobile heavy metals, if properly designed, the selective heavy metal absorption and fixation properties of anaerobic sludge can be exploited to reduce the availability of heavy metals to plants and humans in an aquatic environment. A point worth mentioning is that the heavy metal absorption characteristics of anaerobic digested sludge should not be a big obstacle to its agricultural land application, owing to its slow heavy-metal releasing property and a promising ion- exchange capacity between non-heavy metals and heavy metals.

12.5 The micro-structure of the anaerobic digestion sludge and the structure alterations by microscopical observations

To gather information regarding the micro-structural characteristics of solid anaerobic digestion sludge and the structural alterations of the lignocellulose sample during digestion, the microscopical observations were made with a scanning electron microscope (SEM). The anaerobic sludge was free-dried during the first week and at the end of the digestion process (2 week-thermophilic-4 week-mesophilic) and the fresh lignocellulose sample (tobacco dust) was vacuum-dried at room temperature. The micro-structure observations were made using a Topcon ABT-60 scanning electron microscope. The SEM photos are shown in Fig.12.18.

The micro-structure of lignocellulose (tobacco dust) is presented in Fig.12.18 (A1) and Fig.12.18 (A2). The micro-organism in the early anaerobic digestion process are presented in Fig.12.18 (B1) and Fig.12.18 (B2). The microstructure of the anaerobic digestion sludge is presented in Fig.12.18 (C1) and Fig.12.18 (C2).

As can be seen, the microstructure of the lignocellulose (tobacco dust) has evidently been altered by anaerobic digestion. The arrangement of stem-like structures in tobacco dust not treated by anaerobic digestion was not found in the anaerobic digestion sludge. The anaerobic digestion sludge appeared to be much more porous and seemed to have more open structures than the fresh lignocellulose sample, which was not treated with anaerobic digestion. The anaerobic sludge appeared highly ruffled on the surface, with multi-orientation and dimensions of protrusions extending from the plane of the sheet (flake)-like structure. This highly porous and greatly corrugated surface structure of anaerobic sludge assists in understanding its high water-holding capacity, strong affinity for heavy metals, large heavy metal absorption capacity and slow metal releasing property. This special micro-physical structure of lignocellulose anaerobic digestion sludge is also helpful in understanding the benefits that the anaerobic sludge could bring about to agriculture land by exerting a number of functions that are typical and specific to humified soil organic matter. They include the slow release of nutrients such as N, P, and S, high CEC (cation exchange capacity), pH buffer capacity, specific physiological effects on plant growth, and an extended capacity of interactions with micro-nutritive and/or micro-toxic metal ions and xenobiotic organic molecules such as pesticides⁽⁵⁴⁾.

At the early stage of the anaerobic digestion, the microbial community consisted of well-separated rod-shaped cells of variable length and small rods and cocci with smooth surfaces. However, the cells of these microorganisms could hardly be observed in the end digested sludge (Fig.12.18 (C1,C2)). The survival capacity of pathogens is limited by a thermophilic anaerobic digestion due to the narrow requirements of these organisms for specific substrates and temperatures for optimal activity. Therefore, most of the indicator micro-organisms (total coliform, fecal coliform and fecal streptococci) and viruses can be destroyed after a continuous thermophilic-methophilic anaerobic digestion. The micro-organisms present at the early stage of digestion most likely consisted of lignocellulose hydrolysis/degradation fungi and/or acidic fermentation bacteria. It can therefore be postulated that the final digested sludge is free of most fungi and /or acidic fermentation bacteria and pathogens.

12.6 Discussion and Summary

Like humic substances, the anaerobic sludge consists of a chemically heterogeneous mixture of structure-undefined large molecular humic polymers and a large amount of micro-organisms. It is virtually impossible to describe exclusively chemical structure of the anaerobic sludge. However, it is possible to depict the general structure of the “typical” chemical functions of the anaerobic sludge on the basis of available functional compositions of humic substances. Accordingly ^(236, 148), the macro-molecular structure of humic polymers consists of aromatic, phenolic, quinonic, and heterocyclic “building blocks” that are randomly condensed or linked by aliphatic, oxygen, nitrogen, and sulphur bridges. The macromolecule of HS bears aliphatic, glucidic, amino acidic, and lipidic surface chains as well as chemically active functional groups of various natures (mainly carboxylic, but also phenolic and alcoholic hydroxyls, carbonyls etc.), which render the humic polymer acidic.

The extracellular polymer of the micro-organism (ECP) is another important polymer in the anaerobic sludge. Bacterial ECP is defined as polysaccharide-containing structures of bacterial origin lying outside the integral elements of the outer membrane of Gram-positive cells and the peptidoglycan of Gram-positive cells ^(49, 50). ECP is made up of organic debris, phages, lysed cells and other organic material excreted by the microbial cells. It contains polymers of saccharides, proteins, lipids, phenols and nucleic acids. Because of its porosity

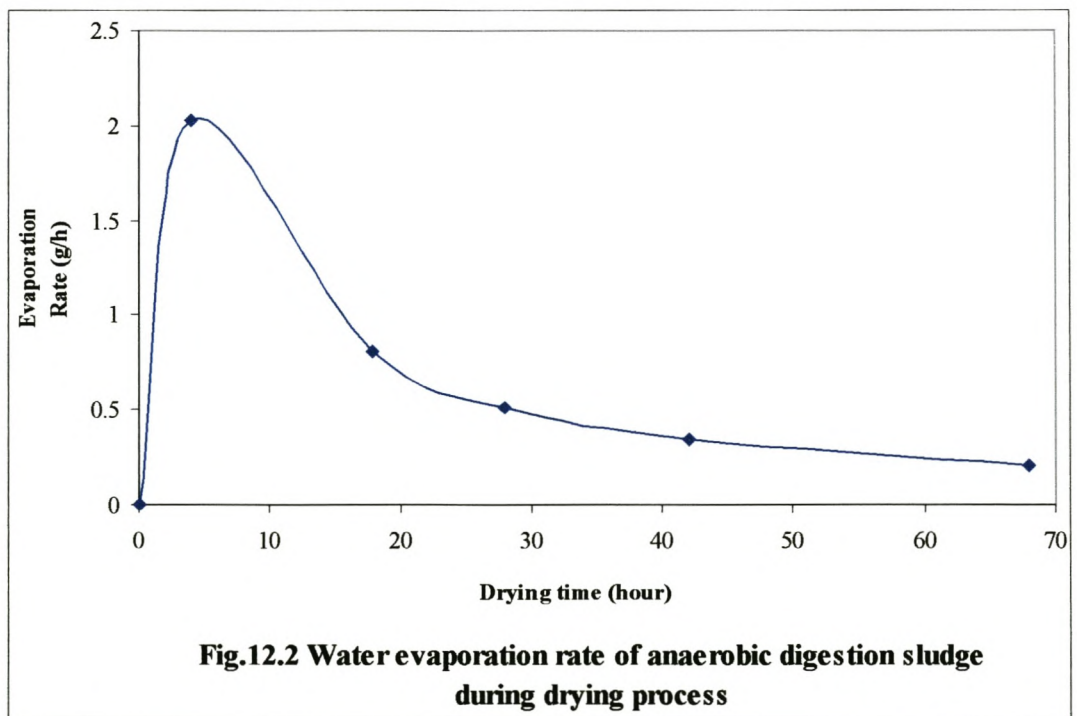
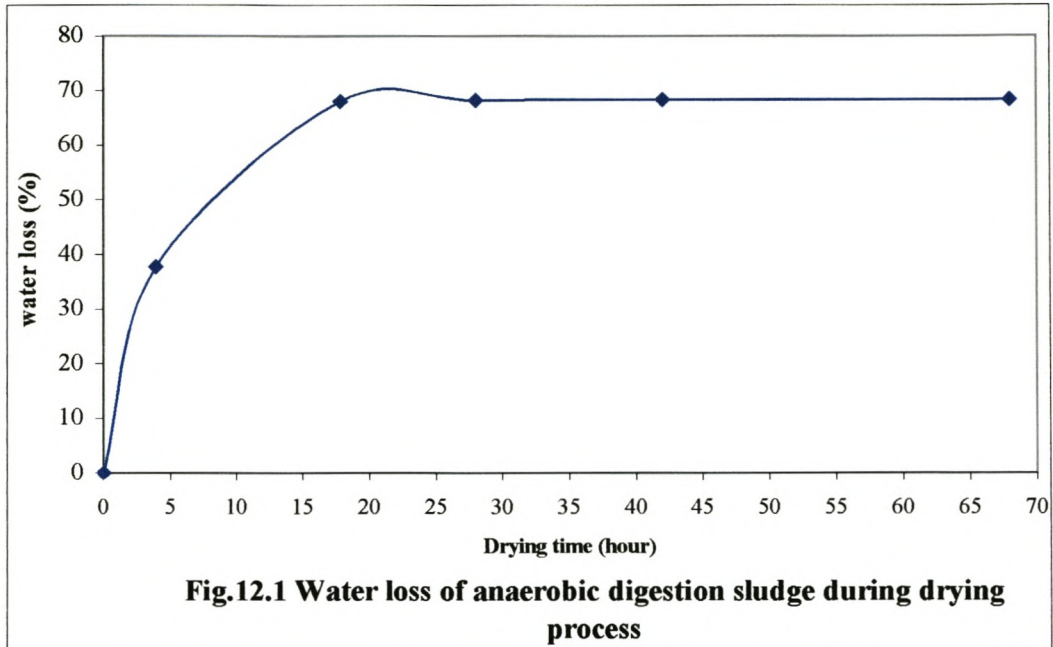
and the multi-functional groups in its physical and chemical structure, ECP can trap soluble nutrients which include trace metals in the environment ⁽²³⁵⁾.

The absorption of dissolved metals by the anaerobic sludge was likely to be based upon several possible mechanisms, such as weak physical sorption, ion exchange or surface complexation. As can be seen, the humic polymers and the ECP of the micro-organisms in the anaerobic sludge are highly poly-electrolic, with several sites and functional groups potentially able to bind with metal ions, mineral surfaces and other organic compounds. As far as the results obtained in this study are concerned, it can be postulated that the selective absorption of heavy metals on the anaerobic sludge was likely through forming multi-dental chelate complexes with the humic polymers and the ECP of the micro-organisms in the anaerobic sludge. However, the absorption of non-heavy metals, such as Ca, Mg, K, Si, on the anaerobic sludge was possible by weak physical sorption or ion exchange.

According to the results obtained in this study, the following conclusions can be made:

- 1) The anaerobic digestion sludge has about 44.5% free water plus immobilized water and 46.9% bound water that is difficult to drain off. The water-holding capacity of this sludge is relatively high and is beneficial for agricultural application in dry-climate areas.
- 2) The anaerobic digestion process under thermophilic conditions has promising disinfecting effects in contrast to normal acidified landfill processes. From a hygienic point of view, the anaerobic digestion sludge is suitable for agricultural lands without extra disinfecting treatment, while further disinfecting treatment, such as composting, for acidified landfill sludge is definitely required before it can be applied to agricultural lands.
- 3) The nitrogen and potassium content of anaerobic sludge are comparable or higher than that of activated sludge and UASB sludge. The anaerobic sludge is suitable to be applied with commercial fertilizer in agriculture land application.
- 4) The anaerobic sludge has a strong selective absorption affinity for heavy metals. The absorption of heavy metals partially resulted from the desorption of non-heavy metals such as Ca, Mg, K and Si from the anaerobic sludge.

- 5) The heavy metal desorption capacity of anaerobic sludge under normal environmental conditions (pH higher than 1.0) was impressively weak. The anaerobic sludge can be considered to be an effective heavy metal fixer rather than a heavy metal spreader. The heavy metal absorption property of the anaerobic sludge should not be seen as an obstacle for its application to agriculture land when its weak and slow heavy-metal-releasing characteristics are considered.
- 6) The highly ruffled surface, with multi-orientation and dimensions of protrusions extending from the plane of the sheet (flake)-like structure of the anaerobic sludge confirmed the high water-holding capacity, strong affinity for heavy metals, large heavy metal absorption capacity and slow metal releasing properties of anaerobic sludge.
- 7) The micro-organisms at the early stage of the anaerobic digestion were composed of well-separated rod-shaped cells of variable lengths and small rods and cocci with smooth surfaces. The final anaerobic digestion sludge appeared to “be sterilized ”.
- 8) To conclude, solid thermophilic-mesophilic lignocellulose anaerobic digestion sludge can be viewed as a humus-rich hygienic product that improves soil fertility and water-holding capacity, nourishes plants and immobilizes heavy metals in the environment.



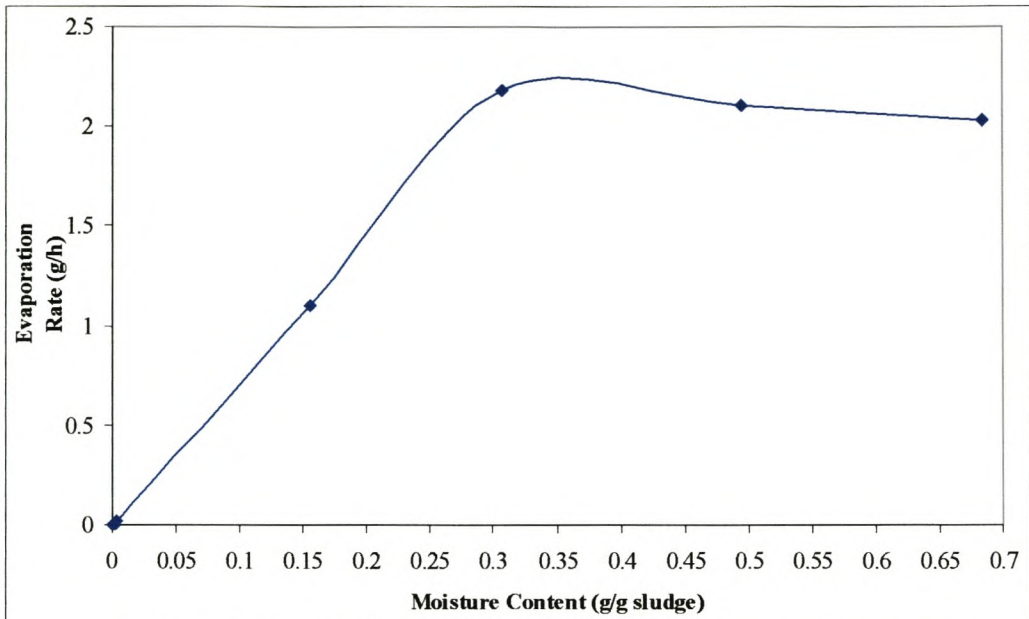


Fig.12.3 The relationship between evaporation rate and moisture content of anaerobic digestion sludge during drying process

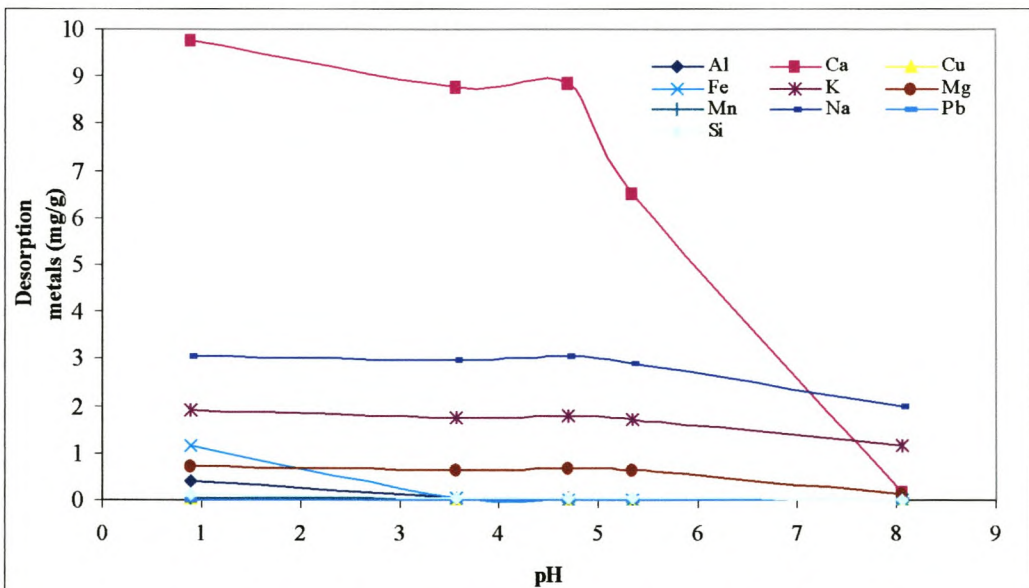
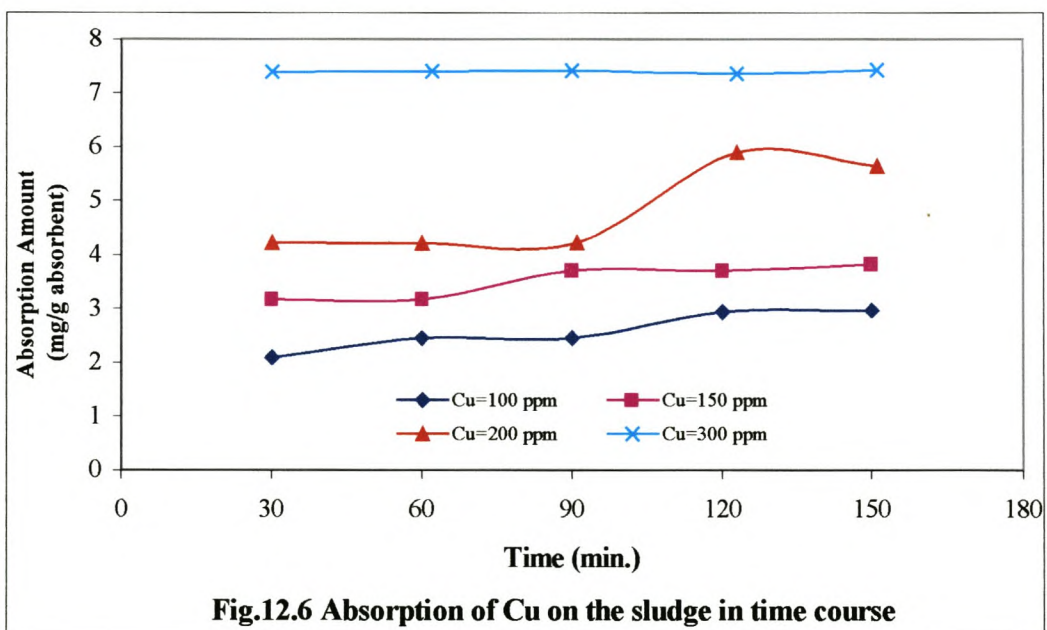
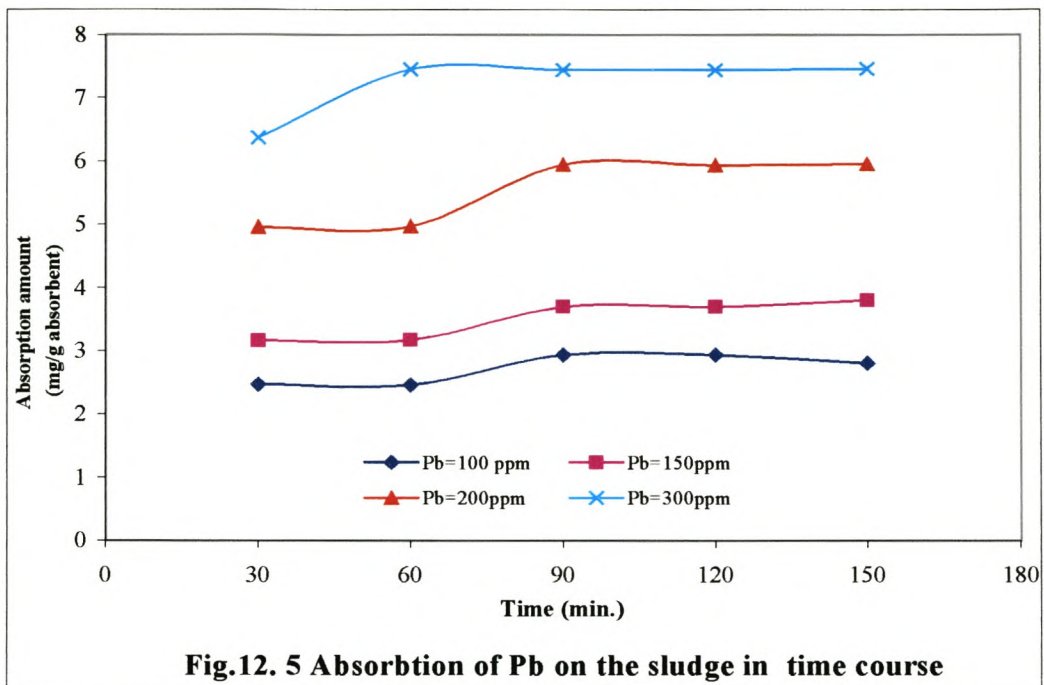
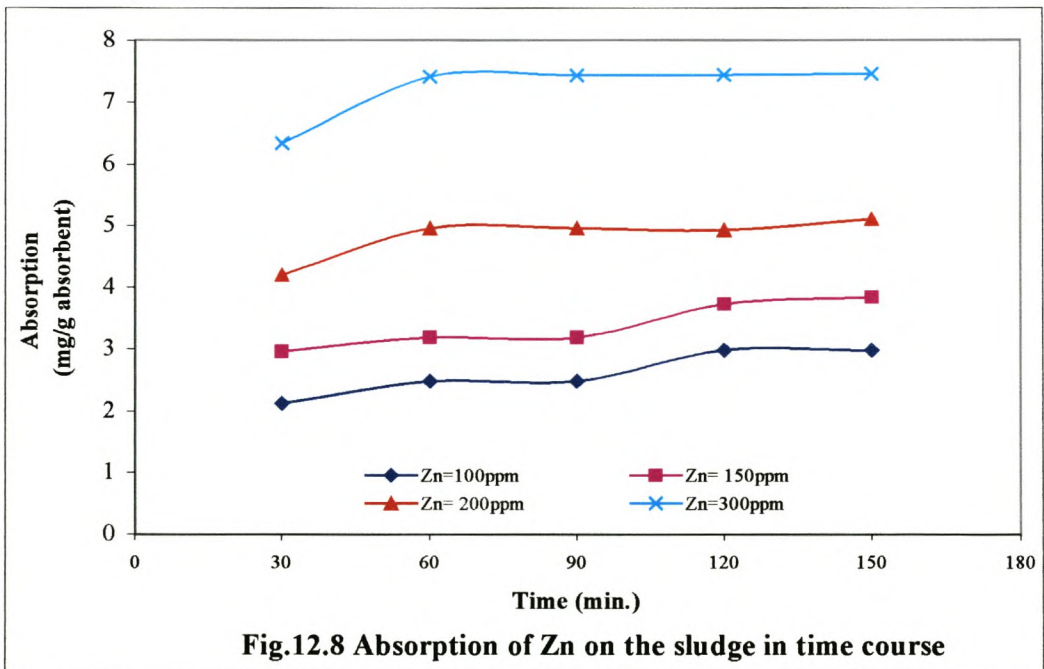
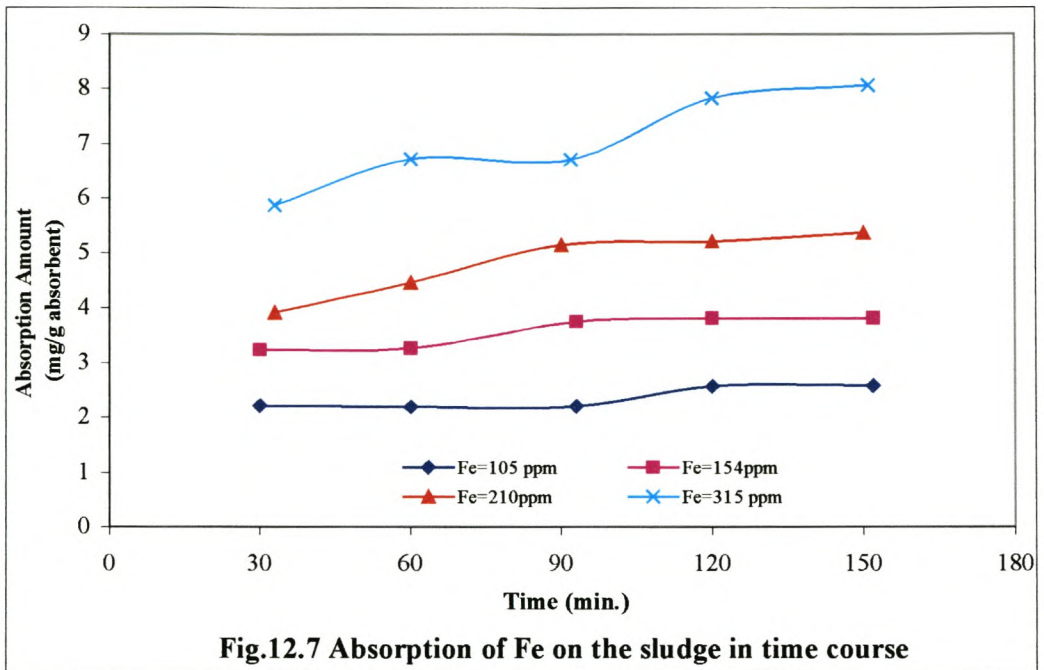
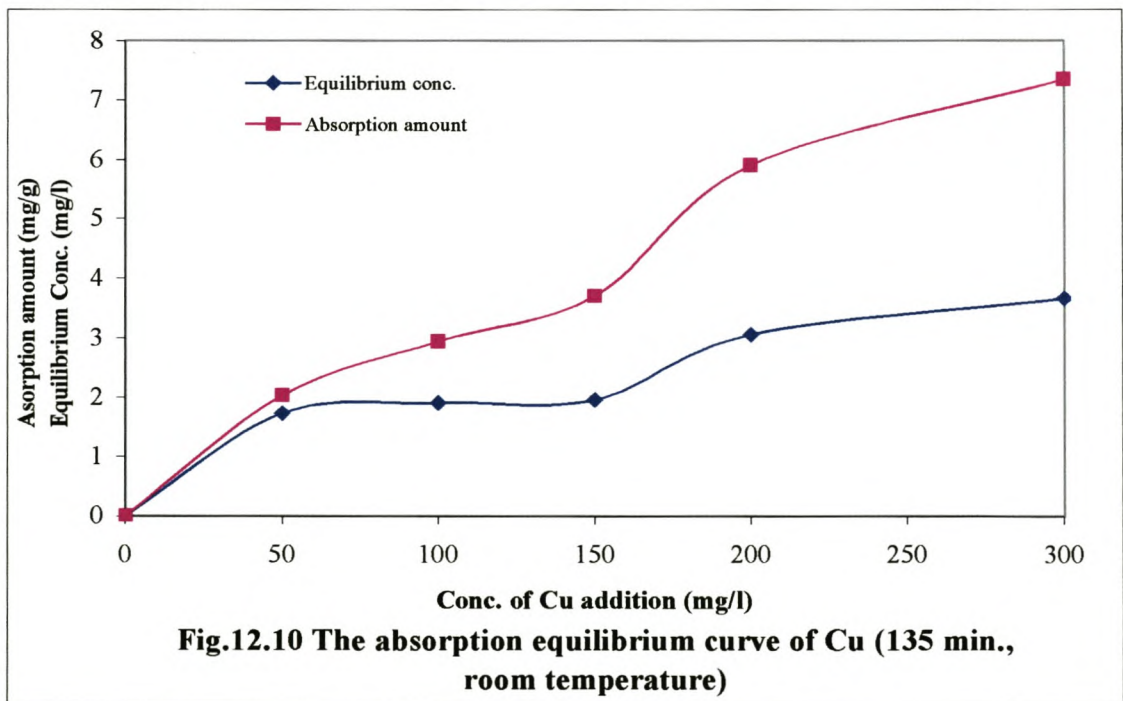
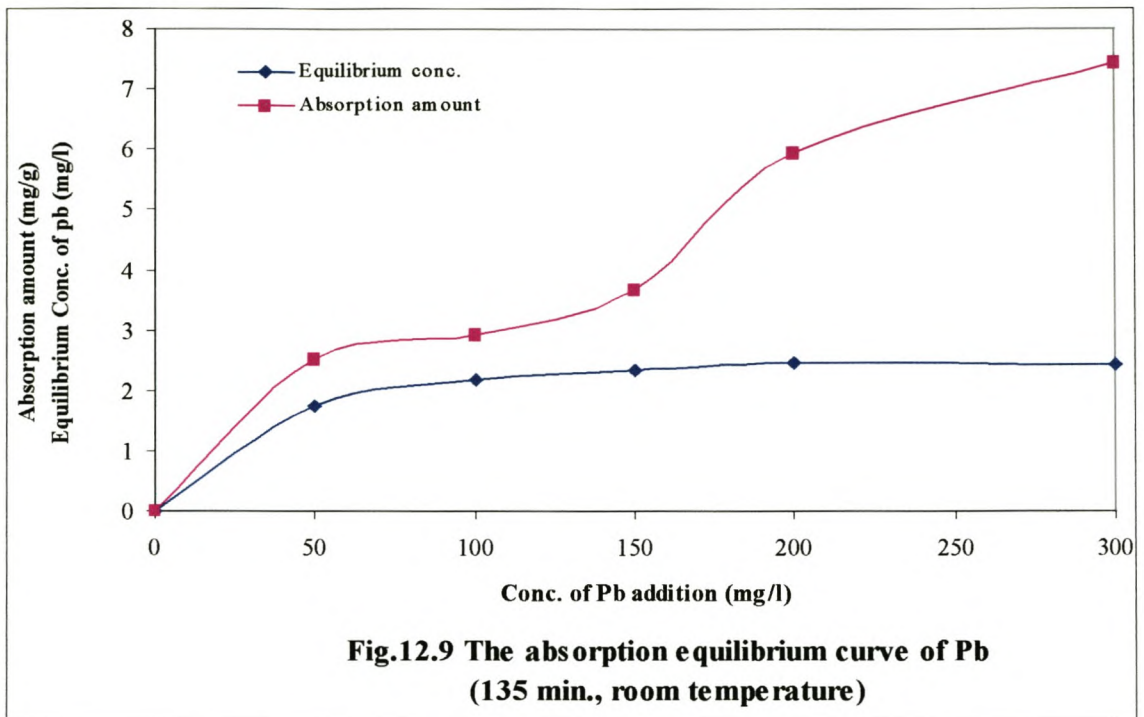
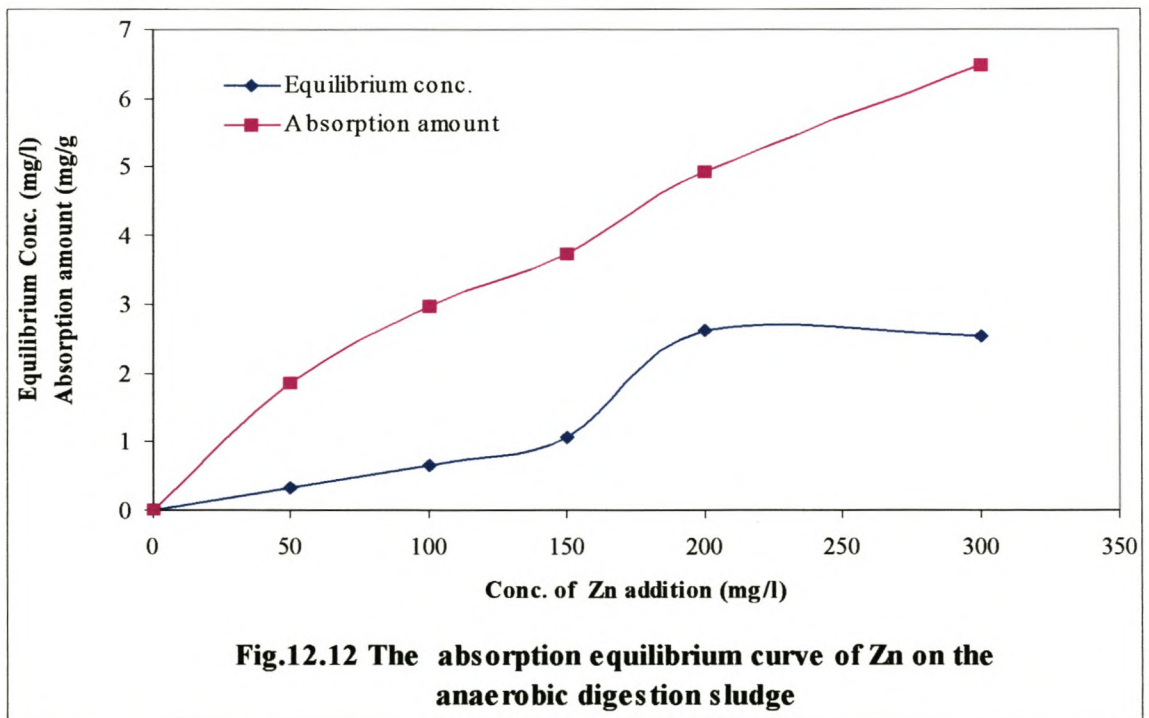
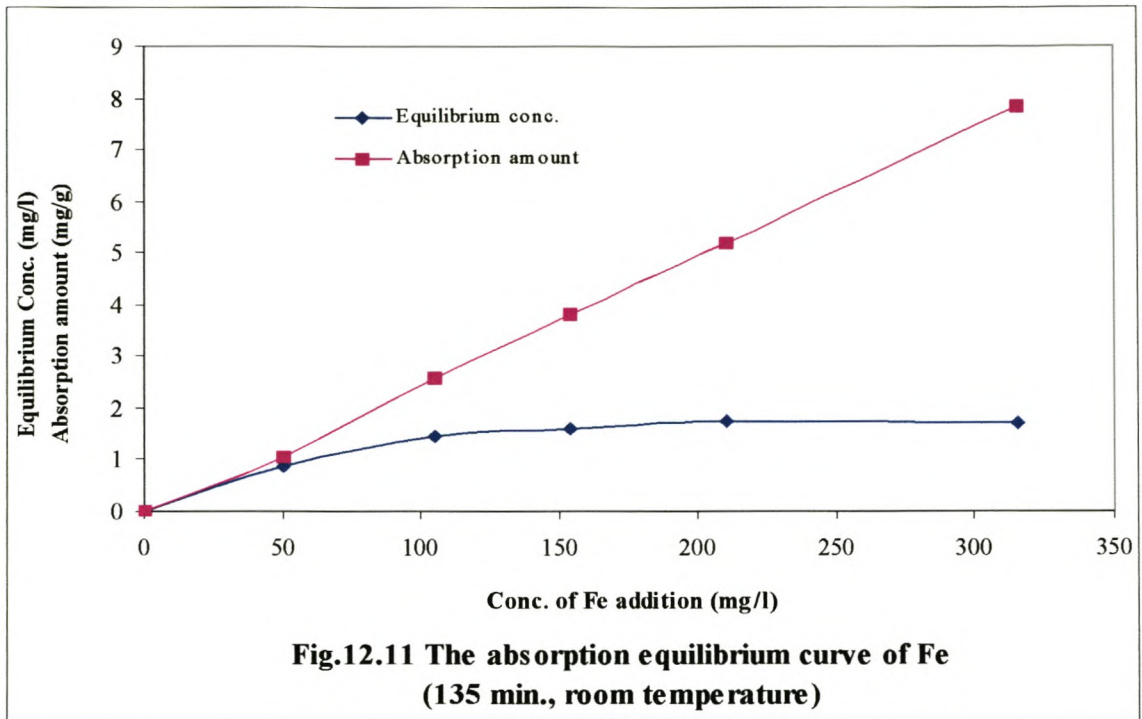


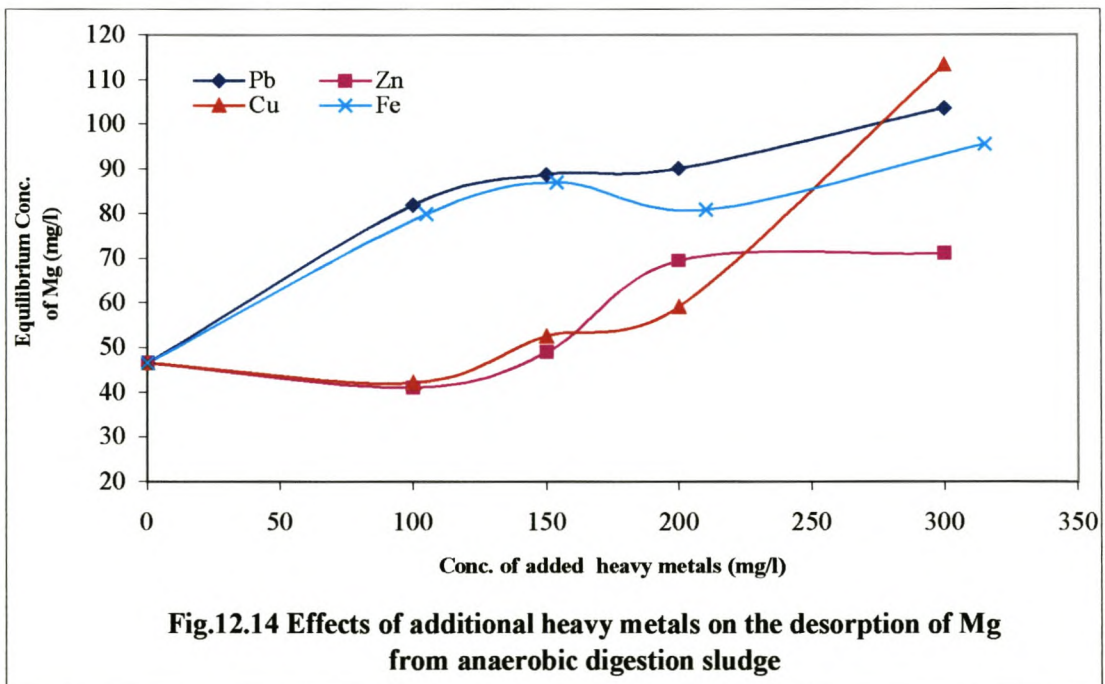
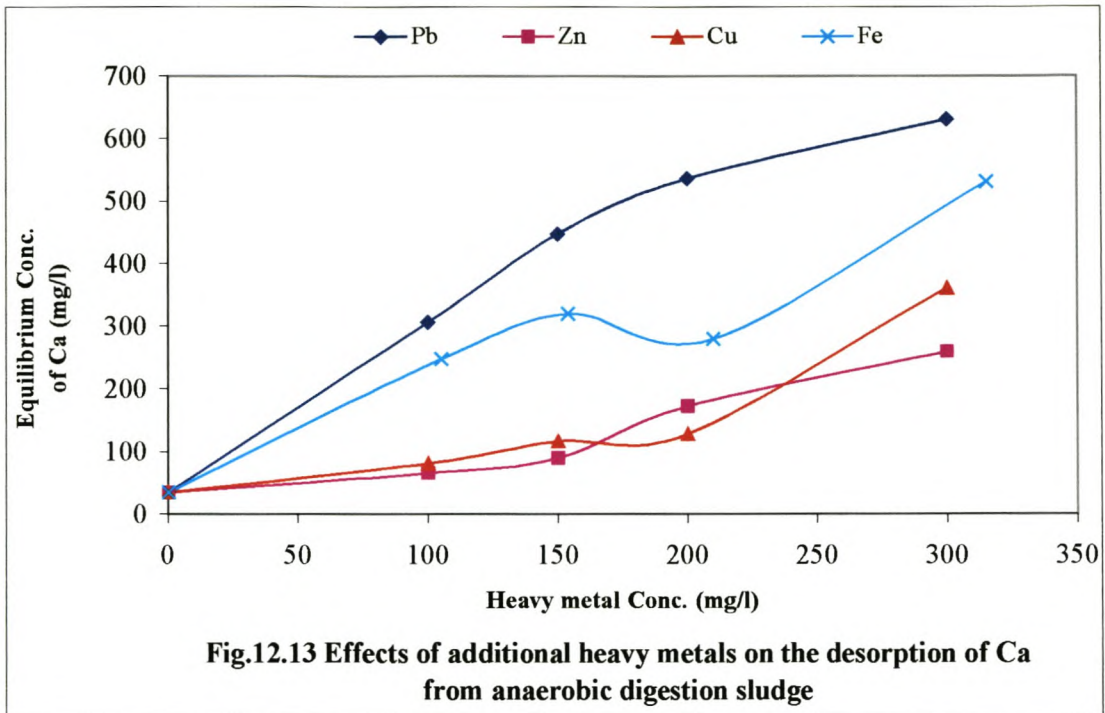
Fig.12.4 Metal desorption property of anaerobic sludge under different pH conditions

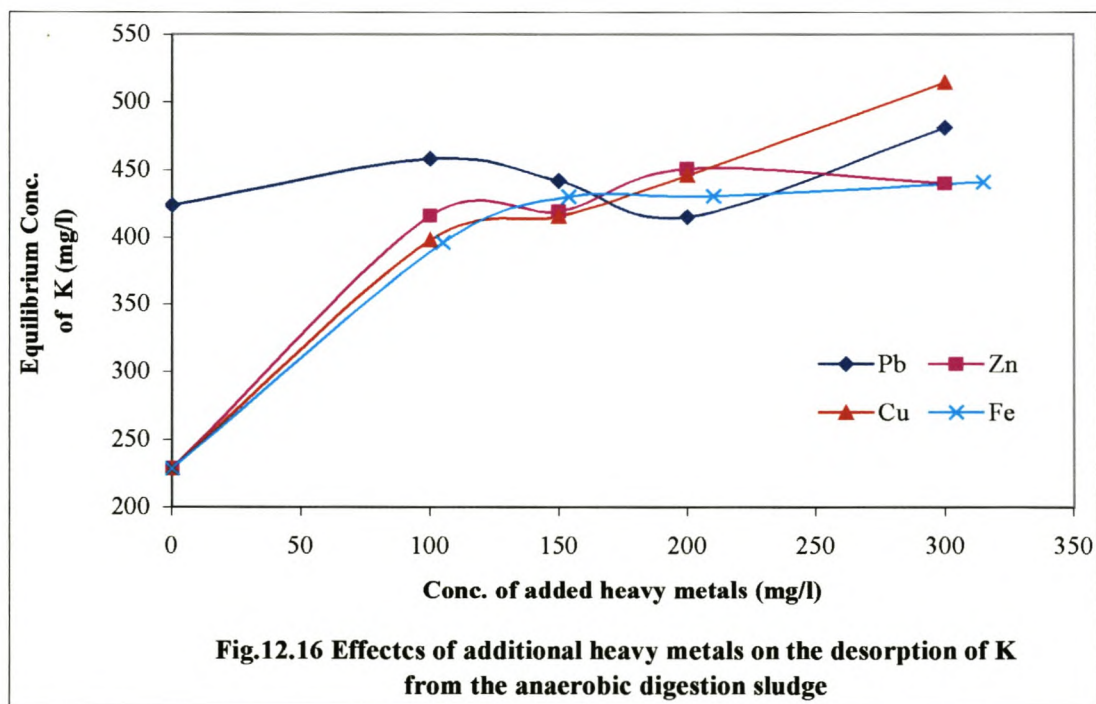
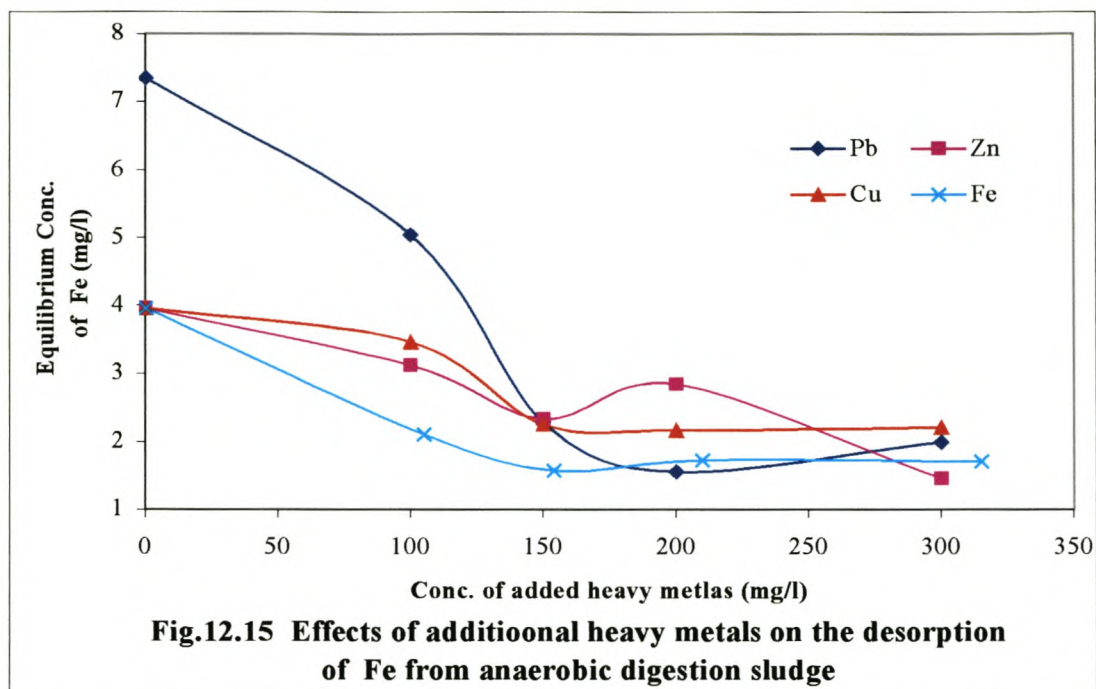












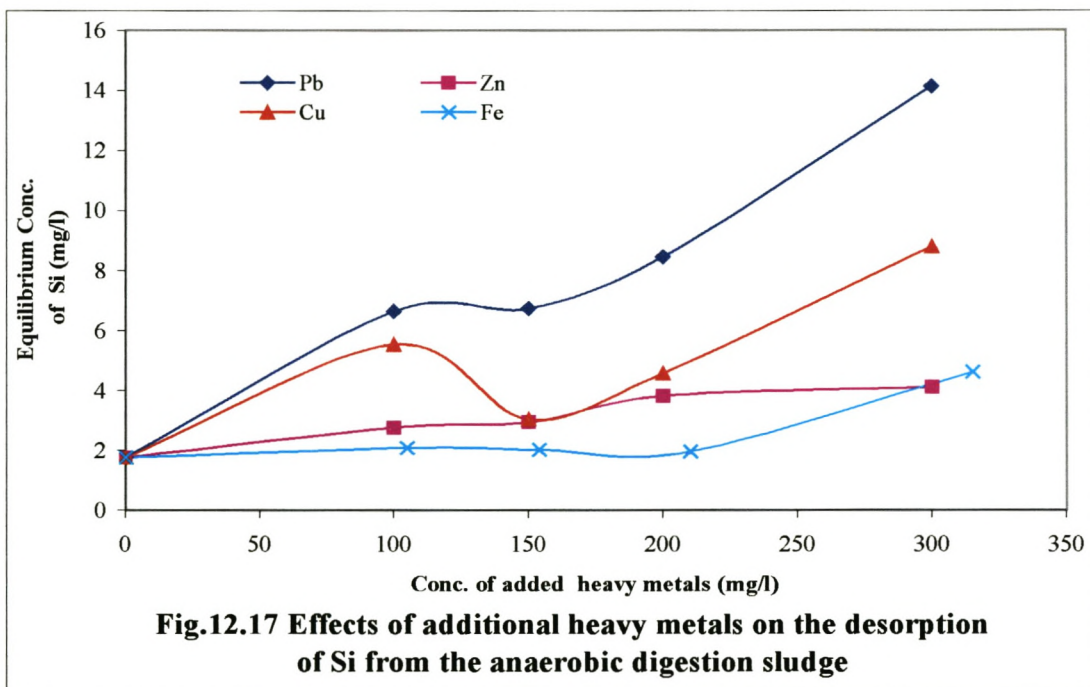




Fig.12.18 (A₁) SEM photos of the fresh lignocellulose substances before digestion (150x)

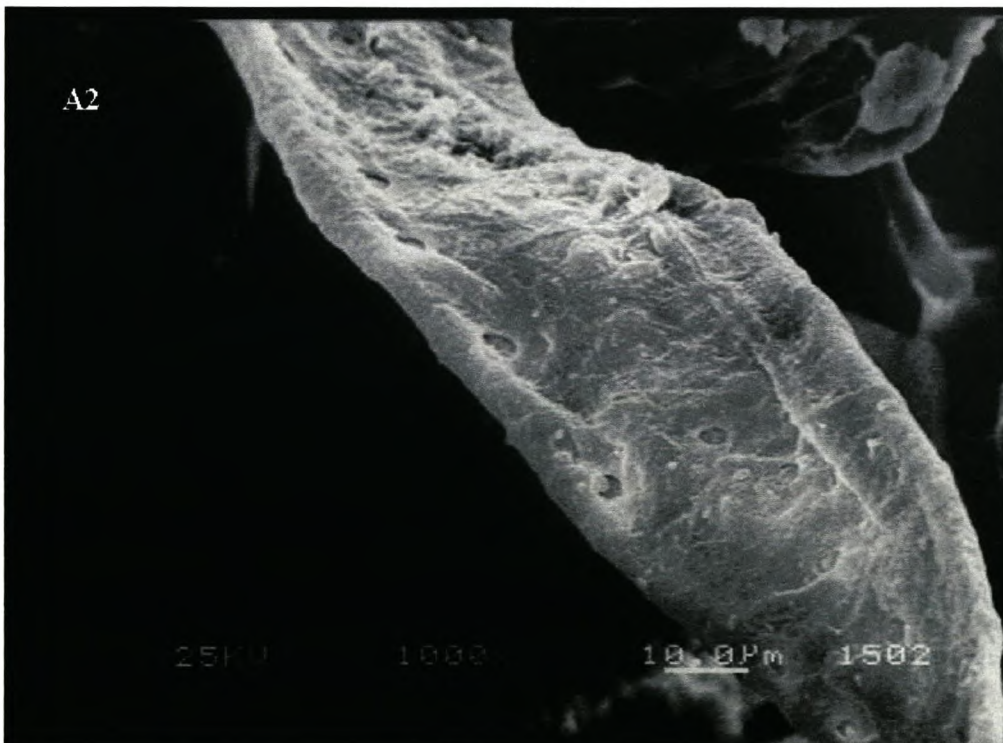


Fig.12.18 (A₂) SEM photos of the fresh lignocellulose substances before digestion (1000x)



Fig.12.18 (B₁) SEM photos of the lignocellulose degrading microorganisms at the early stage of the anaerobic digestion process (1000x)

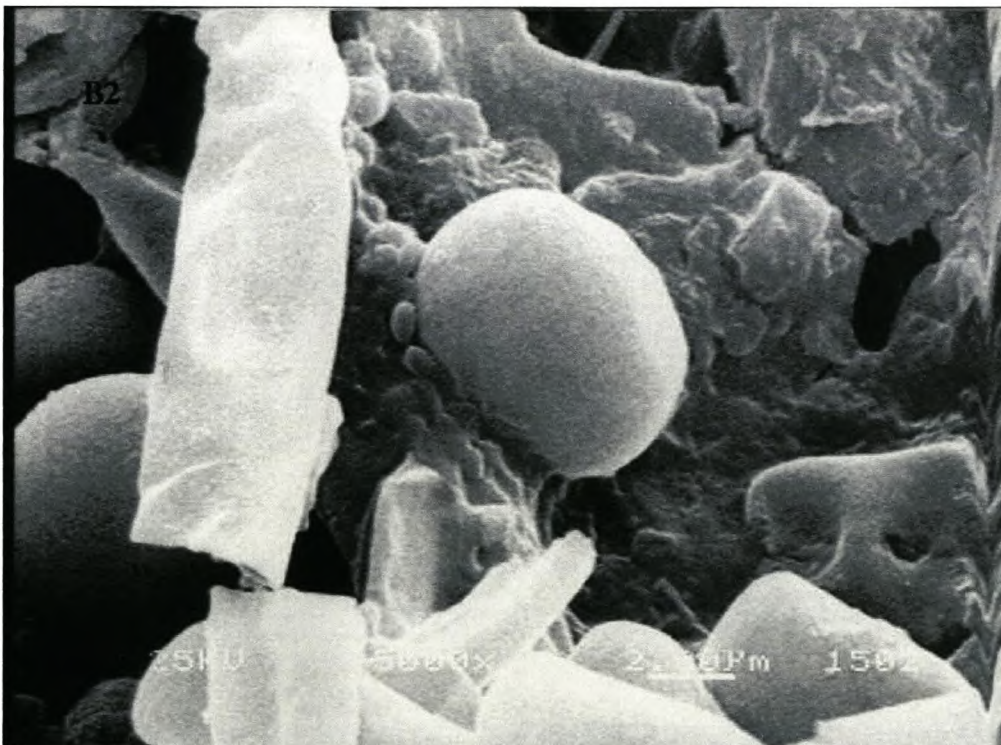


Fig.12.18 (B₂) SEM photos of the lignocellulose degrading micro-organisms at the early stage of the anaerobic digestion process (5000x)

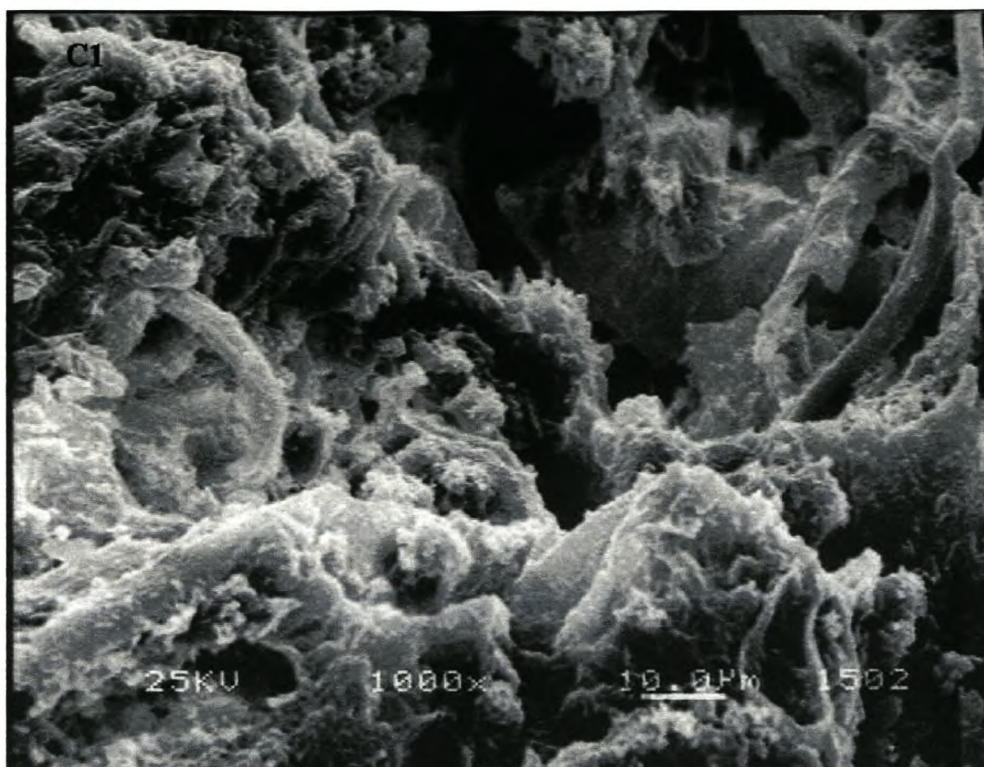


Fig.12.18 (C₁) SEM photos of the anaerobic digestion sludge of the lignocellulose substances (1000x)

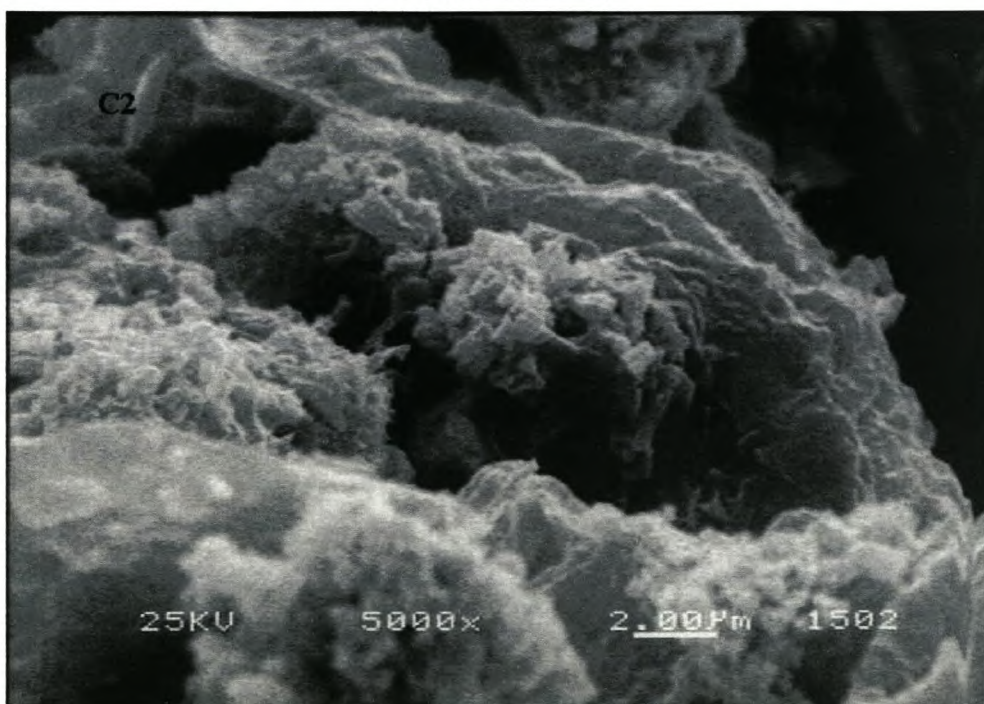


Fig.12.18 (C₂) SEM photos of the anaerobic digestion sludge of the lignocellulose substances (5000x)

Conclusions

With respect to the chemical and structural complexity of lignocellulose substrate, the accomplishment of the bio-degradation of lignocellulose residues is composed of a series of slow microbiological degradation and chemical alteration processes. From the results in this dissertation, it can be concluded that the re-utilization of lignocellulose wastes for energy recovery and agricultural land application by solid anaerobic digestion is plausible. An efficient, environmentally friendly and value-added method of disposal of lignocellulose residues is presented and the following conclusions can be drawn:

- ◆ The biodegradation of lignocellulose substrate can occur in extremely sulphate acidic simulated landfill environments. This process is, however, noticeably slow and unhygienic. As a result of the metabolic activities of the micro-organisms, the initially sulphate acidified habitats were quickly self-neutralized. Although the involvement of individual heterotrophic, prokaryotic acidophiles was not eliminated, the biodegradation of lignocellulose substrate in this special environment probably requires micro-eukaryotic organisms, such as filamentous fungi and yeast and soil actinomycetes. The acidophilic bacteria, filamentous fungi and soil actinomycetes were found to be dominant in the degradation process. The evidence of whether the yeast is detected or not could be the signal which indicates whether sugar was formed from the ligno-polymers or degraded into volatile fatty acid during the lignocellulose decomposition. With the production of soluble organic acids and the consumption of oxygen and other potential oxides, the landfill habitats were acclimatized to the development of anaerobic bacteria (such as sulphate-reducing bacteria). A number of pathogenic bacteria (coliforms and *salomonella sp.*) were consistently detected during the degradation process. It is comprative to enhance the processing efficiency and hygienic effects of the degradation of lignocellulose substance in both environmentally and economically beneficial ways.
- ◆ Regarding the composition and chemical structural complexity of lignocellulose, initial hydrolysis of lignocellulose substrate into soluble forms is critical for a successful anaerobic digestion of lignocellulose substrate.

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- ◆ The influence of digestion time on the destruction of lignocellulose to volatile fatty acid was evident. At least 2-3 weeks of digestion time was required with a batch reactor or a semi-continuous system reactor. The leachate circulation was necessary when the short retention time was used. The highly negative oxidation reduction potential status of the initial feeding medium (such as NaOH solution or cathodic radical waters) improved the acetic acid and the total volatile fatty acid production during the fermentation process. From the point of volatile fatty acid production and destruction of volatile solids, the acidification process of lignocellulose substrate was successfully promoted by a combined pre-treatment method, which involved the mechanical (roll-milling), physical (freezing/thawing) and chemical (alkali wetting agent) pre-treatment and the thermophilic co-digestion with activated sludge.
- ◆ The thermophilic high-rate digestion may accelerate the hydrolysis and acidification digestion process. However, partially because of the high concentration of volatile fatty acid produced in this acidification fermentation process, the methanogens, which was the most sensitive to the accumulation of acidification products and other toxicants, was easily inhibited at this high-rate digestion stage.
- ◆ It was found that diluting the high-rate acidification sludge system, adjusting the pH of the recycled leachate and using a two-phase digestion procedure could recover the methanogenic phase from the high-rate acidogenic phase. Dilution could alleviate the inhibition from the accumulated intermediate acid products and reduce the concentrations of the toxic compounds (such as lignin-related phenolic and HAs) formed in the process of digestion. The integrated digestion configuration composed of a high-rate thermophilic acidogenic stage followed by a pH and dilution controlled-mesophilic stage was desirable to lead the biotransformation of the lignocellulose substrate all the way to the end-product of methane and ultimately stabilize the digestion system.
- ◆ Denitrification during the anaerobic digestion of nitrate-containing lignocellulose residues was unavoidable. There was competition between denitrifiers and

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- ◆ Denitrification during the anaerobic digestion of nitrate-containing lignocellulose residues was unavoidable. There was competition between denitrifiers and methanogens for carbon sources. Increasing the C/N ratio of the substrate in the head loading substrate could assist the accomplishment of the denitrification process and accelerate the methanogenic developing course.
- ◆ Microbial flora respond to their abiotic environment and, as a result, select a favourable community structure. Owing to the complexities of the lignocellulose substrate fed to the digester, a complex diversity of micro-organism species is necessary to facilitate a complete methane fermentation process. The first group of the young microbial community in the decomposition process of lignocellulose substrate might be the indigenous consortia, such as filamentous fungi and soil actinomycetes, from the lignocellulose sample and activated sludge. They previously attacked these complex polymers and secreted extracellular hydrolytic enzymes into the digestion systems. Most of them were aerobic genera, which rapidly exhausted the trace oxygen left in the digester system and generated a proper anoxic haven for the development of anaerobic acidogenic genera. The acidogenic bacteria can be reckoned as the second group of the digestive microbial community that provides the methanogens (the third candidate in the community) with a suitable carbon source (volatile fatty acid, CO₂) and reductants (H₂). The more diverse community endorsed its wider functioning range of degradation and higher adaptability to the fluctuations of the associated environmental factors and the substrate to be digested. Like a diversity of micro-consortia in the vast majority of environments coexisting as communities to degrade the complex substrate by acting in concert, these three functional groups of micro-organisms coexist in the young microbial community of the digestion system to perform the complete degradation and stabilization of lignocellulose substrate. As the anoxic digestion proceeded, the community showed a higher selectivity for the anaerobic bacteria genera.
- ◆ Not all the acidogens produce methane. A densely acidogen-populated community did not always lead to a higher methanogen population, therefore microbiologically adjusting the acidogenic fermentation pathway to methanogenic fermentation was

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compulsory. From this point onwards, the two-phase digestion system is microbiologically more favourable than the single-phase digestion system.

- ◆ The influence of alkaline pre-treatment on the microbial population communities was not as evident as expected. The alkaline pre-treatment effects on the acidification process of lignocellulose can therefore be seen as abiotical rather than as biological.
- ◆ The inhibiting effect of the soluble concentrated organic compounds formed as the sub-products during the high-rate acidification process could be solved to some extent by a dilution process. However, the complete degradation and special treatment of these complicated organic sub-products (especially HAs, the major inhibitory sub-products of lignocellulose digestion and heavy metal spreader) need further investigation to obtain a complete solution to the inhibitory and potential pollution problems. The application of ultrasonic irradiation and flocculation with aluminium salts to the further treatment of HA solutions was investigated. Simultaneously, the degradation of HA by lignocellulose-degradation micro-organisms and the microbial consortia of UASB granules and their toxicity to the anaerobic microbial consortia were evaluated.
- ◆ Both the chemical and physical characteristics of HAs were altered by ultrasonic irradiation, depending on the irradiation time. Ultrasonic irradiation first acted on the lower-molecular-weighted fraction (dominant fraction) of HA. Around 50% of this fraction decomposed into volatile forms and vanished from the system. The higher molecular-weighted fraction (minor fraction) tended to aggregate into a colloidal form and remained in the system. The chemical modifications induced by ultrasonic irradiation happened mainly in the carboxylic acid groups, the methylene groups α to aromatic rings, a variety of aliphatic carbons (mainly polymethylene) and aliphatic carbons β to aromatic rings and carbons α to oxygen. The SEM micrographs showed that the fibre structures of HA macromolecules quickly deteriorated and the aggregation of a closely-woven flake network structure resembling sponge and a thickened sheet structure with finger-like protrusions were frequently observed after ultrasonification.

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- ◆ The introduction of the ultrasonic irradiation into the process of flocculation and/or coagulation of HA by preformed aluminium hydroxide visibly improved the efficiency of HA removal. The effect was more evident when ultrasonic irradiation performed on the HA and preformed aluminium hydroxide flocs simultaneously. Aggregation or condensation of the flocculation system was one of the reasons why ultrasonic irradiation enhanced the HA removal efficiency via preformed aluminium hydroxide flocs. The ultrasonic irradiation could not only provide more complex sites for the neutralization of the positive charges of the flocs but also increased the interaction activities between exchangeable species of the flocs and the anionic functional groups of the HAs during the ion-exchanging process.
- ◆ The fungal and bacterial isolates, except the yeast, from the lignocellulose degradation system were able to partially degrade the HAs. The biological decomposition of HA was found to be enhanced by ultrasonic irradiation. This effect could be attributed to: 1) the chemical oxidation effects of ultrasonic irradiation on the HAs, thus making HA more accessible to the bacterial or fungal exoenzymatical hydrolyzing; 2) the colloidal effects of ultrasound on the polydispersed HA, which could enhance the aggregation of HA in the mycelial net structure of the filamentous fungi.
- ◆ Among the three functional groups of anaerobic bacteria consortia, methanogens are the most sensitive to the toxicity of HA. However, the fermentative consortiums can survive and grow properly on the easily digestible carbon source (such as glucose) in HA solutions where the HA concentration was as high as 0.1 g/l.
- ◆ HAs can be partially hydrolyzed and decomposed by the acid fermentative consortia in the UASB granules. Instead of inhibiting the activities of fermentative consortia of the UASB granule, HA probably played a mediator role in the electron and substrate interspecies transfer among a variety of juxtaposed microniches located along the structure of the UASB granule. Owing to the granules' layered microstructure, the most toxicity-sensitive methanogens populating in the interior of the granule can be shielded from being completely exposed to the HAs by the hydrolytic and acidogenic fermentative bacteria in the outer layer. The methanogenic activity inhibited by the

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HAs can be easily alleviated by feeding with proper substrate (such as glucose, acetate and H_2/CO_2).

- ◆ The inhibitory effects of HAs on the methanogenic activity of the granule probably resulted from the accumulation of fermentative products (such as acetate) at the interfaces between the fermentative acidogenic consortia and the methanogenic consortia due to the high absorbing capacity of HAs at these interfaces or on the surface of the ECP constitutes of the granules.
- ◆ The treatment of HA bearing waste water is possible from this primary study. However, the influence of HAs on the granulation process, the granule microstructure and the interspecies transferral which are closely associated with the core methanogenic process in the UASB granule needs to be investigated extensively before a robust UASB granule, which can handle the HAs containing waste water efficiently, can be developed.
- ◆ The water-holding capacity of the solid anaerobic digestion sludge is relatively high and is beneficial to agricultural application in dry-climate areas. From a hygienic point of view, the anaerobic digestion sludge is suitable for agricultural lands without extra disinfecting treatment, while further disinfecting treatment, such as composting, for acidified landfill sludge is definitely required for the application of the sludge to agricultural lands. Concerning nitrogen, potassium and other trace nutrient contents, it is recommended that the anaerobic sludge be applied together with commercial fertilizer in agriculture land application. In addition, the anaerobic sludge has a strong selective absorption affinity for heavy metals. The absorption of heavy metals partially resulted from desorption of none-heavy metals such as Ca, Mg, K and Si from the anaerobic sludge structure. The heavy metal desorption capacity of anaerobic sludge under normal environmental conditions (pH higher than 1.0) was impressively weak. The sludge can be considered to be an effective heavy metal fixer rather than a spreader. The heavy metal absorption property of the anaerobic sludge should not be a striking obstacle to its application to agriculture land when its weak and slow heavy-

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metal-releasing characteristics are considered. To conclude, solid thermophilic-mesophilic lignocellulose anaerobic digestion sludge can be viewed as a humus-rich hygienic product that improves the fertility and water holding capacity of the soil, nourishes plants and immobilizes heavy metals in the environment.

The complexity of lignocellulose make it difficult to lead the degradation process all the way to methane. The degree of solubilization, the modification and the conversion of the different components are not very clear, also the overall anaerobic degradation of lignocellulose is not yet understood at chemical and micro-organism species level. For example, it is known that various micro-organisms are involved in the degradation process and a diversity of chemically complicated intermediate products are formed during the digestion process. However, the details of the micro-organisms at species level, further degradation or bio-conversion of the complex intermediate products and the extent of between particular fungi and/or bacteria involved in the degradation of lignocellulose (including intermediate products) are not clear. Therefore, further extensive investigations at microbiological, genetical and chemical levels are required to elucidate the mechanisms behind the solid anaerobic digestion of lignocellulose substrate and to promote an advanced technology for the utilization of lignocellulose wastes in accordance with the sustainable development ethos.

In addition to the theoretical achievements, an environmentally and economically beneficial technology to utilize lignocellulose wastes and activated sludge can be established as is proposed in the following flow-sheet (Fig.13.1).

Conclusions

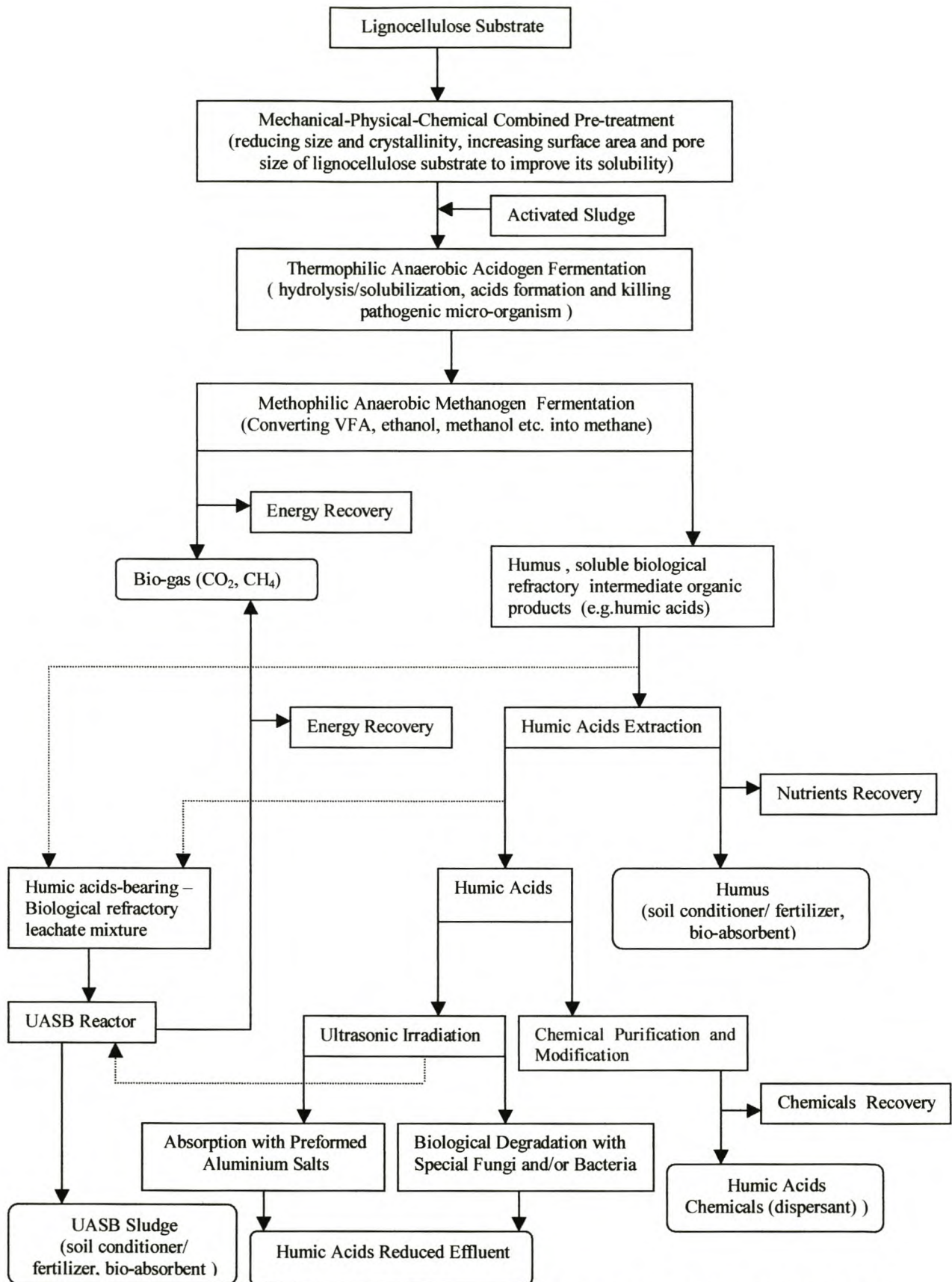


Fig.13.1 The utilization of lignocellulose wastes in the sustainable development ethos

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