Enhancing Anaerobic Degradation of Lipids in Wastewater by Addition of Co-substrate

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DECLARATION

I declare that the work in this thesis is my own account of my research and the contents has not been submitted for a degree at any university.

Yunhua Kuang

PUBLICATION


ABSTRACT

Anaerobic treatment systems are becoming increasingly popular to treat complex organic wastes that contain carbohydrates, proteins and lipids. Lipids are widely found in sewage and industrial wastewaters. Dairy, edible oil, fat refining, slaughterhouse, wool scouring, meat processing plants and grease-trap wastes from restaurants generate wastewater high in lipids. Although it is well known that lipids can be degraded by biological process, they have been reported to inhibit anaerobic processes by causing sludge flotation and wash-out. The inhibitory effect of lipids in anaerobic process has also been attributed to the long-chain fatty acids (LCFAs) which are the hydrolysed products of lipids. It has been shown that LCFA and lipids inhibit the formation of granular sludge in Upflow Anaerobic Sludge Blanket (UASB) reactors and that the adsorption of LCFAs on to the granules can result in its flotation and washout. It was also found that the degradation of LCFA was very poor.

Various techniques have been employed to enhance degradation of lipids and these include physico-chemical pre-treatment, application of two stage treatment employing new reactor designs like Expanded Granular Sludge Bed (EGSB).

This thesis investigated the influence of co-substrates, both in the form of hydrolysed products and polymeric form, on reducing the toxicity and enhancing the degradation of LCFA and lipids in a single stage and two stage upflow anaerobic sludge blanket (UASB) reactors. The investigations were carried out on both microbiological and physico-chemical aspects. A combination of techniques including the use of light...
microscopy (LM), confocal laser scanning microscopy (CLSM), transmission electron microscopy (SEM) and Fluorescent In Situ Hybridisation (FISH) was used to study the characteristics of microbial aggregates and to locate microbial populations within these aggregates. The microbial populations visualised using FISH techniques were Bacteria, Archaea, Methanobacteriaceae, Methanomicrobiales and Methanosarcinaceae. The performance of digesters was also monitored by measuring bulk parameters such as concentration of residual substrates, intermediate products (LCFAs, volatile fatty acids), methane (or gas) production rate and chemical oxygen demand of treated effluent.

Initially batch assays were carried out to determine the effects of glucose (hydrolysis product of carbohydrate) and cysteine (hydrolysis product of protein) on the toxicity of sodium oleate (hydrolysis product of lipid) to methanogenesis. The results showed that glucose and cysteine addition could reduce the toxicity of sodium oleate on the methanogenesis and enhance the degradation of sodium oleate. While the addition of glucose had a better effect than cysteine on decreasing the toxicity of sodium oleate, the combination of glucose and cysteine had the optimal result to stimulate the degradation of sodium oleate.

Secondly the effect of addition of glucose, cysteine and sodium oleate as co-substrates on the characteristics of granules in an LCFA fed single stage UASB were investigated. It was shown that the addition of glucose produced the best results on the formation of granules while both cysteine and sodium oleate adversely affected the granule formation. In a LCFA inhibited digester glucose and cysteine addition enhanced the recoveries of
different anaerobic microbial communities. Although the effects of glucose and cysteine on the various microbial groups were different, the combination of glucose and cysteine had the optimal results on recoveries of all bacterial groups.

The next half of the thesis investigated the influence of starch and yeast extract on the hydrolysis and degradation of canola oil by application of one and two stage UASB reactors. The results showed that the combined addition of protein and carbohydrate had an optimal effect on enhancing the hydrolysis of lipid compared to the addition of only protein or carbohydrate by promoting a balanced growth of the microbial groups. It was also demonstrated that a two-stage UASB reactor performed better in terms of extent of lipid hydrolysis and methanogenesis than a one-stage UASB reactor.
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<table>
<thead>
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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ABR</td>
<td>anaerobic baffled reactor</td>
</tr>
<tr>
<td>CLSM</td>
<td>confocal laser scanning microscopy</td>
</tr>
<tr>
<td>COD</td>
<td>chemical oxygen demand</td>
</tr>
<tr>
<td>CSTR</td>
<td>continuously stirred tank reactor</td>
</tr>
<tr>
<td>EGSB</td>
<td>expanded granular sludge bed reactor</td>
</tr>
<tr>
<td>ELISA</td>
<td>immunosorbent assay</td>
</tr>
<tr>
<td>FISH</td>
<td>fluorescent in-situ hybridization</td>
</tr>
<tr>
<td>GC</td>
<td>gas chromatography</td>
</tr>
<tr>
<td>HPLC</td>
<td>high performance liquid chromatography</td>
</tr>
<tr>
<td>LCFA(s)</td>
<td>long chain fatty acid(s)</td>
</tr>
<tr>
<td>HRT</td>
<td>hydraulic retention time</td>
</tr>
<tr>
<td>SO</td>
<td>sodium oleate</td>
</tr>
<tr>
<td>SEM</td>
<td>Scanning electron microscopy</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission electron microscopy</td>
</tr>
<tr>
<td>UASB</td>
<td>upflow anaerobic sludge blanket</td>
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<tr>
<td>VFAs</td>
<td>volatile fatty acids</td>
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Chapter 1 Introduction

1.1 General introduction

Lipids, which can be traditionally characterised as fats, greases and oils, are widely found in industrial and municipal wastewaters. Lipids in industrial wastewaters are mainly esters of straight-chain, even-numbered long chain fatty acids and linear polyols (triglycerides or phospholipids) and their hydrolysed products (Rinzema, 1988). Physicochemical techniques, such as gravity separation, flotation and emulsion, can effectively treat wastewaters containing lipids. However, even after these processing steps, considerable amount of emulsified or colloidal lipids is left in the wastewater (Rinzema, 1988). Thus, to completely remove the lipid remaining, further treatment is required. Biological techniques, with their low requirement for expensive chemicals or energy and with fewer harmful end products, have been increasingly used in recent years.

Biological treatment of lipids may be accomplished by aerobic or anaerobic digestion. Anaerobic digestion is the biological degradation of organics into carbon dioxide and methane, while aerobic digestion oxidises organics to carbon dioxide and water using oxygen. Anaerobic treatment has advantages over aerobic treatment in that there is less energy required and the end product (methane) can be used for energy production. Reactors can be easily restarted even after several months of shut down (Beccari et al., 1996). However, there are some disadvantages to this method when compared to aerobic digestion. Anaerobic treatment is more susceptible to failure from changes in reactor conditions, as well as from difficulties during reactor operation even under stable conditions (Fongastitkul et al., 1994). Other drawbacks to the use of anaerobic
digestion include a longer retention time and the comparative lack of development of lipid treatment processes (Rinzema, 1988). Furthermore, there is a lack of understanding of the identity and complexity of the microorganisms involved in this process due to the limitation of traditional culture-based methods.

With the traditional culture based methods, only a relatively small fraction of the microorganisms making up a natural community can generally be cultured, and these cultured microorganisms are also difficult to be identified. In recent years, group-specific 16S rRNA hybridization probes have been used to study the microbial populations in anaerobic digesters (Amann et al., 1990a; Raskin et al, 1994a; Stahl, et al. 1988). This technique is attractive due to the specificity of the probes for both cultured and uncultured microbial cells in natural complex microbial communities (Raskin et al., 1995).

In anaerobic digestion, following the hydrolysis of LCFAs from lipids, LCFAs are further degraded, mainly by β-oxidation, to acetate and hydrogen and finally converted to methane or carbon dioxide by methanogenesis (Komatsu et al., 1991). However, even low concentrations of LCFAs can be inhibitory to the process (Hanaki, 1981; Koster, 1986; Angelidaki et al., 1990). It was suggested that LCFAs are toxic to bacteria because of their damaging effect on the transport channel of bacterial cells, inducing the lysis of protoplasts (Galbraith, et al., 1971; Galbraith and Miller, 1973). Considerable work has hence been carried out on decreasing the toxicity of LCFAs on anaerobic microorganisms (Koster, 1986, 1987; Hwu, 1996; Rinzema et al., 1993). These studies were limited to the addition of chemicals and the improvement of reactor technologies and the performance was monitored by indirect parameters, such as
methane production rate, chemical oxygen demand (COD), biological oxygen demand (BOD) and volatile fatty acids (VFA). The effects of LCFAs directly on microbial groups have not been extensively investigated.

Furthermore, although the toxicity of LCFA on anaerobes is well defined, effective methods that are capable of decreasing the inhibition of LCFA are poorly documented. During anaerobic lipid digestion, many factors can affect the growth of microbial communities and interfere with this process. An important factor is the nutrient composition. Ahring et al. (1992) observed that supplementing co-digested lipid waste with other industrial waste enhanced the digestion process. Angelidaki and co-workers (1998) also investigated the effect of co-digestion of complex substrates by using mathematical modelling. Mousa (1998) reported that the presence of trace organics reduced the inhibition of LCFA. The complex microbial communities in this process were not identified. In depth research is clearly necessary to identify and understand the effect of various substrates on the shifts of microbial subpopulations which related to the activities of anaerobic digestion of lipids.

Research has also been conducted on the selection of the optimal pH, temperature, substrate components and loading rate for the anaerobic reactor without characterisation of the real structure of microbial community inside the reactor (Koster, 1987; O'Flaherty et al., 1997). The effect of altering a single variable of digester operation is difficult to ascertain by monitoring bulk parameters. Any effect observed will not be a direct result but one arising from the effect the change has, in conjunction with other factors, on the population dynamics of the digestor microbial community. The complex microbial subpopulations which are involved in the degradation of lipids
in anaerobic digestion are the key factors to maintaining the anaerobic mineralisation process. Knowledge of the presence of different subpopulations during LCFA and lipid anaerobic digestion is very limited due to the fact that traditional techniques are unable to completely identify and quantify the heterogeneous microbial community in anaerobic digestion. Methanogens represent one of the most difficult groups amongst this community to investigate with culture-based methods even though they constitute an important group in forming the final product.
Chapter 2 Literature review

2.1 Lipid anaerobic digestion process

Anaerobic digestion consists of a series of microbiological processes that convert organic compounds to methane in the absence of oxygen. A schema of these processes is displayed in figure 2.1:

![Pathway for the metabolism of organic compound in anaerobic digestion](image)

The overall reaction for this process can be described as:

$$\text{Organic materials} + \text{H}_2\text{O} \rightarrow \text{biomass} + \text{CH}_4 + \text{CO}_2 + \text{NH}_4^+$$
Anaerobic processes occur naturally in the environment, such as in ocean sediments, lakes, river, swamps, soils and the gastrointestinal tract of animals. As one of the oldest forms of biological wastewater treatment, anaerobic digestion has been used for more than a century by human civilisation. The great quantities of organic waste generated by urban and industrial activities and have resulted in a reawakening of interest in the anaerobic process as a means of disposal.

During anaerobic digestion, organic material is converted to biomass, methane and carbon dioxide. Nitrogen is released as ammonia and only very little of it is used for anaerobic bacterial growth. In this process, different kinds of microbial populations are involved in the degradation of organic compounds into methane and carbon dioxide, which include several consecutive and parallel reactions (Fig. 2.1).

In the first step, complex organic compounds (proteins, carbohydrates and lipids) are hydrolysed into simpler compounds by extracellular enzymes that are excreted by fermentative bacteria. Proteins are degraded via (poly)peptides then to amino acids using proteases; carbohydrates are converted to soluble sugars (saccharides) using cellulases; and lipids are transformed into long chain fatty acids (LCFAs) and glycerol using lipases. Pavlostathis and Giraldo-Gomez (1991) suggested that lipid hydrolysis is generally faster than protein or carbohydrate hydrolysis, and considered lipid hydrolysis to be a rapid process in anaerobic digestion (Hanaki, 1981; Angelidaki et al., 1995). Some researchers reported however, that lipid hydrolysis was the rate limiting step in digestion (Beccari et al., 1996). Other research indicated that lipid hydrolysis was not usually the rate-limiting step and the overall conversion rate was limited either by the degradation of LCFAs or by the physical
processes of dissolution and mass transfer of these acids (Rinzema et al., 1993; Hanaki et al., 1987). The apparent discrepancy between the results obtained by different researchers may be attributed to differences in feed concentration and physico-chemical conditions in their experiments.

As the first step of anaerobic digestion, hydrolysis is a very important factor. Although some work has been carried out in this area, the focus was primarily on the changes of influents and effluents with mathematical modelling. The most commonly applied model for lipid as well as carbohydrates and protein hydrolysis is first-order kinetics (Pavlostathis and Giraldo-Gomez, 1991). However, Two-phase and Contois were approximately equivalent and best simulated the hydrolysis data (Batstone, 1999). Rinzema (1993) suggested that higher biomass concentration was necessary for hydrolysis.

Little is known however, about the microbial communities involved in lipid anaerobic hydrolysis. After hydrolysis, LCFAs and glycerol undergo fermentation or acidogenesis. Because LCFA require an external electron acceptor for oxidation, degradation of LCFA happened during acetogenesis. While mainly saturation or hydrogenation of unsaturated LCFAs occurs in this process, glycerol is also mainly degraded to acetate, lactate and 1,3-propanediol (Batstone, 2000).

Syntrophic acetogenesis commences follows hydrolysis and fermentation. During this period, the products of acidogenesis are converted into substrates i.e. acetate, hydrogen and carbon
dioxide for methanogenesis. The reaction can be represented by the following equation (Haandel et al., 1994):

\[ \text{CXHyOz} + \frac{1}{4}(4x-y-2z)\text{H}_2\text{O} \rightarrow \frac{1}{8}(4x+y-2z)\text{CH}_3\text{COOH} + \frac{1}{4}(2z-y)\text{CO}_2 \quad \text{(when } y<2z \text{)} \]

Or

\[ \text{CXHyOz} + (x-y)\text{H}_2\text{O} \rightarrow \frac{x}{2}\text{CH}_3\text{COOH} + \frac{1}{2}(y-2z)\text{H}_2 \quad \text{(when } y>2z \text{)} \]

Normally all LCFA and ethanol require syntrophic acetogenesis before methanogenesis along two pathways. $\beta$-oxidation is the main pathway observed in LCFAs anaerobic digestion:

\[ \text{LCFAs} \xrightarrow{\text{activation}} \text{LCFA-CoA} \xrightarrow{\text{saturation}} \text{saturation LCFAs} \]

\[ \text{Palmityl-CoA} + \text{acetyl-CoA} \rightarrow \text{acetic acid} \]

$\omega$-oxidation is the another pathway to LCFA oxidation by carboxylation of the far methyl carbon.

The final step is methanogenesis which is usually the rate limiting step in the overall anaerobic process of soluble substrates. Methanogenesis consists of acetotrophic and hydrogenotrophic methanogenesis. In acetotrophic methanogenesis, methane is produced from acetate by acetotrophic bacteria, while in hydrogenotrophic methanogenesis, the hydrogenotrophic bacteria covert carbon dioxide and hydrogen into methane.
2.2 Microbiology of anaerobic digestion

The biological systems involved in anaerobic digestion have been studied for more than 80 years. Even since the first description of the degradation of cellulose by methane formation in 1906, microbiological studies of anaerobic bacteria have been proved to be difficult due to the complexity of the anaerobic metabolism (Hwu, 1997). Bryant (1972) and several other researchers used traditional laboratory techniques to isolate and study anaerobic bacteria. These researches showed that anaerobic ecosystems involved complex interactions between microorganisms. It was also found that different steps in the anaerobic process were dominated by different bacterial groups.

In the last 20 years, several biochemical and molecular methods have been developed to characterise the microbial communities involved of anaerobic digestion particularly in anaerobic reactors such as continuously stirred tank reactor (CSTR), anaerobic baffled reactor (ABR), upflow anaerobic sludge blanket reactor (UASB) and expanded granular sludge bed reactor (EGSB).

2.2.1 Molecular techniques

The molecular methods, that have been applied to anaerobic digestion were primarily immunotechniques and hybridisations with oligonucleotide probes targeting the small subunit ribosomal RNA.
Immunological tests for characterization of anaerobic bacteria were first introduced by Conway and Macario (1982) and Archer (1984). These techniques involved the use of polyclonal antibodies which were initially developed for use in defined cultures (Conway and Macario, 1982). This technique has not been developed further due to limitations such as the requirement for pure strains and time consuming production of antibodies. Nevertheless, the utilisation of the enzyme linked immunosorbent assay (ELISA) to methanogens (Archer, 1984; Sorensen et al., 1997) has proved to be highly reliable.

The use of oligonucleotide probes for characterization of anaerobic bacteria was first introduced by Amann and co-workers (Amann et al., 1990a; Amann et al., 1990b) and was further developed by Raskin and co-workers (Raskin et al., 1994a; Raskin et al., 1994b; Raskin et al., 1995). The method was demonstrated to be a highly specific and reliable tool in detection and identification studies. It has been widely applied in the characterization of methanogens, acetogens and sulfate reducing bacteria (SRB) (Amann et al., 1992; Kane et al., 1993; Santegoeds et al., 1999).

Phylogenetically defined groups of bacteria can be quantified and visualized by hybridisation of the $^{32}$P –and by hybridisation of fluorescent-dye-labelled oligonucleotide DNA probes to the 16S rRNA from samples respectively. With this technique, the anaerobes could be studied in their natural environment. The limitation of traditional culture-based techniques, in which only a small fraction of microorganism can be cultured due to the limitation of condition is thus avoided. These microbes that could be cultured were often difficult to quantify and identify. However, the use of fluorescent dye or enzyme-conjugated probes for hybridisation
with specific RNA in whole cells provided clear visualisation of single cells in the natural population. When this technique was used in conjunction with PCR, cloning, and comparative sequencing of the cloned DNA, identification and quantification of uncultured or undescribed strains was also possible. An alternative quantification method for using a $^{32}$P-labelled probe hybridization was shown to involve extensive work, such as RNA extractions, slot-blot hybridisations and data analyses. Although the results of this method were reliable, precision and detectability should be checked when examining species present in low numbers in the natural environment (Raskin et al., 1996).

The oligonucleotide approach is more attractive than immuno-methods because oligonucleotide probes can be designed from DNA or RNA sequences obtained directly from environmental samples and then synthesised on the DNA synthesizer. The production of antibodies required for immuno-methods involve more labour and the use of animal hosts. Furthermore, immuno-methods require the isolation of an immunising strain from a defined culture. The antibodies raised in animals could be to a specific strain, and might not be similar to the majority of this kind of organisms in the environment, and thus they could not detect most of the organisms. These limitations reduce the general applicability immuno-methods in microbial environmental studies.

2.2.2 Taxonomy of anaerobic bacteria

Classification of anaerobic bacteria can be described by two systems (Fig. 2.2). The first system relies on morphological and physico-chemical characteristics, such as the five-kingdom phylogeny system from a classical taxonomy, in which anaerobes are in both
kingdoms of the Prokaryotes - the *Archaea* and the *Bacteria* (Woese *et al*., 1990). Fungi, Animals and Plants are another three kingdoms that belong to Eucaryotes. The *Archaea* include the methanogens and a few thermophilic sulphur-metabolises (Mah *et al*., 1986) while the other obligate anaerobes are *Bacteria* (Table 2.1). The other classification is based upon molecular structure and 16S rRNA sequences (Woese *et al*., 1990) and all living things were included in three domains. Using this method, it was found that anaerobes were present in the *Bacteria* and *Archaea* domains. This latter form of classification has been widely used in recent anaerobic research (Zheng *et al*., 1996; Sekiguchi *et al*., 1999). It appears to provide a more specific system in the highest levels of natural classification. Although some fungi and protozoa may be involved in anaerobic reactions, anaerobic bacteria are the dominant microorganisms in anaerobic digesters.

Fermentative bacteria, acidogenic bacteria, acetogenic bacteria and methanogenic archaeba are the four main bacteria groups that are involved in reactions and operate synergistically. Sulfate-reducing bacteria however, can also act either as acetogenic bacteria or as bacteria that compete for nutrients with methanogenic bacteria (Raskin *et al*., 1996).

Fermentative bacteria are responsible for the two first stages of anaerobic digestion (hydrolysis and acidogenesis). Fermentative bacteria, also called hydrolytic bacteria, break down complex organic molecules into soluble monomer molecules. The function of fermentative bacteria in the anaerobic process depends on both the initial substrate and the environmental conditions (Novaes, 1986).
Fig. 2.2. Tree of life and the situation of three domain (Adopted from Norman, 1997).
ARCHAEA (arkeootit; archbacterians, archeans)

|-- KORARCHAEOTA
|--o CRENARCHAEOTA
|   |--+-- Thermofilum
|   |   `--+-- Pyrobaculum
|   |   `-- Thermoproteus
|   `--+-- Sulfolobus
|       `--+-- Metallosphaera
|       `-- Acidianus
|       `--+-- Pyrolobus
|           `--+-- Hyperthermus
|           |   `-- Pyrodictium
|           `--+-- Thermodiscus
|               `-- Ignecoccus
|               `-- Staphyothermus
|               `--+-- Desulfiurococcus
|               `-- Thermosphaera

|--o EURYARCHAEOTA
|   -- Thermococcales
|   -- Thermoplasmales
|   -- Archeoglobales
|   -- Methanopyrales
|   -- Methanococcales
|   -- Methanobacteriales
|   `--+-- Methanomicrobiales
|   `-- Halobacteriales

Table 2.1 The classification of Archaea (2003) (http://www.palaeos.com/Kingdoms/Prokaryotes/Archaea.htm)
2.2.2.1 Fermentative bacteria

When fermentative bacteria are involved in hydrolysis, different extracellular enzymes such as cellulase, proteases and lipases are secreted (Batstone, 2000). These are released from the cell membrane and break down the organic materials outside the cell. Enzyme secreting rates is usually assumed to be related to microbial growth rate and as well as environmental factors. The mechanism of the reaction between enzymes and substrates is still not very well understood.

Although fermentative bacteria are very important in anaerobic digestion, the conditions required for their growth, as well as cell metabolism and ecology are not completely understood. Toerien and Hattingh (1969) reported that species belonging to the genera of \textit{Bacteroides}, \textit{Clostridium}, \textit{Butyrivibrio}, \textit{Eubacterium}, \textit{Bifidobacterium} and \textit{Lactobacillus} are dominantly found in the hydrolysis process. Of these, \textit{Bacteroides} and \textit{Butyrivibrio} are Gram-negative bacteria, while \textit{Clostridium}, \textit{Eubacterium}, \textit{Bifidobacterium} and \textit{Lactobacillus} are Gram-positive.

Acidogenesis takes place intracellularly where the soluble protein, sugars and LCFA are degraded to short-chain organic acids and alcohols. Lipases convert lipids to long-chain fatty acids. A population density of $10^3$ - $10^5$ lipolytic bacteria per ml of digester fluid has been reported (Zeikus, 1980). Clostridia and the micrococci appear to be responsible for most of the extracellular lipase production. Although the technological aspects of acidogenesis
have received much attention, little is known about the microbial diversity and community structure required for it to occur (Britz et al., 1994).

Britz et al. (1994) isolated 288 acidogenesis strains from the liquid phase of four anaerobic digesters, which were fed with different substrates and kept in different physico-chemical conditions. *Clostridium bifermentans* was the predominant species in four digesters, while *Bacillus, Citrobacter, Enterobacter, Fusobacterium, Pseudomonas, Klebsiella* and *Staphylococcus* were the most numerous species in the other digesters.

So far, the research on fermentative bacteria has been conducted using traditional microbiological approaches. Although both are known as fermentative bacteria, distinctions and subsequent classifications between the hydrolytic and acidogenic bacterial groups are still not clear. The mechanisms used by these bacteria are even less understood. There would hence appear to be a need for the application of molecular techniques to study these bacteria in greater detail. Van Dyke and McCarthy (2002) had applied molecular techniques to check *Clostridium* populations.

2.2.2.2 Acetogenic bacteria

Acetogenic bacteria can be divided into hydrogen producing acetogenic bacteria and homoacetogenic bacteria. Their main function in anaerobic digestion is to convert fatty acids and alcohols into acetate, hydrogen, and carbon dioxide. These are subsequently used by methanogens for their metabolism.
Enhancing Anaerobic Degradation of Lipids in Wastewater by Addition of Substrate - Y. Kuang  
Chapter 2: Literature Review  

Hydrogen producing acetogenic bacteria are essential for the anaerobic digestion because of their action in catabolizing propionate and other organic acids into acetate and hydrogen. A few species have been isolated and some information is known about their main nutritional requirements (Bryant et al., 1967; Boone et al., 1980; McInerney et al., 1981).

The homoacetogenic bacteria are also important in anaerobic digestion and are characterized as chemolithotrophic, H2 and CO2 users. It was reported by Bryant et al. (1967) that methanogenic H2 consuming bacteria and an acetogenic bacteria called “organism S” had a syntrophic association. The methanogenic H2 consuming bacteria used the H2 released from the organism, with the maintenance of low levels of H2 as a result. Boone and Bryant (1980) reported a similar action between acetogenic bacteria Syntrophobacter wolinii and H2 – using bacterium Desulfovibrio in which propionate was degraded into acetate, CO2 and H2. McInerney et al. (1981) showed that the rumen bacteria Syntrophomonas wolfei and rumen methanogens formed a structural and metabolic association enabling them to β-oxidize fatty acids to acetate and H2 or acetate, propionate and H2.

Although acetogenic bacteria play an important role in LCFA degradation, only four syntrophic acetogens have been described so far (Roy et al., 1986; Lorowitz et al., 1989; Angelidaki et al., 1995; Svetlitshnyi et al., 1996). Of these four syntrophic acetogens, Syntrophomonas sapovorans and Syntrophomonas wolfei sub.sp. saponavida were mesophilic, while Thermosyntropha lipolytica and a short rod thermophile were thermophilic.
With traditional culture methods, the syntrophic partners of *Syntrophomonas sapovorans* and *Syntrophomonas wolfei* sub.sp. *saponavida* have been isolated. These microbes were identified as *Methanospirillum hungatei* and *Desulfovibrio* sp. respectively. *Methanobacterium* sp was identified as the syntrophic partner of *Thermosyntropha lipolytica* while *Methanobacterium thermoautotrophicum* was the syntrophic partner of the short rod thermophile.

Current developments in biological science using molecular approaches have widened the scope for studies on the quantification and identification of the natural ecology of acetogenic bacteria (Hansen *et al.*, 1999; Zhao *et al.*, 1993). Hansen *et al.* (1999) used new oligonucleotide probes to characterize the phylogenetic groups of mesophilic members of the family *Syntrophomonadaceae* as well as their syntrophic relationship with the hydrogenotrophic methanogens.

2.2.2.3 *Archaea*

Compared to the other three groups described, methanogenic bacteria have been well studied. The methanogenic group is composed of both gram-positive and gram-negative bacteria with a wide variety of shapes. A number of specific characteristics are present that make this group quite different from other bacteria and cause their inclusion in the domain, *Archaebacteria* (Woese *et al.*, 1990). Methanogens do not have murein in their cell walls and are related to the most primitive microorganism groups while it is common in *Bacteria.*
In 1936, Barker initially classified methanogens into only one family, the *Methanobacteriaceae*, which consisted of four genera: *Methanobacterium*, *Methanosarcina*, *Methanococcus*, and *Methanospirillum*. Based on the 16S rRNA structure, Balch et al. (1979) proposed a new taxonomic classification (table 2.2).

<table>
<thead>
<tr>
<th>ORDER</th>
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<th>GENUS</th>
</tr>
</thead>
<tbody>
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<td>Methanohalophilus</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Methanaeta</td>
</tr>
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Table 2.2 Classification of methanogens
Methanogens grow slowly in wastewater and their generation times range from 3 days at 35°C to as high as 50 days at 10°C. There are two main subcategories, hydrogenotrophic methanogens and acetotrophic methanogens. Hydrogenotrophic methanogens convert hydrogen and carbon dioxide into methane.

$$\text{CO}_2 + 4\text{H}_4 \rightarrow \text{CH}_4 + 2\text{H}_2\text{O}$$

Although it was originally considered that hydrogenotrophic methanogens only used hydrogen as the electron carrier, evidence has been presented that in some cases formate can act as an electron carrier (Boone et al., 1989).

Acetotrophic methanogens convert acetate into CH₄ and CO₂. It is this step that is thought to be the bottle-neck of the whole anaerobic digestion process. This is because the acetotrophic methanogens are slow-growing and do not adapt well to environmental changes.

$$\text{CH}_3\text{COOH} \rightarrow \text{CH}_4 + \text{CO}_2$$

*Methanosarcina* and *Methanothrix/Methanosaeta* are the only two genera that have been confirmed so far to use acetate.

About two thirds of the methane produced is derived from the conversion by acetotrophic methanogens. The other third is the result of carbon dioxide reduction by hydrogenotrophic methanogens.
Species differ in the range of environmental conditions within which they can compete advantageously. Methanogens can develop in an environment where the redox potential is approximately -300 mV with a temperature range of 15-40°C. A few species such as *Methanobacterium thermoautotrophicum* can grow above 60°C. The optimal pH for methanogens is between 6.5-8.0.

While the physico-chemical and biochemical mechanisms of methanogens have been widely studied, the ecology of microbial populations and communities in natural anaerobic digesters remains largely unexplored (Raskin *et al.*, 1994a). The recent development of molecular techniques will allow appropriate new methods to be applied to the research of methanogens.

### 2.2.2.4 Sulfate reducing bacteria

Sulfate reducing bacteria (SRB) are widely found in anaerobic digesters coexisting with methanogens and acetogens. SRB produce acetate, H₂ and sulfide (which are used by methanogens) and will sometimes compete for intermediates, such as hydrogen, formate, acetate, propionate and butyrate with methanogens or acetogens (Isa *et al.*, 1986). The activity of SRB is very complicated. The syntrophic and competitive interactions of SRB with methanogens and acetogens have been widely studied although some aspects remain unclear.
The genera of SRB have been defined on the basis of morphology rather than on physiological grounds (Table 2.3). They include *Desulfovibrio*, *Desulfotomaculum* and *Desulfobulbus* which are those principally found in anaerobic digesters (Amann *et al.*, 1992; Kane *et al.*, 1993; Santegoeds *et al.*, 1999). The results of molecular research of these genera have provided some valuable additional information on SRB. Kane *et al.* (1993) used oligonucleotide probes and polymerase chain reaction (PCR) to isolate and identify the SRB species successfully. Using similar techniques, Amann *et al.* (1992) suggested that the SRB were involved in developing and establishing biofilms. Using molecular methods and microsensors, Santegoeds *et al.* (1999) studied the relation of SRB and methanogens in anaerobic aggregates. Devereux and his colleagues (1992) designed genus- and group-specific hybridisation probes for SRB. These probes were also used by Raskin (1996) and co-workers in their study of the competition and coexistence of SRB and methanogenic populations in anaerobic biofilms. The results of their work showed that 15% of SRB were present in the methanogenic reactor even in presence of sulfate and SRB might functioned as proton-reducing acetogens and/or fermenters. The increase of sulfate in the anaerobic digester could significantly increase the SRB population level and decreased the methanogen population level in the reactor, indicating that the addition of sulfate benefited the competition of SRB with methanogens. However, they also found that some SRB (*Desulfococcus/Desulfosarcina/Desulfobotulus* groups) were more competitive without sulfate.
Enhancing Anaerobic Degradation of Lipids in Wastewater by Addition of Substrate - Y. Kuang
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<table>
<thead>
<tr>
<th>Genus</th>
<th>Morphology</th>
<th>Motility</th>
<th>flagella</th>
<th>Spores</th>
</tr>
</thead>
<tbody>
<tr>
<td>Desulfovibrio</td>
<td>vibrios</td>
<td>+</td>
<td>single, polar</td>
<td>-</td>
</tr>
<tr>
<td>Desulfotomaculum</td>
<td>rods</td>
<td>+</td>
<td>peritrichous</td>
<td>+</td>
</tr>
<tr>
<td>Desulfobacter</td>
<td>rods/cocco-bacilli</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Desulfococcus</td>
<td>cocci</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Desulfosarcina</td>
<td>irregula, in packets</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Desulfobulbus</td>
<td>citron-shaped cocci</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Desulfonema</td>
<td>filaments</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 2.3 Traditional classification of SRB (Levett, 1990).

2.2.3 Anaerobic bacteria and granulation

Microbial aggregation in anaerobic reactors is essential in biological wastewater treatment. Aggregation significantly enhances the settle ability of biomass and subsequent bacterial retention in the reactor. Granulation occurs due to microbial immobilisation which leads to aggregation and growth in anaerobic reactors. It has been reported that syntrophic bacteria and Methanoseta spp. play an important role in the anaerobic granulation process (El-mamouni et al., 1997). The species Methanoseta spp. (former Methothix) and Methanosarcina spp. have been identified as the main methanogens in granulation (Schmidt et al., 1996).
Granules are usually spherical with a diameter ranging from 0.14 to 5 mm (Schmidt et al., 1996). Granules are typically developed from inorganic components and extracellular polymers (ECP) produced by bacteria. The inorganic minerals content in granules was found to range from 10 to 90% of the granular dry weight while the ECP was between 0.6 to 20% in volatile suspended solids (Schmidt et al., 1996). From microscopic observations, it would appear that bacterial ECP were more important than inorganic components in the structure and maintenance of granules. Investigation of the granulation also showed that methanogens were more important than acidogens and acetogens in the granulation process.

Many theories regarding anaerobic granulation have been proposed (Macleod et al., 1990; Fang et al., 1994a). Macleod and Costerton (1990) suggested that the structure of bacterial aggregates in UASB granules was composed of three layers: an exterior layer of acidogens and H₂ consuming organisms, a middle layer dominated by H₂ producing acetogens and H₂ consuming organisms, and a third layer consisting of Methanosaeta - like species and large cavities. There were conflicting observations however, on the different substrates forming the granular bacterial structure. Fang et al. (1994b) suggested that the typical three layered granule was limited to full-scale anaerobic reactors fed with soluble carbohydrates. No layers were evident in the granules from a reactor degrading non carbohydrate substrates.

High rate organic waste treatment anaerobic reactors are more likely to fail due to the presence of lipids and LCFA inhibiting the growth of acidogens, acetogens and methanogens as well as the wash-out of the sludge. Although lipids were shown to enhance biogas production significantly (Angelidaki et al., 1990), the high potency of this substrate
could easily lead to an overloading of the reactor. Furthermore, a long adaptation period for microbial communities in the reactor would be required. Achieving granulation in anaerobic reactors treating lipids and LCFAs has proven difficult. Some work has been undertaken in this field but focussed mainly on the engineering and physico-chemical aspect of the process. Little study has been conducted on the effect of substrates on granulation. Sam-soon et al. (1991) observed that granulation did not take place when oleate, a typical LCFA monomer of lipids, was used as the sole substrate for a UASB system. The result from Batstone (2000) demonstrated that the microbial “layers” were not correct.

2.2.4 Physicochemical factors affecting anaerobic bacteria

Considerable research has been conducted on the many environmental physico-chemical factors related to the growth of anaerobic microorganisms. Physical factors such as temperature, sludge retention time (SRT), hydraulic retention time (HRT), loading rate and reactor design has been widely investigated. Anaerobic digestion has been shown to operate under two temperature ranges: mesophilic conditions (25-40ºC) and thermophilic conditions (> 45ºC). Zinder et al. (1984) reported that the production of methane increased with the temperature increase. When the temperature increased to 70ºC, methane production decreased. Other methanogens and bacterial groups were shown to differ in optimum growth temperatures (Angelidaki et al., 1995; Hwu, 1997). Differences in pH for optimum growth were also apparent. Methanogens required pH values in the neutral range, 6.6-7.6 while the optimum pH for acidogens was 5.5-5.9 (McCarty and Mosey, 1991; Toerien and Hattingh, 1969). Because of the different temperature and pH requirements of acidogens and
methanogens, a two-phase reactor was designed for separating acidogenesis and methanogenesis.

Chemical factors, organic nutrients such as lipids, hydrocarbon and protein, and other trace metals are very important in anaerobic digestion. The knowledge of the nutrient demands of anaerobic bacteria is poor. However, most research on nutrients in anaerobic digestion focused on nutrient removal during the process. The nutrient requirement for the growth of anaerobic bacteria has not been extensively examined. Nutrients are a key factor in anaerobic degradation due to their important function for bacterial growth. Although it can be used as an important energy source if operated successfully, high concentrations of organic matter can result in the reactor inhibition (Becarri et al., 1996). Due to their high energy content, lipids and associated products of hydrolysis, LCFA are also an attractive potential substrate. However, lipids are very difficult to hydrolyse and can result in the flotation and washout of granular sludge and biomass, while LCFAs inhibit anaerobic microorganisms even at low concentrations (Rinzema, 1988; Hwu, 1997).

Most of the research on the physico-chemical factors affecting on anaerobic digestion has been observed using traditional indicators such as volatile fatty acids (VFA), methane production, specific methanogenic activity (SMA), F420 and ATP (Tenorio, 1995). The effects of microbial population structure on the anaerobic digestion are not fully known. The lack of microbial studies on the anaerobic digestion of lipid is due to the limitation of traditional techniques, such as selective enrichment and pure-culture isolation. Natural microbial ecosystems are very difficult to identify and quantify using these techniques.
(Raskin et al., 1994). As discussed in section 2.2.1, the use of molecular techniques may hence aid in the investigation of anaerobic microbial growth under variable conditions.

2.3 The inhibition of LCFAs on anaerobic microorganism

With the increasing world population and rising living standards, disposal of plant oily waste has become increasingly an issue. Industrial wastewater and domestic sewage have different concentrations of lipids ranging from 20% to 65% (Quemeneur et al., 1994; Broughton et al., 1998). Olive mill and palm mill wastewater, domestic kitchen wastewater and other plant oily waste are the consequences of increasing populations. The chief constituents of plant oil are long chain acids of varied colour. Because of the close similarities between triglyceride molecules found in different oils, the densities and viscosities of most oils lie within a narrow band of higher viscosity and higher density oil.

Olive oil is a pale yellow or greenish-yellow liquid consisting primarily of oleic acid, palmitic acid and linoleic acid. Palm oil is a yellow-brown, soft and buttery solid at room temperature and is made up of triglycerides of oleic, palmitic and lauric acid.

In some olive growing countries of the Mediterranean area (Greece, Italy, Lebanon, Portugal, Spain, Syria, Tunisia and Turkey), olive oil mill effluent production was reported as more than 30 million m$^3$ per year (Beccari et al., 1996). There are many difficulties involved in the treatment of this product. Anaerobic digestion is being increasingly used to treat these effluent products due to the process producing considerably less waste sludge.
than aerobic processes as well as energy savings. Moreover, anaerobic digestion could easily be restarted after several months of shut-down (Beccari et al., 1996). Biodegradation of palm oil effluent and organic oil and fats from restaurants and other commercial kitchens by anaerobic digestion to environmentally acceptable products has been reported (Ibrahim et al., 1984; Stoll and Gupta, 1997).

Although animal fat waste is not as toxic as petroleum, the large quantities of animal oily waste produced daily (such as wool scouring effluent and the fat waste of the meat industry), constitute a problem for adequate disposal. There is currently no effective method available to treat this waste. Animal fat is generally a dark brown and fixed oil with a distinct odour. Its chief constituents are hydrocarbons, pyridine bases and amines. Wool grease is comprised of both oxidised and unoxidised grease, both of which have a melting point of around 40 °C. After refining, wool grease (called lanolin) forms a highly complex mixture of lipids with high molecular weight. Some batch experiments performed on the anaerobic digestion of wool wax and sheep tallow showed potential on wax hydrolysis and degradation (Broughton et al., 1998; Gutierrez, 1999).

Treatment of wool scouring effluent by anaerobic bioflocculation (Lapsirikul et al., 1993) indicated potential benefits. Research into the use of anaerobic digestion for petroleum waste treatment (Kleerebezem et al., 1997) and anaerobic digestion of milk-fat waste (Petruy and Lettinga, 1997), manure waste (Ahring et al., 1992), slaughterhouse waste (Salminen et al., 2000) and cheese whey waste (Yilmazer Yenigun, 1999) have been trailed with good results. In the past twenty years, with the development anaerobic digestion, more
emphasis has been placed on LCFA degradation due to lipids and LCFAs forming a major part in domestic and industrial wastewater.

The effects of LCFAs on anaerobic microorganisms were first observed in rumen bacteria (Demeyer and Hendrickx, 1967; Viviani, 1970) the focus being on digestive and nutritional problems. Their effect on rumen bacteria was initially examined using poorly defined mixed rumen culture and/or involved the use of in vivo experiments in which the rumen bacteria could not be monitored. Galbraith et al. (1973) found that low concentrations of LCFAs could induce lysis of bacterial cell protoplasts at pH 7.4. A few studies involved in vitro studies in which pure methanogen groups were used. It was difficult however, to show the real inhibition of LCFAs on anaerobic digestion using this method, furthermore, the toxicity of LCFA was also not investigated for each anaerobic microbial group.

Hwu (1997) reported that LCFA toxicity varies with the type of anaerobic sludge and is more correlated to their physical characteristics, specific surface area and size distribution. He also suggested that oleic acid toxicity was higher in the flocculent sludge than in the granular sludge under the three tested temperatures of 30, 40 and 55°C. This was due to a bigger specific surface area being available for LCFA absorption in flocculent sludge than in granular sludge with a subsequently higher potential for damage of anaerobic bacteria. Rinzema (1988) and Koster and Cramer (1987) also reported that inhibition of LCFA was mainly related to its concentration and that the ratio of LCFA and biomass was less important. Most of the research of lipid anaerobic degradation involved chemical
engineering, mathematical modelling and reactor designing (Lettinga et al., 1997). The detailed microbiological studies of this field were limited to the traditional culture-based techniques, which could not adequately monitor the complex anaerobic microbial communities present (Raskin et al., 1994b).

The inhibition of anaerobic digestion by LCFAs was investigated primarily in the past as its effect on methanogens using the measurement of parameters such as VFA, methane volume and difference of influent or effluent with these parameters (Koster and Cramer, 1986; Hanaki, 1981). Methanogenesis was inhibited by concentrations higher than a specific concentration of LCFAs. There was however, a slight stimulation of methanogenesis when the concentration was lower than this concentration (Hanaki et al., 1983). Numerous interactions of complex metabolic groups have been observed to occur during anaerobic digestion of LCFAs, such as saturation of unsaturated LCFAs (Hanaki et al., 1983), the possible pathway for specific LCFA (Weng and Jeris, 1976). Little is known about subpopulation shifts under LCFA inhibition. Study of anaerobic microorganisms during LCFA digestion is therefore necessary.

2.3.1 The physicochemical characteristic of LCFAs

Fatty acids normally contain even numbers of carbon atoms which are from 14 to 24 in biological systems. Fatty acids with 12 carbon atoms or above are considered to be LCFAs. LCFAs have varying chain lengths and degrees of saturation (Table 2.4). Moreover, different types of raw material will vary in their main constituents (Table 2.5). This results in a range of LCFA ratios occurring in wastewater, depending upon the origin of the waste.
However, oleic (cis-9-octadecenoic) acid and palmitic (hexadecanoic) acid are generally the most abundant unsaturated and saturated LCFAs present in wastewater.

Triacylglycerols are the most abundant lipids and can be hydrolysed to fatty acids and glycerol as shown:

\[
\begin{align*}
\text{Triacylglycerol} & \\
\text{Hydrolysis} & \\
\text{Glycerol} & \quad \text{Fatty acids}
\end{align*}
\]

Fig. 2.3 Pathway of triacylglycerols hydrolysis. \(R_1, R_2, R_3\) present alkyl groups in the equation. Approximately 93-96% of COD in lipids is conserved in LCFAs from the hydrolysis of lipids.
<table>
<thead>
<tr>
<th>Common name</th>
<th>Systematic name</th>
<th>Carbon numbers</th>
<th>Double bond number</th>
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</tr>
</thead>
<tbody>
<tr>
<td>Lauric</td>
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<td>12</td>
<td>0</td>
<td>(\text{CH}_3(\text{CH}_2)_n\text{COOH})</td>
</tr>
<tr>
<td>Myristic</td>
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<td>0</td>
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</tr>
<tr>
<td>Palmitic</td>
<td>Hexadecanoic</td>
<td>16</td>
<td>0</td>
<td>(\text{CH}_3(\text{CH}<em>2)</em>{14}\text{COOH})</td>
</tr>
<tr>
<td>Palmitoleic</td>
<td>cis-9- Hexadecenoic</td>
<td>16</td>
<td>1</td>
<td>(\text{CH}_3(\text{CH}<em>2)</em>{16}\text{CH} = \text{CH}(\text{CH}<em>2)</em>{2}\text{COOH})</td>
</tr>
<tr>
<td>Stearic</td>
<td>Octadecanoic</td>
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<td>0</td>
<td>(\text{CH}_3(\text{CH}<em>2)</em>{18}\text{COOH})</td>
</tr>
<tr>
<td>Oleic</td>
<td>cis-9- Octadecenoic</td>
<td>18</td>
<td>1</td>
<td>(\text{CH}_3(\text{CH}<em>2)</em>{12}\text{CH} = \text{CH}(\text{CH}<em>2)</em>{2}\text{COOH})</td>
</tr>
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<td>2</td>
<td>(\text{CH}_3(\text{CH}<em>2)</em>{14}\text{CH} = \text{CHCH}_2\text{CH}(\text{CH}<em>2)</em>{2}\text{COOH})</td>
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<tr>
<td>Linolenic</td>
<td>cis-9,12,15-Octadecatrie</td>
<td>18</td>
<td>3</td>
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<td>0</td>
<td>(\text{CH}_3(\text{CH}<em>2)</em>{24}\text{COOH})</td>
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</tbody>
</table>

Table 2.4 Different LCFAs in lipids (Hwu, 1998)
Table 2.5  LCFA composition in raw material and wastewater (weight percentage)  (Rinzema, 1988).

Although it is normally considered that LCFAs are degraded via β-oxidation both under mesophilic and thermophilic conditions, ω-oxidation is also thought to be involved in LCFA degradation (Jeris and McCarty, 1965). The pathway can be described as shown in Figure 2.4  (Hwu, 1997):
Released during the hydrolysis of lipids, LCFAs can be degraded to acetate and hydrogen via $\beta$-oxidation. During $\beta$-oxidation, unsaturated and saturated LCFAs are both activated by the acyl-CoA synthetase present in the cytoplasmic membrane of cells. LCFAs enter into the microbial cells before $\beta$-oxidation degradation starts (Fig.2.5). The transport of LCFAs into the cytoplasm is a complex process (Ratledge, 1994). Several hypotheses based on the study of the membrane-associated transport of *E. coli* (Nunn, 1986) were proposed. Nunn (1986) suggested that LCFAs were adsorbed onto the outer membrane receptor or pore, then were transferred across the cytoplasmic membrane to bind to acyl-CoA synthetase (Fig.2.5). The molecules were then released into the cell after activation by acyl-CoA synthetase.

Saturated long-chain acyl-CoA then undergoes $\beta$-oxidation. Unsaturated molecules of long-chain acyl-CoA require saturation before entering $\beta$-oxidation. During the $\beta$-oxidation cycle, the long-chain acyl-CoA is broken down into a two-carbon fatty acetyl-CoA which can be released as acetic acid without any further activation. The remaining shortened long-chain acyl-CoA remains as substrate in the $\beta$-oxidation cycle until all the carbons are broken down to acetyl-CoA, and finally to acetic acid.
Fig. 2.4 The pathway of LCFA degradation in β-oxidation. Adapted from Hwu (1997).

Fig. 2.5 Model of LCFA transformation (Nunn, 1986). FLP: a possible receptor; OM: outer membrane; PG: peptidoglycan; PS: periplasmic space; IM: inner membrane.
Acyl-CoA synthetase is hence important in oxidative degradation, both in LCFA transfer and in β-oxidation. Furthermore, the enzyme appeared to effectively detoxify the fatty acids as the CoA ester was not inhibitory.

2.3.2 The toxicity of LCFA on anaerobic groups

LCFAs are well known inhibitors to various anaerobic microorganisms at millimolar concentrations (Rinzema et al., 1994). It was suggested that the toxicity of LCFAs to bacteria was a result of the absorption of LCFAs by cell walls or membranes, leading to the damage of transport systems or protective functions (Galbraith and Miller, 1973). In this theory, it was thought that areas covered with LCFAs had altered permeability, resulting in the lysis of protoplasts (Galbraith et al., 1971). This assumption was also demonstrated by an experiment in which unsaturated LCFAs (ULCFA) had a higher inhibition level than saturated LCFAs (SLCFA) (Koster and Crimer, 1986; Thies et al., 1994). Due to a bent molecular shape arising from double bonds, unsaturated LCFAs have a greater cover area of cell wall per molecule than saturated LCFAs. Increasing the number of double bonds in LCFAs was shown to increase inhibition in previous studies (Demeyer and Henderrickx 1967). Furthermore, the saturation degree (ratio of SLCFA / ULCFA) was reported as the key factor governing membrane fluidity. Hwu (1997) indicated that microorganisms such as thermophilic methanogens with cell membranes composed of a high proportion of saturated LCFA, were more susceptible to LCFA toxicity than mesophilic methanogens, whose cell membranes contained more unsaturated LCFA (ULCFA). It was also reported (Thies et al., 1994) that the addition of the detergent Tween 80 (mainly comprised of oleic acid) resulted
in a change in the fatty acid composition of the thermophilic cell membrane to higher levels of ULCFA with a subsequent fall in SLCFA. Kodicek and Worden (1945) found that the site of action for toxicity of LCFAs was the wall of the bacterial cell with the effects of unsaturated fatty acids rising with increasing unsaturation in Gram-positive bacteria. It was also found that Gram-positive bacteria were more sensitive to the inhibition of LCFAs than were gram-negative species (Nieman, 1954). However, the actual mechanism of LCFA toxicity is not known.

It was observed that addition of CaCl₂ and other soluble calcium salts reduced the inhibition of LCFAs due to precipitation of the salts with the LCFAs (Hanaki et al., 1983). Angelidaki et al. (1990) found that the toxicity of LCFAS could be decreased by the addition of bentonite due to its capacity to flocculate LCFAs. However, these chemical and clay materials were also toxic to anaerobic bacteria and/or were difficult to degrade.

Mousa and Forster (1998, 1999) found that some growth factors, such as glucose and glycine behaved antagonistically with the inhibitory effects of gallic acid. Hwu (1997) reported that using glucose at 35% in total COD weight enhanced the degradation of sodium oleate. These results indicated that addition of the growth factor or other organic substrates during LCFA degradation might decrease the toxicity of LCFA. However, the effects of addition of LCFA on the growth of the microbial communities are poorly studied. During anaerobic digestion, complex microbial communities are involved in the degradation process. The nutrient requirements for specialized bacterial groups are different.
Fermentative bacteria can hydrolyse organic compounds (protein, lipid and carbohydrates), while syntrophic acetogens and methanogens degrade the fermentation products to methane and carbon dioxide. The difference of composition of the nutrient content may affect the growth of specific bacteria groups. Previous research reported that glucose and cysteine were necessary to the growth of anaerobic bacteria (Balch et al., 1979), while the detailed nutritional requirements during lipid treatment and the nutrient composition balance needed during this process is still not known. Thus, a thorough investigation of the nutritional requirements of anaerobes during lipid treatment using physico-chemical techniques combined with molecular techniques will be of benefit to wastewater treatment industries.

2.3.3 The adaptation of methanogens to the inhibition of LCFA

It was reported that during the start-up of an anaerobic treatment system of lipid containing wastewater, a lag phase in degradation of LCFAs would occur (Rinzema, 1988; Hwu, 1997). This appeared to be an adaptation phase due to an inhibition of LCFA degradation while acetogenic bacteria capable of LCFA degradation grew in number. Reactors with lower concentrations of LCFA feed normally exhibited faster adaptation periods than those fed with higher concentrations (Rinzema, 1988; Hwu, 1997). This indicated that a higher concentration of LCFA had a greater toxic effect. Roy et al. (1986) found that both methanogens and acetogens were greatly inhibited by LCFA. A lag phase while subpopulations of more LCFA resistant microorganisms grew could hence be expected. Usually, the recovery of microorganisms in thermophilic digestion processes was faster than
in mesophilic treatments (Hwu, 1997). This might have been due to the faster growth rate of those thermophiles which survived initial exposure to LCFAs.

Because the adaptation of acetogens and methanogens to LCFA had an unavoidable lag phase, a two-phase treatment of LCFAs in which the acidogenic reactor was separated from the methanogenic reactor was introduced. Phase separation was normally achieved by keeping a short retention time in the acidogenic reactor to wash-out methanogenic bacteria (Ghosh et al., 1975). In an acidogenic reactor, lipids are hydrolysed to LCFAs and then to short chain fatty acids that are later used by methanogens in the methanogenic reactor. Acidogens were shown to be less susceptible to LCFA inhibition and had a faster growth rate than methanogens (Jeyaseelan McCarty, 1995). Some difficulties such as low to nil lipid removal in acidogenic reactors have been reported however (Hanaki et al., 1987; Fongastitkul and Mavinic, 1994). A thorough study of this field is therefore still required.

2.4 Anaerobic treatment of lipids containing wastewater

Oily waste treatment is a new and difficult field that has aroused great interest in the environmental sciences. Because of the LCFA toxicity described previously and the propensity to form floating aggregates, lipids are difficult to degrade biologically. Although some physical methods can be used prior to biological treatment, the use of chemical or physical techniques to degrade lipid containing wastewater is very costly. For this reason, more emphasis has been placed in recent years on improving the methods of biological treatment.
The advantage to treatment of oily wastes by anaerobic digestion is that the method can be exploited on a large scale as a simple and effective biotechnology process to reduce the pollution caused by these wastes. In theory, anaerobic digestion offers significant advantages in energy terms. Aerobic waste treatments require an energy input and produce end products of little value. Anaerobic digestion however, with its valuable main product, methane, not only has the potential to treat wastes but also to reduce the energy usage of petroleum based oils.

As a new technique then, anaerobic digestion of oily wastes is still facing some difficulties. Nevertheless, with more intensive research using advancements in the fields of physico-chemical technology and molecular biology, more progress for lipids treatment will be achieved.

### 2.5 Difficulties and new approach of lipids anaerobic treatment

Anaerobic digestion has long been used for the stabilisation of wastewater sludges and is now widely used in the treatment of lipid containing wastewater. Some of the reported advantages of this process were:

1. Anaerobic digestion of lipid used readily available CO$_2$ as an electron acceptor and had no aeration costs (Lettinga et al., 1980; Beccari et al., 1996).
2. Anaerobic digestion produced lower amounts of sludge because the energy yields of anaerobic bacteria were relatively low (Lettinga et al., 1980).
3. The lipid waste are valuable sources of energy and fertiliser and most of the energy of this process was found in the final product (CH$_4$) that could be used for power generation (Salminien et al., 2000).

4. The process of lipid anaerobic digestion could be easily restarted after several months shut-down and it was very useful for the seasonal oil production plants (Beccari et al., 1996).

As a biological process, anaerobic digestion also had some disadvantages for lipid degradation:

1. Difficulties experienced in anaerobic digestion of oily waste included the fact that lipid compounds, as well as long chain fatty acids and phenolic compounds of the C-7 and C-9 phenylpropanoic family, are difficult to degrade by microorganisms.

2. The high organic load was difficult to dilute (Beccari et al., 1996) and this caused a very slow digestion process in oily waste anaerobic treatment.

3. Flotation or washout of granular sludge and fatty matter occurred at low waste loadings.

4. The hydrolysis products of lipid, LCFAs were shown to be highly toxic to anaerobic microorganisms.

These problems have only partly been overcome by the use of physical and chemical pre-treatments, designing different engineering processes and modelling of digestion kinetics to control the loading rate, HRT or other physico-chemical parameters. The activities of microorganisms in anaerobic digestion however, are difficult to control. Parameters that can be controlled during digestion are pH, dissolved oxygen concentration (DO), oxidation-
reduction potential (ORP), load of organic compounds and nutrients, hydraulic retention time (HRT) and sludge retention time (SRT). The relation between these parameters and the ecology of the microorganisms involved in the process is not clearly defined.

The general performance of anaerobic digestors and the diversity of wastes that can be treated have been increasing steadily due to an array of breakthroughs related to reactor design, operating conditions, and biocatalyst engineering. After UASB, an expended granular sludge bed (EGSB) and a staged multi-phase anaerobic (MPSA) reactor system were rapidly developed.

The two-phase anaerobic digestion process has been widely used in organic wastes, especially lipid containing wastewater (Beccari et al., 1996) but it still has problems in the areas of poor supernatant quality and relatively low methane production (Fongastitkul et al., 1994). However, the two-phase system is very attractive due to its prevention of lipid inhibition of methanogens (Komastsu et al., 1991) and the enhancement of lipid hydrolysis (Beccari et al., 1996).

One new approach reported was the use of granulation-enhancing additives (Lettinga et al., 1997). Biomass retention through adequate granulation is of utmost importance in UASB technology; firstly, in order to obtain a good effluent quality and secondly, to avoid the wash-out of the slow-growing anaerobic bacteria. It was suggested that addition of synthetic
polymers could shorten the onset of sludge granulation after start-up of an anaerobic sequencing batch reactor from four months to one month (El-mamouni et al., 1998). Water absorbing polymer particles were also shown to enhance the formation and stability of the granules (Imai et al., 1997).

Another new approach used anaerobic biomass to degrade toxicants present in wastewater, soil and river sediments. This method used specific biomass with different residence times, anaerobic tunnel reactors and sequential anaerobic-aerobic treatment to treat various toxicants (Londry, 1997; Trevizo et al., 1999). The major limitation to these new applications was the long adaptation period (up to months or years) required before the microbial community achieved maximal conversion rates of a new compound. Nevertheless, the potential for using biotechnology and genetically engineered microorganisms are apparent.

Recently, with the development of integrated municipal waste management, the number of co-digestion plants has been increased due to the advantage of full-scale co-digestion of organic waste (Kubler et al., 2000; Edelmann et al., 2000; Ahring et al., 1992). The lipids added to anaerobic digester treating animal manure could be converted effectively (Ahring et al., 1992). However, studies of anaerobic co-digestion of organic materials were mainly in applying physico-chemical methods (Kubler et al., 2000). The understanding of the microbial mechanisms for co-digestion of three organics is still unknown. Thus a study for the microbial community shifts in co-digestion of lipids, carbohydrates and proteins to find the optimal co-digestion conditions is necessary.
The efficiency of co-digestion might be related to the multiple sources of nutrients that serve a similar physiological function. The term "mixed substrates" encompass all types of nutrients; such as the carbon sources, energy sources, N-sources, etc. Mixed substrates are very relevant in waste management sciences. The great majority of biological waste treatment processes involve the metabolism of mixed substrates.

It was observed that the co-metabolism from hydrocarbon-utilizing bacteria which could grow on methane as the sole source of carbon (Alexander, 1985). They were methanotrophs (methane-oxidizing bacteria) and not able to utilise or metabolise higher alkanes such as ethane or propane as sole carbon sources. When the bacteria were grown on a mixture of methane, ethane and propane, the cells were found to not only utilise methane, but ethane and propane also get oxidized to products such as acetaldehyde, acetic acid, propionic acid and acetone, respectively. The investigation of biodegradation toluene and other toxic pollutants in wastewater treatment showed that these toxicants could be degraded in the presence of mixtures of organic compound which provide source of carbon and energy (Stenstrom, et al., 1989). Alexander (1985) defined co-metabolism as a process in which a toxic compound is mineralised or converted to non-hazardous metabolic products and it does not provide energy or nutrient for microbial growth. With co-metabolism techniques, It was investigated that pentachlorophenol (PCP) could be degraded by mixed microorganisms in an anaerobic, fixed-film reactor and by supplementation with 1g/L glucose. Some toxicants, such as polymers, such as poly (β-hydroxyalkanoates) (PHA) and two carbon
chlorinated aliphatics (eg. TCE) could be degraded by co-digestion with other substrates (Alexander, 1985).

Although the process of anaerobic digestion has progressed considerably since its first reported use, little is known about the microecosystems of anaerobic bacteria and the microbial effects on wastewater treatment processes. Recent advances in this area include the use of 16S rRNA hybridisation probes to describe natural communities of methanogens (Amann et al., 1990; Raskin et al., 1995) and the investigation of microorganism structure with antibodies (Conway and Macario, 1982). These approaches may have potential in improving the current understanding of biological wastewater treatment. All the functional behaviour of anaerobic digestion ultimately rests on the metabolic pathways of its microorganisms and the microecosystem of bacteria. The speed, the end products and the reaction rate of the anaerobic digestion process depend upon the growth of anaerobic microbial populations. Although syntrophic and competitive interaction in microbial populations present in anaerobic decomposition is known to occur, the extent of knowledge in this field is limited. This is a result of the limitations of traditional microbiological techniques, such as selective enrichment, pure culture isolation and number counting. With advancements in molecular techniques, in-depth understanding of the nutrient requirements of microorganisms during anaerobic digestion will be achieved. From this knowledge, it is envisaged that the physicochemical and engineering aspects of anaerobic digestion will further improve.
2.6 Research objective

Current difficulties in lipid anaerobic digestion processes occur during the hydrolysis of lipid. The inhibition of LCFA on methanogens and bacteria can lead to the shut down of anaerobic reactors. The current status of knowledge of the microbiology of lipid anaerobic digestion is mainly about the inhibition of LCFA and lipids on anaerobes. The study of the reduction of the inhibition by mechanism such as substrate addition however, is not exhaustive.

Therefore, this thesis has the following objectives:

- To quantify the effect of substrates, such as glucose and cysteine on LCFA inhibition of specific anaerobic microbial groups

- To define the function of glucose and cysteine on LCFA inhibition on the granulation of anaerobic bacteria

- To clarify the influence of glucose and cysteine on the recoveries of microbial groups under LCFA inhibition

- To examine the effects of addition of proteins and carbohydrates on lipid hydrolysis
To explore the optimum substrate composition as well as other physicochemical conditions for anaerobic co-digestion of lipid with carbohydrate and protein

To compare the efficiency of two-phase and one-phase UASB reactors on lipid degradation when co-digested with protein and carbohydrate

2.7 Outline and outcomes of the thesis

Chapter 1 provides the general introduction and summary of the thesis.

Chapter 2 presents the current knowledge, background and motivation for the research. The microbiology of lipid and LCFA anaerobic digestion as well as the current research on lipid anaerobic digestion is discussed.

Chapter 3 describes the experimental methods and techniques used for the determination of physico-chemical parameters and the microbiological investigation.

Chapter 4 characterises the role of typical nutrients, glucose and amino acid (cysteine) on the reduction of toxicity of LCFA (sodium oleate) in batch experiments. The effects of nutrients were examined by investigating of physicochemical parameters and by fluorescent in-situ hybridisation (FISH).

The effects of LCFA and addition of glucose and cysteine co-substrates on granule formation in lab-scale UASB reactors is presented in Chapter 5.
Investigations of the a recovery or adaptation of anaerobes to LCFA inhibition by adding different substrates (glucose and cysteine) in lab-scale UASB reactors is discussed in Chapter 6.

Chapter 7 compares the efficiency of monitoring the microbial population and physico-chemical parameters to control the lipid anaerobic degradation process. The microbial population shifts of acidogenic and methanogenic bacteria in one-phase reactors during lipid degradation were monitoring by using microbiological and FISH technique.

The influence of protein and carbohydrate addition on lipid hydrolysis in the acidogenic and methanogenic stages of a two-phase UASB reactor under different experimental conditions is given in the Chapter 8.

The optimal ratio of lipid, protein and carbohydrate on the growth of methanogenic and acidogenic bacteria in acidogenic and methanogenic reactors and the effect of the composition of carbohydrate and protein on lipid degradation is discussed in the Chapter 9.

Chapter 10 presents the conclusions and recommendations.
Chapter 3  Materials and methods

This chapter summarises the general materials and methods used in this research. The details of the specific methodology for each experiment can be also found in the related chapters.

3.1 Experimental lab-scale reactors and set up

3.1.1 Anaerobic sludge and substrate feeding

The suspended sludge from the Woodman Point Wastewater Treatment Plant (Woodman Point, WA, Australia) that mainly treated municipal wastewater was chosen as all inoculum for the experiments described in Chapter 4 and 5, which investigated LCFA toxicity and granulation, respectively. The sludge was stored at 4°C in tightly closed plastic containers and used without any preparation. The inocula for the experiments described in Chapters 6, 7, 8 and 9 was obtained from the hybrid UASB reactors of the Spearwood Waste Treatment Plant (Spearwood, WA, Australia). The sludge of the Spearwood Wastewater Treatment Plant was chosen for the acidogenic reactor because this plant was mainly fed with wastewater from a slaughterhouse, rich in lipid and protein. The sludge would therefore adapt more easily to the lipid feeding in the experiment.

In addition to sodium oleate or canola oil as feed (table 3.1), other substrates (table 3.2) were used to feed the reactors for reactivation or start-up. Canola oil was emulsified by sonication...
with an ultrasonic clearer (Branson, Sonifier 450) for 2 h (40% duty cycle and 7 output control) before feeding. The details of feed concentrations varied according to the experimental requirement and can be found in separate chapters.

<table>
<thead>
<tr>
<th>Substrates</th>
<th>Batch reactor</th>
<th>One-phase UASB reactor</th>
<th>Two-phase UASB reactor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Canola oil</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>Sodium Oleate</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Basal Medium</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Starch</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
</tr>
</tbody>
</table>

Table 3.1 Substrates for different reactors
### Table 3.2. Chemical composition of basal medium (Lapsirikul, 1993).

<table>
<thead>
<tr>
<th>NAME</th>
<th>AMOUNT (g/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>KH₂PO₄</td>
<td>0.4</td>
</tr>
<tr>
<td>K₂HPO₄</td>
<td>0.2</td>
</tr>
<tr>
<td>NH₄Cl</td>
<td>0.3</td>
</tr>
<tr>
<td>KCl</td>
<td>0.5</td>
</tr>
<tr>
<td>NaCl</td>
<td>1</td>
</tr>
<tr>
<td>NaHCO₃</td>
<td>1.8</td>
</tr>
<tr>
<td>Na₂CO₃</td>
<td>0.5</td>
</tr>
<tr>
<td>CaCl₂.2H₂O</td>
<td>0.05</td>
</tr>
<tr>
<td>MgSO₄.7H₂O</td>
<td>0.1</td>
</tr>
<tr>
<td>Yeast Extract</td>
<td>1</td>
</tr>
<tr>
<td>Resazurin Indicator</td>
<td>1ml</td>
</tr>
<tr>
<td>Sodium Dithionite</td>
<td>until colourless</td>
</tr>
<tr>
<td>pH 7-7.5</td>
<td></td>
</tr>
</tbody>
</table>

3.1.2 Set up of UASB reactor and two-phase UASB reactor

The glass UASB reactor was operated with a water jacket by circulating water from a water bath at a constant temperature as shown in Figures 3.1 and 3.2. Substrate was fed by peristaltic pump and the feed tank was stirred continuously with the mixer. Biogas was collected from the top of the reactor and led through 10% NaOH before being measured by
water displacement using an inverted cylinder and the gas flow rate was measured using a downward displacement of acidified water (0.05M H₂SO₄). The effluent tube was U shaped and maintained by a liquid seal to prevent gas escaping through the line. The two-phase reactors used in the experiments were made from Perspex (polycarbonate) material. They possessed the same shape and working volume (2.0 L). A water bath was employed to maintain the water jacket temperature around 37°C.

Fig. 3.1 Photograph of single and two-phase UASB reactors.
Enhancing Anaerobic Degradation of Lipids in Wastewater by Addition of Substrate - Y. Kuang
Chapter 3 Materials and Methods

![Diagram showing materials and methods](Image)

- Biogas collector
- NaOH (10%)
- Pump
- Effluent container
- Water bath
- Reactor
- Feed container
- Stirrer

A
Fig. 3.2. Schematic diagram of experimental single-phase (A) and two-phase (B) UASB reactors.
3.2. Batch experiment for LCFA toxicity test

Forty ml of the basal medium (Table 3.2) and 10 ml of the flocculent sludge were pipetted into the serum bottle of 120 ml working volume. The vial was flushed with N₂ /CO₂ (2:8 in v:v) before sealing with a silicon rubber cap and an aluminum clasp.

Subsequently, the reactors were placed in a 37°C water bath stirred at 30 strokes/min. Prior addition of sodium oleate (SO), glucose and amino acid, the reactors were reactivated for 48 h to degrade the residual substrate. After reactivation, 20 ml of the experimental substrate solution (according to the experiment in chapter 4) were injected into the reactors to get different concentrations of different substrates. The control was added with the same volume demineralized water. The vials were incubated in the water bath for 48 h. All the experiments were conducted in triplicate. The headspace of reactors was flushed again before collection of the methane gas for GC. The methane composition of the reactors was measuring using GC (see section 3.6.1). The samples for FISH experiments were gained and fixed freshly for whole cell hybridization and DAPI stain quantification (see section 3.3 & 3.4).
3.3 Quantification and identification of anaerobic bacteria groups by oligonucleotide probe hybridization

3.3.1 Oligonucleotide probes

The four group-specific rhodamine-dyed 16S rRNA hybridisation probes, MB310, MC1109, MG1200 and MSMX860, which target the communities of methanogens were bought from Synthetic Genetics, California (U.S.A). They targeted all three orders of known methanogens, *Methanobacterales*, *Methanococcales*, *Methanomicrobiales*. In them, families *Methanomicrobiaceae*, *Metahosarcinaceae*, *Methacorpusculaceae*, *Methanosarcinaceae* made up the order *Methanomicrobiales*. The three rhodamine-dyed oligonucleotide, ARC915 and EUB338, and SRB385 were purchased from Sigma-Aldrich Pty. Ltd. (Australia). Probes EUB 338 and ARC 915 were used to investigate the two domains *Bacteria* and *Archaea*, separately, while SRB was applied to target Sulfate-reducing bacteria. The other fluorescein-isothiocynate (FITC)-dyed probe, EUB338, was ordered from Synthetic Genetics, California (U.S.A) for granule location analysis. The probes were 5’-end labelled with fluorescent dye and sequences for 7 probes were extracted from the previous reference (Table 3.4).
3.3.2 Cell fixation and whole–cell hybridization

About 10 ml of granule and suspended sludge were taken from the reactors and promptly washed by phosphate-buffered saline (PBS) solution (130 mM sodium chloride, 10 mM sodium phosphate buffer) (Raskin et al., 1994a & b). After washing, the granule sludge was disintegrated by an ultrasonic clearer (Branson, Sonifier 450) with continuously microscopy inspection to monitor disaggregation and possible cellular disruption.

One ml cell suspension was obtained and centrifuged at 13,000 rpm for 4 min. Half of the supernatant was removed and the cell pellets were resuspended in the same volume of
fixative solution (described below) or ethanol (99.5%) depending on the probes used. The suspended cells were incubated at 4°C for about 18h for fixation.

The fixative solution was prepared with 4% Paraformaldehyde in 200mM sodium phosphate buffers (pH 7.2) and not more than 8h before use. After centrifugation 4 min at 6000 rpm, the pellets were washed by centrifugation with 9 volume of PBS (130mM NaCl, 10 mM sodium phosphate) and 1 volume Igepal CA-630. The supernatant was removed and cells were resuspended in 50% ethanol and stored at –20°C for several weeks without significant decrease of rRNA content.

Four µl well suspended samples were spread on the well of six-well Teflon-coated slides purchased from Proscitech Australia. After air drying, the slides were dehydrated by three successive immersions of slide in 50, 80 and 100% ethanol solution for 2 min each. They were air dried again and the slide prepared for hybridisation. Ten µl hybridisation and 1 µl rhodamine-labelled oligonucleotide probe (50-80 ng) were added in the wells and mixed. The slides were incubated in a 42°C moisture chamber for 2 h, rinsed twice with the hybridisation solution and washed 15 min at 42°C. They were washed three times with double distilled water at room temperature and air dried in the dark at room temperature.
3.3.3 Quantification of different microbial groups

The hybridised cells were air dried and viewed immediately under oil immersion objective with a UV light on a Leitz DIAPLAN 307-148 microscope fitted for epifluorescence excited in the green region of the spectrum. Ten fields were counted in each well to determine the number of cells fluorescing in the rhodamine emission region. Black and white Kodak Tri-X Pan 400 film was used for photomicrographs. Exposure times were between 40 s - 6 min for epifluorescence.

3.4 Total cell counts with DAPI method

The fixation of cell was the same as described above. The fixed cells were immobilized on the hybridisation well and stained by DAPI (6-diamidino-2-phenylindole) solution for 7 min by adding 20 µl of 40 µg/ml DAPI solution (30% formamide, 0.9% Nacl, 20mM Tris HCl at pH 7.2, 0.1% SDS) to the hybridisation well. The cells were washed with the same solution without DAPI. Air dried and viewed at Leitz DIAPLAN 307-148 microscope fitted for epifluorescence excited in the UV region of the spectrum. The counting was performed at 10 different microscopic fields (Amman et al., 1990b)
3.5 Investigation of granular structure with scanning and transmission electron microscopy.

Scanning electron microscopy (Philips 501B) and transmission electron microscopy (Philips CM 100) from the Veterinary School, Murdoch University, Western Australia were used for the analysis. The methods of fixation granule samples were modified from Macleod et al. (1990) and Zhu and Gu (1997).

The sludge samples collected from the reactors were gently washed with a 0.1 M phosphate buffer solution and fixed with 2.5% glutaraldehyde in sealed 50 ml serum bottles overnight at 4°C. The fixed samples were washed twice for 10 min, each in 0.1 M cacodylate buffer. The granules were fast frozen in liquid nitrogen with aluminium block and cleaved with a motar and pestle. The granules were dehydrated through graded series ethanol (30%, 50%, 75%, 90%, 100%) for 15 min each. Dehydrated samples were air dried in a desiccator and mounted on a stub and sputter coated with gold. Samples were viewed with a SEM (Philips 501B) at 10 kv.

For TEM, the granular samples were washed with 0.1 M phosphate buffer solution twice gently and quickly fixed with 3.5% glutaraldehyde for 24 h in 50 ml serum bottle which sealed with butyl rubber stoppers and aluminium seals. Treated with 0.1 M phosphate buffer and fixed in 1% OsO₄ for 1.5 h, they were washed twice with buffer then dehydrated in a series of 30%, 50%, 75%, 90%, 100% w/w acetone-water solution for 15 min each. After being washed twice with propylene oxide over 10 min. and left in epoxy resin
overnight on a rotator, they were embedded in capsules by using fresh epoxy resin and
baked at 60-65 °C for 24h. The hardened embedded sludge was cut with a glass knife. Thin
sections (silver or golden colour) were chosen and placed on copper grids. Finally, these
sections were stained first with uranyl acetate then with lead citrate, and dehydrated in 80%,
90%, absolute alcohol. They were critical-point dried and gold coated, and viewed with
TEM.

3.6 Confocal laser scanning microscopy (CLSM).

The confocal laser scanning microscopy (Olympus, Fluoview, BX50) from the State
Agricultural Biotechnology Centre, WA, Australia was used to view the florescence of
rhodamine and FITC from different probes.

3.6.1 Fixation and sectioning of granule

The method for the granule sectioning was modified from Chui and Fang (1994) and
Sekiguchi et al. (1999). A sample of 50-60 granules was washed three times with 0.1 M
phosphate buffer solution at pH 7.2 and then fixed in a phosphate buffer solution with 10% formadehyde at 4 °C overnight. It was then dehydrated with 50%, 80%, 100% ethanol solution twice at 15 min each, and then immersed in a graded series of xylene/ethanol solution, in which the xylene concentration gradually increased from 50% to 100%.
Granules saturated with xylene were immersed in molten paraffin 50% (v/v) for 30 min, and then immersed in 100% paraffin twice. Five to ten granules were transferred to the 10 ml vial and immersed in molten paraffin at 60 – 65 °C overnight, allowing enough paraffin to
penetrate into the granules. Three to four granules paraffin-embedded granules were put into the peel-off mold and cooled to the room temperature. The thin paraffin section were sliced by a microtome (Leitz Model 1400) and mounted on gelatine-coated glass slide. The slide was air-dried, then put back in a 65°C oven for half an hour before dewaxing with xylene.

3.6.2 Hybridisation and CLSM scanning investigation

For only rhodamine dyed probes, 2 µl (25-50 ng/µl) rhodamine labelled oligonucleotide probe and 10 µl hybridisation buffer (0.9 M NaCl, 20 mM Tris-HCl (pH 7.2) 0.01% sodium dodecyl sulfate) were added to each well. Then hybridisation of the 16S rRNA-targeted oligonucleotide probes to granule section were performed at 40°C for 16 hours. After hybridisation, the granule section was washed 4 times with hybridisation buffer, air dried and observed by CLSM.

For double staining hybridisation, FITC labelled probe was hybridised and washed first, and rhodamine labelled probe was followed for the second hybridisation. After the second hybridisation, the granule sections were washed with the hybridisation buffer for 4 times, air dried and observed by CLSM (Sekiguchi, et al., 1999).
3.7 Analysis of gas and effluent of batch and UASB

3.7.1 Gas chromatography (GC)

The methane in gas was analysed by GC (Varian Model 3700) using the following parameters:

- Column ID and length: 1.8 m & 2 mm
- Column temperature: 100°C
- Carrier Gas: Nitrogen
- Injector temperature: 200°C
- Sample volume: 1 ml
- Detector: FID
- Detector temperature: 200°C

The volatile fatty acid (VFA) in samples was prepared by centrifuging at 15,000 rpm for 10 min to remove the suspended solids (Hwu, 1998). The supernatants were acidified by the addition of 1% HCl to convert the VFAs into the free acid form. Then the sample was ready for VFA examination with the same method as methane gas analysis, except for the column temperature (200°C) and sample volume (10 µl). The standard curve of acetate, propionate, butyrate were made with different concentrations, respectively.
The long-chain-fatty acids were determined by GC (SHIMAZU, GC 17A) with a column AT-225 (Altech, Perth, Australia) using the following parameters:

- **Column ID:** 0.25 mm
- **Column Length:** 30 metres
- **Film thickness:** 0.25 µm
- **Detector:** FID
- **Detector temperature:** 275°C
- **Injector temperature:** 250°C
- **Oven temperature:** 125°C
- **Carrier gas:** Helium
- **Flow rate:** 1.2 ml/min
- **Pressure:** 12 psi
- **Split ratio:** 100:1
- **Sample size:** 1.0 µl
- **Solvent:** Hexane

The standard LCFA methyl esters, palmitic acid methyl esters, oleic acid methyl esters, linolenic acid methyl esters and linolenic acid methyl esters were bought from Sigma Aldrich Pty., Ltd. (NSW, Australia).
3.7.2 High performance liquid chromatography (HPLC)

Samples (1 ml) were acidified with 4 drops of 10 N HCl and extracted with 10 ml petroleum ether (Lapsirikul, 1993). A standard concentration of SO was also analysed in parallel. After 1 hour mixing, the ether phase was transferred to another tube and entirely evaporated in 80°C water bath. Then HCl and dry methanol (1:19 v/v) were added and mixed and put back to the 80°C water bath for 1 h. After esterification, 2 ml of petroleum ether were added and vortexed for 5 min. One ml of the ether phase were added into a glass vial and capped for analysis.

The sodium oleate in sample was analysed with HPLC (Varian 2010), equipped with Varian 2050 UV Detector and Varian 4290 Integrator. HPLC was performed with sperical 5 μm LiChrospher-NH₂ column packing. The effluent A and B were mixtures of n-hexane/methanol. The UV detector wavelength was 217 nm.

3.8 Measurements of lipids, carbohydrates and proteins and methods for measurements of COD, total suspended solids (TTS), volatile solids (VS), suspended solids (SS) and volatile suspended solids (VSS).

All the measurements of samples were applied in duplicate or triplicate.

3.8.1 Lipid analysis
The concentration of lipid was determined gravimetrically after extraction of lipid from the samples by petroleum ether, according to the Soxlett extraction method (APHA, 1985).

3.8.2 Carbohydrate analysis

The anthrone method was applied for the analysis of carbohydrate as described by Raunkjaer et al. (1994). The mechanism of this method is based on the colour reaction between carbohydrate and the anthrone reagent. This method has a high specificity for carbohydrate and the colour can be measured accurately by spectrophotometer.

The anthrone reagent was a 0.1% solution of anthrone made up in 75% (V/V) sulphuric acid. It should be prepared freshly each day of analysis. Solution of glucose of 5, 20, 40, 80 and 100 mg/l was prepared as standards for a standard curve. One ml samples or standards were pipetted into Pyrex test tubes. Then 1ml of cold anthrone reagent was added and the test tubes were closed and shaken. The test tubes were put into the 100°C water bath for 20 min and then were placed to the 4°C refrigerator for 10 min. Finally, the samples were measured spectrophotometer at 625 nm.

3.8.3 Protein analysis

The Lowry method was applied in the experiments because of its sensitivity to low concentrations of protein (Raunkjaer et al., 1994). Bovine serum albumin (BSA) was used as standard. The principle behind the Lowry method of determining protein concentrations
lies with the reactivity of the peptide nitrogen[s] with the copper [II] ions under alkaline conditions and the subsequent reduction of the Folin-Ciocalteay phosphomolybdicphosphotungstic acid to heteropolyolymolybdenum blue by the copper-catalysed oxidation of aromatic acids.

Reagents preparation:  A. 2% Na2CO3 in 0.1 N NaOH;  B. 1% NaK Tartrate in H2O;  
C. 0.5% CuSO4.5 H2O in H2O;  D. 48 mL of A, 1 mL of B, 1 mL C, 1ml  
E. Phenol Reagent - 1 part Folin-Phenol [2 N]: 1 part water

To obtain the standard curve, eleven sets of three 16 x 150 mm test tubes in triplicates were set up in rack. 0, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100 µl of BSA was added to these tubes. Two ml of solution D was then added to each test tube. Incubate at room temperature for 30 minutes before addition of 0.2 mL of dilute Folin-phenol solution (E). Each tube was vortexed immediately and incubated at room temperature for 30 minutes. Determination of the absorbance of each sample at 600 nm was plotted against amount of protein (mg) to obtain standard curve.

For the analysis of samples, set up triplicate assays as described for standard curve. The data from the standard curve were used to determine the concentration of unknown samples.

3.8.4   Other analysis

COD, total suspended solids (TTS), volatile solids (VS), suspended solids (SS) and volatile suspended solids (VSS) were analysed as described in the Standard Methods (APHA, 1985).
Chapter 4 Reduction of the toxicity of LCFA on anaerobic bacteria by addition of co-substrates in batch tests

4.1. Introduction

Long-chain fatty acids (LCFA) are products of lipid hydrolysis in anaerobic wastewater treatment. They are mostly degraded into acetic acid and hydrogen by acetogens via the β-oxidation pathway and finally converted to methane by methanogens. Due to the toxicity of LCFA on anaerobic microorganisms, both acetogens and methanogens suffer greatly from inhibition of LCFA in anaerobic digesters (Hwu et al., 1996). It was reported that LCFA inhibition process was so significant that the methanogenic activity was lost only after several minutes of exposure (Koster, 1987). The reason for inhibition by LCFA could be related to the adsorption of LCFA onto the cell membrane resulting in the damage of its transport and protective function and finally in the lysis of the cell (Demeyer and Henderrickx, 1967). It also might be related to the fact that LCFAs change the membrane permeability and affect the surface tension, thus inhibiting the division of the cell (Rinzema, 1988).

The toxicity of LCFA on methanogenic bacteria has been positively correlated to the concentration of LCFA (Hanaki, 1981). Severe inhibition can be caused even at 3 to 5 mM concentration of LCFA (Hanaki, 1981; Hwu et al., 1998). The ratio between LCFA and biomass appears to be of less significance for the toxicity of LCFA (Koster and Cramer,
1986; Rinzema, 1988). Besides the concentration, previous investigations demonstrated that the toxicity of LCFA was also related to the temperature, pH and origin of the sludge (Galbraith and Miller, 1973; Hwu, 1997). Koster (1987) reported that the addition of calcium chloride decreased the inhibition of LCFA by forming the insoluble calcium salt of LCFA and thus reducing the concentration of LCFA. However, this was only a short-term strategy, as the toxicity of LCFA would reappear after several hours. Angelidaki et al. (1990) also found that the addition of bentonite (a clay mineral) reduced lipid inhibition and caused a significant increase in the degradation of lipid. The possible reason for this effect might be the flocculation capacity of bentonite with the lipid. However, it seemed that the bentonite could not be degraded and had no benefit to the bacterial population. Although the simple addition of calcium or bentonite could decrease the toxicity of LCFA in pilot studies, its application to full scale lipid treatment has not been yet reported.

This chapter discusses investigations on the effect of addition of co-substrates on the inhibitory effects of LCFA during anaerobic digestion from physico-chemical and microbial aspects. In the experiments reported here, sodium oleate (SO) was selected as the model compound for LCFA because it is the most abundant of all LCFAs in wastewater (Hwu, 1997). Previous research has reported that glucose and cysteine were necessary for the growth of anaerobic bacteria (Balch et al., 1979). Therefore glucose and cysteine were chosen as the co-substrates for digestion with LCFA. In addition, glucose and cysteine are likely to be produced as intermediates during the digestion of complex substrates containing carbohydrates and protein. The effects of glucose and cysteine as well as their different concentrations and composition on toxicity of sodium oleate were also compared. The
counteraction of glucose and cysteine to sodium oleate toxicity was investigated by checking the production and composition of gas and VFA. The effects of sodium oleate, glucose and cysteine on the shift of specialised anaerobic bacterial population were also monitored by 16S rRNA fluorescence in-situ hybridisation (FISH) technology and linked to the functional changes of batch reactor. All investigations were carried out as batch assays in serum vials.

4.2. Material and Methods

The preparation of inoculum and its reactivation were described in 3.2. The methods of quantification and identification of anaerobic bacteria groups can be found in 3.4. The methane production was examined with the method shown in 3.6.1.

4.2.1. Examination of individual effect of glucose, cysteine and sodium oleate on methanogenic activity

The individual effect of glucose, cysteine and sodium oleate on the anaerobic process was investigated by feeding different concentrations of these substrates separately. The concentrations of glucose and cysteine were chosen as 0, 0.5, 1, 2, 3, 4 g/l, and sodium oleate as 0, 1, 2.5, 5, 10, 20 mg/l. The source of sludge was a suspended sludge obtained from the Woodman Point Wastewater Treatment Plant (WA, Australia). The methods for batch assays were as described in section 3.2. The initial concentrations of glucose, cysteine and SO were set in the different vials with addition of 20 ml substrate solution. A control assay was set up with the same volume of demineralized water.
After 24 h of incubation, the content of methane in the gas phase of the vials was examined by GC as described in 3.7.1, and the methane content was used as an index of methanogenic activity. The loss of maximum methanogenic activity in each reactor was expressed as a percentage of the uninhibited maximum methanogenic activity of control. All the experiments were conducted in triplicate and the results quoted as mean values.

4.2.2. Comparison of toxicity concentration of sodium oleate with the addition of glucose and cysteine

The concentrations of SO were adjusted to 0, 1, 2.5, 5, 10, 20 mg/l by injecting 20 ml SO solution into the vials. The vials were fed with the optimal concentration (determined in 4.2.1) of glucose, cysteine or glucose and cysteine together, respectively. In the vial, which contained both glucose and cysteine, the amount of each was half the optimal concentration. The control reactors were only fed with the same concentration of glucose, cysteine or glucose and cysteine together, respectively without SO. Then the headspace of the vials was flushed with N2 / CO2 and return back to the incubator.

The content of methane gas in the gas phase was determined after 24h of incubation. The relative methanogenic activity was expressed as the percentage of methane volume compared to the control. All the experiments were conducted in triplicate and the results quoted as mean values.
4.2.3. Effect of the addition of glucose and cysteine on the degradation of SO in batch tests

This experiment was performed in four groups. The concentration of SO was chosen higher than the concentration that inhibited the methanogenesis determined in 4.2.1. The adsorption of SO during the experiment was checked separately by adding SO to autoclaved sludge. While one group was taken as a control with fed SO, three groups were fed glucose, cysteine or both glucose and cysteine. The concentration of glucose and cysteine were chosen as 0, 0.5, 1, 2, 3, 4 g/l, respectively. The total concentration when both glucose and cysteine were added was 0, 0.5, 1, 2, 3, 4 g/l with cysteine and glucose being added at 1:1 (w/w) ratio.

The degradation of SO was calculated by subtracting the SO residuals in the batch reactor after 24 h incubation and the adsorbed quantity of SO of sludge from the total amount of SO added into the vial. SO was measured following the methods outlined in 3.7.2.

4.2.4 Effect of glucose and cysteine on degradation rate of SO

The experiment was performed in four groups. The control group was prepared by only feeding SO (5 mg/l). The other three groups contained glucose (2 g/l), cysteine (2 g/l) or both glucose (1 g/l) and cysteine (1 g/l) in addition to SO at a concentration of 5 g/l. The concentration of SO was examined after 5, 10, 15, 20, 25 and 30 h of incubation. SO was determined following the methods described in 3.7.2.
4.2.5. Growth of specialized microbial groups under different substrate composition

The microbial communities were analysed after 30 h by using 16S rRNA probes. Two domain specific probes, ARC 915 (targeting domain Archaea) and EUB 338 (targeting domain Bacteria) were used to investigate the Archaea and Bacteria population which covered all the microorganisms present in the batch reactors. Because the sulfate existing in cysteine could benefit the growth of Sulfate-reducing Bacteria (SRB), the group specific probe SRB 385 (targeting SRB) was used to monitor SRB and evaluate the competition between methanogen and SRB. To study the microbial shifts in domain Archaea, order Methanobacteriales (targeted by MB310)) and four families Methanosarcinaceae (targeted by MSMX860), Methanomicrobiaceae, Methanocorpusculaceae and Methanoplanceae (targeted by MG 1200) were analysed separately. All the probes listed above circumscribed most of the currently known microbial groups which are involved in LCFA, glucose and cysteine anaerobic degradation. The competition and coexistence among Archaea, Bacteria and SRB was observed by comparing the shifts of the total cell number examined by whole-cell hybridization methods as described in 3.3. All the experiments were conducted in triplicate and the error bars indicated the standard deviations.

4.3. Results and Discussion

4.3.1. Effect of co-digestion of glucose and cysteine on the toxicity of sodium oleate

Figure 4.1a presents the methanogenic activities of the vials containing different concentrations of SO and SO along with glucose and/or cysteine (Fig.4.1b). The inhibitive
concentration (IC) of SO which caused 50% methanogenic activity lost was defined as IC50 in the experiment with SO concentration higher than 1mg/l, the methanogenic activity sharply decreased and IC50 was about 2.5 mg/l. In previous research, the reported concentration of LCFA which caused CI50 inhibition on methanogenesis was different. Hanaki et al. (1983) reported that 5 mg/l loading of SO resulted in 50% inhibition for methanogenesis, while it was 130 times higher in Koster and Cramer (1987) experiment. In Hwu’s (1997) experiment, the IC50 was about 4 mg/l. While Koster and Cramer (1987) speculated that this difference might be due to different culture medium applied in the specific experiment. Hwu (1997) suggested that it could also be related to the sludge originals and temperature.

As would be expected, the methanogenic activity increased as the concentration was increased up to 1 g/l for cysteine and 2 g/l for glucose. As the concentration of glucose and cysteine was increased beyond these, the methanogenic activity dropped. At 3 g/l and 4 g/l of glucose or cysteine, the methanogenic activity was only 40% of the activity observed for the control. Even though it would be expected that methanogenic activity increased with the addition of more substrate, at high concentration of substrate, it is expected that the methanogenic capacity of the inocula would have been exceeded, which in turn, caused the VFA to accumulate resulting in a drop in pH. Low pH would inhibit methanogenic activity. Hence it was shown that for the basal medium and sludge used in the assays here, 1 g. L⁻¹, of cysteine and 2 g. L⁻¹ of glucose was optimal.
Chapter 4 Reduction of the toxicity of LCFA on anaerobic bacteria by addition of co-substrates in batch tests

Fig. 4.1. Methanogenic activity from feeding glucose, cysteine and SO at different concentrations.

The toxicity of SO on methanogenic activity and the effects of the addition of glucose and cysteine on this toxicity is shown in Fig. 4.2. The IC50 concentration of SO was about 2.5 mg/l and was very similar to that observed in the batch tests discussed previously (Fig. 4.1a). The addition of glucose increased the IC50 of SO from 2.5 mg/l to about 12 mg/l,
and the addition of cysteine increased the IC$_{50}$ of SO to about 5 mg/l. Addition of both glucose and cysteine increased the IC$_{50}$ of SO to 20 mg/l. This suggested that addition of other organic substrate such as glucose and/or cysteine could relieve the toxicity of SO on methanogenic activity. In terms of extent of enhancement, the addition of glucose and cysteine was most effective in three feeding modes. Following this, glucose gave better results than the addition of cysteine alone.

Fig. 4.2. Methanogenic activity when SO is co-digested with glucose (2 g/l), cysteine (1 g/l), and glucose (1 g/l) together with cysteine (0.5 g/l).

The IC$_{50}$ concentration of SO on the methanogenic activity was about 2.5 mg/l (Fig. 4.1a). In order to investigate the effects of glucose and cysteine on the degradation of SO, the concentration of SO was chosen as 5 mg/l which was higher than the IC$_{50}$ concentration of SO.
Fig. 4.3 shows that the amount of SO degraded increased with the addition of glucose, cysteine or combination of glucose and cysteine. The results indicated that glucose had a better effect than cysteine, while the combination of glucose and cysteine had the greatest effect on enhancing the degradation of SO. It is in accordance with the result of Figure 4.2, which also suggested that the combination of glucose and cysteine had the optimal effect on decreasing the toxicity of SO. The result also showed that the addition of 1 g/l glucose and 1 g/l cysteine had the best effect on increasing the degradation of SO. Increasing the concentration of glucose and cysteine beyond these values did not enhance SO degradation, but on the contrary, the residual SO concentration increased. As explained before, at concentration above 2 g/l, it is likely that VFA accumulation could cause pH to drop, which in turn inhibited methanogenic activity, when glucose together with cysteine was added.

Fig. 4.3. The effects of glucose and cysteine on the degradation of SO. The concentrations of glucose and cysteine in Glu+Cys group were 1:1 in w:w.
Figure 4.4 shows the effect of glucose, cysteine or glucose and cysteine on the rate of degradation of SO. In the vials which were fed only SO, very little of SO was degraded over the 30 h of the test. This is in agreement with results of batch assays discussed in Fig.4.1a in which SO inhibited the methanogenic activity severely. The addition of glucose and cysteine increased the degradability and degradation rate of the SO. The combined addition of glucose and cysteine increased more significantly the degradation rate of SO. By supplementing with glucose and cysteine, about 80% of the initial SO was degraded in 25 hours. In addition, more SO was degraded over the 30 h. Glucose alone increased the degradation rate higher compared to the addition of cysteine alone. This is in accordance with the results shown in Fig.4.2 in which glucose was more effective than cysteine in decreasing the toxicity of SO.

![Fig.4.4. Effect of glucose (2 g/l), cysteine (2 g/l) and combination of glucose (1 g/l) and cysteine (1 g/l) on the degradability of SO (5 mg/l).](image)

Fig. 4.4. Effect of glucose (2 g/l), cysteine (2 g/l) and combination of glucose (1 g/l) and cysteine (1 g/l) on the degradability of SO (5 mg/l).
The experiments above demonstrated that the addition of limited concentration of glucose and cysteine significantly decreased the toxicity of SO on methanogenic activity and enhanced the degradation of SO. The results also showed that the combination of glucose and cysteine was most effective in reducing the inhibition by SO, compared to the separate addition of glucose and cysteine. Glucose has been reported as a growth factor which decreased the inhibition of some compounds both in aerobic and anaerobic digestion (Mousa and Forster, 1999). Beccari et al. (1996) found that oleic acid (3g COD/l) could not be degraded without the supplementation of glucose. Hwu (1997) observed that use of glucose and acetate as co-substrates enhanced the degradation of LCFA mixture in anaerobic reactor. The role of the glucose was that of an exergonic substrate which provided the energy for the microbial populations. As a source of nitrogen, cysteine is also well known to benefit the acidogenesis, acetogenesis and methanogenesis in anaerobic digestion. However, high concentration about 90 mg N/l also inhibited the anaerobic process (Samsøn et al., 1991).

4.3.2. Population shifts during degradation of SO with glucose, cysteine or glucose and cysteine addition.

The microbial community structure analysis carried out in the previous section (Fig. 4.4) at 30 h are shown in Fig.4.5a. The control sample was the original inoculum diluted by addition of demineralised water, the volume of which was the same as the volume of feed used in the other tests. After feeding with 5 mg/l SO, the number of cells of Archaea, Bacteria and SRB all decreased. Among, the decrease of Archaea was the most significant.
This might indicate a higher sensitivity of *Archaea* bacteria to SO compared to *Bacteria* cells and SRB cell. The addition of glucose (2 g/l) alone with the SO significantly increased the numbers of SRB, *Archaea* and *Bacteria* cells compared to the addition of SO only and this might be linked to the enhanced methanogenic activity and SO degradation discussed in Fig.4.2. When adding cysteine (2 g/l), the increase in population of *Archaea* and *Bacteria* cells was not as high as that observed with the addition of glucose. However, the increase of SRB was more significant than that in glucose addition. The presence of sulphur in cysteine might stimulate the growth of SRB. When adding glucose (1 g/l) and cysteine (1 g/l) together, the population of *Archaea*, *Bacteria* and SRB all increased and recovered numbers similar to the control level. Therefore, the co-digestion of SO, glucose and cysteine was most beneficial to the growth of *Archaea*, *Bacteria* and SRB.

Figure 4.5b shows the shifts within the *Archaea* community observed for the different feeds after 30 h. The members of *Methanococcales* (MC1109) were not detected in the inoculum for the different feeds. *Methanobacteriales* (MB310) were the most abundant group with SO feeding alone, suggesting that *Methanobacteriales* were the least inhibited by the toxicity of SO. In contrast, *Methanosarcinaceae* (MSMX860) were the least abundant, indicating that *Methanosarcinaceae* was the most sensitive to the inhibition of SO. When fed with glucose, cysteine or glucose and cysteine, the percentage of *Methanosarcinaceae* increased to the highest value in *Archaea*, suggesting that the three feeding modes all benefited the growth of *Methanosarcinaceae*. When fed glucose and cysteine, the structure of the three methanogenic groups was similar to the control, implying that the
Fig. 4.5. Microbial population shifts for different feeds (a), and methanogenic community structure in batch reactors (b). Data are mean values of triplicate tests and error bars indicate the standard deviations.
feeding of glucose and cysteine promoted the optimal recovery of *Archaea* communities. This might also be linked to the fact that the mixture of glucose and cysteine had the best effect on the reduction the toxicity of SO.

The microbial investigation presented in this study suggested that glucose and cysteine enhanced the degradation of LCFA in anaerobic digestion through the recovery of microbial communities. Addition of glucose increased the growth of sub-populations of the microbial communities, especially *Archaea* cells in anaerobic reactor. The reason might be related to the fact that it provided the necessary growth factors for *Archaea* microbial communities, and hence helped the *Archaea* microbial communities to survive under LCFA toxicity or recover from the inhibition of LCFA. Addition of cysteine was not as effective as glucose in increasing the number of *Archaea* cells but it was more effective in stimulating the growth of SRB. In recovery, even the SRB population in cysteine feeding was higher than glucose or glucose and cysteine feeding, the recovery of methanogenesis and SO degradation were lower there, implying that methanogenesis and SO degradation was more linked to the recovery of *Archaea* cells. Use of cysteine in the experiment provided some information related to the effect of addition of amino acids in LCFA degradation. However, further investigation on different amino acids is required. The addition of a mixture of glucose and cysteine was most effective in enhancing the growth of *Archaea* cells. It optimally increased the growth of the *Methanosarcinaceae* family which is the most important group involved in the methanogenesis (Raskin et al., 1990b). Glucose and amino acids are required in anaerobic digestion as they are important nutrients for the growth of anaerobic microorganisms (Balch *et al.*, 1979). The results suggested that the addition of glucose and
cysteine relieved the toxicity of LCFA by stimulating the growth of microbial communities, which in turn, lead to more degradation of LCFA.

Mousa and Forster (1999) reported that the addition of the growth factor glucose (10 and 20 mg/l) reversed the toxicity of gallic acid. To date, some experiments have also demonstrated that the glucose and other organic substrates could reduce the inhibition of some toxic material in sludges (Hwu, 1997; Mousa and Forster, 1999). However, this mechanism is not clear. It was speculated that some non-growth substrates or toxicant which do not assist in cell division could be transformed by using the energy or enzymes produced during degradation of other co-substrates or growth substrates (http://www.uoguelph.ca/~hlee/418chap6.htm). The growth substrates, such as glucose or amino acids could provide energy to support microbial growth and produce catabolic enzymes which catalyse toxicant transformation. During LCFA anaerobic digestion, the availability of nutrients for anaerobe growth is more important due to the toxicity of LCFA. The optimal ratio among glucose, amino acids and LCFA is very complex due to the changing nutrient requirement as the community shifts. The results of the comparison of microbial community shifts from this experiment showed that only specific concentrations of glucose and cysteine could effectively decrease the toxicity of LCFA on microorganisms. Too high or low concentrations could not decrease the toxicity of LCFA effectively.

It has been shown in industrial situation that co-substrates, such as carbohydrate, protein and lipid could benefit the anaerobic degradation (Ahring et al., 1992). This might be related to the fact that carbohydrate and protein could be hydrolysed to glucose and amino acids and these components could benefit the growth of microorganisms under the inhibition of LCFA.
which hydrolysed from lipids. The study of the effects of co-substrates on lipid anaerobic digestion will be performed in Chapters 7, 8, and 9.

### 4.4 Conclusion

In this chapter, the effects of glucose and cysteine on decreasing the toxicity of sodium oleate and enhancing of its degradability were investigated in batch experiments. The results showed that the addition of glucose and cysteine could reduce the toxicity of SO and enhance the degradation of LCFA. The combination of glucose and cysteine was most effective in reducing the inhibition of LCFA and stimulating the degradation of LCFA.

The microbial studies illustrated that SO seriously inhibited the growth of methanogens and that the addition of co-substrates, glucose and cysteine decreased the inhibition of SO on methanogens. While glucose was more effective than cysteine on stimulating the growth of *Archaea* cells, cysteine benefited the growth of SRB cells. The co-digestion of glucose and cysteine optimally stimulated the growth of *Archaea, Bacteria* and SRB cells and the recovery of the methanogenic microbial groups to the structure prior SO inhibition. The methanogenesis and SO degradation were more likely linked to the population of *Archaea* cells than SRB cells.
Chapter 5 Influence of co-substrate supplementation on granule characteristics in a LCFA fed single stage anaerobic digester

5.1 Introduction

High-rate anaerobic methanogenic systems are becoming increasingly popular to treat wastewater from various industries including dairy, meat processing and beverage industries. Among the various designs that are commercially available the Upflow Anaerobic Sludge Blanket (UASB) design is the most popular (Fang et al., 1994a). The UASB design relies on the granulation of bacterial biomass with enhanced settling properties to enable increased retention of the microbial consortia when compared to continuous stirred tank digesters. The formation of granules is a complex process involving inorganic and organic compounds as well as the bacterial communities.

The microstructure of UASB granules depends on physio-chemical parameters and the feed composition (Fang et al., 1994a, b; Alpenhaar et al., 1994). With TEM and SEM, it was observed that granule microstructures differed depending on the substrate feed. Granules (Fang et al., 1994a) from UASB reactor that was fed with sucrose as the major carbon source was shown to be dense containing diverse bacterial population arranged similar to the three-layered structure proposed by MacLeod et al. (1990). On the other hand granules fed hydrolysed proteins had a coarsely packed structure with multiple cracks with satisfactory settling characteristics (Fang et al., 1994b) and were predominantly smaller than the
granules fed carbohydrates. Data on the nature of microbial aggregation when fed with lipids or LCFA are scarce. It is known that LCFA and lipids inhibit the formation of granules (Sam-soon et al., 1991; Rinzema, 1988; Koster and Cramer, 1987) and that the adsorption of LCFA on to the granules can result in its flotation and washout (Hwu, 1997). Sam-soon et al. (1991) found that a digester fed with oleate could not produce granular sludge and that the degradation of oleate was very poor. The difficulty encountered during lipid and LCFA wastewater treatment has been mainly attributed to the high concentration of these compounds in the wastewater (Beccari, 1996). Hwu (1997) reported that the addition of glucose was likely to stimulate the degradation of LCFA.

Numerous studies have been reported on the microbiology of immobilized anaerobic bacteria due to their important role in the structure and maintenance of granules (Wu, 1996; Zhu et al., 1996). Using traditional microscopic methods including scanning transmission electron microscopy (SEM) and transmission electron microscopy (TEM), several investigators have found that not only methanogens were involved in granulation, but also other bacteria, such as syntrophic bacteria were present in microcolonies (Macleod et al., 1990; Wu, 1996). However, these techniques were not sufficiently specific to differentiate between groups of microorganisms. In recent years, the bacterial distribution within granules has been examined using whole cell in-situ hybridization with molecular 16S rRNA probes (Sekiguchi et al., 1999; Harmsen et al., 1996). Sekiguchi et al. (1999) used 16S rRNA probe in situ hybridization combined with confocal laser scanning microscopy (CLSM) to investigate the spatial distribution of microbes in the granules.
A combination of TEM, CLSM and FISH techniques were applied to investigate the characteristics of microbial agglomerates fed with LCFA in lab-scale UASB digesters. The effects of glucose and cysteine addition on the degradation of LCFA and formation of granules were also investigated in this study.

### 5.2 Materials and Methods

#### 5.2.1 Anaerobic digesters and inoculum.

<table>
<thead>
<tr>
<th>Reactors</th>
<th>Constituents</th>
<th>COD ratio</th>
<th>Total concentration (gCOD.L⁻¹.day⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>R1</td>
<td>Oleate</td>
<td></td>
<td>2</td>
</tr>
<tr>
<td>R2</td>
<td>Glucose, Oleate</td>
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<td>R3</td>
<td>Cysteine, Oleate</td>
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</tr>
<tr>
<td>R4</td>
<td>Glucose, Oleate, Cysteine</td>
<td>1:1:1</td>
<td>2</td>
</tr>
</tbody>
</table>

Table 5.1 The composition of feeding nutrients

Four, 3 litre, mesophilic (37 °C) lab-scale water jacketed anaerobic digesters, with diameter 95 mm and height 1100 mm, were used in the studies reported here. These were seeded with sludge from a full-scale anaerobic sludge digester (35-37°C) at Woodman Point Sewage Treatment Plant (Woodman point, WA, Australia). The inoculum was stored at 4 °C in tightly closed plastic containers for 5 days before use. The digesters were seeded with this sludge and activated by feeding yeast extract (YE) and acetate (1:1 w/w at 1 g COD l⁻¹.d⁻¹) for 19 days until a stable volume of methane gas was achieved per day following...
which each was fed with different substrates as shown in Table 5.1. The pH of the reactors was maintained between 6.9 and 7.2 with the addition of HCl or NaOH (10 M). Feed into the digesters was introduced from below at an upflow velocity of 0.027 m/h. The characteristics of the microbial agglomerates from the digesters were examined 160, 250 and 420 days after start-up.

5.2.2 Performance of reactors.

The performance of different reactors was monitored by measuring the methane gas production rate and total COD removal rate. The methane content of the gas was determined using a Varian Model 3700 gas chromatograph fitted with a thermal conductivity detector. The gas flow rate was measured using a downward displacement of acidified water (0.05M H$_2$SO$_4$). The COD removal rate was calculated by measuring COD of the effluent and in the feed using standard methods (APHA, 1992).

5.2.3 Fluorescence in Situ Hybridization (FISH) of section of granule.

Oligonucleotide probes were used to target most of the microbial populations in the digester. The probes used and the corresponding targeted microorganisms are listed in Table 5.2. Because these oligonucleotide probes had been widely used and demonstrated (Raskin et al., 1994a; Sekiguchi, et al., 1999), the application using reference strain of bacteria was not done. The method used for sectioning granule was as described by Chui and Fang (1994), and the FISH method was modified from the techniques of Sekiguchi et al. (1999).
Hybridization of the 16S rRNA-targed oligonucleotide probes in the granule section was performed at 40°C for 3 h instead of 10h as described by Sekiguchi et al. (1999). This is because too long a hybridization caused difficulties in washing off unhybridised fluorescence probes. Sekiguchi et al. (1999) used Cy-5 and rhodamine labels to show the green and red colour in the different layers. While the distribution of specific groups was clearly visible, the intensity of the fluorescence signal from different groups could not be compared easily. In this study, we chose to use only rhodamine labelled probes to be able to compare the fluorescence intensity of different microbial subpopulations. In order to investigate the syntrophic relation between Archaea members and Bacteria, section of granules were double stained simultaneously by using FITC dyed EUB338 and rhodamine dyed ARC915, MB310, MG1200 and MSMX860.

<table>
<thead>
<tr>
<th>Probe Name</th>
<th>Targeted groups</th>
<th>Probe dye</th>
</tr>
</thead>
<tbody>
<tr>
<td>ARC915</td>
<td><em>Archaea</em> (domain)</td>
<td>Rhodamine</td>
</tr>
<tr>
<td>EUB338</td>
<td><em>Eubacteria</em> (domain)</td>
<td>Rhodamine, FITC</td>
</tr>
<tr>
<td>MB310</td>
<td><em>Methanobacteriales</em> (order)</td>
<td>Rhodamine</td>
</tr>
<tr>
<td>MG1200</td>
<td><em>Methanomicrobiaceae</em> (family)</td>
<td>Rhodamine</td>
</tr>
<tr>
<td></td>
<td><em>Methanocorpusculaceae</em> (family)</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Methanoplanaceae</em> (family)</td>
<td></td>
</tr>
<tr>
<td>MSMX860</td>
<td><em>Methanosarcinaceae</em> (family)</td>
<td>Rhodamine</td>
</tr>
</tbody>
</table>

Table 5.2 Oligonucleotide probes used in the experiment.
5.2.4 Granule Characterization.

The whole granule structure was visualised through light microscope (LM). Granule size was presented by diameter of the granule. TEM and CLSM methods and their sample preparation were conducted as described by Fang et al. (1994a) and Sekiguchi et al. (1999). While TEM provided information on the ultrastructure of bacteria, CLSM illustrated the detailed location of specific bacteria in the granule.

5.3 Results and Discussion

5.3.1 Performance of the digesters.

Fig. 5.1 shows that the digester fed with only oleate achieved about 10% COD removal even after 420 days operation, suggesting that the adaptation of anaerobic bacteria to the toxicity of oleate is very difficult. This is in agreement with other observations (Koster and Cramer, 1986, Rinzema, et al., 1988). The digester fed with oleate and cysteine also showed a lower adaptation process in which only around 50% COD removal was achieved after 420 days. The COD removal of digester fed with glucose and oleate achieved 90% after only 50 days of adaptation, whereas the digester fed with glucose, cysteine and oleate reached nearly 90% of COD removal after 400 days, suggesting that glucose was more effective than cysteine in reducing toxicity of oleate or in enhancing the activities of reactor. Although cysteine has been shown to be a necessary nutrient in anaerobic bacteria and tissue culture, it has also been reported that feeding cysteine might immediately reduce the generation of pelletised mass (Sam-soon et al., 1991). These contradiction results might be related to the concentration of cysteine, as high concentration of cysteine could inhibit the methanogenesis as shown in Figure 4.1 (Chapter 4).
Fig. 5.1. The performance of the four UASB digesters. (a) COD removal efficiency, (b) volumetric methane production.
Enhancing Anaerobic Degradation of Lipids in Wastewater by Addition of Substrate - Y. Kuang

Chapter 5 Influence of co-substrate supplementation on granule characteristics in a LCFA fed single stage anaerobic digester
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Enhancing Anaerobic Degradation of Lipids in Wastewater by Addition of Substrate - Y. Kuang
Chapter 5 Influence of co-substrate supplementation on granule characteristics in a LCFA fed single stage
anaerobic digester
Figure 5.2 Comparison of granular structure of sludge from the different UASB reactors by using LM (a, b and c), TEM (d, e and f) and CLSM (g to u showed the sections hybridised with rhodamine labelled probes; A to L shows sections double hybridised with rhodamine labelled methanogenic probes) and FITC labelled *Bacteria* probes). Left column shows granule from R3 which was fed with oleate and glucose, middle column shows granule from R2 which was fed with glucose and cysteine, and right column shows granule from R4 which was fed with glucose, cysteine and oleate.

<table>
<thead>
<tr>
<th>Feed composition</th>
<th>Size</th>
<th>Structure</th>
<th>Distribution of microbial groups</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oleate + Glucose</td>
<td>1-3mm</td>
<td>Layered structure (a)</td>
<td>ARC915 Whole Granule (g)</td>
</tr>
<tr>
<td></td>
<td>(a)</td>
<td>(a)</td>
<td>EUB318 Outer region (j)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>MG1200 Whole Granule (m)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>MSMX 860 Whole Granule (p)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>MB310 Whole granule (s)</td>
</tr>
<tr>
<td>Oleate + Cysteine</td>
<td>0-1mm</td>
<td>No layered structure (b)</td>
<td>ARC915 Whole Granule (h)</td>
</tr>
<tr>
<td></td>
<td>(b)</td>
<td>(b)</td>
<td>EUB318 Mainly outer &amp; centre region (k)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>MG1200 Sparsely distributed (n)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>MSMX 860 Outer layer (q)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>MB310 Sparsely distributed (t)</td>
</tr>
<tr>
<td>Oleate + Cysteine+ Glucose</td>
<td>1-3mm</td>
<td>No layered structure. Fine outer region (c).</td>
<td>ARC915 Whole Granule (i)</td>
</tr>
<tr>
<td></td>
<td>(c)</td>
<td></td>
<td>EUB318 Whole granule (l)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>MG1200 Mainly outer region (o)</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>MSMX 860 Whole granule (r)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>MB310 Outer region (u)</td>
</tr>
</tbody>
</table>

Table 5.1 Comparison of structure and composition of granules from the digesters. The respective figures from Fig.5.2 from which the observation were made are also indicated in the table.
5.3.2 Comparison of microstructure of granules from digesters.

The digester fed with oleate did not form any ‘typical’ granules even after 420 days. However, small flocs about 20-50µm were seen. This might be related to the inhibition by oleate on the growth of microorganisms, and subsequent wash out of the microorganisms before proper granulation (Yan and Allen, 1997). It is well known that granulation depends on several parameters including the wastewater composition and process conditions (Schmidt and Ahring, 1996). Moreover, the bacteriological composition and structure of granules adapted to different feeds are different (Grotenhuis et al., 1991). In a granular sludge blanket, three distinct zones of behaviour can be identified. They include a lower active zone, a middle active zone and an upper inactive zone (Wentzel et al., 1994). Sam-soon et al. (1991) found that the pelletization and granulation did not develop in the sludge bed when a typical LCFA, (oleate) was used as sole substrate to a digester. The results from this study confirmed the previous observations. It might also imply that the failure of granule development with the use of LCFA as the sole substrate is related to the difficulty of LCFA degradation.

The characteristics and the distribution of microbial groups within the granules from the digesters fed with oleate, and cysteine and/or glucose are given in Table 5.1. The corresponding figures from which these observations were made are shown in Figure 5.2.
A granular sludge bed in the digester fed with oleate and glucose (R2) was established after 160 days of operation and a sample of the granules was taken after 250 days. They had a clear-layered structure, which was very similar to the granules from a UASB treating brewery wastewater (Fang et al., 1994a). The diameter of the granule was about 1-3 mm and had a typical three-layered structure (Fig. 5.2a). The outer layer was loose, the middle and inner layers were dense. The section observed by TEM illustrated that rod shaped microorganisms and cocci microorganisms were densely packed together (Fig. 5.2d). CLSM showed that the members of *Archaea* were distributed all over the granule (Fig. 5.2f).

These observations were very different from those of Sekiguchi et al. (1999) and Harmsen et al. (1996) in which *Archaea* cells were mainly present in the inner and middle layers. Observations showed that the members of *Bacteria* were mainly occupying the loosely packed outer layer (Fig. 5.2j). This was in accordance with the investigation of Sekiguchi et al. (1999) and Harmsen et al. (1996) in which *Bacteria* cells were mainly occupied in outer layers. This is also in agreement with the structure proposed by MacLeod et al. (1990), in which acidogenic bacteria were mainly in the outer layer of granule. The members of families *Methanomicrobiaceae*, *Methanocorpuslaceae* and *Methanoplanaceae* (Fig. 5.2m), and family *Methanosarcinaceae* (Fig.5.2p) and order *Methanobacteriaceae* (Fig.5.2s) were observed in the whole granule (Fig. 5.2s). In three-layered structure model of MacLeod et al. (1990), methanogens were in middle and outer layers, however, granules from this study showed that methanogenic groups were distributed in the whole granule. The reason might be related to the fact that the presence of oleate decreased the methanogen growth rate resulting in high concentrations of acetate in whole granule which may have benefited the growth of methanogen throughout the granule.
A granular sludge bed in the digester fed with oleate and cysteine (R₃) developed after about 250 days of operation. The granules were very small (diameter 500 µm-1 mm). The granules were very loose and lacked layered structure (Fig. 5.2b), and they were very similar to the structure of glutamate-degrading granule structure discussed by Fang et al. (1994b). The granule had a fine outer layer. The centre appeared to have large and thick clumps, which was interspersed with groups of organisms of various morphologies showed by TEM (Fig. 5.2e). There was no evidence of syntrophic microcolonies. There were large numbers of bacterial cells of abnormal morphologies throughout the section, which might have been non-methanogenic bacillus. CLSM images showed that Archaea cells were the dominant cells in the granules (Fig.5.2h). The members of Bacteria (Fig. 5.2k) were located in outer and centre region. The members of families Methanomicrobiaceae, Methanocorpuslaceae and Methanoplanaceae (Fig. 5.2n), and order Methanobacteriaceae (Fig.5.2t) were observed in the whole granule. The cells of family Methanosarcinaceae (Fig.5.2q) mainly occupied in outer region of granules.

In the digester fed with oleate, cysteine and glucose (R₄), a granular sludge bed was formed after about 120 days of operation and granules were taken at day 250 with the same time granules were sampled from the other two digesters. These granules were about 1-3mm in diameter and larger and denser than the granules from the digester fed with oleate and cysteine. The overall structure of these granules (Fig.5.2c) was similar to that of the granules fed with oleate and cysteine (Fig. 5.2b) and did not exhibit the typical layered structure. The granular structure of combined feeding of glucose, cysteine and oleate was very similar to that showed in Batstone’s thesis and the reason for the lack of layered structure could be explained by the slow growth of acidogens, acetogens and methanogens (Batstone, 2000).
In this experiment, there was no layered structure in the granules from reactors fed with cysteine, implying that cysteine inhibited the formation of layered structure. TEM (Fig. 5.2f) indicated that the bacteria distributed in the granules were more complex and looser than the granule from digester fed by oleate and glucose (Fig. 5.2d). CLSM images showed that *Archaea* cells (Fig. 5.2i) existed in the whole space of the granules. The *Bacteria* cells grew throughout the whole granule as different colonies (Fig. 5.2l). The spatial distribution of the members of families *Methanomicrobiaceae*, *Methanocorpuslaceae* and *Methanoplanaceae* (Fig. 5.2o), and family *Methanosarcinaceae* (Fig.5.2r) were observed in the whole granule. The cells of order *Methanobacteriaceae* (Fig.5.2u) mainly occupied in the outer region of the granules.

The comparison of the rhodamine fluorescence intensity signals from three type granules showed that the signal intensity generated by *Archaea* probes were stronger than the intensity from *Bacteria* probe, suggesting that the growth rate of methanogens was the highest. The *Archaea* probe signal generated by in the granules fed with oleate and glucose or oleate, glucose and cysteine were stronger than that from the granule fed oleate and cysteine, suggesting that the growth rate of *Archaea* was lower without glucose feeding. This is in agreement in the activities of the reactors, in which the methanogenic activities were the lowest in reactor fed with oleate and cysteine. In the granules from the three digesters, *Methanosarcinacea* cells (targeted by MSMX860) had the strongest signals compared to the other two methanogenic probes, suggesting that *Methanosarcinace* cells were the dominant methanogens in three digesters.
With the double staining red rhodamine labelled methanogenic probe and green FITC labelled *Eubacteria* probe, the syntrophic relation of *Archaea* members and *Bacteria* cells was visualized as the yellow colour in the CLSM images (Fig. 5.2 A to L). The spatial distribution of syntrophic groups were mainly located in the outer regions of granules, implying that the outer region was more active than the inner region of granule.

It has been documented that the addition of glucose could enhance the biodegradation of LCFA (Hwu, 1997). However, the effects of co-substrate addition on the granulation in digesters fed with LCFA were still unknown. Results of this study showed that the addition of co-substrates could not only decrease the inhibition by LCFA but also promote granulation. However, the extent of granulation depended on the choice of co-substrates.

The rate for granulation was dependent on the feed composition. The study showed that the granulation time in digester fed with oleate, glucose and cysteine was the shortest among the four digesters. Granulation in the digester fed with oleate and glucose was faster than granulation in the digester fed with oleate and cysteine. The reason for this might be that glucose stimulated the growth of different bacterial groups and hence enhanced the rate of granulation. Glucose feed has been found to be excellent for the formation of the pellet or granules in UASB (Sam-Soon *et al*., 1991). Cysteine also improved granulation but concentrations above 12.2mg/l could reduce the specific pellet yield (Sam-Soon *et al*., 1987). Findings from this study indicated that supplementation with cysteine alone did not enhance granulation but addition of glucose was necessary to promote granulation.
5.4 Conclusion

Results indicated that addition of glucose and cysteine along with oleate not only decreased the inhibition of oleate on methanogenic activity but also improved the formation of granular biomass. The presence of glucose and cysteine enhanced the granulation and methanogenic activity of biomass. However, addition of glucose alone established higher COD removal and methane production rate much more rapidly than cysteine alone or in combination, suggesting that glucose addition may have had the best result in relieving toxicity of oleate. Microscopic images also showed that biomass from the digester fed oleate and glucose formed the biggest granules (2-3mm), which in turn may have had better settling properties, and closely resembled granules from a sucrose fed digester (Fang et al., 1994a). As reported by Fang et al. (1994b) the size of granules from digesters which were fed cysteine were smaller than that which was fed glucose but addition of glucose in the presence of cysteine increased granule size. The CLSM images of hybridised granule sections showed distribution of microbial groups was dependent on feed compositions. ARC 915, which targeted Archaea cells including all methanogens showed that methanogens within the granules from digester fed with oleate and cysteine were less abundant than within the other two digesters. This is in agreement with the methane production rate measurements from three digesters.
Chapter 6 Enhancing recovery of methanogenesis from LCFA inhibition by feeding glucose and cysteine in a single stage UASB reactor

6.1 Introduction

Previous studies (Hanaki et al., 1983; Hanaki 1981) and results in chapters 4 and 5 have shown that even low concentrations of LCFAs can inhibit the methanogenic process. It has been suggested that LCFAs are toxic for bacteria because of their damaging effect on the transport channel of bacterial cell by inducing lysis of protoplasts (Galbraith et al., 1971, Galbraith and Miller, 1973). It has been observed that after a period of inhibition, methanogenic activity would reappear suggesting that there was an adaptation of the bacteria to the presence of LCFAs (Angelidaki et al., 1990; McAvoy et al., 1996). However, this adaptation is a prolonged process and could last several months even under low concentrations of LCFAs and was far too long for practical purpose. Furthermore, the only way to recover the inhibited reactor in real plant is to re-inoculate although this is an expensive operation (Rinzema, 1988; Koster and Cramer, 1986). Rinzema (1988) suggested that the adaptation to LCFAs or the recovery of methane production under LCFA exposure could be explained by the re-growth of a small number of surviving bacteria. It has also been shown that the addition of calcium or bentonite can decrease LCFA toxicity as well as enhance its degradation (Koster, 1987). However, the addition of calcium ion or bentonite would only temporarily decrease the concentration of LCFA and the toxicity due to LCFA would appear after several hours (Koster, 1987). These methods may therefore not be
Despite these observations, anaerobic digestion has been successfully used to treat complex wastewater containing mixture of lipids, carbohydrates and proteins (Batstone, 1999). The presence of co-substrates like carbohydrates and protein could reduce the toxicity of lipids, or the hydrolysis products of carbohydrate and protein, i.e. glucose and amino acids could reduce inhibition of LCFA. While Rinzema (1988) suggested that the recovery of bacteria could not be related to the substrate composition, Mousa and Forster (1999) reported that addition of glucose decreased the inhibition of methanogenesis due to gallic acid (C₆H₂(OH)₃CO₂H), a crystalline organic acid. The mechanism of counteraction of glucose on the inhibition of gallic acid and the effect of glucose on anaerobic bacteria re-growth under inhibition of LCFA might be related to the co-metabolism discussed at section of 2.3.2 (Chapter 2).

As previously described in Chapter 4, direct microbial investigation of microbial groups under LCFA inhibition is scarce. Knowledge of re-growth of different microbial groups during LCFA adaptation or recovery may assist to investigate the influence of glucose and amino acids on the recovery process of anaerobic digestion under LCFA inhibition.

The aim of this study was to investigate the effects of addition of substrates on the reduction of toxicity and recovery of anaerobic digestion from LCFA inhibition. FISH techniques were applied to identify and quantify the shifts of main microbial groups. Sodium oleate (SO) was used as the model LCFA in this study. It was used because of its well-understood
biochemical degradation pathway, its predominance in wastewater and its high solubility as a sodium salt (Hanaki et al., 1983; Hwu et al., 1996). Glucose and cysteine were used as the co-substrates. Glucose represents the hydrolysis product of carbohydrate degradation and cysteine represents the product of protein degradation.

6.2 Materials and methods

6.2.1 Anaerobic digesters. Four mesophilic (37 °C) water jacketed bench-scale UASB reactors (labeled A, B, C, D) of 95 mm diameter, 1100 mm height and 2 L working volume, were seeded with granular sludge as described in 3.1.2. The sludge originated from a hybrid UASB reactor (35-37°C) of Spearwood Wastewater Treatment Plant treating wastewater from pig abattoir (Perth, WA, Australia). The sludge was stored at 4 °C in tightly closed plastic containers for 14 days before being used. The four digesters were reactivated for 19 days by continuously feeding a solution of 1.5 g COD. L⁻¹ yeast extract and 1.5 g COD. L⁻¹ acetate at a loading rate of 3 g COD. L⁻¹.d⁻¹ until stable methanogenic activity was attained. After reactivation, the digesters A, B, C and D were fed only with a solution of 100 mg COD.L⁻¹ SO at loading rate of 100 mg COD. L⁻¹.day⁻¹ for 14 days after all digesters lost almost all methanogenic activity i.e. no methane gas was produced. Digester A was treated as the control for the investigation of the adaptation process by continuing to be fed with SO. While the digester B was only fed with glucose 3 g COD.L⁻¹, the digester C was fed with only cysteine 3 g COD. L⁻¹ and digester D was fed with both glucose and cysteine, 1.5 g COD.L⁻¹ of each. The loading rate of reactors B, C and D were all kept at 3 g COD. L⁻¹ day⁻¹. During the experiment, the pH of the reactors was kept between 6.9 and 7.2 with the addition of drops of HCl or NaOH (10M).
The techniques of cell fixation, whole cell hybridization, quantification of microbial groups under different condition and obtaining of Epifluorescence micrographs were as described in 3.3 and 3.4.

6.3. Results and Discussion

6.3.1. Comparison of performance of UASB reactors.

The data in Figure 6.1 illustrates the changes in methanogenic activities during the experiment. After 10 days of reactivation, the production of methane gas from four reactors was similar and about 900 ml. L⁻¹.day⁻¹. On day 19 onwards, reactors were fed SO, consequently the methane production from the four reactors began to drop sharply.

![Fig. 6.1 The methanogenic activities after feeding different substrates](image)

After 14 days of feeding with SO, no methane gas was produced from the four reactors.
From day 33, the feed to three out of four reactors was changed. Reactor A was controlled to be fed with SO and was the control reactor. It did not produce any methane gas during the whole experiment, showing that it was difficult for anaerobic bacteria to adapt or recover from the inhibition of LCFA if LCFA was provided as the only carbon source. Methane production from Reactor B began to increase within 2 days of switching to glucose feed. It increased steadily and produced about 600 ml methane per day (about 65% of daily methane gas production rate prior to inhibition by SO) 10 days after switching to glucose feed, and it was maintained around 600 to 650 ml for the following 70 days. It suggested that glucose could effectively stimulate the adaptation or recovery of anaerobic bacteria from the inhibition of LCFA.

Compared to glucose, cysteine (Reactor C) had less effect on stimulating the methanogenic activities. After 20 days, there was about 20% of daily methane gas production rate prior to inhibition by SO recovered, and only about 30% of daily methane gas production was recovered after 80 days.

When fed with glucose and cysteine (Reactor D), in the first 10 days, the methane gas production was not as high as feeding only with glucose. However, the methane gas producing increased sharply after 10 days and leveled off around 900 ml per day after 30 days. The feeding of combination of glucose and cysteine was not as efficient as only glucose only for short recovery period from SO inhibition. However, it was more efficient than feeding glucose only for long term recovery after SO inhibition. Even though the total COD in different feeding was the same, the methane production were different in different
reactors, indicating that methanogenesis during the recovery of reactors was related to the composition of organic substrates.

The degradation of SO in the control was kept very low (Fig.6.2), however, it increased when fed with glucose, cysteine or glucose and cysteine, suggesting that glucose and cysteine enhanced the degradation of SO. In them, glucose was more effective than cysteine in enhancing the degradation of SO, while the combination of glucose and cysteine had the best result on the SO degradation.

Fig.6.2. The residual of sodium oleate in four UASB reactors.
6.3.2 Identification and quantification of the microbial subpopulations under inhibition by sodium oleate

In the reactor, acidogens, acetogens, and methanogens were expected to mediate conversion of SO, glucose, or cysteine to methane and carbon dioxide. All methanogens are in the domain *Archaea*, and acidogens and acetogens are in the domain *Bacteria*.

The morphological difference of microbial groups before (day19) and after (day33) SO feeding was visualised on epifluorescence micrographs of FISH using rhodamine-dyed domain probes ARC915 (Fig.6.3). After SO feeding, the long curved rod methanogens which might be related to the families *Methanobacteriaceae* and *Methanomicrobiaceae* were rare (Fig 6.3a). Moreover, the epifluorescence intensity of *Archaea* cells before feeding with SO was stronger than that after feeding with SO, suggesting that the number of *Archaea* cells had decreased after feeding with SO. Figure 6.3 (b and c) presents the morphological differences of order *Methanobacterales* (targeted by MB310) and family *Methanosarcinaceae* (targeted by MSMX860) after feeding SO. The fluorescent signals or epifluorescence after feeding SO were all dimmer than before SO feeding with both probes, and the cell numbers also decreased in all groups of methanogens.
Fig. 6.3 Photographs of different probes with FISH before (day 19, left) and after (day 33, right) SO feeding. (a) domain *Archaea* (ARC915). (b) order *Methanobacteriales* (MB310). (c) Family *Methanosarcinaceae* (MSMX860).

Figure 6.4 illustrates the results of the quantification of different subpopulation changes after feeding SO. Compared to *Archaea* cell, *Bacteria* cells suffered less from inhibition by toxicity of SO. This is in good agreement with previous experiments (Beccari *et al.*, 1996;
Rinzema et al., 1994). The results also showed that amongst the methanogen groups, family *Methanosarcinaceae* cells were the most sensitive to the toxicity of sodium oleate. Because the family *Methanosarcinaceae* includes *Methanosarcina* and *Methanosaeta*, the only two genera that have been confirmed so far as acetotrophic methanogens to use acetate, this might be the main reason why LCFA could severely inhibit the methanogenesis.

![Bar chart showing cell numbers in control reactor before and after feeding SO](image)

**Fig. 6.4** The cell numbers in control reactor before (day19) and after (day33) SO feeding.

### 6.3.3 The re-growth of anaerobic microbial subpopulation during recovery with cysteine and glucose addition.

The results of the control experiment (Reactor A) indicated that there was no obvious recovery of both in *Archaea* and *Bacteria* groups even after 80 days (Fig. 6.5). The previous research (Raskin et al., 1994b; Zhang and Noike, 1991) and the data of chapter 4 showed higher quantity of *Archaea* cells than *Bacteria* cells when the anaerobic digester was operating successfully and was efficiently converting substrates to methane. In this experiment, the number of *Archaea* cells was lower than the number of *Bacteria* cells even
after 80 days, there was higher quantity of *Archaea* cells than *Bacteria* cells when anaerobic digester was operating stably and efficiently converting substrates to methane. In this experiment, the number of *Archaea* cells was lower than the number of *Bacteria* cells even after 80 days, indicating that methanogenic activities were more difficult to recover than acidogenesis activities from the inhibition of SO (Becarri *et al.*, 1996). Amongst the members of *Archaea*, the re-growth of family *Methanosarcinacene* was the slowest in these whole methanogenic groups, suggesting that *Methanosarcinaceae* cells were more sensitive to the toxicity of SO.

![Fig.6.5. Analysis of microbial populations recovery from LCFA inhibition in control reactor after 33 days.](image)

In reactor B fed with glucose after SO inhibition, the numbers of *Archaea* cells immediately increased during the first 20 days (fig.6.5), and nearly reached the population level before feeding SO (Fig.6.3). Interestingly, the recovery of number of *Bacteria* cells was not as
significant as that of the number of *Archaea* cells. The members of *Methanosarcinaceae* had the best recovery in methanogenic groups after feeding glucose; implying that glucose could stimulate the acetotrophic methanogens growth effectively, thus accelerate the conversion of acetate into CH\(_4\) and CO\(_2\).

In the reactor C fed with cysteine after SO inhibition, the numbers of *Archaea* did not increase as fast as when fed with glucose, whereas the numbers of *Bacteria* increased faster than when fed with glucose (Fig. 6.5). The numbers of *Archaea* cells as well as the numbers of methanogens were all lower than that in the reactor which was fed glucose. This could be linked to the fact that cysteine could not assist as effectively with the recovery of methanogenesis as glucose (Fig. 6.1). Interestingly, the numbers of *Bacteria* cells were much higher than those in the glucose fed reactor, in fact even higher than the numbers before SO inhibition, implying that cysteine benefited the growth of *Bacteria* cells.

The data of numbers of *Archaea* and *Bacteria* cells from feeding of glucose or cysteine, showed that glucose was more effective to stimulate the growth of *Archaea* cells than cysteine, while the cysteine was more beneficial to the recovery of *Bacteria* cells than *Archaea* cells in the reactor under inhibition of LCFA.
Chapter 6 Enhancing recovery of methanogenesis from LCFA inhibition by feeding glucose and cysteine in a single stage UASB reactor

Fig. 6.6 Recovery of microbial populations under (a) glucose and (b) cysteine feeding.
Figure 6.7 illustrates that feeding a combination of glucose and cysteine could significantly increase both the re-growth of *Archaea* and that of *Bacteria* cells. The re-growth of *Bacteria* cells was very similar to that observed with feeding cysteine only but the increase was slower. It might be related to the lower concentration of cysteine in combined feeding (50% of only cysteine feeding). Although the re-growth of *Archaea* cells was not as fast as observed when fed glucose only, the combined feed promoted higher population level than glucose only after 30 days, suggesting that the combination glucose and cysteine was more effective in stimulating the growth of *Archaea* cells than glucose only. The population levels of re-growth of all methanogenic groups were also higher than those obtained with from feeding glucose or cysteine only. It could be explained by the fact that both glucose and amino acid were essential in bacterial growth and they acted as growth factors to stimulate the growth of microorganisms (Mousa and Forster, 1998).
From the experimental data shown in this study, it illustrated that the methanogenic activities at different feeding were related to the shifts microbial population structure. When fed with SO, the numbers of *Archaea* cells and *Bacteria* cells decreased sharply and this resulted in the lost of methanogenic activities. With the addition of glucose, the recovery growth of *Archaea* cells was higher than that of *Bacteria* cells and this enhanced the methanogenic activity. With the addition of cysteine, the recovery growth of *Bacteria* cells was higher than *Archaea* cells and the methanogenic activity was not as high as with glucose feed, indicating that the high numbers of *Archaea* cells were more important than those of *Bacteria* cells in enhancing the methanogenic activity. When fed with a combination of glucose and cysteine, high recovery of both *Archaea* cell and *Bacteria* cell population were achieved. Highest methanogenic activity was also achieved with this feeding mode, suggesting that the combination of glucose and cysteine was most beneficial for the recovery of methanogenic activities after the inhibition of LCFA.

Although the physico-chemical parameters, such as methane production rate, VFA and pH were measured to evaluate the effect of organic loading rate and feed composition of the anaerobic reactor, the microbial parameters could provide more information than physico-chemical parameters for understanding disturbance of operation on microbial structure. For example, when fed combination of glucose and cysteine, the increase of population of *Archaea* cells and *Bacteria* cells in Fig.6.7 could explain the increase of methane production presented in Fig.6.1. The results of microbial investigation presented detailed reactor changes in the different feeding modes by microbial population shifts that related to the efficiency of the reactors and could not be shown by physico-chemical parameters. The
efficiency of reactor could be predicted and explained by the changes of microbial communities, and this information could be used to speed up the recovery process by adjusting the substrate feeding.

Studies presented in Chapters 4, 5, 6 were focused on the effects of glucose and cysteine on the inhibition of SO using batch or lab-scale reactors. The studies in Chapters 7,8 and 9 will investigate the function of carbohydrate (starch) and protein (yeast extracts) on the degradation of lipids (canola oil) using one or two-phase UASB reactors. These experiments will simulate a real wastewater treatment plant, in which the effluent is likely to contain mixture of protein, carbohydrates and lipids. The comparison of the efficiency of the microbial and physico-chemical indicators on the monitoring of the reactor anaerobic process will also be conducted in Chapter 7.

6.4 Conclusion

Four lab-scale UASB reactors were deliberately inhibited by feeding sodium oleate. Three of them were fed glucose, cysteine or glucose and cysteine together while one reactor was operated as a control reactor and was fed continuously with sodium oleate. Two domains Archaea (targeted by ARC 915) and Bacteria (targeted by EUB 338), one order Methanobacteriales (targeted by MB310) and two family- Methanosarcinaceae (targeted by MSMX860), Methanomicrobiaceae, Methanocorpusculaceae and Methanoplanceae (targeted by MG 1200) DNA probes were used to quantify the specific groups of anaerobic microbes throughout the operation of the reactors to compare the effect of glucose and cysteine on the recovery of reactor.
It can be concluded from the methane production rate that glucose had the better effect than cysteine on recovery the methanogenic activities after inhibition of sodium oleate, while the combination of glucose and cysteine was most effective on this recovery.

The results of detection and quantification of anaerobic microbial community shifts provided more detailed information about the recovery process by feeding with glucose, cysteine or glucose and cysteine. It showed that glucose was more effective in stimulating the re-growth of methanogen groups than cysteine, while the combination of glucose and cysteine could achieve the highest recovery of Archaea cells. Feeding cysteine was more effective than glucose feeding for recovery of Bacteria cells, while the integrated feeding of glucose and cysteine had highest recovery on Bacteria cells. The numbers of “recovered” cells of Archaea and Bacteria was nearly the same as those before the SO feeding, implying that glucose and cysteine had the optimal stimulation on the growth of Archaea and Bacteria cells. The adaptation or recovery of microbial groups to LCFA might also be related to the different biological characteristic, nutrition demand of each group during different periods (Ahring et al., 1992) or other factors still unknown.

From the comparison of physico-chemical parameters and microbial parameters during the recovery of microbial organisms, it is obvious that microbial study could provide more detailed information of the recovery process. The knowledge gained from microbial studies to guide the recovery of different microbial groups from inhibition of lipids or LCFA will benefit the industry of the anaerobic digestion of lipids containing wastewater, which
currently encounters frequent shut-down events due to the inhibition of LCFA or lipids.
Chapter 7 Evaluation of lipid anaerobic digestion when co-digested with carbohydrate and protein in a one-phase UASB reactor by monitoring the microbial and physico-chemical indicators

7.1 Introduction

Anaerobic treatment of wastewater containing very high concentration of lipids is a very difficult task as lipids cause granular sludge flotation and wash-out even at very low organic loading rates due to the adsorption of lipids by biomass (Rinzema, 1988; Hwu, 1997). Lipids also cause some engineering problems, such as clogging of gas and effluent lines (Petruy and Lettinga, 1997). Furthermore, the long-chain fatty acids which are hydrolysed from lipids can also severely inhibit the methanogenic activity. This was shown in Chapters 4, 5 and 6 and previous reports (Hanaki, 1981; Koster, 1987).

Fongastitkul and Lo (1994) investigated two approaches that could enhance performance of UASB reactor in treating wastewater with high solid level: (1) increase the density of bacterial populations in the reactor. (2) increase the bacterial activities by operating at optimum conditions and supplying essential nutrients for the growth of bacteria. From both approaches, it could be concluded that the growth of bacteria in the anaerobic reactors was the key factor to the degradation of complex organic materials in wastewater. To get the optimal growth of biomass, availability of nutrient is important. Because lipids and LCFAs
inhibit the growth of biomass, provision of some other organic materials, such as carbohydrate and protein to support biomass growth during lipid digestion seems to be reasonable. Another approach to enhance lipid digestion is to co-digest lipid containing waste with other organic wastes. It has been speculated that the co-digestion of different wastes would supply sufficient nutrients for bacterial growth thus improving the biodegradability of lipid and increasing the efficiency of gas production (http://res2.agr.ca/initiatives/manurenet/en/man).

Although some work has been performed in lipid anaerobic digestion, its evaluation was limited to the physico-chemical parameters, like on-line or off-line investigation of gas composition, pH, COD and VFA (Rinzema, 1988; Hwu, 1997; Beccari et al., 1996) and the microbial analysis was usually not performed due to the difficulties to apply microbiological techniques. The physico-chemical parameters were used to control digester or diagnose problems in the digestion process. Some research has been carried out on investigating biomass in anaerobic process but indirectly through monitoring the metabolic products related to specific bacterial groups. However, the results were not always reliable because different bacterial groups could produce the same products through different process and at different operating conditions (Rozzi and Remigi, 2002).

Raskin et al. (1994a) reported that knowledge of microbial composition is necessary and may be used as a direct indicator of performance of anaerobic process. But in most wastewater plants, the routine monitoring of anaerobic digestion is performed by analysing
physicochemical indicators, such as total solid, VFA, alkalinity, pH and composition of biogas. In theory, the effect of a disturbance in the process on the microbial population will be seen before the changes of physico-chemical indicators. The changes of these physicochemical indicators are slower than biological indicators and may not be effective for reactor monitoring (Farrell et al., 1988). Primary disturbances that cause failure of anaerobic digestion process are feed overload and presence of inhibitors in feed (Rinzema, 1988). The disturbances may cause the microbial populations to decrease severely that they may have difficulty to recover. If only physico-chemical indicators are applied for monitoring, it will sometimes be very hard to control the failure of the reactor. Thus monitoring the changes in microbial community composition may be beneficial. However, the application of biomass monitoring seems impractical due to the difficulties associated with traditional microbial investigation.

The hybrid UASB reactor of Spearwood Wastewater Treatment Plant (WA, Australia) treating slaughterhouse wastewater was shut down in 1997 due to overloading of wastewater from slaughterhouse. A long period was required to re-start the reactor. The method used for analysing bacterial activity was to measure the gas production after shaking the sludge sample in a closed plastic container for several minutes. The growth and composition of the microbial community was treated as a black box. Fortunately, with the recently developed molecular techniques, which were discussed in the previous chapters, the application of small-subunit (SSU) rRNA probes to investigate the sophisticated microbial communities in the reactor is possible. Application of these techniques will provide an in-depth indication of
the composition of microbial community. Because all methanogen groups presented in anaerobic digestion are included in the domain *Archaea*, while acetogens and acidogens are included in domain *Bacteria*, domain oligonucleotide hybridisation probes were used in the experiment to monitor these groups.

The objective of this chapter was to evaluate the effect of co-digestion of protein and carbohydrate on lipid treatment in the one-phase UASB anaerobic reactor by monitoring the physico-chemical indicators and the shifts of bacterial population. From the results obtained a comparison of the effectiveness of monitoring biological parameters over physico-chemical indicators will be possible.

### 7.2 Materials and methods

#### 7.2.1 Feeding conditions and sampling

Two, one-phase UASB reactors were set up as described 3.1.2 and were seeded with granular sludge from the hybrid UASB reactor of Spearwood Wastewater Treatment Plant (Australia, WA). The hybrid UASB reactor treats wastewater from a pig abattoir and dairy processing plants which are high in fat and protein. Therefore, it was expected that the granular sludge will easily acclimatize to the lipid (canola oil), protein (yeast extracts) and carbohydrate (starch) feed in the studies.
One of UASB reactors was only fed with canola oil and set up as a control reactor. It was operated in parallel to another UASB reactor. This other UASB reactor was fed with varying composition of canola oil, starch and yeast extract in different runs (as shown in Table 7.1). Before feeding, both reactors were reactivated by 2 gCOD L\(^{-1}\) day \(^{-1}\) of starch for 1 week to obtain stable methanogenic activity. Each run lasted 40 days before shifting to the next run. Sampling of sludge for physico-chemical and microbial assays in each run was taken every day for the first 5 days, and then taken once every 5 days. The reactor was operated at a hydraulic retention time (HRT) of 4 days and the loading rate was kept at 2 gCOD L\(^{-1}\) day \(^{-1}\). The temperature of the reactor was maintained at 35 °C using a water jacket connected to a water bath.

<table>
<thead>
<tr>
<th>Component</th>
<th>Control</th>
<th>Run1</th>
<th>Run2</th>
<th>Run3</th>
<th>Run4</th>
<th>Run5</th>
<th>Run6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Starch</td>
<td>0</td>
<td>60</td>
<td>50</td>
<td>40</td>
<td>30</td>
<td>20</td>
<td>10</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>0</td>
<td>30</td>
<td>30</td>
<td>40</td>
<td>40</td>
<td>40</td>
<td>40</td>
</tr>
<tr>
<td>Canola oil</td>
<td>100</td>
<td>10</td>
<td>20</td>
<td>20</td>
<td>30</td>
<td>40</td>
<td>50</td>
</tr>
</tbody>
</table>

Table 7.1 Feed composition for the different runs expressed as the % of 2 gCOD L\(^{-1}\) day \(^{-1}\).

Note: 1 g carbohydrates (starch) is equivalent to 1.01 g COD; 1 g lipid (canola oil) is equivalent to 2.86 g COD; 1 g protein (yeast extract) is equivalent to 1.35 g COD
7.2.2 Monitoring of Physico-chemical indicators

The physico-chemical parameters monitored were total VFAs (acetate, propionate, butyrate) concentration and daily methane production. They were analysed by the methods described in 3.6.

7.2.3 Microbial analysis of UASB reactor

The dynamic changes of *Eubacteria* and *Archaea* during the six runs were analysed by dual staining the sludge samples by FISH and DAPI methods.

The FISH method which was as described in 3.4 and the DAPI (Hicks *et al*., 1992) method were applied to identify and quantify the population changes during the different runs.

7.2.4 Analysis of lipid, carbohydrate and protein

Methods for analysis of oil, starch and yeast extract were as described in the section 3.7. The extent of the adsorption of oil on sludge was determined separately by setting up batch assays containing the same amount of sludge as in the UASB reactor, except that the sludge was autoclaved. The adsorptions of yeast extract and starch were determined with the same method. The extent of the degradation of each component (g COD/L) was calculated according to:
7.3 Results and discussions

7.3.1 Lipid inhibition on UASB reactor

In the control experiment, the addition of canola oil (2 gCOD. L\(^{-1}\) day\(^{-1}\)) caused severe inhibition of methanogenic activity. As shown in Fig.7.1 the methane production rate dropped to zero within 3 days of the oil being introduced. This inhibition lasted for the duration of experiment. Lipid analysis showed that there was about 10% degradation of lipid during the first 5 days, and less than 5% thereafter, suggesting that canola oil did not only inhibit the methanogenic activities, but also inhibited the acidogenic activities as reported previously (Hanaki et al., 1987). The numbers of Archaea and Bacteria cells were very low during the whole process, around \(10^3\) and \(10^6\) /ml, respectively. As reported by Hwu (1997) and Rinzema (1988), the oil clogged the gas and effluent lines of the reactor and the reactor contents turned to a dark brown colour (Hwu, 1998; Rinzema, 1988).
**Fig. 7.1 Methane production rate in the control reactor.**

### 7.3.2 The influence of feed compositions on digester performance.

Figures 7.2 and 7.3 show the methane production rate and the total VFA during the operation of UASB using various feed compositions. Upon introducing lipid into the reactor along with starch and yeast extract after the initial reactivation with starch alone, methane production and VFA both decreased even though the total loading rate was kept the same. This indicated that the higher loading rate of oil or yeast extract decreased the methanogenic and acidogenic activities. When reactor was shifted from 100% starch feeding to starch, yeast extract and oil (Run1, ratio 6:3:1 in w:w:w), the methane production and extent of degradation of starch, yeast extract and oil all slightly decreased (Fig.7.2). When oil feeding was increased from 10 to 20%, starch feeding decreased from 60 to 50% and yeast extract was kept same (Run1 to Run2), the methane production decreased from 500 to 325 ml/l.day, while the production of VFA decreased from 253 to 181 mg/l and extent of degradation of
oil decreased 45 to 26%. This suggested that the increase of oil concentration decreased both the acidogenic and methanogenic activities of the UASB reactor. From Run 2 onwards, the methane production continuously decreased, this could be attributed to the increase of the loading rate of yeast extract or oil and the decrease of starch. However, it is more likely that 20% loading rate of oil exceeded the threshold oil loading rate for the UASB reactor and from 40 days i.e. beginning of Run2, the biomass was being washed out from the digester and leading to a decrease of methane production. The extents of degradation of starch and yeast extract also continuously decreased, suggesting that higher concentration of oil inhibited the degradation starch or yeast extract.

Interestingly, with the decrease of methane, VFA concentration decreased continuously instead of increasing. This could be attributed to the inhibition of oil on acidogenesis and acetogenesis as well (Fig.7.3). The decrease of acidogenesis and acetogenesis caused a decrease of VFA production. The LCFA concentrations at Run1 were the lowest. It increased with the increase of oil loading rate indicating that lower oil loading rate in co-digestion benefitted the LCFA degradation or transformation. Because high concentration of LCFA is toxic to the methanogens as well as to the acidogens, lower methane production and VFA were produced in the high loading rate runs.

Because starch (60%), yeast extract (30%) and oil (10%) were in total 2g COD/l.day feeding, it could be calculated that 1.2, 0.6 and 0.2 g COD /l.day starch, yeast extract and oil were fed to UASB reactor, respectively. From the results of the extent of degradation of
starch (85%), yeast extract (73%) and oil (45%) at Run1 (mean value), it could be calculated that 1.02, 0.43 and 0.09 g COD/l.day hydrolysed from starch, yeast extract and oil, respectively. If all the hydrolysed products proceeded to methane, about 557 ml methane could be produced per day. This value is very near that of methane produced in the Run1 (502 ml/day), suggesting that only acidogenesis was depress in Run1. The methanogenesis rate could be calculated as the methane production (502ml/day) in the reactor divided by theoretical methane production (557ml/day), i.e., 90.1%. These calculations were performed for all runs and shown at table 7.2. It showed that with the increase of oil and yeast extract loading rate, the methanogenesis rate decreased. Taking the LCFA results (Fig.7.5) in conjunction with the methane production data suggested that a higher loading rate of carbohydrate in co-digestion would benefit the methanogenic and acidogenic or acetogenic activities.

<table>
<thead>
<tr>
<th></th>
<th>Run1</th>
<th>Run2</th>
<th>Run3</th>
<th>Run4</th>
<th>Run5</th>
<th>Run6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Theoretical methane production (ml/day)</td>
<td>557</td>
<td>407</td>
<td>283</td>
<td>217</td>
<td>140</td>
<td>70</td>
</tr>
<tr>
<td>Methane production (ml/day) in UASB</td>
<td>502</td>
<td>353</td>
<td>232</td>
<td>135</td>
<td>46</td>
<td>0</td>
</tr>
<tr>
<td>Methanogenesis rate (%)</td>
<td>90.1</td>
<td>86.7</td>
<td>81.9</td>
<td>62.2</td>
<td>32.8</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 7.2. Extent of methanogenesis for different runs
Fig. 7.2 Methane production rate for the different runs.
Enhancing Anaerobic Degradation of Lipids in Wastewater by Addition of Substrate - Y. Kuang

Chapter 7 Evaluation of lipid anaerobic digestion when co-digested with carbohydrate and protein in a one-phase UASB reactor by monitoring the microbial and physico-chemical indicators

![Graph showing VFA concentrations for different runs](image)

Fig. 7.3. VFA concentrations for the different runs.
Fig. 7.4. Degradation of three organic components in feed for the different runs
Fig. 7.5 LCFA hydrolysed in the UASB reactor for the different runs.
Chapter 7 Evaluation of lipid anaerobic digestion when co-digested with carbohydrate and protein in a one-phase UASB reactor by monitoring the microbial and physico-chemical indicators.

Fig. 7.6 Comparison of the shifts of *Bacteria* and *Archaea* population for the different runs.
7.3.3 Microbial communities shifts under different feeding codes

Figure 7.6 shows that the numbers of *Archaea* cells were larger than the numbers of *Eubacteria* cells when only 10% of oil was in feed (Run 1). However, when increasing the oil ratio from 10% to 20% from Run 1 to Run 2, the numbers of *Archaea* cell decreased sharply. However, it was kept stable from Run 2 to Run 3 when the oil loading rate was the same even though the loading rate of starch decreased and that of yeast extract increased. These suggested that the numbers of *Archaea* cells were only related to the concentration of oil, and that the concentrations of starch and yeast extract were less important. With the increase of oil loading rate from Run 4 to Run 6, the numbers of *Archaea* cells decreased, indicating that higher oil loading rate or oil concentration did not benefit the growth of *Archaea*. This is in accordance with the methanogenesis rate shown in the Table 7.2 in which the methanogenesis rate decreased significantly from Run 4 to Run 6.

Unlike *Archaea* cells, *Bacteria* cell were less affected by oil addition. This is in good agreement with previous studies (Galbraith and Miller, 1973) and experiment results described in the Chapter 4. *Archaea* member were more sensitive to the inhibition of LCFA than *Bacteria*. Although both *Bacteria* and *Archaea* cell numbers decreased when the oil concentration in feed was increased from Run 3 to Run 4, the numbers of *Bacteria* cells recovered a significantly in both runs while the numbers of *Archaea* cells were inhibited severely by oil, indicating that *Bacteria* cell was less sensitive to the toxicity of oil than *Archaea* cell. However, even *Bacteria* cells demonstrated some recovery from Run 5 to
Run6, their numbers could not reach the level of Run1, suggesting that too high oil loading would also inhibited the growth of *Bacteria* cells and the recovery of *Bacteria* cell would not be completed. There was not only significant recovery of *Archaea* cell in the all runs, suggesting that the recovery of *Archaea* cells was a long process and more difficult than the recovery of *Bacteria* cell.

### 7.3.4 Comparison of the efficiency of physico-chemical and microbial monitoring

The data depicted in Fig. 7.2 (methane production rate), Fig.7.3 (VFA concentrations), Fig.7.4 (extent of degradation of organics) and Fig.7.5 (LCFA concentrations) showed that physico-chemical parameters responded slowly when the loading rate of oil was changed. However, the shifts of bacterial population, especially for *Archaea* cells were significantly faster in the same conditions. After feeding 10% oil (Run1), the *Archaea* cell numbers exhibited a Log 2 reduction after 18 hours only, while the methane production rate only decreased by 50ml/day after 5 days. The VFA concentrations and the extent of organics degradation did not change appreciably. A Log 4 reduction of the *Archaea* cell numbers was observed when the oil loading rate increased from 20% to 30% (from Run3 to Run 40) compared to a decrease of less than 10 mg/l in the VFA concentration in 5 days. This indicated that investigating *Archaea* cell population shifts would be more effective than were observing the changes of physico-chemical indicators when toxic organic wastes were fed. By the time the significant changes in physico-chemical indicators were observed, the
anaerobic digester would have been severely inhibited and difficult to recover. However, there are still some difficulties in monitoring biological parameters. These techniques are time consuming and require a skilled microbiologist to perform assay whereas the plant operator can be trained to carry out routine analysis of physico-chemical parameters. Apart from this, it seems more difficult to apply biological indicator monitoring techniques on-line or off-line than physico-chemical parameters due to the complexities in bacteria identification and quantification.

7.3.5 Efficiency of one-phase UASB in the degradation of oil combination with starch and yeast extract feeding.

The addition of starch and yeast extract significantly enhanced oil methanogenesis in one-phase UASB reactor when the oil loading rate was lower than 30% in a total of 2 gCOD/l.day. However, less than 50% of the oil was decreased when co-digested with starch and yeast extract even at the lower concentration (10%), and that percentage decreased with the increase of oil concentration. The VFA concentration decreased continuously. It indicated that one-phase UASB reactor had relatively low capacity to degrade oil even though co-digested with starch and yeast extract. Furthermore, the increase of oil concentration decreased the degradation of both starch and yeast extract. Among the three organic components, starch had the best degradation in all runs.

7.4 Summary and conclusion
The physicochemical and biological monitoring of oil degradation combined with starch and yeast extract in one-phase UASB digester was investigated in this experiment. The results showed that the monitoring of biological parameter, such as bacterial numbers to monitor the oil degradation process with different feeding modes was as effective as that of physicochemical parameters, such as VFA, methane production rate and extent of degradation of organic components. Biological monitoring could avoid digester failure resulting from wrong operation by providing early warning. Further research to develop the simple microbial methods for on-line biological parameters monitoring is necessary.

The results of this experiment also demonstrated that the application of one-phase UASB reactor was not beneficial for oil degradation either when oil was fed by itself or in combination with yeast extract and starch. This application of two-phase UASB reactor to enhance degradation of oil when co-digested with protein and carbohydrate will therefore be investigated in the Chapters 8 and 9.
Chapter 8 Lipid hydrolysis in mesophilic and thermophilic two-phase anaerobic treatment systems.

8.1 Introduction

Previous investigations have demonstrated that the hydrolysis of lipid to LCFA and glycerol was a kinetic bottleneck of the anaerobic digestion process (Beccari et al., 1996; Petruy and Lettinga, 1997). Lipid hydrolysis could be enhanced by adding emulsifying chemicals, such as the anionic surfactant sodium dodecylbenzenesulfonate (SDS) before hydrolysis (Charles et al., 1996). This treatment reduces the size of the lipid particles, providing more reactive surface area for hydrolysis (Petruy and Lettinga, 1997). However, these chemicals have been shown to be toxic to anaerobic bacteria (Petruy and Lettinga, 1997).

Yet another approach to enhance lipid hydrolysis is the use of two-phase anaerobic digestion systems which are becoming increasingly popular for wastewater treatment. These consist of two serially connected reactors, with the first reactor known as an acidogenic reactor and the second the methanogenic reactor. In the first reactor the organic matter in the wastewater is hydrolysed or fermented to organic acids. This reactor is normally operated at short hydraulic retention times, which ensures that little or no methanogenesis occurs. Since Ghosh and Pohland (1974) first proposed two-phase reactor system, many efforts had been focused on the studies of physico-chemical parameters in acidogenic and methanogenic reactors during anaerobic digestion of the complex organic materials (Yilmazer and Yenigun, 1999; Jeyaseenlan and Matsuo, 1995). However, the shifts of bacteria populations
in the two reactors under different experimental conditions is less known. It has been demonstrated that two-phase reactors for lipid anaerobic digestion had the apparent advantage over one-phase reactor in that separation of phases could decrease the inhibition of LCFA on methanogenesis (Hanaki et al., 1987; Becarri and Torrisi, 1998). Effluent from the acidogenic reactor was then pumped into the second reactor where methanogenesis mainly occurred. Inhibition of lipid on methanogenic activity could be attributed to the unsaturated LCFA. Saturated LCFA were shown not to cause any inhibition on methanogenesis (Komatsu et al., 1991). The acidogenic reactor in two-phase reactor system could saturate unsaturated LCFA due to the presence of a modest hydrogenotrophic activities (Becarri and Torrisi, 1998). Two-phase anaerobic digesters have been used successfully to treat effluent from olive oil mill and cheese whey (Beccari et al., 1996; Petruy nd Lettinga, 1997; Yilmazer and Yenigun, 1999) containing up to 200 g COD/l in lipids. The extent of lipid digestion in a two-phase operation was shown to be twice as that in a single-phase operation (Jeyaseelan and Matsuo, 1995). Hanaki et al. (1987) found that even though the performance of two-phase and single-phase system were similar when treating carbohydrates and proteins, the two-phase systems was more effective in preventing inhibition by LCFA when treating lipid. It was suggested that LCFA were transformed into less toxic forms (i.e. transformation of unsaturated LCFA to saturated LCFA) by acidogenic bacteria in the first reactor thereby preventing inhibition in the second (methanogenic) reactor (Komatsu et al., 1991). Compared to mesophilic temperatures, operation at thermophilic temperatures (> 55°C) has also been shown to improve breakdown of lipids in the acidogenic reactor (Ibrahim et al., 1984).
The study presented here evaluates the effect of a combination of parameters, like temperature, HRT and feed type, on the extent of hydrolysis of lipids in two-phase anaerobic treatment system. Following the lipid degradation studies in one phase anaerobic reactor discussed in Chapter 7, the effects of addition of protein and carbohydrate on both acidogenic and methanogenic reactors operated at various hydraulic retention times and mesophilic (35°C) or thermophilic conditions (55°C) on the extent of hydrolysis of lipid were investigated. Canola oil was used as the model lipid and yeast extract and starch as protein and carbohydrate respectively. The Archaeal and Bacterial communities in the acidogenic reactor were enumerated using rRNA fluorescence in-situ hybridization (FISH) molecular technique.

8.2 Materials and methods

8.2.1. Acidogenic and methanogenic reactors and their operation. Two sets of lab-scale, two-phase UASB processes were set up in parallel. Each two-phase UASB process was made up of two Perspex, water-jacketed bottles each of 2 litre working volume and served as the acidogenic and methanogenic reactor. Both acidogenic and methanogenic reactors of one set was operated at 35°C, while the other was maintained at 55°C. The acidogenic reactor was inoculated with 1 litre of fine sludge from the acidogenic stage (equalisation tank) and the methanogenic reactor was seeded with 1 litre granular sludge from a full-scale hybrid mesophilic UASB reactor at the Spearwood Wastewater Treatment Plant (Australia, WA). The inoculum was stored at 4°C prior to use.
A set of experiments was performed in parallel on mesophilic and thermophilic systems. Each set included four runs (Table 8.1). Each run lasted 3 months. As listed the concentrations of yeast extract, starch and canola oil in the feed for the different runs were adjusted in such a way that the total COD of the feed was 2 g COD.L⁻¹. Canola oil was emulsified by sonication with an ultrasonic clearer (Branson, Sonifier 450) before feeding and was kept at 1 g COD in run1. The feed was refrigerated at 5°C and fed to the reactor by a peristaltic pump. During each experimental run the reactor was operated at 1, 2, 3, 4 and 5 day hydraulic retention time. The reactor was maintained at each hydraulic retention time for two to three weeks. The inoculum in the reactor was changed to the original inoculum from the full-scale treatment plant at the end of each run so as to keep the starting inoculum for all runs the same.

<table>
<thead>
<tr>
<th></th>
<th>Run1 (mgCOD/l)</th>
<th>Run2 (mgCOD/l)</th>
<th>Run3 (mgCOD/l)</th>
<th>Run4 (mgCOD/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>canola oil</td>
<td>1000</td>
<td>1000</td>
<td>1000</td>
<td>1000</td>
</tr>
<tr>
<td>starch</td>
<td>0</td>
<td>1000</td>
<td>0</td>
<td>500</td>
</tr>
<tr>
<td>yeast extract</td>
<td>0</td>
<td>0</td>
<td>1000</td>
<td>500</td>
</tr>
</tbody>
</table>

Table 8.1. Feed composition for different runs.

8.2.2 Enumeration of Archaeal and Bacterial communities. The microbial population shifts of each run under different HRT and temperature were characterised by FISH
techniques. Analyses were conducted in triplicate and data were averaged. The samples from the acidogenic reactor were taken at the same level of the reactor. Two oligonucleotide probes, ARC915 (Stahl et al., 1991) and EUB338 (Amann et al., 1990a) which targeted to Archaea and Bacteria cells were applied to identify and quantify these organisms.

8.2.3 Measurement of extent of hydrolysis of lipid, protein and carbohydrate. The analysis for the quantification of starch and yeast extract and canola oil was performed according to the methods described in sections 3.7.1, 3.7.2 and 3.7.3. The concentration of canola oil was determined gravimetrically after extraction of lipid from samples by petroleum ether, according to the Soxlett extraction method (APHA, 1985). The anthrone method was applied for the analysis of starch and the Lowry method for the analysis protein (Raunkjaer et al., 1994). The extent of the adsorption of lipid on sludge was determined by subtracting the liquid phase lipid from the total lipid that was added to 1 litre of autoclaved sludge from the full-scale plant.

The extent of hydrolysis (%) of canola oil, starch and protein for each run were calculated using:

$$\text{Hydrolysis} (%) = \frac{\text{Total amount added} - \text{Residual amount} - \text{Adsorbed amount}}{\text{Total amount added}}$$

The organic materials present in sludge before addition of substrates were assumed to be zero.
8.2.4 Analysis of LCFA hydrolysed from canola oil. The four main LCFA, oleic acid (C18:1), linoleic acid (C18:2), linolenic acid (C18:3), and palmitic acid (C16:0) in canola oil were analysed by the method described in 3.7.1. The transformation or saturation of individual LCFA in acidogenic reactor was evaluated by comparing their concentrations in different experimental conditions.

8.3 Results and discussion

It should be noted that there was no methane gas produced in the acidogenic reactor in the studies reported here.

8.3.1 Effects of HRT on hydrolysis of lipid, carbohydrates and protein in acidogenic reactor. At mesophilic temperature (Fig.8.1a), the extent of hydrolysis in acidogenic reactor which was only fed with canola oil was between 6-9% and it appeared to be independent of the HRTs investigated. The highest extent of hydrolysis of canola oil was measured when oil was fed along with starch and varied between 26 and 35%.
Figure 8.1. Hydrolysis of canola oil in acidogenic reactor at different feed composition and HRT at (a) mesophilic and (b) thermophilic condition.

The extent of hydrolysis of canola oil varied between 10 and 14% when fed with yeast extract, and 22 and 27% when fed with starch and yeast extract, suggesting that starch had the better effect on enhancing the hydrolysis of canola oil. Interestingly, increasing HRT beyond 1 day did not have an apparent effect on the extent of hydrolysis when oil was fed
with co-substrates, implying that one day HRT was sufficient for acquiring the maximum extent of hydrolysis of canola oil in co-digestion with yeast extract and starch.

At thermophilic temperature (Fig. 8.1b), the extent of hydrolysis of canola oil was greater than that at mesophilic condition for all feed combinations and HRTs. The extent of hydrolysis of oil without the presence of any other co-substrates feeding was between 5 and 7%. However, when fed with starch, it increased to 55-63%, with yeast extract it increased to 33-38%, and in combination with yeast extract and starch it was around 48%. The higher hydrolysis achieved in thermophilic condition could be attributed to two facts. One is that the high temperature benefitted the growth rate of the microorganisms, especially thermophiles growth (Hwu, 1997). Even though the numbers of thermophiles in the original mesophilic inoculum were very low, they may have become very active at high temperature and thus degraded the lipid effectively. The other reason might be related to the fact that biosorption of lipid increased with the increase of temperature (Riffat and Dague, 1995). They reported that with the increasing of biosorption, the COD removal in the effluent was also increased. The extent of biosorption will be discussed later.

The actual reason for the advantage of co-digestion in lipid degradation is still unclear and it could be related to the increase in the lipid surface area due to the presence of protein and carbohydrate and their fermentation products might better emulsify the lipids. It is known that an increase of particulate surface will enhance the hydrolysis efficiency (Zeeman and Sanders, 2001). Or it might be related to the fact that the complex organics could provide
the more complete essential nutrients for the growth of the hydrolysis acidogens which will be discussed in the microbial experiment of this chapter.

In mesophilic condition, starch (2g COD/l) fed by itself was mostly hydrolysed. The hydrolysis extent was 98 % at HRT of 1 day and 99 % at HRT of 5 days. High hydrolysis of starch could be achieved at short HRT (< 1 day). This is in good agreement with previous reports (Hanaki et al., 1987; Pavlostathis and Giraldogomez, 1991). The easy hydrolysis of starch might be explained by its high solubility. However, the extent of hydrolysis of starch decreased when it was fed with oil or oil and yeast extract at all HRTs (Fig.8.2). At an HRT of 1 day, the lowest percentage of starch hydrolysis measured was 85% and 75% by feeding with oil and oil together with yeast extract, respectively. The hydrolysis of starch increased when HRT increased from 1 day to 2 days both when only oil or oil together with yeast extract was fed. When HRT increased from 2 days to 5 days, the extent of starch hydrolysis varied between 95 to 91 % by feeding with oil, while it increased from 87 to 94 % when oil together with yeast extract was added.

Starch (2g COD/l) was mostly hydrolysed at thermophilic temperature when fed alone and was irrespective of HRT. However, the extent of hydrolysis of starch was lowered and slightly decreased with increase the HRT when fed with oil or oil and yeast extract. At an HRT of 1 day, the hydrolysis extent was 86% when fed with oil and 83% when fed with oil and yeast extract. These figures decreased to 70% and 71% respectively at HRT of 5 days. The presence of oil clearly inhibited the extend of starch hydrolysis even though the addition
of starch increased the extent of hydrolysis of oil. The effect on microbial population will be discussed later.

![Graph](image)

**Fig. 8.2** Hydrolysis of starch in acidogenic reactor at different feed compositions and HRT at (a) mesophilic and (b) thermophilic temperature.

In mesophilic condition, the extent of hydrolysis of protein in yeast extract (2g COD/l) when fed without starch or oil was between 76 and 80% and was independent of HRT when HRT $\geq$ 2 days. However, the extent of hydrolysis of protein was greatly reduced when fed with oil.
(56–58%) and with oil and starch (65–69%) (Fig. 8.3). The hydrolysis extent of protein when fed with oil and starch was higher than when just fed with oil, suggesting that starch could decrease the inhibition of oil on the hydrolysis of protein.

The extent of protein hydrolysis in yeast extract (2g COD/l) was greater (> 80%) at thermophilic temperature than at mesophilic condition. It slightly increased from 83% to 87% when HRT was increased from 1 day to 5 days. Interestingly, at thermophilic
temperature, the hydrolysis of protein in yeast extract with addition of oil (71-75%) was higher than that when both oil and starch was fed (65-68%). The results might be attributed to the fact that different temperature and nutrient composition could benefit the growth of specific bacteria groups, which in return, benefited the hydrolysis.

Fig. 8.2 and 8.3 show that the addition of oil inhibited hydrolysis of both yeast extract and starch. It might be related to the fact that lipids and their hydrolysed products LCFA were toxic to the growth of microorganisms.

8.3.2 Formation and transformation of LCFA in acidogenic reactor. LCFA and glycerol are expected products in the first step of canola oil hydrolysis. These are further transformed into other short chain fatty acids or VFA. The LCFA, oleic acid (C18:1), linoleic acid (C18:2), linolenic acid (C18:3) palmitic acid (C16:0) were the primary LCFA of the canola oil used in the experiment. Upon complete hydrolysis of 1 gCOD/l of canola oil, 320mg/l of LCFA are expected to be produced. In the acidogenic reactor, the expected LCFA concentration could be calculated by multiplying the extent of oil hydrolysed with the amount of LCFA in oil. The extent of LCFA transformation was estimated by subtracting measured LCFA from the expected amount of LCFA then dividing the difference by the expected amount of LCFA.

Figure 8.4 shows that the addition of starch and yeast extract stimulated the transformation of LCFA which was hydrolysed from oil in acidogenic reactor. At mesophilic temperature, the extent of LCFA transformation in acidogenic reactor when fed with starch, yeast extract
or starch and yeast extract were very similar (18 to 20%) at different HRT and slightly increased upon increasing HRT from 1 to 2 to 3 days. At thermophilic temperature, the extents of LCFA transformation in different feeding modes were very similar at different HRT, indicating that the LCFA transformation was independent of HRT at thermophilic temperature. The results showed that the transformations of LCFA in acidogenic reactor at mesophilic and thermophilic temperature were very low, suggesting that the LCFA transformation in acidogenic reactor was suppressed during the oil degradation in two-phase anaerobic process.

![Fig. 8.4. LCFA transformation at (a) mesophilic and (b) thermophilic temperature.](image)
The residual concentration of the four LCFA:s: oleic acid, linoleic acid, linolenic acid and palmitic acid in acidogenic reactor was higher at thermophilic temperature than at mesophilic temperature (Fig.8.5). The concentration of residual LCFA was the highest when the reactor was fed by oil and starch, while the concentration of LCFA was the lowest when it was only fed by oil both at mesophilic and thermophilic temperature. This was in accordance with the results shown in Fig.8.1 in which the oil was best hydrolysed when oil and starch was fed in acidogenic reactor, while it was hardly hydrolysed when only oil was fed.

In the acidogenic reactor, the concentrations of the four LCFA were detected at different HRT, feed compositions and temperature. During the hydrolysis, unsaturated LCFA, such as oleic acid (C18:1), linoleic acid (C18:2), linolenic acid (C18:3) might be saturated into saturated LCFA, such as, stearic acid (C18:0), palmitic acid (C16:0) or other saturated LCFA. Because oleic acid (61%), linoleic acid (21%), linolenic acid (9%) and palmitic acid (4%) made up the LCFA in canola oil used in the experiment, after hydrolysis, the ratio among oleic, linoleic, linolenic and palmitic acids were expected to be at 61:21:9:4 ratio, assuming no further transformation of LCFA occurs or the transformation of each of LCFAs was in the same proportion.
Fig. 8.5. Concentrations of individual LCFA under different feed compositions at mesophilic (left column) and thermophilic temperature (right column).
Fig. 8.5 indicated that the ratio of palmatic acid to the four LCFA increased from 4% to 36% (oil and starch feeding), 38% (oil and yeast extract feeding) and 25% (oil, starch and yeast extract feeding) at mesophilic temperature, and 67% (oil and starch feeding), 24% (oil and yeast extract feeding) and 29% (oil, starch and yeast extract feeding) at thermophilic temperature. There was a significant saturation of unsaturated LCFA to palmitic acid in acidogenic reactor when fed oil with starch, yeast extract or starch and yeast extract. This is in good agreement with the result reported by Komatsu et al. (1991) which suggested that saturation of unsaturated LCFA in acidogenic reactor was one of the main reasons why two-phase anaerobic system could reduce the toxicity of LCFA. However, the ratio of palmitic acid was 6% and 8% under mesophilic and thermophilic conditions when fed only oil, suggesting that the addition of starch and yeast extract stimulated the saturation of unsaturated LCFA in acidogenic reactor.

8.3.3 Microbial population shifts under different feed composition, temperature and HRT in acidogenic reactor. The LogC value of the numbers of Bacteria cells was between 11 and 12 in the inoculum used in the experiments. At the mesophilic temperature, the numbers of Bacteria cells were lowest when only canola oil was fed and decreased with the increase of HRT (Table 8.2), implying that canola oil inhibited the growth of Bacteria cells. When fed with starch, yeast extract or starch and yeast extract, the bacteria numbers increased with the increase of HRT, suggesting that longer HRT benefited the growth of bacteria under co-digestion situation. The population levels when canola oil and starch were fed were higher than that when the canola oil and yeast extracts were fed at every HRT, indicating that starch had the better effects on enhancing
the growth of *Bacteria* cells under the inhibition of canola oil. The numbers of *Bacteria* cells when canola oil, starch and yeast extract were fed was the highest at all HRT. This suggested that the combination of canola oil, starch and yeast extracts had the most effective results on increasing the *Bacteria* population. Even though the highest bacterial population was achieved under feeding of oil, starch and yeast extract, the extent of oil hydrolysis under this condition was lower than that when oil and starch were fed (Fig.8.1a), implying that the hydrolysis of oil was not only related to the total population number, it might also related to the numbers of bacterial subpopulations in the acidogenic reactor.

<table>
<thead>
<tr>
<th>HRT</th>
<th>1 day</th>
<th>2 days</th>
<th>3 days</th>
<th>4 days</th>
<th>5 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>oil</td>
<td>mesophilic 6.3</td>
<td>6.1</td>
<td>6</td>
<td>5.4</td>
<td>5.7</td>
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<td></td>
<td>thermophilic 3.5</td>
<td>4.8</td>
<td>4.8</td>
<td>5.1</td>
<td>5.8</td>
</tr>
<tr>
<td>oil, starch</td>
<td>mesophilic 7.2</td>
<td>9.3</td>
<td>10.2</td>
<td>11.2</td>
<td>11.3</td>
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<tr>
<td></td>
<td>thermophilic 10.3</td>
<td>11.2</td>
<td>11</td>
<td>10.8</td>
<td>10.6</td>
</tr>
<tr>
<td>oil, protein</td>
<td>mesophilic 6.1</td>
<td>9.2</td>
<td>9.1</td>
<td>9.3</td>
<td>10.3</td>
</tr>
<tr>
<td></td>
<td>thermophilic 9.6</td>
<td>9.6</td>
<td>10.2</td>
<td>9.8</td>
<td>8.9</td>
</tr>
<tr>
<td>oil, starch, protein</td>
<td>mesophilic 7.1</td>
<td>9.2</td>
<td>11.2</td>
<td>11.3</td>
<td>12.1</td>
</tr>
<tr>
<td></td>
<td>thermophilic 10.4</td>
<td>12.3</td>
<td>9.9</td>
<td>9.7</td>
<td>9.5</td>
</tr>
</tbody>
</table>

Table 8.2 *Bacteria* population (LogC of cell counts. cm\(^{-2}\)) on varied organic components, temperature and HRT.
At thermophilic temperature, the shifts of the number of *Bacteria* cells varied significantly with that at mesophilic temperature. When only fed with oil, the *Bacteria* population
increased with the increase of HRT. There was no significant change of the number of
Bacteria cells in different HRT when fed with oil and starch or oil and yeast extract, indicating that the number of Bacteria cells was independent of HRT at these two feeding compositions. When fed with oil, starch and yeast extract, the number of Bacteria cells increased up to HRT of 2 days, it decreased when HRT was greater than 2 days.

The LogC value of numbers of Archaea cells in original inoculum was about 8 to 9. Table 8.3 shows that the number of Archaea cells were very low both in mesophilic and thermophilic conditions and had no difference at various HRT when only fed with oil. However, when fed with starch, yeast extract or starch and yeast extract, the number of Archaea cells all increased compared to that when only fed with oil, suggesting that Archaea cells could grow effectively when fed oil with starch, yeast extract or both. This demonstrated that the combination of organic materials is essential for the growth of Archaea cells. When fed by oil and starch or oil and yeast extract, the number of Archaea cells all increased with increase of HRT both at mesophilic and thermophilic temperature. When fed by oil, starch and yeast extract, Archaea population increased with the increase of HRT at mesophilic temperature. However, at thermophilic temperature, it increased with the increase of HRT up to 3 days and started decreasing when HRT>3days.
Table 8.3 *Archaea* population (LogC cell counts, cm\(^{-2}\)) on different organic components, temperature and HRT.

Zeeman and Sanders (2001) suggested that *Archaea* cells were very important to the lipid hydrolysis and because not too many *Archaea* cells existed in acidogenic reactor, two-phase will not benefit lipid hydrolysis. This experiment showed that at HRT of 1 day, the numbers of *Archaea* cells were low and had no significant difference at mesophilic and thermophilic temperature when fed with different feed compositions. However, the extent of oil hydrolysis in different feed compositions and temperature were different, implying that the lipid hydrolysis efficiencies may not be related to the number of *Archaea* cells. The reason might be related to the facts that with the higher *Archaea* population, VFA could be consumed fast and this will enhance the acidogenesis, acetogenesis and hydrolysis process.
8.3.4 The hydrolysis of canola oil in methanogenic reactor. The extent of oil hydrolysis in methanogenic reactor was estimated as the oil concentration of the effluent of methanogenic reactor subtracted from the oil concentration of effluent from acidogenic reactor then divided by oil concentration of effluent from acidogenic reactor. The total extent of oil hydrolysis in the two phase system was calculated by subtracting the oil in the effluent of reactor from total oil fed into the acidogenic reactor and divided by the total oil fed into the acidogenic reactor. The data showed that the extent of oil hydrolysis in methanogenic reactor was very low when only fed oil at both mesophilic and thermophilic temperature. The extent of oil hydrolysis increased with the increase of HRT at all feed compositions, suggesting that longer HRT benefited the hydrolysis of oil in the reactor. At thermophilic temperature, the extent of oil hydrolysis was highest when fed by oil, starch and yeast extract, while feeding with oil and starch achieved highest extent of oil hydrolysis at mesophilic temperature. This was different with the oil hydrolysis in acidogenic reactor in which feeding oil and starch could achieve highest extent of oil hydrolysis both at mesophilic and thermophilic temperature.

However, contradicting these findings, Zeeman and Sanders (2001) observed that lipid hydrolysis cannot proceed in the absence of methanogenesis and the two-phase system will not improve the efficiencies of lipid hydrolysis. In this experiment, with co-digesting with starch, yeast extract or starch and yeast extract the total oil hydrolysis in two-phase UASB anaerobic systems as all higher than 80% and it was much greater than that in the one-phase UASB (below 40%) as shown in Chapter 7.
## Chapter 8 Lipid hydrolysis in mesophilic and thermophilic two-phase anaerobic treatment systems.

### Table 8.4. Extent of oil hydrolysis at various HRT in methanogenic reactor.

<table>
<thead>
<tr>
<th>Feed substrate</th>
<th>HRT</th>
<th>Oil hydrolysis in methanogenic reactor (%)</th>
<th>Total oil hydrolysis in two reactors (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Mesophilic</td>
<td>Thermophilic</td>
</tr>
<tr>
<td>Oil</td>
<td>1</td>
<td>4.2</td>
<td>8.6</td>
</tr>
<tr>
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<td>2</td>
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<td>3</td>
<td>5.6</td>
<td>8.8</td>
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<td>4.8</td>
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<td></td>
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</tr>
<tr>
<td>Oil+yeast extract</td>
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<td>80.5</td>
</tr>
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<td></td>
<td>2</td>
<td>75.5</td>
<td>80.7</td>
</tr>
<tr>
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<td>3</td>
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<td>81.8</td>
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<td>Oil+starch+yeast extract</td>
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<td>76.8</td>
<td>87.2</td>
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<td>83.8</td>
<td>89.9</td>
</tr>
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</table>
Fig. 8.6 Methane gas production in different HRT at (a) mesophilic and (b) thermophilic temperature.

<table>
<thead>
<tr>
<th>HRT</th>
<th>1 day</th>
<th>2 days</th>
<th>3 days</th>
<th>4 days</th>
<th>5 days</th>
</tr>
</thead>
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<tr>
<td>oil</td>
<td>mesophilic</td>
<td>0</td>
<td>0</td>
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</tr>
<tr>
<td></td>
<td>thermophilic</td>
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<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>oil, starch</td>
<td>mesophilic</td>
<td>44.6</td>
<td>43.9</td>
<td>42.8</td>
<td>43.1</td>
</tr>
<tr>
<td></td>
<td>thermophilic</td>
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<td>52</td>
<td>54.8</td>
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<tr>
<td>oil, protein</td>
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<td>49.2</td>
<td>46.9</td>
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<td>thermophilic</td>
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<td>55.7</td>
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<td>53.7</td>
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<td>oil, starch, protein</td>
<td>mesophilic</td>
<td>42.8</td>
<td>49</td>
<td>50.2</td>
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<tr>
<td></td>
<td>thermophilic</td>
<td>45.9</td>
<td>55.7</td>
<td>59</td>
<td>52.5</td>
</tr>
</tbody>
</table>

Table 8.5. The extent of oil methanogenesis at various HRT in the methanogenic reactor.
When the reactor was only fed with oil, no methane gas was produced both at mesophilic and thermophilic temperature. Figure 8.6 shows that at mesophilic temperature, the methane gas production was highest at all HRT when fed with oil, starch and yeast extract, while the methane gas production was high when fed with oil and yeast extract than oil and starch. When HRT >1 days, the difference of the methane gas production at mesophilic and thermophilic was not significant, suggesting that thermophilic temperature was not required for methanogenic reactor when co-digesting oil with starch or yeast extract.

The rate of methanogenesis was estimated as the methane gas produced at different feed compositions divided by the theoretical total methane gas that could be produced by feeding 2 gCOD/l at different HRTs. For example, when fed with oil and starch and HRT of 1 day at mesophilic temperature, 624 ml methane was produced from the methanogenic reactor, the theoretical amount of methane gas that could be produced from methanogenic reactor was 1400 ml (350 ml/gCOD multiplied by 4 g COD), thus 44.6% methanogenesis was achieved. From the data show in Table 8.5, the methanogenesis at mesophilic and thermophilic temperature were around 45 to 55%, suggesting that mesophilic and thermophilic had no significant on methanogenesis in methanogenic reactor of two-phase system and that complete mineralisation of feed to methane was not achieved. One gCOD/l of oil feed might be too high, thus, a detailed study of effects of oil concentration on two-phase UASB anaerobic process was performed and is discussed in Chapter 9.
8.4 Summary and conclusion

The results of this study showed that co-digestion oil with starch and yeast extract was effective on enhancing the oil hydrolysis in two-phase UASB anaerobic process. One reason might be related to the fact that the co-digestion stimulated that growth of Archaea and Bacteria cells under inhibition of oil or LCFAs which was hydrolysed from oil. Another reason might be attributed to the fact that co-digestion enhanced the significant saturation of unsaturated LCFA in acidogenic reactor.

The extent of starch and yeast extract hydrolysis decreased when co-digested with canola oil at both thermophilic and mesophilic temperature. This might be related to the fact that oil inhibited the growth of both Archaea and Bacteria cells. Interestingly, in combination with oil or with oil and yeast extract, the amount of starch hydrolysed at mesophilic temperature was higher than at thermophilic temperature, while the extent of yeast extract hydrolysed was lower at mesophilic temperature than at thermophilic temperature when fed together with oil or oil and starch. At mesophilic temperature, the amount of starch hydrolysed increased when HRT increased from 1 to 2 days under oil or oil and yeast extract feeding. At thermophilic temperature, the extent of starch hydrolysis decreased with the increase of HRT, while the extent of yeast extract was relatively independent of HRT.

In acidogenic reactor, there was no methanogenesis to be achieved and the extent of oil hydrolysis when fed by oil, oil and starch, oil and yeast extract or oil, starch and yeast extract was higher at thermophilic temperature than at mesophilic temperature. Furthermore,
the addition of starch was most effective in the oil hydrolysis compared to the addition of yeast extract or starch and yeast extract. The extent of hydrolysis of the oil was independent of HRT when HRT > 2 days under all feed conditions.

In the methanogenic reactor, high oil hydrolysis was also achieved accompanied with the methanogenesis under co-digestion with starch, yeast extract or starch and yeast extract. More than 80% oil was hydrolysed after the acidogenic and methanogenic reactors, suggesting that two-phase UASB anaerobic systems have the significant advantage on lipid hydrolysis which is currently the most difficult step in wastewater treatment plant. However, the methanogenesis in the methanogenic reactor was not significant.
Chapter 9. Enhancing the lipids anaerobic digestion by stimulating bacteria growth with co-digestion of proteins and carbohydrates in two-phase UASB reactor

9.1 Introduction

Unprocessed urban domestic wastewater carries about 40-100 mg lipids/l (Quemeneur and Marty, 1994) which mainly come from kitchen water and human excreta. In industrial wastewater, lipid concentrations vary, for example, slaughterhouses wastewater could contain 350-520 mg/l lipids and wool scouring wastewater could contain 2 –15 g/l (Rinzema, 1988). Lipid could be successfully degraded by aerobic processes, however, even very low lipid loading rate (1-2 g COD/l·day) resulted in wash-out of granular sludge in an anaerobic expanded granular sludge bed (EGSB) reactor (Rinzema, 1988). Two-phase process is designed to achieve optimum growth of acidogens in acidogenic reactor and methanogens in methanogenic reactor. However, the continuous and constant organic overloaded result in process failure (Fongastitkul and Lo, 1994). Although conventional two-phase anaerobic digesters have been used for sewage treatment, these systems still face problem of relatively poor performance of the methanogenic reactor (Fongastitkul and Lo, 1994). The data of Chapter 8 also showed that even though high extent of hydrolysis could be achieved at a loading rate of emulsified canola oil of 1 g COD/l·day, the methanogenesis of the oil in the methanogenic reactor was poor (30 to 50%) in a two-phase UASB reactor system with co-digestion of starch and yeast extract.
In recent years, a number of large-scale biogas plants have been built to treat organic industrial waste from slaughterhouse, food processing industries and animal manure which are rich in lipids, protein and carbohydrates. These plants have achieved very good results (Ahring et al., 1992; Edelmann et al., 2000). It was difficult to digest wastewater only rich in lipid or lipid and protein. Carbohydrate was necessary in the digestion of lipid and protein rich wastewater (personal communication with operator in Spearwood Wastewater treatment Plant). The Chapter 8 showed that the addition of carbohydrate produced a better result than protein on enhancing the extent of hydrolysis of lipid. Co-digestion of lipid with carbohydrate and protein could decrease the inhibition of lipid on anaerobic digestion and achieve greater acidogenesis and methanogenesis than when only fed with lipid (Chapters 7 and 8). However, the effects of concentration or ratio of lipid, carbohydrate and protein on lipid anaerobic digestion is still unclear. The aim of this study is to investigate the effects of concentrations of protein and carbohydrate on the anaerobic degradation of lipid, as well as on the population shifts of anaerobic bacteria communities during two-phase anaerobic co-digestion. Chapter 8 showed that higher extent of oil hydrolysis could be achieved at thermophilic temperature than at mesophilic temperature in the acidogenic reactor, while the extent of oil methanogenesis was not significantly different at mesophilic and thermophilic temperature in two-phase UASB reactor. Thus, in this experiment, thermophilic temperature was chosen for acidogenic reactor while mesophilic temperature was chosen for methanogenic reactor.
9.2 Materials and methods

9.2.1 Experimental reactor set up. One, two-phase UASB reactor was set up as described in section 3.1.2. The acidogenic reactor was kept at 55°C while the methanogenic reactor was kept at 37°C. Inoculation was performed as described in the section 8.2.1. After inoculation, the reactor was fed 1.5 g COD/l of starch at hydraulic retention time (HRT) of 1 day for 14 days until more than 80% COD reduction had been achieved. After reactivation, the reactors were fed 500 mgCOD/l canola oil for 14 days as the control. Then the system was restarted by inoculating as before and feeding 1.5 g COD/l of starch for 14 days until more than 80% COD reduction was achieved. The system was fed a mixture of lipid (canola oil), carbohydrate (starch) and protein (yeast extract) at various concentrations as shown in the Table1 at hydraulic retention time (HRT) of 1 day.

Eight experiments were carried out to examine the effects of the addition of protein and carbohydrate on the anaerobic degradation of lipid. Each run lasted 30 days. The lipid degradability and microbial population shifts in the methanogenic reactor were investigated. The solutions of three organic materials were freshly prepared and autoclaved before the experiment and stored in the refrigerator at 4°C to avoid the degradation by contamination.

The feed solution was stirred continuously. HRT for acidogenic reactor and methanogenic reactor was controlled at 24 hours. The pH in the acidogenic reactor was controlled at 5 to 6 by addition of 10 N NaOH and 2M HCl.
Chapter 9. Enhancing the lipids anaerobic digestion by stimulating bacteria growth with co-digestion of proteins and carbohydrates in two-phase UASB reactor

<table>
<thead>
<tr>
<th>Component</th>
<th>Canola oil (mgCOD/l)</th>
<th>Starch (mgCOD/l)</th>
<th>Yeast extract (mgCOD/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
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<td>0</td>
<td>0</td>
</tr>
<tr>
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</tr>
<tr>
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<td>Run8</td>
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Table 9.1. Composition of feed in the different runs.

1 g carbohydrates (starch) is equivalent to 1.01 g COD; 1 g lipid (canola oil) is equivalent to 2.86 g COD; 1 g protein (yeast extract) is equivalent to 1.35 g COD

9.2.2 Sampling and analysis. Procedure for sampling and analysis of microbial communities was as described in section 3.4. Procedure for measurement of canola oil, yeast extract and starch was as described in section 3.7. Analysis of LCFA in acidogenic and methanogenic reactor was as described in section 3.7.1.
9.3 Results and discussion

9.3.1 The inhibition of oil on acidogenic and methanogenic reactors.

In the control experiment which was fed only oil, there was no methane produced in acidogenic reactor and the pH was about 6.3-6.9. The extents of hydrolysis and degradation of canola oil in both acidogenic reactor and methanogenic reactor were lower than 20% after two days (Fig. 9.1), indicating that canola oil inhibited the acidogenesis and methanogenesis even at low concentration (500 mgCOD/l) in the feed. This was in good agreement with the reports in the literature (Rinzema, 1988; Angelidaki et al., 1990) and previous experiments (Chapters 7 and 8). Interestingly, the extent of oil hydrolysis at 1000 mg COD/l concentration of oil at HRT of 1 day (Fig.8.1b) in acidogenic reactor at thermophilic temperature was about 5% and it was similar with that at 500 mg COD/L (Fig.9.1) at day 14. Therefore the extent of oil hydrolysis in acidogenic reactor did not increase with the decrease of oil concentration. The hydrolysis of oil in the methanogenic reactor was also very low, this was also in accordance with the data shown in Table 8.4 in which the extent of oil hydrolysis was about 5 % in the methanogenic reactor at mesophilic temperature. The experiment demonstrated that oil was difficult to hydrolyse even at low lipid loading in the two-phase anaerobic reactor.
Fig. 9.1. Degradation of canola oil in the acidogenic and methanogenic reactors.

Fig.9.2 Methane production from methanogenic reactor after feeding with canola oil.

The methane gas production decreased upon feeding 500 mg COD/l of canola oil in the acidogenic reactor (Fig. 9.2). The methanogenic activity decreased to zero after 4 days.
feeding canola oil as indicated by zero-methane production from day 4 onward. From day 3, the sludge in the methanogenic reactor started to wash out. This might be related to the adsorption of lipid on sludge as discussed by Hwu (1997).

The results from Fig.9.1 and Fig.9.2 suggest that in the absence of other organic component in feed, even low concentration of oil (500 mgCOD/l) would inhibit the acidogenesis and methanogenesis in the two-phase anaerobic process.

9.3.2 The influence of organic composition on the oil degradation

Figure 9.3 shows the methanogenic activity when the reactors were fed with different compositions of starch and yeast extract with 500 mgCOD/l of oil. Assuming that all the COD fed was degradable, the extent of methanogenesis was calculated as the mean value of the daily methane gas production during the different runs divided by the theoretical expected daily methane production rate, calculated as 350 ml/gCOD in fed (shown at Table 9.2). For example, when the reactor was fed with 2 g COD/l total COD at oil: starch: yeast extract ratio of 1:2:1 in COD weight (Run1) at HRT of 1 day, 1400 ml gas was theoretically assumed to be produced from the reactor. However, only 655 ml of methane was produced. Thus the extent of methanogenesis was calculated as 655 ml divided by 1400 ml i.e. 47%. With the increase of starch amount, the methane production increased, implying that starch benefited the methanogenesis. This is in accordance with results of previous experiments (Hanaki et al., 1987) which reported that carbohydrates were easily converted to acid during the complex organic material anaerobic degradation. However, when the concentration of
yeast extract increased from 500 to 2500 mgCOD/l, the extent of methane production decreased significantly, suggesting that yeast extract was not as effective as starch in methanogenesis process. With the increase of yeast extract feeding, the extent of methanogenesis decreased, implying that the degradability of yeast extract was not as high as that of starch. This is also in agreement with the experimental results achieved previously, which suggested that protein was difficult to be degraded due to its lower extent of hydrolysis (Hanaki et al., 1987; Batstone, 1999). These results suggested that the higher carbohydrate concentration or ratio in anaerobic co-digestion of complex organic waste treatment would benefit the methanogenesis of the two-phase anaerobic process.

The extent of methanogenesis was the highest when COD weight ratio of Oil:Starch:Yeast extract was 1:5:1, suggesting that higher starch concentration in co-digestion oil, starch and yeast extract was essential. The results indicated that there was an optimal composition of protein, lipid and carbohydrate for methanogenesis although it might be different in different anaerobic reactors. The extents of methanogenesis from Run 1 to Run 4 were all higher than those fed with 1000 mgCOD/l.day of oil as shown in Fig.8.7, indicating that lower oil concentration also benefit the methanogenesis in the two-phase UASB anaerobic system.
Table 9.2 The extent of methanogenesis in methanogenic reactor for different feed compositions.

<table>
<thead>
<tr>
<th>Run</th>
<th>1:2:1</th>
<th>1:3:1</th>
<th>1:4:1</th>
<th>1:5:1</th>
<th>1:1:2</th>
<th>1:1:3</th>
<th>1:1:4</th>
<th>1:1:5</th>
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<tbody>
<tr>
<td>Oil:Starch:Yeast extract (COD g)</td>
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<tr>
<td>Percentage of methanogenesis</td>
<td>47.3</td>
<td>68.5</td>
<td>71.6</td>
<td>80.7</td>
<td>37.2</td>
<td>36.5</td>
<td>35.7</td>
<td>30.</td>
</tr>
</tbody>
</table>
Fig. 9.3. Effect of different ratio of organic composition on methane production in methanogenic reactor.
Fig. 9.4. Extent of oil, starch and yeast extract degradation in methanogenic reactor with different feed compositions
Chapter 9. Enhancing the lipids anaerobic digestion by stimulating bacteria growth with co-digestion of proteins and carbohydrates in two-phase UASB reactor

Fig. 9.5. The concentration of LCFA in the effluent of methanogenic reactor under different feeding modes.
Figure 9.4 shows a comparison of the final breakdowns of starch, lipid and yeast extract after the second reactor under the different feeding modes. With the increase of starch addition from 1000 to 2500 mg COD/l.day (Run 1-Run 4), the lipid degradation increased from 71 to 92%. With increase of protein addition (Run5- Run8) from 1000 to 2500 mg COD/l.day, the degradation of lipid decreased from 82 to 65%. It suggested that higher starch was more effective than higher yeast extract in enhancing the oil degradation.

With the increase the loading rate of starch, the degradation of starch was kept around 85%. However, with the increase of yeast extract, the degradation of starch decreased from 85 to 75%, suggesting that higher yeast extract could also inhibit the degradation of starch. The degradation of yeast extract in the first four Runs was around 55%, and it decreased from 55% to 36% with the increase of yeast extract from 1000 to 2500 mg COD/l.day.

The concentration of LCFA in the effluent of methanogenic reactor decreased from 25 mg/l to 7 mg/l with the increase of starch concentration, suggesting that the addition of starch benefited the LCFA transformation. With the increase of yeast extract feeding, the LCFA concentration increased, indicating that higher yeast extract concentration or lower starch concentration inhibited the LCFA transformation. With the higher LCFA concentration accumulation, the methane production decreased and was kept lower from Run 5 to Run 8. This might explain the result showed in the Chapter 8 in which even though higher hydrolysis of oil was achieved the extent of methanogenesis was low.
Fig. 9.6  *Archaea*, *Bacteria* and SRB shifts under the different feeding compositions in methanogenic reactor.
9.3.3 The effects of addition of different composition substrates on the shifts of microbial subpopulations in methanogenic reactor

Figure 9.6 illustrates the results obtained with oligonucleotide probes ARC 915 (targeting *Archaea* cells), EUB 338 (targeted to *Bacteria* cells including mainly acetogens and acidogens in the reactor) and SRB (targeting to sulfate-reducing bacteria). After reactivating by feeding 1.5 g COD/l carbohydrate, the number of *Archaea* cells was higher than the number of *Eubacteria* cells and the numbers of SRB cells. After feeding with oil, starch and yeast extract, the numbers of *Archaea* cells decreased sharply from LogC value of 13 to 10 and became lower than the numbers of *Bacteria* cells, implying that the growth of methanogens was more sensitive to lipid than acetogens and acidogens. With the increase of starch concentration, the numbers of *Archaea* cells increased from LogC value of 10.2 to 13.8, indicating that starch benefited the recovery of growth of *Archaea* cells. This result could be related to the increase of methanogenic activity and lipid degradation in the methanogenic reactor with the increase of starch feeding. Contrary to the expectation, the numbers of *Bacteria* and SRB cells were relatively stable with the increase of starch, indicating that increasing of addition of starch did not affect the population of *Eubacteria* cells in the methanogenic reactor.

With the increase of addition of yeast extract from Run 5 to Run 8, the numbers of *Archaea* cells decreased from LogC value of 13.8 to 10.4, while both the numbers of *Bacteria* cells and SRB cells increased from 11.4 to 13.6 and 8.2 to 12.3, respectively. Higher LCFA concentration accumulated from Run 5 to Run 8, and higher LCFA inhibited the growth of *Archaea* cells. Furthermore, some of the amino acids hydrolysed from yeast extract, like methaionine and cysteine contain sulphur, therefore, the increase
of addition of yeast extract resulted an increase of the sulphur concentration which benefits the growth of SRB. The competition between SRB cells and *Archaea* cells reported in previous experiments (Raskin *et al.*, 1995; Raskin *et al.*, 1996) showed that the increase of sulphur would benefit the competition of SRB. This experiment showed that *Bacteria* and SRB cells were more competitive than *Archaea* cells in a protein abundant environment. Beside substrate competition, it was reported that SRB and methanogens are also competitive on hydrogen (Lovley *et al.*, 1982).

With the decrease of *Archaea* cells, the LCFA accumulated in the reactor, implying that LCFA transformation was indirectly related to the numbers of *Archaea* cells, although some other parameters, such as hydrogen pressure might indirectly relate to the *Archaea* cells. The Chapter 8 showed that lipid hydrolysis was not related to the numbers of *Archaea* cells, however this chapter shows that LCFA transformation or degradation depend on *Archaea* cells and the transformation of LCFA might be more effective with more *Archaea* cells.

The microbial sub-population shifts observed under the different feeding conditions provided some more information that could not be illustrated by monitoring the physico-chemical parameters. Koster and Cramer (1986) suggested that the inhibition of lipid and toxicity of LCFA was affected by the concentration of lipid or LCFA, and not related to the numbers of anaerobic bacteria. This experiment obviously showed that the numbers of different microbial communities were related to the degradation levels of lipid or LCFA. The microbial investigation during the anaerobic digestion process is therefore essential for the control of the digester performance. The success of co-digestion of lipids, protein and carbohydrates is explained by the fact that these three
organic materials are all necessary in the growth of anaerobic bacteria and providing these nutrients will benefit the microorganisms. The different composition of three organic components could stimulate the growth of different bacterial communities. Microbial shift investigation is very suitable indicator to design or explain the optimal co-digestion composition of lipid, protein and carbohydrate.

9.4 Summary and Conclusion

In this chapter, the influence of concentrations of starch and yeast extract on canola oil degradation in two-phase UASB anaerobic systems was evaluated. The results showed that the concentration of starch higher than that of oil and yeast extract in co-digestion could enhance the degradation of oil. Higher starch feeding probably enhanced the LCFA transformation, while higher yeast extract loading would decreased the degradation of oil.

The microbial population study present more detailed information to explain the experimental results. The addition of higher starch concentrations stimulated the growth of Bacteria cells thus positively increase the Archaea cells number, while higher yeast extract concentrations also benefit the growth of Bacteria and SRB cells. The later cells competed with the growth of Archaea cells, thus decreased the methanogenesis in methanogenic reactor. The microbial data also showed that LCFA transformation or degradation might be related to the numbers of Archaea cells as the LCFA accumulation increased with the decrease of Archaea cell numbers.
Chapter 10. Conclusion and recommendation

10.1 Conclusion

In theory, lipids and their hydrolysis products long chain fatty acids (LCFA) are readily biodegradable. However, two main difficulties have to be overcome before achieving efficient and reliable anaerobic treatment of lipids. First is the inhibition of LCFAs on methanogenic and acetogenic bacteria. Second is the wash-out of the biomass due to the flotation resulting from the adsorption of lipids. Some efforts have been done in developing new reactor designs and loading rate control. However, the effect of influent substrate study seems very limited. Recently, combined biodegradation of complex substrate or full scale co-digestion of organic waste has attracted more and more interest (Ahring et al., 1992; Kubler et al., 2000). It has been noticed that the co-digestion of lipids, protein and carbohydrates would benefit organic wastewater treatment. However, the study of organic co-digestion only in the primary stage due to the fact that co-digestion is a complex process. The relation between combination of organic substrates that are fed and efficiency of lipid hydrolysis is still unclear. Most of the difficulty in the study of co-digestion is the investigation of microbial communities shifts under the different combination of organic substrates. Monitoring or controlling of this process is correctly conducted using physico-chemical parameters, such as VFA, methane volume, pH, temperature, COD, loading rate and HRT. Although the microbial control of co-digestion process is also a very important aspect, the research of this field is scarce.
This study applied microbial techniques as well as physico-chemical parameters to investigate the effects of the combination of substrates on the reduction of toxicity of LCFA on anaerobic bacteria and the enhancement of the anaerobic degradation of lipid. The experimental results can be divided into two groups:

Chapters 4, 5 and 6 dealt with the research of the influence of glucose and amino acid on the toxicity of LCFA on anaerobic bacterial groups, as well as on the granule formation under LCFA and on the recovery from inhibition of LCFA. It was proven in the laboratory scale that glucose and amino acid were effective in decreasing the toxicity of LCFA on the anaerobic bacteria, thus enhancing the degradation of LCFA. Although glucose gave better results than cysteine on reducing the toxicity of LCFA and recovering the regrowth of anaerobic bacteria, the combination of glucose and cysteine was optimal for the reduction of toxicity of LCFA.

Chapters 7, 8 and 9 were directed to investigate the influence of carbohydrates and protein on the hydrolysis and anaerobic degradation of lipids. The results showed that the integrated addition of protein and carbohydrate with lipid had the optimal effect on the hydrolysis of lipid compared to the separate addition of protein or carbohydrate. The main reason might be that the combined addition of protein and carbohydrate benefited the growth of bacteria and thus decreased the loss of biomass. The co-digestion of protein, carbohydrate and lipid decreased the loss of microbial population and thus enhanced the digestion of lipids.
Based on the observations and results of this thesis, the following conclusions could be drawn:

- The addition of glucose or amino acid could reduce the toxicity of LCFA on anaerobic bacteria, thus benefit the anaerobic degradation of LCFA.

- The presence of glucose could benefit the growth of methanogens, acidogens and acetogens, thus enhance the granule formation under the inhibition of LCFA.

- When the reactor was shut down and anaerobic bacteria were inhibited by LCFA, glucose and cysteine could enhance the recovery of anaerobic bacteria growth.

- The co-digestion of carbohydrate, protein and lipid benefited the hydrolysis of lipid and then increased the anaerobic degradation of lipid.

- Two stage digestion was more effective than one stage digestion in lipid hydrolysis but presented low methanogenesis at high lipid loading rate. Lipid hydrolysis occurred both in acidogenic and methanogenic reactors.

- Biological monitoring of lipid degradation combined with other organic materials, carbohydrate and protein in anaerobic digester was necessary and could avoid some failure resulting from wrong operation by early warning and detailed inside microbial information. It should be included in the routine analyses performed in the wastewater treatment plants.
The co-digestion of protein and carbohydrate enhanced the anaerobic degradation of lipid through the stimulating the growth of anaerobic bacteria subpopulation under the inhibition of lipid.

While starch was more effective in stimulating the growth of Archaea cells, yeast extract more benefited the growth of Bacteria cells. The number of Archaea cells might be related to the LCFA transformation during the lipid degradation.

10.2 Recommendation for future works

In this study, cysteine and sodium oleate were chosen to represent the amino acids and LCFA, canola oil and yeast extract to represent lipid and protein. Before the practical application of co-digestion of substrate and lipids or LCFAs, more products need to be tested. The relation between the combination ratio among the lipid, protein and carbohydrate and growth rate of anaerobic bacteria also need further examination and more detailed microbial and physico-chemical modelling, computer software designing before practical application. Because this study is limited to lab-scale experiments, the investigation of integrated addition or co-digestion in the practical wastewater treatment plants is still needed.

Although the physico-chemical as well as microbial parameters have been investigated during the co-digestion of lipid with starch and yeast extract, or LCFA with glucose and cysteine, the pathway and co-metabolism of carbohydrates and protein on enhancing the
lipid degradation, or glucose and amino-acids on stimulating LCFA transformation still have not been clarified. Thus, future works on these fields are still necessary.
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