GUT MICROFLORA OF TERMITES AND ASSOCIATED EMISSION OF GLOBAL WARMING GASES

Thesis submitted in part fulfil ment of the requirements for the degree of Doctor of Philosophy (Agriculture) in Environmental Sciences to the Tamil Nadu Agricultural University Coimbatore

By

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2000

CERTIFICATE

This is to certify that the thesis entitled "**GUT MICROFLORA OF TERMITES AND ASSOCIATED EMISSION OF GLOBAL WARMING GASES**" submitted in part fulfillment of the requirements for the award of **DOCTOR OF PHILOSOPHY (Agriculture) IN ENVIRONMENTAL SCIENCES** to the Tamil Nadu Agricultural University, Coimbatore is a record of bonafied research work carried out by **MS. V.GOMATHI** under my supervision and guidance that no part of this thesis has been submitted for the award of any other degree/diploma/ fellowship or other similar titles and that the work has not been published in part or full in any scientific or popular journal or magazine.

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Acknowledgement

The author is sincerely grateful to **Dr. K.Ramasamy**, Dean, Adiparasakthi College for Agriculture, Kalavai, Chairman of the Advisory Committee for suggesting the problem and inspiring guidance at every state of this endeavour.

The author is extremely thankful to the members of the Advisory Committee, **Dr.J.Prabhakaran,** Professor of Agricultural Microbiology, Department of Environmental Sciences, Dr. **R. Samiyappan**, Professor of Plant Pathology and **Dr.K.Govindarajan,** Professor and Head, Department of Agricultural Microbiology, Tamil Nadu Agricultural University, Coimbatore, for their valuable suggestion and keen interest shown during the course of study. The author is indebted to **Dr. D. Augustine Selvaseelan**, Professor and Head, Department of Environmental Sciences for providing the facilities to carry out the research.

The author sincerely thanks Messrs. Marimuthu, Venkatachalam, Suri, Doraisamy, Ilamurugu, Kalaichelvan, Prasad, Pughlendi, and Mahimairaja for their valuable guidance and suggestion during the course of study.

The author is very much indebted to Kumutha, Anita, Bharani, Suresh Babu and Kannan for their immense help in executing this work. The author gratefully indebted to her parents her sisters and acknowledges the help rendered by Ms. Kokila and Mr & Mrs. Vivekanandan and Ms. Punitha for their constant encouragement through out this investigation.

V.Gomathi

Abstract

ABSTRACT

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Degree : DOCTOR OF PHILOSOPHY IN ENVIRONMENTAL SCIENCES

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Termites are eusocial insects belong to order Isoptera. In the present investigation an attempt was made to study the gut environment of the termites, presence and distribution of anaerobes in the gut of termites and their contribution of green house gases (CH_4 and CO_2) was investigated. The aerobic population isolated from the different region of the guts showed the presence of plysaccharide producing , N_2 fixing bacteria. Based on their characteization they were identified as *Azotobacter* and *Beijerinckia*. Nitrogen fixation in the gut of termites is mediated by bacterial isolates. Salivary gland and hindgut showed the presence of N_2 fixing aerobic and anaerobic *Azotobacter* and *Beijerinckia* (Aerobes) and facultative anaerobes like *Klebsiella* and *clostridium* which play an important role in nitrogen metabolism . Enumeration of total anaerobe and aerobes resulted the maximum population in worker followed by soldier and queen

termites. Whereas the population of anaerobe is more in the foregut followed by midgut and hindgut of soldier termites. Three isolates of methanogenic bacteria was further purified and characterized. They belong to the genus *Methanobacteria* , *Methanosarcina.* All the three isolates produced methane .Among the isolates *Methanobacteria* grows very well in formate and H_2:CO_2 whereas, *Methanosarcina* grow very well in acetate and methanol. The optimum temperature for the methanogenic isolates was 30-37°C and they preferred optimum pH ranged between 7.0-7.5. A fluorescing compound F_{420} was detected from the bacterial isolates which is a main diagnostic tool for the identification of methanogens. The protein content of the isolates *Methanosarcina* showed two low molecular weight. The cellulosic feed ingested by the termites get digested with the help of enzymes *viz* ., cellulase exoglucanase, endoglucanase and other enzymes amylase, invertase and maltase were detected from the salivary gland. , foregut, midgut and hindgut. The maximum enzyme activity was observed in the midgut followed by salivary gland. The present study revealed the vital role of anaerobes in the digestion of cellulosic feed and the emission of methane to the global cycle. *In situ* emission of methane from termites was observed higher in worker termites than soldier and queen. Artificial crumb formed from the isolated polysaccharide producers which enhanced the plant growth. The study revealed that the gut microflora of termites play a major role in the digestion of cellulose in to various metabolic products, which is responsible for emission of CO_2 and CH_4.

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CHAPTER I

INTRODUCTION

Termites are insects belonging to the order Isoptera and are characterized by their colonial behaviour and development of morphologically distinguishable caste member in a colony, their incomplete metamorphosis and the formation of alate of fore and hind wings that are similar in size and venation (Breznak, 1982). The feed performance of termites ranges from wood to leaves, grasses, humus, detritus and herbivore dung and some have evolved the intriguishing habit of cultivating fungus garden as a nutrient resources. Worker larvae dominate the nutrient dynamics with the aid of intestinal microbiota it decomposes plant litter. The alimentary tract of termites consists of three main division, the foregut, (stomodeum) the midgut (mesenteron) and hindgut (proctodeum). Hindgut is the major site of nutrient absorbtion of cellulose and hemicellulose which undergo appreciable degradation on passage through the termite gut. The bulk of polysaccharide dissimilation occurs in the hindgut and all available evidences indicates that the hindgut microbiota is the driving force of such activity .

Many studies revealed that morphologically diverse bacterial flora colonizes the hind gut, due to the diversified microbial activity lot of fermented compound like propionate, acetate and carbondioxide are produced Odelson and Breznak (1983). Number of bacterial isolate found to possess C_x type of cellulase activity however, todate there is no convincing evidence that bacteria are quantitatively important to cellullase hydrolysis. The use of nitrogen by termites as nitrogen source speculated by many workers, Noda *et al.* (1999). Termite contain high C: N ratio the nitrogen requirement may be met out by nitrogen fixing gut microflora. The fungal comb present in the termite gut also play a major role in the digestion of cellulose with the help of enzymes from *Termitomyces* sp.

Anoxic degradation of cellulose is also occur in the termite gut the concentration of terminal electron acceptor other than CO_2 is usually limited., thus the fermentation by the resident microbiota is primarily towards methanogen. Microbial methanogenesis is common in nature and is normally associated with decomposition of organic matter,

(Smith and Hungate, 1956; Miller *et al.* 1985 ; Bryant and Boone , 1987; Breznak and Brune, 1994; Leadbetter and Breznak, 1995). Almost all termite emit methane and early work claims suggested that they made a major contribution to atmospheric methane and the greenhouse effect (Hackstein and Stumm, 1994; Ohkuma *et al.* 1995). Most termites use soil together with saliva and faeces to construct their nest in doing so they form stable soil crumbs with the help of microbes and their microbial products. Such crumb help in receiving and retaining more rain water and helps in increasing soil biological process and ultimately improves the crop yield. Hence the present investigation was carried out to find aerobic and anaerobic microflora of termite and their associated products which involved in cellulose degradation and emission of greenhouse gases (CO_2 and CH_4) on the global carbon cycle and also study their effect on soil health and plant growth.

- Studies on the aerobic and anaerobic gut microflora of termite
- Studies on the digestive enzymes in the termite gut
- Studies on the nitrogen fixing and methanogenic bacteria present in the termite gut
- Studies on the effect of substrate, pH and temperature for the growth of the culture isolated from termite guts
- Studies on the methane emission *in situ* from different termites
- Studies on the microbial products associated with termite gut
- Studies on the effect of artificial crumb on the plant growth

REVIEW OF LITERATURE

CHAPTER II

Review of Literature

Termites constitute an important group of soil animals and play a major role in tropical ecosystems. Microorganisms symbiotic in the hindguts of termites are important in the nutrition of their insect hosts. Gut microbes are important for termite vitality, nutrition derives from the products of microbial metabolism.

Termites or white ants are highly developed social insects and are grouped under the order Isoptera. Termites are an important group of soil organisms and are mostly found in tropical, subtropical and warmer temperate zones of the world. Termites are divided into two groups and their association with microbes are tabulated (Table 2.1)

The termites form colonies of a few hundred to a maximum of seven million individuals. The termites present in a colony consist of several castes, which are morphologically and functionally distinct (Miller, 1969).The caste may be divided in to two broad groupings, reproductive and sterile. The most important of the sterile castes are the soldiers, and the workers. They subsist on a diet rich in cellulose which may be in the form of living or dead wood, woody tissues of plants or dung.

The nutritive metabolism of termites is based upon the exploitation of cellulosic materials by facultative anaerobes and obligate anaerobes which are symbiotically associated with the termites and exist in the hind gut of the insect (Paul *et al* ., 1986)

The digestive system of the termite is divided in to fore gut, tubular mid gut and voluminous hind gut. However, higher termites secrete their own digestive enzymes and are independent of gut microorganisms for their nutrition. The lower termites also possess this ability, but their production of cellulolytic enzymes is apparently inadequate, (Breznak *et al.*,1973). Hence, lower termites mostly depend on the activity of gut microorganisms for their nutrition, which are present in the hind gut region.

2.2. Role of termites in ecosystem:

Termites play a significant role in flow and recycling of nutrients in the tropical ecosystem. In natural environment, termites play a role in the turn over of complex carbohydrates by degrading dead trees and other cellulose containing materials to more

simple carbon compounds. From the tropics to deserts, they stir and mix with the aid of bacteria, protozoa and fungi and thereby effectively recycle cellulosic organic residues.

Table. 2.1 Different types of termites

(Breznak, 1982)

Family	Mutualistic associations
Lower termites	Protozoa, bacteria
Mastotermitidae	Protozoa, bacteria
Kalotermitidae	Protozoa, bacteria
Termopsidae	Protozoa, bacteria
Hodotermitidae	Protozoa, bacteria
Rhinotermitidae	Protozoa, bacteria
Serritermitidae	Protozoa, bacteria
Higher termites	
Termitidae	Bacteria
Apicotermitinae	Bacteria
Termitinae	Bacteria
Macrotermitinae	Bacteria, Fungi
Nasutitermitinae	Bacteria

Termite gut microbiota presents an unique biological system involved in the conversion of woody and cellulosic substrates into useful products through the process of solubilization. Saxena *et al.* (1993) reported that termites are the best cellulose degraders in soil. Termites also play a significant role in soil fertility by replenishing nitrogen compounds in the soil (Breznak *et al.*, 1973), by fixing atmospheric nitrogen with the help of microbial symbionts. Paul *et al.* (1986) reported that aerobic soil inhabiting microbes solubilize the undigested cellulosic materials to glucose and low carbon compounds with the help of facultative coccoid gut bacteria.

Environmental conditions such as light levels, humidity, temperature and CO_2 and O_2 concentrations play a part in CH_4 production. Termites prefer the absence of direct solar radiation, an immobile atmosphere, saturated or near saturated relative humidities, high stable temperature, and even elevated levels of CO_2.

2.3. Methane emission

Methane is an important atmospheric trace gas which affects the chemistry of the troposphere and of the stratosphere. Termites emit large quantities of methane, carbondioxide and molecular hydrogen in to the atmosphere. The production of methane by termites was first reported by Cook (1932). The prevalence of strict anaerobic conditions in the hind gut was recorded in termites. (Cruden and Markovetz ,1984), Oryctes larvae (Bayon, 1980) and Cockroach (O' Brien *et al* ., 1979). Veivers *et al.* (1980) confirmed anaerobic nature of the hind gut of the termites.

The methane was produced from members of the methanogenic archea which are present in the gut either alone or in association with protozoa, and appear to be one of the terminal H_2 sink of hind gut fermentations.

Environmental parameters such as pH, redox potential, temperature of the hind gut are some of the factors that control the methane production from termites. Considerable amount of methane is produced in the guts of insect due to anaerobic decomposition (Kovoor, 1967) and the site of methane production is the hind gut (Bayon, 1980; Cruden and Markovetz, 1984; Gijzen *et al* ., 1991).

Colette and Etievant (1980) reported that in ruminant mammals and xylophagous insects, the breakdown of cellulose by extracellular symbiotic organism is complete and lead to the formation of by products which may act as substrates for methanogenic bacteria.

Table **2.2 Gaseous emission rates per termite**

(Zimmermann *et al.*,1984)

Colony	CH_4 µg/day	CO_2 µg/day	NH_4 µg/day	CO µg/ day
Reticulitermes sp 1	0.447	0.001	0.05	0.006
Reticulitermes 2	0.237	0.107	0.02	0.018
Reticulitermes 3	0.592	0.137	0.03	0.060
Gnathamiterms1	0.456	0.410	0.16	0.091
Gnathamiterms2	0.338	0.210	0.12	0.053

Johannes *et al.* (1994) reported the potential contribution to atmospheric methane by methanogenic bacteria in the hind gut of insects. Breznak and Switzer (1986) reported that the methane generation was due to acetogenesis in cockroaches and termites. Whereas, Miller and Wolin (1983) observed that methane production in cockroaches was mainly from CO_2 and H_2 Table 2.2.

The amount of methane liberated depended on the number of *Methanospirillum* like cells in the gut (Cruden and Markovetz, 1984). Rasmussen and Khalil (1983) estimated the global production of methane by termites. The symbiotic relationship of two species of *Methanobrevibacter* associated with the flagellate in the hindgut of termites *(Reticulitermes flaviceps)* was found to enhance the methane production (Leadbetter and Breznak, 1996). Rasmussen and Khalil (1983) reported that the global production of methane to an extent of 2 to 150 Tg/ year by termites. Rogers and Whiteman (1991) also estimated that contribution of green house gases : methane, nitrogen oxides by termites estimated between 25 and 150 Tg/year. Zimmerman *et al.* (1982) reported that termites emit 1.5×10^{14} g of methane, 5×10^{16} g of CO_2 and 2×10^{14} gms of molecular H_2. Mugedo (1995) reported that termites contribution to atmospheric methane content was between 1.11% and 4.25% per annum. Zimmermann *et al.* (1984) further reported that the average rate of methane production was 0.425 µg CH_4/termite/day for the lower termites and 0.397µg CH_4 / termite/ day for the higher

termites. Zimmermann *et al.* (1984) also reported that there existed variation in the amount of CH_4, CO_2 and CO released by different species of termites (Table. 2.3)

Fraser *et al.* (1986) observed a positive correlation between the amount of biomass consumed and methane generation. Further, they reported that the methane emission was 3.2 mg/g of wood consumed. Termites emitted higher quantities of methane when fed on C_4 plants than on C_3 plants, because of more enriched carbon in C_4 plant material. In the case of cockroach, methane production ranged from 10 – 25 to 165 µmol/h /insect in cockroach (Cruden and Markovetz, 1984 and 300 - 380 µmol /h/insect in scarab beetle larvae (Bayon, 1980). Messer and Lee (1989) reported the effect of 2 bromoethanesulfonic acid treatments on methane emission by the hind gut microbiota in the termite *Zootermopsis angusticollis.*

Table 2.3 Emission of CH_4 by termite gut

(Brauman *et al* . 1992)

Termite	CH_4 emitted µmol/ gut of termites
Wood feeding termites	
Coptotermes formosanus	0.01
Reticulitermes favipes	0.10
Microcerotermes parvus	0.14
Nasutitermes arborum	0.13
N.nigriceps	0.24
Grass feeding termites	
Trinervitermes rhodosiensis	0.18
Fungus growing termites	
Macrotermes mulleri	0.25
Soil feeding termites	
Cubitermes speciosus	0.48
Procubitermes sp	0.39

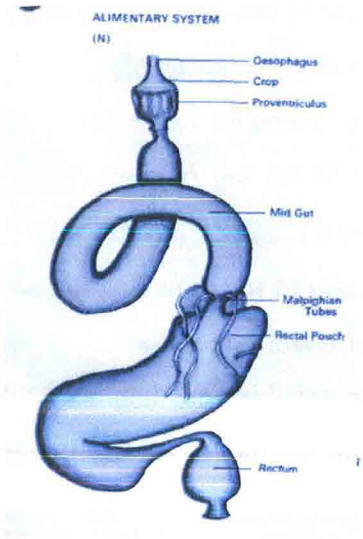
2.4. Insect gut environment

The alimentary system in most of the animal species provides a suitable niche for microflora. However, only some sections of the alimentary system are more amenable than others for the colonization and multiplication of microbial communities, Hungate, (1966). Hungate (1975) reported a complex association of many bacterial types and of protozoa capable of digesting cellulose in rumen.

In animal species, communities of indigenous microorganisms are distributed both vertically and horizontally in the alimentary tract. Animal intestine exihibit a continuous culture process and the microbial population increases from the oseophaegal end to colon. In birds, the intestine is same as that of animals but there are two ceaca, (Holland *et al.*, 1987). In mammalian intestine, stomach is connected to the dueodenum followed by the colon. There are no diversions except for the small appendix. In ruminant mammals, stomach is modified into a four chambered structure, the largest chamber being rumen. The gut of the insect is generally divided in to three regions *viz.*, fore gut, mid gut and hind gut. Foregut (Stomodenum) is a place of storage, Midgut (mesenteron) is a place of digestion and Hindgut (proctodenum) is a place of excretion. The clear bulk of the microbiota is housed in the hind gut usually in a dilated portion of the proctodeum known as paunch which is a major site of nutrient absorption,(O' Brien and Slaytor, 1982).

Bignell (1984) reported that arthropod gut provided a suitable niche for microbes, but the nature of microflora and their distribution depended on the physico-chemical conditions like pH, redox potential, temperature etc of that region. Brune *et al.* (1995) reported that large number of aerobic and facultative anaerobic bacteria occur in the hind gut. Presence of anaerobic microflora showed that hind guts are a purely anoxic environment together with steep axial pH gradients in higher termites.

2.4.1. pH/ Redox potential of termite



pH is one of the important factors that determine the growth of a wide range of micro organisms. Any acidic metabolites excreted by the microorganisms are effectively buffered or removal by the termites O'Brien and Slaytor (1982). pH of the fore and mid gut is around neutrality. Whereas the paunch, colon and rectum appear to be slightly acidic. The pH of the foregut is lower and more variable than in the other regions of the gut. (Eutick *et al.*,1976) reported that the pH of hindgut of termites was between 6.5 to 7.0. However reported that the pH of the gut of cockroach was 5.4, (Greenberg *et al.*, 1970) whereas higher pH values for the mid gut region of scarabaediae larvae (Bayon, 1980).

Redox potential of the gut determines the type of microflora *i.e.,* aerobic, facultative anaerobic or anaerobic and their distribution in the gut ecosystem. The foregut and midgut of termites were aerobic with and E'o in excess of +100 mv. The paunch and colon were anaerobic with and E'o about -230 to -270 mv. Whereas the hindgut of termites showed -125 to -270 mv which proved the anaerobic conditions of the gut. Brune *et al (*1995) reported the role of oxygen and the intestinal microflora in the metabolism of lignin derived phenyl propanoides and other monoaromatic compounds by termites.

2.5. Digestive enzymes of the gut

The gut micoflora also possess enormous potential in aiding the digestion of the ingested feed as reported by Krishnamoorthy (1960).

The termite gut consists of the tubular mid gut which is a key site for secretion of digestive enzymes and for absorption of soluble nutrients. Hungate (1966) observed the enzymes extracts from fore gut and mid gut which are devoid of protozoa, could hydrolyze roughly 1/3rd the weight of cellulose and hemicellulose components of saw dust.

O' Brien and Slaytor (1982) reported that cellulose digestion in higher termites is mediated solely by cellulolytic enzymes secreted by termites Whereas, in lower

termites, cellulose is digested by enzymes secreted both by termites and the gut organisms and the end products of metabolism are used by termites as energy source.

Enzymes of termite origin included an exoglucanase, two endoglucanases, glucosidase and invertase. Amylase and maltase activities which were found in salivary glands and mid gut of *M.darwiniensis* which were able to utilize starch as a nutrient (Veivers *et al.*, 1982). These enzymes were secreted by the salivary glands, fore gut and mid gut and were not of microbial origin.

Higher and lower termites have all enzymes necessary for converting glucose to pyruvate and for oxidizing acetate to CO_2 and H_2O via the tricarboxylic cycle. Potts and Hewitt (1974) found that about 70% of the total cellulase activity was in the mid gut and that about 40 % of this activity was associated with mid gut wall. In another experiment they reported that hind gut was shown to possess about 15% of the cellulase activity. Veivers *et al.* (1982) reported the presence of carboxy methyl cellulase (CMC), cellulose hydrolyzing activity (CHA) and cellobiase in salivary gland, fore gut, mid gut and hind gut of *Mastotermes darwiniensis.* Further, they stated that enzymes secreted by the gut tissues accounted for half of the total cellulase activity of whole termites. However, most of the cellulase activity was found in the hind gut of termite. Veivers *et al* (1982) reported that termites secrete a C_1 type cellulase in *Trinervitermes trinervoides* (higher termite) and *Mastotermes darwiniensis* (lower termites).

Yamaoka and Nagatani (1975) observed that the extracts of salivary glands contained C_1 and C_x cellulase activities , while foregut and midgut region recorded only C_x cellulase activity. Further, they reported that C_x cellulase secretion in gut tissues increased, when the termites were fed on the green leaves. In *Macrotermes natalensis* cellulase activity was mainly in the mid gut (Martin and Martin, 1978). Whereas *Coptotermes lacteus* C_x cellulase activity was associated in fore and mid gut (O'Brien et al. 1979).

Retiff and Hewitt (1973) detected β-glucosidase activity in the fore gut, mid gut and paunch of workers of *Hodotermes mossambicus.* Orlova (1974) has reported β-glucosidase activity in extracts of alate, soldiers and workers of three species of termites. Kovoor (1970) found that cellobiase activity in *Microcerototermes edentatus* with

workers from one nest showed activity only in the mid gut, whereas another nest showed activity in both the hind gut and midgut.

Mc Ewen *et al.* (1980) reported that 90% of the cellobiase activity of *Nasutitermes exitiosus* and *N.walkeri* was located in the midgut, Whereas, in the case of *Macrotermes natalensis,* 78% of the cellobiase activity was present in the hind gut and only 21% was detected in the mid gut of the termite.

Saxena *et al.* (1991) reported the production of carboxy methyl cellulase, xylanase and β- glucosidase activities by the gut of termites belonging to the genus *Odontotermes*. Sara Parwin Banu (1996) reported that the digestion of cellulosic feed is accomplished by the combined action of endoglucanase, exoglucanase and cellobiase in the digestive tract of mango stem borer, *Batocera rufomaculata.*Sara Parwin Banu and Ramasamy (1997) reported the role of *Clostridial* cellulases in the digestion of feed on Wood boring grub.

2.6. Termite gut microbes

Eutick *et al.* (1978) isolated facultative heterotrophs viz., *Streptococcus, Staphylococcus, Enterobacter and Citrobacter* from the /hind gut of *Mastotermes darwiniensis*. Thayer (1976) isolated *Bacillus cereus, Arthrobactr sp, Alcaligenes sp* and *Serratia marcescens* from *Reticulitermes hesperus.* Bignell (1978) reported the colonization actinomycetes type bacteria and nonfilamentous bacteria colonizing the walls of *Procubitermes aburiensis and Cubitermes severus.* Schlutz and Breznak (1979) reported a possible symbiosis between *Streptococcus lactis* and a species of *Bacteroides* in the hind gut of *Reticulitermes flavipes.* Kane and Breznak (1991) isolated *Cl. mayombei sp nov.*, an H_2/CO_2 acetogenic bacterium from the gut of the African soil feeding termite Cubitermes peciosus. Breznak *et al.* (1988) isolated *Sporomusa termitida* from *Nasutitermes nigriceps* termites. Kaul and Breznak (1991) isolated *Acetonema longum* from *Pterotermes occidentis.* Breznak and Blum (1991) isolated mixotrophy in the termite gut acetogen viz., *Sporomusa termitida* from *Nasutitermes nigriceps.* Brauman *et al.* (1992) isolated CO_2 reducing acetogenic bacteria from *Coptotermes formosanus.*

Saxena *et al.* (1995) isolated *Cellulomonas sp., Micrococcus sp* and *Cellovibrio sp.* from termites *(Odontotermes sp)*. Different groups of microorganisms present in the gut of termite is presented in Table 2.4

Table.2.4. Distribution of termite gut bacteria among different termite species

Eutick *et al.* (1978)

Termite species	*Microbial species*	Total population	Hindgut
C. primus	*Streptococcus*	0.50- $9x10^7$	$0.5x10^7$
M.darwiniensis	*Streptococcus*	0.50- $9x10^7$	$0.5x10^7$
R.flavipes	*Streptococcus*		$3.5x\ 10^5$
N.exitiosus	*Enterobacter*	$0.1\text{-}0.4 \times 10^7$	-
N.exitiosus	*Staphylococcus*	$1\text{-}3 \times 10^7$	-
Anacanthotermes ahngerianus	*Bacteroides*		$4.3\ x10^9$
N.exitiosus	*Serratia marcescens*	3×10^5	$3x\ 10^5$
Methanogen			
R. flavipes	*Methanobrevibacter*	3×10^9	$3.5\ x10^9$

Table 2.5. Different groups of microorganisms present in the gut of termites

Microrganisms	Insect	Reference
Aerobes		
Arthrobacter sp	*Reticulitermes Hesperus*	Paul *et al* .(1990)
Arthrobacter sp	*M.darwiniensis*	Thayer (1976)
Bacillus cereus	*Reticulitermes virginicus*	Thayer (1976)
Bacillus sp	*R.Hesperus*	Eutick *et al* .(1978)
Citrobacter freundii	*M. darwiniensis*	Eutick *et al* .(1978)
Micrococcus sp	*Odontotermes* sp	Paul *et al.* (1986)
Serratia marcescens	*Coptotermes formosanus*	Thayer (1976)
Streptomyces sp	*Cubitermes severus*	Bignell *et al.* (1979)
Streptomyces sp	*Soil feeding termites*	Pasti and Belli (1985)
Facultative anaerobes		
Bacteroides sp	*R. flavipes*	Schultz and Breznak (1979)
Cellulomonos sp	*Odontotermes sp*	Paul *et al* .(1990)
Cellovibrio sp	*Odontotermes sp*	Paul *et al* .(1990)
Cl. termitidis	*N. lujae*	Saxena (1993)
Enterobacter sp	*M. darwiniensis*	Eutick *et al* (1978)
Staphylococcus sp	*M. darwiniensis*	Eutick *et al* . (1978)
N fixing bacteria		
Citrobacter freundii	*R. flavipes*	French *et al* .(1976)
E. agglomerens	*Kalotermes minor*	Bennemann (1973)
CO_2 acetogenic bacteria		
Acetonema longum	*Pterotermes occidensis*	Kane and Breznak (1991)
Cl. mayombei	Cubitermes speciosus	Kane and Breznak (1991)
Sporomusa termitida	*N. nigriceps*	Breznak (1988)
Methanogenic bacteria		
Methanobrevibacter sp	*R. flavipes*	Lee *et al* . (1987)
M. curvatus	*R. flavipes*	Leadbetter and Breznak (1996)
M. cuticularis	*R. flavipes*	Leadbetter and Breznak (1996)
M. arboriphilicus	*N. nigriceps*	Yang *et al* . (1985)
M. bryantii	*N. nigriceps*	Yang *et al* .(1985)
Protozoa		
Trichomitopsis termopsidis	*N. nigriceps*	Yamin (1978)
Trichonympha sphareica	*N. nigriceps*	Yamin (1978)

2.6.1. Anaerobic Bacteria

Anaerobic bacteria present in the gut of termites included cellulolytic ,$C0_2$ reducing acetogenic and methanogenic bacteria. They play an important role in the digestion of complex lignocellulosic material in the host nutrition Table 2.5.

2.6.1.1.Acid forming bacteria

Volatile fatty acids were present throughout the intestine and each segment contained a constant volatile fatty acid concentration. Organic acids produced in the hind gut in the order of acetatet> formate> propionate> butyrate. In Orycetes larvae, volatile fatty acids were present at different concentrations in the midgut, hindgut dilation and the rectum respectively. The level of volatile fatty acids present in the midgut, hindgut and rectum was 25 x 10^{-6} moles mg^{-1}, 7.2 x 10^{-6} moles $mg^{-1}$and 1.8 10^{-6} moles mg^{-1} respectively. Kovoor (1967) reported the presence of volatile fatty acids production in higher termites. Volatile fatty acid in Orycetes larvae showed three times greater than the mid gut as in the proctodeum. The reduction of CO_2 during methanic fermentation lead to the release of CH_4. The bacteria have a role similar to that of the protozoa in the lower termites i.e. the formation of volatile fatty acids viz., acetate, propionate and some butyrate from the digestion products of cellulose which in turn used as an energy source by the termites *Microcertermus edentatus* (Kovoor, 1970). Breznak (1973) reported abundance of streptococci in hind gut of *R. flavipes*. Mannessmann (1972) reported that acetate is a dominant volatile fatty acids in the hind gut of *Reticulitermes flavipes*. Schultz and Breznak (1978) reported that the *Bacteroides* in the guts capable of fermenting lactate to propionate and acetate by interspecies lactate transfer occur between *Streptococci* and *Bacteroides*. In *Reticulitermes flavipes* (Kollar) acetate produced at high concentration, they were able to show that about 80% of the acetate was derived from cellulosic carbon and 20% from the hemicellulose. Odelson and Breznak (1983) reported that acetate which occurs in the termite hind gut at a concentration up to 80 mM and which can constitute over 90 mol % of the VFA, and is taken up from the termite tissue for its nutrition.

Odelson and Breznak (1983) reported that acetate, propionate and other organic acids produced during microbial fermentation of carbohydrates in the hindgut are important oxidizable energy sources for termites.

2.6.1.2. Homoacetogenic bacteria

Odelson and Breznak (1985) reported that homoacetogenic bacteria from R. flavipes homo acetic fermentation of glucose units of cellulose ($C_6H_{12}O_6 \rightarrow CH_3COOH$), which of the two of the acetates derived from fermentation by the protozoa $C_6H_{12}O_6 + 2H_2O \rightarrow CH_3COOH + 4H_2 + 2CO_2$ and another acetate most likely derived from CO_2 by H_2 consuming CO_2 reducing acetogens ($4H_2 + 2CO_2 \rightarrow CH_3COOH + 2H_2O$. Tholen and Brune (1999) reported the homoacetogenic bacteria in the highly compartmentalized hindgut of soil feeding higher termites (*Cubitermes sp*) Methanogens outcompete homoacetogens for endogenous reductant. They concluded hooacetogenic population in the hindgut are supported by either substrates other than H_2 or by a cross epithelial H_2 transfer from the anterior gut regions, which may create microniches favourable for H_2 dependent acetogenesis. Methanogenesis and homoacetogenesis occur simultaneously in the hindguts of almost all termites. Wagner and Brune (1999) reported that methanogenesis and reductive acetogenesis in the hind gut of the wood feeding termite (Reticulitermes flavipes is based on the microbial population and relatively high hydrogen partial pressure in the gut lumen.

2.6.1.3.Cellulolytic bacteria:

Cellulolytic bacteria from gut contents of lower and higher termites are significant to cellulose hydrolysis *in situ.* Gut bacteria involved in digestion of a polymer such as cellulose involves more than its hydrolysis to glucose. Enzymes of termites included exoglucanases capable of releasing glucose from cellulose or carboxymethylcellulose, two endoglucanases (each with activity on carboxymethylcellulose) but not on cellulose. Enzymes of protozoa include an exoglucanases and gluconolactone inhibitable cellobiase activity, two endoglucanases and two gluconolactone inhibitable cellobiases. Higher termites synthesis their own cellulases, carboxymethylcellulases and cellobiase activities in the region of midgut. In fungus cultivating termites, fungal comb contributed enzymes for cellulose degradation. Most of the cellulase activity was located in the midgut and consisted of C_1 cellulases, C_x cellulase and ß-glucosidase activity. Rouland *et al* (1986) purified two cellulase from *Macrotermes mulleri* associated with *Termitomyces* sp. Enzymes I is from termites which are monomeric glycoproteins of M_r 34,000 kDa showed hydrolytic activity on microcrystalline cellulose. Whereas, enzyme II from fungal

nodules has monomeric protein of M_r 52,000 kDa had relatively high activity on carboxymethylcellulose. Sara Parwin Banu and Ramasamy (1997) reported cellulase enzyme protein mainly involved in cellulose degradation were originate from the bacterium as well as from the insect gut. They isolated *Clostridial* cellulases from the wood eating grub. The molecular weight of the proteins of the gut was 2.5 to 669 kDa , while extracellular and cell bound proteins of *Cl. Cellulovorans* was 10 to 240 kDa. Most of the energy available to termites from cellulose digestion appears to come from oxidation of the acetate derived from cellulose, (Odelson and Breznak ,1983).

Cleveland (1924) and Dickman (1931) were unsuccessful in isolating cellulolytic bacteria from the gut of termites, but Eutick *et al.*(1978) and Schultz and Breznak (1978) were successful and reported the presence of cellulose degrading bacteria from termites. They further reported that cellulose digestion in the gut of termites was a slow process.

Mannessman (1972) isolated cellulose degrading bacteria from *Reticulitermes* virginicus and *Coptotermes formosanus.* Thayer (1976) isolated *Bacillus cereus, Serratia marcescens* and *Arthrobacter* sp from *Reticulitermes hesperus.* Thayer (1976) reported that *Bacillus* and *Serratia* sp hydrolysed carboxy methyl cellulose to reducing sugars. Pasti and Belli (1985) isolated *Clostridium termitidis* from *N. lujae* isolated cellulolytic actinomycetes *viz.*, *Streptomyces* sp and *Micromonospora* sp from wood feeding, soil feeding and fungus cultivating termites. Hethener et al. (1992) isolated *clostridium termitidis* from *Nasutitermes lujae.* Sara Parwin Banu and Ramasamy (1996) isolated cellulose degrading *Cl. cellulovorans* from the gut of wood boring grub.

Paul *et al.* (1986) isolated *Staphylococus* and S. *saprophyticus* from *Odontotermes obesus.* Saxena *et al.* (1993) isolated cellulose degrading *Cellulomonas* and *Micrococcus* sp from the hindgut of *Odontotermes* sp. which were associated in cellulose digestion .

2.6.1.2. CO_2 reducing acetogenic bacteria:

Acetogenic bacteria are capable of fermenting glucose and or cellobiose to acetate are present in the guts of higher and lower termites. These bacteria are capable of forming acetate by the reduction of CO_2. Acetate is not only an oxidizable energy source for termites, but an important prescursor for synthesis of aminoacids, cuticular hydrocarbons and terpenes (Breznak 1982).

Breznak *et al.* (1988) isolated H_2 oxidizing CO_2 reducing *Sporomusa termitida* sp.nov., acetogenic bacteria from the gut of *Nasutitermes nigriceps* termites. Kane and Breznak (1991) isolated acetogenic anaerobe *Acetonema longum* from the gut contents of wood feeding termites. Brauman *et al.* (1992) stated that acetogenesis from hydrogen and carbondi oxide was little significance in fungus growing and soil feeding termites.

Breznak and Brune (1994) reported that H_2 producing sites/ microbes, thereby g a higher effective concentration of H_2 than their methanogenic competitors. Odelson and Breznak (1983) reported that 77 - 100 per cent of the respiratory requirement of *R. flavipes* met by uptake and oxidation of microbially produced acetate in the hind gut. Breznak and Switcher (1986) reported that 1/3 rd of the respiratory requirement was fulfilled by acetate formed by CO_2 reducing acetogens. CO_2 reducing acetogen was catalysed by gut bacteria and not protozoa and was sensitive to the presence of O_2 (50 per cent inhibition) occurring at $10^{-13.25}$ atm with complete inhibition occurring at 10^{-2} atm of O_2 Microbial H_2 CO_2 acetogenesis occur in the hind gut of R. flavipes at the rate of 0.01-5.96 μ mol acetate per g (fresh wt $^{-1}$) h^{-1} (Brauman *et al* 1992). Tholen and Brune (1999) reported localization and *in situ* activities of homoacetogenic bacteria in the highly compartmentalized hindgut of soil feeding higher termites (*Cubitermes* spp).

Hydrogenases of Termites

Hydrogenases from numerous bacteria are known to contain iron –sulphur protein. Methanogenic bacteria are generally grown as H_2 and CO_2 as sole energy source. Hydrogenase is therefore an essential enzyme for these bacteria. Hence it is shown that the synthesis of active hydrogenases in Methanobacterium is dependent on nickel (Graf and Thauer, 1981). Nickel in hydrogenase enzyme is redox sensitive and is the binding site of the substrate H_2. Albracht *et al.* (1982) reported that nickel is required for the biosynthesis of hydrogenase in Knall gas bacteria. nickel is known to bind non specifically to proteins. Flavins are known to function as electron carriers for hydrogenase, methyl reductase and formate dehydrogenase system. Methyl reductase system is also required for methanogenesis from substrate other than CO_2.

2.6.1.5.Methanogenic bacteria

Methanogenic bacteria have been visualized in association with protozoa in termites. Methanogenic bacteria are morphologically diverse group of bacteria with

filterpaper, and obtain their nitrogen requirements via dinitrogen fixation by their gut symbionts. Hungate (1941) confirmed that termites can fix atmosphere nitrogen. (Table 2.6)

Table.2.6. Nitrogen fixation by different gut microflora

Organism	Cells/ guts	N_2 fixation units nmol of C_2H_4 /h	References
E. agglomerens	$2.3x10^4$	0.611	Potrikus and Breznak (1977)
Klebsiella pneumoniae	$4 x10^8$	4 – 5	Lobo and Zinder (1988)
Citrobacter freundii	$6 x 10^4$	5.5	Potrikus and Breznak (1980)

Krasilnikov and Satdykov (1970) isolated N_2 fixing bacteria viz., *Bacteium aerogens (Klebsiella aerogens)* and *B.cloacae (Enterobacter cloacae)* and *streptococcus faecalis* from the gut of *Kalotermitidae* termites. Breznak *et al.* (1973) isolated seven species of *Enterobacter* and *E.coli* from the gut of Australian termites.

Potrikus and Breznak (1977) isolated two strains of dinitrogen fixing E*nterobacter agglomerans* from termites. *French et al.,* (1976) isolated N_2 fixing bacteria from the gut of termites. They also reported that *Citrobacter freundii showed* nitrogenase positive in three species of termites. E*utick et al.* (1978) isolate putative dinitrogen fixing organisms from the termites.

Breznak (1973) recorded the reduction of acetylene to ethylene by termites. Benemann (1973) reported the presence of acetylene reduction activity in both workers and soldiers of *Kalotermes minor* and higher activity was recorded in workers. N_2 fixation in the gut of termites is mediated by bacteria which play an important role in overall nitrogen metabolism by recycling uric acid nitrogen. Prestwich *et al.* (1980) reported that nitrogenase activity in the soldiers of *N. ephratae* (28.4 µg nitrogen fixed per gram fresh weight/ day was four fold times greater than the workers of *R.*

variation in morphology, gram stain reaction, DNA base composition and physiological characters. In gastrointestinal tract of termites gut, the concentration of terminal electron acceptor other than CO_2 is limitted, thus the fermentation by the resident microbiota is primarily towards methanogenesis. Arthropods hind gut is colonized by free methanogenic bacteria, the methanogens are closely associated with chitinous structure formed by the hosts hind gut. Methanogenic bacteria symbiotic with *Trichomitopsis* produced most of the methane in the hind gut ecosystem reported by Yamin (1981).

Presence of methanogenic bacteria in the hind guts of millipedes (Diplopoda), cockroaches (Blattaria), termites (Isoptera) and scarab beetle (Scarabaediae) has been earlier reported by Cruden and Markovetz (1987). Acetogens are in competition with methanogens for the same reductant H_2. The extent to which H_2 flows to CO_2 reducing methanogenesis versus acetogenesis varies with the feeding guild to which temite belong. In soil feeding and fungus cultivating methanogenesis dominates acetogenesis as an H_2 sink, reverse in wood and grass feeding temites reported by Brauman *et al.* (1992). Within the hind gut ecosystem, acetogenic reactions appear to be the major electron sink whereas methanogenic reactions consume only a small fraction of the available H_2 and CO_2.

Methanogens are generally thought of strict anaerobes, their metabolic responses to the presence of oxygen and their sensitivity to it vary with the species. Lee *et al.* (1987) isolated methanogenic bacteria viz., *Methanobrevibacter sp* from the termite hind gut. Leadbetter and Breznak (1996) isolated *Methanobrevibacter cuticularis and M. curvatus* isolated from the hind gut of the termite *Reticulitermes flavipes*. Yang *et al.* (1996) reported the presence of *M. arboriphilicus and Methanobacterium bryantii* in the guts of wood eating higher termites (*Nasutitermes nigriceps). Wagner* and Bruen (1999) reported that localization of methanogenic bacteria in the hindgut of soil feeding higher termites (*Cubitermes* spp) Methane accounts for only a minor fraction (<10%) of the carbon obtained from the fermentation of cellulose in the hind gut, the methane emission system offer a new tool for studies of symbiosis in termite ecosystem.

2.6.1.6. Nitrogen fixing bacteria :

Termites live on a diet that is poor in vitamins, protein and other sources of nitrogen. Cleveland (1925b) proved that termites live for long periods when fed on

peraramatus had a nitrogenase activity (about 3.5µg nitrogen fixed per gram fresh weight per day). Nitrogenase activity was higher in *R. peraramatus* fed with leaf litter, whereas it was lower in *N. ephratae* fed with woody litter. The rate of biosynthesis of the nitrogenase enzyme decreases in the presence of ammonia. Biological N_2 fixation to be the exclusive domain of the bacteria. Nitrogenase reaction requires the two components of the nitrogenase complex. *A.chroococcum* contains vanadium in the place of molybdenum. *Azotobacter* nitrogenase apparently contains neither molybdenum nor vanadium, Lobo and Zinder (1988). The vanadium nitrogenase purified from *A. chroococcum* reduces at a low rate compared with dinitrogen. The low rate of C_2H_2 reduction due to switched off enzyme or to the nitrogenase having lower activity towards acetylene than towards dinitrogen. *Klebsiella pneumoniae* had reduced acetylene at a rate of 4 to 5 n mol C_2 H_4 formed of $prot^{-1}$ when supplied with ATP reducing power. Termites possess efficient means to acquire or conserve N_2. Termites play a role in complementary side to N acquisition is recycling, there are three main ways storage and recycling of nirogenous metabolic wastes, recycling of termite tissues, and digestion and assimilation of gut microbes or lytic or secretory products.

2.7. Microbial products associated with aggregation

Soil aggregate composed of primary particles and binding agents are the basic units of soil structure. The ability of soil microorganism to produce extra cellular polysaccharides in pure culture is well known (Finch *et al.,* 1971). Martin (1971) reported that soil polysaccharides contribute to soil aggregate stability. Bacteria have been shown to cement soil particles together by forming polysaccharides substances. Burns (1977)reported a positive correlation between the extractable polysaccharides and aggregation. Gomathi and Ramasamy (1992) reported that bacterial polysaccharides and polyuronates are generally present in soils and in sufficient concentration they are particularly effective in enhancing aggregation.

2.8. Effect of crumbs on plant growth

Soil aggregates composed of primary particles and other binding agents which are their basic units. In crop production, the soil aggregation lies in the indirect effect on water and air relationships in the soil.

Erhart (1981) reported that the crumbs formed protect the soil beneath them from erosion and also occur beneath the soil surface, they also conserve soil water in the root zone above the crusted horizon. Kayani and Sheikh (1981) reported that interrelationships of vegetation, soils and termites in pakistan Wielemaker (1984) reported that crumb formation increases the oxygen availability to plants and increase in rootability and potential for moisture uptake by plants.

Literature study revealed the association of few identified bacteria and their role in mineralization of carbon. However, the emission and product analysis have been carried out only in controlled condition. Much of the work reported assessed one or two parameters for reporting. But the whole ecology of the termatorium is missing. Especially the varied observations in the field distribution of termatorium in Indian sun continent need critical investigation so that the myth associated with the termatoria can be elucidated in scientific terms.

MATERIALS AND METHODS

CHAPTER III

Materials and Methods

3.1. Introduction

Termites are eusocial insects with colonies of a few hundred to a maximum of seven million individuals. One reproductive king and queen or several pairs are tended by sterile workers and soldiers. Queens of advanced termites can lay up to 30,000 eggs/ day. Termites are terrestrial insects occurring approximately between 45°N and 45° S. Within these latitudinal limits, vegetations are rarely found at altitudes above 3000 m. The termites' gut was associated with protozoa, bacteria, fungi, actinomycetes thereby acquiring the capacity to fix atmospheric nitrogen to improve digestion and assimilation of plant materials.

3.2. Collection of termites

3.2.1. Transport and storage

Higher, lower and queen termites were collected from different environments. Surface feeders and mound forming termites were collected from Vamban (National Pulses Research Centre), Pudukottai, Tamil Nadu and the emission by live termites of different feeding guilds. Live termites were introduced in presterilized vials, stoppered and flushed with nitrogen gas. Wet cotton was used to base the vial and support them without damage. The stored samples were transported to the Department of Environmental Sciences, TNAU, Coimbatore and further studies were initiated.

3.3. Dissection of the termites

The termites were washed in distilled water and anaesthetised with chloroform. Dissection was carried out under supply of CO_2 or N_2 atmosphere. The gut was separated and other fat tissues were removed. The foregut, midgut and hindgut regions were enriched by transferring to different vials containing prereduced Hungate's broth (Hungate, 1957) using acetate, formate and H_2:CO_2 (80:20) as substrates.

3.4 Studies on enumeration of aerobic and anaerobic bacteria

3.4.1. Enumeration of aerobic bacteria

Polysaccharide producing *Azotobacter* sp. and *Berijerinckia* sp. were isolated by dilution plate technique as per standard method (Parkinson, *et al.*, 1971). The individual

colony developed on the plates were transferred to the Becking's medium (Becking, 1974). After desired growth, these cultures were further purified, mass multiplied in a fermentor (Bio engineering Wald) and used for characterization and extracellular polysaccharide (ECP) production.

3.4.2. Enumeration of anaerobic bacteria

Termites samples were collected from the fungus cultivating termite mount for enumerating the population of anaerobic microflora. Random samples were collected from the field under anaerobic conditions (Ramasamy *et al.*, 1992).

3.4.2.1 Dilution medium

Sodium bicarbonate	0.5%
Sodium carbonate	1.0%
Resazurin	0.001%

To 100 ml serum vial of the cystine HCl reduced dilution medium was added, stoppered and autoclaved at 15 lbs pressure for 20 min. Soil samples collected anaerobically were transferred to the above vials (Ramasamy *et al.*, 1992).

The soil samples were further diluted by using the dilution medium and appropriate dilutions were used for the enumeration of total anaerobes, methanogens, sulphate reducers, proteolytic lipolytic and acid formers.

3.4.2.2 Enumeration by roll tube technique

The appropriate media listed in the appendix were prepared and sterilized. The sterilized medium was placed in a water bath at 45-50°C to maintain the liquid state. The medium was flushed continuously with CO_2/N_2 using the gassing manifold assembly, simultaneously the sterile test tubes were kept under CO_2/N_2 atmosphere.

The samples were also maintained under CO_2/N_2 atmosphere. One ml trace element solution and vitamin solution were added to one litre of the media before transferring to the sample. One ml of the sample was transferred from the desired dilution to the sterile test tube of 25 ml capacity which again was kept under CO_2/N_2 atmosphere. By using a sterile (10 ml capacity) with silicon tube at one end, 5 ml of the medium was transferred to the test tube with sample which was maintained under CO_2/N_2 atmosphere. The test tube was immediately stoppered with sterile rubber corks by simultaneously drawing out the gassing jet. The test

tube was rolled over the foam, soaked in cold water till the medium uniformly solidified on the sides of the test tube. The test tube were incubated in an anaerobic jar (Hungate, 1957).

From the enrichments, the population of total anaerobic bacteria was enumerated using Hungate's medium (Sivakumaran and Ramasamy, 1989) and roll tube technique as detailed earlier. After the incubation period, the number of colonies in the roll tube were recorded.

3.4.2.3. Cellulolytic bacteria

The sample was collected , diluted and enumerated by following the roll tube technique. Hungate medium (1957) was used for the enumeration of cellulolytic bacteria with cellulose as the C- source. The population of cellulolytic bacteria was enumerated by counting the colonies which appeared and clearancezone in the opaque medium which occurred by solubilsation of substrate.

3.4.2.4. Acid forming bacteria

By adopting the methods of Chynoweth and Mah (1977) the population of acid formers were estimated. The acid forming bacterial colonies that showed the characteristics change in colour of the medium from blue to yellow were counted after five days of incubation.

3.4.2.5. Methanogenic bacteria

The population of methanogens were estimated by using Mah's medium (Mah, 1980). The colonies were identified by their fluorescent appearance under UV light (290 nm).

3.4.2.6. N_2 fixing anaerobic bacteria

Nitrogen fixing anaerobes were isolated using Hungate's (1957) roll tube technique using with Hills medium (Bergersson, 1980). The individual colonies developed in the roll tubes were transferred to the vials containing nitrogen free medium and incubated under nitrogen atmosphere. After desired growth, the cultures were further purified using the roll tube technique.

3.5 Studies on the methane emission by termatorium

The sampling device was used for *in situ* collection of mixed gases from termatorium. Termatorium was covered with perplex container and the gas contents were mixed by circulators. The gas samples were collected in transport bags through pulse pump. After collection of the samples, they were identified and quantified by injecting in to GC (Varion

Environmental Analyser Model No. CP 3800 GC), USA with porapak column (6'x1/8") connected with flame ionization detector.

3.6 *Invitro* culturing of termatorium and axenic cultures of termites

A mound of fungus cultivating termites (Macrotermes sp.) with a height of 1.0 m and basal diameter of 0.4 m was collected from National Pulse Research Station (NPRC), Vamban, Pudukottai. The mound was placed in an aluminium frame containing sand and soil . This was connected to a feeder box containing leaf/ wood litter. The mound temperature was maintained at 28, 36, 30-32°C's at surface, core and interior respectively. The relative humidity was maintained at 85-90% which remained constant through out the experiment. The feeder box was completely closed but the observation of termite activity was possible through Perspex lid. The amount of gas produced was estimated as given in 3.5. The termite population was approximately 4000 individuals which was used for the assay. Cultured worker termites were used for this purpose and the homogenate of the termite was used as inoculum for methane production as detailed (Breznak, 1989) and the units of methane emission expressed as micro mole per gram of termite biomass per hour.

3.7 Studies on the digestive enzymes associated with termite gut

3.7.1. Amylase (EC.3. 2.1.1)

The enzyme activity was expressed in µg of glucose per ml of culture per day of incubation (Pancholy and Rice, 1973).

One ml of the culture filterate was transferred in 50 ml of Erlenmeyer flask and 1 ml of acetate buffer (pH 5.2) and two drops of toluene. 1 ml of 8% soluble starch and 3 ml of distilled water was added. The flaks were stoppered and shaken thoroughly and incubated at room temperature (28 ± 1 °C) for 24 hour. At the end of the incubation 1 ml of supernatant from the flask was withdrawn and reducing sugars were estimated (Nelson,1944). The enzyme activity was expressed in µg of glucose per ml of culture for 24 hrs of incubation.

3.7.2. Invertase (EC. 3. 2.1. 26)

One ml of the culture filterate was transferred in 50 ml of Erlenmeyer flask and 1 ml of acetate buffer (pH 5.2) and two drops of toluene. 1 ml of 4% of sucrose solution and 3 ml of distilled water was added. The flasks were stoppered. After shaking ,they were incubated at room temperature (28 ± 1 °C) for 24 hours. The reducing sugar was estimated by drawing 1

ml of supernatant from the sample (Nelson ,1944). The enzyme activity was expressed in µg of glucose per ml of the culture for 24 hr of incubation.

3.7.3. Maltase (E.C. 3. 2.1. 20)

One ml of the culture filterate was transferred in 50 ml of Erlenmeyer flask and 1 ml of acetate buffer (pH 5.2) and two drops of toluene. 1 ml of 4% of maltose solution and 3 ml of distilled water was added. The flasks were stoppered. After shaking they were incubated at room temperature (28 ± 1 °C) for 24 hours. The reducing sugar was estimated by drawing 1 ml of supernatant from the sample (Nelson ,1944). The enzyme activity was expressed in µg of glucose per ml of the culture for 24 hr of incubation.

3.7.4. Endoß glucanase (E.C. 3. 2. 1. 4)

Endoglucanase activity was assayed by the amount of reducing sugars released from CMC (Wood, 1968). In this assay , the reaction mixture contained 10 ml of McIlavin buffer (pH7.0) 5 ml of 1% Carboxymethyl cellulose solution with .01%(w/v) sodium azide as an antimicrobial agent. To this 2.5 ml of the enzyme solution was added. Endoglucanase activity was assayed with Oswald viscosimeter at 30°C. Reaction was started by adding the enzyme and the run out times of the solution were assayed at 15 minutes intervals. The cellulolytic activity was calculated as the increase in specific fluidity per minute according to the formula.

$T = [(1/T_1 - t) - (1/T_0 - t) \times (1/t) \times 10^4]$

Where, t is run out time of the viscometer for distilled water

T_1 and T_2 is run out time at the start and after the enzyme action on polymer

A unit of enzyme activity was define as the amount of enzyme producing an increase in the specific fluidity of 10^{-4} /min / mg of protein.

3.7.5. Exo ß glucanase (E.C. 3. 2. 1. 91)

Exoglucanase activity was assayed by the amount of reducing sugars released from Cellulose equivalent to one µg of glucose. (Wood, 1968). Exoglucanase splits of cellobiose or glucose units from nonreducing ends of cellulose molecule. Add 1.5 ml of McIl avin buffer (pH 7.0, 0.2 M) ,2.5 ml of 1% crystalline cellulose and 0.1 ml of enzyme was added and incubated at 37°C. After desired incubation period, one ml of the mixture was taken and reducing sugars released from cellulose was estimated by the method of Nelson (1944).

3.7.6.Cellulase

One ml of the culture filterate was transferred in 50 ml of Erlenmeyer flask and 1 ml of acetate buffer (pH 5.5) and two drops of toluene. 1 ml of 4% micro crystalline cellulose solution and 3 ml of distilled water was added. The flasks were stoppered. After shaking they were incubated at room temperature (28 ± 1 °C) for 24 hours. The reducing sugar was estimated by drawing 1 ml of supernatant from the sample (Nelson ,1944). The enzyme activity was expressed in µg of glucose per ml of the culture for 24 hr of incubation (Pancholy and Rice, 1973).

3.8 Characterization of N_2 fixing aerobe and anaerobes associated with Termite gut

3.8.1. Cell morphology

The morphology and motility of the cells were observed under the microscope. Gram staining was performed to study the gram reaction.

3.8.2. Growth on different carbon sources

The cultures at log phase with an OD of 0.6 (CFU x 10^6 ml^{-1}) were inoculated in the vials containing different carbon sources viz., glucose, rhamnose, fructose, sucrose, cellulose, cellobiose were incubated anaerobically under N_2 atmosphere. The growth rate was measured by observing OD at 600 nm in ECIL GS5701 Spectrophotometer at periodical interval.

3.9 Studies on the characterization of methanogenic anaerobes associated with termite gut

The isolated cultures were studied for their colony morphology and Gram reaction as described by Gerhard *et al* (1994).

3.9.1. Effect of substrates on the growth of *Methanogens*

The ability of some of the generally reported methanogenic substrates to support the growth of the cultures was determined. Media was prepared anoxically with the substrates viz., acetate, methanol, formate, monomethylamine, trimethylamine and hydrogen : carbondioxide @ 1%. The methanogenic culture with a cell load of 10^3 ml was inoculated and the samples were periodically analysed for its optical density and protein content.

3.9.2. Effect of pH on the growth of methanogens

The pH requirement for the growth of Methanosarcina was determined by using Mah (1980) broth adjusted to various pH ranging from 5.5 to 8.0. The culture was inoculated and incubated at 30± 2 °C and the growth was measured as optical density and protein.

3.9.3.Effect of temperature on the growth of methanogens

The optimum temperature range for the growth was determined in Mah (1980) media at pH 7.0 and 7.5 by incubating at room temperature, 10°C, 30 ±2 °C, 37°C and 55°C respectively.

3.9.4. Effect of Nickel on the growth of Methanogens

The effect of nickel on the growth of Methanosarcina was studied by incorporating different levels of nickel chloride varying from 0.01μM to 1.0μM into the medium and incubating at 30 ±2°C. The growth was measured as protein equivalents.

3.9.5. Protein determination

Protein concentrations were determined by the Bradford's dye binding protein assay with bovine serum albumin (BSA) as the standard (Bradford, 1976).

3.9.6. SDS PAGE – Separation of protein

Total cellular protein was analysed by discontinous sodium dodecyl sulphate gel electrophoresis as described by Laemmli (1970). **Appendix** . whole aceate grown cells were disrupted by boiling for 2 minutes in the cell disruption mixture . The disrupted materials was cooled and loaded immediately on to the gels. Gels were run in the both Horizontal gel electrophoresis in 0.5 mm gel using a multiphor ii(Pharmacia LKB biotechnology,Swedan) and Vertical gel electrophoresis in 2 mm gel (Hoffer,USA).**Appendix**. After completion of the electrophoresis, the gels were stained and destained using the coomassie blue procedure of Noel and Brill(1980).

3.9.7. Assay for coenzyme F_{420}

One gm of *Methanosarcina* cell paste was mixed with 1 ml glass distilled water. To this, one volume of acetone was added and the mixture was boiled for 5 minutes, cooled, shaken vigorously and centrifuged at 9700 x g for 10 minutes. The soup was stored for further analysis.

A aliquot of sample was injected into Hitachi HPLC system with L-6200 intelligent pump connected to a reverse phase Hipersil 5 ODS column (25 cmx 4.6 mm) and the effluent

was monitored in a Varian Prostar Model 320 Fluorescence Spectrophotometer detector coupled to an D2500 chromato – integrator. The mobile phase consisted of a mixture of methanol and 25mM acetic acid in glass distilled water

Adjusted with KOH to pH 6.0 (AppendixIII). The flow rate was 2.0 ml min-1 at a pressure of 10 to 14 Mpa. A linear gradient from 0 to 50 per cent was run in 10 minutes. The excitation and emission wave lengths of the coenzyme F_{420} was at 400 and 470 nm (Van Beelan *et al* ., 1983).

3.9.6. Methane estimation

The total gas production was determined at intervals by measuring the volume of gas produced at room temperature by displacement of syringe. The following equation was used to estimate the formation of methane and expressed in µmoles (Mah *et al*.., 1978).

µmoles formed = [% of CH_4 x (Vf + Vr)/2.24 + M.P.]

where,

% CH_4 – is volume per 100 ml of gas phase

Vf - is the volume of gas phase in vessels

Vr - is the volume of gas phase removed by syringe following equilibriation to atmospheric pressure

Mp - is the sum of µmoles recorded after daily equilibration

Mp = [C (%CH4 x Vr)/ 2.24]

Methane was assayed using Varian Gas Chromatograph

3.9. Studies on microbial products associated with aerobic bacteria isolated from termite gut

The isolated aerobic bacteria from termite gut was enriched in different carbon source for its polysaccharide production. The polysaccharide production was assayed as per the method (Jarman, 1978).

3.11.Artificial aggregate formation by the isolated polysaccharide producers

Air dried soil samples ground and sieved through 52 mesh was mixed with fresh culture with polysaccharide or the purified and dried poysaccharide. For every 100g of soil , 10 ml of the culture or 1 g of extracellular polysaccharide (ECP) was added enough with double distilled water to get a paste. This paste was passed through vermicelli sieve to get long cylindrical threads and dried in shade. Dried materials were processed to get 2-4 nm

diameter aggregate by selective sieving of broken soil rods. These artificially formed aggregates was used for the experiment.

3.12. Studies on the effect of crumbs on plant growth

An experiment was laid out to study the effect of crumb on germination of crop (green gram) using the culture isolated from termites gut.. The germination of gram seeds were observed after 7^{th} day of sowing. Root length, shoot length vigour index and biomass were recorded after 30 days of sowing.

RESULTS

Plate . 1 Termatorium at NPRC, Vamban Tamil Nadu

Plate . 2 Termite castes *viz.*, queen larvae, soldiers and workers of this investigation.

CHAPTER IV

RESULTS

4. Collection of samples

Termites (soldiers, workers and queen) of fungus cultivating were collected from different places of National Pulse Research Centre, Vamban. After collection, the termite samples were surface sterilized and their guts were dissected. Enrichments were made for isolating different group of organisms. From these enrichments and from the direct samples, the population of aerobes, total anaerobes, cellulolyzers, acid formers, methanogens and N_2 fixing anaerobes were enumerated. The enumerated colonies were purified and characterized for further work. Plate 1 and 2

4.1. Isolation of aerobic bacteria

Termites were collected and their guts were surface sterilized and dissected and assayed for the enumeration of aerobes. On isolation, the total number of bacteria observed was maximum in salivary gland (32.0 x 10^4 CFU ml^{-1}) followed by fore gut (24.6 x 10^4), midgut (19.3 x 10^4 CFU ml^{-1}) and hindgut (12.6 x 10^4 CFU ml^{-1}). In the total number of bacterial population, polysaccharide producing bacteria was found maximum comparatively. Thus gram negative extracellular polysaccharide producing bacteria were identified based on their colony morphology and their characterization (Table 1). Salivary gland recorded maximum population of *Azotobacter* (19 x10^4 CFU ml^{-1}) and *Beijerinckia* (13.2 x 10^4 CFU ml^{-1}) followed by foregut. The foregut recorded the population of *Azotobacter* (16x10^4 CFU ml^{-1}) and *Beijerinckia* (11.5x10^4 CFU ml^{-1}). Whereas less population of *Azotobacter* (5.4x10^4 CFU ml^{-1}) and *Beijerinckia* (4.6x 10^4 CFU ml^{-1}) in midgut followed by hindgut were recorded . Plate 3 and 4.

4.2. Characterization of nitrogen fixing aerobic bacteria

The isolated nitrogen fixing aerobic bacteria were purified and characterized (Table2). In the Becking's media the culture smooth glistening colonies. Microscopic observation of the above cultures rod to oval in shape and often in pairs. They are negative to gram variable in staining reaction. They are cyst former. Based on the

Table 1. Enumeration of aerobic bacteria from different gut region of termites

Particulars	Bacteria $x10^4$	*Azotobacter* $x10^4$	*Beijerinckia* $x10^4$
Salivary gland	32.0	19.0	13.2
Foregut	24.6	16.0	11.5
Midgut	19.3	5.4	4.6
Hindgut	12.6	3.2	3.5
SED	0.29	0.13	0.25
CD	1.28	1.17	1.24

Media Becking's media (Becking, 1974)

Cell morphology was identified under direct microscopic observation

revealed the gram negative and polysaccharide producing rods.

The population of the colonies were expressed in Colony forming Units (CFU ml^{-1})

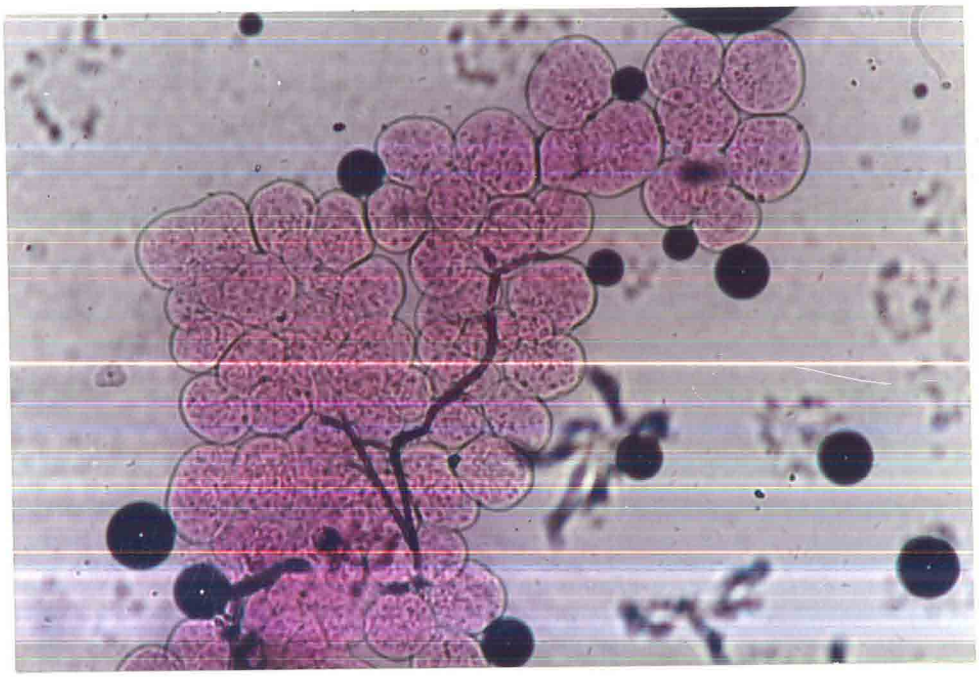


Plate .3 Salivary gland of worker termites

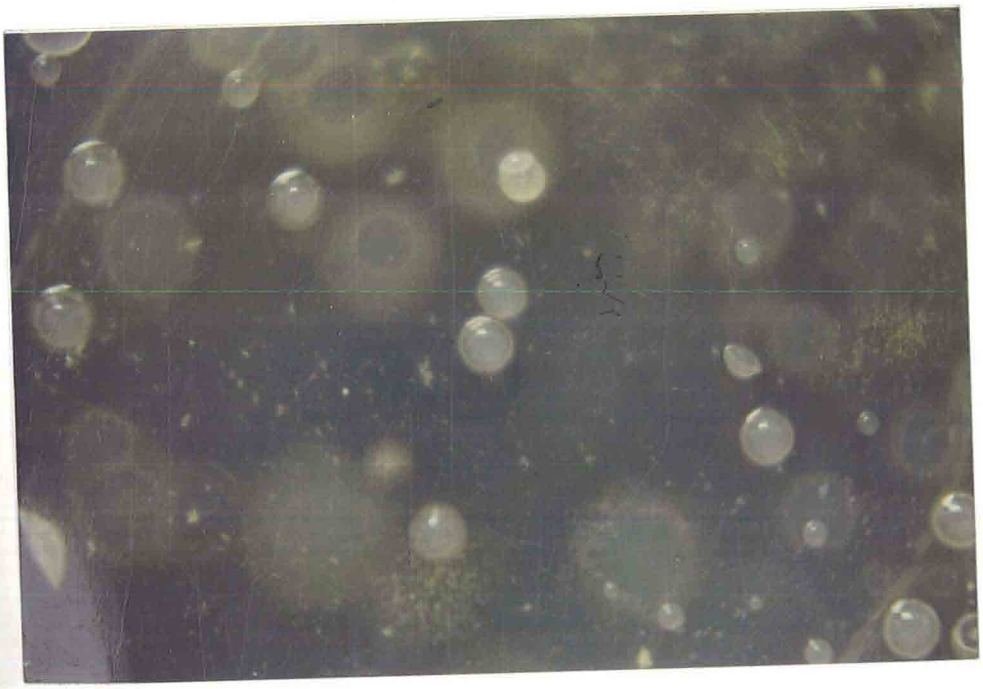


Plate 4. Colony morphology of *Beijerinckia*

Table 2. Characterization of N_2 fixing aerobic bacteria isolated from termites gut

Characteristics	*Azotobacter*	*Beijerinckia*
Cell shape	rod to oval, often in pairs, smooth glistening,	coccal to rods copious slime smooth glistening
Motility	+	-
Green fluorescent	+	+
Acid production	+	+
Catalase activity	+	+
Cellulase activity	-	-
Cellulose hydrolysis	-	-
Starch hydrolysis	-	-
Dye sensitivity	-	-
Cyst formation	+	-
Gram reaction	G –ve to variable	G –ve

Becking media with sucrose (1%) was used as substrate for isolation and characterization, Becking (1974)

character culture 1 was identified as *Azotobacter* in the Becking's medium the culture 2 produced smooth, glistening and slimy colonies. Cells are cocci to rod in shape and gram negative in stain reaction. Both the cultures grow well in the N_2 free medium. Based on the characterization, the culture identified as *Beijerinckia.* For characterization they were tested for acid production, catalase activity, motility, starch hydrolysis, cellulose hydrolysis and dye sensitivity.

4.3. Enumeration of anaerobic bacteria

4.3.1.Isolation of total anaerobes:

Termite samples of worker, soldier and queen were collected and their dissected guts were enriched in Mah medium. From the enrichments, roll tube was done for the isolation of total anaerobes. Total anaerobic population was observed maximum in worker temites than soldier and queen. Hence other work concentrated in worker termites. Maximum total anaerobic population of 44.7 x 10^5 colony forming units ml^{-1} observed in the fore gut, mid gut 22.3 x 10^5 CFU $ml^{-1}$and hind gut 27.7 x 10^5 colony forming units ml^{-1} compared with soldier and queen (Table 3). Fore gut showed the presence of maximum acid forming bacteria 40.3 x 10^3 colony forming units ml^{-1} compared with mid gut (30.3 x 10^3 CFU ml^{-1}) and hind gut 20.4 x 10^3 CFU ml^{-1}) respectively. Methanogenic population was observed higher in the hindgut (28.0 x10^4 CFU ml^{-1}) compared with foregut (10.3x10^4 CFUml^{-1}) and midgut (15.6x10^4 CFU ml^{-1}). Cellulolytic colonies were enumerated from the hindgut and recorded about 21.0 x 10^4 CFU ml^{-1}, whereas midgut and foregut showed 16.3 x 10^4 CFU $ml^{-1}$and 14.0 x 10^4 CFU ml^{-1} respectively.

Soldier termite showed maximum population of total anaerobes in fore gut (30.5 x10^5 CFU^{-1}), while it was in midgut (26.0 x 10^5 CFU^{-1}) and (21.0 x 10^5 CFU^{-1}) hindgut. Hindgut recorded higher methanogenic population of 19.6 x 10^4 CFU^{-1} followed by midgut 11.5 x 10^4 CFU^{-1} and foregut 10.3 x 10^4 CFU^{-1} (Table 4).

Queen termites showed total anaerobic population of 28.3 x 10^5 CFU^{-1} in foregut, 19.5 x 10^5 CFU^{-1} in midgut and 15.8 x 10^5 CFU^{-1} in hindgut. Methanogenic population enumerated from hindgut midgut and foregut exihibited (22.0 x 10^4 CFU^{-1}), (9.6 x 10^4 CFU^{-1})) and (6.5 x 10^4 CFU^{-1}) respectively (Table 5). Isolation and

Table 3. Enumeration of total anaerobes from worker termite gut

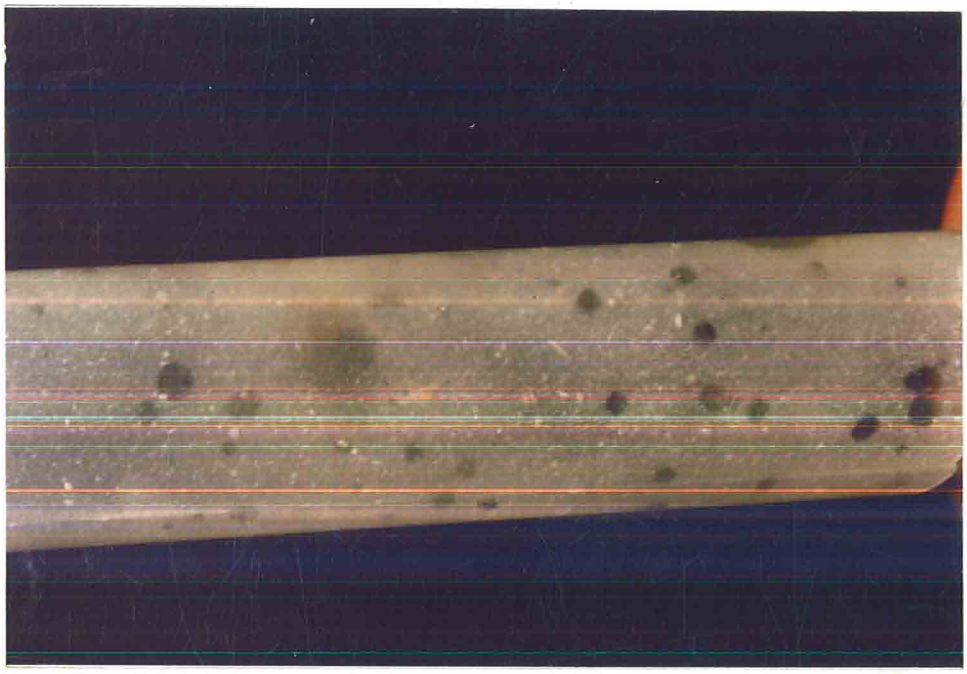
Organisms	Foregut	Midgut	Hindgut
Total anaerobes (10^5)	44.7	27.7	22.3
Cellulolysers (10^3)	14.0	16.3	21.0
Acid formers (10^4)	40.3	30.3	20.4
Methanogens (10^4)	10.3	15.6	28 .0
Klebsiella (10^4)	20.0	17.0	11.6
Clostridium (10^4)	39.7	22.3	26.7
SED	0.81	0.81	0.92
CD(0.05)	1.70	1.77	1.62

Facultative nitrogen fixing *Klebsiella* and *Clostridium* , Bergerssen (1980)

Total anaerobes (Ramasamy *et al* ., 1982), Cellulolytic anaerobes (Hungate, 1957), Acid formers (Chynoweth and Mah, 1977) and Methanogen (Mah, 1980) were enumerated by roll tube technique (Hungate, 1950)

The population of the colonies were expressed in Colony forming units(CFU ml^{-1}).

Plate. 5 Hungate's roll tube used for the enumeration of anaerobes



Cellulolytic colonies

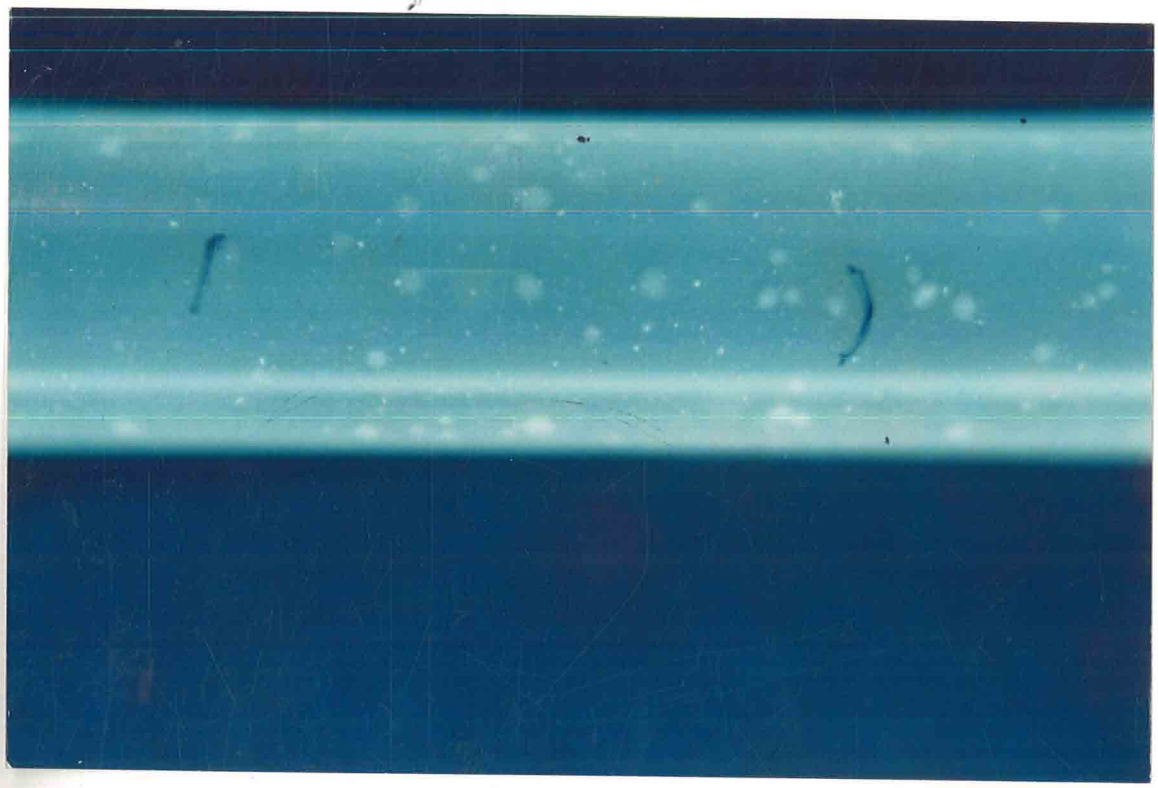


Plate. 6 **Methanogenic colonies**

Table 4. Enumeration of total anaerobes from soldier termite gut

Organisms	Foregut	Midgut	Hindgut
Total anaerobes (10^5)	30.5	26.0	21.0
Cellulolysers (10^3)	28.5	23.3	17.5
Acid formers (10^4)	12.0	16.0	20.5
Methanogens (10^4)	10.3	11.5	19.6
Klebsiella (10^4)	15.0	11.7	8.5
Clostridium (10^4)	26.0	13.0	19.0
SED	1.37	0.88	0.81
CD (0.05%)	2.04	1.69	1.72

Facultative nitrogen fixing *Klebsiella* and *Clostridium(* Bergerssen ,1980)
Total anaerobes (Ramasamy *et al* ., 1982), Cellulolytic anaerobes (Hungate, 1957), Acid formers (Chynoweth and Mah, 1977) and Methanogen (Mah, 1980) were enumerated by roll tube technique (Hungate, 1950)

Table 5. Enumeration of total anaerobes from queen termite gut

Organisms	Foregut	Midgut	Hindgut
Total anaerobes (10^5)	28.3	21.0	17.0
Cellulolysers (10^3)	21.5	19.5	15.8
Acid formers (10^4)	10.3	13.0	18.0
Methanogens (10^4)	6.5	9.6	22.0
Klebsiella (10^4)	12.0	8.6	6.5
Clostridium (10^4)	23.0	18.0	21.0
SED	0.80	0.74	0.76
CD (0.05%)	1.64	1.62	1.63

Facultative nitrogen fixing *Klebsiella* and *Clostridium,*(Bergerssen ,1980)

Total anaerobes (Ramasamy *et al* ., 1982), Cellulolytic anaerobes (Hungate, 1957), Acid formers (Chynoweth and Mah, 1977) and Methanogen (Mah, 1980) were enumerated by roll tube technique (Hungate, 1950)

The population of the colonies were expressed in Colony forming units(CFU ml^{-1}).

enumeration of total anaerobes, acid formers, cellulolyser and methanogenic population from queen, soldier and worker termites showed all the above mentioned microbial activities were found maximum in worker termites. The population of worker should be more and hence the worker termites was selected for further studies.

4.3.2. Nitrogen fixing anaerobic bacteria

Different gut regions of the termites were assayed for the presence of Nitrogen fixing bacteria from fore gut, mid gut and hind gut. Fore gut of worker termites recorded the presence of maximum N_2 fixing anaerobes viz., *Klebsiella* sp (20.0 x 10^4 CFU/ ml) and *Clostridium* sp (39.7 x 10^4 CFU / ml) whereas mid gut showed 17.0 x 10^4 CFU / ml of *Klebsiella* and 22.3 x 10^4 CFU / ml of *Clostridium*. Hind gut recorded comparatively less population of *Klebsiella* (11.6 x 10^4 CFU / ml) and *Clostridium* (26.7 x 10^4 CFU / ml). (Table 6.7)

Nitrogen fixing bacteria from soldier termites of fore gut recorded the presence of maximum N_2 fixing anaerobes viz., *Klebsiella* sp (15 x 10^4 CFU/ ml) and *Clostridium* sp (26.0 x 10^4 CFU / ml) whereas mid gut showed 11.7 x 10^4 CFU / ml) of *Klebsiella* and 13.0 x 10^4 CFU / ml) of *Clostridium*. Hind gut recorded comparatively less population of *Klebsiella* (8.5 x 10^4 CFU / ml) and *Clostridium* (19.0 x 10^4 CFU / ml).

Nitrogen fixing bacteria from queen termites of fore gut recorded the presence of maximum N_2 fixing anaerobes viz., *Klebsiella* sp (12 x 10^4 CFU/ ml) and *Clostridium* sp (23 x 10^4 CFU / ml) whereas mid gut showed 8.6 x 10^4 CFU / ml) of *Klebsiella* and 18 x 10^4 CFU / ml of *Clostridium*. Hind gut showed comparatively less population of *Klebsiella* (6.5 x10^4 CFU / ml) and *Clostridium* (21.0 x 10^4 CFU / ml).

4.3.3. Cellulolytic bacteria

Cellulolytic colonies were present in the hind gut recorded about 21.0 x 10^4 colony forming units ml^{-1} by appearing clear zone colonies in the cellulose enriched media Whereas mid gut showed 16.3 x 10^4 CFU / ml and hindgut recorded 21.0 x 10^4 colony forming units ml^{-1}. For enumeration of cellulolysers modified Hungate media with cellulose was used as substrate . Plate. 5.

Table 6. Characterisation of N_2 fixing *Clostridium*

Cell shape	Rod shaped
Colony morphology	Viscoid
Motility	Motile
Gram reaction	Gram negative
Sporulation	Terminal spores
Volatile fatty acid production	Acetate and propionic
Gas production	acid
Nitrogenase	CO_2 and H_2
Denitrification	Negative
	Positive
Utilization of Sugar	
Sucrose	
Cellulose	Positive
Cellobiose	Positive
Glucose	Positive
Rhamnose	Positive
	Positive

The medium used for the isolation of N_2 fixing bacteria *Clostridium* was Hino and Wilson medium (Bergersson 1980).

Table 7. Characterization of N_2 fixing *Klebsiella*

Cell shape	Straight rods arranged single or pairs
Colony morphology	Dome shaped glistening colonies
Motility	Non motile
Gram reaction	Gram negative
Volatile fatty acid production	Acetic and formic acid
Gas production	CO_2 and H_2
Nitrogenase	Positive
Utilization of sugars	
Glucose	Positive
Cellulose	Positive
Sucrose	Positive
Cellobiose	Positive
Fructose	Positive
Rhamnose	Positive

The medium used for the isolation of N_2 fixing bacteria *Klebsiella* Hino and Wilson medium (Bergersson, 1980)

4.3.4. CO_2 acetogenic bacteria

For enumeration of CO_2 acetogenic bacteria modified Hungate media were used. Foregut recorded 39.7 x 10^4 colony forming units ml^{-1}, whereas mid and hindgut recorded 22.3 x 10^4 and 26.7 x 10^4 colony forming units ml^{-1} respectively.

4.3.5. Methanogenic bacteria

For enumeration of methanogens, acetate was used as a substrate in Mah media. Among the three regions of the termites gut, hind gut showed maximum methanogen (28.0 x 10^4 colony forming units ml^{-1}) compared to fore gut (10.3 x 10^4 colony forming units ml^{-1}) and mid gut (15.6 x 10^4 colony forming units ml^{-1}) respectively. Fluorescing colonies observed under ultraviolet rays were counted as methanogens. From these roll tubes, individual colonies were picked and inoculated in serially numbered vials for further purification. The marked colonies were sub cultured in the Mah medium. The vials which tested for methane, the culture 3 produced more methane showed positive as compared to other vials. Plate. 6.

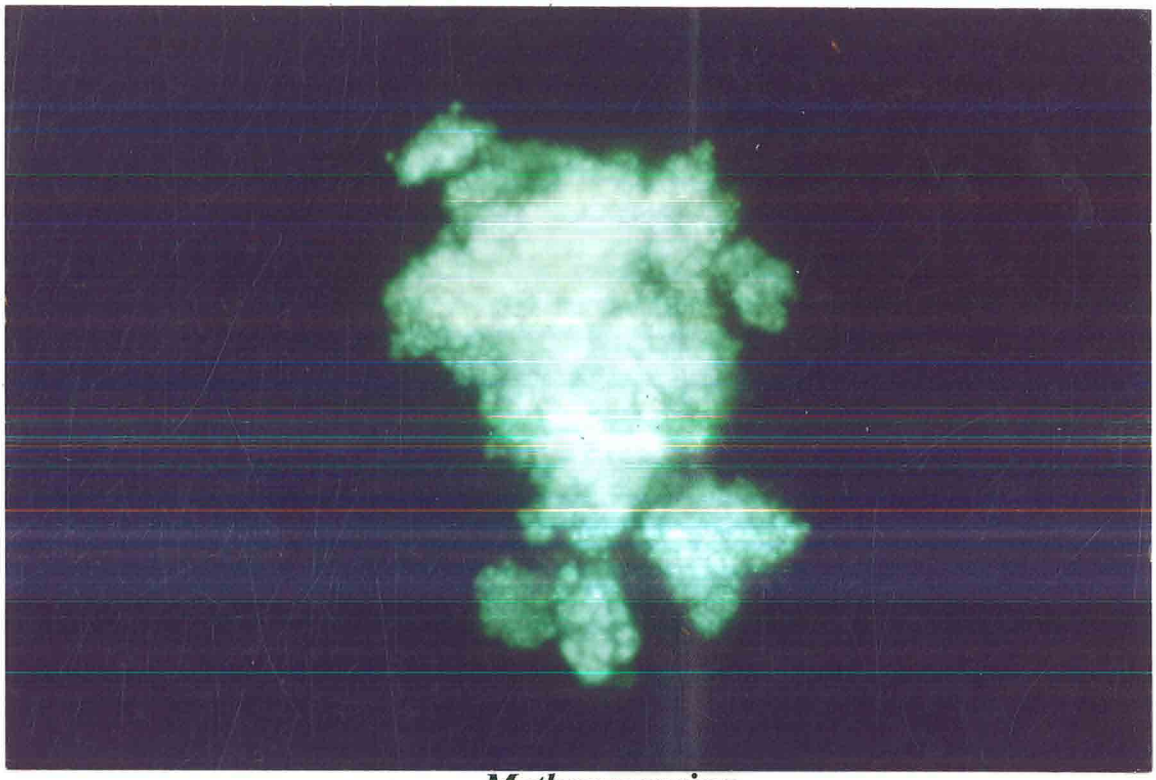
4.4. Charaterisation of Methanogenic culture

The isolates of culture was tested for purity in microscope and it was found to be pure. Further studies were carried out from this isolate and colony morphology was studied in roll tube using acetate (0.1%) as substrate. After 21 days of growth, the colonies were checked for fluorescence in UV lamp (295 nm) and fluorescent colonies were sub cultured and used for further studies (Table 8). Plate. 7, 8 and 9.

4.5. Digestive enzymes in Termites gut

The ecology of the microflora prevalent in the digestive tract of termites showed enzyme activities in the different region of foregut, midgut, hindgut and salivary gland. The result of the estimated enzymes *viz.*, amylase, maltase and invertase were presented in Fig 1. Salivary gland exhibited the maximum activity of amylase, invertase and maltase than other regions of the termite gut. Mid gut of termites showed maximum amount of amylase (0.36 mg/ glucose released/hr) and invertase (0.78 mg/ glucose released/ hr) and maltase (0.56 mg/ glucose released/hr) whereas hind gut and fore gut recorded less enzymic activities of amylase and

Plate 7 **Pure cultures of methanogens from the hindgut of worker termite**



Methanosarcina

Plate. 8.

Methanobacteria

Plate 9. Scanning electron microscope photograph of *Methanobacterium*

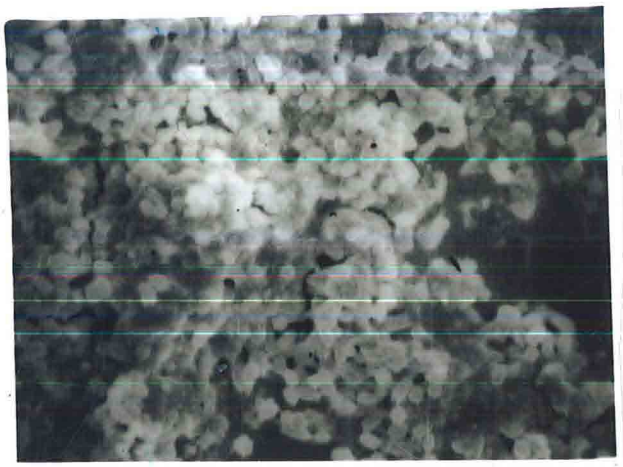


Table.8 Morphological characteristics of isolated methanogens from termite gut

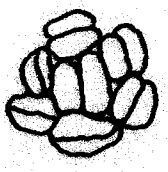
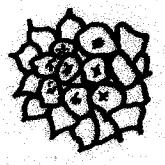
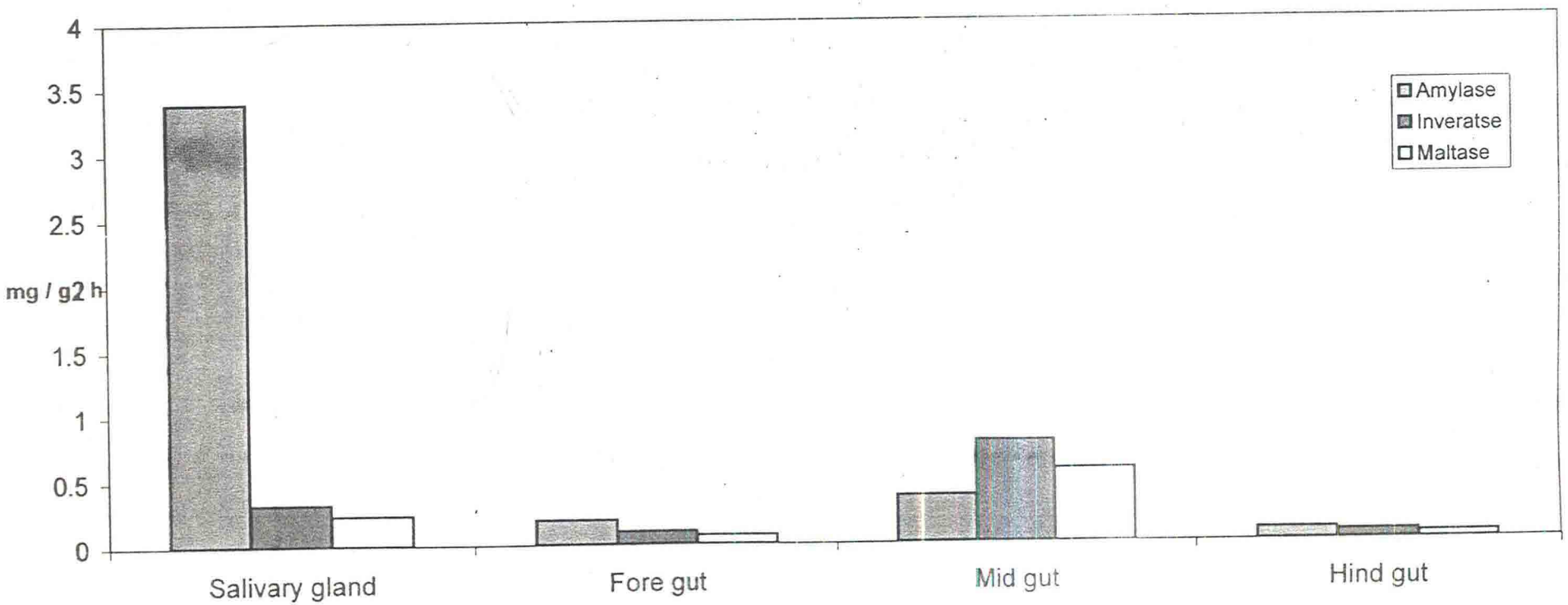
Methanogen	Colony characters	Morphology	Physiology	Related genus	Identified as
	Circular, slimy transparent colony	Short rods	H_2:CO_2	*Methanobacteria* *Methanobrevibacter*	*Methanobacteria* Kluyver and Van Niel (1936)
	Circular slimy transparent colony	Long and lengthy rod	Formate, H_2:CO_2	*Methanobacteria*	*Methanobacteria*
	Circular, slimy transparent with wavy margin	Coccus	Formate, H_2:CO_2	*Methanococcus*	*Methanococcus* Balch and Wolfe (1976)
	Circular waxy colony	Coccus	Formate, H_2:CO_2	*Methanococcus*	*Methanococcus*.Balch and Wolfe (1976)
	Slimy transparent colony with wavy edges	*Sarcina*	Acetate H_2: CO_2 Methanol	*Methanosarcina*	*Methanosarcina* Kluyver and Barker (1936)
	Circular slimy with hard centered concentric circular colony	*Sarcina*	Acetate H_2: CO_2 Methanol	*Methanosarcina*	*Methanosarcina* Kluyver and Barker (1936)

Fig. 1. Distribution of amylase, invertase and maltase activities of termite gut



Amylase, invertase and maltase activity was assayed as per the method, Panchely and Rice, (1979).
Rducing sugar released was estimated as per the method, Nelson, (1944).

invertase. Foregut recorded less enzymic activity of amylase (0.19 mg/ glucose released/ hr), invertase (0.10 mg/ glucose released/ hr) and hindgut (0.07 mg/ glucose released/ hr). Salivary gland recorded maximum of amylase (3.39 mg/ glucose released/ hr) invertase (0.32 mg/ glucose released/ hr) and maltase (0.05 mg/ glucose released/ hr).

4. 5.1 Cellulase activities of the termite gut

The termite gut consists of tubular mid gut which is a key site for secretion of digestive enzymes and for absorption of soluble nutrients. Enzymes of termite origin included an exoglucanase, endoglucanase and cellulase. The activities of cellulase ß 1,4 exoglucanase and ß I,4 endoglucanase activities from the extracts of the termite gut were presented in (Fig 2).

Cellulase activities were found to be maximum in mid gut (2.10 $\mu g\ ml^{-1}\ g^{-1}$) while in salivary gland, it was 1.89$\mu g\ ml^{-1}\ g^{-1}$. But fore gut and mid gut recorded less enzymic activities (0.09$\mu g\ ml^{-1}\ g^{-1}$) whereas mid gut showed 0.12$\mu g\ ml^{-1}\ g^{-1}$.

4.5.2. Endo ß 1, 4 glucanase

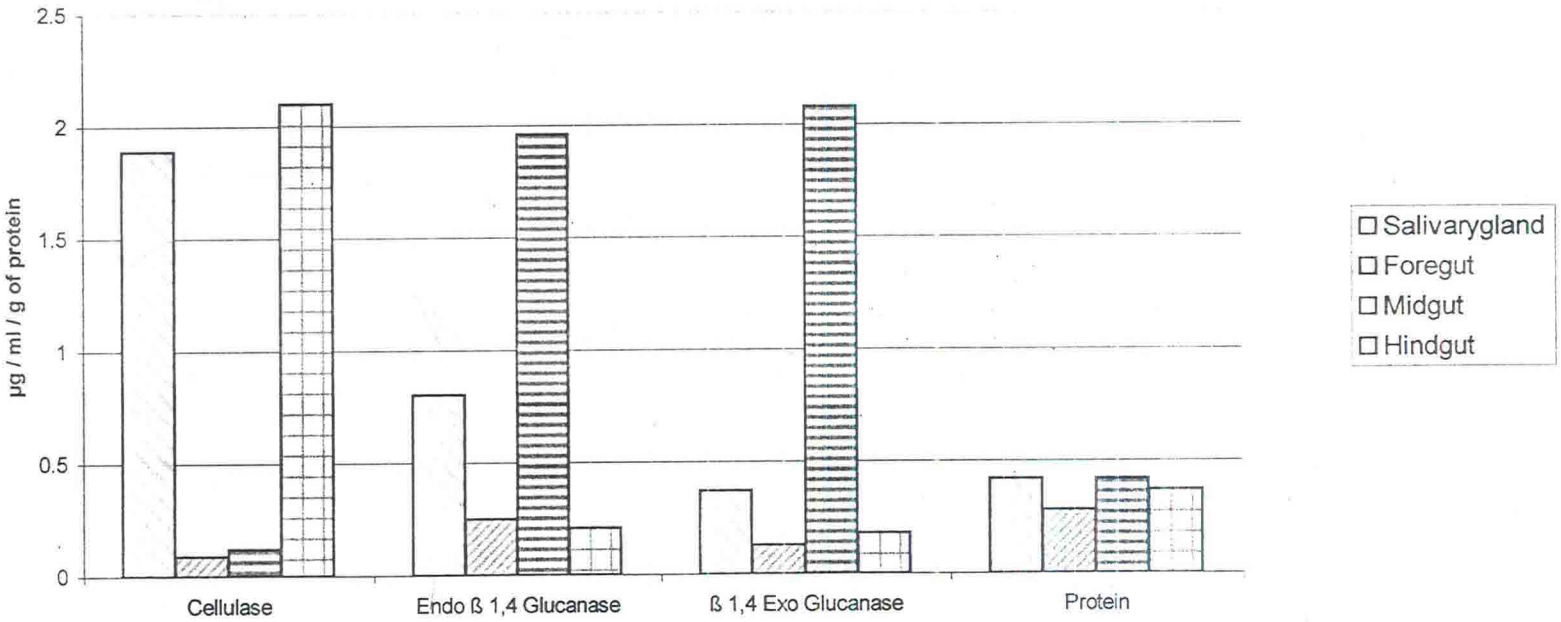
Salivary gland showed maximum amount of Endo ß 1, 4 glucanase 6.8$\mu g\ ml^{-1}\ g^{-1}$ followed by mid gut which showed 1.96$\mu g\ ml^{-1}\ g^{-1}$ .While, fore and hind gut recorded less amount ß 1, 4 endoglucanase (0.25$\mu g\ ml^{-1}\ g^{-1)}$ and 0.21$\mu g\ ml^{-1}\ g^{-1}$ respectively.

4.5.3. Exo ß 1, 4 glucanase

Exo ß-1, 4 glucanase activity in midgut showed the maximum activity of ß 1, 4 exoglucanase 2.08$\mu g\ ml^{-1}\ g^{-1}$ protein followed by salivary gland (0.37$\mu g\ ml^{-1}\ g^{-1}$ protein) while fore gut and hind gut recorded less enzymic activities of 0.13$\mu g\ ml^{-1}\ g^{-1}$ and 0.18 $\mu g\ ml^{-1}\ g^{-1}$ respectively.

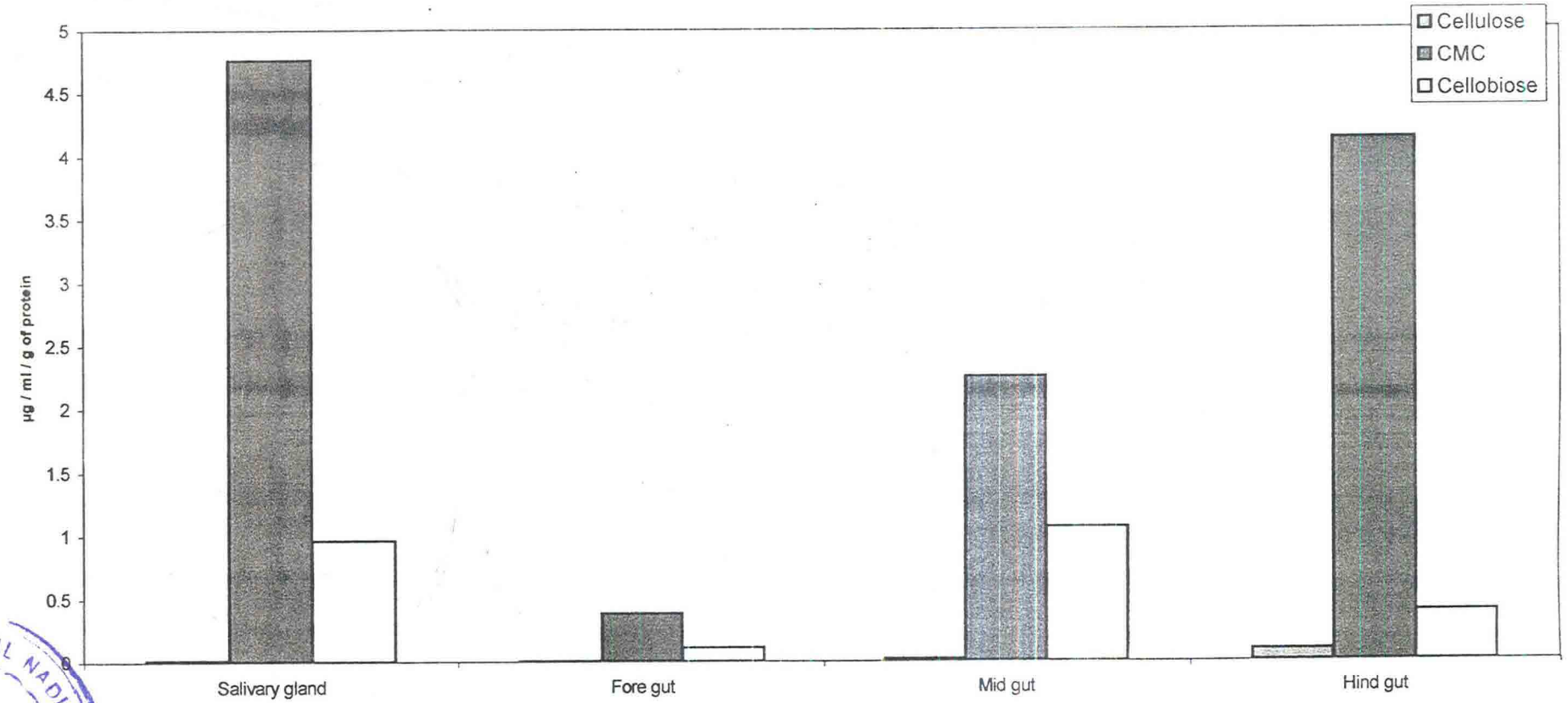
The activities of cellulase in different carbon sources were presented in (Fig3). The activities of cellulase in extracts of the termite gut are compared with the activities found in the fore gut, mid gut, hind gut and salivary gland. The result showed that the cellulase had a higher activity with CMC than with crystalline cellulose. Cellulase activity in CMC as substrate showed maximum in the salivary gland (4.76 reducing sugar equivalent to $\mu g\ ml^{-1}\ \mu g^{1}$) followed by hind gut which

Fig. 2. Enzyme activities of worker termites gut



Endoglucanase activity was assayed by the amount of reducing sugar released from CMC (Wood, 1968)
Exoglucanase activity was assayed by the amount of reducing sugar released from cellulose (Wood, 1968)
Cellulase activity was assayed as per the method (Pancholy and Rice , 1979)
Protein content was estimated by dye binding assay (Bradford,1976.
Reducing sugars was estimated as per the method (Nelson, 1944.
Reducing sugar was equivalent to 1 µg ml-1 µg-1 protein

Fig. 3. Effect of different substrates on cellulase activity of termite gut



Cellulase activity was assayed as per the method (Pancholy and Rice , 1979)
Protein content was estimated by dye binding assay (Bradford,1976).
Reducing sugars was estimated as per the method (Nelson, 1944).
Reducing sugar was equivalent to 1 µg ml-1 g-1 protein

showed 4.13 reducing sugar equivalent to μg ml^{-1} μg^{-1} whereas cellulose as a substrate, cellulase activities were found to be maximum in salivary gland (0.014 reducing sugar equivalent to μg ml^{-1} μg^{-1}) and mid gut (0.019 reducing sugar equivalent to μg ml^{-1} μg^{-1}).

Cellulase activity on cellobiose activities were found, maximum in the mid gut (1.06 reducing sugar equivalent to μg ml^{-1} μg^{-1}) followed by salivary gland (0.96 reducing sugar equivalent to μg ml^{-1} μg^{-1}) while fore gut and hind gut showed less enzymic activity recorded (0.11 and 0.39 reducing sugar equivalent to μg ml^{-1} μg^{-1} respectively.

4.6.Biochemical characterization of microbial products of *Azotobacter* and *Beijerinckia*

Different regions of (Fore, Mid and Hindgut) of the termites were dissected and assayed for the enumeration of N_2 fixing aerobes. On isolation gram negative polysaccharide producing bacteria viz., *Azotobacter* and *Beijerinckia* sp were observed. The isolated cultures were characterized and enriched in Beckings media for further work.

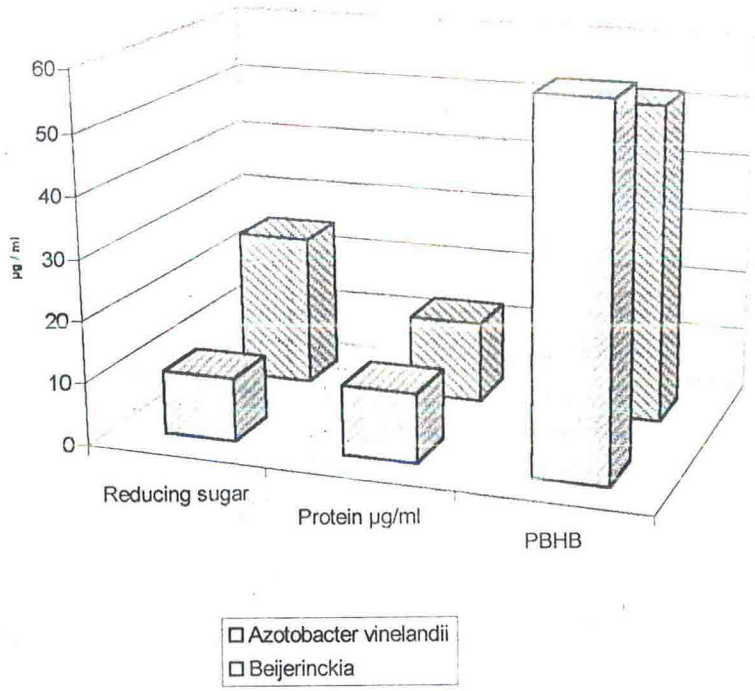
Biochemical characters of the microbial products were estimated from the isolated cultures. The amount of reducing sugars produced were maximum in *Beijerinckia* (25 μg/ ml) and their protein content was 13.0 μg/ ml. while in *Azotobacter,* the reducing sugar observed was 10.0 μg / ml and their protein content was 11.0 μg / ml. The PßHB observed was maximum in *Azotobacter* (58.9 μg/ ml) whereas in *Beijerinckia* showed 52.0 μg/ml. (Fig 4)

4.7. Distribution of enzymic activities of the polysaccharide producing bacteria

The isolated polysaccharide producing bacteria *viz., Azotobacter* and *Beijerinckia* were assayed for amylase, invertase, ß 1, 4 exoglucanase and ß 1, 4 endo glucanase. The result of the enzymic activities were presented in the (Fig 5).

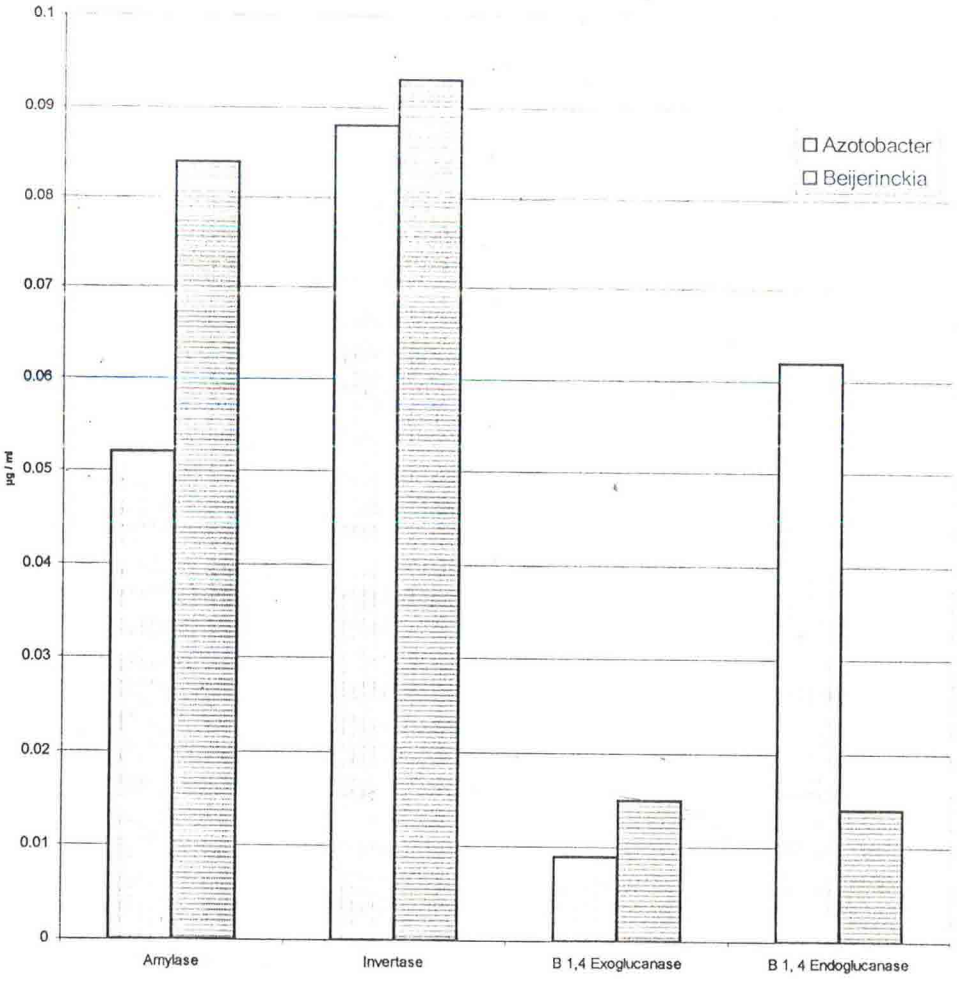
The result showed both *Azotobacter* and *Beijerinckia* sp showed maximum invertase activity (0.088 μg/ ml) and 0.093 μg/ ml whereas amylase activity was higher in *Beijerinckia* (0.084 μg/ ml) while in *Azotobacter* showed (0.052 μg/ ml).

Fig. 4. Biochemical characteristics of microbial products from Azotobacter and Beijerinckia



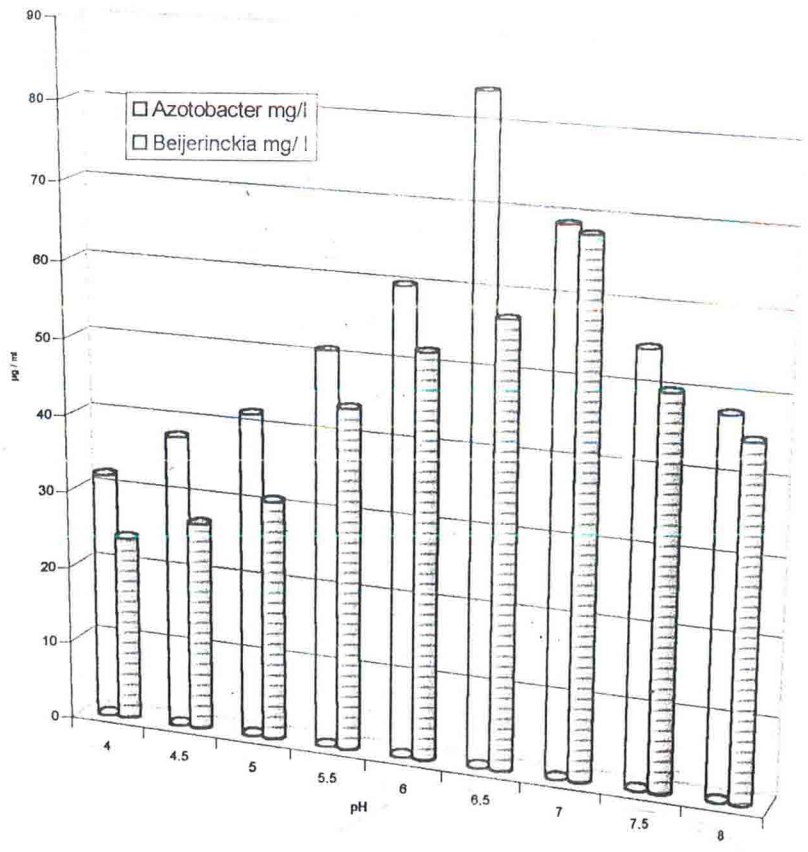
Reducing sugar released from the culture was estimated as per the method , (Nelson, 1944)
Protein was quantified as per the method (Bradford 1976)
Poly ß Hydroxy butyrate was quantified (Jarman, 1978)

Fig. 5. Distribution of different enzyme activities in Azotobacter and Beijerinckia



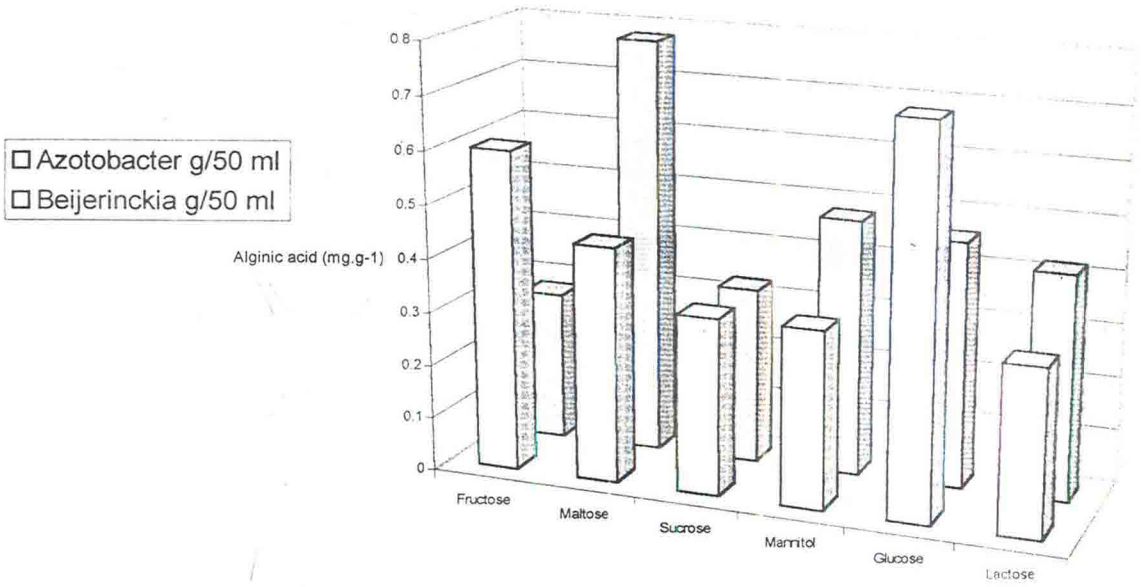
Endoglucanase activity was assayed by the amount of reducing sugar released from the culture (Wood, 1968)
Exoglucanase was assayed by the amount of reducing sugar released from the culture (Wood, 1968)
Amylase and invertase activity was assayed as per the method (Pancholy and Rice (1979)

Fig. 6. Effect of different pH in polysaccharide production by Azotobacter and Beijerinckia



Polysaccharides extracted from the different carbon sources were quantified as per the method Jarman, (1978).

Fig. 7. Effect of different carbon sources in polysaccharide productin by Azotobacter and Beijerinckia



Polysaccharides extracted from the different carbon sources were quantified as per the method Jarman, (1978).

Between ß 1,4 exo and endo glucanase activities, exoglucanase activity was observed maximum in two cultures. *Azotobacter* showed maximum exoglucanase activity of 0.062 µg/ ml whereas *Beijerinckia* showed 0.014 µg/ mlof exoglucanase activity.

4. 8. Effect of different pH levels on polysaccharide production by the cultures

Polysaccharide producing bacteria viz., *Azotobacter* and *Beijerinckia* were enriched in Becking' s media at different pH levels. The result of the experiment was given in Fig 6. The result showed *Beijerinckia* produced more polysaccharide (68.0 mg/ ml) at the level of pH 7.0 whereas *Azotobacter* produced maximum polysaccharide (84 mg/ ml) at the level of pH 6.5.

4. 9. Polysaccharide content extracted from the isolated cultures

The polysaccharide extracted from the two cultures viz., *Azotobacter* and *Beijerinckia* sp were enriched in different substrate like fructose, maltose, sucrose, mannitol, glucose. The polysaccharide extracted from the cultures were quantified and are presented in Fig 7.

The result of the experiment showed *Beijerinckia* sp showed maximum polysaccharide content in maltose (0.78 g/ ml) followed by mannitol (0.48g/ ml) and glucose (0.46 g/ 50 ml) whereas *Azotobacter* showed maximum polysaccharide content in glucose (0.72 g/ ml) followed by fructose (0.60 g/ ml).

4.10. Substrate utilization pattern of the isolated culture

4.10.1.Effect of different temperature on the growth of Methanogenic culture

To find out the preferred substrate and temperature the isolated culture was tested with different temperatures *viz.*, 30 ± 2°C, 37°C, 55°C and 10°C. The isolated three methanogenic cultures were enriched with different substrates like acetate, formate, methanol, monomethylamine, trimethylamine and H_2 and CO_2 were presented in (Fig 8-3 D). The result showed that optimum temperature for the growth of three cultures preferred between 30 ± 2, 37°C. The cultures showed maximum growth in 30 ± 2°C followed by 37°C respectively. Growth was observed comparatively leser than other two temperatures. At temperature 30 ± 2°C the culture1 used maximum H_2 : CO_2 (OD: 0.291 and protein content was 78.64 µg

Fig8. Effect of different substrates on the growth of *Methanobacterium* (Mb1) at 30+ 2°C

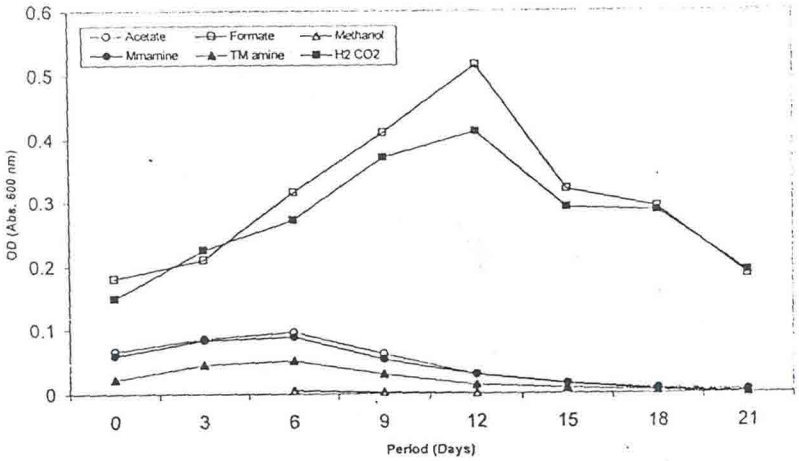


Fig. 9 Effect of different substrates on the protein content of *Methanobacterium* (Mb1) at 30+ 2°C

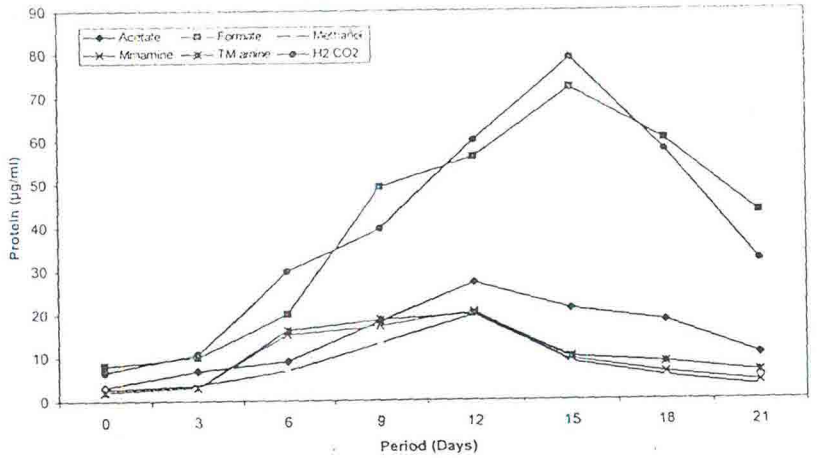


Fig. 10. Effect of different substrates on the growth of *Methanobacterium* (Mb1) at 37°C

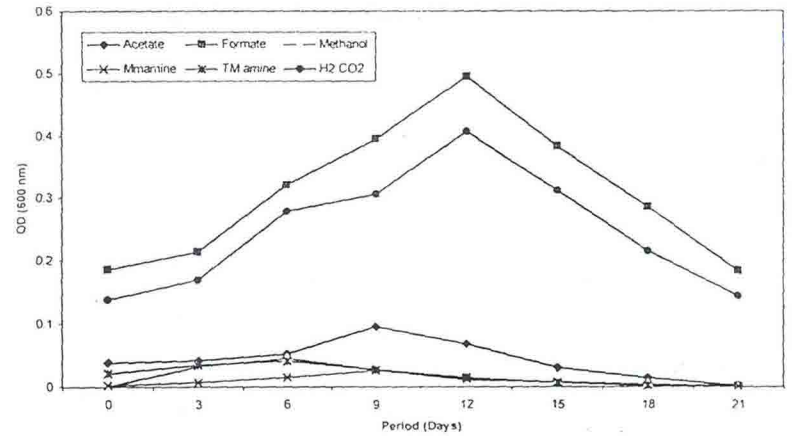


Fig. 11. Effect of different substrates on the protein content of *Methanobacterium* (Mb1) at 37°C

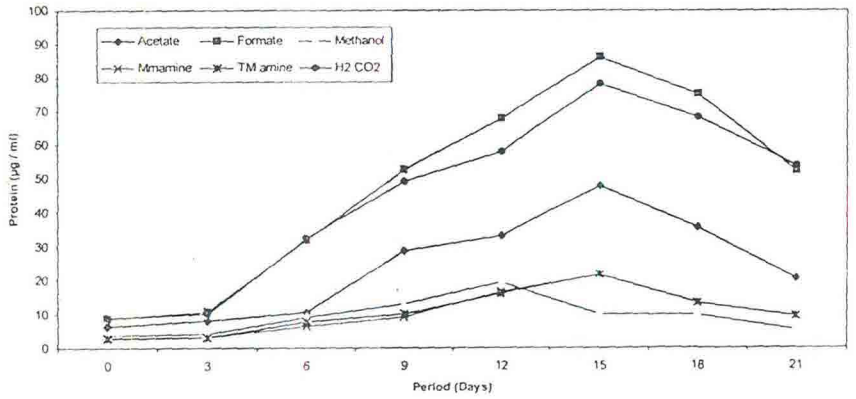


Fig. 12. Effect of different substrates on the growth of *Methanobacterium* (Mb1) at 55°C

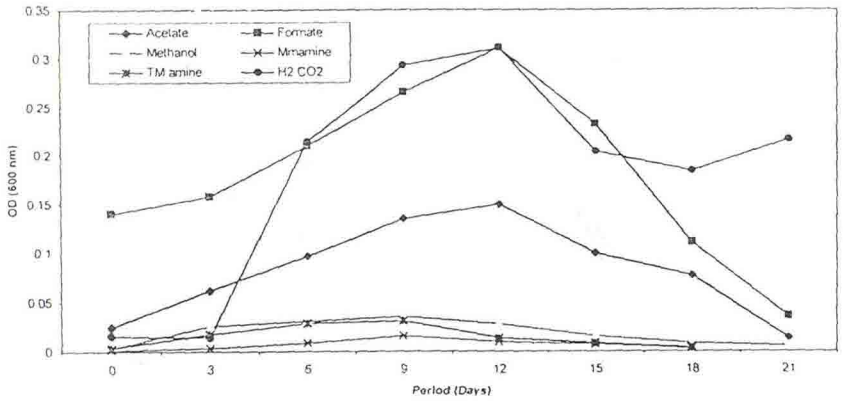


Fig. 13. Effect of different substrates on the protein content of *Methanobacterium* (Mb1) at 55°C

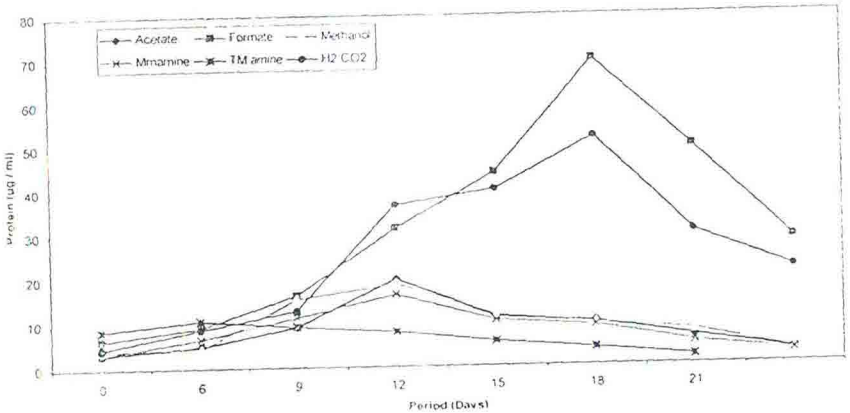


Fig. 14. Effect of different substrates on the growth of *Methanobacterium* (Mb1) at 10°C

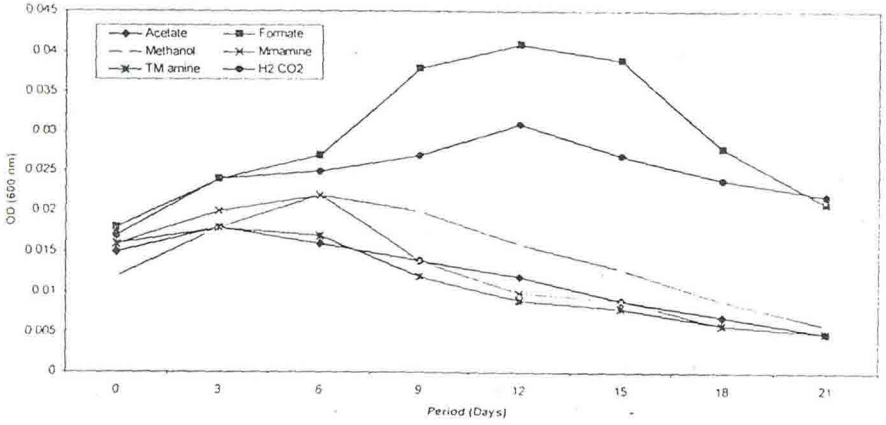


Fig. 15. Effect of different substrates on the growth of *Methanobacterium* (Mb1) at 30+2°C

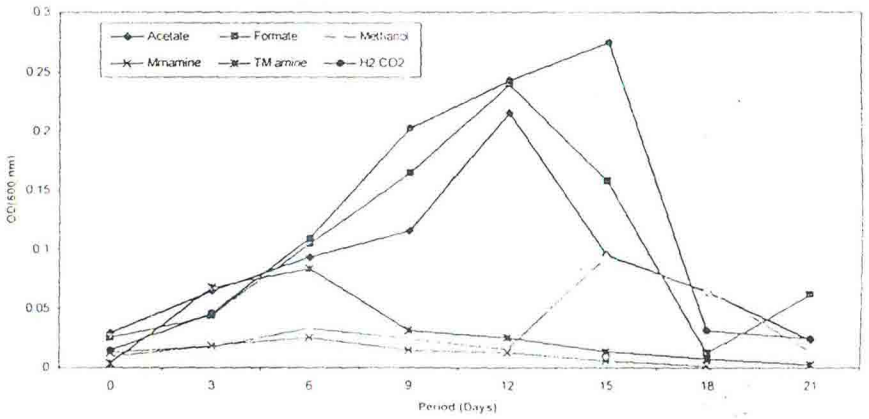


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Fig. 16. Effect of different substrates on protein content of *Methanobacterium* (Mb2) at 30+2°C

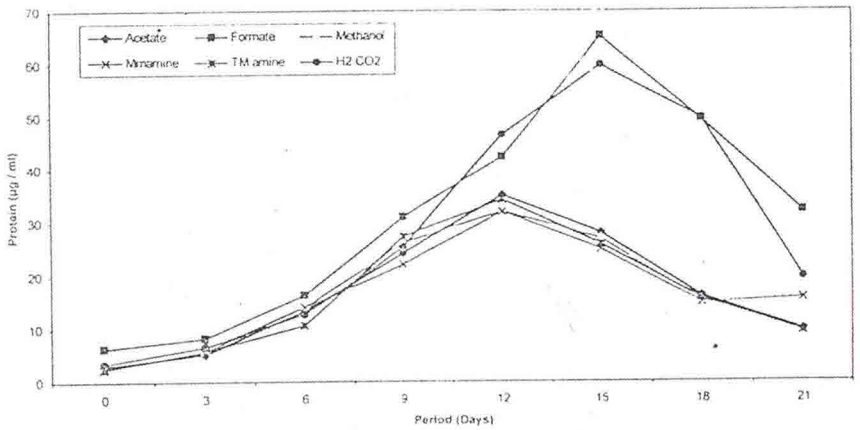


Fig. 17. Effect of different substrates on the growth of *Methanobacterium* (Mb2) at 37°C

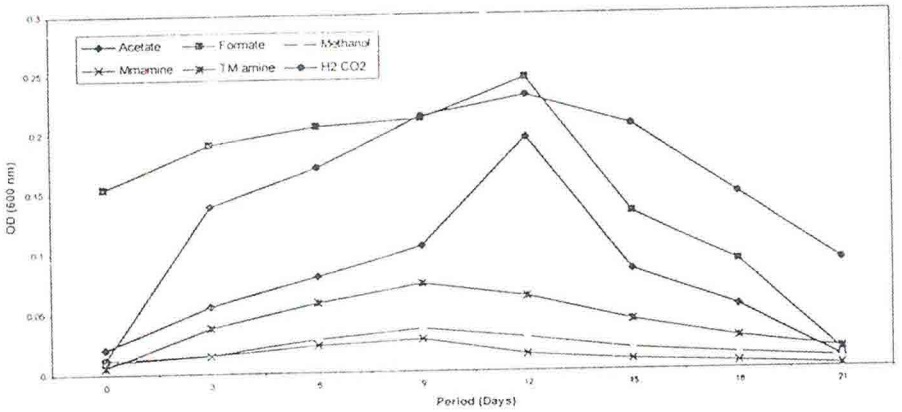


Fig. 18. Effect of different substrates on protein content of *Methanobacterium* (Mb2) at 37°C

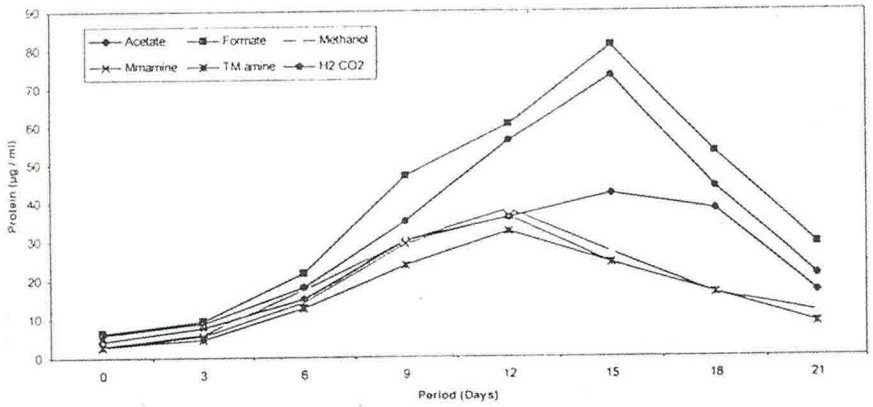


Fig. 19. Effect of different substrates on the growth of *Methanobacterium* (Mb2) at 55°C

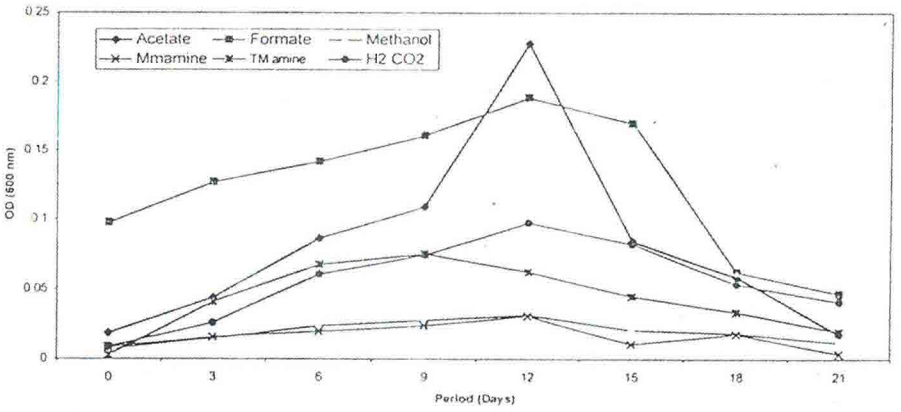


Fig. 20 Effect of different substrates on protein content of *Methanobacterium* (Mb2) at 55°C

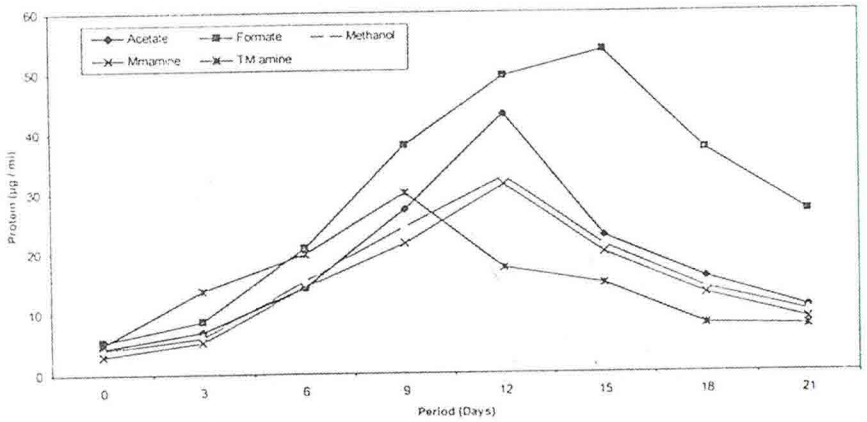


Fig. 21. Effect of different substrates on the growth of *Methanobacterium* (Mb2) at 10°C

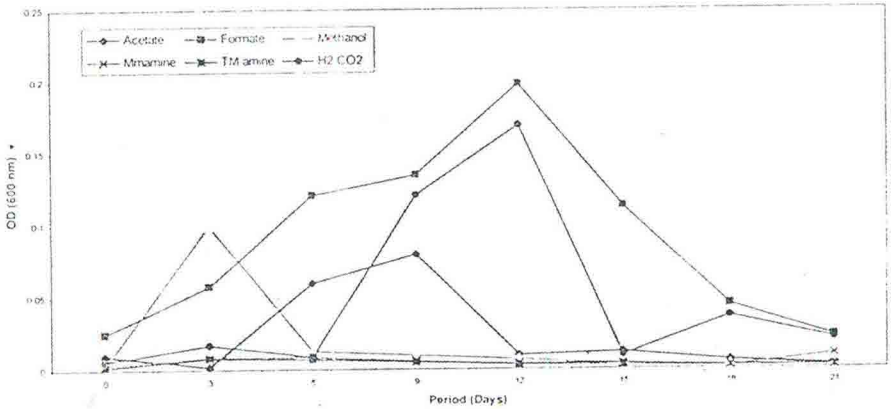


Fig. 22. Effect of different substrates on protein content of *Methanobacterium* (Mb2) at 10°C

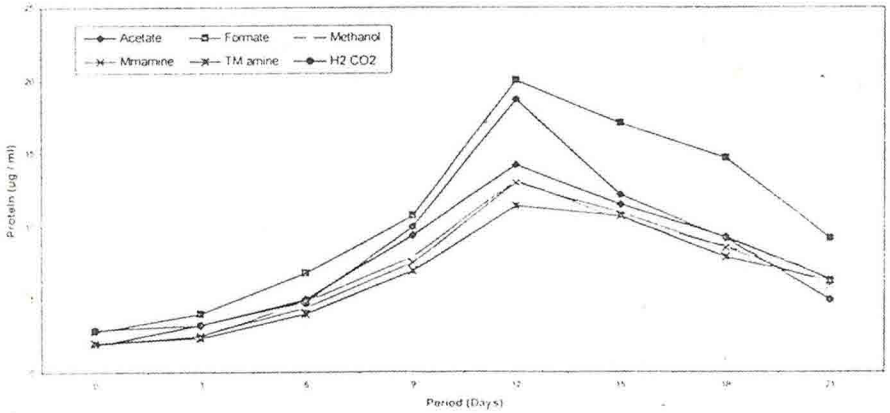


Fig. 23. Effect of different substrates on growth of *Methanosarcina* at 30+2°C

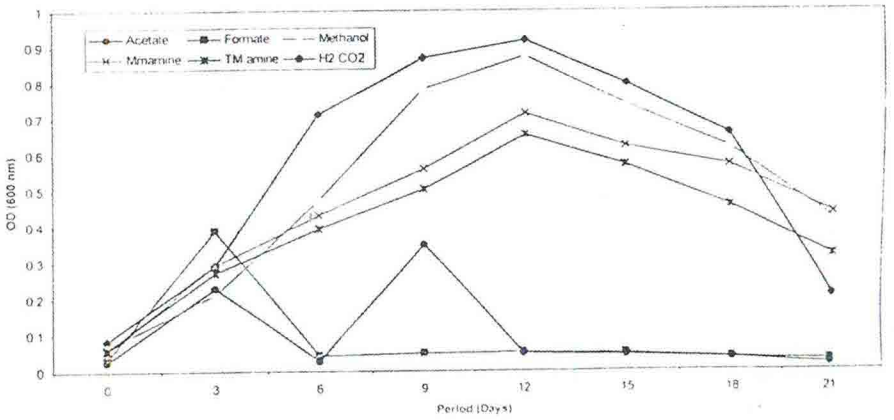


Fig. 24. Effect of different substrates on protein content of *Methanosarcina* at 30+2°C

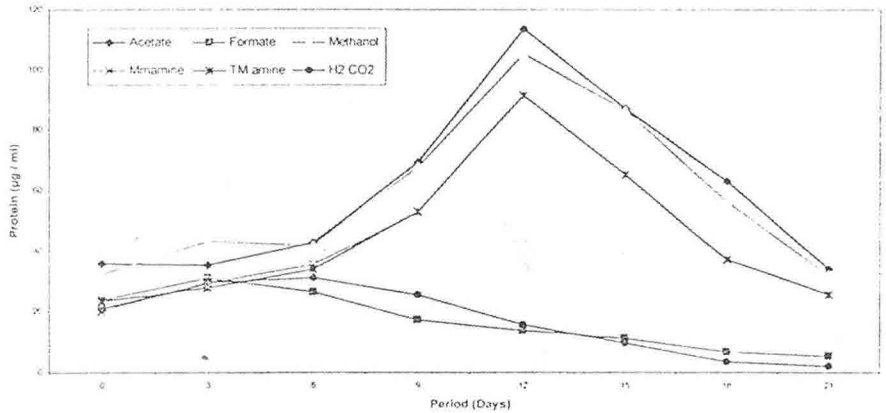


Fig. 25. Effect of different substrates on the growth of *Methanosarcina* at 37°C

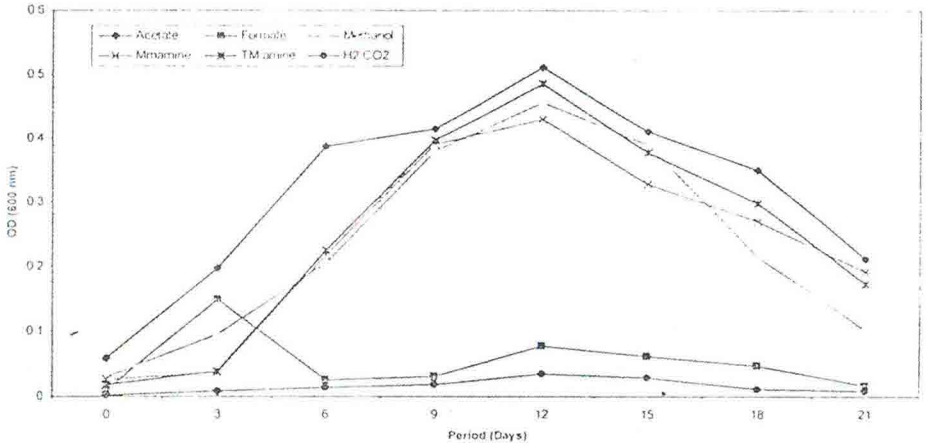


Fig. 26. Effect of different substrates on protein content of *Methanosarcina* at 37°C

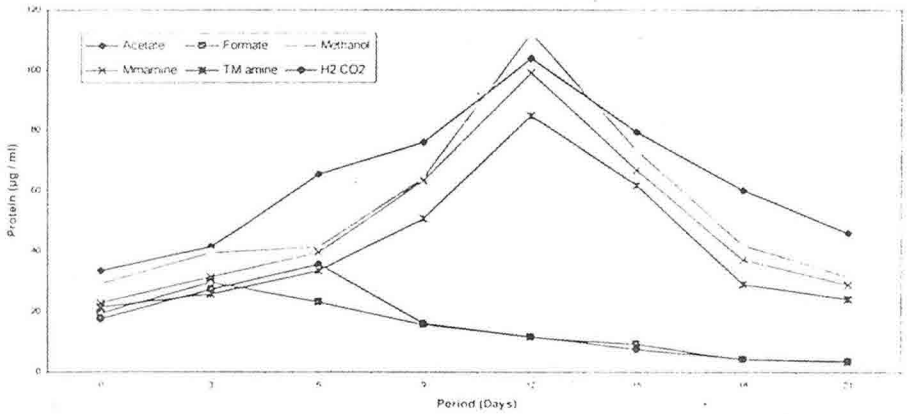


Fig. 27. Effect of different substrates on the growth of *Methanosarcina* at 55°C

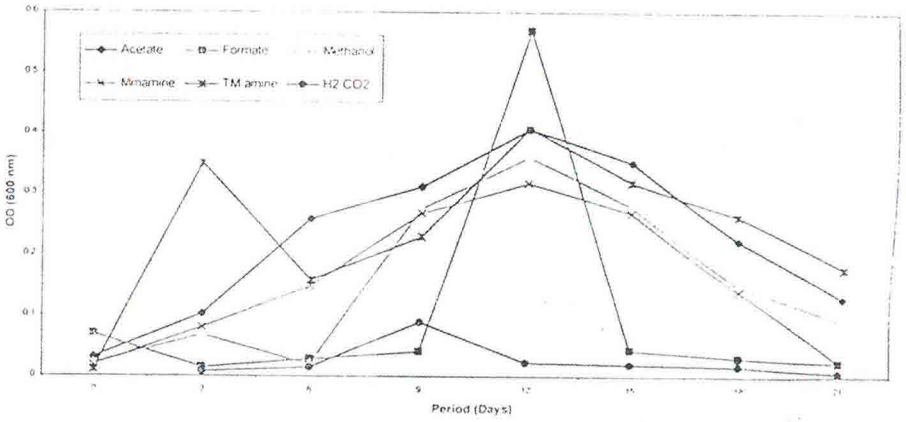


Fig. 28. Effect of different substrates on protein content of *Methanosarcina* at 55°C

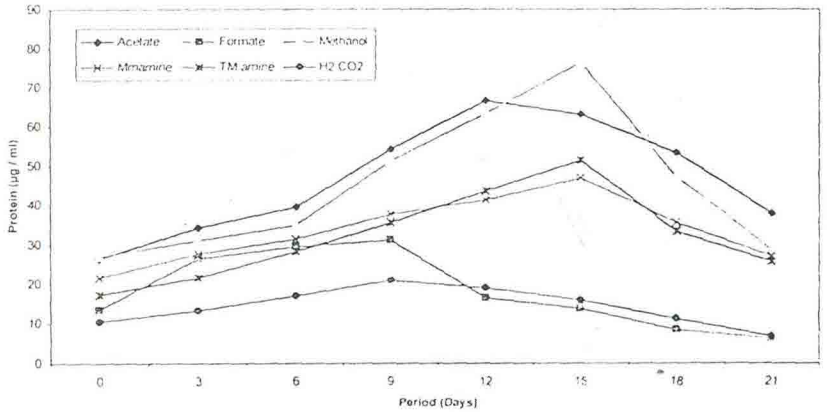


Fig. 29. Effect of different substrates on growth of *Methanosarcina* at 10°C

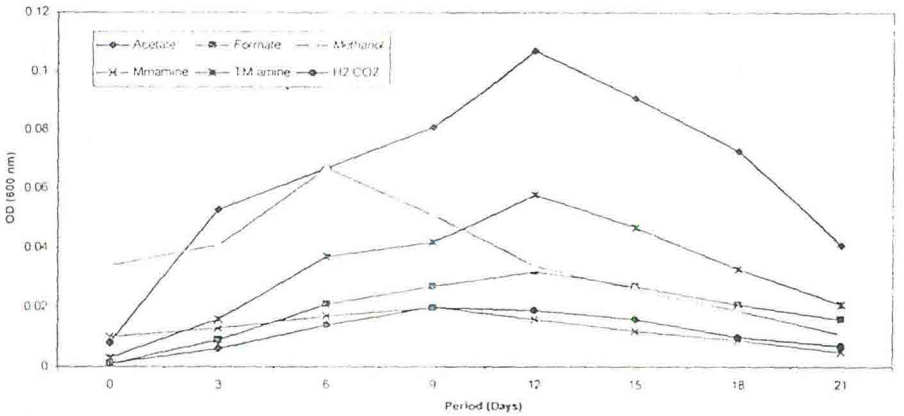
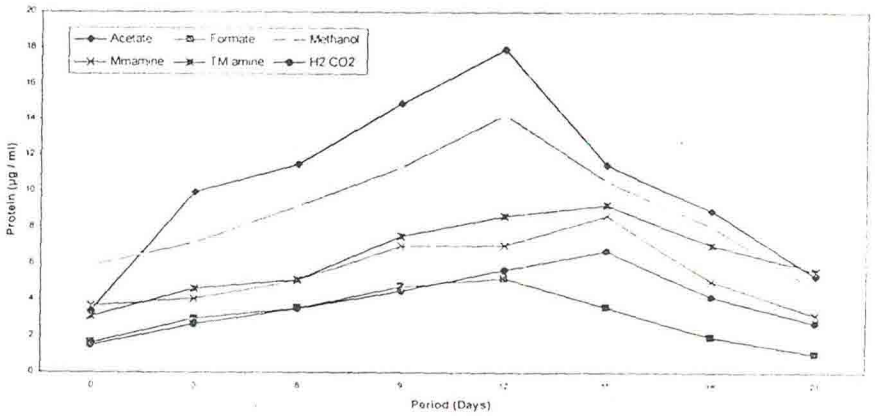


Fig. 30. Effect of different substrates on protein content of *Methanosarcina* at 10°C



protein ml^{-1}), followed by formate (OD: 0.32, protein 72.12 µg protein ml^{-1}) on 15th day of inoculation. On 15th day onwards the growth was observed maximum in all the three cultures, then the growth started declining after 18th day onwards. The culture 2 showed maximum growth in formate (OD: 0.158 ; protein was 65.1µg protein ml^{-1}) on 15th day of inoculation followed by H_2 : CO_2 (OD: 0.275 ; protein was 59.75µg protein ml^{-1}) at the temperature of 30 ± 2°C. The culture 3 showed maximum growth in acetate (OD: 0.918; protein was 113.6 µg protein ml^{-1}) followed by methanol (OD: 0.875 and protein was 105.36 µg protein ml^{-1}) at the temperature of 30 ± 2°C on 15th day of inoculation The acetate grown culture was used as inoculam for further work. (Fig 8-31).

4.10.2. Effect of different concentration of acetate on the growth of *Methanosarcina*

The effect of different concentration of the acetate on growth of *Methanosarcina* was tested (Fig 31). The results showed that the growth of the cells occurred at all the concentration of acetate. But maximum growth was observed at 100 mM acetate concentration (143.00 µg protein ml^{-1}) and OD 0.428 on 15th day of inoculation followed by 75 mM acetate concentration (86.00µg protein ml^{-1}) and OD was 0.395 were presented in (Table.9)

4.10.3. Effect of different levels of pH on the growth of *Methanosarcina*

The studies on the effect of pH on growth of the isolated *Methanosarcina* were presented in Fig.32. The isolate found to grow between 6.5 - 7.5. The maximum growth was measured at pH 7.5 (118 µg protein ml-1) and OD 0.763 on 21st day of inoculation. On 18th day of inoculation pH 7.0 showed (97.00 µg protein ml^{-1}) and their OD: 0.512. At pH 5.5 showed 16.00 µ g protein ml^{-1} and OD 0.009 observed on 21st day, whereas, at pH 8.0 showed the amount of protein measured was (31.5 µg protein ml^{-1}) and their OD was 0.126. Throughout the growth period lowest amount of protein was detected at pH 8.0 on 12th day (2.50 µg protein ml^{-1}). The maximum specific growth activity was found to be at pH 7.5. (Table 10)

Table.9 Effect of different concentration of acetate on the growth of on *Methanosarcina*

Periods (days)	Acetate concentration			
	25mM	50 mM	75 mM	100 mM
0	40.5	41.0	40.5	41.0
3	41.0	43.0	43.5	44.0
6	43.5	45.0	48.0	56.0
9	45.0	49.0	55.0	73.0
12	48.5	56.0	70.5	84.0
15	53.0	68.5	86.0	143.0
18	52.5	62.0	74.5	123.0
21	50.5	59.5	71.0	116.0
24	48.0	56.0	68.5	88.5
27	47.0	48.0	65.0	80.5

Growth measured as protein $\mu g\ ml^{-1}$

Medium Mah (1980)

Substrate sodium acetate

Table. 10 Effect of different pH on growth of *Methanosarcina* .

Periods (days)	pH range					
	5.5	6.0	6.5	7.0	7.5	8.0
0	31.0	33.00	36.00	34.00	31.00	1.00
3	30.50	35.00	35.50	36.00	34.00	7.00
6	28.50	37.00	39.50	42.00	38.00	0.50
9	28.00	37.00	41.50	44.00	39.50	3.00
12	22.50	32.50	45.00	49.00	47.00	2.50
15	18.00	34.00	66.00	95.00	81.00	35.0
18	21.00	35.00	43.50	97.00	104.0	31.0
21	16.00	31.50	38.50	81.00	118.0	31.5
24	20.00	26.00	31.00	79.00	98.50	25.5
27	14.50	11.00	26.50	63.00	83.00	23.0
30	11.00	4.50	20.50	38.00	36.00	18.0

Methanosarcina sp was inoculated (5% v/v) in Mah medium (Mah, 1980) with sodium acetate as carbon source. The pH of the medium was adjusted to required levels before inoculation.

Growth was measured in terms of protein content using dye binding assay (Bradford, 1976).

Fig. 31 . Effect of different concentrations of acetate on the growth of Methanosarcina

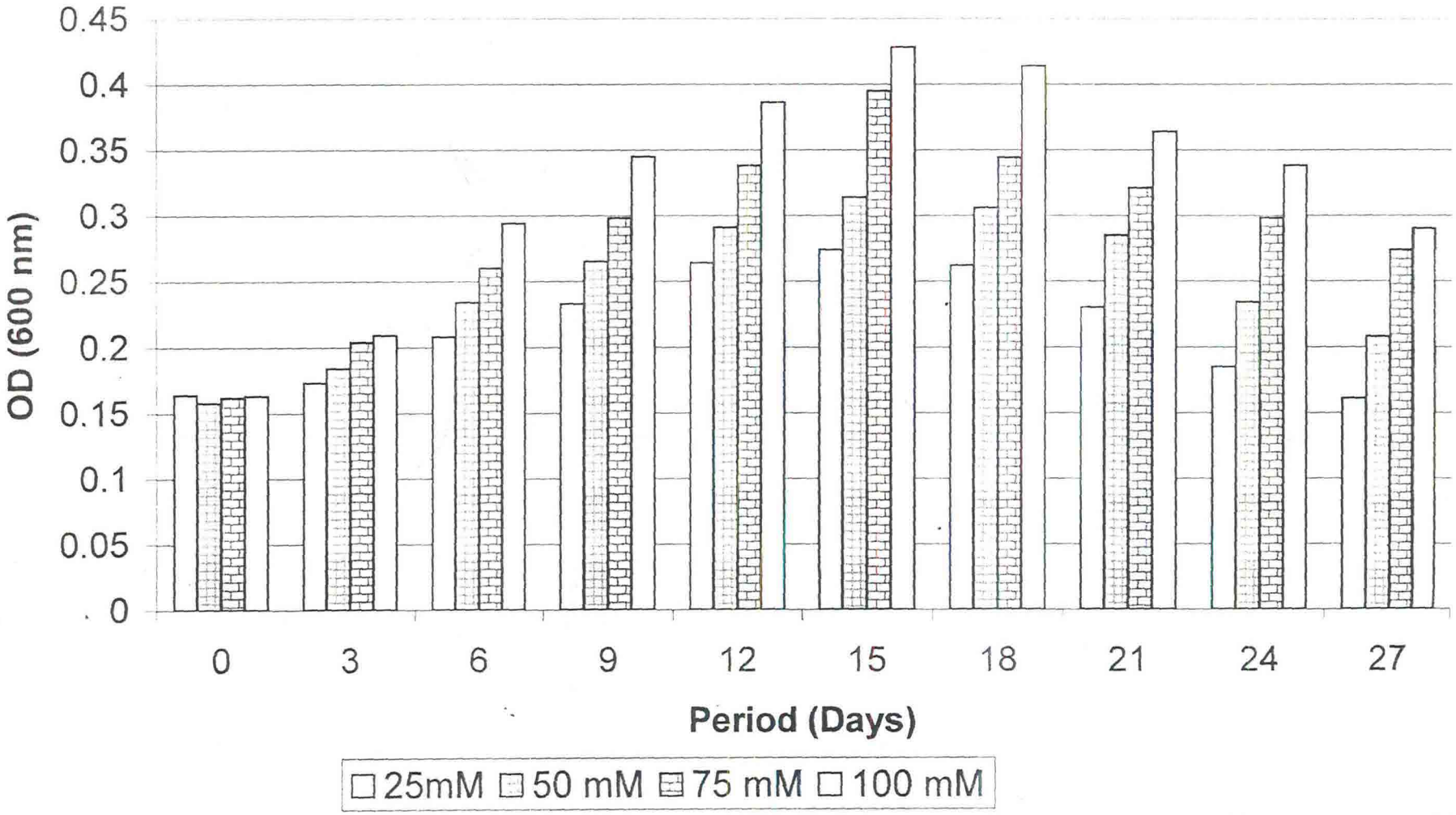
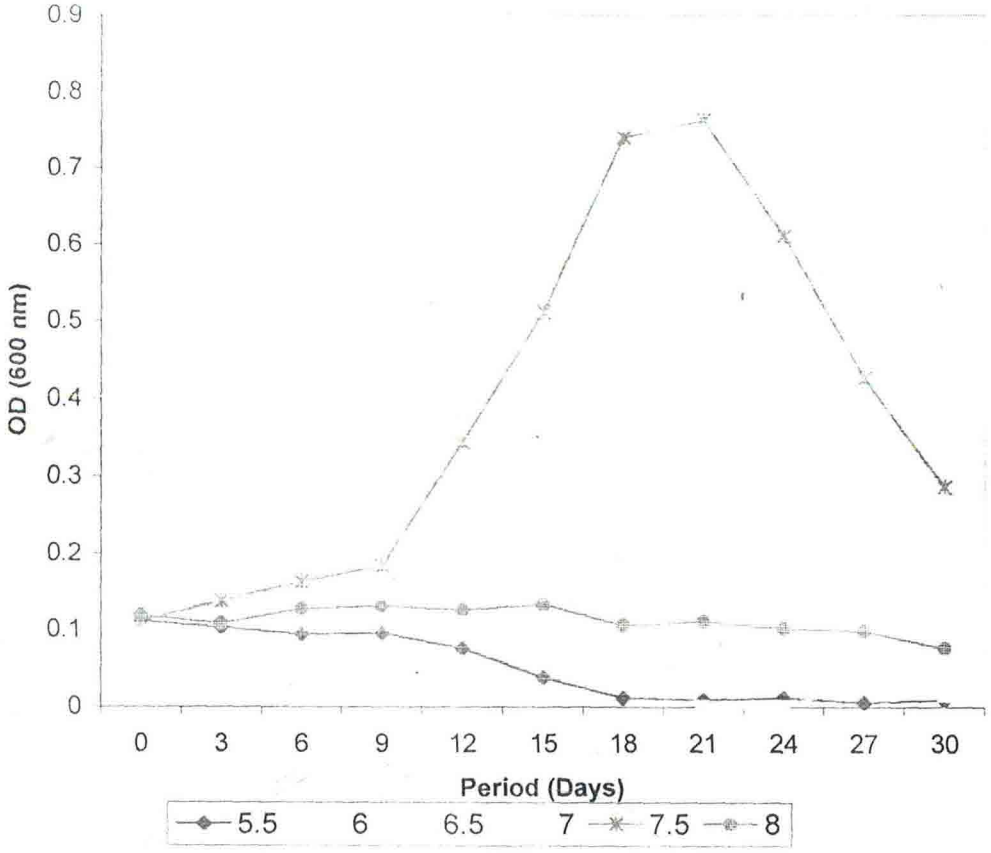


Fig. 32. Effect of different pH on the growth of *Methanosarcina*



4.10.4. Effect of different substrates on methane production by *Methanosarcina* at 30 ± 2 °C

The methane production by *Methanosarcina* in different substrate was presented in Table 11. The culture grown in acetate showed maximum methane production (1258.4 μ mole) on 21st day of inoculation followed by methanol (760.0 μ mole), while in monomethylamine and trimethylamine, it was (547.36 μ mole) and (545.6 μ mole) respectively. Whereas formate and H_2 : CO_2 showed less amount of methane production (6.56 and 5.31 μ mole) respectively.

4.10.5. Effect of different substrates on methane production by *Methanosarcina* at 37 °C

Studies on the effect of temperature on the methane production of isolated methanogenic cultures were presented in (Table 10). At temperature 37°C all the substrates initially produced methane production. The maximum methane production was observed in acetate (1596.32 μ mole) followed by methanol (1259.2 μ mole), while in monomethylamine and trimethylamine it was (1155.68 μ mole) and (996.16μ mole) but formate and H_2 : CO_2 recorded less amount of methane production (4.96 μ mole and 6.24 μ mole) respectively on 21st day of inoculation. From 6th day onwards, the methane emission from the culture showed (63.2 μ mole) in acetate and methanol followed by monomethylamine (62.72 μ mole) and trimethylamine (53.76 μ mole) respectively. The maximum methane production was observed in 21st day of inoculation in acetate (1258.4 μ mole) followed by methanol (760.00 μ mole), monomethylamine (547.36 μ mole), trimethylamine (545.60 μ mole), whereas less methane production was recorded in formate (6.56 μ mole) and H_2 : CO_2 (5.31 μ mole) (Table 12).

4.11. Metal content methanogenic bacteria

The studies on the metal analysis of Iron and Nickel content from the isolated methanogenic culture were presented in Fig.3. Among the three cultures tested, the culture 2 exhibited maximum iron and nickel of 31.88 μ g Fe/ mg protein ml^{-1} and 2.371 μ g Ni / mg protein ml^{-1} followed by the culture 1 which showed Iron and Nickel of 20.54 μg Fe/ mg protein ml^{-1} and 20.54 μ g Ni / mg protein ml^{-1}, whereas

Table. 11 Effect of different substrates on methane production by *Methanosarcina* at 30 ± 2°C (μ mole)

Days of inoculation	Acetate	Formate	Methanol	M amine	T amine	$H_2 + CO_2$
3	28.16	6.24	19.84	20.32	19.84	4.96
6	63.2	11.84	63.2	62.72	53.76	15.84
9	143.04	21.92	107.68	105.44	67.04	16.64
12	244.64	22.72	196..48	179.2	178.56	19.84
15	547.36	28.16	404.16	395.32	336.32	30.56
18	764.32	14.08	601.44	547.36	401.28	11.95
21	1596.32	4.96	1259.2	1155.68	996.16	6.24

Methanosarcina sp was inoculated in Mah medium with acetate, formate, methanol, monomethylamine, trimethylamine and $H_2 + CO_2$ as carbon sources (5 % v/v). Growth was measured at every three days intervals

Methane was detected by injecting gas into Varion Environmental Analyser (Model No.CP 3800GC), USA with Poropak column (6' x 1/8") connected to Flame ionization detector. Nitrogen (30 ml, min^{-1}) was used as carrier gas. Hydrogen (30 ml, min^{-1}) Oxygen (60 ml, min^{-1}) were used for flame. Column, injector and detector temperature were 90°C, 90°C and 90°C respectively.

Table. 12 Effect of different substrates on methane production by *Methanosarcina* at 37°C (μ mole)

Days of inoculation	Acetate	Formate	Methanol	M amine	T amine	$H_2 + CO_2$
3	14.08	3.04	12.16	9.0	11.84	1.6
6	30.56	4.96	29.12	28.16	22.72	3.04
9	63.20	11.60	62.72	61.60	54.88	4.96
12	242.24	18.76	196.48	179.20	178.56	9.24
15	404.16	21.4	395.68	336.32	333.44	11.60
18	601.44	13.56	483.68	452.16	430.72	8.65
21	1258.4	6.56	760.0	547.36	545.6	5.31

Methanosarcina sp was inoculated in Mah medium with acetate, formate, methanol, monomethylamine, trimethylamine and H_2+CO_2 as carbon sources (5% v/v). Growth was measured at every three days intervals.

Methane was detected by injecting gas into Varion Environmental Analyser (Model No.CP 3800GC), USA with Poropak column (6' x 1/8") connected to Flame ionization detector. Nitrogen (30 ml, min^{-1}) was used as carrier gas. Hydrogen (30 ml, min^{-1}) Oxygen (60 ml, min^{-1}) were used for flame . Column, injector and detector temperature were 90°C, 90°C and 90°C respectively.

culture 3 showed the presence of Iron (13.95 μ g Fe/ mg protein ml^{-1}) and Nickel of (2.001 μ g Ni / mg protein ml^{-1}).

4.11.1. Effect of different concentration of Nickel on the growth of *Methanosarcina*.

Nickel was found to influence the growth of *Methanosarcina* sp. Different concentration of Nickel Chloride at the rate of 0.01 μm, 0.10 μm and 1.00 μm were incorporated in acetate containing medium (Fig.3). Medium without Nickel addition served as control. The rate of growth in all the four treatments were initially similar, but the rate of increase in growth varied with treatments. The maximum growth was observed in all the treatments on 15th day, afterwards there was a decline in growth rate. Maximum growth of *Methanosarcina* occurred in treatment containing 1.0 μm Nickel (protein content was 238.00 μ g ml^{-1}) and OD : 0.975 followed by 0.1 μm (protein content was 206.00 μ g ml^{-1}) and OD was 0.928 and 0.01μm nickel showed (protein content was 174.50 μ g ml^{-1}) (Table 13).

4.11.3. Effect of nickel chloride on substrate utilization by *Methanosarcina*

The isolated *Methanosarcina* was inoculated with 1 μm Nickel chloride with different substrate *viz.*, acetate, methanol, monomethylamine and trimethylamine was tested for its absorption capacity. The absorption of Nickel by the culture was examined by observing its protein contents at timely intervals. The absorption of Ni by *Methanosarcina* is initially high in acetate (78.34 μ g Ni/ mg of protein ml^{-1}), while in methanol (62.35 μ g Ni/ mg of protein ml^{-1}), whereas monomethylamine and trimethylamine showed similar results of (46.76 μ g Ni/mg of protein ml^{-1}) and (41.52 μ g Ni/mg of protein ml^{-1}). At 18th day onwards the Ni was maximum mobilised by *Methanosarcina* in acetate (3.84 μg Ni/ mg of protein ml^{-1}) followed by methanol (8.53 μ g Ni/ mg of protein ml^{-1}) (Table 14).

4.12. *In situ* studies of methane emission from termites

Higher and lower termites were assayed for the quantification of methane and carbondioxide were presented in (Fig. 34) Plate 10 and 11.

Plate 10 ***In vitro*** **cultured worker used in the methane emission studies**

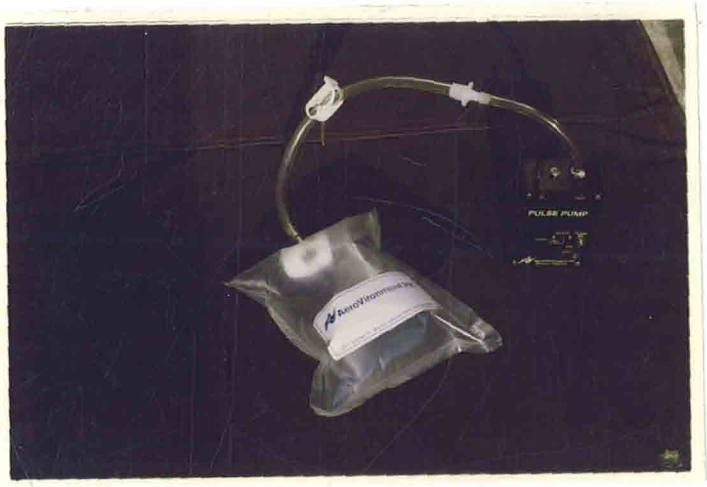


Plate 11 **Methane and Carbon di oxide collection chambers used in this investigation.**

Table.13 Effect of nickel concentration on the growth of Methanosarcina

Periods (days)	Nickel concentration (μm)		
	0.01μm	0.10μm	1.00 μm
0	32.0	38.0	41.5
3	58.0	41.0	54.0
6	76.0	83.5	121.5
9	89.5	134.0	186.0
12	134.0	178.0	194.0
15	174.5	206.0	238.0
18	154.0	184.0	211.0
21	121.0	148.0	187.0
24	108.0	131.0	167.0
27	90.5	108.0	109.0

Growth measured as protein μg ml^{-1}

Medium Mah (1980)

Substrate sodium acetate

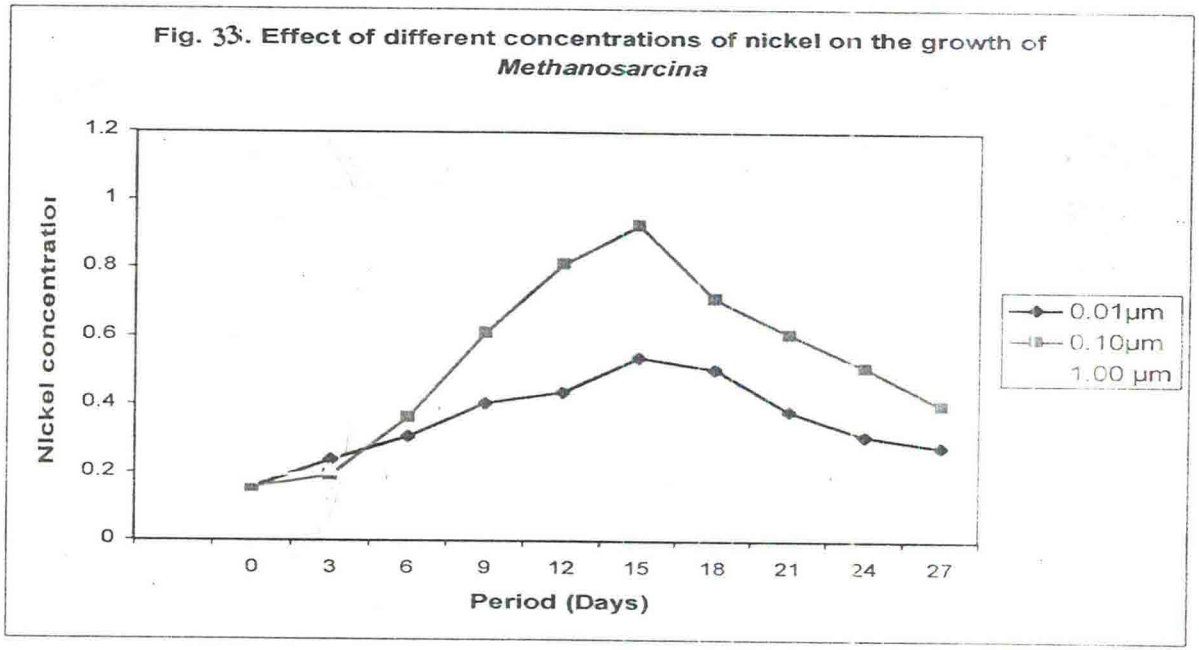
Table 14 Effect of different substrates on Nickel absorption by *Methanosarcina*

Days	Acetate	Methanol	M amine	T amine
3	78.34	62.35	46.76	48.52
6	32.16	36.22	25.59	28.43
9	22.48	31.13	22.62	24.37
12	21.94	28.43	16.79	22.86
15	9.00	14.56	14.89	18.43
18	3.84	8.53	12.98	17.12
21	1.19	4.31	5.60	9.65

Growth measured as protein μg ml^{-1}

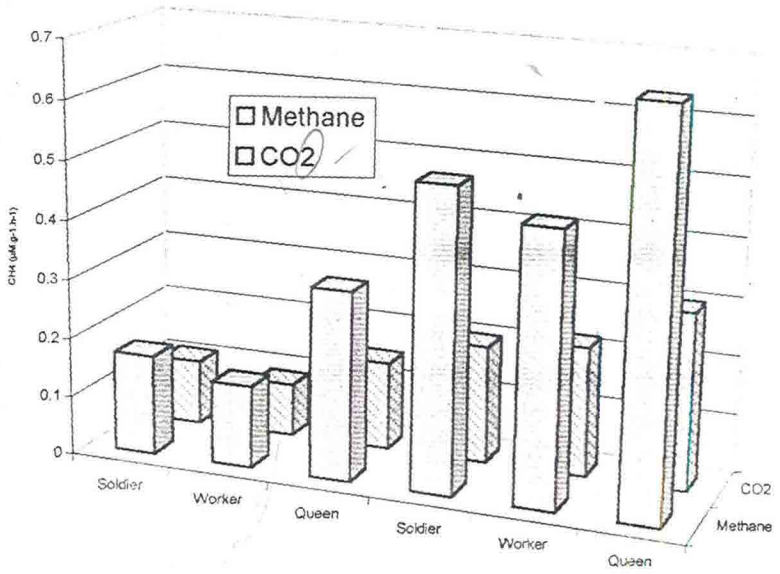
Medium Mah (1980)

Substrate Sodium acetate



Medium : Mah (1980)
Substrate: Sodium acetate

Fig. 34. In situ emission of methane from different termites



Methane quantified through Gas Chromatography (Varion Environmental Analyser Model NO. CP 3800 GC, USA with poropak column (6' x 1/8") .
CO2 through alkali absorption and titration ( )

The results of the experiment showed that, higher termites produced more methane than the lower ones. Queen of higher termites produced 0.67 μ mole/ g/h of methane whereas queen of lower termites produced only 0.32 μ mole/ g/h. CO_2 emission was observed higher in queen of higher termites (0.30 μ mole/ g/h), followed by queen of lower termites (0.15 μ mole/ g/h). All the workers and soldiers of higher and lower termites showed less methane and CO_2 emission.

4.13. Emission and Excitation study of F_{420}

Factor F_{420} was identified from *Methanosarcina* sp by different techniques. HPLC Flourometry analysis disclosed, that F_{420} was eluted at 1.68 minute at a flow rate of 2 ml/min^{-1} (Fig 35).

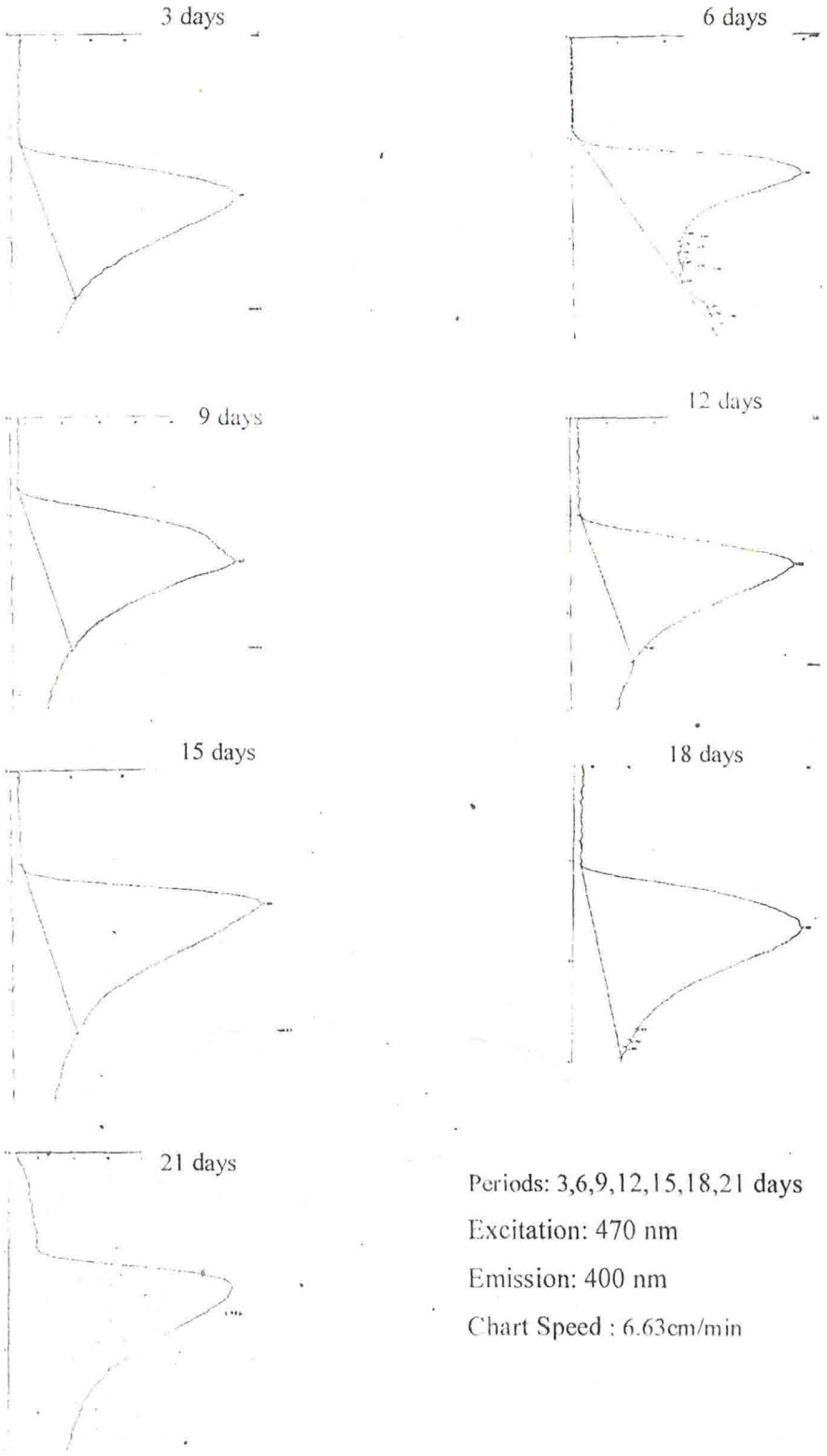
4.14. Protein profile study of Methanogens

The protein profile of Methanogenic cultures were separated in vertical electrophoresis according to Laemmli (1970). Electrophoretic analysis recorded two major protein bands with molecular weights ranging from 14.3 kDa to 43.0 kDa (plate lane 2 and 3). Among the three sample tested, third samples showed higher induction of protein. The molecular weight was around 43.0 kDa and was determined based on mobility of known molecules whereas its induction in 1st and 2nd culture was very mild. In addition to 43 kDa protein, another one low molecular weight protein (14.3) induction was also observed from lane 3.

4.15. Polysaccharide production and artificial crumb performance

Production of polysaccharides and its components sugars were assayed from the isolated cultures. On observation, the study showed that glucose was present in all the samples. *Azotobacter* contained component sugars of mannitol, glucose and fructose. Whereas *Beijerinckia* sp contained less component sugars of inositol, fructose, mannose and glucose. Artificially formed crumbs enhanced the physical parameters and nutrient status of the soil. The effect of crumb on the germination of green gram seeds were observed 7th day after sowing. Germination percentage 94.4% and its root length (2.06, shoot length (21.3) vigour index (3205.18) and biomass (0.220) compared with control.

Fig. 35. **Analysis of F_{420} from isolated *Methanosarcina***



Periods: 3,6,9,12,15,18,21 days

Excitation: 470 nm

Emission: 400 nm

Chart Speed : 6.63cm/min

DISCUSSION

CHAPTER - V

Discussion

Termites are eusocial insects with colonies of few hundreds to a maximum of seven million individuals, one reproductive king and queen or sometimes several pairs are tended by sterile workers and soldiers. Termite gut microbiota represents an unique biological system for the study of the conversion of woody and cellulosic substrates into useful products. The digestive tract of the animal species normally paves a suitable niche for a variety of microorganisms to inhabit and multiply. Microorganisms including fungi, bacteria, actinomycetes and protozoa bring out most of the cellulose degradation occurring in nature. The degradation process takes place under aerobic, anaerobic and mesophilic conditions. The alimentary tracts of almost all animal species are colonized by the microflora (Hungate, 1966). Among insects, the microbial ecology of the gut of termites has been well documented (Cruden and Markovetz, 1978; Schultz and Breznak, 1978). Bignell (1984) reported about the digestive ability of these insects and the role of their gut microflora on digestion. The physico chemical conditions prevalent in the digestive tract determine the distribution of aerobic and anaerobic microflora in the gut of termites.

5. 1. Isolation of aerobic and anaerobic microorganisms from Termite Gut

5.1.1.Isolation of aerobic bacteria

The gut microorganisms of termite consists of bacteria and fungi. The microflora of termite gut are not autochthonous microflora but are transient organisms ingested by the termite or are contaminants from the outside surface of the termite (Collins, 1989). The complex nature of the association of the flora and fauna in the gut, with bacteria adhering to the epithelium of the paunch or to other bacteria or protozoa make it difficult to recover all of the bacteria present in the gut (Bignell *et al.*, 1991 and To *et al.*, 1980). In the present study, aerobic bacteria were isolated and the isolated bacterial cultures were found to produce more of polysaccharides and also they are nitrogen fixers. These polysaccharide producing and nitrogen fixing cultures were identified as *Azotobacter* and *Beijerinckia*. Peklo (1946) also reported that insects like termites have endosymbiotic nitrogen fixing bacteria viz., *Azotobacter* in termites.

Lee and Wood (1971) evidenced that lignin part of the wood get digested in an aerobic portion of the alimentary canal and they concluded that fore and midgut of a number of termites were found to be aerobic. The gut of Australian termite hind guts are oxygen limited, but basically aerobic and it has been supported by Veivers et al. (1980). They reported that the foregut and midgut of all species were aerobic with an E'o in excess of + 100 mV. Mannesmann (1969) isolated cellulose degrading bacteria from the gut of several species of termites (after effective surface sterilization) under both aerobic and anaerobic conditions and also reported that the bacteria grew best under aerobic conditions. Noirot and Timothee (1969) and Krelinova *et al.* (1977) isolated gram negative, cellulose degrading bacteria from several species of termite and they also isolated the nitrogen fixing bacteria from the hind gut of termites. Bergersen and Hipsley (1970) also reported the presence of N_2 fixing bacteria in the gut of termites.

5.2. Isolation of anaerobic bacteria

Termites possess heterotrophic bacteria in their gut regions. The isolation, morphology and substrate specificity of the isolates from termite gut were studied. A high proportion of the bacteria were facultative anaerobes belonging to the genera *Streptococcus, Staphylococcus, Enterobacter* and *Citrobacter (*Schultz and Breznak, (1978) and Eutick *et al.* (1978). In our investigation, anaerobic bacteria were isolated from foregut, midgut and hindgut regions of the termites. The anaerobes isolates were cellulolytic acetogenic bacteria, (*Clostridium*), N_2 fixing facultative anaerobic bacteria (*Klebsiella)* and methanogenic bacteria. Based on the characteristics, the methanogenic isolates were identified as *Methanosarcina* sp and *Methanobacterium.*sp. During the growth, the formation of clumps or aggregates occurred, which is a typical characteristics of *Methanosarcina* sp.

In the present investigation also, the presence of total anaerobes was maximum in the foregut (44.7 $x10^5$ ml^{-1}) followed by midgut (27.7 $x10^5$ ml^{-1}) and hindgut (20.4 x 10^5 ml^{-1}); whereas in cockroaches, the load of anaerobes was around 10^{10} ml^{-1} (Crude and Markovetz, 1987).

5.2.1. Acid forming bacteria

Scheifinger *et al.* (1974) isolated *S.rumunantium* which play important role in the production of volatile fatty acids. The present study also stated that, acid producing

colonies were found maximum in foregut (40.3 $x10^3$ ml^{-1}) followed by midgut (30.3 x 10^3 ml^{-1}) and hindgut (20.4 $x10^3$ ml^{-1}); Bayon and Etievant (1980) reported the microbial fermentation of cellulose to volatile fatty acids and methane in the region of proctodeum and mesenteron which were the active sites of cellulolysis. They also reported that the rate of methane emission by live *Oryctes nasicornis* and it was found similar to the report of Breznak (1975).

5. 2. 2. N_2 fixing bacteria in the gut of termites

Symbiotic microorganisms present in the hindgut of termites are important for the survival of their host. Nitrogen fixation in the gut of termites is mediated by bacteria and these bacteria appear to play an important role in the overall nitrogen metabolism by recycling uric acid nitrogen. Termite possess efficient means to acquire and or to conserve nitrogen with the help of their gut microflora. Nitrogen fixing bacteria or their metabolic products may serve directly as a nitrogen source for the termites. The termites obtain all their nitrogen from ingested wood, and merely obtain growth factors from the nitrogen fixing bacteria or from protozoa which might require nitrogen fixing bacteria. Results showed that they used wide range of substrates and pH for their polysaccharide production and N_2 fixing activity. The intestinal tract of termites because of the wide C:N ratio favour the fixation of gaseous nitrogen. Generally, the nitrogen requirements of termites are low and the average nitrogen content of the termites was high due to the recycling of their uric acid and gut tissues.

The salivary gland possess N_2 fixing aerobes whereas hind gut contains N_2 fixing anaerobes viz., *Klebsiella* and *Clostridium* sp. Both aerobic and anaerobic N_2 fixers helped in N_2 fixation and cellulolytic activity of the termites. *Clostridium* is generally present in wide range of niches viz., soil, rumen and sewage sludge (Hungate, 1950), and in the gut of cockroaches (Cruden and Markovetz, 1981) and Czolij *et al.* (1985).

In the present study N_2 fixing facultative anaerobes viz., *Klebsiella* and *Clostridium* were found to be maximum in worker termites compared with soldier and queen. Hino and Wilson (1958) also reported the nitrogen fixation by facultative Bacilli. Breznak (1973) reported that all castes of termites reduced acetylene (C_2H_2) to ethylene (C_2H_4), but soldiers possessed the lowest activity. This was because the soldiers did not

feed on wood as do workers; instead, they periodically derive stomodaeal and proctodaeal nutrients from colony mates.

5. 2. 3. Cellulolytic bacteria in the gut of termites

Microorganisms that digest cellulose are abundant in nature. They include aerobic, anaerobic bacteria, fungi and actinomycetes. Besides the ruminant organisms, the termites which subsist on a diet rich in cellulose have been found to harbour cellulose digesting microorganisms. The microbes present in the digestive tract of the animals exhibit a symbiotic relation with the host in many of its functions. Besides enzymes secreted by the insects, the gut microflora also possess enormous potential in aiding digestion of ingested feed. The microorganisms were seen throughout the digestive tract or in some specialized organs such as the paunch of termites and the proctadeal dilation in *Oryctes* larvae.

The present study also stated that the cellulolytic population was observed maximum in the hindgut (21.0 $x10^4$ ml^{-1}) followed by midgut (16.3 $x10^4$ ml^{-1}) and foregut (14.0 x 10^4 ml^{-1}).The population of cellulolysers in the hindgut and their metabolic products showed that these anaerobes play a key role in the digestion of cellulose. A number of cellulose solubilizing bacteria was reported from the gut of millipedes (Anderson and Bignell,1980), Cockroach (Cruden and Markovetz, 1979) and from wood eating termite gut (Paul *et al.,* 1986). Hethener *et al.* (1992) isolated anaerobic cellulolytic *Clostridium termitidis* from *N.lujae* which had optimum temperature and pH of 37°C and 7.5 respectively.

5. 2. 4. Methanogenic bacteria in the gut of termites

Bacterial methanogenesis in ubiquitous process in most anaerobic environments. The association of this event with anaerobic decomposition of organic matter in microbial habitats such as rumen and intestinal, tract of animals, sewage sludge disgester, muds of various aquatic habitats etc., was well established (Zeikus, 1977). Thus, gas production commonly observed in nature was mainly due to the growth of methanogens on specific energy sources that are formed as a result of microbial decomposition of organic matter. The anaerobic environment of the gut was supported by the presence of methanogens. Most of the organisms are beneficial for the host and are distributed from oesophagus to anus. The methanogenic bacteria share physiological and biochemical characters such as ability to anaerobically oxidize hydrogen and reduce carbon di oxide to methane.

In the present study, all the three gut regions recorded the presence of both aceticlastic and hydrogenotrophic methanogens. The microscopic observation of the hind gut contents also confirmed the presence of fluorescent rods, filamentous bacteria, cocci in *sarcina* pockets.Methanogen was found to be maximum in the workers hindgut (28.0 x 10^4 ml^{-1}) followed by midgut (15.6 x 10^4 ml^{-1}) and foregut (10.3 x 10^4 ml^{-1}). The hindgut bacteria was responsible for the maintenance of anaerobic conditions in the hind gut and the foreign bacteria cannot remain permanently in the hindgut of termites possessing a normal microbiota. Gijzen *et al.* (1991) reported that in cockroaches, methanogen *viz.*, *Methanobrevibacter* was present in association with protozoa. Sara Parwin Banu and Ramasamy (1996) isolated methanogens viz., *Methanosaeta, Methanobacterium* and *Methanosarcina* from the gut of mango stem borer. Shinzato *et al.* (1999) isolated *Methanobrevibacter* and related symbionts from the hindgut of *Reticulitermes speratus.* Tholen and Brune (1999) reported that both acetogenesis and methanogenesis occur in the hindgut region of *Cubitermes sp.*

5.3. Characterization of Methanogenic bacteria

The methanogenic bacteria comprise a highly specialized *Methanobacterium* and *Methanosarcina* sp which were identified by their morphology and fluorescent property (Schauer and Ferry,1980). The morphology of the *Methanobacterium* varied with growth on formate and H_2:CO_2 and also depend upon the phase of the growth. Individual cells of *Methanobacterium* were rod in shape with diameter ranging from 1-2 µm whereas *Methanosarcina* were coccal shaped cells arranged in pockets and aggregates, in masses of a few to several units. Colonies were circular with entire margin and size ranging from 0.1 to 1 mm. They exhibited fluorescence under UV light and the color varied from cream to tan.

Methanogens metabolize only restricted range of substrates and are poorly characterized with respect to other metabolic, biochemical and molecular properties. All methanogens share a common energy metabolism that involves H_2/CO_2 reduction (Wolfe, 1971). Some species can utilize CO, formate, acetate, methanol as methanogenic substrates (Zeikus, 1977).

In the present study, the isolated methanogens were distinct from other methanogenic bacteria in respect of pH, temperature and substrate utilization pattern. Maximum growth of the isolated *Methanosarcina* was observed with the substrate acetate

at 100 mM concentration Acetic acid is the principal organic acid present in the intestinal contents of the termite *R. flavipes*. Odelson and Breznak (1985) also reported that the isolated methanogens utilized substrates *viz.*, acetate, formate and methanol. Zeikus *et al* (1975) reported that *Methanosarcina* utilized acetate as a substrate for methanogenesis. Acetic acid is the principal organic acid resulting from rumen fermentation that is absorbed by the ruminant and oxidized and used for biosynthesis. Weimer and Zeikus (1978) reported that in *M.* barkeri, yields of both methane and cell material increased as methanol concentration increased from 0 – 200 mM.

The isolated methanogenic population *viz.*, *Methanobacterium* had an optimum temperature ranged from 30 - 37°C and pH about 7.0 - 7.5 while for sarcina, the optimum pH was found to be 7.0 and the doubling time was 24 h. Sowers *et al.* (1984) reported that H_2 : CO_2 utilizing acetogenic bacteria grow optimally at pH 7.2 and little or no growth occurred at pH 6.2 or 8.1. The cells grew within the temperature range of 19-37°C with an optimum of 30°C but no growth occurred at 40 or 45°C. Doubling time of cells at 30°C with substrate H_2:CO_2 was 7.8 h whereas in methanol / CO_2, the doubling time was 7.0 h. Odelson and Breznak (1984) reported endogenous methanogens associated with the protozoa *Trichomitopsis* 6057 for methanogenesis, which utilized the substrates, H_2 - CO_2 and acetate for its growth and cellulose fermentation.

Leadbetter and Breznak (1996) concluded that *Methanobrevibacte. cuticularis* and *Methanobrevibacter curvatus* from *R. flavipes* grow well at an optimum temperature between 30 - 37 °C. The substrates used for their growth were formate and H_2 : CO_2. and their doubling time was 40 h.

In the present investigation, the pH of the gut ranged from 7 - 7.5. All the facultative anaerobes viz., cellulolytic *(Clostridium)*, N_2 fixing (*Klebsiella*) and methanogenic organisms (*Methanobacterium* and *Methanosarcina)* isolated from different gut regions of the termites grow very well at pH 7 - 7.5. Bignell and Anderson (1980) reported that the termite (*N. exitiosus*) hind gut pH was 7 - 7.5. The intestinal cellulose breakdown include acetic, propionic acid and hydrogen inferred that cellulolysis take place in the absence of oxygen. In the gut, oxygen swallowed with the food is diffused from blood vessels are rapidly removed and hence many intestinal bacteria are adapted only to low oxygen levels (Holland *et al.*, 1987). Further they reported that pH and flow rate determined the microflora in the gut and anaerobic microflora developed to

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a level of 10^{12} ml^{-1}. Eutick. (1976) reported that the pH of the gut of *C. lacteus* and *N. exitiosus* ranged from 6.5- 7.5. Cruden and Markovetz (1981) stated that the pH of the gut of cockroach was between 6.5 - 7.2. pH is usually considered as an ideal one for the growth of wide range of microorganisms and indicates that any acidic metabolites excreted by the microorganisms are effectively buffered or removed by the termites, (Mannessman *et al.*, 1972). The pH of paunch,colon and rectum of *N. exitiosus* showed that these regions are alkaline. Most of the bacteria isolated are facultative organisms and they act as an oxygen scavenger, thus maintaining the E'o and the oxygen pressure at values suitable for the growth of protozoa and spirochaetes. Brune *et al.* (1995) reported that the termite gut microflora act as an oxygen sink and maintain their gut at pH 7.0 Leadbetter and Breznak (1996) concluded that *M. cuticularis* and *M. curvatus* from *R. flavipes* grow well at pH 7.1 - 7.7.

Methanogenic bacteria contains a number of unique coenzyme and related compounds. Coenzyme F_{420}, a deazaflavin analog plays a key role in methanogenic bacteria as an electron carrier in both anabolic and catabolic redox reactions (Jones and Stadtman,1980).

The fluorescent on exposure to UV light is a characteristics due to the presence of the coenzyme F_{420} during active metabolism and 80 per cent of F_{420} exists in the oxidized form (Doddema and Vogels, 1978) and they further reported that the presence of these fluorescent coenzymes F_{420} and F_{342} are specific to methanogens. Methanogens generally contain a large amount of unique fluorescent cofactors F_{420} (a deazaflavin) and F_{350} (a pterin derivative) making their cells autoflouresce brightly blue green or blue when illuminated near 420 nm and 350nm. Ferry and Wolfe (1977) isolated F_{420} from *M.hungatii* and it was similar to coenzyme F_{420} from *M.rumunantium.* Leadbetter and Breznak (1996) observed epiflourescence of methanogens due to F_{420}. F_{420} fluorescent cells were quite scarce in contents expressed from hind gut. However an abundance of F_{420} fluorescent cells were associated with the hindgut epithelium, either attached to the cuticle surface directly or mixed among other prokaryotes or attached as epibionts to larger (upto 1.5 μm in diameter) filamentous prokaryotes. In the present study, the F_{420} isolated from *Methanosarcina* under different periods of incubation showed the retention time varied from 1.234 to 1.782 min.

5.4. Effect of Nickel concentration on the growth of *Methanosarcina*

Methanogenic bacteria have been shown to require nickel for growth and hydrogenase enzyme activity. Methanogenic bacteria contain a number of unique coenzymes and related compounds depend upon the nickel content for their enzymic activity.

In the present investigation the nickel requrement for the isolates *Methanosarcina* was 1.00 μM for their methanogenesis. Eirich *et al.* (1979) concluded that *Methanobacterium* and *Methanosarcina* contained a low levels of F_{420} i.e. less than 20 mg / kg of cells, whereas other methanogens tested contained high levels (100 - 400 mg / kg of cells). They also reported that the Ni requirement to the culture was met out with extraneous addition of Ni in the form of Nickel chloride with a concentration of 0.25 μM.

5.5. Digestive enzymes of the gut

Cellulose digestion by termites with a combined action of various enzymes in the salivary gland indicates that saliva is important for both cellulose and starch digestion. Cellulose are digested anaerobically to form a range of organic acids, some of which are absorbed by the host. To know the importance and the potentiality of anaerobic bacteria in cellulose degradation of the feed ingested, the cellulase components were studied further. This study inferred the release of endoglucanase (EC 3.2.1.4) and exoglucanase (EC.3.2.1.91) by the different gut regions and showed that invariably the enzyme activity was observed in both cell bound and extra cellular proteins. Martin and Martin (1978) reported that the cellulase of the salivary gland and the mid gut were more active with CMC which is more in soluble state than with crystalline cellulose.

The present study showed that the cellulase complex was distributed in all the three regions of the gut. Salivary gland and hind gut recorded the maximum cellulase activity in CMC as substrate compared to other substrates such as cellobiose and cellulose. The activity of cellulase in the fore gut was 2 - 3% and in salivary gland was 45%, in mid gut it was 2.52%. whereas in hind gut, it was 50%. The cellulase activity in the salivary gland shown to produce little glucose from crystalline cellulose, whereas hind gut enzyme readily produces glucose. Salivary gland cellulase is an endoglucanase producing cellodextrins by random cleavage of cellulose and the hindgut (protozoal) enzyme is an exoglucanase cleaving glucose molecules from the cellulose. Hindgut

cellulase produced glucose at a rate of 100-140 µg/h, whereas the cellulase from salivary gland produced 0-5 µg/h. Cellulase activity was higher in hindgut due to protozoa. Cellulase activity was higher with CMC than with crystalline cellulose. Yamaoka and Nagatani (1975) also reported that cellulase activity of the salivary gland and mid gut was higher with the substrate CMC than with crystalline cellulose and cellobiose. Sutherland (1933) reported that higher cellulase activity in the part of the hind gut was due to protozoa in *M.darwiniensis.*

In the present study also amylase (EC.3.2.1.1), maltase (EC.3.2.1.20) and invertase (EC.3.2.1.26) activities were assayed. The result showed that the salivary gland exhibited high amylase activity (37.8 %) followed by mid gut (34.9 %). Fore gut and hind gut showed less amylase activity ranging from 8-18.4 %. Hill (1942) evidenced that higher amylase activity in the salivary gland and mid gut indicated the capability of metabolizing starch by termites while fed with flour. Noirot (1969) detected an amylase in the salivary gland and the midgut of *Kalotermes visintin* and *Kalotermes flavicollis* (Zhuzhkor and Korovkina,1972), the fore gut of *Zootermopsis* (Hungate, 1938).

Almost all maltase activity was found in the mid gut (58.3%) and salivary gland (23.9 %) while less activity was observed in the fore gut and hind gut (5-7 %). Most of the maltase activity was detected in the mid gut. This showed that starch is completely hydrolysed before it reach the hind gut and since the products are soluble to the termites to produce their own amylases (McEwen *et al.*, 1980). Invertase activity in salivary gland was 25.2 %, 10 % in fore gut, 61 % in mid gut and 5 % in the hind gut. Veivers (1982) reported that invertase activity was found maximum in the mid gut followed by fore gut and hind gut. Cellulase activity was found higher in mid gut (42.06 %) followed by 38.0% in salivary gland, 15.4 % in hind gut and 4. 3% in fore gut. Veivers *et al.* (1981) reported that in *M. darwiniensis*, the salivary gland exhibited a cellulolytic activity of 37%, mid gut (37%) and hind gut (20%). Having observed the presence of cellulase components, an attempts were made to detect the intermediary products released by the action of cellulase. The environmental conditions are extremely important regulators for the physiological activities of the microorganisms in the natural ecosystem.

5.6. Methane emission

Termites play an important role in global methane cycle. Methane is a minor component of the microbial carbon metabolism in the hind gut of termite digestive

system. In the hind gut ecosystem, an acetogenic reactions appear to be the major electron sink whereas methanogenic reactions consume only a small fraction of the available H_2 and CO_2. Hind gut contents have methanogenic population which enhanced CH_4 production by termites which contributes to global warming

Methanogenic bacteria utilize H_2 as an electron donor for methanoegenesis and growth. Formate is an electron donor for most of the *Methanobacterium* strain (Schauer and Ferry 1980). The present investigation revealed that high methane production was observed at the temperature of 30 ± 2°C. The culture enriched with acetate produced more methane compared to other substrates. The culture enriched with acetate and incubated at temperature 37°C showed more methane production followed by methanol, mono and trimethylamine (Breznak and Switcher 1976). Joseph winter and Wolfe (1979) reported that *Methanosarcina* grew and converted acetate to CO_2 and CH_4 after an adaptation period of 20 days. Growth and metabolism were rapid with gas production being comparable to that of cells grown on H_2:CO_2. In addition to H_2 using methanogens, termites harbour homoacetogenic bacteria (homoacetogens) that use H_2 and CO_2 to synthesize acetate. Weimer and Zeikus (1978) reported that methane production in *Methanosarcina* was decreased in the order of methanol / >methylamine.

The protein profile of the isolated methanogens were studied by SDS - PAGE electrophoresis method. The results clearly showed that among the three methanogenic isolates, the genus *Methanosarcina* showed low molecular weight (43 KDa) of whole cell bound proteins compared with *Methanobacterium* (14.3 KDa).

5.7. *In situ* emission of methane by different termites

Termites produced more methane, carbon dioxide and molecular hydrogen (Collins and Wood, 1986). Termites are the one of contributors of methane to the atmosphere. The present study showed that higher production of methane was observed in queen of higher termites (0.67μm g^{-1} h^{-1}) whereas the lower termites of queen recorded 0.32 μmg^{-1} h^{-1}. Zimmerman *et al.* (1983) stated that methane production was less in higher termites of fungus feeding termites *R. flavipes*, because of the metabolism of their fungal combs.

The metabolism of fungal combs resulted in higher CO_2 production rates for *in situ* colonies. In the present investigation, CO_2 emission in higher termites of queen showed 0.30 μm g^{-1} h^{-1} compared with queen of lower termites which showed 0.14 μm

g^{-1} h^{-1}. Collete and Etievant (1980) concluded that methane emission from the mid gut of cockroach was 26.0 to 97.5 nm of CH_4 when it was fed with saw dust, whereas when the insect was fed with cellulose, CH_4 emission was on an average of 34 and 38.nm h^{-1} g^{-1}. Brauman *et al.* (1992) reported that acetogens from hydrogen and carbon dioxide evolved more methane in fungus and soil feeding termites than wood and grass feeding termites. The rate of CO_2 reduction to acetate by gut contents from wood and grass feeding termites with or without exogenously supplied H_2 were greater than those of fungus growing or soil feeding termites. The evolution of methane by different feeding guilds in termite is varied based on the activity of their gut microbiota. They lack significant levels of bacterial acetogenesis from H_2 + CO_2 and are potentially more important sources of CH_4 emission. The CH_4 emission results from the reduction of CO_2 to CH_4 using molecular H_2 by methanogenic archaebacteria. Methanogens are minor hydrogen consumers in termite hind gut reported by Odelson and Breznak (1983).

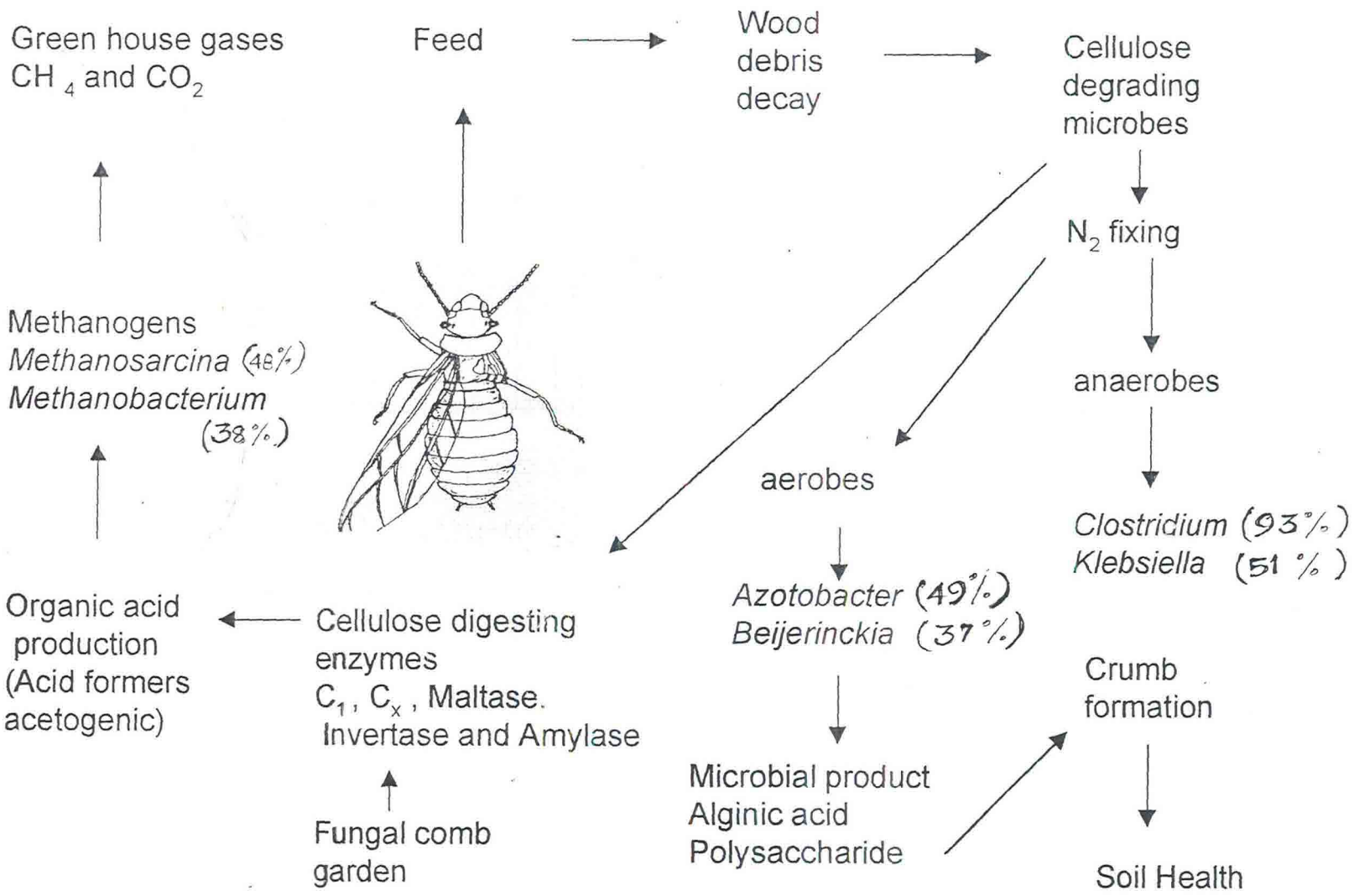
5. 8 Microbial products involved in stable soil aggregates

Most termites use soil together with saliva and faeces to construct their nests. Saliva is also used as a cementing medium for nest and gallery construction. Pomeroy (1982) reported that the nutrient status of the mound was usually better than that of the surrounding top soils. In our present investigation the polysaccharides isolated from the bacterial isolates viz., *Azotobacter* and *Beijerinckia* were found efficient in stable soil aggregate formation. Finch *et al.* (1971) reported that appreciable amount of soil polysaccharides were of microbial origin, and these microbial materials showed high degree of resistance to decomposition. Cooper and Stacay (1948) reported that the polysaccharides of *A. chroococcum* consists of 87 % glucose and 3 % uronic acid. In our study, *Azotobacter* and *Beijerinckia* are known to produce nearly 60 – 80 % of their dry weight as polysaccharides. These extracellular polymeric materials of biological origin originate from products or biological excretions usually found in abundant in natural systems. Harris and Mitchell (1973) reported that the polysaccharide producing bacteria were usually negatively charged at pH values greater than 2 to 3.

5.9. Effect of artificial crumbs on plant growth

To test the effect of organic matter on the aggregate stability of the pure culture of *Azotobacter* and *Beijerinckia* for selected crop, an experiment was conducted. The present study showed that organic matter addition improved the stability of aggregates

Fig. 36. **Gut microflora of termites and associated emission of global warming gases**



and there existed some selectivity to the type of organic matter suited for each organism. Renney *et al.* (1974) reported that the soil aggregation was influenced by microbial level of fertility and kind of crop. Crumbs help in receiving and retaining more rain water and help in increased soil biological processes. The crumb formation increases the rootability of the crop and also improves the nutrient status of the soil. The maximum germination percentage (94.4 %) was observed in crumbs compared with control and ant hill. The results of the present study showed that the addition of *Azotobacter* is required by cow pea and red gram, whereas black gram require *Beijerinckia* for the positive results in acid lateritic soil.

The study clearly revealed that diversified microflora are present in the gut of worker termites. Cellulolytic microorganisms are responsible for the digestion of cellulose and the nitrogen requirements are met out by the presence of nitrogen fixing aerobic and anaerobic microorganisms present in their gut. The biopolymers excrete from the saliva of the termite also enhance the polysaccharide production which help in soil aggregate formation. Aggregate formation by the termite gut microbial products enhanced the vigour index of plants. The different cellulolytic enzymes produced in the gut of termites also play a vital role in the cellulose digestion. The intermediary products released during cellulose digestion augmented the methanogenic population which in turn increases the emission of methane and carbon di oxide, the major contributors of green house effect / global warming. Fig.36

SUMMARY

CHAPTER VI

SUMMARY

Termites of different castes were collected and maintained in laboratory. Samples were enumerated for microbial association with or without enrichment. For detailed assessment samples were enriched with different substrates for isolating total anaerobes, cellulolysers, acid formers and methanogens. Termites harbored a variety of anaerobic bacteria in all the regions of gut. The maximum total anaerobic population was at a level of 44.7 x 10^5 CFU ml^{-1} was observed in the foregut of worker termites compared with soldier and queen. Foregut recorded maximum number of total anaerobes and acid formers, whereas cellulolysers and methanogens were more in the midgut and hindgut region.

Polysaccharide producing bacteria *viz.*, *Azotobacter* and *Beijerinckia* sp were isolated from the salivary glands of termites. Polysaccharide produced by these two cultures was maximum in sucrose compared with other carbon sources. Artificial aggregation formed by these two cultures enhanced plant vigour index.

Facultative N_2 fixing anaerobes *viz.*, *Klebsiella* and *Clostridium* sp from the mid gut and hindgut were also isolated.These organisms aided in digestion of cellulose and also helped in N_2 fixation activity. Both the organisms showed positive nitrogenase activity and are not associated with denitrification

Cellulolysers were enumerated using different carbon sources. Results indicated indicated the maximum population of 14 x 10^4 was recorded in foregut, whereas in midgut (16.3 x 10^4) and hindgut (21.0 x 10^4) respectively was noticed.

Presence of endoglucanase, exoglucanase and cellulase enzyme was observed in the different regions of the termites. Maximum cellulase activity was observed in carboxymethylcellulose than the other substrates. Enzymes like amylase, invertase and maltase from the midgut and hindgut of termites were also observed in termites.

Microscopic observation of isolated culture showed the presence of fluorescing small rods and cocci in *sarcina* pockets in the midgut and hindgut. Three isolates of methanogenic bacteria were further purified and characterised. The rod shape isolates

(Methanobacterium) utilized formate, $H_2 : CO_2$ whereas sarcina utilized acetate, methanol for their growth. They produced more methane at the temperature of 30 ± 2°C and 37 °C, less methane production at the temperature 55°C and 10°C respectively. Fluorescing compoud F_{420} was detected from *Methanosarcina* which is a diagnostic tool for the identification of methanogens. Protein analysis of cell free extract in SDS-PAGE resulted in separation of the two protein molecules. The analysis revealed the presence of protein subunits with following molecular weights 14.3 and 43.0 kDa. The study indicated the contribution of the enzymes by the microorganisms helped in the digestion of the cellulosic feed by the termites and also the gut contents of methanogenic flora which emit methane and carbon dioxide to the atmosphere which leads to global warming.

In vitro culturing of termites and artificial feed showed the differential colonization of microbes

Methane oxidizers in hindgut can be tailored for reducing the methane emission.

Methane production is a mechanism for detoxification of acetate accumulation in the gut and to maintain CO_2 levels in termite garden.

Laboratory studies revealed the efficiency of N_2 fixation by slimy associated heterotrophs and their polysaccharides for soil binding.

Few of the methanogens had high molar ratio turnover of CH_4. These cultures in association with other heterotrophs improved the overall methane production per unit of carbon supply in mini digester. Pilot plant study will help to translate the microbial strains.

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APPENDIX

APPENDIX

Becking's medium

Sucrose	20.0
$K H_2 PO_4$	0.8
$K_2H PO_4$	0.2
$MgSO_4$	0.5
Sodium molybdate	0.005
FeCl	Trace
Agar	15
Dis. Water	1000 mg
pH	6.9

1. DILUTION OF THE SAMPLE

Dilution medium	-	
Sodium bicarbonate	-	1.0%
Resazurin	-	0.0001%

2. DILUTION BUFFER

Glass distilled water	-	500 ml
General salt solution	-	500 ml
Resazurin	-	1 ml
Cysteine hydrochloride	-	0.5%

3. MEDIUM FOR TOTAL ANEROBIC BACTERIA

Modified Hungate's medium (g/l)

Potassium dihydrogen phosphate	-	0.02
Dipotassium hydrogen phosphate	-	0.03
Magnesium sulphate	-	0.01
Calcium chloride	-	0.01
Ammonium sulphate	-	0.10
Sodium chloride	-	0.02
Cysteine HCL	-	0.02
Sodium bicarbonate	-	0.50
Resazurin	-	0.0001
Glucose	-	0.50
Maltose	-	0.25
Cellobiose	-	0.25

Bromothymol blue	-	0.005
Calcium carbonate	-	1.0
Agar	-	20.0

5. Medium for Cellulolytic bacteria (g/ l)

Potassium di hydrogen phosphate	0.02
Di potassium hydrogen phosphate	0.03
Magnrsium sulphate	0.01
Calcium chloride	0.01
Ammonium sulphate	0.10
Sodium chloride	0.10
Cysteine HCl	0.02
Sodium bicarbonate	0.50
Resazurin	0.0001
Cellulose powder	1.0
Agar	20.0
Vitamiñ solution	1.0ml
Trace elemnet solution	1.0ml
PH	7.0

HINO AND WILSON'S MEDIUM FOR MASSIPLICATION OF NITROGEN FIXING ANAEROBES (g/l)

Sucrose	-	20 g
$K_2H\ PO_4$	-	12.06
~~KH_2~~ PO_4	-	3.4
µg $SO_4\ TH_2O$	-	0.5
NaCl	-	0.01
$FeSO_4.TH_2O$	-	0.015
$Na_2Mo\ O_4.2H_20$	-	0.005

Hill **Medium** (g/l)

Sucrose	-	20
$Na_2\ H\ PO_4$	-	10.4
$KH_2\ PO_4$	-	3.4
Fe (III) Citrate	-	36
$Mg\ SO_4$	-	30
$CaCl_2\ 2H_20$	-	26
$Mn\ SO_4$	-	0.3
$Na_2\ Mo\ O4\ .\ 2H_20$	-	7.6

6.MEDIUM FOR METHANOGENIC BACTERIA (g/l)

NH_4 Cl	-	1
K_2HPO_4. $3H_2O$	-	0.4
Mg Cl_2. $6H_2O$	-	0.1
Trypticase/tryptone	-	2
Yeast extract	-	2
Calcium acetate	-	10
Resazurin	-	0.001
Agar	-	20
Dist.water	-	1000 ml

6. GROWTH MEDIUM FOR METHANOGENS

K_2HPO_4. $3H_2O$	-	0.31 g
KH_2PO_4	-	0.24 g
(NH4)2 SO4	-	0.24 g
$MgSO_4$. $7H_20$	-	0.10 g
NaCl	-	0.48 g
$CaCl_2$. $2H_2O$	-	6.40 mg
$FeSO_4$. 7H20	-	2.00 mg
NiCl. $6H_2O$	-	0.50 mg
NiCl. $6H_2O$	-	10 ml
Vitamin solution	-	10 ml **
Mineral solution	-	10 ml *
Resazurin	-	0.50 mg
Trypticase/tryptone	-	1.00 g
Yeast extract	-	1.00g
L-Cystine – HCl. H_2O	-	0.50 g
Na_2 S.$9H_2O$	-	0.50 g
$NaCH_3COO$	-	8.00 g
Na HCO_3	-	4.00 g
Distilled water	-	970 ml
pH	-	7.0

Acetate Buffer

0.2M Acetic acid	11.55g/l
0.2 M Sodium acetate	27.22g/l

For pH 5.2 (7.90 ml of sodium acetate and 2.10 ml of acetic acid was made up to 100 ml.)

McIlavine Buffer

0.2 M Di sodium Hydrogen phosphate	35.61 g/l
0.1M Citric acid	21.10 g/ l

For pH 7.0 10. 30 ml / l of Di sodium hydrogen phosphate and 19.70 ml of citric acid was made up to 100 ml.

APPENDIX II

Solution for Tris/Glycine SDS-polycrylamide gel Electrophoresis

10 per cent SDS ~~poly~~acrylamide gel electrophroesis separating gel

Water	-	15.8 ml
30 per cent Acrylamide mix	-	13.3 ml
1.5 M Tris (pH 8.8)	-	10.0 ml
10 per cent SDS	-	0.4 ml
10 per cent APS	-	0.4 ml
TEMED	-	0.016 ml

Stacking gel

Water	-	5.5 ml
30 per cent Acrylamide mix	-	1.3 ml
1.5 M Tris (pH 8.8)	-	1.0 ml
10 per cent SDS	-	0.08 ml
10 per cent APS	-	0.08 ml
TEMED	-	0.008 ml

Cell disruption buffer

The cell were suspended in cell disruption buffer containing

Glyceral	-	10 per cent
2-mercaptoethanol	-	5 per cent
Sodium dodecyl sulfar	-	5 per cent
Tris HCL (pH 6.8)	-	62 mM

Sample loading buffer for proteins

Dithiothreitol	-	0.3 g
SDS 10 per cent	-	4.0 ml
Stacking gel buffer	-	1.6 ml
Glycerol (87 per cent)	-	2.56 g
Bromophenol blue	-	0.005 g
Tris-HCl (0.08 M pH6.8)	-	14.4 ml

Mobil phase buffer co-enzymes separation

25 mM acetic acid

a.	Acetic acid	-	1.252 g
	Double distilled water	-	1000 ml
	PH (adjusted with KOH)	-	6.0
b.	Same buffer (a) containing 50 per cent methanol filter and degass before use.		

25 mM sodium formate

a.	Sodium formate	-	1.702
	Double distilled water	-	1000 ml
b.	Same buffer (a) containing 50 per cent methanol		

20 mM potassium phosphate buffer

a.	Potassium dihydrogen phosphate	-	2.839 g
	Double distilled water	-	1000 ml
b.	pH (adjusted to 5)	-	5.0
b.	Same buffer (a) containing 3 per cent Acetonitrile		

25 mM sodium formate

a.	A sodium formate	-	1.702 g
	Double distilled water	-	1000 ml
B	pH (adjusted with HCL)	-	3.0
b.	25 mM phosphate buffer containing 50 per cent methanol.		