MANAGEMENT OF BIODEGRADABLE PLANT TISSUE CULTURE LAB WASTES THROUGH BIOMETHANOGENESIS

By

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THESIS

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DECLARATION

I hereby declare that this thesis entitled **"Management of biodegradable plant tissue culture lab wastes through biomethanogenesis"** is a bonafide record of research work done by me during the course of research and that the thesis has not previously formed the basis for the award to me of any degree, diploma, fellowship or other similar title, of any other University or Society.

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Certified that this thesis, entitled **"Management of biodegradable plant tissue culture lab wastes through biomethanogenesis"** is a record of research work done independently by **Mr. Abdulla Fayas T.** under my guidance and supervision and that it has not previously formed the basis for the award of any degree, diploma, fellowship or associateship to him.

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EXTERNAL EXAMINER

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ABBREVIATIONS

A	Absorbance
ABARD	Agro Biotechnology Agency for Rural Development
ATIC	Agricultural Technology Information Centre
cfu	Colony forming unit
CH_4	Methane
CIAE	Central Institute of Agricultural Engineering
cm	Centimeter
CO_2	Carbon dioxide
CPBMB	Centre for Plant Biotechnology and Molecular Biology
DNA	Deoxyribo Nucleic Acid
DNES	Department of Non-conventional Energy Sources
EDTA	Ethylene Diamine Tetra Acetic Acid
g	Gram
H_2	Hydrogen
HRT	Hydraulic Retention Time
IARI	Indian Agricultural Research Institute
KAU	Kerala Agricultural University
Kg	Kilogram
L	Litre
m	Meter
m^3	Meter cube
ml	Millilitre
MW	Mega Watt
ng	Nano gram
O_2	Oxygen

°C	Degree Centigrade
OD	Optical Density
PCR	Polymerase chain reaction
pH	Hydrogen ion concentration
RAPD	Random Amplified Polymorphic DNA
RNA	Ribo Nucleic Acid
RNAase	Ribonuclease
rpm	Rotation per minute
Tm	Melting Temperature
TNAU	Tamil Nadu Agricultural University
V	Volt
% G + C	Molecular percentage of Guanine and Cytosine
%	Percentage
µl	Micro litre
µm	Micro metre

Introduction

INTRODUCTION

Energy is a necessary concomitant of human existence. Although many sources of energy exist in nature, it is coal, electricity and fossil oil which have been commercially exploited for many useful purposes. Methane gas and more popularly known as bio-gas is one such alternate sources of energy which has been identified as a useful hydro-carbon with combustible qualities as that of other hydrocarbons. Though its calorific value is not high as some products of fossil oil and other energy sources, it can meet some needs of household and farms. Unlike these hydro-carbons which are derived from direct chemical processes, bio-gas is produced through a bio-chemical process in which some bacteria convert the biological wastes into useful bio-gas comprising methane through chemical interaction. Such methane gas is renewable through continuous feeding of biological wastes and which are available in plenty in rural areas in the country. Since the useful gas originates from biological process, it has been termed as bio-gas in which methane gas is the main constituent.

Biotechnology is an emerging field and it has its role in almost all the realm of science. Though the modern techniques of crop production using biotechnology has been developed for commercial utilities, it has no much relevance among the common people, since the cost of maintenance of the laboratory is much high for them to afford. So these cost factors act as impediments obstructing growth of the most advanced and productive field of science and its application among the novice. As against the usual practice in developed countries, people of India do hesitate to start biotech laboratories, for reason of fuel deficit and safe disposal of lab wastes. Currently tissue culture labs in our state are run by marginal farming groups like kudumbasree units, co-operative societies, state government etc. Proper functioning of all these units are crippled due to financial stringency and other related reasons. The difficulty rendered in getting gas connection is yet another reason acting as a hindrance in this direction. So the current strategy should be production of tissue

culture plantlets utilizing lab wastes as a source of energy requirement and here we are utilizing these wastes for recycling and cost effective energy regeneration.

Biomethanogenesis of biotechnology wastes have profound significance as it helps in safe disposal of wastes. Normally a biotechnology lab will produce large amount of wastes every day. The lab wastes itself have detrimental effect, of much concern, if these wastes are allowed to accumulate for long period of time. The accumulation of these wastes result in the hampering of hygienic atmosphere, thus effecting the concerned professional and other visitors to the lab. These lab wastes may degrade and cause fowl smell and thus negatively effect the prevalence of aseptic condition for explants, thereby contaminating them and causing their mass destruction. Moreover there may be some chemicals in the residues, which in large quantity become hazardous for human beings. Also, the damping of these wastes in open condition may act as shelter for many bacteria which may sometime act as source of pathogens. By developing a biofuel plant employing anaerobic bacteria and digesting the lab wastes using them can thus eliminate the hazards from improper disposal of these wastes.

In the light of the above facts, the following aspects are taken up for the present study.

1. To produce combustible bioenergy from tissue culture lab wastes through biomethanogenesis.

2. To develop RAPD based molecular markers for identification of methanogens

Review of literature

REVIEW OF LITERATURE

The discovery by Italian physicist Alessandro Volta in "1776" that "combustible air" was formed in the sediments of streams, bogs and lakes rich in decaying vegetation led to the subsequent discovery of a microbiological basis for the origin of methane gas (Barker, 1956). Barker (1956) emphasized on the highly unique physiology of the group and thereby clustered the methanogens into a single family, the Methanobacteriaceae. During the past few decades, studies have established the widespread and fundamental role of methane producing bacteria in anaerobic degrading process in nature (Wolfe, 1971; Mah *et al.*, 1977; Zeikus, 1977 Zehnder, 1978; Wolfe and Higgins, 1979). The methanogens are a morphologically diverse group of organisms; as well as physiologically coherent group of strict anaerobes, sharing the common metabolic capacity to produce methane. The uncertainty concerning their systemic relationships was reflected in the early taxonomic schemes which initially dispersed the methanogenic bacteria among the better characterized bacterial groups according to their morphologies.

Bacterial methanogenesis appears as a ubiquitous process in anaerobic ecosystems in which organic matter is being vigorously decomposed in the absence of excess exogenous electron acceptors (e.g. O_2, NO_3^{2-} and SO_4^{2-}). Methanogenic bacteria have been isolated from a variety of environments that include fresh water and marine surface sediments, gastrointestinal tracts of animals, thermal springs, sewage sludge, anaerobic digestors and wet wood of living trees (Zeikus, 1977). Radioactive tracer and enrichment culture studies have demonstrated microbial methanogenesis associated with deep subsurface natural gas deposits in oil bearing strata and formation waters (Rosanovo and Kuznetsov, 1975; Belyaev *et al.*, 1977; Belyaev, 1979).

Methanogens, a subspecies of archea, are present in numerous species of animals (Lange *et. al.*, 2005). They are a distinct group of organisms which are normal components of animal gastro-intestinal microbial ecosystem. Methanogens belonging to animal gastro-intestinal tract belong to the genera *Methanobacterium*, *Methanobrevibacter*, *Methanosphaera*, *Methanomicrobium*, *Methanogenium* and *Methanosarcina* (Miller and Wolin, 1986; Boopathy, 1996; Jarvis *et. al.*, 2000; Miller, 2001; Miller and Lin, 2002). The most predominant species of methanogens in the animals' intestinal tracts are related to *Methanobrevibacter* (Garcia *et al.*, 2000). A few non *Methanobrevibacter* species, *Methanobacterium, Methanosarcina, Methanosphaera* and *Methanomicrobium*, have also been isolated from animals (Pol and Demeyer, 1988; Boopathy, 1996). Methanogens comprise approximately 0.5 to 3% of the total population of microflora in steers, cows, sheeps, pigs and goat (Lin *et al.*, 1997).

2.1 Biogas

Biogas is a suitable fuel as compared to few other gaseous fuels (Tandan *et al.*, 1988). Comparison has been done between biogas and other fuels and the former has been proved suitable than other gaseous fuels (Tomar, 1984). The biogas is also a safe source of disposal of biological material with less pollution (Tomar, 1991). The generation power option is 2000MW for biomass and 6000MW for agricultural waste (Swarup, 1991).

The biomass which is a direct and renewable source of energy can be converted into various forms of energy such as biogas, producer gas, alcohol etc. (Tomar, 1985). The end products of organic decomposition are carbon dioxide and methane and that of inorganic are hydrogen sulphide and nitrogen (Chawla, 1986). Any organic matter, if digested or decomposed anaerobically generates combustible gas and this gas containing methane, carbon dioxide and few others is known as

biogas. In the digestion process, the complex molecules are broken into simple molecules by action of several types of bacteria.

Presently installed biogas plants can be divided into two categories. First category refers to family size biogas plant of 1 to $10m^3$ capacity and second category refers to community/institutional plants (Tomar, 1995). Amongst the discussed alternative non-conventional source of energy, the biogas can be popularized in any rural or semi urban part of the country, because of the availability of its raw material. Besides animal dung, other raw materials for biogas generation are human excreta, forest waste, agricultural waste, water weeds, kitchen waste etc. These can be used as raw material after some treatment. Hence there is a possibility of installing more number of biogas plants rather than sole dependence on cow dung. The biogas contains pollutants like methane, carbon monoxide, and hydrogen sulphide and it also has higher calorific value as compared to few other gaseous fuels (Tomar, 1995).

2.1.1 Historical background of biogas generation

Sathianathan(1975), Tomar(1985) and Chawla(1986) described the chronological development of energy appliances especially biogas programme in India and abroad. Wan Helme in 1930 had investigated 15 gases which were generated due to anaerobic fermentation of organic matters. Marsh gas was invented by Shirele. In 1776, Voltas had found this gas on base/ bottom of the lakes. Voltas had volumetrically analysed this gas in 1804. First of all Humphrey Davy in 1808 had generated biogas by anaerobic fermentation of animal excreta. Reiset from France academy got it in 1869. The generation of present type of biogas through biogas digester was started first time in 1895 in England where human excreta was anaerobically digested in big tanks. Machbeth and Scale in 1918 have fermented organic matter in moist condition and got CO_2, CH_4, H_2 etc. The real fruitful achievements were started since 1920 by Brusel in England. A bulletin regarding biogas generation was published which is even today regarded as good publication.

The year 1944-45 was the year of climax for anaerobic fermentation. France had installed about 1000 biogas plants in 1950, though these were not a proper design. In India in 1937, Indian Agricultural Research Institute (IARI), New Delhi has conducted successful experiments on biogas in 1939-40.

In our country Ajitmal Itawa(UP), KVIC Bombay, National Environmental Engineering Research Institute (NEERI) Nagpur, Central Institute of Agricultural Engineering (CIAE), Bhopal and about 300 other centres are involved in biogas development programme. Presently we have (DNES, 1990 and 1991) about 1.5 million family size and 1000 community/ institutional biogas plants. Biogas plant is a most economical and popular source of energy of present day. One biogas plant of average $4m^3$ size saves 40 tonnes of wood per year, which is equivalent to saving of 4-5 tree every year from cutting.

2.1.2 Theoretical aspects for biogas generation

For the generation of biogas, theoretical aspects taken into account are: the feed characteristics, hydraulic retention time, soil characteristics and other related parameters. The process of energy generation from biomass can be broadly classified into two groups; thermo-chemical, which produces mainly producer gas, char and oil and biological, producing mainly biogas in biological and alcohol in biochemical. The biomass which is responsible for biogas production in general contains 90 per cent water and 10 per cent dry weight. Of the dry weight materials about 10 percent is nitrogen, 50 percent carbon, 10 per cent hydrogen, 20 per cent oxygen and rest, other matters (Tomar, 1995).

The biomass inside biogas plants are digested in 3 phases. In the first solublizing phase, insoluble organics are converted into soluble complex organics. In second stage or non-methanic phase, the product is converted into acid and carbon dioxide. In third stage, the methanogenic bacteria come in action and convert the

mass into methane and other products. The end products of organic decomposition are carbon dioxide and methane and that of inorganic are hydrogen sulphide and nitrogen (Chawla, 1986).

In the digestion process, the complex molecules are broken into simple molecule by action of several types of bacteria. The process occurs in 2 stages i.e. acid forming stage by acetogen and methane formation stage by methanogenic bacteria. Among the known bacteria the methane forming bacteria are strictly anaerobic. Thus, even a minor crack in biogas plant will disturb the gas production.

The hydrogen ion concentration is a measure of pH of an aqueous solution. pH is a function of bicarbonate alkalinity of the system, the fraction of CO_2 in digester gas and the concentration of volatile acids. The alkalinity is a measure of buffering capacity of the contents and acts as a safeguard against pH fluctuations due to volatile acids (Chawla, 1986). A digester operates well in neutral solution of pH 7.0 or just above. In general, the optimum pH range for quality gas production was 6.8 to 7.2

For methane generation process three temperature range such as thermophilic zone (above 45°C), a mesophilic zone (20-45°C) and a psychrophilic zone (below 20°C) are reported (Khandelwal, 1986). However effective and efficient anaerobic fermentation was carried out at both thermophilic and mesophilic temperatures. The 30-35°C is the optimum temperature means that at this range the rate of volatile acid produced becomes more or less proportional to its utilization by methanogenic bacteria. The temperature of about 35°C and 60°C are considered as the optimum value for operation in the mesophilic and thermophilic ranges, respectively.

The C/N ratio of 20 to 30 is favourable for biogas production in plants (Tomar, 1995). The C/N ratio of cow dung, horse dung and water hyacinth is about 25 while that of poultry manure and night soil is between 5 to 8 and for straw rice

husk and other few agricultural wastes, it is about 70. Therefore, poultry manure and straw in mixture and cow dung separately can be good feed materials.

All feed materials, consist of volatile solids and non volatile solids. The non volatile solids are called fixed solids and are unaffected during digestion process and come out of the digester unchanged. Fresh cattle dung generally consist of 80 percent water and 20 percent total solids. The total solids in turn consist of 70 percent volatile solids and 30 percent fixed solids, approximately. The concentration of total solid is important for easy mixing and handling. Normally 10 percent total solid in the feed is recommended. Hence cow dung is mixed with equal volume of water to bring the total solids concentration to 10 percent (Tomar, 1995).

The rate of gas production is initially high and decreases gradually as the digestion nears completion and stops when slurry is completely digested. The time required to achieve a 70-80 percent digestion is considerably less than that needed to achieve a 100 percent digestion. In the family size biogas plants in most of the cases the fresh slurry is charged and spent slurry is withdrawn daily. Thus the operation of the digester is in semi-continuous fashion. The hydraulic retention time (HRT) is defined as the average time spent by the input slurry inside digester before it comes out. In this time 70-80 percent digestion completes. The HRT in Indian conditions varies from 30-60 days. In colder climate like China it is 100 days. In the laboratory bottle digester in ambient conditions it takes a year or so to complete the digestion (Tomar, 1995).

2.2 Methanogenesis

A large number of Euryarchaeota produce methane (CH_4) as an integral part of their energy metabolism. Such organisms are called methanogens and the process of methane formation, **methanogenesis.**

The biological production of methane occurs through a series of reactions involving novel coenzymes and amazing complexity. The key coenzymes in methanogenesis can be divided into two classes; those involved in carrying the C_1 unit from the initial substrate, CO_2, to the final product and those function in the redox reactions to supply the electrons necessary for the reduction of CO_2 to CH_4 (Madigan and Martinko, 2006).

Methanogenesis or digestion of bio-waste is by anaerobic thermophilic reaction. It involves three biochemical phases. The first phase is called solubilizing phase, where the lignocellulosic materials (carbohydrates, proteins and lipids) undergo partial hydrolysis, carboxylation and oxidation to form soluble organic compound viz. formate, acetate, propionate, butyrate, ethanol, hydrogen and carbon dioxide. In the second stage, the methanogens convert fatty acids to methane (Ramasamy *et al.*, 1992). The second phase is called acidogenesis which produces volatile fatty acids, organic acids, ethanol, CO_2 etc. Acetic acid, lactic acid, succinic acid, formic acid, β-hydroxy butyric acid and β-hydroxy valeric acids are some of the volatile fatty acids formed in the acidogenesis. The final phase is called methanogenic phase. Methanogenic bacteria under strict anaerobic condition reduce the organic acids into CH_4 and CO_2. The decomposition of protein yields ammonia as an end product. Inorganic decomposition occurs generally to produce H_2S, N_2 and H_2 in very small quantities

Methanogens are the key organisms in the production of methane from waste materials by breaking down acetate and hydrogen to gaseous end products. Methanogens are classified into two, aceticlastic and non-aceticlastic. Aceticlastic generate methane using acetate as carbon source. Non-aceticlastic use hydrogen to reduce CO_2 to CH_4.

2.2.1 Substrates for methanogenesis

Methanogenic bacteria (methanogens) produce methane (natural gas; sometimes called biogas) as the end product of their energy-generating metabolism. This biochemical process, termed methanogenesis, is the rate-limiting and final step in the anaerobic biodegradation of organic compounds. It occurs naturally in freshwater and marine sediments, marshes, paddy fields, geothermal springs, and in the digestive tracts of invertebrates and vertebrates. Termites and ruminants are major source of biologically produced methane. Approximately 65% of the methane released to the atmosphere, equal to approximately 1% of atmospheric carbon cycle, is of biological origin; the remainder is produced geologically from wells, mines and natural vents (Enhalt, 1974; Daniels, 1984).

Methanogenic bacteria are strictly anaerobic archaebacteria with a unique form of energy metabolism involving the generation of methane. Their substrate range for methanogenesis is limited to carbon dioxide, formate, methanol, methylamines, and acetate, but no single isolate is able to utilize all these carbon sources (Jones *et al.*, 1987). Elucidation of the methanogenic pathway from carbon dioxide to methane has led to the discovery of several novel biochemical reactions and six new coenzymes specifically involved in methanogenesis (DiMarco *et al.*, 1990), and various genes of the methanogenic pathway have been isolated and sequenced.

At least, 11 substrates have been shown to be converted to methane by pure cultures of methanogens. Interestingly, these substrates do not include such common compounds as glucose and organic or fatty acids. Compounds such as glucose can be converted to methane, but only in cooperative reactions involving methanogens and other anaerobic bacteria. With the right mixed culture, virtually any organic compound, even including hydrocarbons, can be converted to methane plus CO_2. Three classes of compounds make up the list of methanogenic substrates. These

include CO_2 type substrates, methyl substrates, and acetotrophic substrates. CO_2 type substrates naturally include CO_2 itself, which is reduced to methane using H_2 as electron donor. Other substrate here includes formate and carbon monoxide. The second class of methanogenic substrates is methylated substances. The final methanogenic process is the cleavage of acetate to CO_2 plus CH_4, called the acetotrophic reaction. Acetate forms about 70% of the methanogenic substrates in anaerobic digestors (Smith and Mah, 1966; Gujer and Zehnder, 1983; Zinder *et al.*, 1984; Fukuzaki *et al.*, 1990) and is the only dicarbon substrate that methanogenic bacteria can degrade completely (Thauer *et al.*, 1989). The ability to catabolize acetate to methane is restricted to only two genera, *Methanosarcina* and *Methanothrix*, of the kingdom Euryarcheota, which belongs to archaeal domain (Woese *et al.*, 1990). *Methanosarcina* spp. are generalistic organisms, capable of degrading a variety of substrates like H_2/CO_2, methanol, methylamines and acetate (Hutten *et al.*, 1980; Smith and Mah, 1980; Kenealy and Zeikus, 1982; Krzycki *et al.*1982). In contrast, *Methanothrix* spp. is a specialist that can use only acetate as its carbon and energy source (Zehnder *et al.*, 1980; Huser *et al.*, 1982).

2.2.2 Methanogenic bacteria

The biological production of methane, methanogenesis, is carried out by a group of strictly anaerobic archaea called the methanogens. The existence of a third biological kingdom, the Archaebacteria, or Archaea, was recognized in the late 1970s from comparisons of the oligonucleotides generated by RNase digestions of 16S rRNAs (Woese and Fox, 1977; Woese *et al.*, 1978).

All known methanogens are Archaea, their closest non-methanogenic relatives being the extremely halophilic Archaea. Although, the methanogens are unified as a group by their ability to generate energy by the process of methanogenesis, they are otherwise extremely diverse with the G+C content of their DNA ranging from 25 to 60 mol % (Jones *et al.*, 1987). Methanogen genomes are

circular, double stranded DNA molecules of approximately the same size as those of bacteria (Klein and Schnorr, 1984: Sitzmann and Klein, 1991) and methanogens are prokaryotes as they lack a nuclear membrane but in some molecular properties they more closely resemble the Eukarya than the Bacteria (Brown *et al.*, 1989; Iwabe *et al.*,. 1989). Because methanogens have unique metabolic pathways, and many are thermophiles or hyperthermophiles (Stetter *et al.*, 1981; Jones *et al.*, 1983, 1987, 1989; Zhao *et al.*, 1988; Huber *et al.*, 1989), they are also likely to be exploited as source of novel metabolites, pharmaceuticals, and industrial biocatalysts (Konisky, 1989). They are already proven to be a source of restriction enzymes (Schmid *et al.*, 1984; Thomm *et al.*, 1988; Lunnen *et al.*, 1989). Some of the biomethanogenic bacteria used in biodegradation are *Eubacterium tortuosum, Methanobacterium ruminatum, Clostridium butyricum, Selenomonas ruminatum, Methanosarcina barkeri, Methanobacteria soehngeii, Bacterioides ruminicola* (Abubacker, 2005).

Numerous investigators (Reiset, 1863) have demonstrated that much methane is formed in the rumen, suggesting that methanogenic bacteria are abundant in this environment. Information on their nature is sparse. Detection of *Methanosarcina* in goat rumen (Beijer, 1952), and demonstration of *Methanobacterium formicicum*, a methanogenic acetate-utilizer, in cattle (Opperman *et al.*, 1957) has stimulated an attempt to determine their numbers in bovine rumen contents and to isolate the predominant types in pure culture.

Cellulose, the most abundant biopolymer in the biomass, is known for its degradation under anaerobic conditions. Anaerobic bacteria belonging to the genera *Acetovibrio, Clostridium* and *Ruminicoccus* are active anaerobic cellulose degrading organisms (Ramaswamy *et al.*, 1992). Many anaerobic bacteria such as *Bactereoides, Butyrivibrio, Clostridium, and Selenomonas* show proteolytic activity.

2.2.3 Methanogenic bacterial species

Methanobacterium formicum

In *Methanobacterium formicicum*, cells are slender, crooked rods with blunt, rounded ends. They are long and often form chains or filaments (Schnellen, 1947) and are 0.4-0.8μm wide and 2-15μ long. Each strain has a relatively constant diameter, but its length may vary. They are also non motile and form no endospores (Boone and Mah, 1989). This organism has fimbriae (Doddema *et al.*, 1979) and intracytoplasmic membraneous elements.

Surface colonies are white to grey, flat and filamentous. Deep colonies are profusely filamented spheroids. In roll tubes with H_2-CO_2, colonies appear after 3-5 days, and complete growth occurs within 2 weeks, attaining diameter up to 5mm. Appearance of liquid cultures depends on the strain; medium may be uniformly turbid, or growth may occur as highly granular clumps which do not break up even with vigorous agitation (Langenberg *et. al.*, 1968). The molecular percent G+C of DNA is 40.7 (Zeikus, 1977). Bacterial cultures of *Methanobacterium formicicum* can be isolated from sewage sludge digester. They have numerous habitats including anaerobic digesters, anaerobic fresh water sediments, rumen of cattle or as endosymbionts in anaerobic protozoa (van Bruggen et al., 1984).

Methanobacterium ruminatium

They are non motile, nonspore forming, gram positive, encapsulated rod with rounded ends, which can utilize hydrogen and formic acid as oxidisable substrate (Smith and Hungate, 1958). Cells are non-motile to poorly motile with an optimal growth range of 37 to 39°C. They form lancet shaped cocci to short rods which form pairs or chains 0.5 to 1.0μm in width (Balch *et al.*, 1979). It resembles *Methanobacterium formicicum* in utilizing formate but differs in cell morphology, colony morphology, pigmentation and temperature range. The organism is a short rod

0.7 μ in width and 0.8 to 1.8 μ in length. Recently divided cells may be almost spherical. In young cultures the organism occurs predominantly in pairs. Spores were never reported (Smith and Hungate, 1958) *Methanobacterium* requires acetate as major source (60%) of cell carbon (Bryant et al., 1971). The mol% G+C of *Methanobacterium ruminatium* is 30.6 (Balch *et al.*, 1979). Colony fluorescence can be used as presumptive evidence of methanogenic bacteria (Edwards and McBride, 1975).

Methanobacterium soehngenii

A methanogenic bacterium, commonly seen in digested sludge and referred to as the "fat rod" or *Methanobacterium soehngenii*, has been enriched to a monoculture and is characterized (Barker, 1936). Cells are gram negative, non-motile and appear as straight rods with flat ends. They form filaments which can grow to great lengths. The structure of the outer cell envelop is similar to *Methanospirillum hungatii*. The organism grows on a mineral salt medium with acetate as the only organic component. Acetate is the energy source, and methane is formed exclusively from the methyl group. Acetate and carbon dioxide act as sole carbon source and are assimilated in a molar ratio of about 1.9:1. The reducing equivalents necessary to build biomass from these two precursors are obtained from the total oxidation of some acetate. Hydrogen is not used for methane formation and is not needed for growth. Formate is cleaved into hydrogen and carbon dioxide. Coenzyme M was found to be present at levels of 0.35 nmol per mg of dry cells and F_{420} amounted to 0.55 microgram per mg protein. The mean generation time was 9 days at 33 degrees Centigrade.

Methanosarcina barkeri

Coccoid bodies 1.5-2.0μm in diameter, occurring mostly in irregular aggregates ranging from several to several hundred micrometers in size (Barker, 1956). Membranes contain C_{25} isoprenoids as major neutral lipid, but no C_{30}

isoprenoids (Langworthy et al., 1982). They are non-motile, stains gram positive and are not lysed by SDS. Deep colonies in methanol agar with inorganic salts are whitish to light yellow and 0.5-1.0 mm in diameter. In liquid medium, growth may occur as sediment with active gas formation.

Energy yielding metabolism involves methane production. Methanol, monomethylamine, dimethyl amine, and trimethyl amine acetate and CO may be used as substrate. The methyl group of methanol or acetate is reduced to CH_4 without intermediate oxidation to CO_2. Optimum growth is obtained at pH 7.0 and at 30-40°C. They are strict anaerobes. The molecular percent G+C of the DNA is 38.8 (Welmer and Zeikus, 1978). The habitat of their growth includes fresh water and marine mud, rumen of ungulates, animal waste lagoons and sludge from anaerobic sewage-sludge digesters.

Bacteroides ruminicola

The genus *Bactereoides* consist of rod shaped organism of variable size. Many species are pleomorphic and show terminal or central swellings, vacuoles or filaments. They are anaerobic, nonmotile, chemoorganotrophic, metabolizing carbohydrates, peptone, or metabolic intermediates. Hemin and vitamin K are highly stimulatory for the growth of many species and are generally added to media for the growth of *Bactereoides*. The bacteria were isolated from a wide range of anaerobic habitats; gingival crevice, intestinal tract, sewage sludge and infective and purulent conditions in human and animals (Holt *et al.*, 1994). The molecular percent G+C of the DNA is 50.6 (Mannarelli *et al.*, 1991).

Selenomonas ruminatium

Selenomonas meaning "moon shaped unit" (Certes, 1889 and Prowazek, 1913) obviously alludes to the overall morphology of organism in the genus. They are curved, usually crescent shaped rods, 0.9-1.1 x 3.0-6.0μm, with ends tapered (Holt *et*

al., 1994). They occur singly in pairs, or in short chains and are gram negative. Capsules are not present and are non sporing. They have an active tumbling motility due to flagella, up to 16, arranged as a tuft or short line near the center of the concave side of the cell. It has a fermentative type of metabolism. Fermentation of glucose gives mainly acetic and propionic acids with CO_2 and/or lactate. They are found mainly in human buccal cavity, the rumen of herbivores, and cecum of pigs and several rodents (Holt *et al.*, 1994). The mol% G+C of *Selenomonas ruminatium* is 54 (Kingsley and Hoeniger, 1973).

Clostridium butyricum

The cells are rod shaped, 0.3-2.0 x 1.5-20.0 μm and are often arranged in pairs or short chains, with rounded or sometimes pointed ends (Prazmowski, 1880). They are commonly pleomorphic, catalase negative and gram positive (Smith and Hobb, 1974) in young cultures. They are usually motile with peritrichous flagella. Usually they form oval or spherical endospores that usually distend the cell. The optimum temperature of growth ranges from 10-65°C. The mol% of the G+C is 27-28 (Cummins and Johnson, 1971; Matteuzzi *et al.*, 1977).

Eubacterium tortuosum

They form irregular rods often in pairs or chains. Cells are usually irregular, often with swollen or tapered ends and are sometimes curved (Debono, 1912). They are found in intestinal tract of humans and animals, plants and soil. The temperature for optimum growth is 37-41°C. They are strict anaerobes and require special anaerobic techniques for growth and require nutritionally rich media on which colonies are usually low convex or flat. They are catalase negative and indole negative (Holt *et al.*, 1994).

The above mentioned morphological and cultural characteristics of methanogens as described by different authors are given in Table 1.

Table 1. Morphological and cultural characters of methanogens

Species	Gram stain	Shape	Size	Optimum pH	Motility	Ensospore Staining	Optimum temperature	Distinguishing features
Methanobacterium ruminatium	Positive	Curved, crooked to straight rods. Short oval rods	0.7 x 0.8-1.8 µm	6.3-6.8	Non motile	Nonspore forming	37-39°C	Divided cells are almost spherical. Colonies with translucent convex circular entire margin and frequently light yellow in colour
Methanobacterium formicicum	Positive	Cells are long and often form chains or filaments	0.4-0.8 µm wide and 2-15 µm long	6.6-7.8	Non motile	Nonspore forming	37°C	Cells are slender crooked rods with blunt, rounded ends.
Methanosarcina barkeri	Positive	Coccoid bodies occurring mostly in irregular aggregates ranging from several to several hundred micrometres in size	1.5-2.0 µm	7.0	Non motile	Nonspore forming	35-40°C	Irregular spherical bodies, occurring alone or typically in aggregates of cells
Bactereoides ruminicola	Negative	Rod shaped	0.4-0.5 x 1.4 – 6.0 µm	6.8	Non motile	Non spore forming	37°C	Rod shaped, non motile organism of variable size.
Eubacterium tortuosum	Positive	Rod shaped usually arranged in pairs or in chains	0.2-2 x 3-10µm	5.3 to 5.6	Motile	Nonspore forming	37-41°C	Cells are irregular often with swollen or tapered ends and are sometimes curved
Selenomonas ruminatium	Negative	Crescent shaped curved rods with ends often tappered	0.9-1.1 x 3.0-6.0 µm	6.1 to 6.6	Motile	Nonspore forming	35-40°C	Flagella arranged as a tuft or short line near centre of concave side of cell.
Clostridium butyricum	Positive	Rod shaped	0.3-2 x 1.5-20 µm	6.5-6.7	Motile (peritrichous flagella)	Oval or spherical endospores	10-65°C	Cells are arranged in pairs or short chains with rounded or pointed ends. Commonly pleomorphic(rounded colony, peritrichous)

2.3 Molecular and Biochemical features of Methanogenic bacteria

In addition to the normal prokaryotes (eubacteria) and eukaryotes, the archaeobacteria represent the third line of descent in the evolution of life. This indicates that the methanogens belong to a new kingdom; archaeobacteria is based mainly on sequence comparison of the 16S rRNA oligonucleotides. It was found that rRNA changes occur very slowly in the new kingdom.

The archaebacteria are predominantly terrestrial and aquatic microbes, occurring in anaerobic or hypersaline or hydrothermally and geothermally heated environments, also, some occur as symbiotic in animal digestive tracts.

A unique biochemical feature of archaeobacteria is the presence of glycerol isopropanyl ether lipids. The lack of murein in cell walls makes archaeobacteria insensitive to β-lactan antibiotics. The "common arm" of the tRNA contains pseudouridine or 1-methylpseudouridine instead of ribothymidine. The sequences of 5S, 16S and 23S rRNAs are very different from the corresponding ones in eubacteria and eukaryotae.

Archaeobacteria share some molecular features with the eukaryotae: (a) elongation (EF-2) contains the amino acid diphthamide and is therefore ADP-rybosylable by diphtheria toxin. (b) amino acid sequences of ribosomal "A" protein exhibit sequence homologues with the corresponding eukaryotic (L-7/L-12) protein, (c) the methionyl initiator tRNA is not formylated, (d) some tRNA genes contain introns, (e) the aminoacyl stem of the initiator tRNA terminates with the base pair "AU", (f) the DNA- dependent RNA polymerases are multicomponent enzymes and are insensitive to the antibiotics rifampicin and streptolydigin, (g) like the α-DNA polymerases of eukaryotae, the replicating, archaeobacterial DNA polymerases are not inhibited by aphidicolin or butylphenyl-GTP, and (h) protein synthesis is inhibited by anisomycin but not by chloramphenicol.

Autotrophic archaeobacteria do not assimilate CO_2 via the calvin cycle. In methanobacterium, CO_2 is fixed via an acetyl-CoA pathway. Biological N_2 fixation has been demonstrated by some methanogens.

Gram stain results may be positive or negative within the same order, due to very different types of cell envelopes. Gram-positive-staining species possess pseudomurein, methanochondritin and heteropolysaccharide cell walls; Gram-negative-staining cell have (glycol-) protein surface layers. The cells may have a diversity of shapes, including spherical, lobed, spiral, plate or rod shaped; unicellular and multicellular forms in filaments or aggregates also occur. The diameter of an individual cell may be from 0.1 to >15μm, and the length of the filaments can be up to 200μm. Multiplication is by binary fission, budding, constriction, fragmentation or unknown mechanisms. Colors of cell masses may be red, purple, pink, orange-brown, yellow, green, greenish-black, gray and white.

Methanogens have unusual coenzymes such as coenzyme M (Bryant *et al.*, 1971; Taylor *et al.*, 1974), F_{420} (Cheeseman *et al.*, 1972; Eirich *et al.*, 1979), F_{430} (Gunsalus and Wolfe, 1978), methanopterin (Vogels *et al.*, 1982) and methanofuran (Leigh and Wolfe, 1983); their membranes are composed mainly of ether-linked isoprenoids rather than ester-linked phospholipids (Tornabene *et al.*,1978; Tornaben and Langworthy, 1979, Langworthy, 1985), and they contain no peptidoglycan (Kandler and Hippe,1977, Kandler and Konig, 1985)

2.4 Culture technique for anaerobes

For the culture of anaerobes, the problem is to exclude, not provide, oxygen. As oxygen is present in the air, special methods are needed to culture anaerobic microorganism. Obligate anaerobes vary in their sensitivity to oxygen, and a number of procedures are available for reducing the oxygen content of cultures. Some of

these techniques are simple and suitable mainly for less-sensitive organisms, while others are more complex, but necessary for growth of strict anaerobes.

Bottles or tubes filled completely to the top with culture medium and provided with tightly fitting stoppers provide suitably anoxic conditions for organisms not too sensitive to small amounts of oxygen. It is also possible to add to culture media a chemical called a reducing agent that reacts with oxygen and reduces it to H_2O. A typical example is thioglycollate, which is added to a medium called thioglycollate broth, commonly used to test an organism's requirements for oxygen.

After thioglycollate reacts with oxygen throughout the tube, oxygen can penetrate only near the top of the tube where the medium contacts air. Obligate aerobes grow only at the top of such tubes. Facultative organisms grow throughout the tube but best near the top. Microaerophiles grow near the top but not right at the top. Anaerobes grow only near the bottom of the tube, where oxygen cannot penetrate. A redox indicator dye called *resazurin* is added to the medium because the dye changes color in the presence of oxygen (pink when oxygenated, colorless when reduced) and thereby indicates the degree of penetration of oxygen into the medium.

To remove all traces of O_2 for the culture of anaerobes, it is possible to place an oxygen-consuming system in a jar holding the tubes or plates. One of the simplest devices for this is an anoxic jar, a heavy-walled jar with a gastight seal within which tubes, plates, or other containers to be incubated are placed. The air in the jar is replaced with a mixture of H_2 and CO_2, and in the presence of a chemical catalyst the traces of O_2 left in the jar and culture medium are consumed by the H_2 ($H_2 + O_2 \rightarrow H_2O$), eventually leading to anoxic conditions.

For strict anaerobes, such as methanogens, it is necessary not only to carefully remove all traces of O_2 but also to carry out all manipulations of cultures in an anoxic atmosphere (Madigan and Martinko, 2006). Strict anaerobes can be killed by even a

brief exposure to oxygen. In these cases, a culture medium is first boiled to render it oxygen-free, and then a reducing agent such as H_2S is added, and the mixture is sealed under an oxygen-free gas hydrogen or nitrogen that is directed into the culture vessel when it is open, thus driving out any oxygen that might enter. For extensive research on anaerobes, special boxes fitted with gloves, called anoxic glove boxes, permit work with open cultures in completely anoxic atmospheres.

2.5 Techniques for Isolation and Identification of bacteria

2.5.1 Microbiological Sampling technique.

A pure culture may be obtained from a sample containing a mixture of bacteria by serially diluting the sample with sterile broth in cultures tubes. Five fold, 10 fold or 100 fold dilutions may be made to the point of extinction, i.e., to the point where a single cell may be suspended in a tube. On incubation, these tubes must presumably give rise to cultures derived from a single cell. Broth cultures thus obtained are, however, of dubious purity unless tested by the pour plate or streak method (Sullia and Shantharam, 2005). The original sample should be suitably diluted with sterile water or broth to ensure development of isolated colonies. Pour plating can be used for plating of a diluted sample mixed with melted agar medium. Pour plates will exhibit both surface and subsurface colonies as some cells will be trapped deep in the medium. A combination of serial dilution of the original sample with pour plate method is suitable for isolation of soil bacteria, fungi and actinomycetes.

2.5.2 Isolation Technique for Anaerobes

Pour plate or roll tube technique (Hungate, 1969) can be used for isolation of total anaerobes , anaerobic cellulolytic, proteolytic, acid forming and methanogenic bacteria by using appropriate culture medium.

Anaerobic methods for isolation include the use of anaerobic jars, Bio-Bags, the Pre-reduced anaerobically sterilized technique (PRAS method of Hungate) and anaerobic chamber or glove box.

Anaerobic jar can be set up by two different methods. The easiest utilizes a commercially available H_2 and CO_2 generator envelope activated simply by adding 10ml of water. The envelope is placed in one jar with the inoculated plates, water is added and the jar is sealed. Production of heat within a few minutes and subsequent development of moisture on the walls of the jar are indications that the indicator is working properly. Reduced conditions are achieved in 1 to 2 hours, although methylene blue takes longer time to become decolorized. In another method air is removed from the sealed jar by drawing vacuum of 25 inches of mercury. This process is repeated twice or thrice, filling the jar with oxygen-free N_2 between evacuations. The final fill of the jar is made with a gas mixture containing 80% to 90% N_2, 5% to 10% H_2 and 5% to 10% CO_2.

The bio-bag technique utilizes clear, gas impermeable bag, an ampule containing resazurin indicator and a gas generator ampule. One or two plates are placed inside the bag and it is sealed with a heat sealer. The indicator ampule is then crushed; the gas generated is then activated. Reduced conditions are achieved within one and half hours. Organism grows and maintains viability for one week. These bags are especially convenient for incubating primary plates or in other circumstances where few plates are used.

2.5.3 Enrichment culture Technique

This is a special culture method wherein an environment is created to favour a specific group of microorganisms. This may be achieved by introducing a specific nutrient by modifying the pH and temperature. The conditions will be unsuitable for majority of the microorganisms which are to be eliminated. This technique is

particularly useful in isolating organisms which could degrade specific chemical pollutants in soil or water. For very fastidious methanogens, the enrichment medium may also be amended with thirty percent rumen fluid or sludge extract (Mah and Smith, 1981)

2.5.4 Staining techniques for light microscopy

Fixed and stained preparations are used for observation of the morphological characteristics of bacteria. The advantages of these procedures are that the cells are more clearly visible after staining and the cells of different species can be made out by differential staining. However, fixed and stained preparations cannot be used to observe motility of cells in a liquid environment. Differential staining makes possible differentiating the types of cells or parts of the same cell by difference in the staining pattern.

2.5.5 Gram staining

The most widely used differential staining in microbiology is Gram staining. The technique was introduced by Christian Gram in 1884. Gram stain result may be positive or negative within the same order, due to very different types of cell envelopes. Gram-positive staining species possess pseudomurein, methanochondroitin and heteropolysaccharide cell walls. Gram-negative-staining cells have (glycol-) protein surface layers.

2.5.6 Endospore staining

Bacteria such as *Bacillus* spp. and *Clostridium* spp. form endospores which are not stained by routine stains. Barthlomew and Mittwer's endospore staining method is a differential stain which highlights spores in bacterial cells and in free state (Sullia and Shantharam, 2005).

2.5.7 Motility test

Wet mount and hanging drop technique can be used for observing the motility of the bacteria (Sullia and Shantharam, 2005). The size, shape and arrangement of the cells of microorganisms can also be observed by this method. Flagella staining can be done using Gray method.

2.5.8 Fluorescence Microscopy

Fluorescence microscopy employs short wave, usually ultraviolet range waves to excite the specimen which emits a visible wavelength light. Methanogenic species can be tentatively identified and enumerated within a mixed population on the basis of their blue green autofluorescence (Mink and Dugan, 1977). Since methanogens have Factor F_{420}, they fluoresce under UV illumination without the presence of a fluorochrome. F_{420} is a low potential electron carrier (Eirich *et al.*, 1978). F_{420} has been detected in all methanogens but has not been reported elsewhere. (Eirich,1978). Many other bacteria do not fluoresce without the presence of a fluorochrome. Thus when a successful enrichment has been obtained, the methanogens may be observed by fluorescence microscopy (Doddema and Vogels, 1978). This is taken advantage to rapidly screen and observe the methanogens.

2.5.9 Phase contrast microscopy

Phase-contrast microscopy requires a special optical system-a phase contrast objective and a phase contrast condenser. With this system, it is possible to observe structures within the cells which are not stained, based only on the refractive inidices of the structures or the thickness of parts. This is based on the principle that light passing through one material to another of slightly different refractive index will undergo a change in phase. These differences in phase or irregularities in waves are translated into variations in brightness and hence detected by the eye.

2.5.10 DNA base composition

The unique feature of DNA for taxonomic importance is its Guanine plus Cytosine content (mol % G + C). Closely related bacteria have a similar mol % G + C values. Determination of DNA G + C (mol %) is only helpful parameter to distinguish between two species rather than in a taxonomic placement of a new methanogenic species. DNA mol % G + C is determined by thermal denaturation technique (Marmur and Doty, 1962).

Materials and Methods

MATERIALS AND METHODS

A study on "Management of biodegradable plant tissue culture lab wastes through biomethanogenesis" was carried out at the Centre for Plant Biotechnology and Molecular Biology (CPBMB), College of Horticulture, Vellanikkara during 2005-2007. The materials used and the methodology adopted for the studies are described in the following sections.

3.1 MATERIALS

3.1.1 Chemicals and glass wares

Chemicals used for the preparation of various media were procured from Sisco Research Laboratories (SRL) and Himedia Laboratories. Inverted measuring cylinders used in the experiment were designed by M.J. Chemicals, Thrissur.

3.1.1.1 Inverted measuring cylinder with nozzle

Inverted measuring cylinders were specially designed for the calibration of biogas through water displacement technique. About twenty seven inverted cylinders were specially made with the help of M.J. Chemicals, Thrissur.

3.1.1.2 Plastic drum

Plastic drums for the biogas units were procured from Kakkassery stores Vadakkenchery. Small biogas units were designed and assembled.

3.1.1.3 Basin with stand

Plastic basins were fitted with short PVC pipes at the base for anchoring or holding the inverted cylinder.

3.1.2 Equipments

The equipments used for the experiment were those present at Centre for Plant Biotechnology and Molecular Biology. Incubation of the cultures was done in bacteriological incubator. Centrifugation was done in KUBOTA high speed refrigerate centrifuge. Slides of bacterial cultures were observed with the help of a compound binocular microscope (CETI) and fluorescent microscope. The photos were taken using Nikon digital camera attached to the microscope. Eppendorf Master Cycler was used for DNA amplification. Alpha imager TM1200 was used for imaging the gel. Quantification of DNA was done using Nanodrop (ND-1000 spectrophotometer). The anaerobic bacterial cultures were maintained and multiplied using anaerobic system mark IV device from Himedia.

3.2 QUANTITY OF TISSUE CULTURE WASTE AVAILABILITY

Tissue culture lab agar wastes for the experiment on biomethanogenesis were procured from tissue culture labs present in CPBMB, Department of Pomology and Floriculture, Campus Development Centre and ATIC-ABARD, Mannuthy. The weight of the tissue culture wastes from each lab was found and the individual cumulative production for a period of thirty days was determined. The total residues that can be collected for experiment for a month and the site of maximum production was thus found out. The average weight procured from each lab per day was also calculated.

3.3 METHANOGENIC BACTERIAL CULTURES USED IN THE STUDY.

Seven bacterial cultures were used in the present study. They belonged to two broad classes, viz. aceticlastic methanogens and proteolytic anaerobes as described by (Ramasamy *et al.*, .1992). The aceticlastic methanogens used in the present investigation were *Methanobacterium ruminatium*, *Methanobacterium formicicum* and *Methanosarcina barkeri*. These methanogens were abbreviated as A1, A2 and A3

respectively. *Bactereoides ruminicola, Eubacterium tortuosum. Selenomonas ruminatium* and *Clostridium butyricum* were the proteolytic anaerobes studied. They were numbered P1, P2, P3 and P4 respectively.

3.4 PROCUREMENT OF METHANOGENIC BACTERIAL CULTURES

Out of the seven methanogens for the present investigation, three of the cultures were procured from other institutions. Lyophilised cultures of *Clostridium butyricum* were obtained from Microbial Type Culture Collection, IMTECH, Chandigarh. Cultures of *Selenomonas ruminatium* and *Eubacterium tortuosum* were collected from TNAU, Coimbatore. The remaining four bacterial cultures were isolated in the microbiology lab of CPBMB. The methanogen, *Methanobacterium formicicum* was isolated from rumen fluid and was substituted for *Methanobacterium soehngenii* which was earlier mentioned in technical programme.

3.5 ISOLATION OF METHANOGENS

The four methanogens isolated in CPBMB were *Methanobacterium ruminatium, Methanobacterium formicicum, Methanosarcina barkeri* and *Bactereoides ruminicola.* They were isolated from rumen fluid, adjacent paddy fields and biogas unit according to the procedures prescribed by Blackburn & Hobson (1962) and Mah (1980). The identities of the entire bacterial cultures were confirmed by morphological observation, staining reactions and by other molecular parameters.

3.5.1 Collection of samples for isolation

The inoculum for isolation of methanogens consisted of rumen fluid, biomass sample from biogas plant and paddy soil samples. The rumen fluid for isolation was collected from Department of Meat Technology, College of Veterinary and Animal Sciences, Mannuthy. The biomass sample was collected from Agricultural Research Station, Mannuthy.

3. 5.1.1 Rumen fluid

Rumen fluid was collected from the slaughter house of Department of Meat Technology, College of Veterinary and Animal Science, Mannuthy. The rumen fluid was collected by squeezing the biomass from the rumen of cattle. It was immediately filled in a non transparent plastic container and closed air tight.

3. 5.1.2 Biomass sample from biogas plant.

The biogas plant used in Agricultural Research Station, Mannuthy was a dome shaped one. The sample for the experiment was siphoned from the bottom of the biogas plant by using a tube with rubber bulb at the end. It was then transferred to an air tight container.

3.5.1.3 Paddy field soil sample.

Soils from paddy fields were collected from Ollukkara and Agricultural Research Station, Mannuthy. The soil samples were collected from the rhizosphere of the rice plants. They were then filled in plastic containers, covered using aluminium foil and refrigerated.

3.5.2 Transfer area and aseptic manipulations

All the aseptic manipulations such as serial dilution, picking of colonies, streaking and pour plating were carried out under hood of a clean laminar air flow chamber inside microbiology lab. The working table of the laminar air flow chamber was first surface sterilized with absolute alcohol and then by putting on the ultraviolet light for 30 minutes. The inoculation loop and other accessories were flame sterilized before each inoculation. The hands were also scrubbed with absolute alcohol before inoculation.

3.5.3 Dilution of samples

Materials used

1. Dilution media (Annexure I)
2. Slurry sample (Rumen fluid/Biogas biomass/ paddy soil)
3. Serum vials

3.5.3.1 Procedure for dilution of samples

1. Ninety ml of dilution media was added to serum vials and decolourised it with cysteine HCl.
2. Stoppered the serum vials and autoclaved it for 20 minutes.
3. Serially diluted the slurry sample by adding 10ml of the slurry to sterilized and cooled serum vials having 90ml of dilution medium.
4. The diluted sample was used for the enumeration of the organism by roll tube technique.

3.5.4 Culture media for isolation

Mainly two media were prepared for the isolation of methanogens. The composition of medium for aceticlastic methanogens (Mah, 1980) and proteolytic anaerobes (Blackburn and Hobson, 1962) are given in Table 1 and Table 2 respectively.

3.5.4.1 Preperation of media.

Standard procedures were followed for the preparation of media. Stock solution of major and minor nutrients were prepared first by dissolving the required

quantity of chemicals in distilled water and stored under refrigerated conditions in bottles and covered using aluminium foil.

Table 2. Composition of media for culturing aceticlastic methanogens (Mah, 1980)

Ingredients	Quantity
33% culture supernatant from inoculum sources (rumen fluid/biogas biomass)	
67% Barker's salt solution containing	
Tap water	100ml
Ammonium chloride	0.1gm
Dipotassium hydrogen Phosphate	0.04gm
Magnesium chloride	0.01gm
Other nutrients (W/V)	
Tryptone	0.2%
Yeast extract	0.2%
Sodium acetate	1.0%
Sodium bicarbonate	0.15%
Cysteine hydrochloride	0.05%
Sodium sulphide	0.02%
Resazurin	0.0001%
pH	7.0

Table 3. Composition of media for culturing proteolytic anaerobes (Blackburn and Hobson, 1962).

Ingredients	Quantity (/L)
Mineral (a) (Annexure II)	15.0 ml
Rumen fluid	10.0 ml
Casein	0.5g
Tryptone	0.3 g
Cysteine HCl	0.05g
Resazurin	0.0001g
Sodium bicarbonate	0.5 g
Agar	2.0 g
Dipotassium hydrogen phosphate	0.045 g

3.5.5 Procedure for isolating methanogens

Methanogens were isolated using pour plate technique and roll tube technique.

Materials used:-

1. 25 ml test tubes with rubber stoppers
2. Petriplates
3. Appropriate agar media (Blackburn & Hobson medium/ Mah medium)

4. Serial diluted samples (10^{-1}dilution)
5. Pipette (1ml)
6. 10ml measuring jar

3.5.5.1 Protocol for Roll tube technique

1. Sterilized medium was placed in a water bath maintained at 45-50°C to maintain it in liquid state
2. Culture supernatant and trace elements were added before it is transferred to the test tubes.
3. One ml of the sample of desired dilution was transferred to 25ml sterile test tubes.
4. Five ml of the prepared medium (Blackburn & Hobson medium/ Mah medium) was transferred to the test tubes using the measuring cylinder.
5. Test tubes were immediately stoppered with sterile rubber corks.
6. Tubes were rolled immediately on the foam soaked in cold water till the medium solidified on the side of test tubes.
7. When the medium solidified the tubes were kept for incubation in an anaerobic jar (Anaerobic system Mark IV from Himedia).
8. After incubation is over the single isolated colonies were selected and transferred to sterile broth.

3.5.5.2 Pour plate technique protocol

1. The sample containing the inoculum was serial diluted.

2. One ml of sample of desired dilution was added to sterile petri dishes.

3. The agar medium (Hobson & Blackburn medium/ Mah medium) was maintained in a liquid state by maintaining the temperature at 45°C.

4. The medium after pouring to sterile petri dishes were gently shaken to mix with the inoculums.

5. When the medium solidified, the petri dishes were kept for incubation in an anaerobic jar (Anaerobic system Mark IV from Himedia).

6. The single isolated colonies obtained after incubation were selected and transferred to sterile broth.

Rolling tubes and pour plates incubated inside anaerobic jar is presented in Plate 1.

3.5.6 Subculturing and maintaining individual methanogenic colonies

The individual colonies of four methanogenic bacteria isolated by enrichment technique from rumen fluid, biogas biomass and paddy soil etc. were taken from its agar medium using a loop inside the hood of laminar air flow. The colonies were then inoculated in their respective culturing media (Blackburn and Hobson media/ Mah media) containing filter sterilized vitamin solution and trace element solution (Laanbrock *et al.*, 1985) (Annexure III) instead of culture supernatant, in separate test tubes. They are then incubated in anaerobic jar at room temperature.

In the case of bacterial colonies isolated in Mah medium (aceticlastic methanogens), the petri plates or rolling tubes containing colonies were observed under UV lamp for auto-fluorescence. The selected individual colonies were subcultured in the Mah media broth containing vitamins (Annexure IV) and trace elements (Annexure V) instead of other enrichment substances.

Plate 1. Rolling tubes and pour plates incubated inside anaerobic jar

3.6 IDENTIFICATION AND CHARACTERIZATION OF METHANOGENS

3.6.1 Morphological characterization

Morphological characterization was done by observing a wet preparation under microscope. Cell morphology is the key parameter in classification. Morphological characterization was done based on cell shape, motility, presence of endospores and gram staining. Molecular G + C content (%) of the bacterial DNA was calculated and catalase test was also done for identification.

3.6.1.1 Gram staining

Gram staining reaction was done for the identification of methanogenic bacterial species by method prescribed by Gram (1884). The various methanogens stained gram positive and negative based on the composition of their cell wall.

Materials used:-

1. Crystal violet reagent (Annexure VI)
2. Gram's Iodine mordant (Annexure VII)
3. Ethyl alcohol (95%) (Annexure VIII)
4. Safranine counter stain (Annexure IX)

3.6.1.1.1 Procedure for gram staining

1. A smear was prepared on a clean slide and air dried.
2. The smear was heat fixed by gently passing them through a flame.
3. Flooded the slides for one minute with crystal violet staining reagent and washed the film in tap water for 2 seconds.

4. The slides were then flooded with gram's iodine mordant for one minute.
5. Washed the film in tap water for two seconds and then blotted dry with absorbent paper.
6. Flooded the slide with decolourising reagent (95% ethyl alcohol) for 30 seconds, washed the slide with tap water and blotted dry.
7. Again flooded the slides with saffranin counter stain for 10 seconds and washed them off.
8. Blotted dry the film and observed under 100x objective of a microscope.

3.6.1.2 Fluorescence microscopy

Fluorescence microscopy was employed for the characterization of aceticlastic methanogens (Doddema and Vogels, 1978). Wet mounts of various bacterial colonies were prepared and the slides were immediately observed under fluorescent microscope in a dark room. The lenses and filters of the microscope were adjusted and the light path was kept as short as the system allowed. The various aceticlastic methanogen species due to the presence of the factor F_{420} in them, emitted light of visible wavelength on excitation with short waves of ultraviolet range.

3.6.1.3 Endospore staining

Smirnoff staining protocol was employed for the staining of endospores.

Materials used:-

1. 1.5% Amido black
2. 1% Carbol fuchin

3.6.1.3.1 Procedure for endospore staining

1. A loopful of 48 hour old culture grown on thioglycollate agar medium was taken.
2. Heat fixed the smears of culture on a clean dry glass slide
3. Few drops of 1.5% amidoblack was added and allowed to stay for 70 seconds.
4. The slide was then washed under gentle stream of running water, stained for 20 seconds with carbol fuchin (1%) and washed thoroughly under the tap water.
5. The slides were washed under water, air dried and observed under microscope for presence of spores.

3.6.1.4 Motility Test

Hanging drop technique was used for observing the size, shape and arid arrangement of the cells of microorganisms and their motility.

Materials used:-

1. Cavity slide
2. Coverslip
3. Bacterial suspension

3.6.1.4.1 Protocol for Hanging drop technique

1. A drop of microbial suspension was placed at the centre of a coverslip
2. Pasted vaseline on the four corners of the coverslip

3. The cavity slide was then placed over the coverslip in such a way that the concave depression came just above the drop.

4. The slide together with the cover slip was inverted carefully, so that the drop containing the cells hanged from the coverslip without touching the glass slide.

5. The slide was then observed microscopically for motility.

3.6.1.5 Catalase Test

Materials used

1. 3% H_2O_2

2. Glass slides with coverslip

3.6.1.5.1 Procedure for catalase test

1. A smear of bacteria was taken on one of edge of the coverslip

2. Placed a drop of 3% H_2O_2 on the glass slide

3. Kept the coverslip over the drop.

4. Slides were then observed for froth formation

3.6.1.6 Estimation of G+C content

Thermal denaturation technique (Marmur and Doty, 1962) was employed for the estimation of GC content of bacterial DNA.

3.6.1.6.1 Protocol for estimation of G+C content

3.6.1.6.1.1 Isolation of bacterial DNA

1. 1.5ml of culture was centrifuged at 10,000rpm for 5 minutes at 4°C and the supernatant was decanted.
2. The pellet was resuspended in 500µl of distilled water
3. 5µl of lysozyme (1mg/ml) and 120 µl of 0.5M EDTA (pH 8.0) was added and mixed gently and incubated for 5 minutes at room temperature.
4. 70 µl of 10% SDS was added to this mixture and mixed by inversion and incubated at 37°C for one hour.
5. 600 µl of equilibrated phenol: chloroform (1:1) was added and mixed gently by inversion.
6. Centrifuged at 10,000 rpm for 10 minutes and the top aqueous layer was taken
7. Added to it 600 µl of chloroform: isoamyl alcohol (24:1) and mixed gently by inversion.
8. Centrifuged at 7000 rpm for 10 minutes at 4°C
9. Top aqueous layer was transferred to another fresh tube and 60 µl of 3M sodium acetate (pH 5.8) and 1ml of ice cold absolute ethanol were added to precipitate the DNA.
10. Kept it in -20°C for 20 minutes
11. Centrifuged at 15,000 rpm for 5 minutes.
12. The DNA pellet was air dried

13. The pellet was then resuspended in 1.5ml solution containing 0.15 mM NaCl and 15mM sodium citrate.

3.6.1.6.1.2 Measurement of Tm with constant temperature baths.

1. Water baths was set at 30, 35, 40, 45, 70, 75, 80, 85, 90, 95 and 100°C.
2. UV lamp of spectrophotometer was turned on for 20 minutes warm up.
3. Diluted the DNA solution to an A_{260} of 0.4
4. Transferred 3ml of the DNA solution and incubated for 15 minutes
5. Maintained one tube at room temperature, two in 100°C bath and one tube in each of the constant temperature bath.
6. After incubation period quickly cooled the tubes except one in the 100° bath and the one at the room temperature, by placing them in an ice water bath for 10 minutes (25°C)
7. One of the tubes in 100°C baths was allowed to cool to room temperature.
8. Measured and recorded the A_{260} of each DNA solution (25°C)
9. Slow cooled tube was allowed to remain at room temperature for one hour before A_{260} measurement.
10. Calculated the ratio $A_{260}(T)/ A_{260}(25°C)$ at each temperature.
11. Plotted the absorbance ratio versus the temperature graph
12. Connected the points for a smooth temperature profile.

13. Measured Tm by determining the temperature midpoint of the absorbance increases

14. Calculated the GC% content by using the formula %GC = 2.44 (Tm - 69.3) (Marmur and Doty, 1962)

3.7 ENUMERATION OF METHANOGENIC COLONIES.

Plate count technique was used for the enumeration of bacterial colonies. A loopful of bacterial colony was serial diluted to dilutions of 10^{-5} and 10^{-6}. The colonies of 10^{-5} and 10^{-6} dilution were pour plated on appropriate agar medium (Black burn & Hobson medium/ Mah medium) and the colony count was taken after 24 hours incubation in Anaerobic system Mark IV from Himedia.

3.8 MOLECULAR CHARACTERIZATION OF METHANOGENIC BACTERIA

Random Amplified Polymorphic DNA analysis was carried out for identifying the monomorphic and polymorphic bands of different methanogens.

3.8.1 DNA isolation

The protocol used for total DNA isolation of methanogens was that described by Sambrook and Russel (2001). Harvested cells of overnight grown bacterial cultures in the respective broth were used for centrifugation. DNA was isolated from the seven bacterial cultures in three replications.

Materials used:-

1. Lysozyme
2. Freshly prepared lysozyme and SDS (10%)
3. Phenol: Chloroform 1:1 (v/v) mixture

4. Chloroform: isoamyl alcohol 24:1 (v/v) mixture
5. 0.5M EDTA
6. 70 % ethanol
7. 3M Sodium acetate

3.8.1.2 Procedure

1. 1.5ml of culture was centrifuged at 10,000 rpm for 5 minutes at 4°C and the supernatant was decanted.
2. The pellet was resuspended in 500µl of distilled water
3. 5µl of lysozyme (1mg/ml) and 120 µl of 0.5M EDTA (pH 8.0) was added and mixed gently and incubated for 5 minutes at room temperature.
4. 70 µl of 10% SDS was added to this mixture and mixed by inversion and incubated at 37°C for one hour.
5. 600 µl of equilibrated phenol: chloroform (1:1) was added and mixed gently by inversion.
6. Centrifuged at 10,000 rpm for 10 minutes and the top aqueous layer was taken
7. Added to it 600 µl of chloroform: isoamyl alcohol (24:1) and mixed gently by inversion.
8. Centrifuged at 7000 rpm for 10 minutes at 4°C
9. Top aqueous layer was transferred to another fresh tube and 60 µl of 3M sodium acetate (pH 5.8) and 1ml of ice cold absolute ethanol were added to precipitate the DNA.

10. Kept it in -20°C for 20 minutes

11. Centrifuged at 15,000 rpm for 5 minutes.

12. The DNA pellet was air dried

13. The pellet was then resuspended in 150 µl of distilled water.

3.8.2 Assessment of quality of DNA

The quality of isolated DNA was evaluated through agarose gel electrophoresis (Sambrook *et al.*, 1989) followed by gel documentation.

3.8.2.1 Electrophoresis of DNA samples.

A. Reagents

1. Agarose

2. 50x TAE buffer

   Tris base : 242g

   0.5M EDTA, pH 8 : 100ml

   Glacial acetic acid : 57.1ml

   The contents were mixed well, autoclaved and stored at room temperature.

3. 6x gel loading dye

   Bromophenol blue : 0.25%

   Xylene cyanol FF : 0.25%

   Glycerol in water : 30%

The components were mixed well, autoclaved and stored at 4°C

4. Electrophoresis unit, power pack, casting tray, comb etc.
5. Ethidium bromide solution (0.5µg/ml)
6. UV transilluminator
7. Gel documentation and analysis system (Alpha imager TM 1200)

B. Procedure

Gel buffer 1x TAE was prepared from the 50x TAE stock solution. Gel buffer 1x TAE was taken in a conical flask (100ml for large gel and 30ml for small gel. Agarose (1.0% for DNA samples and 1.4% for RAPD samples) was weighed, added to flask, stirred and boiled till the agarose dissolved completely. Ethidium bromide (2µl) was added into the flask, mixed well and it was allowed to cool to 40°C. The open ends of the gel casting tray was sealed with cello tape and placed on a leveled horizontal surface and the comb was placed properly on the tray. The dissolved agarose was poured gently into the tray. The gel was allowed to solidify for 30 minutes and then the comb was removed carefully. The gel was then placed in electrophoresis unit with the wells directed towards the cathode. 1x TAE buffer was added to cover the gel with 2 to 3 millimeters of buffer. The DNA sample (5µl) was mixed with gel loading dye. The samples were then loaded carefully into the well using micropipette. Standard DNA molecular weight markers were loaded in one well and a negative control was also loaded in another gel in the case of electrophoresis for RAPD assay. The cathode and anode of electrophoresis unit were then connected to the power supply and gel was run at constant voltage (100V). The power supply was turned off when the loading dye moved to the required distance.

3.8.2.2 Gel documentation

The gel was taken from electrophoresis unit and viewed under UV light of 320nm. The image of the gel was monitored and stored in a gel documentation system Alpha Imager TM 1200.

3.8.3 Quantification of DNA

Quantification of DNA was done using a NanoDrop® ND-1000 spectrophotometer. For the most consistent result, measurement was begun with a blanking cycle.

Materials used:-

1. Blank sample (autoclaved distilled water)
2. Bacterial DNA samples
3. Tissue paper
4. Micropipette and tips

3.8.3.1 Procedure for quantification using NanoDrop ND-1000 spectrophotometer

1. Loaded the blank sample on to the lower measurement pedestal and lowered the measuring arm in to the 'down' position
2. Clicked on the blank 'F3' button
3. Wiped the blanking sample from both pedestals using the tissue paper
4. Analyzed an aliquot of the blanking solution as though it were a sample, using the 'Measure' button (F1).

5. Obtained result, as a spectrum with a relatively flat baseline.

6. Wiped the blank from both measurement pedestal surfaces and with the sampling arm open pipette the bacterial sample on to the lower measurement pedestal.

7. Closed the sampling arm and initiated the spectral measurement using the operating software on the PC.

8. The sample column was automatically drawn between the upper and lower measurement pedestal and the spectral measurement was made.

9. After completion of measurement, the sampling arm was opened and the sample was wiped from both the upper and lower pedestals.

3.8.4 Assessment of purity of DNA.

The purity of DNA was also assessed using NanoDrop® ND-1000 spectrophotometer. The absorbance of the bacterial DNA was measured at wavelengths of 260 and 280nm. The ratio of absorbance at 260 and 280 was taken for each and every DNA. For good quality DNA the value of A_{260}/A_{280} came in between 1.8 and 2.0. These samples, which have high degree of purity than the rest, were selected for molecular characterization using Random Amplified Polymorphic DNA analysis.

3.8.5 Random Amplified Polymorphic DNA (RAPD) analysis

3.8.5.1 Screening of random primers for RAPD

A total of 8 decamer primers under OPS, Kit C and Kit N series of Integrated DNA Technologies (IDT) were screened for amplification of genomic DNA extracted from bacterial cultures. The procedure of Demeke *et al.,* (1992) was modified and

was used for amplification of genomic DNA. In polymerase chain reaction, the following cycles were followed.

Table 4. Thermal cycles in RAPD

Step no.	Temperature	Duration	Steps involved	No. of cycles
1	94	3	Initial denaturation	1
2	92	1	Denaturation	40
3	37	1	Annealing	
4	72	2	Initial extension	
5	72	5	Final extension	1

Composition of reaction mixture for 25µl was as follows

1. 10x assay buffer
2. 1mM dNTP mix
3. Taq DNA polymerase
4. Primer
5. Template DNA
6. Milli Q water

A master mix was first prepared based on the number of reaction, using the reaction mixture. The master mix did not contain template DNA and primer. From this master mix, 22µl was pipetted into each PCR tube. To this mixture, 1µl of primer and 2.0µl of template DNA were added. Heated lid was used while running PCR. The

PCR tubes were loaded in the thermocycler and the aforesaid programme was run. The amplified products were resolved on 1.4 percent agarose gel using 1x TAE buffer. The gel was viewed under UV light in the transilluminator and then documented.

Table 5. List of primers used for screening

Sl. No.	Primer code	Primer sequence
1	Kit C17	TCCCCCCCAG
2	Kit C18	TGAGTGGGTG
3	Kit C19	GTTGCCAGCC
4	Kit C20	ACTTCGCCAG
5	OPS1	GTTTCGCTCC
6	Kit N5	ACTGAACGCC
7	Kit N11	TCGCCGCAAA
8	Kit N19	GTCCGTACTG

3.8.5.2 Screening of bacterial cultures by RAPD

The primers Kit N5, Kit N11 and Kit N19 which gave about three to ten amplicons in the initial screening were selected. Genomic DNA of the bacterial cultures *Methanobacterium ruminatium* (A1), *Methanobacterium formicicum* (A2), *Methanosarcina barkeri* (A3), *Bactereoides ruminicola* (P1), *Eubacterium tortuosum* (P2), *Selenomonas ruminatium* (P3) and *Clostridium butyricum* (P4) were subjected to amplification using the selected random primers. After amplification using thermal cycler, the products were loaded in the wells of agarose gel together with standard DNA molecular weight markers and run at constant voltage in an electrophoresis unit. The gel was later viewed under UV light and documented.

3.8.5.3 Cluster analysis

The DNA amplification pattern was scored for the 3 primers as 1 or 0, for the presence or absence of bands respectively and using NTSYS pc 2.0 software the data was analysed. A dendrogram was constructed with the Unweighted Pair Group Method of Arithmetic Averages (UPGMA) using NTSYS package for the seven bacterial cultures and extend of similarity between the individual cultures were observed.

3.9 BIOGAS EXPERIMENTAL SYSTEM

System for generation and quantification of biogas was devised by modifying the conventional unit. The system consisted of a plastic drum and an inverted measuring cylinder with a nozzle at the top. The plastic drum was air tight, so as to create anaerobic condition for decomposing substrate (20Kg) and had an inlet and an additional outlet regulated by valves at the top. The inlet of the drum had a larger circumference than the additional outlet. An outlet was present at the lower side of the drum to remove the left over residues after methanogenic degradation. The inverted measuring cylinder was designed so as to measure the quantity of gas evolved during the process of methanogenesis. A rubber tube aided in gas passage from the drum to the inverted cylinder. One end of the rubber tube was connected to the additional outlet of the plastic drum and the other end entered the bottom of the measuring cylinder kept in the basin. The basin and the inverted measuring cylinder were filled with water. The water column in the inverted measuring cylinder did not move downward due to the suction pressure at the top of the measuring cylinder and the upward thrust from the water present in the basin.

The tissue culture wastes and the seven selected methanogenic bacterial cultures were inoculated through the inlet of the drum. The gas evolved was collected through the additional outlet. The volume of gas generated was measured by water

displacement technique. The gas produced inside the drum passed through the tube and got collected at the top of the measuring cylinder. The quantity of gas collected was measured from the calibration on the inverted cylinder which was equal to the amount of water displaced. The nozzle present at the top of the inverted measuring cylinder allowed checking the combustibility of the collected gas. The newly designed experimental biogas unit is shown in Plate 2.

3.10. QUANTITY OF GAS PRODUCED

The quantity of gas produced and its combustibility has been checked for various treatments using bacterial cultures and, cow dung with TC wastes. The treatments done were follows.

1. Inoculation of cow dung slurry alone in biogas unit
2. Tissue culture agar waste + cow dung slurry
3. Inoculation of bacteria *Methanobacterium ruminatium* with TC agar waste
4. Inoculation of bacteria *Methanobacterium formicicum* with TC agar waste
5. Inoculation of bacterial *Methanosarcina barkeri* with TC agar waste
6. Inoculation of bacteria *Bactereoides ruminicola* with TC agar waste
7. Inoculation of bacteria *Eubacterium tortuosum* with TC agar waste
8. Inoculation of bacteria *Selenomonas ruminatium* with TC agar waste
9. Inoculation of bacteria *Clostridium butyricum* with TC agar waste

The quantity of gas produced was directly read from the readings on the inverted measuring cylinder. The combustibility of gas was checked by opening the valve at the nozzle of the inverted measuring jar and by showing a flame at the mouth of the nozzle.

Plate 2. Experimental biogas unit

3.11 PHYSIO-CHEMICAL CHARACTERISTICS

3.11.1 C/N ratio of the Tissue culture waste samples

3.11.1.1 Estimation of Nitrogen

Total nitrogen in tissue culture waste was determined by Kjeldahl method (Tandon, 1994). This essentially involves (i) digestion of the sample to convert the N compounds in the sample to NH_4^+ form and (ii) determination of NH_4^+ in the sample.

Materials used:-

1. Owen dried TC agar waste sample
2. Digestion tube
3. Con. H_2SO_4
4. Digestion mixture ($CuSO_4 + K_2SO_4$)
5. Kjeldahl apparatus
6. Boric acid.
7. Burette containing 0.02N H_2SO_4

3.11.1.1.2 Procedure

Step 1. Digestion

1. Weighed about 1gm of the tissue culture waste sample
2. Dried the sample in a hot air owen at 100°C for one hour.
3. Put the samples in digestion tubes
4. Added 10ml H_2SO_4 + 1gm digestion mixture
5. Kept over night for digestion
6. Digestion carried out at 200°C.

Step 2. Distillation

1. The digest is cooled to room temperature before distillation.
2. 10ml of the digest was taken and transferred to the vacuum jacket of micro-kjeldahl distillation apparatus.
3. In a conical flask, 10ml of boric acid solution is taken containing bromocresol green and methyl indicator to which condenser outlet of the flask is dipped.
4. The ammonia released by distillation got collected in boric acid and as a result the colour turned from brick red to green.

Step 3. Titration

1. The boric acid containing Ammonia was titrated against 0.02N H_2SO_4 taken in the burette till the green colour turns back to brick red.
2. The percentage of Nitrogen was calculated by the formulae.
3. Nitrogen percentage = Titre value x 0.02 x 14/1000 x 100/wt of the sample x 50/10

3.11.1.2 Estimation of Carbon

Materials

1. TC agar waste sample
2. Muffle furnace
3. Silica crucibles

3.11.1.2.1 *Procedure*

1. One gram of tissue culture sample was taken and heated in a hot air oven at 110°C to remove the moisture.
2. The dried sample weight is taken

3. The dried sample was then put in a silica crucible and kept inside a muffle furnace at a temperature of 600°C for 6 hours. The carbon in the sample was oxidized into CO_2 and got removed.
4. Cooled the sample and the weight was taken again.
5. The percentage carbon content was calculated by subtracting the final weight from the initial weight (Amma, 1989).

3.11.1.3 C/N ratio

C/N ratio was found by dividing the percentage content of carbon by percentage content of nitrogen

C/N ratio = percentage of Carbon/ percentage of Nitrogen.

3.11.2 Hydraulic retention time

Hydraulic retention time is the time for which the slurry should be held in the digester for getting eighty percent gas. It was measured in days.

Results

RESULTS

The results of the study on 'Management of biodegradable plant tissue culture lab wastes through biomethanogenesis' undertaken at the Centre for Plant Biotechnology and Molecular Biology are presented in this chapter.

4.1 QUANTITY OF TISSUE CULTURE WASTE AVAILABILITY IN KAU CAMPUS

The total quantity of tissue culture waste produced in Kerala Agricultural University campus, Vellanikkara for a period of thirty days was noted. Tissue culture wastes were procured from Centre for Plant Biotechnology and Molecular biology (CPBMB), Campus Development tissue culture laboratory, Floriculture tissue culture laboratory and ATIC-ABARD tissue culture laboratory. Total quantity of tissue culture waste collected from the entire laboratories came up to 314.5 kilograms for a period of 30 days. From this data, the average weight procured from each laboratory per day was calculated and it counts to about 2.62 kilogram. The highest quantity of the tissue culture waste was produced from CPBMB laboratory (93.7 Kg/ 30 days) followed by floriculture laboratory with a total production of 83.7 Kg for 30 days. The third in production was campus development TC laboratory and finally the lowest quantity of TC wastes were procured from ABARD tissue culture laboratory. Details of the tissue culture waste collected from each laboratory for a period of 30 days are given in Table 6.

4.2 PROCUREMENT OF METHANOGENIC BACTERIA

Three bacterial cultures viz. *Clostridium butyricum*, *Selenomonas ruminatium* and *Eubacterium tortuosum* were procured from other institutions. Lyophilised culture of *Clostridium butyricum* was procured from Microbial Type Culture Collection, IMTECH, Chandigarh. Bacterial cultures of *Selenomonas ruminatium* and *Eubacterium tortuosum* were obtained from TNAU, Coimbatore. The remaining four cultures were isolated in CPBMB.

Table 6. Quantity of tissue culture wastes procured from various tissue culture laboratories of the campus.

Date of Collection	Quantity of wastes (Kg)			
	Floriculture Tissue Culture Lab.	ATIC-ABARD Tissue Culture Lab.	CPBMB Tissue Culture Lab.	Campus Development Tissue Culture Lab.
29-09-06	2	1.6	4.5	2.8
30-09-06	2.4	3.4	3.6	4.2
03-10-06	3.0	-	-	2.8
04-10-06	2.8	4.3	3.2	3.6
05-10-06	4.2	3.6	3.8	2.2
06-10-06	4.8	-	5.0	4.2
09-10-06	-	7.6	1.7	-
10-10-06	4.7	-	-	3.3
11-10-06	2.5	-	4.0	3.3
12-10-06	2.8	4.2	-	5.0
13-10-06	1.7	-	-	-
16-10-06	-	-	4.4	2.6
17-10-06	1.9	-	3.7	-
18-10-06	3.4	1.5	2.6	4.2
19-10-06	4.8	-	4.3	2.8
20-10-06	3.4	3.6	4.1	2.5
25-10-06	3.7	4.3	2.5	-
26-10-06	3.8	3.9	-	4.7
27-10-06	-	5.4	4.6	3.9
28-10-06	3.4	-	5.2	4.0
30-10-06	-	5.2	3.4	-
01-11-06	6.2	-	-	2.8
02-11-06	-	-	1.9	-
11-04-07	5.3	4.4	8.2	4.2
12-04-07	5.1	-	3.3	-
13-04-07	-	-	2.6	4.1
16-04-07	-	-	2.6	3.5
17-04-07	-	3.9	5.3	-
18-04-07	6.1	1.6	4.3	3.5
19-04-07	5.7	-	4.9	4.4
Total	**83.7**	**58.5**	**93.7**	**78.6**

Grand Total: 314.5 Kgs

4.3 ISOLATION AND IDENTIFICATION OF METHANOGENS.

4.3.1 Isolation of methanogens

The four isolated methanogens for the study were cultured specifically to Mah media (A1, A2 and A3) and Blackburn and Hobson media (P1) using anaerobic jar. These selective media were found very effective for these methanogens. Good results were observed in both proteolytic and aceticlastic methanogens incubated media. The incubation of aceticlastic methanogens for two weeks in petri plates and test tubes resulted in gas generation. The cracks and cavities produced in petri plates and rolling tubes due to methane gas generation are shown in plates 3, 4 and 6. The bacterial colonies produced on the sides of the test tubes by roll tube technique are shown in plate 5.

4.3.2 Identification of methanogens

The identity of the seven bacterial cultures were confirmed on the basis of their shape, gram staining characters, fluorescence microscopy, endospore staining, motility test, catalase test and G+C content.

4.3.2.1 Shape of bacteria

The shapes of the bacteria were observed under microscope. In the aceticlastic bacterial cultures, *Methanobacterium ruminatium* (A1) and *Methanobacterium' formicicum* (A2) were rod shaped bacilli, while *Methanosarcina barkeri* (A3) formed round cocci. A2 have long rod shaped character compared to A1. A1 formed chains in which cells were arranged in the form of short oval rods. In A3, both isolated single bacterial cells and also colonies containing number of bacterial cells were found. All the proteolytic bacterial cultures, namely *Bactereoides ruminicola* (P1), *Eubacterium tortuosum* (P2), *Selenomonas ruminatium* (P3) and *Clostridium butyricum* (P4) formed rod shaped bacilli.

4.3.2.2 Gram staining

The bacterial population A1, A2, A3, P2, P4 stained blue and were thus identified as gram positive, while P1 and P3 stained pink indicating gram negative (Plate 7a and 7b).

4.3.2.3 Fluorescence microscopy

Wet mounts of the bacterial colonies were prepared and the slides were immediately mounted and observed under a fluorescent microscope in a dark room. Colonies of all aceticlastic methanogens viz. A1, A2 and A3 showed fluorescence (Plate 8).

4.3.2.4 Endospore staining

Endospore staining was done using Amido black and Carbol fuchin reagents. Colonies of aceticlastic methanogens namely A1, A2, A3 were non-spore forming. Proteolytic bacterial cultures P1, P2 and P3 also did not produce any endospores while P4 produced endospores.

4.3.2.5 Motility test

Motility of the bacteria was checked by hanging drop method. Bacterial colonies A1, A2, A3, and P1 were non-motile. Motile colonies included P2, P3 and P4.

4.3.2.6 Catalase test

Catalase test was done using H_2O_2 reagent. All the bacterial species were catalase negative.

4.3.2.7 G + C content

The molecular G + C content of cultures A1, A2, A3, P1, P2, P3 and P4 were found to be 31, 41, 39, 51, 61, 54 and 28 per cent respectively .

The result of the staining and culture characteristics has been summarized in Table 7.

Table 7. Characteristics of Methanogens studied

Bacterial name	Culture No.	Shape	Gram staining result	Fluoroscence microscopy	Endospore staining	Motility test	Catalase test	Mol G+C content (%)
Methanobacterium ruminatium	A1	Short oval rods	Positive	Fluorescent	Non spore forming	Non motile	Negative	31
Methanobacterium formicicum	A2	Long Rods	Positive	Fluorescent	Non spore forming	Non motile	Negative	41
Methanosarcina barkeri	A3	Cocci	Positive	Fluorescent	Non spore forming	Non motile	Negative	39
Bactereoides ruminicola	P1	Rods	Negative	Not Fluorescent	Non spore forming	Non motile	Negative	51
Eubacterium tortuosum	P2	Rods	Positive	Not Fluorescent	Non spore forming	Motile	Negative	61
Selenomonas ruminatium	P3	Curved rods	Negative	Not fluorescent	Non spore forming	Motile	Negative	54
Clostridium butyricum	P4	Rods	Positive	Not fluorescent	Spore forming	Motile	Negative	28

A = Aceticlastic methanogens; P = Proteolytic anaerobes

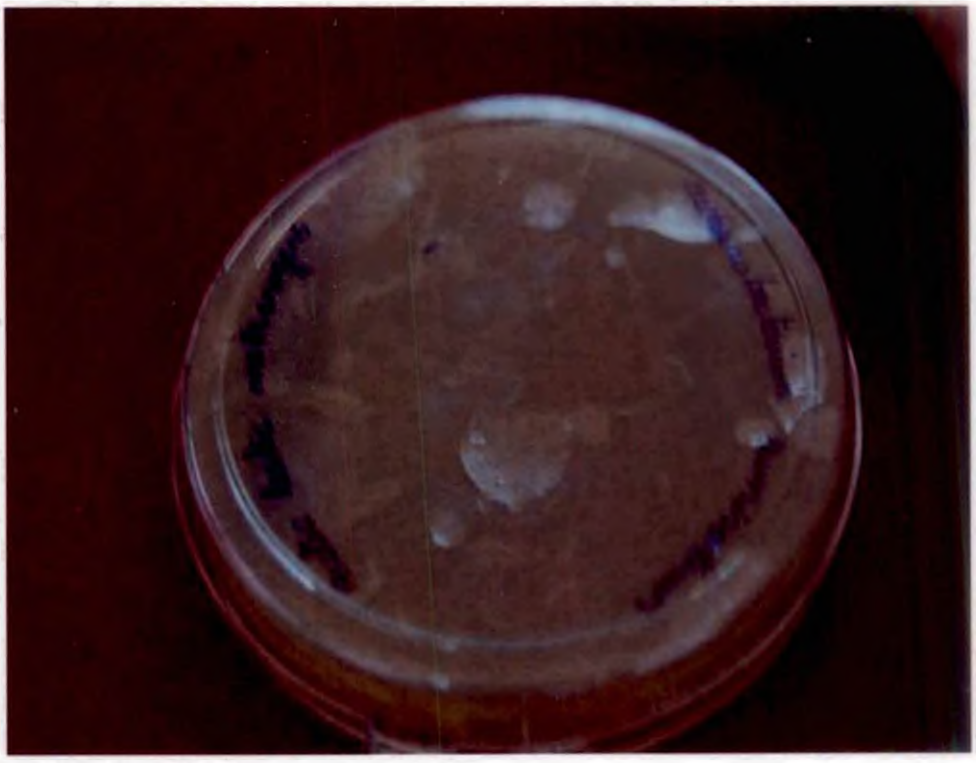


Plate 3. Gas vacuoles produced on the lower side of petri plate due to methane generation

Plate 4. Cracks produced in the agar plate due to methane emission

Plate 5. Bacterial colonies on the sides of test tube

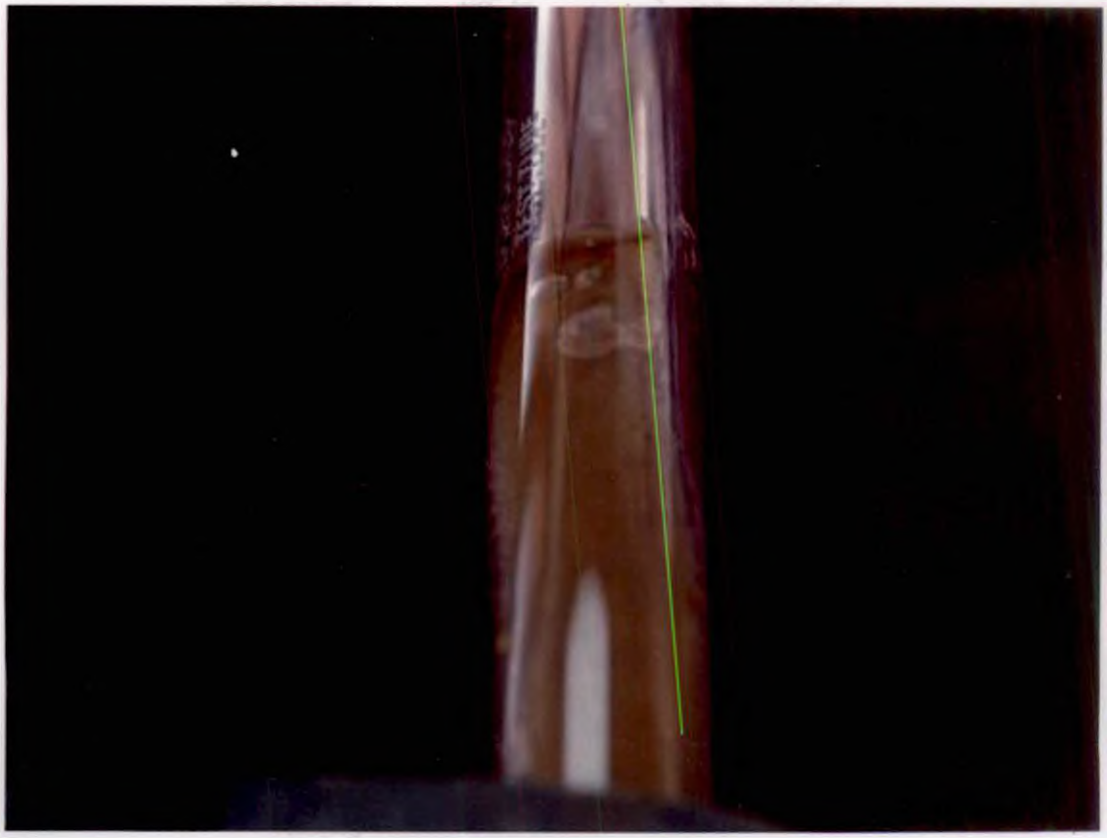
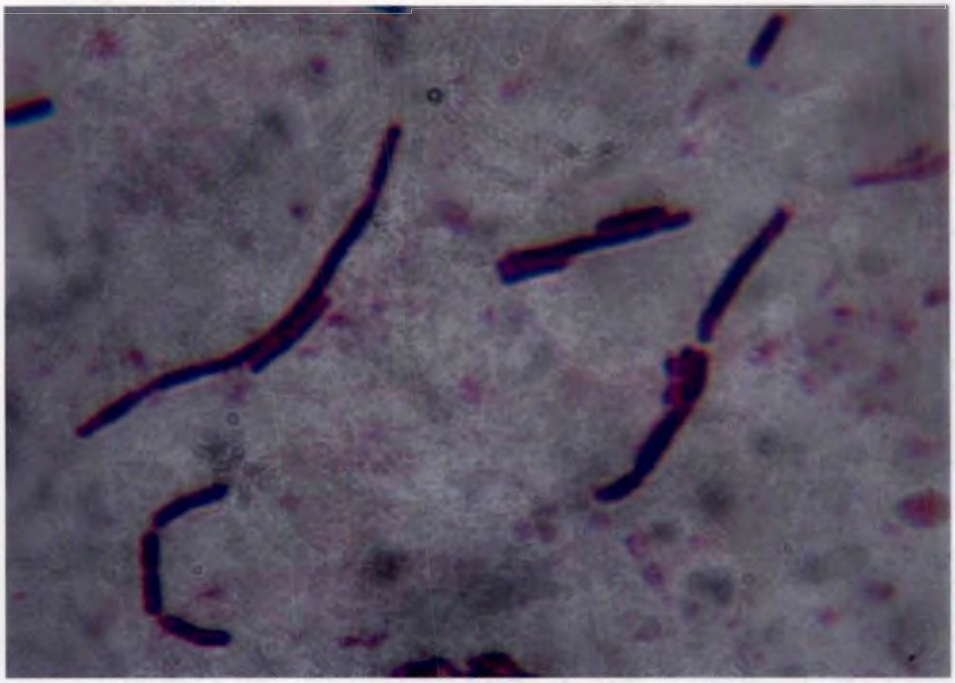
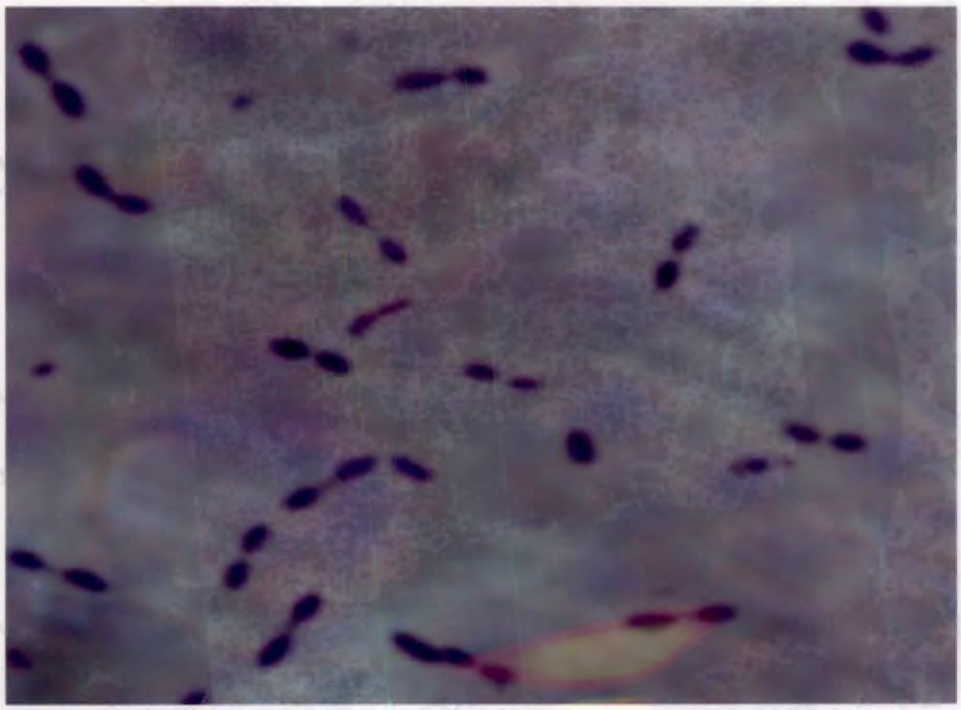


Plate 6. Cavities due to methane production on the sides of test tube.

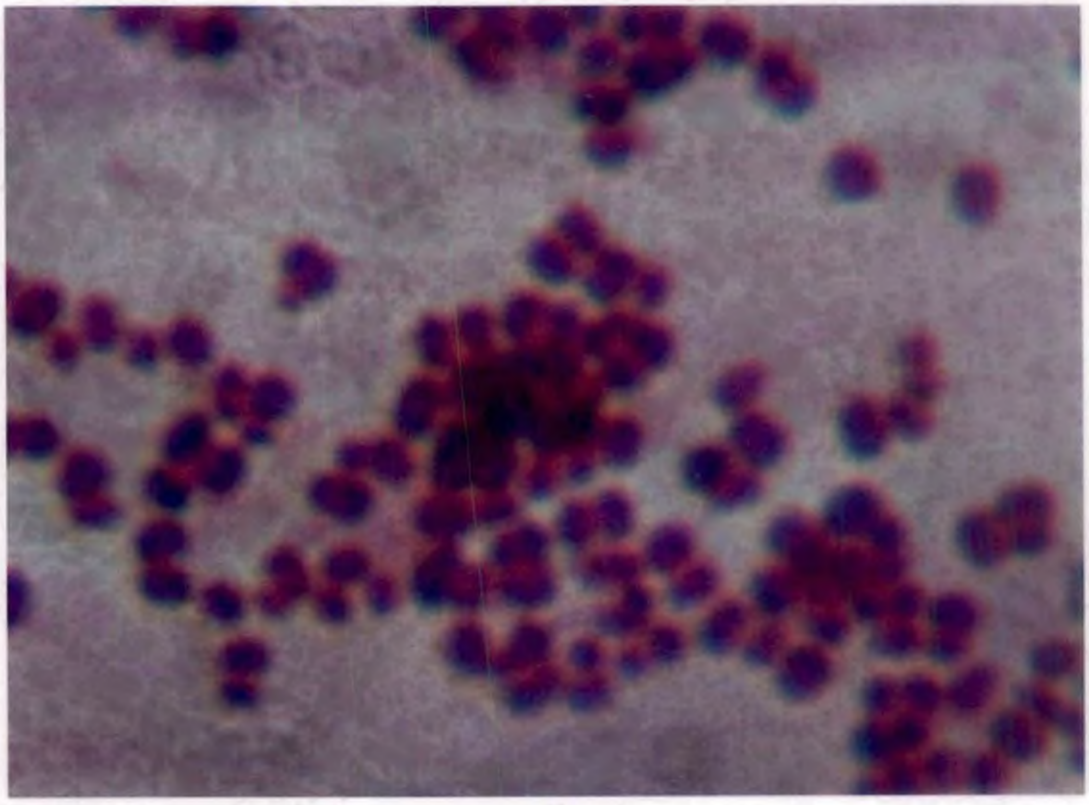


Methanobacterium formicicum

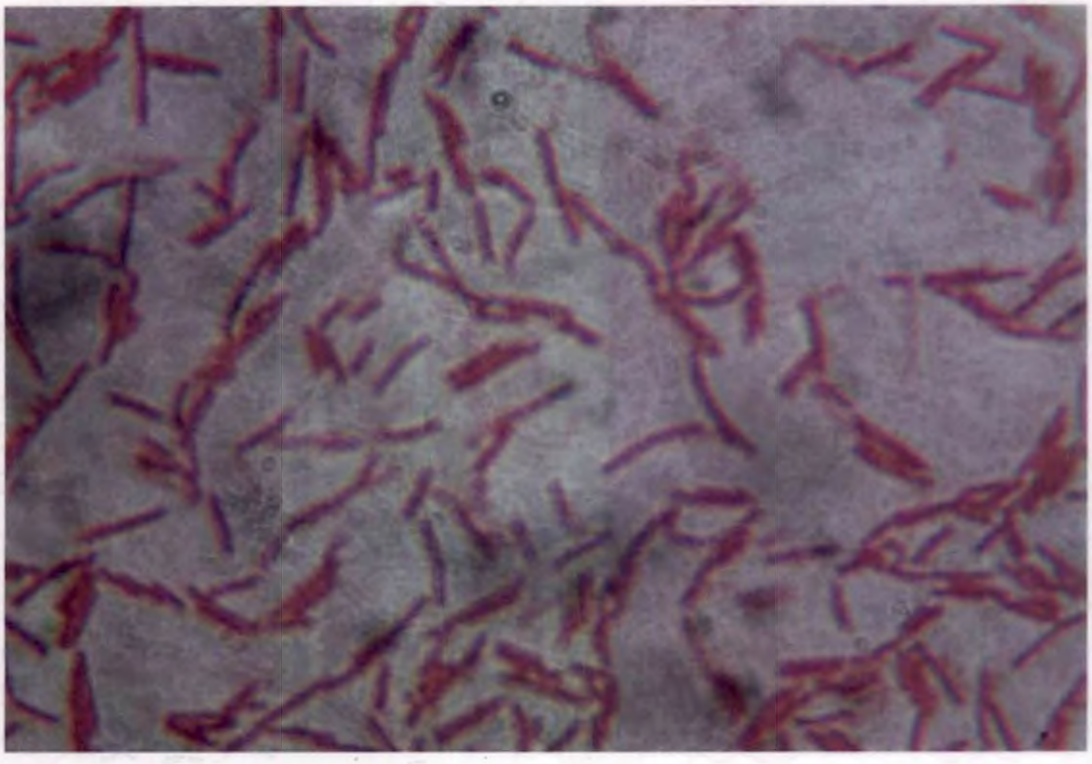


Methanobacterium ruminatium

Plate 7a. Gram staining of different methanogens

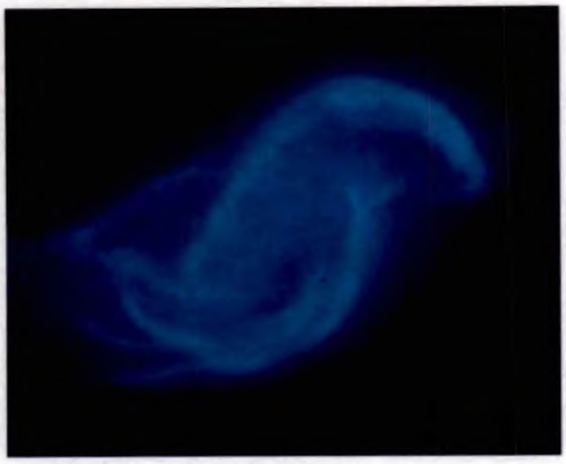


Methanosarcina barkeri



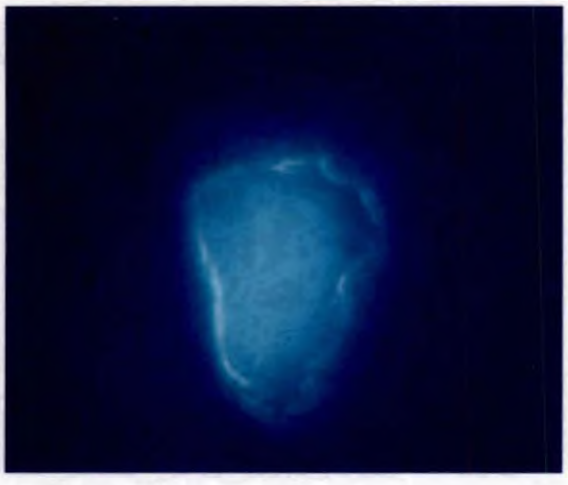
Bactereoides ruminicola

Plate 7b. Gram staining of different methanogens



Methanobacterium formicicum

Methanosarcina barkeri



Methanobacterium ruminatium

Plate 8. Aceticlastic methanogens viewed through fluorescent microscope

4.4 ENUMERATION OF METHANOGENIC COLONIES.

Enumeration of bacterial colonies in petri plates was done by plate count method. The maximum number of colony forming units (cfu) was found for *Selenomonas ruminatium* at 10^{-5} and 10^{-6} dilution. They produced small and isolated colonies. Maximum number of cfu were produced by *Eubacterium tortuosum* followed by *Methanobacterium formicicum* after *Selenomonas ruminatium* in both 10^{-5} and 10^{-6} dilution. The least number of cfu were produced by *Methanosarcina barkeri*. The colonies produced by *Methanobacterium ruminatium*, *Methanobacterium formicicum* and *Methanosarcina barkeri* were pinpointed. Comparatively large sized colonies were produced by *Selenomonas ruminatium, Bactereoides ruminicola* and *Eubacterium tortuosum*. The colonies of *Clostridium butyricum* were large and covered almost half of the petri plate, but the number of colonies produced was very few. The results are provided in Table 8.

Table 8. Enumeration of bacterial colonies by plate count method

Culture No.	Bacterial species	No. of cfu per ml of sample	
		Dilution 10^{-5}	Dilution 10^{-6}
A1	*Methanobacterium ruminatium*	7	5
A2	*Methanobacterium formicicum*	33	12
A3	*Methanosarcina barkeri*	3	1
P1	*Bactereoides ruminicola*	16	8
P2	*Eubacterium tortuosum*	34	23
P3	*Selenomonas ruminatium*	68	36
P4	*Clostridium butyricum*	6	2

4.5 MOLECULAR CHARACTERIZATION OF METHANOGENS

Molecular characterization of different species of methanogenic bacteria were done by RAPD technique.

4.5.1 Genomic DNA isolation from methanogens and gel separation

Genomic DNAs were isolated from all the seven selective methanogenic bacterial cultures using the protocol prescribed by Sambrook and Russel (2001). The isolated DNAs, when subjected to agarose gel electrophoresis followed by gel documentation, showed single sharp bands with varying intensities indicating its intactness (Plate 9). RNA contaminations were also noticed.

4.5.2 Quantification of DNA

Quantification was done for all the seven bacterial DNA samples in three replications using NanoDrop ND-1000 spectrophotometer. The quantity of DNA ranged from 79.16ng/µl to 964ng/µl. The data is presented in Table 9. The absorbance ratio of bacterial DNA (A_{260}/ A_{280}) ranged between 1.34 and 2.24. The best quality DNAs among the three replications, having A_{260}/A_{280} value between 1.8 and 2.0 were selected for molecular characterization by RAPD (Table 10). The graph showing the absorbance of DNA for various wavelengths has been presented in Fig.1. The absorbance peak at 260nm indicated DNA, while the peak at 220nm indicated RNA and protein contamination. The presence of peaks at 220nm and 260nm for all the samples, explicated that the DNA samples isolated were not devoid of contaminations. The highest peak for 4a, 4b and 4c in the graph is also in corroboration with the values of Table 9 which indicated that among all the isolates, *Methanobacterium formicicum* registered the highest quantity of DNA.

4.5.3 RAPD assay

4.5.3.1 Screening of random primers

Eight decamer primers were screened using two bacterial DNA with selected PCR reaction mixture and thermal settings. The primers screened were KitC-17, KitC-18, KitC-19, KitC-20, OPS-1, KitN-5, KitN-11 and KitN-19. Of

these 8 primers, all others, except 3 namely KitN-5, KitN-11, KitN-19 did not show any banding pattern (Plate 10). KitN-5, KitN-11 and Kit N-19 gave three to ten bands on screening. KitN-5 gave nine bands for the first DNA and two bands for the second DNA. Kit N-11 gave ten bands with the first DNA and a single band with the second DNA. Six and four bands were obtained when these two bacterial DNAs were screened with KitN-19. KitN-5 and KitN-11 gave good amplification than KitN-19 for the selected DNA.

4.5.3.2 Screening of methanogens by the selected RAPD primers

The genomic DNA extracted from the seven methanogenic bacteria were amplified using three primers, selected from initial screening. The marker used in RAPD screening was λDNA/ EcoRI/ Hind3 double digested. The details of amplification products obtained and the number of polymorphic and monomorphic bands are given in Table11 and 12. The bacterial cultures screened include, *Methanobacterium ruminatium, Methanobacterium formicicum, Methanosarcina barkeri, Bactereoides ruminicola, Eubacterium tortuosum, Selenomonas ruminatium and Clostridium butyricum*. Amplification pattern of genomic DNA of methanogens using decamer primers in KitN series is shown in Plate 11.

4.5.3.3 Cluster Analysis

Genetic similarity was computed from RAPD profiles as Jaccards coefficient using NTSYS PC.2.0 software (Table 13). A dendogram was also constructed using the UPGMA routine. Strains *Methanobacterium .formicicum* and *Methanobacterium ruminatium* showed maximum similarity with similarity coefficient of 0.8. The coefficient of similarity of *Methanobacterium ruminatium* and *Methanobacterium formicicum* with *Methanosarcina barkeri* was found to be 0.33 and 0.38 respectively. The least similarity between the strains was exhibited by *Eubacterium tortuosum* with a similarity coefficient of zero. The variability between the strains ranged from 8 to 80 per cent with maximum variability of 80 per cent. The dendrogram showing the pooled data of RAPD analysis is presented in fig 2.

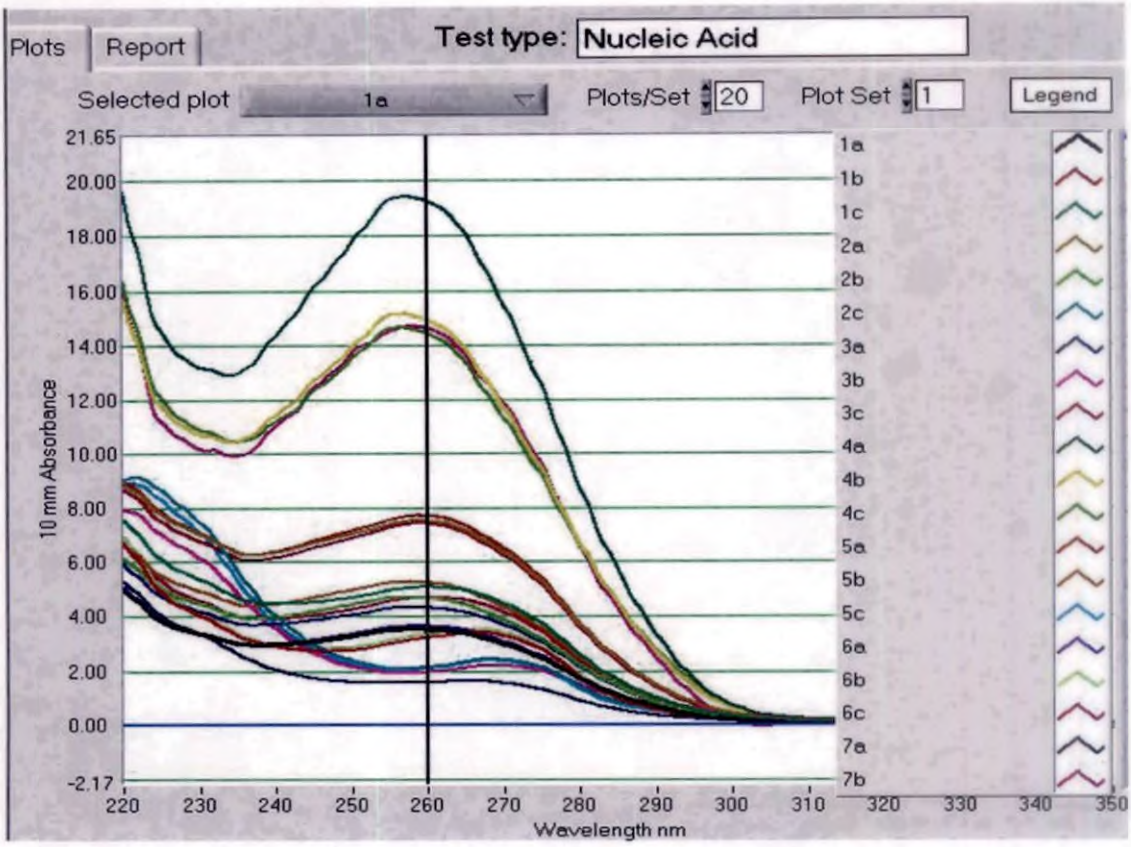


Fig1. DNA quantification by NanoDrop ND-1000

Table 9. DNA quantification in Methanogens

Sl. No.	Bacteria	Quantity of DNA(ng/µl)		
		A	B	C
1	*Bactereoides ruminicola*	176.33	161.10	253.66
2	*Selenomonas ruminatium*	375.92	233.01	106.80
3	*Methanobacterium ruminatium*	215.53	97.45	370.73
4	*Methanobacterium formicicum*	964.01	744.99	720.91
5	*Methanosarcina barkeri*	385.63	261.75	104.23
6	*Eubacterium tortuosum*	181.94	167.25	234.52
7	*Clostridium butyricum*	79.16	730.22	82.48

Table 10. Absorbance ratio (A_{260}/A_{280}) of different methanogenic DNA

Sl. No.	Bacteria	Replications	Absorbance		A_{260}/A_{280}	Remarks	Selected DNA
			A_{260}	A_{280}			
1	*Bactereoides ruminicola*	I	3.527	1.690	2.09	Average	II
		II	3.222	1.733	1.86	Good	
		III	5.073	2.547	1.99	Average	
2	*Selenomonas ruminatium*	I	6.518	3.648	1.87	Good	I
		II	4.660	2.172	2.15	Average	
		III	2.136	1.380	1.55	Poor	
3	*Methanobacterium ruminatium*	I	4.111	2.116	1.94	Good	I
		II	1.949	1.455	1.34	Poor	
		III	7.415	3.563	2.08	Average	
4	*Methanobacterium formicicum*	I	17.28	8.702	1.98	Average	III
		II	14.90	6.662	2.24	Average	
		III	12.42	6.587	1.88	Good	
5	*Methanosarcina barkeri*	I	6.713	3.699	1.81	Good	I
		II	4.535	2.455	1.84	Good	
		III	2.085	1.292	1.61	Poor	
6	*Eubacterium tortuosum*	I	3.639	1.706	2.13	Average	II
		II	3.345	1.790	1.87	Good	
		III	4.690	2.285	2.05	Average	
7	*Clostridium butyricum*	I	1.583	0.870	1.82	Good	II
		II	11.90	6.588	1.80	Good	
		III	1.690	0.904	1.83	Good	

Plate 9. Quality of genomic DNA isolated from methanogens

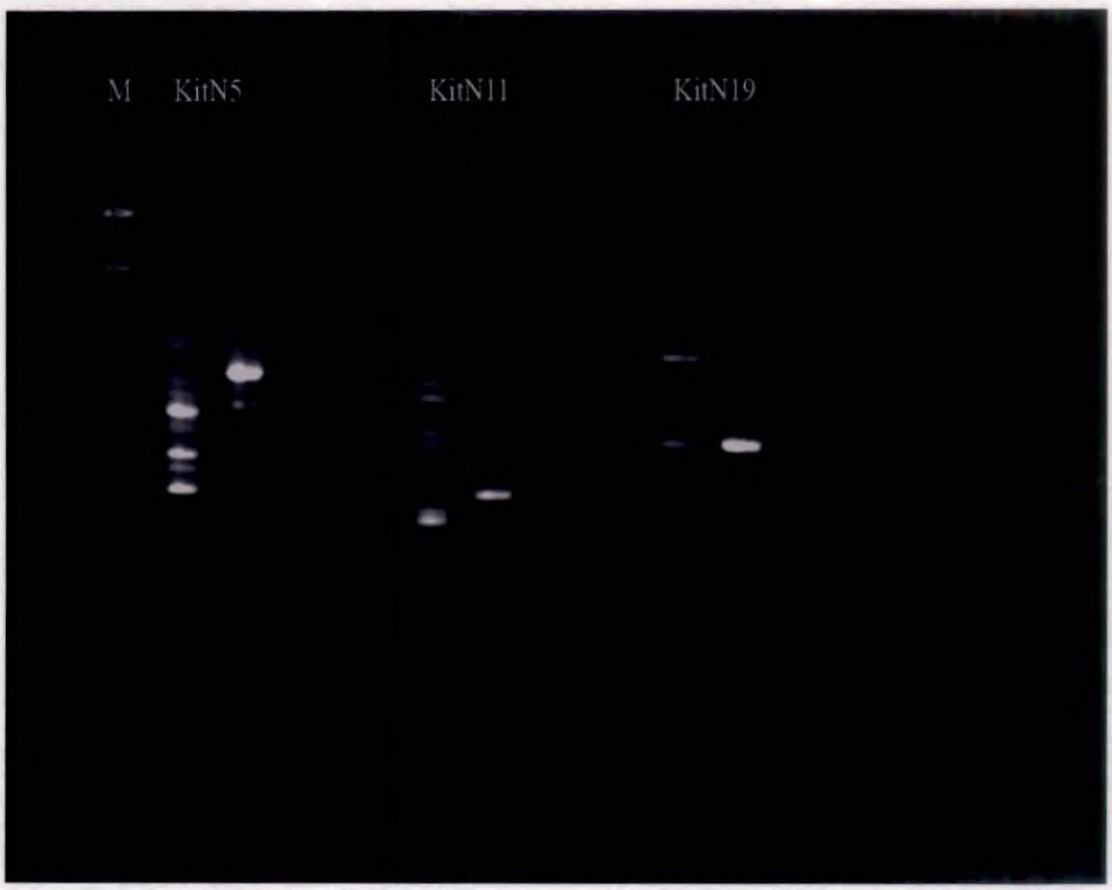
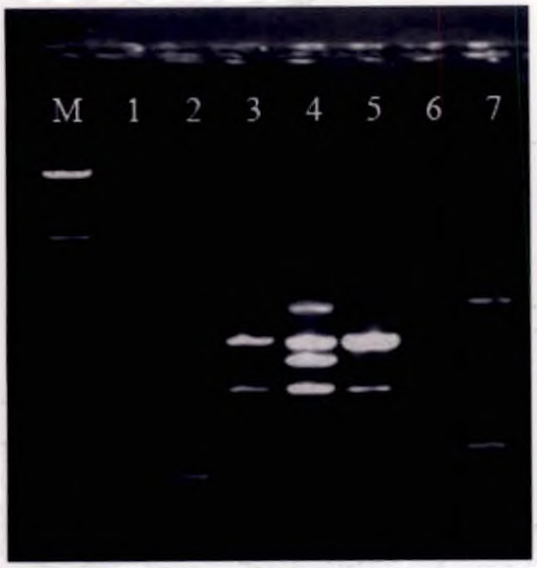


Plate 10. Primer screening for RAPD analysis



KitN5

KitN11

KItN19

M - marker

1-*Bactereoides ruminicola*

2-*Selenomonas ruminatium*

3-*Methanobacterium ruminatium*

4-*Methanobacterium formicicum*

5-*Methanosarcina barkeri*

6-*Eubacterium tortuosum*

7-*Clostridium butyricum*

Plate 11. Amplification pattern of genomic DNA of methanogens using decamer primers in OPN series

Table 11. Amplification pattern of methanogenic genomic DNA with different primers under OPN series

Primer code	Primer sequence	No. of amplicons						
		Bactereoides ruminicola	*Selenomonas ruminatium*	*Methanobacterium ruminatium*	*Methanobacterium formicicum*	*Methanosarcina barkeri*	*Eubacterium tortuosum*	*Clostridium butyricum*
KitN-5	ACTGAACGCC	1	6	8	8	6	0	9
KitN-11	TCGCCGCAAA	0	3	2	1	3	0	6
KitN-19	GTCCGTACTG	0	0	1	3	0	0	1

Table 12. No. of polymorphic and monomorphic bands obtained for each cultivar after RAPD using the selected primers

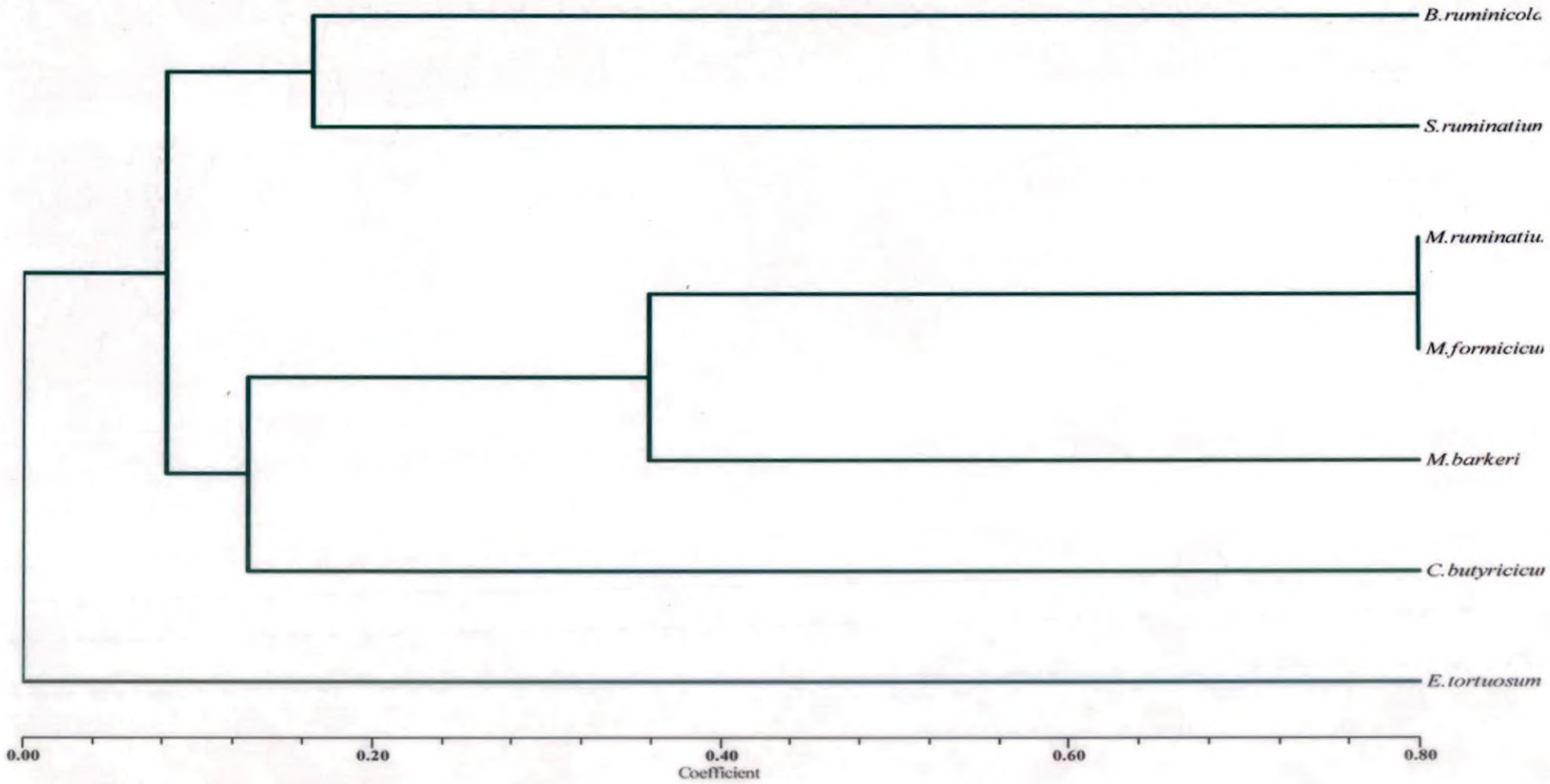
Primer / Culture	KitN-5		KitN-11		KitN-19	
	mb	pb	mb	pb	mb	pb
Bactereoides ruminicola	0	1	0	0	0	0
Selenomonas ruminatium	1	5	0	3	0	0
Methanobacterium ruminatium	2	6	1	1	1	0
Methanobacterium formicicum	3	5	1	0	1	2
Methanosarcina barkeri	3	3	0	3	0	0
Eubacterium tortuosum	0	0	0	0	0	0
Clostridium butyricum	3	6	1	5	0	1

Pb = polymorphic band; mb = monomorphic band

Table 13. Genetic similarity matrix of RAPD profiles

Rows/ columns	*Bactereoides ruminicola*	*Selenomonas ruminatium*	*Methanobacterium ruminatium*	*Methanobacterium formicicum*	*Methanosarcina barkeri*	*Eubacerium tortuosum*	*Clostridium butyricum*
Bactereoides ruminicola	1.0000000						
Selenomonas ruminatium	0.1666667	1.0000000					
Methanobacterium ruminatium	0.1250000	0.0769231	1.0000000				
Methanobacterium formicicum	0.1000000	0.6666667	0.8000000	1.0000000			
Methanosarcina barkeri	0.0000000	0.1666667	0.3333333	0.3846154	1.0000000		
Eubacterium tortuosum	0.0000000	0.0000000	0.0000000	0.0000000	0.0000000	1.0000000	
Clostridium butyricum	0.0000000	0.1250000	0.1111111	0.1000000	0.1764706	0.0000000	1.0000000

Fig. 2 Dendrogram obtained from the pooled data of RAPD profiles



4.6 DEVICE DESIGNED FOR BIO-FUEL EXPERIMENT.

4.6.1 Setting of biogas experimental unit

Experimental system for the generation and quantification of biogas was set in the Environmental Biotechnology Laboratory, Centre for Plant Biotechnology and Molecular Biology, College of Horticulture, Vellanikkara, using plastic drums, basins and inverted measuring cylinder with nozzle. All the operations in the system viz. incorporation and removal of tissue culture wastes, collection of evolved gas etc. were regulated by valves. The designed device for the experiment successfully evolved gas from all the twenty seven drum attachments. Plate 12 shows the conventional biogas experimental system while Plate 13 and Plate 14 presents the improved device designed at CPBMB, Vellanikkara.

4.7 QUANTITY OF GAS PRODUCED.

The quantity of gas produced for the treatment involving cow dung and the seven bacterial cultures on all the three replications were tabulated and the average production was calculated. The evolved gas was also checked for combustibility. Maximum quantity of gas was produced from treatment involving cow dung alone (710 ml). The next highest production was obtained from treatment containing cow dung slurry mixed with TC agar waste (570ml). Among bacterial treatments, highest quantity of gas was obtained from treatment involving *Clostridium butyricum* and TC waste (563.3ml). The least quantity of gas was produced from bacterial treatment involving *Bactereoides ruminicola* and TC waste. Bacterial cultures of *Eubacterium tortuosum Methanosarcina barkeri*, *Methanobacterium formicicum, Selenomonas ruminatium* and *Methanobacterium ruminatium* produced 466.6ml, 416.6ml, 400ml, 366.6ml and 353.3ml of gas respectively. The gas produced from the bacterial treatment with TC waste was non-combustible. Combustible gas was produced from treatments in which cow dung was used. The quantity of gas produced and combustibility of the gas for various treatments are shown in Table 14.

Plate 12. Conventional model of biogas experimental unit

Plate 13. Design improvement over the conventional model

Plate 14. The biogas experimental setup developed for the study in Environmental Biotechnology Laboratory

Table 14. Quantity of gas production with different treatments.

Sl. No.	Treatment	Date of loading	Date of cessation of gas production	Quantity of gas produced (ml)				Combustibility
				R1	R2	R3	Mean	
C1	Cow dung	18.12.06	05.01.07	700	650	780	710	Combustible
C2	Cow dung slurry + TC agar waste	08.01.07	25.01.07	630	580	500	570	Combustible
A1	*Methanobacterium ruminatium* + *TC waste*	15.09.07	02.10.07	550	260	250	353.3	Noncombustible
A2	*Methanobacterium formicicum*+ *TC waste*	05.10.07	23.10.07	550	250	400	400	Noncombustible
A3	*Methanosarcina barkeri* + TC waste	21.10.07	08.11.07	500	450	300	416.6	Noncombustible
P1	*Bactereoides ruminicola* + TC waste	09.11.07	27.11.07	250	200	400	283.3	Noncombustible
P2	*Eubacterium tortuosum* + TC waste	26.11.07	10.12.07	350	500	550	466.6	Noncombustible
P3	*Selenomonas ruminatium* + TC waste	4.01.2008	21.01.08	300	450	350	366.6	Noncombustible
P4	*Clostridium butyricum* + TC waste	21.01.2008	08.02.08	580	480	630	563.3	Noncombustible

4.8 PHYSIO CHEMICAL CHARACTERISTICS

4.8.1 C/N ratio of Tissue culture laboratory wastes.

Total carbon and nitrogen content in the tissue culture laboratory wastes from various TC laboratories was found out and the C/N ratio was calculated and summarized in Table 15. The moisture content in the tissue culture waste was also noted (Table 16). The C/N ratio was maximum (1.26) for the wastes collected from Floriculture TC laboratory under the Department of Pomology and Floriculture. The lowest C/N ratio was observed for the waste collected from ATIC-ABARD TC laboratory (0.17). The C/N ratio of TC waste samples taken from CPBMB and Campus Development TC laboratories were respectively, 0.44 and 0.19.

4.8.2 Physical conditions of the medium and the atmosphere.

The initial, mid and final pH of the bioconversion media was noted. The pH was highest during the initial period and got reduced towards the mid and final stages. Compared to other treatments, the initial pH was highest for the treatment using cow dung alone (7.31). The initial pH of treatment containing cow dung mixed with TC waste was the next highest (6.8). Initial pH was least for treatment using *Bactereoides ruminicola* and TC waste. The relative humidity of atmosphere on the date of loading of tissue culture waste (initial), during the mid period of gas production and on the cessation of gas production (final) was observed. Also the temperatures of bioconversion media during the initial, mid and final stages were noted. The temperature of the bioconversion media also got reduced towards the final stages. The physio-chemical characters observed in different treatments are summarized in Table 17.

4.8.3 Hydraulic retention time

The time for which the slurry should be held in the digester for getting eighty percent gas, Hydraulic retention time, was noted for the experiments involving all the seven bacterial cultures. Hydraulic retention time was least for *Clostridium butyricum* and maximum for *Bactereoides ruminicola.* The hydraulic retention time for various treatments is presented in Table 18.

Table 15. C/N ratio of tissue culture laboratory wastes

Sample	Carbon content (%)				Nitrogen content (%)				C/N ratio
	R1	R2	R3	Mean	R1	R2	R3	Mean	
CPBMB	3.1	3.08	3.03	3.07	6.89	7.03	6.93	6.95	0.44
Floriculture laboratory	3.46	3.51	3.44	3.47	2.7	2.76	2.76	2.74	1.26
Campus Development	1.17	1.15	1.25	1.19	6.3	5.9	6.1	6.1	0.19
ATIC- ABARD	0.8	1.1	1.4	1.1	6.48	6.3	6.54	6.44	0.17

Table 16. Moisture content in the tissue culture laboratory wastes

Sample	Moisture content in sample (%)			
	R1	R2	R3	Mean
CPBMB	97	97.5	96.8	97.1
Floriculture	95.45	94.9	95.4	95.25
Campus Development laboratory	97.8	97.3	97.4	97.5
ATIC-ABARD	97.6	97.9	98.5	98

Table 17. Effect of different treatments on physical conditions.

Sl. No.	Treatment	Date of loading	Date of cessation of gas production	Temperature (°C)			Atmospheric Relative Humidity (%)			pH		
				Initial	Mid	Final	Initial	Mid	Final	Initial	Mid	Final
C1	Cow dung	18.12.06	05.01.07	34	31	29	52.5	50.5	52.5	7.31	7.29	7.1
C2	Cow dung slurry + tissue culture agar waste	08.01.07	25.01.07	34	32	30	52.0	62.0	42.5	6.8	6.63	6.54
A1	*Methanobacterium ruminatium*	15.09.07	02.10.07	33	31	28	91.5	91.5	81.0	2.74	2.53	2.22
A2	*Methanobacterium formicicum*	05.10.07	23.10.07	33	32	29	79.5	79.5	79.5	2.78	2.53	2.24
A3	*Methanosarcina barkeri*	21.10.07	08.11.07	32	31	29	79.5	77.0	71.5	2.79	2.74	2.69
P1	*Bactereoides ruminicola*	09.11.07	27.11.07	33	32	28	71.0	48.5	57.0	2.61	2.53	2.42
P2	*Eubacterium tortuosum*	26.11.07	10.12.07	33	31	29	68.5	57.5	62.0	2.82	2.79	2.77
P3	*Selenomonas ruminatium*	04.01.08	21.01.08	32	31	28	57.0	61.0	47.0	2.76	2.65	2.44
P4	*Clostridium butyricum*	21.01.08	08.02.08	33	31	29	61.5	63.5	67.5	2.91	2.89	2.87

Table 18. Hydraulic retention time of the treatments

Treatment No.	Treatment	Hydraulic retention time (days)		
		R1	R2	R3
C1	Cow dung	14	12	13
C2	Cow dung slurry + tissue culture agar waste	14	12	12
A1	*Methanobacterium ruminatium*	15	16	15
A2	*Methanobacterium formicicum*	14	15	15
A3	*Methanosarcina barkeri*	13	14	13
P1	*Bactereoides ruminicola*	16	16	16
P2	*Eubacterium tortuosum*	11	11	10
P3	*Selenomonas ruminatium*	15	15	15
P4	*Clostridium butyricum*	10	9	9

Discussion

DISCUSSION

Methane fermentation of complex natural organic substrate is the end of a food chain process involving a wide variety of anaerobic bacteria. These methanogenic anaerobes are taxonomically placed in the family of methanobacteriaceae. They are identified primarily on the basis of cell morphology and the ability to produce CH_4 gas from the substrates. The present investigation was aimed to produce biogas from plant tissue culture agar wastes using seven different methanogenic bacteria. The methanogenic bacteria are classified into aceticlastic methanogens and proteolytic anaerobes. Proteolytic anaerobes P2 (*Eubacterium tortuosum*), P3 (*Selenomonas ruminatium*) and P4 (*Clostridium butyricum*) were procured from other institutions and the remaining 4 bacteria, including 3 aceticlastic methanogens A1(*Methanobacterium ruminatium*), A2 (*Methanobacterium formicicum*) and A3 (*Methanosarcina barkeri*) and a proteolytic anaerobe P1 (*Bactereoides ruminicola*) were isolated from rumen fluid, biogas biomass and paddy soils and were identified using morphological, physiochemical and molecular parameters. Morphological characteristics of selected methanogenic bacteria were matched with the findings of Prazmovski (1880), Schnellen (1947), Barker (1956), Smith and Hungate (1958), Mink and Dugan (1977) and Moore and Moore (1986) and hence confirmed their identity.

The total quantity of tissue culture wastes collected for a period of one month from various TC laboratories of Kerala Agricultural University campus, Vellanikkara was 314.5 kgs. These wastes otherwise went to the soil pollution pool that could be utilized for producing bio-energy. According to Tomar (1995) a biogas plant of $4m^3$ capacity with an input of 50 kgs organic wastes could be able to generate biogas for a 4-6 member family per day. He again opined that the biogas generated from such family unit could be burned for atleast one hour. At this rate the available TC wastes from the laboratories in the headquarters would be able to generate bioenergy for 6 hours. Chawla (1986) reported that the organic

end products, CO_2 and methane as well as inorganic end products, H_2S and N_2 were evolved as bye-products of biomass decomposition which leads to soil and air pollution. In case of composting the biomass, all these produced pollutants are directly released to the atmosphere. Anaerobic digestion in biogas plant will act as chemical reactor to clean the atmosphere and generated gas will be utilized as energy source. Moreover, the byproduct of biogas can be used as super compost or enriched manure which does not leave any adverse effect on soil. The TC biomass consists of non-protein nitrogenous compounds, inorganic salts, organic compounds etc and the presence of nitrogen and sulphur in proteins and other compounds are the sources of pollutants. Hence, the biomethanogenesis using the methanogenic bacteria may be a safe disposal process of TC biomass (Tomar 1995). The bioenergy generated from anaerobic digestion of TC wastes might be used as fuel for inoculation works under laminar hood.

Major aspects of the investigation for producing bio-energy from TC wastes were the following.

1. Procurement of methanogenic bacteria
2. Isolation and identification of methanogens
3. Molecular characterization of methanogenic bacteria
4. Modified device design for bio-fuel experiment.
5. Variability in physio-chemical characteristics of different methanogenic bacteria.
6. Variability in gas production by different methanogenic bacteria.

5.1 PROCUREMENT OF METHANOGENIC BACTERIA.

Among the seven methanogenic bacteria selected for the present investigation, the proteolytic anaerobes *Clostridium butyricum* (P4) were procured from IMTECH, Chandigarh and the bacterial cultures of *Selenomonas ruminatium* (P3) and *Eubacterium tortuosum* (P2) cultures from TNAU, Coimbatore. They were cultured by pour plate technique and produced sufficient quantities for replicated trials.

5.2 ISOLATION AND IDENTIFICATION OF METHANOGENS

5.2.1 Isolation of methanogens

Out of the seven methanogens used in the study, one proteolytic anaerobe, *Bactereoides ruminicola* (P1), and the three aceticlastic anaerobes, *Methanobacterium ruminatium* (A1), *Methanobacterium formicicum* (A2) and *Methanosarcina barkeri* (A3) were isolated from rumen fluid, sewage sludge from adjacent paddy fields and biomass of ATIC-ABARD biogas plant. Boopathy (1996), Pol & Demeyer (1988), Lin *et al.* (1997) and Lange *et al.* (2005) also had isolated these anaerobes from gastrointestinal tract of animals.

Both proteolytic and aceticlastic methanogens were isolated by combination of serial dilution with pour plate or roll tube technique as reported by Hungate (1969) and Sullia and Shantharam (2005). The isolated proteolytic methanogenic anaerobes P1 was stained pink indicating gram negative as confirmed by Gram (1884). All the three isolated aceticlastic methanogens were stained blue and thus identified as gram positive. These findings again confirmed the identification of Gram (1884), Mah and Smith (1981) and Sullia and Shantaram (2005).They were of the opinion that the gram positive staining methanogens possess pseudomurein, methanochondroitin and heteropolysacharide cell walls, while the gram negative staining methanogens have (glycol-) protein surface layers. All the three isolated aceticlastic anaerobes and one proteolytic anaerobe were non-spore forming methanogens. The results of endospore staining and motility test were in line with the reports of Smith and Hungate (1958), Macdonald *et al.* (1959), Smith (1961), Zwillenberg (1964) and Sullia and Shantaram (2005).

Isolation, identification and culturing of methanogenic bacteria were done based on technique described by Hungate (1950), Bryant and Robinson (1961), Blackburn and Hobson (1962), Holdeman and Moore (1972) and Mah (1980). Their methodologies provided the environment necessary to grow the extremely oxygen sensitive methanogenic bacteria.

Pour plate and roll tubes were employed for isolation of methanogens. Among the two methods, pour plate was observed for rapid growth, picking

isolated colonies and easiness in detection through dissecting microscope and ultraviolet light fluorescence. This offers a simple, rapid and sensitive method for isolation and enumeration. The fluorescence which was intense on pour plate method may be due to light scattering at the agar petri-plate interface and absorption of light by growth medium. This is in line with the report of Edwards and McBride (1975). All the isolated aceticlastic methanogenic bacteria exhibited autofluorescence in the oxidized state and were nonfluorescent in the reduced form.

Enumeration of bacterial colonies was done by plate count method. Though maximum number of colonies was produced by *Selenomonas ruminatium* followed by *Eubacterium tortuosum* the gas production efficiency of these bacteria registered lower values. It might be due to the production of small and isolated colonies by these methanogens. The bacterium *Clostridium butyricum* which produced large sized colonies was bestowed with maximum gas production efficiency. Bacterial colonies count and gas production was correlated in *Eubacterium tortuosum.*

5.3 MOLECULAR CHARACTERIZATION OF METHANOGENIC BACTERIA

Agarose gel electrophoresis and gel documentation of genomic DNA of seven selective methanogens explicated the intactness of DNA. Regarding the quantification of DNA, the DNA quantity of the selected bacterial cultures and also the absorbance ratio on A_{260}/ A_{280} were noted. From the various replications those with absorbance ratio between 1.8 and 2.0 were selected for RAPD analysis. The variations in absorbance ratio in some of the replications indicated protein or RNA contamination in the samples.

RAPD analysis was carried out for seven different bacteria using eight decamer primers (KitC17, KitC18, KitC19, KitC20, OPS1, KitN5, KitN11, and KitN19). Of which three primers KitN5, KitN11 and KitN19 showing DNA amplification were selected for RAPD assay. The variability in banding pattern was noticed for different species of selected methanogens indicating the clear cut

identifiable difference at species level. The constructed dendrogram computed from RAPD profiles have shown genetic variability between different species ranging from 8 to 80 per cent. *Methanobacterium ruminatium* and *Methanobacterium formicicum* have elucidated maximum similarity with similarity coefficient of 0.8 followed by *Methanosarcina barkeri* with similarity coefficient of 0.38. The least similarity coefficient was registered by *Eubacterium tortuosum.* The relationship between *Methanobacterium formicicum* and *Methanobacterium ruminatium* can be attributed to the fact that both of them belong to the same genus. The similarity of *Methanobacterium formicicum* and *Methanobacterium ruminatium* to *Methanosarcina barkeri* is due to their inclusion within the same taxa, methanogenic archaebacteria. These findings are in line with the reports of Konig and Stetter (1989).

The G + C content of the methanogenic bacteria were found out from the melting temperature (Tm) of the bacterial DNA by thermal denaturation technique prescribed by Marmur and Doty (1962). As DNA was heated, it reached a temperature where the strands separated. For measuring the Tm of the DNA, A_{260} at consecutive range of temperature was noted. The A_{260} increased dramatically when the double stranded DNA began to melt. A graph was drawn and from the mid point of the rapid increase in temperature Tm was noted. The per cent G+C content of the DNA was calculated from the melting temperature using the formula given by Marmur and Doty (1962). Among the selected bacteria, the per cent G+C content was maximum for *Eubacterium tortuosum*, followed by *Selenomonas ruminatium.* These research outcomes were in corroboration with the findings of Cummins and Johnson (1971) Kingsley and Hoeniger (1973), Matteuzzi *et al.* (1977), Welmer and Zeikus (1978) and Balch *et al.* (1979).

5.4 DESIGN OF BIOFUEL EXPERIMENTAL UNIT.

Biofuel experiment for the determination of efficiency in biogas production by various bacterial cultures was carried out in a designed setup. This design was constructed as an improvement over an earlier laboratory model described by Tomar (1995). This apparatus has similar features like the former

one with some added advantage. This is a very cheap and simple design when compared for the cost incurred in the setting of the previous model of Tomar (1995).

The modified biofuel experimental unit used airtight portable plastic drum of about 35.0 litre capacity instead of borosil aspirator bottles. It has two main openings, one for feeding tissue culture wastes and other on the bottom for slurry inspection and removal of residues, as described by Tomar (1995) for his experimental model. In addition, it also has a small outlet regulated by valve at the top for collecting the generated gas and to lead it towards the water displacement unit. Replacement of borosil aspirator bottles with air tight plastic drums helped in reducing the cost incurred in setting biogas experimental setup. This substitution also simplified the activities related to biogas experiments like feeding and removing the biomass. Since they are light in weight and having handles on either side, experienced easiness in waste disposal and transporting without tampering the device. These plastic drum biogas production devices are not vulnerable to the damage caused by sudden rise in pressure (breakage) as in aspirator bottle model reported by Tomar (1995).

The water displacement unit for quantifying the biogas generation was also improved over Tomar (1995) model. The inverted cylinders with nozzle and tap for quantification of biogas generated were erected or held on 2" PVC pipes mounted on the floor of plastic basins. The difference of the new unit from the previous models is that it does not require an additional stand for keeping the measuring cylinder in its vertical position. The nozzle present in the new setup helps for checking the combustibility of the generated gas which was not possible in the previous models. Thus the newly designed laboratory model explicited superiority over the previous model of Tomar (1995).

5.5 PHYSIOCHEMICAL CHARACTERESTICS

The physiochemical characters included for observation in the present study consisted of finding C/N ratio of tissue culture waste, physical condition of the medium and the atmosphere and hydraulic retention time of the treatments.

5.5.1 C/N ratio of the tissue culture lab wastes

All feed materials like cattle dung, kitchen waste and aquatic weeds contain carbon (C), nitrogen (N), Oxygen (O) etc. However, the major nutrients are C and N. As reported by Tomar (1995) for biogas production, the most favourable C/N ratio comes between 20 and 30.

The C/N values for the TC wastes are very low (0.17 to 1.26) and nowhere near the value required for safe biogas production (20 to 30) reported by Tomar (1995). This could be the possible reason for the non production of combustible gas from TC wastes. Tissue culture medium for foliages and other ornamental flowering plants contained lesser quantities of nutrients especially nitrogen. This might be reason for comparatively higher C/N ratio in TC wastes collected from Floriculture TC laboratory. Feeding TC wastes with some other supplements like coir pith, coconut water etc. having higher C/N ratio (Radhika *et al.*, 1983; Tomar, 1995; and Mathew *et al.*, 2000) in a biogas reactor, would ensure production of combustible gas.

5.5.2 Physical condition of medium and the atmosphere.

pH of the medium is the most important physical parameter which effects biogas generation. The hydrogen ion concentration is a measure of pH of an aqueous solution and it is a function of bicarbonate alkalinity of the system, the fraction of CO_2 in digester gas and the concentration of volatile acids. The alkalinity is a measure of buffering capacity of the contents and acts as a safeguard against pH fluctuations due to volatile acids (Chawla, 1986). A digester operates well in neutral solution of pH 7.0 or slightly alkaline condition (Tomar, 1995). Generally for quality biogas production, pH ranging between 6.8 and 7.2 is optimum. In the present investigation, the pH of the biomass was taken in the initial, mid and final stages. It was noticed that pH of medium got gradual reduction towards the final stages. In the case of biogas unit fed with cow dung, the pH was 7.3 and in the reactor containing cow dung mixed with TC waste, the pH was around 6.5. All other units had an initial pH below 3. These findings are in pace with the reports of Tomar (1995).

For methane generation three temperature ranges such as thermophilic zone (above 45 °C), a mesophilic zone (20-45 °C) and psychrophilic zone (below 20 °C) are reported by Chawla (1986) and Khandelwal (1986). However, effective and efficient anaerobic fermentation is carried out at both thermophilic and mesophilic temperatures. As per Hobson *et al.* in Chawla (1986) anaerobic digestion will take place at almost any temperature, essentially similar process goes on river mud and wet soil at near freezing point. The 30 to 35 °C range is reported to be optimum temperature and at this range the rate of volatile acid produced becomes more or less proportional to its utilization by methanogenic bacteria. During the present investigation, the temperature of the TC waste was noted during the initial, mid and final stages. Only a slight difference in the temperature has been noticed. The temperature in the biogas unit came near 30 °C which was very congenial for biogas production. But there was a gradual decrease of temperature from the initial to final stages.

5.6 QUANTITY OF GAS PRODUCED

Methanogenic bacteria under the Archaebacteria order are producing methane gas exclusively from the oxidation of methyl group of acetate (Zehnder *et al.*, 1980, Patel, 1984). In anaerobic methanogens, the CO_2 is the major electron acceptor and reduce the CO_2 by oxidizing H_2 to methane by splitting acetate to methane. The chief pathway of methane formation is the reduction of CO_2 by molecular hydrogen. The non-methanogenic bacterial species produces biogas from precursors like formate, pyruvate and serine (Zehnder *et al.*, 1980, Huser *et al.*, 1982, Fathepure, 1983 and Patel, 1984). The lack of H_2 in the TC agar substrate for CO_2 reduction and low C/N ratio might have hindered the methane production in the present investigation. These findings are corroborating the research results of Bryant *et al.* (1968), Patel (1984), Chawla (1986) and Tomar (1995). According to them, there is however, an absolute dependence of CH_4 formation upon H_2, indicating that an endogenous source of electrons is not available for reduction of CO_2. This is due to the lack of fermentation of organic materials at the acid forming stage of methanogenesis (Ramasamy *et al.*, 1992)

Quantity of gas produced from TC lab wastes on treatments using cow dung and seven bacterial cultures was read using water displacement unit of the biogas system. It was found that mean production of gas was highest for treatment using cow dung alone (710 ml). The second in gas generation was the treatment containing cow dung mixed with TC waste. In treatment containing bacteria mixed TC waste, maximum gas was generated by *Clostridium butyricum* (563.3ml) followed by *Eubacterium tortuosum* (466.6ml). Of all these treatments the least quantity of gas was produced by *Bactereoides ruminicola*. This could be explained on the notion that in the treatment involving cow dung alone, the quantity of cow dung fed comes to about half of the mixture. Moreover, the agglomeration of established bacterial colonies in the cow dung, as compared to the treatment (in which petri plates containing bacterial cultures are added) would be the possible reasons for low biogas production in all the treatments except the control. The bacterial cultures in the petri plates will be in the log phase and their quantity will be few compared to those in the cow dung.

Low C/N ratio of the agar substrate and the lack of H_2 for reducing CO_2 to methane might be the limiting factor for methane production from the treatments using different methanogenic bacteria. The biogas production efficiency or common metabolic capacity to produce methane would be improved by adding substrate with high C/N ratio like coir pith, other solid wastes along with coconut water (waste from copra drier unit) to agar waste disposed from TC laboratories (Radhika *et al.*, 1983; Mathew *et al.*, 2000).

Future line of work:-

1. Substrate with high C/N ratio like coir pith, coconut water etc. fortified with the TC biomass may be dispensed to increase the biogas generation.
2. Use of combinations of methanogenic bacterial consortium for increasing the efficiency of decomposition of TC biomass with the addition of other substrates

Summary

SUMMARY

The study on "Management of biodegradable plant tissue culture lab wastes through biomethanogenesis" was carried out at the Centre for Plant Biotechnology and Molecular Biology, College of Horticulture, Vellanikkara during the period 2005-2007. The objective of the study was to produce combustible bioenergy from tissue culture laboratory wastes through biomethanogenesis and to develop RAPD based molecular markers for identification of methanogens. The salient features of the study are summarized below.

1. The total quantity of tissue culture waste produced in Kerala Agricultural University campus, Vellanikkara for a period of thirty days was noted. Total quantity of tissue culture waste collected from the entire laboratories came up to 314.5 kilograms. The average weight procured from each laboratory per day counts to about 2.62 kilogram. The highest quantity of the tissue culture waste was produced from CPBMB laboratory.

2. Three bacterial cultures viz. *Clostridium butyricum*, *Selenomonas ruminatium* and *Eubacterium tortuosum* were procured from other institutions. Bacterial cultures of *Methanobacterium ruminatium*, *Methanobacterium formicicum*, *Methanosarcina barkeri* and *Bactereoides ruminicola* were isolated. The identity of the entire seven bacterial cultures was confirmed by morphological, cultural, molecular and staining characteristics.

3. Enumeration of bacterial colonies in petri plates was done by plate count method. Though maximum number of colonies was produced by *Selenomonas ruminatium* followed by *Eubacterium tortuosum,* the gas production efficiency of these bacteria registered lower values. It might be due to the production of small and isolated colonies by these methanogens. The least number of cfu per ml were produced by *Methanosarcina barkeri.* The bacterial colonies produced by *Methanobacterium ruminatium*,

Methanobacterium formicicum and *Methanosarcina barkeri* were pinpointed colonies since they are slow growers. The bacterium *Clostridium butyricum* which produced large sized colonies was bestowed with maximum gas production efficiency.

4. Genomic DNAs isolated from all the seven selective methanogenic bacterial cultures showed single sharp bands on agarose gel electrophoresis followed by gel documentation showing the intactness of DNA. Quantification was done for all the seven bacterial DNA samples in three replications using NanoDrop ND-1000 spectrophotometer. The quantity of DNA ranged from 79.16ng/µl to 964ng/µl. The best quality DNAs among the three replications, having A_{260}/A_{280} value between 1.8 and 2.0 were used for molecular characterization by RAPD analysis.

5. RAPD analysis was carried out for seven different bacteria using eight decamer primers (KitC17, KitC18, KitC19, KitC20, OPS1, KitN5, KitN11, and KitN19). Three primers KitN5, KitN11 and KitN19 showing DNA amplification were selected for RAPD assay. Based on the RAPD profile, scoring was done and genetic similarity matrix and dendrogram were generated using NTSYS pc 2.0 software. The variability between the strains ranged from 8 to 80 per cent. *Methanobacterium ruminatium* and *Methanobacterium formicicum* have elucidated maximum similarity with similarity coefficient of 0.8 followed by *Methanosarcina barkeri* with similarity coefficient of 0.38. The least similarity coefficient was registered by *Eubacterium tortuosum.*

6. Experimental system for the generation and quantification of biogas was set in the Environmental Biotechnology Laboratory, Centre for Plant Biotechnology and Molecular Biology, College of Horticulture, Vellanikkara, using plastic drums, basins and inverted measuring cylinder with nozzle. The designed

device for the experiment successfully evolved gas from all the twenty seven drum attachments.

7. The quantity of gas produced for the treatment involving cow dung and the seven bacterial cultures on all the three replications were tabulated and the average production was calculated. The evolved gas was also checked for combustibility. Mean production of gas was highest for treatment using cow dung alone (710 ml) due to agglomeration of established bacterial colonies in the cow dung. Only treatment involving cow dung was combustible. In treatment containing bacteria mixed TC waste, maximum gas was generated by *Clostridium butyricum* (563.3ml) followed by *Eubacterium tortuosum* (466.6ml). Of all these treatments the least quantity of gas was produced by *Bactereoides ruminicola.*

8. Physio chemical characteristics were studied for the various treatments involving TC waste, cow dung and methanogenic bacteria. C/N ratio was calculated for the TC waste procured from various laboratories in KAU campus. The C/N value for the TC waste is very low (0.17 to 1.26) and nowhere near the value required for safe biogas production (20 to 30) reported by Tomar (1995). This could be the possible reason for the non-production of combustible gas from TC wastes. The pH of the biomass was taken in the initial, mid and final stages. It was noticed that pH of medium got gradual reduction towards the final stages. The pH for the treatments involving bacterial cultures were very low when compared to the optimum pH required for safe biogas generation which is 6.5 which could be another reason for the production of non combustible gas. There was a gradual decrease of temperature from the initial to final stages in all the treatments.

9. Hydraulic retention time was noted for the experiments involving all the seven bacterial cultures. Hydraulic retention time was least for *Clostridium butyricum* and maximum for *Bactereoides ruminicola.*

10. Future line of work will be concentrated on fortification of TC biomass using substrate with high C/N ratio like coir pith, coconut water etc. that may be dispensed to increase the biogas generation. Addition of other substrates with methanogenic bacterial consortium will be worth-trying to increase the efficiency of decomposition of TC biomass.

References

REFERENCES

Abubacker, M.N. 2005. Biofuel Technology. In: National Seminar on Emerging Trends In Biotechnology; 18-19, February, 2005. Mahendra Arts and Science College, Namakkal Dt., Tamil Nadu; Department of biotechnology. 13p.

Amma, M.K. 1989. Plant and soil analysis. Rubber Research Institute, Rubber Board, Kottayam, 110p.

Balch, W.E., Fox, C.E., Magrum, L.J., Woese, C.R. and Wolfe, R.S. 1979. Methanogens: reevaluation of a unique biological group. *Microbiol. Rev.* 43: 260-296.

Barker, H.A. 1936. Studies upon the methane-producing bacteria. *Arch. Microbiol.* 7: 420-438.

Barker, H.A. 1956. Bacterial fermentations. John Wiley and Sons, New York. pp. 1–95.

Beijer, W.H. 1952. Methane fermentation in the rumen of cattle. *Nature.* 170: 420-438.

Belyaev, S.S. 1979. Microbiological methane formation in various ecosystems. In: Role of microorganisms in natural turn over of gases. Edition Nann, Moscow, pp.205-219.

Belyaev, S.S., Laurinavichus, U.S. and Gaitan, V.I. 1977. Contemperorary methane formation in quarternary and pliocene sediments of the Caspian Depression. *Geokhimya.* 8: 1251-1256.

Blackburn, T. H. and Hobson, P. N. 1962. Further studies on the isolation of proteolytic bacteria from the sheep rumen. *J. Gen. Microbiol.* 29:69-81.

Boone, D.R. and Mah, R.A. 1989. Genus I Methanobacterium Kluyver and van Niel 1936. 399. In: Staley and Holt (eds.) Bergey's Manual of Systemic Bacteriology, Volume 3, Williams and Wilkins.Co., Baltimore, 2175p.

Boopathy, R. 1996. Isolation and characterization of methanogenic bacterium from swine manure. *Bioresour. Technol.* 55: 231-235.

Brown, J.W., Daniels, C.J. and Reeve, J.N. 1989. Gene structure, organization and expression in archaebacteria. *CRC. Crit. Rev. Microbiol.* 16: 287-338.

Bryant, M. P. and Robinson, L.M. 1961. An improved nonselective culture medium for rumen bacteria and its use in determining diurnal variation in numbers of bacteria in the rumen. *J. Dairy Sci.* 44:1446-1456.

Bryant, M. P., Tzeng, S. F., Robinson, I. M. and Joyner, A.E. 1971. Nutrient requirements of methanogenic bacteria. In: Gould(ed.), Anaerobic Biological Treatment Processes. Advances in Chemistry, Series 105. American Chemical Society, Washington D.C., pp 23-40.

Bryant, M.P., McBride, B.C. and Wolfe, R.S. 1968. Hydrogen-oxidizing methane bacteria. I. Cultivation and methanogenesis. *J. Bacteriol.* 95: 1118-1123.

Certes, A. 1889. Note sur les micro-organismes de la panse des ruminants. *Bull. Soc. Zool. France.* 14: 70-73.

Chawla, O.P. 1986. *Advances in Biogas Technology.* Publication and Information Div., ICAR, New Delhi, 144p.

Cheeseman, P. A., Toms-Wood and Wolfe, R.S. 1972. Isolation and properties of a fluorescent compound, factor$_{420}$, from Methanobacterium strain M.o.H. *J. Bacteriol.* 112: 527-531.

Cummins, C. S. and Johnson, J. L. 1971. Taxonomy of the clostridia: wall composition and DNA homologies in *Clostridium butyricum* and other butyric acid producing clostridia. *J. Gen. Microbiol.* 67: 33-46.

Daniels, L. 1984. Biological methanogenesis: physiological and practical aspects. *Trends Biotechnol.* 2: 91-98.

Davy, H. 1808. Electro-chemical researches on the decomposition of earths; with observation on metal obtained from alkaline earths and on the amalgam produced from ammonia. *Phil. Trans. Roy. Soc.* 98: 333-370

Debono, M. 1912. On some anaerobical bacteria of the normal human intestine. *Zentrabl. Bakteriol. Parasitenkd. Infentionskr. Hyg. Abt.1 Orig.* 62:229-234.

Demeke, T., Adams, R.P. and Chibbar, R. 1992. Potential taxonomic use of random amplified polymorphic DNA (RAPD): a case study in Brassica. *Theor. Appl. Genet.* 84: 990-994.

DiMarco, A.A., Bobik, T.A. and Wolfe, R.S. 1990. Unusual coenzymes of methanogenesis. *Annu. Rev. Biochem.* 59: 355-394.

DNES.1990. *Annual Report 1989-90*, DNES, New Delhi, p.87

DNES.1991. *Annual Report 1990-91*, DNES, New Delhi, p.43

Doddema, H.J. and Vogels, G.D. 1978. Improved identification of methanogenic bacteria by fluorescence microscopy. *Appl. Environ. Microbiol.* 36: 752-754.

Doddema, H.J., Derksen, J.W.M. and Vogels, G.D. 1979. Fimbriae and flagella of methanogenic bacteria. *FEMS Microbiol. Lett.*5:135-138.

Edwards, T. and McBride, B.C. 1975. New method for the isolation and identification of methanogenic bacteria. *Appl. Microbiol.* 29: 540-545.

Eirich, L.D. 1978. The structure of coenzyme F_{420}, a novel electron. carrier isolated from *Methanobacterium* strain M.o.H., Ph.D. thesis, University of Illinois, Urbana.

Eirich, L.D., Vogels, G.D. and Wolfe, R.S. 1978. The structure of coenzyme F_{420}, a novel electron carrier isolated from Methanobacterium strain M.o.H. *Biochem.* 17: 4583-4593.

Eirich, L.D., Vogels, G.D. and Wolfe, R.S. 1979. Distribution of coenzyme F_{420} and properties of its hydrolytic fragments. *J. Bacteriol.* 140: 20-27.

Enhalt, D.H. 1974. The atmospheric cycle of methane. *Tellus.* 26: 58-70.

Fathepure, B. Z. 1983. Isolation and characterization of an aceticlastic methanogen from biogas digester. *FEMS Microbiol. Lett.* 19: 151-156.

Fukuzaki, S., Nishio, N. and Nagai, S. 1990. Kinetics of the methanogenic fermentation of acetate. *Appl. Environ. Microbiol.* 56: 3158-3163.

Garcia, J. L., Patel, B.K.C. and Ollivier, B. 2000. Taxonomic, phylogenetic and ecological diversity of methanogenic archaea. *Anaerobe* 6: 205-226.

Gram, C. 1884. The Differential Staining of Schizomycetes in Tissue Sections and in Dried Preparations. *Fortschitte der Medicin.* 2: 185-189.

Gujer, W. and Zehnder, A.J.B. 1983. Conversion process in anaerobic digestion. *Water Sci.Technol.*15: 49-77.

Gunsalus, R. P. and Wolfe R.S. 1978. Chromophoric factors F_{342} and F_{430} of Methanobacterium thermoautotrophicum. *FEMS Microbiol. Lett.* 3: 191-193.

Holdeman, L.V. and Moore, W. E. C. 1972. Roll-tube technique for anaerobic bacteria. *Am..J. Clin. Nutr.* 25:1314-1317.

Holt, J.G., Krieg, N. R., Sneath, P.H.A., Staley, J.T. and Williams, S.T. 1994. Gram-Negative, Anaerobic, straight curved and helical bacteria. In: Bergey's manual of determinative bacteriology, Ninth edition, Williams and Wilkins, Maryland, USA. 301p.

Huber, R., Kurr, M., Jannasch, H.W. and Stetter, K.O. 1989. A novel group of abyssal methanogenic archaebacteria (*Methanopyrus*) growing at 110°C. *Nature* (London) 342: 833-834.

Hungate, R. E. 1950. The anaerobic mesophilic cellulolytic bacteria. *Bacteriol. Rev.* 14: 1-49.

Hungate, R.E. 1969. A roll tube method for cultivation of strict anaerobes. In: Norris, J.R. and Ribbons, D.W. (eds.), Methods in Microbiology, Vol.3B. Academic Press, New York. pp. 117-132.

Huser, B.A., Wuhrmann, K. and Zehnder, A.J.B. 1982. *Methanothrix soehngenii* gen. nov. sp. nov., a new acetotrophic non-hydrogen oxidizing methane bacterium. *Arch. MIcrobiol.* 132: 1-9.

Hutten, T.J., Bongaerts, H.C.M., Drift, C. and Vogels, G.D. 1980. Acetate, methanol and carbon dioxide as substrates for growth of *Methanosarcina barkeri. Antonie Leeuwenhoek J. Microbiol.* 46: 601-610.

Iwabe, N., Kuma, K., Hasegawa, M., Osawa, S. and Miyata, T. 1989. Evolutionary Relationship of Archaebacteria, Eubacteria, and Eukaryotes inferred from phylogenetic trees of duplicated genes. Proceedings of the National Academy of Sciences of the United States of America 86: 9355-9359.

Jarvis, G. N., Strompl, C., Burgess, D. M., Skillman, L.C., Moore, E. R. and Joblin, K. N. 2000. Isolation and identification of ruminal methanogens from grazing cattle. *Curr. Microbiol.* 40: 327-332.

Jones, J.W., Leigh, J.A., Mayer, F., Woese, C.R. and Wolfe, R.S. 1983. *Methanococcus jannaschii* sp. nov., an extremely thermophilic methanogen from a submarine hydrothermal vent. *Arch. Microbiol.* 136: 254-261.

Jones, W.J., Nagle, D.P. and Whitman, W.B. 1987. Methanogens and the diversity of archaebacteria. *Micobiol. Rev.* 51: 135-177.

Jones, W.J., Stugard, C.E. and Jannasch, H.W. 1989. Comparison of thermophilic methanogens from submarine hydrothermal vents. *Arch. Microbiol.* 151: 314-318.

Kandler, O. and Hippe, H. 1977. Lack of peptidoglycan in the cell walls of Methanosarcina barkeri. *Arch. Microbiol.* 113: 57-60.

Kandler, O. and Koṇig, H. 1985. Cell envelopes of archaeabacteria. In: Woese and Wolfe (ed.), The Bacteria, Vol.VIII, Academic Press, New York, pp 413-457.

Kenealy, W.R. and Zeikus, J.G. 1982. One-carbon metabolism in methanogens: evidence for synthesis of a two-carbon cellular intermediate and unification of catabolism and anabolism in *Methanosarcina barkeri. J. Bacteriol.* 151: 932-941.

Khandelwal, K.C. 1986. *Biogas Technology*: A Practical Hand Book, Vol. I. Tata-McGraw Hill Publishing Company Ltd., New Delhi, 128p.

Kingsley, V.V. and Hoeniger, J.F.M. 1973. Growth, Structure and Classification of Selenomonas. *Bacteriol. Rev.* 37: 479-521.

Klein, A. and Schnorr, M. 1984. Genome complexity of methanogenic bacteria. *J. Bacteriol.* 158: 628-631.

Konig, H. and Stetter, K.O. 1989. Archaeobacteria. In: Staley and Holt (eds.) Bergey's Manual of Systemic Bacteriology, Volume 3, Williams and Wilkins Co., Baltimore, 2171p.

Konisky, J. 1989. Methanogens for biotechnology; application of genetics and molecular biology. *Trends Biotechnol.* 7: 88-92.

Krzycki, J.A., Wolkin, R.H. and Zeikus, J.G. 1982. Comparison of unitrophic and mixotrophic substrate metabolism by an acetate-adapted strain of *Methanosarcina barkeri. J. Bacteriol.* 149: 247-254.

Lanbroek, H. J., Verplanke, J. C., deVisscher, P. R. M. and deVuyst, R. 1985. Distribution of phyto- and bacterioplankton growth and biomass parameters, dissolved inorganic nutrients and free amino acids during a spring bloom in the Oosterchelde basin. The Netherlands. *Mar. Ecol. Prog. Ser.* 25: 1-11.

Lange M., Westermann, P. and Ahring, B.K. 2005. Archaea in protozoa and metazoan. *Appl. Microbiol. Biotechnol.* 66: 465-474.

Langenberg, K.F., Bryant, M.P. and Wolfe, R.S. 1968. Hydrogen-oxidizing methane bacteria. II. Electron microscopy. *J. Bacteriol.* 95:1124-1129.

Langworthy, T. A. 1985. Lipids of archaeabacteria. In: Woese and Wolfe (ed.), The Bacteria, Vol. VIII. Academic Press, New York, pp 459-498.

Langworthy, T.A., Tornabene, T.G. and Holzer, G. 1982. Lipids of archaebacteria. *Zentrabl. Bakteriol. Microbiol. Hyg.1 Abt. Orig.* C3: 228-244.

Leigh, J. A. and Wolfe, R.S. 1983. Carbon dioxide reduction factor and methanopterin, two coenzymes required for CO2 reduction to methane by extracts of Methanobacterium. *J. Biol. Chem.* 258: 7536-7540.

Lin, C., Raskin, L. and Stahl, D.A. 1997. Microbial community structure in gastrointestinal tracts of domestic animals: comparative analysis using rRNA-targeted oligonucleotide probes. FEMS. *Microbiol. Ecol.* 22:281-294.

Lunnen, K.D., Morgan, R.D., Timan, C.J., Krzycki, J.A., Reeve, J.N. and Wilson, G.G. 1989. Characterization and cloning of MwoI (GCN7GC), a new

type-II restriction-modification system from *Methanobacterium wolfei*. *Gene* (Amst.) 77: 11-19.

Macdonald, J. B., Madlener, E. M. and Socransky, S. S. 1959. Observations on *Spirillum sputigenum* and its relationship to Selenomonas species with special reference to flagellation. *J. Bact.* 77: 559.

Madigan, M.T. and Martinko, J.M. 2006. *Brock Biology of Microorganisms* (11th ed.), Prentice Hall, London. 564p.

Mah, R.A. and Smith, M.R. 1981. The methanogenic bacteria. In: Starr. M.P., Stolp, H., Truper, H.G., Balows, A, Schlezel, H.G. (eds.). The Prokaryotes, a Handbook on Habitats, Isolation and Identification of Bacteria. Vol.1. Springer-Verlag, Berlin. 76p.

Mah, R.A., Ward, D.M., Baresi, L. and Glass, T.L. 1977. Biogenesis of methane. *Annu. Rev. Microbiol.* 31: 309-341.

Mah, R.A.1980. Isolation and charachterization of Methanococcus mazei. *Curr. Microbiol.* 3:321-326.

Mannarelli, B. M., Ericsson, L.D., Lee, D. and Stack, R.J. 1991. Taxonomic relationship among the strains of anaerobic bacterium Bactereoides ruminicola determined by DNA and extracellular polysaccharide analysis. *Appl. Environ. Microbiol.* 57: 2975-2980.

Marmur, J. and Doty, P. 1962. Determination of base composition of deoxyribonucleic acid from its thermal denaturation temperature. *J. Mol. Biol.* 5: 109-118.

Mathew, A. C., Singh, T. V. and Bosco, S. J. D. 2000. Technology to produce biogas from coir pith. *Indian Cocon. J.* 31(3): 46-48.

Matteuzzi, D., Trovatelli, L. D., Biavati, B. and Zani, G. 1977. Clostridia from Grana Cheese. *J. Appl. Bacteriol.* 43: 375-382.

Miller T.L. and Wolin, M. J. 1986. Methanogens in human and animal intestinal tracts. *Syst. Appl. Microbiol.* 7: 223-229.

Miller, T.L. 2001. Genus III. *Methanosphaera* In: Boone, D.R., Castenholz, R.N. and Garrity, G.M. (ed.), *Bergeys's Manual of Systemic Bacteriology*, (2nd ed.), vol. 1. Springer, New York. pp.226-229.

Miller, T.L. and Lin, C. 2002. Description of *Methanobrevibacter gottschalkii* sp. nov., *Methanobrevibacter thaueri* sp. nov., *Methanobrevibacter woesei* sp. nov. and *Methanobrevibacter wolinii* sp. nov. *J. syst. Evol. Microbiol.* 52: 819-822.

Mink, R.W. and Dugan, P.R. 1977. Tentative identification of methanogenic bacteria by fluorescence microscopy. Appl. Environ. Microbiol. 33:713-717.

Moore, W. E. C. and Moore, L. V. H. 1986. Genus Eubacterium. In: Bergey's manual of systematic bacteriology. Volume 2. Williams & Wilkins Co., Baltimore, USA 1353p.

Opperman, R.A., Nelson, W.O. and Brown, R.E. 1957. *In vitro* studies on methanogenic rumen bacteria. *J. Dairy. Sci.*, 40: 799-788.

Patel, B. L. 1984. Prospects of utilizing biogas energy in rural India, *Agric. Engng. Tod.* 8:14-17.

Pol, A. and Demeyer, D.I. 1988. Fermentation of methanol in the sheep rumen. *Appl. Environ. Microbiol.* 54: 832-834.

Prazmowski, A. 1880. Untersuchung uber die Entwickelungsgeschichte und Ferment-wirking einiger Bacterien-Arten. Inaug. Diss. Hugo Voigt, Leipzig, pp.1-58.

Prowazekl, S.V. 1913. Zur Parasitologie von Westafrika. Zentrabl. Bakteriol. Parasitenk, Infektionskr. *Abt. I. Orig.* 70: 32-36.

Radhika, L. G., Sheshadri, S. K. and Mohandas, P. N. 1983. A study of biogas generation from coconut pith. *J. Chem.. Technol. Biotechnol.* 33: 189-194.

Ramasamy, K., Kalaichelvan, G. and Nagamani, B. 1992. Working with anaerobes: Methanogens-Laboratory manual, Department of Environmental Science, Tamil Nadu Agricultural University, Coimbatore. 122p.

Reiset, J. 1863. Recherches chimiques sur la respiration des animaux d'une ferme. *Compt. Rend. Acad. Sci.* 56: 740-747.

Rosanovo, E.P. and Kuznetsov, S.I. 1975. *Microflora of Oil Deposits*, Edition Nanna, Moscow. 196p.

Sambrook, J. and Russel, D.W. 2001. *Molecular Cloning*: A Laboratory Manual. Cold Spring Harbor Laboratory Press, New York, USA, 1541p.

Sambrook, J., Fritsch, E.F. and Maniatis, T. 1989. Molecular Cloning: A Laboratory Manual. Second edition. Cold Spring Harbor Laboratory Press, New York, USA, 1322p.

Sathianathan, M.A. 1975. *Biogas Achievement and Challenges*, Association of Voluntary Agencies for Rural Development, New Delhi, 192p.

Schmid, K., Thomm, M., Laminet, A., Laue, F.G., Kessler, C., Stetter, K.O. and Schmitt, R. 1984. Three new restriction endonucleases MaeI, MaeII and MaeIII from *Methanococcus aeolicus*. *Nucleic Acids Res.*12: 2619-2628.

Schnellen, C.G.T.P. 1947. Onderzoekinger over der methaangistung, Ph.D. thesis, Technical University of Delft, Delft, Netherlands, pp. 1-137.

Sitzmann, J. and Klein, A. 1991. Physical and genetic map of the Methanococcus voltae chromosome. *Mol. Microbiol.* 52: 505-513.

Smith, L.D.S. and Hobbs, G. 1974. Genus III Clostridium. In: Buchanan and Gibbons (eds.) Bergey's Manual of Determinative Bacteriology, 8th Edition, Williams and Wilkins Co., Baltimore. pp.551-572.

Smith, P. H. 1961. Studies on the methanogenic bacteria of domestic sludge. *Bact. Prod.* 60p.

Smith, P.H. and Hungate, R.E. 1958. Isolation and chrachterization of methanobacterium ruminatium. *J. Bacteriol.* 75: 713–718.

Smith, P.H. and Mah, R.A. 1966. Kinetics of acetate metabolism during sludge digestion. *Appl. Environ. Microbiol.*14: 368-371.

Smith, P.H. and Mah, R.A. 1980. Acetate as sole carbon and energy source for growth of *Methanosarcina barkeri* strain 227. *Appl. Environ. Microbiol.* 39: 993-999.

Stetter, K.O., Thomm, M., Winter, J., Wildgruber, G., Huber, H., Zillig, W., Covic, D., Konig, H., Palm, P. and Wunderl, S. 1981. *Methanothermus fervidus*, sp. nov., a novel extremely thermophilic methanogen isolated from an Ice-landic hot spring. *Zentrabl. Bakteriol. Mikrobiol.Hyg. 1Abt. Orig.* C2: 166-178.

Sullia, S.B. and Shantharam, S. 2005. General Microbiology. 2nd edition. Oxford & IBH Publishing Co. Pvt. Ltd., New Delhi. 443p.

Swarup, K. 1991. Energy conservation, Presidential address, All Ind. Seminar on Energy conservation, IE(I), Bhopal, June: 1-6

Tandan, M.M. N., Saxena, G.C. and Rathore, D.S. 1988. New Unified Chemistry, Shivlal Agrawal & Co., Indore. 568p.

Tandon, H.L.S. 1994. Methods of analysis of soils, plants, water and fertilizers. Fertilizer Development and Consultation Organization, New Delhi, 143p.

Taylor, C. D., Mc Bride, B.C., Wolfe, R.S. and Bryant, M. P. 1974. Coenzyme M, essential for growth of a rumen strain of Methanobacterium ruminatium. *J. Bacteriol.* 120: 974-975.

Thauer, R.K., Moller, D. and Spormann, A.M. 1989. Biochemistry of acetate catabolism in anaerobic chemotrophic bacteria. *Ann. Rev. Microbiol.*43: 43-67.

Thomm, M., Frey, B., Bolton, G.J., Laue, F., Kesler, C. and Stetter, K.O. 1988 . *Mvn*I: a restriction enzyme in the archaebacterium *Methanococcus vannielii. FEMS Microbiol. Lett.* 52: 229-234.

Tomar, S.S. 1984. *Agricultural Engineering Directory*, Orange book, Ind. Soc. of Agric. Engrs., New Delhi, 327p.

Tomar, S.S. 1985. Performance of IC Engines with Mixture of Fossil and Photosynthetic Fuels. MTech. thesis, Dept. of Applied Mechanics, MACT, Bhopal. 124p.

Tomar, S.S. 1991. Bio-energy and environment, Souvenir, 7th annual BESI Symposium, New Delhi, July:28.

Tomar, S.S. 1995. Energy Agriculture and Environment. Mittal Publications, Mohan Garden, New Delhi. 4p.

Tornabene, T. G. and Langworthy, T.A. 1979. Diphentyl and dibiphantyl glycerol ether lipids of methanogenic archaeabacteria. *Science* 203: 51-53.

Tornabene, T. G., Wolfe, R.S., Balch, W.E., Hulzer, G., Fox, G.E. and Oro, J. 1978. Phytanyl-glycerol ethers and squalene in the archaebacterium Methanobacterium thermoautotrophicum. *J. Mol. Evol.* 11: 259-266.

van Bruggen, J.J.A., Zwart, K.B. , van Assema, R.M., Stumm, C.K. and Vogels, G.D. 1984. *Methanobacterium formicicum*, an endosymbiont of the anaerobic ciliate *Metopus striatus. Arch. Microbiol.* 139: 1-7.

Vogels, G. D., Keltjens, J. T., Hutten, T. J. and Drift, C.V.D. 1982. Coenzyme of methanogenic bacteria. *Zentralbl. Bacteriol. Mikrobiol. Hyg. 1Abt.Orig.* C3: 258-264.

Welmer, P.J. and Zeikus, J.G. 1978. One carbon metabolism in methanogenic bacteria: cellular charachterization and growth of *Methanosarcina barkeri. Arch. Microbiol.* 119: 49-57.

Woese, C. R. and Fox, G.E. 1977. Phylogenetic structure of prokaryotic domain: the primary kingdoms. *Proc. Natl. Acad. Sci. USA*74: 5088-5090.

Woese, C.R., Kandler, O. and Wheelis, M.L. 1990. Towards a natural system organisms: proposal for the domains Archaea, Bacteria and Eucarya. *Proc. Natl. Acad. Sci. USA* 87: 4576-4579.

Woese, C.R., Magrum, L.J. and Fox, G.E. 1978. Archaebacteria. *J. Mol. Evol.* 11: 245-252.

Wolfe, R.S. 1971. Microbial formation of methane. In: Rose, A.H. and Wilkinson, J.F. (ed.), *Advances in Microbial Physiology*, Vol.6. Academic Press Inc., New York. pp. 107-146.

Wolfe, R.S. and Higgins, I.J. 1979. Biochemistry of methane-a study in contrasts. *In:* Quayl, J.R. (ed.), *Microbial Biochemistry*, MTP Press Ltd., Lancaster, England. pp. 267-283.

Zehnder, A.J.B. 1978. Ecology of methane formation, In: R. Mitchell (ed.), *Water Pollution Microbiology*, Vol. 2. John Wiley & Sons, Inc., New York. pp. 349-376.

Zehnder, A.J.B., Huser, B.A., Brock, T.D. and Wuhrmann, K. 1980. Characterization of an acetate-decarboxylating non-hydrogen-oxidising methane bacterium. *Arch. Microbiol.* 124: 1-11.

Zeikus, J.G. 1977. The biology of methanogenic bacteria. *Bacteriol. Rev.* 41: 514-541.

Zhao, H., Wood, A.G., Widdel, F. and Bryant, M.P. 1988. An extremely thermophilic *Methanococcus* from a deep sea hydrothermal vent and its plasmid. *Arch. Microbiol.* 150: 178-183.

Zinder, S.H., Cardwell, S.C., Anguish, T., Lee, M. and Koch, M. 1984. Methanogenesis in a thermophilic anaerobic digestor: *Methanothrix* sp. As an important aceticlastic methanogen. *Appl. Environ. Microbiol.* 47: 796-807.

Zwillenberg, H. H. L. 1964. Bau und Funktion der Forellenmiz. Academic Proefschrift, Verlag Hans Huber, Bern.

Annexures

Annexure I

Composition of dilution medium

Sodium bicarbonate	0.5%
Sodium carbonate	1.0%
Resazurin	0.0001%

Annexure II

Mineral (a) in gm/l of distilled water

Potassium dihydrogen phosphate	3.0g
Ammonium sulphate	6.0g
Sodium chloride	6.0g
Magnesium sulphate	0.6g
Calcium chloride	0.6g
pH	6.5

Annexure III

Filter sterilized vitamin and trace element solution

- Vitamin and trace element solution filtered through filter sterilization unit.
- Filter sterilization unit consist of filter sterilization apparatus with a membrane filter (Millipore GS 0.22μm) inside.
- Vitamin and trace element solution are pumped through this unit using a syringe kept at the nozzle.
- The sterilized solution is collected in a conical flask under the sterile condition of a laminar air flow

Annexure IV

Vitamin solution (Laanbroek *et al.*, 1985)

Biotin	: 10mg
Nicotinic acid	: 100mg
p-amino benzoic acid	: 50mg
Thiamine	: 100mg
Pantothenic acid	: 50mg
Pyridoxamine	: 250mg
Cobalamin	: 50mg

Annexure V

Trace element solution (Laanbroek *et al.*, 1985)

12.5N HCl	: 4.0 ml
$FeCl_3.4H_2O$	: 200 mg
$ZnCl_2$	: 70 mg
$MnCl_2.\ 4H_2O$	: 100 mg
$CoCl_2.6H_2O$	: 190 mg
$CuCl_2.2H_2O$	: 17 mg
$NiCl_2.\ 6H_2O$	: 24 mg
$Na_2MoO_4.2H_2O$	: 36 mg
$Na_2SeO_3.\ 5H_2O$	: 39 mg
$Na_2WoO_4.2H_2O$	: 49 mg

Annexure VI

Crystal violet reagent

Solution A

Crystal violet (90% dye content)	- 2.0 gm
Ethyl alcohol (95%)	- 20.0 ml

Solution B

Ammonium oxalate	- 0.8 gm
Distilled water	- 80.0 ml

Mix solution A and B in equal volume. Store for 24 h and filter before use.

Annexure VII

Gram's iodine mordant

Iodine	- 1.0 gm
Potassium iodide	- 2.0 gm
Distilled water	- 300 ml

Annexure VIII

Ethyl alcohol (95%)

Ethyl alcohol (100%)	- 95.0 ml
Distilled water	- 5.0 ml

Annexure IX

Safranine	- 0.25 ml
Ethyl alcohol	- 10 ml
Distilled water	- 100 ml

MANAGEMENT OF BIODEGRADABLE PLANT TISSUE CULTURE LAB WASTES THROUGH BIOMETHANOGENESIS

By

ABDULLA FAYAS T.

ABSTRACT OF THE THESIS

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ABSTRACT

Generating renewable source of energy from tissue culture laboratory waste by the process of biomethanogenesis is the focal theme of present investigation. Unlike developed countries, the developing countries are hesitant to establish more number of biotechnology/ tissue culture laboratories due to financial constraints. Easy and regular availability of biogas from TC wastes will be a boon to establish self-sustainable TC laboratory in view of present energy crisis.

The biogas experimental units required for the study was designed and various treatments were employed for the biodegradation of tissue culture waste, using the methanogenic bacteria *Methanobacterium ruminatium, Methanobacterium formicicum, Methanosarcina barkeri, Bactereoides ruminicola, Selenomonas ruminatium, Eubacterium tortuosum* and *Clostridium butyricum*. Treatment involving TC waste and cow dung was also conducted for biomethanation in the present study. Quantity of gas production and its combustibility was noticed for various treatments. In bacterial treatments the quantity of gas generation was highest for *Clostridium butyricum*. Only treatments involving cow dung produced combustible gas.

Molecular characterization of methanogenic bacterial cultures was also done for finding the genetic similarity between them. RAPD followed by scoring of the bands by UPGA routine showed maximum similarity between bacterial cultures of *Methanobacterium ruminatium* and *Methanobacterium formicicum* with *Methanosarcina barkeri*.

Physio-chemical characters like C/N ratio of the TC wastes, pH and temperature of medium and Hydraulic retention time was also observed for the various treatments. The C/N ratio of the TC wastes was found to be very low and nowhere near the optimum C/N ratio of 20-30 required for gas production. Other parameters like pH of the treatments and Hydraulic retention time was also

noticed. The pH of the treatments involving bacterial cultures was very low, considering the normal pH of 6.8 to 7.5 required in biogas generation.

The main constraints in the biogas generation were found out to be the low C/N ratio of the TC waste and the low pH of the medium. The present study indicated the possibility of bio-gas generation from TC waste through fortification using various supplements like coconut water and coir pith which have higher C/N ratio.