DOKUZ EYLÜL UNIVERSITY
GRADUATE SCHOOL OF NATURAL AND APPLIED
SCIENCES

BIOHYDROGEN GAS PRODUCTION FROM WASTEWATER TREATMENT SLUDGE BY DARK FERMANTATION

by
Onur BALCAN

October, 2012
İZMİR

BIOHYDROGEN GAS PRODUCTION FROM WASTEWATER TREATMENT SLUDGE BY DARK FERMANTATION
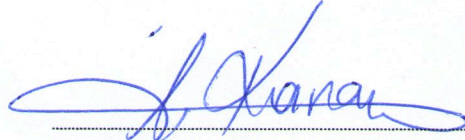
A Thesis Submitted to the

Graduate School of Natural and Applied Sciences of Dokuz Eylül University
In Partial Fulfillment of the Requirements for the Degree of Master of

Science in Environmental Engineering, Environmental Engineering Program

by
Onur BALCAN

October, 2012
İZMİR

M.Sc THESIS EXAMINATION RESULT FORM

We have read the thesis entitled **"BIOHYDROGEN GAS PRODUCTION FROM WASTEWATER TREATMENT SLUDGE BY DARK FERMANTATION"** completed by **ONUR BALCAN** under supervision of **PROF. DR. İLGİ K. KAPDAN** and we certify that in our opinion it is fully adequate, in scope and in quality, as a thesis for the degree of Master of Science.
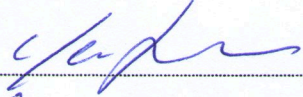
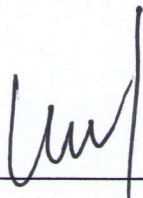Prof. Dr. İlgi K. KAPDAN

Supervisor

Prof.Dr. Nihayet Bizsel

(Jury Member)

Yrd. Doç. ERKAN EKŞİR

(Jury Member)

Prof.Dr. Mustafa SABUNCU
Director
Graduate School of Natural and Applied Sciences

ii

ACKNOWLEDGMENTS

BIOHYDROGEN GAS PRODUCTION FROM WASTEWATER TREATMENT SLUDGE BY DARK FERMANTATION

ABSTRACT

The decrease in fossil fuel resources revealed an urgent need for new and clean energy sources. Hydrogen is a clean and high energy fuel and considered as potential substitute for fossil fuels. Chemical, physical and biological methods are used for the production but, biological methods have certain advantages over the others. Dark fermentation is one of the biological hydrogen gas production processes. It is simple and practical with high rate and yield of formation. Sustainable biohydrogen production requires available and low cost, carbon containing raw material. Wastewater treatment sludge, which is an important environmental problem, could be a suitable raw material for biohydrogen production due to its high organic substance content. Utilization of waste sludge for hydrogen generation may help sludge management and sustainable energy production.

In the light of these facts, the study aimed to determine the significant factors that affect hydrogen production from waste sludge by dark fermentation. For this purpose, sludge hydrolysis method and conditions were determined in the first stage of the study. Three hydrolysis methods as single stage acid, single stage heat treatment and two stage sequential acid-heat treatment were applied to sludge. Two-stage hydrolysis provided 4 times and 0.5 times higher total sugar concentrations compared to acid and heat treatment,respectively, under optimized conditions as t=60 min, pH=2 and T=135 degrees celcius. In the second stage of the study, the effects of media composition and fermentation conditions on hydrogen gas production from filtrate and hydrolyzed sludge were investigated. The most important factors were initial biomass concentration and fermentation pH. The yield of production reached to 3.3 mol/glucose at 5g/L biomass concentration and at pH=5. External addition of nitrogen and protein did not affect the production substantially. The yields and rates of production form filtrate were slightly higher than the rates and yields from sludge.

Keywords: Biohydrogen, dark fermentation, treatment sludge.

ATIKSU ARITMA ÇAMURLARINDAN KARANLIK FERMENTASYON İLE HİDROJEN GAZI ÜRETİMİ

ÖZ

Fosil yakıt rezervlerinin azalması nedeniyle, yeni ve temiz bir enerji kaynağına ihtiyaç vardır. Hidrojen gazı, temiz ve enerji değerinin yüksek olması nedeniyle fosil yakıtların yerini alabilecek bir enerji kaynağıdır. Hidrojen üretiminde fiziksel, kimyasal yöntemler ve önemli avantajları olan biyolojik yöntemler kullanılmaktadır. Karanlık fermentasyonla hidrojen üretimi en bilinen biyolojik yöntemdir. Diğer biyolojik yönetmelere gore basit işletim şartlarında daha yüksek üretim verimi ve hızı elde edilebilmektedir. Sürdürülebilir biyohidrojen üretimi için yüksek karbon içerikli, ucuz ve sürekliliği olan ham madde gereklidir. Önemli bir çevre kirliliği yaratan arıtma çamurları, yüksek karbon içeriği ve atık madde niteliğinden dolayı biyohidrojen üretimine uygun bir kaynaktır. Arıtma çamurunun bu amaç için kullanılması atık yönetimine ve enerji üretimine önemli bir katkı sağlayacaktır.

Bu tez çalışmasında, arıtma çamurundan ışıksız fermantasyonla hidrojen gazı üretimini etkileyen faktörlerin belirlenmesi, hidrojen üretim hızını ve verimini arttırmak amaçlanmıştır. Çalışmanın ilk aşamasında, arıtma çamuruna, tek basamak asit hidrolizi ve ısıl işlemle hidroliz, iki basamak asit-ısıl işlemle hidroliz olmak üzere üç farklı ön işlem uygulanmış, maksimum hidroliz verimi sağlayan yöntem belirlenmiş ve hidroliz şartları optimize edilmiştir. Asit-ısıl işlemle hidrolizde optimum hidroliz şartları olan t=60 dk, pH=2 ve T=135 santigrat derecede toplam şeker derişimi tek basmak asit ve ısıl işlemle hidrolize nazaran, sırasıyla, 4 ve 0.5 kat artmıştır. Projenin ikinci aşamasında, besi ortamı bileşimi ve fermantasyon şartlarının etkisi incelenmiştir. Üretimi etkileyen en önemli faktörlerin pH ve biyokütle derişimi olduğu belirlenmiş ve verim 3.3mol/mol glikoz'a kadar ulaşmıştır. Besi ortamına azot ve protein ilavesi hidrojen gazı üretimine önemli bir katkı sağlamamıştır. Filtrattan hidrolize çamura nazaran daha yüksek üretim verimi ve hızı elde edilmiştir.

Anahtar Kelimeler: Biyohidrojen, ışıksız fermentasyon, arıtma çamuru.

CONTENTS

CHAPTER ONE
INTRODUCTION

1.1 Bio-Hydrogen Gas Production by Dark Fermentation

Despite the increasing fuel need, decrease in reserves each passing day had alerted the world and the interest in hydrogen has increased since 1990s (Kim et al. 2006). Nowadays, nearly 90% of energy demand is provided from fossil fuels in the world these days (Liu & Shen 2004). It was reported that fossil fuel reserves that meets a large part of the world's energy requirements are decreasing day by day, and in the near future it will be unable to meet the demand. In addition, environmental and air pollution caused by fossil fuel is very serious and CO_2 released by combustion may lead to global warming. For this reason, using renewable energy as hydrogen,has become important (Liu & Shen 2004). Hydrogen gas is a cleaner fuel as it only releases water after combustion, it can be produced from a variety of renewable sources and its energy content is 2.75 times higher than conventional fossil fuels (Mizuno et al., 2000) and it can also be converted into electricity (Winter 2005). Moreover, it doesn't produce air pollutants like CO_2, NO_x and S after combusted (Mizuno et al., 2000). But, unlike natural gas and fossil fuels, hydrogen gas cannot be found in the nature and it requires expensive production methods (Kapdan & Kargı, 2006). In order to produce hydrogen gas, there are some methods like, conversion of liquefied gasses, ethanol by steam reforming, partial oxidation of hydrocarbons, pyrolysis of fossil fuels and electrolysis of water (Yu et al. 2009, Seo et al.2010, Qinglan et al, 2010, Ishida et al, 2009, Dubey,et al. 2010). 90% of hydrogen gas production occurs by the reaction of natural gas with steam at high temperatures (steam reforming) (Shirasaki et al, 2009, Roh et al, 2010). Coal gasification and electrolysis of water are the other industrial methods. However, utilization of fosil fuels in large quantities, high temperature and energy requirements are the major disadvantages of these production methods. Under normal conditions, bio-hydrogen gas production doesn't require high amount of energy and it offers economical advantages, such as the use of renewable resources.

Hydrogen production by dark fermentation takes place during the transformation of organic matters to organic acids by microorganisms under anaerobic conditions. The fermentation could be achived at mesophilic (25-40°C), thermophilic (40-65°C) extreme thermophilic (65-80°C or >80°C) conditions (Levin & Chahine, 2010). The most commonly used bacterial culture for hydrogen gas production are spore forming bacteria like *Clostridium* species. Some of the *Clostridium* sp. used for hydrogen gas production so far are *C. butyricum* (Yokoi et al., 2001), *C. thermolacticum* (Collet et al., 2004), *C. pasteurianum* (Lin & Lay, 2004; Lıu & Shen, 2004), *C. paraputrificum* M-21 (Evvyernie, 2001) and *C. bifermentants* (Wang et al., 2003a). Recently, the other species like *Clostridium tyrobutyricum JM1* (Jo et al., 2008)*, Clostridium termitidis* (Ramachandran et al., 2008, Umesh et al., 2008), *Clostridium beijerinckii* (Pan et al., 2008) *C. amygdalinum* (Jayasinghearachchi et al., 2010) have been isolated and hydrogen gas production performances have been investigated. Apart form *Clostridium* sp., hydrogen gas production was carried out by using facultative enteric bacteria (*Enterobacter aerogenes*, *E. cloacae ITT-BY08*) and some thermophilic microorganisms *(T. Thermosaccharolyticum, Desulfotomaculum geothermicum, Thermococcus kodakaraensis* KOD1*, Klebisalle oxytoca* HP1) (Kapdan & Kargı, 2006). Hydrogen gas production potential of *Enterobacter aerogenes* species was comparable to that of the *Clostridium* sp. (Fabiano & Perego, 2002). It was stated that, without using chemical reducing agent to create anaerobic conditions, *E. aerogenes* and *Clostridium* can be used together in hydrogen gas production (Yokoi et al., 1998; Yokoi et al., 2001).

Microrganisms consume organic carbon to obtain energy which is used for growth and cell maintanance. Some of the carbon is converted into other metaobilies as organic acids with the side products hydrogen and methane in anaerobic respiration. Simple sugars like glucose, sucrose and lactose are the most preffered carbon sources by the microrganisms. These carbohydrates are converted to VFA (lactic acid, formic acid, acetic acid, propionic acid, butyric acid), alcohols (ethanol, propanol, butanol) and CO_2 by microrganisms during dark fermentation and hydrogen gas production. The methabolic pathway of the organic susbtance degradation to these end products is given in Figure 1.1. In practice, the highest hydrogen yield is associated with

acetate production. The mixture of acetate and butyrate results in decreasing in the yield. But, low hydrogen yield is associated with the formation of end-products, ethanol, propionate, and lactic (Levin et al., 2004). Theoretically, when the end-product is acetic acid 4 mol H_2 can be produced per 1 mol glucose consumed. If the end-product is butyric acid, then, 2 mol H_2 is produced per 1 mol glucose. However, in practice 4 mol H_2 cannot be obtained from 1 mol glucose. Because some of glucose are used for microbial growth, maintanance and for formation of VFAs (Argun et al., 2009b). In addition, H_2 is consumed in propionate, formate and ethanol formation. In the formation of propionic acid 1 mol H_2 is consumed per mol of propionic acid (Argun & Kargi, 2011). On the other hand, the resulting organic acids may inhibit bio-chemical reactions of metobolic pathways involved in the hydrogen production and represses hydrogen yield. Therefore, the diverting the microbial methabolism to acetic and butyric acid with controlling the their concentrations is the most important factor in increasing hydrogen production yield and rate.

Tablo 1.1 Fermentation end products and hydrogen yields from the main anaerobic glucose degradation pathways.

Fermentation end product(s)	Equation for Anaerobic Glucose Degradation Pathway	Theoretical Hydrogen Yield (mol H_2/ mol glucose)
Acetic acid (CH_3COOH)	$C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COOH + 4H_2 + 2CO_2$	4
Butyric acid ($CH_3CH_2CH_2COOH$)	$C_6H_{12}O_6 \rightarrow$ $CH_3CH_2CH_2COOH + 2H_2 + 2CO_2$	2
Butyric acid ($CH_3CH_2CH_2COOH$) Acetic acid (CH_3COOH)	$4C_6H_{12}O_6 + 2H_2O \rightarrow$ $3CH_3CH_2CH_2COOH + 2CH_3COOH + 10H_2 + 8CO_2$	2.5
Ethanol (CH_3CH_2OH) Acetic acid (CH_3COOH)	$C_6H_{12}O_6 + 2H_2O \rightarrow$ $CH_3CH_2OH + CH_3COOH + 2H_2 + 2CO_2$	2
Propyonic acid (CH_3CH_2COOH)	$C_6H_{12}O_6 + 2H_2 \rightarrow 2CH_3CH_2COOH + 2H_2O$	0
Ethanol (CH_3CH_2OH)	$CH_3COOH + H_2 \rightarrow CH_3CH_2OH + 4H_2O$	0
Lactic acid ($C_3H_3O_3$)	$C_6H_{12}O_6 \rightarrow C_3H_3O_3$	0
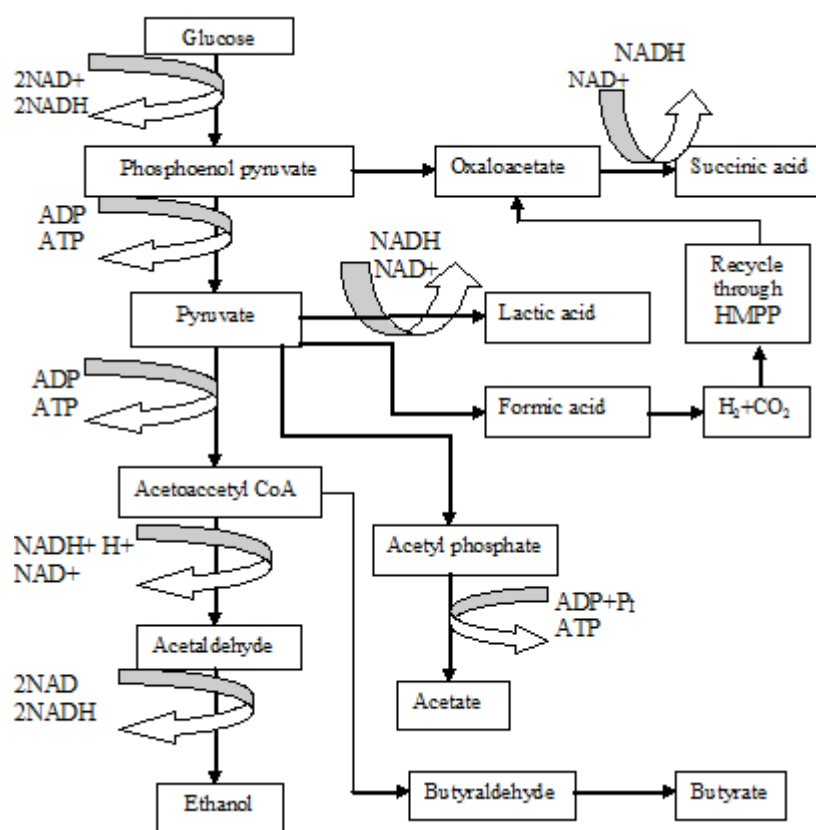
Figure.1.1 Systematic way traces in the transformation of hydrogen to by-products with dark fermentation (Das & Nath, 2004).

Numerous studies have been conducted in order to increase hydrogen production by dark fermentation. It has been indicated that environmental conditions (pH, temperature) gas pressure, type of produced organic acids (VFA), inoculum must be well controlled in order to obtain high hydrogen yields. Among these, pH is considered as one of the important parameters that affect hydrogen yield and specific hydrogen rate. Argun & Kargi (2011) stated that, in order to get high hydrogen yield the most suitable pH range is 5.5-6.5.

Anaerobic treatment is composed of successive acidogenic and methanogenic phases. The gas phase is composed of H_2, CO_2, CH_4, CO, some hydrogen sulphide while liquid phase contains some remaining organic acids. These products occur at different time intervals. At acidohgenic phase, organic acid formtaion and hyrodgen gas generation occur. These products are consumed by the methanogenic bacteria for

methane generation. So, the system must be kept at acidogenic phase to prevent formation of methanogenic phase and methane generation. One of the control mechanism to keep the system under acidogenic phase is controlling pH. In many anaerobic studies, it was observed that final pH value decreased to 4-4.8 value when hydrogen production was done in an uncontrolled pH conditions (Kim et al. 1999). The reason for the decrease in pH is organic acid production. Fermentation pH also affects activity of Fe (iron) containing hydrogenase enzyme. Acidic conditions inhibit hydrogen production (Kapdan & Kargı, 2006). As stated in many studies, hydrogenase activity of hydrogen producing organisms at pH=5.8 is 2.2 times more than that of pH 4.5. Generally, hydrogenase activity is low at pH<5.2 values (Vazquez & Varaldo 2008). The results of these studies indicated that hydrogenase activity is directly related to pH and the defined optimum pH value for hydrogen production is needed to be controlled. Variation of hydrogen, organic acid, pH, solvent, and substrate profile with time without pH control during dark fermentation is shown in Figure 1.2.
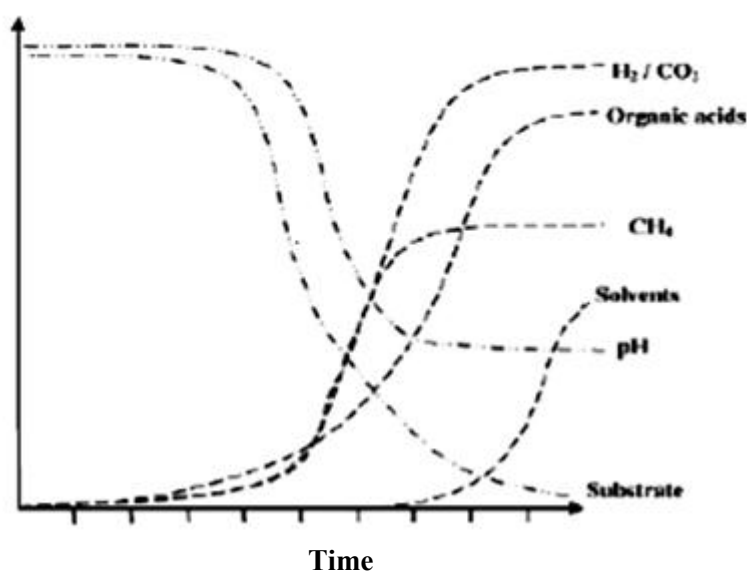

Figure 1.2 Typical presentation of batch hydrogen fermentation (Vazquez & Varaldo 2008).

Another important factor in controlling hydrogen gas production is the partial pressure of hydrogen in the gas phase. It has been reported that hydrogen production decreases with the increase in hydrogen pressure in the gas mixture (Holladay et al,

2009). High partial pressure of hydrogen inhibits further production. Therefore, it must be removed from the system continuously to prevent inhibition effect (Florin et al., 2001). Similarly, oxygen causes inhibition of hydrogen generation. One of the control method for removing oxygen from the system is sparging the fermentation medium with other gases which does not interfere with hydrogen production (Skjånes et al, 2008; Kim et al, 2010).

Finally, the type of the microrganisms and their hydrogen gas production capabilities are the other factors. Selection of the most suitable microbial species growing at mild environmental conditions and in growth media compositions without requiring expensive growth factors is still under investigation. The simple methabolic reactions take place in two different hydrogen producing microbial cultures are given below. The importance of ferrodoksin enzyme and hydrogen generation is indicated in Figure 1.3 (Turner et al., 2007).

1) Enteric bacteria, such as *Escherichia coli*, pyruvate-formate hydrogenlyase enzyme complex.

$$Piruvat + CoA \longrightarrow Asetil\text{-}CoA + format \text{ (Benemann \& Hallenbeck, 2002).}$$

Pyruvate-formate hydrogenlyase
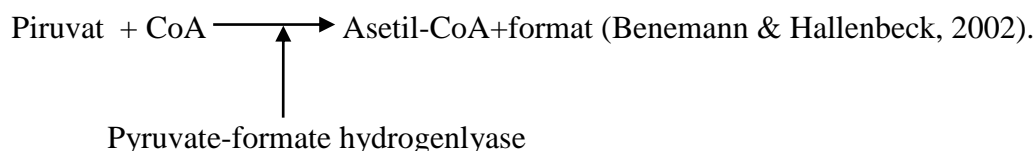
2) Strong anaerobes, such as *Clostridia* species, Pyruvate-ferrodoksin oxidoreductase (Benemann & Hallenbeck, 2002).

$$Piruvat + CoA + 2Fd(ox) \longrightarrow Asetil\text{-}CoA + CO_2 + 2Fd(red)$$

Pyruvate-ferrodoksin oxidoreductase
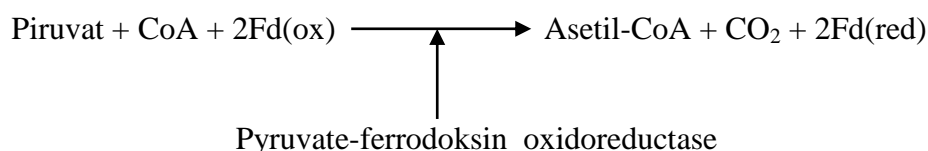
Hydrogen production catalyzed by the hydrogenaz enzyme with the following reactions below.

Hydrogenase
$$2H^+ + 2e^- \longleftrightarrow H_2$$
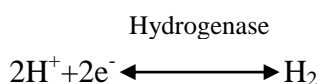
Hydrogen yield produced per 1 mol glucose is 2 mol (2 mol H_2 mol^{-1} glucose) by *Enteric* bacteria. In the presence of NADH, hydrogen yield obtained by *Clostridium* increases. 2 mol more hydrogen can be produced with ferrodoksin oxidoreductase activity.(Turner et al., 2007).
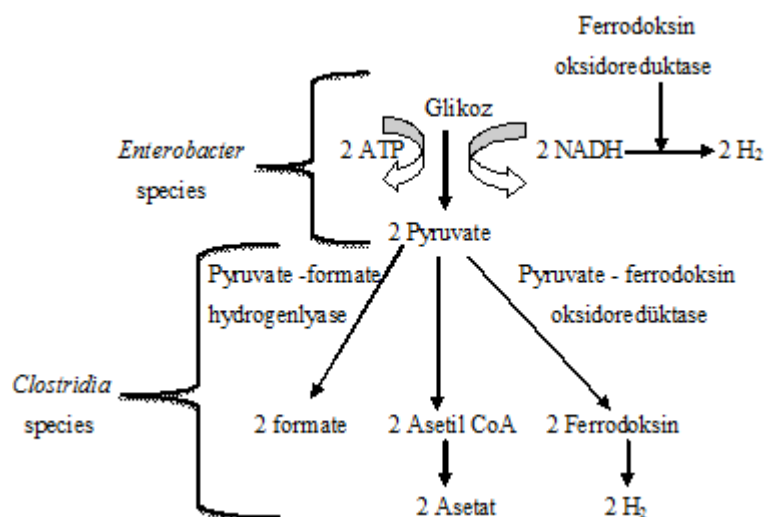
Figure 1.3 Hyrogen formation pathway with glucose fermentation (Turner et al., 2007).

The selection or isolation of these hydrogen generating organisms is another challange. They naturally exist in anaerobic treatment microorganisms mixture, anaerobic sludge of the deep water sediments. These natural microorganism masses are used as source of hydrogen producing organisms. The principle selection method is exposing the culture to exteremelevironmental conditions like high temperatures, acidic conditions or some chemicals to inactivate unwanted microbial cultures such as methanogens and to obtain spores of hydrogen producing microrganisms. This selection process has been so called "pre-treatment of sludge".

Microrganisms need high carbon containing substrate for their metabolism, growth and to generate end product like hydrogen in dark fermentation. For this reason, in order to increase hydrogen production yield and rate, biodegradability of the substrate must be high, it must be available in high quantities, economical and must have high carbohydrate content. Studies have shown that hydrogen production potential of carbohydrate-rich waste is 20 times larger than the waste which is rich in

fat or protein (Bartacek et al., 2007). Pure, simple sugars like glucose and lactose which are easily biodegardable are preferred for this purpose. But, these substances are generally used as food source. That makes them not easily available and economical. The alternative carbon source for this purpose is the waste biomass. Utilization of waste biomass is important in terms of reducing the cost of waste disposal, waste management and providing natural organic matter cycle with the valuable end product as energy. Therefore, instead of pure carbohydrates, wastes with high organic content must be preferred as substarte. Wastes like starch containing agricultural and food industry waste, cellulosic agricultural and food industry waste, carbohydrate-rich industrial waste can be preferred. But, these wastes can only be used for hydrpgen gas production after some pre-treatments. For example, lignin and hemicellulose contents of the agricultural wastes are the unused fraction in production. Therefore, expensive pre-seperation processes are needed (Kapdan & Kargı, 2006). The suggested scheme for hydrogen gas production from agricultural wastes containing starch or cellulose and wastewater is shown in Figure 1.4.
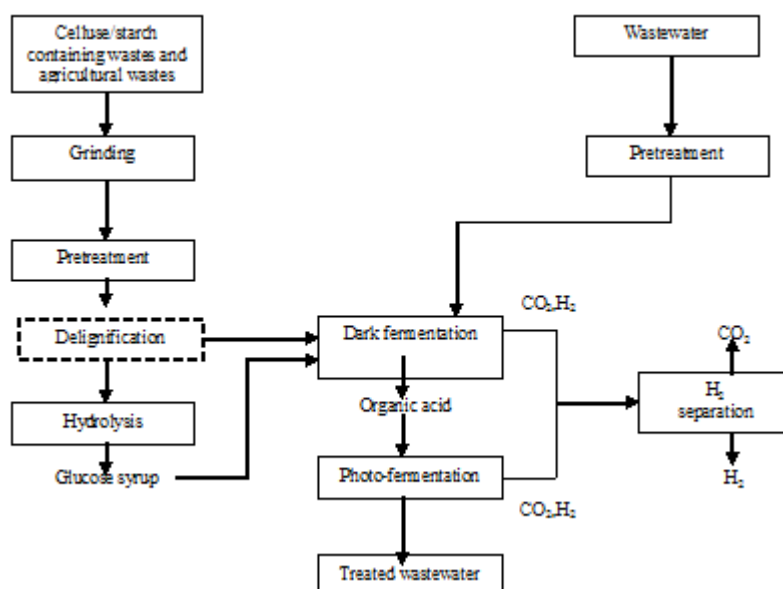

Figure 1.4 Scheme of biohydrogen gas production from agricultural wastes containing starch or cellulose and wastewater (Kapdan & Kargı, 2006).

In addition to agricultural wastes and wastewaters, sewage sludge have high organic mater content. The treatment and disposal of waste sludges require sequential unit processes which cause substantial increase in total cost of wastewater treatment process. The most simple sludge treatment methods are tickening and then drying. Howewer, the recent regulations emphasize that the sludge must be efficiently stabilized, should not contain toxic substances and must be compatible with the nature after disposal. That makes treatment sludges a primary concern in terms of environmental point of view. Therefore, the applicability of advanced and expensive treatment methods like oxidation, microwave, ultrasound is being studied by the reseracher (Wang et al. 2010,. Song et al.2010, Oh et al., 2007, Erden et al., 2010, Tony et al. 2008). However, sludge contain carbon and that carbon can be used for energy generation. The well know process is to obtain methane from sewage sludge by anaerobic digestion. Unfortunately, methane is one of the greenhouse gas. Therefore, methane generation is not in the agenda of the researchers anymore. Hydrogen gas generation form any carbon source is the research area. Treatment sludge is rich in carbon, a waste material and readily avaliable. Utilization of sludge for hydrogen gas generation combines the concepts of sustainable waste management and clean energy production. Although, agricultural wastes are the most preferred substrate for hydrogen gas, they could be used as animal feed and fertilizer after composting. But, sludge is waste and nothing else.

The organic and inorganic content of sludge may vary depending on the wastewater and treatment technology used. Table 1.2 indicates the characterization of domestic raw sludge, pre-treated sludge and filtrate (Guo et al., 2010). Similarly, characterization of primary sludge and biological sewage sludge are given in Table 1.3 (Zhu et al., 2008). As shown in the tables, organic matter content of sludge is considerably high and there are suitable organic matter types to produce hydrogen gas.

Table 1.2 Characterization of domestic raw sludge, pre-treated sludge and filtrate (Guo et al., 2010).

Parameter	Raw Sludge	Pre-treated sludge (Heat treatment)	Filtrate
TCOD (mg L^{-1})	13,050 ± 2530	16,000 ± 2750	3455 ± 705
SCOD (mg L^{-1})	380 ± 60	2840 ± 25	2011 ± 12
pH	6.9 ± 0.2	7.3 ± 0.2	7.8 ± 0.4
Carbohydrate (mg L^{-1})	27 ± 7	203 ± 32	446 ± 43
Protein (mg L^{-1})	33 ± 5	223 ± 21	351 ± 28
Cu (mg/kgDS)	443.2 ± 3.9	NA	19.0 ± 0.2
Pb (mg/kgDS)	110.7 ± 19.0	NA	24.7 ± 15.7
Cd (mg/kgDS)	18.8 ± 0.4	NA	0
Zn (mg/kgDS)	779.3 ± 7.9	NA	0
Ni (mg/kgDS)	49.8 ± 2.6	NA	4.8 ± 3.9

Table 1.3 Characterization of primary sludge (PS) and biological waste sludge (WAS) (Zhu et al., 2008).

Parameter	PS	WAS
TS (g L^{-1})	30.6±5.6	8.8±1.5
VS (g L^{-1})	19.8±3.9	6.5±1.3
Carbohydrates (mg L^{-1})	124±44	31±10
Soluble COD (mg L^{-1})	4480±2160	240±110
Total COD (mg L^{-1})	35900±12600	10600±2890
Acetic acid (mg L^{-1})	1140±516	n.d.
Propionic acid (mg L^{-1})	581±284	n.d.
Butyric acid (mg L^{-1})	289±183	n.d.
TKN (mg L^{-1})	1233±350	709±190
PO$_4$–P (mg L^{-1})	216±130	n.d.
Ba (mg L^{-1})	2.41	0.45
Ca (mg L^{-1})	418	108
Cu (mg L^{-1})	3.22	2.22
Fe (mg L^{-1})	735	558
K (mg L^{-1})	70.9	60.3
Mg (mg L^{-1})	77.9	29.3
Mn (mg L^{-1})	2.05	1.66
Mo (mg L^{-1})	0.06	0.14
Na (mg L^{-1})	148	109
Zn (mg L^{-1})	3.43	1.28
Total acidity as CaCO$_3$	1972	2200
Total alkalinity as CaCO$_3$	1960	840
pH	5.9±0.1	6.8±0.1

1.2 Literature Review

Various biomass wastes (sewage sludge, barley, straw, corn stalks, sugar beet, wheat, sugar beet pulp, sweet corn) are used for bio-hydrogen production as raw marerials. (Guo et al, 2008, Wang et al. 2003-b, Nicolau et.al, 2008, Kotay & Das, 2009; Argun, Kargi, Kapdan, & Oztekin, 2008a, 2008b, 2009a, 2009b; Cao et. al, 2009; Fan et. al, 2008; Guo et. al, 2010; Lin et al., 2008). Hydrogen gas production from wastewater and solid wastes by dark fermentation was also investigated (Dong et al., 2009; Han & Shin, 2004; Gilroyed et al., 2008; Kyazze et. al, 2008; Sivaramakrishna et al., 2009; Thong et. al, 2008).Utilization of waste sludge for hydrogen production is still under investigation.

In order to transform sewage sludge to hydrogen gas, cell structure of microrganisms in sludge must be disrupted and carbon content must be easily available to be consumed by bacteria. In other words the sludge must be hydrolyzed to release its carbon content to the liquid phase. Microwave, ultrasonication, the addition of acid and base, temperature or sterilization, enzymatic hydrolysis were applied to sewage sludge for hydrolysis purpose in hydrogen production studies (Guo et al, 2008; Park et al. 2009; Eskicioglu et al., 2010; Thungklin et al, 2010; Wang et al. 2003-b; Nicolau et.al, 2008; Kotay & Das, 2009).

Wang et al., (2003b) applied pre-treatments like ultrasonication, acidification (pH=3), sterilization, freeze/thaw to domestic sewage sludge. The COD concentration after hydrolysis of sludge in the liquid phase increased about 8%-16% depending on the method applied. After fermentation of the liquid phase, the highest hydrogen production yield was obtained as 1.5 mmol--H_2/g-COD and 2.1 mmol--H_2/g-COD at freeze/thaw and sterilization, respectively. The yields of production from other methods apllied were substantially lower.

Pre-hydrolysis with microwave was applied to wastewater treatment sewage sludge of poltry slaughterhouse wastewater treatment plant for 3 minutes at 850W power. After the pre-treatment temperature rised up to 78°C, significant deterioration

was obtained in microorganisms' cell structures, release of organic matter content of sludge achieved. Dissolved COD amount in raw sludge increased to 25.79 g L^{-1} from 15.34 g L^{-1}after hydrolysis. Similarly, dissolved protein content increased to 7.95 g L^{-1} from 3.38 g L^{-1}. The highest organic substance found in the filtrate was the protein followed by carbonhydrates and fats. Hydrolysis provided about 2.5 times incerase in concentrations of the organic substances released from sludge cell structures. Variation in protein, carbonhydrate, fat, total COD and soluble COD concentrations were monitored during fermentation. It was observed that 4%-20% increase in the final COD concentration at the end of the fermentation. Protein concentration significantly decreased compared to fat and carbonhydrate concentrations. It was determined that protein is the main carbon source consumed in hydrogen production. Hydrogen production yield from raw sludge was 0.18 ml H$_2$ g^{-1} tCOD^{-1}. Hydrogen production yield raised up to 12.77 ml g^{-1} tCOD^{-1} in nutrient added (endonutrient) microwave applied sludge (Thungklin et al., 2010).

Nicolau et al (2008) applied two different pre-treatments to domestic sewage sludge for hydrolysis purpose; 70°C heat treatment for 1 hour and enzymatic treatment. The results indicated that there was no substantial increase in carbonhydrate concentration after single-stage heat treatment. Cellulase enzyme was used in enzymatic hydrolysis. The total carbohydrate concentration rised from 2.6% to 13.5%. However, although total carbohydrate concentration in sludge was about 18000 mg L^{-1}, the dissolved concentration, which could be defined as avaliable carbohydrate, was only 2500 mg L^{-1}. This result shows that enzymatic hydrolysis is less effective compared to other chemical and heat treatment methods in terms of substrate release from sludge.

Xiao & Liu (2008) applied 121°C heat treatment for 30 minutes (sterilization) to domestic raw sludge. After single-stage heat treatment, an increase was observed in soluble COD, protein and carbonhydrate concentrations. Hydrogen productions by dark fermentation of raw sludge and pre-treated sludge were studied to compare the effect of hydrolysis on hydrogen production. The observation during fermentation was further release of COD up to 2800-4000 mg L^{-1}in sterilized sludge. However,

COD release in raw sludge was about 200-700 mg L^{-1} which is substantially lower that that of sterilized sludge. Hydrogen production yields obtained from the experiments were 0.35 ml H_2/VS and 16.26 ml H_2/VS from raw sludge and sterilized sludge, respectively. These result indicate that sterilization for hydrolysis of sludge help increasing the yield of hydrogen formation.

Kotay & Das (2009) applied different pre-treatments to domestic sewage sludge, such as acid (pH 3–4, 24hour 0.1 N HCl, 25°C) base (pH 10–11, 24 h, 4N NaOH, 25°C), sterilization (121°C, 20 min), freeze/thaw (-20°C/25°C), microwave (600W, 2mins.) ultrasonication and chemical supplement BESA (bromoethenesulfonic acid) and $CHCl_3$. The highest protein, carbonhydrate and fat solubilizations were obtained in heat-treated sewage sludge. The highest hydrogen production yield observed from this hydrolysate was 14 ml H_2/ g COD. In another study, among the pre-treatments, as alkaline, freeze/thaw and acidification, the highest organic matter solubilization was provided in alkaline pre-treatment. But the highest hydrogen production potential was obtained by acid hydrolysis (Ting & Lee , 2007).

Since, bio-hydrogen gas production studies applied mostly to carbonhydrate-rich wastes, the contribution of proteins to hydrogen gas production wasn't examined in detail. But, there are evidences about the positive effects of the high protein concentrations, released from the hydrolysis of sewage sludge on hydrogen gas production. Thungklin et al. (2010) reported that protein consumption was higher than carbohydrate and fat consumption and protein was the main carbon source used for hydrogen generation. Similar results were obtained by Cai et al. (2004) and Guo et al. (2008) and it was interpreted that proteins are essential and enhance hydrogen gas production from sludge by dark fermentation.

In the studies summarized above, hydrogen gas production was provided with the fermentation of solid and liquid phase together after hydrolysis. But, Guo and friends (2010) developed a theory that, nutrient (C,N,P) transfer from solid phase to liquid phase will continue throughout the fermentation, although this transfer seems benefical in terms of reducing the organic matter content, it will cause inhibition in

hydrogen gas production due to excessive nutrient loading. It was observed that it is possible to obtain 8% to 40% increase in soluble COD concentration at the end of fermentation. The study was conducted by separating the filtrate and solid phase of the sludge obtained after hydrolysis. Batch fermentation of filtrate and sludge, resulted in 4.44 mg H_2 g^{-1} $tCOD^{-1}$ and 1.34 mg H_2 g^{-1} $tCOD^{-1}$ hydrogen yields, respectively. 3.3 times higher yield was obtained from filtrate and it was determined that filtrate is a more suitable substrate for hydrogen gas production.

There are also studies on production of hydrogen gas from the mixture of sewage sludge and other organic solid wastes. Increase in hydrogen gas production can be provided by sewage sludge addition to domestic organic solid wastes. The main reason for higher hydrogen production from this mixture is expalined as nutrient contents of sewage sludge helps decomposition of organic solid wastes. Especially, it was stated that protein contents of sewage sludge help the growth of *Clostridum sp.* and improve C/N ratio. pH is a determinative factor (butyric or acetic fermentation) for fermentation type in bio-hydrogen production. There was also a rise in bio-hydrogen production pH buffering capacity with the addition of sewage sludge to organic domestic wastes (Zhu et al. 2008, Kim et al.2004). Table 1.5 depicts the hydrogen production yields form raw sludge, and filtrates and solid phases of the hydrolyzed sludges.

Table 1.4 summarizes the the type of the pretreament applied to sludge for hydrolysis purpose, organisms used in fermentation an the yield of hydrogen fromation obatiend from sludge. Table 1.5 compares the yields of hydrogen formation from raw sludge, filtrate and hydrolyzed sludge.

1.3 Objectives and the Scope

Wastewater treatment sludge is a waste material that needs advance, expensive treatment technologies to be converted into environmentally acceptable forms. Hydrogen gas is a clean and renewable source that can be generated from carbon reach substances by biological methods. Carbon rich waste materials must be the substrate for biohydrogen production technologies.

Table 1.4 The effect pretretament methods applied to sludge on hydrogen gas production.

Pre-Treatment	Microorganism	Biyoproces	H_2 Production Yield (ml H_2 g^{-1} $tCOD^{-1}$)	Reference
Sterilization	*Pseudomonas sp.* GZ1	Batch	15.02	Guo et al., 2010
Microwave	*Pseudomonas* sp. GZ1	Batch	11.44	Guo et al., 2010
Ultrasonication	*Pseudomonas* sp. GZ1	Batch	4.68	Guo et al., 2010
Alkaline	Raw sludge	Batch	18.48	Cai et al., 2004
Alkaline	Alkaline treated sludge	Batch	10.08	Cai et al., 2004
Acid	Heat treated sludge	Batch	1 mmol /g COD	Wang et al. 2003-a
Boiling	Thermal treated sludge	Batch	4.48	Wang et al. 2003-a
Raw sludge	*Clostridium bifermentans*	Batch	13.40	Wang et al. 2003-a
Freezing/thawing and sterilization	*Clostridium bifermentans*	Batch	40.32	Wang et al. 2003-a
Aerobic thermophilic digestion	Aerobic thermpphilic sludge digestion sludge	Batch	35.66	Lin & Lay, 2005
Raw sludge	Raw sludge	Batch	1.54	Lin & Lay, 2005
Raw sludge	*Enterobacter aerogenes*	Batch	0.18	Thungklin et al., 2010
Microwawe	*Enterobacter aerogenes*	Batch	12.77	Thungklin et al., 2010
Enzymatic hydrolysis	Heat treated sludge	Continuous	18.14	Nicolau et al., 2008

Table 1.5 Hydrogen production yields from raw sludge filtrates and solid phases of the hydrolyzed sludges.

Raw Sludge	Filtrate	Sludge	Reference
-	-	1.5 mmol-H_2/g-COD	Wang et al (2003-b)
-	-	2.1 mmol-H_2/g-COD	Wang et al (2003-b)
0.18 ml H_2/ g tCOD	-	12.77 ml / g TCOD	Thungklin et al, (2010)
0.35 ml H_2/VS	-	16.26 ml H2/VS	Xiao & Liu (2008)
-	-	14 ml H_2/ g COD	Kotay & Das (2009)
-	4.44 mg H_2/g tCOD	1.34 mg H_2/g tCOD	Guo et al. (2010)

Since, sludge is a carbon reach substances and a waste material to be handled, it perfectly fits to the characteristics of substrate asked for biohydrogen production. By considering these facts, the main aim of this thesis is to investigate hydrogen gas production potential from wastewater treatment sludge by dark fermentation. In order to achieve this aim, the scope of the study was framed as follows;

- Determination of hydrolysis methods and hydrolysis conditions: Single stage acid and heat treatment, two stage sequential acid and heat treatment of sludge were three different approaches for sludge hydrolysis. The significant factors effecting the hydrolysis of sludge were determined for each method, the most efficient hydrolysis method and optimum hydrolysis conditions were selected.

- Investigation of effect of environmental factors on hydrogen gas production potential of hydrolyzed sludge by dark fermentation; the effects of fermentation temperature and pH were investigated and the conditions for maximum hydrogen gas production yield were determined.

- Evaluation of effect of media composition on hydrogen gas production from hydrolyzed sludge: Biomass, nitrogen, substrate and protein concentration were varied and the media composition for the maximum hydrogen gas production yields were determined.

CHAPTER TWO
MATERIALS & METHODS

2.1 Hydrolysis of Sludge

2.1.1 Single Stage Hydrolysis of Waste Sludge

2.1.1.1 Acid Hydrolysis of Waste Sludge

In single stage acid hydrolysis, aerobic sludge taken from Pak-Maya Baker's Yeast Industry in İzmir, Turkey was used without dilution. 10M of H_2SO_4 was added to sludge and hydrolyzed at different pH values between pH=2-6. Sludge was stirred continuously for 24 hours at magnetic stirrer and pH value was controlled at desired value during mixing. Nine samples were taken during the acid hydrolysis at time interval between t=15-1440 min. First eight samples were taken within the first 4 hours of hydrolysis and the last sample was taken at the end of 24 hour. The samples were centrifuged at 8000 rpm and analysis were made in clear supernatant. Two-factor factorial experimental design method was used as statistical experimental design method. Experimental points of factorial experimental design method are given in Table 2.1. pH and hydrolysis time were two factors in experiment design. TOC, COD and total sugar (TS) concentrations at each samples were analyzed for different experimental conditions. Single measurement was done for NH_4-N and protein analysis. PO_4-P analysis was made for three samples taken at different times of hydrolysis.

Table 2.1 Experimental points of factorial experimental design for acid hydrolysis of sludge.

pH	Hydrolysis Time (min)								
2	15	30	45	60	90	120	180	240	1440
3	15	30	45	60	90	120	180	240	1440
4	15	30	45	60	90	120	180	240	1440
5	15	30	45	60	90	120	180	240	1440
6	15	30	45	60	90	120	180	240	1440

2.1.1.2 Hydrolysis of Waste Sludge by Heat Treatment

Single stage hydrolysis at different temperatures was applied to Pak-Maya Beker's Yeast Industry aerobic wastewater treatment sludge. Factorial experimental design method with 2 factors at 3 levels was used as statistical design method. Temperature and time were the factors. Levels for temperature were T=60°C, T=100°C and T=135°C and levels for hydrolysis time was t=30 min, t=45 min and t=60 min. The number of replicate was two at each experimental condition. Experimental points of factorial experimental design method are given in Table 2.2.

Table 2.2 Experimental points of factorial experimental design for hydrolysis of sludge by heat.

Experiment No	Hydrolysis Time (min)	Temperature (^{0}C)
1	30	60
2	30	100
3	30	135
4	45	60
5	45	100
6	45	135
7	60	60
8	60	100
9	60	135

2.1.2 Two-stage (Acid + Heat) Hydrolysis of Waste Sludge

Acid and heat treatment hydrolysis methods were used in two-stage hydrolysis of sludge. Box-Behnken surface response (RSM) method was the experimental design methods. The ranges for the levels of the factors were selected based on the results obtained from single stage hydrolysis experiments. Independent variables were pH (X_1=2-6), temperature (X_2=60-135°C) and hydrolysis time (X_3=15-60min) Dependent variables were COD, total sugar, NH_4-N, PO_4-P and protein concentrations. Surface response method consists of factorial, axial and central points. Factorial and axial points were not repeated, three replicates were conducted

at central points. Sequential hydrolysis of the sludge was achieved as acid hydrolysis at different pH for 4 hours and heat treatment in autoclave according to the experimental conditions given in Table 2.3.

Table 2.3 Experimental points of Box-Behnken experimental design method for two stage hydrolysis of sludge.

Experiment No	Independent variables		
	X_1	X_2	X_3
	pH	Temperature ^0C	Time (min)
1	4.00	60.00	15.00
2	4.00	135.00	15.00
3	4.00	60.00	60.00
4- C*	4.00	97.50	37.50
5	6.00	97.50	60.00
6	2.00	97.50	15.00
7	2.00	97.50	60.00
8	2.00	135.00	37.50
9-C*	4.00	97.50	37.50
10	6.00	135.00	37.50
11	6.00	60.00	37.50
12	4.00	135.00	60.00
13-C*	4.00	97.50	37.50
14	2.00	60.00	37.50
15	6.00	97.50	15.00

*C: center points

2.2 Experimental Procedure

2.2.1 Microorganisms

The mixed microbial culture was obtained from Pak-Maya Baker's Yeast Industry anaerobic wastewater treatment plant. In order to obtain spore forming- hydrogen producing bacterial culture, heat treatment was applied to anaerobic treatment sludge. Sludge was boiled for an hour at 100°C. Heat-treated anaerobic sludge was activated with batch dark fermentation in a rich nutrient medium containing glucose (60 g L^{-1}), peptone (10 g L^{-1}), yeast extract (0.6 g L^{-1}), $MgSO_4 \cdot 7H_2O$ (0.25 g L^{-1}), K_2HPO_4 (1 g L^{-1}), KH_2PO_4 (1 g L^{-1}), l-cysteine $HCl.H_2O$ (0.1 g L^{-1}). Argon gas was passed through the bottles before incubation. The bottles were plugged with gas-

tight silicone stoppers and screw cap. After 2 days of incubation, at pH=7 and T=37°C, organisms were used as inoculum for fermentation experiments.

2.2.2 Batch Dark Fermentation Experiments

Aerobic wastewater treatment sludge was hydrolyzed before dark fermentation at hydrolysis conditions as pH=2, T=135°C, t=60 min which were determined at optimization of two stage hydrolysis experiments. Two types of substrate were used in dark fermentation. The first one is the liquid phase, called as filtrate, obtained after two stage hydrolysis reaction. The second substrate was the mixture of solid and liquid phases of sludge hydrolysis. The mixture is called as "sludge". Control experiments with raw sludge, without any hydrolysis, were conducted in parallel to the experiments with other two substrates.

Batch dark fermentation experiments were done in 310 ml serum bottle (Isolab-Germany Boro 3.3). The bottles were filled with 200 ml of substrate as filtrate and hydrolyzed sludge obtained after hydrolysis. 30 ml heat treated and, then, activated inoculums was added to the fermentation bottles to obtain around 2- 3 g L^{-1} initial biomass concentration. The bottles were closed with gas-tight silicone stoppers and screw cap to prevent gas leakage. Argon gas was passed through the bottles before incubation to exhaust oxygen remained in the head space and in the liquid phase of bottles. Fermentation was conducted at mesophilic conditions 37°C apart from the studies carried out to investigate the effect of fermentation temperature. pH of the fermentation was controlled around 6.5-7.5 in the first part of the studies and then it was kept at 5.0- 5.5 after the effect of pH was determined. Total gas volume, hydrogen gas percentage in the gas mixture were monitored daily. Total sugar, TVFA, COD, NH_4-N, PO_4-P and protein analysis were done in liquid samples. Hydrogen production potentials was evaluated in terms of hydrogen percentages, hydrogen gas production yield and production rate. Effect of environmental conditions as fermentation temperature and pH, effect of media composition as biomass, nitrogen, substrate and protein concentrations were investigated in batch

dark fermentation. Details of conditions for each experiment were given in following sections.

2.2.3 The Effect of Environmental Factors

2.2.3.1 Dark Fermentation Temperature

Aerobic sludge was hydrolyzed at optimum conditions as identified at two-stage hydrolysis. Filtrate and sludge were used as substrate. Batch dark fermentation experiments were done in 310 ml serum bottle (Isolab-Germany Boro 3.3) at 230 ml fermentation volume. Fermentation was done at 37°C, 45°C, 55°C, pH was kept constant at pH=7 during the fermentation.

2.2.3.2 Dark Fermentation pH

In order to investigate the effects of pH on hydrogen gas production, fermentation was carried out at four different pH values as pH= 4, 5, 6, 7. Filtrate and sludge were used as substrate. Dark fermentation was done at 37°C and daily H_2 measurements were conducted.

2.2.4 The Effect Media Composition

2.2.4.1 Protein Supplementation

Peptone was used as protein sources and externally added into the fermentation media at the concentrations between 1 g L^{-1} and 5 g L^{-1}. Fermentation was conducted at 37 °C and pH=7. Filtrate and sludge were used as substrate.

2.2.4.2 Initial Biomass Concentration

Initial biomass concentration was varied between X=2 g L^{-1}- 6 g L^{-1} at five different levels.

Fermentation was done at 37 °C and at pH=5. Filtrate and sludge were the substrates.

2.2.4.3 Initial NH$_4$-N Concentration

In order to determine the effects of nitrogen concentration on hydrogen production, $(NH_4)_2SO_4$ was added to the fermentation media. NH$_4$-N concentrations were 200 mg L^{-1}, 300 mg L^{-1}, 400 mg L^{-1}. Initial biomass concentration was kept constant at 5 g L^{-1} , fermentation temperature was 37 °C and pH was controlled at pH=5.

2.2.4.4 Initial Substrate Concentration

Initial sugar and COD concentrations in the filtrate and sludge can only be increased by increasing the was sludge concentration to be hydrolyzed. The sludge concentration was increased 60 g L^{-1} by concentrating the raw sludge in Imhorff. Then the concentrated sludge was hydrolyzed at the conditions previously determined. Initial biomass concentration was kept constant at 5 g L^{-1}, fermentation temperature was 37 °C and pH was controlled at pH=5.

2.3 Analytical Methods

2.3.1 Sampling

Samples removed from the liquid phase everyday were centrifuged at 8000 rpm and the clear supernatants were used for analysis.

2.3.2 Total Sugar Analysis

Total sugar concentrations were determined by the acid-phenol spectrometric method (Dubois et al., 1956).

2.3.3 Total Vololite Organic Acid Analysis

TVFA analyses were carried out by using analytical kits (Spectroquant, 1.01763. 0001, Merck, Darmstadt, Germany) and a PC spectrometer (WTW Photolab S12).

2.3.4 Total Gas Volume Measurement

The total gas volume produced was determined by water displacement method everyday by using sulfuric acid (2%) and NaCl (10%) containing solution.

2.3.5 H_2 Gas Measurements

Hydrogen gas was sampled from the head space of the bottles by using gas-tight glass syringes. Hydrogen gas concentration in the gas phase was measured by using a gas chromatograph (HP Agilent 6890). The column was Alltech, Hayesep D 80/100 6" × 1/8" × 085". Nitrogen gas was used as carrier with a flow rate of 30 ml min^{-1} and the head pressure was 22 psi. Temperatures of the oven, injection, detector, and filament were 35 ^{O}C, 120 ^{O}C, 120 ^{O}C, 140 ^{O}C, respectively.

2.3.6 Biomass Concentration

Biomass concentration was determined by filtration of aliquots on pre-weighted filter (Whatman GF/C) which was dried at 105°C for 24 h and then weighed after cooled to room temperature in a desicator. (APHA SM 2540 D; 2005).

2.3.7 pH and ORP Measurements

pH and ORP of the fermentation medium were monitored by using a pH meter and ORP meter with relevant probes (WTW Sci., Germany). pH was maintained between 6.5 and 7.5 by manual pH control. ORP values varied between −100 and −300 mV, in general.

2.3.8 NH$_4$-N Analysis

NH$_4$-N was determined by using analytical kits (Spectroquant NH$_4$-N 1.14752.0001, Germany) and a PC spectrometer (WTW Photolab S12).

2.3.9 PO$_4$-P Analysis

PO$_4$-P analyses were carried out by using analytical kits (Merck Spectroquant® Fosfat Reaktif Testi, Orto-Fosfat Tayini İçin-1.14848.0001) and a PC spectrometer (WTW Photolab S12).

2.3.10 Protein Analysis

Protein analyses were carried out by using analytical kits (Thermo Modified Lowry Protein Assay kits (23240) and by Bradford Assay Metod.

2.3.11 COD Analysis

Soluble COD concentrations were determined by the closed reflux method of SM (Standard Methods) (APHA 2005). Total COD concentrations were determined by the open reflux method as stated in APHA SM (2005).

2.3.12 TOC Analysis

TOC measurements were done at Shimadzu TOC anayzer.

2.4 Calculations

The cumulative hydrogen gas production was determined by the following equation (Logan et al., 2002):

$$V_{H2, i} = V_{H2, i-1} + V_W \; C_{H2,i} + V_{G,i} \; C_{H2,i} - V_{G,i-1} \; C_{H2, i-1} \qquad \text{Eqn 2.1}$$

where $V_{H2, i}$ and $V_{H2, i-1}$ are the volumes of cumulative hydrogen (mL) calculated after the ith and the previous measurement; V_W is the total gas volume measured by the water displacement method (mL); $C_{H2,i}$ is the concentration of H_2 gas in the total gas measured by the water displacement method (%); $V_{G,i}$ and $V_{G,i-1}$ are the volumes of the gas in the head space of the bottle for the ith and the previous measurement (mL); $C_{H2,i}$ and $C_{H2, i-1}$ are the percent H_2 in the head space of the bottle for the ith and the previous measurement.

The generalized gas equation presented in Eqn 1.1 was used to calculate the mole number of cumulative hydrogen.

$$PV = nRT \qquad\qquad\qquad \text{Eqn 2.2}$$

where: n is mmol H_2 gas, P= 1 atm, V= Cumulative total hydrogen gas volume (mL), R= 0.082 (L atm / mol K) , T= Temperature in Kelvin (K)

In batch fermentations, cumulative hydrogen versus time data were correlated with the Gompertz equation in Eqn 2.3 and the constants were determined by regression analysis with Statistica 5. The Gompertz equation has the following form (Han & Shin, 2004):

$$H(t) = P \exp \left\{ -\exp \left[\frac{R_m\, e}{P} (\lambda - t) + 1\right] \right\} \qquad\qquad \text{Eqn. 2.3}$$

where, H is the cumulative hydrogen (mL H_2) at any time t; P is the maximum potential hydrogen formation (mL); R_m is the maximum rate of hydrogen formation (mL h^{-1}), λ is duration of the lag phase, „e" is 2.718 and „t" is time (h). The coefficients of the Gompertz equation were determined by regression analysis using the experimental data.

Hydrogen formation yield and specific hydrogen production rate (SHPR) are important parameters indicating the effectiveness of fermentation. The yield was calculated by using the following equation.

$$Y = CHF / V_0 (S_0 - S)$$
<div align="right">Eqn. 2.4</div>

where Y is the hydrogen gas yield (ml H_2 g^{-1} TS or mol H_2 mol^{-1} glucose); CHF is the cumulative hydrogen gas formation (mL); V_o is the initial fermentation volume (L); S_0 and S are the initial and final total sugar concentrations (g L^{-1}).

The SHPR (mL H_2 g^{-1} biomass h^{-1} at certain temperature and 1 atm) were calculated by using the following equation,

$$R_X = R_m / V_O X_O$$
<div align="right">Eqn 2.5</div>

where, R_m is the volumetric hydrogen formation rate as calculated from the Gompertz equation (mL H_2 h^{-1}); V_O is the initial volume of the fermentation broth (L) and X_O is the initial biomass concentration (g biomass L^{-1}).

CHAPTER THREE
RESULTS & DISCUSSION

3.1 Characterization of Raw Sludge

The characterization of Pak Maya Baker's Yeast Industry wastewater treatment plant aerobic sludge was conducted. Stock sludge samples from the industry were taken at different periods of the study. Solid concentrations in these stock sludges were determined and it was observed that it slightly changed. The analysis of some parameters were carried out triplicate and the results of raw sludge characterization are given in Table 3.1. The average solid concentration was 33 ± 2 g L^{-1}. Total COD concentration was about $22\pm$ g L^{-1} but soluble COD concentration in filtrate was 570 mg L^{-1}. Total sugar concentration in filtrate was 216 mg L^{-1} in average. The most important metals in hydrogen production are Mo^{+2} and Fe^{+2}. As seen in the Table, Mo concentration was 4 mg kg^{-1} and Fe was 5.3 g kg^{-1} which were enough to meet required Mo^{+2} and Fe^{+2} concentrations for hydrogen gas production by dark fermentation. Hydrogen generating organisms require high sugars or organic substances for high gas production. However, total sugar concentration and soluble COD concentrations in the liquid phase indicate that there is no enough carbon or sugar for hydrogen gas generation by dark fermentation. On the other hand, total COD concentration is considerably high and it could be a good source of organic substances for the hydrogen gas production if it can be converted into the readly biodegradable form for microorganisms. One of the solutions to achieve this is to hydrolyze the sludge. The following sections contain three different hydrolysis approaches as single stage acid and high temperatures, sequential acid-heat treatment to increase the available organic substance concentrations for hydrogen gas production by dark fermentation.

Table 3.1 Pak-Maya Baker's yeast industry aerobic wastewater treatment plant waste sludge characterization.

Parameters	Raw Sludge		
TCOD (mg L^{-1})	21600	17600	28000
SCOD (mg L^{-1})	516.4	639.8	578.1
Total Sugar (mg L^{-1})	216	217	216
Protein (mg L^{-1})	375	375	375
pH	7.76		
SS (g L^{-1})	33±2		
tCOD (g L^{-1})	22+1		
Zn (mg kg^{-1})	139		
Ba (mg kg^{-1})	211.2		
Cu (mg kg^{-1})	95		
Fe (mg kg^{-1})	5348		
Mg (mg kg^{-1})	2866		
Mn (mg kg^{-1})	312		
Mo (mg kg^{-1})	4		
Na (mg kg^{-1})	8784		
PO$_4$-P (mg L^{-1})	27.3		
TS (g L^{-1})	33.656	34.94	
VS (g L^{-1})	23.524	27.524	
SS (g L^{-1})	33	32	35

3.2 Hydrolysis of Sludge and Hydrogen Gas Production by Dark Fermentation

3.2.1 Single Stage Acid Hydrolysis

Single stage acid hydrolysis of treatment sludge was carried out by using concentrated sulfric acid to adjust the hydrolysis pH between pH=2 and pH=6. Suspended solid concentration stock sludge sludge was 33±2 g L^{-1} and it was not diluted before hydrolysis reaction. After pH adjustment to the required value, sludge was stirred for 24 h on magnetic strrier to obtain homogenous conditions. pH of hydrolysis reaction was monitored for the first 4 h and at the end of the 24 h. At least 9 samples were taken during hydrolysis period between 15 min and 1440 min. The first eight samples represent the hydrolysis period between 15 min and 240 min. The last sample was taken at the end of 24 h (1440 min). Collected samples were santrifuged and TOC; COD, total sugar (TS), NH$_4$-N, PO$_4$-P and protein

concentrations in the liquid phase were determined. The experiments were designed accoding to two factor factorial design. pH and hydrolysis time were the two factors with different levels.

Variations of COD, TOC and TS (total sugar) concentrations with hydrolysis pH and time are given in Tables 3.2-3.3-3.4, respectively. Concentrations of the released products increased considebaly in the first 90 minutes of the hydrolysis reaction. No substantial release of organic substances and nutrients into the liquid phase was observed for the further hydrolysis period and product concentrations remained almost constant. If the concentrations after 24 hours were accepted as the maximum organic matter concentrations, which could be reached after hydrolysis at different pH values, then it could be concluded that 60% to 100% of COD and 70% to 90% of TOC recovery can be achieved within the first 90 minutes of hydrolysis. The increase in the concentration of total sugar started in the first 45th minutes of hydrolysis reaction. Total sugar concentration remained almost constant after 4 hours of hydrolysis for pH=2-5 and only 10% improvement was obtained for further hydrolysis period. However, TS did not significantly changed at pH=6 for the total hydrolysis period and remained around 200 mg L^{-1}.

Table 3.2 Varitaion of soluble COD concentration with time and pH in single stage acid hydrolysis.

COD mg L^{-1}	Time, min								
pH	15	30	45	60	90	120	180	240	1440
2	1972	1729	1681	1778	2112	2177	2436	2500	2759
	1875	1827	1827	1924	2241	2306	2500	2565	2889
3	1778	1681	1584	1875	1918	1853	2047	2047	2306
	1535	1584	1681	1632	2047	1918	1918	1982	2565
4	1681	1827	1729	1972	2021	2069	1972	2555	2069
	1778	1778	1924	1827	2264	2021	2166	2021	2166
5	1476	1476	1671	2124	1768	1865	2092	2092	2901
	1476	1541	1678	1800	1703	1800	1800	1994	2448
6	1541	1379	1606	1444	1606	1541	1509	1574	1785
	1541	1638	1703	1574	1606	1638	1671	1671	1930

Table 3.3 Varitaion of soluble TOC concentration with time and pH in single stage acid hydrolysis.

TOC, mg L^{-1}	Time, min								
pH	15	30	45	60	90	120	180	240	1440
2	423	397	437	427	459	453	458	453	501
	420	394	438	427	456	457	455	453	496
3	319	302	313	325	333	323	363	372	411
	321	299	317	322	330	320	362	373	413
4	281	282	278	282	288	284	309	314	398
	276	284	277	280	288	281	308	315	390
5	250	267	256	261	261	259	264	265	290
	251	260	252	259	258	261	258	269	286
6	176	161	165	176	190	205	238	233	246
	176	161	165	176	189	202	235	233	241

Table 3.4 Varitaion of soluble total sugar concentration with time and pH in single stage acid hydrolysis.

TS, mg L^{-1}	Time, min								
pH	15	30	45	60	90	120	180	240	1440
2	275	273	341	367	358	387	352	393	478
	273	286	343	343	367	355	355	396	454
3	245	231	246	229	214	269	303	287	267
	274	205	253	224	242	246	285	306	319
4	212	209	196	214	214	236	260	282	358
	185	223	194	205	199	240	282	284	341
5	207	187	181	208	199	212	230	221	208
	225	175	203	196	208	248	200	226	203
6	205	144	135	127	175	195	167	157	227
	216	162	147	153	187	229	190	170	269

The effects of pH and hydrolysis time on COD, TOC and total sugar (TS) concentrations were also presented in Figures 3.1-3.2-3.3, respectively. As shown in the figures, when the hydrolysis conditions became more acidic (pH=2), the released organic matter concentration increased. Hydrolysis time also had a positive effect on released organic matter concentration. Hydrolysis pH and contact time positively interact with each other resulting in increasing in organic substance concentration in

the liquid phase or hydrolysate. The maximum COD, TOC and TS concentrations were observed at pH=2 and t=1440 minutes hydrolysis with the concentrations as COD=2800 mg L^{-1}, TOC=500 mg L^{-1} and TS= 470 mg L^{-1}.
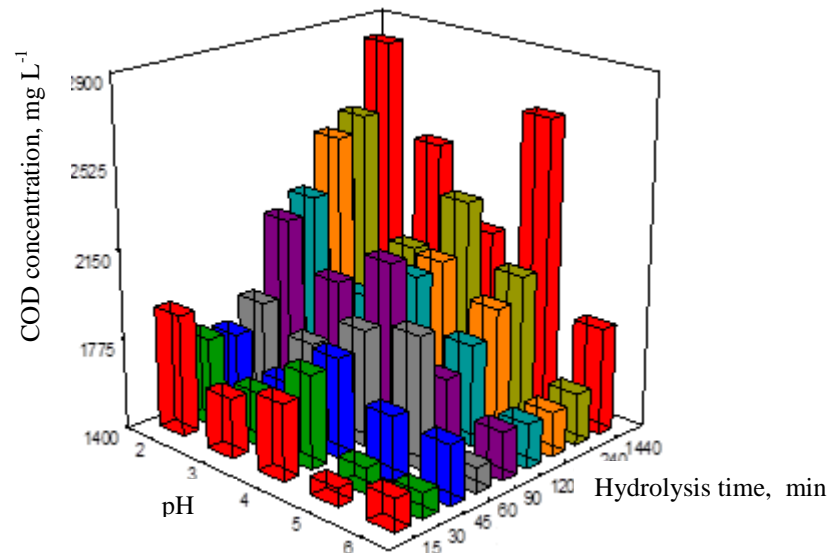
Figure 3.1 Variation of soluble COD concentration with hydrolysis pH and time.
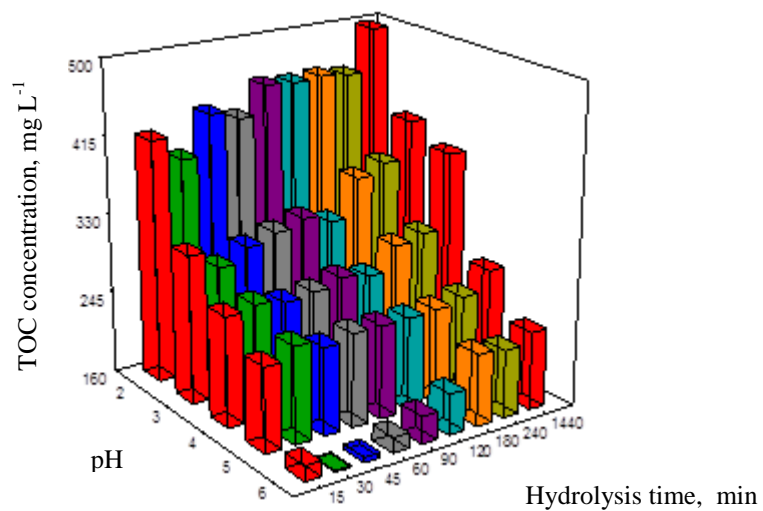
Figure 3.2 Variation of soluble TOC concentration with hydrolysis pH and time.
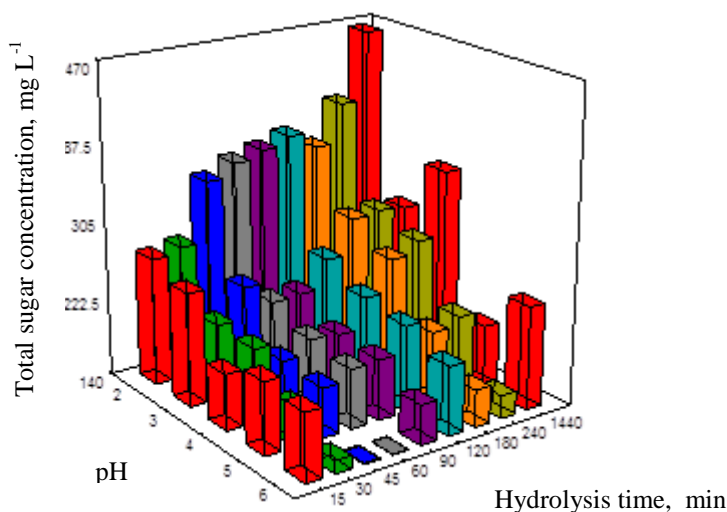
Figure 3.3 Variation of total sugar concentration with hydrolysis pH and time.

The statistical analysis (ANOVA) of the single-stage acid hydrolysis for COD, TOC and TS are given in Table 3.5-3.6-3.7, respectively. Variance analysis showed that pH and hydrolysis time are the two factors that significantly the release of organic matter from sludge. In addition, the interaction between pH and time was significant ($\alpha=0,05$). Increasing the contact time under acidic conditions affected the hydrolysis positively and provided an increase in organic matter content in hydrolysate. Design Expert Software program was used to determine the optimum hydrolysis conditions that maximize COD, TOC and TS concentrations and the results are presented in Figure 3.4. The numbers in the figure represent "desired level" meaning that degree of achievement in reaching the maximum values of the responses. In other words, If the desired level is "1" than there is at least one combination of factors that maximize all responses. The lower values in the figure represent that the conditions are getting away from the maximum concentrations of the responses. The maximum desired level obtained after optimization was 0.96 which indicates that pH=2 and t=1440 min give almost the maximum concentrations for three responses. The improvement in the organic matter concentration was around 10% after 240 min reaction time. Therefore, t= 240 min can be selected as maximum reaction period. However, the desired level for hydrolysis conditions as pH=2 and t= 240 min was 0.80 indicating that not all responses were maximized and may not be considered as optimum hydrolysis conditions. Nevertheless, t=240 min

provides a substantial decrease in hydrolysis time with acceptable level of hydrolysis. For the sake of the economic process and to be practical, t=240 minutes can be accepted as enough hydrolysis period to reach almost maximum concentrations of the organic substances in hydrolysate.

Table 3.5 Variance analysis of hydrolysis pH and time for COD concentration (ANOVA, α=0.05).

Source	Sum of Squares	df	Mean Square	F Value	p-valueProb > F	Evaluation
Model	9176564	44	208558.3	13.34696	< 0.0001	significant
pH	3021985	4	755496.2	48.34895	< 0.0001	significant
Time	4525946	8	565743.3	36.20547	< 0.0001	significant
pH-Time interaction	1628634	32	50894.8	3.257078	< 0.0001	significant
Pure Error	703165.8	45	15625.91			
Cor Total	9879730	89				

Table 3.6 Variance analysis of hydrolysis pH and time for TOC concentration (ANOVA, α=0.05).

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	Evaluation
Model	682847.3	44	15519.26	3150.567	< 0.0001	significant
pH	608441.3	4	152110.3	30879.94	< 0.0001	significant
Time	58490.19	8	7311.273	1484.263	< 0.0001	significant
pH-Time interaction	15915.85	32	497.3704	100.9713	< 0.0001	significant
Pure Error	221.6637	45	4.925861			
Cor Total	683069	89				

The concentrations of protein, NH_4-N and PO_4-P in the hydrolysate at different hydrolysis pH and period are given in Figure 3.5-3.6-3.7, respectively. NH_4-N concentration did not vary significantly with time and pH. It was between 205-250 mg L^{-1} at pH range of pH=2-6 and hydrolysis period of 15 min to 1440 min. (Figure

3.5). ANOVA test support this results that pH and time are not significat factors (α=0.05) for the release of NH_4-N content of sludge to the liquid phase.

Table 3.7 Variance analysis of hydrolysis pH and time for total sugar concentration (α=0.05).

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	Evaluation
Model	456498	44	10374.96	48.68625	< 0.0001	significant
pH	320654.9	4	80163.73	376.182	< 0.0001	significant
Time	80499.99	8	10062.5	47.22	< 0.0001	significant
pH-Time interaction	55343.1	32	1729.472	8.115843	< 0.0001	significant
Pure Error	9589.421	45	213.0983			
Cor Total	466087.4	89				
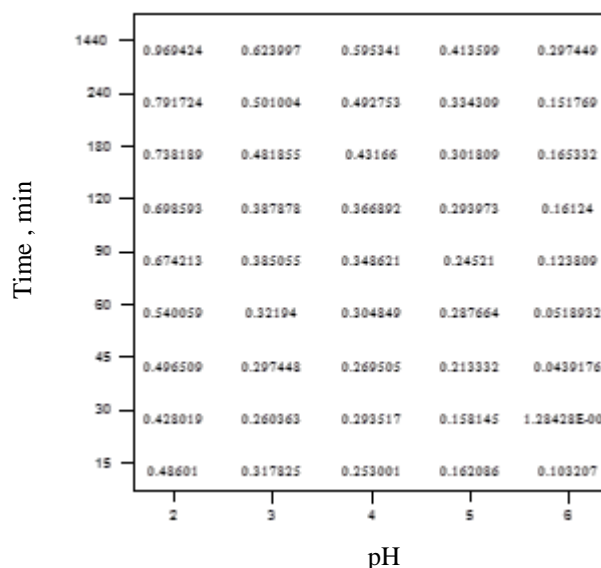
Figure 3.4 Maximization levels of COD, TOC and total sugar (TS)
concentrations in hydrolysate at different pH and hydrolysis time.

PO$_4$-P analyses were carried out on the samples taken at t= 15 min, t=240 min, and t=1440 min to indicate the initial, 4th h and final concentrations obtained during hydrolysis. PO$_4$-P concentration decreased with increasing hydrolysis pH. At low pH values (pH= 2-3), the concentration reached to around 500 mg L^{-1}, but it decreased to

around 50 mg L^{-1} at pH= 6 (Figure 3.6). No substantial effect of hydrolysis period on PO$_4$-P concentration was observed. Release of PO$_4$-P from cell structure to the liquid phase is completed in the first 15 minutes of the hydrolysis.

Variation of final protein concentration with hydrolysis time and pH is depicted in Figure 3.7. Increase in pH value resulted in a substantial decrease in protein concentrations. Under acidic conditions (pH= 2-3), cell membrane is strongly disrupted and then efficient release of intercellular and cell membrane bound proteins is achieved. Moderate acidic condition (pH=4-5) resulted in relatively lower protein concentrations in the hydrolysate. Finally, hydrolysis at pH=6 provided almost no protein release form microbial cells in sludge. Variance analysis (ANOVA) indicated that pH significantly affects (α=0.05) protein concentration in the hydrolysate but, time does not.
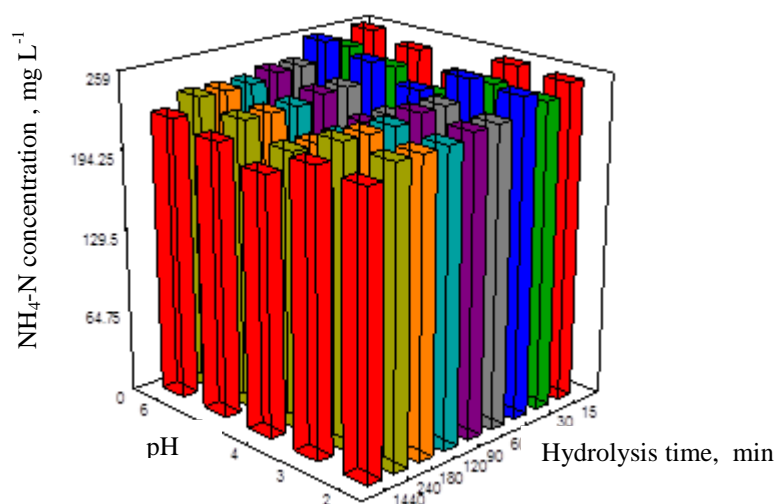
Figure 3.5 Variation of NH$_4$-N concentration with pH and time in single–stage acid hydrolysis of sludge.

The results of the single stage acid hydrolysis indicated that pH has got significant effect on most of the desired products as organic matters and nutrients. Hydrolysis time has got signifcant effect on carbon content but not for the nutrient and protein contents. Based on the optimization study, the most effective hydrolysis conditions in single stage acid hydrolysis were determined as pH=2 and t= 1440 min.
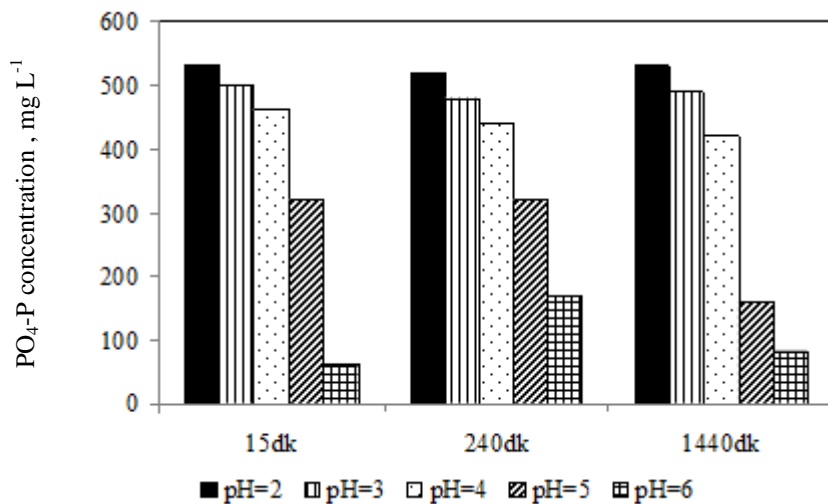
Figure 3.6 Variation of PO_4-P concentration with pH and time in single–stage acid hydrolysis of sludge.
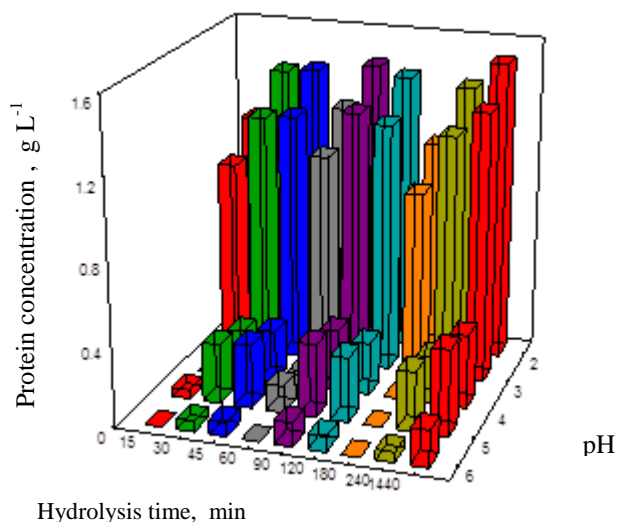
Figure 3.7 Variation of protein concentration with pH and time in single–stage acid hydrolysis of sludge.

3.2.1.1 Hydrogen Gas Production by Dark Fermentation

The hydrolysis under acidic conditions and long hydrolysis periods could cause formation of unknown and unwanted substances which could interfere with the fermentation for hydrogen gas production. Therefore, the effect of hydrolysis conditions on hydrogen gas production was investigated. Two sets of hydrolysis

under the same conditions were carried out. In one of them, liquid phase named as filtrate was used as fermentation media. In the other, liquid and solid phases were not separated after hydrolysis, the mixture called as "sludge" was used as fermentation media. These two substrates were subjected to dark fermentation under the same conditions. A parallel experiment was conducted with raw sludge without applying any hydrolysis process to compare if hydrolysis of sludge has got positive effect on hydrogen production.

3.2.1.1.1 Hydrogen Gas Production from Filtrate. Filtrate of hydrolysis reaction at different pH values at the end of t=1440 min hydrolysis time was collected and then hydrogen gas production studies were conducted. Total gas, hydrogen gas percentage were monitored daily and hydrogen gas volume was calculated (Eqn 2.1). Variations of cumulative hydrogen gas volumes (CHV) with time for filtrate of hydrolyzed sludge at different pH values are depicted in Figures 3.8-3.12. Cumulative hydrogen gas volume obtained on filtrate of pH=2 was only V_{H2}= 5 ml. The volume increased to about V_{H2}=11 ml at hydrolysis pH value of pH=4 and pH=5. But cumulative hydrogen volume decreased to 5 ml for the filtrate obtained at pH=6. Almost the same production trends were observed in all filtrates used. In the first 20 hours of fermentation period a rapid increase in hydrogen gas volume was observed and then it remained almost constant with the maximum volume of production.
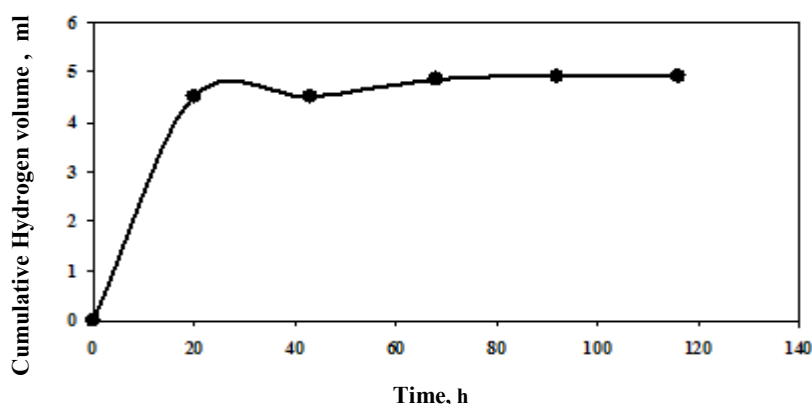
Figure 3.8 Variation of cumulative hydrogen gas volume with time for filtrate
of sludge hydrolyzed at pH=2 for t=1440 min.
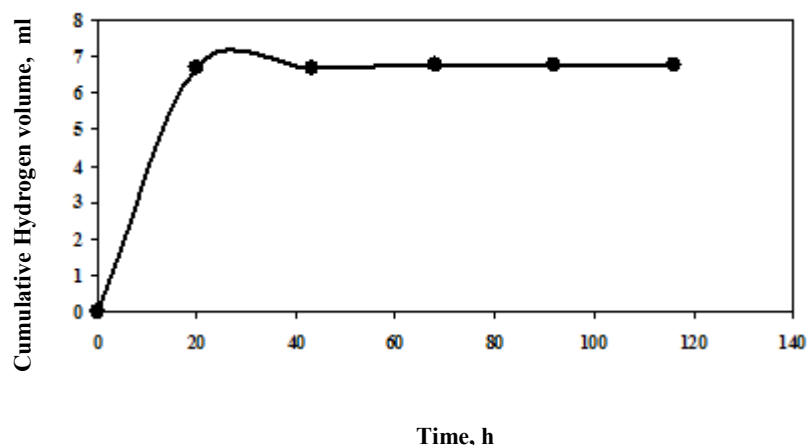
Figure 3.9 Variation of cumulative hydrogen gas volume with time for filtrate of sludge hydrolyzed at pH=3 for t=1440 min.
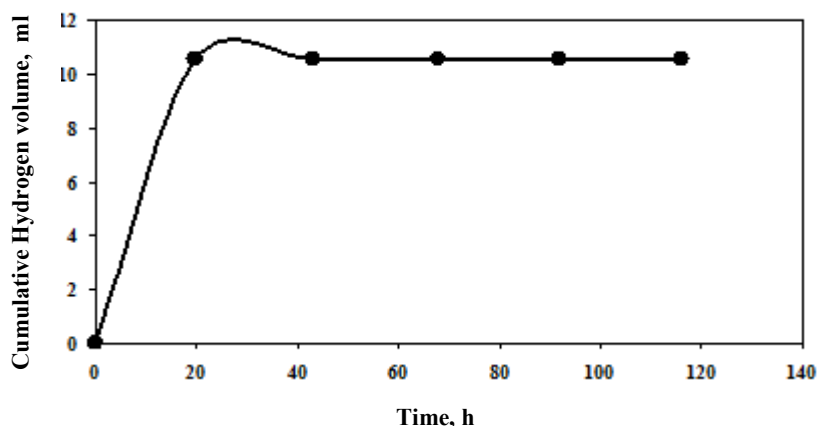
Figure 3.10 Variation of cumulative hydrogen gas volume with time for filtrate of sludge hydrolyzed at pH=4 for t=1440 min.
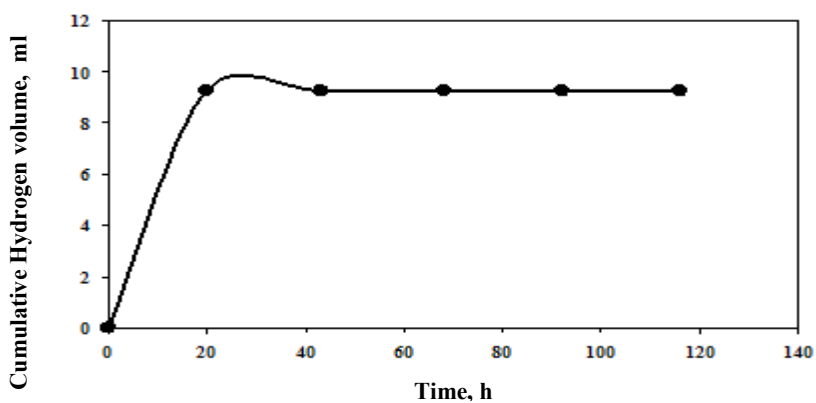
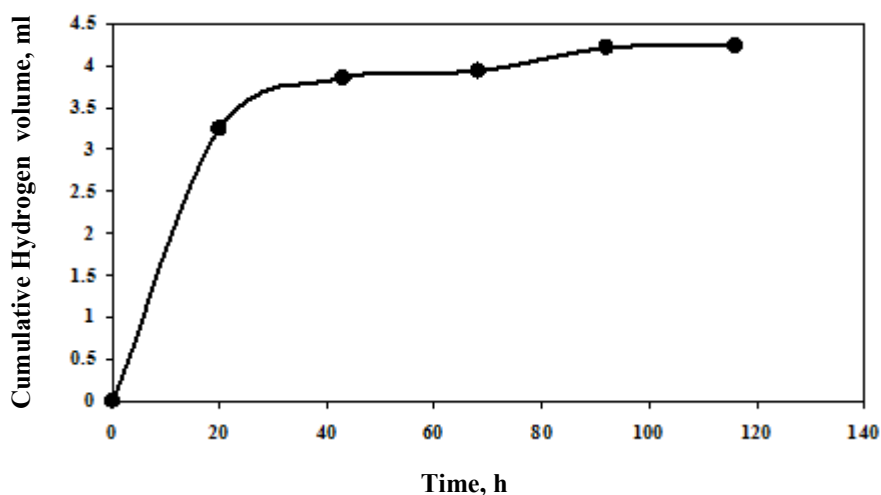Figure 3.11 Variation of cumulative hydrogen gas volume with time for filtrate of sludge hydrolyzed at pH=5 for t=1440 min.
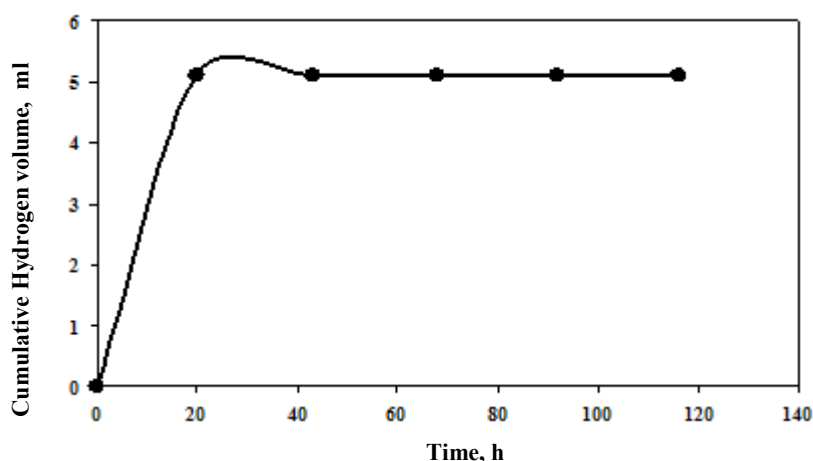
Figure 3.12 Variation of cumulative hydrogen gas volume with time for filtrate of sludge hydrolyzed at pH=6 for t=1440 min.

Dark fermentation in the filtrates of acid hydrolysis reaction was conducted without adding any nutrient, trace elements or growth promoting substances. The purpose was to determine the hydrogen production potentials from these filtrates without external nutrient addition. In addition, it was aimed to determine removal of organic substances and nutrients from the filtrate to understand if treatment of sludge can be achieved simultaneously. For that purpose, removal of carbon, nutrient and protein during fermentation was monitered. Figure 3.13 indicates initial and final COD concentrations obtained in the filtrates of different hydrolysis pHs. A slight decrease in COD concentrations were observed for filtrates of pH=2 and pH=6. But, unfortunaley, an increase in final COD concentration was obtained in the filtrates of pH= 3-4-5. This increase in COD was suspicious and unexpected. The expected result was to get partial but not complete removal of COD. It is partial, because, some of the organic substances are used for growth or metabolized up to CO_2, but the main fraction is biotransformed to organic acids which constitute COD. The analyses were carried out duplicate to minimize the analytical errors. No substantial difference between replicates was observed. Therefore, the increase in the COD concentration during fermentation was not due to analytical errors. It could be because of metabolic problems occurred during fermentation, contribution of organic content of the inoculum due to inactivation. Similar increase in TOC concetrations were observed for the same reasons (Figure 3.14). Total sugar consumption was slightly different then that of COD and TOC. About 40 to 50 % of the sugar was consumed

by the microorganisms. Maximum sugar utilizations were obtained as 51% at pH=3 and 55% at pH= 6 (Figure 3.15).
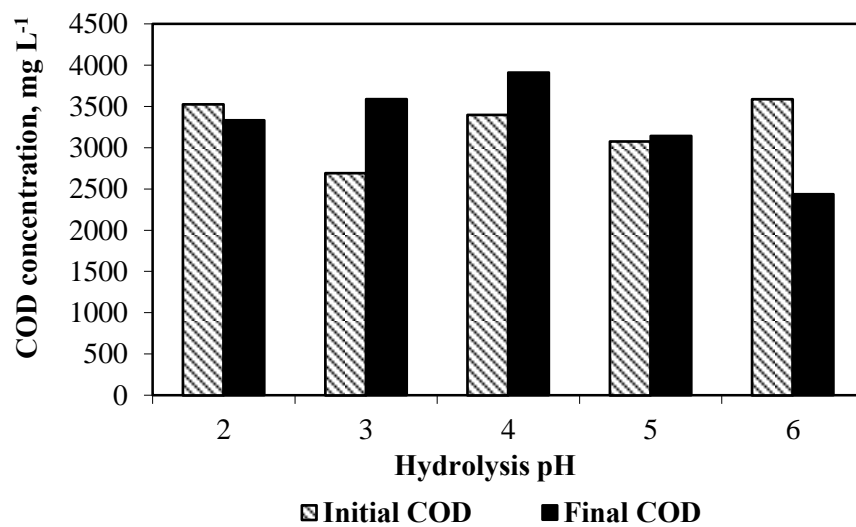
Figure 3.13 Initial and final COD concentrations in the filtrate of sludge hydrolyzed at different pH values.
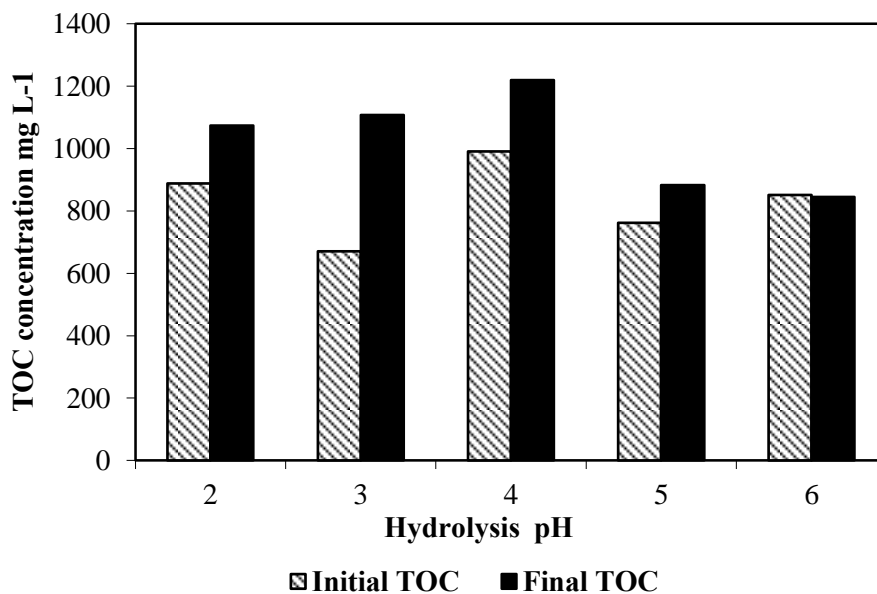
Figure 3.14 Initial and final TOC concentrations in the filtrate of sludge hydrolyzed at different pH values.
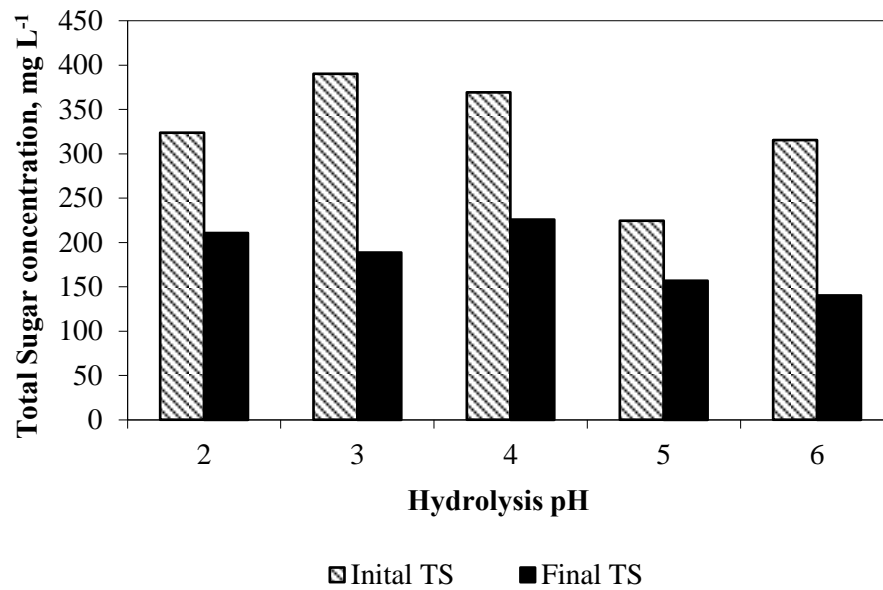
Figure 3.15 Initial and final TS concentrations in the fitrate of sludge hydrolyzed at different pH values.
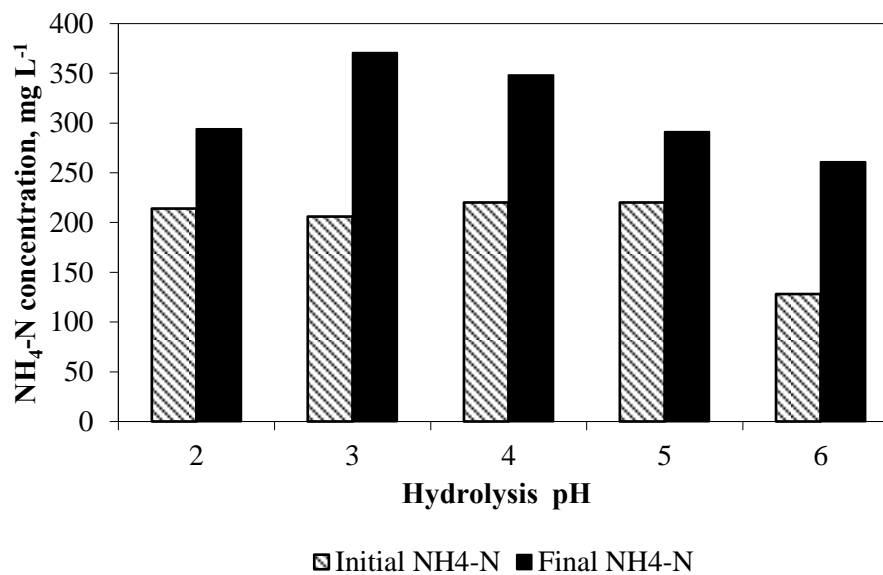
Figure 3.16 Initial and final NH_4-N concentrations in the filtrate of sludge hydrolyzed   at different pH values.

After hydrolysis of sludge at different pH values, initial NH_4-N concentration in filtrate varied between 128-220 mg L^{-1} (Figure 3.16). Similar to COD and TOC concentrations, final nitrogen concentrations increased up to 450 mg L^{-1}.

In the filtrate of sludge hydrolyzed at pH=6, NH_4-N concentration increased from 128 mg L^{-1} to 261 mg L^{-1} resulting in 100% increase.

Initial and final PO_4-P concentrations in the filtrate are given in Figure 3.17 PO_4-P in the filtrates of pH=4, pH=5 and pH=6 was consumed during fermentation period. The maximum consumption was obtained as 71% in the filtrate of pH=5. Figure 3.18 depicts variation of initial N/C and P/C ratios in filtrates. The maximum N/C ratio was observed at hydrolysis pH=3 and pH=5 (N/C=0.3). P/C ratio decreased gradually with the increase in hydrolysis pH. At pH=2, P/C ratio was 0.2 whereas it was 0.04 at pH=6. These results indicated that the fermentation media was nutrient sufficient.
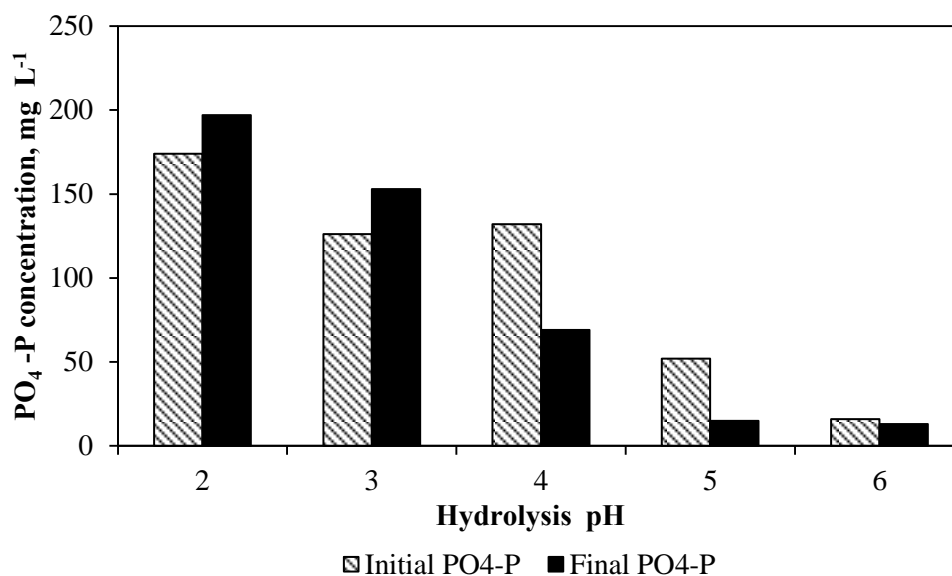
Figure 3.17 Initial and final PO_4-P concentrations in the filtrate of sludge hydrolyzed at different pH values.

Initial and final total volatile organic acid concentrations (TVFA) in filtrate is given in Figure 3.19. The initial concentration was around 500 mg L^{-1}. The main source of initial TVFA is the TVFA content of the inoculums. After the fermentation, there was a significant increase in TVFA. Final TVFA concentration was about 1200-1300 mg L^{-1} in the filtrates of hydrolyzed sludge at pH=2 and pH=3. The highest concentration was observed as 1400 mg L^{-1} in the filtrate of pH=4. However,

it decreased to TVFA=926 mg L^{-1} in the filtrate of pH=6. TVFA production varies with the amount of active microorganism, initial organic matter concentration and pH of fermentation. These parameters also have effect on the type of produced organic acid. High organic substance concentration will result in high organic acid concentration. In addition, TVFA formation will be high if dominant microbial culture is *Clostridum sp.* On the other hand, homoasetogens could generate acetic acids from CO_2 and H_2 formed during fermentation and then cause formation of extra acetic acid without H_2 generation. pH of fermentation media determines the type of the organic acid, as well. It has been clearly stated in the literature that, pH= 4.0-4.5 shifts the microbial metabolism to the formation of butyric acid rather than acetic acid. The results obtained from the fermentation experiment indicated that there was high TVFA formation. Hydrogen gas production was in parallel to TVFA formation. High culumative hydrogen gas production was obtained at high TVFA concentrations as observed at hydrolysis pH values of pH=3 and pH=4.
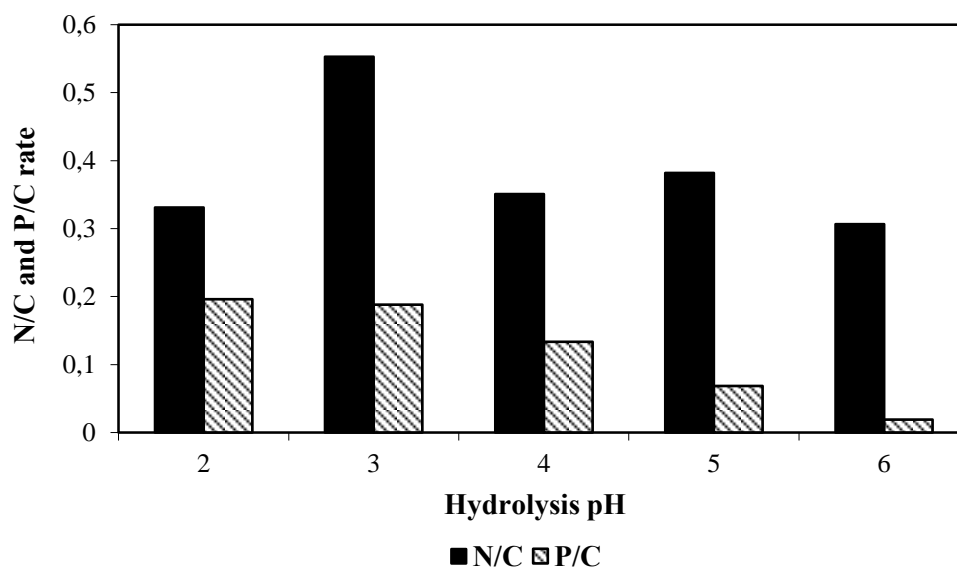
Figure 3.18 Variation of initial N/C and P/C ratios in the filtrate with hydrolysis pH.
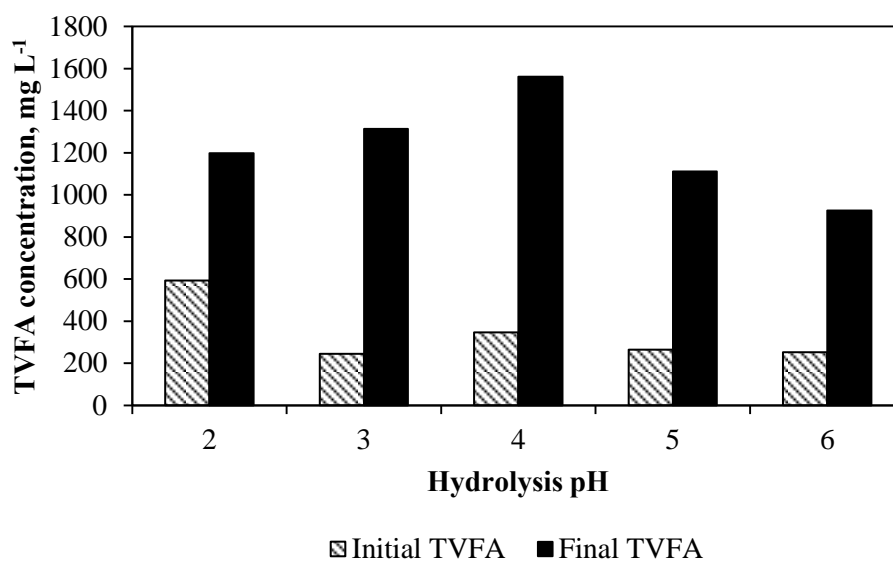
Figure 3.19 Initial and final TVFA concentrations in the filtrate of sludge hydrolyzed
at different pH values.

3.2.1.1.2 Hydrogen Gas Production from Sludge. Dark fermentation was applied to
hydrolized sludge as substrate. The purpose of this study was to determine whether
the content of the organic matter of solid phase after acid hydrolysis would have
contribution to hydrogen gas generation. Hydrogen production from raw sludge
without any pretreatment was conducted in parallel to experiments with hydrolyzed
sludge to compare the effect of hydrolysis on hydrogen production. Variations of
cumulative hydrogen volumes produced from raw sludge and hydrolyzed sludge at
different pHs with time are given in Figure 3.20 and 3.24. CHV did not vary
substantially with the pH of hydrolysis reaction. The maximum CHV was obtained
as V_{H2}=5.5 ml from sludge hydrolyzed at pH=4. In general, hydrogen production was
completed in the first 20 hours of fermentation and no further increase in the volume
was observed for the rest of fermentation period.

COD and total sugar concentrations were monitored for all hydrolyzed sludges.
Variation of COD and TS concentration with time for sludge hydrolyzed at pH=3
was given in Figure 3.25. TS concentration was 400 mg L^{-1} in the beginning of
fermentation. But, an increase to 600 mg L^{-1} at t= 20 h was observed. This result
indicates that some of the carbohydrates in cell structure of sludge were further
hydrolyzed to simple sugars by microbial action.

Figure 3.20 Variation of cumulative hydrogen gas volume with time for sludge hydrolyzed at pH=2 for t=1440 min.
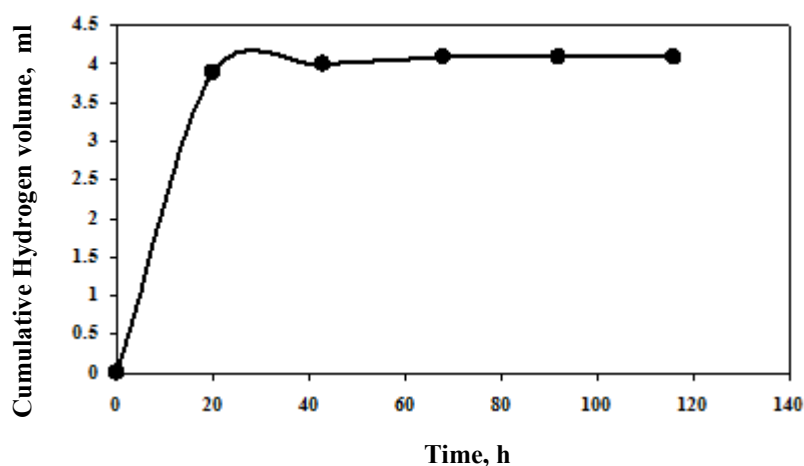
Figure 3.21 Variation of cumulative hydrogen gas volume with time for sludge hydrolyzed at pH=3 for t=1440 min.

COD concentration steadly increased from 3900 mg L^{-1} to 5100 mg L^{-1} for the first 20 hours of fermentation. But, second increasing tendency starting from 92nd h was observed and finally, COD concentration reached to 6150 mg L^{-1} at the end of fermentation period. There was the same increasing trend for sludge hydrolyzed at pH=3 and pH=4. However, there was no increase in COD concentration for raw sludge. Instead COD concentration decreased from 3000 mg L^{-1} to 1800 mg L^{-1} during fermentation. A slight decrease in COD concentration was also observed for sludges hydrolyzed at pH=5 and pH=6. TOC concentrations obtained after the fermentation were similar to the changes in COD (Figure 3.26).
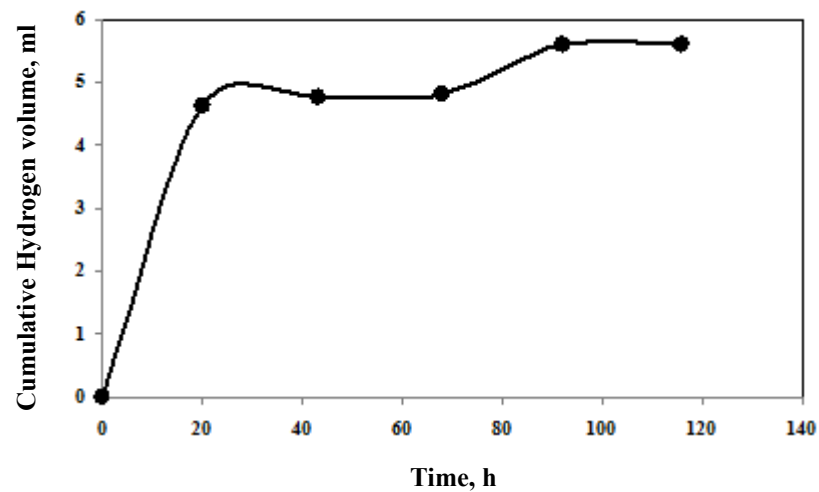
Figure 3.22 Variation of cumulative hydrogen gas volume with time for sludge hydrolyzed at pH=4 for t=1440 min.
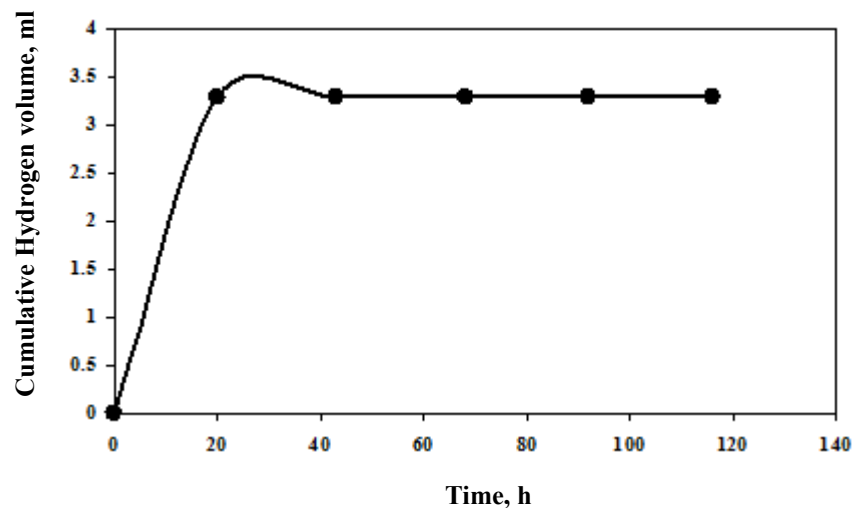
Figure 3.23 Variation of cumulative hydrogen gas volume with time for sludge hydrolyzed at pH=6 for t=1440 min.

The reason for this increase in organic matter concentration can be explained as follows; the partially disturbed cells after acid treatment are further hydrolyzed by the microorganisms in the fermentation media. This continuing hydrolysis results in release of organic matters from the cells and then increase in the COD or TOC concentration in the liquid phase. No organic matter content release from the sludge was observed for pH=5 and pH=6, since cell deterioration was not effective at these pHs during acid hydrolysis.

Time, h
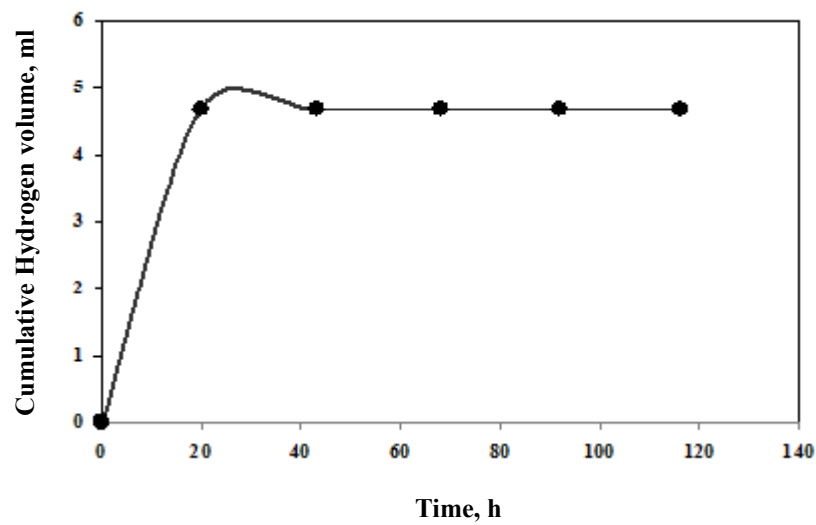
Figure 3.24 Variation of cumulative hydrogen gas volume with time for raw sludge
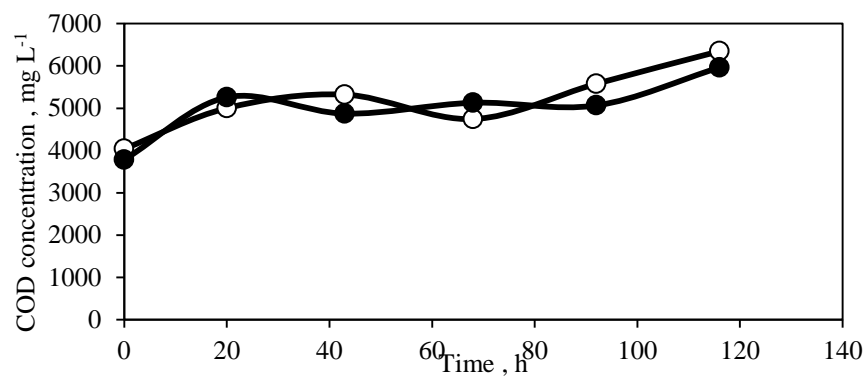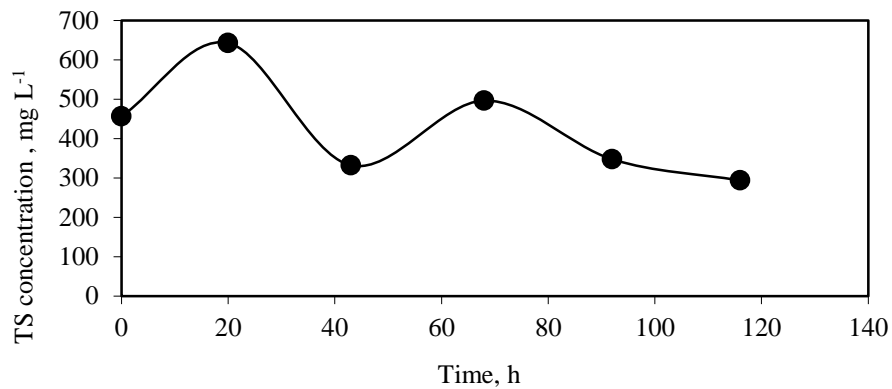
Figure 3.25 Variations of COD and total sugar concentrations with time during

dark fermentation of sludge hydrolyzed at pH=3.
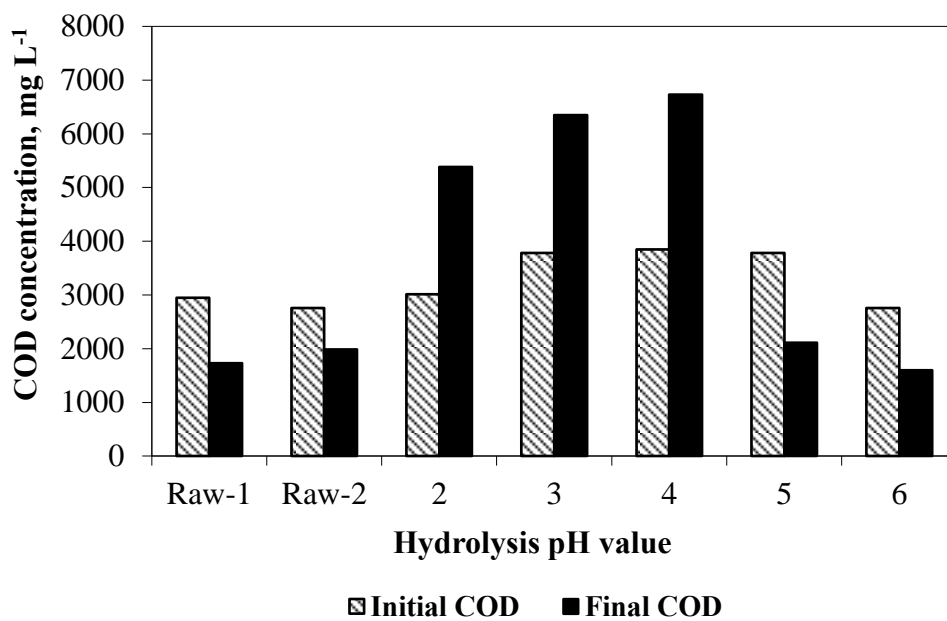
Figure 3.26 Initial and final COD concentrations in hydrogen gas production from sludge hydrolyzed at different pH values.
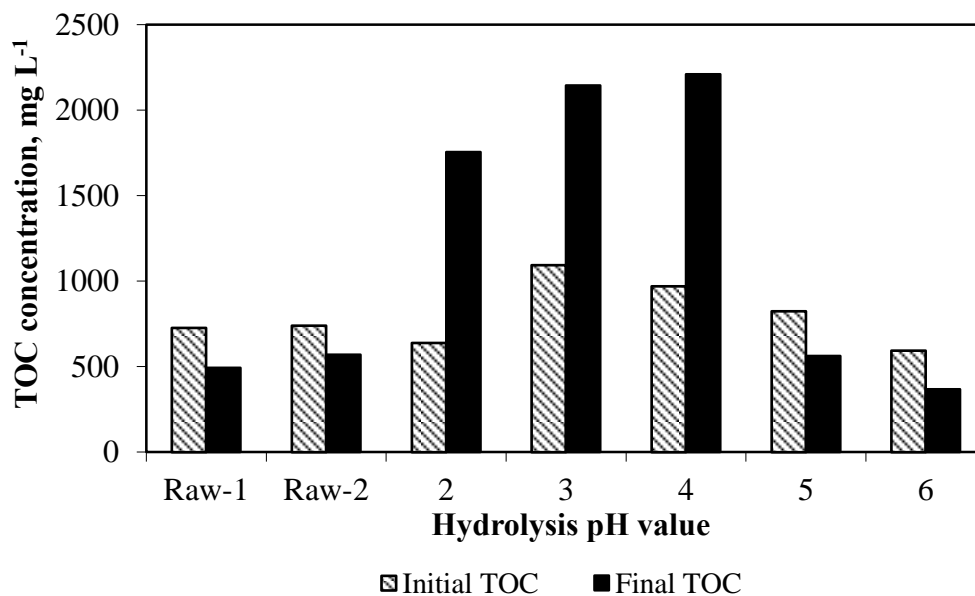
Figure 3.27 Initial and final TOC concentrations in hydrogen gas production from sludge hydrolyzed at different pH values.

Figure 3.28 indicates initial and final total sugar concentrations in dark fermentation of hydrolyzed sludges. As seen from the Figure, total sugar concentration decreased for all hydrolyzed sludges. The same results were observed during hydrogen production from filtrate. Although COD and TOC were not removed and even increased, sugar was consumed in fermentation of filtrate. The same observation was valid for fermentation of sludge. This result shows that among the organic matters, released after the hydrolysis, only sugar is consumed for hydrogen production. The sugar consumption in filtrate was between 30% and 55%, but it was only 5% to 47% for sludge. The main reason of the low sugar consumption for sludge was release of sugar to the liquid phase during fermentation due to further microbial hydrolysis of cells which was partially hydrozed by acid previously.
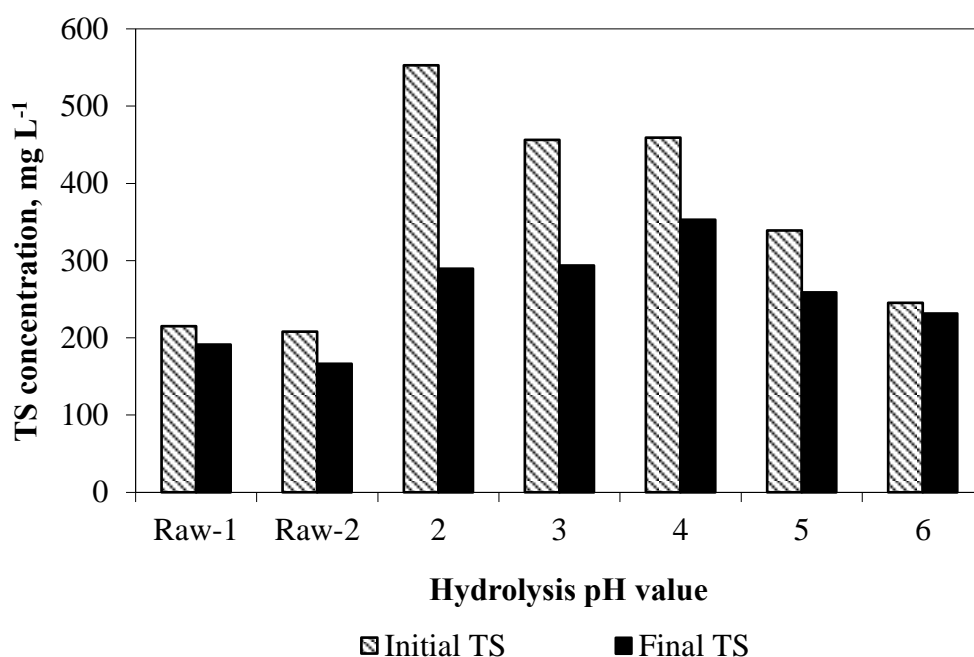
Figure 3.28 Initial and final total sugar concentrations in hydrogen gas production from sludge hydrolyzed at different pH values.

The initial and the final NH_4-N and PO_4-P concentrations of sludge fermentation for hydrogen gas production are given in Figure 3.29 and Figure 3.30, respectively. Final NH_4-N concentration was higher than initial concentration. Similar results was observed in fermentation of filtrate. Higher concentrations of NH_4-N after

fermentation can be explained as further hydrolysis of proteins by inoculum. The decrease in the concentration of PO_4-P after fermentation showed that PO_4-P was used by the microorganisms (Figure 3.30).
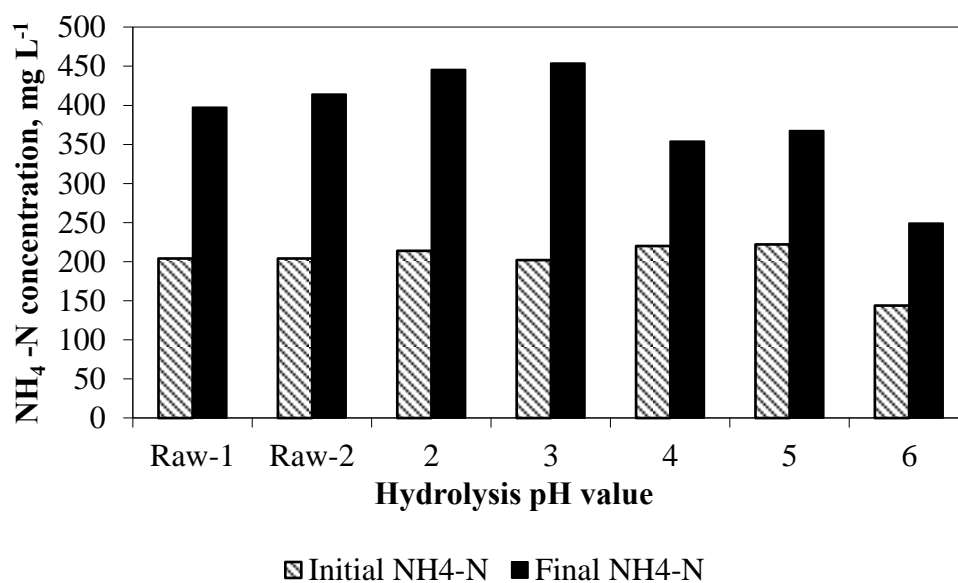
Figure 3.29 Initial and final NH_4-N concentrations in hydrogen gas production from sludge hydrolyzed at different pH values.
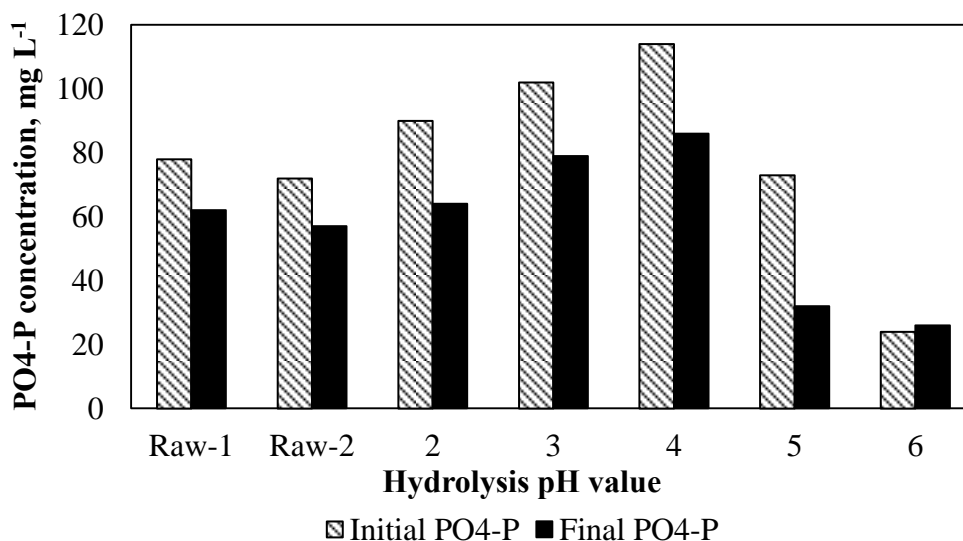
Figure 3.30 Initial and final PO_4-P concentrations in hydrogen gas production from sludge hydrolyzed at different pH values.

Initial and final total volatile fatty acid concentrations (TVFA) are depicted in Figure 3.31. Initial TVFA concentrations varied between 160 and 300 mg L^{-1} due to contribution of TVFA content of inoculum. There was a significant increase in TVFA after the fermentation. The highest TVFA concentrations were observed in the fermentation of the hydrolyzed sludge at pH=2 and pH=3. Maximum TVFA concentration as 2900 mg L^{-1} was obtained for sludge hydrolyzed at pH=2. TVFA production was parallel to the sugar consumptions.
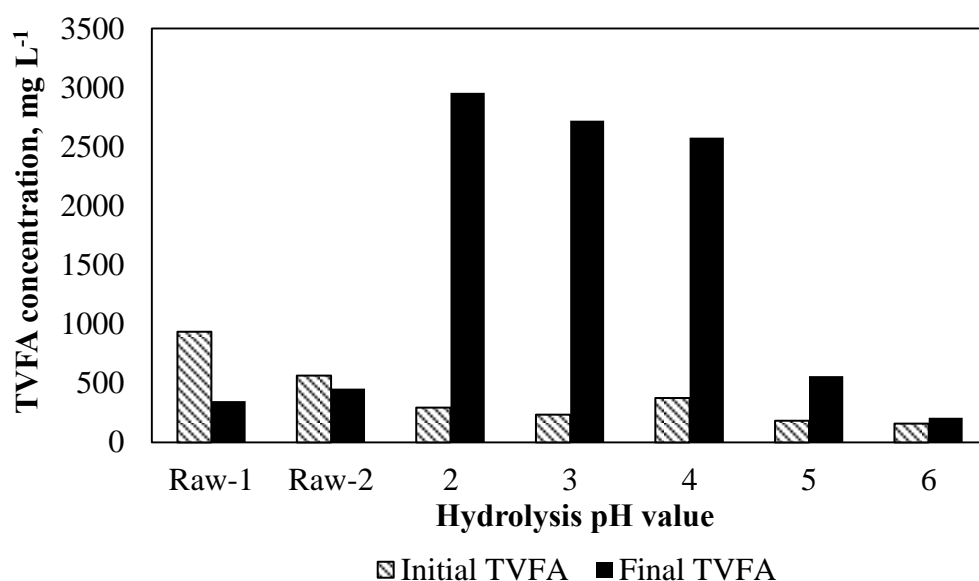
Figure 3.31 Initial and final TVFA concentrations in hydrogen gas production from sludge hydrolyzed at different pH values.

3.2.1.1.3 The Potentials and Yields of Hydrogen Production from Filtrate and Sludge. Gompertz Equation was used to calculate the production potential and production rate. Hydrogen production rate and potential from the filtrate is given in Table 3.8. According to the Gompertz Equation, maximum hydrogen production potential was observed as V=10.5 ml for filtrate of the sludge hydrolyzed at pH=4. Similarly, production rate reached to Rm=6.4 ml h^{-1}. Calculated production potentials (P) were parallel to the cumulative hydrogen volumes observed in experiments. The lag phase was comperatively shorter for hydrolysis pH values at which high production volumes obtained.

In the studies of hydrogen production from sludge, the highest production potential was V=5.2 ml for the sludge obtained at pH=4. Production rate was also lower compared to that of filtrate. Lag phases for hydrogen production from sludge was relatively higher in comparison to lag phases observed for filtrate. These results show that filtrate is a more suitable substrate to produce hydrogen gas from sludge by dark fermentation.

Table 3.8 Gompertz Equation constants of hydrogen gas production from filtrate of acid hydrolysis.

Hydrolysis pH value	P (ml)	R_m (ml hour^{-1})	λ (hour)
2	4.8	0.4	3.6
3	6.7	0.8	3.7
4	10.5	6.4	1.5
5	9.3	4.3	1.7
6	5.1	3.5	3.4

Table 3.9 Gompertz Equation constants of hydrogen gas production from sludge of acid hydrolysis.

Hydrolysis pH value	P(ml)	R_m (ml hour^{-1})	λ (hour)
2	4.1	0.2	3.6
3	4.1	0.4	4.0
4	5.2	0.4	3.5
6	3.3	2.0	1.5
Raw-1	4.7	3.2	3.1
Raw-2	3.3	2.0	1.4

Hydrogen production yields were calculated by considering cumulative hydrogen gas volumes and substrate consumptions. Organic matter parameters that would be used for hydrogen gas production were COD , TOC and total sugar. Both in sludge and in filtrate, total sugar consumption was observed but there was no COD and TOC removal. This result shows that some of the organic matters released after hydrolysis cannot be consumed by microorganisms. Only carbonhydrates or simple sugars generated after hydrolysis or during microbial hydrolysis were used for hydrogen production. For this reason, production yield was calculated by considering

only total sugar consumption. The yield was calculated as mmol H_2 g^{-1} TS^{-1} and ml H_2 g^{-1} TS^{-1}. As seen in Figure 3.32 and 3.33, production yield in filtrate increased when hydrolysis pH was increased form pH=2 to pH=5. Maximum production yield was observed as Y=24 mmol g^{-1} TS^{-1} (596 ml g^{-1} TS^{-1}) at pH=5. The maximum yield obtained from fermentation of sludge was Y=41 mmol g^{-1} TS^{-1} at pH=6. Although filtrate was stated as better substrate, the yield of hydrogen production in sludge was higher. The main reason for this result is low difference between initial and final sugar concentrations. It seems that sugar consumed during fermentation of sludge is diverted to hydrogen gas production. However, as it was mentioned in pervious sections, hydrolysis of carbohydrates in sludge continuous during fermentation and provides extra available sugar which can not be detected during fermentation (Figure 3.25). Therefore, artificially high yield of hydrogen production was observed from sludge. Total sugar consumption was calculated as 262 mg L^{-1} by considering increases and decreases in the sugar concentration of the samples taken during dark fermentation of sludge hydrolyzed at pH=6. If this sugar consumption is taken into account, hydrogen production yield decreases to 2.4 mmol g^{-1} glucose^{-1} from 41 mmol g^{-1} glucose^{-1} value. For this reason, it can be concluded that the real yield of production from sludge is lower than calculated one.
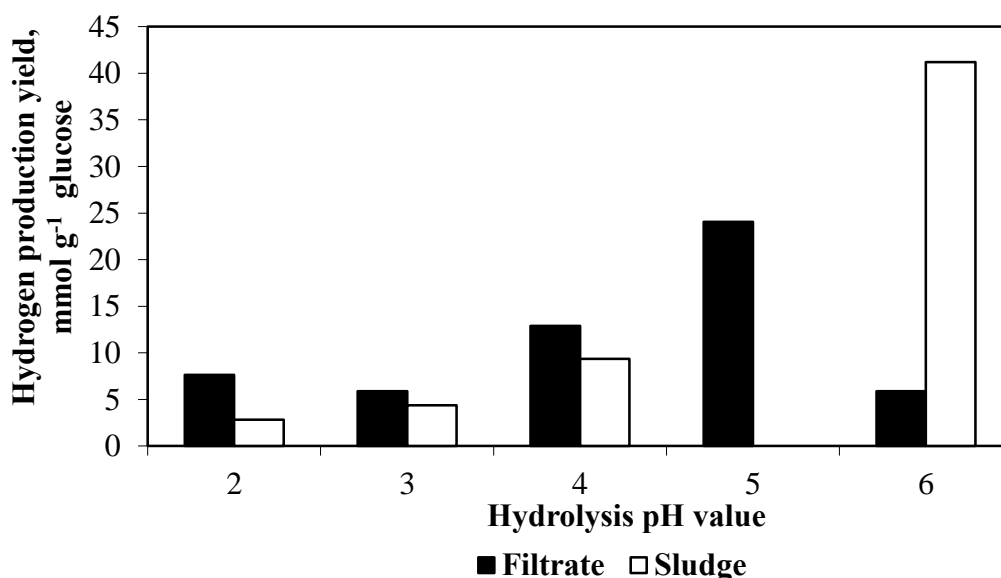
Figure 3.32 The effect of hydrolysis pH on hydrogen gas production yields (mmol g^{-1} TS^{-1}) form sludge and filtrate.

Figure 3.33 The effects of hydrolysis pH on hydrogen gas production yields (ml g^{-1} TS^{-1}) from sludge and filtrate.

3.2.2 Hydrolysis of Treatment Sludge by Single-stage Heat Treatment and Hydrogen gas production

3.2.2.1 Hydrolysis by Single Stage Heat Treatment

In single-stage heat treatment, waste aerobic sludge was exposed to high temperatures in autoclave. Statistical experimental design method used in the study was 3 level-2 factor factorial design. Two factors were hydrolysis temperature (T) and hydrolysis period (t). The levels in temperature were T= 60º C, T=100ºC and T=135ºC. The levels in time were t=30 mins., t=45 mins and t=60 mins. The number of replicate in each experimental points was two. Stock sludge concentration was 33±1 g L^{-1}. COD, TOC ,total sugar, NH_4-N, PO_4-P and protein concentrations before and after hydrolysis and in the raw sludge were determined.

COD, TOC and total sugar concentrations observed in raw sludge and after heat treatment are given in Table 3.11. Average organic matter concentrations in the liquid phase before the hydrolysis were TS=54 mg L^{-1} COD= 750 mg L^{-1} and

TOC=193 mg L^{-1}. Organic matter concentrations observed in the filtrate after the hydrolysis were significantly higher than that in raw sludge. Total sugar concentration was doubled when temperature was increased from T=60º C to T=100º C at hydrolysis time of t= 30 min. Increasing temperature to T=135º C for t=30 min, provided 8 times higher total sugar concentration. The incerase in COD and TOC concentrations were in parellel to the changes in TS concentration from different hydrolysis temperatures and times. The results show that hydrolysis temperature and time are the two important factors that positively affect hydrolysis efficiency. The statistical analysis of experimental results (Table 3.10, 3.12, 3.13) also supports this conclusion. ANOVA analysis indicated that hydrolysis temperature and time significantly affect (α=0.05) the released organic matter concentration and the interaction between these two factors is significant.

Variations of COD, TOC and total sugar concentrations with temperature and time were depicted in Figures 3.34-3.35-3.36, respectively. The increase in temperature and time also caused a significant increase in organic matter concentration. The highest COD concentrations were obtained at treatment temperature of T=135º C. Maximum COD, TOC and TS were obtained as 9800 mg L^{-1}, 2300 mg L^{-1} and 1338 mg L^{-1} respectively at t= 60 mins and T=135º C. Hydrolysis by heat treatment provided at least 3 fold increase in organic matter content compared to single stage acid hydrolysis (TS= 478 mg L^{-1}, COD= 2800 mg L^{-1}).

Table 3.10 ANOVA table for COD concentration of sludge hydrolysis by heat treatment.

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Model	1.46E+08	8	18189378	1583.786	< 0.0001	significant
Time	15753483	2	7876742	685.8437	< 0.0001	significant
Temperature	1.28E+08	2	63912851	5565.02	< 0.0001	significant
Time-Temperature Interaction	1935837	4	483959.3	42.1393	< 0.0001	significant
Pure Error	103362.7	9	11484.75			
Cor Total	1.46E+08	17				

Table 3.11 Factorial design experimental points and observed TS, COD and TOC concentrations in hydrolysis of sludge by heat treatment.

Experiment no	Time, min	Temperature, ^{0}C	Total sugar, (mg L^{-1})	COD, (mg L^{-1})	TOC, (mg L^{-1})
1	30	60	156	993	324
			161	993	350
2	30	100	353	1987	594
			320	2051	595
3	30	135	924	6412	2063
			938	6541	2057
4	45	60	230	1827	341
			244	1698	348
5	45	100	460	3174	704
			478	3110	710
6	45	135	1056	7535	1945
			1096	7696	1951
7	60	60	326	2435	570
			344	2691	546
8	60	100	627	3910	844
			669	4102	832
9	60	135	1338	9683	2302
			1338	9875	2286
Raw Sludge			53	736	190
			55	800	197

Table 3.12 ANOVA table for TOC concentration of sludge hydrolysis by heat treatment.

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Model	9974833	8	1246854	12400.48	< 0.0001	significant
Time	214265.8	2	107132.9	1065.481	< 0.0001	significant
Temperature	9727896	2	4863948	48373.96	< 0.0001	significant
Time-Temperature interaction	32671.66	4	8167.916	81.23328	< 0.0001	significant
Pure Error	904.94	9	100.5489			
Cor Total	9975738	17				

Table 3.13 ANOVA table for TS concentration of sludge hydrolysis by heat treatment.

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Model	2729131	8	341141.4	1098.821	< 0.0001	significant
Time	270631.5	2	135315.7	435.8538	< 0.0001	significant
Temperature	2429907	2	1214954	3913.382	< 0.0001	significant
Time-Temperature interaction	28592.33	4	7148.083	23.02407	< 0.0001	significant
Pure Error	2794.152	9	310.4613			
Cor Total	2731925	17				
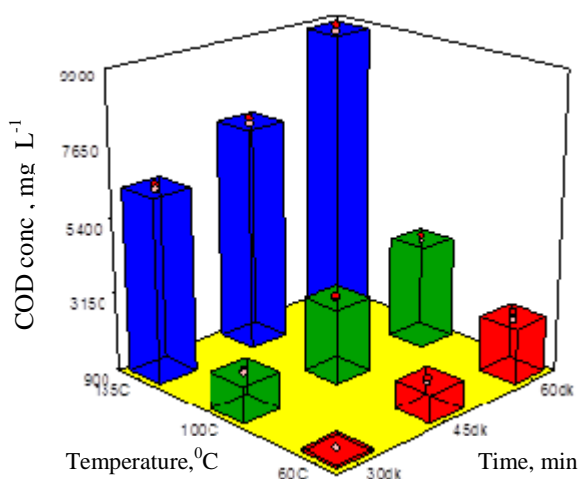
Figure 3.34 Variation of COD concentration with hydrolysis time and temperature.
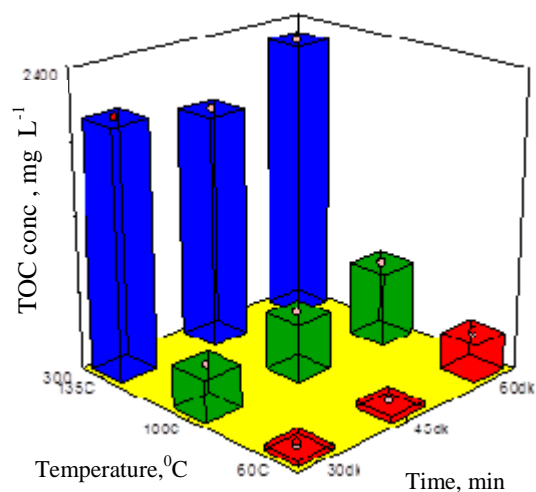
Figure 3.35 Variation of TOC concentration with hydrolysis time and temperature.

Concentrations of NH_4-N, PO_4-P and protein after heat teratment were also determined. Table 3.14 depicts experimental points and observed concentrations of three responses of hydrolysis by heat treatment. The effect of hydrolysis temperature and time on NH_4-N is depicted in Figure 3.37.
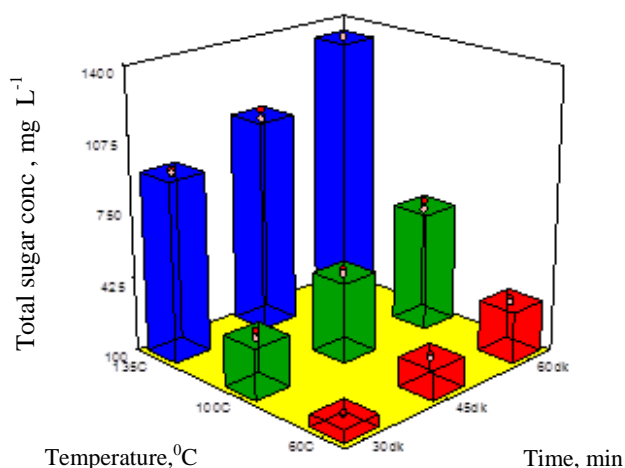
Figure 3.36 Variation of total sugar concentration with
hydrolysis time and temperature.

After hydrolysis reaction, the maximum NH_4-N concentration obtained in the filtrate
was about 240 mg L^{-1}, in average, under the hydrolysis conditions as t= 30 min and
T= 60 ^{0}C. Extending the hydrolysis time to t=60 min at T= 60 ^{0}C, caused a partial
decrese in NH_4-N to around 200 mg L^{-1}. Similarly, hydrolysis temperature affected
NH_4-N concentration negatively. Altough it was 240 mg L^{-1} at T= 60 ^{0}C (for t=30
min), substantially decreased to 140 mg L^{-1} at T= 135 ^{0}C. This nitrogen
concentration is even lower than the concentration in raw sludge. The results of
variance analysis indicated that these two factors have got significant (α=0.05) effect
on final nitrogen concentration in hydrolzed sludge (Table 3.15). Both factors have a
negative effect on NH_4-N concentration. The effect of tempertaure is more
pronounced than the effect of time. The interraction between these two factors is
significant too. Therefore, combined effects of the factors should be considered to
obtain maximum NH_4-N concentrations after hydrolysis.

Variation in protein concentration with hydrolysis time and temperature is given
in Figure 38. No protein was detected in the liquid phase of raw sludge. Therefore,
the protein concentrations observed in the hydrolysate are the results of
disintegration of cells by hydrolysis. As seen form the figure, there was no protein in
the hydrolysate at temperatures T= 60 ^{o} C and T=100 ^{o} C .

Table 3.14 Factorial design experimental points and observed NH$_4$-N, PO$_4$-P and protein concentrations in hydrolysis of sludge by heat.

Experiment no	Time, min	Temperature, ^{0}C	NH$_4$-N, (mg L^{-1})	PO$_4$-P, (mg L^{-1})	Protein, (mg L^{-1})
1	30	60	270	17	0
			220	19	0
2	30	100	216	22	0
			250	28	0
3	30	135	150	108	0.59
			135	66	0.63
4	45	60	229	28	0
			225	19	0
5	45	100	211	32	0
			213	36	0
6	45	135	135	108	0.77
			129	20	0.74
7	60	60	190	23	0
			216	22	0
8	60	100	204	39	0.063
			204	41	0.055
9	60	135	123	120	1.02
			123	108	0.92
Raw Sludge			201	19	0
			201	18.8	0

Extending the hydrolysis time from 30 min to 60 min at these temperatures did not provide a substantial release of proteins form cell structure. Fortunately, increasing the temperature to T=135º C had positive effect on protein concentration. Hydrolysis time had a slight effect in increasing the protein concentration at high temperature. It was 0.6 g L^{-1} at t= 30 min hydrolysis period and increased to 1 g L^{-1} with the incease in time to t=60 mins. at T=135 ^{0}C. Due to the disintegration of the cells at high temperature and time, release of intracellular and membrane bound proteins to liquid phase was provided.
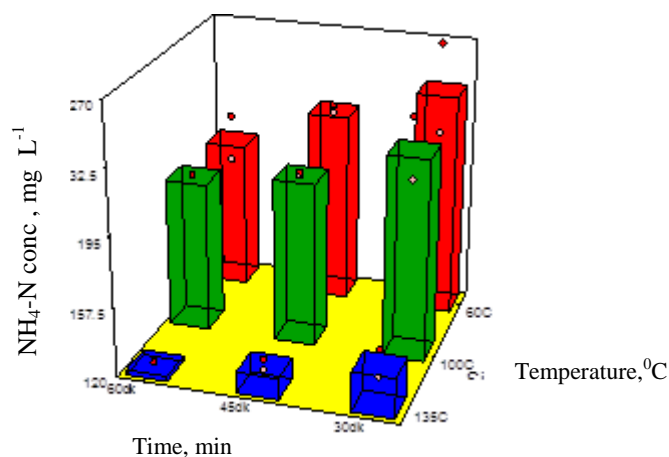
Figure 3.37 Variation of NH_4-N concentration with

hydrolysis time and temperature.

Table 3.15 ANOVA table for NH_4-N concentration in sludge hydrolysis by heat treatment.

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Model	34373.11	8	4296.639	16.76555	0.0001	significant
Time	2738.111	2	1369.056	5.342077	0.0296	
Temperature	31318.78	2	15659.39	61.10319	< 0.0001	significant
Time-Temperature interaction	316.2222	4	79.05556	0.308476	0.8653	
Pure Error	2306.5	9	256.2778			
Cor Total	36679.61	17				

The statistical analysis of the results (Table 3.16) show that hydrolysis time and temperature are the two factors that affect protein release in hydrolysis of sludge by heat treatment. The interaction between factors is also signifcant.

Variation of PO_4-P by hydrolysis time and temperature in single-stage heat treatment is depicted in Figure 3.39. The most significant increase in PO_4-P concentration obtained when temperature was rised form T=100º C to T=135º C.
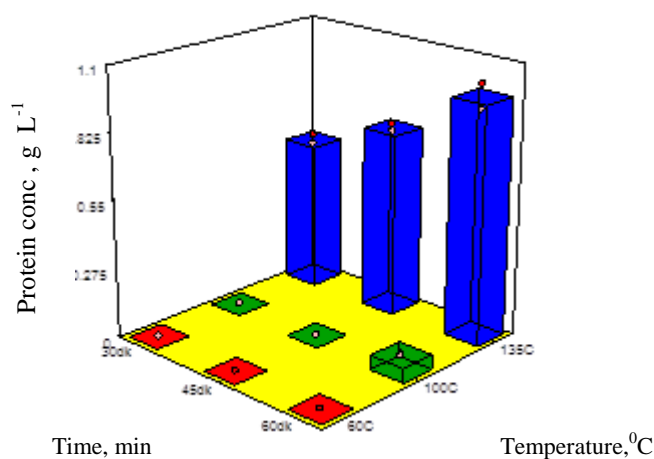
Figure 3.38 Variation of protein concentration with
hydrolysis time and temperature.

Table 3.16 ANOVA table for protein concentration in sludge hydrolysis by heat treatment.

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Model	2.499404	8	0.312426	447.601	< 0.0001	significant
Time	0.060369	2	0.030185	43.24451	< 0.0001	significant
Temperature	2.363529	2	1.181765	1693.073	< 0.0001	significant
Time-Temperature interaction	0.075505	4	0.018876	27.04346	< 0.0001	significant
Pure Error	0.006282	9	0.000698			
Cor Total	2.505686	17				

Similarly, a slight rise was obsreved when hydrolysis time was extended from t=30 min to t=60 min at T=100 ^{0}C and T=135 ^{0}C. However, the effect of time was not substantial at low hydrolysis temperatures (T= 60 ^{o}C). PO_4-P concentration was about 20g L^{-1} at different hydrolysis times and T=60º C. Time has slight effect on concentration at T= 100 ^{0}C. It was about 25 mg L^{-1} for t=30-min and increased up to 40 mg L^{-1} for t= 60 min. The most substantial effect of time was obsered at high tempertaures (T= 135º C). İncreasing time to t=60 mins from t=30 min at T=135 ºC hydrolysis tempertaure provided a rise in PO_4-P concentration from 85 mg L^{-1} to 114 mg L^{-1}.

ANOVA analysis indicated that hydrolysis temperature is a signficant factor wherase time is not.
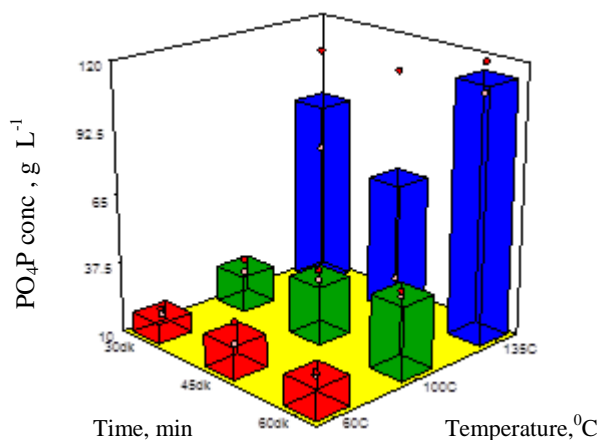
Figure 3.39 Variation of PO_4-P concentration with hydrolysis
time and temperature.

Tablo 3.17 ANOVA table for PO_4-P concentration in sludge hydrolysis by heat treatment.

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Model	18141.44	8	2267.681	4.167679	0.0238	significant
Time	1168.778	2	584.3889	1.074025	0.3817	
Temperature	15373.78	2	7686.889	14.12742	0.0017	significant
Time-Temperature interaction	1598.889	4	399.7222	0.734633	0.5910	insignificant
Pure Error	4897	9	544.1111			
Cor Total	23038.44	17				

3.2.2.2 Hydrogen Gas Production by Dark Fermentation

The sludges and filtrates obtained after heat treatment at different temperatures and periods were subjected to dark fermentation. Dark fermentation was also applied to raw sludge without any pretreatment.

3.2.2.2.1 Hydrogen Gas Production from Filtrate: After heat treatment, the sludge was centrifuged and solid phase was seperated. The obtained filtrate was used in dark fermentation. Hydrogen production studies were carried out at 37º C, mesofilic, conditions and at pH=7. Variation of cumulative hydrogen gas volumes with time for filtrates are depicted in Figures 3.40-3.48. Hydrogen gas production showed a rapid increase in the first 20 hours of the fermentation. Production slowed down or completely stopped for the rest of fermentation period. As seen in Figure 3.49, cumulative hydrogen volumes from filtrates obtained at different hydrolysis temperatures and periods were almost constant around 4 ml. Although there were significant differences in the concentrations of organic matters (COD, TOC and TS), these differences didn't have an effect on hydrogen gas production. As it was presented Figure 3.36, total sugar concentration varied between 230 mg L^{-1} and 1350 mg L^{-1} for the different hydrolysis conditions. The maximum concentration was obtained at t= 60 mins. and T=135º C. There was no variation in the hydrogen production volumes for the filtrates even though sugar concentrations in the filtrates were substantially different. It is accaptable to have low hydrogen volume at low sugar concentrations. But it is not to have 4 ml of hydrogen volume at 1350 mg L^{-1} total sugar concentration. These results show that carbon source wasn't used effectively in hydrogen gas production, no positive or negative relation between nutrient concentration and hydrogen production exist. The most possible conclusion could be a side- product formed during hydrolysis negatively affected the microbial activity or metabolic used followed in hydrogen production.

Initial and the final COD concentrations of filtrate fermentation are given in Figure 3.50. A slight decrease in COD concentration were observed after fermentation. COD removal efficiency was around 10 % in most of the filtrate. But it reached to 34% for the filtrate of hydrolysis reaction conducted at t=45 mins and T=60º C (experiment no:4). Total sugar consumption was much higher compared to COD removal (Figure 3.51) Sugar concentrations obtained at high temperatures (Experiment no:3-6-9 and T=135ºC were higher than the other conditions. Maximum sugar utilization was 83% and obtained from the filtrate of sludge hydrolyzed at T=135º C and t=45 min.
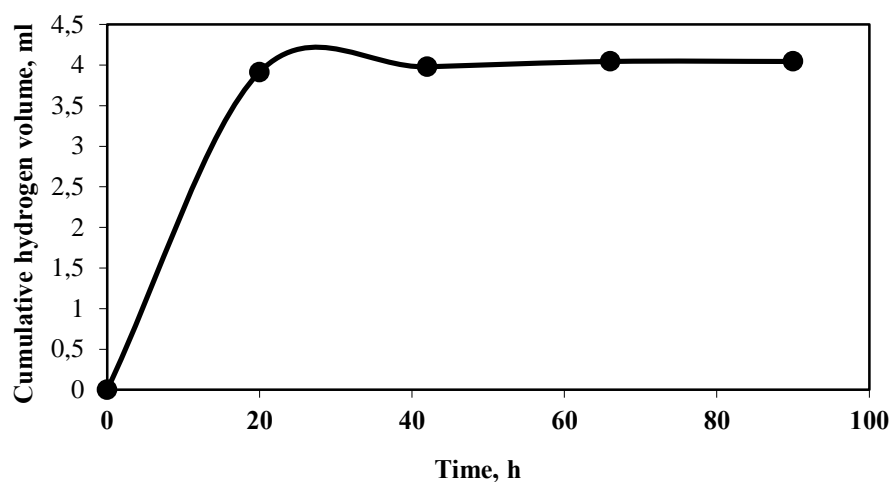
Figure 3.40 Variation of cumulative hydrogen gas volume with time for filtrate
obtained at T=60°C and t=30 min hydrolysis conditions.
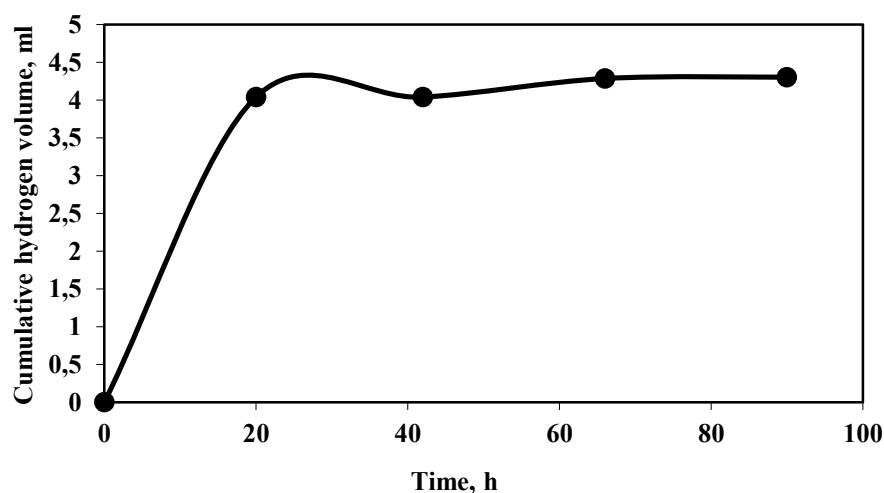
Figure 3.41 Variation of cumulative hydrogen gas volume with time for filtrate
obtained at T=100°C and t=30 min hydrolysis conditions.

NH_4-N concentrations rised at the end of the fermentation as it was observed in fitrates of acid hydrolysis (Figure 3.52). The increase in concentration was parallel to the increase in hydrolysis temperature. PO_4-P concentration also rised after the fermentation (Figure 3.53). The highest increase in PO_4-P in the filtrate obtained at low temperatures and in short hydrolysis times. TVFA concentrations, observed after the fermentation were parallel to sugar and COD consumption profiles (Figure 3.54).
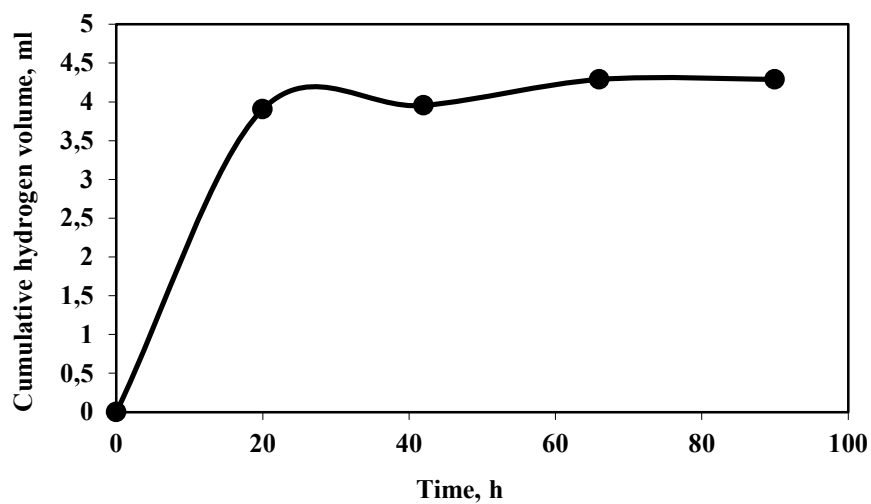
Figure 3.42 Variation of cumulative hydrogen gas volume with time for filtrate obtained at T=135°C and t=30 min hydrolysis conditions.
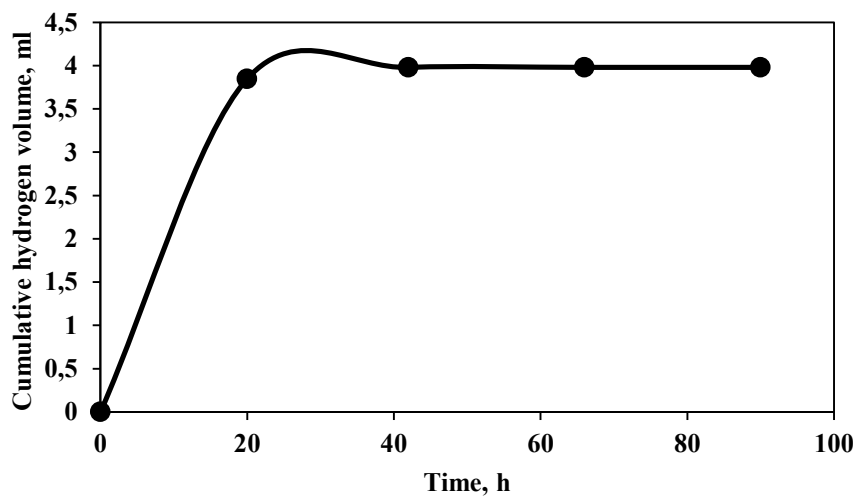
Figure 3.43 Variation of cumulative hydrogen gas volume with time for filtrate obtained at T=60°C and t=45 min hydrolysis conditions.

It was high in experiments 3, 6 and 9 at which high sugar consumption occured. The maximum TVFA concentration (1000 mg L^{-1}) was obtained in filtrate of hydrolyzed sludge at 135° C for different hydrolysis times (experiments no: 3-6 and 9).
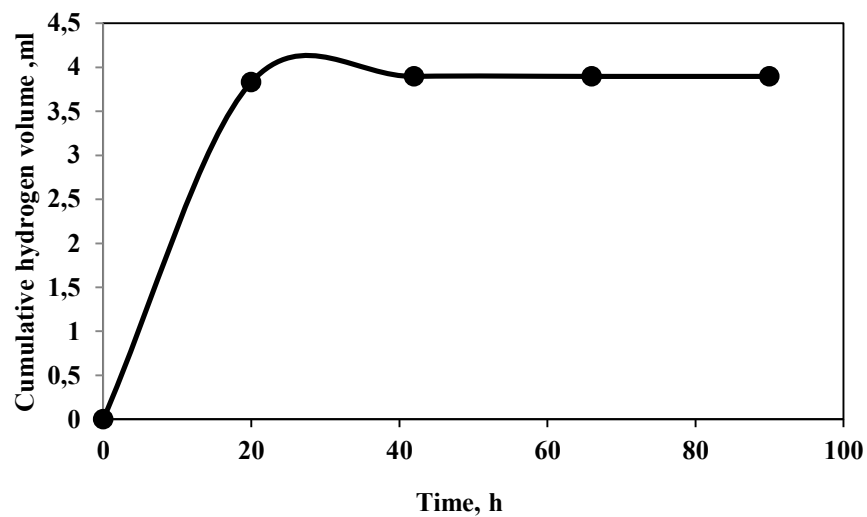
Figure 3.44 Variation of cumulative hydrogen gas volume with time for filtrate obtained at T=100°C and t=45 min hydrolysis conditions.
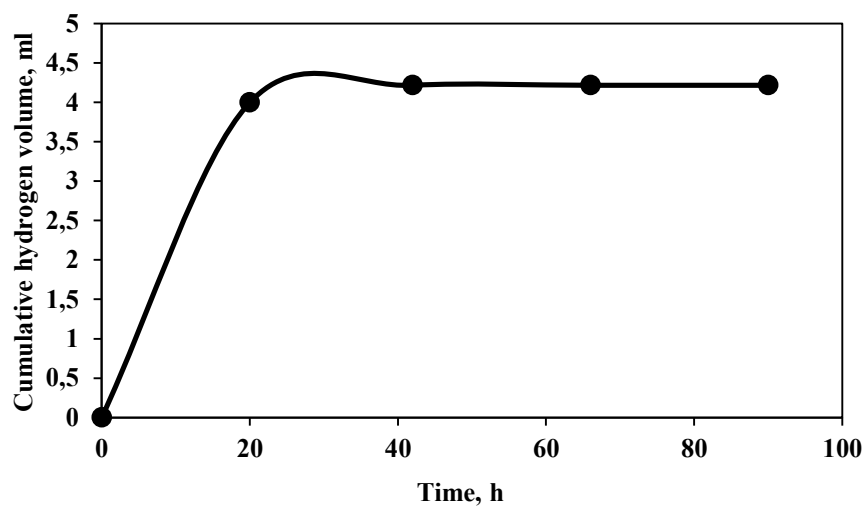
Figure 3.45 Variation of cumulative hydrogen gas volume with time for filtrate obtained at T=135°C and t=45 min hydrolysis conditions.
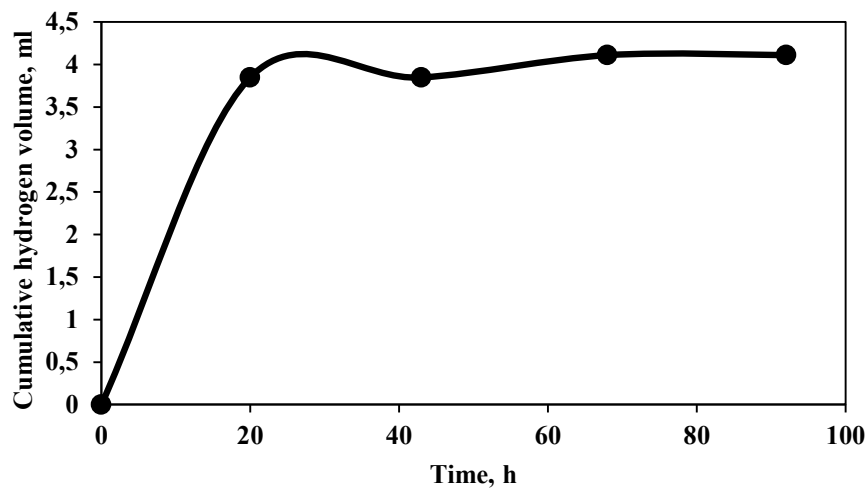
Figure 3.46 Variation of cumulative hydrogen gas volume with time for filtrate obtained at T=60°C and t=60 min hydrolysis conditions.
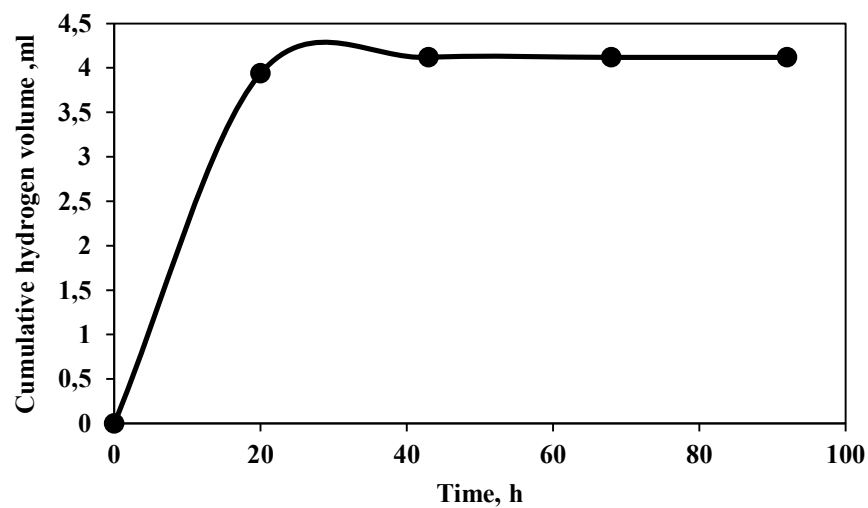
Figure 3.47 Variation of cumulative hydrogen gas volume with time for filtrate obtained at T=100°C and t=60 min hydrolysis conditions.
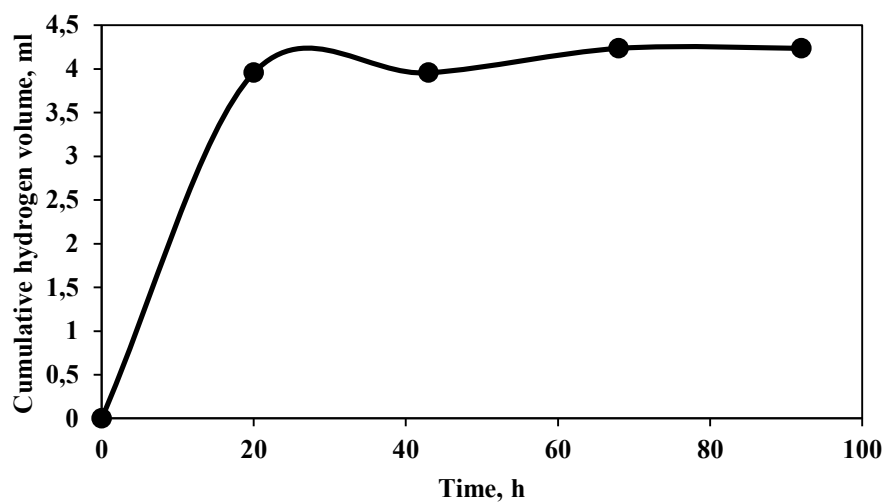
Figure 3.48 Variation of cumulative hydrogen gas volume with time for filtrate obtained at T=135°C and t=60 min hydrolysis conditions.
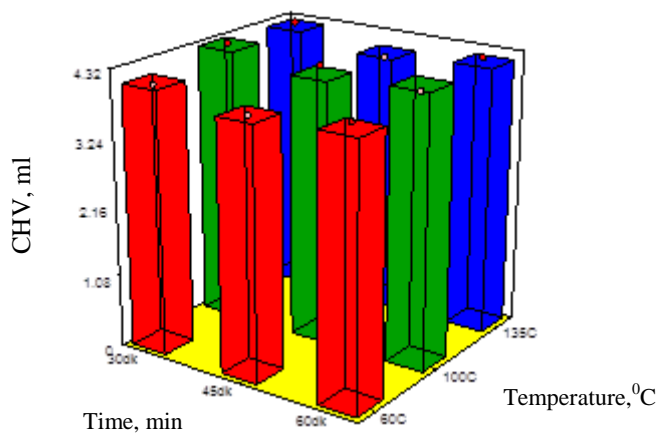
Figure 3.49 Effect of hydrolysis temperature and time on cumulative hydrogen gas volume obtained from fitrate.
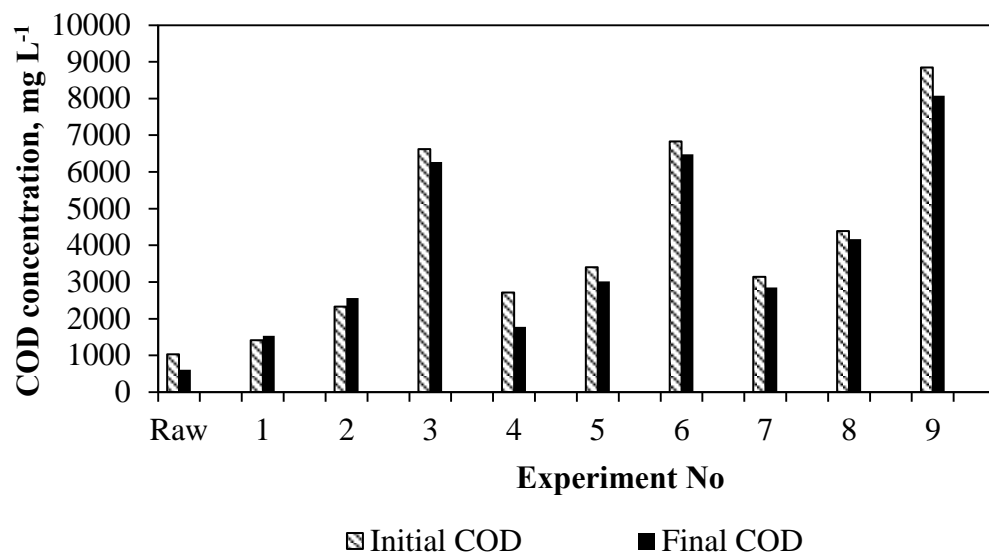
Figure 3.50 Initial and final COD concentrations in hydrogen gas production from filtrate obtained at different hydrolysis conditions.
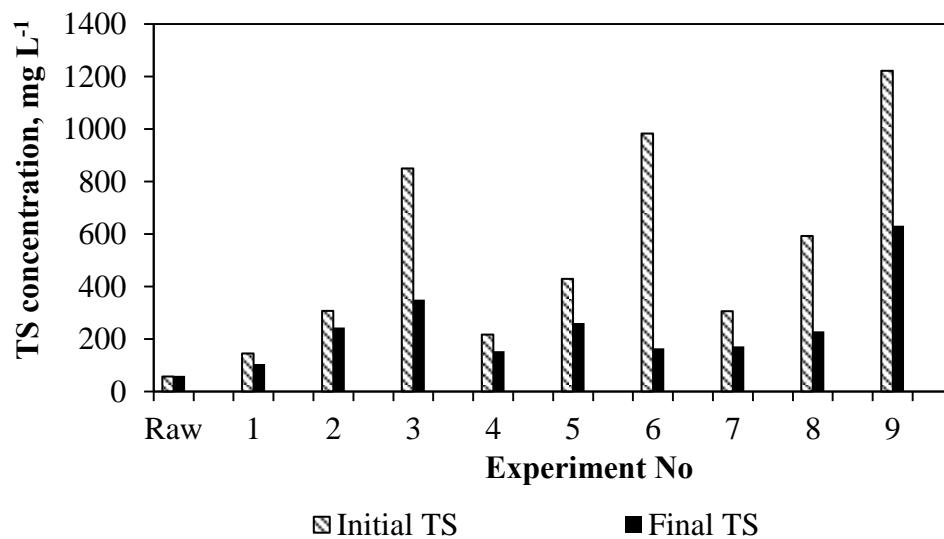
Figure 3.51 Initial and final TS concentrations in hydrogen gas production from filtrate obtained at different hydrolysis conditions.
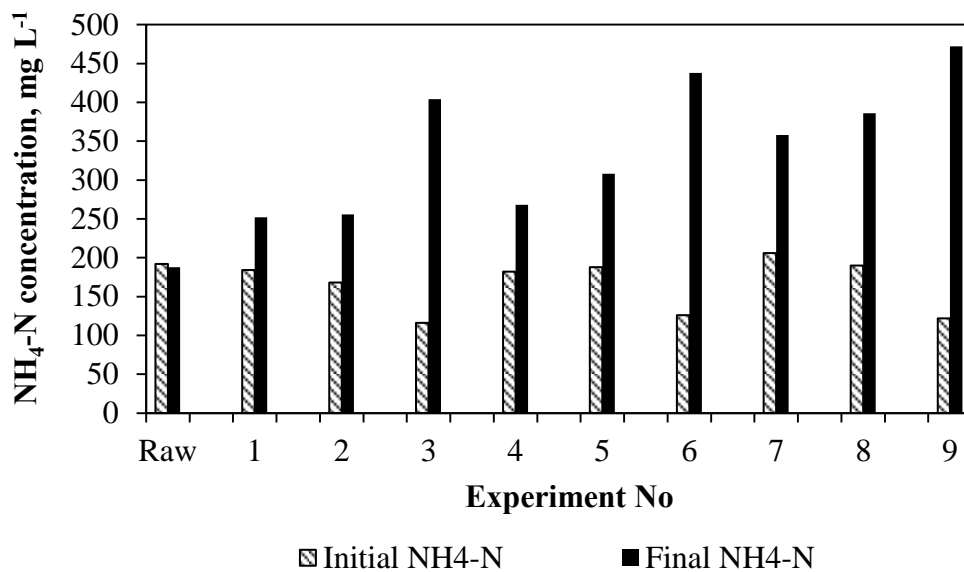
Figure 3.52 Initial and final NH$_4$-N concentrations in hydrogen gas production from filtrate obtained at different hydrolysis conditions.
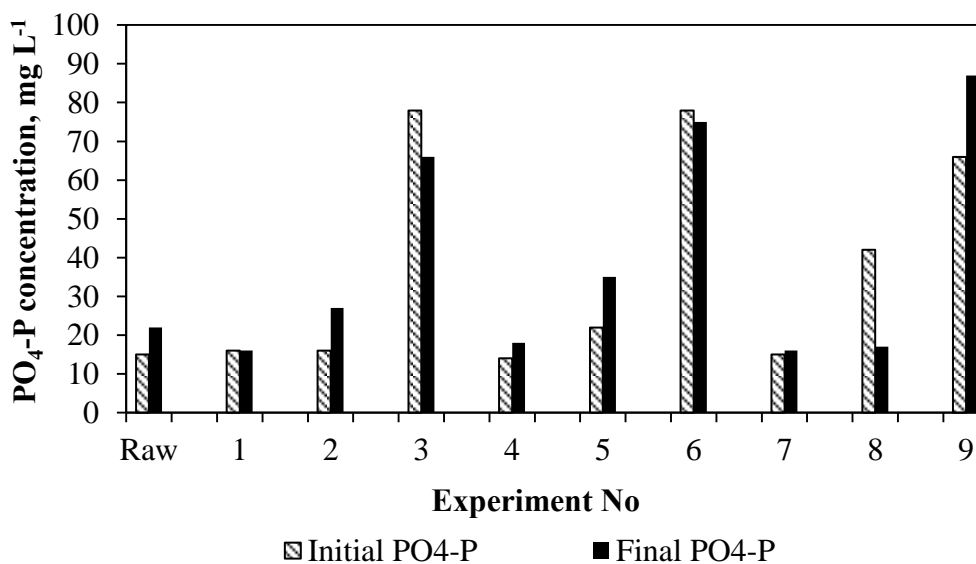
Figure 3.53 Initial and final PO$_4$-P concentrations in hydrogen gas production from filtrate obtained at different hydrolysis conditions.
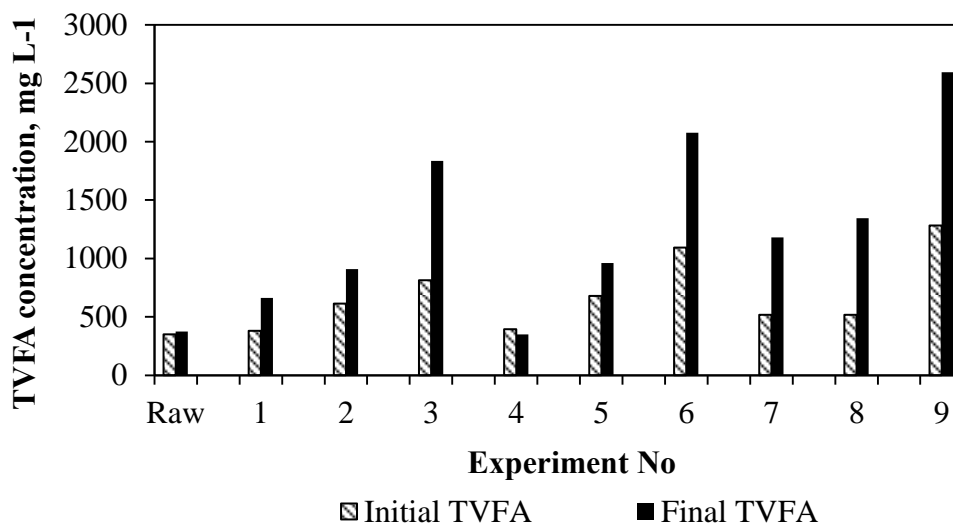
Figure 3.54 Initial and final TVFA concentrations in hydrogen gas production from filtrate obtained at different hydrolysis conditions.

3.2.2.2.2 Hydrogen Gas Production from Sludge. Hydrogen gas production from sludge obtained from single-stage heat treatment hydrolysis was evaluated by subjecting the sludge-filtrate mixture to the dark fermentation. Hydrogen production with raw sludge was carried out in parallel. Variation of cumulative hydrogen gas volume with time for raw sludge and for hydrolyzed sludge are depicted in Figures 3.55-3.64. Hydrogen gas production was completed in the first 20 hours of the fermentation and no substantial changes were observed in cumulative hydrogen gas volume for the rest of the fermentation period. Figure 3.65 summarizes the final hydrogen gas volumes obtained form sludges. As seen from the figure, CHV from all hydolyzed sludge-filtrate mixtures was around 4 ml and the maximum production volume from raw sludge was 4 ml as well.

Initial and the final COD concentrations of sludge fermentations are given in Figure 3.66. As observed in the acid hydrolysis of the sludge, there was a rise in COD concentration after the fermentation. The main reason for this results was transfer of organic matter content of partially disrupted microbial cells in the sludge by heat treatment (Figure 3.67). The amount of increase in COD concentration in fermentation varied depending on the hydrolysis temperature and time.
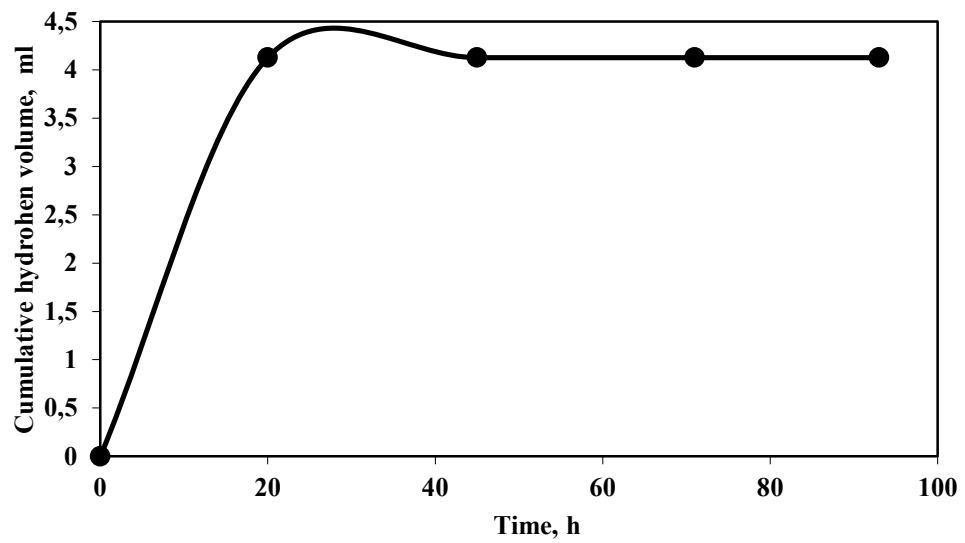
Figure 3.55 Cumulative hydrogen gas volume produced by dark fermentation of sludge hydrolyzed at T=60°C and t=30 min.
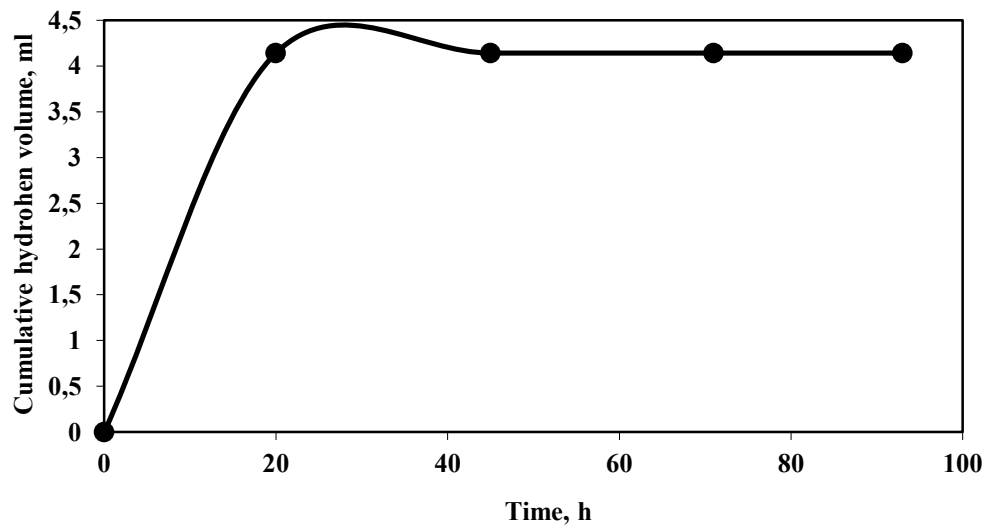
Figure 3.56 Cumulative hydrogen gas volume produced by dark fermentation of sludge hydrolyzed at T=100°C and t=30 min.
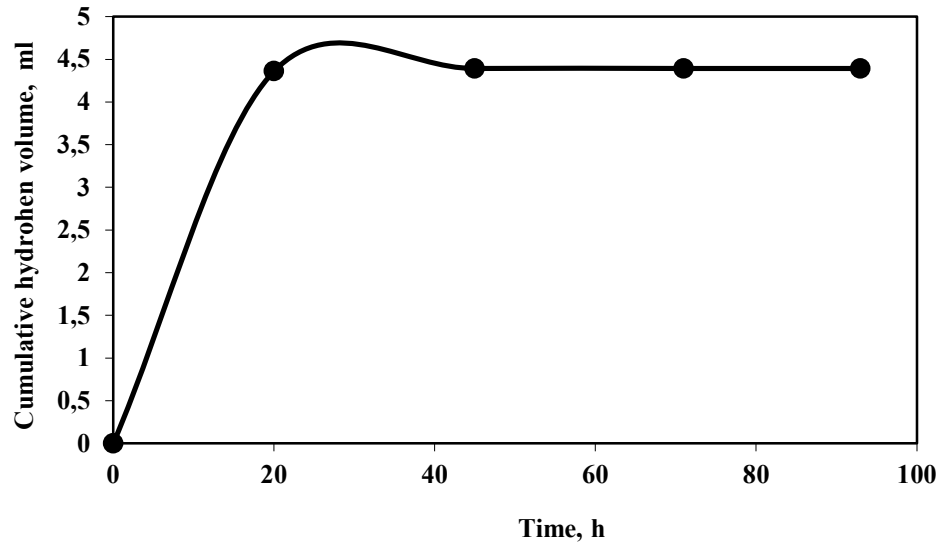
Figure 3.57 Cumulative hydrogen gas volume produced by dark fermentation from sludge hydrolyzed at T=135°C and t=30 min.
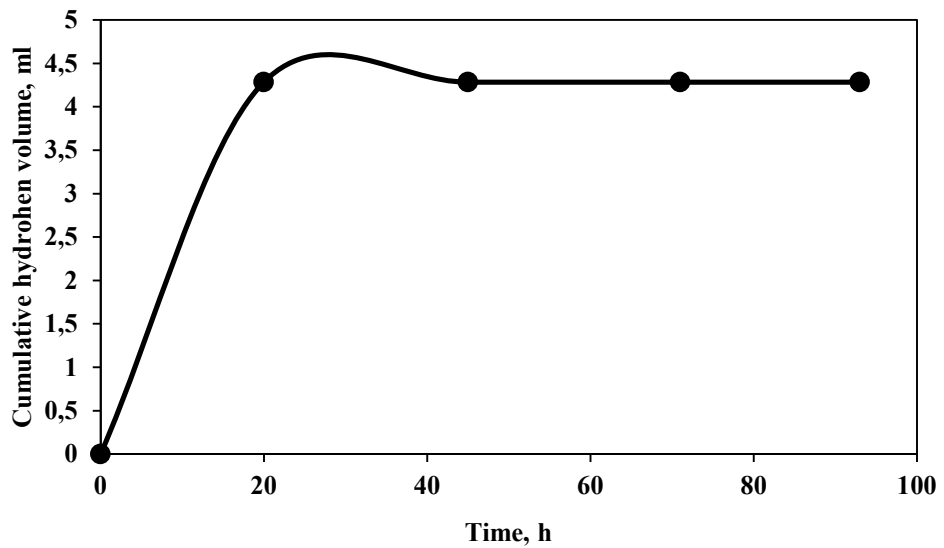
Figure 3.58 Cumulative hydrogen gas volume produced by dark fermentation of sludge hydrolyzed at T=60°C and t=45 min.
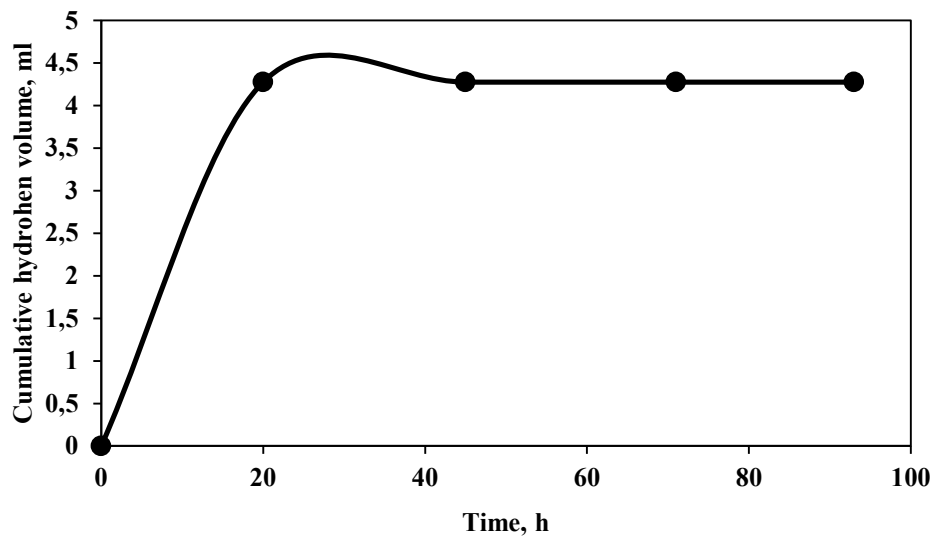
Figure 3.59 Cumulative hydrogen gas volume produced by dark fermentation of sludge hydrolyzed at T=100°C and t=45 min.
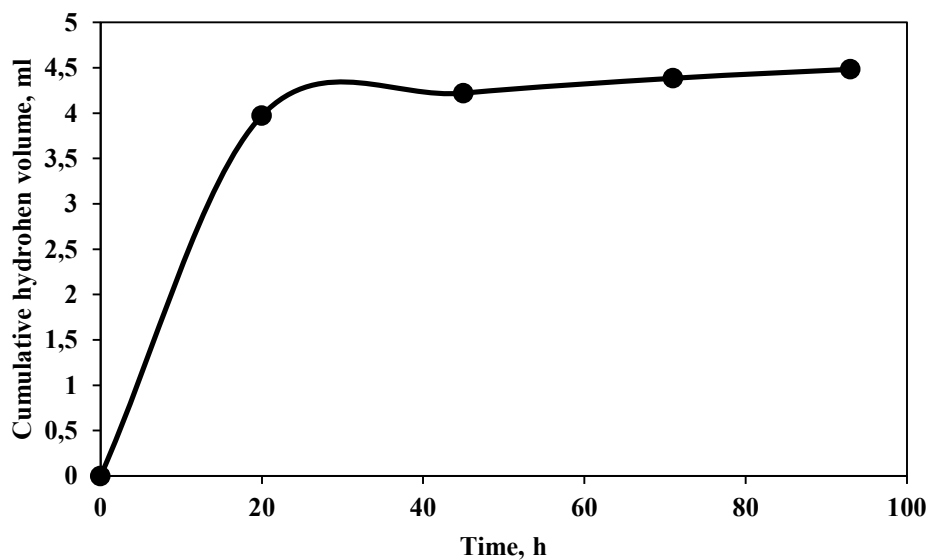
Figure 3.60 Cumulative hydrogen gas volume produced by dark fermentation of sludge hydrolyzed at T=135°C and t=45 min.
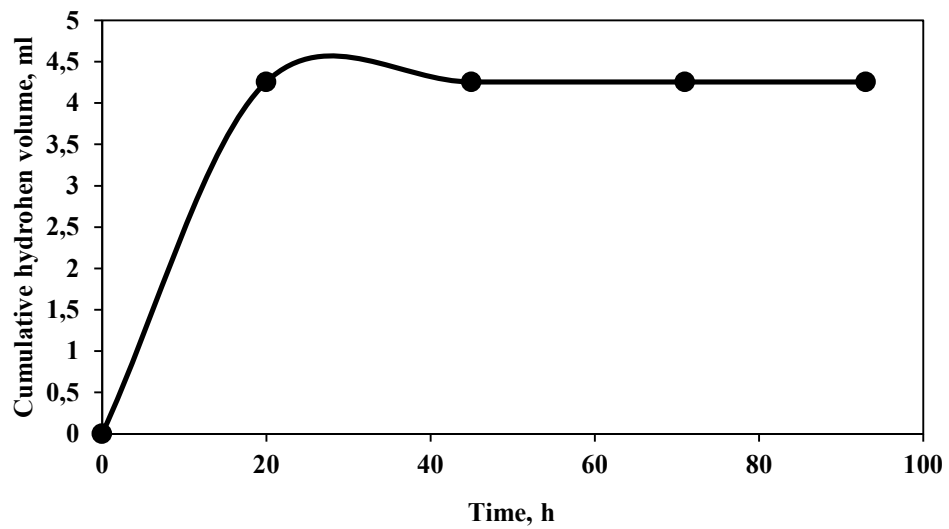
Figure 3.61 Cumulative hydrogen gas volume produced by dark fermentation of sludge hydrolyzed at T=60°C and t=60 min.
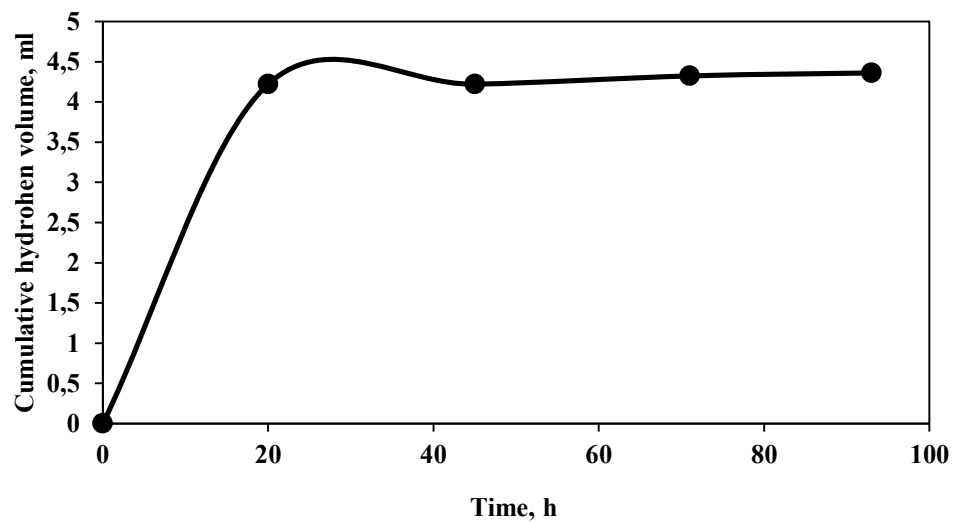
Figure 3.62 Cumulative hydrogen gas volume produced by dark fermentation of sludge hydrolyzed at T=100°C and t=60 min.
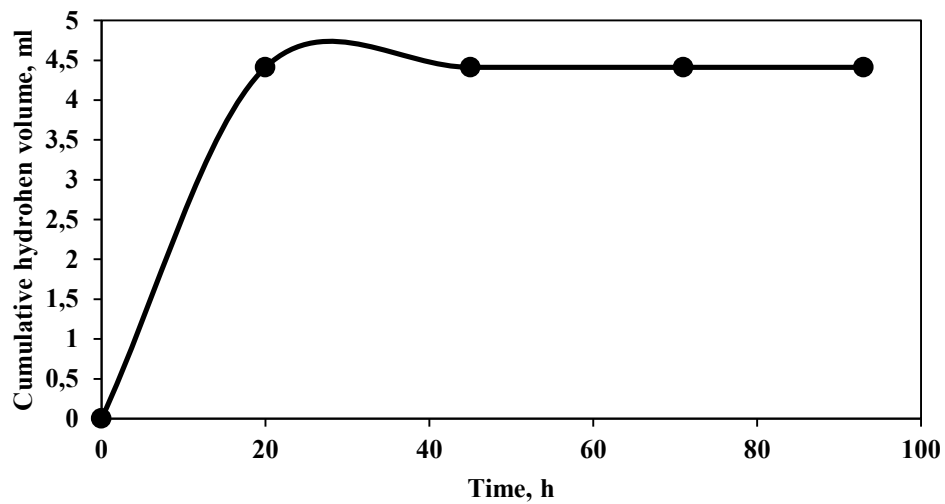
Figure 3.63 Cumulative hydrogen gas volume produced by dark fermentation of sludge hydrolyzed at T=135°C and t=60 min.
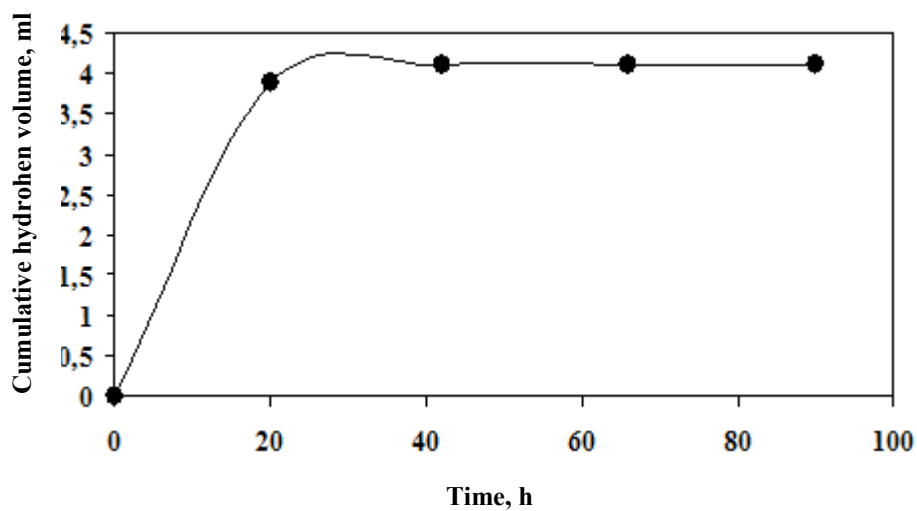
Figure 3.64 Cumulative hydrogen gas volume produced by dark fermentation of raw sludge.

The increase in COD concentration at T=60º C and t= 30 min was 19% whereas in the fermentation of the sludge, obtained by extending the time to t=60 mins it was 33%. But, in the fermentation of hydrolysis sludge at T=135º C and t=30 min, this increase was only 8% while it was 18% in the fermentation of the sludge hydrolyzed at the same temperature for t=60 mins.
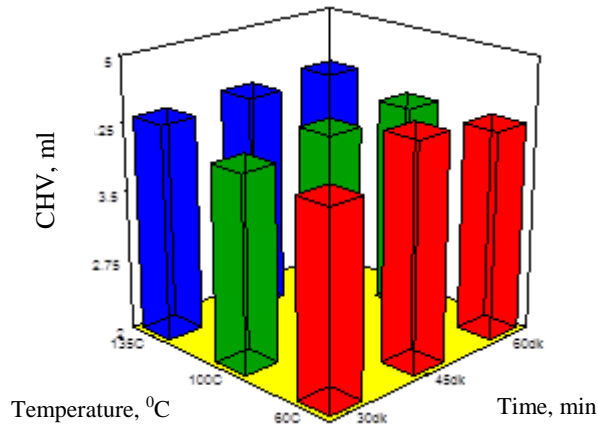
Figure 3.65 Effect of hydrolysis temperature and time
on cumulative hydrogen gas volume obtained from
hydrolyzed sludge.

The observations about final COD concentration indicated that the increase was low
If initial COD was high after hydrolysis, but, if the COD concentration after
hydrolysis was low then the increase in COD after the fermentation would be higher.
Under the efficient hydrolysis conditions as high temperatures and long exposure
time, the release of organic matter from sludge by bacterial hydrolysis was low.
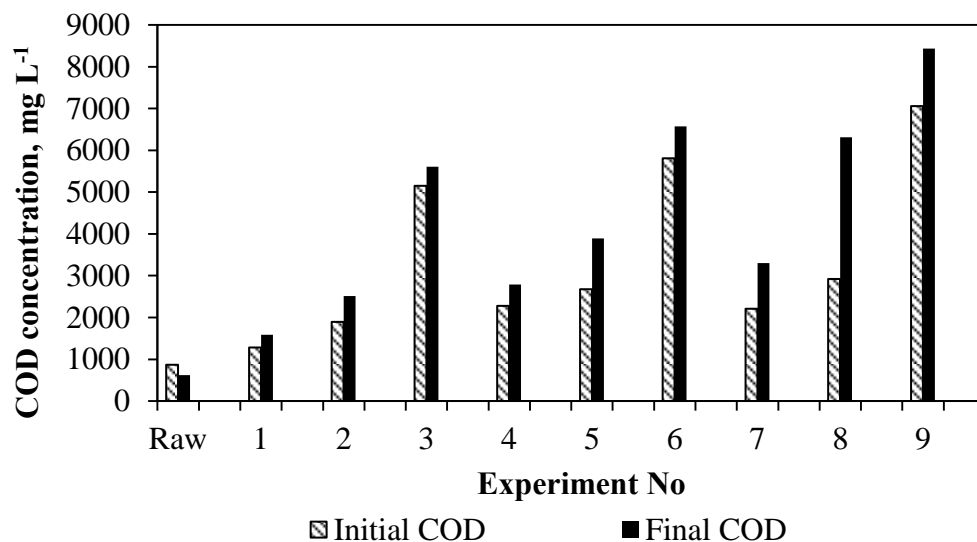
Figure 3.66 Initial and final COD concentrations in hydrogen gas production from sludge
hydrolyzed at different temperature and times.

T=60 ºC t=30 min

T=100 ºC t=30 min

T=135 ºC t=30 min

T=60ºC t=45 min

T=100ºC t=45 min

T=135 ºC t=45 min

T=60 ºC t=60 min

T=100 ºC t=60 min
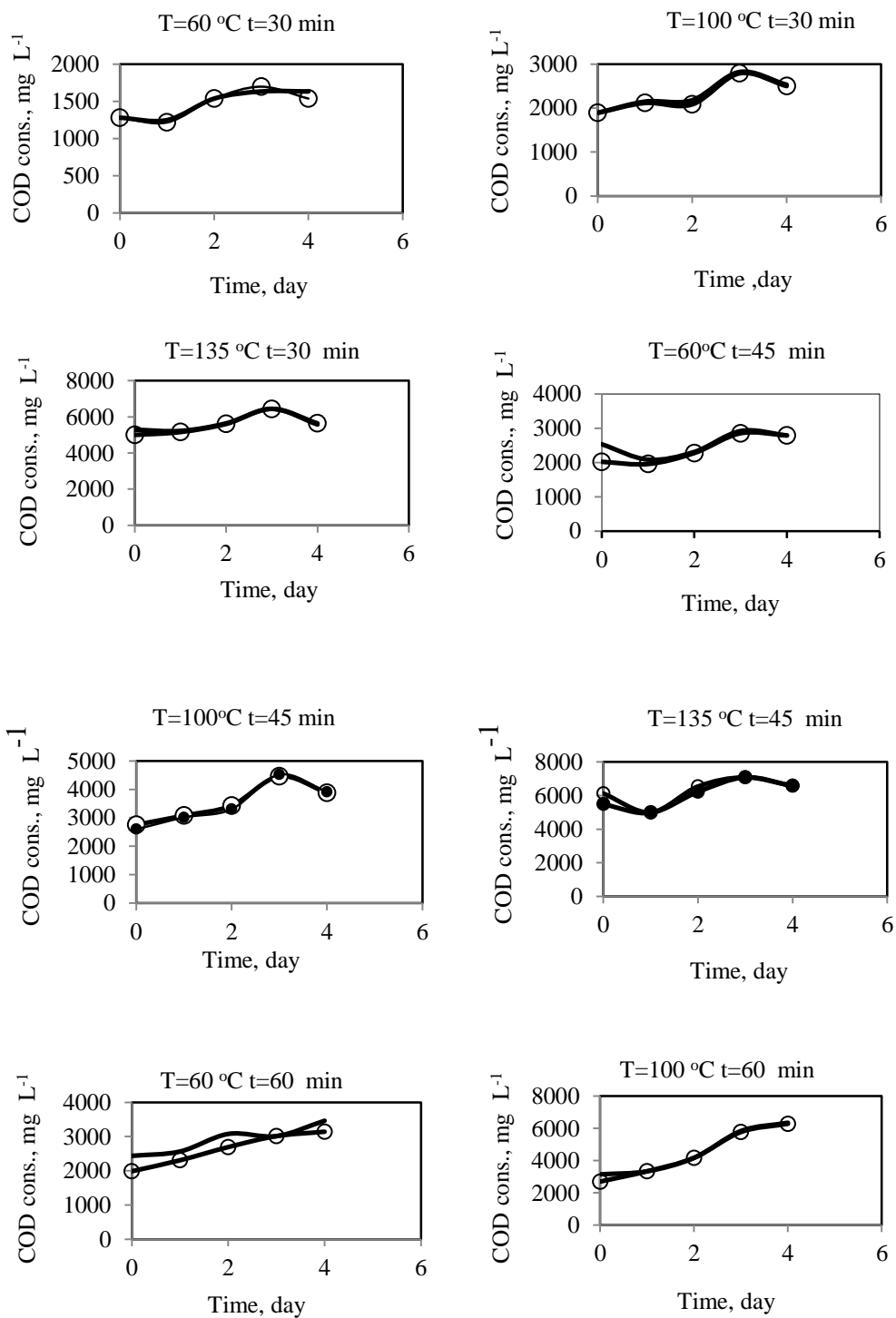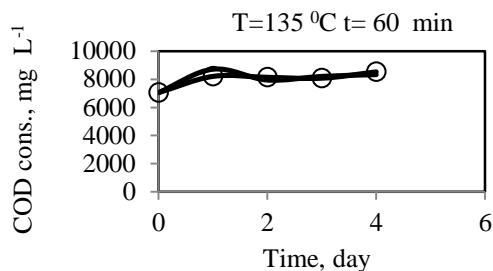
Figure 3.67 Variation of COD concentration with in time during dark fermentation of sludge hydrolyzed at different temperatures and times.

Sugar consumption in hydrolyzed sludge was lower than the consumption observed in the filtrate (Figure 3.68). Maximum consumption was observed as 59% in the sludge hydrolyzed at T=135º C and t=45 mins. However, the maximum sugar consumption obtained from the fermentation of the filtrate reached to 83% levels. When the variation of sugar concentrations with time during fermantation is evaluated, increases and decreases in concentrations are observed. This result indicates that sugar content of sludge is released and then used by the microrganisms simultaneously. Variation of sugar concentration in time was more emhasized for the sludge hydrolyzed at low temperature and short hydrolysis periods. In other words, although sludge hydrolysis under these condtions were not efficient for the release of organic matter but it made the sludge ready for bacterial hydrolysis.

Similar to previous observations, NH_4-N concentration increased after fermentation (Figure 3.69). But the proteins were effectively consumed as seen in Figure 3.70. Therefore, the main reason for the increase in NH_4-N can be explained as utilization of proteins by microorganisms and then release of nitrogen content of proteins. Apart form the experiments 3, 6 and 9, final PO_4-P concentrations was higher than the initial one (Figure 3.71) which indicates further hydrolysis of cellular materials. TVFA concentrations after the fermentation are given in Figure 3.72. TVFA concentration varied between 1000 mg L^{-1} and 3500 mg L^{-1}. The highest TVFA production was observed at the fermentation of the sludge hydrolyzed at t=60 mins and T=135º C which also resulted in the highest total sugar concentration.
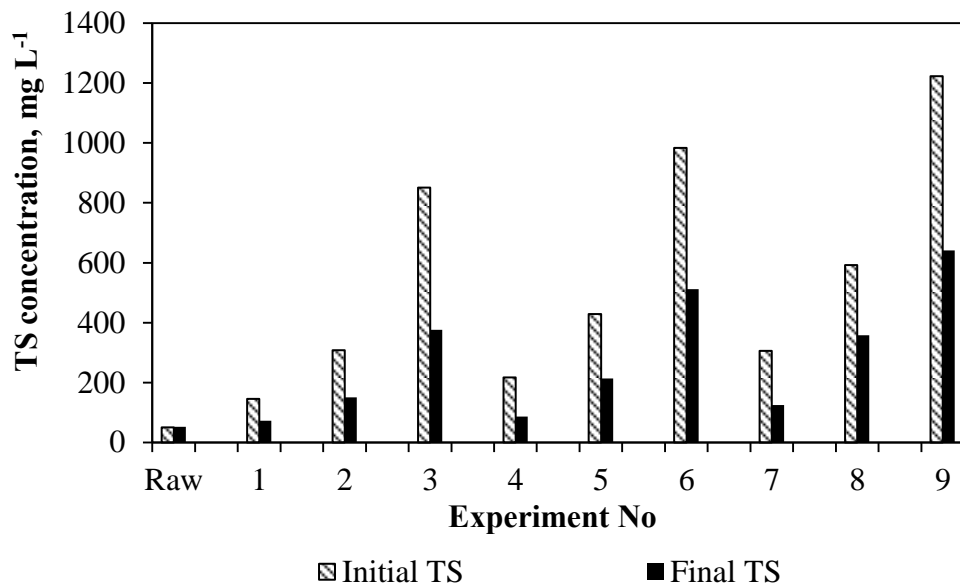
Figure 3.68 Initial and final total sugar concentrations in hydrogen gas production from sludge hydrolyzed at different temperature and times.
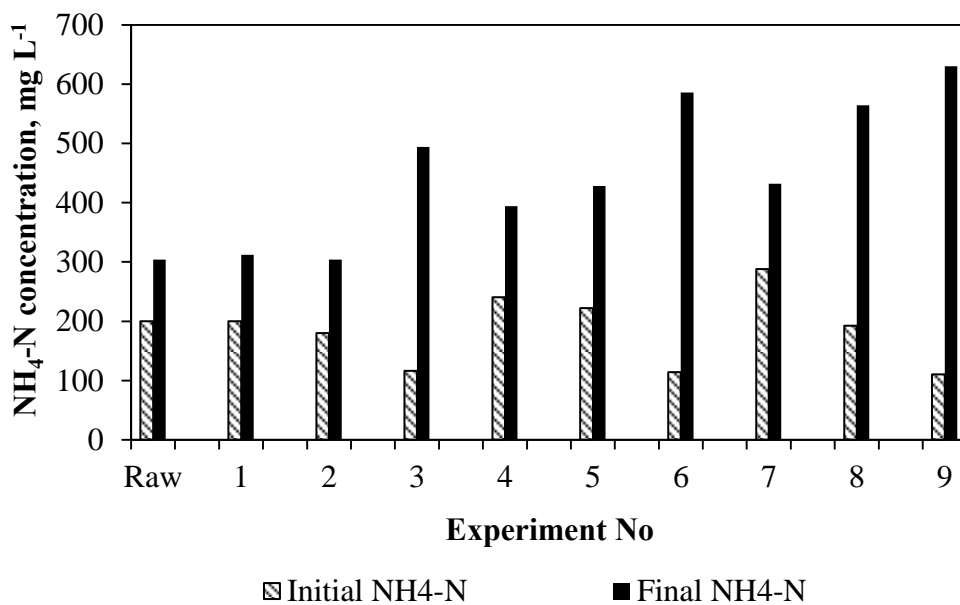
Figure 3.69 Initial and final NH$_4$-N concentrations in hydrogen gas production from sludge hydrolyzed at different temperature and times.
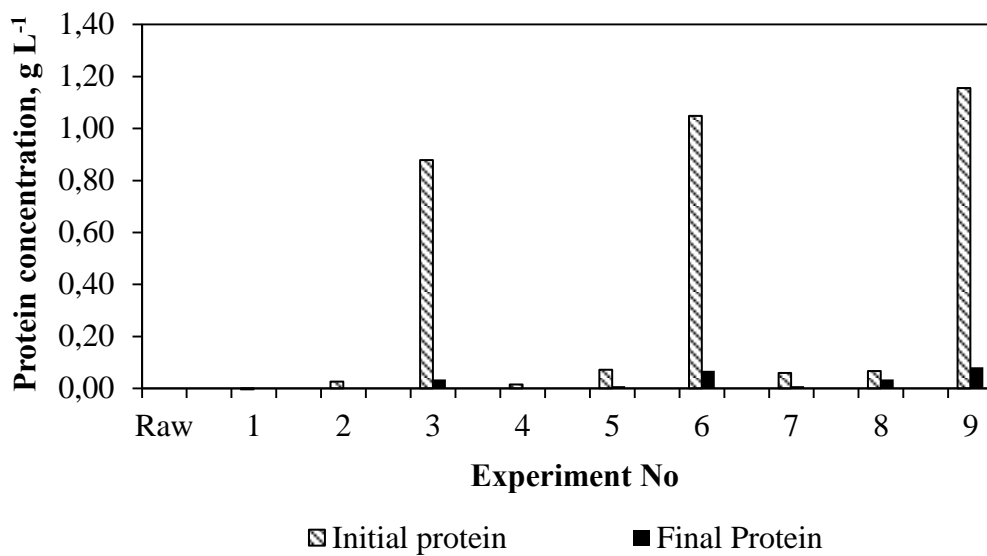
Figure 3.70 Initial and final protein concentrations in hydrogen gas production from sludge hydrolyzed at different temperature and times.
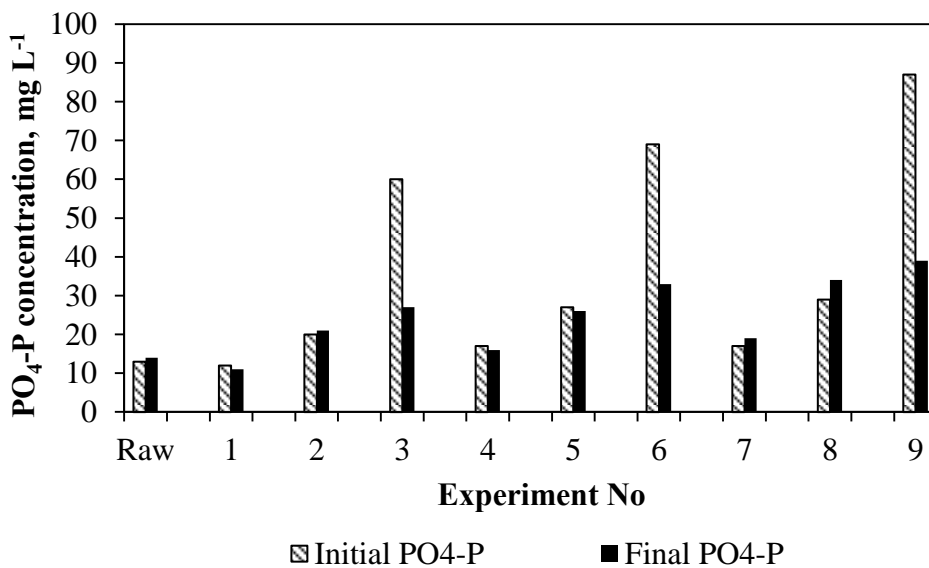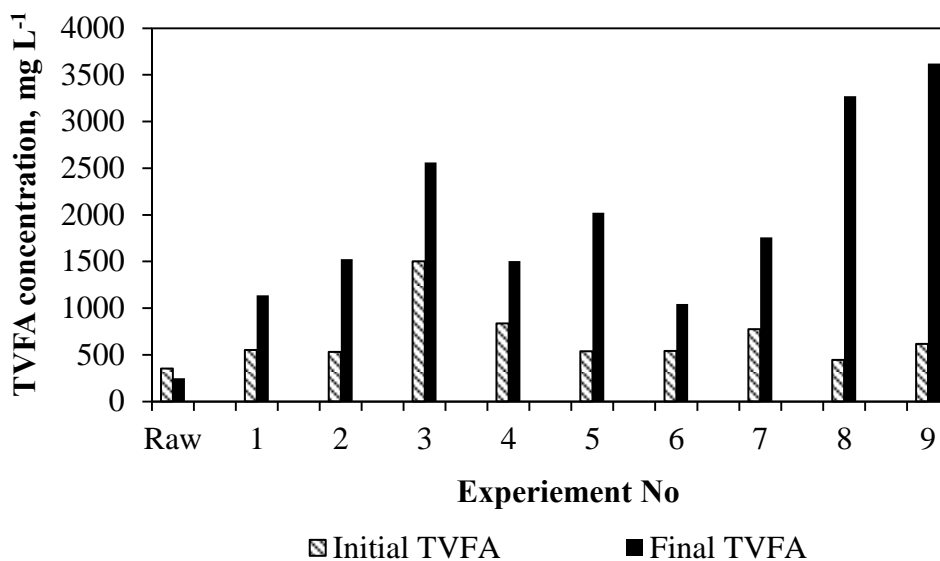

Figure 3.71 Initial and final PO$_4$-P concentrations in hydrogen gas production from sludge hydrolyzed at different temperature and times.

Figure 3.72 Initial and final TVFA concentrations in hydrogen gas production from sludge hydrolyzed at different temperature and times.

3.2.2.2.3 Hydrogen Production Potentials and Yields from Filtrate and Sludge. Hydrogen production yields obtained from the experiments are given in Figures 3.73 and 3.74 in term of mmol g^{-1} glucose ml and mg^{-1} glucose^{-1}, respectively. The maximum hydrogen production yield in the filtrate was obtained as 12.1 mmol g^{-1} glucose (300 ml g^{-1}glucose^{-1}) when sludge was hydrolyzed at t=45 min and T=60° C. The sugar consumption in the filtrate under these hydrolysis conditions was about 29%. It can be concluded that sugar was converted into acetic acid and butyric acid rather than other hydrogen consuming end products. The highest hydrogen production yield from sludge was 10 mmol g^{-1} glucose^{-1} (248 ml g^{-1} glucose^{-1}) obtained form the sludge hydrolyzed at T=60ºC and t=60 min. The yield of hydrogen formation was higher in filtrate in most of the cases compared to that of sludge.

Hydrogen production potentials and rate of formations are given in Table 3.18. Production potential (P) was in parallel to the results obtained from the experimental studies. Production rate reached up to 1.2 ml hour^{-1}. The required lag phase period to start the hydrogen production was 14±1 hours. Hydrogen production potential from sludge was about 4 ml (Table 3.19). No substantial variation in production potential

with regard to the experimental results was observed. However, the rate of prodcution and lag period changed with hydrolysis conditions.
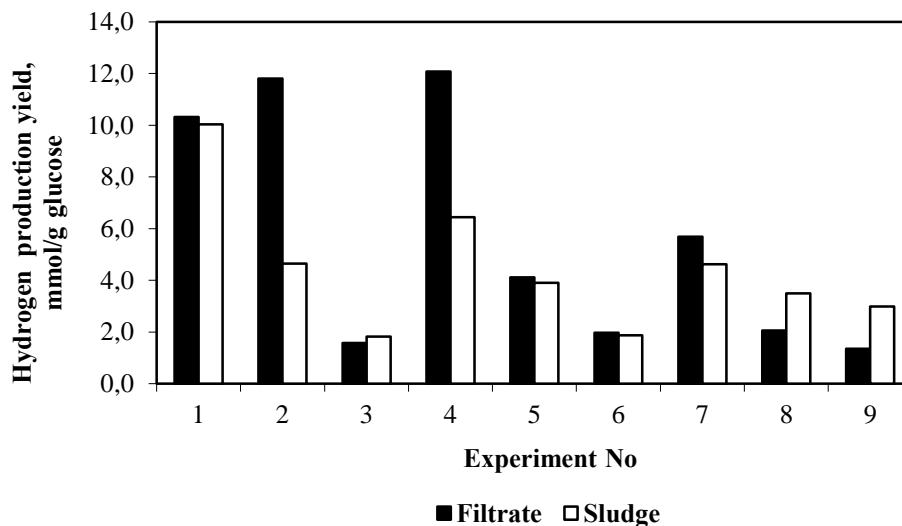
Figure 3.73 Hydrogen gas production yields (mmol g^{-1} TS^{-1}) of sludge and filtrate hydrolyzed at different temperatures and times.
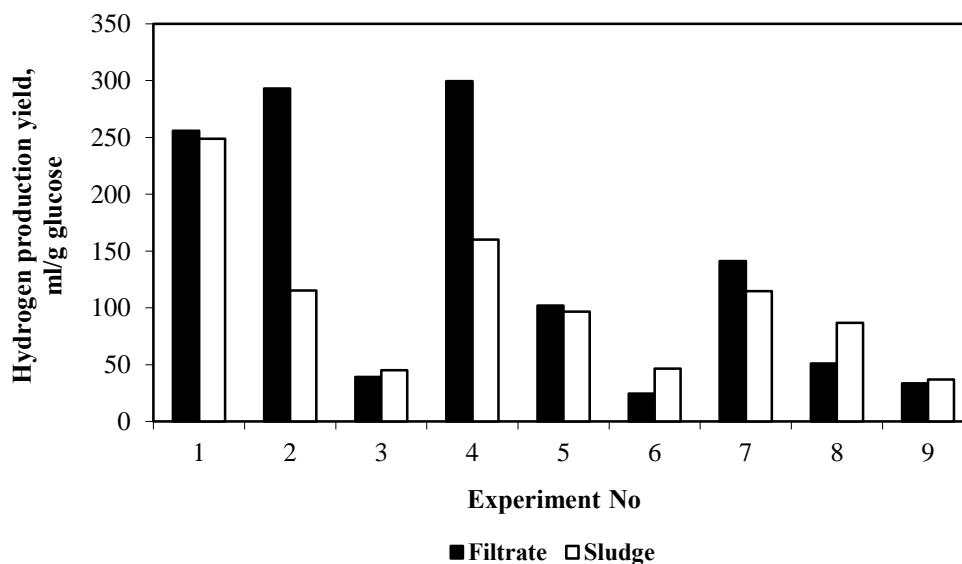
Figure 3.74 Hydrogen gas production yields (ml g^{-1} TS^{-1}) of sludge and filtrate hydrolyzed at different temperatures and times.

This variation can be explained as the substrate releases during fermentation. Since, the amount of substrate released differs according to sludge hydrolysis condition, the

substrate concentration in the liquid phase changes in time and result in variation in the rate of production. The highest production rate was 3.52 ml hour^{-1}, obtained at the hydrolysis conducted at T=135º C and t=60 min.

Table 3.18 Gompertz equatiın constants of hydrogen gas production from filtrate of heat treatment.

Experiment No	P (ml)	R_m (ml hour^{-1})	L (hour)
1	4.02	1.17	14.18
2	4.21	1.21	14.61
3	4.17	1.24	15.43
4	3.97	1.17	14.54
5	3.89	1.14	13.64
6	4.21	1.21	14.97
7	4.02	1.13	14.64
8	4.11	1.19	14.80
9	4.14	1.22	14.91

Table 3.19 Gompertz equation constants of hydrogen gas production from hydrolyzed sludge by heat treatment.

Experiment No	P (ml)	R_m (ml hour^{-1})	L (hour)
1	4.13	2.67	1.47
2	4.14	3.10	4.65
3	4.39	1.16	11.79
4	4.28	2.75	1.51
5	4.28	2.32	1.57
6	4.36	1.29	15.80
7	4.25	2.90	1.91
8	4.30	1.17	13.31
9	4.41	3.52	5.89
Raw	4.10	1.21	15.20

3.2.3 Two-stage (acid+ heat treatment) Hydrolysis and Hydrogen Gas Production by Dark Fermentation

3.2.3.1 Optimization of Two Stage Hydrolysis

Sequential acid and heat treatment was applied to sludge in two-stage hydrolysis. Box-Benkhen respons surface experimental design method was used to determine optimum hydrolysis conditions. Independent variables were pH (X_1=2-6), temperature (X_2=60-135ºC) and hydrolysis time (X_3=15-60 min). Dependent

variables were COD, total sugar, NH_4-N, PO_4-P and protein concentrations. The ranges of the factors were determined based on the results obtained at single-stage acid and heat treatment hydrolysis experiments. It was observed that in acid hydrolysis, 4 hours of hydrolysis was enough for the release of organic matter and there was a slight rise in the concentration of organic matter after 24 hour hydrolysis. For this reason, in two-stage hydrolysis, acid hydrolysis time was kept constant at 4 hours. Response surface methods were consist of factorial, axial and central point. Studies at factorial and axial points were done with no replicates, instead central points were repeated 3 time to test the reproducibility of the results. Box-Benkhen experimenal design points and the observed responses obtained after the hydrolysis are given in Table 3.20. By using Design-Expert (Stat-East 7.0) statistical program, coefficients of the response function were determined and are given in Table 3.21.

The following response function was used for correlation of the hydrolysis yield (Y_H) with independent parameters.

$$Y = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_{12}X_1X_2 + b_{13}X_1X_3 + b_{23}X_2X_3 + b_{11}X_1^2 + b_{22}X_2^2 + b_{33}X_3^2$$

<div align="right">Eqn 3.1</div>

where Y is the predicted response for dependent variable, b_0 is model constant, b_1, b_2, and b_3 are the linear coefficients, b_{12}, b_{13}, and b_{23} are the coefficients of interactions among the variables, and b_{11}, b_{22}, and b_{33} are the quadratic coefficients.

The significance of the coefficients were deterimined by variance analysis and they were found that statistically significant. By using these coefficients and response function, concentrations of the dependent variables were predicted. The observed and the predicted values as given in Table 3.22 were evaluated to determine the fitness of the model coefficients. For COD, total sugar and PO_4-P, the regression coefficient between observed and predicted values were $R^2 > 0.95$ indicating that model coefficient can be used to estimate the concentrations at COD, total sugar and PO_4-P. Regression coeffcient for NH_4-N concentration was $R^2 = 0.87$ which is also acceptable for utilization model coefficient to predict NH_4-N concentration.

Table 3.20 Box-Behnken experimental design points for two stage hydrolysis of sludge with observed concentrations of dependent variables.

Experiment No	Independent Variables			Dependent Variables				
	X_1 pH	X_2 Temperature, ^0C	X_3 Time, min	COD, mg L^{-1}	Total Sugar, mg L^{-1}	NH$_4$-N, mg L^{-1}	Protein, g L^{-1}	PO$_4$-P mg L^{-1}
1	4.00	60.00	15.00	1153.25	330.89	228.00	0.00	537.00
2	4.00	135.00	15.00	3396.88	1042.43	268.00	0.00	600.00
3	4.00	60.00	60.00	1217.39	333.54	238.00	0.00	519.00
4- C*	4.00	97.50	37.50	1536.82	231.77	248.00	0.00	531.00
5	6.00	97.50	60.00	2563.06	455.60	174.00	0.00	21.00
6	2.00	97.50	15.00	1408.54	772.81	243.00	0.00	522.00
7	2.00	97.50	60.00	2627.20	1034.50	213.00	0.15	519.00
8	2.00	135.00	37.50	7052.86	1774.66	270.00	0.43	735.00
9-C*	4.00	97.50	37.50	1536.82	239.70	212.00	0.00	534.00
10	6.00	135.00	37.50	5192.80	1079.44	176.00	0.20	45.00
11	6.00	60.00	37.50	1538.09	293.88	178.00	0.00	30.00
12	4.00	135.00	60.00	4872.10	1256.55	248.00	0.07	621.00
13-C*	4.00	97.50	37.50	1536.82	233.09	242.00	0.00	516.00
14	2.00	60.00	37.50	767.14	492.61	252.00	0.00	504.00
15	6.00	97.50	15.00	2211.56	350.72	196.00	0.00	21.00

* Center points

Table 3.21 Response function coefficient of dependent variables in two-stage hydrolysis of sludge.

Coefficients	COD, mg L^{-1}	TS, mg L^{-1}	NH$_4$-N, mg L^{-1}	PO$_4$-P, mg L^{-1}
bo	4715.24	2936.81	208.86	20.60
b1	-629.74	-512.26	42.96	431.39
b2	-105.17	-36.27	-0.84	-3.12
b3	18.90	-17.85	0.42	-0.05
b12	-8.77	-1.65	-0.07	-0.72
b13	-4.82	-0.87	0.04	0.02
b23	0.42	0.06	-0.01	0.01
b11	205.45	73.48	-6.75	-62.13
b22	0.91	0.27	0.01	0.04
b33	-0.31	0.25	0.00	-0.02

Table 3.22 Regression coefficients, observed and predicted concentrations of dependent variable in two-stage hydrolysis.

COD, mg L^{-1}		Total Sugar, mg L^{-1}		NH$_4$-N, mg L^{-1}		PO$_4$-P, mg L^{-1}	
Observed	Predicted	Observed	Predicted	Observed	Predicted	Observed	Predicted
1153	1043	330	560	228	237	537	493
3396	2271	1042	334	268	184	600	60
1217	6319	333	1733	238	264	519	704
1536	4916	231	1011	248	190	531	55
2563	1640	455	778	174	248	21	541
1408	1986	772	382	243	180	522	-0.4
2627	2851	1034	1002	213	228	519	540
7052	2330	1774	450	270	169	735	1
1536	644	239	258	212	237	534	527
5192	3898	1079	1077	176	269	45	610
1538	716	293	298	178	237	30	508
4872	5381	1256	1329	248	238	621	630
1536	1536	233	234	242	234	516	527
767	1536	492	234	252	234	504	527
2211	1536	350	234	196	234	21	527
R^2	0.946		0.991		0.868		0.995

Figures 3.75 - 3.77 depict variation of COD concentration after two-stage hydrolysis with independent variables pH, T and time. In these Figures, one of the variables was kept constant and the effect of the two variables were analyzed. Variation of COD concentration with pH and time at a constant hydrolysis time (t=60 mins) is given in Figure 3.75. COD concentration at the end of the hydrolysis increased with the increase in temperature at maximum hydrolysis time (t=60 min). Different responses were observed for the effect of pH and temperature. High temperatures (T=135ºC) and acidic conditions provided more effective hydrolysis. Maximum COD concentration of 7121 mg L^{-1} was obtained at t=60 min, pH=2 and T=135 ºC. An increase about 37% was achieved in COD concentration by decreasing pH value from pH=6 to pH=2 under the conditions of T=135ºC and t=60 min (Figure 3.76). Similarly, by increasing time from t=15 min to t= 60 min at pH=2, there was an improvement about 27% in COD concentration. The effect of the hydrolysis

temperature and pH on COD concentration can be seen more clearly in Figure 3.77. Although, extended hydrolysis period improved the final COD, the most important increases were obtained by the rise in temperature. These results show that the most siginifcant factors to obtain high COD in two stage hydrolysis are primarly temperature and secondly pH. Hydrolysis time is in interaction with the other factors, but its effect on COD is low.
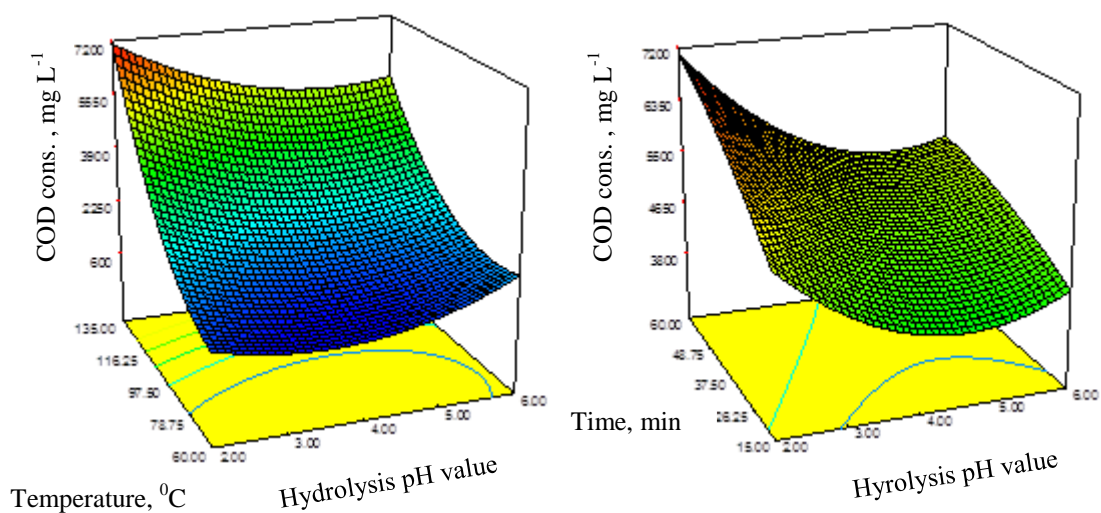
Figure 3.75 Variation of COD concentration with temperature and pH at t=60 min hydrolysis period.

Figure 3.76 Variation of COD concentration with time and pH at T=135 ^{0}C hydrolysis temperature.
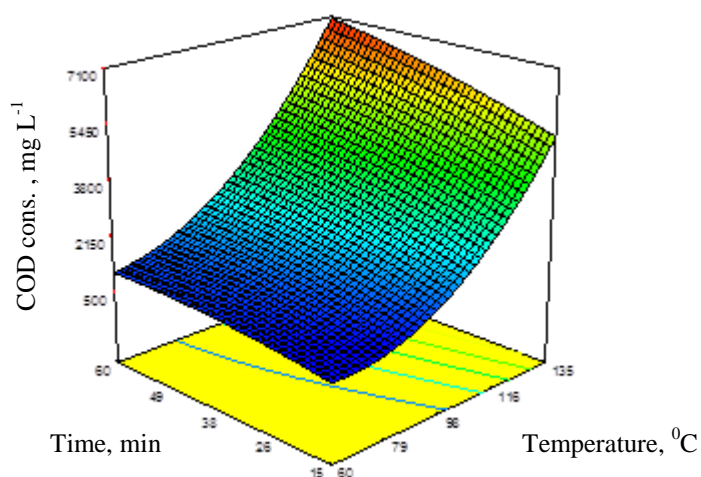
Figure 3.77 Variation of COD concentration with temperature and time at pH=2.

Variation of total sugar concentration with temperature and pH at a constant hydrolysis time of t=60 min is presented in Figure 3.78. Total sugar concentration increased especially with the increase in hydrolysis temperature. The effect of high hydrolysis temperatures and acidic conditions (pH=3-2) became stronger in the released total sugar concentration. But, at the low temperatures (T≥98º C) the effect of pH was low as well. Hydrolysis temperature was more significant factor in total sugar concentration than pH.

The effect of pH and hydrolysis time on total sugar concentration is given in Figure 3.79. There wasn't a significant increase in the total sugar concentration for different hydrolysis time and pH value between pH=6 and pH=4. But, the concentration substantially increased under strong acidic conditions like pH=2 and pH=3, independently from the hydrolysis period. Similarly, when the effect of time and temperature on total sugar concentration was analyzed, it was seen that the main factor was the increase in temperature (Figure 3.80). The hydrolysis conditions which gave the maximum total sugar concentration as 2100 mg L^{-1} were determined as pH=2, T=135ºC and t=60 mins. Under the same temperature and pH values, total sugar concentration was 1691 mg L^{-1} at t=15 min.
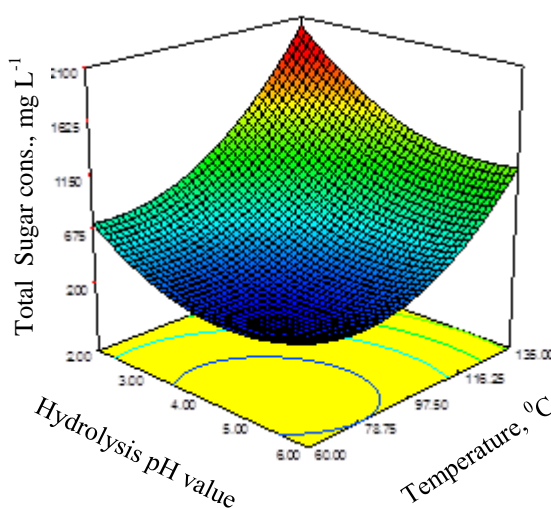
Figure 3.78 Variation of total sugar concentration with temperature and pH at t=60 min.
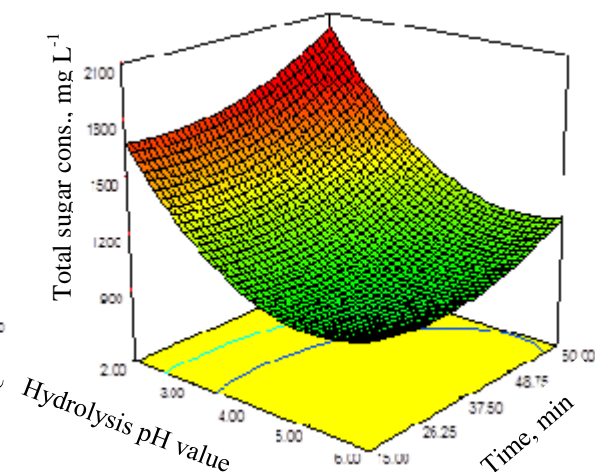
Figure 3.79 Variation of total sugar concentration with time and pH at T=135^{0}C.
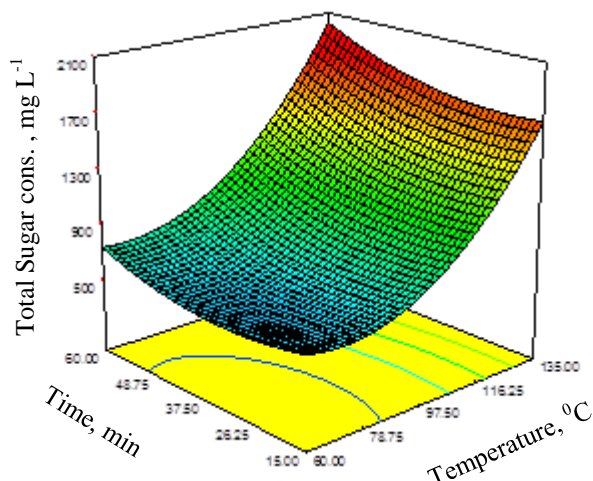
Figure 3.80 Variation of total sugar concentration with temperature and time at pH=2.

Figures 3.81-3.83 summarizes the effect of hydrolysis conditions on NH_4-N concentration. Hydrolysis time was one of the signicant factors in NH_4-N release. At long hydrolysis periods (t=60), there was a decrease in NH_4-N concentration. These results were in parallel to the results obtained from single-stage heat treatment. The highest NH_4-N concentrations were observed at t=15 mins hydrolysis conditions (Figure 3.81). Therefore, it can be concluded that there is no need to extend hydrolysis period to increase NH_4-N concentration. Short hydrolysis periods provide an advantageous for the sake of the process economy. The significance of two factor interaction between time and temperature can be seen clearly in Figure 3.82. Under low temperatures (T≥90ºC), there was no substantial difference in nitrogen concentration for both short and long hydrolysis period. The increase in the concentration was only 5 or 10 mg L^{-1}. However, applying high temperatures decreased the required time to obtain efficient release of NH_4-N during hydrolysis.

The effect of temperature and pH on NH_4-N concentration at t=15 min is given in Figure 3.83. It was observed that NH_4-N concentration rapidly increased at pH=6-4 values under different temperatures. But, there wasn't a significant change for pH>4 and it was almost constant for the strong acidic conditions. The increase in temperature at pH=2-4 values relatively affected the hydrolysis reaction for NH_4-N and provided a slight increase in the concentration. For examples, an increase of

40 mg L^{-1} was obtained in NH$_4$-N concentration by increasing the temperature to T=135° C from T=60 °C at pH=3. But,decreasing pH from pH= 6 to pH= 3 at T=135 °C resulted in 80 mg L^{-1} increase in the concentration. The results indicated that hydrolysis pH value is an important factor in the release of NH$_4$-N as well as time and temperature.
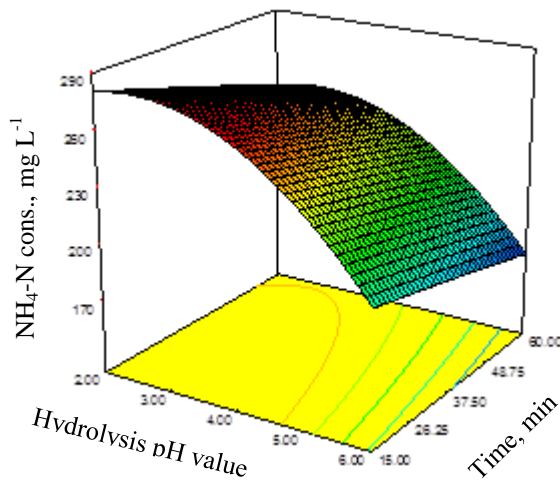
Figure 3.81 Variation of NH$_4$-N concentration with hydrolysis time and pH at T=135^{0}C.
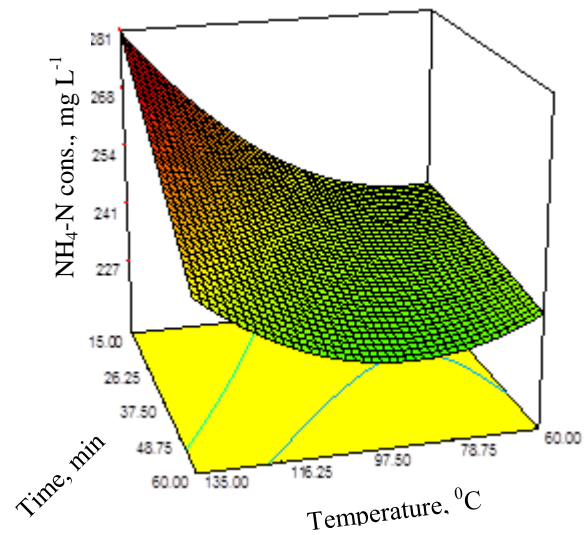
Figure 3.82 Variation of NH$_4$-N concentration with hydrolysis time and temperature at pH=2.
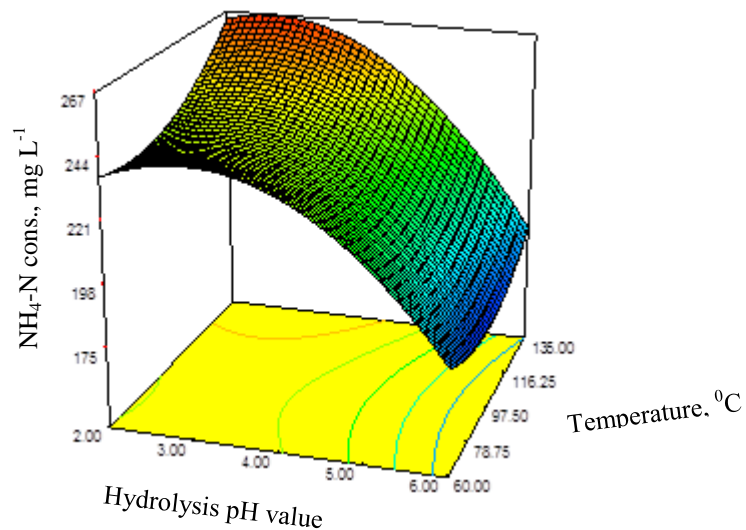
Figure 3.83 Variation of NH$_4$-N concentration with hydrolysis temperature and pH at t=15 min.

Similar results were observed for PO_4-P concentrations released after hydrolysis. While there was a rapid increase in PO_4-P concentration as pH was decreased form pH=6 to pH=4, there wasn't an important change in the concentration for pH range between pH=2 and pH=4 (Figure 3.84). The effect of temperature to the concentration was at low. As seen in Figure 3.85, there was an increase in PO_4-P concentration due to significant effect of pH. But the effect of time was not substantial. For this reason, it can be concluded that the most siginificant factor that affects PO_4-P concentration is hydrolysis pH value. When the effects of time and temperature were evaluated under the strong acidic conditions as pH=2, increase in the temperature had positive contribution to the hydrolysis reaction for PO_4-P (Figure 3.86). For example, by increasing hydrolysis temperature from T=60ºC to T=135ºC, the concentration reached to maximum level. On the other hand, hydrolysis time did not provide any change in the concentration under these conditions. The final results indicated that pH and temperature are two significant factors that affect PO_4-P concentration, but hydrolysis time has no positive or negative contribution to the PO_4- P release.
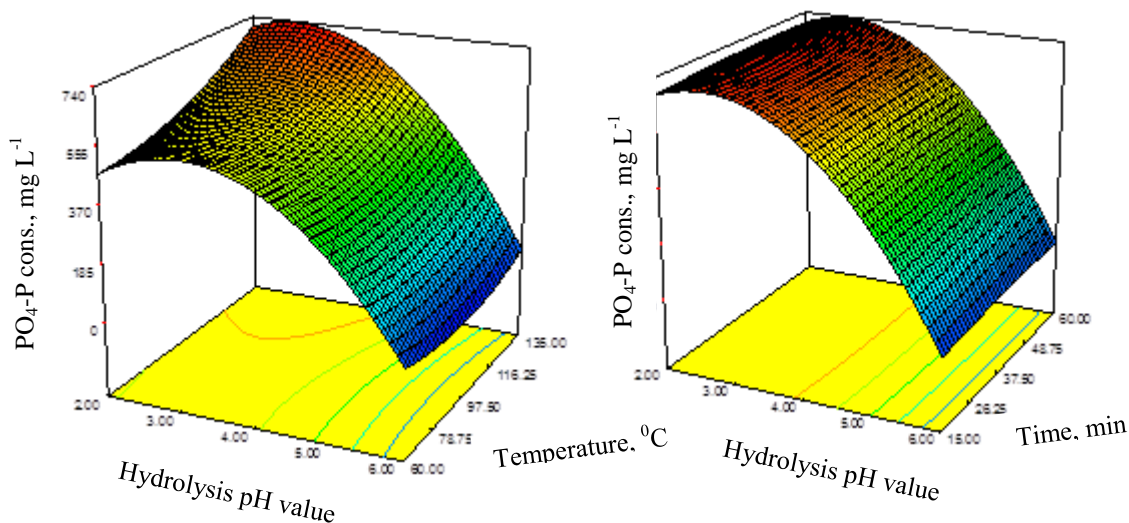
Figure 3.85 Varitaion of PO_4-P concentration with hydrolysis pH and temperature at t=60 min

Figure 3.86 Varitaion of PO_4-P concentration with hydrolysis pH and time at T=135 ºC.
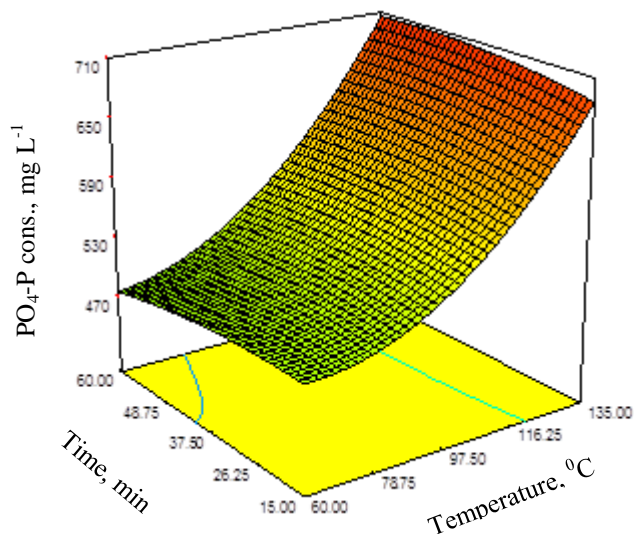
Figure 3.86 Variation of PO$_4$-P concentration with hydrolysis
temperature and time at pH=2.

The optimum conditions of two-age hydrolysis were determined by using Design
Expert 7.0 statistical program. By using response function coefficients, within the
range of factors in experimental design, hydrolysis conditions that maximize COD
and TS concentrations were determined. In addition, fitness of the model coefficients
were tested for some experimental conditions which were different than that of
experimental design. Only COD and TS were selected as the target parameters for
maximization. Because, they are the major substrates in hydrogen gas production, the
others can be externally added into the fermentation media if required. Table 3.23
depicts optimum hydrolysis conditions for the maximization of COD/TS and
predicted concentrations of COD, TS, NH$_4$-N and PO$_4$-P. One of the optimum
condition was obtaines same as experimental point. The observed and predicted
concentrations under this condition was in agreement. The other optimum condition
which is indicated with star in the Table was not experimental condition in design.
Two sets of experiments under the conditions given in the Table were conducted to
evalute if predicted and observed values will be the same. As seen in Table 3.24, the
predicted and the observed values were close to each other for all responses
investigated. These results show that response function coefficients can be used to
determine the concentrations of organic substances and nutrients for examined
ranges of factors.

In brief, maximum COD and total sugar concentrations as COD=7500 mg L^{-1} and TS=1800 mg L^{-1}, in average, were obtained at T=135ºC, pH=2 and t=60 min in two stage hydrolysis of sludge. These were the highest organic substance concentrations obtained compared to the that of other two hydrolysis method. Therefore, two stage hydrolysis was used as pretreatment of sludge in hydrogen gas by dark fermentation.

Table 3.23 The optimum hydrolysis conditions and predicted concentrations of responses.

pH	Temperature (ºC)	Time (min)	Total Sugar (mg L^{-1})	COD (mg L^{-1})	NH$_4$-N (mg L^{-1})	PO$_4$-P (mg L^{-1})
2	135	60	2000	7121	246	705
2*	121	60	1553	5228	236	632

*Experiemental conditions other than conditions determined in experimental design

Table 3.24 Observed concentrations of the responses for the optimized hydrolysis conditions.

pH	Temperature (ºC)	Time (min)	Total Sugar (mg L^{-1})	COD (mg L^{-1})	NH$_4$-N (mg L^{-1})	PO$_4$-P (mg L^{-1})
2	135	60	1846	7636		
			1859	7636	318	776
			1869	7389		
2*	121	60	1584	5661		
			1594	5661	312	720

*Experiemental conditions other than conditions determined in experimental design

3.2.3.2 Hydrogen Gas Production by Dark Fermentation from Filtrate and Sludge at Two Stage Hydrolysis

Hydrogen gas production by dark fermentation studies were carried out from sludge hydrolyzed under optimum hydrolysis conditions. Filtrate and sludge were the two substrate in fermentation. Raw sludge was also used as substrate for the control purpose. Figure 3.87 depicts variation of cumulative hydrogen gas volume produced by dark fermentation of filtrate. The volume of the hydrogen gas raised up to 6 ml in the first 20 hours of fermentation. However, no further incerase in the hydrogen gas volume was observed for the rest of the fermentation period. The maximum

cumulative hydrogen gas obtained from sludge was 10 ml (Figure 3.88) and it was 8 ml from raw sludge (Figure 3.89). Sugar consumption for different substrate can be seen in Figure 3.90. Initial sugar concentration in raw sludge was 250 mg L^{-1}, at the end of the fermentation this concentration decreased to 212 mg L^{-1}. However, initial total sugar concentration after sludge hydrolysis was about 1750 ± 50 mg L^{-1}. The final concentration was 782 ml L^{-1} and 902 mg L^{-1} in filtrate and sludge, respectively, result in 55% and 50 % (Figure 3.90). On the other hand, no efficient COD consumption occurred in this stage either, as it was observed in the first stages of the study (Figure 3.91). While there was a partial removal in the filtrate, there was an increase in the effluent of fermentation of sludge. The main reason for this increase was continuous release of organic content of sludge during fermentation , as it was explained in the prevous sections. Similarly, final NH_4-N concentrations increased as well (Figure 3.92). PO_4-P consumption was over 90% both in filtrate and in sludge.(Figure 3.93). Final TVFA concentrations in all fermentation effluents were about 4200 mg L^{-1}, in average (Figure 3.94). The TVFA production was about 3000 mg L^{-1} and nearly the same both in filtrate and sludge. Yield of hydrogen production was calculated from cumulative hydrogen gas volume and sugar consumption. Production yield for filtrate was 1.23 mmol g^{-1} glucose^{-1} (30.5 ml g^{-1} glucose^{-1}) and it was 2.16 mmol g^{-1} glucose^{-1} (53.5 ml g^{-1} glucose^{-1}) for sludge.
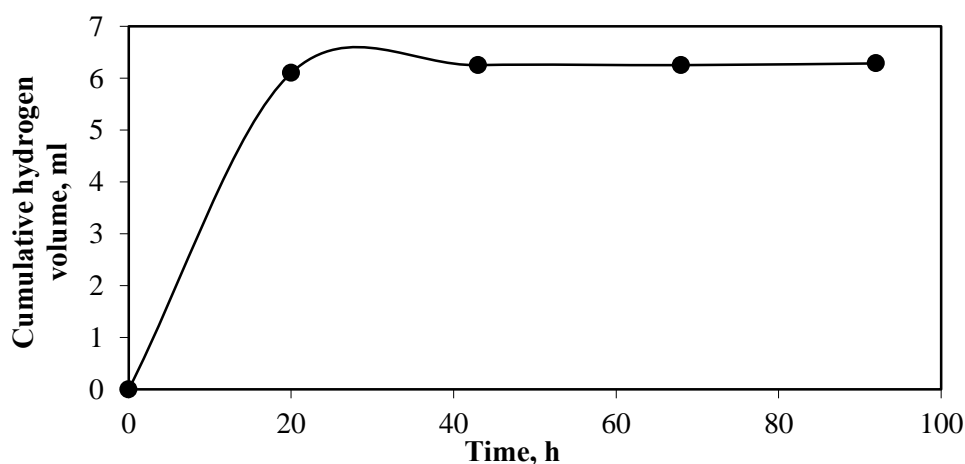
Figure 3.87 Variation of cumulative hydrogen gas volume with time for filtrate obtained at optimized two stage hydrolysis.
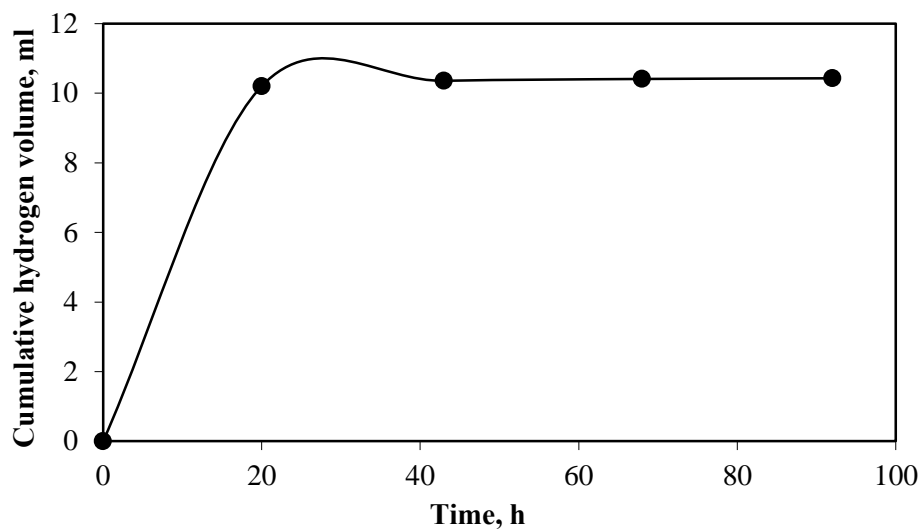
Figure 3.88 Variation of cumulative hydrogen gas volume with time for sludge obtained at optimized two stage hydrolysis.
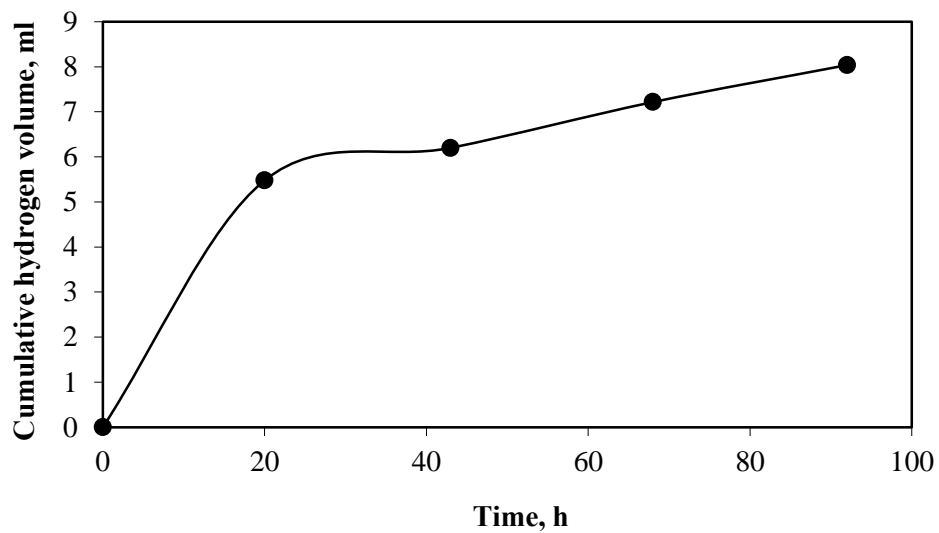
Figure 3.89 Cumulative hydrogen gas volume produced from raw sludge.
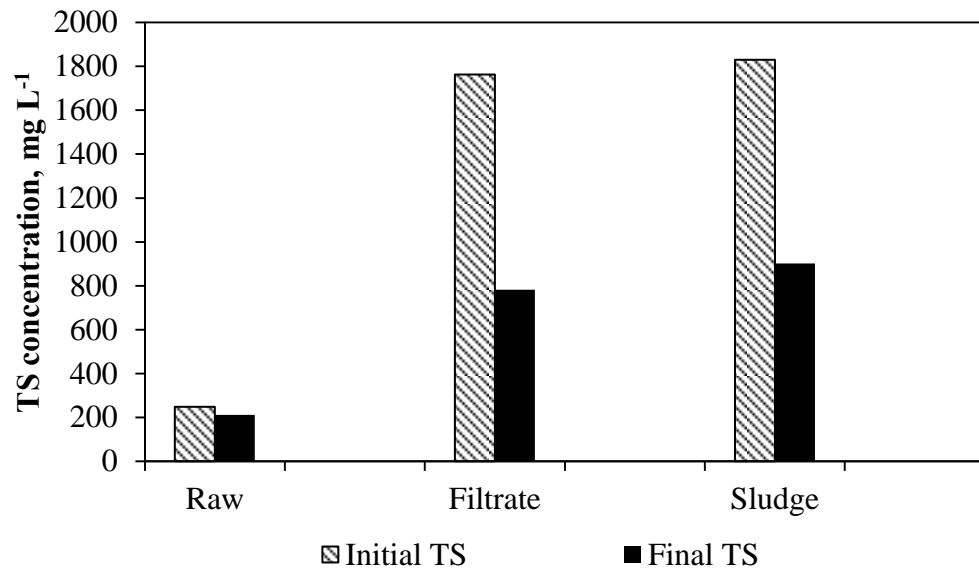
Figure 3.90 Variation of initial and final total sugar concentrations with substrate types.
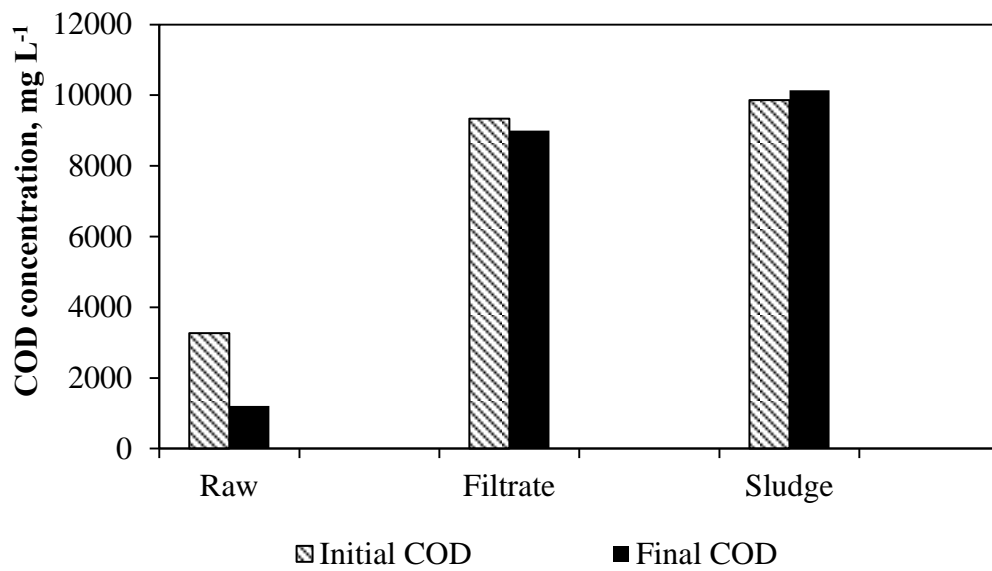
Figure 3.91 Variation of the initial and final COD concentrations with substrate types.
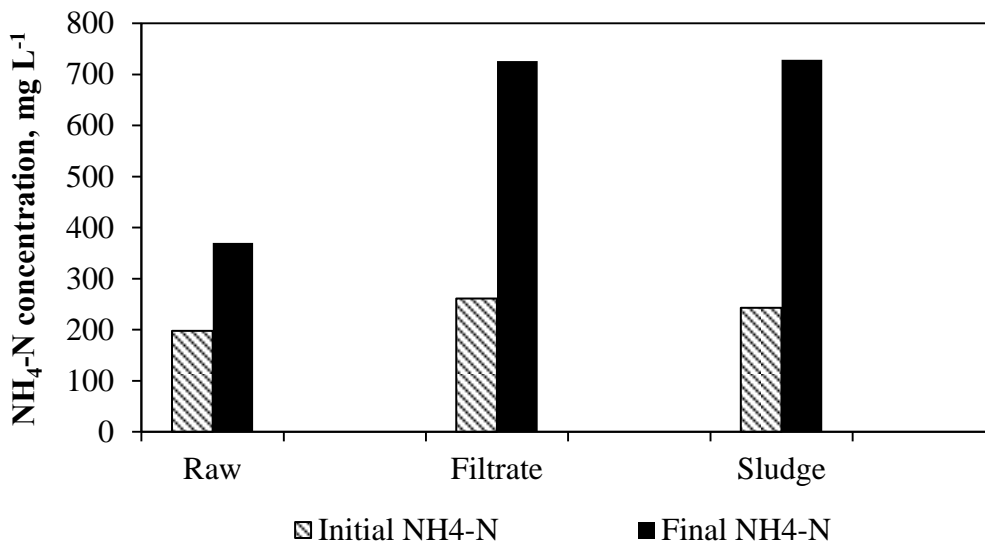
Figure 3.92 Variation of initial and final NH_4-N concentration for raw sludge and substrates of hydrolysis reaction under optimized conditions.
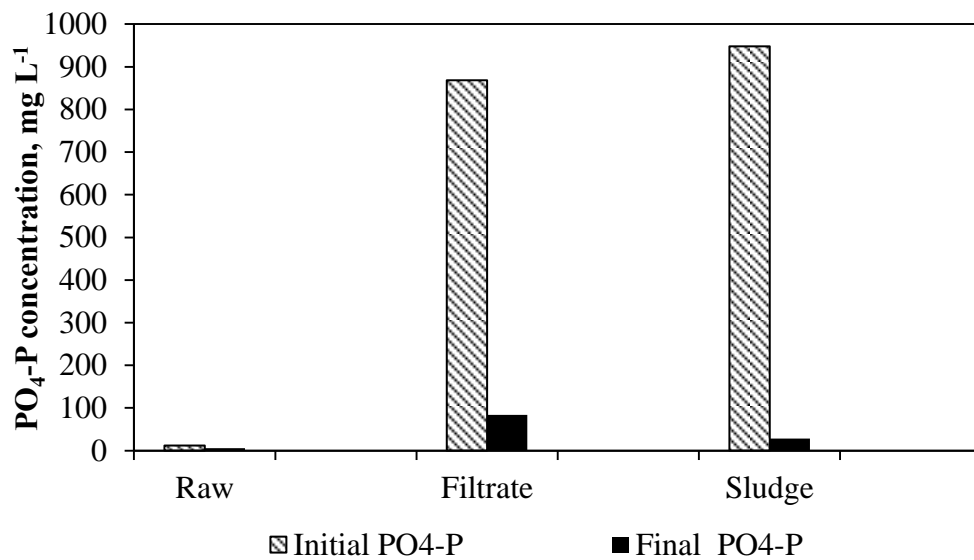
Figure 3.93 Variation of initial and final PO_4-P concentration for raw sludge and substrates of hydrolysis reaction under optimized conditions.
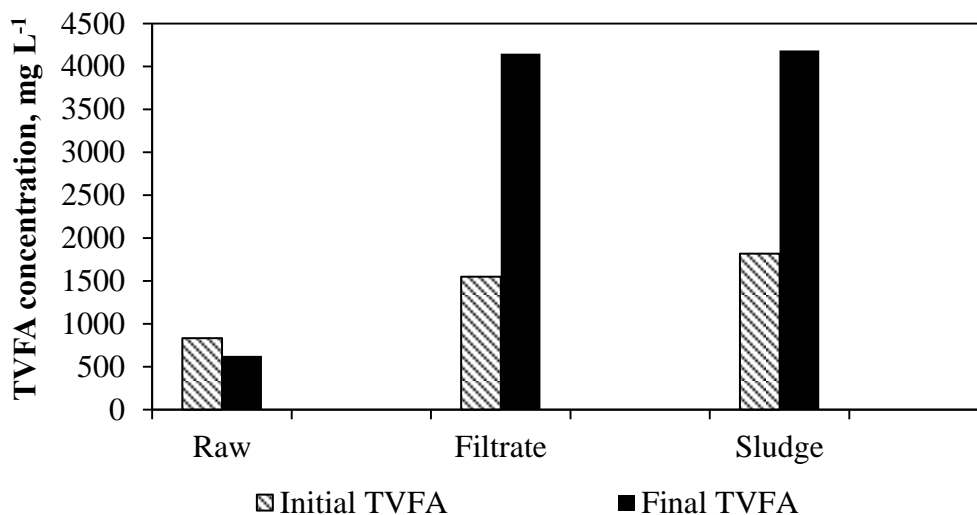
Figure 3.94 Variation of initial and final TVFA concentration for raw sludge and substrates of hydrolysis reaction under optimized conditions.

3.2.4 The Effect of Fermentation Temperature on Hydrogen Gas Production

The effect of fermentation temperature on hydrogen gas production was studied at T=45 ºC and 55 ºC. Sludge was hydrolyzed at optimum conditions determined in two-stage hydrolysis process. Filtrate, hydrolzed sludge and raw sludge were the substrates. Figures 3.95-3.96 depict variation of cumulative hydrogen gas volumes with time for dark fermentation at T=45 ºC for these substrates, respectivley. Maximum production volume from filtrate was V_{H2}=5.8 ml and V_{H2}=8.15 ml from sludge. The CHV did not reach to steady state for raw sludge, but the maximum volume observed was V_{H2}=7 ml.

Total sugar consumptions in filtrate and sludge were about 44% and 50%, respectively (Figure 3.98). As a result of the fermentation of the filtrate and sludge, there was an increase in COD concentration (Figure 3.99) and NH_4-N concentration (Figure 3.100) with regard to the initial ones. PO_4-P consumption efficiency varied depending on the substrate type. The consumption in raw sludge was 70% and 90% in filtrate but rised to 96% for sludge (Figure 3.101). TVFA production amount was 2600 mg L^{-1} in sludge and 1800 g L^{-1} in filtrate.(Figure 3.102). The difference in the TVFA production partly reflected to the hydrogen production. The yields of

productions at this fermentation tempertaure were 1.43 mmol g^{-1} glucose^{-1} (35 ml g^{-1} glucose^{-1}) for the filtrate and 1.77 mmol g^{-1} glucose^{-1} (43.9 ml g^{-1} glucose^{-1}) for the sludge.
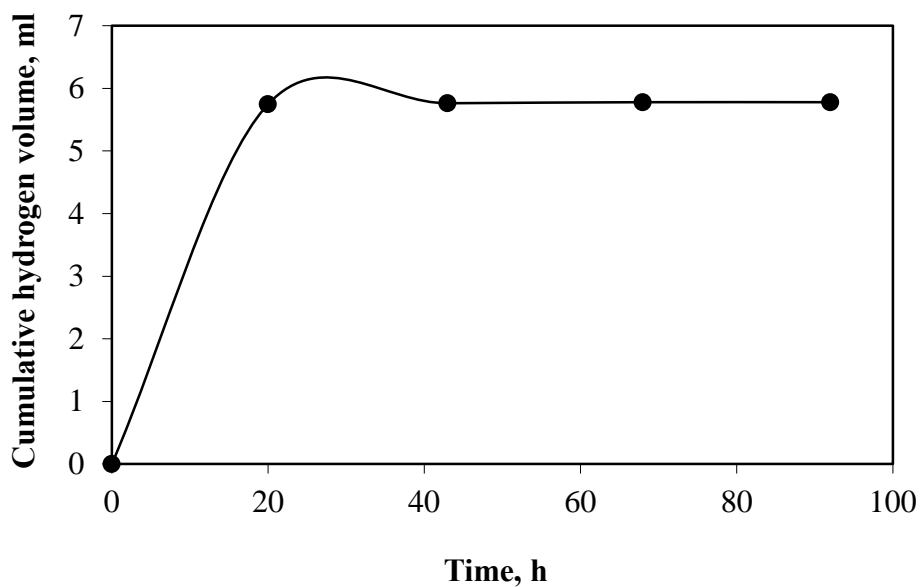
Figure 3.95 Variation of cumulative hydrogen gas volume with time for filtrate at 45°C fermentation temperature.
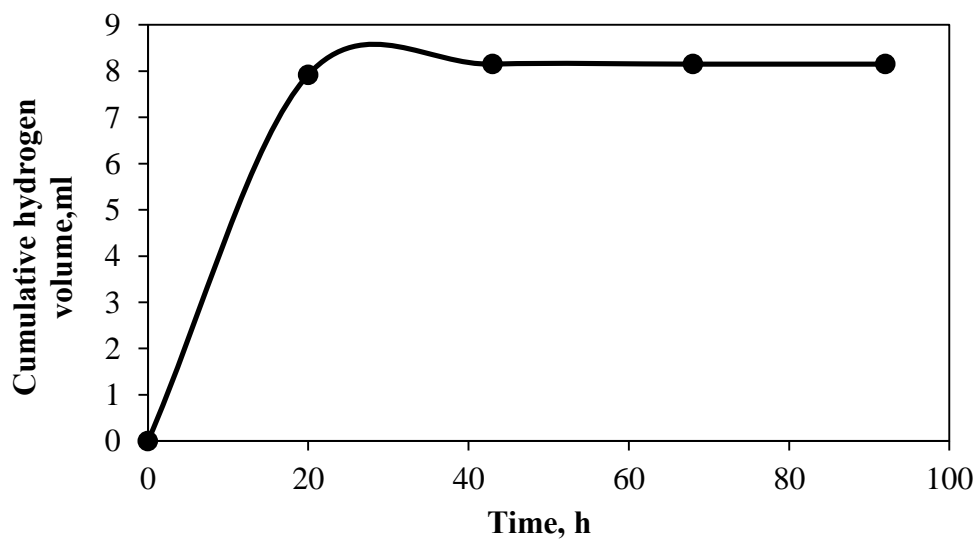
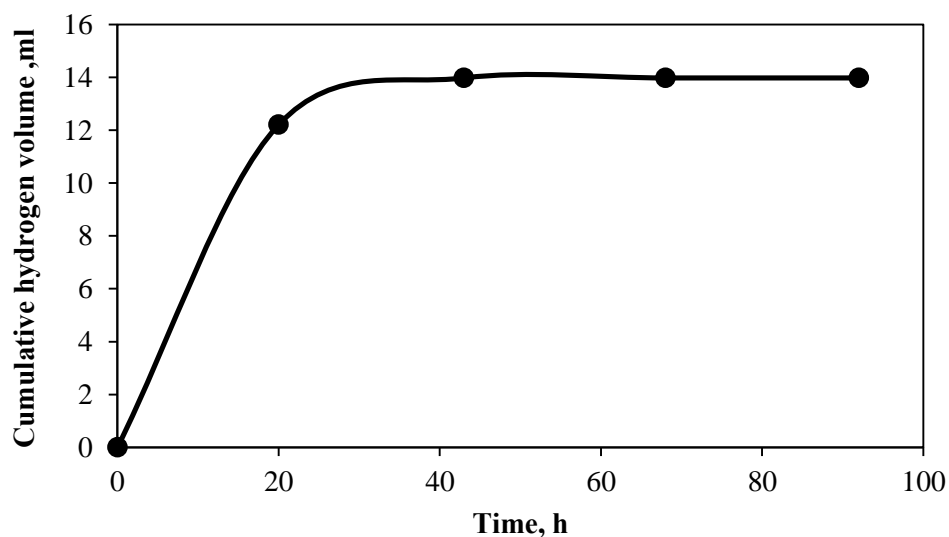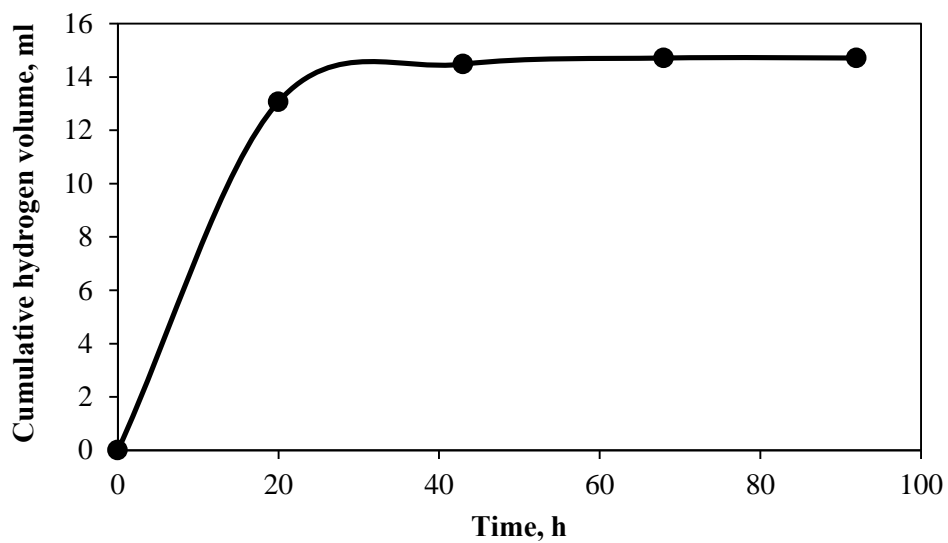Figure 3.96 Variation of cumulative hydrogen gas volume with time for sludge at 45°C fermentation temperature.
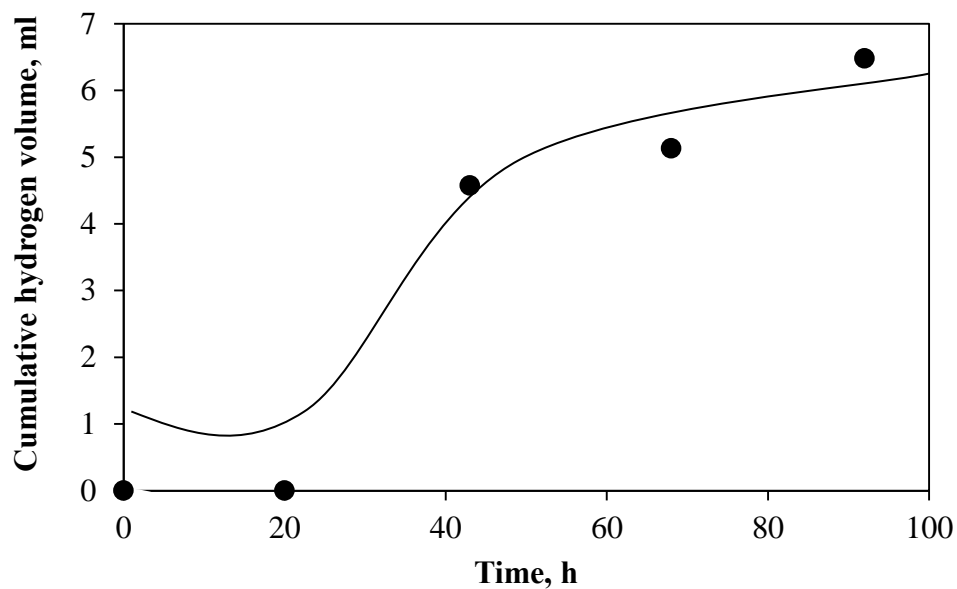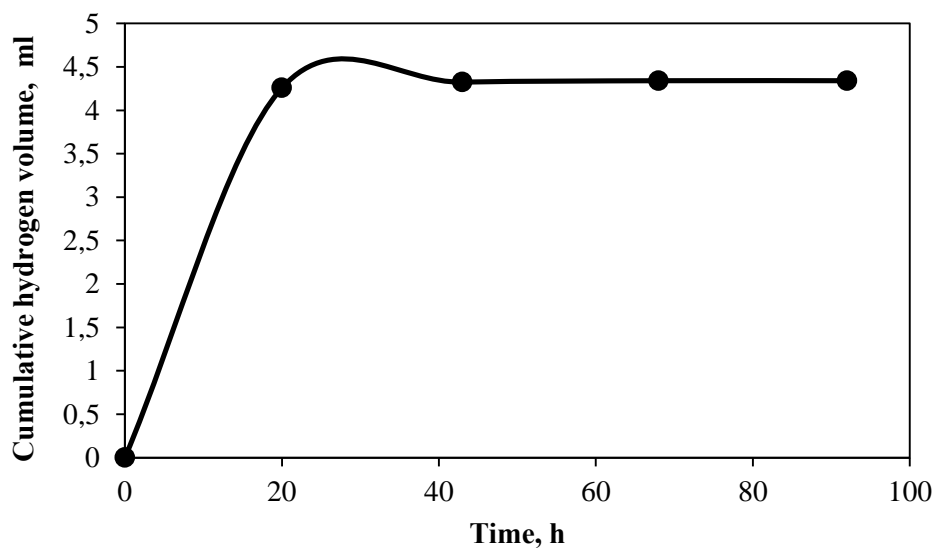
Figure 3.97 Variation of cumulative hydrogen gas volume with time for raw sludge at 45°C fermentation temperature.
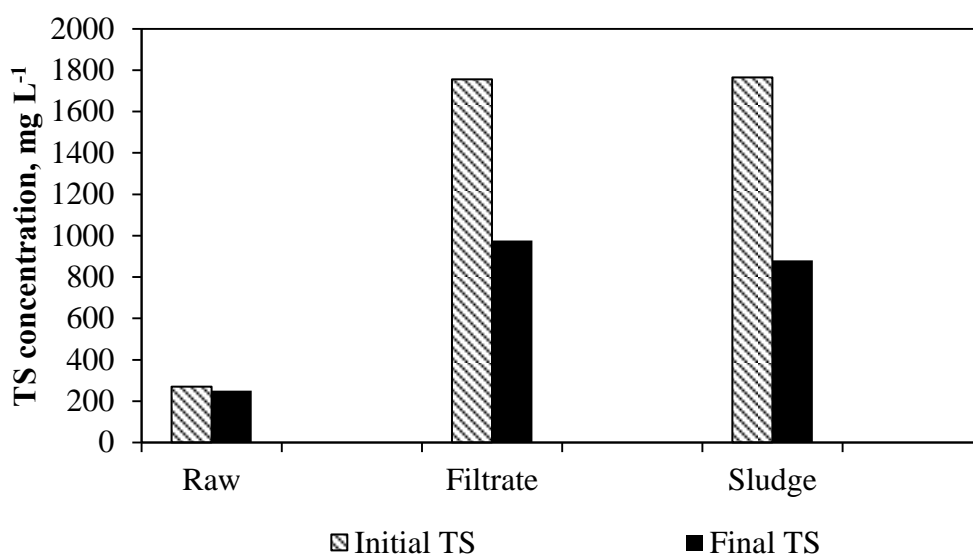
Figure 3.98 Initial and final total sugar concentrations for different substrates at 45°C fermentation temperature.
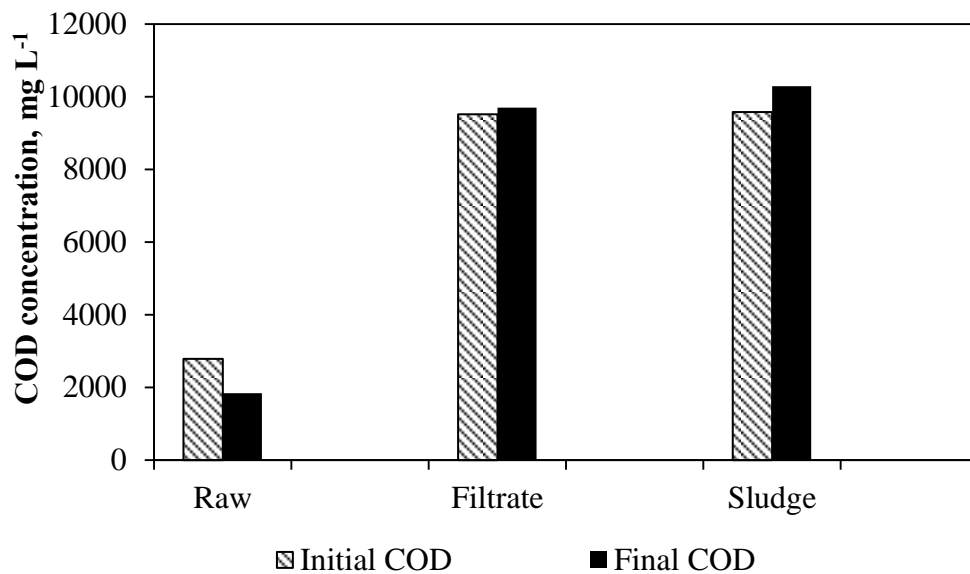
Figure 3.99 Initial and final COD concentrations for different substrates at 45°C fermentation temperature.
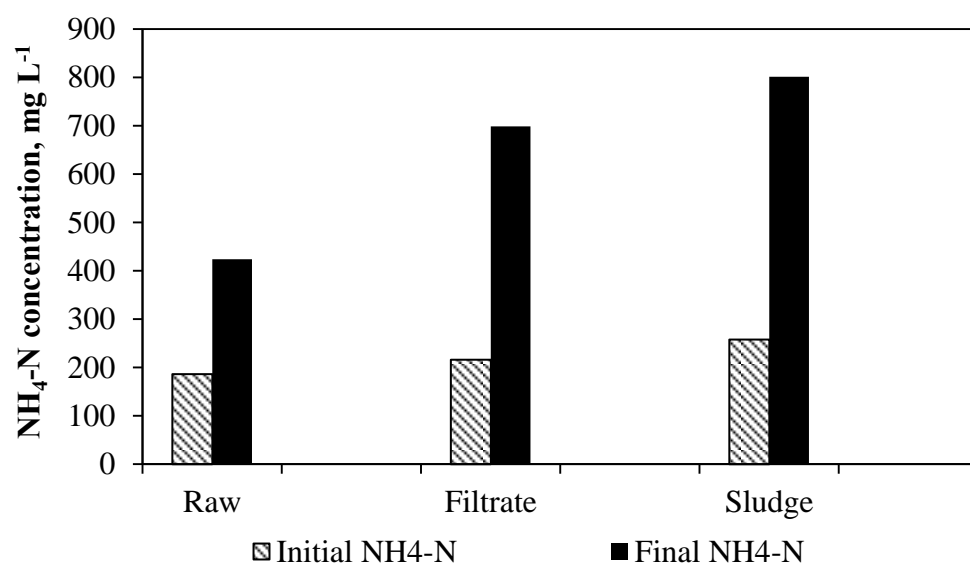
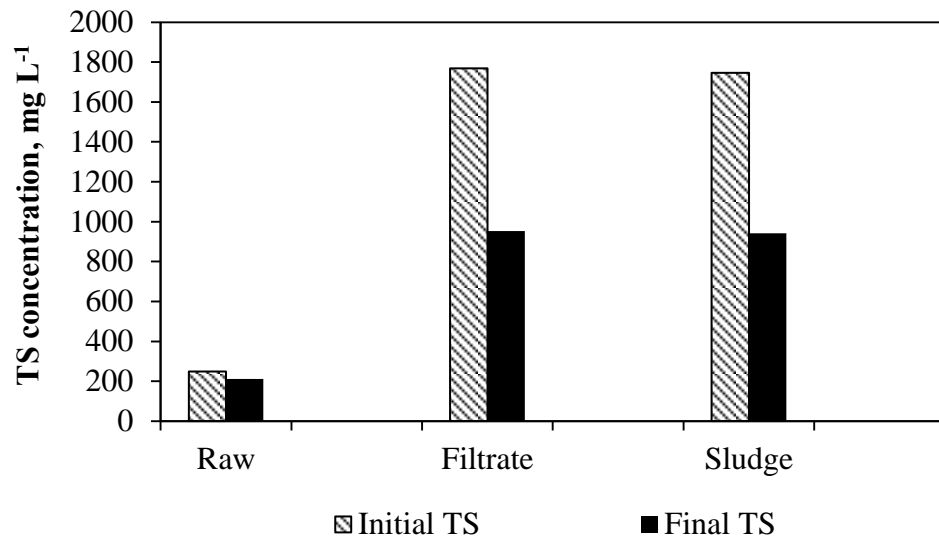Figure 3.100 Initial and final NH$_4$-Nconcentrations for different substrates at 45°C fermentation temperature.
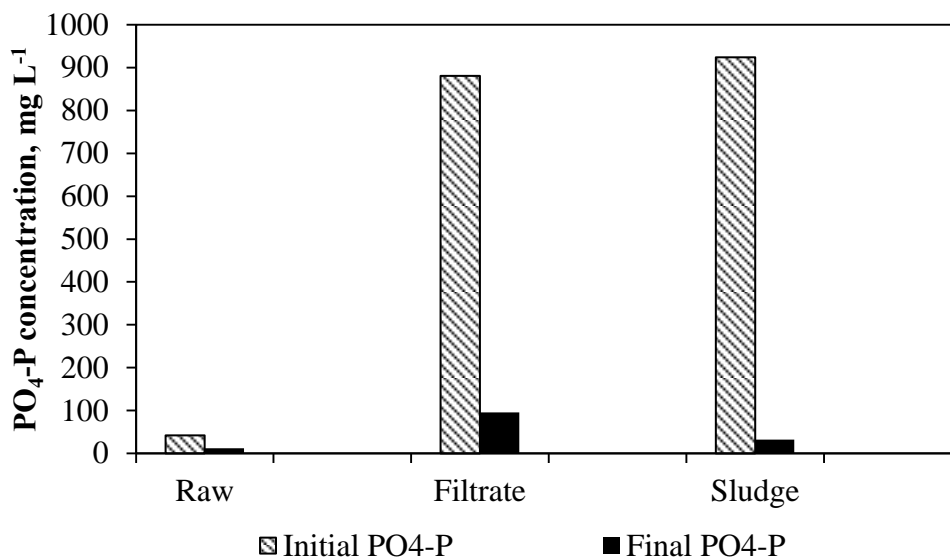
Figure 3.101 Initial and final PO$_4$-P concentrations for different substrates at 45°C fermentation temperature.
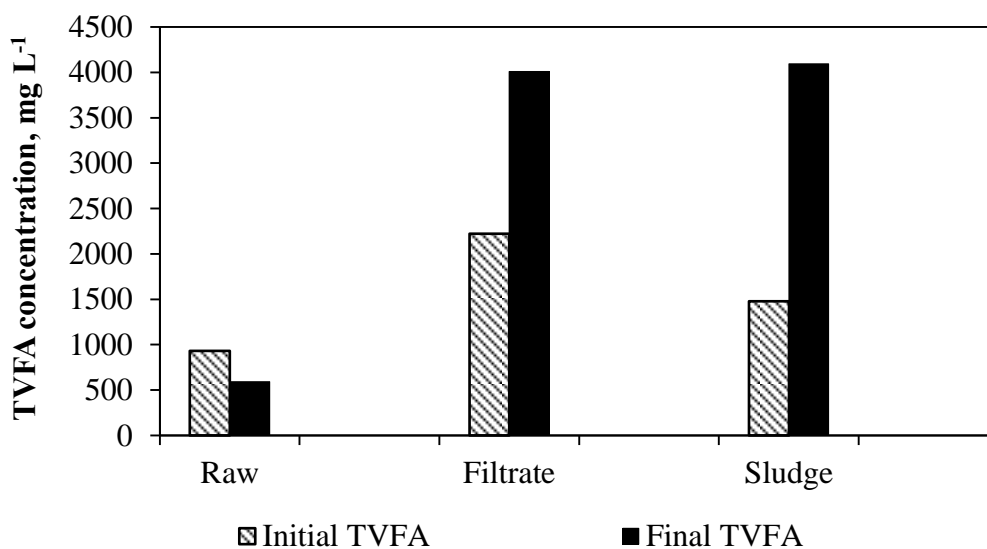
Figure 3.102 Initial and final TVFA concentrations for different substrates at 45°C fermentation temperature.

Fermentation temperature was rised to 55ºC and the cumulative hydrogen gas volumes obtained for different substrates are given in Figures 3.103 - 3.105. The cumulative hydrogen gas volume obtained from the filtrate and the sludge was about 14 ml. In raw sludge maximum production volume was only 4.3 ml. Total sugar consumptions at 55ºC fermentation tempertaure were 46% and 55% for filtrate and sludge, respectively (Figure 3.106). In raw sludge, only 14% sugar consumption was obtained.

COD was not removed effectively in sludge and filtrate (Figure 3.107). The increase in NH_4-N concentration showed the same trend as it was observed in the fermentation of hydrolysates obtained from different pre-treatment processes (Figure 3.108). PO_4-P consumption decreased depending on the substrate type. The consumption in raw sludge was 25% and 87% for filtrate but it was about 90% for sludge (Figure 3.109). The amount of TVFA production was about 2000 mg L^{-1} in sludge and in filtrate. Relatively higher TVFA production was observed for raw sludge and it was about 1300 mg L^{-1} (Figure 3.110). Calculated production yields at this fermentation temperature were 3.3 mmol g^{-1} TS^{-1} (81 ml g^{-1} TS^{-1}) for filtrate, 3.5 mmol g^{-1} TS^{-1} (87 ml g^{-1} TS^{-1}) for sludge.

Figure 3.103 Variation of cumulative hydrogen gas volume with time for filtrate at 55°C fermentation temperature.

Figure 3.104 Variation of cumulative hydrogen gas volume with time for sludge at 55°C fermentation temperature.

Figure 3.105 Variation of cumulative hydrogen gas volume with time for raw sludge at 55°C fermentation temperature.

Figure 3.106 Initial and final TS concentrations for different substrates at 55°C fermentation temperature.
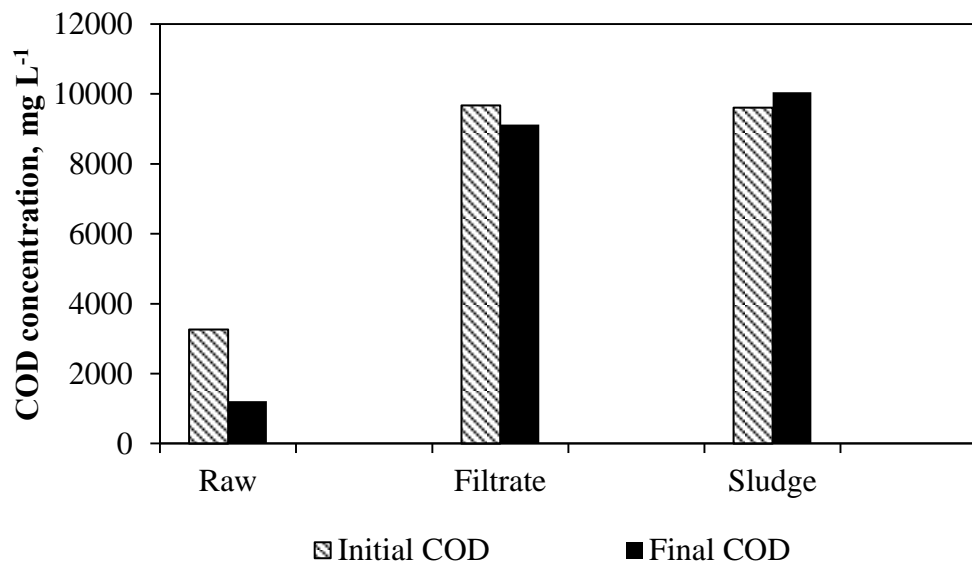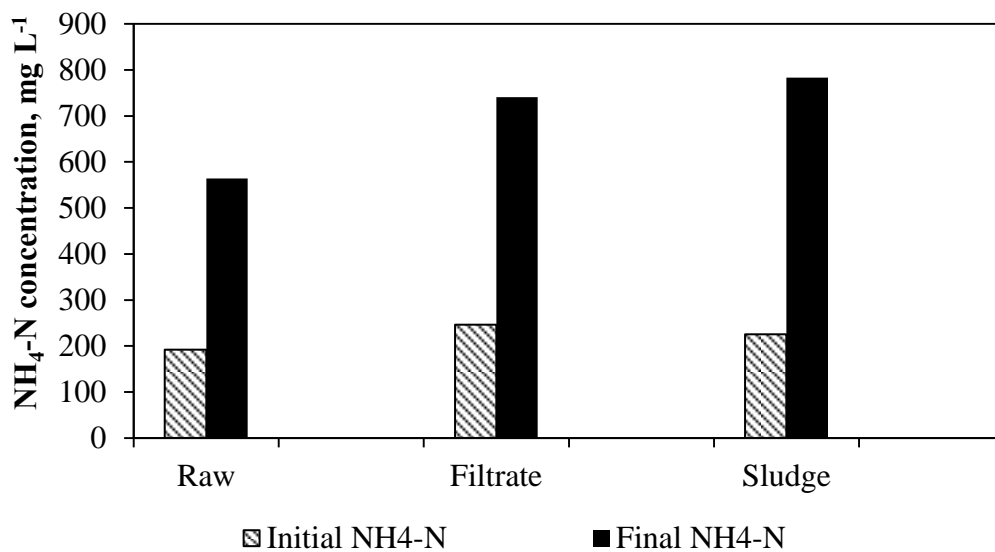

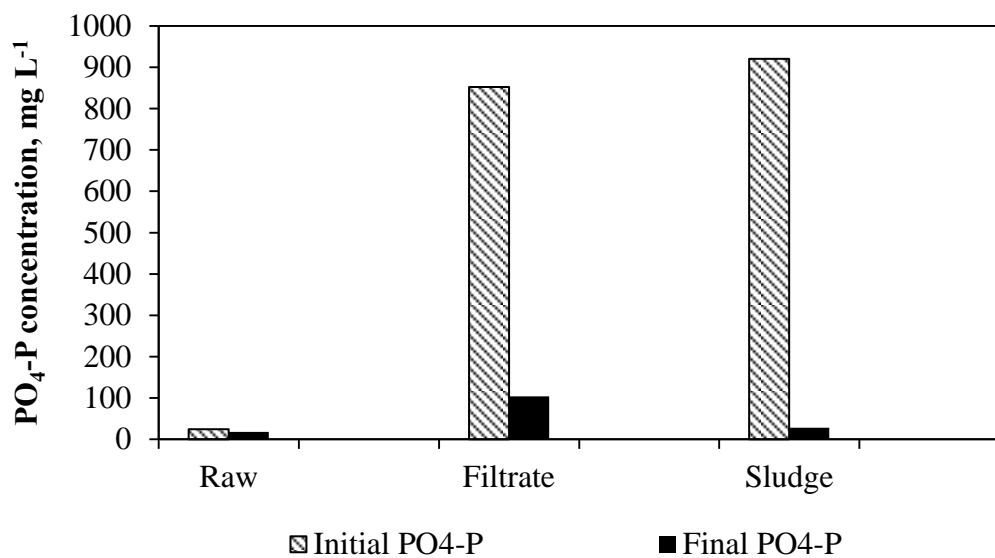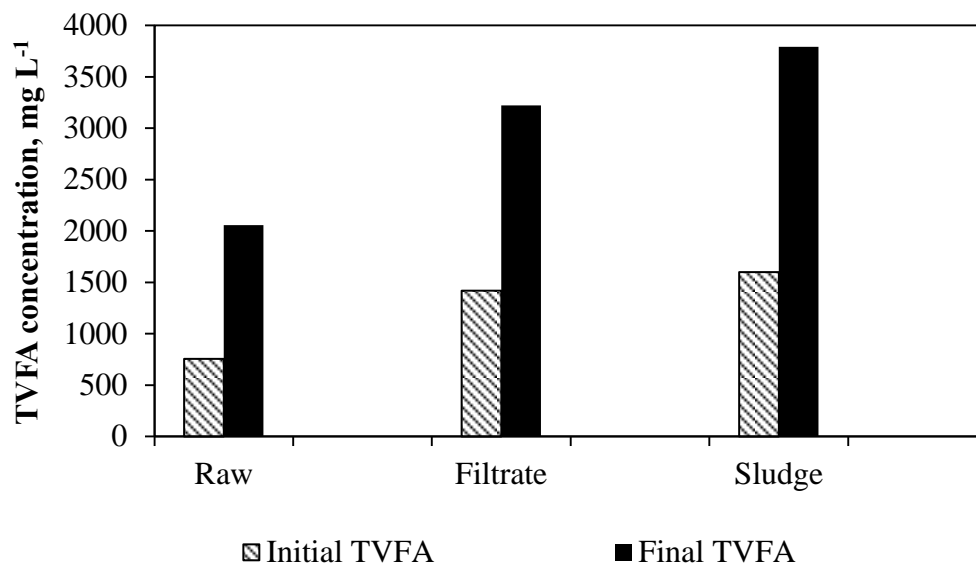Figure 3.107 Initial and final COD concentrations for different substrates at 55°C fermentation temperature.

Figure 3.108 Initial and final NH$_4$-N concentrations for different substrates at 55°C fermentation temperature.

Figure 3.109 Initial and final PO$_4$-P concentrations for different substrates at 55°C fermentation temperature.

Figure 3.110 Initial and final TVFA concentrations for different substrates at 55°C fermentation temperature.

3.2.5 The Effect of Peptone Concentration on Hydrogen Gas Production

It has been reported that protein concentration in the fermentation media could enhance the hydrogen gas production (Bartacek et al., 2007; Cai et al., 2004; Guo et al., 2008; Thungklin et al., 2010). In the light of these studies, the effect of excess amount of protein in dark fermentation of hydrolyzed sludge and its filtrate was investigated. Protein source was peptone and it was added to the media in concentrations between 1 g L^{-1} -5 g L^{-1}. Hydrogen gas volume and removal of COD, total sugar, protein, NH_4-N and PO_4-P were determined at different concentration at protein supplementation.

The effect of peptone concentration on variation of cumulative hydrogen gas volume is given in Figures 3.111-3.115. As seen from figures, the CHVs were around V_{H2}=10±2 ml for all examined peptone concentrations. No substantial change in CHV with peptone concentration was obtained. However, the addition of peptone enhanced total sugar utilization (Figure 3.116). While the initial sugar concentration was about 1200 mg L^{-1}, the final concentration decreased to 360±10 mg L^{-1} and over 75% sugar consumption was obtained. Protein consumption was at the level of

50±2% (Figure 3.117). Because of the high protein concentration and hydrolysis of proteins by the microbial activity during fermentation, final NH_4-N concentration rised up to 834 mg L^{-1} and 1300 mg L^{-1} from 300±30 mg L^{-1}, with the increase in externally added protein concentrations from 1 g L^{-1} to 5 g L^{-1}, respectively (Figure 3.118). An efficient PO_4-P removal was achieved around 68±2 % (Figure 3.119). In summary, peptone supplementation did not effect the hydrogen gas production, substantially.
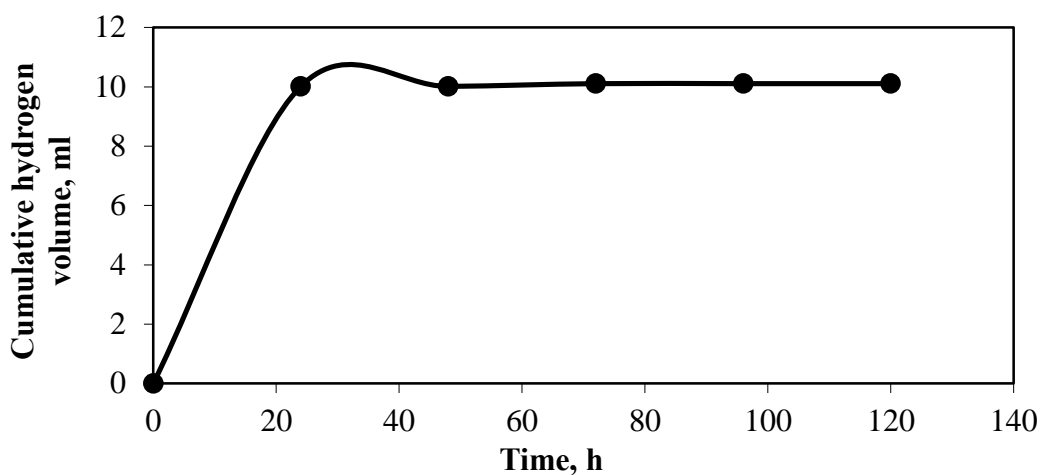
Figure 3.111 Variation of cumulative hydrogen gas volume with time for filtrate at 1 g L^{-1} peptone supplementation.
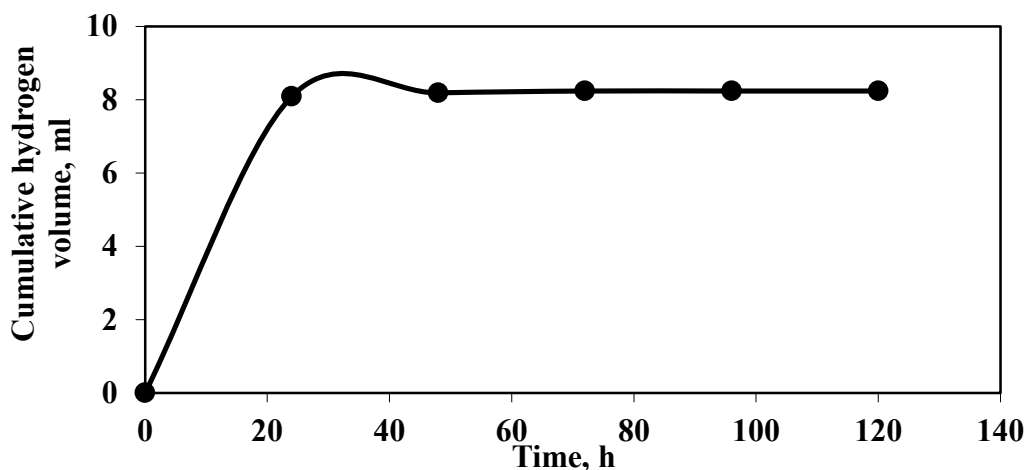
Figure 3.112 Variation of cumulative hydrogen gas volume with time from filtrate at 2 g L^{-1} peptone supplementation.
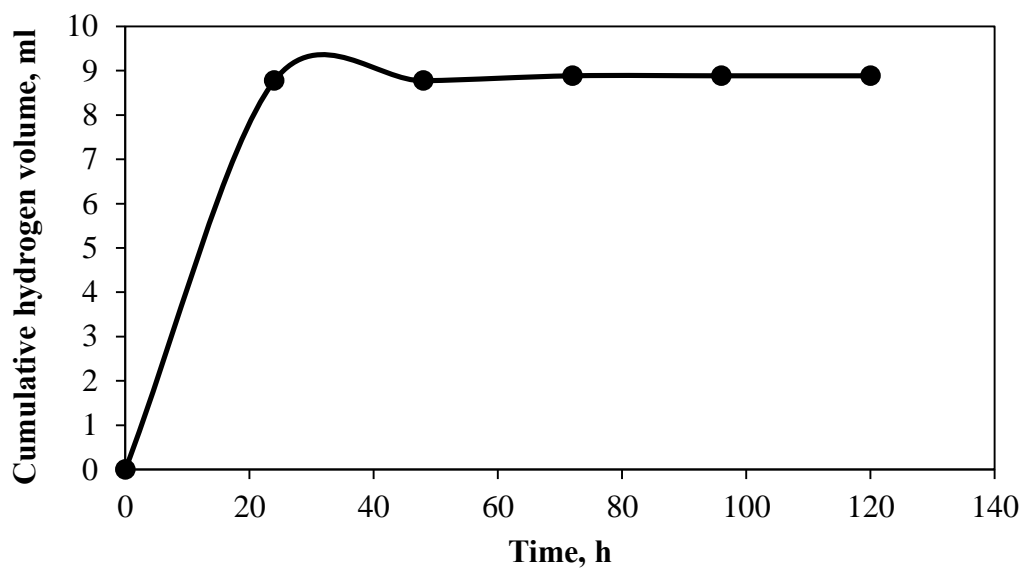
Figure 3.113 Variation of cumulative hydrogen gas volume with time from filtrate at 3 g L^{-1} peptone supplementation.
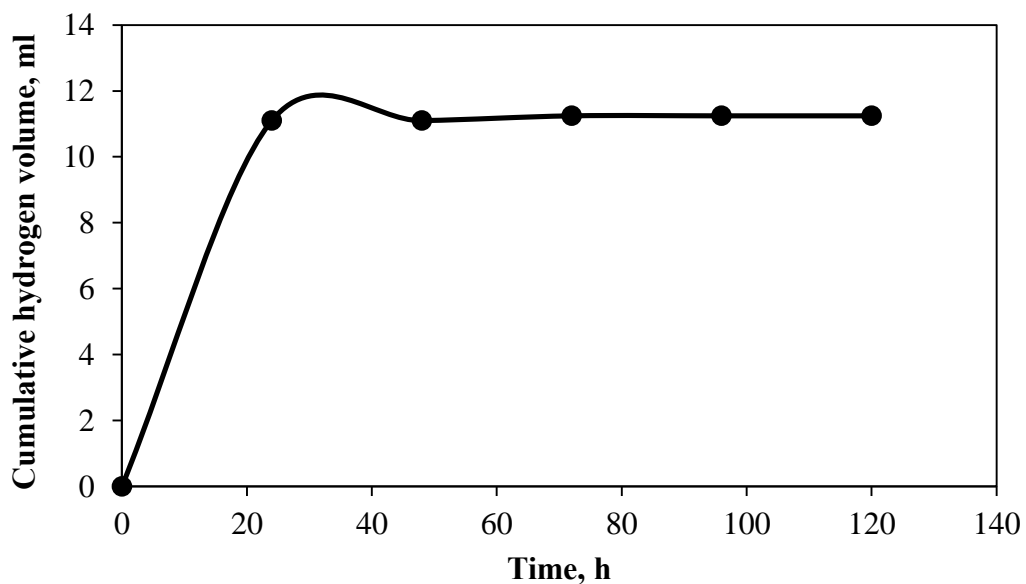

Figure 3.114 Variation of cumulative hydrogen gas volume with time from filtrate at 4 g L^{-1} preptone supplementation
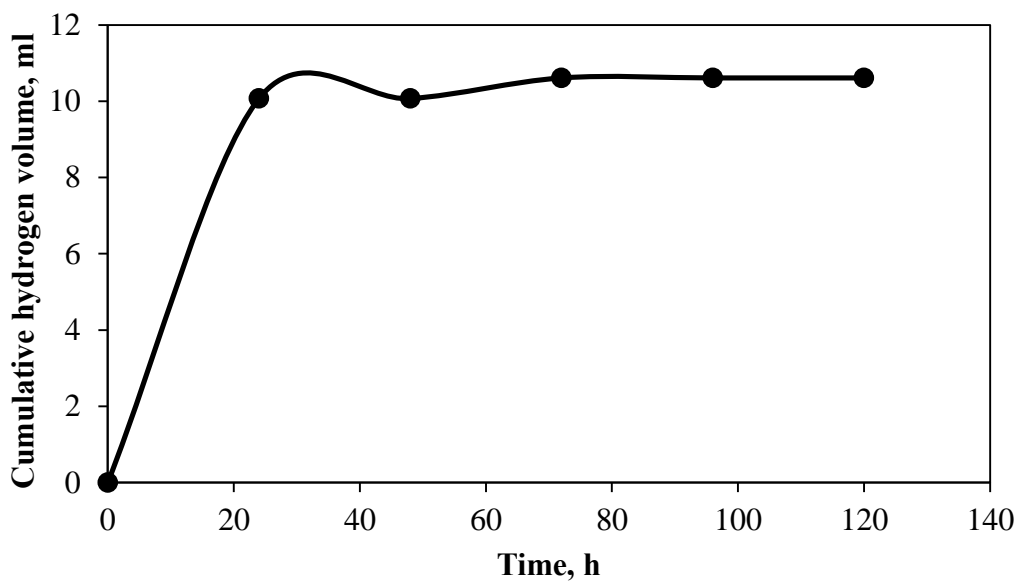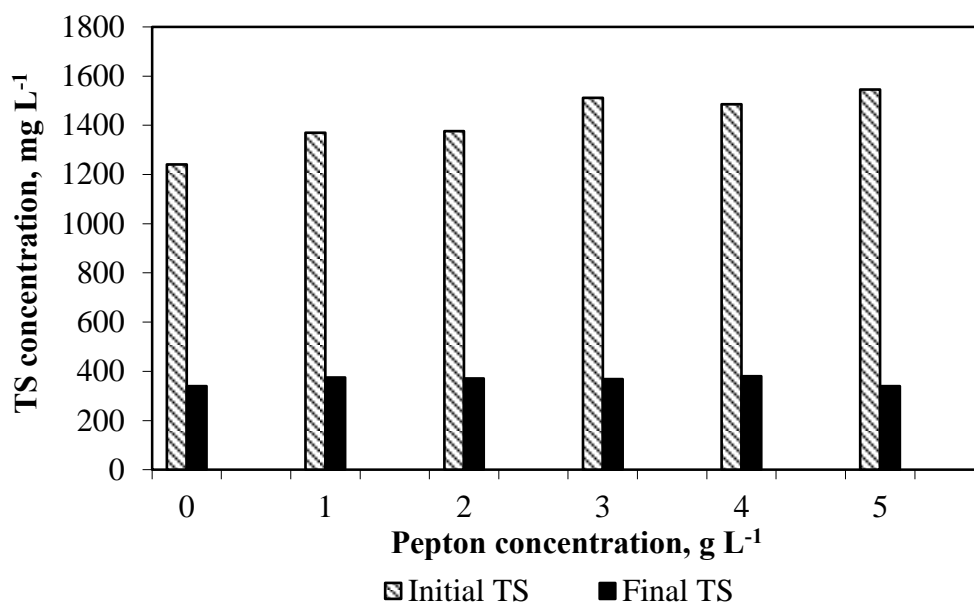
Figure 3.115 Variation of cumulative hydrogen gas volume with time from filtrate at 5 g L^{-1} peptone supplementation.

Figure 3.116 Initial and final total sugar concentrations of filtrate fermentation for hydrogen gas production at different peptone supplementation.
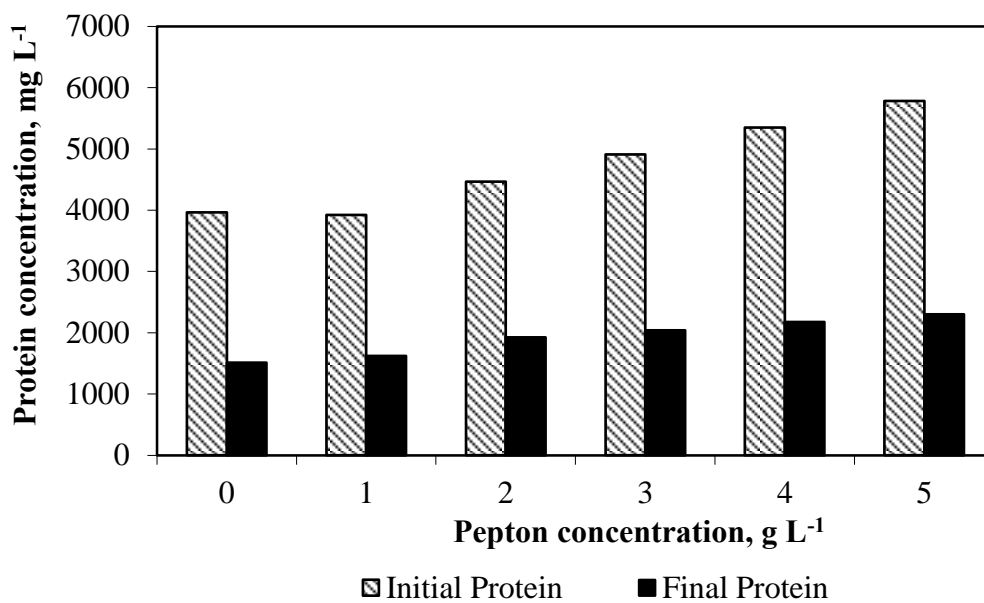
Figure 3.117 Initial and final protein concentrations of filtrate fermentation for hydrogen gas production at different peptone supplementation.
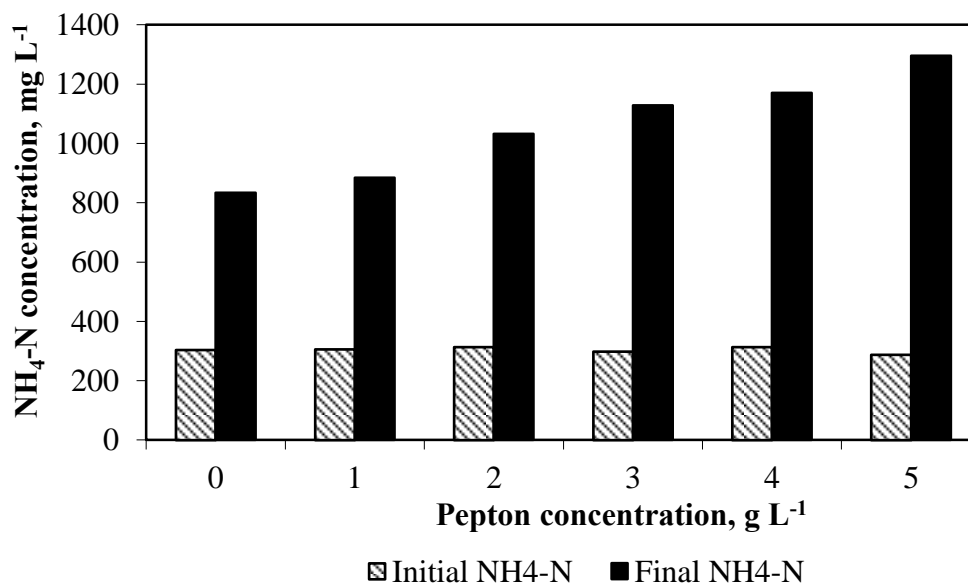

Figure 3.118 Initial and final NH_4-N concentrations of filtrate fermentation for hydrogen gas production at different peptone supplementation.
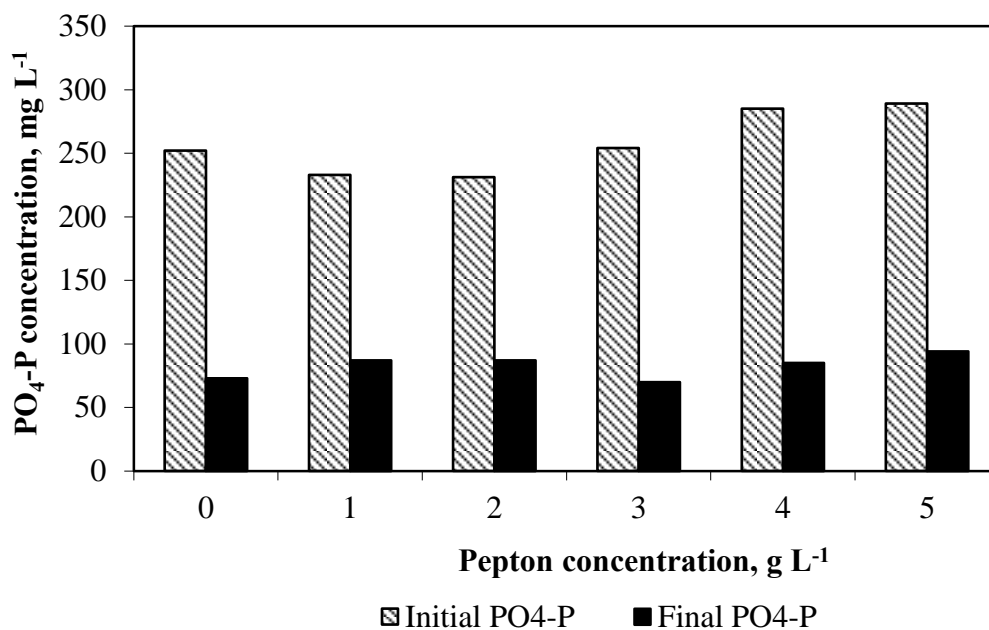
Figure 3.119 Initial and final PO$_4$-P concentrations of filtrate fermentation for hydrogen gas production at different peptone supplementation.

3.2.6 The Effect of Fermentation pH on Hydrogen Gas Production

pH of dark fermentation is one of the most important factor in hydrogen gas production by dark fermentation. The optimal pH value varies between 5.5 and 7 depending on the substrate and microbial culture (Argun & Kargi, 2011). pH of fermentation media was kept constant at pH=7 up to this stage of the study. In order to investigate the effect of pH on hydrogen gas production, the pH of fermentation media was adjusted to pH=4, pH=5 and pH=6. Fermentation temperature was 37 ^{o}C. The substrates were filtrate and sludge obtained form two stage hydrolysis of stock sludge at determined optimum hydrolysis conditions. The head spaces of the bottles were sparged with argon in this set of experiments. The experiment at pH=7 with argon sparging was repeated for control purpose.

Figures 3.120-3.123 show variation of cumulative hydrogen gas volumes with time for filtrate which was subjected to dark fermentation at different pHs. Hydrogen

gas production increased rapidly in the first 150 hours of the fermentation and remained constant for the rest of the fermentation. It was V_{H2}=23 ml at pH=4 and rised up to V_{H2}=41 ml at pH=5. The cumulative hydrogen gas volumes were lower (V_{H2}=30 ml) at pH=6 and pH=7. However, the maximum cumulative hydrogen gas volume obtained from filtrate up to this study at pH=7 was around 10±2 ml. Sparging the head space with argon provided a substantial increase in the total hydrogen gas production. Therefore, this strategy was used in the further experiments.
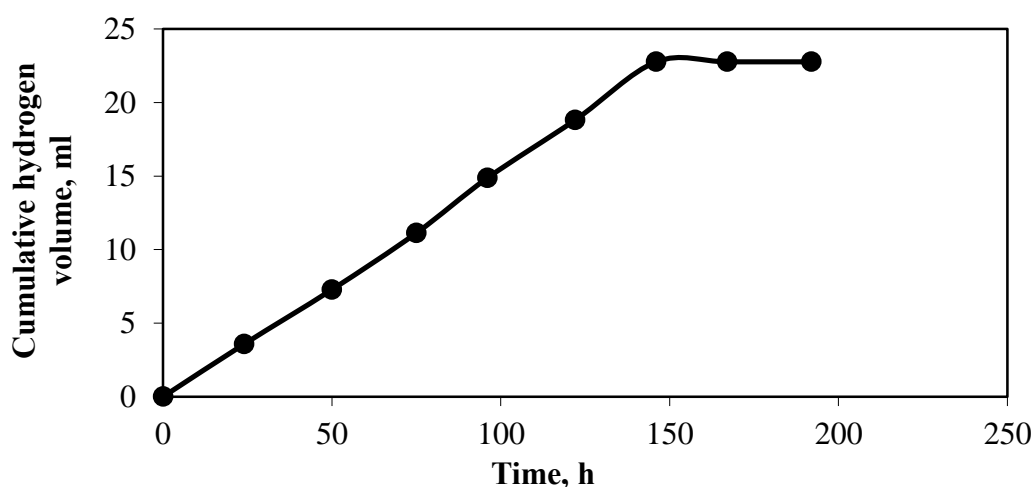

Figure 3.120 Variation of cumulative hydrogen gas volume with time in dark fermentation of filtrate at pH=4.
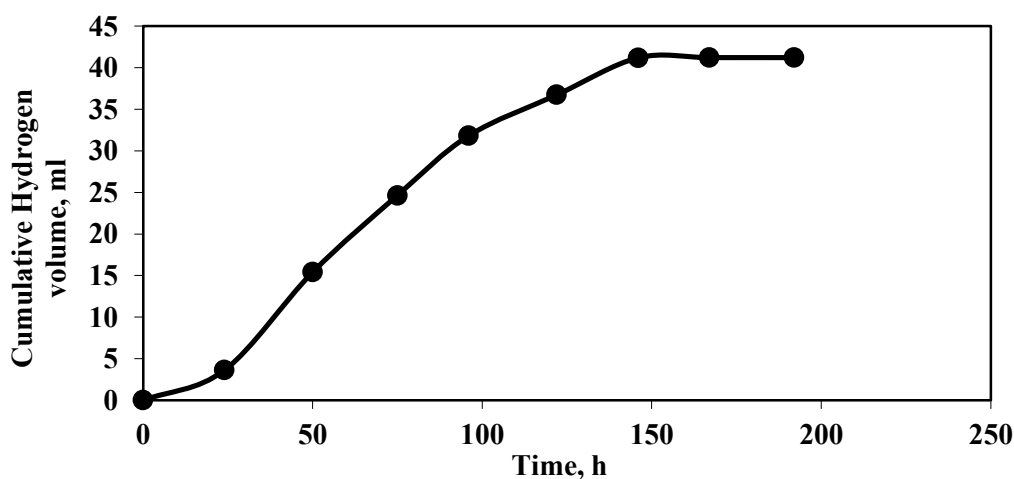

Figure 3.121 Variation of cumulative hydrogen gas volume with time in dark fermentation of filtrate at pH=5.
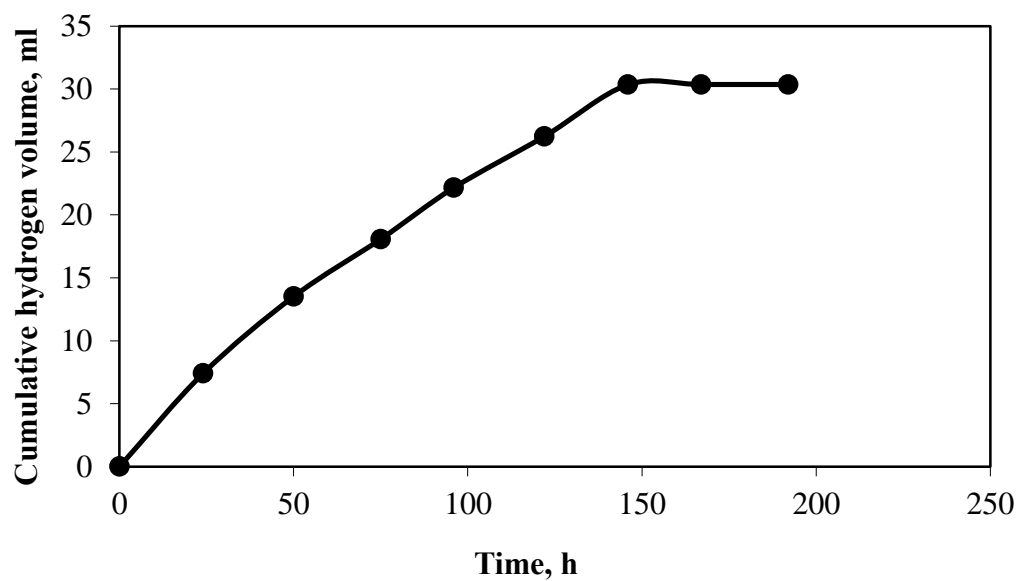
Figure 3.122 Variation of cumulative hydrogen gas volume with time in dark fermentation of filtrate at pH=6.
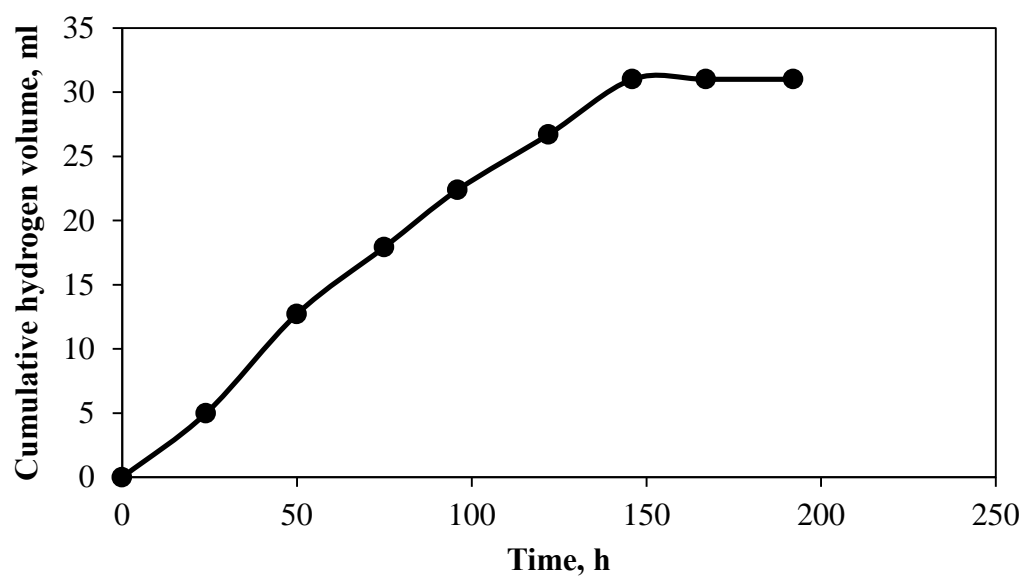

Figure 3.123 Variation of cumulative hydrogen gas volume with time in dark fermentation of filtrate at pH=7.

The effect of pH on initial and final organic matter and nutrient concentrations in the fermentation media is given in Figures 3.130-3.134. The total sugar concentration

was around 1200 mg L^{-1} in filtrate after hydrolysis reaction. Final total sugar concentrations decreased from 1000 mg L^{-1} to 500 mg L^{-1} with the rise in pH from pH=4 to pH=7. In other words, sugar removal during fermentation increased from 30% to 56%, respectively. There was an inverse relationship between sugar consumption and hydrogen gas production. Despite to high hydrogen production such as at pH=5, the sugar consumption was around 30%. This result can be evaluated as microorganisms diverted their metabolism to the organic acid production from organic substances rather than using it for cell growth or cell maintenance. The TVFA profile at different fermentation pH supports this evaluation. As shown in Figure 3.131, TVFA concentrations were higher in the effluent of fermentation conducted at pH=4 and pH=5 at which higher hydrogen productions were obtained. The yields of TVFA formation from consumed total sugar at pH=4 was $Y_{TVFA/TS}$=5.9 and it was $Y_{TVFA/TS}$=3.6 at pH=5. The high yield of TVFA formation at these pH values indicate effective utilization of sugar for organic acid production. Therefore, the optimum pH range for maximum TVFA and hydrogen gas productions was determined as pH=4-5.

The effect of fermentation pH value on NH_4-N concentration is given in Figure 3.126. The initial NH_4-N concentration in filtrate after hydrolysis was about 300±20 mg L^{-1} but the final concentration rised up to 700 mg L^{-1} as it was obtained at pH=6. As seen in Figure 3.127 increasing pH from pH=4 to pH=7, protein concentration decreased from 3500 mg L^{-1} to 1600 mg L^{-1} with almost 50% removal efficiency. Organic matter consumption took place at low levels and no substantial difference was observed between initial and final COD concentrations for different fermentation pH values (Figure 3.128). Organisms can consume sugar but they cannot consume other organic matters occurred after hydrolysis. But, it is well known that high COD removal efficiency is not expected at acidogenic phase of anaerobic treatment. Since, organic substances are mainly biotransformed to organic acids which contribute to COD content of the medium.
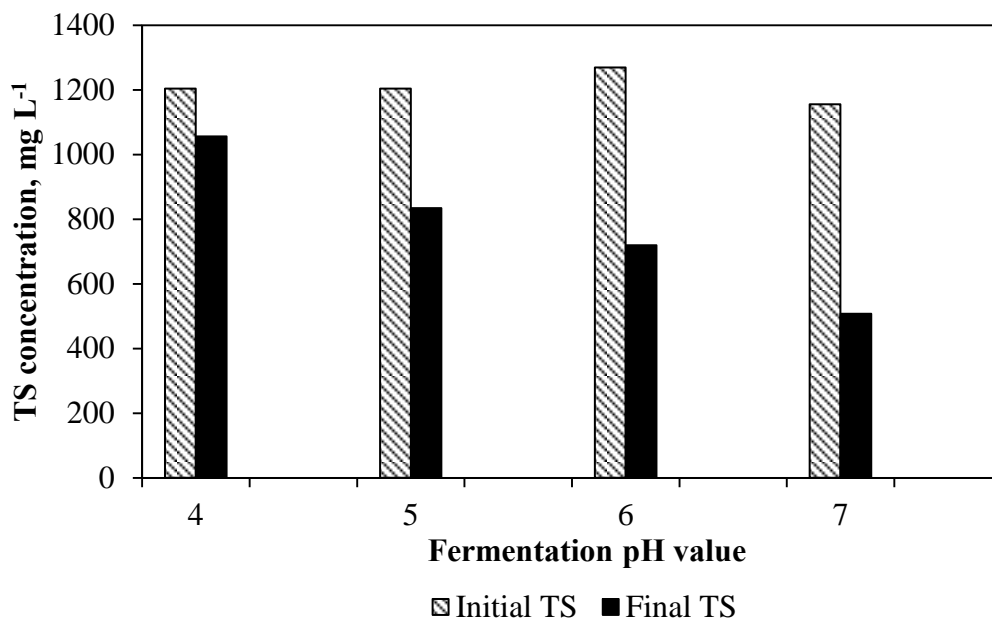
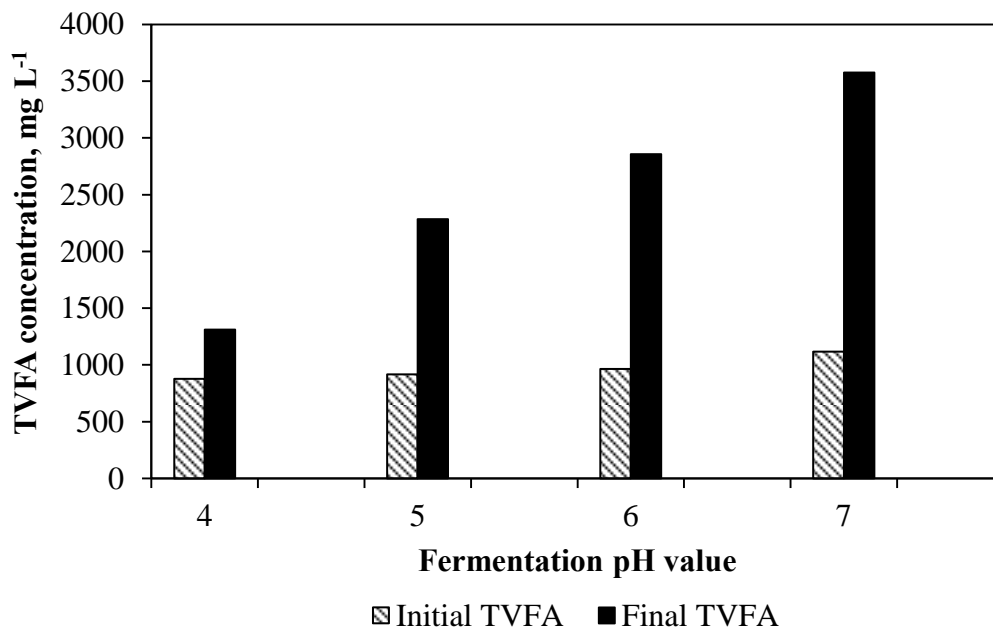Figure 3.124 Initial and final total sugar concentrations of filtrate fermentation at different pH values.

Figure 3.125 Initial and final TVFA concentrations of filtrate fermentation at different pH values.

Figure 3.126 Initial and final NH$_4$-N concentrations of filtrate fermentation at different pH values.

Figure 3.127 Initial and final protein concentrations of filtrate fermentation at different pH values.

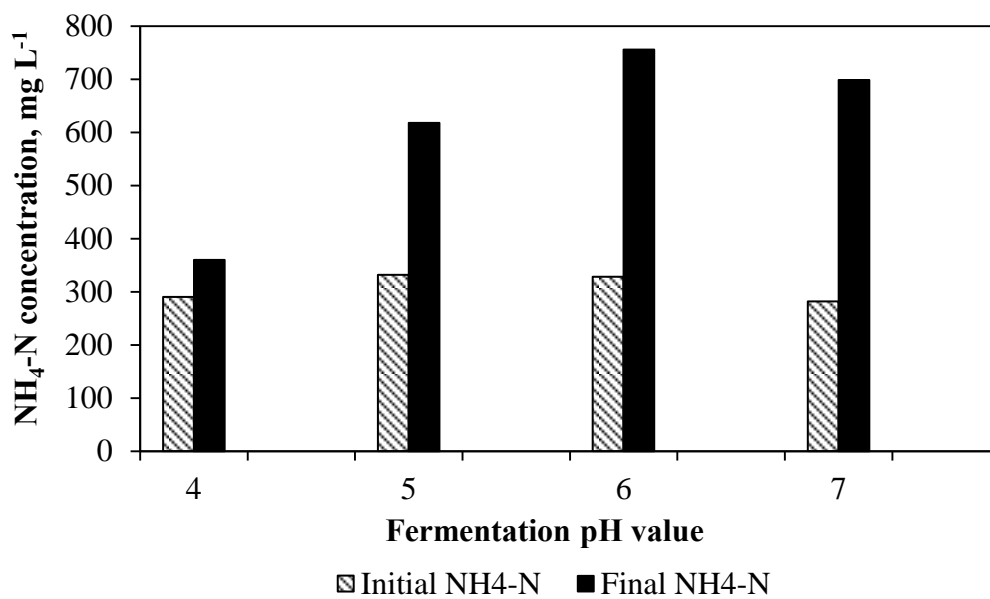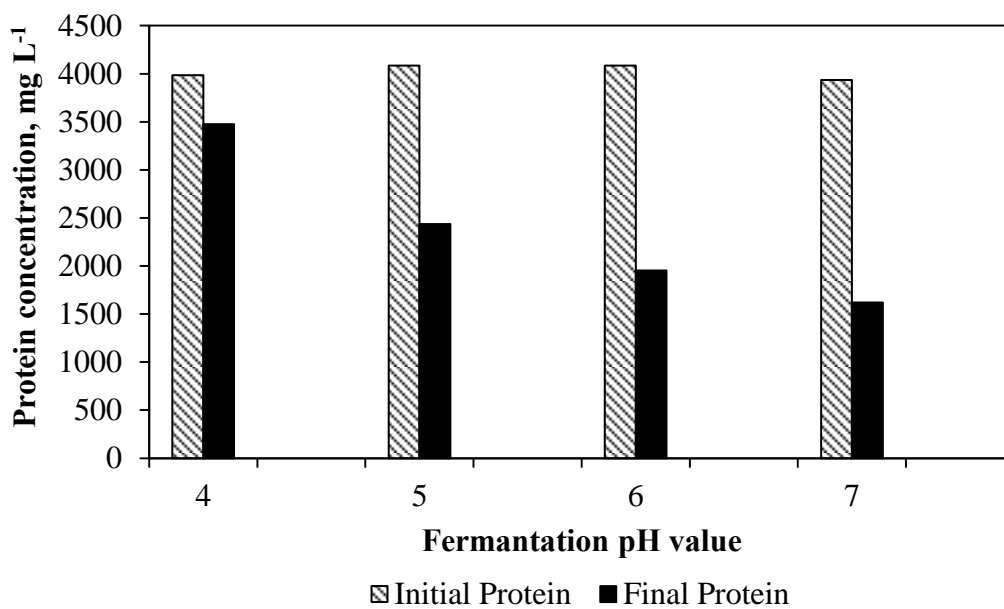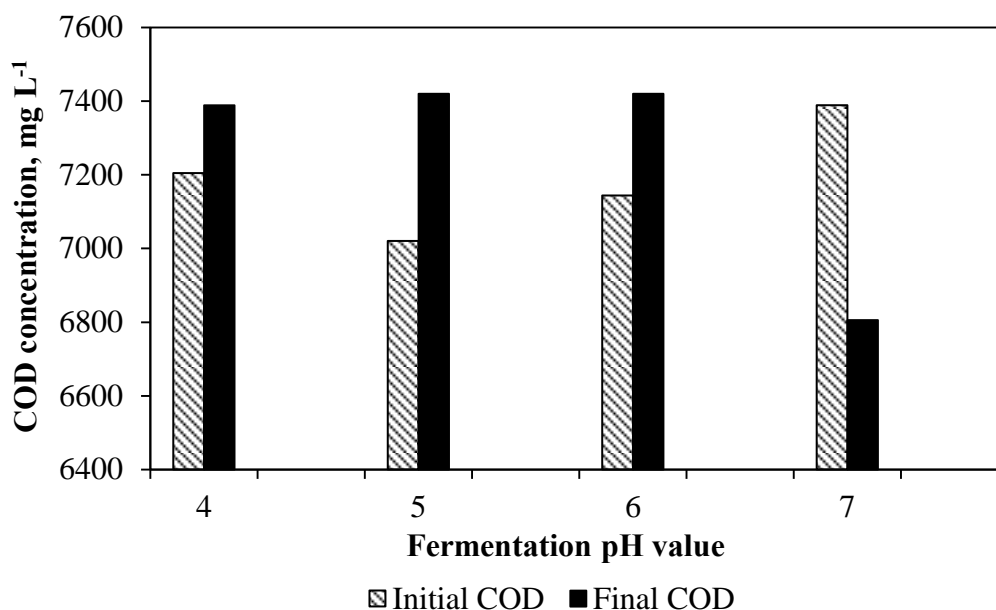Figure 3.128 Initial and final COD concentrations of filtrate fermentation at different pH values.

Hydrogen gas production potentials, production rates and lag phase durations were calculated by using Gompertz Equation (Table 3.25). Production potential calculated from the equation and the volumes observed in experimental studies were in agreement. Although lag phase was as long as $\lambda = 18$ hours at pH=5, hydrogen gas production rate (HPR) reached to maximum level of HPR=0.46 ml h^{-1} and specific hydrogen production rate, SHPR=0.18 ml g biomass^{-1} h^{-1} at pH=5. In Table 3.26 hydrogen production yields for different pH values can be seen. Maximum yield with $Y_{H2} = 3.75$ mol mol^{-1} glucose was obtained at pH=4-5. The results show that pH=5 is the most suitable pH value in producing hydrogen gas from filtrate of hydrolyzed sludge by dark fermentation.

The sludge obtained form two-stage hydrolysis was also subjected to dark fermentation at different pH values. Variations of cumulative hydrogen gas volumes with time at different fermentation pH are presented in Figures 3.129-3.132. Maximum cumulative hydrogen gas volume was obtained as V_{H2}=45 ml at pH=5. Cumulative hydrogen gas volume for other fermentation pH values were as follows; V_{H2}=23 ml. at pH=4, V_{H2}=36 ml at pH= 6 and V_{H2}=32 ml at pH=7

Table 3.25 Gompertz equation constants obtained from fermentation of filtrate at different pH values.

Fermentation pH value	P (ml)	HPR (ml hour^{-1})	Lag phase period (hour)	SHPR (ml g^{-1} biomass^{-1} hour^{-1})	R^2
4	25.49	0.19	13.47	0.08	0.996
5	42.61	0.46	18.43	0.18	0.999
6	32.38	0.26	1.27	0.10	0.995
7	32.94	0.28	8.94	0.11	1.000

Table 3.26 Hydrogen gas production yields obtained from fermentation of filtrate at different pH values.

Fermentation pH value	Production Yield (mg g^{-1} glucose^{-1})	Production Yield (ml g^{-1} glucose^{-1})	Production Yield (mol H$_2$ mol^{-1} glucose^{-1})
4	41.79	518.16	3.30
5	41.63	516.27	3.35
6	31.48	390.41	2.83
7	25.95	321.78	2.34

The nutrient and organic substance removal profile during fermentation of sludge at different pH values are given in Figures 3.133-3.137. Total sugar consumption increased with the increase in fermentation pH value (Figure 3.133). Parallel to sugar consumption, an increase in TVFA concentration was observed (Figure 3.134). The yield of TVFA formation varied between $Y_{TVFA/TS}$=3 g g^{-1} and $Y_{TVFA/TS}$=7 g g^{-1}. The effluent NH$_4$-N was higher than the initial one due to microbial hydrolysis of sludge and protein content in the medium (Figure 3.135-3.136). Finally, the increase in COD concentration was result of the release of carbon content of sludge to the liquid phase through the microbial hydrolysis during fermentation (Figure 3.137). Production rates and production potentials calculated by using Gompertz Equation are given in Table 3.27. Production potential was slightly higher than the observed volumes from experiments. The maximum production potential was V_{H2}=49 ml achived at pH=5. Although lag phase was as long as λ=23 hours at pH=5, hydrogen gas production rate was HPR=0.48 ml hour^{-1} and specific hydrogen production rate

was SHPR=0.19 ml g^{-1} biomass h^{-1}. Table 3.28 depicts the effect of fermentation pH value on production yields. The maximum yield was Y_{H2}=3.35 mol mol^{-1} obtained at pH=5. These results were in parallel to the results of hydrogen gas production from filtrate. As a final conclusion it was determined that the most suitable fermentation pH should be pH=5 in hydrogen gas production by dark fermentation from both filtrate and hydrolyzed sludge.
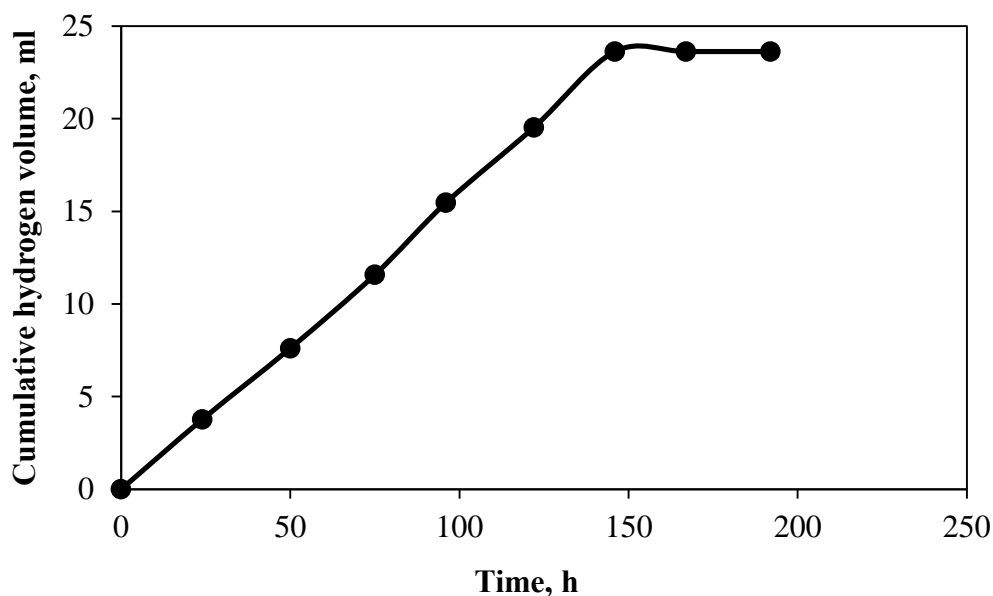

Figure 3.129 Variation of cumulative hydrogen gas volume with time in dark fermentation of sludge at pH=4.

Table 3.27 Gompertz equation constants obtained from fermentation of sludge at different pH values.

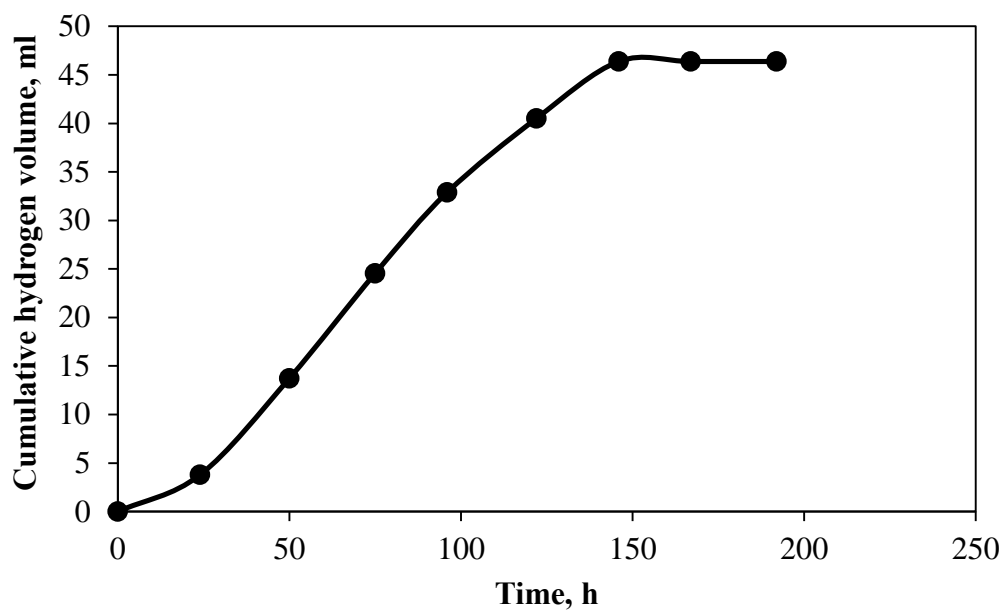| Fermentation pH value	P (ml)	HPR (ml hour^{-1})	Lag phase period (hour)	SHPR (ml g^{-1} biomass hour^{-1})	R^2
4	26.4	0.20	13.1	0.08	0.996
5	49.1	0.48	22.6	0.19	0.999
6	40.4	0.38	2.8	0.15	0.997
7	34.3	0.28	2.9	0.11	0.995

Figure 3.130 Variation of cumulative hydrogen gas volume with time in dark fermentation of sludge at pH=5.
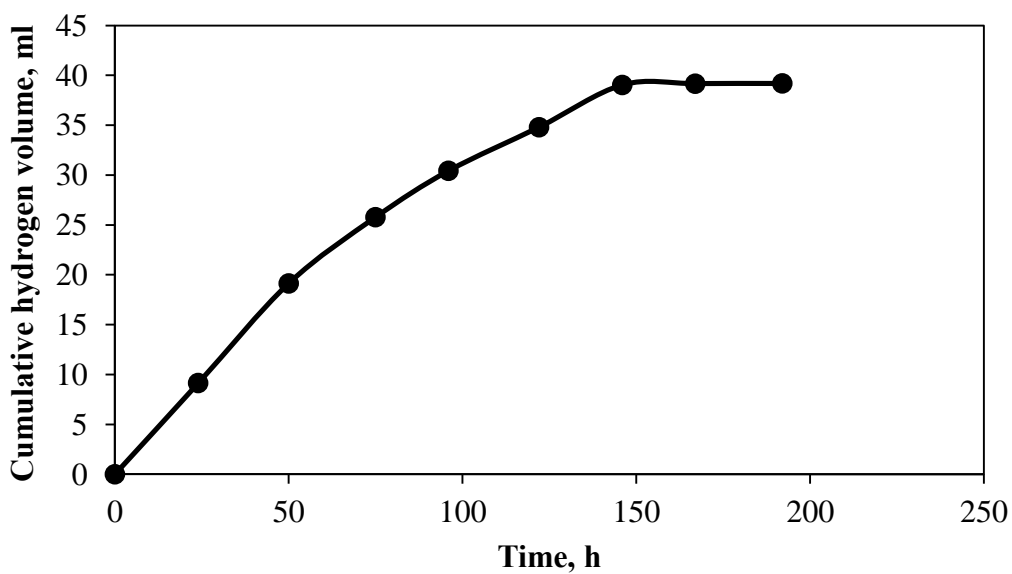

Figure 3.131 Variation of cumulative hydrogen gas volume with time in dark fermentation of sludge at pH=6.
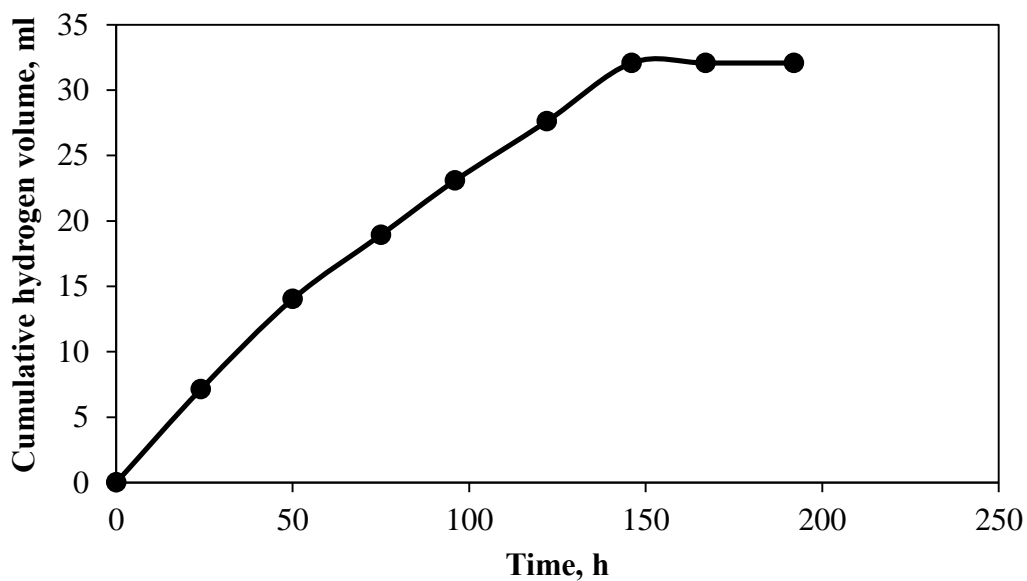
Figure 3.132 Variation of cumulative hydrogen gas volume with time in dark fermentation of sludge at pH=7.

Figure 3.133 Initial and final total sugar concentrations of sludge fermentation at different pH values

Figure 3.134 Initial and final TVFA concentrations of sludge fermentation at different pH values.

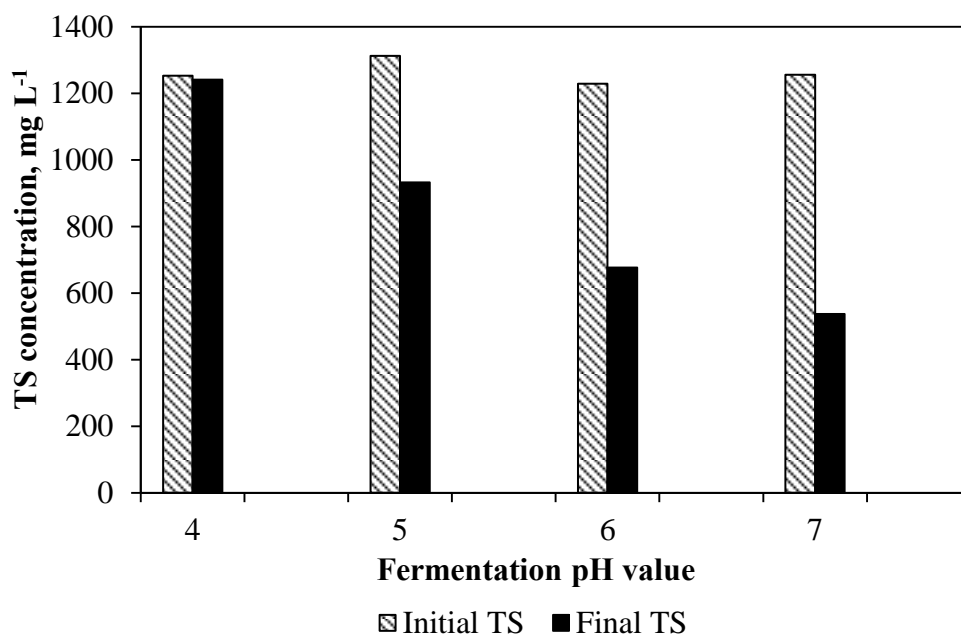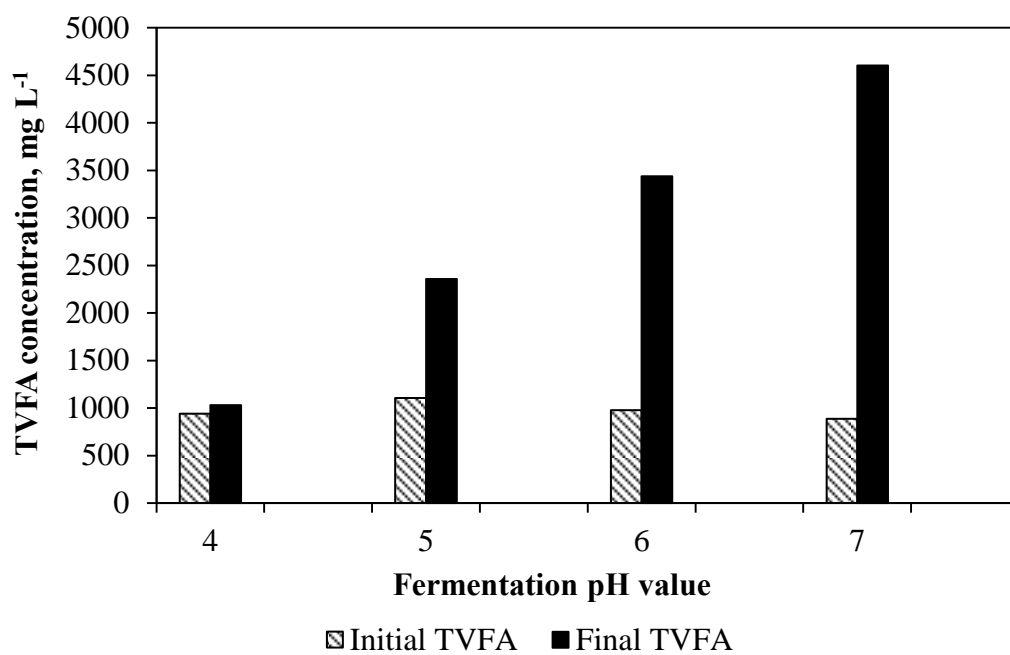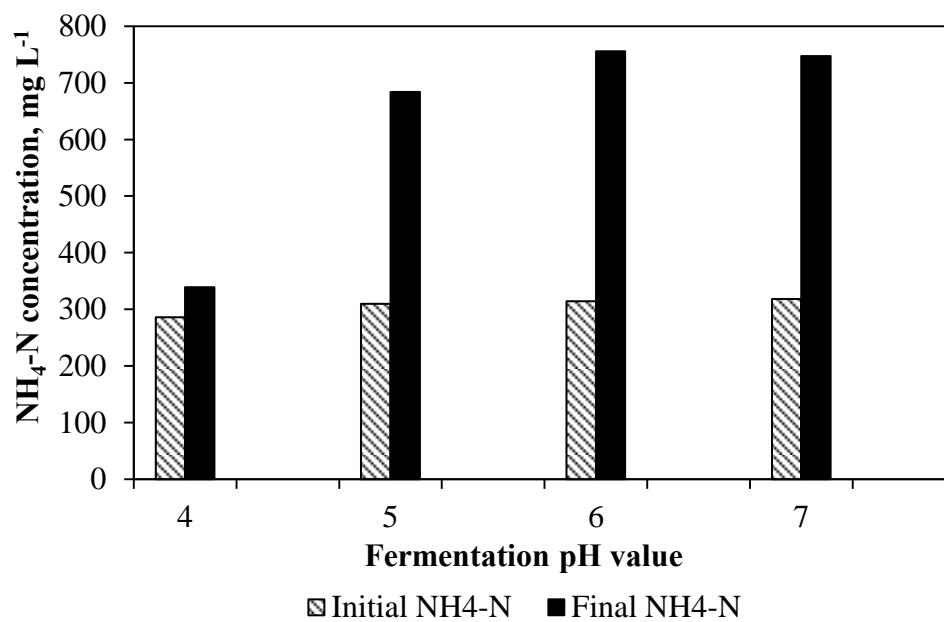Figure 3.135 Initial and final NH_4-N concentrations of sludge fermentation at different pH values.
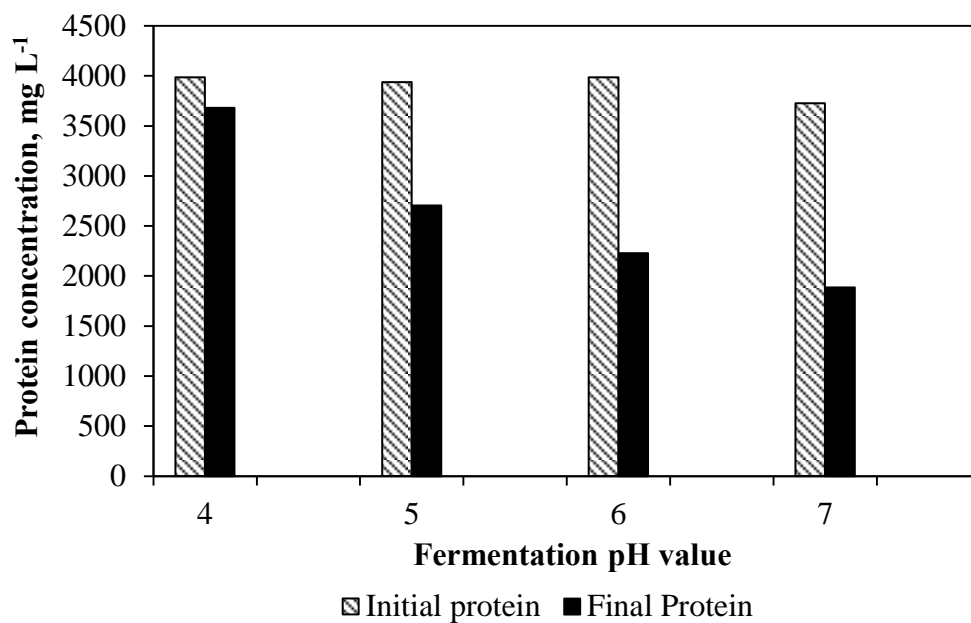
Figure 3.136 Initial and final protein concentrations of sludge fermentation at different pH values.
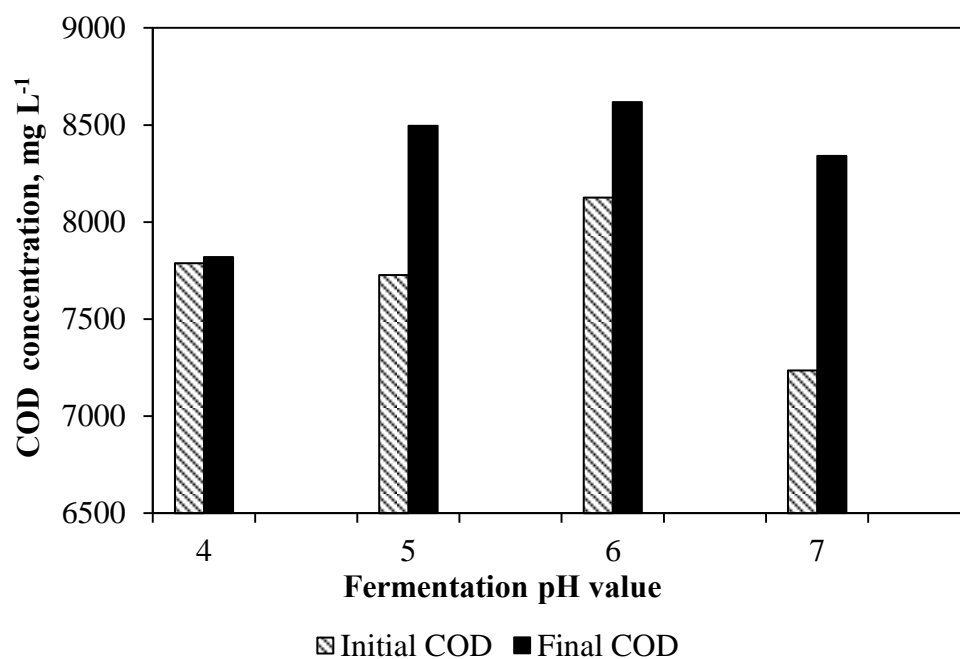

Figure 3.137 Initial and final COD concentrations of sludge fermentation at different pH values.

Table 3.28 Hydrogen gas production yields obtained from fermentation of sludge at different pH values.

Fermentation pH value	Production Yield (mg g^{-1} glucose)	Production Yield (ml g^{-1} glucose)	Production Yield (mol H$_2$ mol^{-1} glucose)
5	41.13	509.97	3.30
6	31.26	387.66	2.81
7	34.73	430.67	3.13

3.2.7 The Effect of Biomass Concentration on Production of Hydrogen Gas

The effect of biomass concentration studies were performed with two different substrates, filtrate and sludge at pH=5 and at 37°C fermentation temperature. Biomass concentration was varied between X=2-6 g L^{-1}. The stock sludge was hydrolyzed by two-stage hydrolysis under the optimized conditions. Cumulative hydrogen gas volumes observed in fermentation of filtrate at different initial biomass concentrations are given in Figures 3.138-3.142. CHV increased from V$_{H2}$=30 ml to V$_{H2}$=43 ml by increasing biomass concentration from X=2 g L^{-1} to X= 5 g L^{-1}. A slight decease in CHV to V$_{H2}$=38 ml at X=6 g L^{-1} was observed. Initial sugar concentration was about 1200 mg L^{-1}. Final sugar concentration did not vary significantly with biomass concentration and it ranged between 640-790 mg L^{-1} (Figure 3.143).

The maximum TVFA productions were achieved as 2500 mg L^{-1} and 2000 mg L^{-1} at biomass concentrations X=2 g L^{-1} and 5 g L^{-1}, respectively. TVFA and hydrogen gas production were in agreement for X=5 g L^{-1}. However, hydrogen gas production was lower with regard to the high TVFA concentration at X=2 g L^{-1}. This is a general problem encountered in the production of hydrogen gas by dark fermentation. It is due to the type of organic acid produced during fermentation. It is well know that the end products like propionic, lactic acid and ethanol do not generates hydrogen gas, while acetic and butric acids do. Therefore, TVFA concentration may not correlate with the hydrogen gas production in some cases. A slight decease in NH$_4$-N concentration (Figure 3.146) and COD concentration (Figure 3.145) was observed in

this set of experiments. The decrease in protein concentration was around of 58±3 % for all examined biomass concentrations (Figure 3.147). According to the Gompertz Equation constants given in Table 3.29, production potentials were parallel to the hydrogen volumes observed in the experiments. The maximum production rate was obtained as HPR=0.58 ml hour^{-1} at X=5 g L^{-1}. But the maximum specific hydrogen production rate was SHPR=0.16 ml g^{-1} biomass^{-1} hour^{-1} achieved at X=2 g L^{-1}. Similarly, yield of production reached to maximum value (Y$_{H2}$=3.15 mol mol^{-1} glucose) at X=5 g L^{-1} (Table 3.30).

Figure 3.138 Variation of cumulative hydrogen gas volume with time in dark fermentation of filtrate at 2 g L^{-1} initial biomass concentration.

The effect of biomass concentration on hydrogen gas production from sludge by dark fermentation was also investigated. Cumulative hydrogen gas volumes obtained at different initial biomass concentrations in dark fermentation of sludge are presented in Figures 3.148-3.152. Cumulative hydrogen gas volume was V$_{H2}$=33 ml at X=2g L^{-1} but when biomass concentration was raised to X=4 g L^{-1}, hydrogen gas volume increased to V$_{H2}$=42 ml. At higher biomass concentrations, there wasn't a significant change in cumulative hydrogen gas volume and it remained constant about 42 ml. While total sugar consumption was 21 % at X=2g L^{-1}, it increased up to

75% at X=5g L^{-1} (Figure 3.153). In parallel to the sugar consumption, the maximum TVFA production occurred as 2100 mg L^{-1} at X=5g L^{-1} (Figure 3.154).
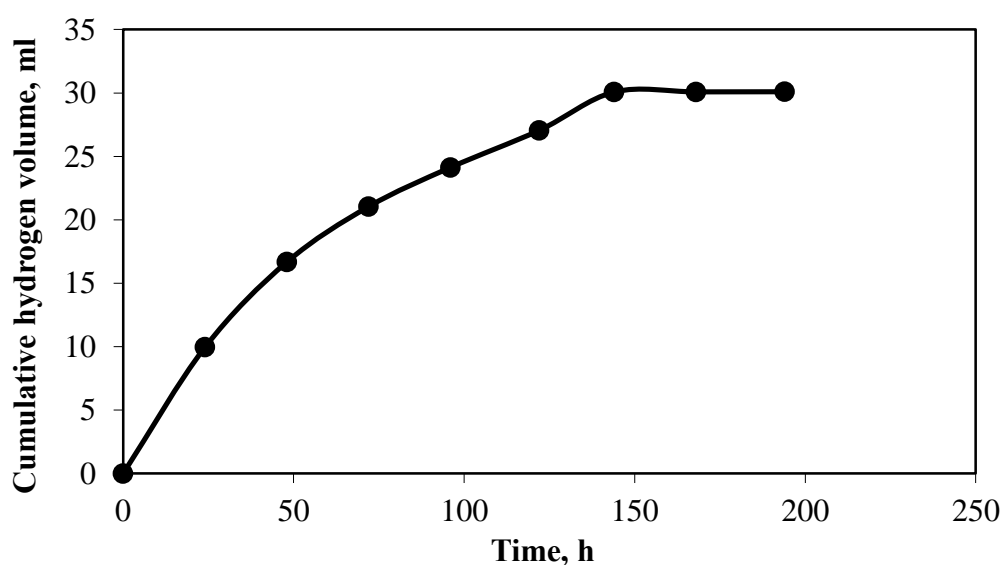

Figure 3.139 Variation of cumulative hydrogen gas volume with time in dark fermentation of filtrate at 3 g L^{-1} initial biomass concentration.
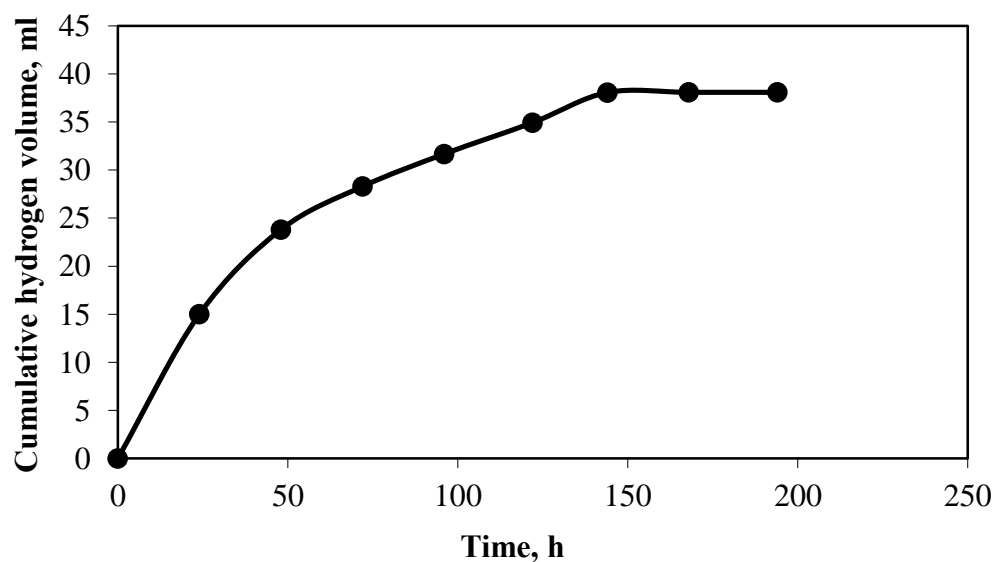
Figure 3.140 Variation of cumulative hydrogen gas volume with time in dark fermentation of filtrate at 4 g L^{-1} initial biomass concentration.
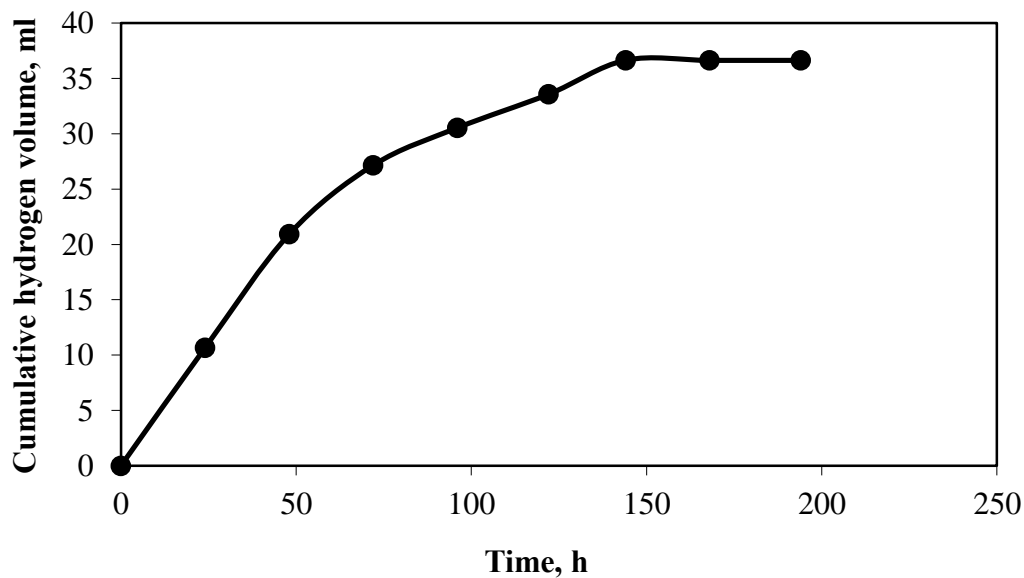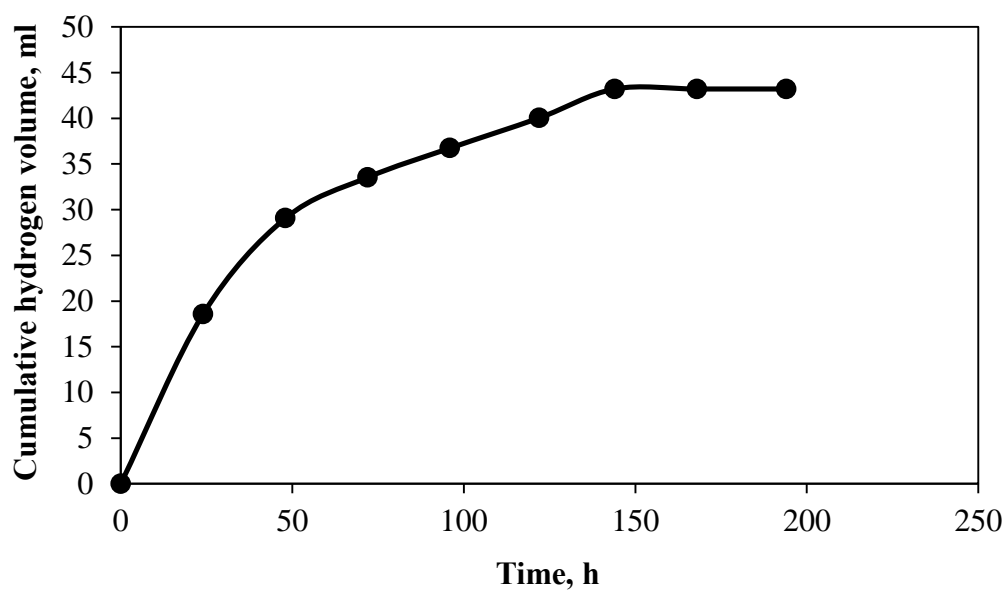
Figure 3.141 Variation of cumulative hydrogen gas volume with time in dark fermentation of filtrate at 5 g L^{-1} initial biomass concentration.
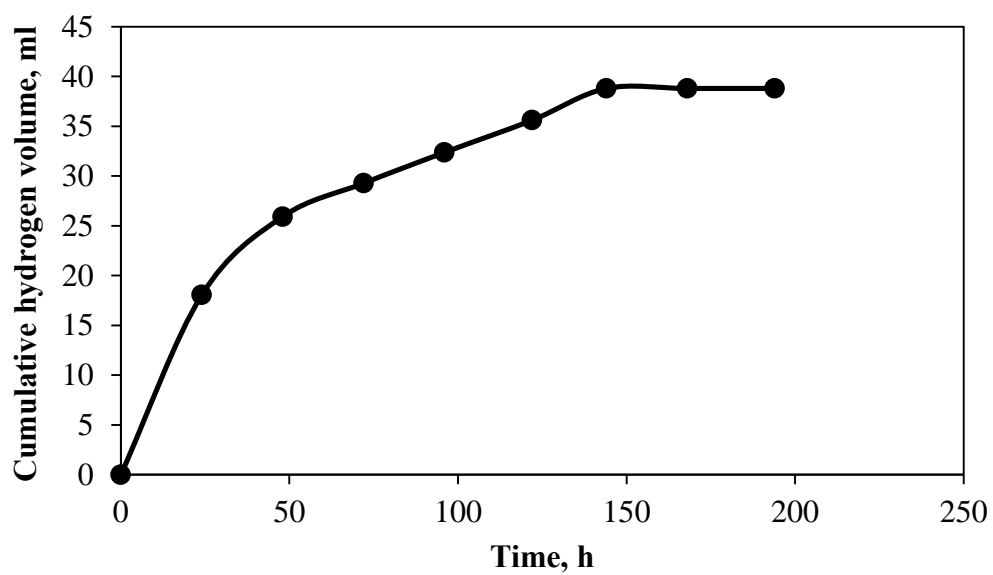
Figure 3.142 Variation of cumulative hydrogen gas volume with time in dark fermentation of filtrate at 6 g L^{-1} initial biomass concentration.
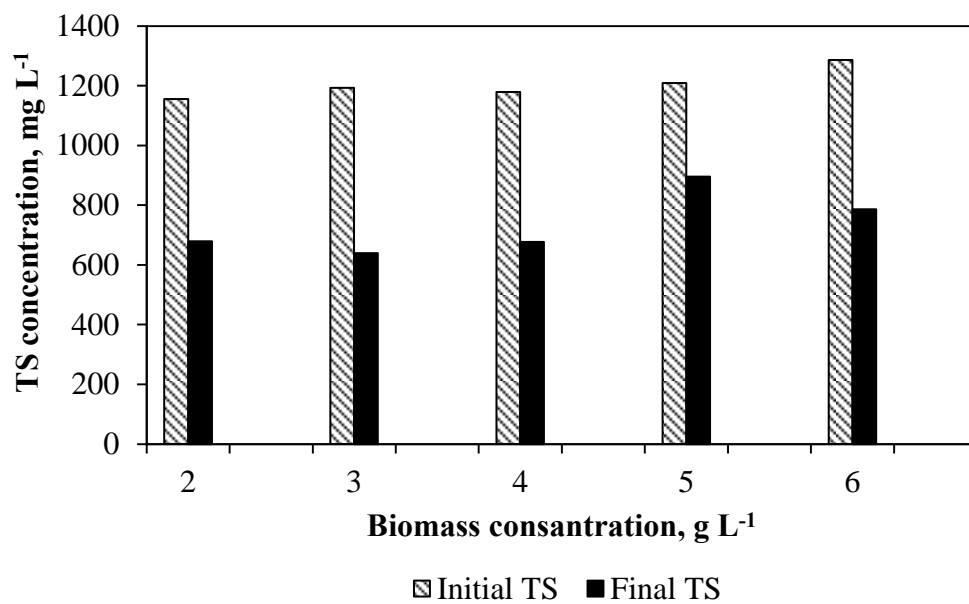
Figure 3.143 Initial and final total sugar concentrations of filtrate fermentation at different initial biomass concentrations.
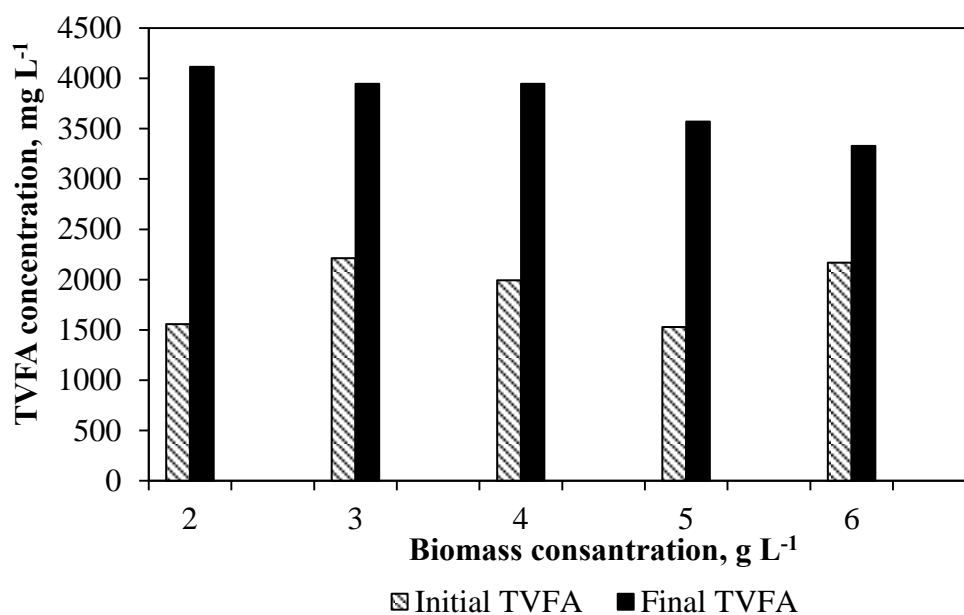
Figure 3.144 Initial and final TVFA concentrations of filtrate fermentation at different initial biomass concentrations.
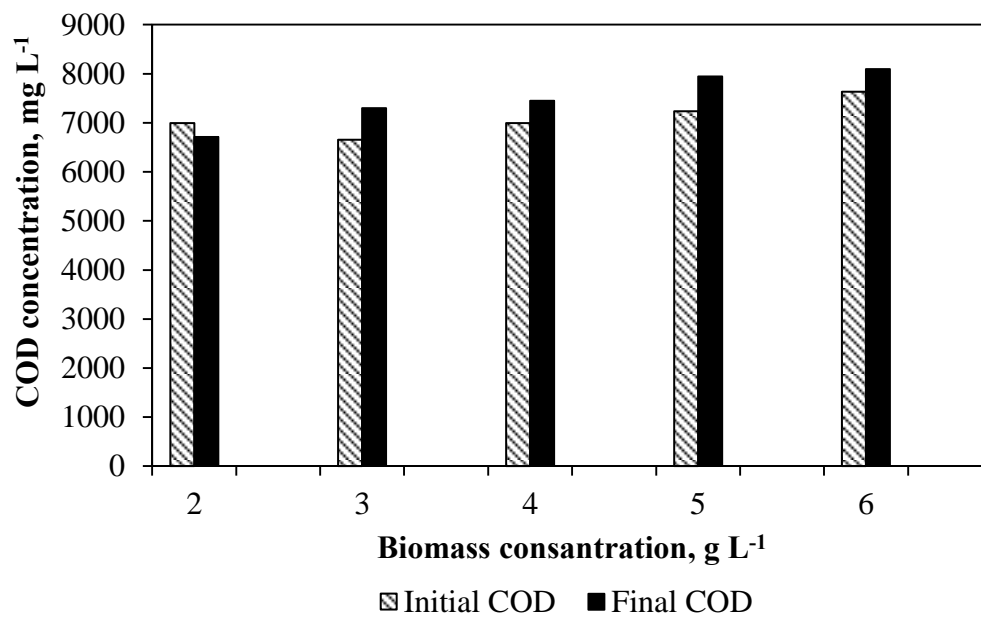
Figure 3.145 Initial and final COD concentrations of filtrate fermentation at different initial biomass concentrations.
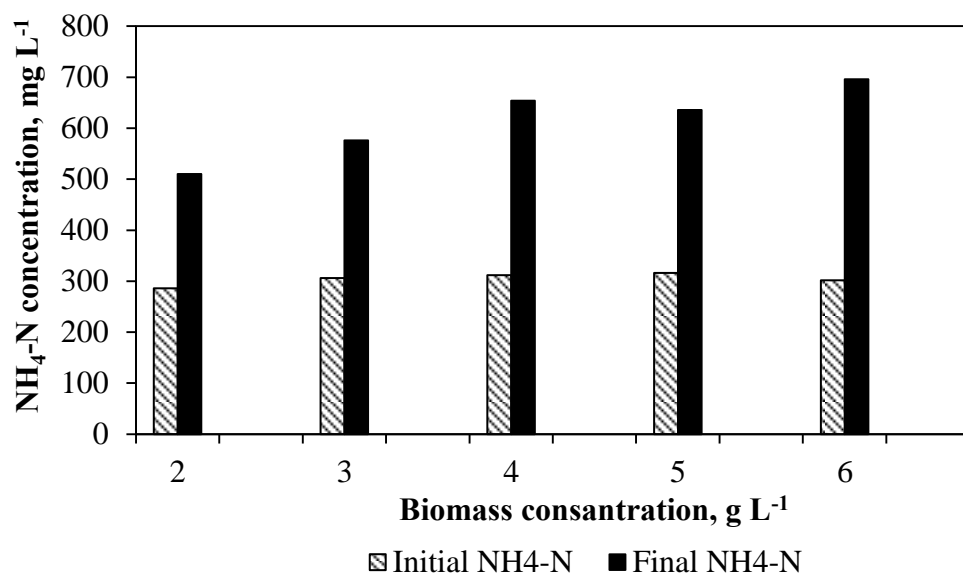
Figure 3.146 Initial and final NH_4-N concentrations of filtrate fermentation at different initial biomass concentrations.
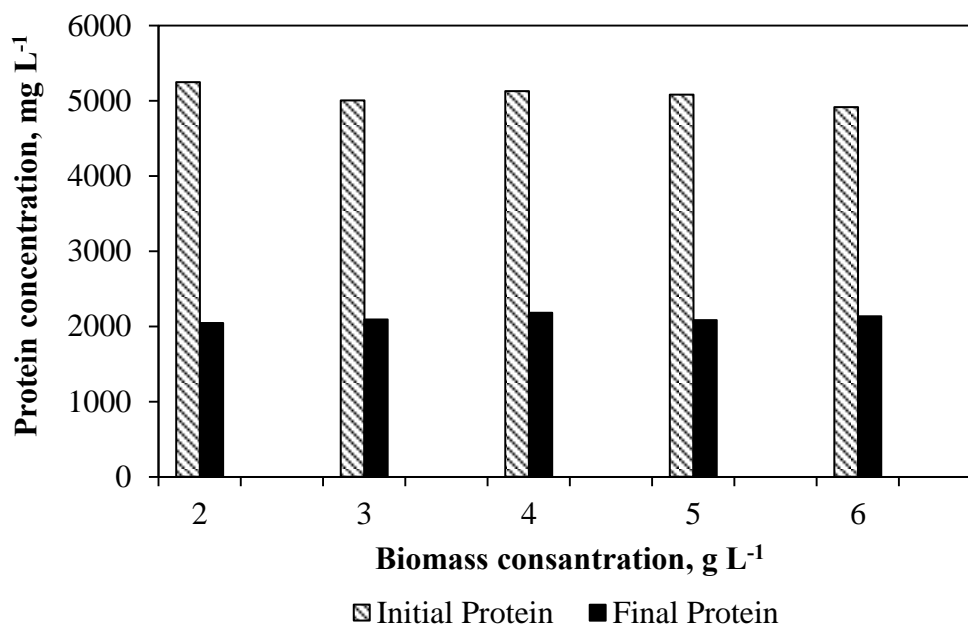
Figure 3.147 Initial and final protein concentrations of filtrate fermentation at different initial biomass concentrations.

Table 3.29 Gompertz equation constants obtained from fermentation of filtrate at different initial biomass concentrations.

| Biomass concentration (X_0), (g L^{-1})	P (ml)	HPR (ml h^{-1})	Lag phase period (hour)	SHPR (ml g^{-1} biomass hour^{-1})
2	30.31	0.31	0	0.16
3	36.56	0.43	1.26	0.14
4	37.59	0.45	0	0.11
5	42.13	0.58	0	0.12
6	37.73	0.49	0	0.08

As observed in previous studies, there was a rise in final COD and NH$_4$-N concentrations (Figures 3.155 and Figure 3.156, respectively). Protein consumption was about 50% (Figure 3.157). Phosphorus removal did not vary with initial biomass concentration and remained constant around at 25 % (Figure 3.158). Gompertz equation constants resulted in maximum production rate of HPR=0.55 ml h^{-1} at X=6 g L^{-1} and maximum specific production rate of SHPR= 0.17 ml g^{-1} biomass hour^{-1} at X=2 g L^{-1} (Table 3.31). Hydrogen production yield from sludge was lower than the

values observed at the filtrate. Maximum production yield was obtained as $Y_{H2}=2.66$ mol mol^{-1} glucose at X=4 g L^{-1} (Table 3.32).

Table 3.30 Hydrogen gas production yields obtained from fermentation of filtrate at different biomass concentrations.

X_0 (g L^{-1})	Production Yield (mg g^{-1} glucose^{-1})	Production Yield (ml g^{-1} glucose^{-1})	Production Yield (mol H$_2$ mol^{-1} glucose^{-1})
2	22	269	1.95
3	23	282	2.05
4	26	323	2.34
5	37	459	3.15
6	27	330	2.40
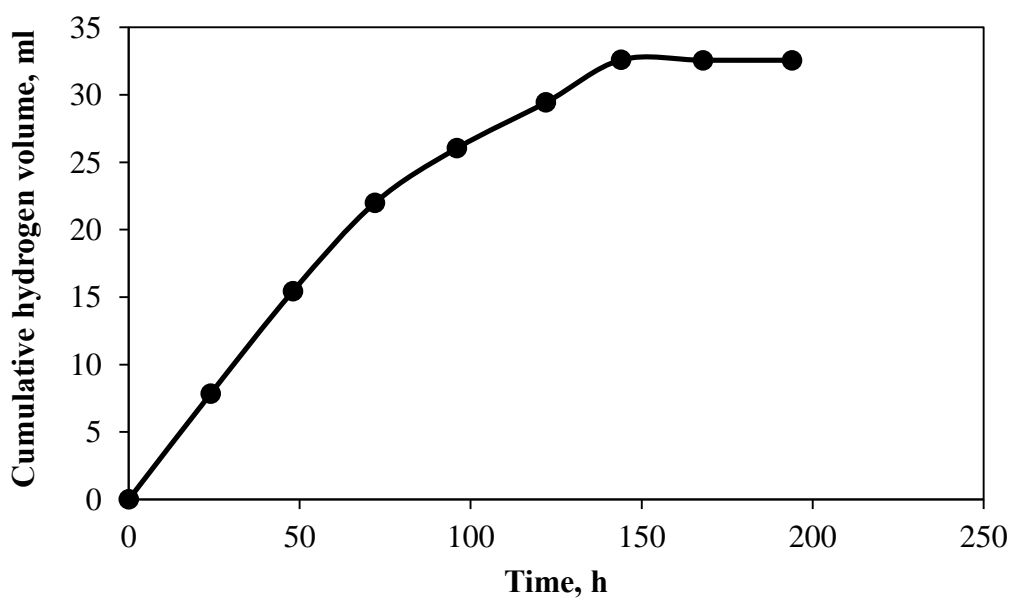
Figure 3.148 Variation of cumulative hydrogen gas volume with time in dark fermentation of sludge at 2 g L^{-1} initial biomass concentration.
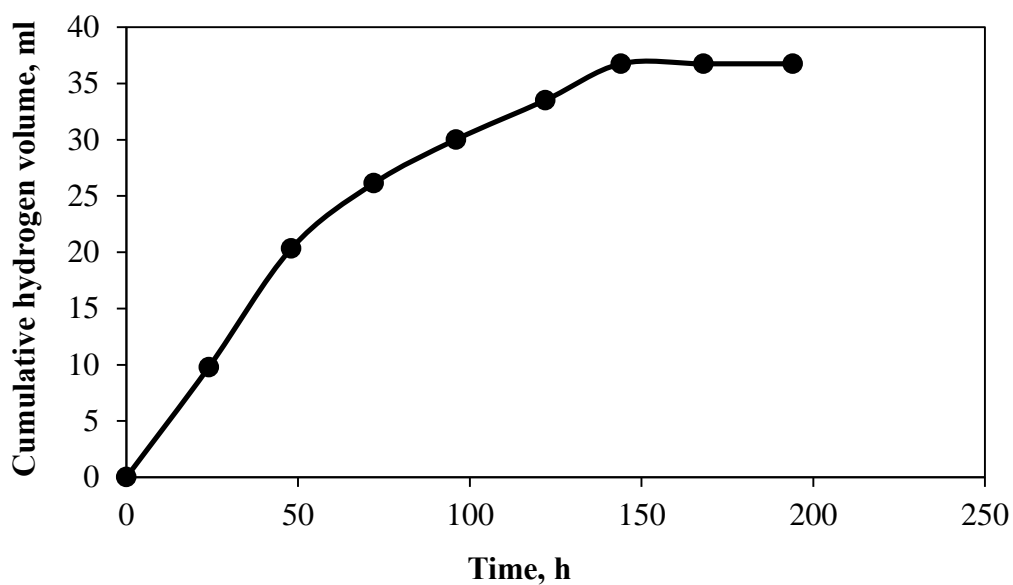
Figure 3.149 Variation of cumulative hydrogen gas volume with time in dark fermentation of sludge at 3 g L^{-1} initial biomass concentration.
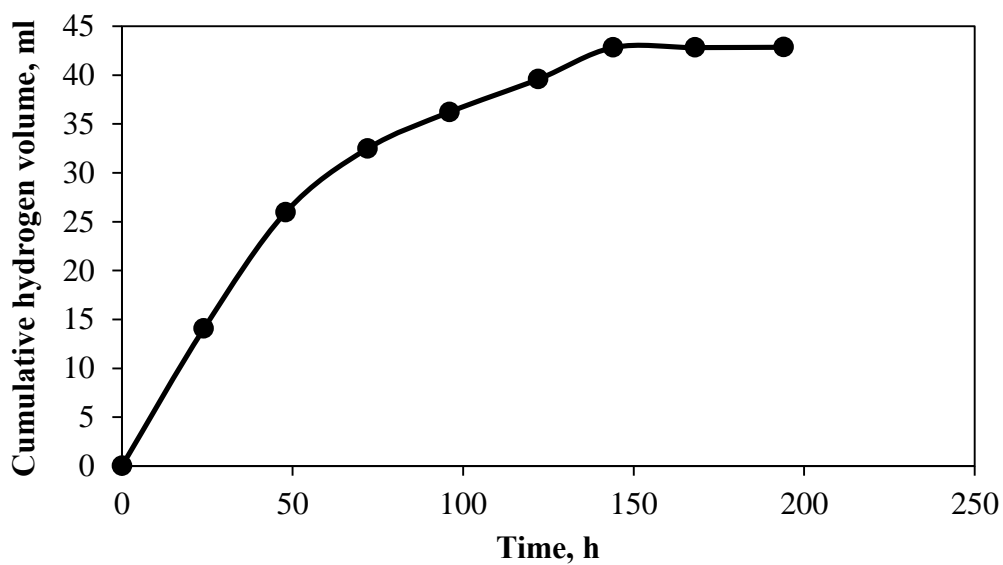
Figure 3.150 Variation of cumulative hydrogen gas volume with time in dark fermentation of sludge at 4 g L^{-1} initial biomass concentration.
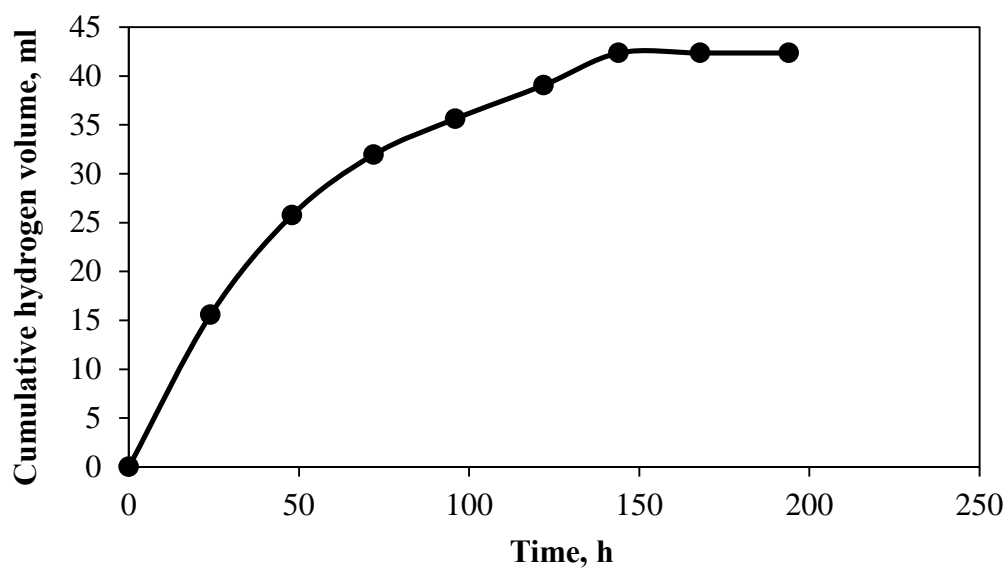
Figure 3.151 Variation of cumulative hydrogen gas volume with time in dark fermentation of sludge at 5 g L^{-1} initial biomass concentration.
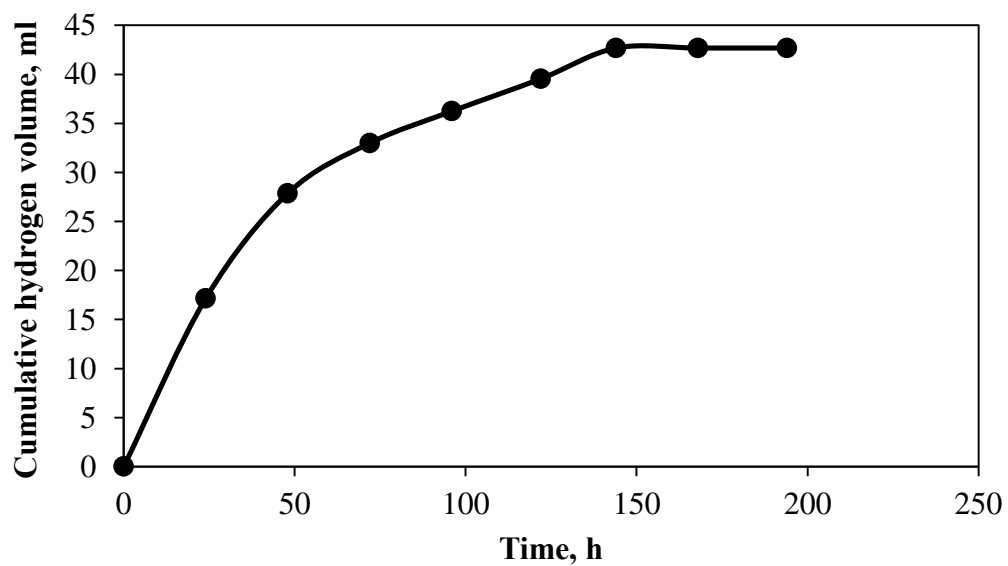
Figure 3.152 Variation of cumulative hydrogen gas volume with time in dark fermentation of sludge at 6 g L^{-1} initial biomass concentration.
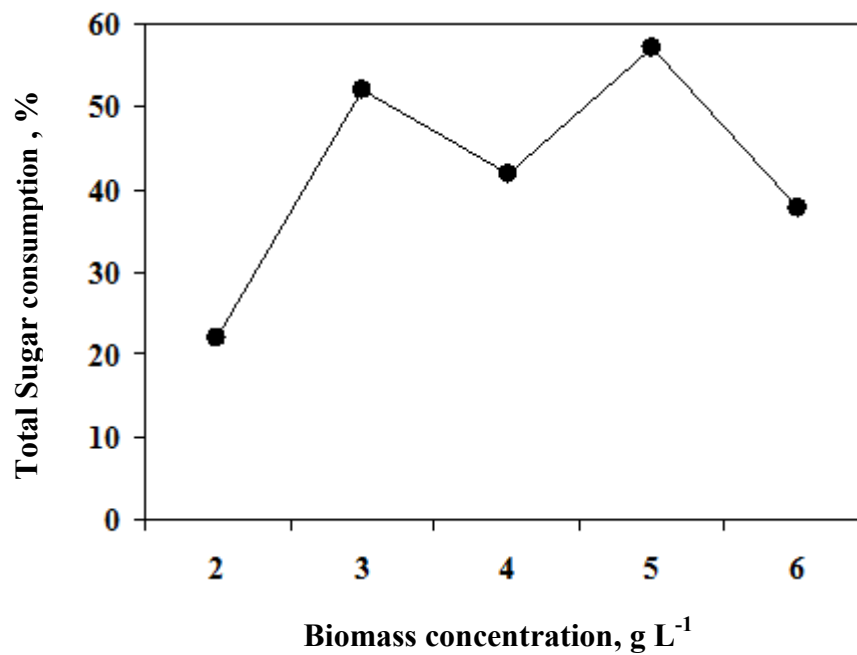
Figure 3.153 Initial and final total sugar concentrations of sludge fermentation at different initial biomass concentrations.
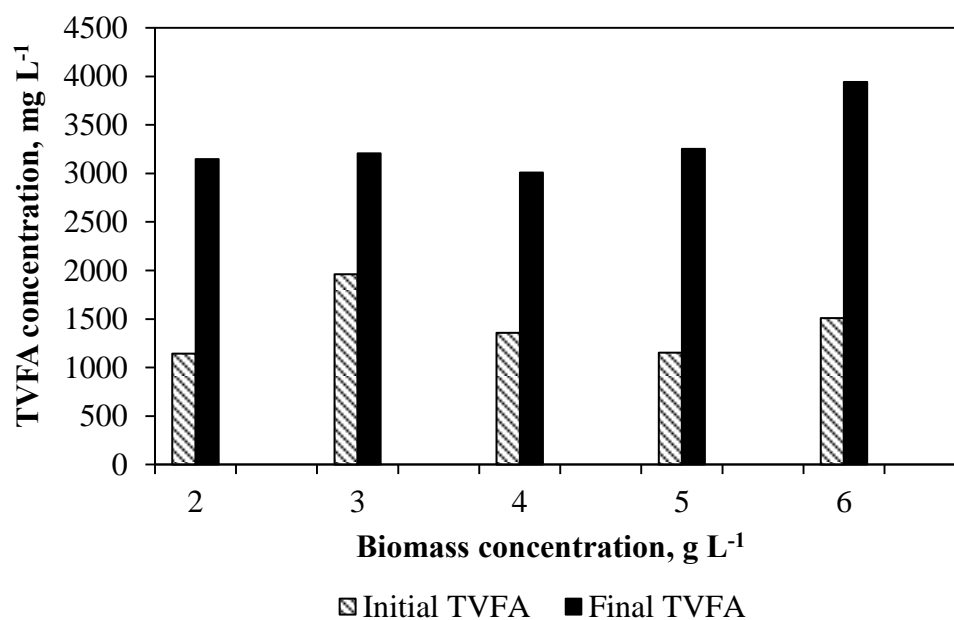
Figure 3.154 Initial and final TVFA concentrations of sludge fermentation at different initial biomass concentrations.
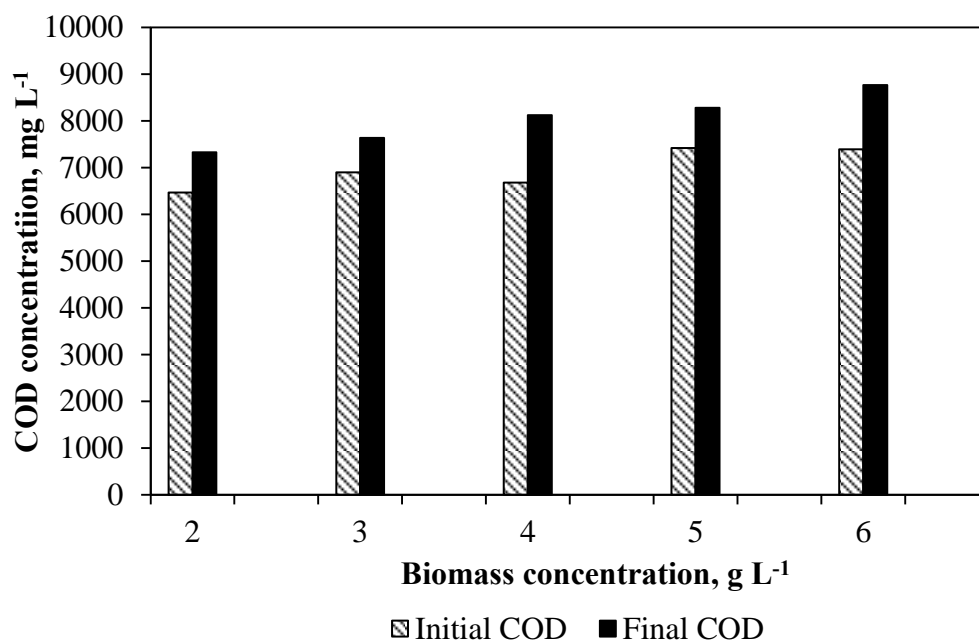
Figure 3.155 Initial and final COD concentrations of sludge fermentation at different initial biomass concentrations.
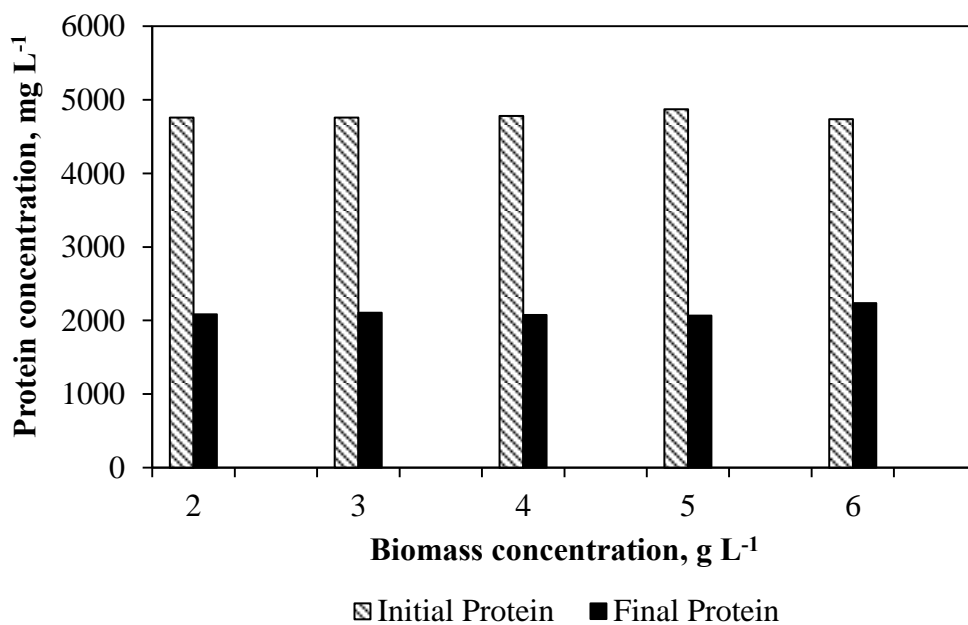
Figure 3.156 Initial and final protein concentrations of sludge fermentation at different initial biomass concentrations.
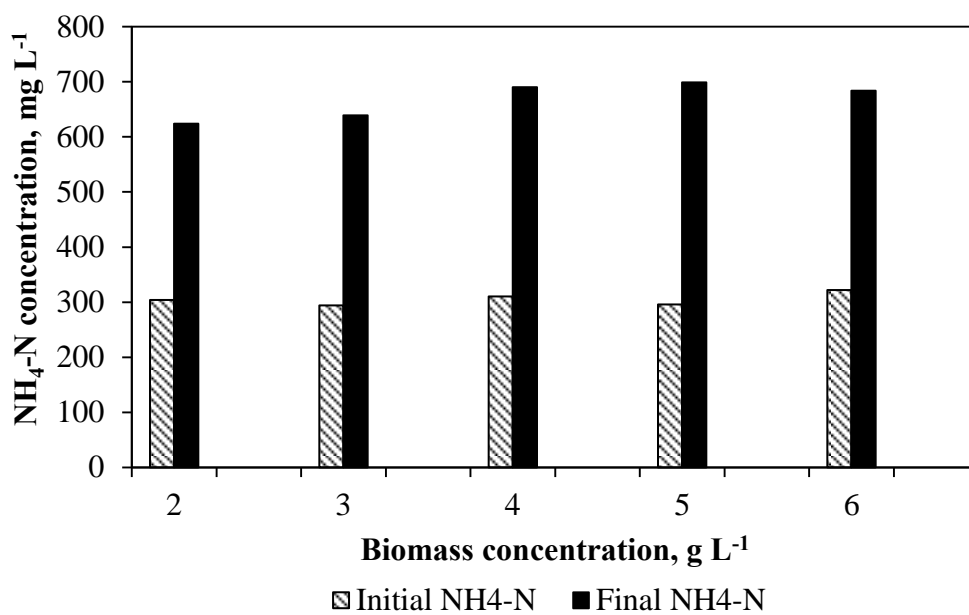
Figure 3.157 Initial and final NH$_4$-N concentrations of sludge fermentation at different initial biomass concentrations.
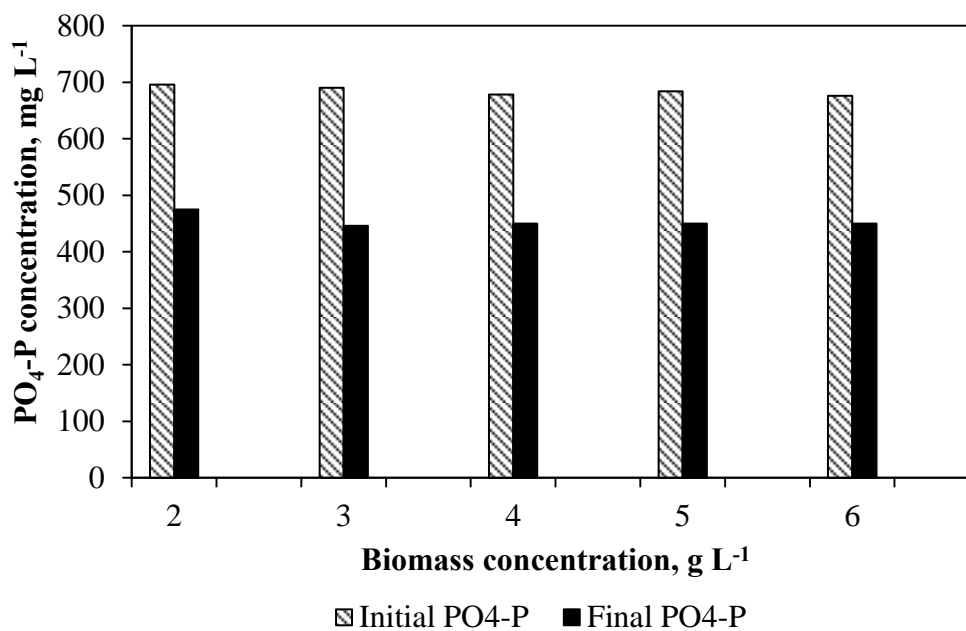
Figure 3.158 Initial and final PO$_4$-P concentrations of sludge fermentation at different initial biomass concentrations.

Table 3.31 Gompertz equation constants obtained from fermentation of sludge at different initial biomass concentrations.

X_0 (g L^{-1})	P (ml)	HPR (ml h^{-1})	Lag phase (hour)	SHPR (ml g^{-1} biomass h^{-1})	R^2
2	33.30	0.34	3.56	0.17	0.997
3	36.91	0.41	1.70	0.14	0.996
4	42.47	0.53	0.00	0.13	0.995
5	41.98	0.50	0.00	0.10	0.992
6	41.86	0.55	0.00	0.09	0.990

Table 3.32 Hydrogen gas production yields obtained from fermentation of sludge at different biomass concentrations.

X_0 (g L^{-1})	Production Yield (mg g^{-1} glucose^{-1})	Production Yield (ml g^{-1} glucose^{-1})	Production Yield (mol H$_2$ mol^{-1} glucose^{-1})
3	18	220	1.60
4	30	367	2.66
5	13	155	1.13
6	28	351	2.55

3.2.8 The Effect of the Initial NH$_4$-N Concentration on the Hydrogen Gas Production

Two stage hydrolysis of the sludge resulted in around 300 mg L^{-1} NH$_4$-N and about 700 mg L^{-1} PO$_4$-P. The concentrations of these nutrients give N/P ratio of about N/P=0.5. This means that the media is nitrogen limited and there is excess amount of phosphorus. In order to shift the ratio toword the excess nitrogen conditions, nitrogen was added into the the fermentation media externally. The amount added into the media were N= 200 mg L^{-1}, 300 mg L^{-1} and 400 mg L^{-1}. Figures 3.159-3.161 depict variation of cumulative hydrogen gas volumes obtained form fermentation of filtrate at different nitrogen supplementation. When the added nitrogen concentration was N=200 mg L^{-1}, cumulative hydrogen gas volume was

V_{H2}=39 ml, whereas it increased to V_{H2}=47 ml when the nitrogen concentration was rised to 300 mg L^{-1}. But, this value decreased to V_{H2}=41 ml for N=400 mg L^{-1}. Final total sugar concentration was around 790±20 mg L^{-1} for all nitrogen concentrations examined (Figure 3.162). Increasing nitrogen concentration didn't have a significant effect on hydrogen gas production volume and sugar consumption. TVFA production was about 2000 mg L^{-1} in these nitrogen concentrations (Figure 3.163). Protein removal was about 50 % (Figure 3.164) and final nitrogen concentration increased up to 1100 mg L^{-1} (Figure 3.166). Different from the previous experiment results, increasing nitrogen concentration affected phosphorus removal in a negative way and only 15 % of removal was obtained (Figure 3.167).

Figure 3.159 Variation of cumulative hydrogen gas volume with time in dark fermentation of filtrate with 200 mg L^{-1} nitrogen supplementation.

Gompertz equation constants determined for this experimental conditions are depicted in Table 3.33. The maximum rate of hydrogen formation was obtained as HPR=0.61 ml h^{-1} when 300 mg L^{-1} NH$_4$-N was added. This value is close to the value (HPR= 0.58 ml h^{-1}) obtained from experimental studies without adding nitrogen. But by adding 300 mg L^{-1} nitrogen, production the yield increased to Y_{H2}=3.5 mol mol^{-1} glucose (Table 3.34). Production yield was Y_{H2}=3.15 mol mol^{-1} glucose in the experimental studies without adding nitrogen (Table 3.30). Although,

there is a slight increase in the yield it is not substantial. Therefore, external nitrogen addition is not necessary in hydrogen gas production by dark fermentation.

Figure 3.160 Variation of cumulative hydrogen gas volume with time in dark fermentation of filtrate with 300 mg L^{-1} nitrogen supplementation.

Figure 3.161 Variation of cumulative hydrogen gas volume with time in dark fermentation of filtrate with 400 mg L^{-1} nitrogen supplementation.
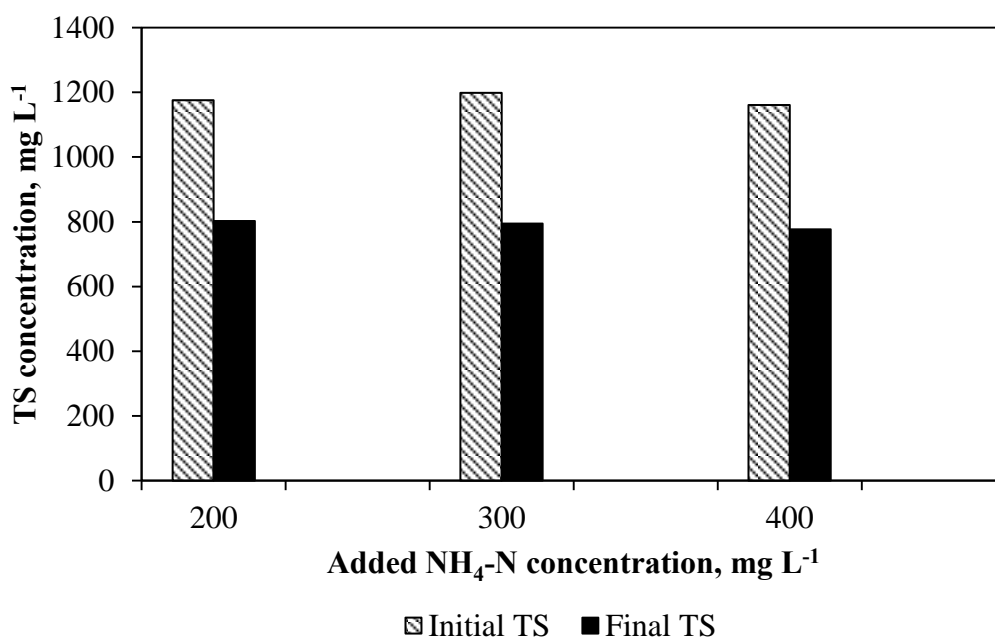
Figure 3.162 Initial and final total sugar concentrations of filtrate fermentation at different nitrogen supplementation.

Figure 3.163 Initial and final TVFA concentrations of filtrate fermentation at different nitrogen supplementation.

Figure 3.164 Initial and final COD concentrations of filtrate fermentation at different nitrogen supplementation.

Figure 3.165 Initial and final protein concentrations of filtrate fermentation at different nitrogen supplementation.

Figure 3.166 Initial and final NH$_4$-N concentrations of filtrate fermentation at different nitrogen supplementation.

Figure 3.167 Initial and final PO$_4$-P concentrations of filtrate fermentation at different nitrogen supplementation.

Table 3.33 Gompertz Equation constants obtained from filtrate at different NH$_4$-N supplementation.

Added NH$_4$-N cons. (mg L^{-1})	P (ml)	HPR (ml h^{-1})	Lag phase period (h)	SHPR (ml g^{-1} biomass h^{-1})	R^2
200	38.9	0.45	0.0	0.09	0.992
300	47.0	0.61	0.0	0.12	0.993
400	40.9	0.48	0.0	0.10	0.993

Tablo 3.34 Hydrogen gas production yield obtained from filtrate at different NH$_4$-N supplementation.

Added NH$_4$-N cons. (mg L^{-1})	Production Yield (mg g^{-1} glucose)	Production Yield (ml g^{-1} glucose)	Production Yield (mol H$_2$ mol^{-1} glucose)
200	36	446	3.2
300	41	514	3.5
400	38	467	3.3

Hydrolyzed sludge was also subjected to dark fermentation at different nitrogen concentrations added into the fermentation media. The increase in nitrogen concentration resulted in a slight decrease in cumulative hydrogen gas volumes. It was V_{H2}=43 with 200 mg L^{-1} nitrogen supplementation (Figure 3.168), but decreased to V_{H2}= 37.5 ml for nitrogen supplementation of 400 mg L^{-1}.(Figure 3.170). There was a significant decrease in sugar consumption at high nitrogen concentration. It was around 37% for 200 mg L^{-1} nitrogen added but decreased to about 18±2 % at N= 300-400 mg L^{-1}.(Figure 3.171). However it was 55 % without nitrogen supplementation (Figure 3.153). TVFA production was about 2500 mg L^{-1} when 300 g L^{-1} nitrogen was added and similar TVFA production was provided without adding nitrogen addition (Figure 3.154). There was a decrease (50%) in protein removal compared to the conditions without adding nitrogen and the removal was about 30%. Final NH$_4$-N concentration reached up to 1000 mg L^{-1}(Figure 3.174). Phosphorus removal was also affected in a negative way fom added nitrogen (Figure 3.175). When the constants of Gompertz Equation were analyzed, maximum production rate was 0.61 ml hour^{-1} and specific production rate was 0.12 ml g^{-1} biomass hour^{-1} obtained at 300 g L^{-1} nitrogen supplementation (Table 3.35)
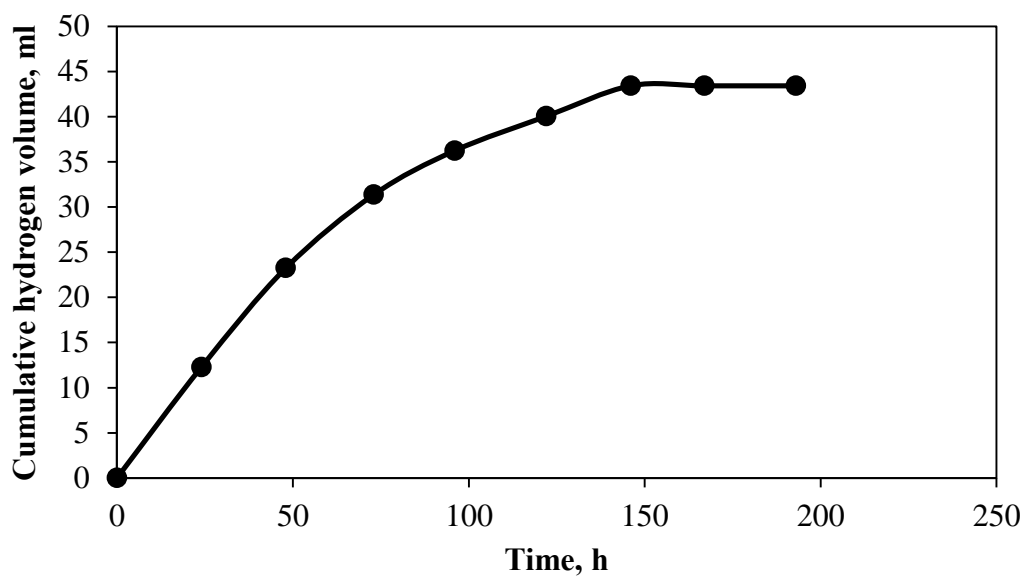
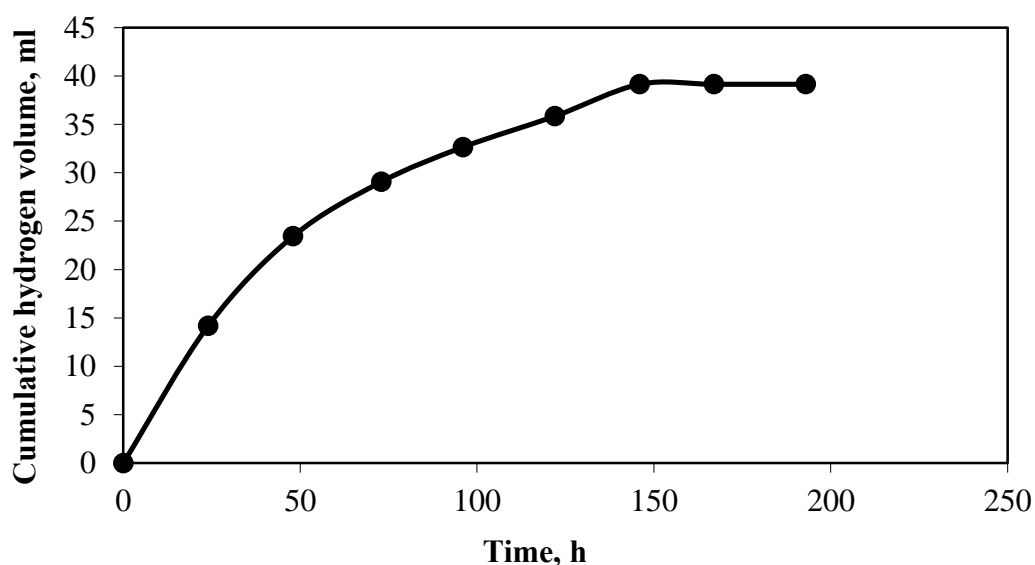Figure 3.168 Variation of cumulative hydrogen gas volume with time in dark fermentation of sludge with 200 mg L^{-1} nitrogen supplementation.
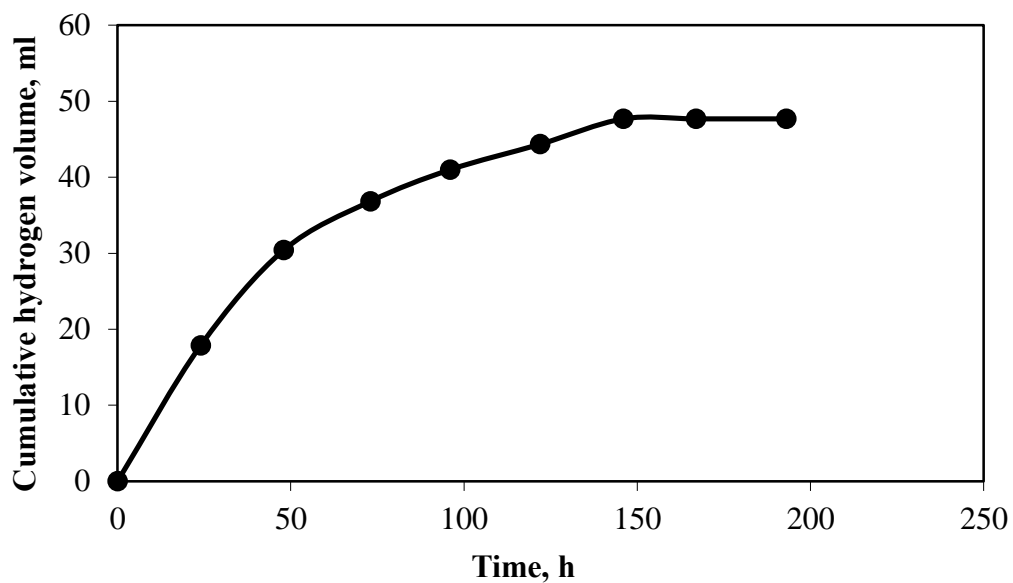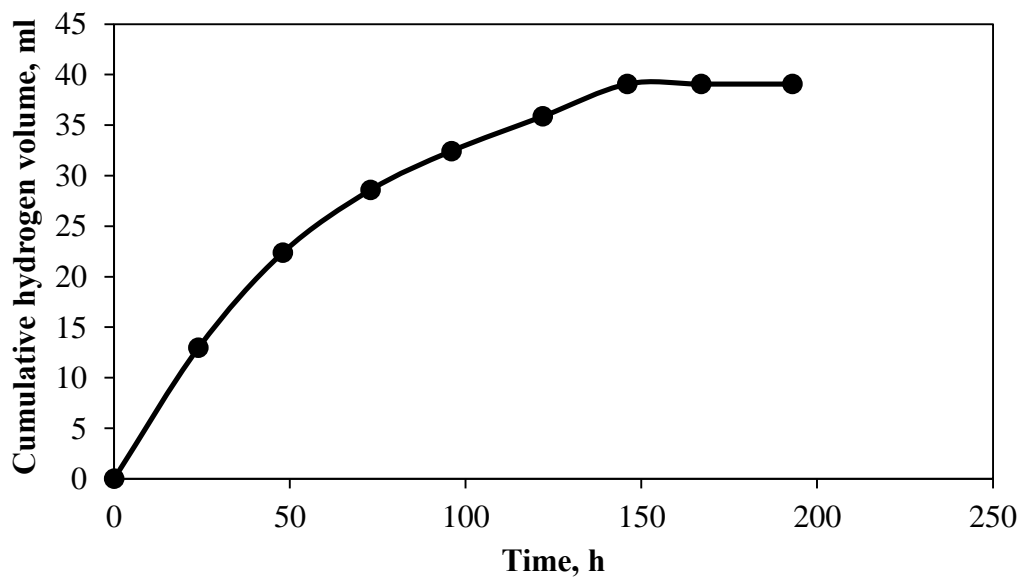

Figure 3.169 Variation of cumulative hydrogen gas volume with time in dark fermentation of sludge with 300 mg L^{-1} nitrogen supplementation.
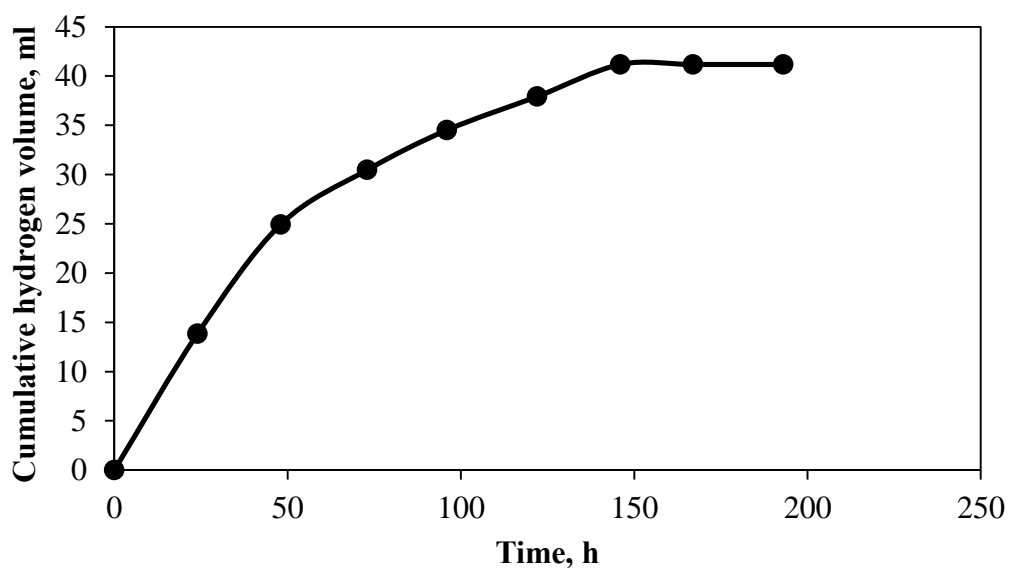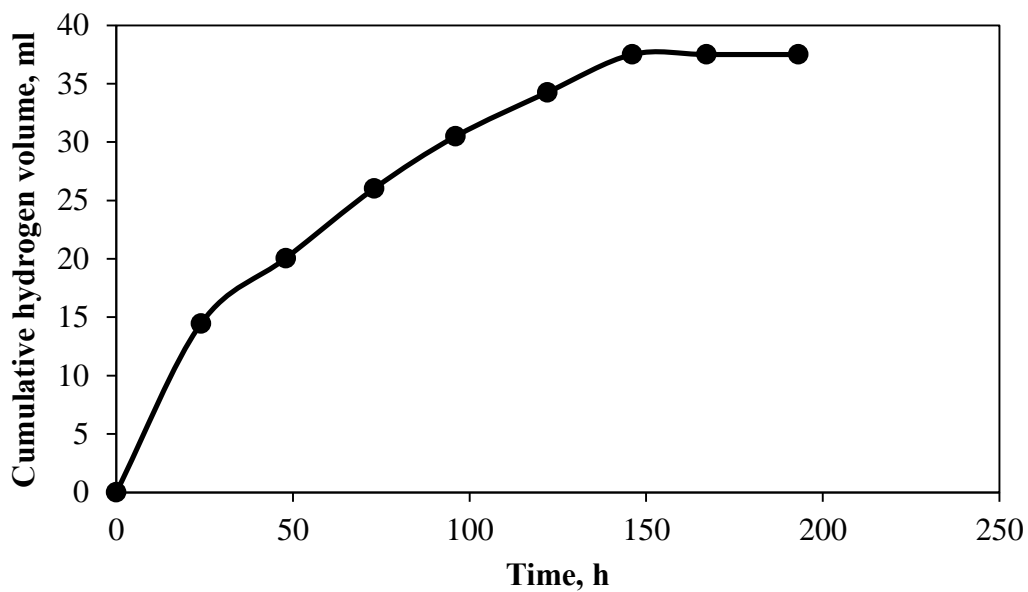
Figure 3.170 Variation of cumulative hydrogen gas volume with time in dark fermentation of sludge with 400 mg L^{-1} nitrogen supplementation.
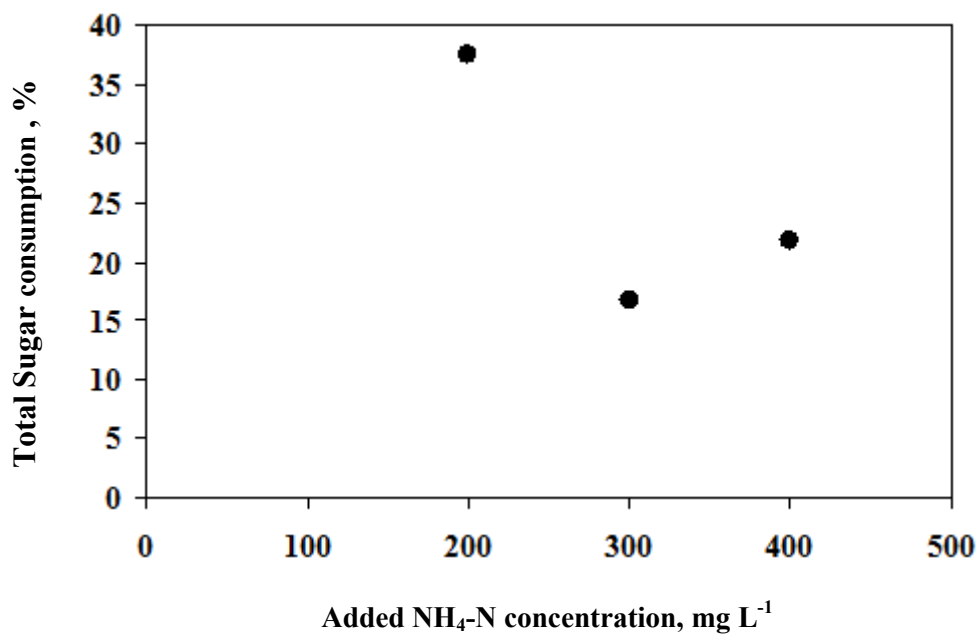
Figure 3.171 Initial and final total sugar concentrations of sludge fermentation at different nitrogen supplementations.
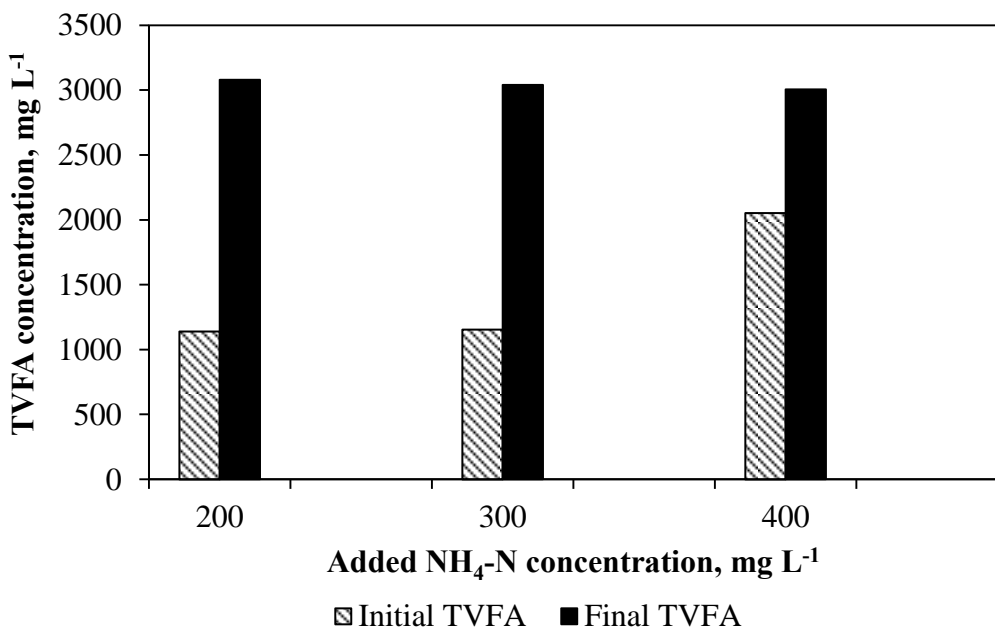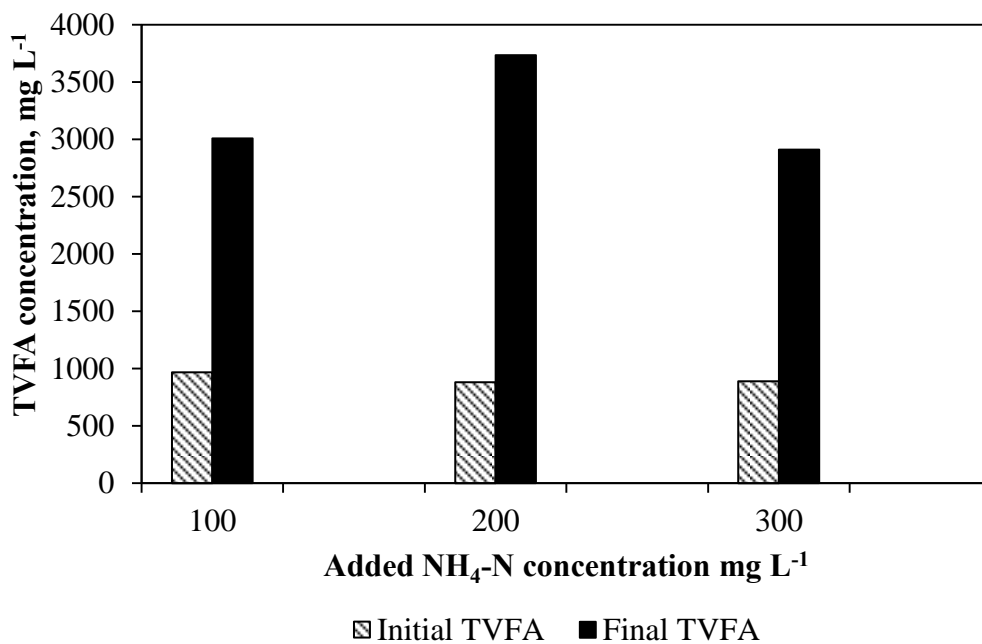
Figure 3.172 Initial and final TVFA concentrations of sludge fermentation at different nitrogen supplementations..
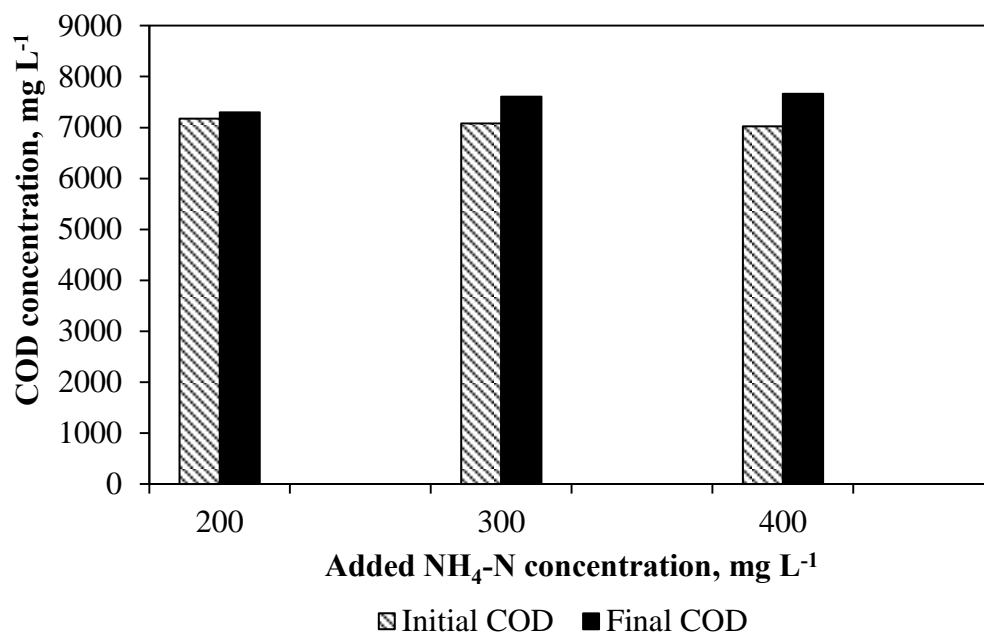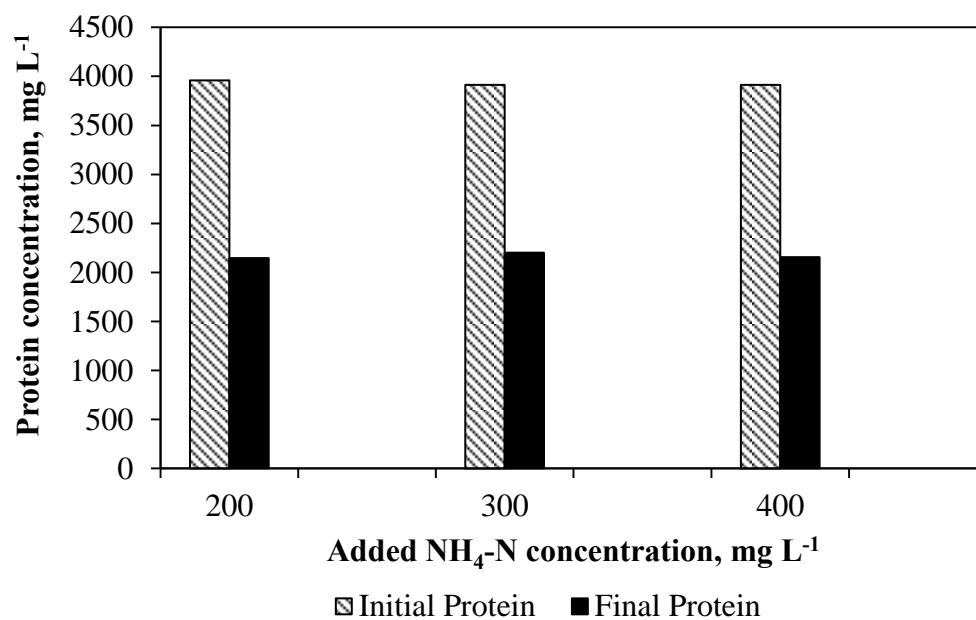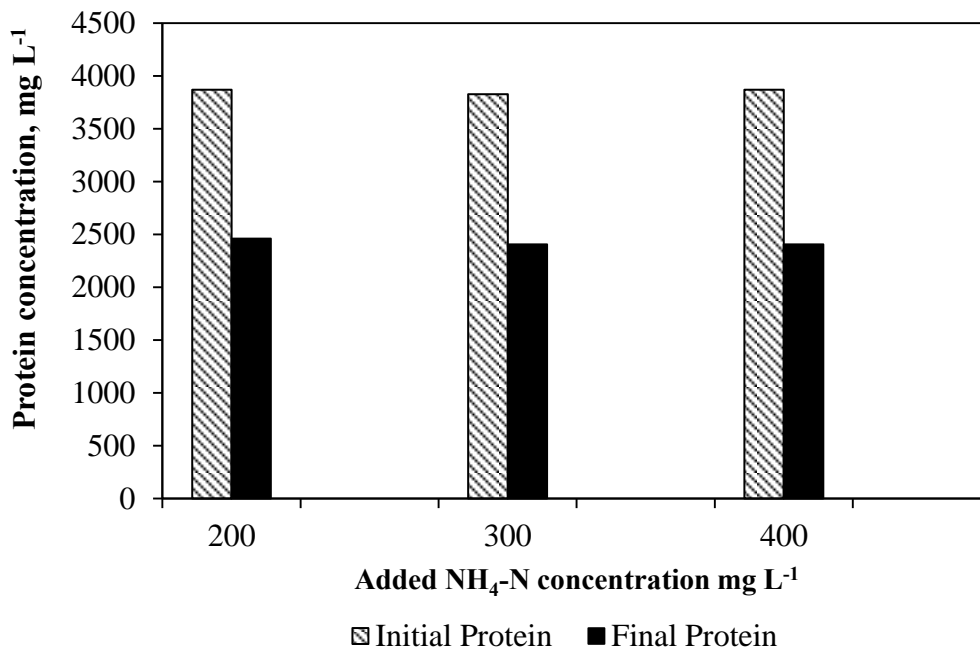

Figure 3.173 Initial and final protein concentrations of sludge fermentation at different nitrogen supplementations.
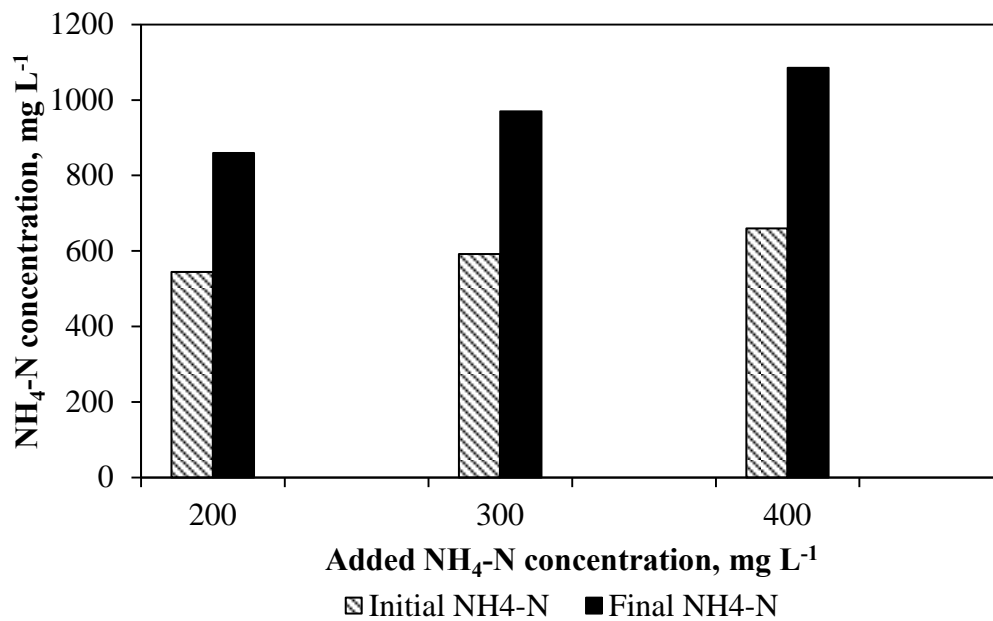
Figure 3.174 Initial and final NH$_4$-N concentrations of sludge fermentation at different nitrogen supplementations.

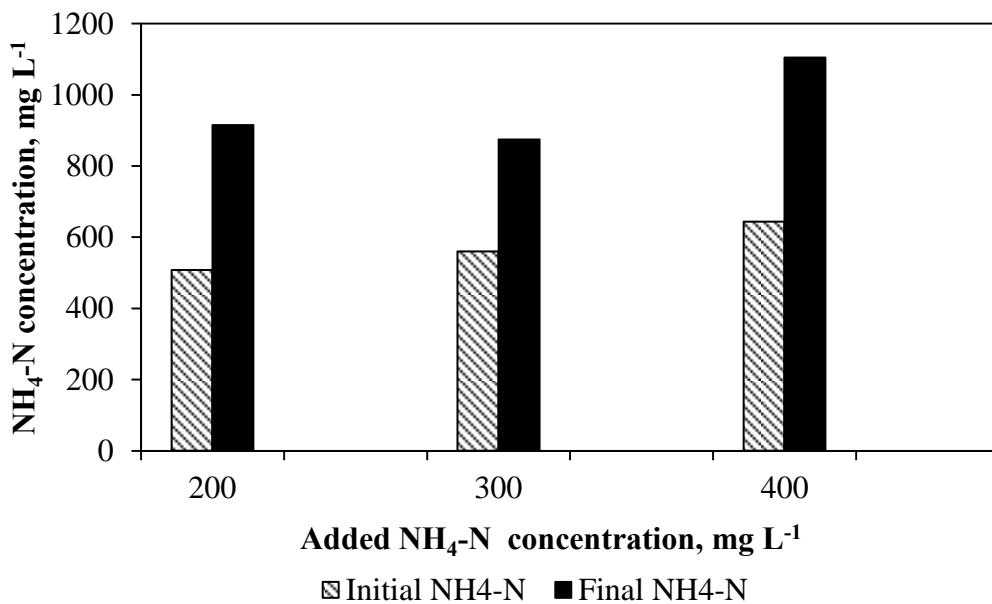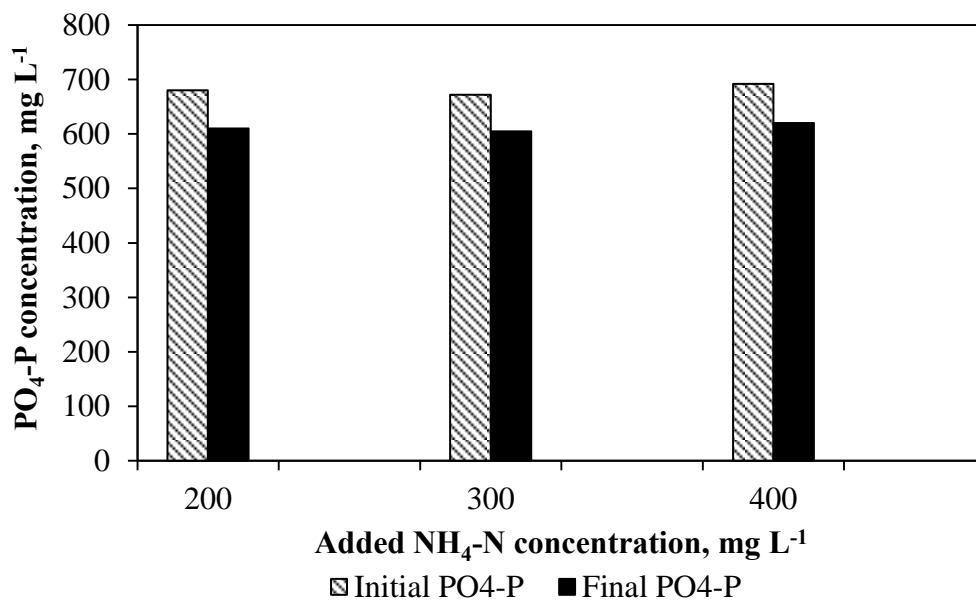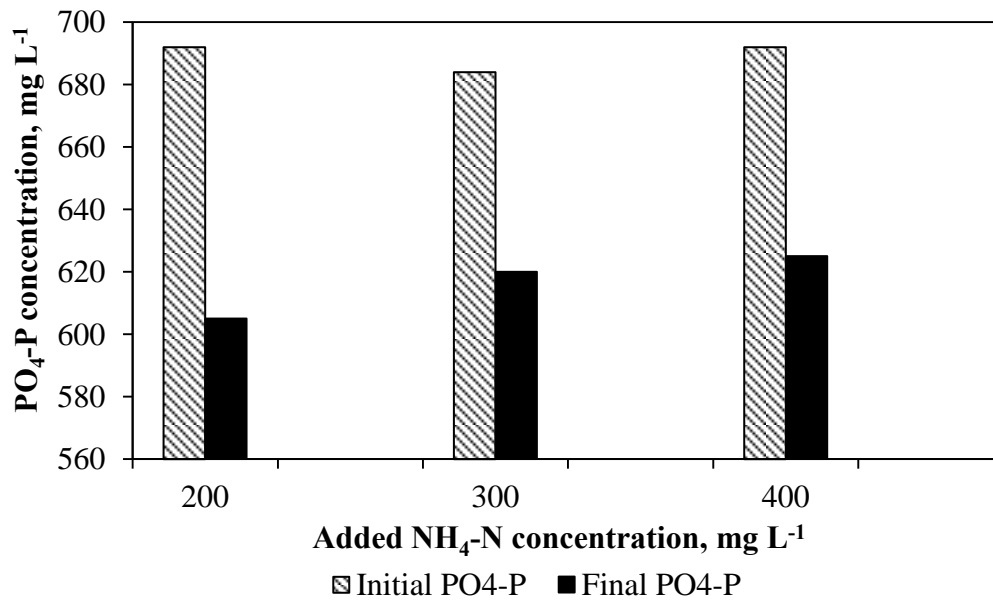Figure 3.175 Initial and final PO$_4$-P concentrations of sludge fermentation at different nitrogen supplementations.

Table 3.35 Gompertz Equation constants obtained from fermentation of sludge at different NH₄-N supplementations.

Added NH₄-N cons.	P	HPR	Lag phase period	SHPR	R²
mg L⁻¹	ml	(ml h⁻¹)	(h)	(ml g⁻¹ biomass hour⁻¹)	
200	38.9	0.45	0.0	0.09	0.992
300	47.0	0.61	0.0	0.12	0.993
400	40.9	0.48	0.0	0.10	0.993

3.2.9 The Effect of Initial Substrate Concentration on Hydrogen Gas Production

Total sugar concentration was the only substrate in the hydrogen gas production from hydrolyzed sludge or filtrate. High hydrogen production volumes by dark fermentation can be achived at high total sugar concentrations. Two stage hydrolysis of 33 g L⁻¹ stock sludge resulted in around 1200 mg L⁻¹. Fermentation was conducted at pH=5, T=37 $^{\circ}$C and at 5 g L⁻¹ biomass concentration. Stock sludge was concentrated to 60 g L⁻¹ obtain higher initial total sugar concentration. The final total sugar concentration reached to 2000 mg L⁻¹ after hydrolysis.

Figures 3.176 and 3.177 depict variation of cumulative hydrogen gas volume with time for filtrate and sludge, respectively. The volume of hydrogen produced from both filtrate and sludge was V_{H2}=48 ml. Sugar consumptions were around 20% in filtrate whereas it was 22% in sludge. But protein consumption was about 25% for both substrates. Increase in nitrogen concentrations were observed in this stage after fermentation (Table 3.36). Relatively higher production was obtained from filtrate as 3.64 mol mol⁻¹ glucose⁻¹ (Table 3.37). Specific production rates for filtrate and sludge were 0.096 ml g-1 biomass h⁻¹ and 0.088 ml g⁻¹ biomass h⁻¹, respectively, (Table 3.38).
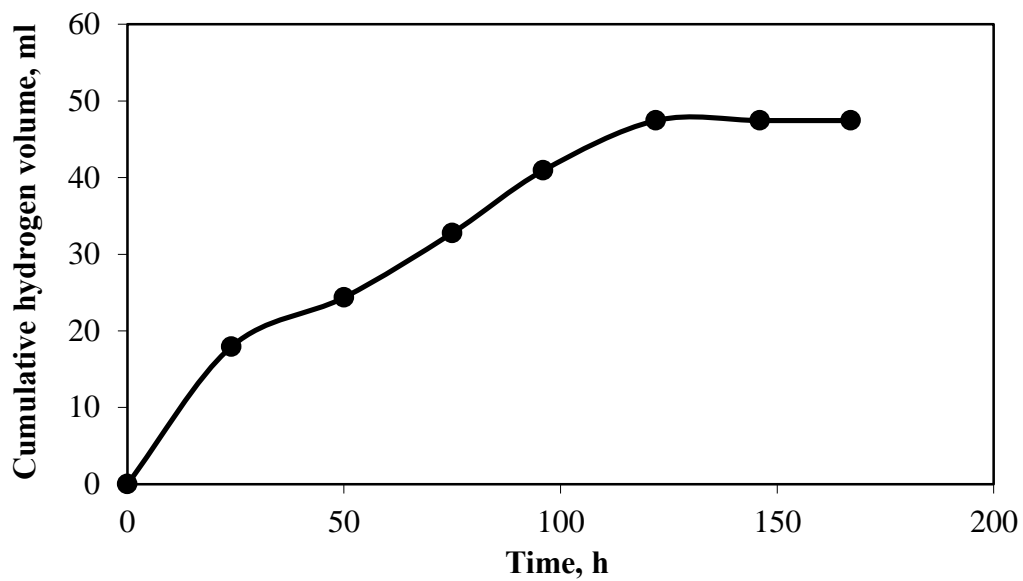
Figure 3.176 Variation of cumulative hydrogen gas volume with time in dark fermentation of filtrate at TS=2000 mg L^{-1} total sugar concentration.
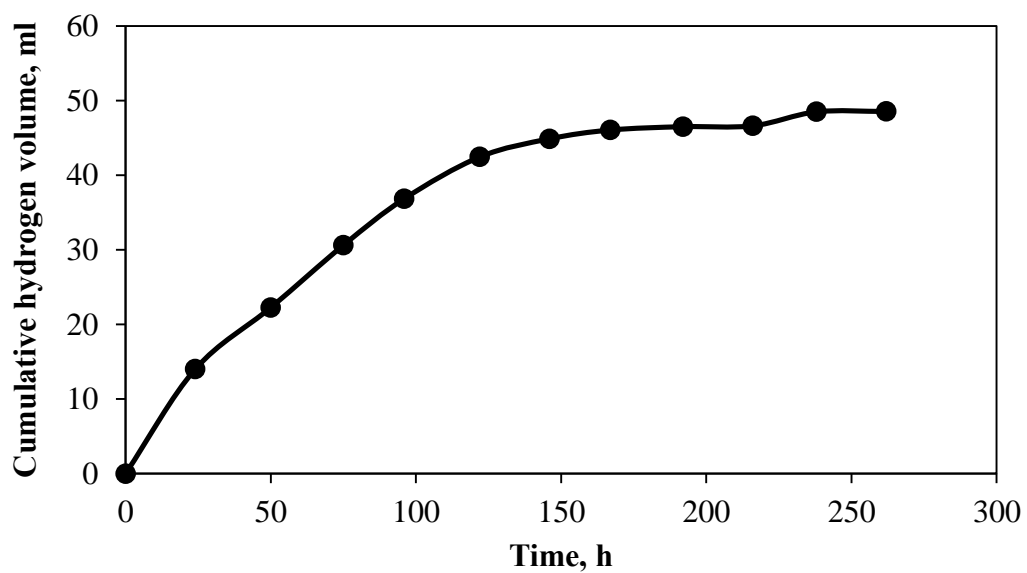
Figure 3.177 Variation of cumulative hydrogen gas volume with time in dark fermentation of sludge TS=2000 mg L^{-1} total sugar concentration.

Table 3.36 Nutrient and organic substance consumptions in fermentation medium when producing hydrogen gas from filtrate and hydrolyzed sludge at TS=2000 mg L^{-1} initial total sugar concentration.

	Filtrate	Sludge
Total sugar consumption,(%)	20	22
Protein consumption, (%)	27	24
$NH_4 - N_0$, (mg L^{-1})	289	684
$NH_4 - N_e$, (mg L^{-1})	684	892
$PO_4 - P$ consumption (%)	2	6

Table 3.37 Hydrogen production yield from filtrate and hydrolzied sludge at TS=2000 mg L^{-1} initial total sugar concentration.

Substrate type	Production Yield (mg/g glucose)	Production Yield (ml/g glucose)	Production Yield (mol H$_2$/mol glucose)
Filtrate	40.4	501	3.64
Sludge	35.6	441	3.20

Table 3.38 Gompertz equation constants for dark fermentation of filtrate and hydrolzied sludge at TS=2000 mg L^{-1} initial total sugar concentration.

Substrate type	P (ml)	HPR (ml hour^{-1})	Lag phase time (hour)	R^2	SHPR (ml g^{-1} biomass hour^{-1})
Filtrate	50.02	0.48	0.0	0.987	0.096
Sludge	47.5	0.44	0.0	0.995	0.088

3.2.10 The Effect of Sparging the Head Space by Argon on Hydrogen Gas Production

High partial pressure of hydrogen in the gas phase could affect the hydrogen production. Decreasing partial pressure of hydrogen could accelerate hydrogen generation and then provides increasing in the total hydrogen volume. The only

method to lower the partial pressure is to sparge the head space with another gas which will not interfere with the hydrogen generation. Argon is one of these gases. The head spaces of experimental bottles were sparged with argon for at leas 3 minutes to exhaust the existing hydrogen gas. Hydrogen gas volumes from dark fermentation of filtrates and sludges obtained from different hydrolysis reactions like single stage acid, single stage heat and sequential acid and heat treatment was too low. The experiments starting from the effect of pH on hydrogen gas production with gas sparging indicated a substantia increase in hydrogen volume. In order to observe the effect of gas sparging on the filtrate and sludge of different hydrolysis reactions, the experiments were repeated with argon sparging. The experimental conditions like, pH, fermentation temperature and initial biomass concentration were the same as the conditions in dark fermentations of substrate without argon sparging to compare the results.

Figure 3.178-3.179 depict cumulative hydrogen volumes obtained from filtrate and sludge hydrolyzed at pH=2 for t=1440 min. Argon sparging in head space of fermentation bottles of filtrate and sludge of acid hydrolysis resulted in over 20% increase in cumulative hydrogen gas volume. The increase in the rate of production was about 0.21 ml h^{-1}(Table 3.39). Similarly, the same strategy was applied to hydrolysate of single stage heat treatment and final cumulative hydrogen gas volume was over 20 ml (Figures 3.180-3.181). There was about 2 times increase in hydrogen production compared to the results of experiments without gas sparging.

Finally, argon sparging in filtrate and sludge of two stage hydrolysis is given in Figures 3.182-3.183. About 20 and 35 ml increases in hydrogen gas volume were obtained in filtrate and sludge, respectively and HPR was 0.30 ml h^{-1} for filtrate and 0.38 ml h^{-1} for sludge (Table 3.41).
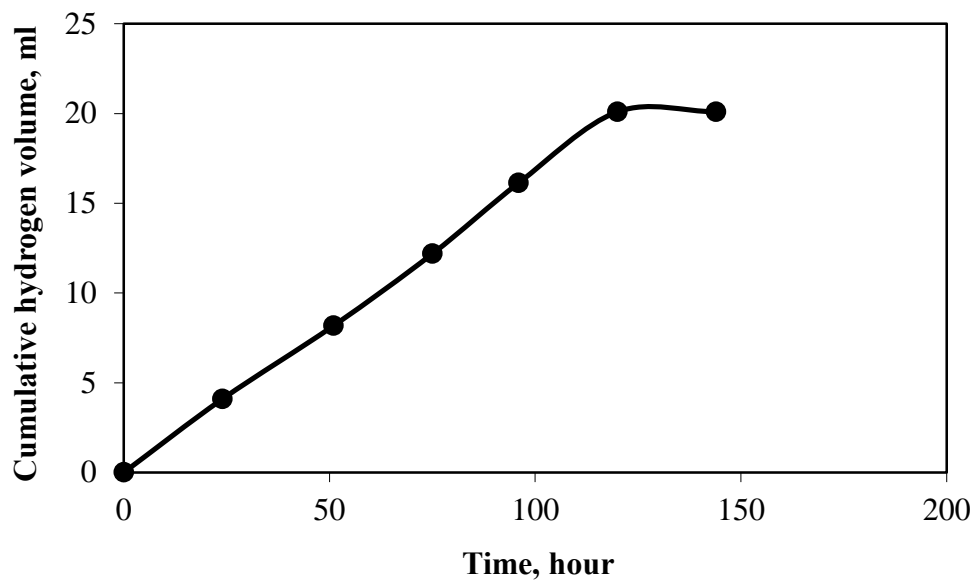
Figure 3.178 Effect of argon sparging on cumulative hydrogen gas volume of filtrate obtained from acid hydrolysis.
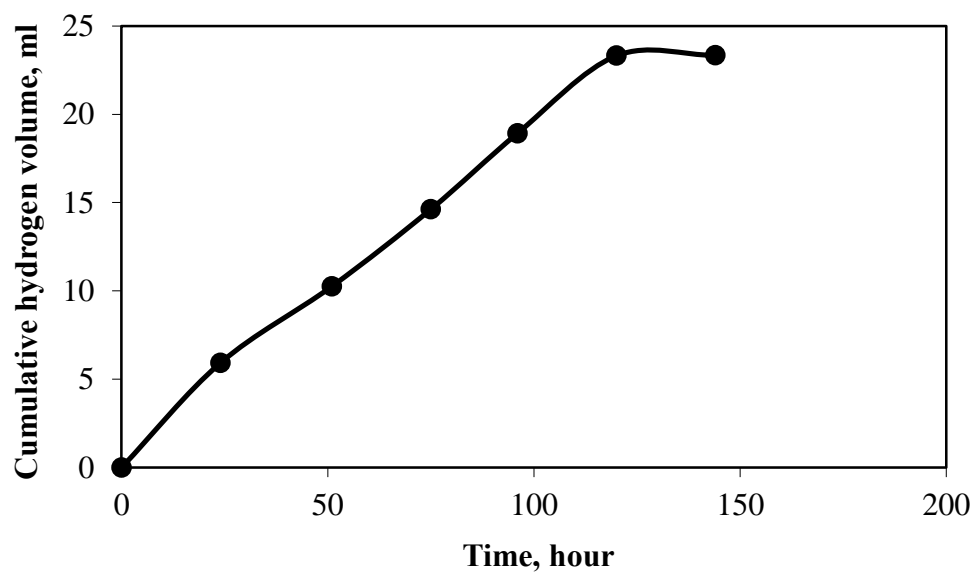
Figure 3.179 Effect of argon sparging on cumulative hydrogen gas volume of sludge obtained from acid hydrolysis.

Tablo 3.39 Gompertz equation constants of argon sparged filtrate and sludge of acid hydrolysis.

Hydrolysis conditions	P (ml)	HPR (ml hour^{-1})	Lag phase time (hour)	R_2	SHPR (ml g^{-1} biomass hour^{-1})
Acid (filtrate)	21.96	0.21	10.32	0.99	0.05
Acid (sludge)	25.44	0.23	5.22	0.99	0.06
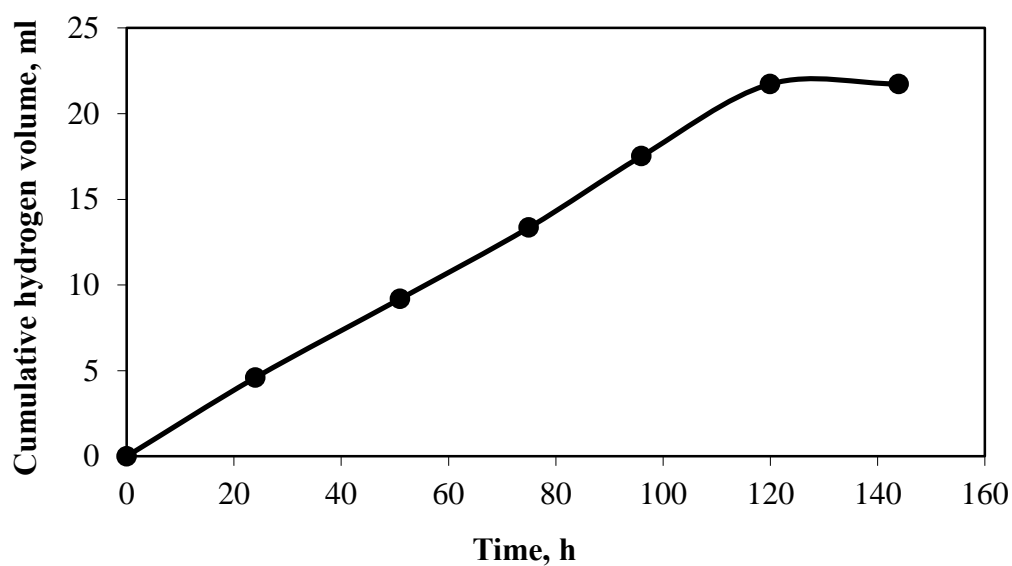
Figure 3.180 Effect of argon sparging on cumulative hydrogen gas volume of filtrate obtained from single-stage heat treatment.
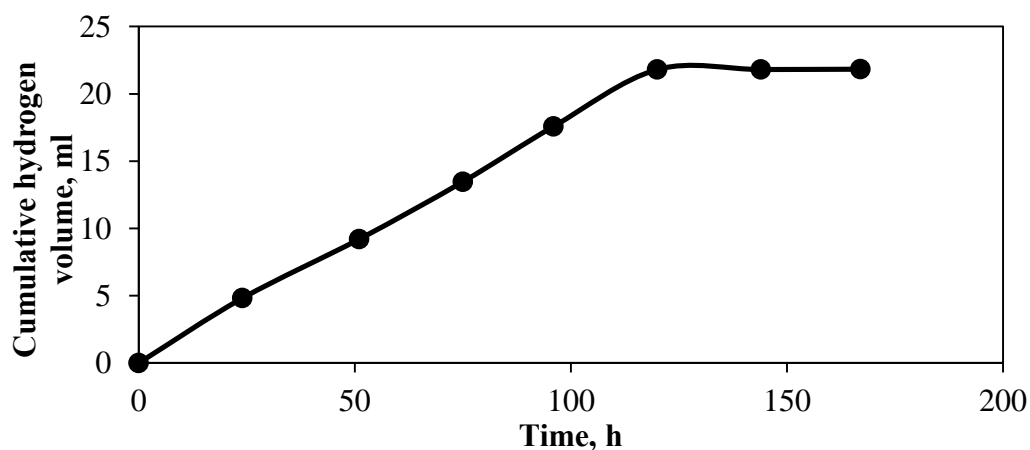
Figure 3.181 Effect of argon sparging on cumulative hydrogen gas volume of sludge hydrolyzed by single-stage heat treatment.

Table 3.40 Gompertz equation constants of argon sparged filtrate and sludge of single-stage heat treatment.

Hydrolysis conditions	P (ml)	HPR (ml h^{-1})	Lag phase period (h)	R_2	SHPR (ml g^{-1} biomass h^{-1})
Heat treatment (Filtrate)	23.74	0.22	9.05	0.99	0.06
Heat treatment (Sludge)	23.80	0.22	8.38	0.99	0.06
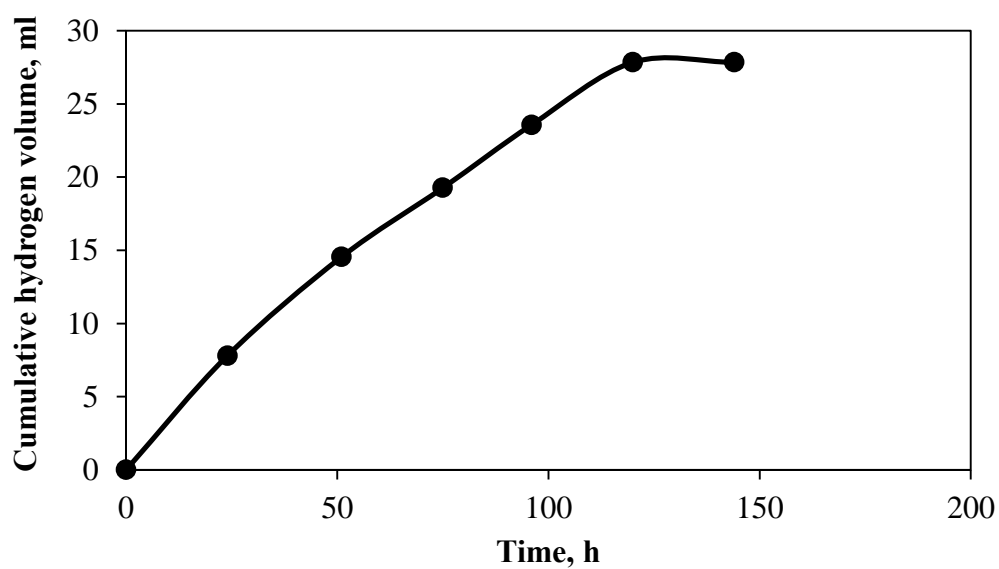
Figure 3.182 The effect of argon sparging on cumulative hydrogen gas volume obtained form filtrate of two-stage hydrolysis.

Table 3.41 Gompertz equation constants of argon sparged filtrate and sludge of two stage hydrolysis.

Hydrolysis conditions	P (ml)	HPR (ml hour^{-1})	Lag phase (hour)	R^2	SHPR (ml g biomass hour^{-1})
Acid+Heat (Filtrate)	29.27	0.30	2.57	0.99	0.08
Acid+Heat (Sludge)	33.09	0.38	7.43	1.00	0.10
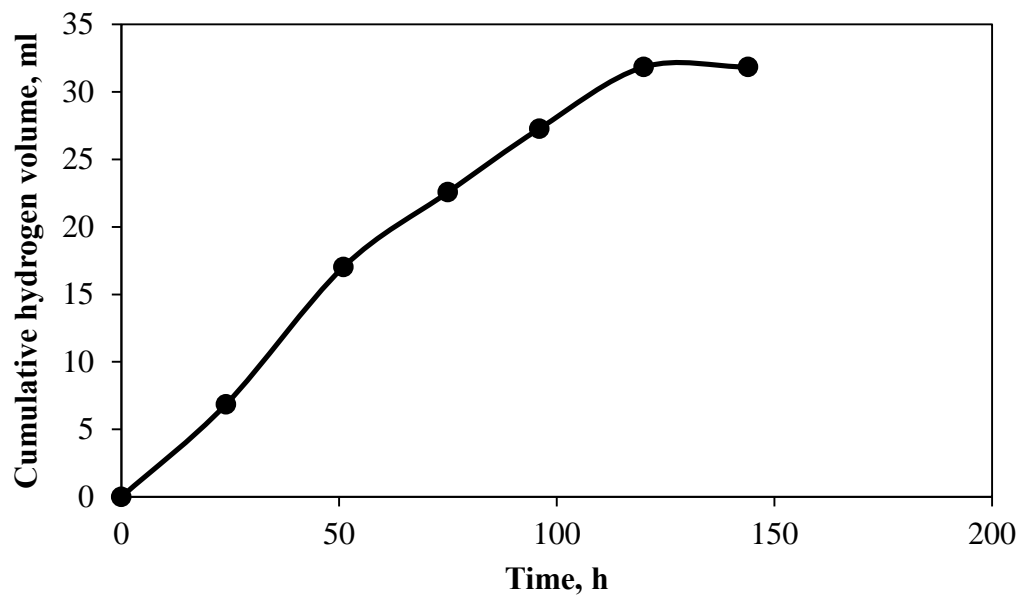
Figure 3.183 Effect of argon sparging on cumulative hydrogen gas volume of sludge obtained from two-stage hydrolysis.

CHAPTER FOUR
CONCLUSIONS

The decrease in fossil fuel resources revealed an urgent need in evaluation and development of alternative energy sources. The major criteria for new energy sources are that they should be reneawable and sustainable to decrease their contribution to the environmental pollution. Hydrogen is a clean and high energy fuel and definitely it is a potential substitute for fossil fuels. Therefore, the studies on hydrogen gas production have been substantially increased in recent years.

Sustainable production of hydrogen gas requires availability of low cost and sustainable raw materials. The handling and management of wastewater treatment sludges are significant problems creating lots of environmental nuisance. From simple to high cost sludge handling and disposal methods are under investigation to solve this problem. However, wastewater sludge could be a good source of low cost raw material in hydrogen gas production due to its high organic matter content. Utilization of wastewater treatment sludge in hydrogen gas production could help sludge handling problem, waste minimization and sustainable energy production.

In the light of these facts, the main aim of this thesis was to determine the factors that affect hydrogen production yield and rate from wastewater treatment sludge by dark fermentation. For this purpose, the sludge was hydrolyzed by acid treatment, heat treatment and sequential acid -heat treatment. After the selection of the most effective hydrolysis method and optimization of the hydrolysis conditions, the effect of environmental factors as fermentation temperature and pH, the effects of media composition as biomass, protein, nitrogen and substrate concentration on hydrogen production were investigated.

Hydrogen generating organisms require high sugars or organic substances for high gas generation. Raw sludge characterization resulted in $22\pm$ g L^{-1} total COD concentration, 570 mg L^{-1} soluble COD in filtrate and 216 mg L^{-1} total sugar concentration in average. These concentrations indicated that total sugar and soluble

COD concentrations in the liquid phase were limited and not enough for hydrogen gas generation by dark fermentation. On the other hand, total COD concentration was considerably high and it could be a good source of organic substances for the hydrogen gas production if it can be converted into the readily biodegradable form for microorganisms. In other words, sludge must be hydrolyzed by somehow to increase the organic substance concentration to be used by hydrogen producing organisms during fermentation. For this purpose, hydrolysis under acidic conditions, at high temperatures and combinations of these two methods were used in the study.

Acid hydrolysis of sludge was the first hydrolysis method used in the thesis. The effects of pH and contact time on acid hydrolysis of waste sludge were investigated. The maximum concentrations of organic matter parameters after the hydrolysis reaction were COD=2800 mg L^{-1}, TOC= 500 mg L^{-1} and TS= 470 mg L^{-1} obtained at pH=2 and t=1440 minutes. COD and total sugar concentrations were increased 5 times and 2 times, respectively, with regard to the concentrations before hydrolysis. NH_4-N concentration did not vary significantly with time and pH. It was between 205-250 mg L^{-1} at pH range for pH=2-6 and for the hydrolysis period from 15 min to 1440 min. PO_4-P concentration decreased with increasing hydrolysis pH. At low pH values (pH= 2-3), the concentration reached to around 500 mg L^{-1}, but it decreased to around 50 mg L^{-1} at pH= 6. Protein concentration is substantially affected by the pH of hydrolysis reaction. No protein was observed in the liquid phase for pH=4-6, but acidic conditions as pH= 2-3 resulted in about 1.5 g L^{-1} protein concentration. Under acidic conditions cell membrane was strongly disrupted and then efficient release of intracellular and cell membrane bound proteins were achieved. Statistical analysis indicated that both pH and contact time in acid hydrolysis of sludge were significant factor (α=0.05) to obtain higher concentrations of organic substance as COD, TOC and total sugar. pH was the only significant factor for PO_4-P and protein concentrations. But, none of the factor had significant effect on release of NH_4-N content of sludge to the liquid phase.

The hydrolysis under acidic conditions and long hydrolysis periods could cause formation of unknown and unwanted substances which could interfere with the

fermentation for hydrogen gas production. Therefore, the effect of hydrolysis conditions on hydrogen gas production was investigated for filtrate and sludge. Maximum hydrogen volume produced from filtrate and sludge was $V_{H2}=11$ ml and $V_{H2}=5$ ml, repecetively, obtained at hydrolysis pH value of pH=4. Raw sludge was also resulted in $V_{H2}=5$ ml cumulative hydrogen gas volume. Although, nutrient and organic matter content of filtrate was rich at hydrolysis pH= 2, the hydrogen volume was $V_{H2}=6$ ml which was lower than that of obtained at pH=4. This could be due to formation of some side products as a result of hydrolysis under strong acidic conditions and these products could cause inhibition of hydrogen generation. The yields of formation form sludge and filtrate support this conclusion. The maxiumum production yields was observed as $Y=24$ mmol g^{-1} TS^{-1} at hydrolyis pH=5 and it was $Y=41$ mmol g^{-1} TS^{-1} for sludge hydrolyzed at pH=6.

As a second hydrolysis method, heat treatment of sludge was investigated. The hydrolysis temperature and exposure time were two factors examined. Average organic matter concentrations in the liquid phase before the hydrolysis were TS=54 mg L^{-1} , COD= 750 mg L^{-1} and TOC=193 mg L^{-1}. The concentrations of organic matters increased to COD=9700 mg L^{-1} , TS= 1338 mg L^{-1} and TOC=2250 mg L^{-1} under the hydrolysis conditions as t=60 mins and 135ºC. Compared to acid hydrolysis, 3-4 times increase in organic matter concentrations were obtained. High hydrolysis temperature (T=135ºC) affected the NH_4-N concentration negatively, Maximum NH_4-N concentration was obtained as 250 m L^{-1} which was the same concentration observed in acid hydrolysis. Protein concentration was substantially affected by hydrolysis temperature and period and reached to maximum level (1 g L^{-1}) at T=135ºC and t=60 min. Statistical analysis of the results indicated both factors and their interactions are significant and have positive effect on the concentrations of organic substances and protein. But only hydrolysis temperature has significant on NH_4-N concentration and increasing temperatures causes decreasing in the concentration.

Dark fermentation on filtrate and sludge obtained after heat tretament was conducted to observe the effect of hydrolysis conditions on hydrogen gas production.

Cumulative hydrogen volumes from filtrates and sludge obtained at different hydrolysis temperatures and periods were almost constant around V_{H2}= 4 ml. Although there were significant differences in the concentrations of organic matters (COD, TOC and TS), nitrogen, phosphorus and protein after hydrolysis, these differences didn't have any effect on hydrogen gas production. It was observed that, carbon source, released as a result of heat treatment, cannot be used effectively in hydrogen gas production. It could be due to an unknown product formed after heat treatment of sludge.

Two stage acid and heat treatment was the third hydrolysis method. Sequential acid and heat treatment was applied to sludge. The maximum COD and total sugar concentrations were obtained as 7121 mg L^{-1} and 2100 mg L^{-1} , respectively, under the hydrolysis conditions of t=60 min., pH=2 and T=135º C. In total sugar concentration with two-step hydrolysis, 4 times improvement was achieved compared to acid hydrolysis and it was 0.5 times compared to single stage heat treatment. PO_4-P concentration reached to maximum concentration of 750 mg L^{-1} under the same hydrolysis conditions. The highest NH_4-N concentration was observed as 280 mg L^{-1} at t=15 mins., pH=2 and T=135ºC. Long hydrolysis times (t=60 min.) resulted in decrease in NH_4-N concentration as it was observed in the single-stage heat treatment results.

The optimum conditions of two-step acid hydrolysis were determined through Design Expert 7.0 statistical program. By using response function coefficients, within the range of factors in experimental design, hydrolysis conditions that maximize COD and TS concentrations were determined. In addition, fitness of the model coefficients were tested for some experimental conditions which were different than that of experimental design. Only COD and TS were selected as the target parameters for maximization. Because they are the major substrates in hydrogen gas production, the others can be externally added into the fermentation media if required. The optimum conditions that maximize COD and total sugar concentrations were T=135ºC, pH=2 and t=60 min in two stage hydrolysis of sludge. The predicted values were COD=7121 mg L^{-1} and TS= 2000 mg L^{-1}. The observed

and predicted concentrations under these conditions were in agreement. An experiment at T= 121 ^{0}C and pH=2 and t=60 min, which were different than the conditions examined in statistical experimental design, was conducted to evalute if predicted responses by using coefficients and observed concentrations form the experiments will be the same. The predicted and the observed values were very close to each other for all responses investigated. These results show that response function coefficients can be used to determine the concentrations of organic substances and nutrients for examined ranges of factors. In brief, organic substance concentrations obtained form two stage hydrolyis of sludge were the highest compared to that of other two hydrolysis method. Therefore, two stage hydrolysis was used as pretreatment of sludge in hydrogen gas by dark fermentation.

Dark fermentation of filtrate and sludge obtained at optimal hydrolysis conditions resulted in V_{H2}= 6 ml and V_{H2}= 10 ml cumulative hydrogen gas volumes, respectively. Production yield for filtrate was 1.23 mmol g^{-1} TS^{-1} (30.5 ml g^{-1} TS^{-1}) and it was 2.16 mmol g^{-1} TS^{-1} (53.5 ml g^{-1} TS^{-1}) for sludge. The yield and hydrogen volumes obtained by dark fermentation of sludge and filtrate of two stage hydrolysis were lower than the values observed in filtrate and sludge of acid hydrolysis. However, total sugar concentration was substantially higher than the one obtained form acid hydrolyis. Therefore, the expectation was to get higher volumes and yield. The reason for this result could be type of the organic acids produced during fermentation. As it is well known that acetic acid and buytric acid production results in hydrogen generation. On the other hand, hydorgen gas is consumed during formation of end products like propionic, lactic acid and ethanol. In addition, homoacetogens are the other factor which causes reduction in hydrogen gas production. Fermentation conditions and media compositions significantly affect both type of the organic acid and activity of the homoacetogens. Improving fermentation conditions and media composition could enhance hydrogen gas production.

The effect of fermentation temperature was investigated at T= 45 ^{0}C and T=55 ^{0}C. Maximum cumulative hydrogen gas volume was obtanied as V_{H2}=14 ml for filtrate

and sludge, V_{H2}=4 ml for raw sludge at T=55 ^{0}C. A slight increase in cumulative hydrogen gas production occurred with respect to the volumes obtained at T=37 ^{0}C. The yield of of formation at this fermentation temperature were 3.3 mmol g^{-1} TS^{-1} (81 ml g^{-1} TS^{-1}) for filtrate, 3.5 mmol g^{-1} TS^{-1} (87 ml g^{-1} TS^{-1}) for sludge. Although there was some increases in hydrogen production potentials by incerasing fermentation temperature, they were not as expected. Therefore, it can be concluded that fermentation temperature does not have a substantial effect on hydrogen production from sludge. Fermentation at T=37 ^{0}C is more economical.

pH of fermentation had more significant effect on hydrogen production. The maximum cumulative hydrogen volumes form filtrate and sludge were V_{H2}=42 ml and V_{H2}=45 ml obtained at pH=5, respectively. A substantial increase in the yields of formation were observed. Fermentation at pH=5 resulted in Y_{H2} =3.75 mol mol^{-1} for filtrate and Y_{H2}=3.35 mol mol^{-1} for sludge. The effect of protein and NH_4-N supplementation, initial biomass concentration at fermentation pH=5 were investigated. It was observed that protein cannot be used as a substrate and protein supplementation didn't have a contribution to the hydrogen gas production. No significant difference was observed in cumulative hydrogen gas volume and production rate with NH_4-N supplementation at 200-400 mg L^{-1} concentrations. Biomass concentration was varied between 2-5 g L^{-1}. Production yield was about 2 mol mol^{-1} at low biomass concentrations (X=2-3 g L^{-1}) whereas production yield for filtrate reached to maximum level with Y_{H2}=3.33 mol/mol glucose at X=5 g L^{-1}. The results indicated that nutrient supplementaion did not provide any improvement in hydrogen gas production. Increasing biomass concentration provided a slight improvement in the yield of formation. Therefore, it can be concluded that fermentation pH is the most significant factor in hydrogen gas production and it sould be pH=5 for the maximum hydrogen gas generation from waste sludge.

An increase in final COD concentrations of dark fermentation was observed in most of the cases. It was expected to get not complete but at least partial COD removal. Some of the organic substances are used for growth or maintanace and metabolized up to CO_2 by microorganisms. Therefore, a decease in COD should

have occurred. But, the main fraction is biotransformed to organic acids which still constitute COD. One of the reasons of increase in COD concentration during fermentation could be contribution of organic content of the inoculum due to inactivation. Another one could be the microbial hydrolysis of proteins during fermentation. Protein concentration decreased but NH_4-N concentration increased after fermentation. Hydrolyzed proteins released their carbon and nitrogen contents resulting in increasing in COD and NH_4-N. The COD concentration in the effluent of sludge fermentation was higher than that of filtrate fermentation. It was due to further microbial hydrolysis of partially disrupted cells in hydrolyzed sludge during dark fermentation.

In summary, two stage hydrolysis of sludge under the optimized conditions resulted in higher organic and nutrient concentration. Optimization of fermentation pH provided a significant increase in the hydrogen gas production and yield. Passing argon gas through the system also had a positive effect on hydrogen gas generation. External addition of nitrogen and protein to the fermentation media is not necessary. The yield and rate of production form filtrate was slightly higher than the rate and yield from sludge.

CHAPTER FIVE
RECOMMENDATIONS

Waste sludge of wastewater treatment plants creates significant environmental problems. It sometimes requires application of expensive, high technologies to be converted to into environmentally acceptable form. The main problem with the sludge is its organic substance and toxic material content. Because, the sludge will be some how disposed to the environment. Wastewater treatment sludge could be a good source of low cost raw material in hydrogen gas production due to its high organic matter content. Utilization of wastewater treatment sludge in hydrogen gas production will help sludge handling problem, waste minimization and sustainable energy production. However, the organic content of waste sludge needs to be converted into a form which can be easily used by microorganisms. In other words, sludge must be pretreated or hydrolyzed. Furthermore, the fermentation conditions must be suitable for the type of substrate. Single stage acid and heat treatment, two stage sequential acid and heat treatment were three hydrolysis methods used in this study and the effect of fermentation media composition and conditions on the production were investigated. The yields of production under the optimized conditions were satisfactory. However, the process needs further improvement to increase cumulative hydrogen gas volumes, efficient removal of organic substances and nitrogen for complete treatment of sludge in order to combine waste minimization and energy production concepts. The following studies are recommended to realize these suggestions.

- Ultrasound and microwave can be used for sludge hydrolysis.
- Pure hydrogen gas producing microbial cultures can be used.
- New isolated or genetically modified microorganisms capable of producing hydrogen gas more effectively can be utilized.

REFERENCES

Argun H., & Kargi F., (2011). Biohydrogen production by different operational modes of dark and photo fermentation: An overview. *Int J Hydrogen Energy*, *36*, 7443-7459.

Argun H., Kargi F., & Kapdan I.K., (2009a). Microbial culture selection for bio-hydrogen production from waste ground wheat by dark fermentation. *Int J Hydrogen Energy*, *34*, 2195–2200.

Argun H., Kargi F., & Kapdan I.K., (2009b). Hydrogen production by combined dark and light fermentation of ground wheat solution. *Int J Hydrogen Energy*, *34*, 4305-4311.

Argun H., Kargi F., Kapdan I.K. & Oztekin R., (2008a). Biohydrogen production by dark fermentation of wheat powder solution: effects of C/N and C/P ratio on hydrogen yield and formation rate. *Int J Hydrogen Energy*, *33*, 1813–1819.

Argun H., Kargi F., Kapdan I.K. & Oztekin R., (2008b). Batch dark fermentation of powdered wheat starch to hydrogen gas: effects of the initial substrate and biomass concentrations. *Int J Hydrogen Energy*, *33*, 6109–6115.

Bartacek, J., Zabranska, J., Lens, P.N.L., (2007). Developments and constraints in fermentative hydrogen production, *Biofuels, Bioproducts & Biorefining*, *1*, 201-214

Benemann, J.R. and Hallenbeck P.C. (2002). Biological hydrogen production; fundamentals and limiting processes. *Int J Hydrogen Energy, 27*, 1185–1193

Cai M.L., Liu J.X., Wei Y.S., (2004). Enhanced bio-hydrogen production from sewage sludge with alkaline pretreatment, *Environ Sci Technol, 38*, 3195–3202

Cao G., Ren N., Wang A., Lee D.J., Guo W., & Liu B., et al. (2009). Acid hydrolysis of corn stover for biohydrogen production using Thermoanaerobacterium thermosaccharolyticum W16. *Int J Hydrogen Energy*, *34*, 7182–7188.

Collet, C., Adler N., Schwitzguébel JP. ve Péringer P., (2004). Hydrogen production by Clostridium thermolacticum during continuous fermentation of lactose, *Inter J of Hydrogen Energy*, *29*, 1479-1485.

Das, D. and Nath, K. (2004). Improvement of fermentaive hydrogen production: various approaches. *Appl. Microbiol. Biotechnol*, *65*, 520–529.

Dong L., Zhenhong Y., Yongming S., Xiaoying K. & Yu Z., (2009). Hydrogen production characteristics of the organic fraction of municipal solid wastes by anaerobic mixed culture fermentation. *Int J Hydrogen Energy*, *34*, 812–820.

Dubey P.K., Sinha A.S.K., Talapatra S., Koratkar N., Ajayan P.M., Srivastava O.N. (2010). Huydrogen generation by water electrolysis using carbon nanotube anode. *International Journal of Hydrogen Energy*, *35/ 9*, 3945-3950

Erden G., Demir O., Filibeli A., (2010). Disintegration of biological sludge: Effect of ozone oxidation and ultrasonic treatment on aerobic digestibility. *Bioresource Technology*, *101/21*, 8093-8098,

Eskicioğlu C., Kennedy K.J., Droste R.L., (2010). Enhanced disinfection and methane production from sewage sludge by microwave irradiation, *Desalination 251*, 279–285

Evvyernie D., Morimoto K., Karita, S., Kimura T., Sakka K., Ohmiya, K. (2001). Conversion of chitinous waste to hydrogen gas by Clostridium paraputrificum M-21. *J Bioscience Bioeng.*, *91*, 339-343

Fabiano B, Perego P. (2002). Thermodynamic study and optimization of hydrogen production by Enterobacter aerogenes. *Int J Hydrogen Energy*, *27*, 149–56.

Fan Y.T., Xing Y., Ma H.C., Pan C.M. & Hou H.W., (2008). Enhanced cellulose-hydrogen production from corn stalk by lesser panda manure. *Int J Hydrogen Energy*, *33*, 6058–6065.

Florin, L., Tsokoglou A. ve Happe T. (2001). A novel type of iron hydrogenase in the green algae Scenedesmus obliquus is linked to the photosynthetic electron transport chain, *Journal of Biological Chemistry*, *276*, 6125-6132.

Gilroyed B.H., Chang C., Chu A. & Hao X., (2008). Effect of temperature on anaerobic fermentative hydrogen gas production from feedlot cattle manure using mixed microflora. *Int J Hydrogen Energy*, *33*, 4301–4308.

Guo L., Li X.M., Bo X., Yang Q., Zeng G.M., Liao .X. et al., (2008). Impacts of sterilization, microwave and ultrasonication pretratment on hydrogen producing using waste sludge *Bior Tech, 99*, 3651–3658,

Guo L., Li X.M., Zeng G.M., Zhou Y. (2010). Effective hydrogen production using waste sludge and its filtrate. *Energy*, *35/9*, 3557-3562

Guo Y.P., Fan S.Q., Fan Y.T., Pan C.M. & Hou H.W., (2010). The preparation and application of crude cellulase for cellulose-hydrogen production by anaerobic fermentation. *Int J Hydrogen Energy*, *35*, 459–468.

Han S.K., & Shin H.S., (2004). Biohydrogen production by anaerobic fermentation of food waste. *Int J Hydrogen Energy*, *29*, 569-577.

Holladay, J. D., Hu, J., King, D. L. ve Wang, Y., (2009). An overview of hydrogen production Technologies, *Catalysis Today*, *139*, 244-260.

Ishıda Y., Kumabe K., Hata K., Tanifuji K., Hasegawa T., Kitagawa K., Isu N., Funahashi Y., Asai T. (2009). Selective hydrogen generation from real biomass through hydrothermal reaction at relatively low temperatures. *Biomass and Bioenergy*, *33/ 1*, 8-13.

Jayasınghearachchi H.S., Singh S., Sarma P.M., Aginihotri A., Lal B. (2010). Fermentative hydrogen production by new marine Clostridium amygdalinum strain C9 isolated from offshore crude oil pipeline. *International Journal of Hydrogen Energy*, *35/13*, 6665-6673

Jo J.H., Lee D.S., Park D., Park J. M. (2008). Biological hydrogen production by immobilized cells of Clostridium tyrobutyricum JM1 isolated from a food waste treatment process. *Bioresource Technology*, *99/14*, 6666-6672.

Kapdan,İ.ve Kargı F. (2006). Bio-hydrogen production from waste materials, *Journal of Enzyme and Microbial Technology*; Review *38*, 569-582.

Kim J.P., Kim K.R., Choi S. P., Han S.J., Kim M.S., Sim S.J. (2010). Repeated production of hydrogen by sulfate re-addition in sulfur deprived culture of Chlamydomonas reinhardti. *International Journal of Hydrogen Energy*, *35/ 24*, 13387-13391.

Kım S.H., Han S.K., Shin H.S., (2004). Feasibility of biohydrogen production by anaerobic co-digestion of food waste and sewage sludge, *Int J Hydrogen Energy* *29*, 1607–1616.

Kim, D., Han, S., Kim, S. and Shin, H. (2006). Effect of gas sparging on continuous fermentative hydrogen production. *Int J. Hydrogen Energy*, *31*, 2158-2169.

Kim, M., Moon, K., Lee, I., Lee, T. and Sung, C. (1999). Hydrogen gas production by fermentation from sugars using Clostridium butyricum NCIB 9576. *Korea Institute of Energy Research*, *27*, 62–69.

Kotay S.M., Das,D. (2009). Novel dark fermentation involving bioaugmentation with constructed bacterial consortium for enhanced biohydrogen production from pretreated sewage sludge. *International Journal of Hydrogen Energy*, *34/ 17*, 7489-7496.

Kyazze G., Dinsdale R., Hawkes F.R., Guwy A.J., Premier G.C. & Donnison I.S., (2008). Direct fermentation of fodder maize, chicory fructans and perennial ryegrass to hydrogen using mixed microflora. *Bioresour Technol*, *99*, 8833–8839.

Levin D.B., & Chahine R., (2010). Challenges for renewable hydrogen production from biomass. *Int J Hydrogen Energy*, 4962-4969.

Levin D.B., Pitt L., & Love M., (2004). Biohydrogen production: prospects and limitations to practical application. *Int J Hydrogen Energy*, *29*, 173-185.

Lin C.Y., Chang C.C., & Hung C.H., (2008). Fermentative hydrogen production from starch using natural mixed cultures. *Int J Hydrogen Energy*, *33*, 2445–2453.

Lın C.Y., Lay C.H., (2005). A nutrient formulation for fermentative hydrogen production using anaerobic sewage sludge microflora, *Int J Hydrogen Energy, 30*, 285–292.

Lin, C.Y.ve Lay, C.H. (2004). Carbon/nitrogen ratio effect on fermentative hydrogen production by mixed microflora, *Inter J of Hydrog Energy*., *29*, 41-45.

Lıu G, Shen J. (2004). Effects of culture medium and medium conditions on hydrogen production from starch using anaerobic bacteria. *J Bioscience Bioeng*.; *98*, 251-256.

Liu, G. and Shen, J. (2004). Effect Of Culture and Medium Conditions on Hydrogen Production From Starch Using Anaerobic Bacteria , *Journal of Bioscience and Bioengineering* , *98(4)*, 251-256.

Mizuno, O., Dinsdale, R., Hawkes, F.,Hawkwes, D. and Noike,T. (2000). Enhancement Of Hydrogen ProductionFrom Glucose By Nitrogen Gas Sparging, *Bioresource Technol*., *73*, 59-65.

Nıcolau J.M., Dinsdale R., Guwy A. (2008). Hydrogen production from sewage sludge using mixed microflora inoculum: Effect of pH and enzymatic pretreatment. *Bioresource Technology*, *99/14*, 6325-6331.

Oh Y.K., Lee K.R., Ko K.B., Yeom I.T. (2007). Effects of chemical sludge disintegration on the performances of wastewater treatment by membrane bioreactor. *Water Research*, *41/ 12*, 2665-2671.

Pan C.M., Fan Y.T., Zhao P., Hou H.W. (2008). Fermentative hydrogen production by the newly isolated Clostridium beijerinckii Fanp3. *International Journal of Hydrogen Energy*, *33/ 20*, 5383-539.

Park W.J., Ahn J.H., Hwang S., Lee C.K. (2009). Effect of output power, target temperature, and solid concentration on the solubilization of waste activated sludge using microwave irradiation, Bior Tech 10.1016/j.biortech .2009.02.062.

Qınglan H., Chang W., Dingqiang L., Yao W., Dan L., Guiju L. (2010). Production of hydrogen-rich gas from plant biomass by catalytic pyrolysis at low temperature. *International Journal of Hydrogen Energy*, *35/ 17*, 8884-8890.

Ramachandran U., Wrana N., Cicek N., Sparling R., Levin D. B. (2008). Hydrogen production and end-product synthesis patterns by Clostridium termitidis strain CT1112 in batch fermentation cultures with cellobiose or α-cellulose. *International Journal of Hydrogen Energy*, *33/ 23*, 7006-7012.

Roh H.S., Lee D.K., Koo K.Y., Jung U.H., Yoon W. L. (2010). Natural gas steam reforming for hydrogen production over metal monolith catalyst with efficient heat-transfer. *International Journal of Hydrogen Energy*, *35/ 4*, 1613-1619.

Seo J.G., Youn M.H., Bang Y., Song I.K. (2010). Effect of Ni/Al atomic ratio of mesoporous Ni–Al_2O_3 aerogel catalysts on their catalytic activity for hydrogen production by steam reforming of liquefied natural gas (LNG) *International Journal of Hydrogen Energy*, *35/22*, 12174-12181.

Shırasaki Y., Tsuneki T., Ota Y., Yasuda I., Tachibana S., Nakajima H., Kobayashi K. (2009). Development of membrane reformer system for highly efficient hydrogen production from natural gas. *International Journal of Hydrogen Energy*, *34/10*, 4482-4487.

Sivaramakrishna D., Sreekanth D., Himabindu V. & Anjaneyulu Y., (2009). Biological hydrogen production from probiotic wastewater as substrate by

selectively enriched anaerobic mixed microflora. *Renewable Energy*, *34*, 937–940.

Skjånes K., Knutsen G., Källqvist T., Lindblad P. (2008). H_2 production from marine and freshwater species of green algae during sulfur deprivation and considerations for bioreactor design. *International Journal of Hydrogen Energy*, *33/ 2*, 511-521.

Song L.J., Zhu N.W., Yuan H.P., Hong Y., Ding J. (2010). Enhancement of waste activated sludge aerobic digestion by electrochemical pre-treatment.*Water Research*, *44/ 15*, 4371-4378.

Thong S., Prasertsan P., Intrasungkha N., Dhamwichukorn S. & Birkeland N., (2008). Optimization of simultaneous thermophilic fermentative hydrogen production and COD reduction from palm oil mill effluent by Thermoanaerobacterium-rich sludge. *Int J Hydrogen Energy*, *33*, 1221–1231.

Thungklın P., Reungsang A., Sittijunda S. (2010). Hydrogen production from sludge of poultry slaughterhouse wastewater treatment plant pretreated with microwave. *International Journal of Hydrogen Energy*, In Press, Corrected Proof, Available online.

Tıng, C.H, Lee, D.J. (2007). Production of hydrogen and methane from wastewater sludge using anaerobic fermentation. *International Journal of Hydrogen Energy*, *32/ 6*, 677-682.

Tony M.A., Zhao Y.Q., Fu J.F., Tayeb A.M. (2008). Conditioning of aluminium-based water treatment sludge with Fenton's reagent: Effectiveness and optimising study to improve dewaterability. *Chemosphere*, *72/ 4*, 673-677.

Turner , J., Sverdrup, G., Mann, M., Maness, P., Kroposki, B., Ghiardi, M., Evans, R. and Blake, D. (2007). Renewable hydorgen production. *Int. Journal of Energy Research, 32/5, 379-407*

Umesh R., Wrana N., Cicek N., Sparling R., Levin D.B. (2008). Hydrogen production and end-product synthesis patterns by Clostridium termitidis strain

CT1112 in batch fermentation cultures with cellobiose or α-cellulose. *International Journal of Hydrogen Energy*, *33/ 23*, 7006-7012

Valdez-Vazquez, I. and Poggi- Varaldo, H. M. (2008). Hydrogen production by fermentative consortia. *Renewable and Sustainable Energy Rewiews*

Wang CC, Chang CW, Chu CP, Lee DJ, Chang BV, Liao CS, Tay, JH. (2003-b). Using filtrate of waste biosolids to effectively produce bio-hydrogen by anaerobic fermentation. *Water Res*., *37*, 2789-2793.

Wang CC, Chang CW, Chu CP, Lee DJ, Chang BV, Liao CS. (2003-a). Producing hydrogen from wastewater sludge by Clostridum bifermentans. *J Biotechnol*., *102*, 83-92.

Wang X., Qiu Z., Lu S., Ying W.(2010). Characteristics of organic, nitrogen and phosphorus species released from ultrasonic treatment of waste activated sludge .*Journal of Hazardous Materials*, *176/ 1-3*, 35-40.

Wınter C.J. (2005). Into the hydrogen energy economy milestones *International Journal of Hydrogen Energy*, *30*, 7, 681-685.

Xiao B., Liu J.,(2008). Biological hydrogen production from sterilized sewage sludge by anaerobic self-fermentation. *Int Journal Hazardous Materials*, *168*, 163–167.

Yokoi, H., Saitsu, A.S., Uchida, H., Hirose, J., Hayashi, S. ve Takasaki, Y. (2001). Microbial hydrogen production from sweet potato starch residue, *J Bioscience Bioeng*., *91*, 58- 63.

Yokoi, H., Tokushige, T., Hirose, J., Hayashi, S. ve Takasaki, Y. (1998). H_2 production from starch by mixed culture of Clostridium buytricum and Enterobacter aerogenes, *Biotechnol. Letters*., *20*, 143-7.

Yu C.Y., Lee D.W., Park S.J., Lee K.Y. Lee K.H. (2009). Study on a catalytic membrane reactor for hydrogen production from ethanol steam reforming *International Journal of Hydrogen Energy*, *34/7*, 2947-2954.

Zhu H., Parker W., Basnar R., Proracki A., Falletta P., Béland M., Seto P. (2008) Biohydrogen production by anaerobic co-digestion of municipal food waste and sewage sludges *International Journal of Hydrogen Energy*, *33/14*, 3651-3659.

ABBREVIATIONS

ATP	Adenosin triphosphate
BESA	Bromoethenesulfonic acid
C/N	Carbon to nitrogen ratio
CHV	Cumulative hydrogen volume
COD	Chemical oxygen demand
GC	Gas choromatograph
HPLC	High performance liquid chromatograph
HPR	Hydrogen production rate
ORP	Oxidation reduction potential
P	Maximum hydrogen formation potential
PS	Primary sludge
Rm	Maximum hydrogen formation rate
SCOD	Soluble chemical oxygen demand
SHPR	Specific hydrogen production rate
SS	Suspended solid
TCOD	Total chemical oxygen demand
TOC	Total organic carbon
TS	Total sugar
TVFA	Total volatile fatty acid
VFA	Volatile fatty acid
VS	Volatile solid
WAS	Biological sewage
WW	Wastewater