CHAPTER 2

LITERATURE SURVEY

2.1 Anaerobic Digestion

2.1.1 Theoretical Overview

The anaerobic digestion of organic material, like cellulosic wastes, is chemically a very complicated process, involving hundreds of possible intermediate compounds and reactions, each of which is catalyzed by specific enzymes or catalysts. However, the overall chemical reaction is often simplified to:

$$\text{Organic matter} \xrightarrow{\textit{Anaerobic digestion}} CH_4 + CO_2 + H_2 + NH_3 + H_2S \qquad (2.1)$$

In general, anaerobic digestion is considered to occur in the following stages (Opasawatchai, 1983) :

1. liquefaction or polymer breakdown,
2. acid formation, and
3. methane formation

Stage 1: Liquefaction

In this stage organic polymer (cellulose) is broken down by extracellular enzymes produced by hydrolytic bacteria, and dissolved in water. The simple, soluble, organic components (or monomers) which are formed are easily available to any acid-producing bacteria. It is difficult to distinguish this stage from what is known as stage 2 (acid-formation stage), because some molecules will be absorbed without further

breakdown and can be degraded internally. The hydrolysis reactions in this stage will convert cellulose into simple sugars. However, the liquefaction of cellulose and other complex compounds to simple monomers can be the rate-limiting step in anaerobic digestion (Op den Camp *et al.*, 1988), since this bacterial action is much slower in stage 1 than in either stage 2 or 3. The hydrolysis rate is dependent on substrate and bacterial concentrations, as well as on environmental factors such as pH and temperature.

Stage 2: Acid formation

The monomeric components released by the hydrolytic breakdown due to bacterial action in stage 1, are further converted to acetic acid (acetates) and H_2/CO_2 by the acetogenic bacteria. Volatile fatty acids are produced as the end-products of bacterial metabolism of cellulose; in which acetic, propionic, and lactic acids are the major products. Carbon dioxide and hydrogen gas are also liberated during cellulosic catabolism, with methanol, and other simple alcohols, being other possible by-products of cellulosic breakdown. The proportion of these different substrates produced depends on the flora present, as well as on the environmental conditions (Archer and Peck, 1989).

Stage 3: Methane formation

The products of stage 2 are finally converted to CH_4 and other end-products by a group of bacteria called methanogens. Methanogenic bacteria are obligate anaerobes whose growth rate is generally slower than the bacteria stages 1 and 2.

The methanogenic bacteria use acetic acid, methanol, or carbon dioxide and hydrogen gas to produce methane (Wiegel, 1986). Acetic acid or acetate is the single most important substrate for methane produced from acetic acid (Oremland, 1988). The remaining methane comes from carbon dioxide and hydrogen. A few other substrates can also be used, such as formic acid (Reeve, 1992), but these are not

important, since they are not usually present in anaerobic fermentation. The methanogenic bacteria are also dependent on the stage 1 and stage 2 bacteria to provide nutrients in a usable form (Wilkie and Smith, 1989). For example, organic nitrogen compounds must be reduced to ammonia to ensure efficient utilization by the methanogenic bacteria.

The methane formation reaction in stage 3 is very important in anaerobic digestion. Besides producing CH_4 gas, the methanogens also regulate and neutralize the pH of the digester slurry by converting the volatile fatty acids into CH_4 and other gases (Peillex *et al.*, 1988). The conversion of H_2 into CH_4 by the methanogens helps reduce the partial pressure of H_2 in the digester slurry, which is beneficial to the activity of the acetogenic bacteria (Colberg, 1988). If the methanogenic bacteria fail to function effectively there will be little or no CH_4 production from the digester, and so waste stabilization is not achieved because the organic compounds will be converted only to volatile fatty acids, which can cause further pollution if discharged into a water-course or on land.

2.1.2 Bacteria in Anaerobic Digestion Process

The current understanding of the microbiology of anaerobic digestion is illustrated in **Figure 2.1**. There are four main groups of bacteria involved in the process, namely (Brown and Tata, 1985):

(i) acid-forming (hydrolytic and fermentative) bacteria,
(ii) acetogenic (acetate and H_2-producing) bacteria,
(iii) acetoclastic (methane-forming) bacteria, and
(iv) hydrogen-utilizing methane bacteria

The acid-forming bacteria are involved in the hydrolysis and breakdown of complex organic compounds into simple products such as CO_2, H_2, and other volatile fatty acids via two main pathways (Gunnerson and Stuckey, 1986):

$$\text{Substrate} \longrightarrow CO_2 + H_2 + \text{acetate} \qquad (2.2)$$

$$\text{Substrate} \longrightarrow \text{propionate} + \text{butyrate} + \text{ethanol} \qquad (2.3)$$

The products from equation 2.2 can be utilized directly by the acetoclastic bacteria (equation 2.4) and the hydrogen-utilizing methane bacteria (equation 2.5) to produce CH_4.

$$CH_3COO^- + H_2O \longrightarrow CH_4 + HCO_3^- + \text{energy} \qquad (2.4)$$

$$4H_2 + HCO_3^- + H^+ \longrightarrow CH_4 + 3H_2O + \text{energy} \qquad (2.5)$$

McInerney and Bryant (1981) reported that the reaction in equation (2.2) will predominate in a digester having a low H_2 partial pressure. At high H_2 partial pressure equation (2.3) will be favored with the formation of volatile fatty acids having more than two carbon atoms (e.g. propionate and butyrate) and ethanol. These products are converted further to methanogenic substrate such as acetate, H_2, and CO_2 by the acetogenic bacteria through the acetogenic dehydrogenation reaction. Some acetogenic bacteria can also convert H_2 and CO_2 to acetate through the acetogenic hydrogenation (**Figure 2.1**).

Brown and Tata (1985) reported that the acetoclastic bacteria have a longer generation time than the acid-forming bacteria (i.e. 2-3 days vs. 2-3 hours at 35°C, under optimum conditions). Thus, anaerobic digesters should not receive too high organic loadings because the acid-forming bacteria will produce volatile fatty acids faster than the rate at which the acetoclastic bacteria can utilize them.

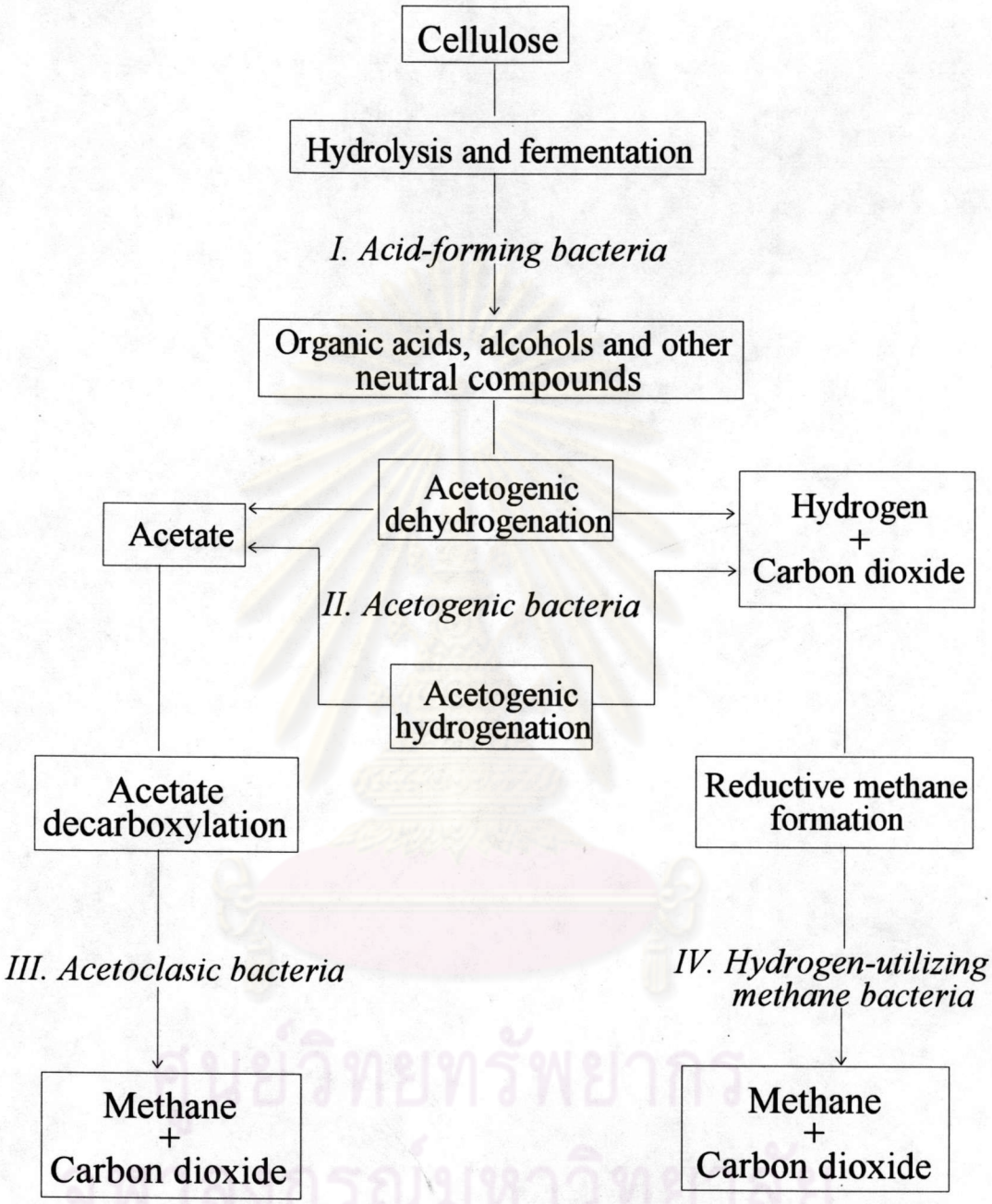

Figure 2.1 Biomethanation of cellulose (Brown and Tata, 1985)

It is currently known that the growth of acetogenic bacteria is sensitive to the H_2 partial pressure in the anaerobic digestion system (McInerney and Bryant, 1981). If the H_2 partial pressure is above 0.0001 atmospheres, the equation (2.3) reaction will take place, and the production of acetate will be minimized. Since about 70 percent of CH_4 is formed by the reaction in equation (2.4) (Wilkie and Smith, 1989), the rate of biogas production will be decreased.

It appears that the reaction in equation (2.5) is important to the anaerobic digestion process because it removes H_2 gas from the system and helps maintain the low H_2 partial pressure.

This process shows that H_2 is essential in the interaction among anaerobic bacteria, which involves a precess termed "interspecies H_2 transfer," where some bacteria produce H_2 and others consume it (Ljungdahl, 1986).

2.1.3 Environmental Requirements

Like any other biological process, anaerobic digestion is a multi-parameter controlled process, each individual parameter having control over the process either through its own effect on the system or through interaction with other parameters. These parameters includes temperature, pH and alkalinity, nutrient concentration, loadings, etc.

Temperature has a pronounced effect on the rate of gas production. Generally two ranges of temperature are considered in methane production. These are mesophilic (25-40°C) and thermophilic (50-65°C). The rate of methane production increases as the temperature increases, but there is a distinct break in the rise at around 45°C, as this temperature favors neither the mesophilic nor the thermophilic bacteria (See **Figure 2.2**).

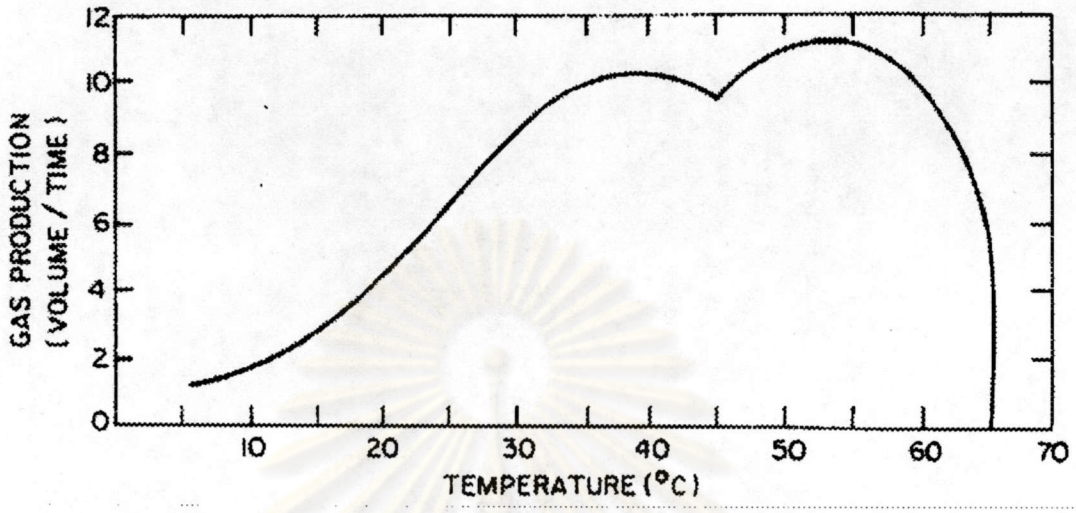

Figure 2.2 Effect of temperature on gas production (Price and Cheremisinoff, 1981)

The operational range of pH in anaerobic digesters should be between 6.6 and 7.6, with the optimum range being 7 to 7.2 (Pfeffer, 1980). Although acid-forming bacteria can tolerate a pH as low as 5.5, the methanogenic bacteria are inhibited at such low pH values (Goodwin and Zeikus, 1987). Appropriate measures should be taken promptly when there is a lowering of pH in an anaerobic digester, due to accumulation of volatile fatty acids, or increase in H_2 partial pressure, and the rate of CH_4 production decreases (Fathepure, 1987).

2.2 Biogas

Biogas (also called 'marsh gas'), a by-product of anaerobic decomposition of organic matters, has been considered as an alternative source of energy. The biogas can be used in small family units for cooking, heating, and lighting, and in larger institutions for heating or power generation. Some variations in the composition of biogas can be noticed, but it approximately conforms to methane (the main product), carbon dioxide, nitrogen, hydrogen, and hydrogen sulfide, depending on factors such

as the composition of raw materials, organic loading, and the time and temperature of anaerobic decomposition.

Of the different gases produced, CH_4 is the most desirable gas, because it has a high calorific value approximately 1009 Btu/ft^3 (Lewis, 1993). The approximate heat value of the biogas is 4,5000-6,300 kcal/m^3, depending on the contents of the other gases besides CH_4.

2.3 Cellulosic Wastes : Sources and Exploitation

2.3.1 General Aspects of Cellulose

Cellulose is the most abundant of all naturally occurring organic compounds, probably compising at least a third of all vegetable matter on earth. It is located in the primary and secondary cell walls of plants and exists as a highly ordered crystalline structure composed of D-(+)-glucose moieties joined via β-1,4-glucosidic linkages (See **Figure 2.3**). There are between 2,000 and 10,000 and on occasion as many as 15,000 glucose units in the molecule which can be hydrolyzed partially to form cellobiose, a disaccharide that contains the β-1,4-bond, or completely to yield glucose by acid or microbial enzyme hydrolysis (Colberg, 1988).

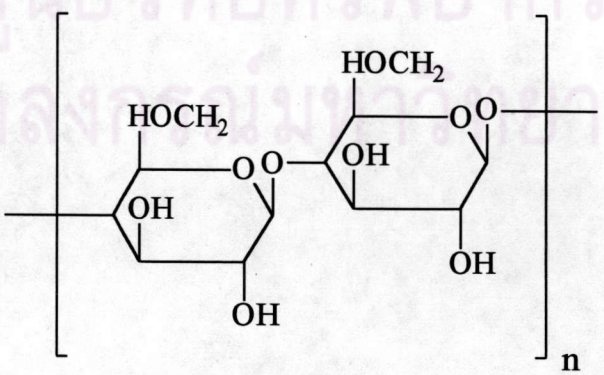

Figure 2.3 Model of cellulose (based on Colberg, 1988)

Cellulose almost never occurs alone in nature, but is usually associated with other plant substances (Kennedy, Phillips, and Williams, 1990). This association may affect its natural degradation. Cellulose fibrils are embedded in a matrix of other polymers, primarily including hemicelluloses, pectin, and proteins. Cellulose imparts tensile strength to the wall to resist turgor pressure. High compression strengths are achieved when lignin, a complex polymer, replaces water in the matrix of cell walls.

2.3.2 Enzymatic Hydrolysis of Cellulose

In recent years, the biodegradation of cellulose has attracted more attention worldwide than other problems of wood chemistry and biochemistry. Among the large amount of information accumulated concerning the cellulolytic activities of enzymes called cellulases during the past decades, the cellulases produced by aerobic white-rot fungi, *Trichoderma reesei*, have been mostly studied, followed by those of anaerobic thermophilic bacteria, *Clostridium thermocellum* (Stafford, Wheatley and Hughes, 1980).

The hydrolysis of cellulose is a very complex process in which a group of cellulolytic enzymes is involved. Cellulolytic enzymes in anaerobic bacteria examined thus far appear to be somewhat different from one another, as well as from those of aerobic organisms. In general, however, most microbial cellulases include the following (Zeikus, 1980) :

(a) endo-β-1,4-glucanases that act ramdomly on crystalline cellulose. In addition, different endoglucanases have different substrate specificities and can attack a variety of substrates.

(b) exo-β-1,4-glucanases that are either glucohydrolases or cellobiohydrolases that act on the nonreducing end of the cellulose chain splitting off cellobiose

(c) cellobiase or β-glucosidase which cleaves cellobiose into glucose, the end product of complete hydrolysis.

These components act synergistically to effectively solubilize crystalline cellulose. Nevertheless, the mechanism for enzymatic cellulose degradation has not yet been completely clarified, despite numerous recent investigations (Beguin, 1990; Bhat and Maheshwari, 1987; Kremer and Wood, 1992; Leschine, 1995).

To date, bacteria represent a promising source for the production of industrial cellulolytic enzymes. Many thermophilic bacterial species produce cellulases that are stable and active at high temperature, resistant to proteolytic attack, and stable to mechanical and chemical denaturation (Hudson, Morgan, and Daniel, 1990, 1991).

Compared with fungal systems, Linden and Shiang (1991) stated that major limitations of cellulases from fungi are low-temperature stability and low rate of enzyme production. Thermophilic bacteria grow rapidly and produce thermostable cellulase but generally have not yielded high productivities (Ng and Zeikus, 1981).

Moreover, there are potential advantages to the isolation of enzymes from, or the use of, thermophilic cellulolytic bacteria. Cellulolytic enzymes from these sources are potentially more economical and anaerobic growth is simpler because of lower oxygen solubility at higher temperature (Reynolds *et al.*, 1986).

2.3.3 The Use of Congo Red in Cellulase Detection

Usually when detecting the cellullolytic activity of bacteria, the clear zones around the bacterial colonies are evident. However, time is required in order to observe the lytic zones. At present, Congo red has been used in the detection of bacterial cellulase. Since the formation of insoluble complex occurs only with β-D-glucan (cellulose), dye binding of Congo red and the associated changes are used to monitor hydrolytic enzyme activity (Wood *et al.*, 1988). When cellulases attack the

bonds that link glucan units, Congo red cannot form a complex with the polymer. Thus, when Congo red is destained, it leaves a yellow halo surrounding the bacterial colony that possesses cellulolytic activity. It provides the basis ofr a rapid and sensitive assay system for cellulolytic bacteria (Teather and Wood, 1982). A vivid contrast between the uniform red color and the halos surrounding the colonies makes this method of differentiation of cellulolytic bacteria superior to other methods (Hendricks, Doyle, and Hugley, 1995).

2.4 Anaerobic Cellulolytic Bacteria

Anaerobic cellulolytic bacteria is a group of bacteria that play an important role in the cellulose decomposition in anaerobic environments. They degrade and consume cellulose as their sole carbon and energy source. Among various fermentation products of cellulolytic anaerobes, they include H_2, CO_2, short-chain volatile fatty acids, and/or ethanol.

2.4.1 Habitat

In anaerobic environments rich in decaying plant material, the decomposition of cellulose is brought about by complex communitites of interacting bacteria. Anaerobic cellulolytic activity starts in rumen, termite guts, cecum of ruminant animals, even in area close to the surface in soils and composts, as well as in freshwater, marine, estuarine sediments, and thermal springs.

2.4.2 Culture Media

More than 40 years ago, Hungate (1950) successfully isolated pure cultures of mesophilic cellulolytic bacteria using a selective media contained rumen fluid and swollen cellulose. Since then, it has been many researches provide medium conditions similar to rumen condition in order to maintain other cellulolytic anaerobes (Pavlostathis, Miller, and Wolin, 1990; Miller and Wolin, 1995). At present, it is

elucidated that rumen fluid is not essential for growth of most cellulolytic anaerobes, except for those rumen cellulolytic bacteria (Coughlan and Ljungdahl, 1988). Jin and Toda (1989) showed the effects of nutrients on cellulase production that the cellulose hydrolysis and endocellulase increased when using a new modified medium, in which urea and K_2HPO_4 concentrations were increased and the yeast extract concentration was decreased. The growth factors required for growing cellulolytic anaerobes are biotin, pyridoxamine, vitamin B_{12}, and *p*-aminobenzoic acid (Zehnder and Brock, 1980). Besides the buffering system, reducing agents, such as cysteine, are added to the culture medium to maintain a low oxidation-reduction potential. Resazurin, the most common used redox indicator, is also added as an oxygen indicator. In the oxidized state, it is blue above pH 6.5 and changes irreversibly to pink below this value. When mixed with reducing agent, the medium should gradually change to colorless. Media that later turn pink have become oxidized and should be discarded (Levett, 1991).

2.4.3 Isolation Methods

A variety of methods is available for the culture of anaerobic cellulolytic bacteria :

(i) Exclusion of oxygen from part of the medium is the simplest method, and is effected by growing the organisms within the medium as a shake or fluid culture

(ii) When an anaerobic atmosphere is required for obtaining surface growths, anaerobic jars and cabinets provide the routine methods of choice.

(iii) A more sophisticated method for the surface culture of anaerobes is the pre-reduced anaerobically sterilized roll-tube technique (Hungate, 1950) and various modification of it. This technique utilizes specialized equipment, is time-consuming, and requires specially trained staff and specialized medium preparation

facilities. It provides the most meticulous anaerobic conditions, and is appropriately used for the isolation and study of exacting anaerobic species that are highly sensitive to oxygen. The method, however is too demanding in time and space for routine use in most diagnostic laboratories where anaerobic work is but one facet of a service commitment.

Comparative studies have shown that pre-reduced anaerobically sterilized methods are not superior to the anaerobic jar and cabinet for the recovery of relevant anaerobes in diagnostic settings (Demain and Solomon, 1986). Throughout this thesis work, an anaerobic chamber is recommended.

An anaerobic chamber, equipped with glove ports and a rigid airlock for transfer of materials, provides an oxygen-free environment (See **Figure 2.4**) in which conventional bacteriological technique can be applied to the isolation and manipulation of obligate anaerobes in conditions of strict and continuous anaerobiosis.

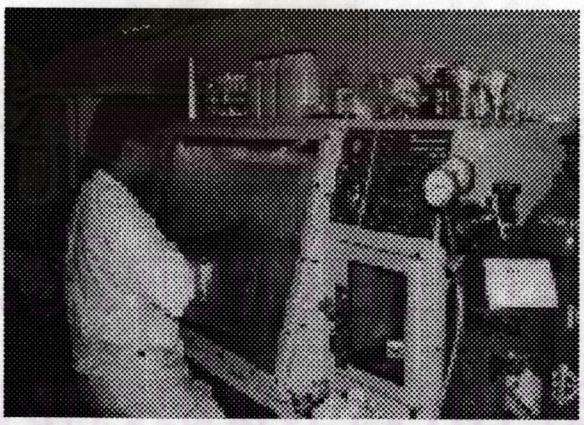

Figure 2.4 Anaerobic chamber

Anaerobiosis in the chamber is achieved with palladium catalyst pellets and a non-explosive gas mixture (10% hydrogen + 5% carbon dioxide + 85% nitrogen) maintained at a slight positive pressure. The interchange is programmed automatically to evacuate and flush with gas mixture prior to access being gained to the main

working chamber; traces of oxygen entering the cabinet from the interchange are rapidly removed by catalysis (Ferrara-Guerrero, Marty, and Bianchi, 1993).

2.4.4 Known Anaerobic Cellulolytic Bacteria

The cellulolytic systems of anaerobic bacteria have been reviewed recently by Coughlan and Ljungdahl (1988). It is worth knowing to concentrate on the transcription regulation systems of *Acetivibrio cellulolyticus*, *Bacteroides* sp., *Butyrivibrio fibrilsolvens*, *Clostridium* sp., *Eubacterium cellulosolvens* and *Ruminococcus* sp. (See **Table 2.1**). To date, however, few have been known which decompose cellulose under extremely thermophilic and obligately anaerobic conditions except for the reports of Reynolds *et al.* (1986) and Sissons *et al.* (1987) in which cellulolytic anaerobes were able to grow at temperature higher than 75°C .

Table 2.1 Some known anaerobic cellulolytic bacteria

Organism	Fermentation Product	Habitat	References
Bacteroides succinogenes	Succinate, propionate, acetate, formate	Rumen	Russell, 1987
Butyrivibrio fibrisolvens	Lactate, butyrate, acetate, formate, H_2, CO_2	Rumen	Buchanan and Gibbons, 1974
Ruminococcus albus	Succinate, acetate, formate, H_2	Rumen	Miller and Wolin, 1995
Ruminococcus flavefaciens	Succinate, lactate, acetate, ethanol, formate, H_2, CO_2	Rumen	Linden and Shiang, 1991
Eubacterium cellulosolvens	Lactate, butyrate, acetate, formate	Rumen	Buchanan and Gibbons, 1974
Clostridium thermocellum	Succinate, lactate, acetate, ethanol, formate, H_2, CO_2	Soil, marine mud, sewage / dairy waste	Hormeyer *et al.*, 1988
Clostridium cellobioparum	Lactate, acetate, ethanol, formate, H_2, CO_2	Soil, rumen, lake sediments	Buchanan and Gibbons, 1974
Acetivibrio cellulolyticus	Acetate, ethanol, H_2, CO_2	Sewage sludge	Mackenzie *et al.*, 1987

2.5 Biomethanogenesis

2.5.1 Definition

Bacterial methanogenesis is a process by which a wide variety of organic materials can be converted by bacteria into a gas rich in methane. This is a ubiquitous process whereas the environment is in the absence of oxygen. The methanogenic bacteria are the most important in the process to help convert volatile fatty acids and reduce carbon dioxide to methane (Zeikus, 1977). Like other biological processes, serveral conditions and factors are essential in biomethanogenesis.

2.5.2 Biochemistry of Methanogenesis

The metabolic pathway of methane production has been reviewed in detail be many investigators (Jone, Nagle, and Whitman, 1987; Rouviere and Wolfe, 1988; Wolfe, 1990). At present, it is elucidated that the process involves at least six coenzymes of methanogenic bacteria in the reduction of carbon dioxide to methane, namely coenzyme M (2-mercaptoethane sulfonic acid), coenzyme F_{420} (7,8-didemethyl-8-hydroxy-5-deazariboflavin 5' phosphate), coenzyme F_{430} (tetrahydrocorphin), methanopterin , methanofuran, and component B (7-mercaptoheptanoylthreonine phospate or HS-HTP). The propose model for the reduction of CO_2 to CH_4 is shown in the **Appendix A**.

In the methanogenesis pathway, the first coenzyme to be involved is methanofuran. It is a carrier of the first CO_2 reduction step (Rouviere and Wolfe, 1988), but the mechanism is unknown. Formaldehyde is then converted to methane by methanopterin with F_{420} as a cofactor or a 2-electron donor. Coenzyme M, the first enzyme discovered, is the smallest among the six known enzymes. It generates the methyl group in the terminal step of CO_2 reduction to CH_4 (Balch *et al.*, 1979) in which component B acts as an inhibitor of methane formation (Wolfe, 1990) and F_{430} is the cofactor in the reaction (Jone *et al.*, 1987).

2.6 Methanogenic Bacteria

Methanogenic bacteria or methanogen are any of a group of bacteria that obtain energy from the metabolic production of methane gas, basically from carbon dioxide and hydrogen. It was first mentioned by the great Italian physicist Alessandro Voltra in 1776 (Blaut, Müller, and Gottschalk, 1990). Methanogenic bacteria are found in a variety of anaerobic habitats including sediments (Kiene *et al.*, 1986; Zeikus and Winfrey, 1976), sludge (Smith and Frank, 1988), and animal waste digesters (Angelidaki and Ahring, 1993; Boone, 1982), the large bowel of man (Misawa *et al.*, 1986) and animals (Miller *et al.*, 1986), the guts of insects, wetwood of living trees, rumen (Beuvink and Spoetstra, 1992; Sribenjalux and Vejjanukroh, 1984), and extreme environments (Brock, 1985; Da Costa, Duarte, and Williams, 1989; Zhao *et al.*, 1986). They are abundant below Eh values of -200 mV (Jone, Nagle and Whitman, 1987). In general, methanogens get inactivated in the presence of oxygen, although not every species is rapidly killed by oxygen (Kemavuthanon, 1994). There are no reports which indicate if any attempt has been made to study occurrence and abundance of methanogenic bacteria in oxic environments.

2.6.1 Unique Characteristics and Taxonomy

Methanogens are fastidious and occur as microbial consortia. They possess some unique features which distinguish them as a special group of bacteria. These include :

a) the absence of peptidoglycan in the cell wall (Woese, 1981).

b) the presence of mainly ether-linked isoprenoids rather than ester-linked phospholipids in the membranes (De Rosa, Gambacorta, and Gliozzi, 1986; Koga *et al.*, 1993).

c) the presence of unusual coenzymes such as coenzyme M, factor F_{420}, factor F_{430}, methanopterin, methanofuran, and component B of the

methylereductase system (Wolfe, 1990). These coenzymes play important roles in the reduction of CO_2 to CH_4. The structures of these coenzymes are listed in **Appendix A**.

Due to F_{420}, cells of methanogens can be recognized by their strong autofluorescence under oxidizing conditions (Jone, Nagle, and Whitman, 1987).

d) based on the unique 16S rRNA structure that differ from those of other procaryotes, methanogens have been grouped under 'archaeobacteria', the third kingdon of life (Woese, 1981).

There have been a number of methanogens isolated during the last 20 years with the developments and improvements in the techniques and tools for cultivation and isolation of these strict anaerobes (Balch *et al.*,1979; Bernhardt, Jaenicke, and Ludemann, 1987). By using the 16S ribosomal RNA oligonucleotide sequence catalogs, as well as biochemical and morphological characteristics, Balch *et al.* (1979) proposed a major revision of the taxonomy of methanogens. This work was based on the characteristics of the 13 species (17 strains) available at the time. Methanogens were grouped into three orders (*Methanobacteriales*, *Methanococcales*, and *Methanomicrobiales*) comprised of four families and seven genera. Moreover, Bryniok and Trosch (1989) used ELISA techniques to determine the taxonomy of methanogens. Thus, the study in this field is not limited. At the time of this present thesis (1996), the number of formal literature reports of methanogenic species in pure culture has risen dramatically and there are preliminary reports of several new isolates. Two new families (*Methanoplanaceae* and *Methanathermaceae*) and five new genera have been added since the 1979 revision (**Table 2.2**)

2.6.2 Culture Isolation

Methanogenic bacteria can be isolated using the Hungate technique (Hungate, 1950) or anaerobic chamber technique as described in 2.4.3. Since they are

very strict anaerobes, exclusion of oxygen in the selective medium is surely necessary, similar to that of anaerobic cellulolytic bacteria (See 2.4.2).

In addition, the physiologically different groups of methanogens can be cultured in selective media containing restricted carbon sources: formate under H_2-CO_2 (80:20), for hydrogen-utilizing methanogens; acetate under N_2-CO_2 (80:20), for aceticlastic methanogens; methanol and trimethylamine under N_2-CO_2 (80:20), for methylotrophic methanogens (Ferrara-Guerrero, Marty, and Bianchi, 1993).

Table 2.2 Validated genera of methanogenic bacteria (Koga *et al.*, 1993)

Order	Family	Genus	No. of species
Methanobacteriales	*Methanobacteriaceae*	*Methanobacterium*	12
		Methanobrevibacter	3
	Methanothermaceae	*Methanothermus*	2
		Methanosphaera	2
Methanococcales	*Methanococcaceae*	*Methanococcus*	7
Methanomicrobiales	*Methanomicrobiaceae*	*Methanomicrobium*	1
		Methanolacinia	1
		Methanospirillum	1
		Methanogenium	4
		Methanoculleus	4
	Methanocorpusculaceae	*Methanocorpusculum*	5
	Methanoplanaceae	*Methanoplanus*	2
	Methanosarcinaceae	*Methanosarcina*	5
		Methanococcoides	1
		Methanolobus	3
		Methanothrix	2
		Methanohalophilus	4
		Methanohalobium	1
		Halomethanococcus	1
Undefined		*Methanopyrus*	1

2.6.3 Known Thermophilic Methanogens

To date, many novel species of thermophilic methanogens have been isolated with temperature optima from 50°C to 88°C (See **Table 2.3**). They are mostly of interests in biotechnology and biochemistry due to their ability to grow at high or very high temperature. The most hyperthermophilic methanogen, *Methanopyrus* strain AV19, isolated by Huber *et al.* (1989), demonstrates that methanogenesis can occur at temperature above 100°C. The fascinated ability to live in thermal environments becomes very interesting.

Table 2.3 Characteristics of thermophilic methanogenic archaebacteria (based on Lowe *et al.*, 1993)

Organism	Mode of nutrition	Isolation/habitat	Optimal growth conditions
Methanobacterium strain CB12	$H_2 + CO_2$, formate	Sludge sample from a mesophilic biogas plant, China	56°C, pH 7.4
Methanobacterium strain FTF	Formate, $H_2 + CO_2$	Thermophilic digestor	55°C, pH 7.5
Methanobacterium thermoformicicum	$H_2 + CO_2$, formate	Thermophilic manure digestor	55°C, pH 7-8
Methanobacterium wolfei	Obligate autotroph, $H_2 + CO_2$	Sewage sludge and river sediment	55-65°C, pH 7.0-7.5
Methanogenium frittonii	$H_2 + CO_2$, formate	Nonthermal freshwater sediments	57°C, pH 7.0-7.5
Methanogenium thermophilicum	Formate, $H_2 + CO_2$, 0.2 M salt optimum	Marine cooling channel of nuclear power plant	55°C, pH 7.0
Methanogenium stain UCLA	Formate, $H_2 + CO_2$	Anaerobic sludge digertor	55-60°C, pH 7.2
Methanopyrus strain AV19	$H_2 + CO_2$	Sediment samples at Guaymas Basin hot vents	98°C, pH not reported, 1.5% NaCl
Methanosarcina strain CHTI 55	Acetate, methanol, methylamines	Thermophilic digestor	57°C, pH 6.8
Methanosarcina thermophila	Acetate, methanol, methylamine, trimethylamine, $H_2 + CO_2$	Sludge from thermophilic digestor	50°C, pH 6-7
Methanothermus sociabilis	Obligate autotroph, $H_2 + CO_2$	Terrestrial solfataric muds	88°C, pH 6.5

2.7 Thermophily

Life at high temperature has been a subject of interest and studies for many years (Brock, 1978, 1985, 1995) and a considerable number of thermophilic bacteria, including cellulolytic and methanogenic bacteria, has been discovered as metioned above. In order to live within a particular temperature range, organisms have to adapt structurally and functionally to the environmental temperature. This means they must "love" these temperatures: to be alive at high temperatures, they must be thermophilies.

The taxonomy and the molecular mechanism of most thermophilic bacteria are far from complete at the present time. However, there are some group of researchers who try to elucidate the secret of thermophiles and/or their enzymes, including Amelunxen and Murdock (1978), Lowe *et al.* (1993), and Zuber (1979).

In thermophilic bacteria, their adaptation to the temperature means a shift of the temperature optimum for growth of the metabolism and of the biological active cell components to higher temperatures compared to that of the mesophilies. These thermophilic cell components should be more thermostable at high temperatures and less active or effective at low temperatures (Zuber, 1978). It is conceivable that at extreme temperatures, e.g. at the upper temperature limit of a thermophilic bacterium, an increase in denaturation will cause an increase in resynthesis. Yet, this remains to be elucidated.

2.8 Biogas Production by Thermophilic Pure Cultures

Many microorganisms have performed biogas production in the natural environment. They are involed with all types of bioconversion and various cycles of matter on earth. In nature, microorganisms compete with each other and the most suitable species predominate in certain niches. At any one time one would expect to

find a mixed population interacting in the environment in antagonistic or symbiotic relationships.

In order to maximize the performance of desirable microorganisms, it is imperative to isolate pure cultures from the environment and study their cultural characteristics in great detail.

To explain the beneficial use of selected thermophilic bacteria, this sample of cellulose degradation may be appropriate. First, the fermentative species that possess the requisite enzymes (invariably extracellullar) act to attack macromolecules and convert them to simpler monomers and oligomers such as acetate, alcohols, fatty acids, H_2 and CO_2, e.g. *Clostridium thermocellum* converts cellulose to hexoses and ethanol, lactate, acetate, H_2 and CO_2 (Hormeyer *et al.*, 1988); *Clostridium thermohydrosulfuricum* and *Thermoanaerobium brockii* (Lowe, Jain, and Zeikus, 1993) convert amino acids and sugars to acetate, ethanol, lactate, H_2, CO_2 and long-chain fatty acids (longer than acetate). Hydrogen-evolving acetogenic bacteria oxidize propionate, butyrate and other fatty acids to acetate, H_2 and CO_2. These bacteria require low hydrogen tensions although they often live in habitats that have high and continuous rates of hydrogen evolution. This paradox may be resolved by the discovery of thermophilic obligately autotrophic H_2-oxidizing bacteria such as *Hydrogenobacter autotrophicus* and *Calderobacterium hydrogenophilum* (Ferguson and Mah, 1983). The end result of these activities is the fermentation of complex cellulose to H_2 and CO_2, acetate and formate that are converted to methane by the methanogens. These include *Methanobacterium thermoautotrophicum* (H_2 and CO_2 to CH_4); *Methanococcus thermolithotrophicus* (H_2, CO_2 and formate to CH_4 and some CO_2) and *Methanothrix* sp. (acetate to CH_4 and CO_2) (Peillex *et al.*, 1988; Zinder *et al.*, 1984). Methanogenesis in defined co-cultures has been studied on fermentation of sugar beet pulp. *C. thermocellum* alone produced acetate, succinate, methanol, ethanol, H_2 and CO_2. A coculture with a species of *Methanobacterium*

resulted in only trace amounts of ethanol and succinate (Weimer and Zeikus, 1977). Acetate concentration was about three times that produced by *C. thermocellum* alone. Association of this coculture with *Methanosarcina* resulted in 75% of total carbohydrate in the pulp being converted to methane (Ahring and Westermann, 1987; Yang and Tang, 1991).

In this study, the coculturing technique were conducted at 55°C using thermophilic cellulolytic bacteria and thermophilic methanogen to convert paper waste to biogas. Although this thesis was conducted in a laboratory scale, some indications mentioned above showed that the techniques of pure culture and coculture would be indubitably implied and could provide a know-how to the country.