CHAPTER 3

MICROBIAL CELLUOSE DEGRADATION FOR OPTIMAL VFA PRODUCTION AND BIOLOGICAL SULPHATE REDUCTION

3.1 INTRODUCTION

Elevated sulphate concentrations present in mine and other industrial effluents can be treated using biological sulphate removal technology. A disadvantage of this treatment method is the high cost of the carbon and energy sources. Therefore, cheaper alternatives have to be found and investigations into the use of the fermentation products of organic wastes have been initiated (Coetser *et al*. 2000; Rose, 2000; Dill *et al*. 2001). Waste utilisation, rather than treatment, reduces waste pollution and provides a source of energy (Sonakya *et al.* 2003). The hydrolisation of organic waste products, produce soluble intermediates due to the presence of exoenzymes e.g. cellulases, amylases, proteases and lipases (Sonakya *et al.* 2001). Anaerobic degradation of organic wastes in the presence of sulphate is a complex process since the SRB compete with MB for compounds such as acetate and hydrogen, whilst AB compete for compounds like propionate and butyrate (Oude Elferink, 1998). During biological sulphate removal, SRB utilize propionate and butyrate, of which some SRB oxidize these fatty acids completely to carbon dioxide, while others oxidize butyric acid (C4) and propionic acid (C3) to acetic acid (C2). SRB can also degrade the branched and long chain fatty acids to short chain volatile fatty acids (C4, C3 and C2). Since anaerobic hydrolysing/fermenting microbes can assist in the degradation of organic material, the investigations in this chapter focussed on detecting the naturally occurring microorganisms, which produce the highest concentration of VFA to be used for biological sulphate reduction.

The aim of the studies in this chapter was to investigate whether 1) VFA can be produced from a plant biomass using naturally occurring microorganisms and 2) the acids produced can function as the carbon and energy sources for biological sulphate removal.

The following microorganisms were sourced:

1) Natural occurring cellulose degrading microorganisms attached to grass cuttings
2) Microorganisms obtained from a municipal ww treatment Anaerobic Digester
3) SRB from a sulphidogenic demonstration reactor

3.2 MATERIALS AND METHODS

3.2.1 Plant biomass

Mainly Kikuyu grass cuttings (abbreviated as GC in the following text) was obtained from the CSIR, Pretoria Garden Service. After cutting, the GC were collected and kept at 4 ℃. GC used for the experiments described in this thesis came from the same stockpile from the cold room. The size of the GC was between1-2 cm. The weight of the grass in the thesis' text refers to air dried grass, due to storage at 4 ℃ (dry weight). Kikuyu grass *(Pennisetum clandestinum)* is a low growing, deep-rooted perennial with stolons and rhizomes, and forms a dense turf, which is very resistant to heavy grazing (Partridge, 2003). When the grass is degraded by cellulose fermenting organisms, nutrients, e.g. nitrate and phosphate are released. Generally, the average nitrate (NO_3^- N) concentration in the reactor was measured at ca. 20 mg/ℓ, while the phosphate (PO_4-P) concentration averaged 25 mg/ℓ in the grass degrading reactors.

3.2.2 Microbial biomass

For the cellulose degrading study, hydrolyzing microorganisms (essentially microorganisms occurring in and attached to decaying grass), as described in *3.2.3.1,* were used. A SRB mixture obtained from the CSIR-o-Sure demo plant (Navigation Mine, Witbank, South Africa) and an anaerobic sludge mixture obtained from the Daspoort Sewage Works, Pretoria, South Africa, were used for the following fermentation studies. The CSIR demo plant is a biological sulphate removing, one stage reactor system, which treated AMD at the Anglo Coal Navigation mine from 2000-2004 (Maree *et al*. 2004). The biomass from this sulphidogenic reactor was suitable seed sludge for the sulphate containing reactors in this study.

3.2.3 Experimental

All studies described in this chapter were executed using batch test conditions. The origin of the microbial populations used in the three studies is captured in Table 3.1.

3.2.3.1 Study 1: Hydrolysis study

The hydrolysis of cellulose in grass was investigated using two reactors (G1 and G2), which comprised two 5 ℓ plastic containers open at the top. G1 and G2 contained 30 g dry GC per ℓ tapwater (150 g GC/5 ℓ tapwater). G2 contained additional naturally occurring microorganisms obtained from a previous grass hydrolysis experiment. These were obtained by settling the contents of the reactor, mainly comprising the

partly degraded GC and microorganisms and discarding the supernatant from a previously used 5 ℓ reactor. The hydrolysis experiment was conducted over 42 days at room temperature. Fresh GC (30 g each time) were added to the reactors contents (5 ℓ) on days 1, 2, 6, 7, 22, 39 and 40. Samples for VFA analysis were taken daily.

Table 3.1. Overview of the three studies in this chapter

Study	Method	Microbial population	Study reactor	Control reactor
1	Hydrolysis	Natural occurring grass microbes	Hydrolysis biomass	No addition of biomass
2	Anaerobic degradation,	Digester sludge	See Table 3.2	
3	Anaerobic degradation,	SRB	See Table 3.3	

3.2.3.2 Anaerobic degradation/ SO_4 removal study

Three anaerobic reactors (F1, F2 and F3) for which the experimental conditions are given in Table 3.2 were operated at 35 °C to evaluate the VFA production using different cellulose degrading microbes. The supernatants of the three GC fermentation reactors (F1, F2 and F3) were used in sulphate removal batch tests, for which four stirred, anaerobically operated, glass bottles (volume 2 ℓ) with rubber stoppers, B1-B4, were used. Samples were taken from the bottoms of the four reactors, through an outlet fitted with a clamp. All reactors received 250 mℓ SRB biomass mixture (obtained from the CSIR sulphate removing demonstration plant in Witbank, (VSS was 10 g/ℓ), 2 mℓ/ℓ macro and micro nutrient mixture, as well as 2 ℓ of the supernatant from the fermentation reactors (F1, F2 and F3). The carbon sources for B1-B4 are given in Table 3.3. All four reactors received sulphate rich feed water ($MgSO_4$), so that the final SO_4 concentrations in B1-B4 were approximately 1 500 mg/ℓ SO_4. Reactors B1-B4 were operated at room temperature (25 °C).

Table 3.2 The experimental conditions in F1, F2 and F3

Reactor	Conditions
F1	30 g Grass Cuttings
F2	30 g GC + 100 mℓ Daspoort anaerobic sludge
F3	30 g GC+ 100 mℓ SRB mixture

Table 3.3 The carbon sources used in the different batch reactors

Reactor	B1	B2	B3	B4
Carbon Source	1 g sucrose/ℓ	Supernatant F1	Supernatant F2	Supernatant F3

3.2.4 Analytical

The sulphate, sulphide, alkalinity, COD, and pH were determined manually according to analytical procedures as described in Standard Methods (APHA, 1985). The analyses were all carried out on filtered samples, except for the COD analysis on feed water, the redox potential and the sulphide samples. Alkalinity was determined by titrating with 0.1N HCl to a pH of 4.3. Prior to the COD measurement, the sulphide in the samples from the reactors was removed by adding a few drops of 98% sulphuric acid and flushing the sample with nitrogen. The redox potential of the samples was calculated from the mV and stabilization temperature measured with a pH/redox meter (Metrohm 744) applying the following formula:

226 - (18 x temperature of reactor contents/25) = Value

Redox potential = Value + mV measurement of sample, where

226 is a Constant.

All VFA analyses were done using a gas chromatograph (Hewlett Packard. HP 5890 Series II) equipped with a flame ionisation detector (GC/FID), while the data analyses were done using the Chem Station (Hewlett Packard, software package). The column used was an HP-FFAP, 15 m x 0.530 nm, 1 micron. The N_2 flow rate was set at 1 mℓ/min. An outline of the GC/FID programme used is depicted in Table 3.4.

Table 3.4. The GC/FID programme for the detection of VFA

Parameter	Setting
Initial oven temperature (℃)	30
Initial time (Min)	2
Temperature programme: (℃)	80
Rate (℃/min)	25
Final temperature (℃)	200
Final time (min)	1
FID temperature (℃)	240

3.3 RESULTS AND DISCUSSION

3.3.1 VFA production from grass-hydrolysis by natural occurring microorganism on grass

The VFA concentrations (acetic, propionic and butyric acids) in the two hydrolysis reactors, G1 and G2, are given in Figures 4.1 to 4.3.

3.3.1.1 Acetic acid

The acetic acid concentrations in G1 and G2 were similar (Figure 3.1), showing that the addition of supplementary naturally occurring hydrolytic bacteria had no influence on the acetic acid production. Fresh GC were added on days 1, 2, 6, 7, 22, 39 and 40, after which no remarkable increase in the acetic acid concentration was observed. From day 22-39, no GC were added, which resulted in a decrease in the acetic acid concentration, which increased slightly on days 39 and 40 when fresh GC were added to the reactor.

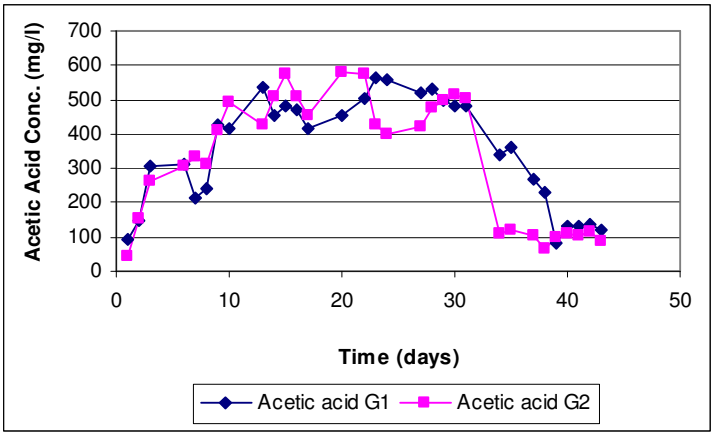

Figure 3.1. Acetic acid production in reactors G1 and G2.

3.3.1.2 Propionic acid

The propionic acid concentration in G1 increased from day 1-14 (Figure 3.2), whereafter it decreased to concentrations < 100 mg/ℓ up to day 22, on which day fresh GC were added to the reactor. The propionic acid concentration in G1 remained stable thereafter till day 31, after which it decreased. It increased again after day 34, to increase sharply on days 39 and 40, when fresh GC were added, indicating that the naturally grass occurring microbes degraded GC to propionic acid. The propionic acid in G2 followed a similar pattern as that in G1, except between day 28-38. The overall propionic acid concentration was higher in G2. This result

indicated that the addition of the natural cellulose degrading microorganisms resulted in a small increase in C3 acid concentration. The average propionic acid concentration in G1 was 129 mg/ℓ, while it was 193 mg/ℓ in G2, which was an improvement of 30%. This result indicated that the addition of hydrolytic microorganisms resulted in an increase of microorganisms. The results indicated that an increased microbial population proved beneficial for additional propionic acid production. Since SRB prefer propionic acid above acetic acid as the electron donor, this information is valuable as it showed that natural occurring microorganisms on grass can degrade to VFA , especially propionic acid.

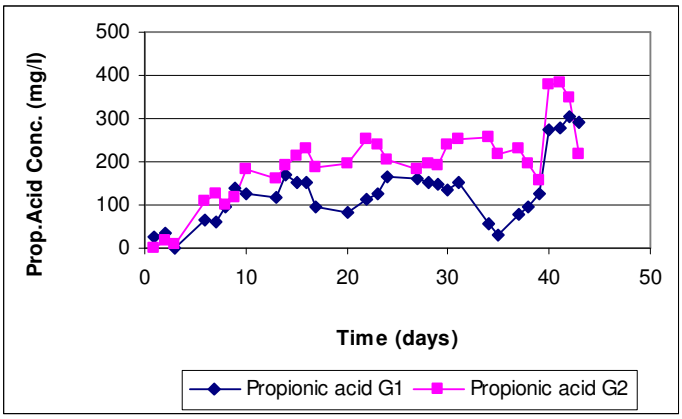

Figure 3.2. Propionic acid production in G1 and G2

3.3.1.3 Butyric acid

The results of the butyric acid production experiment are given in Figure 3.3. The butyric acid concentration in G2 was slightly higher than in G1, as was observed for propionic acid formation. The average butyric acid production in G1 during the total experimental period was 54 mg/ℓ, while it was 69 mg/ℓ in G2, which was 22% higher than in reactor G1. As in the case of the propionic acid production, the addition of the natural cellulose degrading microorganisms attached to grass appeared advantageous as it resulted in a slightly improved butyric acid production. It was noted, however, that only low concentrations of the acids were produced. The rate and extent to which a substrate is hydrolyzed is influenced by the accessibility to the substrate by the exoenzymes (Hobson, 1982). This is especially true for the anaerobic digestion of fibrous materials, in which the cellulosic and hemicellulosic microfibrils are aggregated and embedded within the liquefied cell wall matrix. The crystallinity and surface area of the fibres are the most important features which determine the accessibility for exoenzymes (Fan *et al.* 1980). In the hydrolysis step of the total degradation process, the particle size of the organic waste product influences the speed of the hydrolysis, due to the increased accessibility for the

exoenzymes. It seemed likely that when cellulose degrading microorganisms were adapted to the fermentation of the cellulose fibres in GC and subsequently added to the hydrolyzing reactors, it resulted in a small increase in C3 and C4 acid production (30 and 22%, respectively). This finding indicated that cellulose degrading microbes attached to grass naturally ferment cellulose to VFA, especially propionic and butyric acids, albeit in lower concentrations than the acetic acid concentration.

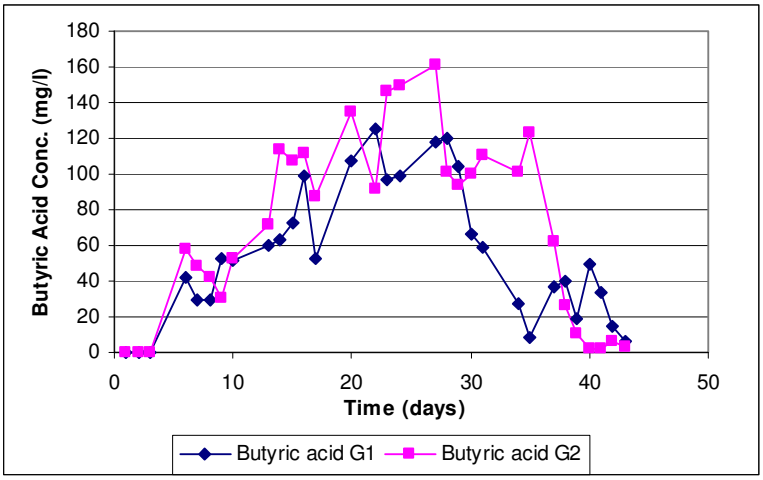

Figure 3.3. Butyric acid production in G1 and G2.

3.3.2 Effect of cellulose degrading anaerobic microorganisms on the VFA production, followed by SO_4 reduction

3.3.2.1 VFA production

The total VFA concentrations in F2 and the butyric acid concentrations in F1-F3 are given in Figures 3.4 and 3.5, respectively. The graphs in Figure 3.4 show the VFA production in F2 and those in Figure 3.5 show the butyric acid production in all three reactors during the first 80 hours of the experiment. It was observed from Figure 3.4 that the acetic acid concentration in F2 increased between 22-56 h and that it decreased thereafter, most likely due to methane production. The propionic acid concentration in F2 was the lowest. The graphs in Figure 3.5 show that the butyric acid production followed the same pattern in all three reactors during the first 50 h. After 72h, the butyric acid concentration was the highest in F2. This finding confirmed the result of Sonakya *et al.* (2003), who reported that the butyric acid concentration was higher than that of the C2 and C3 acids, when measured over the same period.

Zoetemeyer *et al.* (1982) showed that the degradation process depends on environmental conditions. When fermenting glucose in a separate, acid producing reactor, the main products were butyric acid, acetic acid, hydrogen and carbon dioxide. However, when interrupting the feed supply for a period of 1 – 24 h, the fermentation pattern changed to an increased production of propionic and acetic acid. They furthermore showed that at pH values < 6, the main fermentation product of glucose was butyric acid, and when the pH was increased, the product pattern changed, to lactic and acetic acid and to formic acid and ethanol. The product pattern of the fermentation also depends on the type of organic waste (Cohen, 1983). Wolin (1976, 1979) demonstrated the importance of hydrogen production and utilisation in the fermentation reactor. Removal of hydrogen, e.g. by the hydrogenotrophic bacteria such as hydrogen consuming SRB (HSRB), can influence the kinds of products formed by the fermentative bacteria. When hydrogen is consumed by the HSRB or HMB, the fermentative bacteria can produce further oxidized products than they otherwise would do at increased hydrogen levels, which supply more energy per unit of substrate to the bacteria. This finding indicates that when syntrophic bacteria, such as HSRB, are present in the reactor system, keeping the hydrogen partial pressure low, other organisms use the electrons generated in the fermentation process, for hydrogen production rather than for the production of ethanol (Reddy *et al.* 1972). This observation is of importance when using the fermentation products of cellulose for the biological reduction of sulphate in the fermentation tank, when sulphate and SRB are present, since SRB use hydrogen as energy source for sulphate reduction. Harmsen (1996) showed that SRB can participate in the degradation of organic material, to produce propionic acid, even when no sulphate is present.

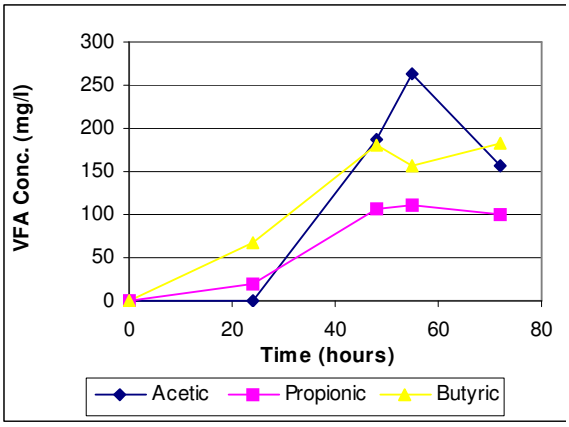

Figure 3.4 The VFA production in F2

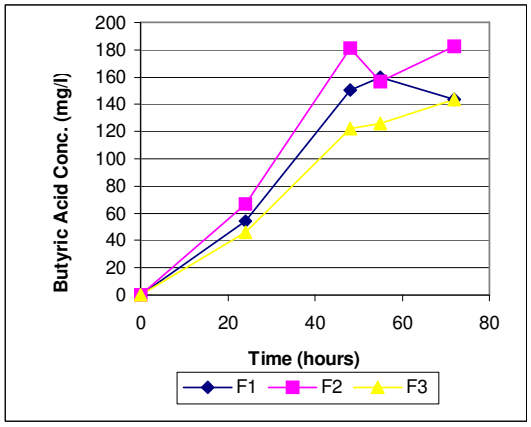

Figure 3.5 Butyric acid production in F1, F2 and F3

3.3.2.2 Sulphate reduction and sulphide production

The sulphate removal and sulphide production using sucrose (control) and the VFAs produced (from reactors F1-F3) are given in Figures 3.6 and 3.7. The results showed that the sulphate reduction rates in all four reactors B1-B4 were similar. However, when linear regression was applied to the four graphs, the concentrations of SO_4 removed were 279, 287, 266 and 349 mg SO_4/d for B1-B4, respectively. These results indicated that during the degradation process in the fermentation reactors (F1-F3), the most favourable fermentation products for sulphate removal were produced in F3, the reactor containing GC and the SRB biomass mixture. The sulphate reduction using sucrose (two step reaction) is given in equation 3.1 and for propionate and butyrate in 3.2 and 3.3, respectively:

$$C_{12}H_{22}O_{11} + 5H_2O \rightarrow 4CH_3COOH + 4CO_2 + 8H_2 \quad (3.1)$$

$$8H_2 + 2SO_4^{2-} + 2H^+ \rightarrow 2HS^- + 8H_2O$$

$$C_{12}H_{22}O_{11} + 2SO_4^{2-} + 2H^+ \rightarrow 4CH_3COOH + 4CO_2 + 2HS^- + 3H_2O + H_2O$$

$$CH_3CH_2COO^- + \tfrac{3}{4}\, SO_4^{2-} \rightarrow CH_3COO^- + HCO_3^- + \tfrac{3}{4}\, HS^- + \tfrac{1}{4}\, H^+ \quad (3.2)$$

$$CH_3CH_2CH_2COO^- + \tfrac{1}{2}\, SO_4^{2-} \rightarrow 2\, CH_3COO^- + \tfrac{1}{2}\, HS^- + \tfrac{1}{2}\, H^+ \quad (3.3)$$

Equations 3.1-3.3 show that acetate is the degradation product of the biological sulphate reduction of most carbon and energy sources. It is considered the most recalcitrant VFA (Lens *et al.* 1998; Vallero *et al.* 2003) and the rate limiting factor (Visser *et al.* 1993) in a sulphidogenic reactor and usually represents a final COD concentration of 200-500 mg/ℓ (Greben *et al.* 2000).

The highest sulphide concentration was obtained in the control reactor B1, followed by reactor B4, containing the carbon and energy source produced in F3.

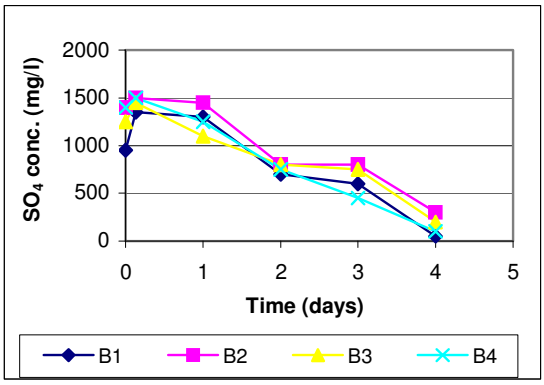

Figure 3.6 Sulphate reduction in B1-B4

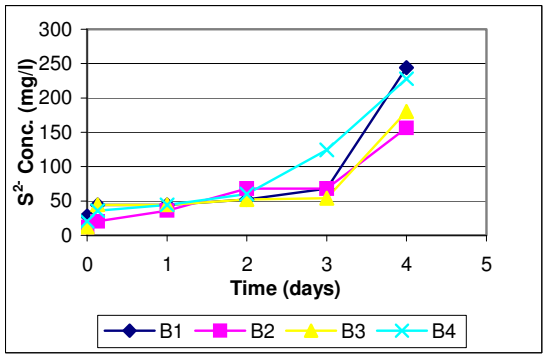

Figure 3.7 Sulphide production in B1-B4

3.3.2.3 VFA utilisation in reactors B1 to B4.

The VFA concentrations in B1 to B4 are given in Figures 3.8 to 3.11

3.3.2.3.1 Reactor B1

The graphs in Figure 3.8 showed that on day 0, acetic, propionic, butyric and valeric acids were present in B1, but that on days 1-3, most of the acids had been utilized, except for acetic acid. When glucose is fermented by the SRB, the final product is H_2, with many intermediate products, such as organic acids. Butyric and propionic acids can be used by SRB as the carbon and energy source. O'Flaherty *et al.* (1998) studied the population structure of biomass from a full-scale anaerobic reactor after 5 years of operation. The results showed that the SRB incompletely oxidised propionate to acetate. The SRB produce four moles of acetate from four moles of propionate for the reduction of three moles of sulphate. It was observed that the SRB and MB competed for butyrate and ethanol. It was suggested that in the presence of sulphate, compounds, such as alcohols, lactate, propionate and butyrate, may be oxidized directly by the SRB (Oude Elfering, 1998). The acetic and propionic acid concentrations had increased in the reactor by day 4 (Figure 3.8). Figure 3.6 showed that the SO_4 concentration decreased to <100 mg/ℓ, indicating that no further COD (VFA) was required by SRB in B1, thus the residual COD (1 394 mg/ℓ) in the reactor consisted mainly of acetic acid and propionic acids (Figure 3.8), higher fatty acids, alcohols and other organic matter.

3.3.2.3.2 Reactor B2

Reactor B2 contained the supernatant from F1, the reactor to which no additional microorganisms had been added. The graphs in Figure 3.9 showed that the C3, C4 and C5 acids were utilized by the SRB, producing the C2 acid as was also shown for reactor B1.

3.3.2.3.3 Reactor B3

Reactor B3 was operated on the supernatant from F2 (GC + Daspoort anaerobic biomass). The results for the VFA concentrations in B3 are given in Figure 3.10, while the graphs in Figure 3.11 show the VFA concentration in B4. The graphs in Figure 3.10 show that on day 2, a high concentration of propionic acid was present in B3, which decreased to zero on day 4. Usually, when propionic acid is utilised, the acetic acid concentration increased. The low acetic acid concentration in B3 may indicate that the acetic acid was utilised by the MB present in the anaerobic sludge obtained from Daspoort. A marginal increase in the butyric acid concentration from days 2-4 could be seen. It seems that when the propionic acid concentration decreased, the butyric acid slowly increased. This finding may indicate that when SRB used propionic acid, the butyric acid increased in the reactor.

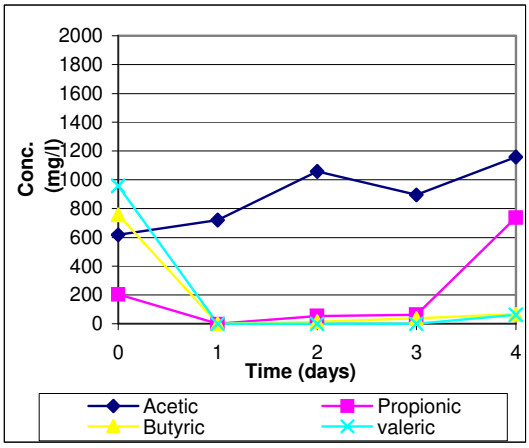

Figure 3.8 The VFA concentration in B1

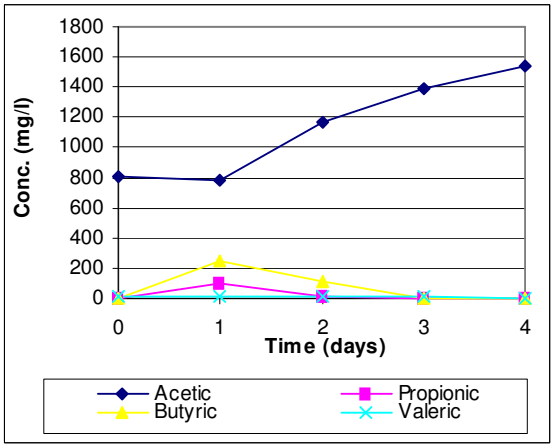

Figure 3.9 The VFA concentration in B2

3.3.2.3.4 Reactor B4

From Figure 3.11, it was seen that initially propionic acid, acetic acid and valeric acids were present in the reactor at concentrations ca. 1000, 700 and 450 mg/ℓ, respectively, which decreased to practically zero on day 1 and subsequently all

available VFA were used up during the experimental period. As soon as the butyric acid was produced (days 1-2), it was utilized (days 2-3). The graphs in Figure 3.11 furthermore showed that the acetic acid concentration decreased from a concentration of > 500 mg/ℓ to values < 100 mg/ℓ. This finding did not correspond with the result obtained from reactors B1 and B2, where an overall increase in the acetic acid concentration was observed. Acetic acid utilisation was observed in B3 as well. The acetic acid degradation in B3 and B4 can possibly be ascribed to the fact that during the fermentation process, bacteria from the SRB mixture were present in the fermentation tank F3, while the supernatant of F2 contained the sludge from the Daspoort anaerobic digester. The MB (present in the Daspoort sludge) most likely degraded the acetate to methane gas in B3, while the SRB in B4 may have used the acetate for further sulphate removal in the absence of the C3 and C4 acids. No gas analyses were conducted to substantiate this theory.

No acetic acid reduction was observed in reactor B1 (glucose reactor) and B2 (supernatant F1: containing only GC) to which no additional bacteria had been supplemented. Omil *et al*. (1996) reported that from a thermodynamic and kinetic point of view, the SRB out-compete the MB on hydrogen, acetic, propionic and butyric acids. Hydrogen is completely consumed by SRB, while propionic and butyric acids are used faster by SRB than by syntrophic consortia. For acetate, the sulphidogenic acetate conversion has been found predominant in marine and freshwater sediments and in mixed reactors, as was the case in this study. The SRB will use hydrogen, propionic- and butyric- and acetic acid as electron donors in that order. Thus, it can be assumed that when no other substrates are available, SRB will utilise acetate. ASRB are slow growers, but when no other substrate is available, SRB will use acetate. Generally, several factors can affect the acetate utilisation by SRB or MB, such as type of substrate and inoculums used (as in this study), reactor pH and temperature and immobilisation properties (Oude Elferink, 1998).

Omil *et al.* (1996) showed that an acetate rich mixture favoured the MB, even when the reactor was started up with sulphidogenic sludge, while a propionate and butyrate rich mixture promoted the SRB. This finding indicated that even when sulphate adapted sludge is used, as in the case of F3, MB may still be present, which can explain the utilisation of the acetate in B4.

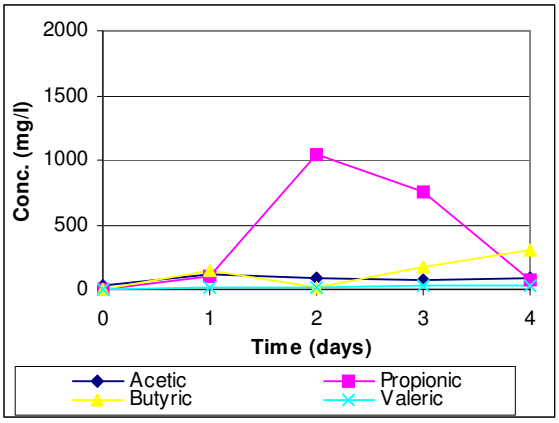

Figure 3.10 VFA concentrations in B3

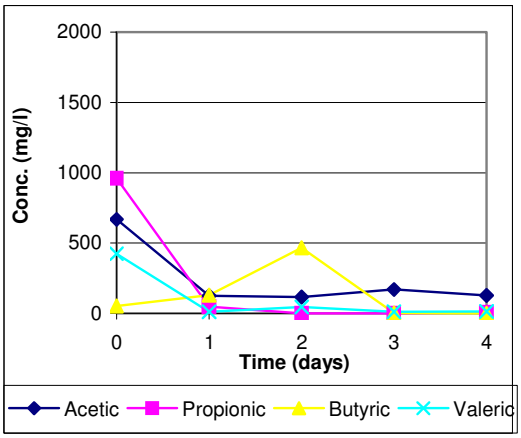

Figure 3.11 VFA concentrations in B4

3.4 CONCLUSIONS

It was concluded from these studies that VFA production from GC was observed. The results showed that VFA production occurred in a hydrolysis tank, using naturally occurring grass-degrading microorganisms. After adding extra hydrolysing bacteria during the hydrolysis process, the C2 and C3 acid concentrations increased by 30 and 22%, respectively. When using cellulose-degrading microorganisms from anaerobic digester sludge and sulphate adapted biomass, acetic acid was produced in the highest concentration (almost 600 mg/ℓ) as opposed to propionic acid at an average of 250 mg/ℓ and butyric acid at about 160 mg/ℓ.

Using the SRB biomass mixture in the fermentation process resulted in butyric acid production at a higher concentration and at a faster rate than when the bacteria from the anaerobic digester were used. This finding showed that SRB can produce VFA, even when no sulphate is present. When using the supernatant from the fermentation tanks, containing the VFA produced, for the removal of sulphate in a second reactor, sulphate reduction was observed over a period of 4 days. A slightly better sulphate reduction (349 mg SO_4/d) was obtained using the supernatant from the fermentation reactor containing GC and the SRB biomass mixture than when using sucrose in the control reactor as the carbon and energy source (279 mg SO_4/d). The VFA concentrations in the four sulphate reducing reactors showed that in the control (sucrose) reactor and in the reactor receiving the supernatant of the fermentation tank without additional biomass, the acetic acid concentration increased, indicating that the acetic acid was not utilized for the biological sulphate removal process. No residual acetic acid was measured in the reactors which received the supernatant

from the reactors to which the anaerobic biomass mixtures had been added. These results appeared to indicate that the MB used the available acetate to produce methane or, alternatively, other bacteria (e.g. AB) used the acetate to produce propionate thus providing the SRB with more substrate for subsequent sulphate reduction. It is possible that in absence of other substrates, acetate is used by the SRB as carbon and energy source.

Although all described microorganisms produced VFA, which could be used for subsequent sulphate reduction, it was observed that the concentrations especially of propionic and butyric acids were relatively low. The focus in the following chapter was therefore on the comparison between using SRB and rumen fluid microbes, natural cellulose degrading microorganisms, for VFA production.

3.5 REFERENCES

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