STUDIES ON THE MICROBIAL PRODUCTION OF FUEL GASES FROM APPLE WASTE AND OTHER SUBSTRATES

A THESIS

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in

BIOCHEMISTRY

By

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Dedicated to my
Parents and Sisters

CANDIDATE'S DECLARATION

I hereby certify that the work which is being presented in the thesis entitled **"STUDIES ON THE MICROBIAL PRODUCTION OF FUEL GASES FROM APPLE WASTE AND OTHER SUBSTRATES"** in fulfilment of the requirement for the award of the Degree of Doctor of Philosophy and submitted in the Department of **Biosciences and Biotechnology** of the University is an authentic record of my own work carried out during a period from **December, 1989** to **August, 1992** under the supervision of **DR. C.B. SHARMA** and **DR. A.P. JOSHI.**

The matter presented in this thesis has not been submitted by me for the award of any other degree of this or any other University.

Signature of the Candidate
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This is to certify that the above statement made by the candidate is correct to the best of our knowledge.

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ABSTRACT

Hydrogen and methane evolving capacities of apple pomace, dumping wheat and vegetable wastes were monitored in both batch culture digestion and continuous daily batch fed culture digestion, in laboratory scale digesters (0.3 - 10 l capacity). Each kg (dry weight) of apple pomace and dumping wheat fed could generate 40 l and 36 l of H_2 followed by 140 l & 218 l of CH_4 respectively in separate stages. In the case of vegetable waste (raddish leaves, cauliflower leaves & stalk and rotten cabbage), subjected to methanogenesis directly, 320 l of biogas containing 68% of CH_4 was generated. Adaptation of methanogens to changing fruit and vegetable feed material was also observed. The direct biomethanation of apple pomace and dumping wheat increased the CH_4 yields, to 157 l and 280 l of CH_4 / kg of total solids fed respectively, whereas recycling of the unutilized solids in the latter case increased the CH_4 efficiency by 27.6%. The digestions led to the maximum reduction of Total solids (TS) by 76.4%, Organic solids (OS) by 80.2% and Chemical oxygen demand (COD) by 70%. Similar biodegradable properties were also seen in other crop grains like maize, rice, pure starch and poultry feed & cattle feed wheat qualities of damaged wheat grains. CH_4 content was found to be upto 80% in the total biogas evolved, whereas maximum H_2% was of the order of 60% in the total biogas-H generated.

The mixed microbial population of H_2 producers and pure isolated culture of *Bacillus licheniformis,* produced H_2 from glycolytic pathway intermediates, like glucose, glucose-6- phosphate and pyruvate, in approximately proportionate

volumes. The mixed and isolated pure culture actively fermented formate to H_2 with a conversion efficiency of 68-85%, thereby following the *Escherichia coli* pathway as one of the biochemical pathways for H_2 generation. The mixed population of H_2 producers showed a nitrogenase activity of 0.460 nmoles C_2H_4 / mg protein / h, when the cells were activated by preincubation for 18-24 h with 0.2% glucose supplementation. Significant inhibitory effect on expression of nitrogenase at O_2 levels of $>0.25\%$ was observed when O_2 sensitivity was monitored. The inhibitory effects of C_2H_2, CO, KNO_3 on nitrogenase and hydrogenase activities for H_2 production were measured. These revealed 63% of H_2 generation through nitrogenase.

The culture fluids were tested for fatty acid and alcohol contents. When mixed culture and pure culture of *B. licheniformis* was grown separately on glucose, dumping wheat and apple pomace, the fermentation products were acetic acid, propionic acid, butyric acid and valeric acid. The concentration of acetic acid produced has been the highest (222.46 mmoles /l) whereas the concentration of other acids has been 1/10 th as high. Maximum ethanol production of 41.75 mmoles / l has been observed in dumping wheat fermentation, whereas methanol production was found only in apple pomace fermentation.

pH changes were monitored during H_2 production. It was observed that during active H_2 producing stages the pH ranged between 4.6-4.8, whereas the pH dropped to 5.3 from an initial pH of 7.0 when H_2 production started.

SO_4^{2-} and Fe^{3+} in the concentration range of 1-10 mM and 50 mM respectively, in the culture medium increased the H_2 production by 15-30%. Ni^{2+} showed neutral effects in the concentration range of 0-200 nM tested.

Activities of the extracellular hydrolytic enzymes were measured during fermentation of dumping wheat and apple pomace on shake and stationary cultures. The maximum activities observed were, $3x\ 10^3$, 2.85×10^3, 3.2×10^2 & 1.8×10^1 units/l of hemicellulolytic, proteolytic, amylolytic and cellulolytic respectively.

Viable cells of H_2 producers (mixed culture and isolated pure cells of *B. licheniformis*) were immobilized on solid support of baked bricks and in calcium alginate beads and were tested for H_2 production using glucose as substrate. In batch culture biotransformations, 2.4 - 4 fold increase in H_2 yields was obtained on bricks support generating a maximum of 1.5 mole of H_2 / mole of glucose fed. On the other hand calcium alginate immobilized cells showed a maximum of 2 -fold increase in the yield, thereby evolving 0.75 moles of H_2 / mole of glucose employed. However, the daily batch fed continuous culture biotransformations of glucose into H_2 by these immobilized cells lead to only 39% improvement of yield. The H_2 evolving activity of the calcium alginate immobilized cells could be maintained over a period of one year of storage under sterile conditions.

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(Ashok Kumar)

CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

α	-	Alpha
β	-	Beta
APS	-	Apple pomace slurry
CDS	-	Cattle dung slurry
DWS	-	Dumping wheat slurry
CF	-	Cattle feed
PF	-	Poultry feed
FW	-	Fresh wheat
CRW	-	Cauliflower raddish waste
RC	-	Rotten cabbage
ARA	-	Acetylene reduction activity
g	-	Gram (s)
mg	-	Milligram (s)
h	-	Hour (s)
l	-	Litre (s)
cm	-	Centimeter (s)
cm^3	-	Cubic centimeter
mM/mmoles	-	Millimolar/ Millimoles
nM/nmoles	-	Nanomolar/ Nanomoles
VPM	-	Volumes per million
NTP	-	Normal, temperature & pressure
SD	-	Standard deviation
$\bar{X}$	-	Mean
μm	-	micrometer
TS	-	Total solids
OS	-	Organic solids
COD	-	Chemical oxygen demand
Kg	-	Kilogram (s)
rpm	-	Revolution per minute

M	-	Molar
N	-	Normal
ml	-	Millilitre (s)
μg	-	Microgram
V/V	-	Volume per volume
W/V	-	Weight per volume
%	-	Percent
°C	-	Degree celcius
min	-	Minute (s)
RT	-	Retention time
μ l	-	Microlitre
Biogas	-	$CH_4 + CO_2 + H_2S$
Biogas-H	-	$H_2 + CO_2 + H_2S$
VFA	-	Volatile fatty acids
pH	-	Hydrogen ion concentration
ID	-	Inner diameter
IA_{25}	-	Incubation assembly 25 ml capacity
IA_{50}	-	Incubation assembly 50 ml capacity
IA_{300}	-	Incubation assembly 300 ml capacity
mm	-	Millimetre (s)
m	-	Metre (s)
Sec	-	Second (s)
CMC	-	Carboxy methyl cellulose
NAD	-	Nicotinamide adenine dinucleotide
FDH	-	Formate dehydrogenase
NADH	-	Reduced NAD
DNSA	-	Dinitrosalicylic acid
pH_2	-	Partial pressure of hydrogen
μmol	-	Micromole
EDTA	-	Ethylene diamine tetraacetate
cum/m^3	-	Cubic metre
Wt	-	Weight

CHAPTER - I

INTRODUCTION

INTRODUCTION

Society is confronted today with dwindling sources of fossil fuels and chemical feed stocks and the proliferation of wastes generated by municipalities, agriculture and industries. The conversion of renewable resources (i.e., biomass) or wastes to chemicals and biofuels by microbial fermentation or enzymes presents a tremendous challenge for microbiologists in the present scientific scenario. Indeed this challenge has helped to stimulate biotechnology as a new scientific discipline, which aims to develop novel processes that are less energy intensive and more efficient than chemical synthetic processes and that are based on abundant renewable resources. It is also clear that the present pattern of energy use cannot possibly continue for long, considering the ever increasing demand of energy on a world scale. Reduced energy consumption is an extraordinarily difficult goal to achieve, especially when the needs of developing countries are considered (Pellizzi *et al.*,1987). On the other hand, fear of possible nuclear accident, the problem of waste disposal and the cost of energy generated by nuclear plants are some of the major problems associated with the nuclear option. Under these circumstances, utilization of renewable energy sources is essential if the world is to have the energy supply it needs. With the exception of tidal energy and geothermal energy, all other potential sources of energy (e.g., solar, wind, water & biomass-based energies, such as biofuel etc.) are derived either directly or indirectly from the sun. Of these renewable energy technologies, biofuel production is one of the most "advanced" in terms of years of use and number of units installed, and it has the potential to alleviate some of the more pressing problems observed in the developing countries.

Biofuel production includes both energy rich gases like hydrogen and methane and liquids like ethanol, butanol, acetone and acids. Hydrogen and methane, being gases, are easier to recover from an effluent stream than any liquid fuel. Fuel gas production is brought about microbially under anaerobic conditions. H_2 has very long been considered as the potential energy carrier. However, the high cost of its production and dependence on conventional resources makes its day-to-day applications unattractive. These limitations have led to the microbial production of H_2 some 50 years back. The production of H_2 by various microorganisms grown on carbohydrates represents a potential alternative energy source that needs to be examined carefully and its economical feasibility and commercialization assessed. While the literature indicates that considerable amount of biochemical and microbiological research has been conducted to elucidate the mechanism of H_2 production, almost no work has been done to optimize the production of H_2 for industrial applications (Zajic *et al.*,1979).

On the other hand, microbial methane production has a long history in anaerobic degradation. Almost any biological material under anaerobic conditions can be converted into methane by microorganisms. The production of methane by anaerobic fermentation has already proceeded successfully on an industrial scale. Today this technology is applied all over the world, though much of the early work on anaerobic digestion was done in developing countries like India and China. However, research work on fuel gas production has become quite popular after the energy crisis of 1973-1974, and has a promising future in terms of cooking fuel, lighting, engine fuel, the use of organic manure in agriculture, improved sanitation, waste treatment and

pollution control. There is however a continuing need for research in this area to make the technology applicable to a greater variety of renewables, to improve product yields, to increase rate of fuel production and to improve reliability.

Biowastes, a potential renewable energy source of different origins have associated with them a negative value due to disposal and pollution costs. Thus, waste utilization, rather than its treatment emphasizes upon shifting the process from reducing the potential for pollution to synthesis of useful products, like chemicals and fuels. Waste materials, which also qualify as biofuels, present a significant source of readily available biomass, and replacement of expensive treatment systems with revenue-producing bioconversion process is an additional financial impetus. Biomass representing a renewable energy feed stock and its amenability to conversion depends largely on the characteristic of the biomass and the process requirements for the conversion technology under consideration.

Disposal of waste materials is not a new problem in the present day affairs. Rapid urbanization all over the world has created a serious problem of solid waste disposal. Large vegetable and fruit markets in big cities, particularly in the developing countries, contribute to the accumulation of this waste which is disposed off by composting, spreading on the land or sometimes as animal feed. Collection, transport and dumping of waste during disposal is uneconomical. One way of solving this problem is to make use of such waste for the production of fuel gases which could be suitably utilized in the surrounding areas and the digested slurry used as organic manure. Research into such high solid anaerobic digestion first began in 1976 at Cornell University U.S.A. to investigate the anaerobic digestion of energy crops. In

1980, an opportunity arose to apply these concepts to apple pomace, a food processing waste of considerable environmental concern (Etheridge and Jewell,1988).

Apple pomace (*Malus pumila*) is the residual solid component when apples are crushed for juice, and is perhaps the most prevalent of fruit wastes and certainly of most concern in all major apple producing countries like India where production has reached over one million tonnes and the proportion devoted to juice production is constantly increasing. The use of pomace as an animal feed has been restricted due to its lower protein content and transportation costs, and because of the low pH and nitrogen values, it has no intrinsic value for land application. Its most viable use as a resource for anaerobic digestion has been evaluated a decade back and still holds a promising future. Approximately, 100 metric tons of apples will produce 75 metric tons of juice, and about 25 metric tons of apple pomace. A typical processing facility using 40,000 tons of apples per year produces 10,000 tons of pomace and leads to a significant environment problem (Etheridge and Jewell,1988). While most of the other apple producing countries like USA and Australia have already taken measures to combat this problem by anaerobic digestion for CH_4 production, in India, the problem is still a concern for the major fruit processing industries. The availability in large quantities at a single place and balanced chemical composition (moisture 66.5-78.2%, carbohydrates 9.5- 22%, protein 1.5-2.5%, pectin 1.03-1.82%, fat 0.82-1.43%, crude fibre 4.3-10.5% and ash 0.56-2.27%) make it a preferable resource for the fuel production (Mohsenin, 1970).

Similar high solid wastes originating from vegetable markets have been tested for potential energy sources. Besides,wastes like damaged wheat grains, can act

as a good replenishable resource of energy in major wheat producing countries. In India, although the exact magnitude of this waste is not known, an approximate estimate, places the damaged wheat grains of the dumping type at about 3-5 tons per godown in approximately 150 godowns. Today, this quality is dumped for land filling and costs money for its disposal.

Thus keeping in view the above aspects, the present piece of work has been carried out with the following aims and objectives.

A I. To determine the hydrogen and methane generating potential of apple pomace and dumping wheat grains under optimum conditions of temperature, pH and substrate concentrations.

II. Adaptation of varying plant materials from fruit and vegetable markets to methanogenic culture in a universal bioreactor.

III. 100-fold upscaled pilot plant experimentation of apple pomace for fuel gas (H_2 and CH_4) production.

IV. Optimization of conditions for hydrogen producing mixed microbial culture.

V. Potential of other pure substrates like glucose, starch, cellulose, rice, maize and wheat grains for H_2 and CH_4 production.

B I. Metabolic studies during the microbial production of H_2, involving the glycolytic and *E. coli* pathway.

II. Elucidation of the role of nitrogenase, hydrogenase and formate hydrogen-lyase in H_2 production.

III. The role of various fatty acids and alcohols during fermentation of different substrates to H_2.

IV. Correlation between pH and maximum rate of H_2 production.

V. Effect of kinetic parameters on the H_2 production.

C I. Studies on whole cell bacterial immobilization for H_2 yield improvement.

These studies were conducted with an emphasis on the improvement of the techniques and optimization of the conditions for the increased and continuous production of H_2, and to understand the mechanism of microbial H_2 production to make the process applicable for the degradation of biological waste materials on large scale. To study the microbial methane production capacity of different plant materials and its application to recover the unutilized energy of the biological material after H_2 extraction has been our other major objective.

CHAPTER - II

REVIEW OF LITERATURE

REVIEW OF LITERATURE

In nature, whereever organic material is degraded microbially, under anaerobic conditions and in the absence of sulfate and nitrate, methane is produced. The discovery of origin of methane has been credited to an Italian physicist A.Volta who in 1776, found the presence of a combustible gas over marshes and ponds (Mudrack *et al.*, 1987). The well known "Will o' the wisps" in marshes and bogs arise through the combustion of methane (marsh gas), although even today it is still not clear how the ignition of this gas comes about. Bechamp is credited with giving the first indication in 1868 that methane was formed by a microbiological process (Winfrey, 1984). Barker (1956) reported that these observations were later confirmed in 1886 by the findings of Hoppe & Seyler.

The microbial formation of methane takes place not only in inland-water and in sea water sediments, in tundras, and flodded rice fields, but also in the rumen of ruminant animals (Klass, 1984). Investigations have shown that in the stomach of a cow about 8-10% of the fodder is converted in to 100-200 l of methane per day (Hungate, 1966 and Wolfe, 1971). It is estimated that on the Earth about 1000 x 10^9 m^3 of methane is formed by microorganisms and passed into the atmosphere each year (Ehhalt, 1976).

A 2.1 BIOLOGY OF THE FORMATION OF METHANE :

It was assumed for a long time that during the anaerobic conversion of organic substances into methane and carbon dioxide only two different groups of bacteria are involved (Barker, 1956). In the last few years it has been possible to show that

there are at least three different groups (Bryant, 1979), which include (a) the hydrolytic bacteria, which degrade various organic compounds such as carbohydrates, proteins and lipids into less complex soluble organic compounds such as fatty acids, alcohols, hydrogen and carbon dioxide (b) the acetogenic bacteria , which convert these alcohols, acids etc. into acetic acid, hydrogen and carbon dioxide, and (c) the methanogenic bacteria which convert hydrogen / carbon dioxide and acetate into methane and carbon dioxide (Fig. 2.1)

2.1.1 HYDROLYTIC AND ACID FORMING (FERMENTATIVE) BACTERIA :

This group of bacteria is very heterogenous . It includes obligate anaerobic strains such as, *Bacteroides, Clostridia, Bifidobacteria* besides facultative anaerobic *Enterobacteria* and *Streptococci* (Hobson *et al.*, 1974; Crowther and Harkness, 1975). These organisms which occur in digestion sludge in numbers of about 10^6 to 10^8 cell/ ml, first hydrolyze the macromolecular compounds such as polysaccharides, proteins and fats with the help of extracellular enzymes (hydrolases) (Mudrack *et al.*,1987). While most biopolymers can be split relatively easily and rapidly, lignocellulose containing materials are degraded only slowly and incompletely, since lignin, which cannot be cleaved anaerobically, has a very pronounced inhibitory effect (Hackett *et al.*, 1977). With the exception of the fatty acids, the hydrolysis products are then fermented by the well known metabolic pathways predominantly to hydrogen, carbon dioxide acetic acid, propionic acid, butyric acid, lactic acid , valeric acid, ethanol, ammonia and hydrogen sulfide (Thauer *et al.*, 1977; Gottschalk, 1979). The influence of the partial pressure of hydrogen on the metabolism of these products has been shown by Thauer *et al.* (1977) and Bryant (1979).

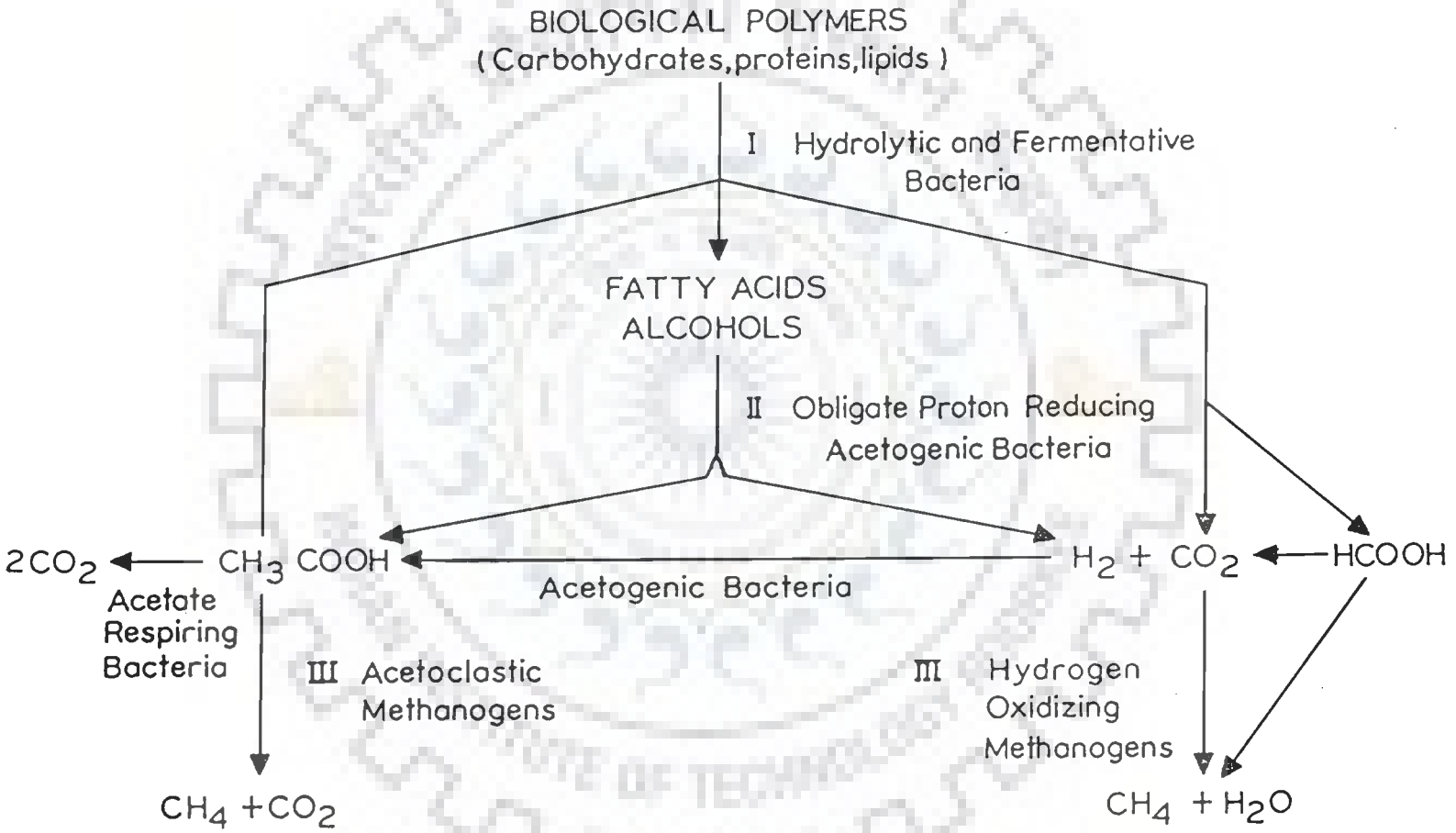

Fig. 2.1: Biochemical reactions for the anaerobic decompostion of organic matter (Winfrey, 1984).

2.1.2 ACETIC ACID AND HYDROGEN FORMING (ACETOGENIC) BACTERIA:

It was believed for a long time that the methane bacteria are capable of utilizing the fermentation products of the first group of bacteria (Bryant, 1979). They were believed to degrade propionic acid and butyric acid and the longer chain fatty acids etc., to acetic acid, hydrogen and carbon dioxide. The findings of Bryant *et al.* (1967) have restricted the knowledge of this group to only a few strains, since these organisms can grow in pure culture only at a very low partial pressure of hydrogen (pH_2). In an extensive review it has been proposed that an intimate contact between the cells of the acitogenic and methanogenic bacteria is necessary to meet the requirement of low pH_2 (Mudrack *et al.*, 1987). The concept has mainly been substantiated by the extensive work of Bryant and his Co-workers (Mc Inerney *et al.*, 1979 & Boone and Bryant, 1980).

2.1.3 METHANE FORMING (METHANOGENIC) BACTERIA :

The third group in turn consists of two physiologically different groups of methane-forming bacteria, one converting hydrogen and carbon dioxide to methane, and the other forming methane from decarboxylation of acetate. Extensive reviews make a mention of more than 20 morphologically diverse methane forming bacterial species, isolated in pure cultures (Wolfe, 1971; Zeikus, 1977 and Balch *et al.,* 1979). Although about 70% of the methane that is formed in nature arises from acetic acid, so far only a few methane bacteria assimilating acetic acid are known (Smith *et al.,* 1980).

This group of bacteria are among the most oxygen-sensitive organisms known today and some are very rapidly killed by oxygen (Paynter and Hungate, 1968).

While the overall conversion of complex substrates to methane requires the synergistic action of all the three groups, the syntrophic association of the hydrogen producers of the second group and the hydrogen oxidisers in the third group is particularly unique leading to the interspecies hydrogen transfer (Mudrack *et al.*,1987). These recent findings emphasize the important symbiotic relationship that must exist in anaerobic treatment. It would appear that our practical consideration of the process should somehow incorporate the three-phase concept. However, the practical consequences of these findings have yet to be demonstrated.

The exact mechanism of methane formation has not been determined, although it is clearly a unique pathway involving a variety of co-enzymes that are absent in other organisms (Balch *et al.*, 1979; Wolfe, 1979; Vogels *et al.*, 1982). Barker (1956) proposed a unifying mechanism to account for methane formation from H_2 plus CO_2, formate, acetate and methanol. He proposed that CO_2 was converted to methane via four 2-electron steps, each mediated by one or more unidentified C-1 carriers. Acetate and methanol entered the scheme at the terminal step and shared the same methyl carrier. At present, methanogenesis is thought to follow the same general scheme proposed by Barker (Wolfe and Higgins, 1979; Vogels *et al.*, 1982).

All the old literature suggested that this unique biological group of microorganisms whether in the pure form or mixed populations from rumen or sewage has been useful in the world whether it is with respect to energy generation or

pollution control. Many applications of anaerobic digestion of organic material by this group of microorganisms are now in use or are being considered. These have different aims, although it is often possible to combine several of these :

i) Sewage purification, including removal of faecal pathogens;

ii) Concentration of sewage sludge before an oxidative biological treatment process (activated sludge);

iii) Disposal of offensive odorous excrement from pig and poultry units;

iv) Production of useful combustible biogas;

v) Production of rich odourless fertilizer;

vi) Disposal of large quantities of cattle excrement;

vii) Disposal of crop residues (straw, palm-oil waste etc.);

viii) Disposal of waste from food processing plants (dairy, brewing, fruit and vegetable processing, sugar beet etc.);

ix) Disposal of waste from wool, textile industry;

x) Disposal of domestic refuse (the organic part), and

xi) Conversion of agricultural crops to biogas fuel;

The use of these processes can not be justified on the sole basis of being an alternative energy source, but these processes can be an economically feasible waste management practice also considering increasingly serious environmental problems (Sharma and Pellizzi, 1991). According to the Royal Commission, 1985, a waste is best defined by a person who wishes to dispose it off.

An extensive review by Golueke and Mc Gauhey (1976), have categorised the wastes as:

a. municipal wastes;

b. industrial wastes;

c. demolition debris;

d. animal manures;

e. agricultural wastes;

f. mining wastes;

g. litters, and

h. junk and automobiles.

The anaerobic digestion process with mixed cultures of bacteria for methane generation is used to treat most of these biological wastes besides the crops grown specifically for it (Archer and Thompson, 1987).

The detailed description of each of the processes mentioned above is beyond the scope of this work. Here we have been interested in the anaerobic digestion of high solid biological wastes originating from the agro-industries and large vegetable and fruit markets (Table 2.1).

The economic success of this process is often dependent on factors other than the value of methane produced; for example, saving the effluent charges or other constraints on disposal of untreated wastes are common factors which determine the feasibility of the process. Thorough studies on the subject have clearly indicated the non viability of this process, if it solely depends on the value of methane (Hawkes, 1985; James and Campbell, 1985).

Anaerobic digestion of liquid effluents, although rendered economically attractive because of savings in effluent charges, has suffered from a reputation of unreliability and has therefore received limited application with respect to fuel production (Archer and Thompson, 1987). But the process is still extensively used with a primary motive

TABLE 2.1: FRUIT, VEGETABLE AND AGRICULTURAL WASTES USED FOR BIOMETHANATION.

WASTE MATERIAL	REFERENCE
FRUIT WASTE:	
1. Apple	Wase & Gordon (1982);Knol *et al.*, (1978); Lane (1984 b).
2. Apple pomace	Lane, (1984 a, b); Etheridge and Jewell (1988).
3. Apple press cake	Lane (1984 b).
4. Apricot fibre	Lane (1984 a).
5. Pineapple	Lane (1984 b).
6. Orange peel	Lane (1984 a).
7. Mango peel	Raju *et al.* (1991).
8. Spinach & Straw berries	Knol *et al.* (1978).
9. Citrus pellets/ peach/pear waste	Lane (1984 a).
VEGETABLE WASTE :	
10. Asparagus	Lane (1984 b); Knol *et al.* (1978).
11. Carrot/Carrot peel/ French bean	Knol *et al.* (1978); Verrier *et al.* (1987).
12. Onion waste	Lubberding *et al.* (1988).
13. Red beet peel	Verrier *et al.* (1987).
14. Sugar beet pulp	Stoppok & Buccholz (1985); Lescure & Bourlet (1984); Labat & Garcia (1986).
15. Tomatoes	Koster (1984); Hills & Roberts (1982).

TABLE 2.1: Continued

WASTE MATERIAL	REFERENCE
16. Plant Materials (leaves of cauliflower, cabbage, tops of sugar beet, mustard, etc.)	Zubr (1986).
17. Mixed vegetable (Rotten vegetables, fruit skins, potatoes, onions etc.)	Ranade *et al.* (1987).
18. Green peas	Knol *et al.* (1978).
19. Organic waste of vegetable market	Mata-Alvarez & Llabres (1992).
AGRICULTURAL WASTES :	
20. Corn cobs	Lane (1984 b).
21. Corn stover	Foutch & Gaddy (1981); Sun *et al.* (1987).
22. Molasses	Hashimoto (1981).
23. Rice straw	Koster (1984); Sun *et al.* (1987); El-Shinnawi *et al.* (1989).
24. Sorghum	Jerger *et al.* (1987).
25. Wheat straw	Dar & Tandon (1987).
26. Maize & cotton stalks	El-Shinnawi *et al.* (1989).
27. Spent coffee grounds/ coffee pulp	Lane (1983); Calzada *et al.*(1981).
28. Honeydew & Peach	Hills & Roberts (1982).

TABLE 2.1: Continued

WASTE MATERIAL	REFERENCE
MISCELLANEOUS BIOLOGICAL WASTES :	
29. Water Hyacinth	Kalle *et al.*(1985);El-Shinnawi *et al.* (1989); Patel *et al.* (1992).
30. *Mirabilis* leaves	Sharma *et al.* (1987).
31. Apple & peach leaf litter	Dar & Tandon (1987).
32. Lantana residue	Dar & Tandon (1987).
33. *Pistia stratiotes*	Nipaney & Panholzer (1987).

of waste water treatment to control the environmental problem (Lebrato *et al.*, 1990). The methane generated is bonus in such cases. Such a process has also been described for the anaerobic digestion of molasses waste water (Hilton and Archer, 1988).

High solid biological wastes originating from agro- industries, fruit and vegetable markets, agriculture sector etc. have been studied in the recent past by many authors (Table 2.1).

Although scientific interest in methane production from such wastes dates back to the 17th Century, the full scale adaptation of these scientific interests have been developed only over the past 30 years (Scharer & Moo-Young, 1979). Some workers developed interest in terms of waste volume reduction and recovery of renewable energy (Zwart *et al.*, 1988). The work of Lane (1983), on anaerobic digestion of spent coffee grounds is aimed at overcoming the problem of the disposal of this bulky waste. The dumping or the incineration of waste presents serious environmental and economic problems. His work has been unique in achieving 99% conversion of organic matter on solid recycling. On the other hand efforts were also directed towards achieving high methane yields and high methane production rates from anaerobic digestion of Sorghum biomass (Jerger *et al.*,1987). High yields from biomethanation of the organic fraction of food wastes from a vegetable market have been presented (Mata-Alvarez and Llabres, 1992).

More biogas generation and large methane content from *Mirabilis* leaves than the conventional cattle dung substrate was established by the work of Sharma et

al (1987). Like other aquatic weeds such as *Pistia stratiota*, water hyacinth has been used as a potential source of methane by many authors (Kalle *et al.*, 1985; Nipaney & Panholzer, 1987; El- Shinnawi *et al.*, 1989; Patel *et al.*, 1992). Besides these, wastes generated in the agricultural sector have been of prime interest to the researchers in the recent years. Large costs incurred on disposal, quantum availability and rich potential for biogas generation make it a suitable substrate for anaerobic digestion. Scharer and Moo-Young (1979) have reviewed in detail the potential of such cellulose containing wastes.

The potential for the commercial exploitation of crop residues like, rice straw, cotton & maize stalks and corn stover for biogas generation was established by the findings of several authors (Sun *et al.*, 1987; El- Shinnawi *et al.*, 1989). The capacity of molasses to generate methane has been worked out, although its potential use has been in ethanol production (Hashimoto, 1981).

Inspite of such a thorough work on agricultural wastes, the literature available today indicates that damaged agricultural grains of rice, maize, wheat and other crops have not been treated for fuel gas production, despite the fact that substantial portion of crop grains get spoiled due to various factors like disease, insects, pests and rains and are unfit for normal consumption. There has not been any report where viable potential of these wastes for gas production was checked.

Dumping wheat grains, which are fit for only dumping purposes are available in large quantities in major wheat producing countries like India. The high cost incurred

in its transportation for land filling, makes it as a choice substrate for fuel gas production by microbial fermentation.

2.2 FRUIT AND VEGETABLE PROCESSING WASTES :

A preliminary study for the fuel gas production from fruit and vegetable wastes by anaerobic digestion has been carried out by Knol and his associates (1978), more than a decade ago. His study was based on an observation that about 10% of the raw material is wasted during processing of fruit and vegetables. The expense involved in its disposal and stringent restrictions from the pollution control authority has been a concern for the industrialists.

Wastes like, apples, asparagus, carrots, green peas etc. have been shown to be a potential source of methane production (Knol *et al.*, 1978). Lane (1979) has established the potential of the anaerobic digestion process for treating these solid wastes. Lane was the first to be credited for publishing his results on scale up studies on digestion of pelletised citrus peel.

Investigations of tomato, peach and honey dew wastes were reported by Hills and Roberts (1982). They also presented the pilot plant results obtained on the seasonal operation of the mobile digestor on these wastes.

Later on it was established that commercial anaerobic digestion of fruit waste is technically feasible (Lane, 1984 a). The conclusion was drawn by successfully operating a larger pilot digestor for 3 years with an operating capacity of 23 m^3 at a fruit and vegetable processing factory in Australia. Apple press cake has been used in his study as a seasonal waste. His efforts were also concentrated on

improving the efficiency of the anaerobic digestion from fruit and vegetable wastes by balancing the chemical composition of the substrate material (Lane, 1984 b). Anaerobic digestion of apples has also been reported by Wase and Gordon (1982).

More recently the findings of Lane on bio-degradation of apple pomace were supported by the results of Etheridge and Jewell (1988). Studies conducted at both laboratory and pilot scale resulted in supporting the feasibility of pomace digestion as a means of stabilizing this wasted resource, while producing a combustible gas.

Two-phase biomethanation of solid wastes like, carrot peels, french bean waste, red beet peels has been conducted by Verrier *et al.* (1987). Influence of pH and temperature on hydrolysis and fermentation of these lignocellulosic wastes has been presented.

Similarly, batch culture studies on other fresh and ensiled vegetable wastes like leaves of cauliflower and cabbage and tops of other plant materials have been carried out (Zubr, 1986).

In India, this type of work has been reported by Ranade *et al.* (1987). The biogas generating capacity of market wastes consisting of rotten vegetables, fruit skins, potatoes, onions etc. have been tested. Full scale studies are yet to be undertaken.

Influence of trace elements on biogas production from mango peel in 1.5 m^3 KVIC digestor in India has more recently been reported by Krishna Nand and his co-workers (Raju *et al.*, 1991).

Inspite of the extensive work on fruit wastes especially apple waste, nothing much has been done in India for its commercial application. Apple pomace produced in large quantities at fruit processing industries has not been studied for its fuel gas generating efficiency.

2.3 FACTORS AFFECTING METHANOGENESIS :

Few microorganisms living in natural habitats grow at optimal rates, and all are subject to a variety of physical and chemical factors that may either inhibit or stimulate their activity. A few methanogenic environments, such as the rumen and sewage digestors, provide relatively constant and nearly optimal conditions for the microorganisms. In the last few decades considerable research has been done on the factors affecting methanogenesis and has provided valuable information for its application (Mah *et al.*, 1977 and Winfrey, 1984). Different biological wastes show variation in their capacities to evolve biogas. These differences are due to either inherent properties of wastes and microbes or due to physical factors like retention time, loading rate, pH, temperature, reactor design etc.

2.3.1 RETENTION TIME :

In continuous-flow anaerobic environments such as the rumen and anaerobic digestors, the retention time is an important factor affecting methanogenesis. As the retention time decreases, a smaller percentage of the organic matter is degraded, but the amount of methane produced per volume of reactor is often increased (Varel *et al.*, 1977; McInerney and Bryant, 1981). There is a minimum retention time however, below which fermentation stops, because of the washout of vital bacterial

groups. Retention time depends upon both the generation time of the methanogens and type of the feed material. The studies of Varel *et al.* (1980) have suggested low retention time of three days at thermophillic temperatures.

Reduced retention time as short as 4 hrs have been used in the studies conducted on waste water treatment with low solid concentration (Lettinga *et al.*, 1980; Colleran *et al.*, 1982; McCarty, 1982). In these studies the development in digestor design has solved the problem of wash out of bacterial culture by retaining the microbial population in a dense sludge or through attachment to an inert support.

Even after such developments, large retention times have generally been used in high solid digestions. Retention times as high as 20-30 days have generally been used for the biodegradation of biological wastes (Lane, 1979; Hills and Roberts, 1982; Ranade *et al.*, 1987). The conclusion drawn by Boone (1982), a decade ago that a rate-limiting step, will, however, depend upon the feed composition stands good even today.

2.3.2 TEMPERATURE :

Methanogenesis occurs in anaerobic environments at temperatures from about 0 to over 100°C (Baross *et al.*, 1982; Zehnder *et al.*, 1982). Most known methanogens are mesophillic, although some thermophiles having optima between 50°C and 80°C have also been isolated (Zeikus and Wolfe, 1972; Zinder and Mah, 1979; Huber *et al.*, 1982; Rivard and Smith, 1982). There is no report of the isolation of

psychrophillic methane producing bacteria, despite the fact that many methanogenic environments exist at cold temperatures.

The optimal temperature of anaerobic digestion is between 35 - 40°C (Zeikus and Winfrey, 1976; Chen *et al.*,1980). Considerable industrial interest in the thermophillic anaerobic digestion has arisen by the fact that methane production and digestion efficiency are greater at the thermophillic range 50 - 60°C (Bryant *et al.*, 1976; Buhr and Andrews, 1977; Chen *et al.*, 1980; Varel *et al.*, 1980; Zehnder *et al.*, 1982). However, the findings of Baross *et al.* (1982) have demonstrated active methanogenesis at 100 ± 2°C in water samples collected from submarine hydrothermal vents. He also revealed that the active growth and methanogenesis was even possible at temperatures as high as 300°C (Winfrey, 1984). This revolutionary observation although limited to his study only, has led to a change in the concept on the upper temperature limits of life and further reveals the extremely unique nature of methanogens.

Despite these findings in the literature of higher efficiency at higher temperature ranges, the bulk of the literature indicates stable, successful and economical anaerobic digestion of biological waste materials within the mesophillic temperature range of 30-40°C (Lane, 1984 a, b; Ueki *et al.*, 1988; El-Shinnawi *et al.*, 1989).

2.3.3 HYDROGEN ION CONCENTRATION (pH) :

Methanogenesis is very sensitive to pH, and virtually all methanogenic habitats are near neutrality (Zehnder *et al.*, 1982). The pH optimum has generally been found to occur between 6.7 - 7.4, and pH values below 6 or above 8 are very restrictive

(McCarty, 1964). Recent studies on methanogenesis of high solid biological wastes have supported these findings (Verrier *et al.,* 1987). Pfeffer (1980) has explained the failure of methanogenesis. In his view, the acid producing bacteria are less sensitive to low pH, and continue acid production until the pH drops to 4.0-5.0, which is highly inhibitory to methanogenic population. Acidophillic methanogens have been reported by several authors (Zehnder *et al.*, 1982) with methanogenesis occurring at pH values as low as 3. This pH sensitivity of the methanogenic bacterial population has led to its separation from acidogenic stage in anaerobic digestion, giving rise to the two- stage digestion process (Pohland and Ghosh, 1971; Cohen *et al.*, 1979; Schwitzguebel and Peringer, 1986).

2.3.4 SUBSTRATE CONCENTRATION :

The literature available so far on methanogenesis clearly indicates that very little attention has been given to the substrate concentration. Only recent findings have shown that high carbohydrate in feed concentrations can inhibit methanogenesis (Roychowdury *et al.*,1988). All the old works with biological waste digestion have aimed at utilizing high solid concentration (3-12%) without checking the efficiency of the process at optimum concentration level (Etheridge and Jewell, 1988). This aspect has been given special attention in the present work.

Other factors affecting microbial methane production include the oxygen tolerance of the microbial population. It has been observed from the literature, that there is a considerable variation in the tolerance to oxygen exposure. Some

methanogens can tolerate exposure upto 100% oxygen for 4 days and still produce methane after reducing conditions were reestabilished (Winfrey, 1984).

Several compounds have been shown to be inhibitory to methanogenesis, like nitrate, sulfate, ammonia, heavy metals, alkaline and alkaline earth metals etc. (Winfrey, 1984; Yadav and Archer, 1989).

B 2.4 PRODUCTION OF HYDROGEN :

The story of hydrogen (H_2) began in 1500 when it was first discovered by Paracelsus and was found to be one of the most abundant elements on Earth. Lemory in 1700 showed explosive property of this gas when combined with air. Cavendish in 1781 proved that it burns cleanly and efficiently producing only water vapour and provides more energy than conventional fuels (Balthasar, 1084). Since then, continuous work on its production has been going on. But in recent years H_2 has received mounting attention in preference to fossil fuels as an energy source (Jones, 1971; Cox & Williamson, 1977; Kumazawa & Mitsui, 1982). Before reviewing the literature on the microbial production of hydrogen, it becomes logical to present some of its properties, which make it a fuel of choice :

1. Crude oil and natural gas are in limited supply. Hydrogen, which is found in water, is very abundant and would be available to both energy-poor and energy rich countries (Cox & Williamsons Jr.,1973; Bockris,1975; Veziroglu,1986).

2. Hydrogen can be substituted for most fuels now in use. It can replace gasoline (2.15 lb H_2 has the same energy content as one gallon of gasoline), diesel fuel, jet fuel, natural gas and propane. It is possible to burn H_2 instead of the fuels now used by trains, trucks, ships, cars, tractors, snow mobiles other ground vehicles and more interestingly by aircraft (Bockris,1975; Cox & Williamsons Jr.,1979; Williamsons Jr. & Edeskuty, 1986).

3. It has a high energy yield per unit weight (122 kj/g) (Khoshoo, 1991).

4. The products of combustion are non-polluting (Cox & Williamsons, Jr., 1979; Veziroglu, 1986).

5. Hydrogen can serve as an agent for the storage and transport of other primary energy sources (nuclear, solar) (Awad & Veziroglu, 1984; Veziroglu, 1986).

6. It is a more efficient route of energy utilization (Dinga, 1989).

   The energy extracted from one ton coal in the form of H_2 fuel can run a bus for 640 miles, whereas energy extracted in the form of gasoline, electricity and methanol fuels will be sufficient to run it for only 440, 480 and 520 miles respectively.

7. Technology has already been developed for the use of hydrogen as an energy source and an enormous amount of work is currently being done on its production (Balthasar, 1984).

Besides its large scale application as a clean fuel, H_2 is produced for industrial use as a chemical feed stock in the manufacture of ammonia, methanol, refined

petroleum fuels, hydrogenated vegetable and animal oils and other chemical (Gregory *et al.*, 1972; Cooperberg, 1979). It has potential applications in the reduction of metals and synthetic natural gas (S N G) production.

In view of its diverse applications in various fields, its large scale and economic production has always been a challenging research area. Today H_2 is commercially produced via the following technologies :

i. Steam reforming of natural gas.

ii. Partial oxidation of heavy oils.

iii. Coal gasification.

iv. Electrolysis and thermolysis of water.

A brief description of each of these processes will be made here.

2.4.1 Steam reforming : As reviewed by Balthasar (1984), the process of high temperature steam reforming has been the most efficient, economical and a practical one available for conversion of light hydrocarbons to H_2 and H_2 and CO mixtures. Raw materials range from natural gas, methane and methane containing refinery gases. Hydrogen is produced by the reforming reaction of these light hydrocarbons with steam at high temperature and pressure.

2.4.2 Partial oxidation : The partial oxidation of hydrocarbons is a major commercial route to H_2 production. The hydrocarbon feed stock may range from natural gas to sulfur containing crude oil or heavy fuel oil. The partial oxidation reaction is the result of a series of reactions when hydrocarbons are burnt with only

30-40% of the stoichiometric amount of oxygen required. The reactions proceed with or without catalysts and the raw gas generated is followed by gas purification.

2.4.3 Coal gasification : The production of H_2 from coal or coke proceeds via coal gasification i.e, reaction of coal with water (Balthasar,1984). In this process a hydrogen rich gas is produced, which is then separated by various techniques into H_2 and the other components. The reaction mechanism of coal gasification very much resembles that of the partial oxidation of heavy oils. However, due to the low H_2 content of coal, much more H_2 of the final product is supplied from water than from the H_2 bound in coal. All coal gasification processes suffer from difficulties in handling a relatively unreactive fuel as a solid and removing a large amount of ash (Balthasar, 1984).

Hydrogen derived from these fossil fuels is obviously not a clean, renewable resource and involves expensive techniques but it still continues to be attractive till low-cost coal is available in abundance as feed stock. Most of the H_2 research community agrees that eventually hydrogen should be produced directly from water but the contribution of this route is still minor compared to fossil based processes (Deluchi,1989).

2.4.4 Electrolysis and thermolysis of water: Water electrolysis is based on the passage of direct current through water that has been made electrically conducting by addition of excess hydrogen or hydroxyl ions, oxygen being liberated at the cathode. Alternate electrodes are surrounded by diaphragms that prevent mixing of gases (Balthasar, 1984).

Water can be decomposed thermally to H_2 at temperatures above 2500 K. However, these thermal decomposition methods are energy intensive and it is difficult to separate dilute H_2 from other gases at high temperatures (Deluchi,1989).

The above mentioned technologies of H_2 production depend on both the fossil fuel and non-fossil fuels and need a good deal of energy-input. The progressively diminishing reserves of fossil fuels and need for development of cost-effective technologies have led researchers to look for newer processes. One of these has been microbial H_2 production.

2.5 MICROBIAL HYDROGEN PRODUCTION :

While other methods of H_2 production have been used on large scale and still persist as the primary choice, great promises have been seen in its generation using unicellular organisms. Microbial production is a relatively new technology and untested on large scale. It is considered to be the method of choice, because H_2 is produced from carbohydrates a renewable resource. (Huang *et al.*, 1985; Kosaric & Lyng, 1988).

Historically, science has known about microbial H_2 production for over fifty years. However, till recently, H_2 gas production by microorganisms has not been studied with a concentrated effort and with the objective of commercializing a process. Almost all the past studies are incidental or are of secondary nature and not aimed at large scale production of H_2 (Zajic *et al.*, 1979).

The review by Zajic *et al.* (1979) mentions that Popoff was the first to observe the evolution of molecular hydrogen by bacterial action in 1875. It was noticed that

fermentation of calcium formate using a mixed culture from pond mud produced H_2 but not methanol. Between 1920 and 1950, chemical activities of bacteria to produce H_2 were envisioned as the result of the activity of the specific enzymes (Gest, 1954). Thereafter microbial H_2 production has been receiving considerable attention. The history of this process is reviewed by several authors (Barker, 1956; Stadtman 1967; Wolfe, 1971; Zeikus, 1977; Zajic *et al.* 1978, 1979; Kondratieva & Gogotov, 1983; Huang *et al.*, 1985; Kosaric and Lyng, 1988).

The ability of microbial species to produce H_2 is a widespread phenomenon and occurs in diverse taxanomic and physiological groupings (Gray & Gest, 1965; Kessler, 1974; Zajic *et al.*,1978). In all these organisms H_2 production is a mechanism for disposing of electrons released during metabolic reaction :

$$2\ e^- + 2H^+ \longleftrightarrow H_2$$

2.6 MICROORGANISMS INVOLVED IN HYDROGEN PRODUCTION :

Both phototrophic and chemotrophic eukaryotes and prokaryotes have been found to produce H_2. Phototrophs producing H_2 include algae, oxygenic cyanobacteria and anoxygenic purple and green bacteia. Chemotrophs are comprised almost entirely of bacteria, though some eukaryotes are capable of H_2 production. Fig. 2.2 presents a survey of the metabolic processes accompanied by either evolution or consumption of H_2.

Although our work in this presentation mainly emphasizes the production of H_2 by fermentative chemotrophic bacteria, a brief description of the other group of microorganisms (phototrophs) follows :

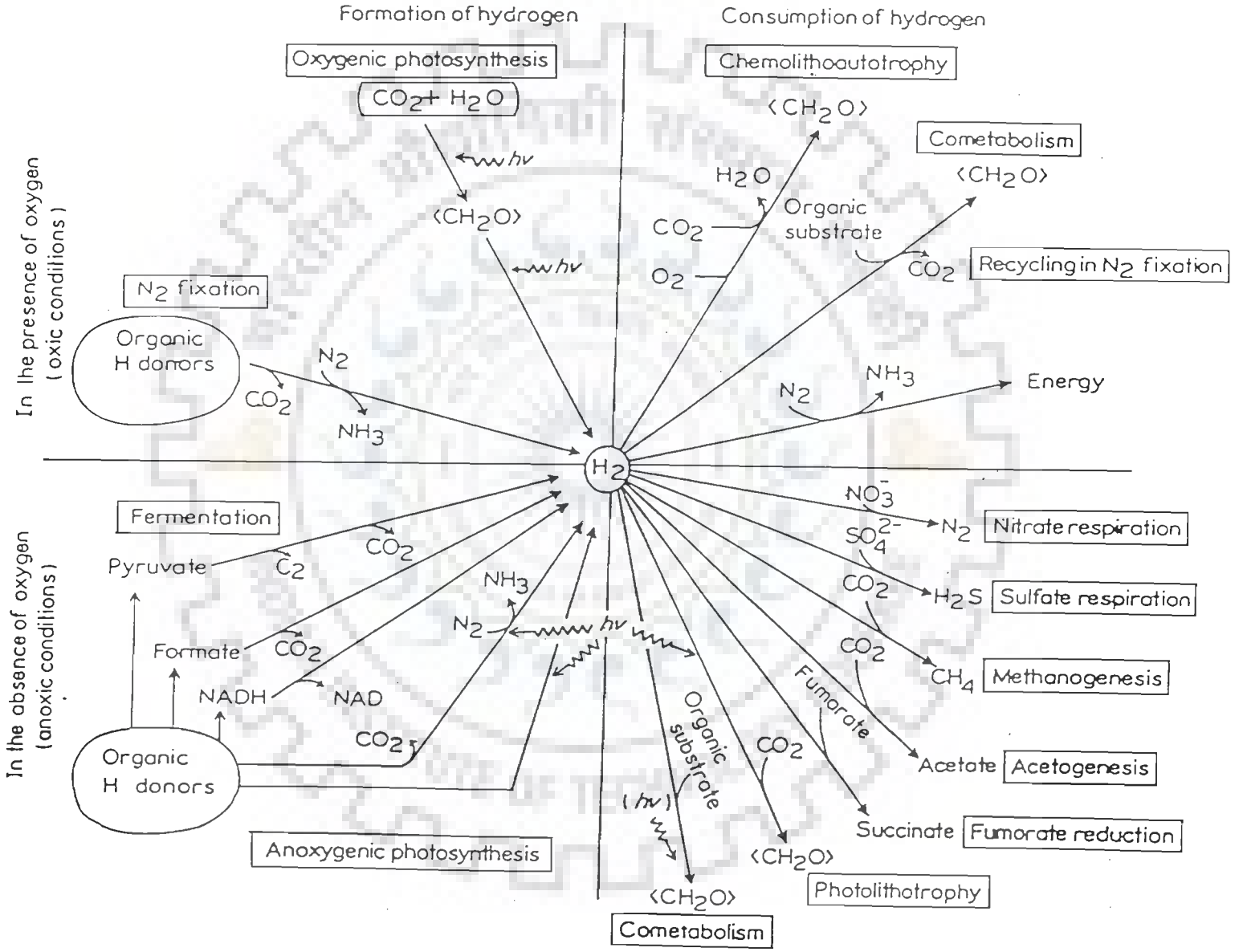

Fig. 2.2: Biological process of hydrogen formation and consumption (Schlegel and schneider, 1985).

2.6.1 PHOTOTROPHS :

ALGAE : Production of hydrogen by algae occurs only when adapted under anaerobic conditions , with adaptation times varying from 30 min to 4 hours (Schelegel & Schneider, 1985). Species most often studied include *Chlamydomonas*, *Scenedesmus*, *Cholerella* and *Spirullina* etc. (Table 2.2).

During the adaptation period hydrogenases are either activated or synthesized de novo. Bothe (1982) has described that in darkness H_2 production arises from the consumption of organic substrates and the pattern of accompanying products (CO_2, ethanol, acetate, glycerol) indicates that H_2 evolution is due to fermentative glucose (polysaccharide) breakdown. Miura *et al.* (1981)proposed the need of microaerobic conditions for H_2 production. The role of formate for H_2 evolution was ruled out (Gfeller & Gibbs, 1984).

When exposed to light, H_2 production is stimulated. At high light intensities, photoproduction of H_2 stops abruptly due to the inhibition of hydrogenase by the photosynthetically produced O_2 (Bothe, 1982).

Two mechanisms for H_2 production have been proposed. One involves photolysis of water coupled to electron transport through photosystems I and II (Pow and Krasna, 1979; Rosen and Krasna, 1980). The alternative pathway involves metabolic breakdown of carbohydrates feeding electrons in to photosystem I only.

OXYGENIC PHOTOTROPHIC BACTERIA (CYANOBACTERIA): The cyanobacteria or blue green algae, under normal growth conditions perform a plant type photosynthesis in the presence of light (Rippka *et al.*, 1981; Stanier *et al.*, 1981).

TABLE 2.2: ALGAE REPORTED TO PRODUCE HYDROGEN (Kosaric and Lyng, 1988):

GENERA	REFERENCES
Ankistrodesmus	Kessler (1967); Kondratieva and Gogotov (1983)
Chlamydomonas	Wang et al. (1971); Gfeller and Gibbs (1984); Healy (1970 a,b)
Chlorella	Healy (1970 b)
Codium	Ben-Amotz et al.(1975)
Corallina	Ben-Amotz et al.(1975)
Kirchneriella	Healy (1970 b)
Porphyridium	Ben-Amotz et al.(1975)
Scenedesmus	Healy (1970 b); Ben-Amotz and Gibbs (1975)
Chrondrus	Kondratieva and Gogotov (1983)

They are able to use water as the primary electron donor and have two photosystems to generate a strong reductant. Hydrogen production occurs in a manner similar to that of algae except that most cyanobacteria produce hydrogen primarily through N_2 fixation (Bothe, 1982).

Some cyanobacteria are unique in their ability to form heterocysts, cells lacking a photosystem and designed primarily to fix N_2 via nitrogenase. Bothe (1982) also demonstrated that the heterocysts, under nitrogen limiting conditions, fix molecular nitrogen and produce H_2. These are the only phototrophs capable of producing H_2 under aerobic conditions. For nonheterocysts cyanobacteria, a deficiency in nitrogen and anoxic conditions are required for derepression of the formation of nitrogenase (Schlegel & Schneider, 1985).

Laczko (1986) recently demonstrated that at high light intensities, H_2 is also produced via hydrogenase in *Anabaena cylindrica*. The non-heterocystous filamentous cyanobacteria can be used for H_2 production when they are periodically exposed to light & dark conditions. However, it is still controversial whether cyanobacteria contain only membrane bound hydrogenases or an additional soluble hydrogenase that are responsible for H_2 production (Peschek, 1979; Bothe *et al.*,1980). Kumar *et al.* (1985) have shown the lethal effects of copper, indicating that the application of cyanobacteria for wastewater treatment is not feasible. Cyanobacteria genera reported to produce H_2 are listed in Table 2.3.

ANOXYGENIC PHOTOTROPHIC BACTERIA : The anoxygenic phototrophic bacteria are well known under their previous name "purple bacteria", derived from

TABLE 2.3:CYNOBACTERIA REPORTED TO PRODUCE HYDROGEN (Kosaric and Lyng, 1988).

GENERA	REFERENCES
Anabaena	Benemann et al. (1973); Kumazawa and Mitsui (1985); Laczko (1986)
Aphanocapsa	Howarth and Codd (1985)
Calothrix	Lambert & Smith (1977)
Gloeobacter	Howarth & Codd (1985)
Lyngbya	Gallon et al. (1974)
Microcystis	Howarth & Codd (1985)
Nostoc	Kumar (1986)
Spirulina	Gu & Wang (1984)
Synechococcus	Howarth & Codd (1985)
Synechocystis	Howarth & Codd (1985)

their red, brown and pinkish colours (Stanier *et al.*,1981). They comprise of at least three groups, the non-sulfur purple bacteria (Athiorhodaceae or Chromatiaceae), the sulfur purple bacteria (Chromatiaceae and Thiorhodacea) and the green sulfur bacteria (Chlorobiaceae). Table 2.4 lists the major genera of phototrophic bacteria reported to produce H_2. Zurrer (1982) has predicted the possibility of waste water treatment in conjunction with H_2 production with the photrophic bacteria beacause these bacteria are able to use either CO_2 or a variety of organic substrates as carbon sources.

The majority of purple and green bacteria produce H_2 at high rates, either in the light or in the dark. Hydrogen may be produced by both hydrogenases and nitrogenases but often nitrogenase is solely responsible (Miyake *et al.*, 1982).

Photoproduction of H_2 by the Rhodospirillaceae is evidently a nitrogenase-dependent reaction. H_2 is produced only under anoxic conditions and when the nitrogen sources limit growth. It is assumed that the production of H_2 serves to dispose of excess electrons derived from organic substrates and provided at a highly negative potential during photosynthesis. Similarly, active H_2 evolution occurs when *Chlorobium* is kept in the light in the presence of thiosulfate. In the dark H_2 is also produced, especially in the presence of pyruvate as carbon substrate. In *Rhodospirillum rubrum* the formate hydrogenlyase and the pyruvate formate lyase systems are apparently involved.

TABLE 2.4: PHOTOTROPHIC BACTERIA REPORTED TO PRODUCE HYDROGEN (Kosaric and Lyng, 1988).

GENERA	REFERENCES
Chromatium	Bennett et al.(1964)
Thiocapsa	Kondratieva and Gogotov (1983)
Rhodomicrobium	Hoare and Hoare (1969)
Rhodopseudomonas	Hillmer and Gest (1977); Kim et al. (1982); Vincenzini et al.(1986)
Rhodospirillum	Benett & Weetall (1976); Yoch (1979); Zurrer & Bachofen (1982)
Chlorobium	Bennett et al. (1964)
Pelodictyon	Pfennig (1967)

2.6.2 CHEMOTROPHS :

Most H_2 producing chemotrophic microorganisms belong to obligate and facultative anaerobes called fermentative H_2 producing bacteria (Table 2.5) (Kondratieva and Gogotov, 1983).

FERMENTATIVE HYDROGEN PRODUCING BACTERIA :

The strict anaerobes are particularly numerous. The widely studied are *Clostridium, Desulfovibrio, Methanobacterium* and *Veillnella* etc. (Zajic *et al.*, 1978). Thermophillic hydrogen producing anaerobic bacteria have also been studied by several authors (Zajic *et al.*,1978; Braun & Gottschalk,1981).

Kosaric & Lyng (1988) have grouped *Enterobacteria* and related microorganisms as the best studied and predominant class of hydrogen producing facultative anaerobes. The other major class includes, *Bacillus*, sp., *Campylobacter* sp. and *Alcaligenes eutrophus* (Laansbrock *et al.*, 1978; Zajic *et al.*, 1978).

Many chemotrophic H_2 producing bacteria are widely distributed in nature. Some of them are symbionts of humans and animals (Kondratieva and Gogotov, 1983). Most characteristic in this report are the microorganisms of the rumen such as *Ruminococcus albus, Megasphaera elsdenii* and others (Wolin, 1979). Several authors have reported hydrogen production in the human intestine (Bigard *et al.*, 1979; Raskin, 1980).

Generally, obligate and facultative anaerobic bacteria evolve molecular hydrogen as a result of degradation of organic substrates, i.e., anaerobic degradation

TABLE 2.5: CHEMOTROPHIC BACTERIA REPORTED TO PRODUCE HYDROGEN (Kosaric & Lyng; 1988).

GENERA	REFERENCES
STRICT ANAEROBES :	
Acetobacterium	Braun & Gottschalk (1981)
Acetomicrobium	Soutschek et al. (1984)
Bacteroides	Joyner et al. (1977)
Butyrivibrio	Wolin (1979)
Clostridium	Wiegel et al.(1979); Zeikus (1980)
Desulfovibrio	Bryant et al. (1977); Thauer et al. (1977)
Eubacterium	Joyner et al. (1977); Kondratieva & Gogotov (1983)
Fusobacterium	Joyner et al. (1977)
Methanobacterium	Graf & Thauer (1981)
Methanosarcina	Bott et al. (1986)
Methanococcus	Zeikus (1977)
Peptostreptococcus	Zajic et al. (1978)
Ruminococcus	Wolin (1979)
Sarcina	Stanier & Cohen-Bazire (1977)
Selenomonas	Wolin (1979)
Syntrophobacter	Boone & Bryant (1980)
Thermobacteroides	Kondratieva & Gogotov (1983)
Veillonella	Devries et al. (1974)

TABLE 2.5 Continued

GENERA	REFERENCES
FACULTATIVE ANAEROBES :	
Aeromonas	Gray & Gest (1965)
Alcaligenes	Friedrich et al. (1981)
Bacillus	Gray & Gest (1965); Stanier & Cohen - Bazire (1977)
Campylobacter	Laanbrock et al. (1978)
Citrobacter	Brosseau and Zajic (1982 a,b)
Escherichia	Azouly and Marty (1970); Brosseau and Zajic (1982 a,b)
Enterobacter	Kondratieva & Gogotov (1983)
Klebsiella	Weetall et al. (1981)
Salmonella	Kondratieva & Gogotov (1983)
Serratia	Kondratieva & Gogotov (1983)
Streptococcus	Kondratieva & Gogotov (1983)
AEROBIC CHEMOTROPHS :	
Azomonas	Kondratieva & Gogotov (1983)
Azospirillum	Berlier & Lespinat (1980)
Azotobacter	Smith et al. (1976)
Mycobacterium	Walker & Yates (1970)
Pseudomonas	Zajic et al. (1978)
Rhizobium	Mahon & Nelson (1986)

of organic compounds resulting in ATP synthesis at substrate level. Proteins, carbohydrates, alcohols, organic acids, amino acids, purines and pyrimidines are among the organic compounds capable of degradation with H_2 evolution (Zeikus, 1980).

The capability of chemotrophs to use various organic substrates and to produce H_2 as the result of degradation is not the same (Zajic *et al.*, 1978). However, literature indicates that many chemotrophs produce H_2 by the degradation of different carbohydrates (Schlegel and Schneider, 1978).

AEROBIC BACTERIA AND HIGHER ORGANISMS :

Other than chemotrophic fermentative bacteria, there are aerobic chemotrophs that are also reported to produce H_2 (Table 2.5) (Kosaric and Lyng, 1988).

The report of Mahon and Nelson (1986), has dedicated these aerobic chemotrophs primarily to reducing fertiliser requirements and not so much for the hydrogen production.

Some *Trichomonads*, symbiotic or parasitic flagellates that live in the digestive or geneto urinary tract are capable of H_2 production (Zajic *et al.*, 1978). *Dasytricha ruminantium* and *Isotricha* species are examples (Hillman *et al.*, 1985). However, major research in this field is directed towards medical treatment and not at potential fuel production (Lloyd *et al.*, 1986).

2.7 METABOLIC PATHWAYS INVOLVED IN THE FERMENTATIVE HYDROGEN PRODUCTION :

Before reviewing the literature on different metabolic reactions involved in H_2 production by fermentation, it is desirable to express the role of different enzymes involved in these reactions and in other reactions for H_2 production.

Extensive literature is available on the role of different enzymes in microbial H_2 production. Different authors have reviewed the literature (Zajic *et al.*,1978; Adams *et al.*,1981; Kondratieva & Gogotov,1983; Cammack *et al.*,1985; Schlegel & Schneider,1985; Kosaric & Lyng,1988).

The enzymes catalyzing biosynthesis of molecular hydrogen can generally be grouped as HYDROGENASES OR NITROGENASES (Kosaric & Lyng, 1988).

2.8 HYDROGENASES :

In 1983, the name hydrogenase was proposed (Stephenson and Stickland ,1931 a,b) for the enzymes that catalyze the reversible oxidation of H_2 to protons and electrons.

$$H_2 \longleftrightarrow 2\,H^+ + 2\,e^-$$

Hydrogenases are known to play a fundamental role in H_2 metabolism in many microorganisms (Adams *et al.*,1981).

All known hydrogenases (EC 1.12) contain iron and acid labile sulfur arranged in iron-sulfur clusters. The literature on these enzymes have shown that some of these also contain nickel (Schlegel & Schneider, 1985).

The reviews on the subject have established that hydrogenases of various microorganisms are very diverse in their relative molecular mass, cofactor composition and spectroscopic properties revealing different function of the enzyme in different microorganisms (Adams *et al.*,1981).

These different functions have led to coining ofthe term "hydrogenase" refers to a class of enzymes and not to a single enzyme. (Table 2.6).

According to Cammack *et al.* (1985), hydrogenases can be subdivided into those that produce hydrogen and those that consume it. Sometimes hydrogenases have been classified as "hydrogen-producing hydrogenases" if they catalyze the evolution of hydrogen with reduced methyl viologen as electron donor, "hydrogen-uptake hydrogenases" if they consume hydrogen with dyes such as methylene blue as acceptor and "bidirectional" hydrogenases if they catalyze both reactions.

On the basis of localization within the cell, hydrogenases are commonly subdivided into soluble and membrane-bound types (Table 2.6). From the available data it is generally concluded that membrane-bound hydrogenases more frequently catalyze the consumption of H_2 by microorganisms, whereas soluble hydrogenases are often involved in H_2 evolution. However, exceptional cases are also reported where enzymes act vice-versa (Schlegel & Schneider, 1978).

H_2: Ferredoxin Oxidoreductase :

The redox potential of the couple $2H^+/ H_2$ is Eo' = - 413 mv. Reduced ferredoxins, present in many anaerobic bacteria, readily donate electrons at this

TABLE 2.6: PROPERTIES OF ENZYMES CATALYZING HYDROGEN PRODUCTION (Kosaric and Lyng, 1988)

HYDROGENASE	LOCATION	FUNCTION	ORGANISMS
H_2: ferredoxin oxidoreductase	Cytoplasm	Disposal of excess electrons	many anaerobes and facultative bacteria
H_2: ferrycytochrome C_3 oxidoreductase	periplasmic space or cytoplasmic membrane	uptake and evolution of hydrogen	*Desulfovibrio*
H_2: NAD^+ oxidoreductase	cytoplasm	reduction of NAD providing reducing power for CO_2 fixation	*Alcaligenes* and *Nocardia*
H_2: coenzyme F_{420} oxidoreductase	cytoplasm	channelize H_2 for CO_2 fixation	Methanogenic bacteria
Membrane bound	cytoplasmic membrane	-	Enterobacteriaceae N_2 fixing bacteria phototrophic bacteria, H_2 oxidizing bacteria
Formate hydrogenlyase	cytoplasmic membrane	H_2 formation from formate, channels electrons to electron transfer chain	Enterobacteriaceae
Nitrogenase	cytoplasm	Nitrogen fixation with release of H_2	N_2 fixing bacteria

potential if the H_2 : ferredoxin oxidoreductase is present. The enzyme is strongly inhibited by oxygen, and its natural function is the disposal of excess electrons associated with anaerobic fermentation through the production of molecular hydrogen.

Reduced ferredoxin is provided from glyceraldehyde-3- phosphate either through NAD : ferredoxin oxidoreductase. (Fig. 2.3) outlines the electron transfer pathway involved in hydrogen production through H_2: ferredoxin oxidoreductase.

In vitro ferredoxin (and flavodoxins) can be substituted by viologen dyes such as methyl or benzyl viologen and these can be reduced by dithionite (Schlegel & Schneider, 1985).

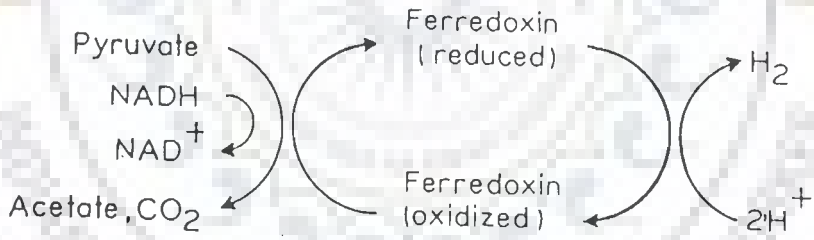

Fig. 2.3. The electron transfer pathway in the production of hydrogen through H_2 : ferredoxin oxidoreductase.

H_2: Ferrycytochrome C_3 oxidoreductase :

The H_2: ferrycytochrome C_3 oxidoreductase is characteristic of the sulfate reducing bacteria, *Desulfovibrio*.

The properties of the enzyme and its role in sulfate reducing bacteria have been recently reviewed by Le Gall *et al.* (1982).

The natural function of the hydrogenase of sulfate-reducing bacteria appear to be both uptake and evolution of the H_2 depending on the growth conditions.

Ferredoxin is reduced through oxidation of pyruvate or NADH and cytochrome C_3 reduced, which in turn donates the electrons to the hydrogenase. (Fig. 2.4).

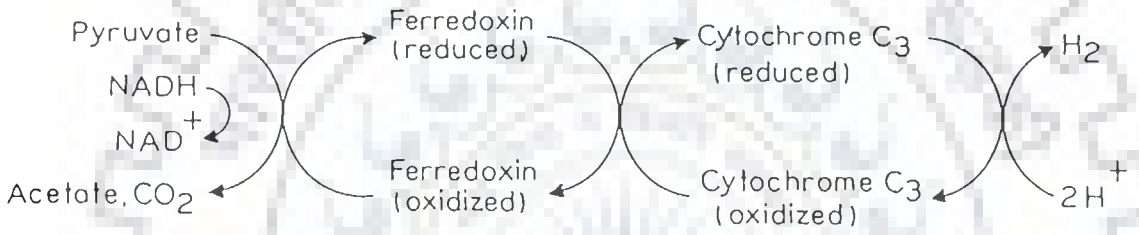

Fig.2.4. The electron transfer pathway involved in the production of hydrogen through H_2 : ferrycytochrome C_3 oxidoreductase.

H_2 : NAD^+ oxidoreductase :

This hydrogenase directly linked to nicotinamide dinucleotide (NAD) has so far been found only in the lithoautotrophic aerobic hydrogen-oxidizing bacteria, *Alcaligenes* and *Nocardia*. It catalyzes the reversible reaction :

$$H_2 + NAD^+ \longrightarrow NADH + H^+$$

The redox couple NAD^+/NADH has a potential of Eo' = -320 mv, the equilibrium of the reaction favours the reduction of NAD. The in vitro production of H_2 from

NADH has been demonstrated (Schneider & Schlegel,1976; Pinchukova & Varfolomeev,1980).

The natural function of H_2: NAD^+ oxidoreductase, therefore is reduction of NAD^+ to provide reducing power for the fixation of CO_2 via the Calvin cycle and channeling of electrons into the electron transport chains (Schlegel & Schneider,1985).

H_2 : Coenzyme F_{420} oxidoreductase : This is an important enzyme of the methanogenic bacteria which grow on H_2 and CO_2 to produce CH_4. The enzymes in these bacteria seem to differ from each other; however, in general they are able to reduce Coenzyme F_{420}, Coenzyme F_{420} derivatives, flavins, or artificial dyes but not NAD or NADP (Schlegel & Schneider,1985).

$$H_2 + \text{Coenzyme } F_{420}(ox) \longleftrightarrow \text{Coenzyme } F_{420}(red).$$

The reaction has low redox potential (Eo' = -340 mv)and favours H_2 consumption.

The natural function of the hydrogenases is to channel hydrogen into the reductive pathways for the assimilation of CO_2 for cell material synthesis and for the formation of methane. Apparently from Coenzyme F_{420} the hydrogen is mediated via NADP into biosynthetic pathways or its provided directly to the last step of methane formation, in which Coenzyme MF_{430} is involved (Keltzens *et al.*,1982).

Membrane bound hydrogenase :

Unlike the soluble, cytoplasmic hydrogenases, the hydrogenases located in the cytoplasmic membrane have not been studied in great detail.

The natural function of membrane bound hydrogenases is to take up hydrogen and feed electrons into the respiratory chain or the photosynthetic electron transport chain.

$$H_2 + \text{acceptor (ox)} \longleftrightarrow \text{acceptor (red)}.$$

Schlegel and Schneider (1985) consider to these enzymes to be less important with respect to hydrogen evolution and unidirectional because of high redox potential. However, they feel that reversibility of these enzymes is not clear because many cytochromes have variable redox potentials.

These enzymes have unique property in nitrogen fixing bacteria to prevent the oxygen inhibition of the extremely sensitive nitrogenase.

FORMATE HYDROGENLYASE :

Formate hydrogenlyase was first described by Stephenson & Stickland (1932), catalyzing the reaction.

$$HCOOH \longleftrightarrow H_2 + CO_2$$

The reaction was later on investigated by several authors (Ordal and Halvorson, 1939; Gest and Peck, 1955; Peck and Gest, 1957 a,b) (Fig. 2.5). Evidence was presented by these authors that formate decomposition to hydrogen and carbon dioxide is catalyzed by a multi-enzyme system, consisting of formic dehydrogenase, hydrogenase and one or two intermediate factors involved in electron transport.

The description of this enzyme will be discussed in detail in the biochemical reaction (*Escherichia coli* system) for H_2 production.

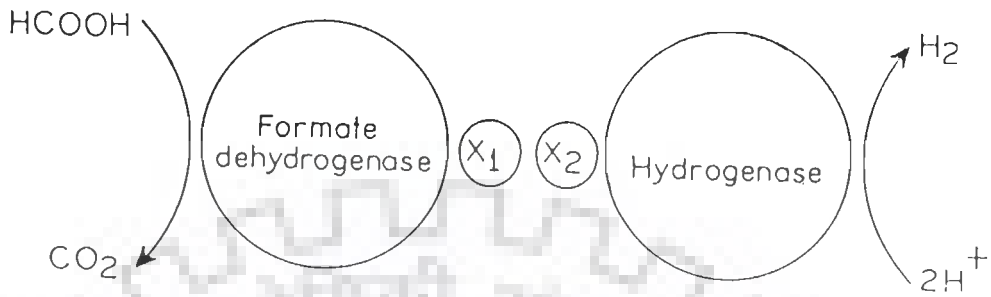

Fig. 2.5: Formic acid degradation and hydrogen production through the formate hydrogenlyase enzyme complex, carrier X_1 and X_2 are not clearly known.

2.9 NITROGENASE :

This enzyme has been studied with respect to nitrogen fixation and not much emphasis has been given with respect to molecular hydrogen production as the consequence of this reaction.

Nitrogenase is present in all nitrogen-fixing bacteria, which include photosynthetic blue-green algae such as species of *Anabaena* and *Nostoc*, bacteria like *Rhizobium* and certain *Bacillus, Clostridium* species etc. (Hardy *et al.*,1973).

In the absence of combined nitrogen, nitrogenase (EC 1.7.99.2) catalyzes the reduction of molecular nitrogen to ammonia for storage in the cell.

$$8H^+ + N_2 + 8\ e^- \longrightarrow 2NH_3 + H_2$$

This enzyme can also catalyze the reduction of a number of other compounds with triple bonds, e.g., C_2H_2 to C_2H_4 and irreversible energy-dependent reduction of protons to molecular hydrogen.

$$2H^+ + 2\,e^- + ATP \longrightarrow H_2 + ADP + Pi$$

Nitrogenases require low potential electrons and ATP to reduce N_2 to ammonia (Zajic *et al.*,1978). The electrons are provided by ferredoxins or flavodoxins, and molecular hydrogen and ammonia are the products of nitrogen fixation. (Fig.2.6).

Fig.2.6 : Hydrogen production associated with nitrogen fixation by nitrogenase.

Nitrogenases are severly inhibited by O_2 and are generally associated with membrane bound hydrogenases. The hydrogenases are believed to protect the nitrogenase from O_2 inhibition through the oxyhydrogen production of water (Schlegel and Schneider, 1985). Nitrogenase is an enzyme complex consisting of two proteins, a molybdenum-iron protein and an iron protein. The pathways of nitrogen fixation have been reviewed by Benemann and Valentine (1972).

The metabolism of hydrogen production in nitrogen fixing bacteria has been worked out by using different inhibitors. The specific inhibitors of N_2 fixation can be

classified as those which are alternative substrates and those which are not. The first group includes N_2O, NaN_3, C_2H_2, HCN, CH_3CN_2, and the second group includes H_2, CO, NO and analogs of these compounds (Burris and Orme-Johnson,1976).

Nitrogenase activity is routinely measured by the ability of the enzyme to reduce acetylene to ethylene (Hardy *et al.*,1973). H_2 is a competitive inhibitor of nitrogen fixation (Burris & Orme-Johnson,1976) and N_2 inhibits H_2 evolution (reciprocal inhibition).

2.10 BIOCHEMICAL REACTIONS IN FERMENTATIVE HYDROGEN PRODUCTION :

Fermentation of carbohydrates and other carbonaceous molecules results in the formation through pyruvate, of a variety of products, including molecular hydrogen. H_2 production is possible with anaerobic chemotrophs grown on a wide variety of complex and simple media including cellulose, hemicellulose and starch (Kondratieva and Gogotov, 1983).

There are two principal biochemical reaction chains involved in the anaerobic fermentation of hydrogen : a) the *Clostridial* system and b) *Escherichia coli* system (Zajic *et al.*,1978; Brosseau and Zajic, 1982 a).

2.10.1 CLOSTRIDIAL PATHWAY FOR ANAEROBIC BACTERIA :

The Clostridial system (Gottschalk *et al.* 1981) shown in Fig. 2.7, employs pyruvate: ferredoxin oxidoreductase to catalyze the reaction :

$$\text{Pyruvate} + \text{CoA} + 2\ \text{Fd (ox)} \longrightarrow \text{Acetyl CoA} + 2\ \text{Fd (red)} + CO_2$$

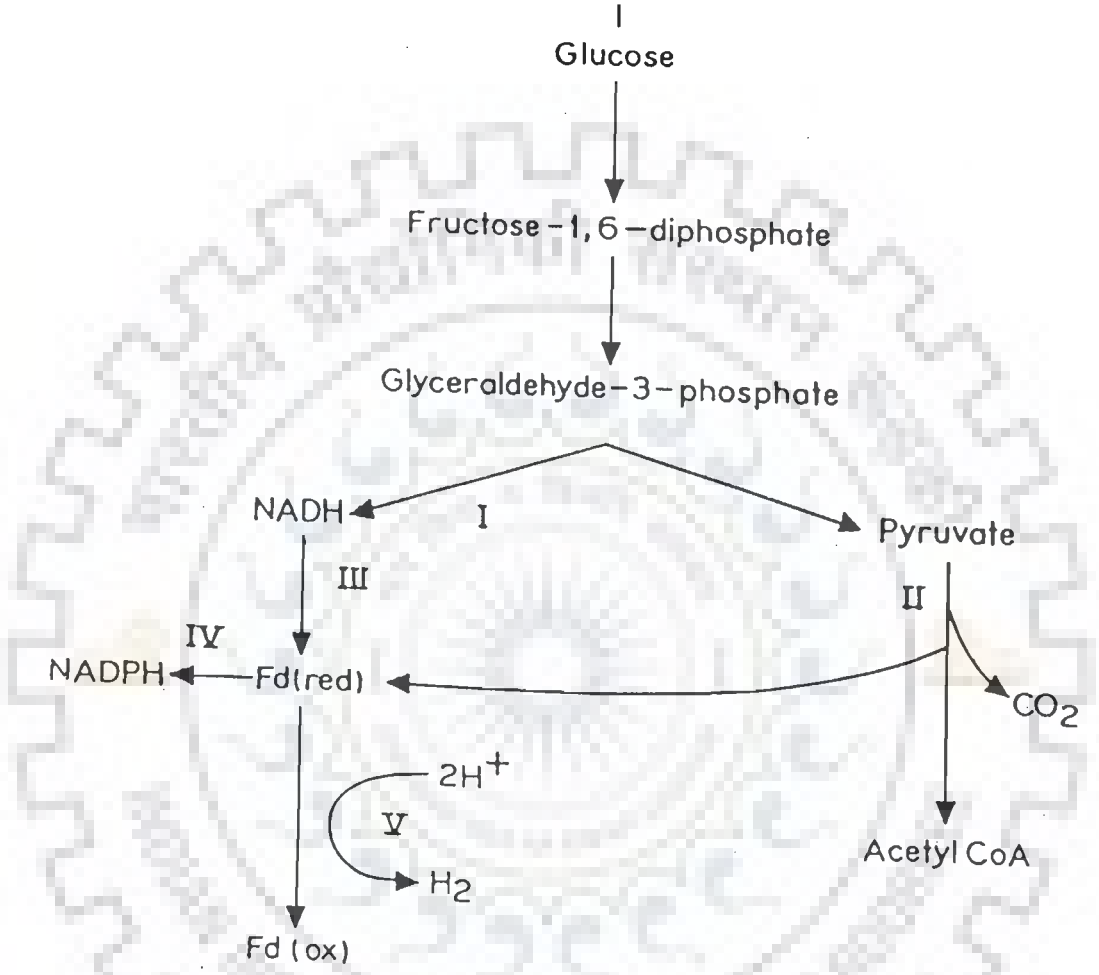

Fig. 2.7 : Anaerobic hydrogen production through Clostridial system

I : Glyceraldehyde-3-phosphate dehydrogenase

II : Pyruvate ferredoxin oxidoreductase

III : NAD : ferredoxin oxidoreductase

IV : NADP : ferredoxin oxidoreductase

V : H_2 : ferredoxin oxidoreductase

The acetyl CoA produced can be transferred in to acetyl phosphate which is used by many anaerobes for the synthesis of ATP by substrate level phosphorylation (Thauer *et al.*1977).

The Clostridial system is found in many bacteria, such as the anaerobic species *Clostridium, Ruminococcus, Megasphaera,* and *Butyrivibrio* and some facultative *Bacillus*, where phosphoclastic cleavage of pyruvate without formic acid intermediate is the mechanism (Huang *et al.*1985; Kosaric & Lyng, 1988).

Hydrogen is formed via H_2 : ferredoxin oxidoreductase according to reaction:

$$2\ H^+ + 2\ Fd\ (red) \longrightarrow 2\ Fd\ (ox) + 2\ H_2$$

In this process, H_2 production is accompanied by the recycling of ferredoxin as well as CoA which may participate again in the oxidation of pyruvate followed by ATP synthesis.

It has been observed that besides bacteria, a few hydrogen producing protozoa are able to oxidize pyruvate in the same-way (Muller,1975). In some of these eukaryotic microorganisms pyruvate oxidation occurs in microbodies called hydrogenosomes (Schlegel & Schneider, 1978).

In some bacteria, such as *Clostridium* species, *Ruminococcus albus, Peptococcus anaerobius*, and *Selenomonas ruminantium*, for example , ferredoxin is reduced through $NADH_2$: ferredoxin oxidoreductase which catalyzes the reaction (Kondratieva and Gogotov, 1983):

$$NADH_2 + 2\ Fd\ (ox) \longrightarrow NAD + 2\ Fd\ (red)$$

The reduction of ferredoxin by $NADH_2$ is a thermodynamically unfavourable reaction, as the redox potential of NAD^+/ $NADH_2$ is Eo' = -320 mv and that of H^+/ H_2 is Eo' = -420 mv. The reaction will only proceed if the reduced ferredoxin is reoxidized by hydrogenase and H_2 is evolved (Schlegel & Schneider, 1985).

Since hydrogenase activity is reversible, accumulation of hydrogen in the environment inhibits hydrogen production and consequently $NADH_2$ oxidation. Therefore, only in an environment where H_2 is continuously removed does $NADH_2$-generated H_2 evolution continue.

A classical example of such type of reaction is given by Soutschek *et al.*(1984), who found that 4 moles of H_2 were produced from 1 mole of glucose by *Actinomicrobium flavidum* (isolated from anaerobic sludge) at 60°C, 2 mol were produced through ferredoxin, and the oxidation of pyruvate, while the additional 2 mol were derived from $NADH_2$ formed during triphosphate oxidation.

2.10.2 *ESCHERICHIA COLI* PATHWAY FOR FACULTATIVE ANAEROBES :

This type of H_2-evolving reaction (Fig. 2.8) is typical of Enterobacteriaceae and related bacteria. According to Zajic *et al.* (1978), such reaction were also found in some facultative *Bacillus* under anaerobic conditions.

The *E. coli* reactions, occurs in two stages (Zajic *et al.*1978). In the first stage pyruvate is converted to acetyl-CoA and formate by pyruvate: formate lyase according to the eqation :

$$\text{Pyruvate} + \text{CoA} \longrightarrow \text{acetyl-CoA} + \text{formate}$$

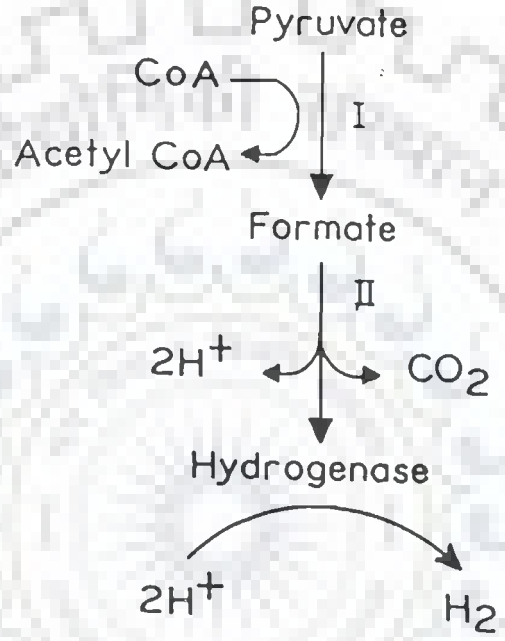

Fig. 2.8 : Anaerobic hydrogen production by the *Escherichia coli* system.

I : Pyruvate : formate lyase

II : Formate dehydrogenase

Although some authors (Huang *et al.*1985) believe that "phosphoclastic" reaction occurs by the production of acetylphosphate and formic acid according to the equation :

$$\text{Pyruvate} + H_3PO_4^- \longrightarrow \text{acetyl phosphate} + \text{formate}$$

Under aerobic conditions, pyruvate : formate lyase is replaced by pyruvate dehydrogenase (Knappe *et al.* 1974). These two alternative enzyme systems accomplish the transformation of pyruvate in to acetyl-CoA and formic acid (Knappe *et al.* 1969). Pyruvate : formate lyase appears in aerobically grown cells within a few minutes upon removal of oxygen (Knappe *et al*,1974).

Hydrogen is formed when formate is cleaved by a formate hydrogenlyase enzyme system (formate dehydrogenase & hydrogenase)

to give CO_2 & H_2.

$$\text{Formate} \longrightarrow CO_2 + H_2$$

Formate hydrogenlyase is a membrane bound multienzyme system consisting of formate dehydrogenase 'H' (after H_2 formation) and hydrogenase linked by intermediate electron carriers (O' Hara *et al.*1967). These electron carriers were identified as cytochrome reductase and cytochrome C_{552} by Wimpenny *et al.*(1963). Although latter studies concluded that cytochromes are not involved in H_2 formation from formate (Ruiz-Herrera & Alvarez, 1972; Douglas *et al.*1974).

The enzymes of the *Clostridium* and *E. coli* systems are not the same. When formate dehydrogenase enzyme from *E. coli* was mixed with the hydrogenase

enzyme from *Clostridium pasteurianum*, no hydrogen was produced (Huang *et al.* 1985).

It has been observed, that in pure cultures, only the reducing power ($NADH_2$) derived from pyruvate via ferredoxin can be released as hydrogen (Schlegel & Schneider, 1985). In appropriate mixed cultures the reducing power of $NADH_2$ and formate can be released as hydrogen.

A third biochemical chain for the production of hydrogen is only found in *Desulfovibrio desulfuricans*. It can catalyze H_2 evolution from $Na_2S_2O_3$ in the presence of cytochrome C_3 or methyl viologen. *D. desulfuricans* hydrogenase shares features with both the *Clostridium* and *E. coli* systems, but differs from both. While structurally similar to the Clostridial hydrogenase, the *D. desulfuricans* hydrogenase reaction does not take place in the presence of ferredoxins ; and as in the *E. coli* system, formate is produced during pyruvate degradation (Huang *et al.* 1985).

D 2.11 HYDROGEN PRODUCTION FROM WASTES BY MICROBIAL FERMENTATION :

In studies of gas production by microbial fermentation of wastes, the emphasis has been on methane. The fact thet hydrogen is also produced in such processes has been largely ignored in practical applications.

Before reviewing the literature on such a subject, let us assess some of its limitations which hindered its widespread applications.

1. Hydrogen is one of only two energy-rich gases produced by microbes which can utilize organic wastes. But unlike methane, however, hydrogen

is not a significant product of organic degradation in the natural environment (Ormerod, 1983).

2. Although it is produced at high rates in some environment, it is normally consumed as it is produced and does not accumulate. So an important constraint on the industrial production of hydrogen from wastes is that hydrogen consuming microbes should be excluded from the reaction vessel (Bollinger *et al.*1985) i.e. waste stream needs tobe sterilized before it enters the reaction vessel.

3. A major problem with microbial hydrogen production via fermentation is that most-microorganisms can ferment substrates only to acetate, CO_2 and H_2 at low partial pressures of hydrogen (Thauer *et al.*1977). At higher concentrations the production of hydrogen is inhibited and other products accumulate (Mah,1982). However, this negative feed-back inhibition effect can be overcome by continuous removal of hydrogen (Archer & Thompson, 1987).

4. Fermentation leads to the production of 4 moles of hydrogen from 1 mole of glucose (Thauer, 1977).

$$C_6H_{12}O_6 + 2\ H_2O \longrightarrow 2\ CH_3COO^- + 2\ H^+ + 2\ CO_2 + 4\ H_2$$

Energetically speaking, even if 4 mol of hydrogen per mol of glucose consumed could be maintained in an industrial process, it only represents a recovery of 33% of the glucose energy (Thauer,1977). This means waste once processed still contains a substantial organic load.

Considering all these limitations, the conclusion drawn, that hydrogen production from wastes via fermentation is not, at the present time, feasible on an

industrial scale, sounds to be reasonable (Archer & Thompson,1987). But on the other hand enormous amount of scientific developments to tackle major scientific problems and dependence on replenishable resources for energy conservation have made other workers to think differently (Zajic *et al.*1979; Huang *et al.* 1985; Tanisho *et al.*1987; Roychowdhury *et al.*1988). All these authors keep an optimistic view, that fermentation is most likely the technique to be adopted for industrial production of hydrogen and all mention that a good deal of work remains to be done in order to optimize microbial hydrogen production and develop an economically feasible process.

Most of the work, has been concentrated on checking the potential of different microorganisms for hydrogen production from glucose, which is the major breakdown product of organic wastes (Zajic *et al.*1978). The gaseous end products of glucose degradation by various H_2-producing microorganisms is shown in Table 2.7. Wide diversity in H_2-producing capability can be seen among these microorganisms.

There is only one report of an organism, which has the capacity to generate 4 moles H_2 from glucose but this cellulolytic organism, isolated from the gut of starving Zebu in Kenya, has since been lost (Hungate,1974; Archer & Thompson, 1987). This amount (4 moles H_2) is theoretically equivalent to 33% of the combustible energy of organic compounds (Thauer,1977).

Greater yields of H_2 (4 moles) were also reported (Iannotti *et al.*,1973) when *Ruminococcus albus* was grown in coculture with *Vibrio succinogenes.*

TABLE 2.7: THE GASEOUS-END PRODUCTS OF GLUCOSE DEGRADATION BY SOME STRAINS OF H_2-PRODUCING MICROORGANISMS

ORGANISM	REFERENCE	GASEOUS END PRODUCTS Mol/mol SUBSTRATE	
		H_2	CO_2
Escherichia coli	Barker (1961)	0.75	NR
Bacillus macerans	De Ley, (1962)	1.35	2.15
B.polymyxa	De Ley, (1962)	0.75	2.03
	& Barker, (1961)	0.7-1.7	NR
Clostridium butyricum	De Ley, (1962)	2.33	1.96
	Zeikus, (1980)	2.35	
C.aceto-butylicum	De Ley, (1962)	1.4	2.2
C.perfrin gens	Wood, (1961)	2.14	NR
Serratia	Neish et al. (1948)	0.60	1.5
Ruminococcus albus	Iannotti et al.(1973)	2.6	2.0
	& Zeikus, (1980)	2.57	NR
R.albus - Vibrio succinigenes	Iannotti et al.(1973) & Hungate, (1974)	4.0	2.0
Enterobacter limosum	Barker, (1961)	0.74	NR
Enterobacter aerogenes	Tanisho et al.(1987)	1.00	NR

High levels of hydrogen evolution capacities were mostly observed with strict anaerobes like *Ruminococcus albus*, different species of *Clostridium* (≤ 2.5 moles). The maximum quantity of hydrogen gas reported to evolve per molecule of glucose among the *E. coli* or related organisms is one (Zajic *et al.*,1978).

Bacillus species have been reported to produce hydrogen in the range of 0.7-1.7 mole / mole of glucose fermented (Kondratieva & Gogotov, 1983).

The recent studies conducted by Roychowdhary and his coworkers has been the only report on the production of hydrogen by microbial fermentation from natural sources rich in either pure sugars or polysaccharides like sugarcane juice, corn pulp and saccharified paper substrates. They were able to evaluate the potentialities of these substrates for H_2 generation by mixed microbial flora from sewage and pure cultures of coliform bacteria atminiaturized level (Roychowdhury *et al.*,1988). They have suggested that the microbial fermentation of sugars and carbohydrate rich biomass should be investigated further as a potentially practical source of hydrogen.

The review by Zajic *et al.* (1978) concluded that following are examples of some industries which could achieve both cost and waste reduction through the utilization of waste materials for hydrogen gas production : a) grain industries, wastes such as chaff, grain dust, water or rodent spoiled grains; b) meat processing industries; c) vegetable oil and margarine manufacturers; d) pulp and paper industries through certain cellulolytic bacteria which can produce hydrogen could be used for the conversion of pulp and paper wastes into H_2 with the reduction of COD (Chemical

oxygen demand) and BOD (Biological oxygen demand) values in the final effluents, contributing to the environmental acceptibility.

2.12 EFFLUENT TRTEATMENT FOR HYDROGEN PRODUCTION BY PHOTOSYNTHETIC BACTERIA :

Extensive literature on algae and photosynthetic microorganisms generating H_2 have been published. Some of these have shown higher potential for H_2 production (4mole/ mole glucose) than via fermentation (Kosaric & Lyng, 1988) (Table 7). However, possible photosynthetic organisms require solar collectors, and engineering analysis has suggested that solar generators would be too costly and thus a cost-effective process is still to be worked out (Huang *et al.*,1985).

There exists, however, a considerable interest in industries in such a process. Waste water from sugar refineries may be suited to this form of treatment because of its high C:N ratio (Bollinger *et al.*,1985). This has been examined experimentally but has yet to be scaled up to an industrial application (Vincenzini *et al.*,1981; Zurrer & Bachofen, 1981). Other wastes which could be treated by photoreduction are those containing lactic acid, i.e. yoghurt and cheese waste (Salih, 1989). Zurrer & Bachofen (1979) have demonstrated continuous hydrogen production from yoghurt waste. This also awaits trial on an industrial scale.

But the literature available today indicates that fermentative hydrogen production has several advantages over photosynthetic hydrogen production (Tanisho *et al.*,1987) :

1. Rate of fermentative hydrogen production is always faster than that of the photosynthetic hydrogen production.

2. It is possible to produce hydrogen by fermentation all day long without light.

3. It is able to use photosynthetic products as substrates of the hydrogen evolution. This is an indirect use of solar energy.

4. It is also able to use industrial and /or agricultural wastes as substrates.

5. Metabolites except hydrogen can also be used.

E 2.13 FUTURE PROSPECTS OF MICROBIAL PRODUCTION BY ENHANCED PRODUCTION TECHNIQUES :

GENETICALLY ENHANCED PRODUCTION :

Perhaps the most significant advances in microbial production of hydrogen will be a result of genetic engineering. Considerable optimism must arise from the work of Karube *et al.*(1984), who cloned the hydrogenase gene of *E. coli* into a plasmid. Transformation of mutant *E. coli* with the plasmid carrying this gene restored hydrogenase activity.

The microbial H_2 production is a multistep process involving several enzymes. The monumental task will be to find out the limiting steps of this pathway and then improvement of that particular step using genetic engineering methods. Since the sequences of most of the enzymes participating is published (Kanayama *et al.*,1987;

Francis *et al.*,1990; Maupin *et al.*,1990), it should be easier to clone the particular gene in suitable vector and transfer it to desired organism.

It appears possible that nitrogenase and cellulase genes may be introduced into marine photosynthetic bacteria through recombination techniques (Matsunaga *et al.*,1986). Another approach would be to promote the growth of the organism with high hydrogen yield through genetic selection. However, direct genetic recombinant manipulation to increase hydrogen evolution is still more desirable.

The plasmids of several hydrogen producing microorganisms have been isolated and stability has also been studied (Nano and Kaplan, 1984).

The prospect for enhanced hydrogen production through genetic engineering is promising.

ENHANCEMENT BY IMMOBILIZATION :

A possible route for the microbial H_2 production and ensuing its stable and dependable supply could be the use of immobilized whole cells (Lee *et al.*, 1989). Both scientifically and industrially, immobilization of whole cells is now a well established method in the field of biotechnology (Chibata *et al.*, 1983). The process offers the following operational advantages associated with the use of immobilized enzymes together with a number of additional merits (Blanch, 1984 and D' Souza, 1989):

a) It eliminates enzyme purification and extraction steps.

b) Higher levels of enzyme activity can be attained after immobilization.

c) It offers the advantages of higher volumetric productivity than conventional fermentation systems.

d) There is a possibility of continuous operation because of prevention ofcell leakage,andhigher stability in general than immobilized enzyme systems.

e) Offers greater potential for multistep process and cofactor regeneration.

f) Greater resistance to environmental perturbations.

However, besides these advantages, the whole cell immobilization poses some limitations in its applications. The cell permeability and diffusional barrier of the cell wall, plasma membrane or subcellular membranes affect the ability of the substrate to reach appropriate enzymes and of product to diffuse out again. However, these have been shown to overcome partly by giving some chemical treatment with organic solvents (D' Souza & Nadkarni,1980; Deshpande *et al.*,1987). Another limitation has been the presence of multiple enzymes in the cell, which leads to unwanted side reactions. These can be obviated to some extent by inactivating such enzymes prior to or after immobilization of the cells (Yamamoto *et al.*,1974 a,b; Godbole *et al.*,1983).

Cells can be immobilized both in the viable and nonviable forms based on the application (D'Souza,1989). In the immobilization of nonviable cells, the multiplication of the cells is restricted whereas the enzyme activities remain intact. These find use in processes, which depend on one or more reaction steps without the need for Co-factor regeneration (Deshpande *et al.*,1987). The immobilization of viable cells is mostly employed in processes, which depend on multienzyme complex reactions,

Co- factor regenration pathways and where enzyme induction can be controlled by the environmental conditions. However, in these cases, leakage of cells due to overgeneration and consumption of carbon, energy and nitrogen sources by the living cells are disadvantages encountered (Fukui & Tanaka,1982).

There is a wide variety of methods which have beentried for immobilization of whole cells, in either viable form or nonviable form. But generally four different types of immobilization methods are commonly in use, such as adsorption, entrapment, covalent binding and cross linking (Messing,1981).

a) Adsorption of cells : Adsorption involves the formation of ionic and hydrogen bonds between the cell surface and the support, primarily determined by the charge distribution of carboxy and amino groups of the cell wall. It is a reversible process and the support may be recovered after the catalyst is denatured. Adsorption of cells on solid surfaces is probably the mildest immobilization technique available and in many instances is a natural phenomena (D'Souza,1989). The properties of the support determine the resulting behaviour of the system, cell loading is determined by the surface area of the support material and its charges and the variable pore size. Among the several supports listed are, gelatin, bricks, PVC, glass fibre pads and modified cellulose (Johnson and Ciegler,1969; Navarro,1976; Blanch 1984; D'Souza & Kamath,1988).

b) Entrapment of cells: This is the most frequently used method for immobilizing whole cell systems. The principle behind the entrapment technique is to form a polymeric network around the material to be trapped. The resulting gel must have sufficient porosity to allow the transport of substrates in and products out while retaining the cells, which have restricted its use to sub-

strates and products having low molecular weights. Various natural polymers like alginate, carragenan, cellulose, agar, hen egg white, gelatin, collagen and synthetic polymers like polyacrylamide and other acrylic polymers, photocross linkable resins etc. have been used for entrapment of whole cell systems (Tampion & Tampion,1987). Alginate has been extensively investigated for the preparation of immobilized viable cells whereas chemical polymerized acrylamide immobilization system has been suitable for nonviable cells (D'Souza & Nadkarni,1980; Deshpande *et al.*,1987; D'Souza, 1989).

c) Covalent binding : The technique of covalent coupling involves the creation of permanent chemical bonds. It has been extensively used in the immobilization of enzymes (Mosbach, 1976; D'Souza, 1989). However, the binding of whole cells to the matrix with commonly used coupling agents has not been very successful, because of the toxic nature of the agents. Low cell loading & leakage of the newly formed cells renders its wide application unattractive.

d) Immobilization through cross linking : Enzymes and cells can be immobilized by cross-linking with bifunctional reagents such as glutaraldehyde, cynuric chloride, isocyanates etc (Mattiasson, 1983; Tampion & Tampion,1987). Cells have been immobilized in the presence of an inert protein like hen egg white, gelatin or cabbage using glutaraldehyde as the cross-linker (Kaul *et al.*,1981; D'Souza,1989). The technique is similar to the entrapment method and has the limitation and advantages of both entrapment and covalent binding.

Application of whole cell immobilization for H_2 production :

The technioque of immobilizing enzymes and whole cells within or on a solid matrix has been applied to a number of biological hydrogen producing systems (Karube *et al.*,1976). Kanayama *et al.*(1988) repeated the continuous production of H_2 by the immobilized cells of recombinant *E. coli*. Egan and Scott (1978) have observed the generation of H_2 with isolated immobilized chloroplasts.

Increased and longer duration of H_2 evolution has been observed with immobilized cells of *Rhodopseudomonas palustris* (Vincenzini *et al.*,1982, 1986).

Application of immobilized cells for effective treatment of industrial wastes with simultaneous production of H_2 yields have been demonstrated (Vincenzini *et al*, 1981). Mitsui *et al.*(1985) established the production of H_2 from the orange processing plant waste by the immobilized cells of *Chromatium* and *Rhodopseudomonas*. Similarly the use of such photosynthetic bacteria like *Rhodospirillum rubrum* and cyanobacteria for H_2 production from waste water treatment system have been reported (Muallem *et al.*,1983; Karube *et al.*,1984). Phlips and Mitsui (1986) have reported an ultimate yield of H_2 twice as large for immobilized cells of *Oscillatoria* species over free cell suspensions. Matsunaga *et al.* (1980) have reported the production of H_2 under aerobic conditions by an obligate anaerobe *Clostridium butyricum*. Effect of different vitamins on the immobilized cells of *C. butyricum* has been observed to increase the efficiency of H_2 production even after repeated use of the cells (Karube *et al.*,1982).

Lee *et al.*(1989), presented the continuous system for the production of H_2 from glucose by calcium alginate immobilized cells of *Rhodospirillum rubrum*.

The enhanced and stable H_2 production by immobilization of *Halobacterium halobium* MMT_{22} coupled to *E. coli* has been recently reported by Taqui Khan & Bhatt (1990).

These studies suggest immobilization as, a possible technique for the continuous and large scale production of H_2.

CHAPTER - III

METERIALS AND METHODS

MATERIALS AND METHODS

A. MATERIALS

3.1 FEED STOCK MATERIAL:

APPLE POMACE (*Malus pumila*) :

It was procured from M/S H.P.M.C., Fruit processing Plant Parwanoo, H.P. The material was freshly dried in the sun and transferred to the laboratory for experimental work. It was stored in a dry place at room temperature.

VEGETABLE WASTE :

Vegetable waste was collected from the Azad Pur Marketing Corporation Complex, Delhi. It consisted largely of cauliflower leaves and stalks (*Brassica oleracea* L. var. botrytis) and raddish leaves (*Raphanus sativus L.*) and occasionally contained rotten cabbage (*B. olerace* L. var. capitata). It was stored at 4°C.

DAMAGED WHEAT GRAINS :

Damaged wheat (*Triticum aestivum*) grains unfit for human consumption, have been categorized as: a) cattle feed, b) poultry feed, c) industrial type, d) manure type and e) dumping type by The Food Corporation of India (FCI), India (FCI Manual 1978).These grains were procured from FCI and the types employed in our study were cattle feed, poultry feed and dumping type. The dirty grains were cleaned as follows: washing under running tap water until no dust particles were

visible. The dust free grains were sun dried and stored in a dry place. These cleaned grains were crushed into powder form before utilization.

Other Substrates:

Fresh rice, maize and wheat grains were purchased from the local market.

3.2 CHEMICALS :

All chemicals used in the study were of analytical grade and were mostly purchased from Merck, BDH and Glaxo (India). Test combination kit for Formate estimation was obtained from Boehringer Manheim (W. Germany). Most of the chemicals for media preparation were from Difco laboratories (USA) and Himedia. Few standards used in this study were purchased from Sigma Chemical Co. (USA).

Gases of high purity (IOLAR-3) grade were supplied by Indian Oxygen Limited, India. Ethylene gas standard of EDT, Research, London was used.

B. METHODS

3.3 MICROBIAL CULTURES :

3.3.1 Methanogenic Bacterial Culture :

a) Cattle dung was the source of bacteria. Fresh cattle dung slurry was used as the unenriched methanogenic culture.

b) Cattle dung slurry incubated at 40°C for 20 days was used as the enriched methanogenic culture.

3.3.2 Hydrogen Producing Bacterial Cultures :

a) Mixed microbial population : Undefined mixed culture was obtained from the cattledung in the following manner : freshly prepared cattledung slurry (3% Total Solids) was filtered through a fine muslin cloth, to remove the debris and heavy particles. The filtrate was allowed to settle down for 5 min to remove any sedimented matter. The supernatant was then centrifuged at 8000 rpm for 25 min. The pellet was collected and given a treatment by which the hydrogen generating bacteria get enriched (Joshi et al.,1986). The pellet extracted from 1 l initial 3% cattledung slurry was diluted upto 100 ml by adding distilled water.

b) Axenic cultures and growth conditions :

i) *Bacillus licheniformis* & strain JK2 : 33 axenic cultures of hydrogen producing bacteria were isolated in our laboratory (Jain, 1992) from hydrogen producing apple pomace fermenter where cattle dung was used as a source of inoculum. Based on the higher activities for hydrogen production from glucose solution, these two organisms were selected for further studies.

The organisms were isolated on defined medium containing

Peptone	5.0 g
Beef extract	3.0 g
Yeast extract	0.2 g
$MgSO_4$	1.5 g
Na_2SO_4	1.5 g
$Fe(NH_4)_2(SO_4)_2$	0.1 g
Glucose	5.0 g
Agar	15.0 g
pH	7.0 with NaOH
Tap water	1 l

The cultures were incubated aerobically at 40°C for 24 h.

Based on the cell morphology, different biochemical tests, fatty acid analysis, phase contrast and electron microscopic studies, the organisms were grouped into the genus *Bacillus.* One of the organism could be identified as *Bacillus licheniformis* which was latter on confirmed from Microcheck Incorporation (USA).

The organisms were subcultured periodically and a pre- culture for conducting the experiments was made by transferring the bacterial colony grown on an agar plate and incubated into pre-culture liquid medium. The cultures were then incubated aerobically at 40°C for 48 h. Cells were harvested by centrifuging the inoculated medium at 10,000 rpm for 20 min, washed twice with sterile saline (0.9% NaCl) before storing them at 4°C in same saline solution for further experiments.

3.3.3 Microscopic studies of mixed and pure hydrogen producing bacterial cultures :

Different microscopic studies such as light microscopic, phase contrast and electron microscopic were done for pure cultures where as for mixed culture only electron microscopic studies were done.

Transmission Electron Microscopy : A drop of fresh broth culture was mixed with a drop of 1% phosphotungstic acid (containing 0.2% bovine serum albumin as a spreading agent) to prepare the negative stain and placed on a Formvar support on a 200 mesh copper grid. After 5-10 minutes the drop in the grid was removed from the edge with a piece of filter paper and the grid was viewed in a Philips 201 C transmission electron microscope. The observations were recorded on 36 mm B/W photographic film.

Scanning electron microscopy : The cells were lyophilized on

Unitop 600 SL, Freeze mobile virtis lyophilizer. The dried cell powder was uniformly sprinkled over a parafilm fixed on the copper blocks. The excess and unfixed cell powder was removed by gentle tapping of the copper blocks. In a "sputter coating" process the fixed cells were covered with a gold layer to increase their electrical conductivity. The gold coated cells were then scanned under the JEOL, JSM-840 scanning electron microscope. The observations were recorded on 20 mm B/W photographic film.

3.4 Miniaturised incubation assembly set ups:

Three different size, incubation assemblies were fabricated indigenously with 25 ml, 50 ml conical flasks and 300 ml BOD bottle respectively. A ground glass neck of B-12 joint was fixed on 25 ml flask where as B-14 joints were fixed on 50 ml and 300 ml assemblies. A ground glass stopper with similar size to fit in these necks were provided with two glass side arms (ID = 3 mm) at the top (Fig. 3.1& 3.2). One of the side arms (out let) was connected with latex tubing for collecting the gases in an inverted measuring cylinder or graduated test tube. The gases were collected by the displacement of water. Another side arm was provided for flushing N_2 gas (an inlet) through the incubation mixture. It was closed at the top after flushing N_2 gas in order to make the system anaerobic and airtight.

Hereafter, these three incubation assemblies will be referred to as follows :

a) 25 ml of incubation assembly set up = IA_{25}
b) 50 ml of incubation assembly set up = IA_{50}
c) 300ml of incubation assembly set up = IA_{300}

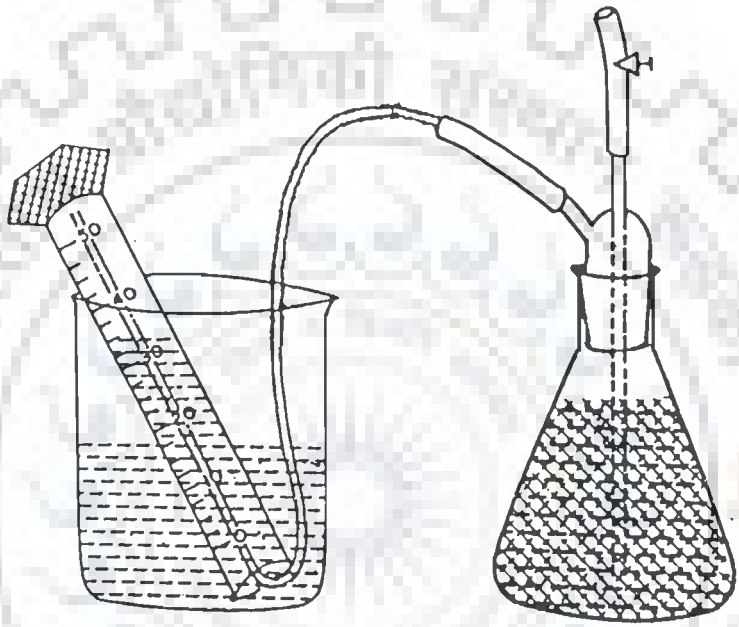

Fig. 3.1: Miniaturised incubation assembly of 25 / 50 ml capacity with gas collecting system.

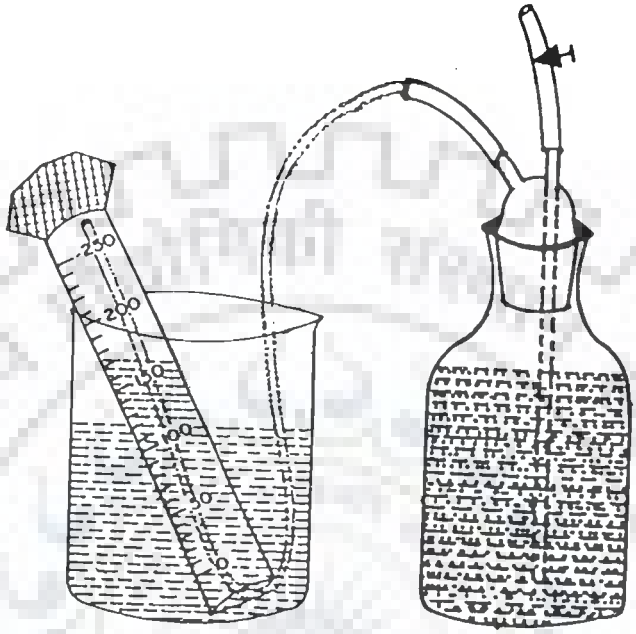

Fig. 3.2: Miniaturised incubation assembly of 300 ml capacity with gas collecting system.

3.5 Incubation conditions :

The following experimental conditions were standardized in this work and followed in all the assays conducted herein (unless otherwise specified).

a) Anaerobic conditions: The anaerobic conditions were maintained in the incubation assembly by flushing with N_2 for 5 min. at a flow rate of 50 ml/min.

b) Neutralization: The incubation mixture was neutralized with 1N NaOH everyday till the gas production ceased.

c) Temperature: All the incubations were done at 40°C.

d) Incubation time: The mixture was incubated till the gas production ceased.

e) Gas analysis: Gases were analysed everyday for their H_2, CO_2, CH_4, N_2, O_2, and H_2S content.

f) Gas collection: Gases were collected over water, maintained at pH 3.0, to avoid absorption of CO_2.

C. ANALYTICAL METHODS

3.6 Determination of C, H, N content in the biological waste material :

The dry matter was ground to a fine powder which was then dried overnight at 100 ± 5°C to remove moisture. The samples were immediately transferred into vacuum desiccator to avoid absorption of water. The dry samples were analysed for C,H, and N content on Heracus CHN-Rapid (Automatic Elemental) analyzer.

3.7 Determination of Chemical Oxygen Demand (COD) :

COD (mg/l) of thoroughly mixed samples was determined according to the method described in the APHA manual (1989).

Principle : Most of the organic matter is converted into carbon dioxide and water when boiled with a mixture of potassium dichromate and sulfuric acid. A sample is refluxed with a known amount of potassium dichromate in sulfuric acid medium and the excess of potassium dichromate is titrated against ferrous ammonium sulfate. The amount of dichromate consumed is proportional to the oxygen required to oxidize the organic matter.

Reagents :

1. Standard Potassium Dichromate (0.25 N)

   It was prepared by dissolving 12.259 g of potassium dichromate (dried at 130°C for 24 h) in distilled water. 120 mg sulfamic acid was added in it and diluted to 1 l.

2. Sulfuric acid :

   22 g silver sulfate was added to 2.5 l of concentrated H_2SO_4 and kept overnight for dissolution. The contents were shaken well before use.

3. Standard Ferrous Ammonium Sulfate (0.1 N) :

   It was prepared by dissolving 39 g of $Fe(NH_4)_2(SO_4)_2$. 6 H_2O in distilled water and 20 ml concentrated H_2SO_4 was added. The mixture was cooled and diluted to 1 l. This solution was standardized daily against the standard $K_2Cr_2O_7$.

4. Ferroin Indicator :

1.485 g of 1,10-phenanthroline monohydrate and 695 mg of $FeSO_4.7H_2O$ was dissolved in water and diluted to 100 ml.

5. Mercuric sulfate crystals ($HgSO_4$).

Standardization of Ferrous Ammonium Sulfate :

10.0 ml of standard $K_2Cr_2O_7$ solution was diluted to 100 ml with distilled water and 30 ml concentrated H_2SO_4 was added to it. The contents were cooled and 3-4 drops of ferroin indicator were added followed by titration with ferrous ammonium sulfate till the colour changed to wine red.

$$\text{Normality of ferrous ammonium sulfate} = \frac{10 \times 0.25}{\text{ml } Fe(NH_4)_2(SO_4)_2}$$

Procedure :

0.2 g of $HgSO_4$ was placed in a reflux flask and 20 ml of diluted sample (1:100 to 1:1000) was added to it. The contents were mixed well and 5 ml of standard $K_2Cr_2O_7$ was added, followed by slow addition of 15 ml of sulfuric acid (containing silver sulfate). The mixture was thoroughly mixed (if the green colour developed, it was discarded and a fresh sample with higher dilution was taken). The raction mixture was refluxed for 2 h. After the mixture was cooled, it was diluted with 40 ml of distilled water by washing down the condenser. This diluted mixture was then titrated against standard ferrous ammonium sulfate using ferroin as indicator. The end point was reached as the colour changed sharply from green blue to wine red.

The reagent blank was refluxed simultaneously with the sample under identical conditions using distilled water.

Calculations :

$$\text{COD (mg/ l)} = \frac{(a-b)\ N \times 8000}{v}$$

Where, a = ml $Fe(NH_4)_2(SO_4)_2$ required for blank.
b = ml $Fe(NH_4)_2(SO_4)_2$ required for sample.
N = Normality of $Fe(NH_4)_2(SO_4)_2$.
V = Volume of the sample.

3.8 Determination of Total solids (TS) and Organic solids (OS) :

Estimation of freshly collected samples was carried out as described by Lane (1984 a).

Thoroughly mixed samples were weighed in a crucible and dried at 100 ± 5°C for 24 h to estimate the total solids (TS). The dried samples were then ignited at 600 ± 25°C for 2 h to determine its ash and organic solid (OS) content.

3.9 Determination of dry weight of the cells :

The dry weight of the cells was determined by overnight drying of the cells at 80°C on Whatman filter paper.

3.10 Determination of whole cell Protein :

The cell extract was produced by the method of Herbert *et al.*,(1971) and protein concentration was determined by the method of Lowry *et al.*, (1951).

3.11 Estimation of glucose :

Glucose was estimated by the anthrone method (Scott & Melvin, 1953).

3.12 GAS AND LIQUID ANALYSIS (Gas Chromatography):

3.12.1 Analysis of H_2, CH_4, CO_2, N_2, O_2 and H_2S:

Gas samples (0.1 ml) were drawn with 1 ml airtight syringes and analysed on a gas chromatograph (GC 5700, Nucon Engineers). A Molecular sieve column of s.s., 1.8 m long and 2 mm inner diameter, at ambient temperature was used for analysing H_2, O_2, & N_2, whereas CH_4, CO_2 and air were analysed on Porapak -Q column (s.s., 1.8 m long & 2 mm inner diameter). Argon at a flow rate of 27 ml/ min was used as carrier. Hydrogen sulphide (H_2S) was estimated by passing a known volume of gas sample through 10% lead acetate solution. The volume of the gas absorbed by the lead acetate solution was used to calculate the quantity of H_2S present in the gas sample. Gas standards were passed before each set of gas analysis. The gases were identified on the basis of the retention time and the percentage was calculated by computing with the standard peak heights (Fig. 3.3 & 3.4).

3.12.2 C_2H_2 and C_2H_4 analysis:

C_2H_4 formed was analysed on Gas Chromatograph (GC 5700, Nucon Engineers) by standard procedures using Flame Ionization Detector mode at an oven temperature of 90°C and injector and detector temperature of 110°C. 0.5 ml of the gas sample was injected in Porapak-T Column of s.s., 2m long with an ID of 2.0 mm which was carried by N_2 gas at a rate of 27 ml / min. The concentration

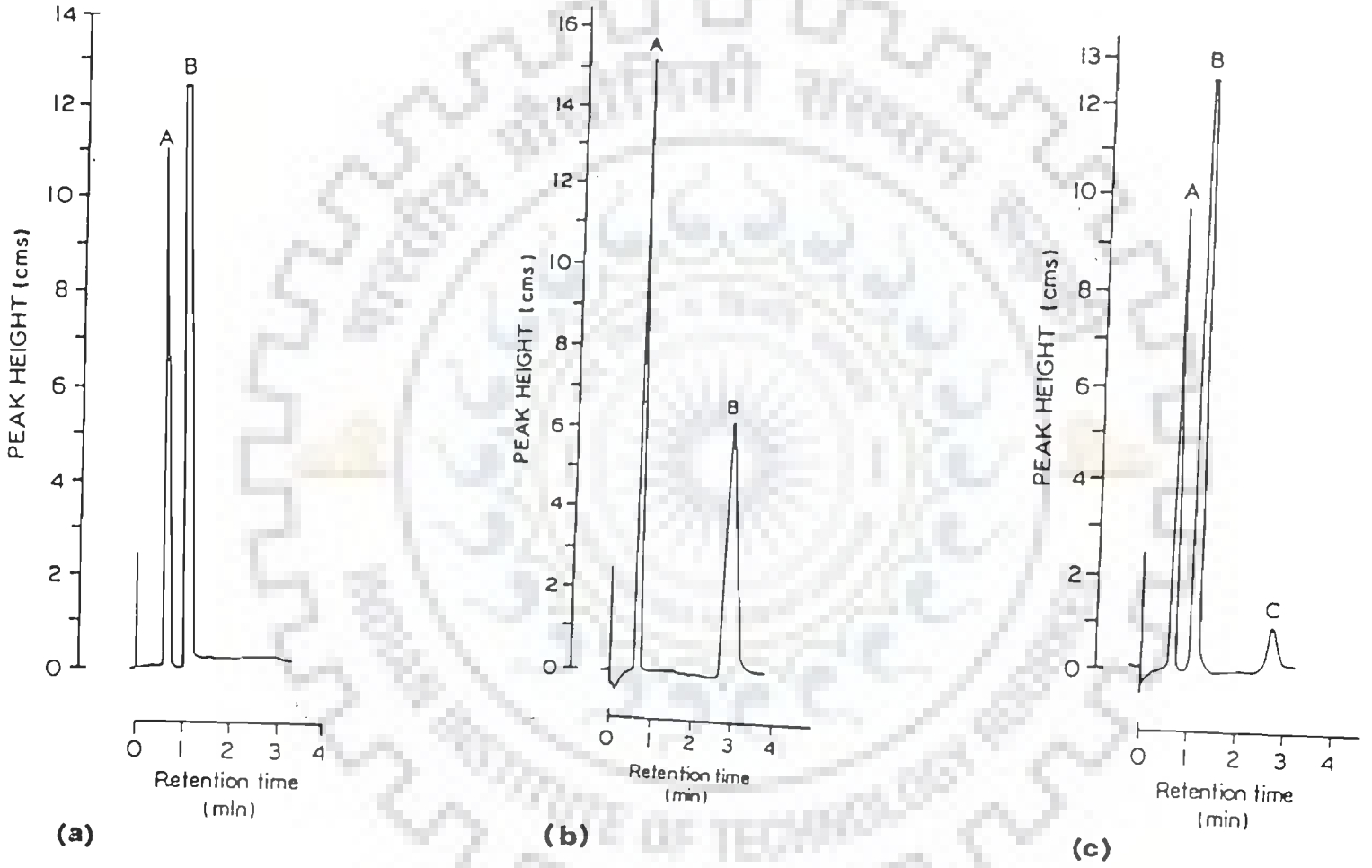

Fig. 3.3: Gas chromatograph analysis showing (a) standard of CH_4, containing A: air B: CH_4; (b) standard of CO_2, containing A: air & B: CO_2; (c) sample from an experiment containing A: air, B: CH_4 and C: CO_2. (TCD mode; attenuation: 8; column: porapak-Q , carrier gas: Ar).

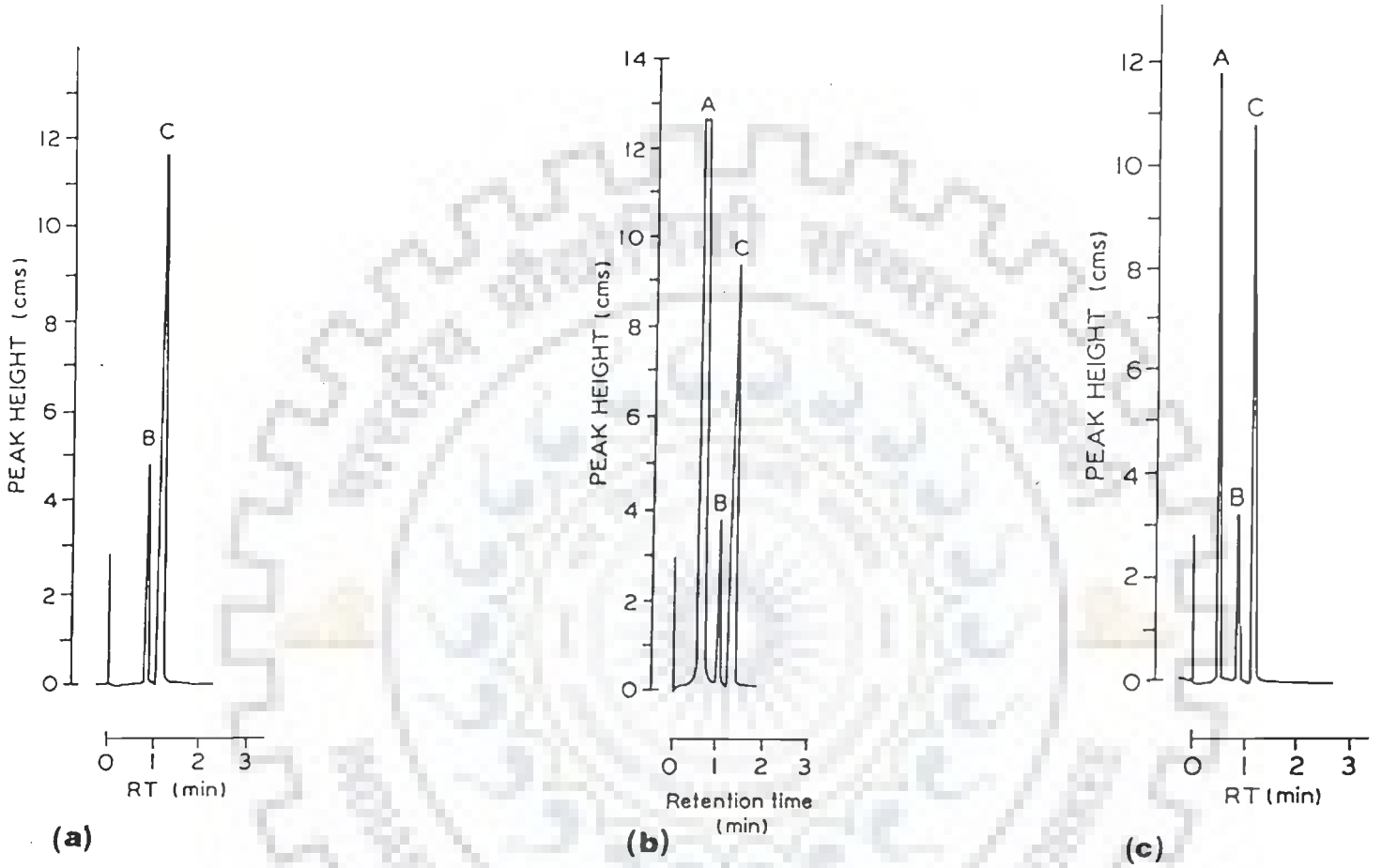

Fig. 3.4: Gas chromatograph analysis showing (a) standard of air containing B: O_2, C: N_2 (b) standard of H_2, containing A: H_2, B: O_2 & C: N_2; (c) sample from an experiment containing A: H_2, B: O_2 and C: N_2. (TCD mode; attenuation: 16; column: Molecular Sieve; carrier gas: Ar).

of ethylene was calculated by computing with the standard ethylene gas sample of 105 vpm (Fig. 3.5).

3.12.3 Acid and alcohol analysis :

The samples were analysed for acids and alcohols on a Gas Chromatograph (GC 5000, Nucon Engineers) using Flame Ionization detector mode. 2 μl of the samples were injected in Chromosorb- 101 glass column [4' (l) x 3mm (ID)]. N_2 gas was used as carrier at a rate of 27 ml/min. Acid analysis was done at an oven temperature of 200°C and injector and detector at 220°C, where as alcohol were analysed at an oven temperature of 130°C and injector and detector at 150°C. The concentrations of different acids and alcohols were caliberated by using standards of acids and alcohols of known concentrations, and comparing the peak areas and retention time (Fig. 3.6 - 3.8).

3.13 FORMATE ESTIMATION :

Formate concentration was determined by the UV method of Hopner and Knappe, as described in the methods of enzymatic food analysis catalogue of Boehringer (Anonymous,1989), using a test combination kit of M/S Boehringer. The difference between the amount of formate fed and the formate unconsumed reveals the amount of formate fermented. The theoretical H_2 gas formation was calculated from it and compared with the experimental data.

Principle : Formic acid (formate) is oxidized in the presence of formate dehydrogenase (FDH) by nicotinamide adenine dinucleotide (NAD) quantitatively to bicarbonate.

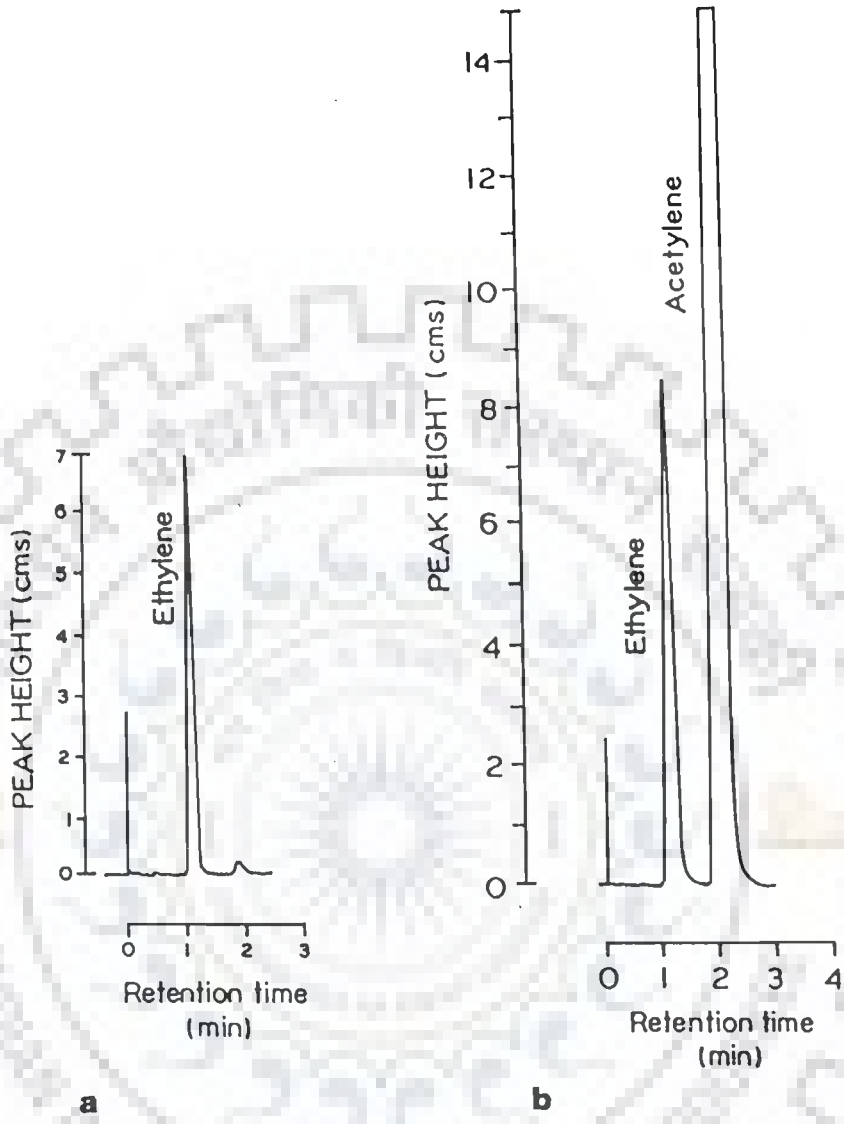

Fig. 3.5: Gas chromatograph analysis for nitrogenase activity (acetylene reduction assay). (a) standard C_2H_4 sample; (b) assay sample of mixed microbial culture at 20 h incubation time on FID mode. (oven temperature: 90°C; injector & detector temperature: 110°C; attenuation:16 for (a) and 160 for (b); sensitivity: 1000 and sample volume: 0.5 ml.)

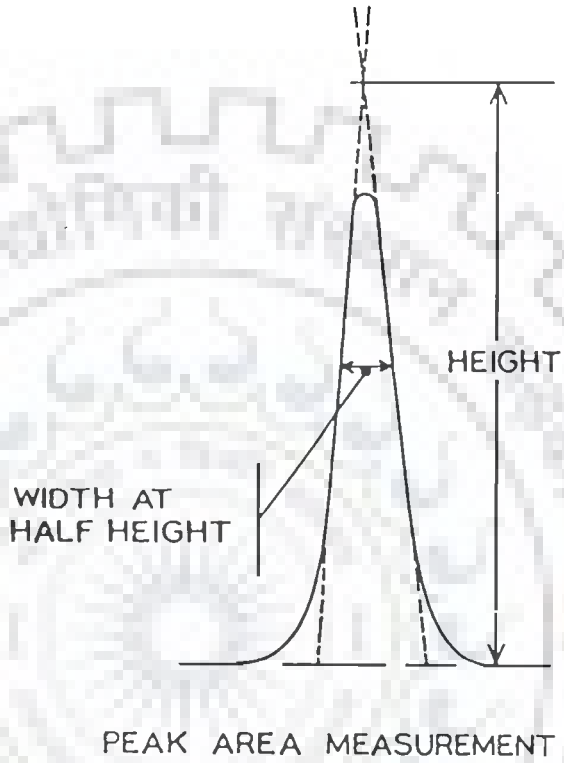

Fig. 3.6: Measurement of peak area of Gas chromatograph.

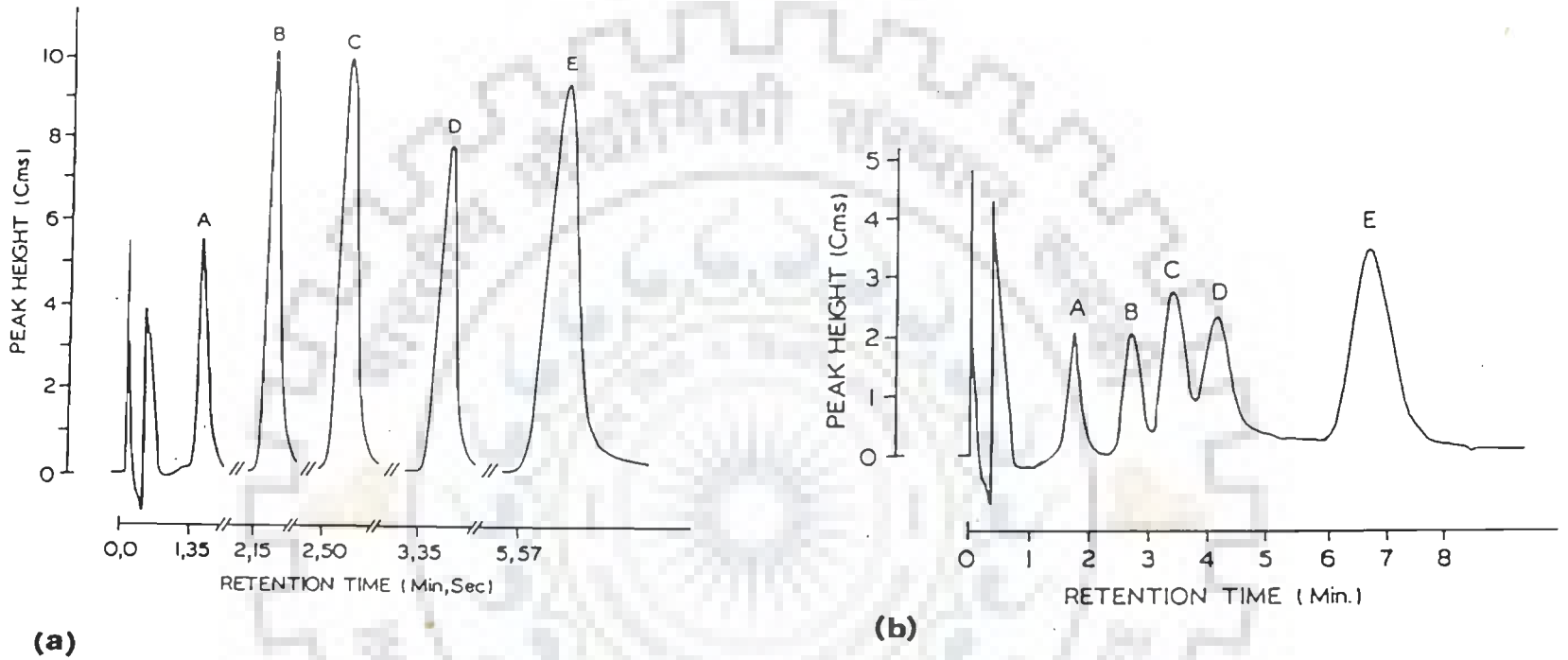

Fig. 3.7: Gas chromatograph analysis of volatile fatty acids showing (a) individual standards of A: 0.2% acetic acid; B: 0.2% propionic acid: C: isobutyric acid; D: 0.2% n-butyric acid and E: valeric acid and (b) sample from an experiment containing different volatile fatty acids on FID mode. (oven temperature: 200°C; injector & detector temperature: 220°C; attenuation: 16; sensitivity: 100 and sample volume: 2 μl).

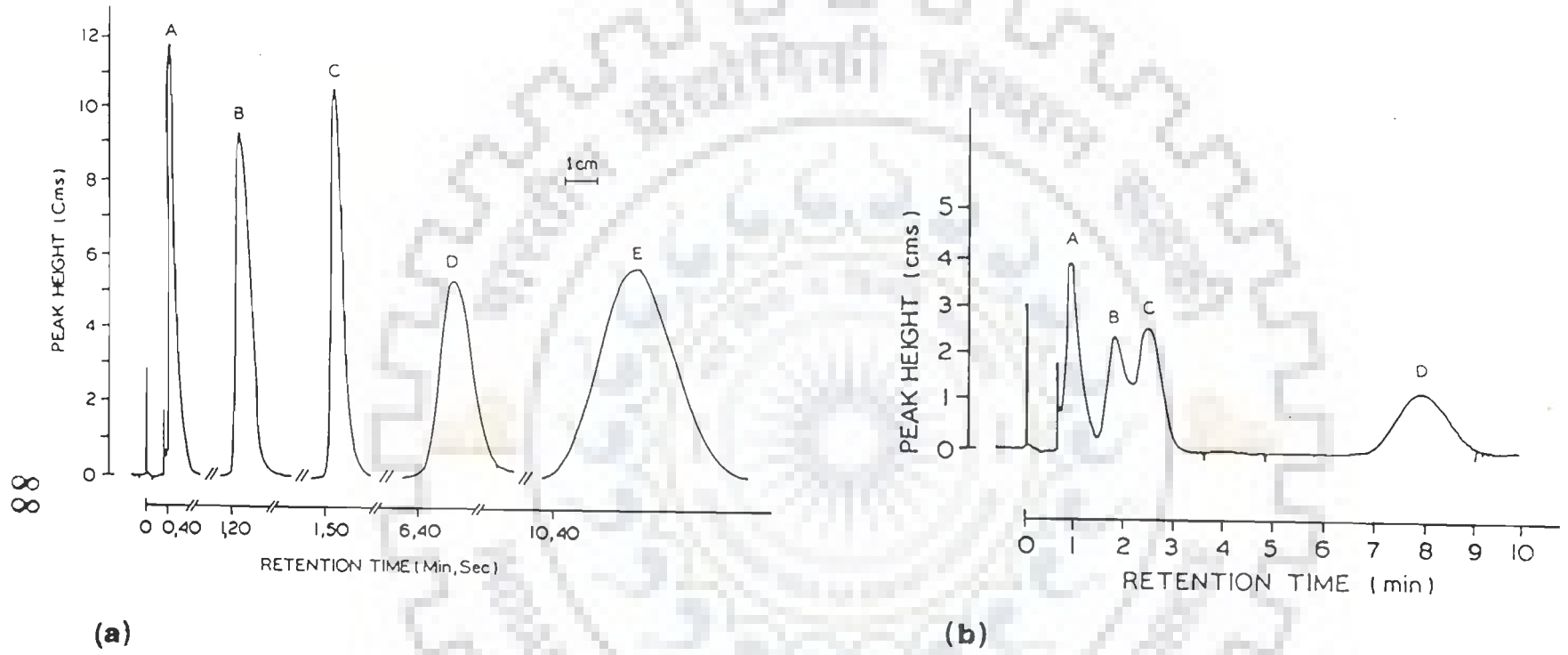

Fig. 3.8: Gas chromatograph analysis of alcohols showing (a) individual standards of A: 0.2% methanol; B: 0.2% ethanol; C: 0.2% propanol; D: 0.2% butanol; and E: 0.2% pentanol and (b) sample from an experiment containing different alcohols on FID mode (oven temperature: 130°C; injector and detector temperature: 150°C; attenuation: 16; sensitivity: 100 and sample volume: 2 μl).

$$\text{Formate} + NAD^+ + H_2O \xrightarrow{FDH} HCO_{3-} + NADH + H+$$

The amount of NADH formed is stoichiometric with the amount of formate reduced. The increase in NADH is measured by means of its absorbance at 340nm.

Reagents : The test combination contains the following :

i) Potassium phosphate buffer, pH 7.5; (20 ml)

ii) NAD, Li salt, lyophilised (420 mg)

iii) Formate dehydrogenase, lyophilised, 80 U (200 mg)

Preparation of solutions :

(a) 420 mg of NAD, was dissolved in 20 ml of potassium phosphate buffer using a magnetic stirrer. This mixture was stable for 2 weeks at +4°C.

(b) 200 mg of formate dehydrogenase was dissolved in 1.2 ml redistilled water. This mixture was stable for 20 days at +4°C, or for longer periods at -20°C. The solution was brought to 20-25°C before use.

Procedure :

The samples were diluted sufficiently (1:20 to1:100) to yield roughly formic acid concentration between 0.01 mg to 0.1 mg/ ml.

Sample : 1.9 ml of redistilled water was added to 1.0 ml of NAD solution [solution No. (a)], followed by 0.1 ml of test sample in a quartz cuvette.

Blank : 2.0 ml of redistilled water was added to 1.0 ml of NAD solution [solution No. (a)] in a separate cuvette.

The cuvettes were closed with parafilm and the contents were thoroughly mixed. The absorbance of blank and sample was read at 340 nm after 5 minutes (A_1).

The reaction was started by adding 0.05 ml of the enzyme solution [solution No.(b)] in both sample and blank cuvettes. The cuvettes were again closed and the contents mixed thoroughly. The reaction was allowed to complete for 20 minutes and the absorbance of blank and sample was read directly one after the other (A_2). The absorbance difference before and after completion of the reaction (A_2-A_1) was determined for both blank and sample (for blank difference = ΔA_b and for sample = ΔA_s).

Δ A formic acid is obtained by subtracting the absorbance difference of the blank from the absorbance difference of the sample.

$$\Delta A = \Delta A_s - \Delta A_b$$

At a standard absorption co-efficient of 6.3 l / mmol / cm for NADH at 340 nm, the concentration of formate can be calculated as:

$$C = 0.329 \times \Delta A \text{ (g/l)}$$

3.14 DETERMINATION OF ENZYMATIC ACTIVITIES:

3.14.1 Amylolytic Activity :

The amylolytic activity of mixed microbial culture during fermentation was estimated by the method of Bernfeld, (1951).

α - amylase catalyses the hydrolysis of, 1-4 links of starch with the production

of reducing sugars. The reaction is followed by measuring increase in the reducing sugars using Dinitrosalicylic acid (DNSA) reagent when its alkaline solution is reduced to 3-amino, 5-nitrosalicylic acid (Miller, 1959).

The results are reported as units of amylase, where one amylase unit corresponds to the amount of enzyme required to release 1 μmol of reducing sugar from 1% starch solution in 1 h at 40°C and at pH 6.7.

3.14.2 Hemicellulolytic (Xylanase) Activity :

Xylanase activity was determined by incubating 0.5 ml of Larch wood xylan (1%) in McIlvaines citrate buffer (pH 6.0) with 0.5 ml of enzyme sample for 30 min at 40°C. Concentration of reducing sugar released was determined by DNSA reagent as described by Miller (1959). The optical density value was calibrated with known concentration of reducing sugar.

One unit of enzyme is defined as the amount which catalyzes the release of 1 μmol of reducing sugar (as xylose) per minute at 40°C.

3.14.3 Cellulolytic Activity:

The exoglucanase [FPase (C_1) & β-glucanase] activity was measured by the method as described by Gascoigne and Gascoigne, (1960) using Whatman filter paper and endoglucanase activity (CMCase glucanase) was assayed according to the method of Olutiola (1976) using carboxy methyl cellulose (CMC) as substrates.

One unit of enzyme is defined as the amount which catalyzes the release of 1 μmol of reducing sugar (as glucose) per minute at 40°C.

3.14.4 Proteolytic activity:

Proteolytic activity was estimated as described by Hyashi *et al.* (1967) and Lowry *et al.* (1951).

One unit of enzyme activity is defined as the amount of enzyme liberating one μg of tyrosine per minute under the defined conditions.

D. EXPERIMENTAL TECHNIQUES

3.15 Optimization of temperature, pH and substrate concentration for H_2 and CH_4 production :

Inoculum : Mixed microbial culture.

Substrate : Glucose

The optimization was conducted in the following range in incubation assemblies (IA_{50}).

For H_2 production:

Temperatures : 20°C, 30°C, 35°C, 40°C, 45°C, 50°C & 60°C.

pH : 3.0, 4.0, 5.0, 6.0, 6.5, 7.0, 7.5 & 8.0.

Substrate concentration : 1%, 2%, 3%, 6%, & 9%.

Glucose solution was inoculated with mixed microbial culture and incubated under anaerobic conditions at different temperatures (pH 7.0, glucose concentration 3.0%).

Similarly 3% glucose solution was incubated at different pH at 40°C.

Substrate concentrations were also varied for concentration optimization (pH 7.0, temp. 40°C).

Similarly the conditions were optimized for CH_4 production by incubating glucose solution with enriched methanogenic culture under the following optimization parameters:

Temperatures : 20°C, 30°C, 37°C, 40°C, 45°C, 50°C, 60°C & 70°C.

pH : 3.0, 4.0, 5.0, 6.0, 7.0, 7.5, 8.0, 9.0.

Substrate : 1%, 2%, 3%, 4%, 5%, 6%, 7% & 8%. concentration

3.16 APPLE POMACE DIGESTION :

3.16.1 Three Stage system :

A three phase digester system for the bioconversion of apple pomace to fuel gases (H_2 & CH_4) has been developed (Fig. 3.9), with 2 l, 5 l and 10 l capacity aspirator bottles as stage I, II and III respectively. The three stages are connected as shown in the Fig. 3.9. In the stage I, apple pomace is fermented under partially anaerobic conditions to yield organic acids. In the stage II, these acids are converted to H_2, CO_2 and acetates and in the stage III, the liquid metabolites from the stage II are converted to CH_4 & CO_2.

Apple pomace and cattle dung slurry (2 l) in the ratio of 9:1 containing 3% TS

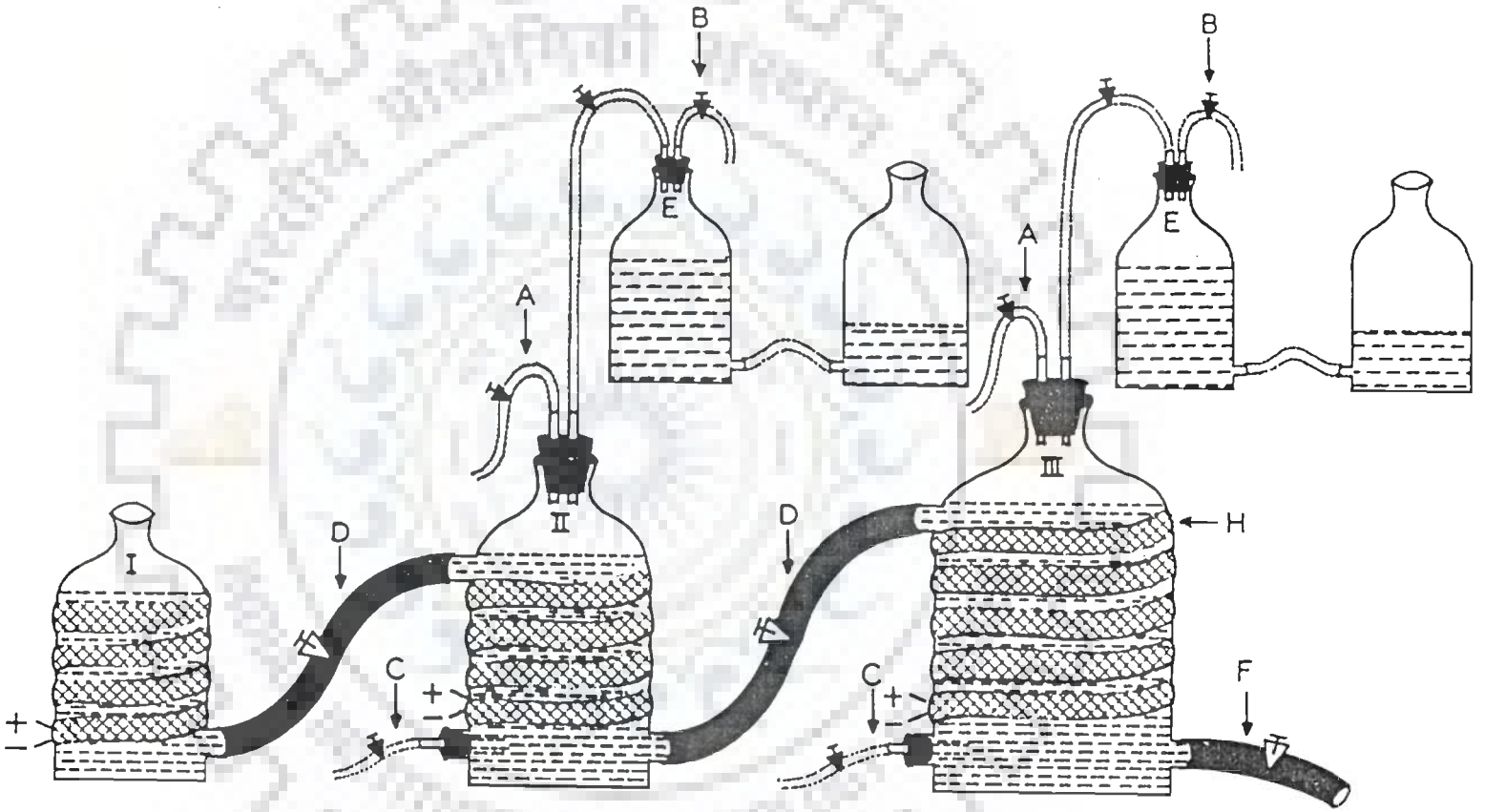

Fig. 3.9: Three stage laboratory set-up for bioconversion of apple pomace into H_2 and CH_4. Stage I, II and III are 2 l, 5 l and 10 l glass aspirator bottles. A: latex tubing inlet for adding NaOH; B: latex tubing outlet for collecting gas sample; C: outlet for slurry sample collection; D: rubber tubings for connections; E: gas collecting assemblies; F: outlet for effluent; H: heating tapes.

was incubated in stage I at 40°C for 4 days for the enrichment of hydrogen producing bacteria. Thereafter everyday 1/4 th of the slurry was transferred to stage II maintained under anaerobic conditions and simultaneously fresh apple pomace slurry was added in stage I to maintain the original volume. After filling the stage II upto 5 l, 1/10 th of its volume was passed on to stage III, which was already filled with 1 l of enriched methanogenic culture. pH neutralization was done in the stage II, when it was initially filled with 1 l of the slurry and latter on after each transfer. The transfer process was continued everyday and 1/10 of the contents were removed every day from stage III, after it got filled upto 10 lts. The standard incubation conditions were maintained.

3.16.2 Direct biomethanation of apple pomace:

Direct biomethanation was carried out in two stage digester system with 4 days and 20 days of retention times respectively. Initially two digesters (2 l and 10 l capacity) were filled with 6% cattle dung slurry, which was progressively replaced by apple pomace (3% TS) slurry.

In similar setup rotten apple pomace was also used as feed material, to check the effect of rotting and contamination which accompany such pomace, etc.

3.16.3 Hydrogen production at ambient temperatures :

Hydrogen production from apple pomace was carried out in two stage digesters (0.8 l & 2 l capacity) in a continuous batch fed system in an open area. The natural temperature variation was from 23 - 43°C. The 3% apple pomace slurry had a retention time of 4 days in stage I and 10 days in stage II. All other incubation conditions were the same as followed for other experiments.

3.17 PILOT PLANT BIOCONVERSION OF APPLE POMACE INTO FUEL GASES (H_2 AND CH_4) :

The studies conducted at laboratory scale level on bioconversion of apple pomace into H_2 and CH_4 were upscaled to a 100-fold level. The experiment was conducted on indigenous three stage H.D.P.E. bioreactor of 200 l, 500 l and 1000 l slurry capacities (Fig. 3.10). Stage I was partially anaerobic, open reactor whereas stages II and III were under anaerobic conditions. A temperature regulatory system was fabricated and placed inside the reactors to maintain the temperature at desired level (Fig.3.10). Slurry in the reactor was thoroughly mixed with mud-pumps and pH was neutralized with 5N NaOH solution.

200 l of 3% apple pomace and cattle dung slurry in 9 : 1 ratio was incubated at 40°C in stage I for 4 days. 50 l of the slurry was then transferred everyday to stage II which was filled upto 500 l in 10 days. Stage III already contained 100 l of active methanogenic culture which was generated by anaerobic digestion of 8% cattle dung slurry for 20 days. Thereafter 50 l of slurry from stage II to stage III was transferred everyday till it got filled upto 1000 l. Stage I was replaced by 50 l of fresh 3% apple pomace slurry. The gases were collected in the gas holders as shown in the Fig. 3.10. The gas volumes were recorded and gas samples analysed everyday.

Direct biomethanation of apple pomace at pilot plant level was also conducted, by directly transferring the slurry from the acidogenic stage to the methanogenic

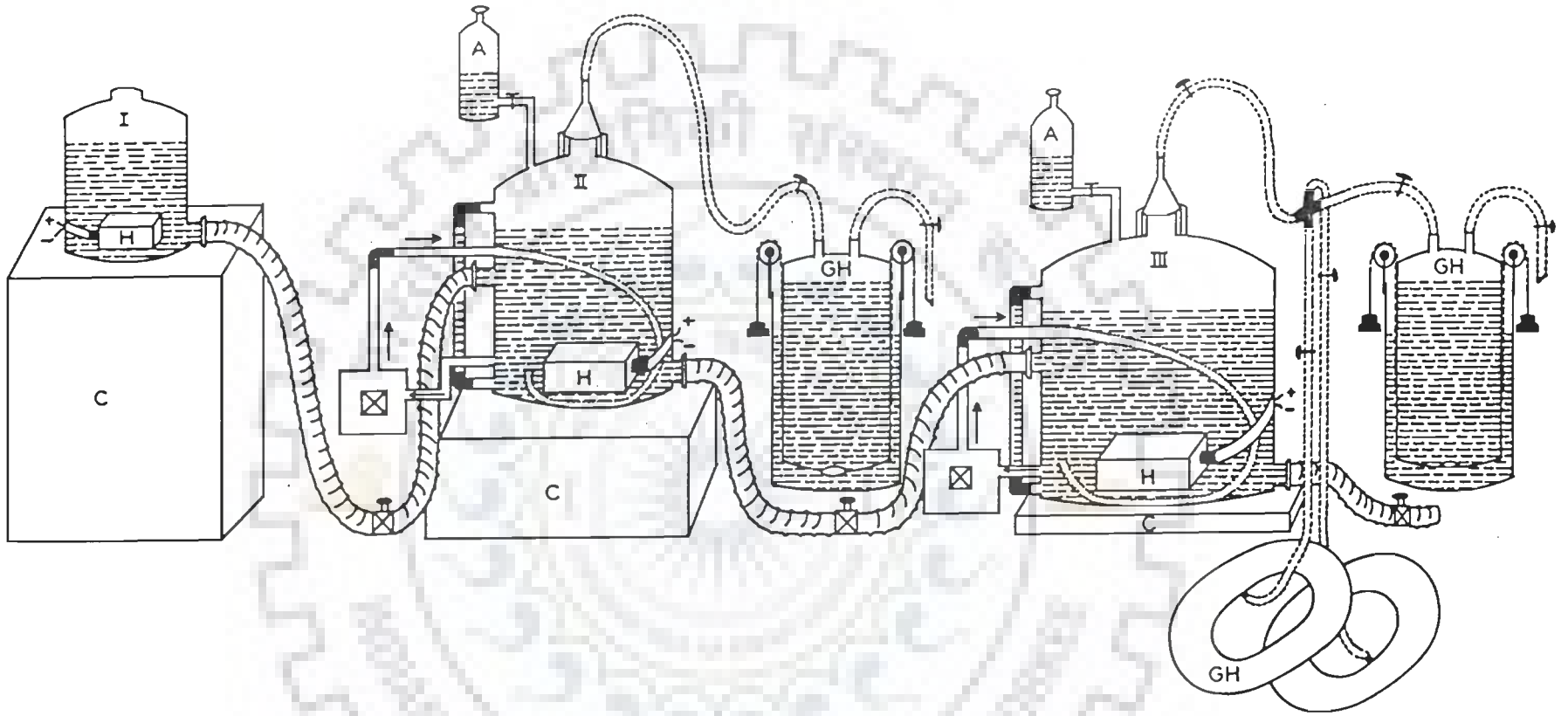

Fig.3.10: Diagramatic representation of pilot plant set-up. I, II, III: HDPE tanks used as bioreactors for the three stages. A: alkali bottles of 2 l capacity; GH: gas holders of 100 l capacity; T: taps; ⊠ : PP ball valve of 1" diameter. Connecting PVC pipes diameter: 50 mm; H: heating box (l x b x h): 30 x 15 x 15 cm in SI; 40 x 20 x 20 cm in SII; and 70 x 30 x 30 in SIII. ⊠ : slurry pump; C: concrete steps.

stage in a continuous daily batch fed system. The plant was continuously run for 80 days.

3.18 BIOMETHANATION OF FRUIT AND VEGETABLE WASTES:

Digestion :

Apple pomace and vegetable waste (raddish leaves, cauliflower leaves & stalk, and rotten cabbage) were subjected independently and also in succession to anaerobic digestion inoculated with cattle dung, on a laboratory scale.

The digestion of these wastes was done in two-stage system (El-Shinnawi *et al.*,1990). The stage I was a glass aspirator bottle of 0.5 l or 1 l capacity which was connected to another aspirator bottle of twice the volume (stage II) through a rubber tubing. During operation these aspirator bottles were left with a 20% head space. In stage I (acidogenic stage) biomatter was converted to organic acids in open conditions. The acids formed were converted into methane in stage II (methanogic stage) in a closed reactor. The hydraulic retention times in the two stages were 4 days and 20 days respectively (Ranade *et al.*,1987; Verrier *et al.*,1987). The subsequent digestion was run as a daily batch fed continuous culture. Fresh waste slurry was added to the acidogenic reactor after an equal volume of slurry was removed to feed the methanogenic reactor once each day. All incubations were done at 40°C. The pH of the methanogenic stage was adjusted to 7.0 with 1N NaOH only when its value fell below 6.5.

To start the digestion, the acidogenic reactor was initially filled with cattle dung slurry (CDS) and incubated for 4 days

before a quarter of its contents were passed on to the methanogenic reactor. The methanogenic reactor was either filled with fresh CDS and was incubated for 4 days (unenriched inoculum) or filled with 16 days preincubated CDS which was further incubated for 4 days (enriched inoculum) before it started receiving material from the acidogenic phase.

The fed substrates (3% TS) were either freshly prepared (apple pomace) or pretreated (vegetable wastes). Vegetable wastes were pretreated as their complete homogenization in water was not possible. The wastes were cut in small pieces (1 cm^3) and inoculated with CDS in 9:1 ratio (vegetable waste : CDS v/v) and incubated at room temperature (30-35°C) for 48 h.

The continuous-culture digestion of the apple pomace and vegetable wastes in four different reactor set-ups was done as follows :

1) The reactor set-up containing 4 days incubated 3% CDS (both stages) was daily batch fed with cauliflower and raddish waste (CRW) for 110 days.

2) In another set-up filled with 4 days and 20 days incubated CDS (3% TS) in Ist and IInd stage respectively was daily batch fed with CRW for 120 days.

3) The daily batch fed digestion of apple pomace (AP, 3% TS) for 113 days, followed by CRW (3% TS) for 38 days and finally for rotten cabbage (RC, 3% TS) was sequentially performed in a single two stage reactor set-up containing 6% CDS incubated for 4 days in stage I and 20 days in stage II.

4) In another similar experimental set-up, AP (3% TS) was daily batch fed digested for 121 days and was followed by the subsequent digestion of CRW (3% TS) for 30 days.

3.19 ANAEROBIC DIGESTION OF DUMPING WHEAT GRAINS FOR H_2 AND CH_4 PRODUCTION :

The digestion of dumping wheat grains for H_2 production was performed by enriched mixed microbial culture of H_2 producers and for CH_4 production either fresh cattle dung or enriched cattle dung was used as inoculum.

Bioreactors and feeding of the system :

Batch culture : Batch cultures were done in IA_{300} incubation assemblies. The dumping wheat slurry (270 ml) was inoculated with 5 ml of mixed microbial culture of hydrogen producers once in the beginning of the experiment and incubated at 40°C. The experiments were conducted with different total solid concentrations of 3%, 6% and 9% under standard incubation conditions (as mentioned in the beginning).

After having exhausted the slurry of its H_2 producing capacity, the same material was inoculated with 20 ml of enriched methanogenic culture and incubated at 40°C till the biogas evolution ceased to occur. The pH was adjusted to 7.0 once in the beginning of the methanogenic phase.

Continuous culture :

Continuous cultures for H_2 production were done in two different manners :

1. Single stage system and 2. Two stage system.

Single stage system : A 600 ml reagent bottle with provision for feeding the substrate and removal of effluent and gases was used for fermenting dumping wheat slurry (DWS). Freshly prepared (100 ml) 6% dumping wheat slurry

was inoculated with 10 ml enriched mixed microbial culture and incubated at 40°C. Here after everyday 100 ml of freshly prepared DWS was added till the total slurry volume became 500 ml. From day 5 onwards, 100 ml of DWS was drained out and fresh 100 ml of DWS was added to maintain the slurry at 500 ml. All standard incubation conditions were followed.

Two stage system : The volumes of stage I and stage II were 400 ml and 1 l, respectively. Stage I was partially anaerobic and stage II was completely anaerobic. Two different loading rates of DWS were employed i.e., 3% TS and 6% TS . In both the cases 360 ml of DWS was inoculated with 40 ml of enriched mixed microbial culture and incubated in stage I at 40°C for 4 days. Thereafter everyday 100 ml of slurry was transferred to stage II followed by adding fresh 100 ml DWS in stage I. The stage II was filled and maintained at a level of 1 l by draining 100 ml from stage II and transferring 100 ml from stage I to stage II. The volume of stage I was made up by adding 100 ml of freshly prepared slurry. All incubation conditions were the same as for other experiments.

Continuous fermentation experiments for methane production :

Continuous fermentation experiments of daily batch fed type were performed in two stages. Volumes in stage I and stage II were 400 ml and 2 l respectively. Two total solid concentration levels of 3% and 6% were employed in two different sets of experiments. Both the reactors were initially filled with cattle dung slurry as an inoculum (3% or 6% TS, depending upon the TS of the DWS to be employed). After 4 days of incubation at 40°C, 100 ml of slurry was drained out from stage II and 100 ml of material from stage I was transferred to stage II. Stage I was filled

to desired volume by adding 100 ml of fresh DWS and the procedure was repeated once every 24 hrs.

Recycling of the effluent : The effluent from the methanogenic stage II was put back into stage I only in the case of 3% TS dumping wheat methanogenesis after it was continuously run initially for 80 days without recycling. The total solids of the effluent were estimated and always made upto the 3% level by adding fresh dumping wheat.

3.19.1 Biomethanation of other types of damaged wheat grains :

Other damaged wheat grains like, poultry feed and cattle feed were also subjected to two stage continuous daily batch fed digestion for methane production at 3% total solid concentration in a similar way as mentioned for dumping wheat grains but the experiments were not subjected to recycling. Fresh wheat was also simultaneously subjected to digestion for comparison of the efficiency of methane production.

3.20 Anaerobic digestion of pure substrates for H_2 production:

The potential of other substrates like rice, maize, wheat, starch and cellulose was checked for H_2 production by mixed microbial culture.

These pure substrates at 3% TS level were inoculated with the cells and incubated in IA_{300} incubation assemblies as batch culture. The standard incubation conditions were maintained. The gases were collected and analysed.

3.21 Hydrogen production at varying glucose concentrations:

40 ml of different glucose concentration solutions were inoculated with 10 ml of the enriched mixed microbial culture in IA_{50} incubation assemblies. The final glucose concentration obtained in these assemblies was 1%, 2%, 3%, 6% and 9%. A control experiment was also run simultaneously without adding glucose. The mixture was incubated at 40°C and the gases generated were analysed for their contents of H_2, CO_2, CH_4, N_2 and O_2. These results were calculated for the total volume of each gas generated in a day. The incubation mixture was neutralized every day and anaerobic conditions also maintained by flushing N_2 every day before setting for incubation.

E. METABOLIC STUDIES OF H_2 PRODUCTION

3.22 Activities of Enzymes involved in the degradation of biowastes :

The efficiency of microbial culture to degrade the biological waste material can be best checked by estimating the activities of different hydrolytic enzymes during fermentation

Microbial culture : Enriched mixed microbial population of hydrogen producing organisms was employed for these studies.

Substrates : a) Apple pomace

b) Dumping wheat

Method : 150 ml each of 3% apple pomace slurry and 6% dumping wheat slurry was inoculated with 2-3 ml (1 g wet wt) of the mixed microbial population in a 300 ml experimental set up. The pH was adjusted with 1 N NaOH to neutrality and N_2 gas was flushed to create anaerobic conditions. The cultures were incubated at 40°C on a shaker at 120 rpm and also as stationary cultures. Liquid samples were withdrawn within 24 h during the following three phases of H_2 production a) at the initiation of H_2 production, b) at maximum H_2 production level and c) at the termination of production. These time intervals were already standardized before conducting the experiments. The samples were centrifuged at 1200 rpm for 5 min to remove the cell mass and the supernatant samples were immediately frozen until analyzed for enzyme activities. Each sample was analysed in triplicate for the following activities: Amylolytic, Hemicellulolytic (Xylanase), Cellulolytic (C_1 and B-glucanase, CMCase glucanase) and Proteolytic. Control samples were prepared by inactivating the enzyme by autoclaving.

3.23 Glycolytic intermediate substrates :

The glycolytic intermediate and end product substrates were used for H_2 production by mixed microbial culture of H_2 producers and pure culture of *Bacillus licheniformis*.

The cells were incubated in IA_{25} and IA_{300} incubation assemblies on glycolytic pathway substrates like, Glucose, Glucose-6-phosphate, pyruvate and lactate (sodium salts) and their combinations to check whether the cells follow this pathway for H_2 production. The concentration of these substrates was in the range of 0.15

- 0.3% and thus H_2 produced per mole of these subtrates was calculated. Incubation conditions were maintained as mentioned earlier.

3.24 *Escherichia coli* pathway in the isolated strains of *Bacillus* and mixed microbial population :

3.24.1 Hydrogen production from formate :

Hydrogen production by formate *hydrogenlyase* system was checked by using the pure isolated cultures of *Bacillus licheniformis* and strain JK_2 and mixed culture on formate substrate.

Induction medium : 1% filter sterilized glucose was added in the sterile growth medium prepared in 0.1 M phosphate buffer pH 7.4.

Washing buffer : 0.1 M sterile phosphate buffer (pH 7.0)

Fermentation buffer :

Sodium formate	2.0 g
$Na_2HPO_4.2\ H_2O$	5.78 g
KH_2PO_4	2.38 g
$(NH_4)_2SO$	44.0 g
NaCl	1.0 g
$Na_2MoO_4.2\ H_2O$	10.0 μg
$FeSO_4.7\ H_2O$	2.9 mg
$MgSO_4.7\ H_2O$	0.5 g
pH	6.85

$MgSO_4.7\ H_2O$ was sterilized and aseptically added separately.

Procedure :

Induction of Formate hydrogenlyase complex for H_2 production from formate :

To induce the enzymes for formate degradation, the strains were grown in 1 l of the induction medium at 40°C under anaerobic conditions. (maintained by flushing N_2 gas for 5 min). The cells were harvested at the end of the log phase (12-14 h of incubation) by centrifugation at 10,000 rpm under aseptic conditions. The purity of the cells was checked by plating and gram staining. The cells were thoroughly washed two to three times with sterile 0.1 M phosphate buffer (pH 7.0).

Fermentation : 1 g (wet wt) of the washed cells was inoculated in 50 ml of fermentation buffer in IA_{50} incubation assembly. The assembly was flushed with N_2 gas to create anaerobic conditions. The mixture was incubated at 40°C and gases were collected and analysed for H_2, CO_2, N_2, and O_2 on GLC Incubaion was continued till the gas production ceased. The amount of H_2 and CO_2 at NTP was calculated in mmol.

Liquid samples were withdrawn after formate decomposition. These samples were centrifuged and supernatant stored frozen till analysed for unconsumed formate.

3.25 NITROGENASE IN H_2 PRODUCTION BY MIXED MICROBIAL CULTURE:

3.25.1 Nitrogenase activity :

Organism : Enriched mixed microbial population.

Source : Cattle dung.

1 l of 3% cattle dung slurry was passed through fine muslin cloth. The filtrate was treated for enrichment of hydrogen producing organisms (Joshi et al.,1986). The enriched cells were recovered from the filtrate by differential centrifugation

as follows : First the filtrate was allowed to stand for 5 minutes, to remove heavy matter. The supernatant recovered was centrifuged at 3000 rpm. The pellet was discarded and the supernatant was again centrifuged at 10,000 rpm to collect the pellet. The enriched cell pellet so obtained was diluted to 50 ml with the same supernatant. The diluted cells were used for conducting further studies.

Nitrogenase assay : The nitrogenase activity in the cells was determined by the acetylene reduction assay method. The assay was conducted in incubation assemblies IA_{50} having total void volume of 65 -70 ml. 3 ml cells (60 mg dry wt or 4.3 mg protein/ml) were inoculated in 22 ml of 2% sterile glucose solution and placed in the incubation assembly. The mixture was neutralized and flushed with argon gas for 5 min to create anaerobic conditions. The system was made perfectly air tight and provided with latex tubing for injecting the gases. The cells were activated by incubating at 40°C for 20-24 h till hydrogen production was maximum which was analysed on GLC.

The cell mixture was again neutralized and whole assembly was flushed with argon gas to create an inert atmosphere. At this stage 0.1 atm (10%) of acetylene environment was created by injecting 4.5 ml of acetylene gas in the assembly after replacing the inner atmosphere by the same amount. The mixture was incubated at 40°C and the gas samples were withdrawn at regular intervals starting from 0 h to 24 h of incubation.

The effect of following parameters was checked on the derepression of the nitrogenase activity.

a) The test was conducted without activating the cells by glucose; where cells were directly incubated with sterile distilled water. In this experiment the effect of pre-incubation for 20 - 24 h was also studied.

b) The effect of different concentrations of oxygen (0.25 - 20%) on the acetylene reduction was studied by injecting different volumes of air in the incubation assembly. The O_2 exposures were performed both on the shake culture and the stationary culture.

c) Acetylene reduction assay was also conducted by varying the inoculum : acetylene ratio. Cell volumes from 5 - 35 ml was taken in the incubation assemblies and acetylene from 3.1 - 6.0 ml was injected to create the appropriate 10% acetylene atmosphere.

The assays were conducted in triplicate and the gases were analysed by C_2H_2 reduction i.e, C_2H_4 formation.

3.25.2 Effect of inhibitors on H_2 production by Nitrogenase and Hydrogenase system :

Carbon monoxide (CO) effect : 3% glucose solution was inoculated with enriched mixed culture at neutral pH. 30 ml of this mixture was placed in individual incubation assemblies (IA_{50}). The mixture was flushed with N_2 gas passed over hot Cu turnings to remove traces of O_2. Different CO gas concentrations (2%, 5%, and 10%) were created in the assemblies by injecting the respective volumes of CO gas after withdrawing an equal volume of gas from inner atmosphere. The void volume in the assemblies was 40 ml. The mixtures with CO atmosphere were incubated at 40°C both in the stationary culture and on shaking at 110 rpm for 24 h. The gases were collected and analysed for H_2 and CO_2 formed and the inhibitory effect on H_2

production was compared by running control experiment under similar conditions without CO atmosphere.

The persistent lethal effect of CO on H_2 production was checked by removing the CO atmosphere from the assemblies by flushing N_2 after 24 h of incubation and neutralization and continued incubation. The amount of H_2 produced was measured.

All the treatments were run in triplicates and the experiment repeated thrice.

Effect of C_2H_2 : The inhibitory effect of C_2H_2 on H_2 production via nitrogenase was checked by using 10% C_2H_2 in a similar manner as mentioned for CO effect.

Effect of KNO_3, $NaNO_3$, NH_4Cl : Similar effects were tested by conducting experiments with KNO_3 & $NaNO_3$ by adding different concentrations 2.5, 6.6, and 10.0 mmol in the incubation mixture. NH_4Cl was checked in the broad range of concentration (6.6 mmol - 0.2 mol).

3.26 FATTY ACID AND ALCOHOL METABOLISM DURING H_2 PRODUCTION :

The fermentation of apple pomace, dumping wheat and glucose was conducted by mixed microbial population and axenic culture of *Bacillus licheniformis*.

Procedure : Incubation assemblies (IA_{300}) were used to conduct the experiments. 270 ml of 3% apple pomace were inoculated separately with 3 g wet wt (5 ml) of cells of *B. licheniformis* and 3 g wet wt (5.5 ml) of biomass of mixed microbial population. Similarly 220 ml each of 6% dumping wheat and 3% glucose solution inoculated with mixed culture and axenic culture, *B. licheniformis.* The conditions

for H_2 production were as already mentioned and kept for incubation. The following parameters were recorded at 1h to 2h regular intervals upto 28 h.

a) pH fall,

b) Volume of gas produced,

c) % of H_2 and CO_2 produced by gas analysis.

Samples for fatty acids and alcohol estimation were collected, centrifuged and supernatant frozen till analysed.

3.27 Study of iron (Fe^{3+}), Nickel (Ni^{2+}) and sulphate (SO_4^{2-}) on H_2 production:

Effect of sulphate was tested on the production of H_2 from glucose by mixed microbial culture whereas iron and nickel effects were checked when apple pomace was used as the substrate.

Method : 3% glucose solution and 3% apple pomace slurry were inoculated with mixed microbial culture in three separate incubation assemblies (IA_{300}). The mixtures were incubated under anaerobic conditions by adding iron, nickel and sulphate separately in the following concentrations.

Fe^{3+} as $FeCl_3$	=	50, 100 & 200 nM
Ni^{2+} as $NiCl_2$	=	50, 100 & 200 nM
SO_4 $^{2-}$ as Na_2SO_4	=	1 - 200 mM

Sulphate reduction was determined by estimating unutilized sulphate and sulfide according to the methods as described by Rossum and Villarruz (1961) and Nusbaum (1965) respectively.

F. ENHANCEMENT OF HYDROGEN PRODUCTION :

3.28 IMMOBILIZATION OF MICROBIAL CELLS :

Both basic techniques of immobilization by absorption of the cells on solid support and entrapment of cells in gel matrix were used.

The organisms used for immobilization were mixed microbial culture of H_2 producers and the pure culture of *Bacillus licheniformis* whereas the immobilizing material was baked brick pieces and sodium alginate. Glucose was used as the substrate in the experiments.

3.28.1 Preparation of immobilized column :

a) **Baked brick column** : Baked red bricks were broken down into small pieces of 4-5 mm size. These pieces were treated with 0.1 N HCl followed by washing under running tap water. These were then packed in a 1 l aspirator bottle occupying a volume of 700 ml.

b) **Calcium alginate beads column :** In separate experiments, cells were immobilized in calcium alginate beads according to the method of D'Souza and Godbole (1989) with some modifications. 25 ml (5 g wet wt) of mixed microbial cells and 10 ml (2g wet wt) of *Bacillus licheniformis* cells were thoroughly mixed with 125 ml and 140 ml of 5% sodium alginate solution respectively. The resulting homogeneous mixture was extruded drop by drop through a syringe into 50 mM $CaCl_2$ solution. The beads were cured in the same solution

at room temperature for 4 h and then washed several times with distilled water. The bead diameter was from 3-4 mm.

3.28.2 Batch culture digestion :

The batch culture digestion were carried out in the following manner in four separate setups.

i) 150 ml of 3% glucose solution was inoculated with approximately 150 ml calcium alginate beads containing mixed microbial population and incubated in IA_{300}.

ii) 150 ml of 3% glucose solution was inoculated with approximately 150 ml of calcium alginate beads containing pure cells of *Bacillus licheniformis* and incubated in IA_{300}.

iii) 300 ml glucose solution (3%) was inoculated with 200 ml of Bacillus *licheniformis* cell culture (5 g wet wt) in 1 l baked bricks column.

iv) 300 ml of glucose solution (3%) was inoculated with 200 ml of mixed microbial culture (5 g wet wt) in 1 l baked bricks column.

Appropriate controls in which the cells were not immobilized were run in parallel experiments. Gases were collected and analysed. Samples were withdrawn at intervals for assaying unconsumed glucose.

Reuse of the immobilized cells :

The columns of alginate beads and bricks were gently washed twice with distilled water, to remove the traces of the unconsumed glucose solution.

The washed columns were supplemented with the 3% glucose solution in the same manner as in the beginning. Again as the hydrogen gas production ceased, the immobilized columns were reused for another cycle of glucose biotransformation.

Long term activity: The alginate immobilized cells after washing thoroughly with sterile distilled water, were kept under aseptic conditions at 4°C for 1 year. The H_2 producing efficiency was checked by again feeding with 3% glucose solution and incubated under anaerobic conditions for H_2 production.

3.28.3 Continuous daily batch fed culture digestion :

It was performed in two of the above cases.

a) Mixed microbial culture on baked brick support

b) *Bacillus licheniformis* entrapped in calcium alginate beads

The digestors were run as batch cultures for the initial period of 4 days and as continuous culture thereafter. 300 ml of glucose solution (3%) was incubated in 1 l of the immobilizing columns. After 4 days of batch digestion, 100 ml of material was drained out and replaced by fresh 100 ml of 3% glucose solution. The procedure was followed once a day till the end of experiment. The baked brick column was supplemented additionally with cell population and nutrients in the middle of experimentation. Control experiments were run simultaneously. Gases were collected and analysed.

All these experiments were conducted under standard incubation conditions for H_2 production and performed in duplicate.

CHAPTER - IV

RESULTS

RESULTS

4.1 Microbiological properties of Hydrogen producing cultures:

The pure cultures of both the strains of *Bacillus licheniformis* and JK_2 appeared as small rods under scanning electron microscope with an average size of 1.3 by 0.5 μm (Fig. 4.1 & 4.2). Under transmission in negative staining, the appearance of single polar flagellum with rod shaped structures was observed (Fig. 4.3 & 4.4). These microscopic studies confirmed the rod shape and single structures of the cells. Nowhere the two cells were attached together end to end but sometimes closely packed cells were observed. The cells were spore former, showed the typical character of acid production as evident by methyl red positive and Vogous Proskeur negative properties. The cultures were oxidase & catalase positive and could produce acids and gases from carbohydrates.

Under electron microscopy, the enriched mixed microbial population of H_2 producers showed very few cells of different cell structures (Fig. 4.5 & 4.6). Some were observed to be rod shaped with and without single polar flagellum. Some circular shaped and some irregular cell structures were also observed.

4.2 Optimum temperature, pH and substrate concentration for hydrogen production.

In a temperature range of 20 - 40°C, the hydrogen producing potential of the mixed microbial culture from glucose increased from 0 to 8.35 l / mole of glucose employed (Table 4.1A). The efficiency was maximum at 40°C and decreased by 35.5% if the temperature was increased to 60°C thus yielding 5.4 l of H_2 / mole of glucose. A variation of 7 - 12% in the H_2 generated was observed within 35 - 45°C (Fig. 4.7A).

The H_2 producing efficiency of the culture varied considerably at different pH levels (pH 3.0 - pH 8.0). The optimum H_2 production was observed at pH of 6.5 - 7.0 generating 8.3 - 8.4 l of H_2/mole of glucose employed (Table 4.1B & Fig. 4.7B).The efficiency level dropped to 84% at pH 6.0 and was almost zero as the pH was changed from 6.0 to 3.0. Also at the pH level of 8.0 the H_2 yields decreased by 18%.

Maximum H_2 yields were obtained at 3% and 6% concentration levels when tested in the glucose concentration range of 1 - 9% (Table 4.1C & Fig. 4.7C). Beyond these concentrations, on either side of the scale, the yields showed noticeable decrease from 42 - 100%. The maximum H_2 produced was 8.47 l/mole of glucose employed at 6% TS level.

It has been observed that all these conversions took a minimum period of 6 days and a maximum of 46 days in batch culture digestion during which biogas-H evolution was evident. H_2 constituted 50 - 55% of this biogas-H besides 45 - 50% of CO_2.

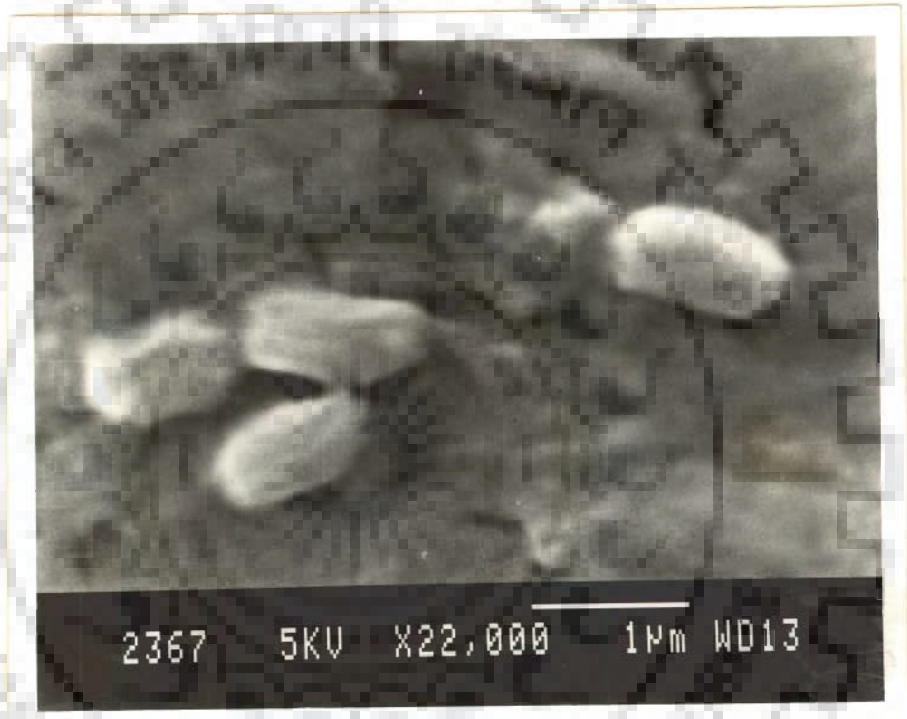

Fig.4.1: Scanning electron micrograph of Bacillus licheniformis.

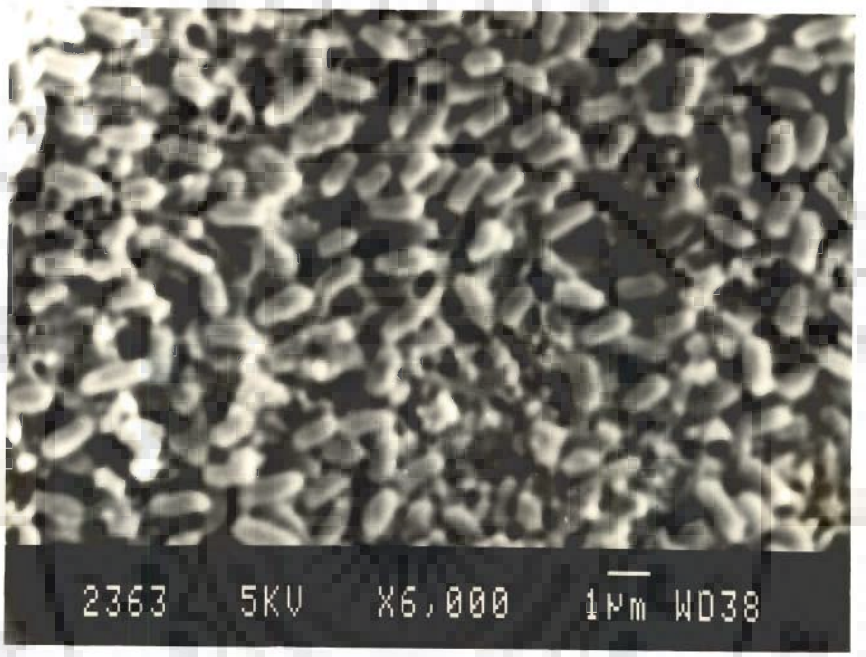

Fig.4.2: Scanning electron micrograph of strain JK2.

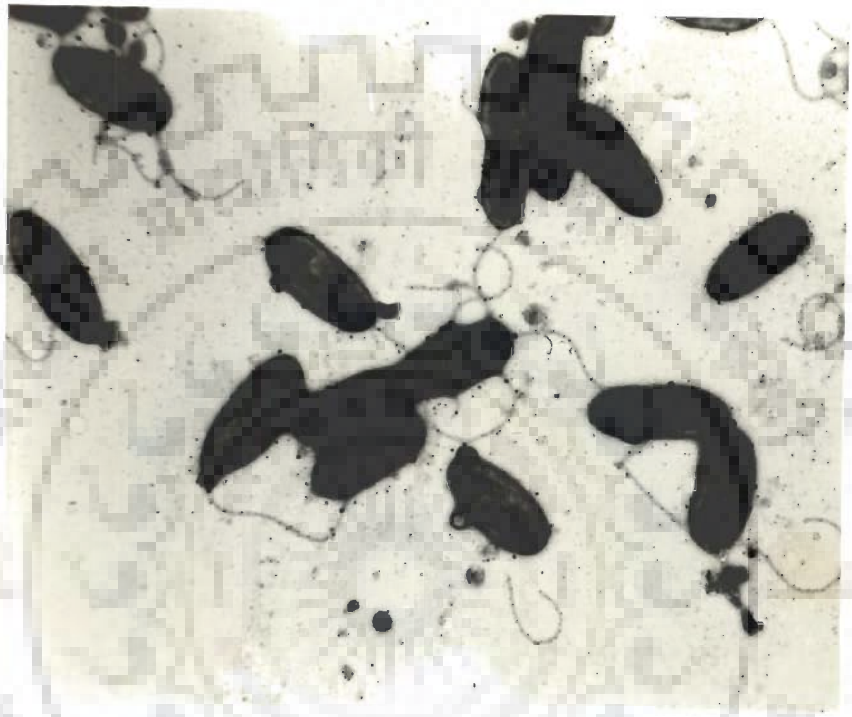

Fig.4.3: Transmission electron micrograph of Bacillus licheniformis. (x 10,933)

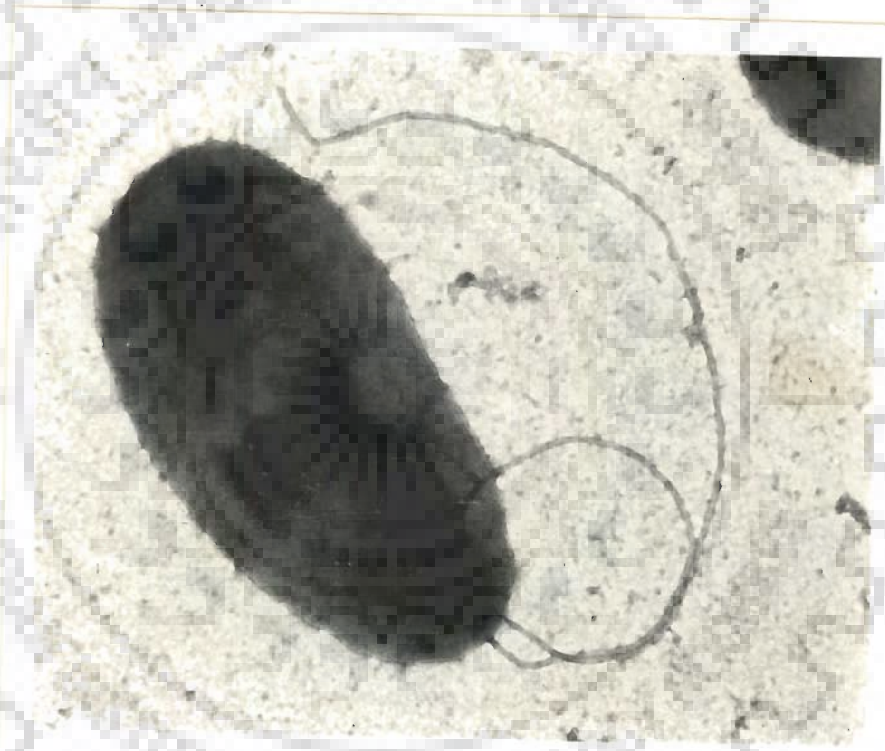

Fig.4.4: Transmission electron micrograph of strain JK2. (x 56,000)

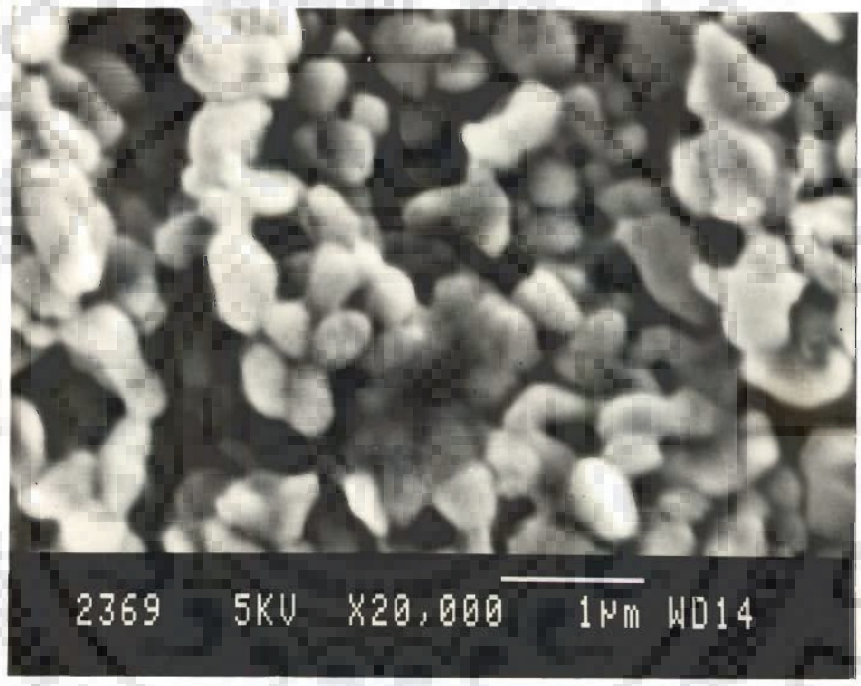

Fig.4.5: Scanning electron micrograph of mixed microbial culture of H_2 producers.

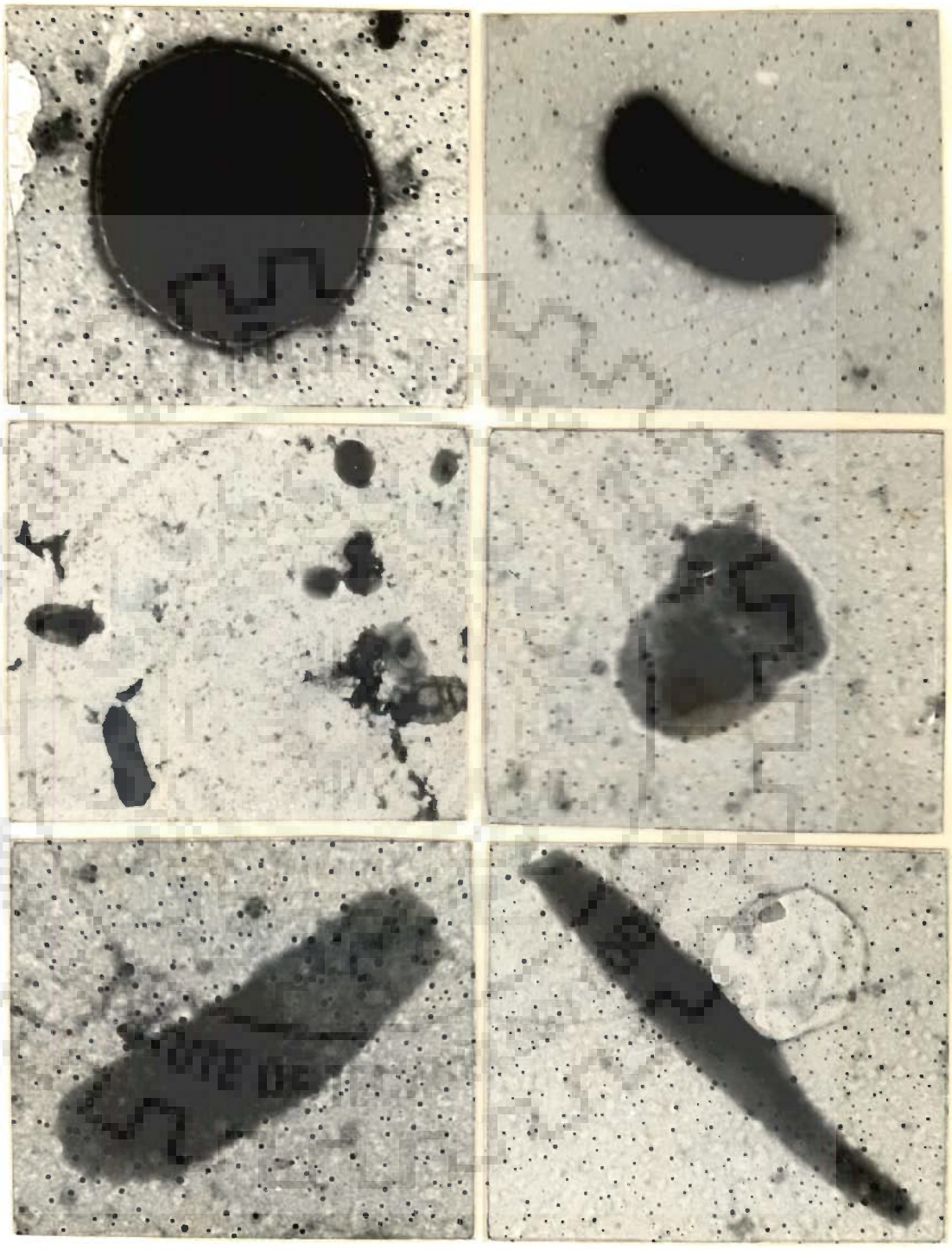

Fig.4.6: Transmission electron micrograph of mixed microbial culture of H_2 producers (x 36,000-73,000)

TABLE 4.1: EFFECT OF TEMPERATURE, pH AND SUBSTRATE CONCENTRATION ON HYDROGEN PRODUCTION BY MIXED MICROBIAL CULTURE.

(A) TEMPERATURE

Temperature (^{o}C)	Days of * Incubation	Hydrogen (l/mole of glucose employed)
20	10	0.00
30	18	3.35
35	15	7.30
40	10	8.35
45	12	7.80
50	13	7.10
60	13	5.40

(B) pH

pH	Days of * incubation	Hydrogen (l/mole of glucose employed)
3.0	9	0.0
4.0	14	1.0
5.0	15	3.0
6.0	11	7.0
6.5	9	8.3
7.0	9	8.4
7.5	12	7.5
8.0	13	6.9

(C) SUBSTRATE CONCENTRATION

Glucose concentration	Days of * incubation	Hydrogen (l/mole of glucose employed)
0.0	6	0.0
1.0	8	2.23
2.0	13	4.90
3.0	16	8.28
6.0	27	8.47
9.0	46	4.66

(A) : pH 7.0, substrate conc. 3% ; (B) : temperature 40^{o}C, substrate conc. 3% ; (C) : pH 7.0, temperature 40^{o}C.
* Total number of days of incubation during which gas volumes were produced in batch culture digestion

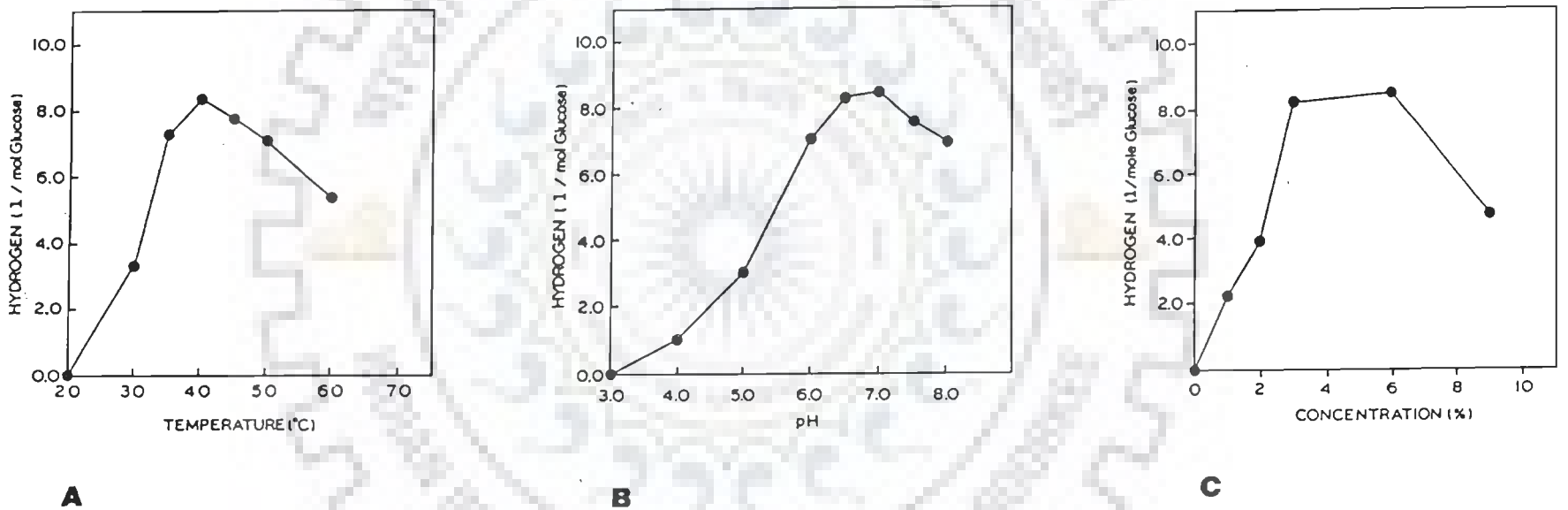

Fig. 4.7: Effect of (A) Temperature; (B) pH and (C) Substrate concentration on hydrogen production from glucose by mixed microbial culture.

4.3 Optimum temperature, pH and substrate concentration for methane production.

Methane evolved during the fermentation of 3% glucose solution inoculated with enriched methanogenic culture at different temperatures, ranging from 20 - 70°C is presented in Table 4.2A. The maximum production efficiency was observed to be at 40°C (Fig. 4.8A), generating 55 l of CH_4 / mole of glucose employed. The results were almost similar at 37°C, but a decrease in the methane production on either side of this temperature range was evident.

The efficiency of methane production was observed to vary within a pH range of 3.0 to 9.0 (Table 4.2B). Methane production yields varied from 10.0 l at pH 5.0 to a maximum of 53.5 l/ mole of glucose fed at pH 8.0, whereas optimum pH range was observed to be 7.0 - 8.0 (Fig. 4.8B). CH_4 production was totally inhibited if the pH was maintained at $\leq$4.0.

Table 4.2C gives observations of CH_4 evolution in experiments conducted by varying the glucose concentrations. The CH_4 produced varied from 1.0 l/ mole of glucose employed at 8% glucose concentration to a maximum of 59.5 l/ mole glucose at 3% glucose (Fig. 4.8C & Table 4.2C). In the concentration range of 1 -4 % glucose, there was not much difference in CH_4 yields. However, with increase in the glucose concentration, the negative impact on yield was dramatic.

All these bioconversions in batch culture digestion took a minimum period of 7 days and a maximum of 30 days during which biogas was evolved. The CH_4 content varied from 50% to 55% and CO_2 constituted rest of the biogas evolved.

TABLE 4.2: EFFECT OF TEMPERATURE, pH AND SUBSTRATE CONCENTRATION ON METHANE PRODUCTION BY ENRICHED METHANOGENIC CULTURE.

(A) TEMPERATURE

Temperature (^{o}C)	Days of * Incubation	Methane (l/mole of glucose employed)
20	15	5.5
30	27	30.0
37	26	54.0
40	24	55.0
45	22	50.0
50	20	42.0
60	17	24.3
70	10	2.5

(B) pH

pH	Days of * incubation	Methane (l/mole of glucose employed)
3.0	7	0.0
4.0	7	0.0
5.0	18	10.0
6.0	27	32.0
7.0	23	52.0
7.5	24	52.0
8.0	26	53.5
9.0	19	29.0

(C) SUBSTRATE CONCENTRATION

Glucose concentration	Days of * incubation	Methane (l/mole of glucose employed)
0.0	8	3.5
1.0	19	44.0
2.0	24	55.5
3.0	26	59.5
4.0	28	50.0
5.0	30	32.5
6.0	22	12.5
7.0	18	6.5
8.0	12	1.0

(A) : pH 7.0, substrate conc. 3% ; (B) : temperature 40^{o}C, substrate conc. 3% ; (C) : pH 7.0, temperature 40^{o}C.
* Total number of days of incubation during which gas volumes were produced in batch culture digestion.

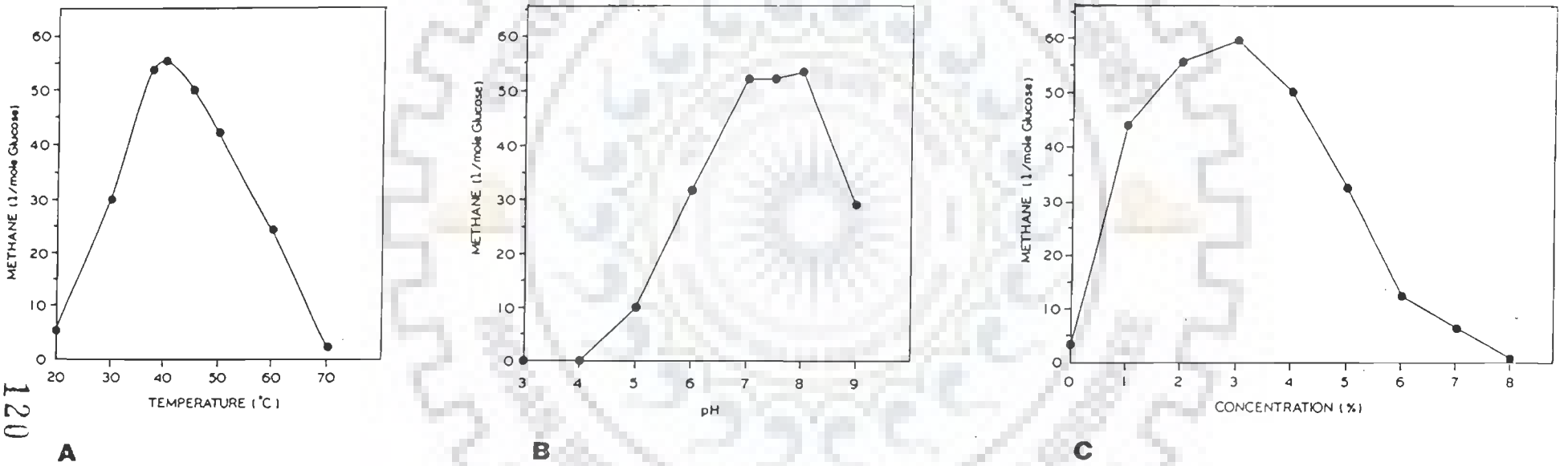

Fig. 4.8: Effect of (A) Temperature; (B) pH and (C) Substrate concentration on methane production from glucose by enriched methanogenic culture.

It has been observed during the course of these experiments that methanogenic cultures and H_2 producing mixed cultures showed their maximum activity at almost similar temperature and pH ranges. These, however, adapt differently to total solid concentrations. A higher activity was observed at 6% TS level for CH_4 production whereas 3% TS was found to be optimum for H_2 production.

4.4 Efficiency of enriched hydrogen producing mixed microbial culture under varying sugar concentrations :

Effect of sugar concentrations : The effect of glucose concentration on stability of the culture for continuous H_2 production has been presented in Table 4.3 & Fig. 4.9A & 9B. At 0% glucose level, the mixed culture of H_2 producers did not evolve any H_2 but produced some CH_4 after 7 days of incubation presumably because of the presence of some unsuppressed methanogens. This inherent behaviour of the culture was reversed as it was fed on glucose. As the concentration of glucose was increased from 1% to 9%, the H_2 production improved from 0.22 l to 3.10 l/mol of glucose added. A delay in the initiation of CH_4 evolution could be observed, for the inoculum took 9 days at 1% to as long as 47 days at 9% glucose concentration. It not only delayed the initiation of CH_4 evolution but also suppressed even its inherent CH_4 producing potential. H_2 production achieved stability and showed maximum efficiency at 3 - 6% level, generating 8.1 l/mol of glucose fed (also see Table 4.1C). Here CH_4 production was only 10 - 74% in comparison to the maximum production rate observed at 2% glucose level where H_2 evolution was practically negligible. During the course of H_2 production from glucose, pH dropped to 5.49 - 5.87, whereas, during CH_4 evolution pH remained always at $\geq$7.0.

TABLE 4.3: EFFECT OF DIFFERENT GLUCOSE CONCENTRATIONS ON THE ABILITY OF MIXED MICROBIAL CULTURE TO PRODUCE HYDROGEN AND METHANE IN BATCH CULTURE.

Parameters	Glucose concentrations					
	Ø%	1%	2%	3%	6%	9%
Initiation of CH_4 production (days) #	7	9	14	17	28	47
H_2 (ml)	Ø.ØØ	Ø.62	1.34	66.65	134.86	77.62
CH_4 (ml)	8.44	138.51	294.75	325.34	91.ØØ	Ø.66
pH during H_2 production *	6.61	5.49	5.87	5.81	5.52	5.52
pH during CH_4 production	7.Ø6	7.34	7.35	7.42	7.28	7.14

The values are based on the results of two experiments.
* pH values were those recorded after 24 hrs of incubation, after initially adjusting to 7.Ø everyday during H_2 production.
# H_2 was produced till 8, 13, 16, 28 & 46 days of initial inicubation at 1, 2, 3, 6 & 9% glucose concentration and thereafter CH_4 evolution started.
Glucose solution = 5Ø ml.

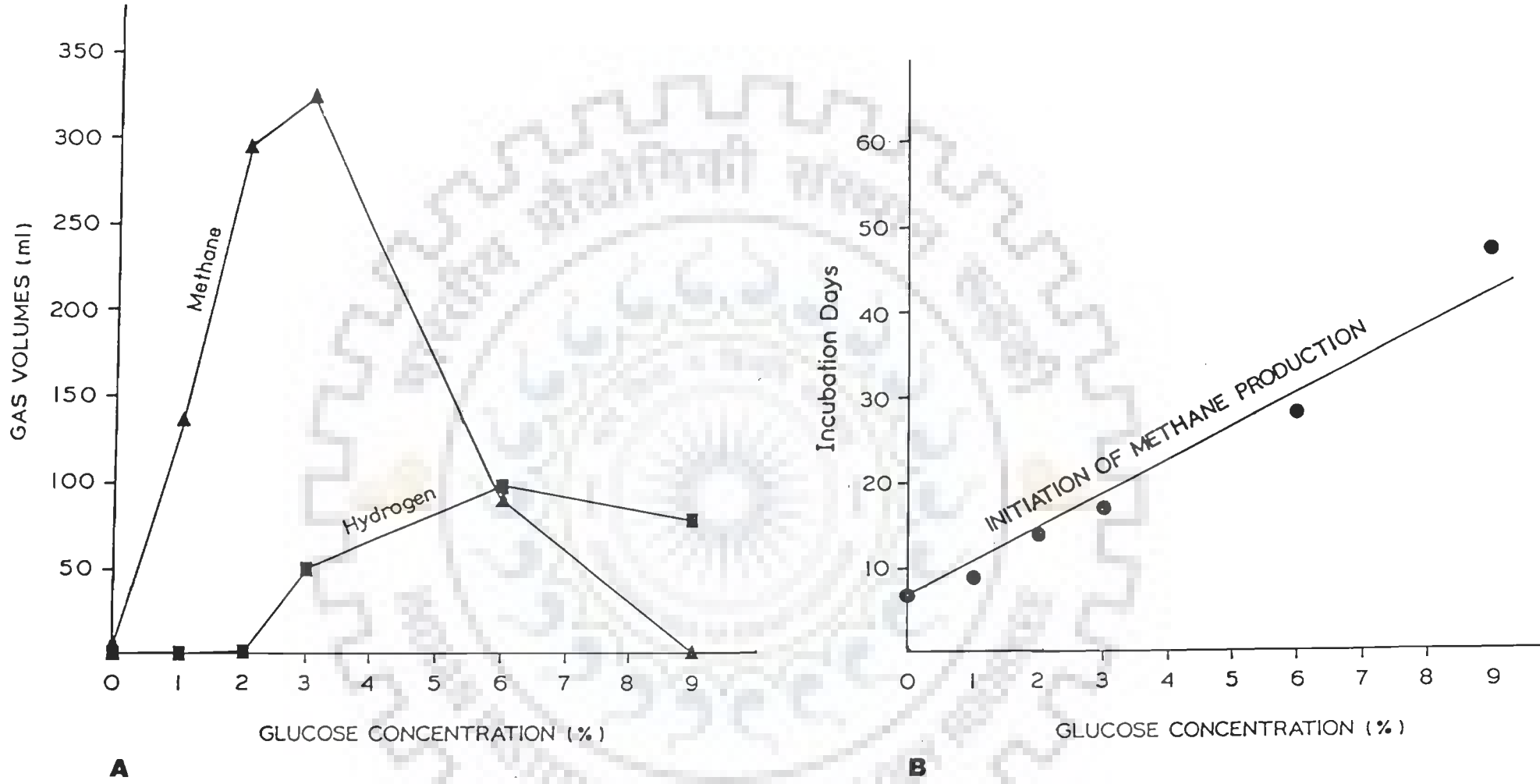

Fig. 4.9: Effect of different glucose concentrations on (A) production of hydrogen and methane; (B) initiation of methane production.

4.5 Bioconversion of apple pomace into H_2 and CH_4:

The apple pomace used in these studies showed a C & H content of 47% & 6% respectively with less than 0.1% of N. Data on the fermentation of apple pomace in a continuous batch fed culture to H_2 and CH_4 under laboratory conditions is presented in Table 4.4. Apple pomace inoculated with CDS (3%) led to the accumulation of acids over a period of 4 days in Ist stage. The acidified slurry was passed on to stage II, where it was retained for 10 days. On neutralization, it could generate 1.0 l of biogas-H from 15 g of dry matter under anaerobic conditions and at a pH of 7.0. Biogas-H so evolved had 60% H_2 and 40% CO_2. The slurry when acted upon by methanogens in a subsequent reactor, evolved biogas at the rate of 3.0 l/15 g of dry matter, which contained 70% CH_4 and 30% CO_2. On an average each kg of dry pomace generated 40 l of H_2, consuming 43.3% TS & 57.2% OS followed by 140 l of CH_4/day with an overall reduction of 73.3% in TS and 76.4% in OS (Table 4.5). The digestion led to the reduction of COD by 22% in acidogenic stage, 40% in the H_2 producing stage and total of 70% in methanogenic stage. The bioconversion capacity of the process was affected to some extent by the quality of apple pomace, which varied from season to season.

Pilot plant studies: The results obtained on the 100-fold upscaled pilot-plant during continuous daily batch fed culture for 60 days are tabulated in Table 4.5. The results reveal that acidification of apple pomace at laboratory and pilot-plant level are almost similar. In the stage II (H_2 generation stage), the total biogas-H yields were 60% of the laboratory scale and the purity of H_2 evolved was also low (40%). Thus the quantity of H_2 decreased from 600 ml/15 g of dry matter at laboratory

TABLE 4.4: BIOCONVERSION OF APPLE POMACE INTO HYDROGEN AND METHANE ON LABORATORY SCALE IN THREE STAGE CONTINUOUS CULTURE.

Stage	Volume (l)	Retention time (Days)	Gas Yield (l/day)	Gas Composition (%) H_2 : CO_2 : CH_4	COD reduction (%)
I	2.Ø	4	-	-	22
II	5.Ø	1Ø	1.Ø	6Ø : 4Ø : Ø	4Ø
III	1Ø.Ø	2Ø	3.Ø	Ø : 3Ø : 7Ø	7Ø

Gas values are based on evolution from 5ØØ ml of 3% Total solids slurry at NTP.

TABLE 4.5: COMPARISON OF BIOCONVERSION OF APPLE POMACE INTO HYDROGEN AND METHANE AT LABORATORY SCALE AND AT PILOT PLANT LEVEL.

PARAMETER	LABORATORY SCALE	PILOT PLANT LEVEL
	STAGE I	
Duration (days)	25	60
pH (*)	2.63	3.29
% TS	2.75	2.78
% OS	2.61	2.64
COD (g/l)	49.4	45.58
	STAGE II	
Duration (days)	24	57
pH (*)	5.09	6.04
Biogas-H (l/15g TS)	1.0	0.6
% H_2	60.0	40.0
% TS	1.7	1.65
% OS	1.27	1.18
COD (g/l)	38.0	35.45
	STAGE III	
Duration (Days)	40	50
pH (*)	ND	6.8
Biogas (l/15g TS)	3.0	2.6
% CH_4	70.0	55.0
% TS	0.8	0.9
% OS	0.7	0.75
COD (g/l)	19.00	25.32

* : pH values are those observed at the end of 24h of incubation after initially adjusting to 7.0.
TS : Total solids; OS : Organic solids; Biogas-H : H_2 + CO_2. The initial TS and OS of the slurry was 3.0% & 2.97% respectively. COD of feed material 3% slurry was 63.3 g/l.

scale level to 240 ml/15 g dry matter at the pilot plant level with a relatively higher reduction in TS & OS i.e., 45% & 60% respectively. In the stage III (CH_4 generation stage), the yield of biogas in the pilot plant was 13% less than laboratory scale but the reduction in its CH_4 content was 21.5%. The net reduction in CH_4 yield was 32%. In the pilot plant the total reduction of TS was 70% and OS was 75%. The process reduced the COD by 28, 44 & 60% in I, II & III stages respectively.

Pilot plant thus worked at an efficiency of 40% for H_2 generation and 68% for CH_4 generation in comparison to the laboratory scale.

A comparison of bioconversion of apple pomace to H_2 under natural temperature conditions (varying from 23-43°C) in an open area on laboratory scale and controlled conditions for pilot plant are presented in Table 4.6. It is observed that the small scale operation was still 36% better than pilot plant. It was also observed that the purity of H_2 was higher (61.8%) during this round of opertion of pilot-plant for 54 days than as earlier observed (Table 4.5) but the total yield of biogas-H was lower by 38% thus making the two operations to work at similar efficiencies. Under uncontrolled temperature conditions the efficiency of H_2 production dropped by 47% when compared with results observed in temperature controlled laboratory experiments.

4.6 Direct biomethanation of apple pomace:

Table 4.7 presents the two stage biomethanation of apple pomace on laboratory and pilot plant level carried out in a continuous batch fed system for 120 and 80 days respectively. In acidogenic phase (2 l capacity), at a retention time of 4 days,

TABLE 4.6: COMPARISON OF HYDROGEN PRODUCTION FROM APPLE POMACE IN PILOT PLANT UNDER CONTROLLED CONDITIONS AND LABORATORY SCALE UNDER NATURAL ENVIRONMENTAL CONDITIONS.

	LABORATORY SCALE	PILOT PLANT LEVEL
	STAGE I	
Duration of experiment (days)	23	56
pH #	2.63	3.29
% TS	2.76	2.71
% OS	2.63	2.59
	STAGE II	
Duration of experiment (days)	32	54
pH #	5.60	6.16
Biogas-H (l/kg TS)	37.80	25.07
% H_2	56.0	61.8
H_2 (l/kg TS)	21.2	15.5
% CO_2	44.0	38.2
% reduction TS	44.1	45.9
% reduction OS	57.2	58.4

: pH values are those observed after every 24 h of incubation (initially after adjusting to 7.0 with 1N NaOH).
TS : Total solids. OS : Organic solids.
The initial TS and OS of apple pomace slurry was 3.0% & 2.91% respectively.

TABLE 4.7: DIRECT BIOMETHANATION OF APPLE POMACE AT LABORATORY SCALE AND AT PILOT PLANT LEVEL IN CONTINUOUS CULTURE.

Parameters	Laboratory scale	Pilot plant level
	STAGE I	
Duration of experiment (days)	120	80
pH	3.60	3.66
% TS	2.78	2.76
% OS	2.66	2.66
COD (g/l of slurry)	40.40	42.30
	STAGE II	
Duration of experiment (days)	120	80
Biogas (l/kg TS)	275.00	178.20
% CH_4	57.00	65.40
CH_4 (l/kg TS)	156.80	116.50
% TS	1.67	1.81
% OS	1.53	1.70
COD (g/l of slurry)	23.30	32.00
	Rotten apple pomace	
Duration of experiment (days)	125	
Biogas (l/kg TS)	241.00	
% CH_4	59.00	
CH_4 (l/kg TS)	142.20	

COD of the feed material is 20.2 kg/kg of TS or 63.3 g/l of slurry (3% TS). TS : Total solids. OS : Organic solids. Biogas = (CH_4+CO_2+H_2S).The initial TS and OS of apple fresh pomace slurry was 3.0% & 2.95% respectively.
* Initially fed with fresh apple pomace for 50 days.

the fermentation of 3% TS slurry of apple pomace mixed with cattle dung (9:1) brought the pH down to 3.6 from an initial value of 5.8, as a result of acid production. This also led to the reduction of COD by 36.18%, consequent to reduction of 7.33% in TS and 9.8% in OS. The acidification stage almost acted in a similar fashion in the 100-fold upscaled pilot plant.

In the stage II i.e., the methanogenic stage the digestion was carried out by the enriched methanogenic culture. Under laboratory conditions, the methanogenesis led to the generation of 275 l of biogas/kg TS, which comprised of 57% CH_4, thus evolving 156.8 l of CH_4/kg TS. Simultaneously the TS and OS reduced by 44.3 and 48.1% respectively. This digestion also helped in reducing the COD by 63%. In comparison, the results of the upscaled experiment indicate slightly lower efficiency, generating 178.2 l of biogas/kg of TS. But the quantity of CH_4 obtained was slightly better, evolving 116.5 l of CH_4/kg TS at a purity of 65.4%. This low gas yield was as a result of lesser reduction of TS and OS i.e., 39.7% and 42.37% respectively. The effluent from the plant had a COD level of 32 g/l i.e., a reduction of 49.5%.

The results indicate that the pilot plant worked at an efficiency of 74.3% with respect to CH_4 generation, when compared with the results obtained on the laboratory scale.

At the same time when the laboratory scale digester being run on FAP for 80 days was fed with rotten apple pomace seperately for direct biomethanation the biogas yield decreased marginally, thus generating 241 l of bigas/ kg TS, with a CH_4 content of 59% (Table 4.7).

4.7 Acclimatization of methanogens to fruit and vegetable wastes:

Apple pomace methanogenesis. The initial cattle dung slurry (CD) was seuentially replaced by AP over a period of 80 days. During this phase, biogas was evolved both by CD and AP. Each addition of 100 ml slurry yielded 1.3 l of biogas.The biogas had 55% CH_4 content. At the end of this period the effluent from the bioreactor contained 1.73% TS.

In the second phase, i.e. from 81 days after start of the digestion the data obtained from bioconversion of AP, carried out in two different bioreactors, upto 113 and 121 days has been presented in Table 4.8. The biogas yield was 275 l/ kg of total solids fed and the CH_4 yield 156 l/ kg of TS fed. The CH_4 content changed from 55% in AP + CD phase to 56.6% in AP phase. Effluent from the methanogenic stage reflected an overall reduction of 44.3% in TS and 48.1% in organic solid (OS) content with respect to the original feed material, which had 3 % TS & 2.9% OS contents. The process reduced the COD level from 63.3 g/l to 23.5 g/l , i.e., a 63% reduction. Fruit waste (apple pomace) when used as freshly dried material evolved 155.6 l of CH_4 / kg (TS) fed.

Vegetable waste methanogenesis:

Fermentation of cauliflower & raddish waste with cattle dung slurry: With fresh CD or enriched CD as inoculum, the parameters of methanogenesis of cauliflower and raddish waste (CRW) are presented in Table 4.9. pH of the methanogenic stage remained at 6.91. It generated 212 l of biogas / kg TS fed, which was composed of 72.8% CH_4, 21.9% CO_2 and 5.3% H_2S. Effluent of these

TABLE 4.8: COMPARISON OF BIOGAS YIELD AND COMPOSITION CHARACTERISTICS OF THE EFFLUENT ORIGINATING FROM BIOCONVERSION OF DIFFERENT PLANT MATERIALS.

Plant material (*)	Yield (l/kg TS)				Biogas Composition (%)						Net reduction (%)					
	Biogas		CH_4		CH_4		CO_2		H_2S		TS		OS		COD	
	N	X	N	X	N	X	N	X	N	X	N	X	N	X	N	X
AP	44	275	27	155.6	27	56.6	27	36.1	6	7.3	18	44.3	18	48.1	5	62.8
CRW	80	210	23	150.7	23	71.8	23	22.5	16	5.7	22	60.0	22	62.4	7	62.8
RC	17	320	4	217.6	4	68.0	4	25.1	4	6.9	4	61.3	4	63.6	4	69.0

AP : Apple pomace. CRW : Cauliflower and raddish waste. RC : Rotten cabbage. N : Number of observations. X : Mean value. TS : Total solids. OS: Organic solids. COD : Chemical Oxygen Demand (g/l of slurry). Net reduction values are the differences between the feed into the acidogenic stage and the effluent from the methanogenic stage.
(*): See Methods for lengths of feeding periods.

TABLE 4.9: CHARACTERISTICS OF BIOMETHANATION OF CAULIFLOWER AND RADDISH WASTE UNDER DIFFERENT CULTURE CONDITIONS.

Parameters of methanogenesis	Cauliflower and raddish biomethanation			
	On cattle dung (#)		On cattle dung fed with AP ($)	
	N	X (!)	N	X
Biogas yield (l/kg TS)(*)	29	212.0	51	208.0
Methane yield (l/kg TS)	10	154.0	13	147.0
% Methane	10	72.8	13	70.8
% Carbon dioxide	10	21.9	13	23.1
% Hydrogen sulphide	10	5.3	13	6.1
pH of the effluent	12	6.91	18	7.22
% Total solids in the effluent	11	1.29	11	1.11
% Organic solids in the effluent	11	0.93	11	0.95
COD reduction	4	63.1	4	61.5

(#) : The values are means of the observations (N) recorded on Cattle dung enriched in two different set of conditions. See Method for details ($) : Cattle dung fed with apple pomace for 80 & 87 days. (*) : Total solids employed. (!) : Standard Errors were < 10 % and have thus not been mentioned individually.

fermented materials had a TS content of 1.29% i.e., 57% reduction and OS content of 0.93 i.e.,62.8% reduction, with respect to feed material (TS 3.0% ; OS 2.5%). The fermentation led to a 63.1% reduction in COD level i.e., from 62.4 g / l of fresh CRW slurry to 23 g/l in the effluent. These observations are quite similar to those recorded on the overall biomethanation of cauliflower and raddish waste. (Table 4.8).

Although bioconversion of these vegetable wastes was possible with cattle dung slurry, an attempt was made to check if the methanogenic culture capable of fermenting apple pomace can be used also on vegetable waste.

Methanogenesis was concurrently observed on inoculum enriched for AP, in two different bioreactors. The switch over of feed material from apple pomace to vegetable waste became evident very soon. The gas generation rate was observed to attain another plateau of 208 l biogas / kg TS fed, which was composed of 70.8% CH_4 (Table 4.9). The effluent reflected a 63% TS reduction, 62% OS reduction and 61.5% COD reduction. These features are quite comparable to bioconversion of vegetable waste observed with CD as such and the overall methanogenesis of vegetable waste (Tables 4.8 & 4.9).

Bioconversion of Rotten cabbage: Rotten cabbage slurry replaced the raddish and cauliflower waste which had previously replaced apple pomace.

Methanogenic stage: The switch over of feed material from raddish and cauliflower waste to rotten cabbage did not affect the pH of the methanogenic stage. pH remained near neutral i.e., 6.87±0.16. In this favourable pH range, methanogenesis continued actively, generating 320 l of biogas / kg TS fed. The

composition of the gas evolved was comparable with that generated from other vegetable wastes (Table 4.9). In the process of bioconversion, the effluent showed a 61.3% reduction in TS, 63.6% reduction in OS content and 69% in COD level, in comparison to the original feed.

4.8 Hydrogen and methane generation from dumping wheat grains:

The C,H,N analysis of dumping wheat grains used in our studies showed C & H content of 40.5% & 6.6% respectively with undetectable N content.

Bioconversion of dumping wheat slurries could be observed after 4 h of incubation, with the evolution of gaseous mixture, which had H_2 , CO_2 and H_2S (Biogas-H).

Batch culture:

The bioconversion of dumping wheat grains was observed initially for H_2 evolution and subsequently for CH_4 generation in incubation assemblies of 300 ml capacity. The results of these two sets of experiments are presented here.

Hydrogen evolution :

The evolution of biogas-H was observed to be affected by the concentration of the substrate i.e., dumping wheat. The variation in the quality and quantity of biogas-H re presented in Table 4.10 and Fig. 4.10a.

At low substrate level of 3% TS, the hydrogen producing bacteria actively evolved biogas-H for a period of 6 days. However, H_2 generation was evident only till the

TABLE 4.10: BIOCONVERSION OF DUMPING WHEAT GRAINS INTO HYDROGEN IN BATCH CULTURES AT DIFFERENT SUBSTRATE LEVELS.

Slurry concentration	Days of incubation	pH (!) after 24 h	Volume (ml)		H_2%
			Biogas-H($)	H_2	
3% TS (*)	0 - 1	4.06	35	23.18	66.24
	1 - 2	5.26	150	69.60	46.44
	2 - 3	5.20	75	23.43	31.25
	3 - 4	5.19	120	43.00	35.90
	4 - 5	6.28	20	1.94	9.73
	5 - 6	6.32	25	0.00	0.00
	Total (ml) =		425	161.15	
6% TS	0 - 1	3.90	200	108.3	54.15
	1 - 2	5.62	420	178.3	42.45
	2 - 3	5.50	380	121.8	32.05
	3 - 4	5.41	500	125.2	25.05
	4 - 5	6.06	150	26.16	17.44
	5 - 6	6.14	100	13.26	13.26
	6 - 7	6.05	75	4.96	6.62
	7 - 8	6.76	20	0.00	0.00
	Total (ml) =		1845	577.98	
9% TS	0 - 1	3.85	75	44.38	59.18
	1 - 2	5.25	260	157.35	60.51
	2 - 3	5.63	910	307.90	33.83
	3 - 4	5.53	700	152.20	21.75
	4 - 5	6.07	320	105.30	21.48
	5 - 6	6.60	180	0.00	0.00
	Total (ml) =		2445	763.13	

(*) : TS = Total solids in a dumping wheat grain slurry volume of 270 mL. (!) : pH was adjusted to 7.0 and the values represent the pH at the end of 24 h of incubation. ($) : Biogas-H represents the mixture containing H_2, CO_2 and H_2 S.

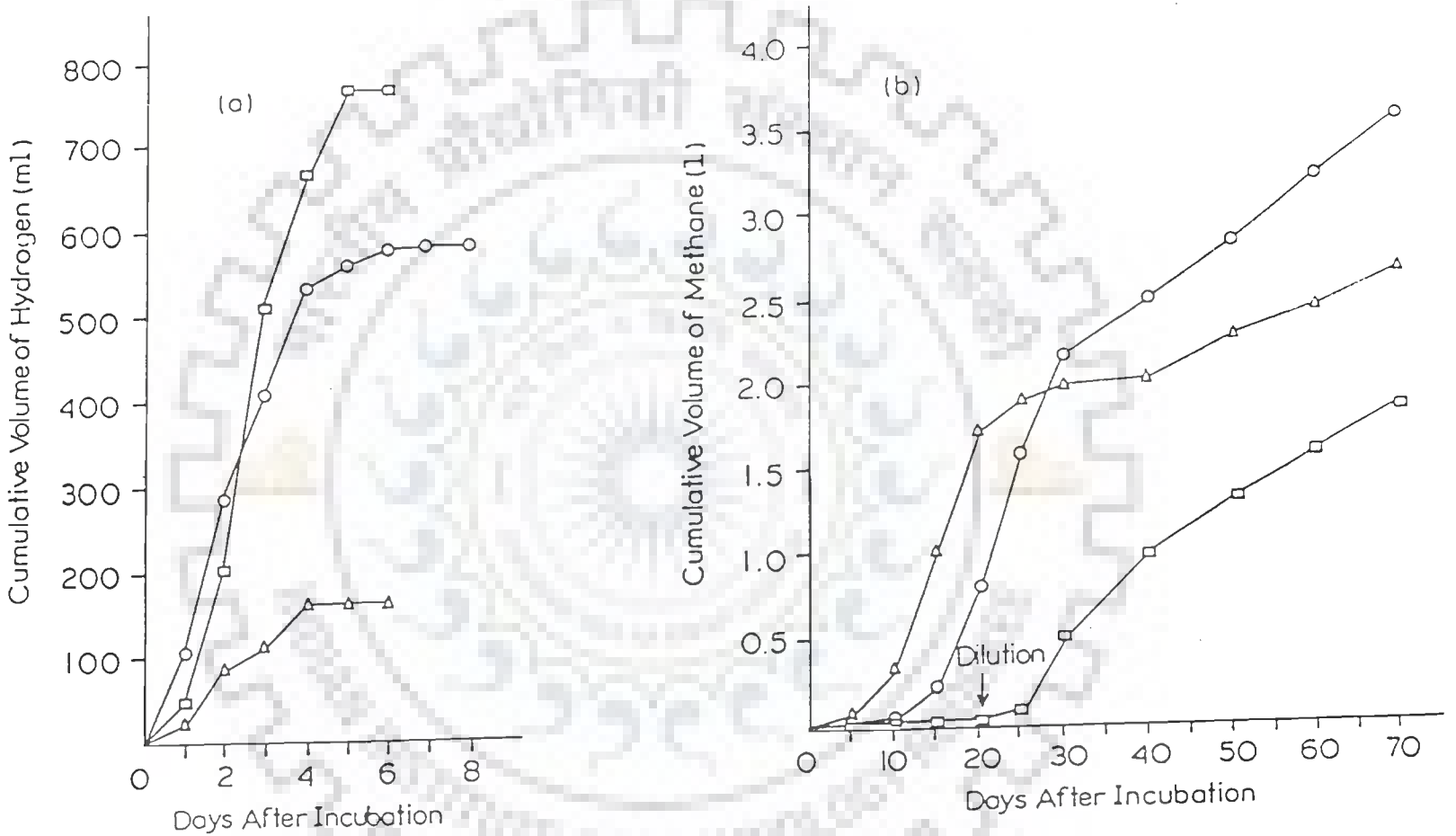

Fig. 4.10: Evolution of (a) hydrogen and (b) methane from dumping wheat at different substrate levels, (Δ)3% TS; (O) 6% TS and (□) 9% TS.

5th day. During this period 425 ml of biogas-H was generated which contained 161.15 ml of H_2 (37.92%). The H_2 evolution rate was quite high since the beginning till the 4th day such that 99% of the total H_2 was evolved in this duration, the maximum being during the 2nd day. In the active H_2 evolution phase, there was a drop in pH values after every 24 h incubation from 7.0 to about 5.2. On day 5 and 6, there was relatively little change in pH and the biogas-H had <1 % H_2 . At the end of this phase of fermenation the DWS showed a net reduction of 45.2% in its organic solid (OS) content (Table 4.11). From each kg of OS reduced, 104 l of biogas-H were evolved, of which 39.4 l was H_2 .

As the concentration of TS in the DWS was increased from 3% to 6%, the duration of biogas-H evolution also increased from 6 days at 3% to 8 days at 6% TS level. The biogas-H evolution rate continued to show a good correlation with the drop in pH of the slurry after every 24 h incubation. From 0-4 days the pH values dropped from 7.0 to about 5.5. However, from day 4 onwards, as there was relatively little pH change, the evolution rate of biogas-H also reduced dramatically. Over a period of 8 days, 1.84 l of biogas-H evolved had 578 ml of H_2. The maximum H_2 evolution was observed during the 24 - 48 h after incubation, as was the case with 3% TS DWS. The trend of fall in H_2 quality was from the maximum of 54.15% at day 1 to 6.62% at the end of day 7. This had a good correlation with changes in pH i.e., larger H_2 evolution was seen when the pH of the slurry dropped to values between 5.41 to 5.62. By the end of 4 days of incubation, 92.5% of the total H_2 evolution had taken place. The biogas-H evolution led to the reduction in OS content of DWS by 57.2%. Thus, at 6% substrate concentration level, it was possible to

TABLE 4.11: COMPARISON OF HYDROGEN EVOLUTION FROM DUMPING WHEAT GRAINS AT DIFFERENT CONCENTRATIONS.

Dumping wheat slurry concentration	Yield (L / kg OS reduced) *				Per cent			% reduction	
	Biogas-H($)	H_2	CO_2	H_2S	H_2	CO_2	H_2S	TS	OS
Batch culture :									
3% TS (!)	104	39.4	60.4	4.2	37.9	58.1	4.0	37.3	45.2
6% TS	178	55.7	104.7	17.6	31.3	58.8	9.9	50.2	57.2
9% TS	159	48.2	93.5	17.3	30.3	58.8	10.9	49.2	62.1
Continuous culture									
6% TS (5 days RT) #	238	48.8	163.5	25.7	20.5	68.7	10.8	41.5	43.1

* OS : Organic solids. (!) TS : Total solids . # RT : Retention time.
($) Biogas-H represents mixture of H_2, CO_2 & H_2S.

generate 178 l of biogas-H /kg of OS reduced (Table 4.11), which was composed of 55.7 l of H_2 i.e., 31.3% H_2 in the total biogas-H. The substrate level of 6% TS therefore evolved biogas-H and H_2 more efficiently than the 3% TS dumping wheat slurry.

At an increased substrate concentration of 9% TS, the hydrogen producing bacteria, fermented dumping wheat grains in a slightly different manner. Although the duration of biogas-H or H_2 evolution was not different from 3% TS DWS, there were differences in the period when the gas evolution rates were at their maximum. The maximum biogas-H and H_2 production occurred during 48 - 72 h instead 24 - 48 h period as in the previous two cases (Table 4.10 & Fig. 4.10 a). Unlike at 3% TS DWS, the biological activity of H_2 evolution was high even on the 5th day. The correlation between changes in pH values and gas evolution during 24 h incubation holds good even in this case. The bioconversion of DWS evolved 2.45 l of biogas-H and 73 ml of H_2 and resulted in 62.1% reduction in its OS content (Table 4.11). The trend in reduction in OS content thus increased from 45.2% at 3%TS DWS to 62.1% at 9% TS DWS. The net evolution of biogas-H was 159 l/kg OS reduced and that of H_2 was 48.2 l/kg OS reduced, which proved to be working at an efficiency level quite similar to 6%TS DWS.

Methanogenic phase :

It may be observed from Table 4.11 that although the slurries were allowed to ferment till biogas-H evolution ceased, the reduction in TS% ranged from 37.3 to 49.2 and in OS% from 45.2 to 62.1. There was thus still scope for more fermentation

to take place. This view was tested with the help of methanogenic bacteria. The results of biomethanation are presented in Tables 4.12 & 4.13 and Fig. 4.10b.

Under similar incubation conditions, the three different TS concentrations of DWS were found to vary considerably in their biogas generation capacities. In 3% TS DWS, methanogenesis was observed to set in very fast. Biogas evolution continued to occur till 70 days after incubation. During this period, 3.65 l of biogas evolved had 2.65 l of CH_4 (Table 4.12). The CH_4 content varied from 71.6% to 92.3%, except during the last 10 days, when it dropped to a low value of 40.8% . The overall value of CH_4 in biogas was 72.67%. Within 20 days of fermentation, about 63% of biogas and 66% of CH_4 evolution had already taken place. During the biomethanation phase there was a further reduction of 56.6% in OS content. The 3% DWS after having undergone two phases of fermentation i.e. biogas-H and biogas, showed a net reduction of 76.22% in its OS content. The biogas yield was 747 l per kg OS reduced and the CH_4 yield was 542 l/kg OS reduced.

With 6% TS DWS, the biomethanation rate was quite different from the 3% TS DWS fermentation (Tables 4.12 & 4.13, Fig. 4.10b). Biomethanation activity was relatively low during the initial period of 15 days of incubation (Table 4.12 & Fig.4.10b). The rate of biogas and CH_4 evolution were quite steady from 30-70 days of incubation. The fermentation of 6% TS DWS by methanogenic bacteria thus led to the evolution of a total of 4.75 l of biogas, which had 3.54 l of CH_4. The CH_4 content was about 50% during the initial period of 10 days but improved to 64-85% during the rest of the biomethanation phase. The process of biomethanation resulted in further decrease in OS content of the DWS by 53.6%. Biogas yield was only 514

TABLE 4.12: PATTERN OF BIOGAS AND METHANE EVOLUTION FROM DUMPING WHEAT GRAINS ($) INCUBATED IN BATCH CULTURES.

Dumping wheat grain slurry concentration	Days after incubation	Volume (ml)		CH_4%
		Biogas	CH_4	
3% TS (*)	0 - 5	90.4	71.9	79.5
	5 - 10	333.2	277.9	83.4
	10 - 15	895.0	705.3	78.8
	15 - 20	933.0	668.0	71.6
	20 - 25	171.4	158.2	92.3
	25 - 30	100.0	92.3	92.3
	30 - 40	57.1	46.4	81.3
	40 - 50	304.1	244.3	80.3
	50 - 60	190.4	152.3	80.0
	60 - 70	571.2	233.0	40.8
	Total(mL) =	3645.8	2649.6	
6% TS	0 - 5	61.9	30.4	49.2
	5 - 10	28.6	14.7	51.5
	10 - 15	115.2	77.6	67.4
	15 - 20	1075.2	687.4	63.9
	20 - 25	1047.2	795.4	75.9
	25 - 30	762.0	563.9	74.0
	30 - 40	419.0	325.6	77.7
	40 - 50	399.8	333.8	83.5
	50 - 60	419.0	355.7	84.9
	60 - 70	428.4	351.2	82.0
	Total(ml) =	4756.3	3535.7	
9% TS	0 - 5	42.8	29.7	69.4
	5 - 10	9.5	6.7	70.5
	10 - 15	0.0	0.0	0.0
	15 - 20	0.0	0.0	0.0
9 (3)% TS (!)	20 - 25	71.4	59.0	82.6
	25 - 30	504.6	409.2	81.1
	30 - 40	599.8	484.0	80.7
	40 - 50	380.8	323.7	85.0
	50 - 60	333.2	264.6	79.4
	60 - 70	295.1	234.3	79.4
	Total(ml) =	2237.2	1811.2	

($) : The slurry represents the one which has previously been used for evolving hydrogen. (*) : TS = Total solids. (!) : As 9% TS level slurry stopped evolving any biogas during 10 - 20 days after incubation, the slurry was diluted to 3% TS level.

TABLE 4.13: BIOMETHANATION CHARACTERISTICS OF DUMPING WHEAT GRAINS AT DIFFERENT SUBSTRATE LEVELS.

Dumping wheat slurry concentration	Yield (L/kg OS reduced)				Per cent			% reduction	
	Biogas	CH_4	CO_2	H_2S	CH_4	CO_2	H_2S	TS(!)	OS(*)
Batch culture :									
3% TS	747	542.3	128.4	76.2	72.6	17.2	10.2	50.3	56.6
6% TS	514	381.9	87.9	44.2	74.3	17.1	8.6	52.6	53.6
9 (3)% TS($)	516	418.9	72.7	24.2	81.2	14.1	4.7	42.3	49.1

(*) OS: Organic solids. (!) TS: Total solids. ($): Value represent the 9% TS slurry after dilution to attain 3% TS level, as 9% TS level slurry evolved only negligible quantities of biogas for the first 20 days of incubation.

l per kg OS reduced in comparison to 747 l/kg OS reduced at 3% TS DWS. Although, the quality of biogas in terms of its CH_4 content was quite similar in 3% and 6% TS DWS, the quantities of CH_4 generated were different (Table 4.13). The biomethanation efficiency (CH_4 content) of DWS 6% (TS) was 29.6% lower than in the case of the 3% TS DWS. The 6%(TS) DWS when subjected to H_2 producing bacteria and subsequently to methanogens led to an overall reduction of 80.2% in its OS content.

The biomethanation of DWS 9% (TS), which had been exhausted of its biogas-H producing potential, was very different from that observed at 3% and 6% (TS) DWS. Inspite of the similar environmental conditions in all the three cases, 9% DWS evolved very negligible quantities of biogas in the initial 10 days of incubation and there was no gas evolution between 10-20 days of incubation. It thus came to a no biomethanation activity phase. At this stage the 9% (TS) DWS was diluted to 1/3 its concentration by adding water (Since 3% (TS) DWS undergoes very active biomethanation even within 20 days). Methanogenesis was found to set in soon (Table 4.12 & Fig. 4.10b). The fermentation of DWS by methanogens evolved 2.24 l of biogas. The evolution of 1.8 l of CH_4 was almost evenly distributed in this phase. The CH_4 content varied from 69.4 to 85%, the overall percentage being 81.2%. The biomethanation process resulted in 49.1% reduction in OS content of DWS. Thus, over the period when DWS was allowed to undergo complete fermentation i.e., biogas-H and biogas evolution, the overall reduction in OS was 80.7%. The total biogas evolution was 516 l/kg OS reduced. Thus, on dilution it

proved to be more efficient than the 6% (TS) DWS biomethanation but not as efficient as the 3% (TS) DWS.

A comparison of the fermentation of DWS at three substrate concentrations reveals that the H_2 evolution occurred at almost similar efficiencies at 6% and 9% TS and at very low efficiency at 3% TS. On the other hand, the efficiency of CH_4 evolution was in the order 3% > 6% >9%.

Continuous culture :

Hydrogen evolution was observed at two different loading rates (3% and 6% TS) and two different retention times of 5 days and 10 days.

1) Two stage culture : The digestion was carried out in two stage fermenters of 400 ml and 2 l capacities respectively. At 10 days retention time (RT) and 3% and 6% TS , H_2 evolution was evident for about 8 - 10 days of incubation. However, in each case CH_4 evolution became evident after 7 days of incubation. At 6% TS DWS, in one of the cases, H_2 evolution continued for a longer period i.e., upto 22 days but once again, CH_4 evolution could not be suppressed, although it was slightly delayed. Thus under these conditions, CH_4 evolution interfered with H_2 generation.

2) Single stage culture : At 5 days RT, 6% TS DWS was chosen on the basis of its efficiency in batch culture experiment. Over continuous period of 30 days, digestion in a reactor of 600 ml capacity, there was regular generation of H_2. No CH_4 was obsered to evolve at all. The biogas-H evolution rate of 238 l/kg of OS reduced was much more than that observed in batch culture

(Table 4.11). However, the H_2 evolution potential in continuous culture was observed to be lower than the batch culture. It was 48.8 l H_2 per kg OS reduced in comparison to 55.7 l H_2/ kg OS reduced in batch culture system. The reduction in OS was only 43.1 %.

4.9 Methanogenesis of dumping wheat grains with recycling of the effluent

Methanogenesis of dumping wheat grains : During 0-60 days of addition of dumping wheat material into the cattle dung slurry the biogas evolved had been a result of fermentation of both the dumping wheat and solids carried over from dung slurry. On an average 1.14 l of biogas was evolved per 100 ml slurry addition every day. It had 57 % methane content. The observations recorded after 60 days of incubation at 40°C have been presented below.

1) At 3% total solids dumping wheat slurry level, (Table 4.14) the methanogenesis was observed to be very active. The biogas evolved composed of 69.1 % methane. Reduction of each kg of organic solids (OS) led to the generation of 544.7 l of biogas. During the bioconversion, a 69.3 % reduction in OS level was evident in the effluent, with respect to the feed material.

2) At 6% total solids dumping wheat slurry level (Table 4.14), after an initial active methanogenesis for 60 days, it was observed that biogas evolution from dumping wheat grains attained a steady state. The biogas during this final period of 30 days had very low level of methane i.e., 24.9 % . At this 6% TS concentration only 195.3 l of biogas / kg of OS reduced was observed. The effluent reflected a 50.1 % reduction in the OS content. Thus from each kg of OS reduced at 3% TS

TABLE 4.14: CHARACTERISTICS OF METHANOGENESIS OF DUMPING WHEAT GRAINS AND THE RECYCLED EFFLUENT.

Parameters	Biogas yield (l/kg OS reduced)	CH_4 yield (l/kg OS reduced)	Per cent		
			CH_4	OS reduced	Efficiency *
At 3 % TS level	544.7	376.3	69.1	69.3	1ØØ.Ø
At 6 % TS level	195.3	48.6	24.9	5Ø.1	12.9
Recycled effluent (At 3 % TS level) :					
(Ø-27 days)	1Ø21.3	692.4	67.8	54.9	184.Ø
(28-66 days)	596.2	416.7	69.9	6Ø.1	11Ø.7
(67-82 days)	288.8	2Ø5.9	71.3	54.7	54.7
(Ø-82 days)	693.2	48Ø.3	69.3	56.6	127.6

TS : Total solids, OS : Organic solids, * : 3 % TS level methanogenesis value has been taken as 1ØØ % efficiency level (On the basis of Methane content). The values represent the steady state methanation.

level, 376.3 l of methane could be obtained in comparison to 48.6 l of methane obtained at 6% TS level.

Recycling of the effluent: The recycling of the effluent was done only in the case of 3% TS dumping wheat slurry methanogenesis. Three different and distinct phases of biogas evolution could be observed over a period of 82 days of recycling (Table 4.14).

On recycling the effluent, during 0-27 days the overall methanogenesis rate was 1021.3 l biogas / kg OS reduced. Its methane content was 67.8%, leading to the evolution of 692.4 l CH_4 / kg OS reduced. Its efficiency (methane content) was 1.84 times more than the preceeding non-recycled system.

From 28-66 days of recycling, the biogas evolution rate was comparable to that observed with non-recycled 3% TS DWS. It evolved 596.2 l of biogas / kg OS reduced. Although the methane content of the biogas did not vary much in comparison to the first phase of recycling, the quantity of methane evolved was less i.e., 416.7 l / kg OS reduced.

In the final stage of 67-82 days the decline in biogas evolution rate was dramatic. Here, it became stable at 54.7% efficiency in comparison to the non-recycled DWS (3% TS). It evolved 288.8 l of biogas or 205.9 l of CH_4/kg OS reduced, although the reduction in OS has been quite similar to that observed during the first phase of recycling (Table 4.14).

Biomethanation of other damaged wheat grains. The biogas generation abilities of other damaged wheat grains like cattle feed (CF) and poultry feed (PF)

in continuous batch fed culture digestion for 90 day in two stage process are presented in Table 4.15. When the 4 days old preincubated slurries were acted upon by enriched methanogenic cultures in stage II, the biogas generated varied from 138 l/kg TS in the case of PF to 240 l/kg in the case of CF. The CH_4 content was quite high at 67.4 and 61.5% respectively. In comparison when fresh wheat (FW) was digested in a similar way, the biogas yield was quite low i.e., 90 l/kg TS (55.2% CH_4). The reduction in TS during the steady state were 39.3% (FW), 50.7% (PF) and 55.7% (CF).

4.10 Hydrogen producing potential of other substrates:

The carbonaceous substrates like, starch, wheat, maize, rice and cellulose were tested for H_2 evolving capacity by mixed microbial culture and the results obtained are presented in Table 4.16. The Pure potato starch could generate 260 l of biogas-H/kg TS when incubated in batch culture at 40°C for a period of 12 days. The gas mixture contained 59% H_2, thus leading to the generation of 153.4 l of H_2/kg TS. On the other hand other starchy substrates like rice, maize and wheat showed 17 - 40% lesser yields of biogas-H as compared to the pure starch. The purity of H_2 also decreased by 15 - 29%, evolving 65.9, 87.5 & 90.3 l of H_2/kg TS of wheat, maize & rice respectively. However, there was not much decrease in H_2 evolution rate as evident by lesser incubation of 7 - 8 days.

In contrast to these starchy substrates, the mixed microbial population could not ferment cellulose to H_2. 50 ml of biogas-H evolved contained mainly CO_2 with traces of H_2.

TABLE 4.15: BIOMETHANATION OF OTHER TYPES OF DAMAGED WHEAT GRAINS AND FRESH WHEAT.

Type of wheat grain slurry	* pH	Biogas yield (l/kg TS fed)		Biogas composition (%)			TS reduction
		Total	CH_4	CH_4	CO_2	H_2S	(%)
Cattle feed (CF)	6.15	240.0	147.6	61.5	32.0	6.5	55.7
Poultry feed (PF)	6.08	138.0	93.0	67.4	27.1	5.5	50.7
Fresh wheat (FW)	5.75	90.0	49.7	55.2	37.8	7.0	39.3

* pH value is that of the slurry in the methanigenic stage, observed after every 24 h of incubation. The initial pH being 7.0.
The digestion was conducted in daily batch fed culture for 90 days.

TABLE 4.16: POTENTIAL OF DIFFERENT SUBSTRATES FOR HYDROGEN PRODUCTION BY MIXED MICROBIAL CULTURE.

Source	Incubation period (days)	Biogas-H (l/kg T.S)	H_2 (l/kg T.S)	% H_2
Starch (potato)	12	260	153.4	59
Wheat	7	157	65.9	42
Maize	8	175	87.5	50
Rice	8	215	90.3	42
Cellulose	10	50	Traces	Traces

Biogas-H = H_2 + CO_2 + H_2S. 250 ml of 3% slurries were used.
T.S = Total Solids.
The experiments were conducted in batch processs.

4.11 Activity of Hydrolytic enzymes during degradation of biowaste by mixed microbial culture :

The enzymatic efficiency of the microbial culture to degrade the biological wastes such as apple pomace & dumping wheat is presented in Table 4.17. During fermentation of apple pomace, on stationary and shake cultures, the activities of the hydrolytic enzymes exuded in the medium was maximum at 48 h of incubation, the time by which maximum H_2 production had come to an end. Further incubation upto 96 h, resulted in the inactivation of cellulolytic, hemicellulolytic (Xylanase) and proteolytic activity by 20 %, 27 % and 40 % respectively. Amylolytic activity remained constant till 96 h, at 0.360×10^2 units/l. The culture showed maximum activity as: 1×10^1 units/l of cellulolytic, 0.360×10^2 units/l of amylolytic, 2.850×10^3 units/l of proteolytic and 0.494×10^3 units/ l of hemicellulolytic enzymes.

On the other hand, fermentation of dumping wheat revealed the activities of these enzymes as: 1.8×10^1 units/ l of cellulolytic, 3.190×10^2 units/ l of amylolytic, 2.390×10^3/ l of proteolytic and 2.983×10^3 units/ l of hemicellulolytic. Again these activities were maximum when H_2 production came to an end i.e., at 24 h of incubation except for cellulolytic activity. In cellulolytic activity, both endoglucanase and exoglucanase activities checked were similar. The results led us to believe that shaking increased the activities of the extracellular enzymes by 19-68 %. In comparison, dumping wheat fermentation showed almost 10 times more hemicellulolytic and amylolytic activities than apple pomace fermentation, whereas proteolytic and cellulolytic activities were almost similar.

TABLE 4.17: ENZYMATIC ACTIVITIES ON FERMENTATION OF APPLE POMACE AND DUMPING WHEAT BY MIXED MICROBIAL CULTURE.

SUBSTRATE	Incubation period (h)	Enzyme activity*							
		Xylanase		Proteolytic		Amylolytic		Cellulolytic	
		St.	Sh.	St.	Sh.	St.	Sh.	St.	Sh.
APPLE POMACE	48	396	494	2370	2850	23	36	8	10
	96	356	361	900	1710	35	36	7	8
DUMPING WHEAT	16	933	1435	1560	1800	13	24	21	18
	24	2276	2983	1710	2390	254	319	9	6

* Activity = Units l^{-1}; St. = Stationary culture; Sh. = Shake culture
The values are the mean of 3 samples each.

4.12 *Escherichia coli* pathway in the production of hydrogen:

4.12.1 Hydrogen production from glycolytic pathway intermediates:

H_2 production via glycolytic pathway intermediates by mixed microbial culture could be best explained by the individual substrates responding to the H_2 evolution, as presented in Table 4.18. Glucose at a concentration of 0.3% in 200 ml solution, produced 83.43 ml of H_2 i.e., 25.03 l/mole of substrate. In order to channel the H_2 evolution through glucose metabolic pathway, the immediate next substrate of the pathway i.e., Glucose-6- phosphate produced 20.81 l of H_2/mole of substrate. It can be seen that the cells could go from glucose substrate to its end product pyruvate for H_2 generation. The volume of H_2 produced is not proportionate for reasons which are not apparent. It has also been observed that lactate in the culture medium can produce H_2 at an efficiency of 0.81 l/mole of substrate. When these substrates were tried in combinations, additive effects on H_2 evolution were evident. Thus glucose + pyruvate and glucose + lactate generated 18.94 l and 14.57 l / mole of these substrates respectively. These gas volumes were produced during 4 - 6 days of incubation at 40°C.

Similar effects were also observed when pure culture of *B. licheniformis* was fed on glucose, glucose-6-phosphate and pyruvate in the culture medium (Table 4.18).

TABLE 4.18: CONTRIBUTION OF GLYCOLYTIC INTERMEDIATES IN HYDROGEN PRODUCTION.

substrate	Conc.	H_2 Vol.(ml)	H_2 Vol.(l/mole of substrate)	Incubation period (days)
		MIXED MICROBIAL CULTURE		
Glucose	0.3%	83.43	25.03	5
Glucose-6-phosphate	0.3%	48.20	20.81	6
Sodium pyruvate	0.3%	22.02	3.85	4
Sodium lactate	0.3%	4.32	0.81	4
Glucose + Sodium pyruvate	0.15% + 0.15%	41.18	18.94	5
Glucose + Sodium lactate	0.15% + 0.15%	31.45	14.57	5
		BACILLUS LICHENIFORMIS		
Glucose	0.3%	11.23	26.95	4
Glucose-6-phosphate	0.3%	7.1	24.52	6
Sodium pyruvate	0.3%	4.2	5.87	6

600 mg (0.3%) of the substrate was used in all the combinations, with mixed microbial culture in a total volume of 200 ml.
With pure culture 75 mg of substrate was used in a total volume of 25 ml.

4.12.2 Hydrogen production from formate via formate hydrogenlyase system.

Data on H_2 production by mixed mirobial culture and pure cultures of *Bacillus licheniformis* and strain JK_2 from formate are presented in Table 4.19. The conversion efficiency of formate into H_2 gas is theoretically 1 mole/mole, because all the available reducing equivalents of formate are converted into H_2 gas (Thauer *et. al.*, 1977). No H_2 gas was detected till the end of the log phase growth of pure cells on glucose supplemented medium whereas traces of H_2 were detected with the mixed microbial culture grown for same period on glucose medium. The induced harvested cells inoculated on 0.2% formate produced 1.32, 0.72 & 0.84 mmoles of H_2 and thus showed a conversion efficiency of 71%, 68% & 85% for mixed microbial culture, *B. licheniformis* & JK_2 respectively. Although fermentation was continued until no more gas production was visible i.e., 72 h in the case of mixed microbial culture and 48 h in the case of pure cultures, formate was not completely exhausted. The conversion efficiencies however, appear to be almost similar. The amount of formate fermented in the case of mixed microbial culture, *B. licheniformis* and strain JK_2 was 65.3%, 72.1% & 67.3%, respectively.

TABLE 4.19: HYDROGEN PRODUCTION FROM FORMATE BY PURE AND MIXED MICRBIAL CULTURES.

Organism	Days of incubation	Formate added (mmole)	Formate unutilized (mmole)	Formate fermented (mmole)	% Formate fermented	H_2 Gas produced (mmole)	Theoretical* H_2 conversion (mmole)	Conversion efficiency
Mixed microbial culture	3	2.94	1.02	1.92	65.3	1.32	1.92	71
Bacillus licheniformis	2	1.47	0.41	1.06	72.0	0.72	1.06	68
JK2	2	1.47	0.48	0.99	67.3	0.84	0.99	85

Values are mean of three experiments.
(*) 1 mmole of formate can produce 1 mmole of H_2 & 1 mmole of CO_2.

4.13 Role of Nitrogenase in Hydrogen production by mixed microbial culture:

4.13.1 Nitrogenase activity :

The nitrogenase activity of the free living cells of mixed culture has been determined by acetylene reduction activity (ARA). The activity estimated at different timings till 70 h of incubation period is presented in Table 4.20 & Fig. 4.11. Maximum activity of 0.460 nmoles of C_2H_4 produced / mg protein / h was expressed during 12-20 h of incubation period. In this range at 20 h the cells showed maximum activity of 0.376 nmoles C_2H_4 / mg protein/ h, whereas at latter stages i.e., till 70 h, the activity decreased by 55% i.e., 0.170 nmoles C_2H_4 / mg protein/ h.

Effect of oxygen concentrations: Oxygen levels of 0.5 to 20% in the gas phase appeared to be inhibitory for acetylene reduction under the stationary and shake culture conditions (Table 4.21 & Fig. 4.12). Concentration of O_2 below 0.5% gave more than 92% of the optimal acetylene reduction when tested with both cultures. In stationary culture, an oxygen level of 1%, significantly decreased the acetylene reduction activity by 55.5% i.e., from 0.409 nmoles at 1% to 0.182 nmoles C_2H_4 / mg protein / h at 20%. In contrast, in the shake culture the inhibitory effect were very prominent. No ARA was observed when the cells were incubated under a gas phase containing 20% O_2, and the inhibition was as high as to 90% when the O_2 levels were increased from 1% to 15%.

Effect of inoculum : acetylene (I : A) ratio: Results obtained by varying the inoculum : acetylene ratio on ARA are presented in Table 4.22. In an I : A ratio of 4.2 - 6.25, the nitrogenase derepression was the optimum, whereas notable

TABLE 4.20: NITROGENASE ACTIVITY OF MIXED MICROBIAL CULTURE AT DIFFERENT INCUBATION PERIODS.

Incubation Time (h)	Nitrogenase Activity * $\bar{X} \pm SD$	Incubation Phases (h)	Nitrogenase Activity * $X \pm \bar{S}D$
0	0.0±0.0	0 - 2	0.069±0.01
2	0.069±0.01	2 - 6	0.280±0.04
6	0.210±0.03	6 - 12	0.433±0.02
12	0.322±0.02	12 - 20	0.460±0.03
20	0.376±0.04	20 - 70	0.088±0.03
70	0.170±0.03	-	-

* Nitrogenase activity is determined by Acetylene reduction assay and is expressed in nmoles C_2H_4/mg protein/h.

$\bar{X}\pm SD$ = Mean ± Standard deviation.
Values are based on six experiments.

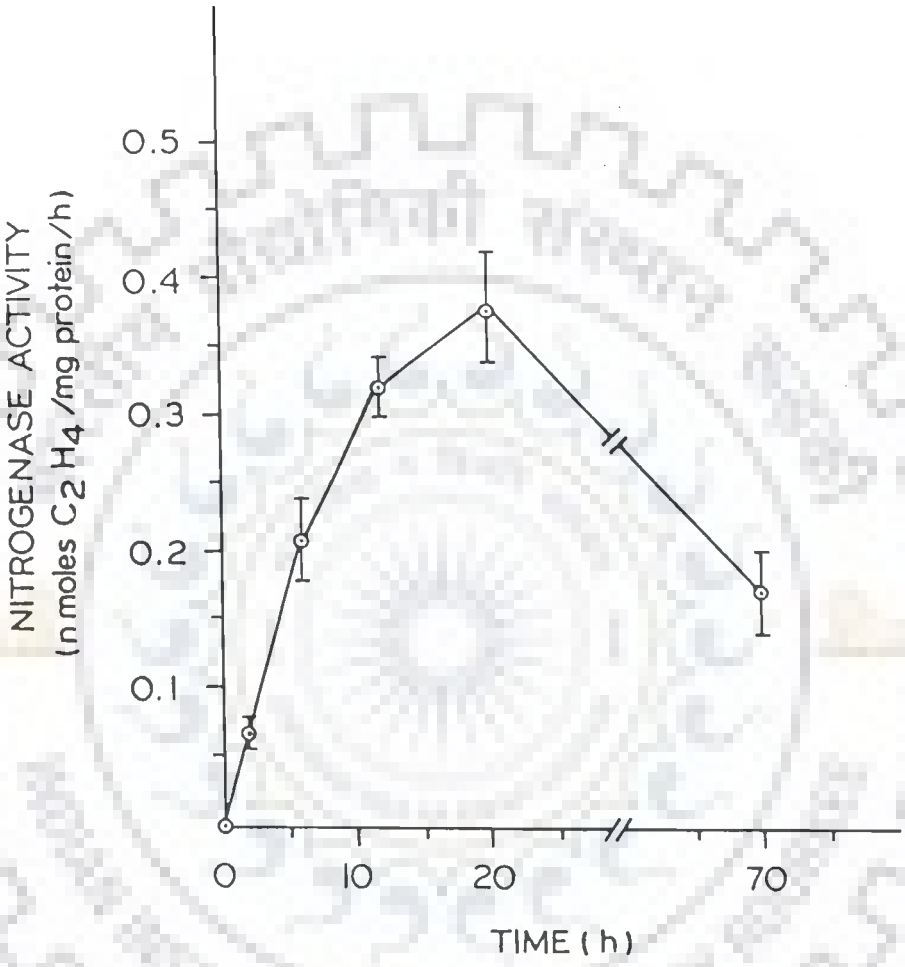

Fig. 4.11: Nitrogenase activity of mixed microbial culture at different incubation time.

TABLE 4.21: COMPARISON OF ACETYLENE REDUCTION (NITROGENASE ACTIVITY) IN MIXED MICROBIAL CULTURE AT DIFFERENT OXYGEN LEVELS.

Initial O_2 levels (%)	Acetylene reduction activity (nmoles C_2H_4/mg protein/h)	
	Stationary	Shaking
$\leq$ 0.25	0.460	0.451
0.5	0.456	0.423
1.0	0.409	0.372
5.0	0.357	0.251
10.0	0.331	0.108
15.0	0.224	0.041
20.0	0.182	0.000

Cells were incubated at 40°C at stationary and shake cultures (50 rpm) during assay. Oxygen consumption over the culture period was negligible. Data are averages of three experiments.

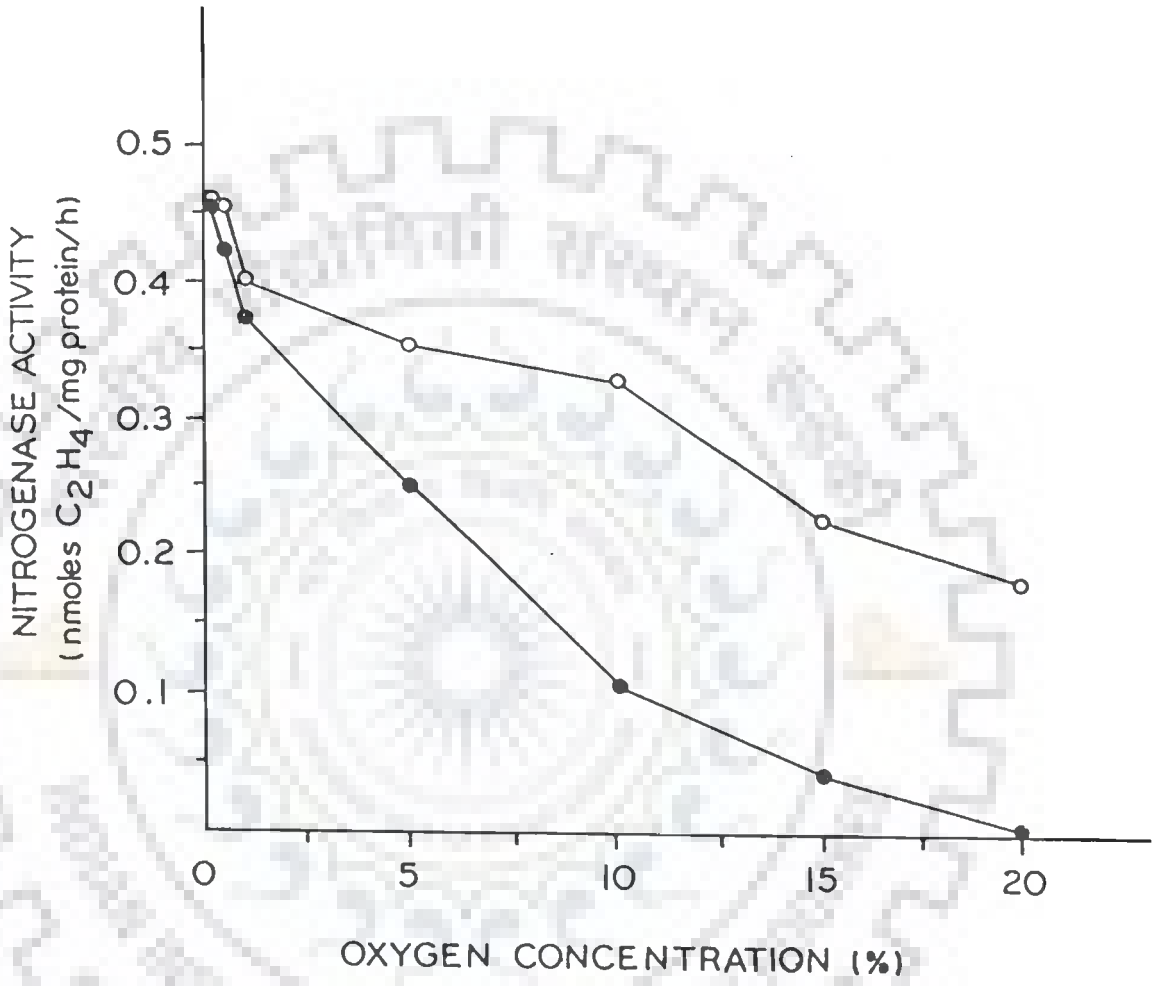

Fig. 4.12: Effect of oxygen concentration on the derepressionof nitrogenase activity. (O): on stationary culture; (●): on shake culture.

TABLE 4.22: COMPARISON OF ACETYLENE REDUCTION (NITROGENASE ACTIVITY) IN MIXED MICROBIAL CULTURE AT DIFFERING RATIOS OF INOCULUM : ACETYLENE (I : A).

Inoculum Volume (ml)	Void Volume (ml)	Acetylene Volume (ml)	I : A ratio	Acetylene reduction *
5	60	6.0	0.83	0.08
10	57	5.7	1.75	0.10
15	51	5.1	2.94	0.22
20	48	4.8	4.20	0.42
25	40	4.0	6.25	0.46
30	35	3.5	8.57	0.32
35	31	3.1	11.29	0.18

* Acetylene reduction was measured in nmoles C_2H_4 /mg protein/h at 20 hours of incubation. The total volume of incubation assemblies varied from 65 - 70 ml. Data are averages of three replicates.

decreases were evident, on deviating from this ratio. At an inoculum volume of 25 ml, when the system was exposed to 10 % (4.0 ml) of acetylene atmosphere in 65 ml incubation assembly the maximum ARA obtained was 0.460 nmoles C_2H_4 / mg protein /h.

Effect of Pre-incubation and glucose activation : Pre- incubation for 24 h in presence of 0.2 % glucose concentration was observed to be essential for the maximum expression of the nitrogenase activity (Table 4.23). In an assay system devoid of these conditions, the derepression of nitrogenase was almost negligible i.e., 0.015 nmoles C_2H_4/ mg protein / h. The acetylene reduction activity was inhibited to the level of 62.6 - 76.7 % if either of the conditions were not observed during nitrogenase development.

4.13.2 Effect on hydrogen production by inhibitors of Nitrogenase and Hydrogenase:

The inhibitory effect of C_2H_2 and CO in the gas phase on inhibition of H_2 production in shake and stationary cultures are presented in the Table 4.24. At 2 % CO level, H_2 production was inhibited by 16 % in the stationary cultures. The effect was almost two-fold when the concentration of CO in the gas phase was increased to 5 %. The shaking of the culture system showed more pronounced effect on the inhibition of the H_2 production because of the better exposure of cells to enzyme inhibitors, thereby inhibiting H_2 evolution to the extent of 75% (Table 4.24). In the shake culture, acetylene reduced H_2 evolution by 63% and the evolution was inhibited upto 95% in combination with 5% CO. The inhibitory effects were again mild in the stationary culture, indicating some cells being unexposed to inhibitors.

TABLE 4.23: EFFECT OF PRE-INCUBATION AND GLUCOSE ACTIVATION ON DEREPESSION OF NITROGENASE ACTIVITY BY MIXED MICROBIAL CULTURE.

Pre-Incubation	Glucose	Nitrogenase activity (nmoles C_2H_4/mg protein/h)
+	+	0.460 ± 0.04
+	-	0.107 ± 0.09
-	+	0.172 ± 0.13
-	-	0.015 ± 0.07

Pre-Incubation time = 24 hrs (before injecting 10% acetylene in the system).
Glucose concentration. = 0.2%

TABLE 4.24: EFFECT OF INHIBITORS OF NITROGENASE AND HYDROGENASE IN GAS PHASE ON HYDROGEN PRODUCTION BY MIXED MICROBIAL CULTURE.

Conc. of inhibitor in the gas phase	Hydrogen production (mmole)					
	Shaking			Stationary culture		
	Control	Treatment	Inhibition (%)	Control	Treatment	Inhibition (%)
2% CO	0.21	0.11	47	0.19	0.16	16
5% CO	0.40	0.10	75	0.39	0.27	31
10% C_2H_2	0.19	0.07	63	0.17	0.11	35
10% C_2H_2 + 5% CO	0.40	0.02	95	0.39	0.20	49

Data is the average of five replicates and SD is less than 10% in each case.
Cells were incubated at 40°C with 0.2% glucose on a shake culture at 110 rpm and also as stationary culture.
Controls were run under argon atmosphere and the variations in the H_2 production in these experiments were because of the different culture and incubation conditions. But every treatment and its control were performed under similar conditions.
Different gas inhibitors were injected after replacing the internal atmosphere by the same amount.

To assess the reversibility of the effect of inhibitors, it has also been observed during the course of these studies, that stationary culture cells showed 50 % of the reversibility of the effect once the inhibitors were removed from gas atmosphere. On the other hand such reversibility was 10 % in the shake cultures.

The effect of NO_3^- and NH_4^+ in the liquid media on H_2 evolution is presented in Table 4.25. H_2 production was reduced by 16 % at 2.5 mM KNO_3 level and no H_2 was evolved at 6.6 mM $NaNO_3$. NH_4Cl in the liquid culture showed unstable effects. In a concentration range of 6.6 m M to 0.2 M, the H_2 evolution was inhibited by 7 - 44 %, whereas increases in H_2 volumes of similar order were also evident in the studies for reasons not understood.

4.14 Metabolism of volatile fatty acids (VFAs) during hydrogen production:

Changes in volatile fatty acids (VFA) and H_2 production from glucose, dumping wheat grains and apple pomace on fermentation by mixed microbial culture are presented in Table 4.26 & Fig. 4.13 & 4.14. With glucose, the concentration of acetic acid varied from 220 mmoles/l at 0 h of incubation to 110 mmoles/l just before the H_2 production was evident. Thereafter, its concentration increased upto 200 mmoles/l, concurrently with H_2 generation rate from 0 - 1.5 ml/h during 11 - 28 h of incubation. On an average, the concentration of acetic acid remained at 202 mmoles/l of slurry in the pre- H_2 producing phase, whereas it stabilized at 126.22 mmoles/l during H_2 producing phase. n- Butyric acid, propionic acid and valeric acid remained at low levels throughout the incubation period. During pre- H_2 producing phase propionic acid levels were 22.54 mmoles/l and n-butyric acid production was 6.01 mmoles.

TABLE 4.25: EFFECT OF INHIBITORS OF NITROGENASE ON HYDROGEN PRODUCTION BY MIXED MICROBIAL CULTURE.

Concentration	Hydrogen production (mmole)		
	Control	Treatment	Inhibition (%)
2.5mM KNO_3	0.44	0.37	16
6.6mM KNO_3	0.49	0.02	96
10.0mM KNO_3	0.49	0.00	100
6.6mM $NaNO_3$	0.44	0.00	100
6.6mM NH_4Cl	0.45	0.53	17.8 #
10.0mM NH_4Cl	0.45	0.39	12.9
18.0mM NH_4Cl	0.45	0.48	6.9 #
25.0mM NH_4Cl	0.45	0.38	14.8
50.0mM NH_4Cl	0.45	0.29	34.0
0.1 M NH_4Cl	0.45	0.65	43.0 #
0.2 M NH_4Cl	0.45	0.25	43.0
6.6mM - 0.2M NH_4Cl	-	-	7 - 43 *

Data is the mean of five experiments for KNO_3 & $NaNO_3$ treatments, whereas NH_4Cl treatment results are from three replicates. SD was $\leq \pm 10\%$ in each case.
Cells were incubated at 40°C with 0.2% glucose with the different concentrations of inhibitors in the liquid phase.
* NH_4Cl showed inconsistent effect. There was inhibition as well as stimulation of the hydrogen production.
# Gain.

TABLE 4.26: FATTY ACID FORMATION DURING HYDROGEN METABOLISM BY MIXED MICROBIAL CULTURE.

Duration	Glucose				Dumping wheat				Apple pomace			
	Acetic acid	Propionic acid	Butyric acid	Valeric acid	Acetic acid	Propionic acid	Butyric acid	Valeric acid	Acetic acid	Propionic acid	Butyric acid	Valeric acid
	(mmoles/l)				(mmoles/ l)				(mmoles/ l)			
Pre-H_2producing phase	202.60	22.54	6.01	7.46	16.55	4.52	0.0	0.0	19.51	5.48	2.17	0.0
H_2producing phase	126.22	11.36	21.02	1.11	47.52	3.83	32.45	0.0	22.72	6.64	7.59	0.0
Post-H_2producing phase (*)	ND	ND	ND	ND	86.80	31.40	77.70	0.0	ND	ND	ND	ND

Values are average concentration of different fatty acids formed during different stages of H_2 metabolism in first 27 hrs of incubation.
ND = Not determined (the stage was not reached during first 27 hrs of incubation)
The samples were analysed in duplicate.
(*) post-H_2 producing phase was considered in the case of dumping wheat fermentation and was taken from the time the H_2 production becomes very low i.e., after 18 h of incubation.

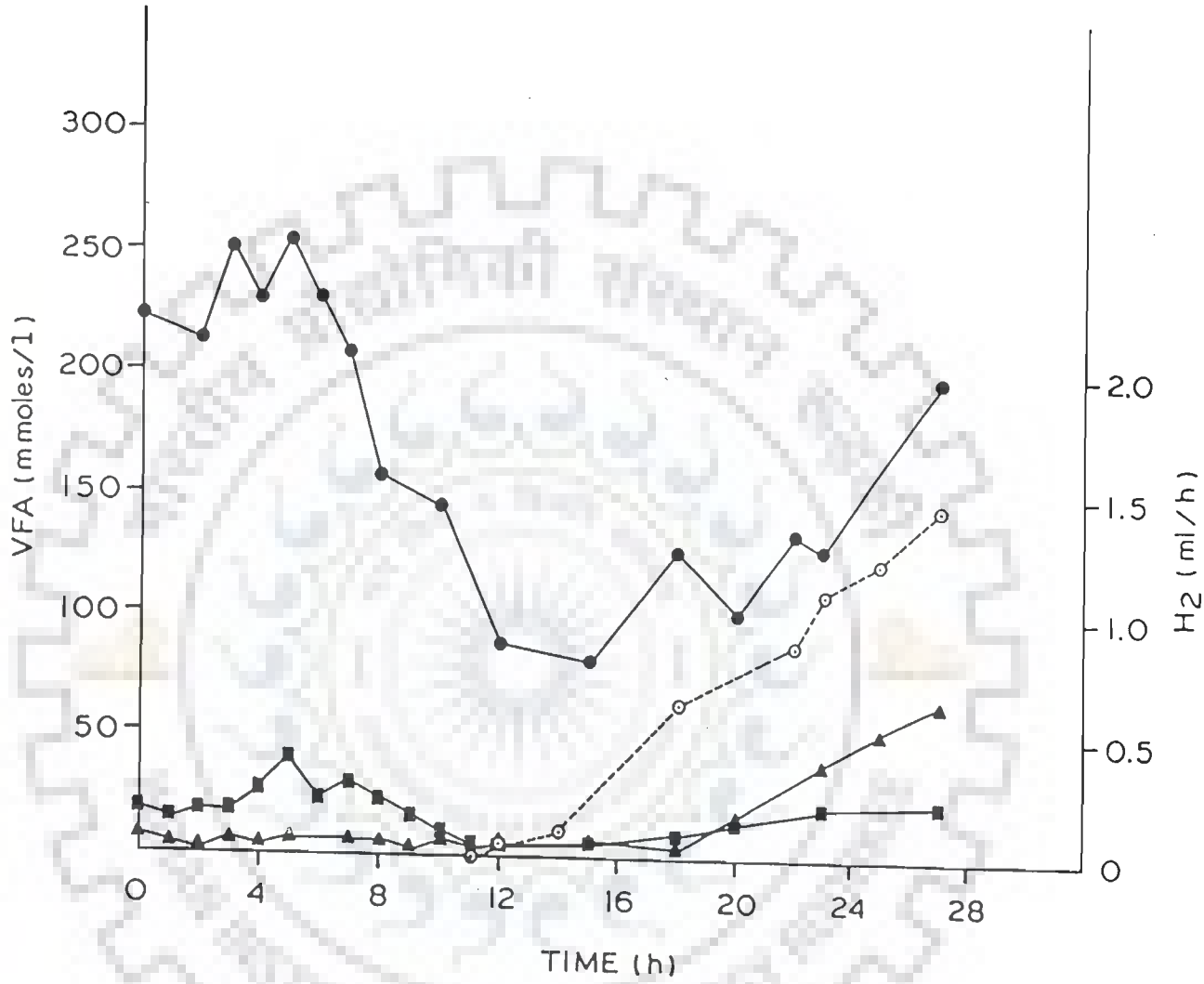

Fig. 4.13: Volatile fatty acid (VFA) concentration and rate of hydrogen production from glucose by Mixed microbial culture during 0 - 28 h of incubation cycle. (●): acetic acid; (■):propionic acid; (▲):n-butyric acid; and (O): rate of hydrogen production.

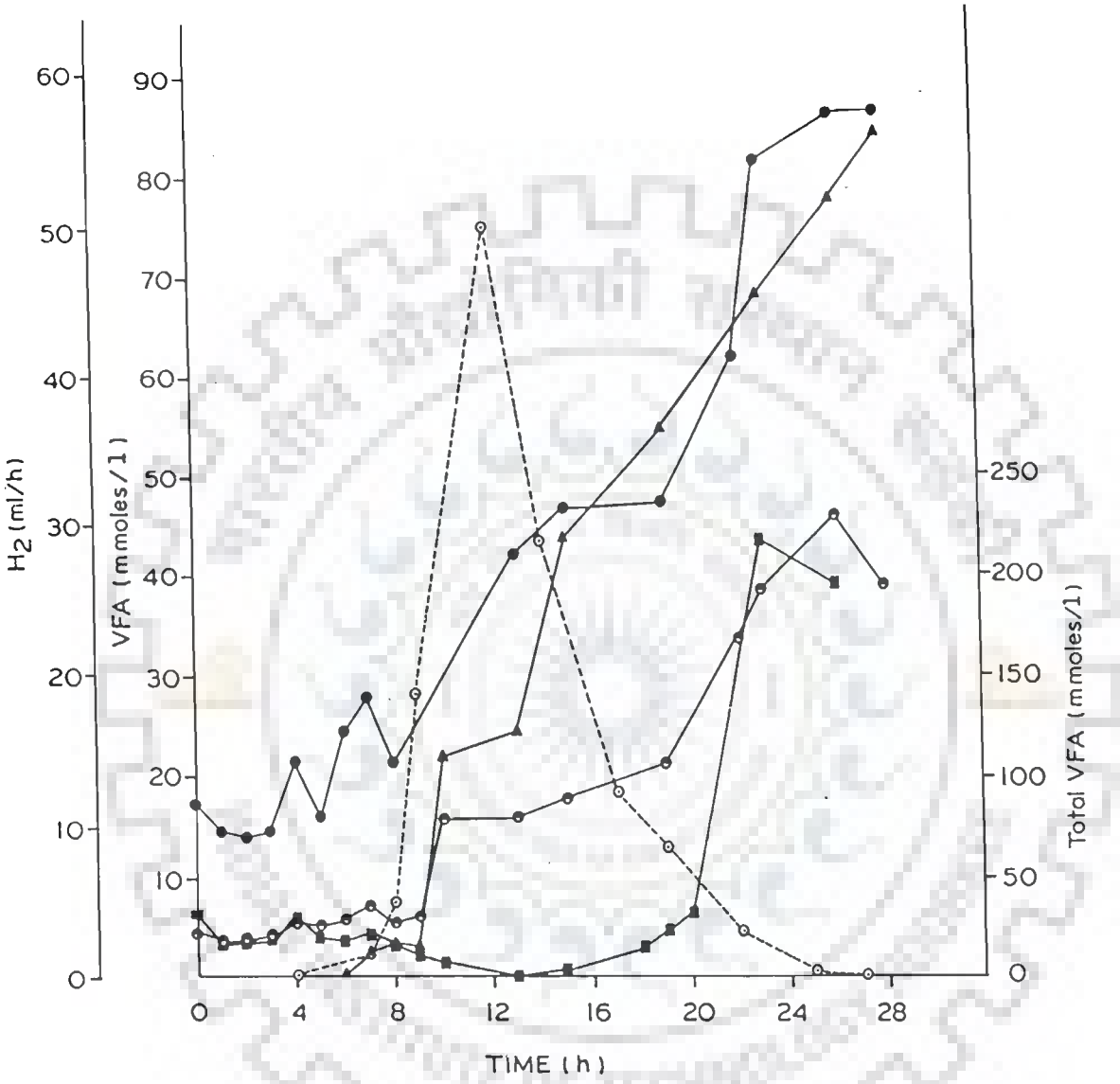

Fig. 4.14: Volatile fatty acid (VFA) concentration and rate of hydrogen production from dumping wheat by mixed microbial culture during 0 - 28 h of incubation cycle. (●): acetic acid; (■): propionic acid; (▲): n-butyric acid; (◓):total VFA (acetic acid + propionic acid + n-butyric acid) and (⊙): rate of hydrogen production.

However, during H_2 production the relative levels of these two acids were reversed i.e., 11.36 mmoles of propionic acid and 21.02 mmoles/l of n-butyric acid. Valeric acid showed a regular decrease from 7.46 to 1.11 mmoles/l in the two phases which probably indicates its conversion to lower fatty acids.

There was a marked change in the fatty acid pattern as the substrate was changed from glucose to dumping wheat grains (Table 4.26 & Fig. 4.14). Acetic acid increased independently of H_2 production i.e., from 16.55 to 86.80 mmoles/l. The maximum accumulation was observed as the H_2 production rate decreased sharply. n-Butyric acid showed remarkable changes, it increased linearly from 0.0 before the start of H_2 generation to as high as 84.0 mmoles/l at the end of the H_2 generation, with an average concentration of 32.45 and 77.7 mmoles / l during pre- and post- H_2 producing phases. Propionic acid maintained a low profile, 3.83- 4.52 mmoles in the first two phases of fermentation, but increased very suddenly to 42 mmoles when the H_2 production was almost negligible. Valeric acid was not detectable at any stage.

In apple pomace fermentation, after an elongated pre- H_2 producing phase of 22 h, H_2 production was noticed from 22 - 27 h of incubation period. During pre- H_2 producing phase the levels of VFAs did not show any major changes. However, with the start of H_2 production there was 15 - 20% increase in the levels of acetic acid and propionic acid, but n-butyric acid concentration increased almost 4-folds (Table 4.26).

A comparison of the fermentation of the three types of substrates at same TS

level of 3%, reveals that although the concentrations of VFAs were in similar range in the case of apple pomace and dumping wheat grains there levels were markedly higher in glucose fermentation. This could be due to lower reducing sugar level available in these wastes as compared to glucose. Also it was observed that valeric acid could be detected only in the case of glucose fermentation. Another point which attracts our attention is that some of the quantity of VFAs were originally present in the apple pomace substrate and do not seem to be metabolized during pre- H_2 phase fermentation.

Fatty acid production pattern was very much similar when glucose, dumping wheat and apple pomace were fermented with pure isolated culture of *Bacillus licheniformis* (Table 4.27 & Fig. 4.15 & 4.16). As indicated in Fig. 4.15, the trend of these VFA from glucose fermentation was identical to that observed with mixed culture in both the pre-H_2 and H_2 producing phases although the duration of the phases varied. H_2 production started at 4 h of incubation as against at 11 h of incubation recorded in the case of mixed culture. The fermentation of apple pomace and dumping wheat grains by *B. licheniformis* was identical to that observed with mixed culture fermentation, both with respect to the pattern of VFAs and duration of H_2 metabolism.

4.15 Fermentation of alcohols during hydrogen metabolism:

Alcohol levels were also monitored along with volatile fatty acids during different phases of H_2 production. Table 4.28 presents the average concentrations of the alcohol produced during the fermentation of glucose, apple pomace and dumping

TABLE 4.27: FATTY ACID FORMATION DURING HYDROGEN METABOLISM BY *BACILLUS LICHENIFORMIS*.

Duration	Glucose				Dumping wheat				Apple pomace			
	Acetic acid	Propionic acid	Butyric acid	Valeric acid	Acetic acid	Propionic acid	Butyric acid	Valeric acid	Acetic acid	Propionic acid	Butyric acid	Valeric acid
	(mmoles/ l)				(mmoles/ l)				(mmoles/ l)			
Pre-H_2producing phase	222.46	23.61	7.29	7.26	16.75	3.93	Ø.Ø	Ø.Ø	23.11	8.Ø9	3.64	Ø.Ø
H_2producing phase	155.78	14.77	12.32	3.8Ø	48.37	6.86	16.98	Ø.Ø	26.Ø7	7.99	11.9Ø	Ø.Ø
Post-H_2producing phase (*)	ND	ND	ND	ND	146.Ø9	11.9Ø	72.95	Ø.Ø	ND	ND	ND	ND

Values are average concentration of different fatty acids formed during different stages of H_2 metabolism in first 27 hrs of incubation.
ND = Not determined (the stage was not reached during first 27 hrs of incubation)
The samples were analysed in duplicate.
(*) post-H_2 producing phase was considered in the case of dumping wheat fermentation and was taken from the time the H_2 production becomes very low i.e., after 18 h of incubation.

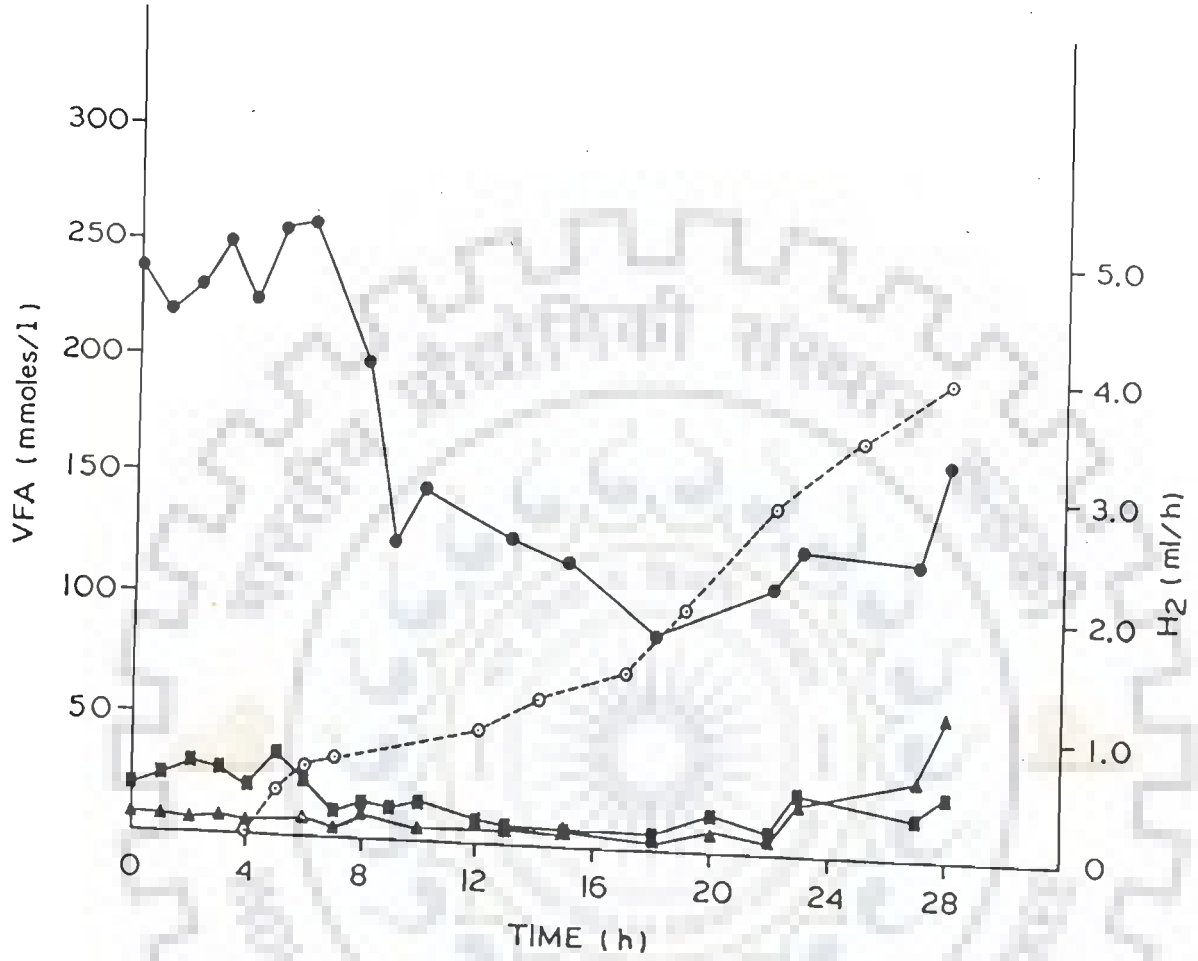

Fig. 4.15: Volatile fatty acid (VFA) concentration and rate of hydrogen production from glucose by *Bacillus licheniformis* during 0 - 28 h of incubation cycle. (●):acetic acid; (■): propionic acid;(▲): n-butyric acid; and (⊙): rate of hydrogen production.

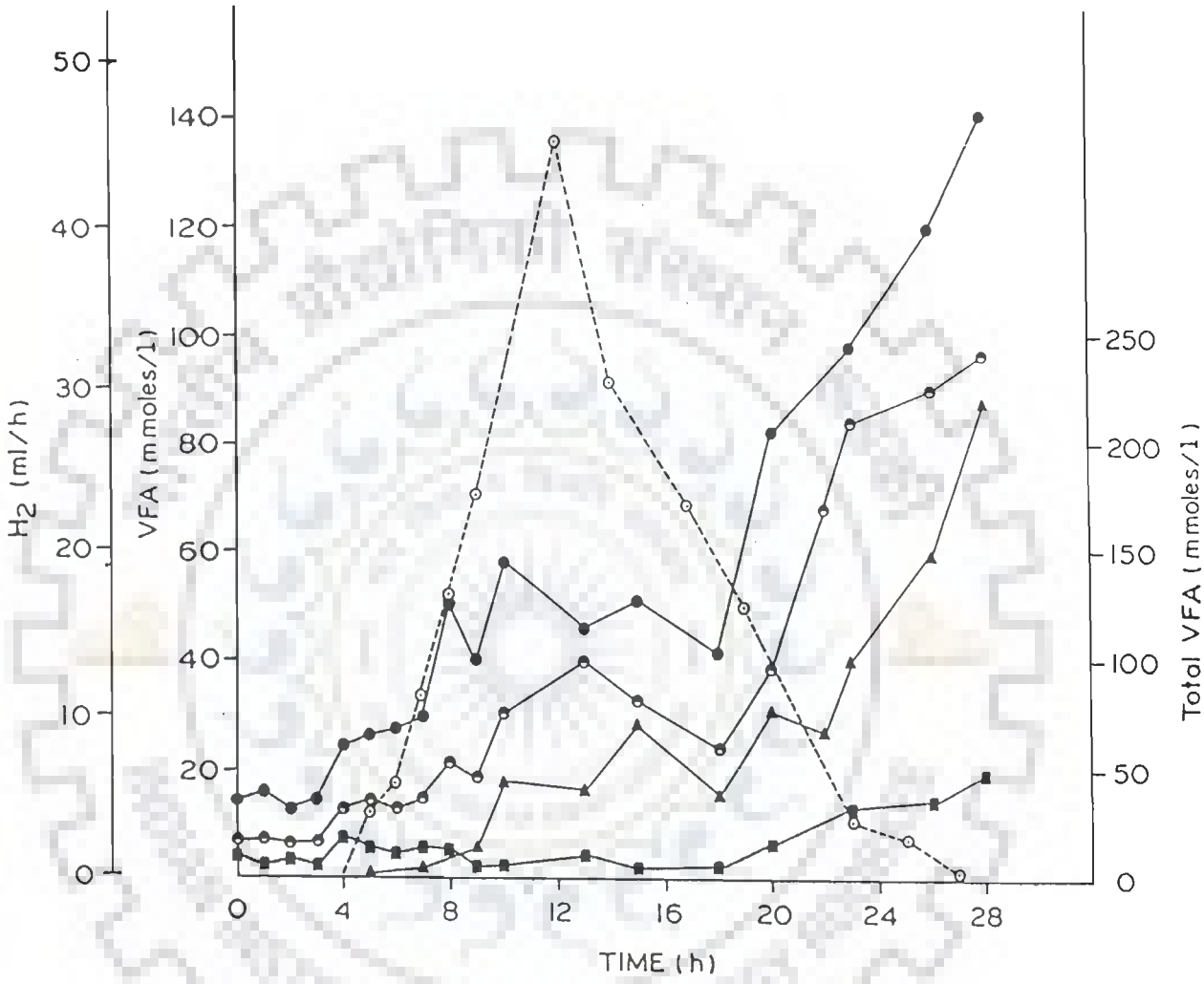

Fig. 4.16: Volatile fatty acid (VFA) concentration and rate of hydrogen production from dumping wheat by *Bacillus licheniformis* during 0-28 h of incubation cycle. (●): acetic acid; (■): propionic acid;(▲): n-butyric acid; (◓): total VFA (acetic acid + propionic acid + n-butyric acid) and (○): rate of hydrogen production.

TABLE 4.28: FORMATION OF ALCOHOLS FROM DIFFERENT SUBSTRATES BY MIXED MICROBIAL CULTURE.

Duration (h)	Glucose			Apple Pomace			Dumping Wheat		
	Methanol	Ethanol (mmoles/l)	Propanol	Methanol	Ethanol (mmoles/l)	Propanol	Methanol	Ethanol (mmoles/l)	Propanol
Pre-H_2 producing phase	0.0	1.13	0.0	12.85	1.97	0.0	0.0	0.0	0.0
H_2 producing phase	0.0	3.04	0.0	11.25	2.61	0.0	0.0	4.55	0.0
Post-H_2 producphase (*)	ND	ND	ND	ND	ND	ND	0.0	41.75	0.0
Average during (0 - 24)	0.0	2.09	0.0	12.05	2.29	0.0	0.0	12.71	0.0

The values are obtained by analysing the samples in duplicate.
The concentrations are the average values of fermented alcohols during different phases of incubation within first 24 hours.
(*) post-H_2 producing phase was considered in the case of dumping wheat fermentation and was taken from the time the H_2 production becomes very low i.e., after 18 h of incubation.

wheat by mixed microbial culture. Ethanol is the main alcohol produced in the fermentation of all the three substrates. The results show no ethanol in the case of dumping wheat grain fermentation during pre- H_2 producing phase, whereas it appeared at a level of 4.55 mmoles/l in the H_2 producing phase, and increased steadily (41.75 mmoles/l) as the post-H_2 producing phase approached. The trend was similar in the case of glucose and apple pomace fermentation, during the first two phases of H_2 metabolism. As the pre-H_2 phases in these cases were longer than the dumping wheat fermentation, ethanol was observed at a low level during this phase i.e., 1.13 mmoles/l in the case of glucose and 1.97 mmoles/l in the case of apple pomace, which increased slightly during H_2 producing phase i.e., 3.04 and 2.61 mmoles/l, respectively.

Methanol was observed in the case of apple pomace fermentation only. The average level was 12.05 mmoles/l. Propanol could not be detected in any of the cases during H_2 metabolism.

Table 4.29 presents the alcohol concentrations during the fermentation of glucose, apple pomace and dumping wheat by *Bacillus licheniformis*. The pure culture was found to produce propanol when the cells were stored in saline solution (0.76 mmoles/l) and this property was also observed in this fermentation process. The average levels of propanol maintained were 1.0, 0.84 and 1.38 mmoles/l during fermentation of glucose, apple pomace and dumping wheat, respectively.

Ethanol production by *B. licheniformis* has been seen to follow the same trend as seen with mixed microbial culture. The concentrations were 1.02, 1.47 and 7.24

TABLE 4.29: FORMATION OF ALCOHOLS FROM DIFFERENT SUBSTRATES BY *BACILLUS LICHENIFORMIS*

Incubation stage	Glucose			Apple Pomace			Dumping Wheat		
	Methanol	Ethanol (mmoles/l)	Propanol	Methanol	Ethanol (mmoles/l)	Propanol	Methanol	Ethanol (mmoles/l)	Propanol
Pre-H_2 producing phase	Ø.Ø	Ø.29	1.Ø1	12.15	1.4Ø	Ø.85	Ø.Ø	Ø.Ø	1.22
H_2 producing phase	Ø.Ø	1.75	Ø.99	1Ø.31	1.54	Ø.83	Ø.Ø	5.35	1.1Ø
Post-H_2 producing phase (*)	ND	ND	ND	ND	ND	ND	Ø.Ø	9.13	1.83
Average during (Ø - 24h)	Ø.Ø	1.Ø2	1.ØØ	11.23	1.47	Ø.84	Ø.Ø	7.24	1.38

The values are obtained by analysing the samples in duplicate.
The concentrations are the average values of fermented alcohols during different phases of incubation within first 24 hours.
(*) post-H_2 producing phase was considered in the case of dumping wheat fermentation and was taken from the time the H_2 production becomes very low i.e., after 18 h of incubation.

mmoles/l with glucose, apple pomace and dumping wheat respectively. Methanol (11.23 mmoles/l) was observed only in apple pomace fermentation, which was initially present in the substrate as was the case in mixed culture fermentation.

4.16 Effect of fall in pH on rate of Hydrogen production:

The influence of pH drop in fermentaion process on rate of H_2 production is depicted in Figs. 4.17-4.20. During H_2 evolution from glucose by mixed microbial culture, the pH dropped from 7.0 to 4.1 in the first 0 - 28 h of incubation cycle. H_2 production started as the pH decreased to 5.3 in the first 11 h of incubation. Thereafter H_2 production rate increased sharply till it reached a maximum of 1.1 ml/h, consequent to the little drop in pH from 5.3 to 4.64. Subsequent fall in pH resulted in the gradual decrease in H_2 production rate. Similar effects were observed by using *B. licheniformis* (Fig. 4.18). The maximum rate of H_2 production was 3.7 ml/h as a result of fall of pH to 4.6 and further decrease in pH to 4.1 slowly decreased the rate of H_2 evolution. In comparison, during pure culture fermentation, the pH sharply dropped to 5.3 within first 4 h of incubation, subsequently leading to the start of H_2 generation.

The observations recorded were somewhat different as the substrate was changed to dumping wheat grains (Fig. 4.19 & 4.20). With mixed culture, the fall in pH was from 7.0 to 3.5 during 0 - 28 h of incubation cycle (Fig. 4.19). Although H_2 evolution started at 4 h of incubation (when pH has come down to 6.3), it was very low till the pH dropped to the favourable level of 5.3. Thereafter, rate of H_2 evolution shot up to the maximum of 51 ml/h at pH of 4.75. Again with further drop in pH,

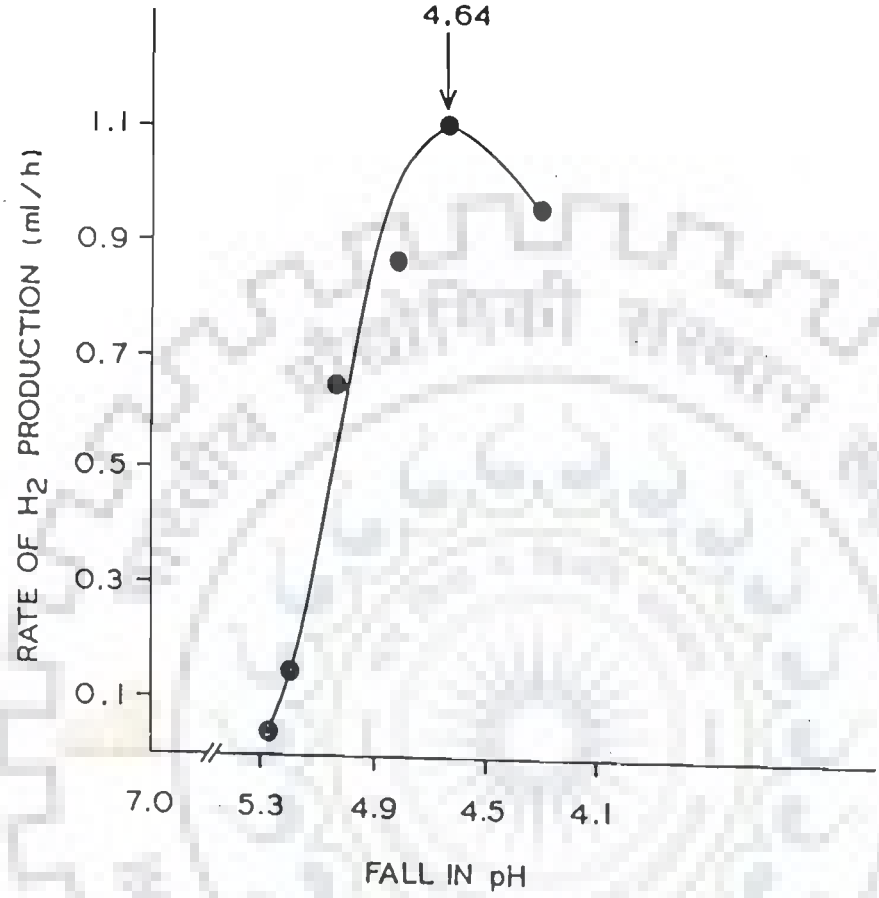

Fig. 4.17: Effect of fall in pH on rate of hydrogen production from glucose by mixed microbial culture during 0 - 28 h of incubation cycle.

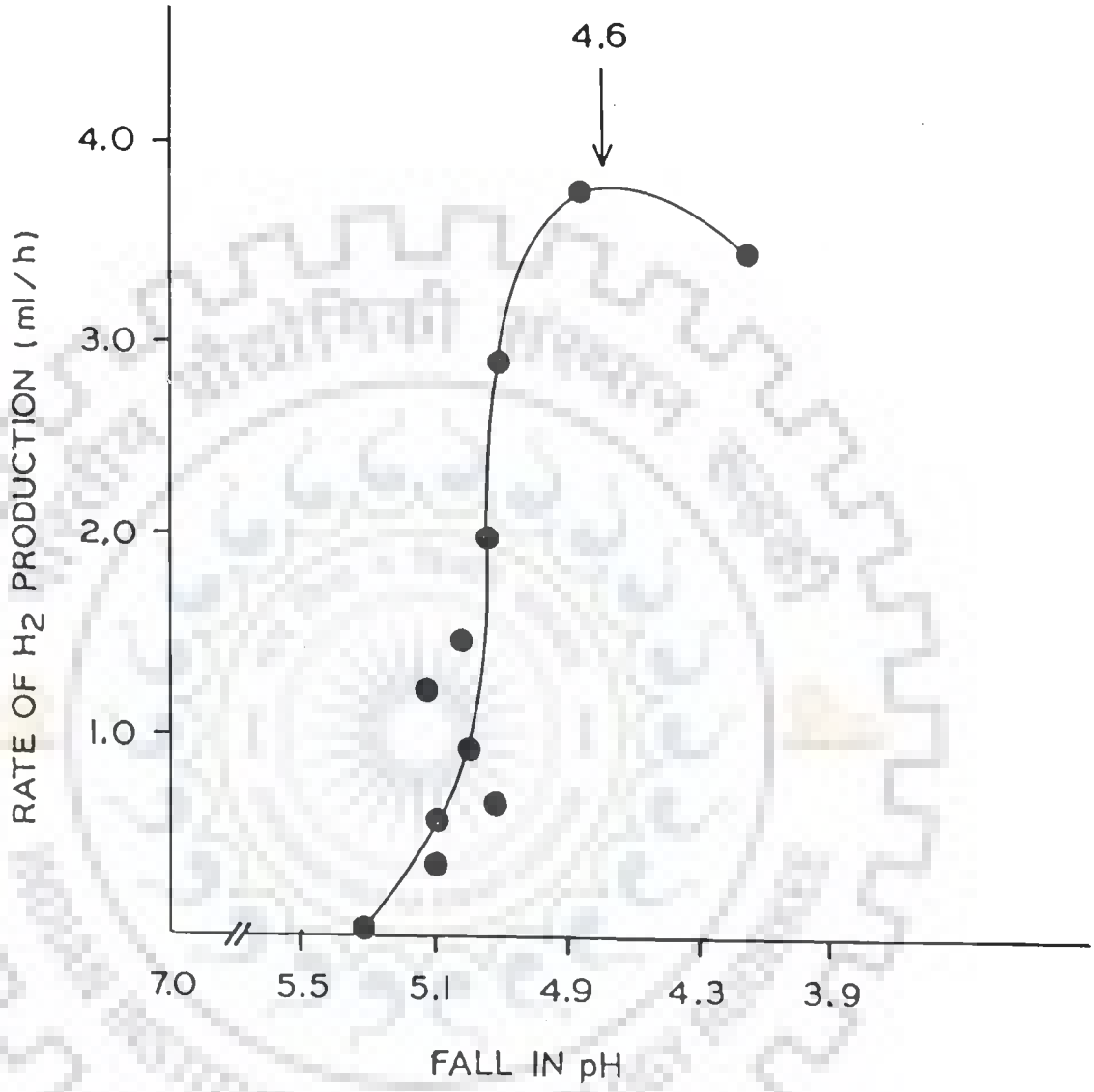

Fig. 4.18: Effect of fall in pH on rate of hydrogen production from glucose by *Bacillus licheniformis* during 0 - 28 h of incubation cycle.

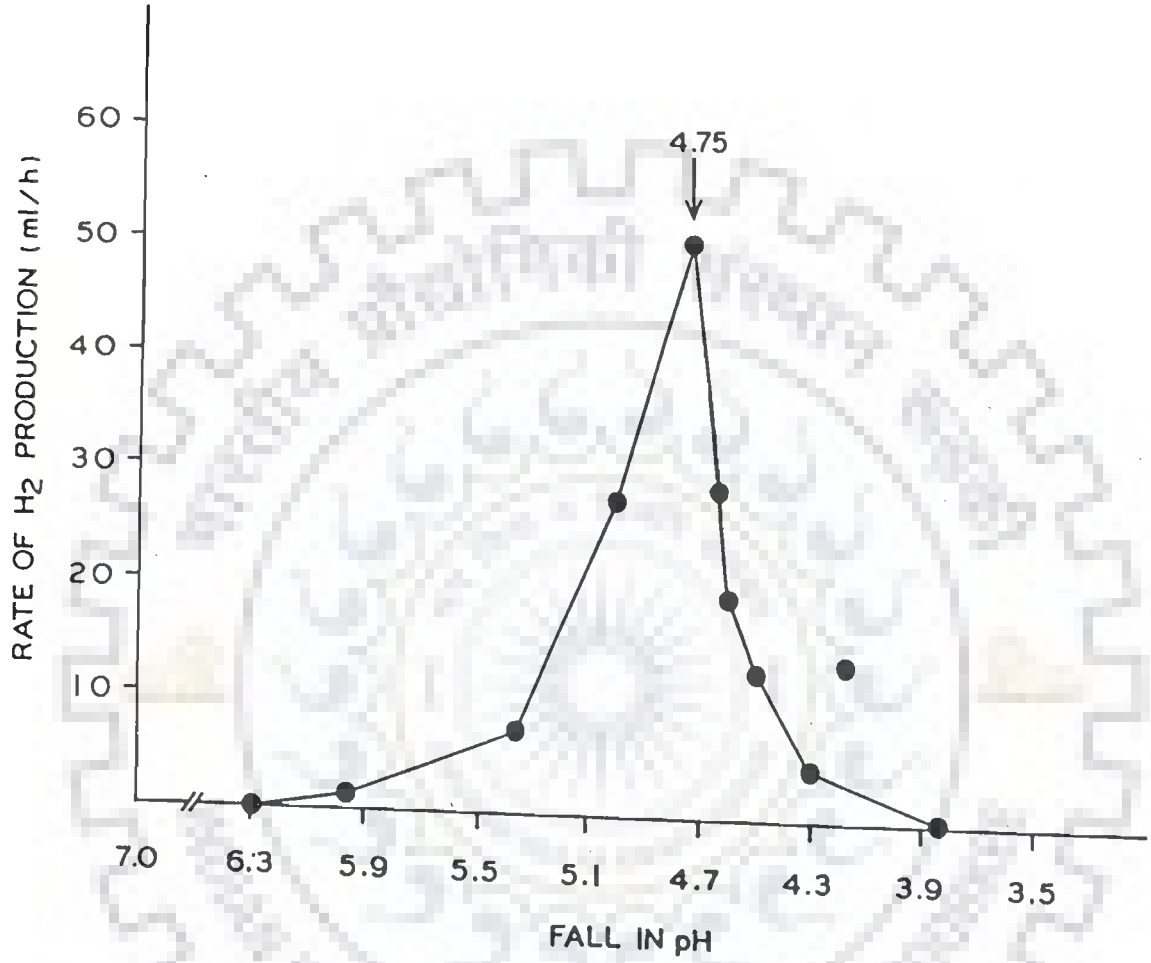

Fig. 4.19: Effect of fall in pH on rate of hydrogen production from dumping wheat by mixed microbial culture during 0 - 28 h of incubation cycle.

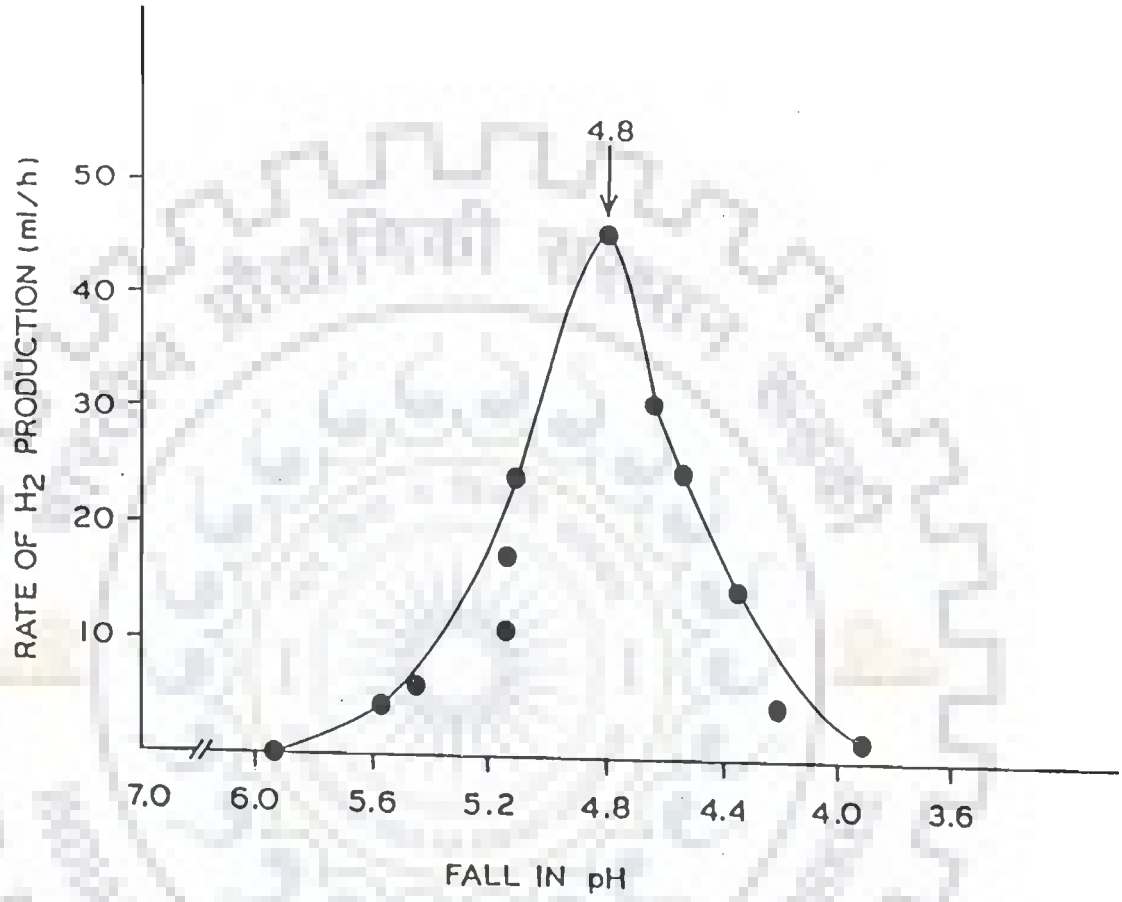

Fig. 4.20: Effect of fall in pH on rate of hydrogen production from dumping wheat by *Bacillus licheniformis* during 0 - 28 h of incubation cycle.

there has been a sudden decrease in the rate of H_2 production. No H_2 was evolved as the pH dropped to 3.9. Similar results were also observed by using pure culture of *Bacillus licheniformis* (Fig. 4.20).

4.17 Effect of SO_4^{2-}, Ni^{2+} and Fe^{3+} on the metabolism of Hydrogen production:

The influence of $SO4^{2-}$, Ni^{2+} and Fe^{3+} on H_2 generation by mixed microbial culture are presented in Table 4.30 & 4.31. H_2 production increased with increasing sulfate concentration from 1 - 5 mM level and thereafter it decreased gradually as the concentration was raised to 200 mM (Table 4.30). The highest yield of 196 ml/g of glucose was recorded with 5 mM sulfate, increasing the yield by 15.3% as compared to the control. It was observed that upto 10 mM level, sulfate in the fermentation medium was favourable for H_2 production. This increase in H_2 production was a consequence of higher sulfate reduction, the maximum reduction of 4.2% was evident at 5 mM of initial sulfate concentration. Higher initial sulfate concentration of 15 - 200 mM resulted in the decrease of H_2 generation by 3 - 87%, whereas sulfate reduction was decreased from 0.95% to 0.17%.

Similar effects were recorded when Fe^{3+} was fed in the apple pomace digester at 50, 100, 200 nM level (Table 4.31). H_2 production enhanced by 30% when 50 nM of $FeCl_3$ was added in the culture. At 100 & 200 nM levels, H_2 evolution decreased by 15% & 24% respectively. Ni^{2+} in the concentration range of 0-200 nM did not show any effect on H_2 evolution.

TABLE 4.30: EFFECT OF SULFATE REDUCTION ON THE METABOLISM OF HYGROGEN PRODUCTION BY MIXED MICROBIAL CULTURE.

Sulfate concentration (mM)	H_2 (ml/g glucose)	Sulfate Reduction (%)
0.0	170	NA
1.0	175	2.8
3.0	187	3.2
5.0	196	4.2
7.0	180	1.7
10.0	174	1.05
15.0	165	0.95
20.0	113	0.88
50.0	69	0.35
100.0	40	0.22
200.0	23	0.17

The values are the mean of two experiments.
Sulfate reduction was estimated on the basis of unconsumed sulfate and sulfide produced.

TABLE 4.31: EFFECT OF Ni^{2+} AND Fe^{3+} ON HYDROGEN PRODUCTION FROM APPLE POMACE.

Concentration (nM)	Ni^{2+}		Fe^{3+}	
	Biogas-H (ml/g substrate)	H_2	Biogas-H (ml/g substrate)	H_2
0	90.7	65.2	96.5	63.2
50	88.7	62.9	115.2	82.3
100	100.0	65.7	80.6	54.0
200	93.0	64.8	70.8	48.2

The values are average of two experiments.

4.18 Bacterial cell immobilization for hydrogen production.

The biotransformation of glucose into H_2 was performed by immobilized mixed microbial population of H_2 producers and an axenic culture of *Bacillus licheniformis*. The observations recorded on the batch culture and daily batch fed continuous culture fermentation by adsorption of the cells on baked bricks and incorporation in the calcium alginate beads are presented here.

4.18.1 Batch culture system:

Table 4.32 presents the observations recorded on the fermentation of 3% glucose into H_2 by immobilized cells, along with the respective controls. Bioconversion of glucose became evident with the evolution of gas, after initial 8 and 20 h of incubation at 40°C with pure and mixed cultures, respectively. The gaseous mixture (biogas-H) largely contained varying amounts of H_2 and CO_2 with an average 6% by volume of H_2S.

The mixed microbial culture yielded 56.80 l and 37.04 l of biogas-H / mole of glucose utilized when immobilized on baked bricks and calcium alginate, respectively. The gases comprised of 58.73% and 46.18% by volume of H_2, thus leading to the evolution of 33.60 l & 17.11 l of H_2 / mole of glucose utilized respectively. The immobilization has improved the productivity of H_2 by 4.1-fold in the case of bricks support and 2.1-fold when immobilized in calcium alginate as compared to the control system which yielded 8.15 l of H_2/mole of glucose. On the other hand, as compared to 13.09 l of H_2 generated in the control by *B. licheniformis*, the bricks & calcium alginate immobilized cells increased the H_2 production by 2.4-fold and 1.2-fold respectively. The total biogas-H evolved in these cases was 48.16 l (64% H_2) and

TABLE 4.32: EFFECT OF WHOLE CELL BACTERIAL IMMOBILIZATION ON HYDROGEN PRODUCTION IN BATCH CULTURE.

Immobilizing culture	Incubation period (days)	Gas volumes (l/mol glucose fed) Biogas-H	Gas volumes (l/mol glucose fed) Hydrogen	Gas volumes (l/mol glucose utilized) Biogas-H	Gas volumes (l/mol glucose utilized) Hydrogen	Hydrogen (%)	Final pH (a)	Unutilized glucose (%) (b)	Advantage (-folds) (C)
Mixed microbial culture									
Control	13	14.67	8.10	14.77	8.15	55.22	4.33	0.67	1.0
Alginate	23	36.29	16.76	37.04	17.11	46.18	5.91	2.03	2.1
Bricks	10	56.39	33.12	56.80	33.60	58.73	4.42	0.72	4.1
Bacillus licheniformis									
Control	16	20.32	12.96	20.52	13.09	63.78	4.28	1.00	1.0
Alginate	20	30.77	15.87	31.22	16.10	51.57	5.65	1.43	1.2
Bricks	9	47.98	30.71	48.16	30.82	64.00	4.84	0.37	2.4

Each value is a mean of two different experiments. Biogas-H = H_2 + CO_2 + H_2S.
(a) pH after 24 h of incubation, (initially adjusted to 7.0 with 1N NaOH)
(b) Initial glucose concentration was 3% (4500mg in alginate experiments & 9000 mg in bricks experiment)
(c) Advantage in H_2 production is with refernce to control taken as 1.0

31.22 l (51.57% H_2) per mole of glucose utilized, thus constitu-ting 1.38 moles & 0.72 moles of H_2/ mole of glucose in respect of bricks and alginate respectively.

The gas generating rate was slow with calcium alginate immobilzed cells, stretching the whole process over a period of 20 - 23 days, whereas better cell-subsrate localization on baked bricks surface markedly improved the production rates. The biotransformation process in this case took only 9-10 days, as compared to 13-16 days in control setups. Glucose utilization efficiency was very high in all the experiments and did not vary much during these fermentations. On an average 0.4 - 2.0% of the initial glucose fed remained unutilized at the end of the fermentation process.

It is also clear from the observations that in alginate immobilized experiments, pH drop was not much (5.91 & 5.65) as compared to the control and backed bricks immobilization.

Fig. 4.21 illustrates the comparative efficiencies of the cultures. The free cells of *B. licheniformis* worked at 60% higher potential for H_2 production than the mixed microbial population. The effect was reversed as the cells were immobilzed thus rendering mixed population comparatively more effective. Cell adsorption on bricks has shown 91-96% higher efficieny when compared with that during incorporation in the gel matrix. On the whole, maximum recovery of H_2 per mole of glucose was almost same with both the cultures thus generating 1.5 moles of H_2 / mole of glucose.

Reuse of the immobilized cells in batch culture: The reuse efficiency of the immobilized cells after gentle washing with distilled water and fed with glucose solution

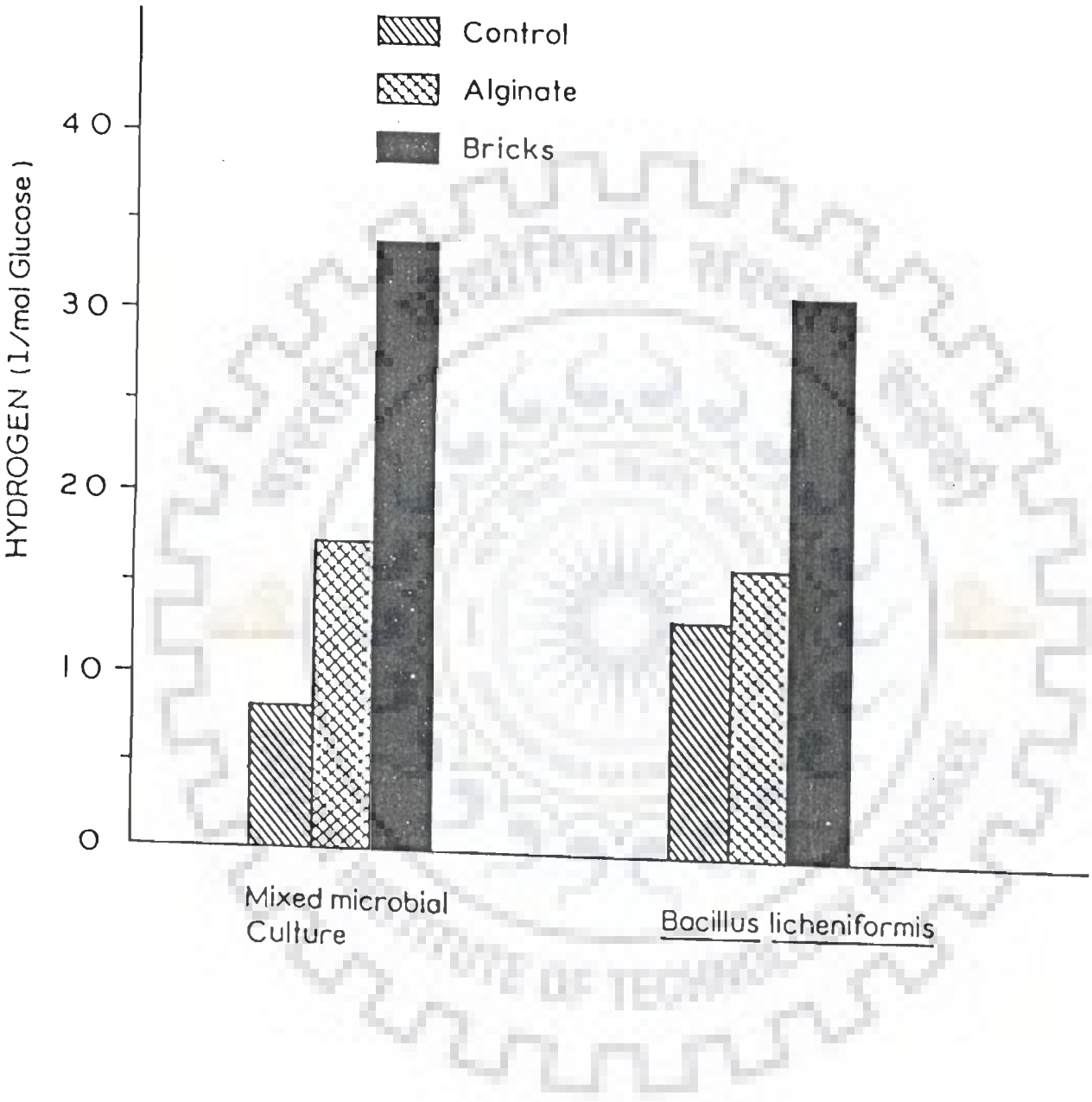

Fig.4.21: Effect of whole cell immobilization on hydrogen production in batch culture.

has been presented in Table 4.33. The cells have been found to bind strongly to the immobilizing material as checked by absence of leakage of cells in the washings. The successive re-use of the immobilized cells was accompanied by a gradual decrease of 17-32% in the net yield of both biogas-H and H_2 (Fig. 4.22). The efficiency continued to be higher as compared to the control till two rounds of reuse. In one of the cases of immobilization of *B. licheniformis* on alginate, the H_2 yield dropped by 6.8%. The reuse of the immobilized cells did not affect the production rates and glucose utilization efficiency. The gas evolution was continued for a minimum period of 8 days in case of bricks support and for 20 days when alginate immobilized cells were reused. Comparatively less drop in pH (5.8 - 6.14) was observed in the latter case.

Long term activity of immobilized cells: Most promisingly long term activity of the alginate immobilized cells was observed when the cells retained 40 - 50% H_2 production efficiency, after one year of storage under aseptic conditions at 4°C.

4.18.2 Continuous culture system:

The results obtained on daily feeding for 60 days of the glucose solution on immobilizing columns and respective controls are presented in the Table 4.34. Batches of 100 ml of 3% glucose solution, fed into the control experimental setups lead to the generation of 189.5 ml and 301 ml of biogas-H i.e., 63.2 ml and 100.36 ml/g of glucose by mixed culture and pure culture respectively. Each day around 600-800 mg of glucose remained unutilized, thus making the biotransformation process work at an efficiency of 6.03 l and 9.52 l of H_2 / mole of glucose employed or 8.22 l and 12.58 l of H_2 / mole of glucose utilized.

TABLE 4.33: REUSE OF THE IMMOBILIZED CELLS FOR HYDROGEN PRODUCTION IN BATCH CULTURE.

Immobilizing culture		Incubation period (days)	Gas volumes (l/mol glucose fed) Biogas-H	Hydrogen	Gas volumes (l/mol glucose utilized) Biogas-H	Hydrogen	Hydrogen (%)	Final pH (a)	Unutilized glucose (%) (b)	Advantage (-folds) (C)
Mixed microbial culture										
Bricks	I round	10	46.84	23.99	47.12	24.14	51.22	4.60	0.60	3.0
	II round	11	33.28	17.06	33.51	17.18	51.26	4.68	0.70	2.1
Alginate	I round	14	28.79	14.59	29.23	14.81	50.68	5.95	1.51	1.8
	II round	15	19.54	9.83	19.93	10.03	50.31	5.80	2.00	1.23
Bacillus licheniformis										
Bricks	I round	8	41.88	24.48	42.33	24.74	58.45	5.38	1.05	1.9
	II round	9	30.08	16.78	30.37	16.94	55.79	5.11	0.95	1.3
Alginate	I round	20	26.43	13.11	26.84	13.31	49.60	6.14	1.15	1.02
	II round	18	23.76	11.96	24.18	12.17	50.34	6.02	1.73	0.93

Each value is a mean of two different experiments. Biogas-H = H_2 + CO_2 + H_2S.
(a) pH after 24 h of incubation, (initially adjusted to 7.0 with 1N NaOH)
(b) Initial glucose concentration was 3% (4500mg in alginate experiments & 9000 mg in bricks experiment)
(c) Advantage in H_2 production is with refernce to control taken as 1.0

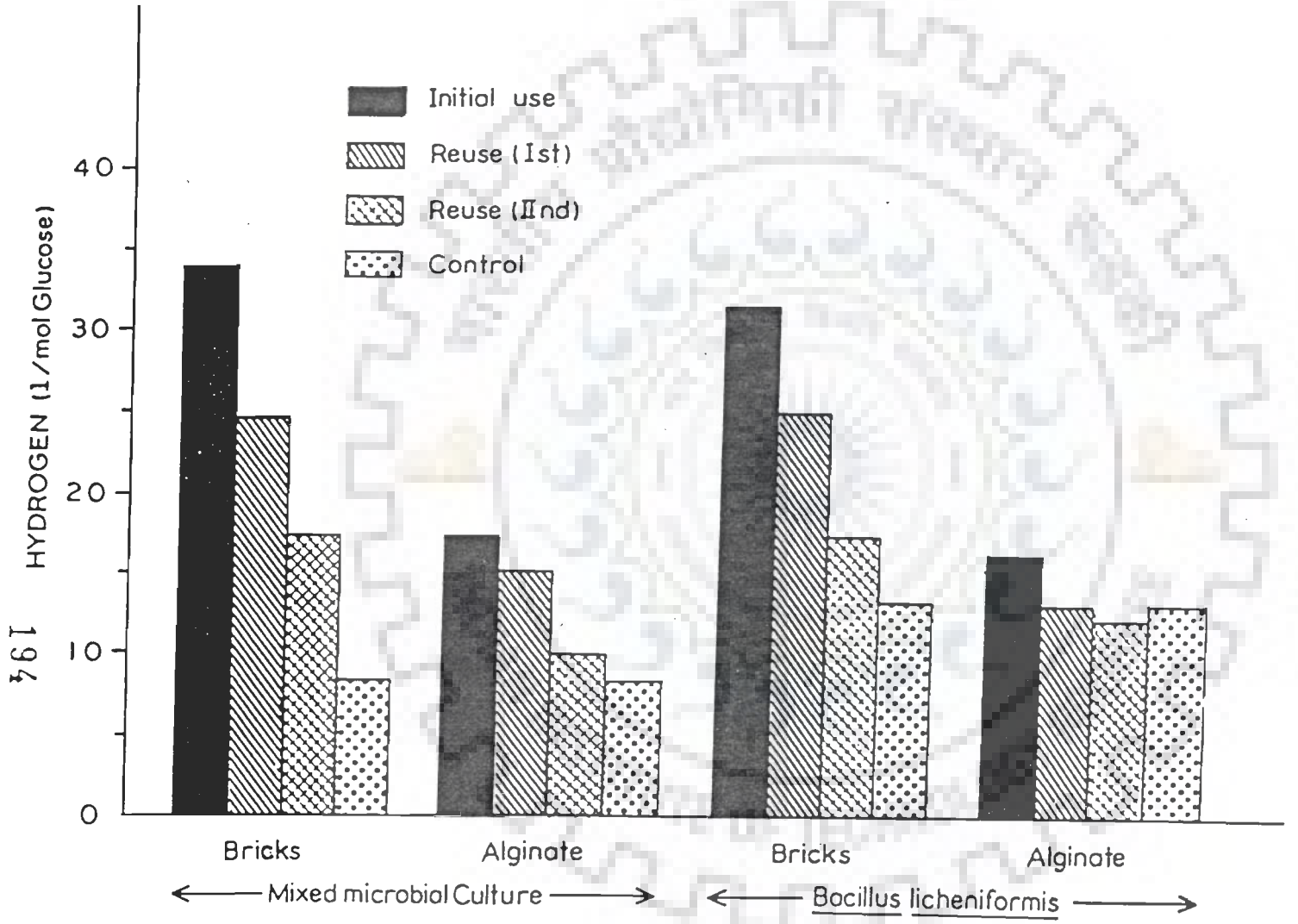

Fig. 4.22: Comparative effect on the improvement of hydrogen production by whole cell immobilization and reuse of the immobilized cells in batch culture.

TABLE 4.34: EFFECT OF IMMOBILIZATION ON BIOTRANSFORMATION OF GLUCOSE INTO HYDROGEN IN A CONTINUOUS BATCH FED SYSTEM.

Immobilizing culture	Incubation period (days)	Gas volumes				Hydrogen (%)		Final pH (a)		Unutilized glucose (%) (b)		Advantage (%) (C)
		Biogas-H		Hydrogen		Biogas-H	Hydrogen					
		N	$\bar{X}\pm SD$	N	$\bar{X}\pm SD$	N	$\bar{X}\pm SD$	N	$\bar{X}\pm SD$	N	$\bar{X}\pm SD$	N	$\bar{X}\pm SD$	
		(l/mole glucose fed)				(l/mole glucose utilized)								
Mixed microbial culture														
Control	60	52	11.38 ± 0.19	52	6.03 ± 1.30	27	15.51	27	8.22 ± 0.27	39	53.00 ± 1.42	50	5.03 ± 0.40	27	26.66 ± 1.61	
Bricks	60	57	15.46 ± 0.13	57	8.16 ± 0.03	29	16.03	29	8.46 ± 0.10	45	52.80 ± 2.25	53	5.24 ± 0.59	29	3.33 ± 0.87	35.3
Bacillus licheniformis																
Control	60	55	18.06 ± 0.54	55	9.52 ± 1.17	25	24.33	25	12.58 ± 1.82	45	52.70 ± 1.52	55	5.14 ± 0.33	25	23.86	
Alginate	60	58	24.35 ± 0.60	58	13.22 ± 1.22	27	10.83	27	14.82 ± 0.35	46	54.30 ± 2.22	58	5.32 ± 0.51	27	27.30	38.87

Each value is a mean of two different experiments. Biogas-H = H_2 + CO_2 + H_2S.
(a) pH after 24 h of incubation, (initially adjusted to 7.0 with 1N NaOH)
(b) The experiment was fed with 100 ml of 3% glucose daily.
(c) Advantage in H_2 production is with refernce to control taken as 100%

On the contrary, the mixed microbial population on bricks support increased the yields of H_2 production and glucose utilization efficiency. 8.46 l of H_2/ mole of glucose utilized was the yield obtained during fermentaion with a H_2 purity of 52.8%. *B. licheniformis* immobilized in alginate showed higher yields in continuous culture fermentation. In this case the immobilized cells generated 135.25 ml of biogas-H/g of glucose employed, which comprised of 54.3% H_2. In the process 330 mg of glucose remained unutilized, resulting in the net yield of 14.82 l of H_2/ mole of glucose utilized.

The immobilization of bacterial cultures in continuous culture system thus led to the better and higher utilization of glucose in comparison to the control setups. In the process a net gain of 35% - 39%, both in the total biogas-H and H_2 production yields was evident.

Other observations which need mention are the total biomass loss and the changes in pH during fermentation. It has been observed that in the effluent of the immobilization experiments, the total biomass lost each day was 16.25% and 18.9% less as compared to the respective controls indicating low leakage of cells. No major difference in the pH drop was observed in these experiments.

CHAPTER - V

DISCUSSION

DISCUSSION

We have found in our work that biological waste materials like apple pomace, vegetable waste and dumping wheat can be actively degraded to methane by mixed methanogenic culture from cattle dung. It has also been established that either pure sugar like glucose or carbohydrate rich waste materials like apple pomace, dumping wheat and pure starch can be microbially converted to hydrogen by undefined enriched mixed microbial population of H_2 producers (cattle dung as source) and isolated pure cultures. The mixed population of H_2 producers from cattle dung have been previously enriched for H_2 producers by Joshi et al. (1986). The microbiological features of the culture studied here have shown very thin population of microbial cells with cocci and bacilli shapes and some undefined structures due to uncleaned biomass.

The studies conducted with two axenic cultures of H_2 producers belonging to the genus *Bacillus* have been isolated in our laboratory from enriched mixed population of H_2 producers (Jain, 1992). One of the strains has been identified as *Bacillus licheniformis*. These cultures have shown the properties of acid and gas production from sugars and under electron microscopy have confirmed their rod shape structures bearing single polar flagellum. Some *Bacillus* species have been previously reported to produce H_2 by various authors (Gray and Gest,1965; Zajic *et al.*,1978). Mixed undefined inocula from landfill sediments and sewage sludge samples have earlier been used by Holmes and Freischel (1978) and Roychowdhury *et al.* (1988) for H_2 production from pure sugar and sugar rich substrates.

The physiological conditions optimized for these cultures for the effective bioconversion of waste materials have resulted in efficient methanogenesis of the glucose substrate are a temperature range of 37-40°C, pH of 7-8 and substrate level of 3%. These observations are well in agreement with the results of the previous studies (Mah *et al.*,1977; Lane,1983; Winfrey, 1984; Kasali and Senior,1989). Optimum conditions for H_2 production by mixed microbial culture of H_2 producers observed in this study include an incubation temperature of 40°C, pH of 6.5-7.0 and substrate level of 3-6%. These findings were supported by the conditions standardized for the pure cultures (Jain,1992). The mixed cultures from the sewage sludge or landfills were able to degrade sugar to H_2 at a pH of 7.0 and temperature of 37°C, at high substrate levels (Roychowdhury *et al.*,1988). During fermentation, pH changed sharply with time. After every 20-24 hrs of incubation, the culture medium became acidic and H_2 production stopped. When the pH was adjusted to neutrality after every 24 h of incubation cycle by addition of NaOH, H_2 production resumed. This observation has also been reported by Roychowdhury *et al.* (1988). However, in their studies, the cultures needed neutralization after 5-7 days of incubation cycle, because of very low rate of H_2 evolution as compared to our results. Fermentative H_2 evolution by pure culture of *Enterobacter aerogenes* has been maximum at pH 6.0 and temperature of 38-40.5°C (Tanisho *et al.*,1987,1989). In our studies, the stability of the mixed culture of H_2 producers is shown to depend on sugar concentration in the substrate fed. Complete suppression of the methanogens in the mixed population, was achieved under constant exposure to high glucose levels. Such a finding has been of utmost importance in the continuous fermentation for

H_2 production by mixed microbial population, where an optimum level of sugars is to be maintained for complete termination of methanogenesis. However, very high concentrations of glucose (above 6%) were inhibitory even for H_2 production. It has been observed during the experiments, that such an effect observed in fermentation at higher glucose concentrations, is probably due to the metabolites produced e.g., acids in large quantities, which could be inhibitory for methanogens. The higher rate of H_2 produced at increased glucose concentrations could itself have been substrate inhibitory for methanogens (McCarty, 1982).

Under such optimum conditions, it has been possible to generate 8-8.5 l of H_2/ mole of glucose fed, by mixed culture under anaerobic conditions. The methanogenic culture could produce 50-60 l of CH_4/ mole of glucose fed under similar conditions.

Bioconversion of apple pomace into H_2 and CH_4 :

A modification of the conventional anaerobic digestion process, described by Joshi et al. (1986, unpublished work) in three stage system was used for anaerobic bioconversion of apple pomace into H_2 and CH_4 in well separated stages. All the earlier work has been directed towards the anaerobic digestion of apples and apple pomace into CH_4 in a single and two stage systems (Lane,1979,1984 a,b; Wase & Gordon,1982; Etheridge & Jewell,1988). In laboratory experiments under optimum conditions, a three stage reactor set-up of 2, 5 & 10 l capacity, 15 gms of dried apple pomace fed daily, generated 1 l of biogas-H (a mixture of 60% H_2 and 40% CO_2) and 3 l of biogas (a mixture of 70% CH_4 and 30% CO_2) in IInd & IIIrd stage

respectively. The overall process reduced the organic matter of the pomace by 76%, when retained for 10 days in H_2 processing stage followed by 20 days in CH_4 generating stage. However, this degradation efficiency of organic matter can be increased by recycling the unutilized solids as observed by Lane (1984 b) for apple pomace digestion. However, when the apple pomace was directly subjected to biomethanation (eliminating the H_2 evolution stage), the increase in CH_4 yields was by12%, thereby generating 156.75 l of CH_4/ kg TS fed. This digestion helped in reducing the organic solids by 48.1% only, probably due to the less retention time of 20 days in the methanogenic stage. Lane (1984 b) has reported slightly higher yields of CH_4 production from apple pomace i.e., 454 l biogas/ kg total solid fed (50-60% CH_4). This increase has been mainly because of the supplementation of various additional nutritional requirements in the digester and also perhaps due to a different source of inoculum i.e., from sewage digester. Temperature has played a significant role in the production of H_2 from apple pomace. Under uncontrolled ambient temperaturres (23-43°C), the yields of H_2 produced decreased by 47%, as compared to the maximum production at 40°C.

Pilot plant studies : The digestion of apple pomace conducted on upscaled pilot plant of 200, 500 & 1000 l capacity (three stage system) showed 40% efficiency for H_2 generation and 68% efficiency for CH_4 generation as compared to the laboratory scale experiments. For direct biomethanation, the pilot-plant worked at an efficiency of 74.3%. In these cases, however, the biodegradation of apple pomace was almost similar to that of laboratory scale experiments i.e., a degradation of 75% of organic solids. The reduction in COD level achieved was of the order of 70%

(25.3 g/l) as compared to reduction of 70% in the laboratory scale, which was substantially high.

The lower efficiency in the pilot plant digestion for H_2 generation has been possibly because of the following reasons :

(i) Very strict anaerobic conditions ($<0.2\%$ O_2) could not be maintained in the digesters. The O_2 contamination was perhaps very lethal to the microorganisms.

(ii) There were some temperature variations from desired optimum temperature of 40°C, with the temperature controlling system employed.

(iii) Proper control of pH was difficult, which has played a very important role for H_2 evolution.

However, we anticipate to achieve the laboratory scale results on a full scale plant, by proper digester designs to control the above problems encountered during our pilot-plant studies.

Fruit and vegetable waste methanogenesis :

Vegetable wastes, largely cauliflower and raddish waste (CRW), generated 210 l of biogas / kg TS fed. However, its CH_4 content was very high (72 %). In comparison to apple pomace this material proved less efficient in terms of biogas production, where the gas generation rate was 275 l/kg TS fed.

An objective of practical importance was to test if a microbial population enriched on a given biological material can also utilize another biological material

of a different composition, in an effective manner over a long period of time. The results of these experiments answer this in affirmative.

It was observed that microbial population enriched for methanogens, acted on apple pomace and led to active and stable biogas generation. The switch over from AP to CRW was reflected in the changed biogas yields. However, the biogas yields were similar to those observed when CRW was fermented independently. The subsequent shift in the feed material from CRW to RC still proved the adaptation phenomenon further. Here, the biogas yields attained a still higher level. It was thus apparent that methanogens have the potential to adapt themselves to different types of feed material.

In terms of energy (CH_4 content) evolved from each type of biowaste, we can say that apple pomace, raddish & cauliflower waste and rotten cabbage are more effective in comparison to cattle dung as feed material for biogas generation. These wastes evolve 151 - 218 l of CH_4 / kg of TS fed in 20 days retention time, where as cattle dung evolves 120 l of CH_4 / kg of TS fed in 40 days retention time (Kumar *et al.*, 1987).

The yield of CH_4 production from apple pomace is slightly less in our studies than that reported by Lane (1984 b). With vegetable waste, however, Ranade *et al.* (1987) have shown market waste (MW) to generate 35 l of gas/kg of MW destroyed in comparison to 350 l biogas/kg of TS destroyed (=> 210 l of gas/kg TS fed) in our set up. These differences are perhaps due to different incubation temperatures (25-28°C in their case & 40°C in our experiments). Since in none of our experiments,

the net reduction in TS and OS has gone beyond 63%, we may infer that there is still scope for more biogas generation, which can be achieved by either recycling the effluent or by increasing the retention time. Such a conclusion finds support from recent study by Mata Alvarez (1992), who reported high gas yields (478 l CH_4/ kg OS added) from the biomethanation of organic fraction of vegetable market.

H_2 and CH_4 generation from dumping wheat grains :

The potential of damaged wheat grains of dumping type, as a source of non-conventional energy has been quite evident in our experiments. It can generate both H_2 and CH_4. It is observed that H_2 evolution alone cannot exploit the total capacity of the material to generate energy (Thauer *et al.*,1977). It is thus necessary and important to make use of the slurry for the generation of CH_4, which is useful to recover the rest of the energy of waste material.

In batch culture system H_2 evolution was less efficient at 3% TS level and was almost equally efficient at 6% and 9% levels. We observe that 6% TS concentration perhaps provides a good physiological environment for H_2 generation. On the other hand at 3% TS concentration, active methanogenesis sets in fast and works efficiently. It is 1.43 times more efficient than 6% TS DWS. Higher concentration level of 9% seems too high for methanogenesis. However, on dilution, the same material worked as efficiently as 3% or 6% TS DWS. The slightly reduced biogas or CH_4 yields on dilution of 9% to 3% are perhaps due to the fact that the material has been quite efficient in H_2 generation, so that its net potential has been diverted more to H_2 evolution, affecting thereby its CH_4 evolving capacity.

In continuous culture system, 10 days of retention time is perhaps too long to prevent CH_4 generation from interferring with H_2 evolution. At 5 days RT, H_2 evolution occurs constantly but less efficiently than its potential, evident in batch culture. This suppression of CH_4 evolution under these physiological conditions finds support from the observation made by Roychowdhury *et al.* (1988) that high concentration of carbohydrates helps in inhibiting the expression of methanogens and consequently suppresses the CH_4 generation. These conclusions also find support from our observations related to inhibition of methanogenesis at high glucose levels.

We have thus a situation where one can exploit waste material like dumping wheat for generating energy (H_2 and CH_4) through fermentation. Our system permits us to get a continuous supply of H_2 without any need for removing H_2 or eliminating hydrogen consuming organisms from the reactor or the instream material.

Continuous culture methanogenesis of dumping wheat and recycling of the effluent :

Our studies indicate that dumping wheat has a good potential for generating biogas. However, methanogens seem to find a concentrationof 3% TS DWS more conducive a physiological environment for their activity than the 6% concentration. Biogas evolution was 2.78 times more with 3% TS content in comparison to the higher TS content, on the basis of reduction in organic solids in continuous culture digestion. It is also remarkable to note that at 6% TS dumping wheat slurry the biogas production has been very poor, inspite of the fact that OS reduction has been of the order of 50.1%. Decline in biogas yields from digestion of apricot waste

after 63 days of incubation for reasons not understood has been reported by Lane (1984 b). However, high concentration of carbohydrates in our case seems to play an important role in inhibiting methanogenesis, as has been observed by Roychowdhury *et al* (1988).

Recovery and recycling of settled solids from the effluent are effective means of reducing the COD level of the final effluent and also in increasing the biogas yields. Lane has shown that by increasing the effective retention time of the sludge solids by recycling has resulted in 99% and 100% conversions in orange peel and spent coffee grounds, respectively (Lane, 1983, 1984 c). Callander and Barford (1983) demonstrated that solids recycling during pig manure digestion resulted in a 36% increase in biogas yield. Recycling of solids has helped in increasing the conversion of 89.7% OS and 93.2% OS in asparagus and pineapple wastes (Lane, 1984 b), respectively in comparison to only 40% conversion of OS to gas (in asparagus) reported by Knol *et al.* (1978). In our case we have emphasized more on water conservation. It can be achieved by recycling of the effluent. Recycling of the effluent has improved the total biogas production efficiency, did not, however, affect the quality of the biogas. Methane content remained fairly constant through out the experiment. The effect of recycling of solids has been overshadowed by the fact that the methanogenesis of dumping wheat grains is more effective at lower total solid concentrations. We have observed that at 1% TS concentration level the dumping wheat methanogenesis is two times more effective than at 3% TS level (data not reported here). This is very well reflected in the first phase of recycling where the fresh infeed contributed little to the total infeed material, as a result of

which the methanogenesis occurred at a rate which was 1.84 times more than the nonrecycled (3% TS) phase. However, this effect was not evident in the third phase of recycling where there was a very low level of methanogenesis. The biogas production efficiency reached a low level of 47%. Here, this negative effect perhaps has its origin in the phenomenon that continuous recycling has led to the accumulation of some metabolites, toxins or ions, which did not prove very helpful to this bioconversion process.

We may, however, conclude that at 3% TS level, methanogenesis can be actively carried out even on the recycled effluent at a high efficiency, at least till a period which is equivalent to that during which the bioconversion was carried out in a non- recycled manner, leading continuously to the saving of water.

The potential biomethanation capacities of other types of damaged wheat grains like cattle feed and poultry feed types have also been established. The efficiencies of conversion were lower when compared with that of dumping wheat grains, but higher than that of fresh wheat grains. The potential for biogas generation from different types of wheat grains has been shown in the order: dumping wheat > cattle feed > poultry feed > fresh wheat. These findings indicate that more the damage, more is its suitability for biogas generation. This probably suggests, that degradation caused by the natural microbial flora in damaged grains has been beneficial for active biomethanation and contamination carried over by these wheat grains adapted well for their degradation.

On the other hand the H_2 producing potential of different starchy substrates

like, fresh grains of wheat, maize, rice and pure starch has established yields of H_2 which were highest with pure starch followed by rice, maize and wheat grains. Under similar conditions, however, the mixed microbial culture could not degrade cellulose to H_2. This wide application of the mixed culture to degrade different types of agricultural grains for H_2 production has clearly confirmed the acceptability of carbohydrate rich waste for microbial H_2 production.

Activity of hydrolytic enzymes during degradation of biowaste by mixed microbial culture :

Most biological waste materials are rich in cellulose, hemicellulose, xylan and to some extent, proteins. The degradation efficiency of these wastes will depend upon the microbial cultures having the activity for corresponding hydrolytic enzymes. Information is not available on any study conducted, where the activities of these enzymes have been checked in a mixed culture, before exploiting them for the anaerobic digestion of waste. However, enzymatic studies of pure cultures of rumen microorganisms have been conducted with an objective to understand rumen metabolism, involving characterization of nutritional requirements and major pathways of carbohydrate metabolism (Joyner and Baldwin, 1966). H_2 producing mixed microbial culture fermentation of apple pomace and dumping wheat have shown good activities of related hydrolytic enzymes. The activities certainly depend upon the nature of the substrate used like, xylanase activity was maximum in the degradation of dumping wheat, whereas proteolytic activity was maximum when apple pomace was used as a substrate. These differences were also evident in

amylolytic and cellulolytic activities estimated. The activities observed were higher on the shake culture, than the one under the stationary conditions.

Hydrogen production by *Escherichia coli* pathway :

Pyruvate is the key intermediate in the microbial production of hydrogen under anaerobic conditions. Microorganisms can use energy derived from the oxidation of pyruvic acid for the generation of ATP through acetyl phosphate, and the protons are utilized as the terminal electron acceptor (Zajic et al.,1979). This intermediate is linked by three well known pathways for its metabolism to H_2 production. This production could follow the route as in anaerobic glycolytic-by-product and then conversion into H_2 either through clostridia pathway in strict anaerobes or through *E. coli* pathway in facultative anaerobic organisms. In clostridial pathway microorganisms evolve H_2 as a result of direct anaerobic oxidation of pyruvate, whereas facultative anaerobes produce formate as an intermediate for H_2 generation by *E. coli* pathway (Kondratieva and Gogotov, 1983).

On this background, results obtained in studies carrried out with the key intermediates of glycolytic pathway (like glucose, glucose-6-phosphate and pyruvate) throw some light on the mode of their conversion into H_2 by our culture system of mixed microbial population and pure culture of *B. licheniformis*. With mixed microbial culture, production of H_2 is almost in proportionate volumes from glucose and glucose-6-phosphate i.e., 25.03 and 20.81 l/ mole of substrate fed, which suggestsits channeling through glycolytic pathway. Although, for reasons not understood, similar proportionality was not apparent when pyruvate was used as the substrate. Such

a conclusion has been supported by the observation of another experiment, when pure culture of *B. licheniformis* was used. Additive effects were observed when the intermediates were tested in combinations. Here, very low quantities of H_2 evolved from lactate (0.81 l/ mole of substrate fed) by mixed culture could not justify the involvement of any other pathway for H_2 evolution. Hillmer and Gest (1977) have also shown, such intermediates to evolve H_2, when used in the culture medium of the photosynthetic bacteria.

Since the pure culture isolated from the mixed population of H_2 producers was a facultative anaerobe, pyruvate conversion to H_2 through formate intermediate was expected by *E. coli* pathway. Stickland (1929) was the first to demonstrate the ability of *E. coli* cells to anaerobically liberate H_2 from formic acid, which was later confirmed by Ordal & Halvorson (1939) while investigating the role of formic hydrogenlyase reaction. The conversion efficiency of formate into H_2 gas is theoretically 1 mole/ mole, because all the available reducing equivalents of formate are converted into H_2 gas (Thauer *et al.*,1977). The cells of B. licheniformis and strain JK2 induced for formate hydrogenlyase complex showed a formate fermentation efficiency of 72% and 67.3% whereas the conversion efficiency to H_2 was 68% and 85% respectively. These observations are supported by the results of De Vos *et al.* (1983), on the basis of higher conversion efficiencies of formate into H_2 by different members of the enterobacteriaceae. Mixed microbial culture of H_2 producers in our studies also lead to higher formate conversion efficiencies, thereby reestablishing the very old concept of mixed culture fermentation of calcium formate from pond mud (Kondratieva and Gogotov, 1983). These studies indicate that, one of the biochemical

pathways followed for H_2 generation by mixed and pure cultures is :

$$\text{CoA Glucose} \longrightarrow \text{Pyruvate} \xrightarrow{\text{CoA}} \text{Acetyl CoA}$$

$$+$$

$$\text{Formate} \longrightarrow H_2 + CO_2$$

Metabolism of volatile fatty acids (VFAs) during H_2 production :

Volatile fatty acid (VFA) relationships are important in the anaerobic digestion of biological waste as they (acetic, propionic, butyric and valeric acids) are direct precursors of methane, either through direct conversion of acetate or through the intermediate formation of H_2 and CO_2. Thus, VFAs are essential for the bioconversion process but can be toxic should they be present in excess quantities. It is thus essential to monitor the levels of VFAs during biogas production (Hill and Bolte,1987; Hill *et al.*,1987). During the digestion of organic matter, acetic acid is invariably found in large quantities (Van den Berg *et al.*,1974). Acetic acid produced by acetogenic bacteria is readily converted into CH_4 particularly, when methanogens are active (Mackie & Bryant,1981). Butyric and propionic acids are not as easily metabolized, and accumulation of these acids is one of the major causes of digester failure during anaerobic fermentation (Henson *et al.*, 1986; Ahring and Westermann, 1987,1988). The work of Ueki *et al.* (1988), has shown that propionic and other larger chain fatty acids accumulated together with acetate and H_2 evolved when methanogenesis was inhibited. It is known that the degradation of propionate and other longer-chain fatty acids to acetate and H_2 by the H_2 producing syntrophic bacteria is an essential step in anaerobic degradation (Boone and Bryant, 1980; Mackie and Bryant,1981;

Lovely and Klug,1982).

The ability of mixed microbial population to digest a wide variety of organic substrates like glucose, dumping wheat grains and apple pomace was exploited to elucidate the role of fatty acids and alcohols in H_2 metabolism, especially when growth and activity of methanogens was suppressed. Acetic acid accumulation was quite high in comparison to other volatile fatty acids, irrespective of the feed materials. But its concentration was higher with glucose as substrate than with apple pomace and dumping wheat as substrates. This difference is believed to be due to high levels of available sugar with glucose as substrate compared to other substrates, since glucose can be readily converted into acetic acid. Propionic and butyric acids were produced in very low concentrations. Perhaps this was because their turnover rate was high. Valeric acid, which has been reported to be a precursor of H_2 (McInerny *et al.*,1979), was not detectable in dumping wheat and apple pomace fermentation and also in the case of glucose fermentation, the concentration decreased from pre-H_2 producing phase to H_2 producing phase. These observations indicate that probably valeric acid is converted into lower fatty acids, simultaneously releasing H_2. All these findings were supported by similar observations recorded, with another experiment, when our isolated pure culture of *Bacillus licheniformis* was used as a source of inoculum. It is concluded, here that although direct correlation of VFAs like acetic acid, propionic acid and butyric acid could not be established with H_2 generation, these acids accumulated in higher concentrations as H_2 production rate decreased or stopped.

Although production of alcohols e.g. methanol, ethanol etc. is a normal

phenomenon during anaerobic fermentations, they have not been reported to participate actively in CH_4 production. In our study we have tried to establish their role in H_2 production. Ethanol was observed to be the main alcohol produced in glucose, dumping wheat and apple pomace fermentation by mixed microbial and pure cultures, but its effect on H_2 production could not be established. Propanol was detected only when fermentation was carried out with *B. licheniformis*. Probably, this alcohol appears in the metabolic pathways unique to this strain. Methanol was detected in apple pomace fermentation only. Its origin may well be the methoxy group of pectin. However, these studies have established production of VFA and alcohol during H_2 generation as evidenced by a similar pattern using pure and mixed cultures. The quantities detected are relatively and it was not felt worthwhile going deeper and studying the possible pathways.

Effect of fall in pH on rate of hydrogen production :

The influence of pH drop on rate of H_2 production during fermentation has been distinctly observed. Active H_2 evolution by mixed microbial culture and *B. licheniformis* was found to initiate when the pH dropped from 7.0 to less than 5.3 and the maximum H_2 evolution rate was between pH 4.6 to 4.8, irrespective of the feed material. Such a phenomenon has been reported previously also during H_2 evolution by *Enterobacter aerogenes* (Tanisho *et al.*,1987,1989). It is argued that pH 7.0 is good for growth of H_2 producing organism whereas, bacteria evolved H_2 vigorously around pH 5.8 (Tanisho et al.,1989). Higher the pH drop (below 4.6) lower is the rate of H_2 evolution. Tanisho et al.(1989) suggested that, that bacteria cannot live at pH values of < 4.4, and are hence obliged to release protons in the

physiological environment prior to active H_2 evolution. These protons perhaps combine with electrons from nitrogenase and hydrogenase to evolve H_2. H_2 evolution has been largely found to be due to electron diversion towards freely available protons and this shift in pH is thus a proton release phenomenon. It may be worth mentioning that at pH values of below 4.8, the rate of H_2 evolution drops sharply to very low levels. Reports have shown that at very low pH values, the growth of microorganisms is inhibited (Zajic *et al.*,1978; Hueting and Tempest, 1979; Bahl et al., 1982; Tanisho *et al.*,1989), the ATP driven electron transport mechanism becomes inoperative and as a consequence H_2 evolution becomes almost negligible.

Role of Nitrogenase in H_2 production by mixed microbial culture :

Hydrogen metabolism in most H_2 evolving prokaryotes involve hydrogenase and nitrogenase enzyme complexes (Kosaric & Lyng,1988). Nitrogenase, in almost all prokaryotes evolve H_2 to different extents while converting molecular nitrogen to ammonia, the process is termed as biological nitrogen fixation (Houchins, 1984). Since our mixed microbial culture metabolized various substrates to H_2, we tested it for its nitrogen fixing ability (acetylene reduction activity). Acetylene reduction activity of 0.460 nmoles of C_2H_4 produced/ mg protein / h revealed the presence of nitrogenase in our microbial culture and raised the possibility of its involvement in H_2 evolution. The culture was able to develop the nitrogenase activity under specific conditions. Oxygen level in the gas phase was the important parameter, which controlled the development of nitrogenase activity in mixed microbial culture. O_2 levels of $>$ 0.25 % were inhibitory for expression of nitrogenase activity and such

effects were most specific in shake cultures. This has mainly been because of large exposure of the cells to the O_2 toxicity. Varying effects of O_2 exposure have also been reported by Mohapatra *et al.* (1983) on different strain of *Parasponia, Rhizobium.* The preactivation of the mixed microbial culture using 0.2 % glucose solution by incubating at 40°C for 20-24 h was essential for maximum expression of nitrogenase activity.

Acetylene: inoculum variation has been another important factor in our study of nitrogenase assay, which has been found to be optimum between 4.2-6.25 (I:A ratio). It can be concluded that our assay method for nitrogenase assay offers reasonable stability under these conditions. No reports are available wherein fermentative H_2 producing mixed culture has been assayed for nitrogenase activity. On the basis of substantial data available in the literature, it is possible through the use of inhibitors of nitrogenase, hydrogenase and uptake hydrogenase to predict their exact role in H_2 evolution (Benemann & Weare,1974; Smith *et al.*,1976; Mortenson, 1978; Kumar *et al.*,1990; Sasikala *et al.*,1990). The total inhibition of H_2 evolution by *Rhodobacter sphaeroides* in the presence of 10% C_2H_2, indicates the presence of nitrogenase mediated hydrogen evolution (Sasikala *et al.*,1990). It has been also suggested that acetylene inhibits conventional hydrogenase activity and thus prevents the uptake of H_2 formed by nitrogenase in the presence of 5% CO (Smith *et al.*,1976). It has been concluded that 5% CO blocks all but the hydrogen evolving function of nitrogenase (Smith *et al.*,1976; Mortenson, 1978).

Ammonium and nitrate salts at their optimum concentrations are well known inhibitors of H_2 evolution through nitrogenase (Gest and Kamen, 1949; Kamen and

Gest, 1949; Kumar *et al.,* 1990). Based upon these findings, it was estimated in our system that mixed microbial culture produced 63% of the total H_2 through nitrogenase. However, in one of our experiments using varying concentrations of NH_4Cl, H_2 evolution was elevated at certain concentrations to very high levels. This inconsistent effect of NH_4Cl may be perhaps attributed to the fact that the culture used was of mixed type and may have helped some microbial population in growth which was responsible for H_2 evolution other than through nitrogenase system. Although H_2 evolution is a unique feature of nitrogenase and is an energy (ATP) dependent phenomenon, it leads to losses in biological nitrogen fixation by diverting electrons towards H_2 evolution rather than towards N_2 reduction (Mortenson and Thorneley, 1979). Since our interest is in H_2 generation, the effect of NH_4Cl is a beneficial one in our system.

Effect of sulfate reduction on the metabolism of H_2 production :

Direct effect of sulfate on microbial production of H_2 has not been reported. However, it is reported, that the presence of high sulfate during anaerobic digestion of wastes creates particular problems because sulfate encourages the growth of sulfate reducing bacteria. These bacteria consume H_2, which leads to lower methane yields (Yadav and Archer,1989). Sulfate reducing bacteria like *Desulfovibrio* are well known H_2 producing bacteria (Thauer *et al.*,1977). Many attempts have been made in the past to overcome the problem of sulfate reduction in the anaerobic treatment, so that methanogenesis is not inhibited (Braun and Huss, 1982; Frostell, 1982; Hilton

and Archer, 1988; Yadav and Archer, 1989). In this work we observe that the influence of sulfate and sulfate reduction was beneficial to H_2 production from glucose by mixed microbial culture. Gradual increase of sulfate from 1 mM to 5 mM, increased the sulfate reduction with simultaneous increase in H_2 yields. Maximum improvement in H_2 production was 15.3 %. Higher concentration of sulfate (> 15 mm) was shown to be inhibitory to H_2 production as well as sulfate reduction. It can be concluded that sulfate in the above range can be helpful in marginally improving the H_2 yield.

Influence of trace elements (Ni^{2+} and Fe^{3+}) on hydrogen production :

Most of the agro-industrial wastes, especially fruit wastes and vegetable processing wastes, are deficient in essential minerals especially trace metals and therefore, standardization of their optimum concentration is needed for anaerobic degradation (Raju *et al.*,1991). The role of these metals is well studied in pure microbial strains with different objectives but its relevance in anaerobic digestion is almost lacking. Fe^{3+} and Ni^{2+} is essential for growth of certain microorgnisms including H_2 producers (Rapaske & Rapaske,1976; Dar and Tandon, 1987; Diekert et al.,1980). Similarly these metal ions are the essential components of the H_2 producing enzymes, hydrogenases and nitrogenase (Adams *et al.*,1981; Mortenson and Thorneley, 1979). We have studied, the role of these trace metals in fermentations using mixed culture. At 50 nM concentration of Fe^{3+} as $FeCl_3$, H_2 production increased by 30% , but higher concentrations were inhibitory. Increase in CH_4 production from mango peel by Fe^{3+} supplementation has been suggested by Raju *et al.* (1991). Ni^{2+} did not show any effect on H_2 production at 50, 100 and

200 nM concentrations, whereas, significant effect has been shown on CH_4 production by this trace element (Raju et al.,1991).

Whole cell immobilization :

The experiments conducted with both mixed microbial culture and pure culture of *B. licheniformis* for the increase in the efficiency of H_2 production using immobilization methods have demonstrated very high yields. The cells were immobilized by entrapment in calcium alginate beads and adsorption on baked bricks support. Ehlinger *et al.* (1988) have used baked clay as the support medium in the anaerobic filter on the basis of material showing good bacterial retention properties. The area of adsorbed cells has been mainly dealt in terms of natural ecosystems as well as some old fermentations (D'Souza *et al.*,1986). On the other hand entrapment has been successful in protecting the cells from the toxic effects of the substrates and products (Keweloh *et al.*,1989) and in case of strict anaerobes, O_2 sensitivity has been minimized by using immobilized cells (Matsunaga *et al.*,1980).

The improvement in the rates of H_2 production using immobilized cells of mixed microbial culture varied with support material. It showed a 4.1 – fold increase with baked bricks and 2.1 – fold increase when calcium alginate beads were used for entrapment in comparison to free cells. Similarly, *B. licheniformis* cells when immobilized on baked bricks and calcium alginate beads improved the H_2 yield by 2.4 – fold and 1.2 – fold respectively. The higher yields were perhaps due to a larger cell mass being available.

Theoretically 1 mole of glucose can be converted to 12 moles of H_2, whereas stoichiometrically, via fermentation, only 4 moles of H_2 can be produced from 1 mole of glucose (Thauer, 1977; Thauer *et al.*,1977) being only 33% of the total available energy. A maximum of 1.5- 2.5 mole of H_2 / mole of glucose has been reported by free cells of facultative anaerobes via fermentation (Zajic *et al.*,1978). Tanisho *et al.* (1987,1989) have also reported 1 mole H_2 / mole glucose with the free cells of *Enterobacter aerogenes*. In our studies we have been able to generate a maximum of 1.5 moles of H_2/ mole of glucose fed in a continuous manner and have observed the actual volume of hydrogen gas collected to be quite large. Although there are studies, where higher yields of H_2 have been reported (Kondratieva and Gogotov, 1983; Kosaric and Lyng,1988) these are meant primarily to check the ability of the organism to produce H_2 rather than demonstrating actual feasibility of production on any worth while scale.

Another aspect of immobilization, the long term stability of the production rates has been achieved in continuous culture systems. The experiments continued for over a period of 60 days by both mixed and pure immobilized cultures. On both baked bricks and calcium alginate supports, yield of H_2 were 8.16 - 13.22 l/ mole of glucose fed with mixed and pure culture respectively. Long term stability has also been observed by Phlips and Mitsui (1986) using *Oscillatoria* sp. immobilized on agar. The continuous removal of H_2 also helped in the high rates of H_2 yield. Immobilization also shifts the response of H_2 production towards oxygen concentration. It was shown by Matsunaga *et al.* (1980) that an oxygen sensitive hydrogenase enzyme of the anaerobe *Clostridium butyricum* can generate hydrogen aerobically when the bacterial cells were immobilized.

Immobilization of cells has been useful in achieving cyclic H_2 production since fresh medium can be added on completion of the H_2 production cycle. In the free cells there is a limitation due to cell dilution. This concept was used to help prolong the period of H_2 production with the cells of *Anabaena cylindrica* (Jeffries *et al.*,1978). It also raises the possibility of reusing the cells without loss of cell concentration. Reuse of immobilized cells, after thorough washing has shown that although the efficiency in the IInd and IIIrd rounds was lower than the initial round, it was still higher than that of free cell systems. In addition, higher cell densities can be achieved by immobilizing the cells as shown by other workers for H_2 producing photosynthetic bacterium (Mitsui *et al.*, 1985; Ikemoto & Mitsui,1984). The support material for immobilization also shows variations in H_2 production (Taqui Khan & Bhatt, 1990). Baked bricks as support material worked more efficiently than the calcium alginate beads. The reason for this may be reduced permeability across the alginate membrane for gases. In our studies it has been observed that the pH drop was not optimum in alginate immobilized experiments as compared to baked bricks, which could also probably be one of the reasons for slow gas production rates in calcium alginate beads. The surface of the baked bricks provides adsorption sites where substrate can accumulate thereby providing high localized substrate concentration. These areas of adsorption provide a more favourable growth environment for the bacterial substrate system (McConville & Maier, 1978).

The immobilized cells have also shown a very interesting phenomenon of long term stability of the cells under sterile conditions. As observed in our experiments with calcium alginate beads which were active even after one year of storage under sterile and aerobic conditions.

LIMITATIONS

Efforts have been made to have an insight into most of the problems connected with microbial production of H_2 and CH_4 from pure and mixed cultures using different feed materials. An attempt has also been made to optimize operational conditions. No work of this type can be brought to a state of total completion and enlisted below are some of the aspects which have not yet been dealt with for a variety of reasons.

1. Methane yield from apple pomace at laboratory scale has been lower than that reported in literature. We have not supplemented our slurry with any nutrients. Bioconversion as evident from low degradation of solids can be improved perhaps through recycling.

2. At pilot plant level, the efficiency of H_2 production had been 40% and of CH_4 production as 68-74% compared to laboratory scale.
   The main reasons envisaged are oxygen contamination, improper mixing, lack of proper pH and temperature regulation and digester design. These were not rectified.

3. In one of our studies, mainly meant for elucidating biochemical pathways, H_2 generation from sugar was quite high at very low TS levels (0.3%). This solid content in the feed was not optimal. Since the objective was to optimize the process for high solid waste utilization, the attention was concentrated upon 3%, 6%, and 9% TS slurries.

4. Improvement in H_2 yields was evident when microbes were mmobilized. Such gains were more in batch culture and could not be maintained under

continuous culture digestions due to lower retention time leading to loss of unutilized glucose and bacterial cells.

5. H_2 generation through nitrogenase is an energy (ATP) dependent process and accounts for 63% of the total H_2 evolved in our systems. As our interest lies in maximizing H_2 yields, we have been able to improve H_2 generation to some extent by addition of NH_4Cl. However, further work is necessary to understand the mechanism fully.

6. H_2 metabolism seems to follow *E. coli* pathway. Such studies can be better performed on pure enzymes. Our bacterial culture was of mixed type and we could not establish if other pathways are also followed and under what conditions.

It is submitted that these are some of the lacunae left in this study. Limitation of time and resources have been major reasons for these. Attempts will be made in future to carry out the work on optimization of the process and other interesting aspects on which initial steps have been taken here.

This work is a small contribution in the vast scientific arena especially in the field of microbial technology.

CHAPTER - VI

SUMMARY AND CONCLUSION

SUMMARY AND CONCLUSION

Microbial hydrogen production was observed to be optimum at a temperature of 40°C, pH of 6.5-7.0 and a substrate concentration of 3-6%, whereas optimum methane production occurs at temperature range of 37-40°C, pH 7-8 and substrate level of 3%. Under these conditions the degradation of biological waste materials to fuel gases has been successfully achieved.

Apple pomace (*Malus pumila*), a major industrial fruit waste having a balanced chemical composition with C & H content of 47% & 6% respectively was found to be suitable for fuel gas production.

Each kg of total solids of apple pomace could generate 40 l of H_2 followed by 140 l of CH_4 in separate stages in a continuous daily batch fed culture system at laboratory scale. The digestion led to the reduction of 73.3% in total solids (TS), 76.4% in organic solids (OS) and 70% in chemical oxygen demand (COD). Fermentation of apple pomace for CH_4 alone could increase its yield by 12% resulting in the reduction of 44.3% TS, 48.1% OS & 63% COD. The H_2 production yield decreases by 47%, when the digestion was carried out at ambient temperatures (23-43°C). At 100 - fold upscaled pilot plant level, the destruction of 70% dry matter of the apple pomace, evolved 16 l of H_2 and 95.33 l of CH_4/kg of substrate, thus working at an efficiency of 40% for H_2 generation and 68% for CH_4 generation in comparison to the laboratory scale. These findings resulted in supporting the feasibility of pomace digestion as a means of stabilizing this wasted resource while

producing combustible natural gases. The total biodegradability of the pomace approached nearly 80%, indicating that the pomace has a higher natural gas value. Thus, an apple pomace processing factory generating 10,000 tons (fresh weight) of apple pomace per year (12% TS) could anticipate generating 330,000 cum of gas per year (55% CH_4) equivalent in calorific value to about 197,000 l of diesel oil or 122,700 kg of liquid petroleum gas [(LPG), Butane] or 173,000 l of kerosene oil. This technology holds immediate promise, not only for these fruit processing industry, but for many other commercial operations producing high solids wastes by generating enough energy to supply an important part of the fuel needed in the processing operation.

The biomethanation of vegetable wastes largely consisting of Cauliflower leaves and stalks, raddish leaves and rotten cabbage have confirmed the production of high methane yields when subjected independently and also in succession in a single bioreactor. After homogenizing the material, each kilogram (dry matter) of fresh vegetable waste generated 210-320 l of biogas (68-73% CH_4) and also showed the adaptation of the culture to changing feed material. On this basis, a fruit and vegetable market complex generating 10,000 tons (fresh weight) of vegetable wastes per year (8% TS) could anticipate generating 171,000 m^3 of gas per year (70% CH_4), equivalent in calorific value to about 117,000 litres of diesel oil or 73,000 kg of LPG or 103,000 litres of kerosene oil.

Thus at a loading rate of 1.5 kg TS m^3/ day, a digester capacity of 667 m^3 /ton TS fed per day would be required for these biological wastes, which can be fed one after another, depending upon their availability, round the year.

Damaged wheat grains, a major crop grain waste, with a rich content of C & H have been shown to be a clean source of microbial H_2 and CH_4 production. The enriched mixed microbial population of H_2 producers, degraded the waste grains satisfactorily, as was evident by generating substantial quantities of H_2. At an optimal level of 6% TS, dumping wheat slurry (DWS) can produce 55.7 l of H_2 /kg OS utilized. The digestion process reduced the dry matter by 50.2% and the remaining unutilized matter could be digested for CH_4 generation, till the total solids and organic solids were utilized upto 76.4% and 80.2% respectively. The higher feeding load of more than 3% TS was inhibitory for methanogenesis, whereas it was favourable for H_2 generation. The active continuous batch fed culture digestion of these waste grains for H_2 and CH_4 generation have made it a probable choice as a source for microbial fuel gas production. Recovery of the unutilized solids after CH_4 production for recycling into the digestor have served the dual purpose of better solid utilization and leading continuously to the saving of water. Viable potential for H_2 generation has also been shown by other crop grains like rice and maize and also by pure starch evolving 66-153.4 l of H_2 /kg TS. The biomethanation potential of other types of damaged wheat grains like cattle feed and poultry feed type have substantiated the viability of these waste grains as a good source for fuel gas production. These results have shown a promise for microbial production of H_2 and CH_4 from such waste resources. In view of the potential benefits, it would be of interest to extend the study on the pilot scale versions before establishing a digester for commercial utilization of these waste crop grains.

The varying glucose concentrations (1-9%), showed inhibition of methanogenesis at higher levels, whereas, H_2 production occurred efficiently. Therefore, in systems producing H_2 by mixed cultures, the suppression of the H_2 consumers (methanogens) could be achieved by feeding on substrates containing high sugar levels.

The mixed microbial population of H_2 producers showed a good degradation capacity of biological wastes as evidenced by their extracellular hydrolytic enzyme activity. Hemicellulolytic, proteolytic and amylolytic were the major enzyme activities being observed besides some cellulolytic activity.

Sulphate reduction was shown to favour the hydrogen production in the concentration range of 1-10 mM whereas Fe^{3+} at 50 mM level was stimulatory for H_2 generation. Ni^{2+} in the concentration range of 0-200 nM did not play any role.

The carbohydrate rich waste materials degraded to evolve H_2 by glycolytic pathway, as was evident by the production of H_2 from glucose-6-phosphate and pyruvate by mixed microbial culture and isolated pure culture of *Bacillus licheniformis*. The cells were shown to follow *Escherichia coli* pathway after pyruvate, with formate as an intermediate. Induced formate hydrogenlyase in the mixed culture and the pure cultures of *Bacillus* strains actively fermented formate into H_2 with a conversion efficiency varying from 68-85%. These findings support the conclusion that, H_2 production has been through *E. coli* pathway after glycolysis. The role of strict anaerobic *Clostridial* system still needs to be evaluated in our system of H_2 production with mixed microbial population.

Nitrogenase activity of the culture of mixed microbial population of H_2 producers has been established under anaerobic conditions. Preincubation conditions and supplementation of the medium with 0.2% glucose was deduced to be essential for expressing the activity. Significant inhibitory effects on expression of nitrogenase activity were evident at O_2 levels > 0.25%. An optimum inoculum : acetylene ratio was essential for better reduction of acetylene by nitrogenase. The contribution of hydrogenases, uptake hydrogenases and nitrogenase studied with enzyme inhibitors like C_2H_2, CO, KNO_3 reveal 63% of hydrogen generation through nitrogenase. These studies concluded the role of the total nitrogenase and hydrogenases in the mixed microbial population for H_2 generation, which have opened the option for yield improvement by regulating these enzymes genetically.

Volatile fatty acids and alcohol production from waste biological materials by both pure and mixed cultures have been observed throughout the fermentation process. Acetic acid has been the major acid produced with substantial quantities of propionic, n-butyric and valeric acid. Higher levels of these acids were produced with pure glucose substrate, whereas biological waste materials (dumping wheat and apple pomace) at the same total solid concentration produced very low quantities. The volatile fatty acids were observed to accumulate as the H_2 production rate starts decreasing. Ethanol production was observed in all substrates, whereas methanol has been produced with apple pomace fermentation only. Pure culture of *Bacillus licheniformis* showed the unique property of propanol fermentation.

pH fall has played an important role in the rate of H_2 production. Optimum H_2 production rate was observed as the pH falls to 4.6 - 4.8. The H_2 production was

independent of cell multiplication, which occurred favourably in a pH range of 6.0 to 7.0. Initiation of H_2 production occurred as the pH falls to a favourable level of 5.3. These studies also led to the conclusion, that H_2 production is more efficient with synergistic mixed cultures, where the association of the cultures can produce the H_2 precursors and simultaneously lead to H_2 generation at low pH, which is inhibitory for H_2 consumers (methanogens).

The current study demonstrated an improvement in the yields of H_2 production by immobilizing the whole cell cultures, by adsorption of the cells on solid support like baked bricks and incorporation in calcium alginate beads. The improvement in the yield of H_2 production with mixed microbial culture was 2-fold when immobilized on calcium alginate, whereas 4-fold increase was evident when immobilized on baked bricks. Similarly, the pure culture of *B. licheniformis* showed a net increase of 1.23-fold when immobilized on calcium alginate, whereas baked bricks support improved the yield by 2.40 times. In a daily batch fed continuous culture system, with mixed microbial population using bricks as support material, 36% improvement in H_2 production was achieved when studied over a period of 60 days, whereas 39% improvement was observed with *B. licheniformis* immobilized in alginate. H_2 yield improvements have broadened the concept of recovery of practically total available energy of glucose (33% or 4 moles) in the form of H_2 which is substantially higher than reports available so far. It may be concluded here that, the maximum H_2 producing efficiency of 1.5 moles / mole of glucose utilized as achieved in our studies is substantially high. These yields however are still far below the theoretical possibility of 4 moles/ mole of glucose. We may thus look forward to one or more of the

options for attaining more efficient fermentation processes for H_2 production before its commercial application: (a) the use of batch culture system for continuous H_2 production; (b) development of bacterial strains, genetically modified with higher hydrogenase and nitrogenase activities (strain improvement); (c) use of pH buffering system which can prevent the drop in pH below certain level; (d) a syntrophic culture where the other partner can prevent inhibitory effects of large accumulation of acids and (e) whole cell immobilizations preferably by adsorption on solid supports.

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Biomethanation of Plant Materials

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Abstract

Apple pomace and vegetable waste (radish leaves, cauliflower leaves and stalk and rotten cabbage) were subjected independently and also in succession to anaerobic digestion inoculated with cattle dung, on a laboratory scale. Each kilogram (dry weight) of apple pomace fed could generate 275 l of biogas (57% CH_4), fresh vegetable waste generated 210 l of biogas (72% CH_4) and rotten cabbage led to the generation of 320 l of biogas (68% CH_4). Adaptation of methanogens to changing feed material was also observed.

Key words: Biogas, biomethanation, biotechnology, environmental pollution, non-conventional energy.

INTRODUCTION

Apple pomace and vegetable wastes are biological materials rich in organic matter and can act as good sources of non-conventional energy, although today these wastes cause environmental pollution. Anaerobic digestion of these materials can serve the dual purpose of reducing environmental pollution and generating energy. Different fruit and vegetable wastes and agricultural wastes (Hobson *et al.* 1975; Hashimoto, 1981) have been exploited for their ability to generate biogas. The origin of these biological wastes ranges from fruits like apple, apricot, orange and pineapple (Wase & Gordon, 1982; Lane, 1984 *a*, *b*) to vegetables like asparagus, carrot, french beans, onion, sugar beet, tomatoes (Koster, 1984; Lane, 1984*a*; Labat & Garcia, 1986; Ranade *et al.*, 1987; Verrier *et al.*, 1987; Lubberding *et al.*, 1988). Apple waste has been used as feed material (Wase & Gordon, 1982; Lane, 1984*a*) but cauliflower leaves and stalk, raddish leaves and rotten cabbage have not been employed for generating biogas.

This study was on municipal vegetable market waste, where the nature of the material changes with the season. As these materials do not carry any methanogens, the possibility of developing a bacterial culture which could adjust itself to regularly changing feed material was investigated. The biomethanation in two different reactor set-ups which were fed initially with fruit waste up to 113 and 122 days followed by vegetable wastes for 60 and 30 days, respectively, is reported. We also report here the direct biomethanation of vegetable wastes under different culture conditions in two separate set-ups.

METHODS

Feedstock material

The fruit waste used was apple pomace (*Malus pumila*) from H.P.M.C. Fruit Processing Plant, H.P., India. It was freshly dried in the sun, and has been designated as AP. Vegetable waste was collected from the Azad Pur Marketing Corporation complex, Delhi. It consisted largely of cauliflower leaves and stalks (*Brassica oleracea* L. var *botrytis*) and radish leaves (*Raphanus sativus* L.), and occasionally contained rotten cabbage (*B. oleracea* L. var *capitata*).

Digestion

The digestion of different waste materials was done in a two-stage system. In stage I (acidogenic stage) biomatter was converted to organic acids in an open reactor and these acids were then converted into methane in stage II (methanogenic stage) in a closed reactor. The hydraulic retention times in the two stages were 4 days and 20 days, respectively (Ranade *et al.*, 1987; Verrier *et al.*, 1987). The digesters used for the methanogenic stage were of 1 litres or 2 litres capacity for the slurry with an additional head space of 0·2 litres or 0·4 litres. The digestions were run in continu-

Bioresource Technology 0960-8524/92/$05.00

ous culture. The waste slurry was added to the acidogenic reactor and an equal volume of slurry removed to feed the methanogenic reactor once each day. All incubations were done at 40°C. The pH of the methanogenic stage was adjusted to 7·0 with 1 N NaOH only when its value fell below 6·5.

For incubation, the acidogenic reactor was filled with cattle dung slurry (CDS) and always incubated for 4 days before a quarter of its contents was passed on to the methanogenic reactor. The methanogenic reactor had been filled with CDS and was incubated either for 4 days (unenriched inoculum) or for 20 days (enriched inoculum) before it started receiving material from the acidogenic phase.

The feed substrates (3% TS) were either freshly prepared (apple pomace) or pretreated (vegetable wastes). Vegetable wastes were pretreated as their homogenization in water was not feasible. The wastes were cut into small pieces (1 cm^3) and inoculated with CDS in 9:1 ratio (vegetable waste:CDS v/v) and incubated at room temperature (30–35°C) for 48 h.

The continuous-culture digestion of the apple pomace and vegetable wastes in four different reactor set-ups was done as follows.

(1) CDS (3% TS) initially incubated for 4 days (both stages) was fed with cauliflower and radish waste (CRW) for 110 days.
(2) CDS (3% TS) incubated for 4 days (stage I) and 20 days (stage II) was fed with CRW for 102 days.
(3) CDS (6% TS) was initially incubated for 4 days (stage I) and 20 days (stage II) and then fed with apple pomace (AP, 3% TS) for 113 days, followed by CRW for 38 days and finally fed with RC for 23 days.
(4) CDS (6% TS) incubated for 4 days (stage I) and 20 days (stage II) was subsequently fed with AP (3% TS) for 121 days followed by CRW for 30 days.

CDS (6% TS) was used as inoculum for apple pomace because CDS (3% TS) did not work well.

Total dry solids (TS) and organic solids (OS)
Estimations on freshly collected samples were carried out as described by Lane (1984*a*).

Chemical oxygen demand (COD)
COD (g/litre) of thoroughly mixed samples was determined according to the method described in the APHA manual (1989).

Gas analysis
All gases were collected over water in graduated aspirator bottles. Gas volumes presented were calculated at 25°C. Gas samples were drawn with 1·0 ml airtight syringes and analysed in a gas chromatograph (GC 5700, Nucon Engineers). A chromosorb 102 column of s.s 1·8 m long and 2 mm inner diameter, at ambient temperature with hydrogen as carrier gas at a flow rate of 27 ml/min, was used for analysis of air, CH_4 and CO_2 in the gas samples. Hydrogen sulphide (H_2S) was estimated by passing a known volume of gas sample through 10% lead acetate solution. The volume of the gas absorbed by the lead acetate solution was used to calculate the quantity of H_2S present in the gas sample. Gas standards were passed before each set of gas analyses. The values of biogas yields have been corrected for the contribution of CDS in vegetable waste:CD slurry. (CDS evolves 200 litres of biogas or 120 litres of CH_4/kg of TS fed; Kumar *et al.*, 1987.) The data reported here represent the steady state.

RESULTS

Apple pomace methanogenesis

The initial cattle dung slurry (CDS) was sequentially replaced by AP over a period of 80 days. During this phase, biogas was evolved both by CDS and AP. Each addition of 100 ml slurry yielded 1·3 litres of biogas. The biogas had 55% CH_4. At the end of this period the effluent from the bioreactor contained 1·73% TS.

From 81 days after the start of digestion, the data obtained from bioconversion of AP, carried out in two different bioreactors is presented in Table 1. The process resulted in the evolution of 155·6 litres of CH_4/kg TS fed or 275 litres biogas and reduced the COD level from 60·5 g/litre to 22·4 g/litre, i.e. 63% reduction.

Vegetable waste methanogenesis
The observations recorded on different parameters of fermentation of cauliflower and raddish waste (CRW) either with 4-day-old or 20-day-old CDS were quite similar (Table 2). On average, 212 litres of biogas was generated from each kg of TS fed. Its CH_4 content was 72·8%. The effluent showed a 63·1% reduction in COD level in comparison to the feed material (COD = 62·4 g/litre).

Bioconversion of CRW was also observed with an inoculum enriched for AP, in two different

Table 1. Comparison of biogas yield and composition and characteristics of the effluent originating from bioconversion of different plant materials[a]

Plant material[b]	*Yield (l/kg TS)*				*Biogas composition (%)*						*Net reduction (%)*					
	Biogas		*CH_4*		*CH_4*		*CO_2*		*H_2S*		*TS*		*OS*		*COD*	
	N	$\bar{X}$	*N*	$\bar{X}$	*N*	$\bar{X}$	*N*	$\bar{X}$	*N*	$\bar{X}$	*N*	$\bar{X}$	*N*	$\bar{X}$	*N*	$\bar{X}$
AP	44	275	27	155·6	27	56·6	27	36·1	6	7·3	18	44·3	18	48·1	5	62·8
CRW	80	210	23	150·7	23	71·8	23	22·5	16	5·7	22	60·0	22	62·4	7	62·8
RC	17	320	4	217·6	4	68·0	4	25·1	4	6·9	4	61·3	4	63·6	4	69·0

[a]AP, apple pomace; CRW, cauliflower and radish waste; RC, rotten cabbage; N, number of observations; $\bar{X}$, mean value; TS, total solids, OS, organic solids; COD, chemical oxygen demand (g/litre of slurry). Net reduction values are the differences between the feed into the acidogenic stage and the effluent from the methanogenic stage.
[b]See Methods for lengths of feeding periods.

Table 2. Characteristics of biomethanation of cauliflower and radish waste under different culture conditions

Parameters of methanogenesis	*Cauliflower and radish biomethanation*			
	On cattle dung[c]		*On cattle dung fed with AP*[b]	
	N	$\bar{X}$	*N*	$\bar{X}$
Biogas yield (l/kg TS)[d]	29	212·0	51	208·0
Methane yield (l/kg TS)	10	154·0	13	147·0
Methane (%)	10	72·8	13	70·8
Carbon dioxide (%)	10	21·9	13	23·1
Hydrogen sulphide (%)	10	5·3	13	6·1
pH of the effluent	12	6·91	18	7·22
Total solids in the effluent (%)	11	1·29	11	1·11
Organic solids in the effluent (%)	11	0·93	11	0·95
COD reduction	4	63·1	4	61·5

[a]The values are means of the observations (N) recorded on cattle dung enriched in two different sets of conditions. See Method for details.
[b]Cattle dung fed with apple pomace for 80 and 87 days.
[c]Standard errors were < 10% and have thus not been mentioned individually.
[d]Total solids employed.

bioreactors. The switch-over of feed material from AP to CRW became noticeable very soon. The biogas generation rate attained another plateau of 203 litres biogas/kg TS fed; the gas contained 70·8% CH_4 (Table 2). The effluent reflected a 61·5% reduction in COD level. These features were quite comparable to bioconversion of CRW observed with 4-day-old or 20-day-old CDS (Table 2).

As rotten cabbage slurry (RC) replaced the CRW, which had previously replaced AP, active methanogenesis continued to occur. With RC as feed material, 320 litres of biogas/kg TS fed were generated (Table 1). It had a CH_4 content of 68%. A 69% reduction in COD was observed.

DISCUSSION

Vegetable wastes, largely cauliflower and raddish waste, and apple pomace, which produced almost similar quantities of methane in 20 days retention time were less efficient than the rotten cabbage as feed material. However, all these proved to be better wastes than the most prevalent source of biogas, the cattle dung which evolves 120 litres of CH_4/kg TS fed in 40 days RT (Kumar *et al.*, 1987). Since the reduction in organic solids was only 48% to 64%, a higher biogas generation might be achieved either by recycling the effluent or by increasing the retention time. Lane (1984*a*), by recycling the effluent, achieved up to 93·4% reduction in OS content and thereby generated 454 litres of biogas/kg TS utilised. On the other hand, temperature seems to have played an important role in increasing biogas yield from 35 litres biogas/kg TS destroyed at 25–28°C (Ranade *et al.*, 1987) to 210 litres biogas/kg TS fed at 40°C (this study).

Our results show the phenomenon of adaptation of microbial population to different biological wastes i.e. AP, CRW and RC. Active methanogenesis at different levels varied with the waste under treatment.

On the basis of the observed bioconversion capacities the following suggestions can be made.

An apple-processing factory generating 10 000 tonnes (fresh weight) of apple pomace per year (12% TS) could anticipate generating 330 000 m^3 of gas/year (55% CH_4) equivalent in calorific value to about 197 000 litres of diesel oil or 122 700 kg of liquid petroleum gas (LPG, butane) or 173 000 litres of kerosene oil.

On the other hand, a fruit and vegetable-marketing complex generating 10 000 tonnes (fresh weight) of vegetable waste per year (8% TS) could anticipate generating 171 000 m^3 of gas/year (70% CH_4), equivalent in calorific value to about 117 000 litres of diesel oil or 73 000 kg of LPG or 103 000 litres of kerosene oil.

At a loading rate of 1·5 kg TS m^3/day, a digester capacity of 667 m^3/tonne TS fed per day would be required for these biological wastes, which can be fed one after another, depending upon their availability, round the year.

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Short communication

Methanogenesis of dumping wheat grains and recycling of the effluent

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ABSTRACT

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INTRODUCTION

Biological waste materials have been used widely for generating energy in the form of biogas. The materials employed had various origins: domestic, agricultural, industrial, etc. Different parts of fruits, vegetables and crops have been used as organic matter-rich sources for methanogens [1–11]. Grain crops have so far not been used for biogas generation, because these are invariably used as animal feed. However, The Food Corporation of India, categorizes wheat grains into two major groups: 1, fit for human consumption and 2, damaged grains unfit for human consumption, based on a certain set of criteria [12]. The damaged wheat grains are then further categorized as 1, cattle feed, 2, poultry feed, 3, industrial use, 4, manure purpose and 5, dumping type. These are based on the following criteria: a, sound grains of wheat, b, broken but sound grains, c, slightly damaged, d, touched/discoloured/chalky grains, e, completely damaged grains, f, foreign matter and also appearance, smell,colour, contamination, etc. The dumping variety of wheat grains are used only for land filling.

We report here the use of dumping wheat grains for methane generation. We also present the results of experiments performed for recycling the effluent of the methanogenic stage.

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MATERIALS AND METHODS

Damaged wheat grains. Wheat (*Triticum aestivum*) grains categorized as "dumping" were procured from the Food Corporation of India. The dirty grains were cleaned as follows: washing under running tap water until no dust particles were visible. The dust free grains were sun dried and stored in a dry place. Each kg of dirty grain had 600 g of "clean" grains. These cleaned grains were crushed into powder form before utilization.

Bacterial culture. Cattle dung was the source of bacteria. The cattle dung slurry was incubated at 40°C for enrichment of methanogenic bacteria.

Bioreactors. Continuous batch cultures were performed in two stages. Volumes in stage I and stage II were 400 ml and 2 l respectively. Stage II had an inlet for receiving slurry from stage I and outlets for gases and effluent. Two total solid (TS) concentrations (3 and 6%) were employed. The slurry was retained for 4 d in stage I and for 20 d in stage II. Initially, both the reactors were filled with cattle dung slurry (3 or 6% TS, depending upon the TS of the dumping wheat slurry (DWS) to be employed). After 4 d of incubation at 40°C, 100 ml of the material from stage II was drained out and 100 ml of the material from stage I was transferred to stage II; the procedure was repeated once every 24 hrs.

Recycling of the effluent. The effluent from stage II was put back into stage I only in the case of 3% TS dumping wheat methanogenesis. The total solids of the effluent were estimated and always made up to the 3% level by adding fresh dumping wheat.

Gas collection and analysis. All gases were collected over water in graduated aspirator bottles. Gas volumes were calculated at 25°C. Gas samples were drawn off using 1.0 ml airtight syringes and analysed by gas chromatography (GC 5700, Nucon Engineers). A Porapak Q column of s.s.1.8 m long and 2 mm inner diameter, at ambient temperature, with hydrogen as carrier gas at 27 ml/min, was used for analysis of methane and carbon dioxide. The methane gas standard was prepared by fermenting cattle dung slurry, 3% TS, at 40°C for 20 d. The biogas produced was passed through 10% lead acetate solution for trapping any hydrogen sulphide present and the gas was then passed through KOH solution for trapping CO_2. The gas collected was used as the methane standard. The air content was calibrated by passing the air sample through the Porapak Q column. A carbon dioxide standard was prepared by taking gas from a CO_2 gas cylinder. Hydrogen sulphide was estimated by passing a known volume of gas sample through 10% lead acetate solution, where a black precipitate indicated the presence of H_2S in the sam-

ple. The volume of the gas absorbed by the lead acetate solution was used to calculate the quantity of H_2S present in the gas sample. The standard gasses were passed before each set of gas analysis.

Total dry solids (TS) and organic solids (OS). Estimations of freshly collected samples were performed as described by Lane (13).

RESULTS

Methanogenesis of dumping wheat grains. During 0–60 d of addition of dumping wheat material into the cattle dung slurry the biogas evolved was the result of fermentation of both the dumping wheat and solids carried over from dung slurry. On average, 1.14 l of biogas (57% methane) evolved per 100 ml slurry addition every day. The observations recorded after 60 d of incubation at 40°C are presented below.

At 3% TS DWS level, (Table 1) the methanogenesis was observed to be very active. The biogas evolved was composed of 69.1% methane. Reduction of each kg of organic solids (OS) led to the generation of 544.7 l of biogas. During the bioconversion, a 69.3% reduction in OS level was evident in the effluent, with respect to the feed material.

At 6% TS DWS level (Table 1), after an initial active methanogenesis for 60 d, biogas evolution from dumping wheat grains attained a steady state. The biogas during this final period of 30 d had very low levels of methane i.e., 24.9%. At this 6% TS concentration only 195.3 l of biogas/kg of OS reduced was observed. The effluent reflected at 50.1% reduction in the OS content.

TABLE 1

Characteristics of methanogenesis of dumping wheat grains and the recycled effluent

Parameters	Biogas yield (l/kg OS reduced)	CH_4 yield (l/kg OS reduced)	Quantity (%)		
			CH_4	OS reduced	Efficiency*
At 3% TS level	544.7	376.3	69.1	69.3	100.0
At 6% TS level	195.3	48.6	24.9	50.1	12.9
Recycled effluent (At 3% TS level):					
(0–27 days)	1021.3	692.4	67.8	54.9	184.0
(28–66 days)	596.2	416.7	69.9	60.1	110.7
(67–82 days)	288.8	205.9	71.3	54.7	54.7
(0–82 days)	693.2	480.3	69.3	56.6	127.6

TS: Total solids, OS: Organic solids, *: 3% TS level methanogenesis value has been taken as 100% efficiency level (On the basis of methane content). The values represent the steady state methanation.

Thus from each kg of OS reduced at 3% TS level, 376.3 l of methane could be obtained in comparison with 48.6 l of methane obtained at the 6% TS level.

Recycling of the effluent. The recycling of the effluent was done only in the case of 3% TS DWS methanogenesis. Three different and distinct phases of biogas evolution could be observed over a period of 82 d of recycling (Table 1).

On recycling the effluent, during 0–27 d the overall methanogenesis rate was 1021.3 l biogas/kg OS reduced; methane content was 67.8%, leading to the evolution of 692.4 l CH_4/kg OS reduced. Its efficiency (methane content) was 1.84 times greater than the preceeding non-recycled system.

From 28–66 d of recycling, the biogas evolution rate was comparable to that observed with non-recycled 3% TS DWS. It evolved 596.2 l of biogas/kg OS reduced. Although the methane content of the biogas did not vary much in comparison to the first phase of recycling, the quantity of methane evolved was less i.e., 416.7 l/kg OS reduced.

In the final stage of 67–82 d the decline in biogas evolution rate was dramatic. Here, it became stable at 54.7% efficiency in comparison to the non-recycled DWS (3% TS). It evolved 288.8 l of biogas or 205.9 l of CH_4/kg OS reduced, although the reduction in OS has been quite similar to that observed during the first phase of recycling (Table 1).

DISCUSSION

Dumping wheat seems to have good potential for generating biogas. However, methanogens seem to find 3% TS DWS a more conducive physiological environment for their activity than the 6% TS DWS. Biogas evolution was 2.78 times greater with 3% TS content in comparison with the higher TS content, on the basis of reduction in organic solids. It is also remarkable to note that at 6% TS DWS the biogas production has been very poor, in spite of the fact that OS reduction was approx. 50.1%. A decline in biogas yields from digestion of apricot waste after 63 d of incubation, for reasons not understood, has been reported by Lane [13]. However, a high concentration of carbohydrates in our case seems to play an important role in inhibiting methanogenesis, as has been observed by Roychowdhury et al. [14].

Recovery and recycling of settled solids from the effluent are effective means of reducing the COD level of the final effluent and also of increasing the biogas yields. Lane has shown that by increasing the effective retention time of the sludge solids by solids recycling has resulted in 99 and 100% conversions in orange peel and spent coffee grounds, respectively [15,16]. Collander and Barford [17] demonstrated that solids recycling during pig manure digestion resulted in a 36% increase in biogas yield. Recycling of solids has helped in increasing the conversions of 89.7% OS and 93.2% OS in asparagus and pine-

apple wastes [13], respectively, in comparison with only 40% conversion of OS to gas (in asparagus) reported by Knol et al. [7]. In our case we have an increased emphasis on water conservation, achievable by recycling of the effluent. Recycling of the effluent, although affecting the total biogas production efficiency, did not, however, affect the quality of the biogas. Methane content remained fairly constant throughout the experiment. The effect of recycling of solids has been overshadowed by the fact that the methanogenesis of dumping wheat grains is more effective at lower total solid concentrations. We have observed that at 1% TS concentration level the dumping wheat methanogenesis is two times more effective than at 3% TS level (data not shown). This is well reflected in the first phase of recycling where the fresh infeed contributed little to the total infeed material, such that the methanogenesis occurred at a rate which was 1.84 times more than the non-recycled (3% TS) phase. However, this effect was not evident in the third phase of recycling where there was a very low level of methanogenesis. The biogas production efficiency reached a low level of 47%. Here, this negative effect perhaps has its origin in the phenomenon that continuous recycling has led to the accumulation of some metabolites, toxins or ions, which did not prove very helpful to this bioconversion process.

We may, however, conclude that at 3% TS level, methanogenesis can be actively carried out even on recycled effluent at a high efficiency, at least until a period which is equivalent to that during which the bioconversion was carried out in a non-recycled manner, leading continuously to the saving of water.

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