CHAPTER 2

LITERATURE SURVEY

2.1 Anaerobic Fermentation and Biogas Production

Anaerobic fermentation or biodegradation has a long history of study in microbiology. The process of conversion or bioconversion of renewable resources, such as agricultural residues, biomass or wastes, to chemicals and biofuels by microbial acitivities, is a result of catabolizing organic matter in the absence of oxygen (12). A variety of catabolic products, such as volatile fatty acids, alcohols and gases, are produced. In case of biogas digestion, methane (CH_4) and carbon dioxide (CO_2) are the main components of gaseous products (13). Several reviews touch on this subject are also available (14, 15, 16).

2.2 Biogas Digestion

2.2.1 Microbes in Biogas Digester

In conventional-type of biogas digester, bioconversion of complex organic matters is carried out by a mixed population of bacteria. Many diverse genera of obligate and facultative anaerobes whose individual metabolic activities perform at different trophic level, and different biochemical reactions proceed in the anaerobic fermentation. Microbial process in biogas digestion was formerly divided into only three stages by three corresponding microbial groups, i.e., hydrolytic stage by hydrolytic group, acetate-

forming stage by acetate-forming group and methanogenic stage by methanogenic bacteria. From the evidences that two corresponding group involve in the second stages, they are : hydrogen-producing acetogenic bacteria and homoacetogenic bacteria, Zeikus had concluded that, not three but four groups of microorganism were corresponding to biogas production process (16). As mention above, the biogas digestion proceeds in anaerobic condition. The effect of oxygen on bioconversion process is quite varied (17). Oxygen is lethal to strict anaerobes, inhibits the metabolism of some anaerobic species and has no effect on some species. It is said that the microbial process is interrupted and the production of methane gas is stopped by the presence of oxygen in the biogas digester.

2.2.2 Environmental Factors

The efficiency of methane production depends on some appropriate conditions, e.g., kinds and temperature of the substrate, loading rate, solid concentration, retention period, acidity/alkalinity (pH), toxic substances, nutrients concentration and type of digesters (18, 19).

2.2.3 Hydrogen Gas as a Key Regulatory Role

Fermentation of carbohydrates, proteins and long-chain fatty acids initially leads to the formation of lower volatile fatty acids, alcohols, other neutral compounds, hydrogen (H_2) and carbon dioxide. Even though hydrogen is rarely detected in the mixed gas (20, 21), but it plays a key regulatory role in the overall digestion

process (22). Hydrogen is rapidly and preferentially oxidized by all known methane-forming bacteria (12, 23) in the process of reduction of CO_2 to CH_4(24).

Hydrogen-producing acetogens degrade the alcohols and long-chain fatty acids produced by the hydrolytic group, and producing acetate, H_2 and CO_2. Without methanogenic bacteria, hydrogen is formed and accumulated in the culture, then growth of hydrogen-producing acetogens will be inhibited. In association with methanogenic bacteria, hydrogen has been removed by the oxidizing activities to methane by the added organisms, good growth of hydrogen producing acetogens can occur. Hydrogen is not only the substrate for methanogenes and regulates the rate of methanogenesis, but it also regulates the rate of acetogenesis and provides the overall mechanism for terminal electron removal, thus permitting the continued breakdown of intermediates (12, 23). The metabolic interactions of non-methanogenic and methanogenic bacteria in the fermentation process are important. Recent investigations have established that utilization of H_2 by methanogenic bacteria may exert a direct effect on the metabolic activities of other bacteria (12, 22, 25). It means that hydrogen formation in non-methanogenic bacteria may occur by proton reduction, either solely or as a supplement to the formation of reduced organic end products for disposing of electrons. This coupled oxidation-reduction reactions between, non-methanogenic bacteria and methanogenic bacteria during fermentation of one initial substrated is termed "interspecies hydrogen-transfer" by Iannotti (22).

2.3 Cellulosic Wastes as Resources

2.3.1 Nature and Availability of Cellulose

Cellulose is a main component of cell-wall material or skeletal polysaccharide of wood and plants, amounting to approximately fifty per cent, and exists as a highly ordered crystalline structure composed of glucose moieties linked by β1-4 glucosidic bonds (26, 27, 28). Cotton is α-cellulose, a form insoluble in 17.5 % NaOH. Plant and wood celluloses generally contain β - cellulose, a material soluble in the indicated solution. Wood cellulose occurs in the presence of hemicelluloses of related structure and lignin, a nonpolysaccharide. Molecular weight of cellulose molecule is generally in the range of 300,000-500,000. When cellulose is partially hydrolyzed, glucose, as well as cellobiose, cellotriose and cellotetraose can be isolated. Complete hydrolysis by acid yields D-(+)-glucose as the only monosaccharide. Other polysaccharides also occur in the presence of cellulose, e.g., pentosan and starch (28).

The cellulose molecule is thread-like, existing as fibrils in long bundles of molecules which is stabilized latterally by hydrogen bonding between hydroxyl groups of adjacent molecules (26). The structure of acid and alkaline swollen cellulose is open ; and this material is readily split by cellulase. Direct conversion of waste cellulose by acid and enzymatic hydrolysis processes yields glucose (26,28, 29, 30).

Waste cellulose is plentiful and offers the most immediate promise for economic utilization. Cellulose is a major component of agricultureal wastes (straw, leaves and stalks of many plants, rice and other hulls, peanut and other shells, corn cobs, bagasse, etc.), food processing wastes (fruit peels, pulp, vegetable trimming, etc.), wood wastes chip, bark, sawdust, paper mill fines, etc.), and municipal wastes (gabages and waste paper). It is expensive to dispose these wastes without undue pollution. The availability of cellulosic wastes, especially in Thailand, ensures abundant cheap substrates for any processes that are developed. Biogas technology is the one of any processes that we are emphasized to develop.

2.3.2 Enzymatic Hydrolysis of Cellulose

It has been found that the degradation of cellulose is a complex process, requiring the participation of a group of enzymes called cellulases. These cellulolytic enzymes are produced by microorganisms that are widely distributed in nature, i.e., bacteria, actinomycetes and higher fungi. There are at least three different types of cellulolytic activities : exo-β-1, 4-glucanse, eno-β-1, 4-glucanase, and β-glucosidase (31, 32, 33). The exoglucanase or β-1, 4-glucan cellobiohydrolase can split off cellobiose from the non-reducing end of the cellulose chain (31). The endoglucanases are the group of enzymes called β-1, 4-glucanglucanohydrolases that randomly hydrolyze β-1, 4-glucans (32). The glucosidase or cellobiase hydrolyzes mainly cellobiose, but also higher cellodextrins

to glucose (34). One of the most important properties of cellulases is heat stable, since the enzymatic hydrolysis of cellulose proceeds faster at higher temperature. Moreover, endoglucanases are more stable than exoglucanases (34).

The mechanism for enzymatic cellulose degradation has not yet been completely clarified. Several investigations (31, 32, 33 34, 35) have proposed that endoglucanases initiate the attack on native cellulose formed hydrolyzed cellulose. Free ends for exoglucanase are released at the sites of non-crystalline regions of the cellulose fibre. Different endoglucanases have different substrate specificities and can attack a variety of substrates. In the second step, exoglucanase acts on hydrolyzed cellulose, and cellobioses are created. Finally, β-glucosidase will attack on cellobiose, and glucose is the end product of complete hydrolysis.

Endoglucanases and exoglucanase have a strongly synergistic action to effectively solubilize crystalline cellulose (34) but not when hydrolyzing phosphoric-acid-swollen cellulose (34, 36, 37, 38).

2.3.3 Biological Utization of Cellulosic Wastes

The biological utization of cellulosic wastes depends on its conversion to glucose, which, in turn, is often converted into other useful products. The biological processes for productions of food, feed, chemicals and fuels from cellulose may be either multiple conversions or single conversion. In general, the multiple

processes may occur naturally, e.g., the conversions of cellulose to meat by ruminants (16), to microbial protein (39, 40, 41), to biofuel : methane or hydrogen gas(39) and to compost (9, 42, 43). Single conversion of cellulose to glucose may be accomplished by either acid hydrolysis or enzymatic hydrolysis. The sugar from these processes were used to produce alcohol (34) and to grow yeasts used as single cell protein for human food (44).

2.4 Anaerobic Cellulolytic Bacteria

A group of anaerobic bacteria that grow well on cellulose as a sole carbon source are called anaerobic cellulolytic bacteria. Many products are found in pure culture of this group, such as H_2, CO_2, formic, acetic, butyric, lactic and succinic acids and ethanol (45). In natural habitats these bacteria have metabolic interactions with other groups of bacteria and may produce other products. In the rumen or the microbial fermentation tank of ruminants, acetic acid, butyric acid and CO_2 are final products. Conversion of lower fatty acids into higher ones has been found in pure cultures and may also occur in the rumen (45). Anaerobic cellulolytic bacteria are also found in anaerobic environments in nature, e.g., sediments, and soils. The early work with anaerobic cellulolytic bacteria has been reviewed by Hungate (43).

2.4.1 Rumen : A Novel Habitat

Anaenobic cellulolytic bacteria are found in many natural habitats, for instance, in soil, in digestive sludge and in

rumen (46). Rumen is a very interesting habitat for studying cellulolytic bacteria, since most of known cellulolytic bacteria are isolated from this organ.

The ruminants differ from other mammals by means of food breakdown process. Its food, such as grasses and other vegetative materials,are degraded by microbial fermentation in the rumen before it passes on to the true stomach and intestinal tract where normal mammalian digestion occurs. Here in the rumen, many microbial processes are conducted including the degradation of carbohydrate such as cellulose which can not be utilized unless digested by microorganisms. Cellulose will be converted through a series of steps, i.e., cellulose→glucose→fatty acids→amino acids→protein. The resulting protein will be further absorbed and existed in animal tissues as meat (16).

Because of a far more constant environment, the rumen is well adapted for maintaining of a large and diverse microbial population. There are relatively constant in supply of food and water, some physical environments such as temperature held at about 39'c, and pH of ingesta, usually slightly acid, is held relatively buffered saliva for adjusting an equilibrium between the ruminal ingesta and the blood stream with regard to hydrogen ions (47). Rumen content is a heterogeneous mixture with various degrees of stratification depending on such factors as the time after feeding, the type of ration and water supply. There are abundent evidences.

that samples taken from different locations in the rumen vary considerably in chemical composition and in rates at which various reactions occur (48). Anyway, data on rates of reactions may very depending on sampling time and water supply (49). By direct microscopic methods, the numbers of bacterial counts may vary all a day long even in animals fed on a constant ration, and also method of feeding, i.e., number of feedings and whether concentrates or roughages are fed separately or not. Hungate (50) obtained almost equal numbers of cellulolytic bacteria in both liquid and solid samples, but, Gall et al (51) found that total counts from solid samples seemed to be higher than liquid samples and the number of bacterial counts from liquid samples might vary depending on method of sampling : that was higher in liquid obtained through a fistula than liquid obtained directly from stomach tube of animal.

Minato and his co-workers (52) studied the technigue for fractionation of bacteria in rumen microbial ecosystem. Generally, it is based on the attachment ability of bacteria to solid substrate encountered in the natural ecosystem. They found that the amount of bacteria attached to cellulose powder were about 8.7-24 per cent of total bacteria in rumen fluid. Attached bacteria were composed of coccal, bacillus and spiral forms. Most of them were gram-negative rods. Furthermore, they found that the specific β-glucosidase activity of those attached bacteria was significantly high compared with amylase and urease activities.

2.4.2 Culture Media

Because of difficulties for pure culture studies, culture media have been developed for isolation and/or enumeration of cellulolytic bacteria. Many researcher had formerly used indirect enrichment technique by using liquid medium (46). This method was more expensive and time consumming compared with direct enrichment technique or semi-solid medium. The latter one has several advantages, i.e., less time and materials in obtaining pure cultures (53). However, the success of culturing was depended on an attempt to provide medium conditions similar to rumen condition (50, 54). A series of studies indicated that bicarbonate and/or carbon dioxide* (46, 50, 55), reducing agents** (46, 55, 56, 57) and growth factors (50, 54) are essential for growth of the anaerobic cellulytic bacteria. Besides that resozurin is also added in the medium as oxygen indicator (46). Containing of mineral mixture, rumen fluid as a source of growth factors, a carbonic acid-bicarbonate buffer, cysteine as a usually reducing agent and cellulose as a subtrate in the medium was recommended (46).

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*For bufferring effect in the medium

**For maintaining a low oxidation-reduction potential in the medium.

2.4.3 Isolation Technique

Method of sampling prior to inoculating on culture medium can assume considerable importance. Speed of sample taking and exclusion of air from the sample have been emphasized (58). Depending on high degree of the reducing capacity of rumen contents when they were exposed to air and held for considerable lengths of time before culture processing, quantitative change in total bacterial counts was not found (54), but quanlitative change was detected (59). From these evidences, it seems best that samples should be processed as rapidly as posible.

In early works, the technique modified by Hungate (46), called Hugate teachnique was widely used, including preparations of oxygen-free gas and recommended medium, inoculation, isolation and transfer of colonies. Although, Hungate roll-tube technique has been widely accepted, but it is limited on utilization of tubes instead of petri dishes, and thus imposes difficulties in single-colony purification of isolates. Reducing these limitations, anaerobic globe boxes have been gradually developed (60, 61). Early performing of anaerobic condition in the box was simple, oxygen from the equilibrated gas was removed by passing through a glass tube containing copper burning at 350°c before delivery to the carbinet and to scavenge residual oxygen in the carbinet by using palladium catalyst. Right now, new technologies are involved in the subject and modern anaerobic chambers are created. It is the convenience and ease in handling which makes

this new equipment used worldwide and as a result many species of anaerobic bacteria have been isolated from natural habitats.

2.4.4 Known Anaerobic Cellulolytic Bacteria

By using the morphology and the ability of spore forming anaerobic cellulolytic becteria are separated into 3 groups, those are spore-forming rods, non-spore-forming rods and cocci (46,53).

Reports on spore-forming cellulolytic anaerobes indicated that these bacteria were not important in the ruminal fermentation (46, 62). It seems possible that this group was not growing or rapidly destroyed in the rumen (63). Total counts of cellulolytic bacteria in cow rumen contentswere quite low and Clostridium lochheadii was predominated (64). Organisms included in spore-forming cellulolytic anaerobes were shown in Table 2.1.

Non-spore forming rods, the second group of cellulolytic bacteria, are comprised of three groups, i.e., cillobacteria group, succinic acid producing bacteriods and butyrivibrio group. Some species of non-spore forming rods were shown in Table 2.1.

On the basis of its being an anaerobic, peritrichous, gram-positive rod, cellobacteria group was placed into genus Cillobacterium (70), and only one species of this genus was found in the ruminal fluid from a cow pastured on clover and named C. cellulosolvens (71, 72). It is suggested that cillobacteria group is not an important ruminal species. Succinic acid-producing

Table 2.1 Known Anaerobic Cellulolytic bacteria.

Microorganism	Reference
Sporeforming rod	
Clostridium cellobioparum	Hungate 1944 (65)
Micromonospora propionici	Hungate 1946 (66)
Non-Sporeforming rod	
Butyrivibrio fibrisolvens	Bryant et al. 1956 (69)
Eubacterium cellulosolvens	Bryant et al. 1958 (67)
Bacteroides ruminicola	Bryant et al. 1958 (67)
Succinimonas amylolytica	Bryant et al 1958 (67)
Bacteroides succinogenes	Hungate 1950 (46)
Butyrivibrio alactacidigens	Hungate 1966 (24)
Cocci	
Ruminobacter parvum	Sijpesteijn 1948 (68)
Ruminococcus flavefaciens	Sijpesteijn 1951 (55)
Ruminococcus albus	Hungate 1957 (64)

bacteriods appear to be among the more important bacteria present in the rumen. This group is strictly anaerobic, non-motile, gram-negative rods that produce large amounts of succinic acid from carbohydrate fermentations. The presence of these and many other succinic acid producing bacteria (46, 67) may involve in the high production of propionic acid in ruminal contents. These bacteria act on carbohydrates including cellulose to produce succinic acid which is further decarboxylated to propionic acid by other ruminal microorganisms (62). Three ruminal species of succinic acid-producing bacteriods utilizing cellulose as a substrate are Bacteroides succinogenes Hungate (46, 70, 73), B. amylophilus Hanalin and Hungate (74) and B. ruminocola Bryant et al (67). The variation of morphological aspect and pigment production can be detected usually in B. succinogeness, on the basis of physiological and cultural characteristics (46, 50, 73, 75) and all strains required certain volatile fatty acid for growth (76).

Butyrivibrio group, the member of non spore-forming rod, was a small gram-negative curved rod that fermented a wide variety of carbohydrates and produced CO_2, H_2, butyric and fomic acids, and showed an uptake of acetic acid in the cellulose fermentation (46). This group may be detected in large number from cattle fed a variety of rations. (69). The genus Butyrivibrio was established with the type species B. fibrisolvens Hungate, which acted on glucose, resulting butyric acid. However, the production of acetic acid may be detected in the carbohydrate fermentation by some strains

of this type species. Only 3 of the 48 strains of B. fibrisolvens were shown to ferment cellulose (53).

Anaerobic cellulolytic cocci have been isolated in a large number from ruminal contents and the species that have ability to ferment cellulose are categorized into genus Ruminococcus (77). It is believed that this group plays an important role in cellulose digestion in the rumen. They are anaerobic, gram-positive, non-motile, non spore-forming cocci that fermented celllulose with the production of large amounts of succinic acid. The first type species R. flavefaciens was established (55). The bacteria in this genus may be gram-negative or variable cocci that fermented carbohydrate to form acetate, a least trace of H_2, and various combinations of ethanol, formate, lactate and succinate (46). This evident is confermed by Bryant et al. (71). Several variety of the species R. flavefaciens may be detected, i.e., production of succinic acid and yellow pigment are usually found from the fermentation of cellulose, or fermented cellulose or cellobiose, not from other sugars (64, 70, 71, 78). A second species, Ruminococcus albus Hungate (64) was established to include other strains of ruminococci that did not produce succinic acid from cellulose. The characteristics of this species were quite variable but most strains differed from the type species in producing little or no yellow pigment, cells were usually arranged singly or in pairs, gram-negative to gram-variable. Fermentation products included H_2, CO_2, ethanol, acetic acid, formic acid and lactic acid in various combinations and

proportion. On the comparison to R. flavefaciens, higher production of H_2 and CO_2 were found in R. albus (53).

2.5 Methanogensis

2.5.1 Definition

Bacterial methanogenesis, a ubiquitous process in most anaerobic environments, has been recognized and documented for more than a century. It is a microbial process of an anaerobic decomposition of organic matters in natural habitats, such as sewage digestive sludge, contents of rumen and intestinal tracts and in sediments and muds of various aquatic habitats, and producing gases, mainly CH_4 and CO_2, as a result. The producing gases or biogas which commonly observed in nature, can often be ascribed to growth of methanogenic bacteria. Similar to cellulolysis, several factors are required in biogas production.

2.5.2 Substrates

As mentioned above, hydrogen plays an importante role in methane formation, i.e., electron receptor, inter species transfer or overall pathway regulator and also hydrogen source (in H_2-CO_2 system). In term of carbon source in the process acetate and carbon dioxide appear to be the major immediate methane precursors in many anaerobic environments (12, 14). The relative importance of these precursors can be compared because acetate conversion to methane occurs by reduction of the intact methyl groups and not via CO_2

as an intermediate (12, 79, 80, 81). In different environments the relative amounts of methane formed from these substrates varies (12, 13, 82). e.g., in anaerobic waste digesters, in rice paddy soil, in lake sediments, percentages of the methane produced from acetate are 73-90, 60 and 70, respectively, and the lest from carbon dioxide. In contrast to the usual importance of acetate in methanogenesis, only 5.3 % of the methane formed in the rumen was derived from acetate (83). In 1981, Sandbeck and Ward (48) examined the fates of acetate and carbon dioxide in several experiments designed to indicate their relative contributions to methane production at various temperature in two low-sulfate, hot-spring algae-bacterial mats, and reported 71 to 80 % of methane were derived from carbon dioxide.

2.5.3 Metabolic Pathways

There are at least two unique coenzymes that involves in the microbial process of methane formation i.e. co-enzyme M and co-enzyme F_{420}. The structures of these two enzymes have been shown in Appendix A. Conenzyme M or Co M has been found only in methane forming bacteria. Co M had been identified as 2-mercaptoethane sulfonic acid.
It is the smallest of all known coenzymes and exceptionally high in sulfur content and acidity (84). Co M is coupled via a newly discovered methyl reductase to the activation of CO_2 and generates methyl group in the terminal step of CO_2 reduction to CH_4 (84, 85, 86, 87, 88). It was concluded that this cofactor does not play a general role in other methyl transfer reactions.

Similar to Co M, coenzyme F_{420} has not so far found in any other living tissues, and has fluorescence at a wavelength of 420 nm. The structure of coenzyme F_{420} or F_{420}, a low potential electron carrier, was elucidated by Eirich et al (89). It has been idenfied as flavin mononucleotide anolog 7, 8-didemethyl-8-hydroxy-5-deazariboflavin-5-phosphate which has an N-(N-L-lactyl-r-L-glutamyl)-L-glutamic acid side chain attached in a phosphodiester linkage. The cofactor operates as a 2-electron donor in both anobolic and catabolic reactions in methane-forming bacteria (15, 90, 91, 92, 93, 94).

Pathways in methane formation can be summerized that H_2 is oxidized via a F_{420} linked hydrogenase and the product of CO_2 reduction or CH_3-CoM is reduced to CH_4 (81, 94, 95). In addition to F_{420}, other chromophoric factors F_{342} (or F_{340} or F_{350}) and F_{430} have been described but the role is presently unknown (96, 97, 98, 99). However, both F_{420} and F_{342} are useful for tentative identification of methanogens by fluorescent microscopy (100, 101). It is apparent that reduction of CO_2 to CH_4 by transfer of eight electrons is a very complicated process, involving a number of new co-enzymes, enzymes and carriers, the mechanisms of which remain to be elucidated.

2.6 Methanogenic Bacteria

This group of bacteria has a specific type of energy yielding metabolism and unique ability to produce methane. The distribution of methanogenic bacteria or, in short, methanogens and activities are

restrictly to anoxic environments where associated microorganisms maintain a condition of law oxidation-reduction energy and also produce substrates for methanogenesis as well as other nutrient factors. The details of methanogens have been reviewed by Zeikus (14) and others (15, 102, 103, 104, 105).

2.6.1 History and Characteristics

It was a long story about combustible air or methane formed in the sediments of streams, bogs and lake rich in decaying vegetation. Many investigations (cited by 106) found that the origin of methane gas based on strictly anaerobic bacteria. From 1950 until 1978, the characteristics and activities of methanogens have been investigated. From 1979, Wolfe and his coworkers and others (15, 102, 103, 104, 105) emphasized that these groups of bacteria have unique characteristics and proposed new groups of bacteria (will be discussed later).

From Bergey's Manual (107) general characteristics of methanogens are strict anaerobes, methane production, morphology varies (rod-shape, spirillum and cocci), gram staining varies, wide ranges of DNA base composition (from 28 to 51 mole percent G+C) and uncertainty of early taxonomic schemes proposed only three genera.

2.6.2 New Trend of Taxonomic Schemes

Methanogens are recognized as members of the family Methanobacteriaceae (107). By physiological specification, Bryant

classified this family into three genera, i.e., Methanobacterium, Methanosarcina and Methanococcus. It is true that an evolution is also occurring in bacterial taxonomy. Because of inadequate taxonomic data from poorly developed methods for phylogenic studies, the result of a qualitative approach provides little insight into the relationships between the various species (15) Fortunately, the successfully molecular approaches to eukaryote phylogeny has been modified and extended to the bacterial domain. Depending on the fact that the ribosome is of ancient origin and has not changed extensively with time (108). Comparative analysis of the 16S ribosomal RNA sequence has been used to explore prokaryote phylogeny. Experimental details of the approach and the methods of data analysis have been decribed (109, 110, 111, 112). Nevertheless, the analysis appears to yield a good approximation to the true phylogenic relationships and posed that there should be 2 major lines of descent, the archaebacteria* and the true bacteria (or eubacteria). The archaebacteria is quite distinct from the latter one in phenotype and should be considered a separate kingdom of prokaryotes. Their unique characteristics are : (i) differences in cellwall structure and composition, none of which contains muramic acid the hallmark of typical cell walls (113, 114), (ii) differences in lipid composition in cellular membrane, major component is a branched chain (phytany),

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* The name archarbacteria implies that these organisms were the dominant ones in the primeval biosphere, and the atmosplere at that time was rich in CO_2 and included some hydrogen but virtually free of oxygen.

ether-linked lipid (115, 116), (iii) unique cellular morphology (113, 114), (iv) differences in transfer RNA (tRNA), devoiding of ribothymidine in the thymine-psendouridine-cystidine or TψC loop (117), (v) differences in RNA polymerase subunit structures (118), and (vi) unique coenzymes are presented (15, 81, 89). The archaebacteria are now known to include three very different kinds of bacteria : methanogens, extreme halophiles and thermo acidophiles, as shown in Figure 2.1, details of archaebacteria have been discussed some where (104, 105, 109, 110).

At any rate, methanogens have been considered to be as old as or older than any other bacterial group. Based on comparative analysis of 16S rRNA sequences and other informations;all known methanogens can be classified into three orders, four families and seven genera, as shown in Figure 2.2. Instead of three genera, the seven proposed genera are Methanobacterium, Methanobrevibacter, Methanococcus, Methanomicrobium, Methanogenium, Methanospirillum and Methanosarcina. Until 1972, only nine species of methanogens were recognized. From newly taxonomic determination, thirteen species have been proposed. Any of former species may be changed, e.g. Methanosarcina methanica to Methanobravibacter smithii. Now, 21 strains of the type species have been known.

2.6.3 Culture Media

All known pure cultures of methanogens utilize hydrogen and/or acetate as a source of energy and NH_4^+ as a nitrogen

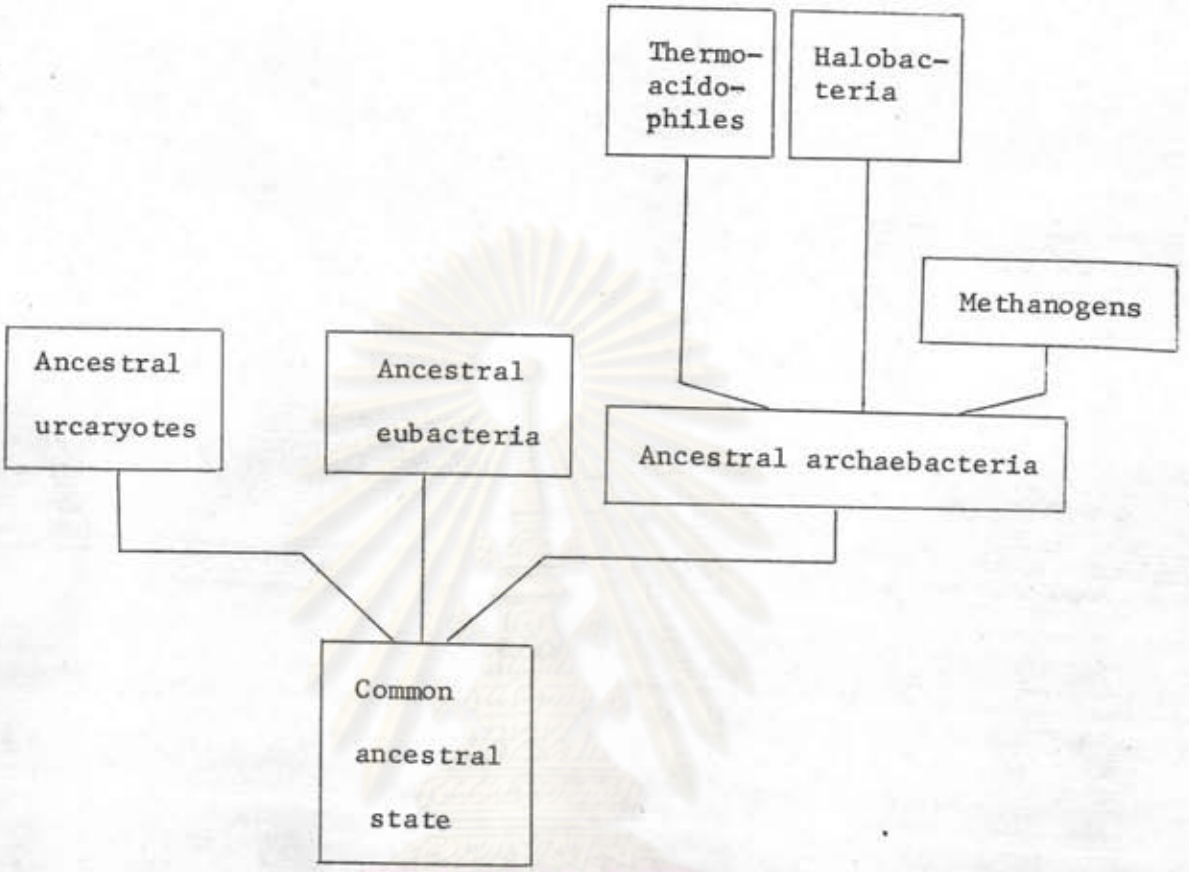

Figure 2.1 Schematic representation of the major lines of prokaryotic descent.

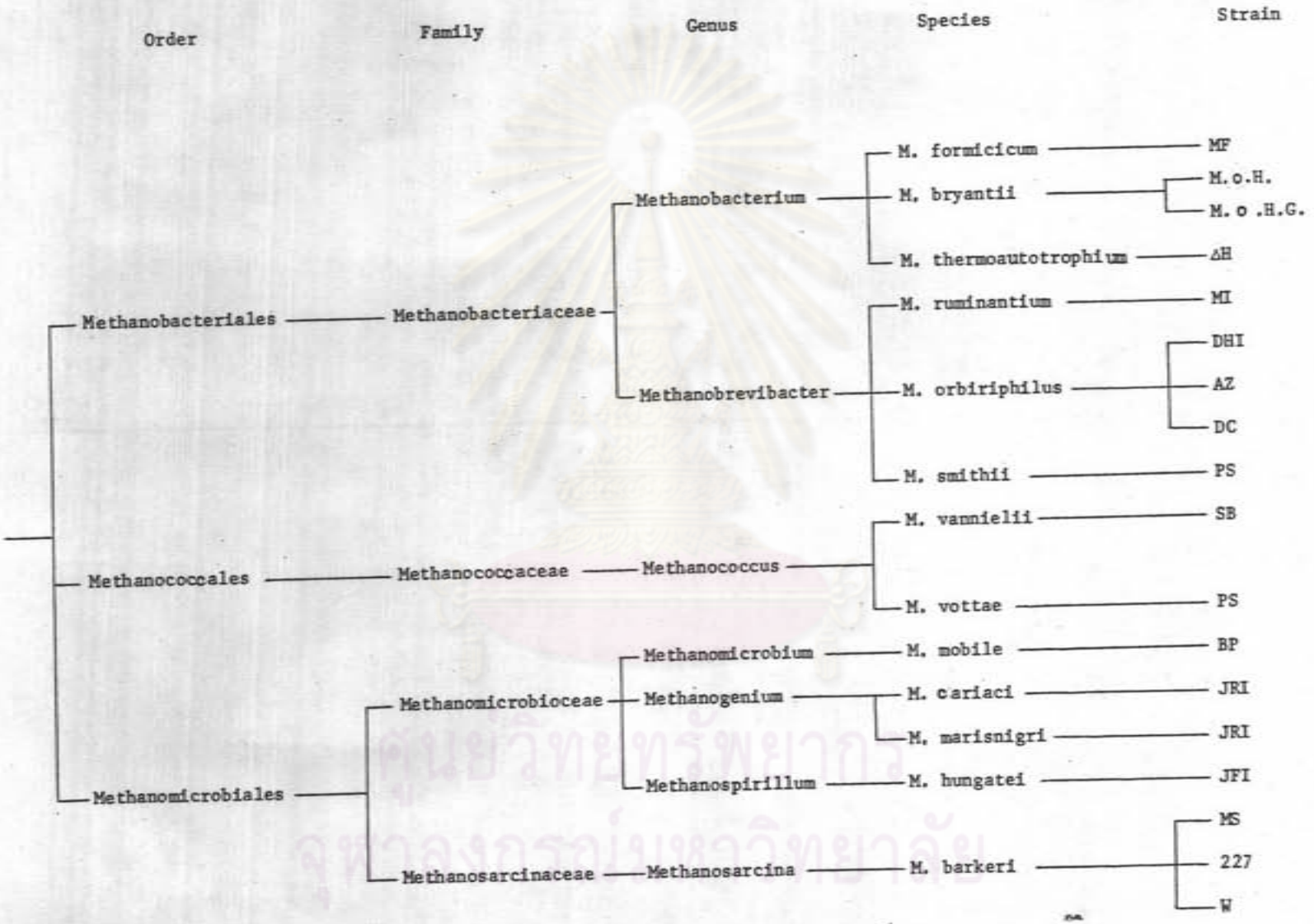

Figure 2.2 New Taxonomic treatment for methanogenic bacteria based on 16S RNA comparative cataloging.

source (12). There are at least three basal media which are successful and widely use in isolation of methanogens (15). As fastidious as cellulolytic bacteria, the basal media should contain trace minerals trace vitamins, sodium bicarbonate, sodium acetate, sodium formate, yeast extract, trypticase and cysteine hydrochloride and also sodium sulfide as reducing agents.

2.6.4 Isolation Technique

By using anaerobic chambers and cylinders have made more advantage in the cultivation of methanogens. These fastidious organisms are easily cultivated on semi-solid basal medium (15). After inoculation in the anaerobic chamber, the semi-solid plates are transferred into a cylinder. Outside the chamber, the cylinder was pressurized to 2 atmospheres with mixed gases of H_2 and CO_2 in the ratio of 80 : 20. Growth can be detected by either methane production or decreasing in cylinder pressure or both (15). Ultralow-oxygen chamber built inside the equipment may be a possible alternative (119).

2.7 Biogas Production from Pure Cultures

Bacterial activities may proceed useful products to gain benefits on the quality of human life. Pure culture technique is one of those which may be performed by using only one, or two or three kinds of selected microbes in the process, which is commonly

called single culture, double or co-culture and triple or tri-culture, respectively. Ethanol produced from molasses is a well-known example of pure culture of one selected strain of yeasts. In co-culture or tri-culture systems, either same or different kinds of microbes are chosen, e.g., all may be bacteria, or fungi and the combinations of each. Metabolic relationships are considerably important to succeed the processes, i.e., the substrate has firstly been degraded by one(s), then resulting product(s) has further been utilized by another or others, and finally, expected or useful product(s) has been found. Several investigations have proved that co-culture technique can be readily be expanded to bio-conversion domain, e.g., the productions of ethanol (16, 123) and fatty acids (22, 122, 124), and those have been summerized in Table 2.2

In general, yields of biogas from certain substrates, especially, cellulosic wastes (as mentioned before in 2.8.1), were not so high by using multiple strains of non-selective microbes in conventional biogas digesters (9, 11, 13). Co-culture and tri-culture have recently been introduced to solve this problem and improve the microbial processes of biomass degradation and gas production in biogas technology (120, 121, 122, 123). By those techniques, selected strain (s) of bacteria and fungi or the combinations of those microbes have been usually utilized in any system. The proper researches have emphasized in cellulose degradation, so that the selected strains of cellulolytic organisms and methanogenic bacteria are included in the biogas

Table 2.2 Examples of some co-culture in the production of biogas, ethanol and some fatty acids.

Co-culture	Substrate	Other Products	Reference
Ethanol			
Acetivibrio cellulolyticus + Yeast	Cellulose	-	Husnair (123)
Clostridium thermocellum + *Clostridium thermosaccharolyticum*	Delignified Wood fibre	-	Zeikus (16)
Fatty acid (acetic acid)			
Ruminococcus albus + *Vibrio succinogenes*	Glucose	H_2, CO_2	Iannotti (22)
Eubacterium limnosum + *Lachnospira multiparus*	Pectin	Butyrate, CO_2	Rode (124)

digester. The summary of this subject has shown in Table 2.3. Even though those studies were conducted just only in a laboratory scale, but some indications show that pure culture technique of co-culture or tri-culture would be possibly implied.

Table 2.3 Examples of some co-culture and tri-culture in the biogas production.

Microorganism	Substrate	Other Products	Reference
Co-culture			
Selenomanas ruminantium + Methanobacterium ruminantium	Glucose + Lactate	acetate	Chen (121)
Clostridium thermocellum + Methanobacterium thermoautotrophicum	Cellulose + Cellubiose	acetate	Weimer (122)
An Acid Producing Bacteria + A Methanogenic Bacteria	Yeast extract	-	Patel (120)
Anaerobic Fungus + Rumen Methanogens	Cellulose	acetate	Bauchop (125)
Tri-culture			
Acetivibrio cellulolyticus Desulfovibrio sp. + Methanosarcina barkeri	Cellulose	-	Laube (43)
Rumen Anaerobic Fungus Methanobrevibacter sp. + Methanosarcina barkeri	Cellulose	-	Mountfort (126)