ORGANIC ACIDS PRODUCTION FROM CHEESE-WHEY

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ABSTRACT

ORGANIC ACIDS PRODUCTION FROM CHEESE-WHEY

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In this study, production of organic acids from cheese-whey was studied. Optimization of organic acids production was performed in semi-batch and batch reactors. Two sets of experiments were performed. First set of experiments were performed in semi-batch reactors for the optimization of organic loading rate (OLR) and hydraulic retention time (HRT). As a result of Set 1 experiments optimum OLR was found to be 15 g COD l^{-1}. Second set of experiments were performed in batch reactors by using the optimum OLR found in Set 1 experiments. Set 2 experiments were conducted to study the effect of using different seed cultures and Basal Media (BM) on Volatile fatty avid (VFA) production. Main acidogenesis products were acetic acid (Hac), butyric acid (Buty) and propionic acid (HPr) with smaller quantities of i-butyric acid (i-Buty), valeric acid (Val) and caproic acid (Cap). It was seen that BM had a suppressive effect on ethanol (EtOH) production while it stimulated the

VFA production. Higher VFA productions and variety of VFA types were observed in Test Reactors seeded with acidogenic culture (R3 and R6).

Key words: Cheese-whey, Volatile fatty acids, Acetic acid, Butyric acid

ÖZ

PEYNİR ALTI SUYUNDAN ORGANİK ASİT ÜRETİMİ

Türkmenoğlu, Seçil
Yüksek Lisans Tezi, Çevre Mühendisliği Bölümü
Tez Danışmanı: Prof. Dr. Göksel N. Demirer

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Bu çalışmada, peynir-altı suyundan organik asit üretimi araştırılmıştır. Organik asit üretimleri yarı-kesikli ve kesikli reaktörlerde uygulanmıştır. Çalışmalar sırasında iki set deney düzeneği kullanılmıştır. İlk set yarı-kesikli reaktörlerde gerçekleştirilmiş ve organik yükleme hızı (OYH) ve hidrolik bekleme süresi (HBS) optimizasyonu yapılmıştır. Birinci set deneyler sonucunda 15 g COD l^{-1} OYH optimum yükleme hızı olarak belirlenmiştir. İkinci set kesikli reaktörlerde, birinci sette elde edilen OYH kullanilarak gerçekleştirilmiştir. İkinci set deneyleri gerçekleştirmenin amacı farklı aşı kültürlerinin ve Besi Kültürünün (BK) uçucu yağ asidi (UYA) üretimine etkilerini araştırmaktı. Ana asidojenesis ürünleri asetik asit (HAc), butrik asit (Buty) ve propionik asit (HPr) iken, az miktarlarda i-butrik asit (i-Buty), valerik asit (Val) ve kaproik asit (Cap) üretimi de gözlemlendi. BK'nin etanol (EtOH) üretimi üzerinde bastırıcı bir etkisi olduğu, ancak UYA üretimini tetiklediği gözlemlendi.

Asidojenik kültür içeren reaktörlerde (R3 ve R6) daha yüksek miktarlarda ve çeşitlilikte UYA üretimi olduğu gözlemlendi.

Anahtar Kelimeler: Peynir-altı, Uçucu yağ asitleri, Asetik asit, Butrik asit

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ABBREVIATIONS

AC	:	Acidogenic Culture
Buty	:	Butyric Acid
Cap	:	Caproic Acid
COD	:	Chemical Oxygen Demand
EtOH	:	Ethanol
HAc	:	Acetic Acid
HMAC	:	Heated Mixed Anaerobic Culture
HPr	:	Propionic Acid
HRT	:	Hydraulic Retention Time
MAC	:	Mixed Anaerobic Culture
OLR	:	Organic Loading Rate
Val	:	Valeric Acid
VFA	:	Volatile Fatty Acids

CHAPTER 1

INTRODUCTION

The recent advances in biotechnology and engineering fields have made a set of new products which are coherent with environmental values and can be produced from agricultural and other renewable resources. In the framework of getting freed from petroleum dependency, the fact that many petroleum-based products can be replaced with their renewable counterparts has placed the bio-based products in the research priorities of not only developed but also developing countries. It has been demonstrated that renewable/clean energy, different industrial chemicals, and other value-added products can be produced from different biomass sources including wastes.

This approach considers wastes not only in terms of their treatment/disposal, but also as a valuable resource for energy production and bio-product formation. The uncontrolled disposal of the municipal and agro-industrial wastes and wastewaters not only results in significant environmental and public health problems such as global warming, acidification, oxygen depletion, eutrophication, odor, etc. but should also be regarded as an economical loss. The conversion of these wastes and wastewaters into industrial chemicals will reduce our foreign dependency, lead to important economical and ecological gains, refresh rural economies through new perspectives and investments, create new employment opportunities, and make Turkey comply with international environmental agreements easier (Klass, 1998, Johnson, 2000, van Wyk, 2001, US BRDB, 2001, NBCO, 2002, CARC, 2003).

According to DPT statistics cheese production in Turkey in 1998 was 313,370 tonnes (DPT, 2001). 90% of the 1 kg milk used in the production of cheese, results in cheese-whey production (Zall, 1979). As a consequence, wastewater produced during cheese manufacture (cheese-whey) in Turkey can be calculated as 2,820,330 tonnes (Demirer et al., 2000). Cheese-whey can be used as an organic acids (Volatile fatty acids – VFA-) source. Turkey has spent 7,160,930 US dollars for importing some common VFAs; namely, acetic acid (HAc), butyric acid (Buty) and propionic acid (HPr), in 2004 (TİK, 2006). Taking all these advances and facts into consideration, production of these acids within the country will reduce our foreign dependency and will lead to important economical gains.

1.1. Aim and Scope of the Study

- The general objective of this study was to investigate the organic acids production potential of cheese-whey by anaerobic acidogenesis, which is produced and disposed in large quantities.

- The second aim of the study was to determine the optimum organic loading rate (ORL) and seed culture for maximum VFA production from cheese-whey. Three different seed types (mixed anaerobic seed with inhibitor, heated mixed anaerobic seed and acidogenic seed culture) were used to investigate the VFA production levels achieved with using these culture types. In addition to these, effect of BM on VFA production was also studied.

CHAPTER 2

THEORETICAL BACKGROUND

A literature survey on manufacture, characteristics, utilization, and treatment of cheese-whey and its acidification products is presented in this chapter.

2.1. Cheese Production and General Characteristics and Utilization of Cheese-Whey

In the following sections brief description of cheese manufacturing processes and the general characteristics and possible utilization methods will be discussed.

2.1.1. Cheese Manufacturing Process

Cheese making is a linear process; however, it involves many factors. There are many types of cheese and many subtle differences of processing methods In general, the production scheme of cheese manufacturing include the following steps; production of a coagulum through the action of rennet and/or lactic acid, separation of the resulting curds from the whey and manipulation of the curds to produce the desired characteristics of the cheese (EPA, 2000). A flowchart of cheese manufacturing and the waste flows from each manufacturing step are presented in Figure 2.1.

Cheese manufacturing consists of seven main steps (Figure 2.1). Firstly, starter cultures are added to the milk to produce lactic acid. The rennet is then used to

coagulate the milk protein. The curds and whey are separated and the curds washed and cut into cubes. Texturisation of the cheese involves compressing and stretching the curds and can be carried out in tower systems. The curd blocks are milled, salt is added, and the curds are pressed. Pressed cheese is wrapped to protect it against moisture loss and mould growth during storage. Cheese is matured to develop flavor and texture in temperature and humidity controlled stores, with regular turning and salting or brine washing of the cheese surface (EPA, 2000).

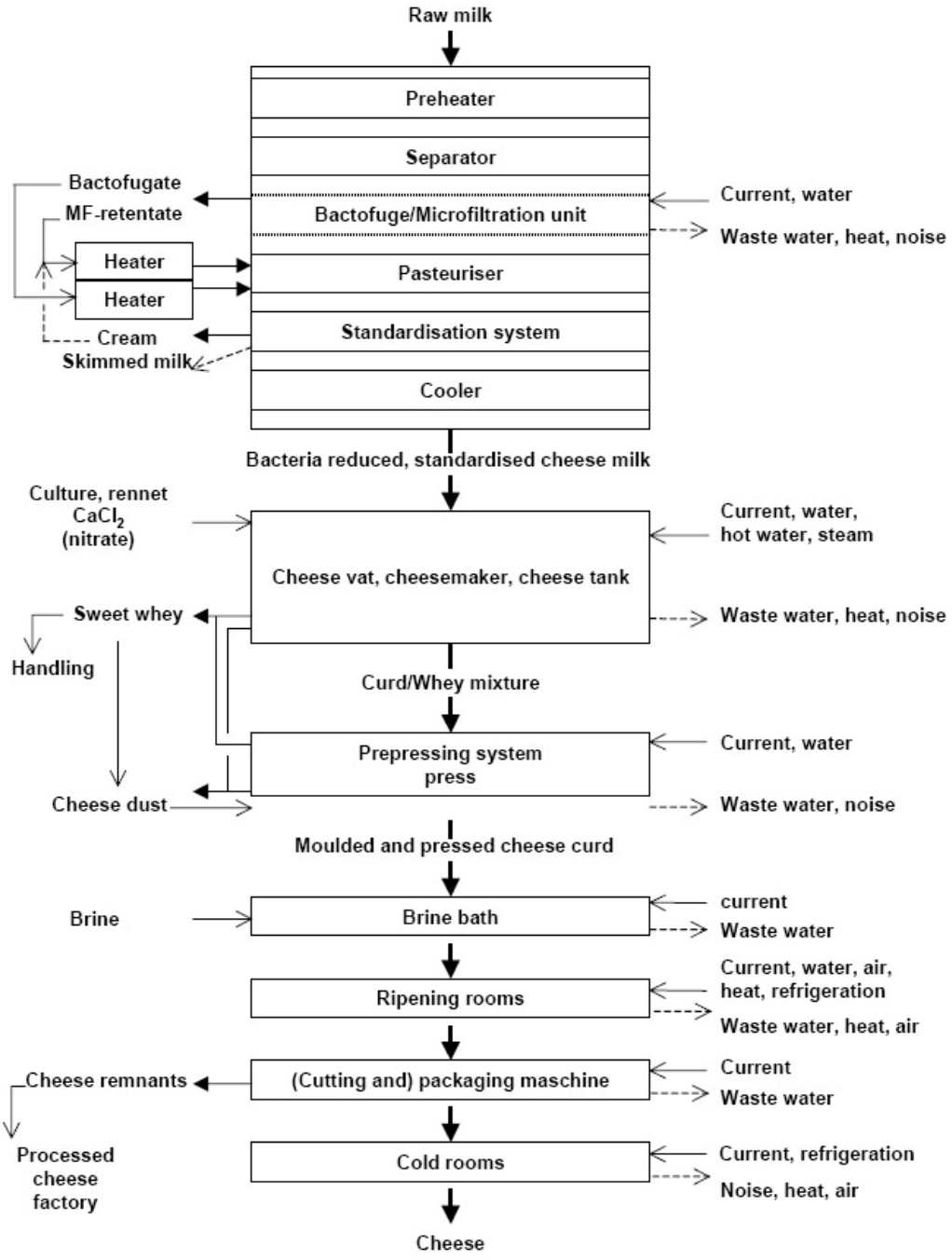

Figure 2.1. Process diagram for cheese manufacture (European IPPC Bureau, 2003).

2.1.2. Characteristics and Composition of Cheese-Whey

Whey is the basic by-product of cheese manufacturing. It is the liquid remaining after the precipitation and removal of milk casein during cheese manufacturing and although there are hundreds of types of cheese, all cheese has to undergo the same basic processes, producing this liquid cloudy water, known as whey.

Cheese-whey represents about 85-95% of the milk volume and retains 55% of milk nutrients. The most abundant of these nutrients are lactose (4.5-5% w/v), soluble proteins (0.6-0.8% w/v), lipids (0.4-0.5% w/v) and mineral salts (8-10 w/v of dried extract). Cheese-whey salts include NaCl and KCl (more than 50%), calcium salts (primarily phosphate) and others. Besides those, cheese-whey also contains lactic (0.05% w/v) and citric acids, non-protein nitrogen compounds like urea and uric acid, B group vitamins and so on (Kosikowski and Wierzbicki, 1973; Coton, 1976; Kosikowski, 1979; Yves, 1979; Anon, 1983; Marwaha and Kennedy, 1988).

There are two main types of cheese-whey; acid and sweet. Acid whey has a pH less than 5 and sweet whey has a pH above 5 (6-7 mainly). The type of the whey produced depends on the procedure used for casein precipitation. Acid wheys have higher ash and lower protein contents than sweet wheys. Thus, their use in alimentation is more limited than that of sweet whey, because of their acidic flavour and high saline content (Weetal et al., 1974; Kosikowski, 1979; Mawson, 1994).

2.1.3. Cheese-Whey – Pollutant Characteristics

Cheese whey is a protein and lactose rich by-product of the cheese industry. It is very biodegradable (~99%) with very high organic content (~ 70 g COD/l) and low alkalinity content (Mawson, 1994).

To produce 1 kg of cheese about 9 kg of whey is generated (Kosikowski, 1979), and because of its low concentration of milk constituents (6-7 % dry matter), whey has commonly been considered a waste product (Sienkiewicz and Riedel, 1990).

The annual world cheese-whey production is increasing and new bio-productions are being sought through biotechnology in order to get full use of the whey produced (Siso, 1996). However, approximately half of the world cheese-whey production is not treated and is being discarded as effluent. Thus, cheese-whey represents an important environmental problem because of the high volumes produced and its high organic matter content, with lactose being largely responsible for the high BOD and COD (Marwaha and Kennedy, 1988; Gardner, 1989; Kemp and Quickenden, 1989; Mawson, 1994).

Cheese-whey utilization has been the subject of much research. BOD reductions of higher than 75%, with the concomitant production of biogas, ethanol, single cell protein or another marketable product, have been achieved (Siso, 1996).Thus, the half of the whey that was seen as a pollutant is now seen as a resource.

2.1.4. Cheese-Whey Utilization

About 50% of worldwide cheese-whey production is treated and transformed into various food products. About 45% of this amount is used directly in liquid form, 30% as powdered cheese-whey, 15% as lactose and its byproducts and the rest is as cheese-whey-protein concentrates. Due to its characteristics cheese-whey is a good source for many products. Many researches is still being conducted with cheese-whey to find new whey products (Marwaha and Kennedy, 1988).

Liquid cheese-whey can be supplied to farmers for either agricultural fertilizer or for supplying proteins and lactose for feeding farm animals. However, it must be noted that the transport of liquid whey is very expensive. Powdered cheese-whey is used in animal feeding and some smaller quantities are used in human foods as sweeteners. However, due to its excessive saline taste its utilization in human foods is not favored. Another possible utilization method of cheese-whey is using it as protein source. It can be converted into whey protein concentrate (WPC) and used as food additive. Whey proteins are also been recently used in the production of iron propionate, an antianaemic preparation. During the manufacture of whey-protein concentrates, permeate with a high lactose content is formed as a byproduct. Mawson (1994) suggested that it is very important to take into account that protein recovery does not solve the BOD problem.

Moreover, cheese-whey can be converted to lactose and used as a supplement in baby milks or pill tablets. Since the amount of purified lactose that is produced worldwide require the use of only 5% of the whey available, some

other utilization methods are being sought. The fermentation of lactose to ethanol has received wide attention nowadays but it is not economically feasible since only low levels of ethanol can be obtained and the distillation process is expensive. Anaerobic digestion producing methane to use as a direct energy source has been employed in industrial waste treatment. Several kinds of digesters and several large scale plants have been established achieving more than 95% COD removal efficiencies. However, the effluents from the anaerobic reactors are generally not suitable for stream discharges. Therefore, some secondary aerobic polishing steps are usually required (Coton, 1976; Kosikowski, 1979; Yves, 1979; Evans and Gordon, 1980; Anon, 1983; Moulin and Galzy, 1984; Marwaha and Kennedy, 1988; Gardner, 1989; Kemp and Quickenden, 1989; Sienkiewicz and Riedel, 1990; Castillo, 1990; Dalev, 1994; Mawson, 1994).

Other whey fermentation pathways provide for the production of materials for chemical, food and textile industries and medical sectors as well as alternative energy sources. In addition to these, some other bioproducts can be produced from cheese-whey, such as several organic acids with food uses (HAc, HPr, lactic, lactobionic, citric, gluconic, and itaconic) (Blanc and Goma, 1989; Nielsen et al., 1990; Fairbrother et al., 1991; Roukas and Kotzekidou, 1991; Zayed and Zahran, 1991; Colomban et al., 1993; Norton et al., 1994), vitamins (B_{12} and B_2) and amino acids (glutamic, lysine, threonine) (Sienkiewicz and Riedel, 1990; Hobman, 1984; Nielsen et al., 1990; Fournier et al., 1993).

2.2. Anaerobic Treatment of Cheese-Whey

In the following sections brief information about anaerobic degradation and anaerobic treatment of cheese-whey will be given.

2.2.1. Anaerobic Digestion Principles

Anaerobic digestion is a biological process naturally occuring in environments with limited or no oxygen. For a long time anaerobic digestion has been used by farmind communities to process cattle slurries into a soil enchanter. However, with better capturing of byproducts, the process is now being used in many sectors of waste industry.

Anaerobic digestion has been used for over 100 years to stabilize municipal sewage and a wide variety of industrial wastes. Increasing environmental pressures on waste disposal has increased the use of anaerobic digestion as a process for reducing waste volumes and generating useful byproducts. Many municipal wastewater treatment plants use anaerobic digestion to convert waste solids to gas.

The anaerobic process removes a wide majority of the odorous compounds. It also significantly reduces the pathogens present in the slurry. Over the past 25 years, anaerobic digestion processes have been developed and applied to a wide range of industrial and agricultural wastes. It is the preferred waste treatment process since it produces, rather than consumes, energy and can be carried out in relatively small, enclosed tanks. The products of anaerobic digestion have value and can be sold to offset treatment costs (Roos, 1991; Lusk, 1995; Speece, 1996; Ghosh 1997; Wilkie, 2000).

2.2.1.1. Stages of Anaerobic Digestion

The digestion of the organic material is done by a range of many different species of naturally occurring bacteria. Each type is responsible of a different

duty in different stages of the digestion process. In general, there are four distinguished metabolic stages of anaerobic digestion of a biowaste; Hydrolysis, acidogenesis, acetogenesis and methanogenesis (Figure 2.2).

1. Hydrolysis – Complex insoluble organic material is solubilised by enzymes excreted by hydrolytic microorganisms.

2. Acidogenesis – soluble organic compounds including the products of hydrolysis are converted into organic acids, alcohols, hydrogen and carbondioxide.

3. Acetogenesis – the products of the acidogenesis are converted into HAc, hydrogen and carbondioxide.

4. Methanogenesis – methane is produced from HAc, hydrogen and carbondioxide as well as directly from other substrates of which formic acid and methanol are the most important (Finstein et al., 2004).

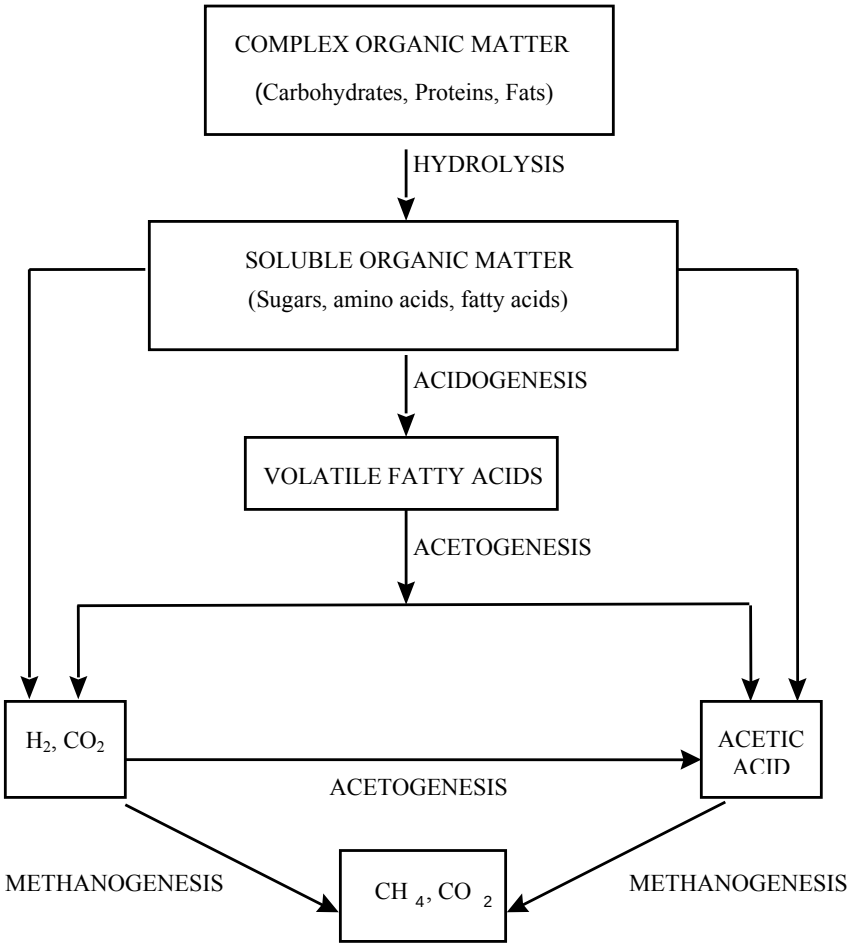

Figure 2.2. Four metabolic stages of anaerobic digestion (Finstein et al., 2004).

The efficient anaerobic degradation of organic matters is dependent upon the coordinated metabolisms of acid-forming and methane-forming bacteria. Imbalances in the metabolic rates of these two bacterial groups have largely been responsible for the instabilities associated with anaerobic digestion. These imbalances can lead to the accumulation of intermediatery acid products which

will eventually cause the inhibition of methanogenic bacteria (Veeken et al., 2000).

2.2.2. Anaerobic Treatment of Cheese Whey

Research on the anaerobic treatment of raw cheese whey started in 1990 with the objective of developing a technology suitable for medium size cheese factories that have growing disposal problems and cannot afford high investment costs for whey valorization technologies (such as whey protein and lactose recovery, spray drying, etc.) (Malaspina et al., 2000). The studies done on treatment of cheese-whey are depicted on Table 2.1.

Extremely high organic content of whey renders the application of conventional aerobic biological treatment mainly due to the cost of oxygen supplementation. Anaerobic treatment does not require any oxygen supplementation and generates significant amount of energy in the form of methane gas.

Raw whey is a quite problematic substrate to treat anaerobically because of the lack of alkalinity, the high COD concentration, the tendency to acidify very rapidly, the difficulty to obtain granulation and the tendency to produce an excess of viscous exopolymeric materials of probable bacterial origin that severely reduces sludge settleability and can be a cause of biomass washout (Malaspina et al., 1995). As can be seen from Table 2.1 most of the studies on anaerobic treatment of cheese-whey dealt with diluted or de-proteinized whey, which is much easier to treat.

The majority of the difficulties in the treatment of cheese-whey arise from its tendency to acidify rapidly. It was reported that a 500 l pilot scale fixed film

reactor receiving raw whey needed addition of NaOH for pH control (Marshal and Timbers, 1982). Moreover, Norstendt and Thomas (1994) observed that without pH control, an anaerobic fixed bed reactor could not achieve stable operation within 30 days. Furthermore, Lo and Liao (1986) observed that anaerobic rotating biological contact reactor fed with cheese whey was not able to sustain a stable operation at hydraulic retention times shorter than 5 days.

It was reported that the cheese-whey concentrations between 25-30 g COD/l were optimal at HRT of 5 days for a stable operation of UASB reactor, while at the influent concentrations of 38.1 g COD/l, an instability of the reactor was observed which is interpreted as the accumulation of volatile fatty acids in the acidogenic stage (Yan et al., 1993). Similar findings were also reported by other authors (Switzenbaum and Danskin, 1982).

Table 2.1. Anaerobic treatment studies on cheese-whey.

Reactor Type	HRT (days)	Initial COD Conc. (g l^{-1})	Loading rate (g COD l^{-1} day^{-1})	RE (%)	Ref.
Fluidized-bed reactor	0.4	7	7.7	90	Boening et al, 1982
Anaerobic attached-film expanded bed reactor	0.6-0.7	5-15	8.2-22	61-92	Switzenbaum et al, 1982
Downflow stationary fixed-bed reactor	5	13	2.6	88	De Haast et al, 1985
Upflow fixed-film loop reactor	5	79	14	95	Wildenauer et al, 1985
Semicontinuous digester with flocculant addition		69,8	16.1	99	Barford et al, 1986
Fluidized-bed reactor	0.1-0.4	0,8-10	6-40	63-87	Denac et al, 1988
UASB	1.5	11	7.1	94	Schroder et al, 1989
UASB	5	5-28,7	0.9-6	97-99	Yan et al, 1989
Anaerobic Filter	4		8.3	85	Viraraghavan et al, 1990
Anaerobic Filter	4			78-92	Viraraghavan et al, 1991
UASB			31	90	Rico Gutierrez et al, 1991
Downflow fixed film	4.9	61	13	75	van den Berg and
	6.6	61	8.3	76	Kennedy, 1992
Rotating biological contact reactor	3			85	Mawson, 1994
Downflow-upflow hybrid reactor	7	68	10	97	Malaspina et al, 1995
Anaerobic pond	8	4,4	0.55	63	Monroy et al, 1995

HRT: Hydraulic retention time; COD: Chemical oxygen demand; RE: Removal efficiency; UASB: Upflow anaerobic sludge blanket

Table 2.1. (Continued) Anaerobic treatment studies on cheese whey.

Reactor Type	HRT (days)	Initial COD Concentration (g l^{-1})	Loading rate (g COD l^{-1} day^{-1})	RE (%)	Ref.
Hybrid	2		Up to 11	>95	Strydom et al, 1995
Two-stage unmixed anaerobic digester	10	69,6	7	32.5	Ghaly, 1996
	20	69,6	3.5	39.5	
UASB	2.3-11.6	5-77	1-28.5	95-99	Kalyuzhnyi et al, 1997
UASB	5.4-6.8	47-55	7-9.5	90-94	Kalyuzhnyi et al, 1997
Upflow anaerobic solid removal reactor	4.5			98 (lipid)	Patel and Madamwar, 1997
Multichamber bioreactor	2			83	Patel and Madamwar, 1998
Batch	-	5.5, 11, 22.1	-	>90	Ergüder et al, 2000
UASB	2.06-4.95	42,7-55,1	10,4 - 24,6	95-97	

HRT: Hydraulic retention time; COD: Chemical oxygen demand; RE: Removal efficiency; UASB: Upflow anaerobic sludge blanket

2.3. Organic Acids Production from Municipal and Industrial Wastewaters

Methane is the final stable product of anaerobic degradation. It is a useful product for energy generation. However, there are some other valuable products which can compete with methane and has a market for itself, like organic acids.

Organic acids are the intermediate products of anaerobic digestion. Hydrolysis and acidogenesis are the first steps in the anaerobic digestion of complex organic materials when they are degraded into methane and carbondioxide. These steps involve the conversion of the polymers present in the organic matter into soluble monomers, which are quickly fermented into volatile fatty acids (VFA), hydrogen and carbondioxide by the rapidly growing and pH-insensitive acidogenic bacteria.

2.3.1. Anaerobic Acidogenesis

Anaerobic acidogenesis is known as the first step in the anaerobic digestion of soluble organic materials to methane and CO_2. Many kinds of bacteria are involved in the acidogenesis and subsequently many kinds of organic acids and alcohols are usually produced.

There are three main acidogenic fermentation pathways through butyrate, propionate and ethanol. Butyrate fermentation is characterized by the production of butyrate and acetate, plus carbondioxide and hydrogen. Propionate fermentation, on the other hand, produces propionate, acetate and some valerate, with no significant gas production. Ethanol fermentation occurs only at low pH of 4.5, producing ethanol, acetate, hydrogen and cabondioxide (Cohen et al., 1984; Ren et al., 1995).

Although the proper operational conditions for the acetogens/methanogens have been extensively studied, little information is available for the acidogenic phase. It is known that product formation by a mixed acidogenic population is a very complex process and is greatly influenced by many factors: wastewater stability, reactor configuration, HRT, influent organic concentration, OLR, pH, temperature, oxidation-reduction potential and nutritional requirements.

The composition of organic acids in the medium influences the quality of the products of fermentation. Thus, it is important to control the product spectrum during anaerobic acidogenesis.

2.3.1.1. Effect of pH on Anaerobic Acidogenesis

pH is one of the major conditions effecting the product formation in anaerobic acidogenesis (Zoetemeyer et al., 1982). However, there are only few studies and little information available on the effect of pH on anaerobic acidogenesis. pH conditions of the system not only influence the product formation but also the product spectrum.

Houriuchi et al. (2002) observed that, under the conditions of pH from 5-7, the main soluble products were Buty and HAc, while the HPr concentration was rather low, in chemostat cultures supplemented with glucose. The main products at pH 8 were HAc and HPr. On the other hand, ethanol concentration was relatively low for all cases. They found that the hyper production of Buty observed at low pH was caused by the high hydrogen content (Buty works as a hydrogen acceptor). Moreover, the reduction of hydrogen production in the acid reactor at pH 8, caused a change in the organic products in the acid reactor. They observed that the molecular hydrogen produced during the production of

HAc and Buty from glucose, was consumed during the production of HPr. Thus, at pH 8, HPr concentration in the acid reactor remarkably increased, resulting in a lower production of hydrogen. However, although the hydrogen content in the reactor was the key factor for regulating the acidogenesis, their results suggested that the microbial population in the acid reactor depended on the culture pH rather than the partial hydrogen pressure. Furthermore, Horiuchi et al. (2002) found that the change in the product formation occurred by the change of the dominant microbial populations in the acid reactor. 120-150 h was found to be enough time to change the dominant microbial populations in the acid reactor. The change in the dominant population occurred because the optimal pH was different for the bacterial groups producing each organic acid. It was found that the shift in products was reproducible and reversible, and was not affected by the dilution rate, and pH control was effective for selective production of various organic acids from organic wastes.

Similarly, Yu and Fang (2003) observed that pH had a more significant effect on acidogenesis than that of temperature. They found that gelatin degradation efficiency substantially increased with pH between 4-7, however, the degree of acidification increased between 4-6.5 but dropped when pH increased to 7. They found that the optimum pH for the overall acidogenic activity was 6. Moreover, they indicated that operation at pH of 4-5 favored the production of propionate and hydrogen, whereas, the operation at pH 6-7 encouraged the production of acetate, butyrate and i-butyrate.

Furthermore, the optimum pH for lactose acidogenesis with respect to VFA distribution was found to lie in the range of 6-6.5 with acetate and butyrate being the major and ethanol, propionate and caproate being the minor products of acidogenesis of lactose (Kissalita et al., 1987).

Dinamarca et al. (2003) found that during the anaerobic acidogenesis of the organic fraction of urban solid waste, it was not necessary to control the pH since the presence of proteins and other compounds provided adequate buffering capacity and that the pH control was thus, not necessary for those type of residues. The pH of the system fluctuated between 6.5-8.2, in the non-pH-controlled reactor.

In another study, done with swine wastewater, the optimum conditions for maximum HAc and Buty production were determined as pH 5.9 and 0.88 days HRT (Hwang et al., 2001).

2.3.1.2. Effect of Temperature on Anaerobic Acidogenesis

Temperature is one of the important parameters affecting the acidogenic activity. Some studies on acidogenesis with respect to the change in temperature have been carried out. However, the temperature effect studies have been focused on overall anaerobic degradation process or methanogenesis, rather than anaerobic acidogenesis.

According to Yu and Fang (2003) lowering operational temperature generally lead to a decrease in the maximum specific growth and substrate utilization rate and that the methanogenic sludge yield decreased with decreasing temperature. They observed that gelatin degradation, efficiency and rate, degree of acidification, and formation rate of volatile fatty acids (VFAs) and alcohols all slightly increased with temperature (Table 2.2). In another study done with solid vegetable wastes to compare the acidogenic fermentation yields in mesophilic and thermophilic conditions, Verrier et al. (1987) obtained higher

yields at 60°C than 35°C and VFA production favored the production of HAc and Buty rather than HPr and Val at 60°C.

Table 2.2. Distribution of VFA and alcohols at various temperatures (Yu and Fang, 2003)

Temp (°C)	HFr	HAc	HPr	HBu	i-HBu	HVa	i-HVa	HCa	Mol	Eol
20	3.2	19.8	18.2	10.3	13.4	10.7	12.3	8.3	0	4.3
25	1.3	22.9	17.3	11.6	15.5	9.7	8.7	8.2	0	5.4
30	1.4	26.1	14.6	11.6	12.7	10.9	11	9.1	0	2.3
37	2	25.4	12.3	12.5	13.4	11.8	12.5	7.3	1.3	3.2
45	0	27.3	13.9	10.7	11.5	11.9	9.6	9.3	2.2	3.3
50	0	23.8	13.3	12.8	10.4	10.6	14.5	9.1	0	2
55	0	21.6	15	14.8	13.3	13.8	9.4	8.5	2.2	3.3

Eol: ethanol; HAc: acetate; HBu: butyrate; HCa: caproate; HFr: formate; HPr: propionate; HVa: valerate; i-HBu: i-butyrate; i-HVa: i-valerate; Mol: methanol; Concentrations as %,

Single volatile fatty acid production from organic urban wastes in mesophilic conditions favored the accumulation of HAc and Buty, while the HPr and Val were produced in minor quantities (Sans et al., 1995). They also found that under mesophilic conditions, the amount of VFA generated and yields tended to increase with increasing retention time in the range between 8 h and 6 days (from 9 g/l up to 23 g/l) with no need of utilizing inoculum. However, in thermophilic temperature conditions and working in the same range of retention times, the addition of inoculum was found to be necessary for optimum VFA production.

Among the four different temperatures studied (26. 35, 37 and 40 ^{o}C), the production of VFAs from akalona, 37°C is found to yield higher amounts of VFAs at shorter time (nearly after 4 days) (Figure 2.3) (Mostafa, 1999).

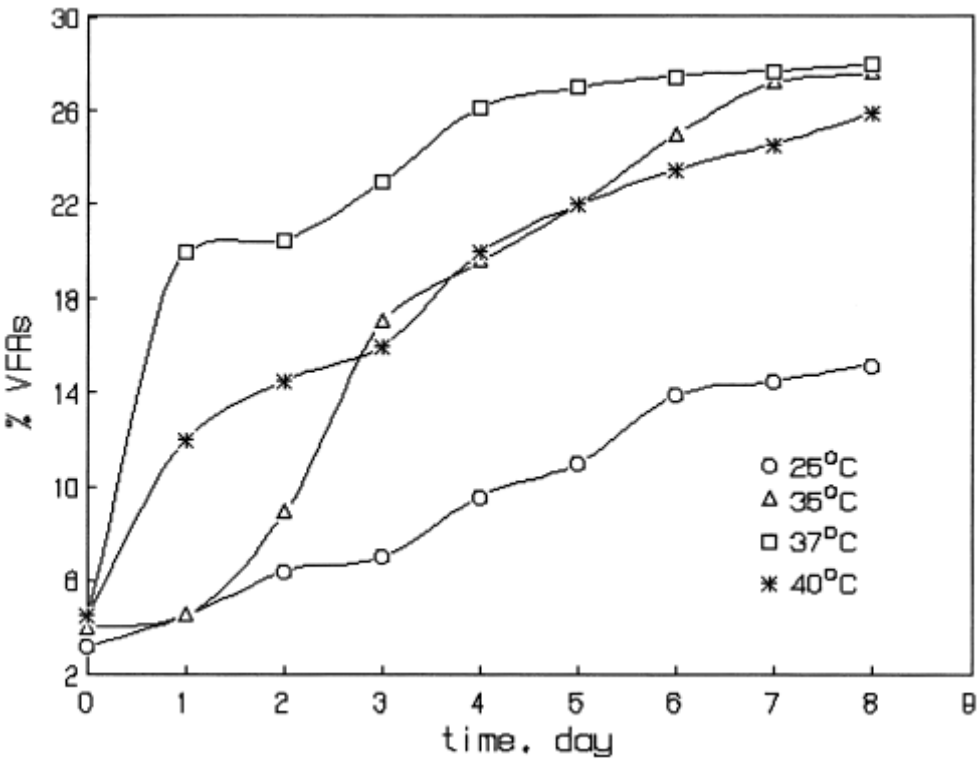

Figure 2.3. Effect of temperature on the production of VFAs from akalona (Mostafa, 1999).

Similarly, according to Maharaj and Elefsiniotis (2001) VFA production rate decreased with decreasing temperature. For the acidogenesis of municipal sludge and combined municipal sludge and starch rich industrial wastewater the optimum conditions appeared to be within the ambient temperature at 30 h

HRT values over the range investigated (18-60 h HRT, 8-35°C). However, despite the drop in lower temperatures, the stability of operation and the concentration levels produced indicate both feasibility as well as the potential of the process at low temperatures.

Furthermore, in a study done with fish meal processing factory wastewater, maximum acidification efficiencies values of 44 and 23% at 55 and 37°C, respectively, were observed (Guerrero et al., 1999).

Cha and Noike (1997) found that the VFA producing bacteria were slightly affected by the temperature changes, whereas number of methanogens decreased significantly with the drop in temperature.

The studies on effect of temperature on anaerobic acidogenesis indicated that although it is possible to operate the system at lower temperatures, higher efficiencies can be obtained at higher temperatures. However, economic studies should be done to determine the relations of yields and energy expenses when working at mesophilic or thermophilic conditions (Sans et al., 1995).

2.3.1.3. Effect of Hydraulic Retention Time (HRT) on Anaerobic Acidogenesis

Another operational condition effecting anaerobic acidogenesis is the HRT and there has been several studies on that.

Kim et al. (2002) observed that VFA concentrations and distributions changed as a function of HRT, in their study done with a membrane coupled fermentor with coagulated raw sludge (Table 2.3). The VFA concentration of permeates revealed at a maximum value at HRT of 12 h. However, despite some

variations in the concentrations of the minor acids, the VFA production was not significantly affected by HRT within the tested ranges of 8, 12, 24, 48 and 96 hours (Table 2.3).

Similarly, Elefsiniotis and Olham (1994) reported that during the acidogenesis of primary sludge at ambient temperatures VFA concentrations increased with HRT up to 12 h and then decreased at an HRT of 15 h with an increase in gas production, indicating the stimulation of methanogenesis.
Sans et al. (1995) observed that when retention time was shorter, the variation of VFA concentrations was more obvious.

Table 2.3. VFAs concentration and distribution as a function of HRT (Kim et al, 2002).

VFAs (mg/l)	HRT 8 h	HRT 12 h	HRT 24 h	HRT 48 h	HRT 96 h
HAc	336	563	406	411	321
HPr	264	430	317	253	280
n-Buty	88	72	99	79	107
i-Buty	64	60	60	52	49
n-Val	24	48	69	44	50
i-Val	25	24	40	35	16
Total	801	1197	991	874	823

Individual VFA concentrations are calculated in carbon base
HAc: Acetic acid; HPr: Propionic acid; n-Buty: n-Butyric acid;
i-Buty: i-Butyric acid; n-Val:n-Valeric acid; i-Val:i-Valeric acid

The studies on HRT showed that lower values favored the production of VFAs while higher values stimulated methanogenic activities and that the VFA

concentration and distribution were affected by HRT changes. However, these changes were not significant.

2.3.1.4. Effect of Organic Loading Rate (OLR) on Anaerobic Acidogenesis

Effect of OLR on anaerobic acidogenesis has been the subject of some studies.

Parawia et al. (2004) observed that the concentrations of VFAs increased with increasing batch concentrations of potato solids in anaerobic reactors and they found that the concentration of the substrate had a considerable effect on the distribution of the acidification products. The concentrations of fermentation products after 300 h digestion of potato were chiefly: 420, 310, 140 and 90 mg g^{-1} of total VFAs for HAc, n-BA, HPr and CA, respectively, with lower amounts of i-BA, n-VA and i-VA when using 500 g potato waste. 410, 400, 110 and 40 mg g^{-1} of total VFAs for HAc, LA, n-BA and CA, respectively, with low amounts of HPr and no n-VA or i-VA when load was 1000g. Moreover, they observed that appearance sequences of VFAs changed slightly with waste loads. Especially the higher molecular weight acids appeared faster when load was lower.

In a study done with dairy wastewater at thermophilic conditions, it was observed that the degree of acidification decreased only slightly when the loading rate was increased (60.8% to 54.9 % when 4 to 8 g COD $l^{-1}day^{-1}$). On the other hand, the degree of acidification decreased drastically at higher loading rates. Only 27.1% of organic matter was acidified at 24 g COD $l^{-1}day^{-1}$ (Table 2.4) (Yu and Fang, 2000). Moreover, they found that OLR was critical to the distribution of VFA/alcohol in the effluent. While the percentage of acetate decreased with the increase in OLR that of propionate increased with

OLR and percentages of butyrate and ethanol were found to be not sensitive to the OLR. They observed that at OLRs less than 12 g COD $l^{-1}day^{-1}$ VFA and alcohols contributed the majority of the effluent COD (Table 2.4).

Similarly, Beccari et al. (1995) observed that the conversion yield to VFAs decreased as the olive oil mill effluent initial concentration increased in anaerobic batch reactors. They also found that the initial concentrations affected the VFA distribution. Lower initial concentration corresponded to a lower percentage of butyrate and higher concentration of acetate. Also, they found that acidogenic yield was less sensitive to the effect of an increase of the substrate concentration than metanogenesis.

To sum up, it was observed that OLR affected the distribution, concentration and production rates of VFAs and alcohols. The increase in OLR caused an increase in VFA concentrations due to the sensitivity of methanogens to high substrate concentrations (Beccari et al., 1995; Yu and Fang, 2000; Parawia et al., 2004).

Table 2.4. Concentrations and percentages of individual VFAs and alcohols in effluent of thermophilically acidified dairy wastewater in an upflow anaerobic reactor (Yu and Fang, 2000).

OLR (g COD l^{-1} day^{-1})	HFr	HAc	HPr	HBu	i-HBu	HVa	i-HVa	HCa	HLa	Mol	Eol	Pol	Bol
4	23	430	202	127	40	60	41	50	76	63	152	0	0
6	26	410	205	149	51	52	62	77	38	52	142	26	0
8	32	344	221	108	50	41	45	74	86	61	123	37	0
12	21	206	179	83	39	32	22	63	54	72	90	36	9
16	23	203	220	79	28	20	14	42	42	69	99	0	17
24	12	103	188	51	22	11	6	46	40	6	68	17	23

Bol: Butanol; Eol: ethanol; HAc: acetate; HBu: butyrate; HCa: caproate; HFr: formate; HLa: lactate; HPr: propionate; HVa: valerate; i-HBu: i-butyrate; i-HVa: i-valerate; Mol: methanol; Pol: propanol; all concentrations are in mg/l

2.3.1.5. Effect of Substrate Type on Anaerobic Acidogenesis

There have been several studies on anaerobic acidogenesis using different substrates. The variation and change of product spectrum can easily be seen when these studies are investigated.

In a study done on the anaerobic acidogenesis of dairy wastewater in thermophilic conditions acetate, propionate, butyrate and ethanol were the main products. The production of propionate was always higher than the production of butyrate, and hydrogen was always present in the biogas. They found that neither butyrate fermentation nor propionate fermentation was predominant in the reactor. Also, although ethanol was present in significant quantities in all runs, it was never a primary end-product. Therefore, they suggested that the three types of fermentation co-existed in the acidification reactor, probably due to the complex nature of the dairy wastewater, and the predominance of a fermentation pathway could be affected by OLR.

While the effects of combining various liquid wastes on the overall anaerobic digestion process have been explored, very little is known about the influence of combining agricultural with domestic wastewaters on the acid-phase step (Carrieri et al., 1993; Maharaj, 1999). It was observed that addition of potato-processing wastewater to primary sludge at 1:1 ratio improved VFA production at the conditions studied (18-30 h HRT and 22-30 ^{o}C) (Banerjee et al., 1998).

For municipal sludge and combined municipal-starch rich industrial wastewaters HAc was the dominant VFA produced followed by HPr. Higer concentrations of n-Buty was observed in combined municipal-industrial

reactor because of the increased carbohydrate concentration coming from starch industry wastewater.

Small amounts of i-Buty, i-Val and n-Val acids were also observed. With respect to both the HRT and temperature experiments, the starch rich industrial wastewater use appeared to facilitate the conversion of soluble organic compounds to VFAs, thereby augmenting the COD concentrations and production rates above municipal-only reactor. Highest VFA concentrations were achieved at an HRT of 30h and at 25°C in both reactors (Maharaj and Elefsiniotis, 2001).

In another study, optimum conditions for HAc production from starch-processing wastewater was determined as 0.56 day HRT, pH 5.9 and 36.1 ^{o}C by modeling and the experimental value at optimum conditions were found as 1681 ± 49 mg HAc/l (Ahn et al., 2004).

Parawia et al. (2004) found that HAc and HPr were most abundant VFAs of potato waste acidogenesis, followed by Buty, i-butyric, Val, i-Val and caproic acids. They observed that HAc, HPr, butyric and i-butyric formed directly from the fermentation of carbohydrates and proteins, as well as during the anaerobic oxidation of lipids. Furthermore, they observed that the high production of Buty was mainly attributed by the large amount of carbohydrates present in the substrate.

Kusel and Drake (1994) studied the acids production from soil from a beech forest, and they observed that it formed significant amounts of acetate when incubated in a bicarbonate-buffered mineral salt solution under anaerobic conditions at 5 and 20 ^{o}C.

Mostafa (1999) found that the maximum percent of VFAs were obtained (in batch fermenters) from akalona (28%) followed by whey (16.3%) and then by akalona hydrolyzate (13%). The higher concentrations of VFAs produced from solid akalona was due to it being free from toxic and undesirable substances such as the toxic compound in akalona hydrolyzate (due to acid hydrolysis) and the salt contained in whey which inhibit the growth of microorganisms and then reduce the rate and yield of VFAs production (Figure 2.4).

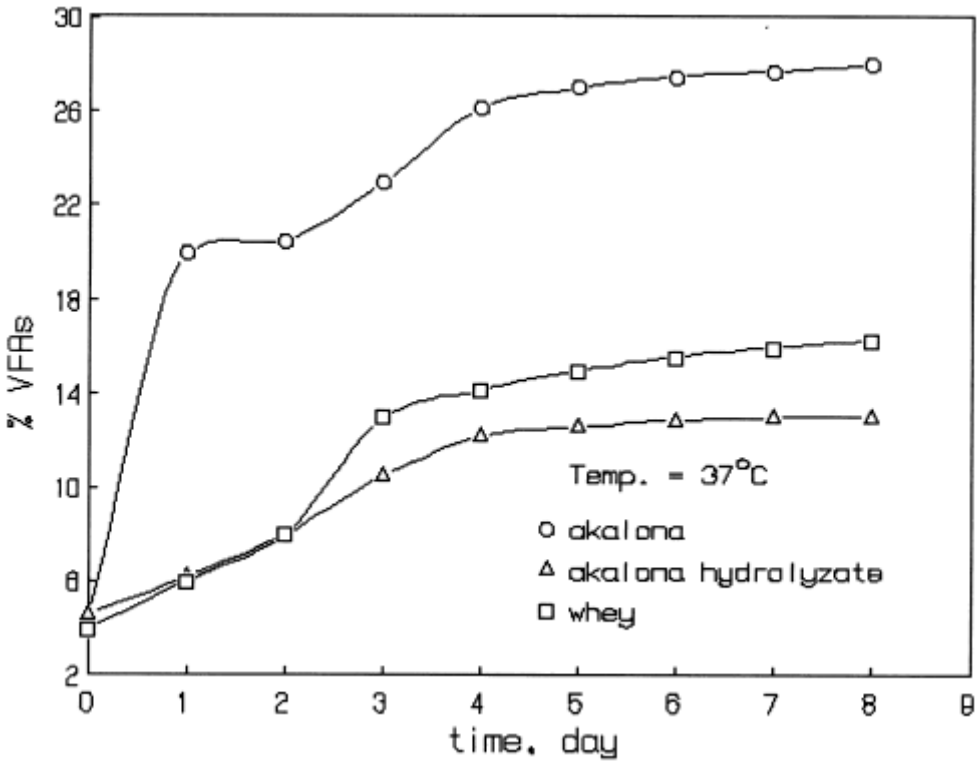

Figure 2.4. Effect of type of substrate on the production of VFAs (Mostafa, 1999).

C2 to C5 straight chain and branched VFAs were the main soluble compounds generated during the acidogenic fermentation of coagulated sludge in a membrane coupled reactor. The most abundant component of volatile fatty acids was HAc and others were produced in the following order: HPr, butyric and Val acid (Kim et al., 2002).

2.3.1.6. Effect of Reactor Type on Anaerobic Acidogenesis

The reactor type used in anaerobic acidogenesis of different substrates indirectly affects the product spectrum and concentrations. Since some reactor types are vulnerable to some operational conditions more than the others (ie. plugflow reactors being more sensitive to shock loads than continuously mixed reactors, due dilution and mixing conditions) the fermentation pathways and conditions affected. Sans et al. (1995) observed that retention time and temperature were two parameters which directly affected the acidogenic fermentation of organic solid wastes in plug-flow reactor. They found that operation of plug flow fermenters in mesophilic conditions would be suitable for slanting the VFA production towards HAc and Butys, for organic urban wastes. Moreover, they saw that the optimal working conditions for the acidogenic fermentation of solid organic wastes in a plug flow reactor included short retention times and recycling of part of the outlet sludge.

The anaerobic hydrolysis and acidification of wastewaters rich in organic suspended solid were studied in continuous stirred tank reactors using fish meal factory effluents. The effect of stirring was studied, and maximum efficiencies

of 44% acidification at 55°C and 24 h HRT were determined (Guerrero et al., 1999).

D'Addario et al. (1993) studied acidogenic digestion of municipal solid wastes in three different reactor types: Batch, semi-continuous stirred tank reactor (SCSTR) and multistage-counter flow reactor (MCFR). They found that on the basis of acid generation from organic fraction of municipal solid waste in batch reactors, after 12 days of operation, a TS concentration of 15% w/v under controlled pH of 6.5 gave better concentrations (23-24 g l^{-1}) and conversion yields (215 g VFA kg^{-1} volatile solids). However, since the conditions caused strong liquefaction of the feedstock, which resulted in highly turbid leachates with elevated content of microbial biomass and suspended material, MCFR system operating under uncontrolled pH conditions was found to be more feasible in spite of lower performances (13 g VFA l^{-1}, 152 g VFA kg^{-1} volatile solids).

2.4. VFA Utilization

As mentioned in the previous sections, different types of VFAs can be produced from different industrial and municipal wastewaters by anaerobic acidogenesis. VFAs can be used in denitrification, dephosphatation or methanisation. They are essential as energy and carbon sources for the microorganisms involved in the biological removal of nitrogens in wastewater treatment. It was reported by Barnard (1993) that 7-9 mg of VFAs is needed to remove 1 mg of phosphorus, while Oldham et al. (1994) have used VFAs to produce effluent phosphorus levels as low as 0.2-0.3 mg/l. VFAs can be produced on-site with low operational costs and no storage or handling problems (Elefsiniotis and Oldham, 1993). Moreover, VFAs produced by the degradation of organic

wastes can be used in the production of biodegradable plastics such as polylactate polymers, an environmentally friendly alternative to non-biodegradable plastics derived from petrochemicals (Chung et. al., 1997; Huang et al., 2003).

Most common VFAs that can be produced from many wastewaters are HAc, Buty and HPr. HAc is an important industrial chemical. As one of the most widely used organic acids, it is often used as a raw material to prepare other valuable products. The largest use of HAc is in the production of vinyl acetate monomer, which is applied in paints and adhesives, closely followed by acetic anhydride and ester production. Acetic anhydride is a strong acetylation agent. As such, its major application is for cellulose acetate, a synthetic textile also used for photographic film. Acetic anhydride is also a reagent for the production of aspirin, heroin, and other compounds. In the form of vinegar, HAc solutions are used directly as a condiment, and also in the pickling of vegetables and other foodstuffs. Furthermore, the major esters of HAc are commonly used solvents for inks, paints and coatings. Dilute solutions of HAc are also used for their mild acidity. Examples of household uses include the use in a stop bath during the development of photographic films, and in descaling agents to remove limescale from taps and kettles. Moreover, HAc is used as a spray-on preservative for livestock silage, to discourage bacterial and fungal growth (Wikipedia, 2006).

HAc production is mainly based on natural gas (Agreda and Zoeller, 1993). However, as a non-renewable resource, and due to current high rates of consumption, natural gas can hardly support the HAc industry. The global demand of HAc is around 6.5 million tonnes per year (Mt/a), of which approximately 1.5 Mt/a is met by recycling; the remainder is manufactured

from petrochemical feedstocks or from biological sources. HAc is produced both synthetically and by bacterial fermentation. Today, the biological fermentation accounts for only about 10% of world production. About 75% of HAc made for use in the chemical industry is made by methanol carbonylation (methanol and carbonmonoxide react to produce HAc). Alternative methods, such as oxidative and anaerobic fermentation, account for the rest (Wikipedia, 2006). As a promising alternative, the production of HAc using biomass materials recently gained more interest primarily attributed to its cost-effectiveness (Shi et al., 2005).

Buty is another common VFA which is a high volume chemical with production exceeding 1 million pounds annually in the U.S. Buty is used in the preparation of various butyrate esters. Low-molecular-weight esters of Buty, such as methyl butyrate, are generally used in food and perfume industry due its pleasant aroma and taste (Wikipedia, 2006).

HPr inhibits the growth of mold and some bacteria. Accordingly, it is mainly used in animal feeds and food for human consumption. For animal feed, it is used either directly or as its ammonium salt. In human foods, especially bread and other baked goods, it is used as its sodium or calcium salt. Also, HPr is useful chemical intermediate, used in pesticide production and in pharmaceuticals. The esters of HPr can also be used as solvents or artificial flavorings (Wikipedia, 2006).

2.5. Turkey's Demand for VFAs

Most common VFAs produced from wastewaters are HAc, Buty and HPr. Demand for these acids in Turkey are increasing parallel to their global demand. Turkish import statistics for these VFAs were examined. It was seen

that there has been an increase in the demand for HAc and Buty within the past few years.

When Turkey's import statistics on HAc were examined, an increase in HAc demand can be realized; such that, 16,910.4 tonnes of HAc was imported in 2003 while this value increased to 17,524.3 tonnes in 2004. Turkey had spent 6,441,900 US dollars in 2003 and 7,122,126 US dollars in 2004, on HAc. Similarly, Turkey had imported 7,705 tonnes of Buty in 2003 while this value increased to 11,250 tonnes in 2004. As a consequence, Turkey had spent 22,257 US dollars in 2003 and 28,245 US dollars in 2004, on Buty. On the other hand, HPr import dropped from 22,459 tonnes to 2.886 tonnes between 2003 and 2004 (TİK, 2006).

The above statistical data shows the importance of VFAs in Turkish economy. At present Turkey in the position of importing these acids for its needs. Production of these acids within the country will reduce our foreign dependency and will lead to important economical gains.

CHAPTER 3

MATERIALS AND METHODS

Characterization of the cheese-whey and seed cultures used in the experiments with the experimental procedure and methods used are presented in the sections below.

3.1. Characterization of Cheese-Whey

150 L of cheese-whey was obtained from a cheese production factory located in the Atatürk Orman Çiftliği in Ankara. It was divided into smaller portions and stored at below 0 ^{o}C for further use in Set 1 (performed in semi-continuously fed batch reactors) and Set 2 (performed in batch reactors). Different proportions were used in each set of experiments.

The characterization of cheese-whey used in the experiments was performed and the parameters are depicted in Table 3.1.

Since the pH values of cheese-whey used in both sets of experiments were above 5, they can be classified as sweet-whey (Weetal et al., 1974; Kosikowski, 1979; Mawson, 1994)

Table 3.1. Characteristics of the cheese-whey used in the experiments.

Parameter*	Unit	Concentration
SET 1		
COD	mg l^{-1}	79867 ± 2581
sCOD	mg l^{-1}	60683 ± 1938
NH4-N	mg l^{-1}	95 ± 8
PO4-P	mg l^{-1}	370 ± 14
SS	mg l^{-1}	4460 ± 168
VSS	mg l^{-1}	4360 ± 127
TS	mg l^{-1}	69245 ± 3161
Alkalinity	mg l^{-1} as $CaCO_3$	584 ± 111
pH		5.92
SET 2		
COD	mg l^{-1}	65267 ± 1159
sCOD	mg l^{-1}	59700 ± 872
NH4-N	mg l^{-1}	41± 16
TN	mg N l^{-1}	126 ± 29
PO4-P	mg l^{-1}	259 ± 16
TP	mg P l^{-1}	953 ± 64
SS	mg l^{-1}	6050 ± 580
VSS	mg l^{-1}	5385 ± 543
TS	mg l^{-1}	79860 ± 330
Alkalinity	mg l^{-1} as $CaCO_3$	761 ± 5
pH		6.2

* COD: Chemical oxygen demand; sCOD: Soluble chemical oxygen demand; SS: Suspended solids; VSS: Volatile suspended solids; TS: Total solids; TN: Total Nitrogen; TP: Total Phosphorus

3.2. Seed Culture

Different seed cultures were used in both sets of experiments. Their characteristics are explained in the following sections.

3.2.1. Seed Culture Used in Set 1 Experiments

Mixed anaerobic culture (MAC) was used in this part of the experiments. The culture was obtained from the anaerobic sludge digesters of Ankara Municipal Wastewater Treatment Plant. Its characteristics are depicted in Table 3.2.

Table 3.2. Characterization of the seed inoculum used in the first stage of the studies.

Parameter*	Unit	Concentration
TS	mg l^{-1}	34393 ± 123
SS	mg l^{-1}	32380 ± 996
FSS	mg l^{-1}	17493 ± 511
VSS	mg l^{-1}	14887 ± 491

*TS: Total solids; SS: Suspended solids; FSS: Fixed suspended solids; VSS: Volatile suspended solids

3.2.2. Seed Cultures Used in Set 2 Experiments

Three different types of seed cultures were used in this part of the experiments; MAC, Heated Mixed Anaerobic Culture (HMAC) and Acidogenic culture (AC).

MAC was obtained from the anaerobic sludge digesters of Ankara Municipal Wastewater Treatment Plant. 2-bromoethanesulfonate (BES) (a methanogenic inhibitor) was used with MAC to hinder methanogenic activity in the reactors.

HMAC was obtained by heating MAC in 80°C for 15 minutes to inhibit methanogenic bacteria (Mostafa, 1999).

AC used in this set was previously prepared in a 2500 ml fill and draw reactor with and effective volume of 2000 ml. Anaerobic seed sludge from Ankara Municipal Wastewater Treatment Plant was cultivated in the system to enrich acidogens. The inoculum system was operated with 10 g COD l^{-1} glucose solution at 1 day HRT. Temperature and pH were maintained at 35°C, and at 5.5 ± 0.5 with 3N NaOH, respectively. The upper part of the reactor was connected to serum bags to measure the total gas production in the reactors. The gas collected in the bags was measured daily with water displacement device (Ergüder et al., 2000). The gas production in the reactor was insignificant. This verified the repression of methanogenic activity in the inoculum system. Daily feed contained necessary nutrients (Basal Media with $NaHCO_3$ (6 g l^{-1})) and glucose (10 g COD l^{-1}). Daily waste was collected and settled for acidogenic activity assay, in order to determine their acidogenic properties (Refer to Section 3.4 for details). Results of acidogenic activity assay are depicted in Table 3.3. Graphs for acidogenic activity assay are provided in Appendix A.

Maximum specific acidogenic activities of the seed cultures used in this experiment were found to be 7.01 ± 1.6, 13.28 ± 4.3 and 6.41 ± 0.6 g COD g^{-1}VSS d^{-1} for acidogenic, mixed anaerobic and heated mixed anaerobic cultures, respectively (Table 3.3).

Table 3.3. Maximum specific acidogenic activity of each seed culture used in the experiments.

Seed Culture	Unit	Activity
AC	g COD.g^{-1}VSS.d^{-1}	7.01 ± 1.6
MAC	g COD.g^{-1}VSS.d^{-1}	13.28 ± 4.3
HMAC	g COD.g^{-1}VSS.d^{-1}	6.41 ± 0.6

Maximum acidogenic activities of pure acidogenic cultures are 13 g COD g^{-1}VSS d^{-1} (Henze and Harremöes, 1983). The specific acidogenic activity of mixed cultures from an anaerobic reactor is usually higher than the 50% of those of pure cultures (Soto et al., 1993). In a study done with two different seed cultures (attached and occulated) in lab-scale single-fed and multi-fed upflow anaerobic filters, treating cheese-whey with organic loading rates (OLR) higher than 20 kg COD m^{-3} d^{-1}, acidogenic activities were found to be changing between 0.5-2 kg COD kg^{-1}VSS d^{-1} along different heights of the reactors. They found that feeding policy affected the acidogenic activities of seed cultures (Punal et al., 1999). In another study, acidogenic activity of sludge from UASB reactor treating a starch based synthetic wastewater at an ORL of 10 kg m^{-3} was found to be 1.12 kg COD kg^{-1}VSS d^{-1}, while sludge from

anaerobic baffled reactor (ABR) treating the same wastewater had a maximum specific acidogenic activity of 38.1 kg COD.kg^{-1}VSS.d^{-1} when OLR was 10 kg m^{-3} and around 6 kg COD kg^{-1} VSS d^{-1} when OLR was 3.5 kg m^{-3}. They found that OLR affected activities of cultures, and that, increasing OLR increased activities of cultures (Hutnan et al, 1999). Since acidogenic activity analysis were performed to the settled sludge, while others were done directly to the active sludge taken from reactors, lower acidogenic activity values were achieved than that of Huntan et al. (1999). However, calculated values lie between values mentioned in literature (Soto, M., 1993, Hutnan et al, 1999, Punal et al., 1999).

The characteristics of each seed culture used in studies are depicted in the Table 3.4.

Table 3.4. Characterization of the seed culture used in the studies.

	Parameter	Unit	Concentration
MAC	TS	mg l^{-1}	23785 ± 156
	SS	mg l^{-1}	23307 ± 562
	FSS	mg l^{-1}	11027 ± 401
	VSS	mg l^{-1}	12280 ± 174
HMAC	TS	mg l^{-1}	25338 ± 265
	SS	mg l^{-1}	22685 ± 550
	FSS	mg l^{-1}	11362 ± 263
	VSS	mg l^{-1}	11322 ± 290
AC	TS	mg l^{-1}	10740 ± 57
	SS	mg l^{-1}	2781 ± 449
	FSS	mg l^{-1}	188 ± 123
	VSS	mg l^{-1}	2593 ± 389

MAC: mixed anaerobic culture; HMAC: heated mixed anaerobic culture; AC: acidogenic culture; TS: Total solids; SS: Suspended solids; FSS: Fixed Suspended Solids; VSS: Volatile Suspended Solids

3.3. Basal Medium (BM)

In order to examine the effect of nutrient addition on VFA production, BM was added to some of the reactors.

The composition of the basal medium used in Set 2 experiments was as follows (mg l^{-1}): NH_4Cl (1200), $MgSO_4{\cdot}7H_2O$ (400), KCl (400), $Na_2S{\cdot}9H_2O$ (300), $CaCl_2{\cdot}2H_2O$ (50), $(NH_4)2HPO_4$ (80), $FeCl_2{\cdot}4H_2O$ (40), $CoCl_2{\cdot}6H_2O$ (10), KI (10), $MnCl_2{\cdot}4H_2O$ (0.5), $CuCl_2{\cdot}2H_2O$ (0.5), $ZnCl_2$ (0.5), $AlCl_3{\cdot}6H_2O$ (0.5), $NaMoO_4{\cdot}2H_2O$ (0.5), H_3BO_3 (0.5), $NiCl_2{\cdot}6H_2O$ (0.5), $NaWO_4{\cdot}2H_2O$ (0.5), Na_2SeO_3 (0.5), cysteine (10). This basal medium contained all the necessary

micro and macro nutrients required for optimum anaerobic microbial growth (Demirer and Speece, 1998).

3.4. Experimental Set-up

In this section, experimental set-up used in the studies is discussed in detail. The aim of the Set 1 experiments was to determine the optimum OLR and HRT for maximum VFA production in semi-continuously fed batch reactors. Set 2 experiments were performed to compare the effects of using different seed cultures and BM on VFA production from cheese-whey in batch reactors using the optimum COD load value obtained in Set 1 experiments.

3.4.1. Experimental Set-up of Set 1 Experiments

In the first part of the studies the optimum of HRT and OLR for maximum VFA production was investigated.

Four different HRTs (2, 3, 4 and 5 days) and three different loads (10, 15.2, 20 g COD l^{-1}) were studied in 15 reactors. The experiments were done in 250 ml semi-continuously fed batch reactors, with an effective mixed liquor volume of 200 ml, using cheese-whey (Table 3.1) as the substrate and MAC (Table 3.3) as the seed culture. Cheese-whey (Table 3.1) was diluted to give the concentrations of 10, 15.2 and 20 g COD l^{-1} for corresponding reactors. Each reactor was seeded with 100 ml of MAC. It was diluted to give 7.4 g l^{-1} VSS concentration. Seed concentration was kept at high levels to obtain sufficient concentration of acidogens after the possible inhibition of methanogens in the reactors due applied conditions (high OLRs and low HRTs).

For the HRTs of 2, 3 and 4 days wastewater concentrations of 10, 15.2, and 20 g $l^{-1}$and for the HRT of 5 days 10 and 15.2 g COD l^{-1} were studied. For each HRT the reactors with 10 g COD l^{-1} of OLR were studied as duplicates. The content of the reactors is depicted in the Table 3.5.

Table 3.5. Experimental set-up for Set 1 Experiments

Reactor:	HRT (day)	OLR (g COD l^{-1} day^{-1})	Cheese-whey (%)	Distilled Water (%)	Seed (%)
R1*	2	10 [(a)]	12.5	37.5	50
R2	2	15.2 [(b)]	19	31	50
R3	2	20 [(c)]	25	25	50
R4*	3	10 [(a)]	12.5	37.5	50
R5	3	15.2 [(b)]	19	31	50
R6	3	20 [(c)]	25	25	50
R7*	4	10 [(a)]	12.5	37.5	50
R8	4	15.2 [(b)]	19	31	50
R9	4	20 [(c)]	25	25	50
R10*	5	10 [(a)]	12.5	37.5	50
R11	5	15.2 [(b)]	19	31	50

*Run as duplicates
Values as volume percent
(a) corresponding to 7.5 g sCOD l^{-1} day^{-1} (b) corresponding to 11.5 g sCOD l^{-1} day^{-1} (c) corresponding to 15 g sCOD l^{-1} day^{-1}

Reactors were fed daily. Daily waste from each reactor was taken after mixing reactors vigorously. 100, 66, 50 and 40 ml of reactor contents were removed daily from reactors with HRT 2, 3, 4 and 5, respectively, and fed accordingly to give the OLR proposed for each reactor.

The experiments were conducted at room temperature (22 ± 3 ^{o}C). They were shaken at 125 rpm throughout the experimental period. The reactors were run for 6 weeks.

Gas production in the reactors was monitored daily, while pH of the reactors was measured every other day. The VFA production in the system was observed (every two days for the first 22 day period and every four days for the remaining period). sCOD concentrations in the reactors were also monitored.

3.4.2. Experimental Set-up of Set 2 Experiments

This set of experiments was performed to compare the effects of using different seed cultures and BM on VFA production from cheese-whey in batch reactors using the optimum COD load found in Set 1 experiments. The experimental set-up for this part of the study is given in the Table 3.6.

Three different sets of reactors were used, namely Blank Reactors, Control Reactors and Test Reactors. Blank Reactors lacked seed cultures, while Control Reactors lacked substrate (cheese-whey). Test Reactors contained both substrate and seed culture. Blank Reactors were prepared to observe cheese-whey's tendency to acidification and effect of 2-bromoethanesulfonate (BES) and BM on this tendency. On the other hand, Control Reactors were prepared to observe the affects of BM and BES on pure seed cultures and also, to set a control point for Test Reactors.

Experiments were conducted in 250 ml serum bottles; with an effective mixed liquor volume of 150 ml. Composition of each reactor is given in Table 3.6.

Cheese-whey (Table 3.1) was diluted to give 12000 mg l^{-1} sCOD concentration in each reactor.

Table 3.6. Experimental set-up for Set 2 experiments

Reactors	Seed		Cheese-whey	BM	BES	Distilled Water
	Type	%	(%)	(%)	(%)	(%)
B	-	-	20	0	0	80
B-BES	-	-	20	0	5	75
B-BM	-	-	20	25	0	55
B-BM-BES	-	-	20	25	5	50
C1*	MAC	20	0	0	5	75
C2	HMAC	20	0	0	0	80
C3	AC	20	0	0	0	80
C-BM1	MAC	20	0	25	5	50
C-BM2	HMAC	20	0	25	0	55
C-BM3*	AC	20	0	25	0	55
R1*	MAC	20	20	0	5	55
R2	HMAC	20	20	0	0	60
R3*	AC	20	20	0	0	60
R4*	MAC	20	20	25	5	30
R5*	HMAC	20	20	25	0	35
R6	AC	20	20	25	0	35

*Run as duplicates; Values as volume percent
MAC: Mixed Anaerobic Culture; HMAC: Heated Mixed Anaerobic Culture; AC: Acidogenic Culture; BES: Methanogenic inhibitor; BM: Basal Medium; B: Blank Reactor; C: Control Reactor; R: Test Reactor

Three different seed cultures (MAC, HMAC and AC) were used to compare the effect of using different seed types on acidification of cheese-whey. Each seed culture was diluted to give 2500 mg l^{-1} VSS concentration in reactors. To inhibit the methanogenic activity in the reactors containing MAC, BES was used since long-term exposure to BES is known to inhibit methanogenesis. BES

is a structural analog of coenzyme M which is found in all methanogens but not in other Bacteria or Archaea (Balch and Wolfe, 1979). Therefore, it is a specific inhibitor for methanogens. 10 mM of BES was injected to related reactors (Chidthaisong and Conrad, 2000). The concentration used has shown to completely inhibit methanogenesis or acetate metabolism in both pure culture of microorganisms and in environmental samples (Oremland and Capone, 1988; Schulz and Conrad, 1996; DeGraaf et al., 1996).

Reactors were subjected to TS, PO_4-P, NH_4-N, COD, sCOD, VFA and pH analysis for initial characterization. Initial TS, PO_4-P, NH_4-N, COD, sCOD and pH values in reactors are depicted in Table 3.7.

Table 3.7. Initial TS, PO_4-P, NH_4-N, COD, sCOD and pH values in reactors.

	TS	PO_4	PO_4-P	NH_4	NH_4-N	COD	sCOD	pH
B	14360 ± 1257	179	58	7	5	15400 ± 1283	12600 ±141	6,4
B-BES	15010 ± 1697	152	50	7	6	13585 ± 626	12800 ± 651	6,4
B-BM	13810 ± 1564	295	95	310	240	12700 ± 2065	11880 ± 1428	7,9
B-BM-BES	16420 ± 1235	320	110	235	180	15620 ± 580	13900 ± 1216	7,8
C1	3300 ± 318	11	4	28 ± 0.7	22 ± 2	1980 ± 161	1000 ± 91	8,9
C2*	1080 ± 25	12 ± 0.7	4 ± 0.4	35	27	935 ± 308	500 ± 262	8,9
C3	2020 ± 256	6	2	39	31	2420 ± 651	1500 ± 707	7,8
C-BM1	4740 ± 65	48	16	305	240	2475 ± 39	1500 ± 187	9,1
C-BM2	2760 ± 89	16	5	405	315	1045 ± 103	900 ± 237	9
C-BM3*	2800 ± 88	9 ± 0.7	3 ± 0.7	420 ± 10	325 ± 14	2420 ± 39	1300 ± 283	8,8
R1*	14930 ± 159	320 ± 11.3	110 ± 7.1	54 ± 2.1	42 ± 2.1	16000 ± 403	13000 ± 838	6,9
R2	11110 ± 365	230	70	45	35	14400 ± 144	11100 ± 109	6,9
R3*	13720 ± 39	260 ± 21.2	80 ± 10.6	51 ± 3	39 ± 4	16900 ± 1442	12200 ± 774	6,9
R4*	16810 ± 53	340 ± 14.1	110 ± 7.1	290 ± 14	230 ± 28	26600 ± 2227	11500 ± 605	7,8
R5*	14660 ± 1220	300 ± 21.2	100 ± 14.1	390 ± 35	300 ± 21	16000 ± 361	12000 ± 636	7,9
R6	14890 ± 1254	290	90	300	240	17000 ± 148	16800 ± 361	7,6

* Analyzed in duplicates (NH_4-N, NH_4, PO_4-P, PO_4)
Concentrations in mg/l

The reactors were run in batch reactors at 35 ± 2 ^{o}C in a constant temperature room. They were shaken at 125 rpm throughout the operation time. 10 ml of samples from each reactor was taken weekly. Samples were taken after mixing reactors vigorously and were stored below 0°C before analysis. Gas production in the reactors was monitored daily, pH of the reactors was measured every other day and VFA, COD and TS measurements were done weekly. The reactors were run for 8 weeks.

3.5. Analytical Methods

pH measurements were performed with a pH meter (Model 2906, Jenway Ltd, UK) and a pH probe.

COD concentration was measured with PC Direct Multiphotometer and AquaLytic COD vials for COD 0–15000 ppm and COD 0–1500 ppm as given in AquaLytic PC Multi Direct Instruction Manual. The basic principal is that oxidizable substances react with sulphuric acid-potassium dichromate solution in the presence of silver sulfate as catalyst. Chloride is masked with mercury sulfate and the reduction in the yellow coloration is evaluated after 2 hr of digestion at 150 ^{o}C.

Total solids, suspended solids, fixed suspended solids and volatile suspended solids were determined according to Standard Methods (APHA, 1995).

Total phosphate and ortho-phosphate were measured with an AquaLytic Photometer and AquaLytic Total Phosphate and Ortho-Phosphate Reagent Sets, respectively. The analyses were performed according to AquaLytic PC Multi

Direct Instruction Manual (Method numbers: 326 and 323 for total phosphate and ortho-phosphate, respectively).

Total nitrogen and ammonium nitrogen were measured with an AquaLytic Photometer and with AquaLytic HR Total Nitrogen Set and Ammonium Nitrogen Reagent Sets, respectively. The analyses were performed according to AquaLytic PC Multi Direct Instruction Manual (Method numbers: 281 and 60 for total nitrogen and ammonium nitrogen, respectively).

Alkalinity was determined according to Standard Methods (APHA, 1995).

TVFA analysis in Set 1 experiments were determined by titration according to Standard Methods (1995). Comparison of titration with gas chromatography (GC) were performed during Set 2 experiments by comparing five different cheese-whey concentrations, which are calculated by both methods (Standard methods and GC Analysis). After the calibration of titration values to GC values, concentrations found in Set 1 experiments were converted to GC equivalents. Calibration curve can be found on Appendix B.

VFA and alcohol analysis in Set 2 epxperiments were performed with a GC unit equipped with a flame ionization detector (0.25 mm) and a 30 m capillary column (Zebron ZB-FFAP). The column temperature was started at 100 ^{o}C with 2 min holding time and then increased to 250 ^{o}C with 8 ^{o}C/min ramping, and the injector/detector temperature was kept at 200/350 ^{o}C with helium as the carrier gas and a flow rate of 30 mL/min. The gas flow rates were gauged at 350 mL/min for air and 35 mL/min for hydrogen. Liquid samples were centrifuged for 15 min at 3,000 rpm and the supernatant was filtered through a 0.22 mm filter. Filtered samples were acidified with formic acid to fatty acids to their undissociated forms (HAc, Buty etc.) before their injection into the GC.

Gas production in the reactors was determined by a water displacement device consisting of a 50 mL of burette and 250 mL water reservoir (Ergüder et al., 2000). Gas composition anaylsis in Set 1 experiments were performed at Turkish Petroleum Corporation (TPAO). Gas composition analysis in Set 2 experiments were determined by a GC unit (Shimadzu 8A) equipped with thermal conductivity detector. Methane, nitrogen and carbon dioxide were separated through a 3 m Porapak Q, 5 mm I.D. column. Column was operated with helium as the carrier gas at a constant pressure of 20 kPa at 40°C. The injector was maintained at 100°C, and the detector temperature was set to 100°C.

Acidogenic activity assays were performed in 250 ml bottles with an effective volume of 100 ml in a constant temperature room (35±2 ^{o}C). Glucose was used as substrate since it's the most common substrate used in acidogenic acitivity determination experiments (Soto et al., 1993). The seed concentration in the reactors was 1.5 g VSS l^{-1} and initial glucose concentration was 1.5 g l^{-1}. Seed inoculum in each reactor was diluted to give the required VSS concentration. Na_2CO_3 was added to each reactor as a reducing agent. Before sealing the reactors their pH were adjusted to 7 and bubbled with N_2/CO_2 (75%/25%) gas mixture. In order to determine if a lag phase was taking place and to observe the substrate utilization more deeply, two feedings were done. After the first addition of the substrate, once it was completely consumed or its consumption had stopped, a second substrate addition was carried out. This step wise feeding was found to permit the formation of a lag phase in the second feeding and was found to overcome observing incorrect values in the second feeding (Soto et al., 1993). Hourly samples were collected for 2 days and were subjected to glucose concentration evaluation. Glucose concentration in acidogenic activity assays were evaluated by determining the amount of reducing sugars in the sample by using di-nitro salycilic acid (DNS) reactive (Miller, 1959).

CHAPTER 4

RESULTS AND DISCUSSION

The experimental outputs of the study are presented and discussed in this chapter in the sections below.

4.1. Results of Set 1 Experiments

Results obtained from this set of experiments are discussed in the following sections.

4.1.1. VFA Potential of the Cheese-Whey Wastewater

During the operation period of reactors, raw cheese-whey's tendency to acidification was monitored. Samples from raw cheese-whey, which was being stored in a refrigerator at 4°C, was taken and subjected to VFA and pH analyses in order to observe the raw wastewaters contribution to the VFA production observed in the reactors (Figure 4.1).

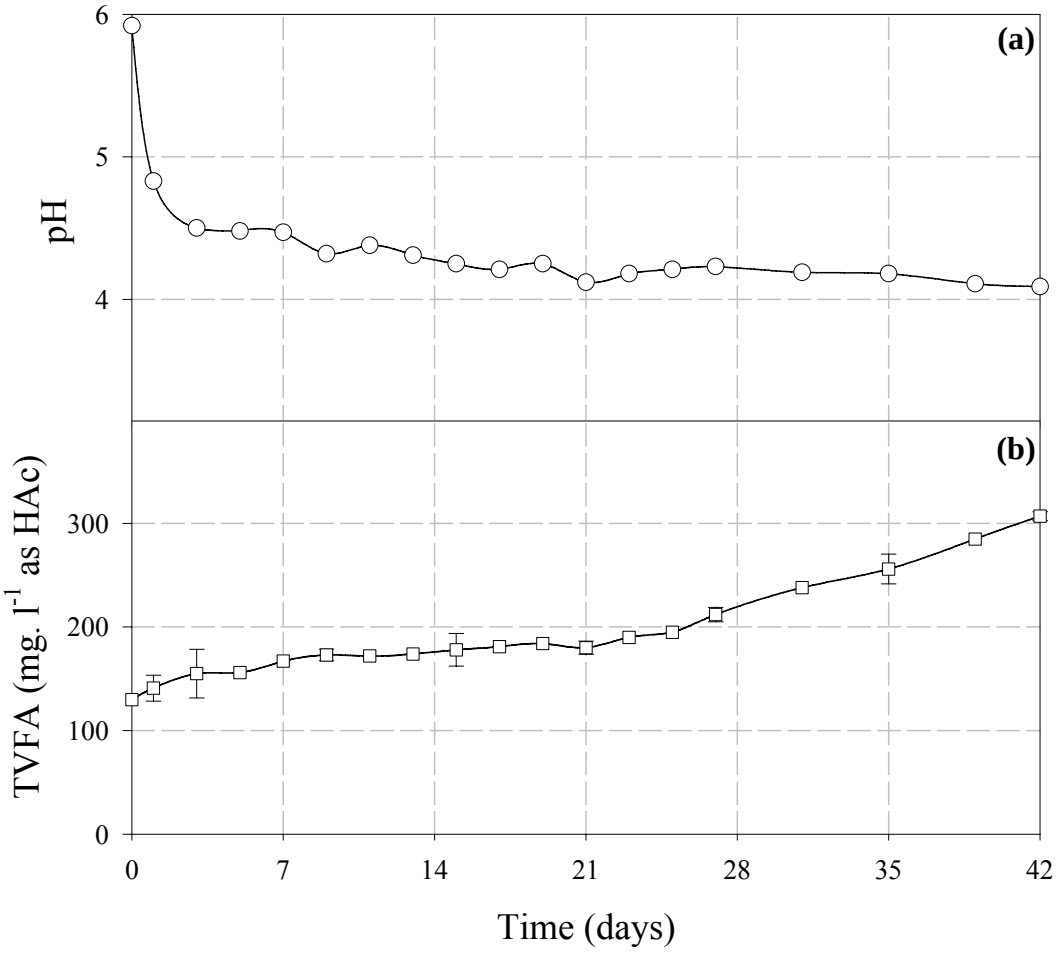

Figure 4.1. (a) pH profile of raw cheese-whey (b) VFA potential of the raw cheese-whey

The pH of the raw cheese-whey dropped from 4.83 to 4.09 in the operation period of the reactors (Figure 4.1a), while its TVFA increased from 130 mg l^{-1} (as HAc) to 307 mg l^{-1} (as HAc) during the same time period.

It was observed that cheese-whey itself is a good source of VFAs. Even without addition of microorganisms, the raw cheese-whey which was stored in refrigerator at 4 ^{o}C acidified and contributed about 307 mg l^{-1} of TVFAs (as HAc), within 42 days.

4.1.2. pH Profile of the Reactors

pH values of the reactors were not controlled during the operation period 6 weeks. pH analyses were done every other day. Effect of HRT and OLR on pH profiles of the reactors were investigated and are discussed in this section.

pH of all of the reactors dropped from initial values around 7.5 to values around 3 within the first few weeks of operation. Then pH of all of the reactors remained at those values till the end of the operation period (Figure 4.1). OLR's effect on pH profile of the reactors was insignificant. pH values showed a similar pattern for all three OLRs of 10, 15 and 20 g COD l^{-1} d^{-1}during the operation period (Figure 4.2). Similarly, effect of HRT on the pH of the reactors was insignificant (Figure 4.2).

Such low pH values may indicate successful acidification in the reactors. Methanogens prefer nearly neutral pH conditions with a generally accepted optimum range of 6.5 to 8.2 (Speece, 1996). Although most methanogens have a pH optima near neutral, there are some methanogens that live in extreme pH environments. Methanogenesis has been shown to occur at low pH's (pH=3) with reduced rates (Ferry, 1993). On the other hand, acidogens grow faster and are relatively less sensitive to low pH conditions than acetogens/methanogens (Cohen et al., 1980). However, that low pH values (between 3-4 in the Test

Reactors) are not the optimum for acidogenic bacteria either. Acidogens are more versatile and have much wider working pH range, 5 to 8, with the optimum level being 5 to 6. For instance, for lactose acidogenesis optimum pH was found to be around 6-6.5 (Kisaalita et al., 1986). On the other hand, Speece (1997) had reported a case in which acidogens were active at pH 3.6 in a starch mill wastewater treatment plant. Therefore, since optimum pH conditions for methanogens are at higher values, it can be said that most of the methanogens were successfully inhibited in the reactors.

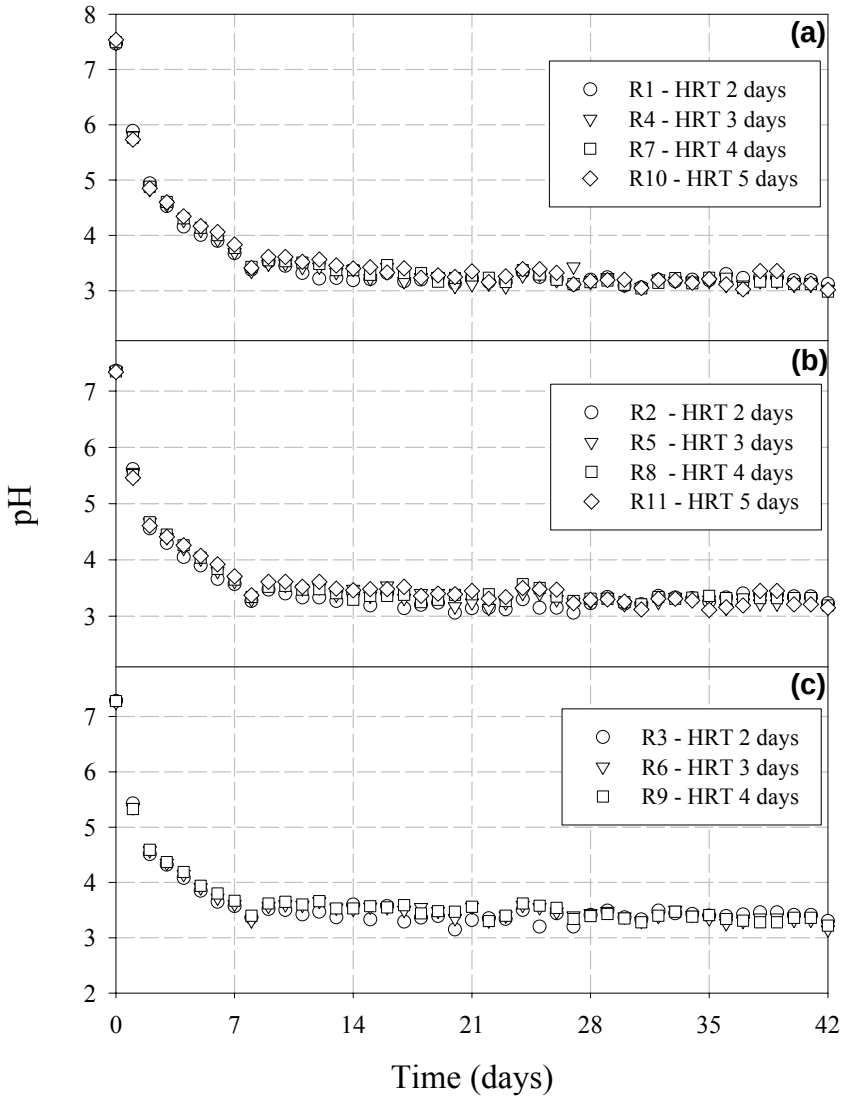

Figure 4.2. pH profile of the reactors at different OLRs

(a) OLR 10 g COD l^{-1} d^{-1} (b) OLR 15 g COD l^{-1} d^{-1} (c) OLR 20 g COD l^{-1} d^{-1}

4.1.3. Gas Production in the Reactors

Gas productions in the reactors were monitored daily during the operation period of 6 weeks (Figure 4.3). The effect of OLR and HRT on gas production was observed.

Gas composition analysis for Test Reactors were performed during the fourth week operation period with gases withdrawn from three randomly picked reactors, R5 (OLR 15 g COD l^{-1} d^{-1}, HRT 3 days), R7 (OLR 10 g COD l^{-1} d^{-1}, HRT 4 days) and R10 (OLR 10 g COD l^{-1} d^{-1}, HRT 5 days) (Table 4.1).

Gas production in all of the reactors continued throughout the operation period (6 weeks). Cumulative gas production achieved at the end of the six weeks was 806, 991, 1124, 1001, 1543, 1617, 1448, 1258, 2005, 1761, 1927 ml for R1, R2, R3, R4, R5, R6, R7, R8, R9, R10 and R11, respectively (Figure 4.3). It was observed that cumulative gas production increased with increasing OLR and HRT. An increase in OLR increased the amount of substrate entering the system, resulting in providing higher amounts of nutrients for microorganisms to ferment, increasing the amount of gaseous products. Similarly, the increase in HRT provided more time to ferment the substrate, an increase in the amount of gaseous products was observed. However, gas production in R5 was higher than that of R8, which might be due to experimental errors. Highest cumulative gas production was observed in R9 (OLR 20 g COD l^{-1} d^{-1}, HRT 4 days) as 2005 ml (Figure 4.3).

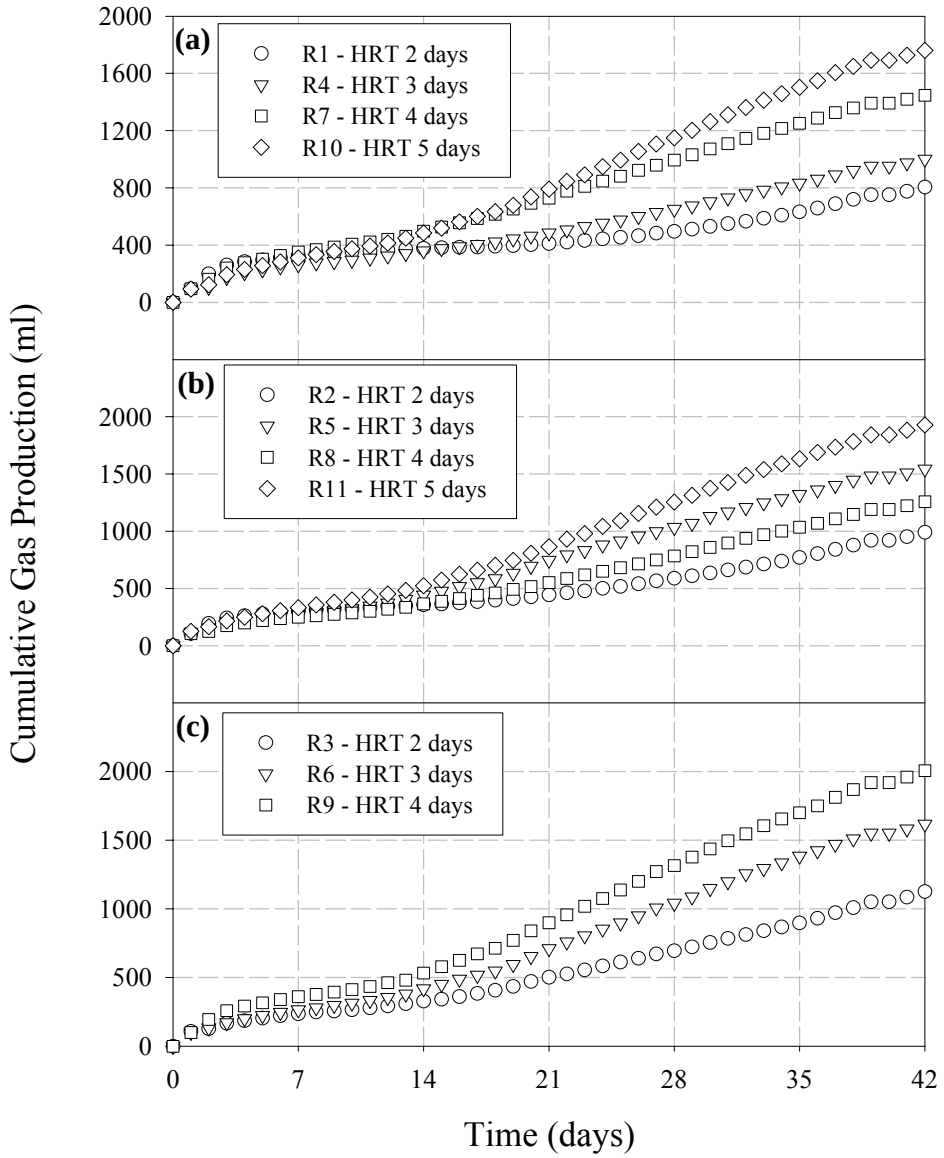

Figure 4.3. Cumulative gas production at different OLRs

(a) OLR 10 g COD l^{-1} d^{-1} (b) OLR 15 g COD l^{-1} d^{-1} (c) OLR 20 g COD l^{-1} d^{-1}

Another reason for the gas production observed in the reactors might be due EtOH fermentation. EtOH fermentation occurs at low pH of 4.5, producing ethanol, acetate, hydrogen and cabondioxide (Cohen et al., 1984; Ren et al., 1995). Since the pH values in the reactors were below 4.5 this might have been the reason for continuous gas production in the reactors. Similarly, Kisaalita et al. (1987) observed that below pH 4.5 gas production (CO_2 and H_2) might be observed along with the VFAs produced. However, EtOH analyses were not performed in this set of experiments, but it was observed that there was a relation between EtOH production and gas production in Set 2 experiments (Section 4.2.2).

When results of gas analysis were observed, it was found that none of the reactors contained CH_4. N_2, CO_2 and trace amounts of H_2 were the only gases detected (Table 4.1). Since no methane could be detected in the gas analysis, it can be said that methanogenic activity in the reactors was successfully inhibited.

Table 4.1. Results of the gas composition analysis of R5, R7 and R10.

Gas Composition*	R5	R7	R10
H_2	0.3	0.3	0.2
Ar	0	0	0
N_2	23.5	21.3	14.9
CO_2	76.2	78.4	84.9
C_1	0	0	0
C_2	0	0	0
C_3	0	0	0

C_1: Methane, C_2: Ethane, C_3: Propane
*All concentrations are in mol, %

4.1.4. VFA Production in the Reactors

In this section, results of TVFA productions in reactors are investigated in detail. Also, effects of OLR and HRT on TVFA production are explored.

TVFA analyses were carried out by titration every two days for the first 22 day period and every four days for the remaining period. TVFA production in the reactors for each OLR (OLR 10, 15 and 20 g COD l^{-1} d^{-1}) is depicted in Figure 4.4. Since TVFA analyses were done with titration, individual VFAs were not determined.

TVFA production in all of the reactors increased greatly within the first two weeks. Thereafter, production stopped or increased slightly in all of the reactors (Figure 4.4). Highest TVFA concentrations observed in R1, R4, R7 and R10 (reactors with OLR 10 g COD l^{-1} d^{-1}) were 404 ± 31, 680, 631 ± 64 and 909 mg l^{-1}, respectively, while that of R2, R5, R8 and R11 (reactors with OLR 15 g COD l^{-1} d^{-1}) were 288, 506 ± 43, 631 ± 32 and 1042 ± 126 mg l^{-1}, respectively. Moreover, TVFA productions in R3, R6 and R9 (reactors with OLR 20 g COD l^{-1} d^{-1}) reached maximum concentrations of 45 ± 13, 644 ± 22 and 853 ± 50 mg l^{-1}, respectively (Figure 4.4).

It was observed TVFA production increased with increasing OLR, which coincided with findings of other authors (Borja and Banks, 1995; Beccari et al., 1995; Yu and Fang, 2000; Parawia et al., 2004). This increase might be due to the sensitivity of methanogenic microorganisms to high substrate concentrations as mentioned in Section 2.3.1.4. Moreover, it was determined that an increase in HRT increased the TVFA production. However, it was expected to have higher production in lower HRTs (Elefsiniotis and Olham,

1994; Borja and Banks, 1995; Kim et al., 2002). This might have been due to low pH conditions observed in the reactors. Although acidogenic bacteria are unusually acid tolerant and able to grow well below pH 5.0, the optimum pH for their growth is 5.4-6.3. (Madigan and Martinko, 2005). Therefore, higher HRTs might have provided time for microorganisms to acclimate to low pH conditions observed in the reactors. This might be the reason for observing higher TVFA productions in higher HRTs.

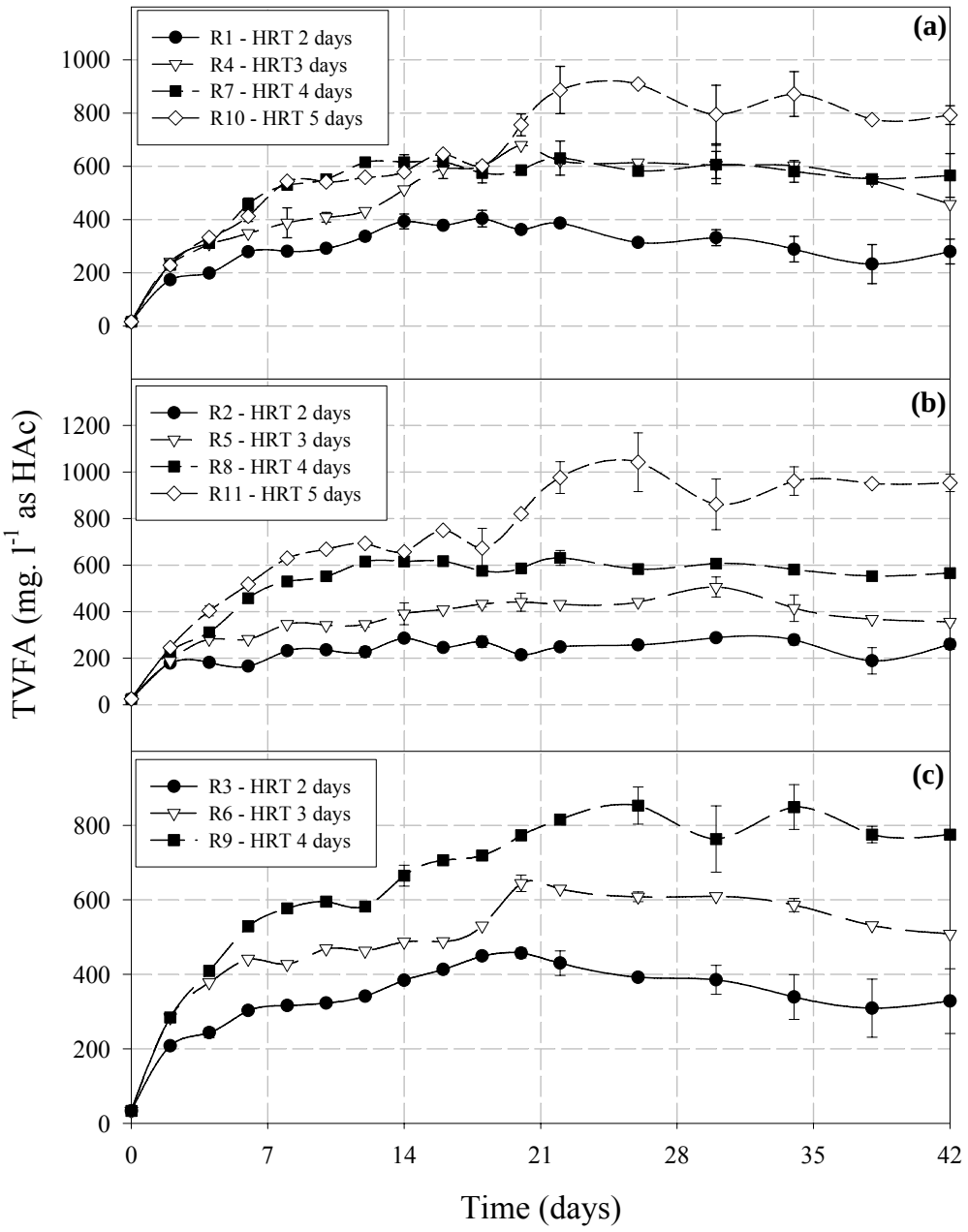

Figure 4.4. Daily TVFA production at (a) OLR 10 g COD l^{-1} d^{-1} (b) OLR 15 g COD l^{-1} d^{-1} (c) OLR 20 g COD l^{-1} d^{-1}

4.1.5. Degree of Acidification in the Reactors

Degrees of acidification in the reactors were calculated by taking the ratio of COD-equivalent of acidogenic products and the wastewater COD. Only TVFAs were included as the acidification products. Degree of acidification determined for each reactor is depicted in Figure 4.5.

Maximum degrees of acidifications achieved in the reactors were 1.41 ± 0.23, 1.74, 1.87 ± 0.16 and 2.13 ± 0.29 % for R1, R4, R7 and R10 (reactors with OLR 10 g COD l^{-1}); 1.7 ± 0.13, 1.94 ± 0.05, 1.84 and 2.15 ± 0.35 % for R2, R5, R8 and R11 (reactors with OLR 15 g COD l^{-1} d^{-1}) and 1.24, 1.39 ± 0.24 and 1.66 ± 0.16 % for R3, R6 and R6 (reactors with OLR 20 g COD l^{-1} d^{-1}), respectively (Figure 4.5). Highest degrees of acidifications were achieved in R10 (OLR 10 g COD l^{-1} d^{-1}) and R11 (OLR 15 g COD l^{-1}). Highest overall acidification degree trend was observed in reactors with 15 g COD l^{-1} d^{-1} OLR (R2, R5, R8 and R11), while lowest values were achieved in 20 g COD l^{-1} d^{-1} OLR (R3, R6 and R9). Yang et al. (2002) had observed that the degree of acidification of the cheese-whey to the short-chain VFAs was less than 20% of the influent chemical oxygen demand (COD) concentration, in their studies for the optimization of HAc and Buty production from cheese-whey wastewater. Moreover, Mostafa (1999) had obtained 28% VFA conversion efficiency from wheat milling waste residues (akalona) to VFA in his studies. Low acidification degrees achieved in this study might be due to inhibition of acidogenic microorganisms due to low pH conditions or due to high substrate concentrations. Moreover, higher acidification degrees could have been achieved in this study if gaseous products were included to the calculations.

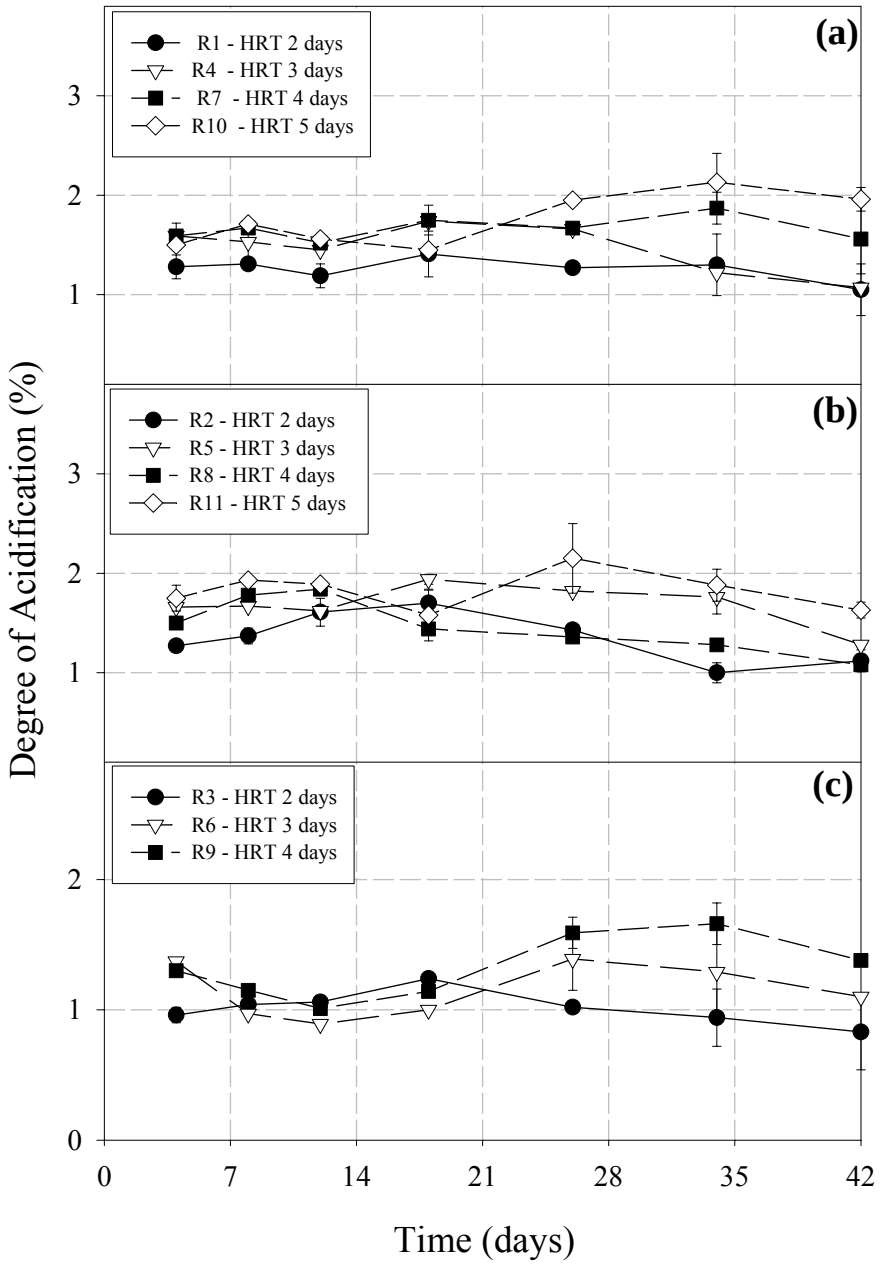

Figure 4.5. Degree of acidification at (a) OLR 10 g COD l^{-1} d^{-1} (b) OLR 15 g COD l^{-1} d^{-1} (c) OLR 20 g COD l^{-1} d^{-1}

4.1.6. sCOD Profile of the Reactors

Periodic sCOD analysis were performed in all of the reactors in order to investigate the solubilisation and degradation of cheese-whey. Results obtained from these analyses are illustrated on Figure 4.6.

An increase in the sCOD concentrations were observed in all of the reactors throughout the operation period. This indicated the solubilisation of particulate matter in cheese-whey. The decrease observed in sCOD concentrations in R7 and R10 during the last two weeks might be due experimental errors (Figure 4.6a). Efstathiou et al. (2003) found that pH affected the hydrolysis yield considerably. At pH levels below 6 and increasing HRT, significant increase in sCOD concentrations were observed due to inhibition of methanogenesis in the system. Since pH levels observed in the reactors were around 3 throughout the operation period, methanogenic activity in the reactors was inhibited, causing an increase in sCOD concentrations. Another reason for this increase might be the solubilisation of particulate matter in the substrate. However, since tCOD analyses were not performed during Set 1 experiments, solubilisation degrees were not determined.

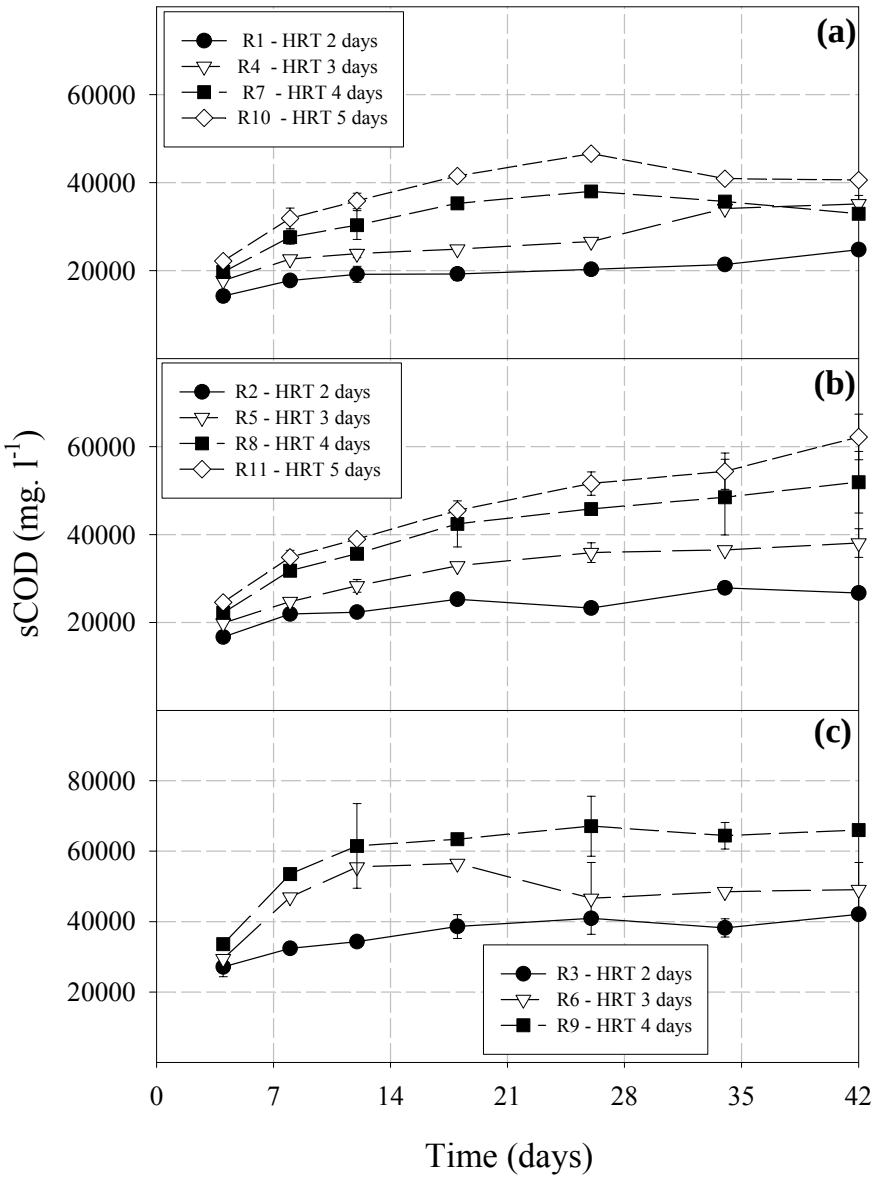

Figure 4.6. sCOD profile of reactors (a) OLR 10 g COD l^{-1} d^{-1} (b) OLR 15 g COD l^{-1} d^{-1} (c) OLR 20 g COD l^{-1} d^{-1}

4.2. Results of Set 2 Experiments

4.2.1. pH Profile of the Reactors

pH values of the batch reactors were not controlled during the operation period of 8 weeks. pH analyses were done every other day. As can be seen in Figure 4.1, pH of all of the reactors dropped drastically at the end of the first day. While pH of the Blank and Test Reactors dropped from initial values given in Table 3.6 to values below 4, pH of Control Reactors dropped to values between 5 - 7. pH values of all of the reactors remained around these values throughout operation period (Figure 4.7).

Similarly to pH profile observations of Set 1 experiments, it can be said that methanogenic activity was successfully inhibited due to the drop in pH, which were well below the optimum pH conditions for the growth of methanogens in all of the reactors.

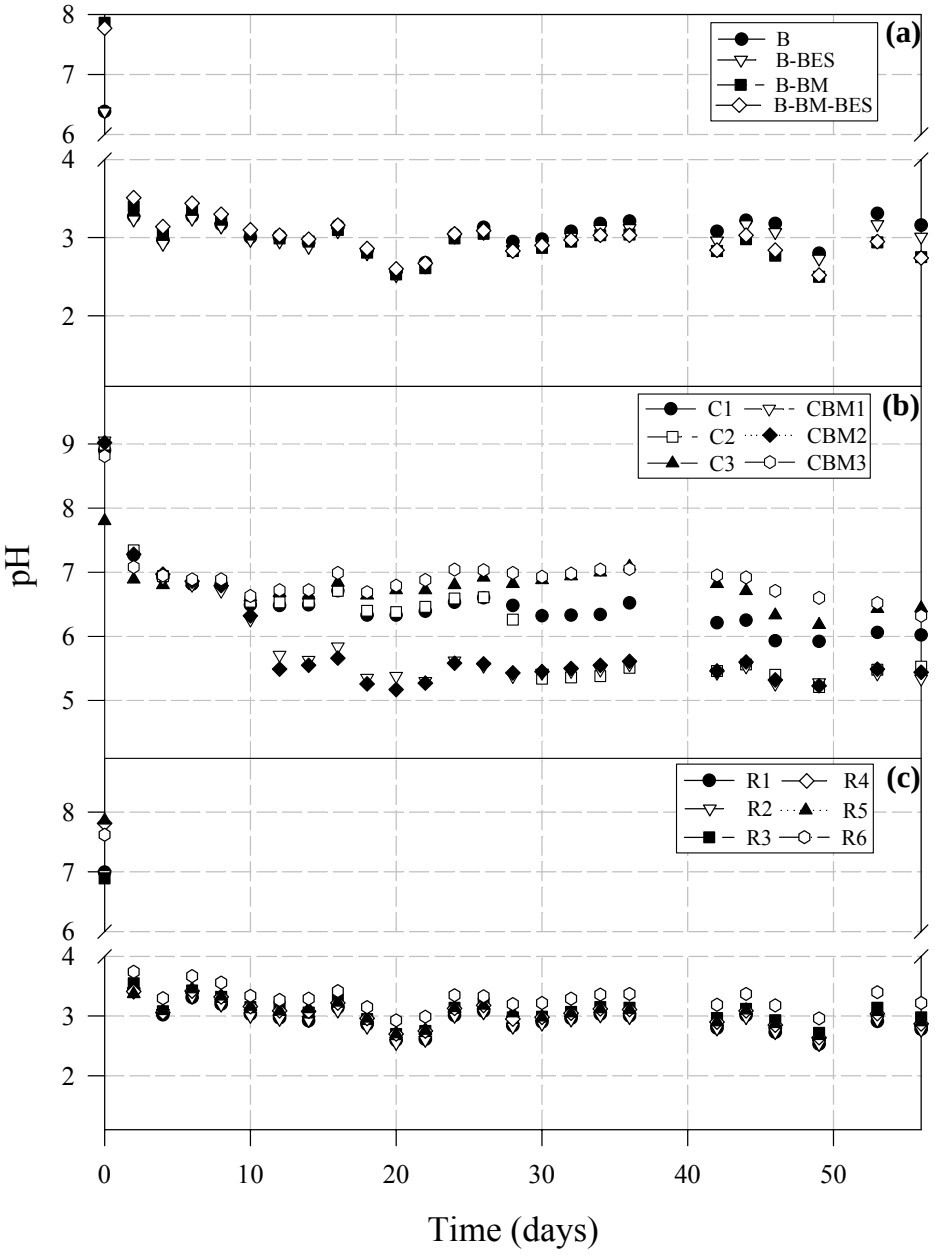

Figure 4.7. pH profile of (a) Blank (b) Control (c) Test Reactors

4.2.2. Gas Production Profile of the Reactors

Gas productions in the reactors were monitored daily during the operation period of 8 weeks (Figure 4.8). Gas composition analysis for Test Reactors were performed at the end of operation period with gases withdrawn from the head space content of each Test reactor since gas production had stopped within the initial few days in most of the reactors (Table 4.2).

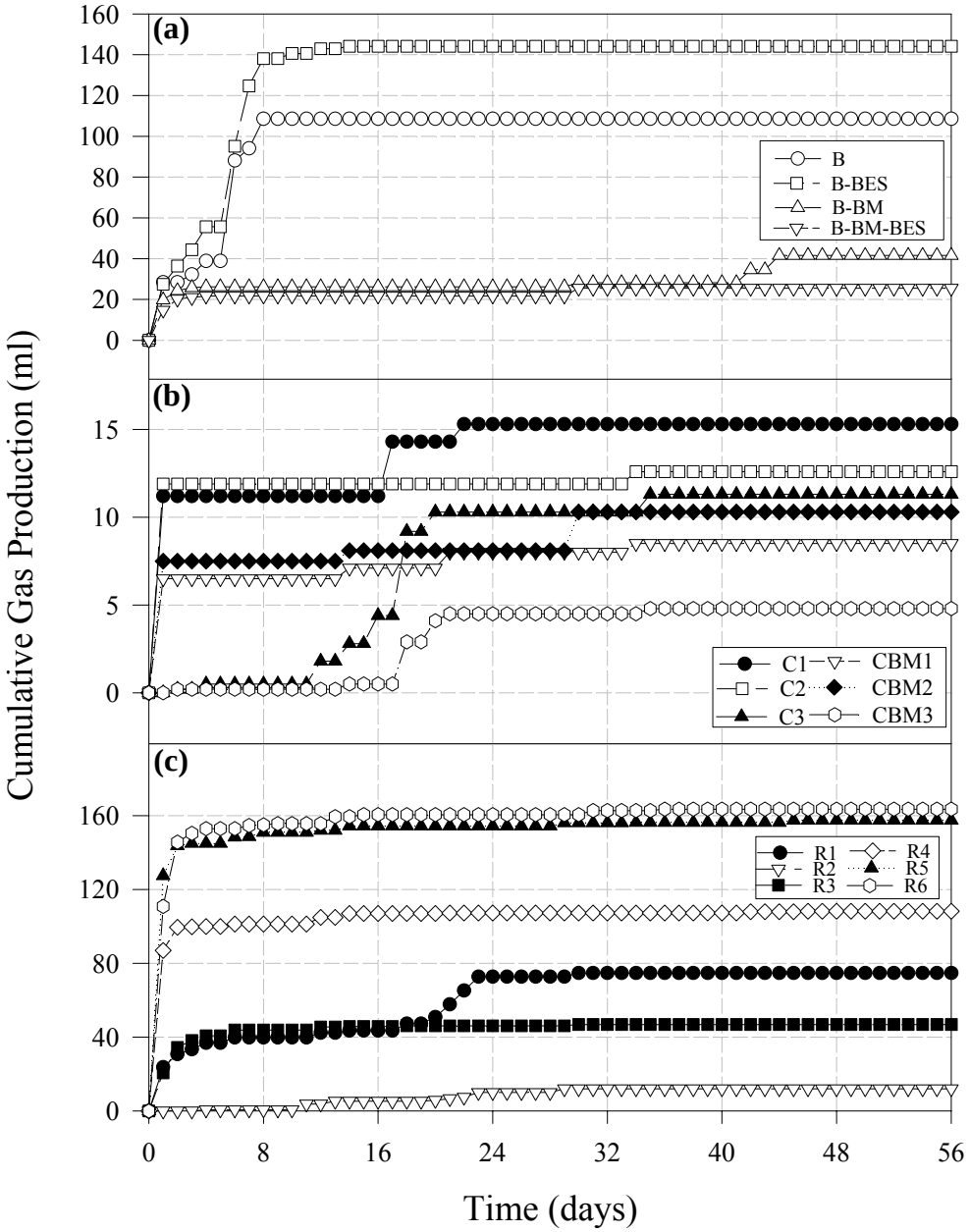

Figure 4.8. Cumulative gas production in (a) Blank (b) Control (c) Test Reactors

Table 4.2. Gas composition of the test reactors.

Reactors →	R1	R2	R3	R4	R5	R6
N_2 (%)	79,9	81,4	78,6	80,5	80,8	80,5
CO_2 (%)	20,1	18,6	20,9	19,5	19,2	19,5

As can be seen in Figure 4.2, gas production in Blank Reactors continued for almost one week (Figure 4.2a) while production in most of the Test Reactors nearly stopped after first few days of operation (Figure 4.2c). Highest overall cumulative gas productions were observed in Blanks without BM (B and B-BES) (Figure 4.2a) even exceeding the production values in some Test Reactors (Figure 4.2c). Cumulative gas production in R1 reached a maximum of 74.7 ml while cumulative gas production in its related blank reactor (ie. B-BES) reached a maximum of 144.1 ml. Similarly, while cumulative gas production in R3 reached a maximum of 46.8 ml, the gas production in its related blank reactor (ie. B) reached a maximum of 108.6 ml during the operation period. Higher gas productions achieved in Blanks might be due greater amount of Ethanol (EtOH) production compared to those of Test Reactors (Figure 4.3a, 4.3c and Figure 4.4a, 4.3c, Section 4.2.3). When gas and EtOH productions in the reactors (Figure 4.3-4.8, Section 4.2.3) were investigated, it was observed that gas production followed a similar path with EtOH production, indicating a possible relation between them, which can be explained by Equation 1. The general composition of sweet-whey consists of 74.4% lactose, 12.9% crude protein, 8.4% ash, 3.2% moisture and 1.1% fat (Dairy Management Inc., 2006). Therefore, based on the assumption that cheese-whey used in our study also

contained around 75% lactose, EtOH produced in our experiments followed the reaction given in Equation 1.

$$\underset{\text{(Lactose)}}{C_{12}H_{22}O_{11}} + H_2O \rightarrow \underset{\text{(Ethanol)}}{4C_2H_5OH} + 4CO_2 \qquad \text{(Equation 1)}$$

In addition to that, due to the pH conditions observed during the operation period, EtOH fermentation might be stimulated, resulting in EtOH, HAc, hydrogen and/or carbondioxide production (Cohen et al, 1984; Ren et al., 1995), which is observed in most of the reactors.

On the contrary, gas productions in Blanks with BM (B-BM and B-BM-BES) were much less compared to B and B-BES, only reaching 41.6 ml and 25.5 ml, respectively. It was observed that BM had a repressive effect on ethanol fermentation reducing the EtOH production while increasing VFA production in the system (Figure 4.6, 4.7 and 4.8 in Section 4.3).

Lowest cumulative gas productions were observed in Control Reactors (Figure 4.2b) since the reactors lacked substrate. Highest gas production was observed in reactor containing MAC (C1), reaching a maximum of 15.3 ml, and lowest was in the reactor with acidogenic seed containing BM (CBM3), only reaching up to 4.8 ml.

Gas production in all of the Test Reactors, but R1 and R2, stopped after first few days of operation. Gas production in R1 continued for about 3 weeks and stopped. On the other hand, slight gas production was observed in R2 during the operation period only reaching 12 ml at the end of the fourth week. Gas production showed similar pattern with VFA and EtOH production in the

reactors, which will be discussed in the following sections in detail (Section 4.2.3).

Furthermore, it was recognized that higher gas productions have occurred in reactors containing BM, which was probably resulting from further nutrient supplementation (Figure 4.2c). This increase in the gas production might be due to the increase of enzymatic activities caused by BM addition (Rittman and McCarty, 2001). The highest gas production in this study was observed in R6 containing acidogenic culture and BM, reaching a maximum value of 163.5 ml (Figure 4.2).

Repression of gas production in the reactors during operation period and final head space gas analysis (Table 4.2) indicated the successful inhibition of methanogenic activity in the reactors.

4.2.3. VFA/Ethanol Production Profile of the Reactors

In the following sections, results of VFA and ethanol productions in Blank, Control and Test Reactors are investigated in detail. Furthermore, effect of BM and using different seed types were examined.

VFA and EtOH analyses were carried out weekly with samples collected from every reactor. Individual VFA and alcohol production in each Test Reactor and their related Control and Blank Reactors are illustrated in Figures 4.9 to 4.14. Only the major acids and ethanol were depicted on figures, while minor components, with concentrations less than 5 mg l^{-1}, were given in Total VFA (TVFA).

HAc, HPr, Buty and EtOH were the expected main products from dairy wastewater acidogenesis, while formic, i-Buty, lactic, Val, i-Val, caproic (Cap) acids and methanol can be categorized as the secondary products (Yu and Fang, 2000). Similarly, in all reactors, the mixed liquor was composed of VFAs and alcohols. The VFAs were mostly HAc and Buty, plus smaller quantities of HPr, i-Buty, Val and Cap. EtOH was the only alcohol analyzed during this study.

The effect of pH on the product types were studied before (Zoetemeyer et al., 1982; Kisaalita et al., 1986; Houriuchi et al., 2002). It is known that lower pH values favors production of ethanol, which was observed in all of the reactors studied (Figure 4.9-4.14). Moreover, the main organic acids produced in the anaerobic acid reactor are strongly influenced by the culture pH due to the change of the dominant microbial populations in the acid reactor (Zoetemeyer et al., 1982). Ethanol fermentation occurs at low pH of 4.5, producing ethanol, acetate, hydrogen and cabondioxide (Cohen et al., 1984; Ren et al., 1995), which represents the cases observed in all of the reactors.

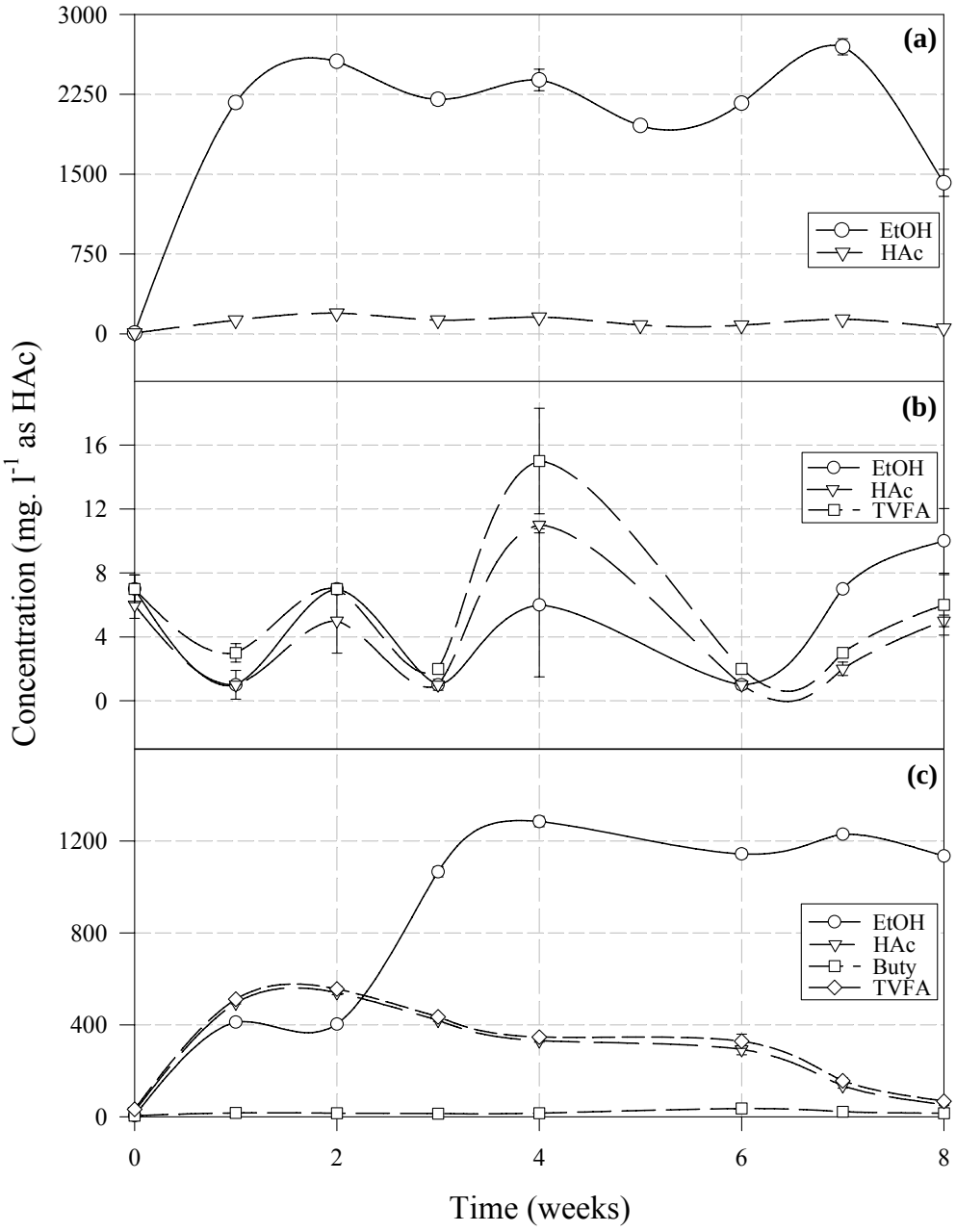

Figure 4.9: Ethanol and individual/total VFA concentrations in (a) B-BES (b) C1 (c) R1

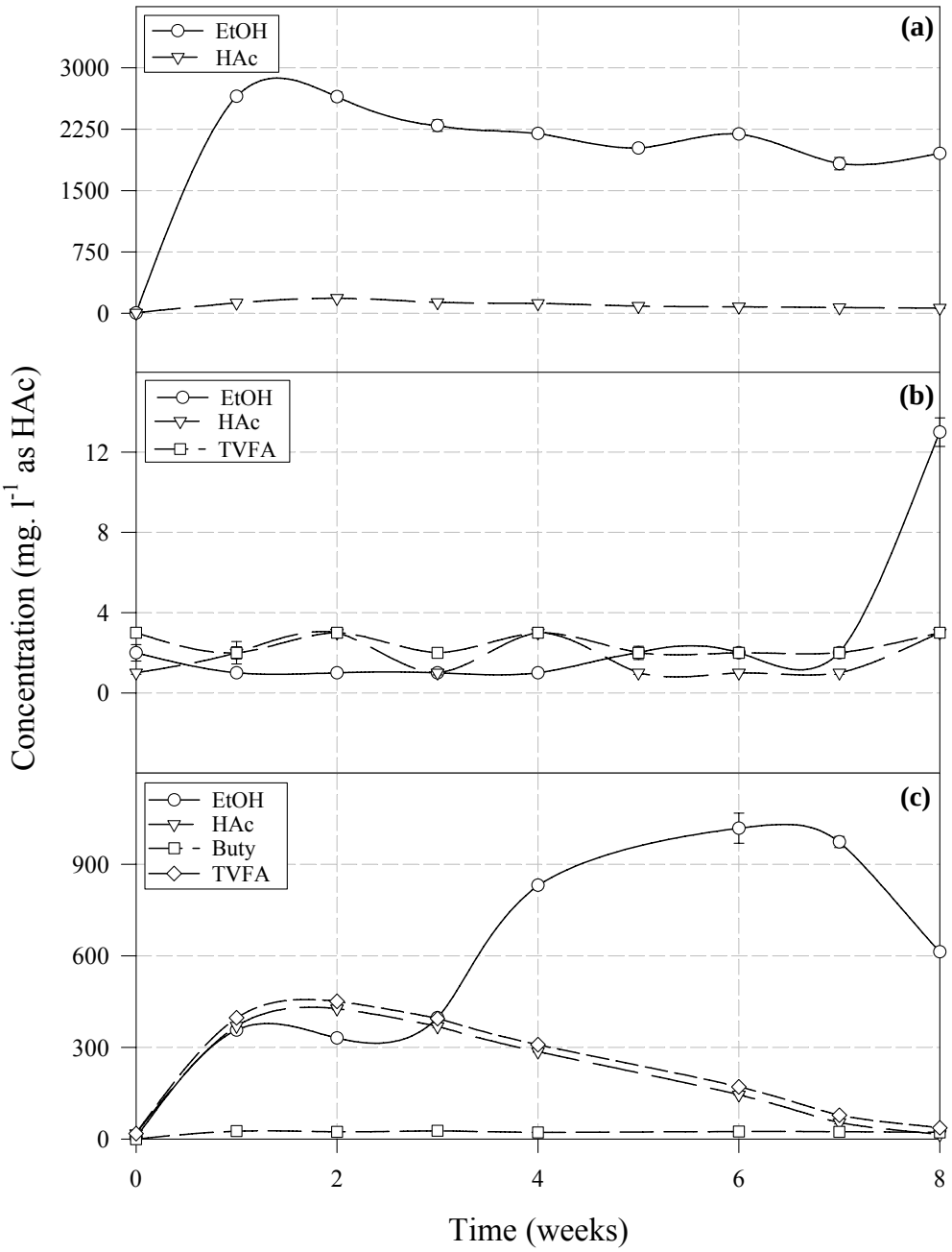

Figure 4.10. Ethanol and individual/total VFA concentrations in (a) B (b) C2 (c) R2

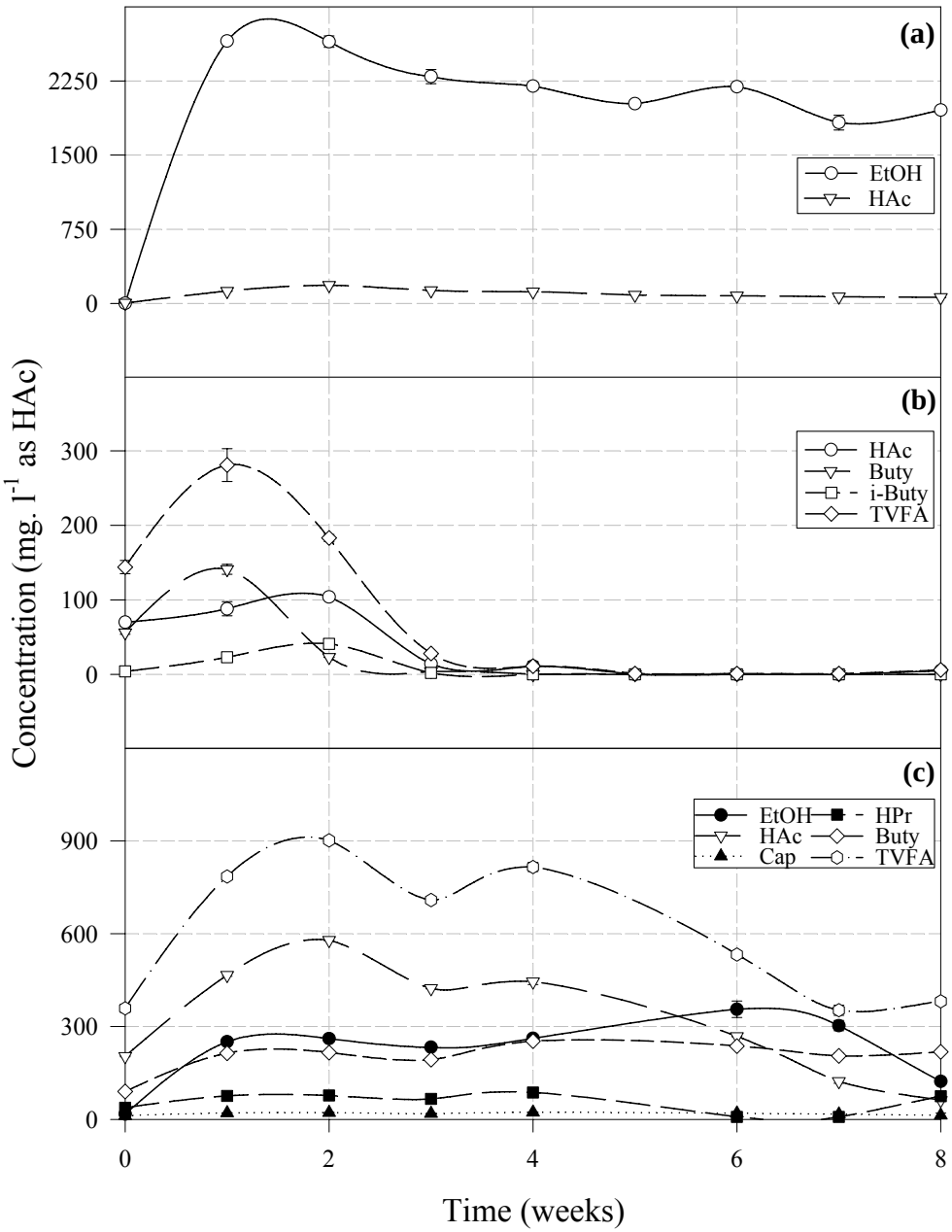

Figure 4.11. Ethanol and individual/total VFA concentrations in (a) B (b) C3 (c) R3

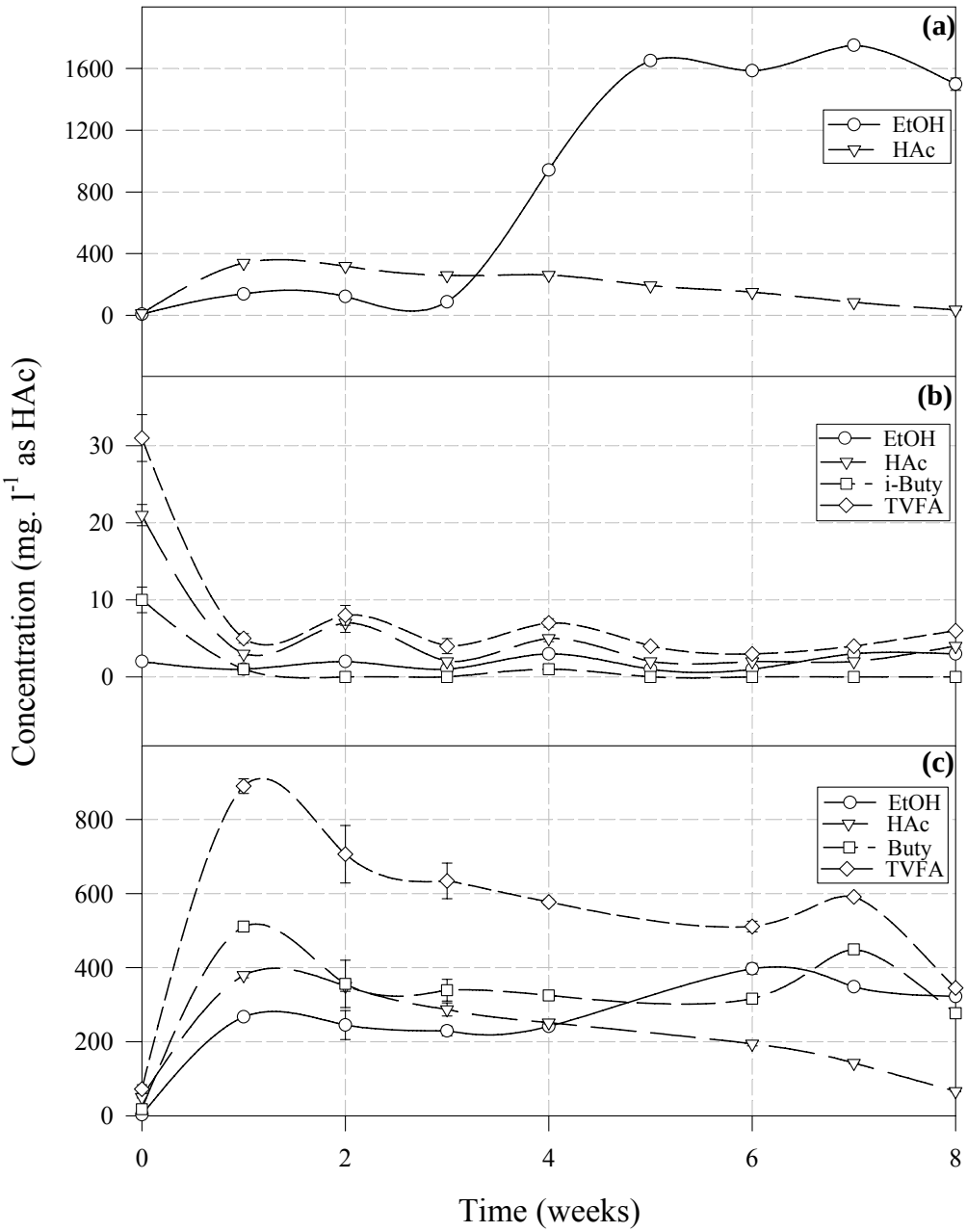

Figure 4.12. Ethanol and individual/total VFA concentrations in (a) B-BM-BES (b) CBM1 (c) R4

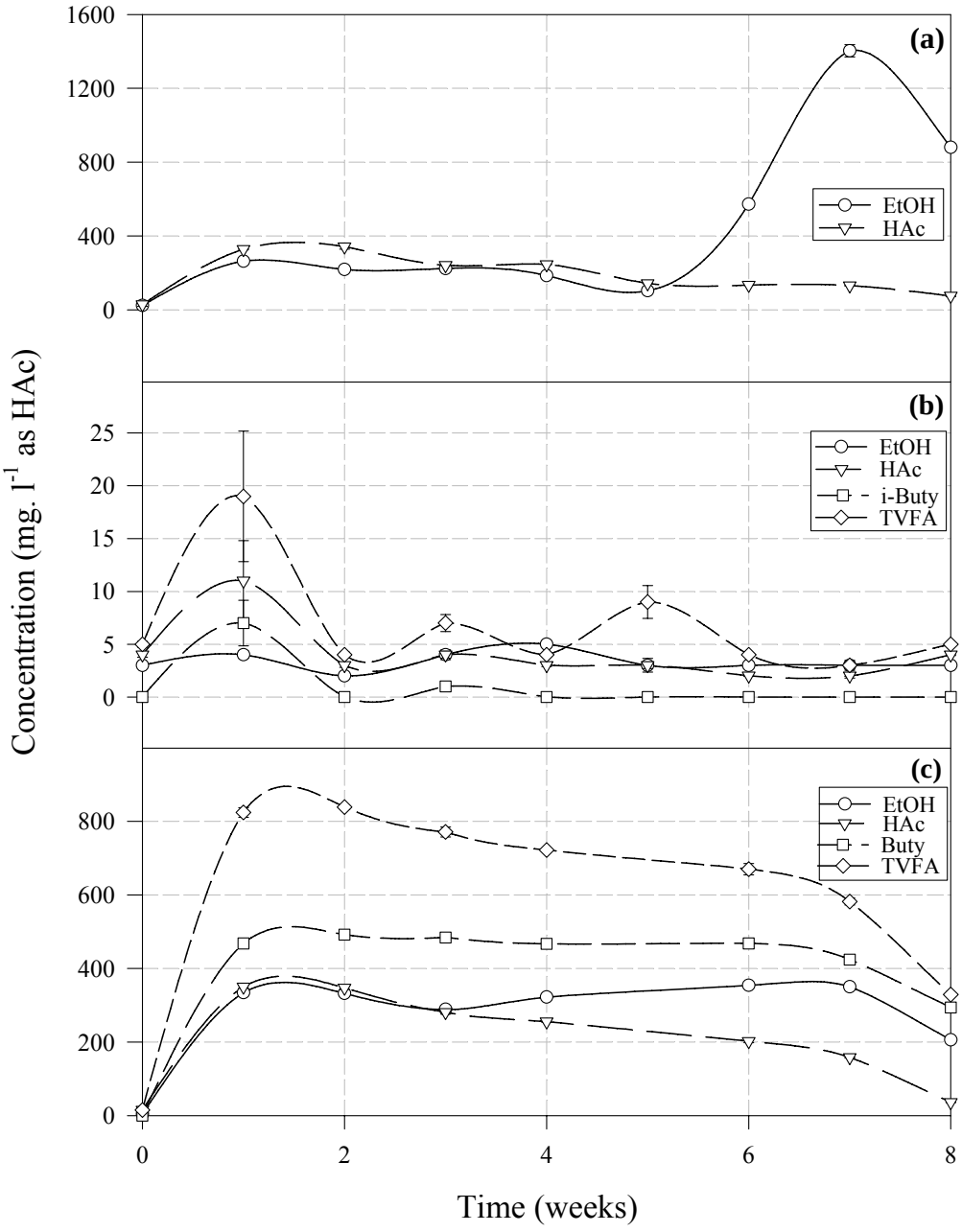

Figure 4.13. Ethanol and individual/total VFA concentrations in (a) B-BM (b) CBM2 (c) R5

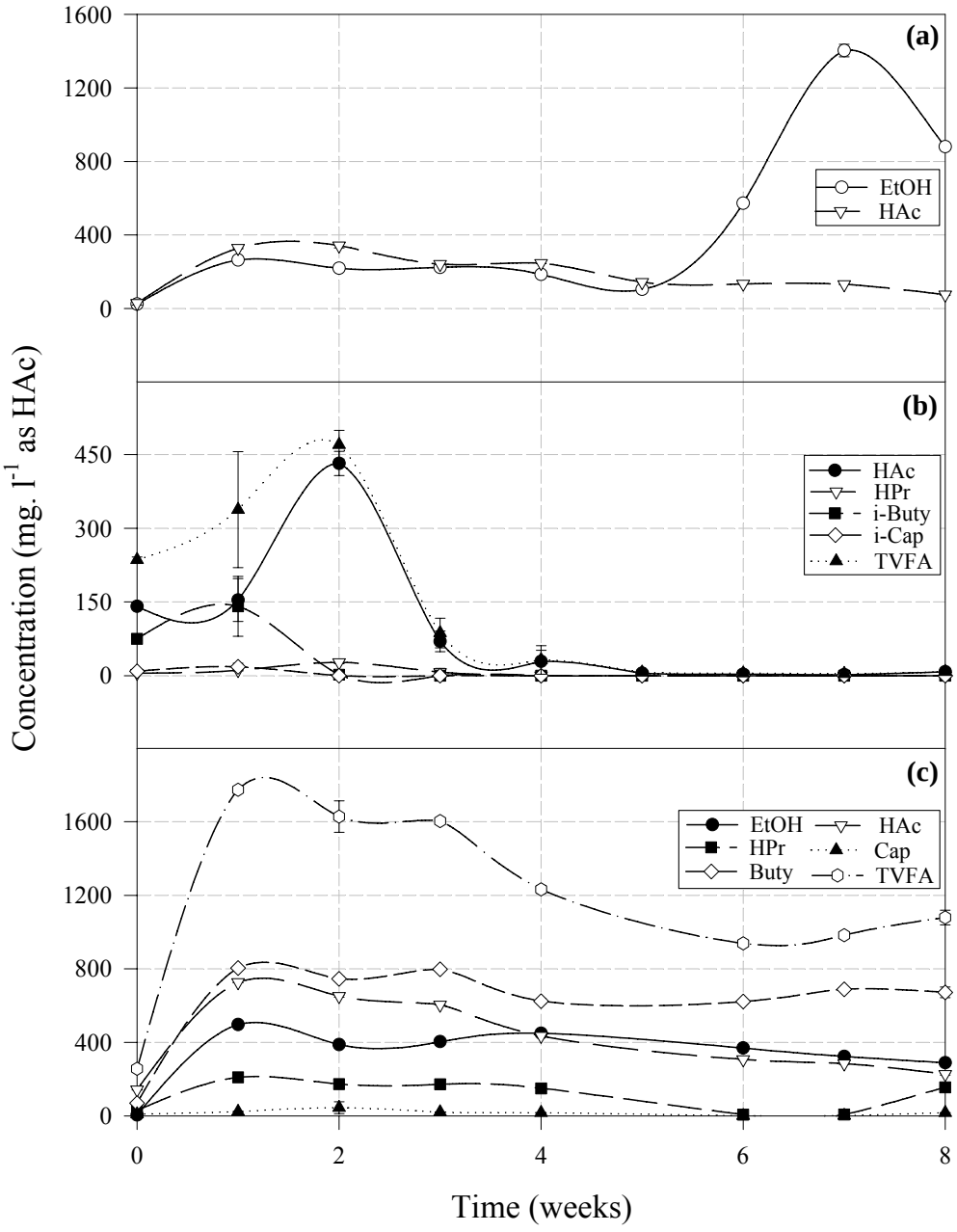

Figure 4.14. Ethanol and individual/total VFA concentrations in (a) B-BM (b) CBM3 (c) R6

4.2.3.1. VFA/EtOH Production in the Reactors

Test Reactors: HAc and Buty were the main VFAs in all of the test reactors (Figure 4.9c-4.14c). Cap and HPr were also present, but in significantly lower quantities not exceeding 5 mg l^{-1} (as HAc) in all reactors except R3 and R6. HAc concentration in reactors R1, R2, R3, R4, R5 and R6 peaked at 540, 427, 579, 379, 350 and 726 mg l^{-1}, respectively. Buty concentration in the reactors R1, R2, R3, R4, R5 and R6 reached 36, 27, 253, 511, 492 and 804 mg l^{-1} (as HAc), respectively (Figure 4.9c-4.14c). Highest total VFA production was observed in R6 reaching 1776 mg l^{-1} (as HAc) at the end of the first week. Keeping in mind that the common organic acids (HAc, Buty and HPr) are completely miscible in water (Wikipedia, 2006), it can be said that there were no mistakes in the reported concentrations concerning their solubilities.

In R1, R2 and R3 (reactors without BM) highest TVFA production were achieved within the first two weeks (Figure 4.9c-4.11c), whereas highest TVFA concentrations in R4, R5 and R6 (reactors with BM) were reached in the first week of operation. VFA productions in all of the reactors were completed within two week, and no VFA production was observed after that, which was also supported with the lack of gas production trend in the reactors after first few weeks of operation (Figure 4.8c). However, EtOH production continued in all of the reactors. EtOH concentrations in the reactors reached 1285, 1018, 356, 397, 354 and 497 mg l^{-1} (as HAc) in R1, R2, R3, R4, R5 and R6, respectively. EtOH concentrations in R1 and R2 increased considerably after third week of operation (Figure 4.9c and 4.11c), which also affected the gas production in the reactors (Figure 4.8). This might be due to the degradation of proteins. It was observed that EtOH production was largely associated with the degradation of proteins, especially in acidogenesis of high strength wastewaters

(Yu and Fang, 2001). A decrease in EtOH production was observed in reactors containing BM which will be discussed in following sections (Figure 4.12c-4.14c).

HAc and Buty were the main VFAs in R1, R2, R4 and R5. Production of these acids were found to be associated with both carbohydrate and protein degradation (Yu and Fang, 2001). They also observed that the degradation of protein was suppressed by the presence of carbohydrate in the system and that protein degradation started only after carbohydrates are fully degraded. Since production of these acids stopped after first few weeks of operation, the degradation of carbohydrates must have taken place within those weeks. The increase in EtOH in the reactors, during the following weeks, on the other hand, might be due protein degradation.

Main VFA products in R3 and R6 were HAc, Buty, HPr and Cap. Val production was also observed in those two reactors, however, in lower quantities. Microorganisms in R3 and R6 were enriched of acidogens and acclimated to acidogenic conditions before. Therefore, higher concentrations and more various types of VFA were observed in those two reactors than that of achieved in R1, R2, R4 and R5, since microorganisms in those reactors were mixed cultures and were not enriched of acidogens and were more sensitive to environmental changes. Similarly to the other reactors, their production stopped after two weeks of operation. Cap in R3 and R6 reached 25 and 45 mg l^{-1} (as HAc), respectively, while HPr concentrations reached up to 87 and 210 mg l^{-1} (as HAc) in R3 and R6, respectively (Figure 4.11c and 4.14c). Val concentrations reached 9 mg l^{-1} (as HAc) in both of the reactor, at the end of fourth week and third week in R3 and R6, respectively. While the productions of HAc, Buty and HPr were associated with both carbohydrate and protein

degradation, productions of Cap and Val were found to be associated with acidification of proteins rather than carbohydrates (Yu and Fang, 2001). Since their production was completed within first two weeks of operation, degradation of carbohydrates and proteins must have taken place by then.

Moreover, it was observed that the concentrations of Buty in all of the reactors remained same while concentration of HAc in the reactors decreased after reaching their peak values. The drop in HAc in the system might be due its consumption for cell growth.

***Blank reactors*:** The fermentation observed in Blank Reactors might be because of the microorganisms/yeast present in raw cheese-whey itself, left from the cheese manufacturing processes. In all of the Blank reactors (B, B-BES, B-BM, B-BM-BES) excessive production of EtOH was observed, while HAc production was much less compared to EtOH produced which was expected at such low pH values (Figure 4.9a-4.14a) (Cohen et al., 1984; Ren et al., 1995). Production of EtOH was much higher in Blanks than in Test Reactors, reaching up to 2657 and 2696 mg l^{-1} (as HAc), while HAc production was much lower, only reaching 184 and 194 mg l^{-1} in B and B-BES, respectively.

Higher concentrations of EtOH were observed in Blank Reactors without BM, than the ones containing BM (B-BM and B-BM-BES) (Figure 4.8a-4.14a). It was observed that in the Blank Reactors with BM, EtOH production was reduced, while HAc production was increased (Figure 4.11.a-4.14a). EtOH production in B-BM and B-BM-BES peaked at seventh week of operation, reaching 1403 and 1750 mg l^{-1} (as HAc), respectively (Figure 4.12a-4.14a). HAc production in B-BM and B-BM-BES was much higher than in B and B-BES, reaching 342 and 339 mg l^{-1} in first few weeks of operation. The shift in

EtOH production to HAc production in the first weeks of operation in Blank Reactors containing BM might be because of encouragement of HAc producing microorganisms that are already present in raw cheese-whey due to BM addition. Moreover, the high concentrations of EtOH produced in Blank Reactors might also be due to cheese-whey's tendency for acidification (Siso, 1996). It can be stated that Ethanol fermentation is the dominant fermentation type occurring in the Blank reactors due to pH conditions (Ren et al., 1995).

Control reactors: Acids and EtOH production in Control Reactors (C1, C2, C3, CBM1, CBM2 and CBM3) were much lower (Figure 4.9b-4.14b). Only the ones with acidogenic seed (C3 and CBM3) had comparable amounts of VFA and EtOH production to Test Reactors, which got depleted within the first 3 weeks of operation period (Figure 4.11b and 4.14b). The higher amounts of VFA observed in acidogenic seed controls (C3 and CBM3), reaching up to 470 mg l^{-1} as HAc in CBM3, initially, might be due to the glucose left in the system from pre-acidification studies (see sCOD data in Table 3.8, Section 3.4.2). EtOH production observed in C1 and C2 on the last few weeks of operation might be due to fermentation of endogeneous breakdown products of microorganisms (Figure 4.9b and 4.14b).

4.2.3.2. Effect of BM on VFA/EtOH production

Effect of BM on VFA production was investigated by comparing the VFA production levels in Test Reactors with and without BM, seeded with same cultures (R1 and R4; R2 and R5; R3 and R6) and the results are illustrated in Figure 4.15-4.17 in terms of TVFA and EtOH production.

While the EtOH production in reactors without BM was greater than that of the ones with BM, TVFA production showed the opposite pattern. VFA productions in the reactors with BM were higher than that of reactors without BM. Use of BM almost doubled the production of VFAs in the reactors (Figure 4.15b-4.17b).

VFA production increased with the addition of BM in all of the reactors (Figure 4.15b - 4.17b). EtOH production in the Test Reactors with BM peaked in the first week and its production stopped (Figure 4.15a - 4.17a). BM addition did not increase EtOH production. When compared with the concentrations achieved in R1 and R2, it can be said that production of EtOH was reduced by the presence of BM, shifting to VFA production in Test Reactors containing BM. This might be because of the inhibition of EtOH producing microorganisms due high VFA production (exceeding 800 mg l^{-1} (as HAc) in the first week), which was stimulated with BM addition. BM addition cultivated the acidogens so that excess VFA in the system did not cause problems.

The shift to VFA production rather than ethanol observed in the Test Reactors with BM might be due to additional nutrients affecting the production of extra-cellular enzymes used in hydrolysis stage. Extra-cellular enzymes catalyze the hydrolysis reactions. Especially the trace metals present in the BM triggers the enzyme production since they are present in the structure of enzymes, as cofactors. Thus, BM might have increased the solubilization of the whey in the reactors, resulting in increased VFA production (Rittman and McCarty, 2001). Another reason for greater VFA production in the Test Reactors with BM might be the nutrients addition to the system, providing microorganisms more suitable conditions for survival, making them able to continue their fermentation in low pH conditions.

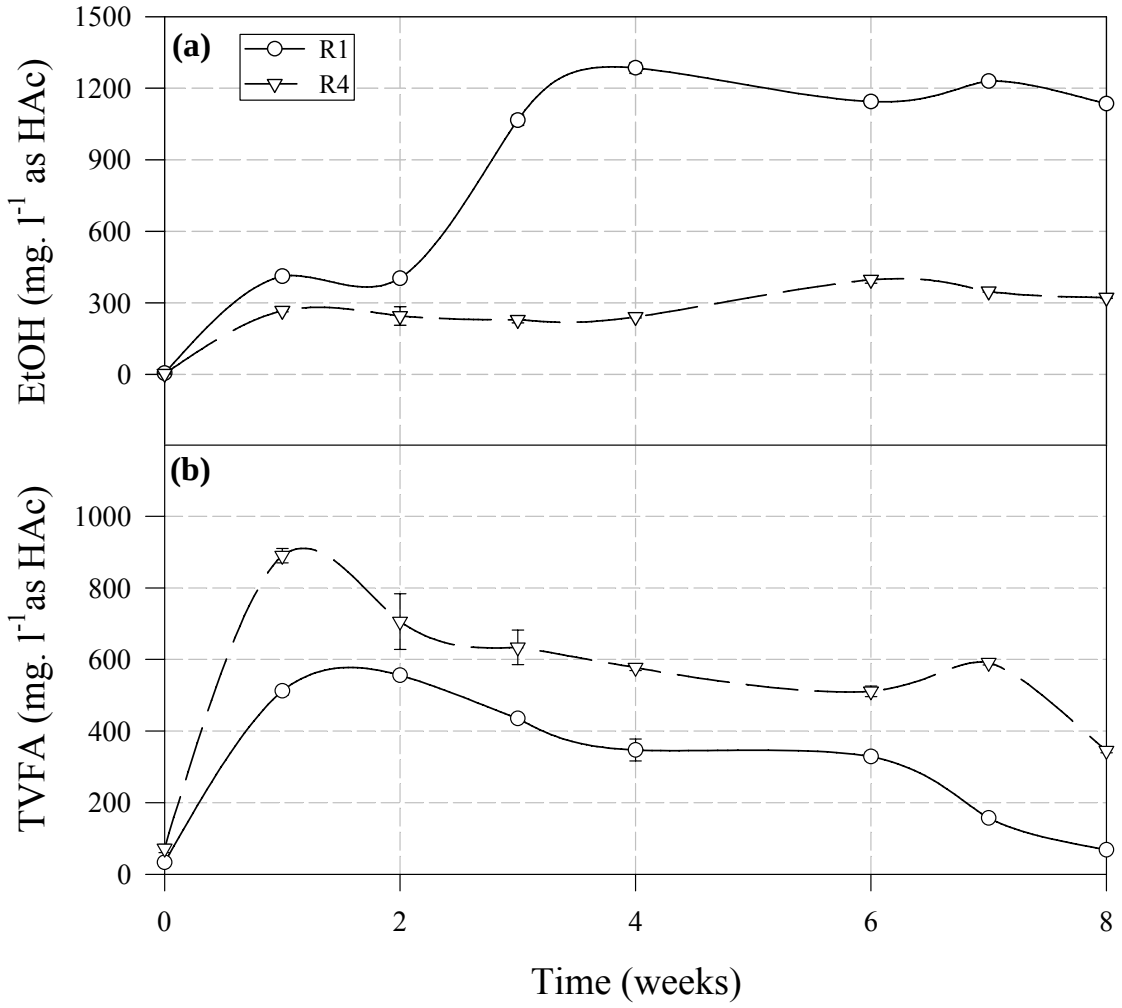

Figure 4.15. Effect of BM in reactors with MAC (R1 and R4)

(a) EtOH production (b) TVFA production

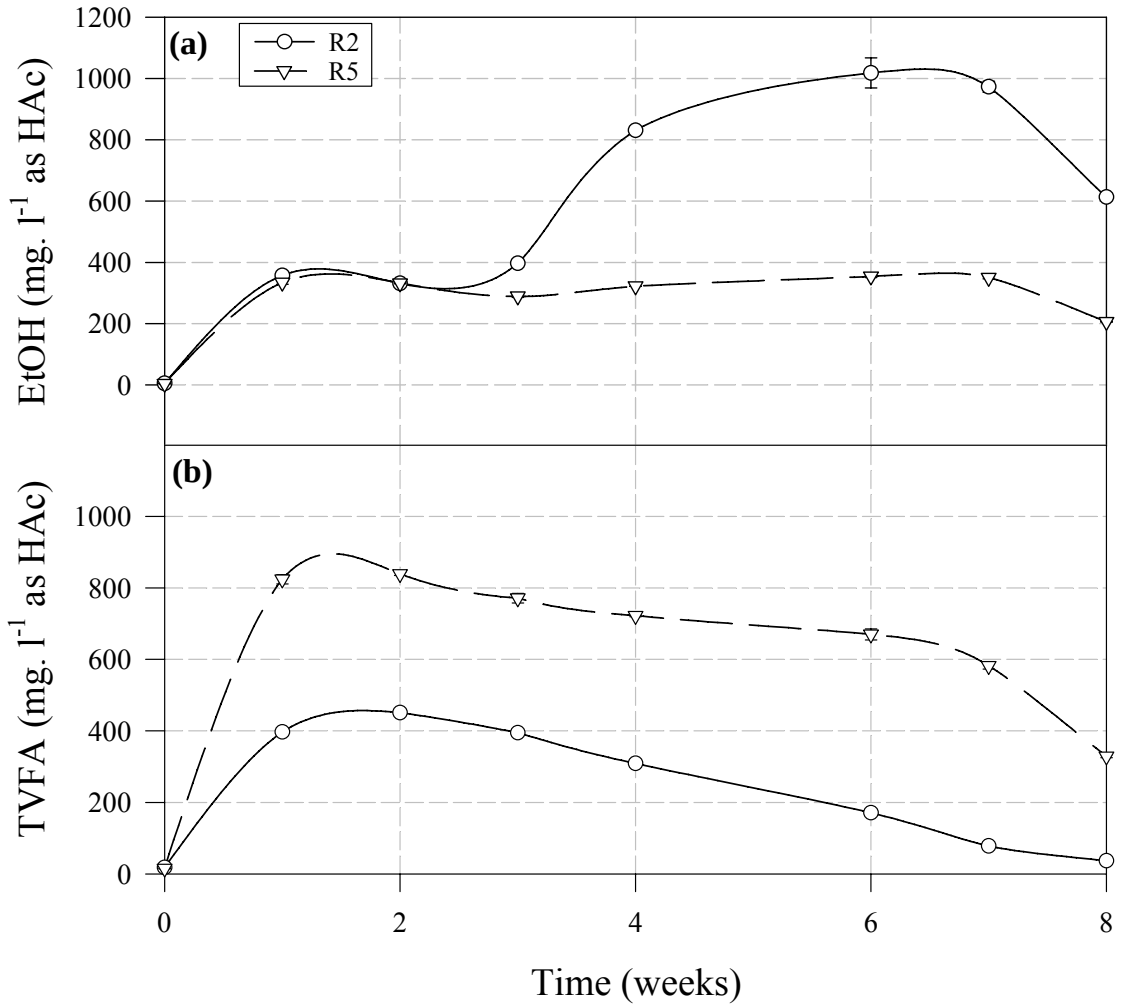

Figure 4.16. Effect of BM in reactors with HMAC (R2 and R5)

(a) EtOH production (b) TVFA production

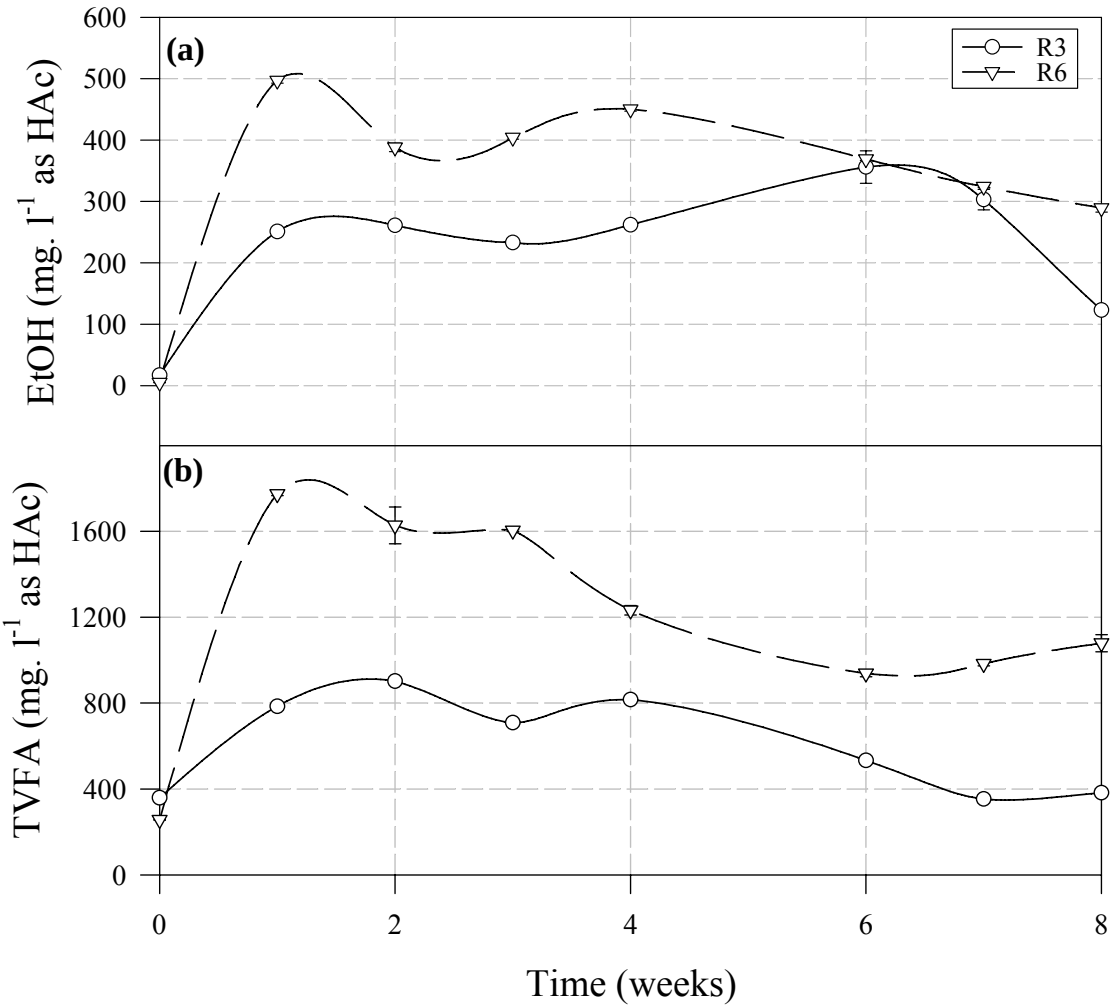

Figure 4.17. Effect of BM in reactors with AC (R3 and R6)

(a) EtOH production (b) TVFA production

4.2.3.3. Effect of Using Different Seed Types on VFA Production

Effect of using different seed types were investigated by cultivating different reactors with three different seed cultures, namely, MAC, HMAC and AC is depicted on Figures 4.18. Characterization of each seed culture was given in Table 3.4 in Section 3.2.

EtOH production was highest in the reactors R1 (seeded with MAC without BM) and R2 (seeded with HMAC without BM) reaching 1230 and 1018 mg l^{-1} (as HAc), respectively (Figure 4.18a). On the other hand, TVFA production was highest in R6 seeded with AC containing BM, reaching 1774 mg l^{-1} (as HAc) (Figure 4.13b). The achievement of highest VFA production in the reactor with acidogenic seed was as expected, since the microorganisms had adapted to acidifying conditions and were enriched before by pre-acidification of the seed.

Acid producing bacteria might be less in R1, R2, R4 and R5, while higher in R3 and R6. BM addition increased hydrolysis and production of acid producing bacteria, in the reactors, as mentioned before in Section 4.2.3.2. Thus, increase in VFA production that was observed in R4 and R5 when compared to R1 and R2, which contained the same seed cultures, respectively, might be due to the dominancy of VFA producing bacteria in these reactors.

The VFA concentrations in R1 and R2 might have become toxic to acid producing bacteria after some point. EtOH production observed in these reactors after second and third week for R1 and R2, respectively, might be due to this. Similarly, slight increase in EtOH concentrations was observed in R3, R4 and R5 after fourth week (Figure 4.18a).

When TVFA productions in the reactors were observed, it was seen that the TVFA in all of the reactors decreased after peaking in first or second week of operation (Figure 4.18b). The decrease in TVFA concentrations might be due to consumption of acids in cell growth.

Furthermore, when VFA varieties in reactors were observed, it was seen that in the reactors with acidogenic seed more types of VFA were produced (Figure 4.5c and 4.8c, Section 4.2.3). Similarly, this variation was due to the enriched acidogens present in the reactors and adaptations to acidic conditions from pre-acidification stage.

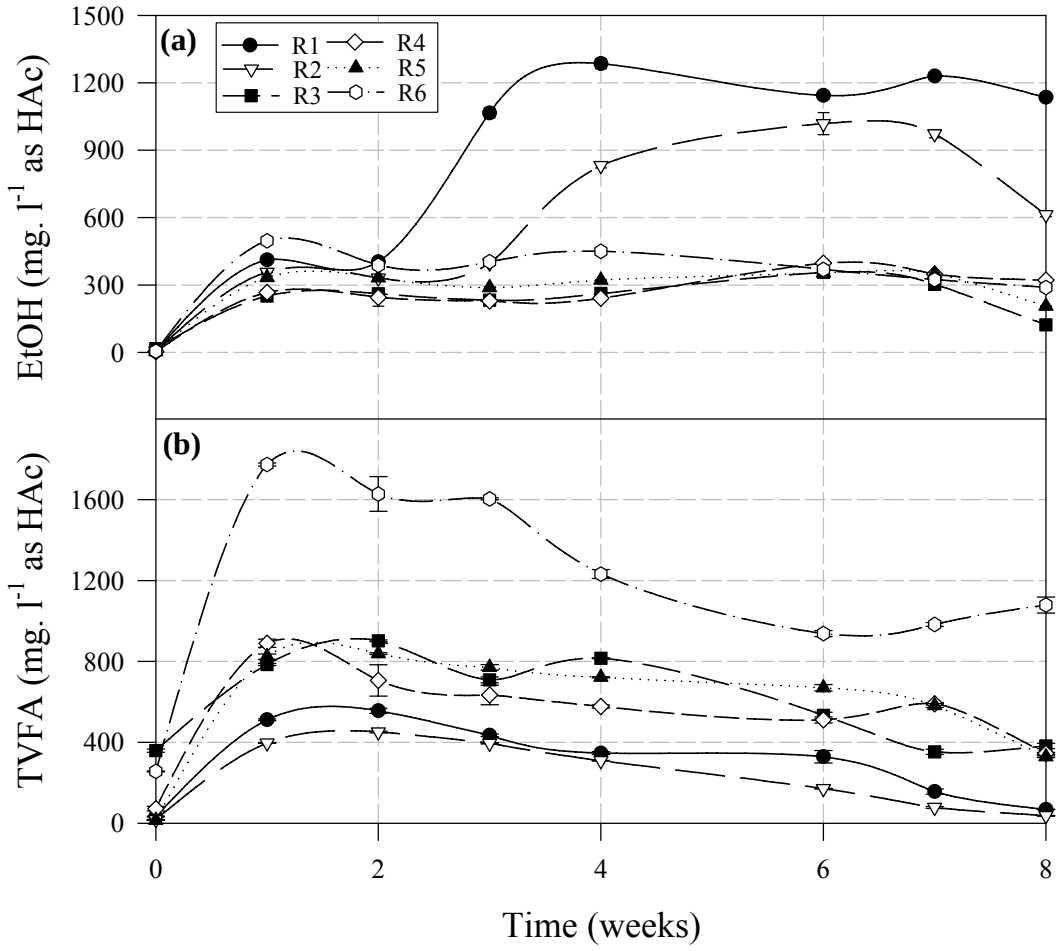

Figure 4.18. Comparison of seed cultures (MAC, HMAC and AC).

Individual major VFA and EtOH productions are depicted in Figure 4.15. More kinds of VFAs were produced in the reactors seeded with acidogenic cultures (R3 and R6). Only HAc and Buty were the products of R1, R2, R4 and R5 with trace amounts of HPr and Val in R4 and R5 (not exceeding 5 mg/l as HAc). HAc, Buty, HPr were the main VFAs in R3 and R6 with Val and Cap as the secondary VFAs in those two reactors.

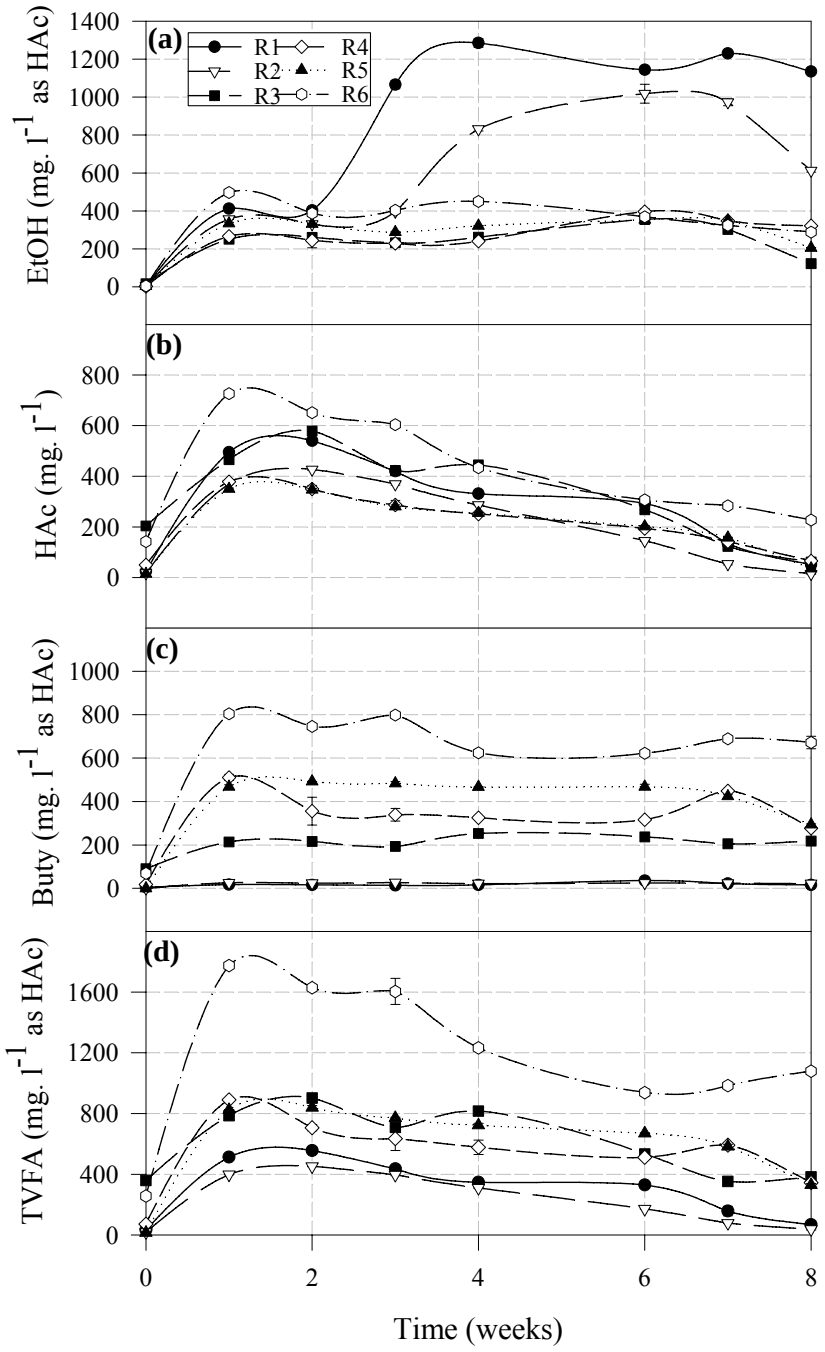

Figure 4.19. Major VFA and EtOH production in Test Reactors
(a) EtOH (b) HAc (c) Buty (d) TVFA profile

EtOH concentration was highest in R1 reaching 1285 mg l^{-1} (as HAc) (Figure 4.19a). EtOH production in R1 and R2 increased after 3 weeks of operation, which might be due to the degradation of proteins. Similarly, in a study done by Yu and Fang (2001), it was observed that EtOH production was largely associated with the protein degredation in the system, especially in high strength wastewaters, and that protein degradation only began after carbohydrates are fully degraded.

HAc and Buty concentrations peaked at 726 and 804 mg l^{-1} (as HAc) in R6, respectively (Figure 4.19b-c). HAc, Buty and HPr production were observed to be in accord with carbohydrate production, as mentioned before. HAc and Buty concentrations increased rapidly in all of the Test Reactors (Figure 4.19b-c). HPr production was observed, as major components, only in the reactors seeded with AC. HPr production peaked at 76 and 210 mg l^{-1} (as HAc) in R3 and R6, respectively (Figure 4.20a). Similarly to HAc and Buty production, HPr production also increased rapidly at first and continued afterwards. However, during sixth week, HPr concentrations dropped to zero and then started increasing in the seventh week, which might be due to the degradation of endogenous breakdown products.

Cap and Val production was observed only in reactors seeded with AC (Figure 4.20b-c). Production of these acids might be either via reductive de-amination of amino acids or by oxidation-reduction reaction between amino acid pairs (Stickland Reaction) because their production is related to protein degradation (McInerney, 1988). However, organisms producing val, i-val, cap or i-cap from amino acids, were found to be producing HAc and Buty in the presence of excess glucose, due to carbohydrate degradation (Saissac et al., 1948). Coinciding with the findings of Saissac et al. (1948), the productions of these

acids were not as significant as those of HAc, Buty or HPr, probably due to the excess carbohydrates present in the wastewater. Cap production reached a maximum of 23 and 45 mg l^{-1} (as HAc) in R3 and R6, respectively, while Val acid production peaked 9 mg l^{-1} (as HAc) in both reactors. Although production of these two acids are largely associated with acidification of protein (Yu and Fang, 2001), their production peaked immediately, in the first few weeks of operation. Cap concentrations in R3 remained almost the same throughout the operation period, while its production in R6 peaked and then dropped almost to zero during sixth week and increased on seventh week (Figure 4.20b). Similarly, the production of Val dropped to zero on the sixth week and increased again on the seventh week of operation in both of the reactors (Figure 4.20c) which might be due to the degradation of endogenous breakdown products, as mentioned before.

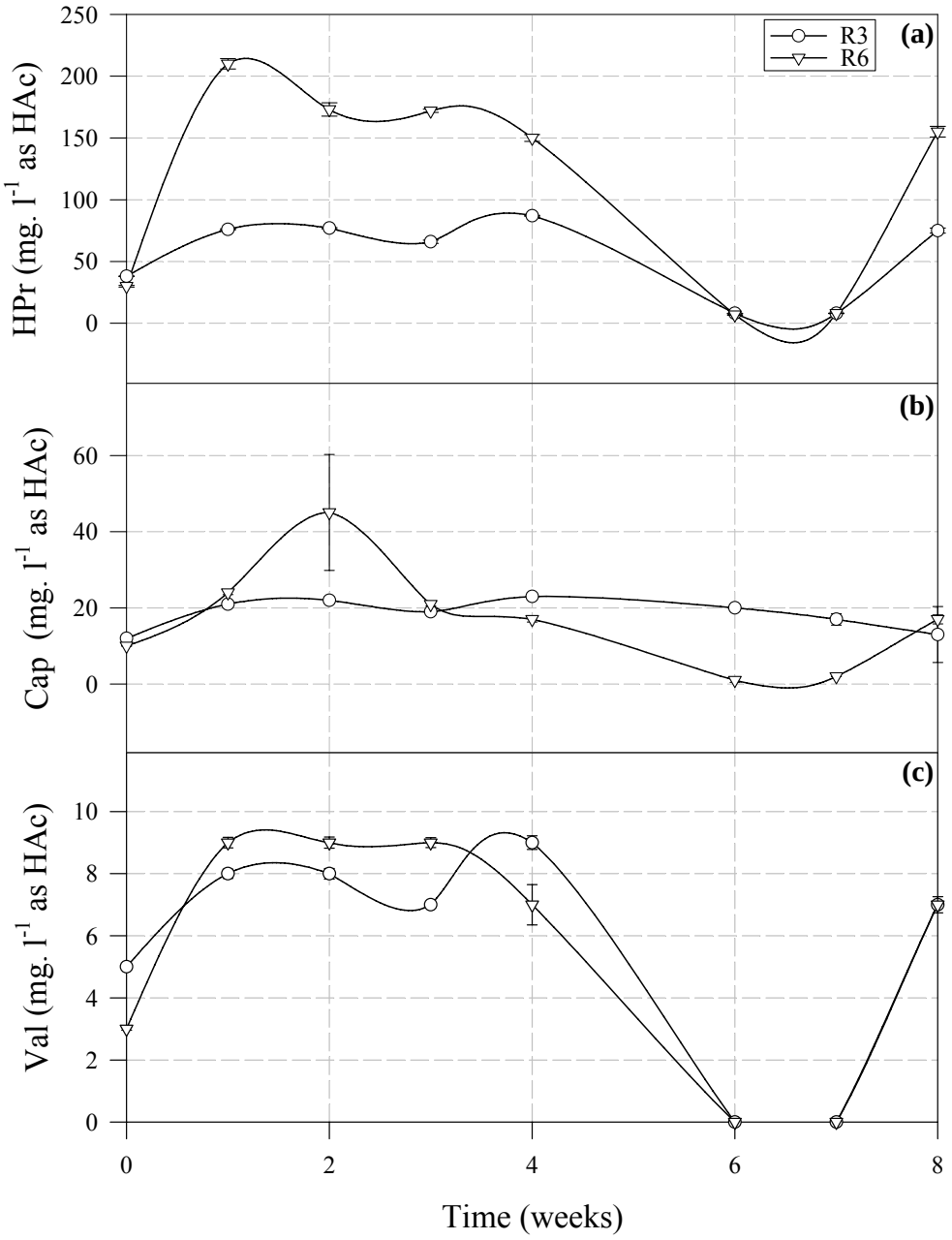

Figure 4.20. Secondary VFAs production profile in R3 and R6

(a) HPr (b) Cap (c) Val

4.2.4. Degree of Acidification in the Test Reactors

Degree of acidification in the reactors were calculated by taking the ratio of COD-equivalent of acidogenic products and the wastewater COD for each week. Acidogenic products determined in reactors were VFAs (HAc, Buty, HPr, Cap and Val) and EtOH. Gaseous products were not included since gas analysis were not performed. Degree of acidification determined for each Test Reactor is depicted in Figure 4.21.

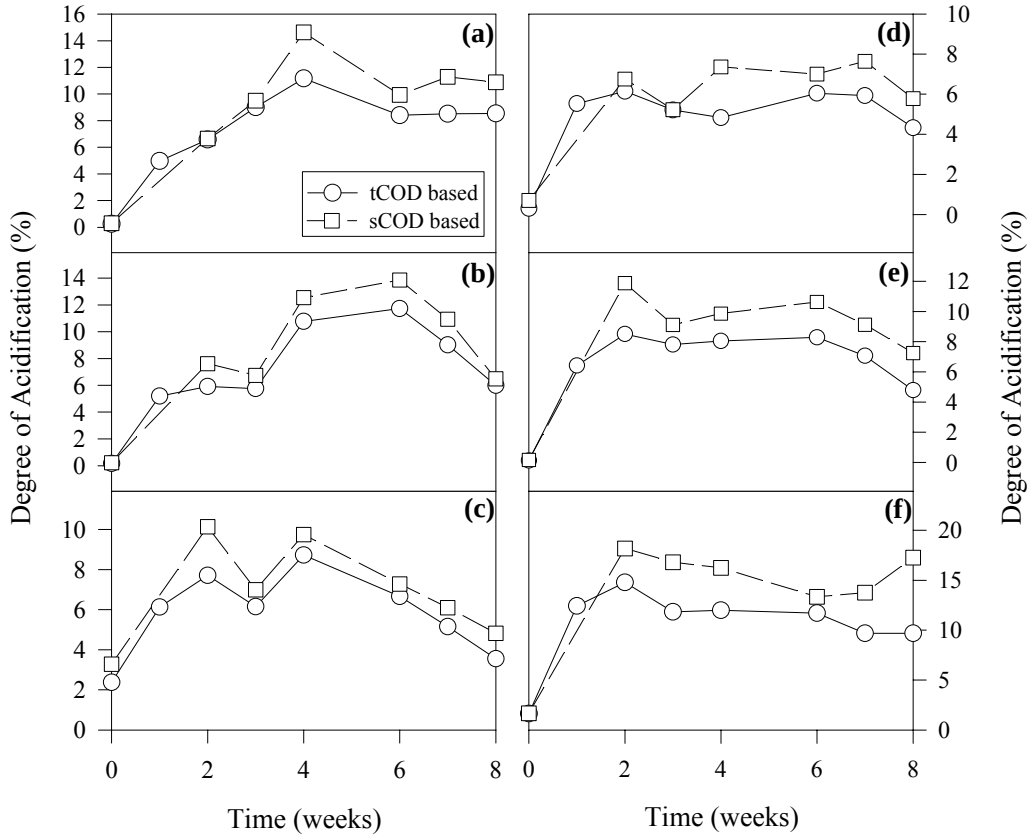

Figure 4.21. Degree of acidification in
(a) R1 (b) R2 (c) R3 (d) R4 (e) R5 (f) R6

Maximum degrees of acidifications achieved in Test Reactors were, 14.6, 13.8, 10.1, 7.4, 11.9 and 18.2 % for R1, R2, R3, R4, R5 and R6, respectively (Figure 4.21). Highest degree of acidification was observed in R6, reactor containing BM and seeded with acidogenic culture. Yu and Fang (2001) had observed that degree of acidification decreased with the increase of wastewater COD and the maximum degree of acidification of 57.1% was observed at 2g COD l^{-1} of initial wastewater concentration. On the other hand, degree of acidification observed at 12 g COD l^{-1} (which was the influent concentration in this study, as mentioned before) was 44.5%. Although higher degrees of acidification have been observed in the literature, reaching up to 57.1 % in a study done with dairy wastewaters (Yu and Fang, 2001), acidification degrees obtained in this study correspond and lie between the values given in literature (Guerrero et al., 1999; Yu and Fang, 2001). Higher degrees of acidification could have been detected if all of the acidification products and gases were included. Another reason for observing lower degrees of acidifications might be the uncontrolled pH values in our experiments. While the pH of the reactors of Yu and Fang (2001) were at pH values around 6.1-6.4, the pH values in our reactors were around 3. Effects of pH on acidification should be investigated on further studies.

4.2.4.1. Maximum Specific Acidogenic Activity of the Effluent from Test Reactors

A final maximum specific acidogenic activity assay was performed with the Test Reactor effluent sludge obtained at the end of the operation period (eight week). While the maximum specific acidogenic activities of the seed cultures used in this experiment were found to be 7.01, 13.28 and 6.41 g COD.g^{-1}VSS.d^{-1} for acidogenic, mixed anaerobic and heated mixed anaerobic cultures, respectively at the beginning of experiments (Table 3.4), the maximum specific

acidogenic activities of seeds from Test Reactor effluents were 18.07, 15.85, 9.9, 9.65, 11.8 and 32.42 g $COD.g^{-1}VSS.d^{-1}$ for R1, R2, R3, R4, R5 and R6, respectively (Table 4.3). Graphs for acidogenic assay are provided in Appendix C. The analysis showed that there had been a comparable increase in the activities of the cultures used, indicating successful acidogenesis in the reactors. The values correspond to values mentioned in the literature (Soto et al., 1993, Hutnan et al, 1999, Punal et al., 1999).

Table 4.3. Acidogenic activities of seed cultures from Test Reactor effluents.

	Activity **g $COD.g^{-1}VSS.d^{-1}$**
R1	18.07 ± 3.2
R2	15.85 ± 4.4
R3	9.90 ± 2.5
R4	9.65 ± 1.2
R5	11.80 ± 1.6
R6	32.42 ± 4.2

4.2.4.2. VFA Potential of Cheese-Whey

Individual acids potential of cheese-whey was calculated taking into account of the values achieved in R6, which was gave the maximum VFA production. For this reason the following calculations were carried out for this reactor.

Unit prices of HAc, Buty and HPr were calculated, by using Turkey's 2004 export statistics (TİK, 2006), as 425.9, 2407.1 and 3613.5 US dollars per m^3 of each product, respectively. Unit price of EtOH was taken as 132,12 US dollars

per m^3 (Renewable Fuels Association, 2005), while the unit price of CH_4 was taken as 0.13 US dollars per m^3 (Demirer, 2005).

In this study it was achieved that 0.00315, 0.00346, 0.00419 and 0.00106 liters of EtOH, HAc, Buty and HPr was produced per 1 L of cheese-whey consumed in R6. According to Ergüder et. al. (2000) 23.4 liters of CH_4 could be produced per liter of cheese-whey.

As a consequence, when the gain from 1 liter of cheese-whey was calculated, the following results were obtained:

- EtOH : 4.2×10^{-4} \$ / liter of cheese-whey
- HAc : 1.5×10^{-3} \$ / liter of cheese-whey
- Buty : 1×10^{-2} \$ / liter of cheese-whey
- HPr : 3.8×10^{-3} \$ / liter of cheese-whey
- CH_4 : 3×10^{-3} \$ / liter of cheese-whey

The results indicated that under the studied conditions the most profitable product is Buty.

4.2.5. COD and Total Solids Profile of the Reactors

In order to investigate the hydrolysis and degradation in the Test Reactors, weekly COD and TS analysis were performed and is illustrated on Figure 4.24 and Figure 4.25, respectively. Unlike COD, TS of Blank and Control Reactors were also monitored (Figure 4.22a-b).

COD in all of the Test Reactors increased in the first week and then decreased with weekly fluctuations till the end of operation period (Figure 4.22).

Although it was expected to observe a decrease in the tCOD in the reactors due to solubilization and degradation of readily biodegradable substrates (Barajas et al., 2003), an increase was observed in all of the Test Reactors, except R4. However, tCOD in the reactors started decreasing after first week of operation. On the other hand, sCOD of most of the reactors increased at first due to solubilization and inhibition of methanogenic activity in the system resulting from low pH conditions and then decreased parallel to tCOD values.

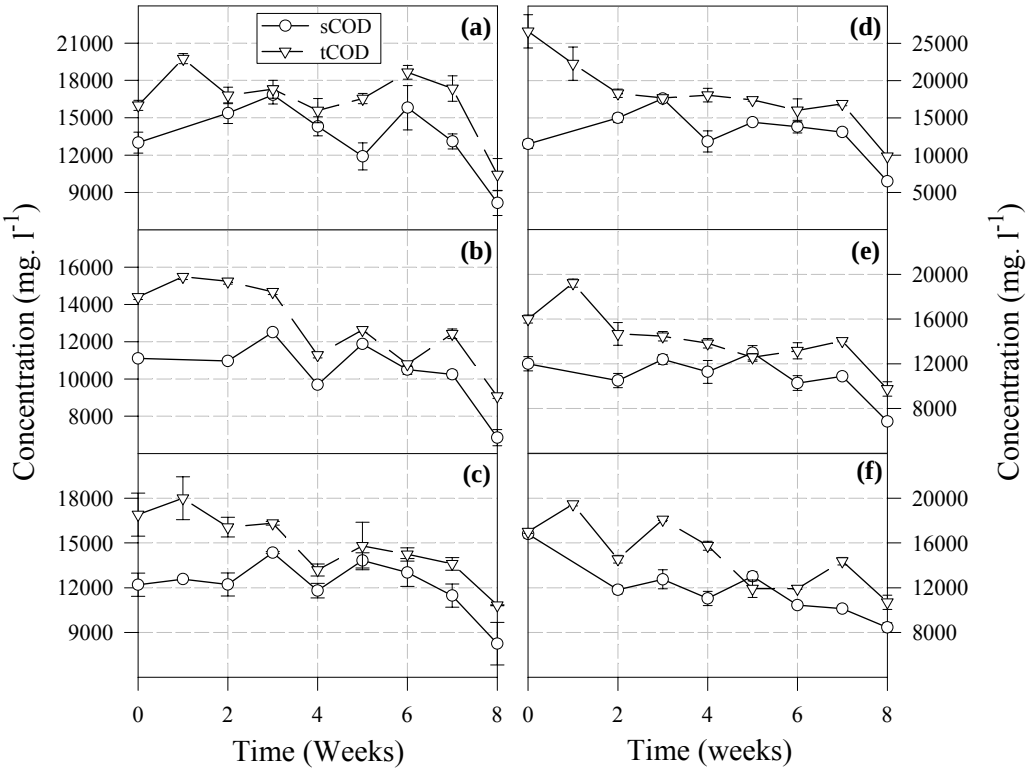

Figure 4.22. tCOD and sCOD profile of the Test Reactors
(a) R1 (b) R2 (c) R3 (d) R4 (e) R5 (f) R6

TS concentration in the Test Reactors decreased during the first week and then remained at almost constant levels till the end of operation period (Figure 4.23c). The decrease observed in the first week might be due to the solubilization and degradation of readily biodegradable substances (Barajas et al., 2003). TS concentration of Blank Reactors showed a similar pattern as Test Reactors (Figure 4.23a), again probably due to hydrolysis and biodegradation, which was probably accomplished by microorganisms present in cheese-whey. TS concentration in Control Reactors were almost constant throughout the operation period (Figure 4.23b).

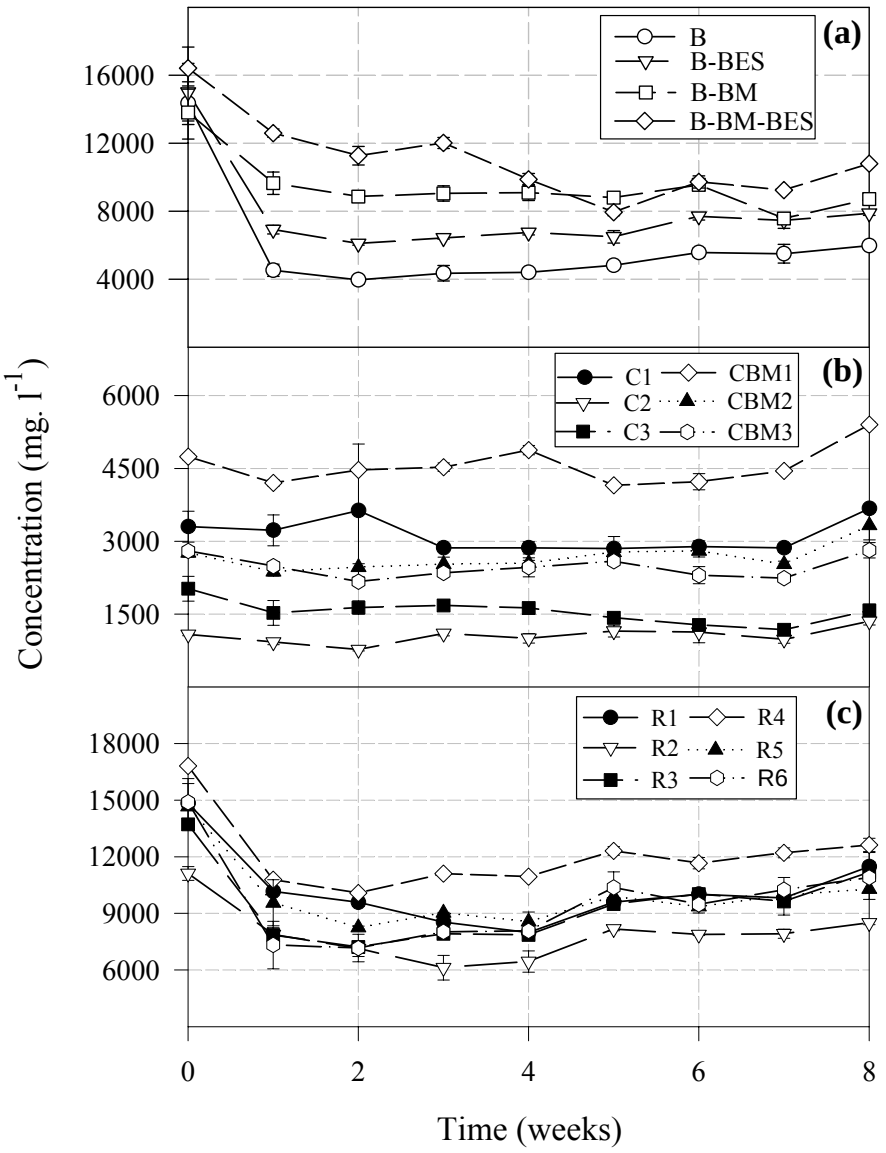

Figure 4.23. TS Profile of (a) Blank (b) Control (c) Test Reactors

4.2.6. Effluent Characteristics

At the end of the operation period of 8 weeks, effluent from reactors were collected and subjected to further MLSS, MLVSS, FSS, NH4-N and PO4-P analysis.

4.2.6.1. Results of MLSS/MLVSS/FSS Analysis

Increase in SS concentrations (especially VSS concentrations) were observed in Blank and Test Reactors. On the other hand, SS concentrations in Control Reactors dropped (Table 4.4). The increase observed in Blank and Test Reactors might be due microbial growth (EtOH forming microorganisms in Blank Reactors, VFA and/or EtOH forming microorganisms in Test Reactors).Therefore, growth of microorganisms, due to the adaptation of microbial species to operational conditions, might have resulted in this increase in the solids concentration in all the reactors.

Table 4.4. Initial and final MLSS, MLVSS and FSS concentrations in Blank, Control and Test Reactors.

	INITIAL			FINAL		
	MLSS	FSS	MLVSS	MLSS	FSS	MLVSS
B	680	10	670	1720	180	1540
B-BES	625	5	620	1660	320	1340
B-BM	905	20	885	1400	180	1220
B-BM-BES	720	10	710	1920	220	1700
C1*	920 ± 5	465 ± 6	455 ± 30	710 ± 42	270 ± 99	440 ± 141
C2	880	340	540	520	120	400
C3	430	10	420	180	40	140
C-BM1	1020	350	670	880	120	760
C-BM2	1108	492	615	920	120	800
C-BM3*	570 ± 40	90 ± 11	480 ± 25	300 ± 5	40 ± 1	260 ± 6
R1*	1900 ± 3	420 ± 127	1480 ± 42	2240 ± 85	250 ± 127	1990 ± 42
R2	2108	369	1738	2900	180	2720
R3*	1350 ± 73	130 ± 18	1220 ± 110	1630 ± 156	60	1570 ± 156
R4*	1970 ± 83	460 ± 34	1510 ± 28	2460 ± 57	250 ± 71	2210 ± 14
R5*	2010 ± 97	520 ± 98	1490 ± 28	2540 ± 113	380 ± 28	2160 ± 85
R6	1390	80	1310	2100	0	2100

* Analyzed in duplicates. All concentrations in mg/l

Drop in SS concentrations observed in Control Reactors (Table 4.7) might be as a result of the death of microorganisms due limited or no substrate present in the reactors.

4.2.6.2. Results of NH_4-N and PO_4-P Analysis

NH_4-N and PO_4-P analyses were performed in all of the Blank, Control and Test Reactors (Table 4.5 and Table 4.6). NH_4-N analysis indicated that there had been a considerable reduction in the NH_4-N within the Test Reactors, reaching up to 90% NH_4-N reduction in R1 –MAC without BM-. The lower percent reduction observed in reactors with BM is probably due to the N

species added to the system with BM addition (Section 3.3) or due protein fermentation (via de-amination or Stickland Reaction) resulting in an increase in NH_4-N concentrations (R4, R5 and R6 –reactors with BM- had greater VFA production than R1, R2 and R3 – reactors without BM -, respectively). However, similar reductions were observed in Blank and Control Reactors too. The reductions in NH_4-N concentration in Blank Reactors were probably due to the presence of microorganisms in the raw cheese-whey left from manufacturing processes. On the other hand, NH_4-N reductions observed in the Control Reactors were probably due to the endogenous breakdown of the microorganisms, for cell growth.

Table 4.5. Initial and final NH_4 and NH_4-N concentrations and % reductions achieved in Blank, Control and Test Reactors.

	INITIAL		FINAL		% Reduction	
	NH_4	NH_4-N	NH_4	NH_4-N	NH_4	NH_4-N
B	7	5	2	2	70	68
B-BES	7	6	1	1	81	79
B-BM	310	240	196	152	37	37
B-BM-BES	235	180	209	162	11	10
C1*	28 ± 0.7	22 ± 2	8 ± 1	6 ± 0.9	72	72
C2	35	27	3	2	92	91
C3	39	31	1	1	96	96
C-BM1	305	240	230	178	25	26
C-BM2	405	315	202	158	50	50
C-BM3*	420 ± 10	325 ± 14	132 ± 6	102 ± 5	69	69
R1*	54 ± 2.1	42 ± 2.1	4 ± 0.4	4	93	90
R2	45	35	4	4	91	89
R3*	51 ± 3	39 ± 4	11 ± 1	9 ± 0.8	78	77
R4*	290 ± 14	230 ± 28	95 ± 35	74 ± 27	67	68
R5*	390 ± 35	300 ± 21	106 ± 22	82 ± 17	73	73
R6	300	240	96	74	68	69

* Analyzed in duplicates
All concentrations in mg/l

Moreover, as can be seen from Table 4.2 around 80% of the gas in all of the reactors was N_2, while the rest was CO_2. At first, it was thought that this might be an indication of denitrification in the system of the nitrogen available in cheese-whey. Denitrification is the process of reducing nitrate, a form of nitrogen available for consumption by many groups of organisms, into gaseous nitrogen. In general, it occurs when oxygen (which is a more favorable electron acceptor) is depleted, and bacteria turn to nitrate in order to respire organic matter. However, denitrifying bacteria are known to be sensitive to low pH conditions (Bremner and Shaw, 1958; Klemedtsson et al, 1977). Furthermore, when initial and final concentrations of NH_4-N in the reactors were observed it was seen that similar NH_4-N reductions were obtained in Blank and Control reactors too (Table 4.5). Therefore, it was concluded that denitrification was not the process occurring here.

Similar results were observed in PO_4-P analysis (Table 4.6). However, % reduction values were lower than that of NH_4-N reduction in the reactors. Maximum reduction was observed in R6 (82 %), the reactor operated with pre-acidified seed and BM. The decrease observed in Blank and Control Reactors were probably due to use of P in cell synthesis. Furthermore, an increase in P concentration in CBM2 and CBM3 was observed. This increase might have been due to the precipitation of P during initial stage due to high pH conditions and solubilisation of P later on.

Table 4.6. Initial and final ortho-phosphate concentrations and % reductions achieved in Blank, Control and Test Reactors.

	INITIAL		FINAL		% Reduction	
	PO_4	PO_4-P	PO_4	PO_4-P	PO_4	PO_4-P
B	179	58	54	18	70	69
B-BES	152	50	67	22	56	56
B-BM	295	95	124	40	58	58
B-BM-BES	320	110	134	44	58	60
C1*	11 ± 0.7	4 ± 0.4	5 ± 0.7	2 ± 0.3	55	52
C2	12	4	10	3	11	4
C3	6	2	5	1.7	17	16
C-BM1	48	16	11	4	77	78
C-BM2	16	5	32	10	inc (100%)	inc (100%)
C-BM3*	9 ± 0.7	3 ± 0.7	26 ± 1.4	8 ± 0.1	inc (200%)	inc (160%)
R1*	320 ± 11.3	110 ± 7.1	81 ± 5.7	26 ± 1.4	75	76
R2	230	70	56	18	76	74
R3*	260 ± 21.2	80 ± 10.6	64 ± 1.8	21 ± 1.8	75	74
R4*	340 ± 14.1	110 ± 7.1	64 ± 17	21 ± 4.2	81	81
R5*	300 ± 21.2	100 ± 14.1	81 ± 29	27 ± 9.9	73	73
R6	290	90	50	16	83	82

* Analyzed in duplicates
All concentrations in mg/l

CHAPTER 5

CONCLUSION

The following conclusions can be made depending on the experimental results of this study:

- pH of the reactors were not controlled during the experiments. Therefore, a drastic pH drop was observed in the system in both sets of experiments due to tendency of cheese-whey to acidification. This drop in pH inhibited the methanogenic activity in the reactors.

- No CH_4 was found in gas composition analysis of both sets of experiments, proving the inhibition of methanogenic activities.

- Effect of HRT and OLR was observed in Set 1 experiments. It was found that TVFA production in the reactors increased with increasing OLRs due to inhibition of methanogens. This might be due to the sensitivity of methanogenic microorganisms to high substrate concentrations.

- TVFA production increased with increasing HRT, which might be due to low pH conditions observed, suppressing acidogenic bacteria.

- The most suitable OLR was determined according to degree of acidification achieved in the reactors in Set 1 experiments. The highest degree of acidification was achieved in reactors with 15 g COD l^{-1} OLR.

- Main acidogenesis products were HAc, Buty and HPr with smaller quantities of i-Buty, Val and Cap. Excessive production of EtOH was observed in Blank Reactors, which was probably due to the microorganisms or yeast present in raw cheese-whey left from the manufacturing processes. Lower quantities of HAc was produced in Blank Reactors when compared to EtOH production. It was seen that, the EtOH production observed in Blank Reactors shifted to VFA production in the Test Reactors, probably due to the enrichment of microorganisms with seed addition.

- Three different seed cultures were used in Set 2; MAC, HMAC and AC. BES was added to reactors with MAC to inhibit the methanogens. Higher VFA productions and variety of VFA types were observed in Test Reactors seeded with AC (R3 and R6).

- BM had a suppressive effect on EtOH production, while it stimulated the VFA production in the reactors. Highest total VFA production was observed in R6 (seeded with AC and containing BM) reaching 1776 mg/l as HAc at the end of the first week. The corresponding degree of acidification for R6 was found as 18.2 %.

CHAPTER 6

RECOMMENDATIONS FOR FUTURE WORK

- Effect of pH in VFA production from cheese-whey should be investigated. It is known that by controlling the pH of the system production schemes of VFA's can be changed (Zoetemeyer et al., 1982, Houriuchi et al., 2001, Kisaalita et al., 1986). Thus, the next step in the studies should be on determining the optimum pH for maximum VFA production and determining its production scheme from cheese-whey. However, high tendency of cheese-whey for acidification should be kept in mind, for the amount of consumables to be used.

- For better optimization of TVFA production or individual VFAs (HAc, Buty, HPr etc.) production determining the most suitable temperatures, reactor types and/or HRTs can be advantageous.

- Recovery of VFAs and/or EtOH from reactor effluents should be studied.

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APPENDIX A

ACIDOGENIC ACTIVITY ASSAY GRAPHS
FOR
SEED CULTURES

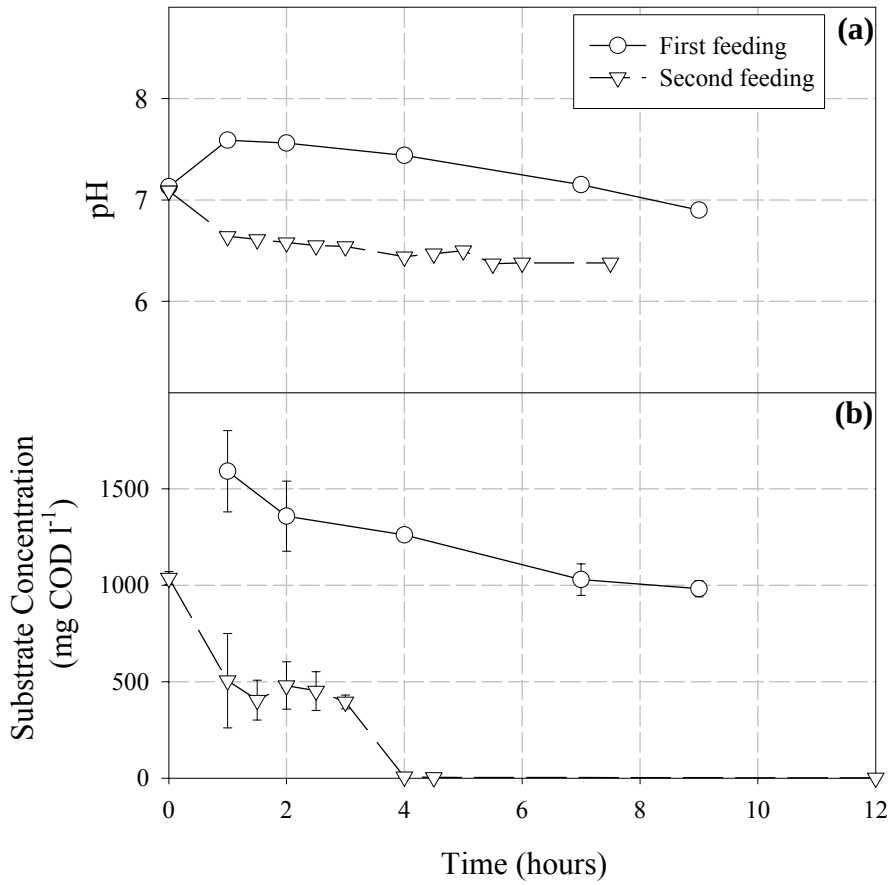

Figure A.1. Acidogenic Activity Graphs for AC (a) pH profile (b) Substrate degradation profile

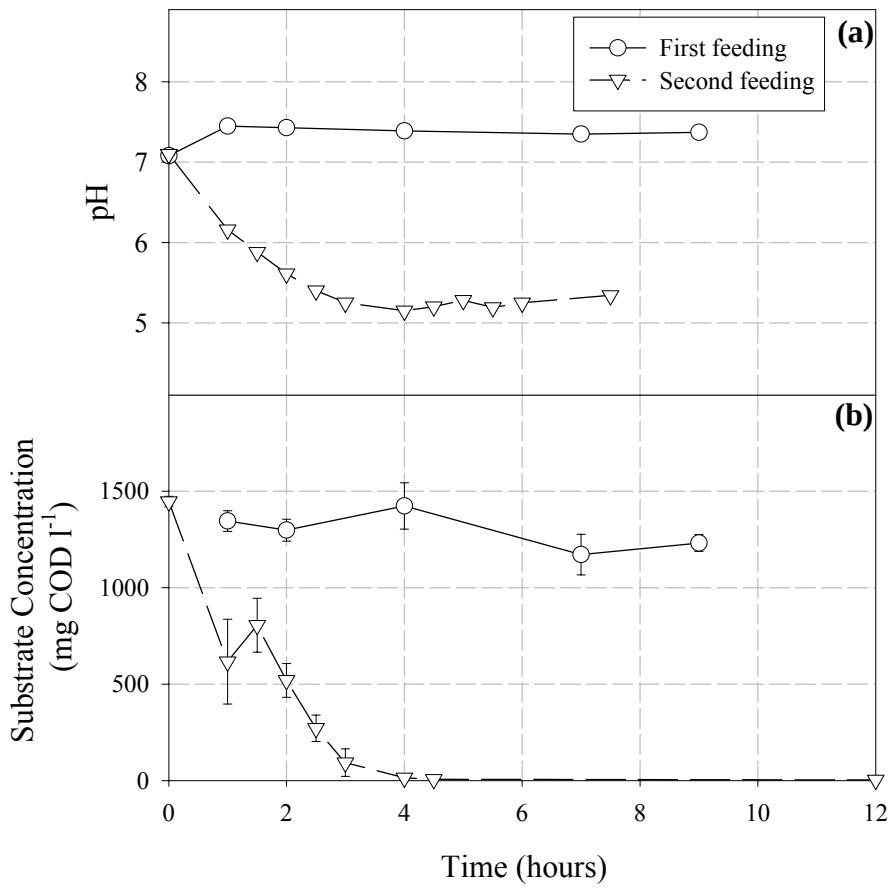

Figure A.2. Acidogenic Activity Graphs for MAC (a) pH profile (b) Substrate degradation profile

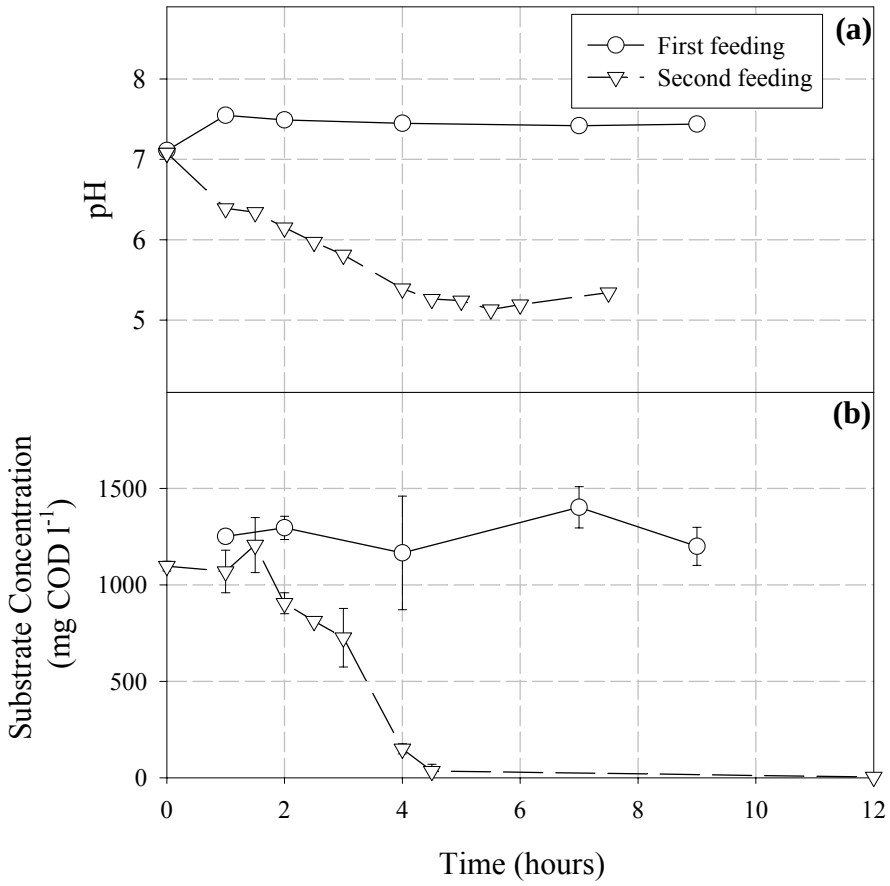

Figure A.3. Acidogenic Activity Graphs for HMAC (a) pH profile (b) Substrate degradation profile

APPENDIX B

CALIBRATION GRAPH
FOR
TITRATION VS GC ANALYSIS

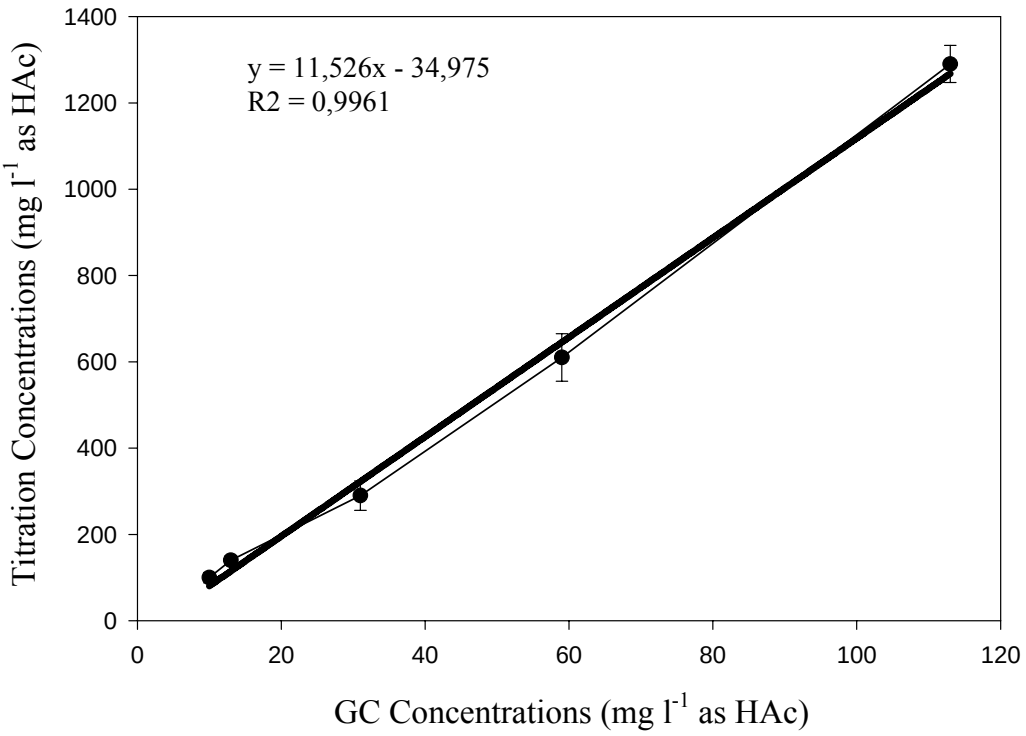

Figure B.1. Calibration graph for titration vs GC analysis

APPENDIX C

ACIDOGENIC ACTIVITY ASSAY GRAPHS
FOR
TEST REACTOR EFFLUENTS

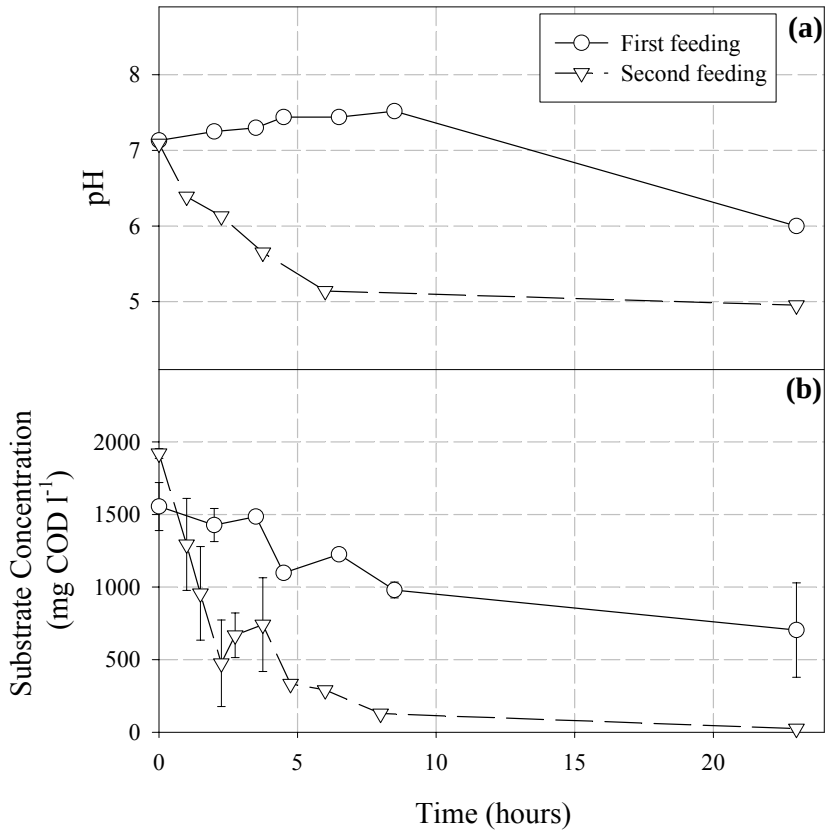

Figure C.1. Acidogenic Activity Graphs for R1 (a) pH profile (b) Substrate degradation profile

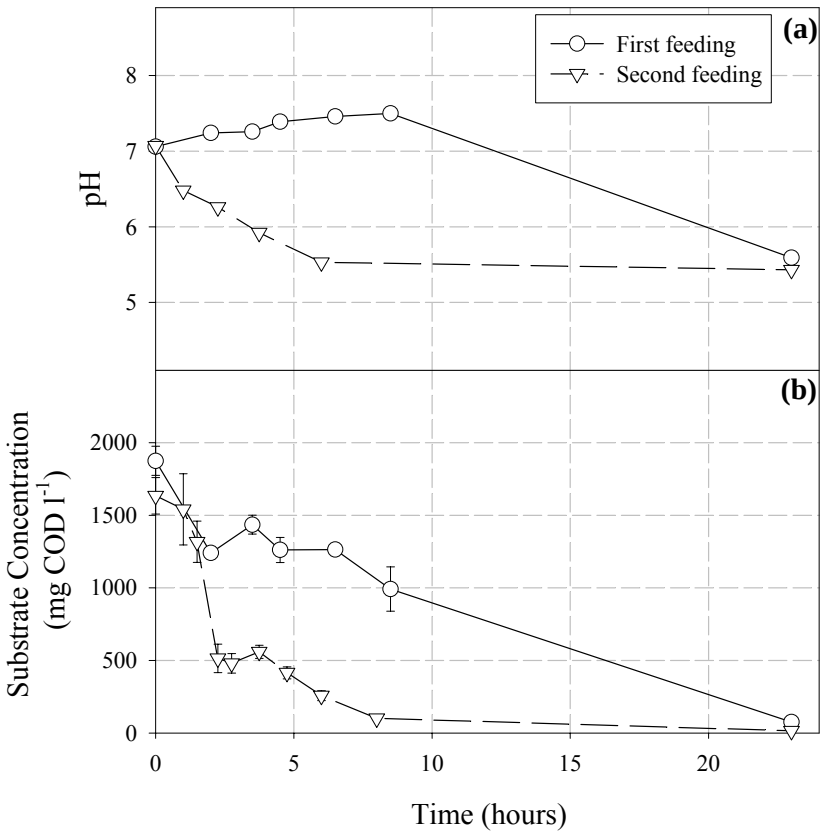

Figure C.2. Acidogenic Activity Graphs for R2 (a) pH profile (b) Substrate degradation profile

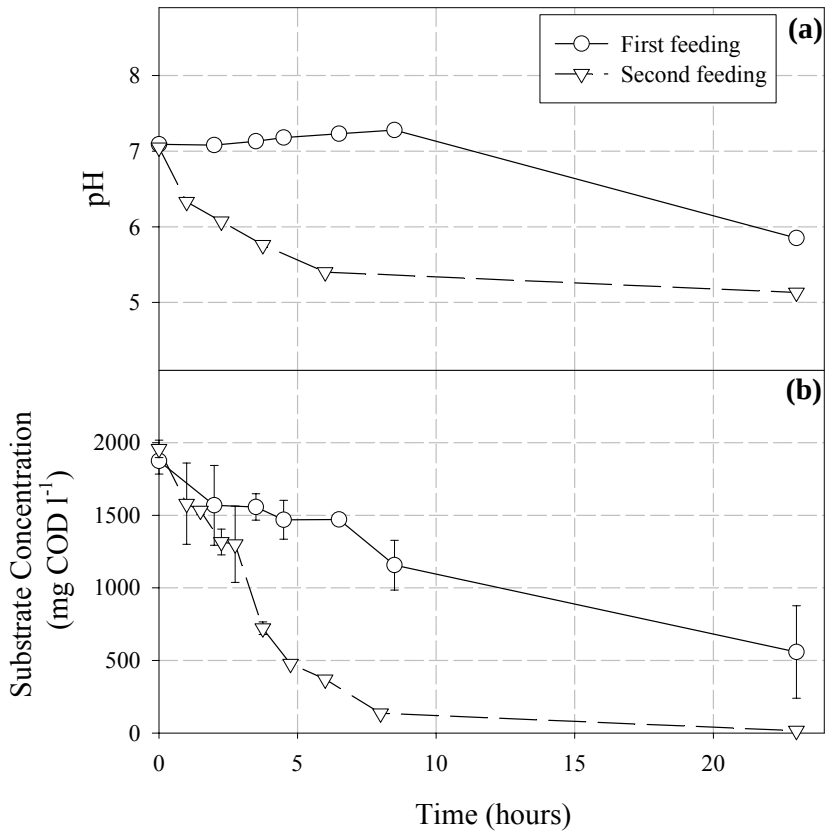

Figure C.3. Acidogenic Activity Graphs for R3 (a) pH profile (b) Substrate degradation profile

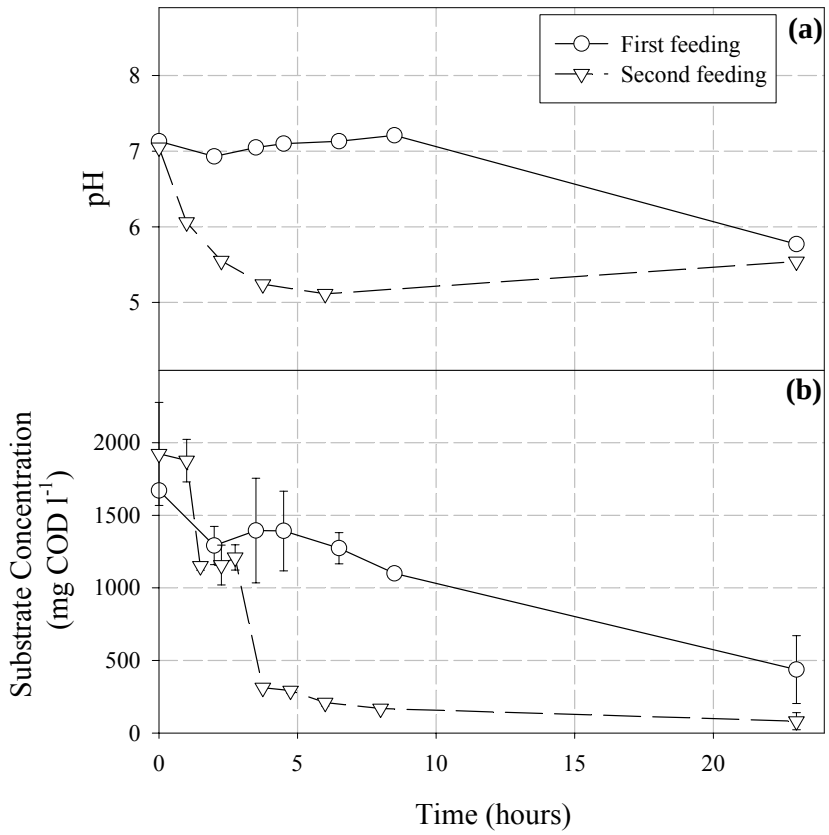

Figure C.4. Acidogenic Activity Graphs for R4 (a) pH profile (b) Substrate degradation profile

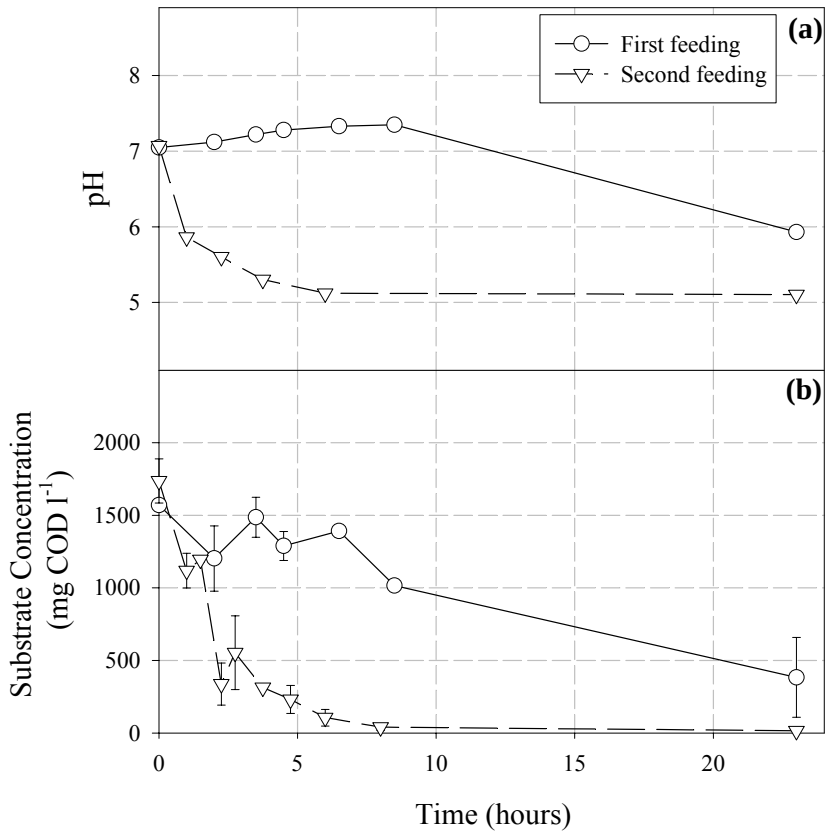

Figure C.5. Acidogenic Activity Graphs for R5 (a) pH profile (b) Substrate degradation profile

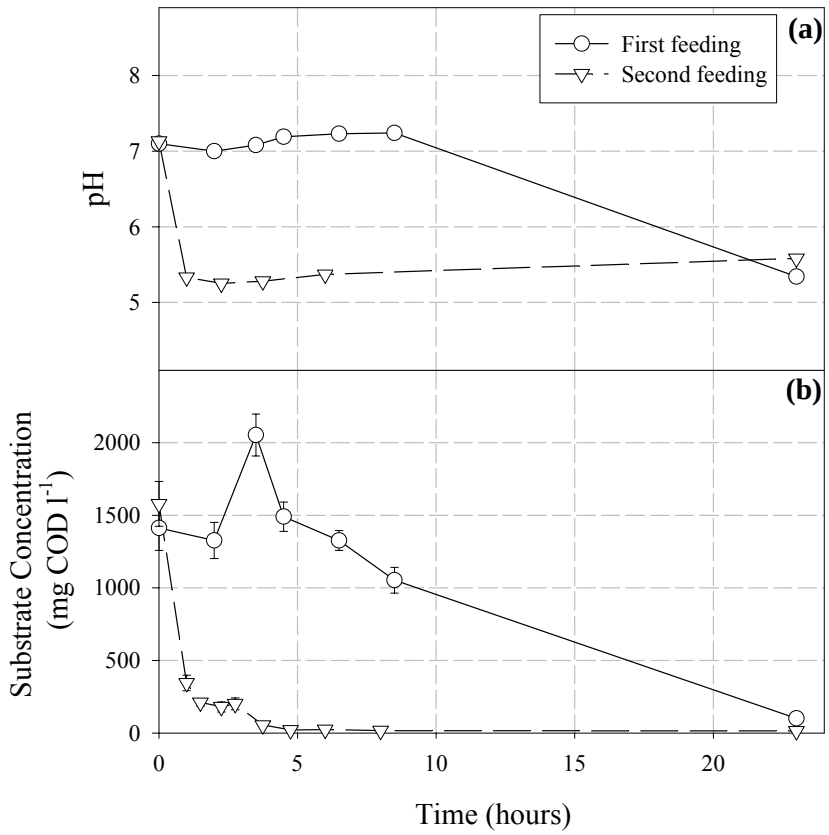

Figure C.6. Acidogenic Activity Graphs for R6 (a) pH profile (b) Substrate degradation profile