İSTANBUL TECHNICAL UNIVERSITY ★ INSTITUTE OF SCIENCE AND TECHNOLOGY

BIOHYDROGEN PRODUCTION VIA DARK FERMENTATION

M. Sc. Thesis by

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Department: Environmental Engineering

Programme: Environmental Biotechnology

JANUARY 2007

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Date of Submission: January 8, 2007

Date of Defence Examination: January 29, 2007

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JANUARY 2007

İSTANBUL TEKNİK ÜNİVERSİTESİ ★ FEN BİLİMLERİ ENSTİTÜSÜ

KARANLIK MAYALANMA İLE BİYOLOJİK HİDROJEN ÜRETİLMESİ

YÜKSEK LİSANS TEZİ

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Tezin Enstitüye Verildiği Tarih: 8 Ocak 2007

Tezin Savunulduğu Tarih: 29 Ocak 2007

Tez Danışmanı : Prof. Dr. İzzet ÖZTÜRK

Diğer Jüri Üyeleri Prof. Dr. Nazik ARTAN (İ.T.Ü.)

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OCAK 2007

ACNOWLEDMENTS

I would like to thank, first and foremost, my supervisor, Prof. Dr. İzzet ÖZTÜRK, for his guidance and support throughout my graduate career and during the completion of this thesis.

There are many people who have directly and indirectly contributed to this thesis. First of all I would like to thank Dr. Mahmut ALTINBAŞ. He has been a great source of encouragement throughout my work. I would like to especially thank him for the amount of patience with which he has worked with me. I am deeply indebted to him for keeping me on course even when things seemed to be going in the wrong direction.

I would like to acknowledge the students and other staff members in the Department of Environmental Engineering Laboratory.

Thanks also to my family for supporting me in my educational pursuits and to my friends for their encouragement. I am particularly indebted to five guys whose creative endeavors allowed me to maintain throughout the writing of this thesis, Cemal, Ercüment, Mehmet Sıddık and Muhammet Übeydullah.

January 2007 **Muhammed İhsan KAHVECİ**

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ABBREVIATIONS

ADP	:Adenosine diphospate
ATP	:Adenosine triphospate
BSR	:Bacterial Stres Respons
COD	:Chemical Oxygen Demand
FAD	:FlavinAdenine dinucleotid phospate
FADH	:Reduced Flavin Adenine dinucleotide
FHL	:Formate Hydrogenlyase
GC	:Gas Chromotograhy
GIGSB	:Carrier-Induced Granular Sludge Bed
GTP	:Goanosine triphospate
HBR	:Hydrogen Bio-produsing Reactor
HTR	:Hydrolic Reteintion Time
NAD	:Nicotinamide Adenine dinucleotid phospate
NADH	:Reduced nicotinamide Adenine dinucleotid phospate
NFDM	:Non-Fat Dry Milk
OLR	:Organic Loading Rate
ORP	:Oxidation-Reduction Potential
PDH	:Pruvate Dehaydrogenase
PFL	:Pruvate Formate Lyase
PFOR	:Pruvate Ferrodaxin Oxidoreductase
RF	:Packing Free
RP	:Packing Rings
SCR	:Semi-continously Operated Reator
SS	:Suspende Solid
TCA	:Tricarboxilic Acid
TOC	:Total Organic Carbon
TS	:Total Solid
VFA	:Volatile Fatty Acid
VSS	:Volatile Suspended Solid
WWTP	:Wastewater Treatment Plant

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BIOHYDROGEN PRODUCTION VIA DARK FEMENTATION

SUMMARY

Although the metabolism of biohydrogen production via dark fermentation is not completely defined, it is basically similar to anaerobic treatment process. The only difference is the inhibition of the methanogenic archaea and thus producing hydrogen. In this study, the effect of the different types anaerobic seed and pH on biohydrogen production was investigated. The efficiency of hydrogen production from glucose was observed by monitoring the total gas production and subsequent determining the hydrogen fraction in total produced gas. Beside that, the fermentation products such as acetate, butyrate and ethanol were also monitored. Identical environmental and operational parameters such as pH and temperature were set for the reactors.

Two different types of seed were used for the assessment of efficient hydrogen production from glucose. The seeds were collected from the anaerobic digester treating municipal wastewater sludges (Reactor A) and the reactor fermenting the olive mill wastes (Reactor B). It was demonstrated that Reactor A did not produce any hydrogen and also the fermentation products like ethanol and butyrate. It could be thought that the seed of Reactor A did not adapt to new environmental conditions and it was inhibited by other environmental factors.

Although the Reactor B was acclimatized to the defined environmental conditions, the hydrogen production was not high enough compared to the expected theoretical hydrogen production. However, if the reactor is operated continuously it could be suggested that the hydrogen production efficiency would be higher. Beside hydrogen, significant amount of ethanol and butyrate production was detected. The average hydrogen production was 1.7 ± 0.2 mol H_2/mol glucose, which is very close the theoretical value of 2.

In conclusion, this study demonstrated that the seed fermenting the olive mill wastes seems more appropriate for the ethanol and hydrogen production and also can tolerate the pH fluctuations.

KARANLIK MAYALANMAYLA BİYOLOJİK HİDROJEN ÜRETİMİ

ÖZET

Organik maddenin fermantasyonu vasıtasıyla gerçekleşen biyolojik hidrojen üretim metabolizması tam olarak bilinmemekle birlikte, temelde anaerobik arıtımdaki metan üretim prosesiyle benzerlik gösterir. En önemli fark, asetik asit ve hidrojenden metan üreten basamağın inhibe edilmesi sonucu anaerobik fermantasyonla üretilen hidrojenin artırılması ve metanojenler tarafından kullanımının engellenmesidir. Bu çalışmada, pH'ın ve karışık mikroorganizma topluluklarının biyolojik hidrojen üretim mekanizmasına etkisi incelenmiştir. Hidrojen üretim veriminin izlenmesi amacıyla; glikozun parçalanması, asetat, bütirat, ethanol gibi ara ürün basamakları izlenmiş, toplam gaz hacmi ve üretilen hidrojen gazı yüzdesi ölçümleri yapılmıştır. Aynı miktarda uçucu katı madde içeren iki farklı tip anaerobik mikroorganizma topluluğu aynı çevresel koşullar altında izlenmiştir. Kentsel atıksu arıtma tesisi anaerobik çürütücüsünden alınan çamur ile aşılanan Reaktör A ve zeytin yağı fermantasyon çamuru ile aşılanan Reaktör B mukayese edilmiştir.

Deneysel çalışmalar sonunda, Reaktör A'da hiç hidrojen gazı çıkışının olmadığı ayrıca asetat, bütirat ve ethanol ara ürünlerinin de kayda değer düzeyde üretilmediği gözlenmiştir. Söz konusu çalışmada hidrojen gazının üretilememesinin sebebi mikroorganizma topluluğunun ortam şartlarına adapte olamaması ve inhibe olmasına bağlı olarak düşünülmektedir.

Reaktör B'de ise anaerobik mikroorganizma topluluğunun sisteme aklime olduğu ancak yüksek miktarda hidrojen üretilemediği gözlenmiştir. Böyle bir aşının sürekli beslemeli reaktörler için, sistem verimini olumlu yönde etkileyeceği düşünülmektedir.

Yürütülen deneysel çalışmanın sonuçları, sistemde baskın olarak üretilen bütirat ve ethanol ara ürünleri ile hidrojen gazı üretildiğini göstermiştir. H_2 üretimi ortalama 1,7± 0,2 mol H_2/mol glikoz olup teorik değer olan 2'ye yakındır. Yapılan çalışma zeytinyağı fermantasyon aşısının ethanol ve hidrojen üretimine son derece uygun olduğunu ve pH salınımlarını daha iyi tolere edebildiğini göstermektedir.

1. INTRODUCTION

1.1 The Importance of the Subject

Energy becomes gradually a critical problem in recent years depending on the increasing population and industry activities. Present energy sources have an adverse effect on the nature of the ecological systems. Natural energy sources such as coal, petroleum and natural gas are neither sufficient enough for energy demand, nor environmentally sustainable for the ecological system. Dependence on fossil fuels as the main energy sources has led to serious energy crisis and environmental problems, i.e. fossil fuel depletions and pollutant emissions. Combustion of fossil fuels produce substantial greenhouse and toxic gases, such as CO_2, SO_2, NO_x and other pollutants, causing global warming and acid rains (Dennis *et al., 2006*). Driving the global energy system into a sustainable path is progressively becoming a major concern and policy objective (Vijayaraghavan *et.al.,* 2004).

The balanced planet readily assimilates pollutants through its natural processes. However, when the planet is overloaded with pollutants and the means by which it assimilates these pollutants are reduced, the planet falls away from equilibrium (Han and Shin, 2003). The balance between energy demand and the carrying capacity of the earth depends on the efficiencies of the various energy chains, mitigating climate effects caused by the use of fossil fuels and developing sustainable energy chains based on the contemporary sunlight-like application of bio-fuels and hydrogen, H_2, from renewable sources (Vijayaraghavan *et.al.,*2004).

Biomass is one of the most abundant renewable resources. It is formed by fixing carbon dioxide in the atmosphere during the process of plant photosynthesis, and therefore, it is carbon neutral in its lifecycle. Biomass has been used for centuries. Currently, biomass contributes about 12% of today's world energy supply, while in many developing countries it contributes 40–50%of energy supply. Biomass research is recently receiving increasing attention because of the probable waste-to-energy applications (Dennis *et al., 2006*). The microbial conversion of agricultural and industrial wastes and residues into hydrogen is attracting increasing interest; this is

due to that hydrogen is an excellent alternative energy source for the future and producing only water instead of greenhouse gases on burning (Fan, Y.T *et.al.,*2005). Microbial hydrogen production from renewable biomass, therefore, plays an important role in bioenergy generation (Lay *et.al.,*1999). Ethanol and methane produced through anaerobic processes are among the best-known microbial products and so far extended research has been conducted on their generation (Gavala *et.al.,*2005). However, H_2 is strategically important as it has low emission, is environment-benign, cleaner and a more sustainable energy system. On the one hand, the introduction of higher efficient and clean H_2-based end-use technologies would help to reduce final energy consumption and also provide regional environmental benefits. H_2 can be produced from carbon-free resources or from fossil fuels combined with carbon separation and sequsestration. Thus, H_2 could contribute substantially to the reduction of greenhouse gas emissions. H_2 from renewable sources might be considered as the ultimate clean and climate neutral energy system (Vijayaraghavan *et.al.,*2004).

When compared with other fuels the benefits of H_2 are based on emissions at the end use, life cycle evaluation, energy needed to retrieve the fuel, relative energy input needed to create the final product and its transition through processing. These energy values can then be equated to demonstrate the overall energy input into the system over its lifetime. H_2 is an effective energy carrier when compared with petrol or natural gas, the energy released per unit mass being twice that of traditional fuels. However, the energy per unit volume is lower than all the other fossil fuels, emphasizing the need for efficient storage. A small amount of NO_x is produced when combusting H_2 in air, however, H_2 utilized in the fuel cell produces no harmful emissions. Combusting fossil fuels produce CO_2, sulfur, NO_x and other harmful by-products. Natural gas is the cleanest of the fossil fuels as it produces a fraction of the CO_2 of heavier fossil fuels like coal and oil. As methane is a powerful greenhouse gas, it is more beneficial to burn natural gas than to release it to the atmosphere (Hawkes *et.al.,* 2002 and Vijayaraghavan *et.al.,*2004).)

Hydrogen can be produced more effectivly from materials which contains high proportion of carbohydrates such as glucose, sucrose hexose etc. Moreover organic waste and wastewater including high level of carbohydrate can be utilized for biohydrogen degradation.

Organic wastes may become a plentiful source of inexpensive organic substrate for fermentative hydrogen production; by which reduction and stabilization of organic wastes also can be accomplished. In recent years, some experimental results using municipal solid waste, foods manufacturing waste, waste activated sludge were reported. The maximum hydrogen production potential and hydrogen production rate were in the range of 49–298 ml H_2/g carbohydrate-COD and 17–142 ml H_2/gVSSh, respectively (Ginkel *et.al.,* 2005).

1.2. Scope and Outline of the Thesis

The mechanism of bio-hydrogen production via dark fermentation is not completely defined. The purpose of this study is able to generate high yields of bio-hydrogen from glucose in butyrate-acetate-ethanol intermediate step, since the butyrate-ethanol pathway is more stable and effective among known pathways such as buthane-buthanol, lactic acid, and acetate, etc. Two different types of anaerobic mixed culture can be compared with respect to their performances. Thus, not only dark fermentation mechanism is able to be investigated but also the performances of two different types of anaerobic complex cultures can be compared by one another. In addition, three different feed volumes (V_{feed}= 1 L, V_{feed}= 2 L, V_{feed}= 3 L) were selected and applied to reactors in order to compare the production of Volatile Fatty Acids (VFA) and biological hydrogen yield of among themselves.

In this study; in order to produce bio-hydrogen via dark fermentation; two different types of anaerobic complex cultures were selected as inoculums. One of them was the digested sludge taken from anaerobic sludge digester of Kayseri Wastewater Treatment Plant; the other was the anaerobic reactor sludge treating olive oil effluent taken from Environmental Engineering Department Laboratory of Istanbul Technical University. These inoculums were boiled at 100^0C approximately for 15 minutes in order to inactivate hydrogen consuming bacteria. Glucose was used as a substrate (10000 mg/L ≈ 10000 mg COD/L Chemical Oxygen Demand).The performances of inoculums were investigated based on both the amount of Volatile Suspended Solid (VSS) ≈ 15000 mg VSS/L) and under same operating conditions (pH 5,9 – 3,3 and temperature= 37 ± 1 ^{0}C). The volumes of total gas, H_2, and CO_2 were measured. Consuming substrate and producing VFA such as butyrate, acetate, propionate and ethanol were determined. These parameters are important, due to the statement of

fertility of bio-hydrogen production via dark fermentation. Thus, not only dark fermentation mechanism was investigated but also the performances of two type anaerobic complex cultures were compared.

2. LITERATURE SURVEY on BIOHYDROGEN PRODUTION PROCESS

2.1 Introduction

Hydrogen gas is seen as a future energy carrier, which is renewable, does not evolve the "greenhouse gas" and is easily converted to electricity by fuel cells. Hydrogen can be produced from fossil fuels and natural gases by chemical, physical and physico-chemical processes (Vijayaraghavan *et. al.*, 2004). However, the hydrogen produced from those sources has adverse affect on the air quality and cause to danger for the environment. Hydrogen, as an environmentally friendly fuel and possessing a high energy yield (122 $kJ.g^{-1}$), provides clean energy generation, without pollution when burned in air and produced no greenhouse gases when combusted (Chang *et. al.*, 2002). Methane and hydrogen are important gaseous fuels produced by the anaerobic processing of organic wastes. However, methane and its combustion product carbon dioxide are both greenhouse gases; hence, hydrogen generates only water (Zhang *et. al.,* 2006).

Processes available for the production of hydrogen gas from non-fossil fuel resources include water electrolysis, thermochemical processes, radiolytic processes, and biological processes. Biological hydrogen production has several advantages over hydrogen production by photoelectrochemical or thermochemical processes. For biological production of hydrogen, several carbohydrates can be used as a carbon end energy source in the fermentation processes. On the contrary, hydrogen production from renewable organic wastes represents an important area for energy production (Han and Shin, 2003).

2.2 Energy Production Processes

The energy production processes from biomass can be divided into two general categories: Thermochemical and biological processes (Dennis *et. al.,* 2006).

2.2.1 Thermochemical Processes

Thermochemical process can be divided four into classes, which are combustion, liquefaction, gasification and pyrolysis. These are explained briefly in the following section.

2.2.1.1 Combustion

Combustion is the direct burning of biomass in air to convert the biomass chemical energy into heat, mechanical power or electricity using equipment such as stoves, furnaces, boilers or steam turbines, respectively. Due to low energy efficiency is low (10–30%) and the pollutant emissions from the processare; combustion is not a suitable hydrogen production method for sustainable development.

2.2.1.2 Liquefaction

In biomass liquefaction, biomass is heated to 525–600 K in water under a pressure of 5-20 MPa in the absence of air. Solvent or catalyst can be added to the process. The disadvantages of biomass liquefaction are difficulty to achieve the operation conditions and low production of hydrogen. Therefore, liquefaction is not favorable for hydrogen production.

2.2.1.3 Gasification

Biomass can be gasified at high temperatures (above 1000 K). The biomass particles undergo partial oxidation resulting in gas and charcoal production. The charcoal is finally reduced to forms of H_2, CO, CO_2 and CH_4. This conversion process can be expressed as follow:

$$\text{Biomass+heat + steam} \rightarrow H_2 + CO + CH_4 + CO_2 + \text{light and heavy hydrocarbons + char} \quad \textbf{(2.1)}$$

This process has taken more attention nowadays due to high yield of hydrogen.

2.2.1.4 Pyrolysis

Pyrolysis is the heating of biomass at a temperature of 650–800 K at 0.1–0.5 MPa in the absence of air to convert biomass into liquid oils, solid charcoal and gaseous

compounds. Pyrolysis can be further classified as slow pyrolysis and fast pyrolysis. Hydrogen can be produced from biomass via heat as given reaction (2.2).

$$\text{Biomass} + \text{heat} \rightarrow H_2 + CO + CH_4 + \text{other products} \quad \textbf{(2.2)}$$

Gaseous products include H_2, CH_4, CO, CO_2 and other gases depending on the organic nature of the biomass for pyrolysis. Liquid products include char and oils that remain in liquid form at room temperature like acetone, acetic acid, etc. Solid products are mainly composed of char and almost pure carbon plus other inert materials.

2.2.2 Biological Process

Although, biological hydrogen production is mainly realized by either photo-fermentative (algae) or dark fermentative bacteria (*Clostridia* spp.), it can be divided into five classes which are direct photolysis, indirect photolysis, biological water-gas shift reaction, photo-fermentation, dark-fermentation (Dennis *et. al.,* 2006). This processes were briefly discussed in following sections, however the dark fermentation processes was discussed in detail in the section of 2.3.

2.2.2.1 Direct Photolysis

Direct biophotolysis is a biological process using microalgae photosynthetic systems to convert solar energy into chemical energy in the form of hydrogen.

$$2H_2O + \text{light} \rightarrow 2H_2 + O_2 \qquad \text{(Microalgea)} \quad \textbf{(2.3)}$$

Water is separated by microalgea and its component with the help of solar energy. However, this reaction occurs slowly (Reith *et. al.,* 2003).

2.2.2.2 Indirect Photolysis

According to Gaudernack, the indirect biophotolysis involves the following four steps: (i) biomass production by photosynthesis, (ii)biomass concentration, (iii) aerobic dark fermentation yielding 4 mol hydrogen and 2 mol acetate per mol of glucose in algae cells, and (iv) conversion of 2 mol of acetate into hydrogen. In a typical indirect biophotolysis, *Cyanobacteria* are used to produce hydrogen via the following reactions (Dennis *et. al.,* 2006 and Reith *et. al.,* 2003):

$$6CO_2 + 6H_2O + light \rightarrow C_6H_{12}O_6 + 6O_2 \quad \text{(microalgea)} \quad \textbf{(2.4)}$$

$$C_6H_{12}O_6 + 2H_2O \rightarrow 4H_2 + 2CH_3COOH + 2CO_2 \quad \text{(cyanobacteria)} \quad \textbf{(2.5)}$$

$$2CH_3COOH + 4H_2O + light \rightarrow 8H_2 + 4CO_2 \quad \textbf{(2.6)}$$

$$\text{Overal reaction: } 12H_2O + light \rightarrow 12H_2 + 6O_2 \quad \textbf{(2.7)}$$

2.2.2.3 Biological Water-Gas Shift Reaction

Some photoheterotrophic bacteria, such as *Rhodospirillum rubrum* can survive in the dark environment by using CO as the sole carbon source to generate ATP by coupling the oxidation of CO to the reduction of H^+ to H_2.

$$CO + H_2O \rightarrow H_2 + CO_2 \quad \text{(fermentative and photosyntetic bacteria)} \quad \textbf{(2.8)}$$

In equilibrium, the dominating products are H_2 and CO_2. Therefore, this process is favorable for hydrogen production (Reith *et. al.,* 2003).

2.2.2.4 Photo-Fermentation

Photosynthetic bacteria have the capacity to produce hydrogen through the stimulation of nitrogenase using solar energy and organic acids or biomass. This process is known as photo-fermentation, as illustrated in Figure 2.1 (Dennis *et. al.*,2006) and reaction 2.9 (Reith *et. al.,* 2003).

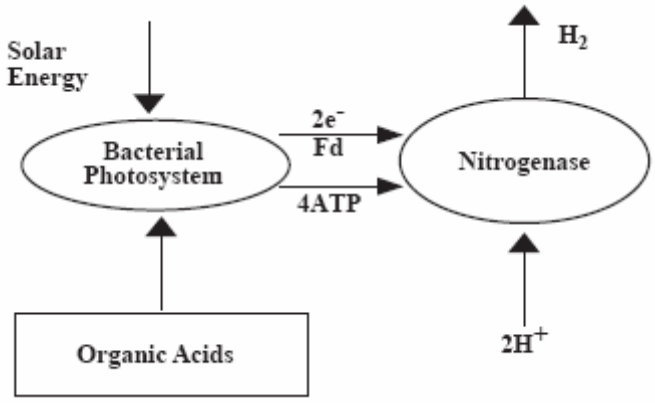

Figure 2.1: Photo-fermentation system (Dennis *et. al.,* 2006).

$$CH_3COOH+2H_2O+light \rightarrow 4H_2+2CO_2 \qquad \text{(microalgea)} \qquad \textbf{(2.9)}$$

Photo-fermentative bacteria can directly utilize the biomass. However, the biomass degrading capacity of these bacteria is lower than acetic acid.

2.2.2.5 Dark-Fermentation

Hydrogen can be generated simultaneously, while organic compound or biomass is broken into organic acids such as acetate, butrate, lactic acid etc. Organic matter, which contains high amount of carbohydrate, can be degraded efficiently by to hydrogen dark fermentation. This process is illustrated in Figure 2.2. Dark fermentation achieves a much higher H_2 production rate and is considered as more applicable for simultaneous waste reduction and H_2 generation (Li L. *et. al.,* 2006).

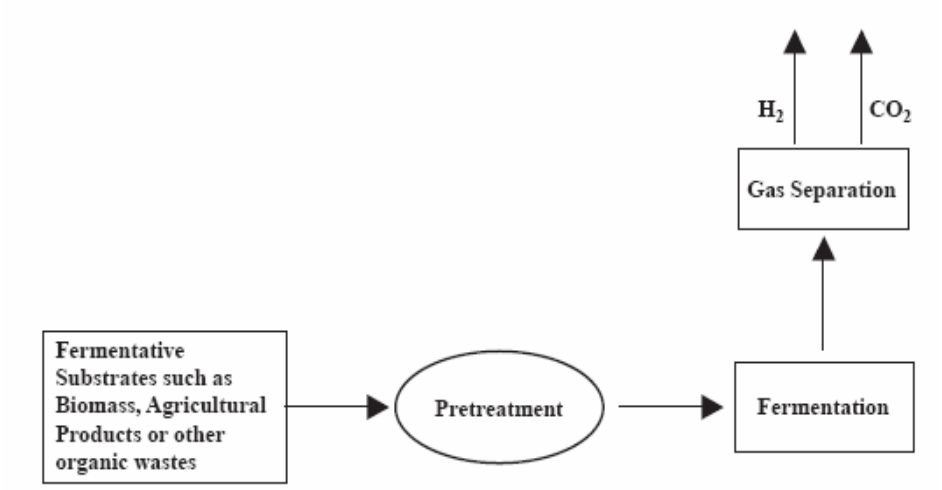

Figure 2.2: Dark fermentation system (Hawkes *et.al.,* 2002).

Producing biohydrogen via dark fermentation has several important advantages, compared to photo-fermentation process; such as independent of light, high level degradation of organic matter, faster and more hydrogen generating yield, valuable metabolites i.e., butrate, acetate, ethanol, and no oxygen limitation (Chen *et. al.,* 2005 and Lee *et. al.,* 2004). Bio-hydrogen production is comprehensively discussed in the section of 2.3.

2.2.3 Hydrogen Storage Systems

Safe storage and transformation of hydrogen are the most important for issues reaching the ultimate goal. Hydrogen is the lightest and the most attractive element on earth. Therefore, it is very difficult to collect, store and transfer. However, there are various transportation means for H_2, including trucking in gaseous form, trucking in liquefied form, trucking in metal hydride storage containers or tanks and low pressure pipeline transportation. H_2 may be stored in one of the following ways: compressed gas bottles, cryogenic liquid, metal hydride, in carbon structure and as chemical storage. Gaseous H_2 storage is a reasonable way of storage, since the H_2 is produced in gaseous form. The compression of H_2 gas in industrial cylinders showes that the energy required for compression from less than 1% (w/w) to 5–10% is quite costly. As H_2 is one of the lightest elements, its energy per unit volume is comparatively low. Currently composite gas cylinders achieve 2.1% compression. The highest price cylinders in the market demand gas pressures of 66.7 bar and the compression process required to reach these pressures is very intensive energy, while liquefied H_2 has a gravimetric density of 7.1% (w/w) and equals to approximately three times the energy per unit mass of petrol. To store H_2 as a liquid, the temperature needs to be lowered to 20°K, which is an energy-intensive process, reducing the net energy value. The energy needed to store liquid H_2 is equivalent to 25–30% of its energy content. Solid storage is an emerging technology that uses rechargeable metallic hydrides; these systems can store H_2 from 1 to several percentages. However, its high capital cost and gaseous impurities may not be tolerated (Vijayaraghavan *et. al.,* 2004; Hawkes *et. al.,* 2002).

2.3 Production of Biohydrogen

Earlier studies demonstrated that the pure cultures of some anaerobic bacteria converted carbohydrates (such as glucose and starch) to hydrogen gas, e.g., *Enterobacter* (Rachman *et. al.,* 1997), *Aspergillus terreus* (Emtiazi *et. al.,* 2001). Later, it was found that especially *Clostridium spp.,* responsible for the conversion of carbohydrates to hydrogen gas (Nandi and Sengupta, 1998; Taguchi *et. al.,*1994). The researches has been recently focused on the fermentative hydrogen-production from biomass using mixed cultures (Fan *et. al.*, 2002; Ginkel *et. al.*, 2001; Lay *et. al.*, 1999). Ueno and co-workers (2001) exhibited the hydrogen-production from an

artificial medium containing cellulose powder by thermophilic anaerobic microflora enriched from sludge compost. Fan and co-workers (2004) have successfully used a heat-shocked cow dung compost to convert a simulated organic wastewater into hydrogen gas. It was also found that the mixed microbial cultures taken from compost pile, a potato field and sludge, generated hydrogen from sucrose or glucose in batch experiments (Lay *et. al.*, 1999). Some investigators used mixed cultures taken from anaerobic digestier sludge and biomass composting, to generate hydrogen from cellulose by a batch culture (Reith *et. al.,* 2003; Kosaric and Lyng,1988; Nandi and Sengupta, 1998). However, only traces of hydrogen were usually evolved in the continuous flow reactors due to the ubiquitous nature of hydrogen consumers and commonly seen inter-specific hydrogen transfer reactions. If the bioactivity of hydrogen consumers could be inhibited, the generation of hydrogen might be expected in anaerobic fermenters.

The mixed cultures of anaerobic digesters or anaerobic bioreactors have advantages over pure cultures due to its inexpensive and practical applications. The seed of the hydrogen-producing fermenters were generally heated around 100°C, before initializing the process. It was necessary to avoid the presence of H_2 utilizing microorganisms, particularly methanogens. Beside heat treatment, operating the continuous reactors at low pH and/or low sludge ages were other techniques used for the elimination of H_2 utilizing microorganisms. It is known that methanogens are slow growing microorganisms and easily affected by low pH. However, non-sterile fermentable organic feed stocks used in continuous processes for a viable H_2 producing technology (Hawkes *et. al.,* 2002).

Several researches displayed that hydrogen production is more efficient using carbohydrates than other materials. Simple sugars, such as sucrose and glucose, are converted to hydrogen with high conversion efficiencies at elevated temperatures. For example, 61% of the maximum possible biological hydrogen production from sucrose (assuming a maximum possible yield of 8 mol-H_2/mol-sucrose) was recovered under the optimum operational conditions (Kim *et.al.,* 2004). Same study, also showed that glucose and sucrose conversion to H_2 were 28% and 26%, respectively, at 30°C. (maximum yield of 4 mol- H_2/mol-glucose). However, hydrogen production from molasses, lactate, and cellulose were 15%, 0.5% and 0.075%, respectively. These results indicated that high-carbohydrate containing

wastewaters would be the most effective operation of fermenters for industrial production of hydrogen.

The seed and operational conditions of the fermenters have a significant effect on H_2 yield, as they influence the fermentation end products. For example, fermentation of hexose to acetate or butyrate produces H_2 and CO_2. On the contrary, fermentation of hexose to propionate or lactate produces no any H_2. The latter reaction can be explained by the usage of H_2 as a reducing power to obtain more reduced fermentation end products such as ethanol and lactate. Therefore, ethanol production gives correspondingly lower H_2 yields. If it is desired to gain more H_2 product, it is important to establish bacterial metabolism resulting in acetate and butyrate as end products (Hawkes *et. al.,* 2002).

As stated before, carbohydrates are the preferred organic carbon source for hydrogen production. Glucose (or in principle its isomer hexoses or its polymers starch and cellulose) fermentation gives a maximum 4 mol H_2 per mol of glucose when acetic acid is the end product.

$$C_6H_{12}O_6 + 2H_2O_2 \rightarrow 2CH_3COOH + 2CO_2 + 4H_2 \quad \textbf{(2.10)}$$

If butyrate is the end product, maximum 2 mol H_2 per mol of glucose can be obtained.

$$C_6H_{12}O_6 \rightarrow CH_3CH_2CH_2COOH + 2CO_2 + 2H_2 \quad \textbf{(2.11)}$$

If ethanol is the end product, maximum 1 mol H_2 per mol of glucose can be obtained with subsequent ethanol fermentation (Mas *et. al.,* 2006).

$$C_6H_{12}O_6 + 2H_2O \rightarrow 2C_2H_5OH + 2CO_2 \quad \textbf{(2.12)}$$

$$2C_2H_5OH \rightarrow 2C_2OH_4 + H_2 \quad \textbf{(2.13)}$$

The H_2-producing metabolism preceded by enzymatic electron transfers via electron-carrying complexes $NADH_2$, $FADH_2$ with the aid of hydrogenase or nitrogenase (Mu *et. al.,* 2006). Hydrogen production occurs from conversion of carbon source to end products such as acetate, butrate and ethanol with the help of both specific enzymes. Environmental factors particularly pH and temperature affects this mechanisms.

Critical factors for the optimal production of hydrogen have been reported as the hydrogen partial pressure, pH and mixing regime of the fermenters, nutrients and carbon source of the feed, and the existence of hydrogenotropic methanogens

(Gavala *et. al.,* 2005). It was found that proper pH control is a key factor to improve the germination of the *Clostridia* spp., as well as to initiate and operate a hydrogen-producing bioprocess (Han and Shin, 2003). Appropriate control of retention time is also an important factor to avoid biomass washout and to increase biomass concentration in the reactor.

In general, attached growth systems, although not suitable for high solids concentration in the influent, allow for better retention of active microbial mass, lower hydraulic retention times, higher substrate conversion efficiency and in many cases suffer from lower product inhibition than the suspended growth systems (Gavala *et*, *al.,* 2005 and Lee *et. al.,* 2004). On the other hand a carrier-induced granular sludge bed bioreactor is able to significantly increase the retention of H_2-producing sludge and thereby being very efficient in biohydrogen production, which have been developed recently (Lee *et. al.,* 2004). However, direct comparison of attached growth systems with conventional suspended growth systems for hydrogen production is not yet available in the literature.

2.3.1 Mechanisms of Biohydrogen Production in Dark Fermentation

Dark fermentation processes resemble to the processes, preceeding in conventional anaerobic treatment systems. So, anaerobic processes firstly clarified before explaining the dark fermentation mechanisms.

The biochemistry and microbiology of anaerobic digestion is a complex biogenic process involving a number of microbial populations, often linked by their individual substrate and product specificities. As shown in Figure 2.3, the conversion of complex substrate ingredients proceeds via the formation of numerous intermediate products. The first group of organisms which take place in anaerobic digestion are the hydrolytic fermentative (acidogenic) bacteria. These bacteria hydrolyze the complex polymer substrate to organic acids, alcohols, sugars, hydrogen, and carbon dioxide. The second groups are hydrogen producing and acetogenic organisms, which convert the fermentation products of the previous step (hydrolysis and acidogenesis) into acetate and carbon dioxide. The third group is the methanogens, which convert simple compounds as acetic acid, methanol, and carbondioxide+hydrogen into methane. In degradation pathway of organic substrates

(proteins, carbohydrates, lipids) in anaerobic processes six distinct steps can be identified (Gujer and Zehnder ,1983). These steps are,

1. Hydrolysis of organic polymers.

2. Fermentation of amino acids and sugars to hydrogen, acetate and short-chain VFA (volatile fatty acids) and alcohols.

3. Anaerobic oxidation of long-chain fatty acids and alcohols.

4. Anaerobic oxidation of intermediary products such as volatile acids (except acetate).

5. Conversion of acetate into methane by acetotrophic organisms.

6. Conversion of hydrogen into methane by hydrogenotrophic organisms (carbon dioxide reduction).

In some literatures, anaerobic degradation process was reprensented by more than six steps. Harper and Pohland identified the process by nine steps. These authors, for example, have separated the groups of obligate hydrogen-producing acetogens, nitrate- and sulfate-reducing bacteria from other trophic groups. Generally, anaerobic process is corresponded to four main steps: hydrolysis, acidogenesis, acetogenesis, and methanogenesis. Considering the feed of anaerobic process is composed of easily biodegradable materials (containing short-chain VFA, monomeric saccharides, etc.) the rate limiting step of anaerobic degradation will be the methanogenesis (step 5 and 6, as mentioned above). On the other hand, during the anaerobic digestion of complex materials (e.g. agricultural wastes, which are mainly composed of cellulose), the rate limiting step of the process will be the hydrolysis. In the design of the anaerobic bioreactors, substrate nature is the decisive mechanism for the selection of optimum configuration. Several analytic parameters, such as the Chemical Oxygen Demand (COD), the Total Organic Carbon (TOC), VSS/SS (Volatile Suspended Solids/Suspended Solids), proteins, fats, and carbohydrates content give enough information about the nature of complex substrates.

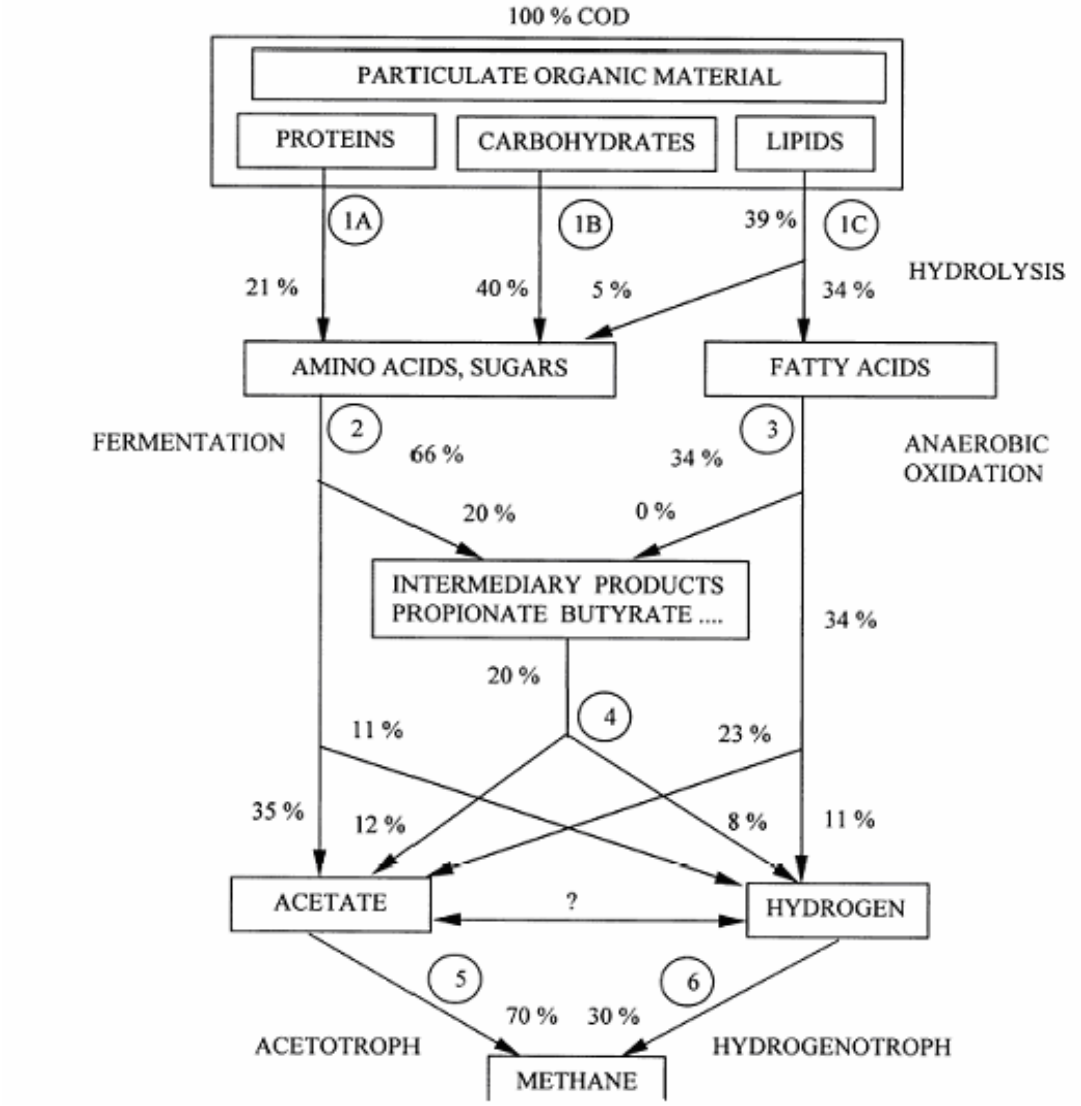

Figure 2.3: Schematic diagram showing the bioconversion reactions taken place in the anaerobic digestion (Hutnan *et. al.,* 1999).

However, from these parameters, it is sometimes difficult to determine exactly of which the steps is rate limiting. It is evident that the experimental method for determining the microbial activity in the different steps of the degradation of complex substrate is necessary (Hutnan *et. al.,* 1999).

Anaerobic treatment processes consist of two main pathways: acidification and methanogenesis. Rapidly biodegradable matters especially glucose and its isomer are frstly converted to volatile fatty acids, such as acetic acid, butyric acid, lactic acid etc. defined as an acidification phase. Hydrogen is also produced simultaneously in this phase. This hydrogen can be used as an electron donor by methanogens at the final stage of the process. However, H_2 itself is of high commercial value and clean

energy source. Therefore, instead of usage of H_2 by methanegens, it can be withdrawn from the system for the energy usage. On the other hand, the production of H_2 from carbohydrates is more complex than methane production. (Kim *et. al.,* 2005) A further advantage of the bio-hydrogen processes is the possibility of CO_2 capturing in the production phase which can be the additional, "bonus", opportunity to reduce overall CO_2 emissions (Reith *et. al.,* 2003).

Hydrogen production ratio is approximately 30% prior to methane production (Hutnan *et. al.,* 1999 and Kim *et. al.* 2005). If hydrogen consuming bacteria are inactiveted and environmental conditions are adjusted to hydrogen producing bacteria, the proportion of generating hydrogen is able to enhance approximately 60% (Lay *et. al.,* 1999, Hawng *et. al.,*2004 and Kim *et. al.,* 2005). The maximal removal efficiency of carbohydrates was approximately 97% and also other organic compounds were converted (Reith *et. al.,* 2003).

However, producing volatile fatty acid and ethanol with dark fermentation are not reached to final their oxidation levels (Ginkel *et. al.,* 2005). These products have two or more carbon atoms. For example acetate and ethanol have two carbon atoms while butrate involve four carbon atoms. Total Soluble Chemical Oxygen Demand is only converted CO_2 and cell products in dark fermentation system as final products. (Vijayaraghavan *et. al.,* 2004; Hawkes *et. al.,* 2002). The proportion of degradation of Total Soluble Chemical Oxygen Demand is approximately 12-19 % , while the proportion of its residual is approximately 80% (Kim *et.al.,* 2004 and Ginkel *et. al.,*2005). To not reach to final oxidation level is main drawback of generating hydrogen via dark fermentation from waste/wastewater (Gavala *et. al.,* 2005). So that hydrogen production by anaerobic system is combined with several applications to reach to final products. COD is degraded as showed schematically.

The other effective application was the combination of dark and photo-fermentations (Figure 2.5). Producing VFA during dark fermentation is good substrate for photo-fermentation bacteria. H_2 and CO_2 produce as final products (Reith *et. al.,* 2003).

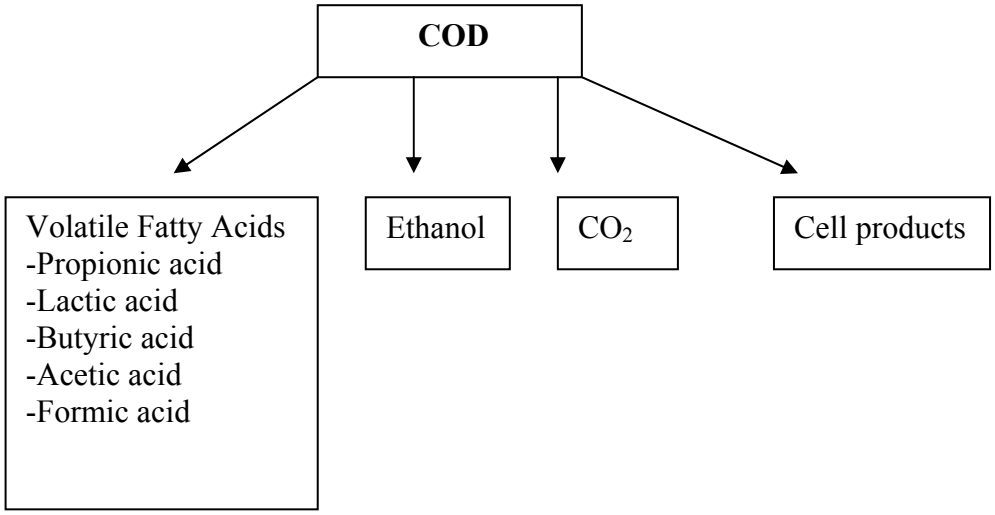

Figure 2.4: Final and intermediate products depending on degradation of substrate by dark fermentative microorganisims.

One of the important experinces gained from the anaerobic processes were the production of ethanol and hydrogen simultaneously in the same reactor (Ahring *et. al.*, 2004 and McMillan, 1997).

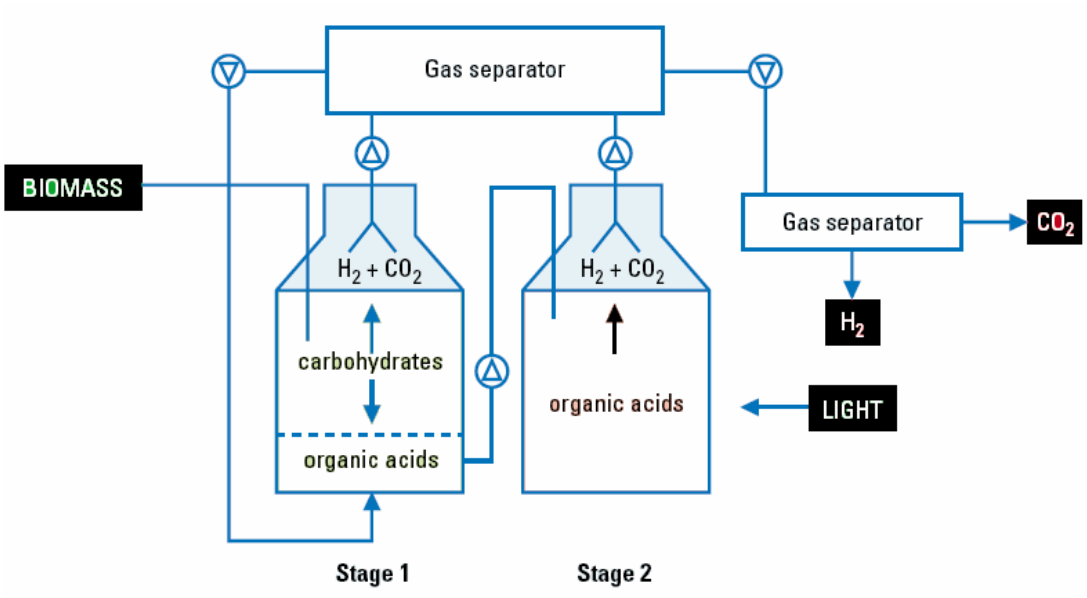

Figure 2.5: Dark and photo fermentation two stage system. Outline of the bioprocess for production of hydrogen from biomass in a 2 stage fermentation. Stage 1 is for heterotrophic fermentation of carbohydrates to hydrogen, carbon dioxide and organic acids. In stage 2 the photoheterotrophic fermentation of organic acids to hydrogen and carbon dioxide takes place (Reith *et. al.*, 2003)

The most remarkable application is that dark fermentation put together to methanization phase (Figure 2.6). Hydrogen production rate increase while methane productions reduce in this two-stage system as being different from conventional anaerobic systems (Vijayaraghavan *et. al.,* 2004 and Hawkes *et. al.,* 2002). H_2, CH_4, and CO_2 produce as final products (Hawkes *et. al.,* 2002). The produced hydrogen from biomass by two-stage fermentation is required for complete conversion of sugars to hydrogen.

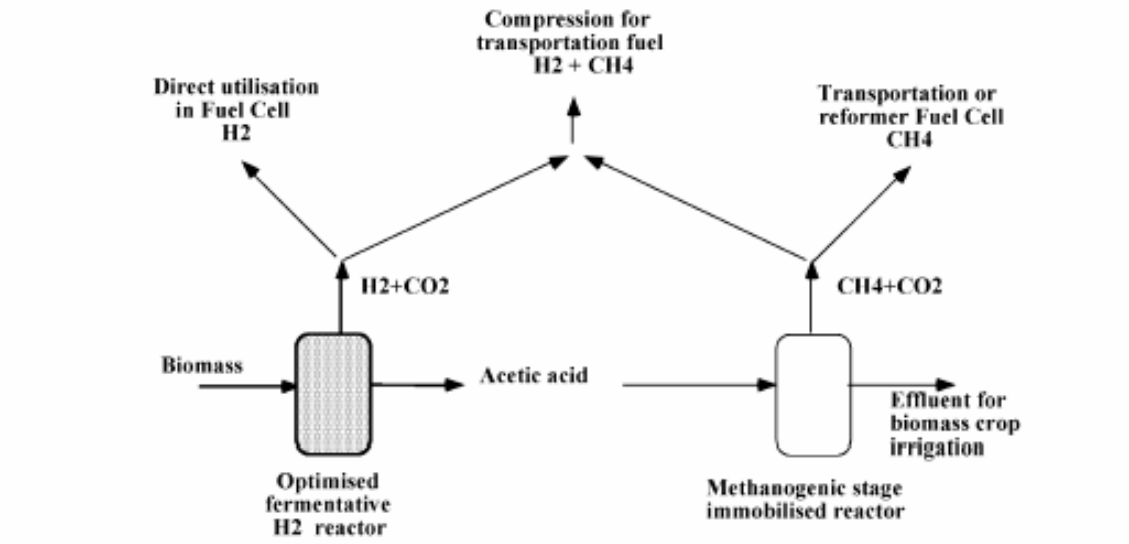

Figure 2.6: Two-stage anaerobic system. While organic acids, carbon dioxide and hydrogen are produced in the first reactor, organic acids are converted to methane and carbon dioxide in the second reactor (Hawkes *et. al.,* 2002)

2.3.2 Hydrogen Producing Microorganisms

There are three main groups of microorganisms, which can produce hydrogen (Reith *et. al.,* 2003; Kosaric and Lyng,1988; Nandi and Sengupta, 1998). These are i)strictly anaerobic, ii)facultatively anaerobic and iii) aerobic microorganisms. Brief descriptions of these organisms were given in the following section.

2.3.2.1 Strictly Anaerobic Microorganisms

Clostridia Spors and Species: Clostridia species are able to product hydrogen from carbohydrates such as glucose, sucrose, hexose and starch (Ginkel *et. al.,* 2005; Kosaric and Lyng,1988; Nandi and Sengupta, 1998). The highest maximal yield of 4 mole H_2 from 1 mole of glucose is produced in acetic acid fermentation. Also,

Clostridia spp. can be used for the production of H_2 from both cellulose and hemicellulose present in plant biomass (Fan Y.T. *et. al.,* 2005; Kim *et. al.,* 2004 and Lay *et. al.,* 2003).

C. butyricum, C. welchii, C. pasteurianum, C. beijerincki, newly isolated *Clostridium* spp. And mixtures of Clostridia have been used in studies dedicated to produce high amounts of hydrogen. Taguchi and colleagues isolated various new Clostridia strains. A growing culture of *C. Beijerincki* AM21B isolated from termites yielded 1.8 to2.0 mole H_2 on glucose. The strain could also utilize a large number of other carbohydrates, such as xylose, arabinose, galactose, cellobiose, sucrose, and fructose with efficiencies from 15.7 to 19.0 mmol/g of substrate in batch fermentations of 24 h. H_2 was also produced from starch with equal efficiencies, but sustained production was not achieved and production ceased before the exhaustion of carbohydrates in the medium (Reith *et. al.,* 2003). Clostridia have also been used in continuous hydrogen fermentations with glucose (Lay *et. al.,* 2003).

Thermophiles: H_2 can be produced from carbohydrates using hyperthermophilic microorganism in an anaerobic fermentation process. Growth and H_2 production by two extreme thermophiles namely *Caldicellulosiruptor saccharolyticus* and *Thermotoga elfii* during sugar fermentation was investigated. The results showed that *C. saccharolyticus* and *T. elfii* reached maximum cell densities of 1.1×109 and 0.8×109 cells/ml, respectively, while their maximum H_2 production rates were 11.7 and 5.1 mmol/g dry weight/h, respectively. The members of the order Thermotogales demonstrated the ability to produce H_2. The investigation with *Thermotoga neapolitana* revealed consistent H_2 production between 25% and 30% along with carbon dioxide (12–15%) as a prominent by product. The results further demonstrated that *Thermotoga neapolitana* can tolerate and utilize moderate amount of oxygen in the gaseous phase of the batch reactor (6–12%), with no apparent decrease in H_2 production (Vijayaraghavan *et. al.,* 2004). However, there is a lack of direct comparison of the thermophilic with mesophilic hydrogen production (Gavala *et. al.,* 2005).

Rumen Bacteria: Other strict anaerobic bacteria producing hydrogen are rumen bacteria.

2.3.2.2 Facultatively Anaerobic Microorganisms

Enterobacter: *Enterobacter* as well as other members of the Enterobacteriaceae can have several beneficial properties favourable for H_2 production. In addition to high growth rates and utilization of a wide range of carbon sources, H_2 roduction by *Enterobacter* is not inhibited by high H_2 pressures. However, the H_2 yield on glucose is normally lower compared to that of e.g. Clostridi (Reith *et. al.,*2003).

***Esesheria Coli* (*E.Coli*):** *E. coli* has been shown to be capable of producing H_2 and CO_2 from formate in the absence of oxygen. The catalytic activity, called formate hydrogenlyase, was shown to be a membrane bound multi-enzyme complex, consisting of a formate dehydrogenase and a hydrogenase (Reith *et. al.,* 2003).

Citerobacter: A newly isolated *Citrobacter* sp. Y19 for CO-dependent H_2 production was studied for its capability of fermentative H_2 production in batch cultivation. When glucose was used as carbon source, the pH of the culture medium significantly decreased as fermentation proceeded and H_2 production was seriously inhibited (Oh *et. al.,* 2003).

2.3.2.3 Mixed Cultures

In industrial applications the use of mixed cultures for hydrogen production from organic wastes might be more advantageous because pure cultures can easily become contaminated with H_2 consuming bacteria (Vijayaraghavan *et. al.,* 2004, Reith *et. al.,* 2003) and are expensive (Ginkel *et. al.,* 2005 and Gavala *et. al.,* 2005). So that some investigators used natural anaerobic microorganisms, taken from anaerobic digestion sludge and sludge compost, to generate hydrogen from cellulose by a batch culture (Ueno *et al.,* 1995). However, only traces of hydrogen are usually evolved with continuous flow digesters due to the ubiquitous nature of hydrogen consumers and inter-specific hydrogen transfer reactions (Kidby and Nedwell, 1991). If the bioactivity of hydrogen consumers can be inhibited, the anaerobic treatment of biowaste may be expected to have a potential to generate hydrogen (Sparling *et al.,* 1997). On the other hand, a continuous fermentation of *Clostridium butyricum* and *Enterobacter aerogenes* in which the higher H_2 yield of the strict anaerobe and the oxygen consumption by the facultative anaerobe were combined. This resulted in fermentations with no need for an expensive reducing agent since the presence of *E.*

aerogenes was sufficient to rapidly restore anaerobic conditions in the fermentor upon short oxygen exposures (Reith *et. al.,* 2003).

Microflora for mixed cultures have been isolated from various sources, such as fermented soybean meal or sludges from anaerobic digesters of municipal sewage or organic waste and sludge from kitchen waste water. These microflora often contain unwanted bacteria such as methanogens which consume the produced hydrogen and convert it to methane. Enrichment cultures of the microflora are prepared by forced aeration of the sludge or by heat treatment which inhibits the activity of the hydrogen consumers while the spore forming anaerobic bacteria survive. Additionally, in continuous fermentations higher dilution rates are used to wash out the slow growing methanogens and select for the acid producing bacteria (Reith *et. al.,* 2003).

Biological H_2 production from organic fraction of municipal solid waste was investigated with two seed microorganisms, namely heat-pretreated digested sludge and H_2-producing bacteria enriched from soybean-meal silo. The contour plots showed that high H_2 - production potentials of 140 and 180 ml H_2 /g TVS occurred with pretreated digested sludge and the H_2-producing bacteria enriched from soybean meal silo was used as a seeding material. A high hydrogenic activity for the pretreated digested sludge (45 ml/g VSS h) was obtained at a high food-to-microorganism F/M ratio; however, that for the H_2-producing bacteria (36 ml/g VSS h) was found at a low F/M ratio. The experimental results showed that the H_2 composition of the biogas was greater than 60% except for initial incubation and no significant methane was found throughout this study (Vijayaraghavan *et. al.,* 2004).

Studies of last years indicated that dispersion of microflora is related with fermentation conditions. Diversity of microorganisms can be changed in anaerobic mixed culture if a environmental condition is changed. Iyer et al. and Xing et al. appear to be the first to follow bacterial population shifts under different conditions in a H_2-producing reactor. Iyer et al. studied a CSTR at pH 5.5 operating on glucose. At 10 h HRT only *Clostridiaceae* were detected while at 30 h HRT the populations were more diverse and included *Bacillaceae* and *Enterobacteriaceae.* At 10 h HRT when the temperature was changed from 30 to 37°C there was a population shift from populations related to *Clostridium acidisoli* to *C. acetobutylicum*. Xing and coworkers followed communities in a CSTR operating on molasses at a low pH with acidophilic bacteria from sewage, which established an ethanol-acetate hydrogen

producing community after 28 days. The hydrogen production rate increased with the increase of *Ethanologenbacterium sp*., *Clostridium sp*. and *Spirochaetes*. Some types of *Clostridium sp*., *Acidovorax sp*., *Kluyvera sp*. and *Bacteriodes* were dominant populations throughout. It appeared that hydrogen production depended not only on hydrogen producers but also on co-metabolism in the whole community (Hawkes *et. al.,* 2006).

Lin et al. (2006) using a CSTR with 20 g l^{-1} sucrose COD at HRTs between 2 and 12 h showed a *Clostridium ramosum* related species present at all HRTs, but a *Clostridium pasteurinum* related species to be present at 12, 8 and 4 hHRT only. A transition in the community structure as HRT was decreased to 0.5 h in a stirred granular reactor showed a dominant species with high similarity to a *C. pasteurianum* strain associated with an increased specific hydrogen production rate and referred to by Wu et al. (2006) as the "superstar" hydrogen-producing species in the consortium used. Kim et al. (2005) studying a CSTR operating at 12 h HRT with different sucrose concentrations found a predominance of clostridial species, one of which at 10 g l^{-1} sucrose COD was related to an acetogen, and at 60 g l^{-1} sucrose COD a band related to the spore-forming, lactic acid-producing *Bacillus racemilacticus*. These species have a deleterious effect on hydrogen production and it is interesting that they predominate at different substrate concentrations (Hawkes *et. al.,* 2006).

2.3.3 Network of Biohydrogen with Anaerobic Mixed Culture

Succinate, malate, formete, valerate, caproate, lactate, propionate, butrate, acetate, ethanol buthanol and propanol can be formed from pyruvate, while glucose is degraded by anaerobic mixed culture (Figure 2.7). However, lactate, propionate, butrate, acetate and ethanol are the main end products of glucose fermentation. In Figure 2.6 demonstrates how these by-products occur through degradation of glucose. Relationship biohydogen production with intermediate products was tried to reveal in the title H_2 generation and intermediate products. In conclusion; hydrogen can be generated by anaerobic mixed culture simultaneously generating intermediate products such as acetate, butrate ethanol from degradation of glucose as shown figure. However hydrogen can not be produced with generating all the intermediate products such lactate and propionate. (Arhing *et.al.,* 2004; Wang *et.al.,*2006).

Pyruvate formation from glucose accompanied byATP and NADH generation via the Embden–Meyerhoff pathway. Only the stochiometry of this conversion is considered, no kinetic description is established and consequently a pyruvate concentration needs to be assumed (Rodruguez *et.al.*, 2006). No detected is hydrogen while pyruvate is produced since generating H_2, ATP and NADH/NAD reduced-couple are consumed to growth of microorganism, and to produce of intermediate products. (Rodruguez *et.al.*, 2006).

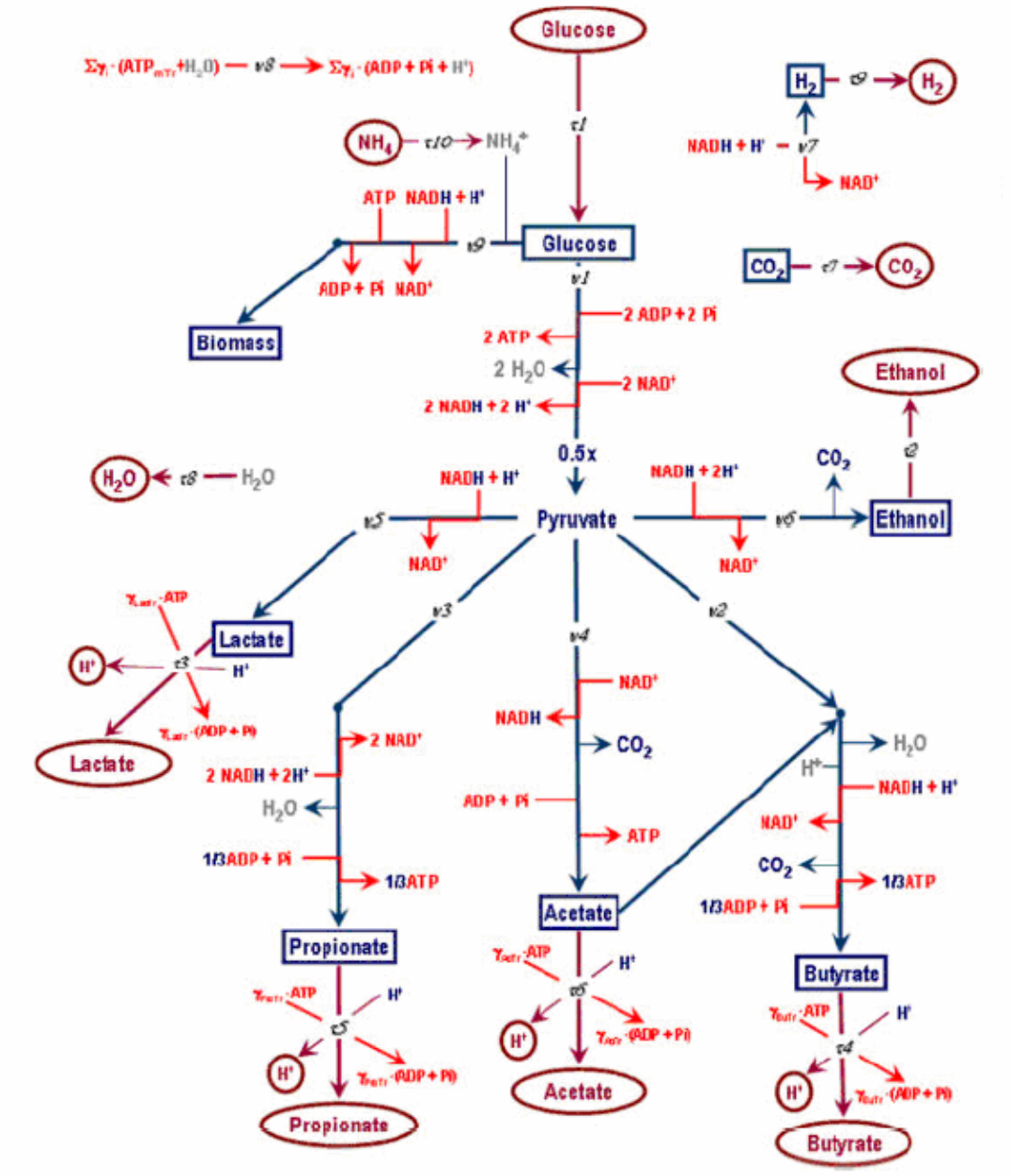

Figure 2.7: Network of glucose degradation in anaerobic process (Rodruguez *et al.*, 2006).

The majority of microbial H_2 production is driven by the anaerobic metabolism of pyruvate, formed during the catabolism of various substrates.

The breakdown of pyruvate is catalyzed by one of the two enzyme systems (Vijayaraghavan *et al.*, 2004). Pyruvate: formate lyase (PFL):

$$\text{(Pyruvate + CoA)} \xrightarrow{\textbf{PFL}} \text{acetyl-CoA + formate} \qquad \textbf{(2.14)}$$

Pyruvate: ferredoxin (avodoxin) oxido reductase (PFOR):

$$\text{Pyruvate + CoA + 2Fd(ox)} \xrightarrow{\textbf{PFOR}} \text{acetyl-CoA + CO2 + 2Fd (red)} \qquad \textbf{(2.15)}$$

Fd(ox): ferrodoxin oxidanse (metal-cluster free hydrogenase)

Fd(red): ferrodoxin reductanse (metal-cluster free hydrogenase)

Butyrate production by reduction and decarboxylation of pyruvate consuming one acetate. One of the bioreactions of the lumped pathway corresponds to the reduction of crotonyl-CoA to butyryl-CoA with $FADH_2$ as electron donor under formation of FAD. Subsequent reduction of FAD is assumed to be accompanied by NADH oxidation by translocation of one proton across the cytoplasmic membrane. Assuming a H^+/ATP yield of 3 this implies that crotonate reduction is accompanied by NAD^+ production and the formation of 1/3 ATP (Rodruguez *et al.*, 2006).

Propionate production by reduction of pyruvate through the succinate–fumarate pathway lumped. Propionate formation is assumed to be accompanied by the production 1/3 ATP during $FADH_2$ dependent fumarate reduction, analogue as in butyrate production. Acetate is produced by pyruvate oxidation and decarboxylation. One ATP is obtained by substrate level phosphorylation. Lactate is generated by reduction of pyruvate. Ethanol is produced by reduction and decarboxylation of pyruvate (Rodruguez *et al.*, 2006).

2.3.4 Metabolism of Biohydrogen Production

Although biohydrogen production metabolism is not completely defined yet, (Lay *et. al.,* 2003; Gavala *et. al.*, 2005) a lot of studies are tired to explain about this station nowadays.

Xi Chen and co-workers had made important study to define metabolism and stochiometry of biohydrogen production with both Clostridium butyricum, a typical

strictly anaerobic bacterium, and *Klebsiella pneumoniae*, a nitrogen-fixing facultative bacteria in 2005. This study has been summarized here in order to provide an understanding of the bio-hydrogen metabolism.

Clostridium butyricum, a typical strictly anaerobic bacterium, is known as a classical acid producer and usually ferments carbohydrates to butyrate, acetate, carbon dioxide and molecular hydrogen. As the metabolic products are concerned, acetate and butyrate are the main products when $NADH_2$ and ATP are generated in the bioprocess. There are two pathways to produce hydrogen superficially, as shown in Fig.2.8. One is the cleavage of pyruvate to acetyl-CoA, CO_2 and H_2, which is catalyzed by pyruvate: ferredoxin oxidoreductase (PFOR). On this pathway, a part of the electrons are transferred to protons to produce hydrogen and the other to NAD^+ to generate $NADH_2$. Then $NADH_2$, involving reducing equivalents generated in glycolysis, is used to produce H_2 on the second pathway, which is carried out by hydrogenase. The two pathways can be classified into one way, i.e., electrons deriving from the oxidation of substrate are transferred to ferredoxin and then to H^+ for H_2 production catalyzed by hydrogenase. In conclusion, hydrogen produced by strictly anaerobic bacteria mainly contributes to hydrogenase.

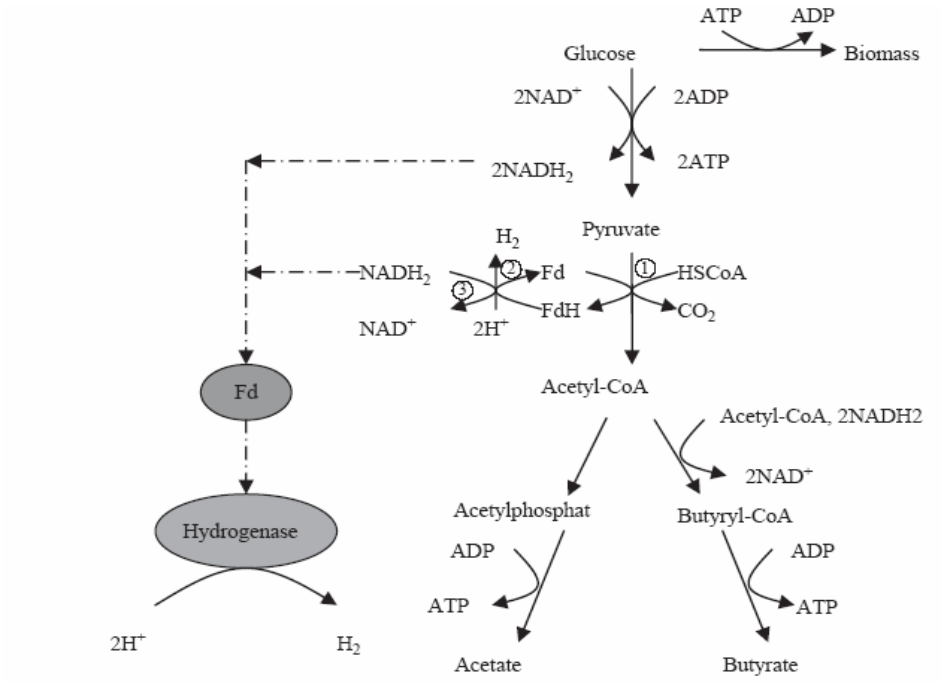

Figure 2.8: Metabolic pathway of glucose by *Clostridium butyricum* under anaerobic conditions. 1 Pyruvate: ferredoxin oxidoreductase (PFOR); 2 Hydrogenase; 3 NADH: ferredoxin oxidoreductase (Chen X.,*et. al.,* 2005).

Generating hydrogen microorganisms are able to produce hydrogen from biomass with help of their enzymes. These enzymes can be divided main two catagories, as hydrogenase and nitrogenase.

2.3.4.1 Hydrogenase

Three kind of hydrogenases enzymes had been described namely(Mertens and Liese, 2004);

1- NiFe hydrogenase (including sub-family of Ni Fe hydrogenase)

2- Fe-hydrogenase and metal free hydrogenase

3- FeS metal-cluster free hydrogenase.

Three fundamental reactions are catalyzed by hydrogenases;

1-Isotopic hydrogen exchange reactions:

$$1H\ \ 2H + 1H_2O \rightleftharpoons 1H_2 + H\ 2HO \qquad \textbf{(2.16)}$$

$$1H\ \ 3H + 1H_2O \rightleftharpoons 1H2 + H\ 3HO \qquad \textbf{(2.17)}$$

$$1H\ \ 2H + 2H_2O \rightleftharpoons 2H_2 + H\ 2HO \qquad \textbf{(2.18)}$$

2- Para-orto hydrogen conversion:

$$p\text{-}H_2 \rightleftharpoons o\text{-}H_2 \qquad \textbf{(2.19)}$$

3- Reversible oxidation of molecular hydrogen:

$$H_2 \rightleftharpoons 2H^+ + 2e^- \qquad \textbf{(2.20)}$$

Hydrogenase have very different biological characteristic and very different physiological role and very complex structure and these enzymes either oxidize H_2 to protons and electrons or reduce protons, thus release molecular hydrogen.(Amstrong, 2004).

All enzymes contain one or more Fe-S clusters to store and transport electrons. The active sites of both Fe-only and NiFe enzymes contain the diatomic p-acceptor ligands CO and CN, which stabilise metals in low-oxidation states. These ligands also provide an important way to monitor reactions at the active site. In below figure is showed that two type of enzymes structure and their active sites and their ligands for hydrogen production.

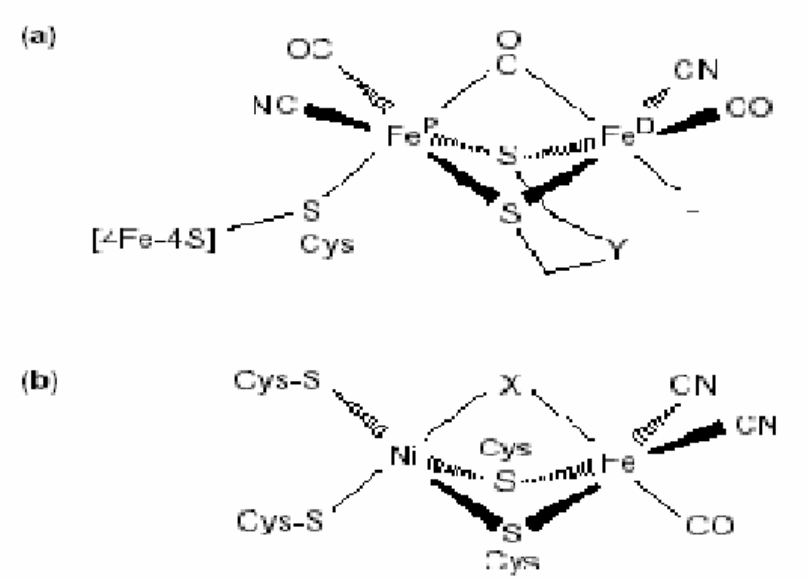

Figure 2.9: Type of hydrogenases and their active site and ligants(Amstrong, 2004). (a) The Fe-only active site is called the H-cluster; FeP and FeD are proximal and distal, respectively, in relation to the [4Fe-4S] cluster; L is an exchangeable ligand (H_2 O) and Y may be an amino-N atom, as recently proposed.
(b) In the NiFe active site, X is an additional bridging ligand, believed to be an oxo or hydroxo group in the inactive forms Ni-A and Ni-B, and a hydride in the active form Ni-C. Because Ni can be oxidized to 3, 2, and 1 levels but Fe can be oxidized 2 levels in this type reaction (Amstrong, 2004).

It should be kept in mind that hydrogenases are enzymes that both produce and consume of hydrogen. Although the catalytic activity is known of these enzymes, corrently there is no evidence on the quantity hydrogen production enzyme being the limiting in any system (Vijayaraghavan, K *et.al.,* 2004).

2.3.4.2 Nitrogenase

This enzyme catalyzes hydrogen production in the absence of molecular oxygen (Koku, H *et.al.,*2002).

$$2H^+ + 2e^- + 4ATP \rightarrow H_2 + 4ADP + 4P_i \qquad \textbf{(2.21)}$$

Effecient operation of nitrogenase requires large amounts of ATP and reducing power. For this reason, synthesis and activity of this enzyme are subject to strict regulatory controls. The primary inhibitor/repressor of nitrogenase is oxygen, which irreversibly destroys this enzyme. Nitrogenase synthesis is strongly simulated by light, resulting in a corresponding increase in nitrogenase activity. In fact that hydrogenase is generally accepted as the metabolic analogist of nitrogenase.

Nitrogenase is required for both energy steps and synthesis reaction. So that, C/N is very important factor. (Koku, H *et.al.*,2002; Eroğlu, E *et.al.*, 2004).

2.3.5 Stochiometry of Biohydrogen Production

The main products of glucose metabolism by *Clostridium butyricum* under anaerobic conditions are biomass, acetate, butyrate, hydrogen, ATP and reducing equivalents. The formations of those main products from glucose can be stoichiometrically expressed as follows:

1) The produsing of biomass of reaction:

$$2C_6H_{12}O_6 + 3NH_3 + 35ATP \rightarrow 3C_6H_7O_2N + 6H_2O \quad \textbf{(2.22)}$$

2) Formation of acetate:

$$C_6H_{12}O_6 + 2H_2O \rightarrow 2C_2H_4O_2 + 2CO_2 + 2H_2 + 4ATP + 2NADH_2 \quad \textbf{(2.23)}$$

3) Formation of butyrate:

$$C_6H_{12}O_6 \rightarrow C_4H_8O_2 + 2CO_2 + 2H_2 + 3ATP \quad \textbf{(2.24)}$$

4) Formation of H_2 through $NADH_2$:

The reducing equivalents generated on all metabolic pathways are used to produce molecular hydrogen by hydrogenase according to:

$$NADH + H+ \rightarrow NAD^+ + H_2 \quad \textbf{(2.25)}$$

Total 4 mole acetate is produced per mole glucose.

In facultative anaerobic bacteria, $NADH_2$ is usually used as a reductant for the production of 2,3-butanediol, ethanol and lactate from pyruvate, but not for H_2. However, as far as a nitrogen-fixing facultative bacterium, *Klebsiella pneumoniae*, is concerned, this microorganism is able to produce hydrogen at significantly high quantities. Hydrogen production by *K. pneumoniae* is associated mainly with the activity of nitrogenase. Nitrogenase can not only reduce N_2 to NH_3, but also catalyze hydrogen production in the absence of molecular nitrogen. Hydrogen production by nitrogenase usually requires a large amount of ATP (at least four ATP/H_2 are produced) and reducing equivalents. The formation of hydrogen can be described by the following equation:

$$NADH_2 + 4ATP \rightarrow H_2 + NAD^+ + 4ADP + 4P_i \quad \textbf{(2.26)}$$

Glucose metabolism in *K. pneumoniae* under facultative anaerobic conditions is shown in Figure 2.10 Tricarboxylic acid (TCA) cycle is taken into account since it plays an important role in facultative cells. The main fermentative products are acetate, ethanol, CO_2 and H_2 whereas $NADH_2$ and ATP are generated simultaneously.

From Figure 2.8, we can draw that the cleavage of pyruvate to acetyl-CoA is catalyzed by two enzymes, namely pyruvate format lyase (PFL) and pyruvate dehydrogenase (PDH). As shown in Figure 2.10, there are three metabolic pathways capable of producing H_2. Firstly, a part of pyruvate to acetyl-CoA catalyzed by PFL produce formic acid, then formate hydrogenlyase, a membrane-bound multi-enzyme complex of which hydrogenase is a part, breaks formic acid down to produce H_2. Secondly, the electrons, generated in the cleavage of pyruvate and catalyzed by PDH, are partly transferred to ferredoxin and then to H^+ to generate H_2 by hydrogenase, and the remaining electrons is transferred to NAD^+ to generate $NADH_2$. Thirdly, a portion of $NADH_2$ is transferred to nitrogenase to generate hydrogen and the remaining part is oxidized completely by oxygen to synthesize ATP through the respiratory chain on the plasma membrane of *K. pneumoniae*. In the second and third metabolic pathways, for simplicity, the electrons are assumed to be all used to produce hydrogen. Therefore, 1 mol H_2 is generated as the cleavage of 1 mol pyruvate to acetyl-CoA catalyzed by two enzymes. Finally, the ATP and $NADH_2$, generated in glycolysis, acetate pathway, and TCA cycle, are transferred to nitrogenase to produce hydrogen.

The formations of main products involved in the glucose metabolism in *K. pneumoniae* can be illustrated as follows:

1) The produsing of biomass of reaction:

$$2C_6H_{12}O_6 + 3NH_3 + 30ATP \rightarrow 3C_6H_7O_2N + 6H_2O \qquad \textbf{(2.27)}$$

2) Formation of acetate:

$$C_6H_{12}O_6 + 2H_2O \rightarrow 2C_2H_4O_2 + 2CO_2 + 2H_2 + 4ATP + 2NADH_2 \qquad \textbf{(2.28)}$$

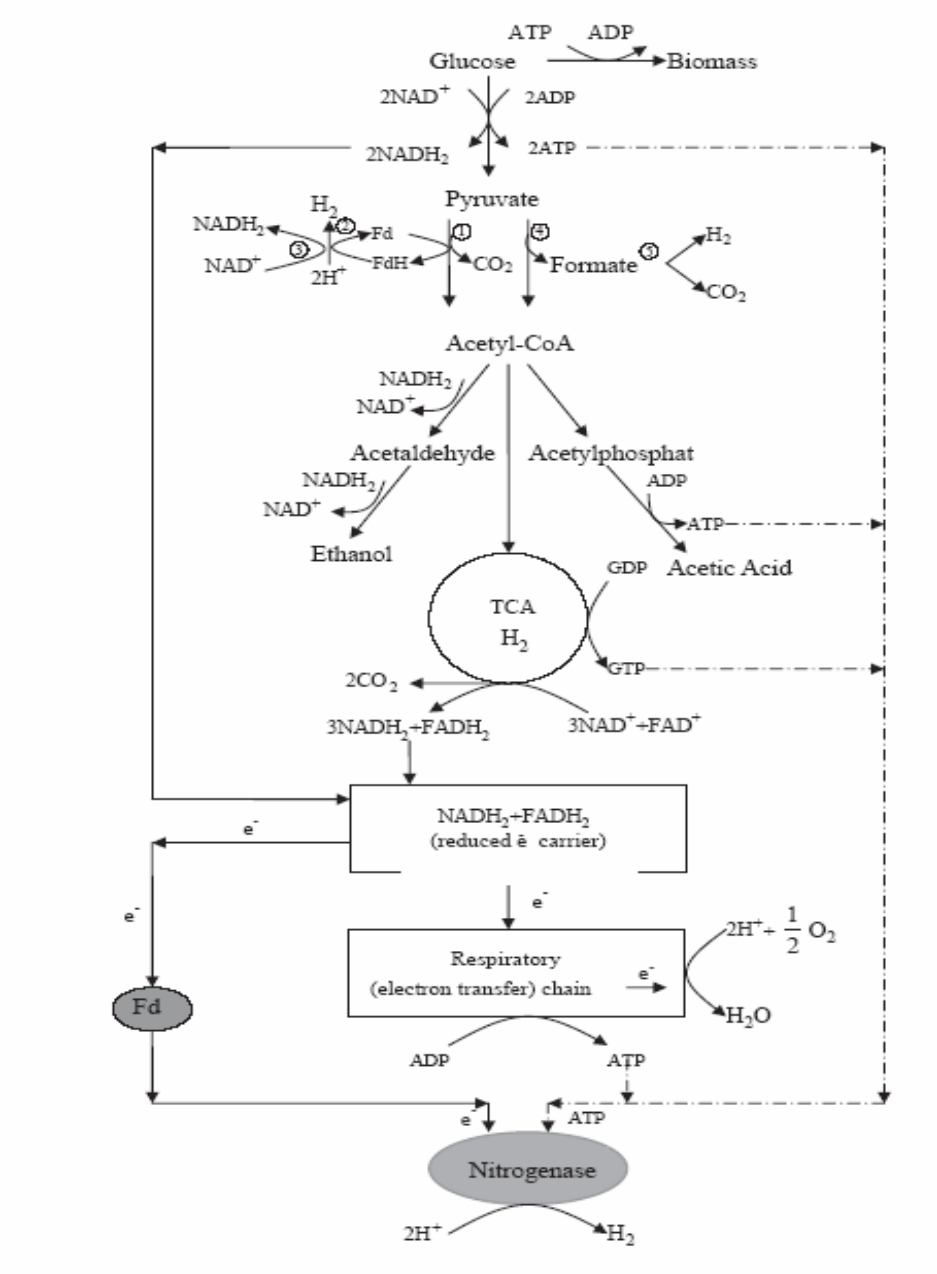

Figure 2.10: Pathway of glucose metabolism in *Klebsiella pneumoniae* under facultative conditions. 1 Pyruvate dehydrogenase (PDH); 2 Hydrogenase; 3 NADH: ferredoxin oxidoreductase; 4 Pyruvate format lyase (PFL); 5 Formate hydrogenlyase (Chen X. *et. al,* 2005).

The $NADH_2$ generated in the acetate pathway would be oxidized completely by oxygen to synthesize ATP through the respiratory chain on the plasma membrane of *K. Pneumoniae.*

The oxidation of $NADH_2$ can be written as follows:

$$NADH_2 + 3ADP + P_i + 1/2\ O2 \rightarrow NAD^+ + H_2O + 3ATP \qquad \textbf{(2.29)}$$

3) Formation of ethanol:

$$C_6H_{12}O_6 + 2NADH_2 \rightarrow 2C_2H_5OH + 2CO_2 + 2H_2 + 2ATP \quad \textbf{(2.30)}$$

4) Glucose metabolism in the TCA cycle pathway:

$$C_6H_{12}O_6 + 6H_2O \rightarrow 6CO_2 + 2H_2 + 2ATP + 2GTP + 8NADH_2 + 2FADH_2 \quad \textbf{(2.31)}$$

As depicted above, ATP would be generated by oxidative phosphorylation of $NADH_2$ as well as $FADH_2$. The oxidation of $FADH_2$ can be described as follows:

$$FADH_2 + 2ADP + P_i + 1/2\ O2 \rightarrow FAD^+ + H_2O + 2ATP \quad \textbf{(2.32)}$$

2.3.6. Relationship Between Biohydrogen Production and Dispersion of Fermentation Products

Generation of bio-hydrogen with anaerobic mixed culture is a very complicated biological system due to the behaviour of the mixed culture system and the sensitivity of hydrogen to environmental conditions. Anaerobic mixed seed sludge consists of variant group's fermentative bacteria species and spores which have distinctive biological properties. Each group follows a different biochemical way and produces by- and/or final product which are particular to its kind. While a group of bacteria produce bio-hydrogen, the other group can consume it. On the other hand; same species of bacteria can both generate and use up fermentative hydrogen, even if environmental conditions are suitable. The best example of this confusion is the acetate pathway for bio-hydrogen production. The well known yield of H_2 is obtained by the acetate pathway theoretically:

$$C_6H_{12}O_6 + 2H_2O_2 \rightarrow 2CH_3COOH + 2CO_2 + 4H_2 \quad \textbf{(2.33)}$$

However some species of clostridia such as *C. aceticum* can lower the H_2 yield by converting H_2 and CO_2 to acetate, or by converting hexose directly to acetate alone with the process of homoacetogenesis.

$$2CO_2 + 4H_2 \rightarrow CH_3COOH + 2H_2O \quad \textbf{(2.34)}$$

$$C_6H_{12}O_6 \rightarrow 3CH_3COOH \quad \textbf{(2.35)}$$

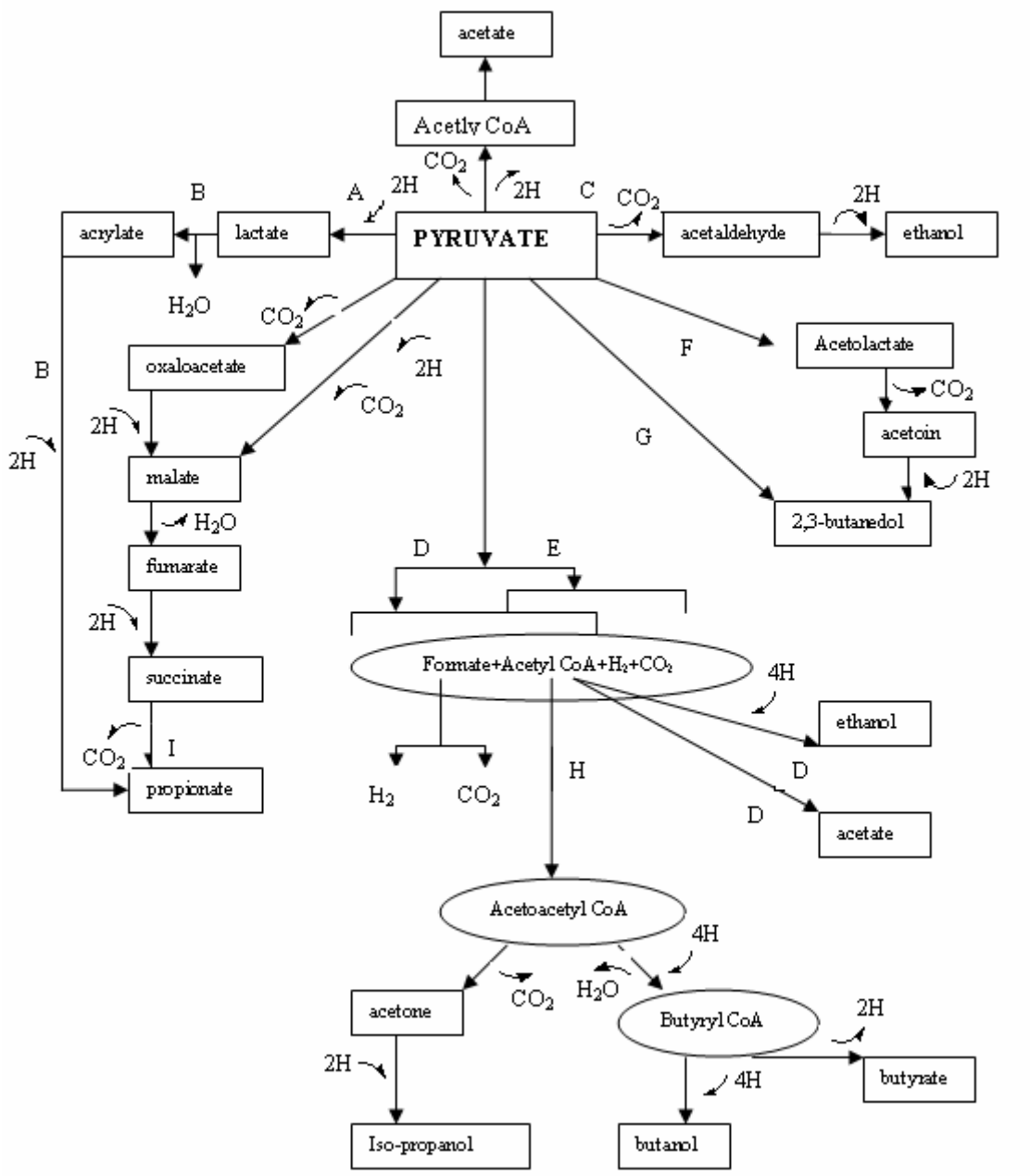

Figure 2.11: Bacterial fermentation products of pyruvate. Pyruvate formed by the catabolism of glucose is further metabolized to some products. The microorganisms involved in this pathway: A. Lactic acid bacteria (*Streptococus, Lactobacillus*), B. *Clostridium proponium,* C. Yeast, *Acetobacter, Zymomonas, Sarcina ventriculi, Erwinia amlyovora,* E. Clostridia, F. *Klebsiella,* G. Yeast, H. Clostridia (butyric, butylic organisms), I. Propionic acid bacteria (Dawes and Large, 1982).

Thus increased acetate production does not necessarily signal an improved H_2 yield (Hawkes *et. al.*, 2006). All pathways and products of anaerobic mixed fermentative bacteria are demonstrated in Figure 2.11 through pyruvate.

Bacterial fermentation requires substrates such as glucose and/or sucrose to obtain energy for growth and maintenance, and produces several intermediate products such as organic acids, alcohols and hydrogen during the metabolic pathways. Gibbs free energy values of these fermentation reactions are all positive, indicating these reactions are not spontaneous. The accumulation of intermediate products in systems can inhibit fermentation. The amount of hydrogen produced from glucose is affected by fermentation pathways and liquid end-products. There is no hydrogen produced when the end-product is propionic acid. In practical operation, high hydrogen yields are associated with a mixture of acetate and butyrate as fermentation products (Ren *et.al.,* 2006). A maximum of 4 mol hydrogen is theoretically produced from 1 mol of glucose with acetic acid as the end-product, while a maximum of 2 mol hydrogen is theoretically produced from 1 mol of glucose with butyrate as the end-product. *Clostridium pasteurianum, Clostridium butyricum* and *Clostridium beijerinkii* are high hydrogen producers while *Clostridium propionicum* is a poor hydrogen producer. In addition, as a intermediate product through fermentation pathways, hydrogen production is affected by fermentation end-products, including, acetic acid, propionic acid, butyric acid, and lactic acid. But there is very limited information of the correlation between fermentation pathways and hydrogen production ability (Ren *et.al.,* 2006).

Depending on the bacterial species and organic nutrients employed, such fermentations result in the generation of an abundance of small organic acids, such as malate, lactate, propionate, butyrate, and/or acetate. The further conversion of these small organic acids to H_2 is not an energetically favourable reaction and cannot be supported by the fermentative metabolism of the anaerobic bacteria. Hence, small organic acids accumulate in the growth medium. As such, they cause inhibition in the rate of growth and limit the yield of H_2 production by the anaerobes. In consequence, the duration of the H_2 production reaction could be relatively short, and yields can be limited because of the accumulating small organic acids (Malis and Melnicki, 2006).

The alcohol type pathway, especially ethanol, is mainly observed with carbohydrate fermentation by yeast, which does not produce hydrogen, whereas the mixed acid type pathway progresses via various microbes such as *Escherichia coli* and *Enterobacter*, and does produce hydrogen. This hydrogen may originate from formate. Formate is found consistently as the product of mixed acid fermentation,

and in the course of the fermentation, is degraded to CO_2 and H_2 by a membrane-associated formate hydorgenlyase (FHL-1) system (Hwang, *et.al.,* 2004).

Lactate and propionate can be produced either by Clostridia themselves or by other bacteria competing in the mixed microflora, lowering the hydrogen yield (Hawkes F.R *et.al.,* 2006).

Bio-hydrogen production is negatively effected by the production of lactate in anaerobic fermentation process. In fact lactate is the main problem for hydrogen production with dark fermentation (Arhing, *et.al.,* 2004) If considerably large amounts of lactate is generated, lactate fermentative bacteria, such as *Lactobasillus*, may dominate in the system. When the lactate producing bacteria dominate, H_2 can not be produced due to the consumption of hydrogen in Pyruvate reactions. Liquid alcohols are the other products which are unfavourable in generating hydrogen. Production of lactate is not possible under adjusted pH and temperature since lactate producing bacteria are able to keep up with other anaerobic fermentative bacteria. Although this situation does not occur often, it is most significant risk for bio-hydrogen production though anaerobic fermentation. Additionally, lactate may be produced by *Colostridia sp*. in anaerobic mixed culture. It is suggested that butyrate and acetate together with H_2 might be produced with lactate production.

In anaerobic conventional systems, the pathway of lactate is favourable to reach final production such CH_4 and CO_2 as shown figure, while H_2 production is unfavourable as demonstrated.

Even if H_2 can be produced with lactate, yield of H_2 is lower, and H_2 production rate is slower than others pathway such as of butyrate and acetate.

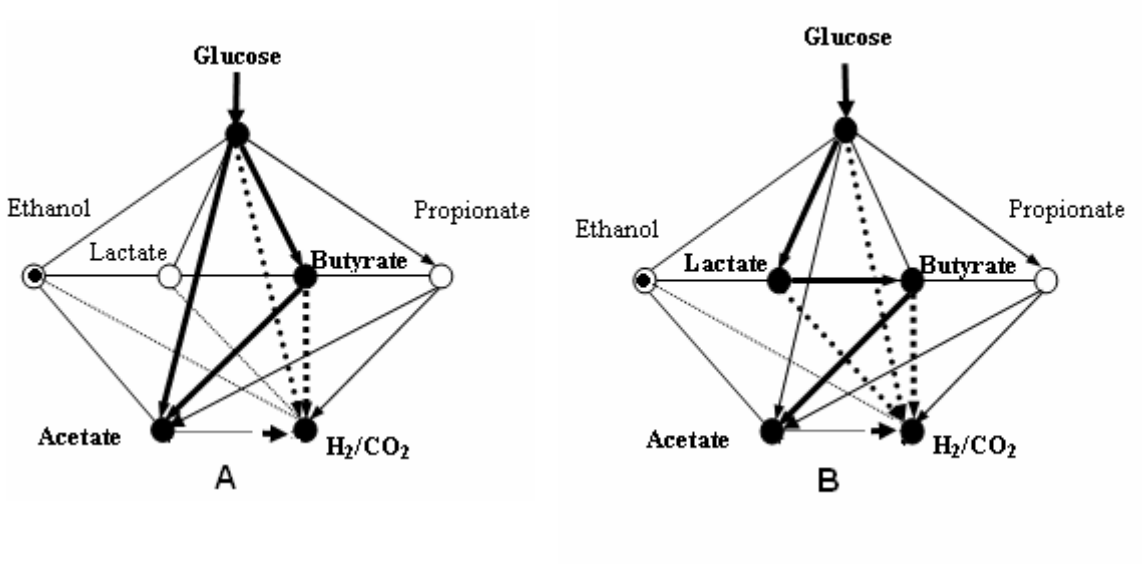

Figure 2.12. A Favorable presentation of acidic fermentation pathways, while **B** unfavorable presentation as shown schematically. (modified Fernandez, 2000).

Generation and accumulation of propionate is another problem for systems due to its toxic effect and infertility in generating hydrogen. Normally, propionic acid is produced with other products but the level of propionate decreases while other products increase in the acidic and conventional anaerobic process. Accumulation of propionate has a high risk for system because nor hydrogen could, neither the other products, be produced under these conditions. A pH of 5.3 or lower levels could prevent the production of propionate in acidic fermentative process (Mu, *et.al.,* 2006).

Hydrogen production is also negatively effected by the generation of some intermediate products, especially acetone and butanol. When acetate is produced, acetone may then produce using the present $2H^+$ (Vortuba, *et.al.,* 1986). Also it is known that butanol may generate using the existing $4H^+$ from the butyrate pathway (Hawng, *et.al.,* 2004). This matter is not clear in the current literature.

H_2 can mainly be produced with butyrate, acetate, and ethanol pathways. Ethanol is an important product for the fermentative hydrogen due to its stable function in the system. Literature research indicates that ethanol is able to take place with all-products in anaerobic mixed culture. It is important to find the optimum level of ethanol generation that affects the H_2 yield. For example Ren et. al., 2006 found that the best yield of H_2 was obtained as acetate**:**ethanol proportion is approximately 1.

Clostridia such as *C. aceticum* can lower the H_2 yield by converting H_2 and CO_2 to acetate or can convert hexose directly to acetate alone by the process of homoacetogenesis. During operation on a particulate starch substrate (pH 5.2, 30°C, 18 h HRT, heat-treated inoculum) (Hussy *et al.*, 2003) observed a doubling in acetate concentration not linked to an increased H_2 yield.

Whether H_2 can be produced or not produced with each of the intermediate products, hydrogen production rate may decrease and/or stop if system generates only one of them due to the inhibition effect. It is suggested that anaerobic hydrogen productivity increase with butyrate and ethanol pathways.

2.3.6.1 Glucose Fermentation to Acetate, Butrate, Ethanol and Hydrogen

Glucose-degrading and butyrate-producing organisms are generally attributed to the *Clostridium* species. However, when given the phylogenetic association between the genera *Eubacterium* and *Clostridium*, it is seen that they have common features. For example, most of the saccharolytic species of *Eubacterium* form butyric acid and hydrogen gas, just as many saccharolytic *Clostridium* species do (Sharak Genthner *et al.*, 1981). In the fermentation of glucose or other sugars, the fate of interspecies electron transfer is an important mechanism for determining the end product formation (Stams, 1994; Thauer, *et al.,* 1977). For example, in the sugar fermentation of *Ruminococcus albus*, acetate, ethanol, hydrogen and CO_2 are the end products, but in the presence of hydrogen scavenger like *Wolinella succinogens*, ethanol would not be formed (Iannotti *et.al.,* 1973). Figure 2.15 also shows that in pure culture *Ruminococcus albus,* the ATP yield is lower than in the coculture. Other examples on the importance of interspecies electron transfer are *Clostridium pasteurianum* and

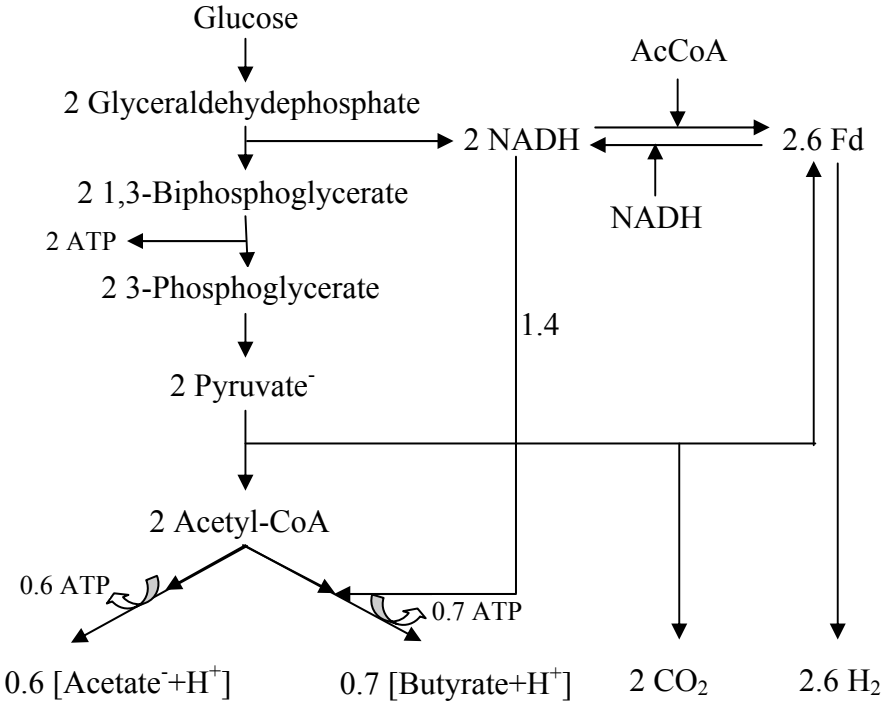

Figure 2.13. Branched glucose fermentation of *Clostridium pasteurianum* and *Clostridium butyricum* (Thauer *et.al.*,1977).

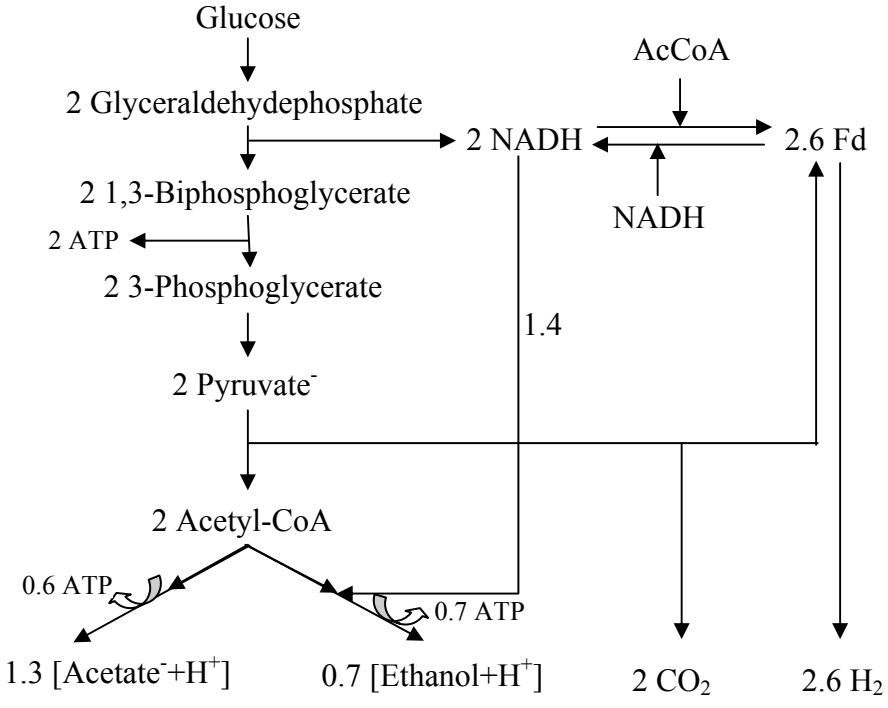

Figure 2.14. Glucose fermentation of *Ruminococcus albus* (Iannotti *et.al.*, 1973).

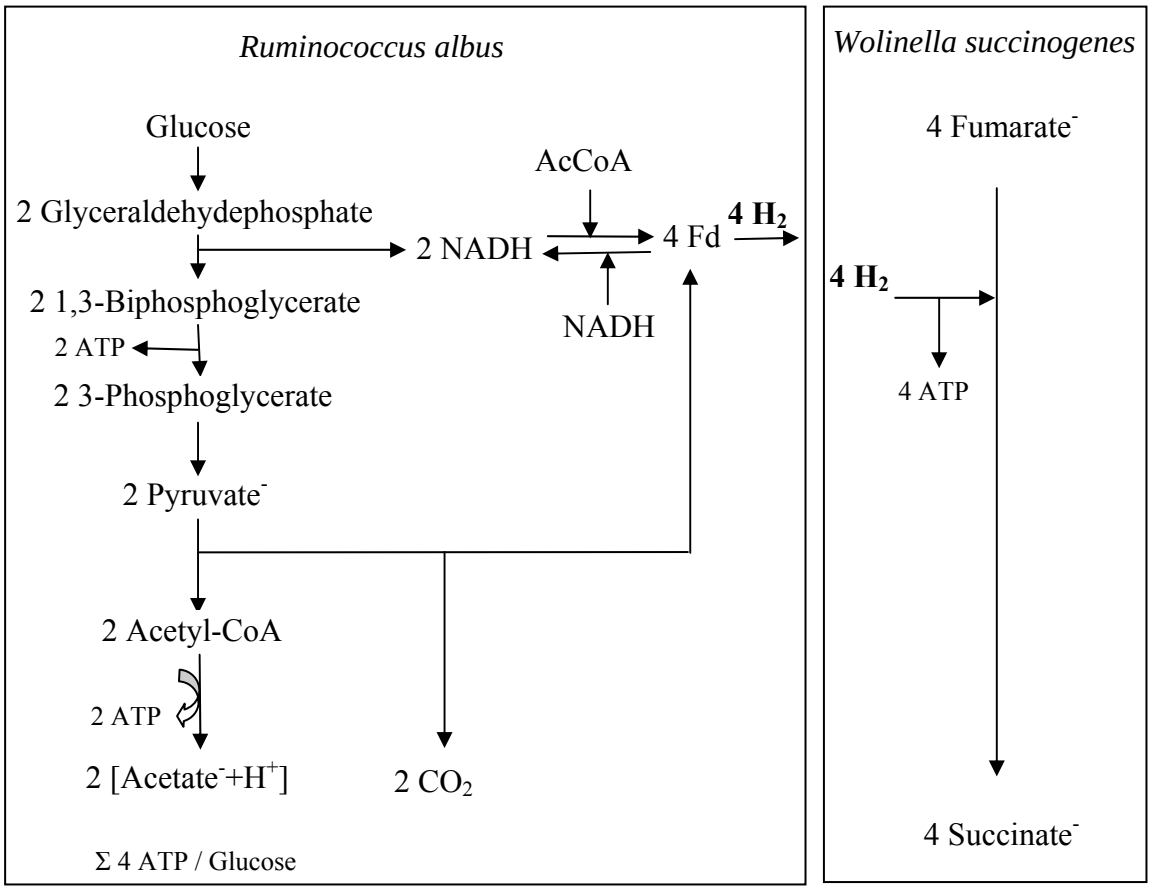

Figure 2.15. Glucose fermentation of *Ruminococcus albus* with and without *Wolinella succinogenes* (Iannotti *et.al.,* 1973).

Clostridium butyricum, that have the same glucose degradation pathway (Thauer *et.al.,*1977). As it can be seen in Figure 3.4, in the absence of hydrogen scavengers, NADH electrons have to be used for reducing acetyl-CoA to butyrate. If the hydrogen partial pressure is pulled to a certain level by one of the hydrogen consumers, the NADH electrons will be released to molecular hydrogen via ferredoxin and butyrate formation will not be observed anymore. For *Clostridium butyricum*, the glucose fermentation that depend on the hydrogen concentration is given in the following equations (Schink, 1997):

$[H_2] > 10$ Pa;

$$C_6H_{12}O_6 + 2H_2O \rightarrow 0.7 \text{ butyrate} + 0.6 \text{ acetate} + 1.3\ H^+ + 2\ CO_2 + 2.6\ H_2\ (\Delta G^{0'} = -233\ \text{kJ.mol}^{-1}) \quad \textbf{(2.36)}$$

$[H_2] \leq 10$ Pa;

$$C_6H_{12}O_6 + 2H_2O \rightarrow 2 \text{ acetate} + 2\ H^+ + 2\ CO_2 + 4\ H_2$$

$$(\Delta G^{0'} = -300\ kJ.mol^{-1}) \quad \textbf{(2.37)}$$

The produced hydrogen, which is the product of the fermentation process, inhibited *Clostridium cellobioparum* rather than *Escheria coli* or *Bacteroides ruminicola* (Chung,1976). After removing the hydrogen by chemical (palladium black), physical (gassing out) or biological electron acceptor (*Methanobacterium ruminantium*), the carbon utilization subsequently increased and the end products were shifted to more acetate and methane. It should be kept in mind that in well-balanced anaerobic conditions, an active hydrogen-utilizing population maintains a low hydrogen partial pressure.

2.3.7 Environmental Factors

Hydrogen generation is in fact a natural phenomena. The ecological niche governs the activity of certain microbial populations and thus the concentration and variety of the final products, i.e. CO_2, water, hydrogen, nitrate, CH_4, etc. No hydrogen is bubbling out of the sewer since in nature there are numerous other bacteria, which readily consume hydrogen as a source of reducing power. When the aim is to produce hydrogen from organic matter, a specific environment needs to be created in which hydrogen producing microorganisms flourish and others perish (Reith, J.H *et.al.,*2003).

When ecological factors change, fermentation bacteria will also change the conversion rate of all terminal fermentation products through normal physiological regulation of organism, even if fermentation-type does not change. Butyric acid-type fermentation in acid-producing phase often occurs during two-phase anaerobic biological treatment (Ren, *et.al.,* 2006).

Many pervious studies have demonstrated that the fermentative H_2-producing process is greatly influenced by many factors, such as wastewater specificity, reactor configuration, hydraulic retention time (HRT), influent organic concentration, organic loading rate, pH, and temperature, oxidation–reduction potential and nutritional requirements. Among these factors, pH has been found to be crucial to the distribution of acidogenic products (Mu, *et.al.*, 2006).

To obtain suitable environmental conditions for hydrogen producing bacteria; pH, temperature, hydraulic retention time (HRT), organic loading rate (OLR) and substrate composition, and mixing can be adjusted artificially to bio-hydrogen (Lay, J.J *et.al.*,2003).

2.3.7.1 Seed Sludge Applications

The ultimate goal is to dominate hydrogen generating species and/or spores in conventional anaerobic mixed cultures. Such an application is important because of fermentation fertility.

Six treatments of natural anaerobic inocula, according to the bacterial stress response (BSR), were applied to evaluate the enrichment of hydrogen-producing mixed microflora. Before these treatments, pre-acid enrichment of natural anaerobic cattle manure sludge was taken at an initial pH of 3, and the pH adjustment was conducted with 10NHCl. After the pH adjustment, the seed sludge was acclimated at 35.5±0.5^{0}C for 48 h. The natural anaerobic sludge without pre-acid enrichment was also prepared for evaluating a single effect due to each BSR technique. The treatment parameters for BSR were as follow: (a) chemical acidification: perchloric acid ($HClO_4$) was mixed with the seed sludge sample for 10 min to alter the pH of the suspension to 2.0; then the sample was stored at 4 ^{0}C for 2–4 h. (b) Chemical methanogenic inhibitor addition: 2-bromoethane sulfonate (BES) has been used as an inhibitor of methanogenesis. Doses of 0.5 and 1.0 M of BES (C_2H_4 BrO_3SNa, Sigma–Aldrich, St. Louis, MO) were mixed for 10 min with the natural seed to inhibit activity of methanogenic bacteria. (c) Dry heat and desiccation: the seed sludge was stored for 2 h in a drying oven at 105 ^{0}C and then desiccated for 2 h. (d)Wet heating (boiling): the seed sludge was boiled for 20 min at 95 ^{0}C, 84.55 kPa pressure. (e) Freezing and thawing: the seed sludge was stored at -10 ^{0}C in a freezer for 24 h and then thawed for another 6 h in a water bath at 30 ^{0}C. The best yield of H_2 is obtained as 419 ml H_2 after pre-acid treatment and, with chemical acidification method. Distribution of VFA and ethanol were recorded as acetate 1711 mg/L, propionic acid 215 mg/L, butyrate 3903 mg/L, and ethanol 2990 mg/L (Cheong, *et.al.,* 2006).

Heat-, acid- and alkaline-treatments were, respectively, performed to inactive hydrogentrophic methanogens and to enrich H_2-producing bacteria. For the heat-

treatment case the seed sludge was heated at 102 ◦C for 90 min. For the acid-treatment, the sludge was adjusted to acidic pH (pH 3.0–4.0) with 0.1N HCl for 24 h, and was then adjusted back to pH 7.0 with the addition of 0.1N NaOH. For the alkaline-treatment, the sludge was pre-treated with adding 4M NaOH and the pH was kept at 12.0 for 24 h, and was then adjusted back to pH 7.0 with the addition of 0.1N HCl. The temperature was around 25 ^{0}C for both acid- and alkaline-treatment (Mu, *et.al.,* 2006).

This study demonstrates that the heat-, acid- and alkaline treated methods were all effective for eliminating methanogenesis and enriching H_2-producing inoculums from mixed anaerobic sludge. The highest H_2 yield of 2.00 mol-H_2/molglucose was achieved with the heat-treated sludge, while lowest yield of 0.48 mol-H_2/mol-glucose was obtained with the alkaline-treated sludge. A butyrate-type fermentation was found with both heat- and alkaline-treated sludge, while a mixed-type fermentation occurred with the acid-treated sludge (Mu, *et.al.,* 2006).

2.3.7.2 The Effect of pH on Biohydrogen Production via Dark Fermentation

pH of the environment has great effects on vital activities of microorganism. One is that it leads to change of cell membrane charge to further influence nutritious substance ingestion of microorganism; another one is that it influences enzyme activity during metabolic process, and the other is that it changes nutritious substance supply and toxicity of harmful substances in habitat (Li, Y. F. *et.al.,* 2006).

pH is also the most important factor in producing hydrogen via dark fermentation. While the low values of pH keeps the hydrogen-consuming bacteria at inactivated form, it supplies suitable growth of hydrogen (Lee, *et.al.,* 2004 and Hawng, *et.al.,* 2004). However pH is not by itself sufficient to inactive the hydrogen-consuming bacteria (Hawng, *et.al.,* 2004). Even if pH is adjusted to 5, hydrogen-consuming species, which are methanogens, could not be destroyed (Spece, 1996). Hydrogen consuming methanogens use 4 mol Hydrogen to produce 1 mol Methane as illustrated in the below reaction (Hawkes, *et.al.,* 2002).

$$CO_2 + 4H_2 \rightarrow CH_4 + 2H_2O \qquad \textbf{(2.38)}$$

If the value of pH is lower that of pH 4, all of the fermentative species are down to high risks (Spece, 1996).

Natural pH (7-7,5) is appropriate for photo-fermentative bacteria (Amstrong, 2004). On the other hand the performances of dark fermentative species alter with the different values of pH, depending on generation of intermediate products such as acetate, butyrate, ethanol, etc.

The change of the pH is effective both on type of and amount of intermediate products, and the resulting amount of hydrogen. For example, the propionate intermediate product occur at the value of pH around 6. However, hydrogen can not be produced with the propionate pathway as like methane (Hawng *et.al.,* 2004). If the pH is in the range of 4-4.5, the proportion of butyrate increases. However the production of butyrate pathway has the potential to change to the butanol production pathway, where hydrogen may be consumed. On the other hand, the number of ethanol and acetate can be increased, when pH is adjusted to pH 5 (Hawng *et.al.,* 2004 and Lay, J.J *et.al.,*2003). Conversely ethanol-acetate pathway is more stable than the others pathways (Hawng *et.al.,* 2004). In addition, the theoretical ethanol is a stable pathway, (Arhing *et.al.,* 2004) while hydrogen yield is at its highest with acetate pathway (Chang *et.al.,* 2002; Lay *et.al.,* 1999).

M. H. Hwang and co-workers tried to explain the effect of pH on the dark fermentation system. Glucose was selected as the carbon source, and Organic Loading Rate (OLR) was depicted 5 g/l. Hydraulic Retention Time (HTR) was taken 3 days, and the temperature was set at 35 + 1 ^{0}C. According to this study; while pH was below 4.5, the production of ethanol and biogas were considerably decreased. This result indicated that the activity of the ethanol producers was slightly suppressed at pH below 4.5, and that of the butyrate producers increased in the pH range 4.5–4.0 than the other microbes. Therefore, the primary product in the reactor in the range of pH 4.0–4.5 was butyrate. A similar result was found in the previous work conducted at pH 4.5 ± 0.2 (Hwang *et al.,* 2002). However, the activity of all the microorganisms was inhibited at around pH 4.0, as shown in Figure. 2.16. These results suggest that pH 4.0 can be regarded as the operational limit for the hydrogen production process. (Lay, *et.al.,* 2003) reported that the hydrogen evolution sharply decreased below pH 4.1 in a batch test, which might be tentatively influenced by un-dissociated VFAs or a low hydrogenase activity.

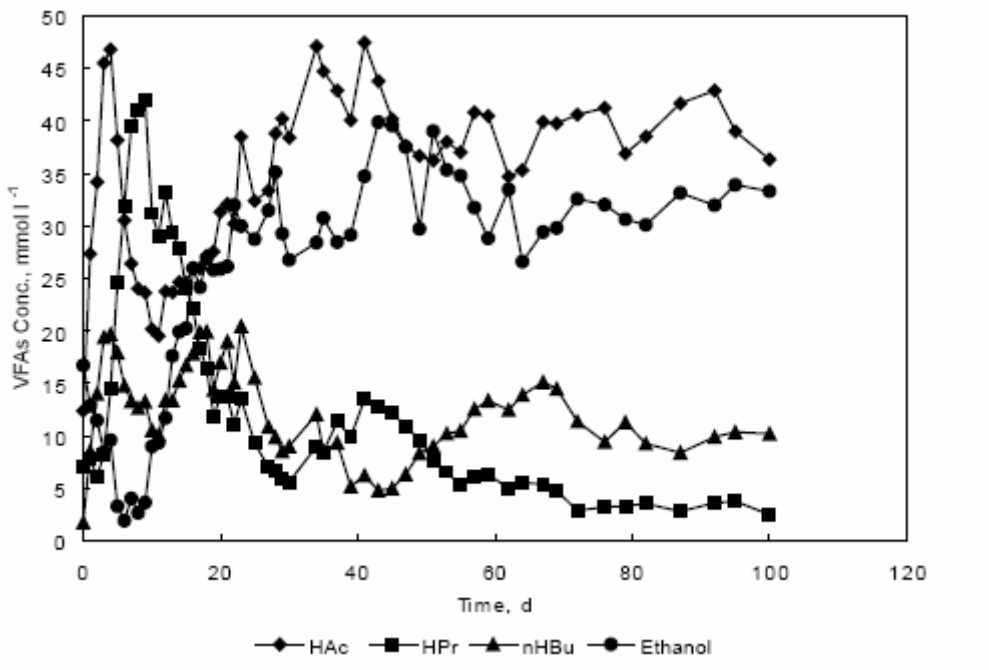

Figure 2.16: The change in the concentrations of the VFAs in the SCR (Hwang *et al.*, 2004).

Moreover, this VFA and biogas production pattern indicates the competition between the ethanol and the butyrate producer in the pH range 4.5–5.0.

At pH 6, the production of hydrogen was insignificant due to the methanogenic activity (Hwang *et al.,* 2002) and propionate production (Wang *et. al.,* 2006). This methanogenic activity was observed over the entire experimental periods. Methane may be produced from both hydrogen and acetate. Hydrogen is not produced in the propionate pathway (Wang *et. al.,* 2006). It was considered that after this period, most of the methane was formed from another source, i.e. acetate.

These results indicate the competition between the microorganisms for the glucose substrate, and the changes in the main fermentation pathway over several pHs. In this study, the main products in the pH ranges of 4–4.5, 4.5–5.0 and 5.0–6.0 were butyrate, ethanol and propionate, respectively. Therefore, an operational pH above 5 may reduce the hydrogen production efficiency of the process where the mixed culture is utilized. Moreover, the pH range 4.5–6 did not completely inhibit the activity of the methanogen, which may negatively affect the hydrogen yield if the reactor is operated long term. Therefore, the pH of the hydrogen production process plays an important role in determining the type of anaerobic fermentation pathway, but is insufficient to inhibit the methanogenic activity (Hawng *et. al.,* 2004).

It has been found that a great amount of intermediate acidic products (such as acetic acid, propionic acid) were produced by anaerobic microorganism, which could cause pH decline in fermentation systems treating highly concentrated wastewater. Therefore, aciduric fermentative bacterial communities, which can tolerate acidic products and low pH, needed to be cultured in order to keep good hydrogen production under high organic loading rate (Ren et. al., 2006).

2.3.7.3 The Effect of Temperature on Biohydrogen Production via Dark Fermentation

Temperature is one of the important factors influencing the biological hydrogen fermentation process. Substrate degradation, H_2 production, product distribution and bacterial growth are all affected by temperature. Furthermore, the microbial community in a fermentative H_2 producing reactor is also greatly influenced by temperature. H_2 production and substrate degradation rate can be increased through elevating the operation temperature. However, for a balance between H_2 production and energy recovery, it seems to be reasonable to operate biological H_2 producing reactors in the mesophilic range (Li,Y. F *et.al.,* 2006).

Hydrogen can be produced by both mesophilic and thermophilic fermentative bacteria (Vijayaraghavan *et.al.,*2004; Reith *et.al.,*2003). However, direct comparison of mesophilic anaerobic fermentative bacteria with thermophilic anaerobic fermentative bacteria for hydrogen production is not yet available in the literature (Gavala *et.al.,* 2005).

Thermophilic condition has a advantage that the activities of methanogens slow at temperature 65 ^{o}C and higher (Reith *et.al.,*2003). Also, thermophilic coditions are more suitable to genereta hydrogen gas by pure culture. For example organic waste bioconversion into H_2 was carried out with *R. capsulatus* and *Thermohydrogenium kirishis* using glucose as substrate (Vijayaraghavan *et.al.,*2004).

Both pure culture and mixed anaerobic culture can be used in mesophilic conditions. Generally a mixed anaerobic culture, taken from an anaerobic treatment or digester system, is preferred.

Yang Mu and co-workers tried to describe the effect of temperature on the anaerobic fermentative mixed bacteria in the mesophilic condition. They have taken complex anaerobic culture from local municipal sewage sludge treatment plant as the seed

sludge. Glucose was selected as the sole carbon source. (10000 mg/l). pH was kept at 5.5, and then the fermentor was operated at 33, 35, 37, 39, 41^{o}C, respectively.

Results showed that glucose degradation rate and efficiency, H_2 yield, and growth rate of H_2-producing bacteria all increased as the temperature increased from 33 to 41^{o}C. However, the specific H_2 production rate increased with increasing temperature from 33 to 39^{o}C, which then decreased as the temperature was further increased to 41^{o}C. The distribution of aqueous products was also greatly influenced by temperature variation. H_2 yield and growth rate of H_2-producing cultures had a linear relationship with temperature (Mu *et.al.,* 2006).

There is a great difference between hydrogen production rates to reach stable state during temperature rise and temperature drop, which do not follow a route. This shows that temperature rise and temperature drop stimulation have different effects on physiological physiochemistry of microorganism. This is easy to explain according to Theory of Dissipative Structure. An organism cell is considered as a microsystem, heat feeding from outside the system (temperature rise) and releasing out of the system (temperature drop) will keep the system at different states. Within temperature limits, heat feeding from outside the system can keep the interior of cell system more orderly. Even within the same temperature limits, heat releasing out of the system will keep the interior of cell system more disorderly to cause the drop of organism metabolism rate in the system (Mu *et.al.,* 2006).

2.3.7.4 Substrate Composition

Hydrogen production from soluble and particulate starch and cellulose from xylose, from sugar beet and the wastewater from a sugar beet refinery and the bottom layer from a beer manufacturing plant has been demonstrated in CSTR experiments using mesophilic mixed microflora. Hydrogen production from other carbohydrate-rich complex materials has been shown mainly in batch conditions, presumably because of the difficulty of delivering particulate matter to continuous reactors at laboratory scale. Hydrogen production from dining hall food waste as a solid has been studied in a mesophilic leaching bed reactor and solid waste as a slurry has been used in CSTRs fed daily by the draw-and-fill method (Hawkes *et.al,* 2006).

Carbohydrate and rich-carbohydrate complex material are best carbon source for anaerobic fermentative bio-hydrogen as substrate. Table 2.1 demonstrates the H_2 yield with several carbohydrate sources in an anaerobic mixed culture.

Table 2.1: The yield of H_2 type of carboydrate. (Hawkes, *et.al.,* 2006)

feedstock	pH	Temperature	hydrogen yield ml-g/carbohydrate	yield %
rice	4.5	37	346	62.6
strach	6	55	92	16.6
cellulose	7	37	72	13
cellulose	7	60	193	34.9
sucrose	5.5	36	280	53.4
glucose	5.5	37	261	52.2
Molasse[a]	5	35	79	40

[a](Ren, *et.al.,* 2006).

Rice, the most common dietary food worldwide, was chosen as the model of carbohydrate-rich food waste after steaming at 100◦C for 30 min. The rice composed of carbohydrate (78.3%), protein (6.6%), lipid (3.2%) and water (11.9%). Batch experiment results showed that hydrogen production from rice slurry was found most effective at pH 4.5, 37◦C treating a slurry containing 5.5 g-carbohydrate/L. An anaerobic digester sludge was used as seed after a 100◦C heat treatment for 30 min. After a 36 h acclimation period, the sludge had a maximum specific hydrogen production rate of 2.1 L/(g-VSS d) and a hydrogen yield of 346 mL/g-carbohydrate, corresponding to 62.6% of stochiometric yield. The effluent was composed mostly of acetate (28.3–43.0%) and butyrate (51.4–70.9%) (Fang *et al.*, 2006).

The growth kinetics of hydrogen-producing bacteria using three different substrates, namely sucrose, non-fat dry milk (NFDM), and food waste were investigated in dark fermentation through a series of batch experiments. The results showed that hydrogen production potential and hydrogen production rate increased with an increasing substrate concentration. The maximum hydrogen yields from sucrose, NFDM, and food waste were 234, 119, and 101 mL/g COD, respectively (pH 5,5 T= 36 0C heat-shock anaerobic mixed culture)(Chen W.H., et al., 2006).

Yield of H_2 is obtained from beer lees wastes as 68.6 mlH2/g TVS. The maximum yield of H_2 was observed, the value is about 10 times as compared with that of raw beer lees wastes.(pH 7 T= 36 0C acid (HCl) pre-treatment anaerobic mixed culture) (Fan Y.T. *et.al.,* 2006).

In another study, carbohydrate- (rice and potato), protein- (egg and lean meat), and fat-rich (fat meat) and (chicken skin) typical solid wastes were selected as experimental materials. The experiments were performed under the mesophilic condition of 37 ^{0}C. The bioreactors were filled with 10% total solid of rice, potato, fat meat, chicken skin, egg, and lean meat. Experimental results indicate that hydrogen-producing potential of carbohydrate-rich HSOW (rice and potato) was approximately 20 times larger than that of fat-rich HSOW (fat meat and chicken skin) and of protein-rich HSOW (egg and lean meat). According to development trends of pH and hydrogen, pH around 6.0 might be threshold for heat-shock digested sludge; that is Clostridium-rich sludge, converting fat- and protein-rich HSOW to hydrogen; (Lay *et.al.,* 2003)

The performance of bio-hydrogen production using the raw wheat straw and HCl pre-treated wheat straw was then compared in batch fermentation tests. The maximum cumulative hydrogen yield of 68.1 ml H2/g TVS was observed at 126.5 h, the value is about 136-fold as compared with that of raw wheat straw wastes. The maximum hydrogen production rate of 10.14 ml H2/g TVS h was obtained by a modified Gompertz equation. The hydrogen content in the biogas was 52.0%. The results showed that the pre-treatment of the substrate plays a key role in the conversion of the wheat straw w (Fan Y.T. *et.al.,* 2006)

Anaerobic co-digestion of food waste and sewage sludge for hydrogen production was performed in serum bottles. The specific hydrogen production potential of food waste was higher than that of sewage sludge. However, hydrogen production potential increased as sewage sludge composition increased up to 13–19% at all the VS concentrations. The maximum specific hydrogen production potential of 122.9 ml/g carbohydrate-COD was found at the waste composition of 87:13 (food waste: sewage sludge) and the VS concentration of 3.0%. The relationship between carbohydrate concentration, protein concentration, and hydrogen production potential indicated that enriched protein by adding sewage sludge might enhance hydrogen production potential. The maximum specific hydrogen production rate was 111.2 ml H_2/g VSS/h. Food waste and sewage sludge were, therefore, considered as a suitable main substrate and a useful auxiliary substrate, respectively, for hydrogen production (Kim *et al.*, 2004).

2.3.7.5 Organic Loading Rate (OLR) and Hydrolytic Retention Time (HTR)

Hydrogen-producing fermentative bacteria can tolerate high levels of organic matter (Lay *et.al.,*1999; Hawkes *et.al.,* 2002).

It has been found that a great amount of intermediate acidic products (such as acetic acid and propionic acid) were produced by anaerobic microorganism, which could cause pH decline in fermentation systems that treat highly concentrated wastewater. Therefore, aciduric fermentative bacterial communities, which can tolerate acidic products and low pH, needed to be cultured in order to keep good hydrogen production under high organic loading rate (Ren *et.al.,* 2006).

In a study; the relationship between organic loading rate and hydraulic retention time is investigated. In order to vary the glucose loading rate over a range of 0.5–18.9 g/h, the glucose concentration in the feed was varied from 2.5 to 10 g COD/L under the conditions where the HRT varied from 1 to 10 h (30 ^{o}C, pH 5.5). Decreasing the glucose loading rate over this range increased the hydrogen yield from 1.7 to 2.8mol-H_2/mol-glucose. High yields of hydrogen were consistent with a high molar acetate:butyrate ratio of 1.08:1 as more hydrogen is produced with acetate as a product (4 mol-H_2/mol-acetate) than with butyrate (2 mol-H_2/mol-butyrate). It was thought that the decrease in yield with organic loading rate resulted from an overall decreased rate of hydrogen production. As the rate of gas production is reduced, H_2 supersaturation in the liquid phase is likely reduced, relieving inhibition due to H_2. Flocculation was also an important factor in the performance of the reactor. At the 5–10 g COD/L influent glucose concentrations, substantial flocculation was observed particularly as the feeding rate was increased due to a reduction in the HRT from 10 to 2.5 h. At the HRT of 2,5 h, biomass concentrations reached as much as 25 g/L. The flocculation nature of the biomass allowed reactor operation at low HRTs with steady H_2 production and >90% glucose removal. However, when the HRT was reduced to 1 h at a glucose feed concentration of 2.5 g COD/L, there was little flocculation evident resulting in wash-out of the culture. These results suggest that hydrogen yields will be optimized for more dilute feeds and lower organic loading rates than have typically been used in bio-hydrogen reactor studies (Zhang *et.al.,* 2006).

It has been found that a high cell density (higher than 5 g/L) in bioreactor is required to keep high hydrogen yield, since low cell intensity cannot efficiently convert

organic substrates to hydrogen, especially at short hydraulic retention time (HRT<4h) (Ren *et.al.,* 2006).

In a hydrogen bio-producing reactor (HBR), temperature, hydraulic retention time and initial biomass concentration were set to be 35 0 C, 11.4 h and 5.74 g MLVSS/L, respectively, together with the loading initiated at 3000 mg molasses COD/L as substrate. The HBR system was operated under the organic loading rates (OLR) of 3.11–85.57 kg COD/m^3 reactor/d with molasses as the substrate. Both biogas and hydrogen yields increased with OLR at the range of 3.11–68.21 kgCOD/m^3 d, but decreased at high OLR (68.21–85.57 kg COD/m^3 reactor/d). The biogas was mainly composed of CO_2 and H_2 with the percentage of H_2 ranging from 40% to 52% in biogas. A maximum hydrogen production rate of 5.57 m3H_2/m^3 reactor/d, with a specific hydrogen production rate of 0.75m 3 H_2/kgMLVSS/d, was obtained in the reactor. The hydrogen yield reached 26.13 mol/kg COD removed within OLR range of 35–55 kg COD/m^3 reactor/d. In addition, it was found that the hydrogen yield was affected by the presence of ethanol and acetate in the liquid phase, and the maximum hydrogen production rate occurred while the ratio of ethanol to acetate was close to 1, due to the adjustment of NAD+/(NADH+H^+) through fermentation pathways (Ren *et.al.,* 2006).

Another study tried to identify the effect of HRT on the yield of producing dark fermentative hydrogen. Fermentative hydrogen production from a synthetic wastewater containing 10 g/L of sucrose was studied in two upflow reactors at 26 ºC for 400 days. One reactor was filled with packing rings (RP) and the other packing free (RF). The effect of HRT from 2 h to 24 h was investigated. Results showed that, under steady state, the hydrogen production rate significantly increased from 0.63 L/L/d to 5.35 L/L/d in the RF when HRT decreased from 24 h to 2 h, the corresponding rates were 0.56 L/L/d to 6.17 L/L/d for the RP. In the RF, the hydrogen yield increased from 0.96 mol/mol-sucrose at 24 h of HRT to the maximum of 1.10 mol/mol-sucrose at 8 h of HRT, and then decreased to 0.68 mol/mol-sucrose at 2 h. In the RP, the yield increased from 0.86 mol/mol-sucrose at 24 h of HRT to the maximum of 1.22 mol/mol-sucrose at 14 h of HRT, and then decreased to 0.78 mol/mol-sucrose at 2 h (Li, L *et.al.,* 2006).

2.3.7.6 H_2 Partial Pressure, Oxidation-Reduction Potential and the Level of NADH/NAD

Bio-hydrogen is generated by the reaction $2NADH+2H^+ \rightarrow H_2+2NAD^+$, catalyzed by hydrogenase under strict anaerobic conditions. Obviously, accumulation of NADH and lower ORP enhances the generation of bio-hydrogen. (Wang *et al.,* 2006)

During the fermentation of glucose, hydrogen was only produced through the catalysis of pyruvate decarboxylation by ferredoxin. Butyric acid, acetic acid and ethanol were the final fermentation products through this pathway. These fermentation pathways were adjusted by NAD^+ (oxidation status)/(NADH + H^+) (reduction status), which existed at certain ratio in microorganisms. Among these fermentation pathways, ethanol and propionic acid pathways have higher ability for the oxidization of NADH +H^+ than butyric acid pathway, since 2 mol NADH + H^+ were oxidized when 1 mol ethanol or propionic was generated. Thus, it can be derived that when acetic acid fermentation (which does not have ability of oxidizing NADH+H^+) is combined with ethanol/propionic fermentation (which has a good ability of oxidizing NADH+H^+), and the mole ratio of ethanol to acetic acid was 1:1, the oxidation/reduction of NAD+/NADH + H^+ could reach a dynamic equilibrium, which can secure a stable fermentation process and thus enhance hydrogen bio-production ability. If the ratio of ethanol to acetic acid was above 1 or below 1, the balance of NAD+/NADH+H^+ was broken, which means fermentation processes were not stable inside microorganisms. As a result, the hydrogen production ability was inhibited (Ren *et.al.,* 2006).

Higher hydrogen partial pressure or higher bio-hydrogen production rate in the anaerobic process would result in the accumulation of propionic acid. However, some other researchers also found that the accumulation of propionic acid seemed to be independent of hydrogen partial pressure, and the accumulation often occurred in the condition of high yield of NADH. Thus they thought higher NADH concentration was the main reason for accumulation of propionic acid in the anaerobic process. This is because, for anaerobic fermentation to proceed continuously, a proper ratio of NADH/NAD^+ inside the cell should be maintained, and propionogenesis can produce more NAD^+ than butyrogenesis in the anaerobic process, so that under condition of high yield NADH, propionic acid fermentation will replace the butyric acid fermentation spontaneously for maintaining a proper ratio of NADH/NAD^+ (Wang

et.al., 2006). If the hydrogen partial pressure is sufficiently low, NADH may be oxidized via hydrogenase, producing H_2 up to the maximum theoretical yield of 4 mol H_2 mol^{-1} hexose consumed and a maximal yield of ATP. However, under normal reactor conditions, most of the NADH will be oxidized in reactions producing reduced fermentation end-products, such as butyrate with a lower molar hydrogen yield and lower ATP yield: (Hawkes *et.al.,*2006).

These phenomena were expected since hydrogen production is required for alcohol production if a positive hydrogen partial pressure was obtained. (Fan Y. *et.al.,* 2004).

There are relationships between $NADH/NADH^+$, ORP and the H_2 partial pressure. With an increase in H_2 partial pressure, the value of NADH/NADH+ increased linearly, but the ORP decreased exponentially. At a H_2 partial pressure of 1 ATM, the maximum $NADH/NAD^+$ is 1514, while the minimum ORP is −414mV. Therefore, the H_2 partial pressure imposes a direct impact on the oxidation state of the NADH/NAD couple and the thermodynamic state of the different product formation pathways. A high H_2 partial pressure leads to a great $NADH/NAD^+$ ratio, making the oxidative processes (e.g., acetate formation) thermodynamically less favourable, and even not feasible under certain conditions. (Mu *et.al.,* 2006).

The composition of VFA depends on the levels of pH and the Oxidation Reduction Potential (ORP). Also, the rate of VFA is directly related to bio-hydrogen production. (L. Wang et al., 2006) found that if the value of pH is 5.3 to 5.6 and level of ORP is approximately – 100 milivoltage (mV), propionic acid dominates in VFA composition and bio-hydrogen production yield is lowest. However, ethanol can rapidly be generated while the range of pH is 4.2-4.6 and level of ORP is – 250 mV, and the hydrogen production yield is 107 mmol L^{-1} d^{-1}. Thus, ethanol-type fermentation is a better selection when using an acidogenic reactor of a two-phase separated anaerobic process to efficiently produce bio-hydrogen with simultaneous organic wastewater pre-treatment. If pH is 5 and the level of ORP is -210 mV, butyrate is the main intermediate product in acid anaerobic reactor. Yi Wang and co-workers found that H_2 partial pressure, H_2 production rate, and H_2 yield were pH-dependent, in the range of $2.8 \times 104–5.2 \times 10^{-4}$ Pa, 61–145 ml-H_2 l^{-1} h^{-1} and 0.68–1.61 mol-H_2 $mol\text{-}glucose^{-1}$. The maximum H_2 partial pressure was observed at pH 3.4, while both maximum H_2 production rate and H_2 yield were found at pH 4.2 (Mu *et.al.,* 2006).

3. MATERIALS and METHODS

3.1 Seed Sludge (Inoculum)

In the two laboratory-scale batch anaerobic reactors that have been set up for the experimental study of producing high rates of ethanol, along with hydrogen, by anaerobic fermentation in the utilization of glucose as the carbon source, two different seeds have been used separately. In addition to monitoring ethanol and hydrogen productions, comparisons of the reactor performances have became possible during the usage of two different seeds. Seed sludge (inoculum) of the first reactor, also referred as *Reactor A*, is a primary sludge of the Kayseri Metropolitan Municipality Wastewater Treatment Plant (WWTP), which has been stabilized in a full-scale mesophilic anaerobic digester. Inoculum of the second reactor, namely *Reactor B*, was taken from a pilot-scale anaerobic reactor at ITU Environmental Engineering Laboratory where the combined treatment of blackwater and prina from an olive oil industry takes place. The seed sludges for the Reactor A and B are referred as inoculum A and B, respectively. Characterization of inoculum A and B are given in Table 3.1.

Table 3.1: Characterization of inoculums

Parameters	**Inoculum from the sludge digester of Kayseri WWTP (ReactorA)**	**Inoculum from digester treating olive oil effluent (ReactorB)**
COD (mgCOD/L)	19824	22619
TS (mgTS/L)	31576	30672
VSS (mgVSS/L)	14600	16042
Alkalinity(mg$CaCO_3$/L)	6500	8200
pH	7,14	7,74

500 mL of inoculum was taken from both Reactors A and B. The seeds were boiled at 100^0C for 15 minutes not only for reducing the activity of methanogens which uses the hydrogen in the anaerobic seeds but also to increase the activity of microorganisms and rate of species that produce hydrogen. Then seeds were cooled through passing nitrogen gas in the flowrate of 20 ml/hour for 1 hour which also maintained the anaerobic conditions. The seeds were then added to the reactors of 5 L capacity, which have been utilized for performing the controlled studies of the experiment.

The most significant difference between the two seeds could be presented as following: inoculum from the sludge digester of Kayseri WWTP was put direct into process, while inoculum from the digester treating olive oil effluent has been waited for ~3 years under anaerobic conditions prior to processing.

3.2 Feed Composition

The sole carbon source of the process has been glucose. The glucose concentration was 10000 mg/L, and 1 mg glucose/L was measured for accounting approximately 1 mg COD/L. Other than the carbon source, the required trace elements have been prepared according to Lay (1999) as given and fed to the two reactors: NH_4CI_2=0.36 g/L, $KHPO_4$=0.125 g/L, $MgCI.6H_2O$=0.10 g/L, $MnSO_4$ $6H_2O$=0.015 g/L, $CuSO_4.5H_2O$=0.005 g/L, $CoCI_2.6H_2O$=0.00125 g/L, $CaCI_2.2H_2O$=0.10 g/L, $NaMoO_4.4H_2O$=0.005 g/L, $FeSO_4.7H_2O$=0.025 g/L, $NiSO_4.6H_2O$=0.02 g/L and $NaHCO_3$=6,7 g/L.

3.3 Operation of the Batch Reactors

Initially, 500 ml of seeds were separately put into the two reactors of 5 L capacity and a feeding solution with 10000 mg COD/L has been added until the volume reached a total of 5 L. Mechanical mixers with speed of 150 revolutions/minute were used in the reactors to provide homogeneous distribution of the inoculum and the feeding solution. As soon as reactors were operated, pH was adjusted to 5 and then set free to vary between 3.3 and 5.9. pH was continuously monitored through a digital pH-meter. While 1 Normal (N) sodium hydroxide (NaOH) solution was fed by peristaltic pumps in order to increase the pH when necessary, 1 N sulphuric acid (H_2SO_4) solution was used to decrease it. Then both of the rectors were put into the

water bath at 37 ± 1 ^{o}C to ensure mesophilic conditions. Experimental setup is given in Figure 3.1.

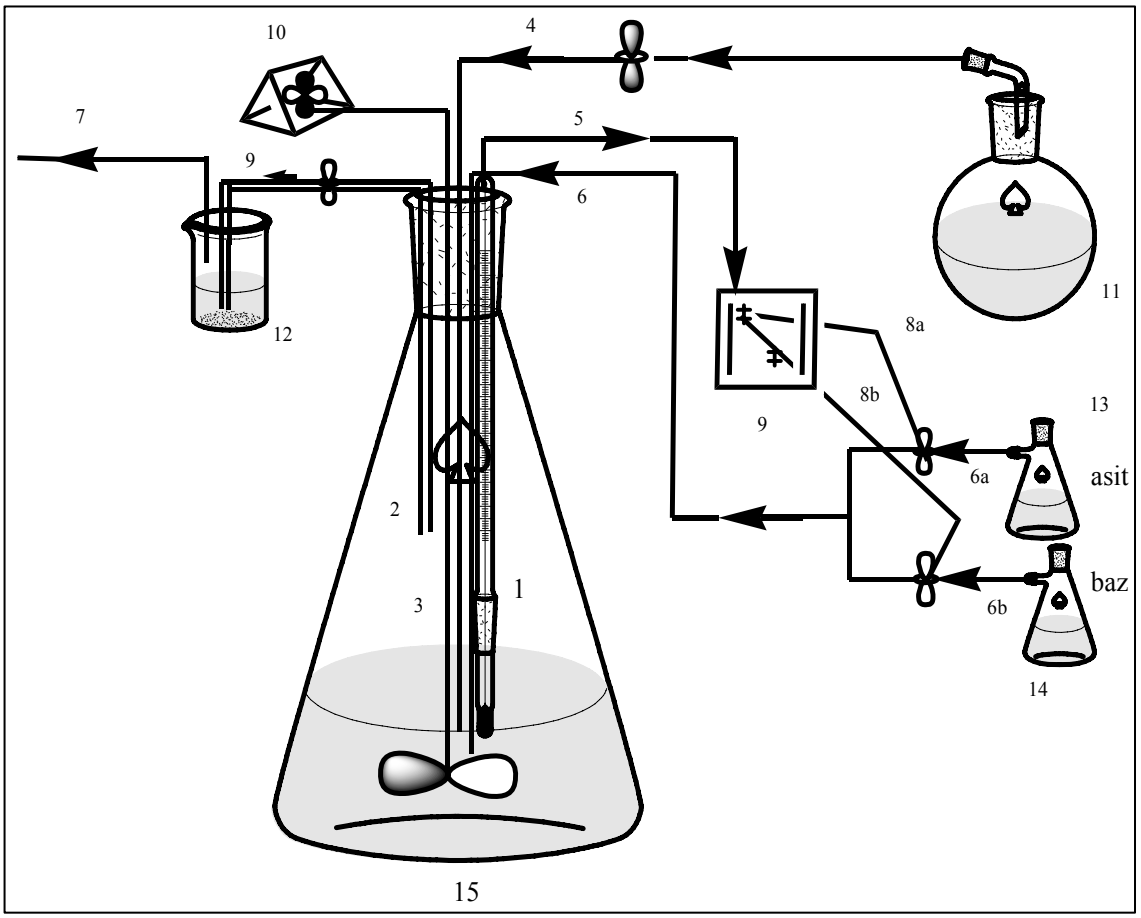

Figure 3.1: Experimental Setup: 1) The probe of the pH meter; 2) Gas outlet; 3) Mechanic stirrer; 4) Feding line; 5) The electronic connection of pH meter; 6)Acid-base lines; 7) Hydrogen outlet line; 8) The electronic connection between pH meter and peristaltic pumps; 9) Digital pH meter; 10) The motor of mechanic stirrer; 11) Gas collecting unit; 12) Substrate solution; 13) Acid solution; 14) Base solution 15) 5 L reactor.

Experimental Series: The system has been subjected to three different types of feedings except for the initial feeding.

1st Period Feedings (Run 1): After 6th day of the 1st feeding, engines were stopped and waited for 4 hours for the settling of microorganisms. Later 1 L liquid phase (liquid phase and/or excessive products) was taken by the injector from the surface (Han, S. K and Shin, H.S. 2003; Lay, J. J. 2003), and 1 L of stock solution was added instead. This application has been given in Figure 3.2.

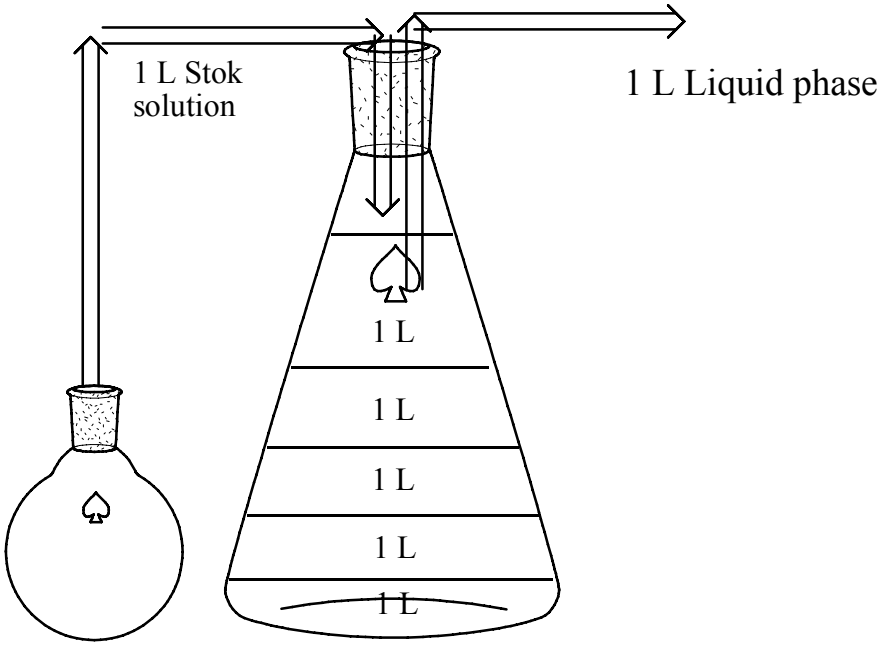

Figure 3.2: Experimental application Run 1.

These experimental parameters were used in this feeding series.

V_{feed} : Feeding volume

$V_{reactor}$: Reactor volume

So(OLR): Organic Loadnig Rate

QV_{feed} : Volumetric Organic Load

Organic Loading Rate (OLR)= So =10,000 mg glucose/L **(3.1)**

$V_{feed} = V_{reactor} * \frac{1}{5} L = 1L$ defined as loading. **(3.2)**

$$QV_{feed} = \frac{So * Vfeed}{Vreactor} = \frac{10{,}000 mg \text{glucose}/L * 1L}{5L} = 2000 mg \text{glucose}/L \quad \textbf{(3.3)}$$

In this period, a total of 8 feedings took place in 48 days. Time span between the feedings was not equal. In other words the interval between any two feedings could be 4 or 5 days as well as 6 days.

2nd Period Feedings (Run 2): After the 54th day of operation without interruptions, the liquid phase and the sludge were separated from each other. 2 L of stock solution was added as the 2 L of liquid phase was taken from surface. This application was processed as shown in Figure 3.3.

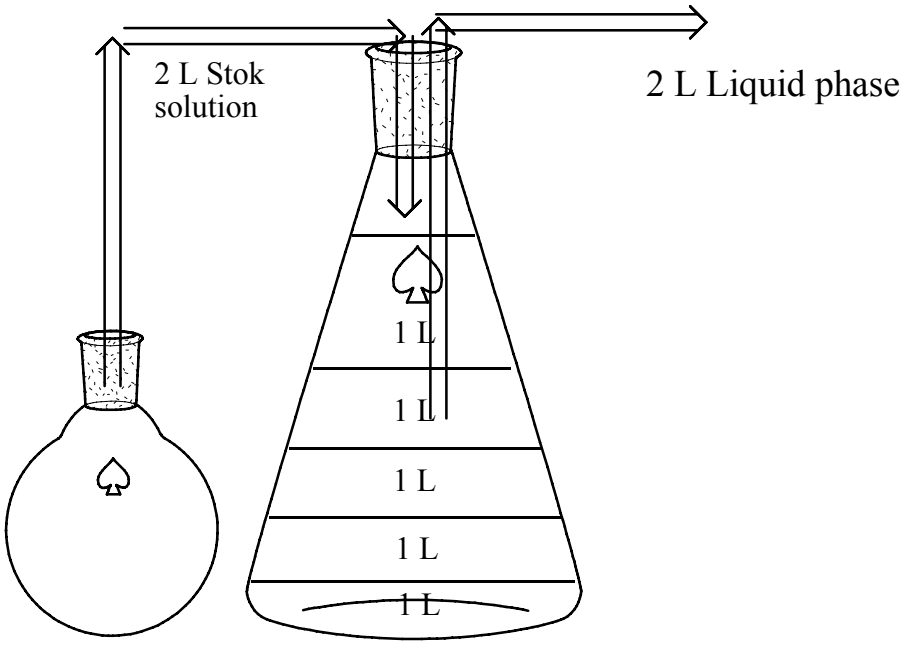

Figure 3.3: Experimental application Run 2.

These experimental parameters were used in this feeding series:

Organic Loading Rate(OLR) = So =10,000 mg glucose/L **(3.4)**

$$V_{feed} = V_{reactor} * \frac{2}{5} L = 2L \quad \textbf{(3.5)}$$

$$QV_{feed} = \frac{So * Vfeed}{Vreactor} = \frac{10{,}000 mglu\cos e / L * 2L}{5L} = 4000 mggluc\cos e / L \quad \textbf{(3.6)}$$

In this period total feeding was 4 throughout the 18 days.

3rd Period Feedings (Run 3): In this period, the total volume of liquid phase taken from surface and the amount of replacing stock solution added to the system were increased to 3 L. This application was processed as shown in Figure 3.4.

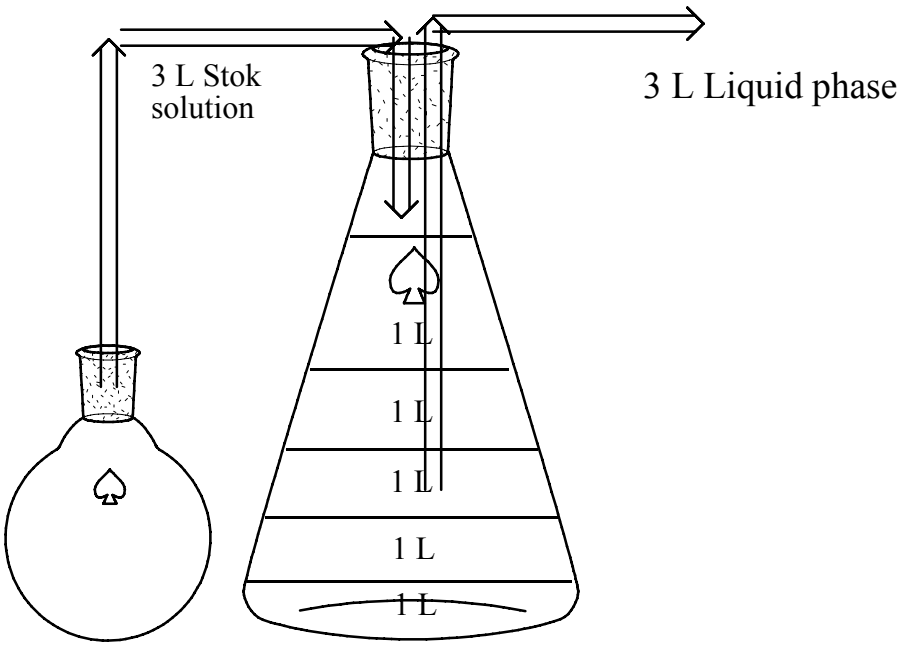

Figure 3. 4: Experimental application Run 3.

These experimental parameters were used in this feeding series:

Organic Loding Rate (OLR) = So =10,000 mg glucose/L **(3.7)**

$$V_{feed} = V_{reactor} * \frac{3}{5} L = 3L \quad \textbf{(3.8)}$$

$$QV_{feed} = \frac{So * Vfeed}{Vreactor} = \frac{10{,}000 mggluc\,ose / L * 3L}{5L} = 6000 mggluc\,ose / L \quad \textbf{(3.9)}$$

2 feedings were done in Reactor A (along 14 days) and 5 feedings were done in Reactor B (along 35 days) in this period.

The total number of feedings was 14 in 88 days for Reactor A and 17 in 107 days for Reactor B. Both of the reactors were applied 3 periods with the first feeding (start-up) unaccounted.

As feedings to the reactors were parallel, sampling from reactors for the analysis of VFA, ethanol and COD was also done concurrently. Nevertheless, the VSS and TS analyses were conducted before and after each period.

3.4 Analytical Methods

The chemical oxygen demand (COD), volatile suspended solids (VSS) and total solids (TS) were measured according to Standard Methods (ALPA, 1995). Volatile fatty acid (VFA) was analayzed by using gas chromatography method (Agilent 6890N). Ethanol was determined by refractometer (Bellingham+Stanley Limited 60/70 ABBE) using a refractive index. Total gas produced was measured from the reactors by water deplacement method. Gas chromatography technique (Helwett Packard) was used to determine the percentage of the hydrogen in the gas.

4. RESULTS and DISCUSSION

4.1 Reactor A

The initial seed sludge of the first reactor, also referred as Reactor A, are taken from the full-scale mesophilic anaerobic digester where the primary sludges of the Kayseri Metropolitan Municipality municipal wastewater treatment plant are stabilized. The COD has been recorded as 9878 mg COD/L as soon as the feeding was done. The initial pH was 8.15 and it has been dropped to the level of 5 using N H_2SO_4. The COD values have been given as dissolved COD throughout the study.

4.1.1 Degradation of Glucose

The glucose concentration has been measured as 9878 mg COD/L after the initial feeding. The values are 9577, 9481, 8764 mg COD/, were recorded respectively at the end of the 2nd, 5th, and 6th days. The COD of the first feeding, COD equivalents of the intermediate products and the difference between the two are given in Table 4.1.

Table 4.1: Distribution of COD of intial loading (So=10,000 mgCOD/L) for Reactor A.

Time	Total COD	Acetate	Propionate	Butyrate	Valerate	Ethanol	Difference COD	pH
day	(mgCOD/L)	(mgCOD/L)	(mgCOD/L)	(mgCOD/L)	(mgCOD/L)	(mgCOD/L)	(mgCOD/L)	
1	9878	252.5	12.8	1738.0	64.3	177.3	-7633.2	5.5
2	9577	67.1	17.8	932.6	130.2	709.2	-7720.0	3.7
5	9481	71.1	19.0	940.4	117.5	1418.5	-6914.6	4.7
6	8764	70.4	17.3	990.8	117.4	709.2	-6858.8	3.95

After 6 days, the 1st feedings series have been started with COD measured as 9483 mg COD/L right after the initial feeding. On the 6th day of the feeding, COD has been recorded as 8929 mg COD/L. A total of 8 feedings have been carried out in 48 days for 1st feeding period. The time spans between the semi-batch feedings were not equal. In other words, the interval between the two feedings could be 4 days as well as 5 or 6 days. The COD removal ratio is 12%. The average COD removal ratio of

the 8 feedings that constitute the 1st feeding period is calculated to be 7.8%. The COD distribution of the period is given in Table 4.2

Table 4.2: Distribution of COD of the1st period (So=10,000 mgCOD/L, V_{feed} = 1 L) for Reactor A.

Run 1.	Time	Total COD	Acetate	Propionate	Butyrate	Valerate	Ethanol	Difference COD	pH
	day	(mgCOD/L)	(mgCOD/L)	(mgCOD/L)	(mgCOD/L)	(mgCOD/L)	(mgCOD/L)	(mgCOD/L)	
1st loading	6	9483	67.5	17.4	921.4	112.5	354.6	-8009.5	5.05
	12	8929	162.8	14.8	1164.8	95.8	1418.5	-6072.3	3.6
2nd loading	12	9462	342.4	13.1	2111.4	69.3	106.4	-6819.4	4.8
	14	9238	301.6	9.3	1918.4	50.1	1418.5	-5540.2	4.13
	18	8040	69.1	11.6	772.3	172.1	354.6	-6660.4	5.3
3rd loading	18	9262	206.8	7.2	1301.0	83.2	133.0	-7530.9	3.83
	19	9256	214.2	10.2	1592.9	49.4	354.6	-7034.8	4.54
	21	8817	199.1	8.3	1523.2	37.4	*	*	5
	22	9003	175.9	11.0	1440.1	51.0	106.4	-7218.6	4.31
4th loading	22	9590	117.0	4.3	655.6	73.7	106.4	-8633.1	4.85
	27	9083	123.6	8.5	1092.8	68.4	159.6	-7630.1	3.97
5th loading	27	9220	92.4	5.3	410.6	12.3	709.2	-7990.1	4.8
	33	8895	111.2	2.9	277.6	23.3	1063.9	-7416.1	4.36
6th loading	33	9100	107.4	4.6	377.4	47.1	177.3	-8386.2	5.21
	49	8761	101.4	4.3	370.9	40.9	1418.5	-6825.1	3.83
7th loading	49	9050	90.2	6.7	391.6	54.0	1063.9	-7443.6	4.7
	52	8684	90.7	7.7	441.4	62.8	1773.1	-6308.4	5.38
8th loading	53	8406	104.5	8.2	454.0	62.3	3723.5	-3969.4	3.73
	54	8322	124.9	9.8	476.6	66.0	4255.4	-3473.3	4.42

At the end of the 54 days, the initial feeding of the 2nd period has been started (V_{feed} = 2 L, Volimetric Organic Load = 4000 mg glucose/L) and the COD has been measured as 10378 mg COD/L. The 5th and the 6th days reveal results of 9978 and 8560 mg COD/L, respectively. The best COD removal efficiency has been calculated to be 17.5% on the 4th and 7th days after the feeding (Table 4.2). Average COD removal ratio is 9.75%. The 2nd feeding period covers a span of 21 days and 4 feedings. Table 4.3 shows the COD distribution of this period.

At the end of the 75th day, the initial feeding of the 3rd period has been made (V_{feed} = 3 L, Volimetric Organic Load = 6000 mg glucose/L) and the COD is recorded as 9884 mg COD/L. Table 4.4 gives the distribution of this period.

Table 4.3: Distribution of COD of the 2rd period (So=10,000 mgCOD/L, V_{feed} = 2 L) for Reactor A.

Run 2.	Time	Total COD	Acetate	Propionate	Butyrate	Valerate	Ethanol	Difference COD	pH
	day	(mgCOD/L)	(mgCOD/L)	(mgCOD/L)	(mgCOD/L)	(mgCOD/L)	(mgCOD/L)	(mgCOD/L)	
1st loading	54	10378	76.3	7.5	57.7	6.9	4255	-5974	3.93
	59	9978	80.7	10.7	993.0	85.4	2127	-6680	4.9
	60	8560	85.5	8.6	723.5	89.5	2659	-4993	3.5
2nd loading	60	9688	94.2	10.8	659.9	106.4	4255	-4561	5.2
	64	9578	91.5	10.8	608.7	102.5	4255	-4509	4.93
3rd loading	64	10015	96.2	12.4	774.6	113.6	2127,	-6890	5.3
	68	9015	101.0	17.0	731.7	122.2	4255	-3787	4.87
4th loading	68	9997	105.8	19.8	685.2	119.7	7092	-1974	5.84
	74	9143	106.0	18.7	657.8	121.3	4432	-3806	4.99
	75	8961	77.8	11.3	651.1	128.9	1063	-7028	4.6

Table 4.4: Distribution of COD of the 3rd period (So=10,000 mgCOD/L, V_{feed} = 3 L) for Reactor A.

Run 3.	Time	Total	Acetate	Propionate	Butyrate	Valerate	Ethanol	Differnce COD	pH
	day	(mgCOD/L)	(mgCOD/L)	(mgCOD/L)	(mgCOD/L)	(mgCOD/L)	(mgCOD/L)	(mgCOD/L)	
1st loading	75	9884	109.8	18.2	109.7	118.5	4078	-5449	4.91
	76	9849	77.7	13.4	964.4	112.1	4432	-4248	5.33
	80	9582	67.0	11.4	774.8	125.9	4255	-4347	4.84
2nd loading	80	10254	60.8	12.1	825.9	118.6	4432	-4803	5.21
	86	9888	62.1	12.0	830.3	124.2	3546	-5313	4.78
	88	9478	244.4	20.1	2130.4	35.8	1063	-5983	5.19

For Reactor A, a total of 14 feedings, excluding the start-up, has been applied for 88 days.

There are two important issues to be considered in hydrogen generation with dark fermentation. The first one is the COD removal efficiency and the second one is the COD equivalences of the intermediate products (acetate, butyrate, ethanol, etc.). While the COD removal ratio is expected to fall between 10-20 % (Hawkes *et.al.,* 2002), the total COD of the intermediate products is projected to be close to the residual COD value. The further investigation of the tables reveals that the COD removal efficiency holds to be around 10% for the total of periods and feedings, however the CODs of the intermediate products is far away from compensating the residual COD value.

The most important reason of this difference is the possibility that there might be intermediate products in the reactor that could not the measured. This situation is tried to be explained in the following section.

4.1.2 Production of VFA and Ethanol

The distribution of produced VFA and ethanol amounts are very important indicators of hydrogen generation with dark fermentation. Therefore, ethanol and VFA analyses have been conducted in parallel with COD experiments. With this purpose, fermentation products of ethanol, acetic acid, propionic acid, iso-butyric acid, normal-butyric acid, iso-valeric acid and normal valeric acid have been measured. Except for ethanol, the VFAs are observed to include mostly iso-butric and butyric acid. The propionic, iso-valeric and n-valeric acid production is close to zero. Even tough the acetate amount turns out to be less than expected, it is the mostly produced fermentation intermediate product after butyric acid.

The maximum amount of acetic acid, 342.4 mg COD/L, has been recorded right after the 2nd feeding in the 1st feeding period. The maximum amount of butyrate, on the other hand, is recorded on the 7th day of the 2nd feeding in the 3rd feeding period with 2130.4 mg COD/L. The concentrations of fermentation intermediate products in terms of COD equivalences for each feeding periods is clearly given in Table 4.1-Table 4.4. The tables provide total butyric acid figures as the addition of iso-butric and butyric acids; valeric acid values have been provided similarly.

The evaluation of ethanol production in Reactor A reveals that it is produced steadily in high concentrations starting from the 8th feeding of the 1st feeding period. The highest ethanol concentration is observed after the 4th feeding in the 2nd feeding period with 7092 mg COD/L. Increasing of the feeding volume and volumetric organic load respectively from 1 L and 2000 mg COD/L to 2 L and 4000 mg COD/L results in increase of the ethanol production. On the other hand, increasing the same parameters respectively to 3 L and 6000 mg COD/L does not have a significant effect on ethanol production. Even tough this reactor has considerable production of ethanol, no hydrogen production has been observed. Except for the ethanol which has a steady production after the final feeding of the 1st period, the other fermentation intermediate products have not been produced at desirable levels.

The studies reveal that if butyrate and/or acetate is produced together with ethanol, the system produces hydrogen with dark fermentation (Hwang, M. H. et. al 2004 and

Fan, Y. T. 2005). However, in dark fermentation conducted with mixed anaerobic culture, some intermediate products are produced which inhibit the production of hydrogen. The most important intermediate product among such are lactate, propanol, butanol and acetone. (Arhing, B.K. and Westermann, P. 2004; Vortuba, J. *et.al*. 1985). The related products have not been measured in this study. In contrast to the considerable and steady production of ethanol starting from the final two feedings of the 1st feeding period, acetate and butyrate production levels have remained to be very low. This is an important disadvantage of mixed-culture studies compared to pure cultures in terms of control and observation difficulties. Another important reason is that the total gas outlet ($CO_2 + H_2$) is recorded to be significantly low in spite of the recorded COD removal efficiency being in harmony with the literature figures and the decrease of pH by itself. This shows that the selected mixed culture is not adaptive to determined environmental conditions. In other words, the most important reason of the lack of hydrogen production is that the selected mixed anaerobic culture could not fully adapt to its environment as the pH has been set free between values of 3.3-5.9.

Another reasonable explanation lies beneath the pre-processes the selected seed has been subjected to before the reactor was operated, as specified in the Material and Method section. These pre-processes could have resulted in elimination of the spores and species in the seed that produce desirable levels of hydrogen. Spores and species of *Closdridia* are the best hydrogen-producing microorganisms known. However, such microorganism communities do not produce hydrogen gas with ethanol. The species that produce hydrogen together with ethanol belong to the species of *E.Coli*, but then their hydrogen generation efficiencies are lower than of the *Closdridia* (Reith *et.al.,* 2003). These results, when evaluated with the results of Reactor B operated under the same conditions, reveal that each mixed anaerobic culture has a significantly different performance and production efficiency.

4.2 Reactor B

In Reactor B, operated with the seed taken from the pilot-scale batch reactor where the combined fermentation of blackwater and prina from olive oil industry takes place, the initial glucose level is measured to be 11592 mg COD/L after the 1st feeding (10000 mg glucose/L). The pH that has been initially measured as 8.37 is

then pulled down to 5. All COD values of this reactor have been measured as dissolved COD.

4.2.1 Degradation of Glucose

The glucose concentration after the 1st feeding is recorded as 11592 mg COD/L and the corresponding values for the 2nd, 5th and 6th days are 10570, 9761 and 9640 mg COD/L, respectively. The COD of the first feeding, COD equivalents of the intermediate products and the difference between the two are given in Table 4.5. The graphical distribution of COD is also shown in Figure 4.1.

Table 4.5: Distribution of COD in the intial loading (So=10000 mgCOD/L) for reactor B

Time	Total COD	Acetate	Propionate	Butyrate	Valerate	Ethanol	Difference COD	pH
day	(mgCOD/L)	(mgCOD/L)	(mgCOD/L)	(mgCOD/L)	(mgCOD/L)	(mgCOD/L)	(mgCOD/L)	
1	11592	92	20	891	114	3724	-6752	5
2	10570	91	18	788	100	4433	-5140	4.6
5	9761	117	13	715	88	3546	-5282	3.7
6	9640	160	12	691	84	4078	-4615	4.8

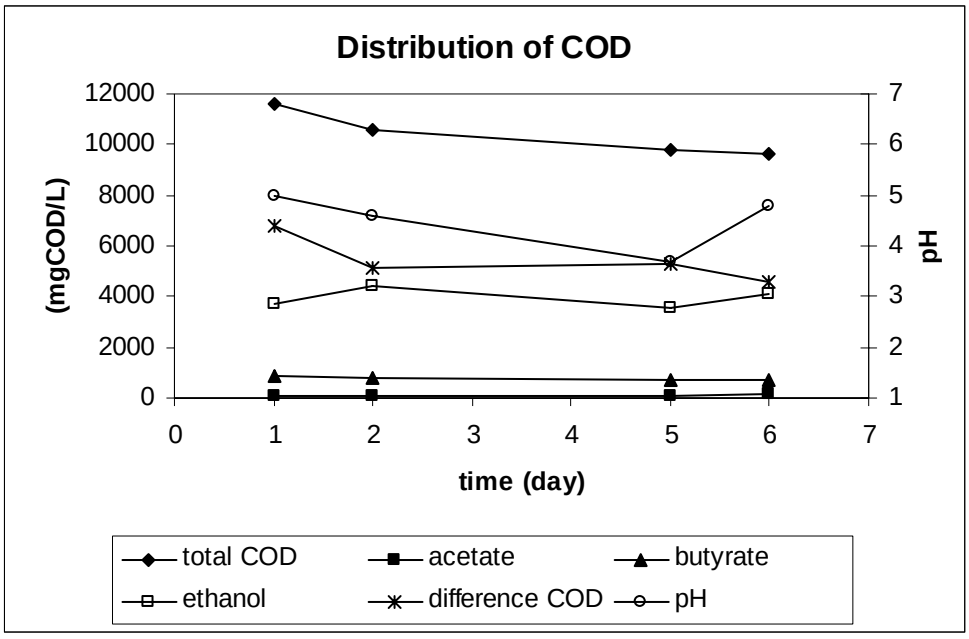

Figure 4.1: The change of COD concentration, production of acetate, butyrate, ethanol and consumed glucose in initial feeding for Reactor B.

1st Period (Run 1): As in the case of Reactor A, firstly (V_{feed} = 1 L, organic concentration = 2000mg glucose/L) has been selected and the 1st feeding of the 1st period has been conducted after 6 days from the initial feeding (start-up). Then the initial COD has been measured as 10180 mg COD/L. This value has changed to 8929 mg COD/L 6 days later. The best COD removal efficiency was observed in the 6th day of the 2nd feeding with 17.4%. The average COD removal ratio has been calculated as 11.3% for the total period. The feedings and the COD equivalents of intermediate products resulting with the COD variation of the feedings are given in Table 4.6. The table also provides the total of fermentation intermediate products and the residual COD value as calculated by subtracting this value from the total COD.

Table 4.6: Distribution of COD of 1st period (So=10,000 mg glucose/L and V_{feed} = 1 L) for reactor B.

Run 1.	Time	Total COD	Acetate	Propionate	Butyrate	Valerate	Ethanol	Difference COD	pH
	day	(mgCOD/L)	(mgCOD/L)	(mgCOD/L)	(mgCOD/L)	(mgCOD/L)	(mgCOD/L)	(mgCOD/L)	
1st loading	6	10810	144	14	980	82	4610	-4980	3.99
	12	8929	200	11	1467	62	4255	-2935	4.9
2nd loading	12	9977	242	9	3369	46	4255	-2056	3.3
	14	9273	213	5	5374	12	3546	-122	4.6
	18	8900	185	5	4825	41	3901	*	4.25
3rd loading	18	9557	173	5	4923	38	1419	-3000	5.2
	19	9393	180	14	5085	15	4433	*	4.03
	21	8950	164	30	5124	19	4255	*	4.56
	22	8630	146	31	4187	18	3546	-702	5
4th loading	22	8864	232	41	6353	58	1419	-761	4.6
	27	7920	212	35	5003	47	355	-2269	4.33
5th loading	27	8600	252	28	4961	66	355	-2938	5.2
	33	7996	531	27	5485	102	355	-1496	4.45
6th loading	33	9090	463	27	4852	91	1419	-2239	4.8
	49	7558	417	26	4116	77	1419	-1504	4.3
7th loading	49	7996	344	23	3273	60	355	-3941	4.7
	52	7811	308	21	2821	52	709	-3900	5.04
8th loading	53	8433	402	28	4021	75	1064	-2844	4.75
	53	8260	371	25	3334	60	1064	-3407	5.09
	54	7987	285	23	2413	43	532	-4691	5.7

*Experimental fault.

Further investigation of the table reveals that the smallest difference of residual COD is in the 1st, 2nd and 3rd feedings. After the 4th feeding this gap starts to increase again.

The best hydrogen production efficiency is obtained in these feedings and this situation is tried to be presented in the Hydrogen Yield section. For each feedings of this period, the graphical distribution of total COD removal and intermediate product concentrations with respect to time and pH have been obtained and are interpreted with respect to Table 4.6. The decreases in pH values have occurred by itself without addition of chemicals.

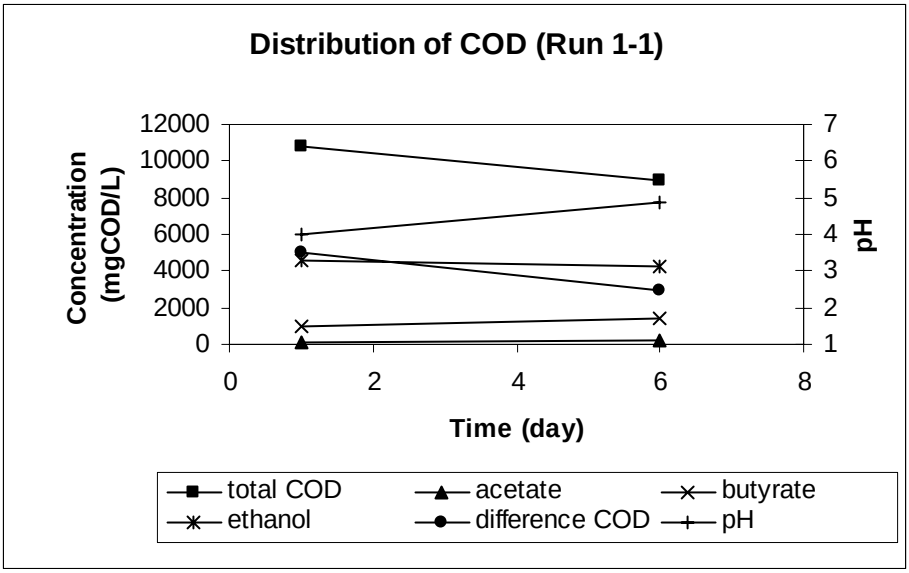

Figure 4. 2: Total COD, production of acetate, butyrate and ethanol versus time and pH graphs the 1st period of the 1st feeding for Reactor B.

1st feeding of the 1st period: In this feeding, COD removal efficiency for 6 days is 17.4% with a drop in residual COD value from 4980 mg COD/L to 2935 mg COD/L. The produced butyrate amount increases together with high ethanol efficiency.

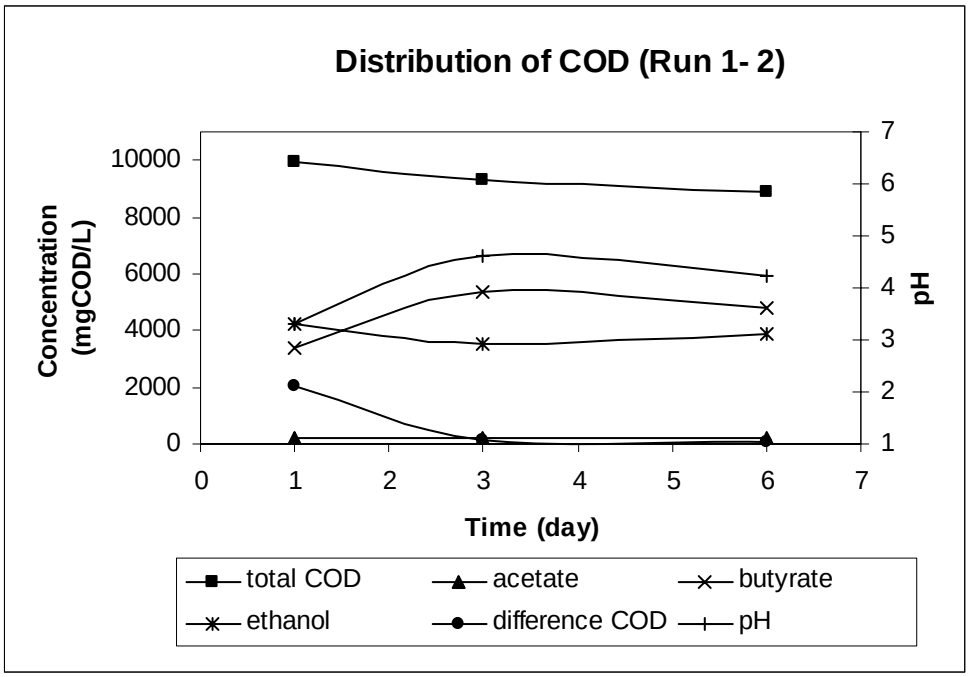

Figure 4. 3: Total COD, production of acetate, butyrate and ethanol versus time and pH graphs the 1st period of the 2nd feeding for Reactor B.

2nd feeding of the 1st period: In this feeding, COD removal efficiency for 6 days is 10.8% with a drop in residual COD value from 2056 mg COD/L to 122 mg COD/L in 2 days. The produced amounts of butyrate and ethanol are high while ethanol:butyrate ratio is calculated to be around 1. The balanced and high production of these two intermediate products is an important indicator in terms of hydrogen efficiency. This situation is clearly explained in the Hydrogen Yield section.

3rd feeding of the 1st period: In this feeding, COD removal efficiency for 4 days is 9.7% with a drop in residual COD value from 3000 mg COD/L to 702 mg COD/L. Again the ethanol and butyrate productions are at balanced and high levels.

4th feeding of the 1st period: In this feeding, COD removal efficiency for 5 days is 10.7% with an increase in residual COD value from 761 mg COD/L to 2269 mg COD/L. This is a result of significantly decreasing ethanol amount. On the other hand, the produced butyrate amount has reached to its highest value all through the study. The fact that the imbalance created by increasing butyrate and decreasing ethanol has negative effects on hydrogen production is explained in the following section.

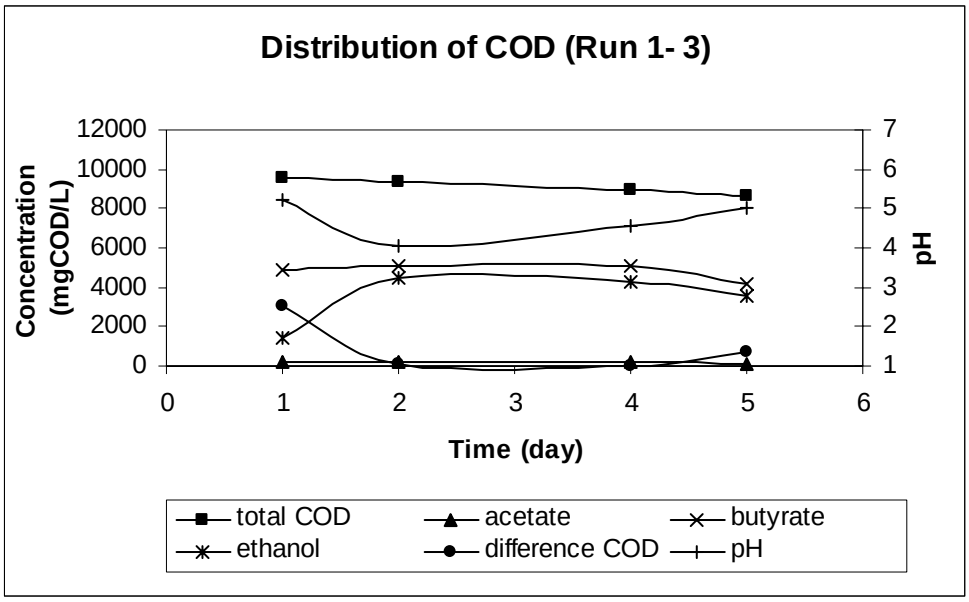

Figure 4. 4: Total COD, production of acetate, butyrate and ethanol versus time and pH graphs the 1st period of 3rd feeding for Reactor B.

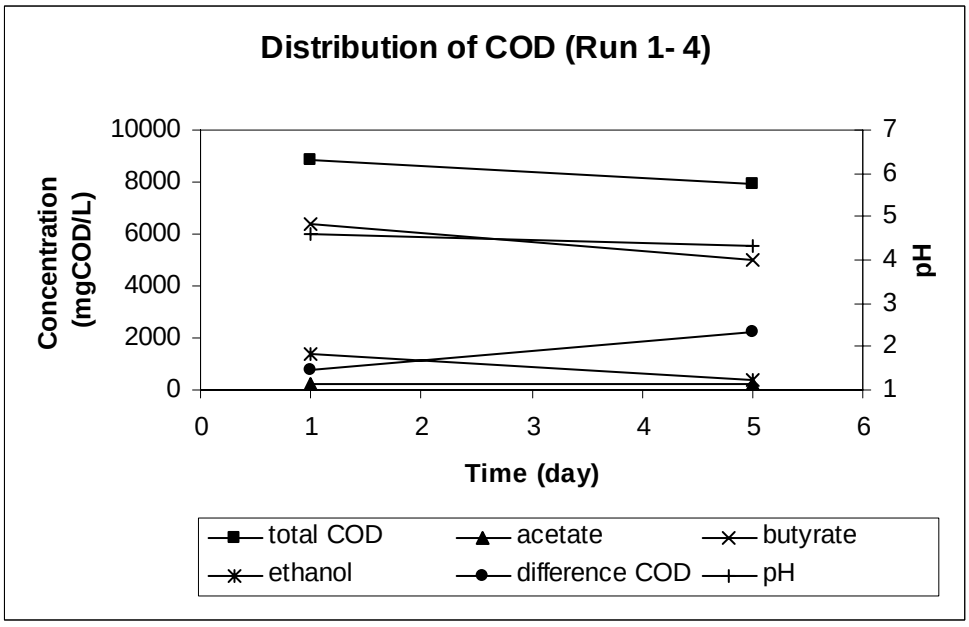

Figure 4. 5: Total COD, production of acetate, butyrate and ethanol versus time and pH graphs the 1st period of 4th feeding for Reactor B.

5th feeding of the 1st period: In this feeding, COD removal efficiency for 6 days is 7% with a drop in residual COD value from 2938 mg COD/L to 1496 mg COD/L. In spite of the COD removal efficiency and the decrease in pH, ethanol-related total gas and hydrogen production efficiency has decreased. Even tough the butyrate efficiency is at high levels, it still remains lower than of the previous feeding. On the other hand, the produced acetate amount is 4 times than of the previous.

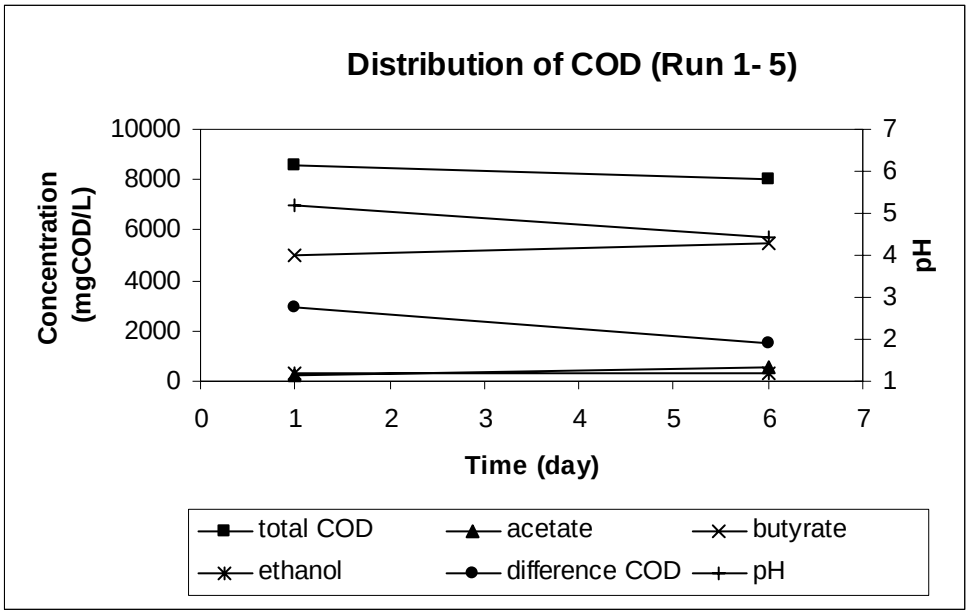

Figure 4. 6: Total COD, production of acetate, butyrate and ethanol versus time and pH graphs the 1st period of 5st loading for reactor B.

6th feeding of the 1st period: During this feeding, a mechanical malfunctioning has occurred in the gas collection unit which took 18 days of repair. Meanwhile feeding has not been made, the reactor has not been opened and it has not been subjected to any external interventions. The experiments for feeding have been continued, however, they could not be interpreted since no gas was collected. The results revealed that the fermentation continued, therefore the remaining feedings were carried on.

7th and 8th feedings of the 1st period: COD removal efficiency together with the produced butyrate ethanol amounts decreased. Therefore 2nd period feeding series were applied to the system. It is considered that there are intermediate products that have accumulated in the system. In conclusion, the liquid phase taken out is decided to be increased.

The 8th feeding has taken a total of 1 day and all analyses have been conducted three times for this single day. The results are given in Table 4.6.

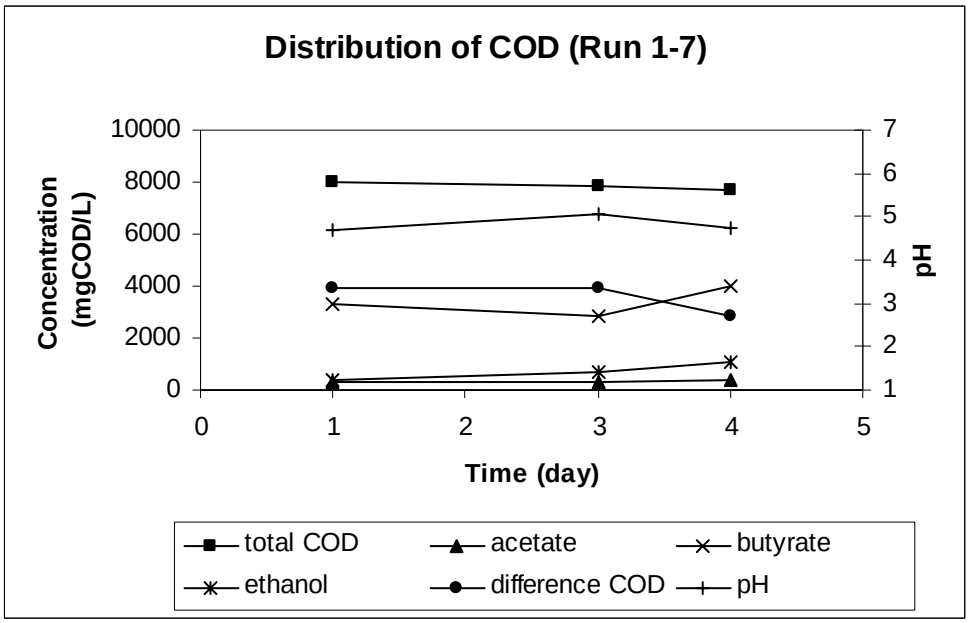

Figure 4. 7: Total COD, production of acetate, butyrate and ethanol versus time and pH graphs the 1st period of 7th feeding for Reactor B.

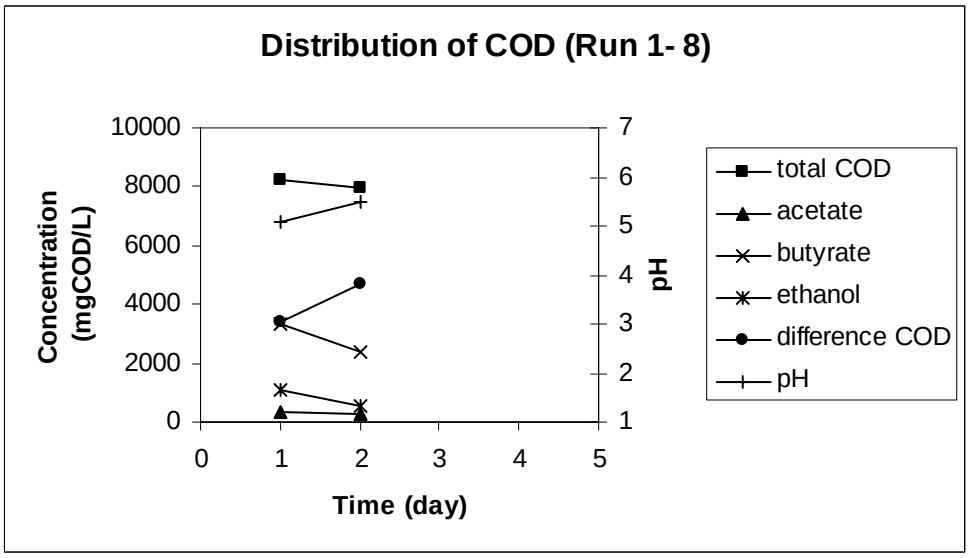

Figure 4. 8: Total COD, production of acetate, butyrate and ethanol versus time and pH graphs the 1st period of 8th feeding for Reactor B.

2nd Period (Run 2): In the 2nd period, the So ratio has not been changed while the feeding volume and the organic concentration has been doubled (V_{feed} = 2 L, Volumetric Organic Rate = 4000mg glucose/L). The 2nd period feedings have been conducted consecutively without gaps. The 1st feeding of the 2nd period has been conducted after 54 days from start-up and the COD value is measured to be 11070 mg COD/L. This value dropped to the level of 8797 mg COD/L after 6 days. The measurement of the COD samples right after the 2nd feeding of the 2nd period has

been eliminated, therefore, it is not included in Table 4.7. The table and the figures show the two feedings together. This period included 4 feedings and lasted a total of 18 days. The 1st, 2nd and 4th feedings have respectively 13.6%, 11.4% and 9.7% of COD removal efficiencies. The change in COD values in this period is as given in Table 4.7 and have been graphed separately for each feeding. The produced butyrate amount has continued to decrease while the COD removal efficiency and the ethanol amount increased. In relation to this, the total gas and hydrogen production efficiency decreased. As the high levels of butyrate produced in the 1st feeding period could not alone result in an increase in total gas and hydrogen production efficiencies, it is concluded that in this period the high level of ethanol production favoured the increase of gas productions.

Table 4.7: Distribution of COD of 2nd period (So=10,000 mg glucose/L and V_{feed} = 2 L) for reactor B.

Run 2.	Time	Total COD	Acetate	Propionate	Butyrate	Valerate	Ethanol	Difference COD	pH
	day	(mgCOD/L)	(mgCOD/L)	(mgCOD/L)	(mgCOD/L)	(mgCOD/L)	(mgCOD/L)	(mgCOD/L)	
1st loading	54	11070	285	23	2413	43	106	8199	4.8
	59	10596	252	22	2233	39	1773	6276	4.91
	60	8797	244	20	2130	36	3723	2643	5.74
2nd loading	60	*	173	18	1237	20	1418	*	4.85
	64	7797	178	19	1247	20	3723	2610	5
3rd loading	64	9215	134	16	747	10	3723	4585	4.55
	68	8161	162	16	663	10	4255	3055	5
4th loading	68	9942	145	17	455	4	3723	5598	5.33
	75	8979	144	18	384	110	4245	4078	4.6

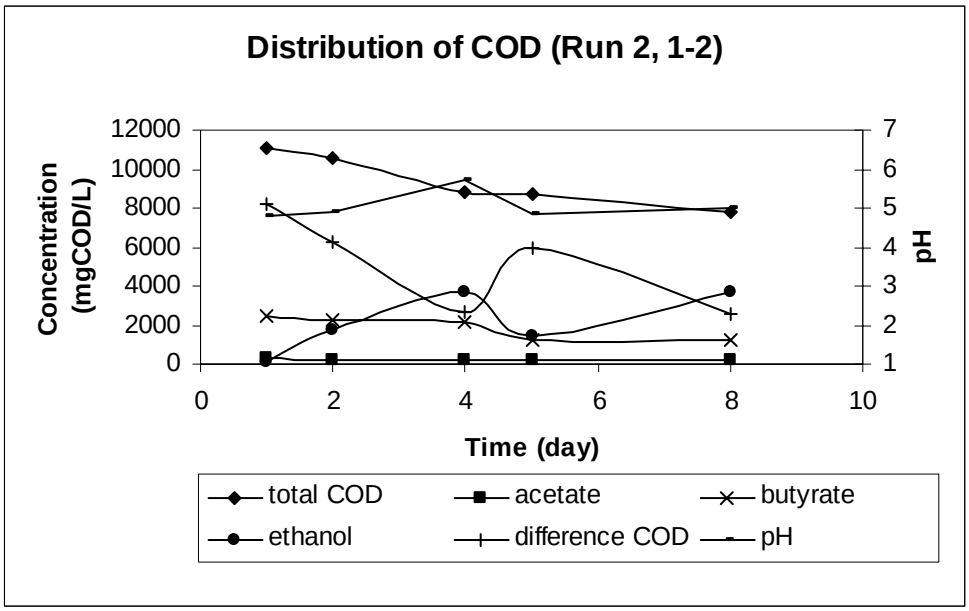

Figure 4. 9: Total COD, production of acetate, butyrate and ethanol versus time and pH graphs the 2nd period of 1st and 2nd feeding for Reactor B.

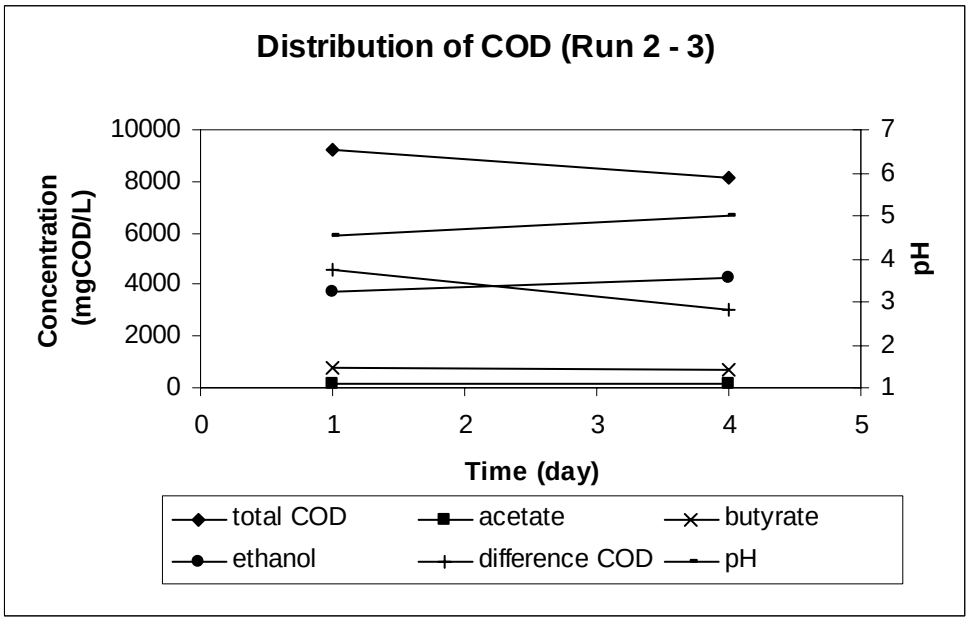

Figure 4. 10: Total COD, production of acetate, butyrate and ethanol versus time and pH graphs the 2nd period of 3rd feeding for Reactor B.

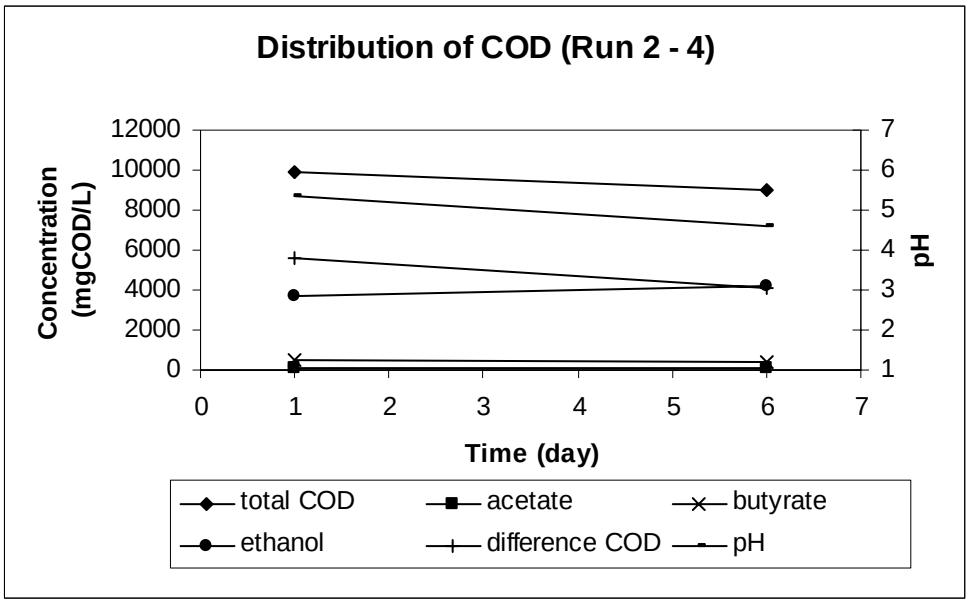

Figure 4. 11: Total COD, production of acetate, butyrate and ethanol versus time and pH graphs the 2nd period of 4th feeding for Reactor B.

3. Period (Run 3): The 3rd period feedings have been initiated with (V_{feed} = 3 L, Volimetric Organic Rate = 6000mg glucose/L) due to decrease in the total gas and production efficiencies. The COD value in this period right after the initial feeding is measured as 10105 mg COD/L and this value changed to 9770, 9331, 9291 mg COD/L, respectively for the 2nd, 3rd and 5th days. The best COD removal efficiency in this period is observed in the 4th and 5th feedings with 11%, while the average removal ratio was 8.4%. The 3rd period had 5 feedings in a total span of 31 days.

1st feeding of the 3rd period: In this feeding, COD removal efficiency for 5 days is 8% with a drop in residual COD value from 5624 mg COD/L to 4405 mg COD/L. The produced butyrate amount has relatively increased, while the ethanol has preserved its high efficiency level.

2nd feeding of the 3rd period: In this feeding, COD removal efficiency for 6 days is 4.5% with a drop in residual COD value from 8560 mg COD/L to 7709 mg COD/L. Produced amounts of butyrate and ethanol has decreased. The total efficiency of gas and hydrogen is recorded to be very low.

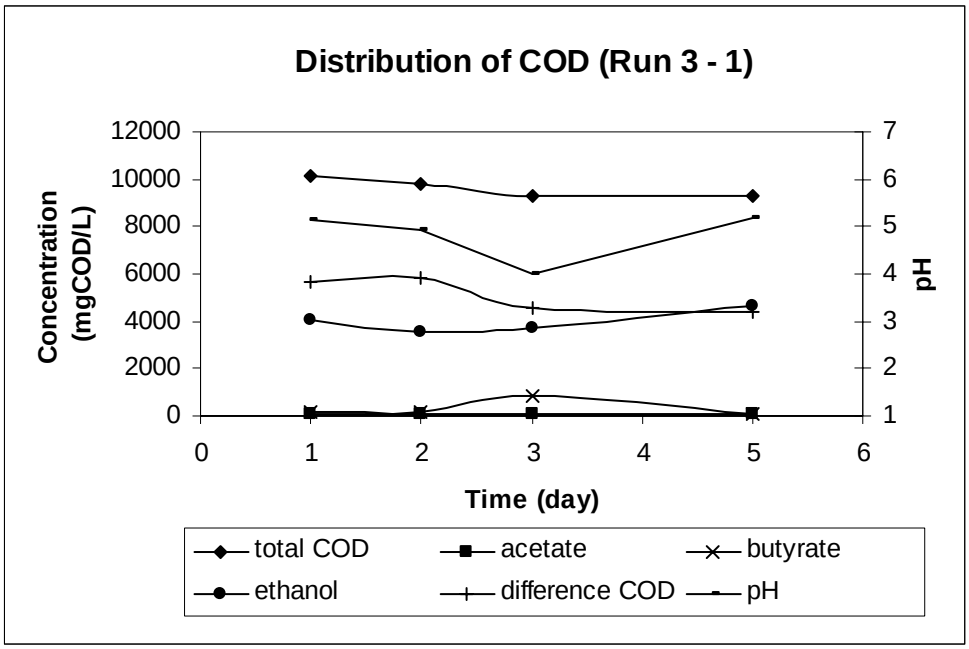

Figure 4. 12: Total COD, production of acetate, butyrate and ethanol versus time and pH graphs the 3rd period of 1st feeding for Reactor B.

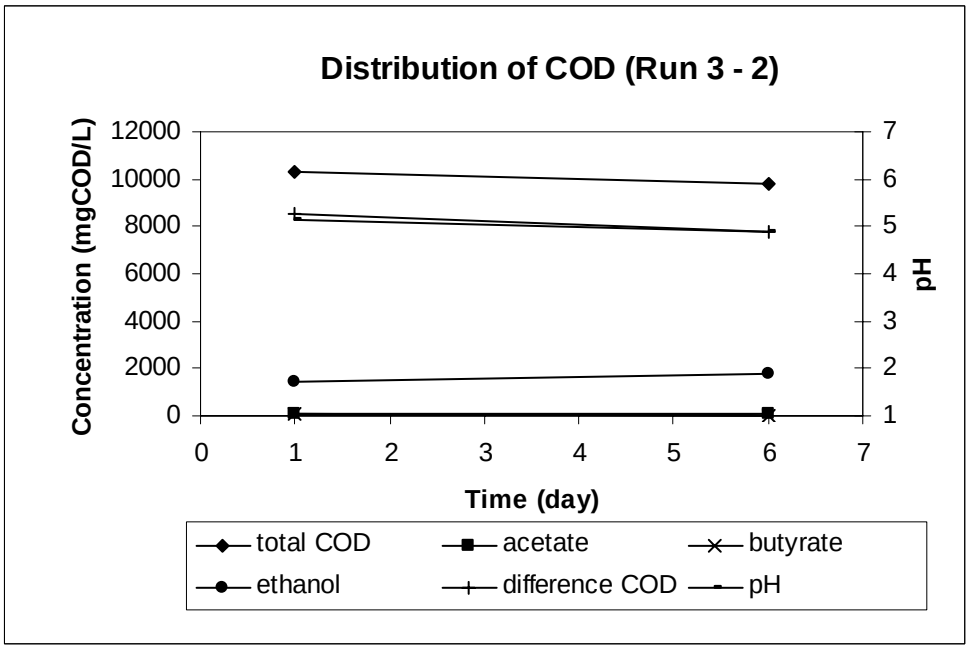

Figure 4. 13: Total COD, production of acetate, butyrate and ethanol versus time and pH graphs the 3rd period of 2nd feeding for Reactor B.

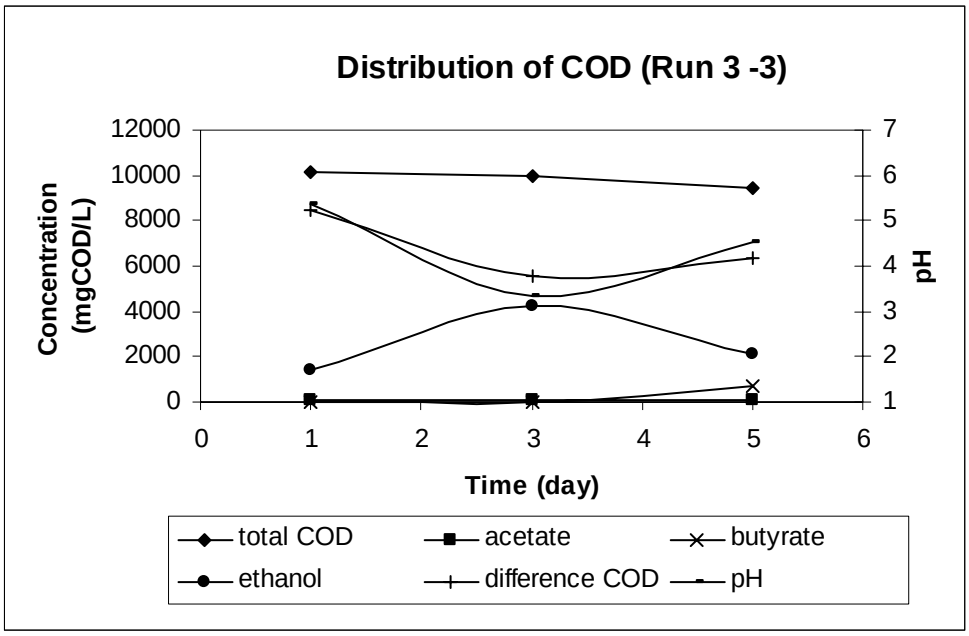

Figure 4. 14: Total COD, production of acetate, butyrate and ethanol versus time and pH graphs the 3rd period of 3rd feeding for Reactor B.

3rd feeding of the 3rd period: In this feeding, COD removal efficiency for 9 days is 6.3% with a drop in residual COD value from 8447 mg COD/L to 5534 mg COD/L in 5 days, and a subsequent increase to 6365 mg COD/L in the next 4 days. This difference is a result of the reduction in ethanol. However, the butyrate amounts started to increase after 9 days. The reason of the 9-day delay is the high amounts of total gas arising after 5 days.

4th feeding of the 3rd period: In this feeding, COD removal efficiency for 4 days is 11% with a drop in residual COD value from 7380 mg COD/L to 6732 mg COD/L. Produced ethanol and butyrate efficiencies have been balanced with the increase in the butyrate amounts. Hydrogen efficiency has increased together with total gas.

5th feeding of the 3rd period: In this feeding, COD removal efficiency for 8 days is 11% with a drop in residual COD value from 6872 mg COD/L to 4923 mg COD/L. The produced butyrate amounts continued to increase. Ethanol, on the other hand, has rapidly increased in the first 3 days, decreased in the last 4 days, and yet remained constant at a certain level at the end.

In the 3rd and the 5th feedings of this period, ethanol production is first significantly increased and then decreased. As given in detail within the literature survey chapter (Section 2 Pages 28-36), this situation is an important indicator that an intermediate product could converted to other products during process in the hydrogen production mechanism with anaerobic fermentation.

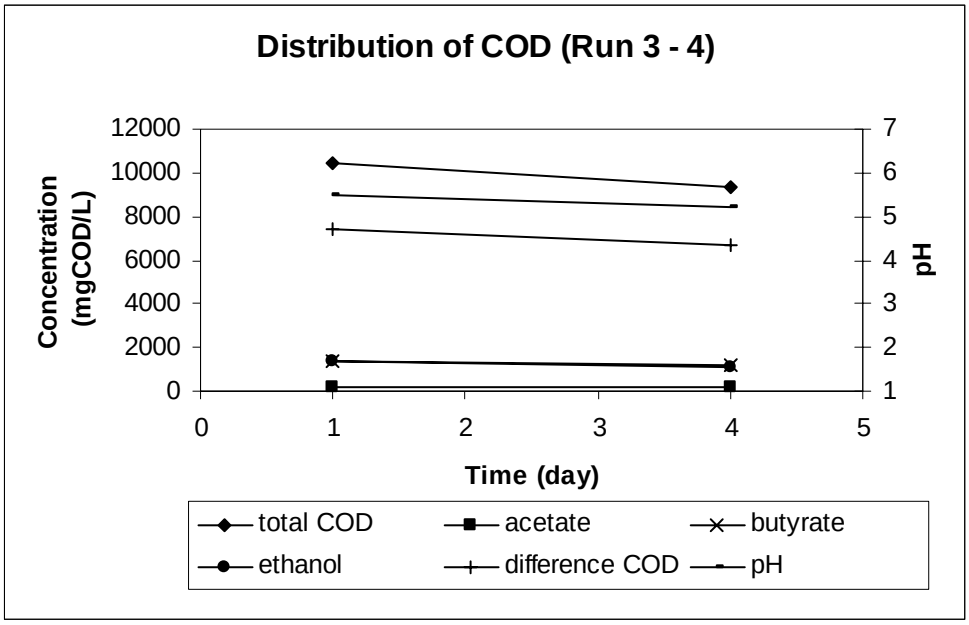

Figure 4. 15: Total COD, production of acetate, butyrate and ethanol versus time and pH graphs the 3rd period of 4th feeding for Reactor B.

The feedings in Reactor B are semi-batch similar to Reactor A. Including the initial feeding, a total of 18 feedings have been conducted in Reactor B for a total of 107 days.

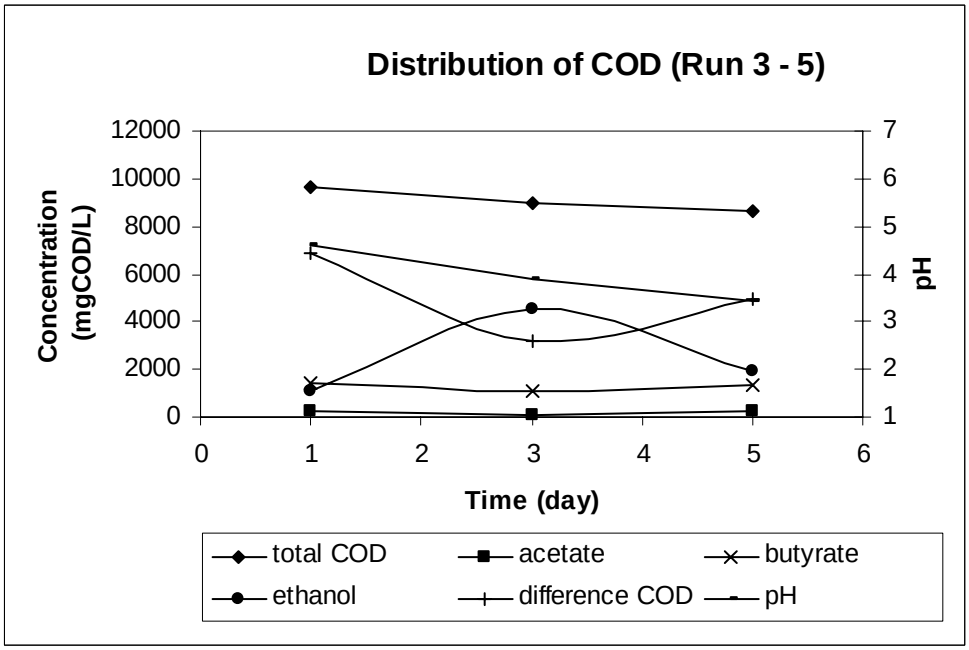

Figure 4. 16: Total COD, production of acetate, butyrate and ethanol versus time and pH graphs the 3rd period of 5th feeding for Reactor B.

Table 4.8: Distribution of COD of 3rd period (So=10,000 mg glucose/L and V_{feed} = 3 L) for reactor B.

Run 3.	Time (day)	Total COD (mgCOD/L)	Acetate (mgCOD/L)	Propionate (mgCOD/L)	Butyrate (mgCOD/L)	Valerate (mgCOD/L)	Ethanol (mgCOD/L)	Difference COD (mgCOD/L)	pH
1st loading	75	10105	98	15	150	140	4078	5624	5.15
	76	9770	94	18	130	131	3546	5851	4.95
	78	9331	64	15	859	134	3723	4536	3.99
	80	9291	79	16	51	130	4610	4405	5.19
2nd loading	80	10280	90	18	59	134	1419	8560	5.12
	86	9819	82	15	27	132	1774	7790	4.9
3rd loading	86	10115	74	11	17	146	1419	8447	5.35
	91	9993	76	15	19	124	4225	5534	3.36
	95	9478	106	12	706	163	2127	6365	4.54
4th loading	95	10474	189	16	1370	102	1418	7380	5.48
	99	9326	188	15	1211	111	1070	6732	5.21
5th loading	99	9675	238	15	1393	89	1069	6872	4.6
	102	9007	71	16	1099	123	4533	3164	3.91
	107	8633	285	16	1365	93	1951	4923	3.45

4.2.2 Production of VFA and Ethanol

In dark fermentation with mixed anaerobic culture, various liquid and volatile intermediate products are generated (Figure. 2,7 Dawes and Large 1982). Malate, fumarate, succinate, propionate, acetate, butyrate, lactate, acetone, ethanol, butanol and propanol are among those intermediate products. The dominant intermediate products generated from 6-carbon sugars in dark fermentation are lactate, acetate, butyrate, propionate and ethanol (Figure 2.11 Rodriquez, J. *et al.* 2006). During the generation of these intermediate products, only ethanol, acetate and butyrate result in hydrogen generation. This situation has been broadly discussed in the literature study.

Acetic, propionic, iso-butyric, butyric, iso-valeric and valeric acids have been measured as VFA similar to Reactor A. Ethanol, on the other hand, is measured as liquid alcohol. The mostly produced VFA in this reactor are iso-butyric and normal-butyric acids. Propionate and total valerate amounts are close to zero.

Acetate; in spite of being generated in amounts far from triggering the acetate interim step for hydrogen production, has been much more and steadily produced than of

Reactor A. The highest acetate figure was measured to be 531 mg COD/L for a pH of 4.45 at the 5th day of the 5th feeding in the 1st period.

The produced amounts of butyrate increased starting from the beginning of the 1st period and reaching a maximum of 6353 mg COD/L in the 4th feeding of the same period. After this point, the amount of butyrate has slowly started to decrease until the 2nd feeding of the 3rd period to a value of 9.4 mg COD/L. Later the amount of produced butyrate has increased through time and is measured to be 1365 mg COD/L in the 107th day. This study reveals that the variation of the produced butyrate has a significant impact on the hydrogen production efficiency.

All analysis results show that when a mixed anaerobic culture is acclimated, the mostly produced intermediate products are firstly ethanol and then butyrate as the pH is set free between 3.3-5.9. In other words, the intermediate product that is least effected by pH variation is ethanol and butyrate.

4.2.3 Production of Hydrogen Gas

Hydrogen gas was generated from glucose by three main pathways such as acetate, butyrate, ethanol pathways (Hwang *et., al.,* 2004).

$$4C_6H_{12}O_6 \rightarrow 3CH_3CH_2CH_2COOH + 2CH_3COOH + 2CO_2 + 2H_2 \quad \textbf{(4.1)}$$

$$C_6H_{12}O_6 + 2H_2O \rightarrow 2C_2H_5OH + CH_3COOH + 2H_2 + 2CO_2 \quad \textbf{(4.2)}$$

$$C_6H_{12}O_6 + 2H_2O_2 \rightarrow 2CH_3COOH + 2CO_2 + 4H_2 \quad \textbf{(4.3)}$$

Experimental results indicated that more butyrate and ethanol were produced than acetate. Even the amount of acetate was not enough to trigger the acetate pathway. For this reason, yield of hydrogen was calculated by using data from butyrate and ethanol.

As indicated in equations (4.1) and (4.2), 1 mol of glucose can produce 2 mol of ethanol, and 3/4 mol of butyrate. (Hwang, *et.al.,* 2004). Also, 3 mol of butyrate can produce 8 mol H_2 and 2 mol ethanol generates 2 mol H_2. Therefore, 5 mol glucose is sufficient to generate 10 mol H_2. 1 mol of glucose can produce 2 mol of H_2 according to butyrate and ethanol intermediate products pathways. Produced hydrogen gas was calculated according to equation (4.4) as related to produced ethanol and butyrate from glucose fermentation.

$C_{H2} = [C_{but} \times (8/3)] + =[C_{et} \times (2/2)]$ **(4.4)**

C_{H2} = Amount of produced total hydrogen gas, mmol

C_{but} = Produced butyrate concentration, mmol

C_{et} = Produced ethanol concentration, mmol

The amount of intermediates obtained from glucose degradation was demonstrated in Tables 4.9-4.12. Propionate and valerate were not shown in tables since these products do not contribute to the generation of H_2.

Table 4.9: Initial loading experimental data. (OLR=10,000 mgCOD/L)

Time day	Consumed glucose mg L^{-1}	Consumed glucose mmol L^{-1}	Acetate mmol L^{-1}	Butyrate mmol L^{-1}	Ethanol mmol L^{-1}	pH
1	loading		1.4	5.6	38.8	5
2	1022	5.7	1.4	4.9	46.2	4.6
5	809	4.5	1.8	4.5	37.0	3.7
6	121	0.7	2.5	4.3	42.5	4.8

Table 4.10: 1st Run experimental data

Time day	Consumed glucose mg L^{-1}	Consumed glucose mmol L^{-}	Acetate mmol L^{-1}	Butyrate mmol L^{-1}	Ethanol mmol L^{-1}	pH
6	1.loading		2.2	6.1	48.0	3.99
12	1881	10.5	3.1	9.2	44.3	4.9
12	2.loading		3.8	21.0	44.3	3.3
14	704	3.9	3.3	33.6	37.0	4.6
18	373	2.1	2.9	30.1	40.7	4.25
18	3.loading		2.7	30.7	14.8	5.2
19	164	0.9	2.8	31.8	46.2	4.03
21	443	2.5	2.6	32.0	44.3	4.56
22	320	1.8	2.3	26.1	37.0	5
22	4.loading		3.6	39.7	14.8	4.6
27	944	5.2	3.3	31.2	3.7	4.33
27	5.loding		3.9	31.0	3.7	5.2
33	604	3.4	8.3	34.2	3.7	4.45
33	6.loading		7.2	30.3	14.8	4.8
49	1532	8.5	6.5	25.7	14.8	4.3
49	7.loading		5.4	20.4	3.7	4.7
52	185	1.0	4.8	17.6	7.4	5.04
53	8.loading		6.3	25.1	11.1	4.75
53	173	1.0	5.8	20.8	11.1	5.09
54	273	1.5	4.5	15.1	5.5	5.7

Table 4.11: 2nd Run experimental data

Time day	Consumed glucose mg L^{-1}	Consumed glucose mmol L^{-1}	Acetate mmol L^{-1}	Butyrate mmol L^{-1}	Ethanol mmol L^{-1}	pH
1	1.loading		4.5	15.1	1.1	4.8
2	474	2.6	3.9	13.9	18.5	4.91
4	1799	10.0	3.8	13.3	38.8	5.74
5	72	0.4	2.7	7.7	14.8	4.85
8	928	5.2	2.8	7.8	38.8	5
8	2.loading		2.1	4.7	38.8	4.55
12	1054	5.9	2.5	4.1	44.3	5
12	3.loading		2.3	2.8	38.8	5.33
21	963	5.4	2.3	2.4	44.2	4.6

Table 4.12: 3rd Run experimental data

Time day	Consumed glucose mg L^{-1}	Consumed glucose mmol L^{-1}	Acetate Mmol L^{-1}	Butyrate mmol L^{-1}	Ethanol mmol L^{-1}	pH
1	1.loading		1.5	0.9	42.5	5.15
2	335	1.9	1.5	0.8	37.0	4.95
3	490	2.7	1.0	5.4	38.8	3.99
5	40	0.2	1.2	0.3	48.0	5.19
5	2.loading		1.4	0.4	14.8	5.12
11	461	2.6	1.3	0.2	18.5	4.9
11	3.loading		1.2	0.1	14.8	5.35
14	122	0.7	1.2	0.1	44.0	3.36
16	515	2.9	1.7	4.4	22.2	4.54
18	4.loading		3.0	8.6	14.8	5.48
22	1148	6.4	2.9	7.6	11.2	5.21
25	5.loadig		3.7	8.7	11.1	4.6
28	668	3.7	1.1	6.9	47.2	3.91
31	374	2.1	4.4	8.5	20.3	3.45

Theoretically, 2 mol of H_2 are produced by fermentation of butyrate-ethanol as shown in Tables 4.13-4.15. The yield of theoretical H_2 production was not calculated in the initial feeding period since total gas was not observed experimentally in this stage. The theoretical amount of hydrogen gas produced in the reactor depending upon consumed glucose and time was shown in Tables 4.13-4.15 for each period.

Table 4.13: Theoretical H_2 yield depending upon butyrate and ethanol in Run 1

Time day	Run 1.	H_2/butyrate ml $mmol^{-1}$	H_2/ethanol Ml $mmol^{-1}$	total ml $mmol^{-1}$	H_2/Glucose ml $mmol^{-1}$
6	1.loading	16.3	48.1	64.4	2
12		24.4	44.4	68.8	2
12	2.loading	56.1	44.4	100.5	2
14		89.5	37	126.5	2
18		80.3	40.7	121	2
18	3.loading	82	14.8	96.8	2
19		84.7	46.2	130.9	2
21		85.3	44.4	129.7	2
22		69.7	37	106.7	2
22	4.loading	105.8	14.8	120.6	2
27		83.3	3.7	87	2
27	5.loding	82.6	3.7	86.3	2
33		91.3	3.7	95	2
33	6.loading	80.8	14.8	95.6	2
49		68.5	14.8	83.3	2
49	7.loading	54.5	3.7	58.2	2
52		47	7.4	54.4	2
53		67	11.1	78.1	2
53	8.loading	55.5	11.1	66.6	2
54		40.2	5.6	45.8	2

Table 4.14: Theoretical H_2 yield depending upon butyrate and ethanol in Run 2.

Time day	Run 2.	H_2/butyrate ml $mmol^{-1}$	H_2/ethanol ml $mmol^{-1}$	total ml $mmol^{-1}$	H_2/Glucose ml $mmol^{-1}$
1	1.loading	49.5	11	60.5	1.9
2		47.2	36,9	84.1	1.9
4		27.4	62,8	90.2	1.9
5		27.6	29,5	57.1	1.9
8		16.5	70,2	86.7	1.9
8	2.loading	14.7	70,2	84.9	1.9
12		10	85	95	1.9
12	3.loading	8.5	70,2	78.7	1.9
21		8.2	85	93.2	1.9

Table 4.15: Theoretical H_2 yield depending upon butyrate and ethanol in Run 3

Time day	Run 3.	H_2/butyrate ml mmol^{-1}	H_2/ethanol ml mmol^{-1}	Total ml mmol^{-1}	H_2/Glucose ml mmol^{-1}
1	1.loading	3.3	77.6	80.9	2
2		2.8	66.5	69.3	2
3		19.0	70.2	89.2	2
5		1.1	99.7	100.8	2
5	2.loading	1.3	29.5	30.8	2
11		0.5	36.9	37.4	2
11	3.loading	0.3	29.5	29.8	2
14		0.4	85.0	85.4	2
16		15.6	44.3	59.9	2
18	4.loading	30.4	29.5	59.9	2
22		26.8	22.1	48.9	2
25	5.loadig	30.9	22.1	53.0	2
28		24.3	85.0	109.3	2
31		30.3	22.1	52.4	2

However, the obtained data from GC (Hewlett Packard) indicated that the yield of hydrogen was not high as calculated. The most significant reason was lower production of butyrate in the reactor when compared to empirical values. The amount of butyrate and ethanol were calculated theoretically and experimentally. In order to determine loss in the amount of hydrogen, experimental data were subtracted from the theoretical ones, and they are shown in Tables 4.16-4.18. Also, obtained hydrogen gas from the fermentation of butyrate-ethanol was shown in Figures 4.17-4.19.

The graphics for hydrogen gas production efficiency has not been drawn separately for each feeding. This is due to the difficulty of determining to which of the substrates the total gas outlet and the portion of hydrogen in this outlet belong to.

Table 4.16: Experimental yield of H_2 obtained in Run 1.

time	Run 1.	H_2/butyrate ml mmol^{-1}	H_2/ethanol ml mmol^{-1}	Total ml mmol^{-1}	H_2/Glucose ml mmol^{-1}
6	1.loading	10.3	42.3	52.6	1.6
12		16.1	39.0	55.1	1.6
12	2.loading	46.0	39.0	85.0	1.7
14		80.6	32.5	113.1	1.8
18		72.6	35.8	108.4	1.8
18	3.loading	74.8	13.0	87.8	1.8
19		77.2	40.7	117.9	1.8
21		78.5	39.0	117.5	1.8
22		63.7	32.5	96.2	1.8
22	4.loading	96.1	13.0	109.1	1.8
27		74.5	3.3	77.8	1.8
27	5.loding	72.1	3.3	75.4	1.7
33		69.2	3.3	72.5	1.5
33	6.loading	61.5	13.0	74.5	1.6
49		51.2	13.0	64.2	1.5
49	7.loading	40.2	3.3	43.5	1.5
52		34.1	6.5	40.6	1.5
53		50.2	9.8	60.0	1.5
53	8.loading	40.1	9.8	49.9	1.5
54		28.3	4.9	33.2	1.4

Table 4.17: Experimental yield of H_2 obtained in Run 2.

Time day	Run 2.	H_2/butyrate ml mmol^{-1}	H_2/ethanol ml mmol^{-1}	total ml mmol^{-1}	H_2/Glucose ml mmol^{-1}
1	1.loading	37.2	9.4	38.9	1.4
2		35.4	31.4	58.2	1.5
4		20.6	53.4	46.8	1.6
5		20.7	25.1	28.5	1.5
8		12.4	59.7	31.6	1.6
8	2.loading	11.0	59.7	23.3	1.6
12		7.5	72.3	20.6	1.7
12	3.loading	6.4	59.7	11.8	1.7
21		6.2	72.3	15.2	1.7

Table 4.18: Experimental yield of H_2 obtained in Run 3.

Time day	Run 3.	H_2/butyrate ml mmol^{-1}	H_2/ethanol ml mmol^{-1}	total ml mmol^{-1}	H_2/Glucose ml mmol^{-1}
1	1.loading	2.4	67.5	69.9	1.5
2		2.1	57.9	60.0	1.7
3		14.3	61.1	75.4	1.7
5		0.8	86.7	87.5	1.7
5	2.loading	0.9	25.7	26.6	1.7
11		0.4	32.1	32.5	1.7
11	3.loading	0.3	25.7	26.0	1.7
14		0.3	74.0	74.3	1.7
16		11.7	38.5	50.2	1.7
18	4.loading	22.8	25.7	48.5	1.6
22		20.1	19.2	39.3	1.6
25	5.loadig	23.2	19.2	42.4	1.6
28		18.3	74.0	92.3	1.7
31		25.7	19.2	44.9	1.7

Data listed in Figures 4.17-4.19 show experimental amount of hydrogen gas production depending upon consumed glucose and produced butyrate-ethanol. Anaerobic fermentation of glucose results in production of lactate, acetate, butyrate, propionate and ethanol. Among them, acetate, butyrate and ethanol are more valuable to generate hydrogen gas. Bio-hydrogen production mechanism has not been completely defined. However, if lactate is the main intermediate product, fermentation system can produce high amount of ethanol and CO_2 leading to lower yield of H_2 (Arhing., *et al* 2004). The experimental results of 2nd feeding period in this study fully support this idea (Figure 4.18). As described earlier, ethanol has been produced in high amounts which resulted in the yields of butyrate and H_2 remaining low. This is probably due to simultaneous production of lactate and ethanol in this period.

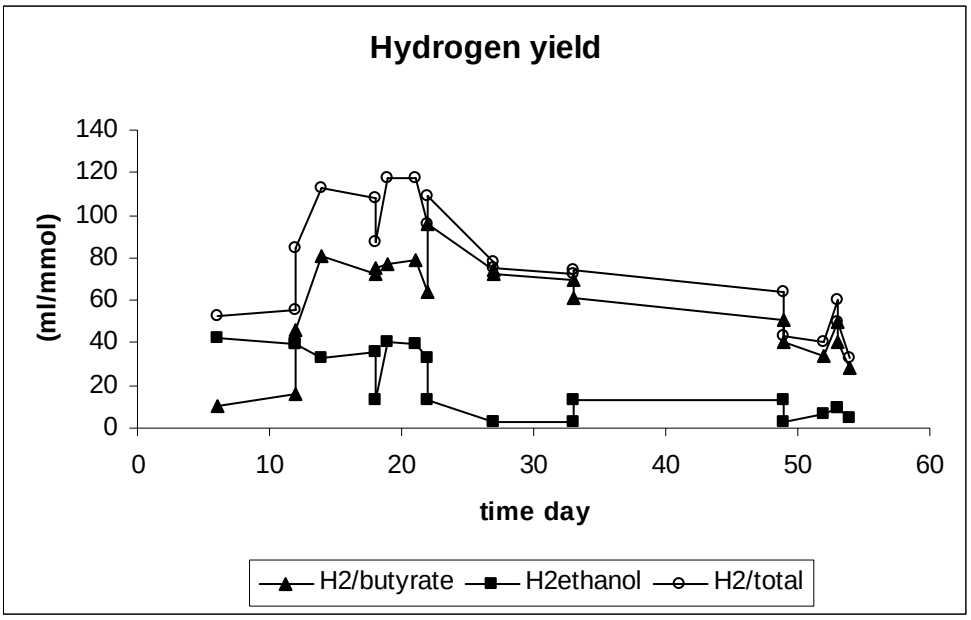

Figure 4.17: The experimental yield of H_2 per consumed glucose depending on butyrate and ethanol in first period for reactor B (H_2/Butyrate = The amount of H_2 per generated butyrate, H_2/Ethanol = The amount of H_2 per generated ethanol, Total H_2 ml $mmol^{-1}$).

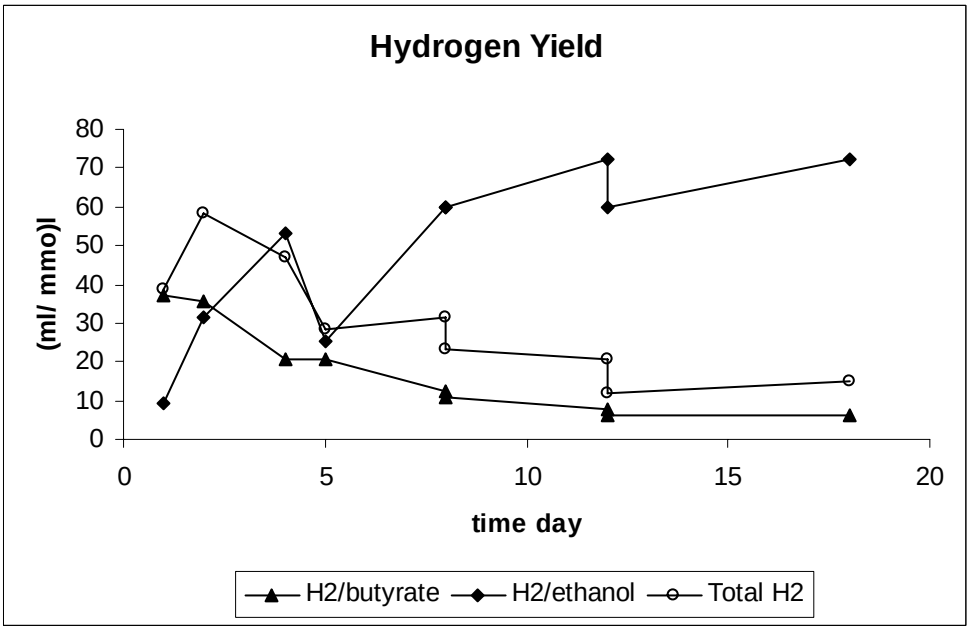

Figure 4.18: The experimental yield of H_2 per consumed glucose depending on butyrate and ethanol in second period for reactor B (H_2/Butyrate = The amount of H_2 per generated butyrate, H_2/Ethanol = The amount of H_2 per generated ethanol, Total H_2 ml $mmol^{-1}$).

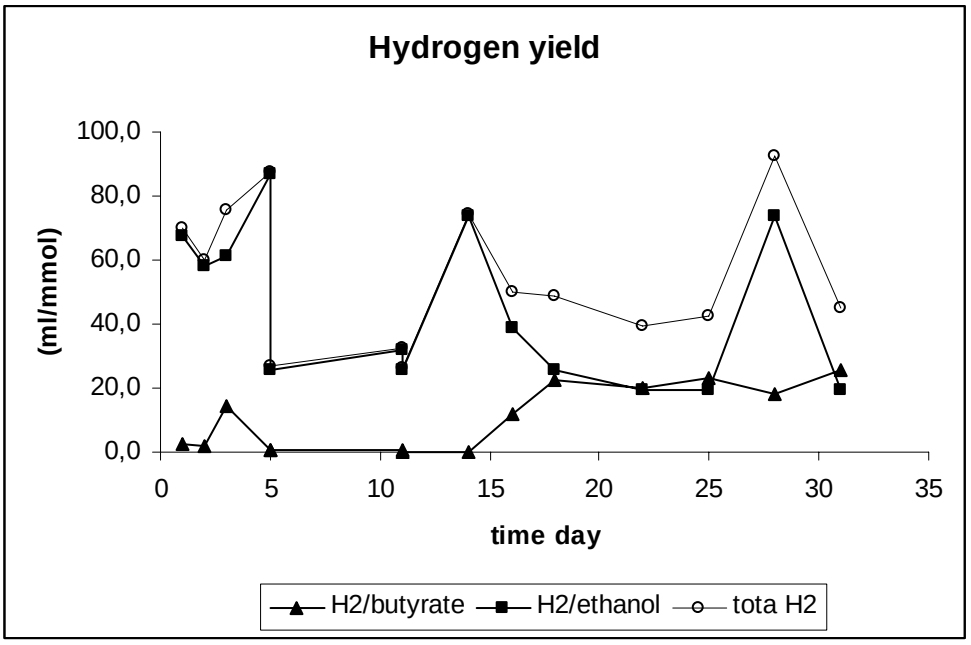

Figure 4.19: The experimental yield of H_2 per consumed glucose depending on butyrate and ethanol in third period for reactor B (H_2/Butyrate = The amount of H_2 per generated butyrate, H_2/Ethanol = The amount of H_2 per generated ethanol, Total H_2 ml $mmol^{-1}$).

It is determined that, propionic acid has a toxic effect on hydrogen fermentative systems. Accumulation of propionic acid stops generation of the other intermediate products such as acetate and butyrate. (Wang, *et.al.,* 2006) However, propionic acid produced in this study was very limited as shown in Tables 4.5- 4.8

As mentioned before, the highest amount of hydrogen production is obtained by generation of acetate, butyrate and ethanol. However, during this study, the amount of acetate produced was lower than the expected level. It is suggested that acetate might be converted to acetone simultaneously (Hwang, *et. al.,* 2004; Vortuba, *et.al.,* 1986). It is also possible that the yield of acetate is sensitive to pH, which has been in the range of 3.3-5.9 during the course of the experiment. The possible reason for generation of insufficient amounts of acetate was lactic acid being produced at high concentrations in the reactor. However, lactate was not measured within the scope of this study.

Table 4.19: The difference of theoretical and experimental H_2 yield for Run 1.

Time day	Run 1	Theoretical ml	Experimental ml	Discrepancy ml	Discrepancy %
6	1.loading	64.4	52.6	11.8	18.3
12		68.8	55.1	13.7	19.9
12	2.loading	100.5	85	15.5	15.4
14		126.5	113.1	13.4	10.6
18		121	108.4	12.6	10.4
18	3.loading	96.8	87.8	9	9.3
19		130.9	117.9	13	9.9
21		129.7	117.5	12.2	9.4
22		106.7	96.3	10.5	9.8
22	4.loading	120.6	109.1	11.5	9.5
27		87	77.8	9.2	10.6
27	5.loding	86.3	75.4	10.9	12.6
33		95	72.5	22.5	23.7
33	6.loading	95.6	74.5	21.1	22.1
49		83.3	64.2	19.1	22.9
49	7.loading	58.2	43.5	14.7	25.3
52		54.4	40.6	13.8	25.4
53		78.1	60	18.1	23.2
53	8.loading	66.6	49.9	16.7	25.1
54		45.8	33.2	12.6	27.5

As a result; in the literature, it is illustrated that acetate-ethanol fermentation is a more productive type (Lay, *et.al.,* 2003 and Ren, *et.al.,* 2006) than the other fermentation types. On the other hand, these experimental results show that butyrate-ethanol pathway is more stable than other intermediate product pathways in dark anaerobic fermentation systems.

H_2 yield was not reached to the expected levels in respect of glucose consumption at all periods. However, the production of butyrate and ethanol are reasonable with the exception of the 2^{nd} period. The difference of theoretical and experimental H_2 yield was shown in alternately in Tables 4.19- 4.21 and Figures 4.20- 4.22.

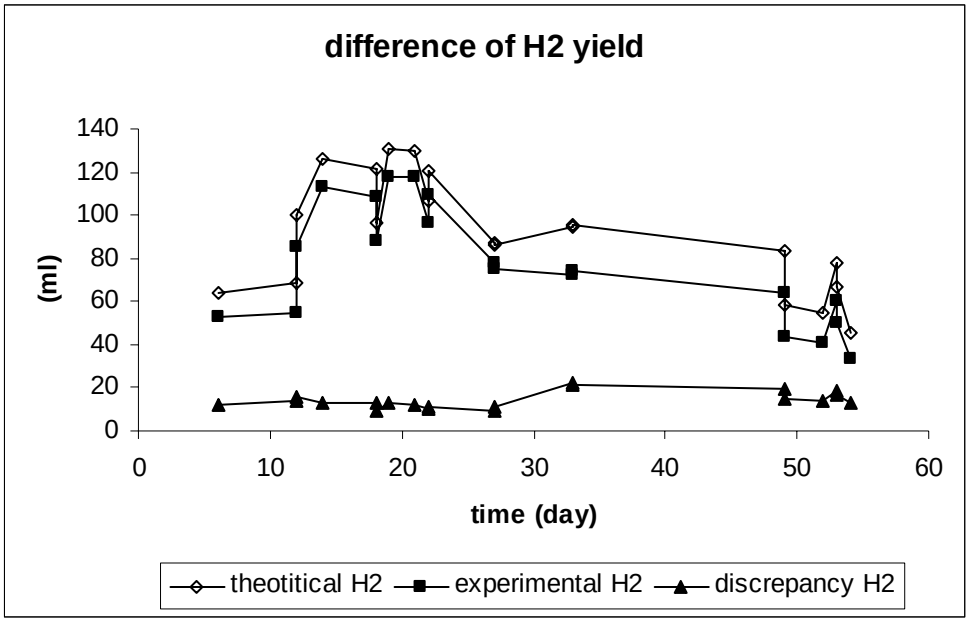

Figure 4.20: Theoretical and experimental H_2 yield according to produced butyrate-ethanol of Run 1 for reactor B.

Table4.20: The difference of theoretical and experimental H_2 yield for Run 2.

Time day	Run 2.	Theoritical ml	Experimental ml	Discrepancy ml	Discrepancy %
1	1.loading	60.5	38.9	21.6	36
2		84.1	58.2	25.9	31
4		90.2	46.8	43.4	48
5		57.1	28.5	28.6	50
8		86.7	31.6	55.1	64
8	2.loading	84.9	23.3	61.6	73
12		95	20.6	74.4	78
12	3.loading	78.7	11.8	66.9	85
18		93.2	15.2	78	84

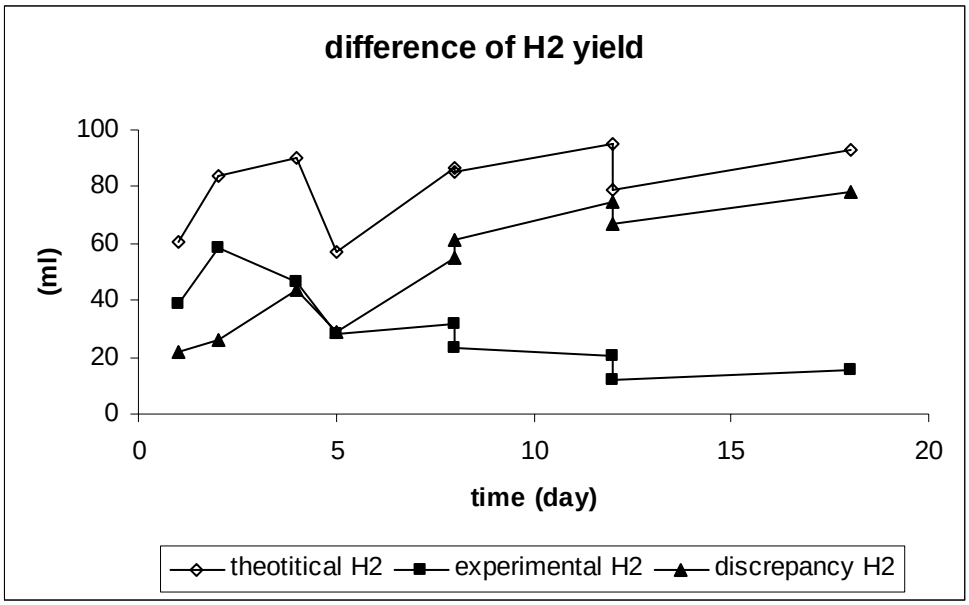

Figure 4.21: Theoretical and experimental H_2 yield according to produced butyrate-ethanol of Run 2 for reactor B.

Table 4.21: The difference of theoretical and experimental H_2 yield for Run 3.

Time day	Run 3.	Theoretical ml	Experimental ml	Discrepancy ml	Discrepancy %
1	1.loading	80.9	69.9	11	14
2		69.3	60	9.3	13
3		89.2	75.4	13.8	15
5		100.8	87.5	13.3	13
5	2.loading	30.8	26.6	4.2	14
11		37.4	32.5	4.9	13
11	3.loading	29.8	26	3.8	13
14		85.4	74.3	11.2	13
16		59.9	50.2	9.7	16
18	4.loading	59.9	48.5	11.4	19
22		48.9	39.3	9.6	20
25	5.loadig	53	42.4	10.6	20
28		109.3	92.3	17.1	16
31		52.4	44.9	7.5	14

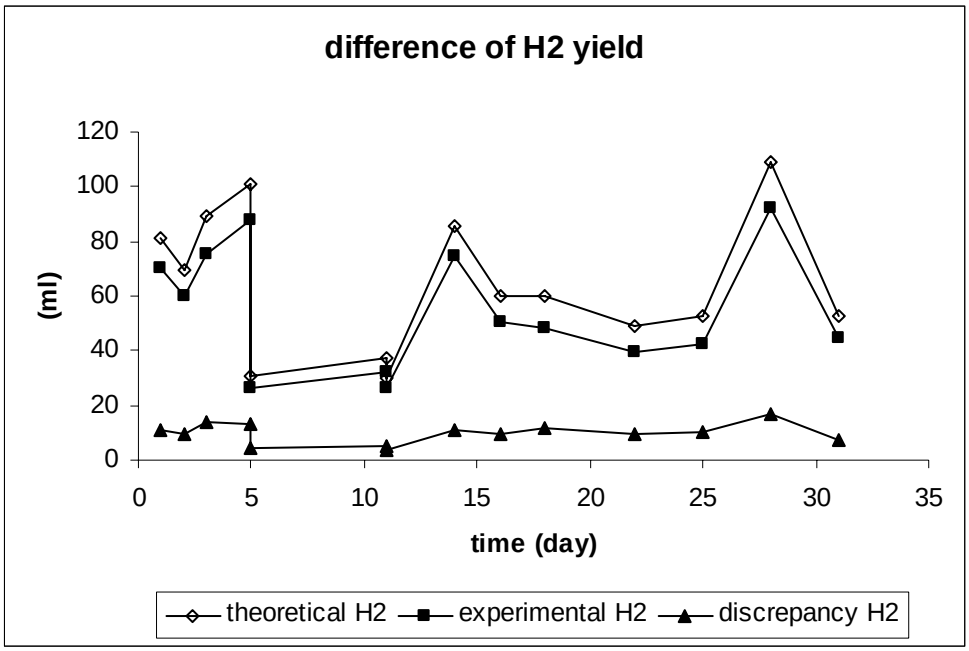

Figure 4.22: Theoretical and experimental H_2 yield according to produced butyrate-ethanol of Run 3 for reactor B.

4.2.4. pH and Total Gas Production

Initial pH was measured as 8 in Reactor B, when glucose (10000 mg/L as substrate), nutrient solution (100 ml), and buffer solution ($NaHO_3$ = 50) ml were fed in the reactor. Then pH value was dropped to 5 by addition of 1 N H_2SO_4. After 3 days, pH was recorded as 3.65 and then it was increased to 4.75 by addition of 1 N NaOH using peristaltic pumps. After one day pH was recorded as 3.99.

pH was not directly interfered in the rest of the study. pH has slightly increased about 3-4 hours after each feeding, but then each feeded in the following days pH decreased to the range of 0.5-1.75. The behaviour of pH versus time is shown in Figure 4.23.

Change in pH was monitored after the 1st feeding was performed. pH value was recorded as 4.5 after 1st feeding of 1st period. 3.5 hours later pH measured again and it is recorded as 4.8. During 4 days, pH decreased gradually and finally it was measured as 3.3. At this level, pH was increased up to 4 through the addition of 1 N NaOH.

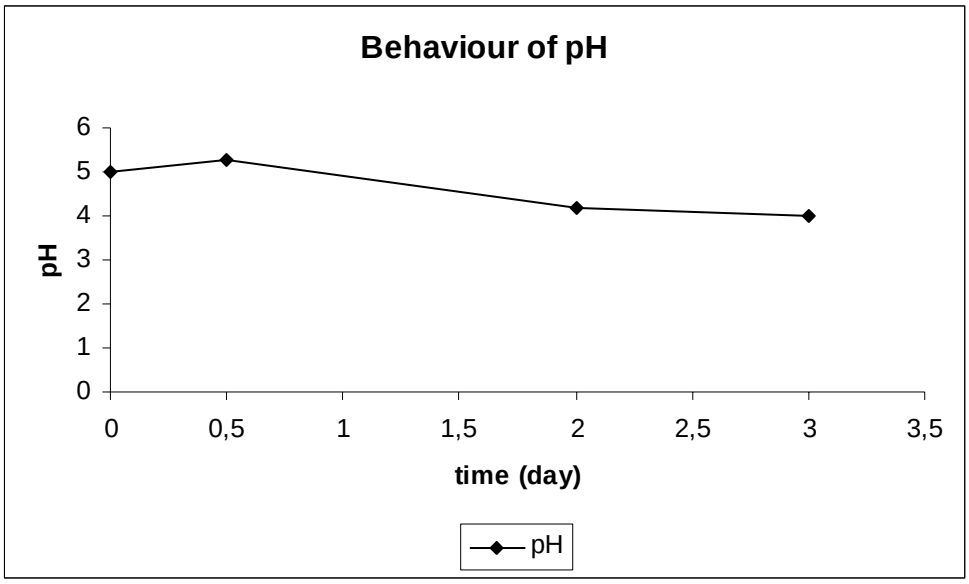

Figure 4.23: General behavior of pH after each loading.

Initial total gas (CO_2 + H_2) production was observed as 20 ml 2 days after the 1st feeding of the 1st period, in other words 8 days after the start-up of Reactor B. At this point, pH was recorded as 4.03. Then the pH was increased to 5 without feeding.

In the following feeding, pH decreased from 5 to 4.6, and 270 ml total gas production was observed at the end of the day. Also VFA and ethanol concentrations were recorded in the reactor (acetate = 169 mg/L, propionate = 9.2 mg/L, i-butyrate 21 mg/L, n-butyrate = 2773 mg/L, i-valerate = 3.3 mg/L, n-valerate = 3.3 mg/L, and ethanol =2125 mg/L).

2 days after the 3rd feeding of the 1st period, 800 ml of gas was collected and pH was recorded as 4.35. At the end of the day, VFA and ethanol concentrations were determined as (acetate = 153 mg/L, propionate = 19.8 mg/L, i-butyrate = 40.4 mg/L, n-butyrate = 2774 mg/L, i-valerate = 4 mg/L, n-valerate = 5.4 mg/L, and ethanol = 1955 mg/L). For this feeding, the total gas production was constant at the end of 4th day. Concentration of intermediate products was measured as follows: (acetate = 136.6 mg/L, propionate = 20.6 mg/L, i-butyrate = 36.3 mg/L, n-butyrate = 2264.2 mg/L, i-valerate = 3.4 mg/L, n-valerate = 5.4 mg/L, and ethanol = 1445 mg/L). For each feeding, total COD, the concentration of intermediate products and difference of COD values were compared with the previous data as given in Tables 4.5- 4.8.

pH decreased from 5.2 to 4.45 and 760 ml of total gas was collected 5 days after the 5th feeding of the 1st period. Concentration of intermediate products were measured (acetate = 198 mg/L, propionate = 22.8 mg/L, i-butyrate = 145.2 mg/L, n-butyrate = 2602 mg/L, i-valerate = 3.9 mg/L, n-valerate = 19.2 mg/L and ethanol = 680 mg/L).

Significant gas generation was not observed in the 1st period. In order to eliminate this inhibition effect in the reactor and to generate gas again, the part (excessive products) that was replaced with fresh medium was increased without any change in OLR.

In the 2nd period, the range of pH was set to be 3.3-5.9 similar to the situation of the 1st period. The difference of the two periods was accomplished through increasing the amount of excessive products from 1 L to 2 L. However, total gas production still decreased during this period. When 3rd feeding in this period was applied to the system, pH increased and gas production was not observed in the 1st day. While pH decreased from 4.75 to 4.55 spontaneously, amount of total gas was measured as 50 ml 2 days later after the 4th feeding. Concentration of intermediate products were measured (acetate = 135.5 mg/L, propionate = 11.4 mg/L, i-butyrate 43.7 mg/L, n-butyrate = 168 mg/L, i-valerate = 0 mg/L, n-valerate = 54 mg/L, and ethanol = 1615 mg/L). Produced ethanol was the most stable product in this period. However, total gas and VFA decreased dramatically.

As the excessive products were replaced with fresh ones in the 3rd period, while the system is continued to be operated under same conditions. Initial pH was measured as 5.14. Then after 2 days, it increased to 5.33 while total gas production could not be observed. pH was dropped to 4.6 using 1 N H_2SO_4. While pH decreased from 4.6 to 4.49, 50 ml of total gas production was observed for the remaining period. When 3rd feeding was applied to the system, gas volume was measured as 940 ml and pH decreased from 5.99 to 5.35 in a day. After 2 days without any interference to the system, the amount of total gas production reduced from 940 ml to 680 ml while pH dropped to 3.36. pH was increased to 4.6 using 1 N NaOH. Then 550 ml of gas production was observed when pH decreased from 4.54 to 4.44.

In the 4th feeding of the 3rd period, 600 ml gas was observed, and the pH decreased from 5.27 to 4.2 during two days. Total gas increased to 910 ml at the end of the 3rd day.

In the last feeding (5th feeding of the 3rd period), pH dropped from 4.7 to 3.86 and 490 ml of total gas was collected in a day. pH was increased to 4.6 by using 1 N NaOH. It again dropped to 3.48 in 2 days and total gas was recorded as 1030 ml.

Without any interference to the system, pH was recorded as 3.29 after 12 days and it remained constant at this value. Methane gas was not detected in total gas at the same period. This situation indicated that pH does not decrease continuously. At this point microorganisms that produce hydrogen gas got adapted to given conditions.

When anaerobic fermentative degradation of glucose (consumption of COD), production of intermediate products (VFA), H_2 yield, pH and total gas are interpreted together, it is possible to attain important results.

Hydrogen gas production was favourable via butyrate-ethanol pathways in the 1st and 3rd period. The highest butyrate production was observed after the 4th feeding in the 1st period. However, total gas and H_2 yield decreased after the 5th feeding in this period. Difference between the value of intermediate products as COD and residual COD is gradually increased after this feeding.

Also, accumulation of intermediate products, such as butyrate, inhibited the system. Therefore, more liquid phase (excessive products) were taken out to lower accumulation of intermediate products.

Although 2 L of excessive products were taken from the system in the 2nd period, H_2 yield, total gas and butyrate were dramatically decreased. On the other hand, ethanol production was reasonable. Lactate may be generated as a main intermediate product together with ethanol. In the literature, it is indicated that H_2 gas can not be produced when the lactate and ethanol are intermediate products (Arhing, *et.al.,* 2004).

Hydrogen and total gas production increased with increasing excessive products in the 3rd period. Also the amount of gas production and yield of H_2 were the most stable at the end of this period.

When butyrate:ethanol ratio equals to 1, the best H_2 yield was obtained for the 1st and 3rd periods.

Experimental results indicated that hydrogen gas production was possible with oscillation of pH. Additionally, pH can be considered as a monitor that presents

microbial activity in the reactor. The results pointed out that the hydrogen-generating microorganisms were resistant to change of pH.

It is known that hydrogen gas can be generated and consumed by the microorganisms present in the reactor. However, it is not possible to monitor the consumed hydrogen level in the reactor.

5. CONCLUSIONS and RECOMMENDATIONS

Although many studies have aimed to explain the metabolism of dark fermentation, mechanism of hydrogen producers in anaerobic mixed culture has not been completely defined. It is known that H_2-generating enzymes are mainly hydrogenase and nitrogenase. However; it is not clear how to produce ATP and NADH with nitrogenases under strict anaerobic conditions, since the functions of this enzyme was explained for photo-fermentation and facultative anaerobic conditions. Such a phenomenon was also observed for hydrogenases, because of the confusion of consumption and production of hydrogen yield. Therefore, it is difficult to determine the best fermentation types, such as acetate-ethanol, butyrate-ethanol, butyrate-acetate, etc., even when the effects of environmental factors (pH, temperature, etc.) are known.

The aim of this research was to obtain better evidence on the mechanism and metabolism of bio-hydrogen production via dark fermentation. The investigation was further extended to examining the effect of pH and seed sludge on H_2 yield and production stability.

Among a set of two reactors, Reactor A was prepared with the seed sludge form a full scale anaerobic digester of the municipal wastewater plant of Kayseri, and Reactor B was filled with fermentation sludge from a lab-scale anaerobic digester treating olive oil wastes. The two reactors were compared in terms of the production of hydrogen and ethanol. At the end of the experiments, it can be said that Reactor A did not produce hydrogen gas and/or with depending intermediate products, such as acetate, butyrate and ethanol. The former studies have shown that the pre-treatment applications of trying to dominate hydrogen generating species, such as heat-shock, acidification, alkalinity, etc., biological hydrogen production was observed at a certain temperature, pH and organic feeding rate. In this study, the reason for no hydrogen production may be due to the un-adapted seed or inhibition of the microorganisms.

Keeping the inoculation sludge for a long time in anaerobic acidic conditions has positive effects on yield of biological hydrogen production. In Reactor B, microorganism population acclimated to the system, however, high amount of hydrogen production was not observed. For continuous feed reactor, such inoculation is considered to have positive effect on the system.

The experimental results from Reactor B, such as total gas production, concentration of intermediate products and amount of H_2 with respect to pH, were also examined. The maximum total gas production increased when pH decreased. Although, the ratio of hydrogen in the gas phase dropped to low levels, it is considered as evidence for the continuity of fermentation. Fluctuations in pH significantly affected acetate production, but ethanol and butyrate were less affected. Experimental results showed that propionate production was inhibited when pH was lower than 5.9. Around pH 3.3, out of order of intermediate products was observed, and yield of H_2 production decreased, whereas there was no change in total gas production. In the pH range of 3.5 - 4, CO_2 gas production dramatically increased. At the end days of the experimental period when pH was 3.45, pH did not decreased below 3.29.1030 ml of total gas containing only 2.2 % H_2 was produced in the system. H_2 can be produced with oscillating pH. Ethanol production is efficient when the pH in range of 3.5-5.5.

Higher amounts of H_2 production were observed when equilibrium was sustained between production of VFA and liquid alcohols. When butyrate:ethanol ratio was approximately 1, both total gas and hydrogen yield increased.

After VFA formation, intermediate products formed by fermentation could be converted to more stable intermediate products using hydrogen. For example, produced butyrate was converted to butanol.

Furthermore, butyrate can be converted to butanol by taking H^+ from medium, and acetate can turn into acetone by using 1 mole of H_2 from NAD/$NADH_2$ electron transporter. High amount of produced ethanol with unbalanced of butyrate and acetate could be resulted from high amounts of lactic acid. Lactic acid production has negative effects on hydrogen production. As a result, 10.5 % of glucose fed to the system was converted to CO_2, and 89.5 % of glucose was converted to intermediate products which were kept in the system.

Opinions and Proposals

Each anaerobic mixed culture chosen as seed sludge may not present the same hydrogen-production capacity.

Organic feedings should be set periodically and short feeding periods should be chosen to avoid usage of produced H_2 gas in the system.

Lactate, butanol and acetone carry risk in the production of anaerobic bio-hydrogen, therefore, these intermediate products must be continuously observed and be controlled in the system.

Hydrogen production is directly related to intermediate products in dark fermentation. There are a lot of intermediate products such as formate, furmarate, succinate, malete, and lactate; not all of them were measured in this study.

The yield of H_2 obtained from acetate–ethanol fermentation is more efficient than other fermentation types. On the other hand, butyrate–ethanol fermentation is more stable than other types. In addition, ethanol is the balancing intermediate product in such systems.

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