CHAPTER 7

STUDIES ON ISOLATION, IDENTIFICATION, CHARACTERISATION AND METHANOGENIC ACTIVITY OF PREDOMINANT METHANOGEN PRESENT IN ANAEROBIC DIGESTER OF TANNERY SOLID WASTE

7.1 INTRODUCTION

Anaerobic digestion is a microbiological process but it is not a unique, manmade process as is the industrial fermentation: the same reactions are carried on in nature, in soils and streams and in the oceans. The objective of the sewage engineer and, later, the microbiologist has been to confine the natural organisms in a man-made system and to optimise the rates and extents of the natural reactions so that polluting substances will be destroyed.

The first application of the system was in the natural decay of human excreta in earth closets, but having observed the overall process men provided conditions in which the process could be more nearly optimised in the septic tank and variations of this. From this came the more efficient anaerobic digester, at first running at ambient temperature and later at elevated temperature. The history of digestion is described in a number of papers, for instance the one by (McCarty 1982), although the presence of microbial reactions was determined quite early on, the 'high-rate' sludge digester was developed without any detailed knowledge of the microbial flora and the digester reactions.

The microorganisms carrying out the reactions in anaerobic digestion are bacteria, and that class of bacteria known as 'anaerobes'; bacteria that live without oxygen and may indeed, be killed by oxygen. There is a gradation in tolerance to oxygen among anaerobic bacteria, but many of the digester bacteria are amongst the anaerobes, which are the least tolerant of oxygen. The investigation, of these bacteria has needed the development of special techniques and media for culture, and these were largely initiated by work on the bacteria of the rumen. The specialised stomach systems of herbivorous animals such as cattle and sheep are akin to the anaerobic digester in the reactions carried out. The modern phase of rumen microbiology, where
the reactions and the bacteria responsible for them have been elucidated and the bacteria isolated and grown in pure culture, started about forty years ago. The advances in digester microbiology and biochemistry have come about since then. The rumen reactions are described in many papers, but a review of the earlier work on the rumen is contained in the book by Hungate (1966) and later work is described in the review by Hobson & Wallace (1982), and in the recent book edited by Hobson, (1988) which also contains chapters outlining the culturing techniques for anaerobes with references for further reading.

Since the metabolic pathways in digesters are similar to those in the rumen, the methods of biochemical investigation of digesters have followed in many ways those of the rumen biochemists. The main biochemical pathways in anaerobic digestion have now been elucidated, with the exception of the methanogenic bacteria, the linkage of these pathways to specific bacteria is nowhere near as complete as it is for the rumen system. Work on the methanogenic bacteria in digesters and other habitats has increased remarkably over the last few years and many new species and genera have been recently isolated. However, although many isolates of methanogenic and other bacteria have been obtained from digesters, there has been comparatively little work on determining the numbers in which the bacteria exist, the variations in these numbers with time and operational factors of the digesters, and the relationships of the different types of bacteria with each other. There is a vast amount of knowledge of the microbial ecology of the rumen system, there is nothing like this information available about digesters.

7.2 Literature Review on Microbial aspects of Methanogenesis and Methane production

The main substrates for methane production in anaerobic digesters are acetic acid and hydrogen plus carbon dioxide. Of these, acetic acid has been generally regarded as the main precursor since Smith & Mah (1966) showed that 73% of the methane in their domestic sewage digester came from acetate. Two moles of methane are produced from acetate and one from hydrogen in glucose digestion. A relatively large amount of lipid material may have contributed significantly to the acetic acid in Smith & Mah digester, as acetic acid can be generated by the degradation of the long-
chain fatty acids of lipids, as well as being a primary product of carbohydrate fermentation. It can also be generated as a secondary product of carbohydrate fermentations from the degradation of the primary fermentation products propionic, butyric or higher volatile fatty acids by the same mechanism as is involved in the degradation of long-chain fatty acids. Acetic and propionic acids are also formed by fermentation of lactic and succinic acids by bacteria such as the *Veillonella*. Hobson et al. (1974) found $3 \times 10^7$ lactic-fermenting bacteria per ml of digesting piggery waste. Sulphate reducing bacteria can also convert lactate to acetate. The lactic and succinic acids are primary products of carbohydrate fermentations. A relatively small amount of acetic acid will be generated by the combination of carbon dioxide and hydrogen, possibly 1 to 4% of the total acetate production (Mackie & Bryant, 1981). Mylroie (1954) observed uptake of hydrogen and carbon dioxide by digesting sludge and isolated a *Clostridium*, which formed acetate but not methane from these substrates (Ohwaki & Hungate 1977). Acetate also appears to be an intermediate in carbon dioxide uptake by methanogens and some seem to leak from the cells has been reported as acetate production by methanogens (Kenealy & Zeikus, 1982; Westerman et al, 1989).

The fermentation of carbohydrates by pure cultures of digester bacteria results in the formation of mixtures of products from amongst the following: acetic, propionic, butyric, valeric, caproic, lactic and succinic acids, and ethanol. Deamination of amino acids can result in some of these acids as well as branched-chain volatile fatty acids such as isovaleric. The formation of fermentation acids is often accompanied by the formation of hydrogen and carbon dioxide. The maximum amount of hydrogen is formed when acetic acid is the only fermentation acid, as follows (formic acid is the equivalent of hydrogen plus carbon dioxide);

$$C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COOH + 2CO_2 + 4H_2$$

When fatty acids higher than acetic, or lactic or succinic acids, are the products the production of hydrogen is lower as some of the hydrogen is combined into the acids, which are more reduced than acetic.
A further source of hydrogen for methanogenesis, or sulphate reduction, is long-chain fatty acids. Lipids in digester feedstocks can come from animal residues, as in slaughter-house and meat-processing effluents, and from faecal and kitchen residues in domestic sewage. Lipids are also present in plant materials and in microorganisms whether from the intestines or from algae grown as digester feedstocks, and they are present in intestinal secretions in faecal residues. It should be noted, though, that hydrocarbons as well as true lipids are sometimes included in the term 'oils and grease' used in the analysis of sewage digesters and hydrocarbon oils are not degraded by anaerobic microorganisms. Plant waxes are also probably little degraded in anaerobic digesters.

In all the reactions, hydrogen transfer plays a leading part. However, it has been suggested that formate (which can be used by many methanogenic bacteria), not hydrogen, is transferred (Thiele & Zeikus 1988). In a study formate was transferred to the methanogen rather than hydrogen (Boone et al 1989) later said they had confirmed this latter suggestion. They grew *Syntrophomonas wolfei* with *M. formicicum* on butyrate and said that the rate of diffusion of hydrogen was not rapid enough for hydrogen to be transferred between bacteria, but that formate was transferred rapidly, so formate transfer was the preferred mechanism in digesters. It should be noted, however, that these were specialised systems. Boone et al (1989) used liquid cultures in which the bacteria were dispersed: in digesters bacteria grow as mixed colonies on substrate or other surfaces and the bacteria are in close proximity and possibly connected by gelatinous polymers rather than liquid.

The numbers of many types of bacteria and the variation in numbers with different digester feedstocks and digester operating conditions have not been investigated; however, bacteria capable of performing most of the reactions in digestion deduced from biochemical analyses have been isolated. Thus a start has been made on the investigation of the microbial ecology of digestion. The importance of the removal of hydrogen from fermentative and other reactions has been stressed. The most important digester bacteria involved in hydrogen removal are the methanogenic bacteria.
In the last few years there has been an 'explosion' in research on Methanogenesis and the number of species of methanogens now recorded is large. Some of these have been isolated from anaerobic digesters; many have their origins in muds, sediments and natural waters. Mesophilic and thermophilic species and varieties are known. There is not space here for a listing and discussion of the methane bacteria, the reader is referred to reviews, a recent one being that of Jain et al. (1988). Zinder (1988) has reviewed thermophilic acetate-utilising methanogens. A previous review, which stated the reclassification of the methanogens, was that of Balch et al (1979) in which it was recognised that the methanogens belonged to the kingdom of the Archaebacteria and differed in many aspects of composition from the true Eubacteria (Woese, Margum & Fox, 1978). Anaerobic fermentative Mycoplasmas are known, in the rumen for instance (Robinson & Hungate, 1973) and Rose & Pirt (1981) described two Mycoplasmas from a sewage-sludge digester one of which, *Anaeroplasma sp.* converted glucose to acids and hydrogen while the, other *Methanoplasma elizabethi*, converted hydrogen and carbon dioxide to methane. Iannotti et al (1982) isolated Mycoplasma-like organisms from a pig-waste digester.

Media and methods for isolation of methanogenic bacteria are described in the review by Jain, et al (1988). The majority of methanogens use hydrogen plus carbon dioxide and many of these also use the equivalent formate, although Belay et al (1986) said that formate utilisation was an adaptive process. Acetate is used by *Methanosarcina* and *Methanoaceta* species. Mono, di and tri methylamine or methanol can be used by some species. These latter substrates, particularly methanol, are probably of little importance in anaerobic digestion. According to some cases the presence of hydrogen inhibits the utilisation of acetate (Ferguson & Mah, 1983). Zehnder et al. (1981) reviewed the kinetics, yield factors, etc., of methane formation from hydrogen or acetate. There have been papers since then dealing with the kinetics of hydrogen, acetate and propionate conversions (e.g., Fukuzaki et al., 1990b; Yang & Okos, 1990).

The methanogens have varied requirements for growth factors, amongst which nickel, which is a component of F₄₃₀, (Dickert et al., 1980), has been suggested as a promotor of methane formation in digesters. However, metals can show stimulatory or toxic effects on digestion according to their concentrations and the presence of
more than one metal. For instance, Kelly & Switzenbaum (1984) found nickel, iron and cobalt to be limiting the digestion of cheese whey. As another example, Wilkie et al (1986) found that addition of Ni, Co, Mo, Se to a Napier grass digester (which had added N and P) improved digestion: the elements were not present in the grass. Like the rumen bacteria, methanogens (Perski et al., 1982) and probably other digester bacteria (e.g. some involved in production of acetate from hydrogen and carbon dioxide; Yang & Drake, 1990) seem to be moderately halophilic and require sodium. The optimum concentration of sodium for digester operation has been reported as 230 mg/l (Kugelman & Chin, 1970). Sodium can reverse the inhibitory effects of high potassium concentrations. The interactive and other effects of metals are reviewed by Oleszkiewicz & Sharma (1990).

Although methanogens have been isolated from digesters there have been few studies on the enumeration of methanogenic populations of digesters. Hobson & Shaw (1974) reported counts of $2 \times 10^5$ to $2 \times 10^6$ hydrogen utilising methanogens per ml liquid in a series of samples from mesophilic piggery waste, stirred tank digesters, and about $2 \times 10^4$ formate utilisers and similar numbers of bacteria using butyrate. In a domestic digester sludge lower numbers of formate and hydrogen utilisers were found ($2 \times 10^2$ and $2 \times 10^3$) and no butyrate utilising bacteria. Acetate-utilising bacteria could not be detected in counting media containing acetate. However, the apparent lack of bacteria using acetate was probably an artefact due to deficiencies in the media used at that time as acetate was used during the start-up of the pig-waste digesters as methane production built up (Hobson & Shaw, 1973). The hydrogen utilising bacteria appeared to be of the same species, *Methanobacterium formicicum*, and this has been isolated from a number of digesters. Counts of hydrogen using methanogens in more highly loaded pig waste mesophilic digesters were of the order of $10^4$ to $10^7$ per ml (Hobson & Richardson, 1985), and similar numbers were also found in a thermophilic pig waste digester. A series of counts of hydrogen using bacteria in mesophilic digesters treating whole and separated dairy cattle wastes gave numbers of about $10^4$ or $10^5$ per ml at different retention times, although there was no correlation between numbers and RT (Summers et al., 1987). Rajasekeran et al. (1986) reported counts of the order of $10^6$ hydrogen utilising methanogens per ml in mesophilic batch digesters fed on mixtures of cattle, poultry and silkworm wastes. Diversity of species and varieties of digester bacteria carrying out a specific reaction, including
methanogenesis, has been mentioned, and Visser et al (1991) also showed that there was a greater diversity in species in mesophilic than in thermophilic methanogenic granules from a wastewater digester and that the species differed in the two types of granules.

7.3 Cultural, Morphological and Taxonomical Characteristics of Methanogens

Methanogenic bacteria are a morphologically diverse group of bacteria unified by their ability to produce methane. This property has been used to distinguish these bacteria from, the other groups of anaerobic bacteria. Methanogens utilize a very limited number of simple carbon compounds as carbon and energy sources for methanogenesis, i.e. H₂ plus CO₂, formate, methanol, methylamines acetate, and CO (1). Methanogens also play an important role in interspecies H₂-transfer in anaerobic ecosystems. Under certain environmental conditions, interspecies formate transfer can be quantitatively more important than interspecies H₂ transfer, but the general importance of this mechanism has not been established in diverse ecosystems. Methanogens also possess some unique features, which distinguish them as a special group of bacteria. These include: the absence of peptidoglycan in the cell wall; the presence of mainly ether-linked isoprenoids rather than ester-linked phospholipids in the membranes; and the presence of unusual coenzymes such as coenzyme M, factor F₄₂₀ (10, II), factor F₄₃₀, methanopterin, and methanofuran. Based on the unique 16S rRNA structure, methanogens have been grouped under archaeobacteria, the third kingdom of life.

Methanogens are very fastidious anaerobes. Their detailed study requires isolation and cultivation in the absence of oxygen and at low redox potentials. Procedures developed by Hungate (1930) have proved most successful for cultivation of methanogens. The development of improved anaerobic techniques stimulated many laboratories to study methanogens and, as a result, novel biochemical information about these bacteria is accumulating in the literature. The purpose of this chapter is to review the methods commonly used to grow and study methanogens so that the necessary information is available. Habitat of Methanogenic bacteria are found in a variety of anaerobic habitats including sediments, sludge, and animal waste digesters, the large bowel of man and animals, the guts of insects, wetwood of living trees,
rumen, protozoa, and extreme environments. They have so far only been isolated from anoxic environments. They are most abundant below Eh values of -200 mV. In general, methanogens get inactivated by the presence of oxygen, although not every species is rapidly killed by oxygen. There are no reports which indicate if any attempt has been made to study occurrence and abundance of methanogenic bacteria in oxic environments.

7.4 Variations on enrichment methods

(a) *Methanobacterium* species can be enriched on H2:CO2 or sodium formate (in a highly buffered medium), but in some enrichments acetate or yeast extract and tryptase are added.

(b) *Methanospirillum* species can be enriched if enrichments are repeatedly incubated at lower temperatures (30-35°C).

(c) Use antibiotics (penicillin, cycloserine, or vancomycin) and methylamines as substrates to inhibit the growth of methylotrophic acetogens such as *Eubacterium limosum* and *Butyribacterium methylotrophicum*.

(d) Use high salt concentrations (> 5% NaCl) to favour enrichment of halophilic methanogens.

(e) Use methanol and H2 to favour and enrich *Methanosphaera*-type of methanogens.

Methanogens, which utilize different substrates as energy sources, are obtained from nature by selective enrichments. Enrichment procedures aid in isolation since some methanogens represent only a small fraction of the total microbial community in the natural environment and can be overgrown by other organisms. Selective pressure can be applied in the isolation of methanogens by the use of substrates, antibiotics which inhibit specifically eubacteria but not archaeabacteria, omission of sulphate, addition of organic components (yeast extract and tryptase, etc.) in very low concentrations, and, at times, supplementation with metal ions such as cobalt, nickel, tungsten, molybdenum. In addition, salt concentration, pH, and temperature can also aid in having a selective pressure in isolation of a specific methanogenic species.
With the enrichment culture technique, the selective medium is inoculated with varying amounts of inoculum source and incubated under selective conditions until growth of organisms appears. Usually, several enrichment cultures are set up with variations in nutrient compositions, substrate, environmental factors such as pH, temperatures, etc. Enrichments showing production of methane in headspace gas are further selected for transfers until a reasonably good population of methanogens develops in the enrichment tube/vial. Different enrichments may provide different methanogenic strains/species. These enrichments are then plated on to a selective medium in plates, incubated anaerobically in an anaerobic chamber. Serum bottles can also be prepared to obtain an isolated colony of a methanogenic bacterial culture.

7.5 Taxonomic characterization

After isolation of a methanogen in pure culture it needs to be characterized taxonomically. There have been a number of methanogens isolated during the last decade with the developments and improvements in the techniques and tools for cultivation and isolation of these strict anaerobes. They have been largely classified as belonging to Methanobacteriales, Methanococcales, and Methanomicrobiales, based on their morphological, physiological, biochemical, and some genetic characteristics, which include:

(a) Morphological characterization: Cell shape, size, arrangement of cells as tetrads, bunches, chains, etc.; colony morphology; motility. Gram-staining; ultrastructure (scanning and transmission electron microscopy).

(b) Physiological characterization: Organic and inorganic carbon, nitrogen, and sulphur nutritional requirements, growth substrates, growth enhancers and growth rate; optimum and range of growth temperature; optimum and range of growth pH; resistance to antibiotics; requirement for NaCl, minerals, sulphide, and vitamins; toxicity and tolerance levels for sulphide and salt.

(c) Biochemical characterization: Total cellular protein profile, i.e. presence or absence of certain peptide band(s) in SDS/PAGE or native gels, DNA G + C ratios; immunological fingerprinting; cell envelope composition, membrane lipids, and key enzyme subunit profile, e.g. methyl reductase.
(d) Molecular characterization: DNA-DNA and DNA-RNA hybridization and 16S rRNA analysis.

Development of a systematic key based on some essential characteristics of the methanogens can help in their identification and classification. Based on the current data a simple schematic key is provided in Figures 1 and 2 for identification, characterization, and classification of methanogens. To speciate a new methanogenic isolate it would be necessary to take into account the biochemical and molecular characteristics of the isolate.

**FIGURE 7.1 – COMPREHENSIVE KEY FOR CLASSIFYING METHANOGENS IN DIFFERENT ORDERS**
7.6 Material and Methods

Culture requirements for selection and screening of methanogens

7.6.1 Oxidation-reduction potential

Removal of oxygen from growth media and from all environments to which the methanogens may be exposed is essential to maintain low oxidation-reduction (redox) potential during cultivation. Hungate (1975) pointed out that it is impossible simply by removal of O2 to obtain a redox potential as low as -330 mV, which is apparently required for methanogens. Although O2 is removed as completely as possible, some will still be present because it remains dissolved in medium, rubber and plastic tubing, and stoppers. Therefore, addition of reducing agents to the medium removes traces of O2 and helps achieve the low redox potentials needed for growth of many anaerobic bacteria including methanogens. Many anaerobes probably prefer to grow in environments having redox potentials of -250 mV or less. This is particularly true for methanogens, which may require redox potentials as low as -330 mV to initiate growth. As calculated by Hungate (1975), to attain a redox potential of -330 mV, the concentration of O2 would have to be of the order of 1.5 x 10^{56} molecules/L.
Thus, reducing agents such as cysteine, sulphide, or titanium salts are included in
cultivation media to obtain low redox potential. Some reducing substances, which are
added to lower the redox potential in anaerobic media, are listed in Table 7.1. The
most commonly used reducing agent solution is a mixture of sodium sulphide and
cysteine hydrochloride to lower the redox potential. Resazurin, which turns red at Eh
values higher than -42 mV, is normally added
to the medium as an indicator.

Table 7.1 - Commonly used reducing agents for lowering the redox potential of
media

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentrations in medium</th>
<th>Redox potential (MV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cysteine - HCl</td>
<td>0.02-0.08%</td>
<td>-210</td>
</tr>
<tr>
<td>Dithiothreitol</td>
<td>0.05%</td>
<td>-330</td>
</tr>
<tr>
<td>FeS, amorphous</td>
<td>0.05 mm</td>
<td>&lt;=-350</td>
</tr>
<tr>
<td>Titanium (III) citrate</td>
<td>0.5-2.0 mM</td>
<td>-480</td>
</tr>
<tr>
<td>Titanium (III) nitrilotriacetate</td>
<td>0.5-5.0mm</td>
<td>-480</td>
</tr>
<tr>
<td>Sodium sulphide or hydrogen sulphide</td>
<td>0.01-0.025 %</td>
<td>-571</td>
</tr>
<tr>
<td>Sodium sulphide +cysteine sulphide</td>
<td>0.025%</td>
<td>-571</td>
</tr>
<tr>
<td>Dithionite</td>
<td>0.001%</td>
<td>&lt;=-600</td>
</tr>
</tbody>
</table>

Source: Anaerobic Microbiology A practical approach edited by P.N. Levett

7.6.2 Nutrient media

Media of different compositions have been used by various research groups to
isolate, grow, and study methanogens. The purpose here is to provide minimal
nutrients to support the growth of methanogens. These media are either phosphate-
buffered or carbonate-buffered. The composition of general media for growing
methanogens is given in Table 7.2. The basal medium is supplemented with solutions
of trace minerals and vitamins. Ammonium is the preferred source of nitrogen but
organic sources such as glutamine and urea can also replace it in certain cases.
Sulphide, which is commonly added to the medium as a reducing agent, also serves as
sulphur source but high concentrations have been reported to be inhibitory to the
growth of methanogens. Mercapto-2-ethanol has also been used as a reducing agent to grow methanogens. Organic sulphur sources (cysteine, methionine) are also utilized by some methanogens, replacing the need for sulphide. Sulphate should be omitted from enrichment medium to eliminate selection of sulphate reducers, particularly when common substrates such as acetate and H₂ are used.

**Table 7.2 - media for cultivation of methanogenic bacteria – Phosphate buffered basal medium (PBBM)**

<table>
<thead>
<tr>
<th>Compound</th>
<th>PBBM Composition (per 1 litre medium)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>0.1 g</td>
</tr>
<tr>
<td>MgCl₂.6H₂O</td>
<td>0.2 g</td>
</tr>
<tr>
<td>CaCl₂.2H₂O</td>
<td>0.1 g</td>
</tr>
<tr>
<td>NH₄Cl</td>
<td>1.0 g</td>
</tr>
<tr>
<td>Trace mineral solution II</td>
<td>10.0 mL</td>
</tr>
<tr>
<td>Resazurin solution, 0.2%</td>
<td>1.0 mL</td>
</tr>
<tr>
<td>Distilled H₂O to</td>
<td>1000 mL</td>
</tr>
</tbody>
</table>

Additions to be autoclaved separately

- Vitamin solution 10 mL
- Phosphate buffer 10 mL
- Reductant 25 mL
- Substrate as required

Source: Anaerobic Microbiology A practical approach edited by P.N. Levett

### 7.6.3 Gas Chromatographic Analysis

Since all methanogens produce methane, analysis of gas phase for production of CH₄ will confirm the growth of methanogens in tubes, vials, or any other inoculated sealed container. Analysis of methane by gas-liquid chromatography (GLC) Poropak Q column with a thermal conductivity detector (TCD). H₂, Ar we used as carrier gas. 1-4 ml standard gas mixture (55% CH₄, 5% H₂ and balance N₂) / unknown
samples were injected into a GLC with oven temperature at 40°C and injector at 70°C. % CH₄ is calculated for the sample based on the reference peak from standard CH₄.

7.6.4 Microscopic analysis

The methanogenic bacteria are structurally diverse and display no unique features by which all species can be characterized. All basic morphological types found among bacteria, including cocci and packets of cocci, rods of different shape and size, spirillum, and filamentous forms, are represented in methanogenic bacteria. Purification examination and morphological investigations of the isolated strain were studied using the phase contrast light microscope.

However, cells of methanogens can be recognized by their strong autofluorescence under oxidizing conditions. The major contributors to this phenomenon are coenzyme F₄₂₀ and the methanopterin derivatives. Coenzyme (or factor) F₄₂₀, has been found in almost all the methanogen cells examined at levels ranging from 1.2mg (*Methanobrevibacter ruminantium*) to 65 mg (*methanobacterium thermoautotrophicum*) of coenzyme per kg of cell dry weight. The compound has an absorption maximum at 420 nm. The absorbance is lost upon reduction. The fluorescence of methanogen cells can be examined using a microscope with a fluorescence attachment.

Scanning Electron Microscopy (SEM) microscopies were performed applying standard procedures for sample preparation, observation and photomicrographic documentation (Zellner et al 1993).

Total cell counts in suspension made from the serum bottles were carried out using an improved Neubauer chamber (Visser et al 1991).

7.6.5 Colony isolation

After the enrichment of methanogenic bacterial population in a liquid medium is obtained, serial dilution of the broth prepared and plated onto an agar medium to isolate single colonies. This was done by the streak-plate and pour-plate method. Methanogens can be plated with high plating efficiencies.
7.6.6 Experimental

A simple methanogenic activity test procedure (Isa et al 1993) was adopted with suitable modifications to suit the requirement of this study. The experimental setup is shown in Fig 7.3. Six serum bottles (volume of 130ml) each containing 100ml of Phosphate Buffered Basal Medium (PBBM) pH 7 containing NH₄Cl 1.0 g/l, MgCl₂.6H₂O 0.2 g/l, CaCl₂.2H₂O 0.1 g/l and NaCl 0.9 g/l was prepared. Trace elements and vitamins were prepared and added at a concentration of 1.0 ml/L respectively (Mahendra K. Jain 1991). Reductants Na₂S.9H₂O and L-cystine HCl were added separately from sterile anoxic stock solutions to final concentration of 0.5g/L. Sodium acetate was used as substrate at the concentration of 5.0 g/L. Deoxidisation of medium was done with purging with mixed gas containing N₂/CO₂/H₂ in the ratio 80:15:5 respectively. The bacteria were cultured at mesophilic temperature (35 ± 3°C) in serum vial closed with butyl rubber stoppers and aluminium crimp seals. Total methane gas production was measured by means of the liquid displacement method once in every day. Contents of the serum bottles were mixed by swirling manually after every gas measurement. One bottle per week was assayed for various parameters mentioned to evaluate the performance of isolated strain. Experiments were repeated thrice before obtaining final result.
7.6.7 Inocula

Anaerobic sludge from a bench-scale mesophilic methanogenic reactor, which had been operated with a mixture of tannery solid and liquid waste as feed, was used as inocula for the studies. Anaerobic sludge, which had been freshly sampled, was distributed anaerobically into serum bottles with medium containing sodium acetate as substrate. Bottles were incubated at 37°C. When gas production had ceased pure aceticlastic methanogens were screened on petri plates containing PBBM Media.
Upon exhibitance of fluorescence isolates were transferred on to a broth containing sodium acetate and used as inoculum. Inoculum strength used for the study was of 1% which contained $8 \times 10^8$ live cells/ml.

7.6.8 Analyses

Chemical Oxygen Demand (COD) was measured according to the procedures recommended in the Standard methods for the examination of water and waste water (APHA 1992). Soluble COD was determined using sample obtained by filtration with GF/A Whatman filter paper. Daily methane gas production of reactors was monitored by means of an inverted bottle containing 40% sodium hydroxide (NaOH). The volume of liquid displaced from the bottle was equivalent to volume of methane gas production. The experiments were carried out in the temperature range of 35 ± 3°C.

7.6.9 Preservation

The isolates were preserved in airtight vials containing 50 % (v/v) glycerol and head-space filled with oxygen free N₂ gas. These anaerobic vials were additionally put in airtight bags containing an anaerobic catalyst (Pd) and stored at -20 °C.

7.6.10 Identification of the isolated strains

Identification was done by fluorescence in situ hybridizations (FISH) technique, samples with high levels of fibers consisting of Methanosacta-like cells were fixed in 4% paraformaldehyde (de los Reyes et al., 1997). A tetra-methylrhodamine-isothiocyanate (TRITC) labeled domain-specific probe for Archaea and a fluorescein isothiocyanate (FITC) labeled species-specific probe for Methanosacta concilii were used for FISH and the fluorescent signal was analyzed with an epi-fluorescence microscope (make Leica).

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7.7 RESULTS AND DISCUSSION

7.7.1 Isolation and growth studies

Pure cultures of aceticlastic methanogenic bacteria were obtained from the stable culture mentioned above and were enumerated on petri plates with the help of anaerobic chamber (Make: Shellab - Bactron).

In order to monitor biomass composition present in the reactors through the methanogenic activity test, the methane production potential of the test biomass is measured under limited substrate and optimal environmental conditions. There are essentially two classes of methanogens namely Hydrogen Oxidising Methanogens (HOM) and Non Hydrogen Oxidising Methanogens (NHOMs). NHOMs are substrate specific and cleave the acetic acid molecule to produce methane. As such the methanogenic activity test with neutralised acetic acid or acetate as sole substrate reflects the activity of NHOM, also known as aceticlastic methanogens and hence referred as Aceticlastic Methanogenic Assay Test (AMA Test) where sodium acetate was used as the main source substrate.

In an effort to monitor reactor biomass in terms of relative population levels, AMA test was carried out on samples drawn from the tannery solid waste digester. The test results obtained for reactor biomass toward the end of each week are presented in Fig.7.4. A gradual increase in methanogenic population was observed during 5-week period. The bacterial growth curve illustrates the slow doubling time of methanogens as reported by several authors (Hobson and Shaw, 1974; Zekius, 1980)
Fig 7.4. Biomass Concentration

Total cells/mL ($10^6$)

Time (Days)
7.7.2 Methane gas production

It is observed from the test that the amount of methane produced during first week was 10 ml, increased to 24 ml in second week, obtained maximum of 39 ml in the third week and declined to 32 ml and 20 ml in fourth and fifth week respectively (Fig.7.5). After fifth week the gas production has almost ceased. The cumulative methane gas produced was 127 ml when 0.5g of sodium acetate was used as substrate (Fig.7.6).
7.7.3 COD Destruction

COD concentration in the reactor reduced from 5296 mg/l to 1780 mg/l during 5-week study period and the total COD removal efficiency was observed as 66%. The observed specific methane production was 0.24 l/g COD$_{fed}$ and 0.36 l/g COD$_{reduced}$.

**Velocity Constant**

Boschoff, applied monomolecular equation to the biogas production to determine reaction rate constant based on the assumption that at any particular time the rate of gas production will be function of the concentration of organic matter yet to be digested. Accordingly, it may be illustrated that

\[
\frac{dy}{dt} = K(A-Y) \quad \text{--- (1)}
\]

where,

\[A = \text{Total amount of gas generated during digestion, ml}\]
\[Y = \text{Amount of gas generated in time ‘t’, ml}\]
\[K = \text{reaction constant, d}^{-1}\]
\[t = \text{time, d}\]

Integrating equation (1) between limits, \(t = 0\) and \(t = t\), it becomes

\[
Y = A(1 - e^{-Kt}) \quad \text{--- (2)}
\]

Substituting \(K = 1/t\) in equation (2)

\[
Y = 0.63A \quad \text{--- (3)}
\]

The value of \(K\) can be calculated from the experimental cumulative gas production curve. Value of \(K\) is the reciprocal of time required for generation of 63% of the total volume of gas produced. In the present study the total amount of gas produced is 127
ml. The time required for generation 63% of the total volume of gas produced was found as 22 d. The reaction rate constant (K) was found as $4.545 \times 10^2$ d$^{-1}$ at 33 ± 2°C.

### 7.7.4 Activity of Isolated Strain

The pure culture used in the experimental study was isolated from a digester having longer retention time of 30 d having long term acetate enrichment. Under this situation, *Methanoaeta* (NHOA) is expected to be present predominantly in the system (Harper and Pohland, 1986).

The AMA test was carried out essentially with this NHOA using 0.5 g of sodium acetate as the sole carbon and energy source to determine its methanogenic activity. Although the energetics of acetate decarboxylation to methane and carbon dioxide are quite poor, Zehender and Brock (1979) hypothesized that a chemi osmotic mechanism might be involved for acetoclastic methanogens to generate sufficient ATP to sustain growth upon acetate.

Methane production in the AMA test is essentially by NHOMs as no substrate is available for HOMs. The microscopic examination of cultures also revealed the presence of *Methanoaeta* that predominate even at high concentration of acetate. This could be due to poor bioavailability of trace metals (Speece, 1996). The low rates of acetate utilisation attained in the study was primarily due to premixing of trace metals especially Fe, Co and Ni with other components of the inorganic media which aided precipitations of sulphide in the media resulting in inadequate bioavailability of trace metals (Speece, 1996).

### 7.7.5 Identification of isolated acetoclastic methanogenic strain

SEM and light microscopy revealed a very dense structure consisting of bundles of filaments resembling *Methanoaeta* cells (Figure 7.7). The specific methanogenic activity (acetoclastic) of the isolated culture samples consisting of these
fibers bundles. *Methanosaeta* have previously been observed with similar bundles in acetic acid fed only systems (e.g., Huser *et al.*, 1982). Furthermore, acetate concentrations were generally above the level believed to provide a competitive advantage for *Methanosarcina* (over *Methanosarcina*). Therefore, isolated strain were surprised to find a high prevalence of fibers apparently consisting of only *Methanosaeta*-like cells. Two oligonucleotide probes, one for Archaea and one for the species *M. concilii*, were combined for FISH analysis of a fiber sample. This experiment confirmed that the fibers consisted of *M. concilii* (Figure 7.9).

### 7.8 Conclusion

Pure culture of aceticlastic methanogen was isolated from the tannery solid waste digester and characterised. The performance results indicate that the isolated methanogenic strain and dominant strain present in tannery waste digester were acetoclastic in nature. Based on substrate utilization it is clear that out of *methanosarcina* and *methanosaeta*, two known genera of methanogens, which can utilize the two-carbon compound, acetate, studies with fluorescence in-situ hybridization (FISH), showed that *Methanosaeta concilii* was the predominant microorganism present. Hence, the utilisation rate of substrate by the isolated strain in the enriched medium and increase in biomass concentration confirm the presence of *methanosaeta* spp. and the maximum possible gas production attained within five weeks with 1% of inoculum indicates reasonably high methanogenic activity of the strain.
FIGURE 7.7 Scanning Electron micrograph of the M.conciliii

FIGURE 7.8 Phase-contrast micrograph of isolates
FIGURE 7.9  Factor F_{420} fluorescence micrograph of the *M. concilii*