CHAPTER 4

THE EFFECT OF INCREASED GRASS CONCENTRATION ON THE VFA PRODUCTION AND SUBSEQUENT SULPHATE REDUCTION USING SRB AND RUMEN FLUID AS FERMENTATION INOCULA

4.1 INTRODUCTION

Cellulose is the major constituent of plant biomass, forming an important component in the carbon cycle. The formation of cellulose can be ascribed to photosynthesis and the CO_2 supply in the atmosphere (0.036%). The carbon cycle is closed as a result of the cellulose utilizing microorganisms present in soil and the guts of animals (Lynd *et al*. 2002). Plant biomass is a sustainable source of energy when cellulose is utilised during anaerobic degradation, resulting in the production of VFA and other degradation products. This process involves many species of bacteria, such as the AB and the MB. The SRB also play a role in the degradation of the complex polymers in the presence of sulphate (Oude Elferink, 1998). Greben and Baloyi (2004) showed that the anaerobic degradation of plant biomass (grass) to VFA could be enhanced when sulphate-adapted biomass was added to the fermentation process, even when no sulphate was present. This outcome indicated that the SRB participated in the degradation of the polymers and monomers to produce VFA. The utilisation of propionic acid in the absence of sulphate was shown by Harmsen (1996).

Fermentation of cellulose also occurs in the rumen of the ruminants. These are herbivorous mammals that possess a special organ, the rumen, within which the digestion of cellulose and other plant polysaccharides occurs through the activity of special microbial communities (Barnes and Keller, 2003, 2004). The energy containing components (carbohydrates, such as cellulose) in the ruminant feed are, during the fermentation process, converted into microbial cells and compounds such as CO_2, CH_4, and acetic, propionic and butyric acids, of which the acids are utilized by the host. The rumen is inhabited by between 10^{10}-10^{11} bacteria and 10^6 protozoa per mℓ rumen fluid. The rumen houses a complex ecosystem where microorganisms live in symbiotic relationships that facilitate fibre digestion. Therefore, it seemed likely that anaerobic degradation of plant material may be executed more efficiently using the bacteria, fungi and protozoa occurring in the rumen (Lee *et al*. 2000). Recent work published by Sonakya *et al*. 2003 demonstrated this concept with the use of digested cattle feed for the production of VFA from grass cuttings.

The results in the previous chapter showed that VFA production was possible from the cellulosic components of plant biomass (GC) using different inocula. However, the results also indicated that the VFA production was low when using the SRB as fermentation microorganisms. In order to achieve higher VFA concentrations it was decided to use grass-cellulose fermenting organisms, obtained from rumen fluid. It also appeared likely that higher grass concentrations could result in improved VFA production. The following studies were conducted with the aim of producing high concentrations of VFA, which subsequently would result in enhanced sulphate removal rates.

The first objective of the study was to investigate whether an increase in GC concentration would result in an increased SO_4 removal rate.
The second objective was to compare the production and utilisation of VFA obtained from grass cuttings (GC) when bacterial communities obtained from
1) Biological sulphate removal systems and
2) Rumen fluid of sheep alone and combined with SRB
were added to the fermentation reactors, containing GC, tap- and SO_4 rich water.
The third objective was to investigate the conditions under which the rumen bacteria would produce VFA consistently for sustained biological sulphate reduction.

4.2 MATERIALS AND METHODS

In order to achieve the above mentioned objectives, three different studies were conducted. Study 1 investigated whether the highest grass concentration would result in the highest VFA production and thus in the highest sulphate removal rate, using an SRB community as the added fermentative microorganisms. During Study 2, the VFA production and utilisation was compared, using microorganisms originating from the rumen (RB) of sheep as well as a combination of RB and SRB as the cellulose degraders. During Study 3, different reactor conditions affecting the VFA production and subsequent sulphate reduction were investigated, using RB as the sole cellulose degrading microorganisms. In the first reactor sulphate rich water was used to which RB were added. In the second reactor tap water and RB were mixed, while tryptone was added to the third reactor, since tryptone can stimulate propionic acid production using RB (personal communication, Professor P.J. Weimer, Dairy Forage Research Center and Department of Bacteriology, Madison, Wisconsin, USA).

4.2.1 Study 1

4.2.1.1 Experimental

This study was carried out under anaerobic conditions (closed to the environment, Dissolved Oxygen concentration of 0 mg/ ℓ) in three 2.5 ℓ Perspex batch reactors: T30, T60 and T90. To each reactor 250 mℓ mixture of SRB (VSS: 10 g/ℓ) obtained from the CSIR Demo plant in Witbank, was added as the fermentation and sulphate removing inocula. All three reactors contained sulphate rich water, made up of Na_2SO_4 (Merck), to which macro and micro nutrients (1mℓ/ℓ) were added (Table 4.4). When the SO_4 concentration in the reactors approached zero, fresh SO_4 solution was added to the reactor (indicated by arrows in the figure), to monitor further SO_4 removal. Different GC concentrations: 30, 60 and 90 g/ℓ in sulphate rich water were used. No fresh GC was added during the experimental period of 42 days. This investigation was conducted at room temperature (25 ˚C). The pH of the reactors was maintained at 7.0-7.5. The experimental conditions are given in Table 4.1.

Table 4.1. Experimental conditions for Study 1

Parameter	**Reactors**		
	T30	**T60**	**T90**
GC (g/ℓ)	30	60	90
SO_4 concentration (mg/ℓ)	1600	1700	1600

4.2.2 Study 2

4.2.2.1 Experimental

Four 500 mℓ batch reactors (R1-R4) were used as the fermentation reactors. R2 and R3 contained 25 mℓ rumen fluid, obtained from fistulated sheep at the University of Pretoria, while R1 and R4 contained a mixture of SRB and RB (12.5 mℓ each). When collecting the rumen fluid from the University, it was transported in a closed vessel, placed in a bucket of warm water (body temperature) and stored in an incubator (37-39 ℃) upon arrival at the CSIR laboratories. It has to be taken into account that shifts in microbial composition of the rumen fluid will occur continuously, due to the "foreign" conditions in the storage vessel as well as in the bio-reactors.

R1 and R2 contained tap water, while sulphate rich water was used in R3 and R4. All four reactors (Vol.: 450 ml) contained 30 g GC (≈ 60 g GC/ℓ). The experimental conditions of Study 2 are given in Table 4.2. The reactors were shaken in an incubator at 39 °C. The pH in the reactors was maintained between 6.6-6.9, to ensure the optimum conditions for the rumen microorganisms. The experimental period was 53 days.

Table 4.2. Experimental conditions of Study 2

Reactors	SO_4 rich water	Tap water	RB	SRB
R1		X	12.5 mℓ	12.5 mℓ
R2		X	25 mℓ	
R3	X		25 mℓ	
R4	X		12.5 mℓ	12.5 mℓ

4.2.3 Study 3

4.2.3.1 Experimental

Three batch reactors (Vol. 2.5 ℓ) were used: L1, L2 and L3. The experimental details are given in Table 4.3. The duration of Study 3 was 25 days.

Table 4.3. Experimental conditions of Study 3

Reactor	Contents
L1	1500 mg/ℓ SO_4 + 30 g/ℓ GC + 250 mℓ RB + nutrients (Table 4.4)
L2	Tap water + 30 g/ℓ GC + 250 mℓ RB + nutrients
L3	Tap water + 30 g/ℓ GC + 250 mℓ RB + 2.5 g tryptone + nutrients

Table 4.4. The chemical composition of the nutrient solution

MACRO NUTRIENTS	MICRO NUTRIENTS
6.5% N ,2.7% P, 13.0% K, 7.0% CA, 2.2% MG , 7.5 % S	0.15% FE, 0.024% MN, 0.024% B, 0.005% ZN, 0.002% CU , 0.001% MO

4.2.4 Analytical

The same analytical procedures as described in Chapter 3 (3.2.4) were followed.

4.3 RESULTS AND DISCUSSION

4.3.1 Study 1. The use of SRB as fermentative and SO_4 removing bacteria

4.3.1.1. Sulphate reduction

Reactor T30

The graphs in Figure 4.1 showed that from days 1-8 sulphate removal occurred and that the sulphate concentration was < 50 mg/ℓ after 8 days. During this period, the propionic and butyric acid concentrations in the reactor were too low to be measured, which indicated that as soon as the VFA were produced they were utilised by the SRB. Generally, when propionic acid is utilised by SRB as the carbon and energy source, acetic acid is produced. The results in Figure 4.1 confirmed the production of acetic acid up to day 15. When the sulphate concentration decreased to < 50 mg/ℓ, fresh sulphate was added to the reactor (Day 8: *arrow*). From days 9-13 the sulphate reduction continued. However, during the following period, the sulphate concentration in the reactor increased, for which no explanation can be given. The decrease in propionate concentration coincided with cessatation of sulphate removal. The graphs in Figure 4.1 showed that no propionic acid was available in the reactor and only a small amount of butyric acid, which most likely resulted in no further sulphate reduction.

Reactor T60

Similarly, as in T30, sulphate removal could be observed during the first 8 days of operation in reactor T60. During that period, the butyric and propionic acid concentrations were very low, while the acetic acid concentration (oxidation product of the propionate utilisation) increased. On day 8, fresh sulphate was added (arrow), which was initially reduced during days 9-14 to 500 mg/ℓ, whereafter sulphate reduction ceased. No further sulphate reduction was ascribed to the low butyric and propionic acids concentrations in the reactor.

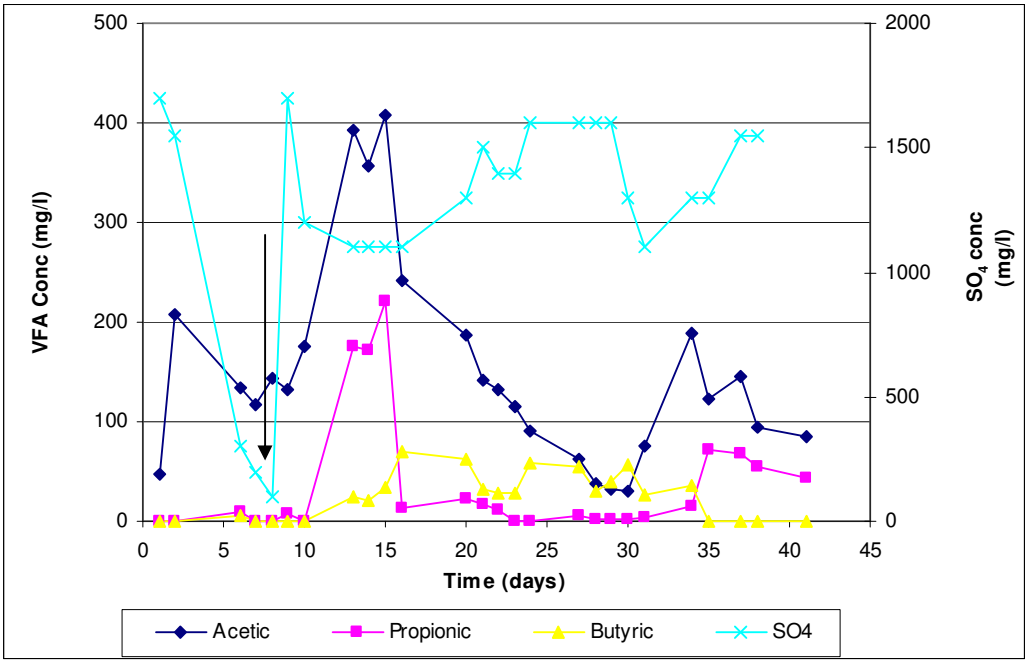

Figure 4.1. The SO_4 reduction and VFA pattern in T30.

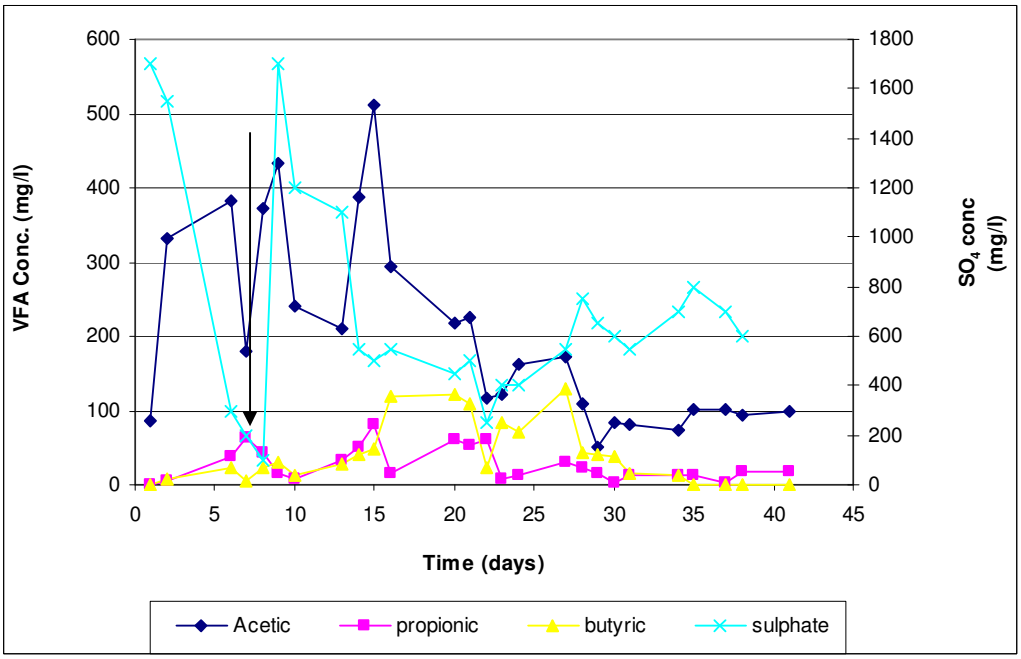

Figure 4.2. The SO_4 reduction and VFA pattern in T60.

When evaluating the VFA concentrations and extents of sulphate reduction in T30 and T60, it was evident that the sulphate reduction could be maintained over a longer period in T60 due to the higher concentrations of the C3 and C4 acids. Furthermore, it was noted that the acetic acid concentration in T60 was higher than in T30. This

result indicated that when sufficient C3 and C4 acids were present in the reactor to reduce the available sulphate load, no acetic acid was used by the SRB. It is interesting to note that as soon as fresh sulphate solution was added to the reactor, the VFA, as well as the SO_4 concentration decreased, showing that the SRB indeed utilised the available VFA produced from the GC for sulphate reduction. After day 30, the sulphate reduction stopped, due to the low propionic and butyric acid concentrations.

Reactor T90

The graphs in Figure 4.3 showed that sulphate reduction in T90 occurred during the first few days of operation and that the VFA production was higher than its utilisation. When fresh sulphate was added to the reactor (Day 8, *arrow*), the sulphate concentration decreased, but not as rapidly as during the first week. This was ascribed to the lower propionic acid concentration in the reactor (from day 16 onwards). The acetic acid concentration decreased when both the sulphate and propionic acid concentrations decreased in the reactor. On day 22 (*arrow*), a fresh supply of sulphate was added to the reactor, which was reduced during the subsequent period (up to day 34), coinciding with a very low concentration of propionic acid in the reactor. During days 22-34 the butyric acid and the acetic acid concentrations decreased in the reactor, which may indicate that the SRB used these substrates for their respiration because of the low propionic acid concentration. In some instances the homoacetogenic bacteria, which normally produce acetate, using H_2 and CO_2, can also produce butyric acid from 2 molecules of acetic acid. It can be assumed that symbiotic interactions between the different microorganisms occurred in the reactor, when SRB require a carbon source to reduce the available sulphate.

Comparing the experimental results obtained from operating T30, T60 and T90, it was observed that when the initial concentration of GC was 30 g/ℓ, all VFA produced were utilized by the SRB, including acetic acid. When, however, the GC concentration was increased to 60 g/ℓ, the sulphate reduction was faster, but not all VFA produced were utilised. When 90 g/ℓ GC was added to the reactor, the sulphate reduction was initially faster and more consistent. It seemed that a shift in the utilisation of the different acids occurred. Initially sufficient C3 and C4 acids were present. After the propionic acid had been utilised, the SRB started utilising the butyric and even the acetic acid. From the graphs in Figures 4.1-4.3, it was noted that the reactor containing the highest concentration of GC (90 g/ℓ) formed the highest concentration of acids and the sulphate removal rate was the highest, e.g.

1500 mg/ℓ sulphate being removed within 5 days in T90, while not all VFA were utilised. This result showed that the SRB utilised the VFA selectively, i.e. the C3 before the C4 acid. This observation is in agreement the findings of Harmsen (1996) and Harada (1994). When enough GC were added to the fermentation reactor, adequate amounts of VFA were produced to reduce the available sulphate in a short time.

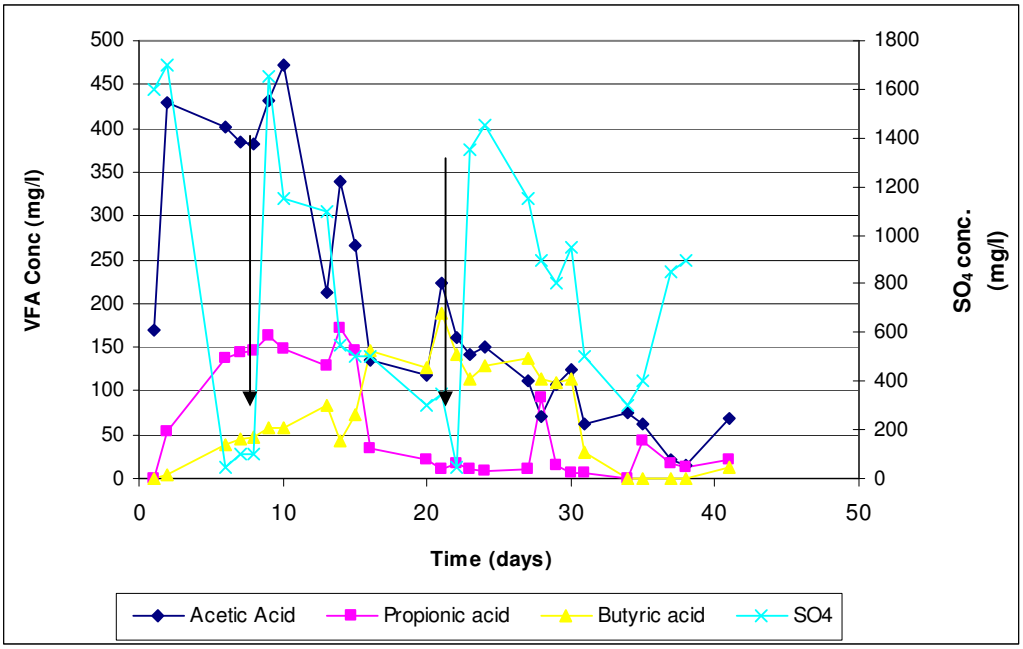

Figure 4.3. The SO_4 reduction and VFA pattern in T90.

4.3.1.2 VFA concentration

The C2, C3 and C4 acid concentrations in T30, T60 and T90, respectively, are given in Figures 4.4-4.6.

4.3.1.2.1 Acetic acid

The acetic acid (C2) production in Reactors T30, T60 and T90 followed a similar pattern: the C2 acid concentration increased during the first two weeks (up to day 15) of the experimental period, whereafter it decreased. This increase in the acetic acid concentration correlated with the sulphate reduction. When the SRB utilised the propionic and butyric acids to reduce the sulphate, acetic acid was produced. The average C2 concentrations from day 1-15 were 214, 312 and 329 mg/ℓ, in T30, T60 and T90, respectively. These results indicated that when the GC concentration was doubled from 30 to 60 g/ℓ, the average acetic concentration did not increase by the same factor. When the GC concentration was increased to 90 g/ℓ, the acid production

hardly increased any further. This irregular acetic acid production/utilisation from an increased concentration of GC can possibly be ascribed to the utilisation of acetic acid by the SRB in the T30 reactor and/or by another microbial population, such as the MB, producing methane gas. Gas production was not measured, though gas production was observed in the reactor during the experimental period.

4.3.1.2.2 Propionic acid

The C3 acid concentration in T30 was low during the first few days of operation, which was in agreement with the sulphate reduction during the first 8 days. After day 10, the propionic acid in reactor T30 increased untill day 15, to decrease thereafter at the same rapid rate. Sulphate reduction was observed in T30 between days 1-8 and days 9-15. The sulphate concentration stabilised after day 15, which can possibly be ascribed to the low propionic acid concentration in T30. No further C3 acid production was observed, except after day 35, despite no fresh GC having been added. However, during that time, butyric acid production decreased and the increase of the C3 acid was ascribed to the possible degradation of butyric acid by microorganisms other than SRB. The propionic acid concentration in T60 was stable at about 50 mg/ℓ for several days, whereafter it decreased. The propionic acid concentration correlated with the sulphate reduction in T60. It was noted that the highest C3 concentration occurred in the reactor together with the highest GC concentration (T90) and that, especially during the first 15 days, a high propionic acid concentration was noticed, even though sulphate was being reduced concomitantly. Barnes and Keller (2003) indicated that an increase in the propionic acid concentration is related to overloading of the reactor. They noted that build-up of cellulose resulted in a significant change in fermentation stoichiometry pattern. The higher concentration of grass-cellulose, when 90 g/ℓ GC were added to the reactor, may have resulted in the high propionic acid concentration from day 0-15 in T90.

The sulphate reduction was of such nature that on days 8 and 22 a fresh sulphate solution was added to the reactor, which was reduced throughout the duration of the experiment. The highest propionic acid concentration was measured at ca. 150 mg/ℓ in T90 from days 6-15, during which time sulphate reduction was observed.

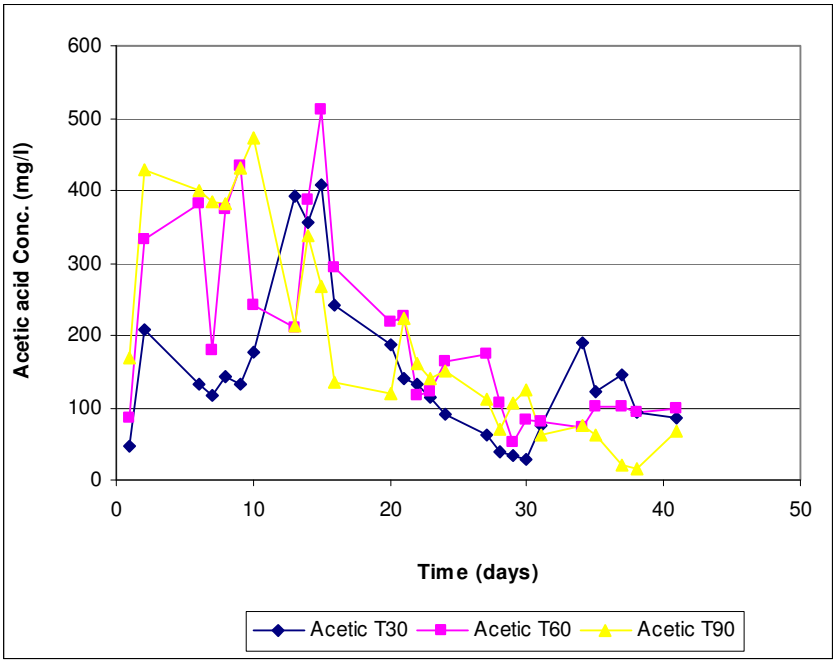

Figure 4.4. Acetic acid concentration in T30, T60 and T90.

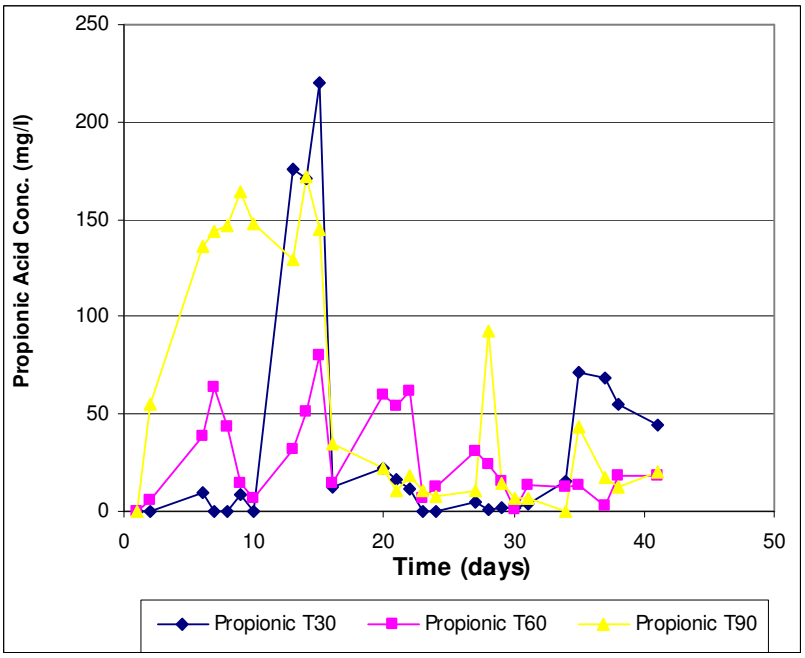

Figure 4.5. Propionic acid concentration in T30, T60 and T90.

4.3.1.2.3 Butyric acid

The butyric acid production in Reactors T30, T60 and T90 is depicted in Figure 4.6. The highest butyric acid concentration was observed during days 10-35 in all three reactors. The average values of the butyric acid concentrations in the three reactors during the full experimental period of 41 days, was 23 mg/ℓ in T30, 39 mg/ℓ in T60

and it was 70 mg/ℓ in T90. These results seemed to indicate that, unlike the acetic acid and propionic acid concentrations, the butyric acid concentration was proportional to the GC concentration. The higher GC concentration yielded the highest butyric acid concentration measured in the reactor. Generally, during the anaerobic degradation process, the SRB utilise the propionic acid, while the Acetogenic Bacteria (AB) use the C4 acid, to produce C2 acid, which in turn is used by the MB to produce methane. This pattern, however, is interrupted when sulphate and SRB are present in the bioreactor (Harmsen, 1996; Oude Elferink, 1998). SRB utilise hydrogen as soon as it is produced by the hydrogen producing microorganisms. This implies that hydrogen is not available to the MB.

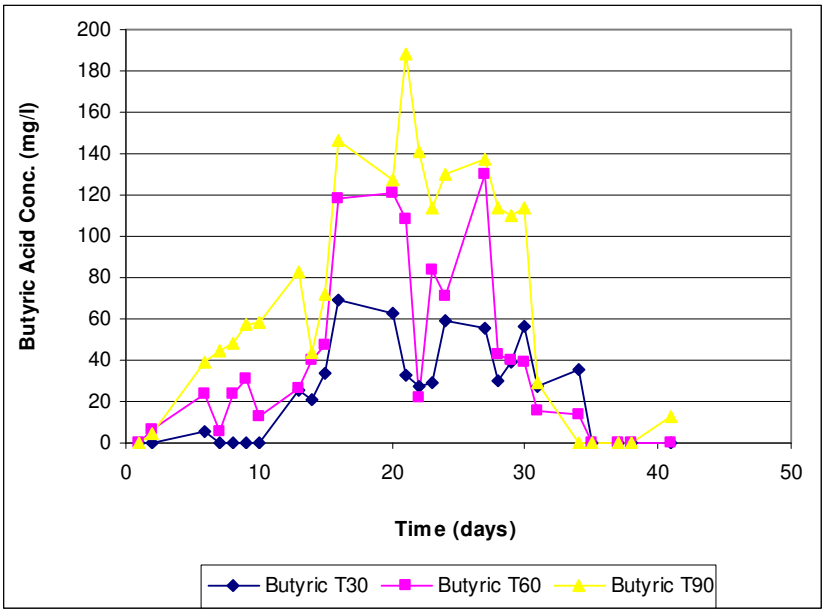

Figure 4.6. Butyric acid concentration in T30, T60 and T90.

4.3.2 Study 2. The use of RB and SRB as fermentative and SO_4 removing microorganisms

4.3.2.1 Sulphate reduction

While in Study 1, the fermentation capabilities of SRB were studied, in this study it was investigated whether RB can ferment cellulose to substrates usable by SRB in the biological sulphate removal process. The sulphate reduction results of Study 2 are given in Figure 4.7. Complete sulphate removal occurred in R3 and R4 during the first 12 days of the experimental period. The microorganisms in R4 consisted of a mixture of SRB and RB which were expected to afford better sulphate reduction than in R3, due to the presence of the SRB mixture. However, the net sulphate reduction

rates in R3 and R4 were similar. Fresh sulphate was added to the reactors on day 14 (*arrow*). These sulphate concentrations decreased at the same rate up to day 32 and day 35, for R3 and R4, respectively. Thereafter the sulphate concentrations reached steady state. No fresh GC were added during this period.

From the results obtained from R3, it was observed that sulphate reducers were present in the rumen consortia. This observation bears out the findings of Matteuzzi, (1964) and Cummings *et al.* (1995), who found that a fairly high count of sulphate reducers is present in rumen fluid. Postgate and Campbell (1965) found a bacterium in rumen fluid that reduced sulphate to sulphide, which they named *Desulfotomaculum ruminis,* while Huisingh *et al.* (1974) isolated *Desulfovibrio* spp. from the rumen fluid of sheep. Several bacteria are able to derive sulphur as nutrient from sulphate (Prescott, 1961) since sulphate is as effective as any form of Sulphur (Block *et al*. 1951)

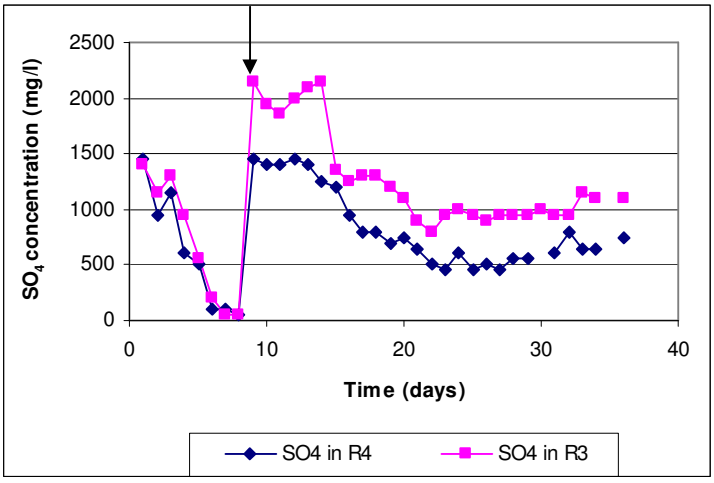

Figure 4.7. The sulphate removal in reactors R3 and R4.

4.3.2.2 VFA concentration

Propionic acid

The propionic acid concentration in reactors R1 and R2 (control reactors, containing no SO_4, Figure 4.8) increased from day 0-15, till values between 500-600 mg/ℓ. This result showed that using a rumen consortium to ferment GC resulted in an improved propionic acid concentration in the reactors, compared to the propionic acid concentration in the fermentation reactors using SRB as the degradation bacteria (Study 1). The reactor containing only rumen fluid produced a higher propionic concentration than the reactor containing the mixture of SRB and rumen, indicating

that the rumen consortia are better cellulose degraders. The principle products of the rumen microbial activity are fatty acids from the fibre and grain part of the food (grass, hay), which the cow uses as energy sources and amino acids from the protein-rich food components such as bean meal or good quality hay. The interaction between the cellulose fermentation and the rumen bacteria was already acknowledged by Hungate (1966). Since the rumen is a highly cellulytic ecosystem with a complex microbial population of bacteria, archaea, protozoa and fungi, rumen research has expanded during the last few decades (Barnes and Keller, 2003). Many researchers are investigating cellulose degradation of plant biomass to generate biogas, while the study in this thesis focussed on the use of the produced VFA and hydrogen for biological sulphate removal.

The propionic acid concentration in R3 and R4 was noticeable lower than in the control reactors (R1 and R2). This result showed the correlation between the propionic acid concentration and the biological sulphate reduction in R3 and R4, which agreed with the SO_4 reduction (day 1-12, Figure 4.7). After day 12, the propionic acid concentration in R3 increased till ca. 200 mg/ℓ, while SO_4 removal occurred concurrently. The SO_4 removal ceased on ca. day 25, which coincided with a propionic acid concentration <100 mg/ℓ. A similar pattern was observed in R4, although the propionic acid in that reactor was lower. This may possibly be ascribed to the difference in microbial origin: R3 contained only rumen fluid and R4 a mixture of rumen fluid and SRB. When comparing the propionic concentrations in R3 and R4, with that in reactors R1 and R2, it can be seen that when no sulphate is present, the propionic acid concentration in the reactors is substantial higher.

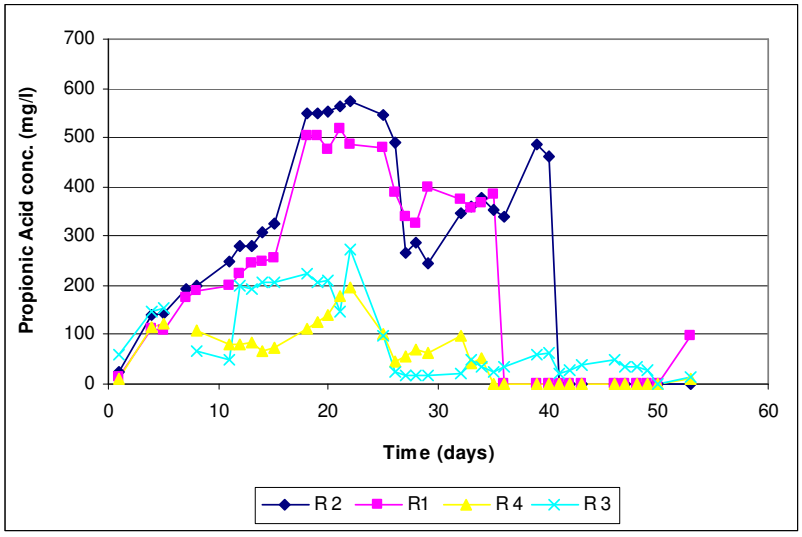

Figure 4.8. The propionic acid concentration in reactors R1-R4

Acetic acid

The acetic acid concentrations in R3 and R4 were higher than in R1 and R2 (Figure 4.9). This result can be expected because the utilisation of propionic acid for the biological sulphate reduction resulted in the formation of acetic acid. The higher acetic acid concentrations corresponded with the data illustrating the sulphate reduction.

The acetic acid concentration in reactor R1 is higher than in R2. This can possibly be ascribed to the fact that in reactor R1 a mixture of micro organisms (SRB and rumen inocculum) is present and that in reactor R2 only the rumen microbes are responsible for the VFA production. This finding seems to indicate that the SRB (in the organisms mixture in R1) favour the acetic acid production, whereas the rumen microbes seem to favour the propionic acid production (Figure 4.8: R2, containing the rumen organisms). The highest acetic acid concentration was measured in R3 at 500 mg/ℓ. The acetic acid concentration increased from day 5 to day15, where after it decreased (days 15 to 25), at which time the propionic acid concentration was the highest. This result confirms the correlation between the propionic and acetic acid concentrations in the reactors. In order to reduce 3 mol of sulphate, 4 mol of C3 acid are used and 4 mol of C2 acid are produced. This observation is in agreement with the acetic acid concentration in the sulphidogenic reactors, where the acetic acid concentration is higher than in the control reactors. This finding indicated that the acetic acid was not ued for the sulphate removal.

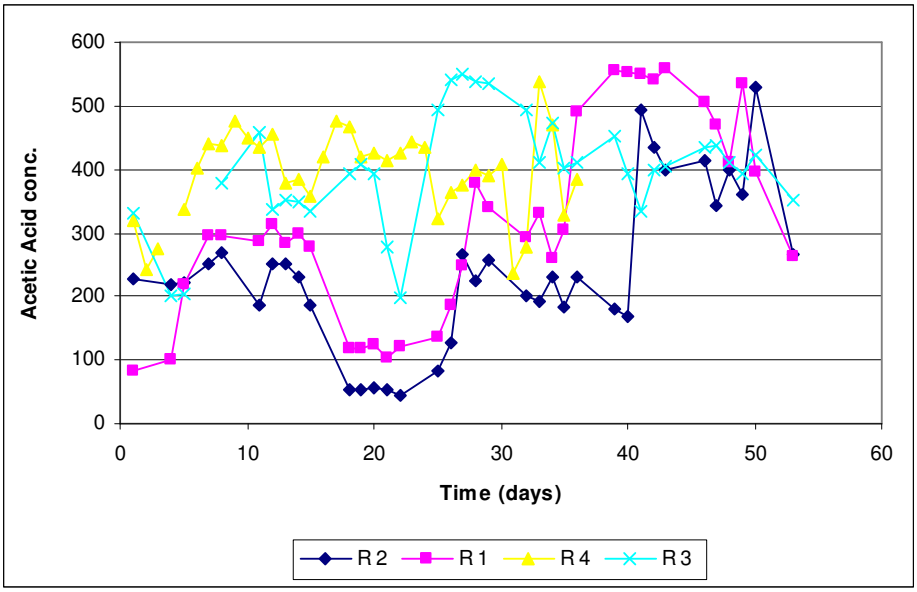

Figure 4.9. The acetic acid concentration in reactors R1-R4.

4.3.2.3 Butyric acid

The butyric acid concentration is presented in Figure 4.10, which shows that in the reactor containing the GC and the rumen microbes (R2), the butyric acid concentration is higher than in the reactor containing a SRB and rumen microbes mixture (R1). The average butyric acid produced in R1 from start to day 15 is 74 mg/ℓ, while in R2 this is 165 mg/ℓ, which is an increase of 55%. This result shows that the rumen microbes can ferment the GC more effectively to butyric acid than the SRB combined with the rumen microbes. After day 15, no further butyric acid was observed in both reactors. The butyric acid concentration in R3 and R4 increased after day 34, which coincided with no further SO_4 removal and with a low propionic acid concentration. This result seems to indicate that the conditions in the reactors favoured the butyric acid production (lower pH) but at the same time that the SRB did not utilise the produced butyric acid for further sulphate removal. The remaining SO_4 concentration in R3 was about 1000 mg/ℓ, while this was on average 500 mg/ℓ in R4. It might be that the increased acetic acid concentration of 400 and 500 mg/ℓ in R4 and R3, respectively, was the rate limiting factor in the reactors.

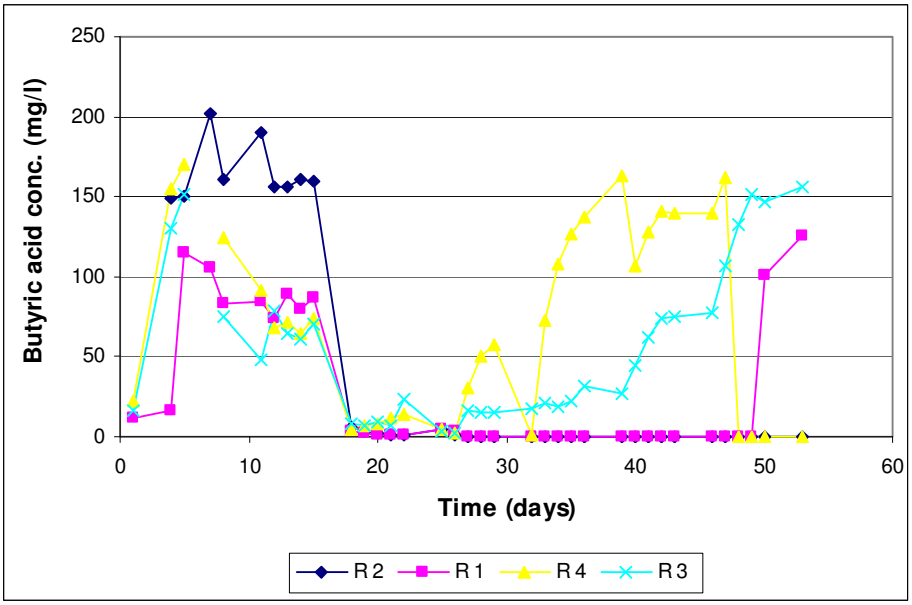

Figure 4.10. The butyric acid concentrations in R1, R2, R3 and R4.

4.3.2.4 Theoretical COD used/Sulphate reduced ratio

When SO_4 is reduced, using the VFA as the carbon and energy sources, equations 1, 2 and 3 can be applied:

Butyrate

$$CH_3CH_2CH_2COO^- + 2{,}5\,SO_4^{2-} + 0{,}25\,H_2O \rightarrow 4HCO_3^- + 2{,}5\,HS^- + 0{,}75\,H^+ + 0{,}25\,OH^- \quad (4.1)$$

Propionate

$$CH_3CH_2COO^- + 1{,}75\,SO_4^{2-} + 0{,}25\,H_2O \rightarrow 3HCO_3^- + 1{,}75\,HS^- + 0{,}5H^+ + 0{,}25\,OH^- \quad (4.2)$$

Acetate

$$CH_3COO^- + SO_4^{2-} \rightarrow 2HCO_3^- + HS^- \quad (4.3)$$

Equations 4.1 to 4.3 show that 1 mol butyrate is needed to reduce 2.5 mol sulphate, that 1 mol propionate is needed to reduce 1.75 mol sulphate and that 1 mol acetate can theoretically reduce 1 mol sulphate, assuming that total sulphate removal is obtained and that no residual VFA is present. Further correlations and calculations between the theoretical COD used and sulphate removed ratios are given in Table 1(Appendix A). A total of 9.5 g/ℓ propionic acid over 40 days was produced in R2, containing 30 g GC, 450 ml tap water and 25 ml of rumen inoculum mixture. Theoretically this amount of propionic acid can remove 21.6 g sulphate. When more GC were added to the reactors, higher VFA concentrations were produced, reducing higher concentrations of sulphate. However, when more VFA and other intermediates are produced the reactor COD/SO_4 ratio will increase, resulting in competition of the MB as well as in a high residual COD concentration in the reactor effluent.

3.2.5 Study 3

The results of Study 2 indicated that the rumen microbial population could ferment grass-cellulose to degradation products, which functioned as carbon and energy sources for SRB reducing sulphate in the feed water of the reactors. In order to understand the conditions under which rumen microbes can produce increased VFA concentrations, Study 3 was conducted. Three reactors were operated under different conditions: with and without the addition of sulphate and with and without the addition of tryptone, a protein rich nutrient, which can enhance propionic acid production rather than C2 or C4 acids from the degradation of cellulose and hemicellulose (personal communication from Paul Weimer, rumen specialist, University of Winconsin, USA).

4.3.3.1 Sulphate reduction

The sulphate removal in L1 is presented in Figure 4.11. The sulphate removal graph shows that initially, from days 0-11, the sulphate concentration decreased from 1250 to 800 mg/ℓ, while by day 14, the sulphate had been reduced to 40 mg/ℓ. Fresh 5.5 g Na_2SO_4 was added to L1 (*Arrows* in Figure 4.11) on days 14-18 (inclusive). This newly added SO_4 solution was removed within 16-18 h. These results indicated a good sulphate removal, which was confirmed by the sulphide concentration in L1, which was 600 mg/ℓ on day 25. Since the high sulphide concentration can inhibit the rumen (fermentation) microorganisms, the experiment was stopped.

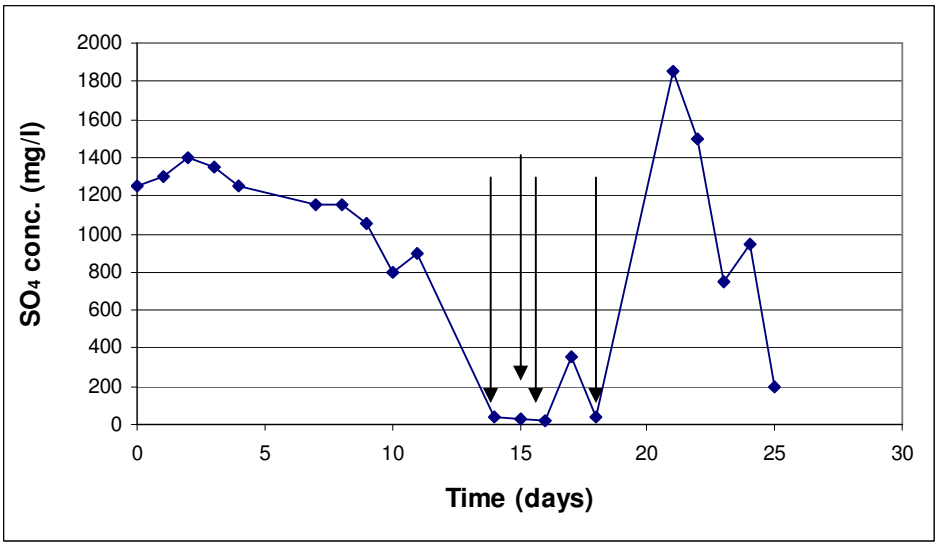

Figure 4.11. The biological sulphate reduction in reactor L1.)

(Arrows indidcate the addition of fresh SO_4: as soon as SO_4 was added it was reduced when next sample was analysed 16-24 hours later, see text

When no metals are present, sulphide accumulation can result in a severe inhibition of the biological sulphate removal process and in some cases may even cause total process failure. Many studies have been dedicated to the effect of sulphide toxicity on the biological sulphate reduction efficiency. In general, these studies demonstrated that, under mesophilic conditions, both granular and suspended sludges are more tolerant of H_2S inhibition at a pH of around 8. At neutral pH values, free H_2S, which is more toxic than HS^-, accounts for 50% of total dissolved sulphide, whereas at pH 8 it is only around 10% (Lens & Hulshoff Pol, 2000). Speece (1996) listed the sulphide toxicity levels investigated by different researchers, which showed that 100-150 mg/ℓ sulphide is toxic for lactate and glucose utilizing SRB in a continuously stirred tank reactor (CSTR). Moreover, 60-75 mg/ℓ sulphide was not tolerated by acetate and propionate utilizing SRB (in a CSTR), while Parkin *et al*.

(1990) reported that when the sulphide concentration was 60-70 mg/ℓ, in an acetate and propionate-fed chemostat, it resulted in process failure. Since the pH in this study operating L1 was > 6.5, the sulphide is not in its toxic form. Greben *et al*. (2004, 2005) have shown that a high sulphide concentration in a sulphidogenic reactor may be beneficial for sulphate reduction when using ethanol as the carbon and energy source. Eloff *et al*. (2004) showed that when sulphide was added to the sulphate rich influent feeding a biological sulphate removal reactor, the sulphate reduction improved compared to feeding sulphide-free influent.

4.3.3.2 VFA production and utilisation

Propionic acid

The propionic acid concentration in L1 was the lowest of the three reactors (Figure 4.12). This was ascribed to the C3 acid utilisation for sulphate removal in L1 (Figure 4.11). Whenever the sulphate concentration decreased to < 100 mg/ℓ, fresh sulphate solution was added to the reactor. The sulphate removing microorgnisms utilised the propionic acid in L1 and resulted in the C3 acid concentration in that reactor being the lowest. The highest propionic acid concentration (Figure 4.12) occurred in reactor L3. Tryptone (1g) was added to L3 daily. This is used as a source of nitrogen (amino acids) and nutrients in many culture media. Tryptone, as well as peptone as amino acid polymers are an excellent choice for bacterial fermentation and most likely will stimulate the rumen microbes to ferment the cellulose in the GC more efficiently. However, the additional costs when adding tryptone to the fermentation reactor have to be taken into account as 1 g tryptone currently costs R 0.50/g. Moreover, tryptone will add to the COD concentration in the reactor and thus to the residual COD concentration in the reactor effluent.

Acetic acid

The utilisation of propionic acid in L1 resulted in the reduction of sulphate and in the production of acetic acid (Figure 4.13). Thus the highest acetic acid concentration was observed in reactor L1. It increased with time up to a concentration of ca. 800 mg/ℓ, which according to Hill *et al*. (1987) can result in process failure. In the reactor which contained the rumen population and tap water, the highest acetic acid concentration obtained was almost 400 mg/ℓ, while it was 450 mg/ℓ in the reactor containing tryptone. These results indicated that the tryptone addition resulted in a 50 mg/ℓ increase in the acetic acid concentration. Moreover, due to the biological

sulphate reduction in L1, the acetic acid concentration increased with about 200-300 mg/ℓ.

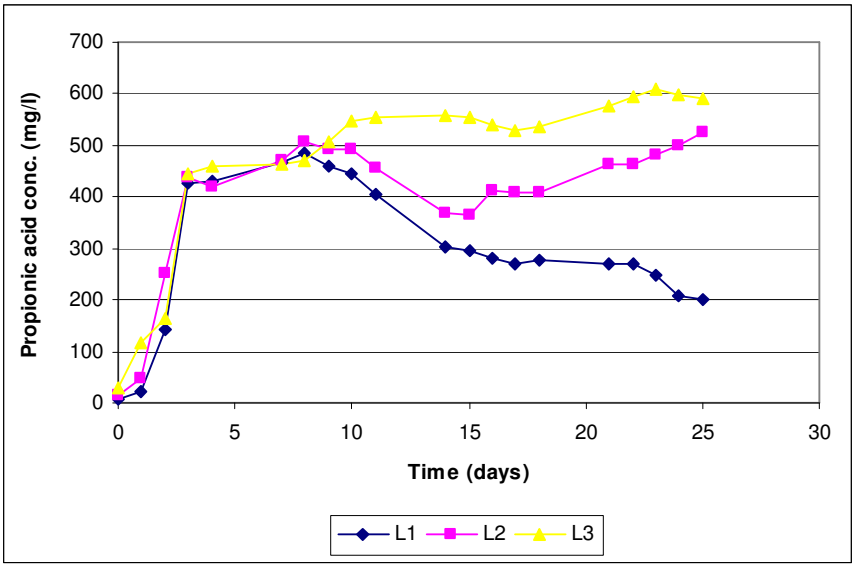

Figure 4.12. Propionic acid concentrations in reactors L1, L2 and L3.

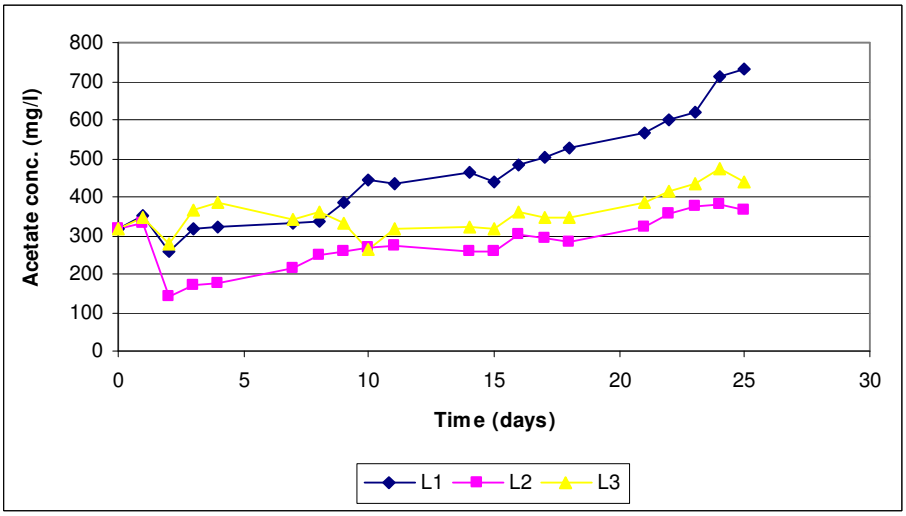

Figure 4.13. Acetic acid concentration in reactors L1, L2 and L3.

Butyrate

The butyrate concentrations in L1-L3 are given in Figure 4.14, which showed that the butyric acid concentration was zero in the three reactors during the first 14 days of operation. This lag in butyric acid production may have been due to an increased C3 production. Thereafter, the butyric acid concentration in L1 was low, as it was being utilised for the sulphate reduction. In L2 and L3 the butyric acid concentration increased due to cellulose degradation. The butyric acid concentration in L2 (control) was higher than in the tryptone reactor (L3). This result seemed to indicate that the

propionate concentration, which is the preferred energy source for the SRB, rather than butyric acid concentration increased when tryptone was added to the fermentation reactor.

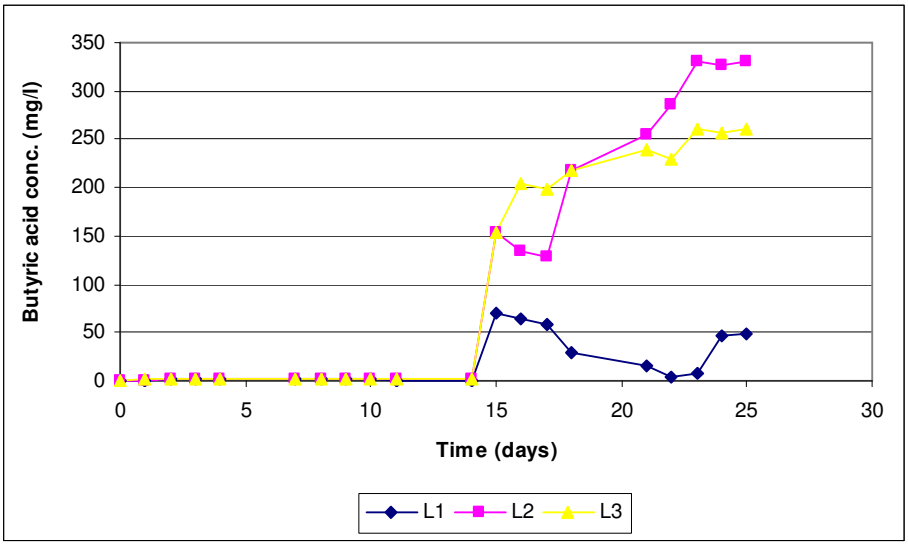

Figure 4.14. The butyric acid concentrations in reactors L1, L2 and L3

4.3.3.3 Sulphate removed/VFA utilised

The high peaks in the graph in Figure 4.11 indicated the sulphate concentration after fresh sodium sulphate was added to the reactor. The sulphate removal, as depicted in Figure 4.11, corresponded to the utilisation of the C3 and C4 acids. An estimate of the total sulphate removal from day 0-21 was calculated, during which period no fresh GC was added to the reactor. During that period ca. 9 g sulphate was removed, while 75 g GC was present in the reactor. Thus it can be deduced that, in order to reduce 1 g sulphate, 8 g GC is needed. The $SO_{4\ removed}$/$grass_{used}$ ratio will again be addressed in subsequent chapters

4.4 CONCLUSIONS

The results from Studies 1, 2 and 3 showed that sulphate reduction was obtained in the sulphate containing reactors in all three studies. It was also shown that when fresh sulphate was added to the reactors, continued sulphate removal occurred. In Study 1, the sulphate reduction was dependant on the concentration of GC: the higher the amount of grass added to the reactor, the faster the sulphate removal. In Study 2, sulphate removal was obtained in both sulphate rich reactors. In Study 3, total sulphate removal was achieved after 14 days, using the rumen fluid

microorganisms. In all three studies it was observed that mainly propionic and butyric acids were used as the carbon and energy sources for the SRB, producing acetic acid.

VFA production in T30, T60 and T90 (Study 1) resulted in removal of sulphate. It was shown that SRB utilised maily propionate. However, the results obtained from reactor T30 seemed to indicate that acetic acid was used during the period that the C3 or C4 acid concentrations were insufficient. It was furthermore hypothesized that the C2 acid was used by other microorganisms to produce the C3 and C4 acids for further sulphate reduction. Sulphate reduction occurred in the reactors T30 and T60 during the first 8 days of operation**,** while in T90, full SO_4 reduction took place during the first 5 days. Thus a higher GC concentration resulted in increased SO_4 removal.

Sulphate reduction was observed in the reactor containing SO_4 rich water and only rumen fluid microorganisms. This finding indicated that sulphate reducers form part of the rumen consortium. The results of Study 2 formed the basis for Study 3 as it demonstrated that the rumen inocula could ferment the grass cuttings to produce VFA and that the VFA produced could be utilised for biological sulphate removal. When comparing the sulphate reduction in reactors T30 (study 1) and in L1 (study 3), containing the same amount of grass cuttings and biomass mixture, it was seen that the sulphate reduction in T30 was higher than in L1. However, the rumen microbes produced more C3 and C4 acids. The improved sulphate reduction in T30 over that seen in L1 was ascribed to the sulphate adapted biomass. When tryptone was added to L3, the propionic acid concentration increased while the butyric acid concentration did not.

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