Role of *Metopus es* in the anaerobic degradation of organic matter and biomethanation process

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By

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To my Family

DECLARATION

I hereby declare that the work presented in this thesis is based on the original work done by me under the guidance of Dr. V. B. Manilal, Principal Scientist, Process Engineering and Environmental Technology Division, National Institute for Interdisciplinary Science and Technology, and that no part of this has been included in any other thesis submitted previously for the award of any degree.

Nimi Narayanan

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Certificate

This is to certify that the work presented in the thesis entitled "**Role of *Metopus es* in the anaerobic degradation of organic matter and biomethanation process**" submitted herewith by Mrs. Nimi Narayanan for the award of Doctor of Philosophy in Microbiology of the University of Kerala is an authentic scientific record of research work carried out under my guidance and supervision, at Process Engineering & Environmental Technology Division, National Institute for Interdisciplinary Science and Technology (CSIR), and no part of this work has been submitted for any degree or award.

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2. **Nimi Narayanan**, B. Krishnakumar, V. B. Manilal. Methane production in *Metopus es* culture by the endosymbiotic *Methanosaeta* sp. FEMS 2009- 3rd Congress of European microbiologists, June 28-July 2, 2009, Gothenberg, Sweden.

Abbreviations

ASCT	Acetate-succinate Co-A transferase
ATP	Adenosine triphosphate
AZM	Adoral zone of membranelles
BES	Bromoethane sulfonic acid
BSA	Bovine serum albumin
CCAP	Culture collection of algae and protozoa
CMC	Carboxy methyl cellulose
COD	Chemical oxygen demand
DAPI	4′, 6-diamidino-2-phenylindole
FISH	Fluorescent In Situ Hybridization
GDH	Glutamate dehydrogenase
LC_{50}	Median lethal concentration
LCFA	Long chain fatty acid
NADH	Nicotinamide adenine dinucleotide
NBT	Nitroblue tetrazolium
OHPA	Obligate hydrogen producing acetogen
PAGE	Polyacrylamide gel electrophoresis
PC	Prescott’s and Carrier solution
PFO	Pyruvate: ferredoxin oxidoreductase
PJ	Prescott’s and James’s solution
MP	Modified Pringsheim’s solution
PMS	Phenazine methosulphate
SEM	Scanning Electron Microscopy
SES	Soil extract with added salts
SOD	Superoxide dismutase
TLC	Thin Layer Chromatography
TOC	Total organic carbon
UASB	Upflow anaerobic sludge blanket reactor
VFA	Volatile fatty acids

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Abstract

Today's energy demanding lifestyle emphasize the need for exploring and exploiting alternate energy sources which are renewable as well as eco-friendly. Among the renewable energy sources, energy recovery from biomass by the process of biomethanation is perceived as a potential alternative. Biomethanation process not only provides renewable sources of energy but also utilises the recycling potential of degradable organic fraction in biomass. The biomethanation process consists of several independent, consecutive and parallel biochemical reactions, which occur through the mutualistic interaction between different groups of bacteria in the absence of oxygen. During the process, the complex organic compounds are converted into gaseous mixture, mainly methane and CO_2 by the combined and coordinated activity of consortia of bacterial populations.

Research on biomethanation is mainly focused on bacterial populations and various extra or intracellular bacterial processes are considered as primarily responsible for organic matter degradation and production of methane. The direct participation of anaerobic ciliates in biomethanation did not receive much attention, though their occurrence has reported in many biomethanation systems. The anaerobic ciliate, *Metopus es*, is the least studied species among the genus *Metopus* in terms of its functional role in anaerobic environment. The major aim of the present study was

to investigate the specific role of anaerobic ciliate, *Metopus es* in organic matter degradation and subsequent contribution to biomethanation process.

Chapter 1 deals with a brief introduction of biomethanation process and anaerobic protozoa, especially ciliates in the genus *Metopus*. Chapter 2 is divided into two parts. Part A describes the population dynamics of *Metopus* sp. in anaerobic reactor and its isolation. The development of monoculture of *Metopus* sp. in a bacteria controlled environment and optimization of cultural conditions are also discussed in this part. The species level identification of isolated ciliate as *Metopus es* is presented in Part B. Chapter 3 describes the tolerance level of isolated *Metopus es* to various important factors in biomethanation process. The occurrence of endosymbiotic methanogens in *Metopus es* discussed in chapter 4. Chapter 5 describes with the direct involvement of *Metopus es* in the degradation of organic matter. The hydrolytic enzyme profile of *Metopus es* and its participation in biomethanation process are discussed in this chapter. Chapter 6 includes summary and conclusions of the present study.

CHAPTER 1

Introduction

Over the last century, world's energy demand increased by over half, and a similar increase is expected in future also. The ever increasing demand for energy and rapid exhaustion of conventional energy sources like fossil fuels have necessitated the search for alternate energy sources. This situation demands the development of other innovative processes for exploiting the renewable energy sources to meet the day by day increasing energy requirements. Several attempts are being made to harness the renewable energy sources like solar, wind, tidal, hydropower and biomass (Ambulkar and Shekdar, 2004). Among them, energy recovery from biomass is achieved through the biological process called biomethanation. Biomethanation process has been widely used as an effective measure to treat organic materials in agricultural, municipal and industrial wastes with the subsequent recovery of energy (Kashyap et al., 2003). It is potentially regarded as one of the most attractive methods for solving the twin problems of energy crisis and pollution.

1.1 Biomethanation process

Biomethanation process involves the conversion of organic matter to methane under anaerobic conditions. The complex organic compounds are converted to a gaseous mixture mainly composed of methane and carbon dioxide through the

concerted action of a close-knit community of bacteria (Lyberatos and Skiadas, 1999). The biogas consists of mainly methane (55 - 65 %) and carbon dioxide (35 - 45 %) and trace amounts of hydrogen, nitrogen and hydrogen sulfide (Milono et al., 1981). Since essential intermediates for certain microorganisms are produced by others, a combined and coordinated metabolic activity of consortia of bacterial populations is required for complete degradation of organic matter to biogas. The biomethanation process consists of several independent, consecutive and parallel biochemical reactions, which occur in four major stages through the mutualistic interaction between different groups of bacteria (Denac et al., 1988) (Fig.1.1.).

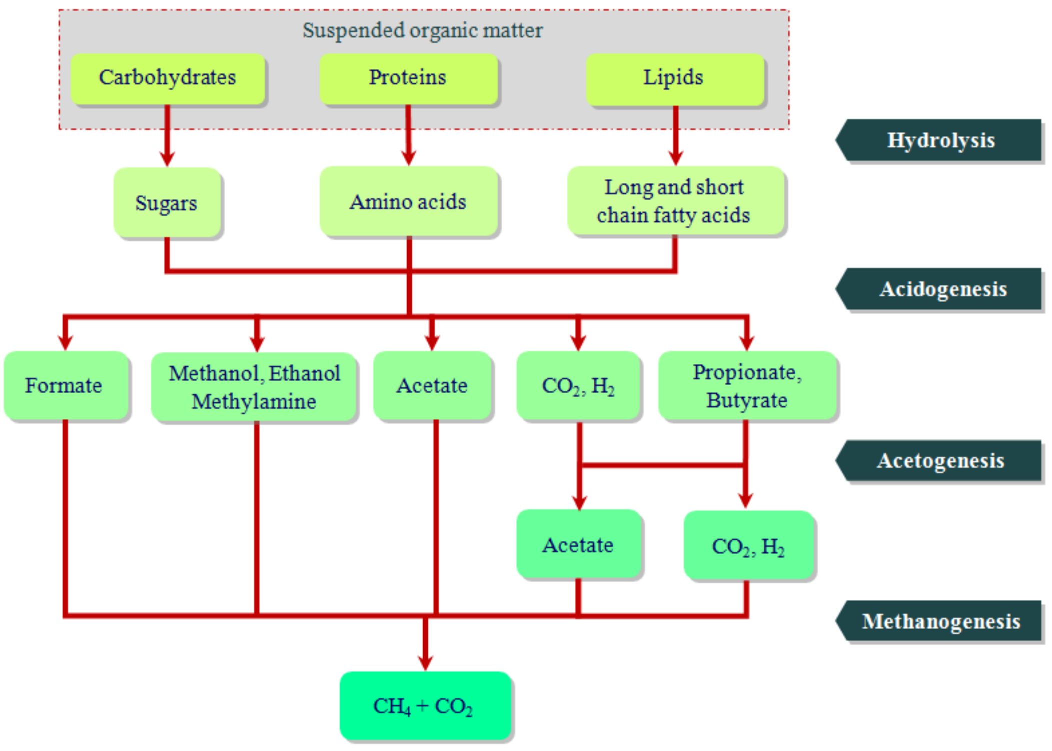


Fig. 1.1. The basic steps in biomethanation process

1.1.1 Hydrolysis

Hydrolysis is the first step in biomethanation process. It is the process of breakdown of complex organic matter to smaller products by hydrolytic bacteria (Morgenroth et al., 2002). In this process, complex macromolecules like proteins, polysaccharides and lipids are converted into simpler water soluble compounds such as amino acids, monosaccharides and fatty acids respectively. Hydrolysis proceeds by the adsorption of extracellular enzymes by fermentative bacteria on the particulate substrate and also by the direct attachment of enzyme producing bacteria on organic particles (Sanders et al., 2000). Hydrolysis is generally a slow process, which is dependent on the nature of particulate matter, thus act as a rate limiting step in biomethanation process (Li and Noike, 1987; Tomei et al., 2009). The rate of hydrolysis is affected by factors like substrate availability, bacterial population density, temperature and pH (Evans, 2001). The involvement of different groups of bacterial populations in the hydrolysis stage plays a significant role in determining the ultimate methane yield.

1.1.2 Acidogenesis

The acidogenesis phase is characterised by the conversion of hydrolysed products to simpler low molecular weight compounds like volatile fatty acids (VFAs), alcohols, aldehydes and gases like CO_2, H_2 and NH_3. The principal products of acidogenesis stage are propionic acid, butyric acid, acetic acid, formic acid, lactic acid, ethanol and methanol. The acidification is carried out by a diverse group of anaerobic

bacteria which metabolise organic matter to volatile fatty acids and bring pH down to 4.

1.1.3 Acetogenesis

During acetogenesis phase, obligate hydrogen producing acetogens break down both long chain fatty acids (LCFAs) and volatile fatty acids to produce acetate, carbon dioxide and hydrogen. Homoacetogens also produce acetate from hydrogen and carbon dioxide. These fatty acids act as electron donors in producing carbon dioxide during their degradation, as well as electron acceptors in transforming H^+ to hydrogen. Hydrogen concentration is significant in this process, since acetogenesis will only occur only at a low hydrogen partial pressure which is thermodynamically favourable for the conversion of all acids. Such lowering of the partial pressure is carried out by hydrogen scavenging bacteria (Mata-Alvarez, 2003).

1.1.4 Methanogenesis

Methanogenesis involves the production of methane from acetate, carbon dioxide and hydrogen, produced in the previous stages. This is carried out by obligate anaerobic methanogenic archaea, having slow growth rate compared to other bacteria involved in the preceding stages. Methanogenesis occur by two processes; one through hydrogenotrophic methanogenesis where methane is produced from hydrogen and carbon dioxide by hydrogen consuming methanogens found in syntrophic association with obligate hydrogen producing acetogenic bacteria. The other process by aceticlastic methanogens, which grow on acetate as substrate and produce methane

and carbon dioxide. The aceticlastic methanogenesis accounts for approximately 72 % of methane production while the remaining 28 % is produced from hydrogenotrophic methanogenesis (Garcia et al., 2000).

1.2 Biomethanation process for wastewater treatment

In earlier days, the biomethanation processe was considered to be sensitive and unstable for wastewater treatment due to the low growth rate of anaerobic bacteria, and it was presumed that high loading rates would not be applicable. But for the last few decades, biomethanation emerged as a well established method for elimination of easily biodegradable organic matter from wastewater, with the development of the high rate and efficient anaerobic reactors such as anaerobic filter reactor, fluidized bed reactor, baffled reactors, fixed film reactors and Upflow Anaerobic Sludge Blanket reactors (UASB) (Iza et al., 1991; Lettinga and Hulshoff Pol, 1992). This successful application of biomethanation process in wastewater treatment could mainly be attributed to the high retention of active biomass in these reactors by uncoupling the biomass retention time and the liquid retention time (Kato, 1994; Visser, 1995). These high rate reactors can accommodate very high loading rates as a result of high retention of dense and active biomass (Van lier, 1995).

Biomethanation process is now successfully used for the large scale treatment of high strength industrial organic wastewater that is not normally suited for aerobic

treatment processes (Kleerebezem and Macarie, 2003). The high-rate anaerobic systems have many advantages over aerobic biological treatment systems (Table 1.1). These treatment systems, which can handle high organic and hydraulic loading rates are simple in construction and operation and are thus cost effective. Moreover, the excess sludge production is low, and the sludge is well stabilized and easily dewatered, and hence does not require expensive post treatment. The most promising feature is the production of the high energy fuel, methane from the organic matter that can be used as an alternate energy source, whereas aerobic processes are energy consuming which require energy supply for aeration (Lettinga, 1995; Sekiguchi et al., 2001).

Table 1.1. Comparison of carbon and energy balances between aerobic and anaerobic degradation processes. (Modified Sahm, 1984)

	Aerobic	**Anaerobic**
Carbon balance	50 % CO_2 50 % biomass	5 % biomass 95 % biogas
Energy balance	40% heat 60% cell growth	5% heat 5 % cell growth 90 % biogas

1.3 Microbial communities in biomethanation process for wastewater treatment

Anaerobic treatment processes are complex microbial ecosystems comprising of several diverse microbial groups that work together in a coordinated manner to convert organic compounds to methane and carbon dioxide. The microbial consortia in biomethanation process result in complete breakdown of organic substrates through series of metabolic reactions, a process involving sequential flow of substrates and products from one microbial group to another (Anderson et al., 2003). The anaerobic reactors possess very high microbial load, with bacteria alone being present up to 10^{10} cells ml^{-1} of the digester content (Yu et al., 2010). It is regulated by the mutual metabolic interactions among three functional groups of microorganisms; hydrolytic, acidogenic and methanogenic groups, in the absence of oxygen (Ariesyady et al., 2007).

1.3.1 Hydrolytic bacteria

This group hydrolyses complex polymeric substances mainly carbohydrates, proteins and lipids to fundamental and structural building blocks such as glucose, amino acids and fatty acids by producing various extracellular hydrolytic enzymes like protease, lipase, cellulase, pectinase, amylase, chitinase etc. Anaerobic reactors contain 10^{8} - 10^{9} hydrolytic bacteria ml^{-1} comprising both facultative and obligate anaerobes (Toerein et al., 1967). The most predominant genera of hydrolytic bacteria that can degrade different types of complex organic substances include: carbohydrate

degraders like *Caldilinea, Anaerolinea*, *Levilinea*, *Leptolinea*, *Acetivibrio*, *Anaerocellum*, *Butyrivibrio*, *Caldicellulosiruptor*, *Clostridium*, *Eubacterium*, *Fervidobacterium*, *Halocella*, *Spirochaeta*, *Thermotoga*, *Fibrobacter*, *Ruminococcus* and *Flavobacterium* (Sekiguchi et al., 2003; Yamada et al., 2006; Cirne et al., 2007), protein degraders like *Bacteroides*, *Bifidobacterium*, *Butyrivibrio*, *Clostridium*, *Eubacterium*, *Lachnospira*, *Peptococcus*, *Selenomonas*, *Streptococcus*, *Thermobacteroides*, *Thermofilum*, *Thermococcus*, *Proteiniphilum*, *Proteiniborus*, *Ruminococcus* and *Eubacterium* (Rollón, 1999; Chen and Dong, 2005; Niu et al., 2008; Díaz et al., 2010) and lipid degraders like *Anaerovibrio*, *Butyrivibrio*, *Clostridium*, *Eubacterium*, *Micrococci* and *Borrelia* (Rollón, 1999).

1.3.2 Acidogenic bacteria

Acidogenic bacteria include a large and diverse group of fermentative bacteria, which metabolise the soluble products of hydrolysis; sugars, amino acids and fatty acids to short chain organic acids (acetic, propionic, formic, lactic, butyric, or succinic acids), alcohols and ketones (ethanol, methanol, glycerol, acetone), acetate, CO_2 and H_2. The cell counts of acidogens in anaerobic reactors vary from about 10^6 - 10^8 ml^{-1} (Archer and Kirsop, 1990). The acidogens predominate in the anaerobic reactors are from diverse genera: *Bacteroides*, *Clostridium*, *Ruminococcus*, *Bifidobacterium*, *Butyrivibrio*, *Propionibacterium*, *Eubacterium*, *Fusobacterium*, *Lactobacillus*, *Streptococcus*, *Peptostreptococcus*, *Pseudomonas*, *Micrococcus*, *Bacillus*, *Citrobacter*, *Enterobacter*, *Klebsiella* and *Esherichia* (Zeikus, 1980; Riedel and Britz, 1993; Britz et al., 1994; Riffat et al., 1999; Anderson et al., 2003). Among these, strict

anaerobes of the genera *Clostridium* and *Butyrivibrio* produce butyrate from a variety of sugars (Gerardi, 2003). The major genera of lactate producing bacteria include *Bifidobacterium*, *Lactobacillus*, *Leuconostoc*, *Pediococcus*, *Sporolactobacillus* and *Streptococcus* while bacteria in the genera *Bacteroides*, *Clostridium*, *Peptostreptococcus*, *Ruminococcus*, *Selenomonas*, *Succinivibrio* and *Veillonella* produce propionate and succinate (Gerardi, 2003).

1.3.3 Acetogenic bacteria

In anaerobic system, acetogenic bacteria function as the producers of acetate, hydrogen and carbon dioxide that can be further metabolized by methanogens. Based on their metabolism, two distinct groups of acetogenic bacteria exist; obligate hydrogen producing acetogens (OHPAs) and homoacetogens.

The obligate hydrogen producing acetogens convert propionate and other higher fatty acids (butyrate, valerate, isovalerate, stearate, palmitate and myristate) to acetate, carbon dioxide and hydrogen through ß- oxidation (Anderson et al., 2003). These include members from the genera *Syntrophomonas*, *Syntrophobacter*, *Smithella*, *Pelotomaculum* and *Syntrophococcus* (Bryant et al., 1967; Boone and Bryant, 1980; McInerney et al., 1981; Liu et al., 1999; Zhang et al., 2005; de Bok et al., 2005; Wu et al., 2006). Thermophilic acetogens in the genera, *Thermosyntropha* and *Pelotomaculum* were isolated from thermophilic anaerobic digesters (Svetlitshnyi et al., 1996; Imachi et al., 2002). They live in close proximity with hydrogenotrophic methanogens which rapidly consume the hydrogen produced by these bacteria, thus

ensuring a low partial pressure of hydrogen. Many of the reported species carrying out acetogenesis are incapable of independent growth when separated from their methanogenic partners (de Bok et al., 2005).

The second group of acetogenic bacteria is the homoacetogens that can utilise carbon dioxide and hydrogen to produce acetate as the end product. They are also capable of heterotrophic growth on a wide range of sugars and other organic compounds producing acetate (Ryan et al., 2004). The homoacetogens include both gram positive and gram negative bacterial genera such as *Moorella*, *Acetobacterium*, *Acetogenium*, *Acetoanaerobium*, *Butyribacterium*, *Clostridium*, *Pelobacter*, *Ruminococcus*, *Sporomusa* and *Eubacterium* (Slobodkin et al., 1997; Kotsyurbenko et al., 2001; Anderson et al., 2003; Drake et al., 2006). They are known to be present in the digester sludges within the range of 10^5 - 10^7 cfu ml^{-1} (Zhang and Noike, 1994).

1.3.4 Methanogens

The methanogens, grouped under the domain archaea, are strict anaerobes producing methane as the major end product of their metabolism. They are the key organisms involved in production of methane from the metabolic end products of other bacterial groups in anaerobic digestion. Methanogens are particularly sensitive to pH values above 7.5 and below pH 6.0 (Moosbrugger et al., 1993) and require a lower oxidation-reduction potential for growth (-300 mV) than most anaerobic bacteria (Forday and Greenfield, 1983). Methanogens have long generation time and thus long retention time is required in an anaerobic bioreactor to ensure the development of a

large methanogenic population to facilitate the degradation of organic compounds (Lettinga, 1995). In 1936, Barker initially classified methanogens into only one family, the *Methanobacteriaceae*, which consisted of four genera: *Methanobacterium, Methanosarcina, Methanococcus* and *Methanospirillum*. The recent taxonomic classification of methanogens based on morphological, physiological and nutritional studies as well as 16S rRNA sequence analysis is given in Table 1.2 (Boone et al., 1993; Garcia et al., 2000).

Table 1.2. Classification of methanogens (Garcia et al., 2000)

Order	**Family**	**Genus**
Methanobacteriales	Methanobacteriaceae	*Methanobacterium*
		Methanothermobacter
		Methanobrevibacter
		Methanosphaera
	Methanothermaceae	*Methanothermus*
Methanococcales	Methanococcaceae	*Methanococcus*
		Methanothermococcus
	Methanocalcococcaceae	*Methanocaldococcus*
		Methanoignis
Methanomicrobiales	Methanomicrobiaceae	*Methanomicrobium*
		Methanolacinia
		Methanogenium
		Methanoculleus
		Methanoplanus
		Methanocalculus
	Methanocorpusculaceae	*Methanocorpusculum*

	Methanospirillaceae	*Methanospirillum*
Methanosarcinales	Methanosarcinaceae	*Methanosarcina*
		Methanolobus
		Methanococcoide
		Methanosalsus
		Methanohalobium
	Methanosaetaceae	*Methanosaeta*
Methanopyrales	Methanopyraceae	*Methanopyrus*

Methanogens use a limited number of substrates that include acetate, H_2, CO_2, formate, methanol and methylamines. All of these substrates are reduced to methyl CoM (CH_3-S-CoM), which is then converted to methane by methyl CoM reductase (Ritchie et al., 1997). Based on their substrate specificity, methanogens are divided into two categories, hydrogenotrophic and acetotrophic methanogens. The hydogenotrophic methanogens convert hydrogen and carbon dioxide to methane and belong to the orders *Methanobacteriales*, *Methanomicrobiales* and *Methanococcales*.

$$CO_2 + 4H_4 \rightarrow CH_4 + 2H_2O$$

These types of methanogens have been detected in various anaerobic reactors from diverse genera; *Methanobacterium*, *Methanospirillum*, *Methanococcus* and *Methanosarcina* sp. (Zeikus, 1980). These organisms help to maintain the low partial pressure of hydrogen inside the anaerobic reactors as required by acetogenic bacteria and they are responsible for 28 % of total methane production in anaerobic reactors (Garcia et al., 2000).

The acetotrophic methanogens convert acetate to methane and carbon dioxide through aceticlastic methanogenesis.

$$CH_3COOH \rightarrow CH_4 + CO_2$$

Methanosaeta and *Methanosarcina* are the only two genera of aceticlastic methanogens described so far (Britton, 2005). *Methanosarcina* sp., such as *M. barkeri*, *M. mazeii* and *M. thermophila* can utilise other substrates like methanol, methylamines, hydrogen and CO_2 in addition to acetate (Smith and Mah, 1978); while *Methanosaeta* sp., such as *M. concilii* and *M. thermophila* can grow only on acetate (Boone et al., 1993; Anderson et al., 2003). *Methanosaeta* sp. possesses high affinity for acetate and has a lower growth rate at high acetate concentrations than *Methanosarcina* sp. *Methanosaeta* sp., therefore, dominate at low steady-state acetate concentrations whereas *Methanosarcina* sp. dominate when acetate concentrations are higher (Jetten et al., 1992; Zinder, 1993). This kind of aceticlastic methanogenesis accounts for 72 % of methane production in anaerobic reactors (Garcia et al., 2000).

Prokaryotic communities have received most of the focus, while little is known about anaerobic eukaryotes participating in biomethanation process. In addition to the prokaryotes, fungi and protozoa have also been observed among the microbial community in anaerobic digesters (Godon et al., 1997; Anderson et al., 2003). Anaerobic fungus, *Neocaliismastix* is a cellulase, xylanase and esterase producer, thereby capable of converting cellulose directly to methane on co-culturing with

methanogens (Nakashimada et al., 2000). Other fungi involved in the digestion of cellulose are close relatives of *Neocallismastix*, namely *Orpimomyces*, *Anaeromyces*, *Piromyces* and *Caecomyces* (Lynd et al., 2002).

1.4 Protozoa in biomethanation processes

Biomethanation is considered as the process principally driven by bacteria. The various extracellular or intracellular bacterial activities were considered responsible for enhanced degradation of complex compounds to methane. Most of the works were mainly focused on bacterial activities in anaerobic system based on the concept of retaining high viable biomass by bacterial sludge immobilisation (Hulshoff Pol and Lettinga, 1986). Anaerobic protozoa have been detected from different biomethanation systems such as marine sediments, sulphide rich lakes, rumen ecosystem, municipal landfills and wastewater treatment plants (Fenchel et al., 1977; Williams and Coleman, 1988; Finlay and Fenchel, 1991a; Xu et al., 2005). But the exact participation of these microbes in anaerobic organic matter degradation is not elucidated yet, except reports from rumen environment. In rumen ecosystems, anaerobic protozoa especially ciliates are directly involved in the degradation of organic matter through the production of a wide variety of hydrolytic enzymes (Gutierrez et al., 1962; Williams and Coleman, 1985; Lockwood et al., 1988; Gijzen et al., 1988; Marcin et al., 1998; Belzeckia et al., 2008). The ability of rumen protozoa to produce hydrolytic enzymes was successfully employed for the efficient degradation of different kinds of cellulosic and lignocellulosic wastes. Based on this, attempts have

been made to develop high rate anaerobic reactors with rumen protozoa (Gijzen et al., 1986; Gijzen et al., 1987; Gijzen et al., 1990). The occurrence of anaerobic protozoa, mainly flagellates, amoebae and ciliates was reported in UASB reactor treating wastewater from gelatin industry (Vieira et al., 2001). The granular sludge of UASB was also reported to contain protozoa like zooflagellates, rhizopods and ciliates (Navarrette et al., 1999). The anaerobic ciliates, *Metopus* and *Colpidium* have been reported from the UASB reactor treating low strength wastewater and anaerobic filter bed respectively (Agrawal et al., 1997; Xu et al., 2005). However, the significance of these organisms, especially ciliates in these environments has not received much attention.

1.5 Protozoa

Protozoa (In Greek *protos* means first and *zoon* means animal) are unicellular eukaryotic microorganisms belonging to the kingdom protista. As unicellular eukaryotes, protozoa cannot be easily defined, because they are diverse and often only distantly related to each other. Within its single cell, protozoa contain all structures required for performing its various functions for which vertebrates have many organ systems.

1.5.1 Habitats

Moisture is essential for the survival of protozoa and have adapted to all environments in which moisture and nutrients are present. Free living protozoa are

found in a wide variety of habitats like freshwater and marine environments, hot springs, organic matter containing soil and even in snowy and sandy habitats. Parasitic protozoa are adapted to different host species with more restricted physio-chemical requirements.

1.5.2 Morphology

Since protozoa are eukaryotes, they possess some similar features of multicellular animals. But at the same time, they have unique morphological features that enable them to carry out all life's function within an individual cell. They substantially vary in their size and shape. Their cells range in size from 1 - 300 μm usually, but some larger ciliates like *Stentor* and *Spirostomum* reaches up to 3 - 4 mm. Typically, they lack cell wall and bounded only by plasma membrane. But some are bounded by a flexible covering called pellicle and others encased in shells or skeletal structures. The cytoplasm under the plasma membrane can often been differentiated into an outer layer of semisolid, relatively homogenous ectoplasm and a more granular inner endoplasm. The ectoplasm contains organelles associated with locomotion, feeding, respiration and protection while the endoplasm houses the nucleus and various cellular organelles. The nucleus enclosed within the nuclear membrane is usually single, but some have two or more nuclei. In protozoa having two nuclei, the larger one is called the macronucleus and is associated with the metabolic and developmental activities of the cell, while smaller diploid micronucleus is involved in genetic recombination during reproduction. Normally the protozoa live in hypotonic environment possess contractile vacuoles that serve to maintain the osmotic balance

within the cell by continuous water expulsion. They also contain phagocytic and secretory vacuoles that function in food digestion and secretion of enzymes respectively. The other cellular organelles like mitochondria, endoplasmic reticulum and golgi bodies are present in the endoplasm. In anaerobic protozoa, mitochondria are replaced by another organelle called hydrogenosomes.

1.5.3 Nutrition

Most of the protozoa are heterotrophic which exhibit different modes of nutrition including holozoic, saprozoic, parasitic and mixotrophic types. Some of them like euglena are holophytic, synthesizing their food autotrophically and at the same time can perform saprozoic nutrition (mixotrophic). Their ability to obtain their food through different modes makes them suitable for diverse habitats where they exist as predators upon bacteria, algae and microfungi. Thus protozoa act as both herbivores and carnivores in the food chain. They also serve as a major food source for micro invertebrates, thus carrying an important role in the transfer of bacterial and algal production to successive trophic levels.

1.5.4 Formation of cyst

Most protozoa form a resting stage called cyst that enables prolonged survival under unfavourable conditions. The cysts are dormant forms with a hard external covering in which metabolic activity has ceased. The cyst formation is triggered by changes in the environment such as nutrient deficiency, drought and variations in oxygen concentration, pH or temperature. Cyst formation is an important survival

mechanism in many aquatic and parasitic protozoa. When favorable conditions returns, the protozoa emerge from the cyst and resume the metabolic activities.

1.5.5 Reproduction

Reproduction in protozoa occurs by both asexual and sexual means. Protozoa can reproduce asexually by binary fission, multiple fission and budding. The most common mode of asexual reproduction in protozoa is binary fission, a process in which the nucleus first undergoes mitotic division, followed by the division of cytoplasm, thus producing two essentially identical individuals. Some species reproduce by multiple fission, a form of cell division in which the parent nucleus divide into many nuclei followed by cytoplasmic division resulting in the formation of a number of identical individuals. In budding, a smaller individual called bud is formed from the parent cell after nuclear division. Budding can occur endogenously, in which the bud forms within the parent and is ejected when mature; or exogenously, in which the bud is formed on the surface of the parental cell. Some protozoa, especially ciliates can reproduce sexually through conjugation. During conjugation, individuals from opposite mating pair undergo temporary pairing and exchange of genetic material takes place between them. In parasitic protozoa, some species mainly undergo syngamy, in which fusion of two haploid gametes results in a diploid zygote that on meiosis produces haploid individuals.

1.5.6 Classification of protozoa

The zoological classification of protozoa is complex and subjected to frequent revisions. According to the Committee of Systematics and Evolution of the Society of

Protozoologists (Levine et al., 1980), the subkingdom Protozoa contains seven phyla in kingdom Protista (Table 1.3). This classification scheme is mainly based on the type of nuclei, mode of reproduction and mechanism of locomotion.

Table 1.3. Abbreviated classification of the subkingdom Protozoa (Prescott et al., 2002; Pelczar et al., 1986)

Taxonomic groups		Characteristics
Phylum I	**Sarcomastigophora**	• Locomotion by flagella or pseudopodia or both • Single type of nucleus • Sexual reproduction by syngamy
Suphylum	Mastigophora	• One or more flagella • Divide by longitudinal fission; sexual reproduction in some groups
Class	Phytomastigophora	• Plant like free living flagellates with chromatophores • Amoeboid forms in some groups
Class	Zoomastigophora	• Animal like with one to many flagella • Amoeboid forms with or without flagella • Chromatophores absent • Mainly parasitic
Suphylum	Opalinata	• Binary fission occurs between the rows of flagella which covers the entire body surface • Life cycle involves syngamy

		• All parasitic
Suphylum	Sarcodina	• Locomotion by pseudopodia • Shells (tests) often present • Flagella restricted to developmental stages when present • Asexual reproduction by fission
Superclass	Rhizopoda	• Locomotion by pseudopodia • Include naked and testate amoebae and foraminifera
Superclass	Actinopoda	• Planktonic and spherical • Pseudopodia delicate with axopodia • Reproduction is asexual and/or sexual
Phylum II	**Labyrinthomorpha**	• Spindle shaped cells capable of producing mucous tracks • Trophic stage as ectoplasmic networks • Nonamoeboid cells • Parasitic or saprozoic on algae and seagrass
Phylum III	**Apicomplexa**	• Contain an apical complex • Spores are present at some stage • Cilia are absent • Sexuality by syngamy • All are parasitic

Class	Sporozoea	• Locomotion of mature organisms by body flexing, gliding or undulation • Both sexual and asexual reproduction • Oocysts contain infective sporozoites, flagella present only in microgametes
Subclass	Gregarinia	• Tophozoites are large and extracellular • Parasites in invetebrates
Subclass	Coccidia	• Trophozoites are small and typically intracellular
Subclass	Piroplasmia	• Small, piriform, round, rod shaped, or amoeboid parasites of vertebrate blood cells with ticks as vector
Phylum IV	**Microspora**	• Unicellular spores with sporoplasm containing polar filaments • Obligatory intracellular parasites
Phylum V	**Acetospora**	• Spores with one or more sporoplasm without polar filament • All parasitic
Phylum VI	**Myxozoa**	• Spores are multicellular • One or more polar capsules • All parasitic, especially in fish
Phylum VII	**Ciliophora**	• Simple cilia or compound ciliary organelles in at least one stage of life cycle • Two types of nuclei • Contractile vacuole present

		• Binary fission transverse • Sexuality involves conjugation • Most are free living, with some commensals and parasites
Class	Kinetofragminophorea	• Oral infraciliature is only slightly different from somatic infraciliature • Cyclostome often apical or midventral • Compound ciliature absent
Class	Oligohymenophorea	• Oral infraciliature is clearly distinct from somatic infraciliature • Cyclostome ventral and/or midventral • Colony formation in some groups.
Class	Polymenophorea	• Well developed adoral zone of membranelles • Somatic ciliature complete or reduced or appeared as cirri • Cyclostome at the bottom of the buccal cavity • Large free living forms

However, a major drawback of this classification is that, only few molecular data of protozoa were available when this classification was made. Over the past three decades, molecular data have been increasingly used to infer phylogenetic relationships among various protozoa. The results of molecular phylogenetic studies supported the monophyly of some taxa reported by Levin et al., (1980), but several

other taxa in this classification was found to be paraphyletic or even polyphyletic. A revised classification of the protozoa at higher taxonomic levels is currently being deliberated by another committee of the Society of Protozoologists considering the phylogenic characteristics.

1.6 Broad functional groups of free living protozoa

According to Finlay and Esteban (1998), the major functional role of protozoa in ecosystems, as grazers, is closely related to their morphology, especially the food capturing organelles. Though they are grazing upon the same bacteria, they may differ in the mechanism of food capture. The free living protozoa can be divided into three broad functional groups; ciliates, heterotrophic flagellates and sarcodines, based on their mechanisms of food capture (using cilia, flagella and pseudopodia respectively).

1.6.1 Ciliates

The ciliates, which are the most important grazers in sediments, feed on bacteria, unicellular algae and other protozoa (Fenchel, 1987). There are three subgroups of ciliates based on their feeding mechanism.

a) Filter feeders: In filter feeding ciliates, food particles and small prey organisms of different size and nature are driven into the oral funnel and towards the cyclostome by ciliary action that acts like a filter. There are two different modes of filtration, namely, upstream and downstream. In upstream mode, the membranelles not only generate

water current but also function as filter. The membranelles pump water out of the oral cavity, while the particles too large to pass between the neighboring membranelles are retained. eg: *Spirotrichea* and *Colpodea*. In downstream filtration, the water currents generated by the membranelles pass through an array of parallel and motionless cilia which act as a sieve. eg: *Oligohymenophora*. In *Peritrichea*, one membranelles and paraoral membrane encircling the apical pole of the cells constitute the filter. The undulating movements of membranelles create water currents towards the oral cavity and when the water flows between the cilia of paraoral membrane filtration occurs and concentrated food suspension enters the oral funnel (Verni and Gualtieri, 1997).

b) Raptorial feeders: They capture relatively large food items often using special devices. The hunting devices include appropriate types of extrusomes, which secrete paralytic enzymes to immobilise the prey, such as small flagellates, algae, cellulose fibrils and other protozoa. eg: *Lintonotus*, *Lacrymaria*. Some others feeding on cyanobacteria exhibit three stages in feeding: contact swimming, prey capture and ingestion. eg: *Nassula*, *Chilodonella*. Some ciliates are able to switch from filter to raptorial mode when relatively large food particles are available. eg: *Frontonia*, *Oxytrichia* (Verni and Gualtieri, 1997; Finlay and Esteban, 1998).

c) Diffusion feeders: These ciliates are often sessile and possess sticky tentacles with special extrusosomes. The motile prey becomes immobilised on contact with these tentacles which then penetrate into prey and draw its contents. eg: Suctorians, *Acineta*.

In the case of filter feeding ciliates, the capture rate is proportional to the concentration of food particles and prey, area of the filter and velocity of the water current generated by the cell. However, in the case of raptorial feeding, the capture rate is proportional to the size and concentration of prey. When food particles and prey are smaller than the ciliate, filter feeding is the most efficient mechanism, but when the prey is up to three to four times the size of the predator, raptorial feeders predominate. In the case of diffusion feeding, the capture rate depends essentially on prey motility. If the prey is motile (e.g. flagellated bacteria) the effectiveness is comparable to that of the filter or raptorial feeding mechanism, whereas if prey is immotile, effectiveness becomes two to three times lower than that of the filter feeding mechanism (Fenchel, 1987).

1.6.2 Heterotrophic flagellates

Heterotrophic flagellates use flagella for both locomotion and feeding. They are recognised as the main consumers of suspended as well as attached bacteria and the size spectrum of consumed particle can range from 0.2 µm to > 50 µm. Most of pelagic flagellates feed on suspended bacteria by different mechanisms: filter feeding (eg: chaonoflagellates), sedimentation (eg: Choanoeca), interception feeding (eg: chrysomonads, *Bodo*), raptorial feeding supported by a pharynx (eg: *Rhyncomonas*) or by pseudapods (eg: *Cercomonads*). Most of the large flagellates may switch between filter feeding and grasping upon contact with smaller and larger food particles respectively. But in the case of small flagellates, other forces like Brownian and

diffusion becomes generally important because even the raptorial feeders get some profit from swimming velocity of the bacterial prey and diffusion (Boenigk and Arndt, 2002).

1.6.3 Sarcodines

Sarcodines engulf prey with pseudopodia and based on their functional morphology they can be categorised into three broad groups: naked amoebae (diffusely organized cells without permanent external and internal compartmentalisation), testate amoebae (transitional cytoplasmic forms with an external shell having openings through which cytoplasm emerges out) and heliozoans (zonal organized forms with a distinct nonliving capsule containing several pores through which cytoplasm extends peripherally forming the axopodia). The naked amoebae capture prey largely by pseudopodial engulfment and formation of food vacuoles. In testate amoebae, the shell supports pseudopodia for food capture and cytoplasm flowing out of the shell increases the surface area of peripheral pseudopodia while in heliozoans, the emerging axopodia capture the prey (Anderson, 1996). Their prey principally includes bacteria, unicellular filamentous algae and small protozoa, but some like *Pelomyxa* feed on organic detritus materials (Finlay and Esteban, 1998).

1.7 Anaerobic protozoa

Anaerobic protozoa, mainly ciliates are widely distributed in reducing environments such as marine and freshwater sediments, rumen ecosystem, sulphide

rich lakes, municipal landfills and wastewater treatment plants (Fenchel et al., 1977; Williams, 1986; Dyer, 1989; Finlay and Fenchel, 1991a; Finlay and Fenchel, 1991b; Finlay et al., 1993; Narayanan et al., 2007; Priya et al., 2007). In these environments, they are mainly responsible for the biological methane production owing to their physiological association with endosymbiotic methanogens (van Bruggen et al., 1983). They usually form consortia with anaerobic bacteria in these environments and their abundance varies in different anoxic environments reaching upto 10^5 to 10^6 ml^{-1} in rumen (Williams, 1986). Major groups of anaerobic protozoa and their representative genera are listed in Table 1.4.

Table 1.4. Protozoan groups with anaerobic representatives

(Fenchel and Finlay, 1995)

	Protozoan group	**Representative genera**	**Lifestyle**
I	**Flagellates orders**		
	Rhizomastigidae	*Pelomyxa*, *Mastigella* *Mastigamoeba*	free living
	Diplomonanida	*Hexamita*, *Trepomonas*, *Giardia*	free living intestinal commensal
	Retortomonanida	*Chilomastix*	free living
	Trichomonadida	*Pseudotritrichomonas* *Trichomonas*, *Tritrichomonas*	free living intestinal commensal
	Oxymonadida	*Monocercomonas*, *Oxymonas*, *Saccinobaculus*	intestinal commensal
	Hypermastigida	*Barbulanympha*	intestinal

			commensal
	Heterolobosa	*Psalteriomonas*	free living
	Chrysomonadida	*Paraphysomonas*	free living
	Kinetoplastida	*Bodo*	free living
	Choanoflagellida	*Salpingoeca*	free living
	Chytridiomycetes	*Neocallimastix*	intestinal commensal
II	**Ciliates orders**		
	Karyorelictida	*Loxodes*	free living
	Prostomatida	*Prorodon*	free living
	Haptorida	*Lacrymaria*	free living
	Trichostomatida	*Plagiopyla, Trimyema*	free living
	Scuticociliatida	*Cyclidium*	free living
	Heterotrichida	*Metopus, Brachonella*	free living
	Oligotrichida	*Strombidium*	free living
	Hypotrichida	*Euplotes*	free living

Most of these anaerobic protozoa are in fact descended from their aerobic ancestors and have later adapted to a life without oxygen (Fenchel and Finlay, 1995). They possess many structural and functional peculiarities, which are evolved independently as adaptations to anaerobic and micro aerobic environments. This is evident from the hypothesis about the evolution of hydrogenosomes, the membrane bound redox organelles found in many anaerobic protozoa. Several physiological, morphological and molecular studies support the hypothesis that hydrogenosomes derived from a modification of pre-existing mitochondria (Biagini et al., 1997a). Such an evolutionary ancestry of hydrogenosomes further points the emergence of

anaerobic eukaryotes from the ancestral aerobic groups with mitochondria (Embley, 2006).

1.7.1 The metabolism in anaerobic protozoa

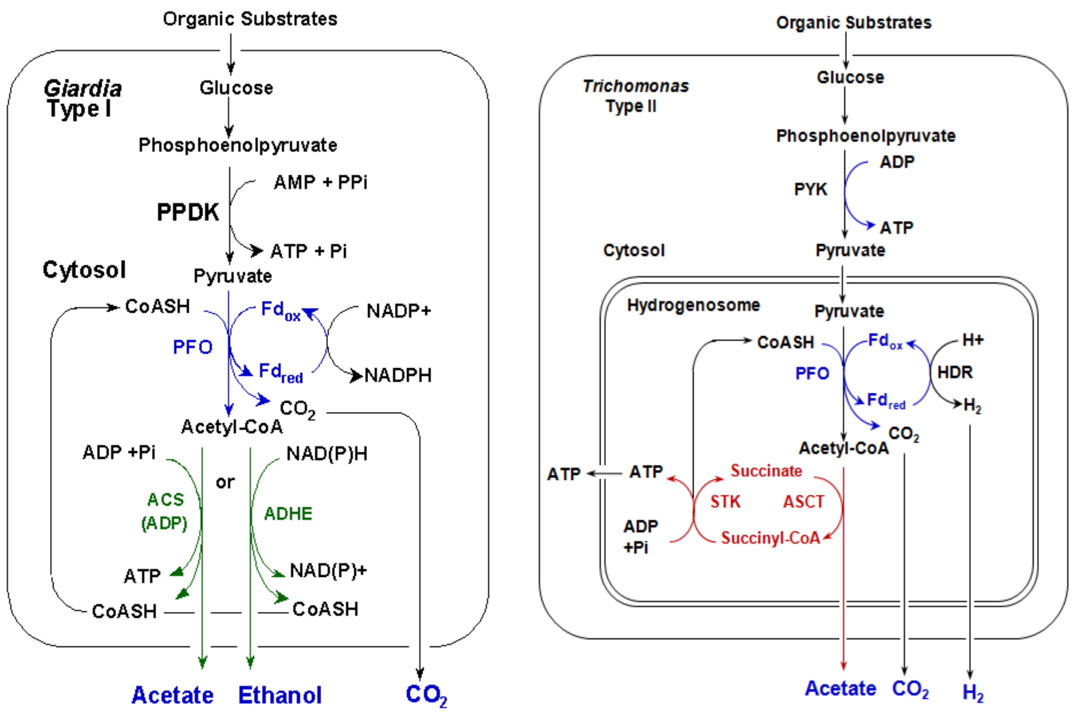
Anaerobic protozoa possess some of the most divergent types of core metabolism among eukaryotes. They do not possess mitochondria and therefore incapable of electron-linked oxidative phosphorylation (Biagini and Bernard, 2000). In aerobic eukaryotes with mitochondria, complete oxidation of one molecule of glucose is achieved through Kreb's cycle and energy generation is coupled with mitochondrial electron transport leading to the formation of 36 ATP molecules per molecule of glucose. The amitochondriate protozoa are essentially fermentative; thus incapable of oxidizing their substrates completely to carbon dioxide and water. The backbone of hexose utilisation is the glycolytic pathway (Embden-Meyerhof-Parnas pathway) and pyruvate is converted to acetyl CoA by pyruvate:ferredoxin oxidoreductase (PFO) and 2 molecules of ATP are generated by substrate level phosphorylation (Hackstein et al., 2001).

The main source of energy is carbohydrate, mainly glucose, its oligomers and polymers. The major end products of carbohydrate fermentations are primarily organic acids (acetate, succinate and lactate), ethanol and CO_2, but they differ with species. Usually two products are formed simultaneously indicating that the existence of branched fermentative pathways, thus permitting the regulation of carbon flow under various environmental conditions.

Anaerobic eukaryotes essentially can have two types of metabolic organisation (Fig.1.2.). In Type I amitochondriate eukaryotes, all processes of core energy metabolism occur in the main cytosolic compartment of the cell (Ellis et al. 1993). In them, cytosolic pyruvate:ferredoxin oxidoreductase (PFO) catalyses the decarboxylation of pyruvate to acetyl Co A which is then primarily converted to a mixture of ethanol and acetate. Ethanol is produced with the help of a bifunctional acetyl-CoA reductase and acetate is produced by an acetyl-CoA synthase that yields the synthesis of one additional ATP through substrate level phosphorylation. The relative amount of ethanol or acetate produced depends on environmental conditions, so that the additional ATP yield is 0 to 2 additional mol ATP per mol of glucose (Martin et al., 2001).

The Type II organisms possess double membrane bound organelles called hydrogenosomes (Muller, 1993). In them, the PFO is localised in the hydrogenosomes where it converts pyruvate to CO_2, acetyl-CoA and reduced ferredoxin. The ferredoxin is reoxidized by hydrogenase, producing H_2 characteristic of the organelle and hence the name hydrogenosomes. The CoA moiety of acetyl-CoA is transferred to succinate by an acetate-succinate CoA transferase (ASCT) yielding acetate and succinyl-CoA. Coenzyme A is regenerated by succinate thiokinase that synthesizes ATP utilising the energy of the thioester bond in succinyl-CoA. Per mol of glucose, pyruvate metabolism in hydrogenosomes thus yields two additional mol of ATP and two mol

each of H_2, CO_2 and acetate as end products. Thus a total of 4 mol of ATP is produced per mol of glucose in hydrogenosome bearing eukaryotes.



Type I amitochondriate eukaryote Type II amitochondriate eukaryote

Fig. 1.2. Basic kinds of compartmentalised energy metabolism in anaerobic eukaryotes
(Martin et al., 2001)

1.7.2 The hydrogenosomes

Hydrogenosomes are special organelles present in variety of unrelated free-living and parasitic protozoa living in anoxic environments. They are membrane bounded organelles, approximately 1 - 2 µm in size, which compartmentalise the terminal reactions of anaerobic cellular metabolism in eukaryotes. They were first described in the parasitic flagellate *Tritrichomonas foetus* (Lindmark and Muller,

1973). Hydrogenosomes seems to be related with a very diverse family of organelles such as mitosomes or mitochondrial remnants which are believe to share a common ancestor with the present day mitochondria. They differ in their ultrastructural morphology in different species varying from single membrane bound microbody-like structures in trichomonads and rumen ciliates to more elaborate double membraned structures resembling mitochondria in free living anaerobic ciliates (Müller, 1993). The metabolic reactions inside the hydrogenosomes involve oxidative decarboxylation of pyruvate to acetyl CoA which is finally converted to acetate, CO_2 and H_2 with the generation of ATP (Fig. 1.3).

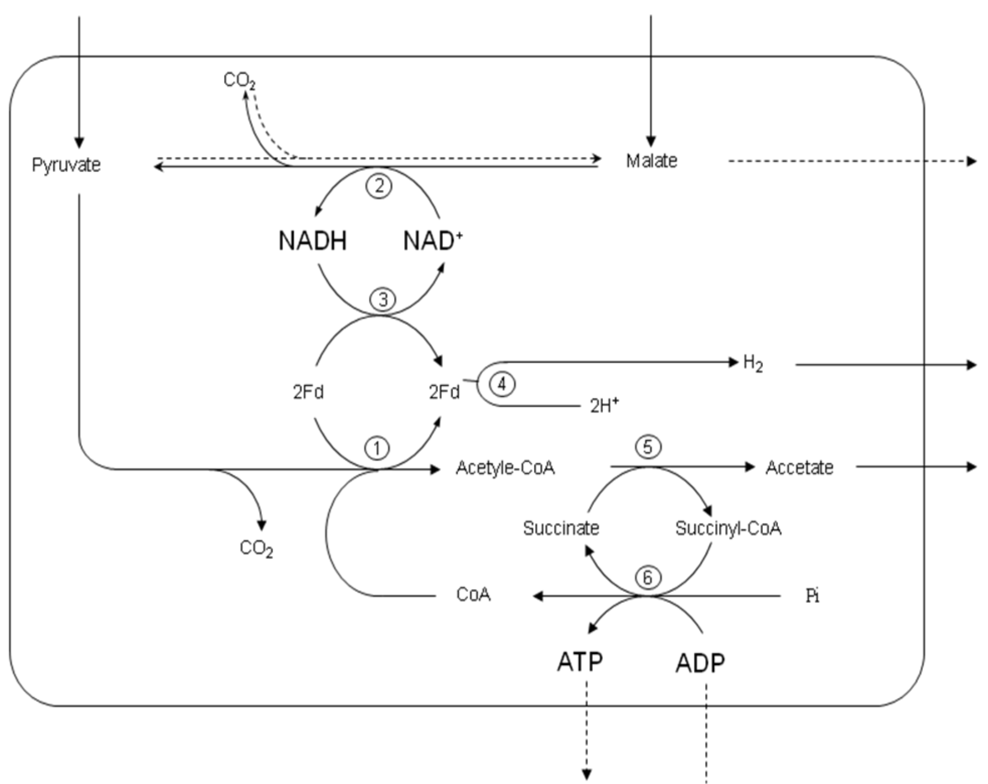


Fig. 1.3. Schematic metabolic map of hydrogenosomes.
1. pyruvate:ferredoxin oxidoreductase; 2. malate dehydrogenase; 3. NAD:ferredoxin oxidoreductase; 4. H_2:ferredozin oxidoreductase; 5. acetate:succinate CoA transferase; 6. succinate thiokinase (Müller, 1993).

The key enzymes in hydrgenosome are the pyruvate:ferredoxin oxidoreductase and hydrogenase which are oxygen sensitive. The pyruvate: ferredoxin oxidoreductase enzyme catalyses the oxidative decarboxylation of pyruvate with the formation of acetyl-CoA, or, in the reverse direction, the formation of pyruvate from acetyl-CoA and CO_2. It is a homodimer of two 120 kDa subunits and contains thiamin pyrophosphate and iron-sulphur centres. The hydrogenase enzyme catalyses the production of hydrogen, using the reduced ferredoxin as electron donor. An acetate:succinate coenzyme transferrase and a succinate thiokinse are also present in hydrogenosomes that mediate the formation of acetate and ATP. In some anaerobic protozoa malate dehydrogenase is also present which interconverts malate to pyruvate (Embley et al., 1997; Embley and Martin, 1998; Hackstein et al., 2001).

Hydrogenosomes and mitochondria share not only the general function of pyruvate metabolism and ATP production but also a number of other characters. Despite their divergent metabolism, several proteins including ferredoxin (Johnson et al., 1990), succinate thiokinase (Lahti et al., 1992), acetate: succinate CoA transferase (Steinbüchel and Müller, 1986), malic enzyme (Hrdy and Müller, 1995) and adenylate kinase (Länge et al., 1994) have been identified in hydrogenosomes as well as in mitochondria. In mitochondria the reducing equivalents are transferred to O_2, whereas in hydrogenosomes, they reduce protons to H_2. Mitochondria act as electron sink and produce ATP by electron transport phosphorylation while hydrogenosomes also acts

as primary sink for reducing power and produce lesser amount of ATP through substrate level phosphorylation (Fenchel and Finlay, 1995).

1.7.3 Symbiotic association with prokaryotes

A large number of anaerobic protozoa live in symbiotic association with prokaryotes and many anaerobic ciliates from a variety of anoxic environments harbour ecto- and endosymbiotic bacteria (Fenchel et al., 1977; Vogels et al., 1980; van Bruggen et al., 1983; Lloyd et al., 1996). They are also unique in the types of symbiotic partners, which include endosymbiotic purple non-sulfur photosynthetic bacteria and methanogens and ectosymbiotic sulfate reducing bacteria. Hydrogen consumption is a common feature of all these symbiotic prokaryotes and this point to the significance of hydrogen production in anaerobic protozoa for maintaining the symbiotic relation. This interspecies hydrogen transfer, from anaerobic protozoa to symbionts, forms the basis of their symbiotic relation (Fenchel and Finlay, 1995).

1.7.3.1 Phototrophic endosymbiotic bacteria in anaerobic protozoa

The phototrophic endosymbiotic bacteria are found in the oligotrich ciliate, *Strombidium purpureum*. Though oligotrich ciliates are typical inhabitants of oxygenated waters, the *Strombidium purpureum* is a benthic ciliate inhabiting the anoxic sandy sediment. This ciliate has purple colored cytoplasm due to the presence of endosymbiotic photosynthetic non-sulfur bacteria, which is closely related to *Rhodopseudomonas*. This ciliate possesses photosensory behaviour, which make them

to accumulate in anoxic water when illuminated and they migrate to oxic anoxic boundary in dark. The endosymbiotic bacteria use hydrogen evolved from ciliate metabolism for photosynthesis under anoxic condition and in the dark at low oxygen levels, the symbionts are capable of oxidative phosphorylation using H_2 and fatty acids. Thus the symbionts protect this ciliate from oxygen toxicity under microaerobic condition (Fenchel and Finlay, 1995).

1.7.3.2 Methanogenic endosymbiotic bacteria in anaerobic protozoa

Anaerobic protozoa with hydrogenosomes frequently host methanogenic symbionts, but such protozoa seem to be restricted to those anaerobic environments that are populated by free-living methanogens (Fenchel and Finlay, 1991a; Fenchel, 1993; Hackstein and Stumm, 1994; Fenchel and Finlay, 1995). These endosymbiotic methanogens can utilise H_2 generated in hydrogenosomes of the protozoa to produce methane and this interspecies hydrogen transfer, helps to maintain the symbiotic relationship. The advantages to the host from the symbionts are not fully understood, but some larger ciliates like *Metopus contortus* and *Plagiopyla frontata* grow faster in the presence of symbionts (Fenchel and Finlay, 1991b; Fenchel and Finlay, 1991c). But in ciliates like *Metopus palaeformis*, endosymbiotic methanogens have only little impact upon the growth of the host ciliate (Finlay and Fenchel, 1991a).

Endosymbiotic methanogenic bacteria are detected in diverse group of sapropelic protozoa like *Pelomyxa palustris* (van Bruggen et al., 1983) and in ciliates like *Metopus*, *Plagiopyla*, *Trimyema*, *Caenomorpha, Brachonella* and *Cyclidium* (Finlay and Fenchel, 1991a; Fenchel and Finlay, 1991b; Fenchel and Finlay, 1991c; Finlay et al., 1993; Embley and Finlay, 1993). Generation of methane by methanogenic endosymbionts has been reported in anaerobic ciliates by Fenchel and Finlay (1992). The ecological importance of ciliate and methanogen endosymbioses is difficult to estimate. In anoxic rice field soils, the symbionts' contribution to overall methane production was only 6.4 % (Shwartz and Frenzel, 2005) and in freshwater sediments they account for 2 % of methane production (van Hoek et al., 2006). However, this symbiotic association is assumed to be responsible for up to 90 % of total methane production in marine sediments (Fenchel, 1993) and 9 - 40% of total methane in rumen environment (Newbold et al., 1995).

1.7.3.3 Ectosymbiotic sulfate reducing bacteria in anaerobic protozoa

The sulfate reducing bacteria are seen as ectosymbionts in anaerobic ciliates like *Sonderia*, *Parablepharisma* and *Metopus* surviving in sulfate rich environments (Fenchel et al., 1977; Fenchel and Finlay, 1991a). These sulfate reducers make use of substrates diffusing out of the ciliates which help them to carry out a high energy yielding metabolism, thereby escape from substrate competition with the free living methanogens (Fenchel and Finlay, 1995).

1.8 *Metopus*

Anaerobic ciliates constitute a very species rich taxon of unicellular organisms. They are characterized by a conspicuous nuclear dimorphism and a complex infraciliature. They occupy the most divergent ecological niches and can thrive as symbionts, commensals and parasites in benthic, sapropelic and intestinal ecosystems. Their morphological complexity, their sensory capabilities and their behavioural repertoire make them unique among the unicellular organisms. Corliss (1979) recognizes 22 orders of ciliates and anaerobic species occur in 11 orders. The strict anaerobes are present in orders ectodiniomorphids and odontostomatids. The order heterotrichida contain many anaerobic families including Metopidae that includes only anaerobic ciliates.

Ciliates of the genus *Metopus* have shown to be strict anaerobic organisms living in many anoxic habitats including marine sediments, municipal landfills, anoxic paddy fields and anaerobic reactors (Finlay and Fenchel, 1989; Finlay and Fenchel, 1991a; Agrawal et al., 1997; Shwartz and Frenzel, 2005; Priya et al., 2007; Narayanan et al., 2007). Most of the species in the genus *Metopus* were discovered and named by Kahl (1935), are ubiquitous filter feeders of bacteria in anoxic environments (Esteban et al., 1995). All the species are asymmetric due to the twisting of anterior region of the cell which gives them the characteristic S-shape.

1.8.1 Classification of *Metopus*

The history of the genus can be traced to the description of *Trichoda es*. The current generic name was erected by Claparede and Lachmann in 1858. Kahl (1935) discovered and named most of the currently known *Metopus* species. Thereafter, the genus continued to absorb new species, Corliss (1979) considered splitting it and Jankowski (1964) carried out a partial taxonomic revision - allocating some species to the restored genera *Bothrostoma*, *Cirranter* and others to the newly-created *Brachonella* and *Tesnospira*. Thus the remaining *Metopus* species became morphologically more homogeneous. The taxonomic position of the genus *Metopus* is given in Table 1.5.

Table 1.5. Taxonomic classification of the genus *Metopus* (Lynn, 2010)

Phylum	Ciliophora
Subphylum	Intramacronucleata
Class	Armophorea
Order	Armophorida
Family	Metopidae
Genus	*Metopus*

1.8.2 General morphology of *Metopus*

All the species in the genus *Metopus* are characterized by a twisted anterior part and a frontal lobe which overhangs an obliquely ascending adoral zone of membranelles. The anterior twisted part includes 5 - 10 kineties, some of them are closer to each other at the edge of overhanging lobe and they form the perizonal ciliary

stripe (Jankowski, 1964). The overhanging lobe together with the torsion of the cell makes these ciliates in this genus S-shaped. Most of them have a group of distinctive intracellular particles close to the anterior end of the cell. The size and shape of many *Metopus* species vary greatly with changes in physiological states.

Kahl (1935) divided *Metopus* into five groups of species, according to general cell morphology and the size of the adoral zone of membranelles (AZM). Esteban et al., (1995) too divided the 76 nominal species of *Metopus* into five broad categories based on cell shape. Thus the 76 nominal species of *Metopus* have been reduced to 22 morphospecies. The groups are as follows:

Group I: *Metopus palaeformis*-like organisms. The equatorial part of the cell is as wide as or narrower than the posterior part. These ciliate are usually thin and elongate.

Group II: *Metopus striatus*-like organisms. Bell-shaped organisms, resembling spine less *Caenomorpha*. Cells are wider at the anterior and equatorial parts of the cell than in the posterior half with or without caudal protuberance or projection. This projection is not spine-like, but a round short projection.

Group III: Cells are wider at the equator than anterior and posterior ends. The posterior end can vary between species, from very narrow (e.g. *M. es*) to less so (e.g. *M. contortus*)

Group IV: Approximately ovoid cells. e.g. *M. ovalis*.

Group V: *Metopus* with the posterior part of the cell narrower than both the cell equator and the anterior end and with a conspicuous and distinctive spine-like posterior extension, e.g. *M. vestitus*. The size and shape within the five groups of *Metopus* along with the species reported so far in each group are given in Table 1.6.

Table 1.6. Size, shape and reported species in different groups of *Metopus* (Esteban et al., 1995).

Morphological group	Morphospecies	Shape	Size (µm)	
			length	breadth
Group I	*Metopus palaeformis* *Metopus hasei* *Metopus setosus* *Metopus laminarius* *Metopus mucians*		101	18
Group II	*Metopus striatus* *Metopus turbo*		51	44
Group III	*Metopus contortus* *Metopus es* *Metopus acidiferus* *Metopus contractus* *Metopus nivaaensis* *Metopus halophila* *Metopus major*		155	42

Group IV	*Metopus ovalis* *Metopus barbatus* *Metopus fuscus* *Metopus tortus*		65	30
Group V	*Metopus spinosus* *Metopus propagatus* *Metopus vestitus* *Metopus verrucosus*		70	32

1.8.3 Culturing of *Metopus*

Unlike the bacteria, anaerobic protozoa are difficult to cultivate in vitro for long periods. Metabolic studies on free-living anaerobic protozoa have been hampered by the limitations to develop their axenic cultures. In the genus *Metopus,* a limited number of species have been cultured successfully in the laboratory condition. *Metopus palaeformis* isolated from the municipal landfill was able to grow on anoxic soil extract medium and ciliate mineral medium under strict anaerobic condition (Finaly and Fenchel, 1991; Biagini et al., 1998a). *Metopus striatus* from anoxic freshwater sediments was able to grow in CM8 medium and monoculture of this species was developed by several repeated enrichments and isolation (van Bruggen et al., 1984). The monoculture of marine species, *Metopus contortus* was also developed in anoxic medium supplemented with sea water and dried grass (van Bruggen et al., 1986; Dyer, 1989). Except these three species, all other members were studied only in

their natural habitats and the difficulties involved in the development of monoculture have limited the more detailed studies on other species.

1.8.4 Endosymbiotic interactions in *Metopus*

All members in this genus found to harbour endosymbiotic methanogens (Esteban et al., 1995) and this symbiotic association forms the basis of increased methane production in many natural anoxic environments inhabiting these ciliates. The *Metopus contortus* from sulfureta was found to contain approximately 28×10^3 methanogens cell^{-1} (Fenchel et al., 1977) and *M. striatus* isolated from sapropelic environment had 2×10^3 methanogens cell^{-1} (van Bruggen et al., 1983). The number of methanogens reported in *Metopus palaeformis* is 360 cell^{-1}. In the case of *M. contortus,* the methanogenic density per ciliate ranges from 6×10^3 - 10×10^3 (Fenchel and Finlay, 1992), where as *M. es* possesses an average number of 637 ± 12 methanogens cell^{-1} (Shwartz and Frenzel, 2005). The average number of methanogenic symbionts was positively correlated with average volume of different *Metopus* species in anoxic rice field soil and the symbiotic methanogens produce methane at average rate of 0.97 fmol methane methanogen^{-1} h^{-1} which account for the 6.4 % of total methane in that habitat (Shwartz and Frenzel, 2005). *M. contortus* with 6000 - 10000 methanogens per cell produce methane at a rate of 8 pmol cell^{-1} h^{-1} where as *M. palaeformis* with 360 endosymbiotic methanogens produce only 0.35 pmol methane ciliate^{-1} h^{-1} in laboratory cultures (Fenchel and Finlay, 1992). The symbionts bearing *Metopus* sp. were reported to be responsible for about 70 % of methane production in flooded rice field soil. In *Metopus*, detailed studies on

endosymbiotic methanogens are limited to only three species, *M. contortus*, *M. palaeformis* and *M. striatus*. All these three species contain *Methanobacterium* as endosymbiont while *M. striatus* has *Methanoplanus* as additional symbiotic methanogen. *M. contortus* contain *Methanoplanus endosymbiosus* and *Methanocorpusculum parvum* in addition to *Methanobacterium* (Embley and Finlay, 1993). In *M. contortus,* the endosymbionts affect the growth rate of host as indicated by 30 % reduction in its growth yield in the absence of symbionts, whereas in *M. palaeformis* they do not exert significant effect on ciliate growth (Fenchel and Finaly, 1991b).

1.8.5 Organic matter degradation by *Metopus*

The successful cultivation and maintenance of three species of *Metopus*; *M. palaeformis, M. striatus* and *M. contortus* in laboratory conditions by supplying exogenous organic matter in artificial cultural medium indicates its ability to take part in organic matter degradation directly. But the exact participation of these ciliates in organic matter degradation has not studied in detail. *M. palaeformis* cultivated in dried grass medium produced an increased amount of methane and reduction in bacterial population (Biagini et al., 1998a). The endosymbiotic methanogens in *M. palaeformis* did not contribute significantly to the methane production; instead the excretions of this ciliate, mainly the organic acids promoted the bacterial growth and thus the methane production. The three species of *Metopus*; *M. striatus, M. contortus* and *M. palaeformis* were cultured along with their prey bacteria which makes it difficult to estimate the direct participation of these ciliates in organic matter degradation (van

Bruggen et al., 1984; van Bruggen et al., 1986; Biagini et al., 1998a). The difficulties in developing the axenic culture of these ciliates without bacteria have hindered elaborate studies on their ability to degrade organic matter.

1.9 The objectives and relevance of the present study

In recent years, biomethanation process has emerged as a successful method for the treatment of high strength industrial wastewater since the development of high rate anaerobic reactors. This method solves the twin problems of pollution and energy crisis as the biofuel, methane is generated from the anaerobic degradation of organic matter present in the polluted wastewater. But most of the studies on this aspect focus mainly on bacterial populations and bacterial metabolic processes that are considered as primarily responsible for organic matter degradation and subsequent production of methane in such conditions. The existence of another trophic group of organisms in these environments, the anaerobic protozoa is rarely recognized in the literature (Gijzen et al., 1990; Agrawal et al., 1997; Xu et al., 2005) and their trophic role is not studied in detail. These anaerobic protozoa, mainly ciliates are abundant in anaerobic reactors with higher removal of chemical oxygen demand and increased methane production with the indication of its possible participation in biomethanation process. The anaerobic ciliate, *Metopus es*, a common inhabitant of anaerobic environments, has been found in higher density in anaerobic reactors receiving complex substrates. This ciliate, *Metopus es*, is one of the least studied species among the genus *Metopus*.

The functional role of *Metopus es* in anaerobic environment is not studied yet, due to the difficulties in developing its axenic culture in the absence of bacterial prey. The major aim of the present study was to investigate the specific role of anaerobic ciliates in organic matter degradation, choosing *Metopus es* as a model organism. In this aspect, monoculture of *Metopus es* was developed in a bacteria controlled environment and optimized the cultural conditions. This monoculture enables to study the specific role of *Metopus es* in biodegradation and biomethanation process. The extra and intra cellular enzyme profile of the ciliate was studied to get an idea about its direct participation in the mineralization of organic matter together with its endosymbiotic methane production.

CHAPTER 2

Isolation, culturing and identification of *Metopus* sp. from the anaerobic reactor

PART A

Isolation of *Metopus* sp. from anaerobic reactor and development of monoculture

2.1.1 Introduction

Anaerobic protozoa, mainly ciliates, are widely distributed in reducing environments such as rumen ecosystem, municipal landfills, sulphide rich lakes, marine sediments and wastewater treatment plants (Williams and Coleman, 1988; Finlay and Fenchel, 1991a; Fenchel and Finlay, 1995). The anaerobic ciliates are often represented as the main bacterial consumers in anoxic environments (Fenchel, 1969) and their predation reduces the bacterial abundance (Fenchel and Finlay, 1990).

In aerobic environment, the ciliates are found to be essential for the treatment and clarification of sewage effluent through their involvement in the regulation of bacterial biomass, thereby causing considerable reduction in biological oxygen

demand and suspended solids in the effluent (Curds et al; 1968; Madoni, 1994; Salvado´ et al., 1995; Madoni, 2003). The significance of ciliates in anoxic environments is still largely ambiguous in the aspect that their bacterial grazing can remove the desirable bacterial population important in biodegradation (Tso and Taghon, 2006). However, Biagini et al., (1998a) reported that grazing behaviour of anaerobic ciliates results in the recycling of many nutrients. Together with organic acid secretions from the ciliates, this exerts a stimulatory effect on bacterial community, which results in enhanced degradation of organic matter.

In order to understand the involvement of anaerobic ciliates in organic matter degradation, it is necessary to monitor their growth in anaerobic systems. The detailed studies on free-living anaerobic protozoa are hindered due to the difficulty in the development of their axenic culture. The isolation of anaerobic protozoa and development of its axenic culture are the major limitations for accessing their role in anaerobic process. The attempts to develop pure culture are restricted to a very few anaerobic protozoan genera (Allen et al., 1966; Wagener and Pfenning, 1987; Broers et al., 1991; Broers et al., 1992; Dehority, 1998; Biagini et al., 1998a; Biagini et al., 1998b). Ciliates of the genus *Metopus* have shown to be strict anaerobic organisms found to occur in many anoxic habitats (Finaly and Fenchel, 1989; Finlay and Fenchel, 1991a; Agrawal et al., 1997; Schwarz and Frenzel, 2005). The presence of *Metopus* has already been reported in landfills and laboratory UASB reactor receiving low strength wastewater (Finlay and Fenchel, 1991a; Agrawal et al., 1997). But the reason

for their presence and involvement in anaerobic process is not elucidated yet. In this aspect, the present study aims to investigate specific roles of anaerobic protozoa in degradation, by developing the monoculture of *Metopus* sp. in a bacteria controlled environment.

Most of the species in the genus *Metopus* were discovered and named by Kahl (1935). *Metopus* species are asymmetric due to the twisting of anterior region of cell which gives them the characteristic S-shape. In this study, *Metopus* sp. was found as the prominent ciliate in the anaerobic reactor treating plant material and high population density of the ciliate was observed during the steady state condition of the reactor in terms of higher COD removal and methane production. In this context, *Metopus* sp. isolated from the anaerobic reactor is chosen as a model organism to investigate the specific roles of this ciliate in organic matter degradation. The present study involves isolation of *Metopus* sp. from anaerobic reactor and optimization of media and cultural conditions in the laboratory.

2.1.2 Materials and methods

2.1.2.1 Population dynamics of *Metopus* sp. in anaerobic UASB reactor

Metopus sp. was isolated from a laboratory scale up flow anaerobic sludge blanket reactor (UASB) used for closed retting process developed at National Institute for Interdisciplinary Science and Technology (NIIST), Trivandrum. The reactor had a working volume of 9 L and provided with a gas-liquid-solid separator at the top. A

closed acrylic tank of volume 0.342 m^3 with a gas tight cover was used for soaking the coconut husk in water under anaerobic condition. The liquid from the tank, rich in organic matter leached out from the coconut husk, was fed to the UASB reactor by peristaltic pump (Watson Marflow 505 U, England). The degradable fraction in the liquid was collected as biogas which was measured using gas flow meter (Insref, India). The effluent from the reactor was recycled back to the leach bed for continuous biodegradation.

The samples from the reactor were taken regularly for microscopical examination to observe the presence of *Metopus* sp. The population dynamics of *Metopus* sp. in the reactor was regularly monitored by direct counting of the cells after fixation. The major parameters of the anaerobic reactor; pH, volatile fatty acid (VFA) and chemical oxygen demand (COD) were determined on a regular basis.

2.1.2.2 Microscopic observations

Samples collected from the reactor were immediately examined under microscope (Nikon-alphaphot2 YS2, USA) to observe the presence of *Metopus* sp. The initial level identification of the ciliate as *Metopus* sp. was done according to the keys given by Patterson (1995).

2.1.2.2a Fixation of *Metopus* sp.

The *Metopus* sp. cells were fixed in Schaudinn’s fixative (Martindale et al., 1982) before counting. Schaudinn’s fixative, widely used for fixation of protozoa, has

the composition of saturated $HgCl_2$ in 0.9 % saline, 60 ml; ethanol, 30 ml and acetic acid, 10 ml.

2.1.2.2b Enumeration of *Metopus* sp.

The population density of *Metopus* sp. in samples was determined by direct counting of samples using Neubauer slide under a compound microscope after fixing them in Schaudinn's fixative. Counting was done in triplicates and the mean count was accounted as the population density of *Metopus* sp.

2.1.2.3 Isolation of *Metopus* sp. from reactor samples

Isolation of *Metopus* sp. from the reactor samples was carried out by centrifugation followed by micromanipulation. The reactor sample of 10 ml was centrifuged at optimized centrifugal force (156 g for 2 min.) to sediment the ciliates. The pellet containing *Metopus* sp. cells was suspended in 10 mM sodium phosphate buffer of pH 6.9 and the *Metopus* sp. alone was carefully picked up by micromanipulatory method. In this method, the sample suspension was placed as small droplets on a glass slide, and individual *Metopus* sp. cell was drawn into a microcapillary tube by capillary action under the microscope at 100x magnification. The presence and identity of the ciliate was confirmed by direct observation of the cells under the microscope before transferring into fresh culture medium. Each time, 20 cells were picked and subjected to repeated washing with anoxic sterile medium to obtain the *Metopus* sp. cells alone with least or no bacteria. The microscopical

examination and micromanipulation were carried out rapidly so that the ciliate gets minimum exposure to oxygen.

2.1.2.4 Cultivation of *Metopus* sp.

The isolated *Metopus* sp. was inoculated into anaerobic culture media and cultivated under strict anaerobic condition in the laboratory.

2.1.2.4.1 Screening of culture media

Six different culture media previously reported successful for the culturing of protozoa were screened for their ability to support the growth of *Metopus* sp. The composition of each medium is given in Table 2.1.

Table 2.1. Compositions of different media used for culturing *Metopus* sp.

Sl. No	Compositions of different basal culture media (g l^{-1})		
1.	Soil extract with added salts (SES) (Biagini et al., 1998a)		
	K_2HPO_4	:	0.02
	$MgSO_4.7H_2O$	:	0.02
	KNO_3	:	0.2
	Soil extract	:	1 l
	Soil extract was prepared by autoclaving 33g sieved soil in 1 l distilled water for 1 h. The supernatant was decanted and filtered through Whatman No:1 filter paper to get the soil extract.		

2 Modified medium M (Dehority,1998)

Sodium acetate	:	0.075
$NaHCO_3$	:	0.49
NaCl	:	0.3
$MgSO_4.7H_2O$	:	0.01
$CaCl_2.2H_2O$	:	0.013
KH_2PO_4	:	0.1
Reactor fluid	:	10 ml

Reactor effluent made free of organisms by filtering through 0.22 µm filter constituted the reactor fluid.

3 Modified Pringsheim's solution (MP) (Culture Collection of Algae and Protozoa)

$Ca(NO_3)_2.4H_2O$	:	0.2
$MgSO_4.7H_2O$	:	0.02
$Na_2HPO_4.2H_2O$	:	0.02
KCl	:	0.026
$FeSO_4.7H_2O$	:	0.002

4 Prescott's and Carrier solution (PC) (Culture Collection of Algae and Protozoa)

$MgSO_4.7H_2O$	:	0.002
KCl	:	0.005
$CaCl_2$	:	0.01
NaCl	:	0.01

	$CaHPO_4$	:	0.0036
5	Prescott's and James's solution (PJ) (Culture Collection of Algae and Protozoa)		
	$CaCl_2.H_2O$	:	0.0043
	KCl	:	0.0016
	K_2HPO_4	:	0.0051
	$MgSO_4.7H_2O$	:	0.0028
6	Ciliate mineral medium (Holler and Pfenning, 1991)		
	KH_2PO_4	:	0.125
	NH_4Cl	:	0.025
	NaCl	:	0.4
	$MgCl_2.6H_2O$	:	0.2
	KCl	:	0.15
	$CaCl_2.2H_2O$	:	0.25

The pH of all the above basal media was adjusted to 7.0 with anoxic 1N NaOH or HCl. Each of the above basal medium was supplemented with different kinds of carbon sources as described in the following section (2.1.2.4.2a). The media were prepared in culture bottles (Schott Duran, Germany) of 500 ml capacity and autoclaved for 15 min at 121 °C. The screw capped bottles were sealed with silicon septa (Thomson scientific, USA) and the headspace was filled with N_2 in order to maintain the anaerobic condition. Complete anaerobiosis was achieved by the addition of 0.67 ml of 3 % (w/v) cysteine hydrochloride solution and resazurin (2 mM)

was used as redox indictor. The bacterial growth was arrested by the addition of 1 ml antibiotic solution (6000 U penicillin G sodium and 200 U streptomycin sulfate ml^{-1}). Initial screening of the culture media was done by inoculating each media in triplicates with 20 washed cells of *Metopus* sp. and incubated at 30 °C. The growth of *Metopus* sp. was monitored regularly in samples withdrawn from the culture bottles using hypodermal syringe and growth was estimated by direct counting as described in section 2.1.2.2b. Increase in the number of viable cells indicated the growth of *Metopus* sp.

2.1.2.4.2 Optimization of nutritional parameters for *Metopus* sp. growth

2.1.2.4.2a Carbon source

The growth pattern of *Metopus* sp. on different carbon sources was studied by adding carbon sources like dextrose, sucrose, starch, cellulose and wheat powder at 1 % concentration. Each carbon source was added separately to all the media listed in Table 2.1, except SES and modified medium M. SES and modified medium M were excluded from this study since these two media already had complex carbon sources, soil extract and reactor effluent respectively. Each medium with different carbon sources were inoculated and incubated as previously described in section 2.1.2.4.1. The ciliate mineral medium with wheat powder suspension was selected as the most suitable one on support of better growth of *Metopus* sp. Different concentrations of wheat powder ranging from 0.1 to 1.5 % were added to ciliate mineral medium to

optimize the concentration supporting the maximum growth. The ciliate mineral medium with 1 % wheat powder suspension was used for further experiments including optimization of inorganic nutrients and physical factors.

2.1.2.4.2b Inorganic nutrients

The optimization of major inorganic nutrients needed for growth such as nitrogen, phosphorus and sulfur were carried out by adding varying concentrations of NH_4Cl, KH_2PO_4 and Na_2S as ammonia-N (2 - 30 mg l^{-1}), phosphate-P (2 - 30 mg l^{-1}) and sulphide-S (0.5 - 5 mg l^{-1}) respectively in the ciliate mineral medium. Each experiment was carried out in triplicates in 500 ml amber coloured bottles inoculated with *Metopus* sp. cells (0.25×10^4 ml $^{-1}$) and growth was monitored up to 9 days by direct enumeration.

2.1.2.4.3 Optimization of physical factors

The influence of physical factors like temperature, pH and dissolved oxygen on growth of *Metopus* sp. was studied under anaerobic condition. The influence of temperature on *Metopus* sp. growth was determined by placing the inoculated bottles at different incubation temperatures ranging from 20 °C to 40 °C. The pH of ciliate mineral medium with 1 % wheat powder was adjusted with sterile anoxic solutions of 1N NaOH or HCl to obtain pH values between 2.0 and 10.0. Experiments on the survival and behavior of cells exposed to different levels of oxygen were carried out by injecting the desired amount of oxygen into the headspace of the culture bottle with

a hypodermic syringe (Broers et al., 1992). Oxygen concentrations ranging from 0.1 - 10 % were maintained in the initial set of cultures and survival of *Metopus* sp. was monitored by direct counting in samples at definite time intervals. The *Metopus* sp. culture kept at anaerobic condition served as control (Goosen et al., 1990a; Broers et al., 1992).

2.1.2.4.4 Bacteriological examination of *Metopus* culture

Bacterial count in *Metopus* cultures was determined by plating 100µl of the culture solution on thioglycolate agar. The thioglycolate agar has the composition (g l^{-1}): peptone, 15; yeast extract, 5; D-glucose, 5.5; L-cysteine, 0.5; NaCl, 2.5; sodium thioglycollate, 0.5; resazurin, 0.001 and agar, 20 with a final pH of 7.0 (Wolf et al., 1975). Plates were then incubated at 37 ^{0}C in N_2 filled anaerobic jars (Oxoid, Japan). After 6 - 7 days of incubation, plates were observed for bacterial growth using a colony counter.

2.1.2.5 Analytical procedures

The pH was measured with a pH meter having temperature correction (Systronics 361). COD was estimated by using open reflux method following standard methods (APHA, 1998). An alkalimetric method was used to determine the total VFA concentration by a two stage sequential titration (Anderson and Yang, 1992). The concentration of ammonia-N was determined by ion selective electrode (Orion, Thermo Fisher Scientific, USA). The phosphate concentration was estimated by

ascorbic acid method and sulphide concentration was determined by iodometry (APHA, 1998). The inorganic nitrogen and phosphorous content in wheat powder was in samples digested by kjeldhal method for nitrogen and perchloric acid - nitric acid method for phosphorous (APHA, 1998). Generation time (h) of *Metopus* sp. was calculated using the exponential growth equation (Creager et al., 1990): Generation time = transfer interval / [(log final concentration – log initial concentration) / log 2].

2.1.3 Results and discussion

2.1.3.1 Population dynamics of *Metopus* sp. in anaerobic UASB reactor

The *Metopus* sp. selected in this study was the predominant ciliate in the laboratory UASB reactor treating plant wastes which is shown in Fig. 2.1.

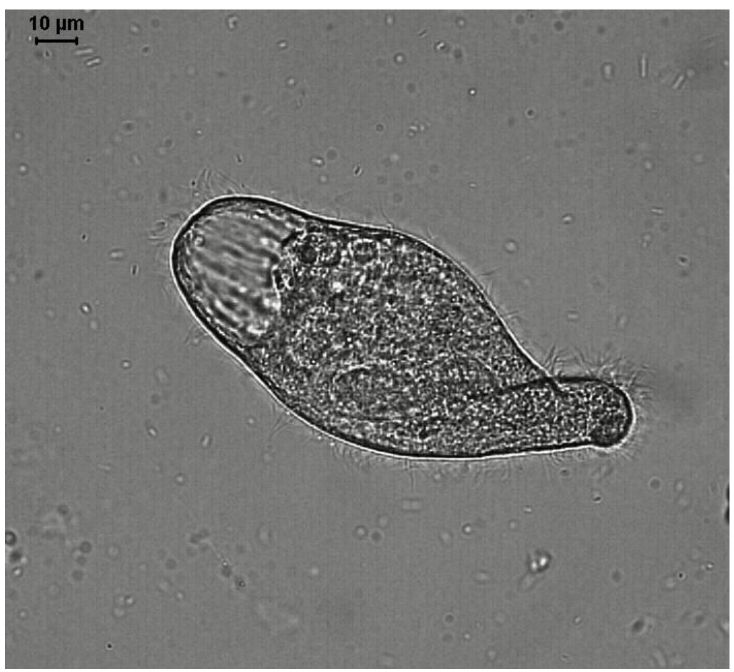


Fig. 2.1. *Metopus* sp. found in the anaerobic reactor under bright field.

The changes in the population of *Metopus* sp. were monitored by direct enumeration of cells during one full cycle of reactor operation. The study shows variations in the population of *Metopus* sp. during different stages of reactor operation. At the initial stage of anaerobic process, characterised by increased production of VFAs and acidic pH, *Metopus* sp. was completely absent in the reactor. The ciliate begin to appear when the reactor reached a steady state condition, characterised by low VFA concentration, stable pH around neutral and effective COD removal. Higher count of *Metopus* sp. was always found to be associated with best reactor performance in terms of COD removal and methane production. The changes in *Metopus* population with various operational parameters of reactor are given in Figures 2.2 - 2.4.

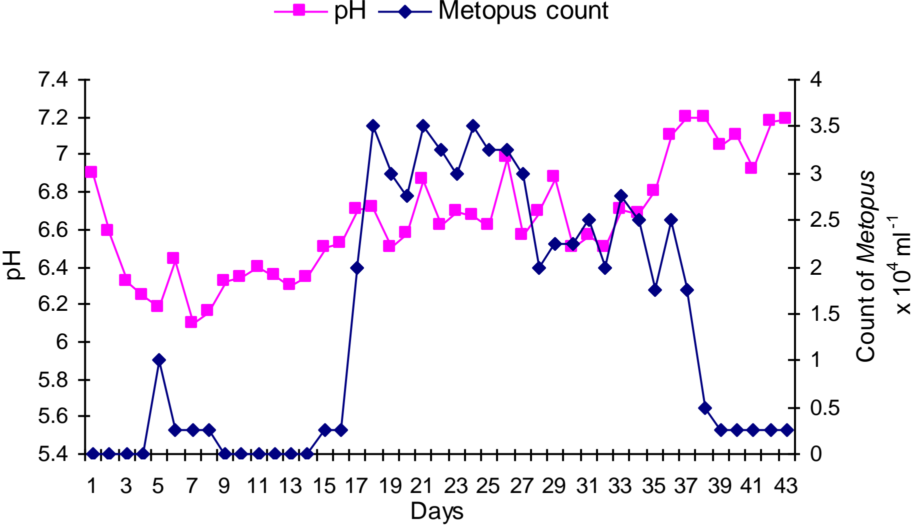


Fig. 2.2. Population dynamics of *Metopus* sp. with reactor pH during one operation cycle.

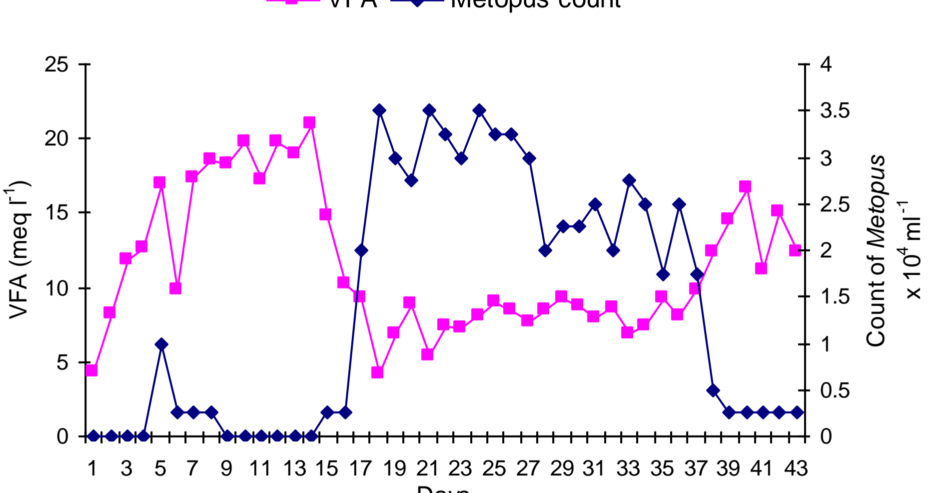


Fig. 2.3. Population dynamics of *Metopus* sp. with reactor volatile fatty acid concentration during one operation cycle.

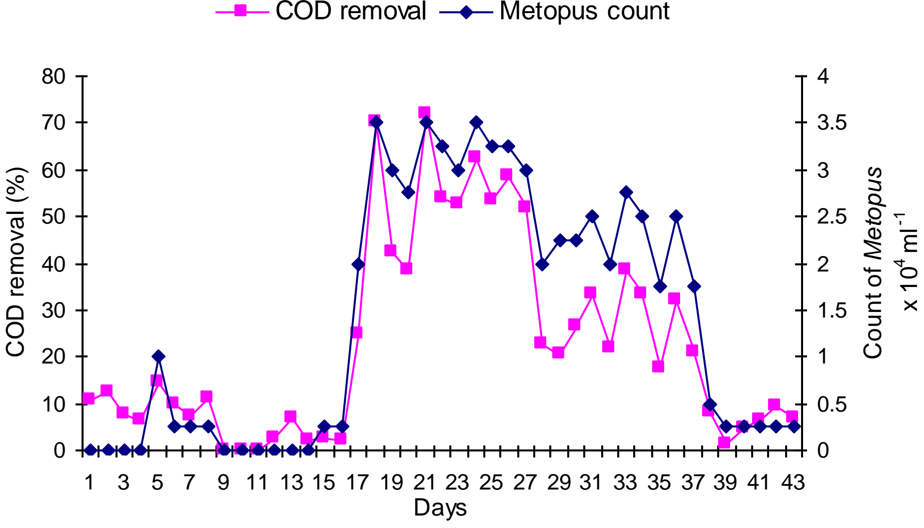


Fig. 2.4. Population dynamics of *Metopus* sp. and COD removal in the reactor during one operation cycle.

The higher population of *Metopus* sp. (2.25 - 3.5 × 10^4 ml^{-1}) was observed during stable pH and VFA. The neutral pH (6.5 - 7.1) and VFA concentration of < 10

meq l^{-1} were found to be characterized by higher counts of *Metopus* sp. (Fig. 2.2 and Fig. 2.3). Fig. 2.4 shows the *Metopus* population and COD removal efficiency of the reactor. During the start up of the reactor operations, when COD removal was poor, less number of *Metopus* sp. was observed. This stage was also noted by the acidic pH (< 6.5) and higher VFA (>15 meq l^{-1}) which could adversely affect the *Metopus* growth. At steady state performance of reactor, above 50 % COD removal was observed along with high population of *Metopus* sp. The highest count of *Metopus* sp. observed in the reactor was 3.5×10^4 ml^{-1} with the corresponding COD removal of 70 %. This relation between COD removal and *Metopus* count indicates that either this ciliate enables COD removal or organics formed during COD removal supports its growth. Only a few reports are available on the presence of anaerobic ciliates and their subsequent contribution to biodegradation process in reactor systems. The anaerobic ciliates were reported to occur in a UASB reactor treating wastewater from gelatin industry (Vieira et al., 2001). The ciliate genera of *Metopus* and *Colpidium* were reported from another UASB reactor treating low strength wastewater (Agrawal et al., 1997). But the role of ciliates in anaerobic process was not clearly elucidated. Certain efforts have been made to develop anaerobic reactors with rumen ciliates possessing hydrolytic enzymes for the treatment of lignocellulosic wastes (Gijzen et al., 1987; Gijzen et al., 1990). However in the above cases, the question of how these ciliates enhance anaerobic degradation is unaddressed. In this contest, the occurrence of a highly dense population of *Metopus* in the anaerobic reactors and its correlation with the best reactor performance deserves special attention.

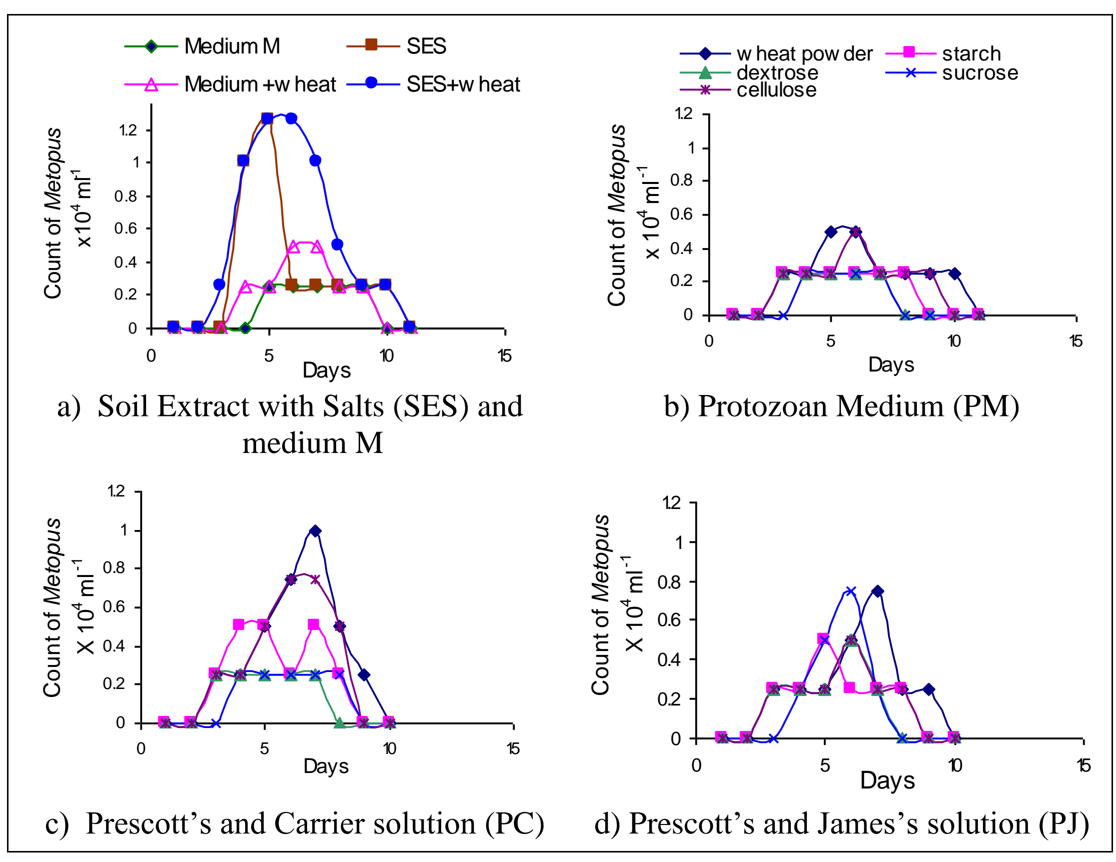
2.1.3.2 Isolation of *Metopus* sp. from reactor samples

Separation by centrifugation followed by direct picking the ciliate by micromanipulation mentioned in this study was effective to isolate *Metopus* sp. from anaerobic sludge. Earlier methods of isolation for anaerobic ciliates have mainly employed either centrifugal separation or micromanipulation independently and had limitations to obtain monocultures for further studies (Massana and Pedros-alio, 1994; Biagini et al., 1998a; Dehority, 1998). The combined method of centrifugation followed by direct picking and repeated washing with fresh media adopted here was tedious but it found to be highly efficient in developing the monoculture without undesired organisms.

2.1.3.3 Cultivation of *Metopus* sp.

2.1.3.3.1 Screening of culture media for *Metopus* sp.

Highly suitable culture medium was selected by monitoring the growth of isolated *Metopus* sp. in different growth media under the same culture conditions. The modified ciliate mineral medium was found to be the best for culturing *Metopus* compared to MP, PC, PJ, medium M and SES as the growth in others was either delayed or limited to a maximum number of 1.25×10^4 ml^{-1} (Fig. 2.5 and Fig 2.6). The ciliate mineral medium was used earlier for the cultivation of anaerobic ciliates, *Trimyema* and *Metopus* (Holler and Pfenning, 1991; Biagini et al., 1998a).



a) Soil Extract with Salts (SES) and medium M

b) Protozoan Medium (PM)

c) Prescott's and Carrier solution (PC)

d) Prescott's and James's solution (PJ)

Fig. 2.5. *Metopus* growth in different anaerobic basal media with varying carbon sources.

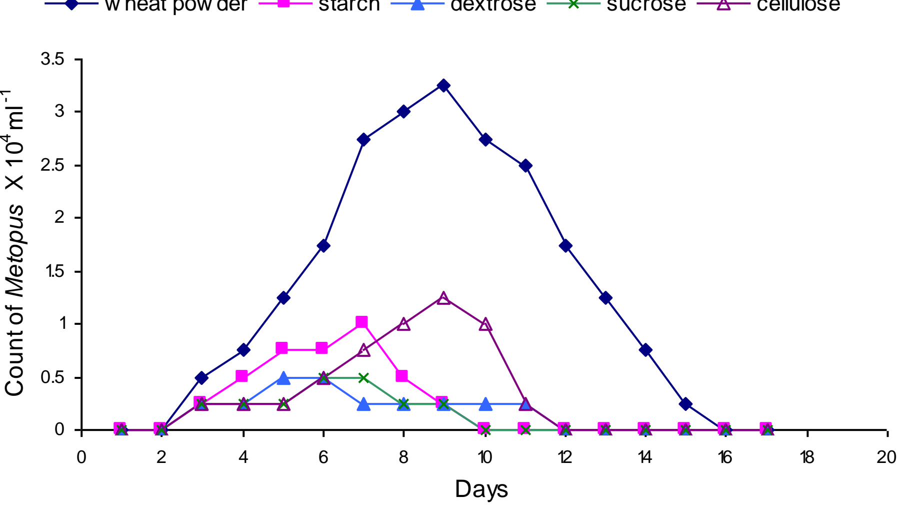


Fig. 2.6. *Metopus* growth in ciliate mineral medium with different carbon sources.

2.1.3.3.2 Optimization of nutritional parameters

2.1.3.3.2a Carbon source

All basal media were tested with different carbon sources, except in SES and medium M. Since the SES medium with soil extract as sole carbon source was reported successful for the cultivation of freshwater protozoa (CCAP), it was tried for culturing protozoa in anaerobic condition. The medium M originally developed for rumen protozoa (Dehority, 1998) had rumen fluid and in this study, it was modified by adding reactor fluid as the *Metopus* sp. was isolated from the reactor. Reactor fluid was added to the medium in order to find out whether the growth of *Metopus* sp. depends on any of the compounds or factors in the reactor effluent. Both SES and Medium M could support the growth of *Metopus* sp. (Fig 2.5a), and they were tested with the addition of wheat also, but the lesser counts were obtained (1.25×10^4 ml^{-1} and 0.5×10^4 ml^{-1} respectively) when compared to ciliate mineral medium supplemented with wheat. The lesser population of *Metopus* sp. in medium M with reactor fluid further indicates that the organism is not strictly depend upon any of the reactor components for its growth.

Among the different carbon sources tried, wheat powder supported the maximum growth of *Metopus* sp. in all the basal media used in this study (Fig. 2.5 and Fig. 2.6). The highest count of *Metopus* sp. (3.25×10^4 ml^{-1}) and sustained growth (more than 2 weeks) was observed in the ciliate mineral medium with wheat powder when compared to other wheat supplemented media (Fig. 2.6). In all the basal media

tried, towards the end of culturing period, the viable cells of *Metopus* sp. changed into cysts. At this level, a reduction medium pH (5.6) was also observed. The wheat powder supplemented medium was characterized by higher growth rate (0.013 h^{-1}) and sustained growth pattern of *Metopus* sp. In this medium, cysts began to appear only after 12 days of growth whereas with all other carbon sources, growth rate was low (0.005 - 0.009 h^{-1}) and there was rapid formation of cysts. Considering the above reasons, ciliate mineral medium with wheat powder was selected as the suitable one for growth of *Metopus* sp. It was selected from the experiments that 1 % wheat powder is the optimum level required for growth of *Metopus* sp. (Fig. 2.7). The suitability of wheat powder as a good carbon source for protozoan growth was successfully tried earlier for the *in vitro* growth of the rumen protozoa, *Entodinium exiguum, Ophryoscolex purkynjii* and *Eudiplodinum maggii* (Dehority, 1998; Dehority, 2004).

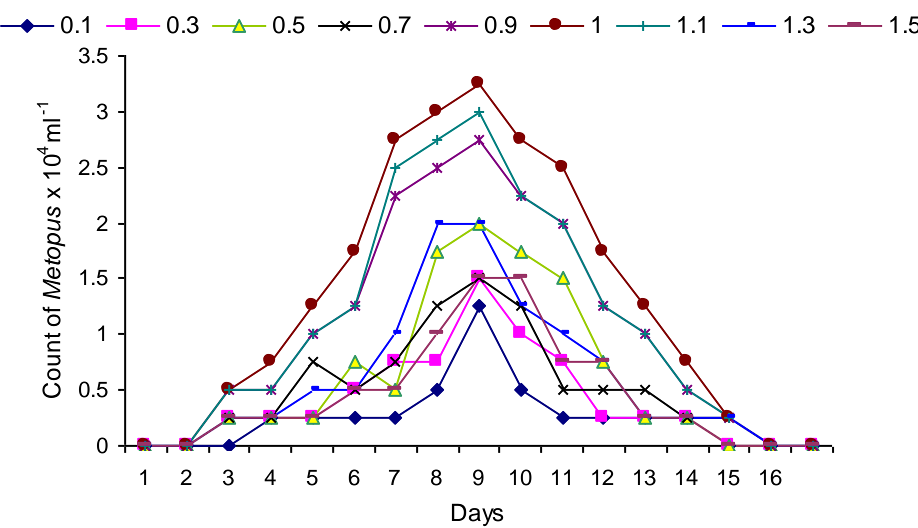


Fig. 2.7. *Metopus* growth in ciliate mineral medium with 0.1 - 1.5 % wheat powder concentrations.

The generation time estimated for *Metopus* sp. in ciliate medium with wheat powder was 53 h which falls within the reported range of 15 to 130 h in the natural environment (Massana and Pedros-Alio, 1994).

2.1.3.3.2b Inorganic nutrients

Along with the carbon source, sufficient quantities of nitrogen, phosphorous and sulfur are essential for better growth of microorganisms. Although these elements may be acquired from the same source that supplies carbon, microorganisms utilise inorganic sources as well. The effect of major nutrients; ammonia-N, phosphate-P and sulphide-S, on the growth of *Metopus* sp. was studied in ciliate mineral medium with varying concentrations.

Ammonia-N

Nitrogen, along with hydrogen, carbon and oxygen is essential for microbial growth. Nitrogen is of central importance in the formation of amino acids, since they are the essential components of proteins. It is also an integral part in biomolecules such as DNA, RNA and ATP. Microorganisms vary in their ability to utilise different nitrogen sources like nitrates, nitrites, ammonia and atmospheric nitrogen. The ability of *Metopus* sp. to utilise ammonia-N for its growth is studied here. The effect of different concentrations of ammonia-N on *Metopus* growth is presented in Fig. 2.8. Maximum growth was observed at 6 - 10 mg l^{-1} of ammonia-N concentration.

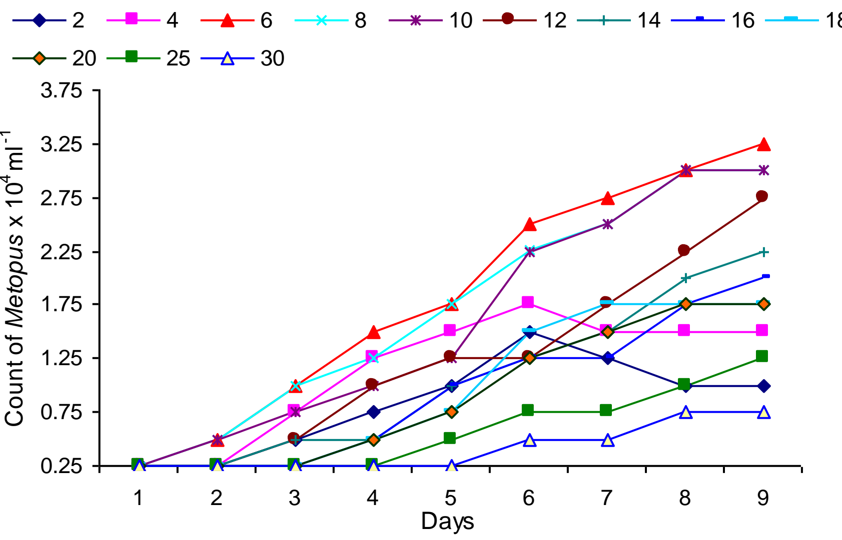


Fig. 2.8. Growth of *Metopus* sp. in different ammonia-N levels (2 - 30 mg l^{-1})

The higher growth rate (0.013) and lower generation time (53.33 h) was observed in presence of 6 mg l^{-1} of ammonia-N. In all other concentrations, *Metopus* had lower growth rates ranging from 0.0124 h^{-1} to 0.006 h^{-1} and higher generation time ranging from 55.71 h to 123.08 h. The changes observed in growth rate and generation time of *M. es* in presence of different ammonia-N concentrations is given in Table 2.2. Statistical analysis revealed that growth dynamics of *Metopus* sp. exposed to higher (>10 mg l^{-1}) and lower (< 6 mg l^{-1}) ammonia-N were significantly different from the one with highest growth rate, ie, 6 mg l^{-1} ammonia-N ($P < 0.05$). But no significant difference exists between the growth rate of *Metopus* sp. in 6, 8 and 10 mg l^{-1} of ammonia-N ($P > 0.05$). However, more than 10 mg l^{-1} ammonia-N was found to affect the growth of *Metopus* adversely.

Table 2.2. Growth rates and corresponding generation time of *Metopus* sp. measured for 9 days at the supply of different ammonia-N concentrations.

Ammonia-N (mg l^{-1})	Growth rate (h^{-1})	Generation time (h)
2	0.01	60.56
4	0.0121	57.01
6	0.013	53.33
8	0.0125	54.10
10	0.0124	55.71
12	0.0122	56.73
14	0.0118	58.55
16	0.011	61.23
18	0.0107	64.59
20	0.0104	66.40
25	0.009	72.62
30	0.006	123.08

Phosphate-P

Phosphorous is present in nucleic acids, phospholipids, nucleotides likes ATP, several cofactors, some proteins and other cell components. Almost all microorganisms can use inorganic phosphate as their phosphorous source and incorporate into the cell directly. Fig. 2.9 describes the variation in growth pattern of *Metopus* sp. in medium supplemented with different concentrations of phosphate-P (2 - 30 mg l^{-1}). Highest growth of *Metopus* was observed at 8 mg l^{-1} phosphate-P. No significant variation in growth rate was seen at 6 and 8 mg l^{-1} phosphate-P ($P > 0.05$) which infers that the optimum phosphate-P concentration needed for the growth falls

in this range. Higher phosphate-P in the medium (> 12 mg l^{-1}) had significant negative effect on the growth of *Metopus* sp.

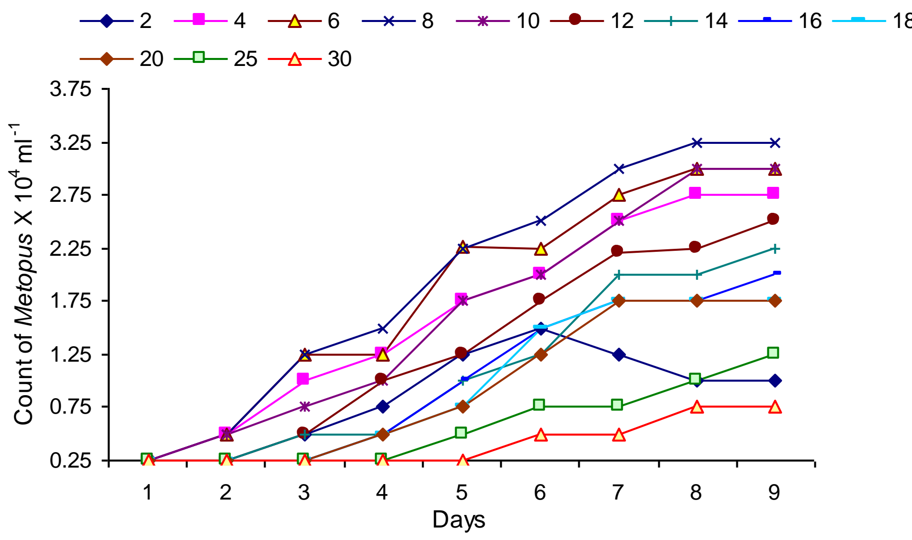


Fig. 2.9. Growth of *Metopus* sp. in different concentration of phosphate-P (2 - 30 mg l^{-1})

Sulphide-S

Sulphur is needed for the synthesis of amino acids like cystiene and methionine and is an important constituent of thiamin, biotin, lipoic acid and coenzyme A. Most microorganisms use sulfate as the source of sulfur and many are capable of using hydrogen sulphide. The growth of *Metopus* sp. in different concentrations of sulphide-S is depicted in Fig. 2.10. The optimum concentration of sulphide-S that supported the maximum growth of *Metopus* sp. was in the range of 1 - 2 mg l^{-1}. Lower (< 1 mg l^{-1}) and higher (> 2 mg l^{-1}) concentrations of sulphide-S significantly affected growth when compared with 1 - 2 mg l^{-1} of sulphide-S ($P < 0.005$).

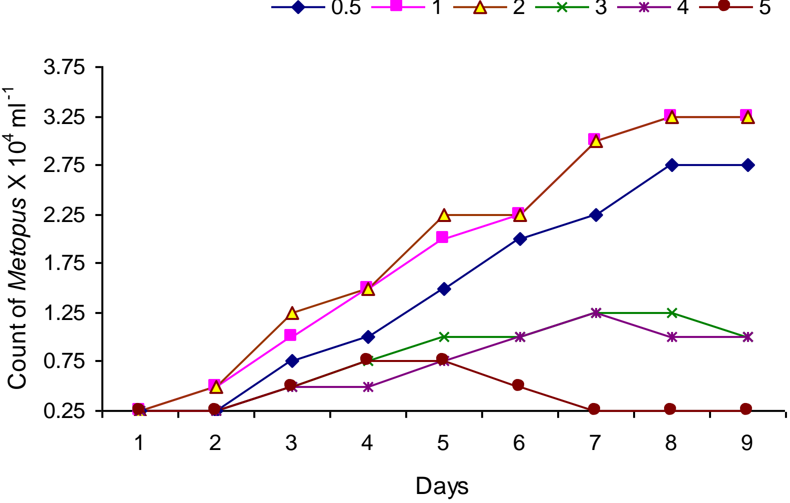


Fig. 2.10. Growth of *Metopus* sp. in different concentration of sulphide-S (0.5 - 5 mg l^{-1})

Anaerobic ciliates were reported to occur in sulfureta (Dyer, 1989) and many of them have been maintained in mixed culture under high sulphide conditions (Fenchel, 1969). Although sulphide is usually toxic to most eukaryotic organisms at very low concentrations (Vismann, 1991), sulphide-tolerant or sulphide-requiring ciliates exist in anaerobic environment. Massana et al., (1994) have reported a decrease in feeding rate in *Plagiopyla nasuta* at a sulphide concentration > 1 mM (32 mg l^{-1}) and it stops feeding above 4.5 mM (144 mg l^{-}). Although *Metopus contortus* was found to be capable of growing upto 5 mM (160 mg l^{-1}) sulphide concentration, its growth was inhibited at 10 mM (320 mg l^{-1}) sulphide (van Bruggen et al., 1986), while *Metopus striatus* died at 2 mM H_2S (van Bruggen et al., 1986).

The wheat powder was reported as a good substrate supporting the growth of microorganisms. It also contains inorganic nutrients like nitrogen, phosphorous and sulphur. The inorganic nitrogen, phosphorous and sulphur content of the wheat powder were estimated as 0.25 mg, 0.34 mg and 0.08 mg g^{-1} respectively. Thus in addition to these amounts of inorganic nutrients present in wheat powder, *Metopus* sp. requires 6 – 10 mg nitrogen, 6 - 8 mg phosphorous and 1 - 2 mg sulphur for its maximum growth.

2.1.3.3.3 Optimization of physical factors

In addition to different nutrients, the growth of microorganisms is greatly influenced by physical parameters. An understanding of the physical factors aids in the control of microbial growth under laboratory condition. Hence evaluating the role of environmental factors on microbial growth is highly relevant. Among the various environmental parameters, the important ones affecting the growth of anaerobic ciliates include temperature, pH and oxygen level.

Temperature

The effect of temperature on *Metopus* growth was studied by incubating the cultures at different temperatures and monitoring their population density at definite intervals. The different incubation temperatures exhibited significant effect on the growth of *Metopus* sp. as shown in Fig. 2.11. The favorable temperature range which supported the growth of *Metopus* sp. was found to be 30 - 35 °C. Both higher (>

35 °C) and lower temperatures (< 30 °C) had adverse effect on growth. The adverse effects on the organism begin with cyst formation at 50 °C (Fig. 2.12).and lead to cell lysis at 60 °C.

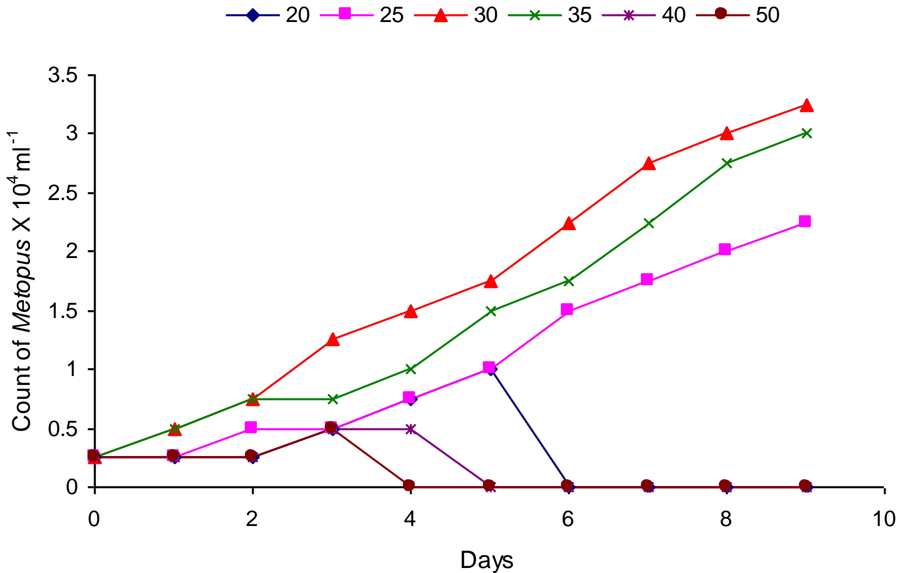


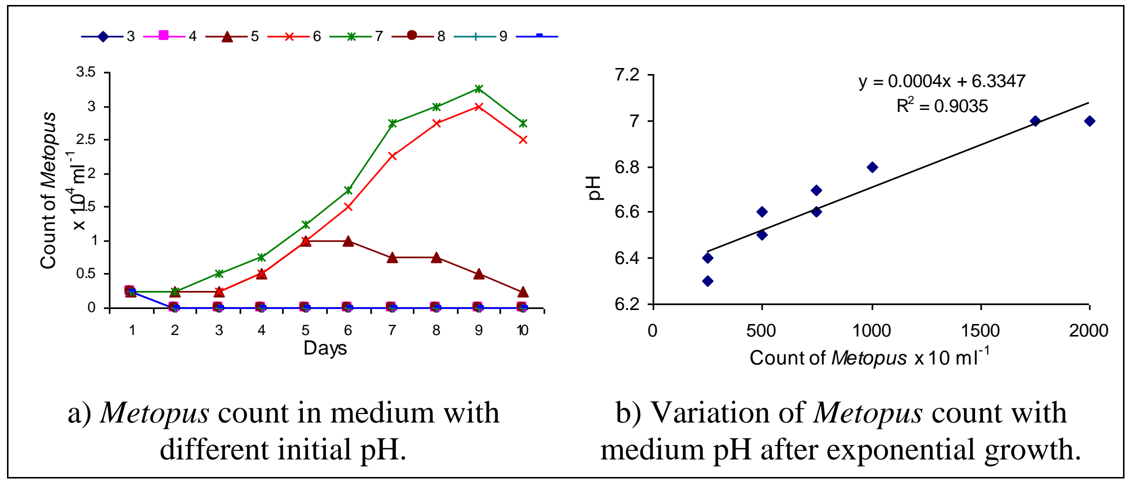
Fig. 2.11. Growth of *Metopus* sp. at different experimental temperatures (20 - 50 °C).

Fig. 2.12. Cyst of *Metopus* sp. formed on exposed to 50 °C for 10 min.

Temperature was reported as an important factor controlling the growth rate of anaerobic ciliates and the favorable temperature varies according to the species. The anaerobic ciliate, *Plagiopyla nasuta* exhibited a maximum growth at 18 °C and further rise in temperature did not produce significant effect on growth (Massana et al., 1994). In case of rumen ciliate, *Epidinium ecaudatum*, the normal growth temperature was 39 °C (Coleman et al., 1972), whereas *Trimyema compressum* was capable of growing at a temperature range of 15 - 35 °C with an optimum at 28 °C (Wagner and Pfenning, 1987). Biagini et al., (1998a) have reported the growth of *Metopus* sp. isolated from landfill at a temperature of 20 °C. Thus most of anaerobic ciliates prefer mesophilic temperature ranges and are sensitive to high temperatures. The existence of ciliates in thermophilic anaerobic reactors is not reported yet, suggesting that organic matter degradation by anaerobic ciliates is rather limited at high temperature conditions in such reactors. The mesophilic biomethanation system normally functions well at a temperature range of 30 - 37 °C and *Metopus* sp. isolated from such a system exhibits temperature preference in that range.

pH

Optimum pH range for *Metopus* growth in ciliate mineral medium was found to be 6 - 7 (Fig. 2.13). A slight decline of pH from 7.0 - 6.7 and further decrease to 6.4 was observed after 10 days of growth (Fig. 2.13b) which can be attributed to an increase in VFA concentration (14 meq l^{-1}). Since acetate and hydrogen are the major metabolites of *Metopus* (Biagini et al., 1998a); acetate-VFA may have accumulated in the medium which otherwise could be utilised by bacteria.



a) *Metopus* count in medium with different initial pH.

b) Variation of *Metopus* count with medium pH after exponential growth.

Fig. 2.13. *Metopus* growth in ciliate mineral medium having varied pH conditions.

The *Metopus* sp. is usually detected in methanogenic environments of neutral pH. Acidic or basic pH had adverse effect on *Metopus* growth which resulted in the formation of cyst at extremes. Earlier reports suggest the occurrence of *Metopus* growth in its microcosm at pHs of 7.0 and 7.2 (Massana et al., 1994; Biagini et al., 1998a).

Oxygen

The exposure to atmospheric oxygen affected the *Metopus* cells adversely as indicated by the drastic decrease in its count within 48 h in cultures received higher amounts of oxygen (Fig. 2.14). The study revealed that the oxygen level upto 0.5 % caused reduction in *Metopus* count. *Metopus* sp. survived upto 0.5 % oxygen level, but their count was drastically reduced from the initial level of 3×10^4 cells ml^{-1} to

0.5×10^4 cells ml^{-1} (Fig. 2.14). Thus strict anaerobic condition is essential for the growth of isolated *Metopus* sp.

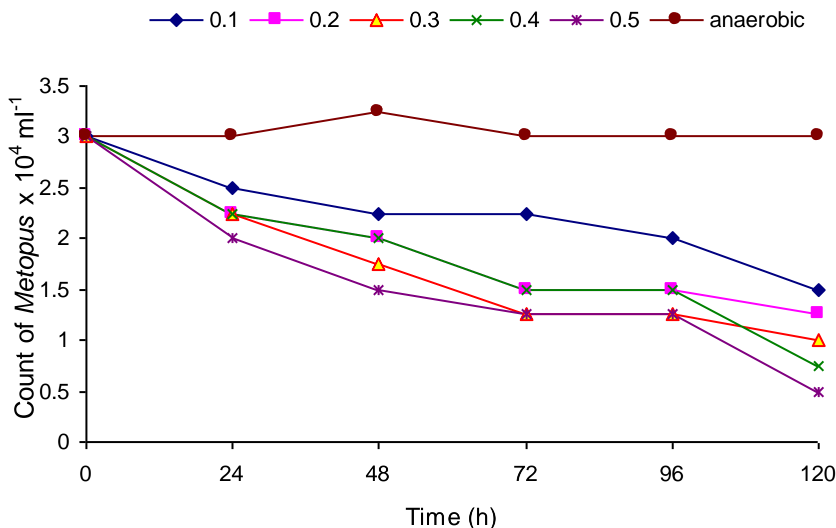


Fig. 2.14. *Metopus* sp. count in culture bottles incubated at 0.1 – 0.5 % levels of headspace oxygen and anaerobic control.

The anaerobic *Metopus* was found to survive at modest levels of atmospheric oxygen as evidenced by the earlier reports (Fenchel and Finlay, 1990b; Finlay and Fenchel, 1991a). *Metopus contortus* from marine sediment displayed considerable aero tolerance with the survival of some cells for two days exposure to atmospheric oxygen (Fenchel and Finlay, 1990b). *Metopus palaeformis* from municipal landfill material had survived for about 6 days in presence of oxygen (Finlay and Fenchel, 1991a).

2.1.3.3.4 Bacteriological examination of *Metopus* culture

Bacteriological examination in *Metopus* sp. cultures with antibiotics (6000 U penicillin G and 200 U streptomycin sulphate ml^{-1}) was done in wheat powder supplemented media. The results showed that there is not much variation in bacterial population in different basal media with wheat powder. The maximum count of bacteria was only 30 cfu ml^{-1} on 2nd day in the PC medium. It declined after a week and remained low in all media for the rest of the experiment (Fig. 2.15), whereas in the control without antibiotics had an increase in bacterial count from 25×10^3 cfu ml^{-1} to 38×10^3 cfu ml^{-1} on 2nd day followed by gradual decline to 5×10^3 cfu ml^{-1} on 7th day and maintained at that level in the second week. Thus bacterial number in the culture was reduced significantly (< 0.2 %) within 24 hours from an initial count of 25×10^3 cfu ml^{-1} by adding penicillin G potassium and streptomycin sulfate at concentrations of 6000 U and 200 U respectively . Increased dose of the antibiotics could eliminate bacteria completely, but it adversely affected *Metopus* growth also. There are many reports on the successful culturing of protozoa with the addition of antibiotics such as penicillin, ampicillin, neomycin, chloramphenicol, streptomycin etc. (Williams and Coleman, 1988). Although the use of penicillin and streptomycin in rumen protozoan cultures had increased the generation time in the absence of live bacteria (Fondevila and Dehority, 2001a; Fondevila and Dehority, 2001b), they have no major effect on protozoan metabolism (Fondevila and Dehority, 2001b). The present study reveals that the antibiotics, penicillin G and streptomycin sulfate at doses of 6000 U and 200 U ml^{-1} respectively, had no adverse effect on *Metopus* sp.

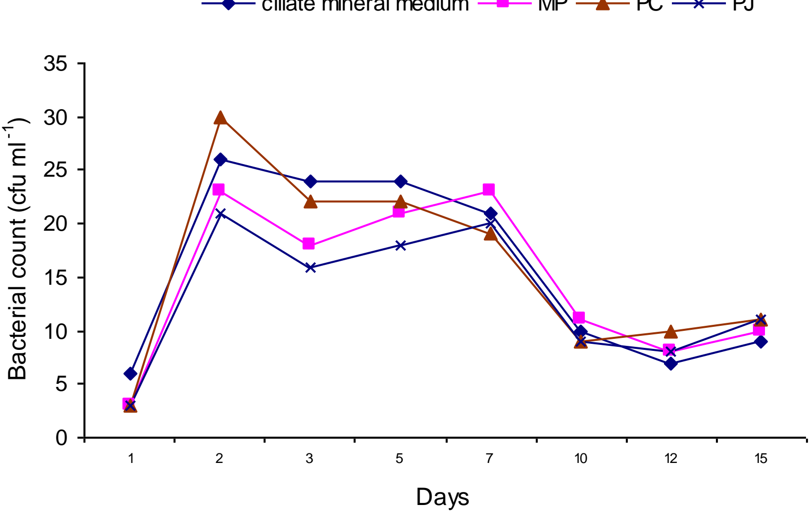


Fig. 2.15. Count of anaerobic bacteria in *Metopus* sp. cultures supplemented with wheat powder.
Keys: MP- Modified Pringsheim's solution; PC- Prescott's and Carrier solution; PJ- Prescott's and James's solution.

Most of the studies reveal that the trophic role of ciliate is intricately related to the interaction between ciliate, its symbiotic bacteria and bacterial prey. However, the direct involvement of protozoa in the digestion of food in the absence of bacterial intermediates was confirmed in rumen ciliates such as *Entodinium caudatum* and *E. exiguum* (Marcin et al., 1998; Fondevila and Dehority, 2001b). In the present attempt, the role of *Metopus* sp. in the anaerobic process was studied by eliminating or reducing the bacterial population with antibiotic treatment. The growth of *Metopus* sp. without bacterial prey indicated that its growth was not entirely dependent on bacterial population; and in the absence of prey bacteria, it can grow on organic matter provided in the medium.

2.1.4 Conclusions

- The present study reveals that monoculture of *Metopus* sp. with less bacterial count can be obtained by centrifugation followed by micromanipulation. The bacterial contamination in the culture can be further reduced by the use of antibiotics in culture media, penicillin and streptomycin.
- Wheat powder serves as the best carbon source for *Metopus* growth in all basal media. The growth of *Metopus* sp. on different kinds of carbon sources provided in the medium indicates its ability to directly utilise organic matter in a bacteria controlled environment.
- Ciliate mineral medium with 1 % wheat powder suspension is found to be the suitable medium for the growth and maintenance of the isolated *Metopus* sp. The generation time of *Metopus* sp. grown in this medium is 53 h.
- The concentration of micronutrients was found to affect the growth of *Metopus* sp. The optimum concentration of inorganic nutrients required for growth was 6 - 10 mg l^{-1} ammonia-N, 6 - 8 mg l^{-1} phosphate-P and 1 - 2 mg l^{-1} sulphide-S, in addition to the inorganic nutrients present in wheat powder. Higher amount of nutrients above the optimum level exerted adverse effect on growth.
- Temperature proves to be an important factor affecting the growth of *Metopus* sp. with an optimum temperature range of 30 - 35 °C.
- pH also has a significant effect on growth of *Metopus* sp. The optimum growth takes place at neutral pH (6 - 7).

- The isolated *Metopus* sp. is very sensitive to oxygen; even slight exposure to oxygen can arrest its growth.

CHAPTER 2

Isolation, culturing and identification of *Metopus* sp. from the anaerobic reactor

PART B

Species level identification of isolated *Metopus*

2.2.1 Introduction

Metopid ciliates can live and reproduce only under anaerobic or microaerophilic conditions (Capar, 2007). They inhabit environments such as polluted water and flooded soil where oxygen is highly deficient (Jankowski, 1964; Foissner et al., 2002). Ciliates of the genus *Metopus* comes under the class armophorea of the phylum ciliophora. They are bacterivorous ciliates with an oral apparatus composed of an adoral zone of membranelles as well as para oral membrane and with somatic kineties built from dikinetids (Vďačný, 2007). These ciliates are characterized by the liar shape of their cell with torsion of the anterior part of the cell and a frontal lobe which overhangs the adoral zone of membranelles. They exhibit strong variability in cell morphology among populations from various biogeographical regions.

The taxonomic classification of ciliates is based on structural, morphogenetic, molecular and phylogenetic data (Cavalier-Smith, 1998). The last edited proposal of the Society of protozoologists (Lee et al., 2000) takes into account the taxonomical characteristics such as morphology, morphogenesis, ecology, etc. along with ultrastructural and phylogenetic characters. Ciliate identification is traditionally based on the morphology and involves microscopical examination of live or fixed cells followed by staining. The live observation and staining is necessary to identify the ciliate upto species level. Staining shows the arrangement of ciliature and infraciliature in somatic and oral structures, and these characters are always mandatory to identify the species. In the present study, the taxonomic investigation of the isolated *Metopus* sp. was done to identify it upto species level.

2.2.2 Materials and methods

The *Metopus* sp. cells at different physiological stages were collected from the monoculture developed in laboratory. The cells fixed in Schaudinn's fixative were observed under microscope as described in section 2.1.2.2a.

2.2.2.1 Size measurements

The length and breadth of each cell is measured with the aid of an ocular micrometer (Erma, Japan) and also by using Leica Qwin software connected along with Leica epiflourescent microscope (Leica DM2500, Leica, Germany).

2.2.2.2 Staining

2.2.2.2a Silver carbonate staining

The silver carbonate staining to reveal infraciliature was performed according to Foissner (1992) with modification in the fixation process. Instead of fixing with 1 % formalin, Schaudinn's fixative was employed because formalin often resulted in bulging and rupture of the cell. In the optimized protocol of silver carbonate staining, the fixed specimens were washed several times with milliQ water. 100 µl of formalin (1 %) and 100µl of Fernandez-Galiano's fluid (It contained pyridine, 0.3 ml; Rio-Hortega ammoniacal silver carbonate, 2.4 ml; peptone solution (4 %), 0.8 ml; milliQwater, 16ml) were added to 30 µl of this ciliate rich suspension and mixed well. Rio- Hortega solution was prepared by adding 150 ml of Na_2CO_3 solution (5 %) drop by drop to 50 ml silver nitrate solution (10 %) under constant stirring. The greenish yellow precipitate of silver carbonate formed was dissolved by the addition of 25 % liquid ammonia and the clear solution obtained was made upto 750 ml with milliQwater.

The staining mixture was then incubated at 60 ^{0}C in water bath for 1 - 2 min with frequent mixing till cognac brown colour appeared. To check whether ciliate was sufficiently impregnated with the silver carbonate, sub samples were taken and observed under compound microscope. On attaining sufficient impregnation, further staining of the ciliate was stopped by the addition of sodium thiosulfate solution

(2.5 %). The stained specimens were observed under bright field in different magnifications and the images were taken.

2.2.2.2b Fluorescent staining

The fluorescent dyes, DAPI (4,6-diamidino-2-phenylindole) (0.5 mg ml^{-1}) and Sybr green (0.5 µg ml^{-1}) which specifically stain the DNA were used to find out the nuclear material inside the cell, both macro and micronucleus. After incubation, the cells were viewed under epifluorescent microscope at specific excitation wavelengths for the dyes.

2.2.2.3 Scanning electron microscopy

The surface features of the ciliate were studied by Scanning Electron Microscopy (SEM). In order to prepare cells for SEM, cells fixed in Schaudinn's fixative were washed twice with 1x Phosphate Buffered Saline (PBS) with the composition (g l^{-1}): NaCl, 8; KCl, 0.2; Na_2HPO_4, 1.44; KH_2PO_4, 0.24. 30 µl of sample was taken on cover slips coated with gelatin which facilitates adhesion of the specimen. After drying at 37 ^{0}C, samples were dehydrated in ethanol series: 30 %, 50 %, 70 %, 80 %, 90 %, 95 % and 100 % for 10 min each. Followed by dehydration in ethanol series, the slides were dipped in 100 % ethanol:hexamethyldisilazane (1:1 (vol/vol) for 20 min. Final dehydration was achieved by dipping the slides 100 % hexamethyldisilazane for 20 min (Stoeck et al., 2003b). After dehydration, samples were mounted on stubs and sputter coated with 22 carrot gold. Samples were

examined with a Scanning Electron Microscope (GSM 5600, Jeol, Japan) and micrographs were taken for reference.

2.2.3 Results and discussion

The classification of *Metopus* species into five broad categories was based solely on their cell shape. Further identification of the species was achieved by considering the following important characteristics of the genus *Metopus*.

- Size (length × breadth).
- Shape
- Somatic infraciliature (number of kineties)
- Peristome (number of kineties in perizonal ciliary stripe)
- Adoral Zone of Membranelles(AZM) (number of membranelles in adoral zone)
- Contractile vacuole
- Caudal cilia
- Macronucleus
- Micronucleus

In addition to the above morphological features, the presence of symbionts was also considered for species identification (Esteban et al., 1995).

2.2.3.1 Size

The isolated *Metopus* species has an average dimension of 167 × 62.6 µm. This was obtained by measuring more than 100 cells drawn at different ages (Fig. 2.16a). Three known species of *Metopus*; *M. es*, *M. contortus* and *M. major* possess similar size range (Table 2.3).

Fig. 2.16a. The dimensions of *Metopus* specimen with 160.5 µm length and 47.9 µm breadth.

Fig. 2.16b. The *Metopus* specimen with characteristic "S" shape due the twisting of the anterior part.

2.2.3.2 Shape

The isolated *Metopus* sp. was wider at anterior and equatorial part than the posterior end, and has a distinctive "S" shape as given in Fig. 2.16a and Fig. 2.16b. This is a prominent feature of Group III *Metopu*s (Esteban et al., 1995) and the important species comes under this group are *M. es* and *M. contortus*. The isolated *Metopus* exhibits a characteristic elegant motion on swimming which is a highly specific feature of *M. es.*

2.2.3.3 Somatic infraciliature

The infraciliature is the arrangement of cilia over the body of ciliate. Cilia can be of two types: monokinetids, single cilia arising from a single kinetosome and dikinetids in which paired cilia arise from two kinetosomes. These kinetids present on

the body and in the oral part constitute the infraciliature. The monokinetids and dikinetids are arranged in rows called kineties which run from the anterior to posterior part of the cell. The presence of these kineties is a peculiar feature of ciliates and each genus varies in the type and arrangement of cilia which aid in their classification. The number of kineties is taken into account while considering the somatic infraciliature for classifying the ciliates. In the present study, the isolated *Metopus* was found to possess 30 - 40 kineties. Two of the reported species possessing same number of 40 kineties are *M. es* and *M. contortus*. The silver carbonate stained specimens with ciliary arrangement are given in Figures 2.17a - c.

Fig. 2.17a. *Metopus* stained with silver carbonate: anterior end.

Fig. 2.17b. *Metopus* stained with silver carbonate: posterior end with caudal cilia indicated by arrow.

Fig. 2.17c. *Metopus* stained with silver carbonate: ciliary rows are shown by the arrow.

2.2.3.4 Peristome

Peristome is another important feature of ciliates. It is the region surrounding cell mouth or cytostome through which food is taken in and food vacuoles are seen in this region. In the case of *Metopus* the perizonal area runs above the adoral zone of membranelles (AZM). The number of kineties in this anterior twisted part ranges from 5 - 10 in different species in the genus *Metopus*. At the edge of overhanging lobe, some of them are arranged closer to each other and form the so called perizonal ciliary stripe (Jankowski, 1964). The number of kineties in the perizonal ciliary stripe is an important feature in species identification. The SEM image of anterior twisted part of the ciliate reveals 5 kineties as shown in Fig.2.18a and silver stained image with the same feature is given in Fig. 2.18b. The number of kineties in peristome has been reported as 5 in three major species; *M. es*, *M. contortus* and *M. major*. However, the number of somatic kineties of isolated *Metopus* did not match with somatic kineties of *M. major*. *M. major* has 50 – 55 somatic kineties and possess the mean dimensions of 146 × 39 µm, whereas isolated *Metopus* possesses only 40 somatic kineties with an average size of 167 × 62.6 µm. Based on these features, the possibility of isolated ciliates as *M. major* can be ruled out.

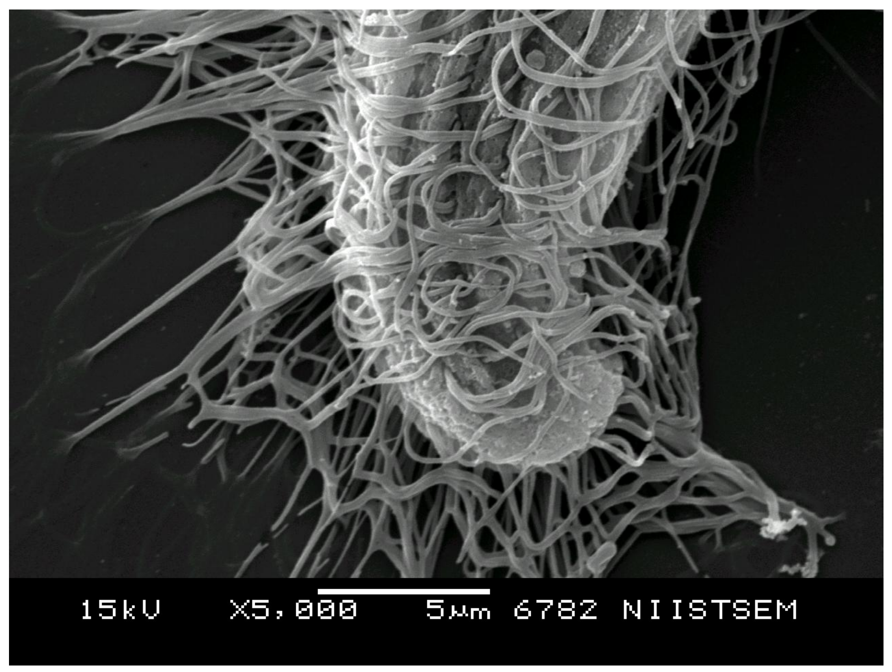


Fig. 2.18a. SEM image of the anterior twisted part of the ciliate with perizonal ciliary stripe.

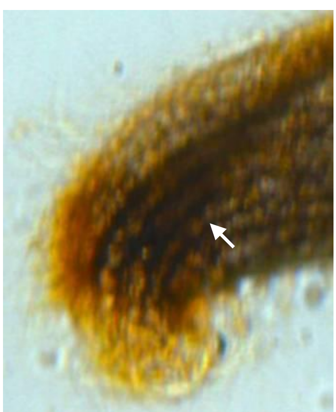


Fig. 2.18b. Microphotograph of *Metopus* stained with silver carbonate: anterior part with perizonal ciliary stripes as indicated by arrow.

2.2.3.5 Adoral Zone of Membranelles (AZM)

This region is associated with the left side of the oral cavity, particularly in heterotrich and spirotrich ciliates. The AZM is characterized by the presence of

prominent membranelles of fused cilia which are serially arranged and hence the name adoral zone of membranelles. The number of membranelles in adoral zone is also considered for the species identification of heterotrich ciliate like *Metopus*. Silver stained *Metopus* cells with AZM is given in Fig. 2.19a and Fig. 2.19b. The presence of fused cilia in AZM is clearly visible in SEM image of the same region (Fig. 2.20a). The number of membranelles in AZM of *Metopus*, isolated in this study, ranges from 40 - 50. This feature is also in agreement with reported number of membranelles in *M. es*, ie, 40 - 50. The chances of the isolated species as *M. contortus* can be eliminates based on this feature. *M. contortus* has 30 - 35 membranelles, while the isolated species possesses 40 -50 membranelles in AZM.

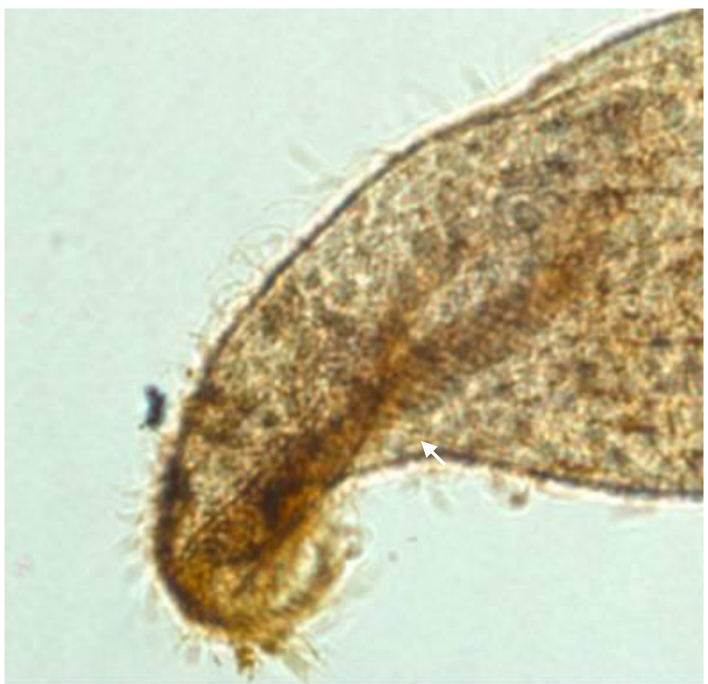


Fig. 2.19a. Microphotograph of silver carbonate stained *Metopus* with adoral zone of membranelles as indicated by arrow.

Fig. 2.19b. SEM image with fused cilia acting like membranelles in the adoral zone of *Metopus.*

2.2.3.6 Contractile vacuole

All the reported species of *Metopus* are characterized by a single terminal contractile vacuole at the posterior end as shown in Fig. 2.21. The contractile vacuole helps the ciliate for maintaining osmotic balance inside the cell.

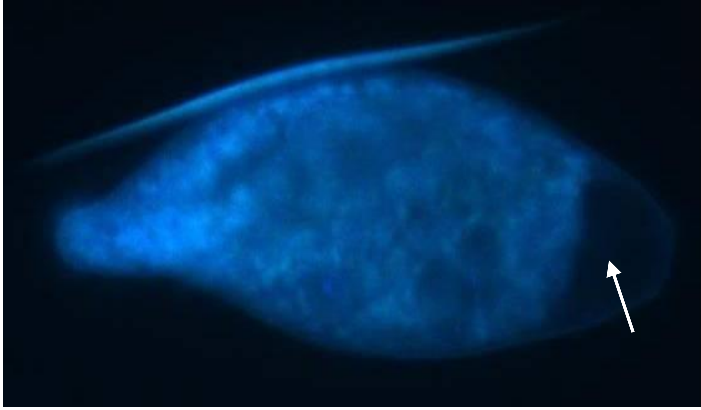


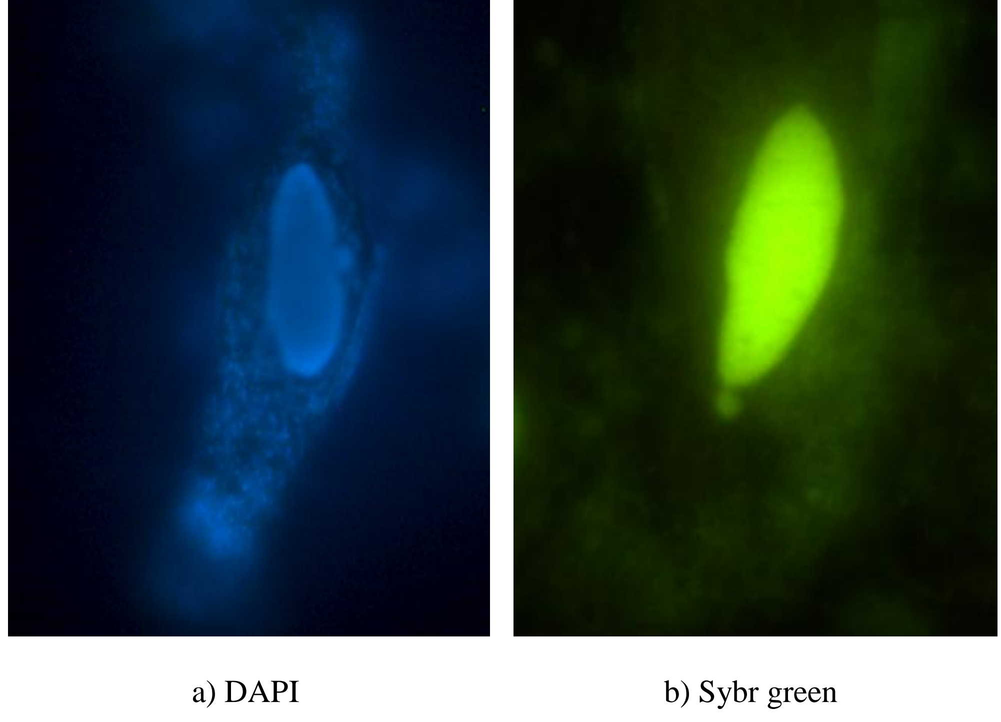
Fig. 2.20. The contractile vacuole in *Metopus* sp.
indicated by arrow and the blue colour denotes the autofluorescing endosymbiotic methanogens.

2.2.3.7 Caudal cilia

The cilia present at the posterior part of the cell constitute the caudal cilia and its number and nature varies from species to species in *Metopus* genus. The ciliate under study shows distinct caudal cilia as indicated in Fig. 2.17b. The presence of caudal cilia was reported in both *M. es* and *M. contortus*.

2.2.3.8 Macronucleus

The presence of two nuclear bodies, macronucleus and micronucleus which differ in their size and function, is the common feature of higher ciliates. The micronucleus is small and diploid which divides by meiosis and is involved in reproduction. The large polyploid macronucleus divides mitotically and control metabolic and developmental activities of the cell. The latter is usually generated from the macronucleus. The shape and position of these two nuclear bodies can vary within species of the same genera which helps in identifying them. The nuclei stained with DNA binding florescent dyes showed one large ovoid macronucleus close to equator in Fig. 2.21a and Fig.2.21b. The oval shape and equatorial position of macronucleus are the features specific to *M. es*.



a) DAPI b) Sybr green

Fig. 2.21. *Metopus* cell stained with the fluorescent dyes showing macro and micronucleus.

2.2.3.9 Micronucleus

The small micronucleus is positioned next to macronucleus as seen in Fig. 2.22a and Fig. 2.22b. *M. es* has an oval macronucleus at the equator region with the micronucleus lying close to it. This feature further confirms the isolated ciliate as *Metopus es*.

The presence of endosymbionts is also taken into consideration for identifying the anaerobic ciliates (Esteban et al., 1995). The *Metopus* sp. under study was found to contain endosymbiotic methanogens which is another matching character with *Metopus es* (Fig. 2.20). All the morphological characters of different ciliate species in

the genus *Metopus* are summarized in the Table 2.3 and are compared with the isolated species in this study. Comparing all the above discussed features that aid in ciliate identification it can be concluded that the *Metopus sp*. under study has the characteristic features matching with *Metopus es* (Esteban et al., 1995). This ciliate is described as the common inhabitant in various anoxic habitats.

Table 2.3. Comparison of morphological features of different species in the genus *Metopus* with the isolated species in this study. Each character showing similarity with isolate is given in bold letters.

Feature	*M. contortus*	*M. es*	*M. nivaaensis*	*M. major*	*M. palaeformis*	Isolate in this study
Length (µm)	**89-165 (119)**	**112-203 (155)**	80-100	**113-191 (146)**	70-200 (101)	118 - 205 (167)
Breadth (µm)	**26-51 (41)**	**23-66 (42)**		**26-49 (39)**	8-31 (!8)	25 - 65 (62.6)
Shape	Typical *Metopus* shape Wide at equator Narrow peristome	**Distinctive "S" shape** **Elegant motion on swimming**	Elongate, ellipsoid, Wide anterior part		Elongate, flattened Ribbon like	Typical "S" shape. Wide anterior and equator than posterior end.
Somatic infraciliature (Number of kineties)	**40** Kineties paired, each bear a cilium	**35-40**	50	50-55	8-14	30 - 40
Peristome	5	**5**	4	**5-6**	2-3	5 - 6
AZM	30-35	**40-50**	32-35	80	10-20	40 - 45
Contractile vacuole	**Single Terminal**	**Single Terminal**	**Single Terminal**	**Single Terminal**	**Single Terminal**	Single terminal
Caudal cilia	**Present**	**Present**	10 cilia in a group	A group of cilia Straight and stiff in living ones	Absent	Present
Macronucleus	Single Anterior	**Single** **Ovoid** **close to equator**	Single Central	Single Elongate at centre	Single Ellipsoid anterior	Single oval shaped close to equator
Micronucleus	Lies near to macronucleus	**Lies next to macronuleus**	Single above macronucleus	Single posterior half of it.	Single anterior adjacent to macronucleus	Single, Lies near macronucleus
Symbionts	Both ecto and endosymbionts	**Endosymbiotic methanogens**	Purple sulfur bacteria		Rod shaped methanogens	Methanogens are present
Habitat	All anaerobic habitats	Anoxic habitats	Anaerobic marine sands	Sulfur rich environments	All anaerobic habitats	Anoxic habitats

2.2.4 Conclusions

The taxonomic studies on the isolated *Metopus* sp. reveal the matching characteristics of *Metopus es*. It has the characteristic "S" shape with mean dimensions of 167 x 62.6 μm. The somatic infraciliature consists of 30 - 40 kineties over the body and 5 - 10 kineties are present in the region surrounding the mouth or peristome. It possesses caudal cilia and a single terminal contractile vacuole. The number of membranelles in the adoral zone is 40 - 45. The ciliate contains a single, ovoid, equatorially placed macronucleus and small round micronucleus near to it. Like other members in the genus, this species harbours endosymbiotic methanogens also. Considering all these resemblances, the isolated ciliate is identified as *Metopus es*.

CHAPTER 3

Tolerance studies of *Metopus es*

3.1 Introduction

The biomethanation system designed for the treatment of wastewater is characterized by the formation various chemical compounds at different stages of the process. During anaerobic treatment processes, organic nitrogen compounds are converted into ammonia, sulfur to hydrogen sulphide, phosphorous to orthophosphates, whereas calcium, magnesium and sodium ions are converted into a variety of salts (Dennis and Burk, 2001). Some of these end products like ammonia, hydrogen sulphide and accumulated organic acids can adversely affect the digestion process. Among these, ammonia and hydrogen sulphide exert strong inhibitory effect upon methanogenic population. Ammonia mainly affect acetate utilising methanogens, and to a lesser degree, hydrogen utilising methanogens and syntrophic bacteria (Heinrichs et al., 1990). Sulphide is more toxic than ammonia to methanogens, inhibiting the growth of hydrogenotrophic methanogens than aceticlastic methanogens (Rinzema and Lettinga, 1988b). Volatile fatty acids (VFAs), the important intermediaries of anaerobic digestion, usually exert stimulating effect on methanogenesis, but at higher concentrations they are inhibitory to many anaerobic bacteria (Zoetemeyer et al., 1982). In addition to the above byproducts, heavy metals

usually present in wastewater or sludge also reduce the methane generation potential of biomethanation process by producing structural disruption of enzymes and protein molecules within the bacterial cells (Hickey et al.,1989). Along with chemical compounds, oxygen poses another important biological inhibition in anaerobic digesters by preventing the growth of strict anaerobic organisms, especially methanogens, thus reducing the methane yield.

Most of the studies regarding the inhibitory action of byproducts of anaerobic metabolism target upon anaerobic bacteria, mainly methanogens (Zoetemeyer et al., 1981; Nandan et al., 1990; Borja et al., 1996). The studies on the effect of these chemicals on anaerobic ciliates have not received much attention, as these organisms are not considered important in anaerobic processes. The action of different byproducts of anaerobic metabolism on ciliates becomes important since they form an additional community in biomethanation systems (Priya et al., 2007). In this aspect, the anaerobic ciliate *Metopus es*, is exposed to the major byproducts of anaerobic system and its tolerance level is assessed in batch culture.

3.2 Materials and Methods

3.2.1 Organism and culture conditions

The anaerobic ciliate, *Metopus es* used in this study was isolated from a UASB reactor treating plant biomass. The culture was maintained in ciliate mineral medium

with 1 % wheat powder. Ciliate culture at exponential phase was used for the experiments.

3.2.2 Ammonia tolerance studies of *Metopus es*

3.2.2.1 Toxicity tests with ammonia-N

The ammonia concentrations ranging from 10 to 500 mg l^{-1} were used for the experiment. The toxicity test was done by adding 0.5 ml of the NH_4Cl solution having different ammonia-N concentration to each well of Lab Tek chamber slides (Nunc, Denmark). The *M. es* cells were separated from the culture, washed with milliQwater and 20 individual ciliates were added into each well. Triplicates were run for each ammonia-N concentration. The control had 20 ciliates in 0.5 ml of milliQwater without added ammonia-N. The ciliates were starved during the experiment. The number of live cells was counted using compound microscope in every 2 h upto 12 h. From this, the percentage of mortality was calculated. The median lethal concentration (LC_{50}) was determined by the probit method (Finney, 1971). The lethal concentrations were calculated by straight-line interpolation and toxicity curve was obtained by linear regression analysis.

3.2.2.2 Effect of ammonia-N on endosymbiotic methanogens in *M. es*

The presence of methanogens inside *M. es* cell was detected by using epifluorescence microscopy according to Doddema and Vogels (1978). To detect the autofluorescence in methanogens, the living *M. es* cells were observed directly under

the fluorescent microscope (Leica DM2500, Leica, Germany) at an excitation of 350 nm. The effect of ammonia-N (5 - 50 mg l^{-1}) on endosymbiotic methanogens was studied by observing the presence or absence of autofluorescence inside the surviving *M. es* cells at regular time intervals.

3.2.3 Sulphide tolerance studies of *Metopus es*

3.2.3.1 Toxicity tests with Sulphide-S

The toxicity of sulphide-S on *Metopus es* was studied by exposing cells to different concentrations of sulphide-S ranging from 1 to 100 mg l^{-1} and mortality percentage was calculated at every 2 h intervals upto 12 h. The experiment was conducted as described in section 3.2.2.1 and the median lethal concentration (12 h LC_{50}) was determined by the probit method.

3.2.4 Heavy metal tolerance studies of *Metopus es*

Different heavy metals Cu, Cd, Cr, Pb, Zn and Hg at concentrations ranging from 0.1 to 150 mg l^{-1}were used to analyse their effect on *M. es*. Exponential phase culture of *M. es* was exposed to different concentrations of each metal and incubated at anaerobic condition. The percentage of mortality and median lethal concentration (LC_{50}) were calculated as described in section 3.2.2.1.

3.2.5 Volatile fatty acids (VFAs) tolerance studies of *Metopus es*

The survival of *M. es* in the presence of different concentrations of VFA was analysed by the addition of acetic acid, butyric acid and propionic acid (0.001 M - 1.0 M) to the exponential phase culture and its count was monitored upto 24 h. The inhibitory level of VFA concentration was obtained from the results.

3.2.6 Oxygen tolerance studies of *M. es*

3.2.6.1 Toxicity tests with oxygen

A pilot experiment to determine the oxygen tolerance was carried out by pouring the exponential phase culture into a petri dish in order to expose it to oxygen; *M. es* was taken at regular time intervals in oxygen exposed culture and compared with that of unexposed anaerobic control culture. The control culture was kept in bottles sealed with butyl rubber septa and the head space was filled with nitrogen. The survival of *M. es* in the presence of oxygen was determined by counting them at regular time intervals as previously described by Finlay and Fenchel (1991a). Morphological changes in *M. es* cells exposed to oxygen were observed using an optical microscope.

The survival and behaviour of cells exposed to different levels of oxygen was studied by injecting desired amount of oxygen to the headspace of the culture bottle with a hypodermic syringe (Broers et al., 1992). Oxygen concentrations ranging from 0.1 - 10 % were maintained initially in the culture bottles and the survival of the ciliate

was monitored by direct counting of *Metopus* cells in samples withdrawn at definite time intervals (Broers et al., 1992; Goosen et al., 1990b). The morphology of the cells was analysed periodically using microscope. Another set of experiment was repeated by introducing oxygen at lower concentrations (0.1 - 0.5 %). The ciliates that survived in presence of oxygen (< 0.5 %) were selected for enzymatic analysis.

3.2.6.2 Preparation of cell extracts

The *M. es* cells were collected from the liquid medium by filtration and concentrated by centrifugation (156 *g*, 2 min). The cells were washed with 50 mM phosphate buffer (pH 7.0) and collected by centrifugation at 156 *g* for 2 min. Cell free extracts of *M. es* were prepared in 50 mM phosphate buffer by disrupting the ciliate by three consecutive freeze-thaw cycles (-20 ^{0}C for 10 min and 35 ^{0}C for 20 min), followed by centrifugation at 204 *g* for 15 min at 4 ^{0}C. The supernatant was collected and kept in sealed tubes at 4 ^{0}C was used for enzyme assays. Protein content of the extract was determined according to Lowry's method using as BSA standard (Lowry et al., 1951).

3.2.6.3 Enzyme assays

Catalase activity was assayed by the method of Beers and Sizer (1952). Superoxide dismutase activity was measured spectrophotometrically using the PMS-NBT assay (Nishikimi et al., 1972). In this, the reduction of nitroblue tetrazolium (NBT) occurs by the superoxide radicals generated by a mixture of

nicotinamide adenine dinucleotide (NADH) and phenazine methosulphate (PMS) at non-acidic pH. Assay was carried out by adding 0.1 ml of the extract from *M. es* to eppendorf tubes containing 50 µM NBT and 78 µM NADH in 100 mM sodium phosphate buffer at pH 7.4. The reaction was started by adding 100 µl of PMS solution (5 µM PMS in 100 mM sodium phosphate buffer at pH 7.4). Both assay mixture and control having milliQwater, instead of the cell extracts, were incubated at 25 ^{0}C for 10 min. The blue formazan, resulted from the reduction of NBT by superoxide radicals generated from the autooxidation of PMS, was measured spectrophotometrically at 560 nm. The reduction in the blue color was taken as the measure of SOD activity in the extract. One unit of activity is defined as the quantity of enzyme that reduces NBT by 50 % (Nishikimi et al., 1972). The aerobic bacterium, *Escherichia coli* was kept as the positive control. The prosthetic group of SOD was identified by incubating the extract with KCN (1 mM) or H_2O_2 (0.5 mM) separately for 1 h and changes in enzyme activity was measured (Biagini et al., 1997c).

3.3 Results and Discussion

3.3.1 Ammonia tolerance studies of *Metopus es*

3.3.1.1 Toxicity tests with ammonia-N

Ammonia in anaerobic digestion process can be of two forms; ionized ammonium (NH_4^+) ions and un-ionized ammonia gas (NH_3), which exists in equilibrium between each other at neutral pH. The un-ionized form is considered as more toxic as it can readily diffuse through the cell membrane and can create harmful

physiological effects on microorganisms including inactivation of enzymes and alteration of intracellular pH. The increase in ammonia concentration within the reactor can cause significant reduction in biogas production by decreasing 56.5 % of the total methanogenic activity (Koster and Lettinga, 1988).

In this experiment, *M. es* was exposed to ammonia-N concentrations of 10 - 500 mg l^{-1} and the number of live cells remaining were enumerated in every 2 h for a period of 12 h. The ammonia concentrations $\geq$ 100 mg l^{-1} began to affect *M. es* adversely within 2 h. The percentage of mortality observed in *M. es* following exposure to ammonia-N within 2 h is given in Table 3.1. The probit units corresponding to each percentage were obtained from Finney's table (Finney, 1971). The log dose mortality curve obtained by plotting probit scale against the $\log_{10}$ of ammonia-N concentrations is given in Fig.3.1. Regression analysis revealed a linear relationship between mortality probit units and ammonia-N concentrations as revealed by the equation, $y = 3.9348x - 3.8749$ ($r^2 = 0.9511$; y = probit scale; x = $\log_{10}$ concentration of ammonia). The LC_{50} value for ammonia-N for 2 h obtained by extrapolation from this equation is 180.08 mg l^{-1}.

Table 3.1. Mortality response of *M. es* to different ammonia-N concentrations in 2 h.

Ammonia-N concentration (mg l^{-1})	Log $_{10}$ concentration	Mortality (%)	Probit scale
10	1	0	-
50	1.67	0	-
100	2	20	4.16
150	2.18	35	4.61
200	2.30	45	4.87
250	2.34	75	5.67
300	2.48	80	5.84
350	2.54	90	6.28
400	2.60	100	-
500	2.70	100	-

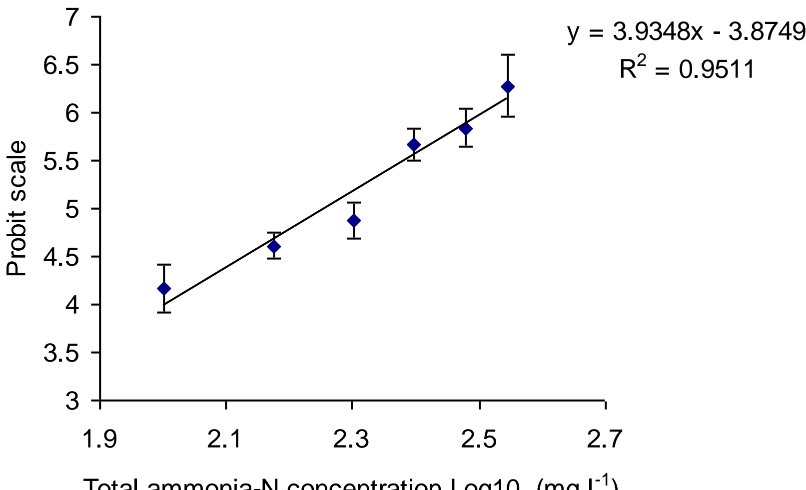


Fig. 3.1. Response of *M. es* on dosing of ammonia-N for 2 h.

In 12 h toxicity studies, the mortality rate in each ammonia-N concentration was calculated upto 12 h. The log dose toxicity curve at each exposure time was calculated by regression analysis and the LC_{50} value for each exposure time was obtained by straight line interpolation as given in Fig 3.2.

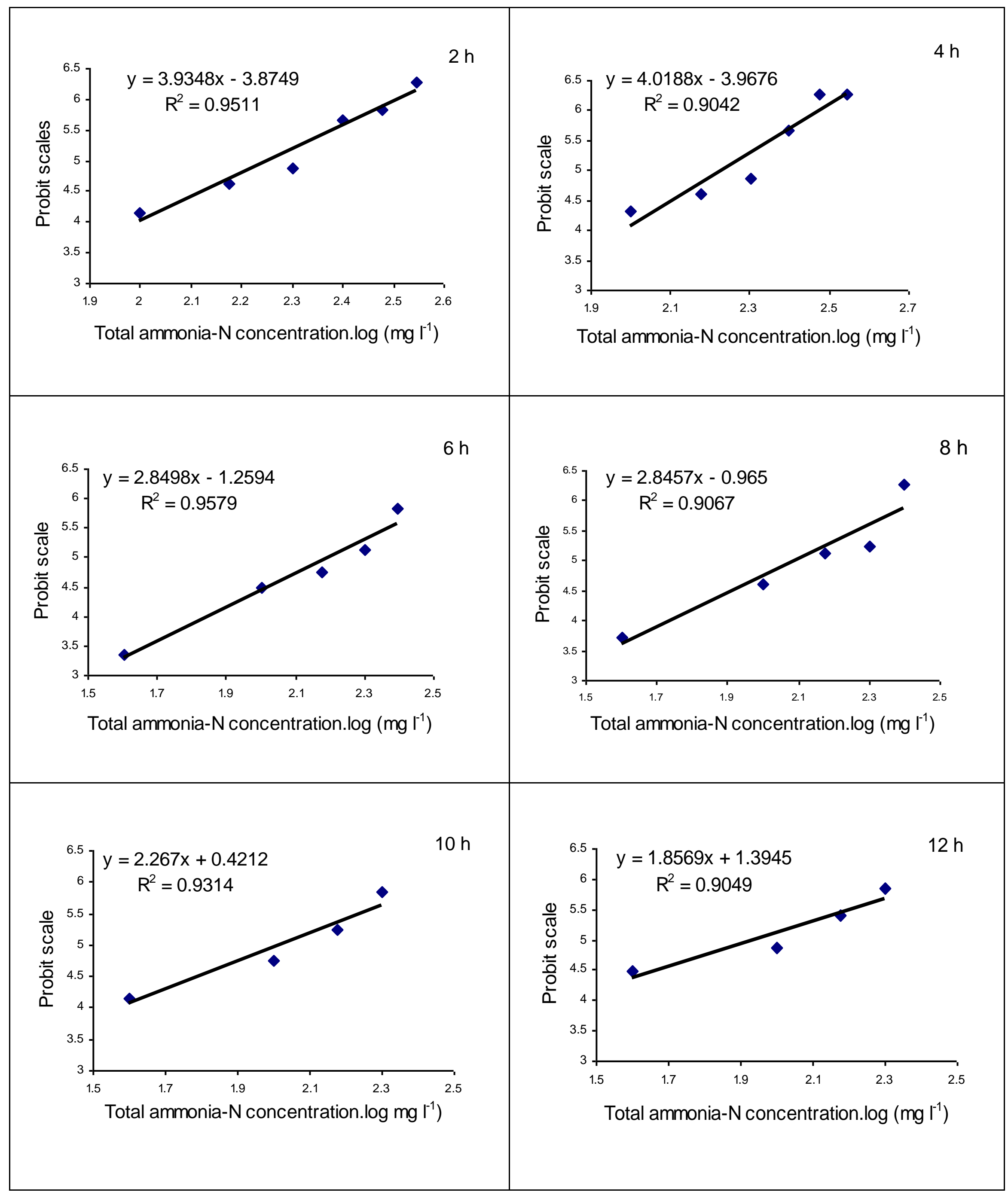


Fig. 3.2. Log dose mortality curve after exposure to ammonia-N for 2 h, 4 h, 6 h, 8 h, 10 h and 12 h.

There was a reduction in the LC_{50} value with the exposure time as shown in Table 3.2. The toxicity curve obtained by plotting exposure time against the LC_{50}

value is given in Fig. 3.3. A linear relationship between the two was obtained by regression analysis as revealed by the regression equation, y = 56.989 $^{e-0.0164x}$ (r^2 = 0.86; y = exposure time; x = LC_{50} value). Using this equation, the LC_{50} value can be predicted for a range of exposure time.

Table 3.2. Mortality response of *M. es* to different ammonia-N concentrations during 12 h.

Time (h)	control	Mortality (%) 50 (mg l^{-1})	100 (mg l^{-1})	150 (mg l^{-1})	200 (mg l^{-1})	250 (mg l^{-1})	300 (mg l^{-1})	350 (mg l^{-1})	400 (mg l^{-1})	LC_{50} (mg l^{-1})
2	0	0	20	35	45	75	80	90	100	180.09
4	0	0	25	35	50	75	90	90	100	170.38
6	0	5	30	40	55	80	100	100	100	157.19
8	0	10	35	55	60	90	100	100	100	124.78
10	0	20	40	60	80	100	100	100	100	104.66
12	0	30	45	70	80	100	100	100	100	87.43

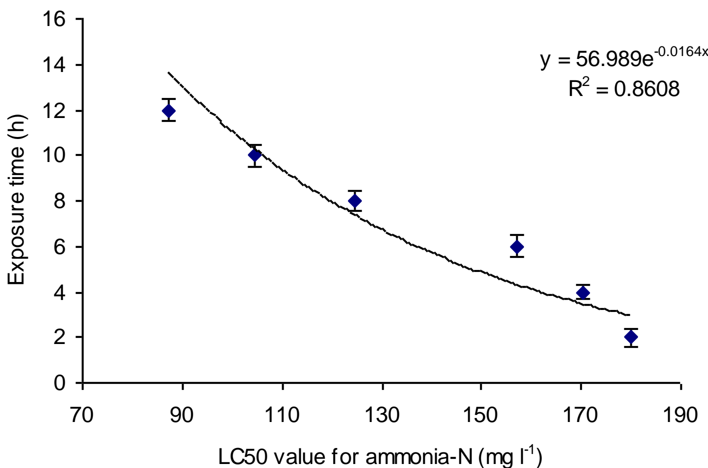


Fig. 3.3. Toxicity curve of *M. es* to ammonia-N during 12 h**.**

M. es found to possess LC_{50} values of 180.09 mg l^{-1} and 87.43 mg l^{-1} at 2 h and 12 h respectively (Table 3.2), which are higher compared to the reported ammonia-N

tolerance of (95.94 mg l^{-1} and 72.86 mg l^{-1}) in the aerobic ciliate, *P. bursaria* (Xu et al., 2005). But the values are lower compared to *E. vannus* which is reported to have LC_{50} values of 7870.46 and 5100 mg l^{-1} at 2 h and 12 h respectively (Xu et al., 2004). Toxicological studies on aerobic ciliates from activated sludge, report that ciliates are able to survive up to 50 mg l^{-1}ammonia-N for 48 h though there is a decrease in their abundance (Puigagut et al., 2005). Long term studies on activated sludge microfauna also reported variation in the abundance of ciliates with increase in ammonia-N concentration. Significant changes in the ciliate density was not found upto 60 mg l^{-1} ammonia-N concentration, but higher concentrations caused considerable reduction in ciliate abundance (Puigagut et al., 2009). On considering the above reports, *M. es* possess a significant level of ammonia tolerance with the LC_{50} value of 52.73 mg l^{-1} of ammonia-N for 24 h as calculated from the equation, $y = 56.989e^{-0.0164x}$, obtained from Fig.3.3.

The exact mechanism of ammonia tolerance exhibited by ciliates is unknown. In multicellular organisms, under high levels of exogenous ammonia and carbon, glutamate dehydrogenase (GDH) plays an important role by converting excess ammonium to glutamate; thus reducing its toxic effect (Melo-Oliveira et al., 1996). In case of anaerobic ciliates, the occurrence of GDH is reported in *Entodinium caudatum* and *Trichomonas vaginalis* (Newbold et al., 2005; Kleydman et al., 2004). GDH in *Entodinium caudatum* acts as anabolic enzyme catalysing the assimilation of ammonia by the ciliate in rumen (Newbold et al., 2005). The occurrence of similar kind of

mechanism in ammonia detoxification can be possible in the case of *M. es* also. But further studies including the detection of various ammonia detoxifying enzymes and their metabolites in *M. es* are needed to elucidate the exact mechanism behind its ammonia tolerance.

3.3.1.2 Effect of ammonia-N on endosymbiotic methanogens of *M. es*

M. es usually reported from the anaerobic habitats was found to harbour endosymbiotic methanogens (Esteban et al., 1995). The endosymbiotic interaction in *M. es* is discussed in detail in the following chapter. The methanogens exhibit autofluorescence owing to their specific cell components, the coenzyme F_{420} and cofactor F_{342}. The detection of autofluorescence by selecting appropriate excitation and barrier filters for these compounds has been widely used for the detection of methanogens in environmental samples (Doddemma and Vogels, 1978). The effect of ammonia-N on endosymbiotic methanogens becomes important with respect to methane generation potential of this ciliate in biomethanation systems. Effect of ammonia-N on endosymbionts was studied by observing the changes in autofluorescence of methanogens inside the survived *M. es* cells exposed to different ammonia-N concentrations. The methanogens retained the fluorescence upto 10 mg l^{-1} ammonia-N concentrations, but >10 mg l^{-1} ammonia-N exposure caused disappearance of the fluorescence in 8 h (Table 3.3).

Table 3.3. Changes in methanogenic autofluorescence in *M. es* exposed to different ammonia-N concentration with time.

Exposure time (h)	Fluorescence at different ammonia-N (mg l^{-1})					
	5	10	20	30	40	50
2	+	+	+	+	+	+
4	+	+	+	+	+	+
6	+	+	+	+	-	-
8	+	+	-	-	-	-
10	+	+	-	-	-	-
12	+	+	-	-	-	-
24	+	+	-	-	-	-

+ indicates normal fluorescence; - indicates fluorescence absent.

One of the mechanisms of ammonia toxicity in methanogens is due to its inhibitory action on cytosolic enzymes (Kadam and Boone, 1996). The ammonia-N concentration of > 40 mg l^{-1} was found to reduce the methanogenic activity in swine wastewater (Belmonte et al., 2011). The reports on the effect of ammonia on endosymbiotic methanogens in ciliates are limited except in rumen ciliates, where ammonia exhibits toxic action upon endosymbiotic methanogens (Tokura et al., 1997).

3.3.2 Sulphide tolerance studies of *Metopus es*

3.3.2.1 Toxicity tests with Sulphide-S

Sulphide is usually toxic at higher concentrations to many microorganisms. The inhibitory effect of sulphide is presumably caused by the unionized hydrogen sulphide which can permeate well through the cell membrane of microorganisms. It

may interfere with assimilatory metabolism of sulfur, while it may also affect the intracellular pH (Oude Elferink et al., 1994). The LC_{50} values for sulphide for each exposure time were calculated by straight line interpolation (Fig.3.4).

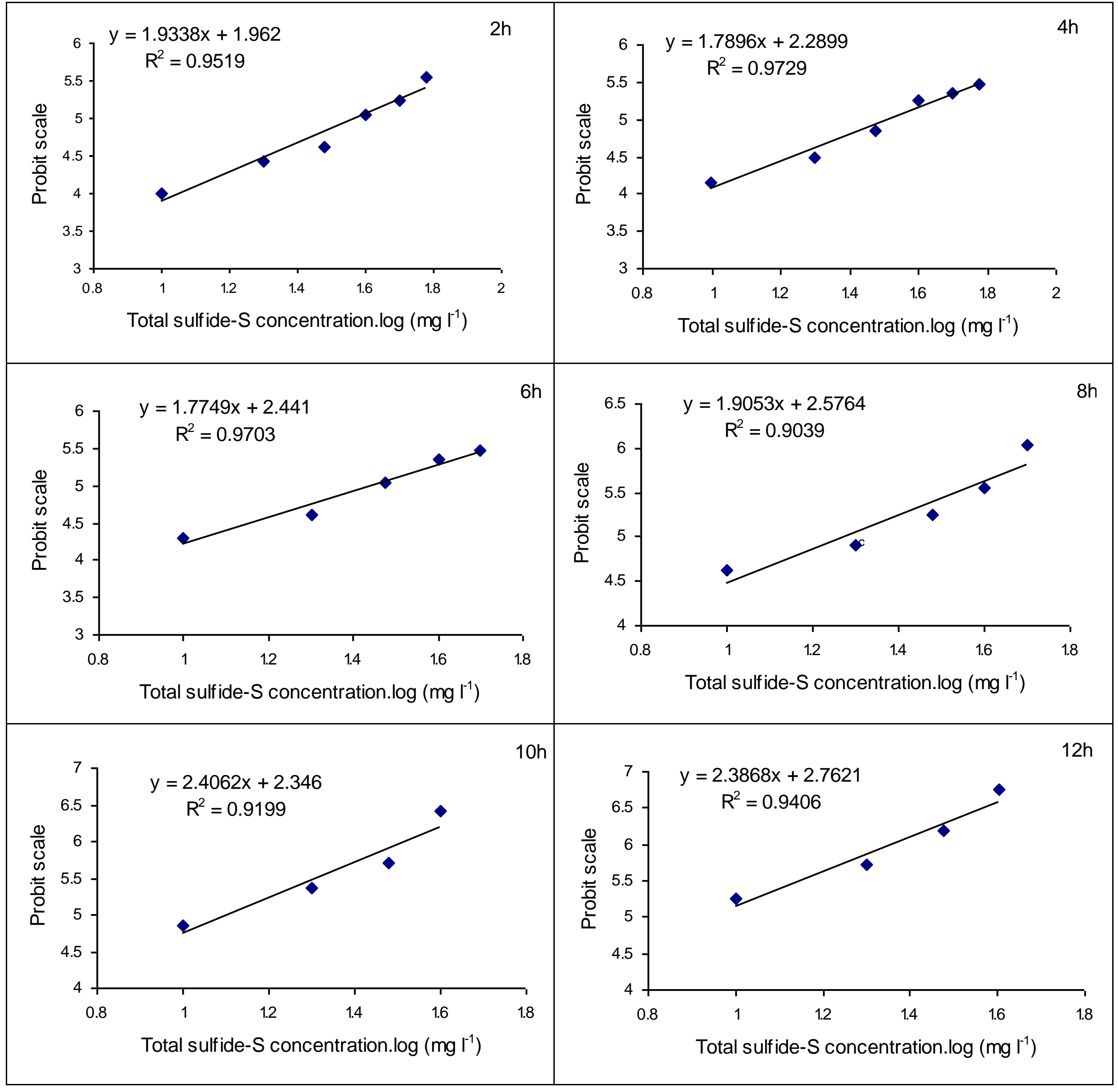


Fig. 3.4. Mortality response of *M. es* on sulphide exposure at 2 h, 4 h, 6 h, 8 h,10 h and 12 h.

The LC_{50} values calculated from the above graphs show considerable reduction with increase in exposure time as shown in Table 3.4. *M. es* is very sensitive to sulfide than ammonia with a 12 h LC_{50} value of 8.66 mg l^{-1}. The toxicity curve is obtained by plotting each exposure time against the corresponding LC_{50} values (Fig. 3.5). It is possible to calculate the LC_{50} value for any exposure time from the graph by using the exponential regression equation, $y = 21.408e^{-0.0555x}$.

Table 3.4. Mortality rates and corresponding LC_{50} values of *M. es* for each concentrations of sulphide-S.

Time (h)	control	Mortality (%) 10 (mg l^{-1})	20 (mg l^{-1})	30 (mg l^{-1})	40 (mg l^{-1})	50 (mg l^{-1})	60 (mg l^{-1})	70 (mg l^{-1})	LC_{50} (mg l^{-1})
2	0	16	28	36	52	60	72	100	37.24
4	0	20	32	44	60	64	68	100	32.69
6	0	24	36	52	64	68	100	100	27.65
8	0	36	48	60	72	84	100	100	18.71
10	0	44	64	76	92	100	100	100	12.7
12	0	60	76	88	96	100	100	100	8.66

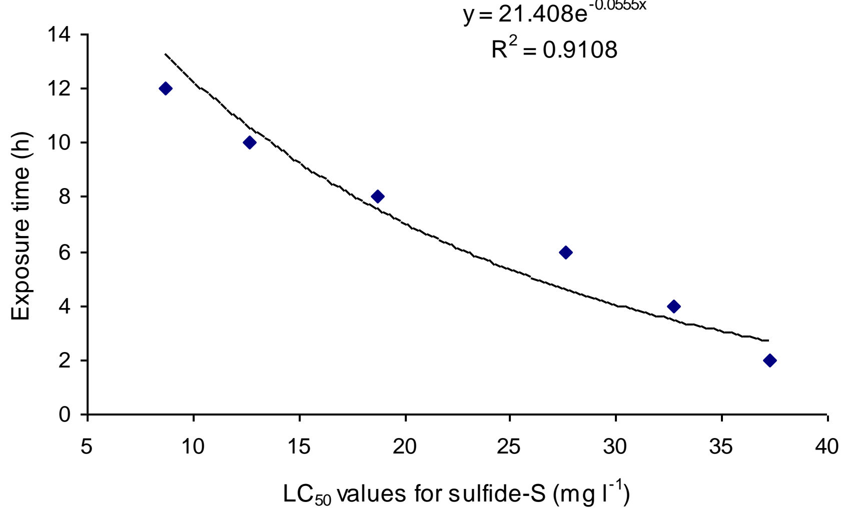


Fig. 3.5. Toxicity curve of *M. es* to sulphide-S within 12 h.

Anaerobic eukaryotes, particularly many ciliates from marine sediments, are surprisingly tolerant to high sulphide conditions. Fenchel (1969) has reported that a number of free living ciliate species of genera, *Metopus*, *Caenomorpha*, *Sonderia*, *Saprodinium* and *Myclostoma* can grow with sulphide concentrations up to 320 mg l^{-1}. But the toxicity studies with sulphide using anaerobic ciliates are rather limited. Studies on the aerobic ciliate, *Strombidium sulcatum* have revealed that 50 % of the cells could survive only for 1 min at 5 mM, but could survive for 40 minutes in 0.2 mM sulphide concentration. The microaerophilic *Euplotes* is reported to survive infinitely at sulphide concentrations less than 5 mM, while 5mM sulphide caused 50 % mortality within 24 h (Fenchel and Finlay, 1995).

The ability of anaerobic ciliates to survive in sulphide conditions was found to vary with the species. In *Palgipyla nasuta*, sulphide concentrations upto 1 mM did not affect feeding activities, but concentrations greater than 2 mM was found to be inhibitory (Massana et al., 1994). The *Euplotes* sp. isolated from the anaerobic sediments grew at rapid rate in sulphide free medium while the addition of sulphide upto 10 mg l^{-1} markedly inhibited its growth (Matsuyama and Moon, 1999). *M. contortus* proved to be more tolerant with ability of growth at 5 mM sulphide, where as *M. striatus* could not survive at 2 mM sulphide concentration (van Bruggen et al., 1986). The sulphide tolerance capacity in anaerobic ciliates was found to be associated with their actual habitats. The ciliates like *Sonderia*, *Cyclidium*, *Metopus*, *Caenomorpha*, *Saprodinium* and *Plagiopyla* isolated from marine sediments, sulfureta

and other sulphide rich habitats were found capable of growing at high sulphide conditions (Fenchel, 1969; Dyer, 1989; Massana et al., 1994).

3.3.3 Heavy metal tolerance studies of *Metopus es*

Heavy metals are considered to be very harmful to microorganisms. Most metals have rapid effect on enzymes; inactivate them by binding to the sulfhydral, amino and imino groups of enzymes (Albergoni and Piccinni, 1983). They can also destroy the integrity of cell membrane causing its lysis. The toxic effects of heavy metals on protozoa include reduction in food uptake, growth inhibition and reduction in the rate of endocytosis which influence the survival (Nilsson, 1981). The toxicity of heavy metal on *Metopus es* was studied by exposing it to different heavy metals like Cd, Cu, Cr, Zn, Hg and Pb and the survival was noticed by direct counting under microscope. The median lethal concentration (LC_{50}) for each metal was found out by plotting the mortality response curve (Fig.3.6) and the calculated LC_{50} values are presented in Table 3.5.

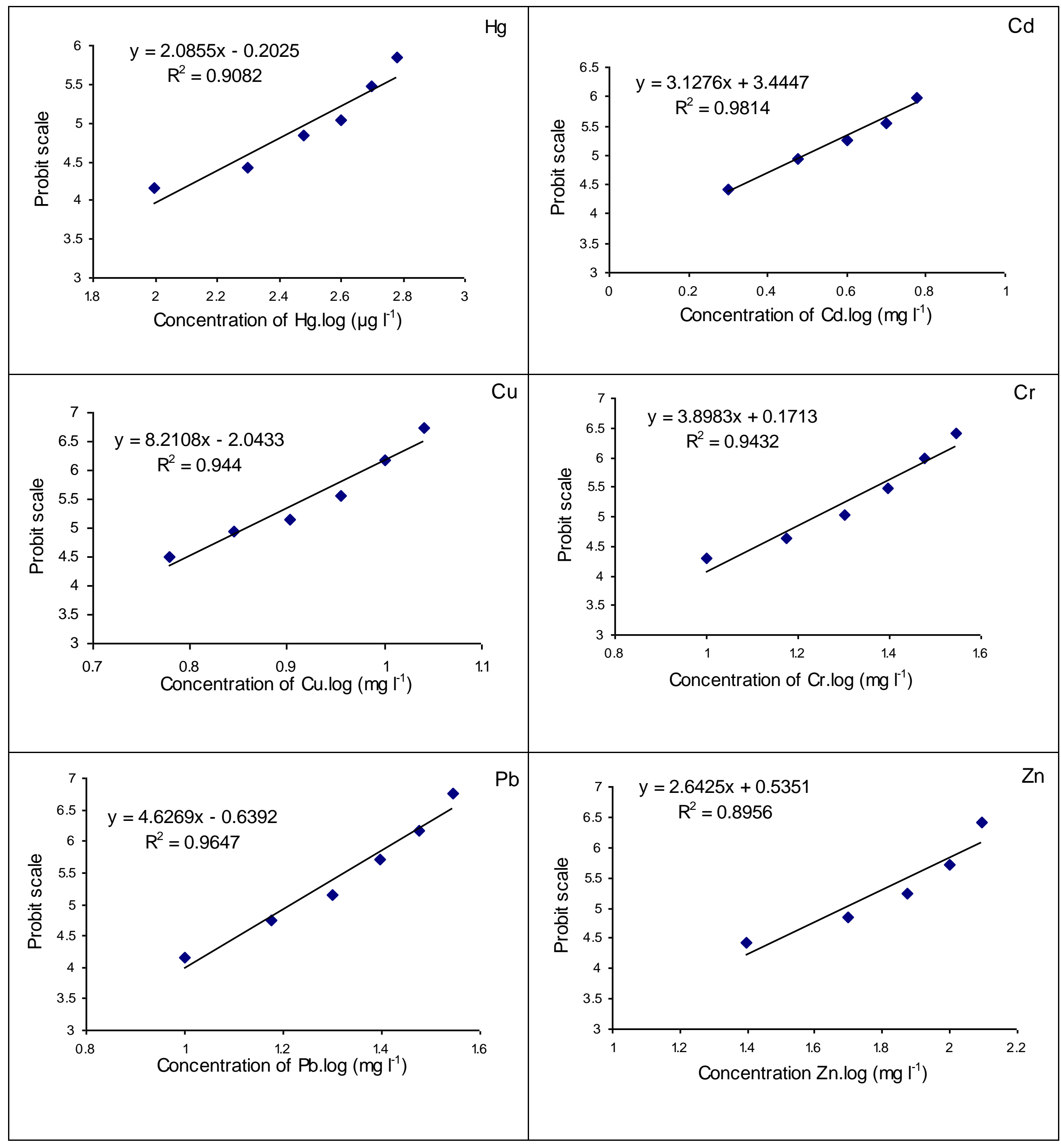


Fig. 3.6. Mortality response of *M. es* exposed to Hg, Cd, Cu, Cr, Pb and Zn.

Table 3.5. Median lethal concentrations (LC_{50}) of important heavy metals on *M. es* within 4 h.

Heavy metals	LC_{50} (mg l^{-1})
Hg	0.31
Cd	3.14
Cu	7.21
Pb	16.55
Cr	17.32
Zn	48.94

M. es was found to be highly sensitive to mercury as indicated by the LC_{50} value of 0.31 mg l^{-1}. Among the studied metals, zinc was least toxic with the LC_{50} value of 48.94 mg l^{-1} and the order of heavy metal toxicity on *M. es* was Hg > Cd > Cu > Pb > Cr > Zn.

The heavy metal toxicity of ciliates was extensively studied in aerobic wastewater treatment systems. Many aerobic and micro aerobic ciliates like *Euplotes*, *Colpidium*, *Paramecium* and *Uronema* were found to be surviving in treatment plants receiving heavy metals (Madoni et al., 1996; Coppellotti, 1998; Martín-González et al., 2006; Rehman et al., 2010). Among these, *Euplotes*, *Vorticella* and *Paramecium* were found to be more tolerant and can uptake multiple heavy metals. This ability can find application for metal detoxification and environmental cleanup operations (Rehman et al., 2009; Rehman et al., 2010).

The heavy metal toxicity in anaerobic ciliates is not studied previously, though these metals are very common in anaerobic wastewater treatment systems. The anaerobic ciliate, *M. es* was found to be very sensitive to heavy metals, particularly to mercury. Generally, the ciliates are reported to be more sensitive to heavy metals than other microorganisms due to the absence of cell wall. This capacity makes them good candidates for using as whole-cell biosensors to detect the presence of certain toxic heavy metals in environmental samples (Martín-González et al., 2006). Because of the limited tolerance of *M. es* to heavy metals, the involvement of this ciliate in anaerobic degradation can be affected by the presence of such heavy metals beyond the tolerance level. Since the *M. es* was reported as a common ciliate in anaerobic wastewater treatment plants (Narayanan et al., 2007; Priya et al., 2007), it has relevance and convenience to use as a bio indicator for evaluating the toxicity of wastewaters polluted by heavy metals.

3.3.4 Volatile Fatty acid tolerance studies *Metopus es*

Volatile fatty acids, mainly acetate, butyrate and propionate, are the byproducts of the non-methanogenic phase of anaerobic digestion. Accumulation of VFA at concentration of 4,000 - 5,000 mg l^{-1} can cause complete inhibition of anaerobic process unless adequate buffering capacity is maintained in the system (Veeken et al., 2000). In order to find out the effect of VFA on *M. es*, different concentrations of acetic acid, butyric acid and propionic acid were added to a set of exponential phase cultures. Figures 3.7a - c illustrate the effect on addition of different VFAs to *M. es*

population. Increased survival of *M. es* was observed in this study where the VFA level was lower in the medium (< 0.025 M). The *M. es* was adversely affected by VFA concentrations greater than 0.05 M and its population density was drastically reduced to 0.5×10^4 ml^{-1} from an initial count of 4×10^4 ml^{-1} within 4 h (Figures 3.7a - 3.7c). The results clearly showed that higher levels of acetate, butyrate and propionate have adverse effects on the growth of this anaerobic ciliate and inhibited its growth beyond 0.05 M. The VFA tolerance of *M. es* is not reported yet. Some rumen ciliates are reported to survive at VFA concentrations > 0.1 M. Whielaw et al., (1972) have reported the presence of ciliate populations including *Eudiplodinium maggii, Epidinium* and *Ostracodinium spp*. at a VFA level of 0.12 M in rumen fluid, and a ciliate count of 2.9×10^4 ml^{-1} was noticed at a VFA concentration of 0.128 M in cattle rumen (Naziroglu et al., 2002). A negative relation was observed in the regression analysis of the data between VFA and number of *M. es* cells in batch cultures (Fig. 3.7d). An increased concentration of VFA concentration upto 14 meq l^{-1} in the cultures was associated with low density of *M. es* (0.75×10^4 ml^{-1}). The increased VFA could be due to accumulation of acetate, which is a major metabolite in *Metopus* sp. (Biagini et al., 1998a). Short chain organic acids are found to be the major metabolic end products in the anaerobic ciliate, *Trimyema* also (Goosen et al., 1990a; Holler and Pfenning, 1991). It was found that the acetic acid concentration of above 0.01 M (equivalent to 10 meq l^{-1} VFA) could cause considerable reduction in the *M. es* count (Fig 3.7a). The VFA concentrations (> 0.05 M) also affected the activity of

endosymbiotic methanogens as detected by the absence of autofluorescence in *M. es* cells within 4 h.

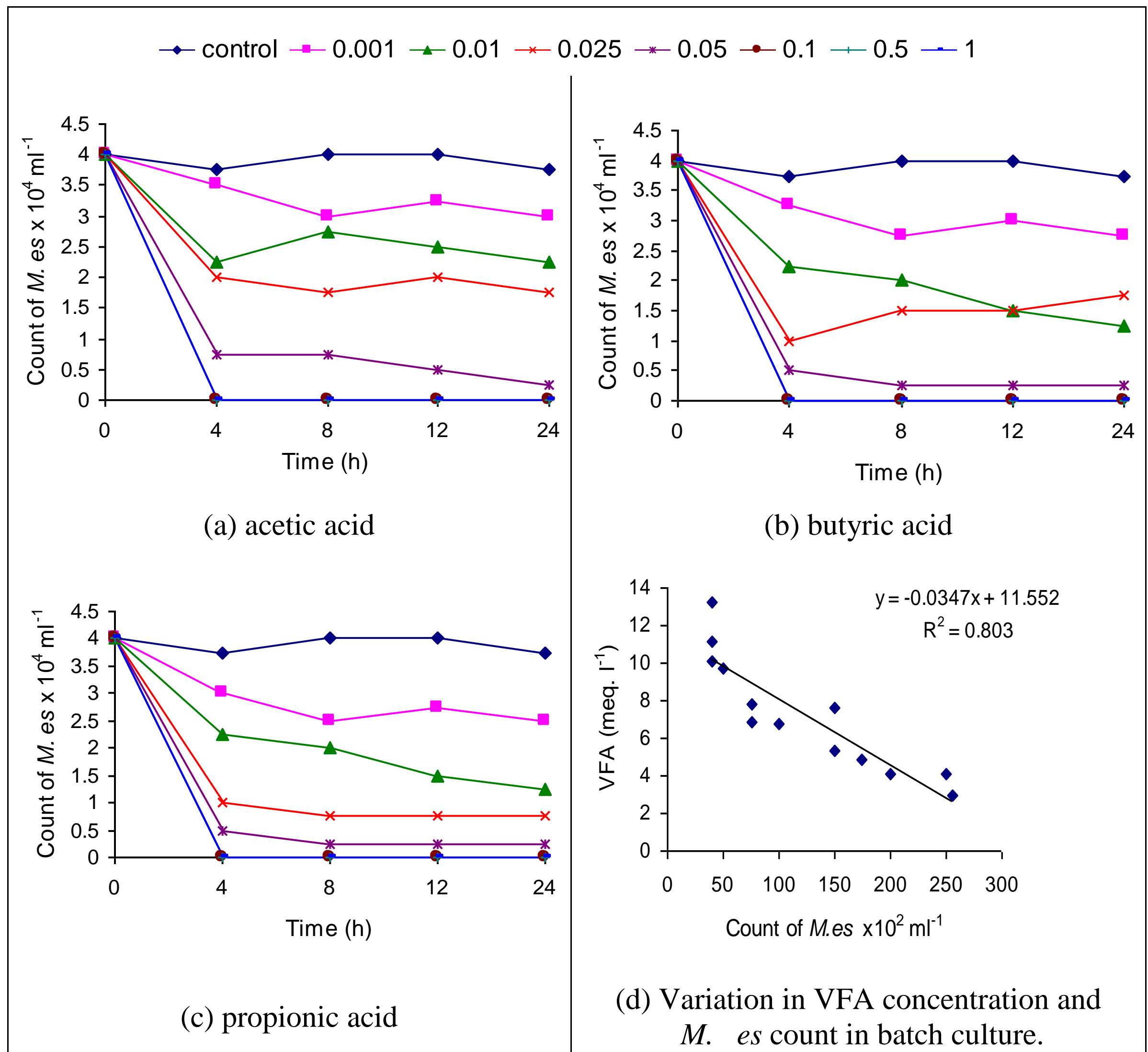


Fig. 3.7. Population of *M. es* in batch cultures added with varying concentrations of different VFAs. Control (0.0005M VFA) without added VFA.

It is presumed that volatile fatty acids in their undissociated form may freely permeate cell membrane. They will dissociate inside the cell, resulting in the lowering of intracellular pH. The excess protons produced then will be pumped out in

exchange of potassium ions to prevent the physiological changes. Because of this so called proton/potassium pump is an energy consuming process, less energy will be available, per unit of substrate consumed, for the synthesis of biomass and therefore the growth will be prevented (Tempest et al., 1968).

3.3.5 Oxygen tolerance studies of *M. es*

3.3.5.1 Toxicity tests with oxygen

The direct exposure to atmospheric oxygen affected *M. es* cells adversely (Fig. 3.8). It was observed that more than 80 % of the cells died on the first day and the remaining cells survived for four days with a reduced motility. However, characteristic morphological changes were developed in the survivors within 72 h. The cells became more prominently vacuolated as shown in Fig. 3.9. Such morphological changes on exposure to oxygen are well documented in many anaerobic protozoa like *Psalteriomons lanterna* (Broers et al., 1992), *Hexamita* (Biagini et al., 1997c) and *Polyplastron multivesciculatum* (Ellis et al., 1991).

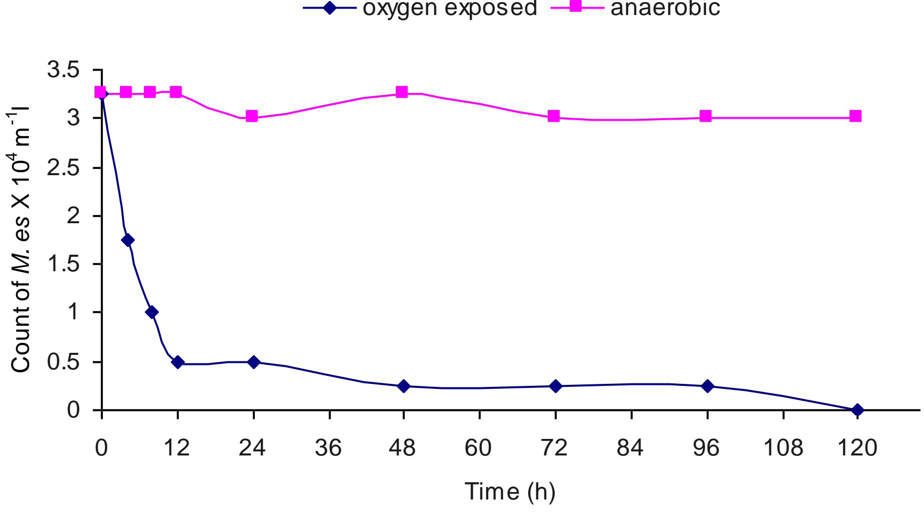


Fig. 3.8. Change in count of *M. es* on direct exposure to atmospheric oxygen.

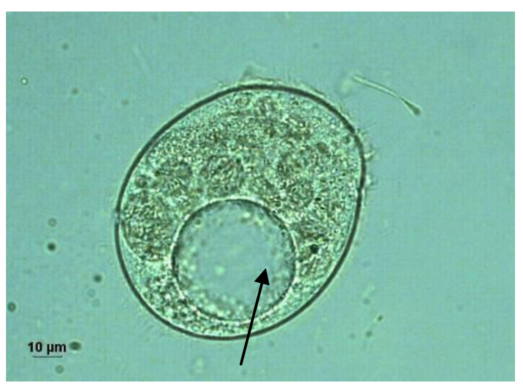


Fig. 3.9(a) Morphological changes observed in *M. es* on direct exposure to atmospheric oxygen, the arrow indicates the enlarged vacuole

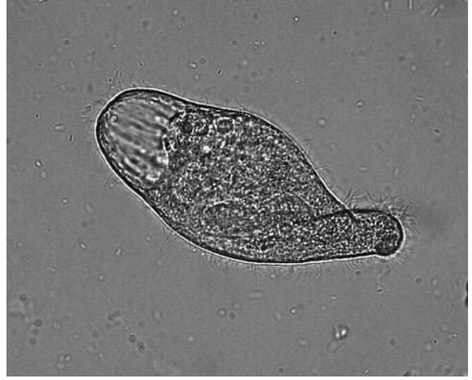


Fig. 3.9(b) The unexposed *M. es* in anaerobic culture

The anaerobic *Metopus* has been found to survive at modest levels of atmospheric oxygen as evidenced by the earlier reports (Fenchel and Finlay, 1990b; Finlay and Fenchel, 1991a). *Metopus contortus* from marine sediment has displayed considerable aero tolerance with the survival of some cells for two days on exposure

to atmospheric oxygen (Fenchel and Finlay, 1990b). *Metopus palaeformis* from municipal landfill material survived for about 6 days in presence of oxygen (Finlay and Fenchel, 1991a).

Our observation is also in agreement with the earlier reports. Further studies in *M. es* revealed that an oxygen level of more than 0.5 % is detrimental and led to the death of ciliates within 24 h of exposure (Fig. 3.10). Survival of *M. es* cells was observed only for oxygen levels up to 0.5 % with a reduction in its count from 3×10^4 ml^{-1} to 0.5×10^4 ml^{-1} within 24 h (Fig. 3.10). The survival rate of the ciliate was increased with less oxygen concentration as evidenced by 50 % and 16 % survival in oxygen levels of 0.1 % and 0.5 % respectively (Fig. 3.11).

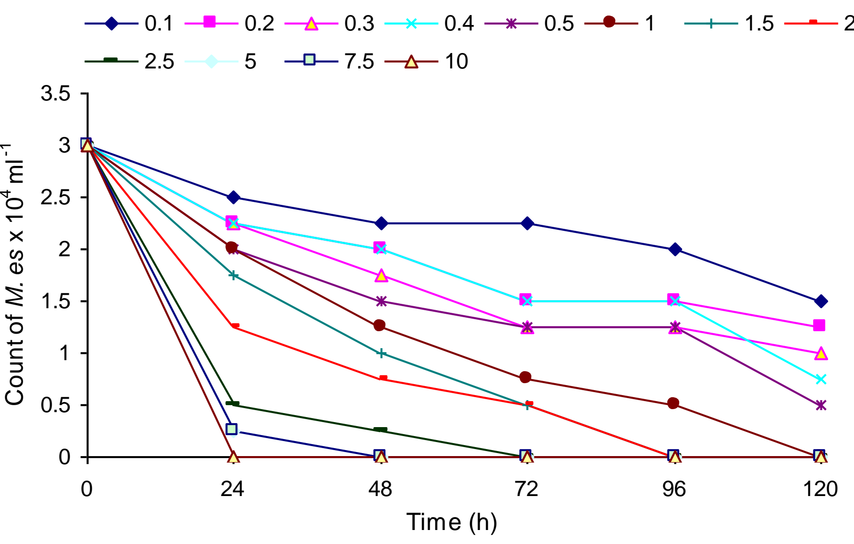


Fig. 3.10. *M. es* count in culture bottles incubated with different levels of headspace oxygen (0.1 - 10 %).

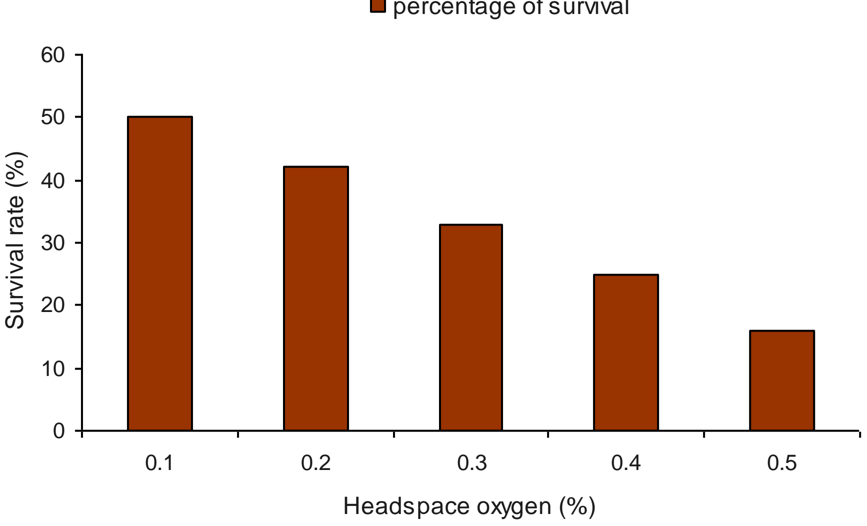


Fig. 3.11. Survival of *M. es* at different levels of oxygen exposure (0.1 - 0.5 %).

Anaerobic protozoa exhibit varied behavioural responses to oxygen. *M. es* exhibits a chemosensory response of clumping together on exposure to atmospheric oxygen (Fig. 3.12). The same phenomenon has been noticed in other anaerobic ciliates, *M. contortus* and *Plagiopyla* (Fenchel and Finlay, 1990b) and in the flagellate, *Hexamita* (Biagini et al., 1997c). This kind of chemosensory response to oxygen acts as a defense mechanism against oxygen toxicity by creating a micro environment of depleted oxygen tensions and it has been observed in motile anaerobes (Fenchel and Finlay, 2008). The chemosensory response of ciliates to oxygen can be better explained on the basis of kinetic response, a kind of chemotactic mechanism that allows protozoa to orient themselves in chemical concentration gradients. A kinetic response means that the motility pattern like swimming velocity and frequency of change in swimming direction is a function of ambient concentration of attractant or

repellent (Schnitzer et al., 1990). In this case, the ciliates remain at minimum motility to avoid the repellent (the oxygen) and eventually clump together in patches.

Fig. 3.12. Clumping of *M. es* cells resulted from direct exposure to oxygen.

3.3.5.2 Enzymatic detoxification of oxygen

Among the two important oxygen detoxification enzymes, superoxide dismutase catalyses the dismutation of the superoxide radical (O_2^-) to hydrogen peroxide (H_2O_2) and oxygen, whereas the catalase breaks down H_2O_2 to oxygen and water. In the assay, the intracellular extract of *M. es* did not have catalase activity whereas the surviving cells at oxygen concentrations of 0.1 - 0.5 % had a mean SOD activity of 1.38 U mg protein^{-1} (Table 3.6). The level of SOD in the cells was not found to be affected significantly by the varying concentrations of oxygen exposure. Such a relation between the SOD activity and oxygen concentrations was reported in the anaerobic bacterium, *Desulfovibrio gigas* (Fareleira et al., 2003). The SOD activity observed in *M. es* was not inhibited by both H_2O_2 and cyanide, which is characteristic for MnSOD.

Table 3.6. Mean SOD activity of *M. es* survived under different oxygen levels.

Headspace oxygen concentration (%)	SOD activity [a] (U mg $protein^{-1}$)
0.1	1.52 ± 0.4
0.2	1.38 ± 0.5
0.3	1.31 ± 0.9
0.4	1.34 ± 0.7
0.5	1.35 ± 0.7

[a]Mean ± SD (n=3).

Metopus ciliates perform an anaerobic metabolism and fail to grow under oxygen exposure at atmospheric pressure (Finlay and Fenchel, 1991a). But their survival and reestablishment of growth was observed after restoring the anaerobic condition (Finlay and Fenchel, 1991a). Also in the case of *M. es*, the survival of very few cells was observed at low oxygen concentrations (< 0.5 %). The important element of oxygen toxicity is due to the formation of the superoxide anion, peroxide and hydroxyl radicals produced by the oxidation of reduced compounds inside the cells by oxygen (Janssen et al., 1993). The formation of peroxide radicals by oxygen induction, in conjunction with the absence of catalase, could be a major reason for its growth inhibition and reduced survival of *M. es* on oxygen exposure. The primary targets of oxygen toxicity in anaerobic ciliates are hydrogenase, pyruvate: ferredoxin oxidoreductase and endosymbiotic methanogens (Fenchel and Finlay, 1990b). On

exposure to prolonged oxygen tension, these enzymes are inactivated and the reactive oxygen species produced probably surpass the detoxification capacity of the cell, which leads to irreversible damage to biomolecules and eventually cell lysis.

The results indicate that superoxide dismutase activity may be rendering protection to *M. es* against the toxic superoxide radicals generated on exposure to low oxygen tensions. The superoxide dismutase in *M. es* was identified as MnSOD, which is a common class of SOD widely distributed among prokaryotes, algae, bryophytes, obligate anaerobes, facultative aerobes and protozoa. The SOD activity (48.31 ± 9.00 U mg protein^{-1}) in anaerobic flagellate *Hexamita* sp. belongs to FeSOD, which helps this flagellate to grow at low levels of oxygen (Biagini et al., 1997c).

According to McCord et al., (1971), the aerotolerance depends on the presence of superoxide dismutase and not necessarily on the presence of catalase which can be the reason in the case of *M. es* also. In general, the superoxide dismutase activity reported in most of the anaerobic protozoa is higher than in *M. es* (Tables 3.6 and 3.7).

Table 3.7. SOD activity reported in some anaerobic protozoa.

Organism	SOD activity (U mg protein^{-1})	References
Loxodes	1.8 ± 0.16	Finlay et al., 1986
Hexamita sp.	48.31 ± 9.0	Biagini et al., 1997c
Trirtrichomonas foetus	5.5 ± 1.0	Lindmark and Müller, 1974

Monocercomonas sp.	1.5 ± 0.5	Lindmark and Müller, 1974
Entamoeba	3.06 ± 0.12	Samarawickrema et al., 1997
Acanthamoeba	3.2 ± 0.5	Choi et al., 2000
Trypanosoma cruzi	22.27 ± 1.72	Mateo et al., 2008
Leishmania tropica	23.00	Meshnick and Eaton, 1981
Plasmodium vinckei	13.60 ± 3.83	Bécuwe et al., 1993

It has been suggested that the higher SOD activity relates to a better oxygen tolerance, as the high SOD activity (48.31 ± 9 U mg protein^{-1}) in *Hexamita* sp. allows its growth in oxygen concentration up to 100 µM (Biagini et al., 1997c). Other anaerobic protozoa like *Loxodes* with low SOD activity (1.8 ± 0.16 U mg protein^{-1}) manages to survive but does not grow in presence of oxygen (Finlay et al., 1986). In the light of these data, SOD activity is responsible for the survival of *M. es* at low oxygen levels.

3.4 Conclusions

- The present study gives an idea about the level of tolerance exhibited by *M. es* to critical parameters involved in anaerobic digestion process. *M. es* possesses different level of tolerance to the parameters studied. This ability can be utilised for considering this ciliate as a bioindicator organism to assess the level of these factors at various stages of biomethanation process.
- The level of ammonia tolerance exhibited by *M. es* is comparable to those reported in aerobic ciliates, which helps it to grow in ammonia contaminated

environments. Furthermore, the higher growth of *M. es* upto 10 mg l^{-1} ammonia-N levels enables it to participate in anaerobic degradation of organic matter in wastewater treatment systems with ammonia.

- The sulphide tolerance level of *M. es* is lesser compared to other sulphide tolerant anaerobic ciliates, with its growth being significantly reduced in presence of 3 mg l^{-1} of sulphide-S.
- The order of heavy metal toxicity on *M. es* was found to be Hg > Cd > Cu > Pb > Cr > Zn with mercury being more toxic one in microgram quantities. The sensitivity exhibited by *M. es* to heavy metals makes it a suitable bioindicator organism to detect the presence of soluble heavy metals in anaerobic treatment systems.
- VFA concentration more than 0.05 M affects the growth of *M. es* in batch cultures. The VFA inhibition studies on the survival of *M. es* reveal that, like methanogens, anaerobic ciliates are also sensitive to the build up of VFAs in anaerobic systems.
- The isolated *M. es* is capable of surviving at oxygen tensions, ranging from 0.1 - 0.5 %, with a substantial decrease in growth. The presence of superoxide dismutase activity (1.31 - 1.52 U mg^{-1} protein) in *M. es* becomes significant since SODs are the first-line defense systems in various organisms against reactive superoxide radicals and forms one of the main components of the antioxidant defense system. The superoxide dismutase activity is not yet reported in the commonly found anaerobic ciliate, *M. es*. The presence of Mn

type of SOD in *M. es* is an important finding which may be significant for accounting its survival at low levels of oxygen.

- Being strict anaerobes, ciliates in the genus *Metopus* are widely used as bioindicator of anaerobic conditions. This study reveals that the various parameters important in biomethanation process affect the growth and survival of *M. es* in different extents. This makes *M. es* a suitable bioindicator organism whose presence or absence can indicate the level of these factors in anaerobic system.

CHAPTER 4

Endosymbiotic interactions in *Metopus es*

4.1 Introduction

Intracellular bacteria in certain sapropelic protozoa have been observed initially by staining experiments and described as symbionts or food reserves for ciliates (Liebmann, 1936). Many anaerobic ciliates reported from a variety of anoxic environments harbour ecto- and endosymbiotic bacteria (Fenchel et al., 1977; Llyod et al., 1996). Most of the endosymbiotic bacteria have shown to be methanogens (Fenchel and Finlay, 1992). These endosymbiotic methanogens can utilise H_2 to generate methane and this interspecies hydrogen transfer, from anaerobic protozoa to symbionts, becomes the basis of symbiotic relationship. The advantages for the host by the symbionts are not fully understood, but some larger ciliates like *Metopus contortus* and *Plagiopyla frontata* grow faster in the presence of symbionts (Fenchel and Finlay, 1991b; Fenchel and Finlay 1991c). Generation of methane by methanogenic archaea was reported in anaerobic ciliates by Fenchel and Finlay (1992). The ecological importance of ciliate and methanogen endosymbioses is difficult to estimate. However, this symbiotic relation is assumed to be responsible for up to 90 % of total methane production in marine sediments (Fenchel, 1993), whereas

only a minor contribution by methanogenic endosymbionts to total methanogenesis is reported in freshwater sediments (van Hoek et al., 2006).

Endosymbiotic methanogenic archaea have been detected in a diverse group of sapropelic protozoa like *Pelomyxa palustris* (van Bruggen et al., 1983; Embley and Finaly, 1993) and in ciliates like *Metopus*, *Trimyema*, *Plagiopyla*, *Cyclidium, Caenomorpha* and *Brachonella* (Fenchel et al., 1977; van Bruggen et al., 1983; Wagener et al., 1990a; Fenchel and Finaly, 1991a; Fenchel and Finlay, 1991b; Fenchel and Finlay 1991c; Fenchel and Finlay, 1992; Embley and Finlay, 1993; Esteban et al., 1993; Finlay et al., 1993; van Hoek et al., 2000). The endosymbionts of intestinal ciliates and free living freshwater ciliates belong to only two different taxa, namely the *Methanobacteriales* and *Methanomicrobiales* respectively (van Hoek et al., 2000). In *Metopus*, detailed studies on endosymbiotic methanogens are limited to only three species, *M. striatus*, *M. contortus* and *M. palaeformis* in which *Methanobacterium formicium*, *Methanoplanus endosymbiosus* and *Methanocorpusculum parvum* were identified as symbionts (van Bruggen et al., 1984; van Bruggen et al., 1986; Embley et al., 1992b; Embley and Finlay, 1993; Embley and Finlay, 1994). The nature and type of methanogenic endosymbionts in *M. es* are not clearly understood, although their presence throughout the cytoplasm of this ciliate was reported (Esteban et al., 1995). The present work aims to study the methanogenic endosymbionts in *M. es* using fluorescent probes specific for archaea and *Methanosaetaceae* in addition to direct microscopic examination.

4.2 Materials and methods

4.2.1 Organism and culture conditions

The isolated *Metopus es* cultured in ciliate mineral medium with 1% wheat powder under neutral pH at 35 ^{0}C were used for the experiments. Scanning electron micrographs were taken with the cells fixed in Schaudinn's fixative as described in section 2.1.2.2a. (chapter 2).

4.2.2 Direct examination of endosymbionts

The presence of methanogenic archaea was detected by means of epifluorescence microscopy according to Doddema and Vogels (1978). The live cells were observed directly under the fluorescent microscope at an excitation of 350 nm. In live cells, fluorescence faded away within one minute, but fixing with a mixture of 12 mg formaldehyde and 3 mg glutaraldehyde per ml was effective to retain the fluorescence upto 5 min (van Bruggen et al., 1983). Enumeration of methanogens was done by direct counting of autofluorescing symbionts in 20 *M. es* cells using Leica Qwin software and mean number was calculated. The ciliate biovolume was calculated with the aid of the method proposed by Sun and Liu (2003). Images were taken with a CCD camera attached to the microscope.

4.2.3 Methane production in *M. es* culture

M. es was cultured in 50 ml bottles sealed with butyl rubber septa by keeping 10 ml headspace (Narayanan et al., 2007). Samples were taken at regular intervals and ciliate counts were determined by direct counting in a Neubauer slide as described in

section 2.1.2.2.b. Methane concentration in the head space was determined at definite time intervals by gas chromatography (FISIONS 8000, FID, 2mm i.d.silica gel column, carrier gas 30 ml min^{-1}, oven 110 ^{0}C, detector 130 ^{0}C and injector 130 ^{0}C).

4.2.4 Whole cell Fluorescent In Situ Hybridization (FISH) and microscopic observations

The *M. es* cells were collected from culture by filtration and concentrated by centrifugation at 156 *g* for 2 min. The cells were then fixed with Schaudinn's fixative and subjected to FISH analysis using fluorescent labelled oligonucleotide probes: ARCH 915, complementary to the region of 16S rRNA (915-934) conserved in the domain Archaea (5′-GTGCTCCCCCGCCAATTCCT-3′) (Stahl and Amann, 1991), MX 825, complementary to the region of 16S rRNA (825-847) conserved in *Methanosaeta* (5′-CGCACCGTGGCCGACACCTAGC-3′) (Raskin et al., 1994) and EUB 338, complementary to a region of 16S rRNA (338-355) specific for the domain Bacteria (5′-GCTGCCTCCCGTAGGAGT-3′) (Amann et al., 1990). The probes were purchased from Integrated DNA Technologies (IDT, USA).

The standard protocol for FISH (Amann, 1995) was used with some modifications in the fixation step. The fixation of sample with paraformaldehyde was not followed as it altered the ciliate morphology and most of the cells ruptured even at half of the recommended concentration of paraformaldehyde (4 %). In this case, cells were fixed in Schaudinn's fixative and washed thoroughly with milliQ water to

remove all traces of fixative before proceeding to hybridization. The hybridization was performed on 14 well Hydrophobic Teflon Coated (HTC) slides (Cel-line, Erie Scientific Company, Germany) coated with gelatin (0.1 % gelatin in 0.01 % $KCr(SO_4)_2$) for adhesion of the specimen. After drying at 37 ^{0}C, slides were dehydrated sequentially in 80, 90 and 100 % (v/v) ethanol (3 min each) and dried at room temperature. Three hybridization buffers were prepared with NaCl, 0.9 M; Tris-HCl, 20 mM (pH 7.2); SDS, 0.01% and probe concentrations of 5 ng μl^{-1} for ARCH 915 and 2.5 ng μl^{-1} for both MX 825 and EUB 338. Formamide was added to the hybridization buffers in concentrations of 35, 50 and 20 % for ARCH 915, MX 825 and EUB 338 respectively (Stahl and Amann, 1995; Crocetti et al., 2006; Amann et al., 1990). 10 µl of hybridization buffer containing one of the three different oligonucleotide probes was added to each well and incubated at 46 ^{0}C for 2½ h in a closed hybridization oven (HB-500 Minidizer^{TM}, UVP, USA). Three washing buffers were also prepared with Tris-HCl, 20 mM (pH 7.2); SDS, 0.01 % and NaCl in the following concentrations: 39 mM for ARCH 915; 9 mM for MX 825 and 198 mM for EUB 338.

After washing the slides at specific washing conditions to remove the unbound probe (50 ^{0}C for 30 min for ARCH 915 and 48 ^{0}C for 30 min for both MX 825 and EUB 338), slides were rinsed with milliQ water and allowed to air dry. The counter staining was performed with DAPI (4′, 6-diamidino-2-phenylindole) solution (0.5 µg ml^{-1}) and mounted with Vectashield mountant (Vector laboratories, CA). The prepared

slides were examined under epifluorescent microscope equipped with CCD camera for imaging.

4.2.5 Isolation of endosymbiotic methanogens from *M. es*

M. es cells were collected with a micropipette, after repeated centrifugation (156 g for 2 min) and washing, cells were suspended in sterile 10 mM sodium phosphate buffer (pH 7.0). The endosymbionts were released by homogenization (Potter S Homogenizer, Sartorius, Germany) of the thoroughly washed and cleaned *M. es* cells. The homogenized cell suspension was inoculated into crimp cap bottle (Supelco, USA) containing sterile (autoclaved for 15 min at 121 ^{0}C) liquid medium having the following composition (g l^{-1}): KH_2PO_4, 0.2; NH_4Cl, 0.25; NaCl, 1.0; $MgCl_2.6H_2O$, 0.4; KCl, 0.5; $CaCl_2.2H_2O$, 0.15 (Mizukami et al., 2006) and lysozyme (1mg ml^{-1}) to prevent the growth of anaerobic bacteria other than methanogens (van Bruggen et al., 1984). The effectiveness of the washing procedure was tested by inoculating a comparable volume of the final washing fluid. The basal medium was supplemented with $NaHCO_3$, 30 mM; Na_2S, 0.5 mM; 2 ml trace element solution with the composition (mg l^{-1}):$ZnCl_2$, 70; $MnCl_2.4H_2O$, 100; $Na_2MoO_4.2H_2O$, 36; $CuCl_2.2H_2O$, 17; $FeCl_2.4H_2O$, 1500; $CoCl_2.6H_2O$, 190; H_3BO_3, 62; $NiCl_2.6H_2O$, 24; 1 ml of selenite and tungstate solution with the composition (mg l^{-1}): Na_2SeO_3. $5H_2O$, 3; $Na_2WO_4.2H_2O$, 4; NaOH, 500 and 1ml vitamin solution having the composition (mg l^{-1}):4-aminobenzoic acid, 4; D(+)- biotin, 1 and vitamin B_{12}, 50. Sodium acetate was prepared as a 1M stock solution, sterilized by autoclaving, and added to the basal

medium before inoculation at a concentration of 20 mM. Ampicillin and vancomycin (each at 30 mg l^{-1}) were added to the medium. The gas phase was N_2 and pH of the medium was adjusted to neutral range. Inoculated bottles were incubated in the dark without shaking at 30 ^{0}C. After fifteen days of incubation, growth and purity of the culture were examined by detecting autofluorescing cells having typical morphology of *Methanosaetaceae.*

4.2.6 Acetate production by *M. es*

Qualitative detection of acetate production in the *M. es* culture was done by Thin Layer Chromatography (TLC). The culture fluid filtered through 0.22 µm and intracellular extract of *M. es* was analysed for the presence of acetate at definite time intervals. The intracellular extract was obtained by disruption of ciliates in three cycles of freezing and thawing (-20 ^{0}C for 20 min and 35 ^{0}C for 10 min) followed by centrifugation at 5600 g for 15 min. The fluid was acidified with 6 M HCl and extracted with ether. The ether extract of 10 µl was spotted on silica gel G coated plates (Silica gel 60 F54, Merck, Germany) and developed in solvent system of n-butanol: methanol (1 : 2). The presence of acetate was detected as red spot formed after spraying with 1 % neutral ferric chloride in methanol: water (1 : 1). The starved *M. es* cells were served as the control. The amount of acetate in intracellular extract was quantified by ion chromatograph (Dionex ICS 1100; ARS column 4 mm; mobile phase, sodium carbonate and bicarbonate in the ratio 4.8 mM: 1 mM; flow rate, 1.5 ml min^{-1}). Cytochemical localization of hydrogenosome in *M. es* was done by staining with NBT according to Zwart et al., (1988).

4.2.7 Effect of oxygen exposure on endosymbionts in *M. es*

The effect of oxygen exposure on endosymbiotic methanogens in *M. es* was studied by exposing the *M. es* culture to different headspace oxygen levels (0.1 – 10 %) as described in section 3.2.6.1. of chapter 3. The autofluorescence inside the surviving *M. es* cells were checked at regular time intervals by direct examination of the cells under the epiflourescent microscope as given in section 4.2.2. The presence of methanogens in cysts of *M. es* was detected by FISH analysis with probe, ARCH 915.

4.2.8 Effect of endosymbiotic methanogens on *M. es* growth

The activity of endosymbiotic methanogens was blocked by using 2-bromoethane sulfonic acid (BES), a specific inhibitor of methanogenic archaea. *M. es* was grown in medium with BES (10 mM) to detect the effect of methanogens on its growth. The inhibitory activity of BES on endosymbiotic methanogens was tested by observing the characteristic autofluorescence inside the *M. es* cells as described in section 4.2.2. The growth of *M. es* in the absence of methanogens in BES added culture was monitored by regular counting and compared to the control culture without BES.

4.3 Results and discussion

4.3.1 Fluorescent endosymbionts of *M. es*

The epifluorescence microscopy technique showed the presence of methanogens directly by capturing the emission light from their specific cell components, the coenzyme F_{420} and compound F_{342}. This method enables the detection of methanogenic archaea even in complex ecosystems (Doddema and Vogels, 1978). F_{420} is a 5-deazaflavin analog reduced by hydrogenase in methanogens and detected by its blue green fluorescence at 420 nm (Eirich et al., 1982). The cofactor F_{342} is a pterin compound which is involved in the oxidoreduction electron transport and emits blue light at 342 nm (Doddema and Vogels, 1978). The fluorescent methanogens in *M. es* cell are presented in Figures. 4.1 - 4.4.

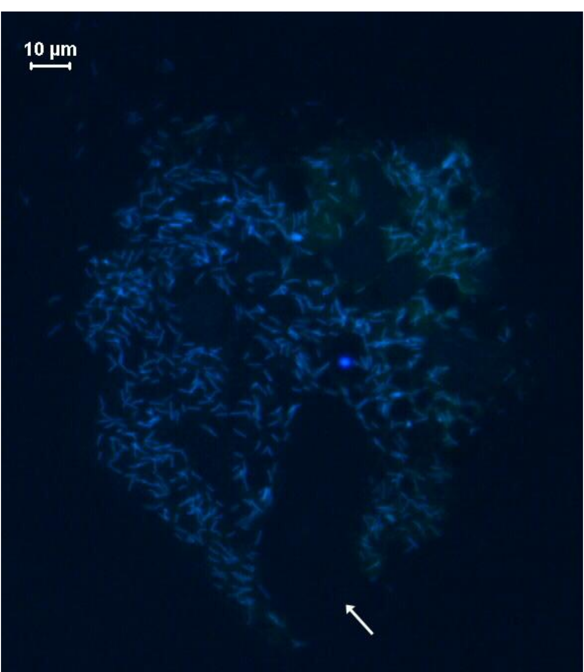


Fig. 4.1. Endosymbiotic methanogenic archaea in *M. es*: the portion indicated byarrow denotes the macronucleus.

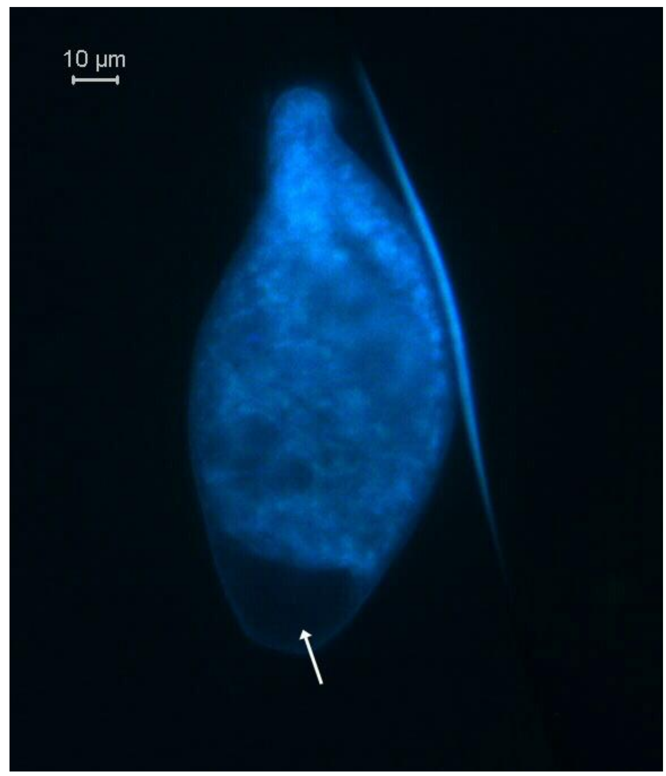


Fig. 4.2. Fluorescing region of methanogenic archaea in a fixed cell of *M. es* with the contractile vacuole (indicated by arrow) devoid of symbionts.

As evidenced from the Figures 4.1 and 4.2, the *M. es* cell possesses endosymbiotic methanogens, which were about to be released on rupture of ciliate cell during observation (Fig. 4.1). In Fig. 4.2, the intact fixed cell shows the fluorescing region of methanogens and the non fluorescing contractile vacuole region. The fluorescent bacteria were seen abundantly throughout the cytoplasm of the ciliates except in the regions of the macro and micro nuclei and the contractile vacuole (Figures 4.1 and 4.2).

The fluorescing methanogens were 2.9 - 4.4 µm long slender rods (Fig. 4.3). The average number of methanogens is 792 ± 12 in a *M. es* cell having the biovolume of 3.44×10^5 μm^3 (Fig. 4.4). In the earlier studies, symbiotic rods ranging from 2 - 7 µm were reported as symbionts in *M. striatus* (van Bruggen et al., 1984) whereas *M. palaeformis* harbours polymorphic bacterial endosymbionts (Finlay and Fenchel, 1991a).

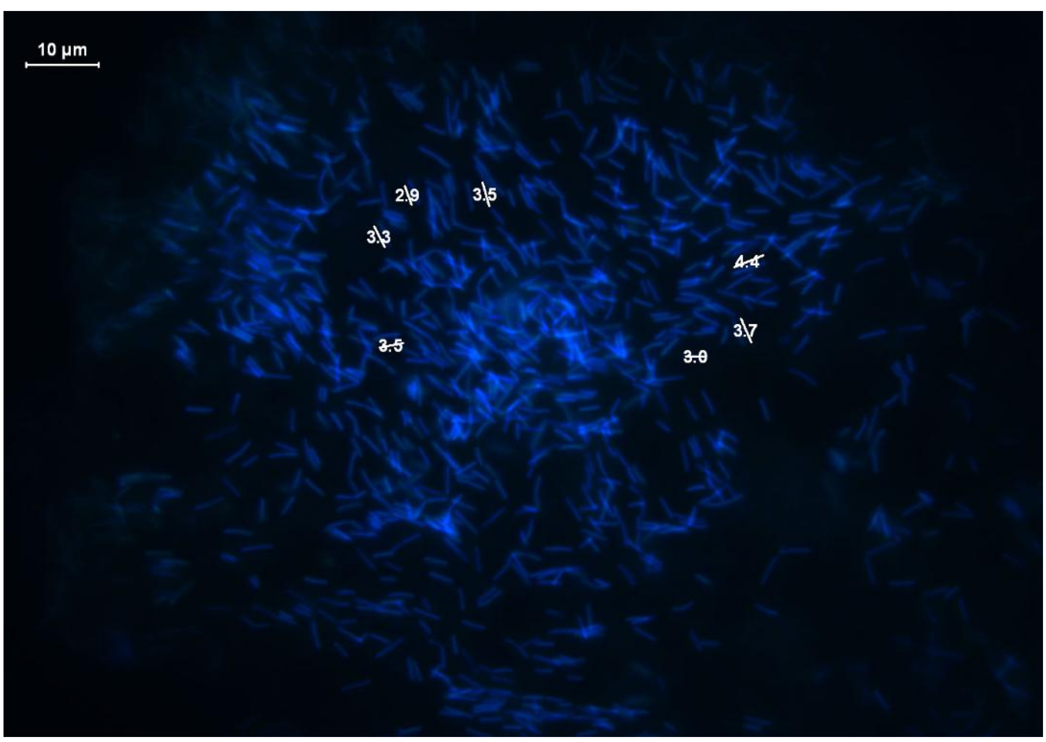


Fig. 4.3. Methanogenic symbionts (3.4µm mean length) released from *M. es.*

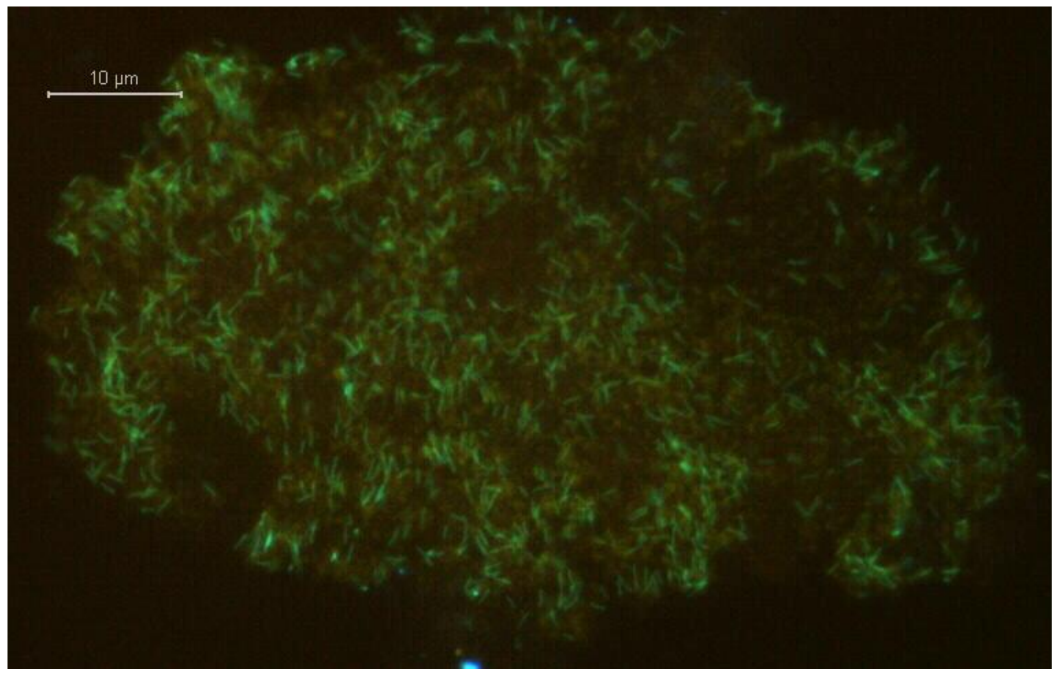


Fig. 4.4. The methanogenic endosymbionts observed in a *M. es* cell.

Most of the anaerobic protozoa are associated with bacteria. In the rumen of cows and sheep, the entodiniomorph ciliates exhibit an episymbiotic association with methanogenic archaea (Vogels et al., 1980). These bacteria are attached to the surface of the ciliate cell whereas sapropelic ciliates and the giant amoeba *Pelomyxa palustris* have endosymbiotic bacteria (van Bruggen et al., 1983). In sapropelic environments, free-living methanogenic archaea constitute only a smaller number compared to methanogenic archaea present in the anaerobic protozoa (van Bruggen et al., 1983). The anaerobic amoeba *Pelomyxa palustris* and *M. striatus* isolated from such an environment found to contain 9.8×10^8 and 2×10^3 methanogens per cell respectively (van Bruggen et al., 1983). The number of methanogens reported in *Metopus*

palaeformis and *Plagiopyla frontata* are 360 and 3500 per cell respectively (Fenchel and Finaly, 1992). In the case of *M. contortus,* the number of methanogens per ciliate ranges from 6×10^3 -10×10^3 (Fenchel and Finaly, 1992) where as *M. es* possesses an average number of 637 ± 19 methanogens $cell^{-1}$ (Schwarz and Frenzel, 2005). The number of methanogens per ciliate in the present study (792 ± 12) is comparable to the previous reports on methanogenic symbionts.

Symbiotic methanogens of different genera are found in *M. palaeformis*, *M. striatus* and *M. contortus* (Embley and Finlay, 1993; Embley and Finlay, 1994). Anaerobic ciliates harbor redox organelles known as hydrogenosomes which are believed to be modified mitochondria generating ATP, hydrogen and carbon dioxide from the decarboxylation of pyruvate (Biagini et al., 1998a). Through the symbiotic association, methanogens utilise these metabolic products for methane production and help to maintain a low internal hydrogen pressure inside the ciliate cell (Fenchel and Finlay, 1992). The occurrence of hydrogenosomes was reported in various anaerobic ciliates (Finlay and Fenchel, 1989) and both *M. palaeformis* and *M. contortus* were found to harbour these redox organelles (Finlay and Fenchel, 1991a; Biagini et al., 1997b).

4.3.2 Methane production in *M. es* culture

Fig. 4.5 depicts the growth of *M. es* in batch culture and methane production in the corresponding period. It was found that maximum methane production rate of 0.85 pmol $ciliate^{-1}$ h^{-1} was observed during the early stages of the exponential

phase and remained almost in that range during the entire exponential growth period. Then the rate of methane production decreased significantly during the later period of growth in batch culture. Since anaerobic ciliates have low growth efficiency, it is difficult to maintain a high population density in batch cultures and it could be a possible reason for the maximum methane production rate observed at the particular period of growth characterized by highest ciliate count.

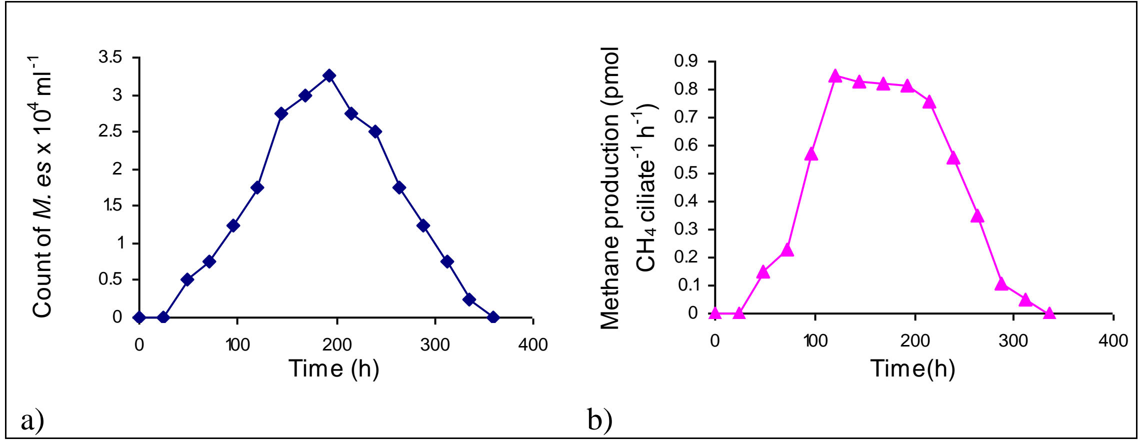


Fig. 4.5. (a) Growth of *M. es* in batch culture and (b) measured methane production rate.

Methane production rates vary in different anaerobic ciliates depending on the number of endosymbiotic methanogens present in them. A single ciliate with 1000 - 5000 methanogens was able to produce 1 - 5 pmol methane h^{-1} (van Hoek et al., 2006). Rumen ciliates carrying a higher number of methanogens produce more methane (83 - 250 pmol CH_4 ciliate^{-1} h^{-1}) (Kisidayova et al., 2000).

The characteristics of *M. es* in the present study are summarized in Table 4.1. It is possible to calculate the methane production rate of endosymbiotic methanogen by dividing the maximum methane production rate of single *M. es* by the average number of methanogens (792) per cell.

Table 4.1. Characteristics of *M. es* and its methanogenic endosymbionts.

Characteristics studied	Values
M. es biovolume (μm^3)	3.4×10^5
Number of methanogens per cell	792 ±12[a]
Single methanogen cell volume (μm^3)	0.35
Total methanogens volume (μm^3)	277.2
Volume of methanogen in % of *M. es* biovolume	0.8
Maximum rate of methane production by ciliate (pmol CH_4 ciliate^{-1} h^{-1})	0.85
Methane production rate by methanogen (fmol CH_4 methanogen^{-1} h^{-1})	1.07

[a] the value is obtained by taking the average of methanogenic endosymbionts in 20 *M. es* cells.

It was observed that methane production by symbiotic methanogen is about 1 fmol CH_4 methanogen^{-1} h^{-1} which is close to the reported value of 0.97 fmol methane methanogen^{-1} h^{-1} in *M. palaeformis* (Schwarz and Frenzel, 2005). Earlier studies on *Metopus* revealed a lower number of methanogens than in rumen ciliates, but the number of methanogenic symbionts was positively correlated with average volume of different *Metopus* species (Schwarz and Frenzel, 2005). *M. contortus* with 1000 - 6000 methanogens per cell produces methane at a rate of 8 pmol ciliate^{-1} h^{-1}

whereas *M. palaeformis* with 360 endosymbiotic methanogens produce only 0.35 pmol methane ciliate^{-1} h^{-1} (Fenchel and Finlay, 1992). The methane production rate of 0.85 pmol CH_4 ciliate^{-1} h^{-1}obtained for *M. es* makes it a significant contributor in biomethanation process.

4.3.3 In Situ Analysis using Fluorescent oligonucleotide probes

Among three oligonucleotide probes used in the study, ARCH 915 and MX 825 showed positive hybridization within the *M. es* cells while cells hybridized with EUB338 did not show any fluorescence. This confirms that *M. es* harbours archaea, particularly the family *Methanosaetaceae*, as the major symbionts. The host ciliate with fluorescent archaeal endosymbionts on hybridization with ARCH 915 is shown in Fig. 4.6. It can be concluded from the autofluorescence study and the positive hybridization with ARCH 915 that the symbionts in *M. es* are methanogens in the domain archaea.

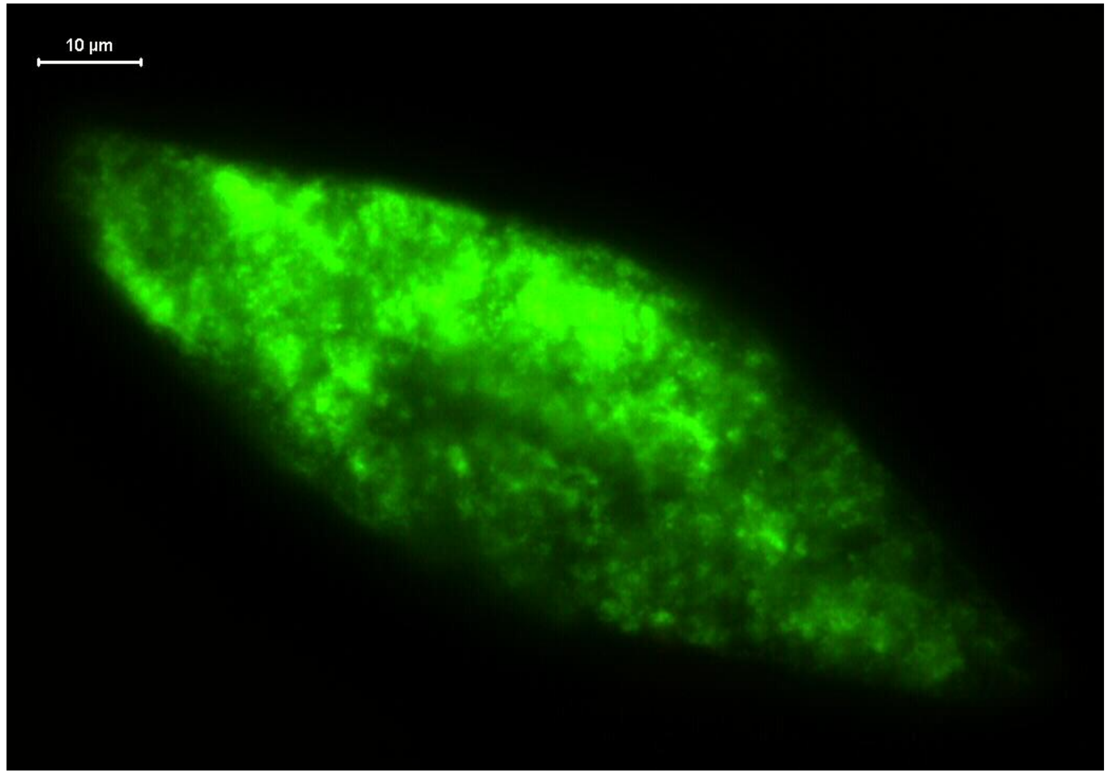


Fig. 4.6. FISH image of *M. es* on hybridization with ARCH 915

The *Metopus* cell with *Methanosaeta* showing red fluorescence on hybridizing with MX825 is given in Fig. 4.7. Till date, the reported endosymbiotic methanogens in sapropelic ciliates are from three different genera; *Methanobacterium, Methanocorpusculum, Methanoplanus* and *Methanolobus* in various ciliate genera like *Metopus, Trimyema, Cyclidium* and *Plagiopyla* (Embley and Finlay, 1993; Embley and Finlay, 1994). The presence of *Methanosaetaceae* as endosymbionts in anaerobic ciliates is confirmed for the first time in the present study.

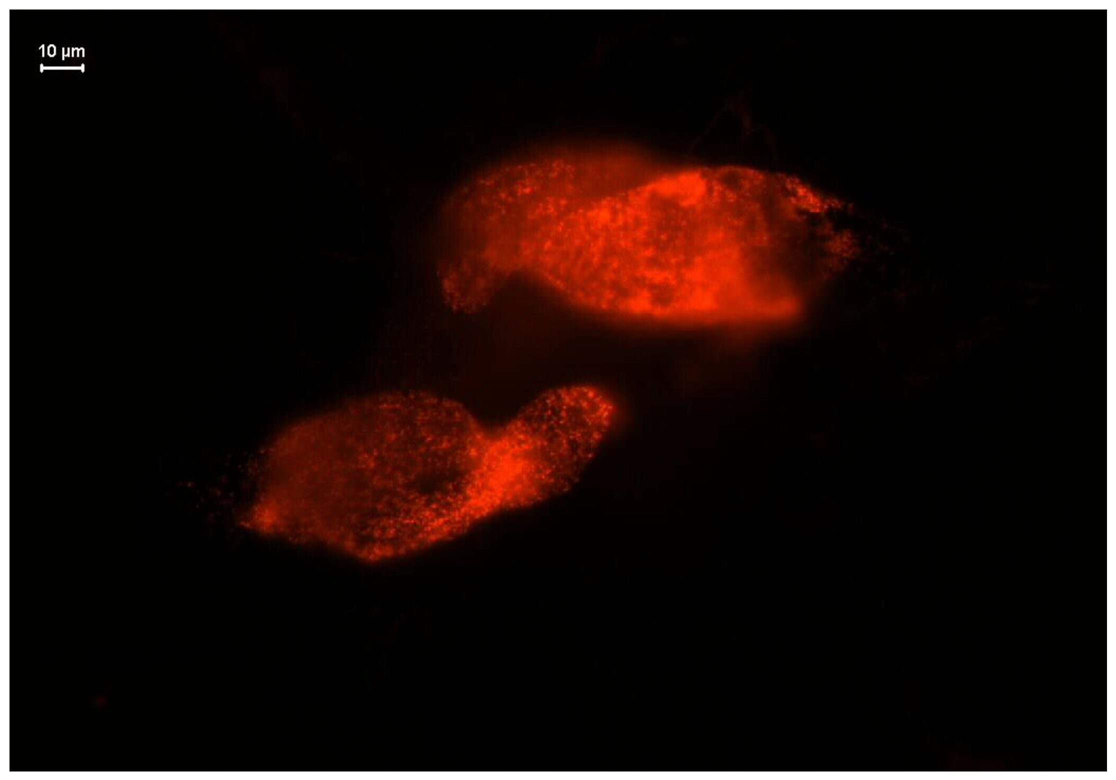


Fig. 4.7. FISH image of *M. es* on hybridization with MX 825

In the case of *M. es*, the presence of endosymbiotic methanogens was reported by Esteban et al., (1995). There is no further information about the nature of the endosymbionts whereas some other studies have been done in congeneric ciliates like *M. palaeformis, M. striatus* and *M. contortus*. *M. contortus* contains *Methanocorpusculum parvum* and *Methanoplanus endosymbiosus* as endosymbionts (Embley and Finlay, 1993). *Methanobacterium formicium* is found as endosymbiont in *M. striatus* (Embley and Finlay, 1994) while *M. palaeformis* harbors endosymbiotic *Methanobacterium* sp. that is closely related to, but distinct from, *Methanobacterium formicium* (Embley et al., 1992a).

The presence of *Methanosaeta* as endosymbiont in *M. es* was further confirmed by performing MX 825 hybridization with released endosymbionts from the homogenized *M. es* cells. It also showed the presence of rod shaped cells showing fluorescence as seen in Figures 4.8a-b. The culture grown in anaerobic medium by inoculating homogenized *M. es* cells also showed the presence of long autofluorescing rods (Fig. 4.9). These rods had mean length of 3.6 µm which is almost in the same range of autofluorescing rods obtained from ciliate (Fig. 4.3). The possibility of *Methanosaeta* as ectosymbionts on *M. es* was ruled out by electron microscopy, since such bacteria could not be detected in Scanning Electron Micrograph (SEM) (Fig. 4.10), as the ciliate was provided with the same kind of pretreatments, fixation and washing for both SEM and FISH.

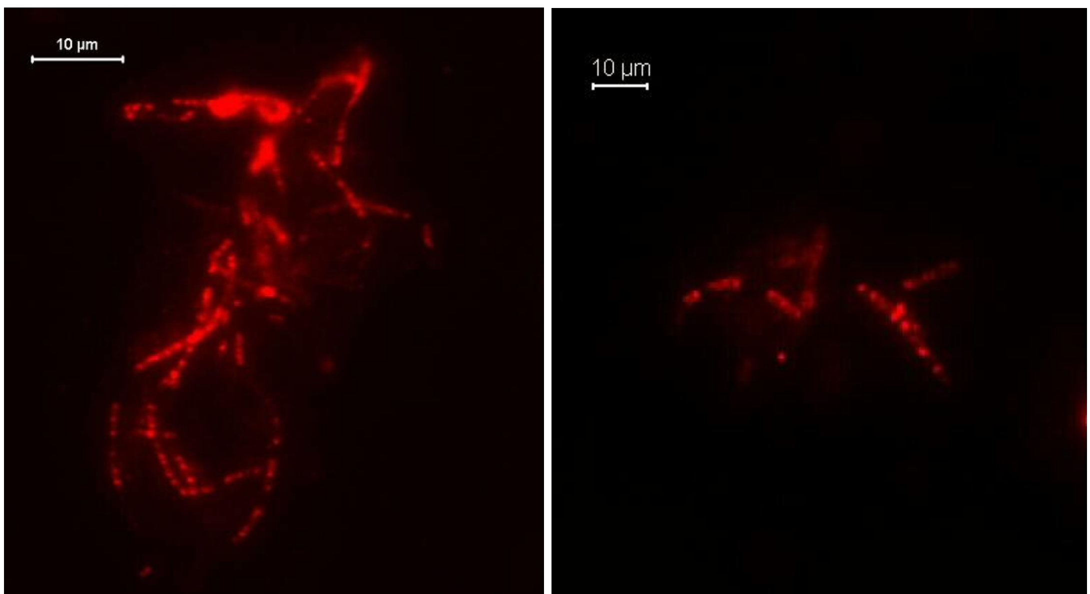


Fig. 4.8a-b. Fluorescing long rods on hybridization with MX 825 in *M. es* cells after homogenization to release the endosymbionts.

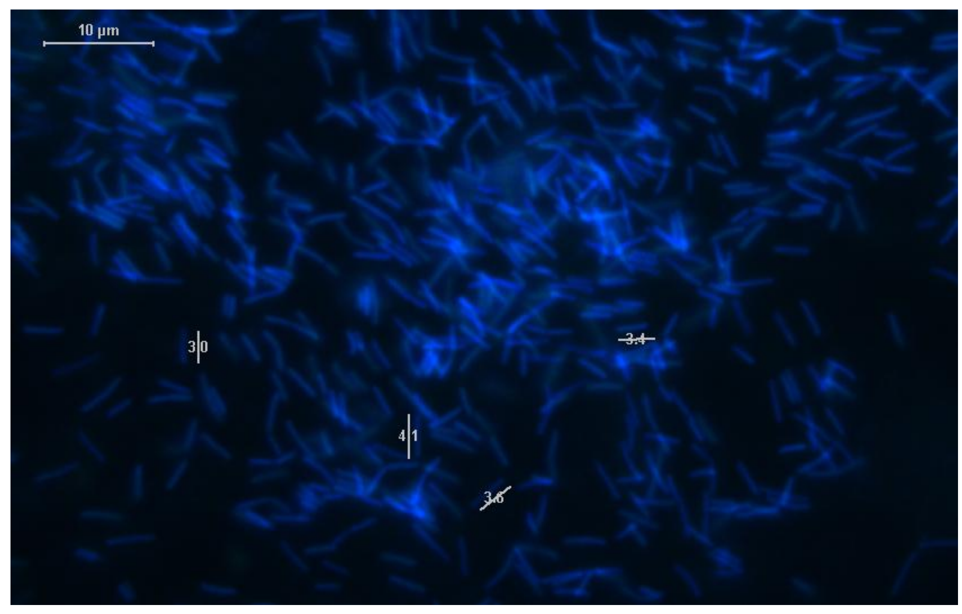


Fig. 4.9. Autofluorescing long rods in anaerobic culture on inoculation with homogenized *M. es* suspension.

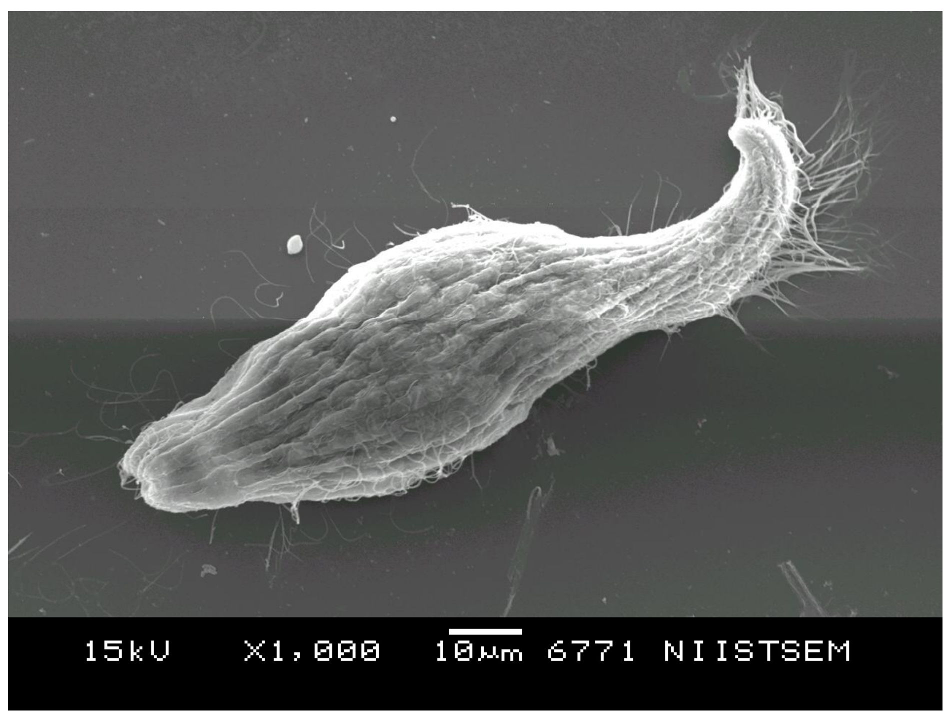


Fig. 4.10. Scanning electron micrograph of *M. es* devoid of any external associates.

4.3.4 Acetate production by *M. es*

The presence of *Methanosaeta* as endosymbionts is interesting because of their characteristic acetotrophism. Observable level of acetate was detected in the *M. es* culture fluid during the exponential growth stage from the 9th -10th day as revealed by the presence of a red spot on TLC plate (Fig. 4.11). In early and later stages of growth, acetate was not detected, probably due to the amount excreted to outside would be less than the detectable range in TLC analysis. However, the intracellular extract of *M. es* showed the presence of acetate from early exponential phase throughout the growth period (Fig 4.12). The acetate was not detected within and outside of the cells maintained free of added carbon source (starved cells).

Fig. 4.11. Extracellular acetate in *M. es* culture (the number corresponds to the age of culture in days).

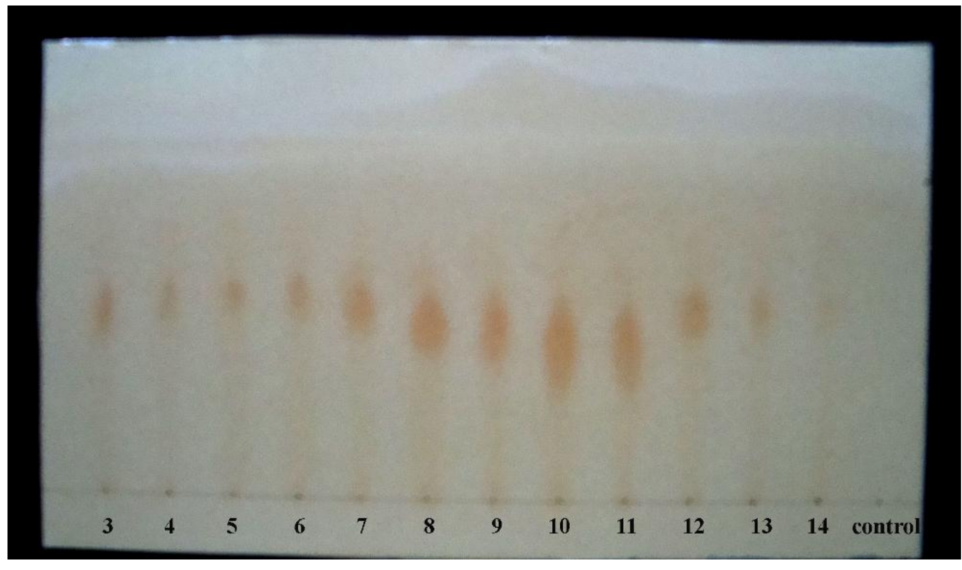


Fig. 4.12. Qualitative detection of intracellular acetate in *M. es* extract (the number corresponds to the age of culture in days).

The amount of acetate detected in *M. es* extract was given in Fig. 4.13. The amount of acetate in *M. es* extract ranged from 20.7 - 58.95 ppm during the exponential growth phase of ciliate. Higher amount of acetate (58.95 ppm) was detected in cells collected on 10th day.

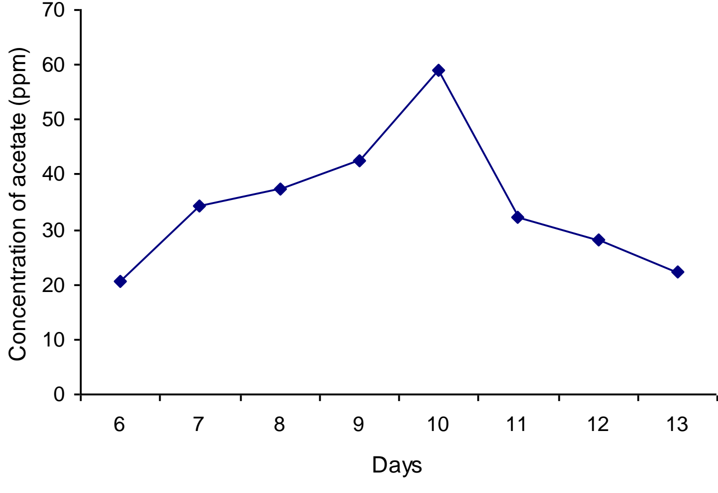


Fig. 4.13. Quantitative detection of intracellular acetate in *M. es* extract.

The *M. es* cells were found to produce intracellular hydrolytic enzymes such as amylase, cellulase and lipase in batch culture (chapter 5). This suggests that *M. es* produces acetate from intracellular digestion of a complex carbon source obtained through its particulate feeding. The acetate produced by *M. es* could be utilised by the harboured *Methanosaeta* and converted to methane.

So far, there are no reports on the occurrence of *Methanosaeta* as endosymbionts in anaerobic ciliates. *Methanosaeta* species are distinguished from members of the other genera by their exclusive use of acetate as a substrate for producing methane and by their rod-shaped cells, instead of the coccoid or pseudosarcinal cells of other aceticlastic methanogens, such as *Methanosarcina* species. These rod shaped cells form long sheathed filaments that often form floc-like aggregates in culture. In addition, in contrast to *Methanosarcina* species, *Methanosaeta* strains are favored in environments with low concentrations of acetate (Mizukami et al., 2006). This is confirmed by the present study that the endosymbiotic methanogens inside the *M. es* cells help to manage the acetate formed from anaerobic degradation of organic matter.

The symbiotic relation between this aceticlastic methanogens and *M. es* can be described on the basis of earlier reports on acetate production by anaerobic ciliates. The accumulation of acetate was noticed in a *M. palaeformis* culture on comparison with a ciliate free culture (Biagini et al., 1998a). The increased acetate level in ciliate

culture can be due to the presence of hydrogenosomes, which are capable of fermenting pyruvate into acetate and hydrogen (Fenchel and Finlay, 1992). In most anaerobic ciliates, endosymbiotic methanogens are found in close proximity of hydrogenosomes by which they can convert the hydrogenosomally produced acetate, hydrogen and carbon dioxide to methane and water (Biagini et al., 1997b). The presence of hydrogenosomes resembling mitochondria was reported in both *M. palaeformis* (Finlay and Fenchel, 1989) and *M. contortus* (Biagini et al., 1997b). The presence of hydrogenosome in *M. es* was confirmed by the formation of blue color in cytoplasm on staining with NBT (Fig. 4.14). This further indicates the occurrence of hydrogenosomal metabolism in *M. es* leading to the secretion of acetate outside these organelles, which is utilised by the endosymbiotic *Methanosaeta* species.

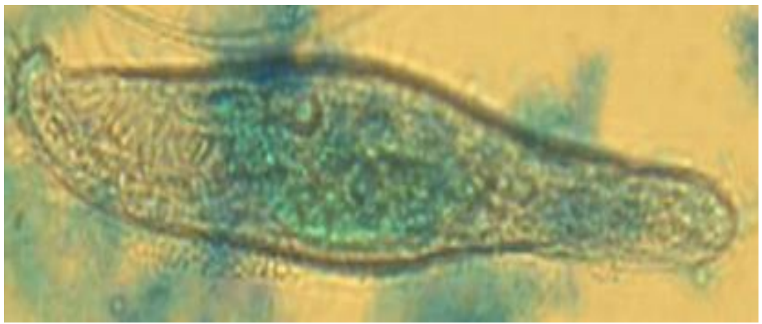


Fig. 4.14. Cytochemical localization of hydrogenosome in *M. es*.

The dependence of endosymbionts on fermentation products of ciliates has already been reported in *Trimyema compressum* (Goosen et al., 1990a; Wagner et al., 1990a; Holler and Pfennig, 1991). *Methanosaeta* always prefer a low acetate concentration (< 1mM) for its growth (Zinder, 1993). Since this culture has low

bacterial count due to antibiotic treatment, acetate production could occur mainly from *M. es* cells. The amount of acetate in intracellular extract of *M. es* ranges from 0.35 – 1 mM. In such a condition, the presence of *Methanosaeta* in *M. es* is a possible indication of its dependence on the low level of acetate produced by the ciliate.

4.3.5 Effect of oxygen on endosymbiotic methanogens of *M. es*

Methanogens are extremely sensitive to atmospheric oxygen due to oxygen liability of their enzymes involved in methanogenesis. In this experiment, the oxygen sensitivity of endosymbiotic methanogens were tested by observing autofluorescence inside *M. es* exposed to different oxygen levels (0.1 - 10 %). The autofluorescence of endosymbionts in *M. es* was affected by oxygen exposure. The fluorescence was observed up to an oxygen level of 0.5 %, but higher oxygen concentrations (1 - 10 %) and longer exposure caused its disappearance (Table 4.2).

Table 4.2. Decrease in methanogenic autofluorescence in *M. es* at different oxygen levels with time.

Exposure time (h)	Fluorescence at different oxygen levels (%)						
	0.1	0.2	0.3	0.4	0.5	0.6	0.7
2	+	+	+	+	+	+	+
4	+	+	+	+	+	-	-
6	+	+	+	+	+	-	-
8	+	+	+	+	+	-	-
10	+	+	+	+	+	-	-
12	+	+	+	+	+	-	-
24	+	+	+	+	+	-	-

+ indicates normal fluorescence; - indicates fluorescence absent.

This results from the inactivation of F_{420} hydrogenase enzyme complex by oxygen and presumably indicates inactivation of methanogenesis (Oremland, 1988). It is observed as a temporary effect as the fluorescence reappeared in a few hours on restoring the anaerobic condition. Restoring of fluorescent methanogens during the subsequent growth of surviving *M. es* cells was observed in anaerobic medium. These cells possessed a similar number of endosymbionts (792 $ciliate^{-1}$) as cells from an oxygen free control culture. The autofluorescing methanogens of *M. es* exposed to different oxygen levels is given in Figures 4.15a-c. Despite the change in ciliate morphology, the fluorescing endosymbiotic methanogens of *M. es* was intact after

exposure to oxygen at lower concentrations (< 0.5 %) (Fig. 4.15b-c) similar to those observed in the unexposed anaerobic cells (Fig. 4.15a).

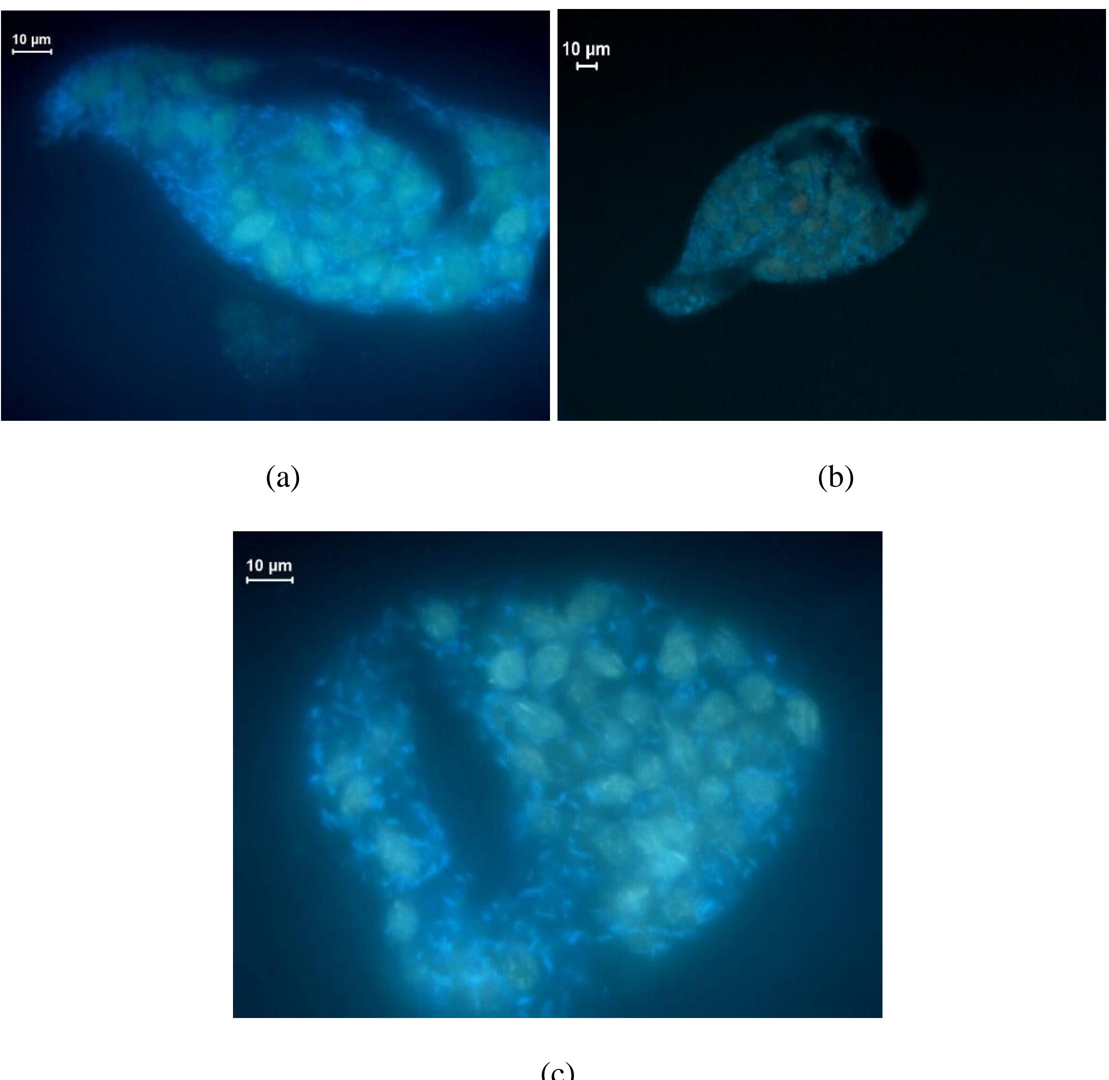


Fig. 4.15. Autofluorescing endosymbiotic methanogens inside *M. es* kept under different oxygen tensions.

a) Anaerobic condition b) Exposed to oxygen concentration > 0.5% for 1 h c) Exposed to oxygen concentration > 0.5% for 2 h.

The methanogens are retained within the cysts of *M. es* that formed on exposure to higher oxygen tension (1%). The fluorescing methanogens could be seen inside the *M. es* cyst (Fig.4.16) as they were protected from oxygen tension.

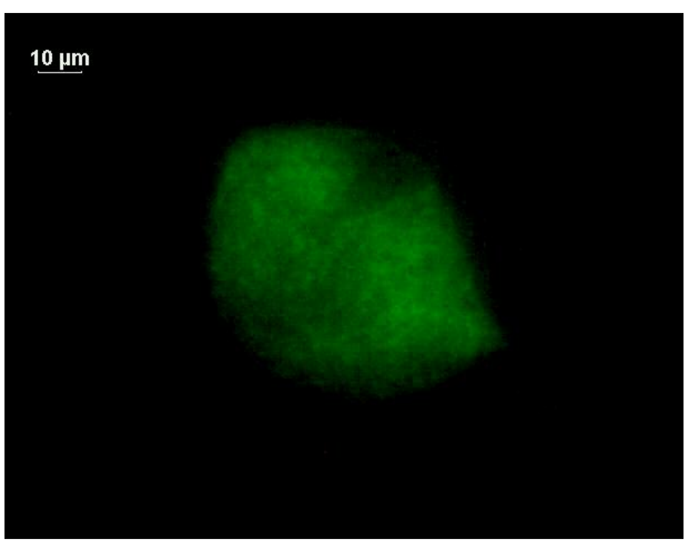


Fig. 4.16. Methanogenic archaea inside *M. es* cyst on hybridization with ARCH 915

At higher oxygen concentrations, oxygen may diffuse into the *M. es* cell and can inactivate the methanogens which are extremely sensitive to oxygen (Oremland, 1988). The activity of methanogens was found to be suppressed at very low oxygen concentrations (> 30 nM); however, that suppression was reversible and the degree of reversibility was reported as a function of exposure duration (Gerritse and Gottschal, 1993). Long term exposure to high oxygen levels could adversely affect methanogens by inactivating the F_{420} hydrogenase enzyme complex (Oremland, 1988)

The disappearance of autofluorescence in methanogens on oxygen exposure can result from the conversion of cofactor F_{420} to F_{390} (Kiener et al., 1988). On exposure to oxygen, a phosphodiester bond is formed between the phosphate group of adenosine 5´-monophosphate or guanosine 5´-monophosphate and the 8-hydroxy group of deazaflavin ring in the F_{420}. These F_{390} derivatives have a maximum absorbance at 390 nm and do not react with the F_{420} dependant hydrogenase and exhibit very little fluorescence at neutral pH. This fluorescence quenching at pH 7.0 may be due to stacking between purine and deazaflavin or due to the solvation of the 8-hydroxyphosphodiester anion (Hausinger et al., 1985). On restoring anaerobic condition, these F_{390} derivatives are capable of converting back to F_{420} (Kiener et al., 1988); thus retaining the fluorescence.

The endosymbionts of other *Metopus* species have also been reported to possess this kind of fluorescence quenching on exposure to oxygen. It was reported that except the temporary effect, exposure to oxygen produces no lasting damage in *M. palaeformis* and its symbionts which was found normal after restoring the anaerobic conditions (Finlay and Fenchel, 1991a). The endosymbiotic methanogens of *M. contortus* also did not suffer permanent damage on oxygen exposure and were capable of growing after retaining the anaerobic conditions (Fenchel and Finlay, 1990b). The regaining of fluorescence on establishing the anaerobiosis together with the maintenance of similar number of endosymbionts per *M. es* cell indicates that the

viability of methanogens was not permanently affected by short term oxygen exposure.

4.3.6 Effect of endosymbiotic methanogens on *M. es* growth

The endosymbiotic methanogenic activity was blocked by adding 2-bromoethane sulfonic acid. This is a bromine analogue of coenzyme-M and interferes with methanogenesis by inhibiting the methyl coenzyme-M reductase in methanogens (Balch and Wolfe, 1979a). The autofluorescence inside *M. es* cells disappeared within 48 h of BES addition to the medium indicating the inactivation of endosymbionts. When BES was added to the cultures, total count and growth rate of *M. es* were reduced (Fig. 4.17). The normal *M. es* cells could grow upto a number of 3.25×10^4 ml^{-1}, but its number was reduced to 2×10^4 ml^{-1} in BES added culture. It is estimated that the population of endosymbiont free ciliate was reduced by 34 % when compared to normal cells with endosymbionts. Growth rate was also reduced from 0.013 h^{-1} in normal cells to 0.009 h^{-1} in endosymbiont free ciliates. This corresponds to 30 % reduction in growth rate in the absence of endosymbionts in *M. es*.

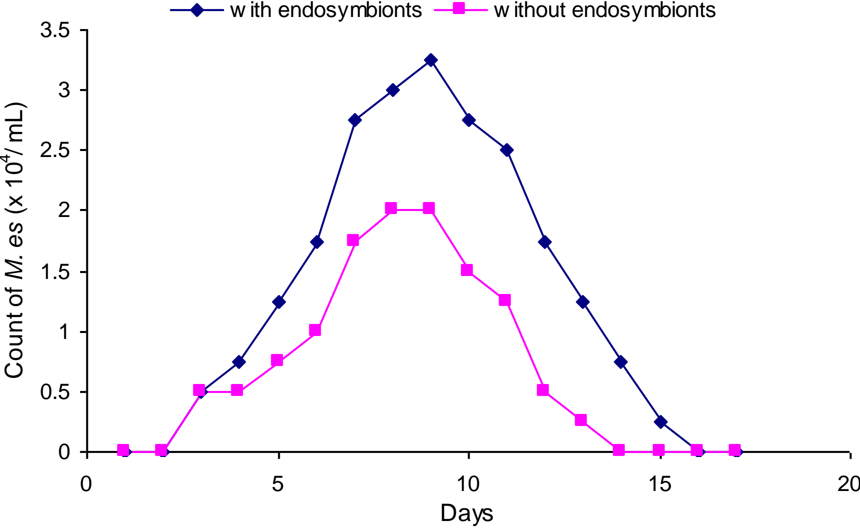


Fig. 4.17. Comparative growth pattern of *M. es* cultures with and without endosymbiotic methanogens.

The significance of endosymbiotic methanogens for the growth efficiency of ciliates was studied in two anaerobic ciliates, *Plagiopyla frontata* and *M. contortus*. The absence of endosymbiotic methanogens has reduced growth rate and yield by approximately 30 % in both the ciliates (Fenchel and Finlay, 1991b). Similarly, the isolated *M. es* also exhibited reduced yield and growth rate by about 30 %. The reduced growth in the absence of endosymbionts can be resulted from the adverse effect on the physiology of the ciliate. The hydrogen produced by metabolic activities in anaerobic ciliates is utilised by the endosymbiotic methanogens as substrate for methane production. The increased partial pressure of hydrogen in the absence of methanogens leads to a lower hydrogen production further and a lower energetic efficiency for the ciliate host (Fenchel and Finlay, 1991b). Thus, the hydrogen transfer

from ciliate to the endosymbiotic methanogen not only supports methanogens but brings low hydrogen partial pressure in the host cell.

4.4 Conclusions

- The presence of intracellular autofluorescing endosymbiotic methanogens in *Metopus es* cells and methane production in batch culture indicates that the ciliate can act as a potential producer of methane in biomethanation systems.
- Single *M. es* cell harbours 792 ±12 methanogens which constitute 0.8% of ciliate biovolume. The methane production rate of 0.85 pmol CH_4 ciliate^{-1} h^{-1} in *Metopus es* is comparable to other anaerobic ciliates.
- The endosymbiotic methanogens in *M. es* are found to be sensitive to oxygen, but they are not permanently affected by oxygen exposure as indicated by the restoration of the autofluorescence under anaerobic conditions. Moreover, the endosymbionts retained their activity even in *M. es* cysts. This ability of endosymbionts to restore its activity is useful in methane production systems.
- Fluorescent in situ hybridization analysis confirms the presence of methanogens as indicated by the fluorescence observed in *Metopus* cell after hybridization with ARCH 915 and MX 825. The presence of *Methanosaeta* as symbionts is an interesting finding and was not known earlier.
- The detection of acetate in *M. es* suggests the possible production of acetate in the hydrogenosomes. This creates a favorable environment for the aceticlastic *Methanosaeta* to enter into a symbiotic association.

- Around 30 % reduction in cell yield and growth rate of *M. es* in the absence of methanogens suggests the significance of their symbiotic association.

CHAPTER 5

Metopus es mediated organic matter degradation and its role in biomethanation process

5.1 Introduction

Biomethanation process is characterised by the microbial degradation of organic matter into methane and carbon dioxide in the absence of oxygen. The organic matter degradation is biomethanation process is often considered as entirely dependant upon different bacterial populations. The different functional groups of bacterial communities; hydrolytic, acidogenic, acetogenic and methanogenic groups are extensively studied for their involvement in organic matter degradation (Gujer and Zehnder, 1983; Keyser, 2006).

In anaerobic environments, bacterial grazing by ciliates alone is considered as significant in organic matter degradation. The grazing activity of ciliates has both negative and positive aspects. Grazing activity produces a negative impact on biodegradation in the sense that selective grazing eliminates some desirable bacterial populations and decrease the rate of biodegradation. The positive effects include increased recycling of limited nutrients, removal of senescent bacterial cells, reduction in competition among different bacterial populations and excretion of bacterial growth

factors, thereby enhancing the biodegradation (Tso and Taghon, 2006). The ability of ciliates to take part directly in organic matter degradation is not elucidated yet, except reports from rumen ciliates. In rumen ecosystem, ciliates play an important role by directly participating in the degradation of various organic compounds like cellulose and lignocellulose (Williams and Coleman, 1985; Santra and Karim, 2002; Takenaka et al., 2004). The ability of rumen ciliates to degrade variety of organic compounds like starch, cellulose and lignin is effectively utilised for anaerobic degradation of lignocellulosic wastes (Gijzen et al., 1987; Gijzen et al., 1988; Gijzen et al., 1990). These rumen ciliates of the genera *Epidinium*, *Entodinium* and *Diplodinium* harbour endosymbiotic methanogens also, which helps in the efficient conversion of their degradation products into methane (Newbold et al., 1995; Ushida and Jouany, 1996). The isolated ciliate, *Metopus es* is capable of growing on organic matter in the absence of bacterial prey and harbours endosymbiotic methanogens (chapter 2 and 4). The possible mechanism for its growth in bacteria controlled environment might be its ability to direct organic matter directly. In this aspect, enzymatic degradation of organic matter by *Metopus es* and its involvement in biomethanation are discussed in this chapter.

5.2 Materials and methods

5.2.1 Organism and culture conditions

The *Metopus es* culture maintained in the ciliate mineral medium in which bacterial growth was controlled by the addition of antibiotics, was used for studying its direct participation in organic matter degradation.

5.2.2 Degradation of organic compounds by *M. es*

Different organic compounds like glucose, sucrose, starch, cellulose, xylan, pectin, tannic acid, protein and lipid were used for biodegradation studies. Each of the above compounds (0.1 %) was added separately to the basal ciliate mineral medium as the sole carbon source. The bacterial growth was controlled by adding penicillin G sodium and streptomycin sulfate, 6000U and 200U ml^{-1} respectively (chapter 2). The inoculation of *M. es* and maintenance of the culture under anaerobic condition was carried out as per methods described in section 2.1.2.4.1 of chapter 2.

5.2.2.1 Assay of enzymes produced by *M. es*

The intra and extracellular enzymes in *M. es* were assayed by analysing the enzyme activity in culture fluid (filtered through 0.22 μm) as well as in intracellular extract of *M. es*.

5.2.2.2 Preparation of intracellular extract

The *M. es* cells alone were collected from the culture by filtration and centrifugation (156 g, 2 min). After washing with 50 mM phosphate buffer (pH 7.0), cells were subjected to three consecutive cycles of freezing (-20 ºC for 10 min) and thawing (35 ºC for 20 min) to obtain the intracellular extract. The cell free extract was obtained by centrifugation at 204 g for 10 min at 4 ºC and the supernatant was collected in 50 mM phosphate buffer (pH 7.0). The supernatant kept in sealed tubes at 4 ºC were used for enzyme assays.

5.2.2.3 Enzyme assays

Glucosidase: The activity of glucosidase was measured by spectrophotometric rate determination using p-nitrophenyl D-glucoside as substrate. The assay mixture contained 1 ml of p-nitrophenyl D-glucoside in 0.05 M sodium acetate buffer (pH 5.0). After equilibration at 40 °C, reaction was started by the addition of 0.1 ml of extract at room temperature. Reaction was then stopped by adding 2.0 ml of 1 M Na_2CO_3 after incubation at room temperature for 10 min. Absorbance was measured at 405 nm (Shimadzu, UV-2401) and one unit of glucosidase activity was defined as the amount of enzyme liberating 1 μmol of p-nitrophenol per minute under the above conditions (Herr et al., 1978).

Amylase: The reaction mixture used for amylase activity determination consisted of 1.25 ml of 1 % soluble starch, 0.25 ml of 0.1 M acetate buffer (pH 6.0), 0.25 ml of milliQ water and 0.25 ml of enzyme extract. After 10 min incubation at 35 °C, the liberated reducing sugars were measured by dinitrosalicylic acid method of Miller (1959). The colour developed was read at 575 nm using glucose as standard. One unit of enzyme activity was defined as the amount of enzyme releasing 1 μmol of glucose per minute under the assay conditions (Okolo et al., 1995).

Qualitative detection of amylase activity was done as per the method of Stark et al., 1953. In this method, 200 μl of crude extract was applied on paper discs on solid

media with 0.2 % starch and 1.0 % agar and incubated at 37 ^{0}C. The starch hydrolysis was detected by the presence of clearance zone on adding 0.1 % iodine in 0.2 % KI.

Cellulase: The assay mixture contained 0.5 ml of crude enzyme extract and 0.5 ml of 1 % carboxymethylcellulose in 0.05 M acetate buffer (pH 7.0). The reaction mixture was incubated for 30 min at 50 °C and the released reducing sugar was estimated by dinitrosalicylic acid method (Miller, 1959). One unit of enzyme activity was defined as the amount of enzyme releasing 1 μmol of glucose per minute under the assay conditions (Grajek, 1987).

Cellulase hydrolysis was detected by Congo red staining procedure (Hendricks et al., 1995). The cellulose-Congo red agar consisted of (g l^{-1}): K_2HPO_4, 0.5; $MgSO_4$, 0.25; cellulose powder, 1.88; Congo red, 0.20 and agar, 10. The clear hallows on Congo red agar on incubation with crude extract at 35 °C for at least 72 h indicated the production of cellulase.

Xylanase: Xylanase activity was assayed by incubating 0.5 ml of extract with 0.5 ml of xylan (0.5 %) in 0.01 M phosphate buffer (pH 7.0) at 50 °C for 30 min. The reaction was stopped by the addition of dinitrosalicylic acid (Miller, 1959) and absorbance was read at 575 nm using glucose as standard. One unit of enzyme activity was defined as the amount of enzyme releasing 1 μmol of glucose per minute under the assay conditions (Asha Poorna and Prema, 2006).

Pectinase: Pectinase activity was determined by 2- cyanoacetamide method. The crude enzyme extract (0.5 ml) was added to 0.5 ml of 0.5% polygalacturonic acid in 0.01 M McIlvaine buffer (pH 7.0) and incubated at 45 °C for 1 h. The reaction was then stopped by adding 2 ml of 0.01 M borate buffer (pH 7.0) and 1 ml of cyanoacetamide (1 %). After boiling the mixture in a water bath for 10 min, absorbance was measured at 276 nm using spectrophotometer. One unit of enzyme activity was defined as the amount of enzyme nedded to release 1 µmol of galacturonic acid per minute under the assay conditions (Jacob et al., 2008).

Pectinase activity was visualised on plates containing 0.2 % pectin and 1 % agar. The plates were incubated for 48 h after adding 100µl of crude extract on filter paper discs. The pectinase activity was visualised by the zone of clearance by flooding the plates with 0.05 % aqueous solution of ruthenium red (Gainvors et al., 1994)

Tannase: Tannase activity was determined by colorimetric method of Mondal et al., 2001. The enzyme extract (0.5 ml) was incubated with 0.3 ml of substarte, tannic acid (0.5 % in 0.2 M acetate buffer, pH 5.0) at 40°C for 10 min. After incubation, the reaction was stopped by adding 3 ml of bovine serum albumin (BSA) solution (1 mg ml^{-1} prepared with 0.17 M sodium chloride solution in 0.2 M acetate buffer, pH 5.0) which precipitates the residual tannic acid. A control was done using heat denatured enzyme. The preparations were centrifuged at 5,000 g for 10 min at 4 °C and the sedimented precipitate was dissolved in 3 ml of SDS–triethanolamine

solution (1 % SDS (w/v) in 5 % triethanolamine (v/v). After adding 1 ml of $FeCl_3$ (0.01 M $FeCl_3$ in 0.01 N HCl) reagent, absorbance was measured at 530 nm (Shimadzu, UV-2401). The specific extinction coefficient of tannic acid at 530 nm is 0.577. Using this coefficient, one unit of tannase activity can be defined as the amount of enzyme which is able to hydrolyze 1 mM of substrate tannic acid in 1 min under assay conditions.

Protease: The assay mixture consisted of 1 ml of 0.1 M phosphate buffer (pH 7.0), 0.25 ml casein (1 %), 0.25 ml of enzyme extract and 0.5 ml milliQ water. The mixture was incubated for 2 h at 39 ^{0}C followed by adding 2 ml 10 % trichloroacetic acid to stop the reaction. The supernatant fluid was collected by centrifugation and hydrolyzed protein was estimated by the method of Lowry et al. (1951). Enzyme activity was defined as 1 µmol tyrosine released per minute (Santra and Karim, 2002).

Lipase: Lipase activity was measured by spectrophotometer using p-nitrophenyl laurate as substrate. In this assay, amount of released p-nitrophenyl was measured at 400 nm. The reaction mixture contained 980 µl 100 mM phosphate buffer including 150 mM sodium chloride (pH 7.2) and 0.5 % triton X-100, 10 µl 50 mM p-nitrophenyl laurate and 10 µl enzyme solution. A blank was kept with milliQwater instead of enzyme source. One unit of enzyme activity was defined as the amount of enzyme liberating 1 µmol p-itrophenol per minute (Lesuisse et al., 1993).

The estimation of protein was done by Lowry's method (Lowry et al., 1951) using bovine serum albumin as standard.

5.2.2.4 Native polyacrylamide gel electrophoresis

Native polyacrylamide gel electrophoresis (PAGE) was performed with each enzyme extract with 4 % stacking and 10 % resolving gel. The separation of proteins was carried out at 60 mV and activity staining was performed by different methods depending on the type of enzyme.

The gel was washed with milliQ water and incubated with 50 mM Tris-HCl buffer (pH 7.5) containing 1 % starch for 1 h at 30 ºC for detecting amylase activity. The amylase activity was then visualised by staining the gel in a solution containing 0.15 % iodine and 1.5 % KI (Chung et al., 1995).

Cellulase activity was detected in 10 % polyacrylamide gel containing 0.1 % carboxy methyl cellulose (CMC). After electrophoresis, gel was incubated with 10 mM sodium phosphate buffer (pH 7.5) and soaked in 0.1 % Congo red solution to detect the presence of clear bands against red background (Nozaki et al., 2008).

Xylanase activity was visualised by the same method that of cellulase except that the proteins were separated on polyacrylamide gel containing 0.1 % xylan.

Pectinase activity was detected on gel containing 0.1 % pectin. After washing with 10 mM Tris buffer (pH 7.0), gel was incubated in citrate buffer (pH 5.0) for 60 min at 30 °C. The pectinase activity was revealed as clear zone on staining with 0.1 % ruthenium red (Acufia-Argfielles et al., 1995).

Protease activity was visualised by staining gel with Coomassie brilliant blue. The gel was immersed in 50 mM sodium phosphate buffer (pH 7.0) containing 1 % gelatin for 1 h at 37 °C, in order to allow the substrate to diffuse into the gel. The gel was then transferred to a clean petri dish and incubated at 37 °C for 1 h for digestion of the gelatin by active protease. After incubation, gel was stained with Coomassie brilliant blue. The protein band with protease activity in gel was visualised as clear zone of hydrolysis (Divakar et al., 2010).

For detecting lipase activity, gel was incubated with 50 mM Tris buffer (pH 8.0) for 30 min at room temperature. The gel was then overlaid with molten chromogenic substance containing phenol red (1 %), tributyrin (1 %), $CaCl_2$ (10 mM) and agar (2 %) at pH 7.2. Following solidification of the substrate, gel was incubated at 30 °C for 20 min. Lipase activity was observed as yellow region over a pink background (Singh et al., 2006).

5.2.3 Isolation of protein from wheat powder

Protein was isolated from wheat powder as per the methods of Wu, 1993. 25 g wheat flour was stirred with 150 ml of 0.03 N NaOH for 25 min. The slurry was

centrifuged at 3300 g for 20 min and supernatant was adjusted to pH 6.2 by 0.03 N HCl to precipitate most of the protein. The precipitated protein was separated by centrifugation (3300 g for 20 min) and extracted again with 0.03 N NaOH followed by precipitation with 0.03 N HCl to get the isolated protein.

5.2.4 Isolation of starch from wheat powder

Starch was isolated from wheat powder by alkali extraction method. The wheat powder (10 g) was mixed with 50 ml of 0.01 M NaOH, stirred continuously for 30 min at 25 ºC. It was then sedimented by centrifugation at 1400 g for 10 min and supernatant was discarded. The precipitate was mixed with 50 ml water and was filtered through nylon cloth (50 µm). The filtrate was neutralized with 1M HCl and supernatant was discarded. The tailings layered on the top of starch were carefully removed to get isolated starch. It was washed three times with water, collected by centrifugation and dried overnight at 40 °C prior to use (Lim et al., 1992).

5.2.5 Contribution of *M. es* in biomethanation process

The ability to degrade organic matter together with the presence of endosymbionts gives an indication about the positive role of *M. es* in biomethanation process. Experiments were conducted to find out the contribution of this ciliate to biomethanation process.

5.2.5.1 Role of *M. es* in COD removal and methane production

Batch experiments were conducted by culturing *M. es* in ciliate mineral medium to attain a maximum growth as described in chapter 2. The COD removal and methane production in *M. es* culture was measured at definite time intervals. The COD removal efficiency in *M. es* cultures having different population density was compared by inoculating same number of *M. es* cells to different basal media PM, PJ, PC and ciliate mineral medium as described in chapter 2. All media were supplied with the same initial COD of 5g as wheat powder. The number of *M. es* in different media was monitored at regular intervals and compared with the COD removal after its growth.

Batch experiments were conducted to find out contribution of *M. es* to biomethanation process. The COD removal and methane production in *M. es* culture was compared to that of batch system containing bacteria alone and system containing bacteria and *M. es*. The experiments were conducted in 500 ml bottles sealed with silicon septa with headspace filled with nitrogen. Complete anaerobiosis was achieved by adding 0.67 ml 3 % cysteine hydrochloride (w/v). Both *M. es* and bacterial cultures were supplied by an initial COD of 5 g COD. The growth of other anaerobic protozoa in bacterial system was prevented by adding cycloheximide, a selective inhibitor of eukaryotic protein synthesis as it has been employed successfully for inhibiting the growth of anaerobic ciliates in soil microcosms without affecting the methanogens (Schwarz and Frenzel, 2005). Cycloheximide at a concentration of 200 mg l^{-1} was effective in eliminating all anaerobic protozoa including *M. es* in bacterial system. The

batch system with bacteria and *M. es* was achieved by inoculating the anaerobic sludge with *M. es.* The methane production was measured by liquid displacement method. The total number of methanogens in bacterial system was estimated by most probable number method (Ranade and Gadre, 1988).

5.2.5.2 Size selective predation by *M. es*

Ciliate mineral medium provided with suspended food particles of different sizes were used to study the size selective predation by *M. es*. Fine suspension of wheat was prepared from powder by milling in a double rack jar mill with zirconium balls. Particles of different sizes were separated from the suspension by selective filtration using filters having different pore size. The size of separated particles varied in the range of 0.45 - 30 µm and growth of *M. es* was monitored in each experimental set up with different sized particulate feed**.**

5.2.5.3 Bacterial grazing by *M. es*

The anaerobic bacteria were isolated from reactor sludge using thioglycolate agar (Wolf et al., 1975). Heat killed bacterial suspensions were added to ciliate mineral medium as food and inoculated with *M. es*. The grazing activity of *M. es* was monitored by enumerating ciliate and bacteria remaining in the medium at regular time intervals. Bacterial count was taken using epifluorescence microscope after staining with DAPI.

5.2.5.4 Nutrient recycling by *M. es*

The nutrient secretion by *M. es* was studied in cultures supplemented with live and heat killed bacteria as food. Both these bacteria were added at an initial count of 4×10^6 ml^{-1} to ciliate mineral medium before inoculating with *M. es*. Maintenance of anaerobic conditions and incubation were carried out as described in section 2.1.2.4.1 of chapter 2. *M. es* count and amount of nutrients (ammonia and phosphate) were estimated in samples taken at regular intervals. Each experiment was conducted in triplicates and average value was taken. The bacterial culture maintained in the same condition without *M. es* served as control to estimate amount of nutrients released by bacteria.

5.2.6 Chemical analysis

5.2.6.1 Thin layer chromatography

The intermediate products of organic matter degradation by *M. es* were qualitatively analysed by thin layer chromatography (TLC). The intracellular extract of *M. es* (100 µl) was incubated with same volume of each organic compound studied (1 mg ml^{-1}) at 30 ºC. At definite time intervals, samples (10 µl) were spotted on silica gel coated TLC plates (Silica gel 60 F54, Merck, Germany) and developed in suitable solvent systems depending on the substrate. The metabolites were detected by comparing with standard compounds run under same conditions.

Sugars were detected in a solvent system, n-butanol:isopropanol:water (10:5:7) and visualised by spraying with 5 % H_2SO_4 (v/v) in ethanol and heating at 104 °C for

10 min (Barnett et al., 1969). Degradation products of pectin were separated in solvent system consisting of butanol:acetic acid:water (3:2:2) and visualised by 10 % H_2SO_4 (v/v) in ethanol and heating at 104 °C for 10 min. The intermediates of tannin degradation were analysed in solvent system of chloroform: ethyl acetate: acetic acid (50: 50: 1) and the detection was done using iodine vapours (Sharma et al., 1998). A solvent system of hexane:ether (9:1) was used for analysing lipids and visualised by iodine vapours.

5.2.6.2 Organic carbon content

Total organic carbon content (TOC) was measured by TOC analyser (Schimadzu TOC-5000 analyser). Chemical oxygen demand (COD) was estimated as per standard methods (APHA, 1998).

5.2.6.3 Gas chromatograph

Methane concentration in head space was determined at definite time intervals by gas chromatography (FISIONS 8000, FID, 2mm i.d.silica gel column, carrier gas 30 ml min^{-1}, oven 110 ^{0}C, detector 130 ^{0}C and injector 130 ^{0}C). Organic acids were detected in gas chromatograph (FISION 8000, FID, Carbovax column, carrier gas 100 KPa each, injector 150 ^{0}C, oven 130 ^{0}C and detector 175 ^{0}C).

5.3 Results and discussion

5.3.1 Degradation of organic compounds by *M. es*

M. es used in this study was isolated from wastewater treatment reactor that received various organic compounds. The growth and higher counts of *M. es* in such conditions points out its capability to utilise organic compounds. Furthermore, it is capable of growing on wheat powder in a bacteria suppressed condition which indicates its ability to uptake the organic matter directly. The efficient utilisation of organic matter by microorganisms is purely dependant on the presence of enzymes that can cleave the complex molecule to simpler ones which are further used for release of energy. In this context, detection of different enzymes in *M. es* was done by developing its culture with different substrates.

5.3.1.1 Degradation of glucose by *M. es*

Glucose is the most common monosaccharide used as the primary source for energy by microorganisms. *M. es* was able to grow at glucose supplemented cultures. The population density of *M. es* in glucose supplemented culture was less when compared to other carbon sources. However, reduction in the amount of glucose was detected during the growth of *M. es* in the absence of bacteria (Fig. 5.1). This indicates direct utilisation of glucose by *M. es*.

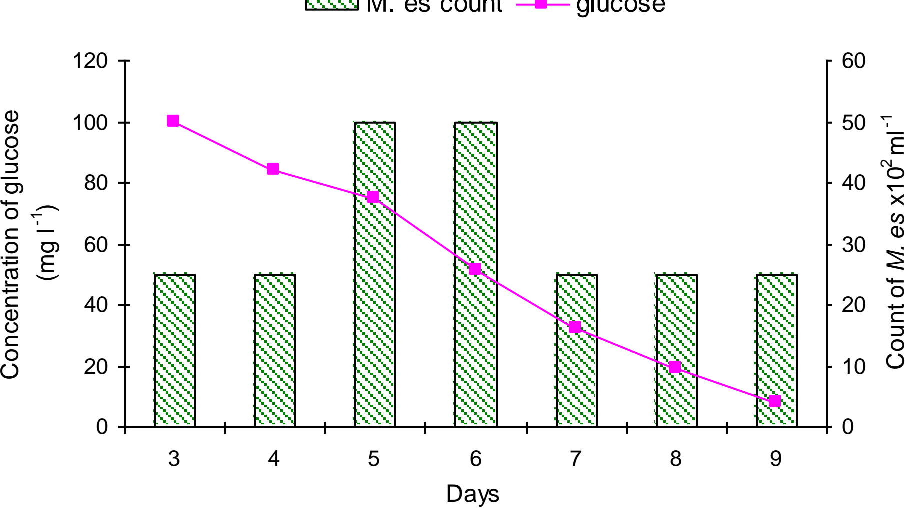


Fig. 5.1. Glucose removal in *M. es* culture.

Methane and acetate along with traces of butyrate were the end products of glucose metabolism detected by gas chromatography in *M. es* culture. Anaerobic ciliates are capable of carrying out glycolytic pathway like other anaerobic organisms. Rumen holotrich ciliates like *Isotricha and Dasyricha* ferment glucose into lactic, acetic, propionic and butyric acid along with carbon dioxide and hydrogen (Heald and Oxford, 1953). The anaerobic flagellate *Hexamita* also converts glucose to acetate, ethanol, lactic acid, alanine and CO_2 through fermentative metabolism (Biagini et al., 1998b). The growth of *M. es* on glucose and the nature of end products indicate the existence of a similar kind of fermentative metabolism in *M. es*.

5.3.1.2 Degradation of sucrose by *M. es*

Sucrose is an easily fermentable disaccharide, composed of glucose and fructose molecules linked together by glycosidic bond. The rate of sucrose degradation

(0.61 mg l^{-1} h^{-1}) by *M. es* is slightly lower than the rate by which glucose was degraded (0.64 mg l^{-1} h^{-1}). The highest intracellular glucosidase activity found in *M. es* is 15.2 U ml^{-1} when its count reached at 0.5×10^4 ml^{-1} (Fig. 5.2).

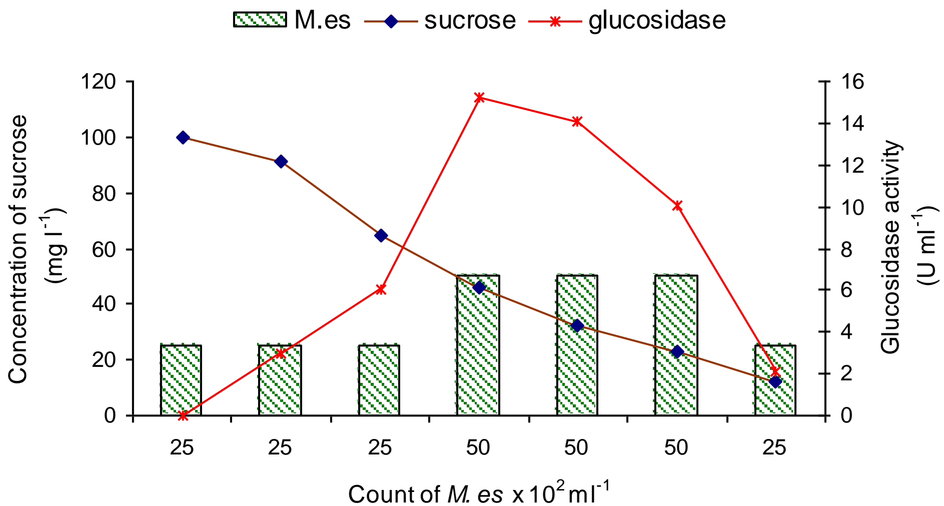


Fig. 5.2. Glucosidase activity and decrease in residual sucrose in *M. es* culture.

The intermediate products of sucrose degradation by *M. es* were detected by thin layer chromatography. The samples were drawn at regular intervals and spotted on TLC plates to detect the degradation products of sucrose (Fig. 5.3). Sucrose was found to be degraded to glucose as detected by its presence on TLC plates within 48 h. It indicates the ability of *M.* es to degrade sucrose to glucose which then enters into the fermentative metabolic pathway.

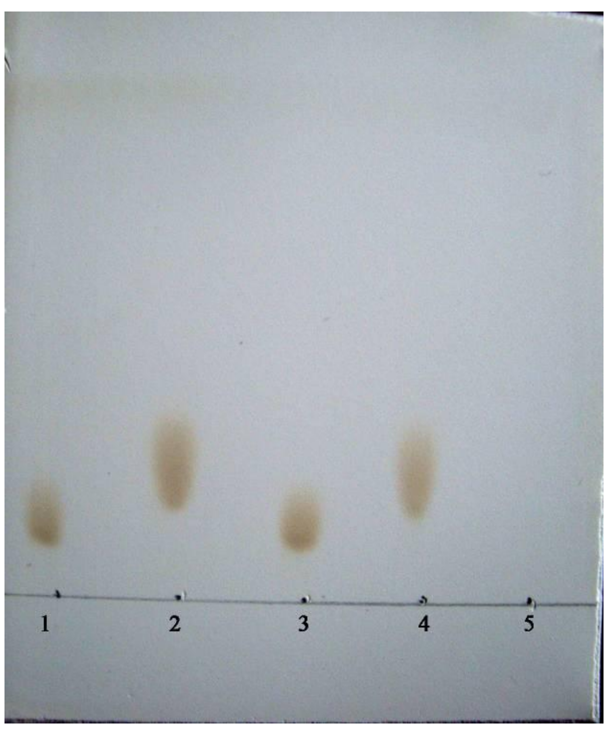


Fig. 5.3. Degradation products of sucrose by *M. es* on TLC plates
1. sucrose (standard) 2. fructose (standard) 3. glucose (standard) 4. sample at 48 h 5. sample at 96h

Acetate and methane were the major end products of sucrose metabolism. Butyrate and traces of propionate were also detected. The ability of anaerobic ciliates to utilise sucrose is well documented in rumen ciliate like *Dasytricha ruminantium* (Clarke and Hungate, 1966).

5.3.1.3 Degradation of starch by *M. es*

Starch is the most common polysaccharide composed of large number of glucose molecules joined together by glycosidic bond. The ability of microorganisms to utilise starch depends on the production of enzyme, amylase which hydrolyses

starch into simple monosaccharide units, glucose. There are two types of amylase: α-amylase that cleaves the α 1-4 linkage in the starch and ß-amylase acting on ß 1-3 linkage at branching point of starch side chain. Starch is the major component in waste water from food processing industries. The maximum count of *M. es* was in media with wheat powder as carbon source (chapter 2). Since wheat flour contains more than 50 % starch, *M. es* was grown on starch extracted from wheat powder. The growth of *M. es* in wheat starch medium and reduction in amount of starch is given in Fig. 5.4. The reduction in amount of was correlated to amylase enzyme production by *M. es.* At the initial stage of growth, having the cell count of 1.25×10^4 ml^{-1}, amylase activity in *M. es* was 9.3 U ml^{-1}. The amylase activity was increased to 29.5 U ml^{-1} with the maximum cell number of 3×10^4 ml^{-1}. The degradation rate of starch by *M. es* (0.55 mg l^{-1} h^{-1}) was lower than that of glucose and sucrose.

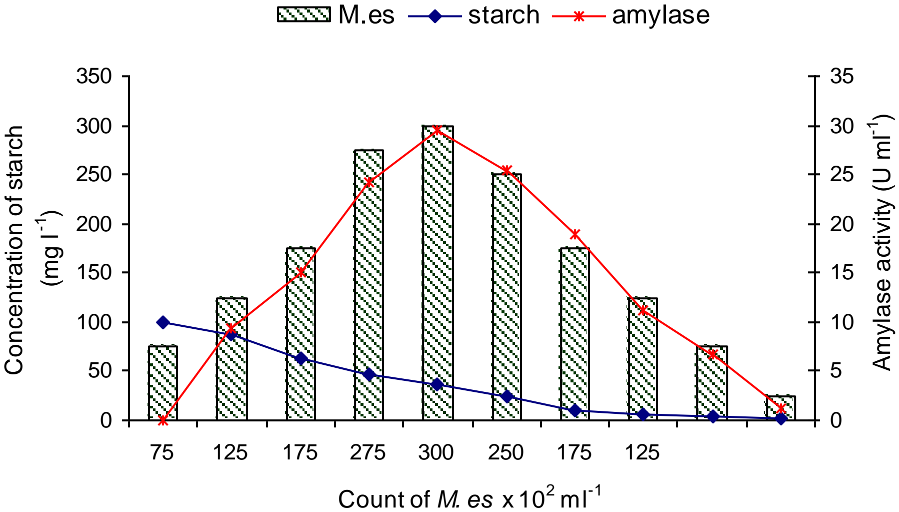


Fig. 5.4. Amylase activity and starch degradation in *M. es* culture supplemented with starch.

The amylase activity of *M. es* extract was visualised as clear zones in starch agar medium flooded with Gram's iodine solution (Fig. 5.5). The activity staining in native PAGE gel also revealed the presence of colorless zone (Fig.5.6).

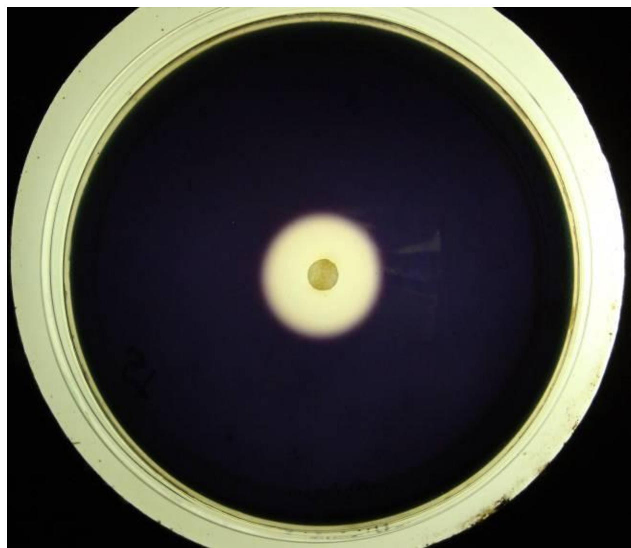


Fig. 5.5. Amylase activity in *M. es* extract on starch agar plates indicated by zone of clearance.

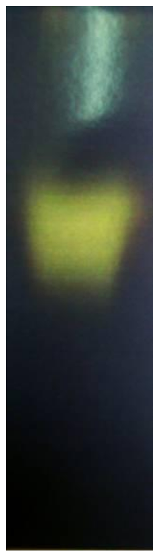


Fig. 5.6. Amylase activity in native PAGE gel stained with iodine.

The digestion of starch by *M. es* resulted in the intermediate products, maltose and glucose as revealed by TLC (Fig. 5.7).

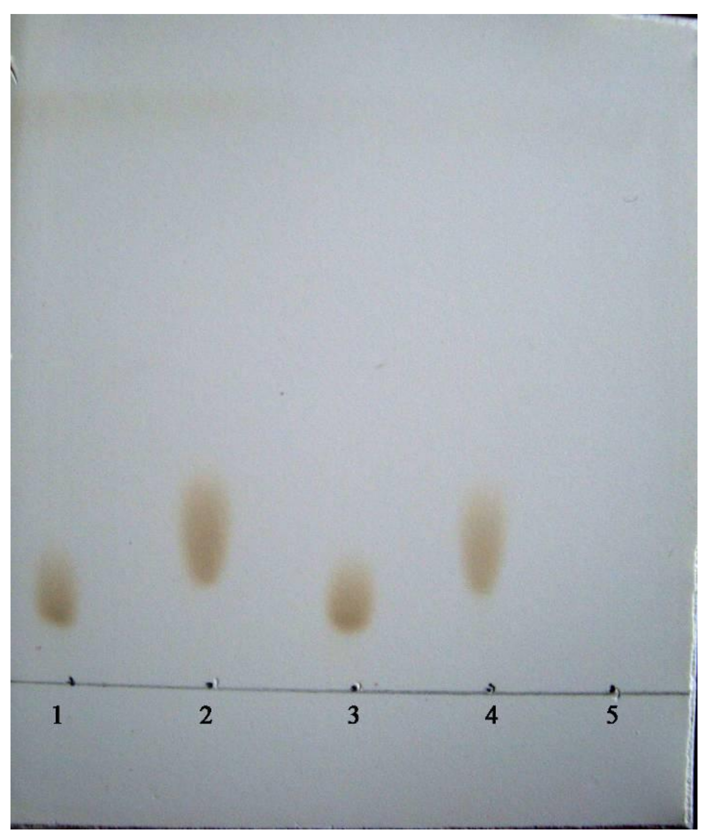


Fig. 5.7. Intermediate products of starch degradation by *M. es* on TLC plate. 1. maltose (standard) 2. glucose (standard) 3. sample at 24 h 4. sample at 72 h 4. sample at 120 h.

The end products of starch metabolism by *M. es* were acetate, methane and butyrate as detected by gas chromatography. Increased activity of amylase was reported in rumen ciliates and found to play an important role in the digestion of starch in ruminant diet (Jouany et al., 1988; Hristov et al., 2001). Many rumen ciliates like

Epidinium ecaudatum, *Entodinium maggii*, *Entodinium caudatum* and *Entodinium exiguum* are capable of direct uptake and digestion of starch (Coleman and Laurie, 1976; Coleman, 1978; Coleman and Sandford, 1980; Fondevila and Dehority, 2001b). Like rumen ciliates, amylase production in *M. es* enables it to grow in starch containing medium.

5.3.1.4 Degradation of cellulose by *M. es*

Cellulose is the major carbohydrate synthesized by plants. Therefore, cellulosic biomass represents an important part of the carbon cycle within the biosphere. It is a partially crystalline, linear polysaccharide made of glucose monomers linked by ß-1,4-glycosidic bonds. Microorganism brings about most of the cellulose degradation occur in nature. Cellulose degrading microorganisms are capable of producing a group of enzymes system, collectively known as cellulase (Lynd et al., 2002). The microbial degradation of cellulose becomes important in the treatment of lignocellulosic wastes.

The growth of *M. es* in cellulose medium was slow and delayed when compared to starch supplemented medium. The maximum number of *M. es* in cellulose medium was only 1.25×10^4 ml^{-1} whereas in starch medium the highest count was 3×10^4 ml^{-1}. The maximum cellulase activity (15 U ml^{-1}) was found when *M. es* reached at higher count (1.25×10^4 ml^{-1}) (Fig. 5.8). The rate of degradation of cellulose was 0.46 mg l^{-1} h^{-1} which is lower than starch degradation rate.

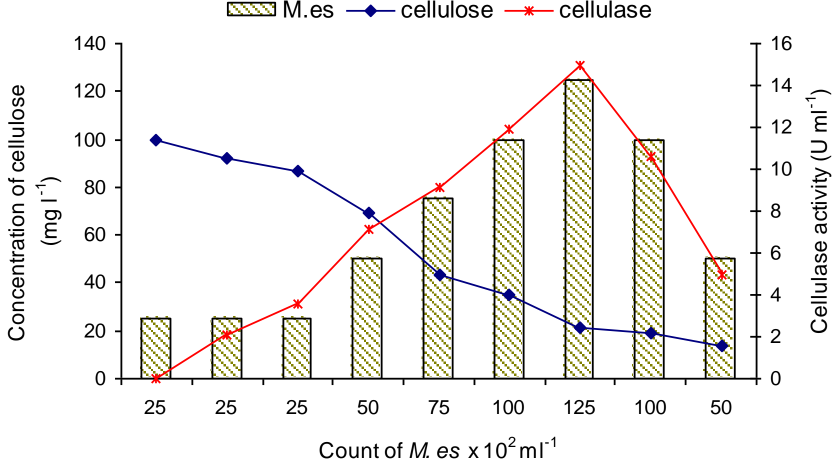


Fig. 5.8. Cellulase activity and degradation of cellulose by *M. es* in cellulose supplemented culture.

The cellulase activity in *M. es* was confirmed by zone of clearance produced on cellulose-congo red agar on incubation with extract of *M. es* (Fig. 5.9). It was further confirmed by activity staining by congo red in native PAGE (Fig. 5.10).

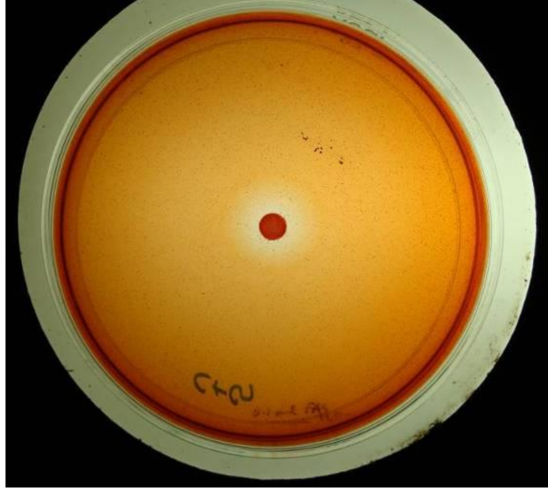


Fig. 5.9. Zone of clearance in cellulose congored agar on incubation with *M. es* extract.

Fig. 5.10. Cellulase activity of *M. es* extract in congored stained native PAGE gel, clear band shows the cellulase activity.

The intermediate products of cellulose degradation by *M. es* were detected on TLC plates. Glucose was found as the product of cellulose degradation in *M. es* (Fig. 5.11).

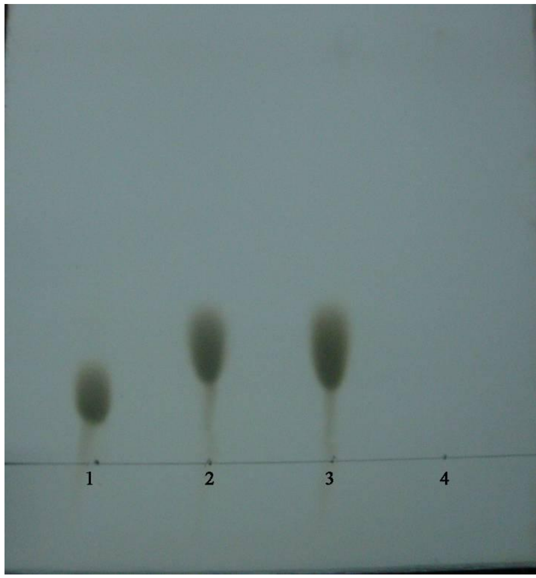


Fig. 5.11. Degradation products of cellulose by *M. es*.
1. cellulose 2. glucose 3. sample at 48 h 4. sample at 120 h.

Methane and acetate were detected as major metabolic end products with traces of butyrate and propionate. Among anaerobic protozoa, rumen ciliates are described as the potent producers of this enzyme. They inhabit an environment with higher supply of lignoclellulosic fibre and higher cellulase activity that helps them to utilise cellulose for their energy metabolism (Gijzen et al., 1988; Santra and Karim, 2002). Most of the ciliate genera in the rumen like *Epidinium, Polyplastron*, *Eudiplodinium* and *Diplodinium* can degrade cellulose by the production of cellulase (Coleman, 1978). The presence of cellulase, enzyme in *M. es* indicates that it can be effectively used in anaerobic reactors treating plant biomass.

5.3.1.5 Degradation of xylan by *M. es*

Xylans, composed of backbone consisting of ß (1–4)-linked xylose units with varying degrees of polymerization, represent the most abundant polysaccharide in hemicelluloses (Hespell and Whitehead, 1990). Xylan contains a variety of chemical linkages and thus its degradation requires a group of enzymes collectively called xylanase. Xylanases produced by microorganisms are of great importance in treating wastewater from paper and pulp industries as the hydrolysis of xylan releases lignin from lignocellulosic material.

The growth pattern of *M. es* in xylan supplemented medium was similar to growth pattern in cellulose containing medium. At initial stage, the growth was delayed with reduced number of *M. es* cells (0.25×10^4 ml^{-1}). A maximum xylanase

activity of 13.8 U ml^{-1} was observed when the cell count reached at 1.25×10^4 ml^{-1} (Fig. 5.12). The rate of degradation was more or less similar to cellulose (0.42 mg l^{-1} h^{-1}).

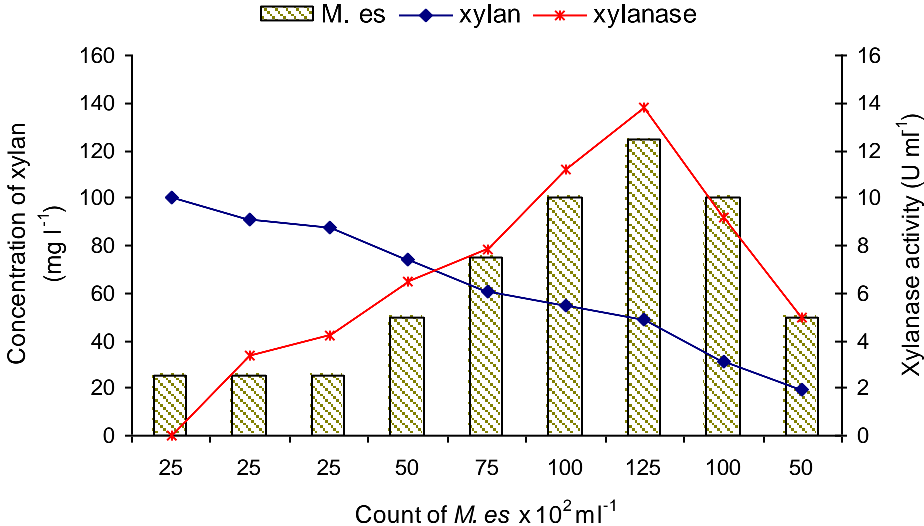


Fig. 5.12. Xylanase acivity and degradation of xylan by *M. es* in xylan supplemented culture.

TLC analysis revealed the presence of xylose and glucose as the intermediate products of xylan degradation (Fig. 5.13). The xylanase activity of *M. es* extract was visualised in native polyacrylamide gel by staining with congo red (Fig. 5.14).

Fig. 5.13. Degradation products of xylan by *M. es*.
1. xylose (standard) 2. glucose (standard) 3. sample at 48h 4. sample at 72 h 5. sample at 120 h.

Fig. 5.14. Xylanase activity seen as clear band in native polyacrylamide gel.

Xylanases are reported in different rumen ciliates; *Eremoplastron boris, Ostracodinium*, *Diploplastron*, *Ophryoscolex*, *Polyplastron multivesiculatum* and *Epidinium ecaudatum* (Williams and Coleman, 1985; Devillard et al., 1999; Michalowski et al., 2001). The xylanase enzyme helps in degradation of hemicellulose by these ciliates in rumen ecosystem effectively. Xylan is found between lignin and cellulose and is thought to be important in fibre cohesion and for the integrity of the cell wall (Iwamoto et al., 2008). Xylan helps to protect cellulose from degradation through its covalent interactions with lignin and non-covalent interactions with cellulose (Bauer et al., 1973). Hence degradation of xylan becomes important in the efficient utilisation of lignocellulose, an inexpensive and renewable energy source obtained from agricultural wastes, municipal solid wastes and from pulp and paper industries.

5.3.1.6 Degradation of pectin by *M. es*

Pectin is the natural polymer present in the middle lamellae and primary cell wall of plants. The predominant structure of pectin consists of esterified D-galacturonic acid residues in an α -(1-4) chain. The acid groups along the chain are largely esterifed with methoxy groups at carboxyl moieties and there can also be acetyl groups present on the free hydroxy groups. Pectinases are an enzyme group that catalyses pectic substance degradation through depolymerization (hydrolases and lyases) and deesterification (esterases) reactions. Pectin also supported the growth of *M. es* with a highest count of 2.5×10^4 ml^{-1}. The maximum amount of pectinase

activity observed was 15.9 U ml^{-1} (Fig. 5.15). The rate of pectin degradation (0.48 mg l^{-1} h^{-1}) was higher than cellulose and xylan.

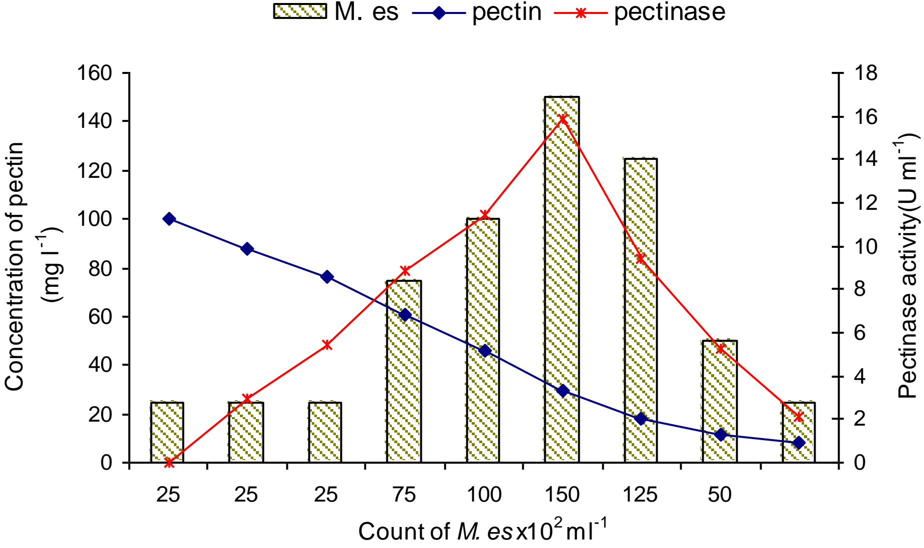


Fig. 5.15. Pectinase activity and degradation of pectin by *M. es* in pectin supplemented media.

The extract of *M. es* showed pectinase activity as indicated by the zone of clearance in pectin agar stained with ruthenium red (Fig. 5.16).

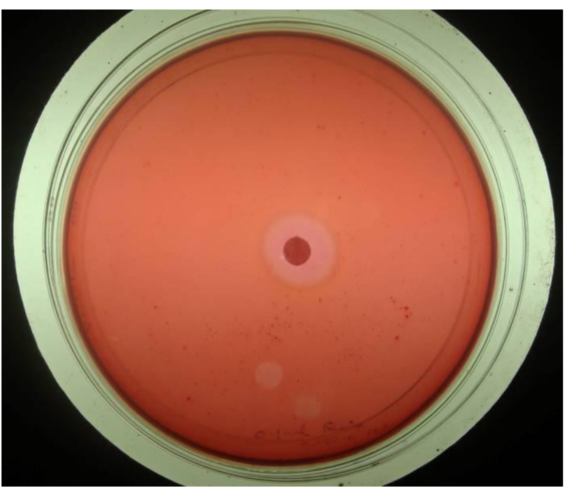


Fig. 5.16. Zone of clearance in pectin agar on incubation with *M. es* extract.

TLC of culture samples showed the presence of galacturonic acid as the major intermediate product of pectinase activity (Fig. 5.17).

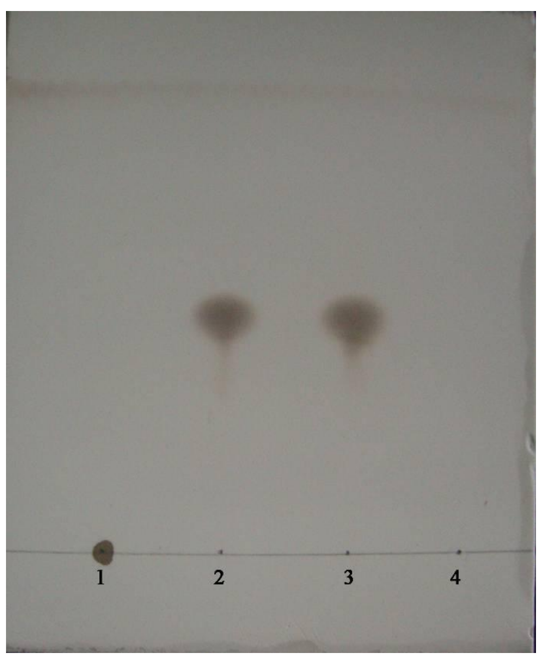


Fig. 5.17. Degradation product of pectin by *M. es*.
1. pectin (standard) 2. galacturonic acid (standard) 3. sample at 48 h 4. sample at 120 h.

The pectinase activity of *M. es* grown in pectin supplemented medium was further confirmed by clear band formed in native polyacrylamide gel on staining with ruthenium red (Fig. 5.19).

Fig. 5.18. Pectinase activity of *M. es* extract in native polyacrylamide gel.

Unlike other carbon sources like sucrose, starch, cellulose and xylan; there are not many reports on pectin utilisation by anaerobic ciliates. Pectinolytic activity has been reported in rumen ciliate, *Epidinium ecaudatum* (Bailey and Clarke, 1963). Coleman et al., (1980) have reported the occurrence of polygalacturonase in rumen ciliates like *Eremoplastron* and *Ostracodinium* that helped them to utilise polygalacturonic acid.

The degradation studies with different carbohydrates show that *M. es* is capable of utilising different categories of carbohydrates, viz, mono-,oligo-and polysaccharides. The growth pattern and population density of *M. es* differ with the complexity of carbohydrates supplied. Simpler carbohydrates like glucose and sucrose are more rapidly utilised as indicated by higher rate of degradation. A lower number of

M. es (0.25×10^4 ml^{-1}) was found in polysaccharide supplemented media upto 3 days. But during subsequent days, *M. es* had higher growth rate and could grow upto 14 days without additional supply of carbon source. Usually, hydrolysis of carbohydrates with different degrees of complexity takes place at initial stages of biomethanation process and is often considered as the rate limiting step controlling overall rate of biomethanation. In bacteria mediated hydrolysis, the enzymatic activity is often extracellular since bacterial cells are non permeable to larger molecules. While large anaerobic ciliates like *M. es* can engulf complex macromolecules very rapidly and degrade them intracellularly by hydrolytic enzymes. Thus maintaining a high population of *M. es* in biomethanation systems can enhance the degradation of organic matter.

5.3.1.7 Degradation of protein by *M. es*

Proteins are degraded by microorganisms and they utilise the degradation products as nutrients for growth. The growth of *M. es* was monitored in wheat protein supplemented medium and residual protein was estimated at regular intervals. The highest protease activity of 14.3 U ml^{-1} was found when growth of *M. es* reached at maximum level of 2.5×10^4 ml^{-1}. The amount of wheat protein in the medium was reduced during the growth of *M. es* (Fig. 5.19) and the rate of degradation was 0.49 mg l^{-1} h^{-1}. Correspondingly, the presence of protease in *M. es* extract was confirmed by zone of clearance produced on gelatin agar (Fig. 5.20). The protease activity in *M. es* was also visualised in gel stained with commassie blue (Fig.5.21).

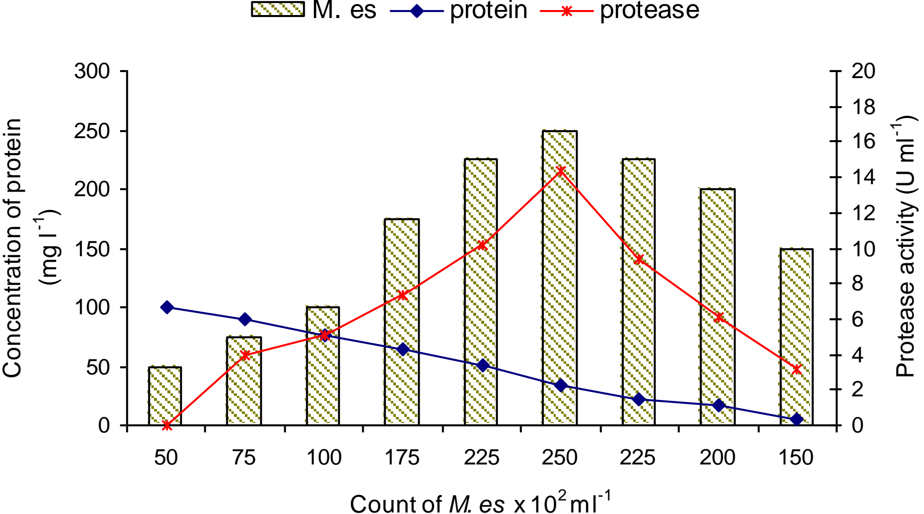


Fig. 5.19. Protease activity and degradation of protein by *M. es* in wheat protein supplemented medium.

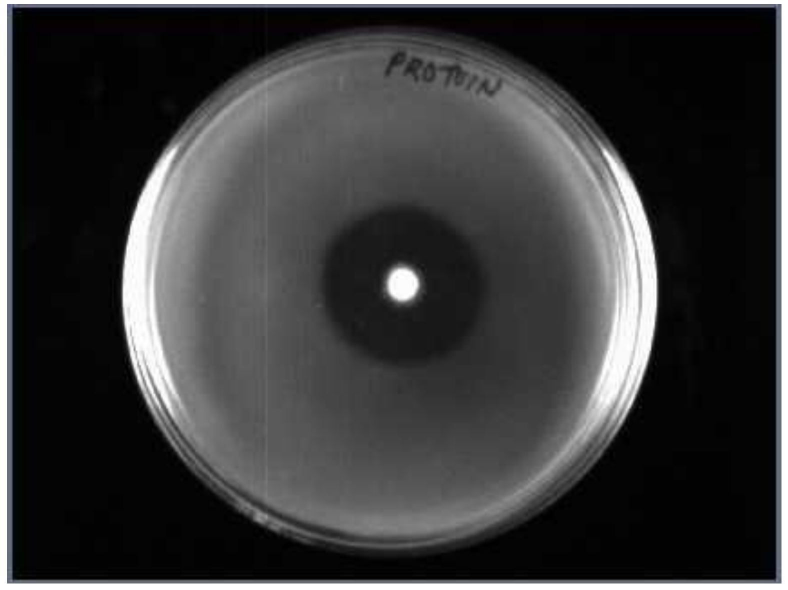


Fig. 5.20. Zone of clearance in gelatin gar on incubation with *M. es* extract.

Fig. 5.21. Clear area of protease activity of *M. es* extract in native polyacrylamide gel.

Proteases are enzymes which catalyse the hydrolysis of peptide bonds in proteins or peptides. They have important role in physiological processes as well as in commercial applications. They also play an important role in aerobic as well as anaerobic food webs by facilitating nitrogen recycling. The rumen protozoa were found to possess high level of endogenous protease activity (Forsberg et al., 1984; Warner, 1956) and rumen ciliates from diverse genera such as *Isotricha*, *Dasytricha*, *Entodinium*, *Ophryoscolex*, *Epidinium* and *Polyplastron* were able to degrade protein component in ruminal diet by producing proteases (Lockwood et al., 1988). Being the principal grazers of bacteria in anaerobic environment, protozoa have the ability to degrade bacterial proteins (Fenchel and Finaly, 1995). In biomethanation process, protein degradation is mainly accounted from the extracelluar protease activity by different groups of bacteria. However, the presence of protease activity in ciliate like

M. es is significant in this aspect since it can directly participate in the degradation of protein containing wastes.

5.3.1.8 Degradation of lipid by *M. es*

Lipids, which are traditionally characterized as fats, grease and oils are widely found in industrial and municipal wastewater. Lipids in industrial wastewater are mainly esters of straight chain, even numbered long chain fatty acids (LCFA) and their hydrolyzed products (Rinzema, 1988). Lipase is a water soluble enzyme that catalyses the hydrolysis of ester bonds lipid substrates. The growth of *M. es* in presence of oleic acid, a long chain fatty acid and lipase production are given in Fig. 5. 22. There was high lipase activity (19.5 U ml^{-1}) during the exponential growth of *M. es* and rate of lipid degradation of lipid was 0.47 mg l^{-1} h^{-1}.

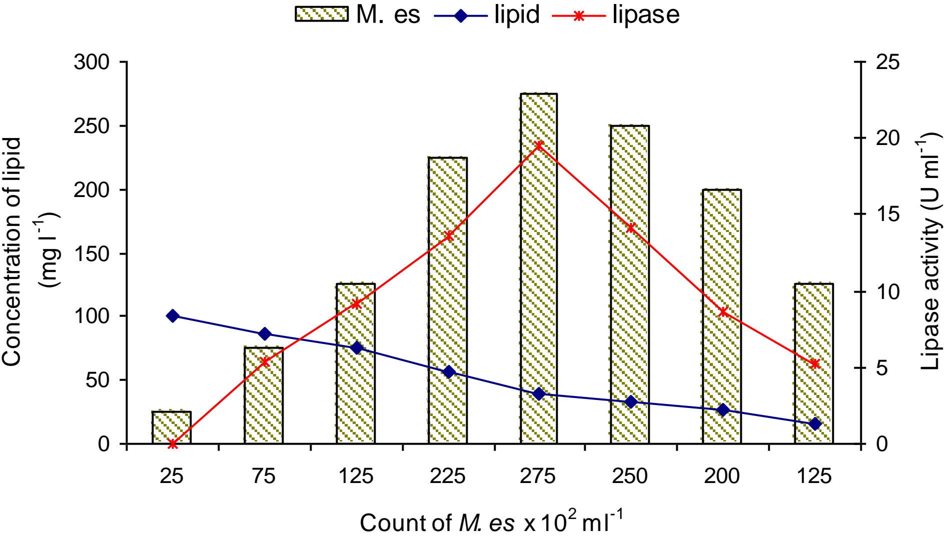


Fig. 5.22. Lipase activity and degradation of lipid by *M. es* in oleic acid supplemented medium.

The intermediate of oleic acid degradation was analysed by TLC (Fig. 5.23). Oleic acid, an 18 carbon unsaturated fatty acid (C18:1) was degraded to palmitic acid (C16:0) by *M. es*.

Fig. 5.23. Degradation product of oleic acid by *M. es*.
1. oleic acid (standard) 2. palmitic acid (standard) 3. sample at 48 h.

The presence of lipase in *M. es* extract was further confirmed by the presence of yellow zone in native PAGE gel stained with chromogenic substance (Fig.5.24).

Fig. 5.24. Lipase activity of *M. es* extract as indicated by yellow zone in pink background.

The uptake of fatty acids was previously demonstrated in rumen ciliates like *Epidinium* and *Isotricha*, where oleic acid was hydrogenated to strearic acid through ciliate metabolism (Guttierrez, 1962). The aerobic ciliate *Tetrahymena pyriformis* is capable of producing lipase when grown on olive oil (De Coninck et al., 2004). In anaerobic digestion, LCFAs are toxic to bacteria because of their damaging effect on the transport channel of bacterial cells, including lysis of protoplasts (Galbraith and Miller, 1973). Hence, bacterial degradation of lipids is possible mainly by extracellular enzymes. The lipids being insoluble in water will form particles of different size in the medium on emulsification. *M. es* is described as a partiuclate feeder in anoxic environments and it can ingest lipid particles directly from the medium and degrade by the intracellular lipase. The detection of lipase production in this ciliate strengthens the above view of direct intake of lipid rich suspended matter by *M. es*.

5.3.1.9 Degradation of tannin by *M. es*

Tannins are defined as naturally occurring water soluble polyphenols of varying molecular weight, abundant in the leaves, fruits, bark and wood of plants. After lignin, they are the second most abundant group of plant phenolics. They are considered as secondary metabolites of plants as they are not involved in metabolic pathways (Bhat et al., 1998). Based on their structures and properties, they are distributed into two major groups – hydrolysable and condensed tannins. Hydrolysable tannins are composed of esters of gallic acid (gallotannins) or ellagic acid

(ellagitannins) with a sugar core which is usually glucose and are readily hydrolysed by acids or enzymes into monomeric products. Condensed tannins are composed of flavonoid units and are usually more abundant in tree barks and woods than their hydrolysable counterparts (Skein and Brooker, 1995). Tannic acid is gallotannin consists of esters of gallic acid and glucose. Though tannins have toxic effects on various organisms, some microorganisms are resistant to tannins and have the ability to degrade them by producing tannase enzyme. It catalyses the breakdown of hydrolysable tannins into gallic acid and glucose.

The growth of *M. es* in tannic acid supplemented medium and tannic acid degradation are given in Fig. 5.25. A maximum tannase activity of 9.4 U ml^{-1} was observed with *M. es* count of 2.25×10^4 ml^{-1}. There was reduction in tannase activity to 3 Uml^{-1} after the exponential growth period. The rate of degradation of tannic acid was 0.46 mg l^{-1} h^{-1}.

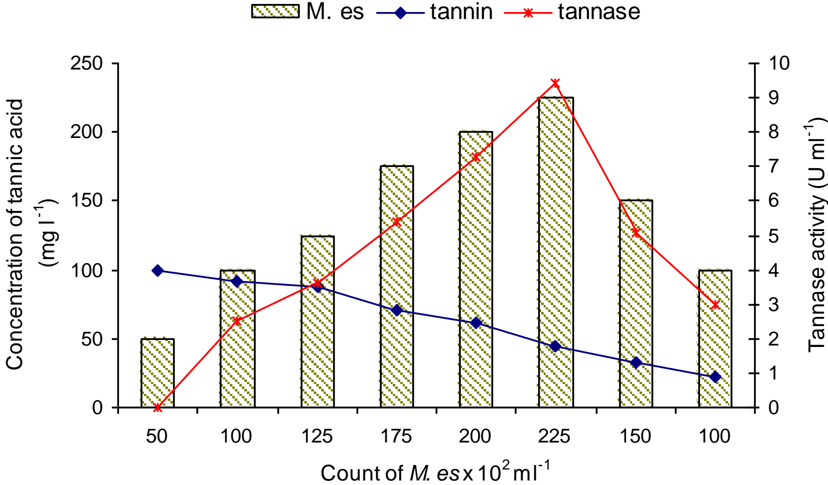


Fig. 5.25. Tannase activity and degradation of tannic acid by *M. es* in tannic acid supplemented medium.

The intermediate products of tannc acid degradation by *M. es* were gallic acid and pyrogallol as detected by TLC (Fig.5.26).Gallic acid could be detected with residual tannic acid in the samples at 48h of incubation. After 96 h, gallic acid and pyrogallol were detected without residual tannic acid.

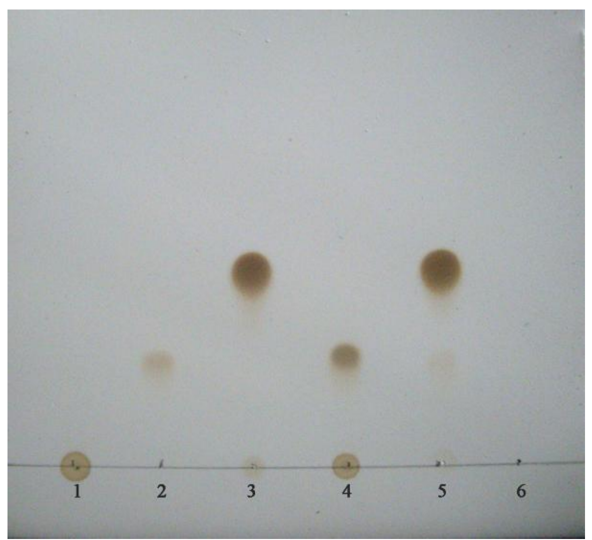


Fig. 5.26. Degradation products of tannic acid by *M. es*
1. tannic acid (standard) 2. gallic acid (standard) 3. pyrogallol (standard) 4. sample at 48 h 5. sample at 96 h 6. sample at 120 h.

Polyphenols and other phenolic compounds are major components in many industrial wastes like oil extraction, leather and wine industries. Anaerobic treatment was successfully employed for the removal of phenolic compounds from wastewater (Chen et al., 2009). A number of microorganisms including bacteria, fungi and yeasts were reported to have the capability of tannin degradation in anaerobic system. Field

and Lettinga (1987) have observed degradation of tannins by a consortium of bacteria in anaerobic sludge. A variety of bacteria capable of degrading tannin was reported in the rumen also (Krumholz and Bryant, 1986; Zhu and Filippich., 1995; Skene and Brooker, 1995). Fungal species of different genera are capable of degrading tannins by producing tannase (Bhat et al., 1998).

The ability of anaerobic protozoa to degrade polyphenols is not recognised yet. *M. es* used in this study was isolated from an anaerobic reactor treating plant material rich in polyphenols. The ability of this ciliate to utilise the tannins in the medium could be the major reason behind its survival in such an environment and treatment systems rich in *M. es* can be utilised for the removal of phenols from wastewater.

5.3.1.10 Extra and Intracellular enzyme profile in *M. es*

As discussed above**,** a spectrum of hydrolytic enzyme was detected in *M. es* cells and culture medium. The levels of extra and intracellular enzymes at exponential growth phase of *M. es* are given in Fig. 5.27. Higher intracellular enzyme activity indicates the ability of intracellular digestion of ingested organic matter by the ciliate. Obviously, it shows the capability of this ciliate to degrade organic matter in anaerobic digestion process.

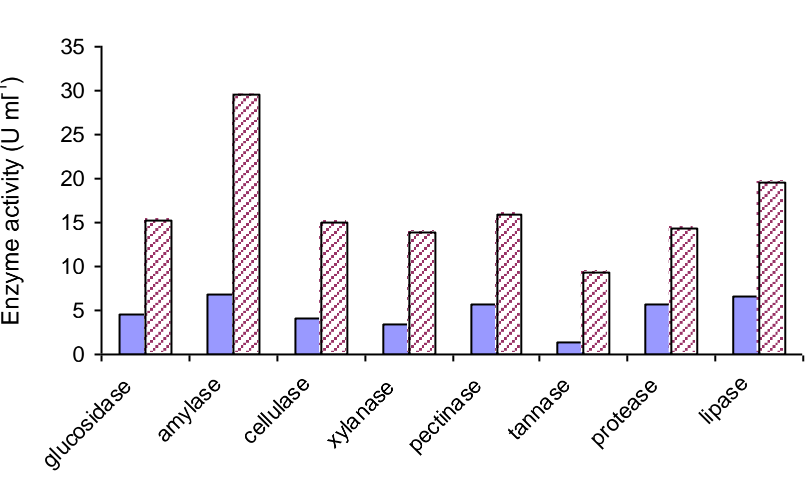


Fig. 5.27. Presence of extra and intracellular enzymes in *M. es* culture.

The presence of higher intracellular enzyme activity in *M. es* put forth the question of whether the endosymbionts are making significant contribution in intracellular production of enzymes. However, there was no evidence of eubacterial symbionts in *M. es* cell as shown by negative hybridization with EUB338 (chapter 4). The endosymbionts of *M. es* were found to be methanogens. The methanogens can not directly utilise complex molecules like starch or even simpler molecules such as glucose and they depend on other fermentative bacteria for energy yielding substances like acetate, formate, methanol and hydrogen. Only eubacteria are capable of degrading the complex polymers by their enzyme machinery. Since *M. es* did not contain eubacterial endosymbionts, the possible contribution of endosymbionts to enzyme production can be ruled out. The presence of various hydrolytic enzymes in *M. es* becomes important in conjunction with the presence of higher number of

endosymbiotic methanogens. In biomethanation systems, these methanogens form the significant members in anaerobic consortia as they produce methane in the course of their energy yielding metabolic process. The presence of endosymbiotic methanogens, together with its capability of providing energy source for methanogens makes *M. es* an efficient member in this consortia helping in the production of methane.

5.3.2 Role of *M. es* in COD removal

Chemical oxygen demand (COD) is an important indicator of organic matter in wastewaters. It is the measure of oxygen equivalent to the chemically oxidizable organic matter present in the sample. During the growth of *M. es*, the change in the organic matter content in the medium was measured in terms of COD at regular intervals. It gives an indication about the organic matter degradation by *M. es*. The growth of *M. es* in batch culture with the corresponding COD removal (%) is given in Fig. 5.28.

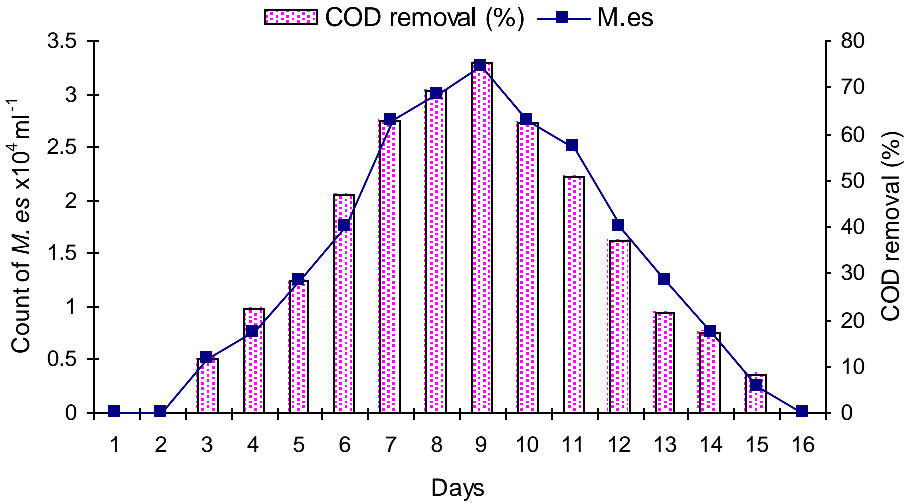


Fig. 5.28. Growth of *M. es* in ciliate mineral medium and corresponding COD removal

The removal of COD was observed in batch culture from 3rd day of *M. es* inoculation into the medium. The COD removal was less (11.85 %) at this stage, characterised by low count of *M. es* in the culture (0.5×10^4 ml^{-1}). The exponential growth of *M. es* was from 3rd to 9th day and this period was characterised by the sudden increase in COD removal. The highest COD removal was upto 75.3 % by 9th day, when *M. es* had the maximum growth of 3.25×10^4 ml^{-1}. In subsequent days, the growth of *M. es* declined and reached a count of 0.25 x 10^4 ml^{-1} on 15th day. This period was noted by decrease in COD removal and had only 8 % COD removal on 15th day.

In batch culture, COD removal was found to be positively correlated with *M. es* growth ($r^2 = 0.9736$) (Fig. 5.29). This suggests the direct involvement of *M. es* in organic matter utilisation. The reports on relation between anaerobic ciliates and organic matter degradation are scarce, though the presence of ciliates like *Metopus* and *Colpidium* is reported from different anaerobic reactors (Agrawal et al., 1997; Xu et al., 2005). According to Gijzen et al (1986), the rumen fluid containing protozoa was employed for the treatment of lignocellulosic waste by maintaining an active ciliate population of 9×10^4 ml^{-1} in a continuous system. Sound reports on the contribution of anaerobic ciliates are limited except studies on the influence of the ciliate count on COD removal in a continuously stirred tank reactor receiving sodium oleate feed (Priya et al., 2007).

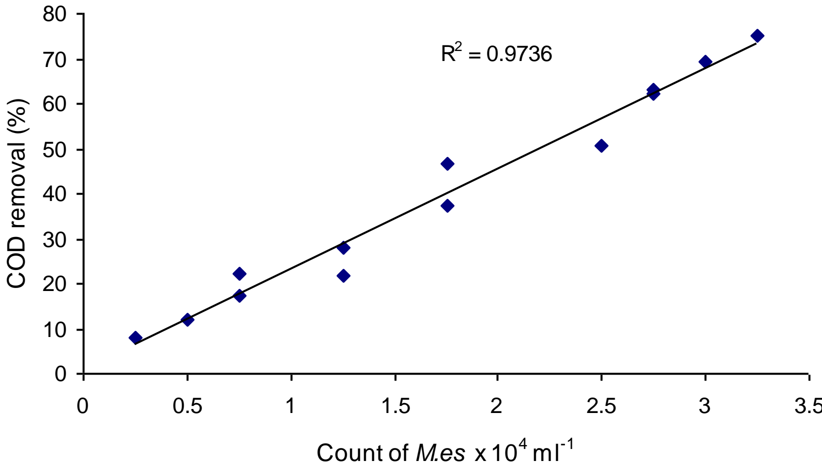


Fig. 5.29. Correlation between the growth of *M. es* and COD removal in ciliate mineral medium with wheat powder.

The isolated *M. es* was found to be capable of growing in different basal media like MP, PJ, PC and ciliate mineral medium with wheat powder as carbon source. Among them, ciliate mineral medium with wheat powder was found to be the most suitable one in terms of *M. es* growth (chapter 2). The maximum COD removal in different basal media and corresponding higher counts of *M. es* is given in Fig. 5.30.

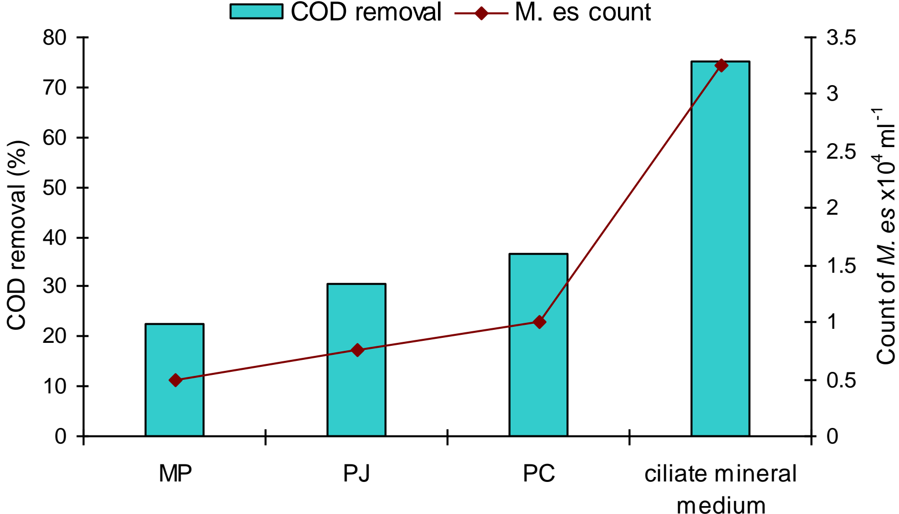


Fig. 5.30. Maximum growth of *M. es* and corresponding highest COD removal in different basal media with wheat powder.
Keys: MP- Modified Pringsheim's solution; PJ – Prescott's and Carrier's solution; PC – Prescott's and James's solution.

The COD removal was found to be related with *M. es* count in different basal media with wheat powder. The ciliate mineral medium having highest *M. es* count of 3.25×10^4 ml^{-1} was characterized by high level of COD removal (75.26 %). In PM, PJ and PC media, the maximum count of *M. es* were 0.5×10^4 ml^{-1}, 0.75×10^4 ml^{-1} and 1×10^4 ml^{-1} respectively. The COD removal was in the order of MP < PJ < PC corresponding to the count of *M. es*. As the bacterial number was less in different media (Fig. 2.1.16 of chapter 2), the higher COD removal in the culture with larger *M. es* population could be due to the direct consumption of organic matter by this ciliate. It is known that in aerobic environment the cropping activity of protozoa stimulates the decomposition of detritus material (Fenchel and Harrison, 1976), but the significance of protozoa in anaerobic environment is largely equivocal except in rumen ecosystem where many protozoa participate directly in the consumption and

fermentation of organic matter entering into the rumen (Marcin et al., 1998; Fondevila and Dehority, 2001; Santra and Karim, 2002).

The relative contribution in COD removal by *M. es* was further analysed by comparing the COD removal with two systems; bacteria alone and bacteria with *M. es* (Fig. 5.31). The mixed community containing both bacteria and *M. es* was characterised by higher total COD removal (93.4 %) while individual systems with *M. es* and bacteria alone had total COD removal of 75.3 and 78.9 % respectively. The order of COD removal observed among these systems was *M. es* with bacteria > bacteria alone > *M. es*. The higher COD removal in mixed system containing both bacteria and *M. es* is a common feature of anaerobic degradation systems, where synergistic activity of different microbial groups enables more efficient degradation. Statistical analysis showed that there is no significant difference between COD removal in individual cultures of *M. es* and bacteria (P > 0.05).

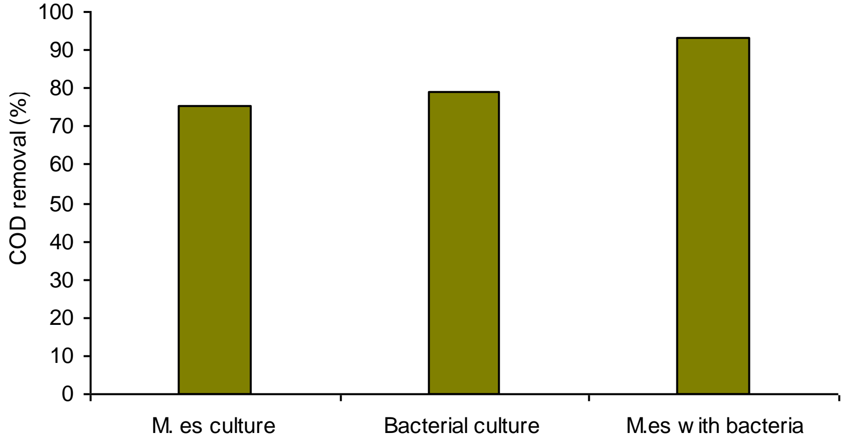


Fig. 5.31. Extent of COD removal in different anaerobic systems; *M. es* culture, bacterial culture and culture of *M. es* with bacteria.

The substrate removal analysed in terms of total organic carbon (TOC) was also found to be varying in the above three systems (Fig.5.32). The mixed community having both *M. es* and bacteria was more efficient in organic matter removal (90.9 %) in five days whereas individual cultures of *M. es* and bacteria had 73.2 and 74.5 % TOC removal respectively. The absence of predator protozoa as well as lower generation time allowed the bacteria to reach the higher population density of 6.3×10^{7} ml^{-1} within 3 days (Fig. 5.36). This was slightly advantageous for TOC removal in bacterial system compared to *M. es* culture at the initial stage. Due to higher generation time of *M. es* compared to bacteria, its exponential growth starts from 3rd day with the cell count of 0.5×10^{4} ml^{-1}. Thereafter the count of *M. es* was increased and that could be the reason for more or less similar pattern of TOC removal compared to bacterial system at this stage.

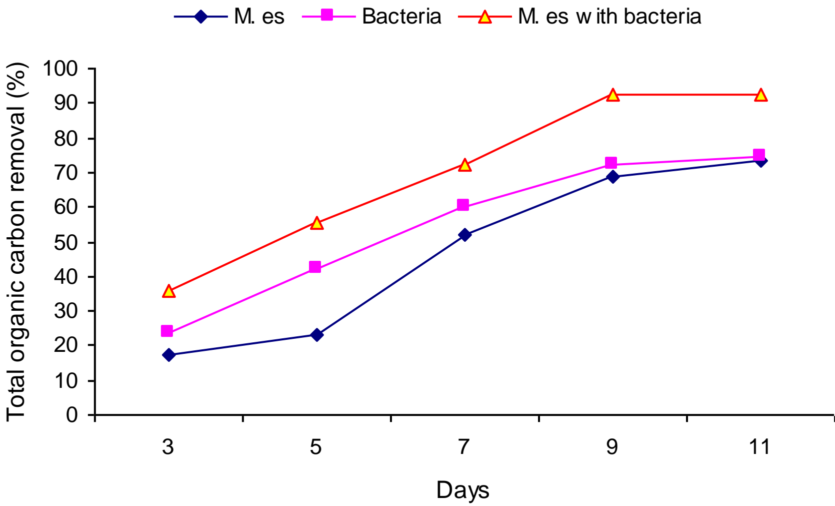


Fig. 5.32. Total organic carbon removal in different anaerobic systems; *M. es* culture, bacterial culture and culture with *M. es* and bacteria.

The ability of organic matter degradation by *M. es* was comparable to a system containing bacteria alone in terms of COD and TOC removal. The isolated *M. es* was cultured in a bacteria controlled environment by providing an alternate carbon source in the absence of prey bacteria (chapter 2). The growth of *M. es* on external carbon source in a bacteria suppressed condition and subsequent removal of organic matter during its growth indicates its active participation in direct degradation of organic matter. The direct participation of ciliates in the removal of organic matter content was extensively studied in aerobic environment. The presence of free swimming aerobic ciliates in activated sludge resulted reduction in COD and suspended matter content when compared to bacterial cultures (Curds et al., 1968; Macek, 1991). Similarly, a strong correlation between COD and population density of ciliates was observed in activated sludge plants (Sudo and Aiba, 1984). But in anaerobic environment, the role of ciliates in the organic matter degradation was not well understood especially using individual ciliate culture. The organic matter degradation by *M. es* culture helps to understand and explains its importance in anaerobic process.

5.3.3. Role of *M. es* in methane production

Methane is the major end product of organic matter mineralisation in biomethanation process and methanogens are the key organisms responsible for methane production. In biomethanation systems, measure of methane production is the most sensitive way to evaluate the efficiency of the process. The methane production by *M. es* was measured in batch systems (Fig. 5.33). There was an increase in amount

of methane produced with increasing number of *M. es* in culture and maximum amount of methane (10.6 ppm) was produced at highest population density of *M. es* (3.25×10^4 ml^{-1}). The exponential growth phase of *M. es* was characterised by maximum methane production rates of 0.13- 0.44 ppm day^{-1}. After exponential growth, methane production reduced along with decline in *M. es* count. Due to low growth efficiency of anaerobic ciliates, it is difficult to maintain higher population density in batch cultures. This might be the reason of decrease in methane production after exponential phase.

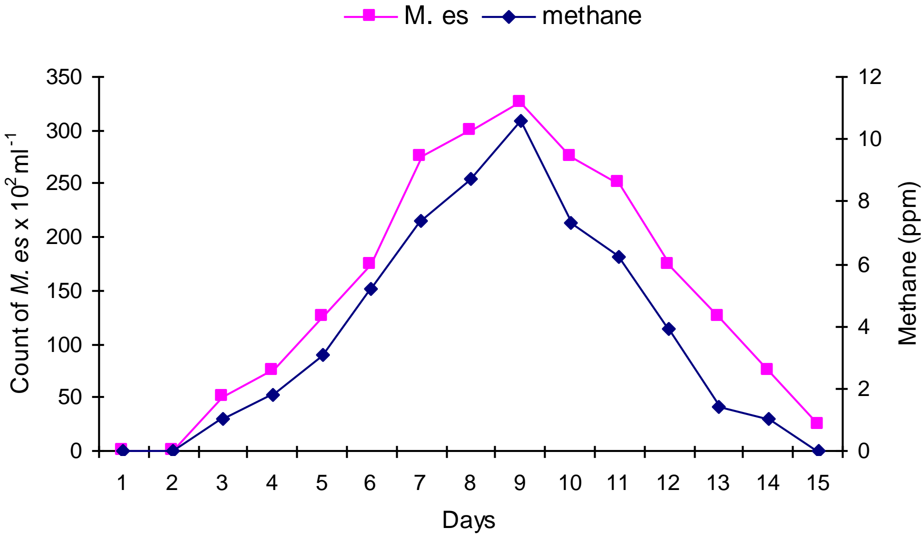


Fig. 5.33. *M. es* growth and methane production in batch culture.

The *M. es* count in batch cultures was found to be positively correlated (r^2 = 0.9478) with the amount of methane produced (Fig.5.34). This indicates the ability of *M.* es to act as potent producer of methane in biomethanation systems with its endosymbiotic methanogens.

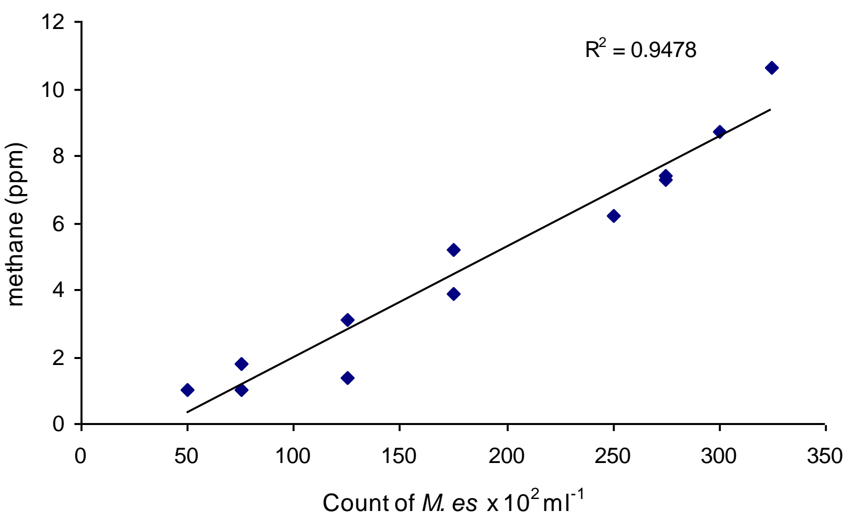


Fig. 5.34. Correlation between methane production and count of *M. es*.

The methane production was compared in bacterial culture and mixed culture containing bacteria and *M. es* (Fig. 5.35). Higher methane production was observed in system containing mixed culture of *M. es* and bacteria (275 ± 5 ml methane) compared to bacterial culture (201 ± 3 ml methane).

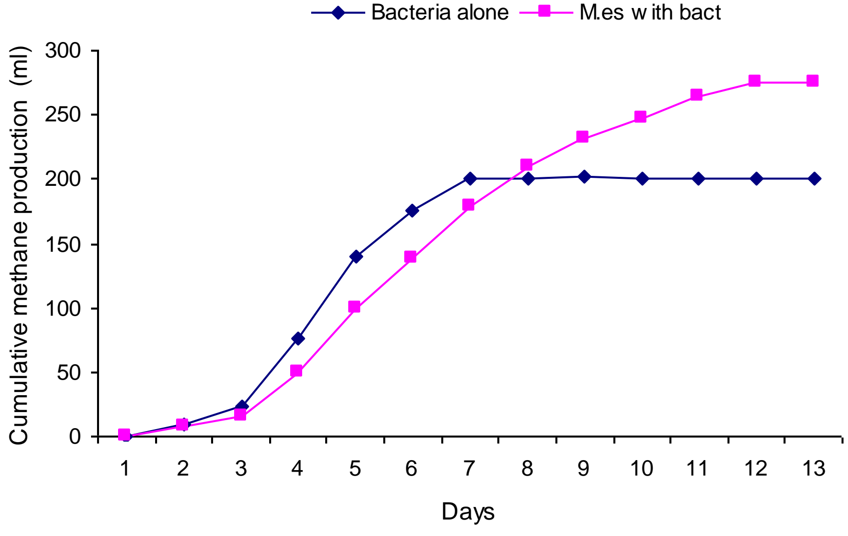


Fig. 5.35. Methane production in different anaerobic systems; bacterial culture and *M. es* with bacteria.

The initial amount of methane produced was higher in bacterial culture which could be due to high bacterial count (9×10^7 ml^{-1}) in the absence of *M. es* whereas in mixed culture, maximum bacterial count was only 6.7×10^7 ml^{-1} (Fig. 5.36). The growth of *M. es* was also increased in presence of bacteria with its population density of 3.75×10^4 ml^{-1} compared to the maximum count of 3.25×10^4 ml^{-1} in the absence of bacteria. At later stages, mixed culture with higher number of *M. es* produced more methane than bacterial culture. In presence of *M. es*, there was 26 % increase in methane production, when compared to bacterial culture. Thus the combined activity of both bacteria and *M. es* might be responsible for increased methane production.

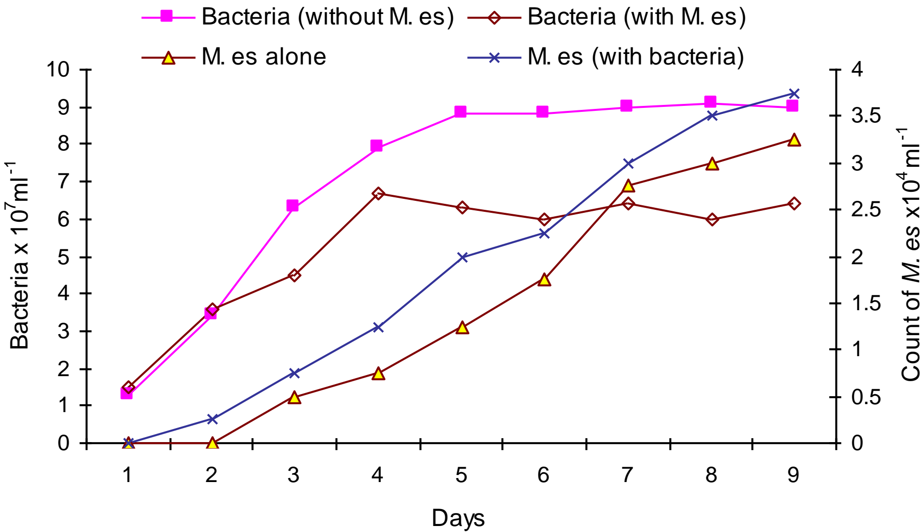


Fig. 5.36. Bacteria and *M. es* counts in different anaerobic systems; *M. es* culture, bacterial culture and culture of *M. es* and bacteria.

Biagini et al., (1998a) reported an increased rate of methane production in presence of anaerobic ciliate, *M. palaeformis* and count of *M. palaeformis* was

correlated with stimulation in methane production. The possible explanation for the increased activity in presence of ciliates is due to recycling of nutrients which would otherwise get immobilised in bacterial biomass. Anaerobic ciliates possess a fermentative metabolism producing substrates like organic acids, CO_2 and hydrogen which can serve as the substrates for methanogens (Goosen et al., 1990a; Holler and Pfenning, 1991), thus contributing to methane production. In anaerobic bacterial cultures, without predation and particulate matter degradation by ciliates, inorganic nutrients and organic acids get immobilised in bacterial biomass, thereby limiting their growth rate. This can be the reason for reduction in amount of methane produced in bacterial system after an initial rise in methane level.

Methanogenic symbionts are found in many anaerobic protozoa, particularly in ciliates (Fenchel and Finlay, 1995). Considerable portion of methane released from rumen livestock was produced by ciliates and their endosymbionts (Ushida et al., 1997). Ciliates have a substantial contribution of 90 % of total methane production in shallow marine sediments (Fenchel, 1993). Thus the presence of endosymbiotic methanogens in anaerobic ciliates helps complete mineralisation of organic matter within their cell. In the present study, *M. es* found to contain endosymbiotic methanogens distributed throughout the cytoplasm of the ciliate except in nuclear and vacuole regions (chapter 4). A single *M. es* has 792 ±12 methanogens which occupy 0.8 % of total biovolume the ciliate. The average count of *M. es* in mixed culture containing both bacteria and methanogens was found to 1.86×10^4 ml^{-1} whereas free methanogens in the same system had an average count of 5.3×10^7 ml^{-1} (Fig.5.35).

Since single *M. es* had 792 methanogens, the total number of endosymbiotic methanogens in 1.86×10^4 cells was 1.5×10^7 ml^{-1}. This accounts 28 % of the free methanogens in batch culture.

The *M. es* culture had maximum amount of methane on 9th day when *M. es* count was 3.25×10^4 ml^{-1} (Fig. 5.33). The maximum rate of methane production by single *M.* es calculated from this data was found be 0.85 pmol CH_4 $ciliate^{-1}$ h^{-1}. Methane production rate of single endosymbiotic methanogen was 1.07 fmol CH_4 $methanogen^{-1}$ h^{-1} as single *M. es* cell carries 792 methanogens (Table 4.1 of chapter 4). Methane production rate per ciliate was found to be varying in different species of anaerobic ciliates. It is related with ciliate biovolume as well as the number of endosymbiotic methanogens per single cell (Schwarz and Frenzel, 2005). Rumen ciliates with higher number of endosymbiotic methanogens produce 83 - 250 pmol CH_4 h^{-1} (Ushida and Jouany, 1996). *M. palaeformis* with 360 methanogens $cell^{-1}$ produces methane at a rate of 0.35 pmol methane $ciliate^{-1}$ h^{-1} whereas *M. contortus* having higher number of methanogens (1000 – 6000 $cell^{-1}$) produces methane at a rate of 7 pmol $ciliate^{-1}$ h^{-1} (Fenchel and Finlay, 1992). The *M. es* cell harbouring 28 % of free methanogens and having a methane production rate of 0.85 pmol CH_4 $ciliate^{-1}$ h^{-1} can contribute significantly to methane generation in anaerobic process.

5.3.4 Size selective predation by *M. es*

The *M. es* was grown in cultures provided with different sized wheat particles as carbon source and highest count of the ciliate was compared with particle size of the feed (Fig. 5.37).

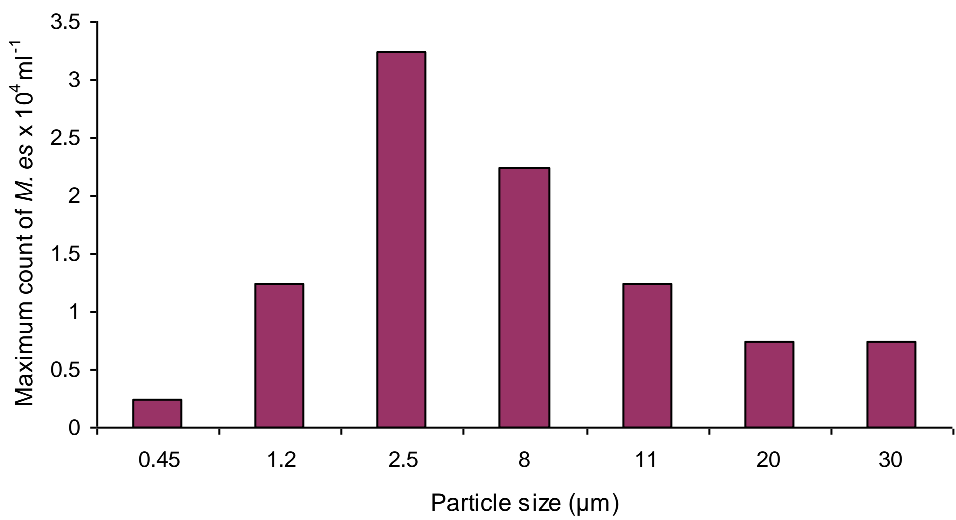


Fig. 5.37. Maximum growth of *M. es* in medium with different sized particulate feed.

The smaller (0.45 - 1.2 µm) as well as larger sized (20 - 40 µm) particulate matter in the medium was associated with low number of *M. es* compared to feed particles in the size range of 2.5 - 11 µm. This suggests that *M. es* feeds on the organic material present in the medium whose optimum size ranges from 2.5 - 11µm. This is further supported by the presence of different enzymes in *M. es*. The particulate feeding nature of *M. es* makes it significant in biomethanation process where suspended organic matter is present in higher amounts.

5.3.5 Grazing of bacteria by *M. es*

The Metopid ciliates are often regarded as prominent filter feeders of bacteria in anaerobic environment. Bacterial grazing by *M. es* was studied by supplying anaerobic bacteria of different sizes, isolated from anaerobic sludge. The isolated bacteria of different shapes were categorized based on their size (Table 5.1). The bacterial suspensions of different size categories were added as sole carbon source to the ciliate mineral medium to study the size selective predation by *M. es*.

Table 5.1. Anaerobic bacteria of different size ranges isolated from sludge

Sl.No	Shape	Size(µm)	average size (µm)
1	cocci	1-3	2
2	rod	2-4	3
3	rod	3.4-5	4
4	rod	4.6-5.3	5
5	long rod	5.6-6.3	5.9
6	rod (filamentous)	7-7.5	7.1
7	long rods	7.8-8.1	7.9

The growth of *M. es* in medium with different sized bacterial prey is given in Fig. 5.38. The bacterial feed of all given size range was found to support the growth of *M. es*, but higher count of *M. es* was observed in medium supplemented with bacteria of average size 3 µm. This is in agreement with results showing highest growth of *M. es* in medium containing suspended particle of the size 2.5 µm (Fig.5.37). The *M. es* growth was less in medium with bacterial prey of < 3 µm and > 4 µm sizes.

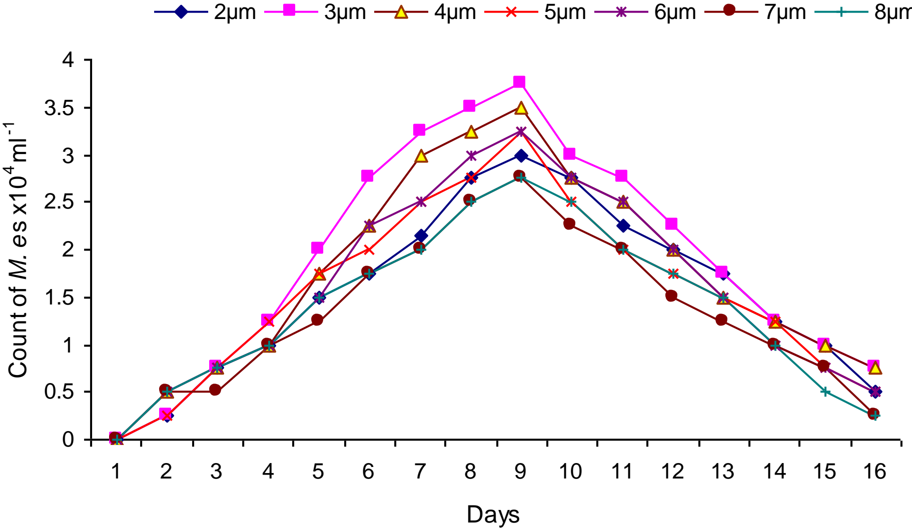


Fig. 5.38. Growth of *M. es* in medium supplied with bacteria of different size (2 – 8 µm).

Selective grazing on specific bacteria is a common feature among protozoa which can directly influence the bacterial community composition, size structure and its productivity in natural environments (Hahn and Hofle, 1999; Posch et al., 1999). Different species of ciliates show a characteristic spectrum of particle size which they can retain and ingest. The holotrich ciliates prefer food in the size range 0.1 - 1 µm while spirotrich ciliates feed on 1 - 2 µm sized food particles (Fenchel, 1980a). Small ciliates like *Cyclidium* and *Colpidium* grows best on food particles of size 0.3 - 1 µm while larger ones like *Stentor* grows best on particles in the size range of 5 - 30 µm (Fenchel, 1980b). In the present study, *M. es* could grow on food with size range of 2 - 8 µm with its highest growth on food particles having size 3 - 4 µm.

In order to study the feeding rate in *M. es*, it was grown in batch culture with known number (4×10^6 ml^{-1}) of heat killed bacteria (average size 3μm) as food. The population density of *M. es* and number of bacteria remaining in the medium were counted at regular intervals. The heat killed bacterial suspension without *M. es* served as control. Mean uptake rate of *M. es* was calculated from these data as number of bacteria $ciliate^{-1}$ h^{-1}. The bacterial uptake by *M. es* at definite time intervals is given Table 5.2.

Table 5.2. The uptake rate of *M. es* grown on bacterial prey of average size 3 μm.

Time (h)	Uptake rate ($\times$ 10 bacteria $ciliate^{-1}$ h^{-1})
1	11.2
2	14.3
3	15.4
4	19.8
5	25.3
6	27.6
7	32.4
8	38.6
9	40.2

The average uptake rate of *M. es* obtained from the above data was 249.7 bacteria $ciliate^{-1}$ h^{-1}. The clearance rate of *M. es* was calculated by dividing mean uptake rate with initial bacterial count ml^{-1} (Massana and Pedros-Alio, 1994). The *M. es* was found to possess a clearance rate of 62 nl $ciliate^{-1}$ h^{-1} on feeding with 3μm sized food bacteria. The bacterial count in control without *M. es* remained the same after 9 h (4×10^6 ml^{-1}) indicating that reduction in bacterial count in *M. es* was not due to bacterial lysis. Clearance rate is the volume of water cleared per individual ciliate per

hour and both uptake and clearance rates have importance in ecological context (Fenchel, 1980b). Earlier studies reported clearance rates of 100, 48.8 and 1.71 nl ciliate^{-1} h^{-1} for *Plagiopyla*, *Euplotes* and *Cyclidium* respectively (Massana and Pedros-Alio, 1994; Tso and Taghon, 1999). Massana and Pedros-Alio (1994) repoted a clearance rate of 32 nl ciliate^{-1} h^{-1} for *M. es* in natural environment. The higher clearance rate obtained in the present study (62 nl ciliate^{-1} h^{-1}) than reported value is significant in biomethanation process in the sense that the ciliate can act as important grazer on suspended particles in these systems. This would help to remove suspended matter including bacteria thereby reducing turbidity and clarify the effluent.

5.3.6 Role of *M. es* in nutrient cycling

In general, bacteria can assimilate large amounts of dissolved organic matter, which is not degraded into inorganic forms and converted into bacterial biomass. Indeed, these nutrients are getting immobilised temporarily in bacteria, if not eaten by their predators, mainly protozoa. Thus by their grazing activity, protozoa act as an important link in mineralisation, the release of organically bound nutrients to inorganic forms. The excretion of nitrogen and phosphorous by phagotrophic protists is the major source of regenerated nutrients in aquatic ecosystems (Sherr and Sherr, 2002). The ability of *M. es* to take part in mineralisation of nutrients was studied in cultures supplemented with bacteria as food source. Cultures of *M. es* were fed with heat killed as well as live bacteria separately to find the generation of nutrients by it. The growth of *M. es* in presence of live and heat killed bacteria is given in Fig. 5.39. The live bacteria were found to support the growth of *M. es* more as indicated by

higher count (3.75×10^4 ml^{-1}) than in heat killed bacteria supplemented cultures (3.5×10^4 ml^{-1}). But no significant difference exists between *M. es* growth pattern among live and heat killed bacteria ($P > 0.05$).

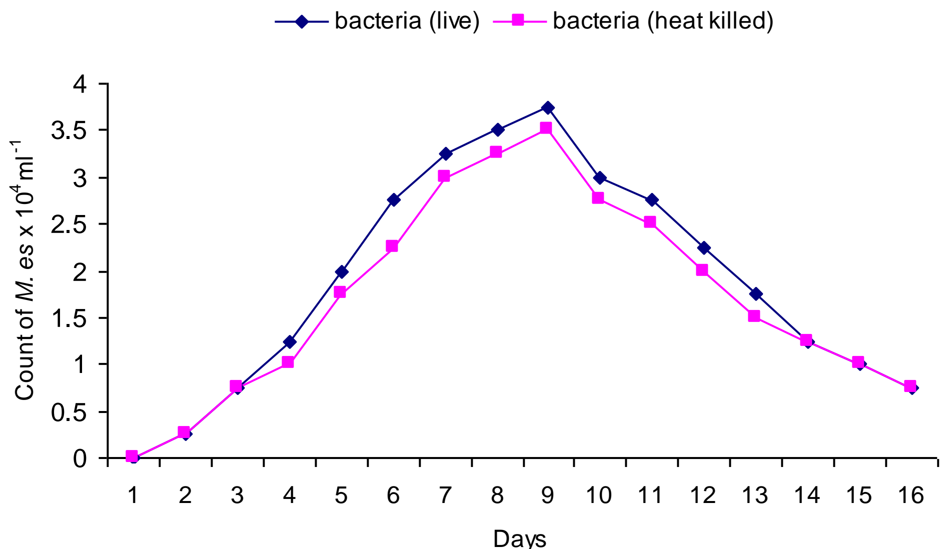


Fig. 5.39. Growth of *M. es* in medium supplemented with live and killed bacteria as food.

The excretion of nutrients by *M. es* was measured as ammonia and phosphate at regular intervals. The amount of ammonia released was found to be related with the number of *M. es* as shown in Fig 5.40. The ammonia concentrations ranged from 1.0 - 4.56 ppm in *M. es* cultures with heat killed bacteria and the maximum concentration was 4.56 ppm during the exponential growth stage of *M. es*. In cultures with live bacteria as food, ammonia generated was less (0.24 – 3.20 ppm). However, in both cases, amount of ammonia produced was related to population density of *M. es* in

culture. Live bacteria utilise released metabolic products and this could be the reason for low level of ammonia in presence of live bacteria.

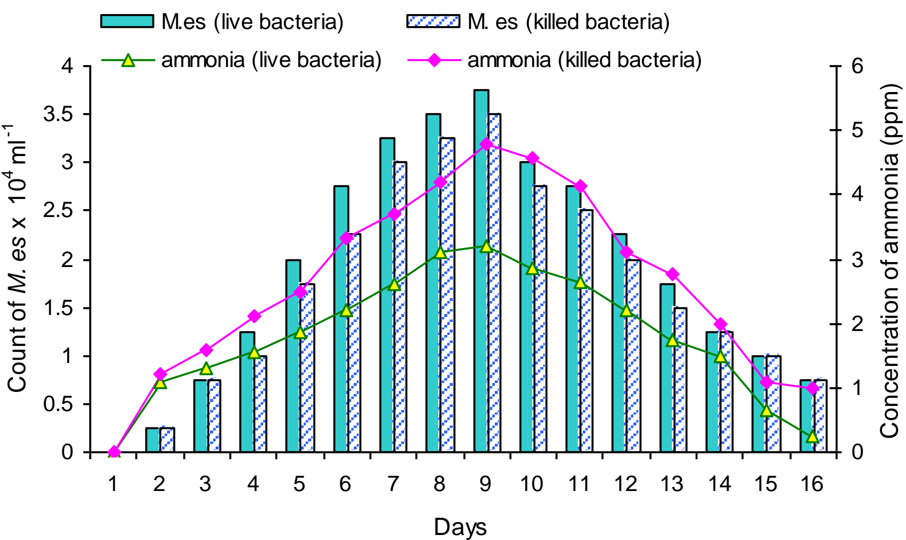


Fig. 5.40. Growth of *M. es* and ammonia released in presence of live and heat killed bacteria.

The phosphate levels detected in *M. es* cultures also followed the same pattern as that of ammonia (Fig. 5.41). The amount of phosphate was higher during the exponential growth phase of *M. es* like ammonia, but its concentration was less. It ranged from 0.02 – 1.32 ppm and 0.05 -1.61 ppm in presence of live bacteria and dead bacteria respectively. Here also, maximum amount of phosphate (1.61 ppm) was observed in *M. es* culture with dead bacteria compared to the live bacteria (1.32 ppm).

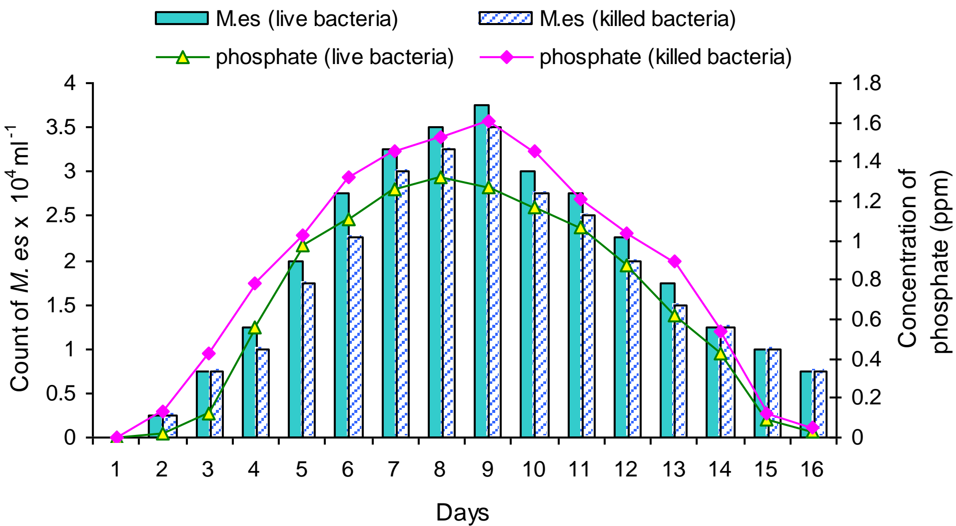


Fig. 5.41. Growth of *M. es* and phosphate released in presence of live and heat killed bacteria.

Ammonia and phosphate concentrations in live bacterial cultures and heat killed bacterial suspension were measured in the absence of *M. es* to determine whether bacteria are participating in release of these nutrients by metabolism or cell lysis. The ammonia concentrations of heat killed and live bacteria were 0.056 and 0.062 ppm respectively; where as the phosphate concentrations were 0.015 and 0.018 ppm. These values are much less when compared to the amount of ammonia and phosphate detected in presence of *M. es* and remained constant during the entire period of bacterial growth which eliminates the chance of bacterial production of nutrients. Both ammonia and phosphate generation by ciliate was higher in presence of heat killed bacteria than live one. The lower concentration of both ammonia and phosphate in presence of live bacteria could be due to utilisation of these nutrients by bacteria during their growth.

Protozoa can re-mineralise organic matter and regenerate nutrients in aerobic environment through their grazing activity (Taylor, 1982). In aquatic ecosystem, protozoa are actively taking part to release nutrients by grazing which would otherwise get immobilised in bacteria, picocyanobacteria and small detrital particles; thereby serve as mediators of nutrient cycling (Caron, 1991; Berman, 1991; Sherr and Sherr 2002). Protozoa, especially ciliates help in more effective operation of aerobic sewage treatment plants through enhancing the growth by recycling the limiting nutrients and making them available to organic matter degrading bacteria (Curds, 1982). The anaerobic ciliate, *M. es* was also capable of releasing limiting nutrients as its metabolic products that can enhance the growth of other microbes involved in biomethanation process.

5.4 Conclusions

The degradation of organic matter in anaerobic environment is considered mainly as bacteria mediated process. Among eukaryotes, some fungi participate directly in the degradation of complex substances like cellulose and lignin (Lynd et al., 2002). Till date, only the grazing activity of anaerobic protozoa is gained importance as it alters the composition and density of bacterial population, thereby affecting the degradation. In this context, the presence of hydrolytic enzymes in the anaerobic ciliate, *M. es* indicates the existence of an additional pathway of organic matter degradation.

- The presence of various hydrolytic enzymes in *M. es* is of great importance in anaerobic digestion indicating its direct participation in organic matter degradation.
- In addition to soluble sugars, different polysaccharides are also degraded by *M. es* by its elaborate enzyme machinery.
- The ability of *M. es* to degrade polyphenols helps it to survive in polyphenols containing environment.
- The uptake and degradation of protein and lipid by *M. es* enable the effective removal of these substances in biomethanation process.
- Bacteria mainly depend on extracellular enzymes for the degradation of complex molecules to simpler ones since its cell membrane is not permeable to large molecules. The *M. es* has high level of intracellular enzyme activity indicating that unlike bacteria, it is capable of engulfing large molecules from the surroundings which will increase the degradation of complex substances.
- The initial breakdown of complex substances like carbohydrates, proteins and lipids is considered as the rate limiting step in biomethanation process. The presence of hydrolytic enzymes in *M. es* and its capability to degrade different substances can be used to enhance the initial rate of degradation by maintaining higher number of this ciliate at this stage.
- The increase in COD removal is correlated with increasing population of *M. es* ($r^2 = 0.974$) in culture in the absence of bacteria shows its capability of organic matter degradation.

- The mixed system containing both bacteria and *M. es* is more efficient in degrading organic matter in terms of COD and TOC removal. The COD removal in *M. es* culture is more or less similar to that of bacterial system which implies that *M. es* has got equal importance to that of bacteria in terms of organic matter degradation.
- The study reveals that amount of methane produced is positively correlated with *M. es* count in culture. The methane production is correlated to the number of *M. es* cells ($r^2 = 0.948$) and there is 26 % reduction in methane production in the absence of *M. es*.
- The endosymbiotic methanogens in *M. es* make a substantial contribution in methane production with a methane production rate of 1.07 fmol CH_4 methanogen^{-1} h^{-1}. They occupy 0.8 % of biovolume of single *M. es* and at exponential growth stage of *M. es*, the total endosymbiotic methanogens accounts to 28 % of free methanogens in culture.
- Size selective feeding on particulate matter by *M. es* points out its ability to remove suspended matter in biomethanation systems. The direct consumption of particulate matter makes this ciliate significant in anaerobic systems.
- *M. es* is an active bacterial grazer which prefer on bacteria of 3 – 4 μm size range as food. It possesses a mean uptake rate of 249 bacteria ciliate^{-1} h^{-1} and clearance rate of 62 nl ciliate^{-1} h^{-1} which is important in the sense that *M. es* can remove the suspended particles from medium substantially.

- The excretion of limiting nutrients (ammonia and phosphate) by *M. es* culture in presence of bacteria indicates its role in recycling of these nutrients in biomethanation systems. Immobilisation of nutrients in bacterial biomass can limit the growth rate and adversely affect degradation in the absence of protozoa.

CHAPTER 6

Summary and conclusions

The organic matter degradation in biomethanation process is driven by the coordinated activity of different functional groups of microorganisms. Among them, bacteria are considered as the important group in the degradation of biomass, though eukaryotes like fungi and protozoa are known to exist in such systems. In case of protozoa, only the grazing activity of ciliates has received attention since it can reduce the bacterial biomass. The present study reports the occurrence of the anaerobic ciliate, *Metopus* sp. in the anaerobic reactors and its growth is linked to the reactor performance in terms of Chemical Oxygen Demand (COD) removal and Volatile Fatty Acids (VFA) conversion to methane. Since *Metopus* sp. was abundant at the steady state condition of the reactor, characterized by low VFA concentration, around neutral pH and effective COD removal, its culture was developed to assess its participation in organic matter degradation.

Metopus sp. was isolated from a laboratory scale reactor by centrifugation followed by micromanipulation. This combined method was found to be more effective in obtaining monoculture with less bacterial contamination than the earlier isolation methods employing either centrifugation or micromanipulation independently. The addition of antibiotics, penicillin and streptomycin was found to be

effective in controlling the growth of bacteria in ciliate culture. Among the different basal media, ciliate mineral medium was selected as the most suitable one for *Metopus* sp. The optimum level of physiochemical and nutritional requirements for the isolated *Metopus* sp. is given in Table 6.1.

Table 6.1. The optimum level of growth conditions of *Metopus* sp. ciliate mineral medium

Parameters	Optimum range
Carbon source	wheat powder (1 %)
Ammonia-N	6 - 10 mg/L
Phosphate-P	6 – 8 mg/L
Sulfide-S	1 – 2 mg/L
pH	6 -7
Temperature	30 – 35 °C
Oxygen	Nil

The species level identification of ciliates is done mainly based on the morphological features of live and stained specimen. The isolated *Metopus* sp. was identified as *Metopus es* based on morphological features for which silver carbonate staining was the key technique used to reveal the morphological features.

Ciliates are considered as effective biological indicators since they respond more quickly to environmental contaminants than other organisms due to their increased permeability to contaminants, high reproduction rate and short life cycle cycles compared to metazoa. The toxicity studies of *M. es* reveal that it is sensitive to

different compounds in biomethanation process and hence organic matter degradation by this ciliate can be affected if these components are present beyond the tolerance level. The level of ammonia tolerance exhibited by *M. es* is comparable to those reported in aerobic ciliates (12 h LC_{50} = 87.43 mg l^{-1} ammonia-N). *M. es* is very sensitive to sulfide (12 h LC_{50} = 8.66 mg l^{-1} sulphide-S) and heavy metals (4 h LC_{50} of 0. 31 mg l^{-1} for Hg) which makes it a good candidate to determine the bioavailable concentration of these compounds. Like methanogens, *M. es* is sensitive to higher VFA concentrations with its growth being affected by > 0.05 M VFA. *M. es* survived at low oxygen levels (0.1 – 0.5 %) owing to the presence of superoxide dismutase (1.31 - 1.52 U/mg protein). Superoxide dismutase is one of the important protective enzymes in antioxidant defense system and occurrence of an Mn type of SOD in *M. es* enables it to survive at low oxygen levels. The determination of tolerance level of *M. es* to important compounds in anaerobic digestion indicates that these compounds can affect the biodegradation potential of this ciliate when present beyond the tolerance limit.

The endosymbiotic methanogens harboring 0.8 % biovolume of *M. es* enables production of methane significantly in biomethanation systems. *M. es* is capable of producing methane at a rate of 0.85 pmol methane ciliate^{-1} h^{-1} which is comparable to methane production rate of other anaerobic ciliates. The endosymbiotic methanogens in *M. es* are not permanently affected by oxygen exposure and retained their activity within cilaite cysts. This symbiosis also acts as a mechanism to protect the strict

anaerobic methanogen from low level of oxygen exposure. The study reveals the presence of acetate utilising methanogen, *Methanosaeta* as endosymbiont in *M. es*. The presence of *Methanosaeta* as symbionts is an important finding and the existence of this aceticlastic methanogen as symbiont in anaerobic ciliates has not been reported in anaerobic ciliates previously. The acetate generated in *M. es* cells supports symbiotic association with this aceticlastic methanogen resulting in the production of methane. *Methanosaeta* prefers a low acetate concentration (< 1 mM) for its growth than the other aceticlastic methanogen, *Methanosarcina*. The intracellular acetate production by *M. es* ranges from 0.35 – 0.9 mM and this low concentration of acetate within the ciliate cell creates a suitable environment for this aceticlastic methanogen. The endosymbionts are significant for the growth efficiency of *M. es* as indicated by reduction in yield and growth rate (30 %) in the absence of endosymbionts. This shows that the symbiotic association between *M. es* and its endosymbionts is significant not only in terms of substrate supply for symbionts but also with the growth efficiency of *M. es*.

The organic matter degradation in biomethanation process is considered as a bacteria-driven process and anaerobic ciliates are often represented only as grazers on bacterial population. In contrast, present study reveals the direct involvement of anaerobic ciliate, *M. es* in organic matter degradation in biomethanation system. In the bacteria controlled environment, *M. es* is capable of ingesting and degrading the organic matter directly. The COD removal was well correlated with the *M. es* growth

($r^2 = 0.974$). The COD removal in *M. es* culture was almost similar to bacterial system suggesting that degradation potential of *M. es* is comparable with bacteria. The hydrolytic reaction is the rate liming pathway of biomethanation process with solid organic wastes. The direct consumption potential of organic matter by *M. es* can overcome the rate limitation which makes it significant in biomethanation process. The methane production was also positively correlated with *M. es* count ($r^2 = 0.948$) owing to the presence of endosymbiotic methanogens in it and thereby producing methane in the anaerobic environment. The average number of endosymbiotic methanogens in *M. es* constitutes 28 % of total free methanogens in anaerobic system containing bacteria and *M. es*. It was observed that the methane production was increased by 26 % in the presence of *M. es*.

Size selective predation studies indicates that *M. es* feeds on particles of both organic matter and bacteria in the size range of 3 - 4 µm, ideally in the anaerobic system. The mean uptake rate is 249 bacteria ciliate^{-1} h^{-1} and clearance rate of 62 nl ciliate^{-1} h^{-1} is significant in biomethanation process in the sense that the ciliate can act as important grazer on undissolved particles in these systems. This would help to remove suspended matter including bacteria which can result in turbidity removal. The *M. es* releases limiting nutrients, ammonia and phosphate when grown on bacterial feed. Thus the growth of this ciliate enhances the mineralization of organic matter and recycling of nutrients, which would otherwise get immobilised in bacterial biomass.

The presence of various hydrolytic enzymes in *M. es* strengthens its capability of organic matter degradation. The direct utilisation of carbohydrates, proteins, lipids and polyphenols by hydrolytic enzymes by *M. es* suggests the existence of an additional route of organic matter degradation. *M. es* can engulf particles of suitable ranges directly from the environment and digest them intracellularly while bacteria need to depend on extracellular enzymes for the degradation of suspended particles.

The significance of the present work is that the anaerobic ciliate, *M. es* participates directly in organic matter degradation which makes this ciliate relevant in biomethanation process besides its grazing activity. This is the first report on the development of monoculture of *M. es* in bacteria controlled condition and enzymatic degradation of organic matter by *M. es*. Through this monoculture development, role of protozoa in anaerobic processes could be studied in detail for understanding the complex ecosystem. The future implication of this study is that anaerobic reactors rich in *M. es* can achieve higher rate of organic matter mineralization and methane production.

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