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NITROGEN FIXATION IN ANAEROBIC DIGESTION

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John A. Eastman

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NITROGEN FIXATION IN ANAEROBIC DIGESTION

By

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ABSTRACT

NITROGEN FIXATION IN ANAEROBIC DIGESTION

By

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Biological fixation of nitrogen gas in anaerobic digestion was investigated to evaluate the potential for utilizing molecular nitrogen for bacterial growth and to compare the effect of molecular nitrogen with ammonia nitrogen. Four laboratory scale batch-fed digesters were operated at 35°C using nitrogen gas and ammonium chrolide as nitrogen sources with glucose, cellulose, and acetate as substrates. With nitrogen gas as the nitrogen source, cellulose and acetate were not degraded. Glucose was almost completely degraded using either nitrogen gas or ammonia, with a delay of about 20 hours for glucose degradation when nitrogen gas was used. Lower cell growth was found in the case of nitrogen gas compared with ammonia: yield coeffieients were 0.047 and 0.122 mg VSS/mg COD consumed in the nitrogen gas and ammonia reactors, respectively. The nitrogen content of the bacterial cells changed from 0.08 mg N/mg VSS when ammonia was used to 0.11 mg N/mg VSS when nitrogen gas was used. No significant change was found in the production of volatile acids from glucose comparing reactors using nitrogen gas and ammonia.

ACKNOWLEDGMENTS

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Shuzo Tanaka

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LIST OF SYMBOLS

Symbol	Meaning
$\Delta G^{o'}$	Standard free energy change with $\{H^+\} = 10^{-7}$
Nc	Organic nitrogen content in cells
P	Pressure in gas phase
Ps	Standard pressure (1 atm)
T	Temperature
Ts	Standard temperature (273^oK)
V	Observed gas volume
Vs	Gas volume at standard conditions
Y	Yield coefficient

I. INTRODUCTION

Anaerobic digestion has been commonly used to treat wastewater sludges -- both municipal and industrial. In addition, it can be used for treating the high strength liquid wastes produced by many industries. Anaerobic digestion has a number of distinct advantages for the treatment of strong wastes and sludges as well as some disadvantages. The advantages include low cell growth, no oxygen requirement, and production of methane gas which can be utilized as a fuel. Disadvantages include the need for heating and long detention times. The advantages, however, generally outweigh the disadvantages for concentrated wastes such as organic sludges.

Low cell growth in anaerobic digestion is significant in waste treatment. It will greatly reduce the sludge disposal problem resulting from conversion of organic matter to bacterial biomass. Low bacterial growth also means low requirements for auxiliary nutrients essential for biological metabolism. In other words, anaerobic treatment will minimize the need to supply deficient nutrients. This is of specific importance for treatment of industrial wastes often deficient in nitrogen.

A variety of nitrogen sources are available for bacterial growth, including ammonia, nitrate, nitrite, and molecular nitrogen. Several investigations on nitrogen sources in anaerobic treatment have been made with combined nitrogen (Gasser and Jeris, 1969, and Brezonik and Lee, 1966). Ammonia nitrogen was the most readily utilized form of combined nitrogen. On the other hand, biological utilization of

molecular nitrogen (uncombined nitrogen) has been investigated primarily from the microbial and agricultural points of view, as regarding nitrogen fertilizers. Since microorganisms cannot directly incorporate molecular nitrogen for cell growth, molecular nitrogen must first be reduced to ammonia. This reduction is called nitrogen fixation. Then, the ammonia can be assimilated into cell organic nitrogen. Biological utilization of molecular nitrogen in anaerobic treatment, however, has never been studied to the best of my knowledge.

The overall objective of this study is to evaluate the potential for utilizing molecular nitrogen for bacterial growth in anaerobic digestion. In addition, the effects of nitrogen sources will be evaluated. If nitrogen fixation is to occur at all, it must, as a minimum, occur in the acid fermentation phase. Accordingly, the emphasis of this study will be in the acid phase. More specifically, the objectives of this study are:

1) to evaluate the effects of nitrogen source on anaerobic digestion with regard to substrate degradation, cell growth, organic nitrogen content, and production of volatile acids. The nitrogen sources used are nitrogen gas and NH_4Cl.
2) to discuss the composition of products formed during anaerobic decomposition with respect to a COD balance.
3) to evaluate the effects of substrates on biological nitrogen fixation and assimilation during anaerobic digestion. The substrates used are glucose, cellulose, and acetate.

II. BIOCHEMICAL AND MICROBIOLOGICAL BACKGROUND

Anaerobic digestion is accomplished by complex biochemical reactions through metabolism of anaerobic microorganisms. Microbial metabolism is affected by many environmental factors , such as nutrients, pH, and temperature. This study is designed to investigate biological fixation and assimilation of nitrogen, which is one of the essential nutrients for biological growth. To effectively perform this study, it is necessary to have an understanding of basic biochemical and microbiological principles of anaerobic digestion. This chapter will present and review basic background materials of (A) the fundamentals of anaerobic metabolism, (B) the general scheme of anaerobic digestion, (C) environmental factors, and (D) the biochemistry of nitrogen fixation.

The organic materials of primary concern in anaerobic digestion are carbohydrates, lipids, and proteins. The biochemical pathways for metabolic activity of each component are substantially different and must be treated separately. However, proteins are not discussed in this chapter; they are not relevant to this study due to their nitrogen content. See Section B of Chapter III for details.

A. FUNDAMENTALS OF ANAEROBIC METABOLISM

Heterotrophic microorganisms, primarily bacteria, accomplish the degradation of organic materials in the anaerobic digestion process. Heterotrophic bacteria use the same organic material as sources of energy and cell carbon. A portion of the organic material

is degraded to obtain energy while the remaining portion is used for cell synthesis. The oxidative degradation process to obtain energy is called catabolism, and the reductive synthesis process is called anabolism. The energy resulting from catabolism is used to drive anabolic reactions.

Under anaerobic conditions, heterotrophic bacteria generally obtain energy in two ways: fermentation and anaerobic respiration. The energy released by these biochemical reactions is stored in the form of phosphate-bond energy and used for cell synthesis and maintenance. This section will present a brief discussion of the energy transfer system and the above two energy-yielding processes. Further information can be obtained from a number of textbooks such as Gandy and Gandy (1980), Bailey and Ollis (1977) or Lehninger (1975).

Because cell synthesis requires many different inorganic, as well as organic, nutrients it will be discussed in Section 6 of this chapter.

1. ENERGY TRANSFER SYSTEM

The energy released by catabolic reactions is stored by bacteria in the form of the phosphate-bond energy of adenosine triphosphate (ATP). The stored energy of ATP is used to drive anabolic reactions, and the ATP molecule changes to a discharged state called adenosine diphosphate (ADP). The reaction of ATP to yield ADP and phosphate is accompanied by a standard Gibbs free energy change of -7.3 kcal/mol at 37^{o} C and pH7. The ADP molecule can then capture the energy resulting from catabolic reactions and again become an energized state as

the ATP molecule. The ADP-ATP cellular-energy system is shown schematically in Figure 1.

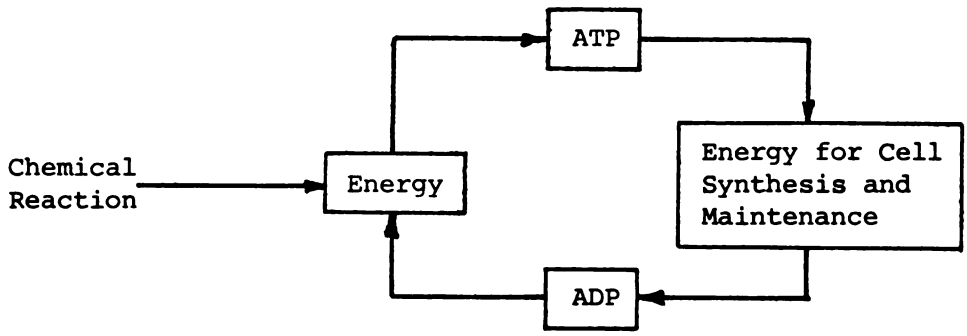


FIGURE 1. ADP-ATP CELLULAR-ENERGY TRANSFER SYSTEM (Source: Metcalf & Eddy, 1979).

Thus, the ATP production in catabolism has much to do with biological growth. In fermentation and anaerobic respiration, the ATP production is much less than that in aerobic respiration. Hence, a lower percentage of organic matter will be assimilated to cell tissue in anaerobic growth compared with aerobic growth. The low cell growth results in low sludge production, an advantage of anaerobic digestion.

2. FERMENTATION

Fermentation is an energy-yielding process in which organic compounds are oxidized with no external electron acceptor under anaerobic conditions. The terminal electron acceptor is an organic molecule.

The Embden-Meyerhof-Parnas (EMP) pathway, simply called glycolysis, is the major route of the carbohydrate fermentation for a variety of microorganisms. As shown in Figure 2, a molecule of glucose is converted to two molecules of pyruvic acid by a series of enzyme-mediated reactions. In this process, two molecules of ATP are produced and two molecules of nicotinamide adenine dinucleotide (NAD) are reduced to $NADH_2$. NAD is one of the most important coenzymes in this pathway. This coenzyme functions as an electron carrier. In anaerobic cells, pyruvic acid may serve as the terminal electron acceptor to reoxidize $NADH_2$ to NAD, forming lactic acid as the end product of the fermentation pathway, or it may be converted to other end products, as shown in Figure 3.

Although other pathways, as the Herose-Monophosphate (HMP) pathway and the Entner-Douderoff (E-D) pathway, can be used to oxidize glucose, they are limited to a small group of bacteria. These pathways are beyond the scope of this study.

3. ANAEROBIC RESPIRATION

Anaerobic respiration is an energy-yielding process in which organic or reduced inorganic compounds are oxidized using inorganic compounds as terminal electron acceptors in the absence of oxygen. In the absence of oxygen, major terminal electron acceptors are carbon dioxide, sulfate, and nitrate. Figure 4 shows that carbon dioxide can be reduced to methane when carbon dioxide is used as the terminal electron acceptor. Likewise, sulfate can be reduced to sulfide and nitrate to molecular nitrogen, ammonia, and nitrous oxide.

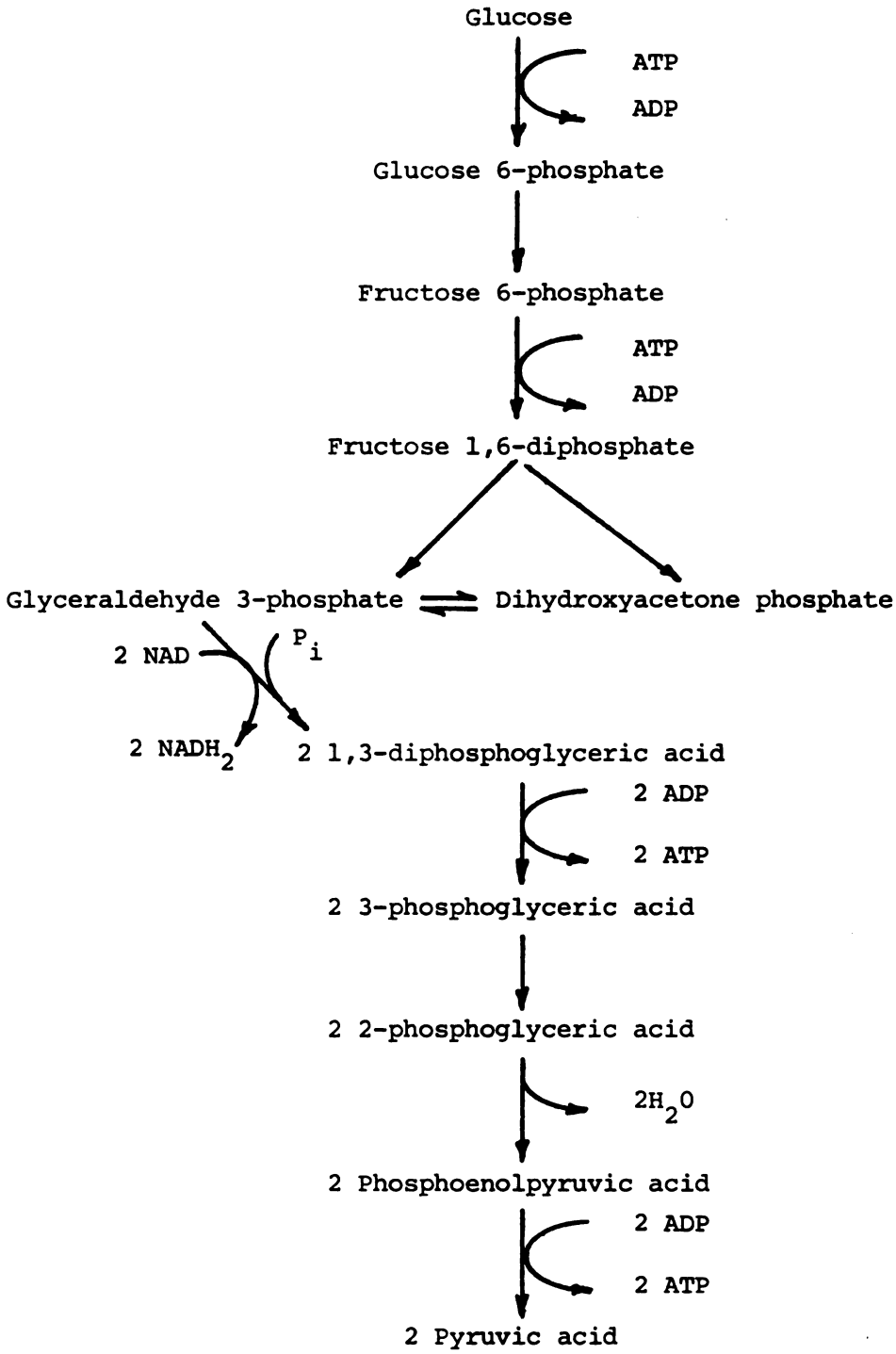


FIGURE 2. Embden-Meyerhof-Parnas (EMP) pathway for catabolism of glucose. (Source: Gandy and Gandy, 1980).

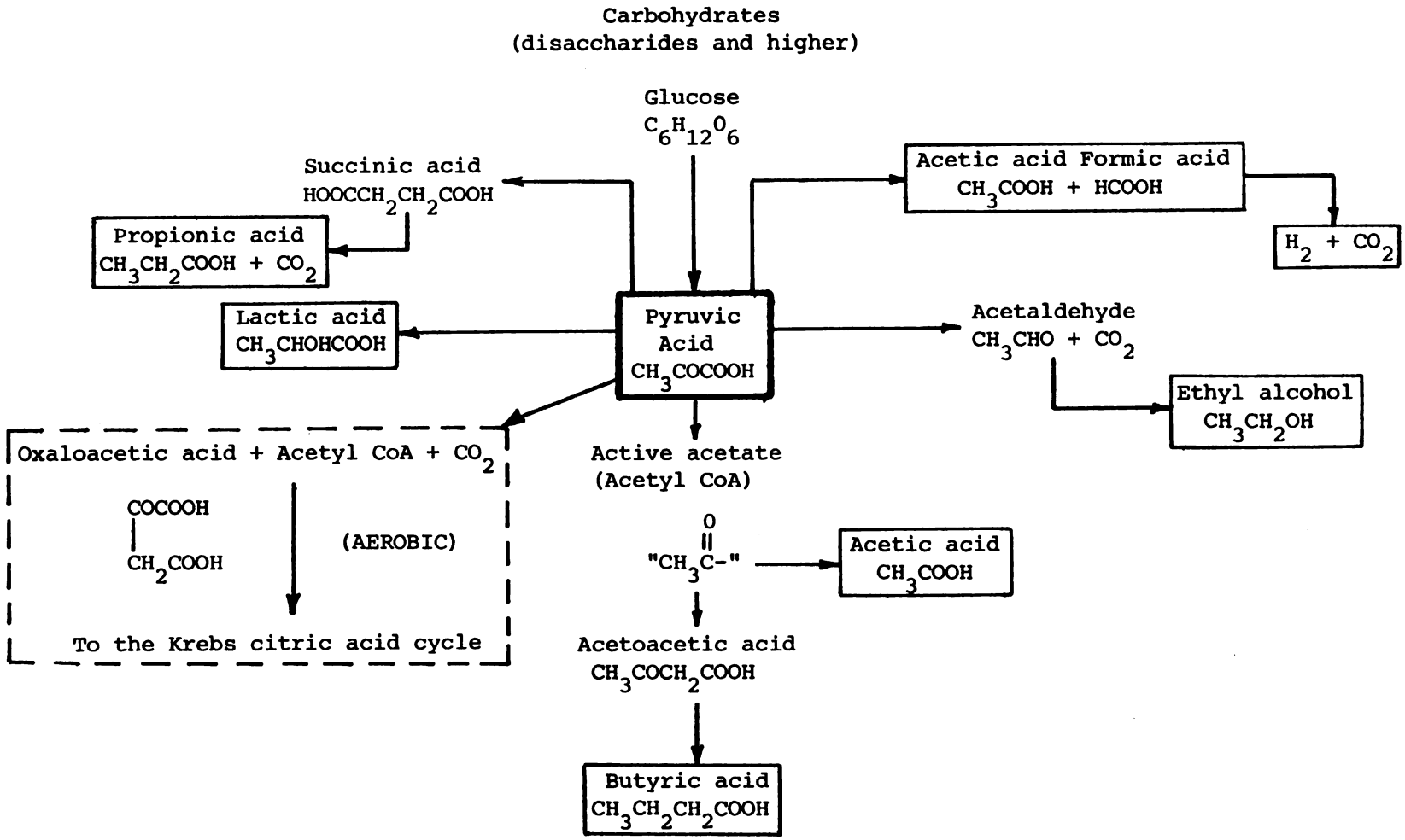


FIGURE 3. Representation of the Pivotal Nature of Pyruvic Acid. (Adapted from Pelczar and Reid, 1972.)

The primary purpose of the electron transport system is to reoxidize the reduced coenzymes formed as a result of oxidation reactions of substrates. The phosphorylation reaction of ADP

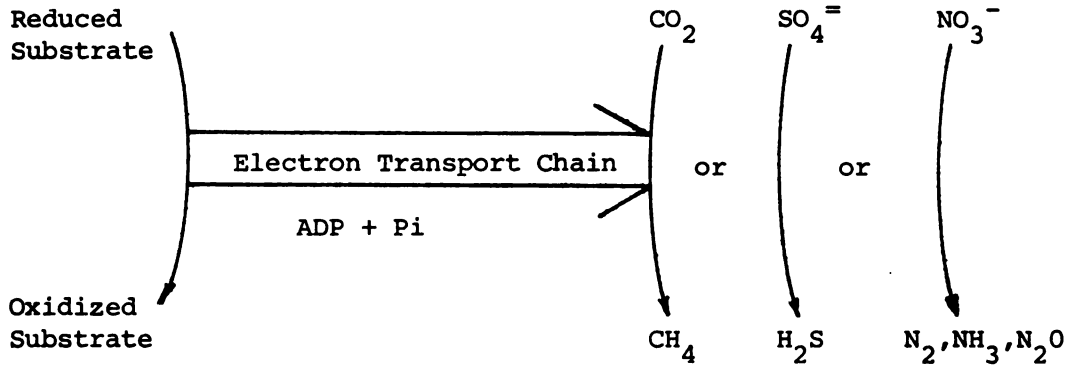


FIGURE 4. Electron Transport Chain for Anaerobic Respiration. (Source: Wilkinson, 1975)

to ATP in this system, however, is not completely understood. It is thought that the electron transport to inorganic acceptors yields less ATP than that to oxygen, i.e., anaerobic respiration yields less ATP than aerobic respiration does (Stouthamer, 1976 and Wilkinson, 1975). Here, again, lower ATP production leads to less biological growth.

B. GENERAL SCHEME OF ANAEROBIC DIGESTION

Wastes containing fermentable organic materials can be treated biologically in the anaerobic digestion process. The organic materials are decomposed by heterotrophic bacteria and utilized to support both bacterial life and growth functions through anaerobic metabolism. In this process, the organic materials are converted ultimately to methane, carbon dioxide, cell material, and a non-degradable fraction.

This biological conversion of organic material generally occurs in three steps: (1) hydrolysis, (2) acid fermentation, and (3) methane fermentation. As shown in Figure 5, the hydrolysis is the breakdown of large insoluble organic molecules into smaller soluble molecules suitable for use as a source of energy and cell carbon. The acid fermentation is the conversion of simple organic molecules to volatile acids, new bacterial cells, and other products, while the methane fermentation is the conversion of volatile acids and other products to simpler end products, principally methane and carbon dioxide. This section will provide the description of these three processes in anaerobic digestion.

1. HYDROLYSIS

The majority of organic material flowing to the anaerobic digestion process is generally in the form of large insoluble molecules which cannot pass through cell membranes. Therefore, it seems that the breakdown of the large molecules into small molecules is necessary before metabolic use in cells. As the first step of anaerobic digestion, the large insoluble organic molecules are hydrolyzed into small soluble molecules by extracellular enzymes produced by heterotrophic bacteria. The soluble organic molecules formed outside the cell membranes during hydrolysis can then diffuse into cells and be utilized for metabolic activity.

The extracellular enzymes to cleave the glucosidic linkage of carbohydrates are called carbohydrases. Such glucose polymers as glycogen, starch, and cellulose are hydrolized generally into glucose

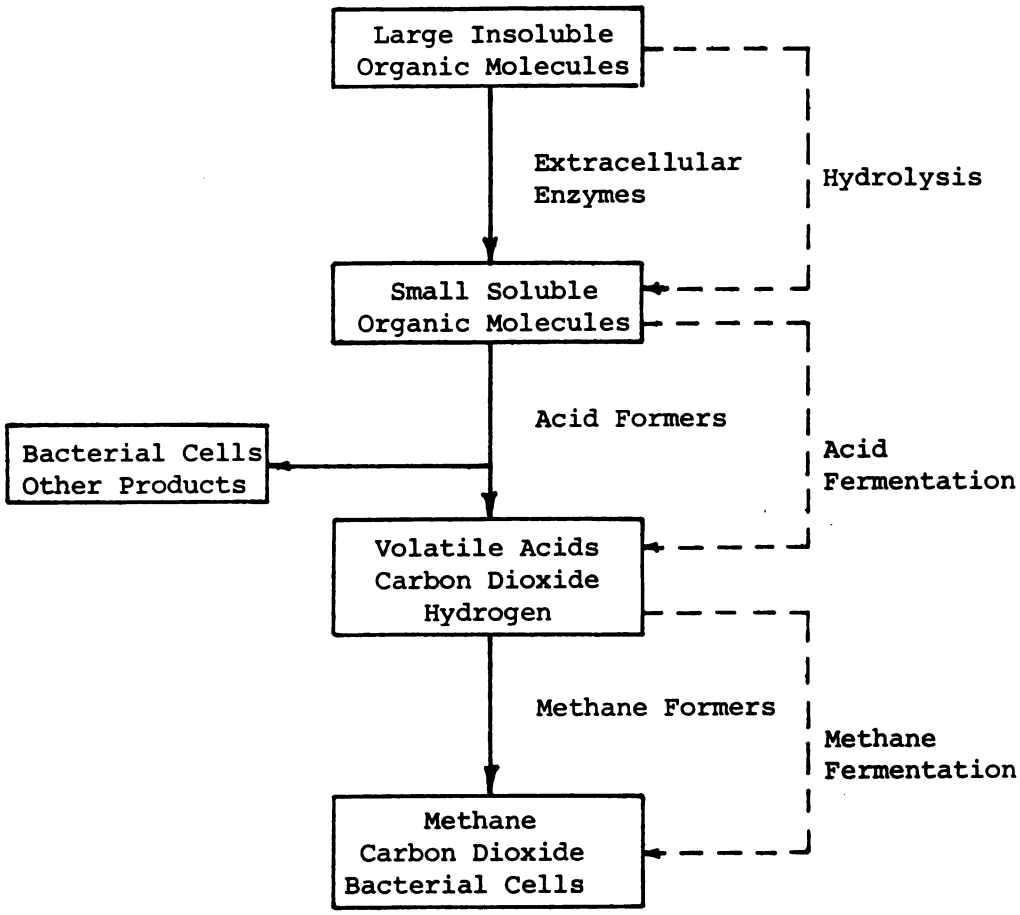


FIGURE 5. Overall Mechanism of Anaerobic Digestion.

and cellobiose, the disaccharide fragment of cellulose.

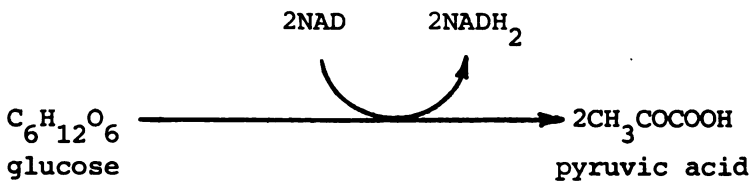
Cellulose is the most abundant carbohydrate in the biosphere. In municipal wastewaters, paper comprises the largest portion of the cellulose. The hydrolytic enzyme of cellulose, denoted as cellulase, splits the polymer into simpler units of cellobiose. Cellulose hydrolysis is thought to depend on three steps: (1) removal of the noncellulosic casing of lignin which surrounds native cellulose; (2) attack of the insoluble form of cellulose; and (3) conversion of soluble cellulose into cellobiose. A major limiting factor of cellulosic hydrolysis is the lignin that binds cellulose fibers together and is not readily biodegradable. Cellulose that has undergone the removal of lignin, as in the chemical pulping of wood, is more readily degraded than is native cellulose.

2. ACID FERMENTATION

The soluble organic materials resulting from the hydrolysis of large insoluble molecules can diffuse through cell membranes and serve as nutrients for the metabolism of facultative and obligate anaerobic bacteria. As the result of microbial catabolism, these organic nutrients are converted to simple organic compounds, mainly short-chain volatile acids and alcohols. This conversion of organic nutrients into volatile acids and alcohols is generally referred to as acid fermentation, and the microorganisms responsible for this conversion are often called acid formers. Also, the products of the acid fermentation phase include new bacterial cells resulting from anabolism. In acid fermentation, organic materials are simply

converted to volatile acids, alcohols, and bacterial cells so that little stabilization of BOD or COD is realized. The true stabilization of the end products of this stage occurs in the methane fermentation phase which will be treated in a later section of this chapter. In the paragraphs below, the acid fermentation of carbohydrates will be presented in detail.

Glucose is the most important product resulting from the carbohydrate hydrolysis. The central pathway of glucose fermentation is the EMP pathway (glycolysis) which was mentioned in Section A of this chapter. Hence, the fermentation of carbohydrates starts with the conversion of glucose to pyruvic acid:



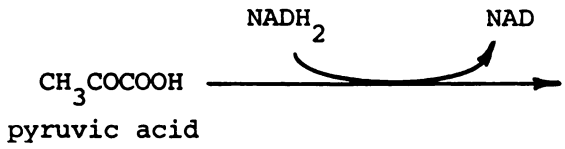
(1)

where $NADH_2$ represents the reduced form of the electron carrier, NAD.

Pyruvic acid is the pivotal compound in acid fermentation. When no external electron acceptors are present, pyruvic acid may undergo any of several alternative reactions to reoxidize $NADH_2$ to NAD. Representation of the most common end products which are derived from pyruvic acid was shown in Figure 3. Possible pathways of major end products will be reviewed as follows:

Formation of Lactic Acid

The production of lactic acid is the simplest way to reoxidize $NADH_2$ to NAD, since pyruvic acid itself is the electron acceptor.

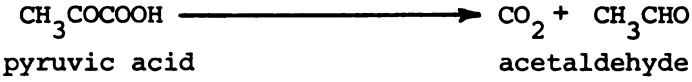


(2)

Decarboxylation of Pyruvic Acid

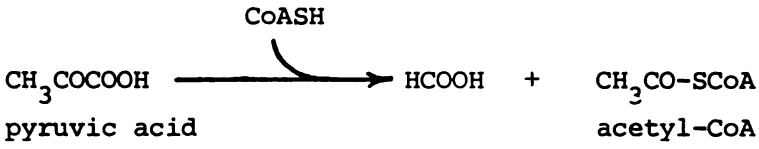
The formation of ethanol, acetic acid, and carbon dioxide results from the decarboxylation of pyruvic acid.

Pyruvic acid can be decarboxylated to form acetaldehyde and carbon dioxide.



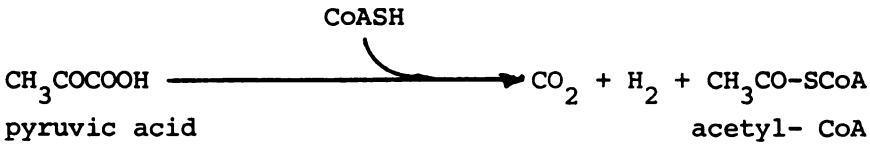
(3)

Alternatively, pyruvic acid can be decarboxylated to form formic acid and acetyl-CoA.



(4)

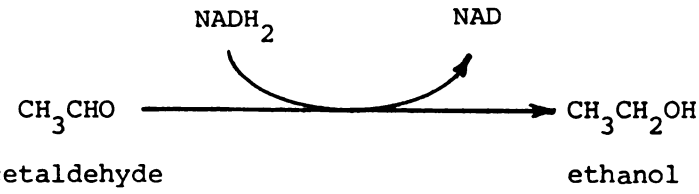
Finally, pyruvic acid can be decarboxylated to form carbon dioxide, hydrogen, and acetyl-CoA.



(5)

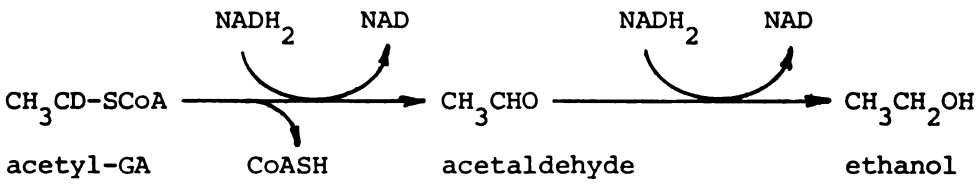
Formation of Ethanol

The acetaldehyde formed by decarboxylation of pyruvic acid (Fig. 3) is used as the electron acceptor for reoxidation of the $NADH_2$ resulting in ethanol formation.



(6)

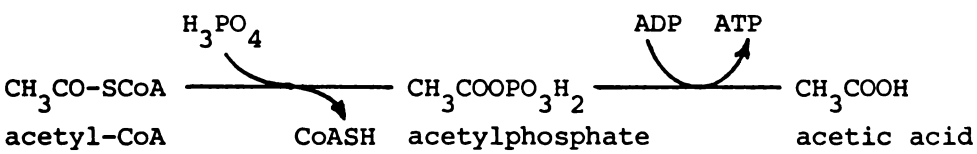
Also, ethanol can be formed by reduction of acetyl-CoA produced by decarboxylation of pyruvic acid (Eq. 4 and 5).



(7)

Formation of Acetic Acid

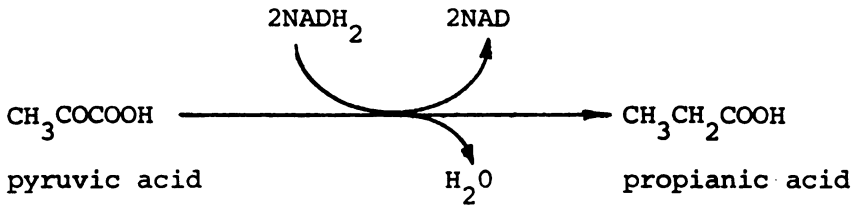
In glycolysis, each glucose molecule is converted to two molecules of pyruvic acid, with the reduction of two molecules of NAD. When both molecules of pyruvic acid are converted to acetyl-CoA through the reactions of Eq. 4 and/or Eq. 5, and one molecule of acetyl-CoA is reduced to form ethanol (Eq. 7), the other molecule is not needed as an electron acceptor. As a result, the organism can conserve the bond energy of acetylphosphate by synthesizing ATP with the production of acetic acid.



(8)

Formation of Propionic Acid

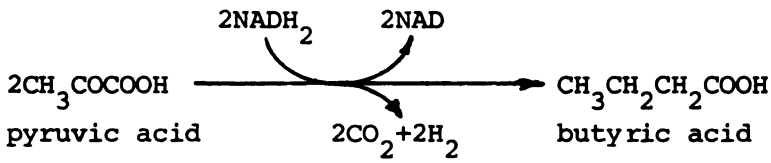
Propionic acid can be formed from pyruvic acid by a cyclic pathway. The overall reaction is:



(9)

Formation of Butyric Acid

Butyric acid can be formed from pyruvic acid through the following overall reaction.



(10)

The various reactions for the formation of end products from pyruvic acid can be combined with glycolysis, the conversion of glucose to pyruvic acid. These are shown in Table 1 along with the associated standard free energy changes.

3. Methane Fermentation

The volatile acids formed in the acid fermentation phase are converted mainly to methane and carbon dioxide which are major end products of complete anaerobic digestion. Methane gas is highly insoluble and escapes from the liquid medium. Here, the actual stabilization of organic matter occurs. This phase is usually referred to as methane fermentation. The bacteria responsible for the methane fermentation are strict anaerobes and collectively called methane formers or methanogens. The principal genera of methanogenes are the rods (Methanobacterium, Methanobacillus) and spheres (Methanococcus, Methanosarcina) (Higgins and Burns, 1975).

TABLE 1. Overall Glucose Fermentations Including Free Energy Changes.

Products	Fermentation Reaction	$\Delta G^{o'}$ kcal/mole
Ethanol	$C_6H_{12}O_6 = 2CH_3CH_2OH + 2CO_2$	-56.1
Lactate	$C_6H_{12}O_6 = 2CH_3CHOHCOO^- + 2H^+$	-47.2
Acetate	$C_6H_{12}O_6 = 3CH_3COO^- + 3H^+$	-76.4
Acetate + H_2	$C_6H_{12}O_6 + 2H_2O = 2CH_3COO^- + 4H_2 + 2CO_2 + 2H^+$	-53.0
Acetate + Propionate	$C_6H_{12}O_6 = 4/3\ CH_3CH_2COO^- + 2/3\ CH_3COO^- + 2/3\ CO_2 + 2/3\ H_2O + 2H^+$	-76.5
Acetate + Butyrate	$C_6H_{12}O_6 = CH_3CH_2CH_2COO^- + 1/2\ CH_3COO^- + CO_2 + H_2O + 3/2\ H^+$	-74.8
Butyrate + H_2	$C_6H_{12}O_6 = CH_3CH_2CH_2COO^- + 2H_2 + 2CO_2 + H^+$	-63.1
Acetate + Propionate + H_2	$C_6H_{12}O_6 = CH_3CH_2COO^- + CH_3COO^- + H_2 + CO_2 + 2H^+$	-70.6

Source: Eastman, 1977

There is a two-step process in methane formation.

1) The volatile acids, long chain fatty acids, and alcohols are converted to acetic acid and hydrogen.
2) Acetic acid and/or hydrogen plus carbon dioxide are converted to methane.

Step 1 can only occur if the hydrogen is quickly removed to very low level by hydrogen utilizing bacteria. Under the normal conditions of anaerobic digestion, the partial pressure of hydrogen is kept low enough to convert fatty acids and alcohols to acetic acid. Approximately 70 percent of the methane production derives from acetic acid and most of the remaining methane from hydrogen and carbon dioxide.

C. ENVIRONMENTAL FACTORS AFFECTING ANAEROBIC DIGESTION

The nutritional and physical environment in which microorganisms are contained greatly affects their growth and treatment efficiency in any biological treatment process. In order to obtain optimum treatment efficiency in the anaerobic digestion process, a proper environment for heterotrophic bacteria must be provided. This section summarizes nutritional requirements, temperature, and pH, which are probably the most significant environmental factors affecting anaerobic digestion.

1. Nutritional Requirements

The chemical composition of a typical bacterial cell provides useful information to estimate the nutritional requirements for

biological growth. The typical composition of organic and inorganic components of bacteria are shown in Table 2. Carbon and nitrogen make up about 60 percent of the dry weight of the cell. In general, the organic portion of the cell consists of carbon, nitrogen, oxygen, and hydrogen. The organic portion is commonly expressed as $C_5H_7O_2N$ and includes 90 percent of the dry weight (Metcalf & Eddy, 1979). The remaining portion of the dry weight of the cell is inorganic and includes a number of elements -- phosphorus, sulphur, potassium, magnesium, sodium, calcium, iron, copper, manganese, molybdenum, and others.

Of the four major elements composing the cell, only carbon and nitrogen must be externally provided. The hydrogen and oxygen of the cell are derived from water or from other compounds used by the cell. Also, all of the inorganic cell components can be obtained from inorganic salts by almost all microorganisms. Thus, the major differences in nutritional requirements of microorganisms are based on the sources of carbon and nitrogen used for cell synthesis.

Heterotrophic bacteria utilize organic compounds as the carbon source for cell synthesis. The energy needed for anabolism is also obtained from the organic carbon through oxidation or fermentation processes.

The organic compounds utilized as carbon sources commonly include carbohydrates, proteins, or lipids. Since carbon is the essential element in these organic compounds and comprises about 50 percent of the dry weight of the cell, the amount of carbon

TABLE 2. Typical Composition of Organic and Inorganic Components of Bacteria.

Organic (*) % Dry Weight			Inorganic (+) g/100g Dry Weight	
Component	Average	Range	Component	Range
Carbon	48	46 - 52	Phosphorus	2.0 - 3.0
Nitrogen	12.5	10 - 14	Sulphur	0.2 - 1.0
Protein	55	50 - 60	Potassium	1.0 - 4.5
Carbohydrate	9	6 - 15	Magnesium	0.1 - 0.5
Lipid	7	5 - 10	Sodium	0.5 - 1.0
Nucleic Acid	23**	15 - 25	Calcium	0.001 - 1.1
Ash	6	4 - 10	Iron	0.02 - 0.2
			Copper	0.01 - 0.02
			Manganese	0.001 - 0.01
			Molybdenum	-----
			Total Ash	7 - 12

** Values this high are observed only with rapidly growing cells.

Sources: * Peppler, 1967
+ Aiba, Humphrey, and Millis, 1973

present is the major factor determining the biomass which can grow in a given habitat.

The nitrogen source may be organic or inorganic. In most microorganisms, the major source of cellular nitrogen is inorganic. Nitrogen exists in a variety of inorganic forms, the most common being ammonia, nitrate, nitrite, and molecular nitrogen. Ammonia nitrogen is more readily utilized than is nitrate in anaerobic digestion. (Gasser and Jeris, 1969, Brezonik and Lee, 1966). The bacterial use of nitrogen gas will be discussed in Section D of this chapter.

2. TEMPERATURE

Microorganisms possess no system for controlling internal temperature and the cellular temperature is therefore determined by the external temperature. Each microorganism can grow only within a specific range of temperature. The temperature at which biological growth is most rapid is called the optimum temperature.

Based on the optimum temperature, microorganisms can be classified as psychrophilic, mesophilic, or thermophilic. Typical temperature ranges of each class are shown in Table 3.

TABLE 3. Typical Temperature Ranges for Various Microorganisms.

Group	Minimum	Optimum	Maximum
Thermophiles	40 to 45	55 to 75	60 to 80
Mesophiles	10 to 15	30 to 45	35 to 47
Psychrophiles			
Obligate	-5 to 5	15 to 18	19 to 22
Facultative	-5 to 5	25 to 30	30 to 35

Source: Stanier, 1970

3. pH

The hydrogen ion concentration, expressed as pH, influences the biological growth rate and can limit growth if it is outside the range an organism can tolerate. For most bacteria, the optimum pH is near neutrality with minimum and maximum pH values near 4 and 9, respectively. In anaerobic digestion, acid formers function best in a range of pH from 4.0 to 6.5 and methane formers in a range of pH from 7.0 to 7.8 (Graef and Andrews, 1974).

D. BIOCHEMISTRY AND MICROBIOLOGY OF NITROGEN FIXATION

Molecular nitrogen, Na, is the most abundant form of nitrogen in nature: it makes up approximately 79 percent of the atmosphere. Although nitrogen is one of the major constituents of the cell, the biological utilization of molecular nitrogen is limited to some specialized microorganisms which can reduce molecular nitrogen to ammonia. This is because only nitrogen at the ammonia (NH_3) or amino ($-NH_2$) level can be assimilated biologically into cellular material. The reduction of nitrogen from N_2 to NH_3 is referred to as nitrogen fixation. Microorganisms which can fix N_2 into NH_3 are called nitrogen fixers.

There has been intense interest in biological fixation of atmospheric nitrogen for fertilizer production in agriculture. Moreover, geneticists have attempted to increase the number of species that can fix atmospheric nitrogen by transferring the nitrogen-fixation genes from a nitrogen fixer to others. Although no references were found with regard to nitrogen fixation in anaerobic

digestion, it is easy to find papers presenting and reviewing nitrogen fixation from the agricultural and genetic viewpoints, e.g., Shanmugan (1978), Schneider (1976), or Streicher (1973). Referring to these papers, this section will discuss the general biochemistry and microbiology of nitrogen fixation.

1. COMMON FEATURES OF BIOLOGICAL NITROGEN FIXATION

The biological fixation of atmospheric nitrogen is an important part of the nitrogen cycle shown in Figure 6. Two general types of nitrogen fixers have been identified: one that can fix nitrogen in symbiotic association with plants, e.g., legumes such as soybeans, peas, and clover, and the other that can fix nitrogen asymbiotically. The latter include many blue-green algae and a few aerobic and anaerobic bacteria. Some nitrogen fixers are listed in Table 4.

The nitrogen fixation reaction accomplished by these microorganisms is an enzymatic, endothermic, reductive chemical reaction. The enzyme system responsible for N_2 reduction is called nitrogenase. All nitrogenases of nitrogen fixers isolated so far are composed of two types of proteins; one contains molybdenum, iron, and acid-labile sulfur, the other acid-labile sulfur and iron only (Streicher, 1973 and Evans, 1972). They are called most commonly Mo-Fe-protein and Fe-protein, respectively. These two types of proteins are dependent on each other in their catalytic activity.

Hardy (1968), Winter (1968), and other authors point out that the activity of nitrogenases is dependent upon the concentrations of both Mo-Fe-protein and Fe-protein and decreases with decreasing

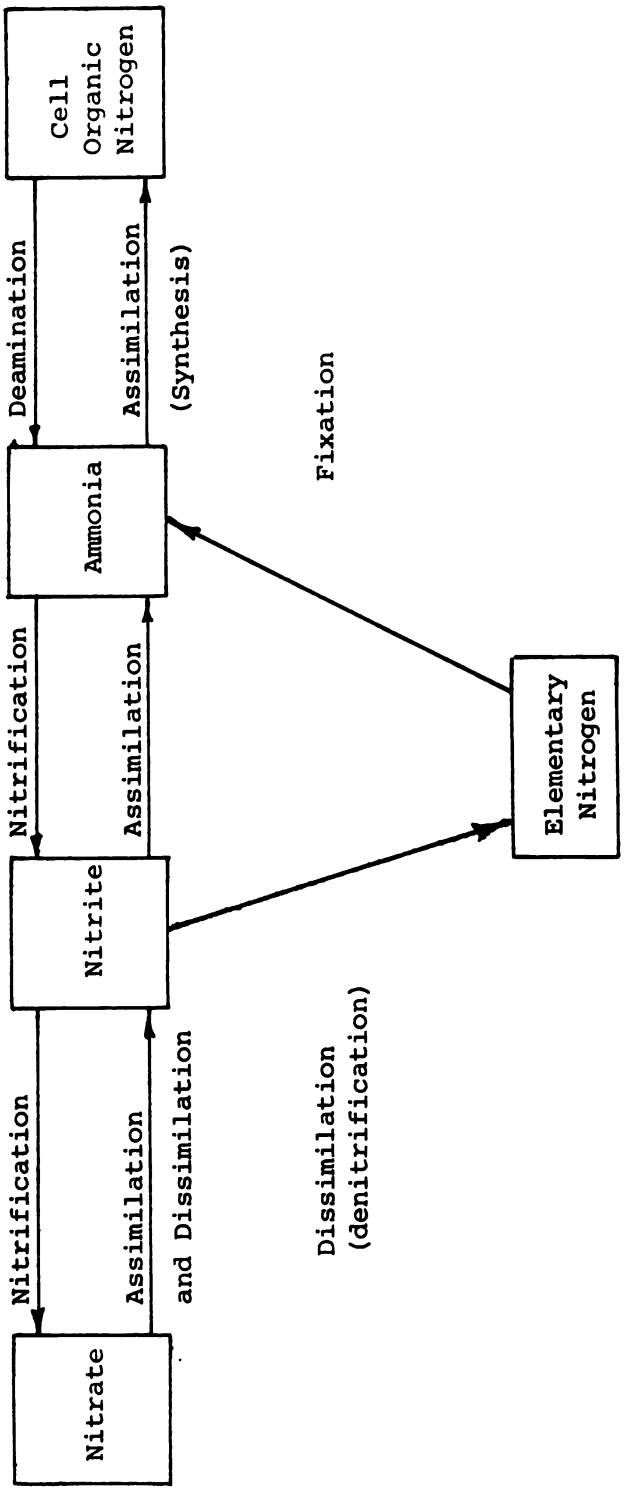


FIGURE 6. Major Biological Processes in the Nitrogen Cycle. (Source: Painter, 1970)

TABLE 4. Classification of Nitrogen Fixing Microorganisms.

System	Examples	Remarks
Asymbiotic		
Bacteria	*Clostridium pasteurianum*	Anaerobic
	Azotobacter vinelandii	Aerobic
	A. chroococcum	Aerobic
	Mycobacterium flavii	Aerobic
	Bacillus polymyxa	Facultative
	Klebsiella pneumoniae	Facultative
	Rhodospirillum rubrum	Photosynthetic
	Chromatium	Photosynthetic
Blue-green algae	*Anabena cylindrica*	Photosynthetic
	A. virabilis	Photosynthetic
	Nostoc commune	Photosynthetic
	N. muscorum	Photosynthetic
	Trichodesmium	Photosynthetic
	Calothrix	Photosynthetic
Symbiotic		
Root nodules of legumes	*Rhizobium*	
Root nodules of nonlegumes	Actinomycetes	(Probably)
Leaf nodules	*Klebsiella rubeacearum*	Free-living also

Source: Schneider, 1976

protein concentration. The formation of active nitrogenase from the two protein components is in dynamic equilibrium according to the following equation (Mortenson, 1972 and others):

X Mo-Fe protein + Y Fe protein $\rightleftharpoons$ Active nitrogenase

The ratio of X to Y differs with the activity of nitrogenase and with the parent organism. Maximal activity for N_2 reduction in Klebsiella pneumoniae preparations was observed at a 1:1 ratio; excess of the Mo-Fe-protein was inhibitory (Huang, 1973). Thus, the reaction of nitrogen fixation is catalyzed by a concerted action of both Mo-Fe-protein and Fe-protein.

The nitrogenase activity is affected by pH as well as protein concentration. The optimum pH values are close to neutrality but differ somewhat with the parent organism. The optimum pH of two anaerobic and facultative organisms are shown in Table 5.

In order to reduce N_2 to NH_3, nitrogenase requires a suitable reductant as electron donor as shown in Figure 7. The energy required for N_2 reduction will be obtained from the phosphate-bond energy of ATP. The reductant and ATP requirements will be discussed in the following paragraphs.

TABLE 5. Optimum pH for Nitrogenase Activity.

Organism	Optimum pH
Clostridium pasteurianum (anaerobic)	6.7 - 6.8
Klebsiella pneumoniae (facultative)	7.8

Source: Schneider, 1976

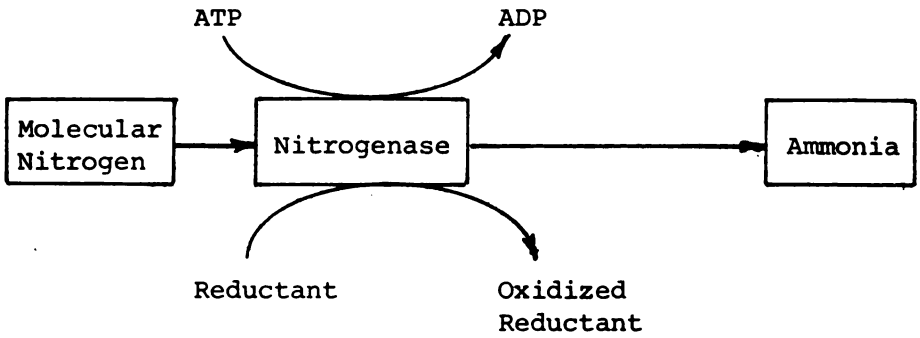


FIGURE 7. Scheme of Nitrogen Fixation.

2. REDUCTANT REQUIREMENT

The origin and nature of reductants used in N_2 fixation vary among anaerobic, aerobic, and photosynthetic organisms. For anaerobic bacteria, the most common reductant is pyruvate which is produced from fermentation of sugars (Gray, 1965 and Hardy, 1964). Electrons provided by pyruvate must be transferred to nitrogenase by electron carriers, such as ferredoxins and flavodoxins. Ferredoxins are iron- and sulfide-containing proteins which have no enzymatic function of their own. Flavodoxins are also proteins but contain no iron and sulfide. Pyruvate supplies, not only electrons, but ATP to nitrogenase through the phosphoroclastic reaction as shown in Figure 8.

Gaseous hydrogen, H_2, can also serve as a reductant for nitrogenase (Mortenson, 1968).

Gaseous hydrogen reduces ferredoxins or flavodoxins under the action of hydrogenase, which is classified as an iron-sulfur enzyme. This reduction reaction is reversible; excess reduced ferredoxin or flavodoxin is converted to molecular hydrogen. Electrons

of reduced ferredoxin or flavodoxin are then transferred to nitrogenase for N_2 reduction. The thermodynamic properties of H_2 as a reductant will be discussed in a later paragraph.

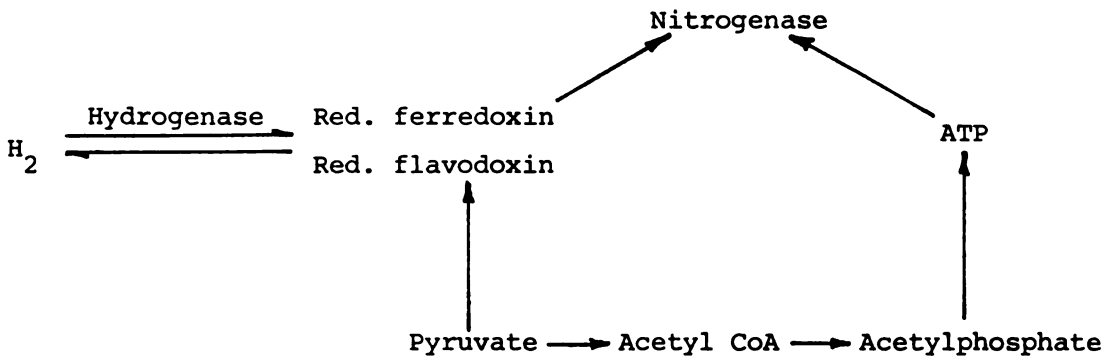


FIGURE 8. Transfer System of Electron and ATP to Nitrogenase.

3. ATP REQUIREMENT

Electron transfer from reductant to substrate is an endothermic reaction and strongly dependent on the hydrolysis of ATP, the energy releasing process which is termed specifically reductant-dependent, ATP hydrolysis to distinguish it from the ATP hydrolysis which is not reductant-dependent.

There are two pathways for ATP hydrolysis (Hadfield, 1969 and Silverstein, 1970): substrate reduction (nitrogen reduction) and H_2 evolution as shown in Figure 9.

The reduction of protons of water to H_2 is termed ATP-dependent H_2 evolution, which is catalyzed by nitrogenase, while the H_2 evolution in Figure 8 is catalyzed by hydrogenase. It seems that ATP-dependent H_2 evolution is thermodynamically an important factor contributing to the high energy requirement for N_2 fixation. Although

it is difficult to measure H_2 evolution mediated by nitrogenase *in vivo* because of the presence of the hydrogenase-catalyzed H_2 evolution, Hill (1976) estimated that 45 percent of the total energy flow through nitrogenase probably resulted in H_2 evolution in *Klebsiella pneumoniae*.

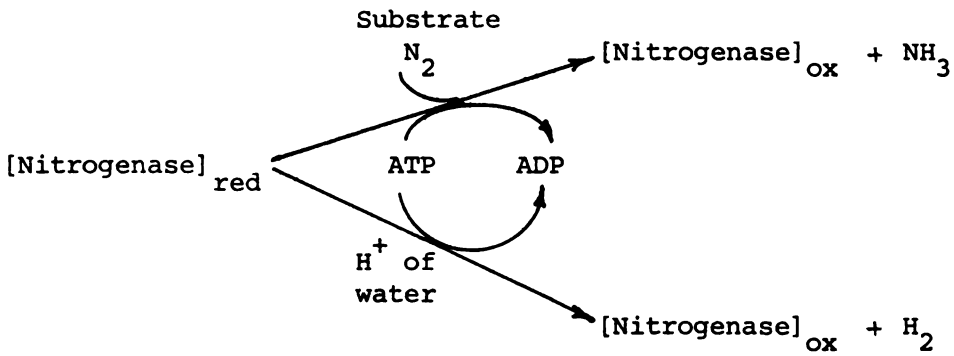


FIGURE 9. A Dual Pathway of ATP Hydrolysis in Nitrogenase-Catalyzed Reaction.

The distribution of electrons between ATP-dependant H_2 evolution and N_2 reduction is altered by controlling the partial pressure of N_2 over the reaction mixture (Hadfield and Bulen, 1969). Table 6 shows a wide electron distribution between H_2 evolution and N_2 reduction but a constant $2e^-$ total. Although the largest decrease of H_2 evolution occurs between 0 and 25 percent N_2 in Ar, the H_2 evolution continues to proceed even at 100 percent N_2.

4. INHIBITORS

Inhibitors of N_2 fixation may be grouped into two major types: one that is reduced by nitrogenase, the other not reduced.

The nitrogenase-reducible inhibitors include azide, N_2O, acetylene, cyanide, nitrites, and isocy anides. They may compete

TABLE 6. ATP Utilization as a Function of Electron Distribution Between H_2 Evolution and N_2 Reduction.

N_2 in Ar(%)	H_2 Evolution (μmoles)	N_2 Reduction (μmoles)	H_2:N_2	$2e^-$ (μmoles)	Pi (μmoles)	ATP:$2e^-$
100	3.50	2.40	1.46	10.7	52.9	5.0 ± 0.1
75	3.64	2.22	1.64	10.3	51.2	5.0 ± 0.1
50	4.23	2.09	2.03	10.5	52.3	5.0 ± 0.2
25	4.85	1.76	2.76	10.2	49.7	4.9 ± 0.3
0	9.47			9.5	47.5	5.0 ± 0.2

Source: Hadfield and Balen, 1969

with N_2 for positions at the active site and for electrons provided by ATP hydrolysis, but nitrogenase exhibits a higher affinity for N_2 than for any of the other reducible substrates (Schneider, 1976).

The second type of inhibitor can be further divided into two groups: one that inhibits by occupying the substrate binding site, the other that destroys nitrogenase, partially or totally. The former group includes CO, H_2, and NO; the latter, O_2, Cu^{2+}, and $8n^{2+}$.

III. EXPERIMENTAL PROCEDURES

The purpose of this chapter is to describe the laboratory apparatus, experimental program, analytical techniques and conversion factors necessary to investigate nitrogen fixation in anaerobic digestion. Also included is a description of the culture media and method of pH control

A. DESCRIPTION OF APPARATUS

A bench-scale, completely mixed anaerobic digestor assembly was used for laboratory investigations. The assembly, shown in Figure 10, consisting of four batch-fed reactors, was similar to that of Eastman (1977). The main features of this experimental apparatus are: (a) continuous introduction of N_2 gas; (b) complete mixing of the batch-reactor contents by paddle stirrers; (c) gas collection using inverted graduated cylinders; and (d) maintenance of a constant temperature by a circulating water bath. The above features of the experimental apparatus will be described in detail in the following paragraphs.

Nitrogen gas was continuously introduced into three of four reactors using syringe needles inserted through a serum cap near the bottom of the reactor. The flow rate was controlled by needle valves. The N_2 gas flow rate was measured with a soap bubble flow meter prior to operation of the digester assembly and monitored by the number of gas bubbles rising in a glass tube partly filled with water as shown in Figure 11. The syringe needle was used to make

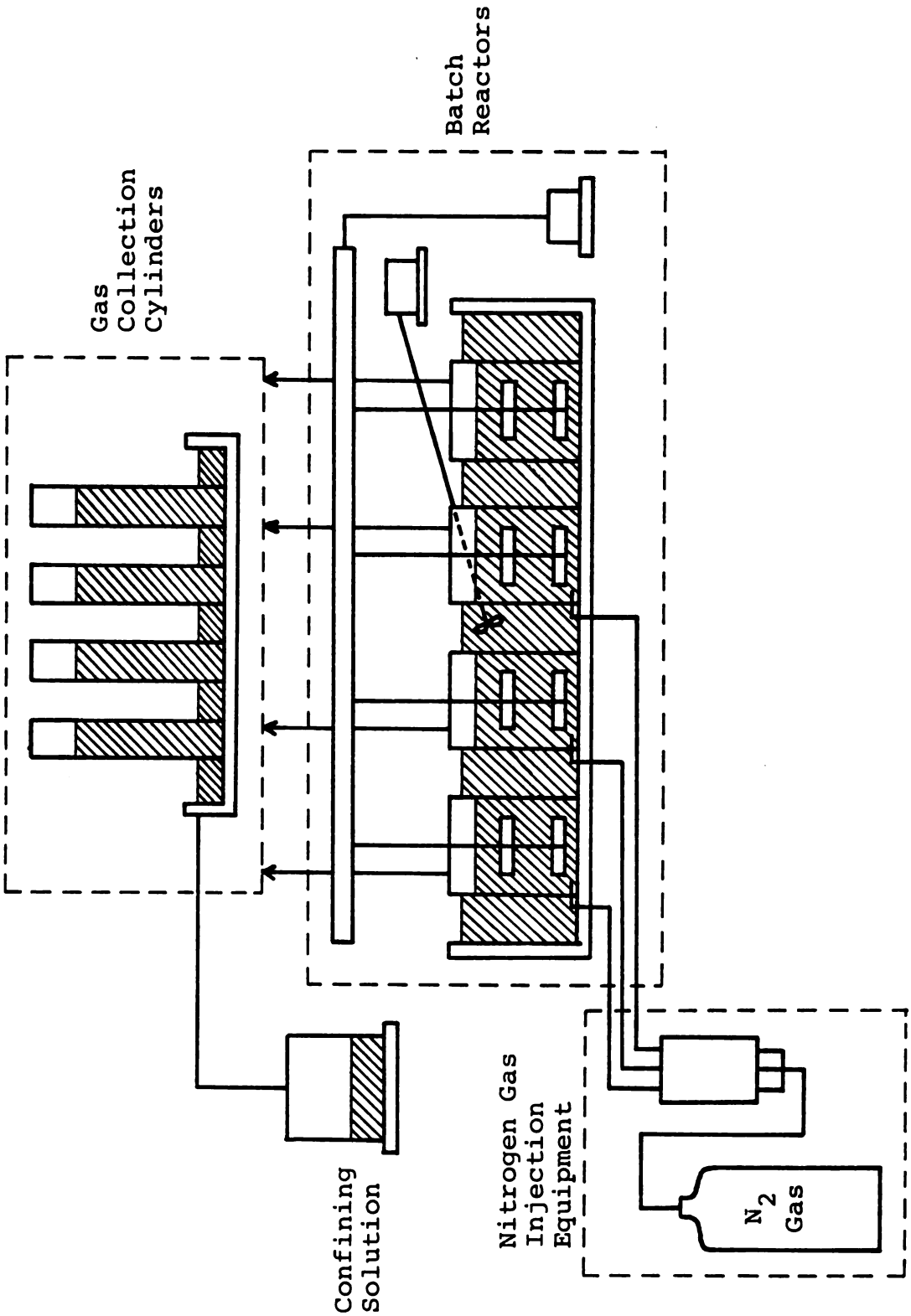


FIGURE 10. Schematic Diagram of Laboratory Digester Assembly.

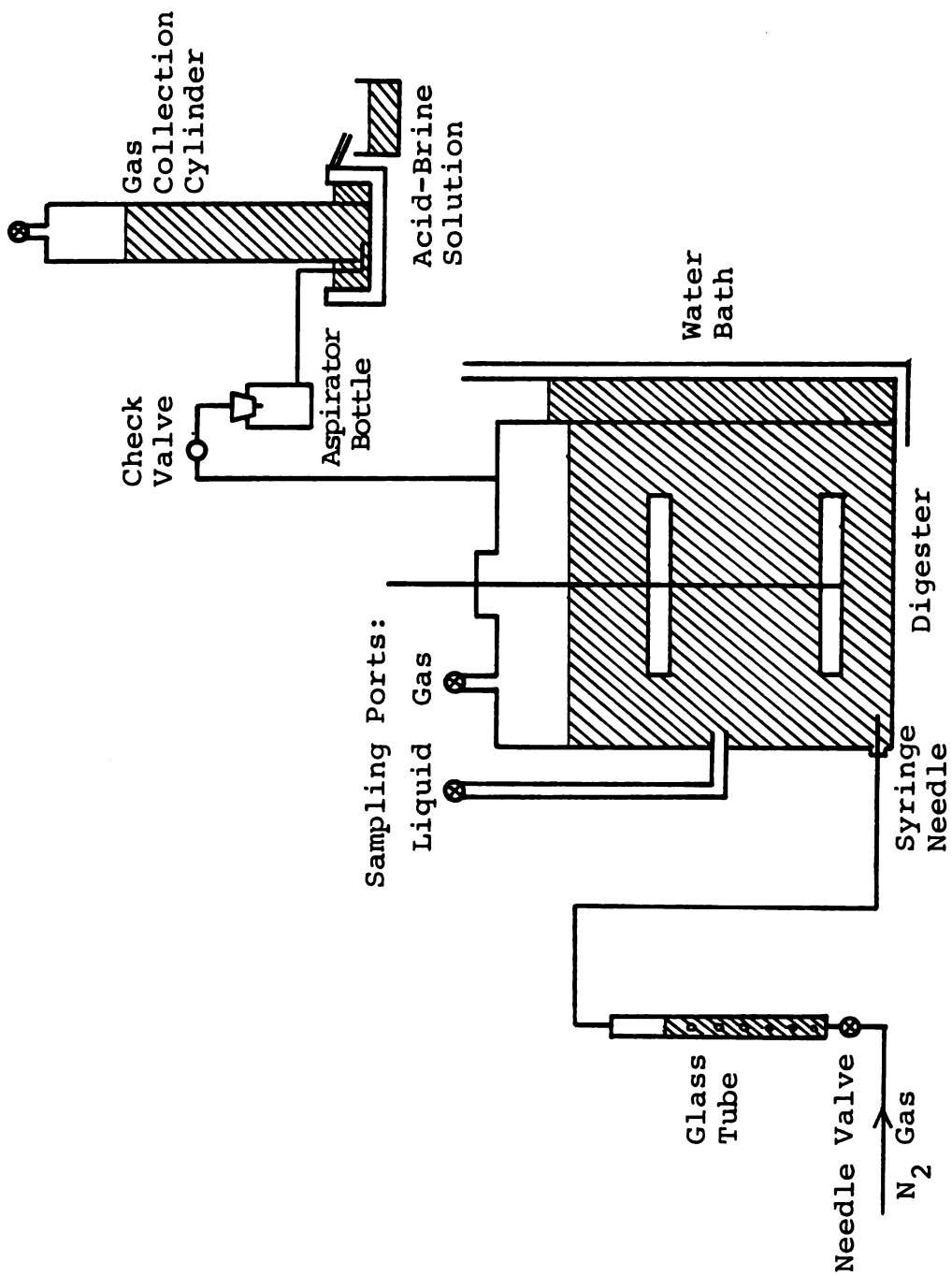


Figure 11. Individual Laboratory Digester.

the gas bubbles in the liquid of the reactor as small as possible, in order to obtain a larger surface area of bubbles.

Each digester, as schematically shown in Figure 11, consisted of a six-inch diameter, plexiglas cylinder having a liquid volume of 2.50 liters and a gas space of about 0.5 liter. A stirrer with two flat paddles was used for complete mixing of the digestor contents. The stirrers of all four digesters were driven with belts from a single variable speed motor (Model 565 with Model 903 Controller, Bodine Electric Co., Chicago, Illinois). The stirring shaft passed through an O-ring seal to prevent gas from leaking. Nitrogen gas, introduced from the gas cylinder, and other gases produced by anaerobic digestion were collected in an inverted, polypropylene graduated cylinder for measurement of gas production volume. The gas collection cylinders were submerged about two inches into the acid brine solution which served as a retaining fluid. The acid brine solution contained 10% NaCl and 2% H_2SO_4. When necessary, the gas collection cylinder was refilled with acid brine solution by removing gas from the top with a handpump. Since the pressure within the digester is reduced when fluid samples are withdrawn, a 250 ml aspirator bottle and a check valve were used to prevent reverse flow of acid brine solution into the digester. The aspirator bottle and the check valve were installed between the digester and the gas collection cylinder as shown in Figure 11. The pressure in the digester gas space was maintained at about 1.5 to 2 inches of water above atmospheric pressure by submerging the outlet of the gas line to the gas collection cylinder below the free surface

of the acid brine solution.

The temperature of the four digesters was maintained at about 35°C in a circulating water bath. A thermostat of the expanding gas type was used to control the temperature of the water bath. When necessary, auxiliary water was added to prevent the water level of the bath from evaporating below the fluid level of the digesters.

Liquid samples were withdrawn using a 50 ml plastic syringe with an enlarged opening. The liquid sampling port was located at mid-depth and extended one inch into the reactor. Gas samples were withdrawn from the digester head space by inserting a gas-tight syringe through a serum stopper which capped a tube through the top of the reactor.

B. EXPERIMENTAL PROGRAM

In this section, the overall experimental program is described. The program used bench-scale experiments to demonstrate and evaluate biological nitrogen fixation in the anaerobic digestion process. Completely mixed, batch-fed reactors were operated with two types of nitrogen source, N_2 gas and NH_4Cl, and three different substrates, glucose, cellulose, and acetate. Since nitrogen is one of the essential nutrients for cell growth, nitrogen fixation must occur during the acid fermentation phase if it is to take place at all. One purpose of this study is to investigate the assimilation of the fixed nitrogen to bacterial cell as well as nitrogen fixation. Because of this, the experiments were continued until acid fermentation appeared complete according to gas production data. There was never a

subsequent rise in gas production indicative of methane formation from volatile acids. The laboratory analyses performed are pH, total and volatile suspended solids, ammonia and organic nitrogen, carbohydrate, gas composition, individual volatile acids, and alcohols.

1. SUBSTRATES AND NUTRIENTS

In order to control the biochemical conditions required for nitrogen fixation and acid fermentation in the anaerobic digestion, four batch-fed reactors were operated with surrogate wastes which will be described in this section. Although this study ultimately aims at anaerobic treatment of industrial wastewater, surrogate wastes were used in this experiment so that the nitrogen source could be easily controlled and other nutrients required for biological growth be satisfied without doubt. As mentioned in Chapter II, heterotrophic bacteria require an oxidizable substrate as a source of carbon and energy and such nutrients as nitrogen, phosphorus, sulphur, and potassium. Also required are the micronutrients: magnesium, sodium, calcium, iron, copper, manganese, and molydenum. Once the elemental requirements have been calculated, choices still remain of the chemical compounds which are used to supply the required elements. In the choice of chemical compounds, much attention should be paid that no nitrogen is contained in these chemicals except for the nitrogen source.

Three different substrates, i.e., glucose, cellulose, and acetate, were used to evaluate the effects of carbon source on nitrogen

fixation and assimilation. Glucose and cellulose were chosen as representatives of short and long chain carbohydrates, respectively. Acetate was used as fatty acid material. Organic nitrogenous material should not be used because it would also serve as a nitrogen source. The initial substrate concentration of the surrogate wastes was 5,000 mg/l as COD, representative of moderate strength industrial wastes. Substrate concentrations are summarized in Table 7.

Preparation of the cellulose and acetate substrate required special attention. Initially, a finely milled cellulose was used but it was not biologically degraded during a primary experiment. Then Whatman filter papers were used for the cellulose substrates after being thoroughly blended in a Warning Blender. In order to obtain an initial pH of 7.0 in the acetate reactor, a combination of acetic acid and sodium acetate was used as the substrate.

Nutritional requirements for biological growth are dependent on the amount of biomass present in the reactor.

TABLE 7. Substrate Concentration in Batch-fed Digesters.

Reactor	Substrate		
	Source	mg/l*	mg/l as COD
I	Glucose	4686	5000
II	Cellulose	4219	5000
III	Acetate	4686	5000
IV	Glucose	4686	5000

* See the section of Conversion Factors and Sample Calculations, p.

The biomass can be estimated by assuming a yield coefficient and mass of substrate utilized. Once the biomass is determined, the required amount of each nutrient can be calculated from the typical composition of inorganic components of microorganisms shown in Table 2. Assuming 0.20 and 0.05 as yield coefficients for carbohydrate and acetate, respectively, a 1,000 mg/l biomass was estimated for the carbohydrate substrate and a 250 mg/l biomass for the acetate substrate. Actual amounts of chemicals added in this experiment, however, were estimated by 1.5 times the calculated biomass as a safety factor. The same amount of chemicals were added to both the acetate and carbohydrate reactors. Table 8 shows the nutrient requirements and chemicals added.

2. NITROGEN SOURCES

Two nitrogen sources, N_2 gas and NH_4Cl, were used to evaluate the effect of nitrogen source on biological growth.

Like other nutrient requirements previously mentioned, the required amount of NH_4Cl was estimated from biomass present and the nitrogen composition of microorganisms. The calculated amount of NH_4Cl is shown in Table 8.

The required amount of N_2 gas, however, cannot be easily estimated in the same way as that of NH_4Cl. Because of this, initial experiments have been performed to determine the injection rate of N_2 gas. The nitrogen gas flow rate was set at about 15 ml/hr. This rate supplied enough N_2 gas for biological requirements and reduced the need for frequent gas withdrawal from the gas

TABLE 8. Nutrient Requirements

Nutrients	Composition %	Requirements (mg/l)	Chemicals	Chemical Requirements (mg/l)
P	3	45	K_2HPO_4	252.6+
S	1	15	$MgSO_4$	56.3
K	2	30	K_2HPO_4	66.9+
Mg	0.5	100*	$MgCl_2 \cdot 6H_2O$	750.6
Na	1	15	Na_2HPO_4	46.4+
Ca	1	150*	$CaCl_2 \cdot 2H_2O$	551.3
Fe	0.2	3	$FeCl_3 \cdot 6H_2O$	14.5
Co	0.02	0.3	$CoCl_2 \cdot 6H_2O$	1.2
Mn	0.01	0.2	$MnSO_4 \cdot H_2O$	0.5
Mo	0.02	0.3	$Na_2MoO_4 \cdot 2H_{20}$	0.8
N	12.5	187.5	NH_4Cl	716.6

* These cations were determined to provide antagonistic control of potential Na and K cation toxicity (see Table 10).

+ These nutrient requirements were satisfied through the buffer system.

collection cylinders.

3. pH CONTROL AND CATION TOXICITY

One of the most important variables to control the anaerobic digestion process is pH. The optimum pH for most bacterial growth is close to neutrality. Table 9 shows optimum pH ranges for bacteria concerned with anaerobic treatment.

TABLE 9. Optimum pH Range for Various Bacteria.

Bacteria	Optimum pH Range	Reference
Nitrogen Fixer	6.7 - 7.8	Schneider, 1976
Acid Former	4.0 - 6.5	Graef and Andrews, 1974
Methane Former	7.0 - 7.8	Graef and Andrews, 1974

The general chemical system controlling pH in anaerobic treatment is the carbon dioxide-bicarbonate buffer system. Lime and bicarbonate buffers are widely used for controlling pH. However, the carbon dioxide-bicarbonate buffer system will yield carbon dioxide if the pH drops. This is undesirable for this experiment because the carbon dioxide production from substrate degradation cannot be determined with accuracy. To avoid the production of carbon dioxide from the buffer system, salts of phosphoric acid were used for pH control in this study. Since the pK value for the second ionization of phosphoric acid is very near 7, salts of phosphoric acid are quite useful substances to buffer solutions near a neutral pH. A mixture of the dibasic potassium salt (K_2HPO_6) and

sodium salt (Na_2HPO_4) were used as buffers to help control cation toxicity.

Cation toxicity must be taken into account in determining buffer requirements. The relative effects of some common cations on the rate of methane fermentation, as summarized by McCarty (1964), are given in Table 10. High concentrations of cations are frequently the cause of inefficiency in anaerobic treatment. However, combinations of these cations show more complex effects as some of the cations act antagonistically, reducing the toxicity of other cations, while others act synergistically, increasing the toxicity of the other cations. Sodium and potassium are good antagonists if present at stimulatory concentrations.

TABLE 10. Effects of Cations on Methane Fermentation.

Concentrations in mg/l

Cation	Stimulatory	Moderately Inhibitory	Strongly Inhibitory
Sodium	100 - 200	3500 - 5500	8000
Potassium	200 - 400	2500 - 4500	12000
Calcium	100 - 200	2500 - 4500	8000
Magnesium	75 - 150	1000 - 1500	3000

Source: McCarty, 1964

Based on the ranges of moderately inhibitory concentrations shown in Table 10, a combination of potassium phosphate and sodium phosphate were used as buffers with a cation ratio of $1K^+$ to $2Na^+$. Buffer concentrations used were 4.299 mg/l K_2HPO_4, 7.017 mg/l Na_2HPO_4 and 345 mg/l KH_2PO_4. These buffer concentrations were

used for all reactors. Calculations of buffer requirements are described in the Appendix.

4. BATCH TEST PROGRAM

Four batch-fed anaerobic digesters were operated for the purpose of this study. All the experiments in this study were conducted with completely mixed reactors. The temperature of every reactor was maintained at 35°C, optimum temperature for mesophiles, in a circulating water bath. Phosphate buffers were added to surrogate wastes for maintenance of pH between 8.0 and 6.5. Operational conditions of the four batch reactors are summarized in Table 11.

TABLE 11. Operational Conditions of Batch-fed Reactors.

Reactor	Substrate	Nitrogen Source	Initial pH	Temperature °C
I	Glucose	N_2 gas	8.1	35
II	Cellulose	N_2 gas	8.1	35
III	Acetate	N_2 gas	7.0	35
IV	Glucose	NH_4Cl	7.9	35

The reactors were seeded with supernatant from digested municipal wastewater sludge which had been centrifuged at 1,000 rpm for 2 minutes. The digested sludge was obtained from the primary digester at the Mason Wastewater Treatment Plant, Mason, Michigan. Since the main purpose of this study is to investigate the biological growth in surrogate wastes whose only nitrogen source is N_2 gas, a very small amount of seed, 2 ml supernatant per 2ℓ surrogate waste, was added into each reactor. The amount of organic nitrogen

contained in the seed is negligible and hence the organic nitrogen measured in this experiment can be considered as fixed and assimilated nitrogen from N_2 gas. trial experiment confirmed that 2 ml of superatant of centrifuged digester sludge contained enough bacteria to grow under the given conditions.

The procedure of the laboratory start-up was as follows. The media in the digesters were allowed to warm in the water bath adjusted to 35° C in advance. To provide reducing conditions, 20 ml of 8 g/l Na_2S was added to each reactor. The media contained no substrates or seed at this step. The N_2 gas flow rate was adjusted to about 15 ml/hr. for Reactors I, II, and III. All units were purged with nitrogen gas to exclude oxygen and then substrates and seeds were added to the digesters.

The first samples were taken immediately after adding substrates and seeds to measure initial pH values. According to trial experiments, the decomposition of glucose was completed very rapidly after 15 hours in Reactor IV and after 30 hours in Reactor I. Cellulose and acetate were not decomposed within a 4-day running period of trial experiments. Therefore liquid samples were taken every 8 hours until the second day and once a day thereafter. The liquid samples were analyzed for pH, solids contents, nitrogen, carbohydrates, and volatile acids. Gas production was frequently measured over the experimental period. The analysis of gas composition was performed twice a day after 30 hours. Sampling was discontinued after 14 days due to lack of additional gas production.

C. ANALYTICAL TECHNIQUES

This section will detail the analytical techniques used for determining the parameters of interest in this study.

1. pH

The value of pH was measured using a portable battery-operated pH meter equipped with a combination electrode (Beckman Chem-Mate, Beckman Instrument, Inc.). A standard phosphate buffer (pH 7.00 at 25°C) was used for calibrating the electrode and meter; a potassium acid phthalate buffer (pH 4.01 at 25 C) used for verifying the correct response of the electrode. The measurement procedure was based on Standard Methods, 14th Edition. Since ± 0.1 pH unit represents the limit of accuracy under normal conditions, pH values were reported to the nearest 0.1 pH unit.

2. TOTAL AND VOLATILE SUSPENDED SOLIDS

The determination of total suspended solids was basically in accordance with Procedure 208 D, Total Suspended Matter, described in Standard Methods except for the filtering method of the samples. Samples were filtered through a 0.45 micron membrane filter (Millipore Type HA) because the filtrate passed through a glass fiber filter still contained some amount of colloidal material. The filtrate passed through a membrane filter, was acidified with concentrated H_2SO_4 to below pH2 and stored in a capped glass tube in a refrigerator for subsequent analysis of volatile acids.

The volatile components in the dried solids from the above

analysis were determined by igniting the sample in an electric muffle furnace at about 550°C for 15 minutes (Standard Methods, 14th Edition). The aluminum weighing dish was capped by the same type of dish in order to avoid the scattering of samples due to the violent ignition of a membrane filter in the furnace.

3. AMMONIA AND ORGANIC NITROGEN

Measurements of ammonia and organic nitrogen were based on Standard Methods, 14th Edition. First, ammonia nitrogen was determined by the distillation-acidimetric method. Then, organic nitrogen was found by the Kjeldahl method using the residue in the distilling flask. The final ammonia in the Kjeldahl method was determined by titration as in the distillation-acidimetric method for ammonia nitrogen.

Samples whose contents of ammonia and organic nitrogen were known were analyzed before measuring the samples. The recovery was more than 98% in both ammonia and organic nitrogen. This recovery rate is high enough for the purpose of this study.

4. CARBOHYDRATES

The carbohydrate content was measured using the phenol-sulfuric acid method. The following paragraphs are a summary of the phenol-sulfuric acid method described by Eastman (1977).

Strong sulfuric acid hydrolyzes polysaccharides to their constituent sugars and dehydrates the sugars to form furfural and hydroxymethyl furfural. These form colored complexes with phenol.

Since the carbohydrates of concern in this study are glucose and cellulose, their concentrations can be estimated by standardization with glucose using a spectrophotometer. The measurement procedure is outlined below.

1) Pipet a 2.0 ml sample containing 0-50 mg glucose/liter into a test tube.
2) Add 1.0 ml 5% Phenol solution. CAUTION: Do not mouth pipet.
3) Using an automatic pipet, add 5.0 ml concentrated sulfuric acid. Add quickly to cause mixing.
4) Immediately, mix thoroughly on a vortex mixer. Wear insulated gloves as the solution becomes very hot.
5) Allow to cool to room temperature.
6) Read absorbance at 485 nm against distilled water.
7) Correct absorbance for reagent blank.
8) Find concentration from a standard curve prepared from analyzing glucose standards.

The calibration curve was linear up to an abosorbance of 1.4 (about 100 mg/l as glucose), but great care was required above an absorbance of about 0.7. Thus, samples were diluted to fall within the range of 0 to 50 mg glucose/liter.

5. GAS COMPOSITION

The composition of the fermentation gases was determined using a gas chromatograph (Model 3700, Varian) equipped with a integrator (CDS 111, Varian) and a strip chart recorder (Model 9176, Varian).

A 12 foot copper column, 1/8" O.D., was packed with 80/100 mesh Porapak Q (Waters Assoc., Milford, Mass.) and connected to a thermal conductivity detector. One milliliter samples were drawn from the digester head space with a one milliliter gas-tight syringe (#1001 N, Hamilton Co.) and suitable volumes of the samples were immediately injected into the gas chromatograph. The standardization of peak area responses was accomplished by injecting various volumes of the pure gases and a commercial calibration mixture.

The temperatures of the injection port and the detector were maintained at 120°C, while the column was operated at 50°C for hydrogen, nitrogen, methane, and carbon dioxide and at 80°C for water vapor, using a temperature program. Since the retention time required for the gas detection is dependent upon the column temperature, the temperature program allowed shortening of the long retention time of water vapor. The detector must be operated at a higher temperature than the column in order to prevent condensation of moisture.

Helium (30 ml/min.) was used as the carrier gas for the determination of all gases. Hydrogen would be expected to give a negative response because of its thermal conductivity being slightly higher than that of helium. However, it does not necessarily give a negative peak. At low concentrations of hydrogen in helium, up to about 3% to 6%, its response is positive. Therefore, the sample volume to be injected was chosen to make the concentration of hydrogen low enough to obtain a positive response.

The gas composition results were normalized to exclude nitrogen because of the nitrogen derived from purging the unit with this gas at the beginning of the experimental run in Reactor IV and from injecting this gas as the nitrogen source in addition to purging in Reactors I, II, and III.

6. INDIVIDUAL VOLATILE ACIDS

The individual volatile acids from acid fermentation were analyzed gas-chromatographically. The volatile acids analyzed here were acetic, propionic, butyric, iso-butyric, and iso-valeric acids.

A 6 foot stainless steel column, 1/8" O.D., was packed with 80/100 mesh Chromosorb 101 (Johns-Manville, Denver, Colorado) and connected to a flame ionization detector. Before packing the column, the column was washed using 50% H_3PO_4 solution, and the Chromosorb 101 was acidified using 10% H_3PO_4 in order to prevent a reaction with the volatile acids. Both were rinsed with distilled water after washing or acidifying. The column oven was maintained isothermally at 195°C. The injection port and the detector temperatures were 210°C. The injection port was lined with a glass insert which was changed periodically to prevent buildup of organic char.

Nitrogen (40 ml/min.) was used as the carrier gas to which formic acid vapor was added to prevent ghosting and peak tailing. The formic acid vapor was added by bubbling carrier gas through liquid formic acid contained in a 5 ml glass chamber. The column was conditioned overnight after injecting formic acid into the

glass chamber through a septum. Hydrogen and air flows to the flame ionization detector were adjusted to obtain maximum sensitivity. Samples for volatile acid analysis were filtered by a Millipore membrane filter and acidified before measurement. One microliter samples were injected using a 10 1 syringe (#701 N, Hamilton Co. Reno, Nevada). The C_2 to C_5 acids were eluted within 4 minutes, showing sharp peaks.

For the standardization of peak area responses, standards were prepared from a stock mixture whose fraction of five volatile acids was known. The fraction of the volatile acids was determined reviewing the reference (Eastman 1977), i.e., HAc: HP: iHB: HB: iHV≃40:40:5:10:5 by weight. The purity of each standard acid was determined by titration of weighed aliquots with standardized base. The results are given in Table 12.

TABLE 12. Purity of Volatile Acid Reagents.

Acid	Concentration (g/100 ml) by Titration	Concentration (g/100 ml) by Weight	Purity %
Acetic	1.4758	1.5048	98.1
Propionic	1.4920	1.5043	99.2
i-Butyric	1.9370	1.9969	97.0
Butyric	1.9492	1.9914	97.9
i-Valeric	2.4533	2.5031	98.0

The peak area response of each standard volatile acid was linear over the entire range of the sample concentrations and also proportional to the number of methyl groups as shown in Figures 12

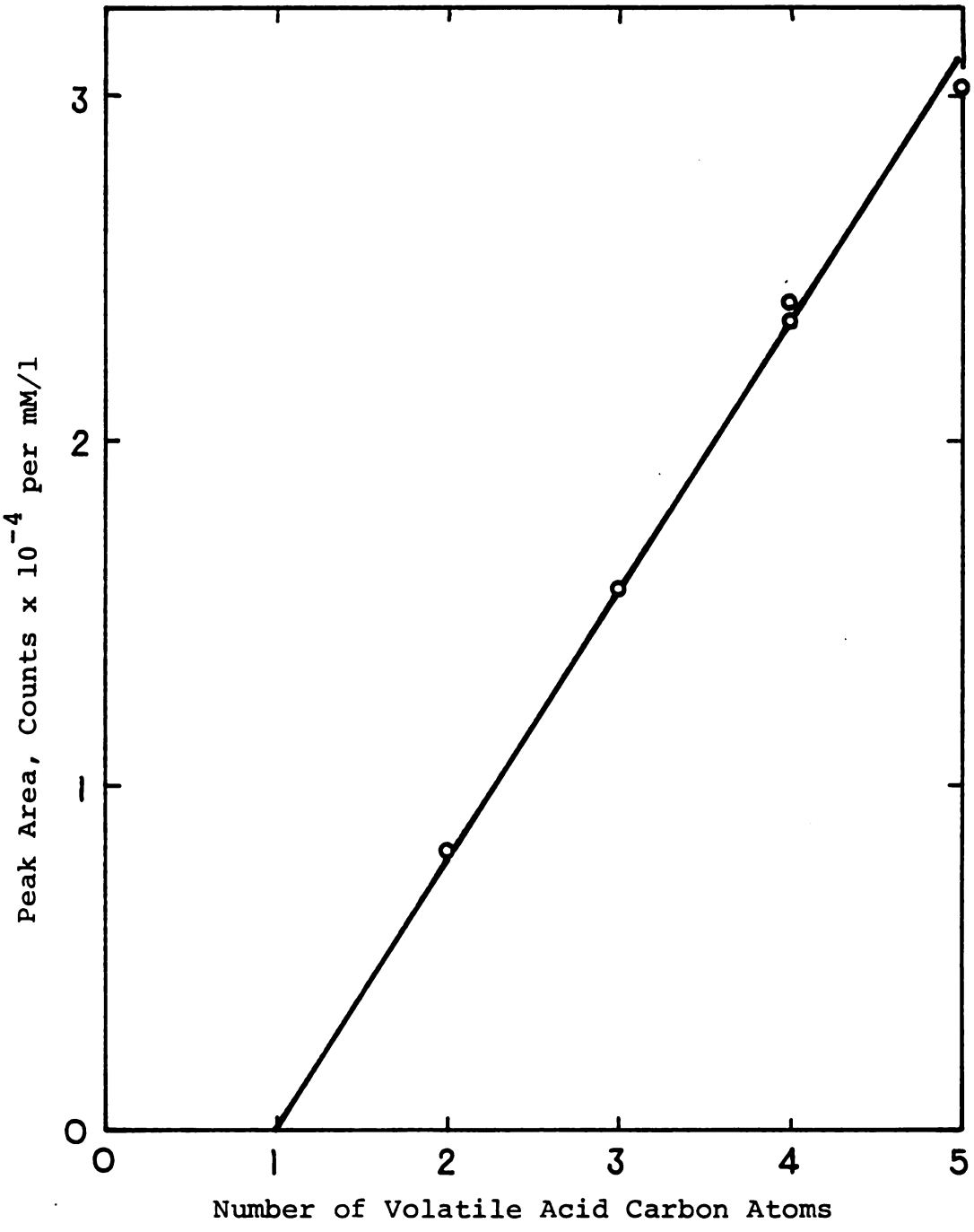


FIGURE 12. Response of the Flame Ionization Detector to the Number of Volatile Acid Carbon Atoms.

and 13. These results correspond to those of Eastman (1977).

7. ALCOHOL

Alcohol can be analyzed gas-chromatographically following the same experimental procedure as that of the individual volatile acids. Ethanol was measured in this study. The response of the standard ethanol was also linear over the entire concentration range of the samples.

D. CONVERSION FACTORS

The experimental results obtained in this study were expressed on an equivalent chemical oxygen demand (COD) basis to facilitate their comparison. In order to convert the analytical results to an equivalent COD, conversion factors were determined on the basis of the theoretical COD of the material in question. Calculation of such conversion factors is shown in this section for carbohydrates, volatile acids, ethanol, and gases.

CARBOHYDRATES

The conversion factor of glucose to equivalent COD is:

$$C_6H_{12}O_6 + 6O_2 = 6\ CO_2 + 6\ H_2O$$

$$F.W. = 180,\quad O_2 = 192$$

Therefore, COD = 192/180 = 1.067 g/g glucose. In the case of cellulose, the conversion factor is:

$$(C_6H_{10}O_5)x + x6O_2 = x6CO_2 + x5H_2O$$

$$F.W. = 162x,\ O_2 = 192x$$

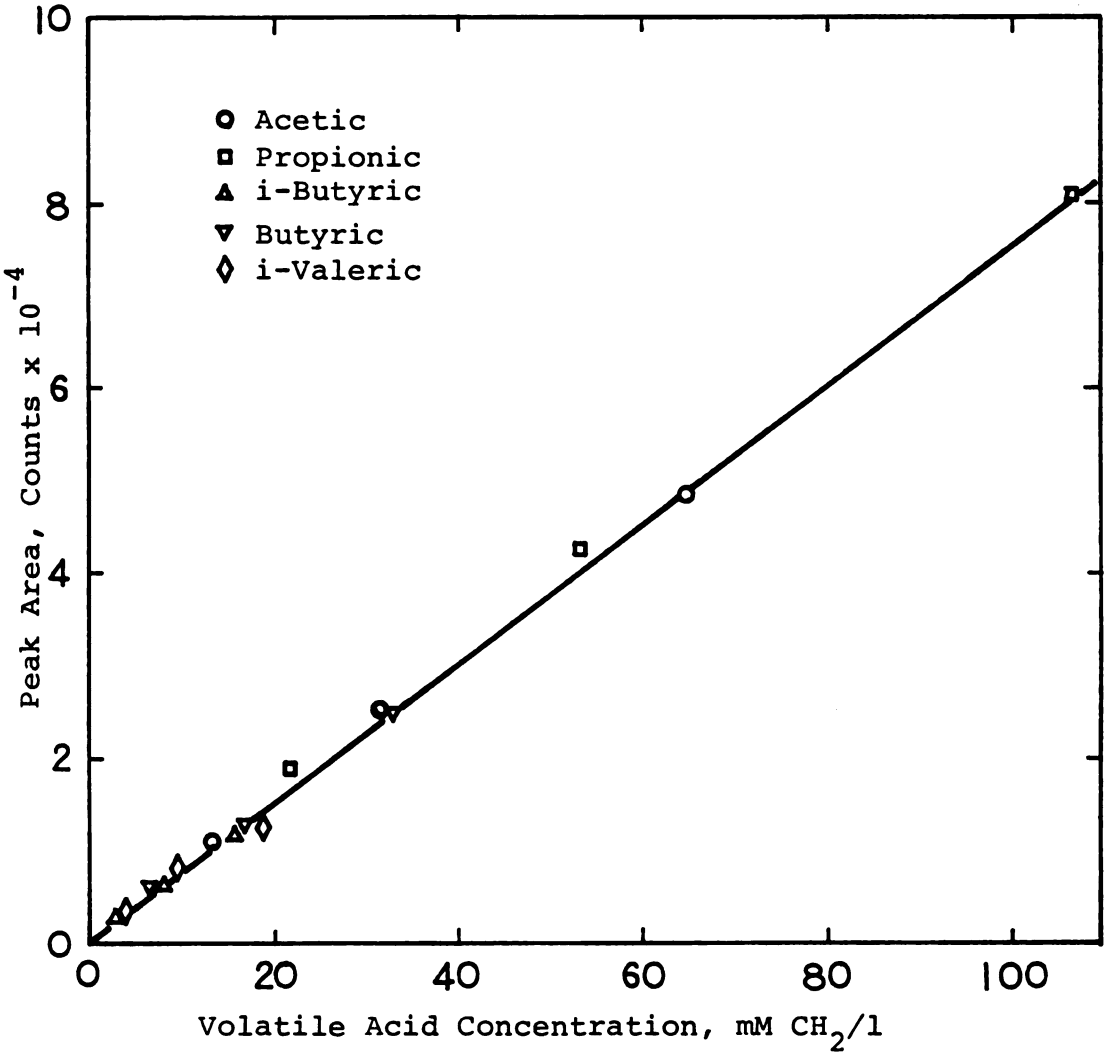


FIGURE 13. Response of the Flame Ionization Detector to the Concentration of Individual Volatile Acids.

Therefore, COD = 192/162 = 1.185 g/g cellulose.

Volatile Acids and Ethanol

The conversion factors of the volatile acids and ethanol can be calculated in the same manner as shown in carbohydrates. The results are shown in Table 13. The conversion factors for branched chain acids are the same as those for the straight chain acids.

TABLE 13. Conversion Factors for Volatile Acids and Ethanol.

Acid	F.W.	O_2 required/mole	g COD/g acid
Acetic	60.05	64	1.066
Propionic	74.08	112	1.512
Butyric	88.10	160	1.816
Valeric	102.13	208	2.037
Ethanol	46.07	96	2.084

Gases

Methane and hydrogen can be converted to equivalent COD on a molar basis.

$$CH_4 + 2O_2 = CO_2 + H_2O$$

$$COD = 64 g/mole\ CH_4$$

and

$$H_2 + 0.5\ O_2 = H_2O$$

$$COD = 16\ g/mole\ H_2$$

One mole of gas occupies 22.4 liters at standard temperature and pressure. Therefore, at standard conditions:

$$COD = 2.86 \text{ g/liter } CH_4$$

and $$COD = 0.714 \text{ g/liter } H_2$$

Determination of Gas Production

Gas production was measured by the change of gas volume in the inverted collection cylinders and expressed at standard conditions. The gas volume at standard conditions (Vs) is calculated using the ideal gas law:

$$Vs = V \frac{P}{Ps} \frac{Ts}{T}$$

where: V = observed gas volume

P = pressure in the gas space (atm.)

T = absolute temperature in the gas space (^{o}K)

Ps = standard pressure (1 atm.)

Ts = standard absolute temperature (273^{o}K)

The following assumptions were considered to simplify the above equation:

(1) The change of the observed gas volume caused by the change of the atmospheric pressure is negligible.

(2) The pressure in the gas space is equal to the standard pressure (1 atm.).

(3) The temperature in the gas space is equal to that in the surrounding air (25^{o}C).

Therefore, the Vs is given by,

$$Vs = V \frac{1 \text{ atm}}{1 \text{ atm}} \frac{273\ ^{o}K}{298\ ^{o}K} = 0.916\ V$$

IV. EXPERIMENTAL RESULTS

This chapter presents the experimental results obtained from operating the bench-scale anaerobic digesters. The experimental data show the extent of substrate degradation and organic nitrogen formation considering the different nitrogen sources. Data are also included on the products of the acid fermentation phase. A complete summary of the experimental results can be found in the Appendix.

A. SUBSTRATE DEGRADATION AND pH

The extent of substrate degradation over a 14-day digestion period is shown in Figure 14. Of the four reactors, only those containing glucose, Reactor I with nitrogen gas and Reactor IV with NH_4Cl, showed substrate degradation. No degradation of cellulose and acetate was found in Reactors II and III with nitrogen gas as the nitrogen source.

Glucose was almost completely decomposed after 30 hours from seeding in Reactor IV, but after 50 hours in Reactor I with a 20 hour delay. The biological degradation of glucose in Reactor I implies the biological fixation and assimilation of molecular nitrogen, because nitrogen is indispensable to cell growth and nitrogen gas was the only nitrogen source. The biological utilization of ammonia nitrogen was implied in Reactor IV. The experimental results on cell growth and organic nitrogen contents will be given in Section B.

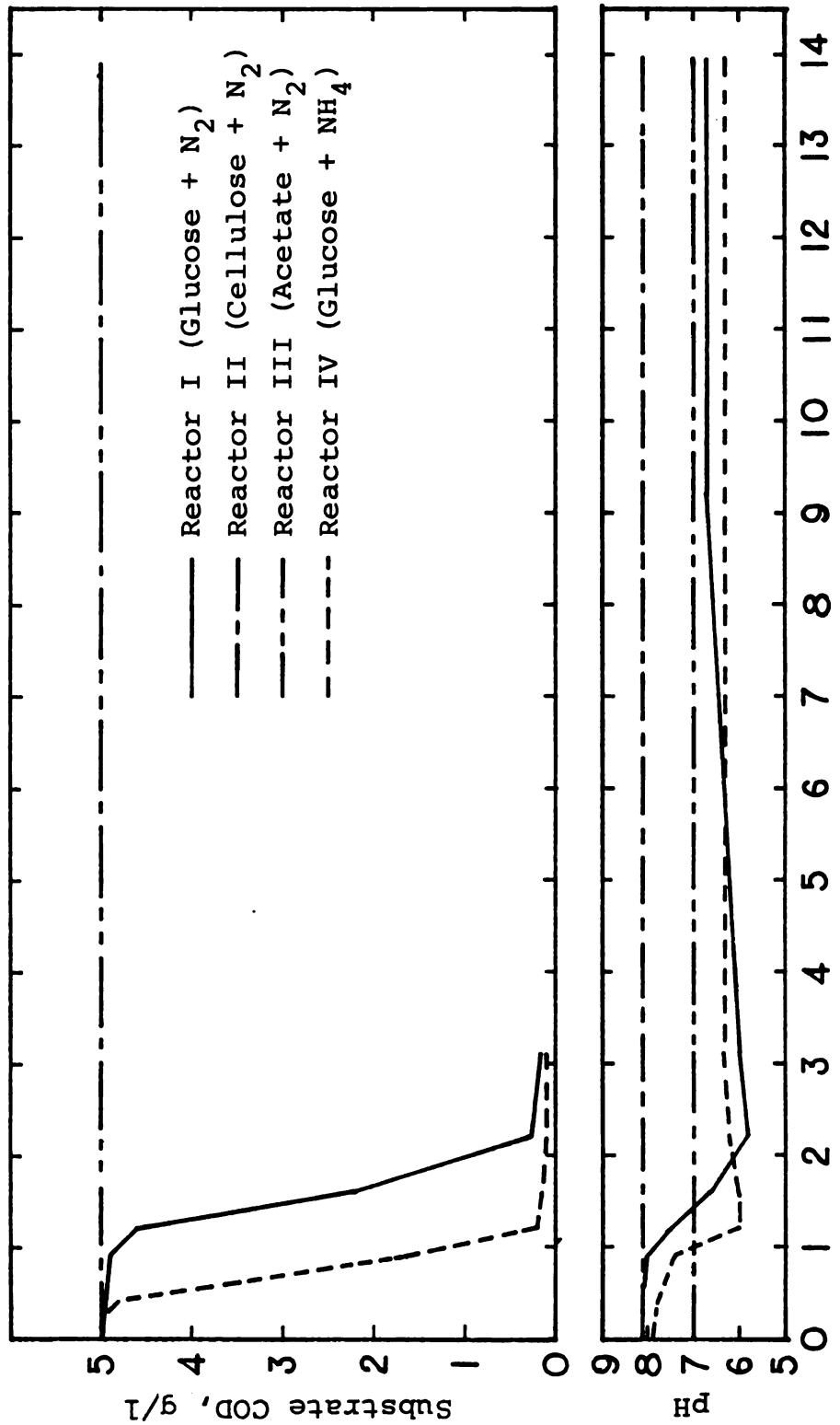


FIGURE 14. Substrate Degradation and pH Change over a 14-day Digestion Period.

The change of pH with substrate degradation is also shown in Figure 14. The drop of pH was found in Reactors I and IV corresponding to the glucose degradation, but no change of pH found in Reactor II and III during a 14-day digestion time. In Reactor I, the pH dropped from 8.1 to a minimum of 5.8 at 2.2 days but rose to 6.7 by day 14. On the other hand, Reactor IV showed a pH drop from 7.9 to 6.0 and a rise to 6.3 at day 14. The slight recovery of pH values was observed after the completion of the glucose degradation in both reactors. The pH values of Reactors II and III were constant at 8.1 and 7.0, respectively.

B. CELL GROWTH AND ORGANIC NITROGEN

Volatile suspended solids (VSS) and nitrogen, ammonia and organic, were measured in order to estimate the biomass and the cellular nitrogen content. Ammonia was measured in expectation of indicating the intermediate product in nitrogen fixation and assimilation for Reactors I, II, and III, while confirming the utilization of ammonia nitrogen as a nitrogen source for Reactor IV. The results of VSS and nitrogen measurements are shown in Figure 15. The measurements were continued until the gas production leveled off indicating degradation of the substrate. However, as shown subsequently, metabolism of intermediate compounds continued beyond this time.

In Reactor I, both VSS and organic nitrogen concentrations were almost constant after 40 hours from seeding. The amount of cells (VSS) and organic nitrogen were 230 mg/l and 18 mg/l at each

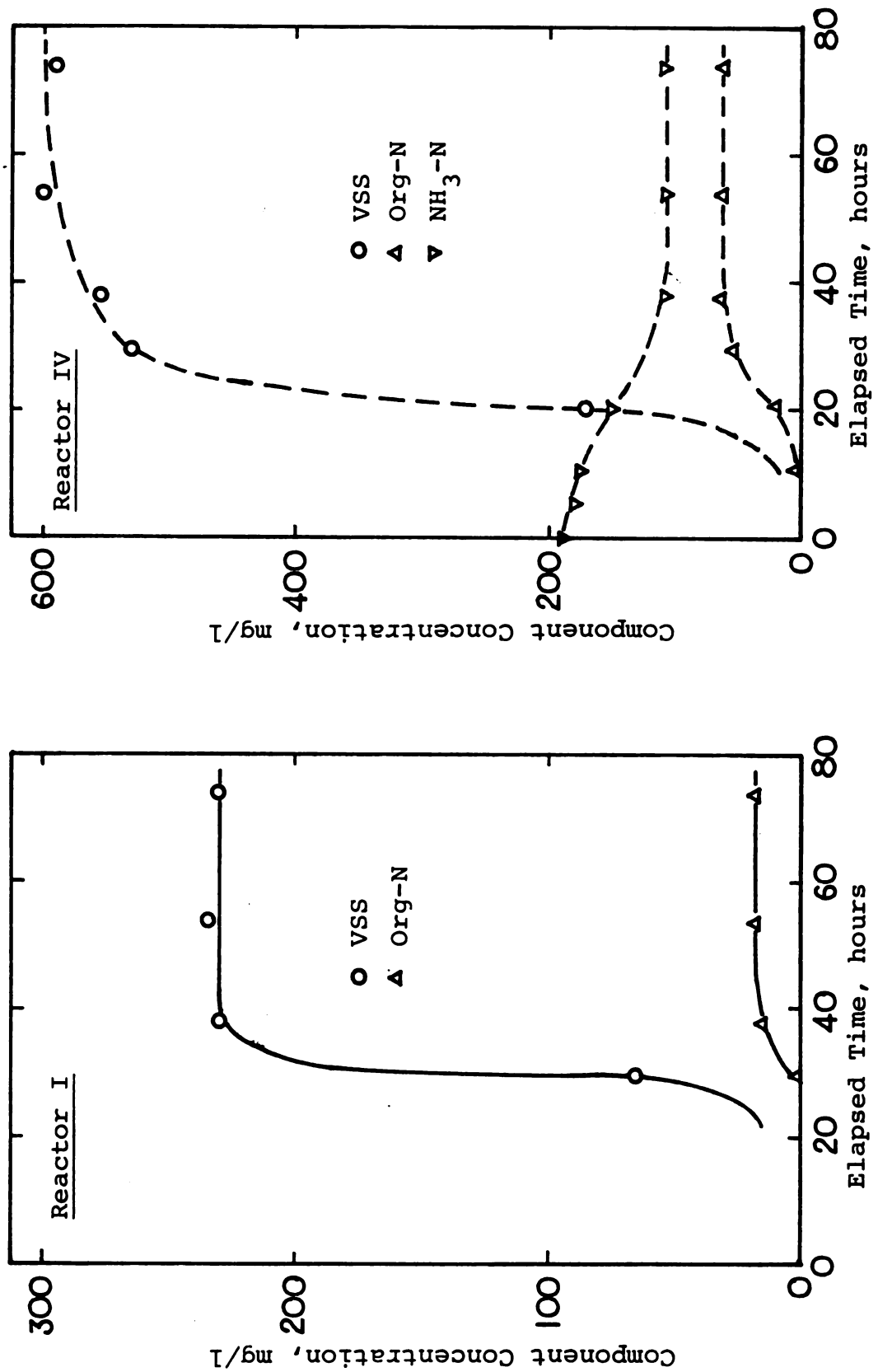


FIGURE 15. Relationship Between Cell Growth (VSS) and Organic Nitrogen.

plateau, respectively. There is no doubt that the organic nitrogen was derived from biological fixation and assimilation of molecular nitrogen. No ammonium intermediates were found. In Reactor IV, the concentrations of VSS and organic nitrogen became almost constant after 30 hours at 600 mg/l and 63 mg/l, respectively. The corresponding drop of ammonia nitrogen concentration confirmed its assimilation into organic nitrogen.

C. PRODUCTS OF ACID FERMENTATION PHASE

As products characterizing the acid fermentation phase of anaerobic digestion, individual volatile acids, alcohols, and gas composition were measured using a gas-solid chromatograph. The experimental results are presented in this section.

1. INDIVIDUAL VOLATILE ACIDS

Although the individual volatile acids were measured for the liquid samples of all reactors, only Reactors I and IV showed the production of volatile acids. The chromatographic analyses detected 5,000 mg/l as COD of acetic acid in the liquid sample of Reactor III, but this is the initial content of acetate as a substrate. This result verified that no degradation of acetate occured in Reactor III.

From Figure 16 showing the production of the individual volatile acids as a function of digestion time, it seems that acetic acid is the main product during the early stage of acid fermentation in both Reactors I and IV. Propionic and butyric acids in Reactor IV

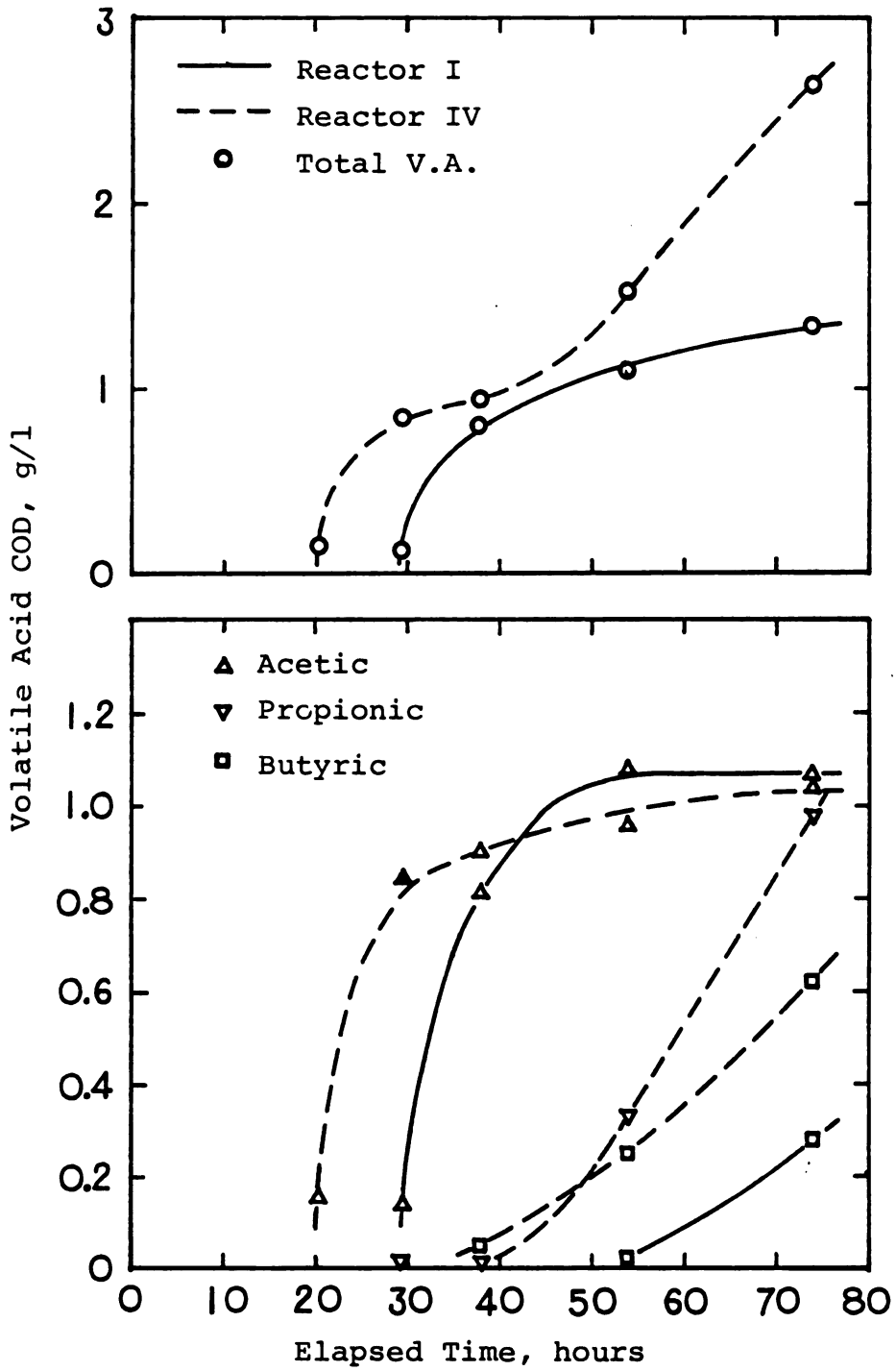


FIGURE 16. Production of Volatile Acids and Distribution of Individual Volatile Acids as a Function of Digestion Time.

and butyric acid in Reactor I began to be produced when the production of acetic acid had almost stopped. There was no big difference between the production pattern of individual volatile acids of Reactor I and that of Reactor IV except for propionic acid which was not observed in Reactor I within the analysis period.

2. ALCOHOL

Ethanol was found in both Reactors I and IV by the gas chromatographic analyses. Figure 17 shows the production of ethanol during an 80-hour digestion time . The curves of ethanol production are very similar to those of acetic acid in both reactors.

3. GASES

The quantity and composition of gas produced during a 14-day digestion period in Reactors I and IV are summarized in Table 14 and Figure 18. The results have been normalized to exclude nitrogen which resulted from purging the units at the beginning of each experimental run and from injection as a nitrogen source. Also, they were expressed at standard temperature and pressure. No gas was produced in Reactors II and III.

The highest gas production rates per unit volume were 356 ml/l·day in Reactor I and 309 ml/l·day in Reactor IV. As a whole, the production of both hydrogen and carbon dioxide in Reactor I are greater than in Reactor IV. On the other hand, the hydrogen is a greater portion of the gas phase than carbon dioxide in Reactor I, but they are almost equal in Reactor IV during the time the gas

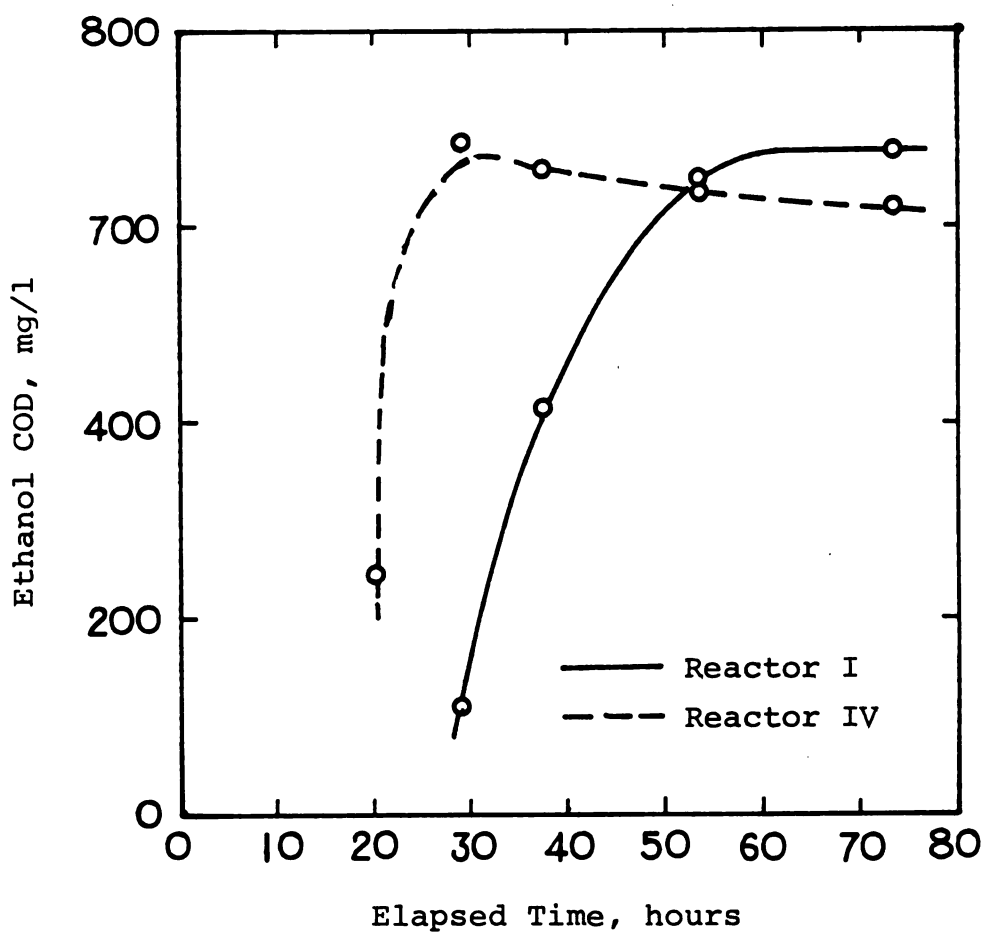


FIGURE 17. Ethanol Production as a Function of Digestion Time.

TABLE 14. Gas Composition and Production Rate.

Reactor	Digestion Time (day)	Gas Production (ml gas/ 1 media/day)	% Composition H_2	CH_4	CO_2
I	1.3	-	-	-	-
	1.7	-	-	-	-
	2.3	23	68	-	32
	3.0	86	67	-	33
	3.2	356	67	-	33
	4.0	328	56	-	44
	8.3	98	45	-	55
	9.1	42	25	-	75
	10.1	30	13	-	87
	11.1	27	5	14	81
	14.0	24	2	10	88
IV	1.3	128	52	-	48
	1.7	304	43	-	57
	2.3	309	55	-	45
	3.0	187	52	-	48
	3.2	133	56	-	44
	4.0	-	57	-	43
	8.3	-	6	3	92
	9.1	-	4	5	91
	11.1	-	-	5	95
	14.0	-	-	6	94

Note: Gas compositions after day 4.0 in Reactor IV show the compositions of the remaining gas in the gas phase of the reactor.

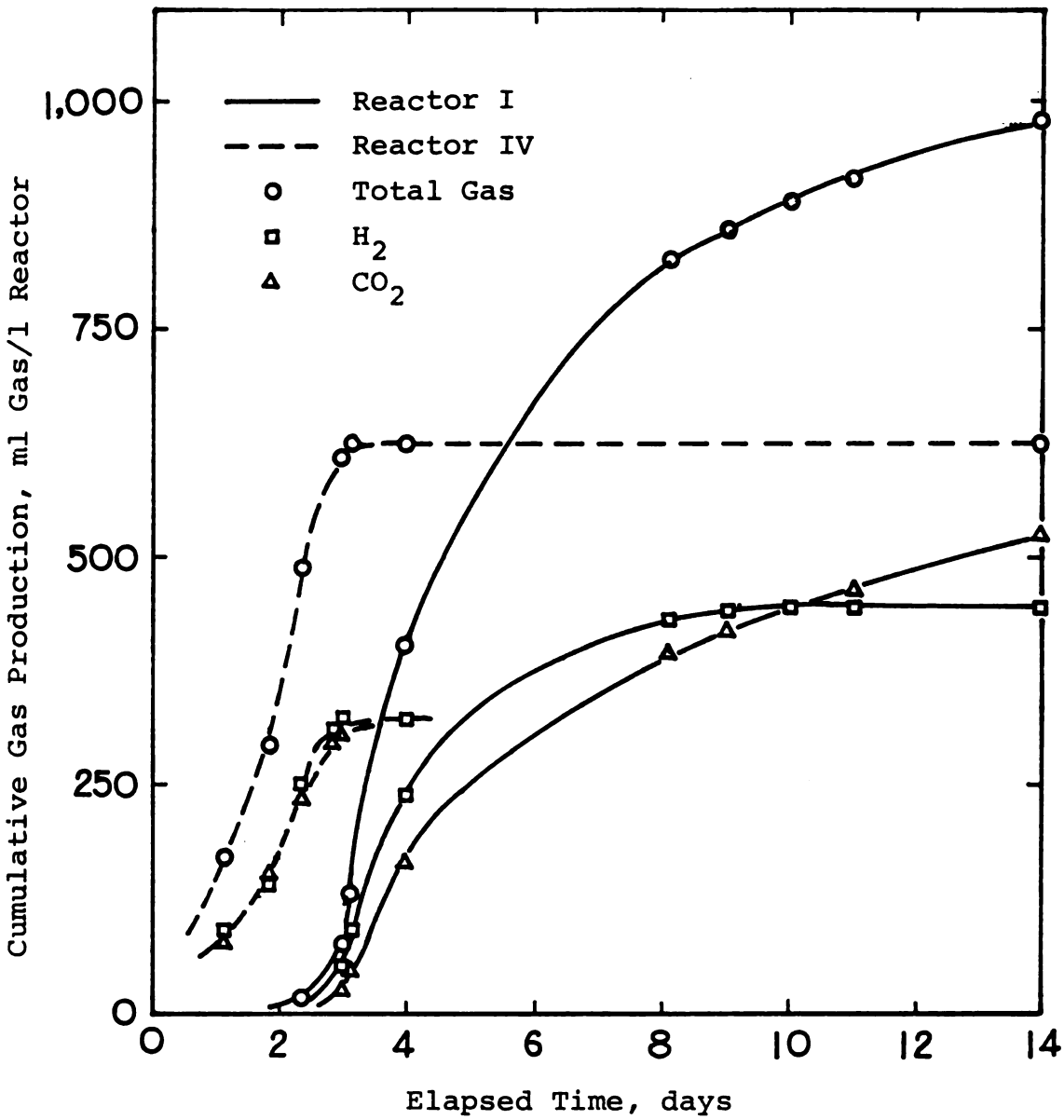


FIGURE 18. Cumulative Gas Production During the Acid Fermentation Phase.

production rate was high. The proportion of carbon dioxide in the gas phase of Reactor I was related to the total gas production, increasing as gas production decreased. An interesting feature of the gas composition data is the shift from hydrogen to methane at longer digestion times (Table 14) although additional gas production was small.

The measurement of gas production was continued until day 40 by reading the gas volume of the inverted gas collection cylinders. The results are shown in Figure 19. Unfortunately, no data points could be plotted after day 14 on Reactor I because nitrogen gas could not be excluded for normalization due to lack of gas-chromatographic measurement of gas composition. Additional gas production began after day 14 in Reactor IV. Although the gas composition was not measured, it is reasonable that the additional gas production derives from hydrogen produced by the oxidation of ehtanol to acetic acid and/or from methane by the stabilization of volatile acids.

Acetate was used as a substrate in order to evaluate nitrogen fixation in the methane phase because the decomposition of acetate is attributed to the methane phase rather than the acid phase. However, no methanogenic activity was found in Reactor III even during a 40-day experimental period.

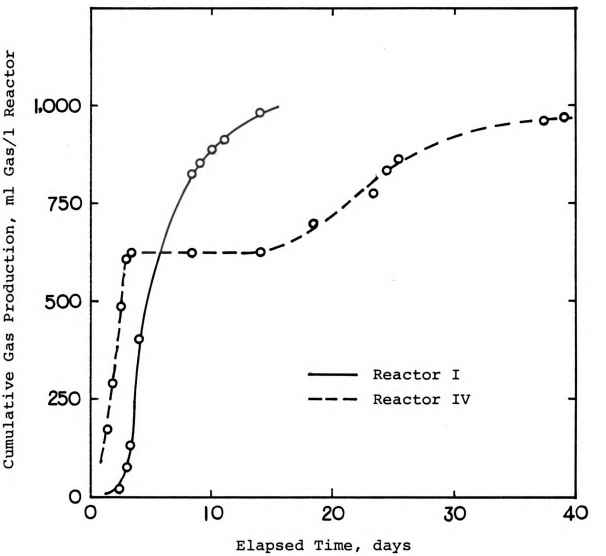


FIGURE 19. Cumulative Gas Production During a 40 Day Digestion Period. Note: No data points were plotted after 14 days in Reactor I because the proportion of N_2 to total gas volume was not measured.

V. DISCUSSION OF RESULTS

The purpose of this chapter is to discuss and develop the experimental results, taking the objectives of this study into account. The objectives of this study include: 1) evaluation of the effects of nitrogen sources on anaerobic digestion concerning substrate degradation, cell growth, organic nitrogen content, and production of volatile acids; 2) discussion of the composition of acid fermentation products with respect to a COD balance; 3) evaluation of the effects of substrates on biological nitrogen fixation.

A. EFFECTS OF NITROGEN SOURCES ON ACID FERMENTATION

As mentioned in the previous chapter, anaerobic fermentation proceeded only in Reactors I and IV with glucose substrates and nitrogen gas and NH_4Cl, respectively. Using the experimental results of these two reactors, the effects of molecular and ammonia nitrogen as nitrogen sources for cell growth will be discussed in this section.

1. DELAY IN GLUCOSE DEGRADATION

It was previously mentioned that a delay of about 20 hours in the degradation of glucose was found between Reactors I and IV. This is again shown in Figure 20, which combines data from Figures 14 and 15. The possible reasons for this delay will be discussed in the following paragraphs.

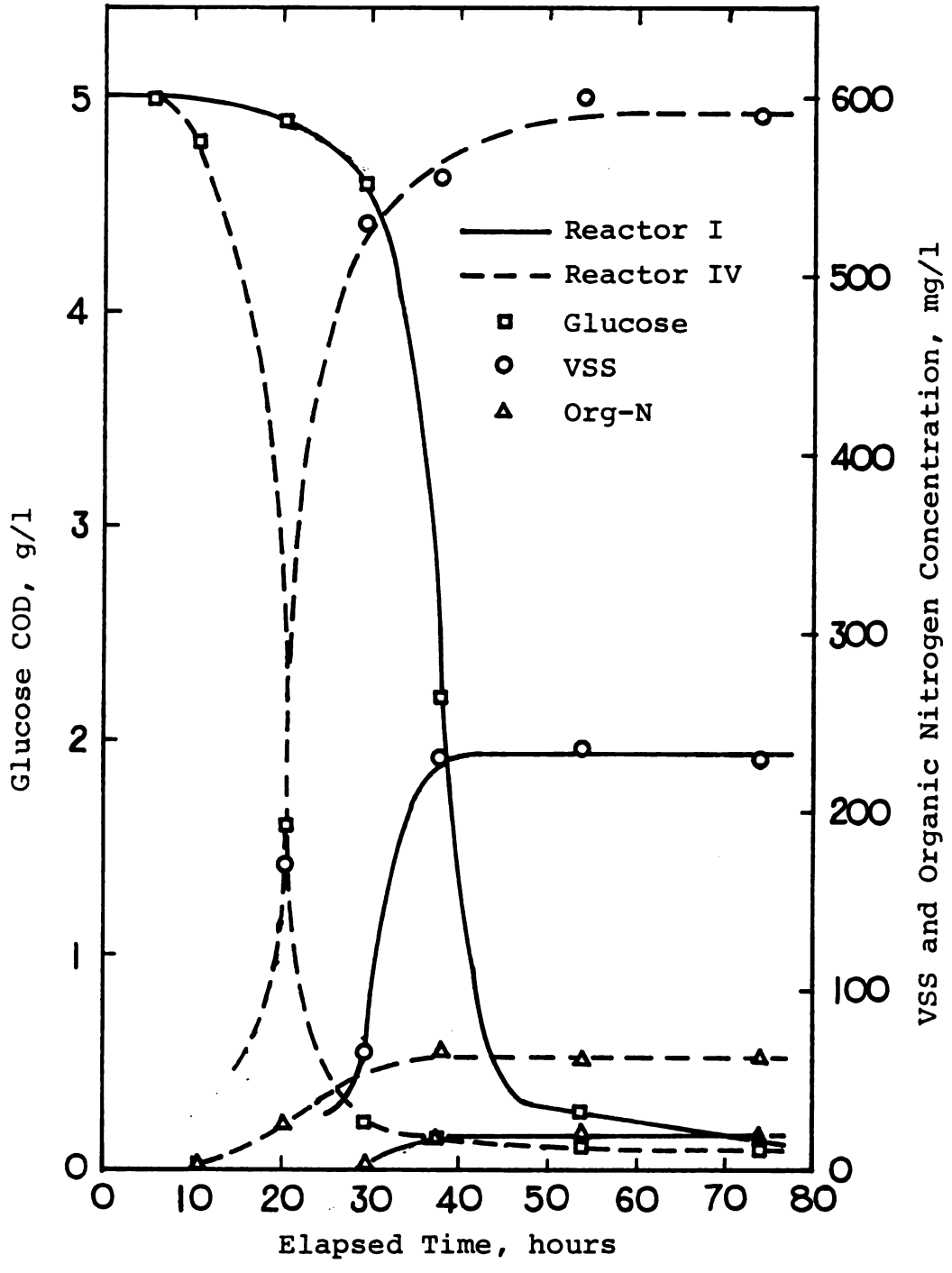


FIGURE 20. Relationship Between Glucose Degradation and Cell Growth (VSS) Including Organic Nitrogen.

In general, anaerobic digestion consists of three biological processes: hydrolysis, acid fermentation, and methane fermentation. However, one more process, nitrogen fixation, is required for cell growth if the only nitrogen source is molecular nitrogen. It can be assumed that bacteria need some extra time to reduce molecular nitrogen to ammonia nitrogen before assimilating it into the cell. Becasue of this, the glucose degradation in Reactor I might have been delayed about 20 hours compared with that in Reactor IV in this investigation.

Another possible factor causing the delay is a shift in predominance of bacterial species between Reactors I and IV. It is therefore possible that the species of bacteria capable of growing in Reactor I have lower growth rates than those in Reactor IV due to metabolic differences other than nitrogen fixation. If so, the decomposition rate of substrate by the former species will be lower than by the latter.

2. YIELD COEFFICIENT AND ORGANIC NITROGEN CONTENT

In the discussion of the relationship between substrate degradation and cell growth, yield coefficients are frequently used. If the yield coefficient is defined as mass of cells (VSS) produced per mass of COD consumed, yield coefficients (Y) in Reactors I and IV can be computed using data from the plateaus in Figure 20. Thus, Y_1 = 230/4.900 = 0.047 mg VSS/mg COD consumed in Reactor I, and Y_4 = 600/4.900 = 0.122 mg VSS/mg COD consumed in Reactor IV. The ratio of Y_1 to Y_4 is 0.385.

As indicated by the above yield coefficients, the cell growth in Reactor I is very low compared with that in Reactor IV in spite of the complete degradation of glucose in both reactors. It seems that the low cell growth in Reactor I has much to do with nitrogen fixation. Since nitrogen fixation is a very energy consuming reaction, much of the energy produced and stored in the form of ATP by fermentation could be consumed to fix nitrogen (see Section D in Chapter II for details). As a result, the energy available for cell growth will be decreased. The ATP-dependent H_2 evolution is thermodynamically an important factor contributing to the high energy requirement for nitrogen fixation. From this point of view, the larger amount of hydrogen production in Reactor I, (see Table 14 or Figure 18) supports the high energy consumption by nitrogen fixation and the low yield coefficient.

The cellular organic nitrogen content can be computed using data in Figure 20 in a similar fashion as the yield coefficients. The nitrogen content (Nc) is: N_{c1} = (18 mg/l org-N)/(230 mg/l VSS) = 0.08 in Reactor I, and N_{c4} = (63 mg/l org-N)/(600 mg/l VSS) = 0.11 in Reactor IV. N_{c4} is within the normal range of organic nitrogen contents shown in Table 2, (10 to 14 percent of cell dry weight) while N_{c1} is smaller than this range. The lower cell nitrogen content in Reactor I is probably because of the difficulty of utilizing molecular nitrogen for cell growth compared with ammonia nitrogen. The utilization of ammonia requires no oxidation or reduction, because the nitrogen in the cell such as amino acids, purines, ammonia, i.e., the valence of N is -3. Molecular nitrogen, however,

must be reduced to the level of ammonia, i.e., nitrogen fixation, and then utilized for amination.

It can be concluded, therefore, that nitrogen fixation is a major factor causing low cell growth and low organic nitrogen content in cells when the nitrogen source is molecular nitrogen only.

3. PRODUCTION OF VOLATILE ACIDS

As regards the production of individual volatile acids, some time delay was found between Reactors I and IV, and no propionic acid was produced in Reactor I during an 80-hour digestion period. The lack of propionic acid production, however, seems to be attributed to the delay of acid production and the short digestion time. The similarity of acid production patterns between Reactors I and IV as shown in Figure 16 indicates that propionic acid might have been found in Reactor I as well if the measurement of volatile acids had continued after 80 hours.

As a conclusion, it is reasonable to say that no particular change caused by nitrogen sources, nitrogen gas and NH_4Cl, was found in the production of volatile acids. The lack of propionic acid in Reactor I is inconclusive.

B. COMPOSITION OF ACID FERMENTATION PRODUCTS

During the acid fermentation phase of anaerobic digestion, substrates are converted to several products such as cell tissue, volatile acids, alcohols, and gases. From the concept of mass

balances, the initial COD of the substrate must balance with the COD of fermentation products and substrate remaining. In this section, the composition of acid fermentation products will be discussed considering such a COD balance.

The COD balances of Reactors I and IV are shown as a function of digestion time in Figure 21. The equivalent COD of each fermentation product was computed using a conversion factor described in Section D of Chapter III. The unknown COD in Figure 21 is the substance which was not characterized by the experimental measurements of this study and was calculated by subtracting the COD of the characterized products plus the remaining glucose from the initial COD of glucose.

As a common feature of the COD balance, half of the glucose was converted to the unknown COD and the other half mainly to cells, volatile acids, and ethanol when the degradation of glucose was almost completed, i.e., at hour 54 in Reactor I and at hour 30 in Reactor IV. Subsequently, the unknown COD began to be converted, mainly to propionic and butyric acids. In the gas chromatographic analysis, this unknown product tailed badly like lactic acid but had a different retention time from that of lactic acid: the peak retention time of the unknown product was approximately five and one half minutes, while that of lactic acid was about twelve minutes under the chromatographic conditions used in this study. Since the production of propionic and butyric acids started just when the unknown COD began to be converted to volatile acids as shown in Figure 16 and 21, it can be assumed that the unknown COD derives

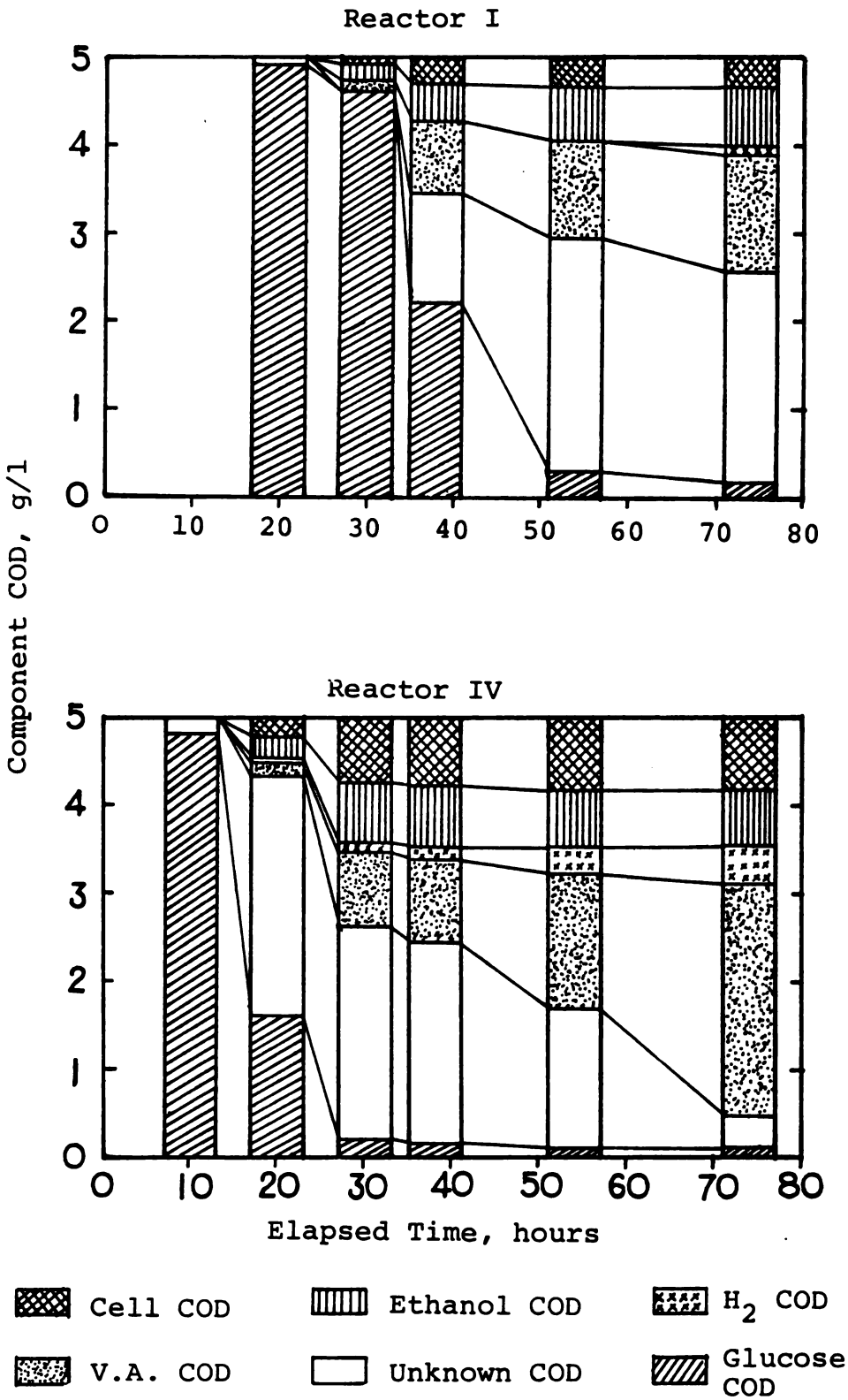


FIGURE 21. COD Balance as a Function of Digestion Time.

from precursors of propionic and/or butyric acids, e.g., succinic and acetoacetic acids (see Figure 3).

Another interesting feature of the COD balances is that the change of composition with time is roughly similar between Reactors I and IV. This implies that the COD compositions of Reactor I will continue to change in a manner similar to that of Reactor IV (shown in Figure 21) beyond 80 hours.

C. EFFECTS OF SUBSTRATES ON NITROGEN FIXATION

Three different substrates, glucose, cellulose, and acetate, were used to compare their effects on nitrogen fixation and assimilation. Substrate degradation, however, occurred only for glucose, nothing happened to cellulose and acetate. In this section, the possible reasons for the lack of cellulose and acetate degradation will be discussed in association with utilization of molecular nitrogen for cell growth.

As mentioned in Section D of Chapter II, the biological nitrogen fixation requires ATP and an electron donor, commonly pyruvate. Both can be theoretically obtained from glycolysis using cellulose. The utilization of cellulose, however, is accomplished with difficulty compared with glucose. Since cellulose is a large insoluble molecule which cannot pass through cell membranes, it must be enzymatically hydrolized into small soluble molecules before metabolic utilization in cells. If cellulose is the only source of pyruvate and ATP required for nitrogen fixation, the hydrolysis of cellulose must occur before nitrogen fixation. It seems that

the hydrolysis requirement has much to do with lack of cellulose degradation as well as non-fixation of nitrogen.

One possible reason for lack of cellulose degradation is that there were no organisms that could hydrolize cellulose under the conditions of this experiment. Another reason is that nitrogen fixers have no enzyme to hydrolize cellulose and the fixed nitrogen cannot be utilized by organisms that can hydrolize cellulose but not fix nitrogen.

On the other hand, no hydrolysis is required for the utilization of acetate because it is soluble. The decomposition of acetate, however, is attributed to the methane phase rather than the acid phase, and the methanogenic activity cannot produce pyruvate. Non-degradation of acetate is probably due to lack of an acceptable electron donor such as pyruvate for nitrogen fixation. It must be emphasized, however, that this experiment does not prove there cannot be nitrogen fixation in the methane phase.

VI. CONCLUSIONS

Based on the results of this investigation, the following conclusions can be made about nitrogen fixation during anaerobic digestion.

1. Molecular nitrogen can be biologically utilized as a nitrogen source with glucose as the substrate during the acid fermentation phase.
2. A delay of about 20 hours was found for glucose degradations using nitrogen gas compared to NH_4Cl as the nitrogen source. Glucose was almost completely decomposed using either nitrogen gas or NH_4Cl as the nitrogen source for cell growth.
3. Lower cell growth and lower cell nitrogen content were found in the experiment with nitrogen gas compared with the experiment with NH_4Cl.

   For the experiment with nitrogen gas:

   Yield coefficient - 0.047 mg VSS/mg COD consumed.

   Organic nitrogen content - 8% by dry weight of cell.

   For the experiment with NH_4Cl:

   Yield coefficient - 0.122 mg VSS/COD consumed.

   Organic nitrogen content - 11% by dry weight of cell.
4. No significant difference was found in the production of volatile acids comparing nitrogen gas with NH_4Cl.
5. A larger amount of hydrogen gas was produced in the experiment with nitrogen gas compared with the experiment

with NH_4Cl. It can be assumed that ATP-dependent H_2 evolution was occurring during the nitrogen fixation process.

6. When glucose was almost completely decomposed, about half of COD of the fermentation products consisted of cell, volatile acid, and ethanol COD; the other half was unknown components which were precursors of propionic and butyric acids.

7. No degradation was found in the experiments using cellulose and acetate as substrates and nitrogen gas as the nitrogen source. Possible reasons for this are: 1) nitrogen fixers have no enzyme to hydrolyze cellulose and the fixed nitrogen cannot be utilized by other organisms that can hydrolize cellulose but not fix nitrogen; 2) pyruvate required for nitrogen fixation cannot be obtained from acetate.

VII. SUGGESTIONS FOR FUTURE WORK

For further development of the findings of this study, the following topics are suggested for future research.

Elucidation of non-degradation of cellulose and acetate using nitrogen gas is an important topic including studies on complex substrates. The reasons for non-degradation of cellulose and acetate were not made clear experimentally in this study. This and studies using industrial wastewater deficient in nitrogen should be conducted for the potential engineering application of nitrogen fixation.

In future work, experiments similar to those described here should be run for longer time periods. Also, procedures should be developed to identify the unknown degradation products.

Overall studies using continuous flow units are strongly recommended to obtain information on the reactor kinetics in association with nitrogen fixation.

APPENDIX

APPENDIX

Calculation of Buffer Requirements for pH Control

In order to maintain the optimum pH for nitrogen fixers and acid formers, phosphate buffer requirements were calculated with the following assumptions:

1) one mole of glucose yields two moles of H^+ on the average (See Table 1),

2) initial pH values of the media should be 8.0.

The total amount of H^+ yielded by 4,686 mg/l of glucose is

$$H^+ = 2 \left(\frac{4{,}686 \text{ mg/l}}{180 \text{ mg/m mole}}\right) = 52.06 \text{ mM}$$

If the final pH is 6.5, the allowable production of H^+ can be computed on the basis of the concept of chemical equilibrium. The following relationships are obtained for a 50 mM phosphate buffer (Weast, 1974)

$$\frac{46.7 \text{ mM } (HPO_4^=)}{3.3 \text{ mM } (H_2PO_4^-)} \quad \text{at initial pH 8.0}$$

$$\frac{13.9 \text{ mM } (HPO_4^=)}{36.1 \text{ mM } (H_2PO_4^-)} \quad \text{at final pH 6.5}$$

Allowable H^+ = 46.7 mM - 13.9 mM = 32.8 mM

Therefore, the buffer requirement to allow the production of 52.06 mM H^+ is

$$46.7 \text{ mM } (\frac{52.06}{32.8}) = 74.12 \text{ mM } HPO_4^{=}$$

$$\text{and } 3.3 \text{ mM } (\frac{52.06}{32.8}) = 5.24 \text{ mM } H_2PO_4^{-}$$

The requirement of metal ion (Me^+) for $HPO_4^{=}$ is

$$Me^+ = 2(74.12 \text{ mM}) = 148.24 \text{ mM}$$

Let 1/3 Me^+ be K^+ and 2/3 Me^+ be Na^+ in order to obtain the antagonistic effects of K^+ and Na^+ (See Table 10).

Metal ion requirements are given by

$$K^+ = 1/3 \text{ (39 A.W.) (148.24 mM)} = 1,927 \text{ mg/l}$$

$$Na^+ = 2/3 \text{ (23 A.W.) (148.24 mM)} = 2,273 \text{ mg/l}$$

Finally, the amounts of K_2HPO_4 and Na_2HPO_4 required are

$$K_2HPO_4 = (1,927 \text{ mg/l}) \ (\frac{174 \text{ M.W.}}{2x39 \text{ A.W.}}) = 4,299 \text{ mg/l}$$

$$Na_2HPO_4 = (2,273 \text{ mg/l}) \ (\frac{142 \text{ M.W.}}{2x23 \text{ A.W.}}) = 7,017 \text{ mg/l}$$

The small amount of $H_2PO_4^{=}$ was added as the K^+ salt:

$$KH_2PO_4 = (5.24 \text{ mM}) \ (136 \text{ MW}) = 345 \text{ mg/l}$$

TABLE A1. Summary of Digester Characteristics for Reactors I and IV.

Elapsed Time (hr)	Reactor I pH	Reactor I VSS (COD) (mg/l)	Reactor I Glucose (COD) (mg/l)	Reactor I NH_3-N (mg/l)	Reactor I Org-N (mg/l)	Reactor IV pH	Reactor IV VSS (COD) (mg/l)	Reactor IV Glucose (COD) (mg/l)	Reactor IV NH_3-N (mg/l)	Reactor IV Org-N (mg/l)
5.5	8.1	--	5000	N.D.	--	7.8	--	5000	178	N.D.
10.5	8.1	--	5000	N.D.	--	7.8	--	4800	174	3
20.5	8.0	--	4900	N.D.	2	7.4	241	1600	150	22
29.5	7.5	92	4600	N.D.	1	6.0	753	200	88	56
37.8	6.6	327	2200	N.D.	16	6.0	788	140	107	66
53.5	5.8	334	270	N.D.	18	6.2	852	100	108	62
73.7	6.0	327	150	N.D.	17	6.3	838	90	114	63
219.7	6.7	--	---	---	--	6.3	---	---	---	--
334.5	6.7	--	---	---	--	6.3	---	---	---	--

N.D. = Not Detected

TABLE A2. Summary of Individual Volatile Acids and Ethanol.

Elapsed Time (hr)	Acids	Reactor I		Reactor II		Reactor III		Reactor IV	
		(mM/l)	COD (mg/l)	(mM/l)	COD (mg/l)	(mM/l)	COD (mg/l)	(mM/l)	COD (mg/l)
20.5	HAc	--	--	--	--	77.75	4977	2.38	152
	Ethanol	--	--	--	--	--	--	2.54	244
29.5	HAc	2.11	139	--	--	78.67	5036	13.20	845
	HP	--	--	--	--	--	--	0.06	6
	Ethanol	1.16	111	--	--	--	--	7.16	687
37.8	HAc	12.67	811	--	--	80.00	5121	14.12	904
	HP	--	--	--	--	--	--	--	---
	HB	--	--	--	--	--	--	0.31	49
	Ethanol	4.31	414	--	--	--	--	6.85	658
53.5	HAc	16.90	1082	--	--	75.50	4833	14.92	955
	HP	--	--	--	--	--	--	2.91	327
	HB	0.13	20	--	--	--	--	1.54	247
	Ethanol	6.78	651	--	--	--	--	6.70	643
73.7	HAc	16.76	1072	--	--	77.88	4986	16.24	1039
	HP	--	--	--	--	--	--	8.78	983
	HB	1.72	276	--	--	--	--	3.87	619
	Ethanol	7.08	680	--	--	--	--	6.47	621

Note: HAc = Acetic Acid, HP = Propionic Acid, HB = Butyric Acid. Acids not listed were not detected. A dash means none detected in that reactor.

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