İSTANBUL TECHNICAL UNIVERSITY ★ INSTITUTE OF SCIENCE AND TECHNOLOGY

POPULATION DYNAMICS IN BUTYRATE DEGRADING COMMUNITIES IN ANAEROBIC BIOREACTORS

Ph.D. Thesis by
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Department : Environmental Engineering
Programme : Environmental Engineering

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POPULATION DYNAMICS IN BUTYRATE DEGRADING COMMUNITIES IN ANAEROBIC BIOREACTORS

SUMMARY

An important intermediate of organic matter conversion under methanogenic conditions is butyrate; which may account for up to 60% of methanogenesis in anaerobic bioreactors. The aim of this research was to get a deeper and better insight into the population dynamics of syntrophic butyrate oxidizing microbial consortia in anaerobic bioreactors using conventional and molecular techniques. Moreover, the investigation was extended to examining the combined cultivation under methanogenic and sulfidogenic conditions. For this purpose, biomass adapted to butyrate using Upflow Anaerobic Sludge Blanket (UASB) bioreactors and different incubation techniques and then molecular techniques were used to characterize this butyrate adapted biomass. The use of combination of conventional and molecular techniques (MPN: Most Probable Number, PCR-RFLP: Polymerase Chain Reaction-Restricted Fragment Length Polymorphism, DGGE:Denaturing Gradient Gel Electrophoresis, Q-PCR: Quantitative PCR, and SIP:Stable Isotope Probing) enabled us to identify several species in an anaerobic syntrophic butyrate degrading-consortium, both qualitatively and quantitatively. The main player for the butyrate degradation was attained into several species demonstrated that this functional group of organisms is not fallen into the phylogenetically consistent groups, rather spread out in to several lineages. Therefore, it was evident that the syntrophic butyrate degrading community is larger than it was thought before. Better understanding of this community structure shed some light on anaerobic treatment of wastewaters and may lead to improvements this process as well.

ANAEROBİK BİYOREAKTÖRLERDE BÜTİRATI AYRIŞTIRAN MİKROORGANİZMA TOPLULUKLARININ POPÜLASYON DİNAMİĞİ

ÖZET

Bütirat, organik maddenin ayrışmasında metanojenlerin mevcut olduğu havasız arıtma koşullarında önemli bir ara üründür. Havasız reaktörlerde metan üretiminin %60'a varan kısmı bütirattan kaynaklanabilir. Bu tezde sunulan çalışmanın amacı havasız biyoreaktörlerde önemli bir ara ürün olan bütiratı ayrıştıran sintrofik mikrobiyal topluluğun popülasyon dinamiğini detaylı bir şekilde ortaya çıkarmaktır. Bu kapsamda konvansiyonel kültür çalışmalarıyla birlikte kültür çalışması gerektirmeyen moleküler teknikler kullanılmıştır. Ayrıca, atıksularda yaygın olarak gözlenen sülfatın sintrofik bütirat ayrışmasına etkisi de incelenmiştir. Yukarı akışlı Havasız Çamur Yataklı (UASB) Reaktörler ve farklı inkübasyon teknikleri kullanılarak biyokütle bütirata alıştırılmış ve moleküler biyolojik teknikler ile bu biyokütlenin karakterizasyonu yapılmıştır. Sintrofik butirat ayrıştıran mikrobiyal topluluktaki türler konvansiyonel ve moleküler teknikler ile kalitatif ve kantitatif olarak belirlenmiştir. Bu teknikler, MPN: En Uygun Sayı, PCR-RFLP: Polimeraz Zincir Reaksiyonu-Restriksiyon Parça Uzunluk Polimorfizm, DGGE: Denatüre Gradyan Jel Elektroforezi, Q-PCR: Kantitatif PCR, ve SIP: Stabil İzotop İşaretlemesi'dir. Yapılan bu çalışmada bütiratı ayrıştıran topluluğun bilinen organizmalardan farklı ve çeşitliliğinin fazla olduğu bulunmuştur. Aynı zamanda bu çeşitlilik filogenetik olarak da farklı gruplara düşmektedir. Bulgular, sintrofik olarak bütiratı ayrıştıran mikrobiyal topluluğun düşünülenden daha geniş olduğunu göstermektedir. Bu sintrofik topluluğun yapısının anlaşılması atıksuların havasız arıtılmasına ışık tutmakta ve havasız prosesin geliştirilmesinde önemli bir adım olabilir.

1. INTRODUCTION

Anaerobic treatment is presently accepted as a sustainable technology for a wide range of wastewater and waste types; and its applicability is validating and growing each year. The complete conversion of these waste materials requires a complex microbial community and a sequence of reactions performed by different physiological types of microorganisms. An important intermediate of organic matter conversion under methanogenic conditions is butyrate; which may account for up to 60% of methanogenesis in anaerobic bioreactors. The degradation of this intermediate depends on the syntrophic interaction with hydrogen utilizing organisms. However, the information on the degradation of butyrate is limited to the pure cultures and butyrate degradation was also always evaluated within the mixture of short chain fatty acids. Since the source of information depends on more laboratory conditions, ecologically significant data are needed to evaluate the communities correctly. To account, seven butyrate as well as some long-chain fatty acids (up to C18) oxidizing bacteria have been isolated in co-culture with a hydrogen utilizing partner under mesophilic conditions. These were mainly affiliated with the the family of *Syntrophomonadaceae*. Although some butyrate-oxidizing syntrophs were isolated in pure culture, most of them, still uncultured, play important roles in the degradation of butyrate.

The natural ecosystem is highly diverse and still cultured microorganisms occupy only a piece of the iceberg. In this respect, diversity and ecology of syntrophic butyrate- degrading bacteria is sharing this unknown characteristic and is waiting to be explored. However, cultivating the microorganism is a highly fastidious guild and the chance of ending up with the already cultured microorganisms is quite high. The most challenging parts for resolving the mystery about microbial box are also the inability in obtaining disturbance free field samples and conducting the laboratory experiments as they occur in real field conditions. This is particularly important for the types of data and their interpretation to what extent of ecologically relevant information is obtained from microbiological analysis.

Within this context, the species diversity of butyrate degrading anaerobic consortia has been subject to both the conventional culture dependent and the novel culture independent molecular techniques. A better understanding of such a consortium may lead to improvements in the anaerobic treatment of wastewaters.

2. SCOPE AND OUTLINE OF THE THESIS

The aim of this research is to get a deeper and better insight into the population dynamics of syntrophic butyrate oxidizing microbial consortia in anaerobic bioreactors. Moreover, the investigation was extended to examining the combined cultivation under methanogenic and sulfidogenic conditions. For this purpose, different molecular techniques were used to characterize the microbial biomass. The use of combination of molecular techniques (PCR-RFLP: Polymerase Chain Reaction-Restricted Fragment Length Polymorphism, DGGE:Denaturing Gradient Gel Electrophoresis, Q-PCR: Quantitative PCR, and SIP:Stable Isotope Probing) enabled us to identify several species in an anaerobic syntrophic butyrate degrading-consortium, both qualitatively and quantitatively. To find out the identity of key microorganisms, which are responsible for the butyrate degrading communities occurring in the complex environments, was the principal motivation of this thesis.

The outline of this thesis is as follows: In Chapter 3, the literature review on butyrate degradation, mainly physiological and phylogenetic characteristics of syntrophic butyrate utilization under methanogenic and sulfodogenic conditions was provided. In Chapter 4, an extensive review on the applications, the advantages, and the drawbacks of molecular techniques used in ecological studies were given. In Chapter 5, the materials and the methods used in this thesis were given in detail.

In Chapter 6, to find out the dominant and active butyrate degrading microrganisms in sludge communities, three reactors were operated with and without the addition of sulfate. The impact of sulfate as an electron acceptor on syntrophic butyrate degradation was studied with a COD/SO_4^{2-} of 10 and 0.5. The activity of the sludge was determined in terms of total, sulfidogenic and methanogenic for all three reactors. The research was also extended to clarify the effects of different propionate, acetate, formate and hydrogen concentrations on the butyrate degradation rate.

The objective of Chapter 7 was to gain a deeper insight into the syntrophic butyrate-oxidizing bacteria of the Eerbeek sludge with the application of enrichment and isolation techniques. Therefore, a combination of cultivation-based methods with a

molecular biological approach was used to investigate the dominant syntrophic butyrate degraders in the lab-scale anaerobic bioreactors and the seed of the bioreactors. The highest dilution cultures that yield positive growth were studied using molecular techniques to identify the dominant species by cloning and sequencing.

In Chapter 8, to find out the dominant and active butyrate degrading microrganisms in sludge communities`, molecular culture-independent methodology, polymerase chain reaction combined with denaturing gradient gel electrophoresis (PCR-DGGE) and cloning-sequencing, was applied to characterize the reactor sludges. Due to the activity observed, which was related with ribosome production, ribosomal RNA (rRNA) based methods were used to detect and quantify phylogenetically defined groups of microorganisms. Population dynamics linkage to the stable operating conditions, in three continuous UASB reactors was also assessed.

In Chapter 9, the result of linking microbial community structure of syntrophic butyrate degradation with the functioning using stable isotope probing technique was represented. To understand the genetic diversity of the complex microbial diversity of syntrophic butyrate oxidation, the SIP experiment was carried out to mimic in situ conditions to approach real conditions as much as possible.

In Chapter 10, population dynamics of anaerobic sludge at different temperatures with different feed mixtures were characterized. The objectives of this study were monitoring and evaluating the bacterial population shifts, which were strongly dictated by substrate composition and temperature using DGGE. Instead of feeding single carbon sources individually, thus introducing a bias in favor of the members of the mixed populations that could utilize that particular C-source; mixtures of different C-sources were fed to the batch mixed cultures to mimic the real-world conditions as much as possible, and to correlate the biochemical performance to population dynamics.

In Chapter 11 the comparative evaluation of the different techniques applied on syntrophic butyrate degrading consortia was given.

In Chapter 12 the major findings described in the previous chapters were discussed.

3. BUTYRATE DEGRADATION: A LITERATURE REVIEW

3.1 Methanogenic Conditions

In anaerobic degradation process, complex organic compounds, such as polysaccharides, proteins and lipids are hydrolyzed to monomers like sugars, amino acids and fatty acids (Figure 3.1). These intermediate products are then degraded by acidogens, forming volatile fatty acids, which are further degraded by acetogens, forming acetate, carbon dioxide, and hydrogen. Finally, both acetate and H_2/CO_2 are converted to methane by methanogens (Gujer and Zehnder, 1983). Complete conversion of these organic compounds requires a complex microbial community. In the sequence of these reactions performed by different physiological types of microorganisms, butyrate is one of the major intermediates in the anaerobic degradation pathway of the complex organic matter.

Failure of the anaerobic biodegradation processes has always been claimed to result in the deterioration of methanogenic activity. Even Speece (1996) emphasized this issue in the preface of his book as "Scientific details and bacterial names, not essential to the understanding of the process and operation involved, have been omitted with the exception of the *Methanosaeta* (*Methanothrix*) and *Methanosarcina*, due to their unique characteristics" (Speece, 1996). On the contrary, many researches as described in the following sections, revealed that different physiological types of bacteria playing important role in the anaerobic process, besides methanogens. This brings the necessity of knowing the each members of the total community realizing the curial bioconversions for better understanding and controlling the entire anaerobic degradation process.

3.1.1 Butyrate as an intermediate

Depending on the waste composition, intermediate concentrations could change for acetogens and methanogens in a wide range. Within this respect, in some reports, the significance of butyrate was highlighted; but the detection of this intermediate is not possible in well-balanced anaerobic processes due to having the high turnover rates.

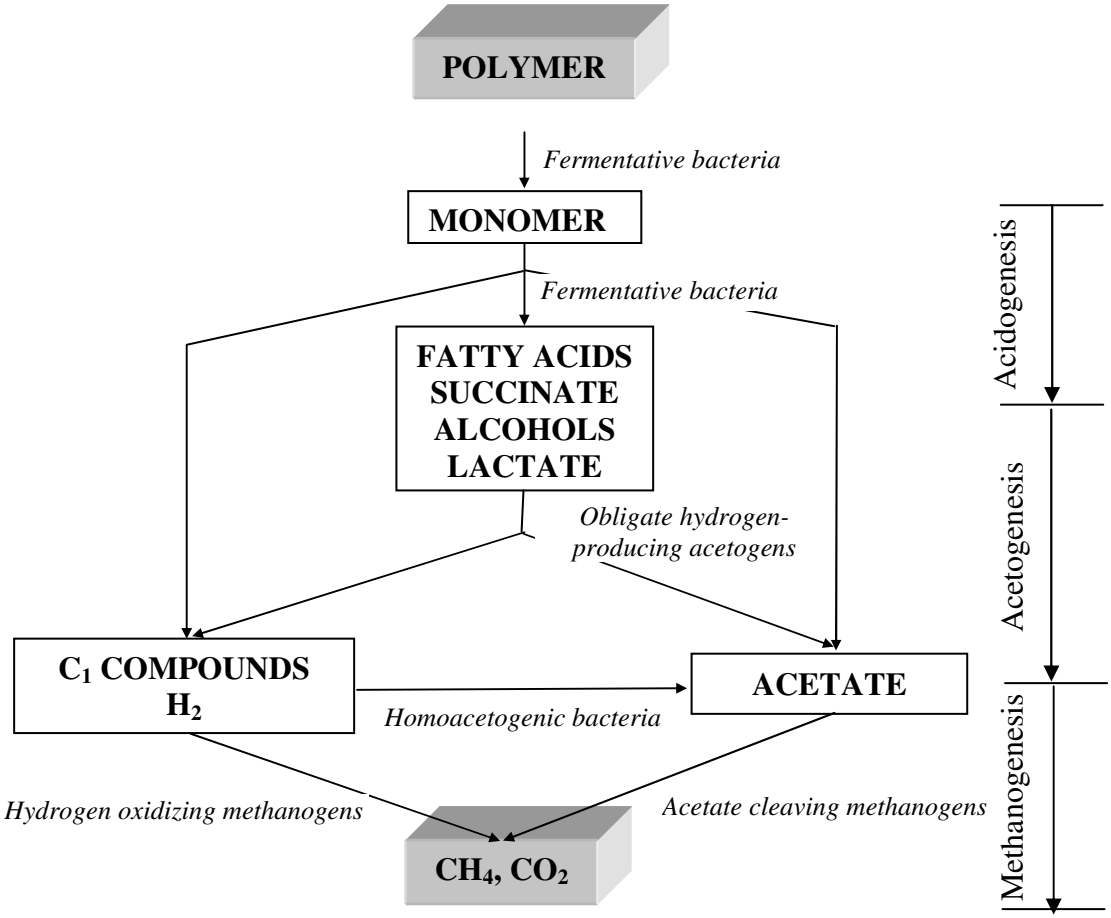


Figure 3.1 : Carbon and electron flow through various trophic groups of microorganisms involved in the degradation of complex organic matter (adapted from Gujer and Zehnder, 1983)

However, it was detected in some studies. For example, the study on anaerobic degradation of soft-drink wastewater, demonstrated that 60% of the glucose was reduced to methane through the butyrate (Fang, H.H.P. *et al.*, 1995a). In addition to this, 20% of the methane was produced from propionate and butyrate for anaerobic digester (Mackie and Bryant, 1981). During hydrogen fermentation of food waste, 30% of the wastewater consisted of butyrate, which was the main VFA component (Han *et al.*, 2005). It was also proposed that butyrate was formed by reductive carboxylation of propionate (Tholozan *et al.*, 1988). Moreover, intervonversion of propionate and butyrate was found to be in the range of 1-20 % (Lens *et al.*, 1996). Fernandez and his co-workers searched the effect of shock substrate loading on population dynamics to find out the relationship between functional stability and the community structure in a methanogenic bioreactor (Fernandez *et al.*, 2000). They found that less functionally stable community degrades the glucose mainly to acetate

and butyrate in parallel; however, functionally stable community degrades glucose to lactate, butyrate and acetate in a serial order (Figure 3.2). In Figure 3.2, the bold lines showed the relative contribution of the pathway. In both cases, butyrate was found as the main intermediate in the shock loading of the glucose into the anaerobic bioreactors.

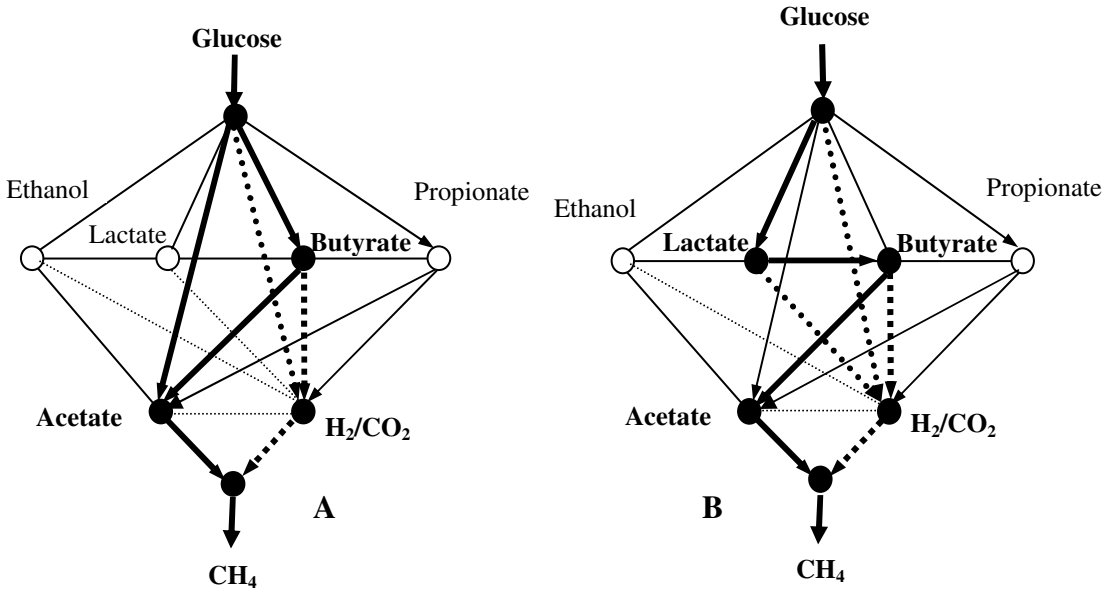


Figure 3.2 : The proposed network model for glucose perturbed anaerobic bioreactors; the less functionally stable community pathway (A), and the more functionally stable community (B) (Fernandez *et al.*, 2000)

3.1.2 Glucose fermentation and butyrate formation

Glucose degrading and butyrate producing organisms are generally attributed to the *Clostridium* species. However, when given the phylogenetic association between the genera *Eubacterium* and *Clostridium*, it is seen that they have common features. For example, most of the saccharolytic species of *Eubacterium* form butyric acid and hydrogen gas, just as many saccharolytic *Clostridium* species do (Sharak Genthner *et al.*, 1981). In the fermentation of glucose or other sugars, the fate of interspecies electron transfer is an important mechanism for determining the end product formation (Stams, 1994; Thauer *et al.*, 1977). For example, in the sugar fermentation of *Ruminococcus albus*, acetate, ethanol, hydrogen and CO_2 are the end products, but in the presence of hydrogen scavenger like *Wolinella succinogens*, ethanol would not be formed (Iannotti *et al.*, 1973). Figure 3.3 also shows that in pure culture *Ruminococcus albus,* the ATP yield is lower than in the coculture. Other examples on the importance of interspecies electron transfer are *Clostridium pasteurianum* and

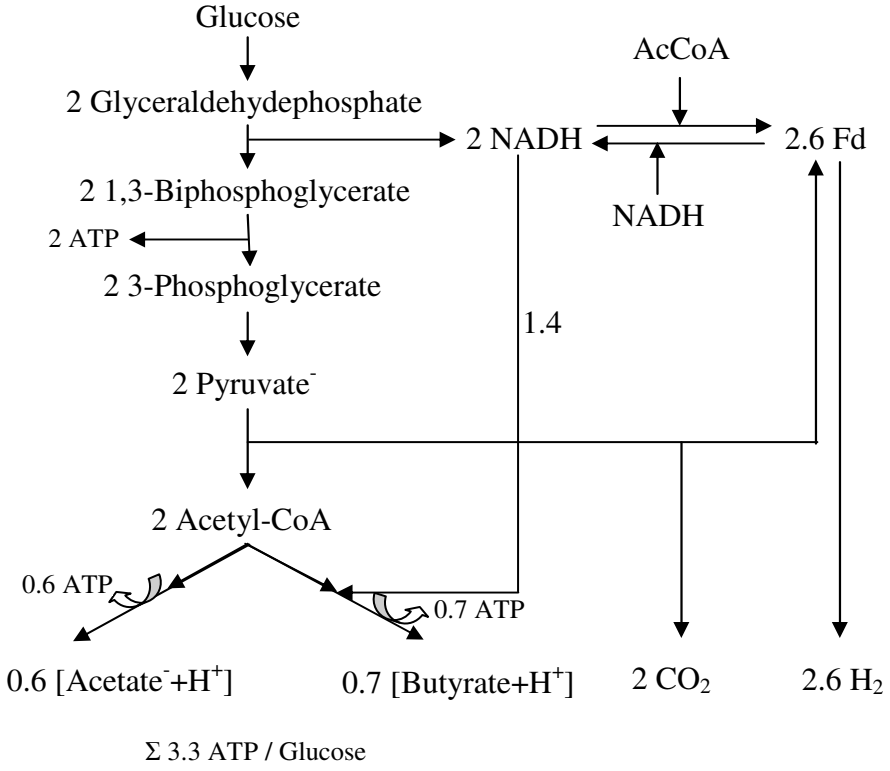


Figure 3.3 : Branched glucose fermentation of *Clostridium pasteurianum* and *Clostridium butyricum* (Thauer *et al.*, 1977)

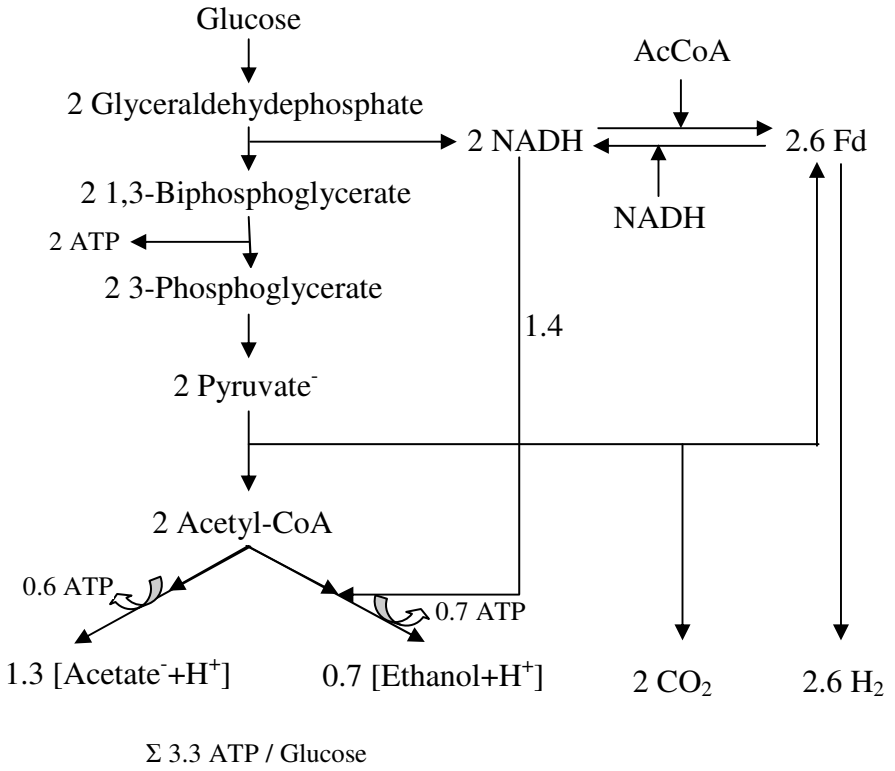


Figure 3.4 : Glucose fermentation of *Ruminococcus albus* (Iannotti *et al.*, 1973)

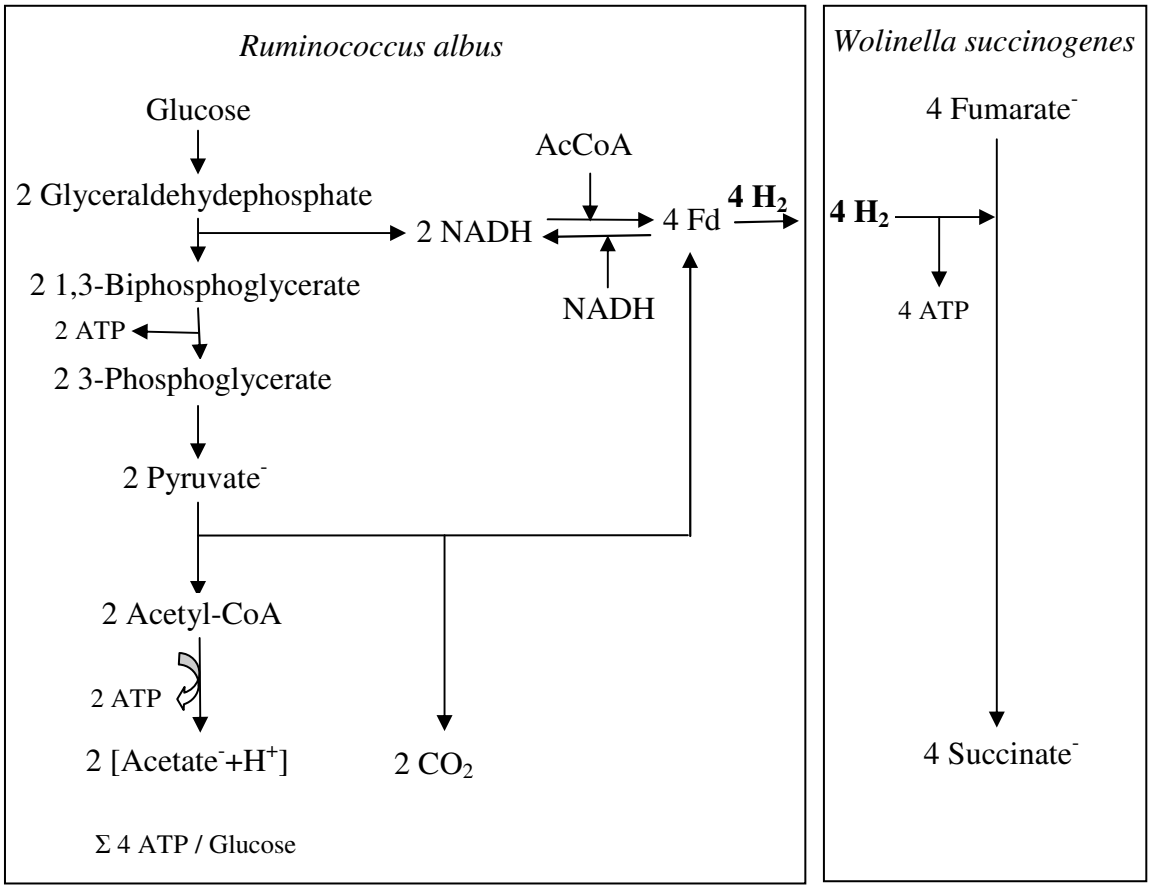


Figure 3.5 : Glucose fermentation of *Ruminococcus albus* with and without *Wolinella succinogenes* (Iannotti *et al.*, 1973)

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

$[H_2] > 10$ Pa;

$C_6H_{12}O_6 + 2H_2O \rightarrow 0.7$ butyrate $+ 0.6$ acetate $+ 1.3\ H^+ + 2\ CO_2 + 2.6\ H_2$

($\Delta G^{0'} = -233$ kJ.mol^{-1}) **(3.1)**

$[H_2] \leq 10$ Pa;

$C_6H_{12}O_6 + 2H_2O \rightarrow 2$ acetate $+ 2\ H^+ + 2\ CO_2 + 4\ H_2$ ($\Delta G^{0'} = -300$ kJ.mol^{-1}) **(3.2)**

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

3.1.3 Syntrophic butyrate degradation

The conversion of butyrate is endergonic under standard conditions. The degradation of butyrate is thermodynamically unfeasible unless the H_2 and/or formate can be removed by one of the hydrogen consumers (Schink and Friedrich, 1994). To proceed this reaction, the hydrogen level should be kept below 10^{-4} to 10^{-5} atm (McInerney *et al.*, 1981). In addition, to make this butyrate degradation reaction favourable, low acetate concentration, either by diffusion or by syntrophic reactions, is the other possible circumstance. However, this possible effect was not investigated in detail. Consequently, butyrate oxidation requires syntrophic interactions between β-oxidizing, hydrogen producing bacteria and hydrogen and/or acetate utilizers (Stams, 1994). The term "syntrophic" should be reserved to describe the metabolic process rather than the organisms that are involved (Schink and Stams, 2004). Degradation pathway of butyrate together with involved enzymes and free Gibbs energy of the intermediate steps were given in Figure 3.6.

In β-oxidation pathway, the activation of butyrate occurs by the transfer of the CoA from the end product acetyl-CoA to butyrate by CoA transferase reaction. After the degradation of butyryl-CoA to two molecules of acetyl-CoA that have two high-energy bonds; one of them was used for the activation of butyrate, while the other one yielded one ATP in the conversion to acetate. The reducing equivalent of the degradation pathway is transferred by $FADH_2$ and NADH, produced at the step of butyry-CoA to crotonyl-CoA and 3-hydroxybutyryl-CoA to acetoacetyl-CoA conversion steps respectively. As a result of these conversions, it was found that when 1 mol butyrate is degraded, about 2 mol acetate and 2 mol hydrogen is produced. As it was observed from the degradation pathway that the conversion of

Butyrl-CoA to Crotonyl-CoA, that has the fee Gibb's energy of +74.1 kJ/mol, is a thermodynamically difficult step (Wofford *et al.*, 1986). Therefore, in butyrate degradation pathway, this step is the crucial and difficult step to proceed in order for the reaction to be realized. Depending on the energetic condition of this pathway, the isolation or cell structure studies is carried on by fermentative growing of the culture on crotonate (McInerney and Wofford, 1992).

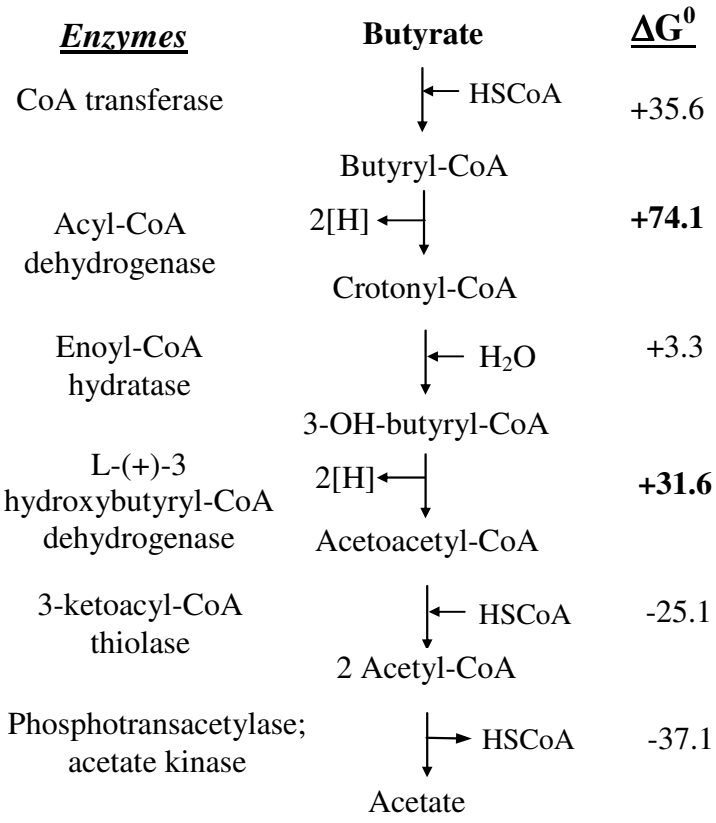


Figure 3.6 : The degradation pathway of butyrate (Wofford *et al.*, 1986)

3.1.4 Thermodynamics of the syntrophic butyrate degradation

The overall equation (Eq. 3.6) for butyrate oxidation can be obtained by three different reaction chains. These reactions are as follows:

$CH_3CH_2CH_2COO^- + 2H_2O \rightarrow 2CH_3COO^- + H^+ + 2H_2$ +48.3 kJ/reaction **(3.3)**

$2CH_3COOH + 2H_2O \rightarrow 2CH_4 + 2HCO_3^-$ -62.0 kJ/reaction **(3.4)**

$2H_2 + 0.5HCO_3^- + 0.5H^+ \rightarrow 0.5CH_4 + 1.5H_2O$ -67.8 kJ/reaction **(3.5)**

$CH_3CH_2CH_2COO^- + 2.5H_2O \rightarrow 2.5CH_4 + 1.5HCO_3^- + 0.5H^+$ -81.5 kJ/reaction **(3.6)**

The obligate syntrophism is the result of energetically unfavourable thermodynamic conditions of the butyrate oxidation to acetate and hydrogen (Eq. 3.3). If the reactions of Eq.3.4 and Eq.3.5 were realized, this unfavourable butyrate oxidation would turn out to be the favourable reaction with the negative values of the free Gibb's energy.

The minimum amount of Gibbs free energy needed to sustain growth and/or conversion of substrates e.g. ethanol, butyrate, propionate, lactate or benzoate is very close to the thermodynamic limits under anaerobic conditions; with a range that varies from -2 to -30 kJ/mol. It was postulated that the minimum amount of Gibbs free energy should be around -20 to -30 kJ/mol, corresponding to 1/3 to 1/4 ATP. ATP can be synthesised in these small amount via ion translocation across the cytoplasmic membrane (Schink, 1997). On the contrary, anaerobic fermentations have been observed at Gibbs free energy change is much closer to 0 kJ/mol. It may be suggested that any exergonic reaction may sustain growth (Thauer and Morris, 1984).

Syntrophic cultures partially degrade their substrate until the threshold concentration occurs (Jackson and McInerney, 2002). Jackson and McInerney reported the reason on how thermodynamically limited anaerobic metabolism proceeds. The average $\Delta G'$ value for butyrate degradation of *Syntrophomonas wolfei* and *Syntrophus aciditrophicus* in coculture with methanospirillum hungatei was -4.5±1.9 under methanogenic conditions. Syntrophic bacteria conserve their energy when they are thermodynamically close to the equilibrium ($\Delta G' \approx 0$ kJ.mol^{-1}) by proton motive force instead of phosphorylation. The different $\Delta G'$ values at threshold between syntrophic butyrate and benzoate degradation for the same species were observed because of activation energies of these compounds, which affect the threshold values. They found that the accumulation of acetate (end product) controls the extent of butyrate degradation thermodynamically.

3.1.5 Pure cultures of the butyrate degradation

To date, several butyrate as well as some long-chain fatty acids (up to C18) oxidizing bacteria have been isolated in co-culture with hydrogen utilizing partner (Table 3.1). *Syntrophomonas wolfei* (subsp. *wolfei*) was the first strain isolated in co-culture with

Table 3.1: Physiological characteristics and growth rates (1/day) of syntrophic butyrate degrading species

Butyrate degrading strains	Strains	Coculture	Origin	Substrate utilization	Syntrophic coculture (methanogens)	Syntrophic coculture (sulfate reducers)	References
Syntrophomonas wolfei	Boneyard (wolfei)	*Desulfovibrio* strain G11	isolation from sediment of a Winogradsky column obtained from the Boneyard creek, Urbana, Ill. at 35 °C	Monocarboxylic, saturated, C4-C8 fatty acids and isoheptanoate	0.2	0.3	(McInerney *et al.*, 1981)
Syntrophomonas sapovorans			Strain OM isolated on calcium laurate medium from an oleate enrichment	Ferments all linear saturated fatty acids with C4 to C18, also oleate, elaidate and linolenate	0.6		(Roy *et al.*, 1986)
Syntrophosphora (Clostridium) bryantii	CuCa1	H_2-utilizing bacteria.	isolation from marine sample, growth on caproate	Fatty acids with C4-C11 and 2-methylbutyrate	0.25		(Stieb and Schink, 1985)
Syntrophomonas curvata	GB8-1^T	*M. Formicicum*	UASB Reactor treating beer wastewater	linear saturated fatty acids with C4 to C18	2.2		(Zhang *et al.*, 2004)
Syntrophomonas erecta	GB4-38^T	*M. Hungatei*	UASB Reactor treating bean-curd farm wastewater	C4-C8 fatty acids	4.8		(Zhang *et al.*, 2005)
Syntrophomonas sp.	TB-6	*M. Formicicum*, TM-8	Flooded paddy soil	C4-C8 fatty acids			(Zou *et al.*, 2003)
Syntrophus aciditrophicus	SB^T	*M. Hungatei*	Benzoate degrading anaerobic digester sludge	Benzoate, methyl esters of butyrate. C4 to C18 fatty acids, heptanoate			(Jackson *et al.*, 1999)

a hydrogenotrophic methanogen or sulfate reducer (McInerney *et al.*, 1981). Moreover, *Syntrophosphora bryantii* (Stieb and Schink, 1985; Zhao *et al.*, 1990). *Syntrophomonas sapovorans* (Roy *et al.*, 1986), *Syntrophus aciditrophicus* (Jackson *et al.*, 1999), *Syntrophomonas* TB-6 (Zou *et al.*, 2003), *Syntrophomonas erecta* (Zhang *et al.*, 2005), *Syntrophomonas curvata* (Zhang *et al.*, 2004) and other mesophilic *β*-oxidizing syntrophs have been isolated (Wu *et al.*, 1992; Zhao *et al.*, 1993).

These mesophilic syntrophic butyrate-degrading bacteria fall into two lineages family *Syntrophomonadaceae*; within the phyla of *Firmicutes* and the family of *Syntrophaceae* in the δ-*Proteobacteria* subphylum. While *Syntrophomonas curvata*, *Syntrophomonas sapovorans*, and *Syntrophus aciditrophicus* are capable of degrading C4-C18 linear saturated fatty acids, for *Syntrophosphora bryantii*, *up to 11,* for *Syntrophomonas erecta* and *Syntrophomonas sp.* TB-6 up to 8 carbon fatty acids, are possible to be degraded. The growth and degradation of fatty acids occur only in syntrophic association with H_2-utilizing bacteria or methanogens. The pure culture of these species can be obtained by using crotonate as a fermentation process converted as 1 mol crotonate to 0.5 mol butyrate and 1 mol acetate; however, pure culture on butyrate plus pentenoate is obtained for *Syntrophomonas erecta* (Zhang *et al.*, 2005). Since, even-carbon-numbered fatty acids were converted to acetate, odd-carbon-numbered fatty acids were converted to acetate, propionate and H_2 in the β-oxidation of long chain fatty acids. While most of the syntrophic propionate degraders have shown to be able to reduce sulfate (Harmsen *et al.*, 1996a; Wallrabenstein *et al.*, 1995b), there have not been any report published on syntrophic butyrate degraders having the same feature so far.

3.1.6 Effect of hydrogen and acetate on acetogenesis

In syntrophic degradation of organic materials, reducing equivalent of the substrates is discarded by the reduction of protons or bicarbonate, leading to the formation of hydrogen and formate, respectively. Interspecies hydrogen or formate transfer plays an important role in the product distribution of the fermentation process as explained in glucose fermentation. In anaerobic ecosystems, hydrogen concentration can increase for any reason, e.g., excess supply of fermentable substrate and inhibition of hydrogenotrophic methanogens due to a drop in pH (<6) or to the presence of toxic

compounds. The increasing hydrogen levels can easily affect the overall anaerobic ecosystem in a different degree for each different trophic group even to the individual member of the community. For example, while in butyrate degradation, hydrogen partial pressure has to be decreased to lower than 100 Pa, for propionate degradation, this has to be less than 10 Pa (Schink and Stams, 2004). Moreover, the hydrogen partial pressure was in the range of 500-2000 Pa for the syntrophic ethanol oxidizing culture (Stieb and Schink, 1987). The hydrogen concentration in the gas phase of the EGSB reactor treating malting wastewater ranged between 85 – 355 ppm as well, under psychrophilic conditions (13-20º) (Rebac *et al.*, 1997).

Acetate removal by methanogens can affect the energetics of various reactions to different extent as explained in the previous section of 3.1.4. In addition, acetate accumulation delay even stops the degradation of propionate and butyrate degradations. Propionate degradation study demonstrated that 20 mM acetate had a modest inhibition on the methanogenic propionate enrichment culture (Boone and Xun, 1987). Another study showed that the presence of 3.3 mM acetate decreased the propionate degradation (Gorris *et al.*, 1989). Also, for mesophilic sludge digester, 80 mM acetate was required to inhibit propionate degradation, entirely (Kaspar and Wuhrmann, 1978).

In tricultures of isolated thermophilic butyrate degrading bacterium and *Methanobacterium thermoautotrophicum* and thermophilic acetate-utilizing methanogen, more than $2x10^{-2}$ (2.026 kPa) H_2 concentration was detected as a total inhibitor to the butyrate consumption. However, the butyrate degradation continued with the same rate as the H_2 untreated cultures even when H_2 was removed from the ambient (Ahring and Westermann, 1988). The effect of acetate on the same defined culture was also tested and was found that concentration of 81.4 mM stopped the degradation of butyrate, and the inhibition could not be reversed even after prolonged incubation time. To demonstrate the importance of pulling out the acetate from an environment for syntrophic butyrate degradation, *Mehanosarcina* was added to the coculture of *Syntrophomonas wolfei* and *M. Hungatei,* and 30% increase was observed on the butyrate degradation rate (Beaty and McInerney, 1989).

3.1.7 Effect of formate

Formate, the form of the transferring the reducing equivalent from the organic matter by reduction of bicarbonate, was proposed as an electron carrier (Bryant *et al.*, 1967). It was postulated that the interspecies formate transfer is a more valid mechanism than the transfer of hydrogen, due to its relatively high solubility (Dong, 1994). Reversible conversion between formate and H_2 was observed in many batches as well as the production of one these substrates or both appeared from proton producing bacteria. Due to the thermodynamic equilibrium between H_2 and formate, the conversion resulted in methane production rates as in the same order of magnitude.

Formate production was detected in the H_2-CO_2 incubation of brewery granules, when 0.34 atm. hydrogen added, 0.6 mM formate accumulated and was degraded concomitantly with hydrogen (Wu *et al.*, 1991). In the presence of sulfate, no formate was detected for the similar brewery granules, though only a low level of sulfate (~1mM) was reduced. The same author also found formate formation (up to ~50µM) and hydrogen (~0.1 kPa) in the syntrophic ethanol degradation.

The formate (20 mM) addition to the methanogenic propionate enrichment caused the H_2 pressure to increase to 177 Pa, resulting in the inhibition of the enrichment culture (Boone and Xun, 1987).

It was reported that the treatability experiments anaerobic filter reactors fed with acetate, acetate and ethanol, and formate for each reactor under the loading rate of 10 kg $COD.m^{-3}.day^{-1}$, demonstrated a higher amount of H_2 (0.13 %) and CO_2 (40%) production in the reactor biogas fed with formate compared to other acetate (0.09% H_2 and 10% CO_2) and acetate+ethanol reactors (0.03% H_2 and 17% CO_2). (Isa *et al.*, 1986b)

3.1.8 Spatial distribution of archaea and bacteria within flocs or consortia

The different trophic groups of anaerobic microorganisms in the degradation of complex organic matters grow under very limited available conserved energy. This energy has to be shared between two to four groups of bacteria. Although the main attention was driven on the degradation of reduced fermentation products, in terms of syntrophic degradation, there was also syntrophism in fermenting bacteria. The metabolic interaction between these species was explained with close spatial

localization of H_2 and acetate producers (acetogens) and consumers (methanogens) based on efficient diffusion of metabolites. This interaction was described in Figure 3.7.

Concentration of dissolved hydrogen for several anoxic ecosystems showed different levels, for example, Sewage sludge digester 10-30 Pa (Conrad *et al.*, 1985; Kaspar and Wuhrmann, 1978); Lake Mendota sediment 1.5 Pa (Conrad *et al.*, 1985); Fresh-water sediments 1 Pa (Robinson and Tiedje, 1984; Strayer and Tiedje, 1978). For obligatory syntrophic degradations, however, high hydrogen values prevent the reaction to take place. However, the floc or granule formation, which causes the juxtapositioning of syntrophic partners, can protect these syntrophs from relatively high hydrogen levels and precede the realization of reactions.

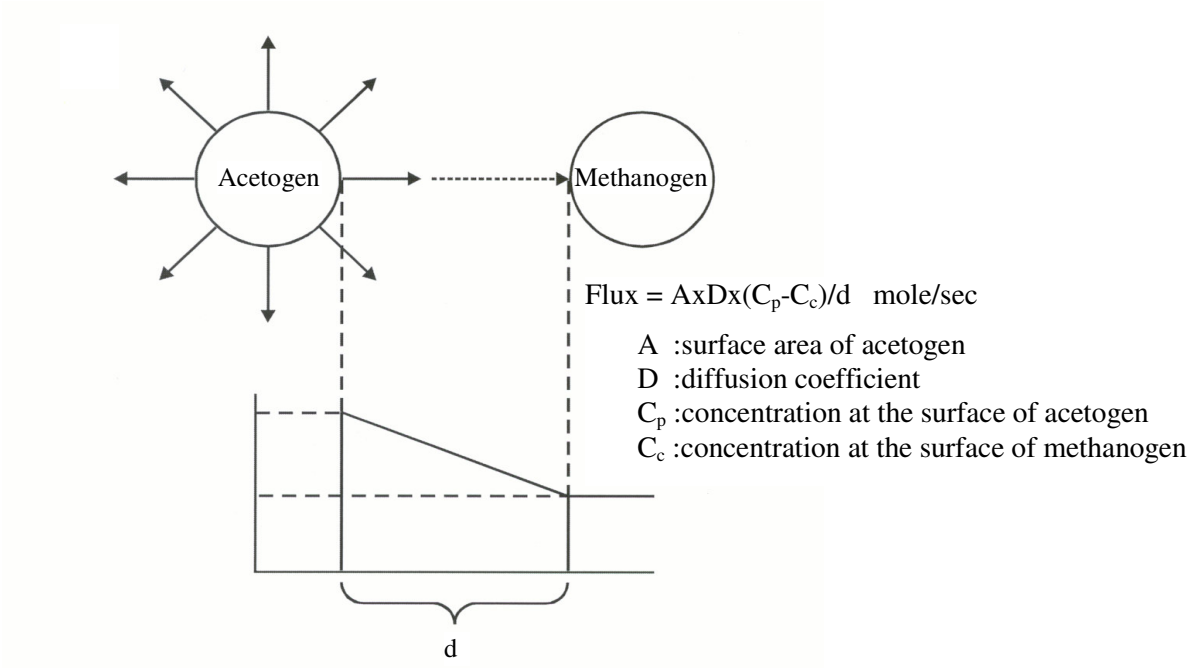


Figure 3.7 : Spatial localization of H_2 and acetate producers (acetogens) and consumers (methanogens) (Stams, 1994)

3.1.9 Butyrate degradation in mixed cultures

Since then, there have been several researches on degradation of VFA mixture, Fang and his co-workers (1995) focused on the butyrate degradation in a UASB reactor. They were able to load the butyrate to the reactor up to 31 g COD.l^{-1}.day^{-1} with 97-99% COD removal efficiency and 94.5% of this removed COD was converted to methane. The activity tests of this butyrate degrading sludge granules demonstrated that the SMA was 1.57 g CH_4-COD.g VSS^{-1}.$day^{-1}$and the sludge was unable to

degrade propionate. The microstructure analysis of granules showed a distinct morphology configuration along the granules. The skin layer of granules was arranged mainly with cocci shape bacteria and closely associated structures. Interior part of the granules mostly composed of Methanosaeta-like archaea.

Syntrophic butyrate degrading consortium which was acclimatized in the fluidized-bed reactor displayed three different functional groups(Zellner *et al.*, 1991). The butyrate degradation was performed by gram negative, thin, curved, spore-forming rods resembling *Syntrophosphora bryantii* and by *Desulfovibrio* sp. SWB1 that was low in number. The reduction of acetate to methane was mainly achieved by *Methanosaeta concilii* and *Methanosarcina mazei*. The hydrogen consumption was done by *Methanobrevibacter arboriphilus* or *Methanogenium* sp.; and *Methanocorpusculum* sp., and *Methanobacterium* sp., were also present in low numbers.

When butyrate or propionate was introduced to the thermophilic anaerobic digester, methanogenic inhibition was observed at the concentrations of 25 mM (Henson *et al.*, 1986). Another work on butyrate degraders was the inhibition tests of methanogenic archaea by using 2- Bromoethanosulphonate (BRES) and chloroform demonstrated that butyrate degrading bacteria are dependent on methanogens (Ahring and Westermann, 1987).

3.2 Sulfidogenic Conditions

The presence of sulfate in the environment permits the growth of sulfate reducing microorganisms (SRM) by coupling the oxidation of organic or inorganic electron donors to the reduction of sulfate to sulfide (H_2S, HS^-). This process is called Dissimilatory Sulfate Reduction. Beside this process, sulfate is also reduced to sulfur for biosynthesis, which is called assimilatory sulfate reduction. This functional group of microorganisms have the capability of degrading a wide range of organic compounds. The typical scheme of sulfate dependent anaerobic degradation of complex organic matter is given in Figure 3.8.

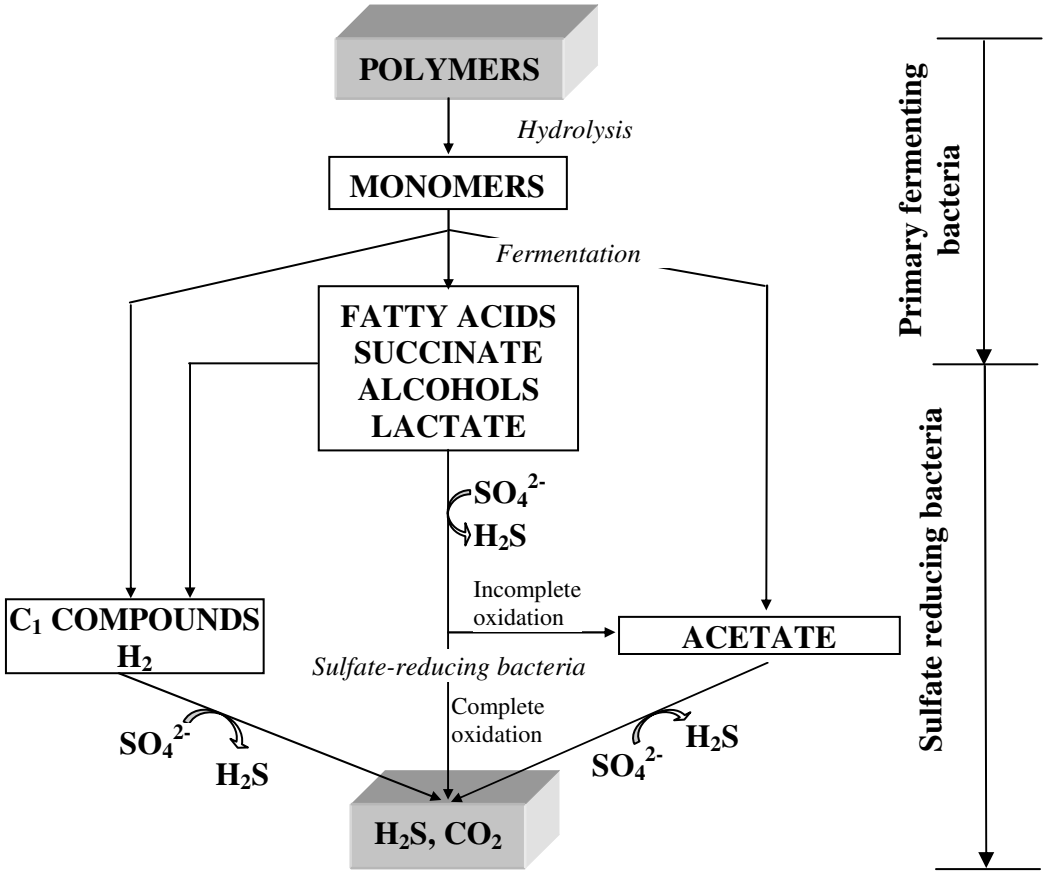


Figure 3.8 : Carbon and electron flow through various trophic groups of microorganisms involved in sulfate dependent degradation of complex organic matter

Sulfate-reducing bacteria are able to use a broad range of compounds as electron donors. Utilization of lactate and pyruvate is almost universal among sulfate reducers, and many species that oxidize energy sources incompletely to excreted acetate can utilize malate, formate, and certain primary alcohols. Those capable of complete oxidation can oxidize electron donors, such as fatty acids, lactate, succinate, and benzoate. These compounds are the end products of hydrolysis and fermentation of complex polymeric compounds, which are likely to be produced by other members of the microbial community utilizing materials produced by the organisms.

Theoretically, all organic matter can be degraded by sulfate reducers, if the COD/SO_4^{2-} (g/g) ratio equal or lower than 0.66 (mol/mol ≤ 0.5). If the ratio is above 0.66, sulfate reducers have to compete with methanogens and acetogens for carbon sources. Factors known to be influencing this competition are COD/SO_4^{2-} , substrate type, free energy of the reaction, maximum specific utilization rate (μ_{max}), half velocity constant (K_s), nutrient availability, adhesion properties, proximity of cells, temperature, long term shifts, toxicity (Speece, 1996).

For K_s, which shows the affinity of microorganisms to the substrate, the lowest value gives the highest affinity. Comparing the K_s values of SRB (Sulfate Reducing Bacteria) and MPA (Methane Producing Archaea), clearly indicated that SRBs are 15 and 6 times lower than MPAs, for acetate and hydrogen, respectively (Isa *et al.*, 1986a).

The proportion of electrons distributed to SRB and to MPA is important to elucidate the competition of these microorganisms. In most cases, it is difficult to distinguish the physiology of fatty acids degrading bacteria, which are involved in this competition. It can be the syntrophic interaction in which the reducing equivalents will be used by SRBs or be the direct oxidation by the SRBs. Under non limiting sulfate conditions, it was always observed that all the formed reducing equivalents of acetogenic bacteria were used up by SRBs (Visser, 1995). This situation can be easily observed in batch tests for granular sludge, since microbial consortia prefer growing outside of granules, due to the limitation of mass transfer of sulfate into the granules and the effects of hydraulic flow pattern differences from original *in-situ* enrichments of the consortia.

However, under continuous flow conditions, it was reported that in high rate anaerobic filter reactor fed with acetate or acetate+ethanol together with sulfate, did not change methane production remarkably (Isa *et al.*, 1986b). Even though the reactor was operated at COD/SO_4^{2-} of 0.33, the inhibition of methanogenesis was only 12%. In case of the suspended sludge, SRB has played important role by taking over the task for the degradation of 75% carbon source (carbohydrate mixture) with the operation of 180 days of the UASB reactor at COD/SO_4^{2-} of 0.83 (Harada *et al.*, 1994). In different ecosystems (such as freshwater and marine sediments), the dominance of SRB ranged between 75 and 99% in terms of electron flow (Isa *et al.*, 1986a; Winfrey and Zeikus, 1977). For these kinds of environments, propionate and butyrate degradation was directed by SRB, under the conditions of excess sulfate (Banat and Nedwell, 1983; Sorensen *et al.*, 1981).

The other reported decisive mechanism for this competition was related to the organic loading rate of acetate (Yoda *et al.*, 1987). The lower organic loadings resulted in dominating the consortia by SRB, which K_s values of SRB and MPA were also proved with values of 9.5 and 32.8 $mg.l^{-1}$ respectively. However, higher organic loadings (9-14 kg $COD.m^{-3}.d^{-1}$) affected the competition in favor of MPA. At low acetate loading rates, on the contrary, the dominance of SRB was also observed. In the operation of reactors at low hydraulic retention times, methanogens have greater advantage over SRB due to the adhesion properties of MPA (Isa *et al.*, 1986a).

Based on thermodynamics and kinetic properties of sulfate reducers, it can be suggested that SRB dominates the MPA for this competition. However, for some certain types of substrates, this estimated out-competition of SRBs is working in an opposite way. Visser (1995) operated a UASB reactor feeding with acetate and sulfate close to non-limited sulfate with a COD/SO_4^{2-} of 2, 1, and 0.62, for 1200 days. At the day of 400, 77% of the acetate was converted to methane by acetotrophic methanogens and complete acetate degradation by SRBs observed at the end of the operation. At that time, total sulphide concentration reached up to 750 $mg.l^{-1}$ at the pH of 7.5-8.0 and sulfate of 3400 $mg.l^{-1}$, and sulfidogenic sludge activity was around 0.5 g $COD.l^{-1}VSS.day^{-1}$. Same author also operated the UASB reactor, which is partly adapted to sulfate, for VFA mixture, consisting of acetate, propionate, butyrate (5:3:2, respectively, based on COD) and non-limited sulfate (8.3 $g.l^{-1}$). It

took approximately 600 days for the methanogens to be out competed by the SRBs. The sulfidogenic activities of this sludge for acetate, propionate and butyrate were 0.79, 0.75, and 0.75, respectively. The sulfide concentration was also around 2000 $mg.l^{-1}$at the pH of around 8.

A possible pathway for sulfate reduction to sulfide is given in Figure 3.9. The redox potential of SO_4^{2-}/HSO_3^- couple is -516 mV, which is too low to perform the reaction. Realization of this reaction is possible by the activation of sulfate to adenosine phosphosulfate (APS) with ATP-sulfurylase (Stams, 1985).

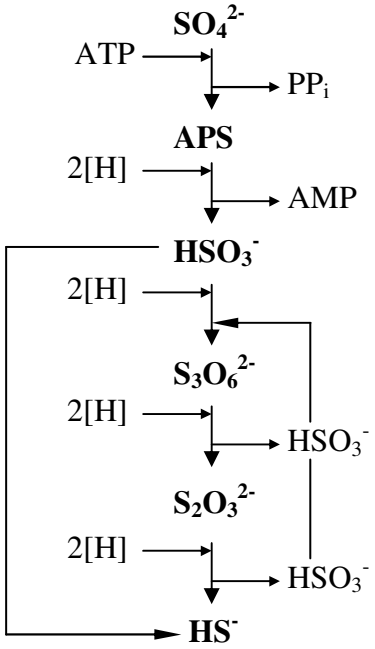


Figure 3.9 : Possible pathway for sulfate reduction to sulfide

Dissimilatory sulfate reducers may be distinguished metabolically in two aspects as complete and incomplete oxidizers. The distribution of the species phylogenetically according to complete and incomplete oxidizing SRBs is given in Figure 3.10. Butyrate and longer fatty acids can be degraded under sulfate reducing conditions by complete (to CO_2 such as *Desulfonema*, *Desulfobacter* species) or incomplete oxidizers (to acetate and/or propionate, such as *Desulfobotulus*, *Desulfofustis*, *Desulfocella* and some *Desulfotomaculum* species). However, in the degradation of butyrate with complete oxidizers, acetate production was also detected. As observed in *Desulfobacterium autotrophicum*, and *Desulfobacterium cetonicum* degradation from 1 mol of butyrate; the products of 1 mol acetate and 1.5 mol hydrogen sulphide

were obtained; meaning 1 mol acetate was consumed and the remaining 1 mol was excreted from the same species. Due to being short of terminal acetyl-CoA oxidation, 1 mol of acetate out of 2 acetyl-CoA was excreted and the excess 1mol CoA was used to activate butyrate. Enzyme activities of cell free extracts of *Desulfobacterium cetonicum* demonstrated that while the butyryl-CoA:acetate CoA transferase activity is present, the butyryl-CoA synthetase activity is not detected.

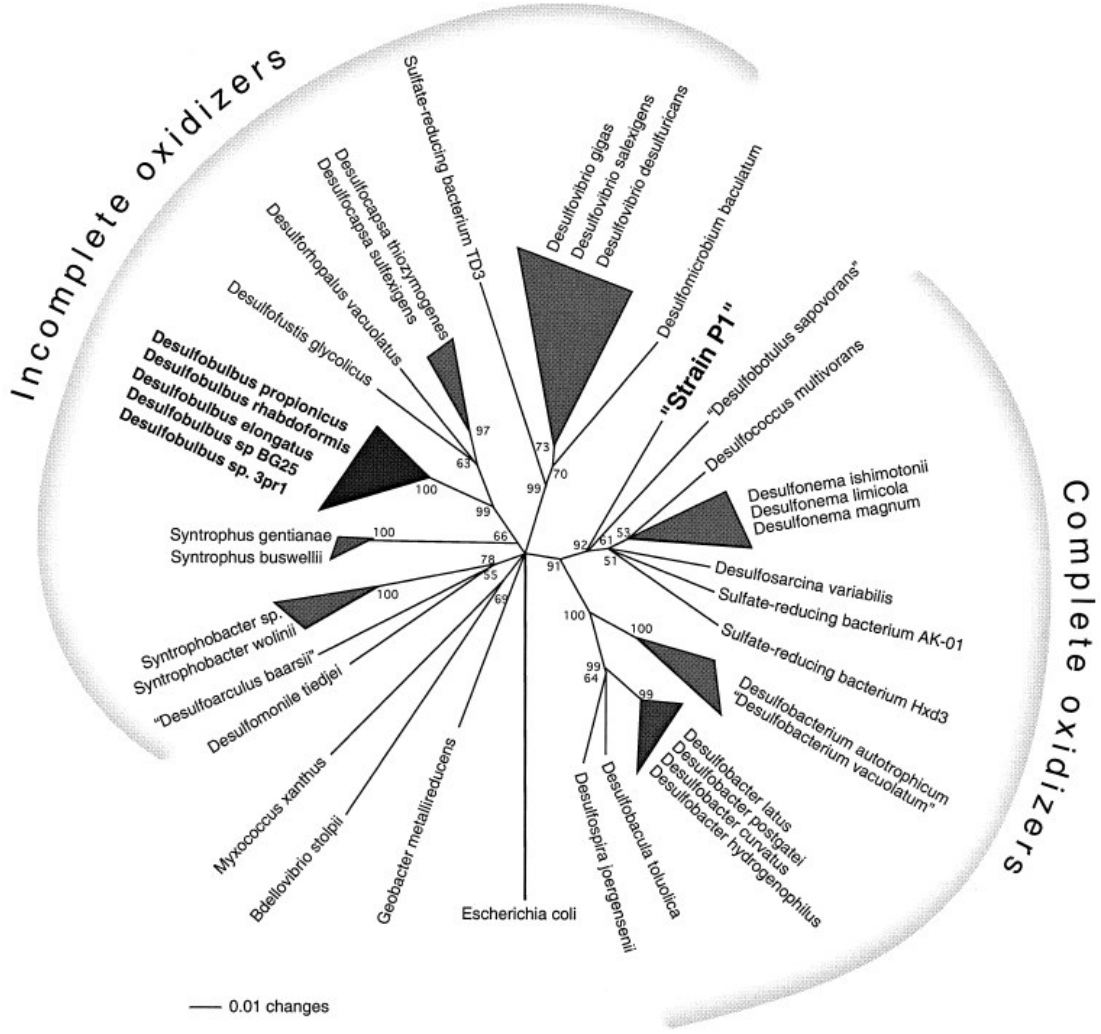


Figure 3.10 : Phylogenetic tree of complete and incomplete oxidizers in SRB (Finster *et al.*, 2001)

At the same time, these disproportional ratios of acetate production were also observed for *Desulfococcus multivorans*. Since then, the acetate concentrations were not as much secreted as *Desulfobacterium autotrophicum*, and *Desulfobacterium cetonicum*, 0.35 mM acetate were produced from *Desulfococcus multivorans* that fed 5 mM butyrate concentration. This low acetate concentration can be attributed to the low free Gibb's energy change and, consequently, the threshold level of the species.

In the competition study for complete (*Desulfobacter* sp.) and incomplete (*Desulfovibrio sp.*) oxidizers for ethanol demonstrated that the incomplete oxidizer was dominant in the system even though the complete oxidizer still remained (Widdel, 1988). This situation can be explained with the thermodynamic conditions, which is more favourable for incomplete oxidation. In addition, the growth rate of incomplete oxidizers is higher than the complete oxidizers, which also shows the oxidation of high-energy substrates to the acetyl-CoA level being faster than the terminal oxidation step. Moreover, the mixture of short chain VFA fed reactor sludge showed the same physiological properties described for incomplete SRBs (Visser, 1995). The activity tests for butyrate and propionate resulted in the production of sulfide of about 20 and 43% of these substances, respectively, which corresponds to the theoretic sulfate reduction for hydrogen consumption.

As it was observed in the syntrophic degradation of fatty acids, C-even fatty acids produce only acetate, whereas C-odd fatty acids generate acetate and propionate. This channelling through different end products depending on the type of long chain fatty acids is due to the presence of β oxidation (Widdel, 1988). The equations of these incomplete reactions are as follows:

$$CH_3(CH_2)_{2n}COO^- + n/2\ SO_4^{2-} \rightarrow \quad (n+1)CH_3COO^- + n/2\ H_2S \qquad \textbf{(3.7)}$$

$$CH_3(CH_2)_{2n+1}COO^- + n/2\ SO_4^{2-} \rightarrow nCH_3COO^- + CH_3CH_2COO^- + n/2\ H_2S \qquad \textbf{(3.8)}$$

Even though completely oxidizing sulfate reducers degrade acetate, their growth rate is considerably low compared to specialized acetate users, which is known as *Desulfobacter* species (with a doubling time around 20 h).

Although, the major part of the SRMs was known to be in the domain of bacteria, some sulfate reducers were also found in the domain of archaea. Bacterial sulfate reducers phylogenetically fall into three main groups (Rabus *et al.*, 2000) (Figure 3.11). These groups are: i) the δ subclass of proteobacteria (gram-negative bacteria) with more than 40 genera, ii) *Desulfotomaculum* and *Desulfosporosinus* genera within Firmicutes (gram-positive bacteria), iii) *Thermodesulfobacterium* and *Thermodesulfovibrio* within Thermodesulfobacteria.

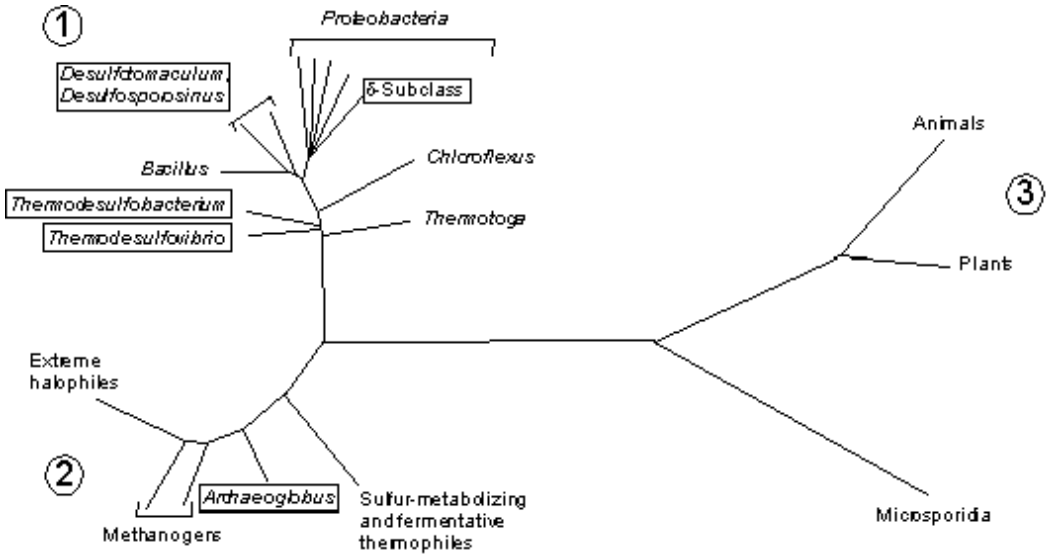


Figure 3.11 : Where the SRBs located in the three domains of life (Rabus *et al.*, 2000)

3.2.1 The effect of pH on SRBs

pH and sulphide concentration play an important role in the SRB and MPA competition. Acetate and fatty acids degrading SRB can live in a wide range of pH (6-9), compared to other bacteria fed with the same carbon sources (Lowe *et al.*, 1993; Visser, 1995). For acetate, SRB can tolerate higher pH values than methanogens. In the competition of SRB and MPA for acetate, above the pH of 7.7, acetogenic SRB have a bigger growth rate than MPA. If pH values are between 7.4-7.7, the competition depends on the acetate:sulfate ratio. However, pH lower than 7.4, methanogens have higher growth rate (Visser, 1995).

3.2.2 Hydrogen and formate production and their utilization in SRB

Many ecosystems demonstrated that SRB is involved in interspecies hydrogen transfer rather than direct oxidation to CO_2, which also gives an idea for the competition of methanogens and sulfate reducers (Phelps *et al.*, 1985). Hydrogen production was observed in some species of *Desulfovibrio* during fermentation on pyruvate as well as during growth on lactate in the presence of sulfate (Traore *et al.*, 1981).

In the ethanol degrading mixed culture, which was cocultured with H_2 utilizing methanogens, H_2 was detected as much as 1.2 and 0.6 mM, in the presence and the absence of sulfate, respectively (Bryant *et al.*, 1977).

With the addition of sulfate into the feed of mixed cultures adapted mainly to methanogenic conditions, firstly, hydrogen utilizing methanogens decrease and hydrogen utilizing SRB take over their mission in a short time. This can easily be explained by the advantage of SRB over MPA, in terms of thermodynamics and kinetics. Other reason favoring the SRB for H_2 utilization will be the spatial localization and closeness of the hydrogenases and dehydrogenases enzymes (Bryant *et al.*, 1977). In SRB, the production and consumption of H_2 was carried out in the same cell, by cytoplasmic and periplasmic hydrogen cycling (Haveman *et al.*, 2003; Odom and Peck, 1981), which shorten the time necessary for movement of the electrons comparing to the discretion of H_2 towards outside of the cell where to be reduced by methanogens.

(Isa *et al.*, 1986b) observed that addition of hydrogen (94 mM) in to the anaerobic filter reactor fed with acetate, improved the sulfate reduction extremely, increasing reduced sulfate from 30% to 80%. (Isa *et al.*, 1986b) also found that sulfate reduction ratio was 47% and 31% in a formate fed anaerobic filter reactor at different SO_4^{2-}/COD ratios of 0.83, 3.3, respectively and at pH of 7. For higher pH(~9), without controlling the reactor pH, a lower ratio of sulfate reduction was observed (13% and 24%, respectively).

3.2.3 Acetate utilization by SRB

Unlikely to hydrogen utilization, the competition between SRB and MPB for acetate utilization takes more time to be out competed to each other. For longer incubations, acetate utilizing SRB out compete MPB, due to having higher affinity, higher free Gibb's energy and higher removal rate of the SRB.

Sludge Activity (SA) tests, which are performed with batch incubations showed that acetate utilizing SRB was not active as much as methanogens, though a high sulfate utilization ratio over methanogens was observed for the sludge enriched in the continuous reactors (Isa *et al.*, 1986b; Visser, 1995). This means that acetate-utilizing SRB did not take over all the consortia, albeit the large fraction of carbons and electrons flow through sulfate in continuous systems.

Hydrocarbon contaminated aquifer, which is known as presence of sulfate, no SRB activity was detected for acetate. *Methanosaeta* was the predominant species for methanogens (Struchtemeyer *et al.*, 2005).

3.2.4 Propionate and butyrate utilization by SRB

Competition studies between acetogenic bacteria and SRB demonstrated that propionate degradation is always directed by SRB at the excess of sulfate along with the significant decrease in the activity of the acetogenic bacteria (Rebac *et al.*, 1996; Visser, 1995; Wu *et al.*, 1991). On the contrary, the competition for butyrate occurred at low COD/SO_4^{2-} (Rebac *et al.*, 1996; Visser, 1995). Therefore, the degradation of butyrate seems less sensitive to sulfate reduction than that of propionate. Butyrate can be converted to acetate and H_2 (or formate) in the absence of sulfate. Then these products are used up by methanogens. In the presence of sulfate, SRB takes over the mission firstly to accomplish the hydrogen utilization and after that starts to utilize the acetate depending on the COD/SO_4^{2-} ratio. Therefore, methanogens are out competed by SRB (Lovley *et al.*, 1982).

3.2.5 Syntrophic associations of SRB and MPA

Desulfovibrio species, isolated from ethanol enriched culture as of anaerobic sewage digester, could metabolize ethanol and lactate in coculture with H_2-utilizing methanogens in the absence of sulfate (Bryant *et al.*, 1977). Unless H_2 was reduced to a very low level, the degradation of acetate by *Methanosarcina barkeri* was not possible in the syntrophic association of *Desulfovibrio* spp. and *Methanosarcina barkeri* for complete oxidation of lactate to CO_2 and CH_4 (McInerney and Bryant, 1981).

3.2.6 Comparison of granular and crushed granular sludge for the SRB

Because of the mass transfer limitations of sulfate into the biofilm or granules, methanogens will dominate over SRB. (Nielsen, 1987) observed this limitation at 0.5 mM (48 $mg.l^{-1}$) SO_4 concentration, for a few micrometers of biofilm. However, Visser, (1995) found 350 $mg.l^{-1}$ SO_4 for granular sludge, which was a relatively higher concentration than the biofilm. The latter author also found that the granular sludge had the growth for a wide pH range compared with the crushed granular sludge (Visser *et al.*, 1996).

3.2.7 Sulfide inhibition

Sulfide, which is the product of dissimilatory sulfate reduction under anaerobic conditions, is toxic at higher concentrations. In bioreactors, sulfide is present in the form of H_2S, HS^- S^{2-} in solution and as H_2S in gas phase. Sulfide toxicity is always related to the presence of undissociated H_2S in the bulk liquid and it inhibits the methanogens as well as itself by means of the crossing of the uncharged molecule through the cell membranes by reacting with the cell components (Isa *et al.*, 1986b; Stucki *et al.*, 1993; Widdel, 1988).

The inhibition of methanogenesis was reported for many different ecosystems (Table3.2). For marine and freshwater sediments, this inhibition occurred due to the presence of 18 $mg.l^{-1}$ SO_4, and for the anaerobic digester, it was at 960 $mg.l^{-1}$ for SO_4 concentrations (Winfrey and Zeikus, 1977). Sulfide, also, however, is a vital substance and it increases the growth rate of some hydrogenotrophic methanogens. (Ronnow and Gunnarsson, 1981) reported that sulfide is required for the growth of isolated methanogens, resembling *Methanobacterium thermoautotrophicum*. The presence of sulfide less than 0.1 mM, the growth was very poor and other sources of sulfur such as cysteine, sulfate, or thiosulfate could not be replaced with sulfide.

Presence of sulfide compounds may also cause beneficial or adverse effects, indirectly on methanogenic systems, in addition to an undissociated H_2S effect. Its beneficial effect can be by preventing the heavy metal inhibition in means of precipitating the metals with sulphide (Jin *et al.*, 1998). The adverse effect will sweep away the metals, for which the trace elements are necessary for the assimilation of microorganisms (Maillacheruvu *et al.*, 1993; Speece, 1996).

The equilibrium between H_2S in the gas phase and H_2S in the solution was regulated by Henry's law (Isa *et al.*, 1986b);

$$[H_2S]_s = \alpha\,[H_2S]_g \qquad \textbf{(3.9)}$$

where α (Henry`s Law Constant or absorbsion coefficient) is 1.83 $mg.l^{-1}$ at 35°C

Table 3.2: Sulfide inhibition on different types of enriched cultures

Culture	Operational conditions	substrate	H_2S inhibition concentrations, $mg.l^{-1}$			Reference
			50%	Complete	Failure	
Desulfovibrio species	pH (6.2-6.7)	Acetate		550		(Reis *et al.*, 1992)
	pH 7			350		(Okabe *et al.*, 2003)
Mixture of *Desulfotomaculum acetoxidans*, *Desulfobacter postgatei* and digester biomass fed with high level sulfate	8.0-8.5, biofilm, LR:60 $kgSO_4^{2-}.l^{-1}.d^{-1}$ under carbon limit cond. >95%removal	Acetate			Total sulphide: 1200 $H_2S > 50$	(Stucki *et al.*, 1993)
Mixed	Biofilm	Acetate	~1200			(Isa *et al.*, 1986b)
Acclimitated on sugar beet molasses	55ºC, crushed, pH 7.2	H_2/CO_2	225			(Pender *et al.*, 2004)
		Acetate	29			
Acclimatized on VFA mixture	10ºC, granular	Propionate			320 (65%)	(Rebac *et al.*, 1996)
		Butyrate			320 (30%)	

The concentration of undissociated hydrogen sulphide can be calculated by using these chemical and physical equilibriums.

$$H_2S \quad \xleftrightarrow{k_1} \quad HS^- + H^+ \qquad \textbf{(3.10)}$$

$$H_2S \quad \xleftrightarrow{k_2} \quad H_2S(g) \qquad \textbf{(3.11)}$$

At the neutral pH $K_1 = 1.49 \times 10^{-7}$ at 35ºC

$$[H+]\,[HS^-] / [H_2S]_s = K_1 \qquad \textbf{(3.12)}$$

The free fraction of H_2S (f) of total sulphide concentration that depends on the pH can be calculated as,

$$f = (1+K_1/10^{-pH})^{-1} \qquad \textbf{(3.13)}$$

In the suspended culture of butyrate degraders, which is acclimated to be low in sulfate concentration, were inhibited at >200 $mg.l^{-1}$ H_2S concentrations at the pH of 7.7-7.9 (Oleskiewvicz *et al.*, 1989). They also found different inhibition levels for different electron donors, in which the order of highest to lowest inhibition concentrations is as follows: lactate, butyrate, acetate, and propionate. In case of loosing the activity of SRB due to sulphide inhibition, the recovery of the reactor performance can take up to 2 months (Stucki *et al.*, 1993).

The ways of preventing H_2S inhibition are: stripping the H_2S with carrier gases (Stucki *et al.*, 1993); increasing the pH; adding heavy metal to form metal-sulfide precipitates; and, increasing the organic content of the influent in order to raise the COD/SO_4^{2-} ratio.

In conclusion, depending on the operational and physiological conditions of the methanogenic and sulfidogenic cultures, different environments react to the hydrogen sulfide inhibition in different ways. The acetate degradation was inhibited in a wide range of 29-1200 $mg.L^{-1}$ sulphide concentrations (Table 3.2). However, this was about 300 $mg.L^{-1}$ for butyrate and propionate degrading enrichments.

3.2.8 Salt inhibition

Because of osmotic pressure, at high salt concentrations, bacteria will dehydrate and subsequently, die. Several researches have been made to explore the inhibition level of the biomass. It was experienced the effect of NaCl on methanogenic microbial consortia fed mainly to acetate and ethanol, and at 30 $g.l^{-1}$(12 g $Na^+.l^{-1}$), 35 $g.l^{-1}$ (14 g $Na^+.l^{-1}$) NaCl concentrations, the initial and 50% inhibition in terms of biogas production, respectively, were observed for shock loading (De Baere *et al.*, 1984). For stepwise increment of NaCl addition, meaning adapting the sludge slowly to high NaCl values, the author found 65 $g.l^{-1}$(26 g $Na^+.l^{-1}$), 95 $g.l^{-1}$ (37 g $Na^+.l^{-1}$) NaCl concentrations, inhibited the methanogens, the initial and 50% inhibition, respectively. Same kind of inhibition was also observed by (Isa *et al.*, 1986b) under the competition of SRB and MPB. Feeding the continuous anaerobic reactor with acetate and sulfate at a concentration of about 45 $g.l^{-1}$ as Na_2SO_4 (corresponding to 14.4 g $Na^+.l^{-1}$), biomass was inhibited at the rate of 88%, decreasing the biogas production. This inhibition was related to the high level of Na^+ concentration, instead of the sulphide inhibition.

4. CHARACTERIZATION of MICROBIAL COMMUNITIES

4.1 Molecular Ecology

To find out the identity of key microorganisms, which are responsible for the important bioconversions that occur in the complex environment, was the principal motivation for the microbiologists for a long time. However, the microbial black box, which exceeds the limits of the current data and information in many aspects, is still awaiting for discoveries. The most challenging part on resolving the mystery about microbial box is the inability to obtain disturbance free field samples and conducting the laboratory experiments as occurred in real field conditions. This is particularly important for interpreting and determining the types of data to what extent ecologically relevant information is obtained from microbiological analysis. An outline of commonly used approaches for microbial ecology and their ecological relevance is summarized in Figure 4.1 (Madsen, 2005). Since the source of information depends more on the laboratory conditions, the risk of achieving ecologically significant data increases, as it was seen from Figure 4.1. To unlock the microbial black box and to resolve the relationships between organisms and their environments, many attempts have been made using molecular techniques, which was pioneered in the1980s.

The novel molecular techniques uncovered the microbial diversity, that was previously unknown, with culture-based techniques (Amann, 1995). The analysis of ribosomal RNA sequences enabled to assess microbial diversity in natural habitats and their classification as a standard. Together with advance techniques, this enables us to determine the structure and dynamics of microbial communities. Considerable progress and generation of data has been implemented on the determination of microbial diversity in complex ecosystems as well as engineered environments (Dumont and Murrell, 2005). Since both molecular and traditional cultivation techniques may have biases, ecological validations are necessary to be certain about their significance. For that purpose, comparative *in situ* and different parallel

techniques are applied to overcome the biases in most researches (Ginige *et al.*, 2004).

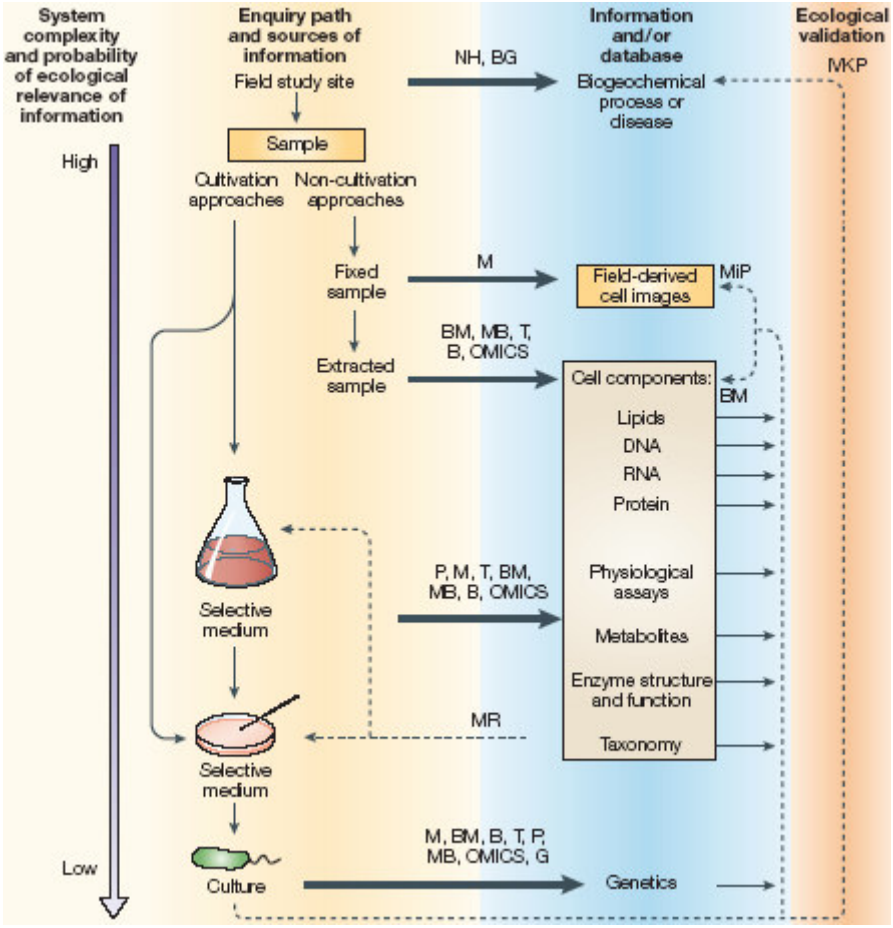


Figure 4.1 : Approaches in microbial ecology and their ecological relevance (Madsen, 2005)

4.2 Cultivation of Microorganisms

Besides these advantages of the molecular biological tools, cultivation based methods should not be underestimated. Latter one makes the baseline for molecular tools. With the accumulation of knowledge of physiology and the identity of pure cultures make it meaningful for the application of molecular techniques. In the meantime, expanding databases on cultivation based microbial diversity will make it easier to interpret the results coming from the cultivation-independent molecular analysis.

There are still many uncultivated microorganisms which were characterized by using cultivation-independent molecular techniques (Hugenholtz *et al.*, 1998). Most of the

lineage of phylogenetic tree is poorly represented by the related pure culture, which is necessary in order to get a better understanding of physiological features of the microorganisms. For example, in the database of 16S rRNA genes, the phylum of green nun-sulfur microorganisms seem very diverse, by having more than a thousand sequences; however, this phylum has only ten pure cultures to understand the existence in the complex ecosystems. This and this kind of phylums may lead to misinterpretation of the complex ecosystem.

Within different habitats, cultivability is estimated averagely less than 1% of the total microorganisms observed in nature. For activated sludge this cultivability changes between 1 and 10%, which is relatively high compared to the other environments such as soil, seawater etc. (Amann *et al.*, 1995). The number of the existing microorganisms in the ecosystem is estimated to be around one million (Kaeberlein *et al.*, 2002). Excessive effort should be given to increase the number of as-yet-uncultivated organisms in order to grasp a complete understanding of microbial physiology and metabolic pathways. On the contrary, cultivation of microorganisms present in the natural environment is limited to the artificial laboratory conditions, which underestimates the diverse indigenous environments. The cultured microorganisms represent only minor fraction of natural microbial communities and also mislead the richness of species of all the ecosystem (Wintzingerode *et al.*, 1997).

Cultivation techniques present limitations on the number and diversity, known as the selectivity of any given medium and cultivation conditions as well as of the interaction and communication between the organisms. The isolates, therefore, may not truly represent the organism's habitat. Besides, cultivation is laborious and time-consuming. To overcome these limitations, isolation of pure cultures should be conducted under the conditions, which reflect the real environment, and nutritional conditions, as much possible. There are some strategies applied successfully to obtain pure cultures; such as: relatively low nutrient concentrations, inhibitors for undesired microorganisms, relatively longer incubation period, directly from inoculums obtained from natural environment (Stevenson *et al.*, 2004). In addition, some high-throughput and novel techniques are used to cultivate previously uncultivated microorganisms. Some of those comprise the application of optical tweezers (Frohlich and Konig, 2000), manipulation in a simulated natural

environment (Kaeberlein *et al.*, 2002), encapsulation of cells in gel micro droplets (Zengler *et al.*, 2002), encapsulation of cells in gel microcapsules (Zengler *et al.*, 2005) high-throughput random isolations (Joseph *et al.*, 2003), and plate-wash PCR (Stevenson *et al.*, 2004).

4.3 Microbial Phylogeny

Phylogenetic relationships between organisms are used to classify evolutionarily branched and diverse populations. Phylogeny is determined by comparing the properties of organisms according to both the physiological and the genetic information. It was found that life falls into three domains, *Bacteria*, *Eucarya*, *Archaea;* based on the comparison of small-subunit ribosomal RNA together with other gene sequences (Woese, 1987). The advances in recombinant DNA and other molecular biological methods let us identify the type of complex microorganisms existing in different ecosystems without the need for cultivation (Amann *et al.*, 1995). After these revolutionary advances in biology, number of molecular works increased sharply for different ecological niches. One of the cursor of this increment for such molecular works is the submission of sequences of organisms in the data banks. DNA sequencing efforts yielded 65,000 bacterial 16S rRNA sequences, based on the numbers published in GenBank at the end of the year 2005(http://www.ncbi.nlm.nih.gov). rRNA gene sequences obviously show huge underestimated diversity, which was not discovered within the *Bacteria* and *Archaea*. Bacterial domain has more than 40 divisions (Hugenholtz *et al.*, 1998) (Figure 4.2); however, most of the divisions poorly or entirely represented yet even the uncultivated microorganisms. As shown in figure 1, 13 out of 36 divisions so called “candidate divisions” were represented only by environmental sequences.

Proteobacteria, *Actinobacteria*, Low G+C gram positives and *Cytophageles,* which are the phylum level divisions within the domain bacteria, are represented by 90% of all cultivated bacteria characterized by the 16S rRNA sequences. On the other hand, a big part of the other divisions exposed to 16S rRNA sequences characterized less cultivated microorganisms such as *Acidobacterium*, *Verrucomicrobia*, Green Non Sulfur (GNS) bacteria. The remaining divisions do not have any pure isolation to be able to give some information about their accurate physiological properties.

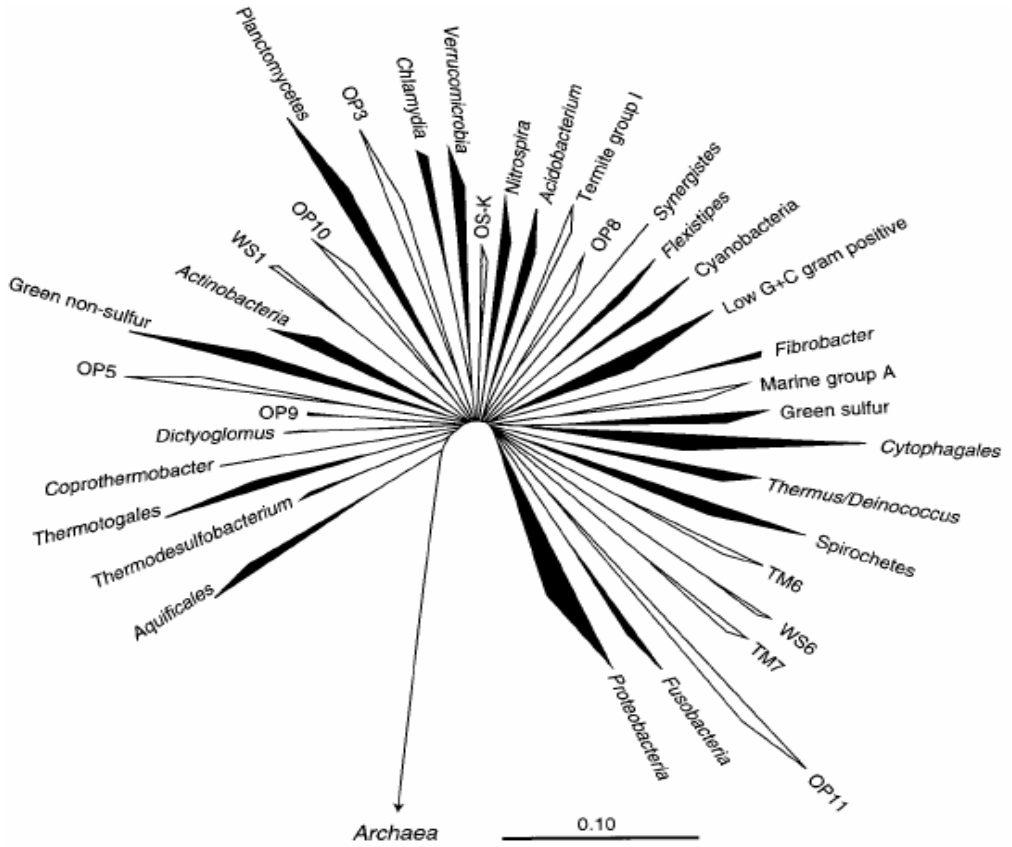


Figure 4.2 : Evolutionary distance tree of the bacterial domain showing the currently recognized and the candidate divisions (Hugenholtz *et al.*, 1998)

GNS bacteria, detected in wastewater treatment plants, soils, sub surfaces, were represented by only a few isolates. Nevertheless, physiological characterization demonstrated a wide trophic nutrition ranging from anoxic photosynthesis to thermophilic organotrophy, which makes it very difficult to interpret the link between functions and the 16S rRNA gene sequences.

4.4 Molecular Tools

The cultured microorganisms are only a minute fraction of the number, which is probably more than a million (Amann *et al.*, 1995). To overcome the limited information known about organisms, many different molecular biological tools were developed and are still being developed. Although the molecular techniques may reflect a large part of microbial diversity, the application of those in different ecosystems should be carefully examined. Microorganisms, that infrequently exist in ecosystems, may provide a functional diversity in a narrow ecological range (Godon *et al.*, 2005).These tools also enable the characterization of several recognized but

poorly understood microorganisms. In this section, an overview of the main methods used in molecular microbial ecology was summarized. In this scope, the techniques used to determine the microbial diversity in complex ecosystems were summarized in Figure 4.3. Briefly, environmental samples can be subjected to direct *in situ* analysis, such as FISH based techniques or nucleic acid, can be extracted for microarray, hybridization, or stable isotope probing analysis. After nucleic acid extractions, the target genes can be amplified using polymerase chain reaction; and then the products could be used for a wide range of techniques, like pattern analysis, cloning and sequencing, microarrays and probe hybridizations.

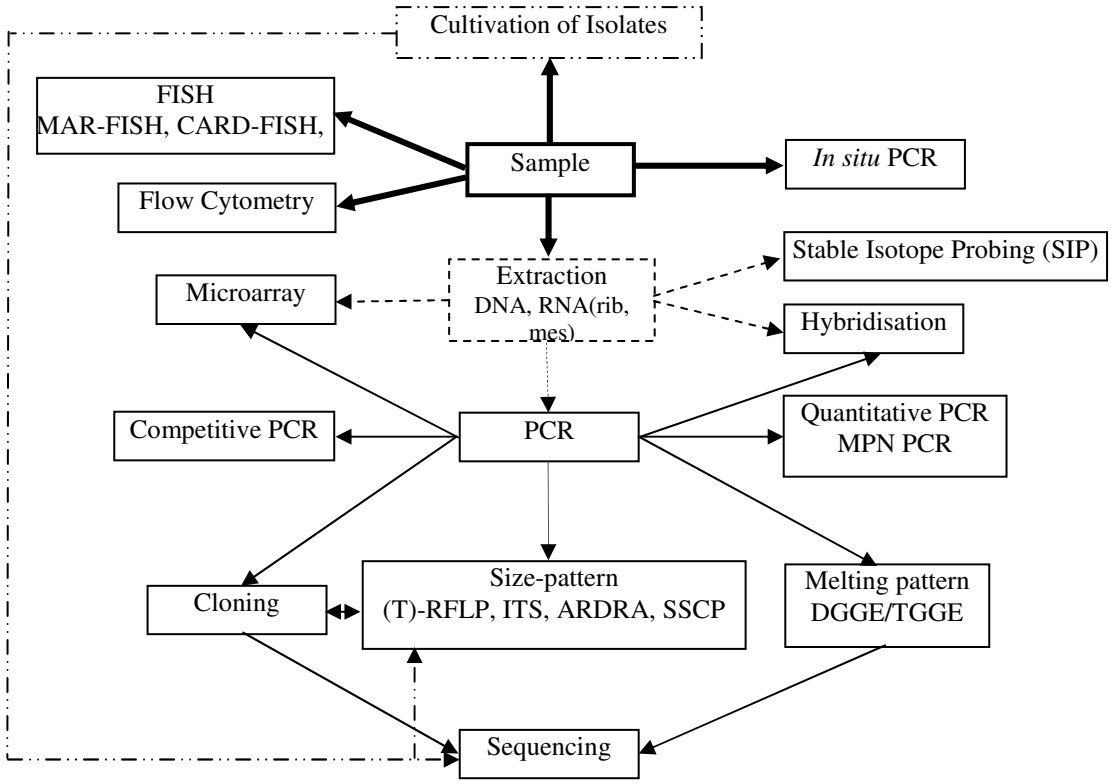


Figure 4.3 : The techniques used to determine the microbial diversity in complex ecosystems

4.4.1 SSU rRNA gene sequences as a biomarker of microorganisms

Working on rRNA genes is a valuable technique for identifying the microbial communities. rRNAs demonstrate the important functional reliability for all organisms (Woese, 1987). Their presence in all cells, when enough in size and variability to discriminate their identity, and tracing the evolution, makes ideal

phylogenetic and taxonomic markers (Amann *et al.*, 1995). Additional advantages of using rRNA as markers come from their functional stability, easiness of sequencing, and specific features of their primary and secondary structures (Tourova, 2003). The sequence of the 16S rRNA gene has been widely used as a biomarker of microorganisms to estimate relationships between the bacteria (phylogeny), and to identify an unknown bacterium. This type of sequence also allows statistically significant comparison between different sequenced information of microorganisms. 16S is a small subunit of the ribosomal RNA molecules, with a length of about 1500 nucleotides in most bacteria. 16S (Swedbergs) refers the sedimentation coefficient and is related to its sedimentation speed (Voet and Voet, 1995).

The higher and lower evolutionarily conserved patchworks of 16S rRNA enable amplification or hybridization to identify the organism from domain to individual species level; on which they are evolutionary based in their complex natural environments.

There are also some disadvantages to using rRNA as a marker, like having different 16S copies within one species (Crosby and Criddle, 2003; Shimizu *et al.*, 2001; Tourova, 2003). Although we assume that each microorganisms is represented by a single and unique 16S rRNA, the probability of having different copy numbers have to be primarily considered for estimating microbial diversity.

The DNA of SSU rRNA genes reflect all existing organisms in the community and do not enable the distinction between active and resting cells. On the contrary, RNA based SSU rRNA gene sequences have the potential ability to target active microorganisms and high sensitivity as a high number cellular ribosome do (Felske *et al.*, 1998; Wagner, 1994). It was also found that growth of bacteria was nearly proportional to the amount of ribosomes in the cells in a pure culture, which demonstrates the ability of using the RNA for analyzing community structures. Nevertheless, the RNA content of some cells did not decrease rapidly during the process of starving of the organisms (Wagner *et al.*, 1995).

4.4.2 Amplification of the SSU rRNA genes using PCR (Polymerase Chain Reaction)

Polymerase Chain Reaction (PCR), in principle, is used to amplify the double stranded DNA. In this technique, the target DNA is first melted, then the set of

primers are annealed and elongated using thermostable polymerase. Repeating this cycle for about 20-35 times, millions of copies of DNA can be obtained at the end of process. Although PCR products are mainly used for successively performed molecular tools due to the requirements of a high number of copies of DNA, they are also used to check whether the presence of organisms even as a species level in the environmental samples.

In the case of using RNA as a template for PCR, the single strand RNA has to be first converted to double strand by adding the complementary part using reverse transcriptase. After that, the double stranded RNA can be preceded as a DNA for PCR.

As it can be seen from the Figure 4.3, after the extraction of the nucleic acids, many molecular techniques cannot be preceded without the application of the PCR technique. This clearly shows that PCR is an initial and important step for other molecular tools. On the contrary, some factors may easily influence the amplification reactions and several problems may arise. PCR-associated problems are; differential amplification of different rRNA templates, sensitivity to rRNA gene copy number, primer specificity, sensitivity to template concentration, amplification contaminant rRNA, formation of chimeric sequences, the secondary structure of the target, G+C content of the target (Becker *et al.*, 2000; Wintzingerode *et al.*, 1997). However, several methods were developed to prevent these PCR biases. Especially, for preferential amplification, it was suggested to i) exclude the degenerated primers in the case of using domain level primers ii) use high concentration of target DNA iii) mix the separately amplified same PCR mixture and iv) decrease the number of amplification cycles (Polz and Cavanaugh, 1998). For other important bias, the formation of chimeric sequence, it was suggested to keep the longer elongation time (Wang and Wang, 1996).

These factors also bring the question whether the PCR based molecular analysis precisely reflects the microbial diversity of different habitats and consequently the phylogenetic based taxonomy. For that reason, especially planning the experimental procedure and interpreting the results of PCR based molecular tools, and the abovementioned factors should be considered very carefully.

4.4.3 Cloning, sequencing and phylogenetic analysis

To identify and classify each member of ecosystems individually, it is necessary to know many properties of organisms. 16S rRNA sequencing analysis of organisms has become powerful tool for that purpose together with physiological properties. This sequencing analysis in environmental samples includes the steps in the order of cloning, screening, sequencing and the construction of the phylogenetic tree.

In the cloning step, the extracted DNA samples, firstly, are subjected to PCR to amplify the target genes of organisms. Later, this amplified DNA fragments can be cloned into a plasmid vector or a bacteriophage. The plasmid vector is the much preferable way for cloning. Then the vector is transformed into an *E.Coli* with a ligase. The *E.Coli* cells are grown on selective agar plates and screened for the presence of vectors in the cells. Afterwards, the vectors that have the target genes are exposed to additional screening to separate the identical genes for subsequent sequencing analysis. This screening can be done by several fingerprinting methods, but the most common ones are the Amplified Ribosomal DNA Restriction Analysis (ARDRA) (Massol-Deya *et al.*, 1995) and the Restricted Fragment Length Polyphormism (RFLP) (Weidner *et al.*, 1996). These methods are based on restriction endonuclease digestion of the genes and then the visualization and comparison of the restriction fragment pattern. After the selection of the different clones, the genes are sequenced to provide information about the nucleotide structure. Mostly, as mentioned in the above sections, the identification of microorganisms is managed by sequencing of the 16S of rRNA. The obtained sequencing codes are then put through the computer programs, which are available on internet sites and determine the closest relatives. These programs use homology searches provided by the BLAST database. Tens and thousands of 16S rRNA sequences are published in this database, which is easily accessible online (http://www.ncbi.nih.gov/BLAST/) (Altschul *et al.*, 1990). The comparison of the sequences with this database using computer programs is fast and reliable, which is very important in terms of finding the highest homology.

The results of highest homology obtained do not clearly determine the relationships between the species when many sequences are compared. The similarities and dissimilarities between the species can easily be visualized by the phylogenetic trees (Stackebrandt and Rainey, 1995). Generation of phylogenetic trees can occur in two

different ways, as distance matrix or as discrete character data. While the distance matrix calculations are based on evolutionary distances, the discrete character data calculation consider the nucleotide position independently (Zoetendal, 2001). Although selecting the most suitable program for constructing the tree is not easy, it was found that trees constructed with these two ways gave almost the same results for *Frankia* strains (Wolters, 1998). In most cases, the selection of methods is based on the speed and the easy use of the programs for the users.

4.4.4 Detection and quantification with hybridization

The rapid and specific detection, identification and quantification of the organisms in their natural habitats can be achieved with hybridization techniques based on high fidelity bonding of the nucleic acid probes into complementary regions on the genes (Sayler and Layton, 1990). Probes are the short piece of the single strand DNA that is complementary to a specific piece of DNA in the cell. Short nucleotides, with lengths ranging between 15 and 30, are used for probes. By marking the probe, it is possible to visualize the presence of the probe target sequence of the genetic material. Although probes can be designed for the most variable regions to detect the organisms at the species level, probes can be designed for group, genus, even domain level of organisms by targeting the more conserved regions. It provides flexibility for detecting the different taxonomic groups of organisms. The other important advantage of oligonucleotide probes is the discrimination of single nucleotide difference on the target sequence. This increases the sensitivity and the accuracy of hybridization techniques.

The applicability of this technique depends on the previously characterized nucleic acid sequences. SSU or LSU rRNA targeting oligonucleotide probes used in the experiments is to determine diversity, composition and the relative abundance of the microbial communities in many ecosystems. This technique is cultivation independent and uses directly the data of rRNA sequences. Therefore, it can be used to identify and quantify the uncultured and rare microorganisms, which can easily be overlooked with other molecular or traditional microbiological methods.

Hybridization techniques can be applied shortly in two different ways according to the marking of the probes for visualization and fixation of the genetic materials. If the hybridization is realized on i*n situ* fixed cells with fluorescently dyed probes,

then the hybridization process is called Fluorescence *In Situ* Hybridization (FISH). If target nucleic acids are immobilized on solid supports, and probes are labelled with radioisotopes, or chromogenic, chemiluminescent, bioluminescent dyes, then the process is called Blot Hybridization (Nakatsu and Forney, 1995).

4.4.5 Blot hybridization

In blot hybridization experiments, the first step is the attachment of single-stranded target nucleic acids to the membranes or filters. Then, the membrane surface is prehybridized to block non-specific nucleic acid binding sites. Later, hybridization is realized with labelled probe binding to the complementary target sequences. After hybridization, excess unbound labeled probe is washed off and hybridization is detected by one of the visualization techniques, depending on the type of probe labelling. Standard application for labelling is the radioactive material. Generally, phosphorous 32 are used as a label, due to its high-energy emission and consequently, its short exposure times. However, the radioactive works need special attention because of their hazardous nature and waste disposal problems. Therefore, nonradioactive labelling methods started to be common for different environmental samples. Although the application of non-radioactively labeled probes has increased rapidly, radioactively labeled probes still give very reliable, sensitive and accurate quantification values (Cunningham, 1995). Various groups of microorganisms were detected and quantified with blot hybridization techniques; such as methanogens (Raskin *et al.*, 1994a; Raskin *et al.*, 1994b; Zheng and Raskin, 2000), sulfate reducers (Oude Elferink, 1998; Raskin *et al.*, 1996), syntrophic acetogens (Hansen *et al.*, 1999; Oude Elferink, 1998) in anaerobic sludge.

4.4.6 Fluorescence *in situ* Hybridization (FISH)

Since 1989, rRNA targeted FISH have become a common tool for many applications (DeLong *et al.*, 1989). This tool consists of four main steps: the fixation and permeabilization of the cells; hybridization with fluorescently labeled oligonucletide probes; washing to remove the unbound probes; and detection the hybridized cells by epifluorescent microscopy or flow cytometry (Amann, 1995). Advantages and disadvantages of the application were summarized in Table 4.1. This technique is directly applied to intact cells and gives information about their localization, morphology and abundance without the limitation of culture-dependent methods. For

example, the internal structure of anaerobic granules were determined with FISH experiment and obtained important hints about the interaction between different trophic groups of organisms, in terms of localization, morphology and abundance (Harmsen *et al.*, 1996b; Sekiguchi *et al.*, 1999). Relative abundance is determined with the ratio of specifically labeled probes to the total cell counts, counter-staining with DAPI (4',6'-diamidino-2-phenylindole hydrochloride), and a DNA-specific fluorochrome. Beside DNA staining, it is possible to use seven different types of probes labeled with different dyes on one slide at a time.

Table 4.1: Advantages and disadvantages of the FISH technique

Advantages	**Disadvantages**
• Rapid phylogenetic identification with staining • Direct, cultivation independent • Identification of individual microbial cells • Oligonucleotides (length 15-30) enough to discriminate the cells • High copy numbers of RNA increase the sensitivity • Gives information about localization and morphology of the organism • Quantification	• Need specialized camera and image capture system • Limited number of commercial probes available • Low accessibility • Cell wall impermeability • Limit the identification of low cellular ribosome content • Specificity of probes • Limited information (probe being tested) • Quantification from images takes time

Despite, FISH is widely used in many different areas, it still suffers from several limitations (Wagner *et al.*, 2003). Variable and low accessibility to the probe target sites are the important disadvantages of this technique.

Even though these hybridization techniques offer microbiologists a practical way for detecting and quantification organisms, they do not necessarily infer their functional character. A new technique has been developed to link the identity and the function using the FISH method. Parallel probing of rRNA and mRNA molecules will provide us with the possibility of determining a link to the identity and the function, together with the improvements of in situ hybridization technique for detection of lower-copy number nucleic acids (Amann *et al.*, 2001).

4.4.7 Community fingerprints

Fingerprinting techniques are generally employed for screening clone libraries, monitoring the population shifts over time, comparing the microbial diversity between samples, and estimating the microbial diversity for different ecological samples. The commonly applied fingerprinting techniques are the Denaturing Gradient Gel Electrophoresis (DGGE), the Temperature Gradient Gel Electrophoresis (TGGE), the Restriction Fragment Length Polymorphism (RFLP), the Terminated RFLP (T-RFLP), and the Single-Strand Conformation Polymorphism (SSCP). The principle of these techniques is based on the separation of DNA of different species in mixed cultures using gel electrophoresis.

4.4.7.1 The DGGE and the TGGE

In principle, DNA fragments of the same size but different base-pair sequences are subjected to electrophoresis on an acrylamide gel with a linearly increasing gradient of denaturing agents (a mixture of formamide and urea). During electrophoresis, the DNA fragments melt in unique segments, called melting-domains, with an identical melting temperature. Since the melting-domains, which are sequence-specific, reach their melting temperature, partly denatured double-stranded DNA amplicons stop migrating at different levels of the gel, thus get separated. This separation, if it is based on linear gradient of denaturants at constant temperature is called DGGE(Muyzer *et al.*, 1993), if based on linear temperature gradient at constant denaturants is called TGGE (Rosenbaum and Riesner, 1987). DNA bands in the DGGE and the TGGE profiles can be visualized by ethidium bromide, SYBR Green I or silver staining (Muyzer and Smalla, 1998). To separate the most stable domains together with the others in complex samples and to prevent complete the melting of the amplicons, a GC-rich sequence of approx. 40 nucleotides (GC-clamp) is preferred to be attached either to the 5'-end of the forward primer or the 3'-end of the reverse primer in the process of PCR or by cloning (Sheffield *et al.*, 1989). The amplicon size is also limited with the 500 base pairs (Myers *et al.*, 1985). DGGE involves identification of single base changes in DNA fragments through the alteration of electrophoretic behaviour. This adds an important advantage to the technique of gradient gel electrophoresis for the detection of the species that have less sequence variations.

Muyzer et al. (1993) introduced the DGGE technique into the ecological works. After his works, it became a widely-used community profiling technique for mainly monitoring the population shifts, and also a comparative evaluation for the different community ecosystems (Lapara *et al.*, 2000; Roest *et al.*, 2005). Once the identity of the organisms in the DGGE profile is determined by the fluctuations in microbial population over time, after that they can easily be assessed. This would be important, especially when studying microbial populations in biological wastewater treatment plants, where the influent characterization changes and, depending on populations, are very dynamic. Besides, it is used to analyze the enrichment in cultures and the isolation of bacteria; by detecting sequence heterogeneities of 16S genes, the comparison of DNA extraction methods, the screening of the clone libraries, and also the determination of PCR and cloning biases (Muyzer *et al.*, 1995).

The fingerprint of mixed bacterial populations via allowing the analysis of PCR products were amplified from SSU rDNAs in a complex microbial community. Together with using universal primers, groups or species of specific primers can also be used for particular interests of groups in PCR. The individual PCR products can be separated by DGGE. The result profiles give the several bands corresponding to the predominant members in the communities. To get the sequence information about dominant individuals, bands can be cut out from the profile and subsequently subjected to reamplification and sequence analysis. The other way of obtaining information about the community members is the hybridization with oligonucleotide probes after blotting the DGGE profiles onto nylon membranes (Muyzer *et al.*, 1993).

One of the limitations of the application of DGGE is its capability of separating relatively small DNA fragments up to 500 base pairs. Subsequently, the sequence analysis of this fragment could not be enough for phylogenetic examination or probe design. Retrieving the complete sequence is also possible, by separately constructing the 16S clone library, comparing each clone with the main profile, and identifying the corresponding bands (Felske *et al.*, 1998). This solution may also help the problem of co-migration of the DNA fragments. Cutting out the co-migrated fragments and sequencing may result in ending up with the wrong sequence information. Therefore, the DNA fragments cut from the gels have to be cloned and then analyzed for sequence. If all bands in one profile were wanted to be identified, it

would be very laborious in terms of cloning each band fraction. Hence, Felske *et al.* (1998) approach seems more feasible to take an advantage for full 16S rRNA sequence information and for overcoming the limitation of co-migration.

Another problem in the study of community diversity is the presence of multiple *rrN* operons for an individual (Nubel *et al.*, 1996). This will lead to an overestimation of microbial diversity. In some cases, it was observed that it was not possible to separate DNA fragments, even if they had significant sequence variations (Vallaeys *et al.*, 1997).

DNA based DGGEs shows the changes in species composition and changes in profiles because of growth and death and the indiscrimination of the resting cells. To make this discrimination happen, and to achieve active fraction of a community, RNA copies of the 16S rRNA were used in some studies (Teske *et al.*, 1996; Zoetendal *et al.*, 1998). New bands were detected in the profiles of the RNA based amplifications.

4.4.7.2 The RFLP and the T-RFLP

Fingerprinting can be obtained by digesting the amplified DNA fragments using restricted enzymes with 4 base pairs recognition sites and running on the agorose gels. This technique is called by the RFLP or ARDRA. These techniques measure the size of polymorphism in the restriction fragments from the PCR amplicons. This has been used for long time as a method of comparison and for screening of DNAs (Chauhan *et al.*, 2006; Earl *et al.*, 2003). To increase the resolution of the gels and to use this technique for distinctive microbial community analysis, the T-RFLP method was developed. In this method, one of the primers used was labelled with fluorescent dye and the restriction step applied was similar to those that are used for RFLP. Each terminal-restriction fragment obtained from enzymatic digestion of PCR amplicons can be defined as a single population in microbial community. The terminated DNA fragments were then run in an automated sequencer analyzer using polyacrylamide gels. Consequently, only terminal restriction fragments were visualized and the intensity of these could be quantified by using computer programs (Liu *et al.*, 1997). New quantitative fingerprinting method, called the real-time-t-RFLP, was also developed for determining the abundance of microbial communities (Yu *et al.*, 2005). The selection of the type and the number of restriction enzymes are the

critical parameters for representing accurate microbial diversity (Engebreston and Moyer, 2003).

T-RFLP, together with DGGE technique, became a popular method for rapid comparison of microbial communities. This technique, like other fingerprinting methods, can be used for comparing community structures, for identifying the organisms, quantitatively in complex environments, and for determining the microbial diversity (Marsh, 1999). Due to its high throughput and sensitivity, T-RFLP has been successfully applied to many ecosystems for different reasons, such as; for assessment of anaerobic wastewater treatment failure (Scully *et al.*, 2005); for molecular characterization activated sludge communities (Eschenhagen *et al.*, 2003); for quantitative fingerprinting of estrogen-degrading consortia in wastewater (Yu *et al.*, 2005); and, for the effect of temperature on the structure and function of the methanogenic archaeal community in a rice field soil (Chin *et al.*, 1999)

4.4.7.3 The SSCP

This technique is based on the separation of single-stranded DNA fragments by mobility, up to 300 bases in size. The mobility of the single strand mainly depends on the secondary structure of the fragments and the physiological properties of the environment (e.g., temperature, pH, and ionic strength). Theoretically, SSCP profiles contain two single-stranded DNA fragments and one double stranded DNA for an individual organism. This can be seen as an advantage over other fingerprinting techniques; because the identification of these two fragments can be done with the control isolates based on the sequence differences without the need of the sequencing of the bands(Clapp, 1995). The limitations of this technique are the same as DGGE except co-migration, which is not observed as commonly as is in DGGE. In one of the studies, 15 *Mycoplasma* species were subjected to SSCP based on the analysis of spacer region between the 16S rRNA and the 23S rRNA gene and the variable size of the PCR products discriminated the populations in SSCP gels (Scheinert *et al.*, 1996). On the contrary, DGGE gel would not be able to separate the populations, due to the compression of the profile pattern. Therefore, the selection of the techniques mainly depends on the scope of the work, indicating the more sensitive separation requiring the use of the SSCP technique.

This technique has been developed for the detection of DNA polymorphism and point mutations in genes in the area of human genetics (Orita *et al.*, 1989). These features are important advantages, in terms of effective separation of the sequences even having one nucleotide substitutions. Although, SSCP has not been applied as widely as DGGE or T-RFLP, there are a few applications for the ecological studies such as constructed wetlands , (Vacca *et al.*, 2005), activated sludges (Dabert *et al.*, 2005), and anaerobic reactors (Chachkhiani *et al.*, 2004; Delbes *et al.*, 2001; Leclerc *et al.*, 2004). In these studies, SSCP was used for investigating the effect of substrate changes on the bacterial populations(Chachkhiani *et al.*, 2004), for monitoring the bacterial and archaeal communities during the start-up of anaerobic reactors (Delbes *et al.*, 2001), and for searching the diversity of archaeal community in 44 anaerobic digesters worldwide (Leclerc *et al.*, 2004).

4.4.8 Quantitative real time PCR

To detect low-level populations in environmental samples, several quantitative PCR methods were recently developed. The quantitative real-time PCR method uses a fluorescence labeled probe and continuously measures fluorescence emitted during the amplification reaction (Heid *et al.*, 1996). Theoretically, there is a relation between the starting amount of DNA and the product of DNA. Therefore, the appearance of the initial exponential phase gives quantitative values for the calculations. The method does not require the use of a competitive sequence in the reaction, nor does it require post-PCR manipulation or gel electrophoresis. Real-time PCR has been used to quantify the 16S rRNA genes.

4.4.9 Linking identity and function

Nevertheless, the most microorganisms are uncultivated, their role in the ecosystem is not clearly known (Gray and Head, 2001). It should be kept in mind that, since the database of the 16S rRNA genes are expanding rapidly at the rate of over 2000 sequence submission each month (Benson *et al.*, 2005), the gap between identity and function increases considerably in terms of functional obscurity of these organisms. Together with the advent of molecular tools, attentions were drawn to solving the problem of functional assignment of as-yet-uncultivated organisms (Gray and Head, 2001). Due to the necessity of a link to be made between specific organisms and their

functional properties, and to get an insight in the occurrence, diversity and the interactions between the consortia, different methodologies were developed.

Cultivated isolates always bring the question up whether physiological and biochemical properties found as a single existence in laboratory conditions reflect the role of the microorganisms in natural environments. Another eccentric point is the different physiological features of the microorganisms having the identical sequences of SSU RNAs (Wolterink *et al.*, 2002). Therefore, 16S rRNA sequences of identical, of closely related strains, of subspecies, or even of different species cannot be used as differentiating markers (Amann *et al.*, 1997).

Functional assignment will probably require a number of diverse and complementary experimental approaches. For that purpose, combination of novel molecular techniques (microarrays, FISH, SIP) and different incubation techniques (MAR, stable isotope) may enable us to assign *in situ* functions to bacteria that exist in their natural habitats at species level. In recent years, FISH-MAR (Micro Auto Radiography), Isotope arrays and SIP (Stable Isotope Probing) techniques are commonly used to demonstrate a link between metabolic activity and phylogenetically characterized organisms. The outline of the Isotope array, FISH-MAR, and SIP techniques for linking the identity and function is given in Figure 4.4.

FISH-MAR is a cultivation technique, which determines the uptake of radioactive chemicals at the single cell level and identifies the cells taken up by the radioactive labeled substrate with FISH. This powerful technique allows the *in situ* detection of active organism(s) in complex communities for the utilization of specific substrate (Wagner *et al.*, 2006). On the other hand, there are two major limitations to the application of this technique. The first one is the laborious experimental procedure to the assignment of all organisms' functional properties. With the advanced techniques, up to seven groups or specific organisms can be analyzed at one run during the experimental procedure (Amann *et al.*, 1996). Characterizing all members of the population becomes a very laborious task. The second one is the identification of microorganisms with limited number of FISH probes; but not all organisms can be detected by FISH due to the inaccessibility of the probes in the target sites. In complex habitats, this may cause the underestimation of the major part of the active population.

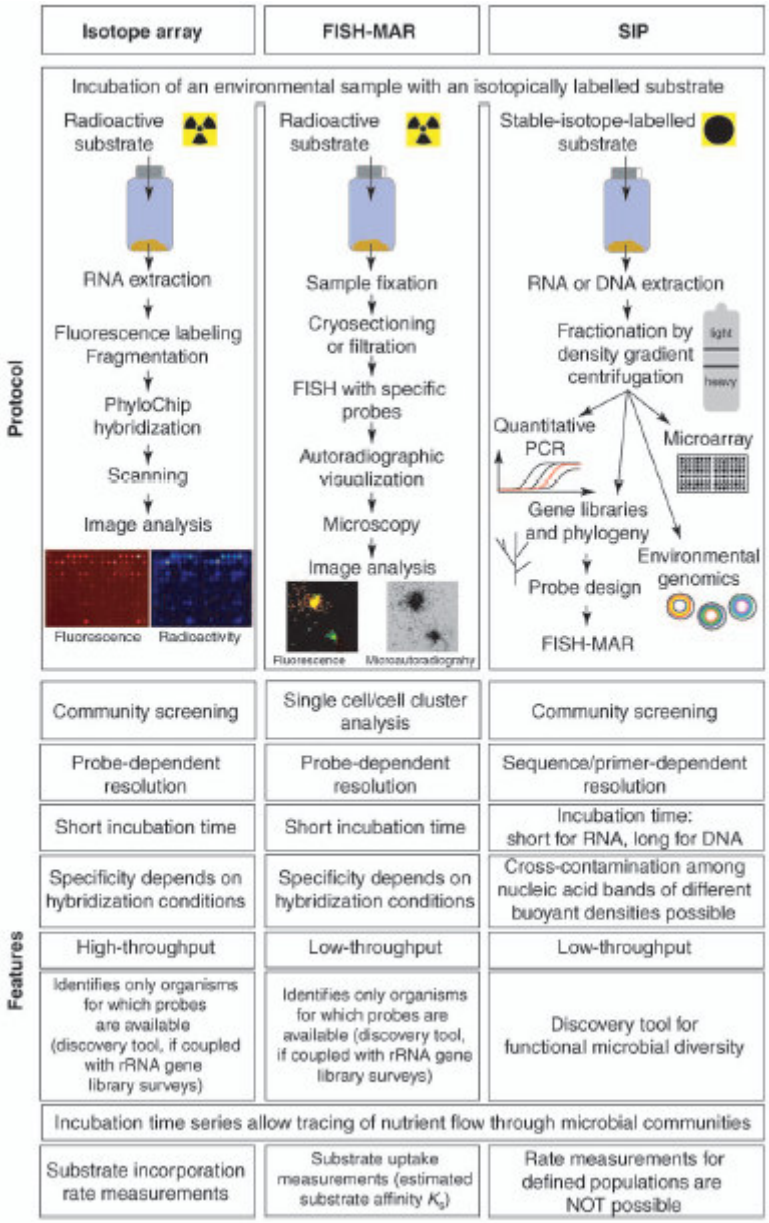


Figure 4.4 : The outline of the isotope array, FISH-MAR, and SIP techniques (Wagner *et al.*, 2006)

To overcome these FISH limitations, isotope arrays were developed to screen more organisms in a single run during the experiments (Adamczyk *et al.*, 2003). With the incorporation of radioactively labeled substrate into the rRNA of the target organisms, the hybridization of these rRNAs with fluorescently labeled cDNA and oligonucleotide probes affixed on surfaces (planar, channel glass, gel element, porous membranes and microbead) occurs (Small *et al.*, 2001; Zhou and Thompson, 2002). Then the detection of the cross labelling of fluorescence and radioactive provides information on the activity of the probe hybridized species and groups.

Construction of microarrays for monitoring the dynamics of ecosystems is a rapid and high throughput tool (Southern *et al.*, 1992). The arrays can include either 16000 or more than 65000 different 20-mer oligonucleotides in an area of 1.6 cm^2 (Lockhart *et al.*, 1996). With such feature, they are expected to accelerate the analysis of microbial community structure, function and dynamics considering the complex ecosystems.

4.4.9.1 Stable Isotope Probing (SIP)

Stable Isotope Probing (SIP) is a cultivation-independent technique, which is used to determine active indigenous bacterial communities (Radajewski *et al.*, 2000). A schematic overview of the stable isotope probing technique illustrating the link between microbial process and taxonomic identity is as outlined in Figure 4.5. This technique enables us to combine the degradation and assimilation of isotopically (^{13}C, ^{2}H, ^{15}N) labeled substrate, and then the separation of labeled nucleic acids from unlabelled nucleic acids by isopycnic density-gradient centrifugation. Afterwards, labeled and unlabeled fractions of gradient are fingerprinted by DGGE or T-RFLP and characterized by sequence analysis of the SSU rRNA genes (Ginige *et al.*, 2004; Lu *et al.*, 2005; Lueders *et al.*, 2004b; Mahmood *et al.*, 2005; Manefield *et al.*, 2002; Rangel-Castro *et al.*, 2005) and/or the functional genes (Gallagher *et al.*, 2005; Hutchens *et al.*, 2004; Lin *et al.*, 2004; Morris *et al.*, 2002; Radajewski *et al.*, 2002). The incorporation of an isotope ideally to genomes of microorganisms allows us to identify active microorganisms, which gives an important advantage over other techniques of linking identity and function. This technique is very sensitive for the separation of heavy (^{13}C) and light (^{12}C) part of the nucleic acids. Separation is done by ultracentrifuge and is based on the mass difference between ^{13}C and ^{12}C. In this separation, the heavy gradient fractions must have included only the ^{13}C enriched nucleic acids fraction in terms of reliance of the technique. The optimal way of detaching the heavy and light fractions was found to be at lower centrifuge speed (140 000 g) and longer centrifugation time (69 h) (Hutchens *et al.*, 2004). Although it is not easy to check the precision of this separation, there is a proposal for background inspection of gradients, like ^{13}C labeled pure cultures DNA and/or as an internal reference control (Singleton *et al.*, 2005). There are also other factors influencing the efficiency of separation for light and heavy fractions, such as the G + C content of the organism's nucleic acids. Organisms that have high G + C content

have a relative high mass amount, which can be close to the mass of ^{13}C labeled nucleic acids; and they will move towards the heavy part of the gradient (Lueders *et al.*, 2004a). Such possibility should be taken into account while interpreting composition of the heavy fraction of the gradient.

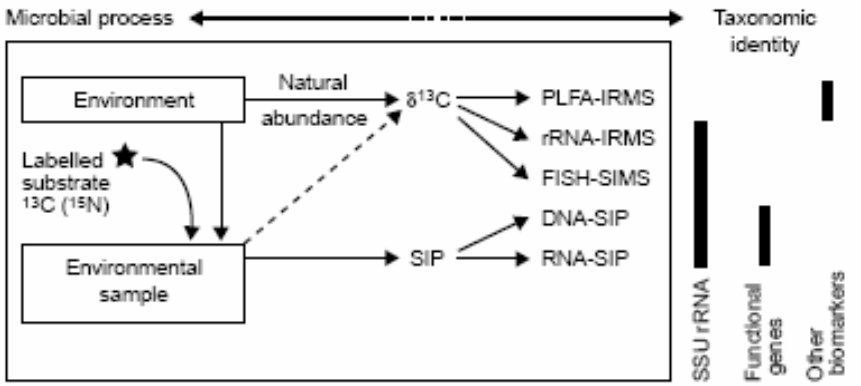


Figure 4.5 : Scheme of the stable isotope probing illustrating the link between microbial process and taxonomic identity (Radajewski *et al.*, 2003)

Both DNA and RNA can be used in nucleic acid based SIP. Selection of the nucleic acid type depends on what kind of microbial consortia we are dealing with. The incorporation rate of ^{13}C labeled substrate in the cells and the DNA synthesis, depends on the growth rate of organisms. This can be a problem especially in slow growing consortia and low cell yielding conditions. Incorporation rate of ^{13}C labeled substrate in RNA is much faster than DNA, due to a higher turnover and the lack of need for the replication of the cells. RNA reflects the activity, by having more copy numbers and being independent from replication, which provides a significant advantage on low cell yielding conditions (Manefield *et al.*, 2002). Using RNA-based SIP, it is possible to reduce the length of incubations and the amount of the labeled substrate (Radajewski *et al.*, 2002). Consequently, RNA-based SIP seems to be a very practical way for linking the identity and function of complex habitats.

SIP was initially used for the detection of microbial Phospho Lipids Fatty Acids (PLFAs) profiles (Boschker *et al.*, 1998; Bull *et al.*, 2000; Pelz *et al.*, 2001; Roslev and Iversen, 1999). This method has limitations to identify each microorganism individually, by only looking at PLFA profiles of microorganisms in complex environments (Radajewski *et al.*, 2000). The first application of the DNA-based SIP technique, which includes the effective separation of ^{12}C and ^{13}C labeled nucleic

acids, was on the methanol-degrading microorganisms in soil (Radajewski *et al.*, 2000). It was found that the active methanol utilizing population falls into only two phylogenetic groups: α-proteobacterial and *Acidobacterium* lineages. Although methanol-utilizing microorganisms were known to be widely distributed in domains of Bacteria and Eukaryotes, these lineages were unusually associated with methanol degraders. This finding clearly shows the power of the technique that reveals the real players in complex ecosystems.

Later, the first RNA-based SIP was applied on aerobic phenol-degrading community in an industrial wastewater treatment plant (Manefield *et al.*, 2002). In contrast to earlier findings for phenol degraders (Whiteley and Bailey, 2000; Whiteley *et al.*, 2000), it was shown that members of the *Thauera* genus are the responsible bacteria in the diverse community. *Thauera* species were previously known to be typical denitrifying bacteria.

RNA based SIP was successfully applied to find and identify the microorganisms responsible for propionate degradation, which is the process of gaining low energy yield in flooded soil (Lueders *et al.*, 2004b). The active population consisted of syntrophic consortia. Sixty percent of the clones were related with syntrophic propionate oxidizers; *Syntrophobacter* spp., *Peletomaculum* spp. and *Smitella* spp.. Six percent of the clones were associated with *Syntrophomonas* and the remaining part (34%) was diverse and not known as a syntroph. In addition, their syntrophic partners e.g., *Methanomicrobiaceae* (35%, hydrogenotrophic methnanogens) and *Methanosarcina* spp. (56%, acetotrophic methanogens) were detected.

Many reports have been published since the introduction of RNA-SIP. Other SIP applications were on: root exudates in soil (Rangel-Castro *et al.*, 2005), degradation of xenobiotic; pentachlorophenol in soil (Mahmood *et al.*, 2005), degradation of polycyclic aromatic hydrocarbons (Singleton *et al.*, 2005), methane oxidizing bacteria in groundwater and cave mat (Hutchens *et al.*, 2004) in peat soil microcosm (Morris *et al.*, 2002) in soda lake sediment (Lin *et al.*, 2004), methylotroph populations in acidic forest soil (Radajewski *et al.*, 2002) in oxic rice field soil (Lueders *et al.*, 2004c), methanogens in rice roots (Lu and Conrad, 2005; Lu *et al.*, 2005), methanol and acetate fed denitrifying bioreactors (Ginige *et al.*, 2005; Ginige *et al.*, 2004), benzoate degradation in coastal sediments coupled to denitrifification (Gallagher *et al.*, 2005), C_1 compounds metabolism in lake sediment (Nercessian *et*

al., 2005), phenol degradation in activated sludge (Manefield *et al.*, 2005), and methyl chloride-utilizing bacteria in terrestrial environment (Borodina *et al.*, 2005).

There are three important limitations to the application of the SIP method. The first one is the dilution of a labeled substrate before incorporation in the cells. During the incubation of microorganisms with unlabelled (^{12}C) substrate, causing the relatively less incorporation of labeled (^{13}C) substrate into the cells makes the detection of the labeled cells difficult.

The second limitation is the utilization of the labeled substrates by different trophic groups of microorganisms. After the utilization of the labeled substrate, organisms may produce a labeled intermediate, and this intermediate can be used by other trophic groups, creating confusion and mislead about the target active populations (Radajewski *et al.*, 2000). Thus, this kind of cross feeding effects may be detected and alleviated with nucleic acids fingerprinting of time series incubations (Radajewski *et al.*, 2002; Whiteley *et al.*, 2005). The above mentioned limitation, dilution of a labeled substrate, will also be an advantage for lessening the cross-feeding effect on mixed populations. Moreover, the intermediately formed labeled substrate could be diluted with the unlabeled intermediates. If the labeled intermediates are only assimilated and not dissimilated, this dilution would be higher and, subsequently, the cross-feeding effect would be much lesser.

The third limitation is the inability of reflecting the exact *in situ* conditions and (Madsen, 2005) concentration of substrates to the incubations. In the lab, in many studies, incubations of labeled substrate are achieved under batches or semi-batches with higher substrate concentrations than normally found in their ecosystems (Lueders *et al.*, 2004b). Additionally, incubation of the environmental samples with the ^{13}C labeled substrate will not be identical to the incubation of ^{12}C labeled substrate in terms of growth rate, quantity and the distribution of nucleic acids (Uphaus *et al.*, 1967; Zengler *et al.*, 1999); even though there was no effect observed on the microorganisms that assimilate the isotopic labels for many substrates (Matwiyoff and Ott, 1973). Such factors also easily affect the nature of the microbial characteristics of the samples and easily stimulate the irrelevant populations. These limitations of SIP should be considered primarily before the application and during interpreting of the results.

The techniques linking the identity to function mentioned above can be used individually or complementary to explain the different insights or features of different microbial communities. For example, some communities will require short incubation times to prevent cross-feeding of substrates by other organisms; in such a case, FISH-MAR will be the best and the most sensitive option in detecting the primary substrate consumers (Wagner *et al.*, 2006). In addition, if the interest is channelled into the determination of spatial localization of the organisms, FISH-MAR would allow us to get information about the active organisms and their locations.

Ginige et al. (2004) showed the use of a combination of SIP and MAR-FISH that more solid results were obtained for methanol-fed denitrifying microbial community. Firstly, the SIP was applied to obtain a 16S rRNA clone library of the active population. Then FISH probes were designed based on the result of sequence analysis. Secondly, FISH-MAR provided visual evidence with the in situ identification of organisms in their complex habitats, using the specific probes. Such an approach provided an effective means to address the presence and physiology of microorganisms in their natural environments.

5. MATERIALS AND METHODS

5.1 Operation of Reactors

5.1.1 Source of inoculums

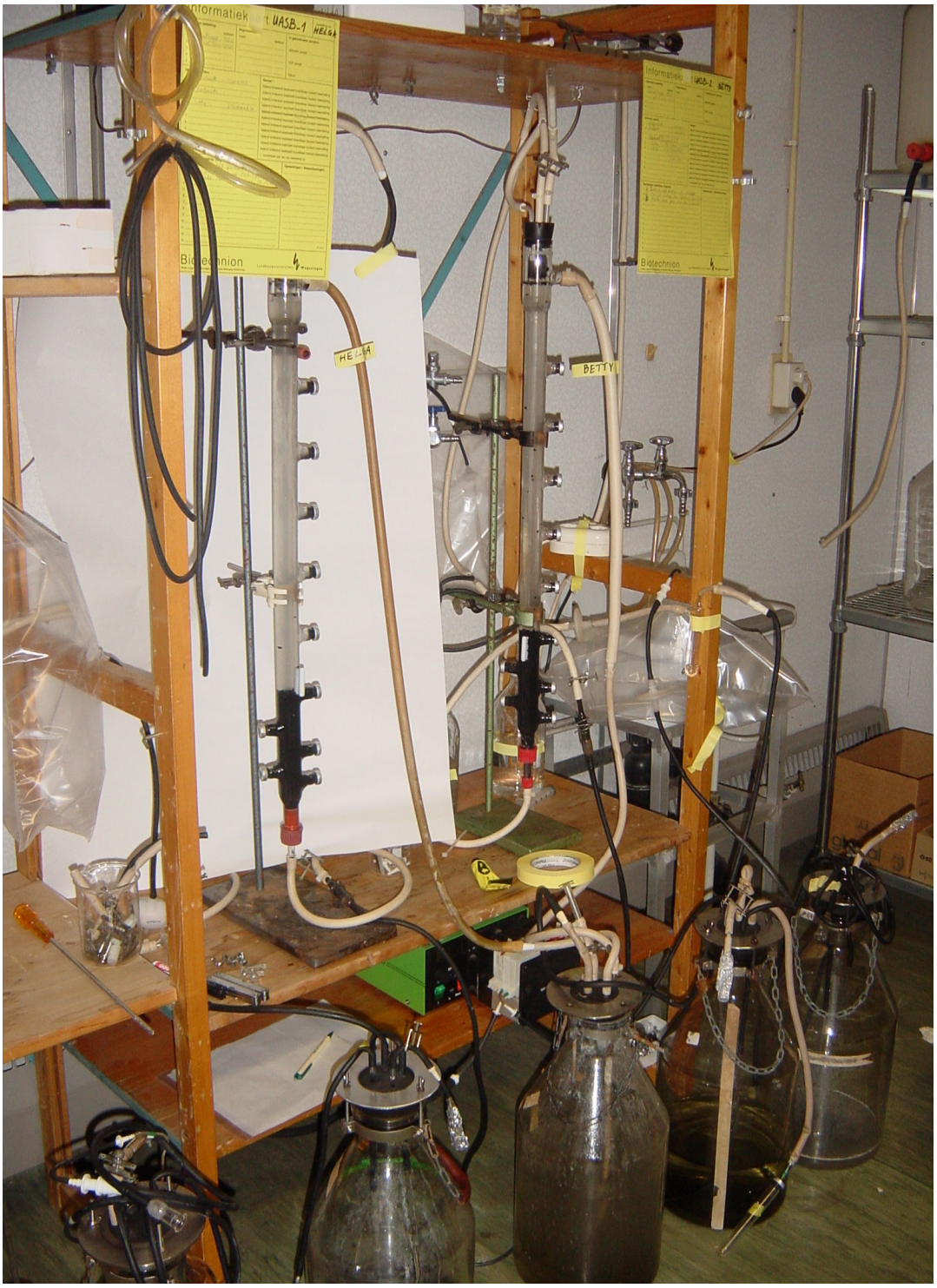
The inoculums for all three reactors were collected from the paper mill wastewater treatment plant (Paques Environmental Technology, BV, Balk, The Netherlands) exhibiting ~70-80% COD removal, in which both sulfate reduction and methanogenesis were observed. In the gas composition, CH_4, CO_2, and H_2S were detected as being 80%, 19%, and 1%, respectively. The COD/SO_4^{2-} of the wastewater was ~9.5-10; the total COD and pH of the wastewater was 1.7 $g.l^{-1}$ and 6.9, respectively. The constituents of the wastewater were given in the Table 1.

Table 5.1: The constituents of the paper mill wastewater fed to the UASB reactor

Constituents	Unit	Concentration
Starch	mg COD.l^{-1}	850
Acetate	mg COD.l^{-1}	500
Propionate	mg COD.l^{-1}	300
Butyrate	mg COD.l^{-1}	45
Formate	mg COD.l^{-1}	20
Sulfate	mg.l^{-1}	180

5.1.2 Reactors

The three identical lab-scale glass UASB reactors consisted of a 500 ml working volume (Picture 5.1). The substrate was pumped from the feeding bottle to the bottom of the reactors. The reactors were operated at a hydraulic retention time of 12 h and at constant temperature room of 37±1°C. The schematic representation of the reactors was given in Figure 5.1. The gas was collected from the reactor and was monitored with a wet gas meter (Meterfabriek Dordrecht, The Netherlands). The reactor was equipped with a liquid separator that consists of an upside-down funnel. Synthetic preparation of the feed with a total COD concentration of 3 $g.l^{-1}$ was used in this study for all three reactors. The inoculations VSS of the reactors were adjusted to 15 $g.l^{-1}$. Operational conditions were kept constant during the experimental period.



Picture 5.1 : The UASB reactors used in this study

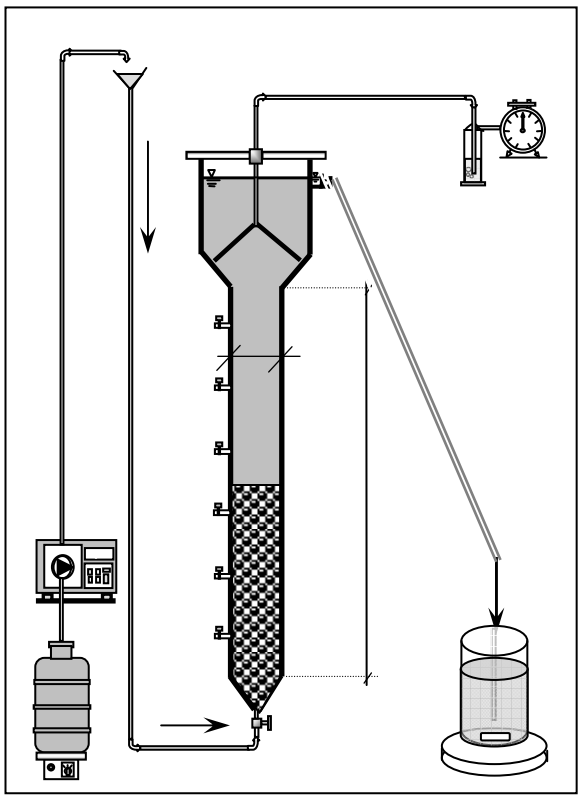


Figure 5.1 : Schematic representation of the UASB reactors used in this study

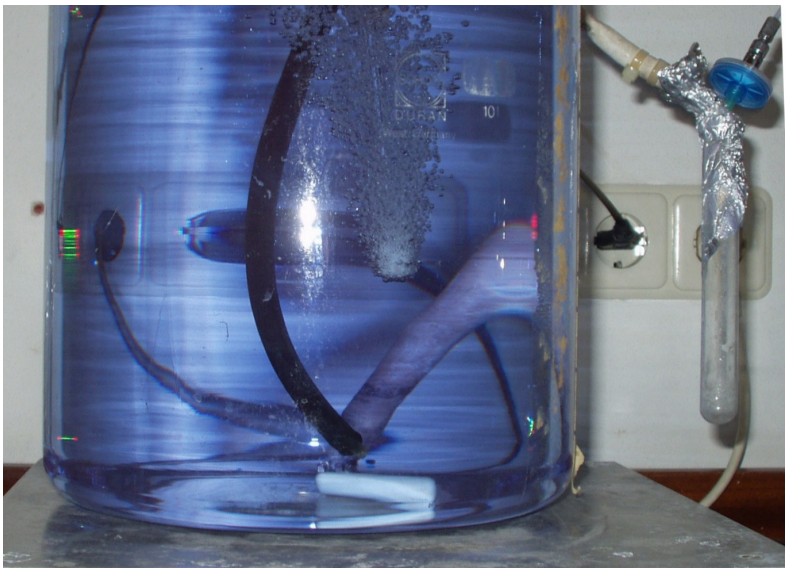
5.1.3 Media composition

Butyrate was the sole carbon and energy source for all three reactors; and the concentration of butyrate was 18 mM, corresponding to 3 g COD. l^{-1}. The BUT (Butyrate) reactor was fed only with butyrate; the BLS (Butyrate + Limited Sulfate) reactor was fed with butyrate together with 3 mM sulfate; and the BUS (Butyrate + Unlimited Sulfate) reactor was fed with 45 mM sulfate and butyrate. The total compositions of the feed of the reactors were given in the Table 1. According to Hungate, the media was anaerobically prepared (1969). The medium for the reactors were prepared in 10-liter bottles. Before the sterilization of the feeding bottles, all ingredients were added in the bottles except bicarbonate, sulfide, vitamins and the substrates. As soon as the bottles were taken out of the sterilization chamber, they were purged with N_2 until the temperature came down to the room temperature. Later, the remaining components of the reactors were added under sterile conditions purging with an N_2 0.2µ filter. All of the compounds added to the reactors were heat sterilized except for the vitamins, which were filter sterilized.

Table 5.2: The composition of the UASB reactors influents

Substances	**Concentrations, mg.l^{-1}**
Nutrients	
KH_2PO_4	408
$Na_2HPO_4.2H2O$	534
NH_4Cl	360
NaCl	360
$MgCl_2.6H2O$	120
$CaCl_2.2H2O$	132
$NaHCO_3$	4000
Reducing Agent	
$Na_2S.9\ H_2O$	240
Anaerobic Control Agent	
Resazurin	5
Acid Stock Solution (I)	
HCl	1.800
H_3BO_3	0.062
$MnCl_2$	0.061
$FeCl_2$	0.944
$CoCl_2$	0.065
$NiCl_2$	0.013
$ZnCl_2$	0.068
$CuCl_2$	0.050
$AlCl_3$	0.050
$(NH_4)_6Mo_7O_{24}$	0.050
Alkaline Stock solution (II)	
NaOH	0.400
Na_2SeO_3	0.017
Na_2WO_4	0.029
Na_2MoO_4	0.021
Vitamins	
Biotin	0.02
Nicotinamid	0.20
p-Aminobenzoic acid	0.10
Thiamin (Vit B1)	0.20
Panthotenic acid	0.10
Pyridoxamine	0.50
Cyanocobalamine (Vit B12)	0.10
Riboflavine	0.10





Picture 5.2 : The general view of the feed bottles a) before autoclaving b) purging with N_2 in order to cool down the temperature of the bottles and removing the oxygen after autoclaving

5.1.4 Calculations for substrate utilization by sulfidogens and methanogens

The equations for calculating butyrate conversion rates by sulfidogens and methanogens were given as follows:

$COD_{conv,mb} = p_{CH4}Q_g f_{CH4} + gQ_l$

$COD_{conv,srb} = 2\ Q_l/V\ (SO_{4,i} - SO_{4,e})$

$COD_{conv,mb}$: COD (g/L) converted by methanogens

$COD_{conv,srb}$: COD ($g.l^{-1}$) converted by methanogens

p_{CH4} : partial pressure of methane

Q_g : gas production ($l.day^{-1}$)

f_{CH4} : conversion factor from liter to g COD

g : solubility of methane in water (g $COD.l^{-1}$)

Q_l : flow rate ($l.day^{-1}$)

V : effective volume of the reactor (l)

$SO_{4,I}$: influent sulfate concentration ($g.l^{-1}$)

$SO_{4,e}$: effluent sulfate concentration ($g.l^{-1}$)

5.2 Sludge Activity Tests

The inoculums for all enrichments were collected from the UASB reactor unit of a paper mill wastewater treatment plant and from three lab-scale UASB reactors operated in the constant temperature room of 37°C, as described previously. Three different activity measurements were used for this collection: the specific methanogenic activity, the specific sulfidogenic activity, and the simultaneous measurement of the methanogenic, sulfidogenic and the total sludge activity.

The basic medium was prepared by adding KH_2PO_4, Na_2HPO_4 trace elements I, II, resazurin (Table 2) and then was boiled in order to remove any O_2 and in order to provide anaerobic conditions; later, it was cooled down by the application of continuous N_2-purging and was transferred immediately and anaerobically into the serum vials by using a dispenser. The bottles were sealed with butyl-rubber stoppers (Rubber BV, Hilversum, The Netherlands) and aluminium caps. The bottles were purged with a 1.7 atm (172.2 kPa) N_2-CO_2. After purging, the bottles were autoclaved at 121°C for 20 minutes. Then the salts, the vitamins and the bicarbonate solutions were added to the serum bottles (Table 2). Later, $Na_2S.9H_2O$ was added as a reducing agent to assure complete anaerobic conditions. All compounds were sterilized with autoclave except vitamins, which were sterilized with filter. Yeast extracts addition was omitted to eliminate the growth of the primary fermenters. After that, washed granular sludge was crushed and homogenized using a potter tube; and 1ml of this crushed sludge was transferred into serum bottles by sterile syringes to give the final VSS concentration of approximately 1 $g.l^{-1}$. All inoculations were carried out by plastic, sterile, and disposable syringes together with the suitable

needles. After connecting the syringe and the needle under sterile conditions, they were flushed several times with sterile N_2 gas in a 120 ml serum bottle.

Substrates were added to the basal medium from sterile and completely anoxic concentrated stock solutions (1 M) flushed with nitrogen by sterile syringes, to give the desired final concentrations. The serum bottles consisted of approximately 2g of $COD.l^{-1}$ of sodium butyrate, propionate, sodiumacetate, sodium formate and hydrogen+carbondioxide as substrates.

Enrichments were cultivated at 37°C in 120 ml serum bottles that contain 50 ml medium. All enrichments were cultivated independently in triplicates. The average values of the triplicates together with the standard deviations were presented in the graphs and tables. Utilization of substrate was followed by monitoring the decrease in each individual substrate, in addition to the monitoring of methane and hydrogen production. Additionally, autoclaved bottles that included cell suspensions and substrates were incubated as abiotic controls, and the bottles that included the suspended sludge without substrates were incubated as negative controls for each different feed mixture and for different temperatures. The produced methane values were corrected for all enrichments. All incubations were conducted in the dark and under non-shaking conditions except the hydrogen series.

At various periods, the gas (0.2 ml) and the liquid samples (1ml) were taken from the serum bottles by syringes. Then, the gas samples were directly injected to the GC and the liquid samples were centrifuged at 14000 rpm for 10 minutes. Later, the supernatant was analyzed for the VFA components at HPLC.

After the termination of each experiment, the exact amount of cell dry mass values were determined after drying the cell suspensions for 24 hours at 100ºC. The dried samples were burnt further for 2 more hours at 550ºC to find out the volatile part of the mass. The mass of the medium components were taken into account for the correction of the biomass values. The pH was determined upon the completion of the experiment.

5.2.1 Methanogenic activity

The cumulative methane production versus time was plotted and the maximum specific activity was calculated from the linear increase of methane production.

Then, specific methanogenic activity values were converted to COD-CH_4.g^{-1} VSS.day^{-1}.

5.2.2 Sulfidogenic activity

In the case of sulfate enrichments, the amount of sulfate was adjusted to the concentration of the reducing equivalent of the electron donor. Each series of substrate incubations were also realized with BRES (Bromoethane sulfonic acid, 30 mM), which is a specific inhibitor of methanogenesis that is used to find out the sulfidogenic activity alone. Sulfidogenic activity was determined from the sulfate depletion curve, which was determined by each sampling period. Then, it was corrected with the hydrogen sulphide measurements, which were done at the end of the incubation period.

5.2.3 Substrate depletion activity tests

The maximum specific activities of the sludge grown in the reactor were determined by the substrate depletion in the serum bottles. The activity was calculated from the course of the substrate concentration and the amount of VSS, which was determined after the termination of each experiment. The Specific activity was calculated from the linear decrease of the substrate concentration, which corresponds to the zero order region of the curve. All of the experiments were performed in triplicates.

5.2.4 The effect of different substrates on the butyrate degradation rate in the reactor sludges

Independent substrate depletion experiments were carried out, starting with different initial concentrations of substrates. The initial substrate concentrations for the three reactor sludges were varied between 5-150 mM (516-15489 mg COD.l^{-1}) for propionate; 5-150 mM for acetate (385-9630 mg COD.l^{-1}); 0.5-30 mM for formate ; 0.15-18 mM for hydrogen+CO_2 .

5.3 The Most Probable Number (MPN) Counting and Enrichment Procedures

MPN counting was used to assess the number of specific bacteria presents in the sludge of the reactors. Three different physiological types of bacteria (acetogenic bacteria, methanogenic archaea, sulfate reducing bacteria) were counted using the MPN technique (n=3). 10 ml of the reactor sludge granules were disintegrated by the Potter

homogenizer (Tamson, Zoetermeer, The Netherlands). Serial dilutions were made as described in Figure 5.2. The MPN counts were made in 120 ml serum vials containing 50 ml of medium. Serum vials contained 20 mM butyrate, 20 mM propionate, 20 mM acetate, and 20 mM formate as substrate, with and without 20 mM sulfate. In the case of butyrate or propionate in the absence of sulfate, the H_2-grown culture (*Methanosprillum hungatei*) alone or together with the acetate grown culture (*Methanosaeta conciili*) was added to establish a steady hydrogen and acetate consumption. After 6 months of incubation, methane production, sulfide formation and substrate depletion were determined. The cultures were also microscopically examined using a phase contrast microscope. The highest dilutions, where growth was observed, were subjected to further molecular characterization and were also subcultured to obtain pure cultures or defined cocultures.

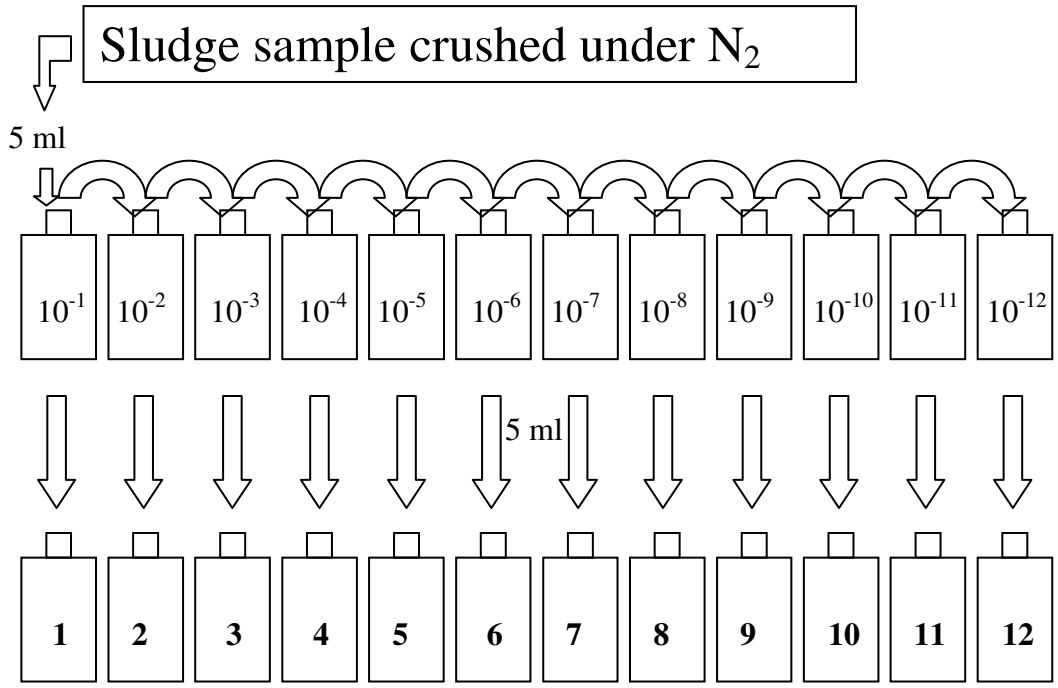


Figure 5.2 : The flow diagram of the most probable numbering of the sludges

5.3.1 The molecular characterization of enrichments

Sampling for molecular analyses was performed during the entire period of the incubation at each temperature. Suspensions of the cultures from the batches were withdrawn with syringes under sterile conditions and were transferred into 10 ml pre-autoclaved screw-cap vials immediately. The aliquots were centrifuged at 10,000

rpm, +4^{o}C, for 15 min (centrifugation). Supernatants were discarded and the pellets were washed with phosphate buffered medium. The biomass samples, as well as the inoculums samples from the Eerbeek paper mill anaerobic wastewater treatment plant, were directly subjected to DNA extraction; during which, mechanical disruption by bead beating and the according phenol/chloroform/iso-amyl-alcohol extraction were performed as outlined somewhere else (Oude Elferink *et al.*, 1997).

Aliquots (1 µl) of the DNA, isolated from the biomass samples, were used for subsequent bacterial and archaeal PCR amplifications. For bacterial PCR U968-GC (5′-CGCCCGGGGCGCGCCCCGGGCGGGGCGGGG GCACGGGGGGAACGCG AAGAACCTTAC-3′) forward and L1401 (5′-GCGTGTGTA CAAGACCC-3′) reverse primers (Nubel *et al.*, 1996) were used to amplify the region between the V6 and the V8 regions of the bacterial 16S rRNA. For archaeal PCR, the V2-V3 region, was amplified using A109 (5′-ACT GCT CAG TAA CAC GT-3′) (Grosskopf *et al.*, 1998) forward and 515-GC (5′-ATCGTATTACCGCGGCTGCTGGCA-3′) (Lane, 1991) reverse primers. All primers were provided from MWG-Biotech (Ebersberg, Germany).

The reaction mixture was subjected to PCR under the following conditions: denaturation at 94°C for 5 min followed by 30 cycles of denaturation at 94°C for 30 s, primer annealing for 1 min (annealing temperatures for bacteria and archaea are 56 and 52, respectively), and DNA extension at 68°C for 1 min. After the last amplification cycle, samples were kept for the final extension at 68°C for 7 min and were immediately cooled down to 4°C. The size and the yield of the amplicons (5 µl) were checked by electrophoresis on a 1% (wt/vol) agarose gel (Invitrogen Breda, The Netherlands) in 1xTAE buffer for 20 min at 100 volts. A DNA ladder (GeneRuler^{TM} 100bp DNA Ladder Plus, MBI Fermentas, Lithuania) was used to check the quality and the quantity of the products. The agarose gels were stained with ethidium bromide (BioRad, Hercules, CA) and the images of the gels were captured using a transilluminator (GelDoc 2000, BioRad, Hercules, CA).

GC-clamped amplicons from the PCR experiments were run in the 8% polyacrylamide (37.5:1acrylamide-bisacrylamide) gels containing urea and formamide using DCode TM System apparatus (BioRad, Hercules, CA). The gradients were prepared with the 40% acrylamide/bis solution (37.5:1 acrylamide/bis, BioRad, Cat. No: 161-0148) using the 0% and the 100% stock

solutions. The 100% solution contained 7 M urea (UltraPure™, Invitrogen, Life Technologies, Cat. No: 15505-027) and 40% (vol/vol) formamide (Sigma, 200-842-0) and also glycerol (Sigma, 200-289-5). Right before pouring the gel, 10% ammonium persulfate (Sigma, 231-786-5) and TEMED (Invitrogen, Life Technologies, Cat. No: 15524-010) were added to the working solutions in order to initiate and accelerate polymerization. The PAG was casted (DCode System, BioRad, Hercules, CA) with the help of the gradient delivery system, from which the working solutions were gradually pumped into the casting sandwich assembly with a flow-rate of 4 $ml.min^{-1}$, providing a denaturing gradient ranging from 55% to 38% (bottom to top) for bacterial applications, and from 50% to 30% for archaeal applications. Gelbond® PAG film (Amersham Pharmacia Biotech, 80-1129) was placed on the rear glass plate of the casting sandwich assembly prior to casting to physically support the polymerized gel and to allow an ease in handling the gel after electrophoresis.

After loading 10 µL of the PCR products together with 3 µl loading buffer to the polymerized PAG, electrophoresis was performed first at 200 Volts for 5 min, then at 85 Volts for 16 hours in 1xTAE buffer. Electrophoresis temperature was 60°C. After the completion of electrophoresis, the gel supported by Gelbond® PAG film was removed from the casting assembly and was silver-stained, following the protocol given by Sanguinetti *et al*. (1994). Images of the DGGE bands were captured in the ".jpg" format by scanning the gels with a scanner.

5.3.2 Cloning and sequencing

Representative samples for each condition (given in italic and bold in the figure of DGGE profiles) were selected for further cloning and sequencing. The amplified V6-V8 regions (bacteria) and V2-3 regions (archaea) of the 16S rRNA (~400 bp) were purified with a QIAquick PCR purification kit (Qiagen GmbH, Hilden Germany) following the manufacturer's instructions. The quality and the amount of the purified PCR products were determined by electrophoresis on a 1% (wt/vol) agarose gel with a DNA marker (GeneRuler™ 100bp DNA Ladder Plus, MBI Fermentas, Vilnius, Lithuania). These amplicons were ligated into the pGEM®-T Easy Vector System I (Promega, Madison, WI) and were sequentially cloned into XL1-Blue Competent Cells (Stratagene, US) following the manufacturer's instructions. Clones, screened

with ampicilin selection and blue-white colony screening, were transferred into 20 µl TE buffer and then were incubated at 95°C for 10 min to lyse the cells. The lysed cells were amplified with the pGEM®-T specific PG1 (5′-TGGCGGCCGCGGGAA-3′) and the PG2 (5′-GGCCGCGAATTCACTAGTG-3′) primers, and these amplicons were digested with a mixture of *Alu* I, *Cfo* I, and *Msp* I (Promega, Madison, WI) restriction enzymes at 37°C for 90 min. To differentiate these digested clones, RFLP analysis was conducted by using Elchrom Submerged Gel Electrophoresis System (Cham, Switzerland). The digested PCR products were run in the 12% (wt/vol) pre-cast agarose gels (Elchrom, Cham, Switzerland) with a DNA marker (GeneRuler™ 50 bp DNA Ladder Plus, MBI Fermentas, Vilnius, Lithuania) at 100 V, 55 °C, for 45 min and were visualized with ethidium bromide staining. Some clones that have different profiles in RFLP runs were selected for further DGGE and sequencing analyses. The sequencing primer was T7 (5′-TAATACGACTCACTATAGGG-3′) (Promega, Madison, WI), when only one side read.

Homology searches of the databases were conducted by using the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST) (Altschul *et al.*, 1997).

5.4 Molecular Characterization of The Reactor Sludge

5.4.1 Extraction of DNA and RNA

DNA was extracted from 1 ml of concentrate biomass with the FastDNA SPIN kit for soil (Bio101) following the manufacturer's instructions and was eluted in nuclease-free water (50 µl) and was stored at -20°C.

Total RNA was extracted from 1 ml of concentrate biomass by using a slightly modified TRIZOL (Invitrogen, Breda, The Netherlands) protocol. First of all, RNAlater™ (Ambion Inc., USA), which was used for storing the samples, was removed from the samples before the extraction by centrifugation (23,645 × *g*, 15 min, 4°C) (Rotina 35R; Hettich, Tuttlingen, Germany). The resulting pellet was washed with phosphate-buffered saline (PBS) and was transferred into a RNase-free 1.5-ml bead beating tube consisted of 300 mg of zirconium beads (diameter, 0.1 mm; Sigma-Aldrich). The homogenization of the cells was carried out by bead beating (FastPrep120; Bio 101) in 1.5 ml of TRIZOL two times of 40 s at a speed of 4.5, were left to chill on ice for 1 min in between samples. After homogenization, the

insoluble material from homogenate was removed by centrifugation at 12,000xg for 10 minutes at 4°C. The supernatant was transferred into a new RNase free tube. The homogenized samples were incubated for 5 minutes at room temperature. Later, 0.3 ml of chloroform was added to the substance and the tube was shaken vigorously in hand for 15 seconds.

Then, it was incubated at room temperature for 2 min. After centrifugation (12,000 x g for 15 minutes at 4°C), the aqueous phase was transferred into a fresh tube and RNA from the aqueous phase was precipitated with 0.75 ml of isopropyl alcohol (J. T. Baker, Deventer, The Netherlands) by incubating the tubes at room temperature for 10 min. Then, the sample was centrifuged at 12,000 x g for 10 minutes at 4°C, was washed with 1ml of 75% ethanol, was vigorously vortexed for 1 min to mix the sample, was centrifuged at 7,500 x g for 5 minutes at 4°C, was dried for 5 min at room temperature and was dissolved in 100 µl RNAse free water. Aliquots of nonpurified total RNA were analyzed by electrophoresis on a 1% (wt/vol) agarose gel in a buffer that contained 40 mM Tris, 10 mM sodium acetate, and 1 mM EDTA (pH 8.0) which was stained with ethidium bromide.

RNA purification together with DNase treatment was performed with RNeasy-Mini kit (Qiagen, Hilden, Germany). All manipulations were made following the manufacturer's protocol except the DNase treatment. At the column treatment of the purification step, the sample was digested with 30 U of RNase-free DNase Set (Qiagen, Hilden, Germany) and RDD buffer was digested at room temperature for 15 min. This step was repeated one more time to make sure that the entire DNA was removed. At the last step, RNA was eluted from the column two times with 50 µl of RNase-free water and was stored at -80°C. The amount of purified RNA was quantified using the RiboGreen® RNA Quantitation Reagent and Kit (Molecular Probes, Eugene, Oregon, USA).

5.4.2 Complementary DNA synthesis

The single-stranded DNA, that is complementary to 16S ribosomal RNA, has been synthesized by SuperScript™ III RNase H^+ reverse transcriptase (Invitrogen, Breda, The Netherlands) following the manufacturer's instructions. RT-PCR analyses were performed with 10 ng of the total RNA. The first reaction mixture of 13 µl contained an aliquot of the total RNA, 10 pmol of universal reverse primer 1492R (5′-

GGTTACCTTGTTACGACTT-3′) (Lane, 1991), 10 pmol dNTP mix and sterile, and RNase-free water (Qiagen GmbH, Hilden, Germany). To denature the secondary structure of 16S rRNA, the mixture was incubated at 65°C for 5 min and then was immediately incubated on ice for 1 min. For the cDNA synthesis, the following agents were added to the previous denatured 13 µl reaction mixture: 4 µl 5× First strand buffer, 1 µl 0.1 M DTT, 1 µl (40 units) RNaseOUT^{TM} RNase inhibitor (Invitrogen, Breda, The Netherlands), and 1 µl (200 units) Superscript III^{TM} RT. The reaction was performed at 50°C for 1 h and then was incubated at 70°C for 15 min to inactivate the reaction. To remove the RNA complementary to the cDNA, the reaction mixture was incubated with RNAse H (2 units) at 37°C for 20 min. The mixtures were also prepared without the target RNA to check a possible contamination at each RT reaction time.

5.4.3 PCR amplification

Aliquots (2 µl) of the cDNA solution were used for subsequent bacterial and archaeal PCR amplifications. PCR was performed in 50 µl reaction mixtures using *Taq* DNA polymerase (Invitrogen, Breda, The Netherlands). The PCR mixture contained 5 µl of 10× PCR buffer 3 µl of 50mM, 1 µl of deoxynucleoside triphosphate (dNTP)s containing 10 mM each dNTP, 1 µl of 10 mM forward and reverse primers, 0.5 µl of the *Taq* DNA polymerase (5 U/µl), and nuclease free water for a final volume of 48 µl. Later, 2 µl target cDNA was added. Beside cDNAs, the RNA samples, which were treated with DNase, were directly subjected to PCR analysis to check whether there was still any DNA remaining in the samples. The samples were amplified using the Biometra Thermocycler (Goettingen, Germany). Complete bacterial 16S rRNA was selectively amplified for cloning and sequencing using Bact-7-forw (5′-AGAGTTTGAT(C/T)(A/C)TGGCTCAG-3′) and 1492-rev- (5′-CGGCTACCTT GTTACGAC-3′) primers (Lane, 1991). The bacterial PCR U968-forw-GC (5′-CGCCCGGGGCGCGCCCCGGGCGGGGCGGGGGCACGGGGGGAACGCGAA GAACCTTAC-3′) forward and L1401-rev- (5′-GCGTGTG TACAAGACCC-3′) reverse primers (Lane, 1991) were used to amplify the V6 to V8 regions of the bacterial 16S rRNA. For the archaeal PCR, the V2-V3 region was amplified using A109-forw- (5′-ACT GCT CAG TAA CAC GT-3′) (Grosskopf *et al.*, 1998) forward and 515-rev-GC (5′-ATCGTATTACCGCGGCTGCTGGCA-3′) (Lane, 1991)

reverse primers. All primers were collected from MWG-Biotech (Ebersberg, Germany).

The reaction mixture was subjected to PCR under the following conditions: denaturation at 94°C for 5 min followed by 30 cycles of denaturation at 94°C for 30 s; primer annealing for 1 min (annealing temperatures are for bacteria and archaea, 56 and 52, respectively); and DNA extension at 68°C for 1 min. After the last amplification cycle, samples were kept for final extension at 68°C for 7 min and were immediately cooled to 4°C. The size and the yield of the amplicons (5 µl) were checked by electrophoresis on a 1% (wt/vol) agarose gel.

5.4.4 Cloning and sequencing

The amplified V6-V8 regions for bacteria and V2-3 regions for archaea of the 16S rRNA (~400 bp) were purified with a QIAquick PCR purification kit (Qiagen GmbH, Hilden Germany) following the manufacturer's instructions. The quality and the amount of purified PCR products were determined by electrophoresis on a 1% (wt/vol) agarose gel with a DNA marker (GeneRuler^{TM} 100bp DNA Ladder Plus, MBI Fermentas, Vilnius, Lithuania). These amplicons were ligated into pGEM$^{®}$-T Easy Vector System I (Promega, Madison, WI) and were sequentially cloned into XL1-Blue Compotent Cells (Stratagene, US) following the manufacturer's instructions. Clones that were determined with ampicilin selection and blue-white color screening were transferred into 20 µl TE buffer and were then incubated at 95°C for 10 min in order to lyse the cells. The lysed cells were amplified with pGEM$^{®}$-T specific PG1 (5′-TGGCGGCCGCGGGAA-3′) and PG2 (5′-GGCCGCGAATTCACTAGTG-3′) primers, and these amplicons were digested with *Alu* I, *Cfo* I, and *Msp* I (Promega, Madison, WI) restriction enzymes mixture at 37°C for 90 min. To differentiate these digested clones, RFLP analysis was conducted using Elchrom Submerged Gel Electrophoresis System (Cham, Switzerland). Digested PCR products were run in the 12% (wt/vol) pre-cast agarose gels (Elchrom, Cham, Switzerland) with a DNA marker (GeneRuler^{TM} 50 bp DNA Ladder Plus, MBI Fermentas, Vilnius, Lithuania) at 100 V, 55 °C and for 45 min, and were visualized with ethidium bromide staining. Some clones, which have different profiles in RFLP, were selected for further DGGE and sequencing analysis. Plasmids of selected clones were purified using the QIAprep Spin Miniprep Kit (Qiagen

GmbH, Hilden, Germany). The sequencing analysis was carried out with the Sequenase Sequencing Kit (Amersham, Slough, UK). The sequencing primers labeled with IRD800 (MWG-Biotech, Ebersberg, Germany) were as follows: T7 (5′-TAATACGACTCACTATAGGG-3′) (Promega, Madison, WI), Sp6 (5′-GATTTAGGTGACACTATAG-3′) (Promega, Madison, WI), Uni-519-Rev (5′-G(A/T)ATTACCGCGGC(G/T)GCTG-3′) (Lane, 1991), Uni-533-Forw (5′-GTGCCAGC(A/C)GCCGCGGTAA-3′) (Lane, 1991), Bact-1100-Rev (5′-GGGTTGCGCTCGTTG-3′) (Lane, 1991). For archaea, the IRD800 labeled sequencing primers were A109-Forw and A915-Rev. The sequences were automatically analysed on a LI-COR (Lincoln, NE) DNA sequencer 4000 L, and were checked and edited manually.

The assembly of DNA sequence data was performed with the DNA STAR, Seqman II program (expert sequence analysis software). The homology searches of the databases were conducted using the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST) (Altschul *et al.*, 1997). The alignment studies were performed on Clustal X program (Chenna *et al.*, 2003). The alignments were manually checked using GeneDoc Multiple Sequence Alignment Editor& Shading Utility Version 2.6.002 (http://www.psc.edu/biomed/genedoc). A phylogenetic tree was constructed in the ARB program package (Ludwig *et al.*, 2004). All sequences were checked for chimeric artifacts using the CHIMERA_CHECK program version 2.7 in the Ribosamal Database Project II (RDP II) (Maidak *et al.*, 2001).

5.4.5 DGGE

GC clamped amplicons were run in the 8% polyacrylamide (37.5:1acrylamide-bisacrylamide) gels containing urea and formamide using DCode TM System apparatus (BioRad, Hercules, CA). For this purpose, two aliquots of the 8% polyacrylamide gel (PAG) stock solutions, one with a 0% denaturing property, and the other with a 100% denaturing property, thus containing 7 M urea (UltraPure™, Invitrogen, Life Technologies, Cat. No: 15505-027) and 40% (v:v) formamide (Sigma, 200-842-0), were prepared from the commercially available 40% acrylamide/bis solution (37.5:1 acrylamide/bis, BioRad, Cat. No: 161-0148). Glycerol (Sigma, 200-289-5) was also added to the solutions. From these stock solutions, working aliquots with 38% and 55% for bacteria and from 30% to 50% for

archaea denaturing capacity, the optimum for the resolution of the desired DGGE bands were prepared. Right before pouring the gel, the 10% ammonium persulfate (Sigma, 231-786-5) and TEMED (Invitrogen, Life Technologies, Cat. No: 15524-010) were added to the working solutions to initiate and accelerate polymerization. The PAG was casted (DCode System, BioRad, Hercules, CA) with the help of the gradient delivery system, from which the working solutions were gradually pumped into the casting sandwich assembly with a flow-rate of 4 $ml.min^{-1}$ providing a denaturing gradient. Gelbond® PAG film (Amersham Pharmacia Biotech, 80-1129) was placed on the rear glass plate of the casting sandwich assembly prior to casting to physically support the polymerized gel and to allow an ease in handling the gel after electrophoresis. After loading the 10 µL of PCR products to the polymerized PAG, electrophoresis was performed first at 200 Volts for 5 min, then at 85 Volts for 16 hours in the 1xTAE buffer. The electrophoresis temperature was 60°C. By the completion of the electrophoresis, the gel supported by Gelbond® PAG film was removed from the casting assembly and was silver-stained (Sanguinetti *et al.*, 1994). Images of the DGGE bands were captured in the ".tif" format by scanning the gels with a scanner.

5.4.6 The real-time PCR

Archaea and Bacteria were quantified with a LightCycler system (Roche) and SYBR Green I master mix (Roche) in the 10 µl final volume reactions. The archaeal specific primers were A109F (ACKGCTCAGTAACACGT) and 519R (GTATTACCGCGGCTGCTGG), 0.5 mM each. The amplification involved one cycle at 95°C for 10 min for initial denaturation and then 40 cycles of 95°C for 10 s followed by annealing at 52°C for 10 s and extention at 72°C for 20 s. Bacterial specific primers were GM3F (AGAGTTTGATCMTGGC) and EUB338R (GCTGCCTCCCGTAGGAGT), 0.5 mM each. The bacterial amplification involved one cycle at 95°C for 10 min for initial denaturation and then 40 cycles of 95°C for 10 s followed by annealing at 55°C for 10 s and extention at 72°C for 20 s. The detection of the fluorescent product was set at the last step of each cycle. To determine the specificity of the amplification, an analysis of product melting was performed after each amplification. The melting was obtained by slow heating with a 0.1°C/s increment from 60°C to 95°C, with continuous fluorescence collection. The

archaeal samples were run in triplicate and the bacterial samples in duplicate. A triple dilution standard of the archaeal 16S rRNA gene plasmids and a quadruple dilution standard of the bacterial 16S rRNA gene plasmids were used with every respective real-time PCR run. These calibration curves were used to calculate the absolute quantities of Archaea and Bacteria, and to obtain the ratio of these microorganisms in the samples.

5.5 Stable Isotope Probing Experiments

5.5.1 Sludge incubations

Approximately 500 µl of fresh sludge (wet weight) was transferred into the 35 ml serum vials containing the 15 ml basal medium (Plugge, 2005). The vials were stoppered, crimp-sealed and incubated up-side-down. The ^{13}C4-Butyrate and/or ^{13}C3-propionate (SigmaAldrich) were added in aliquots of 200 µmol per vial. Based on the original sludge activities, the batches were re-fed with these aliquots just before the substrate was converted completely. For each time point, separate bottles were started. For each series, 4 time points were included. After the incubation, sulfide, methane, butyrate and acetate were analysed according to de Bok et al. (de Bok *et al.*, 2001). The granules were harvested by centrifugation and were stored in RNAlater® (Ambion Inc.) at -20^{0}C following the manufacturer's protocol.

5.5.2 Nucleic acid extraction and quantification

The RNA from the sludge samples was isolated using TRIZOL®, a ready-to-use, monophasic solution of phenol and guanidine isothiocyanate (Invitrogen, Breda, The Netherlands). Approximately 25 mg of the sludge was transferred into a 2-ml tube containing 500 mg Zirconium beads and 1,5 ml TRIZOL®. Sludge granules were disrupted using a bead beater (45 s, 6.5 m/s) followed by a centrifugation at 18.000 g for 5 min. Supernatants were purified using chloroform. Finally, RNA was precipitated with 2-propanol and was re-eluted in 100 µl elution buffer (EB, 10 mM Tris-HCl, pH 8.5). RNA was further purified using the on-column DNAse digestion from the RNAeasy kit (Qiagen) following the manufacturer's protocol. The integrity of the 16S and 23 SRNA was routinely checked on agarose gels and was quantified using a NanoDrop ND1000 Spectrophotometer (Intas, Göttingen, Germany).

5.5.3 Isopycnic centrifugation

Density gradient centrifugation was performed in the 11 ml polyallomer UltraCrimp tubes (Sorvall) using a Kontron TVF 65.13 vertical rotor in a Centrikon T-1065 centrifuge (Kontron Instruments). The centrifugation conditions were 20°C and 65 h at 37,000 rpm (130.000 g_{av}) for CsTFA gradients. The rRNA was resolved in CsTFA gradients with an average density of 1.795 g ml^{-1} . The solutions were prepared by mixing the 2 $g.ml^{-1}$ CsTFA stock solution (Amersham Pharmacia Biotech, Roosendaal, The Netherlands), the gradient buffer (0.1 M Tris-HCl, pH 8; 0.1 M KCl; 1 mM EDTA), and the rRNA extracts (1000 ng) reaching a final volume of 11 ml (rRNA volume was subtracted from gradient buffer). Additionally, 350 µl of formamide was added to the rRNA media to stabilize the RNA. Prior to centrifugation, the average density of all prepared gradients was checked by weighing, and was adjusted by adding small aliquots of the CsTFA solution or the gradient buffer, if necessary.

5.5.4 Gradient fractionation and evaluation

Centrifuged gradients were fractionated from the bottom to the top into up to 11 equal fractions (exactly 1000 µl). A precisely controlled flow rate was achieved with the displacement of the gradient medium with water at the top of the tube using a peristaltic pump (Watson Marlow, Rotterdam, The Netherlands). The density of each collected fraction was determined by weighing a small fraction (75 µl) of each sample. From the CsTFA gradient fractions, nucleic acids were precipitated with 1 volume of iso-propanol. The precipitates from the gradient fractions were washed once with 70% ethanol and were re-eluted in the 30 µl elution buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8,0). The total rRNA was determined using the RiboGreen® RNA Quantitation Kit (Molecular Probes, Leiden, The Netherlands).

5.5.5 Complementary DNA synthesis

The single-stranded DNA, that is complementary to 16S ribosomal RNA, was synthesized by the SuperScript™ III RNase H^+ reverse transcriptase (Invitrogen, Breda, The Netherlands). RT-PCR analyses were performed with 10 ng of the total RNA following the manufacturer's instructions. The first reaction mixture of 13 µl contained an aliquot of the total RNA, 10 pmol of universal reverse primer 1492R (5′-GGTTACCTTGTTACGACTT-3′) (Lane, 1991), 10 pmol dNTP mix and sterile,

and RNase-free water (Qiagen GmbH, Hilden, Germany). To denature the secondary structure of the 16S rRNA, the mixture was incubated at 65°C for 5 min and was then immediately incubated on ice for 1 min. For cDNA synthesis, the following agents were added to the previous denatured 13 µl reaction mixture: 4 µl 5× First strand buffer, 1 µl 0.1 M DTT, 1 µl (40 units) RNaseOUT^{TM} RNase inhibitor (Invitrogen, Breda, The Netherlands), and 1 µl (200 units) Superscript III^{TM} RT. The reaction was performed at 50°C for 1 h and was then incubated at 70°C for 15 min to inactivate the reaction. To remove the RNA complementary to the cDNA, the reaction mixture was incubated with RNAse H (2 units) at 37°C for 20 min. The mixtures were also prepared without target RNA to check a possible contamination at each RT reaction time.

5.5.6 PCR amplification

Aliquots (2 µl) of the cDNA solution were used for subsequent bacterial and archaeal PCR amplifications. PCR was performed in 50 µl reaction mixtures using *Taq* DNA polymerase (Invitrogen, Breda, The Netherlands). The PCR mixture contained 5 µl of 10× PCR buffer 3 µl of 50mM, 1 µl of deoxynucleoside triphosphate (dNTP)s containing 10 mM each dNTP, 1 µl of 10 mM forward and reverse primers, 0.5 µl of the *Taq* DNA polymerase (5 U/µl), and nuclease free water for a final volume of 48 µl. Later, the 2 µl target cDNA was added. Beside cDNAs, the RNA samples, which were treated with DNase, were directly subjected to PCR analysis to check whether there were any DNA remaining in the samples or not. The samples were amplified using the Biometra Thermocycler (Goettingen, Germany).

The bacterial PCR U968-GC (5′-CGCCCGGGGCGCGCCCCGGGCGGGGCGGGG GCACGGGGGGAACGCGAAGAACCTTAC-3′) forward and L1401 (5′-GCGTGTGTA CAAGACCC-3′) reverse primers (Nubel *et al.*, 1996) were used to amplify the V6 to V8 regions of the bacterial 16S rRNA. For archaeal PCR, the V2-V3 region was amplified using A109 (5′-ACT GCT CAG TAA CAC GT-3′) (Grosskopf *et al.*, 1998) forward and 515 GC (5′-ATCGTATTACCGCGGCTGCTGGCA-3′) (Lane, 1991) reverse primers. All the primers were provided from MWG-Biotech (Ebersberg, Germany).

The reaction mixture was subjected to PCR under the following conditions: denaturation at 94°C for 5 min followed by 30 cycles of denaturation at 94°C for 30

s; primer annealing for 1 min (annealing temperatures are for bacteria and archaea, 56 and 52, respectively); and DNA extension at 68°C for 1 min. After the last amplification cycle, samples were kept for final extension at 68°C for 7 min and were immediately cooled to 4°C. The size and the yield of the amplicons (5 µl) were checked by electrophoresis on a 1% (wt/vol) agarose gel.

5.5.7 Cloning and sequencing

The amplified V6-V8 regions for bacteria and V2-3 regions for archaea of the 16S rRNA (~400 bp) were purified with a QIAquick PCR purification kit (Qiagen GmbH, Hilden Germany) following the manufacturer's instructions. The quality and the amount of the purified PCR products was determined by electrophoresis on a 1% (wt/vol) agarose gel with a DNA marker (GeneRuler^{TM} 100bp DNA Ladder Plus, MBI Fermentas, Vilnius, Lithuania). These amplicons were ligated into pGEM$^{®}$-T Easy Vector System I (Promega, Madison, WI) and were sequentially cloned into XL1-Blue Compotent Cells (Stratagene, US) following the manufacturer`s instructions. The clones determined with the ampicilin selection and the blue-white color screening were transferred into the 20 µl TE buffer and were then incubated at 95°C for 10 min in order to lyse the cells. The lysed cells were amplified with pGEM$^{®}$-T specific PG1 (5′-TGGCGGCCGCGGGAA-3′) and PG2 (5′-GGCCGCGAATTCACTAGTG-3′) primers; and these amplicons were digested with *Alu* I, *Cfo* I, and *Msp* I (Promega, Madison, WI) the restriction enzymes mixture at 37°C for 90 min. To differentiate these digested clones, RFLP analysis was done by using Elchrom Submerged Gel Electrophoresis System (Cham, Switzerland). The digested PCR products were run in 12% (wt/vol) pre-cast agarose gels (Elchrom, Cham, Switzerland) with a DNA marker (GeneRuler^{TM} 50 bp DNA Ladder Plus, MBI Fermentas, Vilnius, Lithuania) at 100 V, 55 °C and for 45 min and were visualized with ethidium bromide staining. Some clones, which have different profiles in RFLP, were selected for further DGGE and sequencing analysis.

5.6 Cultivation of the Original Eerbeek Sludge at Different Temperatures and at Different Substrate Compositions

The inoculums for all enrichments were provided from the UASB reactor unit of a paper mill wastewater treatment plant as described previously.

The basic medium was prepared as described in section 5.2. Substrates were added to the basal medium from sterile and completely anoxic concentrated stock solutions (1 M) flushed with nitrogen by sterile syringes, to give the desired final concentrations (Table 5.3). The yeast extracts addition was omitted to eliminate the growth of the primary fermenters.

Table 5.3: Substrate composition of four different enrichments

Substrates	Culture_1	Culture_2	Culture_3	Culture_4
Starch, mg/l	720	720	-	-
Acetate, mM	7.8 (500)	7.8	10 (642)	10
Propionate, mM	2.9 (300)	2.9	5 (516)	5
Butyrate, mM	0.27 (45)	0.27	5 (832)	5
Sulfate, mM	-	1.8	-	31.25

*Values in parentheses are the equivalent of the molar concentrations in mg COD.l^{-1}

Enrichments were cultivated at 10, 25, 37, 45, 55, and 65°C in 580 ml serum bottles, containing 250 ml of medium. All enrichments were cultivated independently in parallel. Since the duplicate experiments resulted in less than 10% of a difference, in terms of biochemical performance, the average values of duplicates were presented in the graphs and tables. The utilization of the substrate mixtures was followed by monitoring the decrease of each individual substrate, in addition to monitoring methane and hydrogen production. Additionally, autoclaved bottles including cell suspensions and substrates were incubated as abiotic controls; in addition, bottles including suspended sludge without substrates were incubated as negative controls for each different feed mixture and different temperatures. The produced methane values were corrected for all enrichments. All incubations were done under non-shaking conditions and in the dark.

10% of active enrichments (vol:vol) were repeatedly transferred into the fresh medium. After 3 subsequent transfers, incubations were used for experiments to determine the population dynamics together with biochemical performance. The growth rate measured after the first transfer to the new media was the same growth rate measured after third transfer of the enrichment culture.

After the termination of each experiment, the exact amount of cell dry mass values and pH were determined. The mass of the medium components was taken into account for the correction of the biomass values.

5.7. Analytical Techniques

0.2 ml of the gas samples were removed from the serum bottles with the 1ml sterile syringes to determine the amount of CH_4 and H_2. Methane and hydrogen levels were measured by gas chromatography with a Packard-Becker 417 GC equipped with a packed column of molecular sieve 13x (60/80 mesh) and a thermal conductivity detector. The column temperature was 50°C, and the carrier gas was argon at a flow rate of 20 ml/min.

Organic acids were measured with an LKB high-performance liquid chromatograph (HPLC) equipped with a Chrompack organic acid column (30 cm by 6.5 mm). The mobile phase was 0.01 N H_2SO_4 at a flow rate of 0.6 ml/min. The column temperature was 60°C. Samples (20 pl) were injected by using a Spectra Physics autosampler (SP 8775). Eluting compounds were quantified by differential refractometry by an LKB 2142 refractometer.

Sulfide was determined as described by Truper and Schlegel (Truper and Schlegel, 1964).

6. THE ANAEROBIC DEGRADATION OF BUTYRATE ALONE AND IN THE PRESENCE OF SULFATE IN UASB REACTORS

6.1 Introduction

The main objective of this chapter was to remark physiological traits of the syntrophic butyrate degraders in continuously flowing UASB reactors at 37°C. The impact of sulfate as an electron acceptor on the syntrophic butyrate degradation was also studied with a COD/SO_4^{2-} of 10 and 0.5. A better understanding of the competition between the MPA and SRBs and the possible control mechanisms for the butyrate degradation was investigated with this study. In this concept, the activity of the sludge was determined sulfidogenically, methanogenically and in terms of total for all three reactors. The research was also extended to examine the effects of different propionate, acetate, formate and hydrogen concentrations on butyrate degradation rate. Using these data, the Inhibition Concentrations (IC) that the compound reduce the butyrate degradation rate by 50% were calculated.

6.2 Operation of the UASB Reactors

A syntrophic butyrate degrading microbial consortium was enriched in an Upflow Anaerobic Sludge Blanket (UASB) reactor with a bicarbonate buffered medium containing butyrate as sole organic carbon in the absence (BUT) and the presence of limited (BLS, COD/SO_4^{2-}:10) and unlimited (BUS, COD/SO_4^{2-}:0.5) sulfate as the electron acceptor.

6.2.1 The performance of the BUT reactor

Over a 500 days operation of the BUT reactor, complete butyrate degradation was observed and no butyrate concentration was detected at the effluent of the reactor (Figure 6.1). Under anaerobic conditions, butyrate readily transformed to methane. At the effluent, acetate was detected to be less than 5 mg COD/L between the days of 101 and 141. The first 20 days, the methane production was in the trend of increase

and reached to the stable level of 1.89±0.23 L/day at the end of the 20 days. From day 20 onwards, the performance of the reactor remained quite stable in terms of effluent concentrations and methane formation.

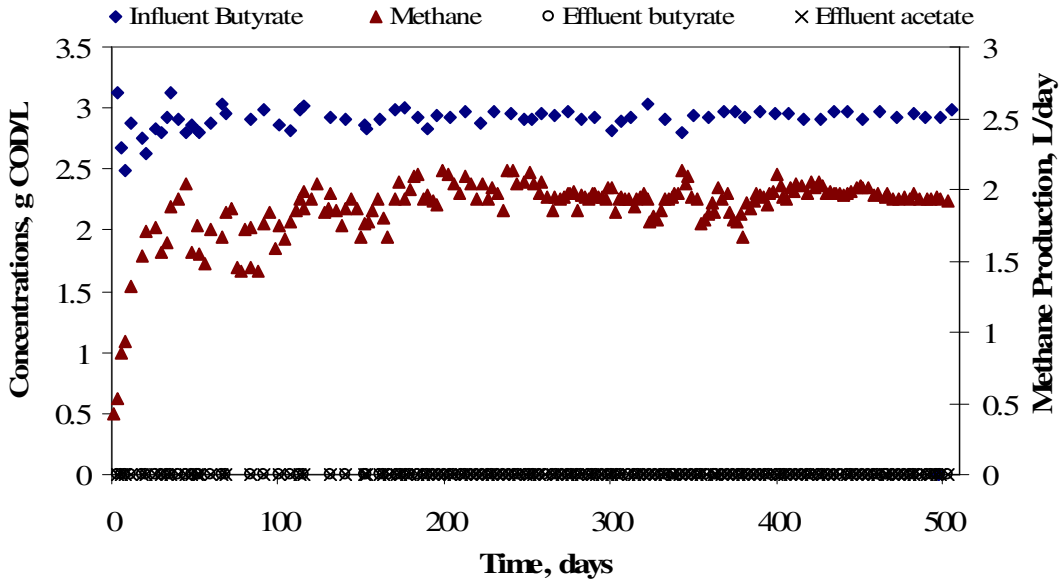


Figure 6.1 : The performance of butyrate degradation and methane formation in the BUT reactor

Loading rate was kept at 5.82 ± 0.19 g COD/L.day for the BUT reactor (Figure 6.2). The conversion rate of the BUT sludge was observed to be unstable in the first 100 days, then kept constant at 5.39 ± 0.57 g COD/L.day for the rest of the operation.

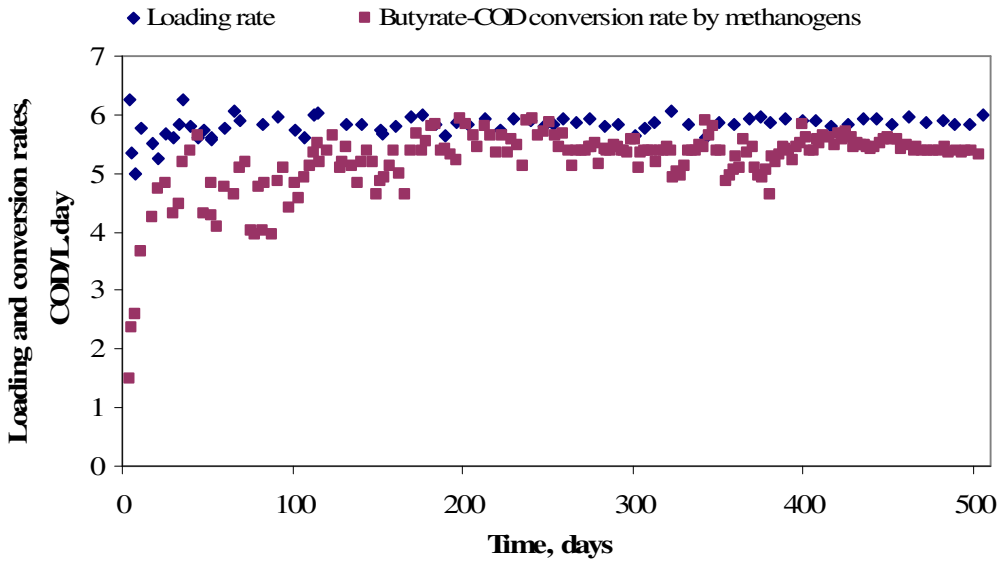


Figure 6.2 : The loading and conversion rate in the BUT reactor

6.2.2 The performance of the BLS Reactor

During the entire period of the operation of the BLS reactor, COD/sulfate ratio of the feed was kept at 10.18 ± 0.79 (Figure 6.3). In this period, butyrate was not observed at the effluent. On the contrary, acetate was detected between the days of 214 and 234 and it was observed to be in the range of 1-4 mg COD/L. Methane production was 1.77 ± 0.17 L/day, 20 days after the start-up period.

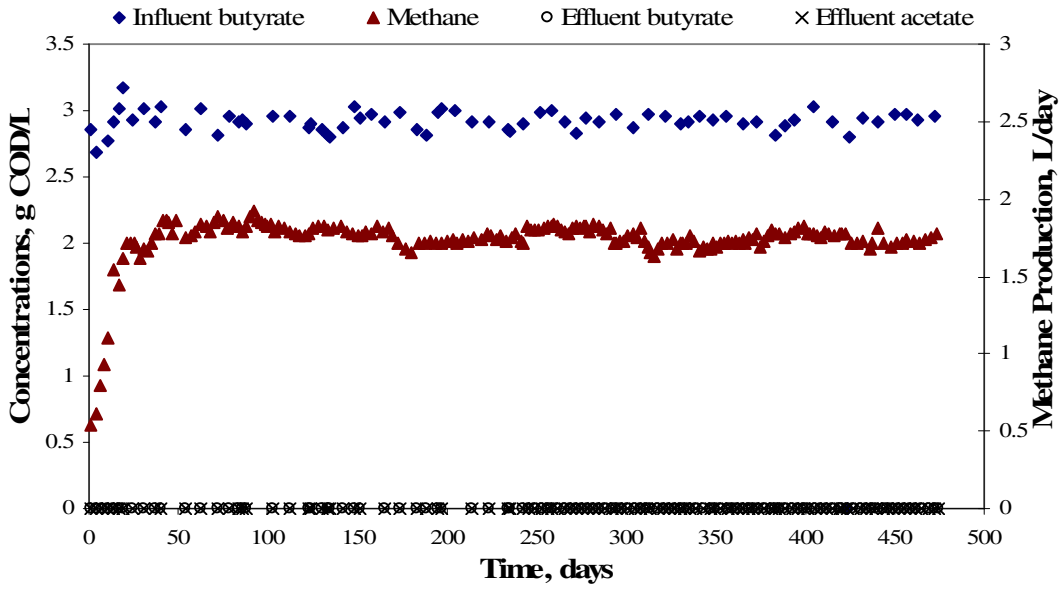


Figure 6.3 : The performance of butyrate degradation and methane formation in the BLS reactor

Sulfate concentrations and removal rate of sulfate was shown in Figure 6.2. The sulfate was reduced from the beginning of the operation of the reactor without the need for the acclimation period, which was the opposite of what had been observed on methanogens. The removal rate of sulfate stayed constant around the level of 93% during the whole period of the operation. Thereupon, not all sulfate was reduced; 19 ± 6 mg/L sulfate was detected at the effluent.

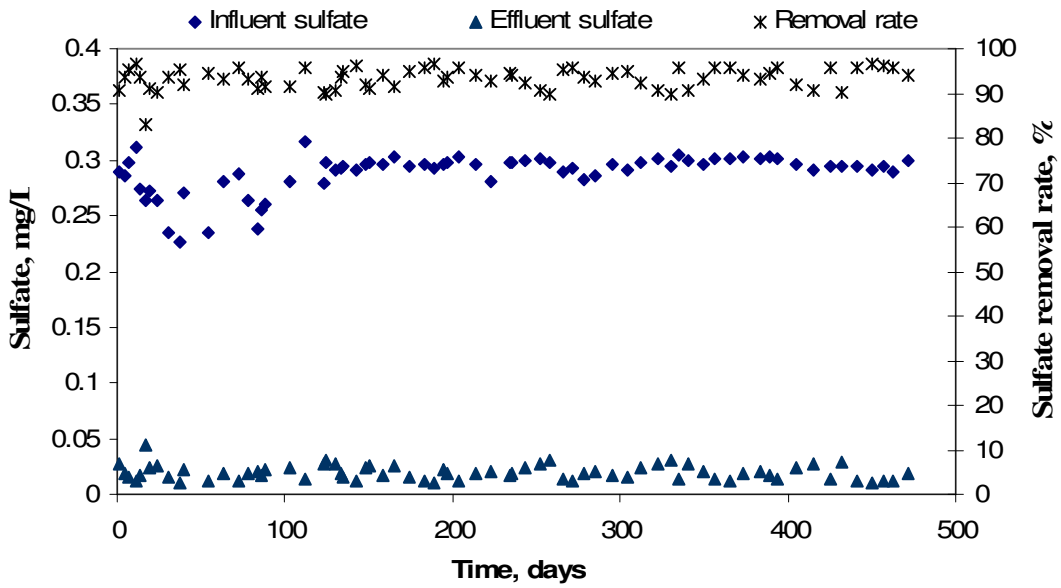


Figure 6.4 : The concentrations of sulfate at the influent and effluent of the BLS reactor together with the removal efficiency

As it can be seen from Figure 6.5, the butyrate degradation ratio by methanogens was higher than sulfidogens, depending on the high COD/sulfate ratio. The 81% of the butyrate was converted by methanogens; whereas, on the contrary, the 9.8% of butyrate was converted by sulfidogens, based on the COD.

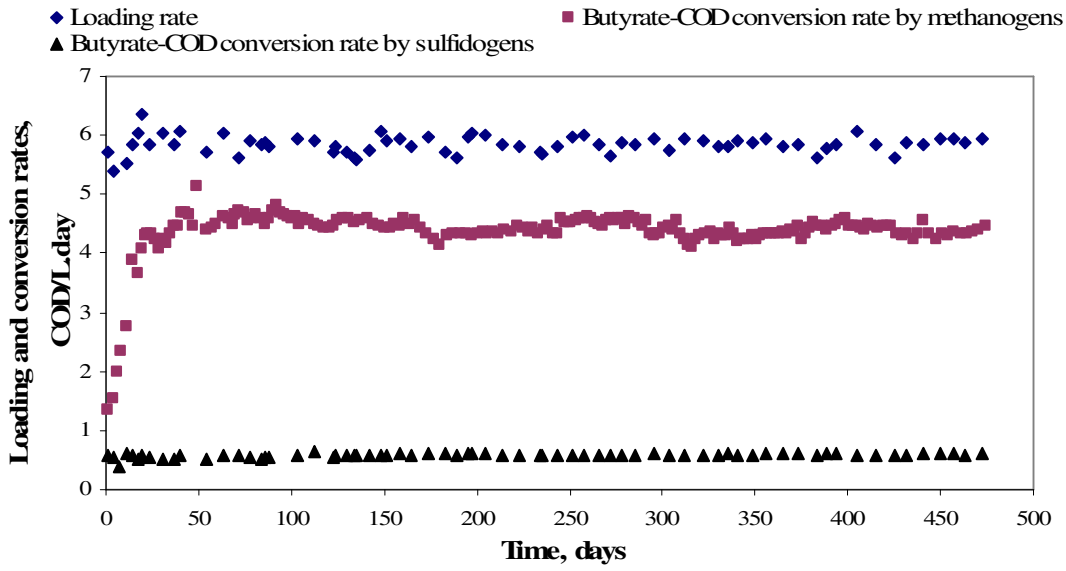


Figure 6.5 : The loading and conversion rates in the BLS reactor

6.2.3 The performance of the BUS Reactor

High removal of the butyrate was accomplished in the BUS reactor and no butyrate was detected at the effluent of the BUS reactor. On the other hand, acetate was observed at some operational period of the reactor until around the first 150 days. The highest observed acetate concentration was around 90 mg COD/L. After this period, acetate was not found at the effluent. The methane production gradually decreased starting from the beginning and reached to around 0.1 L/day for the last 100 days of the operation of the reactor. It never terminated even if the unlimited sulfate concentrations were fed to the reactors, where the COD/sulfate kept at 0.63 ± 0.03. It is known that the ratio of the feed of COD/sulfate is the decisive controlling parameter for the dominance of sulfidogens or methanogens. Decreasing this ratio increases the amount of the sulfate reduction and decreases the amount of the produced methane.

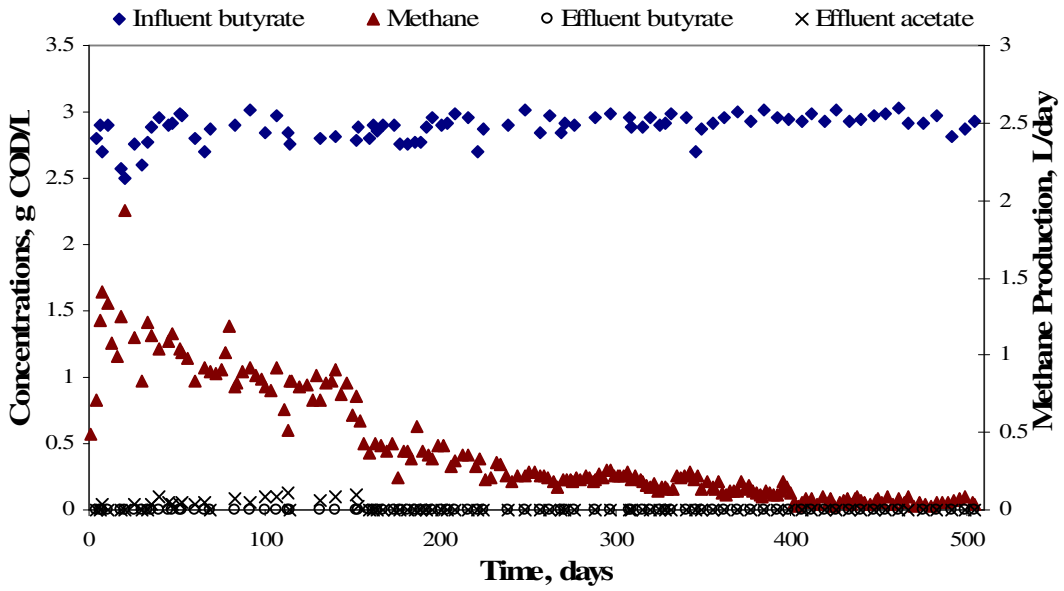


Figure 6.6 : Butyrate degradation and acetate plus methane formation in the BUS reactor

It is clear that from day 170, the reduced sulfate amount remained distinctly stable in the range of 3-3.7 mg/L; corresponding to approximately 83% of the reduced butyrate (Figure 6.7).

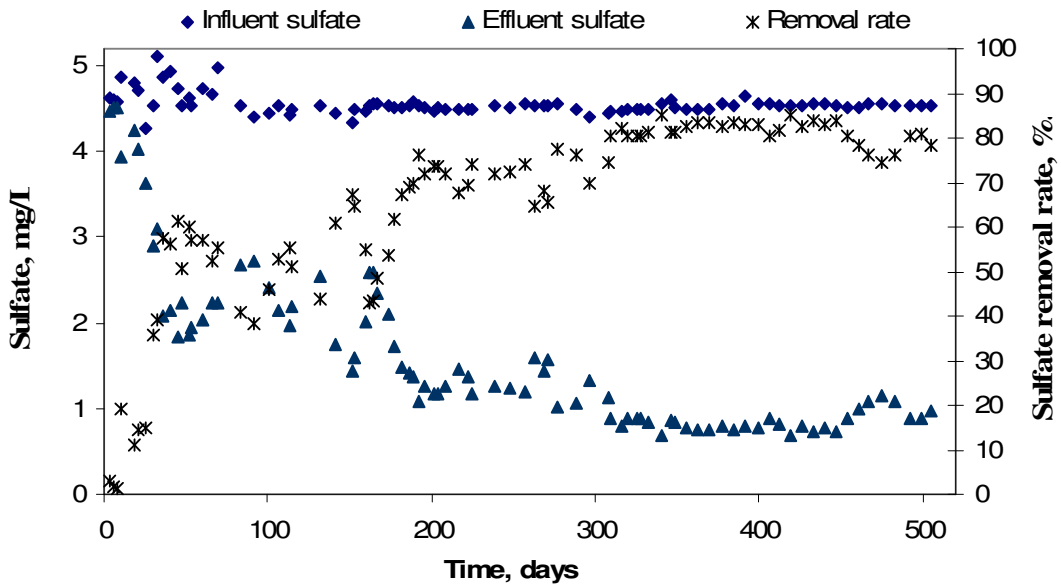


Figure 6.7 : The concentrations of sulfate at the influent and effluent of the BUS reactor together with the removal efficiency

Sulfate reduction increased steadily and reached 4.84±1.26 g COD/L.days from day 170 onwards. During that period, the butyrate conversion rate by methanogens was 0.43 ±1.26 g COD/L.days. These conversion rates demonstrated that the BUS reactor was dominated by the sulfidogens, but it had never been completely taken over by them. The carbon recovery of the butyrate degradation, which was calculated from the conversion rates, was found to be 92%.

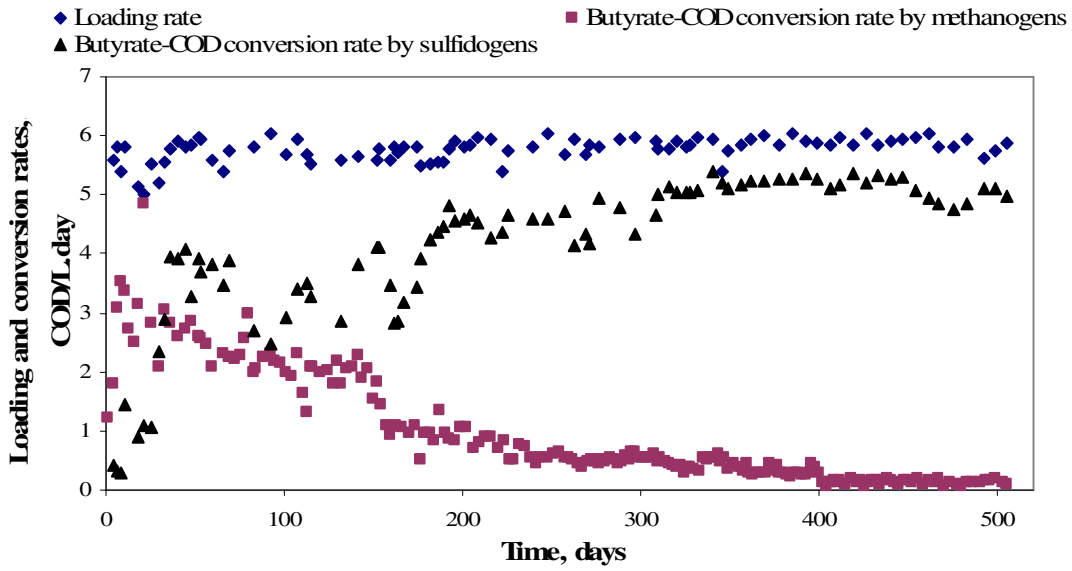


Figure 6.8 : The loading and conversion rates in the BUS reactor

Based on the thermodynamics of the sulfate reduction, and methanogenesis, it is expected that the SRB will out-compete methanogens. However, due to the sulfate transport limitation in the sludge granules, the dominance of the sulfidogens takes time to take over all of the community. This is especially in use for the acetate utilizing sulfidogens. Monod half velocity coefficient (Ks) for sulfidogens is lower than that of methanogens; meaning that the former one has more affinity for the electron donor than the methanogens does. It was found that K_s values of acetate degrading community were 9.5 mg/L and 32.8 mg/L for sulfidogens and methanogens respectively (Yoda *et al.*, 1987). Though the sulfidogens have advantage over methanogens in terms of substrate affinity, most of the researches show that even if the excess sulfate was fed to the anaerobic microbial consortium, the methane producing archaea would exist and would compete for the acetate (Isa *et al.*, 1986b; McCartney and Oleszkiewicz, 1991; Yoda *et al.*, 1987).

6.2.4 Comparative evaluation of the BUT, the BLS and the BUS reactors

The operational and performance data of all of the three reactors were summarized in Table 6.1. From the performance of all of the three reactors under the same hydraulic operational conditions, it was concluded that the sulfate addition influences the butyrate-COD conversion rates whether it is directed by methanogens or sulfidogens. In all of the three reactors, the complete butyrate degradation was observed throughout the whole operation period. Nonetheless, at some periods, a small amount of acetate was detected at the effluent of the reactors. Acetate level was measured to be around 5 mg COD/L for the BUT and the BLS reactors; whereas, 70 mg COD/L of acetate was observed for the BUS reactor.

Since then, there have been several researches made on the degradation of the VFA mixture, Fang and co-workers (Fang, H.H.P. *et al.*, 1995a) worked on the butyrate degradation in a UASB reactor. They were able to load the butyrate to the reactor up to 31 g $COD.l^{-1}.day^{-1}$ with 97-99% COD removal efficiency. 94.5% of this removed COD was converted to methane. Considering the removal efficiency of the reactor, it can be concluded that the major part of the butyrate and its intermediates were converted; which is consistent with the results that have been found in this study.

Table 6.1: The operational parameters and the performance of the mesophilic UASB reactors fed with butyrate and butyrate+sulfate at two different COD/SO_4^{2-} ratios

	BUT	**BLS**	**BUS**
HRT (h)	12	12	12
Volumetric Loading Rate (VLR), g COD/L.day	5.82 ± 0.19	5.84 ± 0.15	5.77 ± 0.2
Organic Loading Rate (OLR), g COD/g VSS.day	0.42	0.41	0.44
COD/sulfate	-	10.18 ± 0.79	0.63 ± 0.03
Butyrate-COD conversion rate by methanogens, g COD/L.day	5.22 ± 0.57	4.37 ± 0.43	0.95 ± 0.93
Butyrate-COD conversion rate by sulfidogens, g COD/L.day	-	0.58 ± 0.03	4.09 ± 1.27

Methane production in the BUT reactor was higher than that of the other two due to the absence of the alternative electron acceptors. However, in the presence of sulfate, methane production was decreased depending on the COD/sulfate ratio. Such an operation is important in controlling the relative growth of sulfidogens and methanogens, which determine the amount of produced sulphide and methane, respectively. If the sludge was not acclimatized to sulfate, the occurrence of complete sulfidogenic conditions could have taken several months at the presence of the stoichiometric amount of sulfate (McCartney and Oleszkiewicz, 1993; Visser, 1995). This retardation of the sulfidogenic conditions is mainly associated with the competition between the sulfodigens and the methanogens for the acetate produced by anaerobic degradation of organic substrates (Visser, 1995). For the BLS, representing the amount of sulfate which was limited in terms of the reducing equivalent of the butyrate, the methane production rate decreased to 16% compared to the BUT reactor. In the BUS reactor, the addition of excess sulfate to the feed, which was more than the reducing equivalent of the butyrate, the methane production was measured to be ~0.1 g COD/L.day at the end of the 505th day of the operation of the reactor. During this period, the hydrogen sulfide production rate reached 5.14 g COD/L.day, which was 89%of the loading rate of the butyrate. The sulfate levels in the effluent also fluctuated over time in the BUS reactor although it was more stable in last periods of the operation than in the rest of the operation. Sulfide has been

found to be toxic towards both sulfidogens (Reis *et al.*, 1992) and methanogens (Isa *et al.*, 1986a), however, such a case was not observed in the reactors operated in this study.

6.3 Sludge Activity

The total and the sulfidogenic sludge activity of the sludge samples of the original Eerbeek UASB reactor and lab-scale UASB reactors (BUT, BLS, and BUS taken on day 271 was determined with possible intermediate substrates revealed with the butyrate degradation.

6.3.1 Specific methanogenic activity

The specific methanogenic activity (SMA)s of the original Eerbeek and the reactors' sludge for butyrate, propionate, acetate, formate and hydrogen as substrates with and without sulfate were given in Table 6.2.

The methanogenic activity of the original Eerbeek sludge showed that SMA of formate and butyrate was higher than those of hydrogen, acetate and propionate with both in the presence and absence of sulfate. The methane production of propionate was affected more than the others in case of sulfate addition, as it reduced the methanogenic activity to as low as 32%. Formate and butyrate were almost not affected by sulfate addition at all. Such a high butyrate and formate methanogenic activity could possibly show the importance of such intermediates in the degradation of paper mill wastewaters.

Table 6.2: The methanogenic activities (mg $COD.g^{-1}.VSS.day^{-1}$) of the original Eerbeek and the the reactors' sludge for butyrate, propionate, acetate, formate and hydrogen as substrates with and without sulfate

	Hydrogen		Formate		Acetate		Propionate		Butyrate	
	$-SO_4$	$+SO_4$	$-SO_4$	$+SO_4$	$-SO_4$	$+SO_4$	$-SO_4$	$+SO_4$	$-SO_4$	$+SO_4$
Original Eerbek Sludge	629 (23)	542 (30)	901 (78)	881 (61)	592 (34)	519 (34)	474 (37)	321 (30)	805 (72)	790 (63)
BUT Reactor Sludge	769 (44)	636 (4)	1033 (10)	949 (3)	347 (15)	313 (10)	84 (17)	38 (2)	1096 (117)	1051 (134)
BLS Reactor Sludge	490 (7)	282 (3)	529 (7)	581 (18)	250 (2)	242 (2)	68 (7)	46 (12)	440 (84)	179 (40)
BUS Reactor Sludge	383 (3)	36 (3)	151 (5)	-	150 (2)	47 (10)	-	41 (2)	631 (99)	35 (2)

*Values are the mean of triplicates. Standard errors (standard deviation) are in parentheses

Butyrate degrading granules taken from the BUT reactor were capable of producing 1096, 84, 347, 1033, and 769 mg of methane COD per day from butyrate, propionate, acetate, formate, and hydrogen, respectively. The methanogenic activity of the BUT sludge did not change significantly for the butyrate, acetate, formate, and hydrogen in the presence of sulfate. However, the methanogenic activity of propionate reduced to around 50% with the addition of sulfate; demonstrating that the syntrophic propionate degraders have the ability to reduce sulfate. Moreover, the growth of the *Syntrophobacter sulfatireducens* (Chen *et al.*, 2005), the *Syntrophobacter fumaroxidans* (Harmsen *et al.*, 1998), the *Syntrophobacter pfennigii* (Wallrabenstein *et al.*, 1995b) and the *Syntrophobacter wolinii* (Boone and Bryant, 1980) on propionate in coculture with methanogens and sulfidogens was observed in pure cultures. Therefore, this low level of methanogenic activity for propionate could easily be shifted to sulfidogens in the presence of sulfate as observed in the BUT reactor sludge. So, it is possible that syntrophic propionate degraders can easily act as an SRB and also vice versa for propionate (Wu *et al.*, 1991). On the contrary, there was not any report that syntrophic butyrate degraders act as an SRB in the presence of sulfate.

The activity tests of butyrate degrading sludge granules taken from the reactor having the loading rate of 31 g $COD.l^{-1}.day^{-1}$ demonstrated that the SMA was 1.57 g CH_4-$COD.g\ VSS^{-1}.day^{-1}$ (Fang, H.H.P. *et al.*, 1995a). In this study, the BUT reactor sludge demonstrated a low methanogenic sludge activity. It might be mainly due to the lower loading rates of the reactors operated in this study. Although the Fang *et al.* (1995a), found that butyrate adapted sludge was unable to degrade propionate, in this study, however, the BUT reactors sludge was able to degrade propionate.

When the SMA of the BUT sludge is compared to the original Eerbeek sludge, it can be seen that the methane activity was higher in the BUT sludge for the hydrogen and formate than it was for acetate; indicating that the methane production was channeled through the hydrogenotrophic methanogens more than the acetoclastic methanogens in the reactor of the syntrophic butyrate degradation.

The order of the SMA was formate>hydrogen>butyrate>acetate>propionate for the BLS reactor sludge. In general, methanogenic activity values were less than those of the BUT reactor sludge due to the adaptation of the BLS sludge to the sulfidogenic conditions. Interestingly, the SMA of butyrate was lower than that of the BUS

sludge, where the BLS sludge was expected to be directed to more methanogenic conditions rather than the BUS sludge. The decrease in methanogenic hydrogen activity compared to the BUT sludge related to the increasing sulfidogenic activity of butyrate (Table 6.3). This result indicated that sulfidogens were competed by hydrogenotrophic methanogens for substrate.

For the BUS sludge, a maximum SMA was observed in butyrate incubations as 637 mg CH_4-COD.g $VSS^{-1}.day^{-1}$ in the absence of sulfate. In the presence of sulfate, it dropped to 35 mg CH_4-COD.g $VSS^{-1}.day^{-1}$, which shows the consistency in butyrate conversion rates of methanogens and sulfidogens at the moment of the sludge taken from the reactor for activity tests. The SMA of formate and hydrogen in the BUS sludge was lower than those of the BLS and the BUT sludge. In the SRBs, the production and consumption of H_2 and/or formate were carried out in the same cell: cytoplasmic and periplasmic hydrogen cycling (Haveman *et al.*, 2003; Odom and Peck, 1981), which makes the time necessary for movement of the electrons shorter compared to the discretion of H_2 towards the outside of the cell where it is to be reduced by methanogens. Therefore, under complete sulfidogenic conditions, the external H_2 acceptors decreased by substituting the electron flow into the same sulfidogenic cells.

6.3.2 Sulfidogenic activity

The sulfidogenic activity (SA) of the reactors' sludge for butyrate, propionate, acetate, formate and hydrogen as substrates with sulfate and sulfate+BRES were given in Table 6.3. The total SA was observed only for propionate in the BUT reactor sludge because of the electron acceptors' flexibility of propionate utilizing bacteria. The decreasing activity of SA, in the BRES inhibited sludge could be partially attributed to the effect of BRES inhibition as well as to the sulfidogens. In BRES inhibited SA, only formate, butyrate and propionate were utilized by the BUT sludge. Formate had the higher SA compared to the other utilized substrate. It was clear that methanogens had the competition advantage for the butyrate adapted sludge even in the presence of sulfate. Sulfidogens were only active in the presence of BRES, which is known as a typical methanogen inhibitor (Ahring and Westermann, 1987).

Table 6.3: The total and BRES inhibited sulfidogenic activity (mg COD.g^{-1}.VSS .day^{-1}) of the reactors' sludge for butyrate, propionate, acetate, formate and hydrogen as substrates

	Hydrogen		Formate		Acetate		Propionate		Butyrate	
	$+SO_4$	$+SO_4$ +BRES	$+SO_4$	$+SO_4$ +BRES	$+SO_4$	$+SO_4$ +BRES	$+SO_4$	$+SO_4$ +BRES	$+SO_4$	$+SO_4$ +BRES
BUT Reactor Sludge	-	-	-	678 (18)	-	-	37 (1)	24 (4)	-	46 (5)
BLS Reactor Sludge	58 (12)	-	-	510 (12)	-	-	69 (14)	42 (16)	284 (55)	133 (48)
BUS Reactor Sludge	126 (10)	40 (2)	343 (10)	406 (32)	371 (5)	279 (2)	204 (4)	254 (2)	424 (13)	332 (11)

*Values are the mean of triplicates. Standard errors (standard deviation) are in parentheses

In the BUT the BLS reactors' sludge, no acetotrophic sulfidogenic activity was detected; indicating that only a low number of acetotrophic SRB were present and, suggesting that acetoclastic methanogens dominated the population regarding the SMA results.

The SA of the BLS sludge demonstrated the same character as the BUT sludge except for the butyrate and hydrogen. While butyrate SA was 284 mg COD.g^{-1}.VSS.day^{-1}, in the presence of BRES, the SA dropped to 133 mg COD.g^{-1}.VSS.day^{-1}, corresponding more than 50% of SA loss. This might also show the importance of the methanogenic activity in the syntrophic degradation of butyrate degradation even under sulfate reducing conditions. As a result, the inhibition of methanogens significantly affected the SA and the BRES addition and lowered most of the SA values. The reason might be the abovementioned possible BRES inhibition to the sulfidogens.

The order of SA was butyrate>acetate>formate>propionate>hydrogen for the BUS reactor sludge in the case of competition between sulfidogens and methanogens. While the butyrate SA was 424 mg COD.g^{-1}.VSS.day^{-1}, the SMA was 35 mg COD.g^{-1}.VSS.day^{-1} under the same conditions; which clearly showed that the SRB out competed the methanogens in time with respect to the degradation of butyrate. The same situation was also observed for the propionate degradation. Therefore, it can be concluded that for syntrophic degradation of butyrate and propionate, SRB out competes the methanogens under excess sulfate conditions. For acetate, both a pre dominance of the methanogens (Isa *et al.*, 1986a; Yoda *et al.*, 1987) and sulfidogens (McCartney and Oleszkiewicz, 1993) has been reported. In this study, the dominance

of sulfidogens was observed for the BUS reactor sludge taken from the reactor at day 271, though the reactor performance demonstrated that ~20-30% of the converted butyrate COD was derived from methanogens. Dominance of sulfidogens could be also due to the limitations of batch experiment conditions for the granular sludge. The batch tests are not stationary and substrate concentrations change over time. Since microbial consortia prefer growing outside of granules due to the limitation of mass transfer of sulfate into the granules, and effects of hydraulic flow pattern differences from original *in-situ* enrichments of the consortia, existing microbial population may not represent the real conditions.

Though formate did not show any methanogenic activity for the BUS sludge, the SA was remarkably higher for formate demonstrating that it was utilized only by sulfidogens. Under the circumstances of methanogen inhibition with BRES, formate SA increased slightly from 343 to 406 mg $COD.g^{-1}.VSS.day^{-1}$. However, the hydrogen SA activity dropped sharply in the presence of methanogenic inhibitor, mainly because of the sensitivity of sulfidogens against BRES.

6.3.3 Substrate depletion activity

The substrate depletion rates of butyrate, propionate, acetate, formate and hydrogen for the three different reactors' sludge in the absence and presence of sulfate and BRES were given in Table 6.4. According to those depletion rates, formate was the substrate that was consumed very fast for all three reactors and under different incubation conditions. This finding was in agreement with that observed by the methanogenic and sulfidogenic activity results. A maximum substrate depletion rate was obtained to be 74 $mM.g^{-1}.VSS.day^{-1}$ for formate utilization of the BUT reactor sludge. Although the formate utilization rate remained as 72 $mM.g^{-1}.VSS.day^{-1}$, BRES addition caused the depletion rate to drop to 48 $mM.g^{-1}.VSS.day^{-1}$. This decrease in the rate demonstrated the significant contribution of the methanogens to the degradation of formate. The same situation was also observed for hydrogen degradation, but the degradation rates were lower than those of formate. Interspecies hydrogen and/or formate transfer is an important mechanism in the degradation of syntrophic degradation of organic substrates. In this study, significant part of the electron transfer might be realized by the reduction of bicarbonate to formate; taking into account the high formate depletion activity. This result was also consistent with

the other research where they found that formate transfer might be even more important than H_2 transfer (Thiele and Zeikus, 1988). The low hydrogen depletion rates might also be due to the hydrogen gas transfer limitations through the inner part of the granules, even though the incubations were done under shaking conditions. For The BLS and BUS sludge, the formate depletion rates were almost the same as the BUT sludge in the presence of sulfate. However, BRES inhibited incubations remained at the same level as the incubations which were without BRES inhibited. This situation clearly showed that all formate was used up by sulfidogens in the presence of sulfate. The hydrogen depletion rates for the BLS and the BUS sludge were remarkably lower than those of the BUT sludge in the presence of sulfate. Noteworthy, BRES inhibited incubations of BLS the BUS sludge depletion rates were much lower than those of the uninhibited ones. For the BUS sludge, the hydrogen depletion rate decreased to 3 $mM.g^{-1}.VSS.day^{-1}$. It might be concluded that, a significant part of the electrons were discarded to reduce the bicarbonate to formate according to the result of depletion rates together with SMA and SA tests.

Table 6.4: The methanogenic, total and BRES inhibited substrate depletion rates ($mM.g^{-1}.VSS.day^{-1}$) of the reactors' sludge for butyrate, propionate, acetate, formate and hydrogen as substrates with and without sulfate

	Hydrogen			Formate			Acetate			Propionate			Butyrate		
	$-SO_4$	$+SO_4$	$+SO_4$ +BRES	$-SO_4$	$+SO_4$	$+SO_4$ +BRES	$-SO_4$	$+SO_4$	$+SO_4$ +BRES	$-SO_4$	$+SO_4$	$+SO_4$ +BRES	$-SO_4$	$+SO_4$	$+SO_4$ +BRES
BUT Reactor Sludge	50.1 (0.8)	47.9 (0.6)	16.5 (3.0)	73.9 (1.2)	71.6 (1.8)	48.0 (0.8)	6.30 (0.29)	6.19 (0.30)	-	0.79 (0.05)	0.46 (0.02)	0.69 (0.01)	10.04 (0.21)	9.73 (0.31)	2.32 (0.24)
BLS Reactor Sludge	38.4 (0.2)	29.4 (1.6)	19.9 (0.4)	70.0 (2.9)	69.4 (0.6)	66.4 (3.0)	6.38 (0.15)	6.20 (0.13)	-	0.52 (0.04)	0.51 (0.01)	0.83 (0.10)	3.87 (0.22)	5.97 (0.27)	4.44 (0.39)
BUS Reactor Sludge	30.7 (0.5)	11.0 (0.1)	3.4 (0.1)	29.2 (1.1)	64.6 (1.4)	68.4 (3.5)	3.58 (0.41)	8.87 (0.25)	6.80 (0.27)	-	2.53 (0.10)	2.52 (0.08)	5.08 (0.17)	4.67 (0.04)	4.86 (0.07)

*Values are the mean of triplicates. Standard errors (standard deviation) are in parentheses.

The acetate depletion rates were around 6.3 $mM.g^{-1}.VSS.day^{-1}$ for the BUT and BLS sludges, and there was not detected any sulfidogenic activity for those sludge. This result confirmed that the acetoclastic methanogens were dominant in the BUT and the BLS reactors. However, the acetate depletion rate increased slightly to 6.8 $mM.g^{-1}.VSS.day^{-1}$ for the BUS sludge, which was the BRES inhibited incubation. This value increased to 8.9 $mM.g^{-1}.VSS.day^{-1}$ without inhibiting the methanogens. It could

be concluded that acetoclastic sulfidogens had a slightly higher acetate degradation rate than acetoclastic methanogens do.

Propionate degradation rates were more or less in the same range: 0.52-0.83 $mM.g^{-1}.VSS.day^{-1}$ for the BUT and the BLS sludge. Nonetheless, they increased up to 5.2 $mM.g^{-1}.VSS.day^{-1}$, in the presence of sulfate for the BUS sludge. It was clear that propionate degradation in the presence of excess sulfate was more active. It was also known from pure cultures of sulfidogens that acetogenic sulfidogens, both butyrate and propionate, were able to be degraded in the same sulfidogenic bacteria. Those propionate depletion rates might also be the indication of the role of propionate in the butyrate degradation pathway.

Butyrate depletion rates were, in general, less than hydrogen and formate depletion rates and higher than acetate and propionate depletion rates except for the BLS sludge. For the latter one, the butyrate depletion rate was 3.9 $mM.g^{-1}.VSS.day^{-1}$ in the absence of sulfate. This might show that the butyrate degradation was channeled more to the sulfidogenic conditions; because, the depletion rate increased to 6 $mM.g^{-1}.VSS.day^{-1}$ when incubated with the sulfate.

For the BUS reactor sludge, the butyrate depletion rates were 5.1, 4.7 and 4.9 $mM.g^{-1}.VSS.day^{-1}$ in the absence of sulfate, in the presence of sulfate and sulfate + BRES inhibited conditions, respectively. Such close values might also show that sulfidogenic butyrate degraders act as syntrophic butyrate degraders under methanogenic conditions and in the absence of sulfate. When the BUT sludge was subjected to the sulfidogenic conditions the rate was as low as 2.3 $mM.g^{-1}.VSS.day^{-1}$. Though, the degradation rate was not as high as the BUS sludge, it could be taken as evidence that some syntrophic butyrate degraders can also reduce sulfate.

6.3.4 Comparative evaluation of the activity tests

All of the three reactors' sludge adapted to butyrate as the sole substrate was also able to degrade other fatty acids such as propionate and acetate. The SMA on the degradation of butyrate for all three reactors' sludge were comparable with other studies that have the SMA value of 258-3057 mg COD.g $VSS^{-1}.d^{-1}$ (Table 6.5). Those high values obtained for the sludge mostly adapted to the VFA mixtures comprising the butyrate; however, propionate (Fang, H.H.P. *et al.*, 1995b; Grotenhuis, JT *et al.*, 1991) and phenol (Fang and Zhou, 2000) adapted sludge also

had significantly higher SMA on the butyrate degradation. This could be attributed to the importance of the butyrate as an intermediate in degradation pathway of the propionate and phenol. In some sludge, the SMA on the butyrate degradation illustrated higher values than the corresponding SMA of other tested substrates (Collins *et al.*, 2005a; Han *et al.*, 2005), although the butyrate was the indirect methanogenic substrate which was expected to have lower or the same rate as direct methanogenesis. Therefore, it could be suggested that high butyrate degradation rates would trigger the methanogenesis in a positive way.

Although the butyrate adapted sludge did not show methanogenic activity on propionate (Fang, H.H.P. *et al.*, 1995a), this activity was observed without lag phase in this study. But the SMA value on the degradation of propionate was as low as 64 and 84 g COD.g $VSS^{-1}.d^{-1}$ for the BUT and the BLS sludge, respectively in the absence of sulfate. These low values might be correlated to the minor contribution of the syntrophic propionate degraders to the syntrophic butyrate degradation pathway. It might also be the indirect relation of propionate degraders to the butyrate degraders.

Since the acetate was known as the rate limiting step in anaerobic treatment systems (Speece, 1996), the degradation of acetate might also demonstrate some variability in terms of activity depending on the microbial community structure as well as the types of feed character. As it can be seen from Table 6.5, the SMA on the degradation of acetate was almost equal or higher than the counterpart of the acetate which is hydrogen and/or formate in terms of direct source of methanogens. Moreover, the activities of the direct methanogenesis substrates were higher than the other SMA on the degradation of VFAs. This implies that in the degradation of VFAs, hydrogenotrophic and acetorophic methanogens play an important role in terms of syntrophic interaction between the methanogens and acetotrophic bacteria.

In this study, the SMA on formate degradation was almost equal to the butyrate degradation rate; however, the SMA on hydrogen degradation was 30% lower than that of formate for the BUT reactor sludge. Additionally, SMA on acetate was as low as 70% than the SMA on butyrate; which was not observed in any other studies shown in Table 6.5. In the presence of limited sulfate (COD/sulfate:10), called BLS reactor sludge, SMA on acetate was 40% lower than the SMA on butyrate; which was a higher ratio compared to the BUT reactor sludge. SMA on both formate and

hydrogen degradation was at the same level and they were higher than the SMA on butyrate degradation in the absence of sulfate for the BLS sludge. SMA on formate was still higher in the presence of sulfate, whereas, SMA on hydrogen dropped to 50%; demonstrating that interspecies electron transfer via formate occurred much more rapidly regarding the both SMA and SA results in the presence of sulfate.

In the BUS reactor sludge, SA on acetate was quite close to the SA on butyrate; meaning that the acetotrophic sulfidogens were the same rate as the butyrate degrading sulfidogens. It was also observed that the SMA on acetorophic methanogens for the BUT and the BLS sludge and SA on acetorophic sulfidogens for BUS sludge was in the same level. The activity of the BUS sludge on formate was still high in the presence of excess sulfate; even the SA value was as high as the SA on acetate degradation. It can be concluded that formate activity was very high compared to the hydrogen activity for all three reactors. It could also be suggested that formate was the dominant mechanism in the interspecies electron transfer.

Table 6.5: SMA of the anaerobic sludge using fatty acids as substrate

Growth conditions of original sludge		SMA conditions	mg COD.g $VSS^{-1}.d^{-1}$							References
			H_2/CO_2	Formate	Acetate	Propionate	Butyrate	Valerate	Caproate	
VFA:Alcohol mixture, (60.9:39.1), (3104 mg $COD.l^{-1}$), VFA: (Formate, 1.5%; Acetate, 17.2%; Propionate, 6.4%; Butyrate, 49.5%; Valerate, 16.8%; Caproate, 1.7%; Lactate, 6.9%) Alcohol: (Ethanol, 88.7%; Propanol, 3.8%; Butanol, 7.5), 21 g $VSS.l^{-1}$	UASB, 40 L, 37±1°C LR:6 g $COD.L^{-1}.d^{-1}$ >96% COD removal	Granular, 37±1°C	-	620	700	590	1010	720	780	(Han *et al.*, 2005)
Acetate:Propionate:Butyrate: Ethanol mixture (1:1:1:1) (5 g $COD.l^{-1}$), 20 g $VSS.l^{-1}$	EGSB-AF, 3.5 L, 15°C LR:2.5 g $COD.l^{-1}.d^{-1}$ >97% COD removal	Granular, 15°	907(2.6)		1723(6.5)	1611(11)	1758(4.3)			(Collins *et al.*, 2005b)
		Granular, 37°C	2556(3)		2003(3.8)	1779(3.3)	1940(6.7)			
Acetate:Propionate:Butyrate: Ethanol mixture (1:1:1:1) (5 g $COD.l^{-1}$) + 50 mg $TCP.l^{-1}$ 20 g $VSS.l^{-1}$	EGSB-AF, 3.5 L, 15°C, LR:2.5 g$COD.l^{-1}.d^{-1}$+25 g TCP $l^{-1}.d^{-1}$, >97% COD removal No TCP and DCP detected at the effl.	Granular, 15°C	552(1.5)		1005(2.4)	645(3.9)	791(2.3)			
		Granular, 37°C	1157(4.1)		1503(3.3)	1138(3.1)	1434(2.5)			
Acetate:Propionate:Butyrate: Ethanol mixture (1:1:1:1) (5 g $COD.l^{-1}$), 20 g $VSS.l^{-1}$	EGSB-AF, 3.5 L, 15°C LR:5 g $COD.l^{-1}.d^{-1}$ >90% COD removal	Granular, 15°C	1652		1620	547	2620			(Collins *et al.*, 2005a)
		Granular, 37°C	2121		2073	1774	3057			
Acetate:Propionate:Butyrate: Ethanol mixture (1:1:1:1) (2.12 g $COD.l^{-1}$) + 1.2 g $Phenol.l^{-1}$ 20 g $VSS.l^{-1}$	EGSB-AF, 3.5 L, 15°C LR:2.12 g $COD.l^{-1}.d^{-1}$ + Phenol LR:2.88 g $COD.l^{-1}.d^{-1}$ >90% COD removal <50 mg $Phenol.l^{-1}$ at the effl.	Granular, 15°C	940		870(lag 73h)	395(lag 382h)	1343			
		Granular, 37°C	1283		1004	526	1638			
Sugar beet molasses 12 g $COD.l^{-1}$ 11.5 g $VSS.l^{-1}$	Hybrid, 8.5 L, 37°C LR:48 g $COD.l^{-1}.d^{-1}$	Chrushed, granular, 37°C	1001		264					(Pender *et al.*, 2004)
	Hybrid, 8.5 L, 55°C LR:48 g $COD.l^{-1}.d^{-1}$	Chrushed, granular, 55°C	716-842		102-171					
Sugar beet molasses 12 g $COD.l^{-1}$ 11.5 g $VSS.l^{-1}$ + 3 g $sulphate.l^{-1}$	Hybrid, 8.5 L, 37°C LR:48 g $COD.l^{-1}.d^{-1}$	Chrushed, granular sludge, 37°C	425		229					
	Hybrid, 8.5 L, 55°C LR:48 g $COD.l^{-1}.d^{-1}$	Chrushed, granular sludge, 55°C	1282-2290		36-45					

Table 6.5: (Continued)

Growth conditions of original sludge		SMA conditions	mg COD.g $VSS^{-1}.d^{-1}$							References
			H_2/CO_2	Formate	Acetate	Propionate	Butyrate	Valerate	Caproate	
Acetate:Propionate:Butyrate: Ethanol mixture (1:1:1:1) 10 g $COD.l^{-1}$ 20 g $VSS.l^{-1}$	EGSB-granular, 3.5 L, 18±1°C LR:5 g $COD.l^{-1}.d^{-1}$ ~800 mg $.l^{-1}$ prop and acetate, ~500 mg $.l^{-1}$ butyrate at the effl.	Chrushed, 15°C	61	-	222	20(lag 780h)	215			(Collins *et al.*, 2003)
		Chrushed, 22°C	332	-	347	129(lag 590h)	455			
		Chrushed, 30°C	1096	-	767	181(lag 214h)	697			
		Chrushed, 37°C	1641	-	1974	206(lag 215h)	1121			
	EGSB-chrushed, 3.5 L, 18±1°C LR:5 g $COD.l^{-1}.d^{-1}$ ~800 mg $.l^{-1}$ prop and acetate, ~400 mg $.l^{-1}$ butyrate at the effl.	Granular, 15°C	85	-	180	34(lag 830h)	225			
		Granular, 22°C	161	-	293	41(lag 430h)	575			
		Granular, 30°C	669	-	263	377(lag 200h)	324			
		Granular, 37°C	1424	-	1161	218	1326			
Acetate, 5 g $COD.l^{-1}$ 37.5 g $VSS.l^{-1}$ (Eerbeek) ($Ca_{influent}$:390 mg $.l^{-1}$)	UASB, 0.16 L, mesophilic LR:13 g $COD.l^{-1}.d^{-1}$, 97-99% COD removal	Granular, 37°C			1.8					(van Langerak *et al.*, 2000)
Acetate:Glucose (3:1) 5 g $COD.l^{-1}$, 37.5 g $VSS.l^{-1}$ ($Ca_{influent}$:390 mg $.l^{-1}$)	UASB, 0.16 L, mesophilic LR:13 g $COD.l^{-1}.d^{-1}$, 97-99% COD removal	Granular, 37°C.			1700 dropped to 600					
VFA mixture Acetate:Propionate:Butyrate, 3:1:0.3 1550-1650 VFA-$COD.l^{-1}$	UASB, 0.4 L, 86°C 2.5-4.3 g $COD.l^{-1}.d^{-1}$ Eff.COD:922 mg $.l^{-1}$	Granular, 86°C			96					(Lepisto and Rintala, 1997)
	1.5-4.4g $COD.l^{-1}.d^{-1}$ 76°C Eff.COD:726 mg $.l^{-1}$	Granular, 76°C			191					
	3.8-20.7 g $COD.l^{-1}.d^{-1}$ 70°C Eff.COD:449 mg $.l^{-1}$	Granular, 70°C			355					
VFA mixture; Acetate:propionate:butyrate 2:1:1, 6.5 g $COD.l^{-1}$	UASB, 2.8 L, 37°C LR: 12 g $COD.l^{-1}.d^{-1}$, 97-99% COD removal	Granular, 37°C		730	1170	350	810			(Fang, H. H. P. *et al.*, 1995)
46% acetate + 54%propionate	UASB, 30 L	Granular, 30°C	1428(321)	1843(369)	1373(175)	636(111)				(Dolfing and Bloeman, 1985)
42% acetate + 48%propionate+10%sucrose			968(193)	783(186)	636(38)	406(120)				
2% acetate + 3%propionate+95% sucrose			968(175)	580(58)	461(74)	184(83)				
46% acetate + 54%propionate	UASB, 12 L		1299(224)	1539(102)	1512(46)	433(46)				

Table 6.5: (Continued)

Growth conditions of original sludge		SMA conditions	mg COD.g $VSS^{-1}.d^{-1}$							References
			H_2/CO_2	Formate	Acetate	Propionate	Butyrate	Valerate	Caproate	
Liquid sugar factory, containing sucrose, ethanol, lactate, acetate, propionate, butyrate, caproate.	UASB, 30 m^3, 35°C	Granular, 35°C			304	314	276			(Grotenhuis, JT *et al.*, 1991)
Ethanol, 26 mM	UASB, 5 L, 35°C, HRT:10 h				2599	433	166			
Propionate, 44 mM	UASB, 5 L, 35°C, HRT:10 h				1889	1779	1318			
Sugar wastewater	UASB, 30 m^3, 35°C	Granular, 35°C	783(288)	1014(83)	378(19)	256(9)				(Dolfing and Mulder, 1985)
Ethanol	UASB, 1 L, 35°C 16.8 $g.l^{-1}$		3631(1107)	3207(416)	746(83)	4				
Propionate			1106(250)	2599	553(83)	516(58)				
Acetate			184(45)	184(32)	1161(416)	6				
Acetate+propionate			903(646)	2175(480)	1060(141)	341(64)				
Acetate+ethanol			2359(666)	1770(205)	424(120)	2				
Phenol/p-cresol, 800/300 $mg.l^{-1}$ 2.92 g $COD.l^{-1}$	UASB, 2.8 L, 37°C, LR: 7 g $COD.l^{-1}.d^{-1}$,~80% COD removal	Granular, 37°C			1020	390	890			(Fang and Zhou, 2000)
Phenol/p-cresol, 1430/500 4.3 g $COD.l^{-1}$	UASB, 2.8 L, 37°C, LR: 4.3 g $COD.l^{-1}.d^{-1}$,~90% COD removal	Granular, 37°C			1100	370	900			
VFA mixture Acetate:Butyrate:Propionate 1.3:1:1	UASB, 4 L, 37°C, HRT:12 h 1.7 g $COD.VSS^{-1}.d^{-1}$	Granular, 37°C	111-765		304-774	378-737	258-1152			(Gorris *et al.*, 1988)
VFA mixture Acetate:Butyrate:Propionate 3:1:1, 1:3:1, 1:1:3	AF, 0.65-5 L, 37°C, HRT:1.5 h 1.7 g $COD.VSS^{-1}.d^{-1}$		28-498		1198-3640	580-1567	884-1843			
Formate 5.25 g $COD.l^{-1}$	UASB, 2.8 L, 37°C LR: 10.5 g $COD.l^{-1}.d^{-1}$ 95-97% COD removal and acetate was detected at the effl.	Granular, 37°C		2900	N.D.	N.D.	N.D.			(Chui *et al.*, 1994)
Butyrate 5.3 g $COD.l^{-1}$	UASB, 2.8 L, 37°C LR: 10 g $COD.l^{-1}.d^{-1}$	Granular, 37°C		960	1510	N.D.	1310			(Fang, H.H.P. *et al.*, 1995a)
Propionate 5 g $COD.l^{-1}$	UASB, 2.8 l L, 37°C, LR: 10 g $COD.l^{-1}.d^{-1}$, at the effl., <50 $mg.l^{-1}$ acetate, 100 $mg.l^{-1}$ prop.	Granular, 37°C		1170	1020	490	580			(Fang, H.H.P. *et al.*, 1995b)

EGSB:Expanded Granular Sludge Bed; AF:Anaerobic Filter; UASB:Upflow Anaerobic Sludge Blanket. N.D.: Not Detectable, N.A.: No Activity

6.4 The Effects of Propionate, Acetate, Formate and Hydrogen on Butyrate Degradation

The effects of propionate, acetate, formate and hydrogen on butyrate degradation were examined in this section. The purpose of this investigation was to determine the interrelationships of the different throphic groups on VFAs and butyrate degradation rate in all of the three reactors' sludge exposed to the abovementioned substrates encountered in the butyrate degradation pathway. The degradation profiles were given in detail for each substrate concentrations in Appendix I.

6.4.1 BUT reactor sludge

Each substrate subjected to incubation together with butyrate to observe the effects of formers to syntrophic butyrate degraders for BUT reactor sludge. In this regard, the effect of different propionate concentrations to the butyrate degradation was given in Figure 6.9. At 5mM propionate, the butyrate degradation rate increased slightly from 11.8 to 12.2 $mM.g^{-1}$ $VSS.day^{-1}$. However, increasing the amount of propionate further decreased the rate gradually to around $3mM.g^{-1}$ $VSS.day^{-1}$ at 80 mM propionate, and remained almost the same at a higher amount of propionate up to 150 mM. Simultaneously, propionate degradation rate increased from 0.27 to 0.5 $mM.g^{-1}VSS.day^{-1}$ for the propionate concentration of 5 to 150 mM respectively.

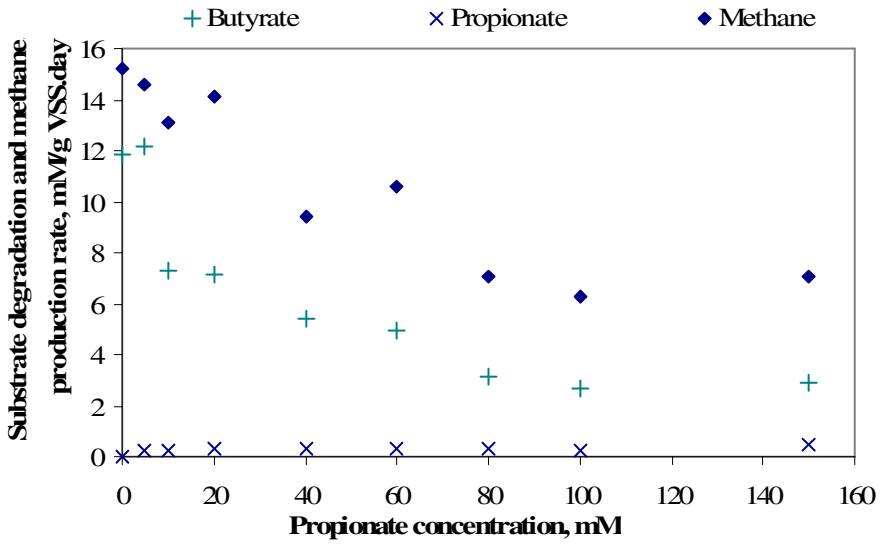


Figure 6.9 : The effect of different propionate concentrations on butyrate degradation and methane production for the BUT reactor sludge

Butyrate degradation rate was adversely affected by the addition of acetate. 5mM acetate addition decreased the butyrate degradation rate as 24% (Figure 6.10). Continuing the acetate addition reduced the degradation rate linearly and the 150 mM acetate addition caused a 78% fall of the butyrate degradation rate. Acetate degradation rate decreased from 6.5 to 5.4 $mM.g^{-1}$ $VSS.day^{-1}$ when 5 mM acetate was added. Then, the acetate degradation rate reached to the peak value of 12.9 $mM.g^{-1}$ $VSS.day^{-1}$ at 80 mM acetate concentration.

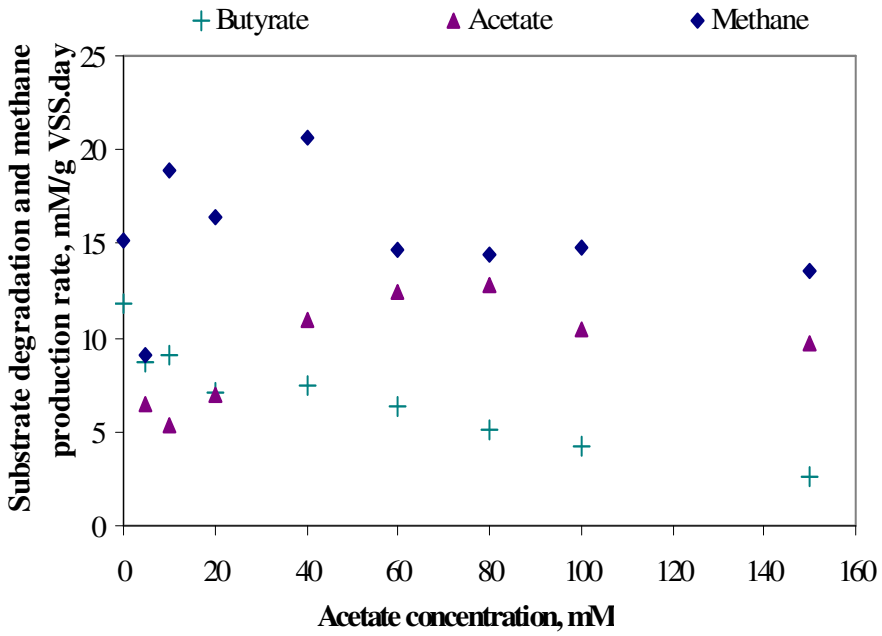


Figure 6.10 : The effect of different acetate concentrations on butyrate degradation and methane production for the BUT reactor sludge

Butyrate degradation rate was not affected excessively until the 20 mM formate addition, which lowered the butyrate degradation rate as 30% at the range of formate 0.5-20 mM (Figure 6.11). However, 30 mM addition of formate decreased the butyrate degradation rate as 72%; bringing down the butyrate degradation rate to 3.4 $mM.g^{-1}VSS.day^{-1}$. The formate degradation rate increased linearly up to 20 mM of formate with the degradation rate of 21.7 $mM.g^{-1}VSS.day^{-1}$. The 30 mM addition of formate decreased this value to 15 $mM.g^{-1}VSS.day^{-1}$.

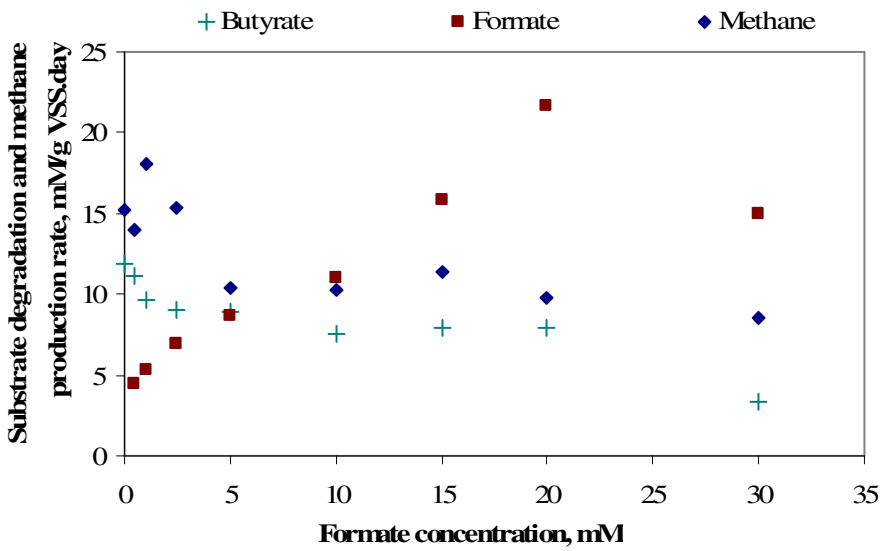


Figure 6.11 : The effect of different formate concentrations on butyrate degradation and methane production for the BUT reactor sludge

Incubation together with hydrogen did not change the rate of butyrate degradation rate and it remained almost constant with the addition of hydrogen up to 0.5 mM (Figure 6.12). Then, the degradation rate gradually declined to 5.1 $mM.g^{-1}VSS.day^{-1}$ at the 15 mM hydrogen concentration. Hydrogen degradation rate always showed an increment trend in terms of its degradation rate. As it was observed for formate degradation, the peak value of hydrogen degradation rate was 21.2 $mM.g^{-1}VSS.day^{-1}$ at the 15 mM of hydrogen.

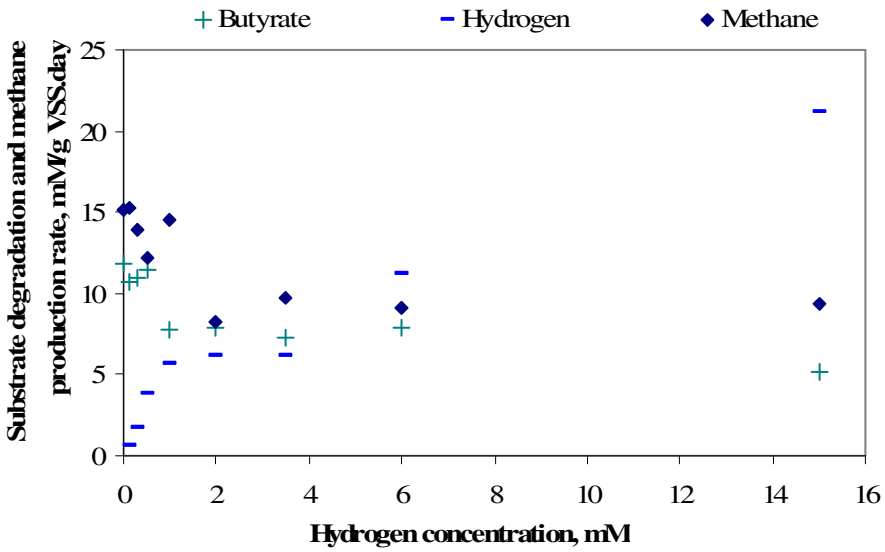


Figure 6.12 : The effect of different hydrogen concentrations on butyrate degradation and methane production for the BUT reactor sludge

6.4.2 BLS reactor sludge

The effect of propionate on the degradation rate of butyrate for BLS sludge was given in Figure 6.13. The butyrate degradation rate decreased linearly from 6.4 to 0.3 $mM.g^{-1}VSS.day^{-1}$ for the propionate concentrations of 0-200 mM. The effect of propionate on BLS sludge was much higher than that of BUT sludge for the butyrate degradation rate. While the rate decreased as 75% for BUT sludge, the decrease was 92% for BLS sludge at the propionate concentration of 150 mM. The propionate degradation rate increased linearly up to 0.77 $mM.g^{-1}VSS.day^{-1}$ at 80 mM propionate concentration, and then subsequent addition of propionate caused the rate decreasing down to the rate of 0.2 for the 150 mM propionate. Compared to the BUT sludge, it was clearly shown that propionate degradation was much more favorable for the BLS sludge. Also the sulfate reduction rate fell gradually from 1.8 to 0.06 $mM.g^{-1}$ $VSS.day^{-1}$ with the increasing amount of propionate ranging from 0 to 200 mM.

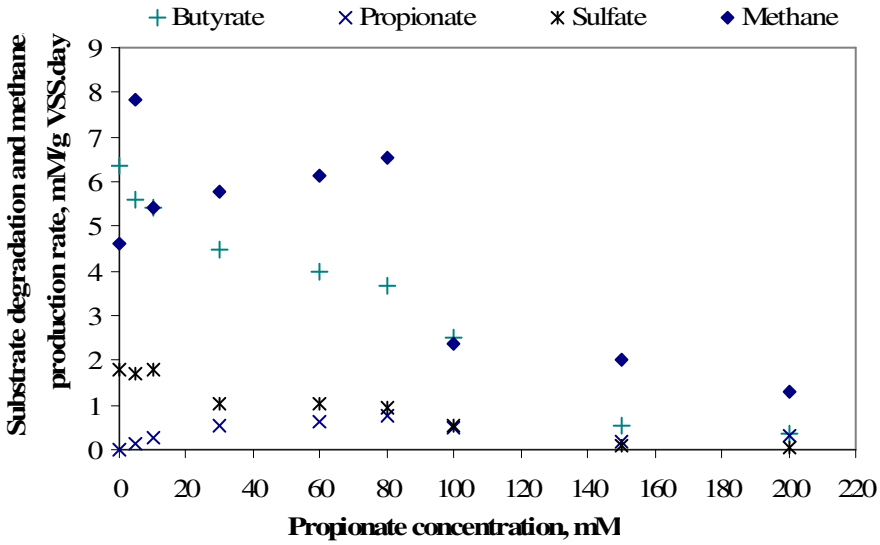


Figure 6.13 : The effect of different propionate concentrations on butyrate degradation and methane production for the BLS reactor sludge incubated together with 3 mM sulfate

The effect of acetate on the butyrate degradation rate was much more than that the BUT sludge for the BLS sludge. The butyrate degradation rate dropped to as low as 1.3 $mM.g^{-1}VSS.day^{-1}$ for the 150 mM acetate concentration (Figure 6.14). Although the acetate degradation rate dropped to 4.8 $mM.g^{-1}VSS.day^{-1}$ at the 70 mM acetate concentration, it remained almost constant for the rest of the acetate concentrations

with a rate of around 6 mM.g^{-1}VSS.day^{-1}. The sulfate reduction rate decreased from 1.1 to 0.5 mM.g^{-1}VSS.day^{-1} for the increment acetate concentration from 0 to 30 mM, respectively. Further addition of acetate did not change the rate and made it remaining constant. In addition, sulfate reduction rate was not affected as much as propionate oxidation for BLS sludge.

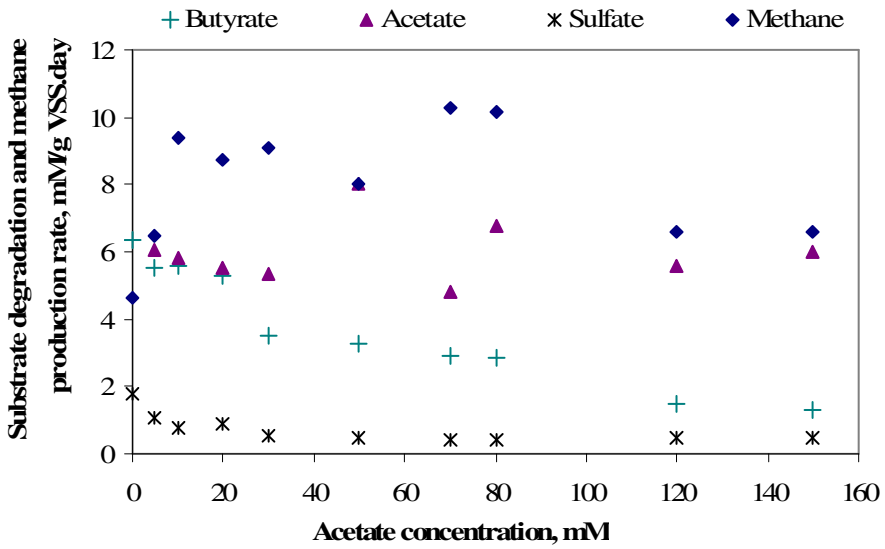


Figure 6.14 : The effect of different acetate concentrations on butyrate degradation and methane production for the BLS reactor sludge incubated together with 3 mM sulfate

Since then the butyrate degradation rate increased with the addition of 1.5 mM formate to 7.6 mM.g^{-1}VSS.day^{-1}; successive addition of formate up to 30 mM; caused the rate to decline to 1.6 mM.g^{-1}VSS.day^{-1} (Figure 6.15). Although formate addition unfavorably affected the butyrate degradation rate and concomitantly the formate degradation rate for BUT degradation, it was favorable for both the butyrate and formate degradation rates for the BLS sludge. Formate degradation rate showed the same trend as butyrate degradation, but the addition of formate more than 1.5 mM did not change the formate degradation rate and let it remain stable at the rate of around 6 mM.g^{-1}VSS.day^{-1}. The sulfate reduction rate stayed constant at 1.5 g^{-1} VSS.day^{-}1 with up to 15 mM formate addition, and then it decreased to 0.2 g^{-1} VSS.day^{-1} at 30 mM formate concentration.

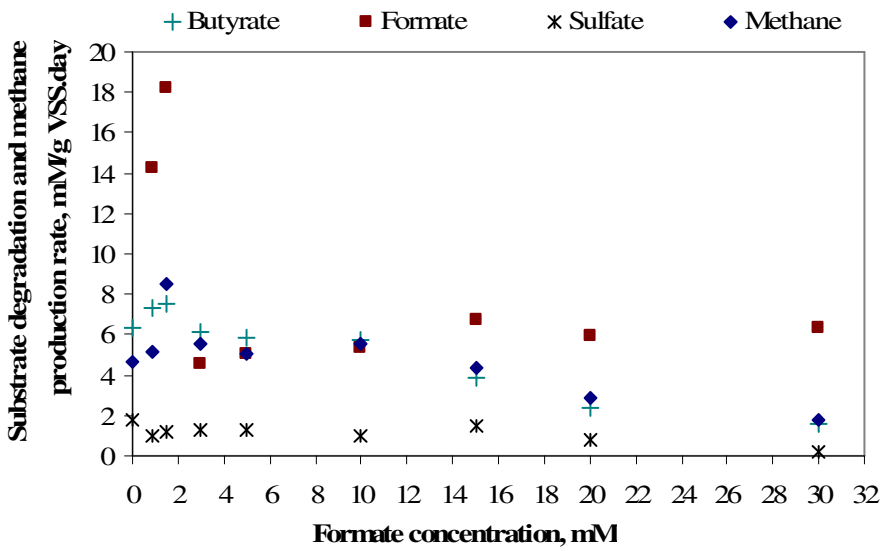


Figure 6.15 : The effect of different formate concentrations on butyrate degradation and methane production for the BLS reactor sludge incubated together with 3 mM sulfate

Hydrogen's effect to the butyrate degradation was higher compared to formate. Since incubating the butyrate together with 15 mM hydrogen decreased the butyrate degradation rate as low as 70%, the addition of the same amount of formate decreased it to as low as 40%. On the contrary to formate, hydrogen degradation rate increased linearly; but it reached the same degradation rate as formate at 15 mM hydrogen concentration level. Sulfate reduction rate fluctuated between 0.15 and 1 mM of hydrogen concentration and then remained stable at the 0.4 g^{-1}VSS.day^{-1} with the increasing hydrogen amount.

6.4.3 BUS reactor sludge

The effect of propionate on the degradation rate of butyrate for the BUS sludge was given in Figure 6.17. The butyrate degradation rate decreased linearly from 8 to 1.8 g^{-1}VSS.day^{-1} for the propionate concentration increasing from 0 to 200 mM, respectively. Nonetheless, propionate degradation rate increased linearly up to 20 mM propionate and then continued increasing with the decreasing rate until the 100 mM propionate concentration. The addition of more than 100 mM propionate decreased the propionate degradation rate. The sulfate reduction rate fluctuated slightly around 6 g^{-1}VSS.day^{-1} for all range of the propionate concentrations.

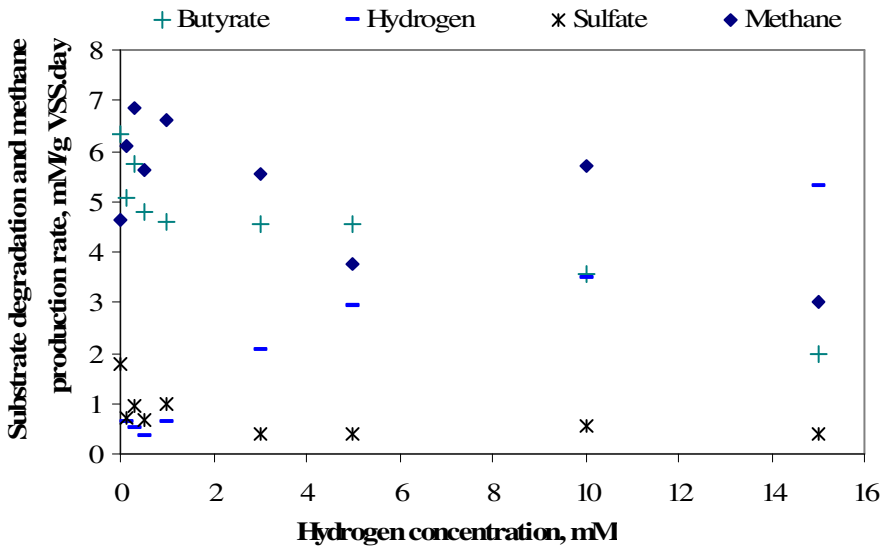


Figure 6.16 : The effect of different hydrogen concentrations on butyrate degradation and methane production for the BLS reactor sludge incubated together with 3 mM sulfate

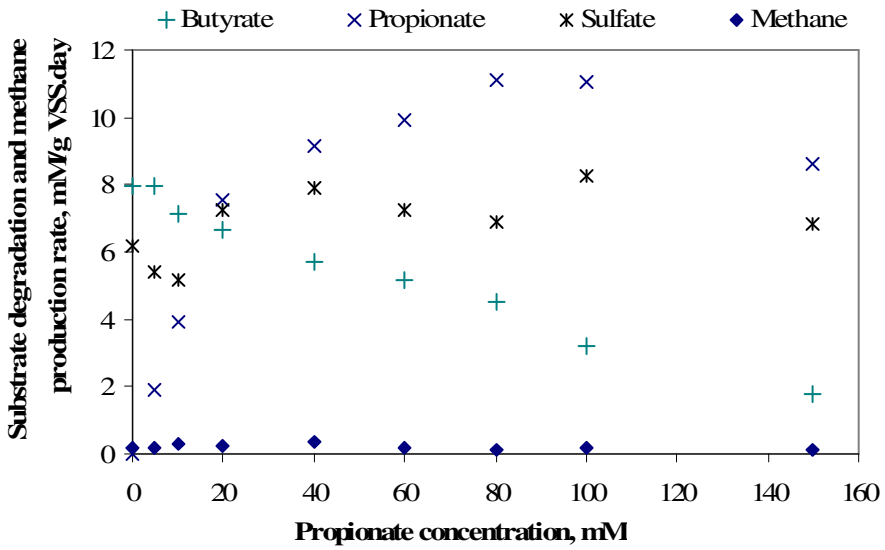


Figure 6.17 : The effect of different propionate concentrations on butyrate degradation and methane production for the BUS reactor sludge incubated together with 45 mM sulfate

The effect of different acetate concentrations on butyrate degradation was given in Figure 6.18. The variation of butyrate degradation rates was small for the acetate concentrations of ranging from 5 to 150 mM. Despite this, the effect of 150 mM acetate on butyrate showed similarity with the BUT sludge with the degradation rate of 2.5 $mM.g^{-1}VSS.day^{-1}$. Likely, to butyrate degradation, acetate degradation rate did

not change for different acetate concentrations either, and remained around 4 mM.g^{-1} VSS.day^{-1}. The sulfate reduction rate was a little less compared to the effect of propionate on the butyrate degradation by varying around 5.5 mM.g^{-1}VSS.day^{-1}

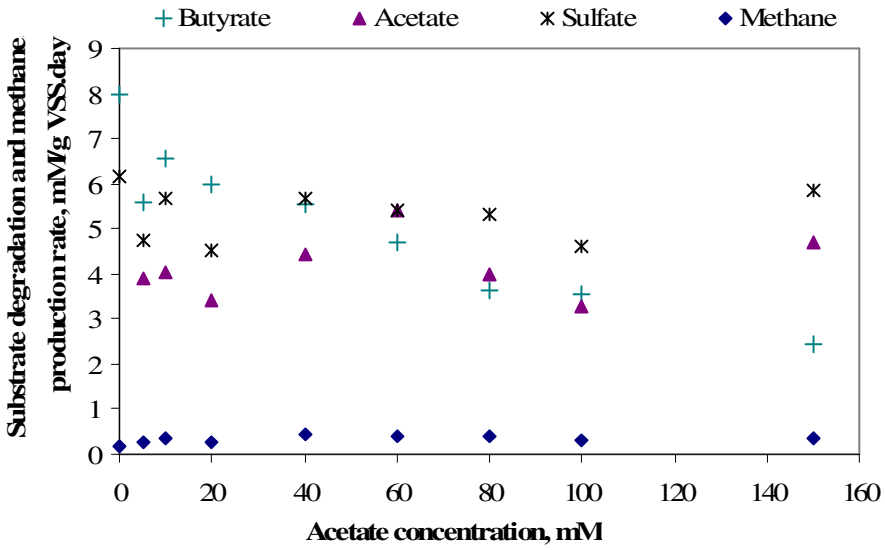


Figure 6.18 : The effect of different acetate concentrations on butyrate degradation and methane production for the BUS reactor sludge incubated together with 45 mM of sulfate

The butyrate degradation rate demonstrated a linear decrease until it reached 20 mM of formate concentration and remained the same rate for 30 mM of formate (Figure 6.19). Formate degradation rate increased with the increasing amount of formate concentration and it stayed stable around 6 mM.g^{-1}VSS.day^{-1} after the addition of 15 mM of formate. Sulfate reduction rate was around 4 mM.g^{-1}VSS.day^{-1} and it was less than that of propionate, acetate and hydrogen.

Although the hydrogen addition to the BUS sludge did not show a meaningful trend for the butyrate degradation rate, it was possible to remark that above 6 mM hydrogen concentration, the rate starts decreasing as around 35%. Such inconsistent change could be attributed to the structure of granular sludge of the BUS reactor, which had a more flabby construction compared to other BUT and BLS granules. It is known that sulfidogens out-compete the methanogens in time and it has also been observed that in such conditions sulfidogens prefer to outgrow the granules and they loose the ability to form intact granular sludge(Visser, 1995). In this regard, it could be concluded that this heterogeneous physical structure of the granules might cause this incoherent fluctuations of the degradation rates.

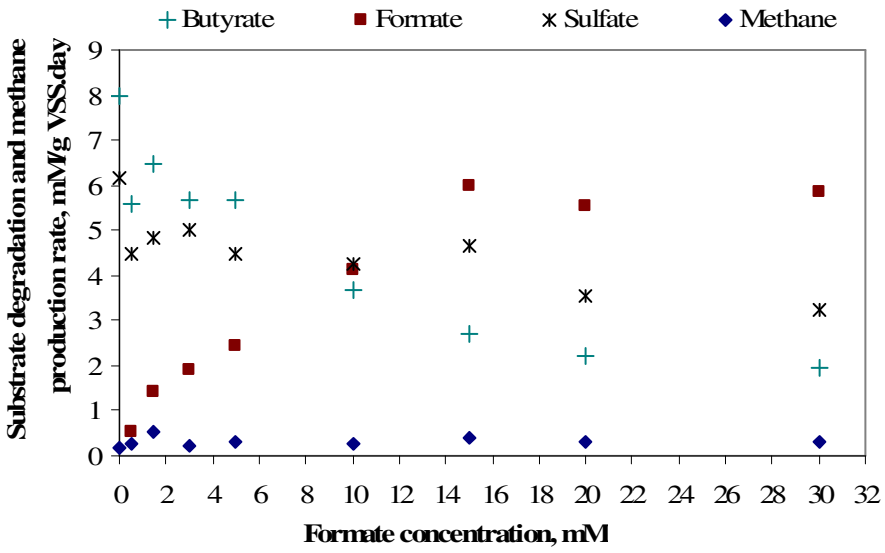


Figure 6.19 : The effect of different formate concentrations on butyrate degradation and methane production for the BUS reactor sludge incubated together with 45 mM sulfate

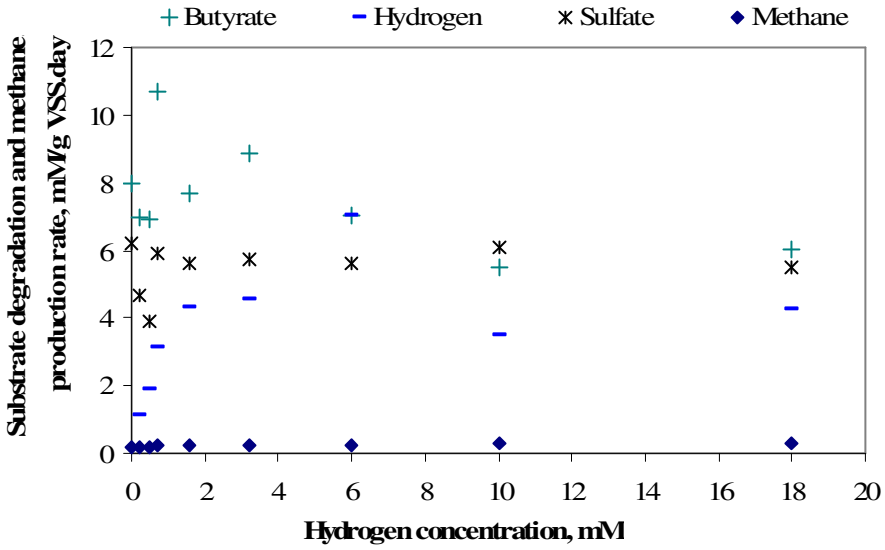


Figure 6.20 : The effect of different hydrogen concentrations on butyrate degradation and methane production for the BUS reactor sludge incubated together with 45 mM sulfate

Hydrogen degradation rate exhibited a linear increase till the 2 mM hydrogen, and then it varied around 6 $mM.g^{-1}VSS.day^{-1}$ with the increasing hydrogen concentrations. The rate of sulfate reduction did not change significantly and

remained stable around 5 $mM.g^{-1}VSS.day^{-1}$ as observed in acetate degradation study for the BUS sludge.

6.4.4 The effect of substrates on the butyrate degradation rates: expression with IC_{50}

Table 6.6 summarizes the IC_{50} values observed for three different butyrate degrading granules. The effect of propionate, acetate, formate and hydrogen on the butyrate degradation rate was different for each substrate. There was, on the contrary, not much difference between the three reactors' sludge for each individual substrate. For the BLS and the BUS reactors' sludge, butyrate degraders had somewhat better resistance to propionate toxicity; however for the BUT reactor this resistance was much lower, though the BUT reactor sludge had higher methanogenic activity on propionate than the BLS sludge.

Table 6.6: IC_{50} values (mM) based on the effect on butyrate depletion rates during exposure of the BUT, the BLS and the BUS reactors' sludge to propionate, acetate, formate and hydrogen as substrates with (BLS and BUS) and without sulfate (BUT)

	Propionate	Acetate	Formate	Hydrogen
BUT Reactor Sludge	59.6 (6154)	71.6 (4597)	22 (352)	11.2 (179)
BLS Reactor Sludge	86.4 (8922)	72.7 (4667)	19.8 (317)	10.4 (166)
BUS Reactor Sludge	89.5 (9241)	88.6 (5688)	11.2 (179)	6.3 (101)

*Values in parentheses are the equivalent of the molar concentrations in mg $COD.l^{-1}$

IC_{50} values showed that butyrate utilizing organisms were very sensitive through the low hydrogen concentrations rather than the formate for all three reactors. Normally, such a high concentration of hydrogen and formate could inhibit the reactors' sludge according to other researches. The high IC_{50} values could probably attributed to the granule structure of the sludge, which makes a concentration gradient along the structure that was lessening the effect of substrate through the inner layer.

7. MOST PROBABLE NUMBER (MPN) COUNTINGS OF UASB REACTORS AND THEIR MOLECULAR IDENTIFICATIONS

7.1 MPN Counting of the Seed and the Reactors' Sludge

A combination of cultivation-based methods with a molecular biological approach was used to investigate the dominating syntrophic butyrate degraders in the anaerobic bioreactors. The application of MPN dilution enrichment techniques allowed estimating the numbers of different tropic groups in the environmental samples. The highest dilution cultures that yield positive growth were examined to identify the dominant species using molecular techniques. The objective of this research was to gain a deeper insight into syntrophic butyrate-oxidizing bacteria of the Eerbeek sludge with enrichment and isolation techniques. The MPN dilutions were applied on the original Eerbeek sludge, the BUT, the BLS and the BUS reactors' sludge. These sludges were incubated in a bicarbonate buffered growth media with butyrate and possible intermediates of butyrate degradation, which were propionate, acetate, formate and hydrogen with or without sulfate (stoichiometric ratio of reducing equivalents). Additionally, before inoculation of the disintegrated sludge for MPN dilutions, acetate and/or hydrogen consuming methanogens were grown in the dilution series for butyrate incubations. In the case of propionate, only the hydrogen consuming methanogens were grown in the incubation bottles.

7.1.1 MPN counting for original Eerbeek sludge

The methanogenic co-culture was constructed by pre-growing the *Methanosprillium Hungatei* in each incubation bottle for butyrate and propionate MPN dilution series. In the original Eerbeek sludge, the butyrate and propionate were the same amount as $3.6x10^6$ acetogens per g VSS (Table 7.1). The population level of the butyrate and propionate had the same order of magnitude ($2.2\text{-}3.6x10^7$), and the sulfidogenic conversion rate of them were 23% and 47% respectively in the presence of sulfate. These values demonstrated that methanogenesis was the predominant group for acetogenic bacteria for the original Eerbeek sludge. There was no significant

difference among the number of cells in the butyrate and propionate under both sulfidogenic and methanogenic conditions individually.

Table 7.1: MPN of acetogens and methanogens in the original Eerbeek sludge incubated with methanogens and different substrates

Incubation conditions	MPN, cells/g VSS			% substrate used by SRB and AB/MA	
	Sulfidogens	Acetogens	Methanogens	Sulfidogens	Acetogens or methanogens
Butyrate+HC		$3.6x10^{10}$			
Butyrate+SO_4^{2-}	$3.6x10^{7}$			23.3	71.1
Propionate+HC		$3.6x10^{10}$			
Propionate+SO_4^{2-}	$2.2x10^{7}$			46.5	47.6
Acetate			$3.6x10^{8}$		
Acetate+SO_4^{2-}	$7.4x10^{4}$			9.8	81.4
Formate			$7.4x10^{9}$		
Formate+SO_4^{2-}	$3.6x10^{9}$			9.5	87
H_2/CO_2			$3.6x10^{12}$		
H_2/CO_2+SO_4^{2-}	$2.2x10^{12}$			3.7	95.2

* HC: Hydrogen Consumer

Since the number of acetate utilizing methanogens was $3.6x10^{8}$, acetate utilizing sulfidogens were as low as $7.4x10^{4}$, demonstrating that the latter one was insignificant in the original Eerbeek sludge. The number of hydrogenotrophic methanogens was 10 and 10,000 times higher for hydrogen and formate respectively, compared to the acetotrophic methanogens. The formate and hydrogen utilizing methanogens were enumerated in the order of 10^{9}-10^{12} per g VSS, both in the presence and absence of sulfate. Also, these substrates were used up mostly by methanogens, 95 and 87% of hydrogen and formate respectively; indicating that major parts of the population were dominated by them.

7.1.2 MPN counting for the BUT reactor sludge

The methanogenic co-culture was constructed by pre-growing *Methanosprillium Hungatei* with and/or *Methanosaeta concillii* in each incubation bottle of MPN dilution series. In the meantime, the number of butyrate utilizing acetogen was $3.7x10^{10}$, where the presence of *M. Hungatei* increased the number by 10^{4} times. However, the presence of both *M. Hungatei* and *M. concillii* in the same series did not change the number of butyrate utilizers. Sulfidogenic butyrate degraders were the same number as the one observed for methanogenic butyrate degraders. In the MPN

series of butyrate and sulfate, the 22.4% of butyrate was used for the sulfidogens; corresponding almost to the theoretical reducing equivalent of the hydrogen (20%). The same situation was also detected for the MPN series of propionate with 43.9% of the butyrate was converted by SRB. This ratio was also very close to the value of 43%, matching to the reducing equivalent of hydrogen for propionate.

Table 7.2: MPN of acetogens and methanogens in the BUT reactor sludge

Incubation conditions	MPN, cells/g VSS			% substrate used by SRB and AB/MA	
	Sulfidogens	Acetogens	Methanogens	Sulfidogens	Acetogens or methanogens
Butyrate		$3.7x10^{10}$			
Butyrate+HC		10^{14}			
Butyrate+HAC	-	$1.9x10^{14}$	-	-	-
Butyrate+SO_4^{2-}	$3.7x10^{10}$			22.4	73.4
Propionate+HC		$6x10^{11}$			
Propionate+SO_4^{2-}	10^{10}			43.9	N.D.
Acetate			$1.2x10^{11}$		
Acetate+SO_4^{2-}	N.D.			N.D.	94.9
Formate			$6x10^{11}$		
Formate+SO_4^{2-}	$6x10^{8}$			6.7	90.2
H_2/CO_2			$6x10^{12}$		
H_2/CO_2+SO_4^{2-}	$1.6x10^{9}$			22.3	75.7

*HC: Hydrogen Consumer; HAC: Hydrogen and Acetate Consumer; N.D.: Not Detected.

In the presence of sulfate, sulfide production was not detected and acetate utilizing methanogens were the dominant population with the number of $1.2x10^{11}$. Both the hydrogen and formate consuming methanogens were enumerated in the order of 10^{12} and 10^{11} respectively; which were comparable to those of the seed sludge. Under sulfidogenic conditions, those numbers dropped to 1000 times lower than the methanogenic consortia had. Population levels of the BUT sludge and the seed sludge had the same order of magnitude for hydrogen utilizing methanogens.

7.1.3 MPN counting for BLS reactor sludge

The presence of *M. Hungatei*, *M. concillii* and 3mM sulfate increased the number of butyric acid utilizing acetogens from $6.8x10^{12}$ to $6.3x10^{14}$. The presence of low level of sulfidogenic population might be the reason why such an increment was observed. This low level of sulfidogens also demonstrated that the methanogenic acetogens were the dominated trophic group in the presence of excess sulfate. The population level of the propionate degraders in the BLS sludge was in the same order as those in

BUT sludge. The degradation ratio of the butyrate and propionate by methanogens and sulfidogens were almost equal to the reducing equivalent ratio of hydrogen utilizers and acetate utilizers as observed in the BUT sludge.

Table 7.3: MPN of acetogens and methanogens in the BLS reactor sludge

Incubation conditions	MPN, cells/g VSS			% substrate used by SRB and AB/MA	
	Sulfidogens	Acetogens	Methanogens	Sulfidogens	Acetogens or methanogens
Butyrate+HC		$6.8x10^{12}$			
Butyrate+ SO_4^{2-} (3mM)+HAC	$2.1x10^{14}$	$6.3x10^{14}$		4.3	93.4
Butyrate+SO_4^{2-} (unlimited)	$1.4x10^{10}$			27.3	70.2
Propionate+HC		$6.8x10^{10}$			
Propionate+SO_4^{2-}	$4.6x10^{9}$			52.2	47.1
Acetate			$2.1x10^{11}$		
Acetate+SO_4^{2-}	N.D.			-	96.2
Formate			$6.8x10^{11}$		
Formate+SO_4^{2-}	$4.2x10^{8}$			36.1	63.2
H_2/CO_2			$6.8x10^{11}$		
H_2/CO_2+SO_4^{2-}	$1.4x10^{11}$			18.9	80.9

*HC: Hydrogen Consumer; HAC: Hydrogen and Acetate Consumer

The number of acetate utilizing methanogens increased to $2.1x10^{11}$ compared to the number of $3.6x10^{8}$ per g of VSS detected in the seed sludge; though remained the same for the BUT sludge. The number of both formate and hydrogen utilizing methanogens were in the same order being 10^{11}, except for the formate utilizing sulfidogens having the number of $4.2x10^{8}$. There were significantly fewer cells of formate utilizing methanogen in the BLS sludge, demonstrating the insignificance of formate utilization by sulfate reducers in the BLS sludge consortia.

7.1.4 MPN counting for the BUS reactor sludge

There was no significant difference among the number of cells in the butyrate and propionate under both sulfidogenic and methanogenic conditions except for the propionate, which was 100 times lower under methanogenic setting (Table 7.4).

Acetate degrading sulfidogens were considered to be the most important group, because the number was 10^{4} times higher than that of the methanogens. More than 94 percent of the butyrate was degraded by sulfidogens, not showing any methanogenic activity. While the number of the population did not change for hydrogen and

formate utilizing methanogens, the sulfidogens increased 10 times for the same community; which can be considered as a variation of no particular significance.

Table 7.4: MPN of acetogens and methanogens in the BUS reactor sludge

Incubation conditions	MPN, cells/g VSS			% substrate used by SRB and AB/MA	
	Sulfidogens	Acetogens	Methanogens	Sulfidogens	Acetogens or methanogens
Butyrate+HC		$3.6x10^{12}$			
Butyrate+HAC		$2.2x10^{13}$	-		
Butyrate+SO_4^{2-}	$6x10^{12}$			94.7%	-
Butyrate+SO_4^{2-} +HAC	$3.6x10^{13}$			98.5%	-
Propionate+HC		$2.2x10^{10}$			
Propionate+SO_4^{2-}	$2x10^{12}$			99.1%	-
Acetate			$4.1x10^{9}$		
Acetate+SO_4^{2-}	$2.2x10^{13}$			98.8%	-
Formate			$3.1x10^{11}$		
Formate+SO_4^{2-}	$7.3x10^{12}$			98.4%	-
H_2/CO_2			$3.6x10^{11}$		
H_2/CO_2+SO_4^{2-}	$7.3x10^{12}$			99.3%	-

*HC: Hydrogen Consumer; HAC: Hydrogen and Acetate Consumer

It can also be concluded that in all incubation conditions, substrates were converted with a higher ratio by the sulfidogens in the presence of excess sulfate.

7.1.5 Comparative evaluation of the MPN counting

It was clear that the number of acetogens were increased in the reactor sludge compared to the seed sludge under both methanogenic and sulfidogenic conditions. Although the addition of hydrogen utilizers to the incubations significantly increased the numbers of acetogens, the addition of acetate utilizers did not change the number of them. This indicated the importance of hydrogen consumers in the syntrophic degradation of butyrate and propionate.

The number of acetate degrader was in the order of 10^8 and 10^4 in the methanogenesis and sulfidogenesis, respectively. While this methanogenic number increased to the order of 10^{11} for the BUT and the BLS sludge, the sulfidogenic number increased to the order of 10^{13} for the BUS sludge. Besides, no acetotrophic sulfidogens were detected in the BUT and the BLS sludge.

Since the formate utilizing methanogens seemed less important for the seed sludge, the number of them increased in the reactor sludge and reached the same importance

as the seed sludge. The other important point was the different numbers observed between the hydrogen and formate utilizing sulfidogens for the BLS sludge. In the presence of limited sulfate, the reducing equivalent of the electrons was carried via hydrogen instead of formate.

If the number of acetogens, methonogens and sulfidogens found in this study compared to other studies, it could be concluded that, since they were in the same order with some methanogenic and sulfidogenic environments (Visser, 1995), most of the results in the other studies were much lower (Ahring *et al.*, 2001; Grotenhuis, JT *et al.*, 1991; Oude Elferink *et al.*, 1998) than what we have found for the reactors' sludge. However, the seed sludge had more or less same number with the anaerobic reactors' sludge (Table 7.5).

Table7.5: The MPN counting of different types of mixed cultures

Growth conditions of sludge				NB.g^{-1} VSS						References
				H_2/CO_2	Formate	Acetate	Propionate	Butyrate	Glucose	
Acetate:Propionate:Butyrate mixture (1:1:1) 18.8 g VSS.l^{-1} crushed granular 99% COD removal	UASB, 1.7L, 37±1°C LR: 14 g COD.L^{-1}.d^{-1}	COD/SO_4=10.4	-	4.10^{13}-7.10^{14}		7.10^{10}-2.10^{12}	4.10^{10}-8.10^{11}	7.10^{13}-9.10^{14}		(Visser, 1995)
			SO_4	4.10^{13}-7.10^{14}		5.10^{11}-8.10^{12}	6.10^{10}-9.10^{11}	4.10^{13}-8.10^{14}		
	UASB, 1.7L, 37±1°C LR: 20 g COD.L^{-1}.d^{-1}	COD/SO_4=3.6	-	2.10^{13}-5.10^{14}		4.10^{11}-8.10^{12}	4.10^{10}-8.10^{11}	5.10^{13}-8.10^{14}		
			SO_4	1.10^{13}-4.10^{14}		8.10^{9}-2.10^{11}	2.10^{11}-6.10^{12}	2.10^{13}-5.10^{14}		
	UASB, 1.7L, 37±1°C LR: 13 g COD.L^{-1}.d^{-1}	COD/SO_4=0.6	-	7.10^{13}-9.10^{14}		3.10^{11}-7.10^{12}	4.10^{10}-8.10^{11}	5.10^{13}-8.10^{14}		
			SO_4	7.10^{12}-9.10^{13}		7.10^{10}-1.10^{12}	7.10^{12}-10^{14}	5.10^{13}-8.10^{14}		
Lab-scale treating cattle manure (70 g COD.l^{-1}) 45.4±1.9 g VS.l^{-1}	CST,3 L, 55°C LR:3 g VS.l^{-1}.d^{-1}	28% VS removal, effluent VFA 0.1-0.3 g acetate.l^{-1}		$4.6x10^9$		$3.3x10^5$	$2x10^4$	$3.3x10^5$	$9.4x10^6$	(Ahring *et al.*, 2001)
	CST, 3 L, 65°C LR:3 g VS.l^{-1}.d^{-1}	22% VS removal, effluent VFA 1.8-2.4 g acetate.l^{-1}		$1.7x10^{10}$		$2x10^2$	0	$9.5x10^2$	$<9.5x10^2$	
Shredded: Vegetable, 27%; Fruits,5.4%; Meat, 4.5%; Rice, 12%; Paper, 0.01%; Dewatered sludge cake, 51.1%	CR 31 L, 41°C Leachate with recycle	41°C		$8.5x10^8$		$2x10^7$	10^7			(Lay *et al.*, 1998)
	Leachate without recycle, 41°C	41°C		$5.2x10^8$		$1.1x10^8$	$2.5x10^8$			
Digested sewage sludge		35°C	-	$5x10^5$		$4.7x10^6$	$1.6x10^4$			(Agrawal *et al.*, 1997)
		+ 700 mg SO_4^{2-}.l^{-1}	SO_4			$3x10^4$	$1.3x10^4$			
Dilute wastewater (sewage)	UASB at a moderate temperature	35°C	-	$3.8x10^8$		$3.8x10^7$	$6.2x10^7$			
		+ 700 mg SO_4^{2-}.l^{-1}	SO_4	$3.8x10^8$			$1.2x10^{10}$			
Concentrated sewage sludge, 50 g TS.l^{-1}	ESD, 36°C (6800 m^3) 1.7 g TS.l^{-1}.d^{-1}	Granular 44.2% TS and 60% COD removal, No VFA detected at the effl.		$3.5x10^9$		$5.9x10^7$				(Li *et al.*, 1997)

Table7.5: (Continued)

Growth conditions of sludge				NB.g^{-1} VSS						References
				H_2/CO_2	Formate	Acetate	Propionate	Butyrate	Glucose	
Liquid sugar factory Containing sucrose, ethanol, lactate, acetate, propionate, butyrate, caproate.	UASB, 30 m^3, 35°C	Granular		$9x10^{10}$	$2x10^{9}$	$2.4x10^{8}$	$2.4x10^{8}$		$2.1x10^{12}$	(Grotenhuis, JT *et al.*, 1991)
Ethanol, 26 mM	UASB, 5 L, 35°C HRT:10 h			$3x10^{9}$	$2.4x10^{8}$	$9x10^{9}$	$3x10^{7}$		$2x10^{8}$	
Propionate, 44 mM				$2x10^{12}$	$2.4x10^{11}$	$4.6x10^{6}$	$4.6x10^{6}$		$2x10^{8}$	
Sulfate rich papermill wastewater, carbohydrates, 850; acetate, 500; propionate, 300; butyrate, 45; formate, 20 mg COD/L	UASB, 2500 m^3, HRT:4.6 h, 70% COD and 95% sulfate removal 37°C	Granular COD/SO_4=9.5	-	$7.3x10^{12}$	$5.8x10^{11}$	$9.7x10^{8}$	$2.9x10^{12}$	$3.4x10^{9}$		(Oude Elferink *et al.*, 1998)
			SO_4	$4.1x10^{12}$ (M) $1.8x10^{11}$ (SRB)	$4.9x10^{11}$(M) 10^{10}(SRB)	$9.7x10^{6}$ (M) $2.5x10^{5}$ (SRB)	$7.6x10^{7}$ (M) $7.6x10^{8}$ (SRB)	$6.1x10^{6}$ (M) $4.6x10^{6}$ (SRB)		

Values are the mean of triplicates. Standard errors (standard deviation) are in parentheses.
UASB: Upflow Anaerobic Sludge Blanket, CST: Continuously Stirred Tank, CR: Column Reactor, ESD: Egg-Shaped Digesters

7.2 Genetic Diversity within the Highest Dilutions of the Reactors' Sludge

7.2.1 Genetic diversity within the highest dilutions of BUT reactor sludge

7.2.1.1 Butyrate MPN series in the presence of hydrogen consumer

Bacterial sequences from the highest dilution of the BUT sludge incubated together with the hydrogen consuming archaea (*M. Hungatei*) clustered in the *Firmicutes*. Three bacteria were observed from these consortia. They included: two butyrate-oxidizing bacteria (clone:BHB04 and clone:B10), which were obtained from mesophilic butyrate-degrading methanogenic community during continuous cultivation, and Expanded Granular Sludge Bed (EGSB) reactors treating oleic acid (Pereira *et al.*, 2002). The other one, *Exiguobacterium acetylicum*, was cultivated from the Siberian permafrost (Rodrigues *et al.*, 2006)(Table 7.6).

Table 7.6: The prevalent bacteria in the higher dilutions of the BUT reactor sludge in the presence of *Methanospirillum Hungatei*, according to the highest 16S rRNA gene sequence similarity to the database (GenBank) search result

Clone	Length (bp)	Closest phylotype	% Seq. sim.	Closest cultured species	% Seq. sim.	Taxonomic position (class, family, or genus)	# Clones	% Frequency
BUT-HC_45	434	clone:BHB04	98	*Syntrophomonas sp.* TB-6	95	Firmicutes, Syntrophomonadaceae	31	64.6
BUT-HC_37	328	clone B10	98	*Syntrophomonas flectens*	97	Firmicutes, Syntrophomonadaceae	14	29.2
BUT-HC_14	449	*Exiguobacterium acetylicum*	99			Firmicutes, Bacillaceae	2	4.2

The most abundant syntroph in butyrate enrichment was related to *Syntrophomonas* sp. TB-6. The second abundant syntroph was the *Syntrophomonas flectens*. Both of them are known as the typical butyrate degraders. These bacteria are isolated from flooded paddy field soils and up-flow anaerobic sludge blanket reactor treating beer wastewater respectively. Members of the family Syntrophomonadaceae are predominantly found in methanogenic environments in syntrophic associations with methanogens (Sobieraj and Boone, 2004). The species of Syntrophomonadaceae are extremely sensitive to oxygen and their pathway proceeds through classical fatty acid β-oxidation which ends up with two moles of acetate and half a mole of protons in the case of butyrate degradation (Wofford *et al.*, 1986).

Exiguobacterium acetylicum is gram-positive and facultatively anaerobic, with non-spore-forming rods and the growth range of 7-43 °C (Fruhling *et al.*, 2002). Although the sequence similarity was high (99%), this organism was isolated from permafrost soil. The presence in the syntrophic and mesophilic environment is not clear. According to (Rodrigues *et al.*, 2006), it consumes both acetate and propionate; whereas (Fruhling *et al.*, 2002) found only the weak acetate degradation for this species. These characteristics make it difficult to interpret the function of them in the syntrophic degradation pathway.

7.2.1.2 Butyrate MPN series in the presence of hydrogen and acetate consumer

Microorganisms from Firmicutes, Betaproteobacteria and Spirochaetes divisions were found in the presence of both hydrogen and acetate consumers (Table 7.7). The diversity was quite high when it is compared to the enrichment grown with only the hydrogen consumer. In addition to the *Syntrophomonas* species, *Dechloromonas hortensis, Spirochaeta stenostrepta, Aminobacterium colombiense* and *Thermovirga lienii* were found differently. Although a higher diversity was observed, *Syntrophomonas* occupied an important part (74%) of the community in terms of the clones' abundance. The remaining clones shared the abovementioned diversity. In these clones *Dechloromonas hortensis* showed a higher frequency with 20%. They included: one dechlorinating bacterium (strain DCB-1), one benzoate-oxidizing bacterium (strain BZ-2), two butyrate-oxidizing bacteria (strains SF-1 and NSF-2) and two H_2-consuming sulfate-reducing bacterium (Desulfovibrio sp. strain PS-1).

The organisms represented by BUT-HAC _43 and BUT-HAC _51 clones sequences were close to *Dechloromonas hortensis* strain MA-1 and they were not able to grow in anoxic medium reduced with sulfide (Wolterink *et al.*, 2005). Therefore, the presence of these species in our culture is unclear. BUT-HAC _20 clone was 99% similar to the eubacterium RFLP5 clone which was isolated from dechlorinating microbial community, and it was also close to the cultured *Aminobacterium colombiense* species with 91%. These species are known to be growing under strictly anaerobic conditions and the amino acid degraders (Baena *et al.*, 1998). Based on this low similarity, it is difficult to predict or suggest the presence reason of these species in this enrichment. Similarly, clone BUT-HAC _60 was the same as the clone BUT-HAC _20 and it was close to the cultured species of *Thermovirga lienii*,

utilizing the proteinous substrates and some amino acids (Dahle and Birkeland, 2006). BUT-HAC _29 clone was related to the clone:MST2, isolated from the mesophilic anaerobic sludge treating lipids. The closest cultured bacteria was *S. stenostrepta* and it fermented a variety of carbohydrates, but did not ferment amino acids or other organic acids (Hespell and Canale-Parola, 1970). Thus, the presence of these amino acids and carbohydrate fermenters were not understandable.

Table 7.7: The prevalent bacteria in the higher dilutions of the BUT reactor sludge in the presence of *Methanospirillum Hungatei* and *Methanosaeta concilli*, according to the highest 16S rRNA gene sequence similarity to the database (GenBank) search result

Clone	Length (bp)	Closest phylotype	% Seq. sim.	Closest cultured species	% Seq. sim.	Taxonomic position (class, family, or genus)	# Clones	% Frequency
BUT-HAC_50	434	clone:BHB04	98	*Syntrophomonas flectens*	95	Firmicutes; Syntrophomonadaceae	28	29.2
BUT-HAC_12	436	*Syntrophomonas* sp. TB-6	94			Firmicutes, Syntrophomonadaceae	31	36.5
BUT-HAC_84	438	clone:TSBW01	97	*Syntrophomonas flectens*	93	Firmicutes, Syntrophomonadaceae	1	1.0
BUT-HAC_68	352	bacterium clone A3	97	*Syntrophomonas sapovorans*	95	Firmicutes, Syntrophomonadaceae	1	1.0
BUT-HAC_21	442	clone:TSBW01	96	*Syntrophomonas flectens*	93	Firmicutes, Syntrophomonadaceae	2	2.1
BUT-HAC_91	441	clone:BHB03	94	*Syntrophomonas sporosyntrophas* strain Y4-1	93	Firmicutes, Syntrophomonadaceae	4	4.2
BUT-HAC_51	435	beta proteobacterium SBRH147	99	*Dechloromonas hortensis* strain MA-1	97	Betaproteobacteria, Rhodocyclaceae	18	18.8
BUT-HAC_43	433	clone B4M56D1	98	*Dechloromonas hortensis* strain MA-1	98	Betaproteobacteria, Rhodocyclaceae	1	1.0
BUT-HAC_20	436	eubacterium RFLP5	94	*Aminobacterium colombiense*	91	Firmicutes, Syntrophomonadaceae	1	1.0
BUT-HAC_60	447	eubacterium RFLP5	99	*Thermovirga lienii* strain Cas60314	90	Firmicutes, Syntrophomonadaceae	1	1.0
BUT-HAC_75	448	clone TTA_B6	98	*Thermovirga lienii* strain Cas60314	93	Firmicutes, Syntrophomonadaceae	1	1.0
BUT-HAC_29	439	clone:MST2	98	*Spirochaeta stenostrepta*	90	Spirochaetes, Spirochaetaceae	3	3.1

7.2.1.3 Butyrate MPN series in the presence of unlimited sulfate

In the case of only butyrate adapted sludge inoculated with sulfate resulted in the selection of members of the *Desulfovibrionaceae* and *Syntrophomonadaceae* (Table 7.8). It clearly shows that sulfate addition affected the H_2 metabolizing

microorganisms causing a change in the pathway of the sulfate reducing bacteria. It was also known from the thermodynamic and kinetic data of hydrogenotrophic methanogens and sulfidogens that if sufficient amount of sulfate was provided, hydrogen utilizing sulfidogens would outcompete the methanogens (Visser, 1995).

Table 7.8: The prevalent bacteria in the higher dilutions of the BUT reactor sludge in the presence of unlimited sulfate, according to the highest 16S rRNA gene sequence similarity to the database (GenBank) search result

Clone	Length (bp)	Closest phylotype	% Seq. sim.	Closest cultured species	% Seq. sim.	Taxonomic position (class, family, or genus)	# Clones	% Frequency
BUT-S_36	423	clone:MOL6	99	*Desulfovibrio sulfodismutans*	98	Deltaproteobacteria, Desulfovibrionaceae	2	4.2
BUT-S_67	435	clone:MPA4	99	*Desulfovibrio sulfodismutans*	98	Deltaproteobacteria, Desulfovibrionaceae	16	33.3
BUT-S_75	435	clone:BHB04	98	*Syntrophomonas* sp. MGB-C1	98	Firmicutes, Syntrophomonadaceae	30	62.5

63% of the sequences clustered with the *Syntrophomonas* spp., known as butyrate degraders. *Desulfovibrio sulfodismutans* remained a part of the clones. These were grown very slowly by sulfate reduction and were also observed to grow very slow on hydrogen but not on formate (Bak and Pfennig, 1987). It was also observed that acetate, propionate or butyrate was used as carbon sources for growth by disproportionation of thiosulfate. Therefore, *Desulfovibrio sulfodismutans* could act as a biological electron acceptor using hydrogen and also could use butyrate as a carbon source in the butyrate degrading consortia if excess amount of sulfate were provided.

7.2.2 Genetic diversity within the highest dilutions of the BLS reactor sludge

7.2.2.1 Butyrate MPN series in the presence of hydrogen consumer and limited sulfate

81.3% of the clones were associated with the *Syntrophomonas* species for the BLS sludge. The remaining clones were clone:MST2 and *Gemmatimonas* sp. with an abundance of 16.7 and 2.1, respectively.

Table 7.9: The prevalent bacteria in the higher dilutions of the BLS reactor sludge in the presence of *Methanospirillum Hungatei* and limited sulfate, according to the highest 16S rRNA gene sequence similarity to the database (GenBank) search result

Clone	Length (bp)	Closest phylotype	% Seq. sim.	Closest cultured species	% Seq. sim.	Taxonomic position (class, family, or genus)	# Clones	% Frequency
BLS-HC_32	435	clone:BHB04	98	*Syntrophomonas* sp. TB-6	97	Firmicutes, Syntrophomonadaceae	30	62.5
BLS-HC_48	437	clone:MST2	99	*Spirochaeta stenostrepta*	90	Spirochaetes, Spirochaetaceae	8	16.7
BLS-HC_12	392	*Syntrophomonas* sp. TB-6	85			Firmicutes, Syntrophomonadaceae	9	18.8
BLS-HC_56	436	*Gemmatimonas* sp. clone HPIRR2E01	94	*Thiocapsa roseopersicina*	93	Gammaproteobacteria, Chromatiaceae, Thiocapsa	1	2.1

As mentioned in the section of 7.2.1.2, *Spirochaeta stenostrepta* fermented a variety of carbohydrates, but did not ferment amino acids or other organic acids (Hespell and Canale-Parola, 1970). However, clone:MST2, isolated from the mesophilic anaerobic sludge treating lipids. In the degradation of long chain fatty acids, syntrophism might play important role; hence, the BLS-HC_48 clone could be related with one of the syntrophic players. The BLS-HC_56 clone was related to *Gemmatimonadetes* phylum, having only one representative pure culture. This pure culture was the aerobic polyphosphate-accumulating bacteria and no fermentative growth was observed (Zhang *et al.*, 2003). The closest cultured species to this clone was the purple sulfur bacterium *Thiocapsa roseopersicina* utilizing certain carbohydrates, amino acids, volatile acids, and Krebs cycle intermediates photolithotrophically on the sulfide (Holm and Vennes, 1970).

7.2.2.2 Butyrate MPN series in the presence of hydrogen and acetate consumer and limited sulfate

Microbial community comprised 43.7% *Syntrophomonas*; 39.5% *Syntrophospora*; 9.1% *Dehalococcoides*; 3.1% *Syntrophobacter fumaroxidans;* 2.1% *Geobacillus thermoparaffinivorans*; 1% *Clostridium* and 1% *Aeromonas simiae* in the presence of hydrogen and acetate consumer for the BLS sludge (Table 7.10). Significant changes in the bacterial composition were observed if the MPN dilution series incubated with the acetate consumer comparing with the non-incubated series. The new clones obtained differently from the previous section were explained as follows:

Table 7.10: The prevalent bacteria in the higher dilutions of the BLS reactor sludge in the presence of *Methanospirillum Hungatei*, *Methanosaeta concilli* and limited sulfate; according to the highest 16S rRNA gene sequence similarity to the database (GenBank) search result

Clone	Length (bp)	Closest phylotype	% Seq. sim.	Closest cultured species	% Seq. sim.	Taxonomic position (class, family, or genus)	# Clones	% Frequency
BLS-HAC_38	435	clone:BHB04	98	*Syntrophomonas* sp. MGB-C1	98	Firmicutes, Syntrophomonadaceae	37	38.5
BLS-HAC_23	442	clone:BHB03	91	*Syntrophomonas wolfei* subsp. methybutyica strain 5J-1	92	Firmicutes, Syntrophomonadaceae	4	4.2
BLS-HAC_87	379	clone:MPA	93	*Syntrophomonas wolfei* subsp. methybutyica strain 5J-1	92	Firmicutes, Syntrophomonadaceae	1	1.0
BLS-HAC_31	439	clone:BHB03	92	*Syntrophospora cellicola* strain 19J-3	91	Firmicutes, Syntrophomonadaceae	37	38.5
BLS-HAC_55	400	clone:MPA	93	*Syntrophospora cellicola* strain 19J-3	92	Firmicutes, Syntrophomonadaceae	1	1.0
BLS-HAC_7	423	clone CR99-7-70	95	*Dehalococcoides* sp. BHI80-15	86	Chloroflexi, Dehalococcoidetes	9	9.4
BLS-HAC_94	437	*Syntrophobacter fumaroxidans*	97			Deltaproteobacteria, Syntrophobacteraceae	3	3.1
BLS-HAC_47	434	clone 1A12	99	*Clostridium* sp. (BN II)	95	Firmicutes, Clostridiaceae	1	1.0
BLS-HAC_39	272	bacterium sk.prop8	93	*Geobacillus thermoparaffinivorans* strain DM2	92	Firmicutes, Bacillaceae	2	2.1
BLS-HAC_62	437	clone:A-8	96	*Aeromonas simiae*	96	Gammaproteobacteria, Aeromonadaceae,	1	1.0

BLS-HAC_7 clone, which was the third abundant species, was affiliated with the green non-sulfur bacteria. Although many retrieved clones fell into this phylum (Boomer *et al.*, 2002), little is known about their physiological properties. The closest cultured relative, chlororespiring anaerobe *Dehalococcoides* sp., used hydrogen as an electron donor to reduce the chlorinated compounds (Waller *et al.*, 2005). Since the abundance of BLS-HAC_94 closely related well characterized the strains of *Syntrophobacter fumaroxidans* was only 3.1, the presence in the butyrate culture was not clear. It oxidized propionate syntrophically in co-culture with hydrogen utilizing methanogen or with sulfate as the electron acceptor (Harmsen *et al.*, 1998). Whereas the 16S rRNA sequence of BLS-HAC_47 clone nearly identical to the clone1A12 (similarity >99%) related to the sulfate reducing anaerobic bioreactor, the closest cultured *Clostridium* sp. with the similarity of 95% brought up

the questions on the reasons of the presence of such saccharolytic microorganisms in the syntrophic butyrate degradation environment. The low abundance clones, BLS-HAC_39, and BLS-HAC_62 were closely related to the clones where retrieved from district heating plant, and degradation of algal bloom environments, respectively. Their existence in the syntrophic culture appeared to be unrelated due to the clear distinction between the environments.

7.2.2.3 Butyrate MPN series in the presence of unlimited sulfate

The microbial composition together with the abundance was 70.9% for *Desulfovibrio putealis*; 20.8% for *Syntrophomonadaceae*; 4.2% for Clostridium; 2.1% for *Gemmatimonas*; 2.1% for *Anaerolinea thermophila*; and 2.1% for *Petrimonas sulfuriphila* for the BLS sludge.

The most abundant sequence of deltaprotoebacteria, represented by BLS-S_9 clone fell into the SRB group; and it was 94% identical to the sequence of cultivated sulfate reducer, *Desulfovibrio putealis* (Basso *et al.*, 2005).It could be inferred that sulfate reduction was realized by using the hydrogen as an electron donor in this syntrophic environment. There were also three more *Desulfovibrio* species in this population with minor abundance.

The *Syntrophomonadaceae* family was the second most abundant group in this clone library. But the members found in this clone library were completely different from the other incubations. The syntrophomonas was the main butyrate degrader found in the other batches; however, in this case, the clones belonged to the same family although they were different on the basis of the genus level. Common properties of these clones were that they were all isolated from anaerobic habitats. Among these, *Anaerobranca californiensis* was the dominant one and the abundance was 8.3%. Nonetheless, it grows preferentially on proteins and peptides and does not reduce the sulfate (Gorlenko *et al.*, 2004). Therefore, the presence with high abundance in this culture was not clear. In addition, the *Anaerobranca bogoriae* grows also on proteineous substrates and on a variety of mono and polysaccharides; which might reduce the sulfate (Prowe and Antranikian, 2001). Moreover, *Aminiphilus restrictus* and *Aminobacterium colombiense* are known as amino acid fermenting bacteria, beside, the latter one can degrade aminoacids in coculturing with hydrogenotrophic methanogens (Baena *et al.*, 1998). Although there were no signs to suspect about

them being the butyrate degraders considering physiological properties; phylogenetically, they could be thought as being syntrophic butyrate degraders that all fall in the family of Syntrophomonadaceae with more than >89% similarity. As was explained in section 7.1.2, 27.3% of the butyrate was converted by the SRB. This ratio was also very close to the value of 20%, matching to the reducing equivalent of hydrogen for butyrate; which meant that butyrate degradation was realized by methanogenic acetogens.

Table 7.11: The prevalent bacteria in the higher dilutions of the BLS reactor sludge in the presence of unlimited sulfate, according to the highest 16S rRNA gene sequence similarity to the database (GenBank) search result

Clone	Length (bp)	Closest phylotype	% Seq. sim.	Closest culture species	% Seq. sim.	Taxonomic position (class, family, or genus)	# Clones	% Frequency
BLS-S_9	439	clone:BSN44	95	*Desulfovibrio putealis*	94	Deltaproteobacteria, Desulfovibrionaceae	30	62.5
BLS-S_26	355	*Desulfovibrio putealis*	96			Deltaproteobacteria, Desulfovibrionaceae	1	2.1
BLS-S_65	438	clone:MPA4	99	*Desulfovibrio* sp SA-6	98	Deltaproteobacteria, Desulfovibrionaceae	1	2.1
BLS-S_41	404	clone:BSN44	94	*Desulfovibrio* sp A-1	94	Deltaproteobacteria, Desulfovibrionaceae	1	2.1
BLS-S_2	438	clone:sediment-2	98	*Desulfovibrio ferrireducens*	95	Deltaproteobacteria, Desulfovibrionaceae	1	2.1
BLS-S_57	446	bacterium BB48	97	*Aminiphilus restrictus*	92	Firmicutes, Syntrophomonadaceae	1	2.1
BLS-S_81	447	eubacterium RFLP5	98	*Aminobacterium colombiense*	89	Firmicutes, Syntrophomonadaceae	2	4.2
BLS-S_17	435	clone DR546BH1103 001SAD28	93	*Anaerobranca californiensis* strain Paoha-1	89	Firmicutes, Syntrophomonadaceae	4	8.3
BLS-S_33	441	clone DR546BH1103 001SAD28	95	*Anaerobranca bogoriae*	91	Firmicutes, Syntrophomonadaceae	2	4.2
BLS-S_49	435	clone CARB_ER2_6	99	*Petrimonas sulfuriphila* strain BN3	99	Bacteroidetes, Bacteroidales	1	2.1
BLS-S_73	423	clone 44a-B1-15	98	*Anaerolinea thermophila*	90	Chloroflexi, Anaerolinaceae	1	2.1
BLS-S_89	434	bacterium MTCE-T2 1G	98	*Clostridium* sp. (BN II)	93	Firmicutes, Clostridiaceae	2	4.2
BLS-S_18	409	*Gemmatimonas* sp. clone HPIRR2E01	94	*Thiocapsa roseopersicina*	93	Gammaproteobacteria, Chromatiaceae	1	2.1

BLS-S_73 clone which were the less abundant species were affiliated with the green non-sulphur bacteria. The closest cultured relatively with a similarity of 90% was *Anaerolinea thermophila* growing on carbohydrates and amino acids. The other less abundant clone was BLS-S_89 related to the genus of *Clostridium*, known as a

typical saccharolytic microorganism. Most of the species were affiliated with carbohydrate and amino acid degraders; which make it difficult to interpret the situation of the existence of those in a syntrophic environment.

7.2.3 Genetic diversity within the highest dilutions of the BUS reactor sludge

7.2.3.1 Butyrate MPN series in the presence of unlimited sulfate

In this library, clones belonging to the phylum Deltaproteobacteria were the dominant group with the abundance of 71.8% (Table 7.12). As it was explained in the previous chapter, the seed of this MPN series was dominated by the sulfidogens. Most of the clones were retrieved from sulfate reducing groups except some minor abundance of the consortia. *Desulfovibrio* genus was the dominant genus in this group retrieved as 53.2%. The presence in this sulfidogenic environment could be attributed to the oxidation of hydrogen to the hydrogen sulfide; however, also other possible substrates such as lactate, ethanol, pyruvate etc. would be the electron donor using other unknown pathways in the degradation of butyrate. (Basso *et al.*, 2005).

The second most abundant genus was 99% identical to the sequence of cultivated anaerobic fermentative bacterium: *Petrimonas sulfuriphila* (Grabowski *et al.*, 2005b). Mainly carbohydrates and some organic acids are degraded in pure cultures. *Desulforhabdus amnigena* with less similarity as 93% was retrieved in this clone library. Although its abundance was 6.3%, it was the only known species degrading butyrate degrading sulfidogens in this MPN dilution series (Oude Elferink *et al.*, 1995). Another frequently observed phylotype grouped in the genus *Dehalococcoides*, known as strictly anaerobic dehalorespiring microorganisms by reducing the hydrogen. It might be the important player in this consortium in the interspecies electron carrier, but the familiar physiological properties do not support such possibility.

BUS-S_15 and BUS-S_54 clones were related to *Syntrophus* genus with the similarity of 96% and 93% respectively. This group was also known as syntrophic butyrate degraders. Even in the presence of excess sulfate, this group was active with the abundance of 6.3%. The clone BUS-S_85 was similar to the cultivated culture of *Thermacetogenium phaeum* with the value of 92%. Although these species grow optimal at the thermophilic range, they might also be able to grow at 37°C at a very low rate. This bacterium oxidizes acetate in co-culture with a thermophilic

methanogen and also oxidizes acetate with the reduction of sulfate or thiosulfate as the electron acceptor (Hattori et al., 2000). Considering the presence of excess sulfate, it might be suggested that acetate was consumed by these related species under sulfate reducing conditions.

Table 7.12: The prevalent bacteria in the higher dilutions of the BUS reactor sludge in the presence of unlimited sulfate, according to the highest 16S rRNA gene sequence similarity to the database (GenBank) search result

Clone	Length (bp)	Closest phylotype	% Seq. sim.	Closest cultured species	% Seq. sim.	Taxonomic position (class, family, or genus)	# Clones	% Frequency
BUS-S_78	332	*Desulfovibrio putealis*	96			Deltaproteobacteria, Desulfovibrionaceae	3	5.3
BUS-S_69	439	*Desulfovibrio* sp. A-1	94			Deltaproteobacteria, Desulfovibrionaceae	23	47.9
BUS-S_22	440	clone: PLB01	95	*Desulforhabdus amnigena*	93	Deltaproteobacteria, Syntrophobacteraceae	3	6.3
BUS-S_53	435	clone CARB_ER2_6	99	*Petrimonas sulfuriphila* strain BN3	99	Bacteroidetes, Bacteroidales	7	11.6
BUS-S_54	438	clone R4b9	99	*Syntrophus gentianae*	93	Deltaproteobacteria, Syntrophaceae	2	4.2
BUS-S_15	439	*Syntrophus* sp. clone KB-1	96	*Syntrophobacter* sp. strain TsuA1	88	Deltaproteobacteria, Syntrophobacteraceae	1	2.1
BUS-S_29	423	clone 44a-B1-15	98	*Anaerolinea thermophila*	91	Chloroflexi, Anaerolinaceae	1	2.1
BUS-S_7	336	clone BMSB4	96	*Dehalococcoides* sp. BHI80-15	92	Chloroflexi, Dehalococcoidetes	1	2.1
BUS-S_46	423	clone RFLP U19	97	*Dehalococcoides* sp. BHI80-15	91	Chloroflexi, Anaerolinaceae	1	2.1
BUS-S_85	448	clone SHA-110	99	*Thermacetogenium phaeum*	92	Firmicutes, Thermoanaerobacteriaceae	2	4.2
BUS-S_94	446	clone:BHB22	98	*Thermovirga lienii* strain Cas60314	90	Firmicutes, Syntrophomonadaceae	1	2.1

7.2.3.2 Butyrate MPN series in the presence of hydrogen and acetate consumer and unlimited sulfate

Desulforhabdus amnigena was the predator and did not face with other bacteria for the MPN incubation of the BUS sludge, which was counted together *M. Hungatei* and *M. concillii* (Table 7.13). These bacteria originally isolated with the substrate of propionate and grew on organic acids including butyrate in a wide range (Oude Elferink *et al.*, 1995). The presence of acetate and hydrogen consumer as syntrophic partner might cause a shift in the population community through methanogenic conditions. However, such species cannot grow in the absence of sulfate even if the

syntrophic partner is present in the incubations. Also, as it was shown in Table 7.4, the butyrate degraded completely degraded by the sulfidogens. Therefore it can be concluded that *M. Hungatei* and *M. concillii* stimulated the revealing of *Desulforhabdus amnigena* selectively under sulfate reducing conditions.

Table 7.13: The prevalent bacteria in the higher dilutions of the BUS reactor sludge in the presence of *Methanospirillum Hungatei*, *Methanosaeta concilli* and unlimited sulfate, according to the highest 16S rRNA gene sequence similarity to the database (GenBank) search result

Clone	Length (bp)	Closest phylotype	% Seq. sim.	Closest cultured species	% Seq. sim.	Taxonomic position (class, family, or genus)	# Clones	% Frequency
BUS-HAC-S_27	438	isolate WB-25	98	*Desulforhabdus amnigena*	98	Deltaproteobacteria, Syntrophobacteraceae	48	100

7.2.3.3 Butyrate MPN series in the presence of hydrogen consumer and without sulfate

The presence of *M. Hungatei* and absence of sulfate caused the population change to a more methanogenic environment. Therefore, *Syntrophomonas* species became dominant with the abundance of 87.5% (Table 7.14). Although sulfate was absent in the incubations of the MPN series, *Desulfovibrio* species were detected as 8.4%. The closest cultured bacteria to the BUS-HC_90 was *S. stenostrepta* and it fermented a variety of carbohydrates, but did not ferment amino acids or other organic acids (Hespell and Canale-Parola, 1970). Beside *Syntrophomonas* species, the presence of others was not clear and not reasonable to be in this consortium.

7.2.3.4 Butyrate MPN series in the presence of hydrogen and acetate consumer and without sulfate

The presence of *M. Hungatei* and *M. concillii* in the MPN incubations formed a single diverged cluster in the sub-phylum Deltaproteobacteria. BUS-HAC_68 and BUS-HAC_5 BUS-HAC_21 were likely to be sulfate-reducing bacteria closely related to the genus *Desulfoarculus* (Schnell *et al.*, 1991) and *Desulfovibrio* that has been known to grow by sulfate reduction under anaerobic conditions. *Desulfoarculus* can oxidize in a wide variety of fatty acids and organic acids with concomitant reduction of sulfate to sulfide. However, it cannot degrade fatty acids with a syntrophic partner in the absence of sulfate. In spite of its 98% similarity to the

Desulfoarculus baarsii, it will still able to degrade the butyrate in a syntrophic cooperation with methanogens.

Table 7.14: The prevalent bacteria in the higher dilutions of the BUS reactor sludge in the presence of *Methanospirillum Hungatei*, according to the highest 16S rRNA gene sequence similarity to the database (GenBank) search result

Clone	Length (bp)	Closest phylotype	% Seq. sim.	Closest cultured species	% Seq. sim.	Taxonomic position (class, family, or genus)	# Clones	% Frequency
BUS-HC_34	435	clone:BHB04	98	*Syntrophomonas* sp. MGB-C1	98	Firmicutes, Syntrophomonadaceae	41	87.5
BUS-HC_90	438	clone:MST2	99	*Spirochaeta stenostrepta*	91	Spirochaetes, Spirochaetaceae;	2	4.2
BUS-HC_42	439	clone:MPA4	99	*Desulfovibrio* sp. SA-6	98	Deltaproteobacteria, Desulfovibrionaceae;	2	4.2
BUS-HC_74	438	clone:MOL6	98	*Desulfovibrio* sp. SA-6	98	Deltaproteobacteria, Desulfovibrionaceae	2	4.2

Table 7.15: The prevalent bacteria in the higher dilutions of the BUS reactor sludge in the presence of *Methanospirillum Hungatei*, and *Methanosaeta concilli,* according to the highest 16S rRNA gene sequence similarity to the database (GenBank) search result

Clone	Length (bp)	Closest phylotype	% Seq. sim.	Closest cultured species	% Seq. sim.	Taxonomic position (class, family, or genus)	# Clones	% Frequency
BUS-HAC_21	422	clone:MOL6	99	*Desulfovibrio* sp. SA-6	98	Deltaproteobacteria; Desulfovibrionaceae	2	4.2
BUS-HAC_5	434	*Desulfoarculus* sp. BG74	92			Deltaproteobacteria; Desulfoarculaceae	2	4.2
BUS-HAC_68	434	*Desulfoarculus baarsii* DSM 2075	98			Deltaproteobacteria, Desulfoarculus	44	91.7

7.2.4 Comparative Evaluation of the microbial diversity in butyrate degrading communities

The aim of this study was to define the groups of microorganisms which completely occur in the syntrophic butyrate degrading consortia using MPN dilution series. Most of the clones were composed exclusively of uncultivated microorganisms. These results indicate that these sequences might represent the yet-uncultivated microorganisms on the basis of genus level. Also, yet-uncultivated microorganisms reflected an important role in the synytrophic butyrate degradation.

There are potential experimental artefacts starting from extraction of the nucleic acids through the sequencing of the clones. To prevent those potential errors, many precautions were taken in the process of experimental works. For the clone libraries, PCR is the crucial step due to primer selectivity in the amplification and have the variable rRNA gene copy numbers. Thus the relative abundance given for the samples may not represent the correct abundance of the species present in the actual sample.

The general properties of these retrieved clones under different conditions were that they were all isolated from anaerobic environments even in bioreactors, with the exception of minor populations recovered from different habitats. Intriguing result found in the study was the detection of unusual species affiliated with the syntrophic butyrate degraders. These diverse microorganisms different from known (reference organisms) were also detected in other studies (Chauhan and Ogram, 2006; Zellner *et al.*, 1997).

In this study, the tri-culturing effects on the butyrate degrading microbial consortia was also experienced by pre-growing the acetate utilizing methanogen (*Methanosaeta concilli*) in addition to the hydrogen utilizing methanogen (*Methanospirillum Hungatei*) in the MPN dilution series. This effect was noticeably observed for the BUT sludge. In the presence of hydrogen utilizing methanogen, the diversity mostly comprised of *Syntrophomonas* species known as a typical butyrate degrader. However, for the incubation with both hydrogen and acetate utilizing methanogens for the BUT reactor sludge, the diversity increased and *Dechloromonas hortensis* species came up with an abundance of 20%; even though the dominant species was still *Syntrophomonas*. The same manner was also observed for the BLS sludge. In contrast to the BUT and BLS sludge, the addition of these methanogens reduced the diversity through more methanogenic consortia in the absence of sulfate even in the presence of sulfate. Strong influence of the presence or absence of acetate consumers was observed in the dilution series of the BUS sludge without the addition of sulfate. Interestingly, in the absence of sulfate, the consortia contained the sulfate reducer, *Desulfoarculus,* for the BUS sludge. Until now, there was no report on the degradation of butyrate consumer under neither sulfidogenic nor methanogenic conditions.

Many wide range carbohydrate and proteinous fermenters were also detected in these batches. The appearance of the species in this syntrophic environment was also unclear.

8. POPULATION DYNAMICS IN SYNTROPHIC BUTYRATE DEGRADING COMMUNITIES

The involvement of the butyrate degrading microorganisms in the chain of methane production process was an important step in the anaerobic processes. Revealing the identities and functions of these microorganisms are necessary in terms of understanding and improving the performance of the anaerobic bioreactors. By conducting a molecular culture-independent methodology, the polymerase chain reaction combined with denaturing gradient gel electrophoresis (PCR-DGGE); and cloning-sequencing was applied in order to characterize the reactors' sludge fed with butyrate in the presence and absence of sulfate. The operational conditions and feed characteristics of the reactors were ascribed as in Chapter 5.1. The PCR amplified V6-V8 and V2- V3 regions of 16S rRNA for bacteria and archaea, respectively were analyzed by DGGE to visualize the prominent bacteria in the reactors' sludge. DGGE analysis was also used to observe the population dynamics together with diversity along the operational period of the reactors. The DGGE patterns derived from the RNA and DNA were compared and all patterns were represented by the RNA approach instead of DNA to reveal the most active populations' presence in the syntrophic butyrate degradation consortia. In this respect, the 16S rRNA clone libraries were assessed to gain more insight to the identification of the syntrophic players. Besides, the quantitative measurement of the bacteria and archaea in the reactors' sludge were determined using the technique of quantitative real time PCR to expose the relative contribution of the bacteria and archaea to the syntrophic interaction.

8.1 Fingerprinting of the Reactor Communities by the DGGE

The DGGE profiles derived from RNA and DNA amplicons of the reactors' sludge were given in Figure 8.1. The aim was to compare the expression levels of the 16S rRNA genes of the most prominent and active bacteria. The DGGE patterns derived from RNA and DNA did not turn out to be the same as there were quite some

differences in the patterns. Some bands were more prominent in the RNA-derived profile, whereas in the DNA-derived profile these bands were faint, even disappearing, indicating that these bacteria could be very active metabolically. On the contrary, while some of the bands were very prominent in the DNA derived profiles; in the RNA derived profiles these bands disappeared. This manner was most likely due to the sludge history, which was active at previous time or conditions and inactivated in the presence conditions, still protecting the DNA. Therefore RNA derived profiles were taken into account for further analysis in order to bring forth the active population of the reactors' sludge.

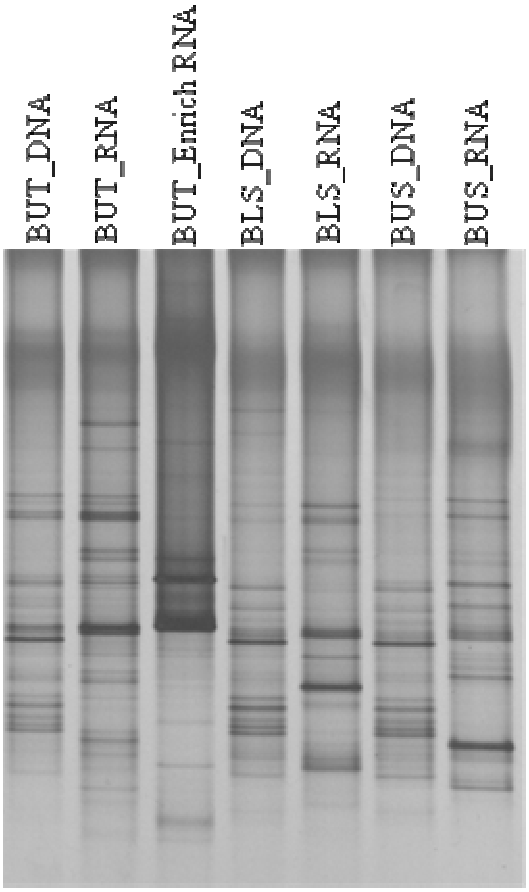


Figure 8.1 : Comparison of the DGGE patterns between the DNA profiles derived from DNA and RNA of the reactors' sludge and the RNA of one of the butyrate enrichment

Population shifts of bacterial communities were assessed by comparing the DGGE banding patterns of 6 samples taken during the operation of all reactors (1-500 days). (Figure 8.2-8.4). In this figures, BUT, BLS and BUS, numbers and M indicated the representative reactor profiles, sampling days, and the marker for the DGGE

analysis, respectively. Direct ribosome isolation was used for RT-PCR and products were separated by high resolution DGGE. Differences in bacterial communities were observed for each reactor separately. A representative pattern of each reactor together with band numbers was shown in the same DGGE gels.

DGGE profiles of the bacterial PCR amplification products obtained from the BUT reactors' sludge at different time intervals were shown in Figure 8.2. Although there was not observed any change in the metabolic properties of the sludge by checking the chemical character of the reactor influents and effluents, the community profile moderately changed after the 271 days of the operation of the reactor. It was also observed that some bands could represent more than one phylotype even from different bacterial divisions due to the comigration of the amplicons in the same profile. For example, OTU41, 19 pairs; OTU68, 78 pairs (Figure 8.2); OTU26, 65 pairs (Figure 8.3); OTU46, 61 pairs and OTU54, 70 pairs (Figure 8.4) were represented by the same bands although they were affiliated with different phylotypes (Table 8.1).

The prominent bands in the DGGE pattern should give the dominant members of the microbial community. So, the dominant isolations, such as OTU77 (Figure 8.2) and OTU26 (Figure 8.3), were represented by the dark bands in the corresponding DGGE patterns. However, other important players could have been underestimated for complex environments. Such a statement could be derived from the PCR biases and and might cause the weakening or absence of the bands in the patterns. Suchlike situation was observed for the OTU25 for the BUT sludge. Since it covered the clone library with 18%, it showed a weak band in the DGGE profile representing the sludge sample taken at the day of 271. These differences between the DGGE profiles and the 16S clone library could be due to the bias of the PCR amplification. Several researches also previously revealed that more than 1% of the total clone library could be visualized at the DGGE patterns (Felske *et al.*, 1998; Muyzer *et al.*, 1993).

Another important observation was the detection of multiple bands corresponding to one species. For example, OTU67 was represented by two differently located bands on the same DGGE profile (Figure 8.2). The same situation was observed for the OTU5 and 26 clones for the BLS sludge and OTU63 for the BUS sludge for the profiles to which subjected clone library.

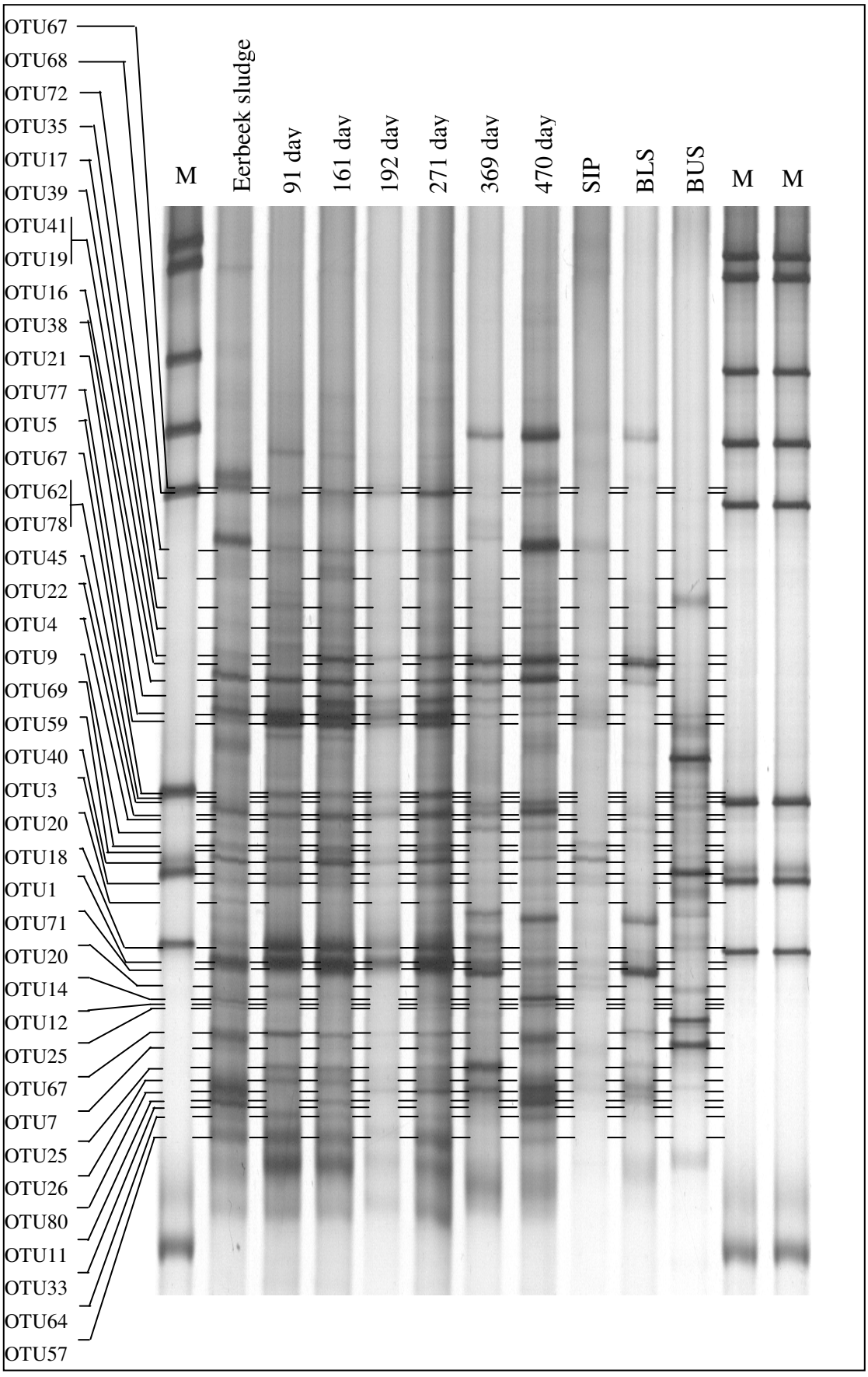


Figure 8.2 : DGGE profiles of the bacterial 16S rRNA of the BUT reactor sludge

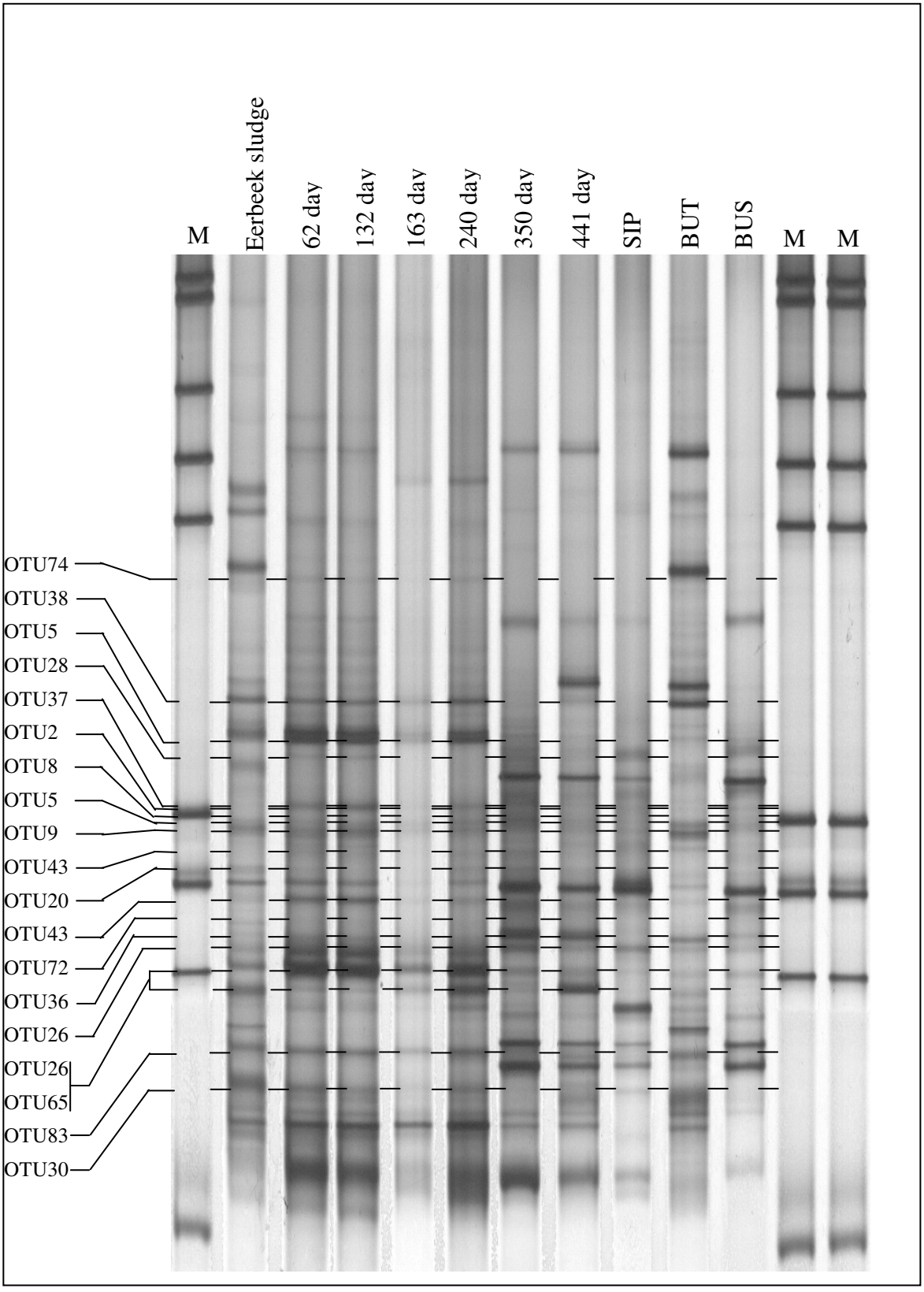


Figure 8.3 : The DGGE profiles of the bacterial 16S rRNA of the BLS reactor

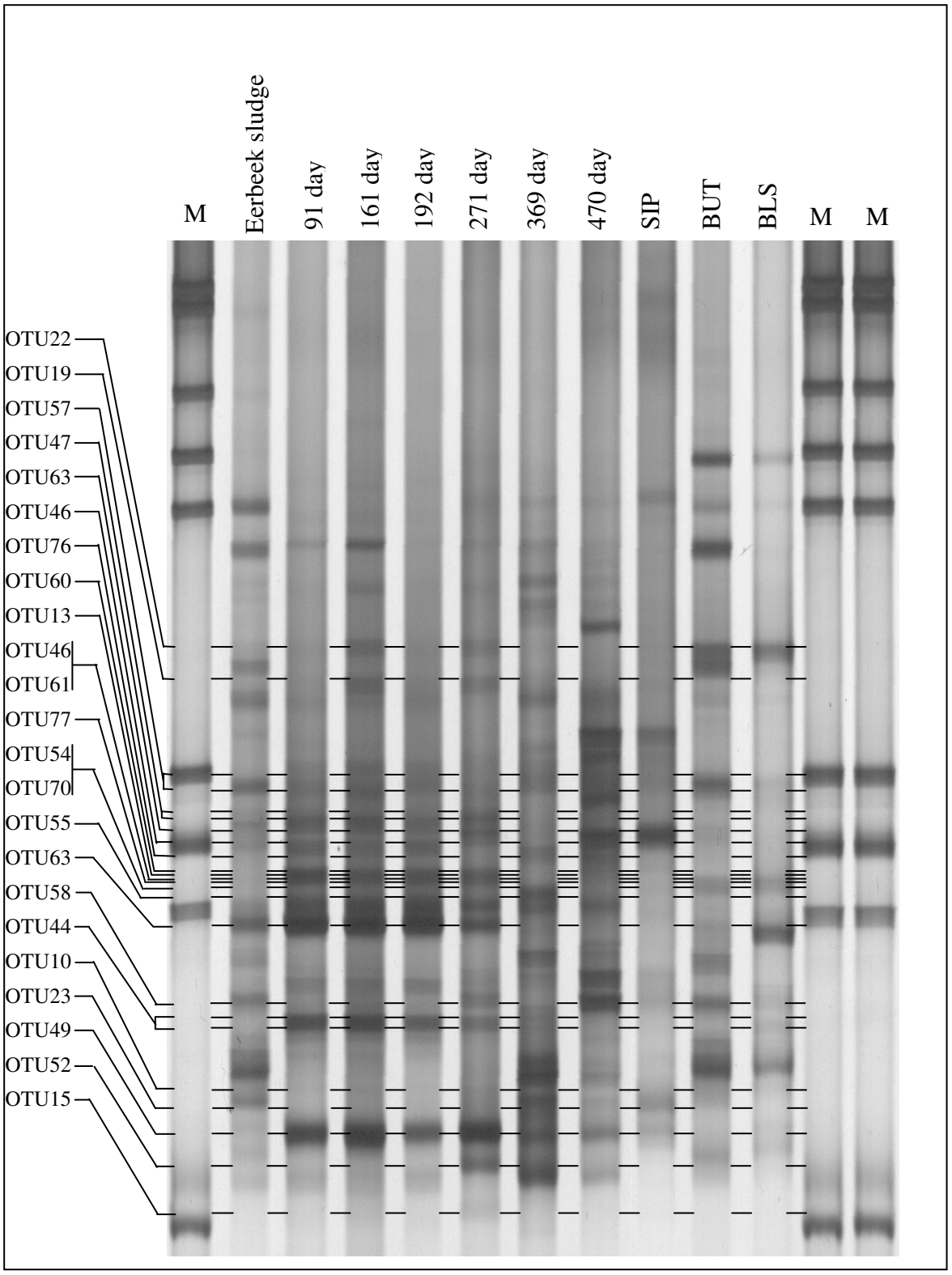


Figure 8.4 : The DGGE profiles of the bacterial 16S rRNA of BUS reactor sludge

8.2 The Identification of the Community Members by 16S rRNA and Phylogenetic Analysis

The biomass samples collected from the BUT, the BLS, and the BUS reactors at the days of 271, 240, and 271, respectively, were subjected to cloning and sequencing analysis. In this respect, 300 bacterial clones were randomly picked for each reactor and these clones were screened by RFLP. 48 out of 900 clones were found as a shorter fragment compared to the 16S rRNA length and were eliminated for further analysis. From the BUT, the BLS and the BUS reactors, 111, 68, and 87 transformants, respectively, were selected for approximately 500 bp (*E.coli* position 1-519) sequencing using RFLP screening. Based on these partial 16S rRNA sequences, 266 clones were compared with each other and, and a total of 63 OTU (Operational Taxonomic Unit) were attained for bacteria. Representative clones for each OTU was sequenced completely. The total OTU distribution for the BUT, the BLS, and the BUS reactors were 37, 18, and 20 respectively. Two of these sequences were detected as chimera and were taken aside for further analysis.

96 16S rRNA archaeal gene clones selected from each reactor were subjected to screening and subsequenty to cloning and sequencing as described above. It was found that all clones were closely related to the lineages of *Euryarchaeota*; including *Methanosarcinales*, *Methanomicrobiales*.

Although, complete 16S rRNA nucleotide sequences showed a small similarity with the cultured bacteria, they showed great similarity with the cultured Archaea species. Table 8.1-8.3 lists the percentage of similarities between transformant sequences of all three reactors and BLAST database after analysis using BLAST (www.ncbi.nlm.nih.gov/blast/Blast.cgi). A homology search showed that the main OTUs could be classified into 3 major phylotypes that belong to *Proteobacteria*, *Firmucutes*, and *Chloroflexi* phylums. In addition, some minor OTUs were in *Chlorobi, Bacteroidetes, Planctomycetes, Thermotogae* phylums (Tables 8.1-8.3).

8.2.1 The clone library of the BUT reactor sludge

The expected dominant group in this syntrophic butyrate degradation consortium was Firmucutes represented by the members of Syntrophomonadacae. However, the most frequently encountered microbial group found in this study was Deltaproteobacteria, if the unclassified bacteria which constitute the 45% of the total clones were omitted

(Figure 8.5). The significant part of the clones could not be categorized on the phylum level. The classified clones were more widely distributed among several phylogenetic groups, including *Chloroflexi*, *Firmicutes*, *Deltaproteobacteria*, *Epsilonproteobacteria*, *Betaproteobacteria*, *Alphaproteobacteria*, *Bacteroidetes*, and *Chlorobi*. These results suggested that various microbial populations were able to degrade butyrate. Similar observations were reported for bioreactors that composed of diverse syntrophs (Zellner *et al.*, 1997).

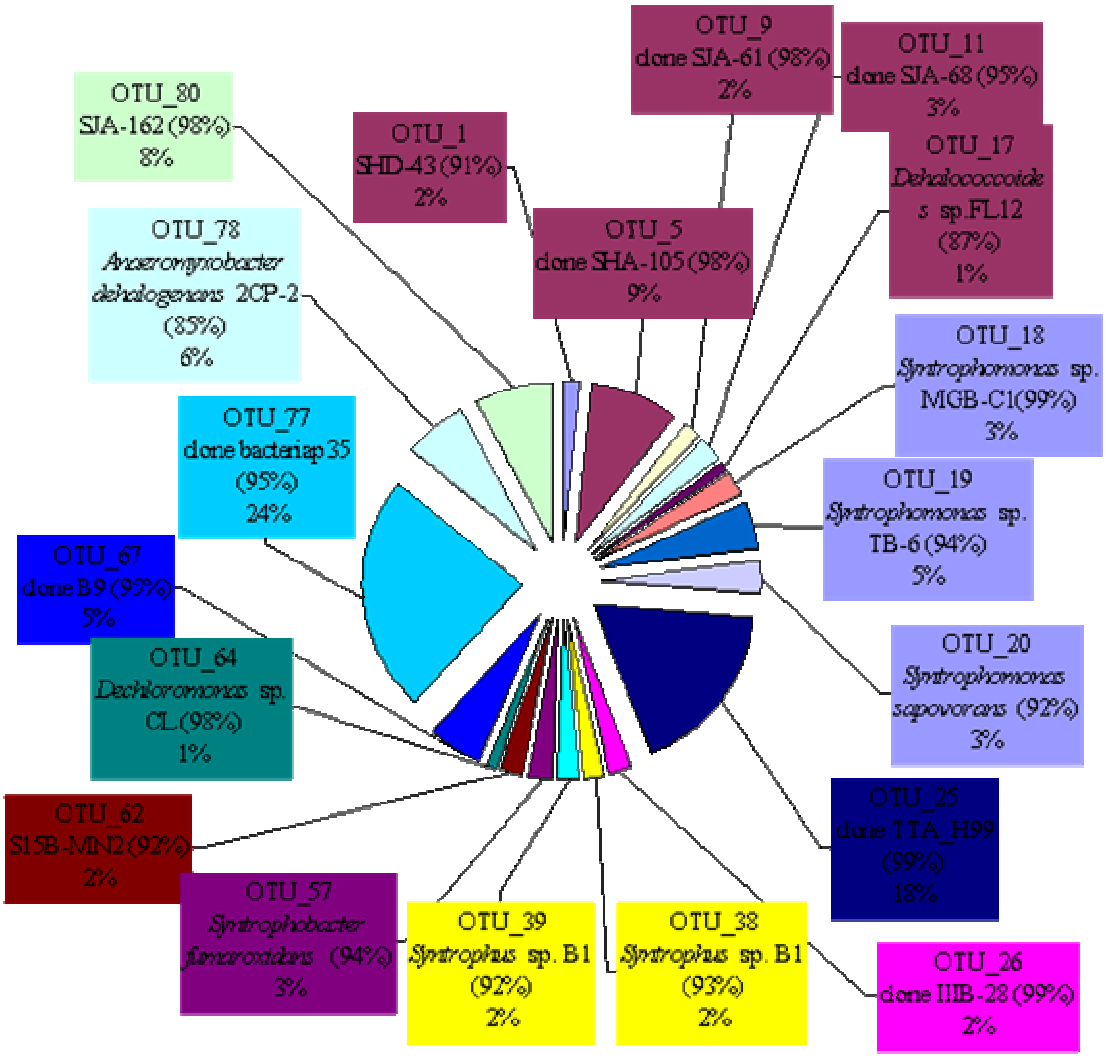


Figure 8.5 : Phylogenetic distribution of the bacterial 16S rRNA clones derived from the BUT reactor

24% of the sequence of the BUT reactor sludge clones (OTU77) was distantly affiliated with the unidentified bacterial sequence retrieved from the DNA of hot pool environmental that has the temperature of (~78°) (Sunna and Bergquist, 2003). The closest culture species of the OTU77 was *Desulfuromonas michiganensis*; however, the length of the sequence taken in to account for the homology search was only ~400 bp (Table 8.1). Therefore, it was hard to comment on the physiological properties of this clone. The abundance of this clone suggested that the organisms represented by this sequence and similar ones may play crucial but unknown roles in the syntrophic interaction of butyrate degradation. The second most abundant clone called OTU25 was closely related to the clone retrieved from thermophilic anaerobic hybrid reactor degrading terephthalate (Chen *et al.*, 2004). The closest culture species of the OTU25 was *Brumimicrobium glaciale*; however, the length of sequence was only ~400 bp as observed for the OTU77. So, these clones were unclassified regarding the homology search.

The following abundant group was green non-sulfur bacteria with a relative abundance of 17%. 10 distinct *Chloroflexi*-related phylotypes were detected. These 10 OTU (OTU1, 3, 4, 5, 7, 9, 11, 14, 16, 17) with the similarity of less than 90%, were included in this phylum. In the database of 16S rRNA genes, the phylum of green nun-sulfur microorganisms seem very diverse; having more than a thousand sequences; however, this phylum has only ten representative pure cultures to understand the characteristic features. The green non-sulfur bacteria, detected in wastewater treatment plants (Lefebvre *et al.*, 2006), soils (Cavaletti *et al.*, 2006), subsurfaces (Morris *et al.*, 2004) and physiological characterization, demonstrated a wide trophic nutrition ranging from anoxic photosynthesis to thermophilic organotrophy; which makes it very difficult to interpret the link between functions and 16S rRNA gene sequences. Therefore, the pure cultures of the green non-sulfur bacteria may not be representative and may lead to misinterpretation of the complex ecosystem.

Table 8.1: Phylogenetic affiliation of bacterial 16S rRNA nucleotide sequences of the BUT reactor

Clones	First Hit	Similarity	Culturable hit	Similarity	Taxonomic position (class, family, or genus)
OTU1	clone SHD-43	1153/1256 (91%)	*Anaerolinea thermophila*	1132/1249 (90%)	Chloroflexi; Anaerolinaceea
OTU3	FilamentousYMTK-2	1395/1423 (98%)	*Anaerolinea thermophila*	1121/1247 (89%)	Chloroflexi; Anaerolinaceea
OTU4	clone SHA-56	1443/1460 (98%)	*Anaerolinea thermophila*	1105/1247 (88%)	Chloroflexi; Anaerolinaceea
OTU5	clone SHA-105	1441/1464 (98%)	*Anaerolinea thermophila*	1115/1251 (89%)	Chloroflexi; Anaerolinaceea
OTU7	clone SHA-105	1261/1375 (91%)	*Anaerolinea thermophila*	1035/1164 (88%)	Chloroflexi; Anaerolinaceea
OTU9	clone SJA-61	1449/1467 (98%)	*Anaerolinea thermophila*	663/750 (88%)	Chloroflexi; Anaerolinaceea
OTU11	clone SJA-68	1183/1245 (95%)	*Caldilinea aerophila*	851/967 (88%)	Chloroflexi; Anaerolinaceea
OTU12	Clone Eb59	1375/1420 (96%)	*Dehalococcoides sp.* BHI80-15	1039/1170 (88%)	Chloroflexi; Anaerolinaceea
OTU14	clone SHA-26	1390/1463 (95%)	*Dehalococcoides sp.* BHI80-15	477/558 (85%)	Chloroflexi; Dehalococcoidetes
OTU16	clone FW21	1198/1235 (97%)	*Dehalococcoides sp.* CBDB1	684/759 (90%)	Chloroflexi; Dehalococcoidetes
OTU17	Clone t0.6.f	1063/1168 (91%)	*Dehalococcoides sp.*FL12	1103/1259 (87%)	Chloroflexi; Dehalococcoidetes
OTU18	*Syntrophomonas sp.* MGB-C1	1421/1430 (99%)			Firmicutes; Clostridia; Syntrophomonadaceae
OTU19	clone A3	1444/1491 (96%)	*Syntrophomonas sp.* TB-6	1235/1302 (94%)	Firmicutes; Clostridia; Syntrophomonadaceae
OTU20	AA64	1122/1222 (91%)	*Syntrophomonas sapovorans*	961/1037 (92%)	Firmicutes; Clostridia; Syntrophomonadaceae
OTU21	clone AC007	1318/1368 (96%)	*Clostridium septicum*	669/743 (90%)	Firmicutes; Clostridia; Clostridiaceae
OTU25	clone TTA_H99	1378/1387 (99%)	*Brumimicrobium glaciale*	349/391 (89%)	Bacteroidetes; Cryomorphaceae
OTU26	clone IIIB-28	1479/1486 (99%)	*Cytophaga sp.*	698/778 (89%)	Bacteroidetes; Flexibacteraceae
OTU35	clone PBg1-037	819/902 (90%)	*Clostridium intestinale*	679/770 (88%)	Firmicutes; Clostridia; Clostridiaceae
OTU38	clone SHA-79	1503/1530 (98%)	*Syntrophus sp.* B1	1166/1250 (93%)	Deltaproteobacteria; Syntrophaceae
OTU39	clone TTA_H101	1207/1227 (98%)	*Syntrophus sp.* B1	1407/1522 (92%)	Deltaproteobacteria; Syntrophaceae
OTU40	clone SHA-42	1506/1527 (98%)	*Syntrophus sp.* B1	1425/1528 (93%)	Deltaproteobacteria; Syntrophaceae
OTU41	clone WCHB1-12	1327/1375 (96%)	*Syntrophus sp.* B1	1331/1401 (95%)	Deltaproteobacteria; Syntrophaceae
OTU45	clone HMMVBeg-45	1425/1521 (93%)	*Desulfofaba gelida*	928/1030 (90%)	Deltaproteobacteria; Desulfobacteraceae
OTU57	clone R1p32	1517/1521 (99%)	*Syntrophobacter fumaroxidans*	885/941 (94%)	Deltaproteobacteria; Syntrophobacteraceae
OTU59	*Desulfovirga adipica*	1165/1242 (93%)			Deltaproteobacteria; Syntrophobacteraceae
OTU62	Clone S15B-MN2	1301/1401 (92%)	*Hyphomicrobium hollandicum*	809/914 (88%)	Alphaproteobacteria; Hyphomicrobiaceae

Table 8.1: (Continued)

OTU64	*Dechloromonas sp.* CL	1471/1500 (98%)			Betaproteobacteria; Rhodocyclaceae
OTU67	clone B9	859/897 (95%)	*Lactobacillus sakei*	406/472 (86%)	Firmicutes; Lactobacillaceae
OTU68	clone vadinBA43	959/1027 (93%)	*Geobacter sp.*	345/373 (92%)	Deltaproteobacteria; Geobacteraceae
OTU69	clone SJA-88	1371/1515 (90%)	*Nitrospina gracilis*	336/369 (91%)	Deltaproteobacteria; Desulfoarculaceae
OTU71	clone SHA-261	885/938 (94%)	*Paenibacillus polymyxa*	334/373 (89%)	Firmicutes; Paenibacillaceae
OTU72	clone 19	954/1035 (92%)			-
OTU73	clone TTA_B21	1353/1383 (97%)	*Chlorobium vibrioforme*	338/379 (89%)	Chlorobi; Chlorobiaceae; Prosthecochloris
OTU74	clone SJA-4	1473/1505 (97%)	*Nitrococcus mobilis*	276/318 (86%)	Gammaproteobacteria; Ectothiorhodospiraceae
OTU75	clone FW32	1232/1303 (94%)	*Desulfuromonas michiganensis*	440/502 (87%)	Deltaproteobacteria; Desulfuromonadaceae
OTU77	clone bacteriap35	1183/1236 (95%)	*Desulfuromonas michiganensis*	333/373 (89%)	Deltaproteobacteria; Desulfuromonadaceae
OTU78	clone PL-11B10	1407/1446 (97%)	*Anaeromyxobacter dehalogenans*	654/759 (86%)	Deltaproteobacteria; Myxococcaceae
OTU80	clone SJA-162	1495/1524 (98%)	*Desulfobacterium cetonicum*	420/469 (89%)	Deltaproteobacteria; Desulfobacteraceae

The *Syntrophomonas* species covered only 11% of the total community, based on the clone abundance. The presence of these species in low frequency for the BUT reactor sludge was remarkable considering that these were the only known butyrate degraders under methanogenic conditions until now. Although OTU18 was very closely related to the *Syntrophomonas sp.* MGB-C1 species, OTU19 and 20 were related to *Syntrophomonas sp.* TB-6 and *Syntrophomonas sapovorans* with the similarity of 94% and 92%, respectively. They were typically known as the typical butyrate and long chain fatty acid degraders (Roy *et al.*, 1986; Zhang *et al.*, 2004; Zou *et al.*, 2003). In the isolation studies of the butyrate degraders, these species were the pure cultures always coming out as a typical organism encountered in the anaerobic environments (Schink and Stams, 2004).

The OTU38-41 clones were associated with *Syntrophus sp.* B1, also known by its ability to use both aliphatic and aromatic acids (Zengler *et al.*, 1999). These species were grown and interacted syntrophically in a co-culture with *M.Hungatei* or *D. desulfiricans*. The presence of *M. concilii* also increased the utilization rate of substrate, demonstrating the syntrophic relationship of this culture (Schocke and Schink, 1997). These four clones were affiliated with the clones retrieved from hydrocarbon and chlorinated solvent contaminated aquifer (OTU41)(Dojka *et al.*,

1998): anaerobic 1,2-dichloropropane-dechlorinating mixed culture (OTU38 and 40) (Schlotelburg *et al.*, 2000) and thermophilic terephtalate treating anaerobic bioreactors (OTU39) (Chen *et al.*, 2004). Common properties of these sources were all dechlorinating or degrading aromatic compounds under anaerobic conditions. Thus far, *Syntrophus* sp. was not known as a syntrophic butyrate degrading bacteria.

OTU21 and 35 were related to the cultivated Clostridium sp. with a similarity less than 90%. Besides, only one type of clone belonged to Bacteroidetes found in this clone library. These group of microorganisms, which are possibly involved in the degradation of carbohydrates or the cell residuals, were also encountered in the seed (Eerbeek) sludge, (Roest *et al.*, 2005).

Since the OTU 45 and OTU59 covered only 0.5% of the total community, OTU80 occupied 8% of the total community as a significant part in terms of sulfate reducing bacteria. The closely related cultured relatives, *Desulfofaba gelida*, *Desulfovirga adipica* and *Desulfobacterium cetonicum* for OTU45, OUT 59 and OTU80 respectively, were present even when no any sulfate was added to the cultures. It could be suggested that these organisms can accomplish both syntrophic growth and sulfate reduction functions. While this property was observed for propionate degrading organisms, it was not observed for the butyrate degradation so far.

The other unusual finding was the existence of *Syntrophobacter wolinii,* which was the closest relative to OTU57. The presence of this syntrophic propionate oxidizing species in the butyrate degrading consortia might be the indication of conversion of butyrate to propionate in the degradation pathway, which was previously not, reported either. A similar situation was observed in the propionate degrading consortia, in which the intervonversion of propionate and were found to be in the range of 1-20% (Lens *et al.*, 1996).

Clone PL-11B10 (6%) isolated in production waters of a low-temperature biodegraded oil reservoir was attributed to OTU78 and was also distantly related to *Anaeromyxobacter dehalogenans*. This cultured species carry the ability to grow using acetate, hydrogen, and formate as an electron donor and 2-chlorophenol (2-CPh) as an electron acceptor (Sanford *et al.*, 2002). However, they do not use butyrate as an electron donor. Therefore their roles in the syntrophic culture were complicated. If they use the abovementioned electron donors, the electron acceptor is needed to reduce them. In the synthetic medium, some compounds such as

Riboflavin, might act as an electron acceptor with a high turnover rate and shuttling mechanism. However, such a possibility has to be investigated in detail.

OTU67 was affiliated with the clone B9 (5%) which was retrieved from the mesophilic oleic acid degrading methanogenic consortium (Pereira *et al.*, 2002). Although it could be suggested that this species might have a direct role in the degradation of butyrate; they turned out to be in the group of unclassified bacteria and distantly related to *Lactobacillus sakei* with a similarity of 86%.

OTU64 was closely related to *Dechloromonas sp.* (1%), which was isolated from anoxic environments with the duty of complete oxidation of acetate in order to reduce (per)chlorate (Coates *et al.*, 1999). These isolates could use butyrate as an electron donor, concomitantly reducing chlorate, by carrying the hydrogen from butyrate to chlorate in order to reduce the butyrate. This reducing equivalent of the butyrate might also be used by biological electron acceptors such as hydrogen utilizing methanogens. This suggestion might explain the existence such a reaction in the syntrophic environment.

OTU62 related to *Hyphomicrobium hollandicum* (2%) are restricted to facultative methylotrophs capable of growth on reduced C1 compounds such as methanol, methylated amines or formate and also to toxic waste compounds that are not metabolized by other methylotrophs (Rainey *et al.*, 1998). They might be involved in the degradation of C1 compounds produced in the oxidation of butyrate. In this regard, the possible competition between methanogens and methylotrophs should also be mentioned.

OTU68, 69, 71, 73 and 74 were the minor group which were closely related to the cultured *Geobacter sp.*, *Nitrospina gracilis*, *Paenibacillus polymyxa*, *Chlorobium vibrioforme*, and *Nitrococcus mobilis* species respectively, with an abundance of 0.5% for each. However, these were scattered phylogenetically over several lineages and the relationship of these clones with the syntrophic butyrate degradation was unclear.

The archaeal composition of the BUT sludge was given in Table 8.2. Archaeal methanogenic community showed low diversity and did not change during all operational periods (data not shown). The major member of the Archaea detected in the BUT sludge was *Methanosaeta concilii* (formerly *Methanothrix soehngenii*)

known as acetate consuming methanogens. These species were commonly found in the anaerobic systems which were operated under steady-state continuous reactors (Grotenhuis, J. T. C. *et al.*, 1991; McMahon *et al.*, 2001; Schmidt and Ahring, 1999).

Table 8.2: Phylogenetic affiliation of archaeal 16S rRNA nucleotide sequences of the BUT reactor

Clones	First Hit	Similarity	Cultured hit	Similarity	Taxonomic position (class, family, or genus)
OTUA13	clone:BHA05	774/790 (97%)	*Methanosaeta concilii*	608/613 (99%)	Euryarchaeota; Methanosaetaceae
OTUA15	*Methanospirillum hungatei*	786/790 (99%)			Euryarchaeota; Methanospirillaceae
OTUA17	*Methanobacterium beijingense* strain 4-1	788/791 (99%)			Euryarchaeota; Methanobacteriaceae

The second dominant archaeal species in the BUT reactor were *Methanospirillium hungatei* (41%) known as the hydrogen consuming methanogens (Figure 8.6). The other member of the archaeal community was *Methanobacterium beijingense* with an abundance of 5%. This specie was also known as a hydrogen utilizing methanogen. Considering the anaerobic food chain completed with the methane production, these acetate and hydrogen utilizing methanogens were complementary to each other to accomplish the mission of producing methane. From these, rRNA sequences of archaeal clones showed homologies more than 97%.

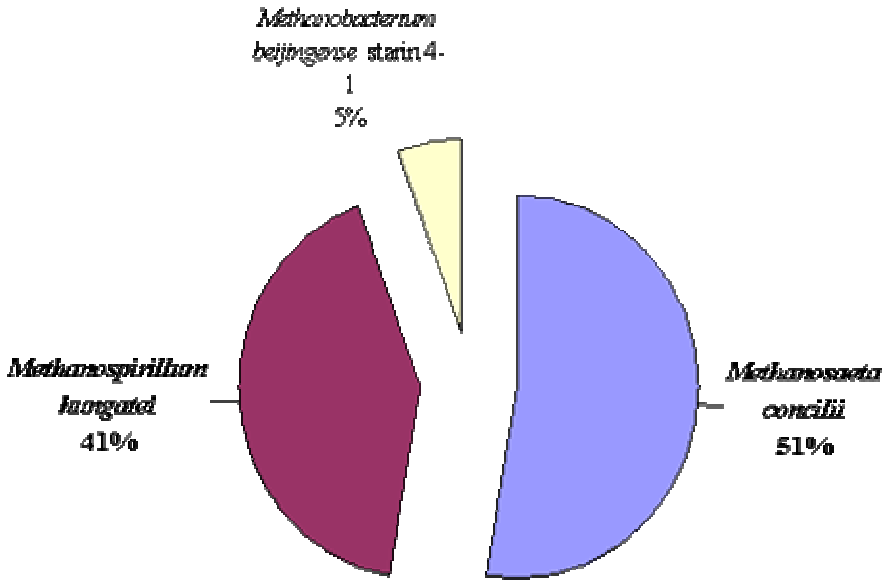


Figure 8.6 : Phylogenetic distribution of 16S rRNA archaeal clones derived from the BUT reactor

8.2.2 Clone library of BLS reactor sludge

Phylogenetic distribution of 16S rRNA clones derived from the BLS reactor was given in Figure 8.7. Phylogenetic analysis of the BLS clones revealed a community clearly dominated by diverse populations of bacteriodes (61% of all clones; Table 8.3);but *Azovibrio restrictus* (21%), *Chloroflexi* (8%), *Nitrococcus mobilis* (3%), Deltaproteobacteria (2%); *Trichlorobacter thiogenes* and *Syntrophobacter fumaroxidans*, *Syntrophomonas sapovorans* (1%), *planctomycete* (1%), *Ruminobacillus* (1%) and unclassified bacteria OTU72 (1%), *Fervidobacterium islandicum* (0.5%) were detected as well (Figure 8.7).

Sequences related to microorganisms, not known as syntrophs, were detected to be 98% of the clone library and the remaining 2% consisted of the *Syntrophomonas sapovorans* and *Syntrophobacter fumaroxidans*. Since this known syntrophic population was 18% in the BUT sludge, it dropped to 2% if the sludge was fed with limited amount sulfate called the BLS reactor.

In this library, 60% of all clones were related closely to clone IIIB-28 retrieved from polluted river sediment reducing dechlorination of polychlorinated dibenzo-*p*-dioxins/ dibenzofurans (Yoshida *et al.*, 2005). Moreover, in this sediment, Bacteriodes were the second dominant group with an abundance of 16%. Although the closest relative was *Cytophaga* sp. according to the full length of 16S rRNA, 778 bp was taken into account for the homology search and a similarity of 89% was found. In such a case, to determine the role of this clone in this consortium was uncertain. This homology result reflects the classification of the dilemma encountered for these clone libraries of the reactor sludges. OTU28, OTU31, OTU30, and OTU32 were also related to *Cytophaga* sp and the closest relatives were revealed from the source of anaerobic digester bacterial community (Delbes *et al.*, 2000), rumen and consortia removing predominantly singly flanked chlorine substituents from chlorobenzenes, for the last two clones, respectively. The Bacteriodes related genus was 99% identical to the sequence of cultivated anaerobic fermentative bacterium, *Petrimonas sulfuriphila* (Grabowski *et al.*, 2005b). Mainly carbohydrates and some organic acids are degraded in pure cultures by such isolate.

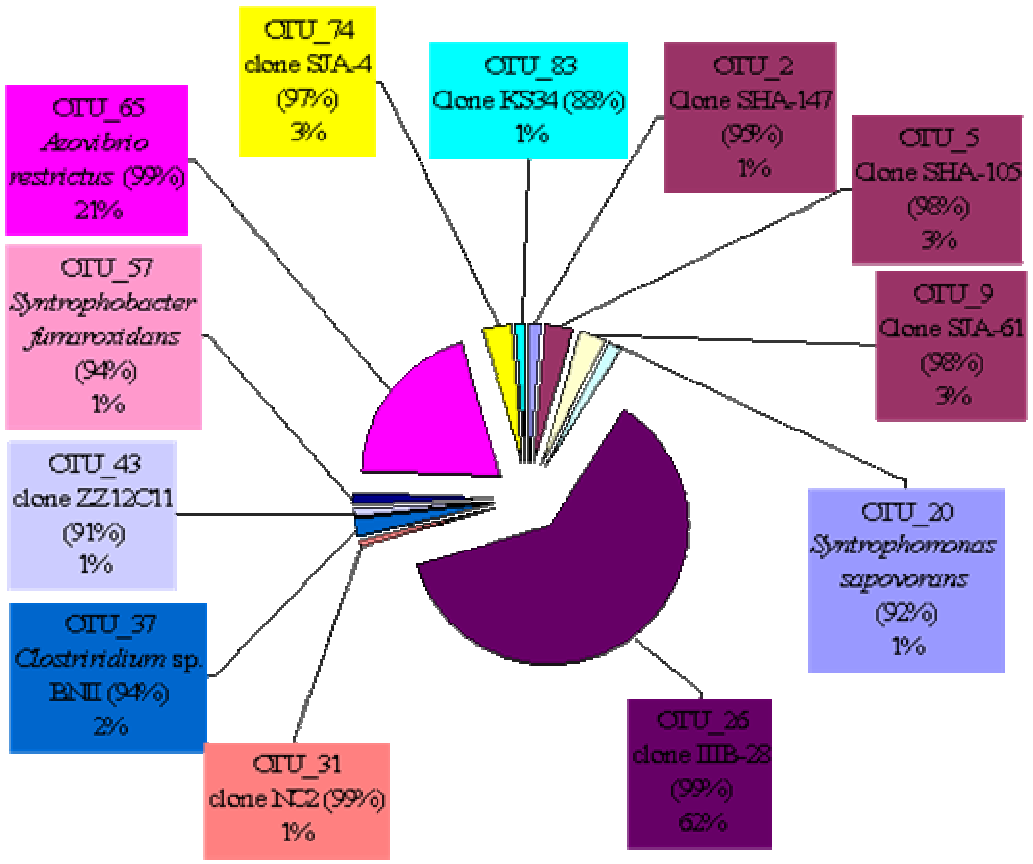


Figure 8.7 : Phylogenetic distribution of 16S rRNA clones derived from the BLS reactor

The second most abundant isolate 99% identical to *Azovibrio restrictus,* was diazotrophic bacteria and it was isolated from the roots of the Kallaer grass (Reinhold-Hurek and Hurek, 2000). It was chemoorganoheterotrophic and capable of growth in atmospheric nitrogen; therefore it could be suggested that butyrate might use the reducing equivalent to reduce the nitrogen to ammonium. The other abundant clone was the OTU74 and was closely related to the isolate SJA-4 from trochlorobenzene-transforming microbial consortium (Von Wintzingerode *et al.*, 1999). This clone was distantly related to the nitrite oxidizer *Nitrococcus mobilis* (Teske *et al.*, 1994). Due to such distant relation, it was difficult to be interrelated each other in terms of physiological properties.

Table 8.3: Phylogenetic affiliation of bacterial 16S rRNA nucleotide sequences of the BLS reactor

Clones	First Hit	Similarity	Culturable hit	Similarity	Taxonomic position (class, family, or genus)
OTU2	clone SHA-147	1398/1459 (95%)	*Anaerolinea thermophila*	1257/1375 (91%)	Chloroflexi; Anaerolinaceea
OTU5	clone SHA-105	1441/1464 (98%)	*Anaerolinea thermophila*	1115/1251 (89%)	Chloroflexi; Anaerolinaceea
OTU8	clone SJA-61	1452/1464 (99%)	*Anaerolinea thermophila*	726/828 (87%)	Chloroflexi; Anaerolinaceea
OTU9	clone SJA-61	1449/1467 (98%)	*Anaerolinea thermophila*	663/750 (88%)	Chloroflexi; Anaerolinaceea
OTU20	AA64	1122/1222 (91%)	*Syntrophomonas sapovorans*	961/1037 (92%)	Firmicutes; Clostridia; Syntrophomonadaceae
OTU26	clone IIIB-28	1479/1486 (99%)	*Cytophaga* sp.	698/778 (89%)	Bacteroidetes; Flexibacteraceae
OTU28	eubacterium AA26	1393/1394 (99%)	Cytophaga sp.	923/1021 (90%)	Bacteroidetes; Flexibacteraceae
OTU30	clone IA-12	1307/1408 (92%)	*Cytophaga* sp.	598/701 (85%)	Bacteroidetes; Flexibacteraceae
OTU31	Bacteroides sp. SA-11	1434/1445 (99%)	*Ruminobacillus xylanolyticum*	969/1022 (94%)	Bacteria; Ruminobacillus
OTU32	clone IIB-29	1477/1493 (98%)	*Petrimonas sulfuriphila*	1398/1409 (99%)	Bacteroidetes; Petrimonas
OTU36	clone R5p5	1471/1490 (98%)	*Fervidobacterium islandicum*	338/381 (88%)	Thermotogae; Thermotogaceae
OTU37	clone PBg1-03 PBg1-0377	1376/1424 (96%)	*Clostridium sp.* (BN II)	1258/1328 (94%)	Firmicutes; Clostridia; Clostridiaceae
OTU43	*Trichlorobacter thiogenes*	1331/1382 (96%)			Deltaproteobacteria; Geobacteraceae
OTU57	clone R1p32	1517/1521 (99%)	*Syntrophobacter fumaroxidans*	885/941 (94%)	Deltaproteobacteria; Syntrophobacteraceae
OTU65	*Azovibrio restrictus*	1451/1464 (99%)			Betaproteobacteria; Rhodocyclaceae
OTU72	clone 19	954/1035 (92%)			-
OTU74	clone SJA-4	1473/1505 (97%)	*Nitrococcus mobilis*	276/318 (86%)	Gammaproteobacteria; Ectothiorhodospiraceae
OTU83	clone KS34	414/470 (88%)	*planctomycete* str. 337	341/386 (88%)	Planctomycetes; Planctomycetaceae

As observed in the BUT reactor sludge, green non-sulfur bacteria were detected in the BLS reactor sludge to be 8% of the total clones. Only a small fraction of the clone library corresponded to *Trichlorobacter thiogenes* that grow by oxidizing acetate with concomitant reduction of thrichloroacetic acid (De Wever *et al.*, 2000). It could be speculated that the corresponding organisms were directly or indirectly involved in the butyrate degradation

Phylogenetic affiliation of archaeal clones, as shown in Table 8.4, fell into two main metabolic groups: hydrogen and acetate utilizing methanogens. The majority of the 16S rRNA archaeal gene clones (62% of the total clones) belonged to the *M.*

Hungatei. The remaining of the clones was acetate utilizing methanogen: *M. concilii* (Figure 8.8).

Table 8.4: Phylogenetic affiliation of archaeal 16S rRNA nucleotide sequences of the BLS reactor

Clones	First Hit	Similarity	Culturable hit	Similarity	Taxonomic position (class, family, or genus)
OTUA25	archaeon clone 5LOC5	788/793 (99%)	*Methanosaeta concilii*	788/793 (99%)	Euryarchaeota; Methanosaetaceae
OTUA22	*Methanospirillum hungatei*	785/786 (99%)			Euryarchaeota; Methanospirillaceae

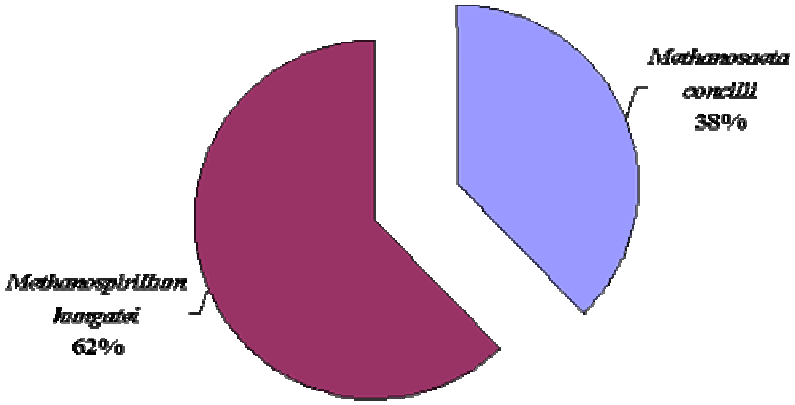


Figure 8.8 : Phylogenetic distribution of 16S rRNA archaeal clones derived from the BUT reactor

8.2.3 Clone library of the BUS Reactor sludge

Cloning of the BUS reactor sludge clearly showed that Deltaproteobacteria was the main dominant phylum in this clone library. The clones belonging to this subphylum phylogenetically were scattered over several genus and were specialized on the guild of sulfate reducers. Beside this, clones belonging to Firmicutes, Deferribacteres and Chlorobi were detected a minor amount which accounts as 5% of the total library (Figure 8.9).

OTU49, 13 and 76 occupied 55% of the total clone library. This dominant group was related to the *Desulfobotulus sapovorans* with a similarity of less than 94% (Table 8.5). This sulfate reducing bacteria can oxidize a variety of substrates completely to CO_2. It has the ability to use fatty acids from butyrate through palmitate, with the inability to use H_2 or ethanol (Rabus *et al.*, 2000). Therefore, it could be suggested

that this dominant group of sulfate reducing bacteria can mainly accomplish the butyrate degradation by the complete oxidation to CO_2.

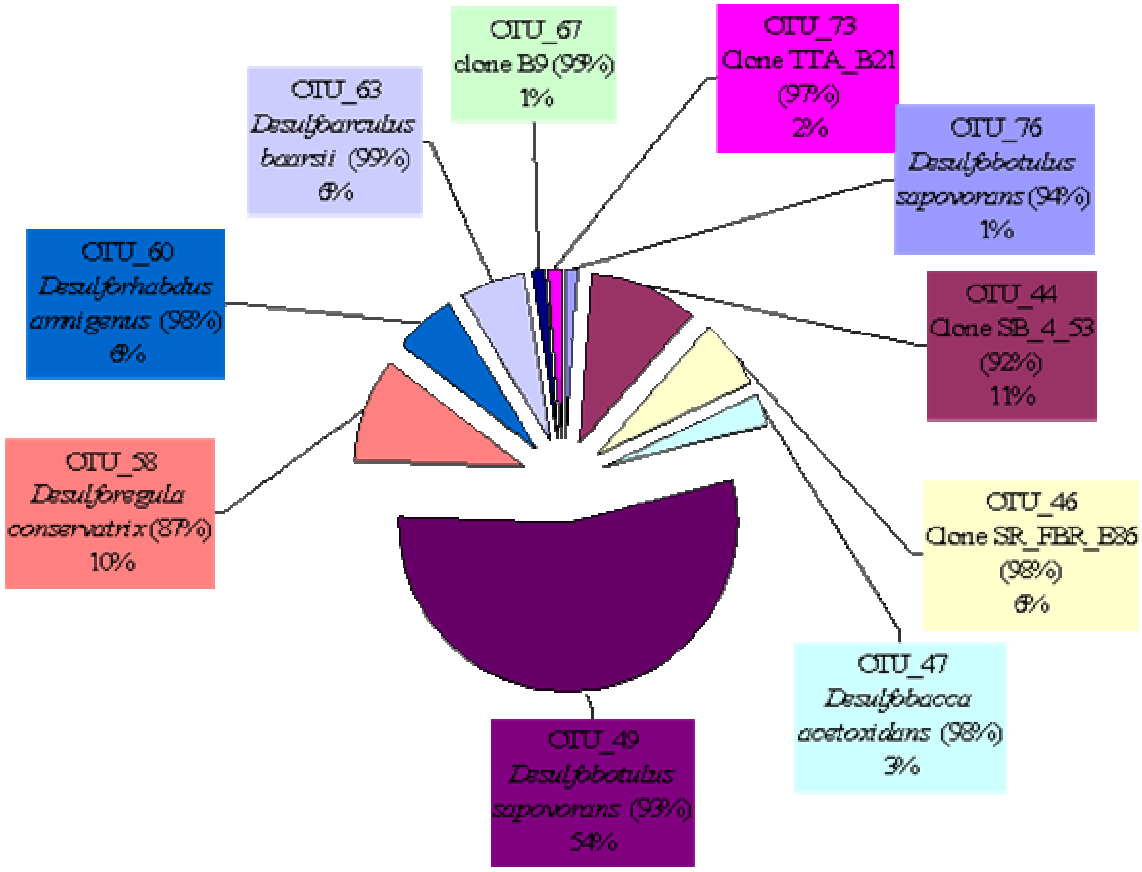


Figure 8.9 : Phylogenetic distribution of 16S rRNA bacterial clones derived from the BUS reactor

The OTU44, coming from Antarctic sediment, marine bay, sulfate reducing bacterium-related phylotype clone SB4_53, was the second most numerically abundant phylotype in the BUS bacterial library (11%).The closest cultured relative, *Desulfatibacillum aliphaticivorans*, was isolated from hydrocarbon polluted marine sediment (Cravo-Laureau *et al.*, 2004). Moreover, it has the ability to degrade butyrate completely to CO_2 if the sulfate is provided to the culture.

The third most frequently detected group (OTU58) of phylotypes (10% of all rRNA gene clones) was most closely related to the fatty acid oxidizing syntrophic consortia and distantly related to the cultured *Desulforegula conservatrix* (87%). While this culture degrades butyrate incompletely in the presence of sulfate, no growth was observed in the absence of sulfate (Rees and Patel, 2001).

Table 8.5: Phylogenetic affiliation of bacterial 16S rRNA nucleotide sequences of the BUS reactor

Clones	First Hit	Similarity	Culturable hit	Similarity	Taxonomic position (class, family, or genus)
OTU10	clone R3p4	728/774 (94%)	*Desulforegula conservatrix*	856/977 (87%)	Deltaproteobacteria; Desulfobacteraceae
OTU13	*Desulfobotulus sapovorans*	1014/1075 (94%)			Deltaproteobacteria; Desulfobacteraceae
OTU15	clone Kazan-3B-15/BC19-3B-15	961/1044 (92%)	*Caldilinea aerophila*	341/388 (87%)	Chloroflexi; Anaerolinaceea
OTU19	clone A3	1444/1491 (96%)	*Syntrophomonas sp.* TB-6	1235/1302 (94%)	Firmicutes; Clostridia; Syntrophomonadaceae
OTU44	clone SB4_53	1165/1253 (92%)	*Desulfatibacillum aliphaticivorans*	902/961 (93%)	Deltaproteobacteria; Desulfobacteraceae
OTU46	clone SR_FBR_E86	951/967 (98%)	*Desulfomonile limimaris*	945/1017 (92%)	Deltaproteobacteria; Syntrophaceae
OTU47	clone SR_FBR_L13	1429/1442 (99%)	*Desulfobacca acetoxidans*	1474/1501 (98%)	Deltaproteobacteria; Syntrophaceae
OTU49	*Desulfobotulus sapovorans*	1305/1393 (93%)			Deltaproteobacteria; Desulfobacteraceae
OTU52	clone SR_FBR_E11	847/873 (97%)	*Syntrophobacter wolinii*	426/466 (91%)	Deltaproteobacteria; Syntrophobacteraceae
OTU54	*Desulfovibrio putealis*	1327/1411 (94%)			Deltaproteobacteria; Desulfovibrionaceae
OTU55	clone E14	1459/1474 (98%)	*Desulfovibrio sulfodismutans*	1435/1481 (96%)	Deltaproteobacteria; Desulfovibrionaceae
OTU57	clone R1p32	1517/1521 (99%)	*Syntrophobacter fumaroxidans*	885/941 (94%)	Deltaproteobacteria; Syntrophobacteraceae
OTU58	clone R3p4	739/777 (95%)	*Desulforegula conservatrix*	848/964 (87%)	Deltaproteobacteria; Desulfobacteraceae
OTU60	*Desulforhabdus amnigenus*	1496/1517 (98%)			Deltaproteobacteria; Syntrophobacteraceae
OTU61	*Desulfococcus multivorans*	855/940 (90%)			Deltaproteobacteria; Desulfobacteraceae
OTU63	*Desulfoarculus baarsii*	1340/1350 (99%)			Deltaproteobacteria; Desulfovibrionaceae
OTU67	clone B9	859/897 (95%)	*Lactobacillus sakei*	406/472 (86%)	Firmicutes; Lactobacillaceae
OTU70	*Geobacter metallireducens*	910/1023 (88%)			Deltaproteobacteria; Geobacteraceae
OTU73	clone TTA_B21	1353/1383 (97%)	*Chlorobium vibrioforme*	338/379 (89%)	Chlorobi; Chlorobiaceae
OTU76	*Desulfobotulus sapovorans*	1050/1143 (91%)			Deltaproteobacteria; Desulfobacteraceae
OTU77	clone bacteriap35	1183/1236 (95%)	*Desulfuromonas michiganensis*	333/373 (89%)	Deltaproteobacteria; Desulfuromonadaceae

OTU60 was affiliated with *Desulforhabdus amnigenus* with a similarity of 98%. This pure culture was isolated from the Eerbeek sludge, which was the same sludge used for reactor sludges as a seed It was known that these species can degrade butyrate (Oude Elferink *et al.*, 1995). Additionally, these species were also dominant in the MPN Series of the BUS sludge incubated in the presence of hydrogen and acetate consumer, and excess sulfate as given in Chapter 7.

Desulfoarculus baarsii was the representitative clone of OTU63 with a 99% identical sequence similarity. *Desulfoarculus* can oxidize a wide variety of fatty acids and organic acids with the concomitant reduction of sulfate to sulfide. It was also found that these species are capable of degrading butyrate in syntrophic cooperation with methanogens from MPN Series of the BUS sludge, which was incubated in the presence of hydrogen and acetate consumer, and in the absence of sulfate as described in Chapter 7.

OTU46 and OTU47 were both related to the clones retrieved from lactate and ethanol utilizing, sulfate-reducing fluidized-bed reactor treating acidic metal-containing wastewater (Kaksonen *et al.*, 2004). The closest cultured relatives were *Desulfomonile limimaris* (Sun *et al.*, 2001) and *Desulfobacca acetoxidans* (Oude Elferink *et al.*, 1999) for OTU46 and OTU47, respectively. Although *Desulfomonile limimaris* was capable of using butyrate and H_2 as electron acceptors for growth, *Desulfobacca acetoxidans* only restricted acetate in the presence of sulfate. When their presence in the BUS reactor sludge with the abundance of 9% was considered, their contribution to the butyrate and acetate degradation might be remarked to be significant.

OTU54 and OTU55 were represented by *Desulfovibrio* species accounting a total of 1.8% of the consortium. The presence in this sulfidogenic environment could be attributed to the oxidation of hydrogen to hydrogen sulfide; however, also other possible substrates such as lactate, ethanol, pyruvate etc. would be the electron donor using other unknown pathways in the degradation of butyrate. (Basso *et al.*, 2005).

In contrast, OTU73 with the abundance of 2% and the *Chlorobi*-related clone which represented 2% of the total DNA-derived clone library, were detected with a similarity of 97%. This TTA_B21 clone was retrieved from thermophilic anaerobic hybrid reactor degrading terephthalate.

Although OTU67 the representative clone B9 was the minor species by 1%, as mentioned in the BUT sludge, it was originated from the mesophilic oleic acid degrading methanogenic consortium (Pereira *et al.*, 2002). This unclassified (potentially novel) species might be designated as a syntrophic group under the bacteria domain.

OTU52 and 57 were related to *Syntrophobacter wolinii* and *Syntrophobacter* fumaroxidans, respectively, which made up 1.5% of the BUS community. Two different species of syntrophic propionate oxidizers were present at the same time. It was known that propionate degraded syntrophically by *Syntrophobacter wolinii* (Boone and Bryant, 1980) also degraded under sulfate reducing conditions, but *Syntrophobacter fumaroxidans* (Harmsen *et al.*, 1998) were not capable of realizing dissimilatory sulfate reduction.

The *Syntrophomonas* species and green non sulfur bacteria were also detected in this clone library, but their richness was less than 1%. Their role in the sulfate reducing community was unclear and arguable.

Although the archaeal specific primers picked one bacterial species out of 96 clones, only one type of archaeal clones dominated the entire sulfate reducing community (Table 8.6). The archaeal clones were closely related to *M. Concilii*. If the bacterial species were kept out for the determination of abundance, it could be concluded that the acetate utilizing methanogen, *M. Concilii*, covered up all clone library (Figure 8.10).

Table 8.6: Phylogenetic affiliation of archaeal 16S rRNA nucleotide sequences of the BUS reactor

Clones	First Hit	Similarity	Culturable hit	Similarity	Taxonomic position (class, family, or genus)
OTUA5	archaeon clone 5LOC9	788/790 (99%)	*Methanosaeta concilii*	785/790 (99%)	Archaea; Euryarchaeota; Methanosaetaceae
OTUA9	archaeon clone AR1R2_2	431/431 (100%)	*Methanosaeta concilii*	427/430 (99%)	Archaea; Euryarchaeota; Methanosaetaceae
OTUA11	clone SR_FBR_E2	320/338 (94%)	*Spirochaeta caldaria*	311/338 (92%)	Bacteria; Spirochaetes; Spirochaetaceae

8.3 Real Time PCR Quantification of the Seed and the Reactor Sludges

Table 8.7 presents the quantitative results for the Eerbeek sludge (granular anaerobic paper mill wastewater treatment sludge used as a seed for reactor sludges) taken at the years of between 1999-2004, three different granular anaerobic paper mill wastewater treatment sludges (Nieuwerschans, Hoogezand, and Sappemeer), and the three reactor sludges (the BUT, the BLS, and the BUS) characterized in this study. Except the Hoogezand sludge, the abundance of the bacteria/archaea ratio changed between 0.16 and 0.29. While these results were consistent with the other work

conducted on the anaerobic granular sludge, some of them contradicted with them as observed for the Hoogezand sludge.

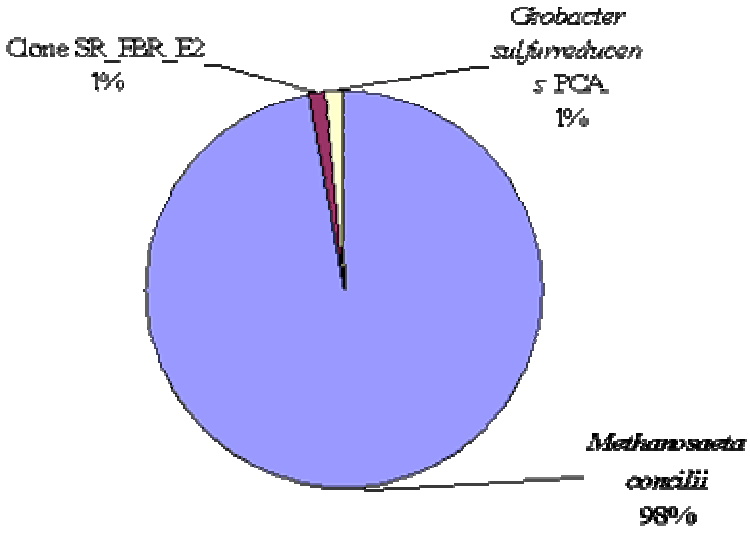


Figure 8.10 : Phylogenetic distribution of 16S rRNA archaeal clones derived from the BUT reactor

These findings were in accordance with other studies of anaerobic bioreactors, where archaea population was always higher than bacteria (Gonzalez-Gil *et al.*, 2001; Oude Elferink *et al.*, 1998; Raskin *et al.*, 1994a). On the contrary, some sludges showed more bacteria than archaea (Griffin *et al.*, 1998; Zheng and Raskin, 2000). In this study, it was found that archaea was the dominant group in the syntrophic butyrate degradation consortia for all of the three different operational conditions.

Table 8.7: Domain level abundance of full-scale anaerobic paper mill wastewater and reactors (operated in this study) sludges by real time PCR

Anaerobic sludges taken from	% abundance of		Bacteria/Archaea
	Bacteria	Archaea	
Eerbeek 1999	22.6	77.4	0.29
Eerbeek 2001	19.5	80.5	0.24
Eerbeek 2002	13.7	86.3	0.16
Eerbeek 2004	18.1	81.9	0.22
Nieuwerschans[a]	24.3	75.6	0.32
Hoogezand[a]	66.6	33.4	1.99
Sappemeer[a]	16.9	83.1	0.20
BUT Reactor	14.2	85.8	0.17
BLS Reactor	22.9	77.1	0.30
BUS Reactor	22.0	78.0	0.28

[a]Samples from other anaerobic paper mill wastewater treatment systems

8.4 Discussion

The fingerprints of DNA and cDNA from RNA led us to generate the clone library from RNA instead of DNA due to significant differences between the DNA and RNA derived profiles. The clone library composed of 16S, which is the conserved region for all bacteria and archaea. Although RNA derived molecular analysis has some disadvantages, it is still a powerful tool in identifying the active population in complex ecosystems. One of these disadvantages was the occurrence of short fragments during synthesis of complementary DNA due to unwanted termination of reverse transcriptase activity (Weller *et al.*, 1991). In addition, when dealing with ribosomes, it must be considered that rRNA contains posttranscriptionally modified ribonucleotides which may also increase the diversity. This was especially important when interpreting the diversity of the clone libraries. The abundance of a 16S rRNA clone library does not necessarily correspond to the same amount in which the 16S rRNA of the clones presented in a natural community. However, the qualitative complexity of a microbial community can be assessed by using different molecular techniques such as quantitative real time PCR, as used in this study.

The low sequence similarity of reactor microorganisms to the cultured bacteria demonstrated a lot of unknown information about microbial population of syntrophic butyrate degradation. Understanding which organisms are responsible in the syntrophic degradation of butyrate can shed some light on anaerobic treatment of wastewaters.

The diversity of the microbial community of the BUT reactor was higher than that of the BLS and the BUS reactor in terms of OTU numbers. Since the BUT reactor was fed with butyrate in the absence of sulfate, methanogenic syntrophy gained importance and microorganisms inhabited in the granules were diverse. This diversity of the consortium and the functional surplus within the butyrate degrading syntrophs might increase the reactor stability and affect the sustainability of these syntrohic consortia in the anaerobic bioreactor. Conversely, in the presence of sulfate, diversity decreased depending on the COD/sulfate ratio. While in the presence of limited amount of sulfate, corresponding to COD/sulfate:10, 18 OTU was observed, in the presence of excess amount of sulfate, corresponding to COD/sulfate:0.5, 20 OTU was observed. Although the OTUs observed in both the BUT and the BLS sludges were more common, that made up the 20% of the BUT

clones, the BUS clones showed a more separated path than those of the BUT and the BLS sludges, where the BUS clone library shared its clones as 10% of the BUT clones.

Molecular analysis of the microbial diversity within the UASB reactors fed with butyrate showed absolute importance for specific unidentified microorganisms. The dominant isolates, such as OTU26 and OTU77, were related to the unclassified bacteria. The major part of other isolates was classified into different groups and they were not known as the potential to degrade butyrate with syntrophic interaction for the BUT and the BLS sludges. Nevertheless, we cannot rule out that these microorganisms are not capable of syntrophic butyrate oxidation. However, for the BUS sludge, 95% of the clones belonged to the Deltaproteobacteria; which gave a more reasonable explanation due to having closely related cultured species. It was noteworthy that clones retrieved in this study that belong to *Chloroflexi*, *Firmicutes*, *Deltaproteobacteria*, *Epsilonproteobacteria*, *Betaproteobacteria*, *Alphaproteobacteria*, *Bacteroidetes*, and *Chlorobi* were closely related to the clones isolated from bioreactors, contaminated environments or consortia dechlorinating chlorinated pollutants.

The numerical analysis of the reactor sludges demonstrated that methanogens that belong to the domain of archaea had greater abundance than the ones that belong to the domain of Bacteria. As expected in the BUT reactor sludge, archaeal population was as high as 86% due to complete metabolic dependence of the acetogens to methanogens; however, the presence of sulfate in the bioreactors decreased this value to 77 and 80% for the BLS and the BUS reactors' sludge respectively, due to the competition between methanogens and sulfidogens.

In conclusion, the culture independent identification methods showed that the diversity besides the known *Syntrophomonas* sp. covered the consortia of syntrophic butyrate degradation. Since these methods cannot comprehensively elucidate the information on the identity linking to the function in complex environments alone, additional advanced molecular tools might be necessary to attain the decisive functional features of the microorganisms for each individual. In addition to this, further studies on the isolation of the syntrophic butyrate degradation with different techniques would support what we found here to understand the physiological

properties of these unclassified microorganisms dominating the syntrophic butyrate degrading consortia due to unknown growth requirements of the bacteria.

9. LINKING MICROBIAL COMMUNITY STRUCTURE OF THE SYNTROPHIC BUTYRATE DEGRADERS WITH THE FUNCTION USING THE STABLE ISOTOPE PROBING TECHNIQUE

9.1 Introduction

The principal motivation of this chapter was identify the key microorganisms, which are responsible for the important butyrate bioconversions that occur in complex environments. The most challenging part to resolve the mystery of the microbial box is obtaining disturbance free field samples and of conducting laboratory experiments as they occur in real field conditions. This is particularly important for the types of data and their interpretation to what extent of ecologically relevant information could be obtained. Therefore, the RNA based Stable Isotope Probing (SIP) technique was applied in this study. This technique is a cultivation-independent technique, which is used to determine the active indigenous bacterial community structure using growth substrates (Radajewski *et al.*, 2000). This technique enables us to combine the degradation and assimilation of isotopically (^{13}C, ^{2}H, ^{15}N) labelled growth substrate and then enables us to determine the separation of labeled nucleic acids from unlabelled nucleic acids by isopycnic density-gradient centrifugation. Labeled and unlabeled fractions of the gradient are fingerprinted by DGGE or T-RFLP and characterized by the sequence analysis of SSU rRNA genes (Ginige *et al.*, 2004; Lu *et al.*, 2005; Lueders *et al.*, 2004b; Mahmood *et al.*, 2005; Manefield *et al.*, 2002; Rangel-Castro *et al.*, 2005) and/or functional genes (Gallagher *et al.*, 2005; Hutchens *et al.*, 2004; Lin *et al.*, 2004; Morris *et al.*, 2002; Radajewski *et al.*, 2002). The incorporation of an isotope ideally to the genomes of microorganisms allows us to identify active microorganisms; which gives an important advantage over other techniques of linking identity and function. This technique is very sensitive for the separation of heavy (^{13}C) and light (^{12}C) part of the nucleic acids. Separation is done by ultracentrifuge and is based on the mass difference between ^{13}C and ^{12}C. In this separation, the heavy gradient fractions must have included only the ^{13}C enriched nucleic acids fraction in terms of the reliance of the technique. At lower centrifuge

speed (140 000 g) and longer centrifugation time (69 h), the optimal way of detaching the heavy and light fractions was found (Hutchens *et al.*, 2004). Although it is not easy to check the precision of such a separation, there is a proposal for the background inspection of the gradients, such as ^{13}C labeled pure cultures DNA and/or RNA as an internal reference control (Singleton *et al.*, 2005). There are also other factors influencing the efficiency of the separation for light and heavy fractions, such as the G + C content of the organism's nucleic acids. Organisms, that have high G + C content have a relative high mass amount, which can be close to the mass of ^{13}C labeled nucleic acids, and they will move towards the heavy part of the gradient (Lueders *et al.*, 2004a). This possibility should be taken into account when interpreting composition of the heavy fraction of the gradient.

Both DNA and RNA can be used in nucleic acid based SIP. Selection of the nucleic acid type depends on what kind of microbial consortia we are dealing with. The incorporation rate of ^{13}C labeled substrate in the cells and the DNA synthesis, depends on the growth rate of organisms. This can be a problem especially in slow growing consortia and low cell yielding conditions. Incorporation rate of ^{13}C labeled substrate in RNA is much faster than DNA, due to a higher turnover and the lack of need for the replication of the cells. RNA reflects the activity, by having more copy numbers and being independent from replication, which provides a significant advantage on low cell yielding conditions (Manefield *et al.*, 2002). Using RNA-based SIP, it is possible to reduce the length of incubations and the amount of the labeled substrate (Radajewski *et al.*, 2002). Consequently, RNA-based SIP seems to be a very practical way for linking the identity and function of complex habitats.

In this study, the incubations fed with the ^{13}C labeled butyrate were carried out on the wild anaerobic granular sludge obtained from Eerbeek paper mill wastewater treatment plant. The incubations were also conducted under two different conditions, which were in the absence and presence of sulfate (3 mM). The original Eerbeek sludge incubated only with the ^{13}C labeled butyrate, which is called the Wild BUT UASB sludge, and incubated with ^{13}C labeled butyrate plus 3 mM sulfate, which is called the Wild BUTS UASB sludge. The enrichments were incubated until 40 days for both series and samples taken for subsequent SIP analysis at the day of 5, 20, 30 and 40 for each series.

9.2 SSU rRNA Distribution in the Centrifugation Gradients

rRNA samples extracted after the days of 5, 20, 30 and 40 days of incubation were subjected to the isopycnic density-gradient centrifugation using cesium trifluoroacetate (CsTFA) to separate the labeled nucleic acids from unlabelled nucleic acids. After the centrifugation of the CsTFA was fractionated into ten different tubes, at each fraction the rRNA content was determined together with the buoyant density. These results were plotted on the graph and light and heavy fractions were analysed for further cloning and sequencing experiments. CsTFA density gradient centrifugation of rRNA extracted from the incubation series of Wild BUT were represented in Figure 9.1. The time series of the incubation showed the distinct difference between heavier and lighter fraction during the 40 days of incubation. Generally, the light fraction that does not contain the labelled nucleic acids represented a buoyant density of less than 1.79 g/ml in CsTFA (Lueders *et al.*, 2004a). After 40 days of incubation, the heavier fraction buoyant density reached 1.82 g/ml in CsTFA, which was higher than the critical value of the attained value for the heavier fraction. Therefore, this fraction, which was represented by the number of four in the DGGE gels (Figure 9.2) was taken as a heavier fraction and was subjected to the following phylogenetic characterization of the consortia. Taking these criteria, the 7th fraction was considered as the lighter fraction.

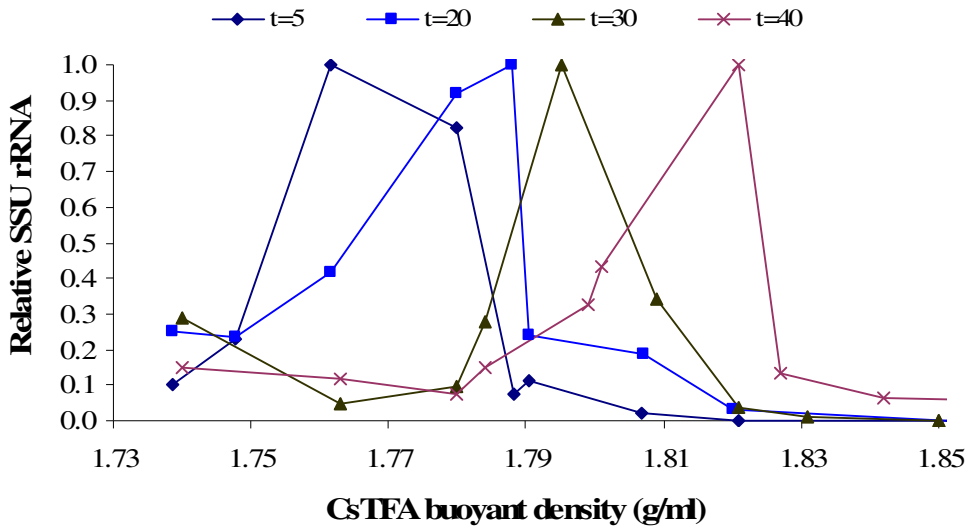


Figure 9.1 : CsTFA density gradient centrifugation of rRNA extracted from the wild UASB reactor sludge incubated with the [^{13}C] butyrate

Each fraction of the centrifugation gradients were analyzed by the DGGE fingerprinting to observe the population change. All gradients of the 5, 20, 30 and 40 days profiles were given in Appendix C (Figure App.C.1 and App. C.2) for the Wild BUT sludge; whereas here, only the incubation of 40 days gradient was given in Figure 9.2. In this figure, lanes 1-10 corresponded to the gradient from heavy to light fractions. As observed from the DGGE profile, there was a slight change between the fractions. However, differences could be observed by detecting the weakness and thickness of the bands between the patterns. Since some bands were prominent in fraction 4, the thicknesses of these bands were weakened at the fraction of 7.

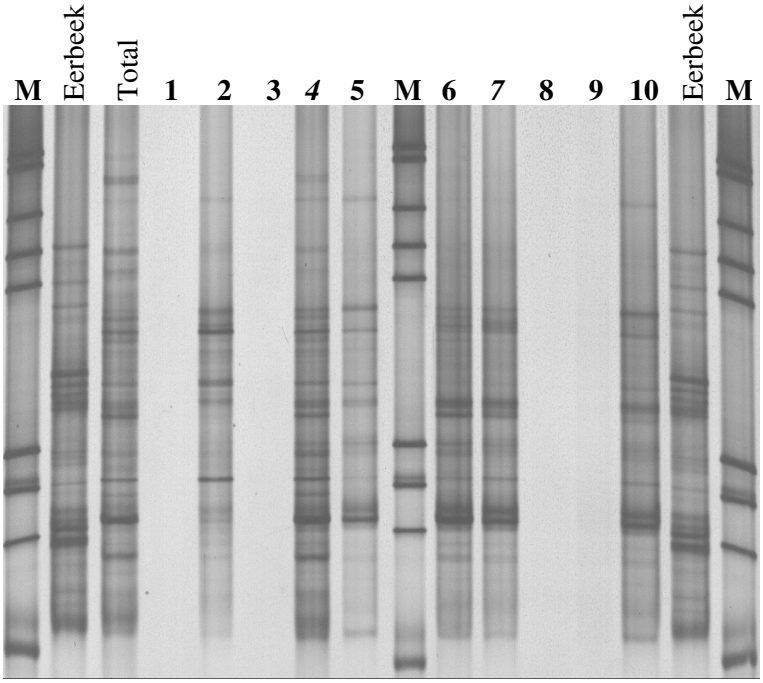


Figure 9.2 : Bacterial DGGE profiles of amplified 16S rRNA from the wild UASB reactor sludge incubated with the [^{13}C] butyrate

CsTFA density gradient centrifugation of the rRNA extracted from the incubation series of the Wild BUTS was represented in Figure 9.3. A similar trend was also observed for the Wild BUTS sludge in terms of the clear distinction between the heavier and lighter fraction of the gradient. Therefore, the 5th and 8th gradients were taken as heavy and light fractions, respectively, for further analysis.

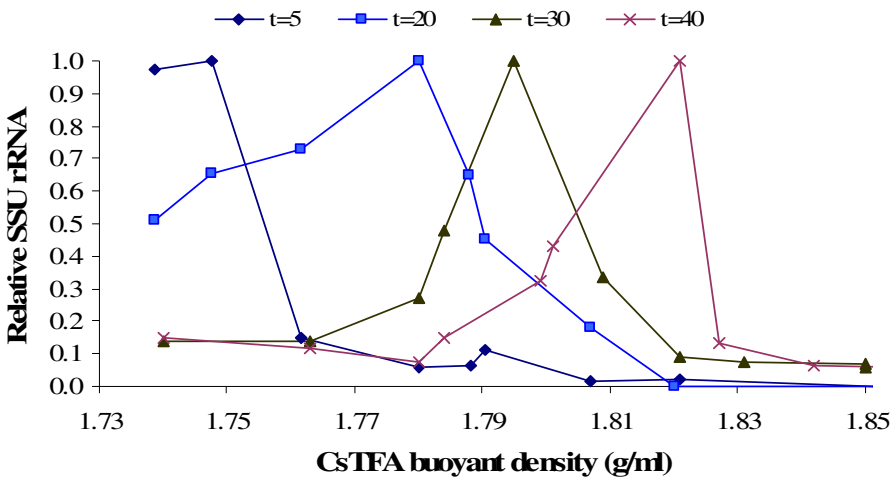


Figure 9.3 : CsTFA density gradient centrifugation of the rRNA extracted from the wild UASB reactor sludge incubated with the [^{13}C] butyrate plus 3mM sulfate

All gradient fractions that belong to the incubation of 5, 20, 30 and 40 days of the profiles for the Wild BUTS sludge were given in Appendix C (Figure App.C.3 and App. C.4). The incubation of the 40 days gradient was given in Figure 9.4. In this figure, lanes 1-10 corresponded to the gradient from heavy to light fractions. As observed in the DGGE profile of the Wild BUT sludge, there was a slight change between the fractions; however the bands were not sharp. Therefore, observing the changes between the lanes was a difficult task for this DGGE gel.

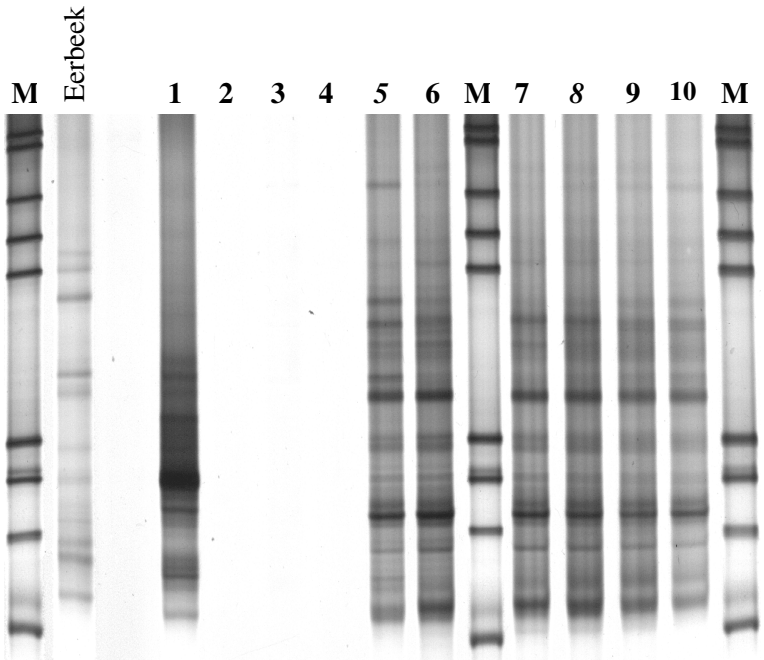


Figure 9.4 : Bacterial DGGE profiles of amplified 16S rRNA from the wild UASB reactor sludge incubated with the [^{13}C] butyrate plus 3mM sulfate

9.3 DGGE Profiles and Sequence Analysis of Density Resolved Nucleic Acids for the Wild BUT Sludge

The bacterial DGGE-PCR amplicons of both heavy and light fractions were separately used for the construction of a clone library and for the sequence analysis. Clones together with corresponding fractions were subsequently subjected to DGGE analysis, and the bands related to the clone libraries were listed in the Table 9.1 and 9.2 for heavy and light fractions of the gradient, respectively. Furthermore, the identification of DGGE bands retrieved from the representative heavy and light fraction of the isopycnic centrifugation of the RNA isolated from the Wild BUT sludge was given in Figure 9.5 and 9.6, respectively.

A total of 238 partial 16S rRNA bacterial gene clones (from 118 heavy and 120 light fractions) were analyzed for the Wild BUT enrichments. The RFLP analysis of the 16S rRNA genes grouped in 43 distinct types, and the analyses of 16S rRNA gene sequences with NCBI database showed that the clones were affiliated with thirteen major bacterial lineages, which are: δ, α, γ- *Proteobacteria, Firmicutes, Chloroflexi, Bacteroidetes, Verrucomicrobia, Spirochaeta, Planctomycetes, Chlorobi, Gemmatimononadetes, Nitrospirae,* and *Acidobacteria.* The heavy fraction of the density resolved nucleic acids revealed that the clones were classified in 11 phyla of the domain *Bacteria*: δ, γ-*Proteobacteria* (44.9, and 4.2%, respectively), *Chloroflexi* (22.9%), *Firmicutes* (10.2%), *Verrucomicrobia* (6%), *Bacteroidetes* (5.9%), *Spirochaeta* (2.5%), *Planctomyces* (0.9%), *Nitrospirae* (0.9%), *Gemmatimononadetes* (0.9%), *Chlorobi* (0.9%). However, in the light fraction of the density resolved nucleic acids, the clones were grouped in 9 phyla: δ, γ, α-*Proteobacteria* (50, 0.8%, and 5%, respectively), *Chloroflexi* (23.3%), *Firmicutes* (8.3%), *Verrucomicrobia* (1.7%), *Bacteroidetes* (8.3%), *Acidobacteria* (1.7%), *Chlorobi* (0.9%); showing similar percentages with the heavy fraction.

According to the clone libraries, *Syntrophobacter* sp., *Syntrophus* sp. and the clones belonging to the phylum of *Chloroflexi* (Green non sulfur bacteria) were dominated in the heavy part, which makes up 44% of the total clone library. Besides these, a substantial number of the sequences in the heavy library (56% of the 16S rRNA bacterial gene clones) were widely scattered into different phyla of the domain bacteria and they were not known as syntrophs. However, our research also showed

that clones that belong to the *Syntrophomonas* related group were not retrieved in the heavy part of the clone library.

When the heavy fraction is taken as actively butyrate degrading population, *Syntrophus* sp. would be attained to the direct butyrate degraders, in this mixed consortium. The sequences of the clones that are related to *Syntrophus acidotrophicus* show growth on certain saturated fatty acids with 4–18 carbons, some unsaturated fatty acids, the methyl esters of butyrate and hexanoate; and benzoate coculturing with *Desulfovibrio* strain G11. *S. gentianae* (Wallrabenstein *et al.*, 1995a) and *S. buswellii* (Mountfort *et al.*, 1984) were the other *Syntrophus* species that were detected in the clone library. These species were different from the *S. acidotrophicus*, with respect to their inability of utilizing butyrate; however, they are able to grow and metabolize crotonate. In the β-oxidizing pathway of butyrate, the free Gibb's energy of the conversion reaction from butyrate to the crotonyl-CoA step is more positive than the downstream of the degradation pathway, which indicates that the reaction from butyrate to crotonate is the difficult when it is meant as the energetically unfavourable step (Schink and Stams, 2004). Depending on the energetic level of the consortia, the syntrophs may also conserve energy from a reduction of crotonate. Therefore, it might be suggested that *S. gentianae* and *S. buswellii* related species can be involved in the step of crotonate fermentation, producing acetate and butyrate.

The presence of the *Syntrophus* species in this clone library demonstrated that the syntrophic butyrate degraders were not limited to bacteria of the family *Syntrophomonadaceae*, but they can be also in the sub-phylum of δ,-*Proteobacteria*.

Syntrophobacter sp. were also detected in this clone library. *S. wolinii* (Boone and Bryant, 1980) and *S. sulfatereducens* (Chen *et al.*, 2005) are only functioned with the syntrophic propionate degradation. These organisms were involved in the butyrate degradation directly or indirectly. It could be suggested that they might utilize the intermediates of the butyrate degradation pathway, when the highest abundant group in the clone library was considered. The function of the *Syntrophobacter* sp. could be dependent on presence of *Desulfobulbus propionicus*, which might have the ability of producing propionate from acetate. While 6 clones related to these species were detected in the heavy fraction, 22 clones were present in the light fraction;

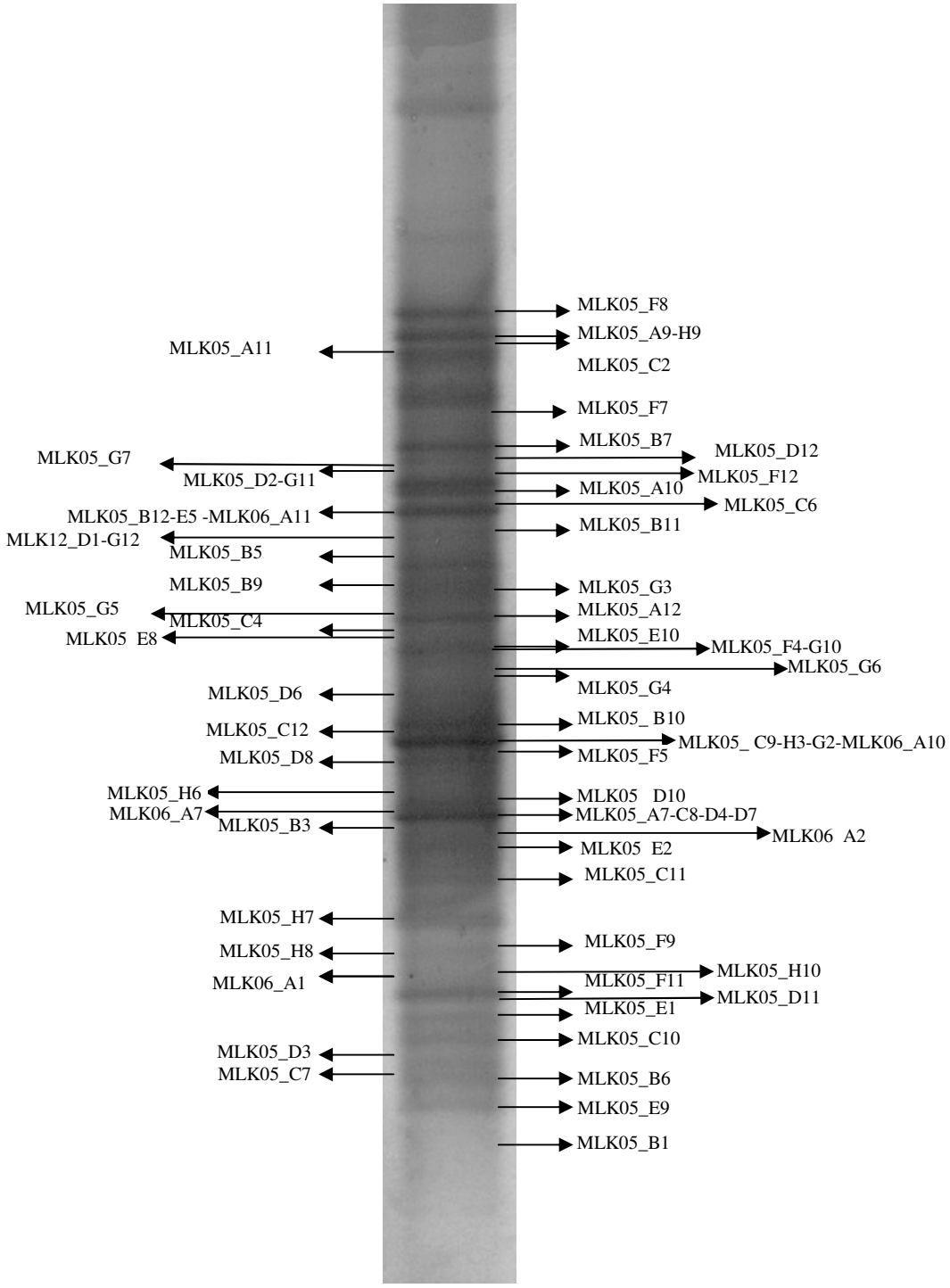


Figure 9.5 : Identification of DGGE bands retrieved from representative heavy fraction of the isopycnic centrifugation of the RNA of wild UASB reactor sludge incubated with the [^{13}C] butyrate

demonstrating that it was not the species that was the one that plays a important role in the butyrate degradation.

Although other sulfate reducing bacteria covered the minor group, they were active in the butyrate degrading community. In the active population, *Desulfacinum* sp. (2.5%), *Desulforegula conservatrix* (2.5%), *Desulfonatronum lacustre* (0.9%), and *Desulfonema magnum* (0.9%) were present and were known as butyrate degrading microorganisms in the presence of sulfate as an electron acceptor. These active microorganisms can probably perform functions of both the syntrophic growth and sulfate reduction. Therefore, these sulfate reducing bacteria may have butyrate oxidizing capabilities in syntrophy with methanogens. However, *Desulfovibrio* sp. (15.2%), *Desulfomicrobium apsheronum* (1.7%), and *Deslfobacterium cetonicum* (0.9%) were present in the clone library of light fraction, which shows the inactive population of the butyrate degrading community.

In contrast to other divisions, *Acidobacterium*, *Verrucomicrobia*, GNS bacteria were not represented enough by the cultivated organisms (Hugenholtz *et al.*, 1998). The abundant *Chloroflexi* (23% in the heavy fraction) related microorganisms were highlighting the impact of *Chloroflexi* group on the syntrophic butyrate oxidizing community. The physiology and in situ function of these microorganisms are unknown because they are poorly represented by the cultivated organisms. This phylum includes more than a thousand clones that were retrieved from different habitats such as geothermal, soil, wastewater, soil etc (Hugenholtz *et al.*, 1998). Since the sequence similarity of the *Chloroflexi* related clones to the cultured species of *Anaerolinea thermophila* was very low (≤93%), functional assignment of this group is hypothetical.

Prosthecobacter related clones (6%) that belong to the phylum of *Verrucomicrobia*, similar to the members of the *Acidobacterium* division, are widespread in the soils (Hugenholtz *et al.*, 1998). All of these isolates preferentially use sugars as growth substrates. Similarly, Bacteriodetes species have the same features and the same abundance.

Table 9.1: Phylogenetic assignment of the bacterial wild BUT clones isolated from the heavy fraction of the SIP experiment

Clone	Closest phylotype	% Seq. sim.	Closest cultured species	% Seq. sim.	Taxonomic position (class, family, or genus)	# Clones	% Freq.
MLK05_B06	R1p32 (E27, B16)	99	*Syntrophobacter wolinii*	93	Deltaproteobacteria; Syntrophobacteraceae	4	3.4
MLK05_E09	R1p32 (E27, B16)	98	*Syntrophobacter wolinii*	92	Deltaproteobacteria; Syntrophobacteraceae	1	0.5
MLK05_F09	R1p32 (B16)	97	*Syntrophobacter wolinii*	93	Deltaproteobacteria; Syntrophobacteraceae	1	0.5
MLK06_A10	R1p32	94	*Syntrophobacter wolinii*	94	Deltaproteobacteria; Syntrophobacteraceae	1	0.5
MLK05_D11	R1p32 (B16, E27)	97	*Syntrophobacter wolinii*	94	Deltaproteobacteria; Syntrophobacteraceae	5	1.4
MLK05_C10	R1p32 (B16, E27)	99	*Syntrophobacter wolinii*	95	Deltaproteobacteria; Syntrophobacteraceae	1	0.5
MLK05_E01	R1p32 (B16, E27)	99	*Syntrophobacter wolinii*	94	Deltaproteobacteria; Syntrophobacteraceae	1	0.5
MLK05_A12	RFLP25	89	*Syntrophobacter sulfatereducens*	88	Deltaproteobacteria; Syntrophobacteraceae	1	0.5
MLK05_F11	*Syntrophobacter sulfatereducens*	94			Deltaproteobacteria; Syntrophobacteraceae	1	0.5
MLK05_B09	clone C13	98	*Desulfobulbus propionicus*	95	Deltaproteobacteria; Desulfobulbaceae	1	0.5
MLK05_C08	clone C13	98	*Desulfobulbus propionicus*	95	Deltaproteobacteria; Desulfobulbaceae	1	0.5
MLK05_G08	clone C13	98	*Desulfobulbus propionicus*	96	Deltaproteobacteria; Desulfobulbaceae	1	0.5
MLK05_E05	clone C13	99	*Desulfobulbus propionicus*	96	Deltaproteobacteria; Desulfobulbaceae	1	0.5
MLK05_A11	LTCE-T2A 4A	90	Desulfobulbus propionicus	89	Deltaproteobacteria; Desulfobulbaceae	1	0.5
MLK05_A09	LTCE-T2A 4A	91	Desulfobulbus propionicus	91	Deltaproteobacteria; Desulfobulbaceae	1	0.5
MLK06_A11	LTCE-T2A 4A	96	*Desulfobulbus elongatus*	94	Deltaproteobacteria; Desulfobulbaceae	1	0.5
MLK05_C11	BSA2B-21	94	*Desulforegula conservatrix*	83	Deltaproteobacteria; Desulfobacteraceae	3	0.5
MLK05_G10	MERTZ_2CM_324	94	*Desulfonema magnum*	91	Deltaproteobacteria; Desulfobacteraceae	1	0.5
MLK05_G07	UASB_TL25	96	*Desulfacinum subterraneum*	87	Deltaproteobacteria; Syntrophobacteraceae	3	1
MLK05_E10	SHA-42	97	*Syntrophus acidotrophicus*	95	Deltaproteobacteria; Syntrophaceae	8	2.4
MLK12_D10	SHA-42	88	*Syntrophus acidotrophicus*	86	Deltaproteobacteria; Syntrophaceae	1	0.5
MLK05_C04	SHA-42	98	*Syntrophus acidotrophicus*	95	Deltaproteobacteria; Syntrophaceae	1	0.5
MLK05_B01	SHA-42	98	*Syntrophus acidotrophicus*	95	Deltaproteobacteria; Syntrophaceae	1	0.5
MLK12_G12	SHA-42	97	*Syntrophus acidotrophicus*	94	Deltaproteobacteria; Syntrophaceae	1	0.5
MLK05_G05	SHA-42	96	*Syntrophus acidotrophicus*	94	Deltaproteobacteria; Syntrophaceae	1	0.5

Table 9.1: (Continued)

Clone	Closest phylotype	% Seq. sim.	Closest cultured species	% Seq. sim.	Taxonomic position (class, family, or genus)	# Clones	% Freq.
MLK05_D12	SHA-42	96	*Syntrophus acidotrophicus*	93	Deltaproteobacteria; Syntrophaceae	1	0.5
MLK05_F04	SSADM_ED8	95	*Syntrophus gentianae*	94	Deltaproteobacteria; Syntrophaceae	2	1
MLK05_H09	SJA-63	93	*Syntrophus buswellii*	90	Deltaproteobacteria; Syntrophaceae	1	0.5
MLK05_D10	SJA-152	92	*Geobacter pelophilus*	95	Deltaproteobacteria; Geobacteraceae	1	0.5
MLK05_G06	PBS-III-24	88	*Thermodesulforhabdus norvegicus*	89	Deltaproteobacteria; Syntrophobacteraceae	1	0.5
MLK05_D06	GZKB33	91	*Malonomonas rubra*	83	Deltaproteobacteria; Pelobacteraceae	1	0.5
MLK05_G03	PG-38	93	*Desulfonatronum lacustre*	87	Deltaproteobacteria; Desulfonatronumaceae	1	0.5
MLK05_G04	BA143	96	*Aquimonas voraii* strain GPTSA 20	90	Gammaproteobacteria; Xanthomonadaceae	1	0.5
MLK05_H10	vadinBA30	94	*Nitrospina gracilis*	90	Deltaproteobacteria; Desulfoarculaceae	1	0.5
MLK05_F07	WCHA1-94	91	*Thiobacillus halophilus*	85	Gammaproteobacteria; Halothiobacillaceae	1	0.5
MLK05_H07	OP5 bacterium clone EUB55	97	*Nitrococcus mobilis*	94	Gammaproteobacteria; Ectothiorhodospiraceae	1	0.5
MLK05_C12	OP8 clone BSR2SA07	96	*Rhodanobacter lindanoclasticus*	85	Gammaproteobacteria; Xanthomonadaceae	1	0.5
MLK05_B07	SUBT-5	90	*Thialkalivibrio* sp. ALMg 2	89	Gammaproteobacteria; Ectothiorhodospiraceae	1	0.5
MLK05_B12	WFeA1-35	90	*Thermoanaerobacter* sp. ToBE	87	Firmicutes; Thermoanaerobacteriaceae	1	0.5
MLK05_A07	WfeA1-35	94	*Thermoanaerobacter* sp. ToBE	88	Firmicutes; Thermoanaerobacteriaceae	3	1
MLK05_H05	WfeA1-35	92	*Thermoanaerobacter* sp. ToBE	88	Firmicutes; Thermoanaerobacteriaceae	1	0.5
MLK05_D07	WfeA1-35	94	*Thermoanaerobacter* sp. ToBE	87	Firmicutes; Clostridia; Thermoanaerobacteriaceae	6	2.5
MLK06_A07	*Natronoanaerobium aggerbacterium*	89			Firmicutes; Clostridiales	1	0.5
MLK05_C07	AKYH1364 (B21)	93	*Anaerolinea thermophila*	87	Chloroflexi; Anaerolinaceae	1	0.5
MLK05_G02	AKYH1364	98	*Anaerolinea thermophila*	92	Chloroflexi; Anaerolinaceae	2	1
MLK05_B05	AKYH1364	95	*Anaerolinea thermophila*	93	Chloroflexi; Anaerolinaceae	12	5.8
MLK05_D04	BVB11	97	*Anaerolinea thermophila*	89	Chloroflexi; Anaerolinaceae	1	0.5
MLK05_C02	SHA-105	98	*Anaerolinea thermophila*	89	Chloroflexi; Anaerolinaceae	1	0.5
MLK12_D01	GCA112	91	*Anaerolinea thermophila*	88	Chloroflexi; Anaerolinaceae	2	1
MLK05_D08	RFLP U19	97	*Dehalococcoides* sp. BHI80-15	87	Chloroflexi; Dehalococcoidetes	5	2.4
MLK05_E02	SHA-135	92	*Dehalococcoides* sp. BHI80-15	86	Chloroflexi; Dehalococcoidetes	3	1.5
MLK05_B11	SHA-25	98	*Cytophaga* sp. strain BD1-16	93	Bacteroidetes; Flexibacteraceae	1	0.5
MLK05_H06	clone A4	99	*Cytophaga* sp. strain BD1	92	Bacteroidetes; Flexibacteraceae	1	0.5
MLK05_H08	BB2_132	90	*Flexibacter aggregans* subsp. Cat.	87	Bacteroidetes; Cryomorphaceae	1	0.5
MLK05_D03	BSA2B-21	99	*Cytophaga* sp. BHI60-57B	83	Bacteroidetes; Flexibacteraceae	2	1

Table 9.1: (Continued)

Clone	Closest phylotype	% Seq. sim.	Closest cultured species	% Seq. sim.	Taxonomic position (class, family, or genus)	# Clones	% Freq.
MLK05_C09	oca44	90	*Adhaeribacter aquaticus*	87	Bacteroidetes; Flexibacteraceae	1	0.5
MLK05_F08	PG-38	90	*Reichenbachia agariperforans*	91	Bacteroidetes; Flexibacteraceae	1	0.5
MLK05_F12	190up	93	*Prosthecobacter* FC1	86	Verrucomicrobia; Verrucomicrobiaceae	1	0.5
MLK05_B10	Verrucomicrobia bact. Clone 9-14	97	*Prosthecobacter* FC2	84	Verrucomicrobia; Verrucomicrobiaceae	1	0.5
MLK05_C06	D14422	95	*Prosthecobacter* FC2	83	Verrucomicrobia; Verrucomicrobiaceae	1	0.5
MLK06_A01	G7-12	92	*Prosthecobacter* FC2	86	Verrucomicrobia; Verrucomicrobiaceae	2	1
MLK05_B03	RB01	92	*Prosthecobacter fusiformis*	84	Verrucomicrobia; Verrucomicrobiaceae	2	1
MLK05_G11	clone 9-14	95	*Nitrospira moscoviensis*	82	Nitrospirae; Nitrospiraceae	1	0.5
MLK05_D02	*Nitrospira* sp.strain RC25	86			Nitrospirae; Nitrospiraceae	1	0.5
MLK05_E08	SHA-41	95	*Spirochaeta asiatica*	87	Spirochaetes; Spirochaetaceae	2	1
MLK05_H03	ALU43	98	Spirochaeta asiatica	86	Spirochaetes; Spirochaetaceae	1	0.5
MLK05_A10	RSD10	94	*Prosthecochloris aestuarii*	86	Chlorobi; Chlorobiaceae	1	0.5
MLK06_A02	PRR-8	93	*Pirellula* sp. (Schlesner 139)	92	Planctomycetes; Planctomycetaceae	1	0.5

The clone related to WFeA1-35 was the isolate from iron or sulfate reduction zone of the aquifer contaminated with hydrocarbons (mainly jet fuel) and chlorinated solvents (Dojka *et al.*, 1998). The closely related cultured species had the similarity of 88% to *Thermoanaerobacter* sp. ToBE was the thermophilic and saccharolytic bacteria in the presence of thiosulfate (Mori *et al.*, 2002). With such low similarity, it was not possible to attain these features to the corresponding clone isolated in this study.

Clone RFLP U19 and SHA-135 (in total 6.8%) were isolated from polycyclic aromatic hydrocarbon degrading methanogenic community and 1,2-Dichloropropane dechlorinating consortia, respectively. These were attributed to only to the clone library of the heavy fraction and were also distantly related to *Dehalococcoides* sp., which are exist in the presence of chlorinated compounds as an electron acceptor where hydrogen as an electron donor (Fennell *et al.*, 2004). However, it does not use the butyrate as an electron donor; therefore its role in the syntrophic culture was complicated. It might also have been involved in the reduction of hydrogen produced in the degradation pathway of butyrate. If *Dehalococcoides* sp. uses hydrogen as an

electron donor, some electron acceptors would be necessary to reduce them. In the synthetic medium, some compounds might act as an electron acceptor with a high turnover rate and a shuttling mechanism.

The clones retrieved from the heavy fraction of the Wild BUT enrichment were closely related to 16S rDNA sequences from microorganisms isolated from anaerobic 1,2-dichloropropane-dechlorinating mixed culture (21.6%), fatty acid oxidizing syntrophs in the granular sludge(12.6%), the terrestrial microbial community (13.5%), the hydrocarbon- and chlorinated-solvent-contaminated aquifer (10.8%), the polycyclic aromatic hydrocarbon-degrading methanogenic community (4.5%), the mesophilic anaerobic protein degradation process (4.5%), the dairy wastewater lagoons (3.6%), the TCE-dechlorinating community (2.7%), the 4-Methylbenzoate-degrading methanogenic consortium (2.7%), the pulp mill effluent treatment system (1.8%), the forest soil, the bay (1.8%), the sequencing batch reactors for bromoamine acid removal (1.8%), the bulk soil and the rice roots of flooded rice microcosms (1.8%). The other phenotypes detected only one time were the isolates from acid-impacted and pristine subalpine stream sediment, the subterranean hot springs, the polluted coastal sediment, the benzoate degrading methanogenic consortium, the psychrophilic anaerobic digesters treating synthetic industrial wastewater, the dechlorination of 2,3,4,5-chlorobiphenyl in sediment culture, the mesophilic acetate-degrading methanogenic community, the oligotrophic microbial fuel cells, the straw degrading populations in anoxic paddy soil, the hydrocarbon- and chlorinated-solvent-contaminated aquifer, the granular sludge treating cassava starch wastewater, a paddy soil, the nitrite-oxidizing bioreactor, an anaerobic digestor, the landfill leachate-polluted aquifer, leachate of a closed municipal solid waste landfill, equine fecal contaminated site, the 2,3,5,6-tetrachlorobiphenyl-ortho-dechlorinating microbial community, the shelf sediment, the seep sediment, the soda lakes, and the sediment of a freshwater lake.

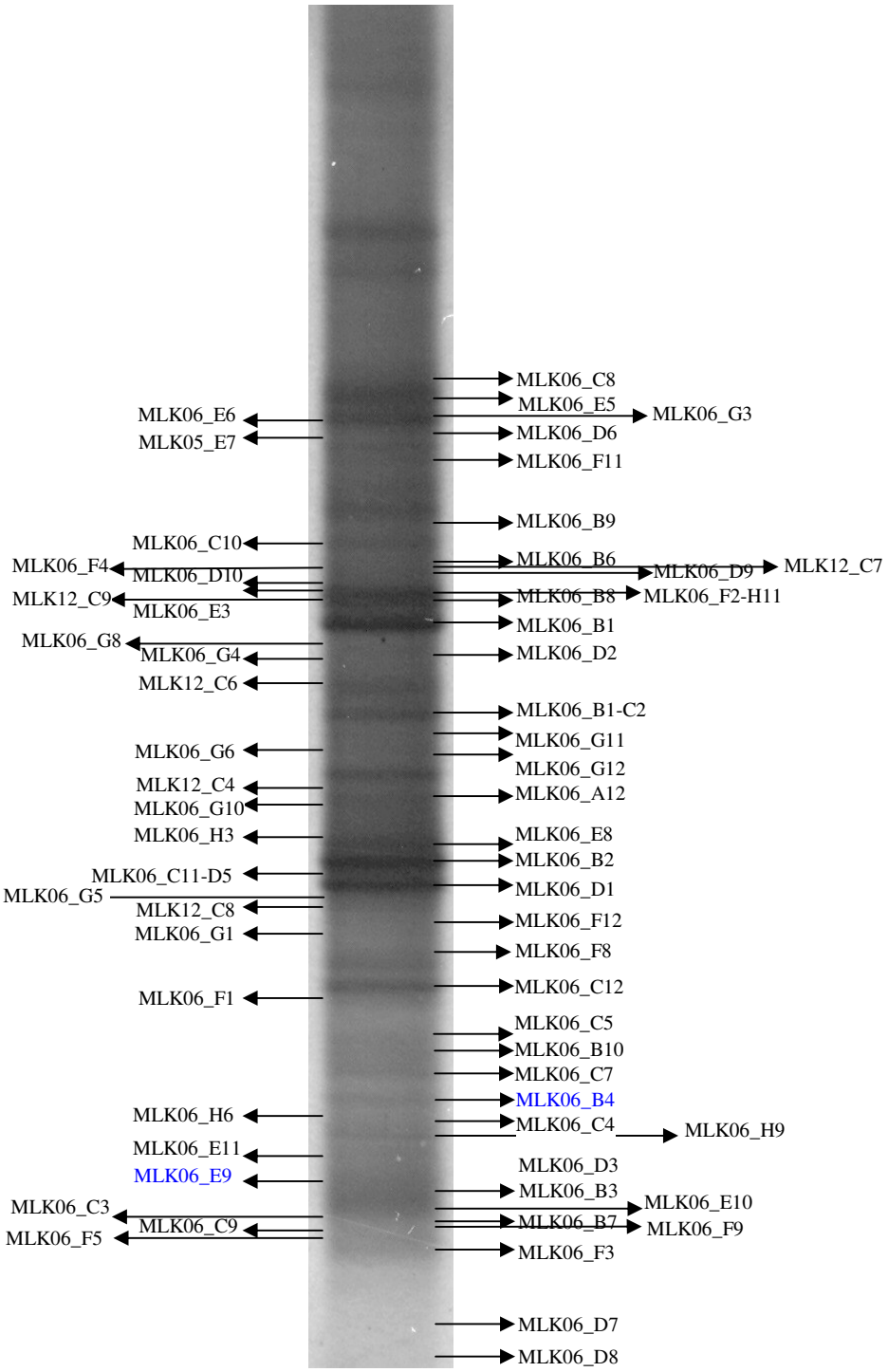


Figure 9.6 : Identification of the DGGE bands retrieved from the representative light fraction of the isopycnic centrifugation of the RNA of wild UASB reactor sludge incubated with the [^{13}C] butyrate

Cytophaga sp. and *Microscilla furvescens* from *Bacteriodetes, Paenibacillus kobensis* and *Bacillus* sp. from Firmicutes were the related cultured organisms of the heavy and light fraction of the Wild BUT enrichment. The presence of these saccharolytic organisms is another interesting feature of the consortium in the syntrophic butyrate degradation. They might use the cell debris or metabolic products, however, the presence of these species in the heavy fraction demonstrated that they were able to use the butyrate as a growth substrate.

9.4 The DGGE Profiles and the Sequence Analysis of Density Resolved Nucleic Acids for the Wild BUTS Sludge

The identification of the DGGE bands retrieved from the representative heavy and light fraction of the isopycnic centrifugation of the RNA isolated from the Wild BUTS sludge was given in Figure 9.6 and 9.7, respectively. The clone libraries related to the bands of the DGGE profiles were listed in the Table 9.3 and 9.4 for the heavy and light fractions of the gradient, respectively.

A total of 230 partial 16S rRNA bacterial gene clones (from 116 heavy and 114 light fractions) were analyzed for the Wild BUTS enrichments. The RFLP analysis of the 16S rRNA genes were grouped in 40 distinct types, and the analyses of 16S rRNA gene sequences with NCBI database showed that the clones were affiliated with only eight major bacterial lineages, which are: δ, α, β, γ- *Proteobacteria, Firmicutes, Chloroflexi, Bacteroidetes, Verrucomicrobia, Spirochaeta, Planctomycetes, Chlorobi, Gemmatimononadetes, Nitrospirae and Acidobacteria.*

In the heavy fraction of the density resolved nucleic acids revealed that the clones were classified into 9 phyla of the domain *Bacteria,* which are: δ, β, γ-*Proteobacteria* (43.1, 0.9%, and 5.2%, respectively), *Chloroflexi* (16.4%), *Firmicutes* (6.9%), *Bacteroidetes* (16.4%), *Acidobacteria* (1.7%), *Actinobacteria* (1.7%), *Chlorobi* (0.9%). However, in the light fraction of the density resolved nucleic acids, the clones were grouped into 11 phyla: δ, α, β, γ-*Proteobacteria* (34.2, 0.9, 1.8, and 0.9%, respectively), *Chloroflexi* (47.4%), *Firmicutes* (7%), *Verrucomicrobia* (0.9%), *Bacteroidetes* (1.8%), *Acidobacteria* (0.9%) *Nitrospirae* (2.6%), *Chlorobi* (1.8%).

Table 9.2: Phylogenetic assignment of the bacterial wild BUT clones isolated from the light fraction of the SIP experiment

Clone	Closest phylotype	% Seq. sim.	Closest cultured species	% Seq. sim.	Taxonomic position (class, family, or genus)	# Clones	% Freq.
MLK06_E08	R1p32	93	*Syntrophobacter wolinii*	89	Deltaproteobacteria; Syntrophobacteraceae	1	0.8
MLK06_E10	R1p32	97	*Syntrophobacter wolinii*	93	Deltaproteobacteria; Syntrophobacteraceae	1	0.8
MLK06_F03	R1p32	98	*Syntrophobacter wolinii*	93	Deltaproteobacteria; Syntrophobacteraceae	1	0.8
MLK06_F09	R1p32	98	*Syntrophobacter wolinii*	93	Deltaproteobacteria; Syntrophobacteraceae	1	0.8
MLK06_D03	R1p32	99	*Syntrophobacter wolinii*	94	Deltaproteobacteria; Syntrophobacteraceae	3	2.4
MLK06_E09	R3p4	99	*Syntrophobacter wolinii*	93	Deltaproteobacteria; Syntrophobacteraceae	2	1.6
MLK06_H09	R1p32	99	*Syntrophobacter wolinii*	94	Deltaproteobacteria; Syntrophobacteraceae	1	0.8
MLK06_F05	R1p32	98	*Syntrophobacter wolinii*	92	Deltaproteobacteria; Syntrophobacteraceae	2	1.6
MLK06_E11	R3p4	95	*Syntrophobacter wolinii*	93	Deltaproteobacteria; Syntrophobacteraceae	1	0.8
MLK06_B11	Desulfobacterales bact. C13	98	*Desulfobulbus propionicus*	95	Deltaproteobacteria; Desulfobulbaceae	15	12.2
MLK06_E07	LTCE-T2A 4A	97	*Desulfobulbus propionicus*	97	Deltaproteobacteria; Desulfobulbaceae	1	0.8
MLK06_D02	LTCE-T2A 4A	97	*Desulfobulbus propionicus*	97	Deltaproteobacteria; Desulfobulbaceae	1	0.8
MLK06_E03	Desulfobacterales bact. C13	89	*Desulfobulbus propionicus*	88	Deltaproteobacteria; Desulfobulbaceae	1	0.8
MLK06_G03	Desulfobacterales bact. C13	98	*Desulfobulbus propionicus*	96	Deltaproteobacteria; Desulfobulbaceae	2	1.6
MLK12_C09	clone C13	97	*Desulfobulbus propionicus*	93	Deltaproteobacteria; Desulfobulbaceae	1	0.8
MLK06_C03	SHA-110	96	*Desulfobulbus propionicus*	88	Deltaproteobacteria; Desulfobulbaceae	1	0.8
MLK06_E06	Amsterdam-2B-17	94	*Desulfomicrobium apsheronum* strain be2.2	88	Deltaproteobacteria; Desulfomicrobiaceae	2	1.6
MLK06_G04	SHA-4	93	*Desulfacinum* sp. M40/2 CIV-2.3	84	Deltaproteobacteria; Syntrophobacteraceae	1	0.8
MLK06_D06	Amsterdam-2B-17	95	*Desulfovibrio africanus*	85	Deltaproteobacteria; Desulfovibrionaceae	1	0.8
MLK06_A12	SHA-42	93	*Syntrophus gentianae*	91	Deltaproteobacteria; Syntrophaceae	2	1.6
MLK06_F04	PD-UASB-5	93	*Syntrophus acidotrophicus*	92	Deltaproteobacteria; Syntrophaceae;	1	0.8
MLK06_C10	cLaKi-StA70	98	*Desulfobacterium cetonicum*	92	Deltaproteobacteria; Desulfobacteraceae	1	0.8
MLK06_D05	SR_FBR_E38	97	*Desulfovibrio* sp. zt10e	96	Deltaproteobacteria; Desulfovibrionaceae	3	2.4
MLK06_B10	SR_FBR_E38	96	*Desulfovibrio* sp. zt10e	95	Deltaproteobacteria; Desulfovibrionaceae	3	2.4

Table 9.2: (continued)

Clone	Closest phylotype	% Seq. sim.	Closest cultured species	% Seq. sim.	Taxonomic position (class, family, or genus)	# Clones	% Freq.
MLK06_B02	SR_FBR_E38	93	*Desulfovibrio* sp. zt10e	92	Deltaproteobacteria; Desulfovibrionaceae	9	7.3
MLK06_G11	Sh765B-TzT-35	94	*Desulfovibrio alaskensis*	85	Deltaproteobacteria; Desulfovibrionaceae	1	0.8
MLK06_C11	LD1-PB1	91	*Desulfovibrio giganteus*	86	Deltaproteobacteria; Desulfovibrionaceae	1	0.8
MLK06_C08	C16T12 D02	92	*Acidithiobacillus* sp. NO-37	86	Gammaproteobacteria; Acidithiobacillaceae	1	0.8
MLK06_D08	055B11_P_DI_P58	87	*Lysobacter brunescens*	93	Gammaproteobacteria; Xanthomonadaceae	1	0.8
MLK06_B01	SHA-4	89	*Beggiatoa* sp. AA5A	80	Gammaproteobacteria; Thiotrichaceae	1	0.8
MLK06_B09	OCG12	96	*Magnetic coccus* MP17	83	Alphaproteobacteria	1	0.8
MLK12_C06	AKYH1364	95	*Anaerolinea thermophila*	90	Chloroflexi; Anaerolinaceae	1	0.8
MLK06_G12	CARB_ESS_12	95	*Anaerolinea thermophila*	92	Chloroflexi; Anaerolinaceae	1	0.8
MLK06_G01	SHA-105	99	*Anaerolinea thermophila*	90	Chloroflexi; Anaerolinaceae	1	0.8
MLK12_C08	SHA-105	98	*Anaerolinea thermophila*	90	Chloroflexi; Anaerolinaceae	1	0.8
MLK06_B08	SHA-105	99	*Anaerolinea thermophila*	90	Chloroflexi; Anaerolinaceae	2	1.6
MLK06_F02	SHA-105	99	*Anaerolinea thermophila*	90	Chloroflexi; Anaerolinaceae	3	2.4
MLK06_D10	SHA-105	95	*Anaerolinea thermophila*	90	Chloroflexi; Anaerolinaceae	1	0.8
MLK06_C02	CARB_ESS_8	98	*Anaerolinea thermophila*	90	Chloroflexi; Anaerolinaceae	1	0.8
MLK06_G06	CARB_ESS_8	99	*Anaerolinea thermophila*	91	Chloroflexi; Anaerolinaceae	1	0.8
MLK06_D01	AKYH1364	98	*Anaerolinea thermophila*	92	Chloroflexi; Anaerolinaceae	8	6.5
MLK06_H11	SHA-105	98	*Anaerolinea thermophila*	90	Chloroflexi; Anaerolinaceae	1	0.8
MLK06_B06	BFA-K16	93	*Anaerolinea thermophila*	89	Chloroflexi; Anaerolinaceae	2	1.6
MLK06_C05	K102	93	*Caldilinea aerophila*	89	Chloroflexi; Anaerolinaceae	1	0.8
MLK06_F12	BMSB4	96	*Caldilinea aerophila*	88	Chloroflexi; Anaerolinaceae	4	3.3
MLK06_F11	WCHB1-71	93	*Syntrophomonas sapovorans*	92	Firmicutes; Syntrophomonadaceae	1	0.8
MLK06_C09	clone 1200-12	93	*Paenibacillus kobensis*	93	Firmicutes; Paenibacillaceae	1	0.8
MLK06_C04	clone 33a	85	*Bacillus* sp. 12B_7	88	Firmicutes; Bacillaceae	1	0.8
MLK06_B07	ALU38	98	*Cytophaga* sp. strain BD1-16	87	Bacteroidetes; Flexibacteraceae	1	0.8
MLK06_H06	PL-34B5	98	*Cytophaga* sp. strain BD1-16	88	Bacteroidetes; Flexibacteraceae	1	0.8
MLK12_C07	SHA-25	95	*Cytophaga* sp., strain BD1-16	89	Bacteroidetes; Flexibacteraceae	1	0.8
MLK06_G05	PL-32B9	96	*Cytophaga* sp. BHI60-95B	91	Bacteroidetes; Flexibacteraceae	2	1.6
MLK06_B03	ALU38	97	*Cytophaga* sp. BHI60-95B	92	Bacteroidetes; Flexibacteraceae	1	0.8
MLK06_G10	ALU38	96	*Cytophaga* sp. I-976	91	Bacteroidetes; Flexibacteraceae	3	2.4
MLK06_F01	TTMF84	97	*Microscilla furvescens*	86	Bacteroidetes; Flexibacteraceae	1	0.8
MLK06_F08	KNA6-EB19	95	Acidobacteria/Holophaga group clone Cart-N4	93	Acidobacteria; Acidobacteriales	2	1.6
MLK06_D09	D14422	93	*Opitutus terrae*	92	Verrucomicrobia; Opitutaceae	3	2.4
MLK06_C07	G7-12	92	*Prosthecobacter fusiformis*	85	Verrucomicrobia; Verrucomicrobiaceae	1	0.8

Table 9.2: (continued)

Clone	Closest phylotype	% Seq. sim.	Closest cultured species	% Seq. sim.	Taxonomic position (class, family, or genus)	# Clones	% Freq.
MLK06_H03	HMMVBeg-25	92	*Gemmatimonas aurantiaca*	87	Gemmatimonadetes; Gemmatimonadaceae	1	0.8
MLK12_C04	RSD10	82	*Chlorobium limicola*	77	Chlorobi; Chlorobiaceae	1	0.8
MLK06_G08	OP5 bacterium clone EUB55	97	*Nitrococcus mobilis*	93	Gammaproteobacteria; Ectothiorhodospiraceae	1	0.8
MLK06_D07	OP5 bacterium clone EUB55	96	*Nitrococcus mobilis*	93	Gammaproteobacteria; Ectothiorhodospiraceae	2	1.6
MLK06_E05	HMMVBeg-25	93	*Thermoanaerobacter* sp. ToBE	88	Firmicutes; Thermoanaerobacteriaceae	1	0.8
MLK06_B04	HMMVBeg-25	93	*Thermoanaerobacter* sp. ToBE	88	Firmicutes; Thermoanaerobacteriaceae	4	3.3
MLK06_C12	WFeA1-35	92	*Thermoanaerobacter* sp. ToBE	85	Firmicutes; Thermoanaerobacteriaceae	2	1.6

We also found that the presence of 3 mM sulfate, which corresponds to COD/sulfate:10, had a little or no effect on the structure of the total clone library; but the abundance of these clones changed. Comparison of the two Wild BUT and the Wild BUTS clone libraries showed that in the latter one, *Desulfovibrio* species was highly abundant in the heavy fraction. Although similar phylotypes were observed for the Wild BUT and the Wild BUTS bacterial clone libraries, distributions of some clones whether they were present in the heavy or light fractions has changed.

A majority of the clones obtained from the metabolically active fraction of the Wild BUTS microbial community were mostly closely related to *Desulfovibrio* sp., the sulfate-reducing bacteria. SRB population probably functioned as proton reducers in association with hydrogen and formate consuming methanogen. The amount of theoretical reducing equivalent produced from 1mmol butyrate is the half mmole of hydrogen (Eq.9.1) or formate (Eq. 9.2). The feed of the enrichment contains 18 mM butyrate, so the produced proton amount will be 9 mM. Therefore, taking into account the thermodynamic advantage of SRBs over methanogens in terms of hydrogen utilization, the presence of 3 mM sulfate was not enough to reduce all this reducing equivalent of the butyrate due to the necessity of 4 mol hydrogen to reduce 1 mol sulfate (Eq 9.3). So, assuming all sulfate is reduced by hydrogen, there still remained 6 mM hydrogen to be reduced by methanogens. This might be the reason of presence of both the SRBs and the methanogens in this consortium for hydrogen utilization.

$$CH_3CH_2CH_2COO^- + 2H_2O \rightarrow 2CH_3COO^- + H^+ + 2H_2 \quad \textbf{(9.1)}$$

$$CH_3CH_2CH_2COO^- + 2HCO_3 \rightarrow 2CH_3COO^- + 2H^+ + 2HCOO^- \quad \textbf{(9.2)}$$

$$4H_2 + SO_4^{2-} + H^+ \rightarrow HS^- + 4H_2O \quad \textbf{(9.3)}$$

The *Syntrophobacter* sp. was found less frequent as 4 clones (3.5%) in the heavy fraction of the Wild BUTS enrichment, while 14 clones (12.3%) were detected in the light fraction. This distribution of the clones clearly showed that the propionate oxidizing bacteria was less significant in the Wild BUTS sludge, since they covered the important part of the Wild BUT clone library. *Desulforegula conservatrix* and *Desulfacinum* sp. were the minor group of SRB in the heavy fraction. *Desulfomonile limimaris* and *Desulfobacterium cetonicum* were also the minor group in the light fraction of the total community. The latter one was also detected in the light fraction of the Wild BUT enrichment, clearly demonstrating its inactivity in the butyrate degrading consortium.

Comparative analysis of microbial communities between the Wild BUT and the Wild BUTS enrichments indicated that the *Syntrophus* sp. were less abundant in the Wild BUTS; indicating other members of the community were more important for the degradation of butyrate.

The second most frequently detected group of phylotypes (16.4% of all rRNA gene clones) was most closely related to hydrocarbon-associated bacteria, a distinct clade within the phylum *Chloroflexi*. However, the number of *Chloroflexi* clustered group of bacteria increased substantially in the light fraction, which makes up 47% of the total light fraction clone library. It should not be forgotten that this phylum has numerous uncultured bacteria and only a small amount representative bacteria. Therefore, evaluating or assigning the functional features of the organisms will bring us wrong suggestions.

Bacteroidetes species; *Taxeobacter* sp., *Reichenbachia agariperforan, Rhodothermus marinus,* and *Saprospira* were detected in the heavy fraction of the Wild BUTS enrichment covering 16.4% of the total clone library. These abundant members of *Bacteroidetes* that have the saccharolytic traits bring doubts about their functions in the sytntrophic culture.

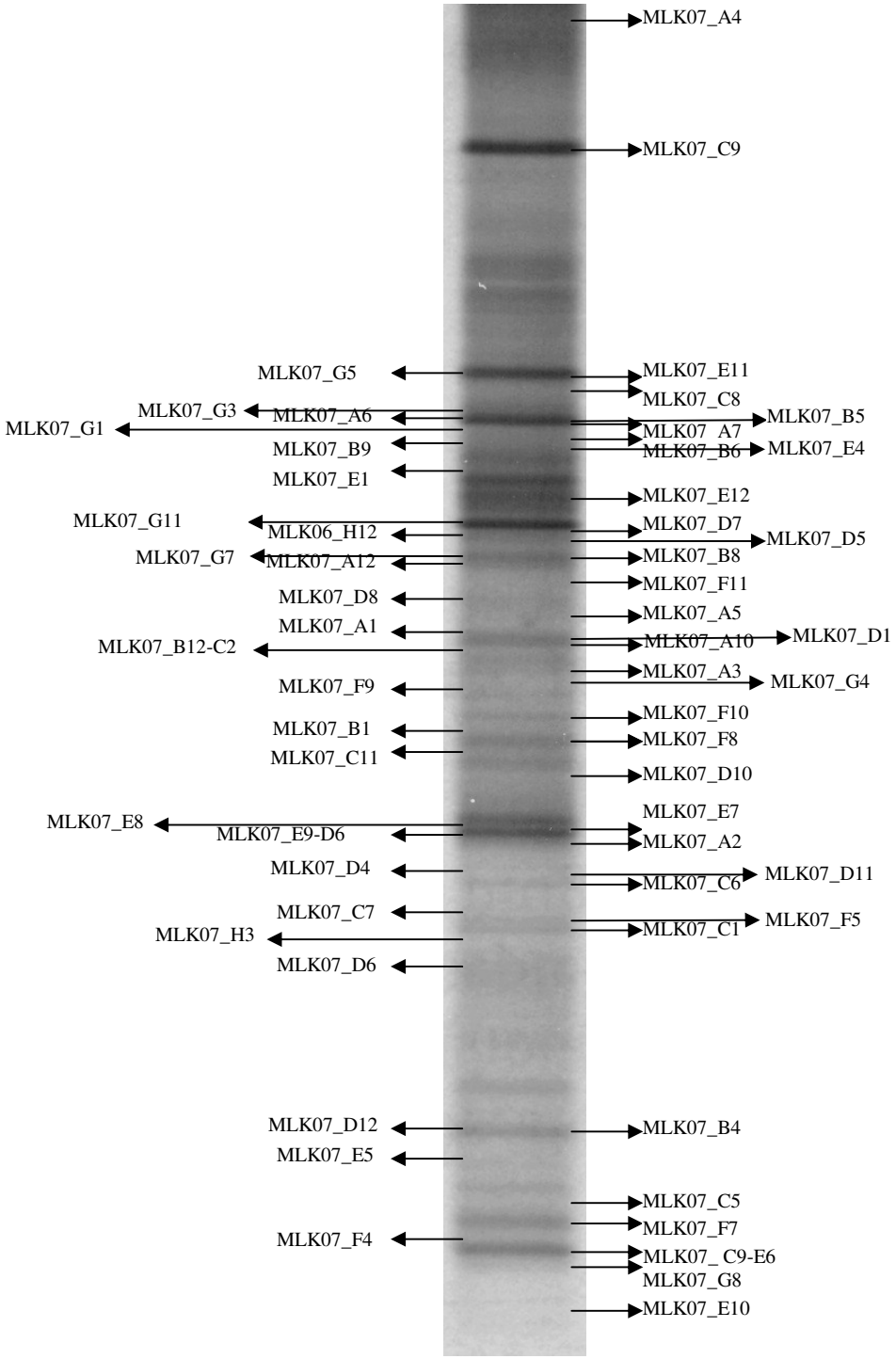


Figure 9.7 : Identification of the DGGE bands retrieved from the representative heavy fraction of the isopycnic centrifugation of the RNA of wild UASB reactor sludge incubated with [^{13}C] butyrate plus the 3mM sulfate

PG-38 clones related to the *Chlorobium* phylum had low similarities (<93%) to 16S rDNA phylotype recovered from the environment of paper mill effluent treatment plant. These clones were also low in similarity to the 16S rDNA sequences of cultured *Chlorobium* sp. isolated from petroleum systems, other subsurface habitats, and marine hydrocarbon-contaminated sites.

A total of 11 clones were affiliated to the sequence retrieved from the equine fecal contaminated site. This *Bacteriovorax*-related cultured phylotypes were identified and were detected only in the heavy fraction of the Wild BUTS enrichment. These species are obligate predators, prey-dependent, and saprophytic form capable of growing on rich nutrient medium (Baer *et al.*, 2000). The presence of these species and their contribution to the butyrate degradation was quite remarkable.

Eubacterium brachy oral asaccharolytic Eubacterium (Cheeseman *et al.*, 1996) was also another heavy fraction clone observed in the Wild BUTS enrichment. As observed for the enrichment of acetate, propionate and butyrate mixture fed enrichment incubated at 25 and 37°C, the presence of these species was detected (Chapter 10). It could be attained that these species might act as a homoacetogenic bacterium in the sulfate reducing consortia. The homoacetogenic bacterium mediates the reaction of hydrogen with carbon dioxide to form acetate.

Thermodesulforhabdus norvegicus (thermophilic sulfate-reducing bacterium from oil field water) were represented by a single phylotype and were also detected in the heavy fraction of the BUT enrichment. These species grow on butyrate in the presence of sulfate. (Beeder *et al.*, 1995). However, the optimal growth temperature of this species does not coincide with the mesophilic temperature ranges.

The other 7 clone sequences were most closely related to non-*Proteobacteria, Chloroflexi,* and *Bacteroidetes* lineages, including the *Acidobacteria*, *Actinobacteria*, *Chlorobi*, and unclassified *Bacteria* group OP8 represented by the sediment and pulp mill effluent treatment system clones. Since they do not exhibit a high degree of similarity to the known cultivated species, Acidobacteria/Holophaga group clones were isolates from air samples and deep-groundwater microorganisms that pass through 0.2-micrometer-pore-size filters.

Table 9.3: Phylogenetic assignment of the bacterial wild BUTS clones isolated from the heavy fraction of the SIP experiment

Clone	Closest phylotype	% Seq. sim.	Closest cultured species	% Seq. sim.	Taxonomic position (class, family, or genus)	# Clones	% Freq.
MLK07_G08	R1p32	97	*Syntrophobacter wolinii*	92	Deltaproteobacteria; Syntrophobacteraceae	2	1.7
MLK07_F07	R1p32	97	*Syntrophobacter wolinii*	91	Deltaproteobacteria; Syntrophobacteraceae	1	0.8
MLK07_E06	R1p32	97	*Syntrophobacter wolinii*	91	Deltaproteobacteria; Syntrophobacteraceae	1	0.8
MLK07_C09	*Desulfovibrio putealis*	91			Deltaproteobacteria; Desulfovibrionaceae	20	16.5
MLK07_B12	PBS-III-24	87	*Thermodesulforhabdus norvegicus*	88	Deltaproteobacteria; Syntrophobacteraceae	1	0.8
MLK07_C11	PBS-III-24	88	*Thermodesulforhabdus norvegicus*	87	Deltaproteobacteria; Syntrophobacteraceae	1	0.8
MLK07_C02	PBS-III-18	89	*Thermodesulforhabdus norvegicus*	88	Deltaproteobacteria; Syntrophobacteraceae	1	0.8
MLK07_F10	BRC1 bacterium clone LD1-PB16	89	*Geopsychrobacter electrodiphilus* strain A2	91	Deltaproteobacteria Geobacteraceae	2	1.7
MLK07_C06	HW71_8	95	*Desulfacinum* sp. M40/2 CIV-2.3	92	Deltaproteobacteria; Syntrophobacteraceae	1	0.8
MLK07_H03	OCG12	98	*Desulforegula conservatrix*	83	Deltaproteobacteria; Desulfobacteraceae	1	0.8
MLK07_A07	SJA-63	96	*Syntrophus gentianae*	94	Deltaproteobacteria; Syntrophaceae	6	5.0
MLK07_B05	*Byssophaga cruenta*	90			Deltaproteobacteria; Polyangiaceae	1	0.8
MLK07_F11	T1-3_CFB	87	*Haliangium tepidum*	87	Deltaproteobacteria; Nannocystinea; Haliangiaceae	1	0.8
MLK07_A05	CR99-35-67	92	*Bacteriovorax* sp. F2	88	Deltaproteobacteria; Bdellovibrionaceae	1	0.8
MLK07_F05	UASB_TL25	96	*Pelobacter* sp.	91	Deltaproteobacteria; Pelobacteraceae	1	0.8
MLK07_A06	265ds10	92	*Bacteriovorax* sp. F2	88	Deltaproteobacteria; Bdellovibrionaceae	7	5.8
MLK07_G07	265ds10	91	*Bacteriovorax* sp. F2	87	Deltaproteobacteria; Bdellovibrionaceae	3	2.5
MLK07_E12	cLaKi-StA59	91	*Luteibactor rhizovicina* isolate OUCZ70	86	Gammaproteobacteria; Luteibactor	1	0.8
MLK07_G01	OP8 clone BSR2SA07	92	*Luteibactor rhizovicina* is. OUCZ70	85	Gammaproteobacteria; Luteibactor	1	0.8
MLK07_D07	Amazon	93	*Aquimonas voraii* strain GPTSA 20	93	Gammaproteobacteria; Xanthomonadaceae	1	0.8
MLK07_E07	*Pseudomonas* sp. CH8	98			Gammaproteobacteria; Pseudomonadaceae	1	0.8
MLK07_D11	PBS-III-18	88	*Achromatium* sp. HK15	84	Gammaproteobacteria; Thiotrichaceae	2	1.7
MLK07_C01	OP8 clone BSR2SA07	92	*Thiobacillus* sp.	86	Betaproteobacteria; Hydrogenophilaceae	2	1.7
MLK07_B01	*Syntrophomonas* sp. MGB-C1	96			Firmicutes; Syntrophomonadaceae	1	0.8

Table 9.3: (continued)

Clone	Closest phylotype	% Seq. sim.	Closest cultured species	% Seq. sim.	Taxonomic position (class, family, or genus)	# Clones	% Freq.
MLK07_G03	*Syntrophomonas* sp. TB-6	95			Firmicutes; Syntrophomonadaceae	1	0.8
MLK07_C07	WFeA1-35	94	*Anoxybacillus flavithermus* strain B	87	Firmicutes; Bacillaceae	1	0.8
MLK07_F08	PBS-III-18	89	*Thermoanaerobacter* sp. ToBE	88	Firmicutes; Thermoanaerobacteriaceae	2	1.7
MLK07_A12	WCHB1-71	95	*Pelospora glutarica*	95	Firmicutes; Syntrophomonadaceae	1	0.8
MLK07_E10	SJA-136	94	*Catabacter hongkongensis*	90	Firmicutes; Clostridiales	1	0.8
MLK06_H12	ML635J-28	92	*Clostridium botulinum*	77	Firmicutes; Clostridiaceae	1	0.8
MLK07_D04	Ad-C1-74	92	*Eubacterium brachy*	91	Firmicutes; Eubacteriaceae	1	0.8
MLK07_D01	BVB11	97	*Anaerolinea thermophila*	89	Chloroflexi; Anaerolinaceae	1	0.8
MLK07_A10	SHA-147	94	*Anaerolinea thermophila*	91	Chloroflexi; Anaerolinaceae	7	5.8
MLK07_G04	TANB37	94	*Anaerolinea thermophila*	86	Chloroflexi; Anaerolinaceae	1	0.8
MLK07_B08	SHA-105	98	*Anaerolinea thermophila*	89	Chloroflexi; Anaerolinaceae	3	2.5
MLK07_E09	CARB_ESS_12	95	*Anaerolinea thermophila*	91	Chloroflexi; Anaerolinaceae	1	0.8
MLK07_D06	AKYH1364	97	*Caldilinea aerophila*	87	Chloroflexi; Anaerolinaceae	6	5.0
MLK07_A02	AKYH929	97	*Caldilinea aerophila*	94	Chloroflexi; Anaerolinaceae	1	0.8
MLK07_E04	44a-B1-13	91	*Dehalococcoides* sp. BAV1	84	Chloroflexi; Dehalococcoidetes	1	0.8
MLK07_A03	Uncultured bacterium	89	*Dehalococcoides* sp. BHI80-52	83	Chloroflexi; Dehalococcoidetes	3	2.5
MLK07_F04	SJA-101	90	*Dehalococcoides* sp. BHI80-52	88	Chloroflexi; Dehalococcoidetes	1	0.8
MLK07_G11	clone A4	99	*Cytophaga* sp. strain BD1-16	93	Bacteroidetes; Flexibacteraceae	3	2.5
MLK07_E01	EUB46-2	96	*Cytophaga* sp. strain BD1-16	91	Bacteroidetes; Flexibacteraceae	1	0.8
MLK07_D10	B-1G	97	*Cytophaga* sp. strain BD1-16	90	Bacteroidetes; Flexibacteraceae	3	2.5
MLK07_E08	clone A4	98	*Cytophaga* sp. strain BD1-16	90	Bacteroidetes; Flexibacteraceae	1	0.8
MLK07_D05	265ds10	88	*Cytophaga* sp., strain BD2-15	83	Bacteroidetes; Flexibacteraceae	2	1.7
MLK07_B09	CR99-35-67	87	*Cytophaga* sp. strain BD1-27	93	Bacteroidetes; Flexibacteraceae	1	0.8
MLK07_C05	ALU38	93	*Cytophaga* sp. BHI60-95B	90	Bacteroidetes; Flexibacteraceae	2	1.7
MLK07_E05	K-273	90	*Taxeobacter* sp. Txc1	83	Bacteroidetes; Flexibacteraceae	3	2.5
MLK07_F09	P. palm A 12	90	*Rhodothermus marinus* strain NR-32	87	Bacteroidetes; Crenotrichaceae	1	0.8
MLK07_A04	P. palm A 12	91	*Rhodothermus marinus* strain NR-32	88	Bacteroidetes; Crenotrichaceae	1	0.8
MLK07_D08	BSA2B-19	97	*Saprospira* sp. CNJ451	88	Bacteroidetes; Saprospiraceae	2	1.7
MLK07_A01	BSA2B-19	96	*Alistipes massiliensis* strain 3302398	86	Bacteroidetes; Rikenellaceae	1	0.8

Table 9.3: (continued)

Clone	Closest phylotype	% Seq. sim.	Closest cultured species	% Seq. sim.	Taxonomic position (class, family, or genus)	# Clones	% Freq.
MLK07_B04	cvf63078	83	Acidobacteria/Holophaga group clone Cart-N4	82	Acidobacteria; environmental samples	1	0.8
MLK07_D12	KNA6-EB19	90	*Acidobacteriaceae* bacterium TAA166	87	Acidobacteria; Acidobacteriaceae	1	0.8
MLK07_C08	WCHA1-94	92	*Prosthecochloris* sp. 4Vi	82	Chlorobi; Chlorobiaceae	1	0.8
MLK07_B06	PG-38	90	*Chlorobium phaeobacteroides*	84	Chlorobi; Chlorobiaceae	1	0.8
MLK07_G05	PG-38	90	*Chlorobium phaeobacteroides*	85	Chlorobi; Chlorobiaceae;	2	1.7
MLK07_E11	PG-38	93	*Chlorobium phaeobacteroides*	86	Chlorobi; Chlorobiaceae;	1	0.8

The clones retrieved from the heavy fraction of the Wild BUTS enrichment were closely related to 16S rDNA sequences from microorganisms isolated from the deep subsurface aquifer (17.1%), anaerobic 1,2-dichloropropane-dechlorinating mixed culture (16.2%) equine fecal contaminated site (10.8%), terrestrial microbial community (6.3%), bulk soil and rice roots of flooded rice microcosms (6.3%), a pulp mill effluent treatment system (4.5%), fatty acid oxidizing syntrophs in granular sludge(3.6%), acetate-degrading methanogenic community (3.6%), acid-impacted and pristine subalpine stream sediments (2.7%), 2,3,4,5-chlorobiphenyl in sediment cultures (2.7%), anaerobic swine lagoons (2.7%), mesophilic anaerobic protein degradation process (2.7%), subseafloor prokaryotic community associated with methane hydrate (2.7%), hydrothermal vent (1.8%), and ammonia-oxidizing bacteria within anoxic marine sediments (1.8%). Other phenotypes detected only one time were isolates from hydrocarbon- and chlorinated-solvent-contaminated aquifer, pilot-scale perchlorate reducing bioreactor, 4-methylybenzoate-degrading methanogenic consortium, a trichloroethene-contaminated deep aquifer, landfill leachate-polluted aquifer, anaerobic biological treatment of whey-based wastewaters at psychrophilic temperatures, granular sludge treating cassava starch wastewater, soda lake, herbivore gastrointestinal tract, suppressive and conducive soils, river, lake, Amazon soil, contaminated mussels, air samples, and deep groundwater microorganisms. In summary, all clones were retrieved from different habitats and mostly from anaerobic environments.

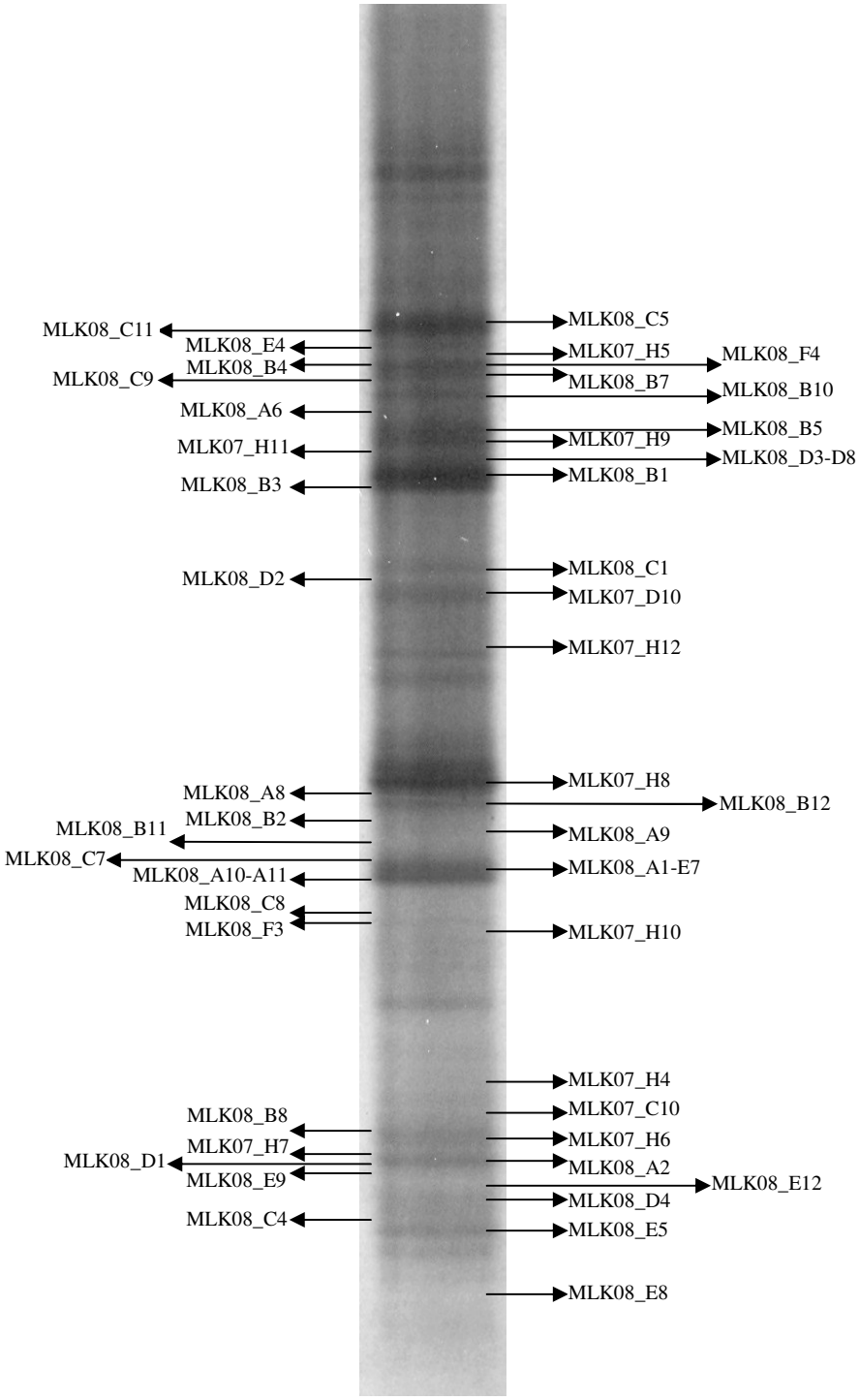


Figure 9.8 : Identification of the DGGE bands retrieved from representative light fraction of the isopycnic centrifugation of the RNA of wild UASB reactor sludge incubated with the [^{13}C] butyrate plus the 3mM sulfate

Table 9.4: Phylogenetic assignment of the bacterial wild BUTS clones isolated from the light fraction of the SIP experiment

Clone	Closest phylotype	% Seq. sim.	Closest cultured species	% Seq. sim.	Taxonomic position (class, family, or genus)	# Clones	% Freq.
MLK08_A11	WFeA1-35	93	*Syntrophobacter wolinii*	84	Deltaproteobacteria; Syntrophobacteraceae	1	0.8
MLK08_D01	R1p32	96	*Syntrophobacter wolinii*	90	Deltaproteobacteria; Syntrophoacteraceae	1	0.8
MLK07_H06	R1p32	98	*Syntrophobacter wolinii*	92	Deltaproteobacteria; Syntrophobacteraceae	7	5.9
MLK08_E12	R1p32	98	*Syntrophobacter wolinii*	93	Deltaproteobacteria; Polyangiaceae	1	0.8
MLK08_B08	R1p32	92	*Syntrophobacter wolinii*	90	Deltaproteobacteria; Polyangiaceae	1	0.8
MLK08_D04	DSB-DSa99-6	92	*Syntrophobacter sulfatereducens*	91	Deltaproteobacteria; Syntrophobacteraceae	1	0.8
MLK07_H04	KB-1	91	*Syntrophobacter sulfatereducens*	91	Deltaproteobacteria; Syntrophobacteraceae	1	0.8
MLK08_E09	*Syntrophobacter sulfatereducens*	94			Deltaproteobacteria; Syntrophobacteraceae	1	0.8
MLK08_B07	SJA-63	96	*Syntrophus gentianae*	93	Deltaproteobacteria; Syntrophaceae	1	0.8
MLK08_E04	SSADM_ED8	98	*Syntrophus gentianae*	94	Deltaproteobacteria; Syntrophaceae	4	3.4
MLK08_B10	cLaKi-StA70	98	*Desulfobacterium cetonicum*	92	Deltaproteobacteria; Desulfobacteraceae	1	0.8
MLK08_C09	cLaKi-StA70	96	*Desulfobacterium cetonicum*	92	Deltaproteobacteria; Desulfobacteraceae	1	0.8
MLK08_B03	cLaKi-StA70	95	*Desulfobacterium cetonicum*	90	Deltaproteobacteria; Desulfobacteraceae	1	0.8
MLK08_D03	*Desulfomonile limimaris*	95			Deltaproteobacteria; Syntrophaceae	1	0.8
MLK07_H12	SHD-100	90	*Geobacter pelophilus*	95	Deltaproteobacteria; Geobacteraceae	4	4.2
MLK08_B11	fcr156	93	*Magnetic coccus* (CS103)	83	Alphaproteobacteria	1	0.8
MLK07_H11	UASB_TL25	96	*Pelobacter* sp. clone GR-WP54-1	91	Deltaproteobacteria; Pelobacteraceae	2	1.7
MLK08_A02	*Desulfovibrio putealis*	94			Deltaproteobacteria; Desulfovibrionaceae	2	1.7
MLK07_H07	*Desulfovibrio putealis*	93			Deltaproteobacteria; Desulfovibrionaceae	1	0.8
MLK08_E05	SR_FBR_E38	95	*Desulfovibrio* sp. zt10e	94	Deltaproteobacteria; Desulfovibrionaceae	3	2.5
MLK08_C05	BA149	97	*Desulfovibrio alkalitolerans*	84	Deltaproteobacteria; Desulfovibrionaceae	6	5.0
MLK08_F04	BA149	97	*Desulfovibrio ferrireducens*	86	Deltaproteobacteria; Desulfovibrionaceae	1	0.8
MLK07_H05	*Chondromyces apiculatus* (str. Cm)	89			Deltaproteobacteria; Polyangiaceae	1	0.8
MLK08_A01	OP8 bacterium clone BSR2SA07	91	*Thiobacillus* sp.	85	Betaproteobacteria; Hydrogenophilaceae	2	1.7
MLK08_B04	OP8 clone BSR2SA07	92	*Luteibactor rhizovicina* isolate OUCZ70	84	Gammaproteobacteria; Luteibactor	1	0.8
MLK08_D10	SHA-105	98	*Anaerolinea thermophila*	90	Chloroflexi; Anaerolinaceae	4	3.4

Table 9.4: (Continued)

Clone	Closest phylotype	% Seq. sim.	Closest cultured species	% Seq. sim.	Taxonomic position (class, family, or genus)	# Clones	% Freq.
MLK08_B01	SHA-105	98	*Anaerolinea thermophila*	90	Chloroflexi; Anaerolinaceae	18	15.1
MLK08_B12	AKYG762	94	*Anaerolinea thermophila*	89	Chloroflexi; Anaerolinaceae	1	0.8
MLK08_D02	TANB37	97	*Anaerolinea thermophila*	88	Chloroflexi; Anaerolinaceae	1	0.8
MLK08_B02	WCHB1-63	95	*Anaerolinea thermophila*	89	Chloroflexi; Anaerolinaceae	3	2.5
MLK08_A06	AKYH1364	95	*Anaerolinea thermophila*	90	Chloroflexi; Anaerolinaceae	2	1.7
MLK08_C01	SHA-147	93	*Anaerolinea thermophila*	90	Chloroflexi; Anaerolinaceae	1	0.8
MLK07_H08	AKYH1364	93	*Anaerolinea thermophila*	86	Chloroflexi; Anaerolinaceae	16	13.4
MLK08_C04	AKYH1364	97	*Anaerolinea thermophila*	91	Chloroflexi; Anaerolinaceae	2	1.7
MLK08_C08	CARB_ESS_5	89	*Anaerolinea thermophila*	88	Chloroflexi; Anaerolinaceae	1	0.8
MLK07_H10	K102	92	*Caldilinea aerophila*	88	Chloroflexi; Anaerolinaceae	2	1.7
MLK08_A08	RFLP U19	96	*Dehalococcoides* sp. BHI80-52	89	Chloroflexi; Dehalococcoidetes	1	0.8
MLK08_A09	SK23	93	*Dehalococcoides* sp. BHI80-15	90	Chloroflexi; Dehalococcoidetes	2	1.7
MLK08_A10	WFeA1-35	92	*Thermoanaerobacter* sp. ToBE	88	Firmicutes; Thermoanaerobacteriaceae	4	3.4
MLK08_D08	WFeA1-35	91	*Anoxybacillus flavithermus* strain B	88	Firmicutes; Bacillaceae	3	2.5
MLK08_C07	*Natronoanaerobium aggerbacterium*	89			Firmicutes; Clostridiales	1	0.8
MLK08_E08	fcr94	97	*Rhodothermus marinus* strain NR-32	85	Bacteroidetes; Crenotrichaceae	2	1.7
MLK08_B05	EUB20-2	98	*Nitrospira* sp.	81	Nitrospirae; Nitrospiraceae	3	2.5
MLK07_H09	clone 9-14	94	*Verrucomicrobia* bacterium clone 38	92	Verrucomicrobia; environmental samples	1	0.8
MLK08_C10	cvf58182	94	*Acidobacteriaceae* bacterium TAA166	87	Acidobacteria; Acidobacteriaceae	1	0.8
MLK08_C11	PG-38	94	*Chlorobium phaeobacteroides*	88	Chlorobi; Chlorobiaceae	1	0.8
MLK08_F03	*Chlorobium* sp. clone OPPB063	85			Chlorobi; Chlorobiaceae	1	0.8

Sulfate-reducing bacteria come from many taxonomic groups, including mainly the delta group of *Proteobacteria;* but some of the members are from *Nitrospira* division. However, the clones found in this phylum were in the light fraction, meaning that they do not function in the syntrophic butyrate degradation.

Phylogenetic affiliation of the syntrophic butyrate community encountered in the UASB reactor of paper mill wastewater treatment plant for both the enrichment of the Wild BUT and the Wild BUTS together with the percentage distribution was given in Table 9.5.

Table 9.5: Phylogenetic affiliation of the clones together with the percentage distribution in the syntrophic butyrate community encountered in the UASB reactor of the paper mill wastewater treatment plant

Taxonomic Classification	Wild BUT, # clones		Wild BUTS, # clones	
	Heavy	Light	Heavy	Light
Deltaproteobacteria	***53 (44.9%)***	***60 (50%)***	***50 (43.1%)***	***39 (34.2%)***
Syntrophobacter sp.	18	13	4	14
Desulfobulbus sp.	6	22	-	-
Desulfovibrio sp.	-	18	20	13
Desulforegula conservatrix	3		1	
Desulfonema magnum	1			
Desulfomicrobium apsheronum	-	2		
Desulfobacterium cetonicum	-	1	-	3
Desulfacinum sp.	3	1	1	
Desulfomonile limimaris				1
Desulfonatronum lacustre	1			
Syntrophus sp.	17	3	6	5
Bacteriovorax sp.			11	
Thermodesulforhabdus norvegicus	1		3	
Others	3		4	3
Gammaproteobacteria	***5 (4.2%)***	***6 (5%)***	***6 (5.2%)***	***1 (0.9%)***
Betaproteobacteria			***1 (0.9%)***	***2 (1.8%)***
Alphaproteobacteria		***1 (0.8%)***		***1 (0.9%)***
Firmicutes	***12 (10.2%)***	***10 (8.3%)***	***8 (6.9%)***	***8 (7%)***
Syntrophomonas sp.	-	1	2	
Thermoanaerobacter sp.	11	7	2	4
Others	1	2	4	4
Chloroflexi	***27 (22.9%)***	***28 (23.3%)***	***23 (19.8%)***	***54 47.4(%)***
Anaerolinea thermophila	18	23	13	49
Caldilinea aerophila	1	5	5	2
Dehalococcoides sp.	8	-	5	3
Bacteroidetes	***7 (5.9%)***	***10 (8.3%)***	***19 (16.4%)***	***2 (1.8%)***
Verrucomicrobia	***7 (6%)***	***2 (1.7%)***		***1 (0.9%)***
Acidobacteria		***2 (1.7%)***	***2 (1.7%)***	***1 (0.9%)***
Actinobacteria			***2 (1.7%)***	
Nitrospirae	***1 (0.9%)***			***3 (2.6%)***
Spirochaeta	***3 (2.5%)***			
Chlorobi	***1 (0.9%)***	***1 (0.8%)***	***5 (4.3%)***	***2 (1.8%)***
Planctomycetes	***1 (0.9%)***			
Gemmatimonadetes	***1 (0.9%)***			

9.5 Archaeal DGGE Profiles and the Sequence Analysis of Density Resolved Nucleic Acids for the Wild BUT Sludge

Archaeal DGGE profiles of all gradients and the identification of the DGGE bands retrieved from the heavy fraction of the isopycnic centrifugation of the RNA isolated from the Wild BUT sludge was given in Figure 9.9. In this figure, lanes 1-10 corresponded with the gradient from heavy to light fraction. The *Archaea* domain contained three OTUs distributed within the archaeal kingdoms *Euryarchaeota.* The three major *Archaea* OTUs are represented by *Methanobacterium beijingense*, *Methanobacterium formicicum* (Bryant and Boone, 1987) and *Methanosaeta concilii*. (Table 9.6). It can be classified into two groups as hydrogen or formate utilizing methanogens: *M. beijingense*, *M. formicicum,* and as acetate utilizing methanogen: *M. concilii*. However, the ratios of acetate and hydrogen utilizing methanogens changed in an opposite way compared to the Wild BUTS enrichment. While the hydrogenotrophic methanogens increased to 62.5%, the acetotrophic methanogen covered the remaining part of the clone library. In contrast to the archaeal component of the Wild BUTS enrichment, the *M. hungatei* was replaced by *M. formicicum.*

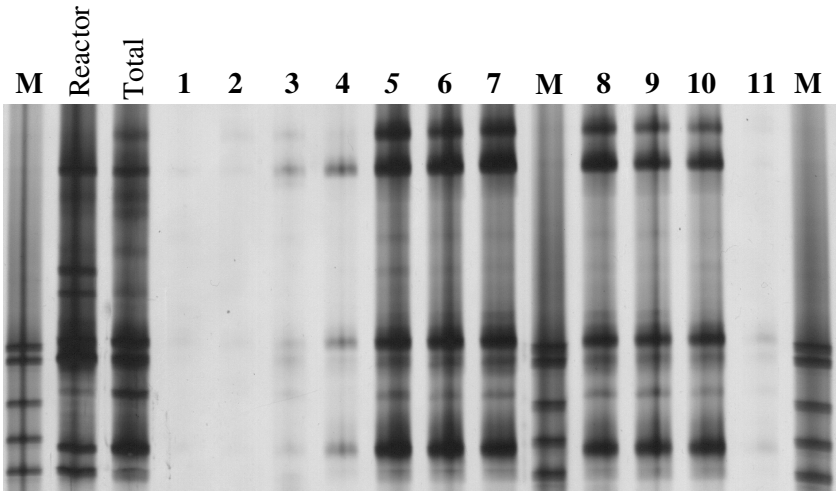


Figure 9.9 : Archaeal DGGE profiles of amplified 16S rRNA from the wild UASB reactor sludge incubated with the [^{13}C] butyrate

Table 9.6: Phylogenetic assignment of the archaeal wild BUT clones isolated from the heavy fraction of the SIP experiment

Clone	Closest phylotype	% Seq. sim.	Closest cultured species	% Seq. sim.	Taxonomic position (class, family, or genus)	# Clones	% Freq.
MLK03_B03	archaeon clone CM17	92	*Methanobacterium beijingense* strain 4-1	92	Euryarchaeota; Methanobacteriaceae	**11**	47.4
MLK03_A08	clone 1G1	99	*Methanobacterium formicicum* strain FCam	96	Euryarchaeota; Methanobacteriaceae	**4**	15.8
MLK03_A11	clone 107	99	*Methanosaeta concilii*	99	Euryarchaeota; Methanosaetaceae	**9**	36.8

9.6 Archaeal DGGE Profiles and the Sequence Analysis of Density Resolved Nucleic Acids for the Wild BUTS Sludge

Archaeal DGGE profiles of all gradients and the identification of the DGGE bands retrieved from the heavy fraction of the isopycnic centrifugation of the RNA isolated from the Wild BUT sludge was given in Figure 9.10. Most archaeal sequences of the Wild BUT heavy fraction were ≥98% similar to the 16S rRNA gene sequences of cultured organisms, except the clone distantly related to *Methanococcus maripaludis* (%83) (Table 9.7). The majority of the prominent bands from the archaeal DGGE profile as well as a major proportion of the clone library (66.7% of the total clone library) was *Methanosaeta* sp. acetate utilizing methanogen (Patel, 1984). *Methanospirillum hungatei* (Ekiel *et al.*, 1983), *Methanobacterium beijingense* (Ma *et al.*, 2005), and *Methanococcus maripaludis* (Keswani *et al.*, 1996) covered the remaining part of the clone library. These isolates shared a set of phenotypic characteristics that included the utilization of H_2 plus CO_2/HCO_3^- or formate as an energy source.

9.7 Discussion

It is very difficult to assess the best method for observing the most active microorganisms in mixed cultures. In this study, to understand the genetic diversity of the complex microbial diversity, the conceptual design of the experiments were conducted to mimic in situ conditions to approach more real conditions as much as possible. For example, the ^{13}C labelled butyrate was fed together with the actual wastewater, which was fed to the full-scale anaerobic bioreactor. Since the feeding conditions were not changed from the actual situation, the syntrophic butyrate

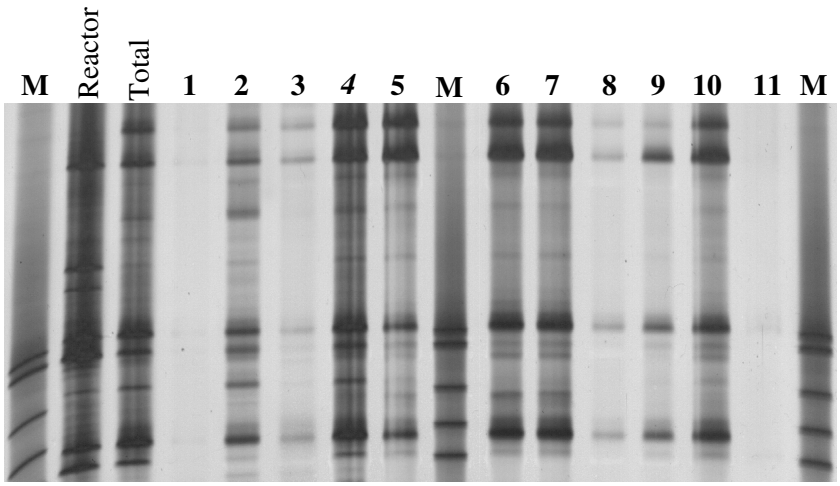


Figure 9.10 : Archaeal DGGE profiles of the amplified 16S rRNA from the wild UASB reactor sludge incubated with the [^{13}C] butyrate plus the 3mM sulfate

Table 9.7: Phylogenetic assignment of the archaeal wild BUTS clones isolated from the heavy fraction of the SIP experiment

Clone	Closest phylotype	% Seq. sim.	Closest cultured species	% Seq. sim.	Taxonomic position (class, family, or genus)	# Clones	% Freq.
MLK03_C08	archaeon F4/B-1	98	*Methanospirillum hungatei*	98	Euryarchaeota; Methanospirillaceae	1	3.8
MLK03_D01	clone CM17	99	*Methanobacterium beijingense* strain 8-2	99	Euryarchaeota; Methanobacteriaceae	5	18.8
MLK03_F09	clone 5LOC9	100	*Methanosaeta concilii* strain FE	99	Euryarchaeota; Methanosaetaceae	7	22.5
MLK03_F11	clone 107	99	*Methanosaeta concilii*	99	Euryarchaeota; Methanosaetaceae	9	36.3
MLK03_F08	clone OuI-15	99	*Methanococcus maripaludis*	83	Euryarchaeota; Methanococcaceae	2	10.0

degraders would be selectively separated by the density of the nucleic acids. These results can be assigned directly to natural systems, assuming the same environmental conditions. Subsequently applied DGGE profiling method was also useful for following the changes of the presence of species along the centrifugation gradient, and was helpful for observing the heavy and light fraction differences.

The composition of the *Bacteria* and *Archaea* community in the syntrophic butyrate degradation environment in the full-scale UASB reactor of paper mill wastewater was determined by the 16S rRNA phylogenetic analyses of clone libraries derived from RNA extracted from the density resolved gradient of the SIP. Taking into account around 120 bacterial and 24 archaeal clones from each heavy and light fraction of the two different enrichments, 16S rRNA gene libraries constructed from

the original sludge.These clone libraries were analyzed by comparing the DGGE and RFLP fragment patterns of the amplified 16S rRNA genes.

This diverse active member of the syntrophic butyrate degraders were represented by grouping at least 10 different phyla, which showed more diversity than recent studies of the bacteria capable of syntrophic metabolism in terms of both phylogenetically and physiologically (Schink, 1997). This functional group of organisms does not fall into the phylogenetically consistent groups; rather, it spreads out into several lineages. In most cases, closest uncultured relatives have been identified from anaerobic ecosystems. These were the majority of the microbial community associated with deep subsurface aquifer, anaerobic dechlorinating mixed cultures equine fecal contaminated sites and bioreactors. Sequence representatives of several bacterial divisions have been identified in a wide range of habitats, suggesting the sophisticated distribution of the corresponding organisms in the environment and, potentially, their wide metabolic capabilities.

The 16S rRNA gene clone library showed that the largest groups of clones belonged to the members of the *Proteobacteria*, which were not what was expected from the community of syntrophic butyrate degradation that belong to the phyla of *Firmicutes*. The main possible role of the butyrate degradation was attained to the *Syntrophus* sp.. Sequence types associated with the genus *Syntrophus* sp. can produce energy from the anaerobic oxidation of organic acids, with the production of acetate and hydrogen. However, it was also found that the *Syntrophobacter* sp., known as propionate degrader, also played an active role in the butyrate degradation.

Since many of the fatty acid degraders can be grown in pure culture as homoacetogens, sulfate reducers, or fermentative anaerobes (Beaty and McInerney, 1987; Chen *et al.*, 2005; Stams *et al.*, 1993) in the mixed cultures, these degraders might be stimulated to function these features, depending on the energetic level of the consortia. Related to these energetic conditions of the consortia, the syntrophs may also conserve energy from an electron-transport-linked (a proton motive force) reduction of an unsaturated intermediate as shown for the reduction of crotonate in *Syntrophomonas wolfei* (Wallrabenstein and Schink, 1994). The presence of cytochromes in *Syntrophomonas wolfei* indicates that these organisms may contain an electron transport chain (McInerney and Wofford, 1992).

If the clone library structures of this study were compared with other butyrate degrading community studies represented in Chapter 7 and 8, main differences would be observed especially in terms of the dominant members of the consortia. Since the dominant species of the butyrate fed reactor was one of the unclassified microorganism (Chapter 8); in this study, *Syntrophus* and *Syntrophobacter* sp. were found as the dominant members of the community. While the *Syntrophomonas* sp. was observed in a different range depending on the incubation type e.g. batch or continuous, none of them were detected in the Wild BUT enrichment. This discrepancy can be explained with the different incubation conditions and the molecular techniques, since other enrichments were acclimatized with butyrate under batch or continuous operations. However, in this study, the sludge was fed with isotopic butyrate together with the original wastewater trying to reflect the real conditions as much as possible according to the original sludge history.

It was also observed that some bands could represent more than one phylotype even from different bacterial divisions due to the comigration of the amplicons for the same profile. Prominent bands in the DGGE pattern were consistent with the frequency of clone percentages observed for most of the clones by detecting the dominant members of the microbial community. However, other important players could have been underestimated in minor cases for these complex mixed cultures. Another important observation was the detection of multiple bands corresponding to one species. Even if a single nucleotide was changed, it would give multiple bands in the DGGE patterns. These multiple bands were also identified in the single profiles of the DGGE profiles of density resolved gradient.

The limitation of the SIP technique is the utilization of the labeled substrates by different trophic groups of microorganisms. After the utilization of the labeled substrate, organisms may produce a labeled intermediate, and this intermediate can be used by other trophic groups, creating confusion and mislead about the target active populations (Radajewski *et al.*, 2000). Though these kinds of cross feeding effects may be detected and alleviated with nucleic acids fingerprinting of time series incubations (Radajewski *et al.*, 2002; Whiteley *et al.*, 2005). The abovementioned limitations were overcome in this study by using the same method explained above.

The techniques linking the identity to the function mentioned above can be used individually or complementary to explain the different insight or feature of different

microbial communities. This approach provided an effective mean to address the physiology and phylogeny of the syntrophic butyrate degrading organisms in their natural environments. By using these techniques, potential roles of the strain-specific microorganisms involved in the syntrophic butyrate degradation *in situ* in the environment were achieved. Additionally, attempts will be made to cultivate these wide range of organisms identified in these heavy fractions of the clone libraries to determine their specific physiology and metabolic traits.

10. POPULATION DYNAMICS OF ANAEROBIC SLUDGE AT DIFFERENT TEMPERATURES WITH DIFFERENT FEED MIXTURES

The objectives of this study were monitoring and evaluating the bacterial population shifts, which were strongly dictated by substrate composition and temperature using DGGE and sequencing. Instead of feeding single carbon sources individually, thus introducing a bias in favour of the members of the mixed populations that could utilize that particular C-source; mixtures of different C-sources were fed to the batch mixed cultures to mimic the real-world conditions as much as possible and to correlate the biochemical performance to population dynamics. For that purpose, the consumption of each C-source in the mixtures were monitored at the supernatants sampled during the course of the entire incubation; and these biochemical results were juxtapositioned with the microbial community shifts determined for the biomass samples harvested at the corresponding time points.

10.1 Biochemical Performance of the Incubations

Biochemical performance data of all batch incubations were given in Table 10.1. Starch was degraded at all temperatures, while acetate, propionate and butyrate were not degraded or were degraded poorly at some temperatures. At 25 and 37ºC, the feed mixture A composed of starch (720 mg/L), acetate (460 mg/L), propionate(212 mg/L) and a little amount of butyrate (24 mg/L), was totally degraded, except the propionate at 25ºC. At 45ºC, although propionate and butyrate were not degraded, a small amount of acetate was consumed. At 55 and 65ºC, the consumption of butyrate, propionate and acetate was not clear, due to the starch fermentation. During fermentation of starch, the products might be in a wide range such as acetate, propionate, butyrate, ethanol, lactate, etc. (Wolff *et al.*, 1993). Therefore, it was not easy to comment on acetate, butyrate and propionate degradations whether they were consumed or not; and if consumed, to what extent they were consumed, because of the accumulation of the fermentation products.

Table 10.1: Experimental and calculated data for the mass balance of the anaerobic degradation in batch cultures enriched at 6 different temperatures (10, 25, 37, 45, 55 and 65ºC) with 4 different feed mixtures (A, B, C, D)

Incubation temperature (°C)	Feed mixture	Max. growth OD	Substrates concentrations at the beginning and the end of incubations (mM)										Product (mM)	Max. observed H_2 and formate (mM)		% carbon degraded by		Electron recovery (%)
			Starch*		Acetate		Propionate		Butyrate		SO_4							
		A_{600}	Beg.	End	Beg.	End	Beg.	End	Beg.	End	Beg.	End	CH_4	H_2	Formate	MPA	SRB	
10	A	0.256	4.98	<0.1	7.51	10.04	2.73	3.56	0.31	0	-	-	9.75	0.41	6.27	100	-	83.1
	B	0.234	5.07	<0.1	7.64	9.25	2.84	3.31	0.29	0.33	2.05	0.11	9.22	0.19	5.45	82.6	17.4	89.3
	C	0.008	-	-	9.89	9.40	5.11	4.94	4.95	4.92	-	-	N.D.	N.D.	N.D.	-	-	-
	D	0.105	-	-	9.93	9.12	5.05	4.36	4.79	4.06	29.58	25.97	N.D.	N.D.	N.D.	-	100	69.5
25	A	0.377	5.13	<0.1	7.79	0	2.78	4.55	0.20	0	-	-	19.12	N.D.	N.D.	100	-	92.9
	B	0.580	5.09	<0.1	7.87	0	3.00	2.45	0.27	0	1.91	0.02	20.25	N.D.	N.D.	91.5	8.5	88.8
	C	0.263	-	-	10.05	0	4.94	4.24	4.93	0	-	-	21.10	N.D.	N.D.	100	-	89.5
	D	0.149	-	-	10.18	0	5.07	0	5.03	0	31.03	18.02	13.74	N.D.	N.D.	51.4	48.6	84.6
37	A	0.524	5.21	<0.1	7.85	0	2.86	0	0.24	0	-	-	29.75	N.D.	N.D.	100	-	102.2
	B	0.444	5.05	<0.1	7.74	0	2.91	0	0.33	0	1.91	0.15	24.93	1.29	N.D.	93.4	6.6	92.6
	C	0.212	-	-	9.99	0	4.94	0	4.93	0	-	-	30.09	N.D.	N.D.	100	-	97.2
	D	0.312	-	-	10.31	0	5.09	0	4.92	0	29.70	15.42	14.61	N.D.	N.D.	50.6	49.4	91.6
45	A	0.213	5.02	<0.1	7.78	14.94	2.78	2.93	0.25	0.27	-	-	6.68	0.23	N.D.	100	-	88.1
	B	0.464	5.12	<0.1	7.75	16.25	2.88	0	0.28	0.30	1.99	0.04	8.60	0.16	N.D.	81.5	18.5	89.0
	C	0.115	-	-	9.96	22.42	5.10	0	4.86	0	-	-	7.87	N.D.	N.D.	100	-	91.4
	D	0.314	-	-	10.29	9.93	5.01	0	4.92	0	30.90	12.41	0.19	N.D.	N.D.	1.1	98.9	87.6
55	A	0.336	5.36	<0.1	7.84	10.06	2.86	2.14	0.27	0.23	-	-	12.75	0.50	N.D.	100	-	84.2
	B	0.316	5.02	<0.1	7.75	11.84	2.91	2.74	0.29	0.21	2.22	0.54	9.15	N.D.	N.D.	84.5	12.5	93.4
	C	0.076	-	-	9.95	8.13	5.08	4.42	4.91	4.87	-	-	0.14	N.D.	N.D.	100	-	-
	D	0.142	-	-	10.09	8.95	4.91	3.87	4.93	4.82	28.95	27.41	2.25	N.D.	N.D.	59.3	40.7	110
65	A	0.221	5.20	<0.1	7.76	12.44	2.76	3.04	0.26	0.53	-	-	4.27	N.D.	12.99	100	-	43.8
	B	0.265	5.14	<0.1	7.71	13.33	2.72	2.74	0.28	0.49	2.04	0.48	2.73	0.02	N.D.	37.2	62.8	45.3
	C	0.012	-	-	9.88	9.25	4.93	4.84	5.03	5.05	-	-	N.D.	N.D.	N.D.	-	-	-
	D	0.087	-	-	9.74	8.78	4.97	4.65	4.96	4.75	29.39	28.24	N.D.	N.D.	N.D.	-	100	56.2

* Starch values given as equivalent of glucose

N.D.: Not Detected, MPA: Methane Producing Archaea, SRB: Sulfate Reducing Bacteria

While the addition of 3 mM sulfate to the feed mixture A (called feed mixture B) did not change the degradation rates compared to the situation at 10, 25, 37 and 45ºC, it significantly affected the culture incubated at 45ºC with the degradation of propionate even in the presence of 16 mM acetate. It was known that at higher acetate concentrations (>5 mM), propionate degradation was easily inhibited under methanogenic conditions (Hyun *et al.*, 1998). In the presence of limited amount of sulfate, which was less than the reducing equivalent necessary for the reduction of propionate, the propionate degradation might be initiated and then completed. This would have to be elucidated with further studies. It was also observed that in all temperatures, more than 75% of the sulfate was reduced.

In feed mixture C, supplemented with only butyrate, propionate and acetate, growth was observed only at 25, 37, and 45ºC. While all constituents of the feed were consumed at 37ºC, at 45ºC, they were not degraded and acetate was produced in stoichiometries indicative of dependence on an acetyl coenzyme A (acetyl-CoA) pathway (Stams, 1994). This result indicated that acetate utilizing methanogens were more sensitive to high temperature than the hydrogen utilizing methanogens. In the anaerobic ecosystem, mesophilic and thermophilic microorganisms do not function at the intermediate temperature of 40 to 45ºC (Rittmann and McCarty, 2001). This statement might be especially true for the acetate utilizing methanogens. However, in this study, it was found that the acetogenic bacteria and hydrogen utilizing archaea were active at 45ºC. In this respect, it could be speculated that the mesophilic range for upper limit would be 45ºC except for the acetotrophic methanogens.

For feed mixture D, competition between sulfate reducers and methanogens for electron donors was influenced by the temperature. A comparison of methanogenesis and sulfate reduction showed that ~50% of the acetate, propionate and butyrate mixture was degraded completely by sulfidogens at 25 and 37ºC. On the contrary, at 45ºC, all propionate and butyrate was degraded by sulfidogens without the production of acetate demonstrating complete oxidation of butyrate and propionate to CO_2 and the present acetate in the feed mixture was not degraded at all. At other temperatures (10, 55, and 65ºC), although some slight decrease was observed for butyrate, propionate and acetate concentrations, such a decrease was not significant to be taken into account.

Based on the biochemical data at 10ºC incubations, although some slight decrease was observed for some components of the feed mixtures, it could be remarked that sludge was not acclimatized at this temperature for short chain fatty acids degradation. However, as observed for all test temperatures, starch was readily consumed. These findings also were observed and were validated for the incubations enriched at 55 and 65ºC, even though the decrease of substrate concentrations was a little bit higher than those of the 10ºC incubations (Table 10.1).

10.2 Comparison of the DGGE Profiles of Eerbeek Sludge Cultivated at Different Temperatures and Different Feed Mixtures

Bacterial community fingerprint of the Eerbeek sludge enriched at 10, 25, 37, 45, 55, and 65°C with different feed compositions A, B, C, and D was given in Figure 10.1. In this figure, while the profiles of the feed mixture A and B showed in upper panel, C and D showed in lower panel. Primers used in PCR-DGGE were F968-GC and R1401. Numbers, letters and M indicated the temperatures, the feed mixtures and the marker, respectively. To assess the changes in the DGGE profiles, similarity index was calculated based on the Pearson correlation coefficient, illustrated with a dendogram (Figure 10.2). Clustering of patterns was calculated using the unweighted-pair group method using average linkages (UPGMA). In Figure 10.2, while the correlation between different profiles of Bacteria of the feed mixture A and B showed in upper panel, C and D showed in lower panel. The first letter B showed the Bacteria. Numbers, second letters and Mark E indicated the temperatures, feed mixtures and the marker (the original Eerbeek sludge), respectively.

Assuming each band in the DGGE profiles being a representative of specific strains, it could be said that the number of distinctive bands is proportional to the diversity of the clone libraries. Although these correlations between clone libraries and corresponded DGGE profiles were observed for some of the incubations, most of them were not correlated well with each other. This might be due to the PCR amplification biases as well as the high 16S rRNA copy numbers of the species which can result in detection of multiple bands corresponding to one species in the DGGE profiles.

For the feed mixture of A and B, the 37 and 45°C enrichments were clustered with more than 50% similarity (Figure 10.2). At 37°C for the feed mixture of A and B, the

profiles were as closely related as 78%, which showed that the presence of sulfate in the starch based feed composition did not significantly change the microbial structure. However, this similarity was a little lower, (58%), for the enrichment incubated at 45°C. Similar clustering properties were also observed for the incubation temperatures 25 and 10°C. Although the enrichment of 55°C-B was similar to 10°C-B, with a similarity coefficient of 56%, 55 and 65°C temperatures made an outgroup with a similarity of less than 40%.

The feed mixtures of C and D showed a different trend than the feed of A and B. While feed mixture C clustered together for the enrichments incubated at 25, 37 and 45°C with a similarity of more than 60%, the feed mixture D also clustered together for the same temperatures with a similarity of more than 65%. This clustering mainly depended on the differences of the feed mixtures just as the C composition shown the methanogenic syntrophs, and the D composition shown the sulfidogens. It was also clearly shown that the mesophilic range of these C and D composition were between 37 and 45°C. At 10, 55, and 65°C, there was not a noticeable degradation of the feed components observed; so, they were not taken into account for further analysis.

To assess the effect of different temperatures and the feed mixture on the archaeal community structure, DGGE profiles were generated (Figure 10.3). In this figure, while the profiles of the feed mixture A and B showed in upper panel, C and D showed in lower panel. Primers used in PCR-DGGE were F109(T) and R515-GC. Numbers, letters and M indicated the temperatures, the feed mixtures and the marker, respectively. Archaeal population dynamics significantly changed during a temperature shift from 45 to 55°C in the feed mixture of A and B. Obviously, different archaeal communities were established at the two different temperatures. However, the archaeal population remained the same along all temperatures in the feed mixture of C and D. This indicates that archaeal populations were more sensitive in the saccharolytic cultures than in the syntrophic cultures.

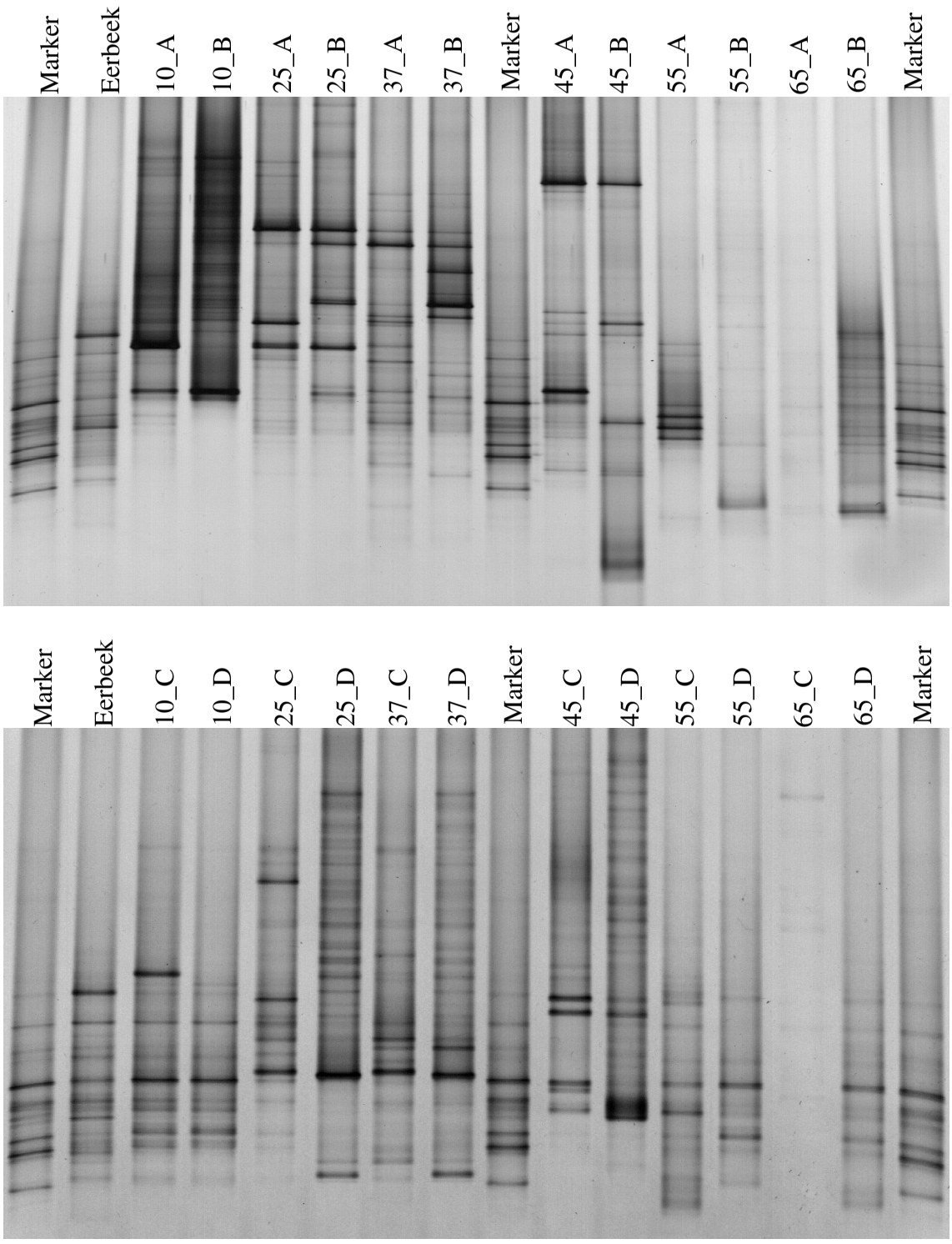


Figure 10.1 : Bacterial community fingerprint of the Eerbeek sludge enriched at different temperatures with different feed compositions

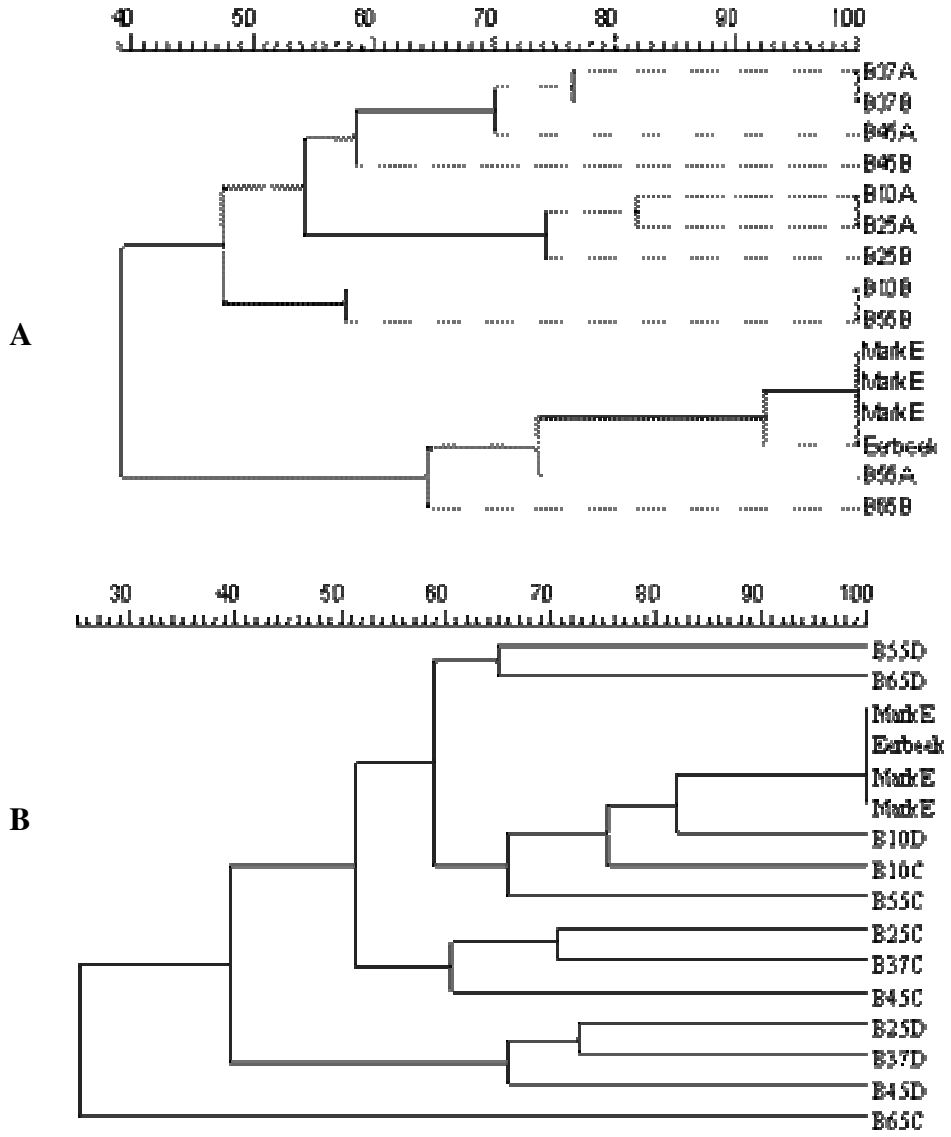


Figure 10.2 : Dendograms of the DGGE gels given in Figure 10.1

In Figure 10.2, while the correlation between different profiles of Archaea of the feed mixture A and B showed in upper panel, C and D showed in lower panel. The first letter A showed the Archaea. Numbers, second letters and Mark E indicated the temperatures, feed mixtures and the marker (the original Eerbeek sludge), respectively.

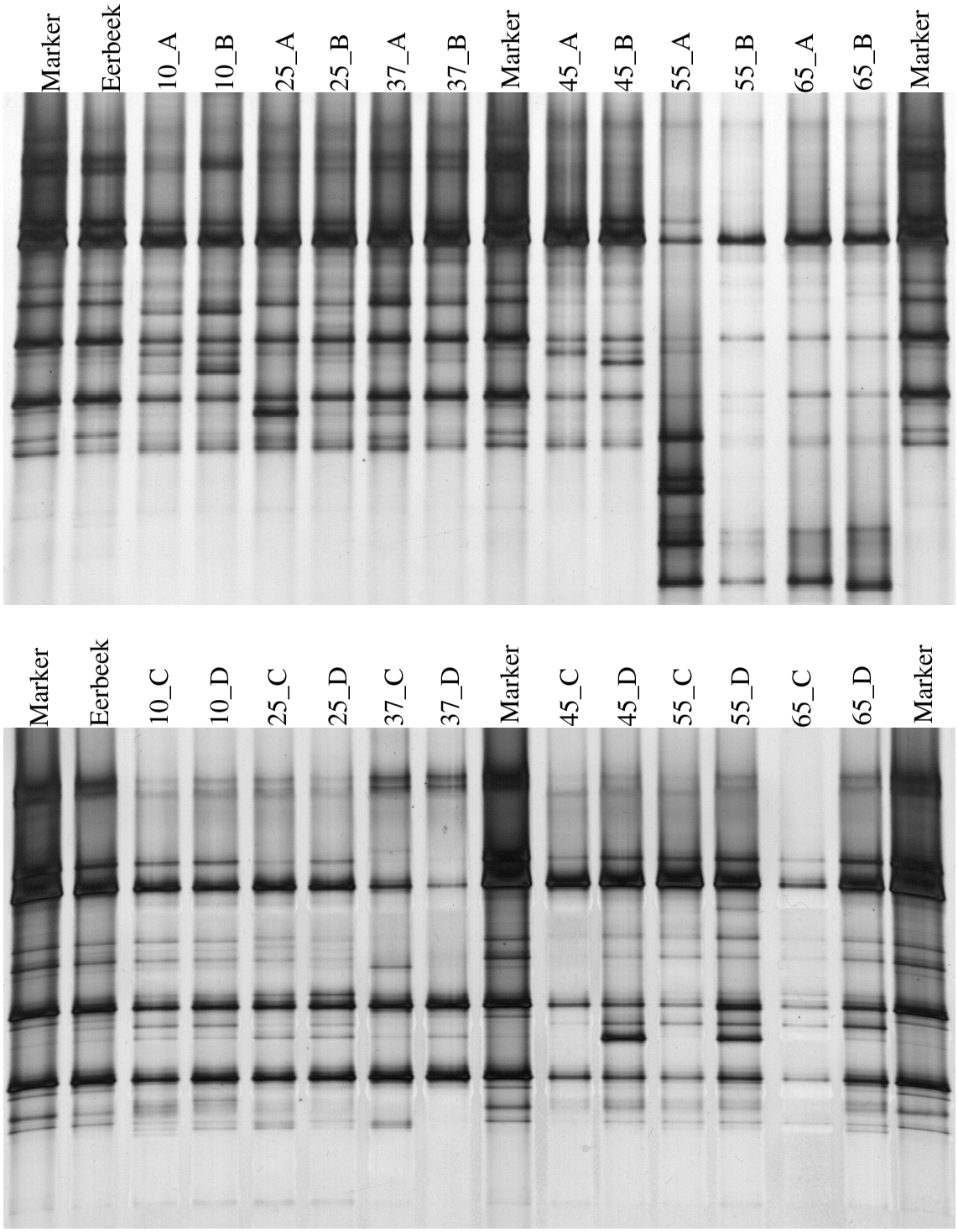


Figure 10.3 : The archaeal community fingerprint of the Eerbeek sludge enriched at different temperatures with different feed compositions

The archaeal profiles showed a high similarity with each other by more than 70% in general (Figure 10.4). However, the feed mixture of A and B at 55 and 65°C demonstrated a significant dissimilarity compared to other incubations. The PCR-DGGE patterns from 25 and 37°C for the feed mixture A showed high similarity to the Eerbeek sludge (90 and 91% for 25 and 37°C, respectively).

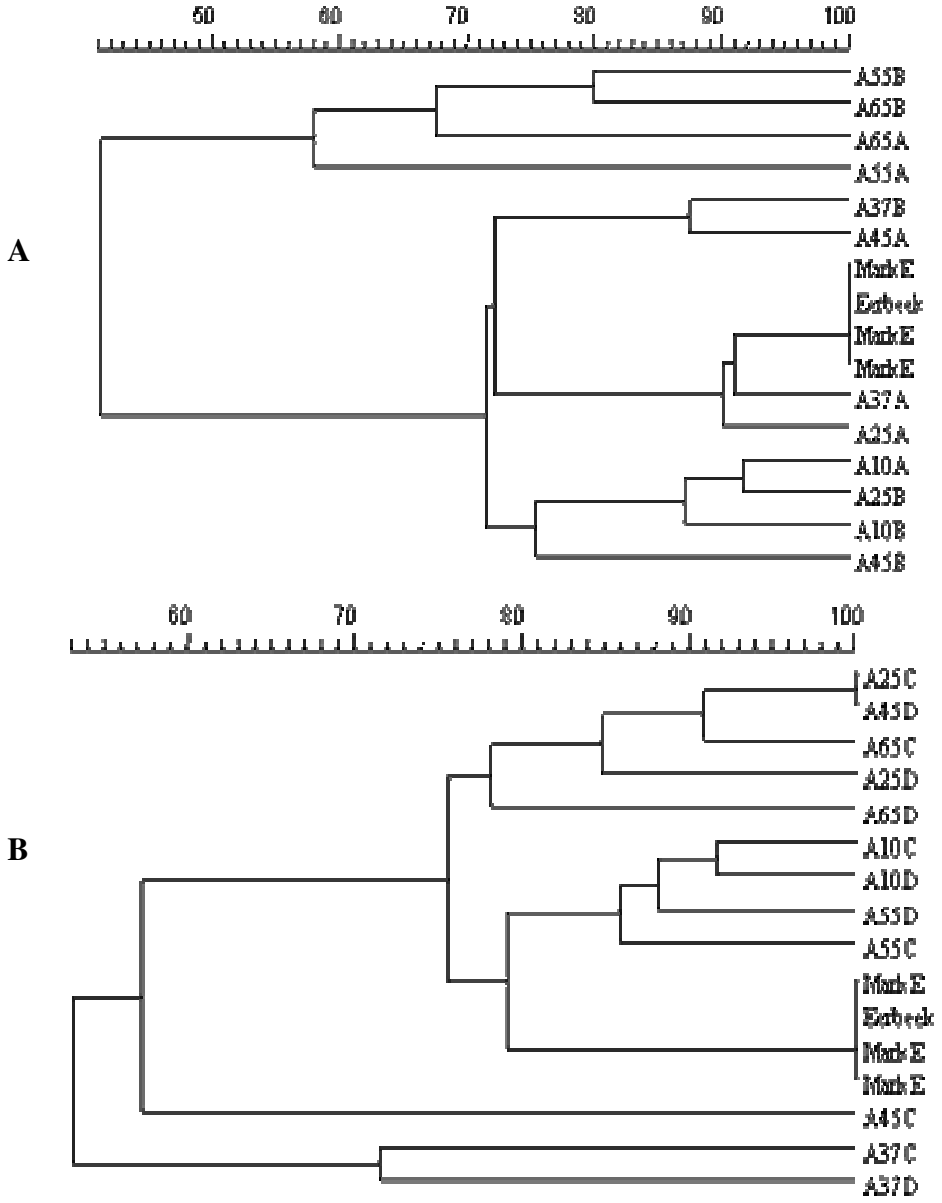


Figure 10.4 : Dendograms of the DGGE gels given in Figure 10.3

10.3 Bacterial Clone Library

10.3.1 Clone library of the batches incubated at 10°C

10.3.1.1 Feed mixture-A at 10°C

The phylogenetic assignment of the bacterial clones isolated from the enrichment incubated at 10°C with feed mixture-A was given in Table 10.2. The majority of the bacterial 16S rRNA gene clones (61%) were related to Firmicutes, and the remaining part of the clones were composed of the lineage of Gammaproteobacteria. The dominant clones Bacterium sp. A15 were the isolates from microbial communities in two production waters of a low-temperature and low-salinity petroleum reservoir, which was consistent with the present temperature conditions (Grabowski *et al.*, 2005a). The cultured filamentous bacterium *Trichococcus flocculiformis* isolated from the bulking sludge showed fermentative properties (Scheff *et al.*, 1984). Considering the feed mainly made up with starch, consequently, the culture would be dominated by the fermentative bacteria.

Another significant member of the psychrophilic fermenting culture was *Tolumonas auensis* associated clones (32%), typically known as the degrader of most common carbohydrates (Fischer-Romero *et al.*, 1996). The minor group of this library were also other fermentative bacteria, *Aeromonas* sp. (7%) retrieved from digestive tract symbionts of animals (Morandi *et al.*, 2005). The biochemical result of this incubation demonstrated that the main fermentative production of the starch was acetate and propionate, and this consortium did not consume these products. Therefore, it can be concluded that this consortium was totally covered up with the anaerobic fermentative bacteria based on the physiological and phylogenetic results.

Table 10.2: Phylogenetic assignment of the bacterial clones isolated from Eerbeek sludge enriched at 10°C with Feed Mixture A

Clone	Length (bp)	Closest phylotype	% Seq. sim.	Closest cultured species	% Seq. sim.	Taxonomic position (class, family, or genus)	# Clones	% Freq.
DF1_049	435	Bacterium sp. A15	99	*Trichococcus flocculiformis*	99	Firmicutes; Carnobacteriaceae	22	49.8
DF1_003	432	Bacterium sp. A15	90	*Trichococcus flocculiformis*	90	Firmicutes; Carnobacteriaceae	2	4.5
DF1_089	439	clone:TSAS03	88	*Trichococcus flocculiformis*	88	Firmicutes; Carnobacteriaceae	1	2.3
DF1_066	219	*Trichococcus* sp. Coc4	99			Firmicutes; Carnobacteriaceae	1	2.3
DF1_049	410	*Bacillus thermoleovorans*	93			Firmicutes; Bacillales; Geobacillus	1	2.3
DF1_073	435	Bacterium H4	99	*Tolumonas auensis*	97	Gammaproteobacteria; Aeromonadaceae	3	6.9
DF1_018	436	Bacterium H4	95	*Tolumonas auensis*	94	Gammaproteobacteria; Aeromonadaceae	6	25.0
DF1_001	435	*Aeromonas* sp. 'CDC 715-84'	98			Gammaproteobacteria; Aeromonadaceae	1	2.3
DF1_090	311	clone W4-B32	95	*Aeromonas salmonicida* subsp. achromogenes	95	Gammaproteobacteria; Aeromonadaceae	1	2.3
DF1_065	224	clone SD164	97	*Aeromonas hydrophila*	96	Gammaproteobacteria; Aeromonadaceae	1	2.3

10.3.1.2 Feed mixture B at 10°C

The presence of the 3 mM sulfate in addition to the feed mixture A, called the feed mixture B, did not affect the diversity of the incubation at 10°C; and even caused *Trichococcus flocculiformis* to increase, being the dominant (91%) of the total community (Table 10.3). Moreover, the biochemical properties did not change significantly either; apart from detecting the hydrogen sulphide, which corresponded to 17% of the carbon source, indicating the presence of the sulfate reducing bacteria. However, the clone library did not include any known sulfate reducers.

Table 10.3: Phylogenetic assignment of the bacterial clones isolated from Eerbeek sludge enriched at 10°C with Feed Mixture B

Clone	Length (bp)	Closest phylotype	% Seq. sim.	Closest cultured species	% Seq. sim.	Taxonomic position (class, family, or genus)	# Clones	% Freq
DF1_012	433	Bacterium sp. A15	100	*Trichococcus flocculiformis*	100	Firmicutes; Carnobacteriaceae	9	20.0
DF1_020	434	Bacterium sp. A15	99	*Trichococcus flocculiformis*	99	Firmicutes; Carnobacteriaceae	32	71.1
DF1_013	285	*Trichococcus* sp. Coc4	100			Firmicutes; Carnobacteriaceae	1	2.2
DF1_068	438	*Paludibacter propionicigenes*	97	*Dysgonomonas shahii*	91	Bacteroidetes; Porphyromonadaceae	3	6.7

Paludibacter propionicigenes species were detected as a different clone in this clone library with an abundance of 7%. These Bactoroidales species were known as anaerobic propionate producing bacteria (Ueki *et al.*, 2006). The presence of these species could be assigned to the biochemical results of this enrichment by observing the increment of the propionate concentration. Besides, as observed at feed mixture A, the members of this clone library were designated as carbohydrate fermenting bacteria.

10.3.2 Clone library of the batches incubated at 25°C

10.3.2.1 Feed mixture A at 25°C

The clone library retrieved from the enrichment of feed mixture A at 25°C demonstrated that the dominant clones were clustered in three different groups (Table 10.4). These were the saccharolytic microorganisms, which covered 78% of the clone library. *Acetobacterium malicum* (29%) (Tanaka and Pfennig, 1988) and *Acetobacterium woodii* (4%) (Sharak Genthner and Bryant, 1987) ferment H_2/CO_2, formate and several sugars to acetate; therefore it could be suggested that it could participate to the degradation of starch in addition to growing autotrophically on H_2/CO_2, formate and CO. These *Acetobacterium* species are psychrophilic acetogens and some strains can grow up to the temperature of 35°C. (Kotsyurbenko *et al.*, 1995). *Ruminococcus* related species are as abundant as 31% of the total clone library. These rumen sourced species were mainly affiliated with saccharolytic microorganisms. As observed at 10°C cultures *Trichococcus flocculiformis*, a fermentative bacterium, was also detected in this clone library.

Table 10.4: Phylogenetic assignment of the bacterial clones isolated from Eerbeek sludge enriched at **25°C** with **Feed Mixture A**

Clone	Length (bp)	Closest phylotype	% Seq. sim.	Closest cultured species	% Seq. sim.	Taxonomic position (class, family, or genus)	# Clones	% Freq
DF1_055	435	*Acetobacterium malicum*	98			Firmicutes; Clostridia	13	28.9
DF1_063	434	equine intestinal eubact sp. PL37	96	*Ruminococcus hydrogenotrophicus*	95	Firmicutes; Clostridia	12	26.7
DF2_017	416	Bacterium sp. A15	100	*Trichococcus flocculiformis*	100	Firmicutes; Lactobacillales	10	22.4
DF1_079	427	*Ruminococcus palustris*	93			Firmicutes; Clostridia	2	4.4
DF2_009	434	*Acetobacterium woodii*	91			Firmicutes; Clostridia	2	6.6
DF1_032	442	*Carnobacterium mobile*	90			Firmicutes; Lactobacillales	2	4.4
DF1_071	435	*Aeromonas simiae*	96			Gammaproteobacteria; Aeromonadaceae	2	4.4
DF1_096	441	clone SD164	85	*Glaciecola mesophila*	84	Gammaproteobacteria; Alteromonadaceae	1	2.2

The saccharolytic bacteria, which covers the minor group in the clone library, were *Carnobacterium mobile* (Collns *et al.*, 1987), *Aeromonas simiae* (Harf-Monteil *et al.*, 2004), and *Glaciecola mesophila* (Romanenko *et al.*, 2003). These bacteria are chemoorganotrophic, having both oxidative and fermentative metabolisms, and hydrolyzing polysaccharides. In general, there were both psychrophilic and mesophilic microorganisms detected in the 25°C culture.

10.3.2.2 Feed mixture B at 25°C

Analyses of the 16S rDNA sequences indicated that all of the isolates retrieved in this clone library were fermentative bacterium, *Trichococcus flocculiformis* (Table 10.5). The dominance of these species were stimulated by the addition of the 3 mM sulfate to the feed mixture A at the same temperature. The similar situation was also observed for the 10°C incubation, as the presence of sulfate increased the *Trichococcus flocculiformis* from 55% to 91% for the same feed mixtures. At 25°C, the abundance of these species increased from 22% to 100%. Although this amount would be affected by the PCR amplification of the partial 16S rRNA, the trend of the increment of these species clearly demonstrated the presence of sulfate, which encouraged them to dominate in the consortium.

Table 10.5: Phylogenetic assignment of the bacterial clones isolated from Eerbeek sludge enriched at **25°C** with **Feed Mixture B**

Clone	Length (bp)	Closest phylotype	% Seq. sim.	Closest cultured species	% Seq. sim.	Taxonomic position (class, family, or genus)	# Clones	% Freq
DF2_081	443	Bacterium sp. A15	100	*Trichococcus flocculiformis*	100	Firmicutes; Lactobacillales	46	100

10.3.2.3 Feed mixture-C at 25°C

The major member of this clone library was closely related to clone IIIA-8 isolated from polluted river sediment reducing dechlorination of polychlorinated dibenzo-*p*-dioxins/ dibenzofurans (Yoshida *et al.*, 2005). The closely affiliated cultured *Geospirillium* sp. (34%) (Heising *et al.*, 1999) ferments fumarate to acetate, succinate, and carbon dioxide (Table 10.6). It could be suggested that these species are possibly participating in the propionate degradation in this habitat; however, biochemical results demonstrated that only 0.7 mM propionate was degraded. Therefore, it would not be right to link the result of this biochemical result and the abundance of *Geospirillium* related species. Therefore, it might be suggested that these species play roles in the butyrate degradation.

Eubacterium aggregans made up 20% of the clone library. This homoacetogenic bacterium mediates the reaction of hydrogen with carbon dioxide to form acetate (Mechichi *et al.*, 1998). Even though no sulfate was present, *Desulfovibrio alcoholovorans* and *Desulfobulbus elongatus* were detected to be 7% of the total clone library. SRB population probably functioned as proton reducers in syntrophic association with hydrogen and formate consuming methanogens. On the contrary, *Desulfobulbus elongatus* most likely contributed to the 0.7 mM propionate degradation in syntrohy with hydrogen utilizing methanogens or SRBs. Only a small fraction (18%) of the clone library corresponded to butyrate oxidizers of the genus *Syntrophomonas*. This would also be an indication of the presence of other bacteria that also take part in this syntrophic interaction.

Table 10.6: Phylogenetic assignment of the bacterial clones isolated from Eerbeek sludge enriched at **25°C** with **Feed Mixture C**

Clone	Length (bp)	Closest phylotype	% Seq. sim.	Closest cultured species	% Seq. sim.	Taxonomic position (class, family, or genus)	# Clones	% Freq
DF2_059	437	clone IIIA-8	99	*Geospirillium* sp. KoFum	95	Epsilonproteobacteria Campylobacteraceae; Sulfurospirillum	15	34.1
DF2_067	359	clone E21	99	*Desulfovibrio alcoholovorans*	99	Deltaproteobacteria; Desulfovibrionaceae	1	2.3
DF2_019	437	clone R1p15	94	*Desulfobulbus elongatus*	94	Deltaproteobacteria; Desulfobulbaceae	1	2.3
DF2_018	437	eubacterium OCG4'	96	*Desulfovibrio aminophilus*	96	Deltaproteobacteria; Desulfovibrionaceae	1	2.3
DF2_026	433	*Trichlorobacter thiogenes*	98			Deltaproteobacteria; Geobacteraceae; Trichlorobacter	1	2.3
DF2_051	434	clone E12	99	*Syntrophomonas* sp. MGB-C1	96	Firmicutes; Syntrophomonadaceae	8	18.1
DF2_074	434	*Eubacterium aggregans*	99			Firmicutes; Clostridia	9	20.3
DF2_083	379	*Acetobacterium fimetarium*	97			Firmicutes; Clostridia	2	4.6
DF2_058	440	*Acetobacterium wieringae*	89			Firmicutes; Clostridia	4	9.1
DF2_050	431	bacterium HB31	96	*Papillibacter cinnaminovorans*	95	Firmicutes; Clostridia; Papillibacter	1	2.3
DF2_066	432	clone LJ8	98	*Clostridium aminobutyricum*	90	Firmicutes; Clostridia	1	2.3

Acetobacterium sp. (14%) ferments H_2/CO_2, formate and several sugars to acetate; therefore, it could be suggested that it could grow autotrophically on H_2/CO_2, formate and CO in the absence of carbohydrate. These *Acetobacterium* species are psychrophilic acetogens, and *Acetobacterium fimetarium* can grow up to the temperature of 35°C (Kotsyurbenko *et al.*, 1995).

Papillibacter cinnaminovorans (2%) was isolated from an anaerobic digester fed with shea cake rich in tannins and aromatic compounds (Defnoun *et al.*, 2000). Growth is limited to some aromatic compounds and crotonate but not to carbohydrates, or organic acids. Crotonate is degraded to acetate and butyrate. However, it might degrade butyrate in syntrophic co-culturing with hydrogen utilizing methanogens.

DF2_066 was low abundant (2%) and similar to *Clostridium aminobutyricum*, 90%. *Clostridium aminobutyricum* (2%) shows extreme growth specificity for γ-aminobutyrate, and two moles of γ-aminobutyrate are converted to two moles of

ammonia, two moles of acetic acid, and one mole of butyrate in an oxidation-reduction reaction (Hardman and Stadtman, 1960).

Only a small fraction (2%) of the clone library corresponded to *Trichlorobacter thiogenes*, which grows by oxidizing acetate with the concomitant reduction of thrichloroacetic acid (De Wever *et al.*, 2000). It could be speculated that corresponding organisms were directly or indirectly involved in the butyrate degradation.

Microbial community observed with the DGGE gels, did not change along the incubations (App. III.3). Biochemical results together with database search of this clone library demonstrated that the butyrate degrading organisms were more diverse than the known *Syntrophomonas* species. Another important phenomenon was the presence of homoacetogenic bacteria, which indicates a possible reaction of the reduction of H_2 and CO_2 to acetate.

10.3.2.4 Feed mixture D at 25°C

The clone library mainly represented by Deltaproteobacteria by occupying 78% of the total clones retrieved from the cultures incubated with feed mixture D at 25°C (Table 10.7). This phylum was composed of sulfidogenic bacteria, Desulfovibrionaceae (55%), Desulfobulbaceae (22%), and Desulfobacteraceae (2%). This abundant SRB was also related with the rate of carbon source used by sulfidogens which made 49% of feed mixture-D. Beside these sulfate reducing related clones, clones clustered to Clostridia were also detected as being 7% of the total clone library.

Table 10.7: Phylogenetic assignment of the bacterial clones isolated from Eerbeek sludge enriched at **25°C** with **Feed Mixture D**

Clone	Length (bp)	Closest phylotype	% Seq. sim.	Closest cultured species	% Seq. sim.	Taxonomic position (class, family, or genus)	# Clones	% Freq
DF2-012	440	*Desulfovibrio putealis*	93			Deltaproteobacteria; Desulfovibrionaceae	24	54.3
DF2-028	441	bacterium LTCE-T2A 4A	99	*Desulfobulbus propionicus*	98	Deltaproteobacteria; Desulfobulbaceae	8	17.2
DF2-085	441	clone TANB52a	93	*Desulfobulbus propionicus*	86	Deltaproteobacteria; Desulfobulbaceae	1	2.2
DF2-044	438	clone R1p15	91	*Desulfobulbus elongatus*	91	Deltaproteobacteria; Desulfobulbaceae;	1	2.2
DF2-004	439	clone TANB52a	96	*Desulfococcus multivorans*	86	Deltaproteobacteria; Desulfobacteraceae	1	2.2
DF2-021	429	clone PL-37B6	87	*Eubacterium infirmum*	84	Firmicutes; Clostridia	1	2.2
DF2-068	433	*Acetobacterium malicum*	91			Firmicutes; Clostridia	1	2.2
DF2-036	436	clone HKT160	98	*Clostridium cylindrosporum*	91	Firmicutes; Clostridia	1	2.2
DF2-045	434	eubacterium RFLP11	95	*Syntrophomonas flectens*	94	Firmicutes; Syntrophomonadaceae	1	2.2
DF2-014	436	bacterium SHA-7	96	*Bacteroides* sp. SA-11	90	Bacteroidetes; Bacteroidaceae	1	2.2
DF2-077	440	clone TANB52a	98	*Spirochaeta asiatica*	87	Spirochaetes; Spirochaetaceae	3	6.5
DF2-084	436	clone SSE9	99	*Treponema* sp. 10:A:C25	92	Spirochaetes; Spirochaetaceae	1	2.2
DF2-069	440	clone IIIA-8	93	*Sulfurospirillum* sp. Am-N	91	Epsilonproteobacteria; Campylobacteraceae	1	2.2

The minor group constitutes of the clone library were summarized as follows: *Acetobacterium malicum* ferments H_2/CO_2, formate and several sugars to acetate (Kotsyurbenko *et al.*, 1995), therefore, the possible role of this organism in this incubation might be acting as homoacetogenic bacteria converting H_2/CO_2 and/or formate to acetate. *Eubacterium infirmum* is known as a saccharolytic bacteria (Cheeseman *et al.*, 1996). Although the homeacetogenic properties were not searched, based on other species of the *Eubacterium* genus found in the clone library of the feed mixture-C, it could be attributed that *Eubacterium infirmum* might act as a homoacetogenic bacteria in this sulfate reducing consortia. The less abundant saccharolytic anaerobic species, *Clostridium cylindrosporum* (Stackebrandt *et al.*, 1999), and haloalkaliphilic *Spirochaeta asiatica* (Zhilina *et al.*, 1996), and *Treponema* sp. isolated from human periodontal samples, were also observed in this clone library. Differing from other clones, the related cultured *Sulfurospirillum* sp. Am-N (Campbell *et al.*, 2001), is able to grow on sulfur lithoauthotrophically using hydrogen as the electron donor. In addition, the low abundance of the

Syntrophomonas flectens related clone (2%) showed that butyrate was mainly degraded by sulfidogens.

10.3.3 Clone library of the batches incubated at 37°C

10.3.3.1 Feed mixture A at 37°C

In this culture, *Ruminococcus hydrogenotrophicus* (90%) dominated the clone library due to the concentration of the type of substrates at the feed; which mainly comprised starch over other VFAs (Table 10.8). The partial 16S rRNA genes showed that four of the six sequences were related to the members of Firmicutes; one being *Victivallis vadensis*, and another a Bacteroidetes.

Table 10.8: Phylogenetic assignment of the bacterial clones isolated from Eerbeek sludge enriched at **37°C** with **Feed Mixture A**

Clone	Length (bp)	Closest phylotype	% Seq. sim.	Closest cultured species	% Seq. sim.	Taxonomic position (class, family, or genus)	# Clones	% Freq
DF2_046	351	clone PeH38	96	*Ruminococcus hydrogenotrophicus*	95	Firmicutes; Clostridia	4	8.5
DF2_054	420	*Ruminococcus hydrogenotrophicus*	94			Firmicutes; Clostridia	38	80.9
DF2_086	434	clone LJ3	91	*Eubacterium oxidoreducens* strain G2-2	91	Firmicutes; Clostridia	1	2.1
DF2_022	420	clone:Pl-10e-26	94	*Clostridium aminovalericum*	89	Firmicutes; Clostridia;	1	2.1
DF2_062	436	eubacterium AA08	98	*Victivallis vadensis*	89	Lentisphaerae; Victivallaceae	2	4.3
DF2_007	426	clone S1-456RC16	97	*Bacteroides merdae*	90	Bacteroidetes; Bacteroidaceae	1	2.1

Victivallis vadensis, cellobiose degrading bacteria, is an isolate from the human faecal sample and is capable of converting glucose to acetate in syntrophic co-culturing with *Methanosprillum hungatei* (Zoetendal *et al.*, 2003). In the absence of this hydrogen utilizing methanogens, the products are acetate, ethanol and H_2. This syntrophic interaction could be the significant mechanism not only for this incubation, but also for all consortia that degrade carbohydrates at different temperatures. This also showed the importance of syntrophic interactions along the anaerobic degradation pathway of organic matter to methane in the anaerobic consortia.

One of the minor abundant groups in this clone library was clone LJ3 (2%) related to the *Clostridia* genus. Aromatic compounds (e.g., gallate, pyrogallol) catabolizing *Eubacterium oxidoreducens*, (Krumholz and Bryant, 1986) were anaerobic chemoorganotroph, that require hydrogen or formate as electron donor. It might be suggested that the intermediates including oxygen occurred at the degradation of starch that was reduced by these organisms using hydrogen or formate to produce acetate or butyrate. Based on this suggestion, the cooperation between chemoorganotroph *Eubacterium oxidoreducens* and saccharolytic *Ruminococcus hydrogenotrophicus* to degrade starch could be mentioned.

The other minor group was the isolate from natural gas pipeline, which was related to the saccharolytic cultured *Bacteroides merdae* species (Sheridan et al., 2003).

10.3.3.2 Feed mixture B at 37°C

Major proportion (44%) of the clone library matched with the sequences from *Ruminococcus hydrogenotrophicus* (Table 10.9). Besides, clones identical to *Trichococcus flocculiformis* (9%) were also significant in this community. Some other members of the clone library were related to the sequence of cultivated anaerobic fermentative bacterium *Clostridium sticklandii* (Stadtman and McClung, 1957), *Victivallis vadensis* (Zoetendal *et al.*, 2003), *Petrimonas sulfuriphila* (Grabowski *et al.*, 2005b) covering 22% of the clone library in total. However, *Clostridium sticklandii*, fermenting amino acids, is distinctly different in both morphological and in several of its biochemical characteristics from other clostridia known as fermenting carbohydrates. It obtains its energy for growth from coupled oxidation-reduction reactions between certain amino acid pairs and also produces hydrogen sulfide.

Table 10.9: Phylogenetic assignment of the bacterial clones isolated from Eerbeek sludge enriched at **37°C** with **Feed Mixture B**

Clone	Length (bp)	Closest phylotype	% Seq. sim.	Closest cultured species	% Seq. sim.	Taxonomic position (class, family, or genus)	# Clones	% Freq
DF2_040	435	*Ruminococcus hydrogenotrophicus*	94			Firmicutes; Clostridia	20	44.4
DF2_024	416	*Trichococcus flocculiformis*	100			Firmicutes; Lactobacillales	9	20.0
DF2_063	438	clone IA-11	99	*Clostridium sticklandii*	97	Firmicutes; Clostridia	4	8.8
DF3_065	336	clone s101	93	*Desulfotomaculum thermobenzoicum*	91	Firmicutes; Clostridia	1	2.2
DF3_089	441	clone:ALU43	91	*Erysipelothrix inopinata*	85	Firmicutes; Mollicutes; Anaeroplasmatales	1	2.2
DF2_080	437	clone FA-PB9	98	*Desulfomicrobium sp. 'Delta +'*	98	Deltaproteobacteria; Desulfomicrobiaceae	1	2.2
DF2_056	435	clone 056F03_P_DI_P58	87	*Desulfovibrio sp. PA35E4*	83	Deltaproteobacteria; Desulfovibrionaceae	1	2.2
DF3_081	426	clone 056F03_P_DI_P58	96	*Akkermansia muciniphila*	82	Verrucomicrobia; Verrucomicrobiaceae	2	4.4
DF2_087	434	eubacterium AA08	99	*Victivallis vadensis*	93	Lentisphaerae; Victivallaceae	5	11.1
DF2_088	434	clone B-1C	99	*Petrimonas sulfuriphila*	99	Bacteroidetes; Bacteroidales	1	2.2

Clone 056F03_P_DI_P58, the isolates from an anaerobic sludge digester, group into the candidate division of OD1 within the phylum of *Verrucomicrobia* (Chouari *et al.*, 2005a). This was the less known bacterial division; therefore, the functional assignment for these species was not possible.

The clone:ALU43, retrieved from an acetate-degrading methanogenic community (Shigematsu *et al.*, 2003), was related to the cultured *Erysipelothrix inopinata* (Verbarg *et al.*, 2004), which is an inhabitant of sterile-filtered vegetable broth.

A difference between the clone libraries of feed mixtures A and B at 37°C was that 7% of the clones affiliated with sulfate reducing bacteria were detected in feed mixture B's library as a result of the presence of limited sulfate.

10.3.3.3 Feed mixture C at 37°C

The consumption of feed mixture C was monitored at the supernatants sampled during the course of the entire incubation; and these biochemical results were juxtapositioned with the microbial community shifts determined for the biomass samples harvested at the corresponding time points, at 7 and 42 days (Figure 10.5). This shift can clearly be observed for the feed mixture C at 37ºC demonstrated with

arrows. In addition, the clone library constructed for the 7th and 42nd days of the incubations were presented in Table 10.10 and 10.11, respectively. As observed from the degradation graphic, at the 7th day of incubation, butyrate, beside acetate, was degraded in the incubation, while the propionate remained almost stable. However, on day 42, propionate was consumed. Therefore, the clone library in the 7th day and the 42nd day was taken into account as the butyrate and propionate dominated clones, respectively.

In this regard, the DGGE profiles demonstrated that band A (7th day of sample) corresponded to the butyrate degrader, *Syntrophomonas* sp. MGB-C1, which was consistent with the biochemical performance that shows mainly the butyrate degradation in addition to acetate (Figure 10.5). Band B of the 42nd day corresponded to *Desulfobulbus propionicus,* known as propionate degrader, which was also coherent with the biochemical results given in Figure 10.5.

The clone library of the sample taken on day 7 was represented in Table 10.10. Sequences most closely related to syntrophic short chain fatty acid oxidizers and sulfate reducers were found. In contrast to the DGGE patterns, where the prominent band corresponded to the *Syntrophomonas* sp., the *Desulfobulbus* propionicus was found as the dominant member in the clone library at the moment when butyrate degradation was observed. This controversy might be originated from the PCR amplification, which could selectively amplify some of the target DNA. Based on these species' properties, it could be attained that the *Syntrophomonas* sp. is responsible for the syntrophic butyrate degradation. Although these *Desulfobulbus propionicus* (44%) *Desulforhabdus amnigena* (2%) *Desulfobulbus elongatus* (2%) and *Syntrophobacter wolinii* (4%) propionate degraders grow under sulfidogenic conditions, only *Syntrophobacter wolinii* (4%) can grow in co-culturing with hydrogen utilizing methanogens in the absence of sulfate (Boone and Bryant, 1980).

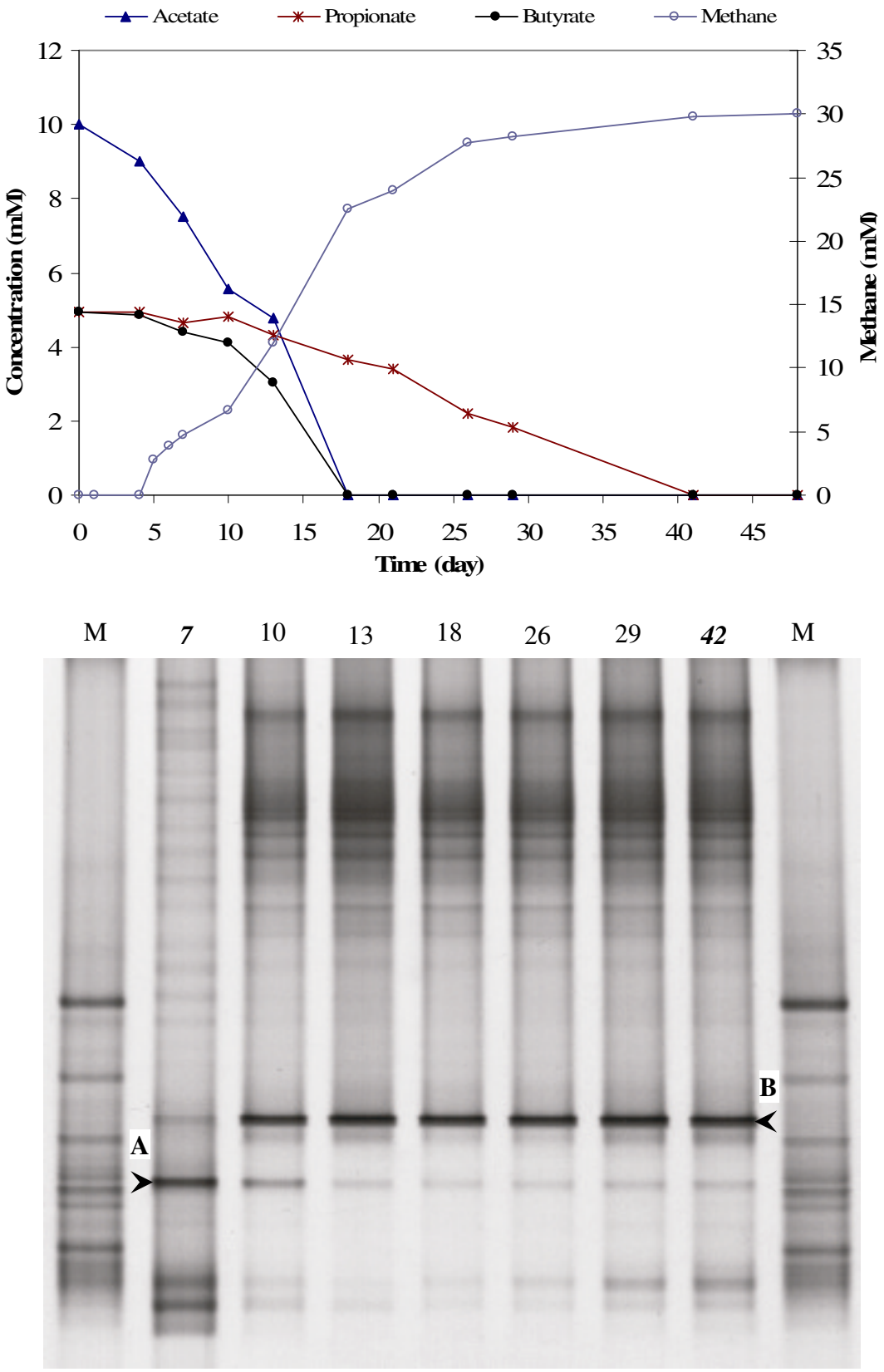


Figure 10.5 : The biochemical performance (upper panel) in comparison with the bacterial population dynamics (lower panel) of Eerbek sludge enriched at 37°C with Feed Mixture C

It is tempting to speculate that the corresponding organisms are directly or indirectly involved in butyrate degradation. However, there is not any result reported so far that these sulfate reducing species are involved in the butyrate degradation.

Geobacter metallireducens is a chemoorganotroph and is capable of degrading butyrate, propionate and acetate with the Fe(III) as the electron acceptor (Lovley *et al.*, 1993). Therefore, in the absence of ferric ion, it might also degrade these short chain acids with possible biological electron acceptor; which could be the hydrogen or formate utilizing methanogens.

Table 10.10: Phylogenetic assignment of the bacterial clones isolated from Eerbeek sludge enriched at **37°C** with **Feed Mixture C** at day 7

Clone	Length (bp)	Closest phylotype	% Seq. sim.	Closest cultured species	% Seq. sim.	Taxonomic position (class, family, or genus)	# Clones	% Freq
DF3_091	442	bacterium LTCE-T2A 4A	99	*Desulfobulbus propionicus*	99	Deltaproteobacteria; Desulfobulbaceae	21	43.8
DF3_027	435	proteobacterium isolate WB-25	95	*Desulforhabdus amnigena*	94	Deltaproteobacteria; Syntrophobacteraceae	1	2.1
DF3_059	433	Uncultured *Geobacter* sp. clone KB-1 2	89	*Geobacter metallireducens*	88	Deltaproteobacteria; Desulfuromonadales	2	4.2
DF3_004	439	*Desulfovibrio* sp. PA35E4	99	*Desulfovibrio alcoholovorans*	99	Deltaproteobacteria; Desulfovibrionaceae	2	4.2
DF3_036	436	clone R1p15	92	*Desulfobulbus elongatus*	92	Deltaproteobacteria; Desulfobulbaceae	1	2.1
DF3_034	439	clone E27	98	*Syntrophobacter wolinii*	92	Deltaproteobacteria; Syntrophobacteraceae	2	4.2
DF3_066	434	*Syntrophomonas* sp. MGB-C1	99			Firmicutes; Syntrophomonadaceae	16	33.3
DF3_028	431	clone W4A-A56	96	*Slackia heliotrinreducens*	90	Actinobacteria; Coriobacterineae	3	6.3

The second most abundant clone was related to *Slackia heliotrinreducens* (30%). An important distinctive feature of *Slackia heliotrinreducens* was their reductive cleavage of hepatotoxic pyrrolizidines, using hydrogen or formate as electron donor. This organism also reduces fumarate and does not grow on carbohydrates (Lanigan, 1976). These abundant species could be attained to the functioning of the propionate degradation pathway due to its fumarate reducing capability.

The clone library of the sample taken on day 42 was given in Table 10.11. The frequently retrieved sequences were closely related to propionate degrading and sulfate reducing bacterium, *Desulfobulbus propionicus* (45%). Although the similarity of the related clone to these species is as high as 99%, the presence of this

culture in the absence of sulfate is not clear. The possibility of its involvement in the syntrophic interaction could be mentioned, however, it was not reported so far. (Widdel and Pfennig, 1982). The syntrophic propionate degraders were affiliated with *Syntrophobacter sulfatereducens* (2%), *Syntrophobacter wolinii* (4%) and *Syntrophobacter fumaroxidans* (9%).

Table 10.11: Phylogenetic assignment of the bacterial clones isolated from Eerbeek sludge enriched at **37°C** with **Feed Mixture C** at the day of 42

Clone	Length (bp)	Closest phylotype	% Seq. sim.	Closest cultured species	% Seq. sim.	Taxonomic position (class, family, or genus)	# Clones	% Freq
DF3_037	441	bacterium LTCE-T2A 4A	99	*Desulfobulbus propionicus*	99	Deltaproteobacteria; Desulfobulbaceae	21	44.7
DF3_085	438	*Syntrophobacter sulfatereducens*	94			Deltaproteobacteria; Syntrophobacteraceae	1	2.1
DF3_005	439	clone E27	98	*Syntrophobacter wolinii*	92	Deltaproteobacteria; Syntrophobacteraceae	2	4.3
DF3_068	436	*Syntrophobacter fumaroxidans*	98			Deltaproteobacteria; Syntrophobacteraceae	4	8.5
DF3_084	431	clone W4A-A56	97	*Slackia heliotrinreducens*	91	Actinobacteria; Coriobacterineae	14	29.8
DF3_045	434	*Syntrophomonas* sp. MGB-C1	95			Firmicutes; Syntrophomonadaceae	3	6.4
DF3_077	437	clone SSE9	99	*Treponema* sp. 10:A:C25	92	Spirochaetes; Spirochaetaceae	2	4.3

Clone SSE9 is the isolate from an anaerobic bioreactor and from a trial to determine the effect of the feed composition performance and the microbial community dynamics. However, the closely related cultured species *Treponema* sp. is known as saccharolytic bacterium, which groups in the phylum of *Spirochaetes*.

10.3.3.4 Feed mixture D at 37°C

Desulforhabdus amnigena and *Desulfobulbus propionicus* covered 77% of the total clone library (Table 10.12). Due to their functional properties of propionate degradation and also the presence of sulfate in this incubation, it could be attained that these species were degrading propionate and concomitantly reducing sulfate.

Table 10.12: Phylogenetic assignment of the bacterial clones isolated from Eerbeek sludge enriched at **37°C** with **Feed Mixture D**

Clone	Length (bp)	Closest phylotype	% Seq. sim.	Closest cultured species	% Seq. sim.	Taxonomic position (class, family, or genus)	# Clones	% Freq
DF3_007	432	isolate WB-25	98	*Desulforhabdus amnigena*	98	Deltaproteobacteria; Syntrophobacteraceae	19	43.2
DF3_006	332	*Desulforhabdus amnigena*	99			Deltaproteobacteria; Syntrophobacteraceae	14	31.8
DF3_054	441	bacterium LTCE-T2A 4A	99	*Desulfobulbus propionicus*	98	Deltaproteobacteria; Desulfobulbaceae;	1	2.3
DF3_015	448	clone:ALU43	88	*Desulfuromonas palmitatis*	84	Deltaproteobacteria; Desulfuromonadaceae	1	2.3
DF3_038	430	Eubacteriaceae oral clone P2PC_29 P2	93	*Eubacterium brachy*	92	Firmicutes; Clostridia	1	2.3
DF3_078	446	bacterium TA19	98	*Aminobacterium colombiense*	89	Firmicutes; Clostridia	1	2.3
DF3_070	436	clone E3	99	*Treponema* sp. 10:A:C25	92	Spirochaetes; Spirochaetaceae	1	2.3
DF3_046	439	bacterium MTCE-T2 1G	98	*Clostridium* sp. (BN II)	94	Firmicutes; Clostridia; Clostridiaceae	4	9.1
DF3_094	435	anaerobic bacterium clone B-1C	91	*Petrimonas sulfuriphila*	91	Bacteroidetes; Bacteroidales	1	2.3
DF3_014	435	clone EUB53-2	98	*Bacteroidales* str. WB4	91	Bacteroidetes; Bacteroidales	1	2.3

The clone, bacterium TA19 isolated from terephthalate-degrading anaerobic granular sludge, will possibly be involved in propionate or butyrate degradation (Wu *et al.*, 2001).

Eubacterium brachy oral asaccharolytic Eubacterium (Cheeseman *et al.*, 1996) detected also in the clone library of the feed mixture D at 25°C. Although, the homoacetogenic features of *Eubacterium infirmum* were not searched; it could be attained that these species might act as a homoacetogenic bacteria in the sulfate reducing consortia, based on the other species features of the *Eubacterium* genus found in the clone library of the feed mixture D at 25°C.

The clone:ALU43 retrieved from the acetate-degrading methanogenic community was related to the cultured *Desulfuromonas palmitatis* dissimilatory Fe(III) reducer, which can oxidize long chain fatty acids but not propionate or butyrate, either not using sulfate as an electron acceptor (Coates *et al.*, 1995). Nevertheless, it can use fumarate and acetate; therefore, it might be involved in the degradation of acetate.

Treponema sp. (2%) *Clostridium* sp. (9%) *Petrimonas sulfuriphila* (2%) and *Bacteroidales* str. (2%) were represented less significantly and were capable of fermenting carbohydrates.

10.3.4 Clone library of the batches incubated at 45°C

10.3.4.1 Feed mixture A at 45°C

The dominant species for the enrichment at 45°C with feed mixture A, was the bacterium E47 isolated from rumen and related to the cultured *Paenibacillus barengoltzii* (59%), which utilizes only gluconate (Osman *et al.*, 2006). Based on the first hit of the NCBI database search, it could be suggested that this abundant clone was a saccharolytic bacteria.

The second abundant clone was affiliated with *Mahella australiensis,* which is a moderately thermophilic anaerobic bacterium isolated from an oil well (Bonilla Salinas *et al.*, 2004). These species mainly ferment carbohydrates, and glucose is fermented into lactate, formate, hydrogen and CO_2.

Table 10.13: Phylogenetic assignment of the bacterial clones isolated from Eerbeek sludge enriched at **45°C** with **Feed Mixture A**

Clone	Length (bp)	Closest phylotype	% Seq. sim.	Closest cultured species	% Seq. sim.	Taxonomic position (class, family, or genus)	# Clones	% Freq
DF3_039	440	bacterium E47	98	*Paenibacillus barengoltzii* strain SAFN-125	96	Firmicutes; Paenibacillaceae	26	59.1
DF3_040	434	*Paenibacillus* sp. S-4-25-10	92			Firmicutes; Paenibacillaceae	1	2.3
DF3_031	271	*Mahella australiensis*	99			Firmicutes; Clostridia; Thermoanaerobacteriaceae	9	20.5
DF3_063	434	*Bacillus thermoamylovorans*	99			Firmicutes; Bacillaceae	6	13.7
DF3_032	438	*Sporomusa sphaeroides*	97			Firmicutes; Clostridiales; Acidaminococcaceae	1	2.3
DF3_071	435	clone 2C	91	*Peptophilum propionigenum* strain TB107	88	Bacteroidetes; Bacteroides Porphyromonadaceae	1	2.3

Bacillus thermoamylovorans, thermophilic, faculatatively anaerobic amylolytic bacteria ferment carbohydrates and produce lactate, acetate, ethanol and formate, but not hydrogen (Combet-Blanc *et al.*, 1995). It could be suggested that the reducing equivalent of the carbohydrate was transferred by formate instead of hydrogen at 45°C.

Sporomusa sphaeroides ferments organic substrates included in N-methyl compounds, such as betaine, N,N-dimethylglycine and sarcosine, and hydroxy fatty acids. In addition, molecular hydrogen and carbon dioxide were fermented into acetate (Moller

et al., 1984). Therefore, these species might act as a homoacetogenic bacterium in this culture.

Clone 2C is the isolate from reductively dechlorinate TCE in contaminated soil (Richardson *et al.*, 2002). Considering the enrichment only consuming starch, the possible function of this clone is not clear.

10.3.4.2 Feed mixture B at 45°C

The dominant *Clostridium* species covered the clone library as much as 54%. However, *Mahella australiensis* covered 21% of the clone library of feed mixture A, it occupied only 4% in the clone library of feed mixture B. Another important clone was related to *Desulfocaldus terraneus*, a sulfate-reducing bacterium isolated from a hot spring. These species might use propionate as well as starch, taking into account the physiological results of the enrichment. The presence of sulfate in the enrichment revealed that sulfidogenic bacteria are noticeably available in the enrichment of feed mixture B; while in other enrichments for feed mixture B, such sulfidogenic bacteria were not observed.

Other minor groups of the clone library were affliliated to the Firmicutes phylum (2%), Gammaproteobacteria (2%), Chloroflexi (6%), Bacteroidetes (4%), and Spirochaetes (2%). A clone was related to *Aminobacterium colombiense*, an amino acid-degrading obligate anaerobe isolated from anaerobic sludge (Baena *et al.*, 1998). When co-cultured with a hydrogenotrophic methanogen, it is able to convert some more amino acids that are not degraded in the absence of such methanogen, indicating the syntrophic relationship of these species. It could also be involved syntntrophically using the products of carbohydrates fermentation in this enrichment.

The chloroflexi clone SHA-135, an isolate from an culture that dechlorinates 1,2-Dichloropropane. Since the representative clones of Chloroflexi phylum are limited in number, making an interpretation about this clone is difficult.

The other less abundant clones, the Bacteroidetes clone 012E03_B_SD_P15 (Chouari *et al.*, 2005b), and bacterium BB48 were found in an anaerobic digester. Similarly, Spirochaetes bacterium related to clone SJA-102 was found in an anaerobic trichlorobenzene-transforming microbial consortium (Von Wintzingerode *et al.*, 1999). Considering the origin of the clones, it might be suggested that the

bacterium was involved in the degradation of starch and propionate in this enrichment.

Table 10.14: Phylogenetic assignment of the bacterial clones isolated from Eerbeek sludge enriched at **45°C** with **Feed Mixture B**

Clone	Length (bp)	Closest phylotype	% Seq. sim.	Closest cultured species	% Seq. sim.	Taxonomic position (class, family, or genus)	# Clones	% Freq
DF4_033	432	*Clostridium* sp. PPf35E10	94			Firmicutes; Clostridia	23	47.9
DF4_002	437	clone BSV54	90	*Clostridium* sp. PPf35E10	90	Firmicutes; Clostridia	1	2.1
DF4_081	330	*Clostridiaceae* str. A4d	95			Firmicutes; Clostridia	1	2.1
DF4_018	434	*Clostridium fallax* isolate VA24831_02	89			Firmicutes; Clostridia	1	2.1
DF4_089	440	*Mahella australiensis*	98			Firmicutes; Clostridia; Thermoanaerobacteriaceae	2	4.2
DF4_034	445	clone TTA_B6	98	*Aminobacterium colombiense*	88	Firmicutes; Syntrophomonadaceae	1	2.1
DF4_017	438	*Desulfocaldus terraneus* strain P6.2	98			Deltaproteobacteria; Desulfocaldus	12	25.0
DF3_088	434	clone SHA-135	89	*Caldilinea aerophila*	85	Chloroflexi; Anaerolinaceae	3	6.3
DF4_073	436	clone 012E03_B_SD_P15	99	*Peptophilum propionigenum* strain TB107	95	Bacteroidetes; Porphyromonadaceae	2	4.2
DF4_065	439	bacterium BB48	97	*Methylohalobium crimeensis*	87	Gammaproteobacteria; Methylohalobium	1	2.1
DF4_001	435	clone SJA-102	98	*Spirochaeta africana*	92	Spirochaetes; Spirochaetaceae	1	2.1

10.3.4.3 Feed mixture C at 45°C

The syntrophic butyrate degrading, *Syntrophomonas flectens,* was the main member of this clone library with an abundance of 91%. Although both butyrate and propionate were consumed in this enrichment, Syntrophomonas sp. was found dominant. *Desulfacinum subterraneum,* thermophilic sulfate-reducing bacterium isolated from a high temperature oil field, and *Desulforhabdus amnigena* and *Syntrophobacter sulfatereducens* covered only 8% of this syntrophic enrichment. While the *Syntrophobacter sulfatereducens* can grow syntrophically in the presence of hydrogenotrophic methanogens (Chen *et al.*, 2005)*, Desulforhabdus amnigena* can grow only in the presence of sulfate (Oude Elferink *et al.*, 1995).

Table 10.15: Phylogenetic assignment of the bacterial clones isolated from Eerbeek sludge enriched at **45°C** with **Feed Mixture C**

Clone	Length (bp)	Closest phylotype	% Seq. sim.	Closest cultured species	% Seq. sim.	Taxonomic position (class, family, or genus)	# Clones	% Freq
DF4_042	435	clone 008D10_B_SD_P15	94	*Syntrophomonas flectens*	93	Firmicutes; Syntrophomonadaceae	10	20.8
DF4_090	418	*Syntrophomonas flectens*	95			Firmicutes; Syntrophomonadaceae	34	70.8
DF4_074	435	*Desulfacinum subterraneum*	90			Deltaproteobacteria; Syntrophobacteraceae	1	2.1
DF4_019	438	isolate WB-25	98	*Desulforhabdus amnigena*	97	Deltaproteobacteria; Syntrophobacteraceae	2	4.4
DF4_058	437	*Syntrophobacter sulfatereducens*	99			Deltaproteobacteria; Syntrophobacteraceae	1	2.1

Syntrophomonas flectens (Zhang *et al.*, 2004) has been reported to be able to grow up to the temperature of 42°C, which was very close to our enrichment temperature. The other mesophilic species *Desulforhabdus amnigena and Syntrophobacter sulfatereducens* can grow with temperature of up to 45°C.

10.3.4.4 Feed mixture D at 45°C

Desulfotomaculum thermobenzoicum affiliated clones of this library were an important group under sulfidogenic conditions at 45°C (90% of the total community). *Desulfotomaculum thermobenzoicum* is thermophilic, syntrophic, propionate-oxidizing (Plugge *et al.*, 2002a). The propionate and butyrate was completely degraded by sulfidogens and methane production was not observed. Therefore, *Desulfotomaculum thermobenzoicum* could degrade propionate completely by the complete oxidation to CO_2 by sulfidogens. However, at lower temperatures of the same feed mixture, incomplete oxidation was observed.

Table 10.16: Phylogenetic assignment of the bacterial clones isolated from Eerbeek sludge enriched at **45°C** with **Feed Mixture D**

Clone	Length (bp)	Closest phylotype	% Seq. sim.	Closest cultured species	% Seq. sim.	Taxonomic position (class, family, or genus)	# Clones	% Freq
DF4_043	411	*Desulfotomaculum thermobenzoicum*	97			Firmicutes; Clostridia; Peptococcaceae	43	89.6
DF4_091	434	clone: SmB60fl	97	Clostridium sp. ANP2	90	Firmicutes; Clostridia; Clostridium	3	6.3
DF4_020	429	clone Lgja-11	95	*Eggerthella sinensis* strain HKU14	92	Actinobacteria; Coriobacteriaceae	1	2.1
DF4_059	438	clone M79	96	*Thermosipho ferriphilus*	84	Thermotogae; Thermotogaceae	1	2.1

Less abundant clones were clone:SmB60fl (6%) isolated from upflow anaerobic sludge blanket granular sludges; clone Lgja-11 (2%) isolated from Landfill Aquifer and Thermotogae clone M79 (2%) isolated from anaerobic biofilms. Even though they were not isolated from sulfidogenic environments, they were present in this enrichment.

10.3.5 Clone library of the batches incubated at 55°C

10.3.5.1 Feed mixture A at 55°C

The dominant member of this clone library was *Caloramator uzoniensis* (40%) thermophilic anaerobic saccharolytic bacterium. The clone related to *Desulfotomaculum thermobenzoicum* was also observed in this starch enrichment. The possible role of these species might be the consumption of the product of saccharolytic organisms such as propionate, lactate or fumarate in a syntrophic interaction with hydrogenotrophic methanogens. This assumption could also be supported with the archaeal clone library of the same enrichment, revealing only the sequences of hydrogenpotrophic methanogens.

The other abundant member of this clone library was *Desulfosporosinus orientis,* which can grow homoacetogenically on methanol and ethanol (Stackebrandt *et al.*, 1997). The production of acetate in this enrichment could be attained to these species.

Table 10.17: Phylogenetic assignment of the bacterial clones isolated from Eerbeek sludge enriched at **55°C** with **Feed Mixture A**

Clone	Length (bp)	Closest phylotype	% Seq. sim.	Closest cultured species	% Seq. sim.	Taxonomic position (class, family, or genus)	# Clones	% Freq
DF4_021	414	*Caloramator uzoniensis*	98			Firmicutes; Clostridia; Clostridiaceae	19	40.4
DF4_060	439	*Desulfotomaculum thermobenzoicum*	*92*			Firmicutes; Clostridia; Peptococcaceae	13	27.7
DF4_045	431	*Desulfosporosinus orientis*	89			Firmicutes; Clostridia; Peptococcaceae	7	14.9
DF4_029	528	Bacterium TGO	97	*Thermacetogenium phaeum*	91	Firmicutes; Clostridia; Thermoanaerobacteriaceae	2	4.3
DF4_037	419	*Clostridium fervidus*	94			Firmicutes; Clostridia	2	4.2
DF4_013	437	clone E5	98	*Spirochaeta taiwanensis*	91	Spirochaetes; Spirochaetaceae	3	6.4
DF4_085	438	clone SR_FBR_E32	98	*Spirochaeta africana*	88	Spirochaetes; Spirochaetaceae	1	2.1

The other constituents (17%) of the library were member of the phylum Firmicutes and Spirochaetes. The clones related to Bacterium TGO, fermenting sugars at thermophilic temperatures (Plugge *et al.*, 2002b); *Clostridium fervidus*, thermophilic saccharolytic bacteria (Rainey *et al.*, 1993); clone E5 from a butyrate+sulfate enrichment of the same origin of sludge that was used in this study (Roest *et al.*, 2005); and clone SR_FBR_E32 retrieved from sulfate-reducing communities in fluidized-bed reactors treating acidic metal- and sulfate-containing wastewater. Due to the absence of sulfate in this enrichment, these sulfidogenic sourced clones might act as fermentative bacteria as the other non sulfidogenic member of this clone library.

10.3.5.2 Feed mixture B at 55°C

The phylum of Firmicutes represented all clones, Clostridia species covered 50% and the remaining 50% was affiliated with the family of *Thermoanaerobacteriaceae*. The most abundant species of latter one was *Mahella australiensis* (38%), detected also in the clone library of feed mixtures A and B incubated at 45°C.

Table 10.18: Phylogenetic assignment of the bacterial clones isolated from Eerbeek sludge enriched at **55°C** with **Feed Mixture B**

Clone	Length (bp)	Closest phylotype	% Seq. sim.	Closest cultured species	% Seq. sim.	Taxonomic position (class, family, or genus)	# Clones	% Freq
DF4_070	557	*Clostridium* sp.	99			Firmicutes; Clostridia	24	50
DF4_094	442	*Mahella australiensis*	99			Firmicutes; Clostridia; Thermoanaerobacteriaceae	18	37.5
DF4_030	515	*Thermoanaerobacter siderophilus*	98			Firmicutes; Clostridia; Thermoanaerobacteriaceae	2	4.2
DF4_031	445	*Thermoanaerobacter wiegelii*	98			Firmicutes; Clostridia; Thermoanaerobacteriaceae	2	4.2
DF4_054	384	*Thermoanaerobacter thermohydrosulfuricus*	94			Firmicutes; Clostridia; Thermoanaerobacteriaceae	2	4.2

Thermoanaerobacter siderophilus (Slobodkin *et al.*, 1999), *Thermoanaerobacter wiegelii* (Cook *et al.*, 1996), and *Thermoanaerobacter thermohydrosulfuricus* (Carlier *et al.*, 2006) occupied 9% of the clone library. They were all known as thermophilic saccharolytic bacteria under anaerobic conditions.

10.3.6 Clone library of the batches incubated at 65°C

10.3.6.1 Feed mixture B at 65°C

The sequences abundantly matched the *Thermacetogenium phaeum* derived from a thermophilic anaerobic reactor treating kraft-pulp wastewater (Hattori *et al.*, 2000) (Table 10.19). This specie is capable of degrading acetate syntrophically as well as oxidizing acetate with the reduction of sulfate. Both of these features might be functioned in this consortium because of the presence of the limited amount of sulfate (3 mM) in the enrichment. The remaining part of the clones could be affiliated with saccharolytic bacteria. In this feed mixture and temperature only starch and a little amount of acetate was consumed. The clones of the bacterium BC13 isolated from an anaerobic digester; bacterium TA19, isolated from a terephthalate-degrading anaerobic granular sludge; *Clostridium* sp. isolated from an UASB reactor; *Bacillus sp.* isolated from light corn steep liquor; clone C583 isolated from intestinal microbial flora; *Microbacterium* sp. isolated from the refusal of a landfill; *Propionicimonas* sp., isolated from biodegraded oil reservoir; and *Rhodocyclus* sp. isolated from phosphorus removing full-scale wastewater treatment plant were saccharolytic bacteria. Although some isolated clones originated from thermophilic environments, most of them originated from mesophilic habitats.

10.4 The Archaeal Clone Library

In the batch culture incubated with feed mixture A at 10°C, the archaeal community was dominated by clones representing the *Methanosarcinaceae* and *Methanobacteriaceae*, a lineage of methanogenic *Euryarchaeota* (Table 10.20). *Methanobacterium beijingense*, and *Methanobacterium formicicum* were the dominant species in the culture (in terms of functional properties); which utilizes hydrogen and formate. These made up 77.3% of the total clone library. The remaining portion of the clone library was represented by the acetate utilizing methanogens, *Methanosaeta concilii*.

Table 10.19: Phylogenetic assignment of the bacterial clones isolated from Eerbeek sludge enriched at **65°C** with **Feed Mixture B**

Clone	Length (bp)	Closest phylotype	% Seq. sim.	Closest cultured species	% Seq. sim.	Taxonomic position (class, family, or genus)	# Clones	% Freq
DF5_021	447	*Thermacetogenium phaeum*	92			Firmicutes; Clostridia; Thermoanaerobacteriaceae	37	77.1
DF5_030	434	bacterium BC13	92	*Paenibacillus polymyxa* strain GBR-462	90	Firmicutes; Bacillales; Paenibacillaceae	2	4.2
DF5_069	446	bacterium TA19	98	*Desulfothiovibrio peptidovorans*	87	Firmicutes; Syntrophomonadaceae	2	4.2
DF5_006	456	*Clostridium* sp. strain P6	94			Firmicutes; Clostridia	1	2.1
DF5_014	437	*Bacillus thermozeamaize*	99			Firmicutes; Bacillales; Bacillaceae	1	2.1
DF5_038	466	eubacterium WFeA1-35	92	*Bacillus* sp. AM-001	89	Firmicutes; Bacillales; Bacillaceae	1	2.1
DF5_022	469	clone C583	94	*Catabacter hongkongensis*	87	Firmicutes; Clostridia	1	2.1
DF5_093	464	*Microbacterium* sp. I33	97			Actinobacteria; Actinomycetales	1	2.1
DF5_029	433	*Propionicimonas* sp. F6	99			Actinobacteria; Actinomycetales	1	2.1
DF5_037	462	beta proteobacterium clone Orbal D54	99	*Rhodocyclus* sp. HOD 5	98	Betaproteobacteria; Rhodocyclaceae	1	2.1

Table 10.20: Phylogenetic assignment of the archaeal clones isolated from Eerbeek sludge enriched at **10°C** with **Feed Mixture A**

Clone	Length (bp)	Closest phylotype	% Seq. sim.	Closest cultured species	% Seq. sim.	Taxonomic position (class, family, or genus)	# Clones	% Freq
DF6_009	383	archaeon clone 5LOC9	99	*Methanosaeta concilii*	98	Euryarchaeota; Methanosaetaceae	5	22.7
DF5_072	385	archaeon clone CM17	98	*Methanobacterium beijingense* strain 8-2	98	Euryarchaeota; Methanobacteriaceae	13	59.1
DF6_017	384	archaeon clone 1G1	99	*Methanobacterium formicicum* strain FCam	95	Euryarchaeota; Methanobacteriaceae	4	18.2

As observed in Figure 10.3, the methanogenic profile has showed a significant change from 10°C to 55°C for the feed mixtures A and B. At 55°C, as it was shown in Table 10.21, thermophilic hydrogen utilizing methanogens dominated the clone library. Since acetate was not used by these consortia related to physiological data, hydrogen was the main source for methanogenic archaea. The clone related to *Methanothermobacter thermautotrophicus* (Zeikus and Wolee, 1972) can grow only on H_2/CO_2. The other member of this clone library was *Methanobacterium*

beijingense, which is a mesophilic Archaea, that can grow up to a temperature of 50°C (Ma *et al.*, 2005).

Table 10.21: Phylogenetic assignment of the archaeal clones isolated from Eerbeek sludge enriched at **55°C** with **Feed Mixture A**

Clone	Length (bp)	Closest phylotype	% Seq. sim.	Closest cultured species	% Seq. sim.	Taxonomic position (class, family, or genus)	# Clones	% Freq
DF6_081	392	Enrichment culture E21A1	98	strain GC-1	98	Euryarchaeota; Methanobacteriaceae	24	92.4
DF6_010	407	Methanobacterium thermoautotrophicum section 146 of 148	95	*Methanothermobacter thermautotrophicus* strain GC-1	95	Euryarchaeota; Methanobacteriaceae	1	3.8
DF6_018	393	archaeon clone CM17	97	*Methanobacterium beijingense* strain 8-2	97	Euryarchaeota; Methanobacteriaceae	1	3.8

10.5 Discussion

Together with the prominent methanogenic archaea, phylotypes that can be affiliated with all the steps along the anaerobic degradation pathway of organic matters to methane have been detected. However, in the presence of excess sulfate, this anaerobic degradation pathway shifted through sulfate reduction concomitantly to the production of hydrogen sulfide in a different ratio together with methane production.

The monitoring and the evaluation of the bacterial population shifts using DGGE patterns demonstrated that the microbial community shifts juxtapositioned with the biochemical results were not clearly observed (Appendix III), except for the incubation enriched at 37°C with feed mixture C. The stable community profiles might be the reason of either the contribution of each member of the community to the degradation of each compound present in the incubations, or nucleic acids being subjected to the community profiles not being sensitive enough to reflect the population changes along the incubation periods. It is known that nucleic acids that belong to specific microorganisms can stay longer in the enrichments; even if the microorganisms are not active in the mixed populations (Wagner *et al.*, 1995). Therefore, the shifts of bacterial community with different types of C-sources were not possible to be observed along the incubation periods. However, a community shift was clearly observed for the enrichment incubated at 37°C with the feed mixture C; including butyrate, propionate and acetate. While the butyrate consumption was observed at the beginning of the incubation, propionate was not

degraded. After consumption of butyrate, propionate degraded. This degradation profile was also helpful in noticing the population structure shift during the course of the incubation. Therefore, it could be correlated with the biochemical results that the *Syntrophomonas* sp. and *Desulfobulbus propionicus* were involved in the syntrophic degradation of butyrate and propionate pathways respectively. Although *Syntrophomonas* sp. is known as butyrate degrading organism, *Desulfobulbus propionicus* can degrade propionate only in the presence of sulfate. Therefore, it could be suggested that this species might be playing an important role in the pathway of propionate degradation.

The enrichment clones were either distinct from any sequence of database or were closely related, even identical, to the sequences cultured or, more, yet-uncultured species. Here, the functional properties of these species were estimated by using the information obtained from the NCBI database search. While using this information for determining the functional properties might not reflect the real assignment, even the identical cultured species do not necessarily have to have the same functional properties due to the possibility of showing differences in functional genes. Caution must also be taken when interpreting the results of molecular methods especially on the quantitative and functional information about mixed communities.

The isolated clones were from a variety of environments such as anaerobic bioreactors, soils, faecal samples, periondental samples and rumens. The distribution of the bacterial clones within the bacterial domain was very diverse.

The archaeal population dynamics significantly changed during a temperature shift from 45 to 55°C in the feed mixture of A and B. Obviously, different archaeal communities were established at the two different temperatures. On the contrary, the temperature dependent change for bacterial clones was observed from 37 to 45 °C for the feed mixture-A and B. For example, the thermophilic saccharolytic species *Mahella australiensis* was observed starting from the temperature of 45 °C and going on through higher temperatures. For the feed mixture C, this situation was not the same; e.g., the mesophilic syntrophic species also survived at 45°C, however at a higher temperature, the growth was not observed; which demonstrates the sensitivity of the syntrophic players to a temperature of more than 45°C. For the feed mixture-D, the sulfidogens were present at all different incubations (25-45°C). While butyrate

and propionate was degraded incompletely in the 25 and 37°C incubations, the complete oxidation was observed for both substrates at 45°C.

For the feed mixture A and B at all temperatures, the clone library profiles are mainly covered up by saccharolytic microorganisms as a result of the feed composition, which mainly composed of starch. Although these mixtures also included propionate and butyrate as well, the relevant organisms in the corresponding clone libraries that degrade these substrates were not detected. This was due to the presence of the fewer amounts of these VFAs in feed A and B.

11. COMPARATIVE EVALUATION OF THE DIFFERENT TECHNIQUES APPLIED ON SYNTROPHIC BUTYRATE DEGRADING CONSORTIA

Metabolic products may be inhibitory to the cells that produce them; subsequent utilization of inhibitory products by commensalisms, therefore, it is necessary for the complete degradation of the substrates. In several cases, the cooperation of two or more organisms is essential, since a substrate would not be degradable at all, if the concentration of the product were not kept very low. Interspecies hydrogen transfer is the most significant example of a symbiosis with only in one direction substrate supply from which mutual benefit is drawn. Because of the low energetic conditions, this metabolic symbiosis called syntrophism is an important mechanism in anaerobic ecosystems for the degradation of wide range of substrates. Due to more unfavorable conditions and the presence of high concentrations compared to other substrates, the degradation of short chain fatty acids especially propionate gained more attention to reveal the syntrophic interaction. However, the research on the syntrophic interaction of the butyrate degradation, which is one of central intermediates in the degradation of carbohydrates, proteins and lipids in anaerobic ecosystems, has received less attention. Metabolic interaction between different trophic groups of organisms should be obtained in order to optimize the anaerobic treatment. Understanding of the microbial aspects of and expanding our knowledge of the extent of microbial communities related to their physiological properties in the syntrophic butyrate degradation was the main motivation of this thesis. The efforts are given to mimic the real ecosystems during the application of the alone or combination of the molecular techniques. In many ecosystems, the important intermediates of anaerobic food chains can not be at the detectable concentrations. The absence of measurable concentrations of a compound does not mean that the compound is not involved in a metabolic reaction. These compounds might be consumed as soon as they are produced. Although they are major products of the predominating anaerobic fermentations, they will be easily underestimated these products in the chain of food web in the anaerobic processes.

The novel molecular techniques uncovered the microbial diversity that was previously unknown, with culture-based techniques. Together with advance techniques, this enables us to determine the structure and dynamics of microbial communities. There is growing evidence for the syntrophic butyrate degrading community is larger than it was thought before. This functional group of organisms is not fallen into the phylogenetically consistent groups, rather spread out in to several lineages. This makes difficult to apply some of the molecular techniques on the practical identification of syntrophs. This could be the explanation of why the abundance of such saturated fatty acid-beta-oxidizing syntrophs, including butyrate degraders, found as low as 1% in the anaerobic co-digesters treating municipal solid waste and sewage sludge. Saturated fatty acid-beta-oxidizing syntrophs, including butyrate degraders, was related with the family of *Syntrophomonadaceae* and the relative abundance was found less tan 1% in the anaerobic co-digesters treating municipal solid waste and sewage sludge (McMahon *et al.*, 2001; McMahon *et al.*, 2004). Between 0.2 and 1% of the microbial community structure of a sample from a full-scale biogas plant was also attributed to the *Syntrophomonadaceae*, of which the majority was accounted for by the genus *Syntrophomonas* (Hansen *et al.*, 1999).

In this thesis, different incubation conditions for the butyrate degradation were accomplished because of the application of different methods. These methods comprised of MPN, UASB reactor, and SIP experiments. Different incubation conditions showed that different microbial consortia were responsible for the butyrate degradation at each condition. The MPN counting study showed that the *Syntrophomonas* species were the dominant group for syntrophic degradation. The number of acetogens were increased in the reactor sludge compared to the seed sludge under both methanogenic and sulfidogenic conditions. Although the addition of hydrogen utilizing archaea to the incubations significantly increased the numbers of acetogens, the addition of acetate utilizing archaea did not change the number of them. This indicated the importance of hydrogen consumers in the syntrophic degradation of butyrate and propionate. Since the formate utilizing methanogens seemed less important for the seed sludge, the number of them increased in the reactor sludge and reached the same importance as the seed sludge. The other important point was the different numbers observed between the hydrogen and

formate utilizing sulfidogens for the BLS sludge. In the presence of limited sulfate, the reducing equivalent of the electrons was carried via hydrogen instead of formate.

Intriguing result found in the molecular characterization of MPN was the detection of unusual species affiliated with the syntrophic butyrate degraders. In this study, the tri-culturing effects on the butyrate degrading microbial consortia was also experienced by pre-growing the acetate utilizing methanogens (*Methanosaeta concilli*) in addition to the hydrogen utilizing methanogens (*Methanospirillum Hungatei*) in the MPN dilution series. This effect was noticeably observed for the BUT sludge. In the presence of hydrogen utilizing methanogens, the diversity mostly comprised of *Syntrophomonas* species known as a typical butyrate degrader. However, for the incubation with both hydrogen and acetate utilizing methanogens for the BUT reactor sludge, the diversity increased and *Dechloromonas hortensis* species came up with an abundance of 20%, even though the dominant species was still *Syntrophomonas*. The same manner was also observed for the BLS sludge. In contrast to the BUT and BLS sludge, the addition of these methanogens reduced the diversity through more methanogenic consortia in the absence of sulfate even in the presence of sulfate. Strong influence of the presence or absence of acetate consumers was observed in the dilution series of the BUS sludge without the addition of sulfate. Interestingly, in the absence of sulfate, the consortia contained the sulfate reducer, *Desulfoarculus,* for the BUS sludge. Until now, there was no report on the degradation of butyrate consumer under neither sulfidogenic nor methanogenic conditions.

The microbial community structures of the UASB reactors demonstrated that the diversity of the BUT reactor was higher than those of the BLS and the BUS reactor in terms of OTU numbers. Since the BUT reactor was fed with butyrate in the absence of sulfate, methanogenic syntrophy gained importance and microorganisms inhabited in the granules were diverse. This diversity of the consortium and the functional surplus within the butyrate degrading syntrophs might increase the reactor stability and affect the sustainability of these syntrophic consortia in the anaerobic bioreactor. Conversely, in the presence of sulfate, diversity decreased depending on the COD/sulfate ratio. While in the presence of limited amount of sulfate, corresponding to COD/sulfate: 10, 18 OTU was observed, in the presence of excess amount of sulfate, corresponding to COD/sulfate: 0.5, 20 OTU was observed.

Although the OTUs observed in both the BUT and the BLS sludges were more common, that made up the 20% of the BUT clones, the BUS clones showed a more separated path than those of the BUT and the BLS sludges, where the BUS clone library shared its clones as 10% of the BUT clones.

Molecular analysis of the microbial diversity within the UASB reactors fed with butyrate showed absolute importance for specific unidentified microorganisms. The major part of other isolates was classified into different groups and they were not known as the potential to degrade butyrate with syntrophic interaction for the BUT and the BLS sludges. Nevertheless, we cannot rule out that these microorganisms are capable of syntrophic butyrate oxidation. However, for the BUS sludge, 95% of the clones belonged to the Deltaproteobacteria; which gave a more reasonable explanation due to having closely related cultured species. It was noteworthy that clones retrieved in this study that belong to *Chloroflexi*, *Firmicutes*, *Deltaproteobacteria*, *Epsilonproteobacteria*, *Betaproteobacteria*, *Alphaproteobacteria*, *Bacteroidetes*, and *Chlorobi* were closely related to the clones when they were isolated from bioreactors, contaminated environments or consortia dechlorinating chlorinated pollutants.

The numerical analysis of the reactor sludges demonstrated that methanogens that belong to the domain of archaea had greater abundance than the ones that belong to the domain of Bacteria. As expected in the BUT reactor sludge, archaeal population was as high as 86% due to complete metabolic dependence of the acetogens to methanogens; however, the presence of sulfate in the bioreactors decreased this value to 77 and 80% for the BLS and the BUS reactors' sludge respectively, due to the competition between methanogens and sulfidogens.

In conclusion, the culture independent identification methods showed that the diversity besides the known *Syntrophomonas* sp. covered the consortia of syntrophic butyrate degradation. Since these methods cannot comprehensively elucidate the information on the identity linking to the function in complex environments alone, additional advanced molecular tools might be necessary to attain the decisive functional features of the microorganisms for each individual.

Diverse active member of the syntrophic butyrate degraders were represented by grouping at least 10 different phyla, which showed more diversity than recent studies

of the bacteria capable of syntrophic metabolism in terms of both phylogenetic and physiology. This functional group of organisms does not fall into the phylogenetically consistent groups; rather, it spreads out into several lineages. In most cases, closest uncultured relatives have been identified from anaerobic ecosystems. These were the majority of the microbial community associated with deep subsurface aquifer, anaerobic dechlorinating mixed cultures equine faecal contaminated sites and bioreactors. Sequence representatives of several bacterial divisions have been identified in a wide range of habitats, suggesting the sophisticated distribution of the corresponding organisms in the environment and, potentially, their wide metabolic capabilities. The 16S rRNA gene clone library showed that the largest groups of clones belonged to the members of the *Proteobacteria*, which were not what was expected from the community of syntrophic butyrate degradation that belong to the phyla of *Firmicutes*. The main possible role of the butyrate degradation was attained to the *Syntrophus* sp., Sequence types associated with the genus *Syntrophus* sp. can produce energy from the anaerobic oxidation of organic acids, with the production of acetate and hydrogen. However, it was also found that the *Syntrophobacter* sp., known as propionate degrader, also played an active role in the butyrate degradation. If the clone library structures of this study were compared with other butyrate degrading community studies represented in Chapter 7 and 8, main differences would be observed especially in terms of the dominant members of the consortia. Since the dominant species of the butyrate fed reactor was one of the unclassified microorganism (Chapter 8); in the SIP study, *Syntrophus* and *Syntrophobacter* sp. were found as the dominant members of the community. While the *Syntrophomonas* sp. was detected in different enrichments especially in batch operated incubations, none of them were detected in the Wild BUT enrichment. This discrepancy can be explained with the different incubation conditions and the molecular techniques, since the enrichments carried on in Chapter 7 and 8 were acclimatized with butyrate under batch or continuous operations.

Acetate was the main product of butyrate degradation. The detection of *Methanosaeta concillii* in all incubations was the evident that all acetate was consumed by this methanogens. However, the presence of excess sulfate in the feed

caused shifting this acetate utilizing methanogen to SRB. This shift takes longer time as observed for also other studies.

Interspecies hydrogen and/or formate transfer is an important mechanism in the degradation of syntrophic degradation of organic substrates as explained in Chapter 6. Based on these results, it can be concluded that formate activity was very high compared to the hydrogen activity for all three reactors. It could also be suggested that formate was the dominant mechanism in the interspecies electron transfer. The MPN results given in Chapter 7, demonstrated that the hydrogen and formate utilizing methanogens were almost in the same order for all three reactors, however the number of formate utilizing methanogens were lower in the seed sludge. Molecular characterization of the archaeal community given in Chapter 8 and 9, showed that hydrogen and/or formate utilizing methanogens were actively presence in the consortia. In this study, significant part of the electron transfer might be realized by the reduction of bicarbonate to formate; taking into account the high formate depletion activity.

The presence of 3 mM sulfate at the influent, which corresponds to COD/sulfate: 10, had an effect on the biogas composition while decreasing the methane ratio, increased the sulphide concentration. The methane production rate decreased to 16% compared to the only butyrate fed reactor. The addition of excess sulfate to the feed, which was more than the reducing equivalent of the butyrate, the methane production was measured to be ~0.1 g COD/L.day at the end of the 505^{th} day of the operation of the reactor. Moreover, the microbial structure has also showed differences especially for hydrogen and/or formate utilizing trophic group. While the methanogens were responsible for hydrogen and/or formate consumption in the absence of sulfate, the presence of sulfate shifted populations towards more *Desulfovibrio* species in terms of hydrogen and/or formate utilization. The amount of sulfate presence in the influent is the decisive mechanism for the selection of different trophic group of the microorganisms. The proportion of electrons distributed to sulfate reducing bacteria and to methane producing archaea is important to elucidate the competition of these species. In most cases, it is difficult to distinguish the physiology of fatty acids degrading bacteria, which are involved in this competition. It can be the syntrophic interaction in which the reducing equivalents will be used by SRBs or be the direct oxidation by the SRBs. Under non limiting sulfate conditions, it was always

observed that all the formed reducing equivalents of acetogenic bacteria which can be hydrogen and/or formate were first used up by SRBs, and then if the amount of sulfate exceeds the theoretical value for the oxidation of electrons, hydrogen utilizing methanogens will be completely eliminated and the acetate will be the other substrate to be competed by sulfidogens and methanogens. Acetate utilizing SRB outcompete MPB as well, due to higher affinity, higher free Gibb's energy and higher removal rate, however, this competition will take a long time.

The amount of theoretical reducing equivalent produced from 1mmol butyrate is the half mmole of hydrogen (Eq.11.1). Considering the COD/sulfate: 10, and the feeding of the enrichments or BLS reactor containing 18 mM butyrate, it can be calculated that the produced proton amount will be 9 mM. Therefore, taking into account the thermodynamic advantage of SRBs over methanogens in terms of hydrogen utilization (Eq. 11.1 and 11.2), the presence of 3 mM sulfate was not enough to reduce all this reducing equivalent of the butyrate (Eq 11.3).

$$CH_3CH_2CH_2COO^- + 2H_2O \rightarrow 2CH_3COO^- + H^+ + 2H_2 \qquad \Delta G^{0`} = +48.3 \text{ kJ.mol}^{-1} \textbf{(11.1)}$$

$$CH_3CH_2CH_2COO^- + 0.5SO_4^{2} \rightarrow 2CH_3COO^- + 0.5HS^- + 0.5H^+ \qquad \Delta G^{0`} = -29.3 \text{ kJ.mol}^{-1} \textbf{(11.2)}$$

$$4H_2 + SO_4^{2-} + H^+ \rightarrow HS^- + 4\,H_2O \qquad \Delta G^{0`} = -151 \text{ kJ.mol}^{-1} \textbf{(11.3)}$$

Therefore, assuming all sulfate is reduced by hydrogen, there still remained 6 mM hydrogen to be reduced by methanogens. This is the reason of presence of both the SRBs and the methanogens in this consortium for hydrogen utilization. Molecular characterization of the sludge incubated under unlimited sulfate conditions demonstrated that *Desulfovibrio* and *Methanosprillum* species were present in the same culture as presented in the MPN numbering, population dynamics and SIP experiments. A majority of the clones in SIP experiment obtained from the metabolically active fraction of the Wild BUTS microbial community were mostly closely related to *Desulfovibrio* sp., the sulfate-reducing bacteria. SRB population probably functioned as proton reducers in association with hydrogen and formate consuming methanogens. If the COD/sulfate < 3.5, all reducing equivalent of the butyrate would be oxidized by sulfate reducers, mainly *Desulfovibrio* species. However, it should be kept in mind that the in the presence of sulfate the reducing equivalents will be used by SRBs or be the direct oxidation by the SRBs. In the case

of direct oxidation, the population was dominated by *Desulfobotulus sapovorans* as found in the population dynamic study under the conditions of excess sulfate.

The effects of propionate, acetate, formate and hydrogen on butyrate degradation were analyzed and results gave significant values for the operational of the anaerobic reactors, considering the butyrate as central intermediate in the anaerobic degradation pathway. Propionate had relatively higher resistance to the butyrate degraders with the IC_{50} values of more than 6 $gCOD.l^{-1}$. Similarly, acetate inhibited the butyrate degraders at high acetate concentrations having the IC_{50} value of around 5 $gCOD.l^{-1}$. However, the IC_{50} of the formate and hydrogen demonstrated that the low concentrations of these compounds inhibit the butyrate degraders. If the hydrogen concentrations exceed the 100 mg $COD.l^{-1}$ values, the degradation of butyrate affected easily.

11. CONCLUDING REMARKS

The aim of this research was to get a deeper and better insight into the population dynamics and functionality of syntrophic butyrate oxidizing microbial consortia in anaerobic Upflow Anaerobic Sludge Blanket (UASB) bioreactors. The investigation was further extended to examining the effect of sulfate on syntrophic butyrate degradation. The integrated application of cultivation-based and molecular techniques (MPN: Most Probable Number, PCR-RFLP: Polymerase Chain Reaction-Restricted Fragment Length Polymorphism, DGGE: Denaturing Gradient Gel Electrophoresis, Q-PCR: Quantitative PCR, and SIP: Stable Isotope Probing) enabled us to identify key players in anaerobic syntrophic butyrate degrading-consortia, both qualitatively and quantitatively.

Based on operational and performance data of three reactors fed with butyrate in the absence or presence of limited or excess concentrations of sulfate, it was concluded that sulfate addition affects the butyrate-COD conversion rates depending on whether processes are directed by methanogenic or sulfidogenic consortia.

Methane production was decreased with increasing COD/sulfate ratio. Hence, availability of sulfate as an electron acceptor is pivotal in controlling the relative growth of sulfidogens and methanogens, which determine the amount of reduced sulfate and oxidized COD.

Complete sulfidogenic conditions were established after around 400 days in the presence of excess amounts of sulfate. This retardation of the sulfidogenic conditions was mainly associated with the competition between the sulfidogens and methanogens for the acetate produced by degradation of butyrate. Although sulfides might have been toxic towards both sulfidogens and methanogens, such a case was not observed in the reactors operated in this study.

The effect of propionate, acetate, formate and hydrogen on the butyrate degradation rate was different for each substrate. There was, on the contrary, not much difference between the three reactors' sludge for each individual substrate. The BLS and the

BUS reactors' sludges showed a somewhat better resistance to inhibition of butyrate degradation by proprionate; however for the BUT reactor this resistance was much lower, though the BUT reactor sludge had higher methanogenic activity than the BLS sludge. For all three reactors IC_{50} values showed that butyrate utilizing organisms were very sensitive to low concentrations of hydrogen, while formate affected butyrate degradation only at higher concentrations. Normally, such a high concentration of hydrogen and formate could inhibit the reactors' sludge according to other researches. The high IC_{50} values could be probably attributed to the granular structure of the sludge, which makes a concentration gradient along the bioparticle structure, resulting in a significantly lower effective concentration of substrate at the inner layer.

All three reactor sludges adapted to butyrate as the sole substrate were also able to degrade other fatty acids such as propionate and acetate. Although the growth on propionate in butyrate adapted sludge was not reported so far, this was observed without any lag phase in this study. This might be correlated to the contribution of syntrophic propionate degraders to the syntrophic butyrate degradation pathway. In the degradation of VFAs, hydrogenotrophic and acetoclastic methanogens play an important role in terms of syntrophic interaction between the methanogens and acetotrophic bacteria. Hydrogen and/or formate utilizing and acetate utilizing methanogens were actively present in the consortia, and were affiliated with *Methanobacterium* and *Methanospirillum,* and *Methanosaeta*, respectively. The SMA and/or SA on formate were always higher than those on hydrogen for all three sludges, suggesting that formate transfer was the dominant mechanism in the interspecies electron transfer.

In this thesis, different incubation conditions for the butyrate degradation were investigated during the application of different methods, namely MPN, UASB reactor, and SIP experiments. Different incubation conditions showed that different microbial consortia were responsible for the butyrate degradation at each condition. MPN incubations showed that *Syntrophomonas* spp. comprised the dominant group for syntrophic degradation. To overcome known limitations of cultivation-based approaches, such as selectivity of different media, MPN incubations were complemented with molecular characterization of the microbial consortia present under the various conditions studied here. The population dynamics study carried on

the butyrate fed UASB reactors demonstrated that yet uncultivated, only distantly related to cultured species, were detected besides *Syntrophomonas* species. Surprisingly, *Syntrophomonas* spp. were not detected as members of the active butyrate degrading community in SIP experiments. These dominant player differences between different incubation conditions clearly demonstrated that special attention should be drawn to select the enrichment and experimental methods to obtain ecologically relevant data. While the butyrate degraders were identified as *Synyrophomonas* spp. in MPN incubations, the UASB reactor and SIP experiments showed that they are more diverse that it was thought before. Therefore, further studies on the isolation of the syntrophic butyrate degraders by applying different techniques are needed to further understand the physiological properties of these unclassified microorganisms dominating the syntrophic butyrate degrading consortia. In the SIP experiment, sludge was fed with 13C-stable isotope labelled butyrate together with the original wastewater, trying to reflect the real conditions as much as possible. The techniques linking the identity to the function mentioned above can be used individually or in combination with different techniques to explain the different insight or feature of different microbial communities. This approach provides an effective means to address the physiology and phylogeny of the syntrophic butyrate degrading organisms in their natural environments. By using these techniques, potential roles of the strain-specific microorganisms involved in the syntrophic butyrate degradation *in situ* in the environment were achieved.

Studies to reveal the effect of different temperatures on anaerobic sludge for different feed mixtures provided important information on composition and physiological features of the communities that established at various conditions. Understanding of the microbial aspects and expanding our knowledge of the extent of microbial communities related to their physiological properties were possible by monitoring the biochemical results juxtapositioned with the microbial community shifts using DGGE fingerprinting, especially for the communities predominantly degrading the substrates sequentially. In doing so, the environmental conditions as occurred in the mixed cultures could be mimicked to determine the structure and dynamics of microbial communities. It was found that the archaeal population dynamics significantly changed during a temperature shift from 45 to 55°C. Obviously, different archaeal communities were established at the two different temperatures.

On the contrary, the temperature dependent change in bacterial composition was observed from 37 to 45 °C for the carbohydrate based feed mixture.

Better understanding of this community structure shed some light on anaerobic treatment of wastewaters and may lead to improvements in this process as well. Additionally, attempts will be made to cultivate these wide range of organisms identified in the heavy fractions of the SIP experiments of the clone libraries to determine their specific physiology and metabolic traits.

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APPENDIX A

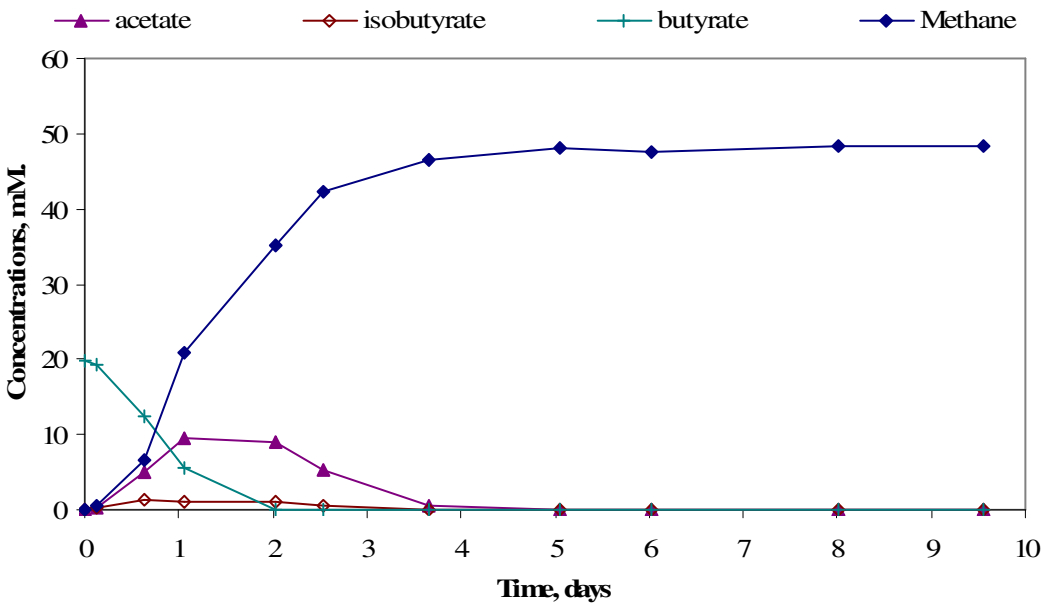


Figure A.1 : Time course of butyrate degradation for **BUT** reactor sludge

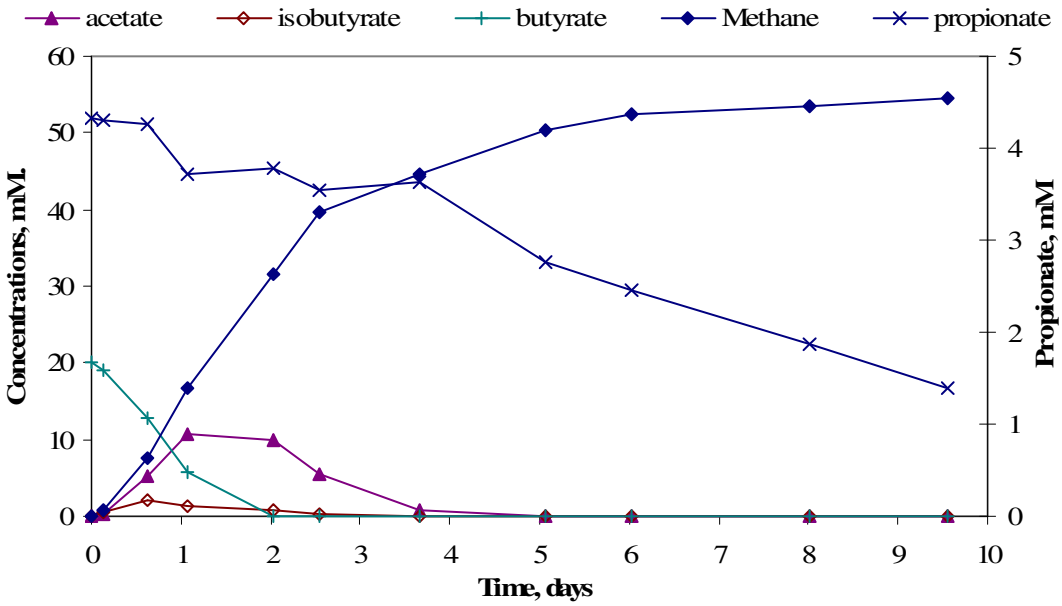


Figure A.2 : The effect of **5 mM propionate** to the butyrate degradation profile of **BUT** reactor sludge

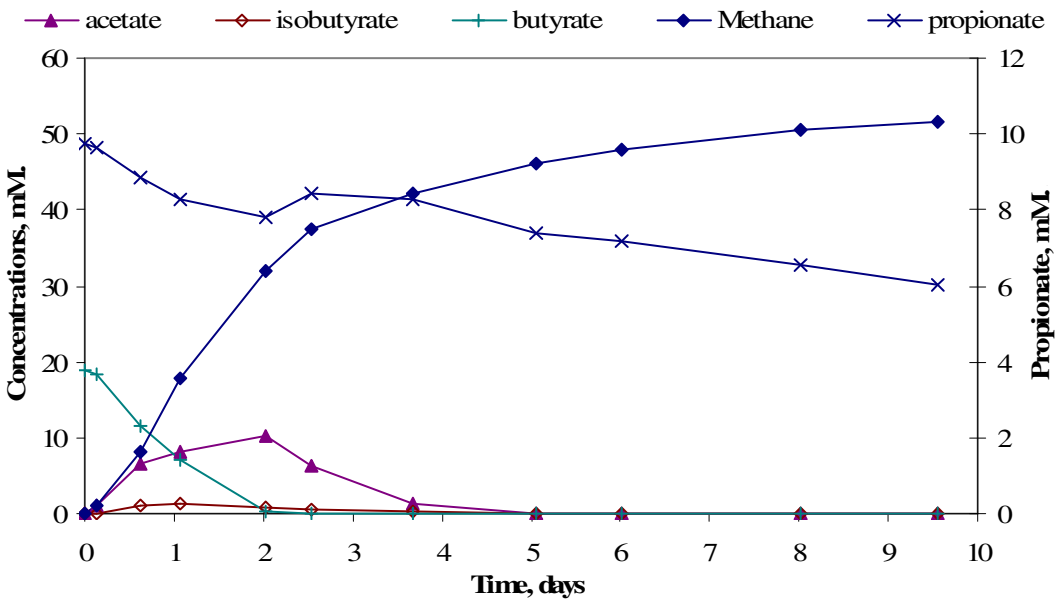


Figure A.3 : The effect of **10 mM propionate** to the butyrate degradation profile of **BUT** reactor sludge

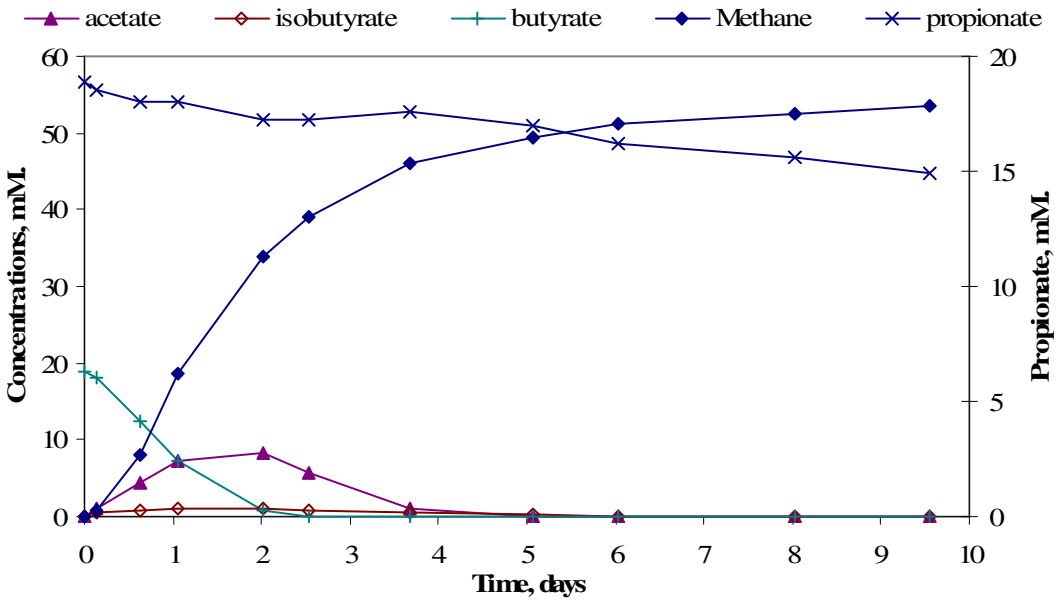


Figure A.4 : The effect of **20 mM propionate** to the butyrate degradation profile of **BUT** reactor sludge

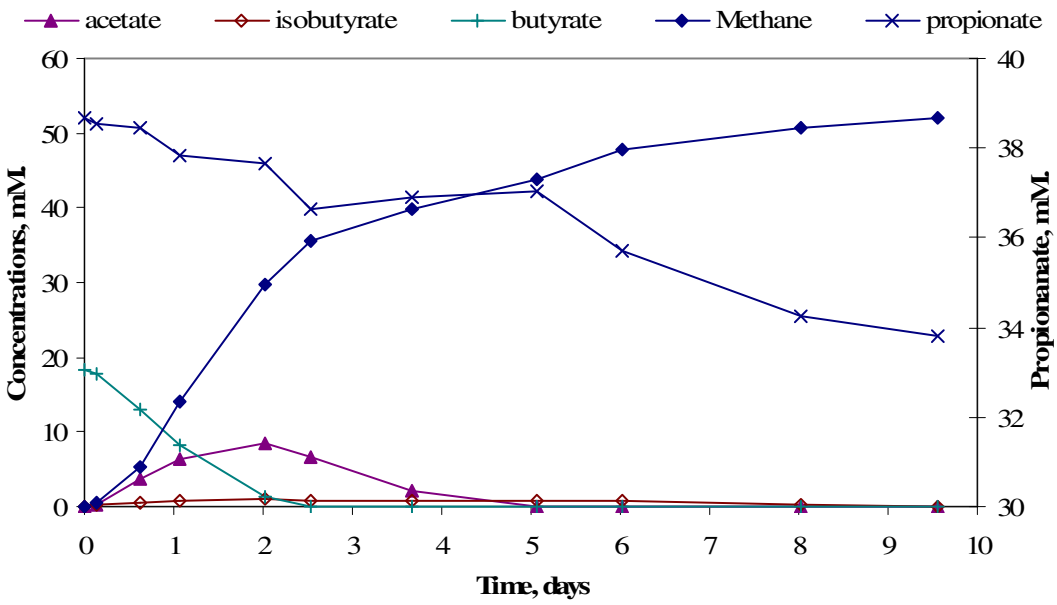


Figure A.5 : The effect of **40 mM propionate** to the butyrate degradation profile of **BUT** reactor sludge

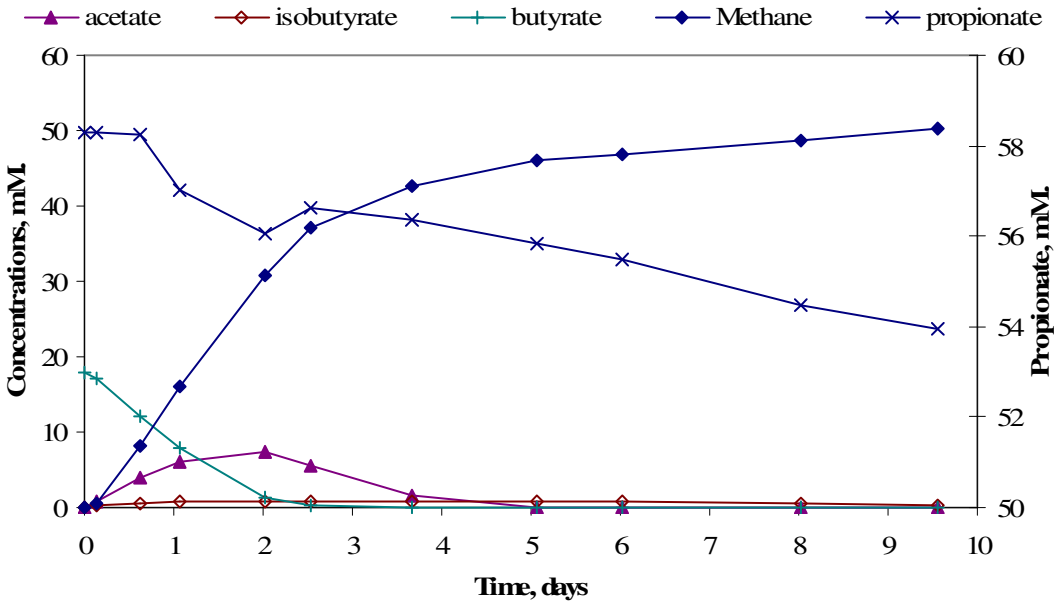


Figure A.6 : The effect of **60 mM propionate** to the butyrate degradation profile of **BUT** reactor sludge

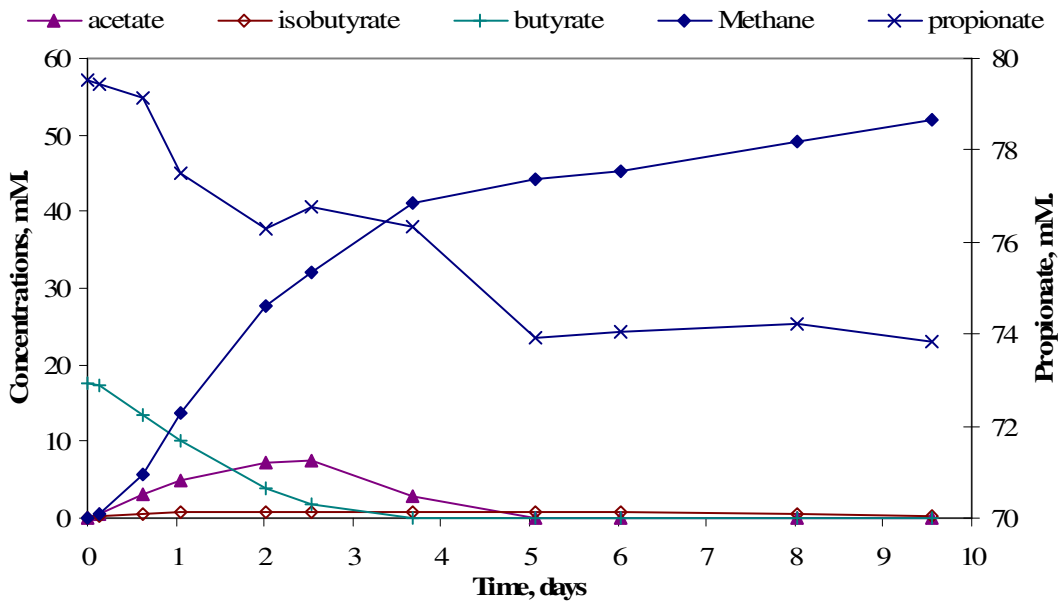


Figure A.7 : The effect of **80 mM propionate** to the butyrate degradation profile of **BUT** reactor sludge

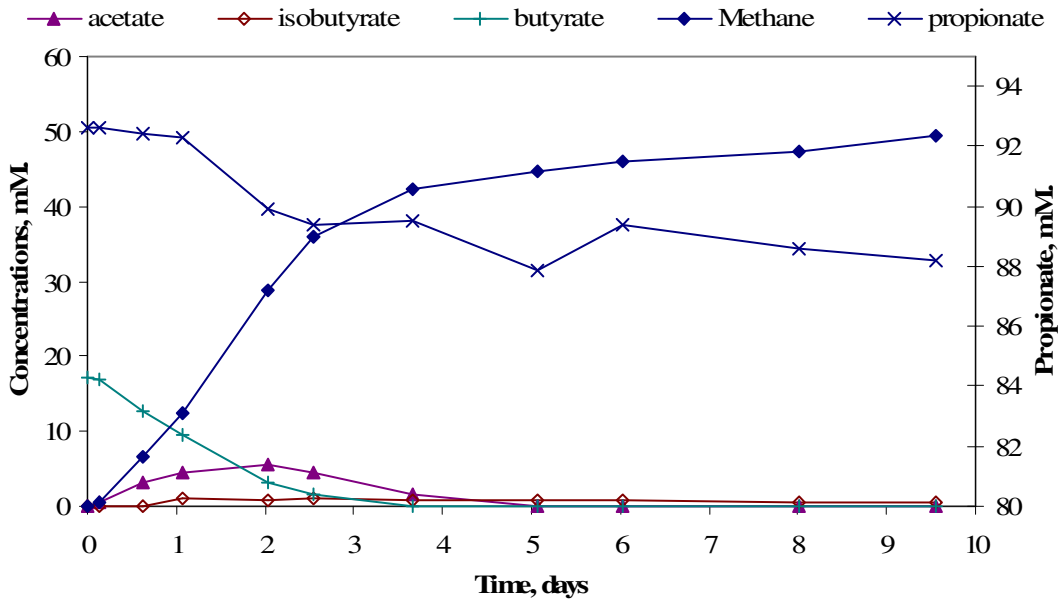


Figure A.8 : The effect of **100 mM propionate** to the butyrate degradation profile of BUT reactor sludge

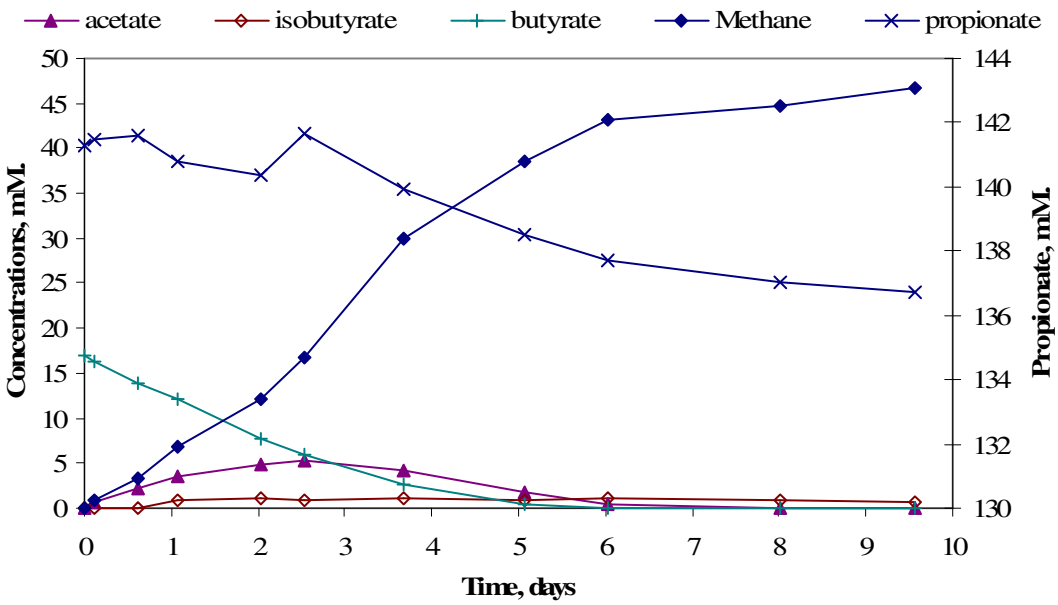


Figure A.9 : The effect of **150 mM propionate** to the butyrate degradation profile of **BUT** reactor sludge

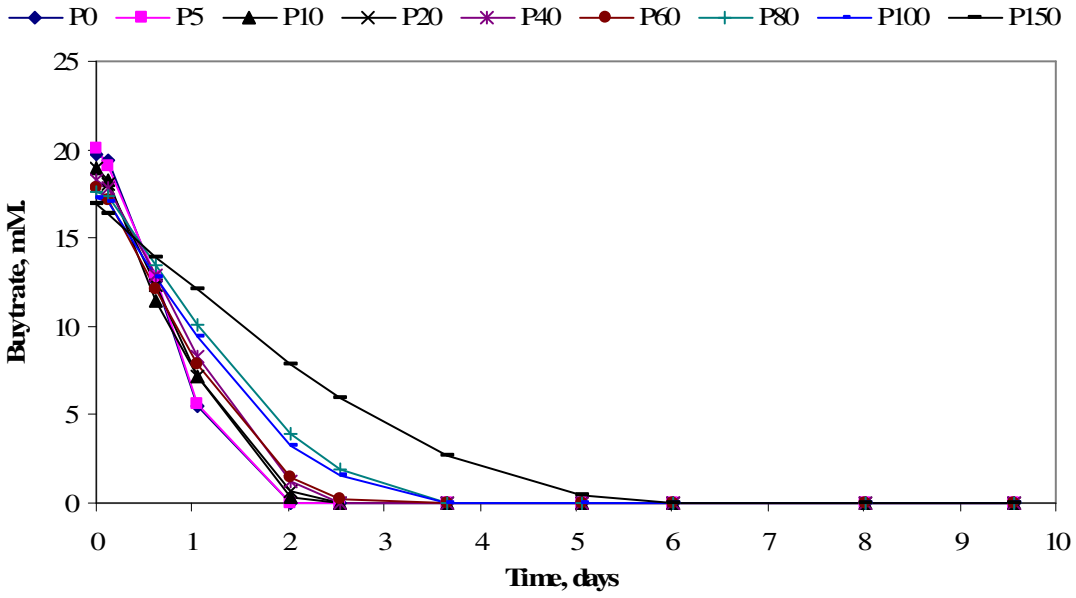


Figure A.10 : The total butyrate degradation profiles of **BUT** reactor sludge incubated at different **propionate** concentrations

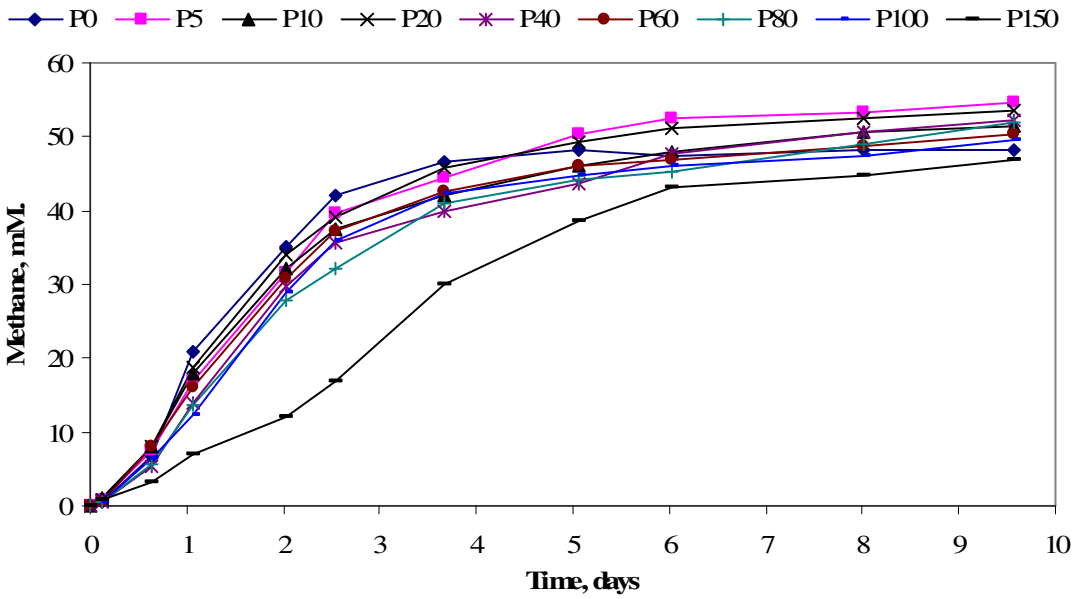


Figure A.11 : The total methane production profiles of **BUT** reactor sludge incubated at different **propionate** concentrations

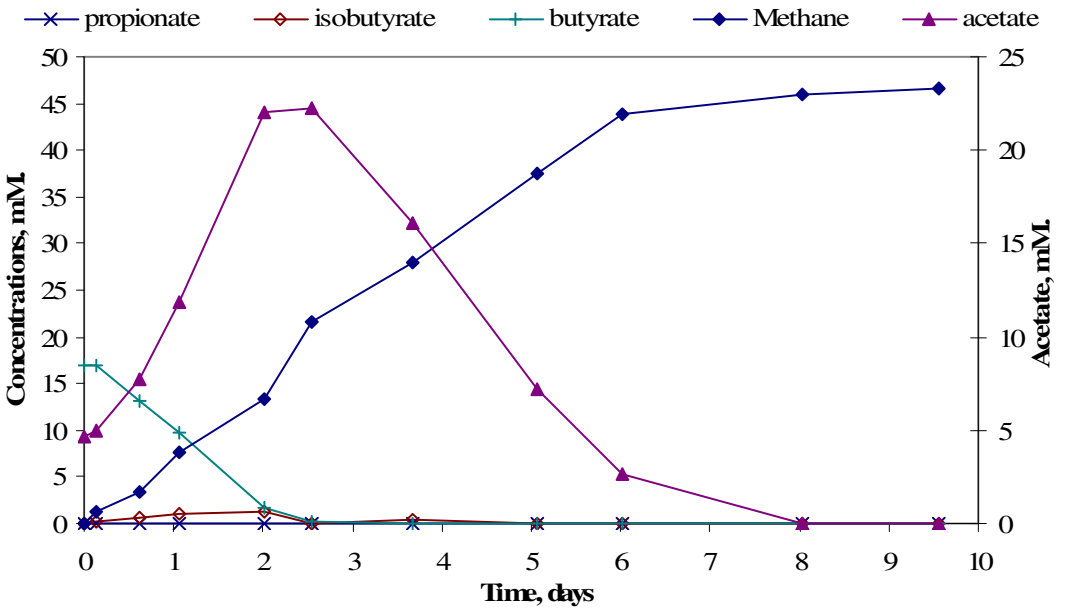


Figure A.12 : The effect of **5 mM acetate** to the butyrate degradation profile of **BUT** reactor sludge

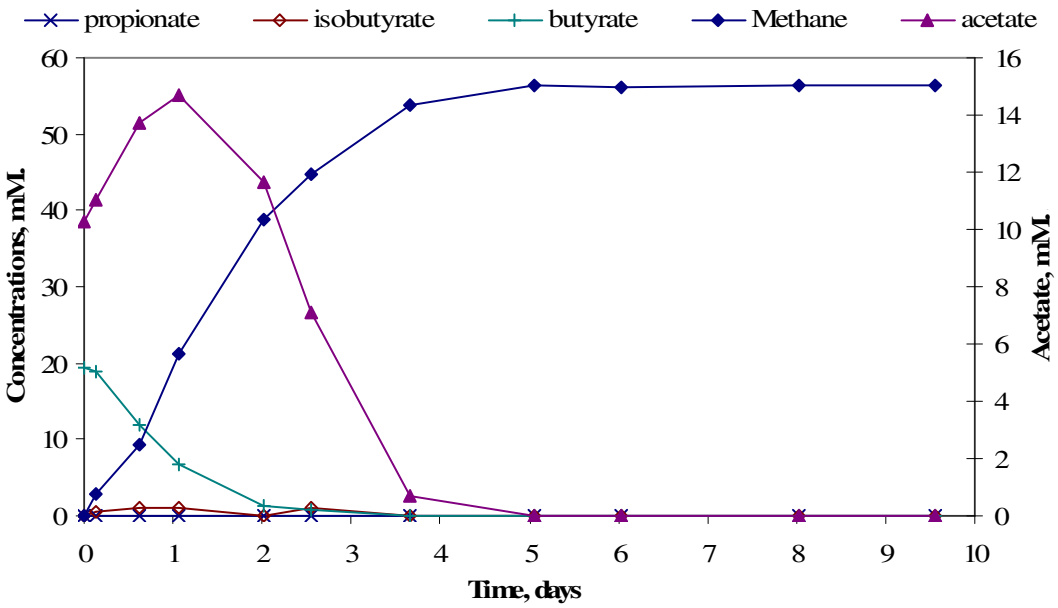


Figure A.13 : The effect of **10 mM acetate** to the butyrate degradation profile of **BUT** reactor sludge

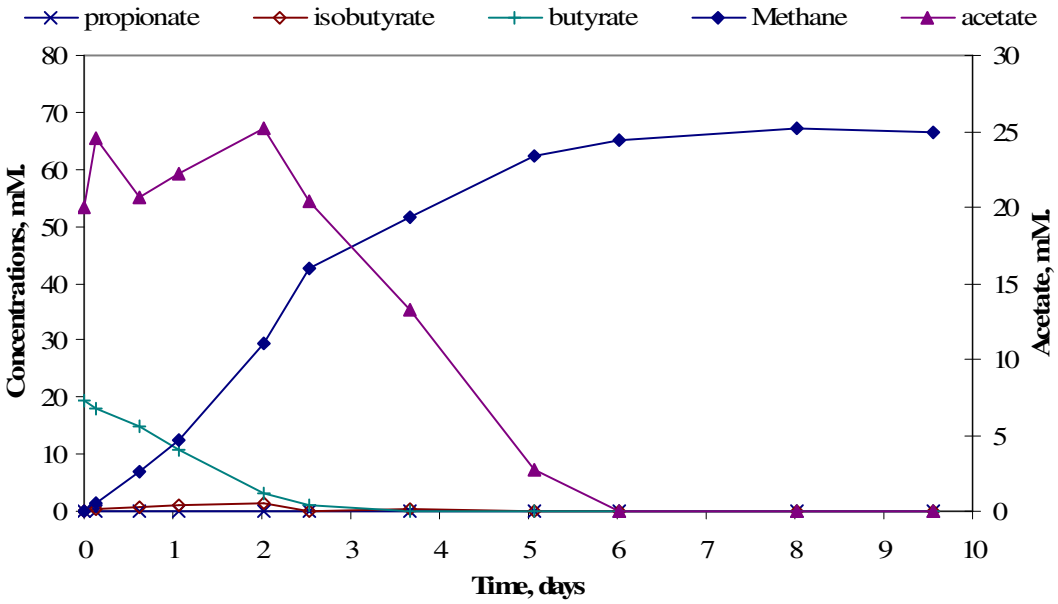


Figure A.14 : The effect of **20 mM acetate** to the butyrate degradation profile of **BUT** reactor sludge

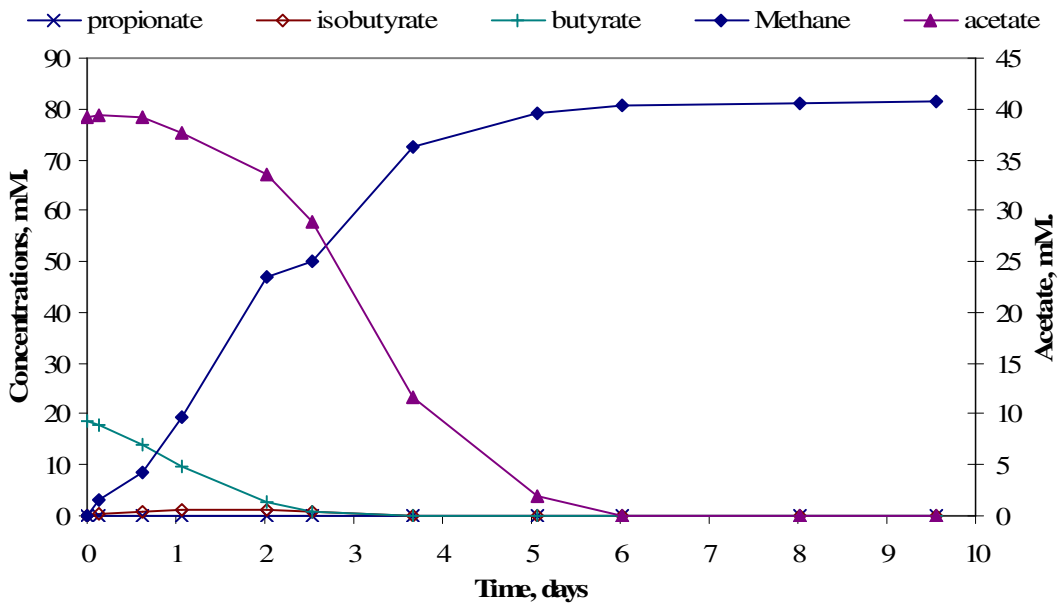


Figure A.15 : The effect of **40 mM acetate** to the butyrate degradation profile of **BUT** reactor sludge

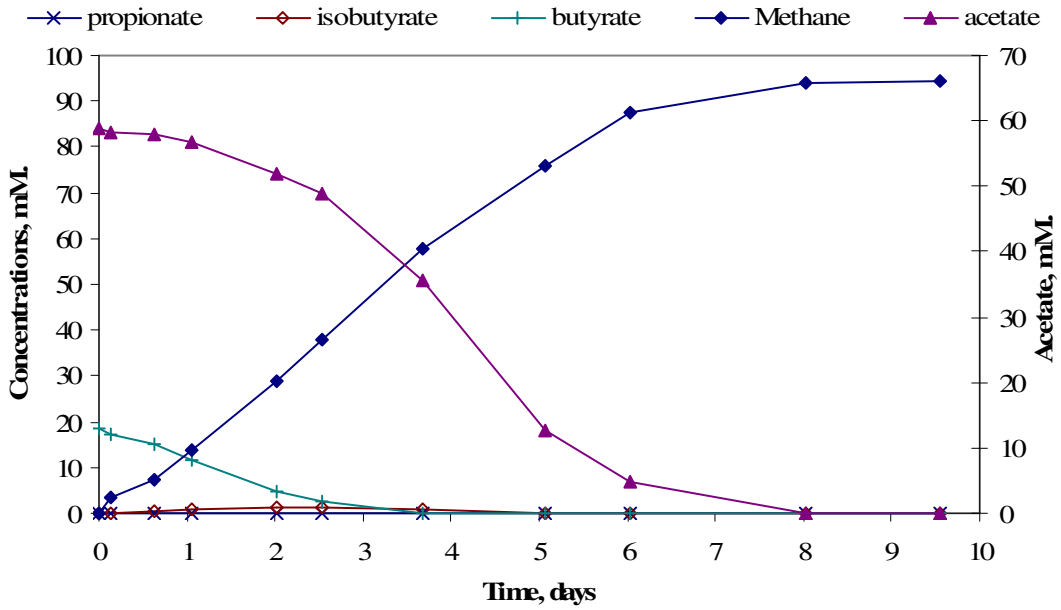


Figure A.16 : The effect of **60 mM acetate** to the butyrate degradation profile of **BUT** reactor sludge

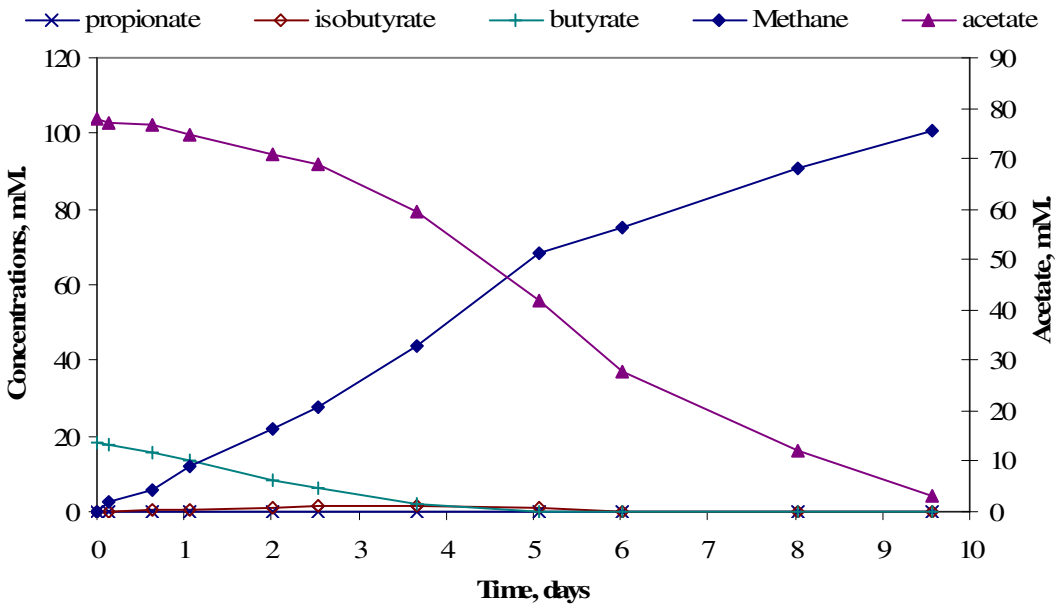


Figure A.17 : The effect of **80 mM acetate** to the butyrate degradation profile of **BUT** reactor sludge

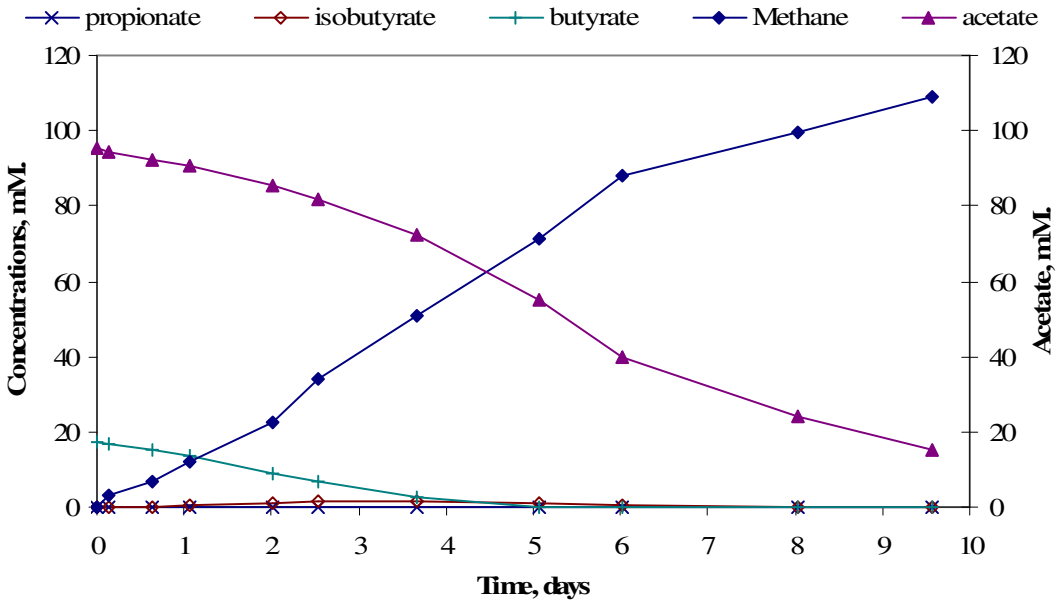


Figure A.18 : The effect of **100 mM acetate** to the butyrate degradation profile of **BUT** reactor sludge

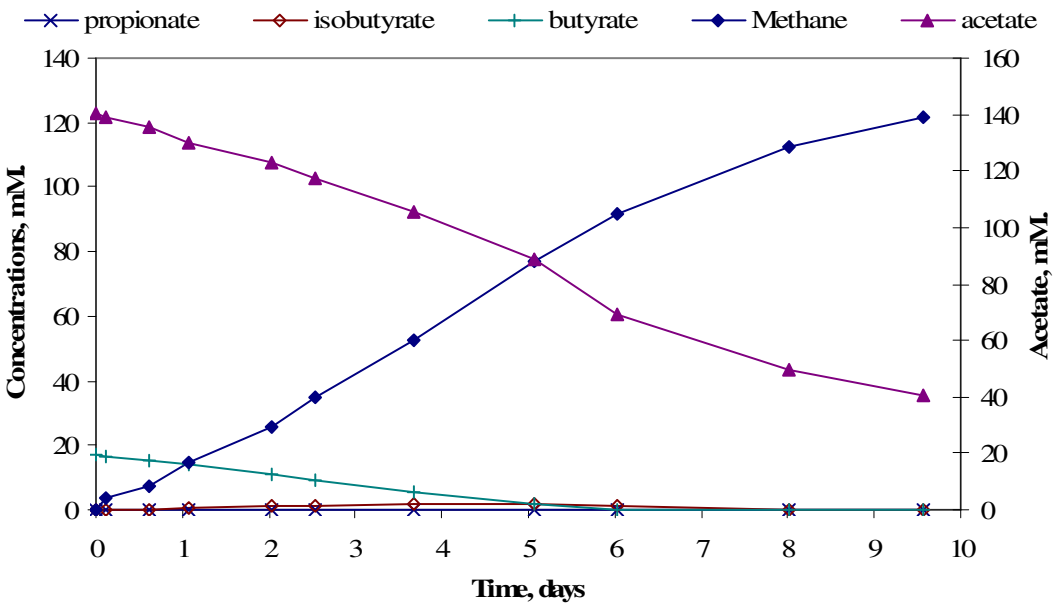


Figure A.19 : The effect of **150 mM acetate** to the butyrate degradation profile of **BUT** reactor sludge

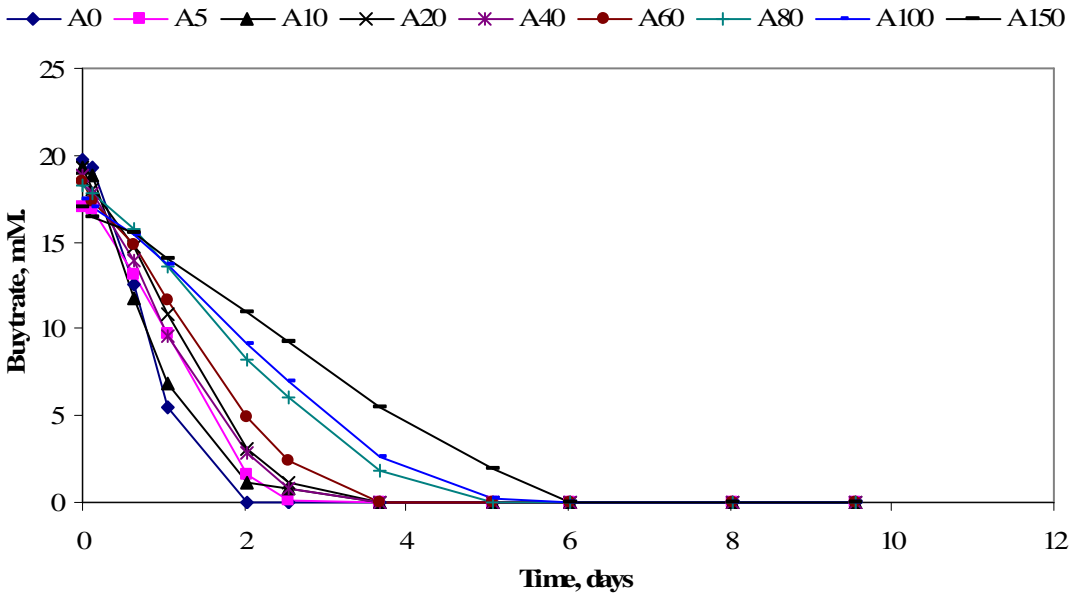


Figure A.20 : The total butyrate degradation profiles of **BUT** reactor sludge incubated at different **acetate** concentrations

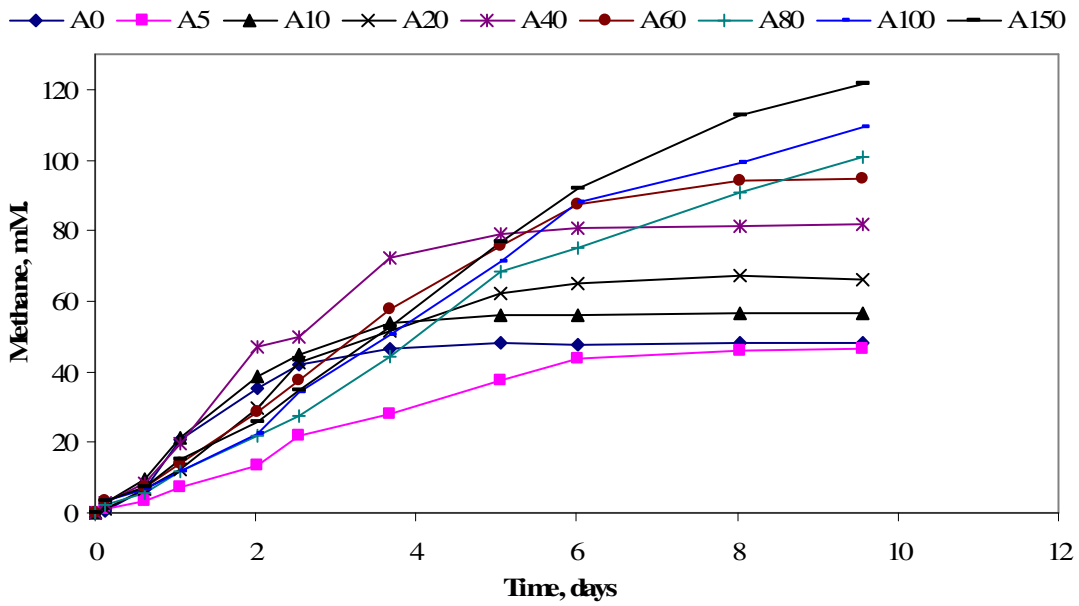


Figure A.21 : The total methane production profiles of **BLS** reactor sludge incubated at different **propionate** concentrations

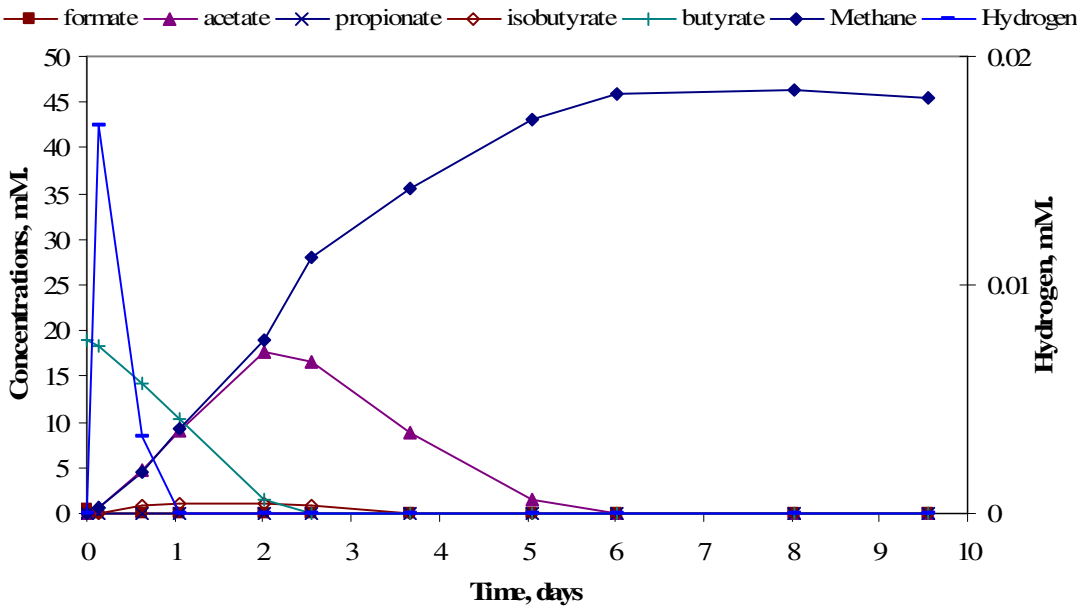


Figure A.22 : The effect of **0.5 mM formate** to the butyrate degradation profile of **BUT** reactor sludge

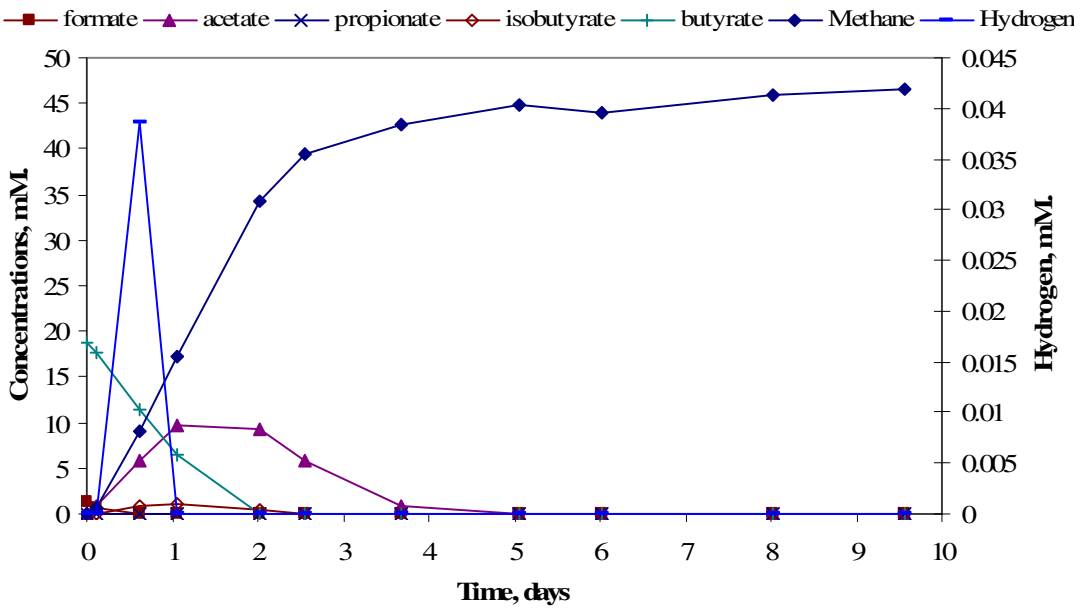


Figure A.23 : The effect of **1 mM formate** to the butyrate degradation profile of **BUT** reactor sludge

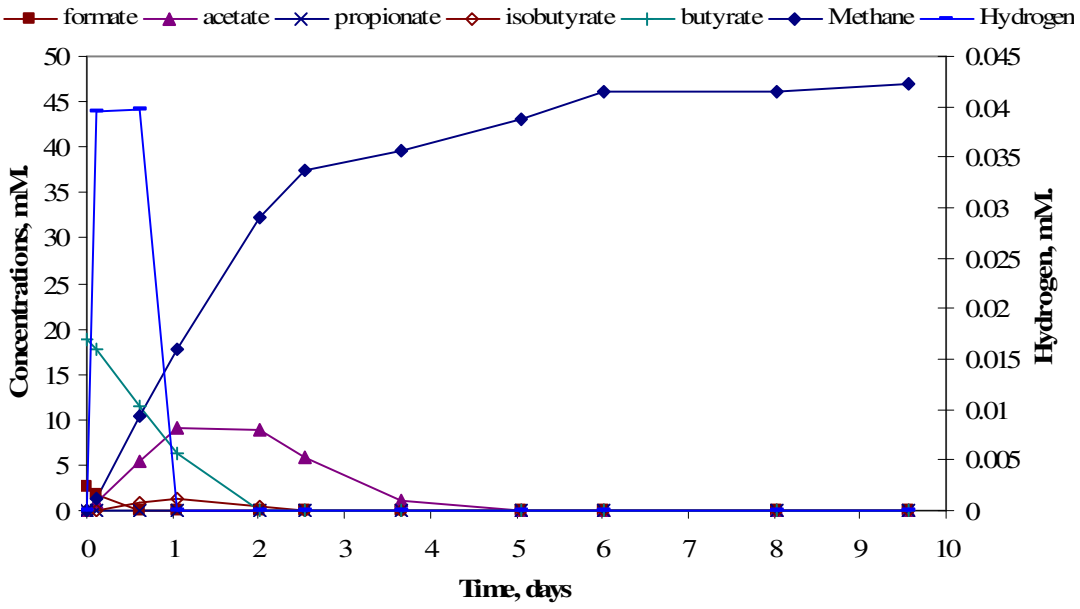


Figure A.24 : The effect of **2.5 mM formate** to the butyrate degradation profile of **BUT** reactor sludge

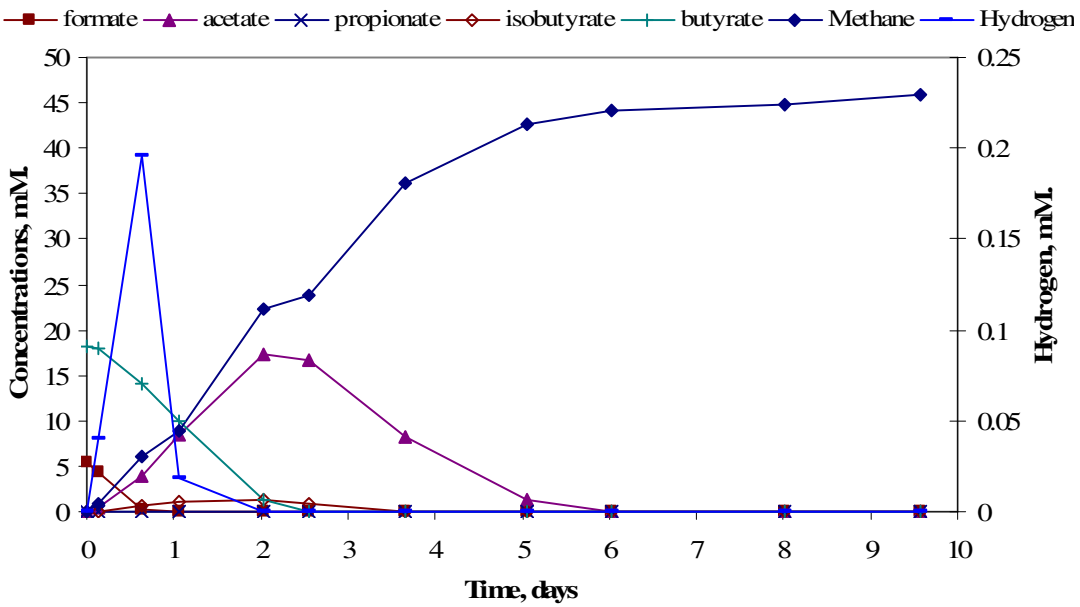


Figure A.25 : The effect of **5 mM formate** to the butyrate degradation profile of **BUT** reactor sludge

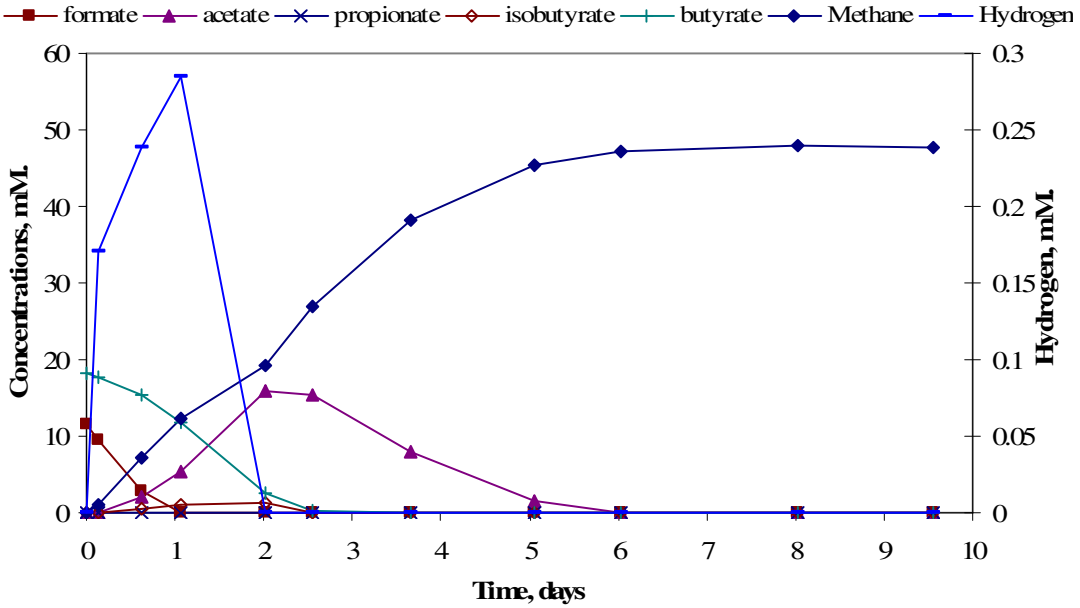


Figure A.26 : The effect of **10 mM formate** to the butyrate degradation profile of **BUT** reactor sludge

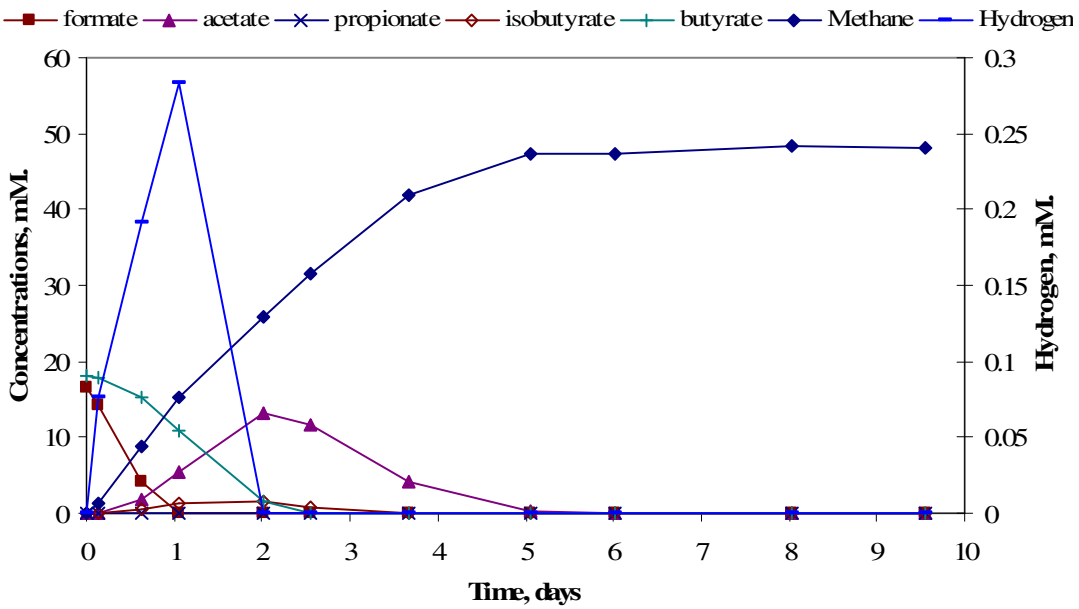


Figure A.27 : The effect of **15 mM formate** to the butyrate degradation profile of **BUT** reactor sludge

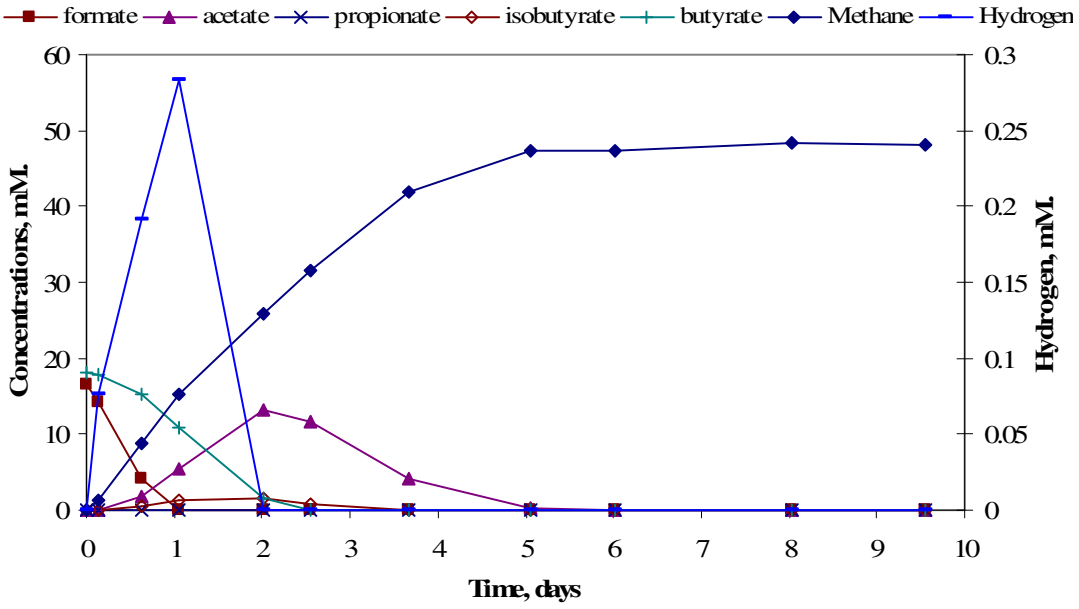


Figure A.28 : The effect of **20 mM formate** to the butyrate degradation profile of **BUT** reactor sludge

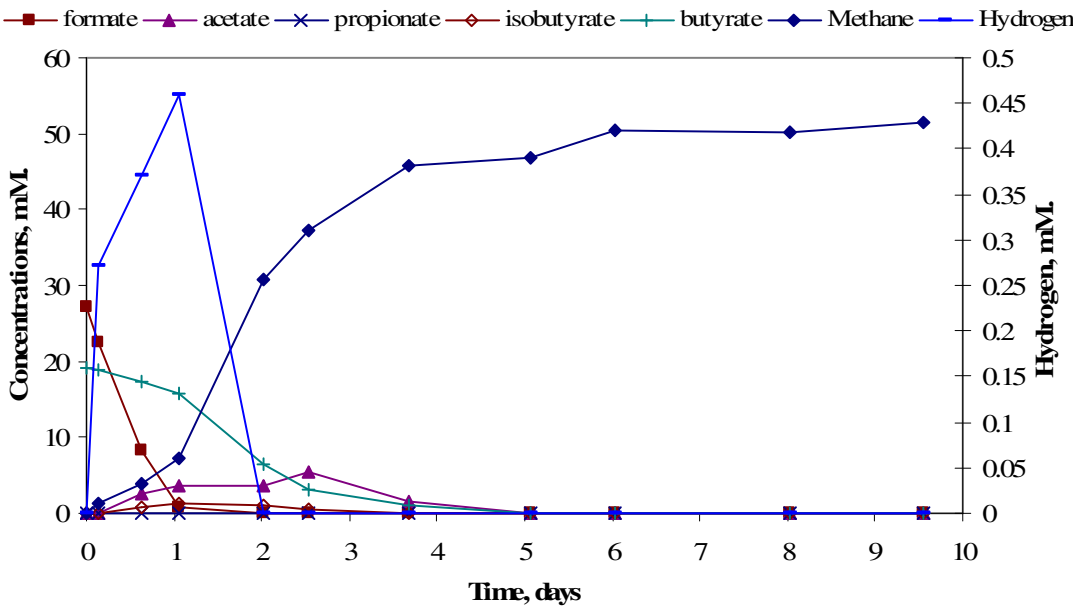


Figure A.29 : The effect of **30 mM formate** to the butyrate degradation profile of **BUT** reactor sludge

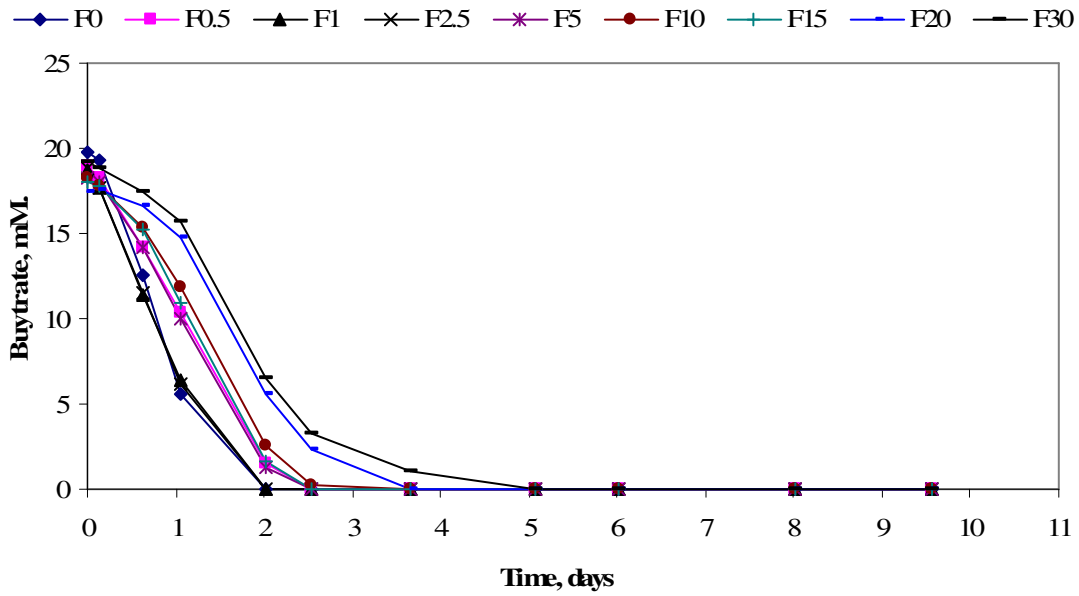


Figure A.30 : The total butyrate degradation profiles of **BUT** reactor sludge incubated at different **formate** concentrations

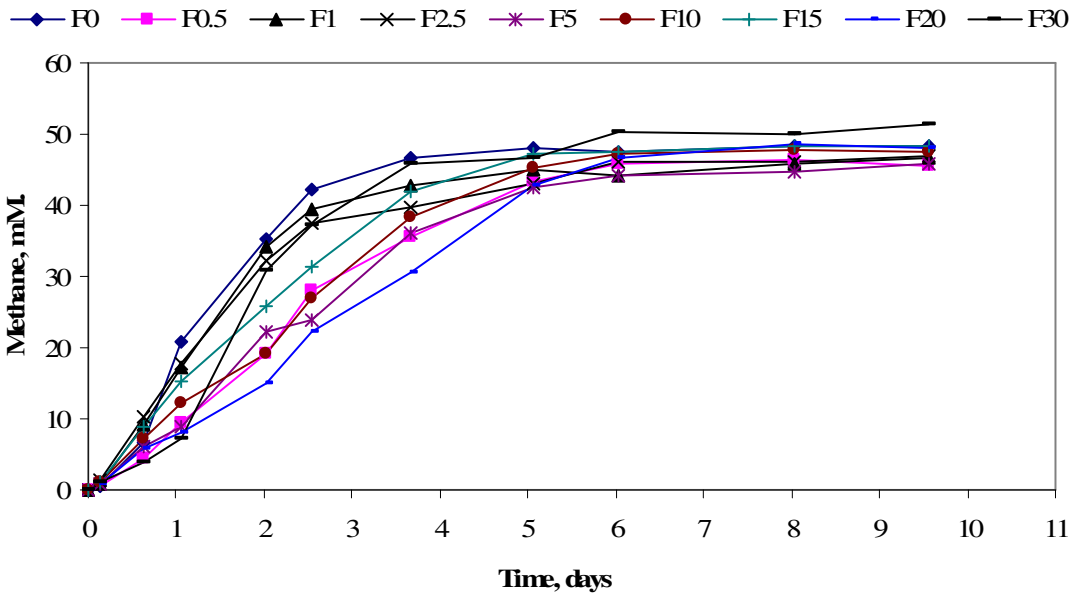


Figure A.31 : The total methane production profiles of **BUT** reactor sludge incubated at different **formate** concentrations

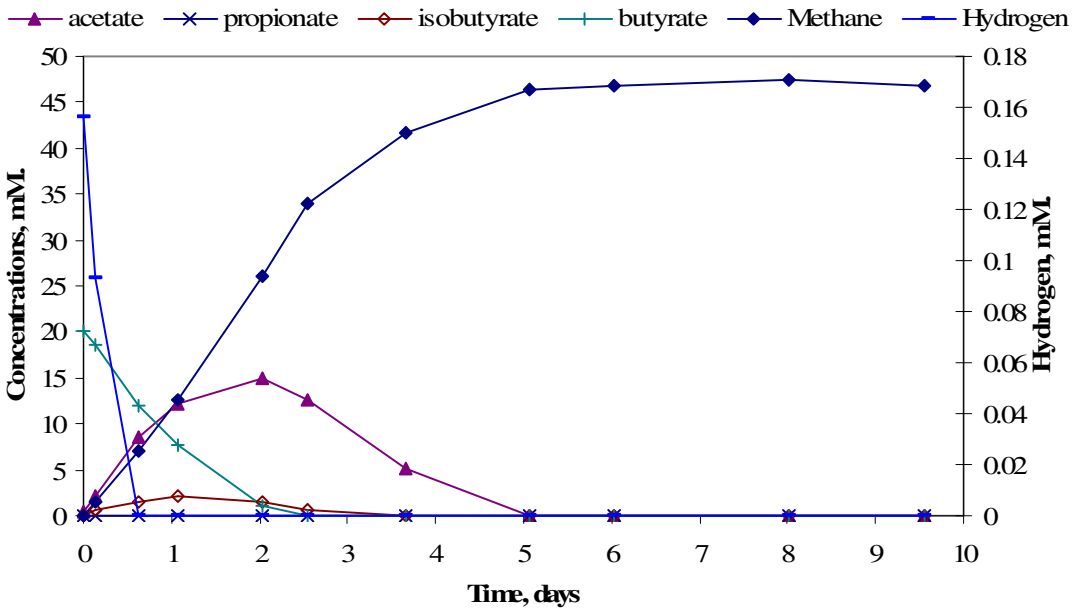


Figure A.32 : The effect of **0.15 mM hydrogen** to the butyrate degradation profile of **BUT** reactor sludge

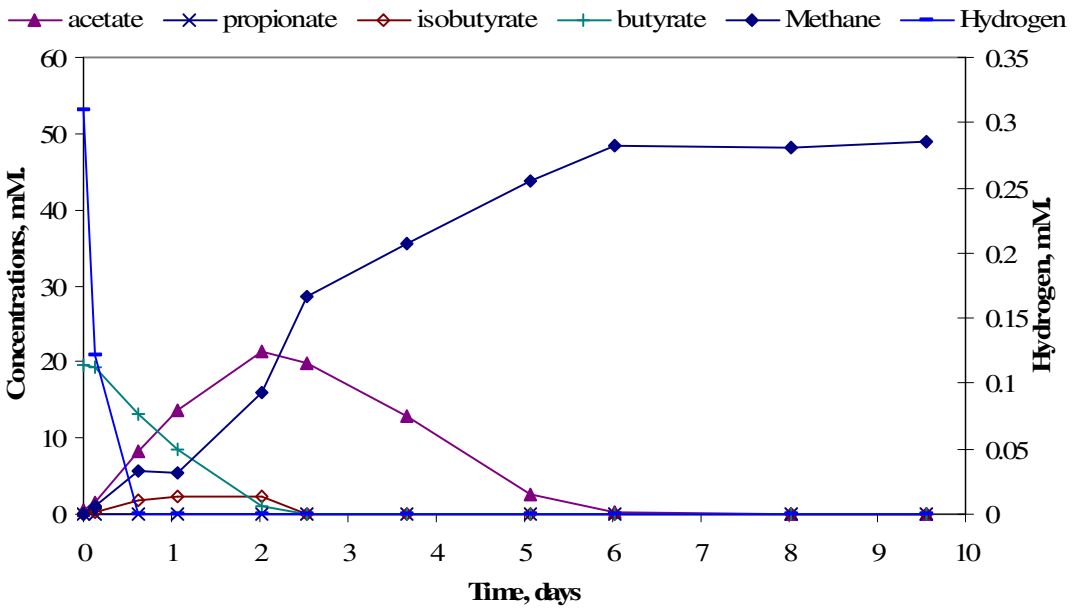


Figure A.33 : The effect of **0.3 mM hydrogen** to the butyrate degradation profile of **BUT** reactor sludge

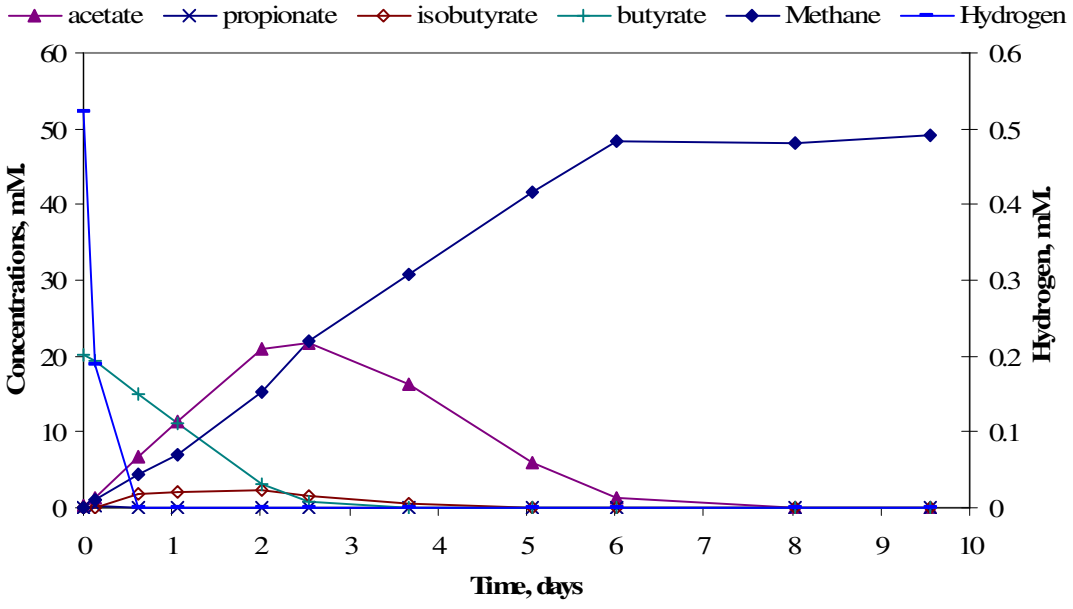


Figure A.34 : The effect of **0.5 mM hydrogen** to the butyrate degradation profile of **BUT** reactor sludge

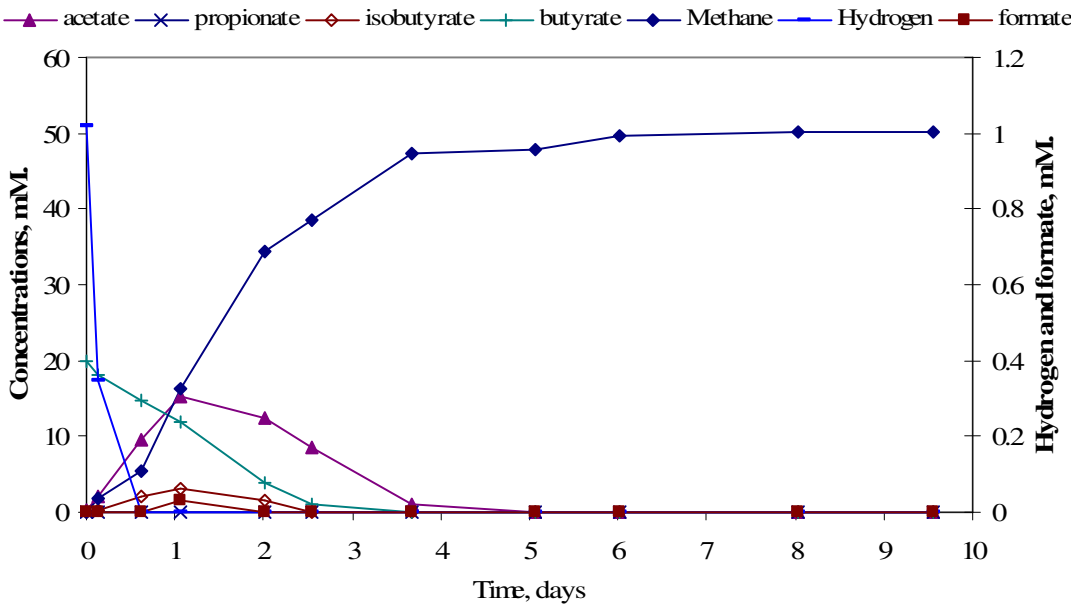


Figure A.35 : The effect of **1 mM hydrogen** to the butyrate degradation profile of **BUT** reactor sludge

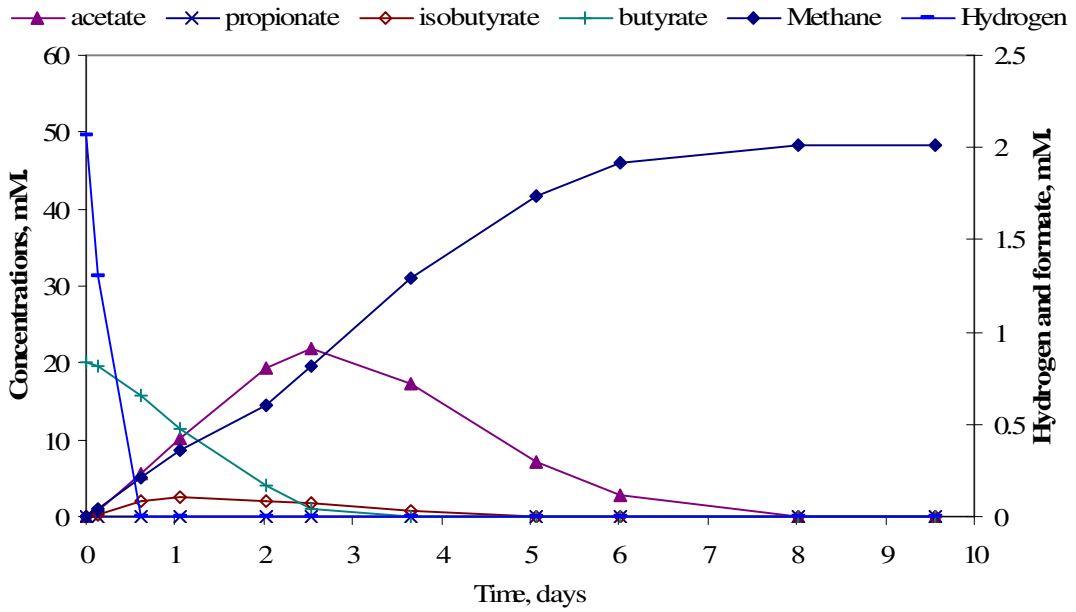


Figure A.36 : The effect of **2 mM hydrogen** to the butyrate degradation profile of **BUT** reactor sludge

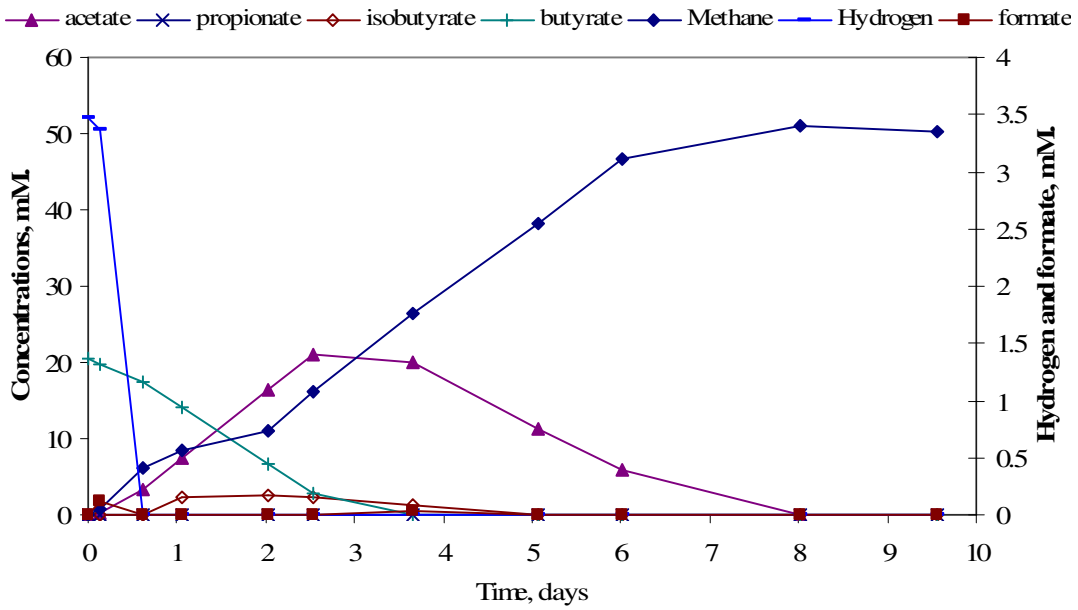


Figure A.37 : The effect of **3.5 mM hydrogen** to the butyrate degradation profile of **BUT** reactor sludge

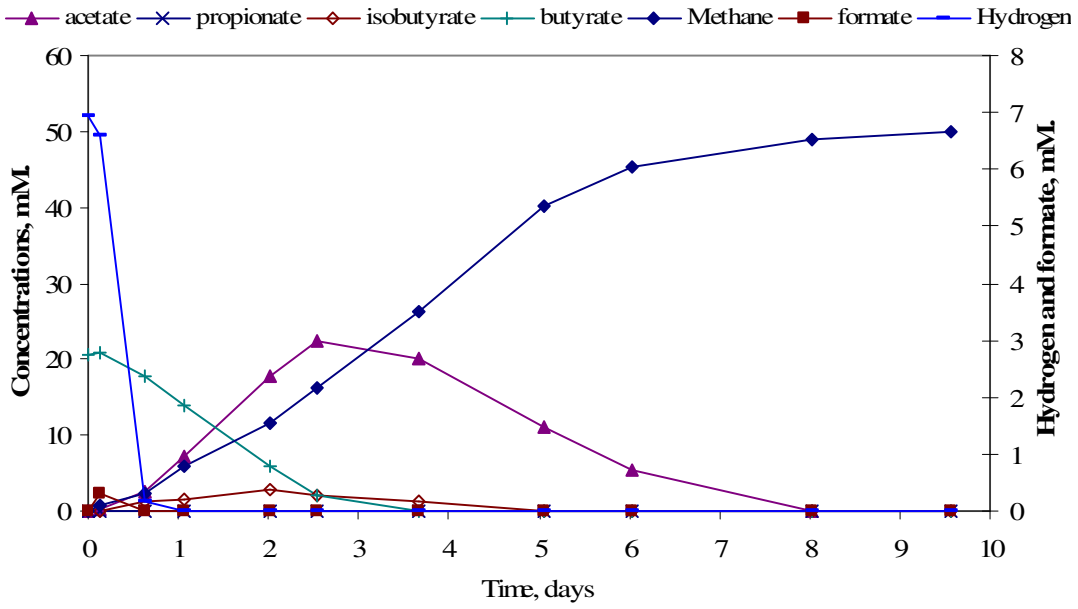


Figure A.38 : The effect of **6 mM hydrogen** to the butyrate degradation profile of **BUT** reactor sludge

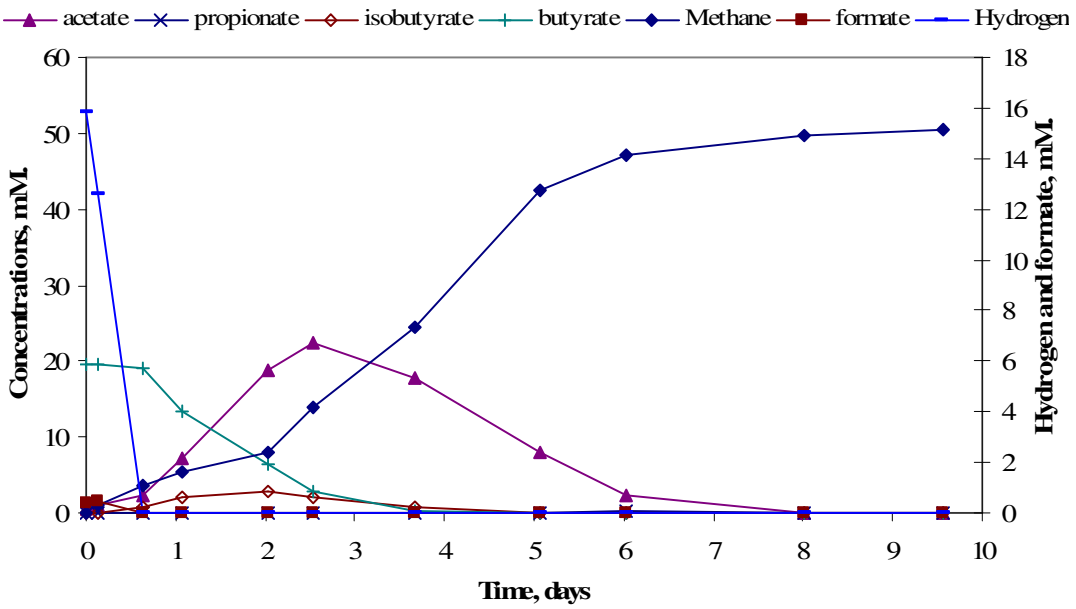


Figure A.39 : The effect of **15 mM hydrogen** to the butyrate degradation profile of **BUT** reactor sludge

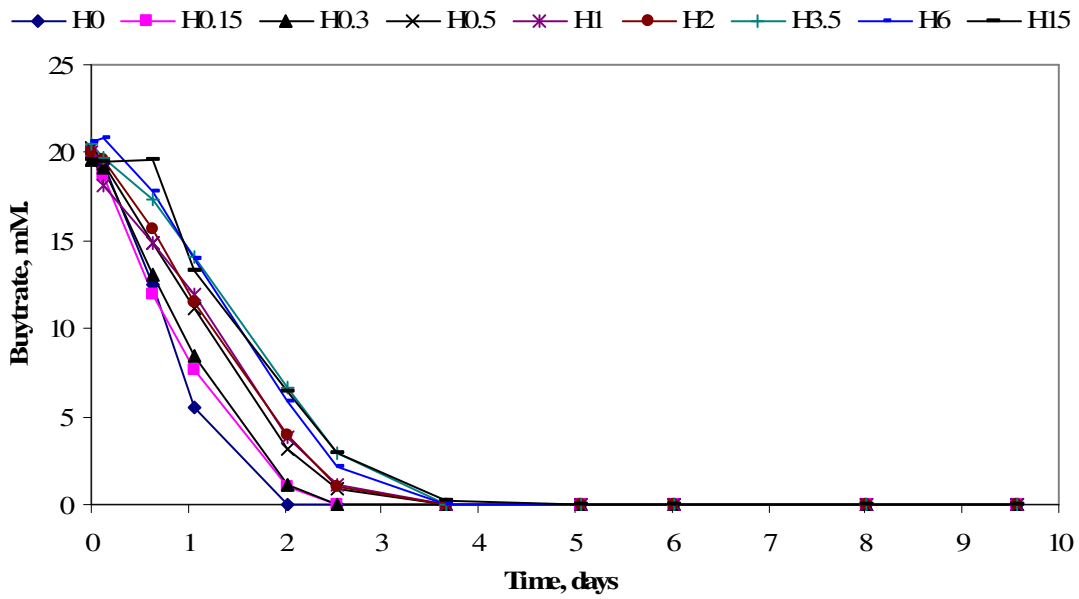


Figure A.40 : The total butyrate degradation profile profiles of **BUT** reactor sludge incubated at different **hydrogen** concentrations

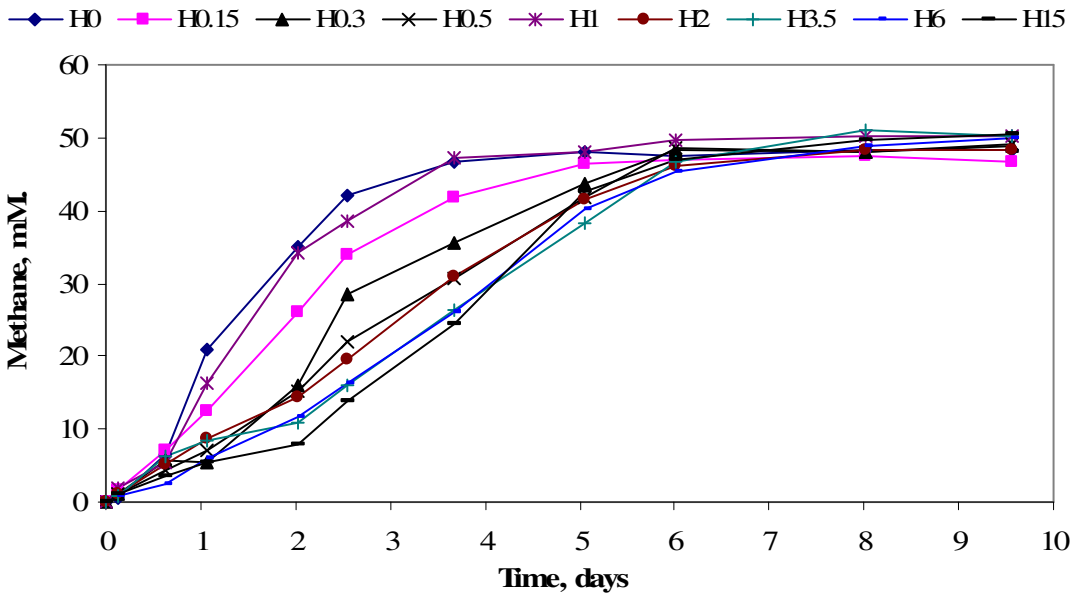


Figure A.41 : The total methane production profiles of **BUT** reactor sludge incubated at different **hydrogen** concentrations

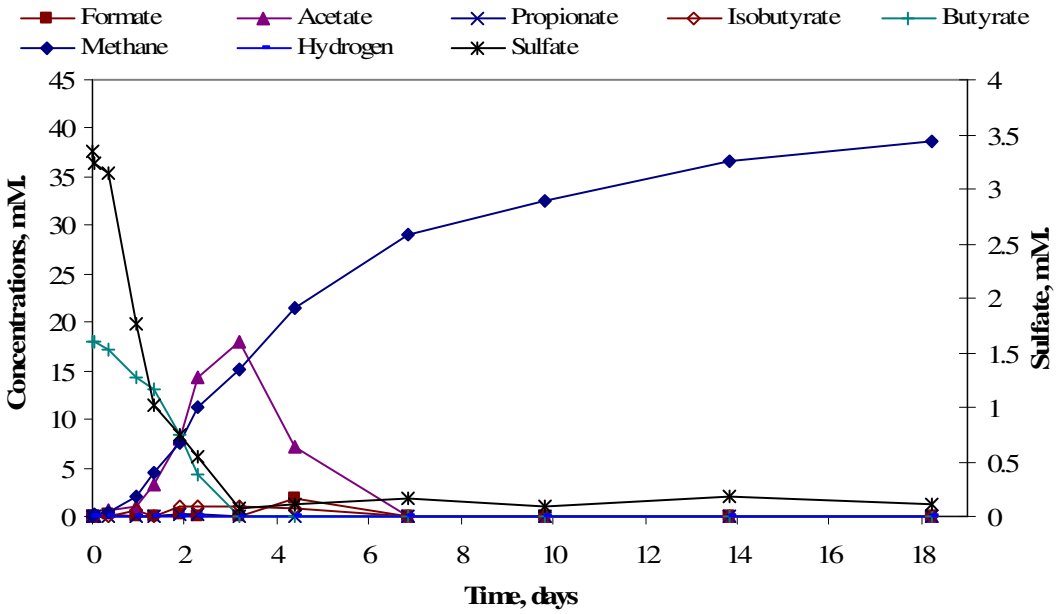


Figure A.42 : Time course of butyrate degradation in the presence of **3 mM sulfate** for **BLS** reactor sludge

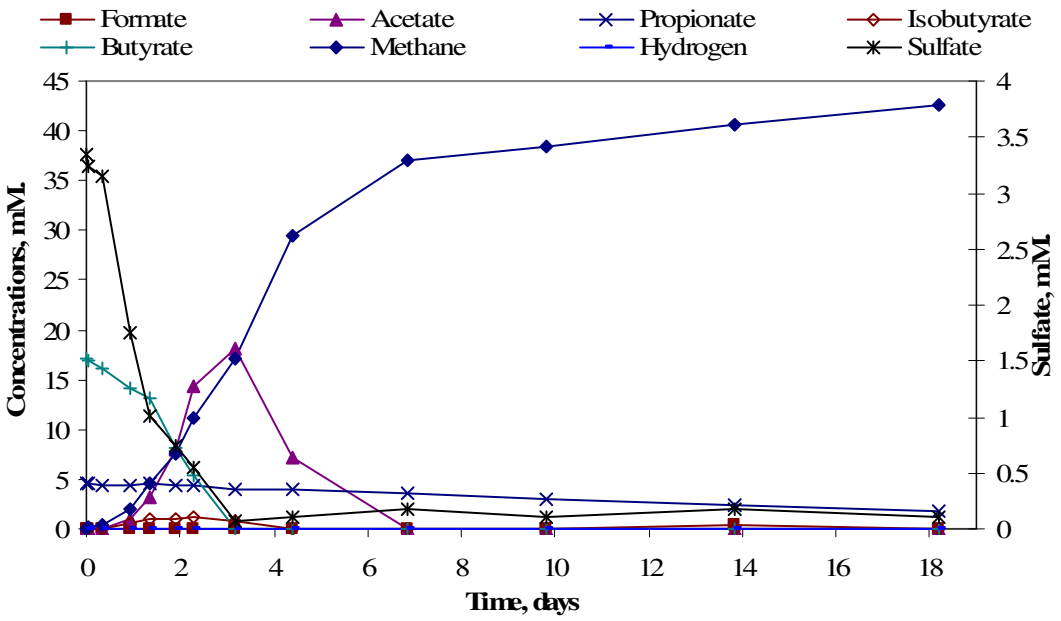


Figure A.43 : The effect of **5 mM propionate** to the butyrate degradation profile of **BLS** reactor sludge in the presence of **3 mM sulfate**

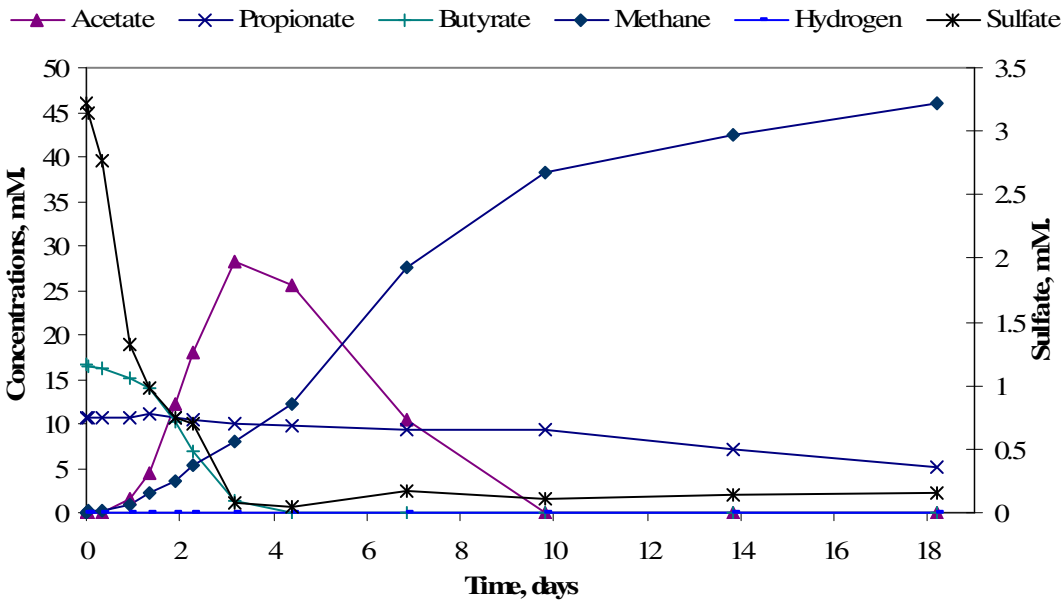


Figure A.44 : The effect of **10 mM propionate** to the butyrate degradation profile of **BLS** reactor sludge in the presence of **3 mM sulfate**

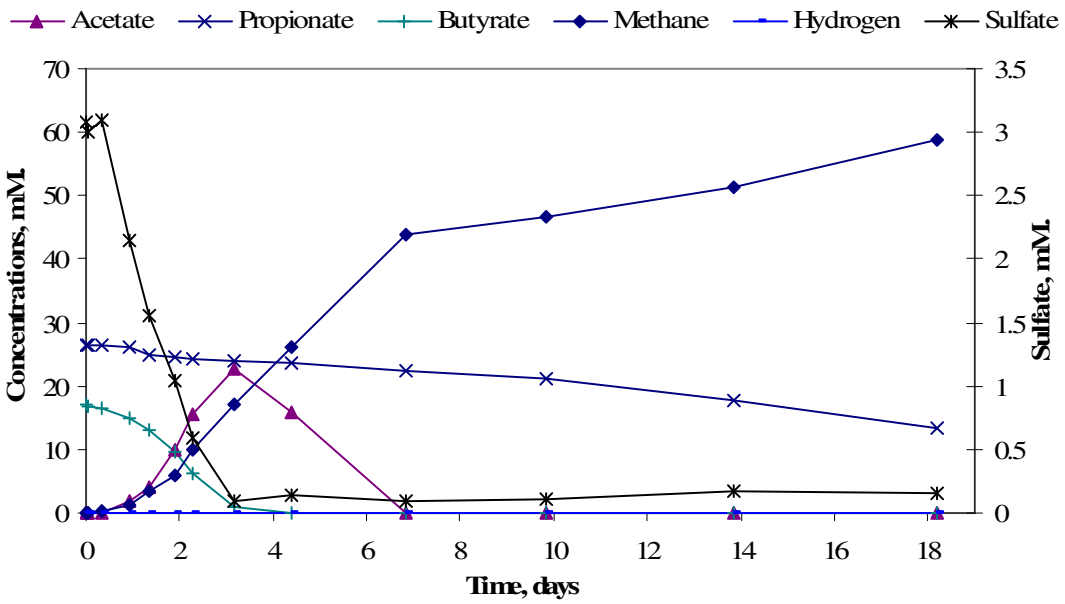


Figure A.45 : The effect of **20 mM propionate** to the butyrate degradation profile of **BLS** reactor sludge in the presence of **3 mM sulfate**

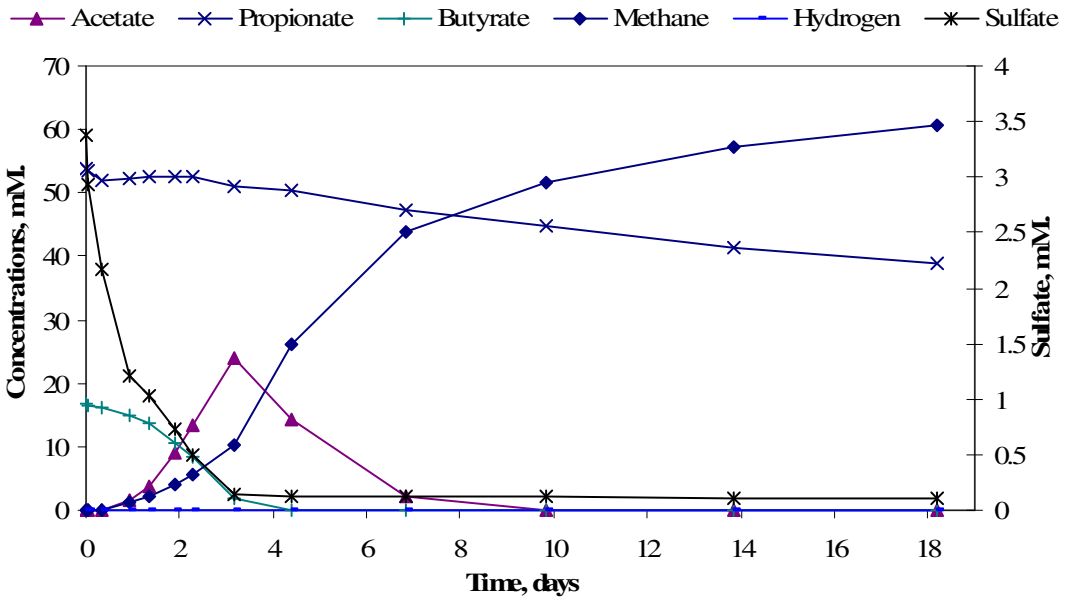


Figure A.46 : The effect of **40 mM propionate** to the butyrate degradation profile of **BLS** reactor sludge in the presence of **3 mM sulfate**

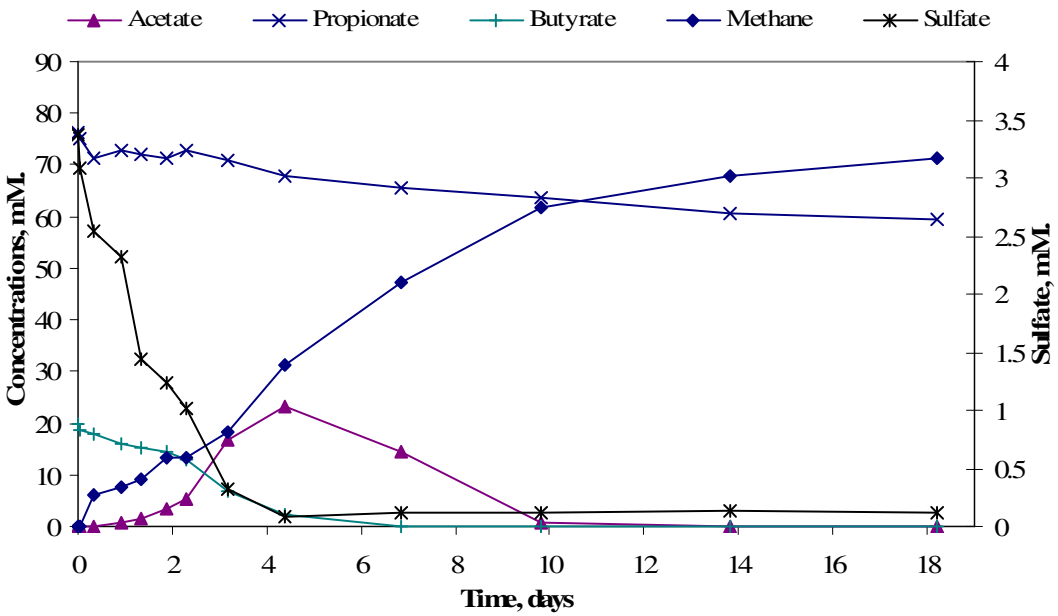


Figure A.47 : The effect of **60 mM propionate** to the butyrate degradation profile of **BLS** reactor sludge in the presence of **3 mM sulfate**

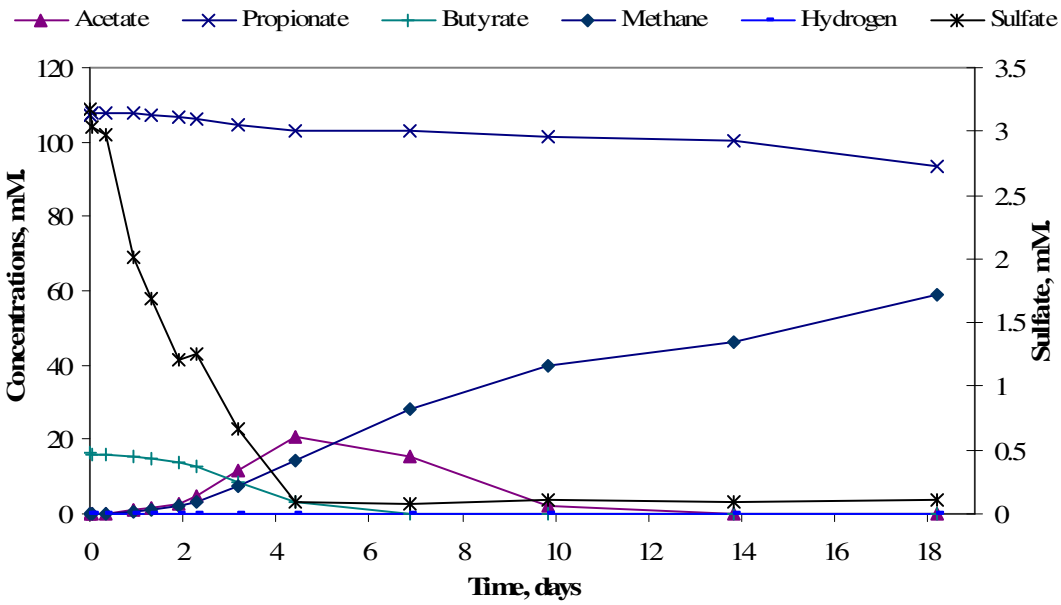


Figure A.48 : The effect of **80 mM propionate** to the butyrate degradation profile of **BLS** reactor sludge in the presence of **3 mM sulfate**

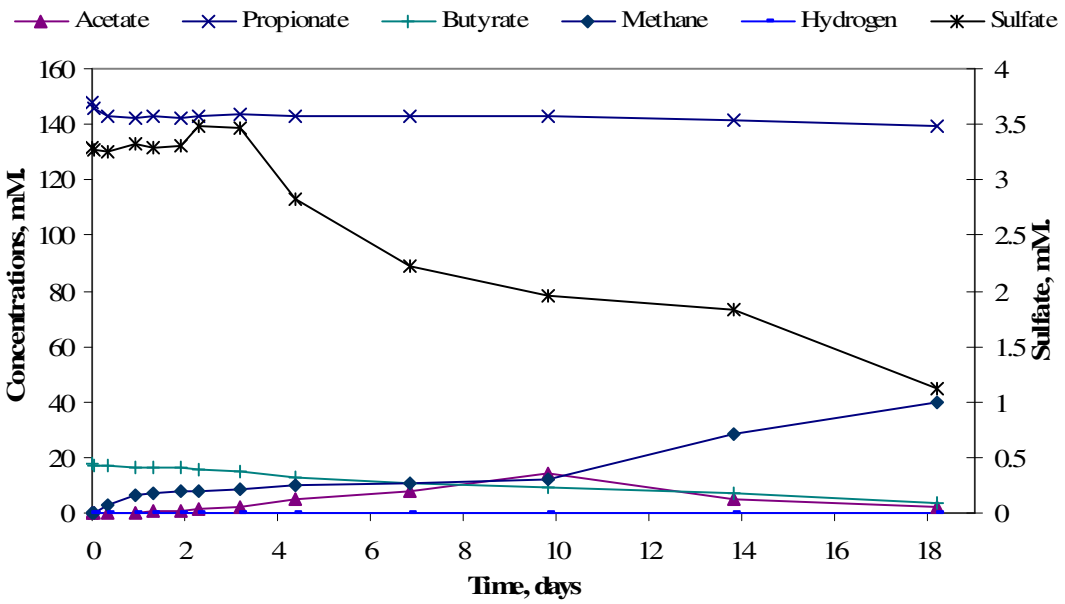


Figure A.49 : The effect of **100 mM propionate** to the butyrate degradation profile of **BLS** reactor sludge in the presence of **3 mM sulfate**

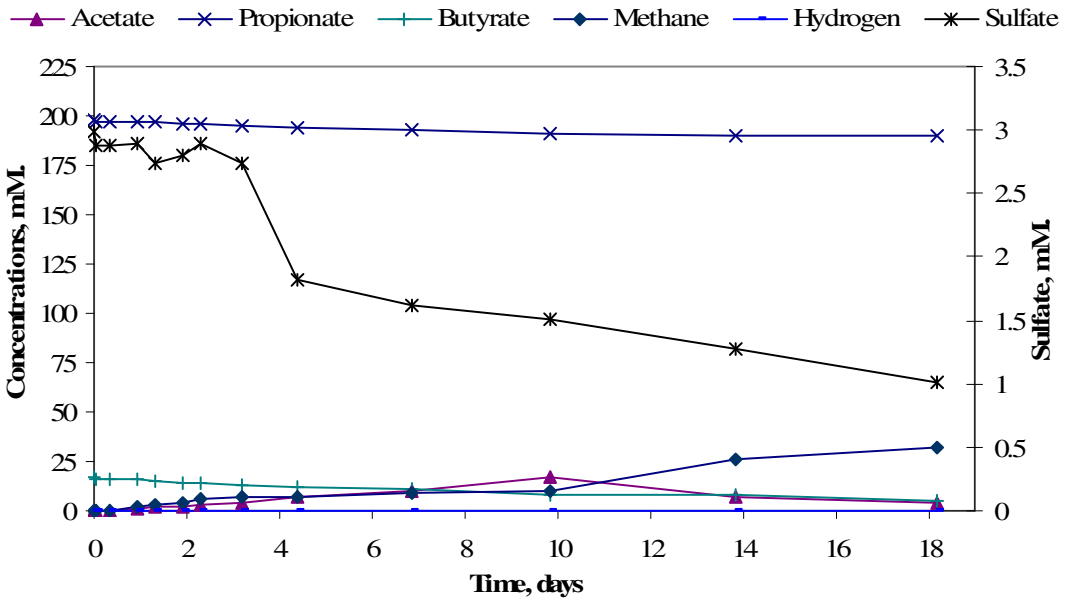


Figure A.50 : The effect of **150 mM propionate** to the butyrate degradation profile of **BLS** reactor sludge in the presence of **3 mM sulfate**

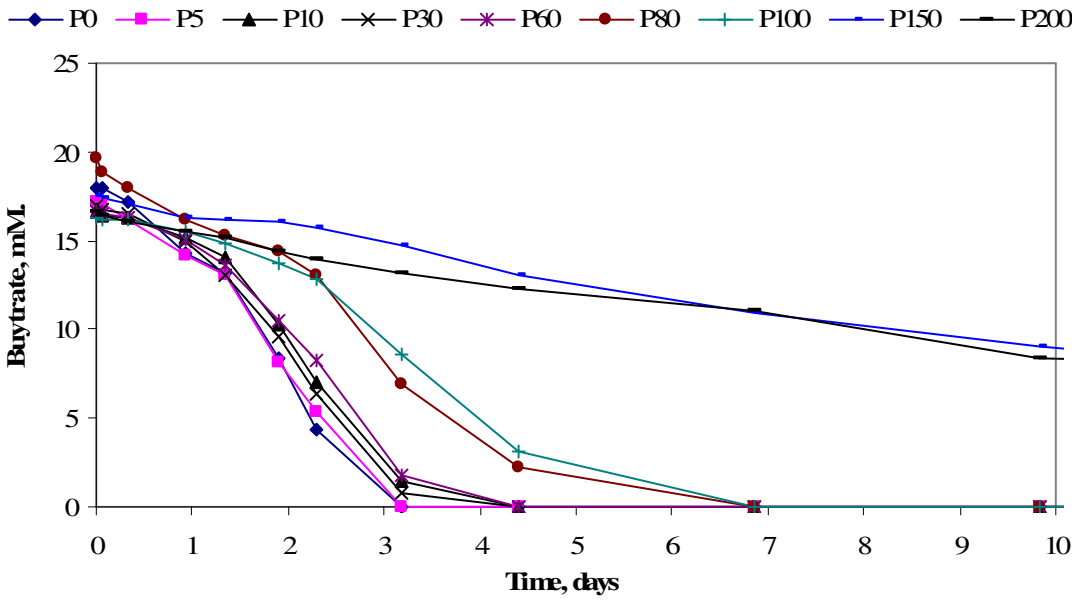


Figure A.51 : The total butyrate degradation profiles of **BLS** reactor sludge incubated at different **propionate** concentrations and in the presence of **3 mM sulfate**

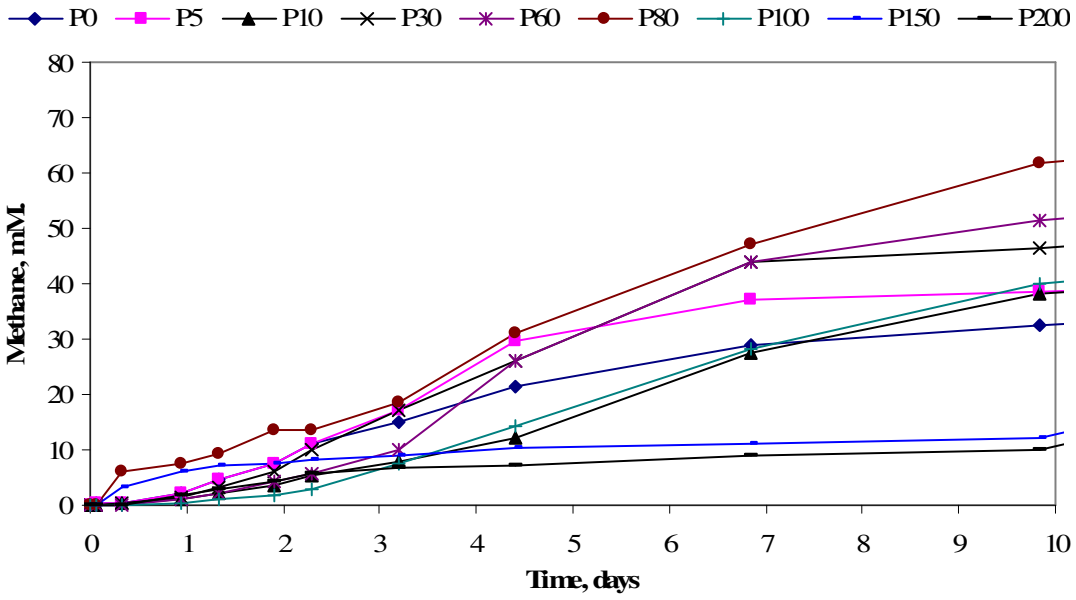


Figure A.52 : The total methane production profiles of **BLS** reactor sludge incubated at different **propionate** concentrations and in the presence of **3mM sulfate**

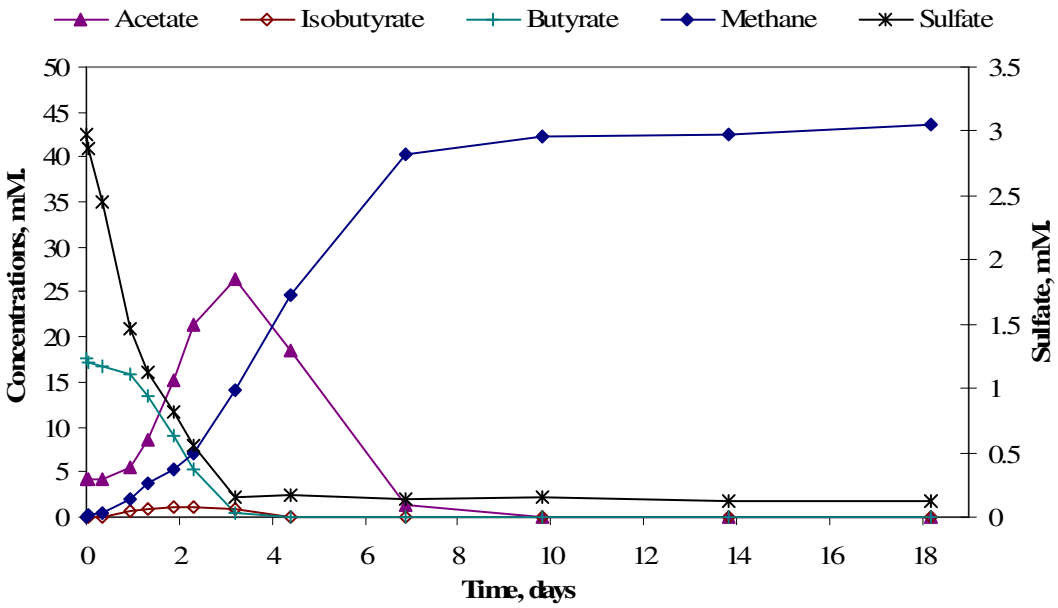


Figure A.53 : The effect of **5 mM acetate** to the butyrate degradation profile of **BLS** reactor sludge in the presence of **3 mM sulfate**

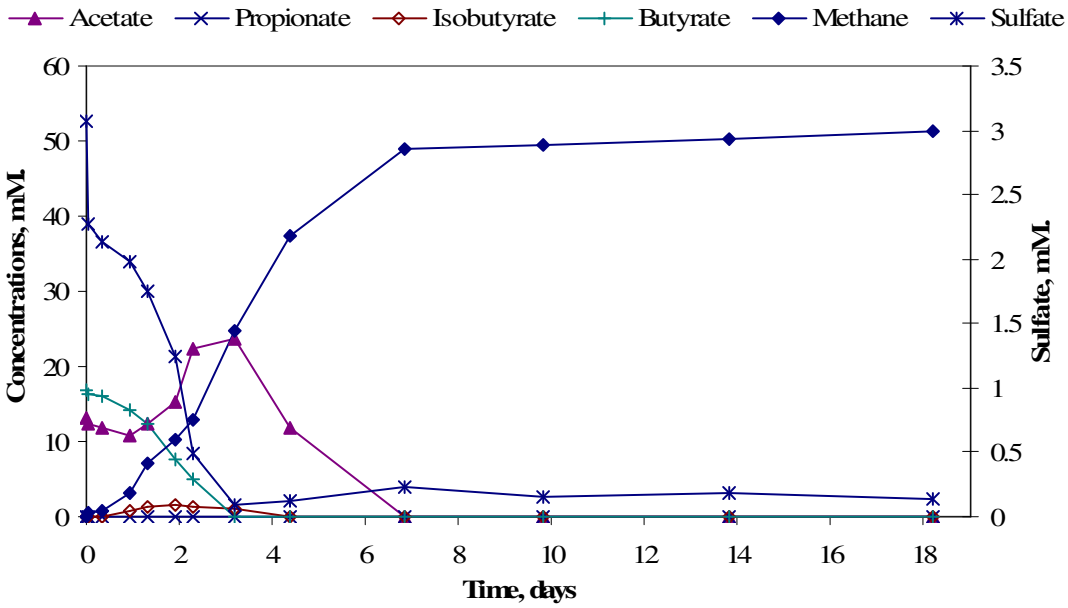


Figure A.54 : The effect of **10 mM acetate** to the butyrate degradation profile of **BLS** reactor sludge in the presence of **3 mM sulfate**

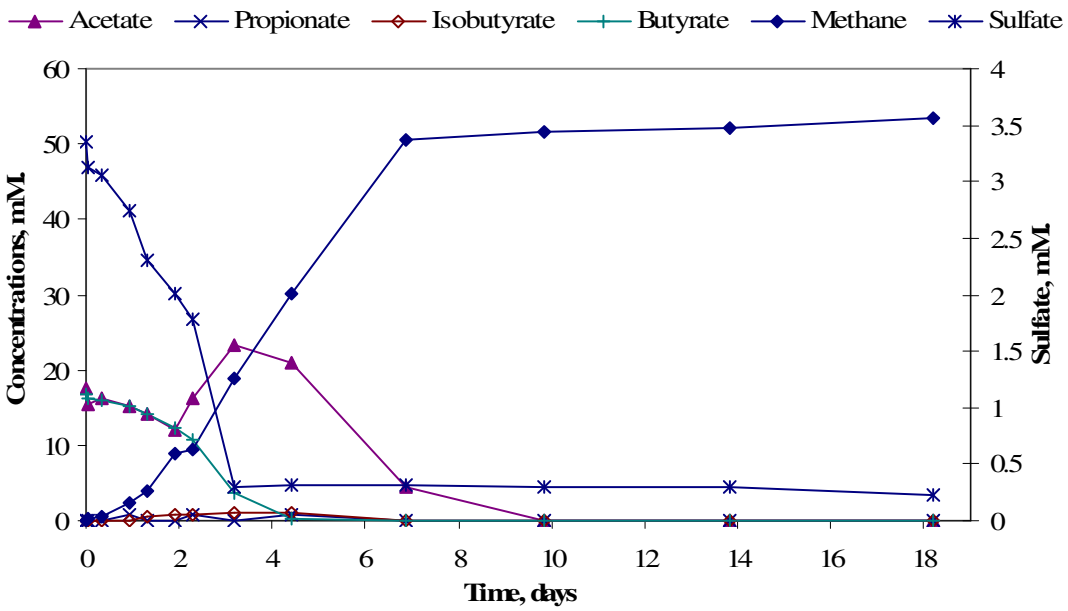


Figure A.55 : The effect of **20 mM acetate** to the butyrate degradation profile of **BLS** reactor sludge in the presence of **3 mM sulfate**

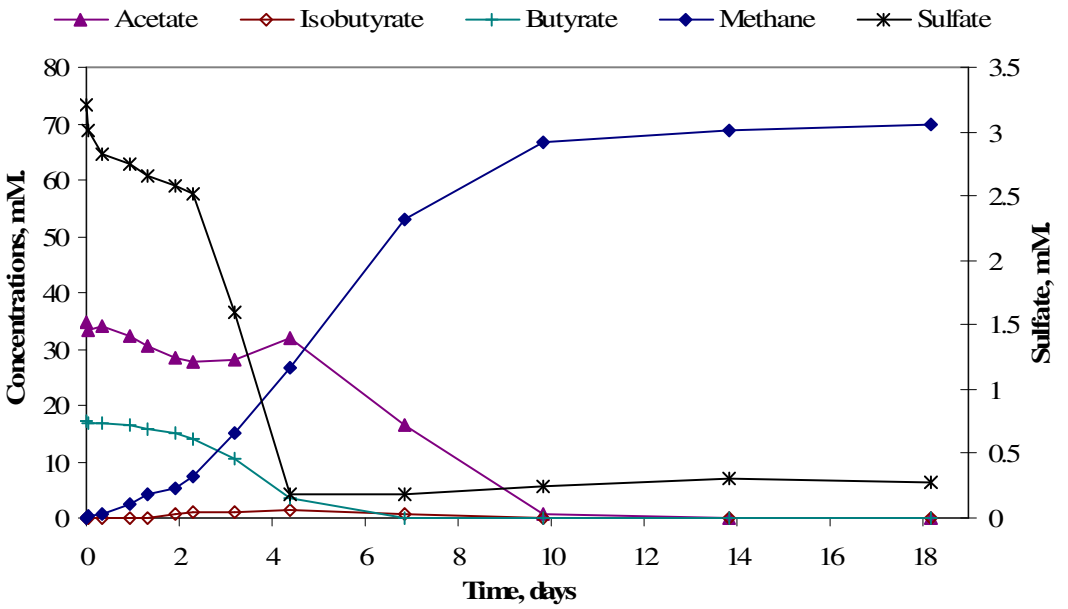


Figure A.56 : The effect of **30 mM acetate** to the butyrate degradation profile of **BLS** reactor sludge in the presence of **3 mM sulfate**

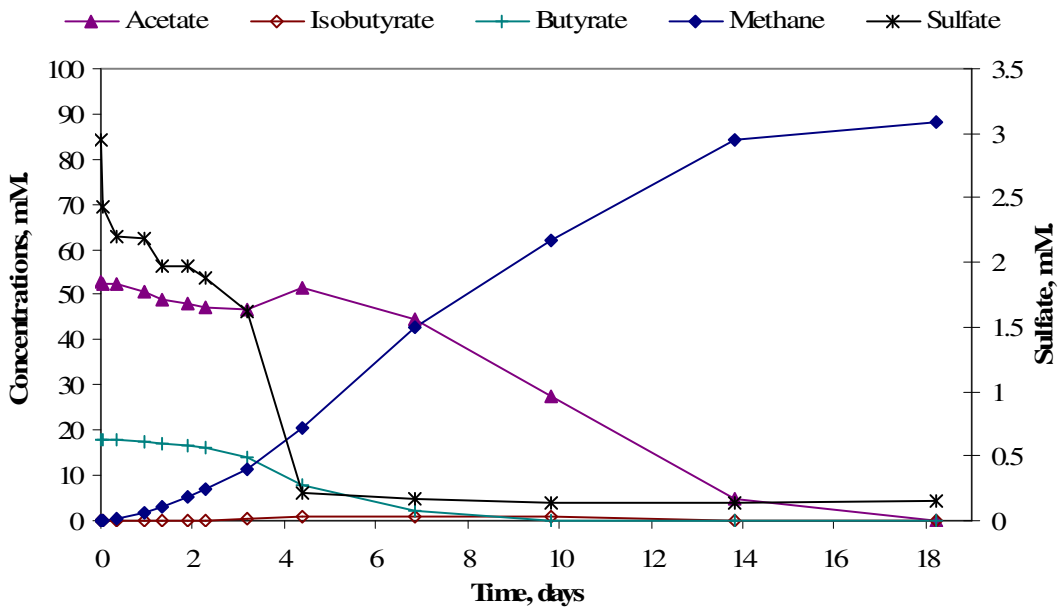


Figure A.57 : The effect of **50 mM acetate** to the butyrate degradation profile of **BLS** reactor sludge in the presence of **3 mM sulfate**

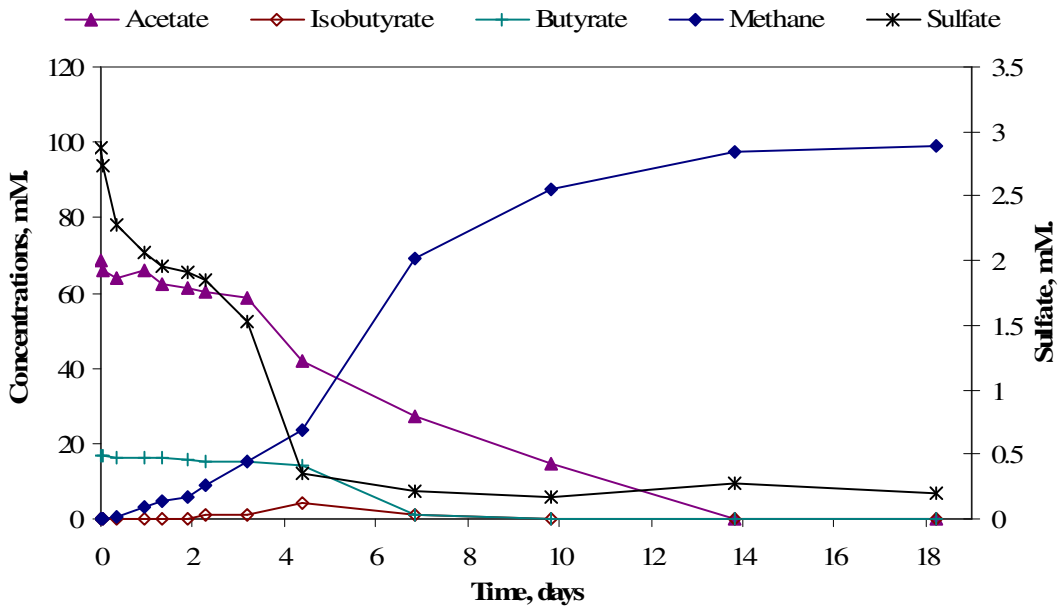


Figure A.58 : The effect of **70 mM acetate** to the butyrate degradation profile of **BLS** reactor sludge in the presence of **3 mM sulfate**

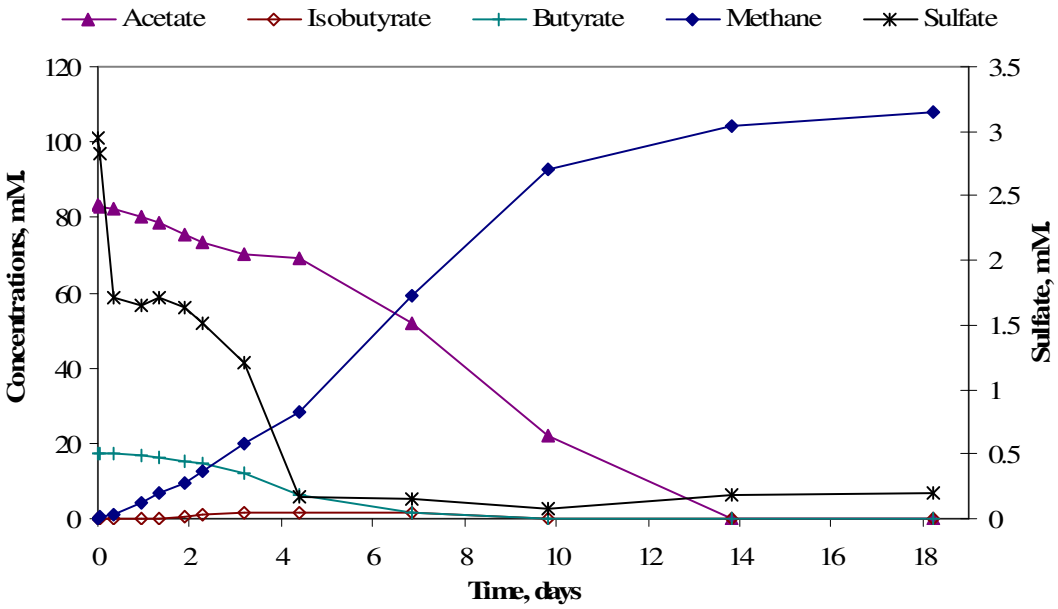


Figure A.59 : The effect of **80 mM acetate** to the butyrate degradation profile of **BLS** reactor sludge in the presence of **3 mM sulfate**

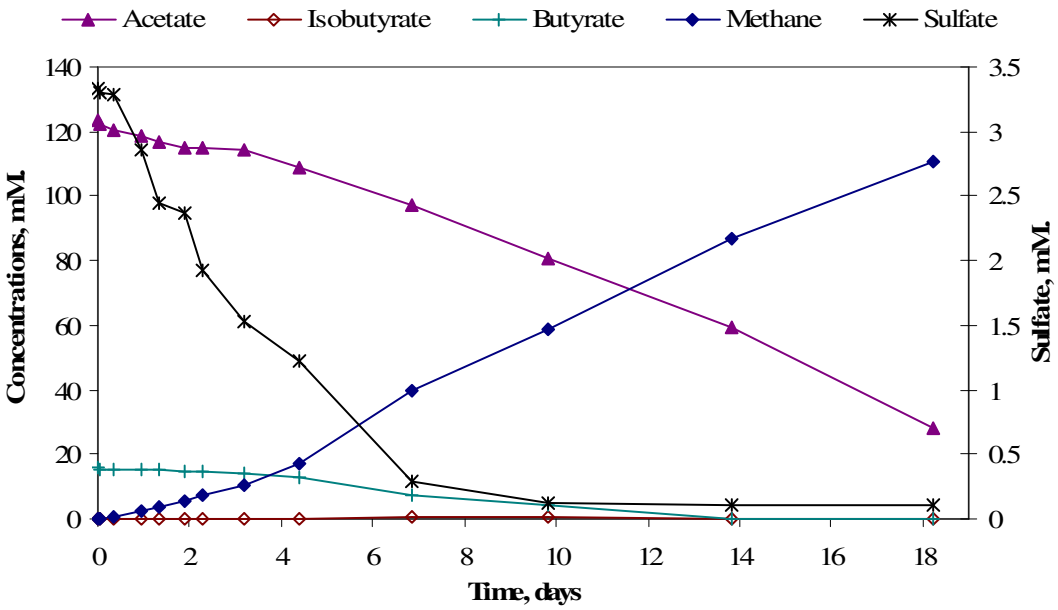


Figure A.60 : The effect of **120 mM acetate** to the butyrate degradation profile of **BLS** reactor sludge in the presence of **3 mM sulfate**

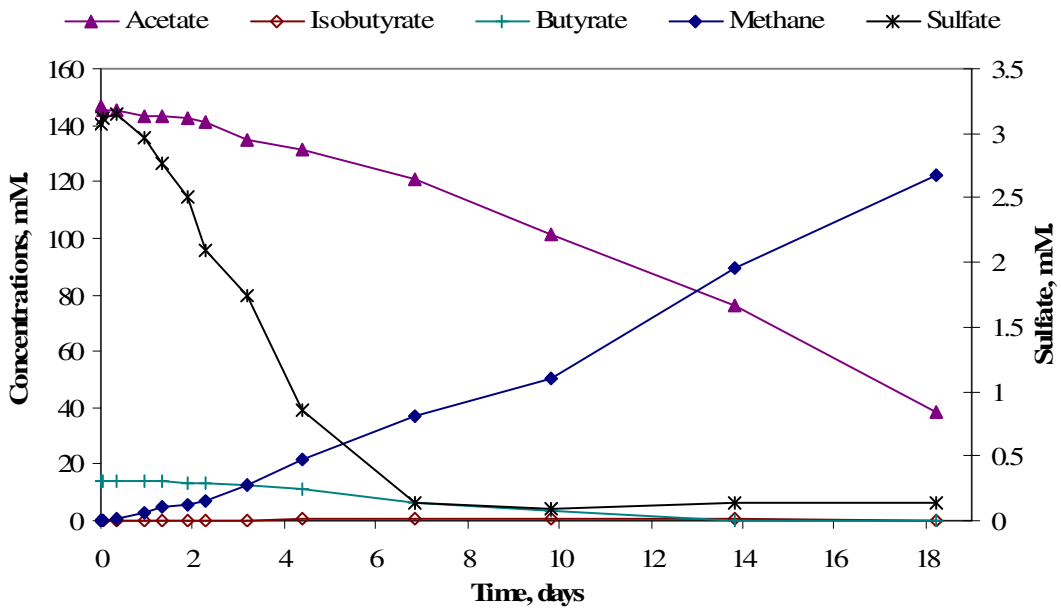


Figure A.61 : The effect of **150 mM acetate** to the butyrate degradation profile of **BLS** reactor sludge in the presence of **3 mM sulfate**

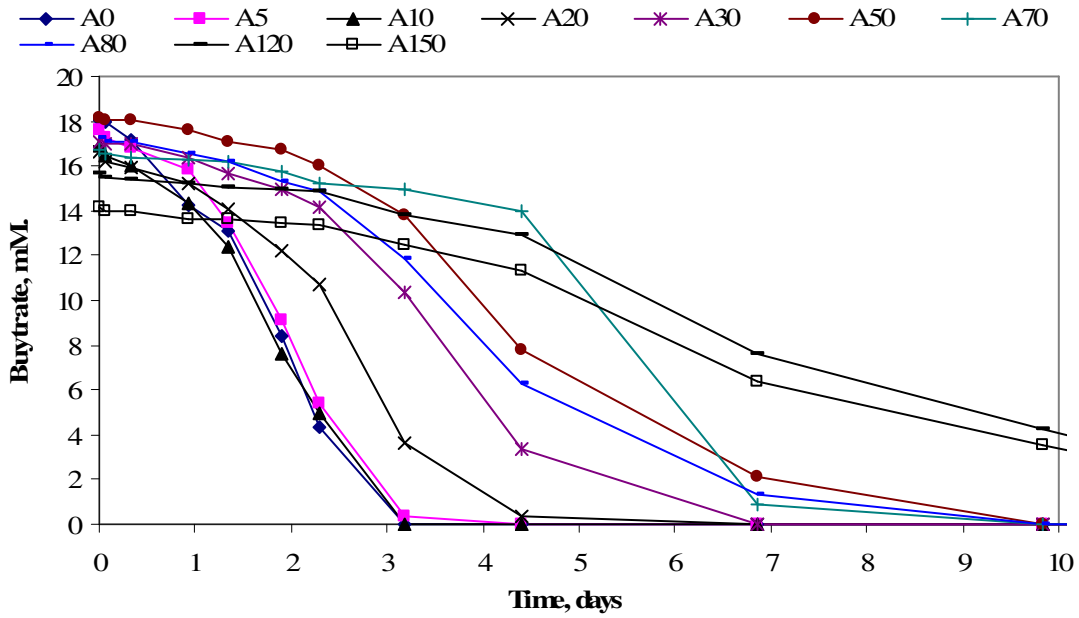


Figure A.62 : The total butyrate degradation profiles of **BLS** reactor sludge incubated at different **acetate** concentrations and in the presence of **3 mM sulfate**

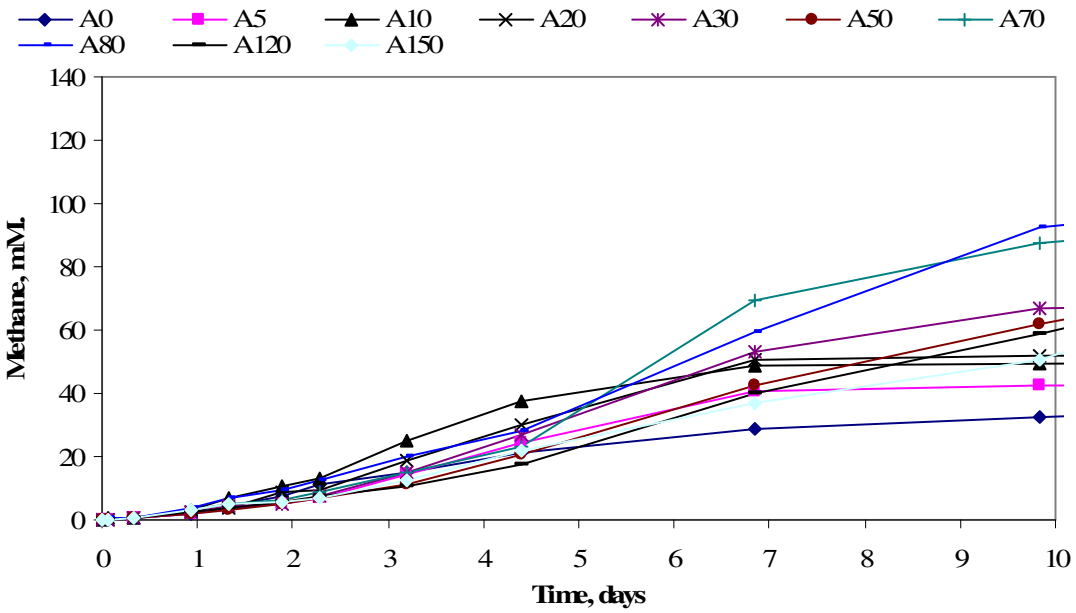


Figure A.63 : The total methane production profiles of **BLS** reactor sludge incubated at different **acetate** concentrations and in the presence of **3mM sulfate**

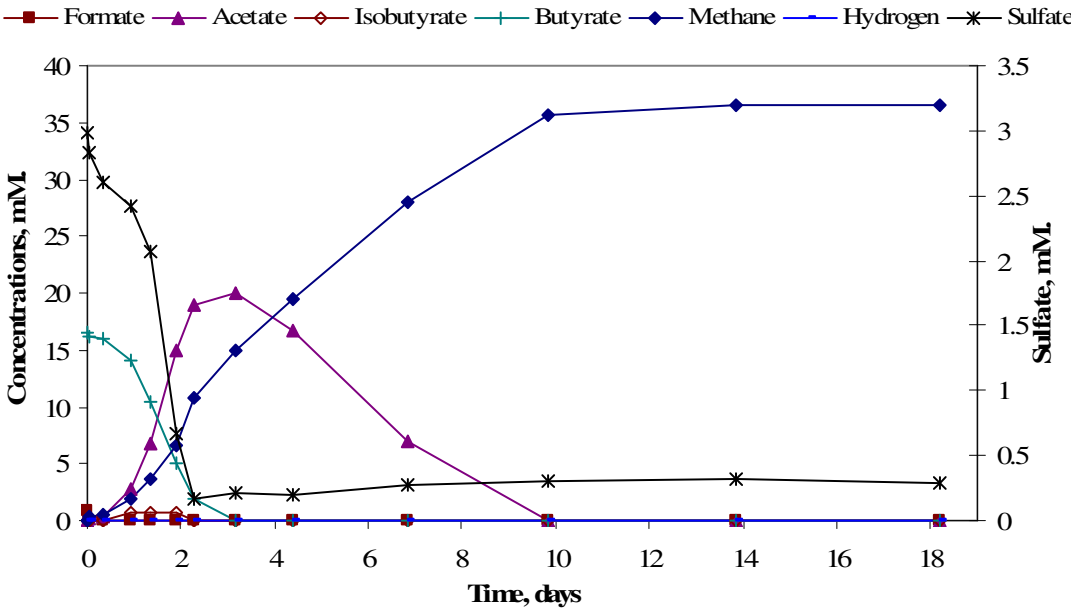


Figure A.64 : The effect of **0.9 mM formate** to the butyrate degradation profile of **BLS** reactor sludge in the presence of **3 mM sulfate**

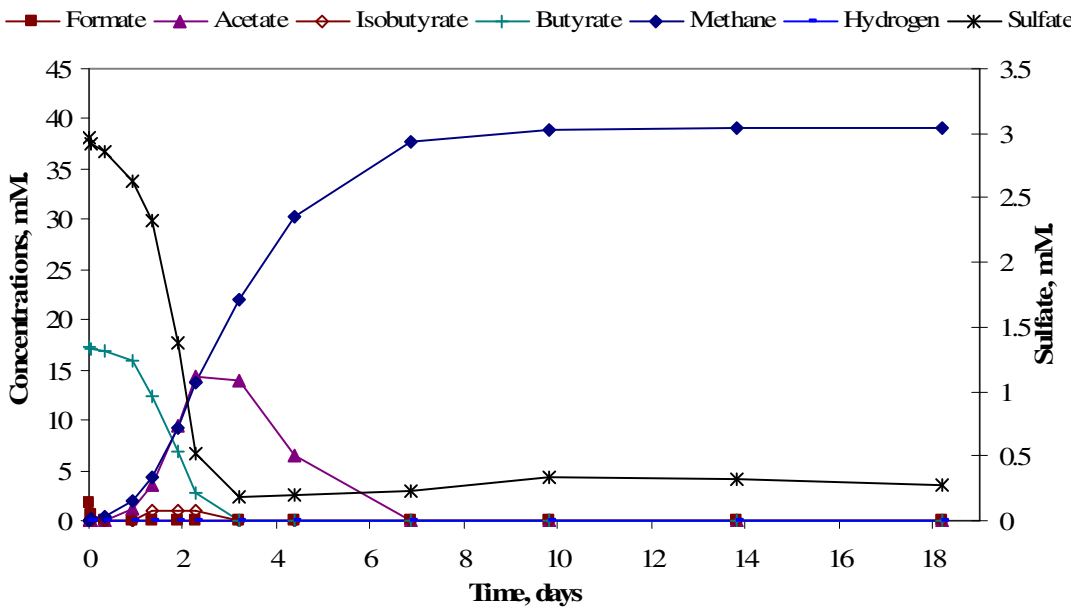


Figure A.65 : The effect of **1.5 mM formate** to the butyrate degradation profile of **BLS** reactor sludge in the presence of **3 mM sulfate**

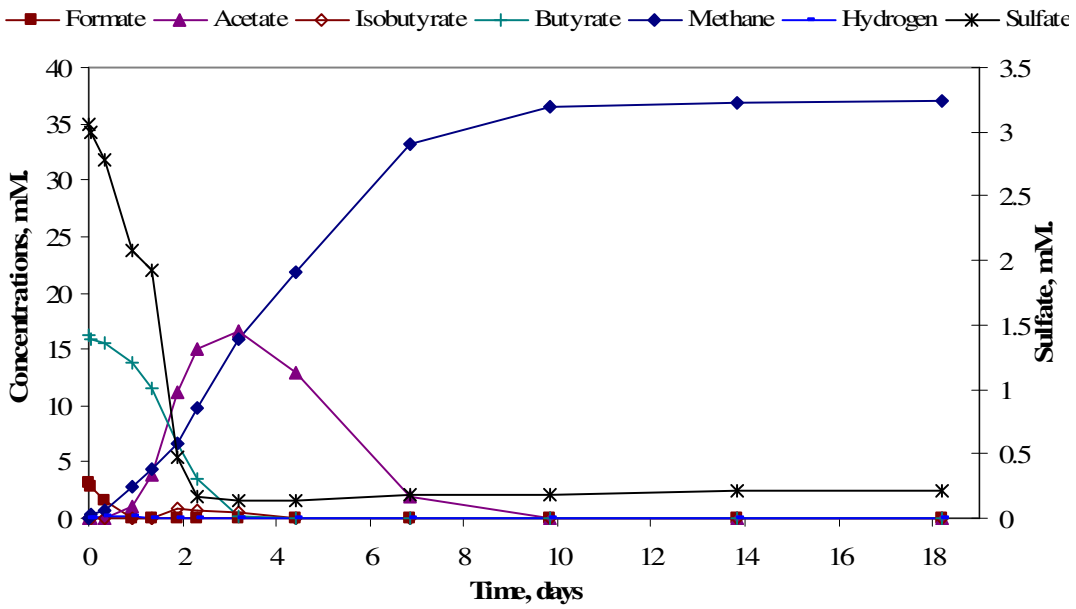


Figure A.66 : The effect of **3 mM formate** to the butyrate degradation profile of **BLS** reactor sludge in the presence of **3 mM sulfate**

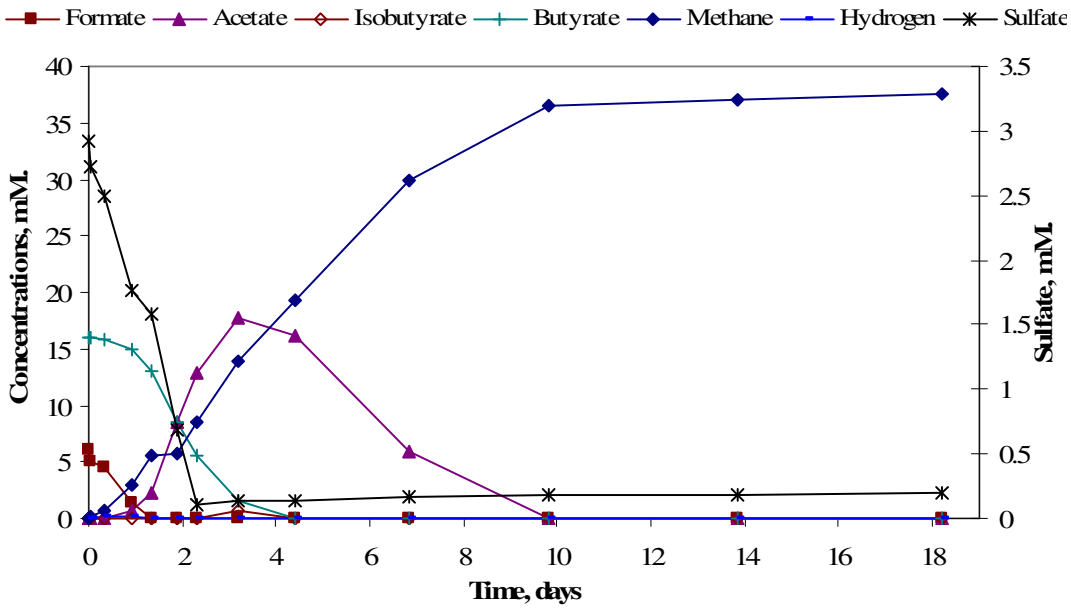


Figure A.67 : The effect of **5 mM formate** to the butyrate degradation profile of **BLS** reactor sludge in the presence of **3 mM sulfate**

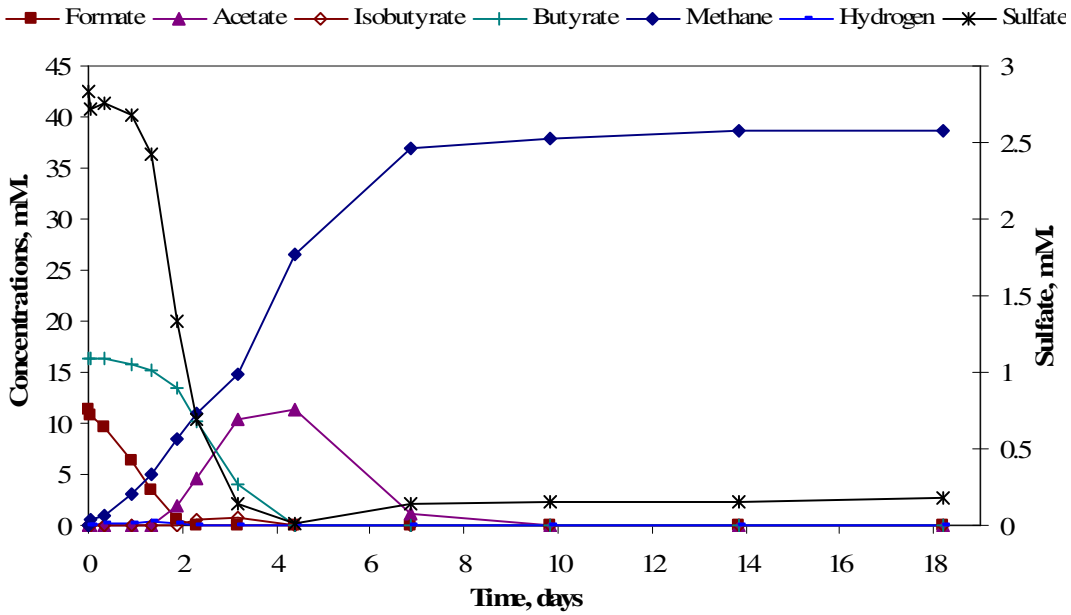


Figure A.68 : The effect of **10 mM formate** to the butyrate degradation profile of **BLS** reactor sludge in the presence of **3 mM sulfate**

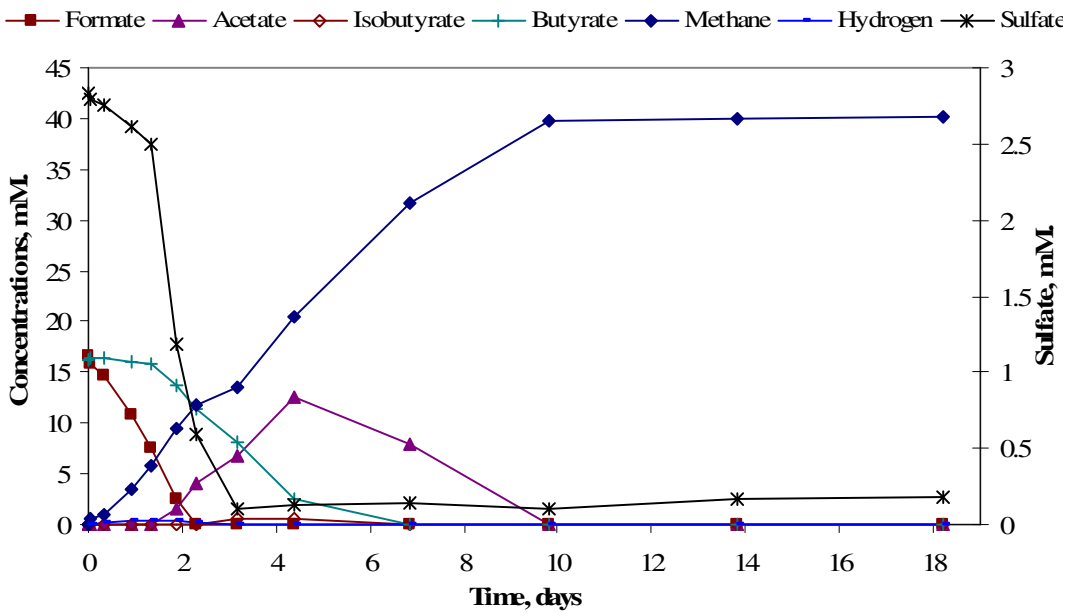


Figure A.69 : The effect of **15 mM formate** to the butyrate degradation profile of **BLS** reactor sludge in the presence of **3 mM sulfate**

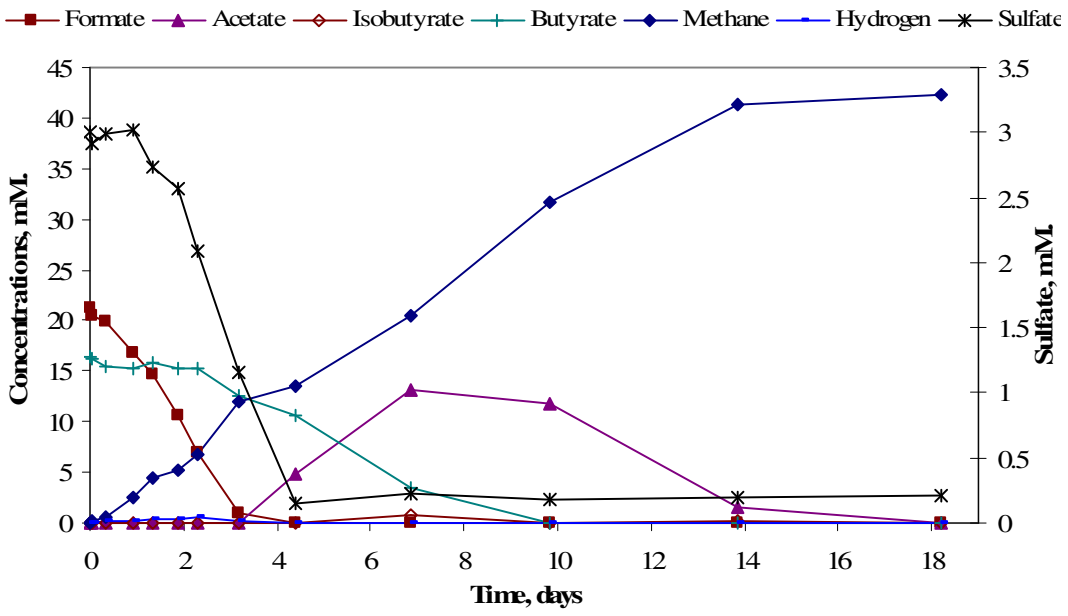


Figure A.70 : The effect of **20 mM formate** to the butyrate degradation profile of **BLS** reactor sludge in the presence of **3 mM sulfate**

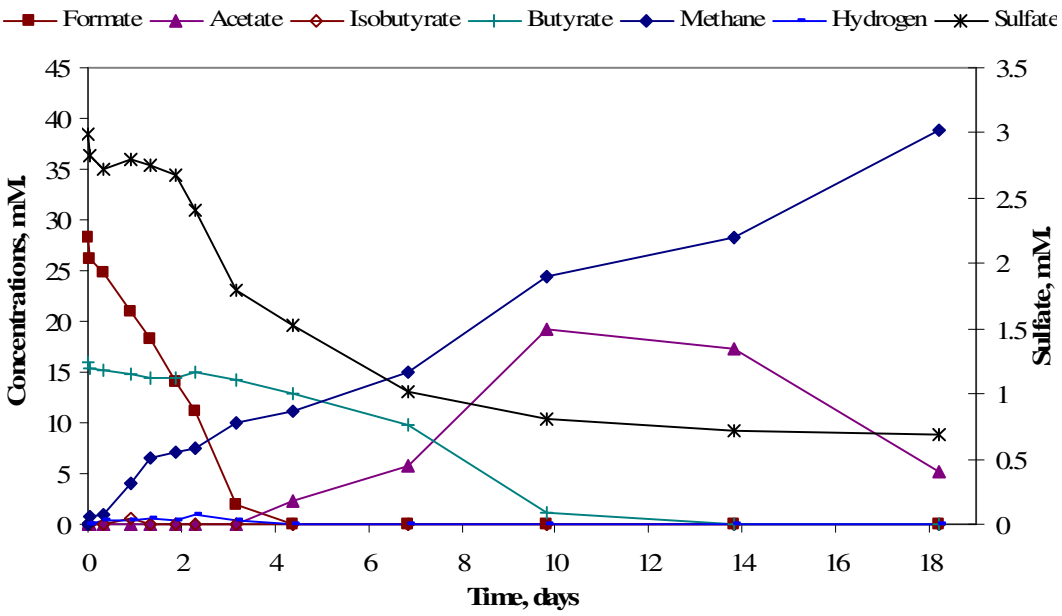


Figure A.71 : The effect of **30 mM formate** to the butyrate degradation profile of **BLS** reactor sludge in the presence of **3 mM sulfate**

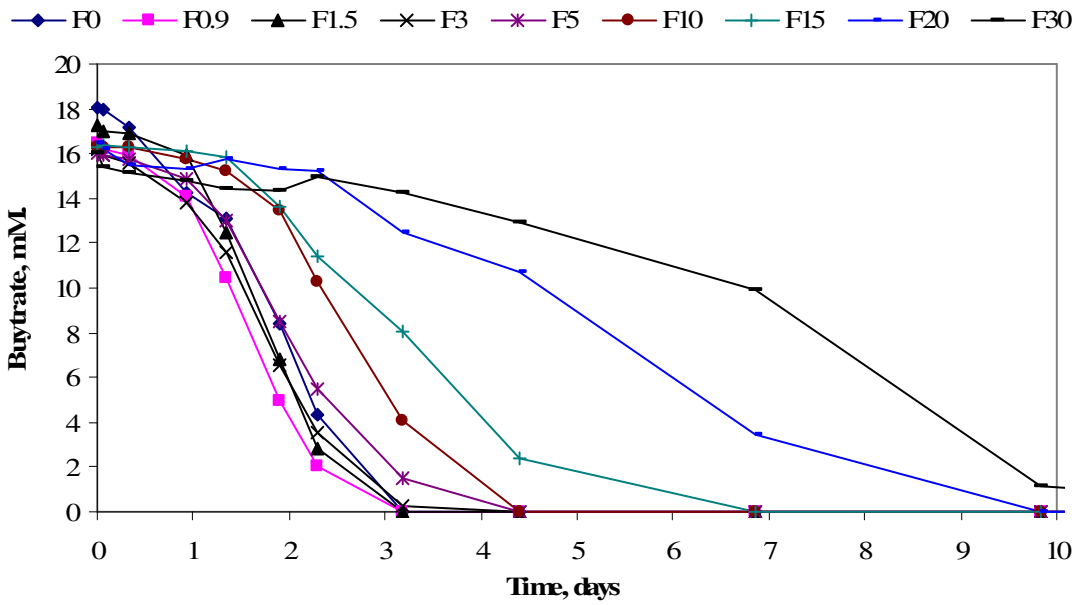


Figure A.72 : The total butyrate degradation profiles of **BLS** reactor sludge incubated at different **formate** concentrations and in the presence of **3 mM sulfate**

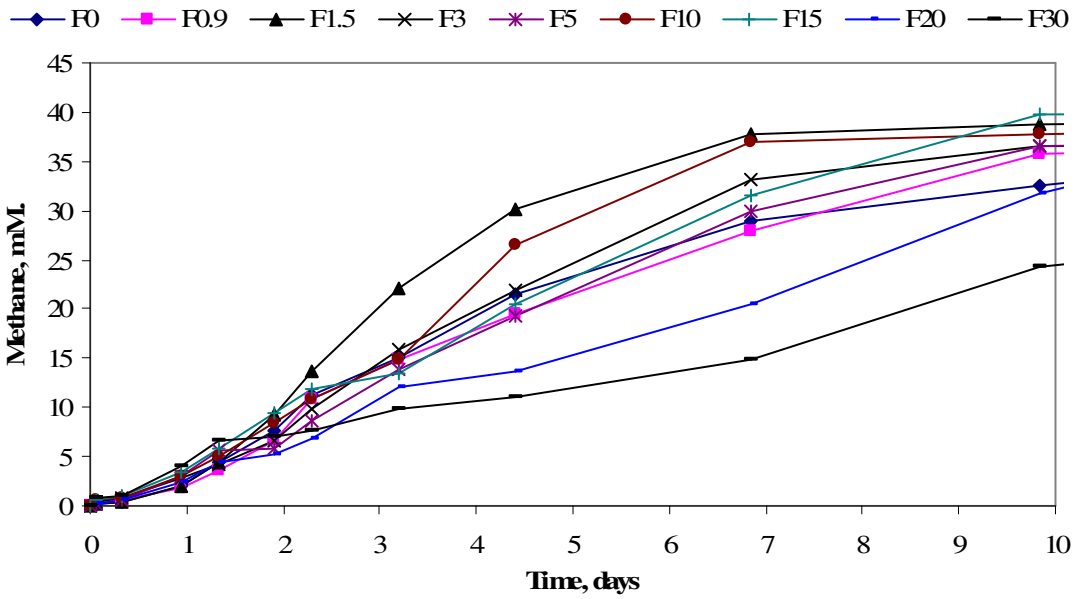


Figure A.73 : The total methane production profiles of **BLS** reactor sludge incubated at different **formate** concentrations and in the presence of **3mM sulfate**

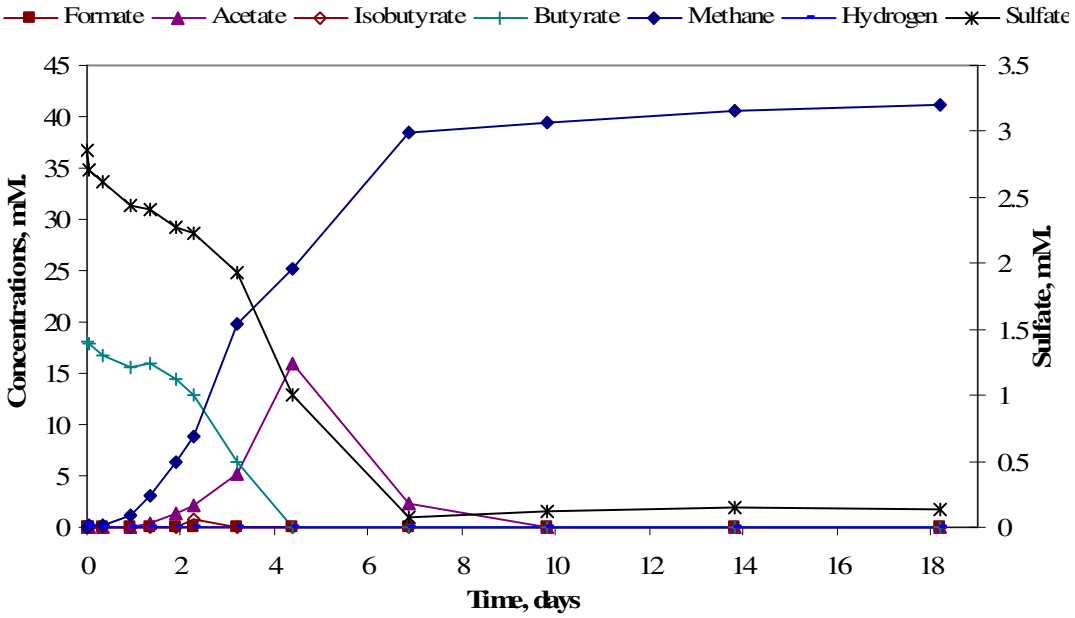


Figure A.74 : The effect of **0.15 mM hydrogen** to the butyrate degradation profile of **BLS** reactor sludge in the presence of **3 mM sulfate**

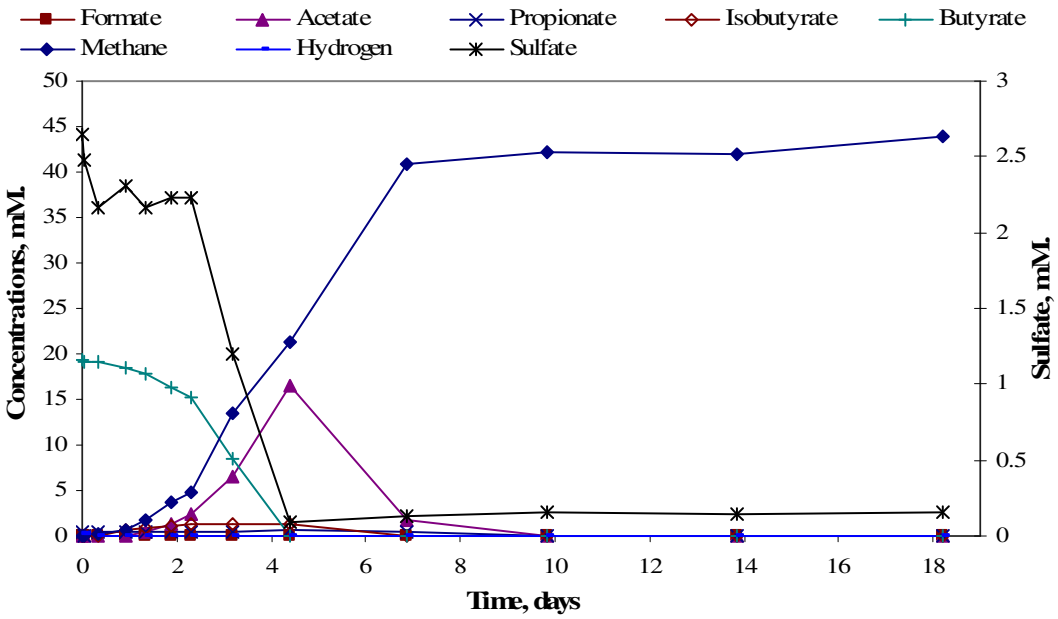


Figure A.75 : The effect of **0.3 mM hydrogen** to the butyrate degradation profile of **BLS** reactor sludge in the presence of **3 mM sulfate**

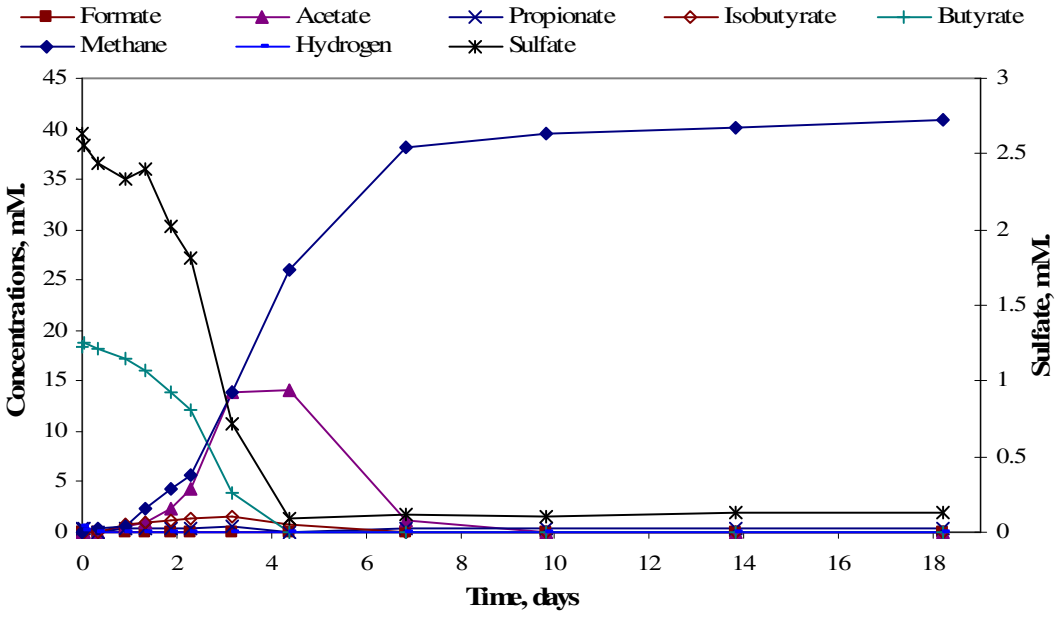


Figure A.76 : The effect of **0.5 mM hydrogen** to the butyrate degradation profile of **BLS** reactor sludge in the presence of **3 mM sulfate**

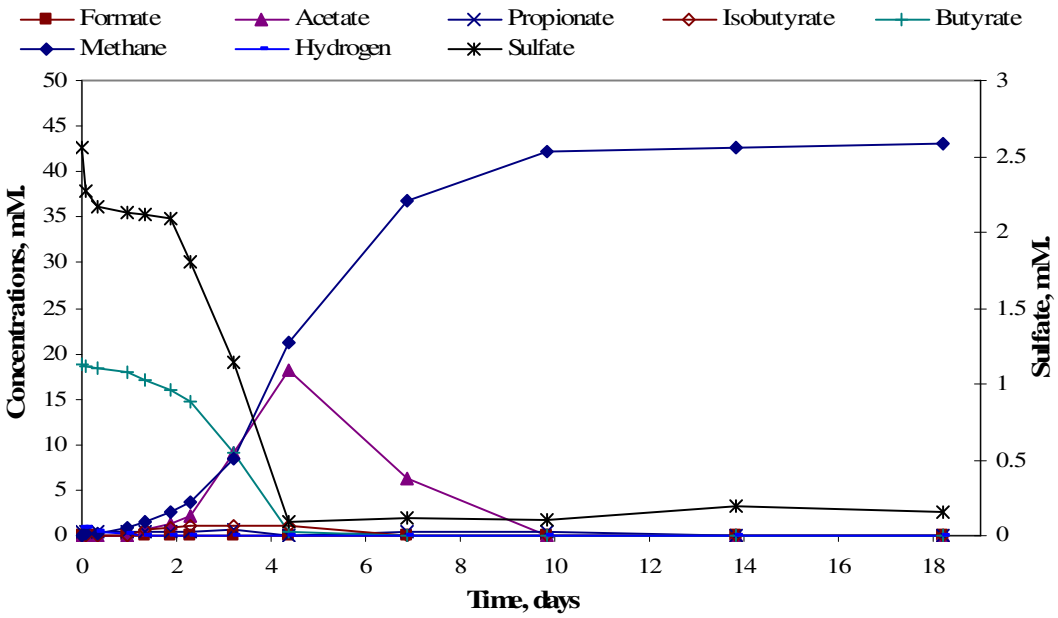


Figure A.77 : The effect of **1 mM hydrogen** to the butyrate degradation profile of **BLS** reactor sludge in the presence of **3 mM sulfate**

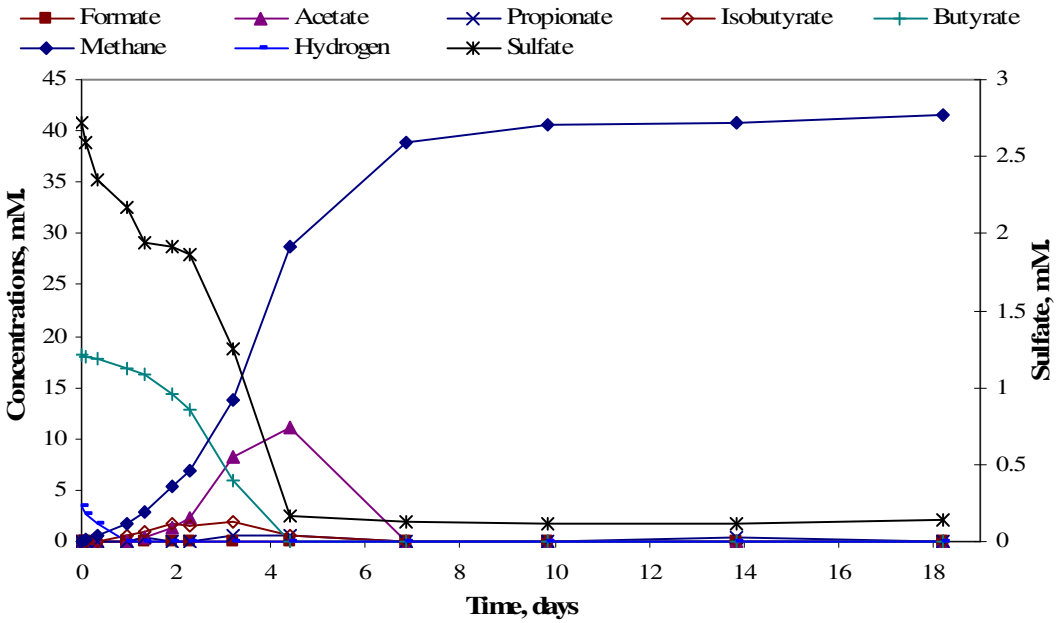


Figure A.78 : The effect of **3 mM hydrogen** to the butyrate degradation profile of **BLS** reactor sludge in the presence of **3 mM sulfate**

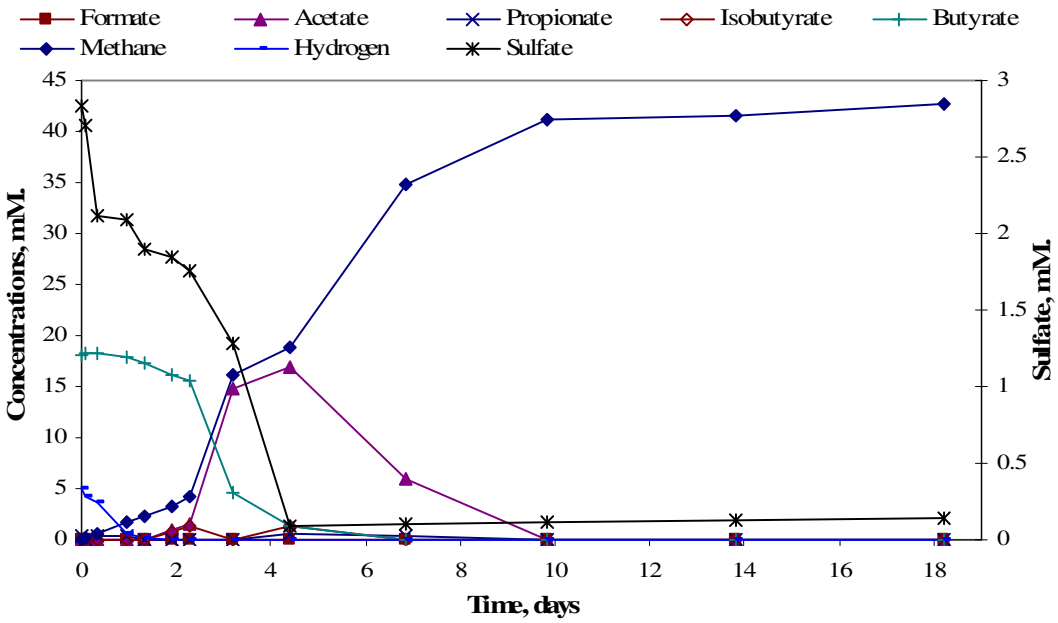


Figure A.79 : The effect of **5 mM hydrogen** to the butyrate degradation profile of **BLS** reactor sludge in the presence of **3 mM sulfate**

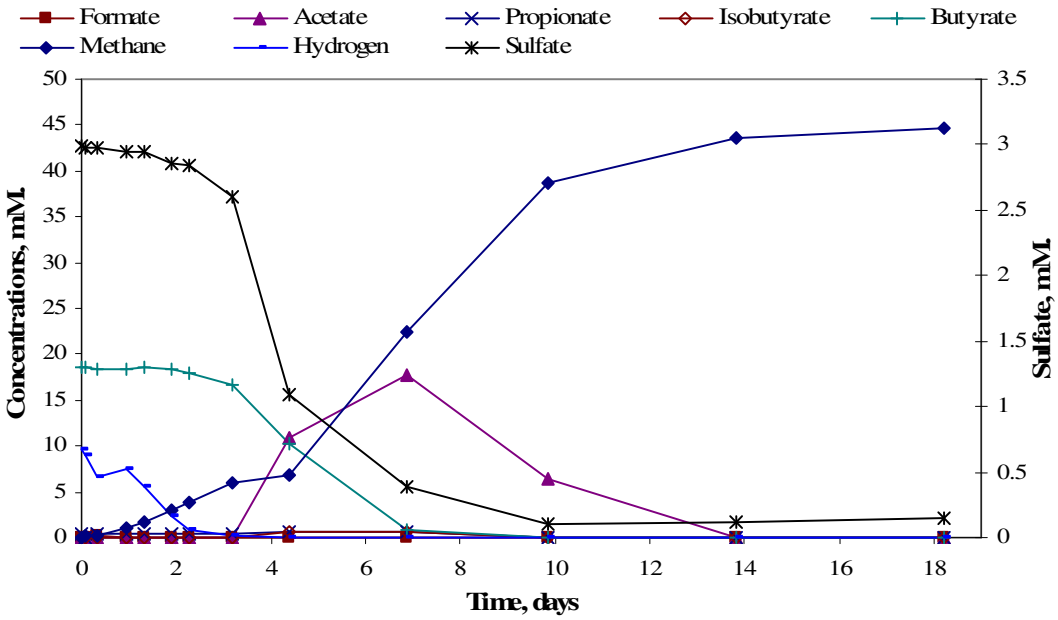


Figure A.80 : The effect of **10 mM hydrogen** to the butyrate degradation profile of **BLS** reactor sludge in the presence of **3 mM sulfate**

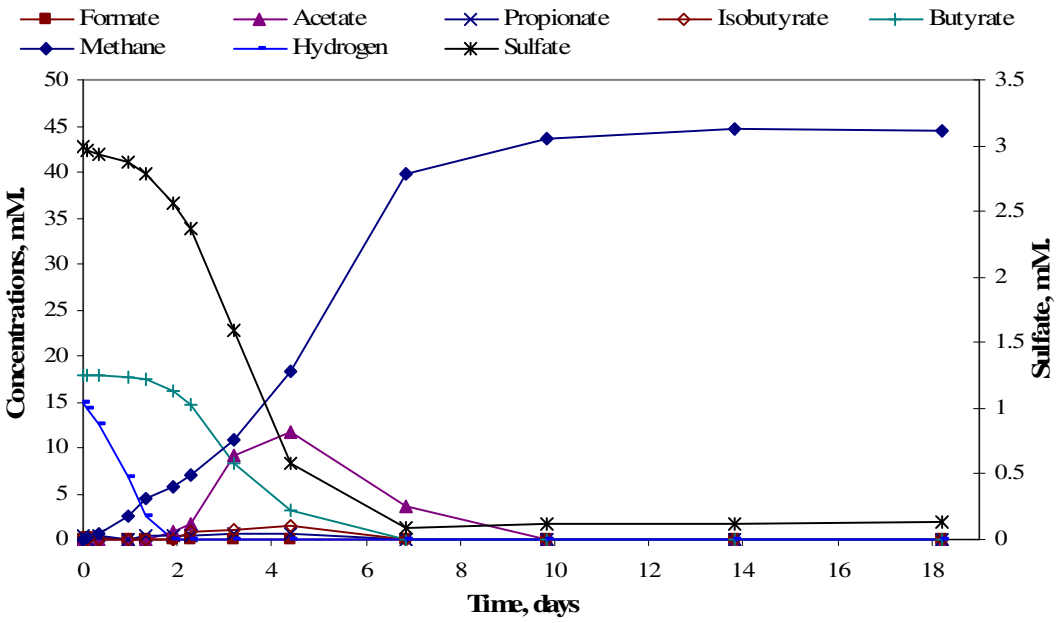


Figure A.81 : The effect of **15 mM hydrogen** to the butyrate degradation profile of **BLS** reactor sludge in the presence of **3 mM sulfate**

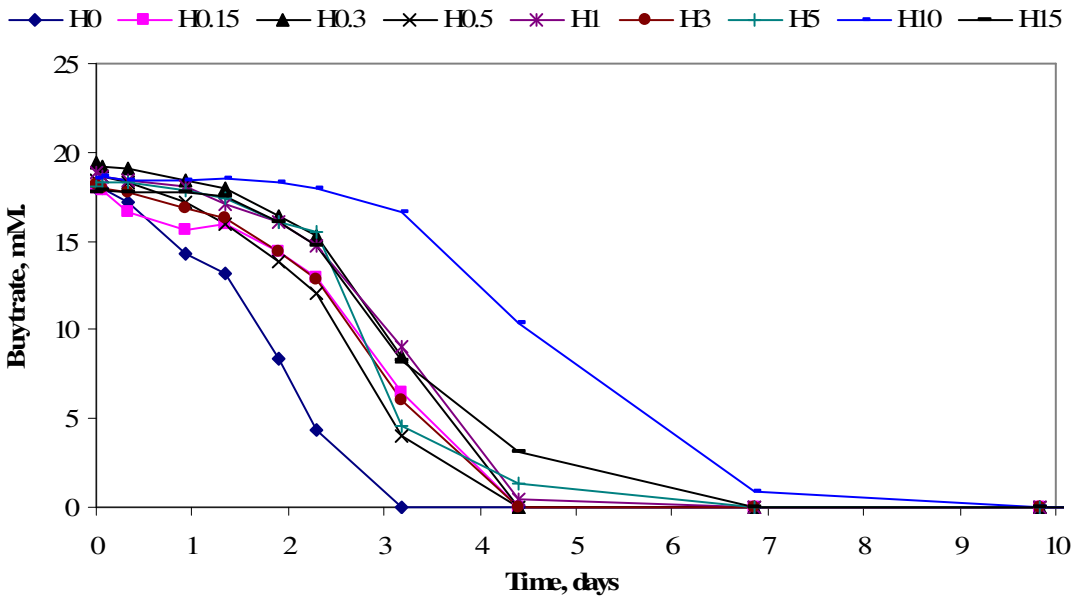


Figure A.82 : The total butyrate degradation profiles of **BLS** reactor sludge incubated at different **hydrogen** concentrations and in the presence of **3mM sulfate**

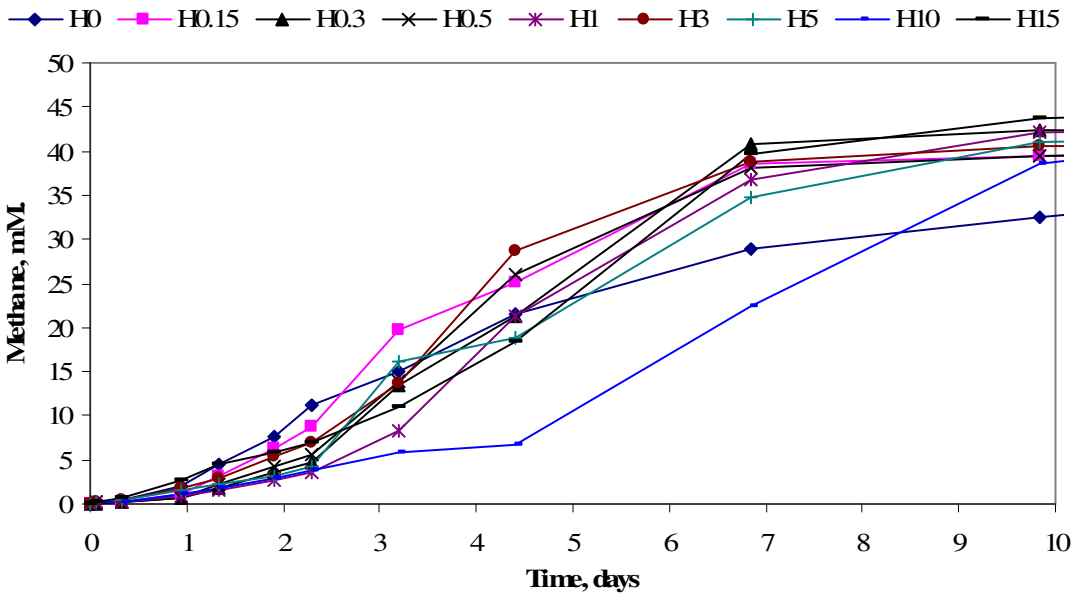


Figure A.83 : The total methane production profiles of **BLS** reactor sludge incubated at different **hydrogen** concentrations and in the presence of **3mM sulfate**

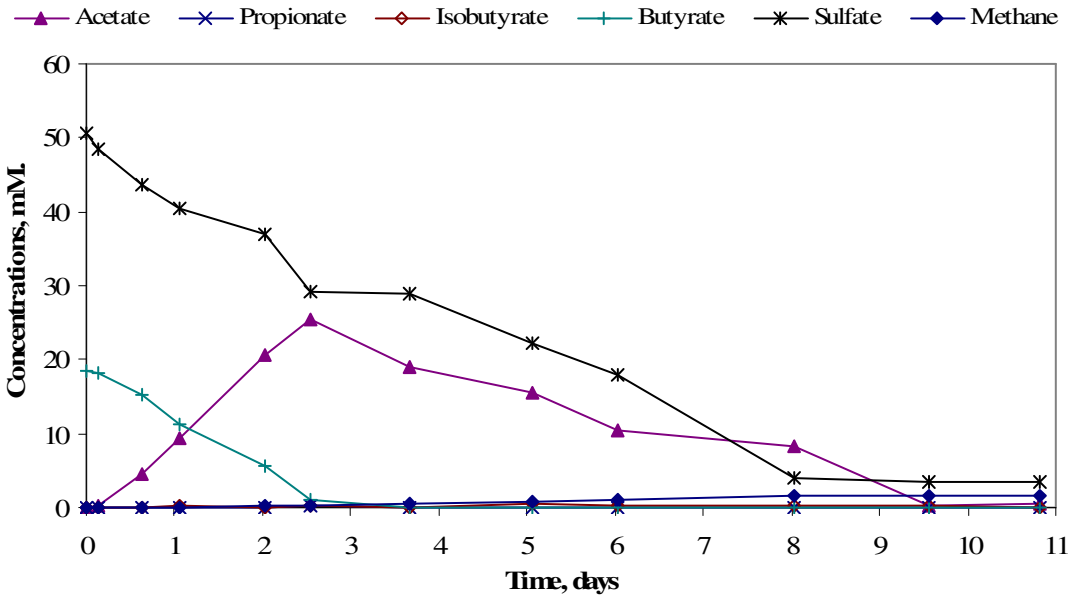


Figure A.84 : Time course of butyrate degradation in the presence of **45 mM sulfate** for **BUS** reactor sludge

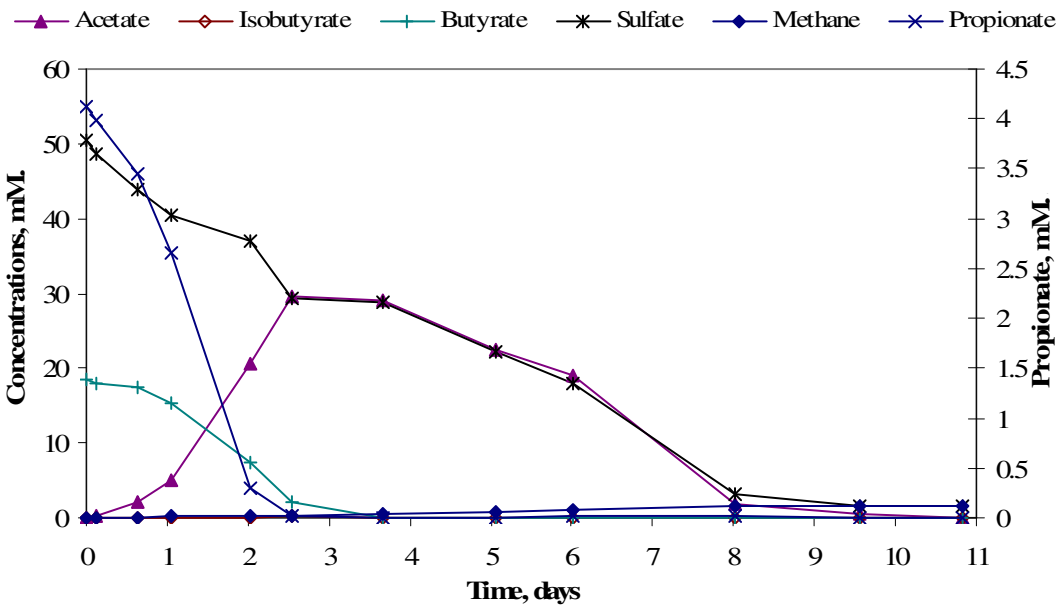


Figure A.85 : The effect of **5 mM propionate** to the butyrate degradation profile of **BUS** reactor sludge in the presence of **45 mM sulfate**

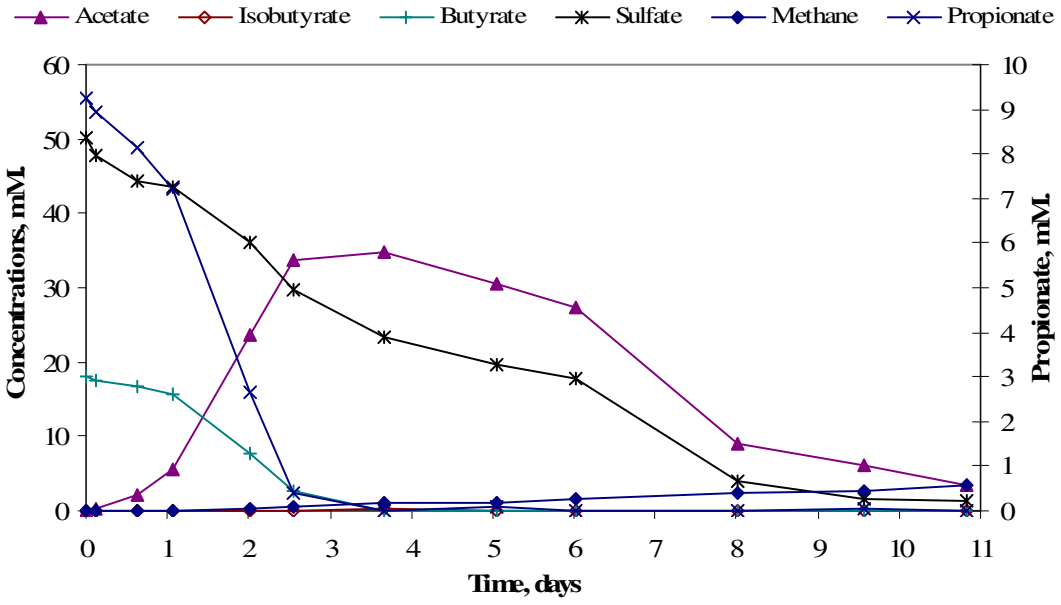


Figure A.86 : The effect of **10 mM propionate** to the butyrate degradation profile of **BUS** reactor sludge in the presence of **45 mM sulfate**

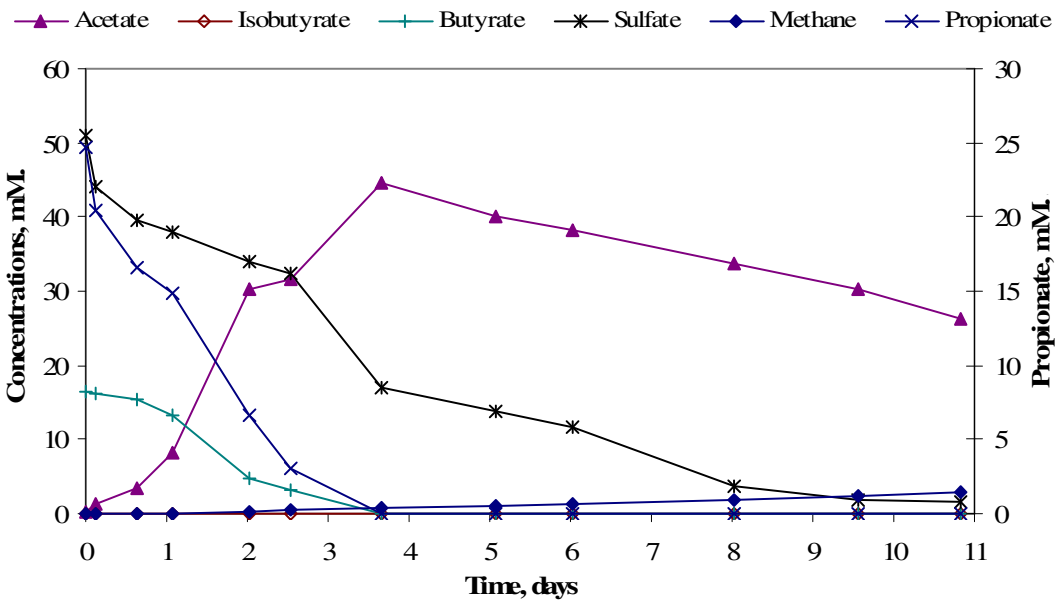


Figure A.87 : The effect of **20 mM propionate** to the butyrate degradation profile of **BUS** reactor sludge in the presence of **45 mM sulfate**

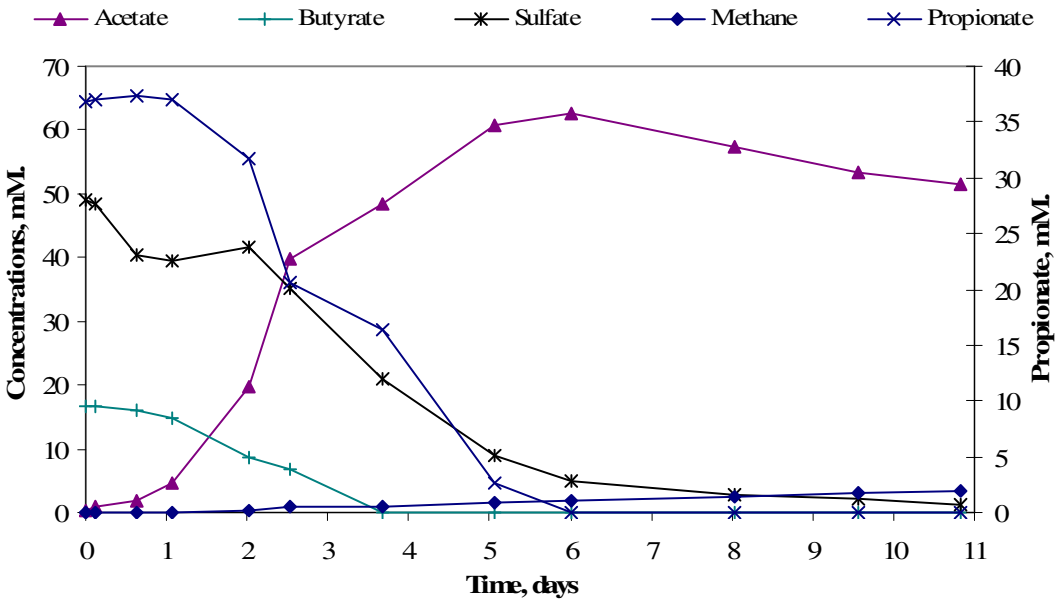


Figure A.88 : The effect of **40 mM propionate** to the butyrate degradation profile of **BUS** reactor sludge in the presence of **45 mM sulfate**

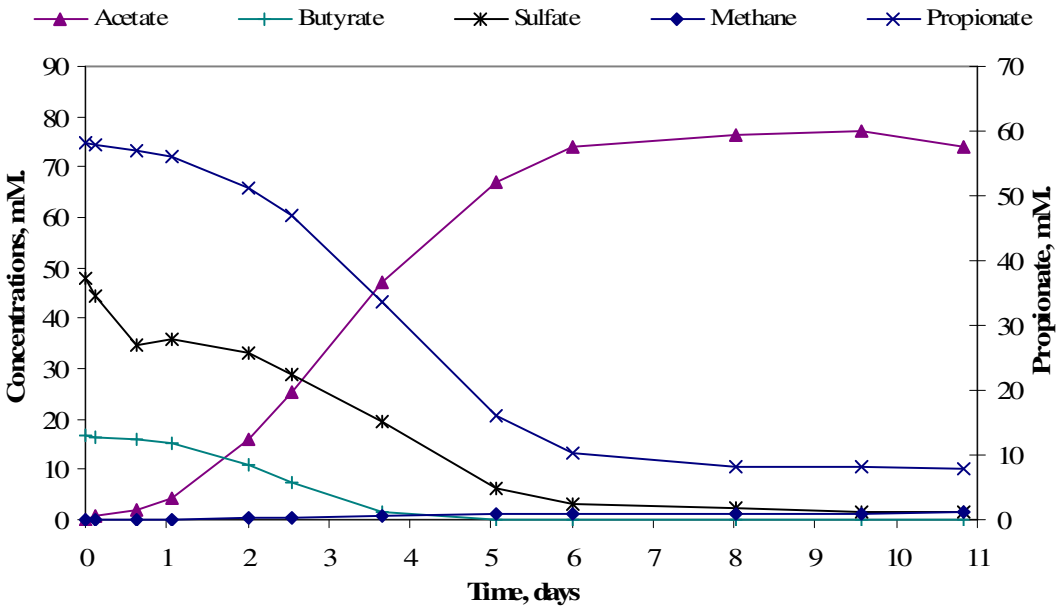


Figure A.89 : The effect of **60 mM propionate** to the butyrate degradation profile of **BUS** reactor sludge in the presence of **45 mM sulfate**

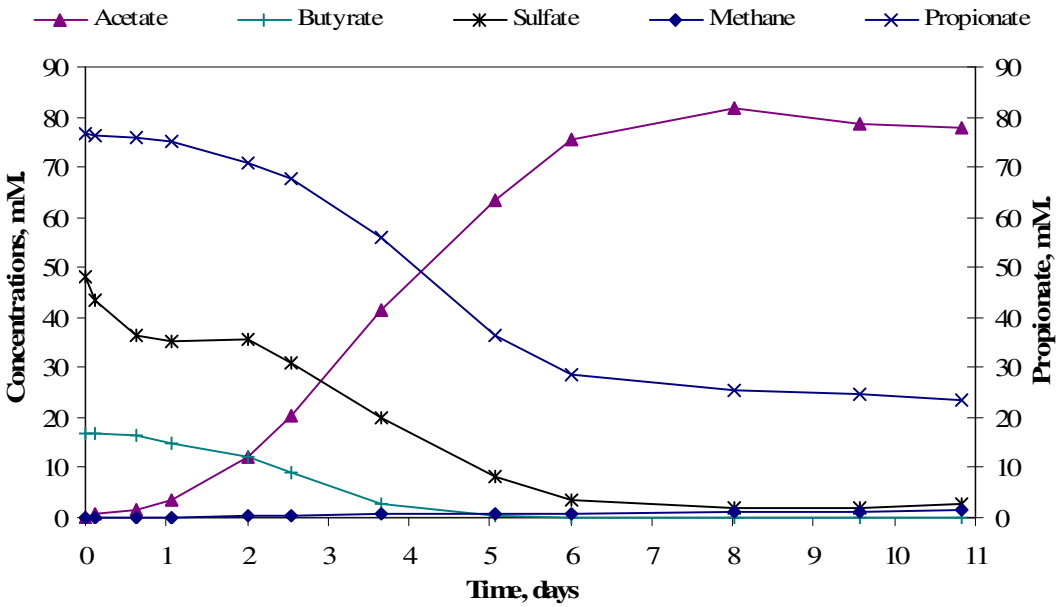


Figure A.90 : The effect of **80 mM propionate** to the butyrate degradation profile of **BUS** reactor sludge in the presence of **45 mM sulfate**

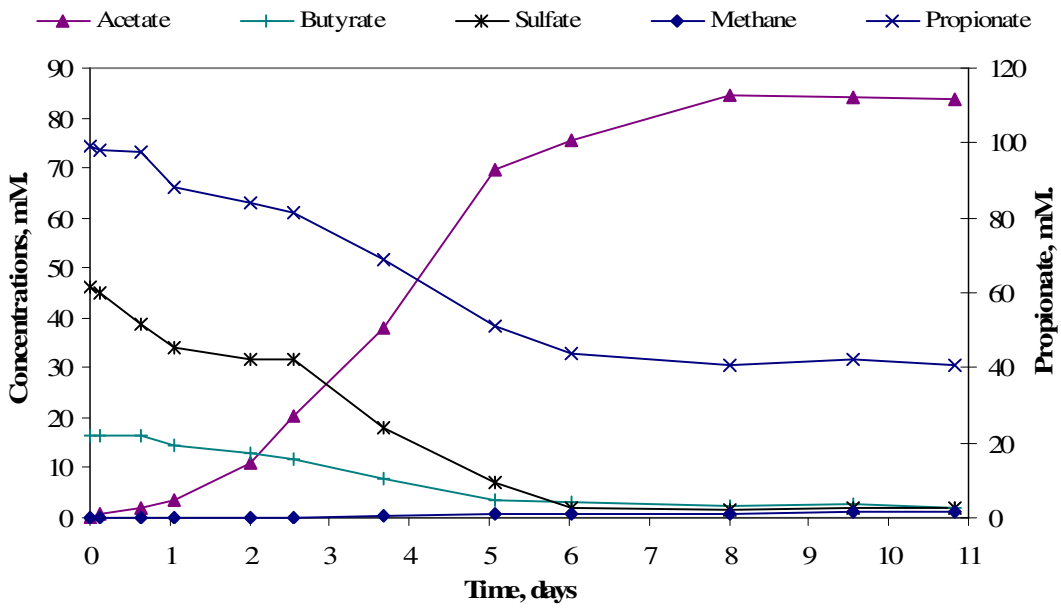


Figure A.91 : The effect of **100 mM propionate** to the butyrate degradation profile of **BUS** reactor sludge in the presence of **45 mM sulfate**

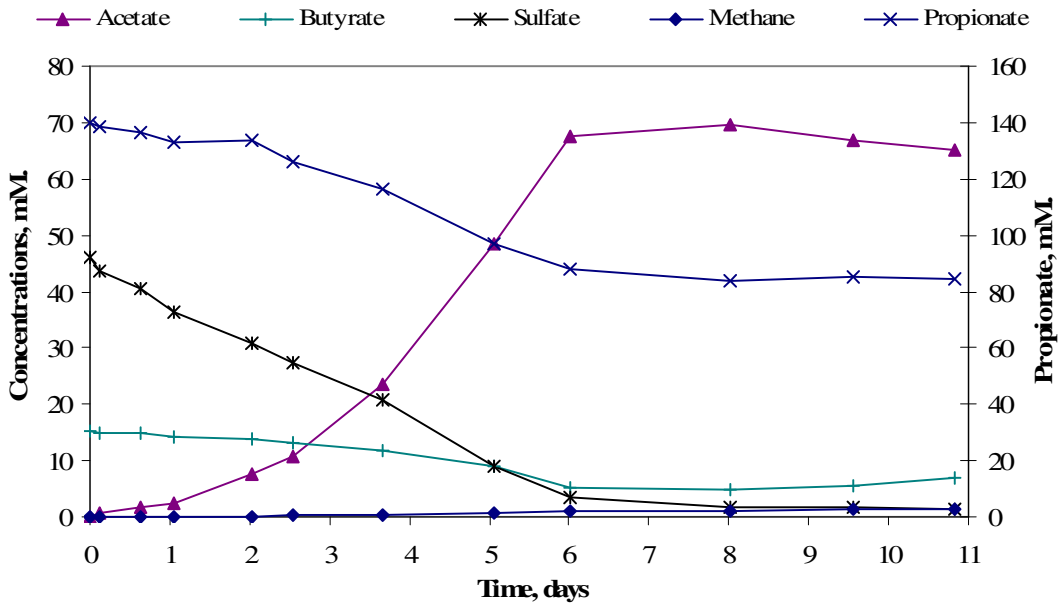


Figure A.92 : The effect of **150 mM propionate** to the butyrate degradation profile of **BUS** reactor sludge in the presence of **45 mM sulfate**

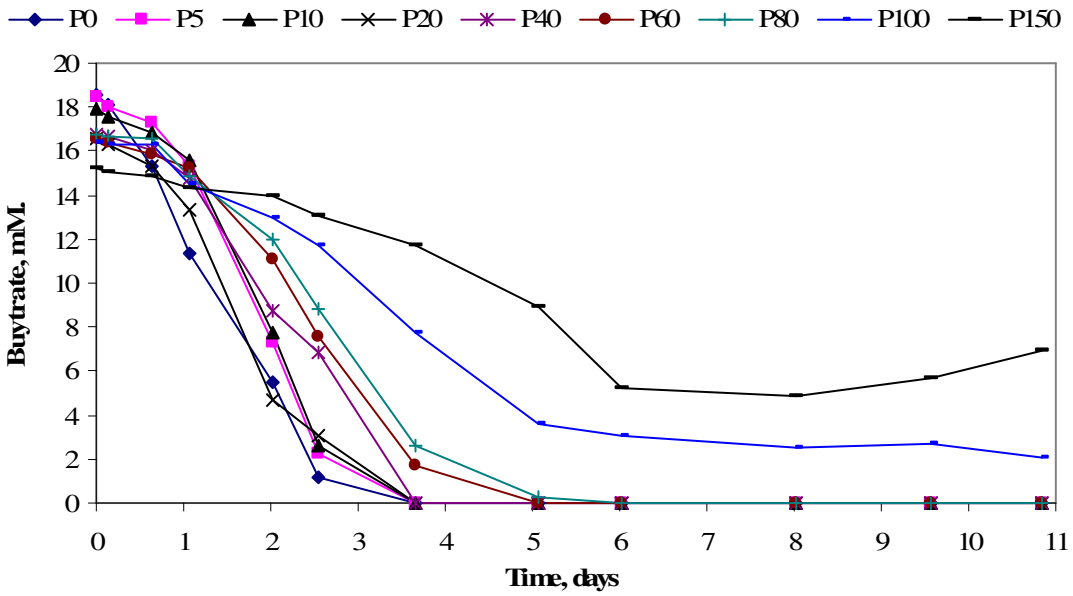


Figure A.93 : The total butyrate degradation profiles of **BUS** reactor sludge incubated at different **propionate** concentrations and in the presence of **45 mM sulfate**

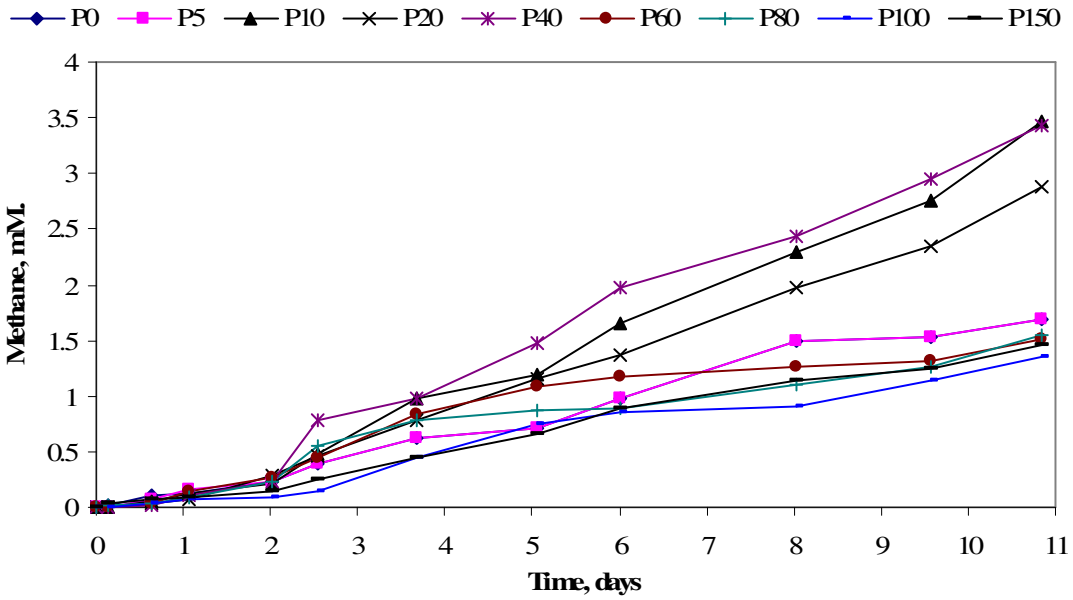


Figure A.94 : The total methane production profiles of **BUS** reactor sludge incubated at different **propionate** concentrations and in the presence of **45 mM sulfate**

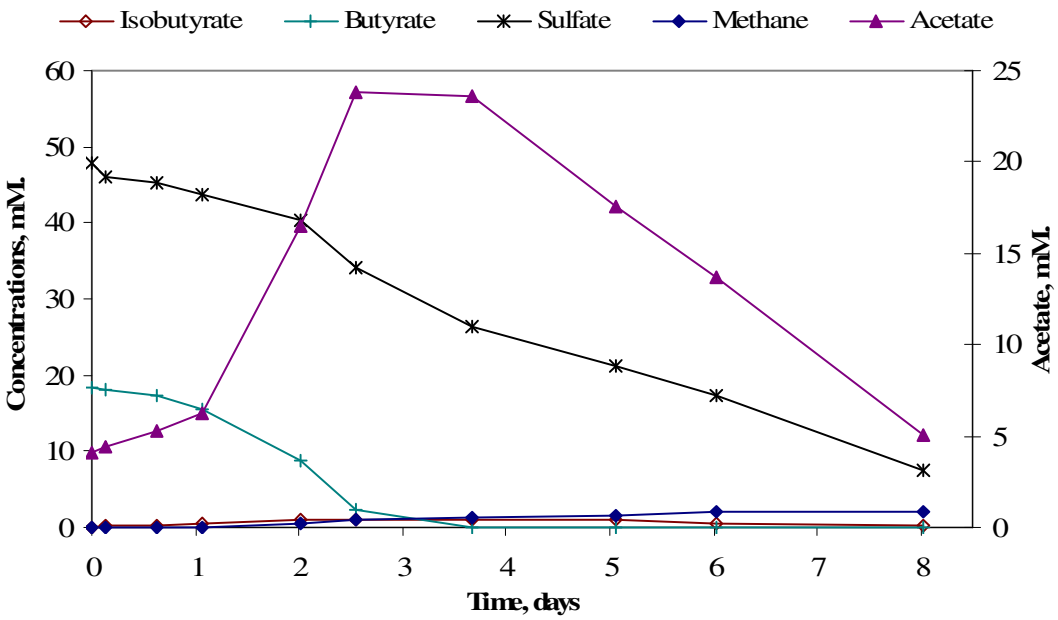


Figure A.95 : The effect of **5 mM acetate** to the butyrate degradation profile of **BUS** reactor sludge in the presence of **45 mM sulfate**

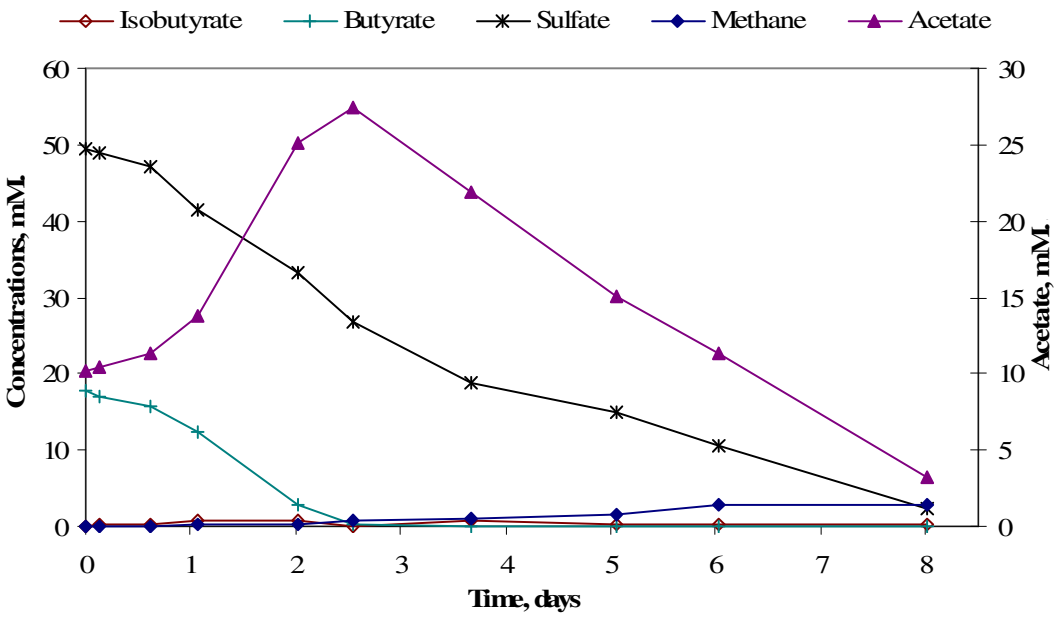


Figure A.96 : The effect of **10 mM acetate** to the butyrate degradation profile of **BUS** reactor sludge in the presence of **45 mM sulfate**

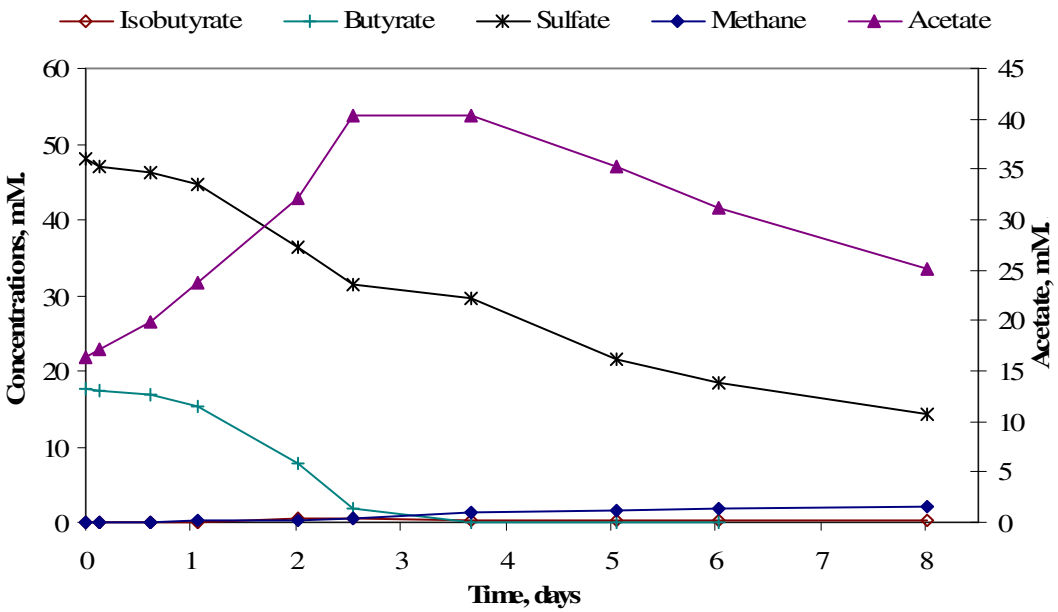


Figure A.97 : The effect of **20 mM acetate** to the butyrate degradation profile of **BUS** reactor sludge in the presence of **45 mM sulfate**

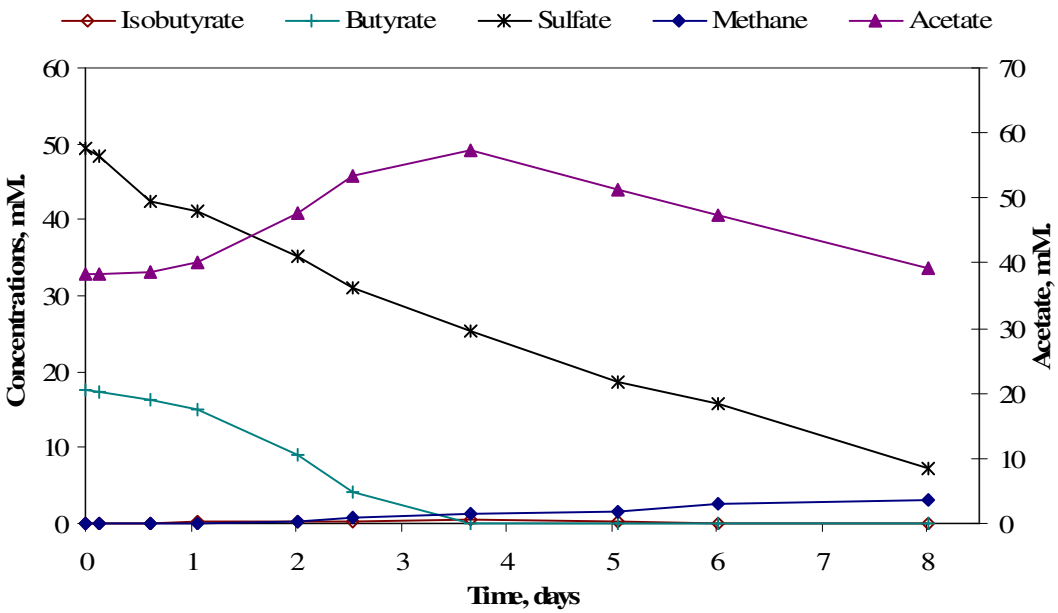


Figure A.98 : The effect of **40 mM acetate** to the butyrate degradation profile of **BUS** reactor sludge in the presence of **45 mM sulfate**

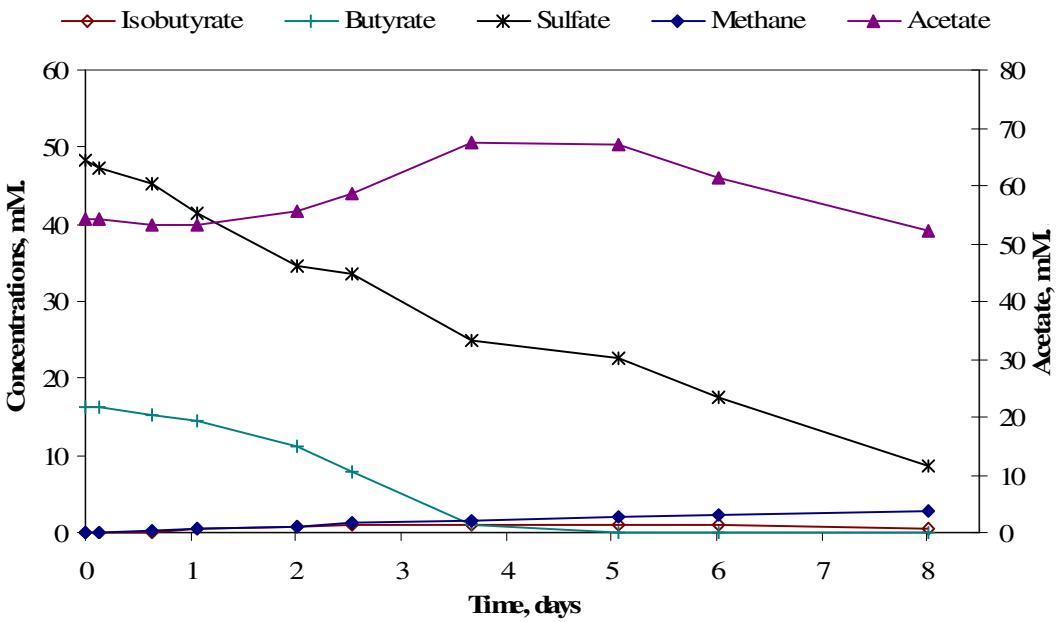


Figure A.99 : The effect of **60 mM acetate** to the butyrate degradation profile of **BUS** reactor sludge in the presence of **45 mM sulfate**

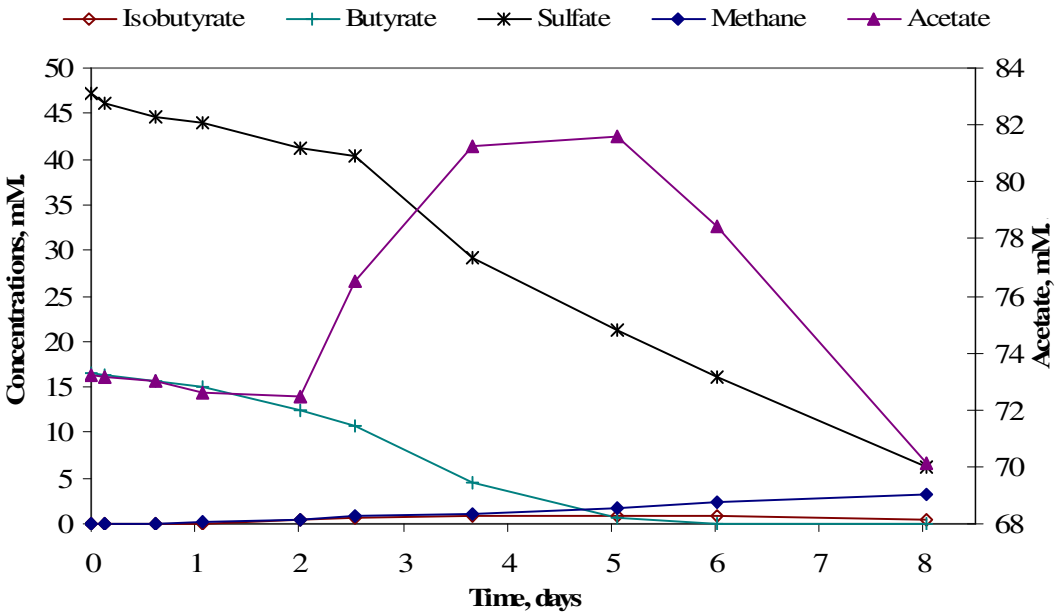


Figure A.100 : The effect of **80 mM acetate** to the butyrate degradation profile of **BUS** reactor sludge in the presence of **45 mM sulfate**

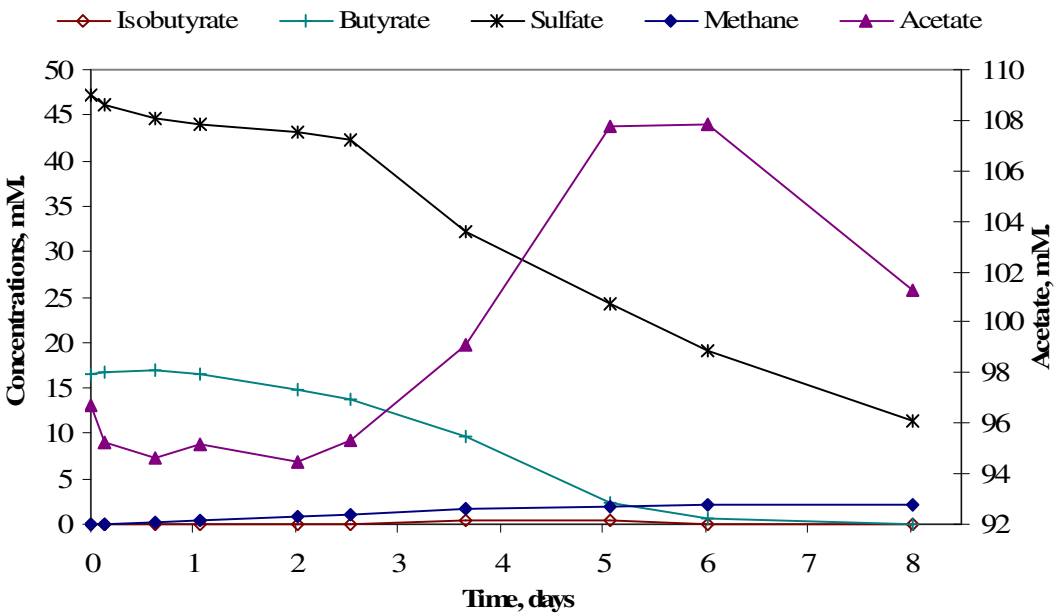


Figure A.101 : The effect of **100 mM acetate** to the butyrate degradation profile of **BUS** reactor sludge in the presence of **45 mM sulfate**

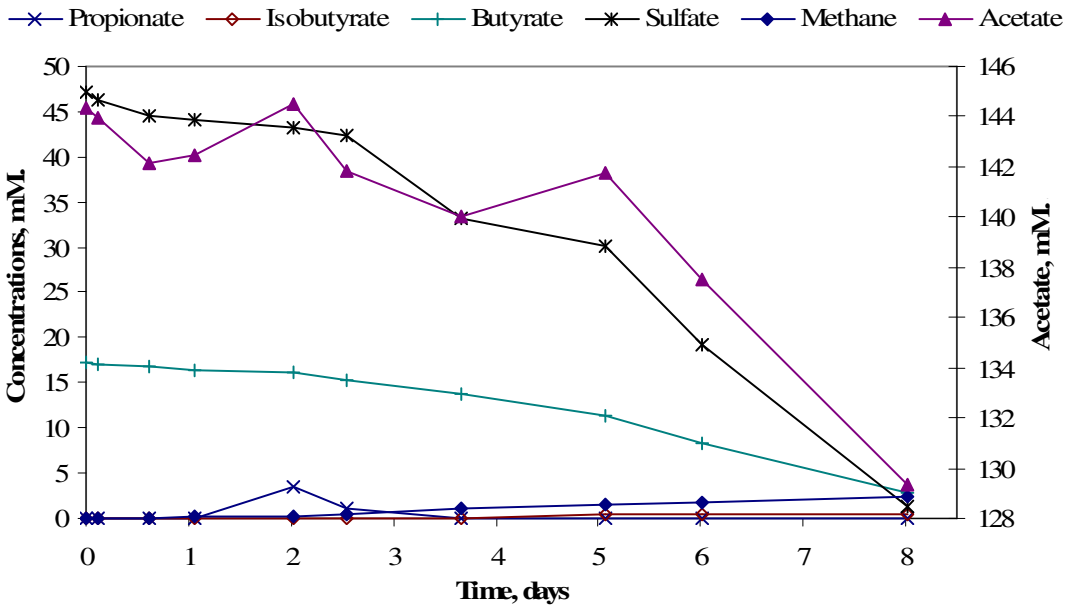


Figure A.102 : The effect of **150 mM acetate** to the butyrate degradation profile of **BUS** reactor sludge in the presence of **45 mM sulfate**

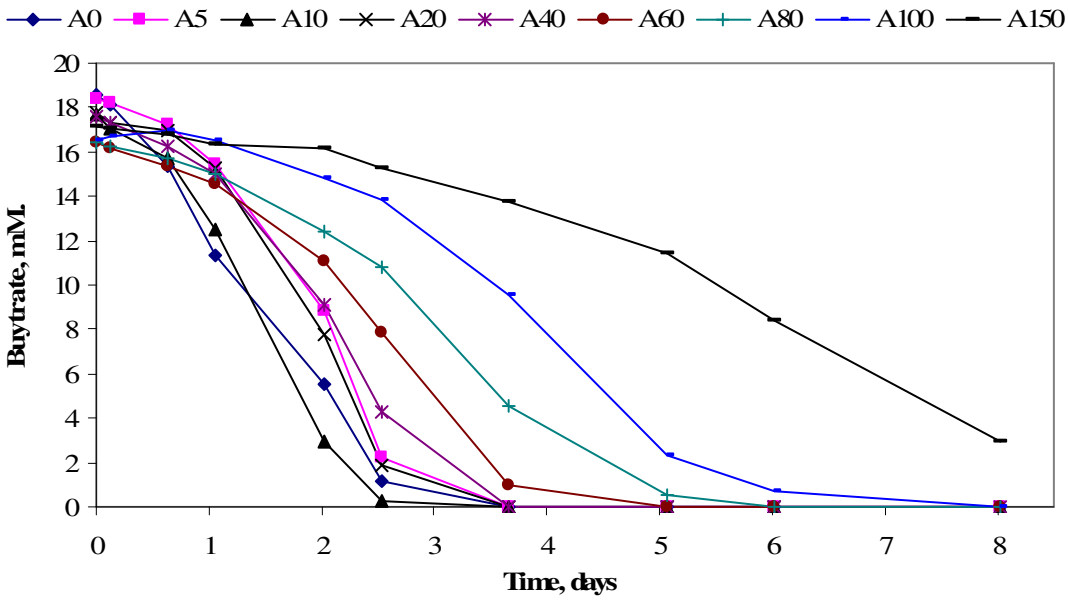


Figure A.103 : The total butyrate degradation profiles of **BUS** reactor sludge incubated at different **acetate** concentrations and in the presence of **45 mM sulfate**

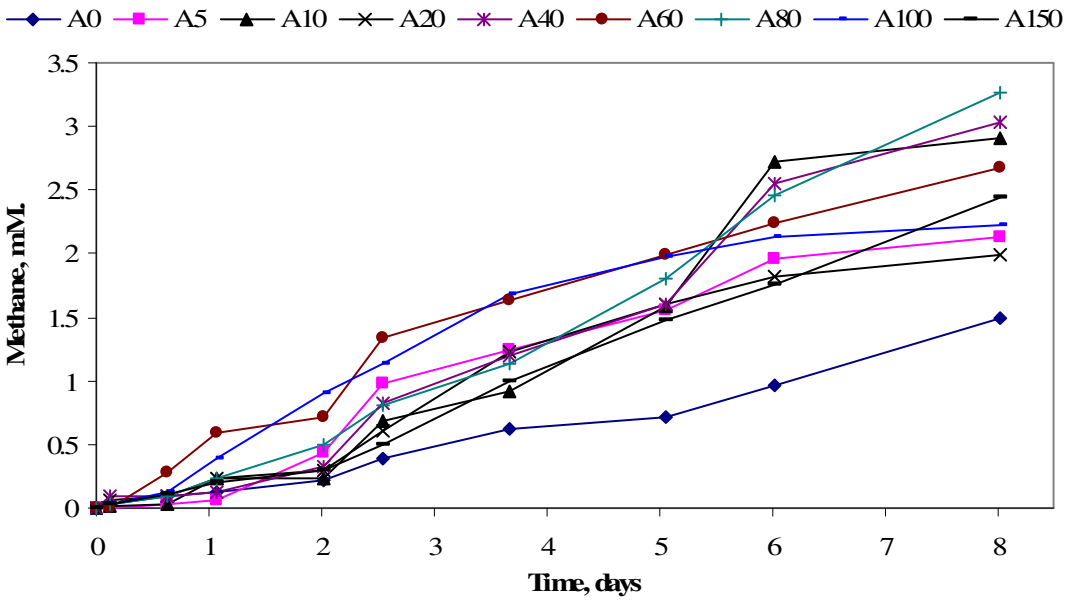


Figure A.104 : The total methane production profiles of **BUS** reactor sludge incubated at different **acetate** concentrations and in the presence of **45 mM sulfate**

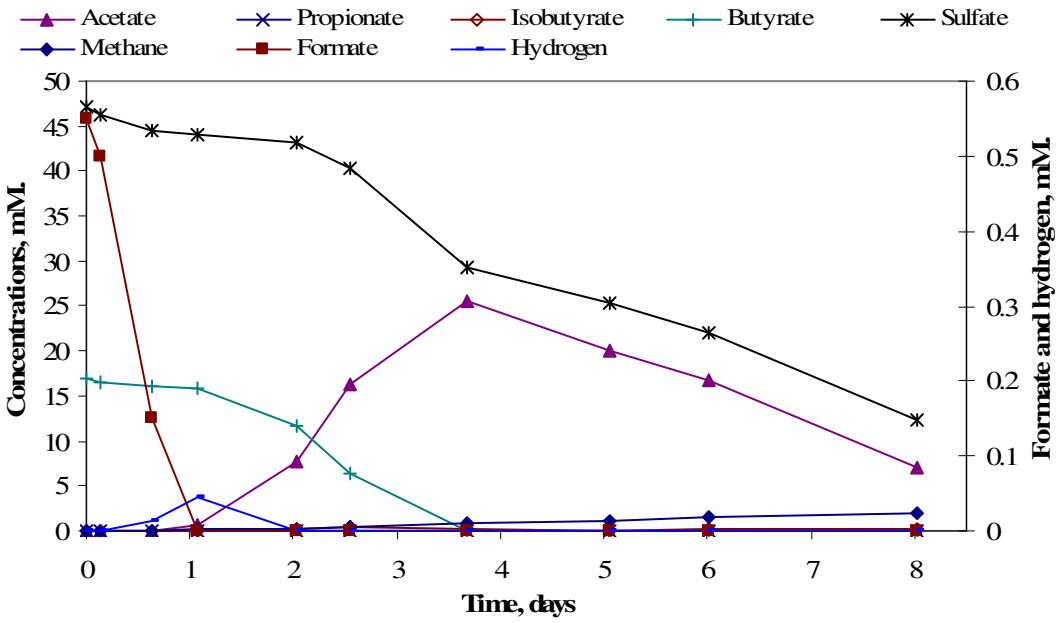


Figure A.105 : The effect of **0.5 mM formate** to the butyrate degradation profile of **BUS** reactor sludge in the presence of **45 mM sulfate**

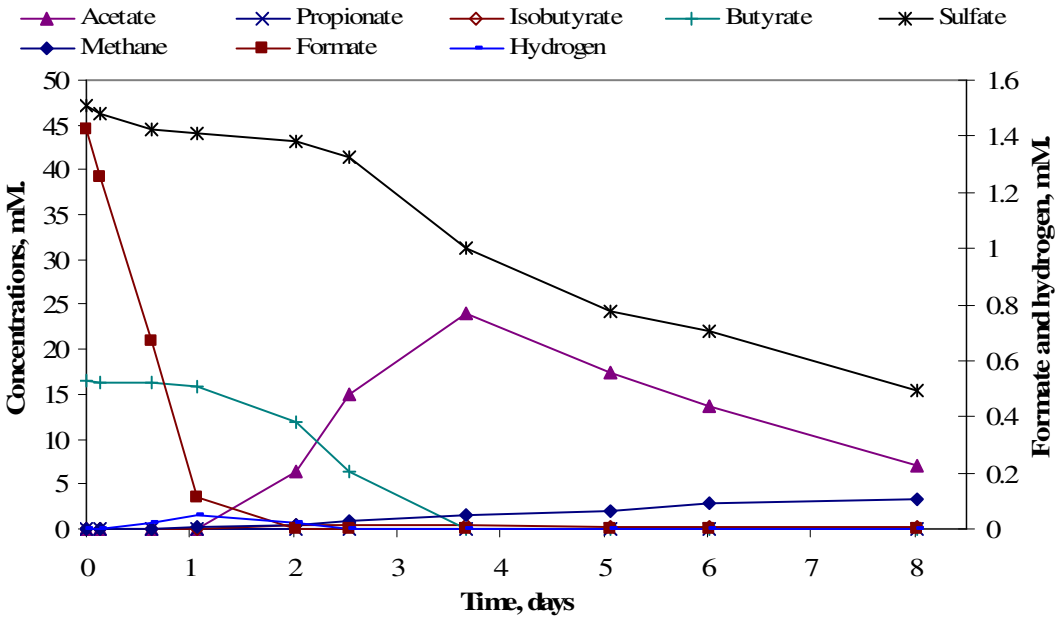


Figure A.106 : The effect of **1.5 mM formate** to the butyrate degradation profile of **BUS** reactor sludge in the presence of **45 mM sulfate**

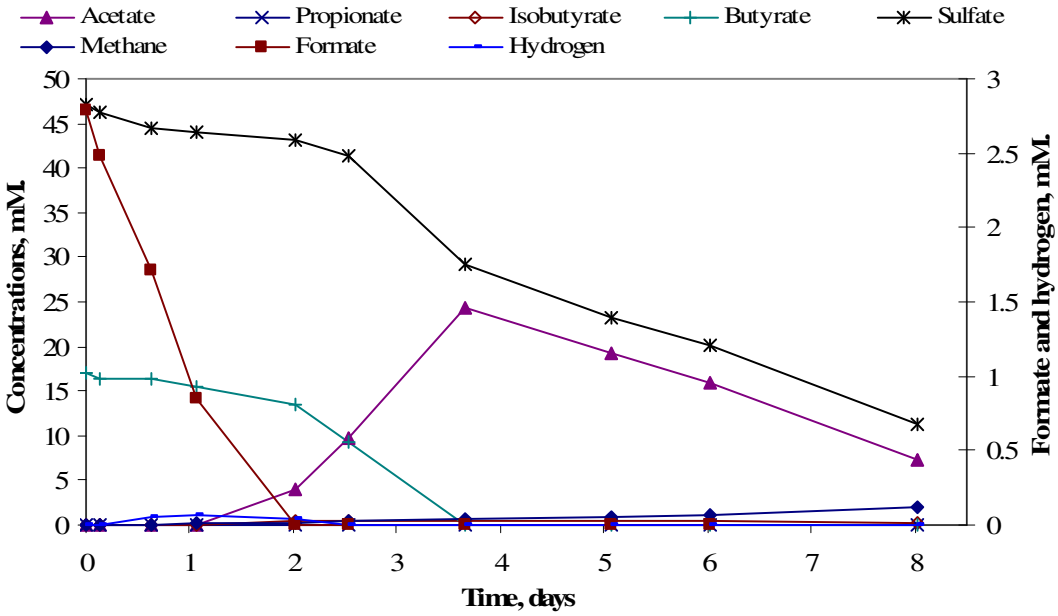


Figure A.107 : The effect of **3 mM formate** to the butyrate degradation profile of **BUS** reactor sludge in the presence of **45 mM sulfate**

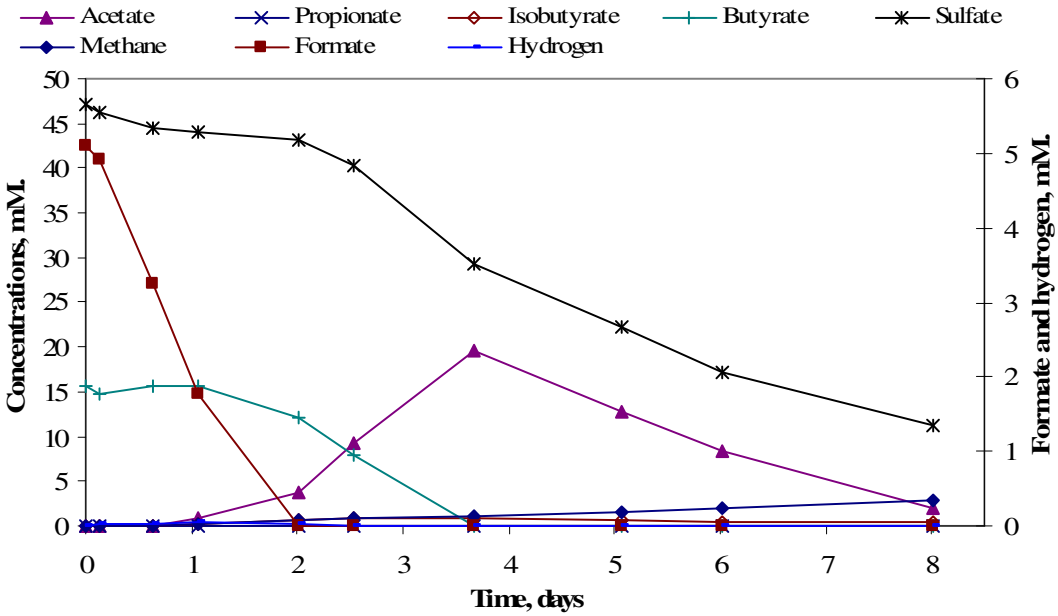


Figure A.108 : The effect of **5 mM formate** to the butyrate degradation profile of **BUS** reactor sludge in the presence of **45 mM sulfate**

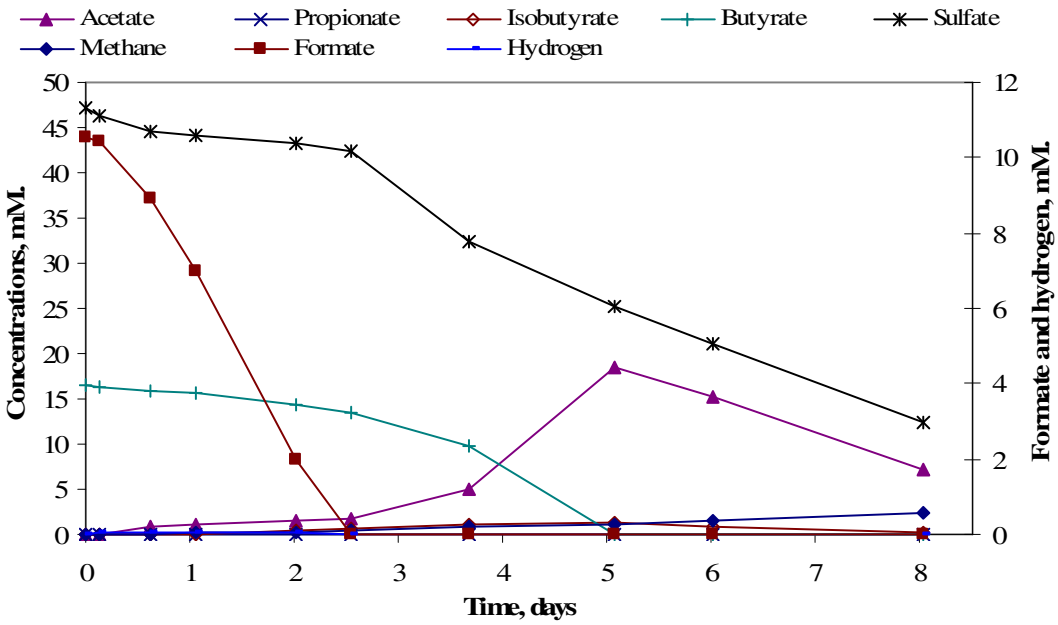


Figure A.109 : The effect of **10 mM formate** to the butyrate degradation profile of **BUS** reactor sludge in the presence of **45 mM sulfate**

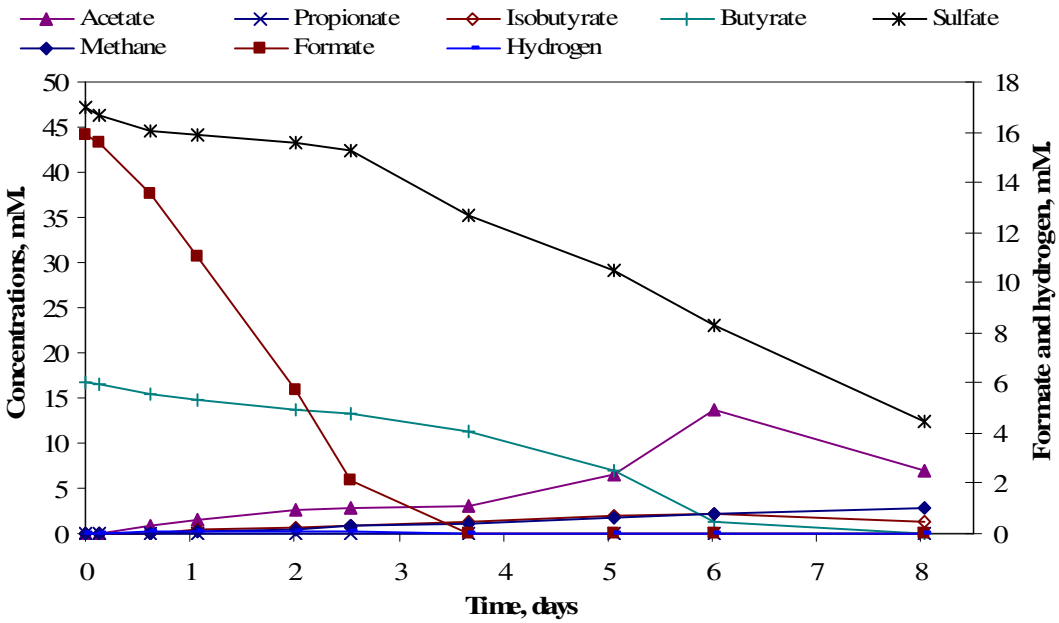


Figure A.110 : The effect of **15 mM formate** to the butyrate degradation profile of **BUS** reactor sludge in the presence of **45 mM sulfate**

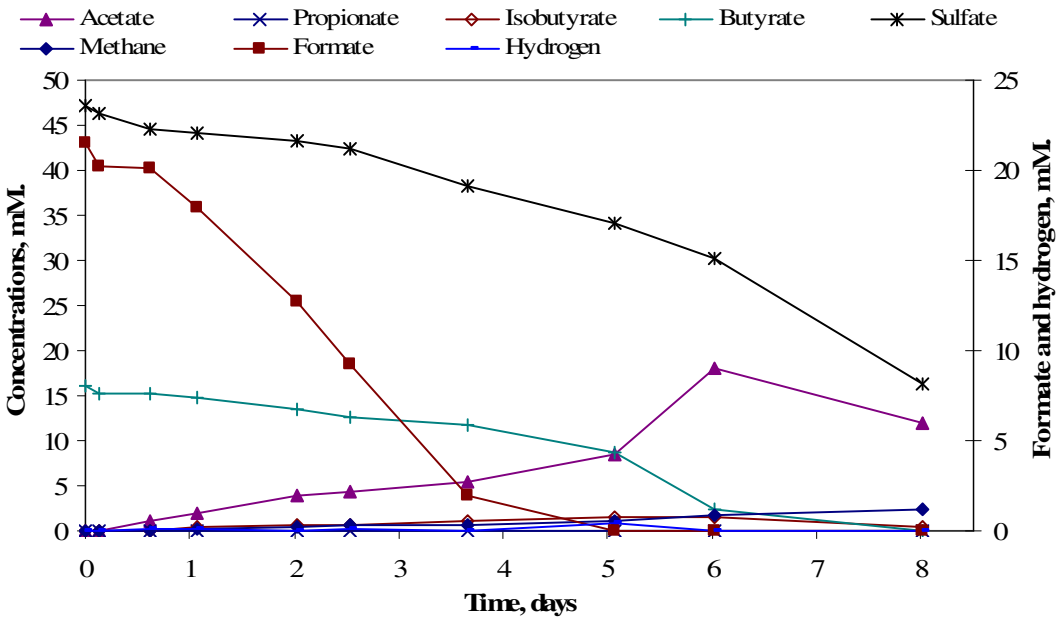


Figure A.111 : The effect of **20 mM formate** to the butyrate degradation profile of **BUS** reactor sludge in the presence of **45 mM sulfate**

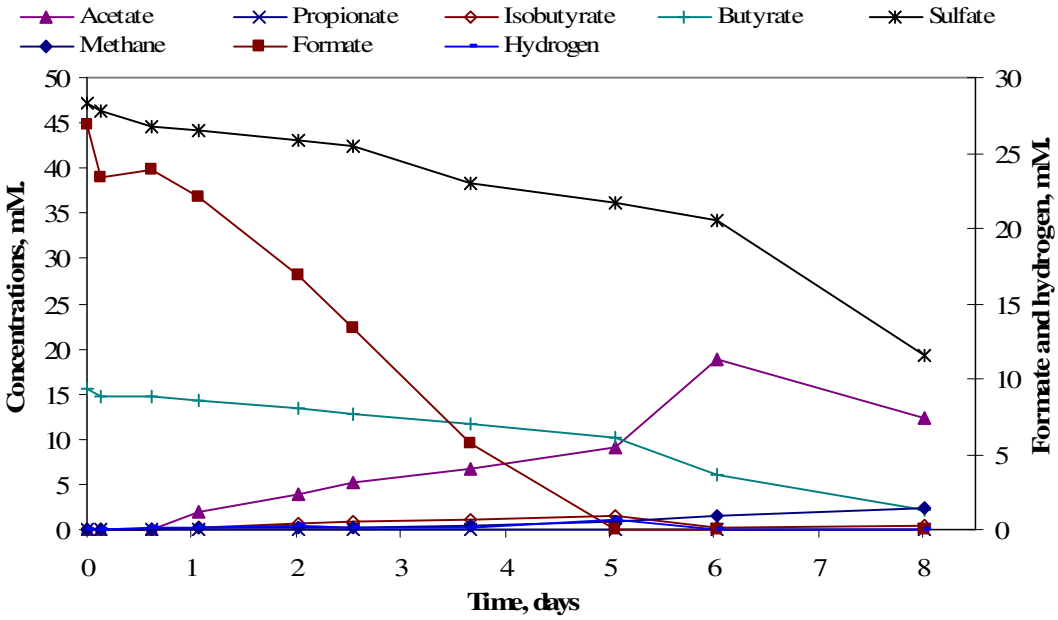


Figure A.112 : The effect of **30 mM formate** to the butyrate degradation profile of **BUS** reactor sludge in the presence of **45 mM sulfate**

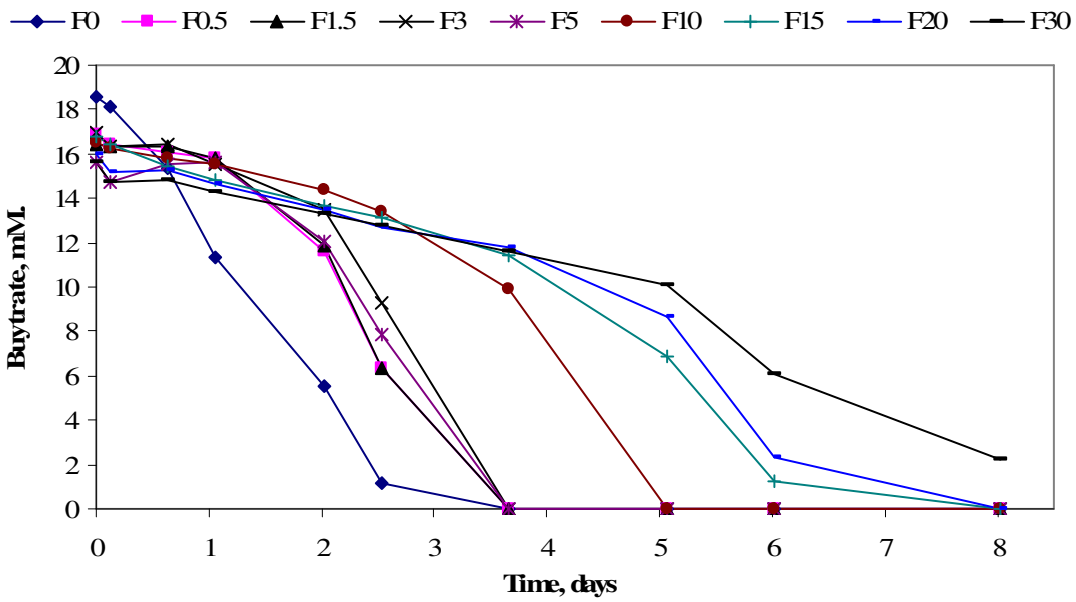


Figure A.113 : The total butyrate degradation profiles of **BUS** reactor sludge incubated at different **formate** concentrations and in the presence of **45 mM sulfate**

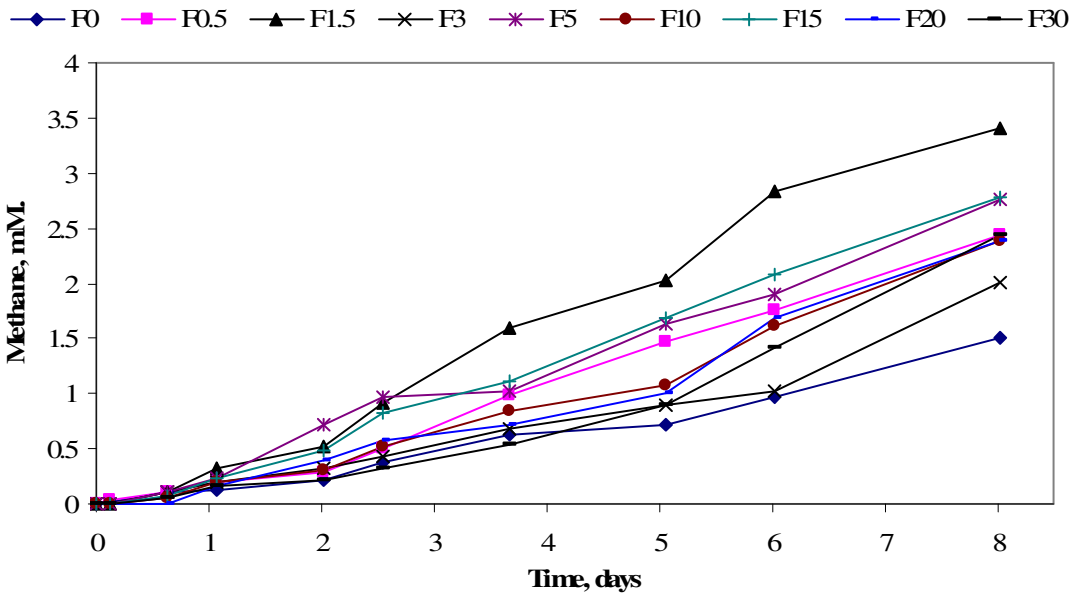


Figure A.114 : The total methane production profiles of **BUS** reactor sludge incubated at different **formate** concentrations and in the presence of **45 mM sulfate**

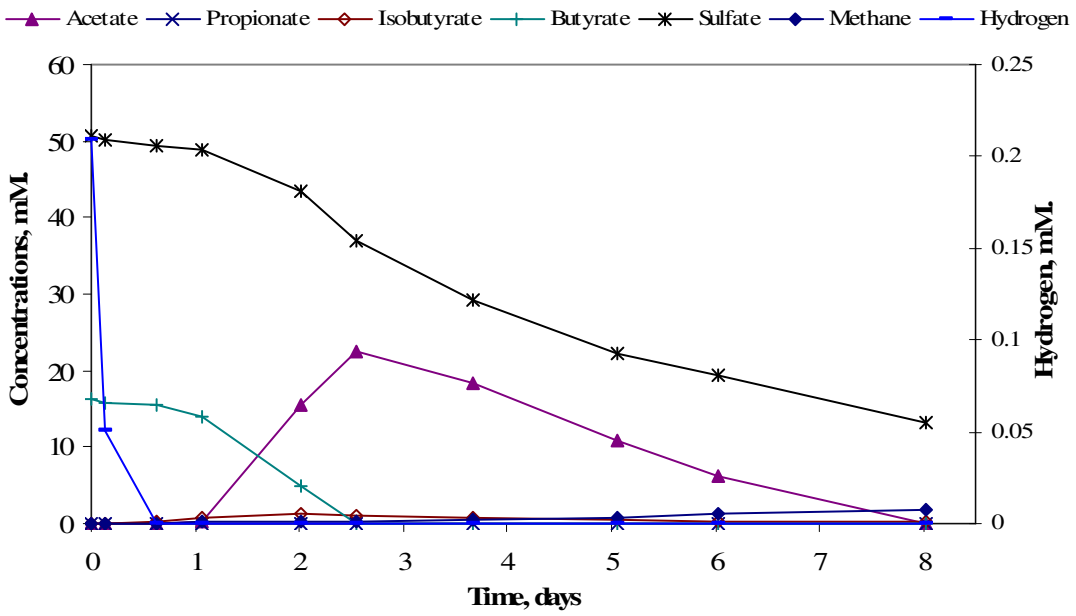


Figure A.115 : The effect of **0.2 mM hydrogen** to the butyrate degradation profile of **BUS** reactor sludge in the presence of **45 mM sulfate**

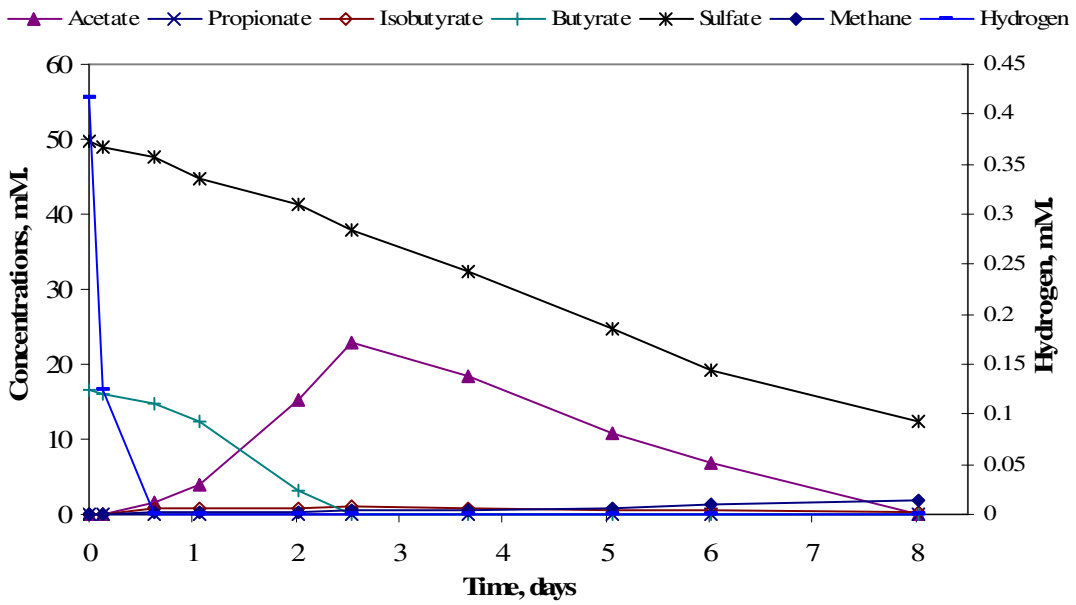


Figure A.116 : The effect of **0.5 mM hydrogen** to the butyrate degradation profile of **BUS** reactor sludge in the presence of **45 mM sulfate**

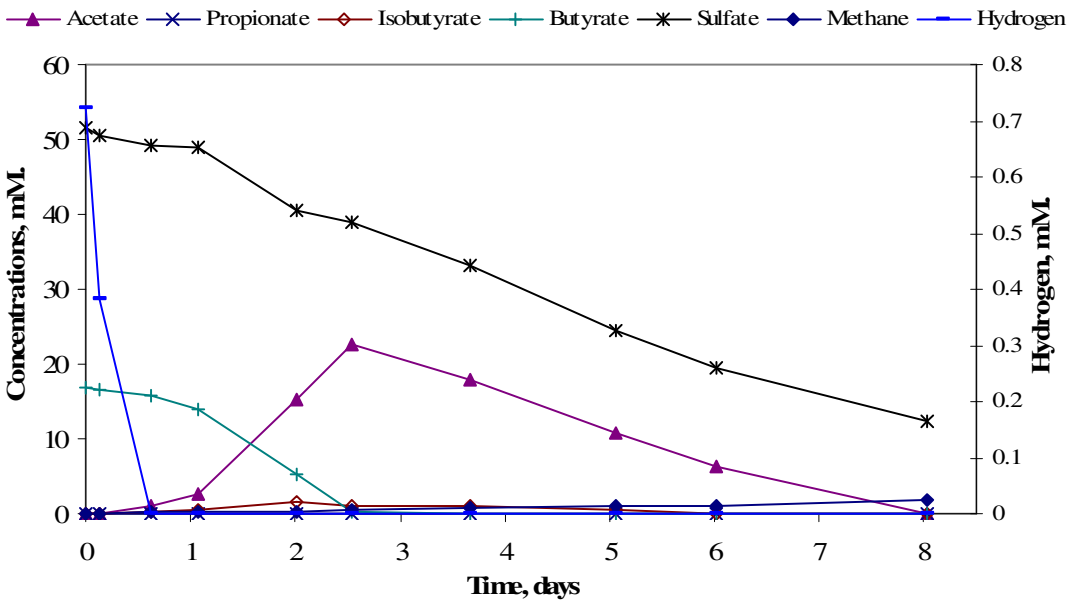


Figure A.117 : The effect of **0.7 mM hydrogen** to the butyrate degradation profile of **BUS** reactor sludge in the presence of **45 mM sulfate**

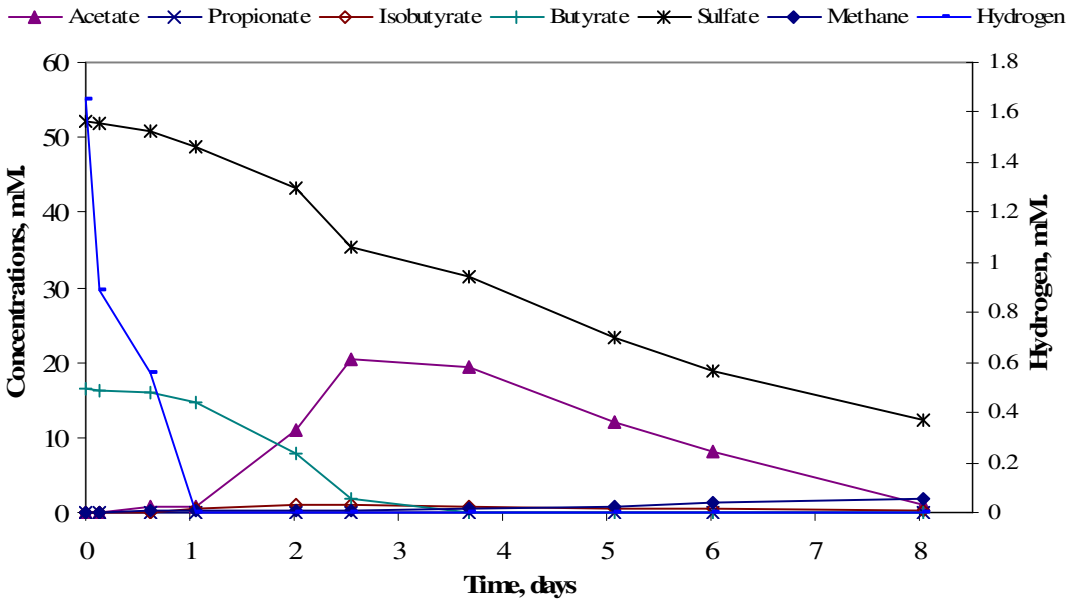


Figure A.118 : The effect of **1.6 mM hydrogen** to the butyrate degradation profile of **BUS** reactor sludge in the presence of **45 mM sulfate**

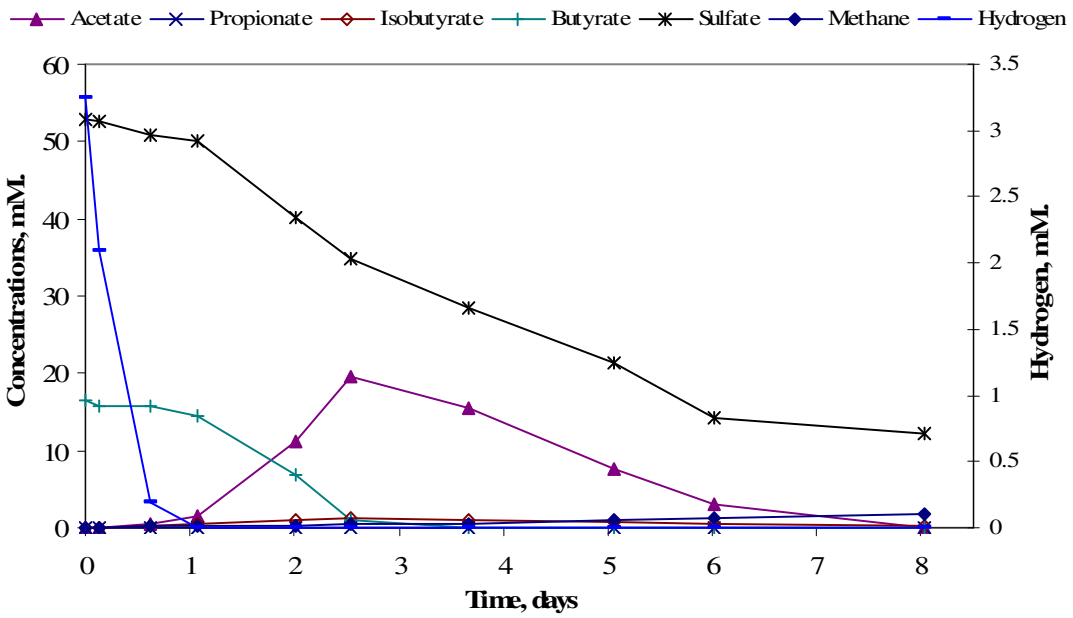


Figure A.119 : The effect of **3.2 mM hydrogen** to the butyrate degradation profile of **BUS** reactor sludge in the presence of **45 mM sulfate**

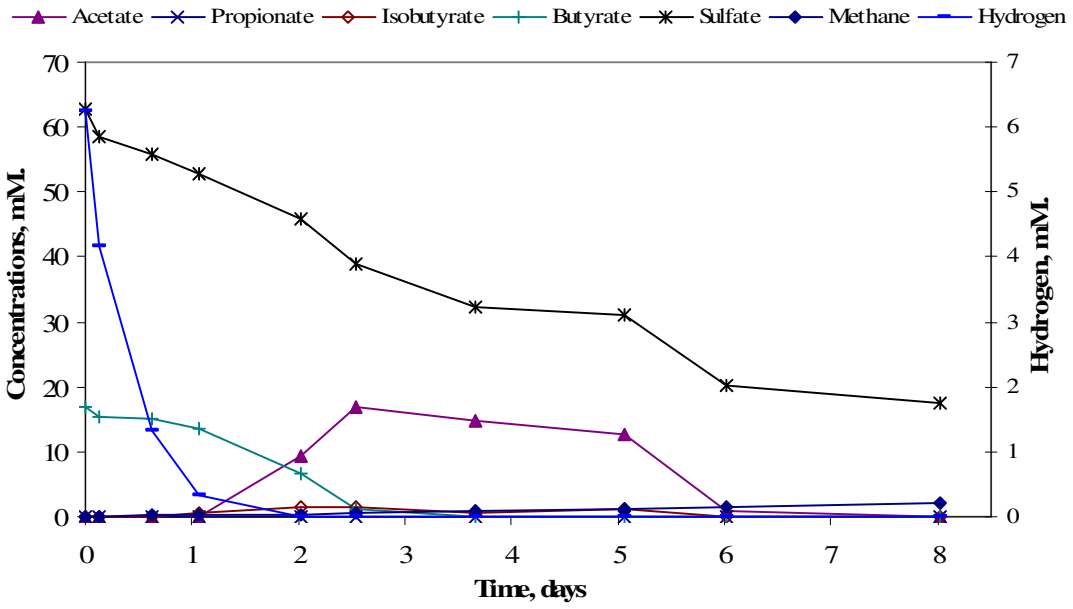


Figure A.120 : The effect of **6 mM hydrogen** to the butyrate degradation profile of **BUS** reactor sludge in the presence of **45 mM sulfate**

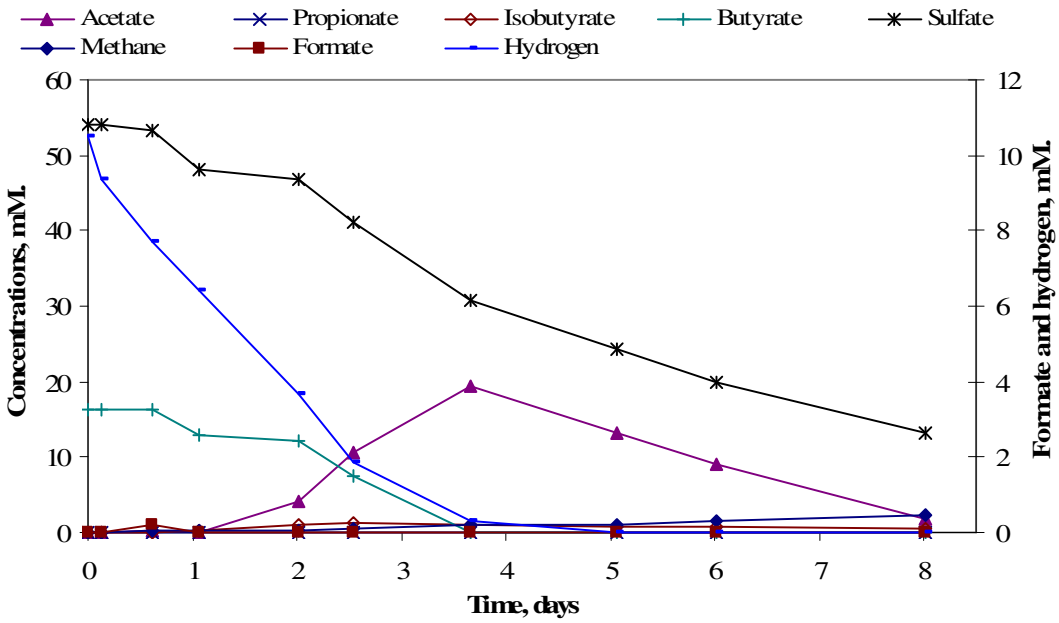


Figure A.121 : The effect of **10 mM hydrogen** to the butyrate degradation profile of **BUS** reactor sludge in the presence of **45 mM sulfate**

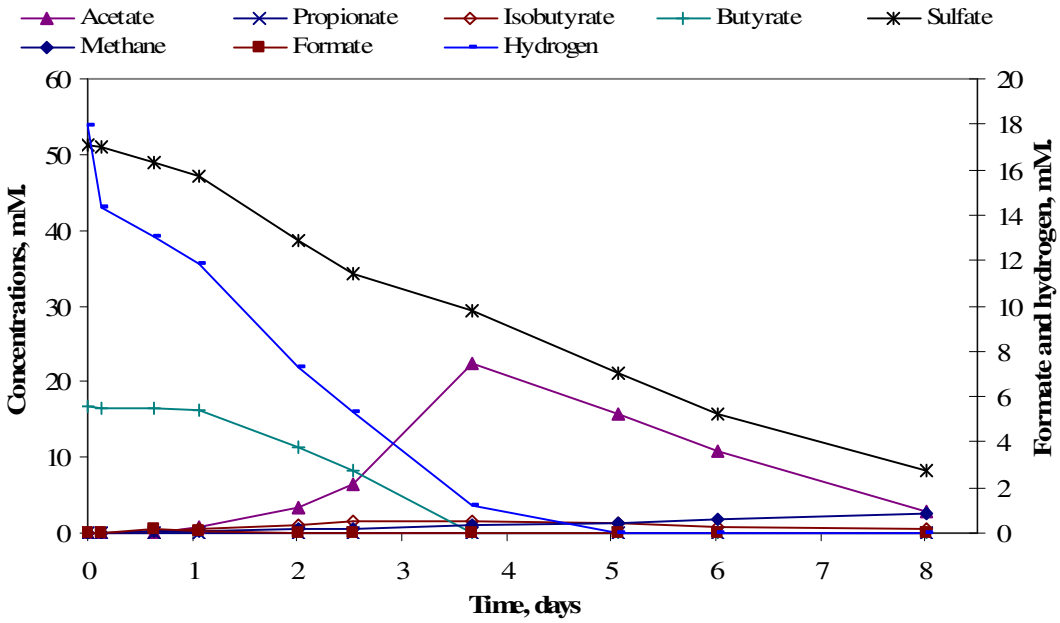


Figure A.122 : The effect of **18 mM hydrogen** to the butyrate degradation profile of **BUS** reactor sludge in the presence of **45 mM sulfate**

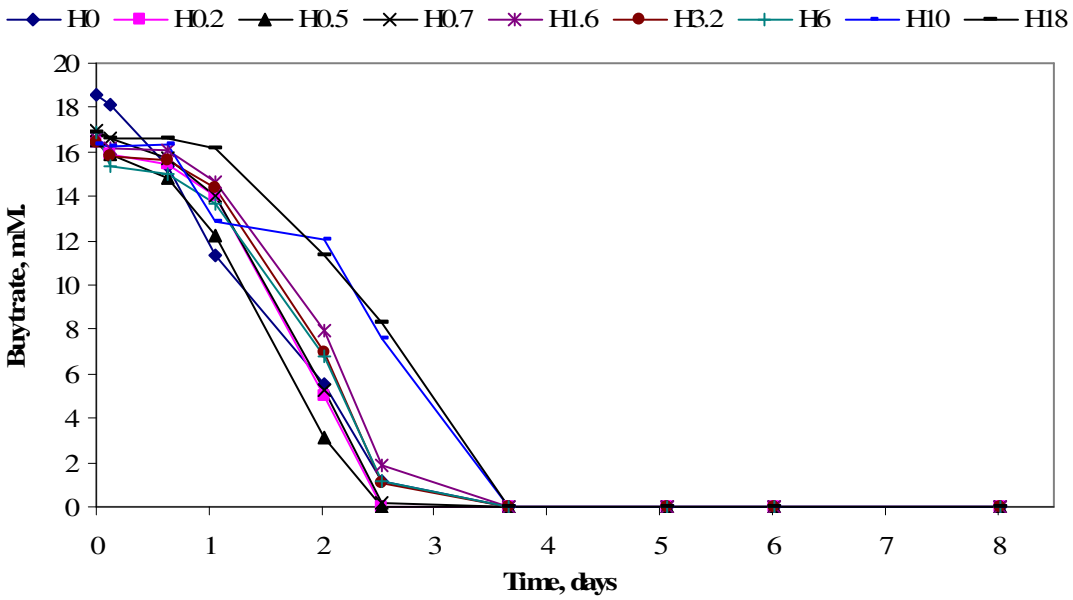


Figure A.123 : The total butyrate degradation profiles of **BUS** reactor sludge incubated at different **hydrogen** concentrations and in the presence of **45 mM sulfate**

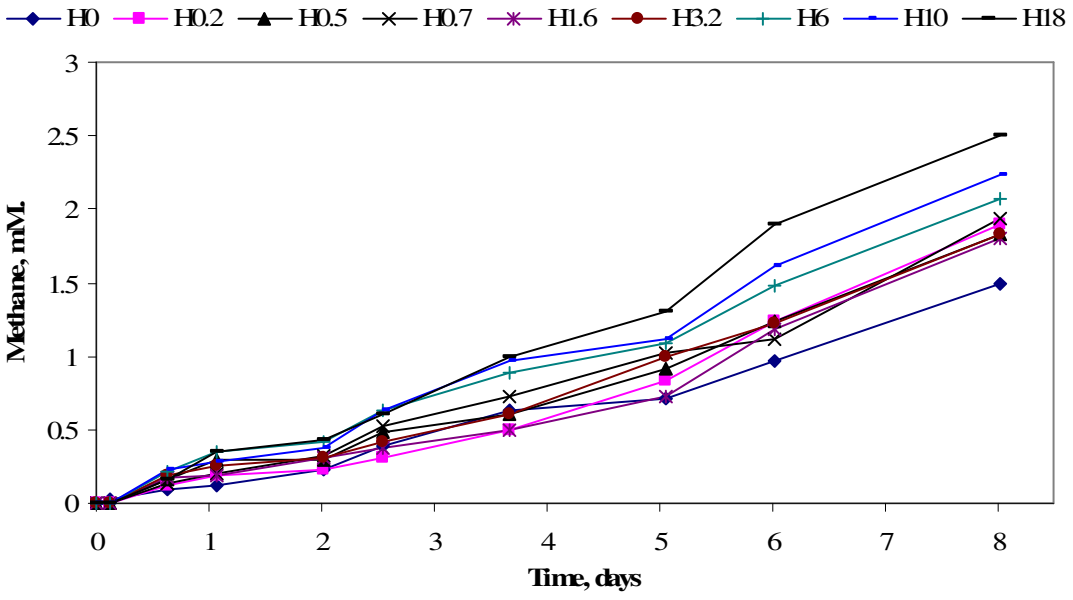


Figure A.124 : The total methane production profiles of **BUS** reactor sludge incubated at different **hydrogen** concentrations and in the presence of **45 mM sulfate**

APPENDIX B

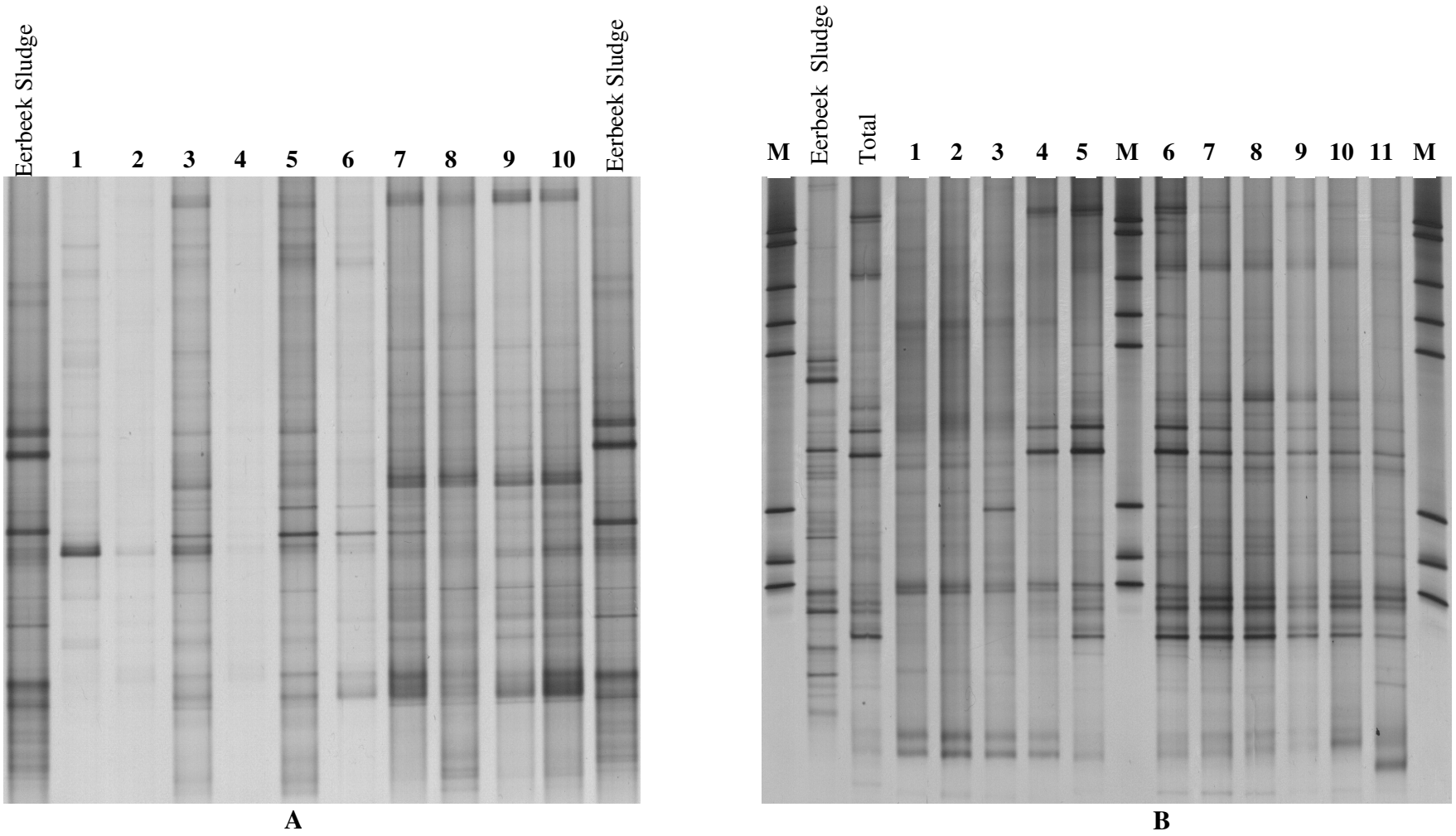


Figure B.1 : Bacterial DGGE profiles of amplified 16S rRNA from **WILD UASB** reactor sludge, after A) 5 days B) 20 days of incubation with [^{13}C] **butyrate**. Lanes 1-10 correspond with the gradient from heavy to light fraction

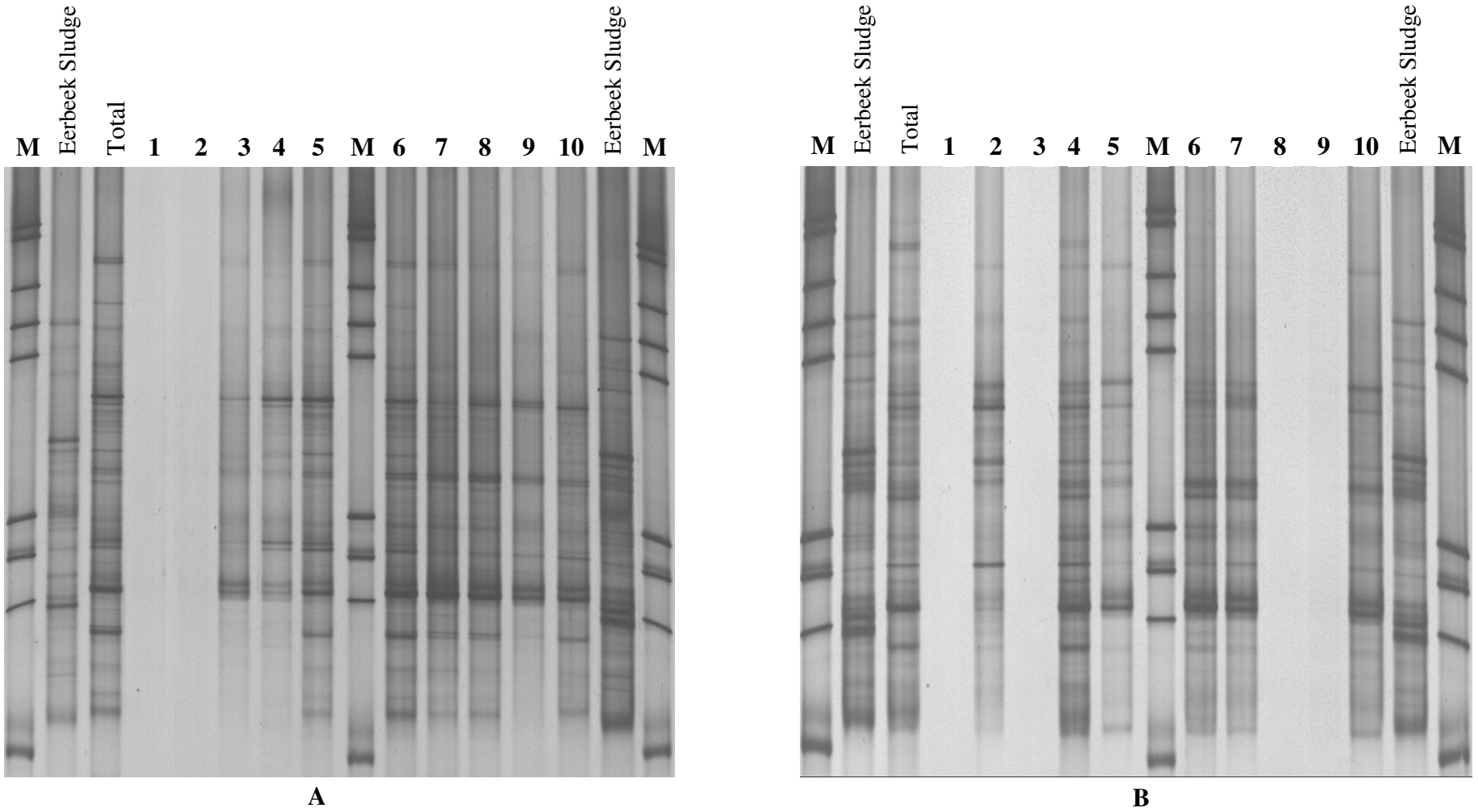


Figure B.2 : Bacterial DGGE profiles of amplified 16S rRNA from **WILD UASB** reactor sludge, after A) 30 days B) 40 days of incubation with [^{13}C] **butyrate**. Lanes 1-10 correspond with the gradient from heavy to light fraction

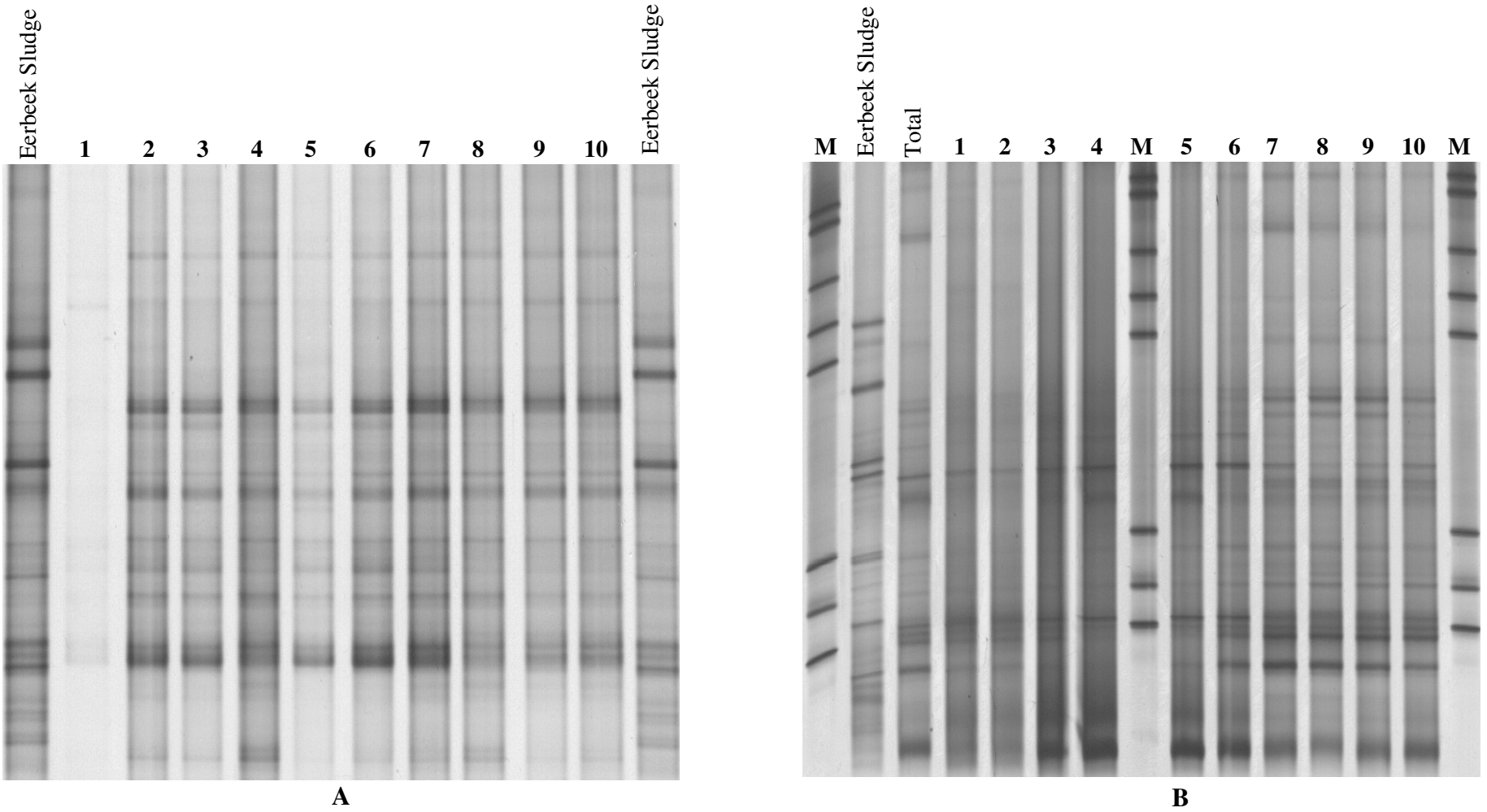


Figure B.3 : Bacterial DGGE profiles of amplified 16S rRNA from **WILD UASB** reactor sludge, after A) 5 days B) 20 days of incubation with [^{13}C] **butyrate plus 3mM sulfate**. Lanes 1-10 correspond with the gradient from heavy to light fraction

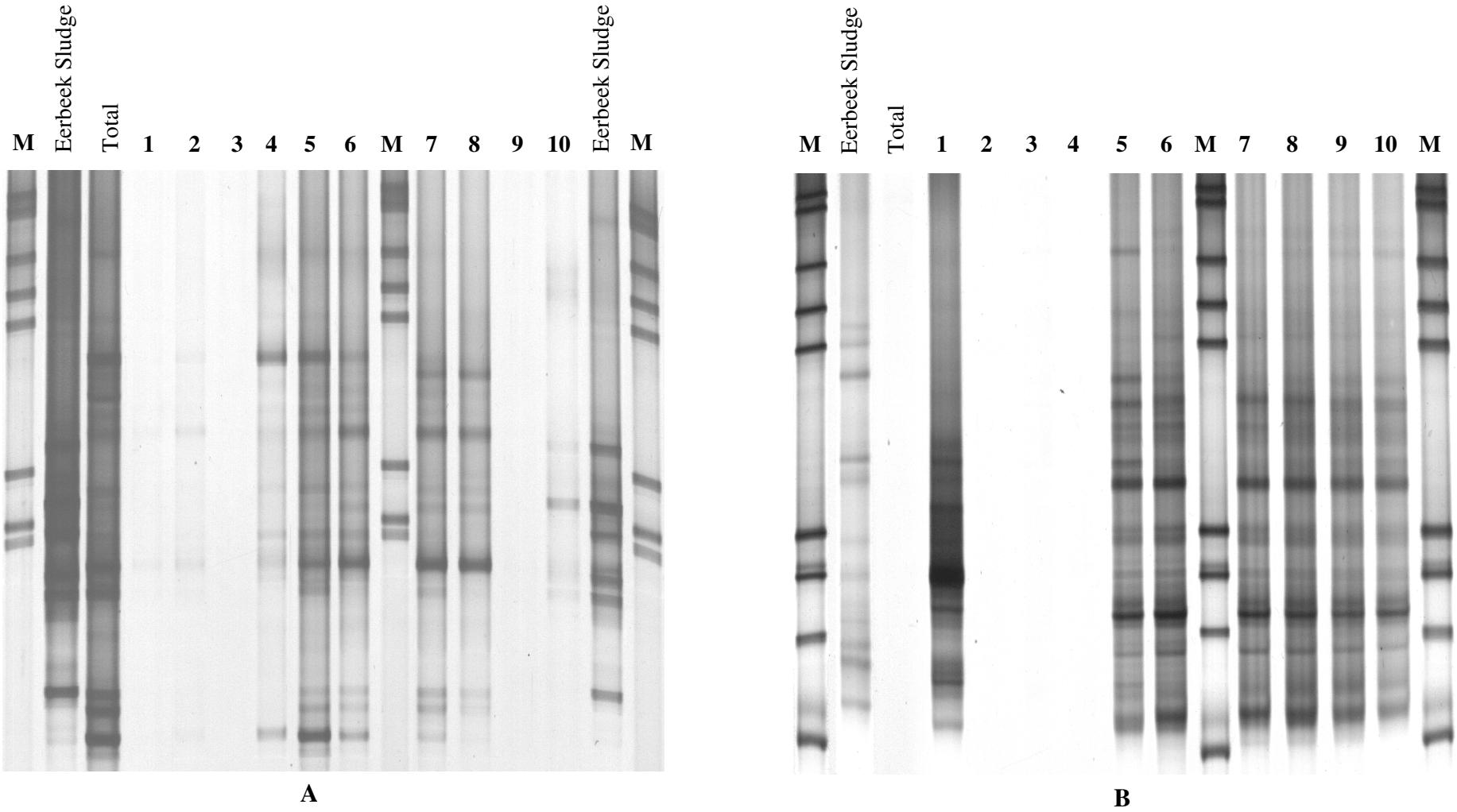


Figure B.4 : Bacterial DGGE profiles of amplified 16S rRNA from **WILD UASB** reactor sludge, after A) 30 days B) 40 days of incubation with [^{13}C] **butyrate plus 3mM sulfate**. Lanes 1-10 correspond with the gradient from heavy to light fraction

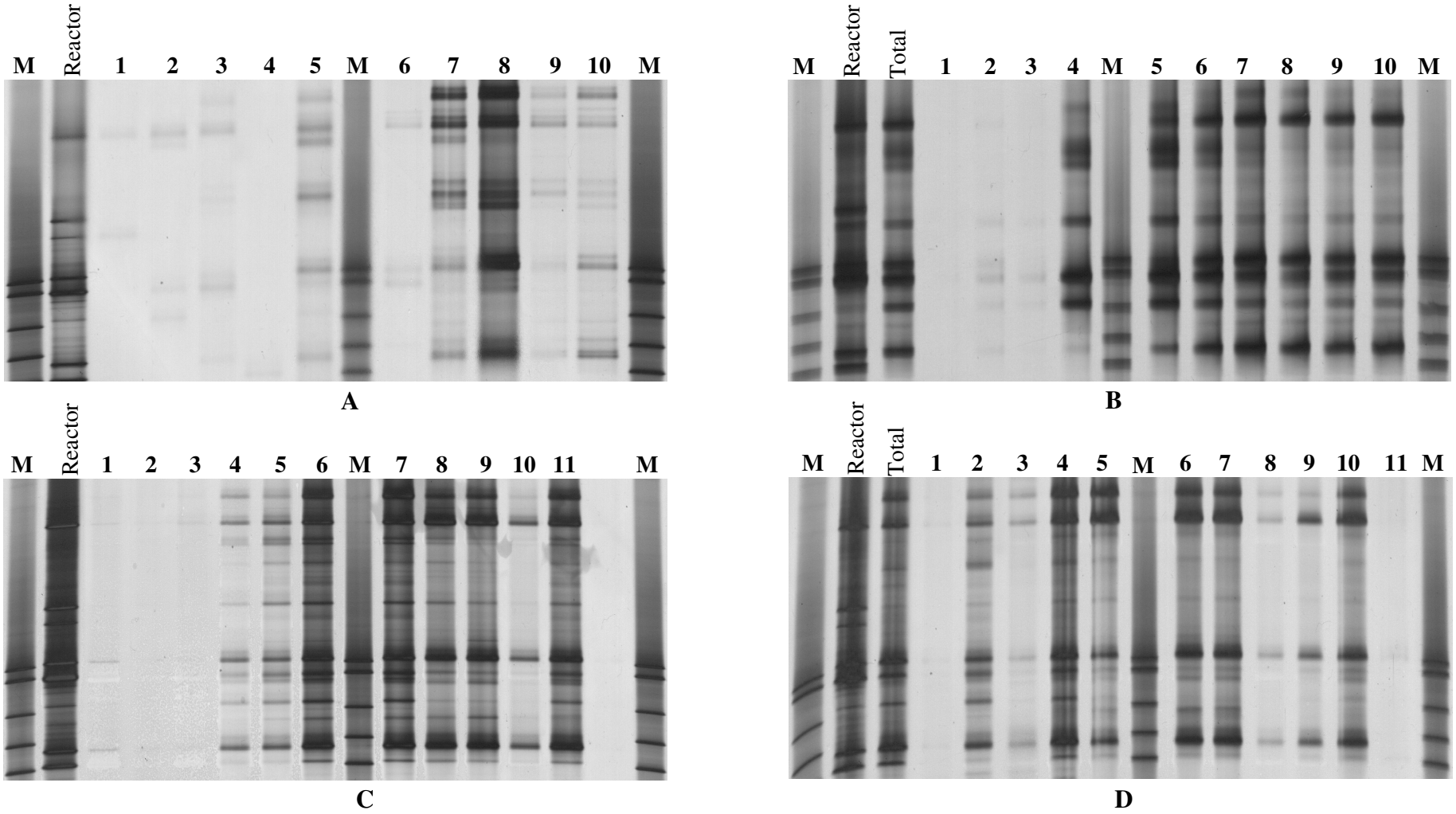


Figure B.5 : Archaeal DGGE profiles of amplified 16S rRNA from **WILD UASB** reactor sludge, after 5, 20, 30 and 40 days (A, B, C and D respectively) of incubation with [^{13}C] **butyrate**. Lanes 1-10 correspond with the gradient from heavy to light fraction

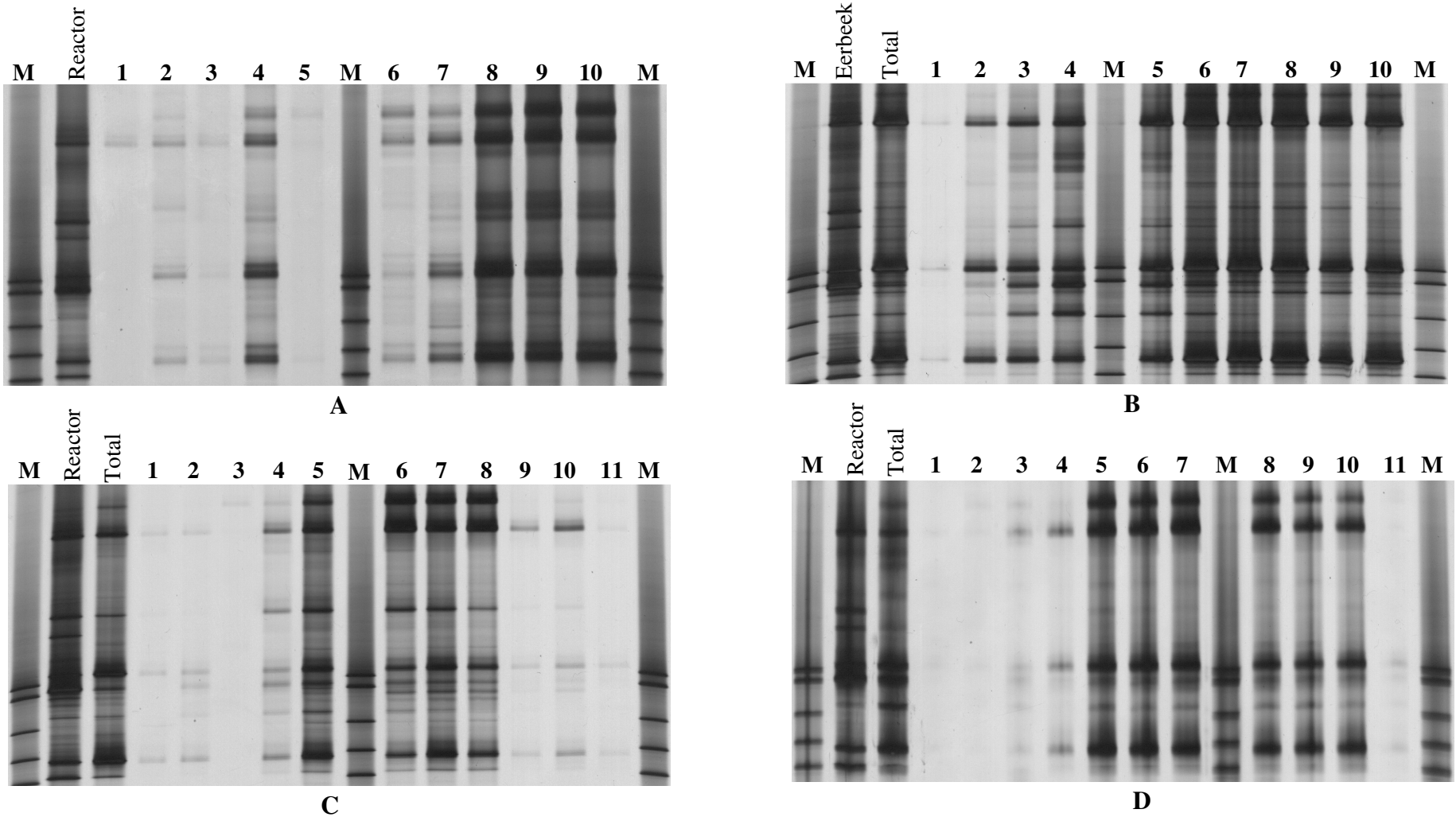


Figure B.6 : Archaeal DGGE profiles of amplified 16S rRNA from **WILD UASB** reactor sludge, after 5, 20, 30 and 40 days (A, B, C and D respectively) of incubation with [^{13}C] **butyrate plus 3mM sulfate**. Lanes 1-10 correspond with the gradient from heavy to light fraction

APPENDIX C

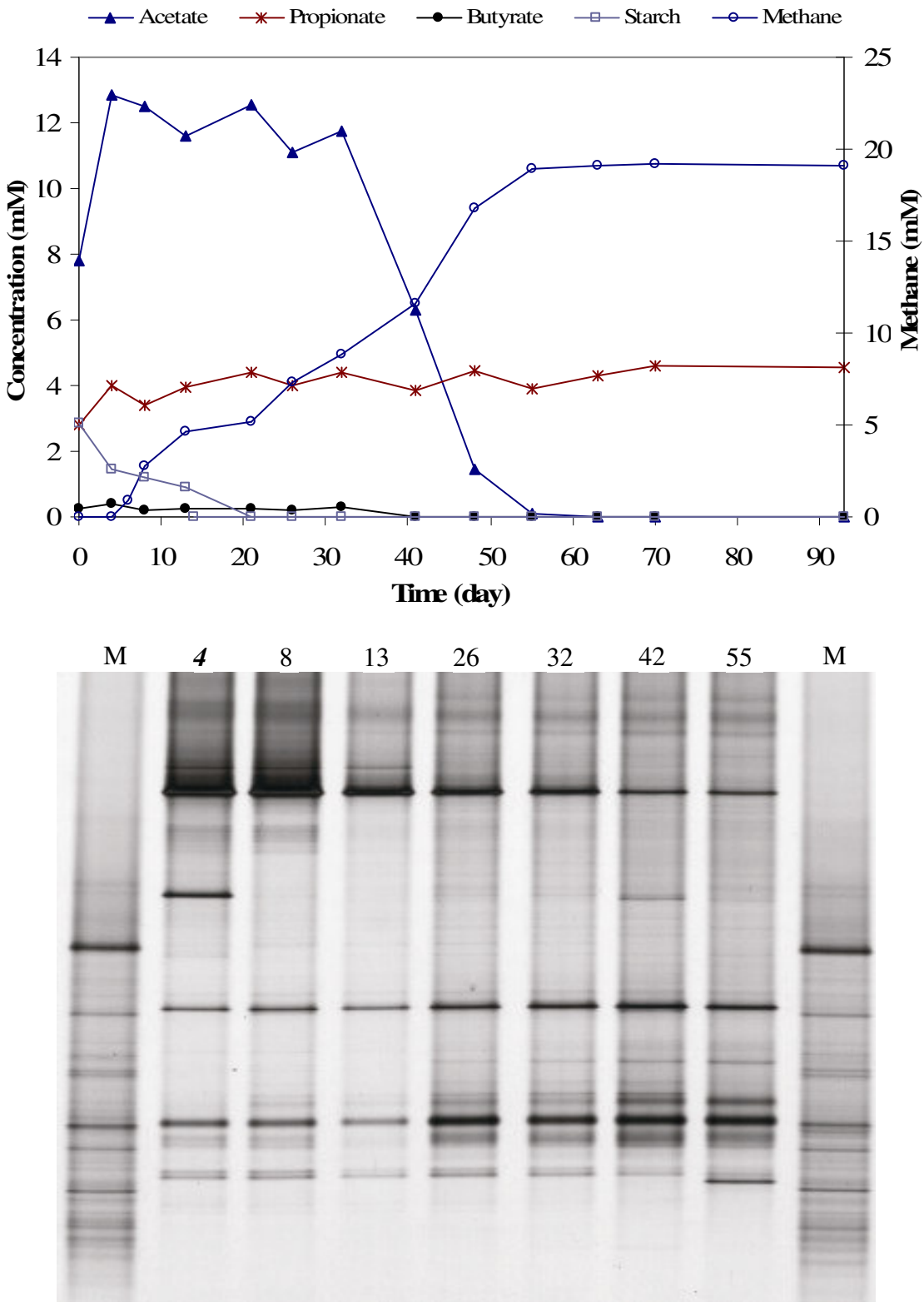


Figure C.1 : Biochemical performance (upper panel) in comparison with bacterial population dynamics (lower panel): Eerbek sludge enriched at **25°C** with the **Feed Mixture-A** (M: Original Eerbeek sludge as Marker, numbers: sampling days)

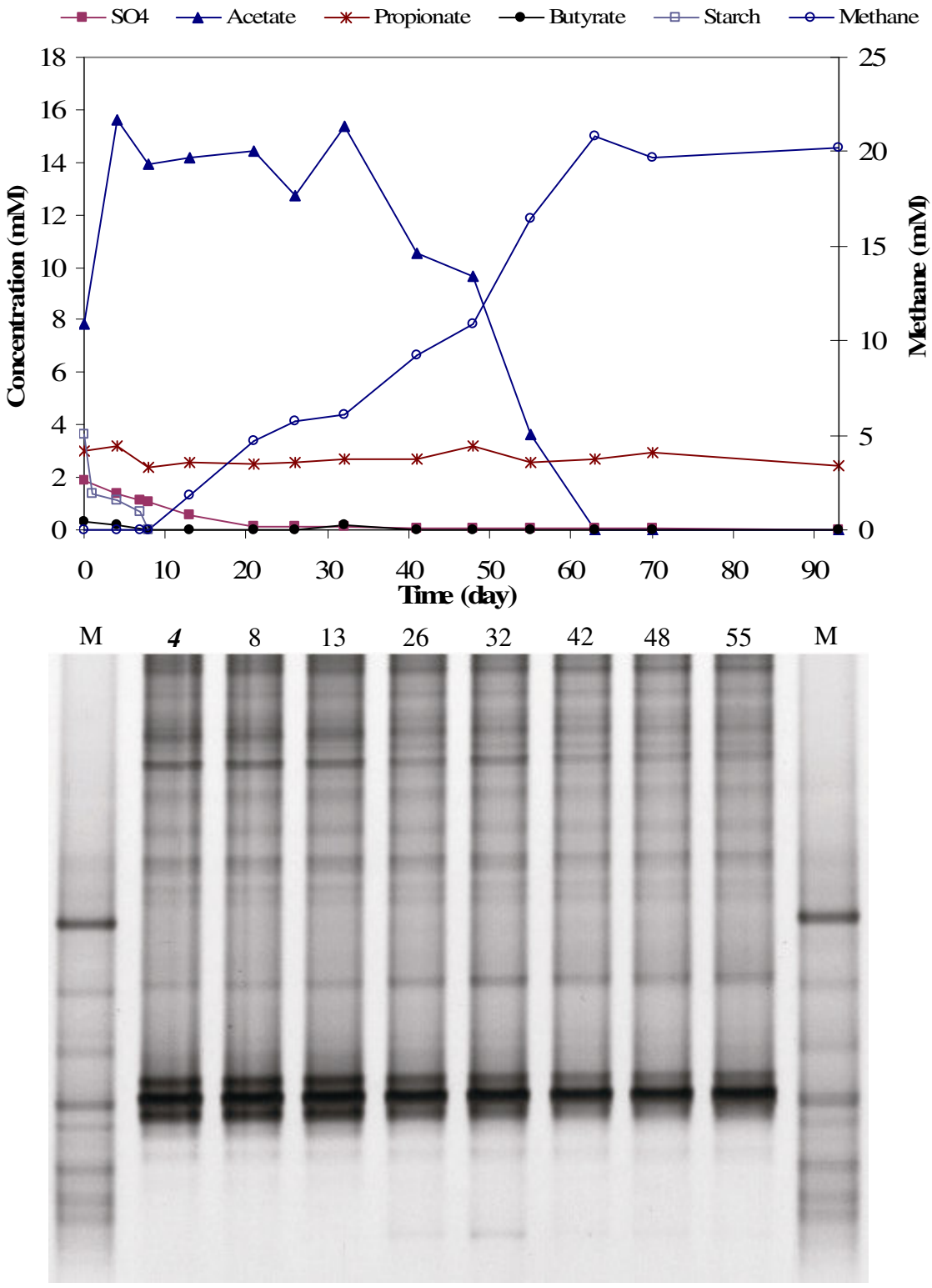


Figure C.2 : Biochemical performance (upper panel) in comparison with bacterial population dynamics (lower panel): Eerbek sludge enriched at **25°C** with the **Feed Mixture-B** (M: Original Eerbeek sludge as Marker, numbers: sampling days)

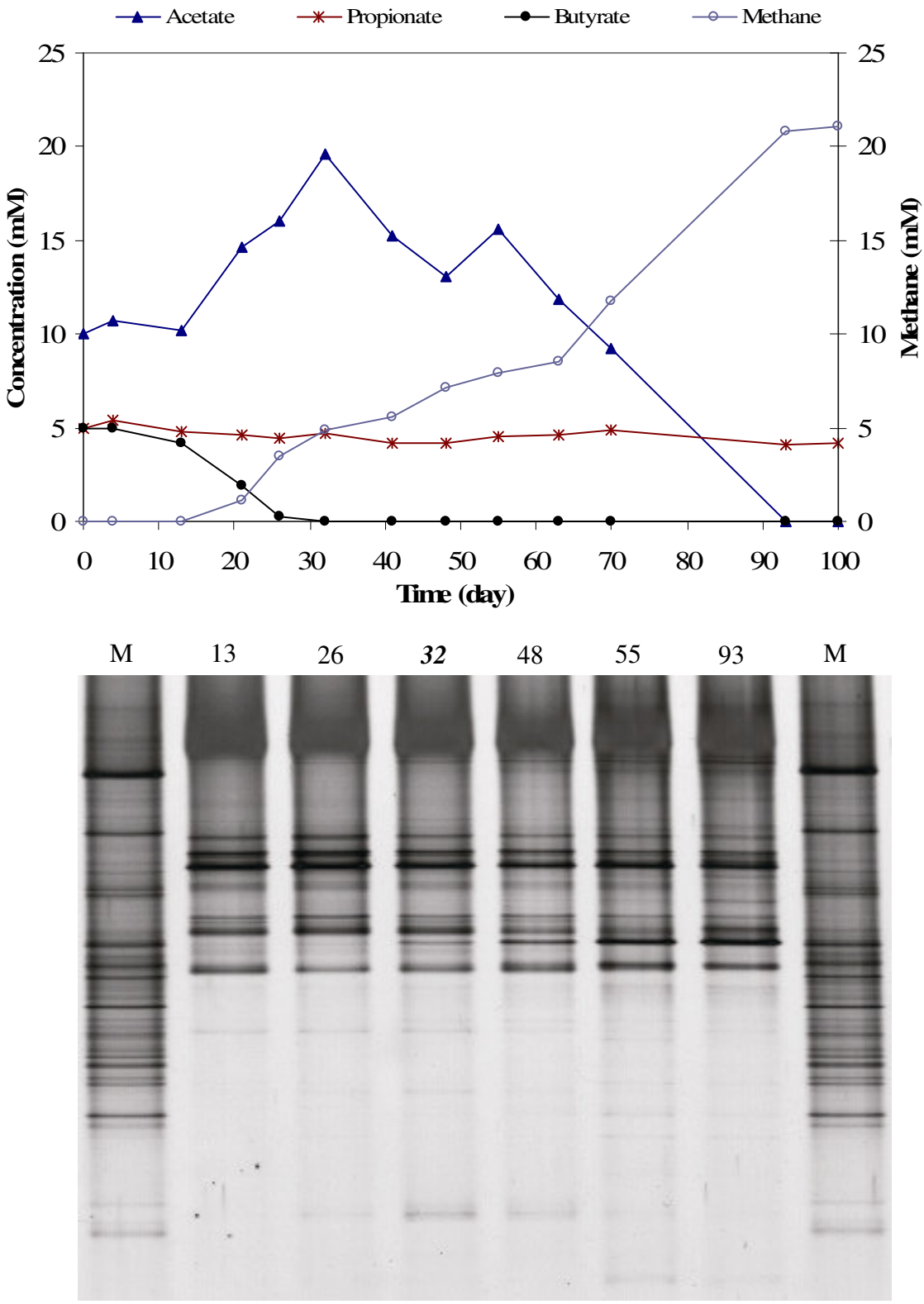


Figure C.3 : Biochemical performance (upper panel) in comparison with bacterial population dynamics (lower panel): Eerbek sludge enriched at **25°C** with the **Feed Mixture-C** (M: Original Eerbeek sludge as Marker, numbers: sampling days)

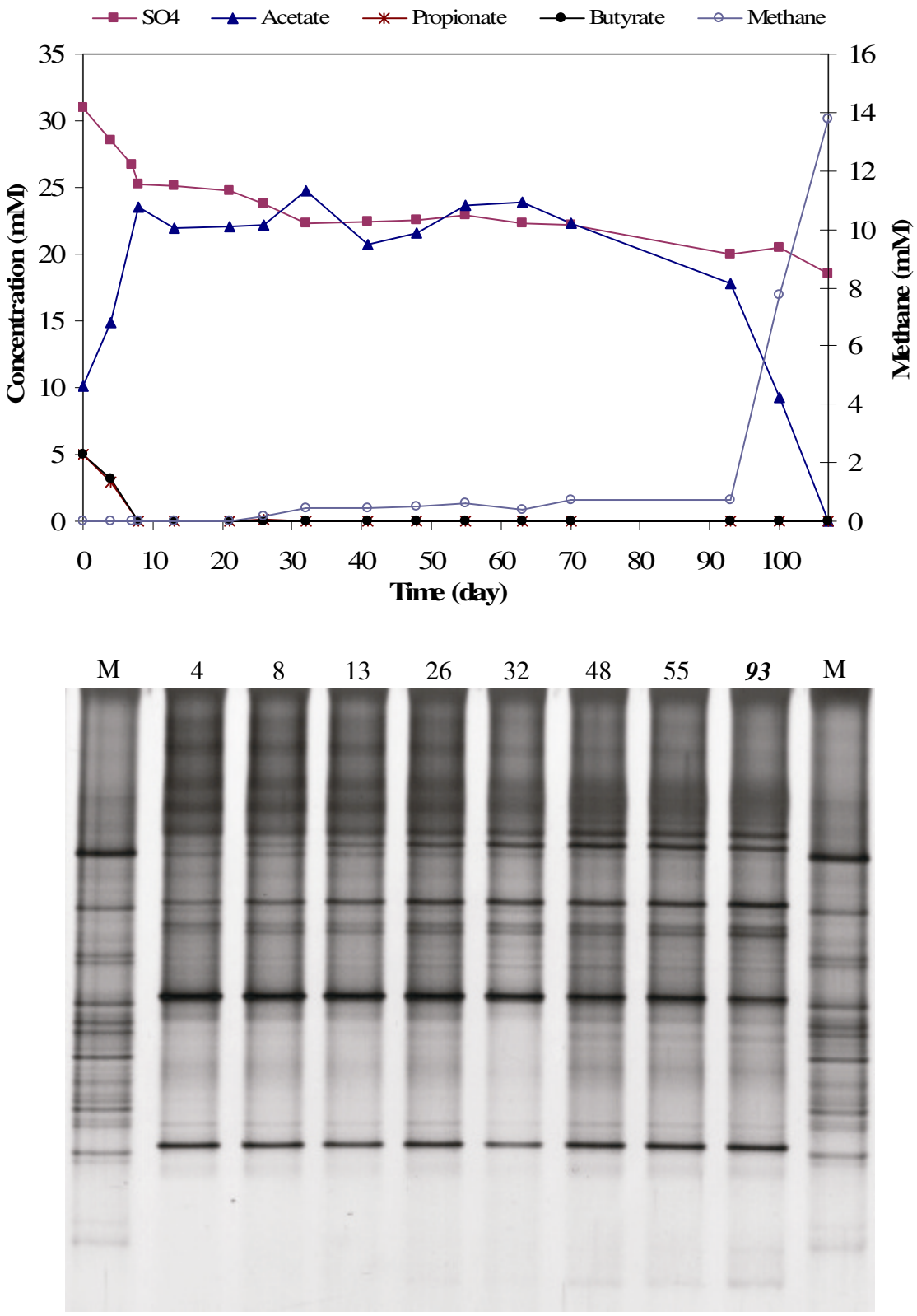
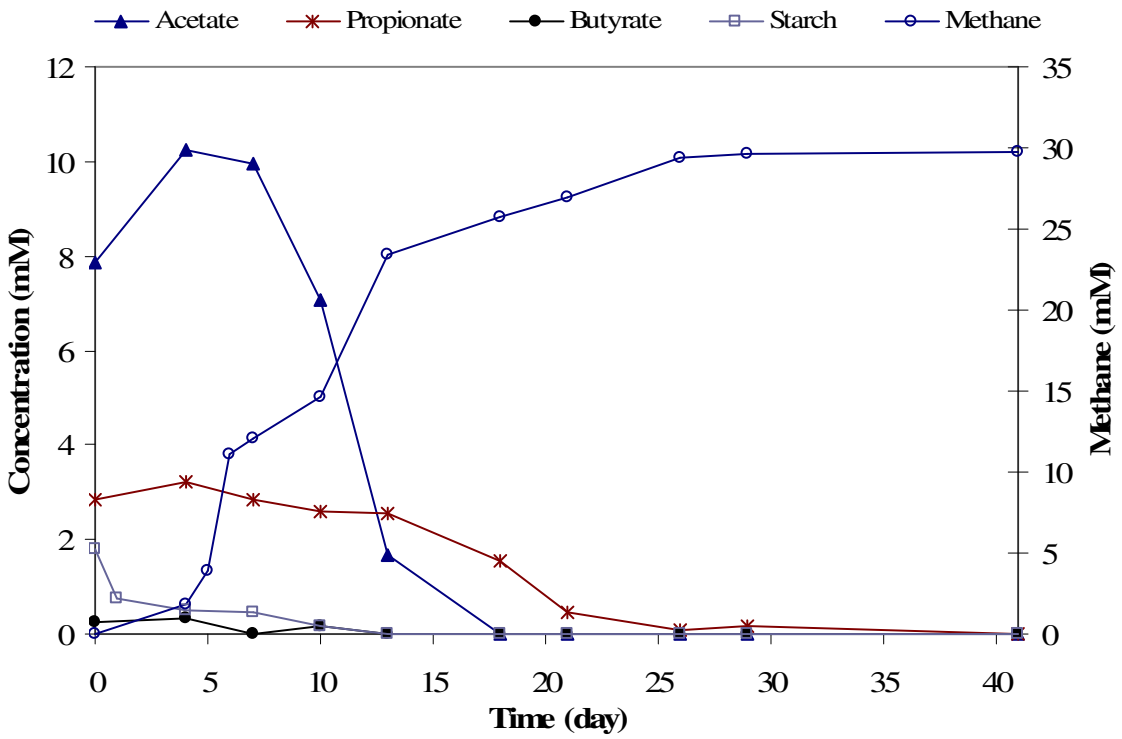


Figure C.4 : Biochemical performance (upper panel) in comparison with bacterial population dynamics (lower panel): Eerbek sludge enriched at **25°C** with the **Feed Mixture-D** (M: Original Eerbeek sludge as Marker, numbers: sampling days)



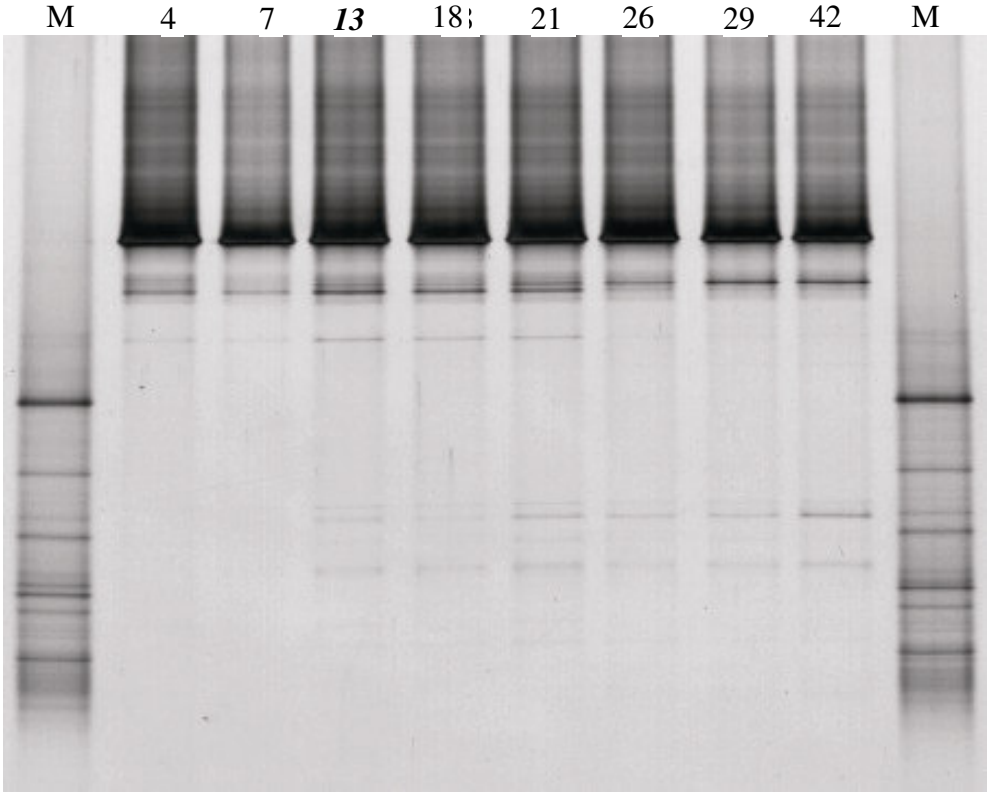


Figure C.5 : Biochemical performance (upper panel) in comparison with bacterial population dynamics (lower panel): Eerbek sludge enriched at **37°C** with the **Feed Mixture-A** (M: Original Eerbeek sludge as Marker, numbers: sampling days)

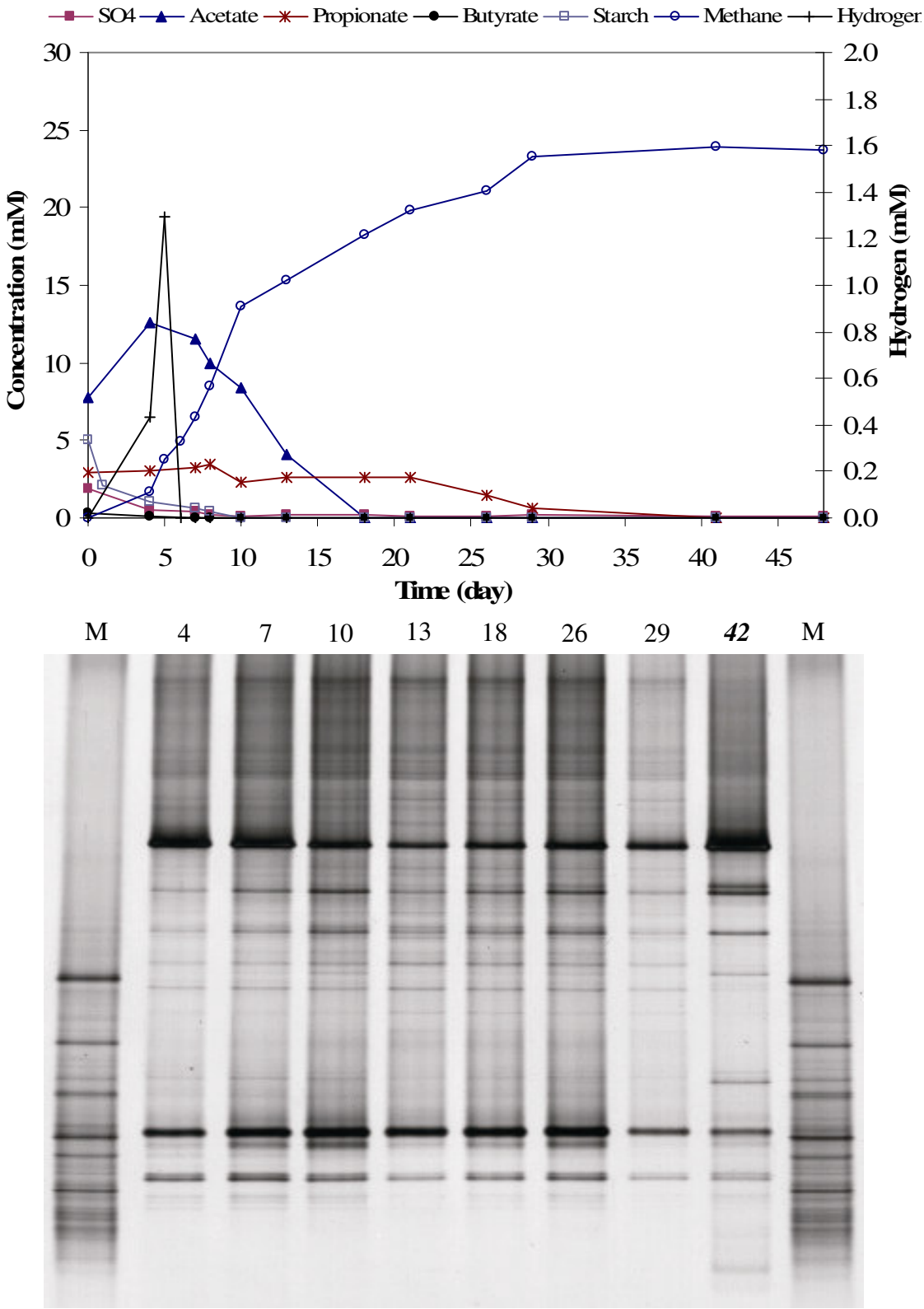


Figure C.6 : Biochemical performance (upper panel) in comparison with bacterial population dynamics (lower panel): Eerbek sludge enriched at **37°C** with the **Feed Mixture-B** (M: Original Eerbeek sludge as Marker, numbers: sampling days)

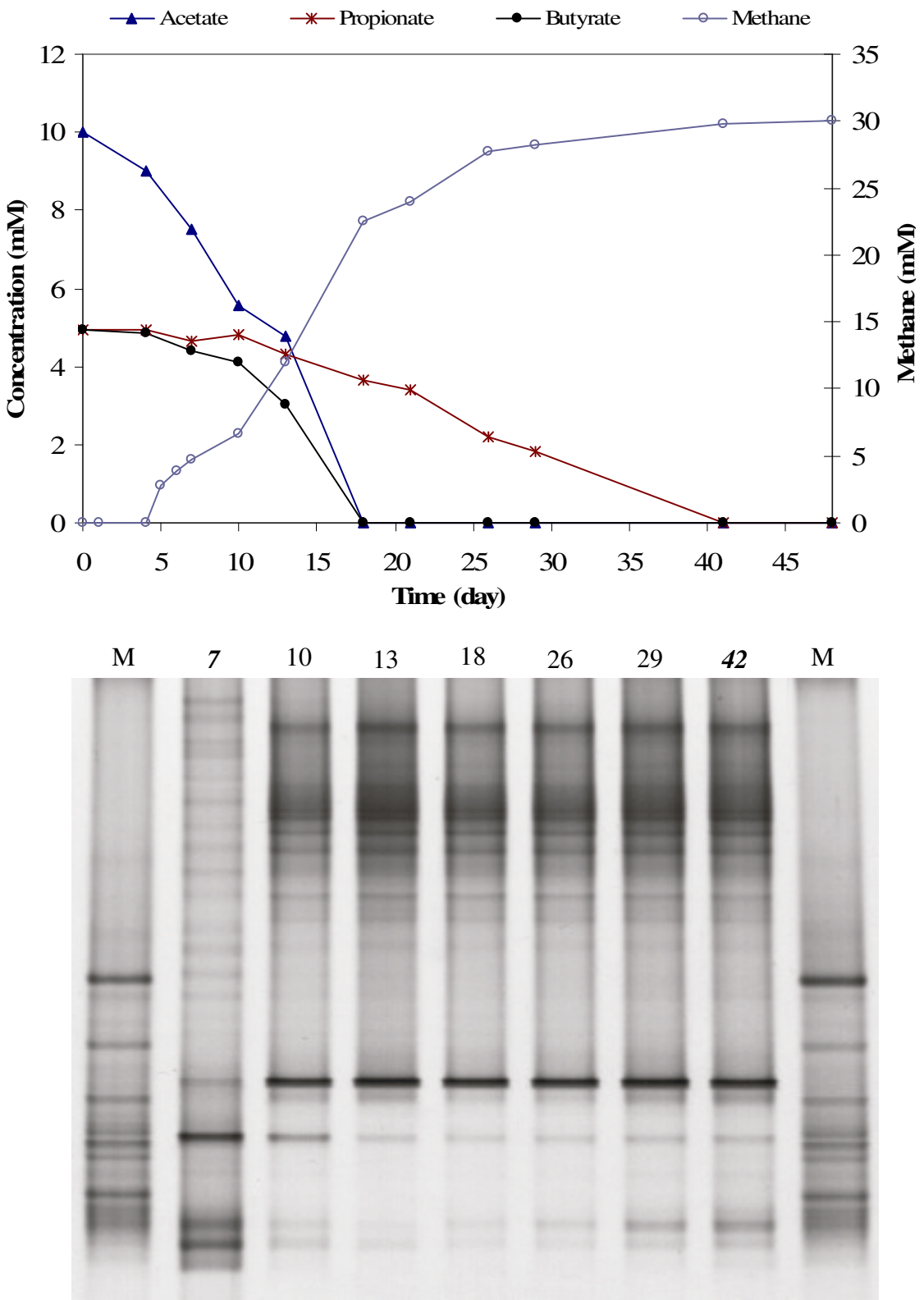


Figure C.7 : Biochemical performance (upper panel) in comparison with bacterial population dynamics (lower panel): Eerbek sludge enriched at **37°C** with the **Feed Mixture-C** (M: Original Eerbeek sludge as Marker, numbers: sampling days)

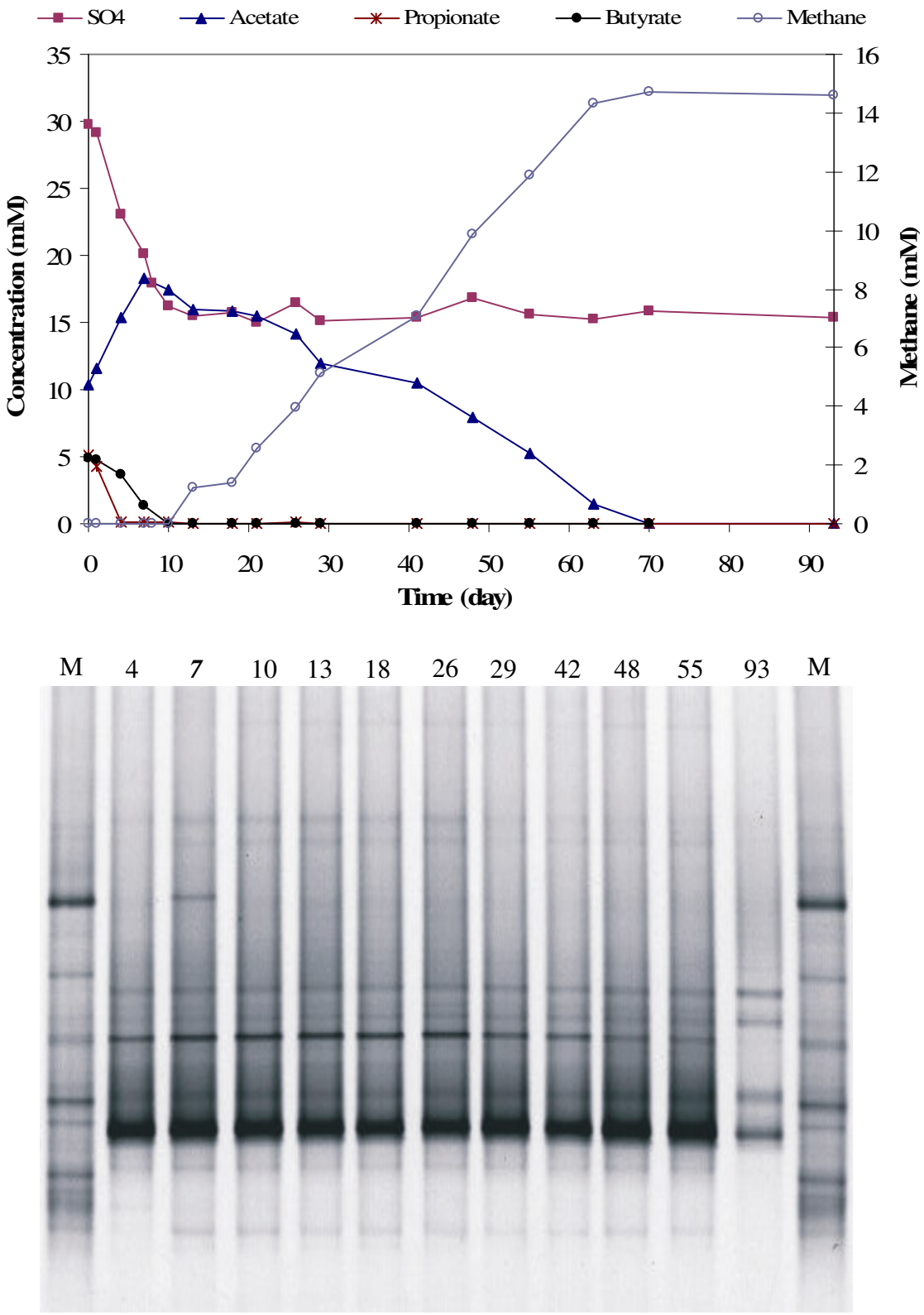


Figure C.8 : Biochemical performance (upper panel) in comparison with bacterial population dynamics (lower panel): Eerbek sludge enriched at **37°C** with the **Feed Mixture-D** (M: Original Eerbeek sludge as Marker, numbers: sampling days)

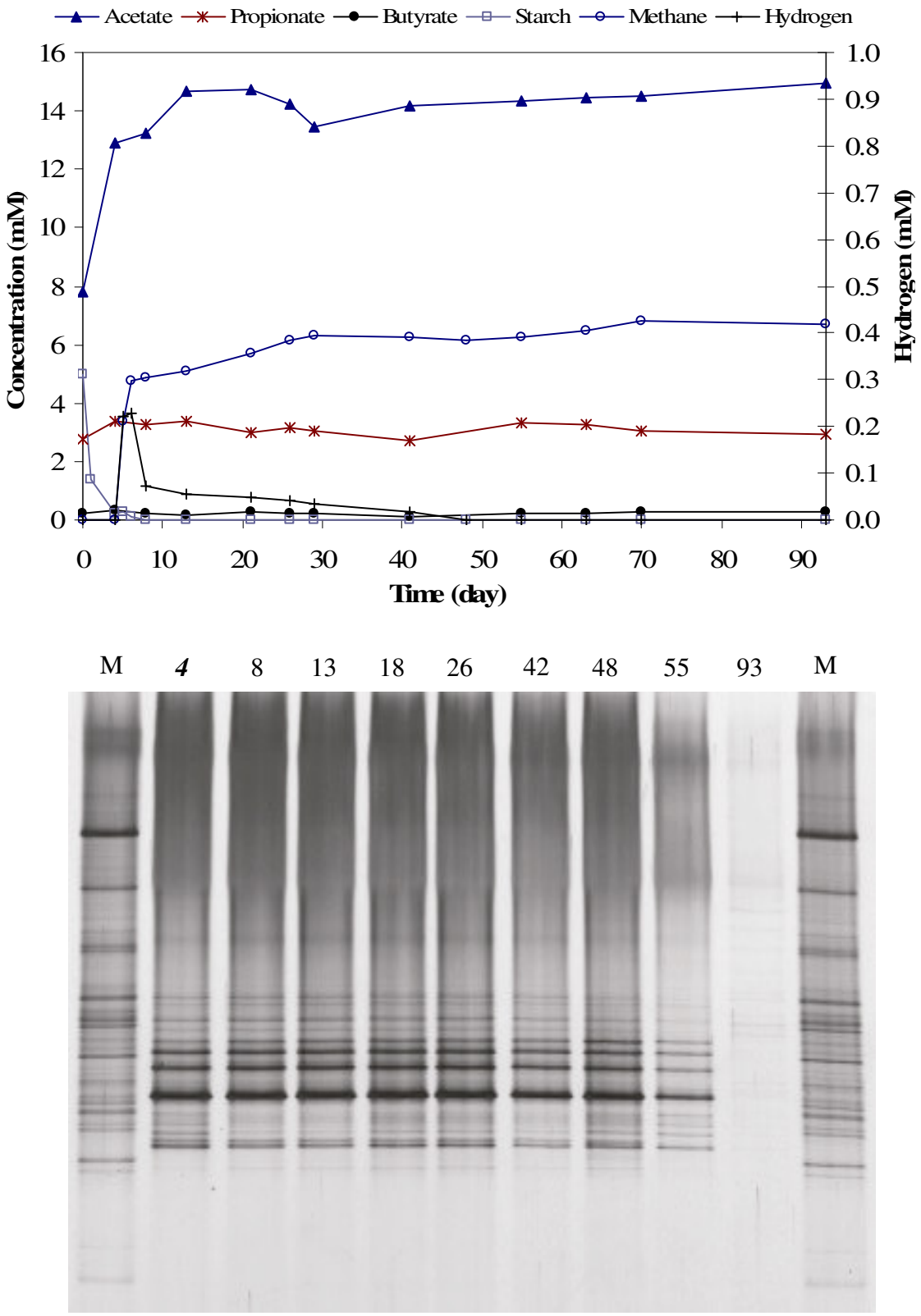


Figure C.9 : Biochemical performance (upper panel) in comparison with bacterial population dynamics (lower panel): Eerbek sludge enriched at **45°C** with the **Feed Mixture-A** (M: Original Eerbeek sludge as Marker, numbers: sampling days)

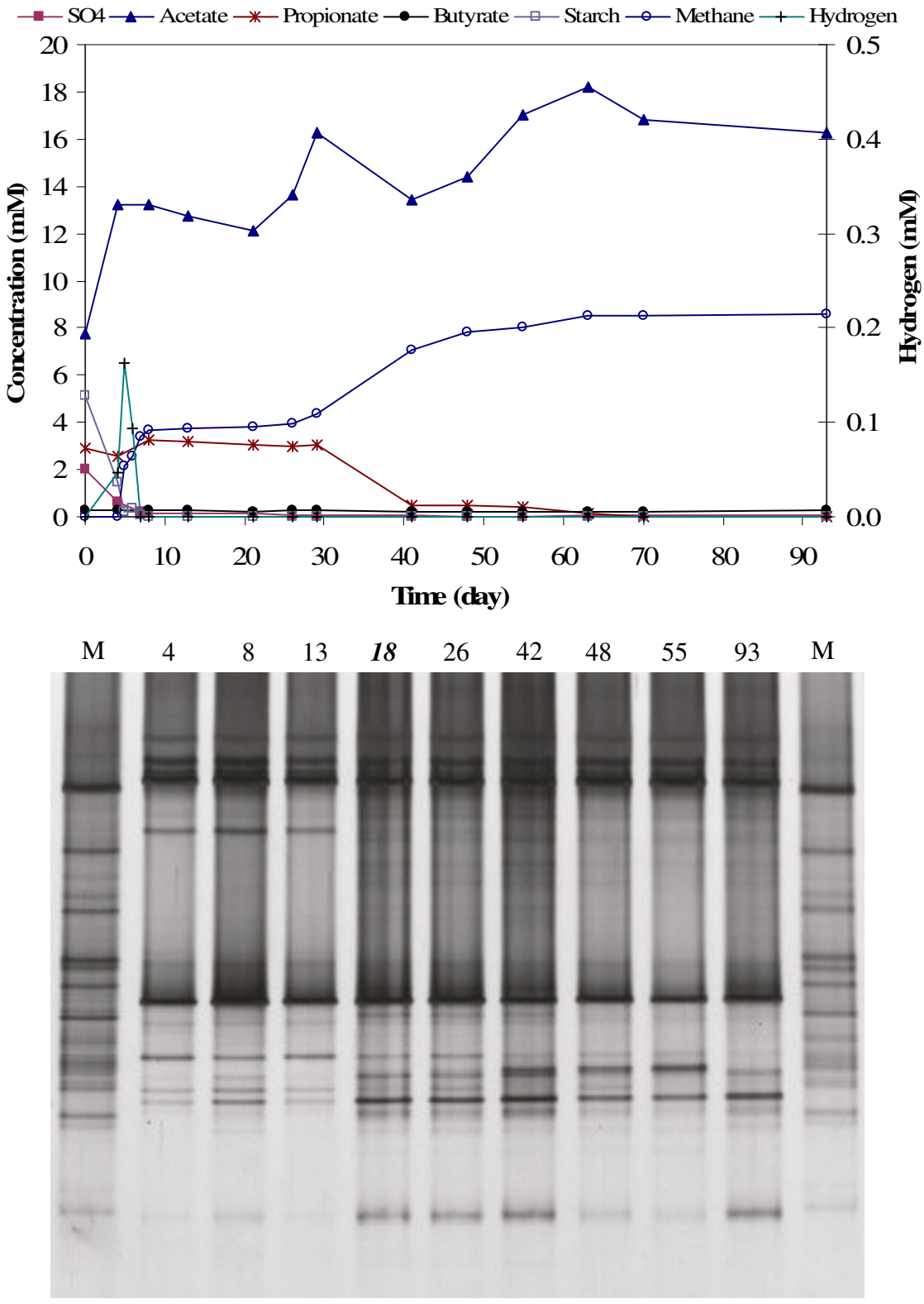


Figure C.10 : Biochemical performance (upper panel) in comparison with bacterial population dynamics (lower panel): Eerbek sludge enriched at **45°C** with the **Feed Mixture-B** (M: Original Eerbeek sludge as Marker, numbers: sampling days)

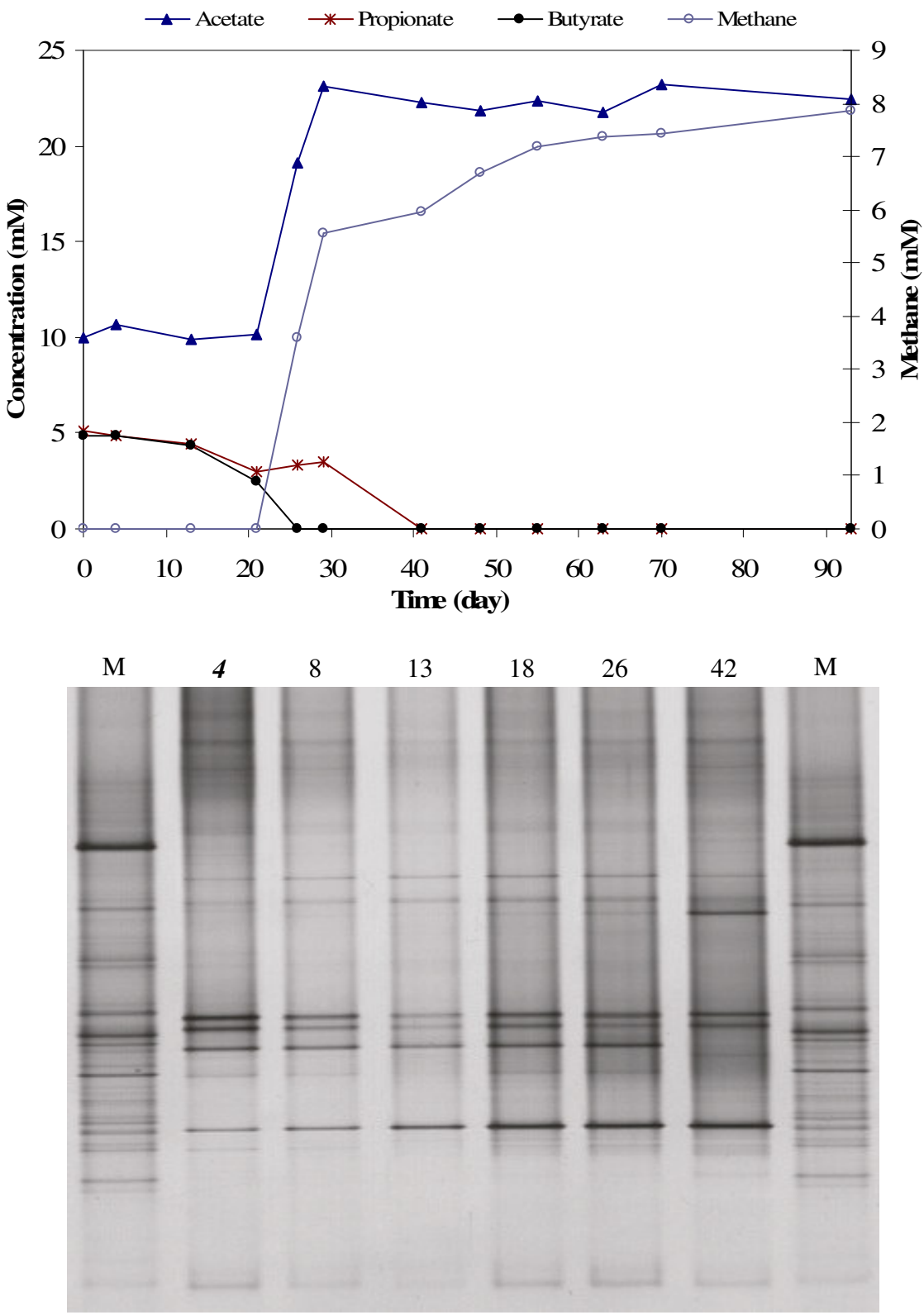


Figure C.11 : Biochemical performance (upper panel) in comparison with bacterial population dynamics (lower panel): Eerbek sludge enriched at **45°C** with the **Feed Mixture-C** (M: Original Eerbeek sludge as Marker, numbers: sampling days)

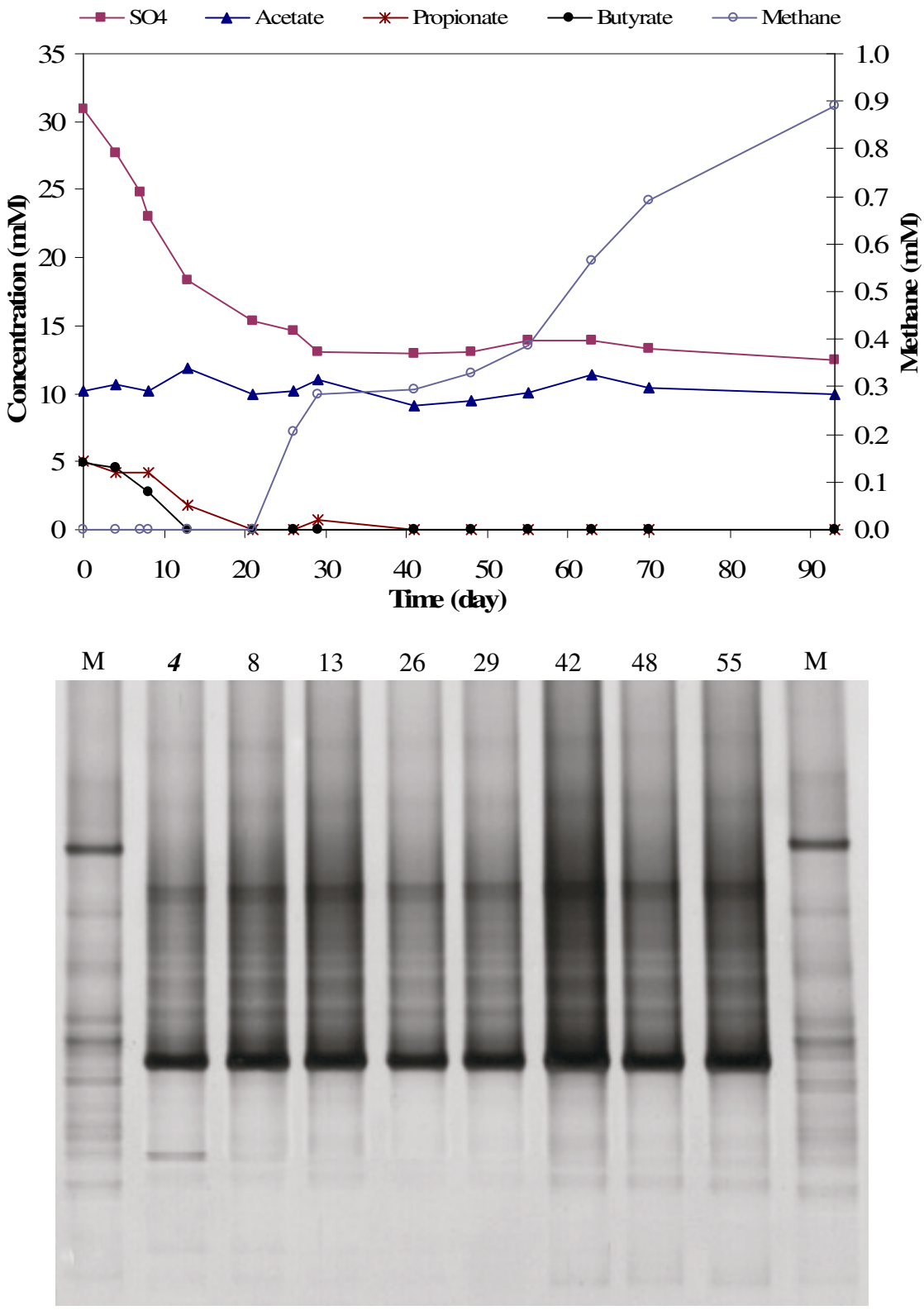


Figure C.12 : Biochemical performance (upper panel) in comparison with bacterial population dynamics (lower panel): Eerbek sludge enriched at **45°C** with the **Feed Mixture-D** (M: Original Eerbeek sludge as Marker, numbers: sampling days)

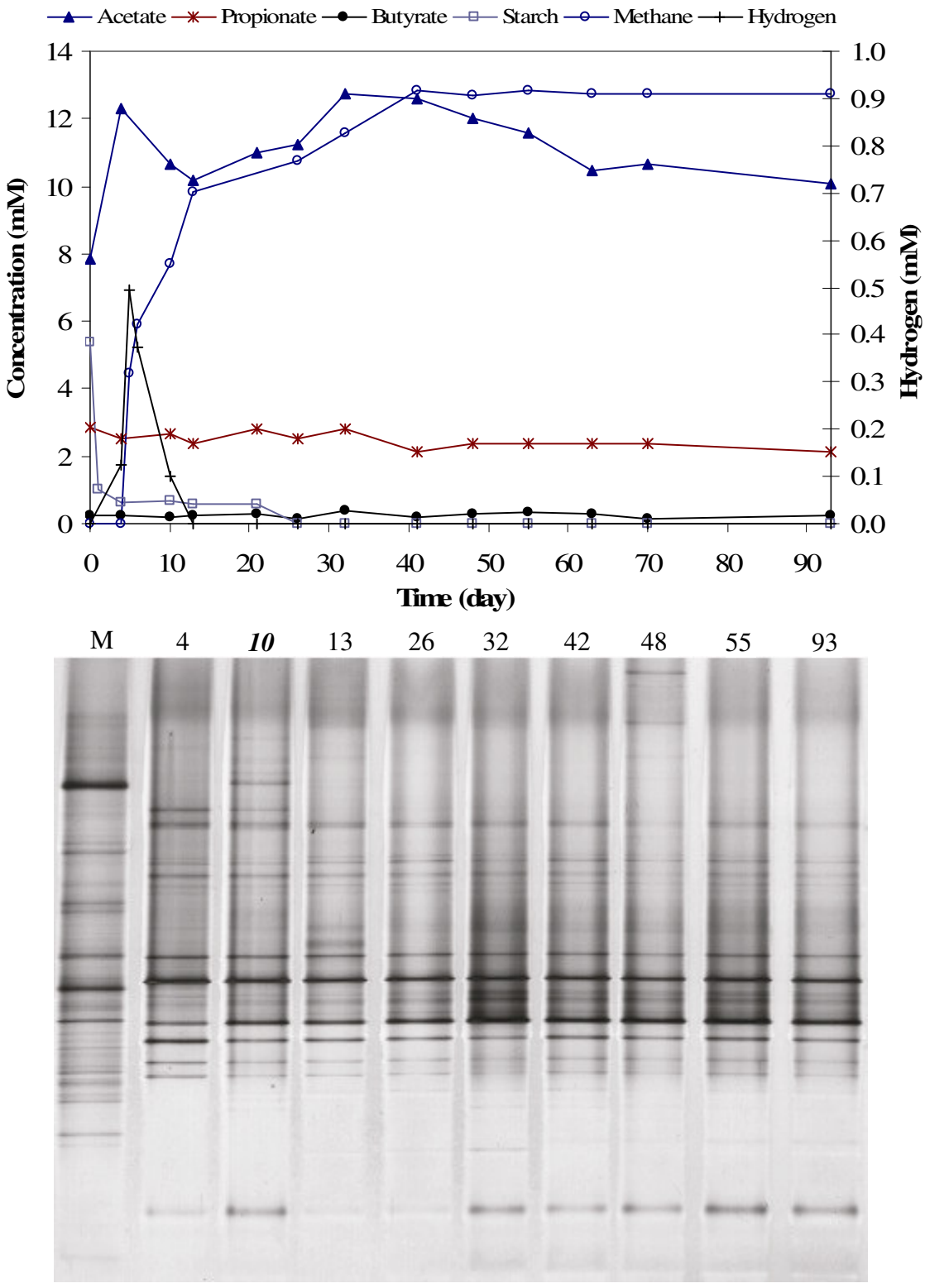
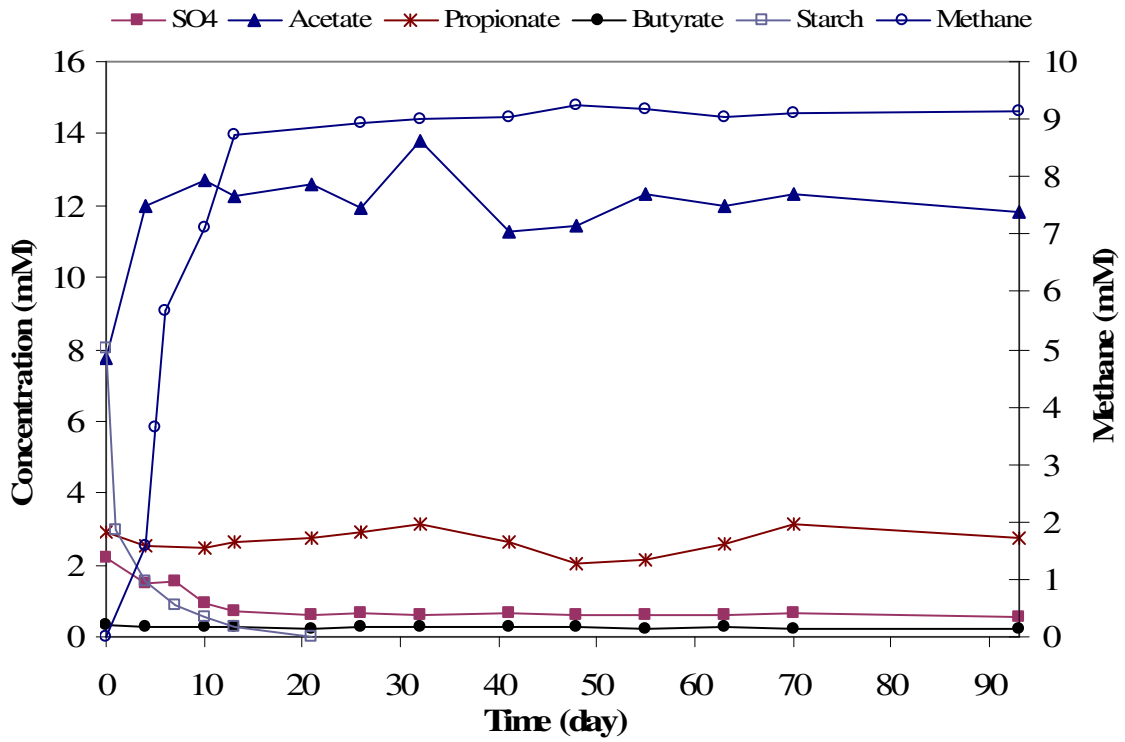


Figure C.13 : Biochemical performance (upper panel) in comparison with bacterial population dynamics (lower panel): Eerbek sludge enriched at **55°C** with the **Feed Mixture-A** (M: Original Eerbeek sludge as Marker, numbers: sampling days)



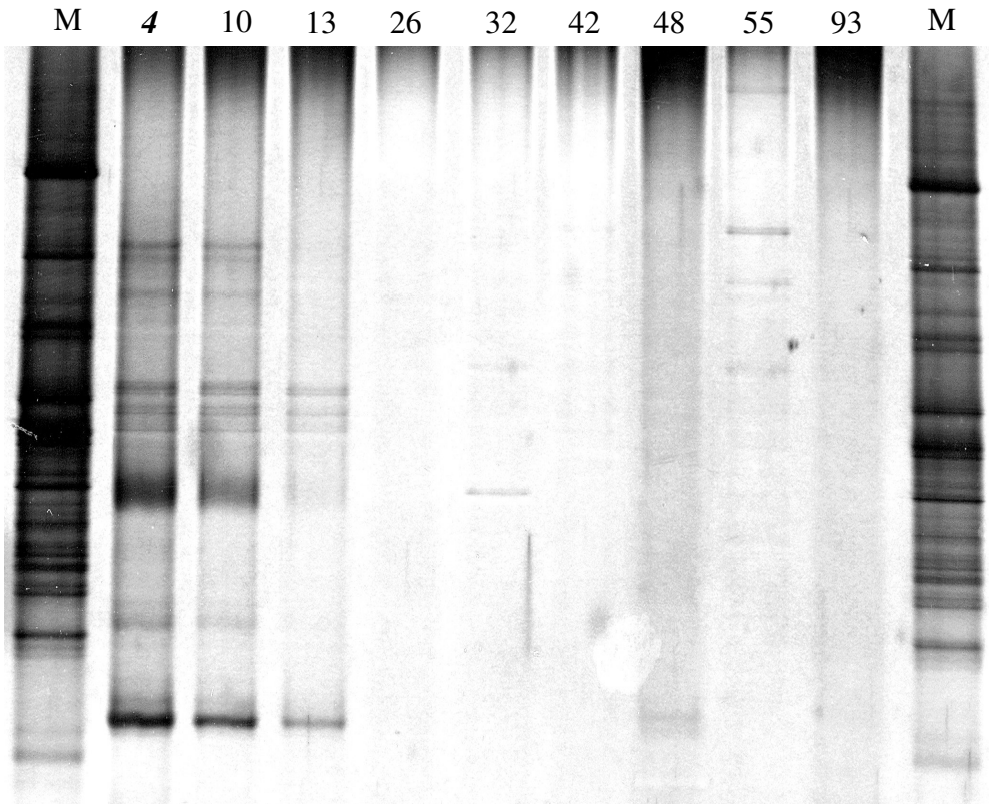


Figure C.14 : Biochemical performance (upper panel) in comparison with bacterial population dynamics (lower panel): Eerbek sludge enriched at **55°C** with the **Feed Mixture-B** (M: Original Eerbeek sludge as Marker, numbers: sampling days)

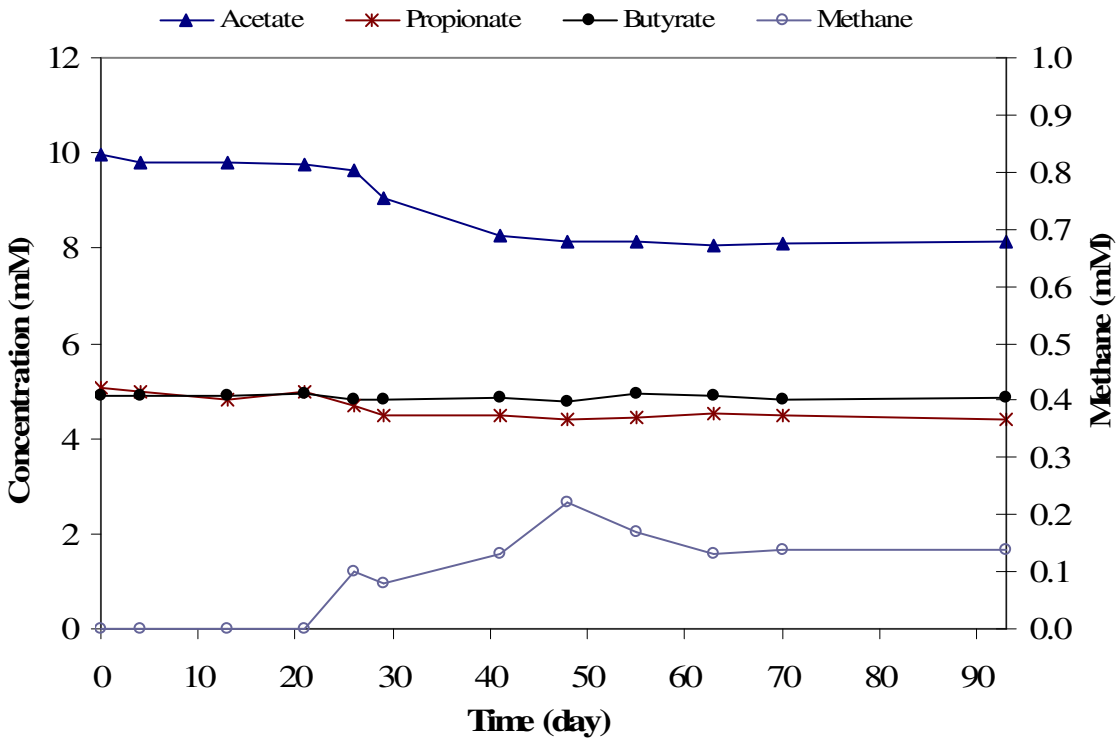


Figure C.15 : Biochemical performance of Eerbek sludge enriched at **55°C** with the **Feed Mixture-C**

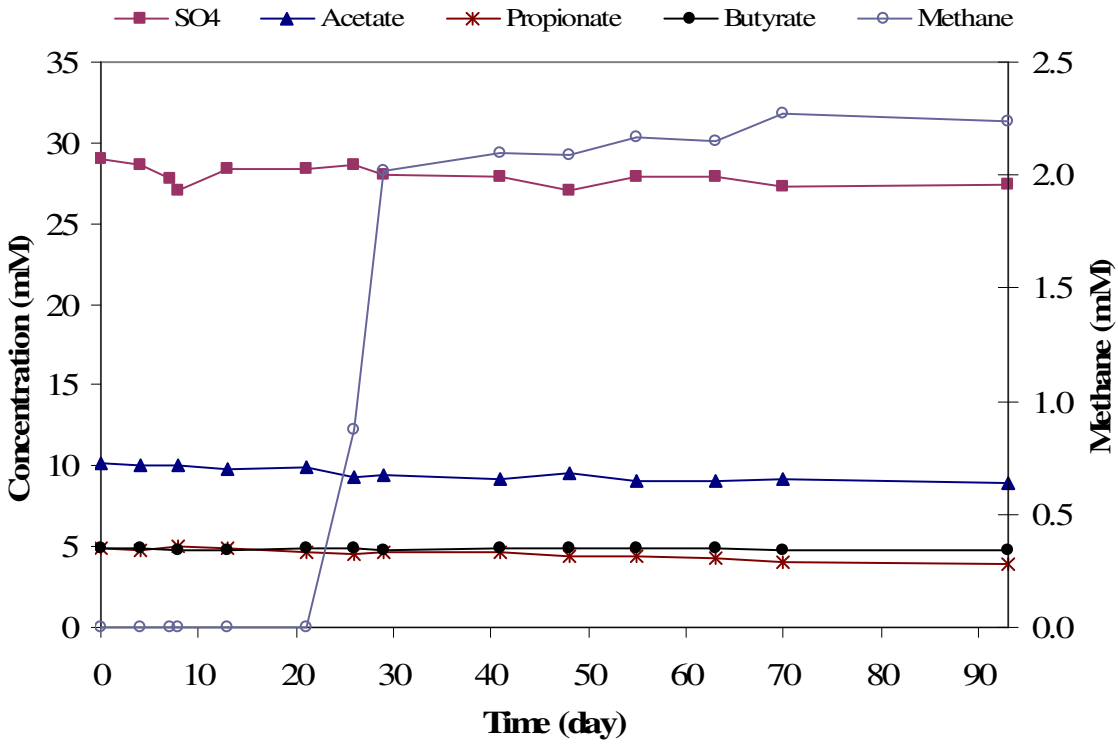


Figure C.16 : Biochemical performance of Eerbek sludge enriched at **55°C** with the **Feed Mixture-D**

CIRRICULUM VITAE

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