INVESTIGATING THE OPERATIONAL CONDITIONS FOR ENHANCEMENT OF DARK FERMENTATIVE HYDROGEN PRODUCTION IN BATCH AND SEQUENCING BATCH REACTORS

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ABSTRACT

INVESTIGATING THE OPERATIONAL CONDITIONS FOR ENHANCEMENT OF DARK FERMENTATIVE HYDROGEN PRODUCTION IN BATCH AND SEQUENCING BATCH REACTORS

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The aim of this master thesis study was to investigate the dark fermentative hydrogen production in batch and sequencing batch reactors (SBRs), and to investigate operational conditions leading to its maximization.

Batch reactor studies were conducted to determine the initial operational conditions for the subsequent SBR operation. Two batch reactor sets, conducted with either sucrose or molasses as substrate, were operated to investigate the effect of initial pH, chemical oxygen demand (COD) and volatile suspended solids (VSS) concentrations and maximize the hydrogen production. These reactor sets were designed with Response Surface Methodology. Results revealed that maximum hydrogen yield (HY) of 2.3 mol H_2/mol $sucrose_{added}$ was achieved at an initial pH of 7 and initial COD concentration of 10 g/L. The studied initial substrate to VSS ratio (S/X_o) values of 4, 12 and 20 g COD/g VSS had no effect on hydrogen production yield. For batch studies conducted with molasses, the change in HY and productivity could not be explained with the studied ranges of three variables; initial pH, COD and VSS values. Maximum HY was achieved at 10 g/L initial COD, as 2.88 mmol H_2/g $sucrose_{added}$. The

decrease in the H_2 and CO_2 percentages of the headspace gas and suction observed in the reactors were attributed to homoacetogenic activity. Molasses, for containing potential intrinsic microorganism, might be more suitable to support and trigger the homoacetogenesis than sucrose.

The SBR experiments consisted of 5 studies with different operational conditions. The objective of these studies was to increase the hydrogen production via each study, by modification of the operational parameters like pH and hydraulic retention time (HRT), with an attempt to maximize the HY. In the course of the research, two more objectives presented themselves. The first one was to suppress the homoacetogenenic activity and, the second appeared as to investigate the effect of Solid Retention Time (SRT) on dark fermentative hydrogen production. Results indicated that continuous dark fermentative H_2 production from sucrose was significantly influenced by pH, HRT, homoacetogenic activity and SRT. Long HRTs (>12 h) and SRTs (>5 days) enhanced homoacetogenic activity and caused low HYs. The pH values higher than 5.5 were inefficient in suppressing the methanogens. The physical interventions done on the reactor increased the hydrogen production significantly. The maximum H_2 yield achieved in SBR studies was 2.52 mol/ mol hexose (13.11 mmol H_2/ g COD) at the conditions of pH of 5.5, HRT of 12 h, cycle time 8 of h, OLR of 22.4 gCOD/L.day and average SRT of 9.5 day. The HPR calculated at these operational conditions was 7.07 L H_2/ (L_{rxr}.day).Yet, dark fermentative SBR systems are hard to stabilize at high HY. The ways to improve the stability of hydrogen production and the dominant microbial population remains to be studied.

Keywords: Dark Fermentation, Hydrogen Production, Homoacetogenesis, Sequencing Batch Reactor, Solid Retention Time

ÖZ

KESİKLİ VE ARDIŞIK KESİKLİ REAKTÖRLERDE KARANLIK FERMANTATİF HİDROJEN ÜRETİMİNİ GELİŞTİRMEK İÇİN İŞLETME KOŞULLARININ ARAŞTIRILMASI

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Bu yüksek lisans tezinin amacı karanlık fermantasyon ile hidrojen üretiminin kesikli ve Ardışık Kesikli Reaktörlerde (AKR) incelenmesi ve maksimum hidrojen üretimini sağlayabilecek işletme koşullarının araştırılmasıdır.

Kesikli reaktör çalışmaları, takip eden AKR çalışmalarının başlangıç işletme koşullarını saptamak amacıyla kurulmuştur. Sübstrat olarak sükroz veya melas ile kurulan iki kesikli reaktör seti, başlangıç pH, başlangıç Kimyasal Oksijen İhtiyacı (KOİ) ve Uçucu Askıda Katı Madde (UAKM) derişimlerinin etkisini araştırmak ve maksimum hidrojen üretimi amacıyla işletilmiştir. Bu reaktör setleri Tepki Yüzeyi Metodolojisi (TYM) kullanılarak tasarlanmıştır. Sonuçlar 2,3 mol H_2/mol $sükroz_{eklenen}$ değerindeki maksimum hidrojen veriminin (HV) pH 7 ve 10 g/L KOİ başlangıç değerlerinde elde edildiğini göstermiştir. Çalışılan 4, 12 ve 20 g KOİ/g UAKM değerlerindeki başlangıç sübstrat/UAKM (S/X_o) oranlarının hidrojen verimine bir etkisi yoktur. Melasla kurulan kesikli reaktör setinde, hidrojen verimi ya da üretim hızındaki değişimler, 3 değişken (başlangıç pH, KOİ ve UAKM) için araştırılan değer aralığı ile açıklanamamıştır.

Maksimum HV, 2,88 mmol H_2/g $sükroz_{eklenen}$ olarak 10 g/L başlangıç KOİ'de elde edilmiştir. Reaktörlerde gözlenen tepe gazındaki H_2 ve CO_2 yüzdelerinin düşmesi ve reaktörlerin vakum yapması homoasetojenik aktivite ile ilişkilendirilmiştir. Melas, yerel mikroorganizmalar barındırma olasılığı ile, homoasetojenisisi tetiklemek ya da desteklemek için sükroza kıyasla daha uygun olabilir.

AKR deneyleri, farklı işletme koşullarına sahip beş çalışmadan oluşmaktadır. Bu çalışmaların amacı, HV'yi maksimuma ulaştırma çabası doğrultusunda, pH ve Hidrolik Bekletme Süresi (HBS) gibi işletme parametrelerini modifiye ederek hidrojen üretimini arttırmaktır. Araştırma sürecinde, iki hedef daha ortaya çıkmıştır. Bunlardan birincisi homoasetojenik aktivitenin baskılanması, diğeri ise Katı Bekletme Süresi'nin (KBS) karanlık fermantatif hidrojen üretimine etkisinin incelenmesidir. Sonuçlar sükrozdan sürekli karanlık fermantatif hidrojen üretiminin pH, HBS, homoasetojenik aktivite ve KBS'den kayda değer şekilde etkilendiğini göstermiştir. Uzun HBS'ler (>12 saat) ve KBS'ler (>5 gün) homoasetojenik aktivitenin etkinliğini arttırmakta ve düşük HV'lere neden olmaktadır. 5,5'ten daha yüksek pH değerleri, metanojenleri baskılamakta yetersiz kalmıştır. Reaktöre yapılan fiziksel müdahaleler hidrojen üretimini kayda değer şekilde arttırmıştır. Maksimum hidrojen verimi pH 5,5, HBS 12 saat, döngü süresi 8 saat, OYS 22,4 g KOİ/L.gün ve ortalama KBS 9,5 gün olan koşullarda, 2,52 mol/ mol heksoz (13,11 mmol H_2/ g KOİ) olarak elde edilmiştir. Fakat, karanlık fermantatif AKR sistemlerinin yüksek HV değerlerinde stabil kalmasının zor olduğu gözlenmiştir. Hidrojen üretiminin stabilitesini ve etkin mikrobial popülasyonu geliştirecek yöntemlerin araştırılması gerekmektedir.

Anahtar Kelimeler: Karanlık Fermantasyon, Hidrojen Üretimi, Homoasetojenesis, Ardışık Kesikli Reaktör, Katı Bekletme Süresi

For all the children I have been touched by…

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ABBREVIATIONS

AnMBR	Anaerobic membrane bio-reactor
ANOVA	Analysis of Variance
ASBR	Anaerobic sequencing batch reactor
BM	Basal medium
COD	Chemical oxygen demand
CSTR	Continuous stirred tank reactor
EGSB	Expanded granular sludge bed reactor
HAc	Acetic acid
HPR	Hydrogen production rate
HRT	Hydraulic retention time
HY	Hydrogen yield
L_{rxr}	Volume of the reactor
MBR	Membrane bio-reactor
MLSS	Mixed liquor suspended solids
MLVSS	Mixed liquor volatile suspended solids
NAD	Nicotinamide adenine dinucleotide
NADH	Reduced nicotinamide adenine dinucleotide
OFMSW	Organic fraction of municipal solid waste
OLR	Organic loading rate
pH_2	Hydrogen partial pressure
R/S	Reaction time/Settle time
RSM	Response Surface Methodology
S	Substrate
SBR	Sequencing batch reactor
sCOD	Soluble chemical oxygen demand
SRT	Solid retention time

tCOD	Total chemical oxygen demand
TAN	Total ammonia nitrogen
TKN	Total Kjeldahl nitrogen
TS	Total solids
TSS	Total suspended solids
TVS	Total volatile solids
UASB	Upflow anaerobic sludge blanket reactor
VFA	Volatile fatty acids
VS	Volatile solids
VSS	Volatile suspended solids
X_o	Initial volatile suspended solids

CHAPTER 1

INTRODUCTION

Finding alternative ways of energy production other than fossil fuels is the most popular research area recently to slowdown the effects of global warming. Hydrogen attracts particular attention because of its high energy content (122 kJ/g), eco-friendly alternative production methods and no greenhouse gas release when burned. In addition, H_2 gas is safer to handle than domestic natural gas. H_2 is universally accepted as an environmentally safe, renewable energy resource and an ideal alternative to fossil fuels since it does not contribute to the greenhouse effect (Das and Veziroglu, 2008).

Hydrogen may be produced through the electrolysis of water, thermocatalytic reformation of hydrogen-rich organic compounds and biological processes. Currently, hydrogen is produced by steam reformation of methane or mostly by electrolysis of water (Atif et al., 2005). Biological production of hydrogen using microorganisms offers the potential production of usable hydrogen from a variety of renewable resources (Levin, 2004). Anaerobic bio-hydrogen may be produced by photosynthetic and chemosynthetic bacteria. Chemosynthetic bacteria generate hydrogen without photoenergy through dark fermentation. The traditional ways of hydrogen production like photolysis of water or photo-fermentation are energy intensive and, thus, not environment friendly (Van Ginkel and Logan, 2005). Hydrogen production from renewable wastes via dark fermentation, therefore, suggests a more sustainable method of hydrogen production.

Dark fermentation is the degradation of organic materials to Volatile Fatty Acids (VFA), H_2 and CO_2 under anaerobic conditions. Although dark fermentative

hydrogen production systems have lower yields compared to the light-initiated processes, they provide a more applicable method for real life applications due to their higher H_2 production rate, no light requirement and potential of utilizing waste material (Das and Veziroglu, 2001). Organic waste can be a very important source for energy production if it is properly managed for dark fermentation applications. This will serve two purposes; clean energy in the form of hydrogen production and the treatment of the organic waste. In addition, since the systems utilize cheap raw materials/waste, they are more compatible with future commercialized processes (Das and Veziroglu, 2008).

In order to increase the bio-hydrogen production, a number of approaches are being investigated like modification of the operational conditions, improvements in reactor design, pre-treatment of the waste, application of two-phase systems and the use of genetically modified organisms (Das and Veziroglu, 2008; Luo et al., 2011; Wang and Wan, 2008a) The strategies to improve stability and maximum yield of dark fermentative hydrogen systems in continuous systems are well reviewed by Van Ginkel and Logan, (2005), Hawkes et al. (2007), Wang and Wan (2009b) and Jung et al. (2011). The main parameter to have significant effect on the yield and stability of the dark fermentative systems, whether batch or continuous, is stated as pH (Wang and Wan, 2009b). Hydraulic Retention Time (HRT) for continuous systems is stated as the next important parameter (Hawkes et al., 2007). Homoacetogenic activity is stated as a significant obstacle before achievement of high yield and stability in dark fermentative hydrogen production systems (Saady, 2013). Homoacetogenesis is carried out by the autotrophic acetogenic microorganisms consuming H_2 and CO_2 which shift their metabolism under stress conditions or when there is depletion of substrate (Saady, 2013). This issue is explored in Saady's (2013) study and mentioned to have important effect in the future of dark fermentation in a number of studies.

There are a limited number of pilot-scale dark fermentative hydrogen production studies in the literature dating back only 5 years. These early pilot-scale applications, each taking one step towards sustainable dark fermentative hydrogen production, provide results that indicate the successful utilization of organic waste and high hydrogen production yields (Cavinato et al., 2012; Faloye et al., 2014; Kim et al., 2010; Krupp and Widmann, 2009; Lin et al., 2011). Within the surveyed literature, the largest pilot-scale application is a 0.76 m^3 continuous stirred tank reactor by Cavinato et al. (2012) with substrate as food waste and there are no municipal or industrial dark fermentative hydrogen production plants. Hydrogen production from dark fermentation systems is still at its early research phase. Important operational conditions and reactor designs are still under development. But it surely has a promising future due to its many advantages stated above and the initial pilot-scale results.

Stability, in terms of reactor gas production amount and operational pH, has been stated as difficult to achieve at dark fermentative hydrogen production by some researches (Dinamarca and Bakke, 2009; Wu et al., 2009; Saady, 2013). Parameters like pH, HRT or Organic Loading Rate (OLR) are frequently investigated in dark fermentation studies (Badie et al., 2011; Fang and Liu, 2002; Won and Lau, 2011) and fairly consistent results are achieved in most studies. But parameters like sludge characteristics and the Solid Retention Time (SRT), or, the combined effects of the operational parameters are still less explored issues.

The scope of this thesis study was to investigate the dark fermentative hydrogen production from sucrose and molasses in batch and Sequencing Batch Reactors (SBRs). The objectives of this thesis were as follows;

1. To investigate dark fermentative hydrogen production from sucrose or molasses in batch reactors,

- to determine the (combined) effects of initial Chemical Oxygen Demand (COD), initial Volatile Suspended Solids (VSS) (as X_o) (or the substrate to VSS concentration amount, S/X_o) and initial pH values on hydrogen production, and
- to determine the optimum values of these parameters for the maximum hydrogen production

2. To investigate dark fermentative hydrogen production from sucrose in SBRs,
    - to determine the effects of initial pH and HRT (or OLR) on hydrogen production and
    - to examine the effects of SRT on hydrogen production

It was also aimed in SBR studies to achieve stable and high-yield dark fermentative hydrogen production. Thus, potential hydrogen consuming reactions such as homoacetogenesis were also taken into consideration. Accordingly, the other objective of this study was set as to examine the effects of the operational parameters such as pH and HRT on homoacetogenesis and how to use them for suppressing homoacetogenic activity.

CHAPTER 2

LITERATURE REVIEW

2.1 Hydrogen Energy

Hydrogen is the ideal clean energy due to its various advantages. Hydrogen is considered as the most intriguing candidate as a future energy carrier due to its high energy content and non-polluting characteristics (Van Ginkel et al., 2001). Hydrogen has a high energy content of 122 kJ/g, which is about 2.75 times greater than the energy content of methane. Unlike methane it does not produce any CO_2 when burned, does not necessarily require fossil sources to be produced or energy intensive methods for production hence, does not contribute further to global warming. It has a lower global warming potential than methane. In fact, Derwent (2006) stated that, "if a global hydrogen economy replaced the current fossil fuel-based energy system and exhibited a leakage rate of 1%, then it would produce a climate impact of 0.6% of the current fossil fuel based system". In addition, it has been widely used in fuel cells to convert chemical energy to electricity with high efficiency.

There has been intensive research interest on biological hydrogen production recently. This is mainly attributed to environmental concerns and energy insecurity (Chen et al., 2009). Some of the conventional hydrogen gas production methods are steam reforming of natural gas, gasification of coal, and electrolysis of water (Nath and Das, 2004). However, these processes do not achieve the coupled goal of waste stabilization and renewable energy production. Biological hydrogen production is the most sustainable method since it provides opportunities for waste reduction as well as energy production

(Ozkan et al., 2010). Since it can take place at ambient temperatures, it only requires a low energy input. It can use different types of organic waste providing a COD decrease in these wastes therefore, its production can also generate waste treatment credits (Keskin et al., 2011). Biological H_2 production processes can be classified as follows (Levin, 2004);

- Biophotolysis of water using green algae and blue-green algae (cyanobacteria)
- Photodecomposition of organic compounds by photosynthetic bacteria,
- Dark fermentation of organic compounds,
- Hybrid systems (through use of fermentative and photosynthetic bacteria or use of bioelectrochemical assisted bioreactor).

2.2. Dark Fermentation

Dark fermentation is degradation of organic materials to VFAs, alcohols, H_2 and CO_2 under anaerobic conditions, with acidogenic and acetogenic bio-reactions; in accordance with Equations 1.1 to 1.5. Spore forming bacteria are the most commonly used species, like *Clostridium* or *Bacillius* and anaerobic acidogenic sludge.

Acidogenic and Acetogenic Bio-reactions are,

Acetic Acid: $C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COOH + 4H_2 + 2CO_2$ (Eq 1.1)

Butyric Acid: $C_6H_{12}O_6 \rightarrow CH_3CH_2CH_2COOH + 2H_2 + 2CO_2$ (Eq 1.2)

Propionic Acid: $C_6H_{12}O_6 + 2H_2 \rightarrow 2\ CH_3CH_2COOH + 2H_2O$ (Eq 1.3)

Lactic Acid: $C_6H_{12}O_6 \rightarrow 2CH_3CHOHCOOH + H^+$ (Eq 1.4)

Ethanol: $C_6H_{12}O_6 \rightarrow 2CH_3CH_2OH + 2CO_2$ (Eq 1.5)

The theoretical maximum Hydrogen Yield (HY) from complete conversion of glucose to hydrogen and carbon dioxide via dark fermentation is 12 mole H_2 / mole glucose. However, this reaction is not thermodynamically feasible (Westermann et al., 2007). In absence of external energy, Equation 1.1 and Equation 1.2 are mostly favoured; providing most common products in the fermentation of carbohydrate are acetate and butyrate. Propionic acid is produced with a H_2 degrading pathway therefore not desired in dark fermentative reactions (Eq. 1.3).

In the biological process, hydrogen can be produced through dark fermentation from organic substances (Saraphirom and Reungsang, 2011). As an aspect of waste stabilization, hydrogen production through dark fermentation couples waste reduction or treatment with production of renewable energy (Chen et al., 2009). The other significant advantage dark fermentative hydrogen production is its use as the first-phase of two-phase anaerobic systems. When phase separation is applied, the efficiency of the second-phase (methanogenesis phase), and in turn the whole system is significantly improved providing increased energy production and also COD removal (Ganesh et al., 2014). Therefore, a dark fermentation system is not only a hydrogen-producing source but also a significant first-step of a two-phase system providing volatile fatty acids to methanogenesis step and improves both energy production and waste reduction.

With its higher synthesis rate, technical simplicity and no additional light energy requirement; dark fermentative hydrogen production is stated to be more suitable for practical application than other bio-hydrogen production methods (Levin and Chahine, 2010). In addition, biological hydrogen production by dark fermentation is of great interest because it can be operated at ambient temperature and pressure. Consequently dark fermentation is less energy intensive compared to traditional thermo and electro-chemical process (Badiei et al., 2011).

Despite its various advantages over other biological hydrogen production methods, dark fermentation has some problems to be solved before it becomes a conventional process. These problems can be listed as;

- Product Inhibition due to
    - Hydrogen Partial Pressure (Guo et al., 2011)
    - Volatile Fatty Acid concentrations (Van Ginkel and Logan, 2005)
    - pH drop (Li et al., 2007).
- Substrate Inhibition (due to high organic loads)
- Low yields and productivity

Therefore a lot of research is underway to determine the most suitable inoculum, substrate and operation type and operational parameters to achieve stable, high yield dark fermentative hydrogen production. To find solutions to the problems listed above, different approaches like modification of operational conditions (Hawkes et al., 2007), pre-treatment of the waste (Ozkan et al., 2011), the use of genetically modified organisms (O-Thong et al., 2008) or reactor configuration change are applied.

2.3. Factors Affecting Dark Fermentation

2.3.1 Substrate Type

The selection of biomass for dark fermentative hydrogen production depends on the cost, availability, carbohydrate content and biodegradability of the material (Kapdan and Kargi, 2006). Synthetic or real wastewater containing carbohydrate substances were the main substrate for the fermentative H_2 production studies. Pure carbohydrate or carbohydrate rich substrates were indicated to be more ideal for hydrogen production by a kinetic analysis of substrate affinity (Chen et al., 2006).

Table 2.1 illustrates the maximum yields achieved by studies using various carbohydrate rich substrates, mostly by mixed anaerobic cultures.

Table 2.1[a] Comparison of various substrates used for fermentative H_2 production

Inoculum	Substrate	Mode of Study	Substrate Concentration (g COD/L)		Maximum Hydrogen Yield	Reference
			Range studied	Opt[b]		
Municipal Sewage Sludge	Xylose	Continuous	10 - 100	20	2.25 mol/ mol xylose	Lin and Cheng, (2006)
Digested Sludge	Glucose	Batch	1.1 – 320.0	2.1	3.1 mol/ mol glucose	Wang and Wan, (2008)
Municipal Sewage Sludge	Sucrose	Batch	10 – 30	10	2.46 mol/ mol sucrose	Wang et al., (2006)
Mixed Cultures	Sucrose	Batch	1.5 – 44.8	7.5	38.9 mL/ g COD-L culture	Van Ginkel et al., (2001)
Anaerobic Digester Sludge	Sucrose	Continuous	10 – 60	30	1.22 mol/ mol hexose	Kim et al., (2006)
Anaerobic Sludge	Starch	Batch	9.8 – 39.0	9.8	67 mL/g Starch	Zhang et al., (2003)
Anaerobic Sludge	Starch	Batch	5 – 60	20	2.2 mol/ mol hexose	Lin et al., (2008)
Municipal Sewage Sludge	Starch	Batch	8 – 32	32	11.25 mmol/ g starch	Fang et al., (2006)
Anaerobic Digester Sludge	Rice Slurry	Batch	2.9 – 23.6	5.9	346 mL/ g carbohydrate	Fang et al., (2006)
Anaerobic Digester Sludge	Food Waste	Batch	0 – 32.3	4.6	101 mL/ g COD	Chen et al.,(2006)
Anaerobic Sludge	Food Waste	Batch	3.2 – 10.7	6.4	1.8 mol/ mol hexose	Shin et al., (2004)

Table 2.1 Continued: Comparision of various substrates used for fermentative H_2 production

Anaerobic Sludge	Non-fat dry milk	Batch	0 – 96	4	119 mL/ g COD	Chen et al., (2006)
Waste Activated Sludge	Food wastewater	Batch	10 – 160	40	47.1 mmol/ g COD	Wu and Lin, (2004)
Municipal Sewage Sludge	Rice Winery wastewater	Continuous	14 – 36	14	1.9 mol/ mol hexose	Yu et al., (2002)
Anaerobic Sludge	Palm oil mill effluent	Continuous	5-13.3	6.6	0.34 L H_2 / g COD	Badiei et al., (2011)
Anaerobic Digester Sludge	Food Waste	Continuous	30	30	0.54 mol H_2/ mol hexose	Kim et al., (2008)
Agricultural Soil	Glucose	Continuous	2.5 - 10	2.5	2.8 mol H_2/ mol hexose	Van Ginkel and Logan, (2005)

[a] Modified from the data of Wang and Wan (2009b)
[b] Opt : Optimal value

2.3.1.1. Synthetic Wastewater

A lot of research on hydrogen production by mixed anaerobic cultures has focused on simple carbohydrate substrates with additional nutrients to imitate carbohydrate rich wastewater (Li et al., 2007). It is evident in Table 2.1 that glucose and sucrose are the most common substrates used for these studies, especially in batch mode.

Sucrose supplemented with nutrients is the most common substrate in batch reactor studies and yields in the range of 1.23 to 2 mol H_2/ mol hexose have been reported (Table 2.1). Yield values as high as 3.1 mol H_2/mol glucose have been reported for batch reactor studies with glucose (Wang and Wan, 2008).

Starch is another very common substrate of the batch reactor studies. The yields reported in these studies are in the range of 11.25 mmol/g starch (Fang et al., 2006) to 2.2 mol H_2/ mol hexose (Lin et al., 2008).

Table 2.1 also illustrates the continuous reactor studies using sucrose as the substrate achieved a yield of 1.22 mol H_2/ mol hexose (Kim et al., 2006), which is compatible to the values achieved in the batch mode. However, continuous reactor studies are more concentrated on real substrates. In the continuous reactor studies, the achieved yields range between 0.5 to 1.9 mol H_2/ mol hexose depending on the substrate or the operational conditions (Table 2.1).

2.3.1.2. Real Wastewater

Real wastewaters, especially the ones with high carbohydrate content, were the most frequently used substrates in dark fermentative hydrogen production studies. Table 2.1 illustrates the batch studies using rice slurry, food waste and non-fat dry milk, as well as, continuous reactor studies that made use of rice winery wastewater, molasses, palm oil mill effluent and food waste. Other continuous mode studies utilized rice winery wastewater (Yu et al., 2002), cellulose containing wastes (Sagnak et al., 2011), starch manufacturing wastewater (Yokoi et al., 2002) or swine manure (Wu et al., 2009). Therefore, there is a great variety on the types of wastewater utilized for dark fermentative hydrogen production.

According to Table 2.1, yields are fairly similar in batch studies and values from 101 to 120 mL H_2/ g COD have been reported. Continuous reactor studies conducted with real wastewater presented a broader range on the achieved yields from 0.54 to 1.9 mol H_2/ mol hexose.

Molasses with its high carbohydrate content stands out as an excellent fuel for dark fermentative hydrogen production in continuous reactor studies. Most of the sugar content of molasses is known to be sucrose (Keskin and Hallenbeck, 2012).

2.3.2. Seed Sludge

The source of the seed sludge and the pretreatment applied to it, have been frequently studied and have significant effect on the yield and the stability of the dark fermentation operation.

2.3.2.1. Seed Sludge Type

A number of different cultures of bacteria have been used to produce hydrogen from a wide range of substrates. Using pure cultures helps to minimize hydrogen losses by excluding methanogens and certain homoacetogens (Park et al., 2005). *Clostridium* and *Enterobacter* were most widely used as pure culture inoculum for dark fermentative H_2 production (Wang and Wan, 2009b). These bacteria capable of producing hydrogen widely exist in natural environments such as soil, wastewater sludge and compost (Cheong and Hansen, 2006; Zhu and Beland, 2006; Hu and Chen, 2007; Wang and Wan, 2008a).

Pure cultures are mostly employed in batch reactors and yields achieved in those studies, with glucose or sucrose as the substrate, reached as high as 2.3 mol H_2/mol glucose in numerous occasions (Kumar and Das, 2000; Levin et al., 2006; Lo, et al., 2008). Random continuous reactor studies employed pure cultures, achieving a variety of yields from 0.3 (Taguchi et al., 1996) to 2 mol H_2/ mol glucose (Turcot et al., 2008).

Despite of the high yield obtained by pure culture, the use of pure cultures is not feasible since waste materials may have insintric bacteria and the prohibitive cost of sterilizing wastewater streams (Park et al., 2005). Also, different bacteria are needed to break down the various components of the organic matter in the waste. At present, the mixed cultures of bacteria from anaerobic sludge, municipal sewage sludge, compost and soil have been widely used as inoculum for dark fermentative H_2 production (Li et al., 2007). Using mixed cultures for dark fermentative H_2 production is much more feasible than using pure cultures,

simpler to operate and easier to control and has a broader source of feedstock (Li et al., 2007). However, with mixed cultures, hydrogen produced by the hydrogen-producing bacteria may be consumed by hydrogen-consuming bacteria (through homoacetogenic process and/or hydrogenetrophic methanogens). Within the surveyed literature, maximum hydrogen yields obtained using mixed cultures were 3.1 and 2.8 mol H_2/mol glucose for batch (Wang and Wan, 2008) and continuous reactor systems (Van Ginkel and Logan, 2005), respectively.

2.3.2.2. Pretreatment Type

There are various methods applied for the inoculum to enrich hydrogen producers in a mixed culture. These methods include acid or base treatment, heat-shock, chemical treatment and freezing/thawing (Wang and Wan, 2008a). Comparison of various pretreatment methods enriching the hydrogen producing bacteria from a mixed culture can be seen in Table 2.2. Hydrogen producer (spore former) enrichment by heat treatment is the most common technique, although there is variation in the temperature attained and the time for which it is applied (Wang and Wan, 2008a). On the other hand, heat treatment of the inoculum at industrial scale would be technically more difficult than acid or alkali treatment (Hawkes et al., 2007) and would also increase the cost of the overall process.

As seen in Table 2.2, toxic chemical addition, like 2-Bromoethanesulfonate, is another practice. But it is seldomly used due to the high cost of the chemicals and their ineffectiveness on non-H_2-producing bacteria such as lactic or propionic acid bacteria which hinder the production (Eq. 1.3) or consume H_2 (Eq. 1.4) (Jung et al., 2011).

Table 2.2[a] The comparison of sludge types and various pretreatment methods enriching hydrogen-producing bacteria from mixed culture inoculum

Inoculum	Pretreatment Methods Studied	Substrate	Reactor Type	Max H_2 Yield	Optimal Pret.[e] Method	Ref [f]
Digested Sludge	Acid, base, heat-shock, aeration and chloroform	Glucose	Batch	1.8 mole/ mole glucose	Heat-Shock	Wang and Wan (2008a)
Cattle Manure Sludge	Freezing and thawing, acid, heat-shock, Na_2-BES[b]	Glucose	Batch	1.0 mole/ mole glucose	Acid	Cheong and Hansen (2006)
Methanogenic Granules	Acid, heat-shock and chloroform	Glucose	Batch	1.2 mole/ mole glucose	Chloroform	Hu and Chen (2007)
Digested Wastewater Sludge	Heat-shock, aeration, acid, base, 2-BESA[c] and iodopropane	Sucrose	Batch	3.06 mole/ mole glucose	Base	Zhu and Beland (2006)
Anaerobic Sludge	Na_2-BES[b], acid, heat-shock and their four combinations	Dairy WW[d]	Batch	0.0317 mmol/ gCOD	Na_2-BES	Venkata Mohan (2008)

[a] Taken without modification from, Wang and Wan (2009b)
[b] Na_2-BES: sodium 2-bromoethanesulfonate
[c] 2-BESA: 2-bromoethanesulfonic acid
[d] WW: Wastewater
[e] Pret: Pretreatment
[f] Ref: Reference

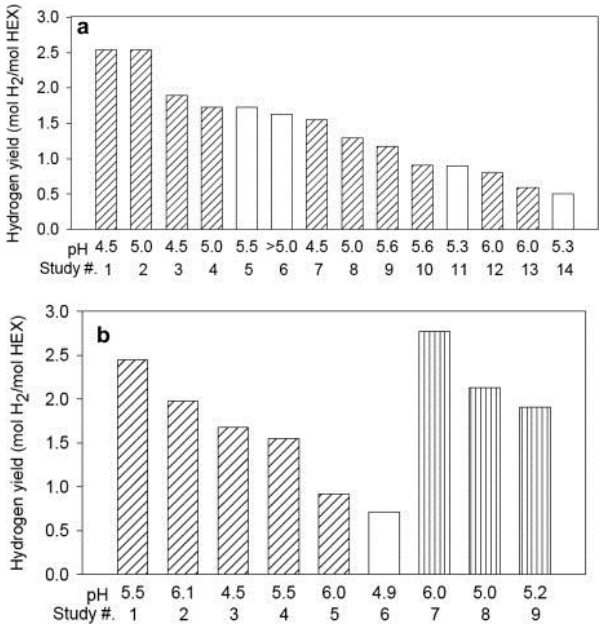
Table 2.2 shows that maximum yield achieved in dark fermentative hydrogen production may depend highly on the pretreatment method used on the seed sludge. The lowest yield achieved was 1.0 mol H_2/ mol hexose with acid treated sludge (Cheong and Hansen, 2006) and highest was 3.06 with base treated sludge (Zhu and Beland, 2006) according to Table 2.2. But optimum treatment method depends highly on the source of the seed sludge to begin with. There is also speculation that although these pretreatment methods provide initial elimination of the methanogenic bacteria that consumes produced hydrogen,

they do not provide a long-term stability and high yield in batch or continuous cultures since they cannot hinder homoacetogenic activity (Saady, 2013). This is further discussed in Section 2.5 of this thesis.

2.3.3. Reactor Configuration

Reactor configuration is important since it puts restrains on the maximum Organic Loading Rate (OLR), Hydraulic Retention Time (HRT), Solid Retention Time (SRT) and cyclic duration time.

Figure 2.1 illustrates the yields achieved in studies using different types of organic substrates, operational pHs used and their mode of operations (Batch, Anaerobic sequencing batch reactor or Continuous stirred tank reactor). Figure 2.1 suggests ASBR studies with lower pHs (4.5-5.0) tend to provide higher yield than batch reactor studies of the same pH range. On average simple substrates like glucose and sucrose provide higher yield than the original carbon sources. The highest yield presented for original substrates in Figure 2.1 is 2.5 mol H_2/ mol hexose by Fang et al., (2006) using rice slurry at a pH of 4.5 and 2.8 mol H_2/mol hexose for simple substrates in the study done by Chen, Sung and Chen (2009) with operational pH of 6.0.



Hydrogen yield reported in other studies: (a) with real waste/wastewater batch ASBR and (b) with synthetic substrates batch ASBR CSTR.

a.

Study #	Authors	Substrates	Inocula
1	Fang et al. 2006	Rice slurry	Anaerobic digested sludge
2	Noike and Mizuno 2000	Bean curd manufacturing waste	Soy bean meal
3	Yang et al 2007	Cheese powder with additives	Sewage sludge
4	Noike and Mizuno 2000	Wheat bran	Soy bean meal
5	Sreethawong et al. 2010	Cassava wastewater	Cassava treating sludge
6	Wu et al. 2009	Swine manure and glucose	Anaerobic digested sludge
7	Fang et al. 2006	Rice slurry	Anaerobic digested sludge
8	Noike and Mizuno 2000	Rice bran	Soy bean meal
9	Lay et al.1999	Mixed waste	Soy bean meal
10	Lay et al.1999	Mixed waste	Anaerobic digested sludge
11	Kim et al. 2010	food waste	Anaerobic digested sludge
12	Logan et al. 2002	Molasses	Soil
13	Logan et al. 2002	Potato	Soil
14	Arooj et al. 2008	Corn starch	Sewage sludge

b.

Study #	Authors	Substrates	Inocula
1	Van Ginkel et al. 2001	Sucrose	Compost
2	Wu et al. 2002	Sucrose	Sewage sludge
3	Khanal et al. 2004	Sucrose	Compost
4	Mu et al. 2006	Glucose	Sewage sludge
5	Logan et al. 2002	Glucose	Soil
6	Chen et al. 2009	Sucrose	Anaerobic digested sludge
7	Hafez et al. 2010	Glucose	Sewage sludge
8	Wu et al. 2010	Glucsoe	Cow dung compost
9	Hussy et al. 2005	Sucrose	Anaerobic digested sludge

Figure 2.1 Hydrogen yields reported in various studies (Won and Lau, 2011).

2.3.3.1. Batch Reactors

Batch reactor studies dominate the hydrogen production with dark fermentation field, due to its simple operation and control (Guo et al., 2010). Especially the studies that concentrate specifically on one parameter or test the feasibility of a specific substrate, batch reactors are mostly used (Guo et al., 2010). Although batch reactors are used to determine bio-hydrogen potential of many organic substrates, they are not suitable for scale-up studies and eventual industrial scale applications.

A number of batch reactor studies were presented in Table 2.1 in Section 2.3.1. These batch studies dealt with a variety of substrates from glucose to food waste, from rice slurry to palm oil mill effluent. Usually optimum pH, temperature, ion concentration or the initial COD concentrations were studied for a specific substrate or seed sludge type. Yields achieved in these batch studies represent the potential maximums of the dark fermentative hydrogen production.

Figure 2.1 illustrates a variety of batch reactor studies. Careful study of this figure indicates the domination of batch reactor studies for real or synthetic wastewater studies. Batch reactor studies have yields in the range of 1.23 to 3.1 mol H_2/ mol hexose for synthetic substrates where they have a range of 101 to 346 mL H_2/ g COD for original substrates.

2.3.3.2. Continuous Stirrer Tank Reactor (CSTR)

Continuous stirred tank reactors (CSTRs) were the most commonly used continuous reactor systems in laboratory scale dark fermentation studies until recently. In the CSTR, it is difficult to maintain high levels of hydrogen-producing biomass at a short HRT due to its intrinsic structure (Chen and Lin, 2003) that causes operational instability and limits the hydrogen production rate (Saraphirom and Reungsang, 2011). In CSTRs without recycle, since biomass has the same retention time as the HRT, washout of biomass may occur at

shorter HRTs (Zhang et al., 2006) to 2.5 days (RenNanqi et al., 2011). Table 2.1 shows that continuos mode studies are mostly applied to original substrates where yields of 0.54 to 1.9 mol H_2/mol hexose range were achieved.

2.3.3.3 Sequencing Batch Reactor (SBR)

The Sequencing Batch Reactor (SBR) operation is composed of 4 periods. First is the feeding period, where the substrate is introduced to the reaction medium. Second is the reaction period, where the medium is continuously stirred and desired reactions take place. Third period is where SBR differs from other modes of operation; it is the settling period where the medium is not stirred and is left to rest for the inoculum to settle down. Fourth period is the withdrawal of the effluent which has been cleared of the microorganisms by the settling period. A new cycle begins with the next feeding period. SBRs started to dominate the continuous dark fermentative hydrogen production studies recently. According to Arooj et al. (2008), the advantages of SBR include a greater biomass retention (hence the ability to decouple solids retention time from HRT) and a higher degree of process flexibility with respect to the changes in Organic Loading Rate (OLR). A single container for reaction and settling is used in SBRs. SBRs have relative ease of production and lower capital investment is necessary. SBRs are known as high biomass-retaining reactor operated with a settling period of biomass. SBRs have been developed to better handle high suspended solids in wastewater and are particularly useful for agricultural waste (Wu et al., 2009). Yet, it has disadvantages such as having an upper limit in OLR and lower biogas production. This lower biogas production is clearly indicated in Figure 2.1 where average maximum yield of the SBRs for synthetic substrates are 0.75 mol H_2/ mol hexose and 2.8 mol H_2/ mol hexose, respectively.

2.3.3.4. Other Reactor Types

There are dark fermentative hydrogen production studies that make use of Anaerobic Membrane Bioreactor (AnMBR) (Lee et al., 2009), Up-flow Anaerobic Sludge Blanket Reactor (UASBR) (Lee et al., 2004), Expanded Granular Sludge Bed Reactor (EGSBR) (Guo et al., 2008) and Anaerobic Fluidized Bed Reactor (Barros et al., 2010). But the number of studies utilizing these types of reactors is limited. Membrane bioreactors for producing H_2 from municipal wastewater treatment are becoming increasingly popular. In MBRs membrane modules are coupled with bioreactors in order to retain the biomass in the reactor and in turn to improve H_2 production.

2.3.4. Retention Times

2.3.4.1. Hydraulic Retention Time

Hydraulic Retention Time (HRT) is a measure of the average length of time that a soluble compound or water remains in a reactor. Therefore it has direct impact on economical operation (Jung et al., 2011). Generally, H_2-producing bacteria prefer short retention time (< 120 h) (Jung et al., 2011). HRTs between 4 to 96 h have been reported as optimal for dark fermentative H_2 production. Short HRT is also preferred for selection of microbial community, since Zhang et al. (2006) observed wash out of propionic acid bacteria, which consume H_2 during their metabolism, upon transition of the HRT from 8 to 6 h.

Arooj et al. (2008) observed that variation of HRT affected metabolic products and microbial population, which led to variation in operating parameters such as H_2 yield and H_2 composition. Shorter HRTs would change the fermentation pattern and suppress the methanogens which generally require relatively longer time to grow compared to the acidogens. Shorter HRT is also preferred by reason of lower capital cost requirement (Won and Lau, 2011). It has been also reported that reduction in hydrogen yield at long HRTs is probably due to the

reuse of H_2 by homoacetogens (Eq. 2.1) which produce acetate form dissolved CO_2 in the presence of H_2 (Wu et al., 2009).

$$2CO_2 + 4H_2 \rightarrow 3CH_3COOH + 2H_2O \quad \text{(Eq. 2.1)}$$

Table 2.3 illustrates the various HRT values studied in the literature. HRT values studied ranges from 96 h to 1 h. There was much dependency of the HRT on other operational conditions, like the operational pH or the substrate type. Despite this dependency, optimum HRTs determined in most studies falls in the range of 8 to 16 h. Decreasing the HRT further than 8 h seem to have a negative effect on the hydrogen yield (Lin and Jo, 2003; Van Ginkel and Logan, 2005). In addition, longer HRTs (> 16 h) surely have a negative effect on the hydrogen yield. Longer HRTs are also associated to promote faster hydrogen consumption (Dinamarca and Bakke, 2009). Generally, HRT of 8 to 20 h is the optimum value for H_2 yield (Chang and Lin, 2004; Cheong, Hansen and Stevens, 2007; Abreu et al., 2010) which overlaps with the doubling time of homoacetogens (1.75 - 29 h) (Zhang and Noike, 1994; Kerby and Zeikus, 1983; Zhang and Noike, 2008).

Table 2.3 Effect of HRT on hydrogen production by dark fermentation

Substrate	Rxr[a] Type	COD[b]	Opt.[c] pH	Max HY[d] (mol/mol hexose)	Studied HRT range (h)[b]	Opt.[c] HRT (h)	Ref.
Pig Manure	SBR	40→120 g glucose/day	-	1.63	24→12→8	12	Wu et al. (2009)
Sweet Sorghum Syrup	SBR	25 g/L COD	-	0.68	96→48→24→12	24	Saraphirom and Reungsang (2011)
Sucrose	SBR	11.8 g/L COD	4.5	2.16	60→30→20	30	Won and Lau (2011)
Palm Oil Mill Effluent	SBR	6.6 g/L.day	-	0.34 L H_2/ g COD	96→72→48→36	72	Badiei et al. (2011)
Starch	SBR	9 g/L total sugar	-	130 mL H_2/g sugar	60→24→6	24	Sagnak et al., (2010)
Molasses + Pig manure	SBR	10 g sugar/ L	5.5	1.57 mol H_2/mol sugar	30→24→18→12→6	16	Wu et al., (2013)
Molasses	SBR	-	5.0-6.0	-	3→6→9→18→48	< 8	Guo et al. (2010)
General Waste	CSTR	-	5.5	1.3	-	8 to 12	Jung et al. (2011)
Corn Starch	SBR	26.7→32→40→ 53.3→80→120	-	0.51	18→15→12→9→9→6→4	12	Arooj et al. (2008b)
Glucose	CSTR	10 and 2.6 g/L COD	-	2.8	10→5→2.5→1	10	Van Ginkel and Logan (2005)
Food Waste	SBR	27 g/L COD	-	1.12	24→30→36→42	30	Kim et al. (2008)
Sucrose	SBR	40→48→60→80→120 g COD/(L.day)	-	1.3	12→10→8→6→4	8	Lin and Jo (2003)

[a] Rxr: Reactor
[b] →represents different values studied
[c] Opt: Optimum
[d] Max HY: Maximum Yield achieved

Wu et al. (2009) used liquid swine manure with glucose supplement for SBR operating at 37 ^{o}C and pH 5, to investigate the effect of HRT. Decreasing the HRT from 24 to 8 h caused an increasing Hydrogen Production Rate (HPR) from 0.05 to 0.15 L/(L.h). They preferred HRT 12 h for high HPR and HY. The highest value of 1.63 mol H_2/ mol hexose was achieved for HY.

Saraphirom and Reungsang (2011) investigated HRTs of 96, 48, 24 and 12 h at 25 g/L total sugar concentration, pH of 5 and 1.45 g/L $FeSO_4$ and at 30 ^{o}C. Their results showed that hydrogen content decreased with the reduction in the HRT from 43% (at 96 h HRT) to 21% (at 12 h HRT). They concluded that 24 h HRT was the optimum condition for ASBR operation indicated by the maximum HY of 0.68 mol H_2/ mol hexose.

Won and Lau (2011) conducted a series of tests in a 6 L ASBR to investigate the effects of HRT along with pH and OLR on bio-hydrogen production at 28 ^{o}C using sucrose and a non-pretreated inoculum. Their findings indicated that the optimum pH value would vary depending on the HRT. Maximum HPR and HY of 3.04 L H_2/ (L_{rxr}.day) and 2.16 mol H_2/ mol hexose, respectively, were achieved at pH of 4.5, HRT of 30 h, and OLR of 11.0 kg/ (m^3.day).

Badiei et al. (2011) studied an ASBR using enriched mixed microflora with POME (palm oil mill effluent) at 37 ^{o}C with four different HRTs ranging from 96 to 36 h at constant cycle length of 24h and 37 ^{o}C. They also tested various OLRs to evaluate hydrogen productivity and operational stability of SBR. Their results showed that higher system efficiency was achieved at HRT of 72 h with maximum HPR of 6.7 L H_2/ (L.day) and HY of 0.34 L H_2/ g $COD_{feeding}$.

Sagnak, Kapdan and Kargi (2010) studied dark fermentation of acid hydrolyzed ground wheat starch. With a feed solution containing 9 g/L total sugar (supplemented with some nutrients), they varied the HRT between 6 and 60. As

the result they observed daily H_2 production increased with decreasing HRT. The highest daily H_2 production (305 mL/d) was obtained at an HRT of 6 h. HY (130 ml H_2 g/L total sugar) reached the highest level at an HRT of 24 h. Effluent total sugar concentration decreased, while biomass concentration and yield increased with increasing HRT from 6 to 24 h, showing more effective sugar fermentation at high HRTs.

Wu et al., (2013) co-fermented molasses with liquid swine manure in an SBR and investigated the combined effects of pH, HRT and TS concentration using central composite design in Response Surface Methodology. They investigated a pH range of 4.5 to 6.5 and HRTs of 6, 12, 18, 24, 30 h and the TS content range of 0.25 to 1.25%. They calculated the optimum operation condition to be pH of 5.32, HRT of 15.62 h and TS of 0.78%. With these optimum values they achieved a HY of 1.52 mol H_2/ mol sugar.

Aguilar et al. (2013) investigated the effect of HRT on the hydrogen production from the organic fraction of municipal solid waste (OFMSW) coming from a full-scale treatment plant. Experiments were conducted in an anaerobic CSTR operating at thermophilic dry conditions (55 ^{o}C and 20% in total solids concentration, respectively). Decreasing the HRTs, from 15 days to 1.5 days in a nine step procedure, was imposed to evaluate its influence on the hydrogen production and the specific hydrogen production. The results have shown that the highest H_2 production was 1.077 L H_2/ (L_{rxr}.day) was obtained at 1.9 day HRT with twice a day feeding.

Lin et al. (2011) operated a pilot system was under different combinations of HRT and substrate concentration to provide different sets OLR to improve bio-hydrogen production efficiency. When operating at HRT of 6 h and substrate concentration of 30 g COD/L (i.e., OLR of 120 g COD/(L/d)), the pilot system obtained the highest HPR, HY and overall hydrogen production efficiency of

1.18 mol H_2/ (L/day), 3.84 mol H_2/mol sucrose and 47.2%, respectively. They concluded this result to be similar to what they had obtained from their previous lab-scale system and to be significantly higher than that from the original pilot tests prior to process optimization.

Recently, it has been illustrated that HRT was a parameter that is more significant than the applied volumetric load to increase the H_2 production (Van Ginkel and Logan, 2005). HRTs less than 16 h are found to be much suitable for dark fermentative H_2 production (Table 2.3).

2.3.4.2. Solid Retention Time

Solid Retention Time (SRT), affects the substrate uptake efficiency, microbial population and metabolic pathway (Kim et al., 2008). SRT is important for maintaining hydrogen-producing bacteria in the process. In H_2 fermentation, it is generally assumed that high SRTs (> 5 days) cause the growth of H_2 consumers, including methanogens and competitors for substrates, such as non-H_2 producing acidogens (Hawkes et al., 2002). Therefore, an SRT in the range of 8-12 h is considered the general operational condition for continuous H_2 production from glucose or sucrose in CSTRs (Zhang et al., 2006). On the other hand, a low SRT may reduce substrate uptake efficiency, active biomass retention and, in turn, the overall process efficiency (Oh et al., 2004). Especially, in the case of complex substrates, a longer SRT may be required due to the slowly degraded organic compounds such as proteins and lipids (Shin, 2004; Vijayaraghavan et al., 2006). Yet, unlike the initial COD, pH or HRT, SRT effects on hydrogen production were not investigated in detail. Within the surveyed literature, there were only a couple of studies in the literature which deal specifically with the SRT effect on dark fermentative hydrogen production (Kim et al., 2008; Tawfik and El-Qelish, 2014). One of the studies, which explicitly focus on the effect of SRT, is by Kim et al. (2008). They operated four ASBRs with food waste (4.4 ± 0.2% VS

containing 27 g COD/L), and aimed to investigate the effects of SRT in the range of 24 -160 h and HRT in the range of 24-42 h. They concluded that achieving high SRT independent of HRT with internal sludge retention contributed to higher H_2 production than previous studies performed with CSTRs. The maximum HPR of 2.73 L H_2/(L.day) was estimated at an SRT of 126 h (5.25 d) and HRT of 30 h, while the maximum yield of 80.9 mL H_2/g VS (1.12 mol H_2/mol hexose added) occurred at an SRT of 126 h and an HRT of 33 h (Kim et al., 2008).

The study by Tawfik and El-Qelish (2014) is the second study that recently investigated the SRT effect. They studied the SRT effect along with the effects of dilution ratio on the continuous H_2 production from co-digestion of OFMSW and kitchen wastewater in a mesophilic anaerobic baffled reactor. They found that HY increased from 83±37 to 95±24 ml H_2/ (g $COD_{removed}$.day), when SRT increased from 3.6 to 4.0 d. Further increase in HY of 148±42 ml H_2 / (g $COD_{removed}$.day) occurred at an SRT of 5.6 days. In short, they concluded that HY was highly dependent on the operational SRT in a dark fermentative reactor system.

Badiei et al. (2011) calculated SRTs for their study. Their calculated SRTs were 19 days at HRT 96 h, 11 days at HRT 72 h and 5.5 days at HRT 48 h. In their study, hydrogen production did not show consistent relationship with SRT and HRT. The longer HRT yielded lower solid removal and showed the longest SRT. They expected long HRTs to yield maximum hydrogen production for this system, however it did not happen and they attributed this to prevalence of H_2-consuming or non-hydrogen-producing bacteria. They stated that, operating conditions at shorter HRT of 72 h with SRT of 11 days supported the retention of active hydrogen-producing bacteria and restored the stability in the system.

Dinamarca and Bakke (2009) stated that significant homoacetogenic H_2 consumption increased with the biomass density and the sludge age in the

bioreactor. Homoacetogenesis (Eq. 2.1, Section 2.3.4.1) is reported not to occur in fresh pretreated inocula but said to increase successively after long term cultivation of repeatedly pretreated inocula (Luo et al., 2011). Therefore, SRT is closely related to the degree of homoacetogenic activity in bio-hydrogen production.

2.3.4.3. Cyclic Duration Time (for SBR type reactors)

Within the surveyed literature, there is only one study specifically focusing on the effect of cyclic duration in the literature. Chen et al., (2009) investigated the effects of pH (4.9, 5.5, 6.1, and 6.7) and cyclic duration (4, 6, 8 h) on hydrogen production in an SBR. They found the operational conditions of 16 h HRT, 25 g COD/L and 4 h cyclic duration resulted in the maximum HY of 2.53 mol H_2/mol $sucrose_{consumed}$ at pH 4.9. Cyclic duration had significant effect on the maximum HY independent of the HRT. Their results showed HY, hydrogen conversion efficieny (mol H_2/ mol $sucrose_{fed}$) and hydrogen content of the produced gas (%) all decreased with the increasing cyclic duration time from 4 to 8 h.

2.3.4.4. Settling Time (for SBR type reactors)

Like cycle duration time, studies conducted to investigate the specific effect of settling time is also limited. It seems to be a significant operational condition that varies greatly among the different studies; from 20 minutes (Saraphirom and Reungsang, 2011) to 85 (Lin and Jo, 2003) and to 100 minutes (Kim and Shin, 2008). Within the surveyed literature, there are only two studies dealing with settling time effect on sludge characteristics and overall efficiency of the dark fermentative hydrogen production (Arooj et al., 2007; Lin and Jo, 2003)

Arooj et al. (2007) investigated the sludge characteristics of SBR to improve the efficiency of the system using starch. They observed the effect of stratification in the settling phase on H_2-producing SBR, which results in settleable and non-settleable sludge in batch experiment. They concluded that specific H_2 activity

of decanting non-settleable sludge was higher than that of settleable sludge, which may be the reason of low yield in H_2 producing SBR. Additionally, the effect of settling time on settleable sludge was also analyzed using another set of batch experiment (Arooj et al., 2007). They obtained the sludge for the batch experiments from an SBR working at 18 h HRT, pH 5.3 and 35 ^oC, with a yield of 0.18 mol H_2/ mol $hexose_{added}$. They performed the batch experiment using sludge from this SBR after 20, 45, and 180 minutes in the settling phase. They concluded that H_2 yield decreased with the increase in settling time. The maximum H_2 yield at settling period of 20 minutes was 0.27 mol H_2/ mol $glucose_{added}$. These results showed that the settleable sludge with long settling phase was ineffective in H_2 production.

Lin and Jo (2003) studied hydrogen production from sucrose in an ASBR. They found hydrogen production was dependent on HRT and reaction period/settling period (R/S) ratio. They studied 5 HRTs (12, 10, 8, 6, 4 h) and 3 R/S ratios (1.7, 3.2 and 5.6). A short HRT, even up to 4 h, gave good H_2 productivity and high HPR values. For each HRT, R/S ratio also increased the hydrogen productivity and HPR. At HRT of 8 h, R/S ratio of 5.6 and an OLR of 0.23 mol-sucrose/ (L.day), they obtained the maximum yield of this study as 2.6 mol H_2/ mol sucrose.

2.3.5. Organic Loading Rate and Initial COD

Organic Loading Rate (OLR) is the amount of substrate applied per volume of reactor per time. Therefore, it is closely related to the maximum hydrogen production potential of the system. There are a number of studies that investigated the sole or the coupled (with pH etc.) effect of OLR. OLR, as well as HRT, SRT or seed sludge source, has significant effect on microbial population diversity (Mariakakis et al., 2011); in turn on the yield and productivity of the dark fermentative hydrogen systems.

The initial COD values studied in batch reactors conducted with anaerobic mixed cultures and carbohydrate based substrates ranged from 0.3 to 103 g/L (Hafez et al., 2010; Wang and Wan, 2009b). The optimum initial COD values, leading to highest hydrogen production, were reported to be between 1.1 and 10 g/L (Guo et al., 2010). At very high substrate concentration process would suffer from product (butyrate) and substrate inhibition (Wu et al., 2010). So, a moderate initial COD value is required to prevent inhibition of the dark fermentative system.

Mariakakis et al. (2011) studied the independent effect of HRT and OLR on bio-hydrogen production with a CSTR of 30 L working volume. The reactor was operated at 37 ^{o}C and sucrose as the substrate at OLRs of 10, 20, 30 g sucrose / (L.day) and HRTs of 0.5 to 5.5 days. They achieved a maximum yield of 1.72 mol H_2/mol hexose for HRT of to 1.6 day and OLR of 20 g sucrose/ (L.day), while no hydrogen gas production was observed for OLRs lower than 10 g sucrose/ (L.day) which they regarded as a threshold value.

Van Ginkel and Logan (2005) tested HRT and glucose loading rate to understand the effect of organic loading on H_2 production in chemostat reactors. Changing the glucose loading rate over a range of 0.5–18.9 g/h, they varied the glucose concentration in the feed from 2.5 to 10 g/L COD under the conditions where the HRT varied from 1 to 10 h (30 ^{o}C, pH 5.5). They found that decreasing the glucose loading rate over their studied range (0.5 to 18.9 g/h) increased the HY from 1.7 to 2.8 mol H_2/ mol glucose. According to the results, they suggested HYs to be optimized for more dilute feeds and lower initial substrate concentrations (2.5 to 10 g/L) than have typically been used in bio-hydrogen reactor studies (Table 2.3). In the range that they studied glucose concentration (2.5–10 g COD/L) had a greater effect on H_2 yields than the HRT (1–10 h).

Zahedi et al. (2013) studied OFMSW under thermophilic acidogenic conditions. with nine different OLRs from 9 to 220 g TVS/(L.day) and HRTs from 10 d to 0.25 d. Increasing the OLR resulted in an increase in H_2 production, except at the maximum OLR tested (220 g TVS/ (L.day). The maximum hydrogen content was 57% (v/v) at an OLR of 110 g TVS/ (L.day) (HRT of 0.5 day). HPR was in the range of 0.1–5.7 L H_2/ (L.day). Their results clearly showed that the increase in OLR was directly correlated with the increase in HPR and increase in microbial activity.

2.3.6. pH

Among all the operational parameters, it has been widely accepted that pH has the most significant effect on dark fermentative hydrogen production, since it directly effects the hydrogenase activity, metabolic pathway and dominant species (Lay, 2000; Fang et al., 2002). pH values lower than 7 are generally accepted as appropriate to prevent methanogenic activity (Hawkes et al., 2007). pH values from 4.5 to 6.0 are usually studied with the optimum value found to be around 5.5 (Won and Lau, 2011). HY increases generally with the increasing pH, within the range of pH 4.5 to 7 in batch reactors (Won and Lau, 2011).

Figure 2.1 in Section 2.3.3 illustrates the pH, reactor configuration and HY relation with various examples. It indicates for real and synthetic wastewaters that the generally studied pH range is between 4.5 and 6.0. SBR studies that used real wastewater generally employ pHs from 5 to 5.5 while continuous reactor studies using synthetic wastewaters were less specified. For continuous reactor studies, optimum pH is strongly dependent on the operating HRT or vice versa. For example, Won and Lau (2011) conducted three sets CSTR experiments with 2.5, 1.25 and 0.83 days of HRTs, with temperature controlled at 28-30 ^{o}C. They found the optimum pH value to vary depending on the HRT. Maximum HPR and HY of 3.04 L H_2/ (L_{rxr}.day) and 2.16 mol H_2/ mol hexose, respectively, were

achieved at pH of 4.5, HRT of 30 h and OLR of 11 kg/ (m^3.day). But they also achieved slightly lower yields with other HRT-pH combinations.

Chen et al. (2009) found that pH 4.9 was more favorable for fermentative hydrogen production in terms of hydrogen yield, hydrogen conversion efficiency and hydrogenic activity. However, less Mixed Liquor Volatile Suspended Solids (MLVSS) concentration existed at pH 4.9 which suggested that low pH could inhibit the growth of mixed microbial cultures.

Low pH is also related to solventogenesis activity and homoacetogenic activity initiation. The relative amounts of the main VFAs (which are the co-products of dark fermentative hydrogen production mechanism) depend strongly on pH (Saady, 2013). pH 4.0 to 5.0 favors propionate production (H_2 sink reaction), while pH 6.0-7.0 promotes acetate and butyrate formation with a transition zone between pH 5.0 to 6.0 (Antonopoulou et al., 2012; Zoetemeyer, Van den Heuvel and Cohen, 1982). *Clostridium* produce acid at medium pH and switch to solvent production at low pH (< 5) (Wiegel et al., 2006). It has been reported that the accumulation of VFAs, and pH values lower than 5.8 shifted the metabolism towards H_2-consuming pathways (Eq. 1.3, Section 2.3.3; Eq. 2.1, Section 2.3.4.1) (Vijayaraghavan et al., 2006) and at low pH homoacetogenic bacteria out-competed hydrogenotropic methanogens for H_2 (Chen et al., 2003). In conclusion, pHs lower than 4.5 and pHs higher than 6.5 hinder hydrogen production in dark fermentative systems (Jung et al., 2011; Won and Lau, 2011; Saady, 2013).

2.3.7. Temperature

Temperature affects the activity of the microorganisms and the conversion rate of fermentation products, and is closely related to economic benefit (Jung et al., 2011). It is stated that sensitivity of mixed anaerobic bacteria to temperature was

significantly high and the optimal temperature for their activity was found to be around 35 ^{o}C (Zhang and Shen, 2006).

It has been demonstrated that in an appropriate range (25 to 70 ^{o}C), increasing the temperature could increase the H_2 production ability of hydrogen producing bacteria. But temperature at much higher levels could decrease it with increasing levels (Wang and Wan, 2008c). Furthermore, highly thermophilic conditions (>70 ^{o}C) have been reported to have an inhibitory effect on methanogenesis (Ueno et al., 2007).

Some of the studies investigating the effect of temperature are listed in Table 2.4. The temperature range studied for most studies falls into mesophilic range (25 to 40 ^{o}C) which is regarded as economically feasible. But the optimal temperatures found in most of these studies usually exceed this range and fall between 40 to 60 ^{o}C.

High temperature (>40 ^{o}C) can promote hydrolysis and simplify microbial diversity in a manner favorable to H_2 production, but it can also bring about monotonous microbial diversity, resulting in incomplete substrate degradation, especially in the treatment of actual waste (Jung et al., 2011). Also, high temperature causes an economic burden, hindering the sustainability of the dark fermentation systems.

Table 2.4[a] Effect of temperature on fermentative hydrogen production

Inoculum	Substrate	Reactor Type	Temperature (°C)		Maximum H_2 Yield	References
			Range studied	Opt[b]		
Anaerobic Sludge	Glucose	Batch	25 – 55	40	275.1 mL/g glucose	Wang and Wan (2008b)
Anaerobic Sludge	Sucrose	Batch	25 - 45	35.5	252 mL /g sucrose	Mu et al., (2006)
Anaerobic Digester Sludge	Rice slurry	Batch	37 - 55	37	346 mL /g carbohydrate	Fang et al., (2006)
Municipal Sewage Sludge	Sucrose	Continuous	30 - 45	40	3.88 mol/mol sucrose	Lee et.al, (2006)
Municipal Sewage Sludge	Starch	Batch	37 - 55	55	1.44 mmol/mol xylose	Lee et al., (2008)
Municipal Sewage Sludge	Xylose	Continuous	30 - 55	50	1.4 mol/mol xylose	Lin et al., (2008)
Cow Waste Slurry	Cow waste slurry	Batch	37 – 85	60	392 mL/L slurry	Yokoyama et al., (2007)
Anaerobic Digester Sludge	Organic waste	Continuous	37 - 55	55	360 mL/g VS	Valdezvazquez et al., (2005)

[a] Modified from the data of Wang and Wan (2009b)
[b] Opt: Optimal Temperature

Higher temperatures are reported to be effective on prevention of homoacetogenic activity on some occasions (Van Niel et al., 2002; Abreu et al., 2007; Zeidan and Van Niel, 2010). Although temperature higher than 70 °C improved HYs, heating the reactor to maintain thermophilic conditions is an energy intensive process which may hinder sustainable H_2 applications (Saady, 2013).

2.3.8 Hydrogen Partial Pressure in the Headspace

Many studies have reported that partial pressure of hydrogen (pH_2) is a restrictive factor in the fermentation of organic waste (Guo et al., 2010). High pH_2 decreases the activity of hydrogenase and making the H_2 production reaction thermodynamically unfavorable (Bahl and Dürre, 2001). At high pH_2, conversion of present CO_2 to acetate, homoacetogenesis, is thermodynamically favored, reducing the performance of the bioreactor. By the increase in the hydrogen concentration in the medium due to microbial metabolism, not only bio-hydrogen production may be affected but also a shift of metabolic pathways towards solventogenesis has been observed, which results in an accumulation of alcohols, leading to further decrease in reactor performance.

Therefore, reducing the partial pressure of hydrogen in reactor vessels can increase hydrogen production (Park et al., 2005). Continuous versus intermittent release of gas pressure in batch tests increased the hydrogen production by 43% (Logan et al., 2002). It was assumed in these tests that this increase in hydrogen production was related to the reduction in the hydrogen partial pressure. However, hydrogen can also be consumed via acetogenesis and it has been proven that changes in CO_2 concentration can also be a factor (Park et al., 2005). The work showed overall hydrogen production increase by 43% with chemical scavenging of the CO_2 (Park et al., 2005).

The widely used methods for decreasing the medium and headspace pH_2 and pCO_2 are agitation (Chou et al., 2008), continuous or intermittent sparging (with argon or nitrogen) (Kim et al., 2006) and CO_2 sequestration, and use of H_2 permeable membrane (Liang, Cheng and Wu, 2002). These techniques in some cases increased the HYs in two to four folds (Chou et al., 2008).

Previous observations have led to the proposition that N_2 sparging in a continuous-flow system might decrease the dissolved concentrations of H_2 and

CO_2 sufficiently to decrease the substrate available to acetogens (Hussy et al., 2003; Park et al., 2005; Kim et al., 2006; Kraemer and Bagley, 2006). Kraemer and Bagley (2008) showed that, N_2 sparging of the SBR at 31 mL / (L_{liqued}.min) increased the H_2 yield from 1.31 to 1.87 mol H_2/ mol glucose but did not change the in-situ rate of H_2 consumption in the SBR operated at 25 ^oC and 10 h HRT at pH of 5.5 with non-heat treated methanogenic sludge. Sparging the reactor with N_2 at a rate around 15 times the hydrogen production rate, increased HY from 0.85 to 1.43 mol H_2/ mol glucose (35 ◦C, pH 6, 8.5 h HRT) (Mizuno et al., 2000). A research using biogas, from an anaerobic digester, stripped CO_2 and H_2S and sparging the first-stage hydrogen producing reactor with the resulting methane-rich gas showed 88% increase in HPR (Liu et al., 2006).

Saady (2013) revealed that eventhough a number of researches managed to lower the pH_2 and observed an increase in the HYs, homoacetogenesis is still a challenge, even at lowered H_2 partial pressure. Saady (2013) suggests that CO_2 scavenging would be the simplest method to hinder homoacetogenesis but goes on to state scavenging CO_2 completely from the headspace during high-rate H_2 production is not possible and using a chemical scavenger in a continuous system will be hard. Effects of H_2 and CO_2 partial pressure during dark fermentative hydrogen production and their removal methods need more investigation (Saady, 2013).

2.3.9 Foam Production / Prevention

Several studies mentioned excessive foam production and the related operational problems during dark fermentative hydrogen production (Aceves-Lara et.al, 2010; Kraemer and Bagley, 2006; Lee et al., 2010) This foaming is suspected to be due to excessive alkalinity added for pH regulation for synthetic wastewaters and due to the nature of the real wastewater. Foaming may cause mechanical problems like clogging of tubes and malfunction of inlets and outlets, as well as chemical problems. Since foaming prevents efficient stirring of the reactor

contents, this might increase H_2 and CO_2 partial pressure in the solution leading to inhibition of the H_2-producing reactions. A variety of antifoaming agents have been used in different studies. Kraemer and Bagley (2008) used mineral oil as anti-foaming. Silicone antifoam emulsion (J. T. Baker, Phillipsburg, NJ, USA) (Cui, Blackburn and Liang, 2012), 2.5% solution of antifoam silicone 426 R (Prolabo) (Infantes et al., 2011) and a an AntifoamA (emulsion, Sigma) (Lee, Li and Noike, 2010) were used in other studies.

2.4 Dark Fermentation Applications

2.4.1. Laboratory Scales

Dark fermentative bio-hydrogen production studies at laboratory scales (lab scale) dates back only 15-20 years. Most of the lab scale studies were in the batch mode for the first 10 years. Now, different types of continuous reactors are being investigated in laboratory scale.

2.4.2. Pilot-Scale and Full-Scale Applications

There are no full scale dark fermentative hydrogen production plants. The pilot scale applications are also limited dating back barely a decade.

One of the leading pilot-scale studies was of Kim et al. (2010), who operated an SBR of 0.15 m^3, (liquid depth 770 mm and diameter 500 mm) to treat food waste. 0.05 m^3 of feed was pumped in every 12 h, corresponding to 36 h of HRT. They heat treated the seed sludge and operated the reactor with 30 g COD/L. During the steady-state operation the highest yield achieved was 0.54 mol H_2/mol $hexose_{added}$ with H_2 content between 43 – 46 %.

In an earlier semi-plot study, Chou et al., (2008) studied stirring speed and pH on hydrogen production in an sequencing batch reactor. Multiple analyses indicated that stirring mainly affected the HPR. Optimum operational conditions

were pH of 6.0 and stirring speed of 120 rpm and production rate and yield were found to be 161 mL H_2/ (g TVS.d) and 13 mL H_2/ g TVS, respectively.

In a recent study, the production of hydrogen from OFMSW was studied on a semi-pilot scale (10 L) and the potential of generating electricity using the process effluents was further assessed using a two-chambered Microbial Fuel Cell (Sekoai and Gueguim Kana, 2014). In this study a maximum hydrogen fraction of 46.7% and hydrogen yield of 246.93 mL H_2 g/ L TVS was obtained at optimum operational set-points of 7.9, 30.29 ^oC and 60 h for pH, temperature and HRT respectively.

A complete high rate hydrogen production plant in pilot-scale is described in the study of Lin et al., (2011). Described pilot-plant system was composed of two feedstock storage tanks (0.75 m^3 each), a nutrient storage tank (0.75 m^3), a mixing tank (0.6 m^3), an agitated granular sludge bed fermenter (working volume 0.4 m^3), a gas-liquid-solid (GLS) separator (0.4 m^3) and a control panel. This pilot-scale fermenter was operated for 67 days at 35 ^oC, an OLR of 40-240 kg COD/ (m^3.d), and the influent sucrose concentration of 20 and 40 kg COD/ m^3. One of their findings was that both biogas and HPRs increased with increasing OLR. The biogas content of the produced gas consisted mainly of H_2 and CO_2 with a H_2 content range of 23.2 - 37.8%. At an OLR of 240 kg COD/ (m^3.d), the hydrogen content in biogas reached its maximum value of 37% with a HPR of 15.59 m^3/ (m^3.d) and a HY of 1.04 mol H_2/mol sucrose. They also found at an optimal pH of 5.5, the bacterial community became simple, while the efficient hydrogen producer *Clostridium pasteurianum* was dominant. They continued to describe their work on another paper, where they worked with different agitation rates, HRTs and substrate concentrations to increase the yields they achieved in early stages of the study. With 25-30 rpm agitation rate and a OLR of 60 g COD/ (L.day) (from combination of 8 h HRT and 20 g COD/L substrate concentration), the HPR of the pilot system reached 0.55 mol/ (L.day)

which is 3.1 fold of that obtained from using a lower agitate rate (10-15 rpm). They also achieved higher HY, with operation at HRT of 6 h and substrate concentration of 30 g COD/L, the pilot system obtained the highest HPR, HY of 1.18 mol/ (L.day), 3.84 mol H_2/ mol sucrose, respectively.

Krupp and Widmann (2009) operated four 30 L CSTRs inoculated with heat treated anaerobic sludge. They studied OLRs in the range 2 -14 kg VS/ (m^3.d) and the corresponding HRTs with pH stabilized at 4 to 5.5. They found the optimum HRT to be 15 h and achieved a HY of 2.93 mol H_2/ mol glucose at 10 kg VS/ (m^3.d). They concluded short retention times and high loads could optimize the biohydogen production process.

There are a limited number of pilot-scale studies on dark fermentative hydrogen production. Operational paramters studied in these pilot-scale applications differ on optimal values hence further research is needed before any full scale application can be attempted.

2.5 Homoacetogenesis

Homoacetogenesis is the autotrophic growth acetogenic microorganisms on H_2 and CO_2 which shift their metabolism, under stress conditions or depletion of substrate (Saady, 2013). Homoacetogenesis occurs through two reactions; either through production of acetate from dissolved CO_2 and gaseous H_2 (Eq. 2.1) or via direct depletion of substrate (Eq. 2.2) (Chen et al., 2009).

$$4H_2 + 2CO_2 \rightarrow CH_3COOH + 2H_2O \qquad \text{(Eq. 2.1, Section 2.3.4.1)}$$

$$C_6H_{12}O_6 \rightarrow 3CH_3COOH \qquad \text{(Eq. 2.2)}$$

Homoacetogenesis is a major concern in dark fermentative bio-hydrogen production (Kraemer and Bagley, 2006; Siriwongrungson et al., 2007; Saady, 2013). One of the biggest problems with homoacetogens is that, they grow on H_2/CO_2 faster than acetogens on organic substrate. Also, they do not depend on

the activity of methanogens because they are not inhibited by high H_2 partial pressure (pH_2) (Saady, 2013). It is known that at high pH_2 (>500 Pa), homoacetogenesis is favored (Demirel and Scherer, 2008).

Methanogenesis is generally regarded as the most prevalent route of H_2 consumption in anaerobic systems. Methanogenic hydrogen depletion reaction is given as Equation 2.3.

$$4H_2 + 2CO_2 \rightarrow CH_4 + 2H_2O \quad \text{(Eq. 2.3)}$$

Methanogens can be killed by heat treatment of the inoculum, but this does not necesserily ensure maximizing the H_2 yield, since it does not prevent homoacetogenesis (Kraemer and Bagley, 2008). As a matter of fact; homoacetogenesis was observed in batch systems for both heat treated and non-heat treated inoculum (Oh et al., 2003). Homoacetogens have a rapid growth rate (doubling time 1.75 - 29 h under favorable conditions). Also, since there is no competition between homoacetogens and methanogens in H_2-producing dark fermentation systems, at high hydrogen partial pressures, homoacetogens dominate in late stages of batch systems (Zhang and Noike, 1994; Oh et al., 2003) and at continuous systems with long HRT (Saady, 2013).

Although homoacetogens growing on H_2 have the same biomass yield as that of acidogens and acetogens (0.07 kg COD/ kg COD) their decay rate (0.015 day^{-1}) is slower than that of acidogens and acetogens (0.02 day^{-1}) (Ni et al., 2011). Thus, they might have an advantage over acidogens and acetogens in systems with long SRTs (Saady, 2013).

The role and activity of the various microorganisms responsible for H_2 consumption during dark fermentative H_2 production are still not well defined or controlled. Until now there is no adequate means to eliminate H_2 consumption (Saady, 2013). Homoacetogenesis during H_2 dark fermentation does not depend on the source of cultures, pre-treatment, inhibition, substrate, or operation

conditions (Van Ginkel et al., 2001; Iyer et al., 2004; Siriwongrungson et al., 2007; Luo et al., 2010). The efficiency of any pre-treatment to eliminate H_2 consuming microorganisms in mixed cultures is questionable as the culture mature during long-term operation (Saady, 2013)

Park et al. (2005) observed that acetogenesis and concurrent H_2 removal in a heat-treated batch culture was prevented when the headspace CO_2 was removed using KOH trap. But controlling CO_2 concentration during dark fermentation needs further investigation as a potential strategy towards controlling homoacetogenesis. Incorporating radioactive labeling technique in H_2 fermentation research could provide information on simultaneous production and consumption of H_2 by homoacetogens.

2.6 Experimental Design Approaches

Experimental design methods used to investigate the effects of various factors on fermentative hydrogen production processes, includes one-factor-at-a-time design, full factorial design, Taguchi design, Plackett–Burman design, central composite design and Box–Behnken design (Wang and Wan, 2009a).

Classical experimental designs that investigate various variables on a particular objective usually depend on one-factor-at-a time way of approaching the issue. But this experimental design approach misses the interaction of various variables that might change the magnitude of the objective (Wang and Wan, 2009a). This experimental design is also time consuming since it requires a number of experiments to cover different levels of a variable (Xing et al., 2011). Therefore using a statistical design approach like Response Surface Methodology (RSM), reduces the number of experiments, includes the interactions of the variables in the resulting objective and provides a statistically sound set of results (Liu et al., 2011). These setbacks present certain needs for the experimental design process. These are;

- Design of Experiments for statistically explanatory data acquisition
- Statistically explain not only the effects of the variables but also the effects of their interactions

RSM is used mostly in situations where several input variables potentially influence some performance measure or quality characteristic of the product or process which is called the response. This response is defined by a function which is a first order or second order polynomial and this equation is called a Response Surface Model. This Response Surface Model makes use of a design method whether it is full factorial design, Taguchi design, Plackett–Burman design, central composite design or Box–Behnken design.

The experiments done in batch mode of this thesis study involved more than one variable and their potential interactions; thus, had been designed with RSM and the results had been evaluated with the tools that RSM presented.

CHAPTER 3

MATERIALS AND METHODS

In this chapter, the seed sludge, details on the substrates, the experimental batch and SBR set designs and details, reactor operations and analytical methods used in each set are described.

3.1. Seed Sludge

The sludge used in all experimental sets was anaerobic digester sludge. It was obtained from the return line of the anaerobic digester of the Greater Municipality of Ankara Domestic Wastewater Treatment Plant. Total Suspended Solids (TSS) and VSS content of the seed sludge used for each stage of the study are given in Table 3.1

Table 3.1 TSS and VSS content of seed sludge used in each batch reactor set and SBR study

	Sludge Properties	
Study	**TSS (mg/L)**	**VSS (mg/L)**
Batch Reactor Set-A	22383±1302	7467±505
Batch Reactor Set-B	42888±702	20288±218
SBR Study-1	43856±1649	21108±520
SBR Study-2	41175±1424	20642±624
SBR Study-3	37675±8622	16542±197
SBR Study-4	41056±2670	20467±1217
SBR Study-5	35022±7489	13511±7185

It is common practice to apply pre-treatment to anaerobic sludge to eliminate methanogens and select acidogenic hydrogen producers (Guo et al., 2010). Heat

treatment has been mentioned to be successful in inactivation of hydrogen consumers and supporting *Clostridium* like hydrogen producers (Kim et al., 2006). All the seed sludge used in this study, whether used in batch reactors or SBRs, was heat-treated at 105^{o}C for an hour (Ozkan et al., 2010).

3.2. Wastewater Composition

Synthetic wastewater was used in all experimental sets except batch reactor Set-B. Synthetic wastewater was composed of a basal medium (BM), involving all the necessary micro- and macro- nutrients for an optimal anaerobic microbial growth, and a carbon source. The composition of the BM is a synthesis of two basal medium recipes (Fang and Liu, 2002; Ozkan et al., 2010). The BM composition is in given Table 3.2. The carbon source, its concentration and alkalinity constituents are given in Table 3.3.

Molasses, a sugar industry by-product, is a renewable source which can be converted to hydrogen by dark fermentation due to its high organic content (>80% sucrose) (Wu et al., 2013). Molasses was obtained from Ankara Etimesgut Sugar Factory and stored in -20^{o}C prior to use. The properties of the molasses can be seen in Table 3.4. In Set-B, molasses was used as the substrate. BM was not added in reactors of Set-B.

Table 3.2 BM compositions used in each experimental set (except Batch Reactor Set-B)

Chemical	Concentrations obtained in the reactor (mg/L)
$MgSO_4.7H_2O$	400
$FeCl_2.4H_2O$	40
KH_2PO_4 and K_2HPO_4 (each)	400
Cysteine	10
NH_4Cl	400

Table 3.3 Modified synthetic wastewater ingredients used in each experimental set

STUDY	COD source	$COD_{influent}$ (mg/L) or OLR (gCOD/L.day)	KH_2PO_4 and K_2HPO_4 each (mg/L)
Batch Reactor Set-A	Sucrose	10/30/50	400
Batch Reactor Set-B	Molasses	10/30/50	400
SBR Study-1	Sucrose	7.5[a]	400
SBR Study-2	Sucrose	7.5[a]	400→2000
SBR Study-3	Sucrose	7.5[a]	2000
SBR Study-4	Sucrose	22.4[a]	2000
SBR Study-5	Sucrose	22.4[a]	2000

Table 3.4 Properties of the molasses used for Set-B

Characterization of Molasses				VFA Composition of Molasses	
Parameter (g/L)[a]	**Values**	**Parameter (mg/L)**	**Values**	**VFA Type**	**Concentration (mM)**
TSS	140 ± 25	**Cu**	0.01	**Lactic**	567±101
VSS	121 ± 24	**Al**	0.00	**Formic**	-[b]
COD	1331 ± 130	**Mo**	0.00	**Acetic**	294±15
sCOD[a]	1153 ± 23	**W**	0.00	**Propionic**	387±17
TKN	16 ± 0	**Se**	0.00	**Iso-Butyric**	25±2
TAN	0.7 ± 0.04	**K**	7300	**Butyric**	28±3
Alkalinity	27 ± 2	**Ni**	0.11	**Iso-Valeric**	-[b]
Sucrose	760.38 ± 1.06	**Fe**	6.88	**Total VFA[c]**	1313 ± 367
Fructose	6.623 ± 0.61	**Co**	0.00		
Glucose	-[b]	**Zn**	0.00		

[a] sCOD: Soluble COD; TAN: (NH4+-N + NH3-N)
[b] Not detected
[c] tVFA, in terms of Acetic Acid (HAc)

3.3. Analytical Methods

3.3.1. Analysis performed to monitor reactor performance

In order to investigate the performance of the reactors operated in this thesis study pH, total gas production, headspace gas composition, effluent VSS concentrations, reactor content VSS concentrations (VSS_{rxr}), effluent VFA, total chemical oxygen demand (tCOD) and soluble chemical oxygen demand (sCOD) analyses were performed. Batch reactor contents were analyzed for tCOD, sCOD, VFA, VSS_{rxr} and pH at the beginning and at the end of their run. SBRs were analyzed for gas composition, VSS, VSS_{rxr} and VFA daily, while they were analyzed for sCOD, tCOD every other day. Alkalinity and alcohol analyses were done whenever presumed necessary.

pH: Continuous pH measurement and regulation was performed with pH-Stat (Eutech Instruments, USA) and pH probe (Eutech Instruments, USA). During the SBR operation pH was continuously and automatically regulated with this pH-stat and via using 2 M HCl and 5 M NaOH solutions.

Total Suspended Solids (TSS) and Volatile Suspended Solids (VSS): TSS and VSS were measured by following Standard Methods (2540 A, B, C, D) (APHA, AWWA and WEF 2005).

*Total Chemical Oxygen Demand (tCOD): t*COD of the unfiltered samples were measured according to an EPA-approved reactor digestion method (for tCOD range of 0-1500 mg/L) (Hach Water Analysis Handbook, 2012). For tCOD measurements, Aqualytic AL 38 heater and PC Multidirect Spectrophotometer (Program 130-131) were used.

Soluble Chemical Oxygen Demand (sCOD): sCOD of the filtered samples were measured according to an EPA-approved reactor digestion method (for sCOD range of 0-1500 mg/L) (Hach Water Analysis Handbook, 2012). Glass fiber

filters were used to filter the samples. For sCOD measurements, Aqualytic AL 38 heater and PC Multidirect Spectrophotometer (Program 130-131) were used.

Volume of the Gas Produced: Daily gas production of the reactors was measured with a water displacement device and recorded in liter. (Ergüder, Güven and Demirer, 2000).

Headspace Gas Composition: The headspace gas composition of the reactors were measured with a gas chromatograph (GC) (Thermo Scientific Co., USA) employing a Thermal Conductivity Detector (TCD). Helium was the carrier gas. The software used for commanding the GC was named Chromquest, and the injector, detector and oven temperatures adjusted were 50^{o}C, 80^{o}C and 35^{o}C, respectively. Calibration of the GC was done whenever there was a change in the operating parts, e.g. a new gas tube, new septums, new ferrules or after a power failure. Calibration curves used for calculation of gas concentrations of the samples are given in APPENDIX A.

Volatile Fatty Acids (VFA): Volatile Fatty Acid (VFA) types and concentrations were measured by an HPLC (SHIMADZU 20A, Japan) using a refractive index detector with a sample volume of 10 μL. VFAs measured were lactic, formic, acetic, propionic, iso-butyric, butyric and iso-valeric acids. HPLC protocol is as follows; oven temperature is 66^{o}C; mobile phase is HPLC grade 0.085 M sulfuric acid solution with the flow rate of 0.4 mL/minute. VFA concentrations were calculated according to the calibration curves (APPENDIX B) prepared by the standard VFA solution (SupelCo VFA Standard Mix).

Total Volatile Fatty Acids (tVFA)(in terms of HAc): To calculate the tVFA concentration, every acid composition is divided by its molecular weight and multiplied by the molecular weight of acetic acid. Therefore the addition of all these values gives the tVFA concentration in terms of acetic acid (HAc) (Yılmaz and Demirer, 2008)

Hydrogen Yield: To calculate the hydrogen yield (HY) as mol H_2/mol hexose, volume of the daily gas production, daily average H_2 percentage in the headspace gas and the ambient temperature is used. This value is divided by daily hexose fed in moles (mol sucrose/2). To calculate moles of H_2, ideal gas law is used.

$$P.V = n_{H_2}.R.T$$

P- Pressure (atm, assumed as 1 atm)

V- Volume of the H_2 gas produced

n_{H_2}-moles of H_2

R- Gas Constant (0.082 atm. L/ (mol. ^oK)

T- Temperature (^oK)

Since the volume of the gas produced is measured at 35 ^oC, the temperature is taken as 308 ^oK (273+35) while calculating moles of H_2 and pressure was assumed as 1 atm. Then daily HY is calculated as the n_{H_2}/ moles hexose.

For Batch Reactor Set-B, the HY values were small. Hence, the values were multiplied by 10^6, and then used in the MiniTab calculations. Therefore to get the actual yield values of the reactors of the Set-B, after the equations are solved with the actual values of the variables, it needs to be divided by 10^6.

Hydrogen Production Rate: To calculate the fermentative hydrogen production rate (HPR) in terms of L H_2/ (L_{rxr}.day), daily total gas production, average H_2 percentage and standard temperature-pressure condition (0^oC, 1 atm) are considered to calculate daily hydrogen production. Then, this value is divided by effective volume of the reactor (3.6 L).

For Batch Reactor Set-B, the HPR value was small in numbers. Hence, the values were multiplied by 10^6, and then used in the MiniTab calculations. Therefore to get the actual HPR value of the reactors of the Set-B, after the equations are solved with the actual values of the variables, it needs to be divided by 10^6.

Sugar Content of the Molasses and Selected Samples: Sugar content of the molasses used in Set-B was tested in the Central Laboratories of Molecular Biology and Biotechnology Research Center of Middle East Technical University. The sugar content analysis was done with HPLC-67C Saccharide-Polysaccharide method of the laboratory. The analyses results are presented in APPENDIX C.

Alcohol Analysis of the Selected Samples: Alcohol content of the selected samples was tested in the Central Laboratories of Molecular Biology and Biotechnology Research Center of Middle East Technical University. The alcohol analysis was done with HPLC-87H Alcohol-Sugar method of the laboratory. The analyses results are presented in APPENDIX D.

3.3.2. Design and Analysis of Batch Reactor Sets with Response Surface Methodology

Classical experimental designs that investigate the effects of various variables on a particular objective (a response- such as HY) usually depend on one factor at a time approach to the issue. But this experimental design approach misses the effects of the interaction of various variables that might change the magnitude of the objective (Wang and Wan, 2009a). This experimental design is also time-consuming since it requires a number of experiments to cover different levels of a variable (Xing et al., 2011). Therefore using a statistical design approach like RSM, reduces the number of experiments, includes the interactions of the

variables in the resulting objective and provides a statistically sound set of results (Liu et al., 2011).

For a set to be designed by RSM, firstly three independent variables (for example pH, biomass concentration (VSS as X_o)) are chosen (as a result of preliminary studies or from literature survey) which might have effect on a response (result parameter; i.e. HY). Then, the (minimum and maximum values (a range) for each of these parameters to be investigated are introduced to the RSM (for example 4 and 7, for the variable pH). These selected ranges are based on the results or optimals of previous studies from the literature. The RSM tool offers a number of modeling methods (i.e. Taguchi design, central composite design, Box–Behnken design). These models determine the values (in other words the levels) of each variable to be tested (within the pre-defined range). In this thesis, Box-Behnken design method was used since it is more commonly used when the reactor sets are to be conducted only once for needing less design points than other methods (Wang and Wan, 2008c). If one wants to run the model with 3 variables (each pre-defined with a minimum and maximum value) and specifies that each variable combination should be processed in two replicas, the model will set 30 design points (i.e. 30 reactors). In other words, such a requirement predefined in the model indicates a total of 13 different reactor types (of different variable combinations) to be conducted or a total of 30 reactors including the replicas.

Two sets of batch reactors, namely Set-A and Set-B, each composed of 30 reactors were experimented for this thesis. The factors and levels used for modeling these sets are given in Table 3.5**.** It is aimed to maximize the the result parameter, called response, which is affected by these three variables. Therefore, this response should be primarily defined.

Table 3.5 Factors and levels used in the Box-Behnken design of Set-A and Set-B

Independent Variable	Symbol	Range and Levels		
		-1	**0**	**1**
pH (for both Batch Reactor Set-A and Set-B	X0	4	5.5	7
COD (for both Batch Reactor Set-A and Set-B)	X1	10	30	50
S/X_o [a] (for Batch Reactor Set-A)	X2	4	12	20
X_o [b] (for Batch Reactor Set-B)	X2	2.5	5.0	7.5

[a] COD/VSS ratio of the reaction at time zero.
[b] VSS content of the reaction medium at time zero (mg/L).

MiniTab Software (Minitab Pro 16.1.0.0) was used to employ RSM. Using RSM, the effect of the independent variables (initial COD, pH and X_o (or S/X_o)) on each response (HY and HPR) was evaluated. In other words, experimental results were used to produce appropriate models and 3-D graphs via RSM which reflect the relationship between the reponses and the variables. To investigate the validity of these models, Analysis of Variance (ANOVA) was performed. ANOVA results were modified for the models to obtain a model with the highest accuracy defining the responses. When ANOVA indicates the insignificance of a specific variable or and interaction variable it is eliminated from the model. Insignificance of a variable means that, a change in its value does not significantly affect the value of the response, for the range of the values studied. Insignificance of a variable is indicated by a p-value of larger than 0.05 in the ANOVA (i.e. it fails to affect the response in the 95% confidence interval). Therefore successive ANOVAs are applied to eliminate the insignificant parameters. Hence an iterative approach is employed to get the model with the highest accuracy in defining the responses. Then, for the defined responses, surface and contour plots are drawn and their corresponding ANOVA's are

studied, and the results are interpreted accordingly. The independent variable points, which result in the maximization of the responses were calculated using the response optimization tool of RSM.

In Batch Reactor Set-A, sucrose was used as the substrate and initial COD, S/X_o, and initial pH were the tested variables. In Batch Reactor Set-B, substrate was sucrose and variables were same except S/X_o. Molasses is known to be mostly consisting of sucrose. Therefore the first substrate to be studied before moving on to the original substrate (molasses) was chosen to be sucrose, and Set-A was conducted. Set-B was designed according to the results of Set-A and molasses was used as the substrate. The variable S/X_o was dropped after the results of Set-A set and X_o was chosen as the third variable.

3.4. Experimental Procedure

The aims of two batch reactor sets conducted and SBR studies employed in this thesis study were defined as follows;

- Batch reactor Set-A: To investigate the effects of initial COD concentration, S/X_o and pH on dark fermentative H_2 production and determine the optimum initial conditions (COD, pH, S/X_o) leading to the maximum dark fermentative H_2 production from sucrose with RSM. The parameters and their values to be studied were decided based on the knowledge gathered from the preliminary batch studies (APPENDIX E).
- Batch reactor Set-B: To investigate the effects of initial COD concentration, VSS concentration (X_o) and pH values on dark fermentative H_2 production and to determine the optimum initial conditions (COD, pH, X_o) leading to the maximum dark fermentative H_2 production from molasses with RSM.
- SBR Study-1: Application of pH=7, HRT=36 h and OLR=7.5 g COD/ (L.day) for dark fermentative hydrogen production in SBR

- SBR Study-2: Application of pH=5.5, HRT=36 h and OLR=7.5 g COD/ (L.day) for dark fermentative hydrogen production in SBR
- SBR Study-3: Application of pH=5.5, HRT=12 h, OLR=22.4 g COD/ (L.day) for dark fermentative hydrogen production in SBR
- SBR Study-4: Application of pH=5.5, HRT=12 h, OLR=22.4 gCOD/ (L.day), SRT≈9 days for dark fermentative hydrogen production in SBR
- SBR Study-5: Application of pH=5.5, HRT=12 h, OLR=22.4 gCOD/ (L.day), SRT≈4 days for dark fermentative hydrogen production in SBR

3.4.1. Batch Reactor Operations

Set-A and Set-B were designed with RSM to investigate the effects of initial pH value, initial COD and initial VSS (or S/X_o) concentrations on dark fermentative hydrogen production.

Literature review was done in order to determine the initial values of the parameters to be studied in the batch reactor sets. Generally, initial COD values studied change between a wide range of 0.27 to 320 g/L in batch reactors (Table 2.1, Section 2.3.1). pH to be studied changes between 4.5 to 8 (Section 2.3.6). But most studies found the optimal values for these parameters as 1 to 60 g/L for COD (Section 2.3.5 and 7 to 5 for pH (Section 2.3.6). In addition to the literature information, the initial values of the parameters to be studied were determined by considering the knowledge gathered from the preliminary batch studies (APPENDIX E), at which hydrogen production ability of the seed sludge was tested under different initial pHs and COD concentrations. The results of the preliminary batch reactor studies indicated that initial pH values from 5 to 7, and initial COD concentrations from 10 to 40 g/L were reasonably effective in hydrogen production. The highest HYs were achieved with the high pH (6 and 7) and low COD (10 g/L) reactors. In addition, product inhibition was highly observed in the results of the batch studies due to VFA production and resulting pH decrease (APPENDIX E). Preliminary studies also indicated that another

parameter concerning the reactor VSS concentration might be incorporated into the study, in order to achieve higher yields and a longer operation period. Therefore, the results of the preliminary batch studies and the literature review provided the basis of deciding on the operational parameters of batch reactor studies.

The design factors and levels of each of these independent variables (initial pH, COD and S/X_o (or X_o)) in both sets are presented in Table 3.6. In other words, in Set-A, initial pH values of 4, 5.5 and 7.0, initial COD concentrations of 10, 30 and 50 g/L, and initial substrate to VSS ratios of 4, 12 and 20 gCOD/ gVSS were studied as independent variables; and in Set-B, initial pH values of 4,5.5 and 7.0, initial VSS concentrations of 2500, 5000 and 7500 mg/L and S/X_o values of 4, 12 and 20.

Table 3.6 Operational Conditions of Batch Reactor Sets

	VSS_{rxr} (mg/L)	**BM addition**	**$COD_{initial}$ (g/L)**	**pH**	**S/X_o (gCOD/ gVSS)**
Set-A	2500	Yes	10, 30, 50	4, 5.5, 7	4, 12, 20
Set-B	2500, 5000, 7500	Yes	10, 30, 50	4, 5.5, 7	4, 12, 20

Thirteen reactor types were conducted for batch Set-A and Set-B, each reactor type with 2 to 6 replicas with respect to the Box-Behnken design approach (Kim et al., 2006). In total, each set was composed of 30 reactors.

3.4.1.1. Batch Reactor Set-A

Set-A was conducted in 100 mL reactors with 60 mL working volumes. All reactors were inoculated with the sucrose, pretreated sludge and the BM (Table 3.4) in required amounts. The initial pH values of the reactors were adjusted with 2M NaOH and HCl solutions. All reactors were incubated at 35±2^{o}C and

constantly stirred at 125 rpm on a shaker. Gas production and headspace gas compositions were measured every day. The reactor types and contents of Set-A are given in Table 3.7.

Table 3.7 The reactor types and contents of Set-A conducted in accordance to RSM

Reactor No	Initial COD (g/L)	Initial S/X_o (g COD/ gVSS)	Initial pH
1	30	20	4
2	30	20	7
3	30	12	5,5
4	10	12	4
5	10	4	5.5
6	50	4	5.5
7	30	4	4
8	50	12	7
9	50	12	4
10	10	12	7
11	30	4	7
12	10	4	5.5
13	30	12	5.5
14	50	4	5.5
15	30	12	5.5
16	30	12	5.5
17	30	12	5.5
18	30	12	5.5
19	10	20	5.5
20	30	20	7
21	30	4	7
22	30	20	4
23	30	4	4
24	10	12	4
25	50	12	4
26	50	12	7
27	10	20	5.5
28	10	12	7
29	50	20	5.5
30	50	20	5.5

3.4.1.2. Batch Reactor Set-B

Set-B was conducted in 250 mL reactors with 150 mL working volume. The reactor types and contents of Set-B reactors are given in Table 3.8. All reactors were inoculated with the molasses and pretreated sludge in required amounts. For using molasses with adequate amount of nutrients (Table 3.4, Section 3.2), BM was not used in Set-B reactors. The initial pH values of the reactors were adjusted with 2M NaOH and HCl solutions. All reactors were incubated at 35±2^{o}C and constantly stirred at 125 rpm. Gas production and headspace gas compositions were measured every day.

As mentioned previously and also seen in Table 3.8, S/X_o was not investigated in Set-b unlike Set-A. The results of Set-A did not provide a satisfactory explaination on the effect S/X_o on the responses and it was suspected it was not an independent parameter to be studied. Thus, instead of S/X_o, initial VSS concentration, i.e., X_o was studied in Set-B, in addition to initial pH and COD values.

Table 3.8 The reactor types and contents of Set-B conducted in accordance to RSM

Reactor No	Initial COD (g/L)	VSS (X_o) (mg/L)	Initial pH
1	30	5000	5.5
2	30	7500	4
3	30	5000	5.5
4	50	2500	5.5
5	30	7500	7
6	30	7500	4
7	30	2500	7
8	10	5000	5.5
9	50	5000	7
10	10	5000	7
11	50	2500	5.5
12	30	2500	7
13	10	5000	4
14	50	7500	5.5
15	10	2500	5.5
16	30	2500	4
17	30	2500	4
18	10	2500	5.5
19	30	5000	5.5
20	30	7500	7
21	10	7500	5.5
22	30	5000	5.5
23	50	7500	5.5
24	50	5000	4
25	10	7500	5.5
26	50	5000	7
27	10	5000	4
28	10	5000	7
29	30	5000	5.5
30	50	5000	4

3.4.2. Sequencing Batch Reactor Operations

3.4.2.1 Reactor Configuration

The SBR used in this study is a 5 L cylinder made of plexiglass material with 15 cm diameter and 30 cm height. The effective volume of the reactor is 3.6 L and the exchange ratio (the volume of the liquid removed in each cycle/total liquid volume of the reactor) used during the operation was 66%. SBR process consisted of 4 periods which are feeding, mixing (anaerobic period), settling and withdrawal, all of which were controlled automatically by timers. Depending on the desired HRT, number and duration of the cycles were arranged. Solenoid valves and other electrical equipment were connected to timers for automated control of the process.

SBR set-up was conducted with the following equipment (1-9) shown in Figure 3.1.

1) Acid and base pumps
2) Influent pump
3) Magnetic stirrer
4) pH control unit
5) Solenoid gas valves
6) Water-Displacement unit
7) Nitrogen (N_2) Bag
8) Cylindrical plexiglas reactor
9) Solenoid liquid valve for effluent removal

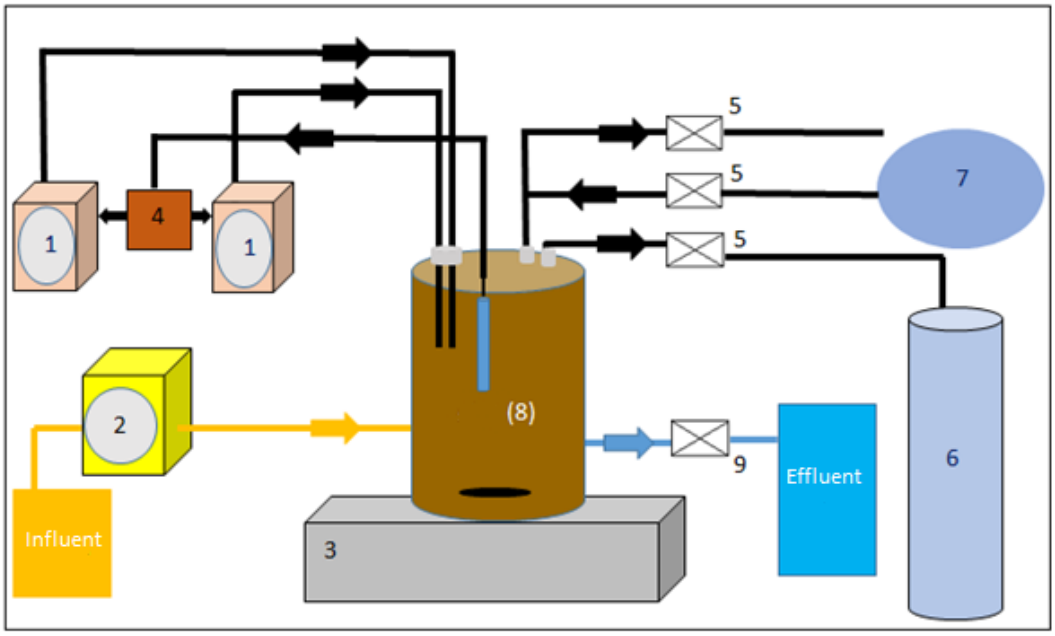


Figure 3.1 Representation of the SBR system used in the study

Acid and base solutions were introduced into the reactor via acid and base pump which are controlled by a pH-stat (control) unit. pH was monitored and controlled instantaneously with this system. The speeds of the pumps were adjusted according to the needs of the reactor operation.

The produced gas was continuously collected in a water displacement device. To measure the daily gas production, the gas production in one cycle was measured and recorded. Assuming that the volume of gas production was also same as those of other cycles of the same day, the gas produced in one cycle was multiplied by the number of cycles per day.

In this thesis study, SBR type reactor was selected because, in SBRs operating at low HRTs, the hydraulic sludge loss is more effectively prevented with the help of the settling period, compared to the systems with no settle phase. With SBR systems a higher culture density, hence higher SRT, values can be achieved. In addition, since reactor content is decanted periodically, dark fermentative end products causing product inhibition of the microbial activity such as VFA and

H_2 can be eliminated. Therefore, SBR is applied to many bench dark fermentative hydrogen production studies (Dinamarca and Bakke, 2009; Hussy et al., 2005; Kim et al., 2010; Won and Lau, 2011). In addition, in the SBR system used in this thesis study, gaseous products as well as the liquid products are decanted periodically with the help of the N_2 bag and automatically controlled gas valves system.

The appearance of the reactor system (with feed pump, pH-stat and the gas collection system) on the very first day of SBR Studies was given in Figure 3.2.

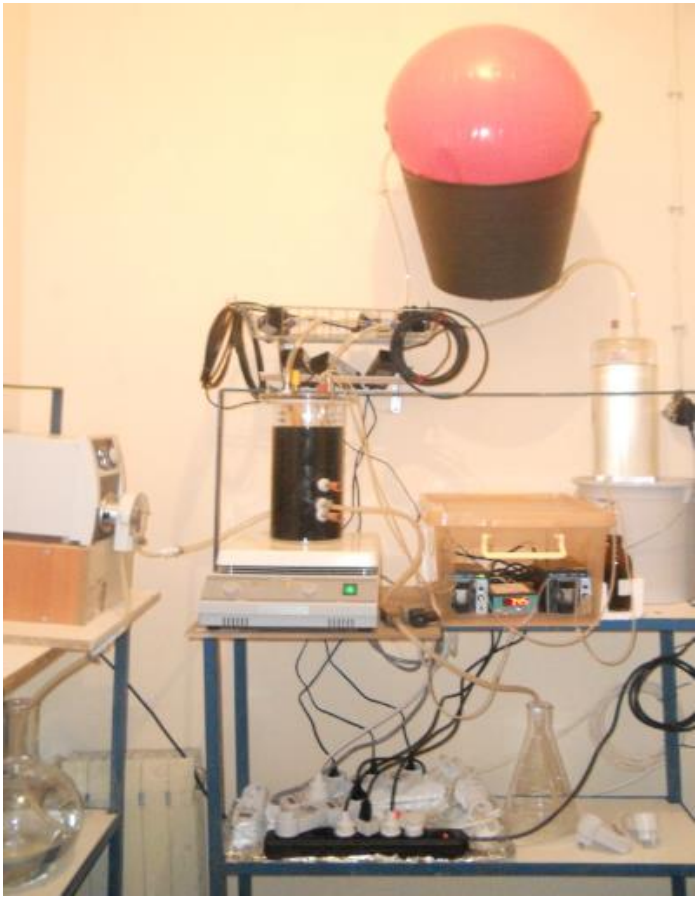


Figure 3.2 Set-up of the SBR in the hot room

3.4.2.2. SBR Study Operational Conditions

HRT and pH are stated as the key parameters for high yield and stable hydrogen production via dark fermentation (Wang and Wan, 2008c; Saady, 2013; Wu et al., 2013). During the experimental study, SRT was also found to be as an important parameter in addition to pH and HRT. Therefore SRT was also selected as a parameter to be investigated. As previously mentioned, 5 SBR studies were conducted with sucrose as the substrate. It was aimed to improve the dark fermentative H_2 production in SBRs via each SBR study. In other words, it was aimed to investigate the optimum SBR operational conditions leading to the maximum and stable H_2 production via each SBR Study. The summary of these SBR studies and operational parameters of each study are given in Table 3.9. The properties and operations specific to each SBR study are described in the following sections (Section 3.4.2.2.1-3.4.2.2.5).

Table 3.9 An overview of the operational properties of each SBR study

SBR Study No	Target pH	HRT (h)	Cycle Period (h)	OLR[a]	Operation time (day)	Number of cycles (and HRTs) operated[b]	$SRT_{average}$ (day)
1	7→[c]5.5	36	24	7.5	17	18 (12)	14.5
2	5.5→[c]4.5	36	24	7.5	48	48 (32)	9.8
3	5.5	12	8	22.4	10	20 (13)	10.3
4	5.5	12	8	22.4	20	40 (27)	9.5
5	5.5	12	8	22.4	34	68 (45)	4.2

[a] OLR, organic loading rate, g COD/L.day
[b] The values in parenthesis indicate the number of HRTs through which the reactor was operated
[c] Operational pH was changed during the study

3.4.2.2.1. SBR Study-1: Application of pH=7, HRT=36 h and OLR=7.5 g COD/L.day

In SBR Study-1, reactor's cycle period was 24 h. Each cycle composed of 5 minutes of feeding, 22 h of mixing (anaerobic period), 6 h of settling and 5 minutes of withdrawal periods. HRT studied was 36 h. This value is in the range of 4-96 h HRT values for dark fermentative hydrogen production (Chen et al., 2009; Won and Lau, 2011; Saraphirom and Reungsang, 2011; Wu et al., 2009, 2013).

Operational pH and initial reactor VSS concentrations were selected and set constant according to the results of the Batch Set-A. The operational pH was controlled at 7, the initial reactor VSS concentration was set at 5000 mg/L and sucrose solution of 10 g/L (a COD equivalence of 11.2 g/L) was introduced to the reactor as influent along with the BM ingredients (in the concentrations stated in Table 3.2 (Section 3.2)). The reactor was operated with 36 h HRT, 24 h cycle time, 7.5 g COD/L.day OLR conditions for 23 days. Operational pH was decreased to 5.5 at the 12^{th} day of operation.

3.4.2.2.2. SBR Study-2: Application of pH=5.5, HRT=36 h and OLR=7.5 g COD/L.day

In SBR Study-2, reactor's cycle period was 24 h. Each cycle composed of 5 minutes of feeding, 22 h of mixing (anaerobic period), 6 h of settling and 5 minutes of withdrawal periods. HRT studied was 36 h. The operational pH was controlled at 5.5, the initial reactor VSS concentration was set at 5000 mg/L and sucrose solution of 10 g/L (a COD equivalence of 11.2 g/L) was introduced to the reactor as influent along with the BM ingredients (in the concentrations stated in Table 3.2 (Section 3.2)). SBR Study-2 lasted 48 days.

3.4.2.2.3. SBR Study-3: Application of pH=5.5, HRT=12 h, OLR=22.4 g COD/L.day

In order to investigate the effect of HRT on dark fermentative hydrogen production, HRT value of 12 h was studied. Therefore each cycle lasted 8 h and was composed of 5 minutes of feeding, 6.7 h of mixing, 72 minutes of settling and 2 minutes of withdrawal. Operational pH was set at 5.5. Hence number of cycles per day was increased to 3 as the OLR increased to 22.4 g COD/L.day. Operational pH was set at 5.5. Third SBR operational period lasted 10 days. The experimental observations led to requirement for further investigation of SRT effect.

3.4.2.2.4. SBR Study-4: Application of pH=5.5, HRT=12 h, OLR=22.4 gCOD/L.day, SRT≈9 days

The operational conditions (HRT, pH, cycle time) for the SBR Study-4 were kept as that of SBR Study-3 (Table 3.9). Only difference was regulation of VSS acuumulation in the reactor, by periodic removal of some reactor content. In other words, to achieve much better control of SRT, required volume of sludge content was periodically withdrawed from the reactor, in a way to achieve a constant VSS concentration in the reactor. Accordingly, the reactors' SRT was kept 8-9 days.

3.4.2.2.5. SBR Study-5: Application of pH=5.5, HRT=12 h, OLR=22.4 gCOD/L.day, SRT≈4 days

The operational conditions for SBR Study-5 were kept as that of SBR Study-4 (Table 3.9). SRT of the reactor was kept at 3-4 days, differing from the operational conditions of the SBR Study-4, of greater SRTs.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Results of the Batch Reactor Studies

Batch reactor studies were conducted in order to investigate the effects of initial COD, initial VSS (X_o) or initial substrate concentration over VSS concentration (S/X_o) and pH on hydrogen production and to determine the parameters leading to the maximum H_2 production.

Considering the literature up to date, unlike the initial COD and pH, the parameters such as X_o or S/X_o and their effects on hydrogen production have not been investigated in detail. Within the surveyed literature, this study is the first to investigate the combined effect of initial COD, S/X_o (and X_o) and pH on dark fermentative hydrogen production. The results of these batch sets are seperately presented below.

4.1.1 Results of Set-A: Determination of Optimum Initial COD, S/X_o Ratio and pH Values

Set-A was conducted according to RSM approach and sucrose was used as the substrate. The objective was to investigate the effects of initial COD, initial substrate concentration over VSS concentration (S/X_o) and pH on hydrogen production and to determine the parameters leading to the maximum H_2 production. This study, within the surveyed literature, is the first study in which S/X_o effect on dark fermentative hydrogen production was investigated.

The reactors in Set-A were operated for 6 days. The results of Set-A are given in Table 4.1. The total produced gas, headspace gas H_2%, HY and HPR results are

presented as the average values of the replicas. Methane was not observed in any of the reactors which indicated the success of the heat treatment to eliminate/suppress methanogens.

It can be seen in Table 4.1 that the highest yield achieved was 2.3 mol H_2/mol $sucrose_{added}$, which was achieved from the reactor pair with the initial COD of 10 g/L, S/X_o of 12 and pH 7. This value equals 1.21 mol H_2 for the more common unit of per hexose. In literature, similar results were presented (Table 2.4, Section 2.3.7) for batch reactors using sucrose. The lowest yield of 0.11 mol H_2 /mol hexose was achieved for the reactor pair at the condition of COD of 50 g/L, S/X_o of 12 and pH 4. Careful study of Table 4.1 showed that reactors with higher initial pH values provided higher yields. There was no significant relation spotted among the yields achieved and the final pHs of the reactors.

Table 4.1 The hydrogen production yield and productivity calculations for the 13 types of reactors of Set-A

Initial			Final pH	V [b,c]	Average Headspace H_2 (%)[d]	V_{H_2} [e]	HY[f]	HY[g]	HPR [h]
COD (mg/L)	S/X_o [a]	pH							
10	12	7	3.65	231	34.6	80.3	9	2.3	10.6
30	20	7	3.6	207	33.7	70.0	2.6	0.67	9.2
50	12	7	3.58	198	35.5	71.0	1.6	0.41	9.3
10	4	5.5	3.39	185	32.7	60.3	6.8	1.73	7.9
50	4	5.5	3.49	153	35.1	53.7	1.2	0.31	7.1
10	20	5.5	3.47	170	35.9	60.5	6.8	1.73	7.9
30	4	7	3.62	215	34.9	75.0	2.8	0.72	9.9
50	20	5.5	3.44	146	28.8	42.3	0.9	0.24	5.6
30	12	5.5	3.7	225 ±29	32±3	72±12	3± 0.5	0.7±0.1	10±1
30	4	4	3.78	117	18.7	21.2	0.8	0.2	2.8
10	12	4	4.17	124	16.4	20.3	2.3	0.58	2.7
30	20	4	3.68	178	15.5	27.6	1	0.26	3.6
50	12	4	3.83	188	9.5	18.8	0.4	0.11	2.5

[a] S/X_o: (mg COD/L) / (mg VSS/L)
[b] V: Total Gas Production, mL (at 35°C)
[c] Values presented are the averages of the two replicas. Only the reactor with conditions COD 30, pH 5.5 and S/X_o=12, was studied in 6 replicas due to the requirement by RSM (therefore, only this reactor type has a standard deviation calculation)
[d] Gas content was analyzed at the 3rd day of operation. Rest of the headspace gas composed of CO_2 and N_2.
[e] V_{H_2}: Total H_2 production, mL (at 35°C)
[f] HY: mL H_2/g sucrose**,** values are calculated by converting the produced gas amount at standard temperature and pressure (0°C and 1 atm)
[g] HY: mol H_2/mol hexose
[h] HPR: mL H_2 / (L_{rxr}. h)

It will be insufficient to evaluate the experimental results of Set-A solely as the raw data since the set was designed with RSM. Therefore, the results should be evaluated by making use of graphical and statistical tools of RSM to find the conditions providing the highest yield. Thus, the effects of the three parameters

on the responses (HY and HPR) were evaluated by means of RSM and presented here.

4.1.1.1 Results of RSM Study for Set-A

As described earlier in Section 3.3.2, the resulting values of the responses were evaluated with graphical and statistical tools that RSM provides. RSM tools can be used to evaluate the effects of each parameter (initial COD, pH or S/X_o) separately or all of them in interaction with one-another; on each of the responses. The results of the ANOVAs and the details of the models developed are presented in Table 4.2. Table 4.2 illustrates the significance of the related variables of each model. The ANOVA results showed that parameter S/X_o and some parameter interactions like COD x pH, COD x S/X_o or $(S/X_o)^2$ were insignificant in terms of their effects on the responses. Therefore successive ANOVAs were applied to eliminate the insignificant parameters (hence, all the parameters in Table 4.2 have p-values lower than 0.05 (Section 3.3.2)). Then, for each response (i.e. HY and HPR), surface and contour plots were drawn and the results were interpreted accordingly to find out the variable (initial pH, COD and S/X_o) values at which HY and HPR maximize.

Table 4.2 ANOVA results of the models of the responses according to the Box-Behnken design with three independent variables and corresponding experimental results of Set-A

for HY[a]			for HPR[b]		
Term	**Coef[c]**	**P-value[d]**	**Term**	**Coef[c]**	**P-value[d]**
Constant	0.6665	0.000	**Constant**	-0.033995	0.000
pH	0.3431	0.000	**COD**	0.000195	0.003
COD	-0.6244	0.000	**S/X**	0.000355	0.025
pH^2	-0.1864	0.003	**pH x COD**	0.011916	0.000
COD^2	0.3036	0.000	**COD^2**	-0.000004	0.001
pH x COD	-0.3312	0.000	**$(S/X)^2$**	-0.000015	0.020
			pH^2	-0.000888	0.000
R-Sq[e] = 94.76%			R-Sq[e] = 89.58%		

[a] HY: mol H_2 / g COD
[b] HPR: mL H_2/ (L_{rxr}.h
[c] Coef: Multiplication coefficient of the related parameter
[d] P-value: Significance (power of effectiveness) of the related parameters
[e] R-Sq: Significance (explainatory power) of the model

The model produced for the response HY (Eq. 4.1) was defined as follows;

$$\text{HY (mol } H_2/\text{mol sucrose}_{added}) =$$
$$-3.30051 + 1.47150 * \text{pH} - 0.0160232 * \text{COD} - 0.0828632 * \text{pH} * \text{pH} +$$
$$0.000758894 * \text{COD} * \text{COD} - 0.0110417 * \text{pH} * \text{COD} \qquad \text{(Eq. 4.1)}$$

This model (Eq. 4.1) is highly significant with all p values smaller than 0.05. R-Sq (R^2) value is 94.76% (Table 4.2), therefore, explaining that much of the response values. According to Eq. 4.1, pH, COD and their interactions pH x pH, COD x pH and COD x COD are the only parameters that have a significant effect on HY. The results of this analysis indicated that S/X_o variable had no effect on resulting HY value.

Contour and Surface Plots of the HY model are given in Figure 4.1. The surface and contour plots form a complimentary couple. Contour plots represent the x-z (COD-pH) plane of the surface plot therefore, making it easier to interpret the graphical results. Figure 4.1-a and b illustrate that as the initial COD value increased from 10 from 50 g/L, hydrogen yield decreased; while pH increased from 4 to 7, yield increased. Supporting these results Wang and Wan (2008a) found maximum yield to occur at pH 7.2 and Won and Lau (2013) calculated maximum yield at lower CODs of 10-20 g/L. According to Figure 4.1, at initial COD concentrations lower than 20 g/L, yield increased with increased pH. This finding was consistent with the results of other studies (Wu et al., 2002; Wu et al., 2013). In addition to the results of the preliminary studies (APPENDIX E), this batch study confirmed the finding that lower initial CODs (10 to 20 g/L) provide higher yields than those of higher initial CODs (25-50 g/L). S/X_o was not a parameter to appear in surface or contour plots of the responses since it was not a significant parameter affecting HY.

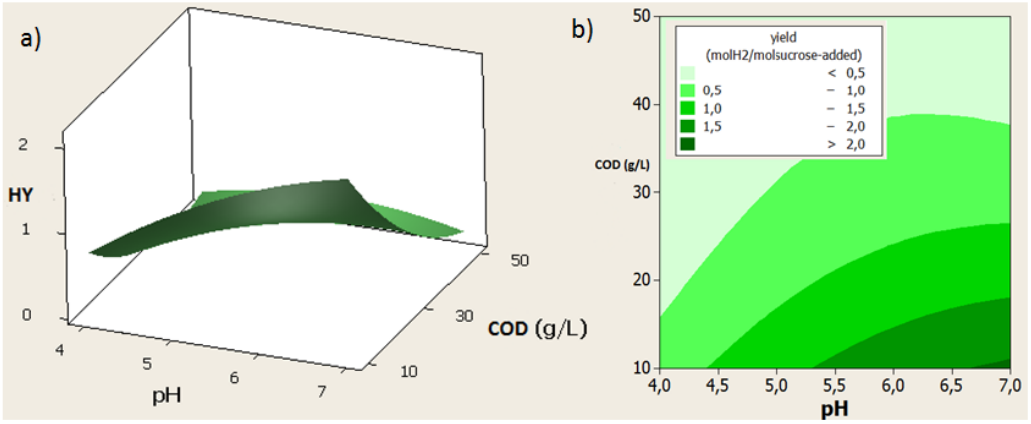


Figure 4.1 Surface and Contour plots for HY of Set-A (HY; mol H_2/mol $sucrose_{added}$)

The RSM results also indicated that S/X_o was ineffective on hydrogen production from sucrose with heat pre-treated sludge in this pre-described range. Another RSM tool (namely the "global solution"-the maximum or minimum points of the model) was used to calculate the maximum point for HY. Maximum HY was calculated by the model to occur at pH 7 and COD 10 g/L, for the range of values studied. The maximum of HY was calculated as 2.08240 mol H_2 / mol sucrose. Chen et al. (2005) used a pure culture of *Clostridium butyricum* CGS5 with sucrose and achieved a yield of 2.78 mol H_2 /mol sucrose, where Wang et al. (2006) used sucrose as substrate with sewage sludge as inoculum and achieved 2.46 mol H_2 /mol sucrose. The latter also find the maximum yield at 10 g/L COD, similar to the findings of Set-A. The yield achieved in various studies depends highly on the culture type and its history.

For the second response, HPR, the following model (Eq. 4.2) was obtained according to the consecutive ANOVAs;

$$\text{HPR (Productivity, mL } H_2/\text{L}_{rxr}.\text{h}) = -0.0333995 + 0.000195 * \text{COD} + 0.000355 * \text{S/X} + 0.011916 * \text{pH} - 0.000004 * \text{COD} * \text{COD} - 0.000015 * \text{S/X} * \text{S/X} - 0.000888 * \text{pH} * \text{pH} \quad \text{(Eq. 4.2)}$$

This model (Eq. 4.2) is highly significant with all p values smaller than 0.05 (Table 4.2). 89.58% of the data can be explained using this model (R-Sq 89.58%) (Table 4.2). Contour and Surface Plots of the HPR model are given in Figure 4.2. The model given in Eq. 4.2 indicated that, unlike for HY, S/X_o parameter had no significant effect on HPR. According to the surface and contour plot pairs (Figure 4.2), HPR increased significantly as pH increased from 4 to 7. The maximum point of HPR was calculated at COD concentration of 26.566 g/L; S/X_o of 11.758 and pH of 6.697. The model calculated the maximum HPR to occur at these three values (initial COD, S/X_o and pH) as 10.7 mL H_2/ (L_{rxr}.h).

From global solution tool of the RSM, maximum HPR was calculated to occur at COD concentration of 26.56 g/L, S/X_o of 11.758, pH 6.697. When these parameters were used in the batch reactor studies, the predicted value of the response is calculated as 10.7 mL H_2/ (L_{rxr}.h) which is a bit lower than previously conducted studies in the literature (Table 2.1, Section 2.3.1).

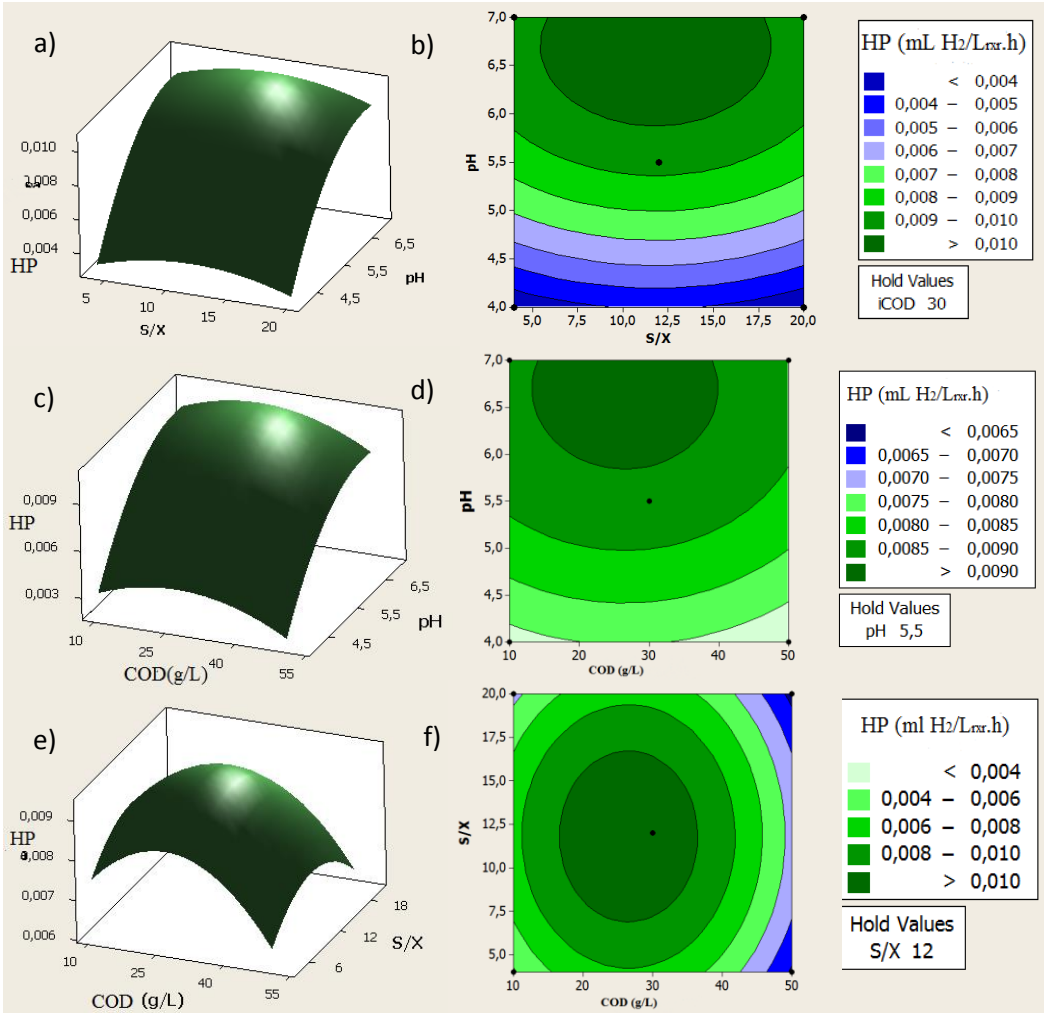


Figure 4.2 Surface and Contour Plots for HPR with respect to various independent parameters of Set-A

Figure 4.2-b and d illustrate that as pH value increased from 4 to 7, HPR significantly increased. On the other hand, although a slight maximum was observed at S/X_o of 12, S/X_o variable had no significant effect on productivity value for the range of studied values (Figure 4.2-e and f). Maximum HPR was observed at COD concentration of 20-25 g/L; at lower or higher COD values the production rate relatively decreased. But the effect of COD concentration on the production rate was not dramatic.

As a result of all the surface and contour plots, it was realized for the values studied, at the middle COD value (20 g/L), as the pH increased, the production rate increased as well. This radical effect of pH on HPR had also been previously verified by other researches (Wu et al., 2002; Lin and Zhu, 2013).

4.1.1.2 VFA Analysis Results of Set-A

Initial VFA concentrations of all reactors at the beginning of the operation were less than 0.1 mM (data not shown). After 6 days of incubation period, the reactors were analyzed for final VFA concentrations. The acid types and their final concentrations detected in all reactor types of Set-A are given in Table 4.3. Results showed that the VFAs produced were dominated by acetic and butyric acids as expected. Acetic acid reached a maximum of 13.64 mM and butyric acid reached 10.69 mM. Lactic acid was produced at the highest amounts in all of the reactors (maximum of 23.33 mM). It is known that lactic acid production is a H_2 production hindering pathway (Eq. 1.4, Section 2.2) (Guo et al, 2010). Therefore, lactic acid production and its accumulation might have resulted in lower hydrogen yield. Lactic acid bacteria dominate during higher organic loads (Oh et al., 2004). Because lactic acid bacteria are resistant to low substrate concentrations and tolerate low pH (pH<5) (Saady, 2013; Adamberg et al., 2003), the low pH values of the reactors (3.4 to 3.8, measured at the end of the study, Table 4.1) might not have negatively affected their growth and abundance.

The highest acetic and butyric acid production was observed in the reactor where the highest HY (2.3 mol H_2 /mol sucrose) was achieved (at the initial COD concentration of 10 g/L, S/X_o of 12, pH of 7; Table 4.3). Disregarding the initial COD concentration or the S/X_o applied, VFA production was in lower amounts than the amount analyzed in the reactors with higher pH values (pH 5.5 and 7).

Table 4.3 VFA results of Set-A using sucrose as the substrate

Initial			**Acid types and their final concentrations (mM)[a]**					**Total VFA (mM, HAc)[c]**
COD (g/L)	**S/X_o[b]**	**pH**	**Lactic**	**Formic**	**Acetic**	**Propionic**	**Butyric**	
10	12	7	19.38	2.72	13.64	0.15	10.39	37.31
10	4	5.5	18.54	0.61	9.02	0.02	9.07	28.37
10	20	5.5	15.92	0.49	6.94	0	6.3	22.49
30	12	5.5	12.1±5	0.33±0.2	6.1±3.1	0.04±0.01	6.49±2.4	21.9±7.4
30	4	7	13.52	1.77	6.77	0.06	5.59	21.95
30	20	7	20.77	3.36	11.04	0.11	6.71	33.93
10	12	4	2.75	0.2	2.63	1.75	0.41	6.41
50	12	7	23.33	3.3	9.83	0.21	7.21	34.77
50	4	5.5	13.57	0.46	7.29	0	7.03	21.73
30	20	4	7.9	0.35	1.49	1.48	0.55	8.79
50	20	5.5	18.74	1.05	6.41	0	6.21	24.5
30	4	4	5.78	0.31	3.39	0.6	0.15	8.23
50	12	4	5.45	0.15	1.19	0.94	0.01	5.78

[a] Molecular weights (g/mol) of the acids are; Lactic 90.08; Formic 46.03; Acetic 60.05; Propionic 74.08; iso-Butyric 88.11; Butyric 88.11; iso-Valeric 102.13.
[b] S/X_o: (mg COD/L) / (mg VSS/L)

At higher pH's (5.5 and 7), acid production was high as is the H_2 production, since they are simultaneously produced. Product inhibition is a major problem in dark fermentative hydrogen production. Acid accumulation in the reaction medium and the consequtive pH decrease, inhibits the hydrogen production reactions. Considering all the reactors had almost the same final pH values (3.4 to 3.8), it can be argued that the higher initial pH values (pH 7) resisted pH drops for a longer time and delayed the system inhibition resultant of VFA production and further pH decrease therefore producing more H_2. This conclusion was also verified with the results of the preliminary batch reactor studies (APPENDIX E).

It was also concluded from Set-A that, as expected, COD removal was not significant for the dark fermentation batch studies (only in the range of 5-10%). Detailed results of these analysis (COD, TSS and VSS) are presented in APPENDIX F.

4.1.2 Results of SET-B: Determination of Optimum COD, VSS and pH values

Set-B was conducted according to RSM design and molasses was used as the substrate. The objective was to investigate the effects of initial COD, initial VSS (X_o) and pH on hydrogen production and to determine the parameters leading to the maximum H_2 production. The photograph of the setup of Set-B in the hot room (35±2 ^oC) on the shaker working at (125 rpm) is given in Figure 4.3. The corresponding values studied for the parameters were COD concentrations of 10, 30, 50 g/L; X_o of 2500, 5000, 7500 mg/L VSS and pH of 4, 5.5, 7. The reactors were incubated for 17 days.

Figure 4.3 Setup of Set-B, incubated at the hot room (35±2 ^{o}C) on a shaker (at 125 rpm)

In this part of the study, a special condition was observed in the reactors that had not been observed in Set-A. Out of 30 reactors conducted, 17 ones started to vacuum after 60 to 400 hours (h) of operation. Table 4.4 presents the results of Set-B in terms of yield and productivity, and indicates the reactors that started to suction.

It is evident in Table 4.4 that the reactors that did suction generally had higher final pH values than the others. This, along with the presence of suction in the reactors, was attributed to the presence of a new pathway; namely, homoacetogenesis (Chen et al., 2009) (Eq. 2.1).

$$4H_2 + 2CO_2 \rightarrow CH_3COOH + 2H_2O \qquad \text{(Eq 2.1, Section 2.3.4.1)}$$

As stated in the Section 2.5, homoacetogenesis is one of the biggest concerns of the stability of dark fermentation studies and continues to be an unresolved issue (Saady, 2013). Most of the recent dark fermentation studies mention its presence and importance in bio-hydrogen producing systems with mixed cultures.

Table 4.4 The vacuum (suction) times for the reactors and final pH values for all reactors

Initial			Code of the reactor	The hour at which the vacuum was initially observed[a]	Final pH
COD (g/L)	X_o (mg/L VSS)	pH			
10	2500	4	M13	109	4.85
10	5000	4	M27	205	4.74
30	2500	4	M16	-	3.38
30	2500	4	M17	-	3.62
30	7500	4	M2	-	3.86
30	7500	4	M6	-	3.89
50	5000	4	M24	282	4.49
50	5000	4	M30	-	3.83
10	2500	5.5	M15	157	4.77
10	2500	5.5	M18	133	4.9
10	7500	5.5	M21	60	5.26
10	7500	5.5	M25	60	5.26
30	5000	5.5	M1	-	4.1
30	5000	5.5	M3	441	4.74
30	5000	5.5	M8	323	3.95
30	5000	5.5	M19	-	4.06
30	5000	5.5	M22	-	4.79
30	5000	5.5	M29	-	4.75
50	2500	5.5	M4	441	3.84
50	2500	5.5	M11	-	3.79
50	7500	5.5	M14	-	4.25
50	7500	5.5	M23	-	3.95
10	5000	7	M10	109	5.6
10	5000	7	M28	85	5.59
30	2500	7	M7	-	3.82
30	2500	7	M12	-	3.95
30	7500	7	M5	282	5.24
30	7500	7	M20	323	5.39
50	5000	7	M9	369	3.85
50	5000	7	M26	282	3.93

[a] The reactors that had showed no suction are indicated with the symbol '-'

Table 4.5 illustrates that, in the reactors where suction was observed, the initial headspace H_2 gas percentages decreased towards the end of the incubation period by 6 to 36%; suggesting the presence of H_2 consuming mechanisms. For example, in the reactors where the suction was observed the latest, M3 (COD concentration of 30 gL, X_o of 5000, pH of 5.5) and M4 (COD concentration of 50 g/L, X_o of 2500, pH of 5.5), the headspace H_2 concentration decreased from 40% to 20% in M3 and from 37% to 8% in M4, in the course of 441 h. It has been stated that a drop in substrate concentration can increase the activity of homoacetogenesis (Wu et al., 2009; Dinamarca and Bakke, 2009). This statement was supported with the results of Batch Reactor Set-B where reactors with lower initial COD concentrations (10 g/L) started to suction earlier than others.

Table 4.5 The HY and HPR calculations for 13 reactor types using molasses as the substrate

Initial			**Final pH**	V [b]	**H_2 %**		V_{H_2} [c]	**HY** [d]	**HY**[e]	**HPR**[f]
COD (g/L)	**X_o** [a]	**pH**			**Beginning Period** [g]	**Ending Period** [h]				
10	5000	4.0	4.8	402	36.3	18.7	97	64.6	2.88	4.67
10	5000	7.0	5.6	333	32.9	25.2	84	56.2	2.51	6.10
10	7500	5.5	5.3	284	30.8	15.3	74	49.6	2.22	8.26
10	2500	5.5	4.8	189	36.4	30	52	34.7	1.55	2.34
30	2500	4.0	3.5	497	11.3	11.6	128	28.5	1.27	3.38
50	5000	7.0	3.9	634	51	16.6	148	19.7	0.88	3.40
30	7500	4.0	3.9	325	23.7	10.6	86	19.2	0.86	2.21
50	7500	5.5	4.1	370	41.7	8.1	118	15.7	0.71	2.33

Table 4.5 Continued: The HY and HPR calculations for 13 reactor types using molasses as the substrate

30	7500	7.0	5.3	197	37.1	20.7	62	13.7	0.61	1.41
30	2500	7.0	3.9	155	42.7	24.2	55	12.2	0.54	1.10
50	2500	5.5	3.8	275	33.6	9.8	78	10.4	0.47	1.18
30	5000	5.5	4.8 ± 0.4	189 ± 101	31.4	21.9	46± 21	10± 4.6	0.46± 0.2	1.17 ± 0.6
50	5000	4.0	4.2	83	34.2	17	24.8	3.3	0.15	0.45

[a] X_o , mg/L VSS
[b] V: Total gas production, mL (at 35^{o}C),
[c] V_{H_2}: Total H_2 production, mL (at 35^{o}C)
[d] Values represent the averages for the 2 replicas. Only the reactor type with the initial COD concentration of 30 g/L, pH of 5.5 and X_o of 5000 mg/L was studied with 6 replicas, therefore only this reactor type was given with standard deviation.
[e] HY, mmol H_2/g COD
[f] HPR, mL H_2 / (L_{rxr}. h), values are calculated by standardizing the gas amounts to standard conditions, 0^{o}C temperature and 1 atm
[g] The values represent the total gas production at the reaction time, after the produced amount reached more than the headspace volume (100 mL). This corresponds to the beginning of the incubation period.
[h] These values are, for each reactor, after the total daily gas production dropped below 20 mL. This corresponded to the ending period of the operation.

In Set-A, where sucrose was used as substrate, the pH values measured at the end of the study was lower than that obtained in Set-B (Table 4.5). In addition, suction was not experienced in Set-A. This finding of Set-B brought forth the idea that molasses is likely to have intrinsic homoacetogenic microorganisms. Oh et al. (2004) stated that heat treatment was not successful in eliminating homoacetogenic microorganisms. Saady (2013) emphasized that no elimination method had been successful in eliminating these microorganisms. This is associated with the similarity of most properties (like spore formation) of homoacetogenic and hydrogen producing bacteria, therefore none of the pretreatment methods eliminate them without hindering hydrogen producing acidogens. Both species have optimum pH at the same range (Oh et al., 2004)

therefore, homoacetogenesis constantly occurs despite of being in small amounts in the course of dark fermentative hydrogen production.

As discussed in detail, although suction was observed, gas productions for all reactors were calculated cumulatively. For each reactor type, operated in duplicates, the cumulative hydrogen production amounts are presented in Table 4.5. As Table 4.5 shows, the cumulative hydrogen production of the reactors changed between 25 and 148 mL. The lowest hydrogen production was observed in the reactor with the reactor pair of initial COD concentration of 50 g/L, pH of 4 and initial VSS of 5000 mg/L. Highest H_2 production on the other hand was observed in the reactor with initial COD concentration of 50 g/L, pH of 7 and initial VSS of 5000 mg/L. Therefore at the same initial COD (50 g/L) and VSS (5000 mg/L) concentrations, high initial pH value (pH 7) supported the hydrogen production, most probably by buffering the potential pH decrease occurring due to VFA production in parallel to H_2 production. But this observation was not true for each COD-X_o pair (Table 4.5). In addition, hydrogen production of the reactors with the same initial COD and pH values was not affected by changing X_o; it did not increase or decrease significantly due to the change in X_o.

Table 4.5 shows the highest yield achieved to be 2.88 mmol/g COD at conditions of initial COD concentration of 10 g/L, X_o of 5000 mg/L and pH of 4. The X_o value of 5000 mg/L affected the yield positively at low COD value. But it can not be said that a certain pH value affected the yield value positively or negatively.

In fact, it will be insufficient to evaluate the experimental results of Set-B solely as the raw data since the set was designed with RSM. Therefore, the results should be evaluated by making use of graphical and statistical tools of RSM to find the conditions providing the highest yield.

4.1.2.1 Results of RSM Study for Set-B

RSM was applied to find out the independent variable (initial pH, COD and X_o) values at which HY and HPR maximize. The first analysis of the raw data indicated that, due to its ANOVA, some variables (X_o and pH) and certain interactions (COD x pH, COD x pH etc.) were insignificant on the responses ($p>0.05$), therefore they were iteratively eliminated from the model. The ANOVA results of the final models for each response, HY and HPR, are given in Table 4.6.

Table 4.6 ANOVA results of the models of the responses according to the Box-Behnken design with three independent variables and corresponding experimental results of Set-B

for HY[a]			**for HPR[b]**		
Term	**Coef[c]**	**P-value[d]**	**Term**	**Coef[c]**	**P-value[d]**
Constant	3669.96	0.000	**Constant**	8723.87	0.000
COD	-156.81	0.000	**COD**	-387.84	0.003
COD^2	1.89	0.001	**COD^2**	5.00	0.017
R-Sq[e] = 64.11%			R-Sq[e] = 38.73%		

[a] HY, mol H_2 /g COD
[b] HP, mL H_2/(L_{rxr}.h)
[c] Coef: Multiplication coefficient of the related parameter
[d] P-value: Significance (power of effectiveness) of the related parameters
[e] R-Sq: Significance (explanatory power) of the model

The final models obtained through iterative ANOVAs, for HY and HPR are presented as Eq. 4.4 and 4.5, respectively.

$$HY\ \left(mol\frac{H_2}{g\ COD}\right) x\ 10^6 = 3369.96 - 156.81\ x\ COD + 1.89\ x\ COD^2$$

(Eq. 4.4)

$$HPR\ \left(mL\frac{H_2}{L_{rxr}.h}\right)x\ 10^6 = 8723.8 - 387.84\ x\ COD + 5.00\ x\ COD^2$$

(Eq. 4.5)

Both of these models (Eq. 4.4) and (Eq. 4.5), are highly significant with all p values smaller than 0.05 (Table 4.6). The HY model can explain 64.11% of the data and HPR model can explain 38.73% of the data (Table 4.6). The low explanatory power of the model developed for HPR was associated to the fluctuation of the expected values of the responses. The values of the responses probably fluctuated due to the activation of homoacetogenic activity. For example, at a certain 3 variable combination, when homoacetogenesis dominates, the produced H_2 amount will change, which will in turn change the values of HY and HPR. Since homoacetogenesis was not an activity that had been incorporated into the model, the models' low explanatory power might be explained as such.

As seen in Table 4.6 and Eq. 4.4 and 4.5, the only parameter that has a significant effect on HY and HPR was initial COD concentration in Set-B. Since both models only include the term initial COD, contour and surface plots for the models cannot be produced. Therefore 2-dimensional graphs for the quadratic models for HY and HPR are drawn and presented as Figure 4.4 and 4.5. Since the HY and HPR values were small in numbers, the values were multiplied by 10^6, and then used in the MiniTab calculations. Therefore to get the actual HY and HPR value, after the equations are solved with the actual COD value, it needs to be divided by 10^6.

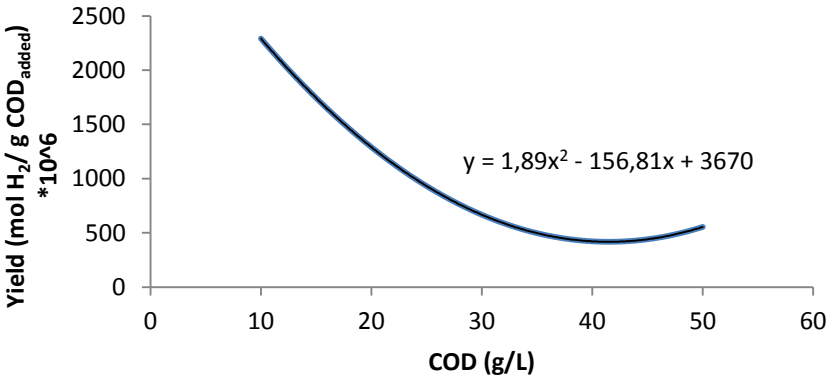


Figure 4.4 Change of HY with respect to COD concentration

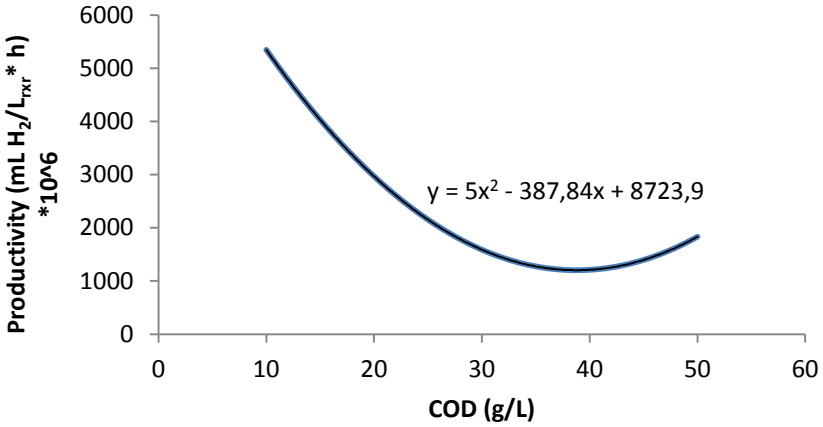


Figure 4.5 Change of HPR with respect to COD concentration

The parabolic shapes of the models presented in Figure 4.4 and 4.5 indicate that, the initial COD concentration of 10 g/L resulted in the maximum HY and HPR among the COD concentrations studied. The maximum HY was calculated as 2.2906 mmol H_2/g COD. Assuming all the COD in the molasses comes from sucrose (Table 3.4, Section 3.2) this yield corresponds to 0.553 mol H_2/ mol glucose (calculation presented in APPENDIX G). Logan et al. (2002) studied with molasses as the substrate and baked soil as the culture, and found a maximum yield of 0.75 mol H_2/ mol glucose. Since molasses is a complex

substrate with potential insintric H_2 depleters in nature, the yields achieved in the dark fermentation studies performed with molasses as the substrate tend to be lower than the ones studied with sucrose or glucose, as expected.

4.1.2.2. Results of the VFA Analysis for Set-B

Table 4.7 displays the VFA results of Set-B. Final VFA concentrations of the reactors with higher initial COD values (30 and 50 g/L) are clearly higher than those of the reactors with lower initial COD values (10 g/L). Acetic acid was the most produced acid in almost all the reactors. On the other hand, for some of the reactors with high initial CODs (30 and 50 g/L), lactic acid concentration is almost as high as the acetic acid concentration. The highest acetic acid concentration was 115.7 mM (6942 mg/L) at the reactor with the initial COD concentration of 50 g/L, X_o of 500 mg/L and pH of 7. The reactors, which had acetic acid concentrations lower than 25 mM, had hydrogen yields above 1.5 mmol H_2/g COD. Whereas, at the reactor that had higher than 25 mM acetic acid concentration, the H_2 yield dropped as low as 0.46 mmol H_2/g COD (Table 4.5 and 4.7). Acetic and butyric acid concentrations higher than 50 and 63 mM, respectively, inhibit the H_2 production (Van Ginkel and Logan, 2005). Therefore, the H_2 production in the reactors with initial COD of 30 and 50 g/L might have been inhibited due to acetic acid concentration. High tVFA may also cause inhibition of H_2 production since dark fermentation is a highly product inhibited process (Saady, 2013).

Table 4.7 VFA types and concentrations for Set-B using molasses as the substrate

Initial			Acid types and final concentrations (mM)[a]					Total VFA (mM, HAc)[c]
COD (g/L)	X_o[b]	pH	Lactic	Formic	Acetic	Propionic	Butyric	
10	5000	4	2.2	2.3	22	2.3	10.1	35.2
30	2500	4	49.6	0.1	69	7.3	0.1	108.2
30	7500	4	43.5	2.3	67.5	4.5	0.2	103.3
50	5000	4	27.5	0.1	56.1	13.8	6.6	90.1
10	2500	5.5	1.9	3.4	15.9	2.7	6.6	28.2
10	7500	5.5	0.2	0.2	24	2.8	12	34.8
30	5000	5.5	24±18	2.4±2	37±12	4.5±2.5	25±7	77±22
50	2500	5.5	72.9	0.1	72.6	6.3	8.6	132.2
50	7500	5.5	95	6	106.4	11.5	20.6	200.9
10	5000	7	2.7	0	26.7	3.6	11.8	39.4
30	2500	7	63.4	7.4	36.6	3.9	14.5	101.5
30	7500	7	0.9	4.8	56.4	5.9	28.1	87.1
50	5000	7	122.7	9.5	115.7	6.4	27.4	233.7

[a] Molecular weights (g/mol) of the acids are; Lactic 90.08; Formic 46.03; Acetic 60.05; Propionic 74.08; iso-Butyric 88.11; Butyric 88.11; iso-Valeric 102.13.
[b] X_o: mg VSS/L

The molasses used in this study was analyzed for its sugar content. The results showed that molasses contained 760.38±1.06 g/L sucrose, 6.623±0.61 g/L fructose and no glucose (Table 3.4, Section 3.2). If sucrose were fermented according to the Eq. 4.6, 5.5 moles of H_2 would be produced per mol of sucrose.

$$C_{12}H_{22}O_{11} + 5/2\ H_2O \rightarrow 4CH_3COOH + 11/2\ H_2 + 4CO_2 \qquad \text{(Eq. 4.6)}$$

Therefore, at standard temperature and pressure (STP) conditions, dark fermentation of one mole of sucrose will result in 123.2 L H_2. For example for

the reactor type with the highest yield of 2.88 mmol H_2/ g COD, total H_2 production of 97 mL and the conditions initial 10 g/L of COD, 5000 mg/L VSS and pH of 4 (Table 4.5), the initially added molasses corresponds to 0.00278 mol of sucrose (calculation presented in APPENDIX H). This amount of sucrose is expected to produce 0.01529 moles of H_2 (0.00278 mole x 5.5 mole H_2/mole sucrose) under the complete fermentation condition, when there is no H_2 depletion. This corresponds to 342.3 mL of H_2 under STP conditions which is much larger than the actually produced amount of 97 mL. Considering all sucrose was depleted, the low amount of H_2 gas measured (only 31% of the theoretical-stoichiometric value) was attributed to the homoacetogenesis. In other words, the produced gas, that is H_2 and CO_2, might have been depleted via homoacetogenesis. If the produced H_2 is used for the homoacetogenic pathway, Eq. 4.7 will take place.

$$4H_2 + 2CO_2 \rightarrow CH_3COOH + 2H_2O \qquad \text{(Eq. 4.7)}$$

Therefore, 1.375 moles of homoacetogenic acetic acid production would be observed per mole of sucrose if all produced H_2 (from sucrose) were used for homoacetogenic activity. In addition to the 4 moles of acetic acid produced during the initial fermentation, the total moles of acetic acid produced will add up to 5.375 moles of acetic acid. For the reactor with the highest yield of 2.88 mmol H_2/ g COD, initial conditions of 10 g/L of COD, 5000 mg/L VSS and pH of 4 (Table 4.5), the calculation above indicates 14.9 mM HAc to be produced (0.00278 mol sucrose x 5.375 mol HAc/mol sucrose. But the actual measured amount was 22 mM. This indicated an even higher rate of homoacetogenesis than expected, probably involving other types of VFAs. Therefore, as a result of homoacetogenesis, VFA concentration might increase unparallel to H_2 production (Saady, 2013).

This calculation accounts the inaccordance of the highest hydrogen producing reactors (COD concentration of 50 g/L, X_o of 5000 mg/L and pH of 7; COD

concentration of 50 g/L, X_o of 2500 mg/L and pH of 5.5) to have middle to low yielding (0.88 and 0.71, respectively) (Table 4.5) ones at the end of the experiments. The reactors with the stated conditions produced 148 and 118 mL H_2 (Table 4.5), respectively but their yields at the end were low. This is due to the fact that the produced H_2 to be depleted as the operation proceeds, hence resulting in lower yields. These reactors also happen to have the highest acetic acid concentrations of 115.7 and 106.4 mM (Table 4.7), respectively, supporting the suspection of homoacetogenic activity domination in these reactors.

Lactic acid production is a known H_2-production-hindering pathway according to Eq. 1.4 (Section 2.2). Substrate inhibition is suspected to be the reason of the lactic acid production (Saady, 2013). Lactic acid producers are known to be favored at lower pH values like homoacetogens (Adamberg et al., 2003). The highest lactic acid producing reactors were the ones with initial COD concentration of 50 mg/L reactors, regardless of the initial X_o or pH value. The reactor with initial COD of 50 mg/L, X_o of 5000 mg/L and pH of 7 produced 122.7 mM of lactic acid where COD 50 mg/L, X_o of 7500 mg/L and pH of 5.5 reactors produced 95 mM of lactic acid (Table 4.7). Lactic acid producers were suspected to become dominant at latter stages of the fermentation, finding suitable environmental condition, due to decreased medium pH by the homoacetogenic VFA production.

VSS, TSS and soluble COD values for each reactor are presented in APPENDIX I. COD removal performance of Set-B reactors are not presented since COD removal was insignificant (<10%) like it was in Set-A.

4.2 Results of SBR Studies

The SBR experiments consist of 5 SBR studies. In other words, SBR has been re-started 5 times with fresh pre-treated inoculum and operated for a certain number of days and each start-up was defined as a SBR study (from 1 to 5). The

objective was to increase hydrogen production with each study, by modification of operational parameters (like pH and HRT) and in turn to find a maximized HY. In addition, as discussed in detail in upcoming pages, another objective became suppressing the homoacetogenic activity as it became an evident challenge upon achieving stable operation with high HY. The appearance of the reactor system (with feed pump, pH-stat and the gas collection system) on the very first day of SBR Studies was given in Figure 3.2 (Section 3.4.2.1).

4.2.1 Results of SBR Study-1: Application of pH=7, HRT=36 h and OLR=7.5 g COD/L.day

For SBR Study-1, the initial values of the operational parameters had been decided by Set-A. As mentioned before in Section 4.1.2, the results of Set-A indicated the optimum initial pH and biomass (VSS) values for hydrogen production from sucrose as 7 and 5000 mg/L, respectively. Therefore, the initial reactor VSS concentration was set as 5000 mg/L, while pH was adjusted to 7. Accordingly, the initial feed of sucrose solution applied was 10 g/L, providing a COD of 11.2 mg/L. The reactor was operated at an HRT of 36 h, cycle time of 24 h and OLR of 7.5 g COD/(L.day) for 23 days in this study. The results of the study have been presented in Figure 4.6.

In the first 6 days of operation, operational and technical difficulties such as pH control and gas collection problems were encountered. Figure 4.6 illustrates the data after these difficulties had been overcome (Zeroth day- Start-up time). The objective of this SBR study was to keep the reactor pH at 7.0±0.1 but rapid VFA production caused pH to go as low as 4.75 (Days 4-5, Figure 4.6-d). To achieve the target pH, the molarity of the NaOH solution which was used to regulate pH, was increased from 2M to 5M and the speed of the base pump was increased to overcome the fast decreasing pH. As a result, although decrease in pH till value of 5 was still observed, pH could be regulated and stabilized much faster within

a cycle. Yet, oscillations in pH still occurred for the following 17 days of operation (Figure 4.6-d).

In this SBR Study, the primary objective was to observe H_2 production. H_2 production was indeed observed, and maximum HY achieved was 0.38 mol H_2/ mol hexose (Figure 4.6-b). Maximum gas production per day was 8 L on Day 6 (Figure 4.6-a). H_2% measured in the headspace of the reactor oscillated from 1.97 to 17.9%, reaching the maximum 17.9% on Day 2 of the operation.

Yet, in addition to H_2 production, methane was observed during operation (Figure 4.6-a). For the days when pH dropped as low as 4.75, methane percentage in the headspace gas was 5% while it rose to 20% for the days when pH was 7 (Days 8-14). In parallel, hydrogen percentage decreased and reached zero at Days 9-11 (Figure 4.6-a). It was suspected that the methanogenesis observed was in fact hydrogenotropic and caused further depletion of the produced H_2. Despite the automated regulation, the reactor pH could not be stabilized at 7.0±0.1 and tended to stay around 5.5 (Figure 4.6-d). In adddition, it was observed that lower pH values better inhibited the methane production (Figure 4.6-a and d). Therefore it was concluded that pH 5.5 was more suitable for hydrogen production. pH for the hydrogen production was changed to 5.5 at Day 12 of the operation. After 36 h (1 HRT) after this change, hydrogen re-appeared in the headspace gas and methane percentages dropped. Headspace methane percentage stayed between 10-15 % for 3 HRTs (4.5 days) (Figure 4.6-a). However, methane production could not be inhibited. Therefore, on the 17th day, the SBR Study-1 was terminated.

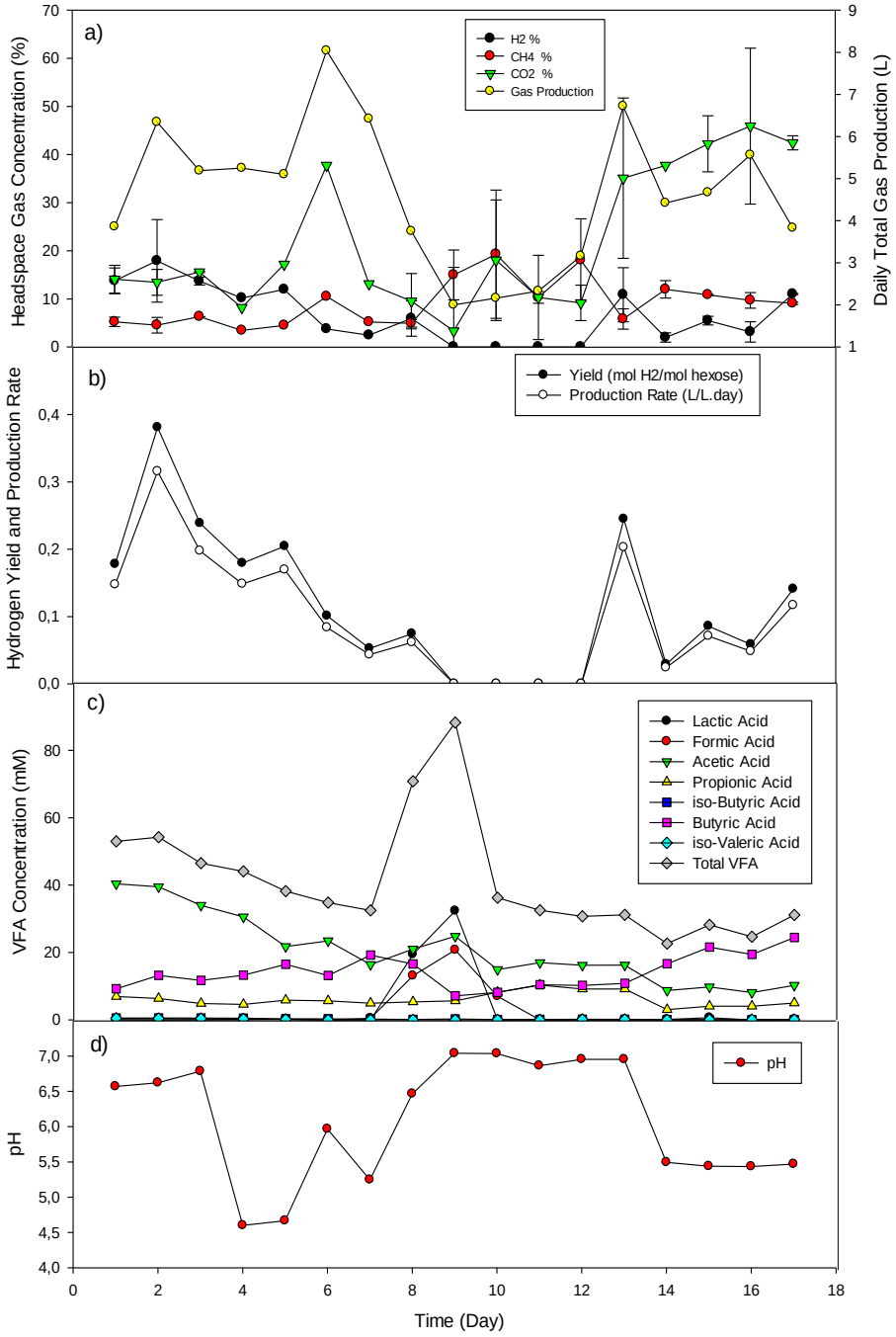


Figure 4.6 SBR Study-1, a) Headspace gas composition and the daily total gas production, b) H_2 production yield and rate, c) Effluent VFA concentrations, and d) Average pH values during the operational period

(HRT: 36 h; Cycle time: 24 h; OLR: 7.5 g COD/L.day; average influent COD: 11.2 g/L; average SRT: 14.5 day; T: 35^{o}C, Target pH: decreased from 7 to 5.5)

In SBR Study-1 HY value changed between 0.05-0.38 mol H_2/mol hexose (Figure 4.6-b). This value was lower than the commonly reported HY range (0.5-2.8 mol H_2/mol hexose) for mixed anaerobic cultures (Van Ginkel and Logan, 2005; Arooj et al., 2008b). HPR was between 0.049-0.310 L H_2/ (L_{rxr}.day). This value was also lower than the rates (0.5-6.7 L/ (L_{rxr}.day)) achieved in similar studies (Badiei et al., 2011; Wu et al., 2009). The lower HY and HPR were attributed to activation and the resulting domination of methane producers due to suitable pH (pH of 7).

The color of the reactor medium was significantly different between the beginning and the end of the SBR Study-1. At the beginning, it was dark, as it was expected to be due to the color of the seed sludge, but as the study preceded it become paler. This was attributed to a change in the dominant microbial community in the reactor medium. This was further discussed in Section 4.2.2.

During this operational period VFAs produced in the highest amounts were acetic and butyric acids (Figure 4.6-c). Acetic acid amount changed between 10-40 mM and butyric acid changed between 20-30 mM. Between Days 7-10, a sudden peak in the lactic acid concentration (32 mM/cycle) (Figure 4.6-c) was observed. Lactic acid production is a known obstacle for hydrogen production (Eq.1.4, Section 2.2). Therefore, peak lactic acid production explains the lack of hydrogen production in the following days. Another hydrogen depletion reaction is the hydrogenotrophic methane production from H_2 and CO_2 (Eq. 2.3, Section 2.5). As previously mentioned, the increase in methane production was well corresponded to the decrease in H_2 production. Therefore, the low yield results obtained in this study might be related to the depletion of hydrogen by methanogens. It is well known that the optimum pH for methane production is 6.8-8.3 (Speece, 1996). But, in this study, methane production was observed at pH values as low as 5.5. It is hypothesized that this might be due to the selection and the resulting dominance of a particular type of methane producer, resistant

to low pH and high VFA concentration. This issue was further investigated in the results of the following SBR Studies.

At the end of the SBR Study-1, it was decided to change operational pH to 5.5 to better inhibit methanogens, increase hydrogen percentage in the headspace gas and to achieve a more stable operation.

4.2.2 Results of SBR-Study-2: Application of pH=5.5, HRT=36 h and OLR=7.5 g COD/L.day

Figure 4.7 illustrates HY, HPR, effluent VFA concentration, operational pH values of the SBR Study-2. In the first day of the operation higher HY (1.88 mol H_2/mol hexose) and H_2 percent in the headspace gas (41 %) were observed compared to those of the SBR Study-1. But just like it was in SBR Study-1, high H_2 yield did not persist. Reactor pH changed between 5.3 and 3.4 from Day 5 to 20 of the operation, while H_2 percent in the headspace gas and the yield decreased and faded on Day 9. Similary, peak HY lasting for 2 HRTs and following radical decrease were also observed in SBR Study-1 (Figure 4.6-b).

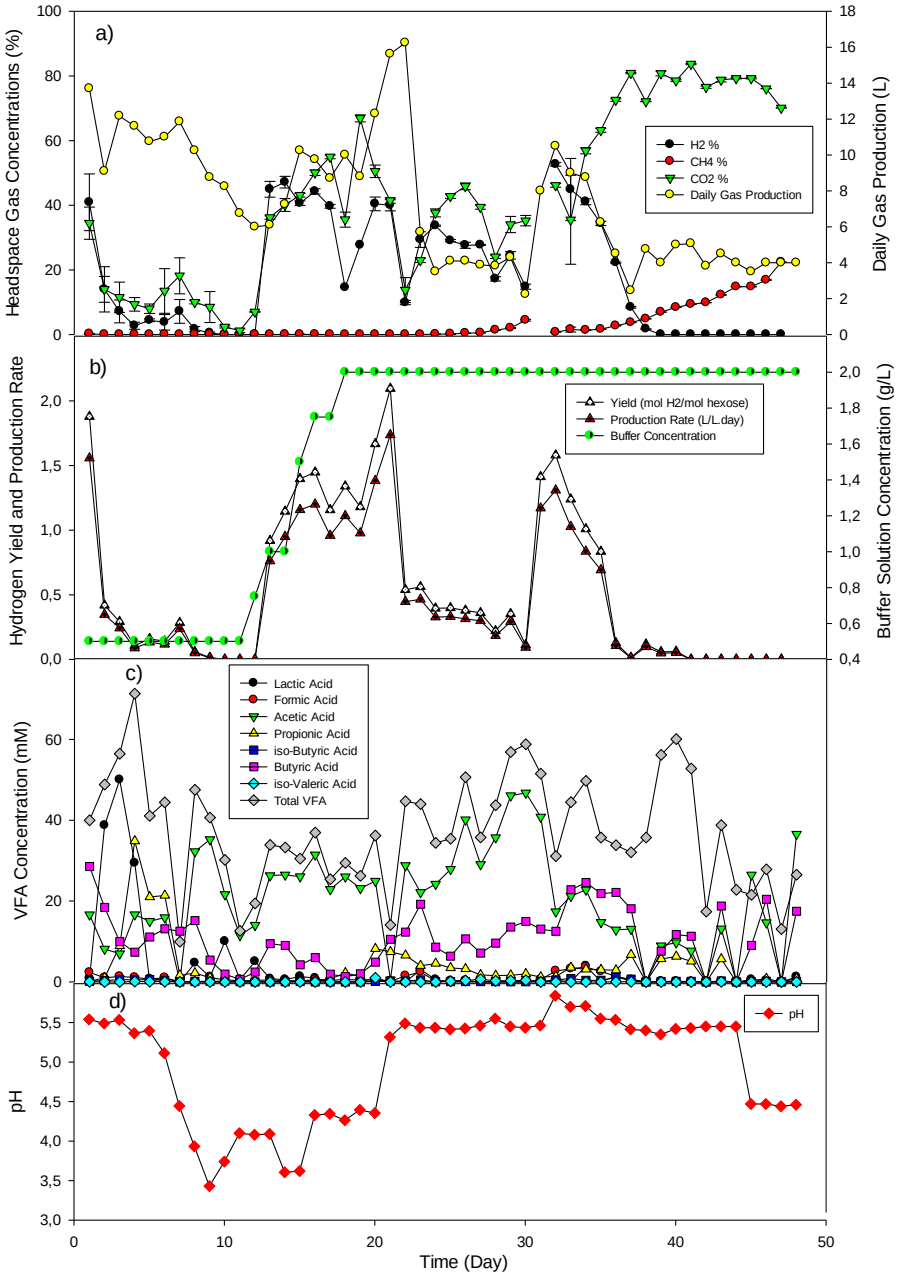


Figure 4.7 SBR Study-2 a) Headspace gas composition and the daily total gas production, b) H_2 production yield, rate and buffer solution concentration, c) Effluent VFA concentrations, and d) Average pH values during the operational period

(HRT: 36 h; Cycle time: 24 h; OLR: 7.5 g COD/L.day; average influent COD: 11.2 g/L; average SRT: 9.8 day; T: 35^{o}C, Target pH: decreased from 7 to 5.5)

There were pronounced stability problems during the first days of SBR Study-2, in terms of pH. Initially, manual addition of concentrated acid was implemented to overcome the problem. But as Figure 4.7 illustrates that these interventations done to bring pH to the desired 5.5 value were not successful enough. Therefore, it was decided to gradually increase the buffer solution concentration (alkalinity) (Figure 4.7-b). This modification performed on Day 12 of operation, increased the H_2 yield (from 0 to 0.91 mol H_2/mol hexose) and percentages (from 0 to 41 %) significantly but reactor pH stabilization at 5.5 was still not achieved. The desired pH was stabilized at 5.5 only when the buffer concentration was increased 4 times (KH_2PO_4 and K_2HPO_4, both at 2 g/L) and applied as long as 2 HRTs (72 h) on Day 21 (Figure 4.7-d). From Day 12 to 21, where pH oscillated between 3.6 and 5.3, the highest yield observed so far in the SBR studies was achieved (2.1 mol H_2/mol hexose). pH was stabilized at 5.5 on Day 21, but stabilization of the pH does not necessarily mean the H_2 production ability of the culture was restored as well. In fact, the culture may already become enriched with H_2 depleters. As a matter of fact, the H_2 percent in the headspace gas changed between 40-10 % and yield decreased from 0.54 to 0.11 mol H_2/ mol hexose from Day 21 to 29.

Between Days 15-22, pH, H_2% in headspace and gas production was relatively stabile; therefore, a kinetic study was made to better understand the chemical changes and progress of the operation within a cycle. Periodic samples were taken from the reactor medium and analyzed on Day 22 (Cycle No: 22) of the SBR Study-2.

The results of the kinetic study are presented in Figure 4.8. Figure 4.8-a show, no methane production within the cycle in a period of 24 h. H_2 production increased after the 7^{th} h and VFA production increased accordingly. After the H_2 concentration reached 30 % at the 12^{th} h, H_2 and CO_2 percent started to decrease and reached 10-14% at the end of the cycle. This decrease observed after the 12^{th}

h of the cycle was associated with H_2 depletion mechanisms, especially with homoacetogenesis. In accordance with this activity, VFA concentration continued to increase after the 12^{th} h and total VFA reached 47 mM at the end of the cycle. The reactions responsible for production and depletion of H_2 and production of VFAs during dark fermentation have been listed in Section 2.2 (Eq.1.1-1.5). It is expected in dark fermentation that most VFAs produced to be acetic acid (Eq. 1.1) followed by butyric acid (Eq. 1.2); which was also observed during kinetic study (Figure 4.8-c). The acid that is known to hinder H_2 production (Arooj, 2008a; Guo et al., 2010) is lactic acid (Eq.1.3) and its production was observed in this cycle. Propionic acid (Eq. 1.4) was produced in low amounts towards the end of the cycle. As a result, at the end of the kinetic study, a yield of 0.54 mol H_2/mol hexose and a rate of 0.45 L/L.day were achieved under the operational conditions of 36 h of HRT, 24 h of cycle time, 7.5 g COD/ (L.day) of OLR and pH of 5.5. VFA production rate of 1.3 mM/h, 16.2 L of total and 1.6 L of H_2 gas production were obtained. Decrease in the headspace concentrations of H_2 and CO_2 were associated with homoacetogenic activity becoming dominant towards the end of the cycle. On the other hand, the concentration of O_2 in the headspace gas was 23% on the average. This was probably due to air leakage into the reactor with the gas depletion due to homoacetogenesis. As a matter of fact, the N_2 concentration started get diluted with H_2-CO_2 production until the 12^{th} h operation but then (in line with H_2-CO_2 depletion) increased to 57 %. Due to O_2 presence in the analyzed gas, a second kinetic study, where suction and in turn O2 intrusion woud not be experienced, was planned for the following studies.

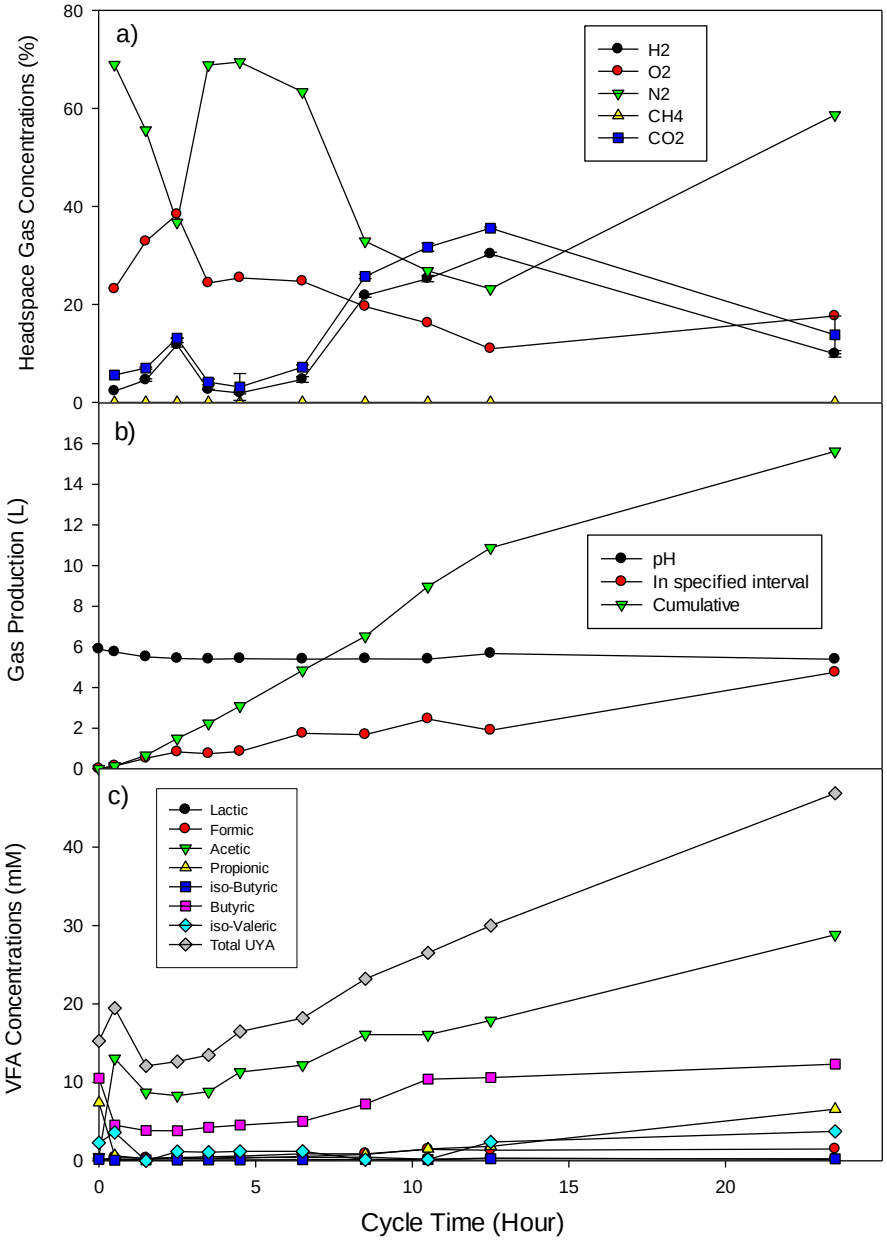


Figure 4.8 The kinetic study performed at Day 22 for SBR Study-2, a) Headspace gas composition, b) the daily total gas production and pH, c) Effluent VFA concentrations changes with respect to time

(HRT: 36 h; Cycle time: 24 h; OLR: 7.5 g COD/L.day; average influent COD: 11.2 g/L; average SRT: 9.8 day; T: 35^{o}C, pH: 5.5)

On Day 30 of the operation, due to mechanical problems observed at the reactor bottom, the reactor was opened, emptied and after the problem was eliminated (the bottom part was changed), the reactor was reestablished, purged with N_2 and closed without any other change. In addition, any problem (such as O_2 intrusion) that may be resulting from the gas collection part was eliminated. After this change, the headspace H_2 content suddenly increased from 18-20% to 40-52% and the yield increased to 1.58 mol/mol hexose (Figure 4.7-a and b). Although H_2 yield decreased after Day 32, it stayed between 0.8-1.3 mol H_2/mol hexose for 4 cycles (days) and then eventually dropped to zero on Day 41. Methane content in the headspace gas was in trace amounts on Day 32; but it increased gradually and reached 12% by Day 41. To repress methane production which was persistant at even pH 5.5, pH was further decreased to 4.5 via manual addition of 5M NaOH (Figure 4.7-d). But this intervention was not successful and methane percentage continued to increase. It was suspected that a specific methanogenic species, resistant to high VFA concentrations, became dominant after reactor got under homoacetogenesis effect. A further pH change to re-select hydrogen producers would not have been successful because lower pHs are known to trigger the high alcohol production (Jung et al., 2011). According to Jung et al. (2011), when in-cell NAD and NADH ratios are considered, the acetate-ethanol type fermentation observed at pH 4.5 is more stable than acetate-butyrate type fermentation observed at pH 5-7. Therefore the tendency of reactor pH values to drop below the desired pH values (7 and 5.5) might be explained by the cultures' selection of more stable fermentation type (Figure 4.6 and 4.7). In addition, pH might have changed according to the dominant fermentation type. For example, during lactic acid production, produced H^+ decreases the medium pH (Equations 1.4c and d). Also, it is well known that lactic acid bacteria tolerate low pH values (pH< 5) well and acclimate to those low pHs (Adamberg et al., 2003). After Day 36 of the operation, CO_2 and CH_4 production was observed exclusively; therefore, it was suspected that all H_2 producers might

have been washed out of the reactor and homoacetogenesis dominated all possible pathways (Eq. 2.1, Section 2.3.4.1).

It is evident in Figure 4.7 that VFA concentrations and compositions oscillate during the 48-day-operation. During the first 4 days of operation, lactic and propionic acids reached their highest production amounts (50 and 35 mM, respectively), then gradually decreased to zero at 5^{th} and 7^{th} days, respectively. Since these acids are known inhibitors of dark fermentative hydrogen production, the decrease in hydrogen production on Day 2 can be associated with high amount of lactic and propionic acids production from Days 2 to 7 (Figure 4.7-c and d). As stated before, alkalinity provided to the reactor was increased on Day 12 and HY increased in line. This situation was explained by to the inhibition of lactic and propionic acid production (Figure 4.7). As a matter of fact, on Days 13 to 20, no lactic and propionic acids were produced, and HY value increased from 0.9 to 1.7 moles H_2/mol hexose. Day 20 marked the reappearance of propionic acid (9 mM) and had the effect of decreasing the yield. Homoacetogenesis was suspected to be the main reason of the decrease in H_2 production on Days 22 to 30. Homoacetogenesis in dark fermentation may occur due to any stress condition for the operation (Saady, 2013). High H_2 partial pressure (>500 Pa) is known to trigger homoacetogenic activity (Demirer and Scherer, 2008). Therefore, high hydrogen production and H_2 content might have been the reason of homoacetogenic activity increase on Days 22 to 30, where HY and percent in headspace gas decreased but H_2 and CO_2 did not followed the same trend. CO_2 concentration percent increased and VFA concentrations (especially acetic acid, from 29 to 47 mM) increased.

As mentioned previously, opening the reactor to make the mechanical change at the bottom of the reactor on Day 30, caused a second production spike on Days 31 and 32 (Figure 4.7-a). This improvement, which happened without any chemical or operational change, was suspected to be due to the decrease in H_2

partial pressure in the system and the decrease in dissolved H_2 concentration in the medium. As a matter of fact, to re-establish anaerobic conditions in the reactor, the reactor was purged with N_2 and dissolved H_2 was flown. The washing of the reactor content and the reactor headspace periodically is a frequently used method to prevent hydrogen related inhibition (Hussy et al., 2003; Kraemer and Bagley, 2008).

In addition to homoacetogenesis, due to high CO_2 content (80%) observed especially after Day 23, it can be speculated that methane producers (Eq. 2.3, Section 2.5) and ethanol producers (Eq. 1.5, Section 2.2) might have dominated the reactor. As previously mentioned, HY and HPR started to decrease after Day 32 (Figure 4.7-b). The high hydrogen percent and partial pressure achieved on Day 32 might have triggered the homoacetogenic activity. After Day 32 as hydrogen percent decreased, CO_2 percent increased. This situation is explained, as Eq. 2.1 illustrates, by the unequal depletion of H_2 and CO_2. In addition to that, hydrogenetrophic methane production (Eq. 2.3) also caused unequal depletion of H_2 and CO_2. Hydrogenetrophic methane production might explain the CH_4 analyzed in the headspace gas. The presence of acetic acid consuming, CH_4 and CO_2 producing microorganisms might explain the unchanging acetic acid concentrations on Days 22-30 despite the potential homoacetogenesis occurred (which is normally expected to increase the acetic acid concentration). High CO_2 percents (70-80%) could also be related to ethanol production (Eq. 1.5). Ethanol producers are usually dominant at low pHs ($pH \leq 4.5$) (Van Ginkel and Logan, 2005); therefore, ethanol producers effective at pH 5.5, as might be the case in this SBR Study, should be further investigated. As a result, due to possible washout of hydrogen producers and possible domination of lactic acid producing and methane producing bacteria, SBR Study-2 was terminated on Day 48.

The results of the batch reactor studies performed with molasses (Section 4.1.2) indicated that, homoacetogenic activity might have occurred. Yet,

homoacetogenesis is not limited to batch reactors. Homoacetogenic activity is stated to be present in a number of continuous studies (Saady, 2013). Although homoacetogenic activity is underestimated in many studies, it occurs simultaneously and continuously, and it negatively affects the overall hydrogen production yield (Saady, 2013). Dinarmarca et al. (2012) concluded that hydrogen depletion rate can change between 4 to 62 mmol $H_2/L_{culture}$.day and could be affected by acetic acid concentration, hydrogen partial pressure, culture type and OLR. In addition, McInery and Byrant (1981) stated that the inhibition of methanogenic activity enhances the homoacetogenic or solventogenic activity. As a matter of fact, in almost all dark fermentation studies in the literature a method to inhibit methanogenic activity had been used (Section 2.5), which might have enhance homoacetogenic activity. As a result, because of the reasons stated above, homoacetogenic activity is suspected to have significant effect on the stability and the production yield of the SBR Study-2.

Arguably, the change in the dominating species, also affected the appearance of the reactor content. It was mentioned before in the results of SBR Study-1 (Section 4.2.1) that the color of the reactor showed dramatic change between the beginning and end of the SBR Study. This phenomenon was observed in the 2nd SBR Study as well and it occurred even more rapidly than it had before. In 72 h, reactors color went from black to brown, then brown to light grey to beige. In addition, the properties of the sludge within the reactor changed. At the beginning of the operation when the seed sludge was still fresh, the culture was more suspended, smaller in particle size and less settleable. At the later stages of the operation as hydrogen production started to decrease, the sludge became more settleable, lighter in color and larger in particle size. Change in sludge characteristics had a particular significance in the study by Van Ginkel and Logan (2005). They observed substantial flocculation at high feeding rates with HRTs in the range of 10 to 2.5 h. At lower HRTs this flocculation dispersed. They concluded that flocculation was present at glucose loading rates greater

than 3.8 g glucose/h and allowed high H_2 production rates and low residual glucose concentrations to be obtained. This result supports the fact that the nature, especially the floating abilities of the sludge strongly affects the H_2 productibility of the culture.

This difference in the appereance of the reactor content was presented in Figure 4.9. Further discussion on these observations was given in Section 4.2.6 of this thesis.

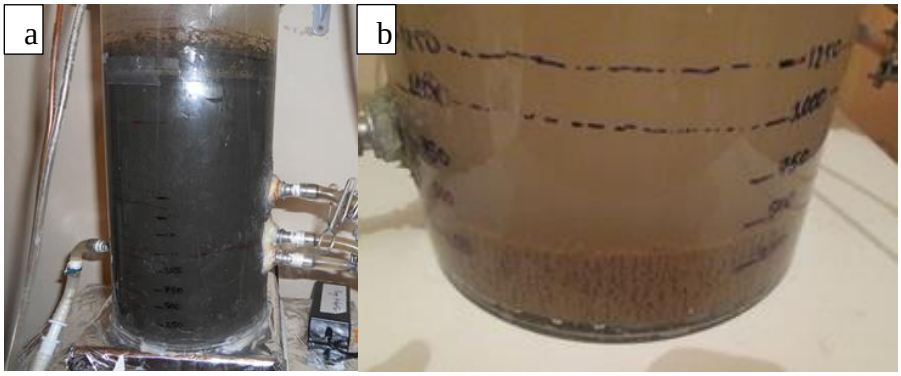


Figure 4.9 The difference between the a) initial and b) final appearences of the reactor content in SBR Study-2 (at the end of the settle phase)

SRT is an important parameter determining the sludge age (Eq. 4.8) and has effect on determination of the dominant microbial group in the reactor medium (Mariakakis et al., 2011). For example, it is known that higher SRTs (15-20 days) support methanogens and other hydrogen consumers (Saady, 2013). Interestingly, among the dark fermentation studies, only a couple of studies have focused specifically on the effect of SRT on the mesophilic, dark fermentative H_2 production. Kim et al. (2008) studied specifically the effect of SRT on dark fermentative hydrogen production in SBR and found 5.25 days as the optimum SRT for efficient H_2 production. But low SRTs may decrease operational parameters like substrate uptake efficiency and active biomass retention (Kim et al., 2008). Arooj et al. (2007) evaluated the influence of sludge withdrawal on

SRT and the change of dominant species, and concluded that non-settleable microorganisms have higher specific H_2 production than the easily settleable ones. They related the result of high SRTs relation with low H_2 yields with this finding. On the other hand, in a thermophilic fermentative hydrogen production study using an immobilized culture with high SRT, the hydrogen amount produced was 5 times the amount produced from a CSTR that had the one-fifth volume of the immobilized reactor (Keskin and Hallenbeck, 2012). In addition, it has been stated that immobilized cultures were more resistant to high OLRs than CSTR cultures. These two different opinions on the effect of SRT on H_2 production might be explained by the difference in the use of suspended or mobilized culture and with respect to studied operational parameters such as temperature, SRT, OLR, substrate properties, reactor type, etc.

$$SRT\ (day) = \frac{(V_{rxr}\ x\ VSS_{rxr})}{(Q_w\ x\ VSS_{effluent} + Q_{sample}\ x\ VSS_{rxr})}$$

(Eq. 4.8)

Where,

V_{rxr}, is the effective volume of the reactor (L)

VSS_{rxr}, is the VSS concentration of the reactor medium (mg/L)

Q_w, is the effluent (withdrawn) volume of the reactor perr each cycle (L/day)

$VSS_{effluent,}$ is the VSS concentration of the reactor effluent (mg/L)

Q_{sample}, is the effluent (withdrawn) volume of the reactor for sampling (L/day)

As stated before, HRT was selected to be the design parameter in SBR studies. It was not aimed to keep the SRT stable. But, nevertheless, the in-reactor VSS and the effluent VSS concentrations were analyzed and monitored daily. Considering the H_2 yield values and VSS analysis, the highest yields (0.2 – 1.88 mol H_2 /mol

hexose) were found to be achieved at the first 8 days of the operation when SRT was calculated as 6.8 days. The lowest yields, on the other hand, were achieved on Days 8 to 13, when average SRT was 17.8 days and yields changed between 0.014 - 0.059 mol H_2/mol hexose. Therefore, the suspicion driven from these findings was that high SRTs (>15 days) promoted the microorganisms other than hydrogen producers and negatively influenced hydrogen production. As a matter of fact, it is well known that SRT of 15 days is an optimum for methanogen promotion (Speece, 1996). SRT value of around 15 days to occur, between Days 8-13, is believed to activate the methanogens. When the alkalinity concentration and pH decrease were controlled and the hydrogen producers were probably supported, a large amount of sludge was naturally discharged from the reactor (data not presented). This decreased the average SRT to 8-9 days immediately and SRT of 8-9 days remained the same (i.e. the in-reactor and effluent VSS concentrations were the same) for the hydrogen producing days (Days 13 to 30). Therefore, the effort was to keep the SRT value at 8-9 days thereafter. This SRT is suspected to have positive effect on hydrogen production but not sufficient to wash-out homoacetogenic microorganisms. As a matter of fact, homoacetogenesis prevailed and prevented stable and long term hydrogen production yield, as previously mentioned (Days 30-38, Fig. 4.8-a and b).

Hydrogen depleting homoacetogens, acidogens and acetogens, have the same microbial yield coefficient for growth on H_2 (0.07 kg COD/ kg COD) (Ni et al., 2011). But since the decay rate of homoacetogens (0.015 day^{-1}) is smaller than that of acidogens and acetogens (0.02 day^{-1}), at relatively higher SRTs they have advantage over hydrogen producers (Ni et al., 2011). Lack of competition between homoacetogens and methanogens and high growth rate of homoacetogens (doubling time: 1.75-29 h), present opportunities for homoacetogens to become dominant at developed sequencing and/or high SRT systems (Saady, 2013).

All these discussions strengthen the homoacetogenesis dominance speculation for this SBR Study-2 and reveal the significance of SRT. As a result, although most SBR-H_2 producing studies do not attribute specific importance to SRT values (Wu et al., 2013; Won and Lau, 2011; Chen et al., 2009), it is evident that SRT should be used to reppress H_2 depleting microorganism and to strengthen H_2 producers. Therefore, it was decided to operate the latter SBR studies at lower SRT values and to investigate SRT effect on H_2 production.

On the other hand, HRT is a much more frequently studied parameter and has been reported as the most crucial parameter in achieving continuous high yield H_2 production. That's why, at the beginning of the SBR studies, it was chosen as one of the parameters (along with pH) to be investigated. In addition, from the results of SBR Study-1 and 2, it was suspected that 36 h HRT might be too long for the studied culture and substrate for dark fermentation operation; since optimum HRTs determined in most studies falls in the range of 8 to 16 h (Table 2.3, Section 2.3.4.1). According to Table 2.3 (Section 2.3.4.1), optimum HRT varies for different studies and operational conditions. HRTs between 4 to 96 h have been reported optimal for dark fermentative H_2 production (Section 2.3.4.1). But, homoacetogens may dominate the late stages of a sequencing operation and systems with high (>12) HRTs (Saady, 2013). A lower HRT was expected to have a better success at oppressing methanogens (Won and Lau, 2011) because, low pH values (pH 5.5) were observed to be not sufficient for this oppression (Figure 4.7-a and d). Therefore, lowering the HRT of the SBR operation was chosen as the next step in the sequence of SBR studies.

4.2.3 Results of SBR Study-3: Application of pH=5.5, HRT=12 h, OLR=22.4 g COD/L.day

The aim of this SBR Study was to investigate the effects of lower HRT (12 h) on dark fermentative H_2 production. To this purpose, HRT was set as 12 h, cycle time was set as 8 h and OLR was set as 22.4 g COD/ (L_{rxr}.day), accordingly. Experimental results are presented in Figure 4.10.

In SBR Study-3, reactor was operated for 10 days. Similar to the observations of the previous studies, an immediate increase in the H_2 production after the start-up, then a catastrophic fall in the H_2 production ability of the reactor was observed. The HY values fluctuated between 0.03 to 1.2 mol H_2/ mol hexose.

The pH was usually around 5.5 as Figure 4.10-d illustrates. Reactor with the operational conditions stated above had dramatic increase in the amount of biogas production per cycle. Along the 10 days of operation period, total gas production per cycle changed between 10 to 15 L (corresponding to 30 L daily, Figure 4.10-a); as opposed to the maximum daily total gas production of SBR Study-2 of 16 L (Section 4.2.1). The maximum yield of 1.2 mol H_2/ mol hexose was observed on the 2nd Day of operation of SBR Study-3. This value was compatible with the maximum yields of other continuous hydrogen production studies in literature using sucrose (Table 2.1). But, on the 4th day of the operation, base pump malfunctioned and pH was not controlled for 12 h, resulting in a decrease in reactor pH to 3.2. Before this problem occurred, the headspace hydrogen gas content was nearly 35% which decreased to 1%, as a results of the pH decrease (Figure 4.10-a). After the problem was solved and the pH was re-regulated back to 5.5, the hydrogen content did not improve. This result might be related to the inactivation of hydrogen producers at pHs lower than 4 (Saady, 2013). Additionally, it was also possible that, as observed in SBR Studies-1 and 2, low pH resistant and H_2-depleting microorganisms might have dominated/activated after the domination of homoacetogenesis. Since H_2

production no significant H_2 was observed after Day 5, SBR Study-2 was terminated at its 10^{th} day of operation.

No methane was detected during SBR Study-3. Because this operation was pre-terminated due to mechanical problems, it did not present a clear data/finding on the effect of HRT on hydrogen production. On the other hand, HRT effect on total gas production and methane oppression was clear. No methane was observed in headspace gas content and total gas production per cycle amount was almost 3 times that of SBR Study-2.

Results of SBR Study-3 and the previous experimental results, indicated a 12 h HRT to be more suitable in terms of high amount of total gas production and hydrogen content in the produced gas. Also, it was evident that combined effect of 12 h HRT and pH of 5.5, had a more successful effect on repressing methanogenic activity than only maintaining pH of 5.5.

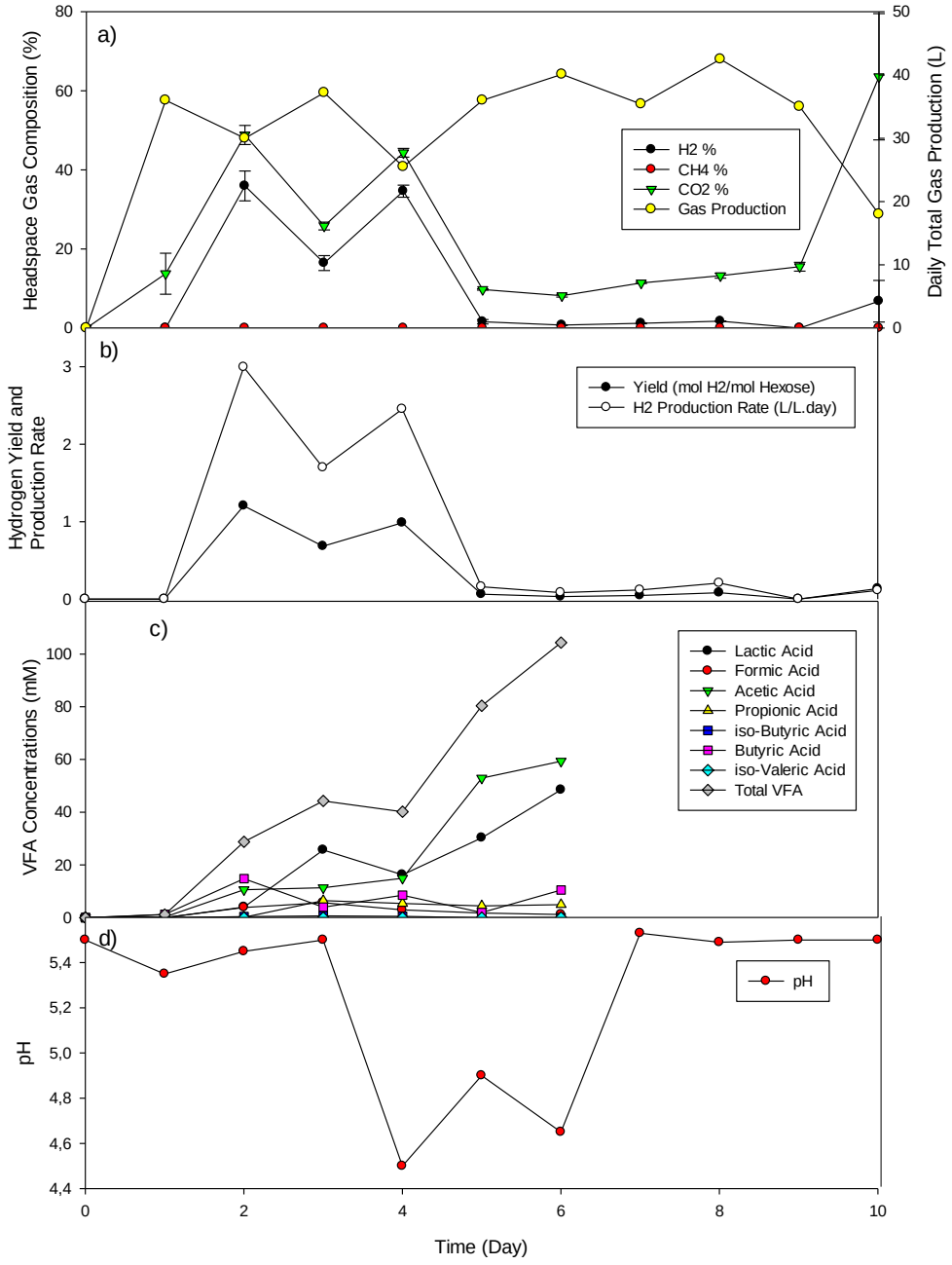


Figure 4.10 SBR Study-3, a) Headspace gas composition and the daily total gas production, b) H_2 production yield, rate and buffer solution concentration, c) Effluent VFA concentrations, and d) Average pH values during the operational period

(HRT: 12 h; Cycle time: 8 h; OLR: 22.4 g COD/L.day; average influent COD: 11.2 g/L; average SRT: 10.3 day; T: 35^{o}C, Target pH: 5.5)

4.2.4 Results of SBR Study-4: Application of pH=5.5, HRT=12 h, OLR=22.4 gCOD/L.day, SRT≈9 days

In the SBR Study-4 all the operational parameters (HRT, cycle time, OLR etc.) were kept as they were in SBR Study-3. HRT was 12 h, cycle time was 8 h and OLR was 22.4 g COD/ (L_{rxr}.day), accordingly. Reactor pH was set at 5.5. The major operational change, due to knowledge gathered from the previous studies, was the periodic removal of some reactor content to keep the reactor VSS, therefore the SRT value stable at 8-9 days. SBR was operated for 20 days. Maximum HY and HPR achieved in this study were, 2.52 mol H_2/mol hexose and 7.07 L/ (L.day), obtained on the third day of operation. The results of the SBR Study-4 are given in Figure 4.11.

Methane was observed until the last 4 days of operation (Figure 4.11-a). pH was oscillating between 5.2 to 5.6, reaching an average of 5.45 (Figure 4.11-d). In this study, the highest total gas production of 22 L/cycle, 8.5 L/cycle H_2; 66 L/day biogas daily, 25.5 L/day H_2 was observed (Figure 4.11-a). Just as was the case in the other SBR studies, H_2 percentage in the headspace gas and hydrogen production reached its peak value in 48 h (47%, 2.52 mol H_2/mol hexose) and then radically decreased. From the knowledge gained from previous studies, the objective was to keep SRT at around 9. Thus, after the 7th day of the operation, 300 mL reactor content was wasted daily to keep the reactor VSS and sludge age at desired values. However, stable reactor operation at the desired SRT could only be achieved after Day 11. As a result of the sludge removal to keep SRT constant, H_2 production did not diminish completely, after the first couple days of operation. HY even increased after Day 14 and reached 0.24 mol H_2/mol hexose and 17% H_2 on Day 18 (Figure 4.11-a and b).

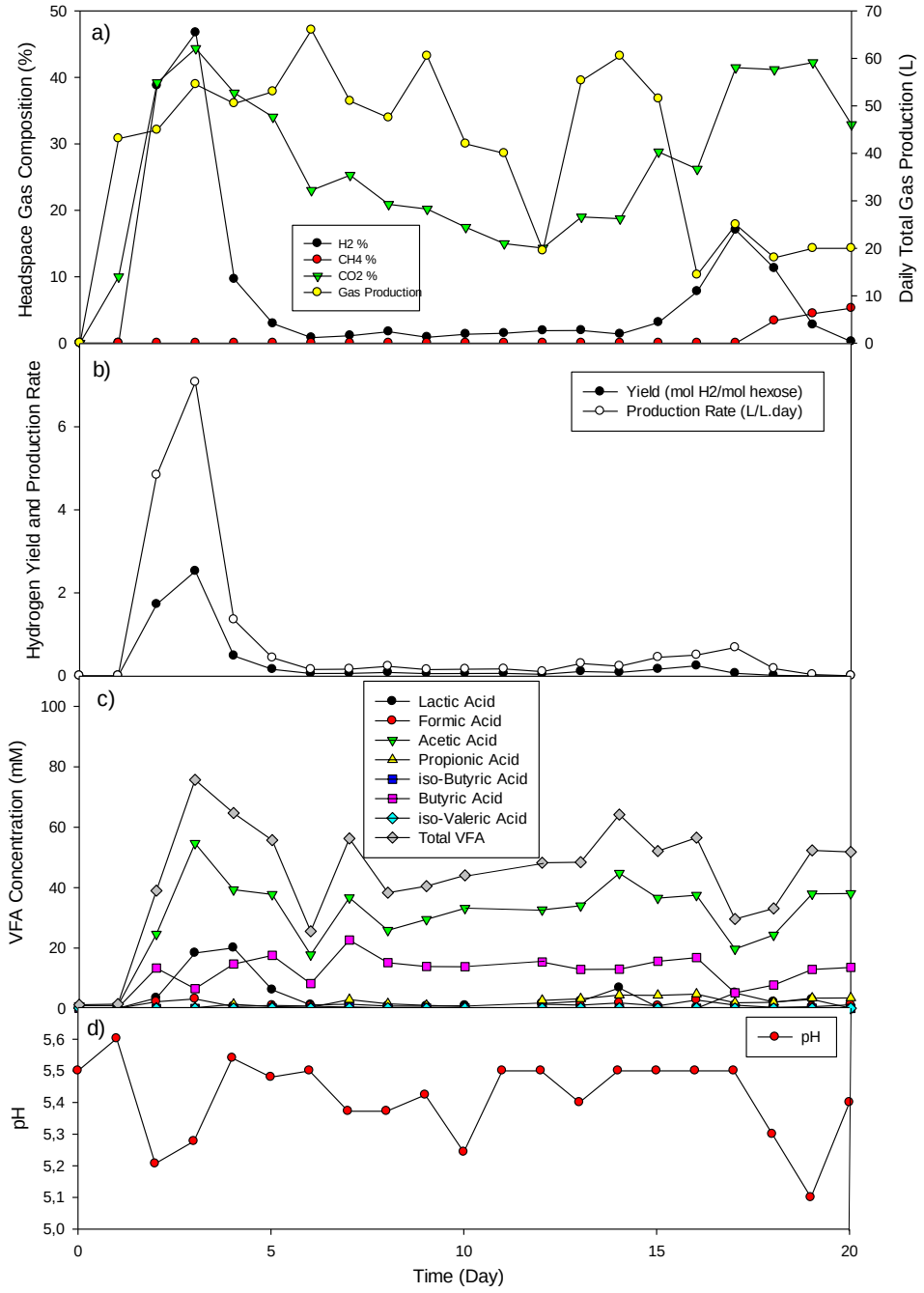


Figure 4.11 SBR Study-4, a) Headspace gas composition and the daily total gas production, b) H_2 production yield and rate, c) Effluent VFA concentrations, and d) Average pH values during the operational period

(HRT: 12 h; Cycle time: 8 h; OLR: 22.4 g COD/L.day; average influent COD: 11.2 g/L; average SRT: 9.5 day; T: 35^{o}C, Target pH: 5.5)

Trends of H_2 yield, productivity and pH observed in SBR Study-4 (Figure 4.11), were almost like the summary of observations of all the SBR studies: when the reactor was initiated with heat-treated fresh culture, system showed maximum hydrogen production, then, right after the hydrogen depleters were activated (due to high hydrogen pressure); hydrogen yield became zero and did not further respond to operational changes. This behaviour of dark fermentative SBR systems have been observed and reported by other researchers (Arooj et al., 2007; Dinamarca and Bakke, 2009; Badiei et al., 2011). The difference between the fourth and the other SBR studies was that, since periodic sludge removal was performed, hydrogen production did not completely vanish (Figure 4.11-a).

During SBR Study-4, a kinetic study was performed in order to better understand the H_2 production process during a cycle. To this purpose, between the days (Days 6-17) when pH, H_2 content of the headspace gas and gas production were relatively constant, on Day 13 (Cycle 37) samples were taken, periodically and analyzed. Figure 4.12 illustrates the results of this kinetic study. As seen in Figure 4.12-c, methane and H_2 were simultaneously observed during the kinetic study. Methane percentage reached 6% by the end of the cycle, where as H_2 reached 4% at the middle of the cycle than dropped to 1%. pH was oscillating between 5.1 and 5.6, achieving an average of 5.38 (Figure 4.12-b). CO_2 and tVFA amount increased strictly from the beginning to end of the cycle, reaching the values of 55% and 54 mM, respectively (Figure 4.12-a and c). Figure 4.12-c shows argon because in order to understand whether there was any leak or air entrance to the reactor, headspace gas had been washed with argon prior to the cycle. Figure 4.12-c shows the dilution of argon gas as the amount of produced gas increased, and therefore, the decrease in Argon content from 91 to 31% during the cycle. No change in N_2% (2.5 – 4%) ensured that there was no air leak in the system. CH_4 reached only 6 % at the end of the cycle. H_2 content stayed lower than 5%. But headspace H_2% increased after 100th minute (2nd h) and decreased after minute 280 (6th hour) of operation, eventually to 1.6%. VFA

production, especially acetic acid, accelerated in 3 phases (Figure 4.12-a). The first increase was observed after 130 minutes. The second increase observed in acetic acid after 300 minutes might be due to homoacetogenic H_2 depletion. End of cycle concentration for acetic acid was 53 mM. When Figure 4.12-a and b were observed together, effects of VFA production on pH decrease can be observed clearly. As pH increased with the automated control of pH-stat, CO_2 % also increased (200-400 minutes) along with the homoacetogenic activity increase. Most produced acids were acetic and butyric acids as expected. Lactic acid production that is known to have a negative effect on hydrogen production was observed at low levels (1-5 mM) for this cycle. Propionic acid reached 3 mM at the end of the cycle. As a result, at the end of the kinetic study, 55 L of total biogas and 1.1 L of H_2 were produced while, VFA production rate of 4.12 mM/h, HPR of 0.3 L/ (L.day) and HY of 0.105 mol H_2/mol hexose was achieved.

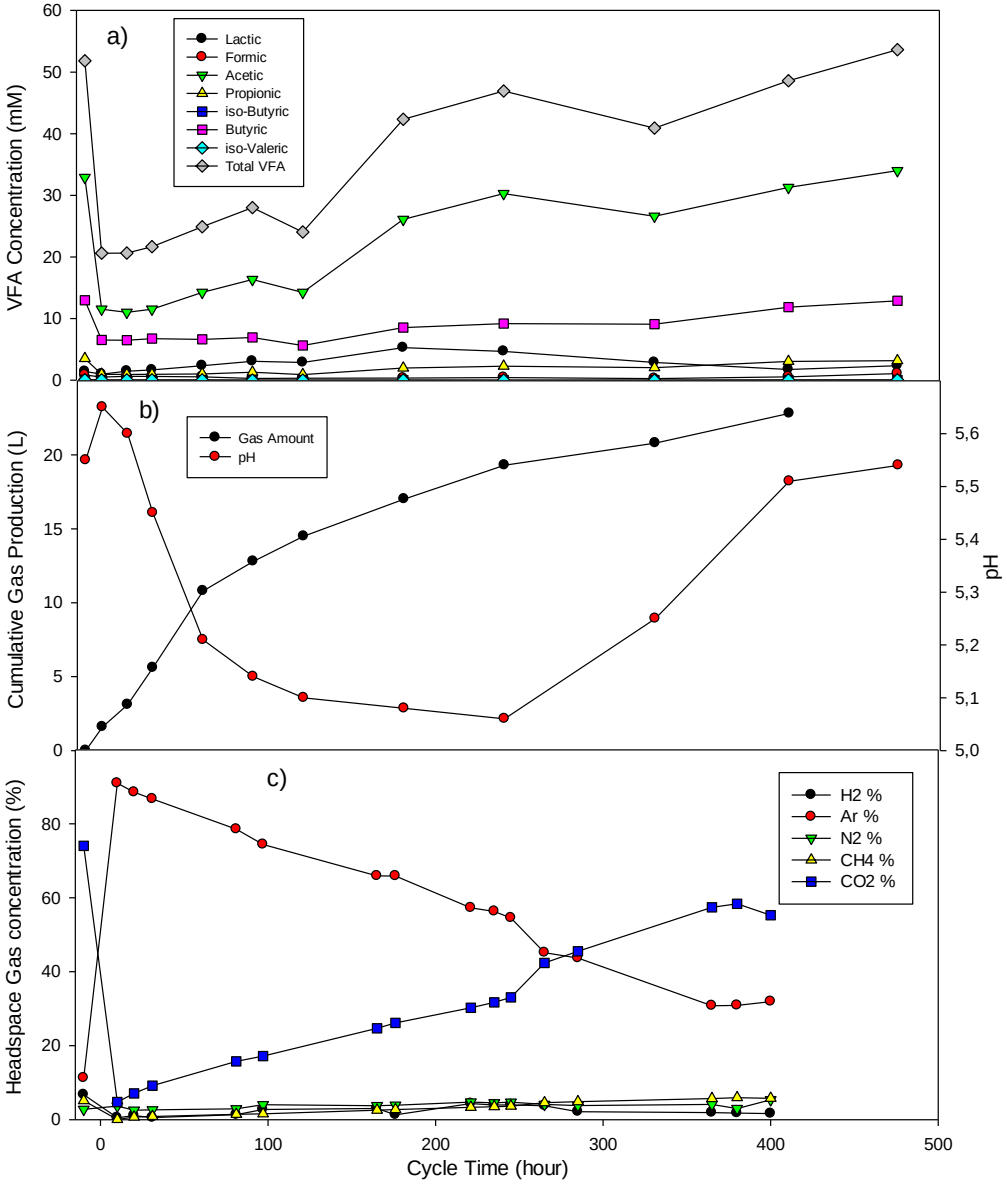


Figure 4.12 The kinetic study performed at Day 13 of SBR Study-4, the changes in a) VFA concentrations, b) the cumulative with respect to time gas production and pH, and c) Headspace gas composition

(HRT: 12 h; Cycle time: 8 h; OLR: 22.4 g COD/L.day; average influent COD: 11.2 g/L; T: 35^{o}C, pH: 5.5)

4.2.5 Results of SBR Study-5: Application of pH=5.5, HRT=12 h, OLR=22.4 gCOD/L.day, SRT≈4 days

In the SBR Study-5 all the operational parameters (HRT, cycle time, OLR etc.) were kept as they were in SBR Study-3 and 4. HRT was set as 12 h, cycle time was set as 8 h and OLR was set as 22.4 g COD/ (L_{rxr}.day), accordingly. Reactor pH was set at 5.5. The major operational change was the periodic removal of some reactor content to keep the reactor VSS constant and, in turn, the SRT value at 3-4 days. The objective of SBR Study-5 was to achieve a more stable operation rather than necessarily increasing the yield and rate of the operation, and observe the effect of lower SRT (3-4 days) on H_2 production.

SBR was operated for 34 days. Steep increase and then steep decrease in H_2 production was observed at the first few days of operation, as it was the case in the previous SBR studies. Making the reactor culture starve for a period of time re-promoted H_2 although long term stabilized production was not achieved. Maximum HY and HPR achieved in this study were 1.66 mol H_2/mol hexose and 4.64 L/ (L.day), obtained on the second day of operation. Total gas production amount changed between 6 to 35 L/day. The detailed results of the SBR Study-5 were given in Figure 4.13.

SBR Study-5, in terms of total biogas produced, was less efficient than the fourth. In SBR Study-5, the hydrogen percentage and yield reached their highest values (50.2 % and 1.67 mol H_2/mol hexose) in 48 h. This time, due to effords to keep the reactor VSS low and SRT value at 3-4 days, the dramatic drop of the reactor performance after 48 h had been hindered (Figure 4.13-a and b). The results of the previous studies led the efford to keep SRT at 3-4 days. As a result, at the first 7 days of the operation, SRT of the reactor was kept at an average value of 3 days. In the following days, the reactor VSS concentration tended to a steap increase (3 to 4.5 g/L of VSS), but due to the intense control, it was kept at an average of 4.2 days for the 34 days of operational period (Figure 4.13-d).

In addition, in this study, pH, which is one of problematic parameters to control due to its radical decrease at high VFA-producing periods, was kept strictly constant between 5.2 - 5.7 at the course of 34 day-operation (Figure 4.13-d).

The most significant problem that occurred in this operational period was the overflow of the reactor on Day 6 due to a malfunctioning valve. This overflow, resulting overload and sludge loss, suspected to be the reason of the yield decrease in Days 7-9 and, can be traced from Figure 4.13-b. Day 10 marked the recovery of the system and yield value reached 1.14 moles H_2/ mol hexose on Day 15. The main reason of this increase was associated to the starvation of the reactor for one cycle time on Day 13, as mentioned previously (Figure 4.13-b). In the previous studies, when there was a technical problem causing the reactor starvation for a certain time; it usually resulted in a sudden increase in reactor productivity. Therefore, after the reactor overflew and overloaded, starvation of the reactor seemed a logical interventation to counter the decrease in H_2 production. The reactor was not fed for a cycle (8 h), after taking the effluent. This intervention initially countered the decrease and increased the yield value; but from Day 15 to 22, hydrogen production gradually decreased again and vanished on Day 23 (Figure 4.13-b). This fail was associated to the uncontrollable increase in the reactor VSS concentration. The analysis conducted in this period indicated an increase in the VSS concentration at the rate of 0.375 to 0.5 g/ (L.h) for an 8 h operation. This rate was surprisingly high, indicating a high rate of microbial growth.

The next operational change made to control the SRT needed due to high microbial growth, was to change the settling time of the reactor from 40 minutes to 20 minutes for more sludge release. This intervention applied on Days 25 to 29 decreased the SRT but did not have an effect on increasing the hydrogen production (Figure 4.13-b). Therefore, another starvation period of 8 h was applied to the reactor on Day 29. Following the intervention, same observations

occurred as before. Yields initially improved (from 0 to 0.5 mol H_2 / mol hexose) for a short while and then reactor collapsed (H_2 production stopped) again. On Day 34, data collection from the reactor was stopped.

Lactic acid inhibition, observed in the previous SBR studies, was also present in SBR Study-5. Under the stress condition that occured after the overflow of the reactor, lactic acid production was initiated on Days 7-9 and as a result hindered the hydrogen production (Figure 4.13-c). In the previous SBR studies, the operational stress conditions providing lactic acid production was the pH decrease. For SBR Study-5, the pH decrease was prevented; but, the overload and the loss of sludge content must probably provide the stress condition and, lactic acid bacteria to thrieve.

In SBR Study-5, daily maximum tVFA and acetic acid concentrations reached 107 and 65 mM, respectively. tVFA and acetic acid concentrations measured were generally higher than the values measured in previous SBR studies. Maximum daily total VFA concentration of the SBR Study-5 was observed 48 h after the reactor overflow. The suspicion was that, the overload and following loss the sludge promoted acidogenic H_2 production; therefore, acetic acid concentration and total VFA concentration increased.

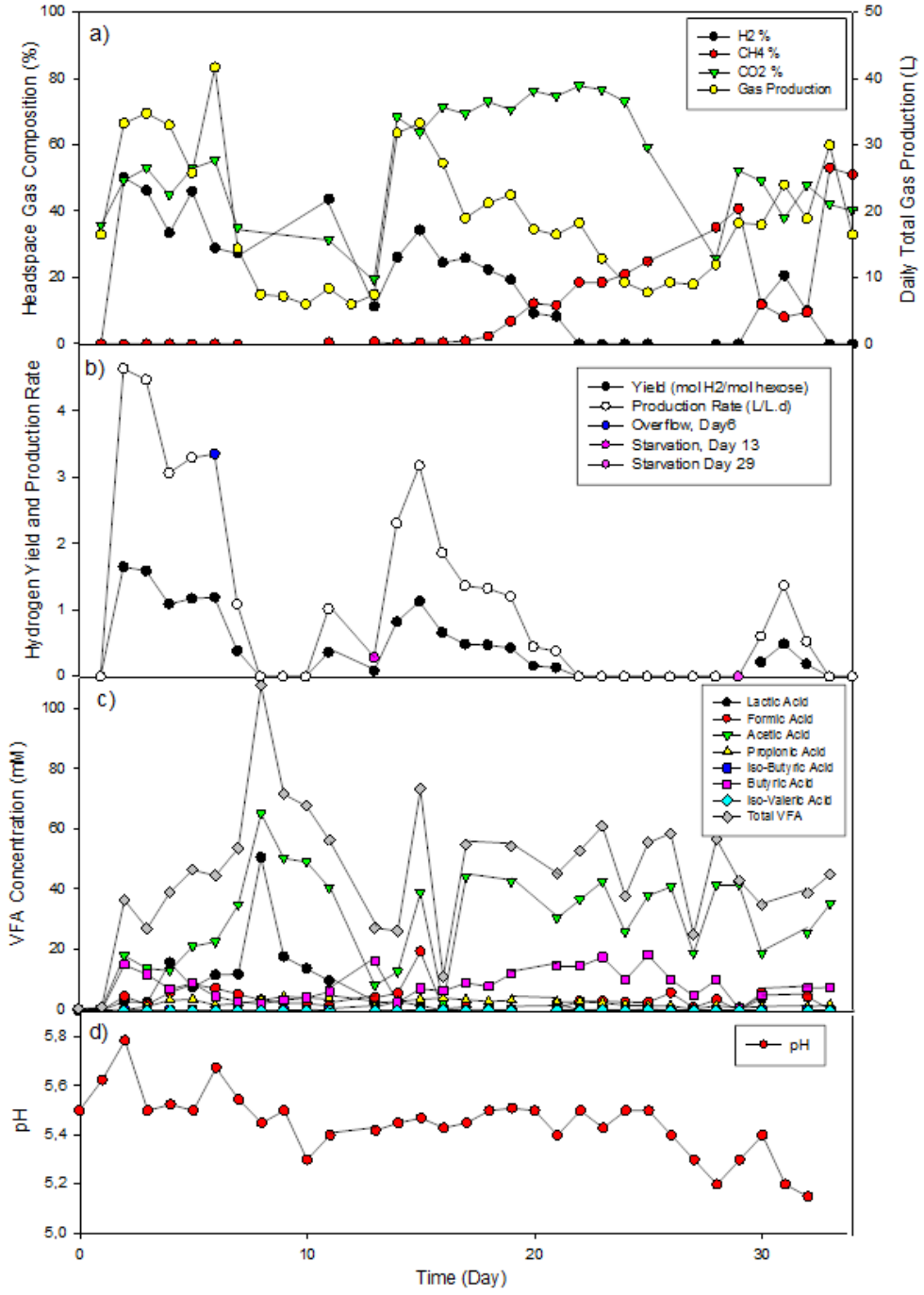


Figure 4.13 SBR Study-5, a) Headspace gas composition and the daily total gas production, b) H_2 production yield and rate, c) Effluent VFA concentrations, and d) Average daily pH values during the operational period

(HRT: 12 h; Cycle time: 8 h; OLR: 22.4 g COD/L.day; average influent COD: 11.2 g/L; average SRT 4.2 days; T: 35^{o}C, Target pH: 5.5)

SRT value providing the maximum hydrogen production may vary due to operational parameters (temperature, HRT, OLR, substrate type, reactor type etc.) (Section 2.3.4.2). Oh et al. (2003) stated that change in the H_2 yield due to SRT might be resultant of physiological change of bacteria or the change of dominant species resulting from SRT. SBR Study-5 was important for understanding the SRT effect that has been previously discussed in SBR Study-3 and 4. The H_2 yield decrease associated with high SRT (>9 day) values, mentioned in the previous operational periods, was also observed in SBR Study-5. This high SRT value differed significantly for different researches. Lee et al. (2010) suggested a metabolic change due to SRT, as it changed from 12.5 to 90 days might be significant; while Kim et al. (2008) mentioned this change to be significant from 3 to 5 days. It is speculated from the results of SBR Study-5 that SRT to occur above 5 days, results in a decrease in hydrogen yield and possibly a microbial change.

Tawfik and El-Qelish (2014) studied organic solid waste and kitchen wastewater for bio-hydrogen production and found out that SRT and solid dilution ratio to be the most significant parameters affecting the hydrogen yield. They studied SRT values of 3.6, 4 and 5.6 days and found the maximum hydrogen yield at SRT of 5.6 days. On the other hand, Oh et al. (2003) studied a membrane bio-reactor, and researched SRTs in the range of 5 to 48 h and found that SRT of 12 h supported the highest hydrogen yield. These results support the knowledge that, for different reactor types and production conditions, different SRT values may provide different hydrogen production abilities in the reactors.

4.2.6 Overall results of the SBR study and related discussions

The results of the SBR studies presented suggest that dark fermentative SBR systems are hard to stabilize in terms of high hydrogen yield and operational steadiness. Steady-state (or pseudo-steady state to be more accurate), at which period hydrogen percentage change within the consequtive periods stays lower

than 10% (Badiei et al., 2011), was hard to achieve. There are a number of studies that came up with the same finding (Dinamarca et al., 2011; Kraemer and Bagley, 2008). The results presented as long-term operational yield values in these studies usually represent the maximum yields achieved throughout the operational periods. Therefore, similar to these studies values presented in Table 4.8 were the maximum yields achieved in the SBR Studies 1 to 5.

Table 4.8 Summary of the dark fermentative hydrogen production studies performed with SBRs

SBR Study No	**Target pH**	**HRT (h)**	**Cycle Time (h)**	**OLR[a]**	**Max HY[b]**	**Max H_2 %**	**Operation Length (day)**	**Stability Period (cycle)**	**SRT_{ave}[c]**
1	7→5.5	36	24	7.5	0.38	17.9	17	5	14.5
2	5.5→4.5	36	24	7.5	2.10	52.7	48	5	9.8
3	5.5	12	8	22.4	2.30	36.0	10	3	10.3
4	5.5	12	8	22.4	2.52	46.7	20	3	9.5
5	5.5	12	8	22.4	1.66	50.2	34	6	4.2

[a] OLR, g COD/L.day
[b] Maximum H_2 yield, ml H_2/mol hexose
[c] SRT_{ave}, average SRT, day

Table 4.8 is the summary of the SBR studies conducted at the course of this thesis study. As Table 4.8 illustrates the highest hydrogen production yield achieved in this study was 2.52 mol H_2/ mol hexose at SBR Study-4. The conditions at which highest yield achieved were pH of 5.5, HRT of 12 h, cycle time of 8 h and OLR of 22.4 g COD/L.day. The yield values between 0.4 to 2.52 mol H_2/ mol hexose were similar to 0.5 - 2.8 mol H_2/mol hexose of the highest yielding continuous studies from literature (Arooj et al., 2008b; Van Ginkel and Logan, 2005). In the last SBR study, by decreasing the system SRT to 3-4 days, the stability of the system was tried to be increased. Although the maximum yield of the complete thesis study was not achieved it was important to understand the importance of SRT on dark fermentation operation.

Three phases were observed in all of the SBR studies. These phases can be summarized as follows;

Phase 1: First 2-3 days of the operation; high hydrogen production, high yield. Hydrogen production phase.

Phase 2: Beginning after Day 3 of the operation; homoacetogenesis domination and hydrogen yield drop. Hydrogen depletion phase.

Phase 3: After Day 5-7 of the operation; methanogenic activity domination. Hydrogen vanishes. Methane takes its place in headspace gas.

Although SBR operation could not be prevented from these phase changes keeping the SRT lower than 15 days stopped complete dissappearance of H_2, at SBR Study-4. With SBR Study-5, SRT effect on yield and stability was investigated, by further decreasing the SRT to 3-4 days. Although maximum hydrogen yield could not be increased with SRT decrease, the improvement in operational stability was clearly observed from the resulting graphs.

The appearance of the SBR was very significant in observing these changes. It was mentioned before in Sections 4.2.1 and 4.2.2 that, the color of the reactor medium changed significantly, indicating the potential change in dominant species. Figure 4.14 illustrates pictures of the reactor medium in consequtive days. Figure 4.14-a and b were taken the same day, Figure 4.14-c was taken 24 h after the start-up and Figure 4.14-d was taken 48 h later. The speed of the color change was quite in line with the steep increase and decrease of the hydrogen yield. It was mentioned before in Section 4.2.5 that the VSS concentration of the reactors increased at quite a high rate of 0.375 to 0.5 g/(L.h). Putting together these two pieces of information, it was speculated that this new color, a much paler one than the initial color of the reactor, presented the dominance of one microorganism type. This type was most probably a methanogen that become stronger at the presence of homoacetogenic environment and was resistant to

lower pHs than average methanogenic species. Further discussion of this subject undoubtfully required microbial analysis of the culture, but molecular studies of the sludge were not in the scope of this thesis.

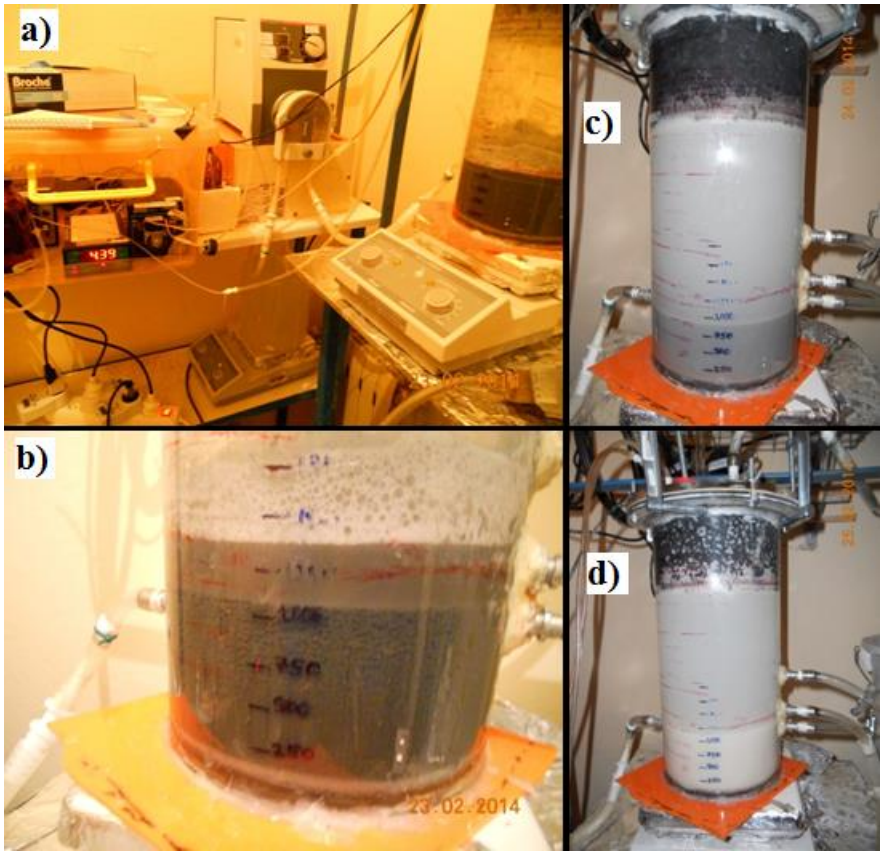


Figure 4.14 Appearance of the reactor medium going though the phase changes in consequtive days a) Phase-1, H_2 production phase; b) Phase-1, closer look of the culture; c) Phase-2, H_2 depletion phase, d) Phase-3, Dissappearence of H_2 and domination of methane in the headspace gas (Pictures are of SBR Study-4)

It can be said that, from the results of the SBR studies, operational pH, HRT and SRT were important parameters for hydrogen production in SBR systems. The rate of growth for acidogenic, homoacetogenic and low pH-resistant methanogenic bacteria should be studied in detail to find an optimum HRT and SRT for hydrogen production. Hydrogen production in SBR systems, as well as

continuous systems in general, should be further investigated for long term stable operation with high hydrogen yields.

CHAPTER 5

CONCLUSIONS AND RECOMMENDATIONS

The aim of this thesis study was to investigate dark fermentative hydrogen production from sucrose and molasses in batch reactors under different operational conditions and to investigate dark fermentative hydrogen production from sucrose in SBRs (by determining the effects of initial pH and HRT on hydrogen production) and to examine the effects of SRT on hydrogen production.

The starting point for this thesis was to investigate the key parameters and their optimal values for the substrate and the inoculum studied, to enhance stable high yielding dark fermentative hydrogen production. The objectives of this thesis study were to investigate; the effect of initial COD, VSS and pH in batch reactors using sucrose and molasses as substrates and the effect of pH, HRT and SRT for the specific substrate and inoculum used in SBR for achieving high yield, stabile hydrogen production in dark fermentation studies.

Batch reactor studies of this thesis confirmed the potential of dark fermentative hydrogen production from sucrose and molasses.

- Maximum HY and HPR values achieved were 2.3 mol H_2/ mol $sucrose_{added}$ (1.15 mol H_2/ mol $hexose_{added}$) and 10.6 mL H_2/ (L_{rxr}.h), respectively; at the batch reactor with the operational conditions of 10 g/L initial COD, S/X_o of 12 and pH of 7. This yield value was right in the middle of the range of the yield values reported by similar previous studies from the literature.

- The optimum conditions, leading to the maximum hydrogen production and yield, were successfully predicted with RSM in batch reactor studies conducted with sucrose (Set-A).
- Set-A results indicated that HY decreased as the initial COD increased from 10 to 50 g/L, while it increased as the initial pH value increased from 4 to 7.
- The studied S/X_o values of 4, 12 and 20 g COD/ g VSS had no effect on hydrogen production yield.
- Maximum HY and HPR achieved in Set-B (batch reactors conducted with molasses) were 2.88 mmol H_2/g COD and 8.26 mL H_2 / (L_{rxr}.h), respectively. Maximum HY was observed at the reactor with the operational conditions of 10 g/L initial COD, X_o of 5000 mg/L and pH of 4. Maximum HPR was observed at the reactor with the operational conditions of 10 g/L initial COD, X_o of 7500 mg/L and pH of 5.5.
- Set-B results showed that the change in HY and HPR could not be explained by the combination of all three variables (i.e. initial pH, COD and VSS values) and with the studied ranges.
- Initial VSS concentration in the reactors had no effect on hydrogen yield and productivity from molasses for the values studied (2.50, 5.0 and 7.5 g/L).
- The maximum HY and HPR values obtained in Set-B were found to be slightly lower than those found in previous studies in literature.
    - These results were attributed to the homoacetogenic activity and in turn its interference with the HY and HPR. This might be due to the substrate type since molasses, for containing potential intrinsic microorganism, might be more suitable to support and trigger the homoacetogenesis than sucrose.

SBR studies confirmed that the optimum pH was 5.5, unlike pH 7 found as optimum for batch reactor types, when the substrate was sucrose. For dark

fermentative SBRs, it is hard to achive a long-term stable operation. The physical interventions done on the reactor (like purging with N_2 during the operation) increases the hydrogen production significantly. It is suggested that after high H_2% in the headspace (around 40%), system was inhibited due to high H_2 partial pressure. This occurance is associated with the activation of homoacetogens, and pH 5.5-resistant methanogens at later stages and, in turn, resulting in low hydrogen yields. HPR decreases in the same trend but since yield was the main response to be maximized, yield was the main parameter for evaluation of the data.

The maximum H_2 yield achieved in SBR studies by optimization of the operational parameters was 2.52 mol/ mol hexose (13.11 mmol H_2/ g COD). This value is in the border of the highest yield defined so far for dark fermentative continuous reactor studies 2.8 mol H_2/mol hexose (Van Ginkel and Logan, 2005). This highest yield was achieved at pH of 5.5, HRT of 12 h, cycle time 8 of h, OLR of 22.4 gCOD/L.day and average SRT of 9.5 day. The HPR calculated at these operational conditions was 7.07 L H_2/ (L_{rxr}.day).

In general, dark fermentative hydrogen production is a high maintainance system requiring detailed control; very suddenly reacting positively or negatively. SRT value of the system is a very important parameter determining the hydrogen production potential and stability of the reactor. It directly affects the domination of hydrogen producers and wash-out of methanogens. Relatively short (3-5 days) SRT values provide a more stable reactor operation with respect to longer (9-15 days) SRT values. Therefore for SBR studies, even shorter SRTs might be investigated. Chemostat systems, equalizing SRT and HRT, might be studied for this purpose. Continuous or intermittent purging of the reactor might also be a topic of investigation for achieving higher yields as well as controlled starvation of the sludge culture to keep it under stress.

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APPENDIX A

CALIBRATION CURVES FOR THE GC ANALYSIS

In the all the graphs x-axis is the peak area calculated by the GC device and y-axis is the amount (moles) of the related gas. Then headspace percentages of the gases are calculated by dividing the moles of the gas by the total moles of the gas sample.

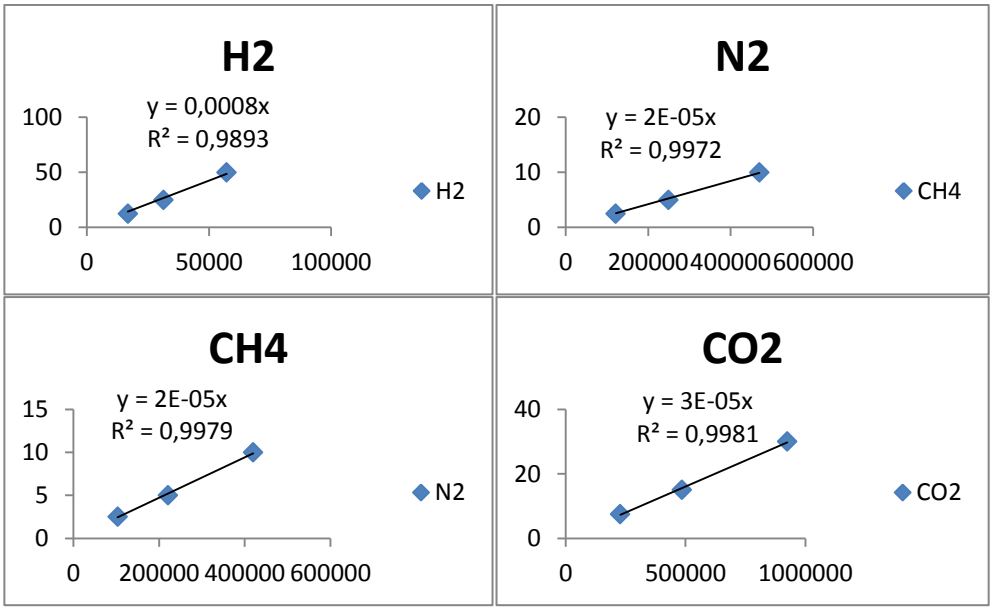

Figure A.1 Calibration curves used for headspace gas analysis of preliminary batch studies, Set-1 and Set-2

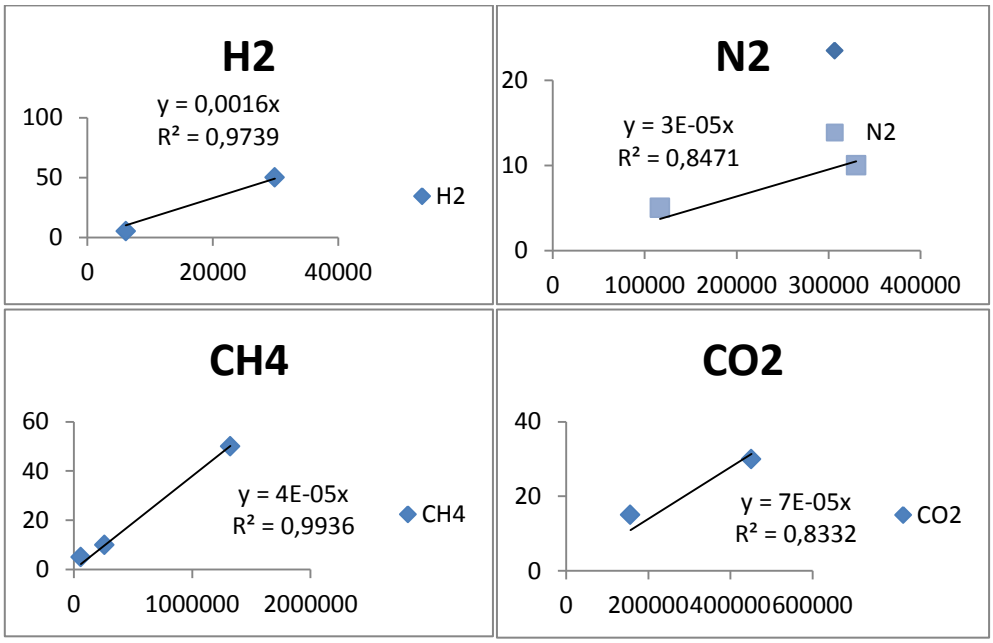

Figure A.2 Calibration curves used for headspace gas analysis of batch reactor studies, Set-A and Set-B

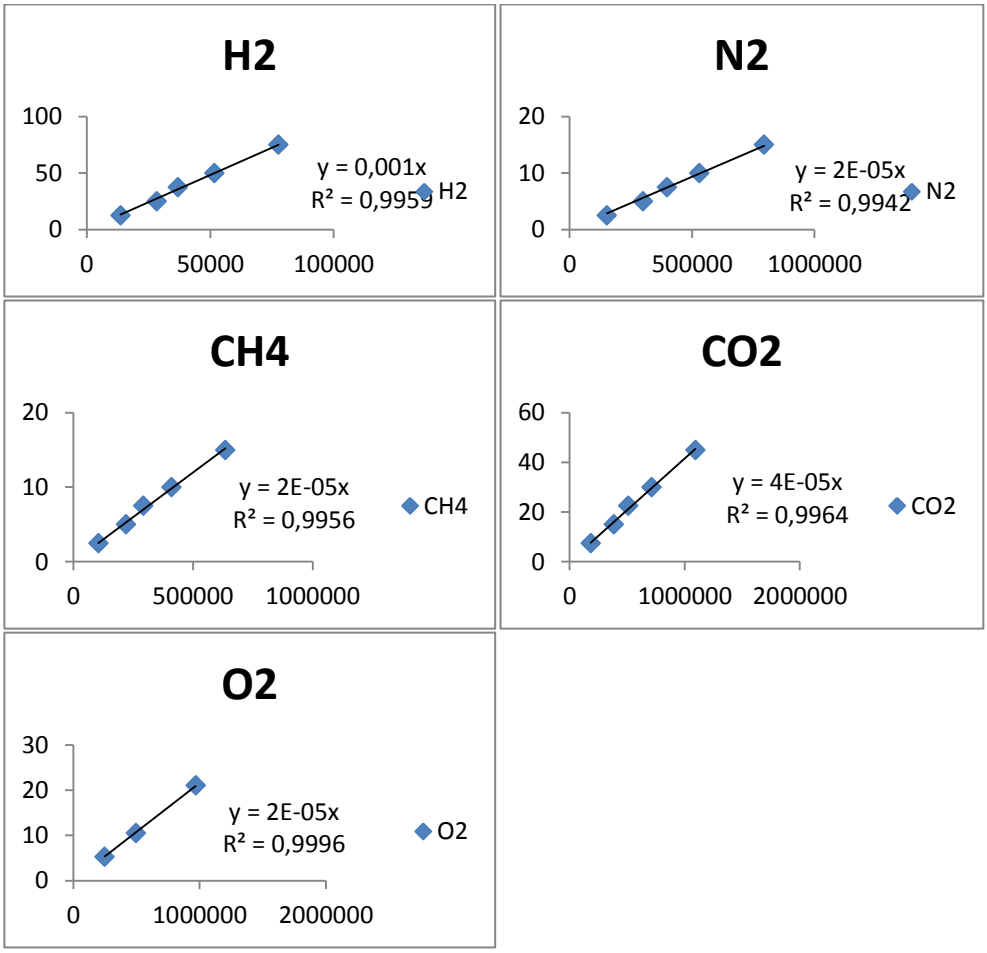

Figure A.3 Calibration curves used for headspace gas analysis of SBR Study-1

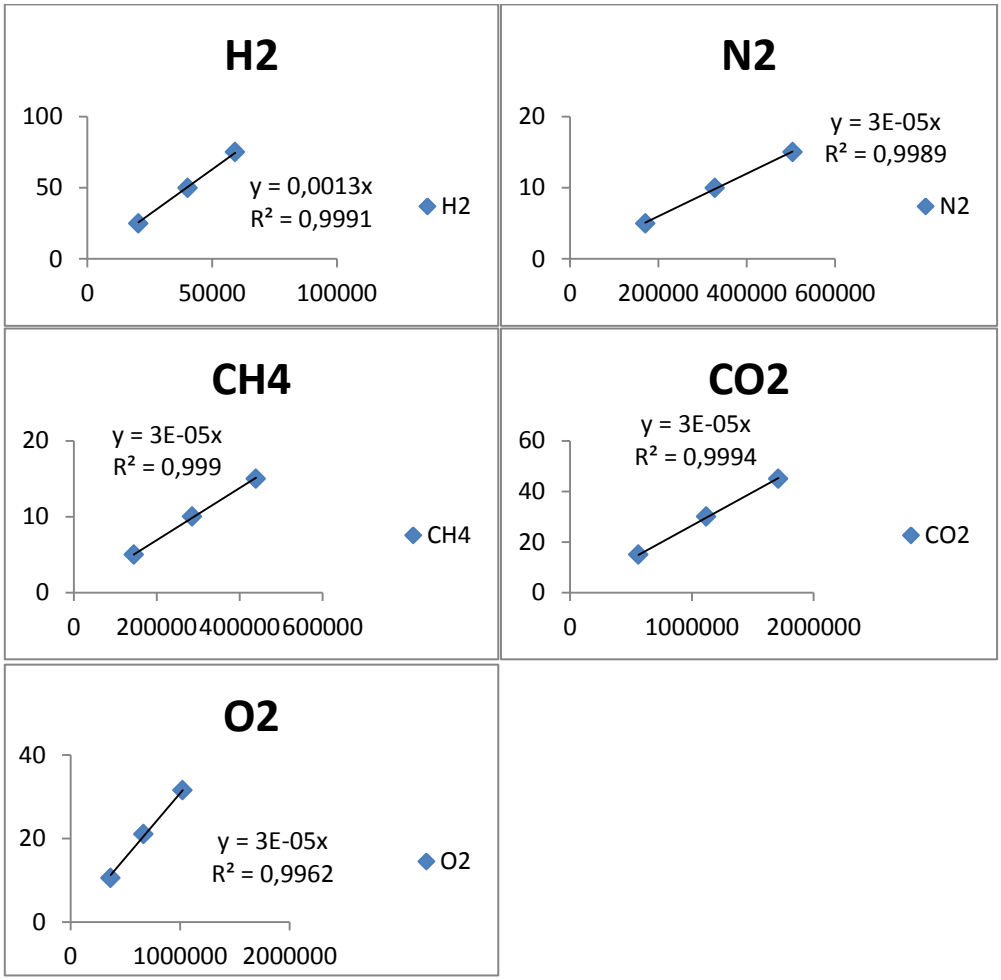

Figure A.4 Calibration curves used for headspace gas analysis of SBR Studies-2 and 3

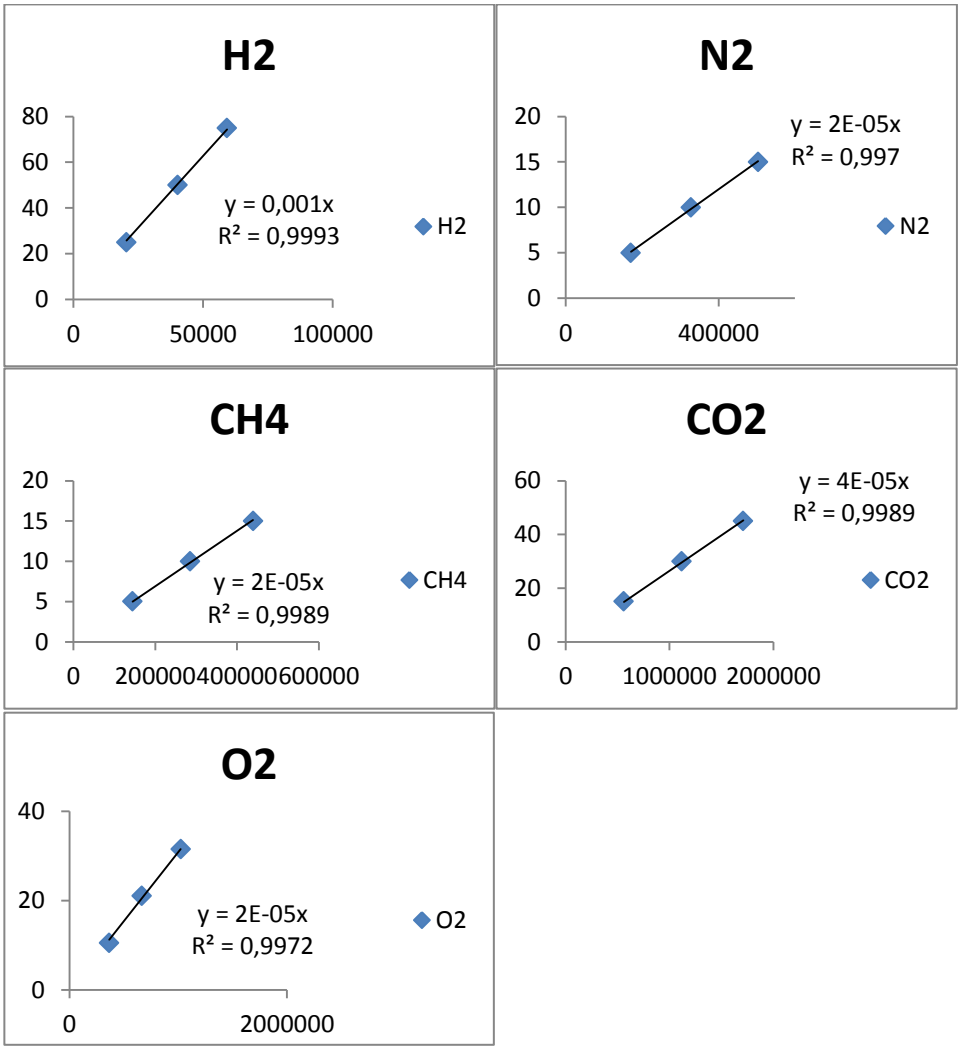

Figure A.5 Calibration curves used for headspace gas analysis of SBR Studies-4 and 5

APPENDIX B

HPLC CALIBRATION CURVES OF THE VFAS

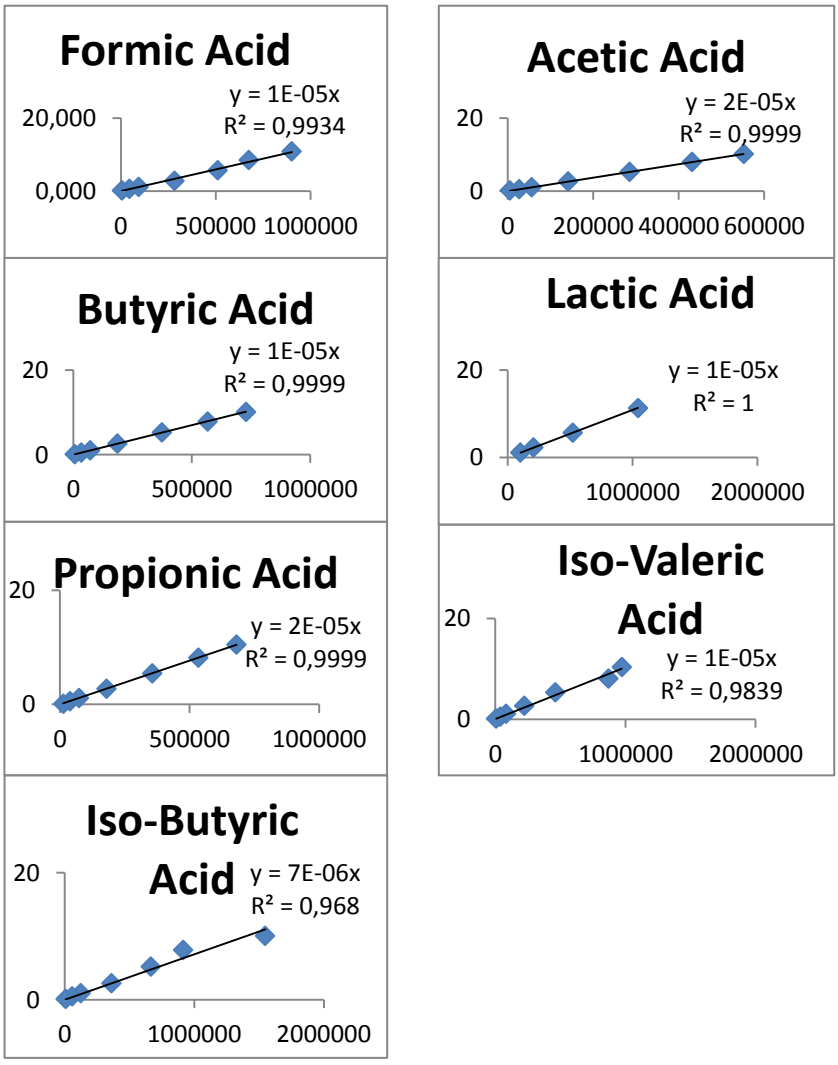

Figure A.6 HPLC Calibration curves used for VFAs (In all the graphs, x-axis is the peak area calculated by the HPLC device and y-axis is the concentration, in mM of the related acid)

APPENDIX C

SUGAR ANALYSIS OF MOLASSES

Table C.1 Sugar content of the molasses used in Set-B (Experiments were performed in Central Laboratories of Middle East Techical University, Record Date and Number; 12.11.2013, 242.)

	mg/mL		
Tag No	**Glucose**	**Fructose**	**Sucrose**
242-01	0.39±0.04	0.46±0.07	211.5±0.6

APPENDIX D

ALCOHOL ANALYSIS OF THE SELECTED SAMPLES

Table D.1 Alcohol content of the selected samples (Experiments were performed in Central Laboratories of Middle East Techical University, Record Date and Number; 16.04.2014, 342.)

Tag No	Ethanol (mg/mL)	Sucrose (mg/mL)
342-01	0.2095 ± 0.0089	n.d.
342-02	0.0797 ± 0.0013	n.d.
342-03	0.3004 ± 0.0032	n.d.
342-04	0.2553 ± 0.0086	n.d.
342-05	0.1202 ± 0.0030	n.d.
342-06	0.1983 ± 0.0036	n.d.
342-07	n.d.	n.d.
342-08	0.4109 ± 0.0000	n.d.
342-09	0.6198 ± 0.0068	0.2028 ± 0.0049
342-10	0.4716 ± 0.0003	0.2182 ± 0.0019
342-11	0.4724 ± 0.024	0.1216 ± 0.0028

APPENDIX E

PRELIMINARY STUDY TO INVESTIGATE THE HYDROGEN PRODUCTION

Preliminary batch reactor studies (Set-1 and Set-2) were conducted in order to test whether hydrogen production could be achieved with the seed sludge used and to come up with the suitable starting operational conditions for the batch reactor studies, namely, Set-A and Set-B.

Table E.1 The summary of the operating conditions of Set-1 and Set-2

	VSS_{rxr} (mg/L)	**BM**	**Initial COD (g/L)**	**Initial pH**	**S/X_o (g/L COD/g/L VSS)**
Set-1	2500	Yes	10	5/5.5/6/6.5/7	4
Set-2	2500	Yes	10/25/40	5/6/7	4/10/16

Set-1: Investigation of optimal initial pH value for hydrogen production

The objective of batch reactor study Set-1 was to evaluate the effect of initial pH and locate the initial pH value at which the highest hydrogen production was observed with dark fermentation from glucose. The TSS, VSS and pH values of the seed sludge used were 23183±922 mg/L and 9433±153 mg/L and pH of 8.6, respectively. The seed sludge was prepared with the same method as the Set-A and Set-B (Section 3.1). The TSS, VSS, COD and gas content analyses were done as described in Section 3.3.1. Reactor set plan was given in Table E.1.

Preliminary batch reactor study Set-1 was conducted in order to evaluate the effect of initial pH on hydrogen production. Because, usually pH values

between, 4 to 7 were considered the range to be studied (Table 2.1, Section 2.3.1), the values to be studied in Set-1 were chosen as, 5, 5.5, 6, 6.5 and 7. Other parameters were all kept same in all reactors, and all pH values were studied in duplicates.

Experiments were conducted in 100 mL glass reactors with 60 mL effective volumes. Reactor VSS concentrations were adjusted to 2500±500 mg/L. The substrate used was glucose and the BM used was as described in Table 3.2 (Section 3.2). For each pH reactor pair, a control reactor that lacked glucose was established. Reactors were incubated at 35±2^{o}C hot room on a stirrer working at 175 rpm, and operated for 7 days. During the operation gas production and gas content was analyzed every 24±2 h. Methane production was not observed in any of the reactors. The results of Set-1 are presented in Table E.2.

Table E.2 The hydrogen production amounts and headspace H_2 percentages achieved in the reactor pairs of Set-1[a]

Initial pH	Total Gas Production (mL)	Total H_2 production (mL)	Hydrogen Gas Percentage (%)
5	242	73	30.2
5.5	215	66	30.6
6	271	92	34.1
6.5	229	73	32
7	256	81	31.7

[a] In the control reactors that were conducted for each pH value, total gas production was between 10-13 mL (which corresponds to the 2-3% of the total gas production amount of the related test reactor type).

Table E.2 showed that the highest total gas production, the highest hydrogen production and the highest percentage of H_2 in the headspace gas were 215-272 mL, 66-92 mL and 30-34%, respectively. The results indicated that the H_2

percentage in the headspace gas and the H_2 gas production amount were not significantly affected by pH values under the studied COD concentration of 10 g/L. It was suspected that at higher COD concentrations, the pH effect would have been more pronounced. Higher carbon concentrations would increase the amount of VFA production, which in turn decreases the medium pH. This pH decrease would probably hinder H_2 production amount and rate. Although hydrogen production amounts of all reactors were similar, the ones with higher pH values obtained higher hydrogen production amounts.

The maximum hydrogen yield achieved was 1.16 mol H_2/ mol glucose, which was achieved at the reactor pair with pH of 6. In line with literate data, higher pH values resisted pH drops for a longer time than the ones with the lower pH values. Therefore reactors with higher pH values provided higher H_2 production than the others.

Set-2: Investigation of initial pH and COD value

The objective of Set-2 was to investigate the combined effect of initial COD concentration and initial pH on dark fermentative hydrogen production and to locate the initial COD and pH combination which provides the highest hydrogen production. For this objective, three initial COD concentrations (10, 25 and 40 g/L) and 3 initial pH values (5, 6, and 7) were investigated.

Experiments were conducted in 100 mL glass reactors with 60 mL effective volumes. Reactor VSS concentrations were adjusted to 2500±500 mg/L. The substrate used was glucose and the BM used was as described in Table 3.2 (Section 3.2). For each pH reactor pair, a control reactor that lacked glucose was established. The reactors were conducted in duplicates and incubated at 35±2^{o}C hot room on a stirrer working at 175 rpm. The reactors were operated for 8 days. During the operation gas production and gas content were analyzed every 24±2 h.

The results of Set-2 were presented in Table E.3. The final pHs of the reactors ranged between 3.6 to 4.74. Independent of the initial pH, as the COD value increased from 10 to 40 g/L, the final pH of the reactors decreased in parallel.

Table E.3 The HYs and HPRs achieved in the reactor pairs of Set-2

Initial pH	**Initial COD (g/L)**	**pH value at the end of incubation period**	**HPR (mL/h)**	**H_2 %**	**HY (mol H_2/mol glucose)**
5	10	3.77	0.72	29.2	0.68
5	25	3.64	0.84	30.2	0.27
5	40	3.65	0.55	40.9	0.22
6	10	4.13	0.72	34.2	1.03
6	25	4.16	0.98	30.6	0.63
6	40	3.6	0.83	35.2	0.41
7	10	4.74	0.95	34.5	1.32
7	25	4.03	0.88	33.5	0.61
7	40	3.74	1.17	33.2	0.54

The highest HPR of 1.17 mL/h was achieved in the reactor with the highest initial COD and pH (COD of 40 g/L, pH of 7). The second highest production rate was observed in the pairs of pH 6-COD of 40 g/L; pH 6-COD of 25 g/L and pH 7-COD of 25 g/L (Table E.3). HYs of the reactor changed between 0.22-1.32 mol H_2/ mol glucose (Table E.3). Independent of the initial pH values, as the COD concentration increased from 10 to 40 g/L, yield value decreased. This finding supported the suspicion that produced VFA concentrations increase in parallel with the increase in COD value, decreasing the medium pH and hindering the hydrogen production. The highest HY of 1.32 mol H_2/ mol glucose was observed in the reactor with initial pH of 7 and COD concentration of 10

g/L. These findings support the results of Set-1, where it was suspected that higher pH values supported higher hydrogen production. Since pH is not controlled in batch reactors, pH drops to 3.5-4.5, at point of which hydrogen production was inhibited. For all the COD concentrations studied as initial pH value decreased, the hydrogen production amount and rate generally decreased (Figure A1-1). The highest HPR of 1.17 mL/h was achieved at the reactor with initial pH 7 and COD of 40 g/L. The H_2 production amounts with respect to time for the reactor pairs are given in Figure E.1. Figure E.1 illustrates that for all the pH-COD pairs, hydrogen production completes in the first 40 h. But, in the reactors with pH 6 and 7, a second production attack was observed. Especially, in the reactors with 25 and 40 g/L initial COD concentrations H_2 production re-started between hours 75 to 100. This occurrence was suspected to be caused by the pH decrease and the consecutive more suitable medium occurrence for the acidogenic bacteria.

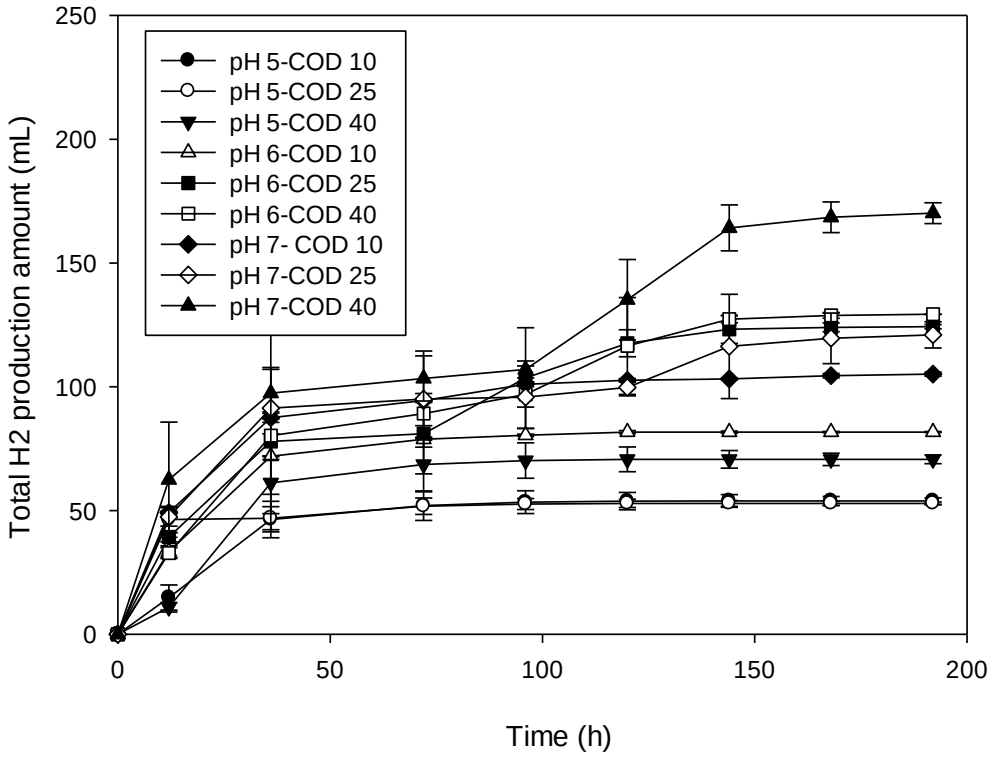

Figure E.1 Total hydrogen production of Set-2

Headspace gas composition analysis shows that H_2% stayed between 29 to 41% (Table E.3). Higher H_2 percentages were generally observed at reactors with higher initial pH values. This was also associated with the change of dominant species due to drop in the pH value and causing the second production attack.

Therefore, for the batch reactor studies to be conducted with the RSM approach (Set-A and Set-B), operating parameters to be investigated were chosen as initial COD, initial pH and the initial S/X_o. The values to be tested in Set-A and Set-B were decided according to the results of preliminary sets, Set-1 and Set-2.

APPENDIX F

RESULTS OF THE FINAL SCOD, TSS AND VSS ANALYSIS OF THE REACTOR PAIRS OF THE SET-A

Table F.1 Results of the final sCOD, TSS and VSS values of the reactor pairs of the Set-A

Initial			Final (mg/L ± Standard Deviation)		
COD (g/L)	**S/X_o**	**pH**	**sCOD**	**TSS**	**VSS**
10	12	4	9604 ± 1225	5941 ± 770	3100 ± 1085
30	4	4	47293 ± 8167	9742 ± 4781	5867 ± 935
30	20	4	25137 ± 2173	7408 ± 1625	6100 ± 2165
50	12	4	49895 ± 3945	11971 ± 232	10571±201
10	4	5.5	11087 ± 779	5025 ± 552	4258 ± 298
50	4	5.5	59876 ± 3156	11058 ± 601	10383 ± 970
30	12	5.5	32800 ± 1423	7964 ± 1080	6272 ± 741
10	20	5.5	10909 ± 525	6883 ± 2774	2954 ± 481
50	20	5.5	40293 ± 1786	10967 ± 1775	7950 ± 1411
10	12	7	9423 ± 262	14817 ± 12830	4675 ± 1433
30	4	7	30108 ± 2686	15506 ± 11288	7656 ± 1576
30	20	7	28506 ± 1285	8058 ± 963	5867 ± 251
50	12	7	63517 ± 3160	11158 ± 1007	9675 ± 1244

APPENDIX G

CALCULATION OF MAXIMUM YIELD OF SET-A IN TERMS OF MOL H_2/ MOL GLUCOSE

According to Eq. G.1, 12 moles of O_2 is required for total combustion of 1 mole of sucrose.

$C_{12}H_{22}O_{11} + 12\ O_2 \rightarrow 12CO_2 + 11H_2O$ (Eq.G.1)

MW of sucrose: 342.3 g/mol

MW of O_2: 32 g/mol , 12 mol O_2 → 384 g

Therefore, (342.3 g sucrose/mol)/ (384 g O_2/ mol) = 0.8914 g sucrose/ g O_2

As a result, 0.8914 g sucrose corresponds to 1 g COD

Calcuted maximum yield of 2.88 mmol H_2/g COD corresponds to;

2.88 mmol H_2/g COD x 1g COD/0.8914 g sucrose x 342,4 g sucrose/1 mol * 1 mol/10^3 mmol

= 1.107 ml H_2/mol sucrose = 0.553 ml H_2/mol glucose

APPENDIX H

SUCROSE EQUIVALENCE OF INITIALLY ADDED MOLASSES TO THE REACTOR WITH THE HIGHEST YIELD (SET-B)

The reactor with the highest reactor had an initial COD of 10 g/L

COD value to be provided in the reactor = 10 g/L

V_m (mL) = The volume of the molasses stock solution to be added to the reactor to achieve 10 g/L COD in 150 mL effective reactor volume

V_{eff} = Effective volume of the reactor, 150 mL

MW_s= Molecular Weight of Sucrose, 342.3 g/mol

COD value of the molasses stock solution = 332.5 g/L

$$V_m = V_{eff}\,(150\ mL)\ x\ 10\frac{g}{L}\ /\ \left(332.5\frac{g}{L}\right) = 4.5\ mL$$

Sucrose content of molasses, according to APPENDIX C = 211.5 mg/mL

M_s = Mass of sucrose added, mg = 211.5 mg/mL x V_m

= 951.75 mg

Moles of sucrose added = M_s / (MW_s x 1000 mg/g)

= 0.00278 moles of sucrose

APPENDIX I

RESULTS OF THE FINAL COD, TSS AND VSS ANALYSIS OF THE REACTOR PAIRS OF SET-B

Table I.1 Results of the final COD, TSS and VSS values of the reactor pairs of Set-B

Initial		Final		
COD (g/L)	**VSS (mg/L)**	**TSS (mg/L)**	**VSS (mg/L)**	**COD (g/L)**
30	5000	11642±620	7575±218	18.7±0.7
30	7500	14175±860	8108±1017	17.2±1.5
30	5000	9558±625	5750±303	18.3±0.2
40	2500	6500±205	4650±25	32±2,6
30	7500	12900±591	8008±505	23.9±1.9
30	7500	13817±485	8050±1339	19.5±4.7
30	2500	6092±265	4275±229	17.6±5.5
30	5000	9725±412	6258±181	14.4±1.4
40	5000	11892±975	8050±733	20.1±1.2
10	5000	9483±1207	5225±715	12.9±1.1
40	2500	6025±109	4142±38	14.5±3.5
30	2500	6042±813	4017±213	17.7±1.1
10	5000	9400±1498	5408±813	10.8±1.7
40	7500	15233±993	9708±600	32.2±2.3
10	2500	4833±208	3075±50	11.5±1.0
30	2500	5592±338	3817±163	21.7±2.6
30	2500	5175±43	3575±66	18.5±1.4
10	2500	4300±354	2717±118	11.3±1.7
30	5000	8608±374	5833±338	19.5±1.6
30	7500	11675±438	6983±326	20.5±0.8
10	7500	9667±743	5367±496	14.3±0.8
30	5000	8825±742	3483±354	19.7±2.0
40	7500	13442±1114	8625±393	32.8±3.0
40	5000	7575±100	4708±414	10.3±1.1
10	7500	10817±1165	5892±389	19±4.5

Table I.1 Continued: Results of the final COD, TSS and VSS values of the reactor pairs of Set-B

40	5000	9442±1411	6775±631	26.4±2.6
10	5000	7000±463	4183±118	17.3±2.0
10	5000	10508±6988	3833±491	11.9±2.5
30	5000	9225±90	5717±336	21.2±1.6
50	5000	11389±945	7372±626	33.7±0.5