Involvement of Protozoa in Anaerobic Wastewater Treatment Systems

Thesis submitted to the University of Kerala for the Degree of Doctor of Philosophy in Environmental science

By

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Dedicated to My Parents

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Certificate

This is to certify that the work presented in the thesis entitled "**Involvement of protozoa in anaerobic wastewater treatment systems**" submitted herewith by Mrs. Priya M. for the award of Doctor of Philosophy in Environmental science of the University of Kerala is an authentic scientific record of research work carried out under my guidance and supervision, at 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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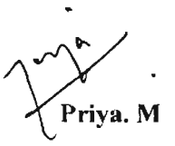
Declaration

I hereby declare that the work presented in this thesis is based on the original work carried out by me under the guidance of Dr. V.B. Manilal, 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.



Priya. M

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Priya.M

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Research articles

1. **Priya. M**, Ajit Haridas and V. B. Manilal. Anaerobic protozoa and their growth in biomethanation systems (**Biodegradation** 19, 179-185 (2007)).
2. **Priya. M**, Ajit Haridas and V. B. Manilal. Involvement of protozoa in anaerobic wastewater treatment processes (**Water Research**. 41, 4639-4645 (2007).
3. Nimy Narayanan, **Priya. M**, Ajit Haridas and V. B. Manilal. Isolation and culturing of a most common anaerobic ciliate, *Metopus* sp. (**Anaerobe** 13(1), 14-20 (2007)).
4. **Priya. M**, Ajit Haridas and V. B. Manilal. Response of anaerobic protozoa to oxygen tension in biomethanation system (Accepted in Journal **Microbiology**).

Poster

1. **Priya . M**, Ajit Haridas and V. B. Manilal. Involvement of anaerobic ciliates in the degradation of particulate organic matter (Poster presented in "**The International Symposium on Ciliate Biology**", University of Delhi, Delhi).

Abbreviations

UASB	Upflow anaerobic sludge blanket
AFBR	Anaerobic fluidized bed reactor
BFBR	Buoyant filter bioreactor
CSTAR	Continuous stirred tank anaerobic reactor
RUDAD	Rumen derived anaerobic digestion
IWA	International water association
SRT	Solid retention time
HRT	Hydraulic retention time
COD	Chemical oxygen demand
BOD	Biological oxygen demand
VFA	Volatile fatty acids
VSS	Volatile suspended solids
MLSS	Mixed liquid suspended solids
TLC	Thin layer chromatography
TOC	Total organic carbon
PDH	Pyruvate dehydrogenase complex
PFO	Pyruvate ferredoxin oxidoreductase
DAPI	4', 6-diamidino -2- phenylindole
TSQ	*N*-(6-methoxy-8-quinolyl)-*p*-toluenesulfonamide
FISH	Fluorescence insitu hybridisation
DNS	Dinitrosalicylic acid
CMV	Ciliate mineral medium
ROS	Reactive oxygen species
SOD	Superoxide dismutases
SDS	Sodium dodecyl sulfate
pNPP	p-nitrophenyl palmitate
TCA	Tricarboxylic acid

DNPH	2,4-dinitrophenylhydrazine
BSA	Bovine serum album
DMP	Dimethylphthalate
LD_{50}	Median lethal concentration
MRD	Maximum resistance dose
PAGE	Polyacrylamide gel electrophoresis

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Preface

Anaerobic wastewater treatment has become a widely accepted technology in the last few decades with the development of high-rate reactors. The process of anaerobic degradation is a complex and dynamic system where, microbiological, biochemical and physico-chemical aspects are closely linked. During anaerobic treatment, a complex natural community consisting of many interacting microbial species degrades natural polymers such as polysaccharides, proteins, nucleic acids and lipids, in the absence of oxygen, in to methane and CO_2. These processes are considered to be bacterial in origin and no other processes are considered in the research literature on anaerobic digestion.

All the high rate biomethanation processes are based on the concept of retaining high viable biomass by bacterial sludge immobilization. The anaerobic digestion model of IWA also proceeds on the assumption that the various reactions are either intercellular or extracellular bacterial process. The role of protozoa in anaerobic digestion process is hardly explained. The objective of this work was to study the population dynamics and functional roles of protozoa in anaerobic treatment process with special emphasis to ciliates.

An introduction to anaerobic digestion and present work are described in chapter 1. Chapter 2 deals with growth of protozoa in anaerobic reactors. The species richness and dynamics of the protozoa communities were studied in different anaerobic reactors. In chapter 3, influence of protozoa on the performance of anaerobic wastewater treatment systems is discussed. Relationship between protozoa and various physicochemical and operational parameters of the anaerobic reactor were studied. Chapter 4 deals with isolation and culturing of protozoa. In order to obtain the basic information necessary to interpret protozoa in anaerobic wastewater treatment, we assessed the effects of physico-chemical and nutritional parameters on the growth of protozoa. 5. The chapter 6 deals with the ability of protozoa to degrade various compounds which are generally present in wastewaters. Finally results are summarized and conclusions are drawn based on the present study.

Chapter 1
Introduction

1.1 Anaerobic wastewater treatment

Anaerobic degradation as a method for treating high and medium strength biodegradable wastewaters, has become a widely accepted technology in the last few decades with the development of high-rate reactors such as the upflow anaerobic sludge blanket (UASB) reactor, anaerobic biofilter and the anaerobic fluidized bed reactor – AFBR (Rajeshwari et al., 2000). The high rate anaerobic systems enables the application of a relatively high loading rate, while maintaining long solid retention time (SRT) at relatively short hydraulic retention time (HRT) due to sludge immobilisation (Stronach et al., 1986).

High rate anaerobic wastewater treatment processes have many advantages over conventional aerobic wastewater treatment processes. Being simple in operation and low in cost, their applicability is particularly feasible in developing countries. High organic loading rates and low sludge production are among the many advantages anaerobic processes exhibit over other biological unit operations. Above all, the important feature emerging as a major driver for the increased application of anaerobic processes is the energy production (Fig. 1.1). Not only does this technology have a positive net energy production but the biogas produced can also replace fossil fuel sources and therefore has a direct positive effect on greenhouse gas reduction (Batstone et al., 2002).

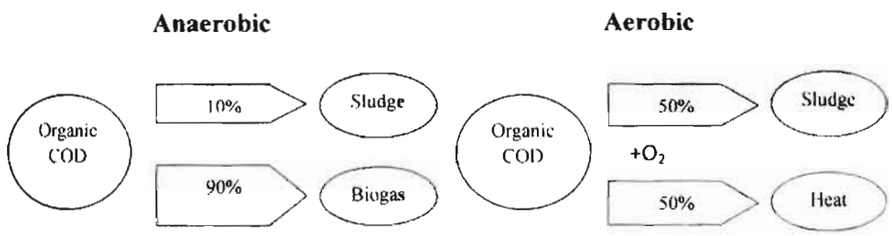


Fig. 1.1. Comparison of COD balance during anaerobic and aerobic treatment of wastewater containing organic pollution (Mahmoud, 2002)

1.2 Anaerobic degradation of organic compounds

The reactions in an anaerobic digester are complex with a number of sequential and parallel steps and can be divided into two main types (Batstone et al., 2002).

(a) ***Biochemical reactions:*** These are normally catalysed by intra or extracellular enzymes and act on the pool of biologically available organic material. Disintegration of composites (such as dead biomass) to particulate constituents and the subsequent enzymatic hydrolysis of these to their soluble monomers are mainly extracellular. Degradation of soluble materials are mediated by organisms intracellularly, resulting in biomass growth and subsequent decay.

(b) ***Physico-chemical reactions:*** These are not biologically mediated and encompass ion association/dissociation, liquid-solid and gas-liquid transfer.

1.2.1 Biochemical reactions

Anaerobic digestion of complex biopolymers is brought about by combined action of a wide range of microorganisms. The degradation is not a sequence of independent reactions, but it is characterized by a complex of mutual interaction between different microbial species (Fig. 1.2). The major microbial groups involved in anaerobic process are hydrolytic bacteria, fermentative bacteria, acetogenic bacteria and methanogens (Gujer and Zehnder, 1983; Mosey and Fernandez, 1989).

Hydrolysis: During the first process of hydrolysis, complex insoluble organic materials are solubilized by enzymes excreted by hydrolytic bacteria. Hydrolysis in most cases, the rate limiting for the overall process of anaerobic degradation of organic matter and it is very sensitive to temperature.

Acidogenesis: Fermentation of smaller subunits in a series of successive reactions is carried out by fermentative bacteria. The fermentation products are composed of a variety of small organic compounds, mainly volatile fatty acids (VFAs), H_2, CO_2, some lactic acids and ethanol. VFAs are the main end products of fermentative organisms which are usually designed as acidifying or acidogenic microorganisms.

***Acetogenesis*:** The products of fermentation under anaerobic conditions are converted to acetate, carbon dioxide and hydrogen by acetogenic bacteria. β-oxidation is the mechanism of anaerobic oxidation of long chain acids with products hydrogen and acetate. The available H_2 and CO_2 are partially converted to acetate by homoacetogenic bacteria. Both propionate and butyrate are the important intermediates in anaerobic digestion, and then are converted by the hydrogen producing acetogenic bacteria into acetate and hydrogen.

Methanogenesis: Final step in anaerobic digestion process is methanogenesis. Methanogenesis is the microbial production of methane through the reduction of carbon dioxide and acetate. Carbon dioxide reduction is coupled to oxidation of hydrogen, with hydrogen gas being one of the most common electron donors generated from acidogenesis and acetogenesis.

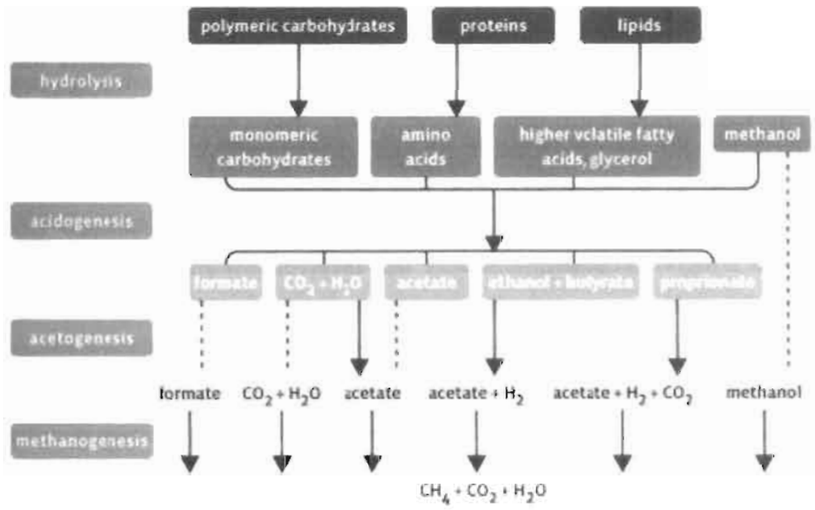


Fig. 1.2. Schematic representation of anaerobic digestion of organic materials

There is a syntrophic association with H_2 producing acetogenic bacteria and H_2 consuming methanogenic bacteria, thereby regulating the H_2 level in anaerobic environment. This has vital importance as these reactions are thermodynamically

unfavorable (+ ΔG^0) unless the H_2 partial pressure is maintained at an extreme low pressure (Zehnder, 1988)

1.2.2 Physico-chemical reactions

Physico-chemical reactions are not mediated by micro- organisms, and which are commonly occurring in anaerobic digester such as: liquid - liquid reactions (ion association and dissociation), gas - liquid exchange (gas transfer) and liquid - solid transformations (precipitation and solubilisation of ions). Only the first two types have been commonly addressed anaerobic digestion modeling, because of the difficulties in implementation of liquid solid transformations. The physico-chemical system is very important when modeling anaerobic systems (Batstone et al., 2002).

1.3 Protozoa in anaerobic wastewater treatment systems

All anaerobic processes are considered to be bacterial in origin and informations available are mainly focused on bacteria in anaerobic treatment process. This has lead to development of many designs based on the bacteria mediated biodegradation The anaerobic digestion model of IWA also proceeds on the assumption that the various reactions are either intercellular or extracellular bacterial process (Batstone et al., 2002). The role of protozoa in anaerobic digestion is completely neglected. Free living anaerobic ciliates similar to *Metopus* has been reported in a high-rate anaerobic reactor that receives low strength wastewater, but the reason for their presence not explained (Agrawal et al., 1997). Gijzen et al., (1988 a,b) have developed a two-phase anaerobic system based on processes and microorganisms including ciliates from the ruminant (Rumen Derived Anaerobic Digestion – RUDAD), however, the specific role of ciliates has not been investigated.

1.4 Protozoa in aerobic wastewater treatment systems

Existing literature regarding protozoa and wastewater treatment deals mainly with aerobic processes, with the focus on activated sludge technology. Activated sludge is a very complicated system, comprised of various populations including heterotrophic bacteria, autotrophic bacteria, fungi and protozoa (Madoni, 1994b).

In activated sludge, protozoa number is reached above 50,000 per ml, which can represent as much as 5-12% of the mixed liquor (Curds, 1982). This is similar to the total mass of bacteria, both viable and non-active in the mixed liquor. Different species of protozoa have been listed by various authors (Pillai and Subrahmanyan, 1944; Curds and Cockburn, 1970; Richard, 1991; Madoni, 1994b; Cereceda et al., 1997). Curds (1982) have listed 228 species of protozoa from activated sludge plants, with 70% of them from the class Ciliata. Although species diversity is dominated by ciliates, testate and naked forms of amoeba species can occasionally dominate numerically (Sydenham, 1971).

The relationship between the microbial population composition and the treatment performance of activated sludge processes has long attracted the attention of microbial ecologists and environmental engineers, as this information might be useful for the proper design and operation of biological wastewater treatment systems. Protozoa have been studied and utilized as an important indicator for judging process performance and effluent quality since the 1970s, because these large sized microorganisms can be directly observed and identified under a microscope (Curds and Cockburn 1970; Curds, 1982; Poole, 1984; Al-Shahwani and Horan, 1991; Esteban et al., 1991; Madoni, 1994a; Fried et al., 2000; Nicolau et al., 2001).

1.4.1 Role of protozoa in aerobic wastewater treatment systems

The efficiency of aerobic wastewater treatment plants is not only linked to the bacterial population but also to the protozoa (Nicolau et al., 1997). In normal conditions, their densities are larger than 10^7 protozoa l^{-1} corresponds to very good pollution abatement (Pillai and Subrahmanyan, 1944; Curds and Cockburn 1970; Drakides, 1978). On the contrary, densities lower than 10^5 protozoa l^{-1} are indicative of a poor efficiency of the plant (Drakides, 1978). In terms of biomass, protozoa represent between 0.17 and 0.44% of the sludge during the colonization phase but can represent up to 9% at steady-state (Madoni, 1994a). Curds and Cockburn (1970) have established relationships between the abundance of some species and the sludge loading: they have associated protozoa to the quality of the effluent depending upon the biological oxygen demand (Table 1.1).

Table 1.1. Effects of ciliated protozoa on the effluent quality of bench-scale activated-sludge plants. Results are given in mg l^{-1} unless otherwise noted (Curds and Cockburn, 1970)

Effluent analysis	**Ciliates**	
	Absent	**Present**
BOD	53-70	7-24
BOD after filtration	30-35	3-9
COD	198-250	134-142
COD after filtration	31-50	14-25
Organic nitrogen	14-21	7-10
Suspended solids	86-118	26-34
Optical density at 620 nm	0.95-1.42	0.23-0.34
Viable bacteria count *	160-160	1-9

*count - millions ml^{-1}

The absence of protozoa in general and ciliate in particular has been described as a factor affecting the performance of activated sludge process. Biological oxygen demands, concentration of suspended solids and the number of viable bacteria in the effluent of the aerobic treatment plants are reduced significantly due to the bacterivorous activity of ciliated protozoans (Curds et al., 1968; Salvado et al., 1995). During the course of treatment, grazing of bacteria and subsequent metabolism of assimilated material result in release of phosphorous (mainly as ortho phosphate) and nitrogen through excretion (Johannes, 1965). Protozoan grazing releases nutrients immobilized in inactive microbial biomass and this enables the remaining population to grow faster and maintain high level of activity. Findings by Wiggins and Alexander (1988) also imply a positive influence of protozoa on bacterial degradation processes with regard to the organic pollutants 2,4-dichlorophenol (2,4- DCP) and 2,4-dichlorophenoxyacetic acid (2,4-D). Although protozoan feeding reduced the mixed culture of freely suspended bacteria by more than one order of magnitude – leading to delayed degradation compared to protozoa-

free cultures – after 15 days the environmental chemicals 2,4-DCP and 2,4-D were mineralized in the presence of protozoa to 70% and 90%, respectively.

1.5 Protozoa

Protozoa cannot be easily defined because they are diverse and are often only distantly related to each other. They are unicellular eukaryotes. Together with the unicellular algae and the slime moulds, they make up the Kingdom Protista.

1.5.1 General characters of protozoa

The protozoa are single celled animals and the smallest of all animals. Most of them can only be seen under a microscope. They do breathe, move, and reproduce like multicelled animals.

- They are simplest, most primitive, cellular organisms of minute size usually microscopic, with one or more nuclei.
- They are cosmopolitan in distribution.
- Solitary or forms loose colonies, in which individuals remain alike and independent.
- Many protozoa are naked or bounded by a pellicle and others are encased in shells or skeletal structures.
- Locomotor organelles are finger like pseudopodia or whip like flagella or hair like cilia or absent.
- Respiration through the general outer surface of the body.
- Excretion through general surface of the body or through contractile vacuoles which also serve osmoregulation.
- Nutrition may be holophytic, holozoic, saprophytic or parasitic.
- Reproduction by asexual or sexual methods.
- Encystment commonly occurs to help in dispersal as well as to resist unfavourable conditions.

1.5.2 Habitats of protozoa

There is hardly any place on earth in which protozoa cannot be found. Protozoan species thrives best wherever it finds a specific combination of environmental conditions, that the same species will be found wherever this

combination occurs worldwide. Therefore protozoan species appear to be cosmopolitan in their spatial distributions (Finlay, 2002). In many places, an individual species will be represented by only a few individuals, or perhaps as cysts, but when appropriate conditions are provided, that species flourishes and becomes abundant (Finlay, 1998).

Protozoa are abundant in terrestrial as well as in aquatic systems. In the latter, they are present in high numbers of species and individuals both in the oceans and in freshwater habitats. Some taxa live attached to solid substrates or within the sediment, some as part of the plankton. An overview of the data about the abundance of protozoa in aquatic habitats gives a first indication that these organisms are not negligible in aquatic environments – although in fact they are still often neglected. In the plankton of highly productive lakes, densities of small flagellates (< 20 mm body size) of more than 10^6 cells ml^{-1} were reported and in studies on the periphyton of small water bodies maximum values of more than 1350 cells ml^{-1} of the much larger testate amoebae specimens were encountered. However, these numbers do not make any statements about the ecological interactions in which the species are involved and the role they play within those processes which mostly are seen as the essence of ecosystem dynamics, namely the fluxes of energy and material (Finlay and Esteban, 1998).

1.5.3 . Classification of protozoa

Phylum protozoa contain four classes – ciliates, flagellates, sarcodines and sporozoa. The locomotor organelles are the primary distinguishing features. Three main locomotor organelles are found in different classes of protozoa and they are cilia, flagella and pseudopodia (Patterson, 1996).

Ciliates: The cilia are used for locomotion in ciliates. They graze bacteria, unicellular algae, filamentous cyanobacteria, other protozoa and occasionally rotifers and microzooplankton. Probably, ciliates are the most important protozoan grazers in many environments. There are three sub-groups- (a) raptorial feeders, (b) true filter feeders and (c) diffusion feeders (Table 1.2).

Table 1.2. Characters of feeding mechanism in ciliates (Finlay and Esteban, 1998)

Raptorial feeding	Catch relatively large food items individually and many have a simple apical mouth (e.g. *Prorodon, Loxodes, Askenasia*).
True filter feeding	A filter to remove microbial food from suspension; some have a relatively large, fine-mesh filter (e.g. *Cyclidium*) The filter is much reduced in ciliates that feed in patches of high bacterial concentrations (e.g. *Colpidium*) The filter is expansive, and encircles the apical end of the ciliate in the peritrichs (e.g. *Vorticella*) In many ciliates, a dense row of membranelles generates a water current for collecting the food particles (e.g. *Oxytricha, Spirostomum, Hypotrichidium*) Some of ciliates generate more effective feeding currents by raising the cell on walking cirri (e.g. *Euplotes, Stylonychia*) or by creating a stalk-like extension of the body, aligned perpendicular to the substrate (e.g. *Stentor*
Diffusion feeding	Swimming prey collide with their sticky tentacles, through which the prey contents are then sucked; they are common in sediments but rarely abundant (e.g. *Podophrya* and other Suctoria)

Flagellates: In flagellates, the flagella are used for locomotion, feeding, or both. Flagellates are the principal consumers of suspended bacteria, and important grazers of the bacteria associated with surfaces and sediments. Those feeding on suspended particles may feed by direct interception (e.g. the chrysomonads), by filter-feeding (e.g. the choanoflagellates), or by diffusion feeding (e.g. *Ciliophrys*). Filter-feeders have relatively large filter areas and the highest volume-specific clearance values - hence competitive superiority over filter-feeding ciliates. It is possible that most suspension-feeding flagellates which create water currents are usually attached to substrates, including suspended particle aggregates and larger planktonic

organisms. Osmotrophic nutrition has been demonstrated in some of the soil flagellates (Ekelund and Rønn, 1994). Osmotrophy in flagellates is probably of no quantitative importance, as the much smaller heterotrophic bacteria will always have a competitive advantage (Finlay and Esteban, 1998).

Sarcodines: These can be divided into three broad functional groups - naked amoebae, testate amoebae and heliozoans. The prey are principally bacteria or algae, depending on the size of the sarcodine grazer, but a great variety of non-living organic particles and aggregates are also often ingested (e.g. *Pelomyxa*). The majority of sarcodines apart from the heliozoans (which are the only truly planktonic group) are usually associated with surfaces, and especially sediments. Amoebae are uniquely able to persist in very thin water films. The testate amoebae are, because of their resistance to desiccation, probably more important in soils than in lake sediments (Finlay and Esteban, 1998).

Sporozoa: Locomotory organs are absent and body is covered with thick pellicle. This class includes parasitic protozoan, which are generally immotile, develop spores, and reproduce by multiple fission.

1.5.4 Nutrition in protozoa

Almost all types of nutrition like holozoic, holophytic, saprophytic, parasitic, mixotrophic are found in protozoa. The protozoan comes with the capacity to engulf the particles and with a suit of digestive enzymes and a metabolic repertoire superior to anything found in the prokaryotes. Digestion occurs mainly intracellularly inside the food vacuoles. Protozoa are efficient at gathering microbes as food, and they are sufficiently small to have generation times that are similar to those of the bacteria particles on which they feed. They are, in quantitative terms, the most important grazers of microbes in aquatic environments, and they probably control the abundance of bacteria (Fenchel, 1987; Sherr and Sherr, 1987; Berninger et al., 1991; Hobbie, 1994).

1.5.5 Reproduction of protozoa

Reproduction of protozoa occurs after a period of growth which ranges, in different species, from less than half a day to several months (certain *Foraminiferida*). Reproductive process and life cycles of protozoa are varied and fall

under two major groups – asexual and sexual. Generally four types of asexual reproduction are recognized, namely binary fission, multiple fission, budding and plasmogamy. Most of protozoa reproduce through asexual reproduction. Sexual reproduction is mainly through syngamy and conjugation.

Simple life cycles of protozoa include a cyst and an active (trophic) stage undergoing growth and reproduction. In certain free-living and parasitic species, no cyst is developed. Dimorphic cycles show two active stages (flagellate and amoeba e.g. Mastigophora and Sarcodina) and polymorphic show several.

1.5.6 . Protozoan diversity and ecosystem function

Protozoa are characteristically phagotrophic, especially on bacteria, unicellular algae and other protists, and their phagotrophy underpins their ecological importance in microbial food webs. In the open water of lakes and oceans (Berninger et al., 1991) and in anoxic sediments (Fenchel and Finlay, 1991a), protozoa are quantitatively the most important consumers of other microorganisms. Heterotrophic protozoa have been studied in lakes, rivers, and oceans and numerous reports have noted the importance of these organisms as links between heterotrophic bacteria and zooplankton grazers (Pace, 1982; Azam et al., 1983). In fact, these organisms are intermediate in remineralization and in recycling of essential nutrients (Sherr and Sherr, 1994). Moreover, protozoa play an important part in systems of prediction and assessment of water quality (Bick, 1968; Sladecek, 1973).

Protozoa are major plankton components in most aquatic systems, feeding on bacteria, auto- and heterotrophic pico- and nanoplankton (Porter et al., 1985, Simek et al., 1997, Hahn and Hofle, 2001). Hence, by their grazing activities, they provide particulate organic matter (POM) to higher trophic levels and dissolved nutrients (DOM) to bacteria (Arndt et al., 1993). Because of their small size, rapid growth and high metabolic rates, they are an important link in microbial food webs even when appearing in low abundance (Fenchel, 1987). High turnover rates and short generation times allow many protozoa species to respond immediately to changing environmental conditions. Thus, protozoa may be sensitive and highly valuable bioindicator organisms in water quality analyses (Sladecek, 1973; Foissner, 1988; Berger et al., 1997).

Bacteriovory by ciliates and flagellates has been shown to enhance the decomposition of leaf detritus in streams through a presumed increased turnover of bacterial populations (Ribblett et al., 2005). This suggests that protozoan predation is likely to play an important role in biodegradation and the majority of microbes as well as microbial energy and nutrient cycling may occur in stream ecosystems (Costerton et al., 1995; Battin et al., 2003; Romani et al., 2004). Protozoa have been identified as a major microbial pathway for the transfer of carbon and phosphorus to higher trophic levels within pelagic systems such as an oligotrophic lake (Heath et al., 2003) and, more recently, running waters such as streams (Marxsen, 2006).

Protozoan grazing releases nutrients immobilized in inactive microbial biomass and this enables the remaining population to grow faster and maintain high level of activity in soil (Ronn, 1994). Excretion of nitrogen and phosphorous compounds and also of trace metals such as iron, by phagotrophic mechanism is a major source of regenerated nutrients in aquatic systems (Sherr and Sherr, 2002). In rumen system many of the protozoa participate directly in digesting and fermenting the plant parts which enters in to the rumen (Coleman, 1992). This direct grazing activity probably contributes to the relatively high ciliate biomass in the rumen (Hungate, 1978).

Protozoa are the largest and most complex of micro-organisms next to bacteria and all other smaller organisms, which are ubiquitous. The number of protozoan species recorded is about 10% of the estimated global species richness (Finlay, 2002). The anaerobic habitats are not exception to protozoa species richness and diversity (Finlay, 2002).

1.6 Anaerobic protozoa

Protozoa consortia are found in various anaerobic environments, including rumen ecosystem, marine and fresh water sediments, wet landfills and anaerobic sewage plants (William and Coleman 1991; Fenchel 1993, Fenchel et al., 1990; Finlay and Fenchel 1991; Fenchel et al., 1977). They can be relatively abundant, free-living anaerobic ciliates typically reach $>10^3$ ml^{-1} of sediment (Fenchel, 1993) and $> 10^5$ ml^{-1} rumen (Hobson, 1988). Anaerobic ciliate with symbiotic methanogens can be relatively abundant in localized patches of decomposing detritus (Finlay and Fenchel,

1991). They are basically aerobic organisms and the anaerobic lifestyle has been evolved independently (Mueller, 1988). Most of them have specific biological and ultra structural adaptations for life in the absence of oxygen. (Finlay and Fenchel, 1989).

Anaerobic protozoa are mainly bacteriovoruos and often represent main bacterial consumers in anoxic environments (Fenchel and Finlay, 1991a). Fenchel and Finlay (1990a) have suggested that through predation anaerobic protozoa could control the bacterial abundance in anaerobic systems, as is frequently the case in aerobic communities. The feeding rates of anaerobic ciliates are comparable to those of similarly sized anaerobic ciliates (Massana et al., 1994), but growth efficiency is low because of its anaerobic fermentative metabolism (Fig. 1.3).

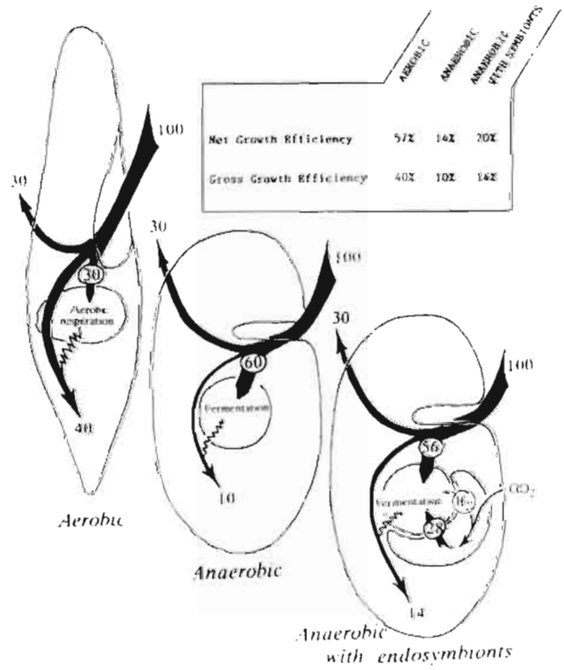


Fig. 1.3. Schematic representation of the carbon allocation in an aerobic ciliate and anaerobic ciliate *Plagiopyla* with and without endosymbiotic methanogens. In all

> cases 30% of the injested material is assumed to be lost or egested. Of the reminder the aerobe will dissimilate 43 % and assimilate the rest. The anaerobe must dissimilate a much larger fraction of its food because the energy yield is much lower (Fenchel and Finlay, 1990a)

The net growth efficiency and gross growth efficiency of ciliates with endosymbionts are only about 20% and 14% respectively compared to aerobic organisms (Fig. 1.3).

1.6.1 The energy metabolism of anaerobic protozoa

Heterotrophic eukaryotes such as protozoa do not posses functional plastids. They satisfy their ATP needs through the oxidative breakdown of reduced organic compounds (Fig. 1.4). Glycolysis (Embden-Mayerhoff pathway) is the backbone of eukaryotic metabolism: one molecule of glucose is oxidized to pyruvate with the help of NAD^+ with a net yield of 2 mol of ATP.

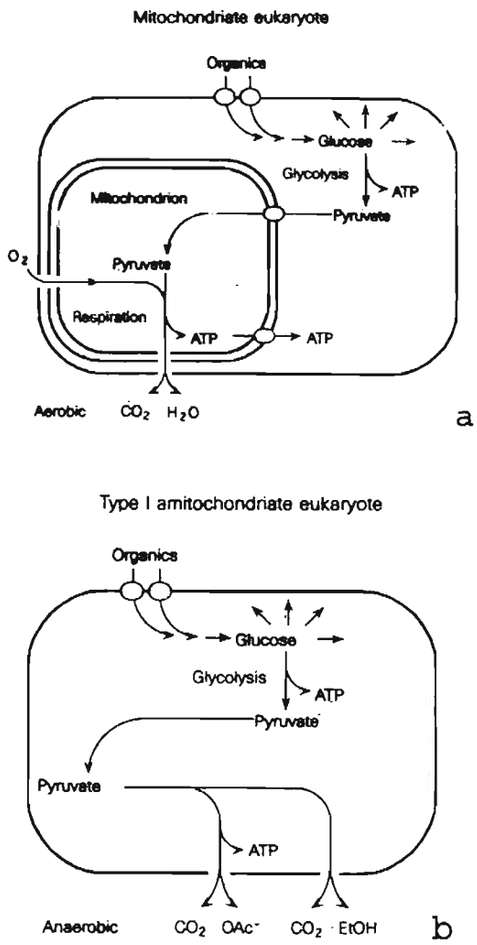
In mitochondriate eukaryotes, pyruvate of glycosis is usually oxidized in the mitochondria through pyruvate dehydrogenase complex (PDH), the Kreb's cycle and oxygen respiration to yield an additional 34 -36 moles of ATP per mole of glucose (Fig. 1.4a).

Amitochondriate eukaryotes meet their energy needs through anaerobic fermentation (Coombs and Mueller, 1998). They also obtain 2 moles ATP from glycolysis but pyruvate is metabolized through pyruvate ferredoxin oxidoreductase (PFO), rather than PDH (Mueller, 1988).

In eukaryotes that lack hydrogenosome (Fig. 1.4b), cystolic PFO carboxylates pyruvate, yielding reduced ferredoxin and acetyl Co A. The latter is converted into a mixture of ethanol and acetate, the relative amount of which depends upon environmental conditions, yielding between 0 and 2 additional mol of ATP per mole of glucose (Fig. 1.4b).

In amitochondriate eukaryotes that harbor hydrogenosomes (Fig. 1.4c), cystolic pyruvate is imported in to the organelle, where PFO converts it to CO_2, acetyl Co A and reduced ferredoxin. Ferridoxin is reoxidised by hydrogenase, producing the H_2 characteristic of the organelle. Per mole glucose, pyruvate metabolism in

hydrogenosomes yields two additional mol ATP and two mol each of H_2, CO_2 and acetate as waste products (Fig. 1.4c). The hydrogenosomes are found exclusively in certain anaerobic, unicellular eukaryotes and some anaerobic fungi (Mueller, 1993).



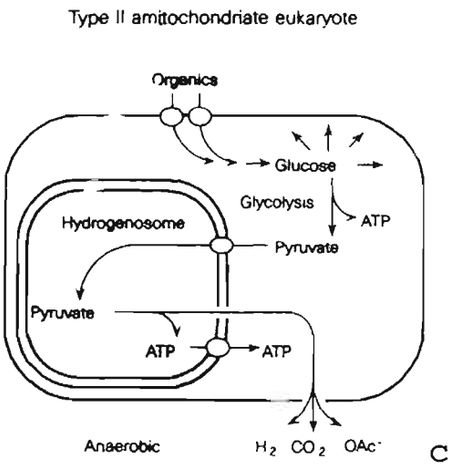


Fig. 1.4. Schematic representation of forms of energy metabolism among heterotrophic eukaryotes (Martin and Mueller, 1998)

1.6.2 Hydrogenosomes in anaerobic protozoa

The physiological adaptations of eukaryotic cells to anaerobic conditions have been widely investigated (Van Bruggen et al., 1983; Finlay and Fenchel, 1989). Some protozoa have special redox organelles, hydrogenosomes and are capable of oxidising pyruvate completely to acetate, CO_2 and H_2 (Fig. 1.5). The hydrogenosomes are membrane-bound organelles about 1 mm in size that, like mitochondria, produce ATP (Mueller, 1993). They compartmentalize terminal steps of anaerobic energy metabolism but, unlike mitochondria, hydrogenosomes cannot use oxygen as an electron acceptor; they reduce protons to molecular hydrogen (Embley and Martin 1998; Martin and Mueller 1998; Mueller, 1988).

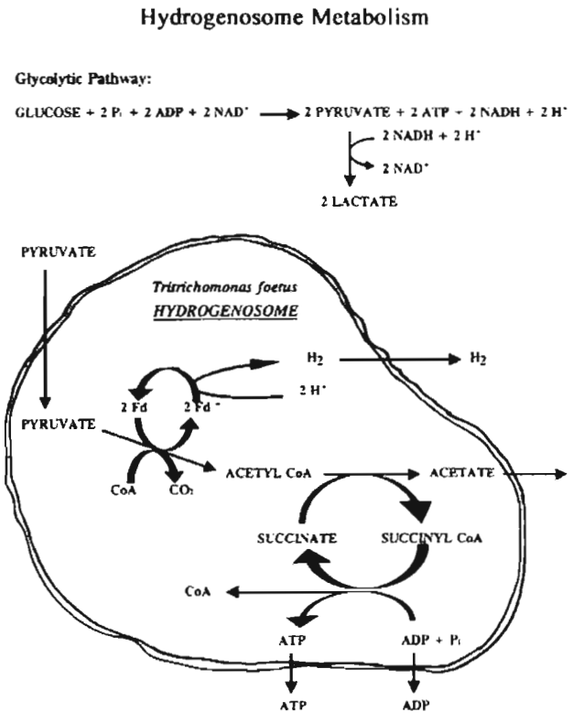


Fig. 1.5. Anaerobic metabolism of glucose in *Tritrichomonas foetus*

Mitochondria and hydrogenosomes share many characteristics, like the surrounding double membrane, protein import machinery and the ATP production. Pyruvate enters to the mitochondria where it is decarboxylated, ATP is produced and reducing equivalents are released as water. The process is similar in the hydorgenosome bearing eukaryote, but the ATP yield is much lower and in many cases, an additional electron sink is present – an endosymbiotic methanogen which releases methane (Fig. 1.6). Hydrogenosomes are characterized by their capacity to produce hydrogen, whereas mitochondria contain an electron-transport chain in their inner membrane and perform oxidative phosphorylation (Embley et al., 1997). The

key enzymes of hydrogenosome are hydrogenase and pyruvate ferredoxin oxidoreductase (Lindmark and Mueller, 1973).

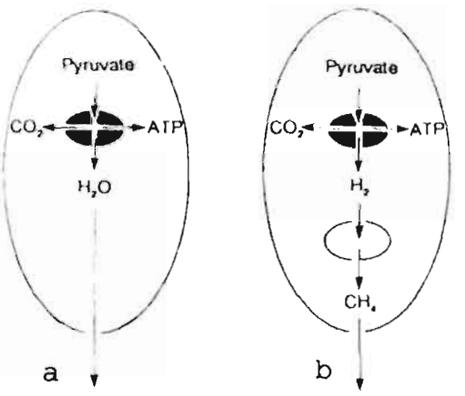


Fig. 1.6. Functional similarities between organisms with (a) mitochondria and (b) hydrogenosome (Fenchel and Finlay, 1995)

The hydrogenase enzyme catalyses the redox equilibrium, $2H^+ + 2e^- \rightleftharpoons H_2$. Despite the simplicity of the reaction, hydrogenase enzymes are complex multi-metal domain proteins of high molecular weight (Fig. 1.7). The Fe-only hydrogenase is primarily responsible for the reduction of protons, this process is thought to occur at the H centre of the enzyme. Crystal structures of the enzymes have shown that the H centre consists of a Fe_4S_4 unit linked via sulfur to a remarkable bimetallic complex, a Fe_2S_2 unit with mixed CO/CN ligands (Razavet et al., 2001).

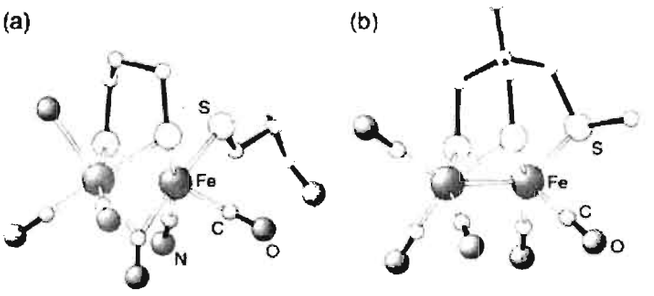


Fig. 1.7. Proposed structure of the H-centre of Fe-only hydrogenases; this is a composite model combining features reported by Peters (PDB code 1FEH) and Nicolet (code 1HFE) (Razavet et al., 2001)

Pyruvate ferredoxin oxidoreductase (PFO) catalyzes the oxidative decarboxylation of pyruvate to acetyl-CoA and CO_2 (pyruvate + CoA + 2 oxidized ferredoxin $\rightleftharpoons$ acetyl-CoA + CO_2 + 2 reduced ferredoxin + 2 H^+). This enzyme belongs to the family of oxidoreductases, specifically those acting on the aldehyde or oxo group of donor with an iron-sulfur protein as acceptor. The catalytic proficiency of this enzyme for the reverse reaction, pyruvate synthase, is poorly understood.

1.6.3 Symbiosis with bacteria

Most free-living anaerobic protozoa, mainly ciliates harbour ecto and endo symbiotic methanogans (Vogels et al., 1980, Goosen et al., 1990, Fenchel and Finlay, 1991b, Fenchel and Finlay, 1995) and some may have ectosymbiotic sulphate reducers (Fenchel and Ramsing, 1992; Fenchel and Finlay, 1995). The benefit of endosymbiotic methanogens seems obvious because they are close contact with hydrogenosomes that supply them the substrates H_2, CO_2 and acetate and they are also protected from predation. The benefit to ciliate is less obvious from endosymbiosis. However, the hydrogen generated in the cells from the result of anaerobic metabolism is removed by symbiosis of methanogens and that can faster the energy metabolism and hydrogen formation. (Fenchel and Finlay, 1989). Thus the presence of endosymbionts might improve the functions of hydrogenosomes and consequently the energy balance of protozoa host.

The hydrogenosome containing free living protozoa often harbour endosymbiotic methanogens which are presumably depend on the H_2 producted in the host cell (Wagener and Pfenning 1987; Goosen et al., 1988; Fenchel and Finlay 1991b).The loss of endosymbionts has been reported with O_2 tension and continuous cultivation (Broers et al., 1992; Yamada et al., 1997). According to Yamada et al (1997), the association of hydrogenosomes and methanogens is not essential for the anaerobic growth of protozoa.

1.6.4 Endosymbiotic methane production

Bacterial endosymbiosis is a widespread phenomenon in protozoa (Liebmann, 1937; Ball, 1969; Fenchel et al., 1977; Lee et al., 1985, Fenchel and Finlay, 1995). However, the presence of methanogenic endosymbionts in anaerobic protozoa has been demonstrated recently (Van Bruggen et al., 1983) and some endosymbionts have been isolated (Van Bruggen et al., 1984, 1986, 1988; Goosen et al., 1988). In sandy sediments anaerobic protozoa play a relatively smaller role in methane production (<2%) due to their low number and limited vertical distribution (Fenchel, 1993). In eutrophic ponds, the ciliates with endosymbiotic methanogenic bacteria is responsible for about 5% methane in surface sediment and anaerobic water column (Finlay, 1993). It has been reported that anaerobic protozoa with endosymbiotic methanogens can contribute a substantial fraction of methane production (15-90%) in anaerobic marine sediments (Fenchel, 1993) and 9-25% of methanogenesis in rumen fluid (Newbold et al., 1995). Studies in wet landfill sites have reported a significant contribution to methane generation by anaerobic protozoa consortia (Finlay and Fenchel, 1991). It is known that ruminants produce 80-100 millon tons of methane per year (Moss et al., 2000) and about 37% of the total methane production from sheep has been attributed to the presence of methanogenic ciliates within these animals (Finlay et al., 1994).

1.6.5 Oxygen toxicity and respiration

Anaerobic protozoa are those whose energy metabolism is independent of free oxygen and are showing varying degrees of sensitivity to the presence of oxygen (Mueller, 1988; Goosen et al., 1988). In the natural environment, a permanent

reducing environment may not always be guaranteed. Fenchel and Finlay (1990b) have found the rates of O_2 uptake in *Metopus contortus* and *Plagiopyla frontata* which maintain an anoxic intracellular environment so long as the external pO2 remains below about 0.4 k Pa. The endosymbionts maintained auto fluorescence at this concentration, but not at higher external O_2 tensions. The decrease in the rate of methanogenesis with increasing pO_2 up to 0.4-0.8k Pa, which probably, reflects the fact that the ciliates spend electrons on the reduction of O_2 at the expense of H_2 production (Fenchel and Finlay, 1990b; Fenchel and Finlay, 1995).

The ciliate *Loxodes striatus* prefers an O_2 tension of about 5% (atm. sat.) and displays a chemosensory behaviour that allows it to accumulate at this level in oxygen gradient (Finlay et al., 1986). *Plagiopyla nasuta* and *Trimyema compressum* has been shown to survive and grow at O_2 tensions up to 5% atm.sat. (Goosen et al., 1988 and 1990 a,b). *Parablepaharisma collare* has so far proven to be the most sensitive species; 100 % cells die when exposed to atm. O_2 tension for about 60 min (Fenchel and Finlay, 1990b). Rumen ciliates tolerate very low O_2 tensions, but their hydrogen production is then inhibited (Yarlett et al., 1983). Anaerobic ciliates are known to be capable of oxygen consumption; this is not coupled to energy conservation, but it is believed to be a mechanism for O_2 detoxification (Fenchel and Finlay, 1990b).

The oxygen toxicity is mainly due to the formation of highly reactive oxygen species (ROS) which are capable of damaging a wide range of biomolecules, including DNA, proteins, and lipids (Imai and Linn, 1988; Janssen et al., 1993). Aerobic organisms have developed several defence mechanisms, involving both enzymatic and nonenzymatic strategies, to detoxify ROS (Cadenas, 1989; Cortez, 1998; Yu, 1994). Several enzymes, including superoxide dismutases, catalases, peroxidases, glutathione synthase and glutathione reductases, are believed to provide the primary protection of cellular components against oxidative stress (Graeff-Wohlleben, 1997; Storz, 1999). Among anaerobic protozoa, SOD has been detected as a protective enzyme under oxygen stress in parasites like *Entamoeba* (Sykes and Band, 1977) *Tritrichomonas* and *Monocercomonas* sp. (Lindmark and Muller, 1974). Free living anaerobic protozoans like *Hexamita* and *Loxodes* have also been reported

to produce SOD under varying levels of oxygen exposure (Biagini et al., 1997; Finlay et al., 1986).

1.6.6 The significance of protozoa in anaerobic environments

Protozoan stimulation of terminal decomposition has previously been reported in natural systems (Fenchel and Harrison, 1976) and in aerobic treatment plants (Curds et al., 1968). The significance of protozoa in anaerobic environments has rarely been studied except in the rumen ecosystem where protozoa is said to enhance the degradation of organic material by direct utilization (Williams, 1991; Santra and Karim, 2002). Finlay and Fenchel (1991) reports grazing and flocculation activity of ciliates leads to an overall stimulation of anaerobic activity and to an increased turnover rate in wet anaerobic landfill sites. Furthermore, symbiotic relation between protozoa and bacteria has been enhancing the anaerobic degradation methane production in the presence of ciliate *Metopus palaeformis* (Biagini et al., 1998a).

1.7 The present study-relevance and objectives

In recent years, considerable attention has been paid towards the development of anaerobic reactors for the treatment of wastewater leading to conversion of organic materials to biogas. All modern high rate biomethanation processes are based on the concept of retaining high viable biomass by bacterial sludge immobilization (Hulshoff and Lettinga, 1986) and works were mainly focused on bacteria in anaerobic treatment process. The latest conference on anaerobic digestion (Anaerobic Digestion X, 2005) was also not mentioned about anaerobic protozoa. Anaerobic protozoa have been very rarely recognised, except some reports of their presence along with bacterial and archeal groups (Agrawal et al., 1997; Gijzen et al., 1988 a,b) and the role of protozoa in anaerobic digestion is hardly explained. However, our microscopic observations in anaerobic Buoyant Filter Bioreactor - BFBR (Ajit et al., 2005) observed an increase in number of protozoa, mainly ciliates with the best reactor performances in terms of COD (Chemical oxygen demand) removal and VFA (Volatile Fatty Acids) conversion to biogas. This has lead to the detailed investigation on anaerobic protozoa in anerobic reactors.

The relationship between the microbial population composition and the performance of treatment processes has long attracted the attention of microbial ecologists and environmental engineers, as this information might be useful for the proper design and operation of biological wastewater treatment systems. The aim of this study was to study the population dynamics and functional roles of protozoa in anaerobic treatment process with special emphasis to ciliates. This is the first attempt to study the involvement of protozoa in anaerobic reactor.

Chapter 2
Growth of protozoa in anaerobic reactors

Abstract

This study was to investigate growth of protozoa in anaerobic treatment systems. The species richness and dynamics of the protozoan communities were studied in different anaerobic reactors in the laboratory. The reactors contain diverse protozoa population such as the rhizopods (*Amoeba, Mayorella, Saccamoeba, Vanella, Mastigamoeba, Amoeba radiosa, Euglypha* and *Filose amoeba*), flagellates (*Rhynchomonas, Cercomonas, Menoidium, Naeglaria, Mastigella, Tetramitus, Peranema, Trepomonas and Bodo)* and ciliates (*Prorodon, Loxophyllum, Plagiopyla, Chilodonella, Colpidium, Brachonella, Euplotes, Colpoda, Discomorphella, Vorticella, Cyclidium, Spathidium, Loxodes* and *Metopus)*. A succession in protozoan species was observed from the start up of reactors and had fluctuations in the protozoan communities during the reactor operations. Our studies show an additional trophic layer of anaerobic protozoa along with bacteria and fungi in the anaerobic reactor ecosystem.

2.1 . Introduction

Most anaerobic environments (including anaerobic sewage, rumen ecosystem, wet landfills, sulphureta, sandy and detritus sediments) harbour protozoa; these are predominantly ciliates, but heterotrophic flagellates and rhizopods also occur (Fenchel and Finlay, 1995). They are mainly considered as bacteriovoruos and often represent main bacterial grazers in anoxic environments (Fenchel and Finlay, 1991a).

The functional role of protozoa in biodegradation are of great interest as protozoan role is generally reported as antagonistic to bacterial growth from grazing activity and thereby unfavorable to bacterial growth and degradation (Kotta et al., 1999). A similar opinion prevails in the case of aerobic treatment, but positive impact of ciliates on the overall purification and performance of aerobic activated sludge has been shown, especially in the reduction of biological oxygen demand, concentration

of suspended solids and control of viable bacteria in the treated effluent (Curds et al., 1968; Salvado et al., 1995; Madoni, 1994b). However, Finlay and Fenchel (1991) have reported that grazing and flocculation activity of anaerobic ciliates lead to an overall stimulation of anaerobic activity and to an increased turnover rate in wet anaerobic landfill sites. In order to understand the anaerobic degradation in detail studies are also needed on the growth of anaerobic protozoa. The present study is on growth of protozoa in biomethanation system related to their involvement in biodegradation.

2.1 . Materials and methods

2.2.1 Anaerobic reactors

Reactors of different configurations were operated with different substrates to study the population dynamics of protozoa in anaerobic wastewater treatment systems.

Buoyant Filter Bioreactor (BFBR): A novel anaerobic reactor developed in the National Institute for Interdisciplinary Science and Technology was used for this study (Ajit et al., 2005). The reactor was made up of glass and divided into two cambers - upper and lower chamber by a fluid tight horizontal partition plate. The lower chamber was seeded with anaerobic sludge. A filter chamber made up of acrylic tube was provided vertically penetrating through the partition plate. Holes were provided at lower part of the acrylic tube, so that flow between the chambers was possible. The filter bed was made from polystyrene balls to form a floating granular filter (buoyant filter). Biomass and biosolids accumulated in the lower chamber when the reaction proceeds. The upper chamber had no active role in the system and except to work as a future second stage treatment and the total active volume of the reactor was 11.9 l (Fig. 2.1).

A gas liquid separator was provided at the top of the upper chamber and a dished bottom with inlet nozzle was fitted at the lower end of the lower chamber. During operation, gas formed due to reaction and gas recirculated by a pump from the gas collection chamber were collected in the upper space of lower chamber. Gas collection displaces liquor from the lower chamber to upper chamber

through the filter chamber. As a result of filtration action, biosolids and sludge were captured in the buoyant filter. After a predetermined quantity of gas has accumulated, it is released using an automatic discharge mechanism. It causes a rapid back flow of filtered liquor from upper chamber to lower chamber. Solid captured in the buoyant filter are washed out in to the lower chamber.

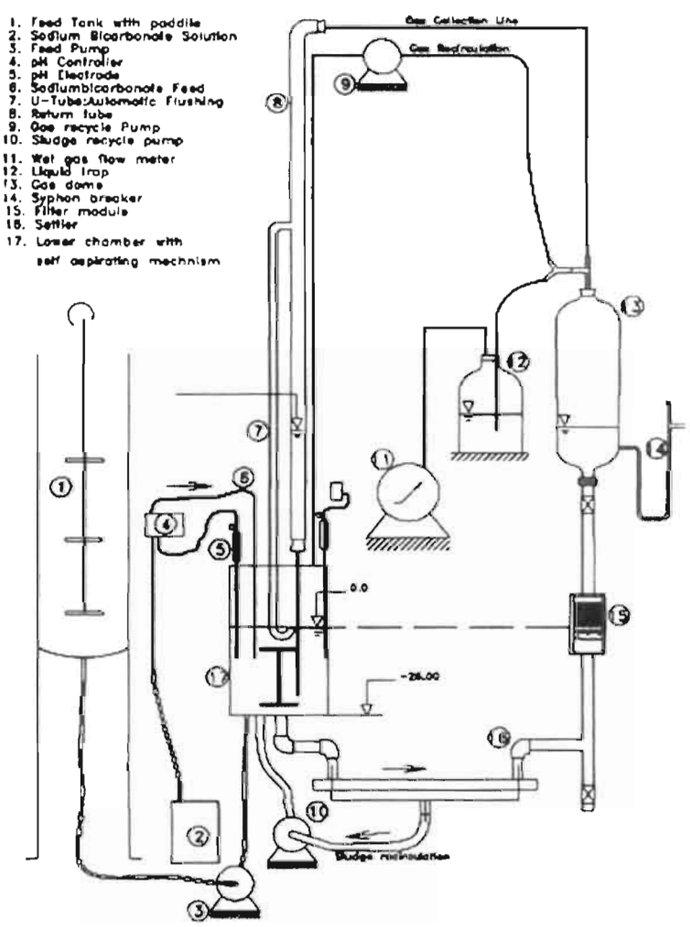


Fig. 2.1. Schematic diagram of the laboratory BFBR

The reactor was operated with synthetic diary waste water. The filter system allows the retention of fine suspended particles in the BFBR compared with other anaerobic reactors. It is hypothesized that the environment in the BFBR is conducive to growth of anaerobic protozoa (Fig. 2.1).

Continuous stirred tank anaerobic reactor (CSTAR): Continuous stirred tank anaerobic reactor (CSTAR), with working volume of 1 l, was operated at different organic loading rates. The reactor was fed with oleic acid suspension as carbon source with Vanderbilt mineral media (Nuri et al., 2001). The feed rate was regulated through a peristaltic pump (Autoclude VT, UK) in order to maintain specific hydraulic retention time. The pH was controlled by adding 1N NaOH or 1N HCl. Biogas generated in the reactors was measured by liquid displacement consisting of a 3% (w v^{-1}) NaOH solution (Fig. 2.2).

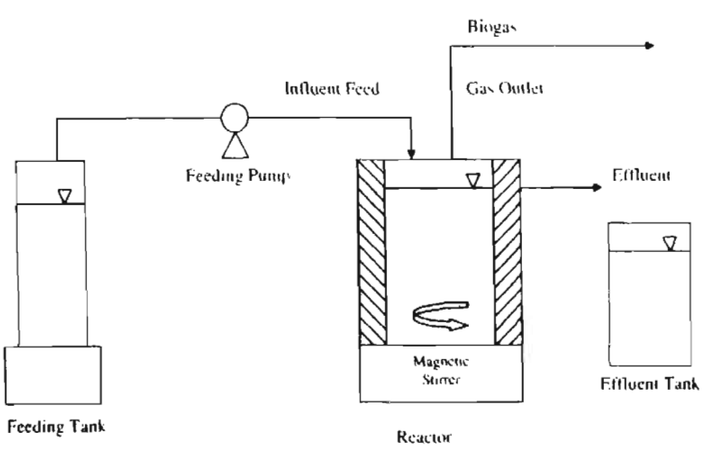


Fig. 2.2. Schematic diagram of the laboratory CSTAR

Upflow anaerobic sludge blanket reactor (UASB): A typical UASB reactor with working volume of 9 l was operated for this study (2.3). The gas –solid- liquid separator fitted on the top had volume of 3 l. A tank of 20 l working volume was loaded with coconut husk in water to leach out organics which was used as the feed

source for UASB. The feed rate was regulated through a peristaltic pump (Watson Marlow 505U, England) in order to maintain specific hydraulic retention time (Fig. 2.3). The methane production was measured using wet gas flow meters (Insref, India).

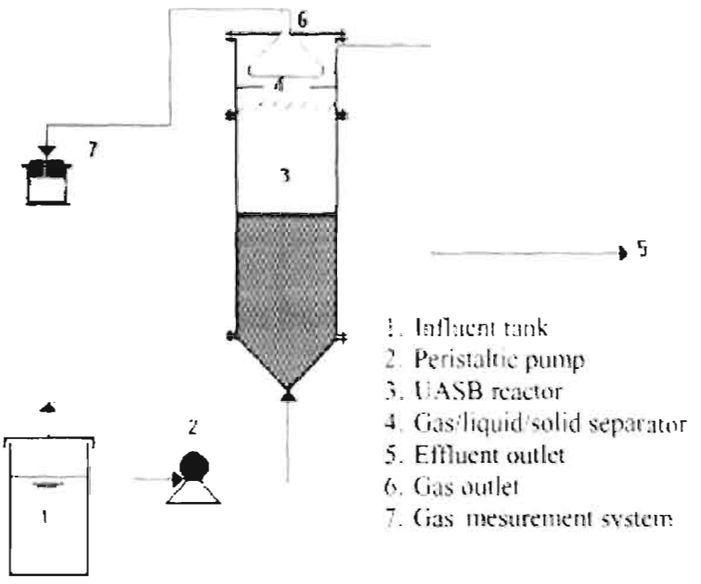


Fig. 2.3. Schematic diagram of the laboratory UASB

Batch anaerobic reactor: The batch reactors were set up with rubber septum capped 500ml glass bottles. The initial COD concentration was set at 1g COD. g^{-1}VSS with the basal medium (Harada et al., 1994) containing sodium oleate as COD source. Anaerobiosis was maintained by the addition of cysteine HCl (0.05%). Experiment bottles were capped and headspace was flushed with nitrogen. Samples were collected at regular intervals using a syringe through the septum for routine analysis. Gas production was measured by liquid displacement (Fig. 2.4).

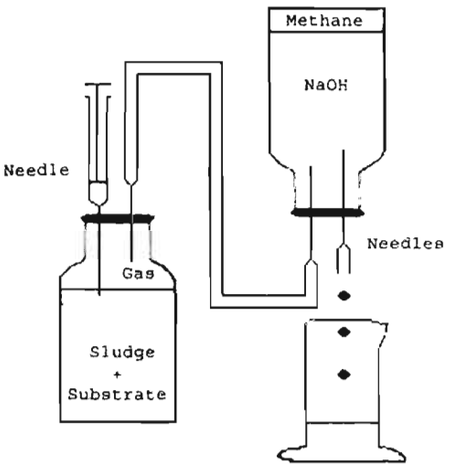


Fig. 2.4. Schematic diagram of the laboratory batch anaerobic reactor

Reactors were seeded with sludge collected from an anaerobic dairy wastewater treatment plant and kept at room temperature (28-32°C). The composition of nutrients supplied to BFBR and UASB reactors are given in Table 2.1. Trace elements were added from a stock solution prepared as per Huser (1980).

Table 2.1. Nutrient composition used in BFBR and UASB reactors

Component	Concentration(mg l^{-1})
NH_4Cl	280
KH_2PO_4	250
$MgSO_4.7H_2O$	100
$CaCl_2.H_2O$	10
$NaHCO_3$	400
Yeast Extract	100

2.2.2 Identification of anaerobic protozoa

The protozoa were identified according to the schemes summarized by Patterson (1995) and Foissner and Berger (1996). Various methods used for identification were the following.

Fixation of anaerobic protozoa: Live samples were collected from reactors and examined under microscope (Nikon -ALPH 4 YS2) for immediate identification. A portion of these samples were preserved for further detailed identification.

Glutaraldehyde : a commonly used fixative for protozoa. A range of 0.25 to 5 % glutaraldehyde was used to get suitable concentration for protozoa.

Schaudinn's Fluid: a broad-spectrum fixative for protozoa. Schaudinn's fluid was prepared by adding two parts saturated aqueous solution of mercuric chloride to one part absolute alcohol (ethanol). Just before use, added 1 ml glacial acetic acid to 99 ml of the fixative.

Immobilization of anaerobic protozoa: In order to view fast-moving ciliates and flagellates under a microscope, it was necessary to slow or immobilize them. There are three basic methods of immobilization: mechanical, chemical and narcotic (WARD'S Natural Science, 2002).

Mechanical: Protozoa has a tendency to cluster around debris. If debris is present, the organisms were found near the debris and studied its morphological characters. If debris is absent (effluent), shredded filter paper was placed in the sample during observation.

Chemical: Fast moving organisms were counted after changing viscosity of medium to slow down their movements. The following chemicals were used for chemical immobilization:

1) Methyl cellulose (1.5%)

2) Polyvinyl alcohol (14%)

Narcotic: Fast movement of protozoa was arrested using various narcotizing agents for the detailed microscopic examination. The narcotics used are the following:

1) Copper chloride (5mM-10mM)
2) Nickel sulfate (0.5 - 1%)
3) Potassium iodide (0.5-1%)
4) Buffered formalin

Formalin (37-40%)	100 ml
Sodium phosphate dibasic	6.5 g
Sodium phosphate monobasic	4.5 g
Distilled water	900 ml

These substances affect the mechanism of ciliary action. The concentrations of narcotizing agents were varied for different organisms.

Staining for anaerobic protozoa: Staining was required for identifying internal and external structures of protozoa.

Methylene Blue: Methylene blue stains the nucleus and cytoplasmic granules of protozoa. The stain solution was prepared by dissolving Methylene blue powder (0.1%) in 0.05% ethanolic water.

Lugol's iodine solution: Iodine stains starches such as glycogen. Lugol's iodine was prepared by dissolving 1g KI and 2g iodine crystals in 10 ml distilled water and used in 1: 10 ratio (1ml sample: 10 ml sample).

DAPI staining: 4', 6-diamidino -2- phenylindole (DAPI) is a fluroscent dye which binds to nuclear DNA and gives exact shape and number of nucleus for identification of protozoa. DAPI of concentration 2 $\mu g\ ml^{-1}$ was used by following the method reported by Stevik et al., 1998. The DAPI stained samples were observed under a epifluorescence microscope (Leica DM 2500).

Silver impregnation: Ciliated protozoa have assume a variety of shapes and sizes, and their phenotypic characterisation is usualy based on the study of oral and somatic infraciliature. Ammoniacal silver carbonate method was used to impregnate and study the specific characteristics of infraciliature in protozoa. Impregnation technique was followed as per the Techniques in microbial ecology (1998).

Microscopy: Leica DM 2500 microscope was used to observe protozoa in Bright field and Phase contrast. Images were taken with a CCD camera attached to the microscope. The cell size was measured with the software Leica Qwin V3.

For Scanning electron microscopy was done by fixing cells in 2% OsO_4 in saline water (15%). The fixed samples were washed in distilled water and stuck to the polylysine covered fragments of glass cover slips. Samples were then transferred to ethanol for critical point drying. Coated with gold in sputter coater (Fenchel et al., 1989). The observations were made with a scanning microscope (JEOL-JSM 5600 LV, Japan).

Methanogenic symbionts in protozoa were detected by autofluroscence which was done directly under the fluorescent microscope (Leica DM2500, Germany) with an excitation at 350 nm (Finlay and Fenchel, 1989).

2.2.3 Enumeration of anaerobic protozoa

Live samples of protozoa were directly observed using a compound microscope, Nikon –ALPH 4 YS2. The number of protozoan was determined by direct counting on a Neubaur counting slide. Triplicates were prepared for each sample. Fast moving organisms were counted after fixing in Shaudinn's fixative (Martindale et al., 1982).

2.3 Results and discussion

2.3.1 Identification of anaerobic protozoa

The growth of protozoa was assessed daily with live samples. Various methods were used for identifying the organisms.

Fixation of anaerobic protozoa: Table 2.2 shows the counts of anaerobic protozoa fixed with glutaraldehyde at different concentrations. Glutaraldehyde concentration of 0.5% was observed as the optimum for fixation and selected as suitable for further studies. Higher concentrations contributed damage to flagella and cilia, and cell distortion.

Table 2.2. Effect of glutaraldehyde on protozoa

Glutaraldehyde Concentration (%)	Number of protozoa ml^{-1} (x 10^4)
0	22 .25
0.1	26
0.25	28.5
0.5	30.75
0.75	30.5
1	30.5
2.5	20
5	12

Undiluted Schaudinn's fluid had caused cell damage and better results were obtained with 25 times dilution in distilled water. On fixation, subsequent shrinkage of cells (35-50%) was observed within a week. It is close to previously reported 30 % shrinkage in aerobic ciliates (Choi and Stoecker, 1989). This shrinkage of organisms posed problems in identification and enumeration. Therefore, studies were mainly done with live samples.

Immobilization of anaerobic protozoa: Free swimming flagellates and ciliates were found to gather near the debris (Fig. 2.5a). The stalked ciliate *Vorticella* was attached directly to debris with their stalk (Fig. 2.5b). The free swimming flagellates like *Bodo* and *Rhyncomonas* had a tendency to attach the debris while feeding (Fig. 2.5c). So it was very easy to observe their morphology. In the absence of debris, placing of tiny pieces of shredded filter paper in the sample was found effective for immobilizing protozoa.

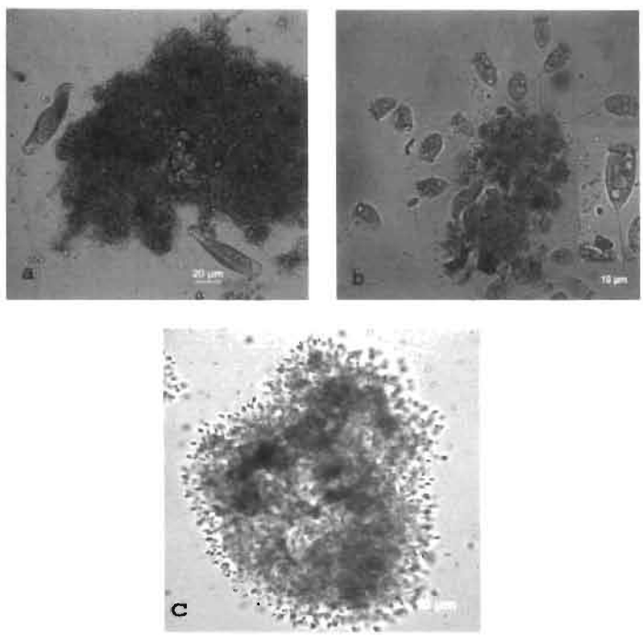


Fig. 2.5. Micrographs showing mechanical immobilization of protozoa

Changing viscosity of medium could arrest the movement of fast moving organisms. Methyl cellulose (1-5% liquid) was an excellent medium for the immobilization of protozoa and had the survival for 24 hours without any shrinkage. While in polyvinyl alcohol, organisms were stable only for 2 hours without any shrinkage. So methyl cellulose was selected for further studies.

The results of various narcotising experiments were interesting, and found to vary with the kind of organisms. Ciliates were more sensitive to narcotic treatments compared to flagellates. The narcotizing with $NiCl_2$ (5mM) needed 5 minutes to slow down the movement of ciliates and 5- 10 minutes for flagellates. In $NiCl_2$ added samples, shrinkage of cells was observed after 1 hour. $NiSO_4$ treatment for 5-10 minutes could arrest the movement of both flagellates and ciliates, but they were distorted in half an hour. The treatment with KI (0.5%) required 25- 30 minutes for arresting the fast moving flagellates and 5-10 minutes for ciliates. The cells found to retain their shape even for a day. Buffered formalin had also the property to arrest the

movement of protozoa, but cells were deprived of transparency. By observing the suitability, $NiCl_2$ and KI were mainly used as the narcotizing agents in this study.

Staining of anaerobic protozoa: Direct addition of stains to the samples caused shrinkage of protozoa. Alternatively, staining after narcotization was better for microscopic identification. Staining after narcotization was performed mainly with $NiCl_2$.

Lugol's iodine was found to be better for both the ciliates and flagellates and stained in brown colour without any change in their characteristic shape (Fig. 2.6a).

Methylene blue was used to stain the nucleus. Methylene blue stained blue to nucleus (Fig. 2.6b) in a contrasting cell background. Methylene blue and Lugol's iodine were selected for further studies.

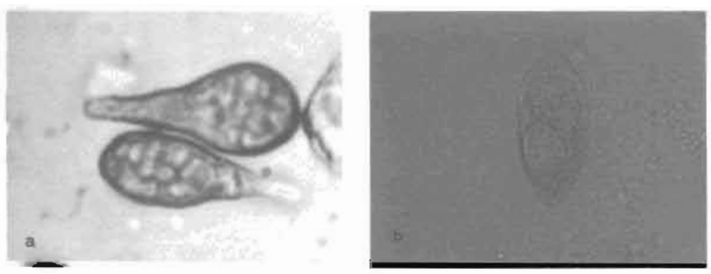


Fig. 2.6. Micrographs of (a) Lugol's stained flagellate, *Menoidium* and (b) Methylene blue stained ciliate, *Prorodon* (nucleus stained blue)

DAPI is a fluroscent dye that bind to nuclear DNA. DAPI staining demontrates shape of nuclei (Fig. 2.7a) and the number (Fig. 2.7b) and, was found to be very useful for identification of protozoa. Staining with DAPI was also helpful to view endosymbionts of protozoa (Fig. 2.7c).

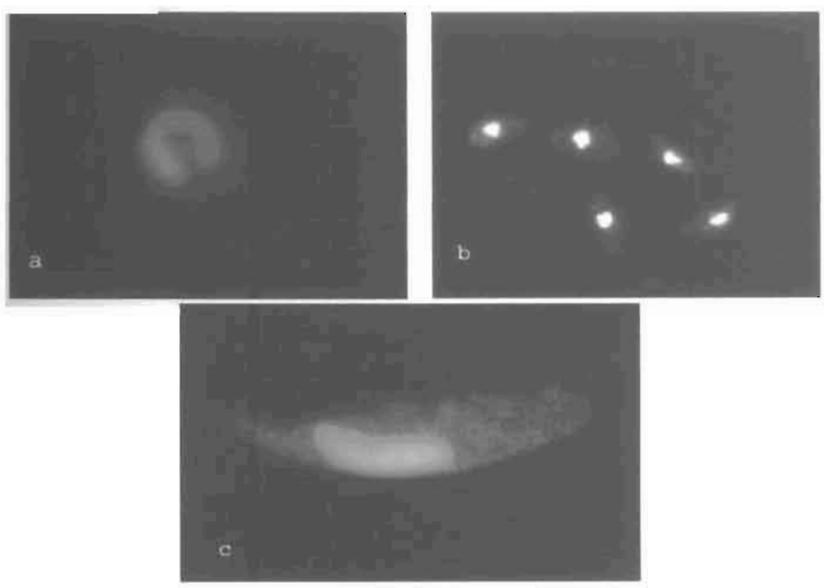


Fig. 2.7. (a) Nucleus of *Vorticella* stained using DAPI (b) DAPI stained cells of *Cyclidium* with micro and macro nucleus and (c) Endosymbionts inside the ciliate *Metopus*

Protozoa, especially ciliated protozoa are the most challenging organisms in the identification studies. They appear in a variety of shapes and sizes, and their phenotypic characterisation is usually based on the study of the oral and stomatic infraciliature. The silver impregnation with ammoniacal silver carbonate was found to be very useful to impregnate and reveal the specific characteristics of the infraciliature in ciliate (Fig. 2.8). The area coloured as dark brown is the nucleus and the brown dots are the originations of cilia which are arranged in a line.

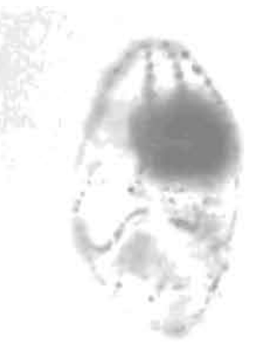


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Fig. 2.8. Morphology of the infraciliature of ciliated protozoa *Cyclidium* (impregnated with ammoniacal silver carbonate)

Micrography: Microphotographs of the sample were taken for detailed study. Bright field photographs were particularly useful to observe the size and shape of protozoa (Fig. 2.9). Number and arrangement of cilia and flagella were also identified by using bright field photographs. These details such as shape, size, cilia and flagella were compared with keys for identification.

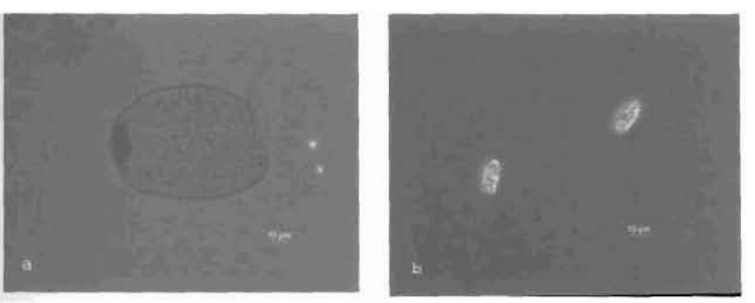


Fig. 2.9. Bright field photographs of (a) ciliate *Brachonella* and (b) flagellate *Menoidium*

Internal cytoplasmic structures were clearly identified by the phase contrast photographs (Fig. 2.10). In some cases, the cilia and flagella were not clearly visible

in bright field but it was clearly seen in phase contrast micrographs. Most of the features of protozoa were more obvious in phase contrast.

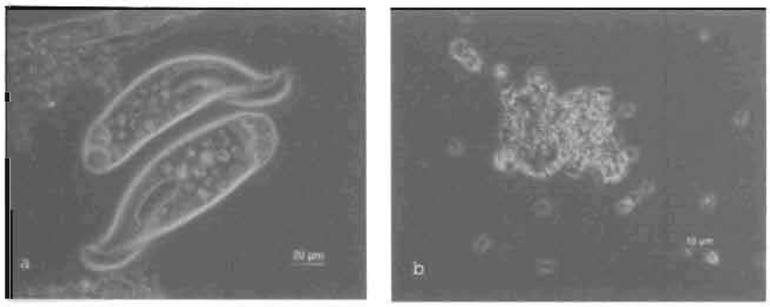
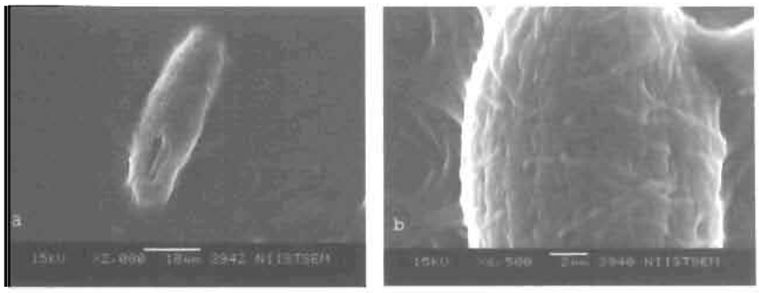


Fig. 2.10. Phase contrast photographs of (a) ciliate *Metopus* and (b) flagellate *Cercomonas*

External morphology of the organism was studied by employing scanning electron macrograph (Fig. 2.11). The shape of organisms with grooves and projections, mouth parts and arrangement of cilia are clearly observed in SEM photographs.



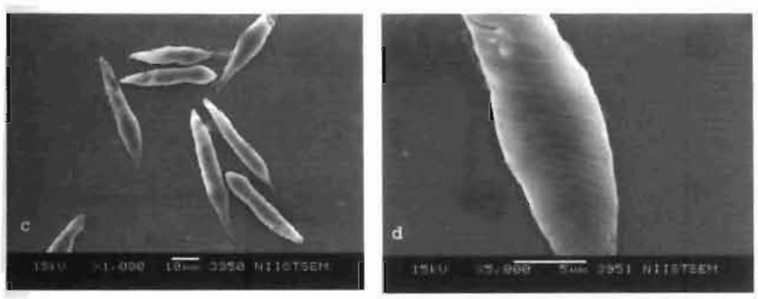


Fig. 2.11. SEM micrographs showing external features of ciliate *Cyclidium* (a) mouth and (b) cilia, and flagellate *Menoidium* (c) mouth and (d) striations on surface

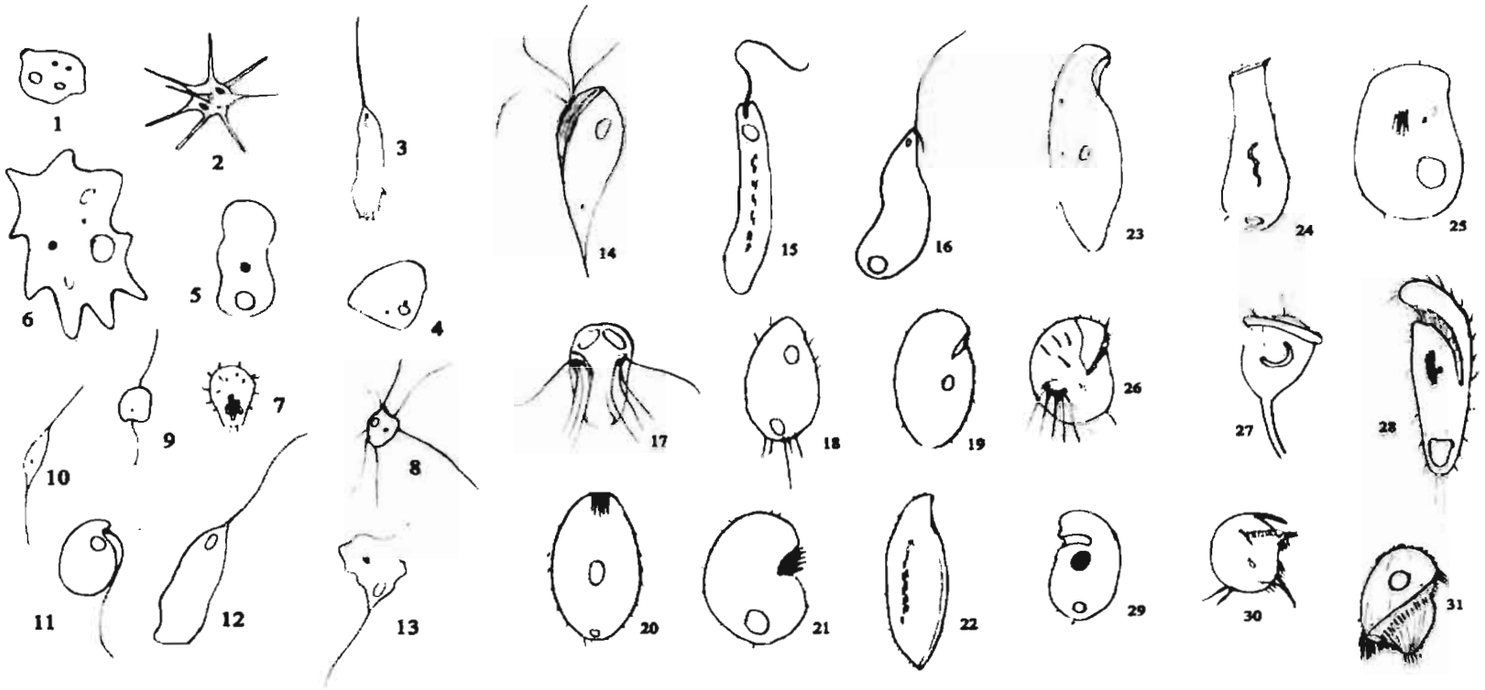
Autofluroscence of the F_{420} co-enzyme in endosymbiotic methanogens was observed by epiflurosence microscopy (Fig. 2.12). The fluorescence was found to fade with time. In living cells, the fluorescence faded away within a minute. Therefore, cells were fixed in 2 % ($v.v^{-1}$) formaldehyde solution to retain the fluorescence for some more time.

Fig. 2.12. Epiflurosence micrograph of *Metopus* showing autofluorescence of endosymbiotic methogens

As there is no single method which could reveal entire details necessary for the identification, several methods including live observations were followed in this study. The details obtained from various microscopic techniques were combined and

drawn schematic diagram of cytological and morphological features of protozoa in the anaerobic reactors (Fig. 2.13) and compared with keys for identification (Patterson, 1995; Foissner and Berger, 1996).

Fig. 2.13. Identified protozoa genera in anerobic reactors- 1. *Amoeba*, 2. *Amoeba radiosa*, 3. *Mastigamoeba*, 4. *Vannella*, 5. *Saccamoeba*, 6. *Mayorella*, 7. *Euglyphids*, 8. *Filose amoeba*, 9. *Bodo*, 10. *Cercomonas*, 11. *Rhyncomonas*, 12. *Peranema*, 13. *Mastigella*, 14. *Tetramitus*, 15. *Menoidium*, 16. *Naeglaria*, 17. *Trepomonas*, 18. *Cyclidium*, 19. *Colpidium*, 20. *Prorodon*, 21. *Colpoda*, 22. *Loxophyllum*, 23. *Loxodes*, 24. *Spathidium*, 25. *Chilodonella*, 26. *Euplotes*, 27. *Vorticella*, 28. *Metopus*, 29. *Plagiopyla*, 30. *Discomorphella and* 31. *Brachonella*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2.3.2 Protozoa identified in anaerobic reactors

Protozoa were observed as an important group of organism in the anaerobic reactors. Protozoa belonging to different genera were observed in anaerobic reactors such as the upflow anaerobic sludge blanket reactor, buoyant filter bioreactor, continuous stirred tank anaerobic reactors and batch reactor. In anaerobic reactors, **fourteen genera of ciliates, nine genera of flagellates** and **eight genera of amoebae** were identified during the study period (Table 2.3).

Table 2.3. List of identified protozoa in anaerobic reactors

Protozoa	Order	Genus
Rhizopoda	Tubulina	*Amoeba*
		Saccamoeba
	Conopodina	*Mayorella*
	Thecina	*Vannella*
	Rhizomastigina	*Mastigamoeba*
		Amoeba radiosa
	Arcellina	*Filose amoeba*
	Euglyphida	*Euglyphids*
Flagellates	Kinetoplastida	*Bodo*
		Rhyncomonas
	Diplomonadida	*Trepomonas*
	Cercomonadida	*Cercomonas*
	Euglenida	*Menoidium*
		Peranema
	Rhizomastigida	*Mastigella*
		Tetramitus
		Naeglaria
Ciliates	Karyorelictida	*Loxodes*
	Prostomatid	*Prorodon*
	Haptorida	*Spathidium*
		Loxophyllum

Protozoa	Order	Genus
Ciliates	Trichostomatida	*Plagiopyla*
	Crytophorida	*Chilodonella*
	Hymenostomatida	*Colpidium*
	Suctociliatida	*Cyclidium*
	Peritrichida	*Vorticella*
	Heterotrichida	*Metopus*
		Brachonella
	Hypotrichida	*Euplotes*
	Colpodida	*Colpoda*
	Epalcida	*Discomorphella*

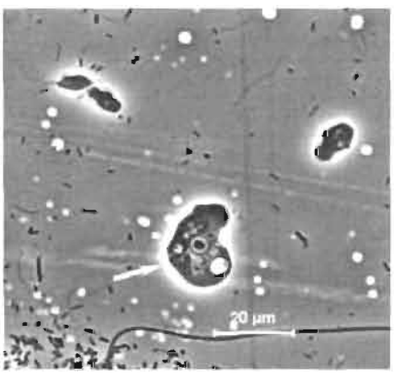
2.3.3 Growth of protozoa

The protozoa in anaerobic reactors showed fluctuations in terms of abundance and diversity during the operation. Their abundance and diversity were significantly varied with respect to feed composition and physicochemical conditions of the anaerobic system. Diverse groups of flagellates and amoebae were observed abundantly at the early stages of reactor operation. The flagellates *Rhynchomonas, Cercomonas, Naeglaria, Tetramitus, Menoidium, Trepomonas and Bodo* were present in all anaerobic reactors throughout and amoeboflagellate *Mastigella* was found occasionally. *Peranema* was observed rarely in the BFBR reactor. The prominent genera of amoeboids observed in the anaerobic reactors were *Amoeba, Myorella, Vanella, Saccamoeba* and *Mastigamoeba*. In addition, *Amoeba radiosa* and *Filose amoeba* were found occasionally. *Euglypha* was observed rarely in anaerobic reactors. The commonly observed ciliates in the reactors were *Prorodon, Cyclidium, Metopus, Spathidium, Colpidium, Plagiopyla, Loxophyllum, Colpoda, Loxodes and Vorticella*, with the maximum prevalence of *Prorodon, Cyclidium, Plagiopyla, Colpidium, Metopus, Vorticella* and *Spathidum*. In addition to this, *Chilodonella, Brachonella* and *Discomorphella* were found occasionally in BFBR. *Euplotes*, a micro aerobic ciliate was found in reactors with oxygen exposure. There was a chance for oxygen exposure in reactors through feeding, but it was totally absent in

strict anaerobic condition. Each of these groups was prominent usually with different reactor conditions and can be considered as reactor performance indicators (Results described in chapter 3).

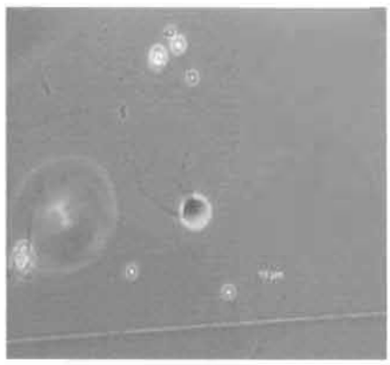
A little information is available on the presence of protozoa in anaerobic reactors. Agrawal et al., (1997) have observed free living anaerobic ciliates similar to *Metopus* in a high-rate anaerobic reactor. Gijzen et al., (1988 a,b) have developed a two-phase anaerobic system namely Rumen derived anaerobic digestion (RUDAD) initiated with micro organisms including ciliates from the ruminant, however, there is no information on the specific involvement of ciliates. Many of the protozoa observed in this study have not been previously described as common inhabitants of various anoxic systems (Fenchel and Finlay, 1995). For example, the ciliate genera of *Colpidium, Loxophyllum, Colpoda, Vorticella* and *Discomorphella,* and flagellates *Cercomonas, Tetramitus, Menoidium* and *Bodo* are not reported as typical ciliates of anaerobic ecosystem. Prominent features of protozoa genera observed in our anaerobic reactor ecosystem are given below.

Amoeba



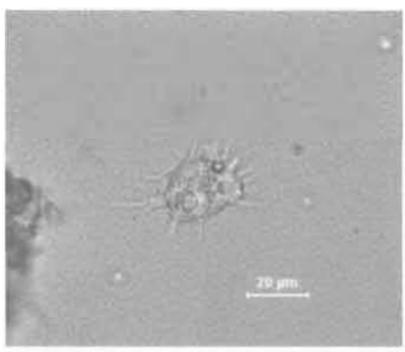
The name amoeba restricted to those species that have a number of broad, rounded pseudopodia and single prominent nucleus. Feeding and motion achieved by extensions from the cell (pseudopodia). Usually measured 20-47 µm long. Moving cells have watery hyaline caps at the tip of pseudopodia.

Filose amoeba



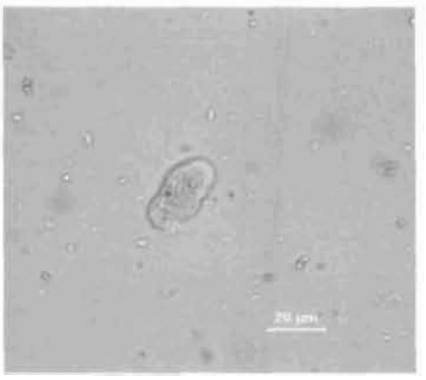
Amoeba with thread like (filose) hyaline pseudopodia, not emerging from the broad, clearly visible from the hyaline zone. This measured approximately 18-29 μm.

Amoeba radiosa



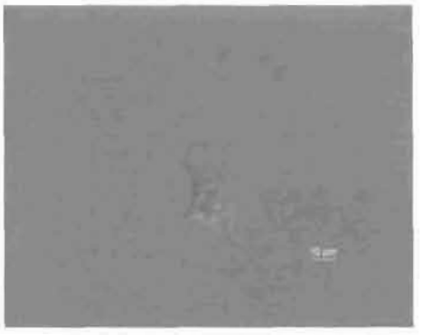
A radiosa is not true species of amoeba. This is a form adopted by many amoebae when they are detached from the substrate. This is a floating form. The amoeboid is contracted to an almost spherical mass, this gives a comet shape to cell. Cell measured 20-34 μm long.

Saccamoeda



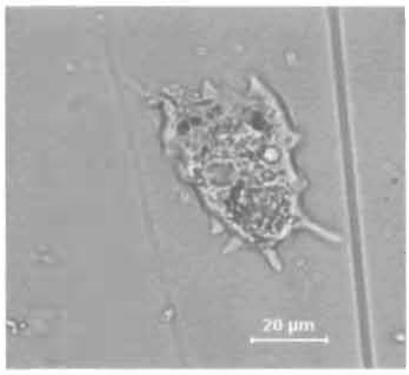
Small amoeba having monopodial appearance; Cell measured 19-32 µm. Hyaline cap reduced or absent. This is characterized with appearance of uroid.

Vannella



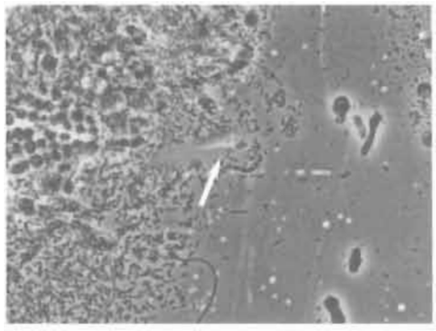
A common genera found in anaeobic environments. Fan shaped cells without folds. Cell measured 17-42 µm. The hyaline zone is prominent.

Myorella



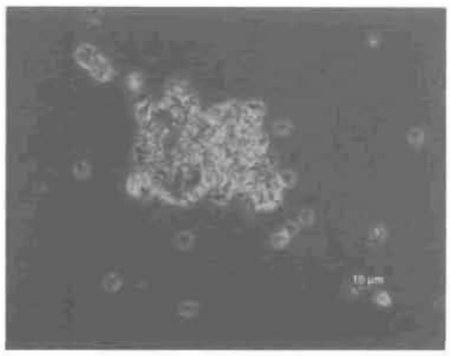
A common voracious scavengers and predators; measured 34-115 µm. A common genus of amoebae with conical pseudopodia that is relatively broad at their base, tapering slightly towards the tip which is rounded. Developing pseudopodia have a small hyaline cap.

Mastigamoeba



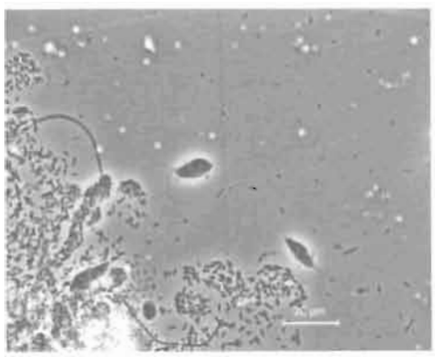
Flagellated cell of myxogastrid slime mould. Body measured 12-19 µm. Cells have one or two or four flagella at the apex of the cell. The flagella beat stiffly and cell is flexible.

Cercomonas



A gliding flagellate with a very fable body. Cells measured 7-16 µm. The anterior flagellum has stiff sweeping motion, and the posterior flagellum adheres to the body surface near its insertions

Tetramitus

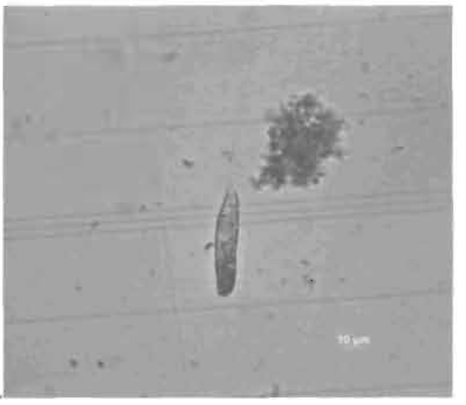


This flagellate is one stage of a polymorphic life cycle that also involves amoebae and cysts. Flagellate cells measured 9-19 µm long with four flagella. A slit extended from the site of flagellar insertion.

Menoidium

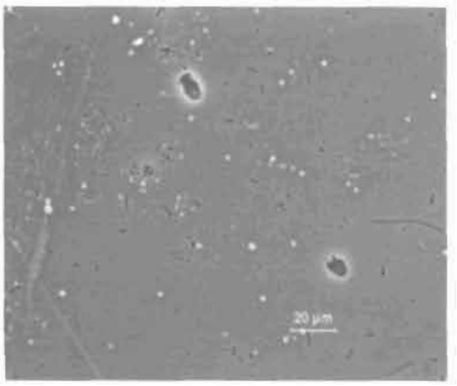
A free swimming euglenoid flagellate. Cells measured 20 - 34 µm. Relatively large flagellum, beating in whiplash fashion.

Peranema



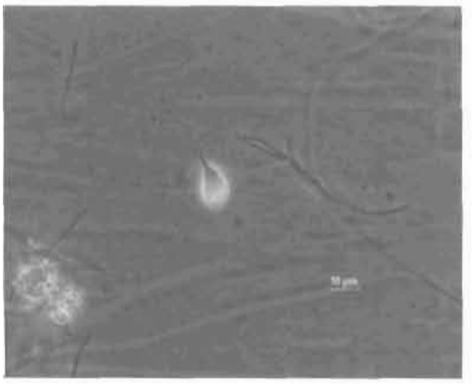
A euglenoid flagellate. Cells measured 23-39 µm long. Cells with prominent anterior flagellum. Slow swimming, but glides along the ground.

Trepomonas



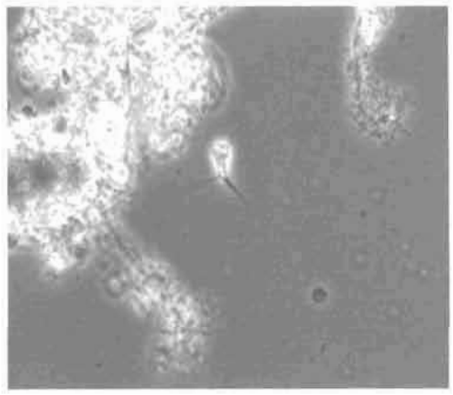
Diplomonad flagellate. Cells are bilaterally symmetrical along their longitudinal axis and 9-18 μm long. With four flagella on either side of the body. One is projecting laterally, while the other three are shorter. The flagella lie in the groove from the point of flagellar insertion.

Naeglaria



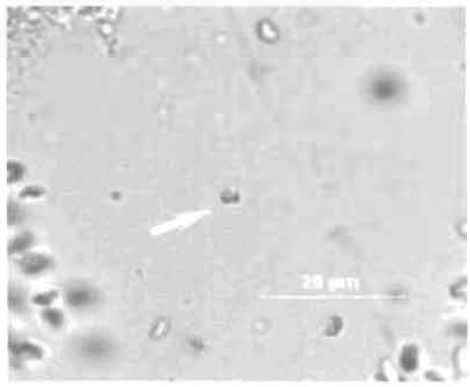
A vahlkampfid amoeba, forming part of heterolobosea. Flagellate is one stage of a polymorphic life cycle, called amoeboflagellate. Flagellate cells measured 9-16 μm long. It is a facultative pathogen.

Mastigella



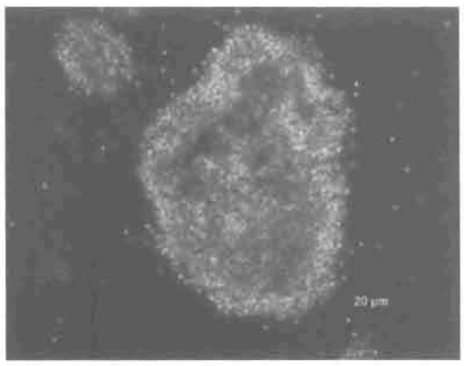
An organism that combines the characteristics of amoebae and flagellate. Body measured 8-24 µm. This has a amoeboid body and a flagellum. The flagellum is thin and beats stiffly like an undulating rod.

Bodo



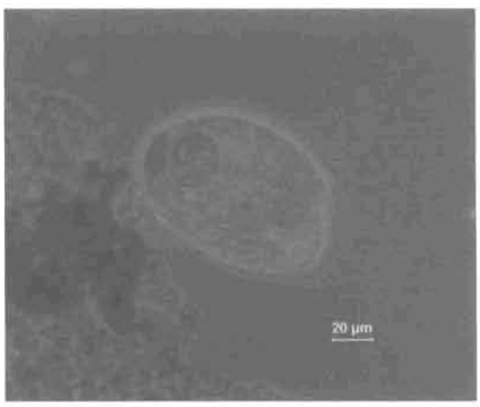
Typical bodonoid. Kidney shaped cells measured 7-9 µm. They move by gliding. Two flagella are inserted to one side of the anterior part of the cell.

Rhyncomonas



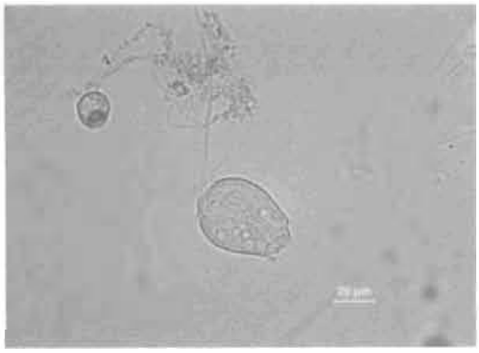
A common bodonoid flagellate. Cell measured 7-9 µm. Cells with single trailing flagellum.

Brachonella



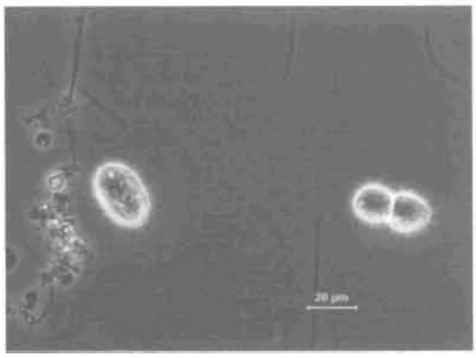
A heterotrich ciliate. Cells flattened; 60-86 µm long; 40 -53 µm wide. The cell has adoral zone of membranelles for feeding follows a spiral band around the anterior end of the cell. Locomotor cilia found axially in the posterior of the cell.

Vorticella



A common peritrich ciliate. Cup shaped with a contractile stalk. Cell measured 46 -98 µm long; 20-32 µm wide. The cilia generates current for feeding. Single large macronuleus present at centre.

Cyclidium

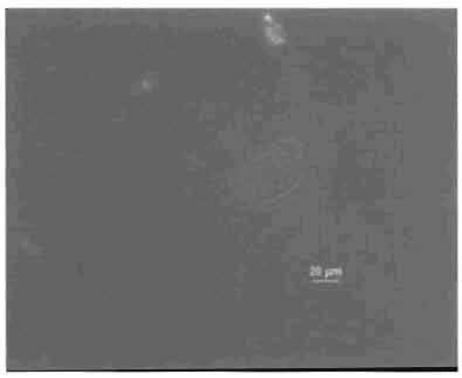


A common suspension feeding scuticociliate. Cells elipsoidal; 19-34 µm long, 12-16 µm wide. Macro nucleus central with adjacent double micronucleus. They were motionless while feeding and otherwise, moving very fast.

Plagiopyla

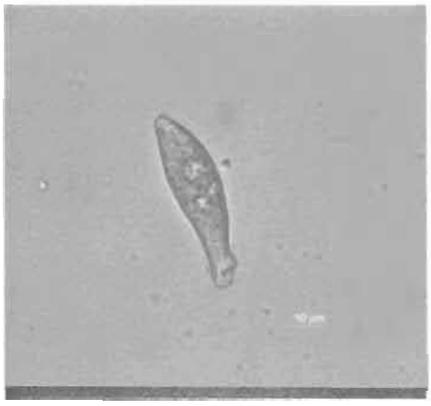
Cell body ovoid, dorso-ventrally flatted; cell body uniformly covered with cilia; a raised lip on the anterior edge of cytostome; 63-95 µm long. A single macronuleus and a single micronucleus present.

Chilodonella



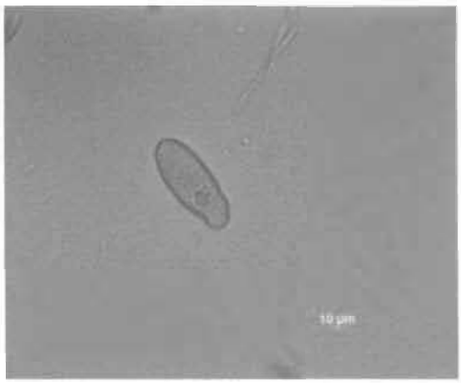
A hypostome ciliate. Flattened cell with well developed basket or nasse. The measured size is 32 -56 µm long and 28-42 µm wide. At the anterior end, the rods have teeth like structures which is surrounded by the cytostome on the ventral side. Micro nucleus attached to macronucleus.

Spathidium



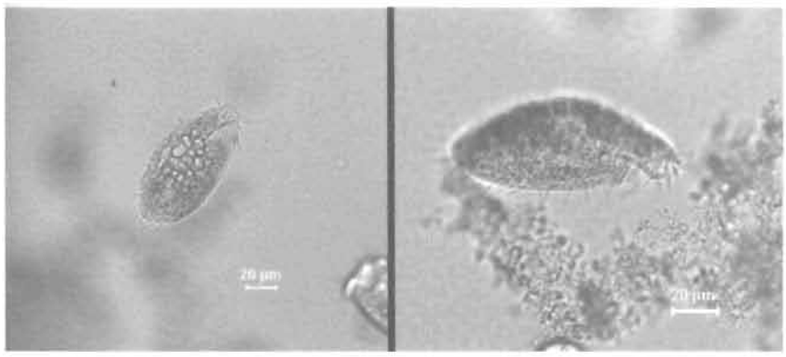
A flattened predatory scavenging ciliate. Body measured 30-86 µm long and 15-26 µm wide. The mouth is broadly spatulate, being widest part of the body. A long spiral like macronucleus present.

Colpidium



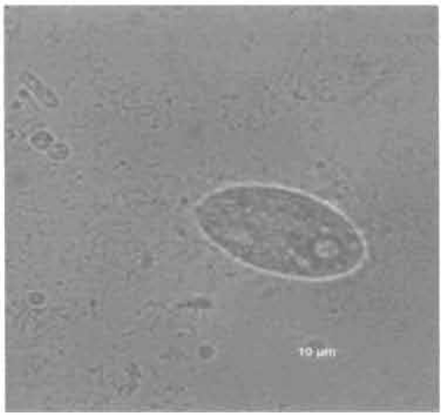
Flattened cell; 33-54 µm long; 20-26 µm wide. Mouth is at the base a short groove located just below a slightly twisted anterior part of the cell. Clearly visible macronucleus and micronucleus. Cells are evenly ciliated.

Euplotes



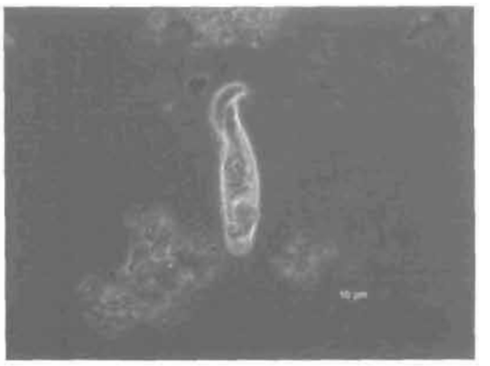
A common hypotrich ciliate. Crawling form; 35-92 µm long; 23-38 µm wide. Adoral membranelles form a collar around the front of the cell. Locomotor cilia are in clusters; which is the line of transverse cirri. Ventral cirri are used for the movement over flocs.

Prorodon



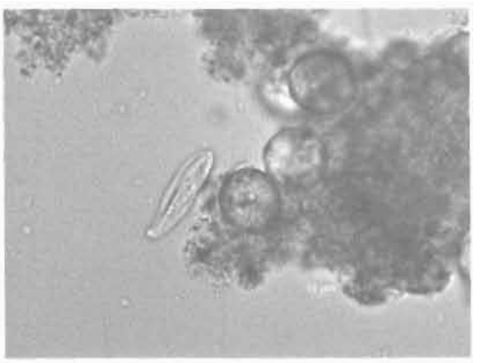
A scavenger ciliate. The cell is egg shaped; 44-87 µm long; 23-38 µm wide. Mouth is located at anterior pole. Microtubular rods lead from mouth into the cell. Macronucleus central and spherical with adjacent micronucleus.

Metopus



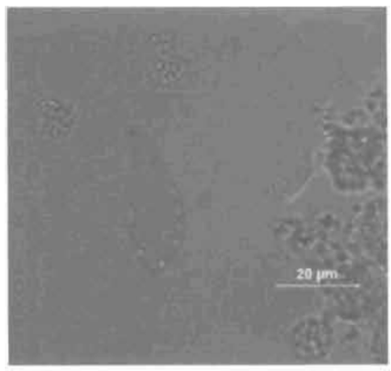
A heterotrich ciliate commonly found in anaerobic environments. An elongate cell with the front end twisted; 80-154 μm; 23-36 μm wide. The cell has adoral membrane for feeding, and conventional cilia for locomotion. Macronucleus found near to twisted end.

Loxophyllum



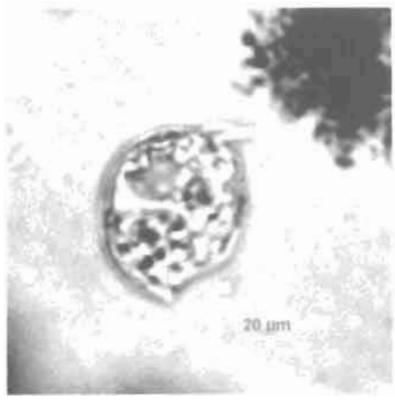
A flattened predatory ciliate. The cell size was 62-82 μm long and 20-36 μm wide. Mouth lies along the outer edge of the body. The adoral edge of the body bears warts. Macronucleus is beaded.

Loxodes



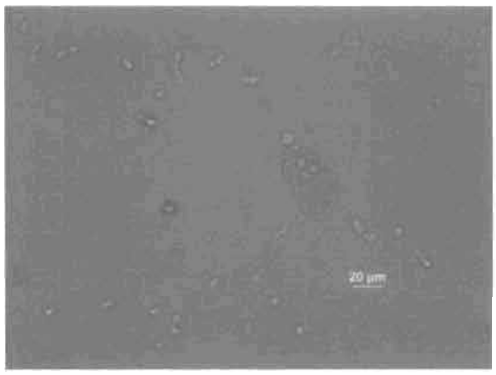
Flattened, with a concave buccal region; 65-14 µm and 14-34 µm wide. The mouth makes the form of a concave depression near the front pole of the cell. Macronucleus present near to posterior end.

Discomorphella



An odontostome ciliate. Cell is flat and body surface is stiff and sculpted, with one spike projecting from the front of the cell. The measured size was 30-46 µm long; 24-34 µm wide. The adoral zone of membranelles is on ledge like a structure that curve around the margin of the body.

Colpoda



A suspension feeder, using the buccal ciliary organelles. Flattened kidney shaped; 32-49 µm; 18-23 µm wide. The cilia emerging in curving kinetics. Large macro nucleus present at anterior end

2.3.4 Diversity and abundance of protozoa in anaerobic reactors

A variety of protozoa were found to associate with the microbial community of different anaerobic reactors which were belonging to 31 genera as described earlier. The number of each protozoa genera was varied in the anaerobic reactors from 0.625 x 10^4 ml^{-1} to 12.5 x 10^4 ml^{-1}. Occurrence of protozoa and their frequencies in anaerobic reactors are listed in Table 2.4. They are in four different categories - scarce (0-0.625 x 10^4 ml^{-1}), moderate (0.625-1.25 x 10^4 ml^{-1}), abundant (1.25-2.5x10^4 ml^{-1}) and most abundant >2.5x 10^4 ml^{-1}.

Table 2.4. Frequency of protozoa occurrence in anaerobic reactors

Genus	Occurrence in each abundance categories			
	Scarce	Moderate	Abundant	Most abundant
Rhizopods				
Amoeba	-	-	-	+
Myorella	-	+	-	-
Vannella	-	-	+	-
Saccamoeba	-	-	+	-
Mastigamoeba	-	-	-	+
Amoeba radiosa	-	+	-	-
Filose amoeba	+	-	-	-
Euglypha	+	-	-	-
Flagellates				
Rhynchomonas	-	-	-	+
Cercomonas	-	-	+	-
Naeglaria	-	-	-	+
Tetramitus	-	-	-	+
Mastigella	-	+	-	-
Trepomonas	-	-	+	-
Bodo	-	-	-	+
Menoidium	-	-	-	+
Ciliates				
Prorodon	-	-	-	+
Cyclidium	-	-	-	+
Metopus	-	-	-	+
Spathidium	-	-	+	-
Colpidium	-	-	+	-
Plagiopyla	-	-	-	+
Loxophyllum	-	+	-	-
Colpoda	-	-	-	+
Loxodes	-	-	+	-
Vorticella	-	-	+	-
Chilodonella	+	-	-	-
Brachonella	+	-	-	-
Discomorphella	+	-	-	-
Euplotes	+	-	-	-

The protozoa genera of *Amoeba* and *Mastigamoeba* were the most abundant amoeboids in all the anaerobic reactors at the initial stages of operation. Occurance of *Vannella* and *Saccamoeba* were abundant in all anaerobic reactors while genera *Myorella* and *Amoeba radiosa* were present moderately. The genera *Filose amoeba* and *Euglipha* were found rarely.

The flagellates *Rhynchomonas, Naeglaria, Tetramitus, Menoidium,* and *Bodo* were the most abundant genera in all the anaerobic reactors. The flagellates *Cercomonas* and *Trepomonas* were observed abundantly. The genera *mastigella* occurred moderately. The genera *Peranema* were found at lower frequency in oleate fed anaerobic reactors.

Ciliates *Metopus, Prorodon, Plagiopyla, Cyclidium and Colpoda* were the most abundant genera in all the anaerobic reactors fed with different substrates. *Spathidium, Colpidium, Loxodes* and *Vorticella* were occured from moderate to abundant. Genera *Loxophyllum* was at moderate frequency. The remaining genera were found with lower frequency (Table 2.4).

2.3.5 Succession in protozoan growth in anaerobic reactors

This study, examined the succession in growth of protozoa in terms of diversity and number with respect to reactor operations. The flagellates and amoeboids were flourished at early stages of reactor operation and then ciliates, after reaching steady state in COD removal and methane production (Fig. 2.14).

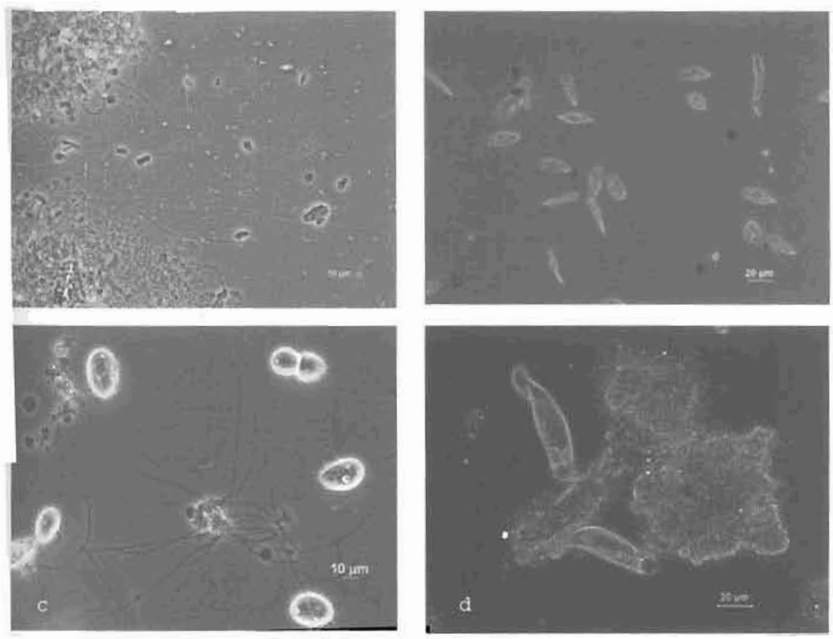


Fig. 2.14. Growth succession of protozoa in anaerobic reactors (a) Small flagellates and amoeboid were flourished at early stages of reactor operation, (b) larger flagellates began to grow, then (c) small ciliates established, and finally (d) larger ones appeared

During start up of anaerobic reactors and with sudden increase in loading rates, fluctuation of protozoa population was high and observed abundance of flagellates and amoeboids. Amoeboids were appeared in all reactors initially, in which *Amoeba*, *Mastigamoeba* and *Vanella* appeared first and followed by *Myorella*. The amoeboids reached a maximum number of 8 x 10^4 ml^{-1} in reactors. Flagellates reached a highest number of $1.6x10^6 \pm 0.5$ ml^{-1} with the maximum occurrence of *Rhynchomonas*, *Naeglaria*, *Menoidium*, *Tetramitus* and *Bodo* at early days of reactor operation. On reaching the anaerobic process stable the density of flagellates declined to the range of 2.0 $x10^4$ to 6.5 $x10^4 \pm 0.4$ ml^{-1} and maintained a steady state thereafter in all reactors (Fig. 2.15). The abundance of flagellates at initial stage could be due to

its shorter generation time compared to ciliates and the energy advantage from the direct utilization of dissolved substrate like VFA as noticed higher levels at initial stage (> 8 meq l^{-1}). Direct uptake of organic matter through osmotrophic nutrition has previously been reported in some of the soil flagellates (Ekelund and Ronn, 1994).

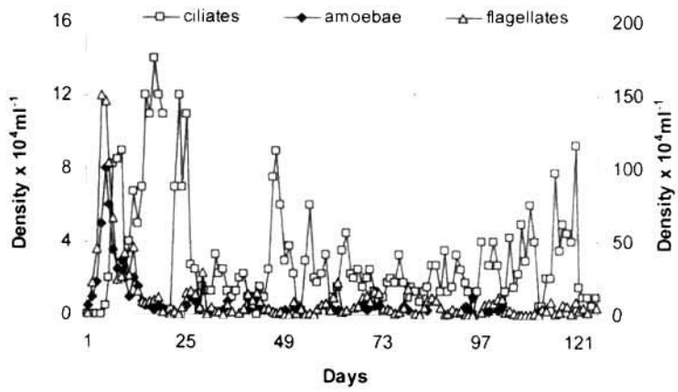


Fig. 2.15. Growth of different group of protozoa in anaerobic reactors operated with loading rate of 1g COD l^{-1} d^{-1} at 5 days HRT

The growth of ciliates was predominant in the reactors on steady state and their count in the reactors were in the range of $2.5x10^3$ to 14 $x10^4$ ml^{-1} (SD $\pm$ 0.6) (Fig. 2.16). Ciliate population also had variations in size and composition at different stages of stable reactor operation. Small ciliates like *Cyclidium* (average size of 24 µm) had greater abundance than larger ones at the early days of stable conditions and ranged in abundance from 3 $x10^4$ ml^{-1} to 9 $x10^4$ ml^{-1}. Later, a diverse assemblage of ciliates with medium size (45 to 89 µm) like *Plagyopyla, Colpoda, Colpidium* and *Loxophyllum* were established with maximum of 14 $x10^4$ ml^{-1}. Finally, genera of larger size (> 80 µm) like *Loxodes, Spathidium, Metopus* and *Vorticella* were observed with stable reactor performance (Fig 2.16).

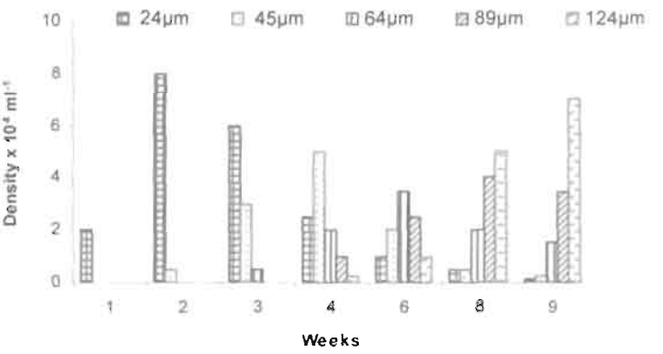


Fig. 2.16. Ciliates with varied size in anaerobic reactor at stable operation

As shown in Fig. 2.17, the maximum number of protozoa were indexed in BFBR reactor ($2.7 \times 10^6 \pm 0.55$ ml^{-1}) and UASB reactor ($2.6 \times 10^6 \pm 0.55$ ml^{-1}) compared to CSTAR ($2.3 \times 10^6 \pm 0.52$ ml^{-1}) at early period of reactor operation. On reaching the anaerobic process stable the density of protozoa were in the range of 32×10^4 to $125 \times 10^4 \pm 0.4$ ml^{-1} and maintained a steady state thereafter in all reactors. These results are revealing a new trophic layer of protozoa in anaerobic reactors. Free living anaerobic ciliates similar to *Metopus* have been observed in a high-rate anaerobic reactor of treating low strength wastewater, which were considered as migrants from other ecosystems (Agrawal et al., 1997). It is suggested that ubiquitous protozoa appear to be an important component of food webs in nature (Finlay and Estaban, 1998).

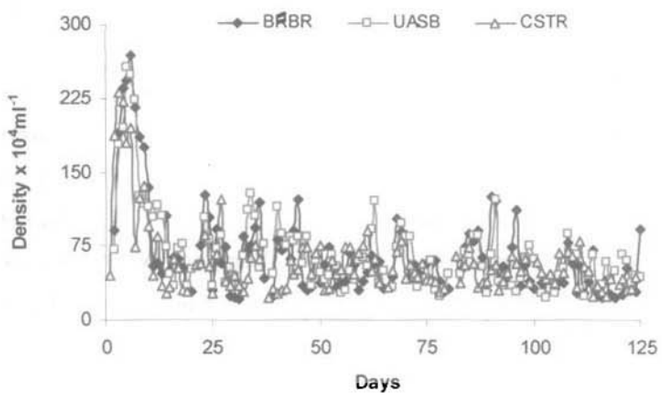


Fig. 2.17. Protozoa population in different kinds of anaerobic reactors

2.3.5 Classification of ciliates in anaerobic reactors

Most abundant group of protozoa found in anaerobic reactors at steady state were the ciliates and are classified into four groups; attached, crawling, free swimming and crawling-free swimming. Aerobic protozoa have been grouped similary in activated sludg process (Cereceda and Guinea, 1996). The ciliate groups observed in the anaerobic are presented in Table 2.5. Attached groups were associated with the flocs by their stalk for firm attachment, while crawling protozoa have cilia for movement on the flocs surface. The free swimming ciliates were freely moving in the liquid phase of reactors. Crawling - free swimming group comprises ciliates that were crawling on flocs surface and freely moving in liquor phase.

Table 2.5. Classification of ciliates according to their association with the sludge flocs

Non floc associated		**Floc associated**	
Free swimming	*Prorodon*	Attached	*Vorticella*
	Cyclidium	Crawling	*Chilodonella*
	Spathidium		*Euplotes*
	Plagiopyla	**Tempoarily floc associated**	
	Metopus	*Swimming -crawling*	*Loxophyllum*
	Discomorphella		
	Brachonella		
	Colpoda		
	Colpidium		
	Loxodes		

2.3.6 Reactor specific ciliate communities

Relative abundance of these groups of ciliates was monitored in anaerobic reactors (Fig. 2.18). Results indicated that only the group of free swimming ciliates was always present, although their proportion varied from reactor to reactor. In all reactors, free swimming group displayed above 90% of total ciliates and the highest frequency (98%) was observed in CSTAR. The floc attaching ciliate-*Vorticella* was observed only in batch reactors BFBR and UASB. *Vorticella* was absent or rare in CSTAR. Presumably, the attachment could be disturbed in the stirred reactor. Crawling and swimming-crawling ciliates were found in all reactors, mostly in minimal numbers, < 3% of the total ciliates. It is evident that each species of protozoa thrives best whenever it finds a suitable environmental condition. According to Finlay (1998) protozoa are cosmopolitan in their spatial distribution.

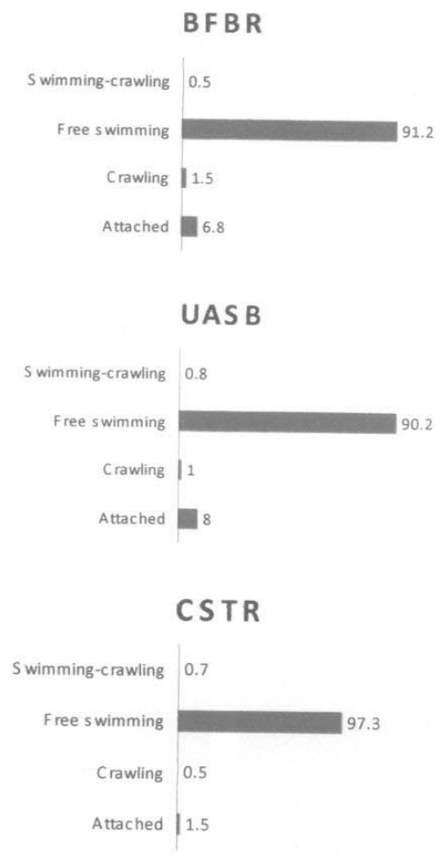


Fig. 2.18. Relative abundance of different ciliate groups in anaerobic reactors

2.3.7 Cyst formation of anaerobic protozoa in anaerobic reactors

Protozoa are characterized by their ability to produce cyst which allows them to survive unfavorable conditions. Under favorable conditions, they rapidly excysted and began to grow. We have observed cysts measuring different sizes at unfavorable conditions such as oxygen toxicity, food scarcity, high organic loading and at the time of changes in pH and VFA concentration (Fig. 2.19).

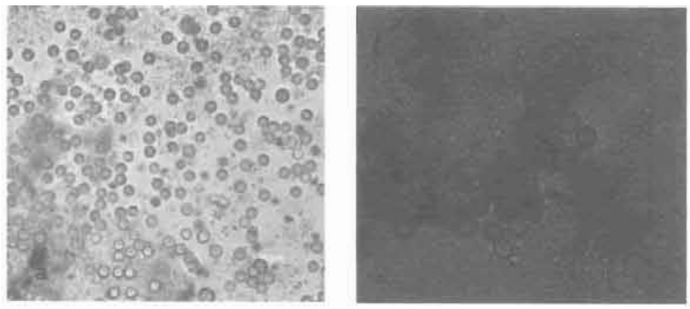
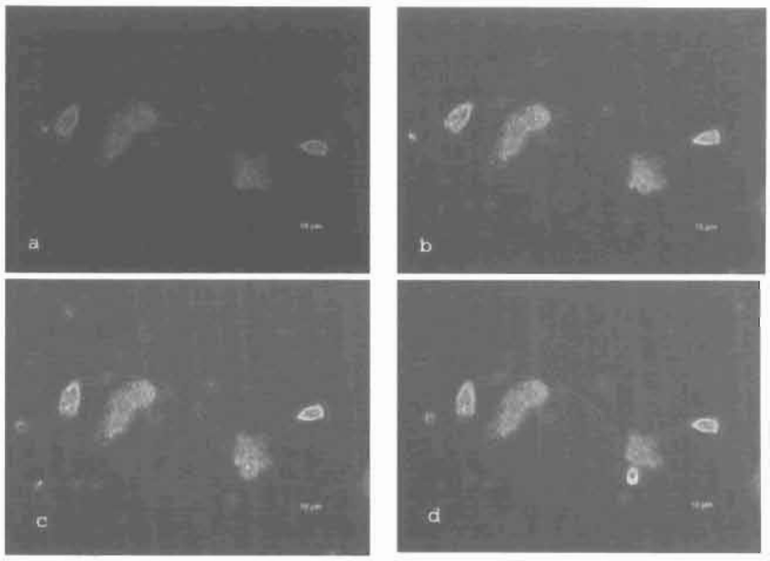


Fig. 2.19. Micrographs of (a) small cysts and (b) large cysts found in anaerobic reactors

The small cysts might be of flagellates or small ciliates. Correspondingly, large cysts might be of larger ones like of *Metopus* and *Vorticella.* Growth of *Vorticella* from a large cyst is presented in Fig. 2.20.



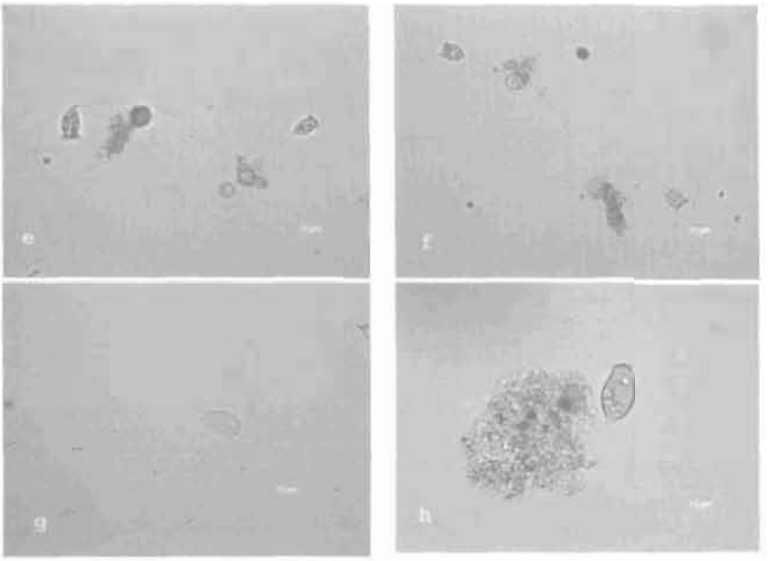


Fig. 2.20. Micrographs of (a) cyst of *Vorticella* inside the sludge, (b-d) began to grow under favorable conditions, (e-f) emerging *Vorticella* from the cyst, (g) wandering for suitable place and (h) finally, attached another sludge particle

Some cells are divided inside the cyst and on rupture grow to many under favorable conditions (Fig. 2.21). The individual cells were found to grow independently.

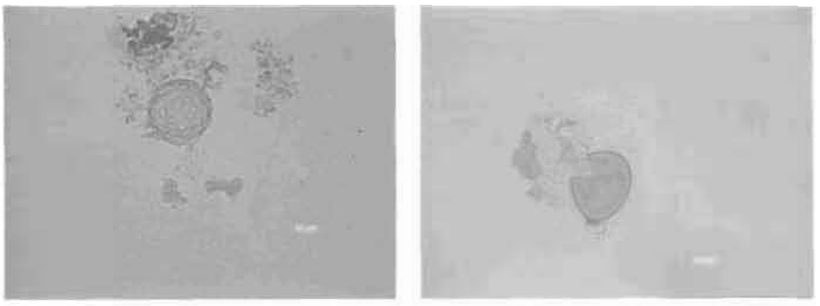


Fig. 2.21. Rupture of cysts under favorable conditions in anaerobic reactors

2.3.8 Reproduction of protozoa in anaerobic reactors

Reproduction of protozoa is two kinds, mainly by asexual and sexual. Various types of asexual reproduction found in protozoa are binary fission, multiple fission, budding and plasmogamy (Patterson, 1995). Binary fission was the main reproductive processes observed in anaerobic protozoa in the reactors (2.22,2.23,2.24).

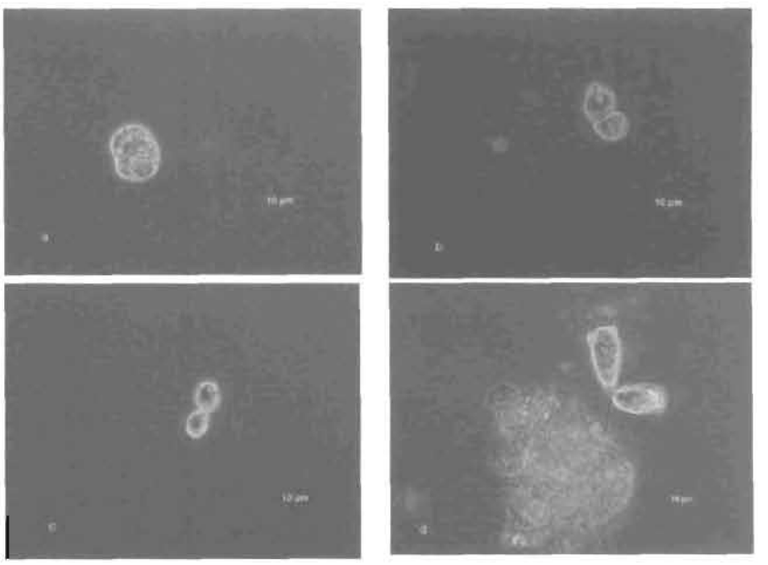


Fig. 2.22. Micrographs of (a-d) cell division and successive growth of *Vorticella* in anaerobic reactor - BFBR

The binary fission and successive growth of ciliate *Vorticella* and *Cyclidium* observed in BFBR are presented in Fig. 2.22 and 2.23 respectively. A rare shot of fission in *Euplotes is* given in Fig. 2.24.

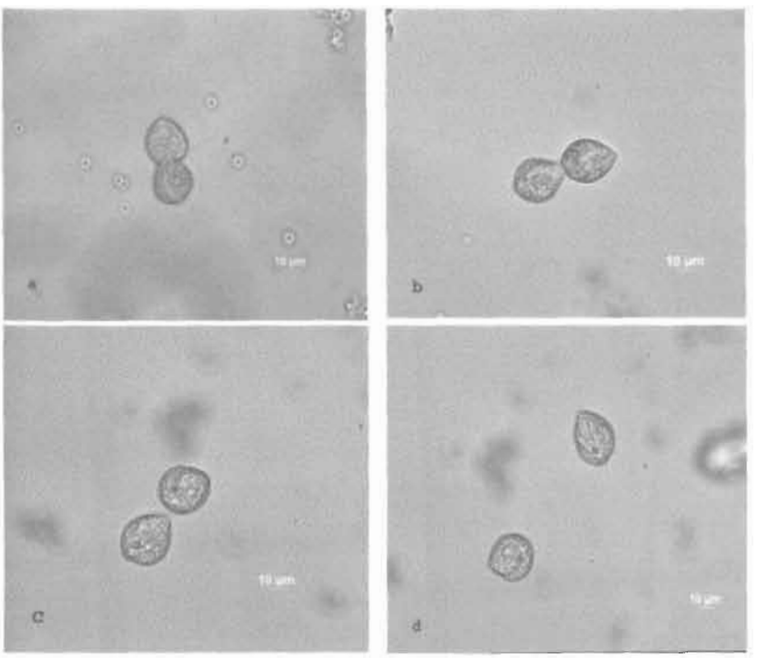


Fig. 2.23. Micrographs of (a-f) binary fission and successive growth of ciliate *Cyclidium* in anaerobic reactors

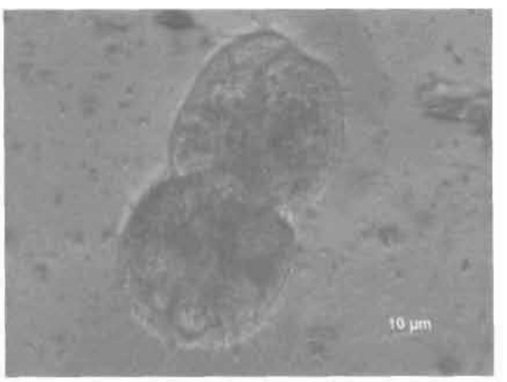


Fig. 2.24. Micrograph of binary fission of *Euplotes* in an anaerobic reactor - CSTAR

Sexual reproduction in protozoa is mainly through syngamy and conjugation. Sygamy was not observed in anaerobic reactors. The main sexual reproduction observed in the anaerobic reactor was conjugation. Conjugation microphotographs of different genera of protozoa are shown in Fig. 2.25.

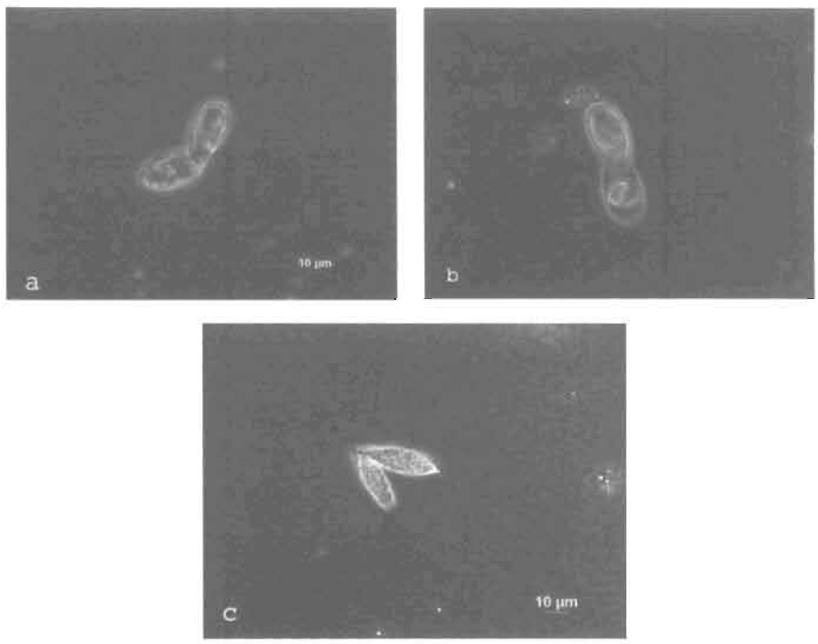


Fig. 2.25. Conjugation in different protozoa genera in anaerobic reactor - CSTAR (a) and (b) *Cyclidium* and, (c) *Menoidium*

2.4 Conclusions

This is the first study on growth of protozoa in anaerobic reactors

- The additional trophic layer of anaerobic protozoa in the anaerobic reactor ecosystem shows importance of eukaryotes in anaerobic degradation along with bacteria in wastewater treatment systems. So far prokaryotes were the groups mainly accounted for anaerobic degradation.
- Studies established a rich fauna of protozoa in anaerobic reactors, in which fourteen different genera of ciliates, nine different genera of flagellates and

eight different genera of amoebae were identified. Ciliates were the abundant organisms and dominant group in the steady state anaerobic reactors.

- Most of the ciliates found in this study have not been previously described in anaerobic systems (the ciliate genera of *Colpidium, Loxophyllum, Colpoda, Vorticella* and *Discomorphella,* and flagellates *Cercomonas, Tetramitus, Menoidium and Bodo)* and this study gives new information about anaerobic micro ecology.
- The succession in growth of protozoa in terms of diversity and number with respect to operation was noticed in all reactors. Amoeboids and flagellates are flourished at the early stages and at unsteady stages, and then the ciliates appear on reaching steady state.
- Change in protozoan abundance and diversity is related to reactor performance. Thus the analysis of protozoa is a useful method to assess the reactor performance.

This understanding of anaerobic reactor ecosystem introduces a new microbial loop in the anaerobic degradation of biomass and energy flow.

Chapter 3
Influence of protozoa on the performance of anaerobic wastewater treatment systems

Abstract

The growth of protozoa related to physicochemical and operational parameters of the anaerobic reactor were studied. The correlation analysis reveal the indicator value of each protozoa group in assessing reactor performance. Ciliates were flourished at the time of optimum loading rate with best performance. Flagellates and amoeboid were dominated at unsteady conditions such as change in pH, variations in loading rates, high VFA concentration, etc. The abundance of ciliates stimulated the rate of COD removal, methane production and MLSS reduction. Abundance and diversity of ciliates were related with good quality of effluent, and abundance of flagellate and amoebae could relate to poor quality of effluent. The efficiency of anaerobic wastewater treatment process has link not only to the bacterial population but also to the protozoa population.

3.1 Introduction

Anaerobic degradation is accepted in many waste treatments because of its unique advantages such as no energy requirement from aeration, produce little biomass, low nutrient requirement, production of bio-fuel from waste organics, etc. Anaerobic conversion of organic matter is recognized as a complex process involving co-ordinated activity of a number of different microbial groups. It is a multistage microbial process where obligate anaerobic bacteria form the dominant population (Gujer and Zehner, 1983). The researchers have mainly studied on bacteria in anaerobic waste treatments. This has lead to understanding the bacterial role in biodegradation and ultimately helped in many designs in anaerobic treatment systems. However, microscopic studies revealed an additional trophic layer of anaerobic protozoa which are widely distributed in anaerobic wastewater treatment reactors (Chapter 2).

There are many reports on protozoa in aerobic wastewater treatment plants (Curds et al., 1968; Madoni, 1994a; Salvado et al., 1995; Nicolau et al., 2001). Protozoa are the common components in activated sludge which represents as much as 5-10% of the total dry weight of the mixed liquor (Curds et al., 1968). The protozoa have an important role for the good balance of the biological ecosystem: they eliminate the bacteria in excess, stimulate bacterial activity and promote flocculation (Gerardi et al., 1995). By consuming the free bacteria protozoa help to decrease the effluent turbidity as well as BOD and suspended matter content (Curds et al., 1968; Salvado et al., 1995). The aim of this study was to investigate the influence of protozoa, and ciliates in particular, in the anaerobic degradation processes.

3.2 Materials and methods

3.2.1 Continuous stirred tank anaerobic reactor

Continuous stirred tank anaerobic reactor (CSTAR) with working volume of 1 litre was operated at different organic loading rates with oleic acid suspension as suspended COD (Table. 3.1).

Table 3.1. Operating parameters of the anaerobic CSTAR

Temperature	30°C ± 2
pH	6.8 - 7.2
Loading rate	1 g COD. l^{-1}. d^{-1} - 2 g COD. l^{-1}. d^{-1}
HRT	5 - 10 days
Redox potential	-300 - -400 mV
VSS	0.15 - 1 g. l^{-1}

3.2.2 Chemical Analysis

Chemical oxygen demand: Chemical oxygen demand was measured according to the open reflux method described in Standard methods (APHA, 1998). Temperature controlled solid block digester (Kel Plus, India) was used for refluxing the sample. The sample was completely oxidised with dichromate (K_2Cr_2O7) in the

presence of concentrated sulphuric acid at 150°C in glass vessels for 2 hour. After digestion, the unreduced dichromate was titrated with Ferrous ammonium sulphate – FAS ($(FeNH_4)_2$ $(SO_4)_2$ $6H_2O$) to determine the amount of dichromate consumed. The organic matter (COD) was calculated in terms of oxygen equivalent. A blank solution with distilled water was also refluxed with same reagents and titrated with FAS.

Suspended solids: Volatile suspended solids and mixed liquid suspended solids were determined as per Standard methods (APHA, 1998). A well mixed sample was filtered through a weighed glass fiber filter and residue retained on the filter is dried to a constant weight at 103 to 105°C. The increase in the weight of filter paper represents the total mixed liquid suspended solids. The residue from the above (after drying) was ignited to a constant weight at 550°C. The weight lost on ignition is the volatile solids.

Tur bidity: Turbidity of the sample was measured using Nephelometric method (APHA, 1998). The method is based on a comparison of the intensity of light scattered by the sample under defined conditions with intensity of light scattered by a standard reference suspension under the same conditions. The higher intensity of scattered light results higher turbidity. Formazin polymer was used as the primary standard reference suspension. The turbidity of a specified concentration of formazin suspension is defined as 4000 NTU.

Volatile fatty acids: Total volatile fatty acids and alkalinity concentration in the samples were estimated by a two stage sequential titration (Anderson and Yang, 1992). Measurements were done immediately after collecting the sample. A pH meter and a magnetic stirrer were used to measure pH and mix the samples during titration.

pH: The pH was measured immediately after sampling using a glass electrode pH meter (Systronic, India).

Redox: The redox potential was measured immediately after sampling using a glass electrode with a conductivity meter (Testo, USA).

Temperature: Temperature measured with mercury filled Celsius thermometer.

Methane: A gas chromatograph (FISION 8000, FID, 2mm i.d silica column, carrier gas 30ml.min^{-1}, oven 110°C, detector 130°C, and injector temperature 130°C) was utilized to measure methane.

3.2.3 Microscopic observations

The method for identification and enumeration of protozoa are presented in chapter 2.

3.3 Results and discussion

The operational conditions and performance of reactor were studied with respect to number and diversity of anaerobic protozoa in laboratory scale CSTAR. Several authors have applied statistical methods to express the relationships between the protozoa and the operational conditions of the aerobic reactors (Curds et al., 1968; Madoni, 1994b; Salvado et al., 1995), but it is hardly explained in anaerobic digestion process.

Table 3.1 illustrates the operating conditions of the reactor. A variety of protozoa were found to associate with microbial community of anaerobic reactors at different stages of operation (Table 3.2). As described in the previous chapter, growth succession of protozoa from the start-up of the reactor was progressed from amoebae and flagellates to small ciliates, and then to high diversity of ciliates. A sudden change in protozoa community was observed with the fluctuations of reactor conditions (Table 3.2).

Table 3.2. Predominant protozoa groups at different stages of CSTAR operation

Conditions	Predominant groups
Starting	Flagellates and amoebae
Steady state	Free-swimming ciliates
Upset	Small flagellates, small amoeboids and cysts

The growth of protozoa related to physicochemical and operational parameters of the reactor was studied using correlation analysis. The observed seven parameters - organic loading rate, pH, COD removal, biomass reduction, turbidity of

effluent. VFA removal and methane production had high correlation with protozoa, mainly the ciliates.

3.3.1 Correlation between protozoa and organic loading rates

Table 3.3 summarizes the predominant groups of protozoa at different organic loading rates. The protozoan communities were varied in the CSTAR with different organic loading rates. At low organic loading rates, small free swimming ciliates like *Cyclidium* and flagellates were the dominating groups. The abundance and diversity of protozoa was low (ciliates 3.75 x $10^4 \pm 1$ ml^{-1}, flagellates 9 x $10^4 \pm 2$ ml^{-1} and amoeboids 0.5 x $10^4 \pm 0.25ml^{-1}$) in such condition. Maximum number of diverse protozoa population was observed at optimum loading rates in the anaerobic reactor (ciliates 12.5 x $10^4 \pm 3$ ml^{-1}, flagellates 3.5 x $10^4 \pm 1.5$ ml^{-1} and amoeboids 0.25 x $10^4 \pm 0.5ml^{-1}$). During this steady operation, the flagellates and amoeboids were flourished at early stages and followed by ciliates, on reaching steady state in VFA generation and utilization by biomethanation. Ciliates genera in the CSTAR were mainly comprised of *Prorodon, Loxophyllum, Colpidium, Brachonella, Colpoda, Plagiopyla, Vorticella, Cyclidium, Spathidium, Loxodes* and *Metopus*. While at high organic loading, abundance of protozoa was higher than optimum loading rates (ciliates 1.5 x $10^4 \pm 1$ ml^{-1}, flagellates 21.5 x $10^4 \pm 5$ ml^{-1}, amoeboids 8.5 x $10^4 \pm 0.5ml^{-1}$ and cysts 17.5 x $10^4 \pm 2.5ml^{-1}$). This abundance was due to the establishment of amoeboids, flagellates and cysts. The number of ciliates was reduced and most of them were transformed into cysts. Cyst formation allows them to survive unfavorable conditions.

Table 3.3. Predominant protozoa groups in function of organic loading in CSTAR

Conditions	Predominant groups
Low organic loading	Small ciliates and small flagellates
Optimum organic loading	High diversity of organism, dominated by ciliates
High organic loading	Dominated by amoeboids, flagellates and cysts
	Few small ciliates like *Cyclidium*.

* g COD. g^{-1} VSS was optimized on the basis of Monad's equation

A similar response of aerobic protozoa communities have been reported in activated sludge plants with respect organic loading (Richard, 1991). However, there was no resemblance in the kind protozoa and they are different from anaerobic system.

Protozoa were observed to be very sensitive organisms and were rapidly changed their communities with shock loads. On applying shock loads (high organic load) to the steady state anaerobic reactor operation with abundance of ciliates and high process efficiency, sudden change in the protozoa community structure were observed in the anaerobic reactor. Flagellates and amoeboids were dominated at this stage, but came back to normal with ciliate abundance on restoring the loading rate (Fig. 3.1). Thus anaerobic protozoa seem to involve in the biological functions of anaerobic reactor (ecosystem balance), and any imbalance can be indicated with their community structure.

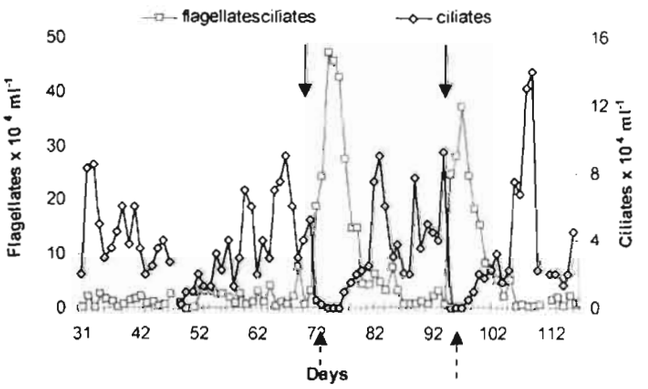


Fig. 3.1. Changes in protozoa population with shock loads (arrows indicate increase of flagellates and decrease of ciliates on shock load. They re-establish after the shock)

3.3.2 Correlation between protozoa and reactor pH

Anaerobic reactor performance is highly pH dependant (Rajeshwari et al., 2000). The optimum pH range for methane producing bacteria is 6.8 to 7.2 while for acid forming bacteria, acidic pH is desirable (Mudrak and kunst, 1986). Neutral pH

(6.8 to 7.2) was the essential condition for maximum growth of ciliates in the CSTAR (Fig. 2). A decline of ciliate count from $4.5x10^4$ to $2.5x10^3 \pm 0.5$ ml^{-1} was observed in the CSTAR with the decrease of pH from 7.0 to 6.5 (Fig.3. 2).

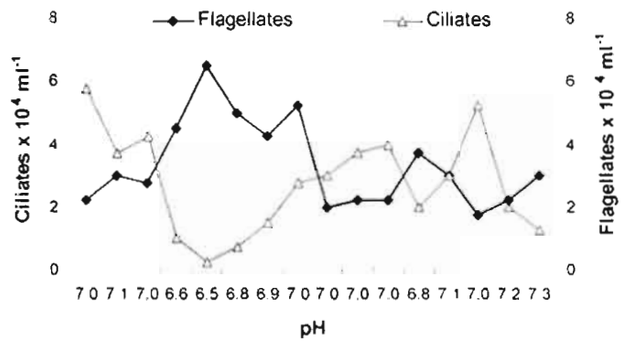


Fig. 3.2. Effect of pH on protozoa in CSTAR operated with loading rate of 1g COD l^{-1} d^{-1} at 10 days HRT

Compared to ciliates, flagellates were less affected in the anaerobic reactors by the acidic condition particularly during the early period of operations where VFA concentration was higher (> 8 meq l^{-1}) and the pH was below 6.8. Flagellates reached a maximum number of $1.5x10^6 \pm 0.56$ ml^{-1} in the anaerobic reactor at early days of reactor operation (Fig. 3.3). On reaching the anaerobic process stable with pH around neutral the density of flagellates declined to the range of $2.0x10^4$ to $6.5x10^4 \pm 0.4$ ml^{-1} and maintained a steady state thereafter in CSTAR. The growth of ciliates was predominant in the reactors from steady state. The flagellated protozoa were the predominant eukaryotic population in the CSTAR under unfavourable conditions and showed a slow decline with increase (above 7.2) or decrease (below 6.8) in pH. Protozoa are suggested to stabilise the pH of the rumen, but their mechanism has not been demonstrated for the pH regulation (Jouany and Ushida, 1999).

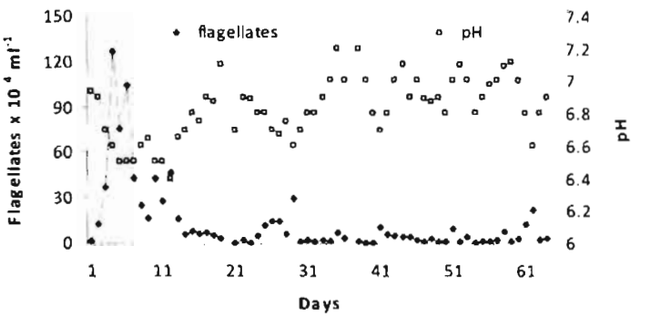


Fig. 3.3. Total flagellate count in CSTAR operated with loading rate of 1g COD l^{-1} d^{-1} at 5 days HRT

3.3.3 Correlation between protozoa and reactor VFA

Flagellates were flourished during the early period of operations and change in loading rates, where VFA concentration was > 8 meq l^{-1} (Fig. 3.4). Amoeba were also survived at high VFA whereas ciliate number was reduced to less than 0.5 x10^4 ml^{-1}. The abundance of flagellates at this stage could be due to their energy advantage from the direct utilization of dissolved substrate like VFA. The direct uptake of dissolved substrate through osmotrophic nutrition has previously been reported in some of the soil flagellates (Ekelund and Ronn 1994).

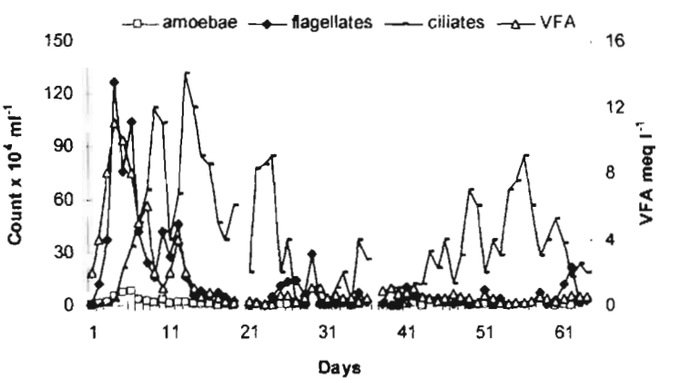


Fig. 3.4. VFA vs protozoa population in CSTAR operated with loading rate of 1g COD l^{-1} d^{-1} at 5 days HRT

During the anaerobic treatment process in CSTAR, protozoa changed its population structure along with changing physical conditions. The most affected group was ciliates and resulted in a sudden change in ciliate population with change in conditions. Flagellates and amoeboids were dominated at unsteady conditions such as change in pH, loading rate and high VFA concentration. The results indicate that protozoa can be considered as good indicators of anaerobic system performance. Similarly, changes in the community structure of protozoa have been reported by the changes in pH and temperature in the aerobic treatment systems (Fried et al., 2003).

3.3.4 Correlation between protozoa and COD removal

At steady state conditions, 70 – 75% of the identified protozoa in the anaerobic sludge were ciliates and they were directly related to the performance of reactor. The removal of COD was higher in CSTAR possessing abundant ciliates (Fig. 3.5) at the studied loading rates and retention time. The COD removal of more than 75 % (SD $\pm$ 2) was observed in the reactors having the highest number of ciliates (in the range of 2.5 to $9.25 \times 10^4 \pm 0.3$ ml^{-1}). While in unsteady states, during reactor start up and change in loading rates the number of ciliates was less than 2.5×10^3 ml^{-1}, and had less COD removal (less than 62 %). In brief, the presence of protozoan community had significant influence on removal of COD.

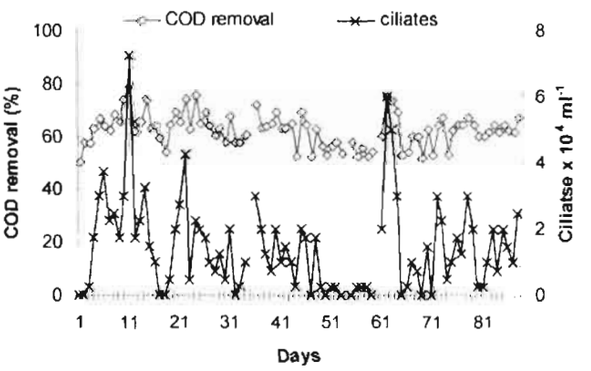


Fig.3.5. COD removal and ciliate density in CSTAR with loading rate of 1g COD l^{-1} d^{-1} at 5 days HRT

The correlation coefficients between COD removal and abundance of ciliates were significantly high (R^2 >0.95) in the studied conditions of hydraulic retention time and feed loading (Fig. 3.5). Stimulation of terminal activity by the presence of protozoa has been reported in natural aerobic systems (Fenchel and Harison, 1976) and in aerobic treatment processes (Curds et al., 1968), but it is unknown earlier in anaerobic systems.

3.3.5 Correlation between protozoa and methane production

The maximum rate of methane production was obtained in the range of 35 to 55 ml day^{-1} with loading rate of 1g COD l^{-1} d^{-1} at 5 days HRT in the anaerobic reactor when the ciliates count was noted high, in the range of 6.25×10^4 to 13.5×10^4 $\pm$ 1 ml^{-1} (Fig. 3.6). The stimulation of methane production with higher counts of ciliates in anaerobic systems also indicating the possible involvement of protozoa in the anaerobic degradation. The production of methane through endosymbiotic methanogens has been reported in anaerobic ciliates (Fenchel and Finlay, 1992) Biagini et al., (1998a) also have demonstrated high rate methane production in the presence of ciliate *Metopus palaeformis* in anaerobic culture.

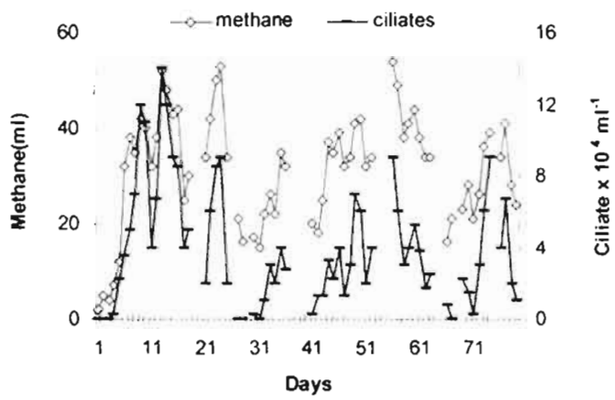


Fig. 3.6. Correlation between methane production and abundance of ciliates in CSTAR operated with loading rate of 1g COD l^{-1} d^{-1} at 5 days HRT

3.3.6 Correlation between protozoa and MLSS

Along with stimulated COD removal, substantial reduction of MLSS was observed with abundance of ciliates in the anaerobic CSTAR. A negative correlation was obtained in correlation analysis between MLSS concentration and ciliate density. More than 25 % (± 5) reduction of MLSS was obtained with $5x10^4$ ml^{-1} ciliates compared to ciliate count less than 2.5 x 10^3 ml^{-1} (Fig. 3.7).

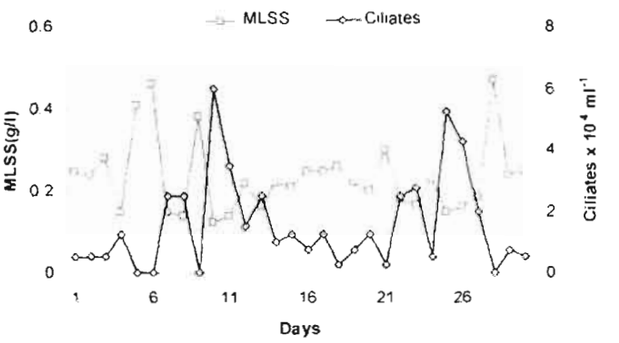


Fig. 3.7. Correlation between MLSS and abundance of ciliates in CSTAR operated with loading rate of 1g COD l^{-1} d^{-1} at 5 days HRT

Importantly, higher growth of ciliates in the anaerobic reactors had increased COD removal and at same time the reduction of MLSS was also found significantly higher. This is a contradiction to the general view on the growth of protozoa, the grazers of bacteria which are considered responsible for biodegradation. It means protozoa may be playing some role in the anaerobic degradation through consumption of biomass for the generation of methane. Protozoa grazing have previously been reported to reduce bacterial biomass in aerobic reactors (Ratsak et al., 1994) and that controls abundance of bacteria in fresh water and marine environments (Sherr and Sherr, 1987).

3.3.7 Correlation between protozoa and effluent turbidity

Fig. 3.8 illustrates the reactor which delivered high quality effluents with the presence of a wide variety of ciliated protozoa in large numbers and reactor which

delivered low quality effluents in the absence of ciliates. In steady state, the reactor had a maximum ciliates of 12 x10^4 ± 1 ml^{-1} and effluent turbidity reached below 0.5 NTU. On decreasing ciliates number to 0.25 x10^4 ml^{-1}, the effluent turbidity was increased to 10 NTU. This shows the effluent turbidity of the reactor decreased with increase of ciliate count. It could be due to the consumption of free bacteria by ciliates and thereby favours reduction of effluent turbidity in reactors. In aerobic treatment plants, ciliated protozoa produce good quality effluents because of their ability to feed on bacteria and suspended particles (Curds et al., 1968; Kuiper, 1973; Wheale and Williamson, 1980).

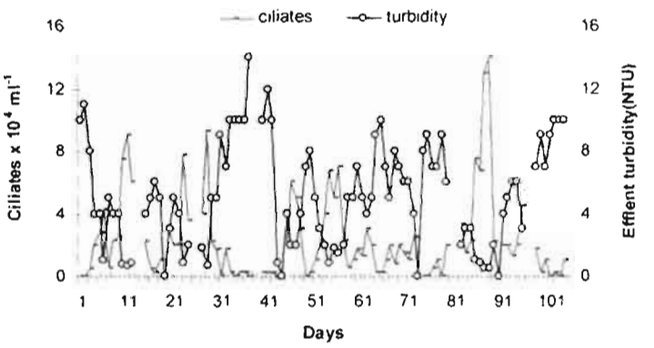


Fig. 3.8. Correlation between effluent turbidity and abundance of ciliates in CSTAR operated with loading rate of 1g COD l^{-1} d^{-1} at 5 days HRT

The present study on protozoa with respect to anaerobic reactor performance tries to explain their presence and significance beyond bacterial grazing.

3.4 Conclusions

The information on eukaryotes is scanty to describe their role in anaerobic degradation. Results obtained from the correlation studies indicate that physicochemical and operational parameters have significant influence on the key groups of protozoa which can be summarized as follows:

- Growth of each protozoa group is associated with different reactor condition and they can be used as performance indicators of the anaerobic process.
- Around 75 % of the anaerobic protozoa in anaerobic reactor belongs t ciliates at steady state, and they are related to the good performance o anaerobic process.
- Abundance of protozoa groups like flagellates and amoebae in the anaerobi system indicates that the process is less efficient.
- The absence of protozoa in general and ciliates in particular can b considered as limiting factor on the performance of anaerobic reactor.
- Protozoa are considered to be an important microbial group taking part to th ecosystem balance in anaerobic digestion process.

Protozoa have been previously been shown to enhance degradation in aerobi system, but this is the first report in anaerobic reactors (Priya et al., 2007a). Thus monitoring of protozoa, mainly ciliates in the anaerobic wastewater treatment systems provides valuable information on their performance.

Chapter 4
Isolation and culturing of anaerobic protozoa

Abstract

Centrifugation and micromanipulation method followed in this study was effective to isolate anaerobic protozoa from anaerobic sludge. The effects of various physico-chemical and nutritional parameters on growth of anaerobic protozoa were studied in batch cultures. The growth rate of anaerobic protozoa increased with temperature from 20-30°C and remained constant in the range between 30-32°C. In batch experiments, very low and high pH adversely affected the growth of protozoa. The optimum pH was found to be in the range of pH 6.8 to 7.2. An increase in VFA above 10 meq l^{-1} had adverse effect on ciliates and above 25 meq l^{-1} affected flagellate growth. Growth of anaerobic protozoa was adversely affected at O_2 tension exceeding 1 % atm. sat. and showed chemosensory behavior in response to O_2 tension. Superoxide dismutase activity was detected in the survived cells under O_2 tension. Catalase could not be found in the cells.

The appearance of different species varied with the different carbon sources. Among the different media tried, suspended carbon source was suitable for growth of anaerobic ciliates and dissolved substrate was best for the flagellate growth. The growth of protozoa required a minimum bacterial density of 3 x 10^4 cfu ml^{-1}. The feeding of bacteria as food source revealed that grazing of protozoa is dependent on size of bacteria (prey) and morphology of protozoa (predator). Maximum grazing of bacteria was obtained on bacterial size in the range of 2-4 µm. The large protozoa species like *Vorticella* and *Metopus* had wide range (2-6 µm) for bacterial grazing.

The optimum concentration of nutrients - ammonia-N, phosphate-P and sulphide-S for the growth of protozoa was found to be in the range of 10-12, 6-8 and 1-2 mg l^{-1} respectively. Deficiency of these nutrients was found to reduce the growth of protozoa in batch experiments and over dosage was harmful to the organisms.

Cytotoxic effect of the heavy metals Cd, Zn, Pd, Cr and Cu were studied on protozoa isolated from wastewater treatment plant. The order of toxicity of heavy metals on protozoa was observed as Cd > Cu > Pd > Cr > Zn. Epifluroscence

microscopy studies revealed the bioaccumulation of heavy metals inside the protozoa which were not seen in controls. Linear regression analysis showed that the median lethal concentration (LD_{50}) for protozoa was different for different heavy metals.

4.1 Introduction

Anaerobic protozoa are widely distributed in anoxic environments (Fenchel and Finlay, 1995). However, little is known about their trophic role in anaerobic food webs except some reports on their bacterial grazing activity and symbiotic association with anaerobic bacteria (Fenchel and Finlay, 1991a; Biagini et al., 1998a). The isolation and culturing of anaerobic protozoa are found as the major limitation for assessing their roles in anaerobic process. The studies on isolation and culturing have been restricted to a few species (Fenchel and Finlay, 1990b; Fenchel and Finlay, 1991b; Smirnov and Fenchel, 1996; Yamada et al., 1997; Biagini et al., 1998a,b). In this context, the isolation and culturing of protozoa is very important to interpret the role of protozoa in anaerobic wastewater treatment. The aim of this study was the isolation and culturing of anaerobic protozoa with negligible number of bacteria and optimization of various physico-chemical conditions and nutritional requirements for the growth of protozoa in anaerobic systems.

4.2 Materials and methods

4.2.1 Preparation of anaerobic protozoa culture

Anaerobic ciliates and flagellates were isolated from laboratory scale anaerobic Buoyant Filter Bioreactor - BFBR (Ajit et al., 2005) fed on dairy wastewater. The samples with higher number of organisms were taken and 10 ml of sample centrifuged at 150 g for one minute. The pellets containing ciliates were suspended in anoxic phosphate buffer (pH 6.9, prepared with sodium phosphate, dibasic and monobasic). This suspension was placed as droplets on a glass slide and at least 100 individual ciliate and flagellate cells were carefully picked up by using micro capillaries under microscope (Nikon –ALPH 4 YS2) at 100 X magnification. Picked cells were resuspended in anoxic ciliate mineral medium (g.l^{-1} 0.125 K_2HPO_4, 0.025 NH_4Cl, 0.4 NaCl, 0.2 $MgCl_2$. 6 H_2O, 0.15 KCl and 0.25 $CaCl_2$. $2H_2O$ buffered

to pH 7) and the centrifugation process was repeated to reduce bacterial number. After washing process about 25-35 cells of different species were remained, as confirmed by direct observation of cells through microscope. Isolated cells were transferred to fresh medium containing ciliate mineral medium and sodium oleate as COD source. All protozoa cultures were incubated in 50 ml amber Schott Duran bottles screw capped with silicon septa (Thomson Scientific USA) and head space flushed with nitrogen. The pH of the cultures was adjusted to the range of 6.8 – 7.2 with 0.1M sterile HCl and NaOH prepared in N_2 stripped distilled water. Cysteine HCl (1mM) was used to achieve complete anaerobiosis. Resazurin (2mM) was used as redox indicator. Bacterial growth was suppressed by the addition of 1 ml antibiotic solution (8000U Penicillin G Sodium and 200U Streptomycin Sulphate (Sarabai chemicals, India) per ml) to the bottle. Cultures were incubated at 30°C ± 2 in triplicates and the mean of results (number of organism) was taken.

After inoculation, the cultures were tested for bacterial contamination. Samples were stained with 4'-6-Diamidino-2-phenylindole (DAPI) and counted bacterial number under epifluorescence microscope (Leica DM 2500). Bacterial numbers were significantly reduced to less than 0.1% from an initial count of 2×10^2 cfu ml^{-1}. The tests were repeated at regular intervals to determine whether bacterial growth was affecting the protozoa test cultures.

4.2.2 Studies on physico-chemical parameters

The cultures with maximum growth of protozoa were subjected to study the effects of various physical and chemical parameters on growth of anaerobic protozoa. The temperature tests were performed by placing the experimental bottles at different incubation temperatures ranging from 20°C to 50°C. The pH of the cultures was adjusted to the range of 3-10 with 0.1M sterile HCl and NaOH prepared in N_2 stripped distilled water. Influence of VFA on the protozoa was studied by adding VFAs -acetic, butyric and propionic acids – at concentrations 0.001, 0.005, 0.01,0.05, 0.1 and 1M.

In order to study the behavioural and adaptive mechanism of cells in reponse to oxygen, the desired amount of oxygen was injected to the culture bottles with a hypodermal syringe. A range of O_2 concentration 0.1- 10% atm. sat. was tested. At

appropriate intervals the bottles were shaken and collected samples with a syringe for counting the cells. Survival of protozoa and its morphological changes with respect to O_2 tension was monitored under a microscope.

Behavioural responses to oxygen: Dense suspensions of protozoa cells in nitrogen sparged **phosphate** buffer (pH 7.2) were dropped on to a slide and the behaviour of the cells was observed during exposure to air.

4.2.3 Studies on nutritional requirements

Carbon compounds: Ciliate mineral medium (CMV) (concentration g l^{-1} 0.125 K_2HPO_4, 0.025 NH_4Cl, 0.4 NaCl, 0.2 $MgCl_2.6H_2O$, 0.15 KCl and 0.25 $CaCl_2.2H_2O$) was supplemented with different carbon substrates such as wheat powder, dextrose, sucrose, starch, peptone, cellulose, oleic acid and sodium acetate at 1% concentration to study the growth of protozoa on these carbon sources. All the media were prepared in 50 ml amber Schott Duran bottles and headspace was filled with N_2. The bottles were screw capped with silicon septa and autoclaved for 15 min at 121°C. Wheat powder medium was autoclaved with one wheat grain (Dehority, 1998).

Isolated bacteria from the anaerobic reactor sludge was supplied as food source. Isolation and culturing of anaerobic bacteria was done as per Laboratory manual - Microbiological aspects of anaerobic digestion (1988). Thioglycollate agar (g.l^{-1} - 15.0 Peptone, 5.0 Yeast Extract, 5.5 D- Glucose, 0.5 L- Cysteine, 2.5 NaCl, 0.5 Sodium Thioglycollate, 0.001 Resazurin and 2.0 Agar with a final pH of 7.0) was used for the isolation of anaerobic bacteria. Samples from the reactor were inoculated on agar plates with minimum oxygen exposure. The inoculated plates were incubated at 37°C in anaerobic jar (Oxoid) filled with nitrogen and examined for appearance of bacterial colonies. The colonies were examined on a colony counting microscope after 3-5 days of incubation. Individual colonies were isolated from the agar plates.

Isolated organisms were incubated anaerobically in acetate and mineral medium in 50 ml Schott Duran amber bottles screw capped with silicon septa. A known amount of bacterial cells was inoculated to protozoa culture as food source.

Bacterial cells were directly observed using compound microscope, Leica DM 2500 and the cell size of the inoculam was measured with the software Leica Qwin V3. Samples were stained with 4'-6-Diamidino-2-phenylindole (DAPI) and counted bacterial number for studying clearance rates.

Macro nutrients: Optimum nutrients concentration required for protozoa growth were studied in serum vials having 10 ml active volume. The test was carried out by using various concentrations of ammonia-N, phosphate-P and sulphide-S ranging from 0.25 mg to 16 mg l^{-1}, with six replicates for each. Growth of protozoa was monitored by sacrificing samples at every 12 hours and enumerated the cell number.

The acute toxicity test was carried out by adding 1ml of nutrient test solution of different concentrations to the serum vials containing known number of protozoa cells. The cells with test solution were incubated at 32°C. The nutrient concentrations tested were 10, 50, 100, 200, 300, 400 and 500 mg l^{-1} for ammonia - N, 5, 50, 100, 200, 300 and 400 mg l^{-1} for phosphate-P, and 1, 10, 20, 30, 40, 50, 60 and 70 mg l^{-1} for sulphide-S. The number of survived cells was observed at selected intervals under a microscope. The median lethal concentrations (LC_{50}) were calculated from these data.

4.2.4 Effects of toxicants (heavy metals)

To study the influence of toxicants on the protozoa, the cells were exposed to different concentrations of metals such as Cd, Zn, Pd, Cr and Cu in serum vials with active volume of 10 ml. The cells with test solution were incubated anaerobically at 32°C. The physiological response of protozoa was assessed in terms of mortality and growth after exposure to toxicants. The number of survived cells was examined at selected intervals under a microscope. The median lethal concentrations (LC_{50}) were calculated from these data. The maximum resistance dose of anaerobic protozoa was extrapolated by analyzing statistically significant differences among the concentrations studied.

Bioaccumulation of Zn in the protozoa was detected by using a specific fluorophores- TSQ (*N*-(6-methoxy-8-quinolyl)-*p*-toluenesulfonamide). The protozoa cultures exposed for 24 h at sublethal concentrations of Zinc were centrifuged at 3000

rpm for 5 min. Then cells were resuspended in Tris -HCl buffer 0.01 M (pH 6.8) and fixed with one drop of pure paraformaldehyde. After fixation, cells were washed in the same buffer (Tris–HCl) twice, and then ciliates were exposed to 10 μg.ml^{-1} of TSQ for 10 min (Ana et al., 2006). Observations were made under a Leica DM 2500 fluorescence microscope.

4.2.5 Enzyme activities

For the demonstration of enzyme activities, the protozoa were concentrated by centrifugation (2min at 150 x g) and washed twice in anaerobic phosphate buffer (pH 7.0). Catalase activity was detected by the transfer of a dense cell suspension to a drop of 3% aqueous H_2O_2. Superoxide dismutase activity (SOD) was determined by illumination of protozoa in a reaction mixture containing riboflavin and nitoblue tetrazolium salt according to Goosen et al., 1990a. After confirming the presence of SOD, the protozoa cell free extract was assayed for SOD activity. Protozoa cell free extracts in 100 mM phosphate buffer were prepared by disruption of organisms in three cycles of freezing and thawing followed by centrifugation at 150 x g for 15 min. The supernatant alone was taken and assayed immediately. SOD activity was measured by following the method of McCord and Fridovich (1969).

4.2.6 Chemical Analysis

Ammonia: Expandable ion analyzer EA 940 (Orion) with ion selective electrode was used to measure ammonia.

Phosphate: The phosphate was determined according to ascorbic acid method described in Standard methods (APHA, 1998). The principle of this method is ammonium molybdate and potassium antimonyltartarate react in acid medium with phosphate to form phosphomolybdic acid – that is reduced to intensely coloured molybdenum blue by ascorbic acid. The intensity of the created colour was detected using a spectrophotometer at a wavelength of 880 nm. Calibration curves prepared with a series of phosphate standard. Distilled water was used a blank with reagent to make photometric readings for calibration curve. Sample concentration was calculated from this calibration graph.

Sulphide: The sulphide was estimated on the principle of reaction between sulphide, iodine and thiosulphate. 10 ml of the sample solution was reacted with standard iodine solution in excess. The sulphide was estimated by titrating the unreacted iodine with standard thiosulphate solution.

Reducing sugar: The dinitrosalicylic acid (DNS) method was used to measure liberated sugar during enzymatic hydrolysis. Total reducing sugars were determined as maltose equivalents by a colorimetric method (Summers, 1924). The reagent was prepared by dissolving 10 g of DNS ($3,5\text{-}(NO_2)_2C_6H_{12}\text{-}2OH\text{-}COONaH_2$) in the 2 M sodium hydroxide solution mixed with 1 M solution of sodium–potassium tartrate ($KNaC_4H_4O_6{\cdot}H_2O$). The resultant liquid yielded a clear orange colour, which was used to measure total reducing sugars. The intensity of the created colour was detected using a spectrophotometer at a wavelength of 540 nm.

4.2.7 Microscopic observations

Protozoan number was determined by direct counting on a Neubaur counting slide. Triplicates were prepared for each sample. Generation time and growth rate (h) were estimated according to the exponential growth equation (Creager et al., 1990). Generation time = transfer interval/ [(log final concentration - log initial concentration)/log 2].

4.3 Results and discussion

Separation by centrifugation followed by picking up of the protozoa by micromanipulation followed in this study was effective to isolate protozoa from anaerobic sludge with negligible number of bacteria. The cultures with highest number of protozoa were further selected for studying the effect of various physico-chemical parameters on growth of protozoa.

4.3.1 Factors influencing protozoa growth

Physical parameters

Effect of temperature on protozoa: During the experiment, population sizes and temperature were monitored at selected intervals. The results show significant influence of temperature on the growth of anaerobic protozoa. Fig. 4.1 shows the exponential regression analysis made with the data for the range from 20-40°C. The density of protozoa was increased with increasing temperature from 20 to 30°C and remained almost constant in the range of 30-32°C. The measured growth rate of protozoa was 0.02 h^{-1} at 20°C, 0.05 h^{-1} at 25°C and 0.06 h^{-1} at 28°C. The calculated growth rate during exponential period at 30-32°C was 0.09 h^{-1} for ciliates and 0.13 for flagellate. The growth was moderate at 35°C with the growth rate of 0.06 h^{-1}. The temperature in the range of 30-32°C was the optimum for growth of protozoa and did not seems to increase at higher temperatures (Fig. 4.1). The growth of anaerobic protozoa was adversely affected by higher temperatures and observed a sharp decline with the increase of temperature above 32°C.

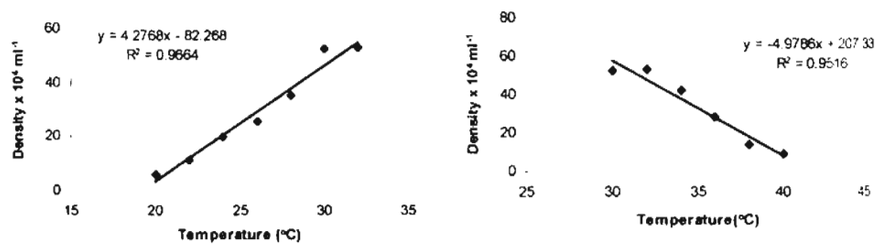


Fig. 4.1. Growth of anaerobic protozoa vs temperature (points in the graph is means of triplicates)

Higher (>35°C) and lower (<20°C) temperatures had adverse effect on the organism (Fig. 4.2) and, lead to cyst formation. Cell lysis was observed with the temperatures above 40°C. However, the cyst could survive at high (>35°C) and low (<20°C) temperatures.

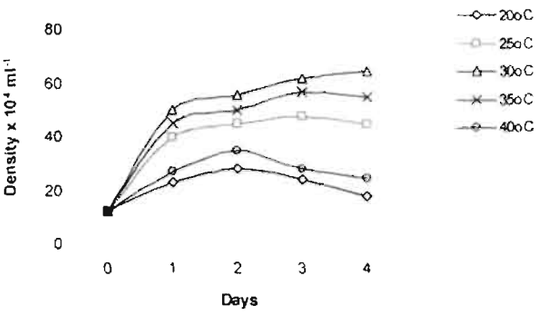


Fig. 4.2. The growth of anaerobic protozoa at different temperature conditions

The temperature range for many protozoa has been studied in detail in aerobic systems (Montagnes, 1996; Ronn, 1994; Weisse and Montagnes, 1998). Masana et al., (1998) have reported increase in the growth rate of ciliate *Plagiopyla* with temperature from 8-18°C and remained constant in the range of 18-24°C. Generally anaerobic digestion and bacterial activity is strongly influenced by temperature for mesophilic organisms, the degradation activity decreases by one half for each 10°C drop below 35°C (Rajeshwari et al., 2000). Similar decrease was also observed in the anaerobic protozoa growth with respect to temperature drop.

Effect of pH on anaerobic protozoa: A sudden change in protozoa count was noticed in anaerobic cultures with variation in pH and VFA concentrations. There was a positive correlation obtained in batch cultures between number of organisms and increase in pH from 6 to 7.2 (Fig. 4.3). During active fermentation in poorly buffered cultures, a sudden drop in pH (pH 3 - 5) was observed with disappearance of active cells mainly, the ciliates. Organic acid (VFA) concentration was measured high during the drop in pH. Wheat, starch and dextrose media had a sudden drop in pH due to fermentation and the measured organic acid was concentration 12.5 meq l^{-1}. Results of experiments conducted further revealed that very low and high pH were adversely affected the growth of protozoa (Fig. 4.4).

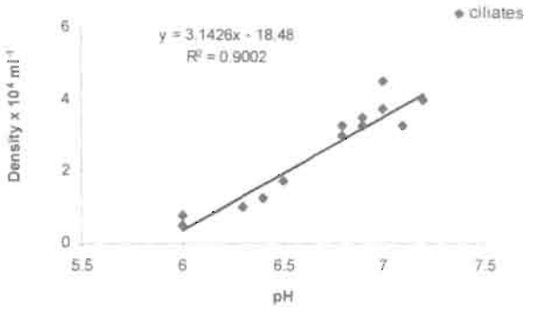


Fig. 4.3. Correlation between pH and count of ciliates in anaerobic cultures

Small amoeboids and cysts were dominant under pH conditions below 6 and above 7.8, and organisms were transformed to cysts at high and low pH. The growth rate of anaerobic protozoa was increased substantially with increasing pH from 6 to 7 (Fig. 4.3). The most favourable pH condition for the growth of protozoan was observed around neutral pH 6.8 to 7.2. pH 6.5 to 7.5 also supported the protozoa with reduced growth (Fig. 4.4). A few flagellates were found to survive at low (5.5) and high pH (7.5) environments showing they have better tolerance to pH conditions than ciliates. Ciliates were the most affected group and could be considered as pH indicators of anaerobic system. Very little information is available on the pH tolerance of anaerobic protozoa. Biagini et al., (1998a) have reported the growth of protozoa on neutral pH. Generally protozoan communities are dynamic and undergo rapid changes like cell division, encyctment and excystment in response to the environmental fluctuations (Patterson, 1995).

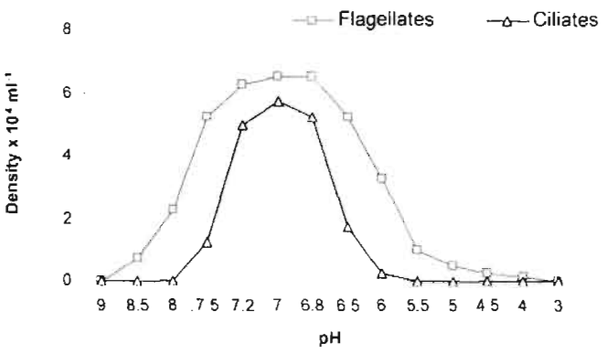


Fig. 4.4. Effect of pH on ciliates and flagellates, in anaerobic batch cultures (the values are the mean of triplicates at exponential stage)

Effect of oxygen on anaerobic protozoa: Fig 4.5a-d shows the morphological and behavioral changes of protozoa in response to oxygen. Exposure to oxygen was found to be very harmful to the cells. Cells prepared as wet mounts or in drops of medium exposed to air, larger species like *Metopus* lost their characteristic shape and rounded up (Fig. 4.5a), and finally lead to cell rupture and lysis (Fig. 4.5b). Small ciliates and flagellates were migrated away from the periphery to avoid air contact (Fig. 4.5c). Clumping of cells was also observed in microscopic preparations (Fig. 4.5d).

The clumping of anaerobic protozoa in response to oxygen tension perhaps allows them to seek out anaerobic micropatches. There are some reports on behavioural response of anaerobic to oxygen. *Hexamita* sp. shows a chemosensory response to atmospheric O_2 by clumping together (Biagini et al., 1997). Studies on the fresh water ciliate *Loxodus striatus* displays a chemosensory behavior that allows it to accumulate at the tolerate level of oxygen gradients (Finlay et al., 1986). Goosen et al., (1988, 1990a,b) have found that anaerobic ciliates *Plagiopyla nasuta* and *Trimyema compressum* could grow with O_2 tensions in the gas phase up to about 5% atm sat.

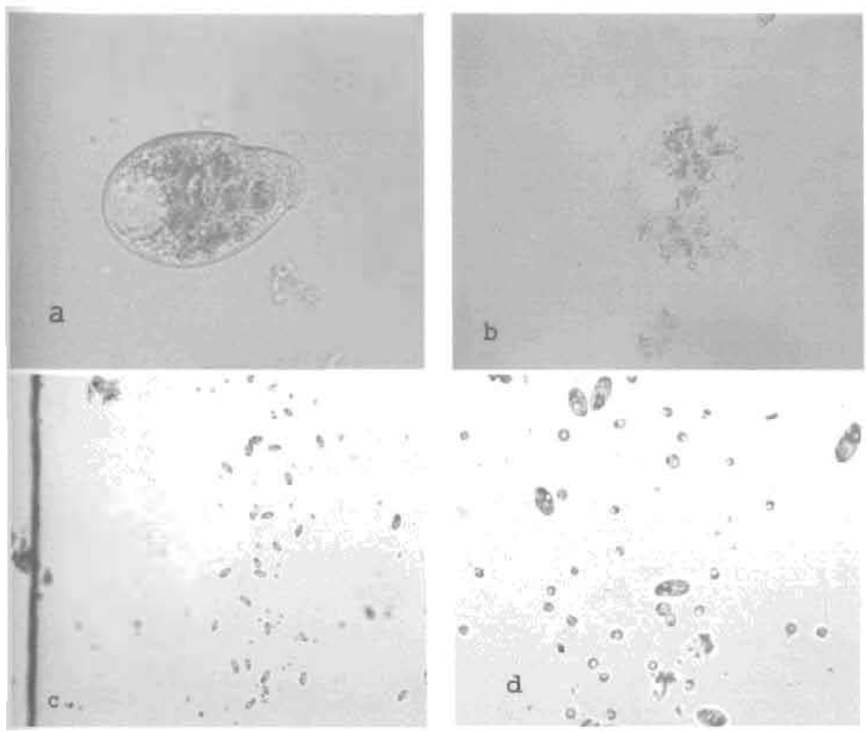


Fig. 4.5. Behavioural and morphological changes were noticed in protozoa on response to oxygen tension. (a) Loss of characteristic shape and vacuolation, (b) lead to cell rupture and lysing and (c) protozoa cells forming a band at a distance from the side of cover slip and (d) clumping of protozoa cells

Fig. 4.6 illustrates the growth of protozoa in response to oxygen tension ranging from 0.1-10 % atm. sat. The maximum ciliate survival was observed in 0.1% atm. sat. of oxygen and 61% of ciliates survived at 72h. Exposure to 0.5% atm. sat. of oxygen had survival of 33% at 72h. 10% atm. sat. was detrimental to ciliates and survival was only 11% at 72h. In the case of flagellates, survival was 71 % in 0.1% atm. sat. of oxygen at 72 h. However, survival rate at 0.5% and 10 % atm. sat. of oxygen was almost equal to ciliates and it was observed as 35% and 14%, respectively. The oxygen level above 1% was inhibitor to most of the anaerobic protozoa and resulted in vacuolation and cell lysing.

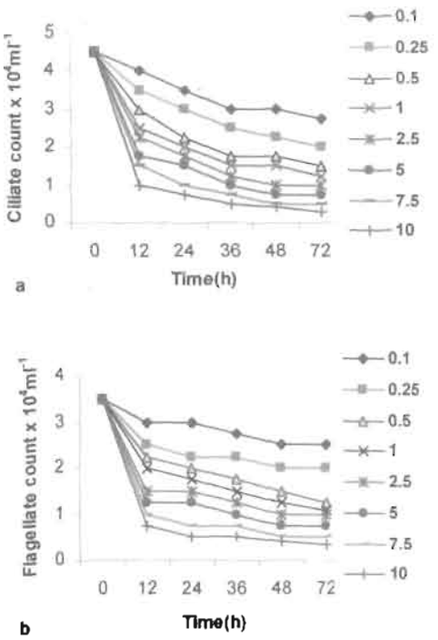


Fig. 4.6. Survival of ciliates (a) and flagellates (b) on response to oxygen tension (0.1-10 % atm. sat.). Initial count of ciliates was 4.5 x 10^4 ml^{-1} and flagellates was 3.5 x 10^4 ml^{-1}

Fig. 4.7 shows the percentage of survival of different genera of anaerobic protozoa in 0.1% atm. sat. of oxygen tension at 72h. Protozoa genera like *Amoeba, Rhyncomonas, Menoidium, Spathidium* and *Cyclidium* were shown to have less adverse effect due to oxygen exposure. While species like *Metopus, Plagiopyla, Brachonella, Trepomonas* and *Vanella* were the least survived. Survival was adversely affected with continuous oxygen tension. The ciliate *Cyclidium* and flagellate *Menoidium* were not affected by the oxygen exposure and their growth rate similar or slightly higher than the anaerobic conditions.

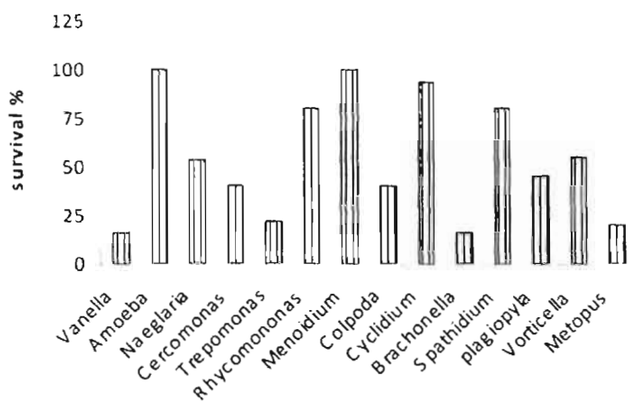


Fig. 4.7. The percentage of survival of different genera of anaerobic protozoa in 0.1% atm. sat. of oxygen tension at 72h

The enzymes responsible for the detoxification of oxygen such as catalase and superoxide dismutase were tested in protozoa cells survived in 0.1 % atm. sat. of oxygen. There was no catalase activity in the protozoa cultures which was confirmed by the absence of O_2 production on addition of 3% H_2O_2 ($v.v^{-1}$) solution. For the demonstration of SOD, cells were illuminated for 45 min in the reaction mixture containing riboflavin and nitoblue tetrazolium salt. In protozoa cells survived at 0.1% atm. sat. of oxygen, the cytoplasm, organelles and the globular body were colourless, indicating the presence of SOD (Fig. 4.8). In contrast, the same protozoa cells grown under strictly anaerobic conditions were found to be SOD negative. After confirming the presence of SOD, the protozoa cell extracts were assayed for SOD activity and was found to be 1.85 ± 0.9 $U.mg^{-1}$ protein.

Oxygen exposure damages the functions of cells in different ways. It includes destruction of specific enzymes such as hydrogenase and pyruvate ferrodoxin oxidoreductase. Oxygen toxicity arising out of oxygen radicals (eg - peroxide, superoxide and hydroxide) produced is managed by cellular detoxification mechanisms (Morris, 1979). The expression of SOD activity for the survival has been

reported in anaerobic flagellate *Hexamita* and in ciliate *Loxodes* (Biagini et al., 1997; Finlay et al., 1986).

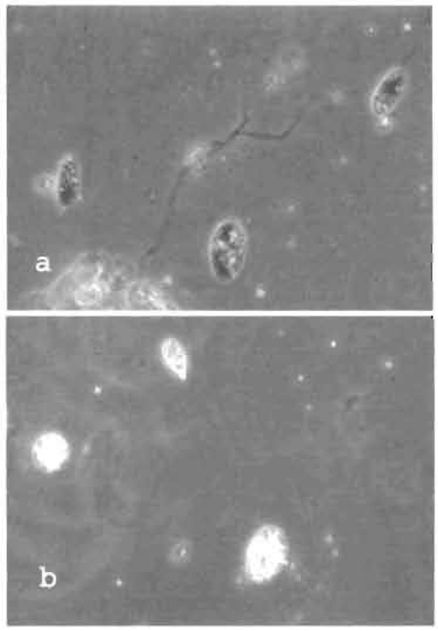


Fig. 4.8. SOD activity in anaerobic protozoa, *Cyclidium and Menoidium* (a) negative under strictly anaerobic condition and (b) Positive on expose to 0.1% oxygen

Chemical parameters

Effect of volatile fatty acids on anaerobic protozoa: The effects of VFA (acetic acid, butyric acid and propionic acid) on anaerobic protozoa were studied by supplying different concentrations to the cultures at exponential growth. The result of the experiments by introducing acetate is presented in Fig. 4.9. Ciliates and flagellates were totally absent in cultures having 1M acetic acid, but amoeboids and cysts present were in the cultures. Increased growth of protozoa was observed in this study where the VFA level was lower in the culture (<0.05M). The VFA concentrations greater than 0.05M caused growth inhibition of ciliates and ciliates were transformed to cysts (Fig. 4.9). Flagellates were found to be survive in 0.1M concentration.

Naziroglu et al., (2002) have reported the presence of ciliates at VFA level of 0.128M in rumen fluid.

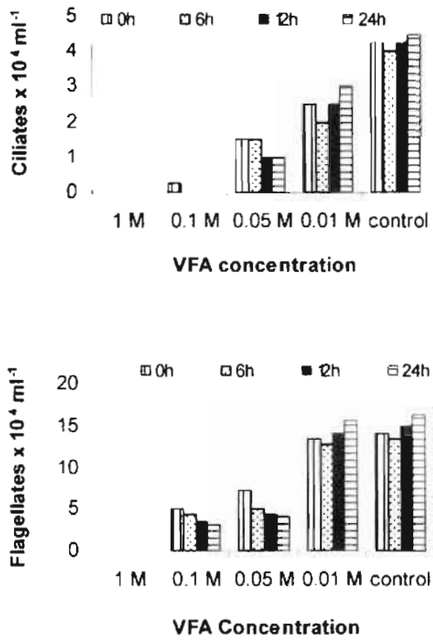


Fig. 4.9. Effect of VFA on ciliates and flagellates in anaerobic batch cultures (control VFA - 0.005 M)

In regression analysis, a negative correlation was noticed between VFA concentration and number of ciliate in anaerobic cultures (Fig. 4.10). Where as an increased growth of flagellates were observed with VFA concentration up to 20 meq l^{-1} in anaerobic cultures (Fig. 4.10). The survival and growth of flagellates at high VFA must be from energy advantage by direct consumption of dissolved material as discussed earlier. Nevertheless the flagellate growth was affected by VFA levels above 25 meq l^{-1}(equivalent to 0.025 M). Results of this experiments are confirming the nature of flagellate growth in anaerobic reactors where flagellate counts were high

with VFA levels were higher than 8 meq l^{-1}(Chapter 3). Therefore an increase in VFA above 10 meq l^{-1} had adverse effect on ciliates and flagellates were affected above 25 meq l^{-1}.

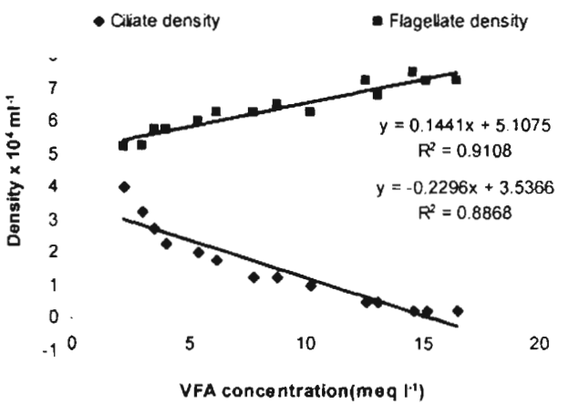


Fig. 4.10. Correlation between VFA concentration and protozoa population in anaerobic cultures

The growth of protozoa with different concentrations of volatile fatty acids (acetic acid, butyric acid and propionic acid) is illustrated in Table.4.1. The results show that higher levels of butyrate and propionate, like acetate have adverse effect on the growth of the anaerobic ciliate and can arrest their growth beyond the concentration of 0.05 M. In fact, the propionic and butyric acids had more adverse effect on protozoa than the acetic acid. The disappearance of ciliates and flagellates may be due to the higher concentration of protozoan metabolic end products. Volatile fatty acids are the major intermediates detected in the breakdown of organic material under anaerobic systems such as rumen, anaerobic digester and aquatic sediments (Finlay and Fenchel, 1995).

Table 4.1. Influence of short chain volatile fatty acids (acetic acid, butyric acid and propionic acid) on protozoa at different concentrations (count at 24 h)

Organisms	VFA used	Control 0.005 M	Count of protozoa at different concentrations VFA($x10^4$ ml^{-1}) 1 M	0.1 M	0.05 M	0.01 M	0.005 M
Ciliates	Acetate	4.25	0	0	1	2.5	4
	Butyrate	4.25	0	0	0	2	3.75
	Propionate	4.25	0	0	0	1.5	3.75
Flagellates	Acetate	4	0	1	4.25	12.5	3.5
	Butyrate	3.5	0	0.25	3	9.5	3.75
	Propionate	4.5	0	0	3.25	8.25	4.25
Amoebae	Acetate	1.5	0	0.5	1.5	7.5	1.25
	Butyrate	0.5	0	0.25	1	6.75	0.75
	Propionate	1	0	0.25	1	5.25	1.25

Nutritional parameters

In addition to a proper physical environment, growth of protozoa also depends on nutrients availability.

Effects of different carbon sources on protozoa growth: An attempt was also made to develop suitable media composition for isolation and culturing of protozoa which is important to conduct studies in understanding metabolic activities of protozoa and biodegradation process. Different feed compositions were tried for culturing anaerobic protozoa. Among these media, wheat powder and oleic acid suspension media supported the maximum growth of ciliates compared to starch, dextrose, peptone, sucrose, cellulose and acetate medium.

Similar to the protozoa growth in anaerobic reactors, a succession in their growth was observed in this study. Flagellates and amoeboids were the prominent groups at early days in cultures and followed by the free-swimming ciliates (Fig. 4.11). Flagellates are considered as lower forms of life compared to ciliates and they

have more opportunity to grow and multiply within short life span (generation time 6-18 h). This adaptability could have helped the flagellates to establish faster than the ciliates which require 20 - 62 h generation time. In the cultures, organisms were declined with utilization of substrate.

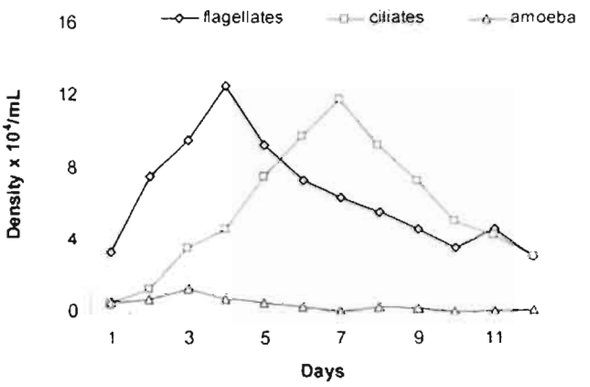


Fig. 4.11. Succession of protozoa growth in anaerobic cultures supplemented with wheat powder

Maximum growth of ciliates and flagellates was supported by wheat powder and oleic acid suspension (Fig. 4.12 and 4.13). Wheat medium supported the growth of ciliates (*Metopus, Cyclidium, Colpoda, Loxodes, Colpidium Plagiopyla, Vorticella and Prorodon)* and flagellates (*Rhynchomonas, Cercomonas, Menoidium, Naeglaria Trepomonas* and *Bodo)* (Fig. 4.14 and 4.15). Oleic acid was found to support the growth of ciliates *Spathidium* and *Loxophyllum* and flagellates, *Mastigella Tetramitus* and *Peranema* in addition to the above mentioned organisms (Fig. 4.14 and 4.15).

The cellulose medium supported the growth of flagellates, *Menoidium, Trepomonas, Rhyncomonas* and *Naeglaria* with maximum number of *Menoidium*. One week was needed to observe the exponential growth of *Menoidium* in the cellulose medium (Fig. 4.14 and 4.15). Presumably, as the cellulose medium did not contain any soluble carbon source, extracellular enzymatic degradation of cellulose to

soluble sugars by flagellates like *Menoidium* might be needed its uptake. Presence of glucose in the medium was 10-32 mg l^{-1} during the study. However, the growth of ciliates was poor in the cellulose medium compared to wheat and oleic acid suspension. Repeated subculturing by using micromanipulation method to fresh medium supported the pure culture of *Menoidium* in cellulose medium.

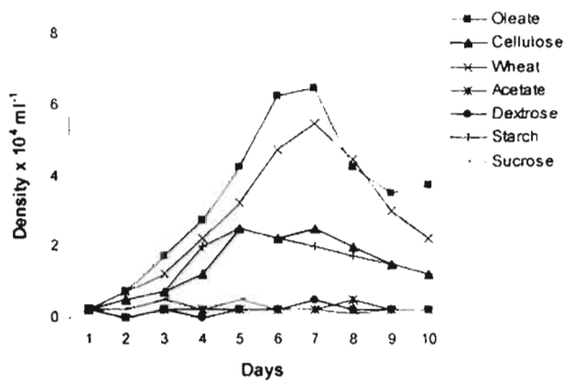


Fig. 4.12. Growth of ciliates in bacteria controlled anaerobic cultures fed on different carbon sources

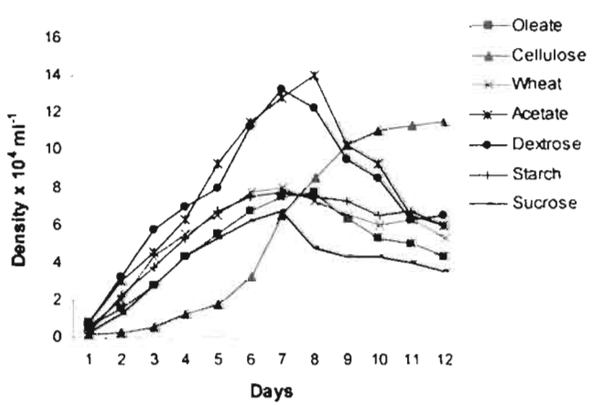


Fig. 4.13. Growth of flagellates in bacteria controlled anaerobic cultures fed on different carbon sources

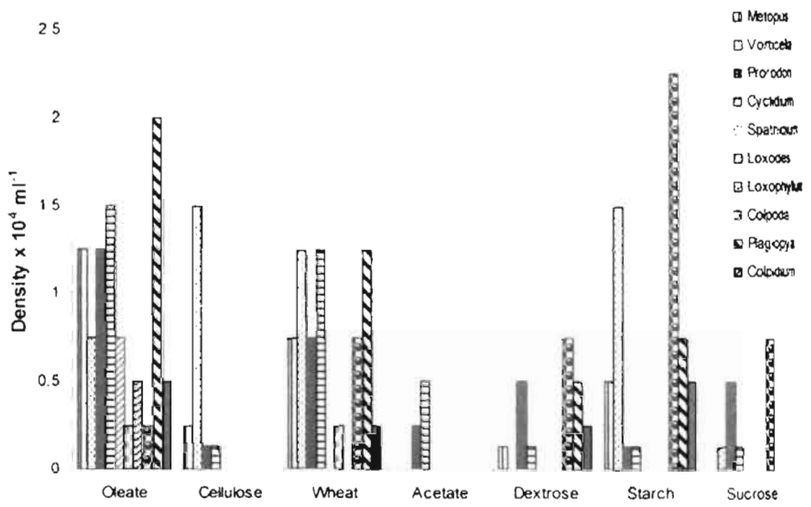


Fig. 4.14. Growth of anaerobic ciliates on different carbon sources

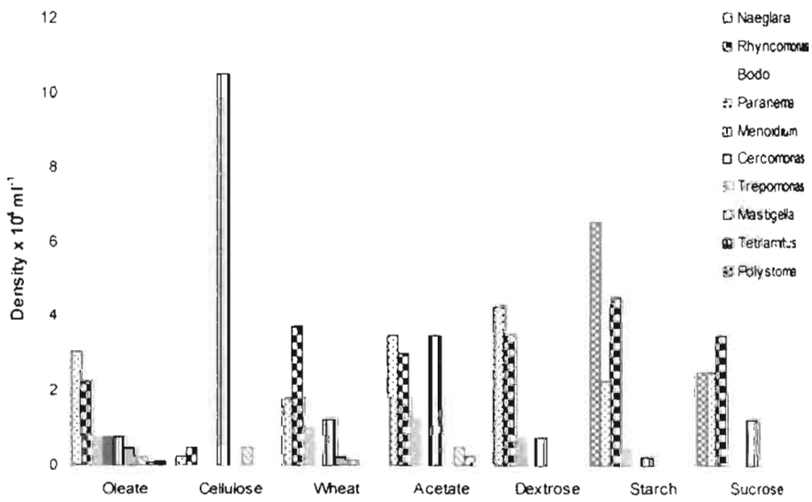
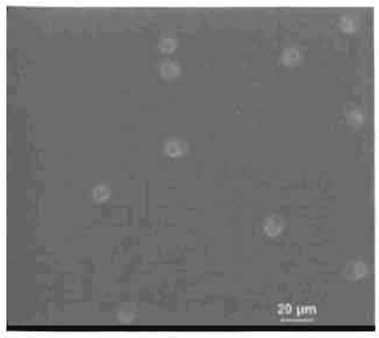


Fig. 4.15. Growth of anaerobic flagellates on different carbon sources

Anaerobic culture with dextrose supported the growth of ciliates mainly, *Metopus, Cyclidium, Prorodon, Colpidium* and *Colpoda* and, flagellates *Bodo, Naeglaria, Menoidium, Rhyncomonas* and *Polystoma* (Fig. 4.14 and 4.15). *Polystoma* was not prominent in seed culture, but it was appeared in dextrose, sucrose and starch media after 2 to 3 days (Fig. 4.16). The starch and sucrose media were supported the growth of flagellates and ciliates with reduced growth rate compared to other carbon sources. Batch culture with the dissolved substrate sodium acetate had no or little growth of ciliate, but flagellates - *Menoidium, Naeglaria, Rhyncomonas, Bodo, Trepomonas,* and *Mastigella* were observed. The diversity and abundance of ciliates were significantly lower in dissolved substrates, except the growth of *Cyclidium* (Fig. 4.14 and 4.15). In peptone medium flagellates and ciliates were absent, and only a few flagellates were appeared occasionally.



Polystoma
A motile coccoid flagellate with granular cytoplasm. Cell measured 10-16 µm. Two flagella with equal length.

Fig. 4.16. Polystoma appeared in sucrose, dextrose and starch media

Growth of the amoeboids - *Mayorella, Saccamoeba, Vanella* and *Mastigamoeba* were observed in all cultures. Most common genera of ciliates in all cultures were *Prorodon, Plagiopyla, Colpidium, Colpoda, Vorticella, Cyclidium, Spathidium, Loxodes* and *Metopus,* and the flagellates were *Rhynchomonas, Cercomonas, Menoidium, Naeglaria, Trepomonas* and *Bodo*. Diversity of ciliates was maximum in undissolved medium especially in oleic acid suspension than other carbon sources (Fig. 4.14 and 4.15). Protozoa population differ in response to the

food source that each can ingest. It is noted that suspended substrate media had high diversity and abundance of organisms than dissolved media.

Effects of bacteria cells (as food source) on protozoa growth: Bacterial cultures of cocci, rod and a mixture of cocci and rod were supplemented to the protozoa culture for studying the size selective grazing of protozoa and growth of protozoa (Table 4.2). Different bacterial cultures (without any other carbon sources) were found to support growth of protozoa.

Table 4.2. Bacteria isolated from the anaerobic sludge as food source for protozoa

Sl .No	Shape	Size(μm)
1	coccus	2-3
2	rod	3-4
3	coccus	1-2
4	coccus	1-3
5	rod	2-4
6	rod	2-5
7	Coci(beaded)	4-10
8	cocci and rods	All above sizes

Size of bacteria was an important factor that could affect growth of different protozoa genera. Growth profile of protozoa was depending on the size and shape of bacteria given as food source. On feeding varied sizes of bacteria cells, the best growth of anaerobic protozoa was in the medium with cell sizes of 2-4 μm compared to <2μm and >4μm (Fig. 4.17). Both smallest and largest size particles showed reduced growth of anaerobic protozoa. The diversity and abundance of protozoa was also depends on size of bacteria given as food source.

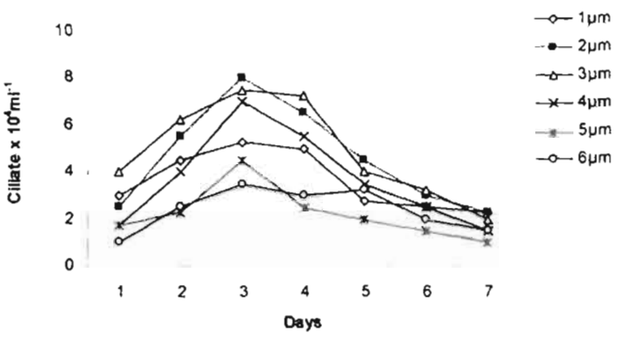


Fig. 4.17. Growth of ciliates in anaerobic culture fed with bacteria as food source

Bacterial density was the other parameter which affects the growth of protozoa. The maximum growth rate was obtained with the bacterial number between 4.5 x 10^6 - 2 x 10^9 cfu ml^{-1}. Bacterial density below 1x 10^3 cfu ml^{-1} had reduced growth in anaerobic cultures. A minimum bacterial density of 3 x 10^4 cfu ml^{-1} was required for the growth of protozoa, mainly the ciliates. In earlier studies, Fenchel (1980) has reported the requirement of bacterial density 4 x 10^6 - 2.5 x 10^7 ml^{-1} for free swimming ciliates.

There was a close link between the size of protozoa and the way in which protozoa function as grazers. The growth of small *Cyclidium* (23-32µm) had maximum growth rate with small cells ranging from 1 to 3µm and it was reduced with the feeding of large cells. The growth of *Spathidium* measuring 55-68 µm was the best in cultures supplemented with small particle of 2 to 4 µm, however, some growth was observed in all particle sizes. The larger ciliates *Vorticella* (72-85 µm) and *Metopus* (80-115 µm) were the prominent members in the cultures fed with 5 and 6 µm bacteria, but they were also able to consume small cells of 2 to 4µm efficiently (Fig. 4.18). Protozoa are considered to be the most important grazers in anaerobic system and probably control the bacterial number (Fenchel and Finlay, 1995).

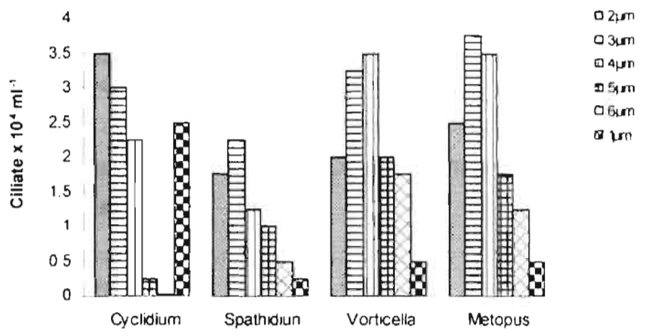
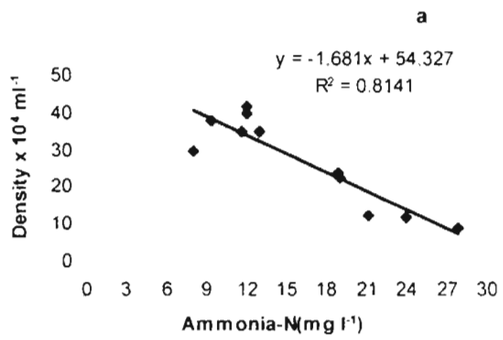


Fig. 4.18. Growth of different genera of ciliates in the anaerobic culture fed with bacteria of different sizes

Effects of chemical nutrients on protozoa

In the reactor studies, we observed a notable correlation between nutrients and protozoa densities along with degradation process. Protozoa had maximum growth at ammonia-N of 9-14 mg l^{-1}, phosphate-P of 4-9 mg l^{-1} and sulphide-S of 0.5 -2 mg l^{-1} (Fig. 4.19). Therefore, experiments were conducted to study the optimum concentration of nutrients for the growth of protozoa in anaerobic process.



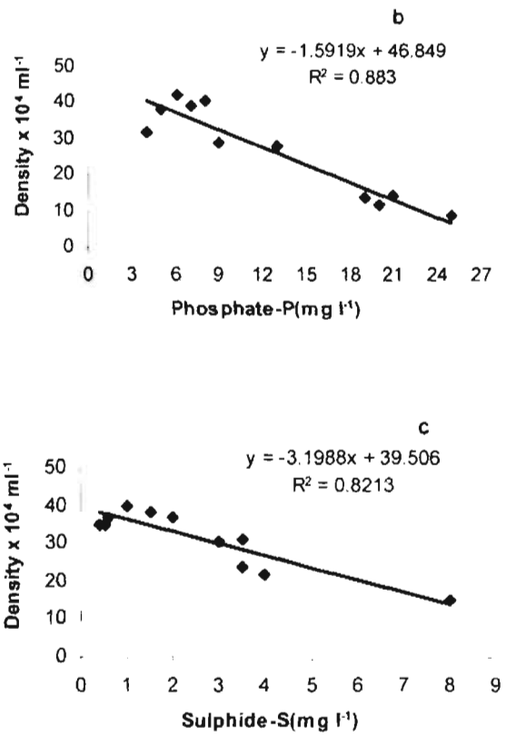


Fig. 4.19. Correlation between protozoa abundance and concentration of (a)ammonia-N, (b) Phosphate-P and (c) Sulphide-S in anaerobic reactors

Effects of ammonia-N on protozoa growth: Nitrogen is needed for the synthesis of molecules such as amino acids, DNA, RNA and ATP. The capability to utilise nitrogen sources and compounds (nitrogen, nitrates, ammonia, or organic nitrogen compounds) varies with organisms. In the present study, protozoa were exposed to different concentrations of ammonia-N in order to determine the optimum range for the protozoan growth and the lethal concentration. The effect of ammonia on the protozoa is shown in Fig. 4.20. The maximum growth was observed in the range of ammonia-N concentration 10-12 mg l^{-1}. The growth rates of protozoa exposed to below 6 and above 16 mg l^{-1} were significantly lower compared with 10-

12 mg l^{-1}. Statistical analysis showed that no significant difference between growth rates at 10 and 12 mg l^{-1} ammonia-N ($p>0.005$). It was confirmed that the optimum amount of ammonia needed for protozoan growth was in the range of 10-12 mg l^{-1} and the observed growth rate was 0.08h at this concentration. Very low and high amount of ammonia-N was not suitable for the growth of anaerobic protozoa

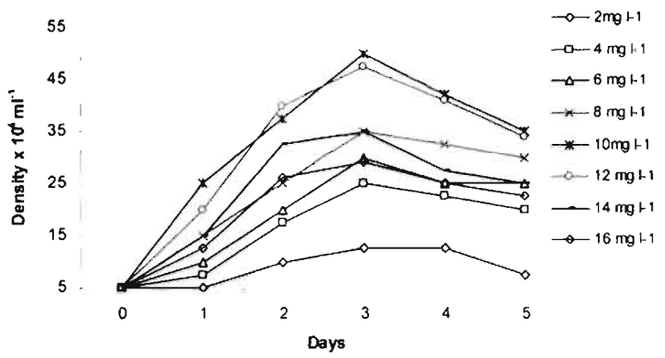


Fig. 4.20. The growth of protozoa on different concentrations of ammonia-N

Growth inhibition of protozoa was observed at ammonia-N concentration above 50 mg l^{-1} and above 100 mg l^{-1} was found as very harmful to the cells. Larger species like *Metopus* lost their characteristic shape and finally lead to cell rupture (Fig. 4.21). The mortality of protozoa, on exposure to a range of concentration of total ammonia-N for 4 hours is shown in Fig 4.22. All the tested ammonia concentrations were adversely affected protozoa growth. Minimum lethal dose of ammonia on protozoa (LC_{50}) was calculated from the equation $y = 0.2035x + 5.2171$, ($R^2 = 0.9807$) was found to be 220 mg l^{-1} on 4h exposure (Fig. 4. 22).

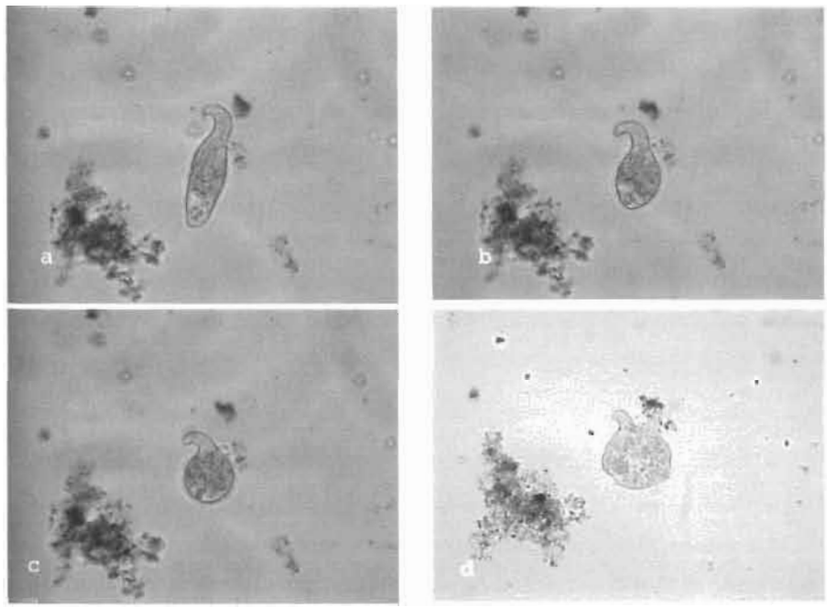


Fig. 4.21. Morphological changes observed in protozoa on response to ammonia-N above 100 mg l^{-1}. (a) normal *Metopus* cell (b) lost characteristic shape, (c) rounded up cel and (d) cell rupture

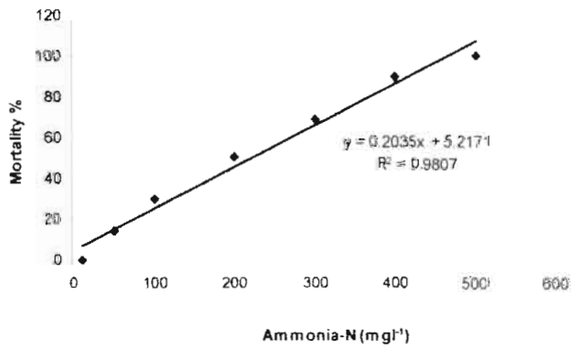


Fig. 4.22. Toxicity curve of ammonia-N for anaerobic protozoa

Ammonia is a common pollutant in waters used for intensive aquaculture and may be present in either un-ionized and ionized forms. Un-ionized ammonia can pass through the cell membrane and is harmful to cells due to its fat-soluble properties. Protozoa are common in most aquatic habitats and are often the dominant consumers of bacteria in wastewater treatment systems (Finlay, 2002; Nicolau et al., 2001). The toxicity of ammonia to protozoa remains poorly-known, despite the fact that the responses of ciliates to ammonia may have wide ecological importance (Legner, 1973). Result of the present studies on the tolerance of anaerobic protozoa to ammonia is therefore useful to gain a better understanding of their function in anaerobic systems.

***Effects of Sulphide-S on protozoa growth*:** Sulphur is essential to synthesise sulphur-containing amino acids and certain vitamins in cells. Depending on the organism, sulphates, hydrogen sulphide, or sulphur-containing amino acids may be used as a sulphur source. In the present study, anaerobic protozoa were exposed to different concentrations of sulphide-S in order to determine the optimum range of sulphur for the protozoan growth. The highest growth rate 0.092h was observed in cultures supplemented with 1-2 mg l^{-1} sulphide-S. Statistical analysis revealed that there is no significant difference between 1-2 mg l^{-1} ($p>0.001$). So the optimum amount of sulphide-S needed for protozoan growth selected was 1-2 mg l^{-1}. Protozoa supplemented with concentrations below 1 mg l^{-1} and above 2 mg l^{-1} had adverse effect on growth of protozoa (Fig. 4.23).

The sulphide-S concentration for growth inhibition of anaerobic protozoa was studied by supplementing different concentrations of sulphide-S. Fig. 4.24 shows that the sulphide-S concentration above 10 mg l^{-1} was found to be very harmful to the cells, but their survival was noticed up to a sulphide concentration of 40 mg l^{-1}. Minimum lethal dose of sulphide on anaerobic protozoa (LC_{50}) was calculated from the equation, $y = 1.533x - 0.925$, $R^2 = 0.981$. The determined LC_{50} value for anaerobic protozoa to sulphide exposure for 4h was found to be 33.245 mg.l^{-1}.

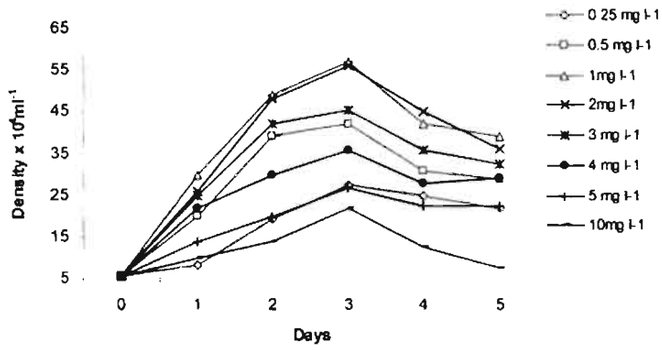


Fig. 4.23. The growth of protozoa on different concentrations of sulphide-S

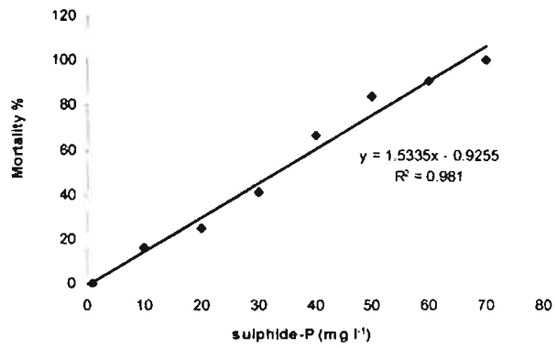


Fig. 4.24. Toxicity curve of sulphide-S for anaerobic protozoa

Anaerobic ciliate always found in environments of sulphide and it is extremely toxic to eukaryotic organisms even at micromolar concentrations (Finlay and Fenchel, 1995). It was observed that growth of anaerobic protozoa decreased at sulphide concentrations above 4 mg l^{-1} and occurred total growth inhibition above 60 mg l^{-1} sulphide. Earlier studies have showed the survival of *Metopus contorts* at 5mm sulphide and *Metopus striatus* died at 2mm sulphide. Earlier studies have showed the survival of *Metopus contorts* at 5mm sulphide (Bernad and Fenchel, 1996). Effect of

sulphide on feeding rates has been studied in detail by Massana et al., 1998 and reported that sulphide concentrations above 1 mM caused decrease in clearance rates and at sulphide concentrations above 4.5 mM, all feeding ceased.

Effects of Phosphate-P on protozoa growth: Phosphorus is needed to synthesize phospholipids, DNA, RNA, and ATP in the cells, which mainly derived from phosphate. Fig. 4.25 illustrates the growth of anaerobic protozoa on supplementing phosphate-P of 2-10 mg l^{-1}, where the highest growth rate was 0.087h with phosphate-P in the range of 6-8 mg l^{-1}. Statistically there is no significant difference in growth rate between 6-8 mg l^{-1} ($p>0.005$). So the optimum amount of phosphate-P needed for protozoan growth was 6-8 mg l^{-1}. Deficiency of phosphate-P was found to reduce the growth of protozoa and methanogenic activity in batch systems. The over dosage of phosphate also had adverse affects on protozoan growth and led to cell destruction.

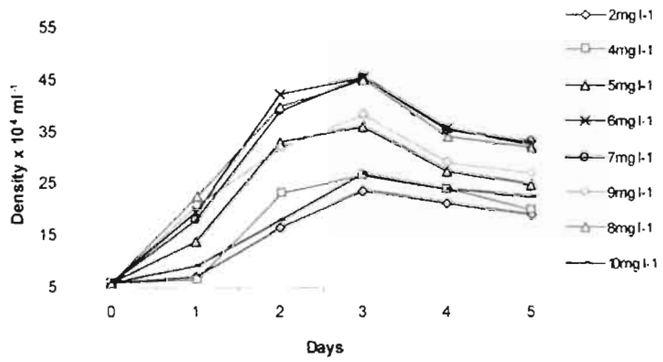


Fig. 4.25. The growth of protozoa on different concentrations of phosphate-P

The phosphate-P concentration above 100 mg l^{-1} was found to be very harmful to the cells. Protozoa exposed to high concentrations of phosphate led to loss of their characteristic shape and cell lysis as in the case of high ammonia exposure. The mortality of protozoa, on exposure to 10-500 mg. l^{-1} of phosphate-P for 4 h is shown in Fig 4.26. Minimum lethal dose of phosphate on anaerobic protozoa (LC_{50})

was calculated from the equation y =0.2035x + 5.2171, R^2 = 0.9807 and it was determined as 164.6 mg l^{-1} (Fig. 4.26).

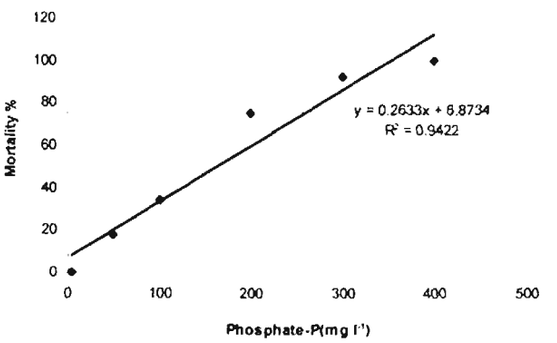


Fig. 4.26. Toxicity curve of phosphate-P for anaerobic protozoa

There was a little information on the requirement optimal phosphorous for the growth of protozoa. In terms of P requirements of the rumen microbial population, Sylvie (1987) has correlated to cellulose digestion, where maximum activity had at P concentrations between 5 and 25 mg. l^{-1}. Obviously this study revealed the optimum concentration of essential nutrients for the growth of anaerobic protozoa.

4.3.2 Effects of toxicants (heavy metals) on protozoa growth

The toxic effect of heavy metals on anaerobic protozoa was studied by introducing Cd, Zn, Pd, Cr and Cu. The result of maximum resistance dose (MRD) of each heavy metal to anaerobic protozoa is given in Table 4.3. Copper had the growth inhibition significantly above 13 mg l^{-1}. Zinc inhibited growth of anaerobic protozoa almost completely above 120 mg l^{-1}. Lead inhibited growth of protozoa above 24 mg l^{-1} and complete growth inhibition was observed with chromium concentrations above 31 mg l^{-1}. Among the five metals studied, cadmium had maximum toxicity and led to complete inhibition at concentration of 8 mg l^{-1} (Table. 4.3). It is important to note that the organisms may survive on the MRD level when the environmental

perturbation or toxic discharge ceases, but they become extinct above the MRD concentration (Walker, 2001).

Table 4.3. Heavy metal resistance of anaerobic protozoa to Cd, Zn, Pd, Cr and Cu (expressed as maximum resistance dose - MRD). Values presented are the mean of 4 replicates

Heavy metals	MRD (mg l^{-1})
Cd	8 ± 1
Cu	13 ±1.2
Pb	24 ± 1.5
Cr	31 ± 0.5
Zn	120 ± 2

Fig. 4.27 illustrates the survival of protozoa cells at different concentrations of heavy metals. Cd was highly toxic to protozoa and zinc was the least, tolerant up to 120 mg l^{-1}. The order of toxicity of heavy metals for protozoa can be stated as Cd > Cu > Pb > Cr > Zn. Madoni et al., (1996) have documented a slightly different sequence for the heavy metals toxicity to aerobic protozoa of activated sludge and is reported as Cu > Hg> Cd > Pb > C r > Zn.

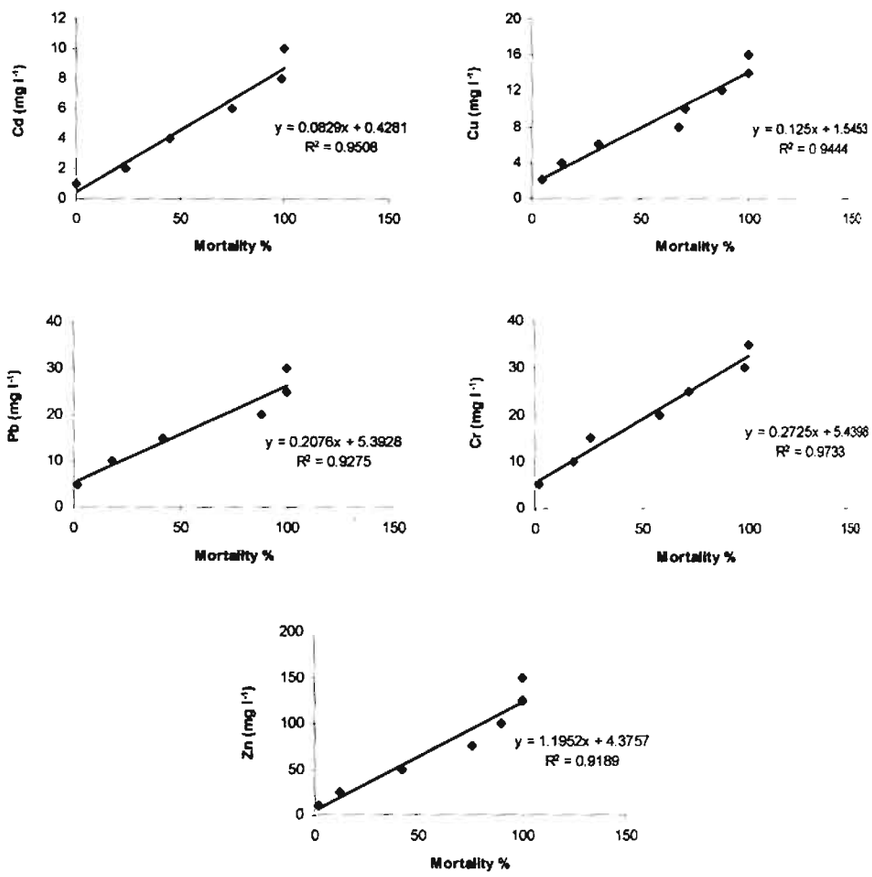


Fig. 4.27. Mortality of protozoa at different concentrations of heavy metals

Minimum lethal dose of heavy metals for anaerobic protozoa (LC_{50}) was calculated from the regression equation (Fig. 4.27) and the values are shown in Table 4.4.

Table 4.4. Minimum lethal dose of heavy metals on anaerobic protozoa (LC_{50})

Heavy metals	LC_{50} (mg l^{-1})
Cd	4.56
Cu	7.79
Pb	15.74
Cr	19
Zn	63.9

The existence of heavy toxicity and its metal bioaccumulation in the ciliate cytoplasm was confirmed by epifluroscence microscopy method. The presence of diverse electron-dense granules could be observed in the cytoplasm of ciliated protozoa exposed to sub lethal concentrations of Zn by epifluroscence microscopy, but was not seen in controls without heavy metals (Fig. 4.28).

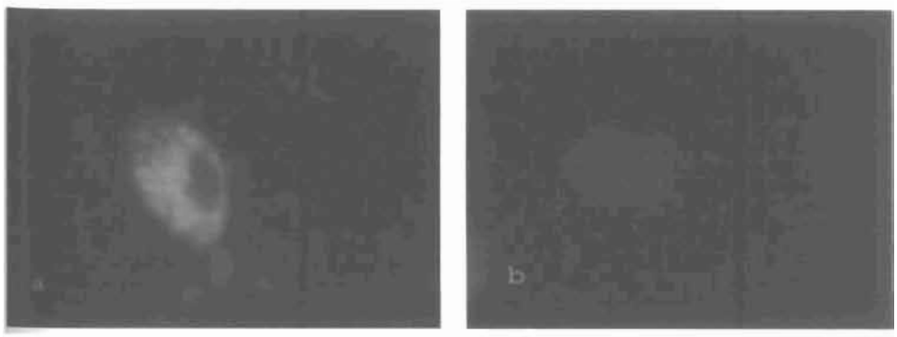


Fig. 4.28. Fluorescence microscopy of Zn bioaccumulation in the ciliate (a) *Prorodon* exposed to Zn (65 mg l^{-1}) and (b) control unexposed zinc

Ciliate strains exposed to heavy metals had different behavioural and ultra structural changes depending on the concentration and type of heavy metals. In general, treated cells were slow in movement and the cytoplasm appeared to be more vacuolated and therefore appeared as bigger in size than the untreated cells. Ciliates exposed to high concentrations of Cd (>8mg l^{-1}), developed outwardly protruding vacuole (Fig. 4.29).

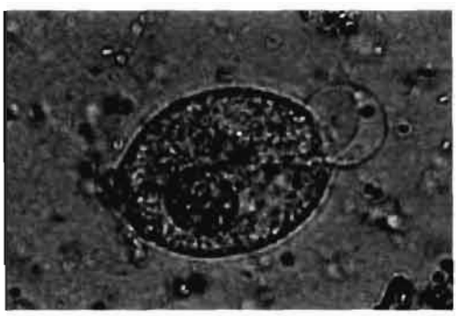


Fig. 4.29. Morphological changes in anaerobic ciliate exposed to >8 mg l^{-1} of Cd

The interactions of heavy metals with bacteria and fungi, and aerobic protozoa have been studied in detail (Gadd, 1986; Silver, 1996; Lovley, 2000; Madoni et al., 1996) and but had no attention with respect anaerobic protozoa. Ciliates are more sensitive to heavy metals than other micro organisms (yeast, micromycetes and bacteria), probably because ciliates have no cell wall at the trophic stage.

4.4 Conclusions

The present study thus shows that growth of anaerobic protozoa is influenced by various physical and nutritional parameters.

- Separation by centrifugation followed by picking up of the protozoa cells by micromanipulation used in this study was effective to isolate anaerobic protozoa from anaerobic sludge.
- Temperature was a significant environmental factor that determines the abundance and growth of protozoa. The mesophilic protozoa seem to be well adapted to grow at temperatures in the range of 30-32°C.
- Hydrogen ion concentration is recognised as an important ecological factor affecting the growth of protozoa and observed maximum growth around neutral pH in the anaerobic environment.
- As the anaerobic process involves formation of VFA, its accumulation leads to adverse effect on protozoa. Ciliates and flagellates were totally absent in 1M organic acids and maximum growth of protozoa was with VFA concentration <0.05M.

- The growth of protozoa on different carbon sources in bacteria controlled anaerobic cultures indicates the direct involvement in anaerobic process. Suspended carbon sources showed higher growth of the ciliates and dissolved carbon sources showed higher growth of flagellates.
- Bacteria density is an important factor that affects the growth of protozoa. The maximum growth rate was obtained with the bacterial density between 4.5×10^6 - 2×10^9 cfu ml^{-1}.
- Cell size of the prey (bacteria) and predator (protozoa) is an important factor affects the grazing activity. The available sizes of bacteria decide the type of protozoa in anaerobic system. The best growth of anaerobic protozoa was obtained with feeding of bacterial cells in the range of 2-4 μm. Large protozoa species can engulf a much larger size ranges than small ones.
- Nutrients were found to limit protozoa growth and the optimum concentration of nutrients required for the growth of protozoa was in the range of 10-12 mg l^{-1} for ammonia-N, 6-8 mg l^{-1} for phosphate-P and 1-2 mg l^{-1} for sulphide-S. The presence of excess nutrients is not advantageous for the growth of anaerobic protozoa.
- Ciliates are good candidates to detect the toxicity and to determine the bio available concentration of certain toxic pollutants, like heavy metals.

In conclusion, requirements for anaerobic bacteria is closely correlated to anaerobic digestion process parameters (Jefferson et al., 2001) and this indicates possible involvement of protozoa in anaerobic process along with bacteria.

Chapter 5
Role of protozoa in anaerobic wastewater treatment process

Abstract

It is only very rarely recognised in literature that anaerobic reactors may contain protozoa in addition to various bacterial and archeal groups. The role of protozoa in anaerobic degradation was studied in continuous stirred tank anaerobic reactor and batch tests. Anaerobic protozoa, especially the ciliated protozoa have direct influence on the performance of CSTAR at all organic loading rates (1 g COD. $l^{-1}.d^{-1}$ to 2 g COD. $l^{-1}.d^{-1}$) and retention times (5 to 10 days). The studies revealed that chemical oxygen demand removal and methane production was strongly correlated to ciliate density in CSTAR fed with oleate (suspended COD) and acetate (soluble COD). Direct utilization of COD by flagellates and ciliates was observed in bacteria-suppressed cultures. In batch tests, increased COD removal and methane production was observed in sludge having ciliates as compared with sludge without protozoa. Methane production was increased linearly with number of ciliates (R^2 = 0.96) in batch tests with protozoa. There was no significant difference in COD removal and methane production between reactors fed with suspended COD and soluble COD. However, diversity and number of ciliates is greater in CSTAR fed with particulate feed. The mixed liquor suspended solids representing biomass was significantly lower (16-34 %) in CSTAR with protozoa. Protozoa, mainly ciliates were found to produce good quality effluents by feeding on bacteria and suspended particles. The protozoa could reduce excess biomass by grazing, and that enabled nutrient recycling. O_2 tension resulted in loss of free methanogens in biomethantion system, while methanogens were observed inside the protozoan cysts. The existence of facultative anaerobic protozoa offers a model for managing temporary exposure to oxygen in anaerobic systems. The facultative anaerobic protozoa did not contain catalase, whereas superoxide dismutase was present. Anaerobic reactors with protozoa rich sludge can detoxify the trace amounts of oxygen toxicity in wastewater. The technological importance of these results is that reactors with protozoa rich sludge

can enhance the rate of mineralisation of complex wastewater especially wastewater containing particulate COD.

5.1 Introduction

The development of high-rate anaerobic wastewater treatment reactors, such as the upflow anaerobic sludge blanket reactor, anaerobic biofilter and the anaerobic fluidized bed reactor has made anaerobic digestion the most competitive treatment technology for high and medium strength biodegradable wastewaters. The process of anaerobic degradation is a complex and dynamic system where, microbiological, biochemical and physico-chemical aspects are closely linked. During anaerobic treatment, a complex natural community consisting of many interacting microbial species degrade natural polymers such as polysaccharides, proteins, nucleic acids and lipids, in the absence of oxygen, into methane and CO_2. The process involves the hydrolysis of high molecular weight carbohydrates, fats and/or proteins into soluble polymers by means of enzymatic action of hydrolytic fermentative bacteria and the conversion of these polymers into organic acids, alcohols, H_2 and CO_2. Volatile fatty acids and alcohols are then converted to acetic acid by H_2 producing acetogenic bacteria and finally methanogenic archaea convert acetic acid and H_2 gas into CO_2 and CH_4 (Gujer and Zehnder, 1983; Mosey and Fernandez, 1989). These processes are considered to be bacterial in origin and no other processes are considered in the research literature on anaerobic digestion.

The anaerobic digestion model of IWA also proceeds on the assumption that the various reactions are either intercellular or extracellular bacterial process (Batstone et al., 2002). Indeed, high-rate reactors operated at short hydraulic retention times are designed on the philosophy of maximum biomass retention assuming that biomass comprises either biofilm or floc forming bacteria. The role of protozoa in anaerobic digestion is completely neglected in the earlier studies. However, there is an additional trophic layer comprising anaerobic protozoa in an anaerobic reactor ecosystem, which our studies show to have significant impact on the digestion process. The aim of this study was to investigate the role of protozoa, and ciliates in particular, in the anaerobic degradation processes.

5.2 Materials and methods

5.2.1 Continuous stirred tank anaerobic reactor

Two continuous stirred tank anaerobic reactors, with working volume of 1litre, each were operated at organic loading rates 1 g COD. $l^{-1}d^{-1}$ and 2 g COD. $l^{-1}.d^{-1}$. One reactor was fed with a suspension (colloidal sodium oleate) and the other a solution (sodium acetate) to study the changes in protozoa population with respect to feed composition. Each substrate was supplemented with Vanderbilt mineral media (Nuri et al., 2001). An HRT variation of 5 to 10 days was obtained with a peristaltic pump (Autoclude VT, UK) with 2mm id tube, cyclic mode operation, 15 seconds on and 10 minutes off. All other conditions were identical for both reactors. The pH was controlled by adding 1N NaOH or 1N HCl. Reactors were seeded with sludge collected from anaerobic dairy wastewater treatment plant. Biogas generated in the reactors was measured by liquid displacement. Protozoa inhibited CSTAR was obtained by adding cycloheximide (200 mg^{-1}) to feed.

5.2.2 Batch experiments

Contribution of protozoa to anaerobic processes was determined by inhibiting growth of protozoa and measuring methane production and COD removal and comparing with a control. The batch tests were conducted in rubber septum capped 500ml glass bottles. The initial COD concentration was set at 1g COD. g^{-1}VSS with the basal medium (Harada et al., 1994) using sodium oleate as COD source. Anaerobiosis was maintained by the addition of cysteine HCl (0.05%). Experiment bottles were capped and headspace was flushed with nitrogen. Samples were collected at regular intervals for routine analysis with a syringe through the septum. Gas production was measured by liquid displacement systems.

Cycloheximide which inhibits eukaryotic protein synthesis was used to control protozoan growth and it has previously been used to remove protozoa without affecting bacterial population in aerobic systems (Hahn et al., 2001). The anaerobic ciliates and flagellates were fully inhibited by the addition of cycloheximide. After testing various cycloheximide concentrations, 250 mg. l^{-1} was chosen as the minimum concentration for fully inhibiting flagellates and ciliates in anaerobic sludge. Control tests were conducted to show that cycloheximide has no effect on anaerobic bacteria.

Anaerobic bacteria was isolated from the anaerobic reactor sludge for the experiment. Isolation and culturing of anaerobic bacteria was done as per Microbiological aspects of anaerobic digestion, Laboratory manual (1988). Isolated organisms were incubated anaerobically in acetate and mineral medium with and without cycloheximide in 50 ml Schott Duran amber bottles screw capped with silicon septa (Thomson Scientific USA). Samples were stained with 4'-6-Diamidino-2-phenylindole (DAPI) and bacterial number counted under epifluorescence microscope (Leica DM 2500). Acetoclastic and hydrogenotrophic methanogens were determined by MPN 3-tube dilution method using basal medium as described earlier (Tseg et al., 1994). MPN tests were done in glass tubes sealed with rubber stoppers and a gas phase of N_2:CO_2 (80:20% $v.v^{-1}$).

5.2.3 Preparation of protozoa culture

Direct uptake of particulate and dissolved COD by protozoa was studied in bacteria-suppressed cultures of ciliates and flagellates. Anaerobic ciliates and flagellates were isolated from laboratory scale anaerobic BFBR fed on dairy wastewater and cultured in ciliate mineral medium with sodium oleate suspension as carbon source (Chapter 4). Anaerobiosis was maintained by the addition of cysteine HCl (0.05%). Resazurin (2mM) was used as redox indicator. Experiment bottles (50 ml Schott Duran amber bottles) were screw capped silicon septa (Thomson Scientific USA) and headspace was flushed with nitrogen. The cultures were incubated at 30°C ± 2 in triplicates and the mean of results (number of organism, COD removal and methane production) were taken.

5.2.4 Size selective uptake of particles

The size selective uptake of suspended particles by anaerobic ciliates was studied giving known COD of sodium oleate suspension of different particle sizes. Sodium oleate suspension was prepared with oleic acid and sodium hydroxide at alkaline pH using homogenizer. The suspension with different particle size was separated by filtering through filter of different pore sizes and the particles that ranged between 0.45 to 20 μm. The particles of varied size with same COD (200 $mg.l^{-1}$) was given to study its uptake and utilization.

5.2.5 Grazing on bacteria

The ingestion of bacteria by anaerobic ciliates was monitored in experimental condition. Suspension of bacteria was obtained and heat killed as described by Sherr et al., (1987) and was added to protozoa culture as food source. Bacterial number was monitored by staining with DAPI and observed under an epifluorescence microscope (Leica DM 2500).

5.2.6 Nutrient recycling by protozoa

For nutrient excretion experiments, the protozoa culture and bacterial culture (as food bacteria) were prepared as described earlier (Chapter 4). Two types of bacterial suspensions were used as food source; heat killed and live bacteria at final concentration of approximately 4 x 10^8 cells ml^{-1}. Protozoa were introduced to these bacterial cultures. The pH of the cultures was adjusted to the range of 6.8 – 7.2 with 0.1M sterile HCl and NaOH prepared in N_2 stripped distilled water. Cysteine HCl (1mM) was used to achieve complete anaerobiosis. Cultures were subsequently incubated in room temperature (30-32°C). Periodically, three samples were sacrificed from each set for measuring cell counts and nutrient excretion by protozoa. Mean of the triplicates were accounted as the results. An estimate of nutrients release due to bacteria was done by conducting protozoa controlled experiments with heat killed and live bacteria cultures.

5.2.7 . Impact of oxygen on anaerobic protozoa

The experimental set up includes two set of batch experiments with same inoculam (sludge from reactor fed with diary wastewater) for studying the changes in anaerobic protozoa population under the stress of oxygen (Table 5.1). One batch setup was kept under permanent anaerobic condition as control. Other set was considered as experimental in which conditions were changed from anaerobic to aerobic and, then re-established the anaerobic condition.

All experiments were carried out in 50ml amber bottles capped with two holed rubber cork. Silicone tube was attached to one hole for feeding and injecting gases (oxygen or nitrogen) in order to get aerobic and anaerobic conditions. A syringe was connected to other hole for sampling. Oxygen was sparged at selected intervals for changing the condition from anaerobic to aerobic. After reaching complete

aerobic environment, nitrogen was sparged to re-establish anaerobic condition. Magnetic stirring was done for effective mixing in the bottle while sparging gases. The initial COD concentration was set at 1g COD. g^{-1}VSS with the basal medium (Harada et al., 1994) using sodium oleate as COD source. Second dose of feed (COD) was given after reaching aerobic condition (12^{th} day). The pH of the cultures was adjusted to the range of 6.8–7.2 with 0.1M sterile HCl and NaOH prepared in N_2 stripped distilled water. Resazurin (2mM) was used as redox indicator. The experiments were performed in triplicates and 3 bottles from each set were sacrificed for analysis at selected days.

Table 5.1. Experimental scheme for studying impact of oxygen on protozoa in anaerobic systems

Anaerobic	Transition period (anaerobic to aerobic)				Aerobic	Transition period (aerobic to anaerobic)				Anaerobic
	Days selected for analysis and oxygen sparging					Days selected for analysis and nitrogen sparging				
Days	1	2	4	8	12*	13	14	16	20	24
D.O (mg. l^{-1})	0.02	0.85	1.8	2.2	3.2	3	2.1	1.5	0.7	0.02
Control	Permanently kept in anaerobic condition									

* The day selected for giving second dose of COD

Sacrificed three bottles of experimental and control for analysing parameters - pH, VFA, Dissolved oxygen, Redox, Methane, COD removal, Protozoa count and Methanogen count.

5.2.8 Chemical Analysis

COD, ammonia, phosphate, VSS, MLSS, VFA, pH and methane were analysed as per the methods described in Chapter 3 and 4. The dissolved oxygen monitored by a DO meter (YSI - 5100, USA) processing temperature correction.

5.2.9 Microscopic observations

The identification of protozoa and enumeration were carried out as per the methods presented in chapter 2. For gram staining, heat fixed cells were stained as per Gram's method (Collins and Lyne, 1984). Autofluorescence of endosymbiotic methanogens in protozoa were detected after fixation in 4% formaldehyde (Chapter 2).

Fluorescence insitu hybridisation (FISH) was done by fixing cells with Schaudinn's fluid for 1h and washed repeatedly with phosphate buffered saline, pH 7.2. FISH was conducted according to the protocols previously described (Amann et al., 1990 and 1991). FISH with rRNA targeted probes specifically for domain archaea - ARCH 915, MX 825 and MS 821 used to examine the distribution of archeal cells. 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% $KCr(SO_4)_2$). After drying at 37°C, the slides were dehydrated sequentially in 80, 90 and 100% ($v.v^{-1}$) ethanol (3 min each) and dried at room temperature. Hybridizations were performed in a closed hybridization oven (HB-500 Minidizer™, UVP, USA) at 46 °C for 2 h with a hybridization buffer (0.9 M NaCl, 20 mM Tris-HCl, pH 7.2, 0.01% SDS) containing each labeled probe (3 $ng.\mu l^{-1}$ for ARCH 915 and 5 $ng.\mu l^{-1}$ for both MX 825 and MS 821) (MWG Biotech). Formamide was added to the final concentrations listed in Table 5.2 to ensure the optimal hybridization stringency. 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 8 mM for MS 821. After washing (50°C for 30 min for ARCH 915 and 48°C for 30 min for both MX 825 and MS 821), 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 $\mu g.ml^{-1}$). The prepared slides were examined under an epifluorescent microscope (Leica DM2500, Leica, Germany) equipped with a CCD camera for imaging.

Table 5.2. Oligonucleotide probes used for FISH

Probe name	Formamide (%)	Sequence	Target group
ARCH 915	0	GTGCTCCCCCGCCAATTCCT	Archea domain
MX 825	35	TCGCACCGTGGCCGACACCTAGC	*Methanosaeta*
MS 821	35	CGCCATGCCTGACACCTAGCGAGC	*Methanosarcina*

5.3 Results and discussion

5.3.1 Role of protozoa in COD removal

Results of control tests to verify whether cycloheximide has adverse effect on bacteria showed insignificant difference in headspace methane concentration, with cycloheximide (36.7 ppm) and control without cycloheximide (36.9 ppm). Bacterial numbers were not reduced by dosing of cycloheximide (16×10^4 ml^{-1} with cycloheximide and 16×10^4 m^{-1} without cycloheximide). MPN index for methanogens in the presence ($11 \times 10^3 ml^{-1}$) and absence ($11 \times 10^3 ml^{-1}$) of cycloheximide in batch experiments indicated that methanogens were unaffected and the cycloheximide did not inhibit bacterial activity (Table 5.3).

Table 5.3. Results of control test to verify the effect of cycloheximide on bacteria in bottles of anaerobic bacterial cultures with and without cycloheximide

	Head space Methane concentration (ppm)	Total bacterial count ($\times 10^4 ml^{-1}$)	MPN index for Methanogens ($\times 10^4 ml^{-1}$)
With Cycloheximide	36.9	16	11
Without Cycloheximide	36.7	16	11

Role of protozoa in COD removal was studied in anaerobic CSTARs fed with oleate and acetate as feed. Fig. 5.1 shows ciliate density and COD removal efficiency in the CSTARs with loading rate of 1 g COD.l^{-1}. d^{-1} at 10 days HRT. Similar results were obtained at all COD loading rates and retention times.

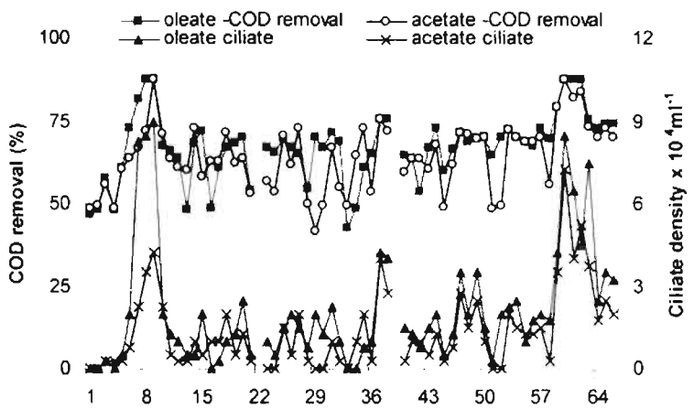


Fig. 5.1. Correlation between number of ciliates and COD removal in CSTARs operated with acetate and oleate feed with loading rate of 1g COD. $l^{-1}.d^{-1}$ at 10 days HRT

At the start up of the reactor operations, the COD removal was poor and the ciliate density was less. At all loading rates, the steady state COD removal efficiency was greater than 75% and high number and diversity of ciliates were seen. The maximum number of ciliates attained was $10.5 \pm 0.4 \times 10^4$ ml^{-1} and the corresponding COD removal was greater than 85 %($\pm$ 5). There was no significant difference in COD removal between reactors fed oleate (suspended COD) and acetate (soluble COD). However, COD removal was more consistent and stable in the case of oleate suspension. This is contrary to what would be the expected from the known microbiology of anaerobic reactors.

COD removal is strongly correlated to ciliate density in the continuously fed reactors were high (R^2 = 0.974 and R^2 = 0.966) at all hydraulic retention times and loading rates as shown in Fig. 5.2. The strong correlation could imply either that

ciliates enable COD removal or that COD removal enable ciliate growth. There is limited information on the contribution of eukaryotes to the degradation processes in anaerobic reactors to either conclusions. Agrawal et al., (1997) observed free living anaerobic ciliates similar to *Metopus* in a high-rate anaerobic reactor, but the reason for presence not studied. The symbiotic relation between protozoa and bacteria reported to enhance methane production (Biagini et al., 1998a) and an increased turn over rate of organic matter due to grazing and flocculation activities of ciliates in wet landfill sites (Finlay and Fenchel, 1991).

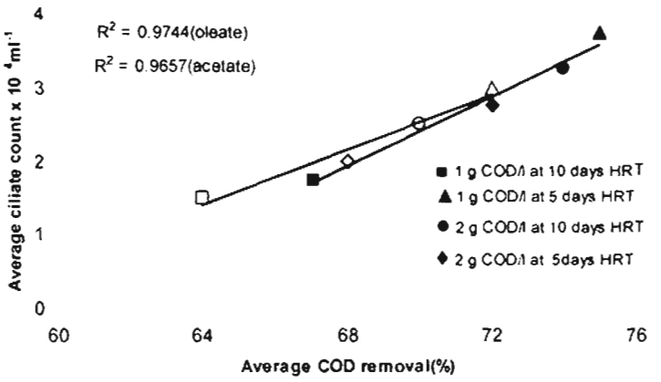


Fig. 5.2. Correlation between number of ciliates and COD removal in CSTARs operated with different loading rates (sodium oleate and sodium acetate were given as COD) and retention times. (■ filled - oleate and□ not filled – acetate)

The diversity of ciliates present is greater in CSTAR fed with oleate (suspension, emulsion) as compared with acetate (solution) as illustrated in Fig. 5.3. Number of larger sized ciliates species such as *Vorticella, Loxophyllum, Metopus, Spathidium, Brachonella* and *Discomorphell*a were more in suspended fed system and *Brachonella* and *Discomorphell*a were completely absent acetate fed CSTAR as shown in Fig. 5.3. These results indicate a possible direct consumption of oleate particle by protozoa. Excepting *Prorodon*, other ciliates were more when given oleate feed. The larger numbers although not significant, may be due to the direct uptake of

colloidal oleate feed by ciliates. Rumen ciliates such as *Entodinium* species, *Eudiplodinium* species can engulf particles directly and digest them intracellulary (Coleman, 1992). Previous studies in rumen system have demonstrated the hydrogenation of lipids by rumen protozoal suspension containing *Epidinium caudatum* (Wright, 1959). Larger diversity in bacterial population for the degradation of oleate (suspension) fed system, leads one to expect larger diversity of predator (protozoa) compared to acetate (solution) feed (Fig. 5.3).

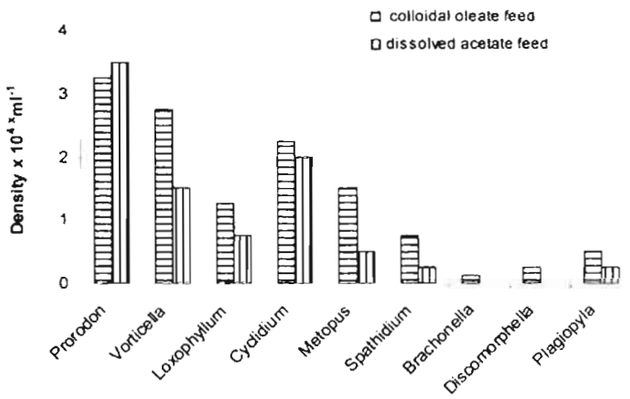


Fig. 5.3. Number and diversity of ciliates in oleate fed and acetate fed CSTARs

In experiments with control, enhanced COD removal was observed in CSTARs with protozoa (more than 75%) compared to the protozoa controlled CSTARs at the same loading rate and retention time(Fig. 5.4). The decreased COD removal could not have resulted from any adverse effect of cycloheximide on anaerobic bacteria, the selective inhibitor used in this study for protozoa did not exhibit any deleterious effect on bacteria as the bacterial number was the same in the presence and absence of cycloheximide. This is a contradiction to the general view on the growth of protozoa, the grazers of bacteria which are considered responsible for biodegradation. It means protozoa may be playing some role in the anaerobic degradation through consumption of biomass for the generation of methane.

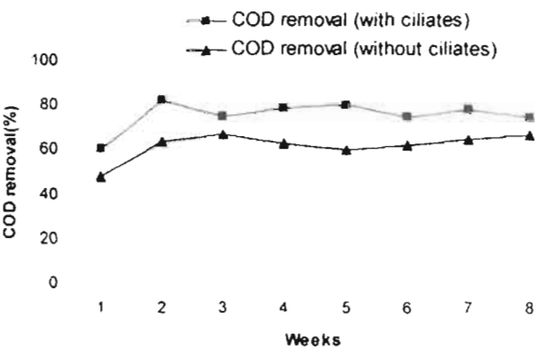


Fig. 5.4. Comparison of COD removal in CSTARs with and without protozoa with loading rate of 1g COD l^{-1} d^{-1} at 5 days HRT

The direct consumption of substrates by anaerobic protozoa was studied in batch experiments. In bacteria suppressed, batch tests with colloidal sodium oleate showed the growth of ciliates species *Prorodon, Cyclidium, Spathidium, Vorticella* and *Metopus* and flagellates species *Menoidium, Trepomonas, Naeglaria, Rhynconomas* and *Bodo* (Table 5.4). In bacteria suppressed, batch tests with dissolved substrate sodium acetate showed no ciliate growth but flagellates species *Menoidium, Naeglaria Trepomonas,* and *Mastigella* (Table 5.4) were observed. The results indicate the direct consumption of colloidal substrates by ciliates. Osmotrophic nutrition has been previously demonstrated in some of the soil flagellates (Ekelund and Rønn, 1994). But the metabolism and energy advantage from acetate to flagellates is unknown. Filtered COD removal was 69 % was in the case of oleate and 65 % (Table 5.4) in case of acetate. These results confirm the direct participation of protozoa in anaerobic degradation. In particular, the direct consumption of particulates by ciliates provides a mechanism to overcome what is generally considered rate-limiting in the treatment of wastewater in anaerobic reactor - the solubilization of solids.

Table 5.4. Growth of protozoa and filtered COD removal in bacteria-suppressed anaerobic culture fed with suspended COD (sodium oleate) and dissolved COD (sodium acetate)

Days	Bacteria-suppressed culture fed with Suspended COD (Sodium oleate)			Bacteria-suppressed fed with Dissolved COD (Sodium acetate)		
	Flagellates ($x\ 10^4\ ml^{-1}$)	Ciliates ($x\ 10^4\ ml^{-1}$)	COD Removal (%)	Flagellates ($x\ 10^4\ ml^{-1}$)	Ciliates ($x\ 10^4\ ml^{-1}$)	COD Removal (%)
2	1.5	1.75	22	3.25	0.125	23
4	3.75	2.5	41.5	4.75	0.25	39
5	2.25	5	53	6.5	0.125	52
6	2	6.75	69	8.25	0.125	65.4

Based on the above findings, the uptake of particulate feed by ciliates was studied with respect to its size. As shown in Fig. 5.5 and 5.6, the abundance and type of ciliates were varied according to the size of particle size supplied. The best growth of anaerobic ciliates was in the medium with particles of 1.2μm and 2.5 μm compared to 0.45μm, 8μm,11μm and 20μm (Fig. 5.5). The growth of *Cyclidium* (measured size 23 to 32μm in the culture) was significantly higher with smaller particles ranging from 0.45 to 2.5 μm and its number was reduced on the feeding of bigger particles. The growth of *Spathidium* measuring 55 to 68 μm had the best in smaller particle (1.2 - 8 μm) fed cultures, however, some growth was observed in the larger particles. Similarly, the growth of *Loxophyllum* (measured size 60 to 72 μm in culture) was best with smaller particle size and it was comparatively low in 20 μm particles fed culture. The ciliates such as *Vorticella* having the size 72 to 85 μm and *Metopus* of the size 80 to 115 μm could grow in cultures fed with bigger particles as well as smaller particles. Obviously, most of the ciliate had the best growth in particles around 2 μm. This size selective grabbing of particulate organic matter by ciliates indicates clearly that the growth of ciliates was dependent on available particle in the suitable size (Fig. 5.5 and 5.6). Small ciliates like *Cyclidium* seems to have a narrow size range for feeding particles (0.45 to 2.5 μm) while large species like *Metopus* and *Vorticella* have wide size range of feeding particles (Fig. 5.6).

The result of the study reveals that ciliates are size selective feeders and their growth is governed by size of particles. Ciliates (count 5.25 x 10^4 ml^{-1}) were capable of consuming 6.6 mg. l^{-1} COD h^{-1}. Thus ciliates can be voracious grazers of particles and each species has a diverse range of preferred particle size which may be depend upon its mouth size and morphology. The utilization of fine particulate plant detritus by a heterotrophic flagellate, *Chilomonas paramecium*, and a ciliate, *Tetrahymena pyriformis* have already been demonstrated (Anja et al., 2005).

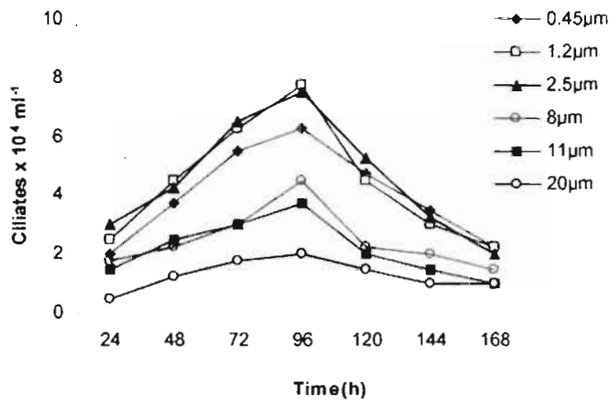


Fig. 5.5. Growth of ciliates in bacteria controlled anaerobic culture fed with suspended particle of different sizes

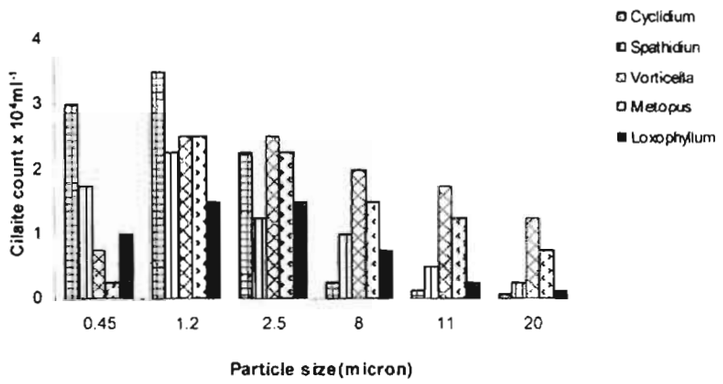


Fig. 5.6. Growth of different kind of ciliates in bacteria controlled anaerobic culture fed with suspended particle of different sizes

As the bacterial number remains almost negligible, then the possible removal of substrate could only be due to the ciliate utilization in bacteria-suppressed anaerobic culture. Thus, the present study on protozoa confirms their ability to utilize suspended organic matter directly. Grazing of particles (mainly bacterial cells) by protozoa has been reported in aerobic treatment systems (Curds et al., 1968; Fried et

al., 2000), but had little evidence in anaerobic systems. This understanding has great importance in anaerobic degradation.

5.3.2 Role of protozoa in methane production

Fig. 5.7 shows ciliate density and methane production in the CSTARs with loading rate of 2 g $COD.l^{-1}.d^{-1}$ at 10 days HRT. The maximum rate of methane production was obtained in the range of 30 to 56 ml day^{-1} in the anaerobic reactors and the corresponding the ciliates count was in the range of $6.25x10^{4}$ to $10.5\ x10^{4} \pm 1$ ml^{-1} (Fig. 5.7). There was no significant difference in methane production between reactors fed with oleate (suspended COD) and acetate (soluble COD). However, methane production was more in the case of oleate suspension as like COD removal.

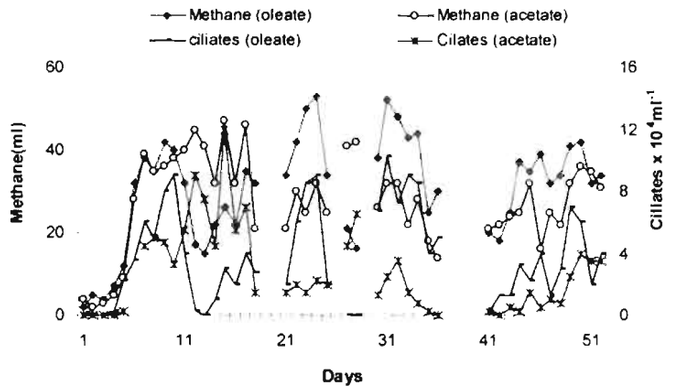


Fig. 5.7. Correlation between number of ciliates and methane production in CSTARs operated with acetate and oleate feed with loading rate of 2 g COD. $l^{-1}.d^{-1}$ at 10 days HRT

The methane production had strong correlation to ciliate density in the continuously fed anaerobic reactors (R^2 = 0.99 and R^2 = 0.97) at all hydraulic retention times and loading rates as shown in Fig. 5.8. The strong correlation indicates the possible involvement of protozoa in methane production.

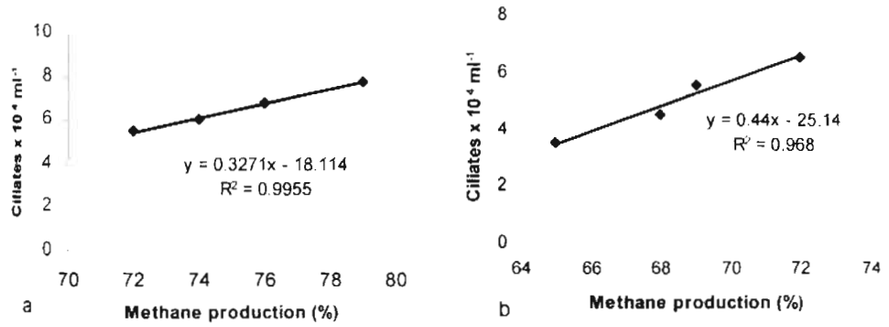


Fig. 5.8. Correlation between number of ciliates and methane production in CSTARs operated with different loading rates ((a) sodium oleate and (b) sodium acetate were given as COD) and retention times

Fig. 5.9 illustrates the changes in methane production in batch experiments with and without protozoa. Batch experiments with oleate as COD source showed increased COD removal (greater than 95%) in sludge having ciliates as compared with sludge without protozoa (less than 66%). Methane produced was significantly lower (31 (+ 2) %) in the absence of protozoa (Fig. 5.9). The observed methane production was 318 ml in experiments with protozoa compared to 227 ml in experiments without protozoa. However, the initial rate of methane production was higher in the absence of protozoa. This could be because of higher bacterial count (6.5×10^7 cfu.ml^{-1} vs 4×10^6 cfu.ml^{-1}) in the biomass in the absence of predators in protozoa inhibited test. Metabolic end products of ciliates such as acetate, CO_2 and H_2 (Goosen et al., 1990b) could serve as growth substrate for methanogenic bacteria. Thus, the enhanced anaerobic degradation of organic matter is brought about by co-ordinated activity of number of different microbial groups including archea, bacteria and protozoa to produce methane.

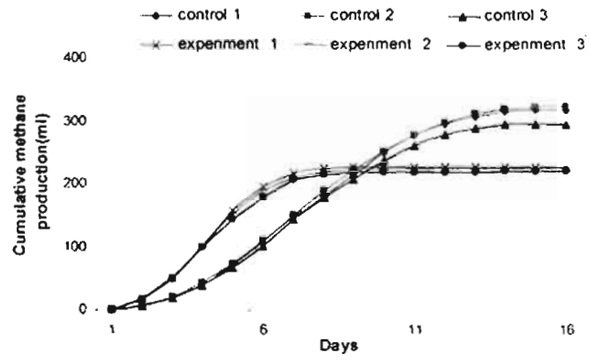


Fig. 5.9. Cumulative methane production in batch system with protozoa (control) and without protozoa (experiment) on adding 1g VSS sludge and 1g COD as sodium oleate

The results of batch experiments also show an increase in methane production with number of ciliates. Methane production increased linearly with number ciliates ($R^2 = 0.96$) (Fig. 5.10).

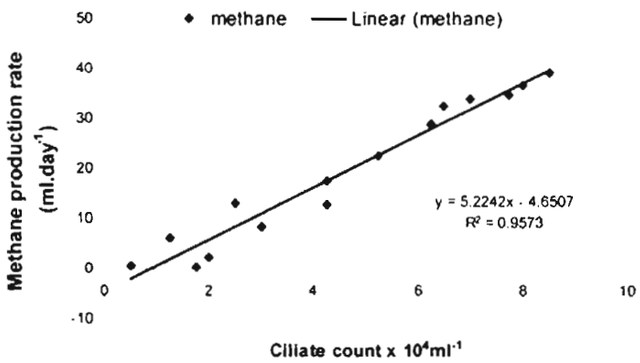


Fig. 5.10. Stimulated methane production proportional to numbers of ciliates in batch experiment

Studies on wet landfill sites have reported anaerobic protozoa consortia are capable of making a significant contribution to methane generation (Finlay and Fenchel, 1991). Furthermore, ciliates have been found to contribute up to 90% methane production in some anaerobic marine sediments (Fenchel, 1993), but total methane production from these sediments is low. The result of the study strongly supports the active participation of protozoa in anaerobic degradation including significant production of methane.

The enhanced methane production with the presence of protozoa, especially ciliates was correlated with presence of endosymbiotic methanogens in anaerobic systems. Epifluorescence microscopy detecting bluish fluorescence of typical methanogenic archaea demontrated that most of the cells had methanogens in their cytoplasm (Fig. 5.11). The fluorescent bacteria were seen abundantly throughout the cytoplasm of the cells.

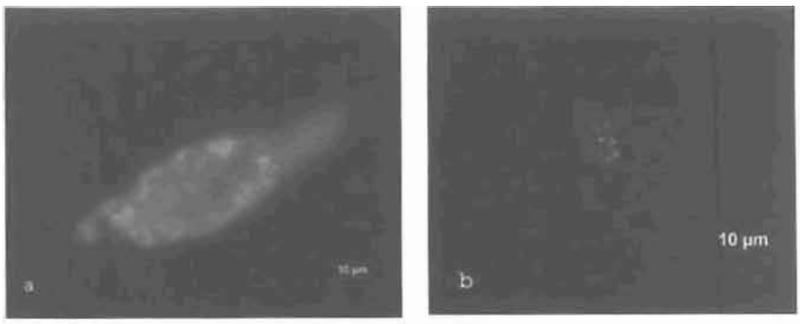


Fig. 5.11. Autofluroscence of endosymbionts in anaerobic protozoa (c) ciliate *Metopus* and (b) flagellate *Menoidium*

Results of FISH analysis also confirmed that protozoa harbor methanogens as the major endosymbionts. The number and type of methanogens in the protozoa were varied with respect to genera. The larger ciliate like *Metpous* had a large number of endosymbionts compared to other ciliates and flagellates harboured minimum number of methanogens (Table 5.5). No methanogen symbionts were observed in small flagellates and amoeba.

Table 5.5. Methanogenic symbionts in anaerobic protozoa from anaerobic reactor at steady state reactor operation (CSTAR operated at 1 g COD. $l^{-1}.d^{-1}$ at 5 days HRT)

Protozoa genera	Average Number of Methanogens
Metopus	818
Spathidium	46
Prorodon	389
Cyclidium	58
Paranema	14
Menoidium	16

Varied number of methanogenic symbionts ranging from 14 in flagellates to 818 in ciliates was accounted in different protozoa genera at steady state reactor operation. Ciliate were heavily colonised by methanogens due to their large cell size and metabolic activities (Fig. 5.12). The numbers observed in this study are comparable to the reports on *Metopus*. In *Metopus contortus*, the number of methanogenic cells ranged from 6×10^3-10×10^3 (Fenchel and Finlay, 1992) whereas *Metopus es* possesses an average number of 637 ± 19 methanogens ciliate $^{-1}$ (Schwarz and Frenzel, 2005). The distribution of symbiotic methanogens inside the protozoa was varied with organisms and growth conditions.

Results of FISH analysis with specific probes could further confirm the presence of *Methanosaetacea* and *Methanosarcina* as endosymbionts in anaerobic protozoa. In Situ Hybridization with MX 825 exhibited strong red fluorescence indicating the presence of *Methanosaeta* as endosymbionts in protozoa *Metopus* and *Cyclidium* (Fig. 5.13). In Situ Hybridization with MS 821 showed strong red fluorescence which confirms the presence of *Methanosarcina* as endosymbionts in protozoa *Prorodon* (Fig. 5.14). *Methanosaeta* and *Methanosarcina* are acetate utilizing methanogen and their presence indicates source of acetate producing metabolism in protozoa.

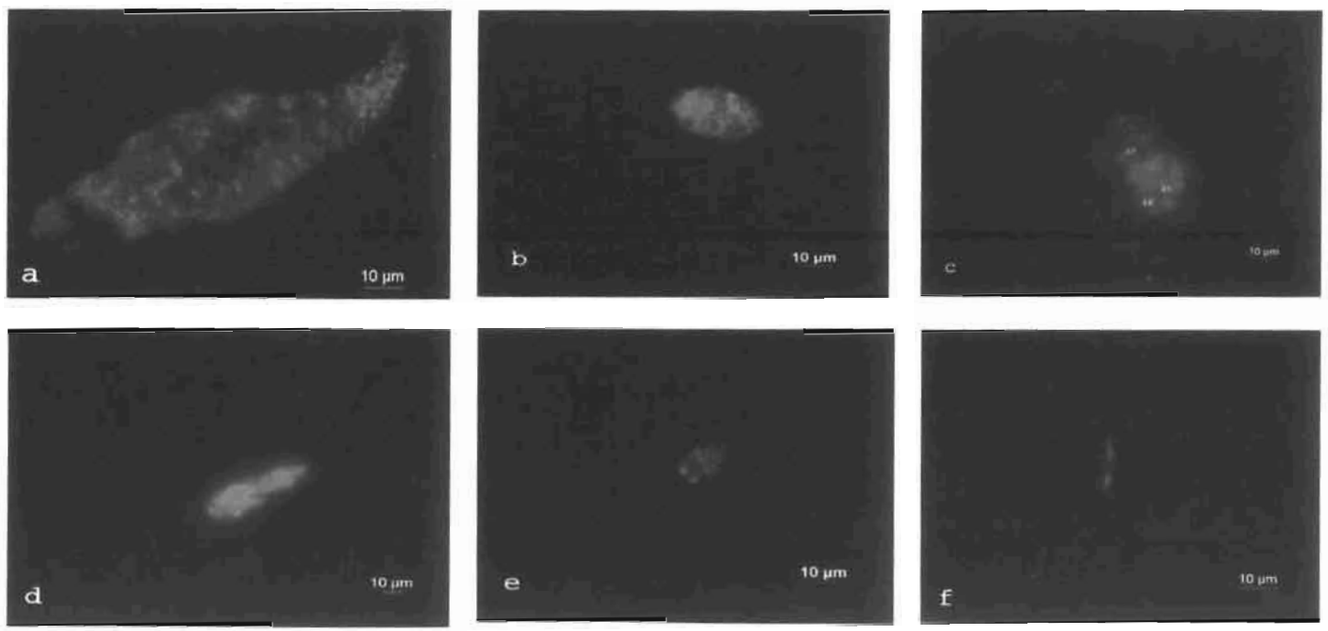


Fig. 5.12. FISH analysis showing endosymbiotic methanogens in (a) *Metopus* (b) *Spathidium* (c) *Prorodon* (d) *Cylidium* (e) *Paranema* and (f) *Menoidium*

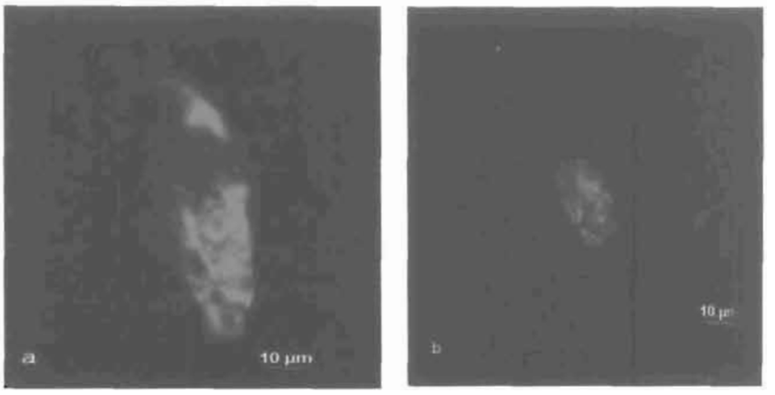


Fig. 5.13. In Situ Hybridization with MX 825 showing the presence of *Methanosaeta* as endosymbionts in (a) *Metopus* and (b) C*yclidium*

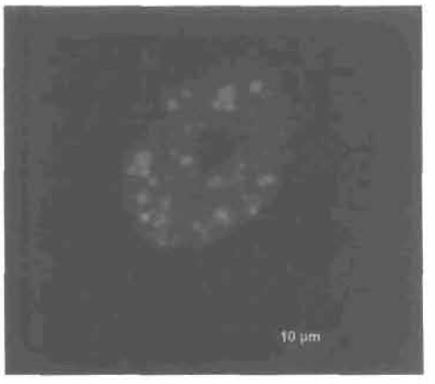


Fig. 5.14. In Situ Hybridization with MS 821 showing the presence of *Methanosrcina* as endosymbionts in protozoa *Prorodon*

Presence of rod shaped methanogens was found in the *Spathidium* when it was subjected to FISH analysis using ARC 915, the universal archeal probe (Fig. 5.11). FISH analysis with specific probes MS 821 and MX 825 did not show red fluorescence in ciliate *Spathidium* confirmimg the absence of *Methanosarcina* and *Methanosaeta* as endosymbionts. The conically pointed gram positive rods in the homogenized cell suspension resembled to M*ethanobacterium sp.* (Fig. 5.15). *Methanobacterium* being a hydrogen utilising methanogen, its presence indicates the availability of hydrogen in S*pathidium*. *Methanobacterium*, *Methanoplanus* and

Methanocorpusculum were the commonly observed endosymbionts in anaerobic ciliates (Embley and Finlay, 1993 and 1994). This is the fisrt report showing *Methanosaeta* and *Methanosarcina* as endosymbiotic methanogenic bacteria in ciliates. It has been reported that fermentative energy metabolism of host protozoa includes the fermentation of pyruvate to acetate, formate and hydrogen (Goosen et al., 1990b). So endosymbionts can utilise the hydrogen and acetate produced by hosts. The symbiotic consortium therefore can produce methane as a metabolic end product. The present study thus confirms enhanced anaerobic degradation and methane production by the presence of protozoa, especially ciliates in the anaerobic systems.

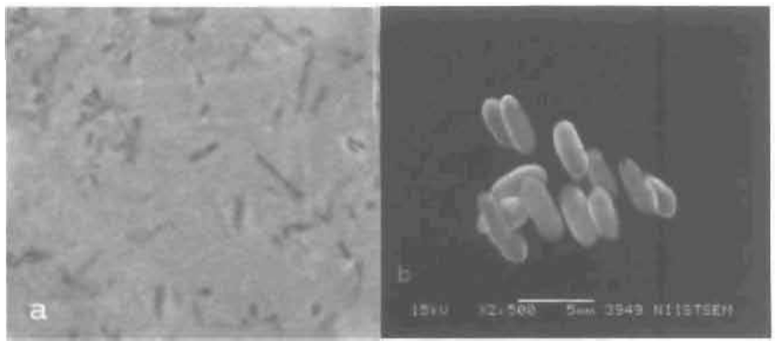


Fig. 5.15 (a) Gram-positive cells in the homogenized cell suspension and (b) SEM showing conically pointed rods resemble to *Methanobacterium*

The results suggest that stimulation of methane production with higher counts of ciliates in anaerobic systems is mainly due to the endosymbiotic methanogens. In the reactor (CSTAR operated at 1 g COD. $l^{-1}.d^{-1}$ at 5 days HRT), an average density of free methanogens at steady state was 9 x 10^7 ml^{-1} and average ciliate density reached up to 8.5 x 10^4 ml^{-1}. Table 5.6 illustrate endosymbiotic methanogens in individual cells of protozoa in anaerobic reactor at steady state. The total number of endosymbiotic methanogens accounted approximately to 2.99 x 10^7 ml^{-1} in anaerobic reactors at steady state (Table 5.6). In brief, the endosymbiotic methanogens reached to about one third of the total free methanogens present in the reactor. The presence

of such a large number of endosymbionts confirms that they are potential producer of methane in biomethanation process.

A cell-specific methane production rate is assumed as 0.97 fmol CH_4 endosymbiont^{-1} h^{-1} (Finlay and Fenchel, 1991) and calculated the cumulative methane production from endosymbionts as shown in Table.4. According to this model, the protozoa had contributed 33% to methanogenesis. Therefore, anaerobic protozoa with methanogens were capable of making a significant contribution to methane generation in anaerobic systems.

Table. 5.6. Anaerobic protozoa and endosymbionts in anaerobic reactors at steady state (CSTAR operated at 1 g COD. $l^{-1}.d^{-1}$ at 5 days HRT)

Protozoa genera	Density of Protozoa (x 10^4) at steady state	Avg.No. Methanogens/ protozoa	Total endosymbionts (x 10^6)
Metopus	2.5	818	20.45
Spathidium	0.5	46	0.23
Prorodon	2	389	7.78
Cyclidium	2	58	1.16
Paranema	1	14	0.14
Menoidium	1	16	0.16
Total	**8.5**		**29.92**

The methanogensis from anaerobic protozoa was measured in batch cultures (Fig. 5.16). In repeated washing steps during the protozoa inoculam preparation, free living methanogens possibly sticking to outer surface of the protozoa were likely to be removed. Therefore, the resulting methane production could be attributed to the methanogens closely associated to protozoa, endosymbionts. The intensity of apparent methane production by anaerobic protozoa was primarily depended on the number of endosymbiotic methanogens. So the contribution of protozoa to methanogenesis was varied among different genera. It is very difficult to quantify methane production of individual genus. So mixed culture of protozoa were used in this study.

The methane production had maximum rates during exponential growth and it was declined after exponential phase due to the low growth efficiency of anaerobic protozoa (Fig 5.16). A maximum of 0.45 ppm was observed with 2.75 x 10^4 ml^{-1} protozoa during exponential stage. The rate of methane production was proportional to cell densities in the anaerobic culture. Initially, the cell density was 0.125 x 10^4 ml^{-1} and the corresponding methane production (at 6 h) was 0.033ppm. Similarly, subsequent to exponential growth, there was a decline of protozoa to 1.5 x 104 ml^{-1} as well as methane production to 0.113ppm (Fig. 5.15).

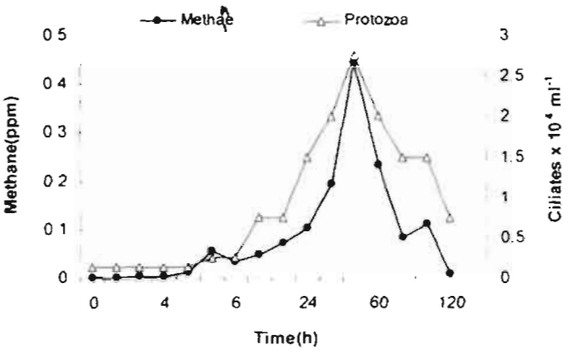


Fig. 5.16. Methane production measured in head space of protozoa culture without free methanogens

The production of methane through endosymbiotic methanogens has been reported in anaerobic ciliates (Fenchel and Finlay, 1992). According to Finlay et al., (1994) about 37% of the total methane production from sheep has been attributed to the presence of methanogenic ciliates.

5.3.3 Role of protozoa in biomass reduction

Fig. 5.17 illustrates the changes in mixed liquor suspended solids concentrations in CSTARs with and without protozoa inhibition. Results showed that MLSS was significantly lower (16 - 34 %) in CSTAR with protozoa. Ciliate numbers were well correlated (R^2 = 0.87) to MLSS in CSTAR with protozoa. This interpreted as grazing activity of ciliates reducing the sludge biomass while enhancing COD

removal (Fig. 5.18). In other words, the specific methanogenic activity of sludge formed in the reactors with ciliates is better than in reactors that do not have ciliates. The removal of MLSS is expected in the reactors since suspended biomass is the major feed for protozoa. Protozoa, mainly ciliates are the only predators in anaerobic environments with reduced growth yields (Fenchel and Finlay, 1990a). Grazing activity of ciliates has been demonstrated to be essential for the clarification and treating of wastewater in aerobic systems (Curds et al., 1968; Holubar et al., 2000). A t-test and f-test (F = 11.5 and F critical = 1.86) showed that the difference of means of MLSS in the presence and absence of protozoa is significant at the 95% confidence interval. Reduction in MLSS concentration with respect to ciliate number denoting that biomass produced in the anaerobic system is converted to biogas by the presence of ciliates.

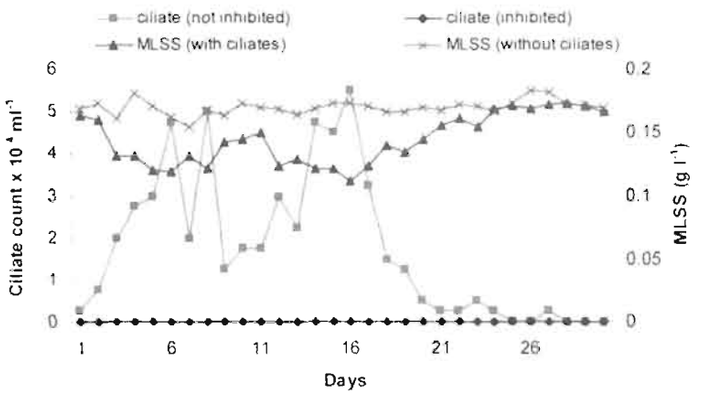


Fig. 5.17. MLSS and ciliate count in CSTARs with and without protozoa at a loading rate 2 g COD. $l^{-1}.d^{-1}$ of sodium oleate and 10 days HRT. (A sudden drop in ciliate count at day 40 was due to the change in pH after 39 days)

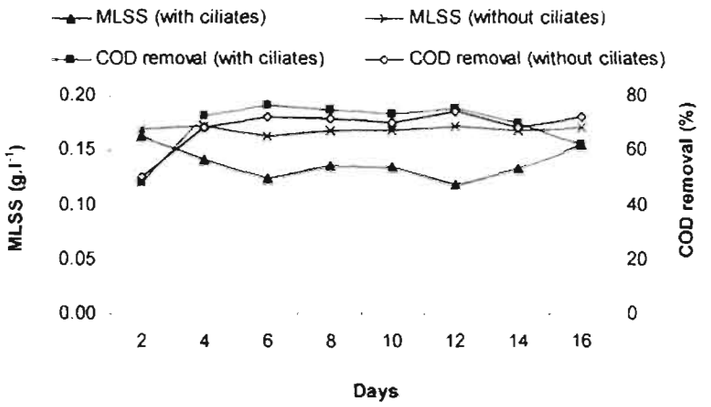


Fig. 5.18. Comparison of COD removal and MLSS in CSTARs with and without protozoa at a loading rate of 2 g COD. $l^{-1}.d^{-1}$ sodium oleate and 10 days HRT

Fig. 5.19 shows the microbial succession in anaerobic CSTAR fed on oleate feed. Initially, bacteria were proliferated rapidly, but their number was subsequently decreased due to grazing by increasing number of flagellates and amoeba. These were eventually displaced by ciliates. Such changes in the microbial population may be due to the predatory advantage of ciliates which ingest large bacteria (escaped from predation by flagellates) more efficiently and the smaller flagellates. Gradually, under steady condition all these organisms maintain a stable and active population. This is resulted in the stimulated COD removal, MLSS reduction and nutrient recycling in CSTARs. A negative correlation was obtained in correlation analysis between MLSS concentration and ciliate density. A reduction of more than 25 % (± 5) MLSS resulted with $5x10^4$ ml^{-1}ciliates (Chapter 3). The result indicates that the protozoa indeed can reduce sludge production in treatment plants. This also leads to conclude active involvement protozoa in anaerobic degradation.

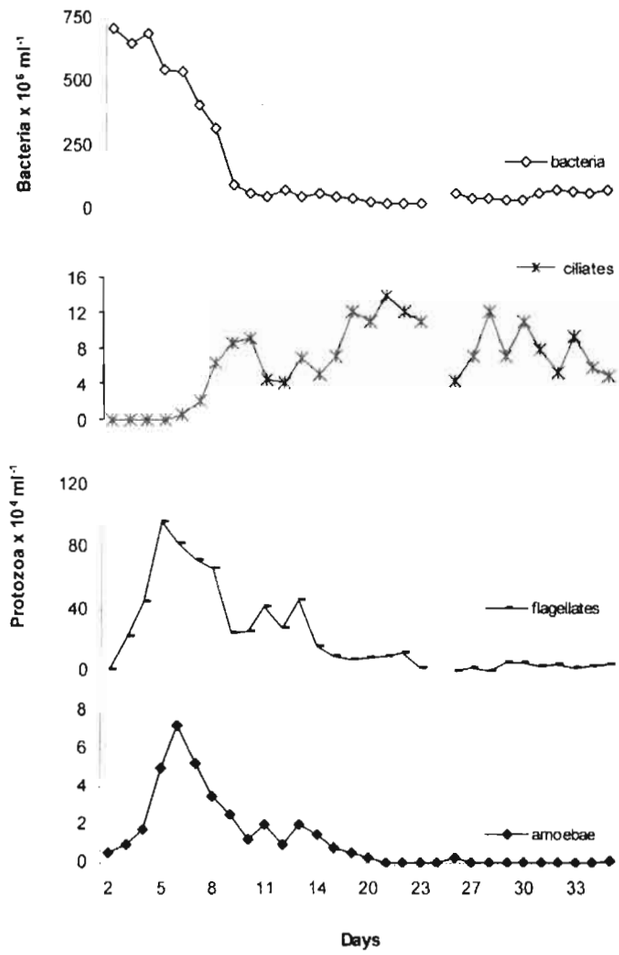


Fig. 5.19. The microbial succession in anaerobic CSTAR fed on oleate feed

5.3.4 Role of protozoa in turbidity removal

Fig. 5.20 illustrates the result of turbidity removal with the presence and absence of protozoa. The turbidity of effluent in the reactor was inversely proportional to the count of ciliates because they were the dominant grazers in anaerobic systems. The effluent turbidity of the reactor decreased with increase of

ciliate count. A t-test and f-test (F = 7.13 and F critical = 0.94) showed that the difference of means of turbidity removal in the presence and absence of protozoa is significant at 95% confidence interval. The bacterial grazing rate was related to size of the protozoa, bacterial density (Chapter 4) and feeding mechanism. Feeding mechanism is the other important factor that determines the grazing rate. The relative size of the protozoan to its prey dictates the most efficient feeding mechanism (Fenchel, 1986). The predator prey size ratio exceeds 10:1, filter feeding prevails and the ratio is smaller than 10:1. Raptional feeding is more common (Fenchel, 1986). Ciliated protozoa are reported to produce good quality effluents in aerobic treatment plants because of their ability to feed on bacteria (Curds et al., 1968).

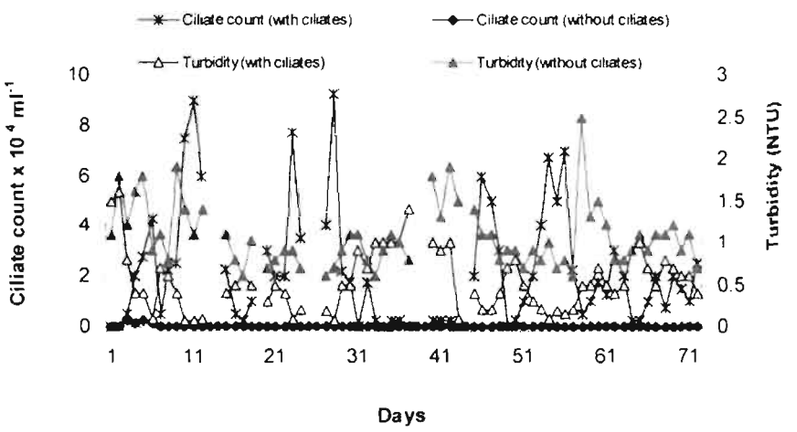


Fig. 5.20. Correlation between effluent turbidity and abundance of ciliates in CSTAR operated with loading rate of 1g COD l^{-1} d^{-1} at 5 days HRT

The bacterial grazing rate of protozoa was determined in batch experiments by feeding heat killed bacteria. The mean ingestion rates of protozoa are given in Table 5.7. The average ingestion of bacteria cells by the protozoa h^{-1} was 91.79 x $10^2 ml^{-1}$ ($\pm$8.5). Live bacteria was not used as food to avoid their multiplication and possible change in population.

Table. 5.7. The bacterial feeding rate (bacteria h^{-1}) of anaerobic protozoa in batch experiments

Time(h)	Feeding rate (x 10^2 bacteria h^{-1})
1	63.42
2	72.53
3	98.76
5	109.99
4	98.78
6	74.98
7	106.13
8	109.78
9	67.85
10	112.43
11	101.21
12	85.7

The maximum grazing was obtained with ciliates as evidenced by Fig. 5.21. A decline in number of protozoa was observed with decrease in bacterial count (Fig. 5.21). This implies that prey concentration was an important factor to decide grazing of protozoa, mainly the ciliates. It was found (chapter 4) that protozoa needed a minimum bacterial density of 3 x 10^4 cfu ml^{-1} in order to maintain their growth and the maximum growth rates of protozoa were reached at bacterial densities in the range of 4.5 x 10^6 - 2 x 10^9 cfu ml^{-1}. Grazing rate of protozoa was showed variation among different genera. It was difficult to distinguish grazing rate of each genus because mixed cultures were used in this study. The results indicate that protozoa, mainly ciliates were capable of producing good quality effluents by feeding on bacteria and suspended particles. The anaerobic protozoa are found to be essential in the operation of biological treatment processes, because they are playing significant roles not only in biodegradation, but also in maintaining the balance of different groups of microorganisms.

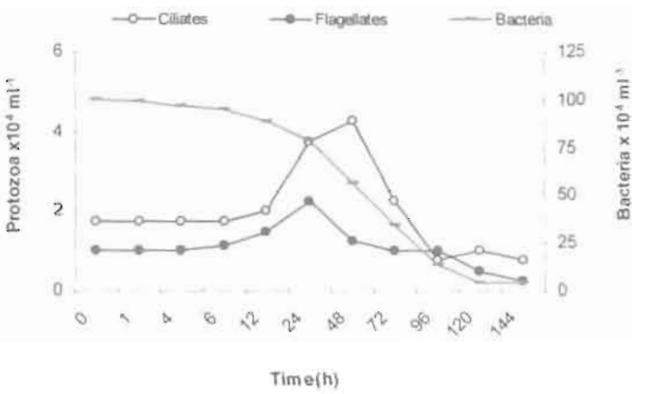


Fig. 5.21. The grazing rate of protozoa in batch anaerobic cultures.

5.3.5 Role of protozoa in nutrient recycling

Bioreactors periodically require nutrients to support stable microbial growth. Fig 5.22 illustrates the performance of CSTAR with and without external addition of nutrients. The results show that performance of the anaerobic reactor was not affected or compare to the nutrient supplemented system. The stable COD removal in CSTARs was observed without any external addition of nutrients for a long time. The continuous reactor performance without any external nutrients could be expected from protozoa nutrient recycling. During the period, the reactors maintained a level of ammonia 34 mg l^{-1} to 68 mg l^{-1} mg without external addition. This might be due to the excretion of nutrients mainly by protozoa through bacterial grazing. Protozoa are mainly considered as bacteriovoruos and often represent main bacterial grazers in anoxic environments (Fenchel and Finlay, 1991a).

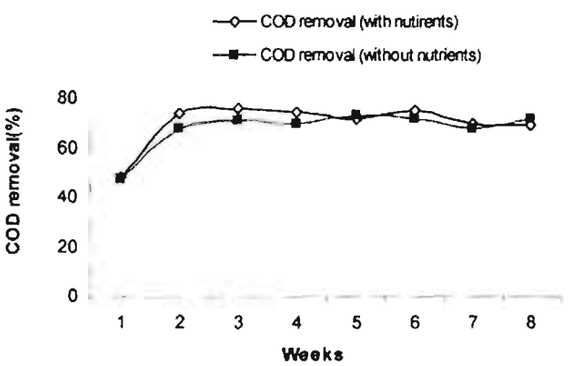


Fig. 5.22. The enhanced COD removal in CSTARs without any external addition of nutrients

Recycling of nutrient by protozoa was studied in batch experiments. Nutrients excretion rate of the protozoa was measured by feeding killed and live bacteria. The results of control tests shows that bacterial cultures without protozoa had no significant excretion of nutrients by bacterial cell lysing or excretion (Table 5.8).

Table 5.8. Nutrients content in control experiments with heat killed and live bacteria in the absence protozoa

Bacteria	Density (x 10^8)	Ammonia-N(ppm)	Phospahte-P(ppm)
Heat killed bacteria	3.2	0.0467	0.0141
Live bactria	4	0.051	0.0168

The protozoa count was higher in live bacteria cultures than in cultures with killed bacteria (Fig. 5.23 and 5.24). Excretion rates of nutrients were measured during the whole growth phase of protozoa population. The amount of ammonia-N and phosphate-P excreted were varied with growth of protozoa. There was a notable difference in ammonia-N and phosphate-P excretion with live and heat killed bacteria. Experiment with live bacteria had low level of ammonia-N and phosphate-P and this could be due to the bacterial absorption of the excretions of protozoa. In heat

killed bacteria, the rates represented the actual excretion rate in the absence of bacterial uptake. So the actual amount of nutrients excretion by protozoa could have obtained in the culture supplemented with heat killed bacteria as food source.

The ammonium-N excretion rates ranged from 1.14 to 5.64 ppm in ciliates culture and 1.09 to 6.2 ppm in flagellates culture. The maximum excretion rates obtained during the exponential phase. The ammonium-N excretion value observed at exponential stage of ciliate culture was 3.15 ppm l^{-1} in live bacteria and it was 5.64 ppm l^{-1} in heat killed bacteria (Fig. 5.23).

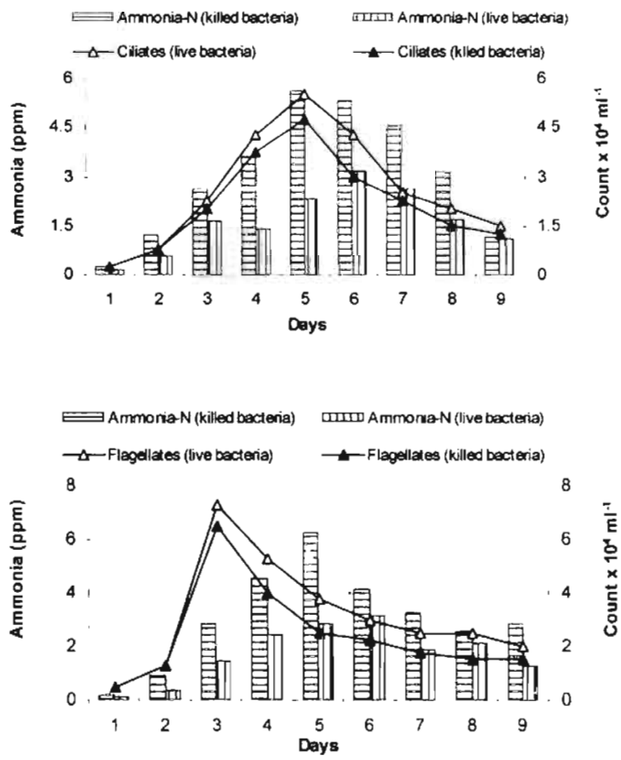


Fig. 5. 23. Growth of protozoa and ammonia-N excretion rates in anaerobic batch experiments (a) ciliates culture and (b) flagellate culture

Trend in phosphate excretion levels in the anaerobic culture was identical to those of ammonia-N. It was ranged from 0.047 to 1.5 in ciliates culture and 0 .034 to 1.7 was in the flagellates culture (Fig. 5.24). The higher phosphate-P excretion values were at exponential phase of culture. The maximum nutrient generation at exponential stage must be due to the higher abundance of organisms. Protozoa grazing on microbes appears to stimulate the activities of microbial community by increasing the turnover rate of essential nutrients that would otherwise locked in bacterial biomass. Excretion of mineral nutrients (phosphorous as phosphate, nitrogen as ammonia or nitrate) by protozoa has been recognized as a means for accelerated activity in rumen (Coleman et al., 1978).

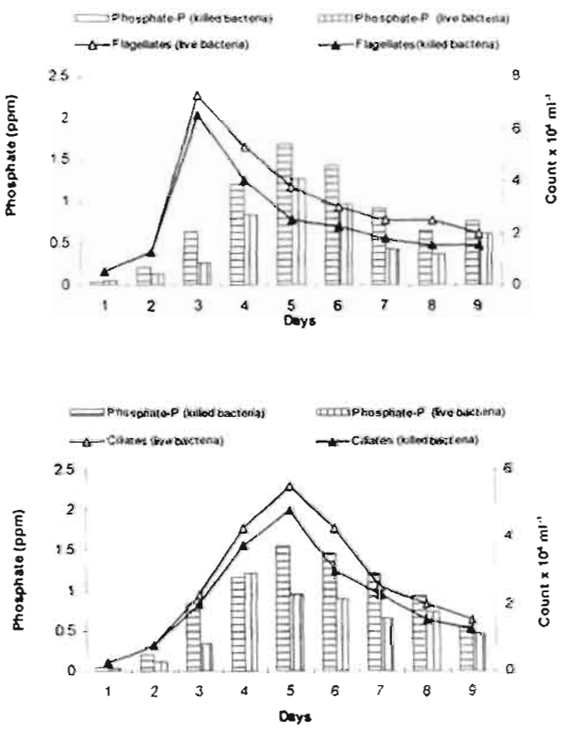


Fig. 5.24. Growth of protozoa and phosphate-P excretion rates in anaerobic batch experiments (a) ciliates culture and (b) flagellate culture

Bacteria are mainly considered as responsible for anaerobic biodegradation that require growth supporting elements in proper ratio of C:N:P - 50:14:3 (Jefferson et al., 2001). The excretion of mineral nutrients such as phosphate-P and ammonia-N by protozoa allows their better availability in the system. The CSTAR experiments infer that consumption of bacteria by protozoa have the highest biomass specific excretion rates of inorganic ammonia-N and phosphorous-P. Thus grazing stimulates the decomposition of organic matter in the anaerobic system. Protist excretions have been the important source of remineralized nutrients, and of the colloidal and dissolved trace metals such as iron, in aquatic ecosystems (Sherr et al., 2002). These observations imply that protozoa have another important role of nutrient recycling in anaerobic reactor.

5.3.6 Role of protozoa on oxygen toxicity removal

Table 5.9 shows the changes in protozoa population from anaerobic to aerobic conditions and, then aerobic to anaerobic conditions. More than 50% of the cells died in the first day, but most of the remainder survived with reduced mortality. It is interesting that different species of protozoa have different preference with respect to oxygen tension. Ciliate genera like *Metopus, Brachonella* and *Plagiopyla* and, the flagellates *Trepomonas* and, *vanella* were more sensitive to oxygen and 100% mortality was observed on 4th day. The ciliate genera *Cyclidium and Spathidium* and, the flagellates *Menoidium* and *Rhynchomonas* and *Amoeba* were shown to survive in complete aerobic condition and the growth was slightly higher or similar to their growth in anaerobic condition. Protozoa genera *Colpoda, Vorticella, Cercomonas* and *Naeglaria* survived under aerobic conditions were not always reproducible, sometimes the cells could only survive and were not multiplied.

Growth of new species with the beginning of aerobic condition was noticed. *Euplotes* observed on 2nd day and was disappeared on 4th day. Another important change was the formation of cysts. The number of thick walled cysts increased with arrival of aerobic condition. When it was returned to anaerobic condition, the resulting number of cells was almost identical to the control kept in permanent anaerobic condition (Table 5.9). These results indicate that some anaerobic protozoa

could be transferred from anaerobic to aerobic condition or aerobic to anaerobic condition without displaying any obvious change in the growth characteristics.

Some of the anaerobic protozoa were able to grow aerobically as well as anaerobically, and which can be considered as facultative anaerobes. When they are returned to anaerobic condition from aerobic, the resulting number of cells was almost identical to that of permanent anaerobic control. So exposure to oxygen produced no lasting damage to the anaerobic protozoa (Table 5.9). The type of energy metabolism employed by these organisms under aerobic conditions is unknown. Bernard and Fenchel (1996) have reported existence of some microaerobic ciliates as facultative anaerobes. In amoeboflagellate, *Psalteriomonas lanterna*, a transformation of the flagellate to a mononucleated amoeba stage have been reported in 2-5 % O_2 tension (Broers et al., 1992). However, the occurrence of facultative anaerobic protozoa offers an important role in anaerobic systems.

Table 5.9. Changes in protozoa population with respect to oxygen stress during 24 days experimentation

	Anaerobic (x 10^4 ml^{-1})	Transition stage (anaerobic to aerobic) (x 10^4 ml^{-1})				Aerobic (x 10^4 ml^{-1})	Transition stage (aerobic to anaerobic) (x 10^4 ml^{-1})				Anaerobic (x 10^4 ml^{-1})	Control (x 10^4 ml^{-1})
Days	0	1	2	4	8	12	13	14	16	20	24	24
Vanella	0.13	0.07	0.25	-	-	-	-	-	-	0.05	0.13	0.063
Amoeba	0.13	0.25	0.13	0.25	0.13	0.13	0.25	0.25	0.13	0.13	-	0.13
Naeglaria	1	0.25	0.5	0.5	0.25	0.25	0.07	0.13	1	0.75	0.5	0.75
Cercomonas	1	0.5	0.75	0.75	0.75	0.5	0.5	1.75	0.5	1	0.5	0.75
Trepomonas	0.25	-	0.03	-	-	-	-	-	0.03	0.5	0.5	0.5
Tetramitus	1.5	0. 5	0.25	0.13	-	-	0.05	0.03	0.13	0.75	0.75	1.75
Rhycomononas	2.75	2	2.5	3.25	3.25	3.5	3.25	3.25	2.5	2.5	2.75	3.5
Menoidium	1.25	1.5	3	6.5	6.75	6	4.5	3	6	7.5	3.5	1.25
Colpoda	0.5	0.03	0.05	0.05	0.05	0.05	0.02	0.05	0.25	0.13	0.13	0.5
Cyclidium	2.5	1.5	2.5	3.25	3.75	3.5	2.75	1.75	3.5	3	2.25	3
Plagiopyla	0.25	0.05	0.05	-	-	-	-	-	0.03	0.13	0.25	0.25
Spathidium	0.25	0.07	0.13	0.25	0.25	0.25	0.25	0.13	0.5	0.25	0.25	0.25
Brachonella	0.5	0.13	0.05	-	-	-	-	-	-	0.13	0.5	0.25
Vorticella	2	0.5	0.25	0.13	0.13	0.25	0.25	0.75	1.25	1	1.75	2
Metopus	2.5	0.5	0.05	-	-	-	-	-	-	0.75	2.25	3
Euplotus	-	0.07	0.13	0.05	-	-	-		0.05	0.07	-	-
Cyst	-	9	14	18	19.5	19	19	15.5	12	4	-	-

*Protozoa count = **x 10^4 ml^{-1}**

Effects of oxygen on methanogens

Another consequence of oxygen toxicity was the loss of methanogens, both free and endosymbiotic. Successive loss of methanogens was noticed under oxygen tension and it was completely absent on 8th day (Fig. 5.25). It is obvious from the results that methane production was also decreased with the loss of methanogens (Fig. 5.25). Methane production was also stopped in the experimental set up under oxygen tension. The COD removal was more or less same with changes from anaerobic to aerobic conditions (Fig. 5.26).

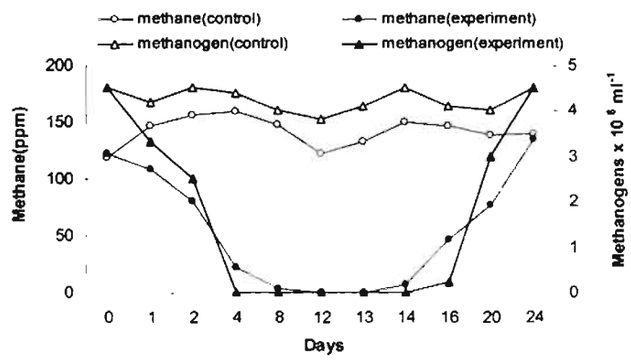


Fig. 5.25. Effect of oxygen on methane production and methanogens in 24 days experimental setup

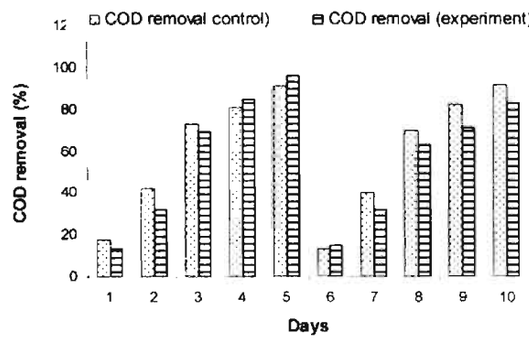


Fig. 5.26. COD removal in 24 days experimental setup

Detailed analysis of the anaerobic protozoa in the experimentation revealed that the decrease of auto fluorescing endosymbionts to about 10% of the original number on 2nd day in the survived ciliate *Metopus* and on the 4th day the fluorescence was very week and difficult to differentiate from the background (Table 5.10). The disappearance of methanogens from the sludge with aeration was confirmed by FISH analysis. However, the methanogens were observed in protozoan cysts formed on oxygen exposure (Fig. 5.27).

Table 5.10. Endosymbiotic methanogen fluorescence as function of oxygen tension in batch experimental setup (+ fluorescence normal, - no fluorescence and ± only a small fraction of cells showed fluorescence)

	Anaerobic	Transition stage (anaerobic to aerobic)				Aerobic	Transition stage (aerobic to anaerobic)				Anaerobic
Days	0	1	2	4	8	12	13	14	16	20	24
Control	+	+	+	+	+	+	+	+	+	+	+
Experiment	+	+	+	±	-	-	-	-	±	+	+

Fig. 5.28 illustrate the survival of methanogens inside the protozoa cyst and its dispersal under favorable conditions. On returning to anaerobic condition, the methanogens were first appeared in the sludge and then in protozoa as endosymbionts. Cysts were disappeared completely on restoring anaerobic condition. Exposure to oxygen has been reported to result in destruction of F_{420} hydrogenase enzyme complex (Zehnder, 1988). However, the methanogens which are retained inside the cyst are protected from oxygen tension (Fig. 5.28). Thus, the results revealed a possible mechanism for methanogens to escape from oxygen toxicity by cyst formation.

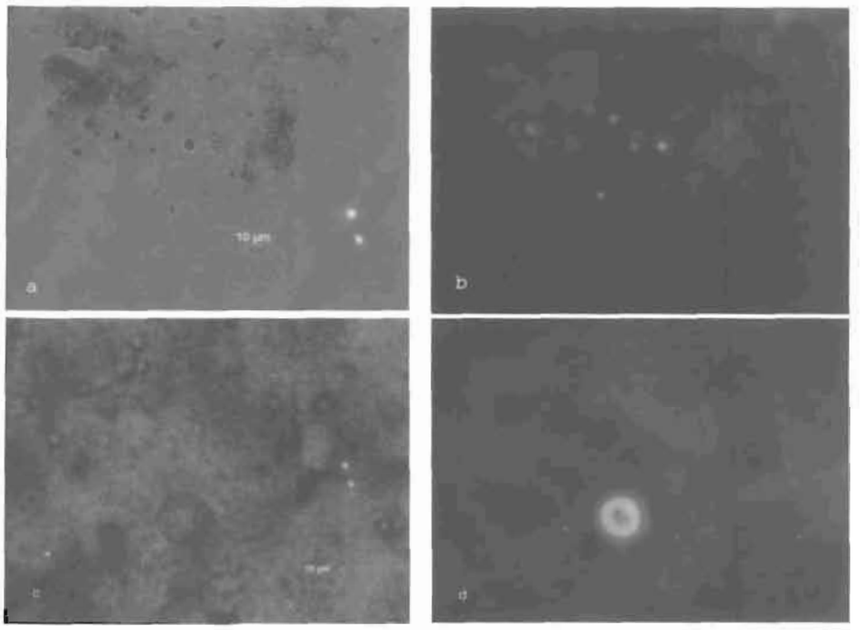


Fig. 5.27 Micrographs of (a) sludge with small cysts under oxygen tension (b) FISH showing methanogens in cyst under oxygen tension (c) sludge with large cyst under oxygen tension (b) FISH showing methanogens in large cyst under oxygen tension

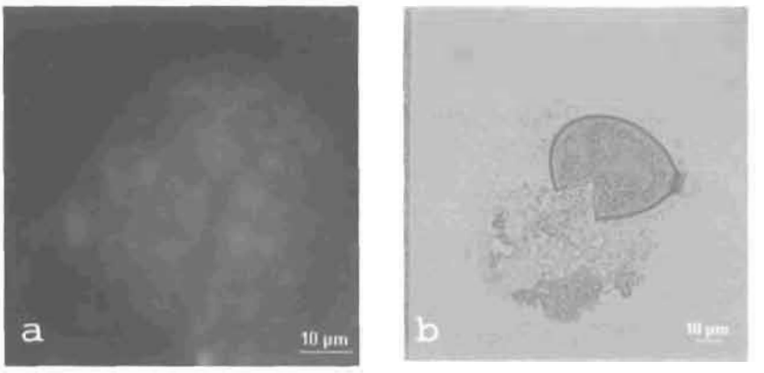


Fig. 5.28 (a) FISH showing methanogens in cyst with oxygen tension and (b) cyst breakage at favourable anaerobic condition.

Enzymatic activities with respect oxygen tension

The enzyme activity was tested in survived protozoa cells (facultative anaerobes). Catalase activity was not detected in the protozoa. In protozoa cells survived at aerobic condition, the protozoa cell free extracts were assayed for SOD and it was found to be 2.5 ± 0.5 U. mg^{-1} protein.

The oxygen toxicity is mainly due to the formation of highly reactive oxygen species (ROS) which are capable of damaging a wide range of biomolecules (Imai and Linn, 1988). Superoxide dismutase in facultative anaerobic protozoa could be a protective mechanism against toxic super oxide radicals generated under low oxygen tensions. The absence of catalase and presence of SOD activity in agreement with the theory of Mc Cord et al., (1971) that acrotolerence is depends on the presence of super oxide dismutase and not necessarily on the presence of catalase.

In anaerobic reactors, there is a probability for oxygen toxicity while feeding. The aerotolerant protozoa with SOD activity could not affect the entry of small level of oxygen and thereby the reactors activities are continued. Thus the oxygen toxicity in the treatment of wastewater in anaerobic reactor is managed by the presence facultative anaerobic protozoa.

5.3.7 Other activities

Stimulation by microturbulanace

Protozoan growth and activity can stimulate bacterial activity due to the microturbulance as it has been reported from aerobic wastewater treatment (Curds et al., 1968). Individual protozoa are too small to influence the activity by water movements. This can be magnified by a group of larger ciliates such as *Vorticella, Metopus* and *Euplotes*.

Good indicators of reactor performance

As discussed in chapter 3, anaerobic protozoa, mainly ciliates are more susceptible to sudden changes in the system. Changes in protozoa genera were observed with changes in condition such as high VFA and pH variations. The populations of protozoa vary considerably with the changes in the anaerobic reactors, which can be considered as an indicator of system performance in anaerobic process.

5.4. Conclusions

The present study reveals the involvement of protozoa, especially ciliates in anaerobic reactors for the first time.

- CSTARs and batch system showed an obvious relation between abundance of ciliates and enhanced biodegradation. COD removal is strongly correlated to ciliate density in both acetate and oleate fed CSTARs (R^2 = 0.974 and R^2 = 0.966) at all hydraulic retention times and loading rates. The enhanced COD removal was observed in CSTARs with protozoa (more than 75%) compared to the protozoa controlled CSTARs at the same loading rate and retention time.
- Direct uptake of dissolved COD by flagellates and particulates by ciliates in bacteria-suppressed cultures confirms that they are not purely dependent on grazing of bacteria as food source and it can also consume organic matter. This understanding has great importance in anaerobic degradation because anaerobic protozoa have been reported as grazers of bacteria in anaerobic environments.
- Size selective feeding of particles confirms that ciliates can be voracious grazers of particles and each species has a diverse range of preferred particle size which may be depend upon its mouth size and morphology.
- The direct consumption of particulates by ciliates in anaerobic system tries to explain their significance beyond bacterial grazing. So the presence of protozoa in anaerobic wastewater treatment is the means to overcome the rate limitation caused by solubilization of solids.
- The present study confirms enhanced anaerobic degradation and methane production by the presence of protozoa, especially ciliates in anaerobic systems. The methane production had strong correlation to ciliate density in the continuously fed anaerobic reactors (R^2 = 0.99 and R^2 = 0.97) at all hydraulic retention times and loading rates. Methane produced was significantly lower (31 $\pm$ 2 %) in the absence of protozoa.
- The enhanced methane production with the presence of protozoa was correlated with presence of endosymbiotic methanogens in anaerobic

systems. The number of methanogens in the protozoa was varied with respect to genera. The larger ciliate like *Metpous* had a large number of endosymbionts compared to other ciliates and flagellates which harboured minimum number of methanogens

- The distribution of symbiotic methanogens inside the protozoa was varied with organisms and FISH analysis with specific probes showed the presence of *Methanosaetacea, Methanosarcina* and *Methanobacterium* as endosymbionts in anaerobic protozoa.
- *Methanosaeta* and *Methanosarcina* are acetate utilizing methanogen, its presence indicates that an acetate producing metabolism can be present in protozoa. *Methanobacterium* being a hydrogen utilising methanogen, its presence indicates the availability of hydrogen in *Spathidium*. Endosymbionts can utilise the hydrogen /acetate produced by the hosts. The symbiontic consortium therefore produces methane as a metabolic end product.
- In the reactor, at steady state an average density of endosymbiotic methanogens are estimated to about one third of the total free methanogens in the reactor and therefore anaerobic protozoa act as a potential producer of methane in biomethanation process.
- Anaerobic protozoa in wastewater treatment plants are capable of contributing a significant contribution in methane production as much as 33%.
- The enhanced growth of ciliates in the anaerobic reactors could increase COD removal with significant reduction of MLSS. Ciliate numbers were negatively correlated (R^2 = 0.87) to MLSS in CSTAR with protozoa and MLSS was significantly lower (16 - 34 %) in CSTAR with protozoa. So protozoa could reduce MLSS and increase sludge activity.
- Bactriovorous activity of protozoa was an important factor in the removal of bacteria from anaerobic wastewater treatment systems. The average ingestion rate of protozos h^{-1} was 91.79 x $10^2 ml^{-1}$ (±8.5).
- In anaerobic treatment plants, anaerobic protozoa can produce good quality effluents because of their ability to feed on bacteria and suspended particles.

- Protozoa plays an important role in self purification and matter recycling in treatment plants. The enhanced activity was observed in CSTARs without any external addition of nutrients for a long time. This is due to the excretion of mineral nutrients such as phosphate-P and ammonia-N by protozoa in anaerobic systems.
- Consumption of bacteria by protozoa have the highest biomass specific excretion rates of inorganic ammonia-N and phosphorous-P. Association of bacteria and protozoa can do a significant role in nutrient recycling in anaerobic treatment systems.
- Some anaerobic protozoa could transfer from anaerobic to aerobic condition or aerobic to anaerobic condition without displaying any obvious change in the growth characteristics. The occurrence of facultative anaerobic protozoa offers an important role in anaerobic systems.
- Consequence of oxygen toxicity was the loss of methanogens, both free and enosymbiotic. However, the methanogen bearing protozoa cyst formation in aerobic environments is a potential mechanism to overcome unfavorable changes.
- Superoxide dismutase in facultative anaerobic protozoa forms a protective mechanism against toxic super oxide radicals generated under oxygen tensions. Significance of facultative protozoa with SOD activity in anaerobic reactors is the ability to overcome possible occurrence of oxygen toxicity.
- The flocculation activities of anaerobic ciliates in particular lead to increased turnover rate of organic matter in anaerobic process

Thus, the protozoa, mainly ciliates prove as significant partner in the biomass of anaerobic wastewater treatment process.

Chapter 6
Protozoa mediated biodegradation in anaerobic systems

Abstract

The ability of protozoa to degrade various compounds which is generally present in wastewaters were analysed in batch reactors. The study revealed, active involvement of protozoa in the degradation of organic matter in the anaerobic system. The co-existence of ciliates and bacteria had higher organic matter removal and methane yield. The protozoa produced acetate and methane as major end products and small amounts of lactate, pyruvate, butyrate, carbon dioxide and hydrogen in cultures with endosymbionts. Loss of endosymbiotic methanogens was observed with continued culturing of the ciliates. They were found capable to ferment food without endosymbiotic methanogen by changing its metabolic pathway. Butyrate and propionate were the major end products along with acetate in symbiont free cells. The studies indicated that protozoa can utilize different saccharides, polyphenols, proteins and fatty acids. The enzymatic degradation of various compounds by protozoa form an additional pathway (Organic matter → anaerobic protozoa) of carbon in anaerobic food along with bacterial degradation. The present study reveals the biodegradation of various organic compounds by protozoa with extracellular and intracellular enzyme activities for the first time.

6.1 Introduction

The degradation of complex organic matter to methane and CO_2 is a widespread process in anaerobic environments. It is considered that the conversion of complex organic matter to simple molecules and finally to methane and carbon dioxide under anaerobic environments is possible only by the combined action of different groups of bacteria (Gujer and Zehnder, 1983; Mosey and Fernandez, 1989). However, a variety of protozoa are found to associate with different anaerobic biodegrading microbial communities (Fenchel and Finlay, 1995; Agrawal et al., 1997, Priya et al., 2007a).

Protozoa have been considered as unfavourable to the biodegradation process and have proposed that protozoa species play only a predatory role, mainly consumers of bacteria (Jouany and Ushida, 1999). On the contrary, studies revealed that reactors with protozoa rich sludge can enhance the rate of mineralization of complex wastewater especially wastewater containing particulate COD (Chapter 5). One of possible mechanism for enhanced degradation might be the direct utilization of various organic matters by protozoa. The aim of this study was to investigate enzymatic degradation of various organic compounds by anaerobic protozoa.

6.2 Materials and methods

6.2.1 Preparation of protozoa culture

Direct uptake of organic compounds by protozoa was studied in bacteria-suppressed cultures of protozoa. Anaerobic ciliates and flagellates were isolated from laboratory scale anaerobic reactor fed on dairy wastewater and cultured in ciliate mineral medium with different carbon source as described in Chapter 4. The organic compounds used to study degradation were glucose, sucrose, raffinose, starch, cellulose, xylan, pectin, tannic acid, lignin and fatty acids (Oleic and stearic acids). Anaerobiosis was maintained by the addition of cysteine HCl (0.05%). Resazurin (2mM) was used as redox indicator. Experiment bottles (50 ml amber Schott Duran bottles) were screw capped with silicon septa (Thomson Scientific, USA) and headspace was flushed with nitrogen. The cultures were incubated at 30 $\pm$ 2°C in triplicates and mean of the results were taken.

To evaluate potential activity and relative contribution to the degradation by specific microbial groups, following cultures were prepared: a mixed system (including both bacteria and protozoa), a bacterial system (only bacteria) and a protozoa system (only protozoa) and examined the removal of organic matter.

6.2.2 Enzyme assays

The extract for ezyme assays was obtained by disruption of cells in three cycles of freezing and thawing (-20°C for 10 min and 35°C for 10 min) followed by centrifugation at 5000*g* for 10 min. 10 ml of sample with known number of cells were

used for centrifugation. The unit of enzyme expressed in the result is the unit ml^{-1} of extract.

Glycosidase: The substrate for the determination of glycosidase activity was p-nitrophenyl D-glucoside. The assay mixture contained 2.0 ml of 1 mM p-nitrophenyl D-glucoside in 0.1 M sodium acetate buffer, pH 5.0, and 0.1 ml of enzyme extract solution. After incubation at 40°C for 30min, the enzyme reaction was stopped by adding 1 ml of 1 M Na_2CO_3 to reaction mixture and the absorbance was read at 405 nm using a UV-spectrophotometer (Shimadzu, UV-2401). One unit of glycosidase activity is defined as the amount of enzyme required to release 1 µmol of p-nitrophenol min^{-1} under the above conditions (Sang et al., 2005).

Galactosidase: Galactosidase activity was determined by the method of Mulimani and Ramalingam (1997). The reaction mixture consisted of 1 ml of 2mM p-nitrophenyl-α-D-galactopyranoside (PNPG) in water, 8 ml of 0.2 M acetate buffer (pH 5.0) and 1ml of enzyme extract. The reaction mixture was incubated at 37°C for 15 min, terminated the reaction by the addition of 0.2 M Na_2CO_3 (3 ml) and the liberated p-nitrophenol was measured at 405 nm. One unit of α-galactosidase activity is defined as the amount of enzyme required to release 1 µmol of p-nitrophenol min^{-1}.

Amylase: For a standard assay of amylase activity, 2.5 ml of 0.01 M acetate buffer (pH 6.0) containing an appropriate amount of enzyme extract solution was incubated with 2.5 of 2% soluble starch at 30°C and the reducing sugar produced was measured by the method of Somogyi and Nelson (1945) using maltose as standard. One unit of enzyme activity was defined as the amount of enzyme which liberates 1 µmol min^{-1} of maltose under the above conditions.

Different concentrations of enzyme extract were impregnated on top of agar plates with 1% starch and the plates were incubated at 40°C for 15-30 min. The hydrolysis zones were visualised by using acidic iodine solution (0.2% I_2 and 2% KI in 0.2 N HCl).

Cellulase: Cellulase activity was assayed (Mandels et al., 1976) by incubating filter paper (Whatman No. 1) 50 mg in 0.5 ml phosphate buffer pH 7 with 0.5 ml of crude extract extract at 50°C for 1 h. The reaction was terminated by adding 2 ml of dinitrosalicylic acid reagent followed by 5 min of boiling. Absorbance was

measured at 540 nm (Shimadzu, UV-2401). One unit cellulase activity was defined as the amount of enzyme that releases 1 µmol of glucose min^{-1}.

Different concentrations of enzyme extract (1-2 U) were impregnated on a small disc of filter paper and placed on top of agar plates with CM-cellulose (0.1%) and the plates were incubated at 40°C for 15 min. The hydrolysis zones were visualised by using Congo red (Teather and Wood, 1982).

***Xylanase*:** Xylanase activity was assayed using oat-spelt xylan (Sigma, USA) of 0.5% as substrate in pH 7 buffer at 50°C (Subramaniyan et al., 1997). One unit of enzyme activity was defined as the amount of enzyme liberating 1 µmol of xylose min^{-1}. Zones of hydrolysis of enzyme extract on agar plates with oat spelt xylan (0.1 %) were detected with Congo red (Teather and Wood, 1982).

***Pectinase*:** Polygalacturonase activity was determined using 2-cyanoacetamide method (Honda et al., 1982). Reaction mixture containing 0.5 ml of enzyme extract was incubated with 0.5 ml of 0.5% polygalacturonic acid in 100mM McIlvaine buffer (pH 7.0) for 30 min at 50 °C. The reaction was arrested by adding 2 ml of 100mM borate buffer (pH 9.0) followed by 1 ml of 1% cyanoacetamide. The mixture was immersed in a boiling water bath for 10 min, cooled and read against a suitable blank at 276 nm using a spectrophotometer (Shimadzu, UV-2401). One unit of Polygalacturonase was defined as 1 µmol of galacturonic acid released min^{-1} under the assay conditions.

***Protease*:** Protease was assayed (Subramaniyan et al., 2001) using 2% casein in 0.1M sodium phosphate buffer pH 7 as substrate. 0.5 ml of casein was incubated at 40°C for 10 min with 0.5 ml enzyme extract. The reaction was terminated by adding 1 ml of 10% ($w.v^{-1}$) TCA. The released tyrosine was estimated by Lowry's method using tyrosine as standard. One unit of protease activity was expressed as 1 µmol of tyrosine min^{-1} ml^{-1} of enzyme under assay conditions.

Protease assay plates containing 1% casein and 1% gelatin, in a solution of 1.5% agar were prepared. Because direct addition of casein to the media resulted in macroscopic casein aggregates and plates with poor readability, the casein was dissolved in 0.02 N NaOH and stirred until it formed a translucent solution. The media were adjusted to pH 7.0 with 1 N HCl (Thomas, 1983). Different

concentrations of enzyme extract (0.5–1.0 U) were impregnated on a small disc of filter paper (diameter 5.0 mm) and placed on top of chromogenic substrate plate. The plates were incubated at 30°C for 30 min. Zones of hydrolysis was observed in discs impregnated with active enzyme extract.

Lignin peroxidase: A standard reaction mixture was prepared with 1 ml of 125 mM sodium tartarate buffer (pH 3.0), 5 ml of 10 mM veratryl alcohol, 5 ml of 2 mM hydrogen peroxide solution and 5 ml of the enzyme extract. The reaction was initiated by adding hydrogen peroxide and the change in absorbance was monitored at 310 nm. One unit of enzyme activity was 1 μ mole of veratraldehyde produced min^{-1} ml^{-1} of the culture filtrate (Arora and Gill, 2001).

Tannase: The substrate was 0.7% ($w.v^{-1}$) solution of tannic acid in 0.2 M acetate buffer at pH 5.5. The reaction was conducted by adding 0.3 ml of substrate solution to 0.5 ml of enzyme extract and incubating at 40°C for 10 min. After incubation, the reaction was stopped by the addition of 3 ml of a 1.0 mg ml^{-1} bovine serum albumin solution (BSA) prepared in a 0.17 M sodium chloride solution in 0.2 M acetate buffer, pH 5.0. The solution was then filtered and centrifuged at 10,000 x g for 15 min at 4°C. The precipitate was dissolved in 3 ml of SDS–triethanolamine, added 1 ml of $FeCl_3$ reagent and held for 15 min for stabilization of the colour. The absorbance was measured at 530 nm and calculated the enzyme activity from the change in absorbance at 530 nm. One unit of tannase activity was defined as the amount of tannic acid hydrolysed by 1 ml of enzyme min^{-1} of reaction (Mondal et al., 2001).

Lipase: Lipase activity was determined spectrophotometrically using p-nitrophenyl palmitate (pNPP) as the substrate (Gupta et al., 2002). 30 g of pNPP dissolved in 10ml solvent (isopropanol) and 90 ml phosphate buffer (pH 7.0) in conjunction with sodium deoxycholate, and gum arabic, making a final concentration of 790 μM pNPP. To 4.8 ml of the above substrate solution, 0.2ml of the enzyme extract is added and the reaction performed at the 30°C for 30 min. The amount of p-nitrophenol released was estimated spectrophotometrically at 405 nm (Shimadzu, UV-2401). One unit of enzyme activity is defined as the amount of enzyme liberating 1 μmol of p-nitrophenol min^{-1}.

Lipase activity was also detected on plates (Rajini et al., 2002). Chromogenic substrate plate was prepared by using phenol red (0.01%) along with 1% lipidic substrate (olive oil), 10 mM $CaCl_2$, and 2% agar. The pH was adjusted to 7.3-7.4 by using 0.1 N NaOH. Different concentrations of enzyme extract (1-2.0 U) were placed on top of different chromogenic substrate plate. The plates were incubated at 40°C for 15–30 min.

6.2.3 Native polyacrylamide gel electrophoresis

Native polyacrylamide gel electrophoresis (PAGE) was performed using discontinuous gel system of Davis (1964). Gels were cast with 4% stacking gel and 8% resolving gel. Proteins were allowed to stack at 40 mV and to separate at 70 mV. The gels were rinsed three times with distilled water and equilibrated in 50 mM Tris/HCl (pH 8.0) for 30 min at room temperature. Protein bands were visualized by using different methods depending on enzymes.

To identify the location of amylase activity (clear zones on a blue background), the gels were incubated for 1 h at 30°C in 2% soluble starch in 0.2 M phosphate buffer (pH 6.5) and stained with an acidic iodine solution (0.2% I_2 and 2% KI in 0.2 N HCl) (Shih and Labbe, 1995).

Samples were subjected to electrophoresis on a gel containing 0.1% xylan for xylanase and 0.1% carboxymethyl cellulose for cellulose. After electrophoresis, the gel was washed three times for 30 min at 4°C in 100mM Na_2CO_3-$NaHCO_3$ buffer (pH 9.0) containing 25% isopropanol for the first two washes to remove SDS, and then incubated in the same buffer for 30 min at 37°C. The zymogram was prepared by soaking the gel in 0.1% congo red solution for 15 min at room temperature, then washed with 1M NaCl, and introduced 0.5% acetic acid to expose cellulase and xylanase active bands that contrasted the dark background (Pochihn et al., 2004).

In order to visualise polygalacturonase the enzyme, the gel was shaken gently at room temperature for 4 h in 2.5% ($v.v^{-1}$) Triton X-100, and then transferred to 0.1 M sodium acetate (pH 5.5) for incubation for 1 h at room temperature. Finally, it was stained with 0.02% ruthenium red and destained with distilled water (Ried and Collmer, 1985). Activity was observed as a white band on a red background. The

background resulted from the binding of ruthenium red to intact pectic acid which was incorporated in the gel.

Gels were stained with coomassie brilliant blue (Weber and Osborn, 1969) for visualizing protease activity.

The gels were overlaid with the molten chromogenic substrate, which was then allowed to solidify and incubated at 37-45°C. Chromogenic substrate plate was prepared by using phenol red (0.01%) along with 1% lipidic substrate (olive oil), 10 mM $CaCl_2$, and 2% agar. The pH was adjusted to 7.3-7.4 by using 0.1 N NaOH. Detection depends on the amount of lipase. The activity was observed as yellow band over a pink background (Rajni et al, 2006).

For lignin peroxidise, the gel was soaked in the solution of 1mM DMP dissolved in 100 ml of 0.1M sodium tartrate buffer (pH 4.5) with 0.1mM H_2O_2 and 1mM $MnSO_4$. The gel was incubated at 30°C until a orange band appeared (Cheng et al., 2007).

The location of tannase activity within the gel was determined as described by Aoki et al., (1979). The gel was washed for 1 h in 100 ml 2.5% ($v.v^{-1}$) Triton X-100 followed by two 45 min washes with 100 ml 10 mM acetate buffer pH 5.5 with constant shaking. This was followed by incubation of the gel containing tannic acid 0.5% ($w.v^{-1}$) in 0.1 M acetate buffer pH 5.5 at 30°C, with constant shaking. The tannic acid solution was discarded and replaced with 100 ml 0.5% ($w.v^{-1}$) quinine hydrochloride solution in 0.05 M acetate buffer of pH 5.5 at room temperature. Tannase activity appeared as a clear band on a white background.

62.4 Assay for lactic acid

Lactic acid was measured by following the method prescribed by Barker and Summerson (1941). In this method, lactic acid was converted to acetaldehyde by the treatment with concentrated sulphuric acid and the amount of acetaldehyde was determined by its colour reaction with p-hydroxy diphenyl in the presence of cupric ions. The colour was read at 560 nm using spectrophotometer (Shimadzu, UV-2401). The lactate concentration of unknown sample was obtained from spectrometer reading with reference to a previously prepared calibration curve (with anhydrous lithium lactate).

6.2.5 Assay for pyruvic acid

2,4-dinitrophenylhydrazine (DNPH) solution (0.0125%) was prepared by dissolving 0.1625 g of wet DNPH powder (~30% water) in 1000 ml 2N HCl. A 2 ml filtered sample was added to 1 ml of a 0.0125% solution of DNPH. After 15 min in a water bath at 37°C, 5 ml of 0.6N NaOH was added and the absorbance measured immediately on a Shimadzu model UV-2401 spectrophotometer (420 nm filter, set at zero absorbance with reagent blank).

The method was calibrated using sodium pyruvate as standard. Pyruvic acid standards were prepared using sodium pyruvate. A 10μM ml^{-1} solution was prepared by dissolving 1.1g sodium pyruvate (M.W = 110.0g) in 1000 ml of distilled water. A subsequent ten-fold dilution gave a 1μM ml^{-1} standard solution which was further diluted to prepare calibration standards (Katsui et al., 1961).

6.2.6 Protein estimation

Protein was measured was estimated according to Lowry's method (Lowry et al., 1951) using bovine serum albumin as the standard.

6.2.7 Isolation of wheat protein from wheat flour

The wheat protein was partly purified from commercially available wheat flour. The flour was defated with acetone, dried and then homogenised in a mortar with Tris-NaCl buffer (0.02 mol l^{-1} Tris and 0.5 mol l^{-1}NaCl). the isolation was repeated twice according to the method of Marcin et al., (1988). The albumin and globulin fractions were isolated from the wheat flour. The homogenate was centrifuged at 1000 g for 20 min and the supernatant fraction containing the extracted protein collected.

6.2.8 Chemical Analysis

Total organic carbon: Microbial degradation of organics was assessed by measuring TOC (Total Organic Carbon) at appropriate intervals using Schimadzu TOC - 5000 analyzer.

Thin layer chromatography: Qualitative detection of intermediate metabolites was done by thin layer chromatography (TLC). The extract was obtained

by disruption of cells in three cycles of freezing and thawing (-20°C for 10 min and 35°C for 10 min) followed by centrifugation at 5000*g* for 10 min. The extract of 10 µl was applied to silica gel G coated plates and run in suitable solvent system. Different metabolites were identified by comparison with the standards run simultaneously.

Sugars were analyzed with the solvent system ethyl acetate/acetic acid/ether/water (12:3:3:2) and naphthoresorcinol reagent for detection (Dennis and Clinton, 1976). The organic acids were analyzed with the solvent system ethanol/ammonium/water (70:4:16) and 2, 6-dichloroindophenol reagent for detection (Kawahara and Obata, 1998). Pectin metabolites were separated by TLC using a solvent system consisting of 1-butanol: acetic acid: water (3:2:2). Products were visualized by heating the TLC plate at 130°C for 5 min after spraying it with 10% ($v.v^{-1}$) sulphuric acid in ethanol. The TLC plates were developed with solvent system consisting hexane: diethyl ether: formic acid, (80:20:2) for detecting lipids. The spots were visualised by using iodine vapours. Polyphenol metabolites were analysed with solvent system of chloroform: ethyl acetate: acetic acid (50: 50: 1) and iodine vapour was used for detection.

Gas chromatograph: End products of anaerobic degradation of organic matter by protozoa were measured in the culture filtrate (VFAs) and in the head space (gases). A gas chromatograph (FISION 8000, FID, 2mm i.d Silica column, carrier gas 27ml min^{-1}, Oven 35°C, Detector 200°C) was utilized to measure ethanol. A gas chromatograph (FISION 8000) with TCD detector was utilized to measure hydrogen. The temperature of the column thermostat and the injection port was 50°C. The flow rate of the carrier gas (helium) was maintained 60 ml min^{-1}. Organic acids were detected by a gas chromatograph (FISION 8000, FID, Carbowax column, carrier gas 100 KPa, H_2/Air 100 KPa each, Injector 150°C, Oven 130°C and Detector 175°C).

6.2.9 Microscopic observations

The methods for enumeration and identification of protozoa are given in Chapter 2.

6.3 Results and discussion

6.3.1 Relative contribution of specific microbial groups to the degradation

Fig. 6.1 illustrates the relative utilization of suspended particles in anaerobic cultures - bacterial system, protozoa system and mixed system based on TOC analysis. TOC removal observed was more or less similar in separate anaerobic cultures of bacteria and protozoa, although the initial rate was higher in bacterial culture than in protozoa culture (Fig. 6.1). The increased removal rate in bacterial cultures at initial stage may be due to the increased number of organisms (6.5×10^6 cfu ml^{-1} against protozoa (1.75×10^3 ml^{-1}). Highest removal of organic matter was observed in the mixed system as recorded more than 90 %(SD $\pm$ 5) in 72 hours compared to less than 75 %(SD $\pm$ 6.5) in the separate cultures of bacteria and protozoa.

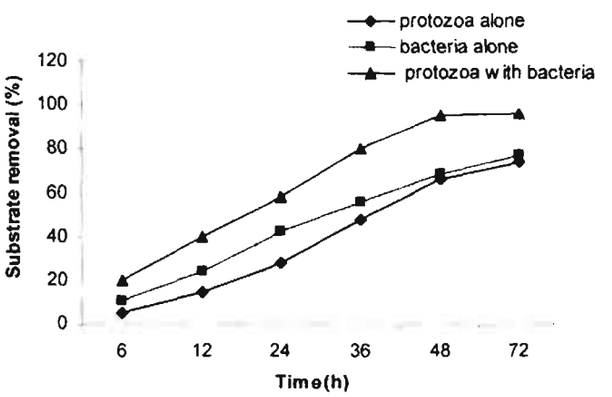


Fig. 6.1. Organic matter degradation rate (%) measured by TOC analysis in the anaerobic cultures of mixed system of both bacteria and protozoa, bacterial system and protozoa system

The organic matter mineralization measured by COD analysis was also more than 95 $\pm$ 2 % in mixed system of bacteria and protozoa, whereas the corresponding values for individual cultures of ciliates and bacteria were 72 $\pm$ 5 % and 76 $\pm$ 4 %respectively (Fig. 6.2). The order of organic matter degradation in the anaerobic

system was as follows: mixed system> bacterial system> protozoa system. Obviously, co-ordinated activity of microbial group was the best for the enhanced degradation of organic matter in anaerobic treatment systems. The present study reveals the active involvement of protozoa in the anaerobic system for higher COD removal along with bacteria.

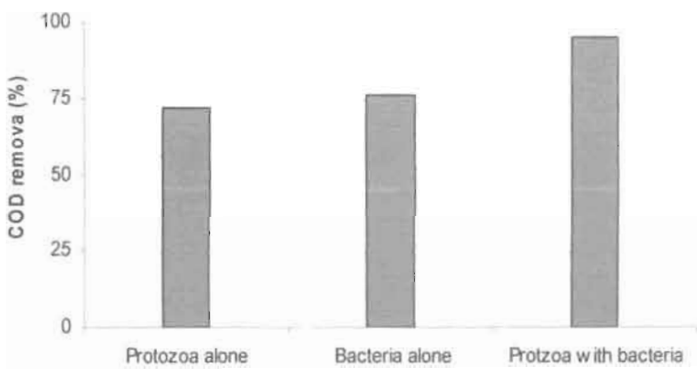


Fig. 6.2. Final COD removal (%) in the anaerobic cultures of mixed system of both bacteria and protozoa, bacterial system and protozoa system

6.3.2 Metabolic end products of protozoa

Loss of endosymbiotic methanogens was observed with the long term culturing of the ciliates. In the initial stges of isolation and culturing, a large number of endosymbionts were observed in the ciliates (Fig. 6.3). The cultures were transferred to fresh medium at every two weeks. In this suculturing, there was no loss of endosymbionts for four months. Thereafter, the number of endosymbionts started to decrease and eventually they were disappeared. No endosymbiotic methanogens were observed after twelve months (Fig. 6.3). The result indicates that the symbiotic association with methanogens is not essential for growth of protozoa, but their growth was affected slightly in the absence of methanogens. After several transfers, the protozoa number was reduced to around 19 x 10^4 ml^{-1} from the initial count of 25 x 10^4 ml^{-1}. Fenchel & Finlay (1991) have reported a decrease of 25-30% in growth rate

in the absence of endosymbionts in anaerobic ciliates. Loss of endosymbiotic methanogens has been observed earlier with continued cultivation of the ciliates (Yamada et al., 1997). Endosymbiont free anaerobic ciliates often exist in natural habitats (Goosen et al, 1990b). In present study, protozoa mainly, flagellates and amoeboids were found free of endosymbionts in the anaerobic reactors.

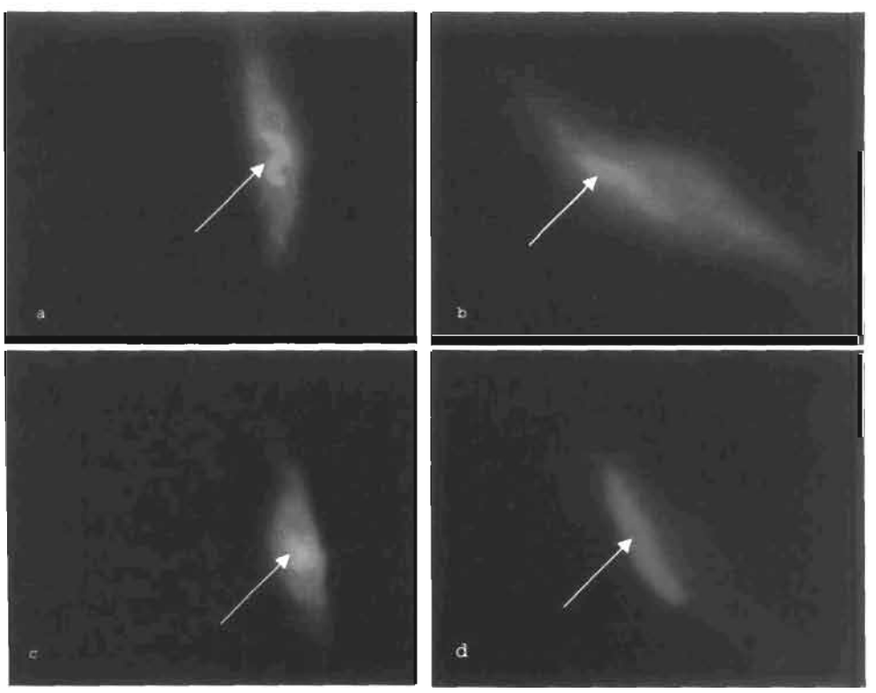


Fig. 6.3. Micrographs of DAPI stained *Metopus* cells show loss of endosymbionts with continued cultivation (a) after four month (b) after six month (c) after nine month and (d) after twelve months - complete loss of symbionts (arrow denotes nucleus of the cell)

The metabolic end products of anaerobic protozoa with and without endosymbionts are presented Table 6.1. When the endosymbiotic methanogens were retained inside the cells, the major metabolic products through digestion of oleic acid suspension were acetate and methane. Small amounts of lactic acid, pyruvic acid,

propionic acid, butyric acid, carbon dioxide and hydrogen were also detected in cultures. However, the profile was significantly changed with disappearance of the endosymbionts. There was no methane. Butyrate and propionate became the major end products along with acetate. Shift in metabolism results production of less oxidised and less energy yielding fermentation products such as propionate and butyrate to manage/reduce hydrogen partial pressure inside the cell. Traces of hydrogen and carbon dioxide were also detected in the absence of endosymbionts. Endosymbiotic bacteria free protozoa were found to metabolize organic matter to short chain acids, carbon dioxide, and hydrogen. Such organic matter degradation of protozoa is shown as their inherent property and is independent of endosymbiotic bacteria. Indeed, protozoa may generally stimulate anaerobic degradation process through common mechanism of VFA excretion.

Table 6.1. Metabolic end products of anaerobic protozoa grown in oleic acid medium

	Protozoa with endosymbionts (ppm)	Protozoa without endosymbionts (ppm)
Lactic acid	0.08	0.12
Pyruvic acid	0.12	0.13
Butyric acid	0.27	1.2
Propionic acid	0	0.8
Acetic acid	1.6	2.2
Formic acid	0	0
Hydrogen	0.202	0.06
Methane	5.1	0
Carbon dioxide	1	0.04
Ethanol	0	0

Metabolic studies on anaerobic protozoa have been limited to ver few organisms, for example axenic cultures of ciliate *Trimyema compressum* (Goosen et al., 1990b; Holler and Pfennig, 1991; Yamada et al., 1997), rumen-dwelling protozoa (Hillman et al., 1995) and parasitic protozoa (Coombs and Mueller, 1995). These

studies have revealed that anaerobic protozoa, unlike their aerobic counterparts, do not generate energy by oxidative phosphorylation but rather have developed fermentative metabolism (Mueller, 1998).

Along with direct uptake of organic matter, metabolic end products of protozoa may serve as growth substrates for bacteria, especially methanogens. Fig. 6.4 shows the presence of ectosymbionts and endosymbionts in ciliates. Ectosymbiotic bacteria attached to ciliates may not be methanogens. Fenchel and Ramsing (1992) have reported ectosymbiotic sulphate reducing bacteria in marine ciliates. Anaerobic protozoa excrete various metabolites from their fermentative energy metabolism and these serve substrates for bacteria and other protozoa, mainly flagellates. Flagellates have the ability to utilize dissolved substrates also (Chapter 5). Methanogens can utilise the hydrogen /acetate produced by the host cells. In this study, hydrogen and acetate utilising methanogens were identified in anaerobic protozoa (Chapter 5). This symbiotic consortium therefore enables to produce methane as a metabolic end product. Earlier studies have reported that anaerobic ciliates can produce acetate, lactate, CO_2 and H_2 as metabolic end products (Googen et al., 1990) and, these could serve as growth substrate for methanogenic bacteria and the removal of electrons in protozoa (Van Bruggen et al., 1983).

Most of the protozoa which live in anaerobic environments harbours ecto /or endo symbiotic bacteria (Fenchel et al., 1977). The adaptive significance of this symbiosis is evident from the view point of bacteria that they receive substrate from the host. Anaerobic protozoa lack mitochondria and instead possess hydrogenosomes (Muller, 1988). Hydrogenosomes are membrane bound organelles generating ATP and H_2 from the decarboxylation of pyruvate (Muller, 1993). The major benefit to the host is that hydrogen partial pressure inside the protozoa cell is maintained to low by symbiotic methanogens that utilizing hydrogen to CH_4

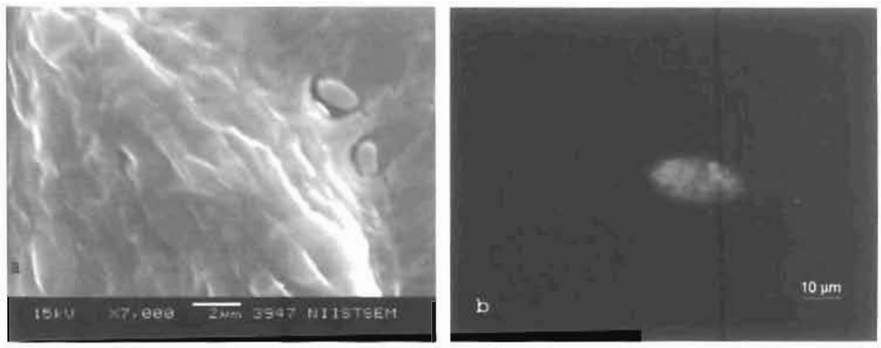


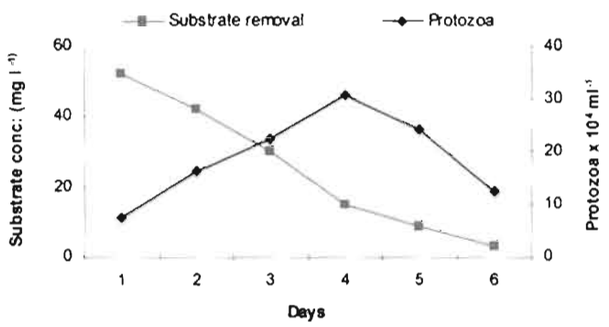
Fig. 6.4. (a) SEM showing ectosymbionts attached to the ciliate *Cyclidium* and (b) FISH showing endosymbiontic methanogens inside the *Cyclidium*

6.3.3 Degradation of various organic compounds by anaerobic protozoa

In the previous chapter, ability of protozoa to utilize organic matter directly was discussed. The enzymatic degradation of various compounds by anaerobic protozoa which is generally present in wastewaters were analysed by supplementing different substrates. The intermediate degradation products were determined by thin layer chromatography and final metabolic end products were analysed by using gas chromatography.

Glucose degradation by anaerobic protozoa

Glucose, one of the most important monosaccharide and the immediate source of energy for cellular respiration. The growth of protozoa and glucose utilization were measured in batch cultures of anaerobic protozoa. A succession in protozoa populations was observed along with the decomposition of glucose. Protozoa had a maximum count of 30 x 10^4 ml^{-1} in batch cultures (Fig. 6.5). Interestingly, flagellates were the prominent genera among the protozoa in the glucose supplemented culture. There was a considerable reduction in sugar was observed in bacteria suppressed protozoa cultures (Fig. 6.5). Osmotrophic nutrition has been previously demonstrated in some of the soil flagellates (Ekelund and Ronn, 1994) which might be the case of flagellates in this system also.



5.5. Growth of protozoa and glucose removal in the anaerobic culture

Fig. 6.6. illustrates the thin layer chromatographic separation of organic acids in anaerobic cultures of protozoa supplemented with glucose. During the growth of protozoa glucose was converted to pyruvate and then to acetate. Glucose was detected in cultures at 12 hours, but it was completely absent on the 3rd day. This implies that the complete utilization of glucose by anaerobic protozoa.

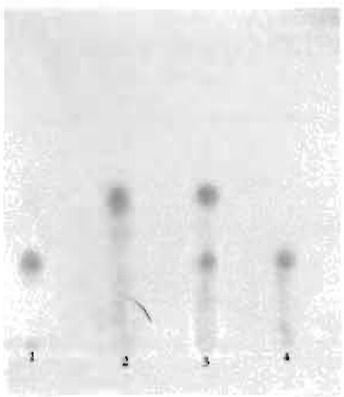


Fig. 6.6. Photograph of TLC- separation of degradation products of glucose by anaerobic protozoa 1. Acetic acid (standard) 2. pyruvic acid (standard) 3. sample of 48h and 4. sample of 120 h

Methane and acetate were the major end products of glucose degradation by anaerobic protozoa and were detected using gas chromatography. Traces of hydrogen, CO_2, butyrate and lactate were also measured as end products of glucose metabolism. The rate of formation of these end products by anaerobic protozoa varied with the amount of glucose given as feed. The degradation rate was also influenced by environmental factors, pH and temperature. Glucose is the major energy source in most life form, despite a limited knowledge is available on glucose degradation by anaerobic protozoa. *Hexamita* is shown to possess a fermentative metabolism: the principle products of glucose fermentation are acetate, ethanol, lactic acid, alanine, and CO_2 (Biagini et al., 1998b).

Sucrose degradation by anaerobic protozoa

Sucrose is the most well-known disaccharide consists of a glucose molecule linked to a fructose molecule. Compared to glucose the anaerobic protozoa had poor growth in sucrose. The rate of sucrose degradation was also low compared to glucose. Flagellates were the main organisms observed in sucrose added culture, mainly the flagellate *Polystoma*. Protozoa had a maximum number of 21 x 10^4 ml^{-1} in anaerobic cultures with highest intracellular glycosidase activity 16.4 U ml^{-1} (Fig. 6.7).

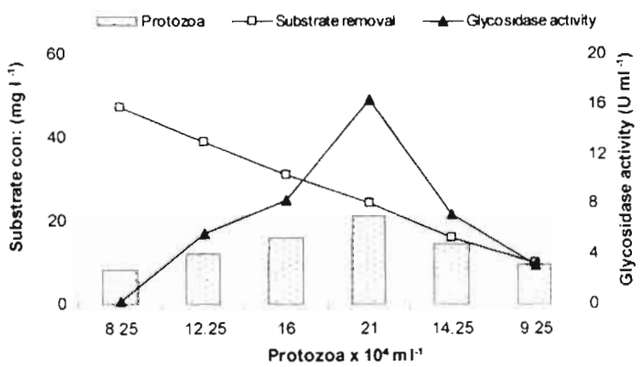


Fig. **6.7.** Enzyme activity and substrate removal observed with the growth of protozoa in sucrose supplemented anaerobic culture

Samples were periodically drawn from the anaerobic culture and analyzed the degradation products on TLC plates (Fig. 6.8). The presence of glucose was observed in 24 h grown cultures, but it was not found in the further growth (120 h).

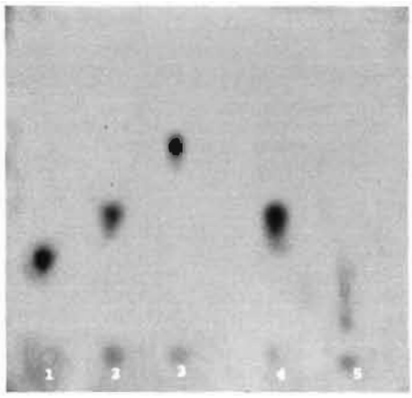


Fig. 6.8. Photograph of TLC- separation of degradation products of sucrose by anaerobic protozoa. 1. Sucrose (standard) 2. glucose (standard) 3. fructose (standard) 4. sample of 48 h and 5. sample of 120 h

As in glucose supplemented anaerobic protozoa culture, acetate and methane were the main metabolic end products from sucrose. Butyrate, CO_2 and traces of propionate and hydrogen were also detected in the gas chromatographic analysis. It was shown that anaerobic protozoa could utilize the soluble disaccahride, sucrose also.

Raffinose degradation by anaerobic protozoa

Raffinose is a trisaccharide composed of galactose, fructose and glucose. Raffinose is almost ubiquitous in the plant kingdom and it is usually found in a large variety of seeds from many plant families. The degree of degradation of raffinose was determined by measuring the amount of carbohydrate utilized and metabolites formed at the end of the culturing period. Protozoa populations and enzymatic activities were observed during the degradation. Protozoa growth reached a maximum number of 26 x 10^4 ml^{-1} in batch cultures (Fig. 6.9). As like other soluble carbohydrates, flagellates

were the prominent genera among the protozoa in raffinose fed culture. The production of intracellular α-galactosidase was observed along with raffinose removal by protozoa. Maximum of 14.8 U ml^{-1} was observed with highest number of protozoa.

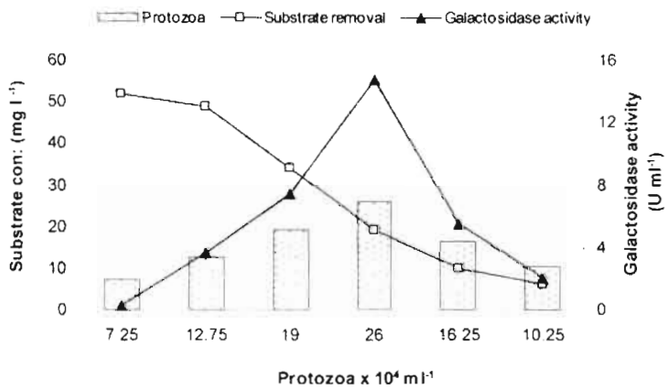


Fig. 6.9. Enzyme activity and substrate removal observed with the growth of protozoa in raffinose supplemented anaerobic culture

TLC analysis for intermediate products showed the presence glucose and galactose. However, presence of fructose was not observed in the anaerobic protozoa culture (Fig. 6.10). Metabolic end products such as methane, hydrogen, CO_2, acetate and butyrate were detected using gas chromatography in 120 h grown culture.

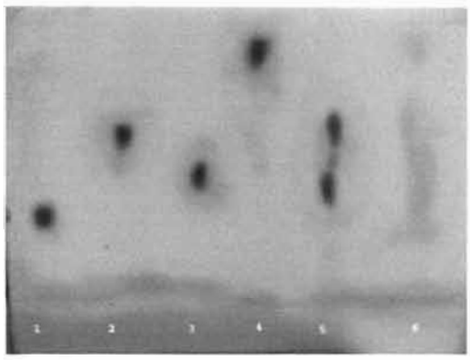


Fig. 6.10. Photograph of TLC- separation of degradation products of raffinose by anaerobic protozoa. 1. Raffinose (standard) 2. glucose (standard) 3. galactose (standard) 4. fructose (standard) 5. sample of 48 h and 6. sample of 120 h

Protozoa are considered to be the most important bacteriovorous grazers in the anaerobic environments. Therefore, soluble organic mattes are not considered to be the source of nutrition for heterotrophic protozoa. According to the report of Howard (1959), rumen ciliates preferentially utilize soluble carbohydrates. In fact, the capability of protozoa to utilise dissolved carbon compounds has great importance in anaerobic degradation.

Starch degradation by anaerobic protozoa

Starch is an abundant carbon source in nature. Starch is insoluble in water and it is serving as storage depots of glucose. The result shows that the growth of flagellates and ciliates were supported by starch. Protozoa had a maximum number of 32 x 10^4 ml^{-1} in batch cultures (Fig. 6.11). Growth of protozoa populations and enzymatic activities were observed during the decomposition of starch. The hydrolysis of starch was confirmed with the enzyme amylase. This enzyme is widespread among aerobes and anaerobes (Vihinen and Mantsala, 1989). However, most of the studies have focused only on bacteria and fungi, and have ignored protozoa for amylase production. Maximum of 34 U ml^{-1} was observed when protozoa were in highest numbers. Subsequently, the enzyme level reduced to 8 U ml^{-1} along with degradation of starch.

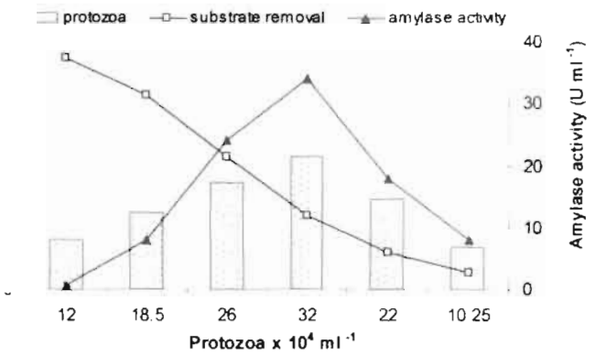


Fig. 6.11. Enzyme activity and substrate removal observed with the growth of protozoa in starch supplemented anaerobic culture

Fig. 6.12 illustrates the hydrolysis zones with enzyme amylase extracted from protozoa cells on agar plates containing starch. The clear zones were visualised by using acidic iodine solution. Amylase activity was also confirmed with nondenaturing electrophoresis (Fig. 6.13).

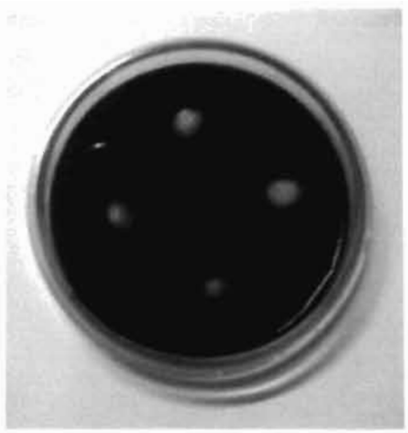


Fig. 6.12. Photograph of hydrolysis zones with protozoa extract on the agar plates with starch

Fig. 6.13. Zymogram of protozoa extract. Clear zones corresponding to protein bands indicating amylase activity in iodine stained gel

Maltose and glucose were the principal intermediate products from starch, as observed from thin layer chromatograms (Fig. 6.14). Maltose could be the primary degradation product and followed by glucose.

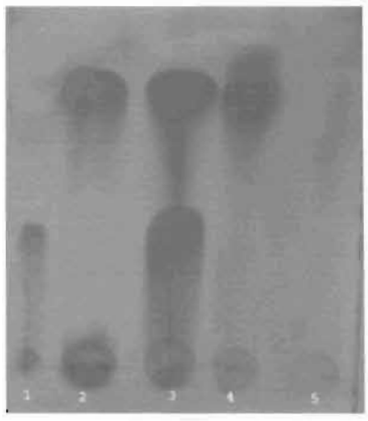


Fig. 6.14. Photograph of TLC- separation of degradation products of starch by anaerobic protozoa. 1. Maltose (standard) 2. glucose (standard) 3. sample of 24 h 4. sample of 72h and 5. sample of 120 h

The end products from anaerobic fermentation of starch by protozoa were methane, hydrogen, CO_2, acetate and butyrate. Among these, acetate and methane were the main metabolites as evidenced from GC. Therefore, it can be concluded that the anaerobic protozoa could utilize insoluble starch. Coleman (1985, 1986) estimated that 50% of the ruminal amylase was present in the protozoal fraction.

Cellulose degradation by anaerobic protozoa

Cellulose, an unbranched β-1,4-linked homopolymer of glucose, is the most abundant renewable carbohydrate on earth, accounting for about half of the organic material in the biosphere, and it is the major polysaccharide found in plant biomass. Cellulose is insoluble and crystalline; hence, it is largely resistant to enzymatic hydrolysis. Cellulose was found to support the growth of flagellate *Menoidium*, *Trepomonas*, *Rhyncomonas* and *Naeglaria* with maximum of *Menoidium*. Protozoa reached a maximum of 42 x 10^4 ml^{-1} in cellulose medium. However, the ciliate growth was very low in cellulose medium (1 x 10^4 ml^{-1}). Bioconversion of cellulose to glucose is catalyzed by a group of enzymes called cellulases. Flagellates had appreciable cellulolytic activity with cellulose culture. Maximum of 22.6 U ml^{-1} cellulase activity was observed at 72 h with culture which had the highest number of protozoa (Fig. 6.15).

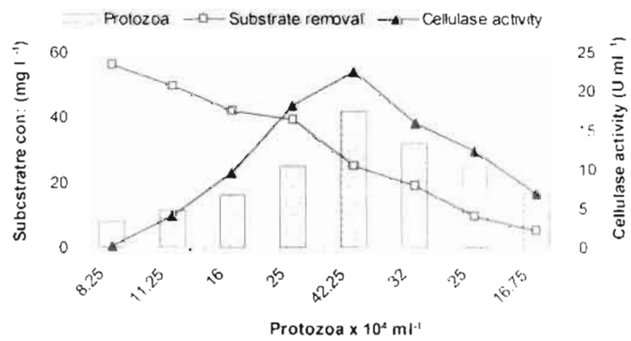


Fig. 6.15. Enzyme activity and substrate removal observed with the growth of protozoa in cellulose supplemented anaerobic culture

To assess the cellulose activities of the culture, protozoa extract was added to agar plates with CM-cellulose (0.1%). Clear zones of hydrolysis were detected on the plates (Fig. 6.16). Thereafter, the cellulase activity was confirmed with nondenaturing electrophoresis (Fig. 6.17).

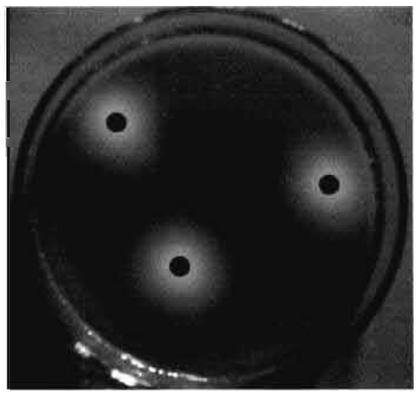


Fig. 6.16. Photograph of hydrolysis zones with protozoa extract on the agar plates with CM-cellulose.

Fig. 6.17. Zymogram of protozoa extract. Clear zones corresponding to protein bands indicating cellulase activity in Congo red stained gel

The anaerobic degradation of cellulose needs a longer retention time than other carbohydrates such as starch. The hydrolysis of cellulose in anaerobic cultures with protozoa could produce glucose, an easily fermentable monosaccharide (Fig. 6.18). Analysis of degradation products at the end of incubation showed that glucose was converted to acetate and methane. Traces of propionate, butyrate and carbon dioxide were also detected in cultures. Therefore, it can be concluded that the protozoa were able to digest the cellulose in the medium. Onodera et al., (1988) have proposed that rumen protozoa population participates in cellulose digestion in the rumen ecosystem with an endogenous 1,4-β glucanase. According to Coleman (1989) much of the cellulolitic activity associated with plant material in rumen may be protozoa origin.

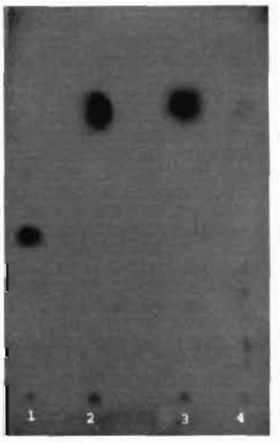


Fig. 6.18. Photograph of TLC- separation of degradation products of cellulose by anaerobic protozoa. 1. Cellulose (standard) 2. glucose (standard) 3. sample of 48 h and 4. sample of 148 h

Xylan degradation by anaerobic protozoa

Xylan is one of the major heterogeneous hemicelluloses present in agro industrial residues. The biological hydrolysis of xylan requires the action of xylanase. Xylan is the most abundant non-cellulosic polysaccharide in hard wood (20-35%) and soft wood (8%), which constitutes approximately one third of all renewable organic

carbon sources on earth. A succession in growth with higher number of ciliate, *Metopus* was observed in anaerobic cultures of protozoa with oat spelt xylan. Protozoa attained a maximum of 29 x 10^4 ml^{-1} in anaerobic culture. Intracellular xylanase activity of 19 U ml^{-1} was observed with the growth of protozoa (Fig. 6.19). Glucose produced (12 $mg.l^{-1}$) in the anaerobic protozoa culture by the enzymatic degradation of xylan was analysed by DNS method. The removal of xylan was also confirmed by the difference in COD removal before and after culturing.

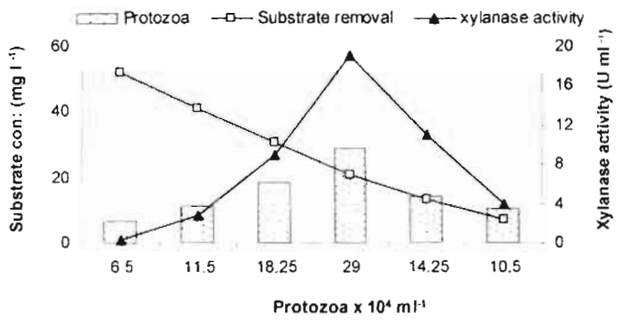


Fig. 6.19. Enzyme activity and substrate removal observed with the growth of protozoa in oat spelt xylan supplemented anaerobic culture

To identify the presence of cellulase activity, the agar plates with oat spelt xylan (0.1%) and protozoa extract was incubated for 15 min and clear zones of hydrolysis on plates were detected with congo red (Fig. 6.20). Gels were stained with congo red for confirming xylanase activity. In zymogram, xylanase active bands were formed in the bright background (Fig. 6.21).

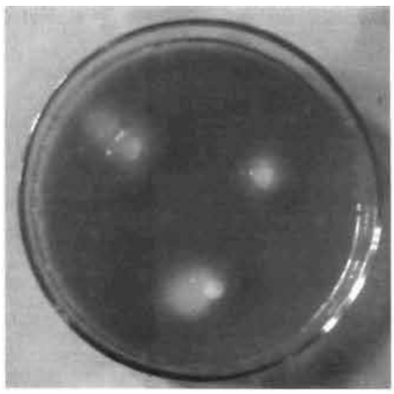


Fig. 6.20. Photograph of hydrolysis zones with protozoa extract on the agar plates with oat spelt xylan

Fig. 6.21. Zymogram of protozoa extract. Clear zones corresponding to protein bands indicating xylanase activity in Congo red stained gel

Hydrolysis of xylan is an important step towards proper utilization of lignocellulosic material in nature. Chemical hydrolysis of lignicelluloses results in hazardous byproducts. Microbial enzymes, which are specific in action for xylan hydrolysis is an environment friendly option (Biely, 1985). Protozoa mediated xylanse activity is important especially, in agro industrial waste treatment process,

but was hardly studied. Devillard et al., (1999) have reported xylanase production by rumen anaerobic protozoa for plant fibre degradation.

Pectin degradation by anaerobic protozoa

Pectin is a structural heteropolysaccharide contained in the primary cell walls of terrestrial plants. Pectin is composed of α-(1-4)-linked D-galacturonic acid. It is present in vegetables and fruits as a component of the plant cell wall. In this study, pectin was found to support the growth of flagellates, mainly *Menoidium* and the ciliates, mainly *Metopus* and *Cyclidium*. Maximum protozoa count of 27.5 x 10^4 ml^{-1} was obtained in the pectin medium (Fig. 6.22).

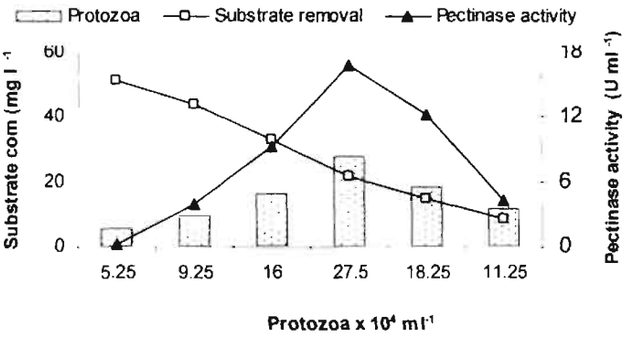


Fig. 6.22. Enzyme activity and substrate removal observed with the growth of protozoa in pectin supplemented anaerobic culture

Galacturonic acid was detected as the intermediate metabolites of pectin degradation by anaerobic protozoa (Fig. 6.23). The galacturonic acids generated were determined by using thin-layer chromatography. Finally, the galacturonic acid was converted to short chain fatty acids and gases. The end products of pectin degradation by anaerobic protozoa were acetate, butyrate, CO_2 and CH_4.

Hydrolysis of pectin was catalyzed by a group of enzymes called pectinases. Maximum of 16.8 U ml^{-1} was observed with the highest number of protozoa. Pectinase activity in the protozoa extract was also confirmed with nondenaturing electrophoresis (Fig. 6.24). Only limited information is available on pectin

degradation by protozoa and it is mainly restricted to rumen protozoa. The presence of pectic enzymes were reported in rumen ciliates that depolymerase polygalacturonic acid (wright, 1961; Abou - Akkada and Howard, 1961).

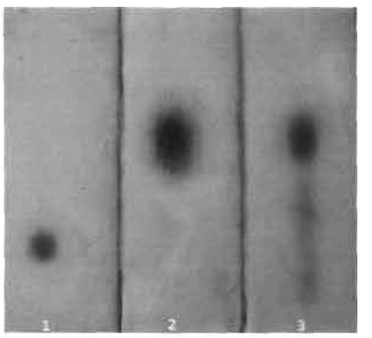


Fig. 6.23. Photograph of TLC- separation of degradation products of pectin by anaerobic protozoa. 1. Pectin (standard) 2. galacturonic acid (standard) and 3. sample of 48 h

Fig. 6.24. Zymogram of protozoa extract. Clear zones corresponding to protein bands indicating pectinase activity in rhuthenium red stained gel

The results of this study show that protozoa can utilize various monosaccharide, disaccharides, oligosaccharides and polysaccharides. Although all the protozoa are not grown on all substrates, many are capable of utilizing a large

range of natural saccharides. Hydrolysis of carbohydrates is generally considered as a rate limiting step in over all degradation process which can be overcome by the presence of protozoa through intracellular degradation. Therefore, an enhancement of the biodegradation activity with protozoa in bioreactors is very important for an economically feasible anaerobic degradation process.

Protein degradation by anaerobic protozoa

Wheat proteins which consist of albumin and globulin fractions were isolated as per the method described Marcin et al., 1998 and applied to cultures in the form of insoluble particles. The rate of wheat protein proteolysis was measured by determining the amount of total wheat protein at selected intervals of incubation. It was observed that proteolytic activity was highest at the exponential growth of the culture. Protozoa attained a maximum number of 23.25 x 10^4 ml^{-1} in the anaerobic cultures (Fig. 6.25). Protease activity and protein degradation are presented in Fig. 6.25. The proteolysis of wheat was mainly intracellular and the maximum protease activity measured was 21 U ml^{-1}.

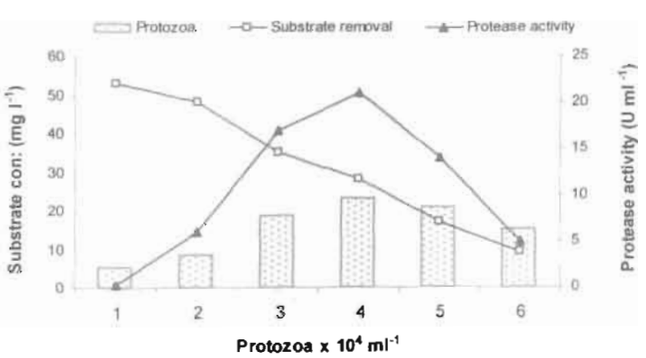


Fig. 6.25. Enzyme activity and substrate removal observed with the growth of protozoa in protein suspension supplemented anaerobic culture

To identify the presence of protease activity in the protozoa extract, the agar plates with 1% casein and 1% gelatin and enzyme extract were incubated at 30°C. A clear zone of hydrolysis was observed after 30 min (Fig. 6.26). Protease activity was

also confirmed with nondenaturing electrophoresis (Fig. 6.27). Gels were stained with coomassie brilliant blue for detecting protein bands.

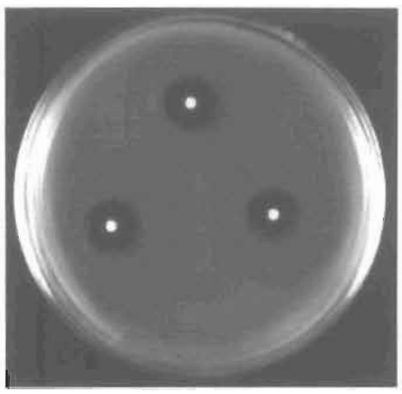


Fig. 6.26. Photograph of hydrolysis zones with protozoa extract on the agar plates with casein and gelatin

Fig . 6.27. Zymogram of protozoa extract. Clear zones corresponding to protein bands indicating protease activity in cumassie blue stained gel

Protease forms an important group of enzymes which are now recognised as being evolved in a wide range of biological processes. The role of protease may vary from general protein degradation to subtle modification involving the hydrolysis of

specific peptide bonds in specific proteins (Holzer and Heinrich, 1980). The results of this study reveal protein degradation by anaerobic protozoa.

Bacteria have been reported to be primarily responsible for the degradation of plant protein, and protozoa were thought to have only a minor role in this metabolic activity (Nugent and Mangan, 1981). However, studies showed that rumen ciliate protozoa can engulf insoluble protein (Onodera and Kandatsu, 1970). Protozoa are the main grazers of bacteria and they can digest microbial protein (Fenchel and Finlay, 1995). The observation on protein degradation and metabolism support the excretion of ammonia-N through bacterial biomass degradation by protozoa as discussed in Chapter 5. The ability of protozoa to degrade both dietary and microbial protein is very important in anaerobic environment, especially for effective and enhanced biodegradation.

Polyphenol degradation by anaerobic protozoa

Polyphenols are a group of chemical substances found in plants, characterized by the presence of more than one phenol group per molecule. Polyphenols are generally subdivided into hydrolyzable tannins, which are gallic acid esters of glucose and other sugars; and phenylpropanoids, such as lignins, flavonoids, and condensed tannins. The phenolic compounds present in wastewater are usually derived from lignin and tannin of plants.

Tannin degradation by anaerobic protozoa: Tannins are phenolic compounds, which can be grouped as hydrolysable and non-hydrolysable tannins. These compounds have a range of effects on various organisms-from toxic effects on animals to growth inhibition of micro organisms. Some microbes are, however, resistant to tannins, and have developed various mechanisms and pathways for tannin degradation. Tannic acid, a heteropolymer of glucose and gallic acid (1 : 9), is one of the most abundant reserve materials of plants (Bhat et al., 1998). Tannin acyl hydrolase (E.C.3.1.1.20) commonly called tannase, hydrolyses only hydrolysable tannins and catalyses the hydrolysis tannic acid.

In tannic acid supplemented anaerobic medium, the protozoa attained a maximum growth of 23.5 x 104 ml^{-1} (Fig. 6.28). Tannic acid could support the growth of flagellates and ciliates, mainly *Metopus* and *Loxophyllum*. The enzyme

tannase production was started from its early phase of growth and reached highest of 12.4U ml^{-1} at exponential period, afterwards it was decreased to 3 U ml^{-1}.

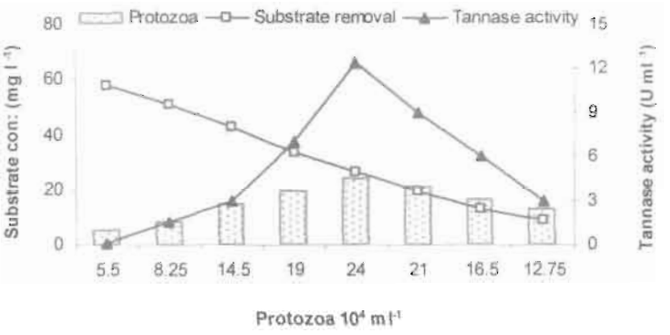


Fig. 6.28. Enzyme activity and substrate removal observed with the growth of protozoa in tannic acid supplemented anaerobic culture acid

Tannase is a key enzyme in the degradation of hydrolysable tannins. The presence of tannase activity was confirmed by nondenaturing electrophoresis and tannase activity appeared as a clear band on a white background (Fig. 6.29). Considerable removal of tannic acid was observed in the anaerobic cultures of protozoa (Fig. 6.28).

The intermediate fermentation products from tannic acid breakdown were gallic acid and pyrogallol as detected by thin layer chromatography (Fig. 6.30). At the end of culturing period phenolic compounds were not present in the system. End products detected were methane, CO_2, acetate, butyrate and propionate. The result indicates that tannic acid is completely converted to simple compounds and further to methane and CO_2

Fig. 6.29. Zymogram of protozoa extract. Clear zones corresponding to protein bands indicating tannase activity

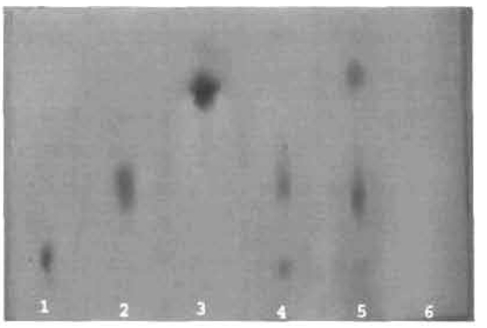


Fig. 6.30. Photograph of TLC- separation of degradation products of tannic acid by anaerobic protozoa. 1 Tannic acid (standard) 2. gallic acid (standard) 3. pyrogallol (standard) 4. sample of 24 h 5. sample of 48 h and 6. sample of 120 h

Generally tannins are toxic as well as bacteriostatic compounds and have non-reversible reaction to protein (Scalbert, 1991). Nevertheless, some micro organisms degrade these compound by producing tannase and play an active role in the soil for nutrient recycling through decomposition of tannin-containing plant materials (Lewis and Starkey, 1969). A number of microbes have also been isolated from the gastrointestinal tract of animals, which have the ability to break down

tannin-protein complexes and degrade tannins, especially hydrolysable tannins (Osawa et al., 1995). A large number of fungi have also been reported to degrade tannins by producing tannase (Bhat et al., 1998; Lekha and Lonsane, 1997). However, this may be the first observation on tannic acid degradation by anaerobic protozoa with key enzyme tannase. This makes them capable to participate actively in the degradation of such plant materials.

Lignin degradation by anaerobic protozoa: The plant cell wall is a composite material in which cellulose, hemicellulose (mainly xylan), and lignin are closely associated. Lignin compounds are polymers of methoxy substituted phenols that are connected by C-O-C or C-C bonds. Growth of protozoa and enzymatic activities were observed during the decomposition of lignin. A maximum protozoa count of 23 x 10^4 ml^{-1} was observed in anaerobic cultures supplemented with ligin (Fig. 6.31). Lignin was found to support the growth of both flagellates and ciliates. Interestingly, *Metopus* was the prominent protozoa in cultures fed with this high molecular weight compound. There was a considerable removal of lignin and observed a maximum of peroxidase activity of 12 U ml^{-1} (Fig. 6.31).

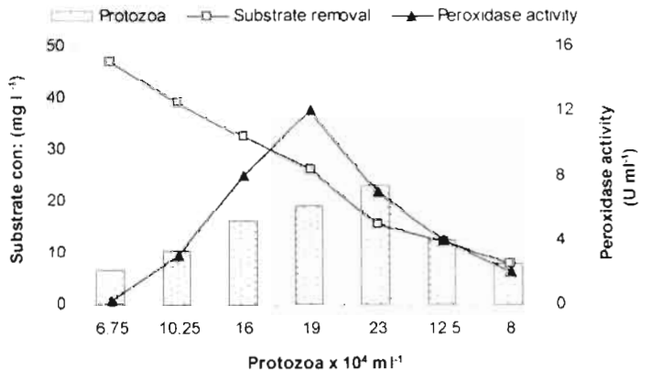


Fig. 6.31. Enzyme activity and substrate removal observed with the growth of protozoa in lignin supplemented anaerobic culture

The protozoa cell extract was applied to the nondenaturing polyacrylamide gel electrophoresis and stained with DMP in the presence of H_2O_2 and Mn^{2+} at pH 4.5 for confirming peroxidase activity. The activity was observed within 5-15 min as orange band over a yellow background (Fig. 6.32).

Tannic acid, gallic acid, and pyrogallol were the intermediate products from lignin biodegradtion, as observed from thin layer chromatograms (Fig. 6.33). On 4th day, no phenolic compounds were visible in the culture.

Fig. 6.32. Zymogram of protozoa extract. Organge zones in yellow background corresponding to protein bands indicating peroxidase activity

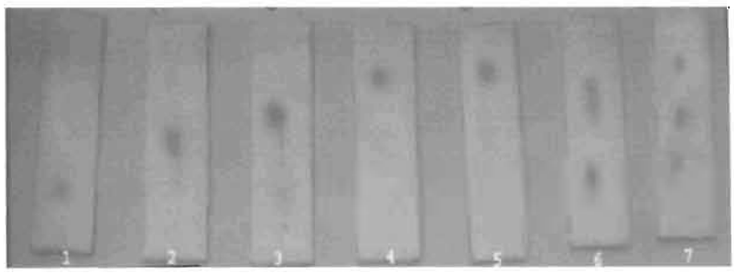


Fig. 6.33. Photograph of TLC- separation of degradation products of lignin by anaerobic protozoa 1. Tannic acid (standard) 2. gallic acid (standard) 3. pyrogallol (standard) 4. resorcinol (standard) 5. ferulic acid (standard) 6. sample of 48 h and 7. sample of 72 h

Acetate and methane were the principal end products of lignin metabolism obtained as evidenced from GC analysis. Small amounts of butyrate, propionate and CO_2 were also detected in the anaerobic protozoa culture. It indicates that complete conversion of lignin to simple compounds and finally to methane and CO_2 by anaerobic protozoa

An intracellular lignin peroxidase detected in the anaerobic cultures might be participated in lignin depolymerization by protozoa. There is no information on the contribution of protozoa to the lignin degradation processes in anaerobic reactors. Although the biodegradation of plant cell wall components by bacteria and fungus have been documented (Akin and Benner, 1988). This is implying that poly phenols are digested not only by bacteria, but also by protozoa in anaerobic systems. Detailed study was required to elucidate the metabolic pathway for lignin degradation by protozoa.

The ability of protozoa to degrade polyphenols is particularly interesting because eukaryotes have not been known to cleave polyphenols. The present results are opening up degradation of polyphenols by protozoa through the synthesis of relevant enzymes. Use of phenol and phenolic compounds are widespread in many industries, such as polymeric resin production and oil refining (Gonzalez et al., 2001). Cmielowski et al., (1964) have first demonstrated the phenol degradation by anaerobic microoranisms. Wang et al., (1986) have reported successful treatment of phenolic compounds using anaerobic fluidised bed reactors and several phenols were degraded to methane and carbon dioxide. However, the role of anaerobic protozoa in the degradation of polyphenols has not been realised so far. Protozoa involvement in phenol degradation would be an added benefit.

Fatty acid degradation by anaerobic protozoa

Long-chain fatty acids (LCFA) are the main products of lipid hydrolysis and are frequently found in wastewaters from various sources, e.g., dairy industry, food processing industry, slaughterhouses, wool scouring industry, and vegetable oil/fat refineries. Long chain fatty acid degradation is considered to be the main problems affecting the anaerobic digestion in reactors. The growth of ciliates species *Prorodon, Cyclidium, Spathidium, Vorticella* and *Metopus* and flagellates species *Menoidium,*

Trepomonas, *Naeglaria*, *Rhyncomonas* and *Bodo* were shown by direct consumption of colloidal sodium oleate particles (Chapter 5). Fig. 6.34 illustrates growth of protozoa and enzymatic activities during the decomposition of oleate given as feed. A maximum of 21 U ml^{-1} intracellular lipase activity was observed during the exponential growth of protozoa. Lipases catalyze the hydrolysis of ester bonds at the interface between an insoluble substrate phase and the aqueous phase.

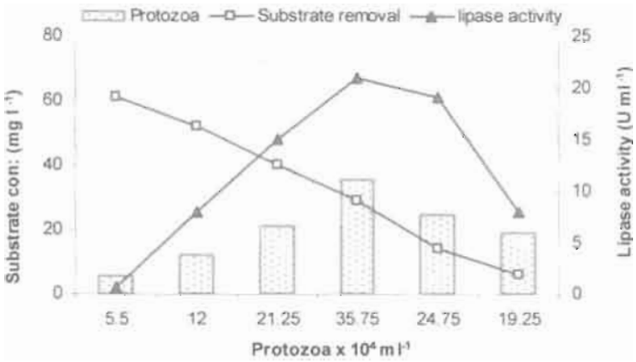


Fig. 6.34. Enzyme activity and substrate removal observed with the growth of protozoa in oleic acid supplemented anaerobic culture

A change in colour from pink to yellow was observed in plates impregnated with protozoa extract within 15 min (Fig. 6.35). Fig. 6.36 shows the zymogram obtained after performing native PAGE with crude lipase enzyme from the anaerobic protozoa. Distinct yellow bands were observed against pink background indicating protein bands.

Fig. 6.35. Photograph of hydrolysis zones with protozoa extract on the agar plates with chromogenic substrate

Fig. 6.36. Zymogram of protozoa extract. Yellow zones corresponding to protein bands indicating lipase activity

In the degradation studies oleic acid, an 18 carbon acid with one double bond (C18 : 1) was degraded anaerobically to palmitic (C16 : 0) acid in batch cultures of anaerobic protozoa (Fig. 6.37). These by-products were further degraded to acetate and ultimately to methane. Small amount of butyrate and CO_2 and, traces of propionate were also observed in cultures.

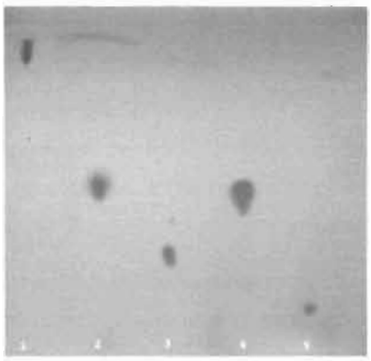


Fig. 6.37. Photograph of TLC- separation of degradation products of oleic acid by anaerobic protozoa. 1. Oleic (standard) 2. palmitic acid (standard) 3 myseric acid (standard) 4. 24 ha sample and 5 120 h sample

Saturated stearic acid was tested for protozoa mediated degradation studies. The growth of protozoa was supported by stearic acid also, but had less diversity and abundance when compared to oleate. Stearic acid degradation was very slow in anaerobic protozoa cultures and lipase activity measured at the exponential stage was only 12 U ml^{-1} (Fig. 6.38). The anaerobic degradation products of the stearate were more or less same as in oleic acid degradation.

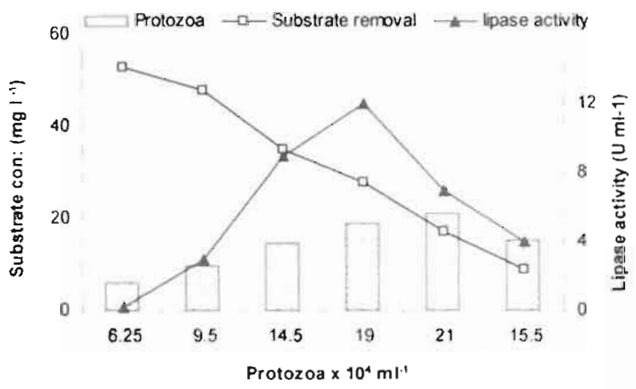


Fig. 6.38. Enzyme activity and substrate removal observed with the growth of protozoa in stearic supplemented anaerobic culture

Previous studies in rumen system have demonstrated the hydrogenation of lipids by rumen protozoal suspension containing *Epidinium caudatum* (Wright, 1959). Guttierez et al., (1962) have reported the hydrogenation of oleic acid to stearic acid in rumen ciliates. The advantage of protozoa is that they would able to digest fatty acids by producing intracellular enzymes while purely bacterial cultures have to first carry out slow extracellular reactions to utilize the substrate.

6.3.4 Extracellular and intracellular enzyme activity in anaerobic protozoa

The enzyme activity in protozoa was mainly intracellular. Intracellular and extracellular amylase activities were quantitatively assessed in four species of free living protozoa in two major groups namely, flagellates and ciliates. The organisms were *Menoidium, Rrhyncomonas, Metopus* and *Plagiopyla* and they were the most common genera in all experimental cultures. Significantly higher intracellular amylase activity was detected compared to extracellular amylase activity for all organisms grown at 30°C (Fig. 6.39). The intratracellular amylase activity was in the order: *Metopus* > *Plagiopyla* > *Rhyncomonas* > *Menoidium*. While, the extracellular amylase enzyme activity for protozoa grown at 30°C followed the order: *Menoidium* > *Plagiopyla* > *Rhyncomonas* > *Metopus*.

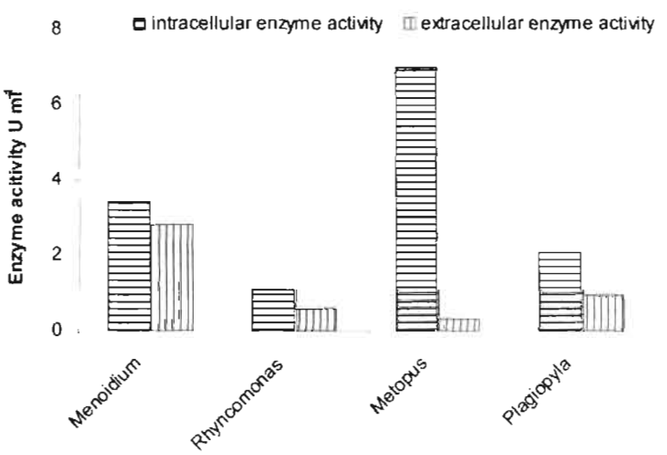


Fig. 6.39. Intracellular and extracellular amylase activites in anaeobic protozoa

The studies show that protozoa were able to consume substantial quantities of organic matter as additional food source along with bacterial grazing. Protozoa with a richer portfolio of intracellular enzymes could able to digest organic particles internally and they profit from minimal wastage of enzyme. Bacteria cannot consume the particulate matter directly and employ extracellular enzymes to hydrolyze the particulate organic matter for absorption. Some of the protozoa can excrete extracellular enzymes and this leads to partial digestion of organic matter around which in turn supports other micro organisms. Present theory of carbon flow in anaerobic system is that both particulate and dissolved matters are degraded by bacteria, which are then grazed by protozoa. Results of present studies pointing to a more efficient pathway of carbon flow in anaerobic food webs, in which direct involvement of protozoa in the organic matter degradation is viewed along with bacteria.

6.4 Conclusions

Most of the studies on anaerobic degradation are based on bacteria, actinomycetes, fungi, and yeast (Wood, 1985). Present study strongly supports the involvement of protozoa in the anaerobic degradation in addition to the above organisms. To our knowledge, this is the first information on the enzymatic degradation of organic matter by protozoa in anaerobic reactors

- Anaerobic conversion of organic matter is brought about by the co-ordinated activity of a number of different microbial groups including archeae, bacteria and protozoa and resulted in higher COD removal and methane yield.
- The symbiotic association with methanogen is not essential for the growth of protozoa and protozoa are capable of digesting food materials without endosymbiontic methanogen but with different end products.
- In the absence of endosymbiontic methanogens, a shift in the metabolism leads to the production of less oxidised and less energy yielding fermentation products such as propionate and butyrate for managing hydrogen partial pressure inside the cell.
- Endosymbionts in protozoa are lost during continuous culturing and protozoa without endosymbiotic bacteria can metabolize organic matter to short chain

acids. Thus, the enzymatic activity of protozoa is an inherent property and it is not dependent on bacteria.

- The capability of protozoa to utilise various soluble carbohydrates has great importance in anaerobic degradation.
- Protozoa can degrade various monosaccharide, disaccharides, oligosaccharides and polysaccharides. Although all the protozoa are not grown on all substrates, many are capable of utilizing a large range of natural saccharides and this lead to enhanced biodegradation in bioreactors with protozoa.
- The ability of protozoa to degrade both dietary and microbial protein is very important in anaerobic environment.
- The ability of protozoa to degrade polyphenols is very important because eukaryotes are considered to be unable to cleave polyphenols.
- Protozoa with intracellular lipase enzyme activity would be able to digest fatty acids intracellularly.
- In the case of carbohydrates, fatty acids, polyphenols and proteins, hydrolysis was found to be rate limiting under anaerobic degradation. Significance of protozoa in anaerobic wastewater treatment technology is the ability to overcome this rate limitation caused by hydrolysis of various compounds.
- The direct utilization of saccharides, proteins, polyphenols and fatty acids by protozoa would represent a more efficient pathway in anaerobic food webs compared with the organic matter →bacteria →protozoa microbial loop.
- Some of the protozoa secrets enzymes into surrounding medium and this may lead to partial digestion of organic matter which can be digested easily.

Bacteria have been recognised as the primarily responsible for the degradation of organic particles, and protozoa were thought to play only a minor role in anaerobic degradation. Present study reveals an additional pathway of carbon flow in anaerobic food webs through protozoa

Summary

Anaerobic protozoa in anaerobic reactor ecosystems

Wastewater provides an ideal growth medium for many groups of organisms and growth of these organisms play key roles in the process of purification through biodegradation. No single organism is capable of utilising the whole variety of compounds, instead a diverse ecosystem develops in the wastewater treatment systems. Such ecosystem typically has degraders, predators, symbionts and competitors. Deep understanding on the ecosystem becomes essential to design engineered reactors for the effective treatment of wastewater.

In anaerobic reactor ecosystem, degradation processes are considered to be bacterial in origin and protozoa are known to play insignificant roles. The findings of the present study force to alter the above views, which could unveil the importance of anaerobic protozoa, an additional trophic layer in the anaerobic reactor ecosystem. The community structure of anaerobic protozoa primarily consists of amoeboids, flagellates and ciliates. Among the constituents of this rich fauna of anaerobic protozoa many were monitored and identified that include fourteen different genera of ciliates, nine different genera of flagellates and eight different genera of amoebae. These organisms are found to be highlty dynamic and sensitive to environmental and operational conditions of the system. A succession in growth of protozoa was observed in this study in terms of diversity and number with respect to reactor operations. Flagellates and amoeboids were flourished at early stages of reactor operation and then ciliates, on reaching steady state conditions in reactors.

Influence of protozoa on anaerobic reactor performance

The populations of protozoa varied considerably with the changes in the anaerobic reactors, which can be considered as an indicator of system performance in anaerobic process. Each protozoa group is associated with different reactor conditions and the predominance of one group or another can be an indicator of wastewater treatment efficiency (Table 1).

Table 1. Relations between protozoa andanaerobic reactor efficiency

Predominant group	Efficiency	Possible cause
Small ciliates and small flagellates	Low	Low loading
Free-swimming ciliates with high diversity	Good	
Small flagellates	Low	High VFA, low or high pH, low loading
Flagellates and amoebae	Very low	High VFA, low pH, high sludge loading
Small flagellates, amoeboids and cysts, a few small ciliates	Low	High loading, not easily biodegradable

The efficiency of anaerobic wastewater treatment process is linked not only to the bacterial population but also to the protozoa. Protozoa, mainly the ciliates in anaerobic reactor are related to the good performance of anaerobic processes. Protozoa have been previously reported to enhance degradation in aerobic system, but this positive involvement in the anaerobic process is the first time (Priya et al., 2007 a,b).

Isolation and culturing of anaerobic protozoa

Anaerobic ciliates and flagellates were isolated from a laboratory scale anaerobic biofilter (Ajit et al., 2005) by using centrifugation and micromanipulation method. The optimum physico-chemical and nutritional requirements for anaerobic protozoa (Table 7) was closely correlated to anaerobic digestion process parameters (Jefferson et al., 2001). The development of anaerobic protozoa culture could facilitate the detailed study of protozoa in anaerobic degradation.

Most of the toxicity studies have focused on elucidating the different metal resistance mechanisms, especially those used by bacteria and fungi (Gadd, 1993; Silver, 1996; Lovley, 2000). It was observed in the present study that ciliates are good candidates to detect the toxicity and to determine the bioavailable concentration of certain toxic pollutants, like heavy metals. They are more sensitive to heavy metals

than the microorganisms such as yeast, micromycetes and bacteria, probably because of the absence of cell wall at the trophic stage.

Table 2. The optimum growth requirements for anaerobic protozoa

Parameters	**Optimum range**
Temperature	30-32°C
pH	6.8-7.2
VFA concentration	<0.05M
Oxygen	Nil or below 0. 03 mg l^{-1}
A ammonia-N	10-12 mg l^{-1}
Phosphate-P	6-8 mg l^{-1}
Sulphide-S	1-2 mg l^{-1}
Bacterial density	4.5 x 10^{6} - 2 x 10^{9} cfu ml^{-1}
Feed	Ciliates prefers suspended organic matter
	Flagellates prefers dissolved organic matter

Role of protozoa in anaerobic wastewater treatment process

COD removal: The understanding of anaerobic reactor ecosystem need to undergo substantial revision, from the traditional view where the anaerobic degradation is mainly a bacterial process. The present study establishes direct involvement of protozoa, especially ciliates in anaerobic reactors. At all loading rates, the steady state COD removal efficiency was greater than 75% with high number and diversity of ciliate. COD removal was strongly correlated to ciliate number (R^2 = 0.974 and R^2 = 0.966) at all hydraulic retention times and loading rates. Direct uptake of dissolved COD by flagellates and particulates by ciliates in bacteria-suppressed cultures confirm that protozoa are not purely dependent on grazing of bacteria as food source and they can consume organic matter. Size selective grabbing denotes that ciliates are size selective feeders and may not distinguishing bacteria and similar sized organic particles.

The methanogenic process is generally limited by the rate of hydrolysis of suspended matter and organic solids, which is of particular importance during the

anaerobic treatment of organic solid wastes. Significance of protozoa in anaerobic wastewater treatment technology is their ability to overcome the rate limitation by direct uptake and intracellular digestion of particulate matter.

Methane Production: Methane production is strongly correlated to ciliate density (R^2 = 0.98 and R^2 = 0.97) at all hydraulic retention times and loading rates. The enhanced methane production with the presence of protozoa, especially ciliates was correlated with presence of endosymbiotic methanogens in anaerobic systems. The endosymbiotic methanogen is reached up to one third of the total free methanogens present in the reactor (free methanogen 9 x 10^7 ml^{-1} and endosymbionts 2.99 x 10^7 ml^{-1}). These endosymbionts accounted to 33% of the total methanogenesis. Obviously, the specific methanogenic activity of sludge formed in the reactors with ciliates is therefore better than in reactors that do not have ciliates (31 ± 2 %) in the absence of protozoa.

Our studies confirmed the presence acetate utilizing *Methanosaeta* and *Methanosarcina* and, hydrogen utilizing *Methanobacterium* as endosymbionts in anerobic protozoa. The energy metabolism of endosymbionts is totally coupled to that of host. The symbiotic consortium enables to produce methane as a metabolic end product.

Biomass reduction: Grazing activity of ciliates is demonstrated to be essential for the clarification of wastewater in anaerobic systems. Grazing activity of ciliates reduces the sludge biomass in the systemwhile enhancing COD removal. MLSS was significantly lower (16 - 34 %) in CSTAR with protozoa. The reduction of MLSS concentration with respect to ciliate number in the anaerobic system denotes that biomass is consumed, digested and converted to biogas by the presence of ciliates.

The treatment and disposal of excess sludge represents a bottle neck of biological wastewater treatment plants all over the world, due to environmental, economic, social and legal factors. Utilization of the potential of protozoa for sludge reduction by promoting their growth in the engineerd anaerobic treatment system is an important advantage. It is shown that both living and dead bacteria can be utilised by protozoa (ciliates, flagellates and amoeba).

Turbidity Removal: The anaerobic protozoa, mainly ciliates were found to scavenge the suspended particles and free moving smaller organisms, and this could help to remove turbidity and to improve the quality of treated effluent. The presence of stable protozoa population in the anaerobic reactor constantly delivered high quality effluents. In brief, anaerobic protozoa have important roles for the good balance of the biological ecosystem: they can stimulate bacterial growth while eliminating excess biomass.

Nutrient recycling: In treatment plants protozoa that consume living bacteria found to have the highest biomass specific excretion rates of inorganic ammonia-N and phosphorous-P. Protozoa grazing thus stimulate the decomposition of organic matter and enhance activity in reactors without any external addition of nutrients. The role anaerobic protozoa is also important to increase the rate of turnover rate of essential nutrients that locked in bacteria

Oxygen toxicity Removal: Facultative protozoa with SOD activity help to overcome possible occurrence of oxygen toxicity in anaerobic reactors. O_2 tension resulted in loss of methanogens in biomethantion system, while methanogens were observed inside the protozoan cysts. The methanogen bearing protozoa cyst formation in under oxygen tension is potential mechanism to overcome unfavorable changes. Anaerobic reactors with protozoa rich sludge can detoxify the trace amounts of oxygen toxicity in wastewater (Priya et al., Accepted paper).

Protozoa mediated organic matter degradation

Bacteria and protozoa are the main microorganisms present in anaerobic treatment systems (Priya et al., 2007a). Higher substrate removal and methane yield obtain from the co-existence of bacteria and protozoa. The protozoa produce acetate and methane as major end products in cultures with endosymbionts and lactate, pyruvate, butyrate, carbon dioxide and hydrogen are also detected in small quantities. Loss of endosymbiotic methanogens in anaerobic protozoa is observed with continuous subculturing, and are capable of fermenting food without endosymbiotic methanogen by shifting their metabolic pathway. Shift in metabolic pathway caused production of less oxidised and less energy yielding fermentation products such as

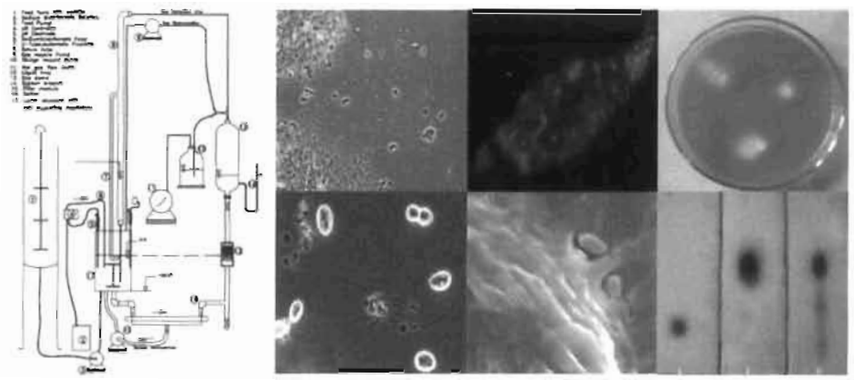
propionate and butyrate to decrease in partial hydrogen pressure inside the cell. Thus, the organic matter degradation of protozoa is an inherent property and is not dependent on bacteria. Metabolic end products - hydrogen, CO_2, butyrate, propionate and acetate serve as substrate for ecto/endo symbiotic bacteria and also for other protozoa, mainly flagellates (VFAs). Dissolved organic matter released in the presence of protozoa is a readily available carbon source for bacteria and may thereby influence bacterial metabolism and growth activity.

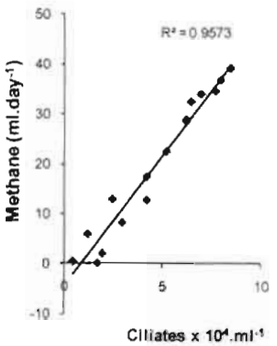
The anaerobic protozoa have the ability to degrade various compounds which is generally present in wastewaters. Present theory of carbon flow in anaerobic system is that both particulate and dissolved matter are degraded by bacteria and then these bacteria are grazed by protozoa. However, direct utilization of saccharides, proteins, polyphenols and fatty acids by protozoa represents a more efficient pathway or additional loop in anaerobic food webs (organic matter → protozoa) compared with the organic matter → bacteria → protozoa microbial loop. Protozoa with a richer portfolio of enzymes are able to digest organic particles internally while purely bacterial cultures have to first carry out slow extracellular reactions to utilize the substrate. Significance of protozoa in anaerobic wastewater treatment technology is the ability to overcome rate limitation caused by hydrolysis of various organic compounds (Priya et al., 2007a,b)

An overview of the data about the abundance of protozoa in anaerobic reactor ecosystem gives the impression that these organisms are not negligible in anaerobic environments – although they are neglected. The present study on protozoa with respect to anaerobic reactor performance explains their presence and significance beyond bacterial grazing. To our knowledge, this is the first reported information on the enzymatic degradation of organic matter by protozoa in anaerobic reactors. The technological importance of these results is that reactors with protozoa rich sludge can enhance the rate of mineralisation of complex wastewater, especially wastewater containing particulate COD.

SUMMARY

The anaerobic reactor ecosystem introduces a new microbial loop in the anaerobic degradation of biomass and energy flow. Short food chain (organic matter → protozoa) adds more efficiency in anaerobic system





Significance of protozoa in anaerobic wastewater treatment technology is the ability to overcome the rate limitation caused by solubilization of organic matters

Protozoa rich sludge enhances the rate of mineralization of complex wastewater, especially wastewater containing particulate COD

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