Université Blaise Pascal
CLERMONT-FERRAND

Laboratoire de Génie Chimique et Biochimique
LGCB

A TOTAL CONVERTING AND BIOSAFE LIQUEFACTION COMPARTMENT FOR MELISSA

TECHNICAL NOTE: 86.2.1

Maximum cellulose breakdown

Catherine CREULY, Lucile PLOURDE-OWOBI, Claude-Gilles DUSSAP

June 2001

CUST - Université Blaise Pascal
24, avenue des Landais - BP 206 - 63174 Aubière cedex France
Tél. 33 (0) 4 73 40 78 38 - 33 (0) 4 73 40 74 30 - fax (0) 4 73 40 78 29

1. 0 INTRODUCTION

Microbial cellulases and hemicellulases are widely used in different industrial activities, such as in textile, detergent, brewery or wood-processing, and also in the treatment of domestic wastes and in biological treatment of fibrous feeds in the non-ruminant livestock industry. However, these enzymes are not very efficient for the degradation of highly lignified plant cell walls because cellulose and hemicelluloses are cross-linked to lignin which is very difficult to degrade and protects cellulose and hemicelluloses against enzymatic hydrolysis (Selinger et al., 1996).
Ruminant animals possesse rumen bacteria (Figure1) which developed a symbiotic relationship to digest lignocellulosic substrates (Hungate,1950).

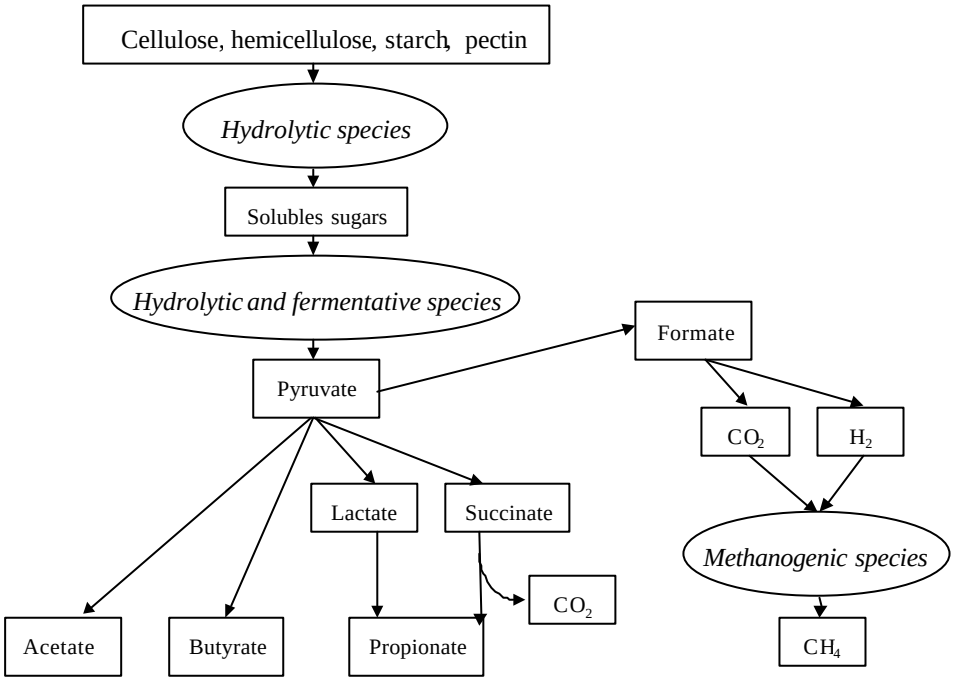

Figure 1: The bacterial ecosystem in the rumen

1.2 The potentialities of *Fibrobacter succinogenes*

Fibrobacter succinogenes is a major fibrolytic bacterium found in the rumens of cattle and sheep.
The enzymatic equipment of *Fibrobacter succinogenes* explains these specific performances. Firstly, this bacterium degrades cellulose due to a very efficient cellulolytic system (Chesson and Forsberg, 1997). Cellulose is depolymerised at the bacterial surface by different cellulases and the released cellodextrins are hydrolysed to glucose and cellobiose in the periplasm (Figure 2). Secondly, it produces ferulic acid and acetylxylan esterase, and arabinofuranosidase that are necessary to cleave ester bonds linking hemicelluloses to lignin, or to debranch xylanes. Finally,

several different xylanases and glucuronidase complete the cellulolytic system (Malburg et al., 1993).

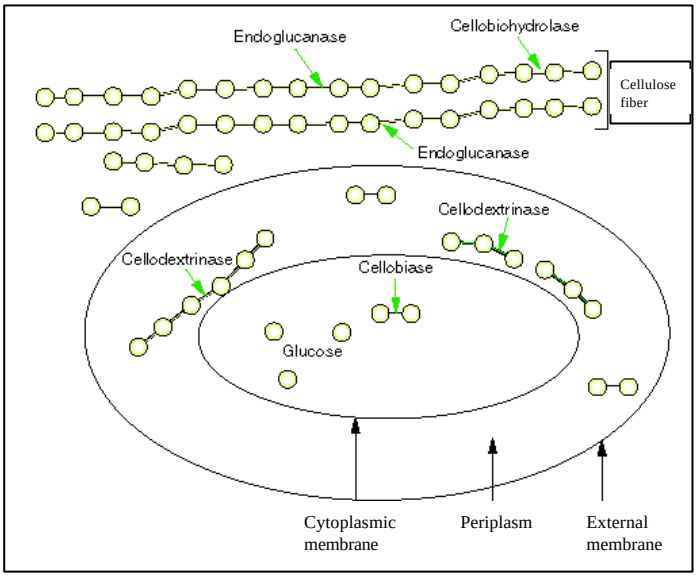

Figure 2: The cellulolytic enzymatic equipment of *Fibrobacter succinogenes*

1.2 The metabolic network

This strictly anaerobic bacterium uses cellulose, glucose and cellobiose as carbon and energy sources, and produces succinate, acetate and few formate (Stewart and Flint, 1989).

The purpose of our previous work was to present a novel approach on this bacterium by developing a detailed stoichiometric model of anaerobic metabolism that includes a complete database of known reactions involved in the central catabolism of glucose and previously established anabolic reactions based on the general knowledge of bacterial metabolism (Gottschalk, 1986) (Figure 3).
The supposed metabolic network involves 96 stoichiometric reactions. Validation of the model is obtained by comparison between the theoretical yields of carbon elements calculated by a data reconciliation technique (Dussap et al., 1997) and the experimental yields measured during anaerobic cultures of *Fibrobacter succinogenes* in a bench scale bioreactor (Creuly et al., 2001).
The major features of catabolic pathways which have been described are as follows :
- glucose is transported across the cytoplasmic membrane through independent constitutive transporters that are sodium dependent. In the cytoplasm, glucose is phosphorylated by a GTP-dependent glucokinase (Glass and Sherwood, 1994). Cultures that were provided with glucose produced cellobiose, and cellobiose gives rise to cellotriose. Gaudet et al (1992) have shown that *Fibrobacter succinogenes* continuously synthesised and degraded glycogen during all phases of

growth, but its dependency on glycogen catabolism was not defined. In fact, when extracellular sugar concentration is high, part of the sugar is stored as glycogen . This glycogen storage can represent a significant part of the total dry mass of the bacteria.

- as it possesses fructose 1,6-biphosphate aldolase and glyceraldehyde 3-phosphate dehydrogenase, *Fibrobacter succinogenes* is assumed to ferment hexoses by the Embden-Meyerhof-Parnas pathway (Joyner and Baldwin, 1966 ; Miller, 1978) until pyruvate. Phosphoenolpyruvate is carboxylated to oxaloacetate by a GDP- specific PEP carboxykinase.
- oxaloacetate is converted to malate by a pyridine nucleotide-dependent malate dehydrogenase (Table 1 ; J20).
- fumarase activity (Table 1; J14) was not demonstrated but it is probably present to produce fumarate from malate. Fumarate is reduced by a flavin-dependent, membrane bound fumarate reductase to produce the major fermentation product, succinate. The reduction of fumarate with reduced flavins is likely to involve cytochrome b (Miller, 1978). Flavin nucleotides mediate electron transport between pyruvate and fumarate (Table 1; three coupled reactions J9, J79, J77).
- in addition, *Fibrobacter succinogenes* possesses the essential enzymes of the non-oxidative branch of the pentose phosphate pathway (Matte et al, 1992), though known to be unable to metabolise pentoses. Enzymatic researchs on the oxidative branch give negative results. This specificity imposes to set glutamate dehydrogenase (Table 1 ; J22) on NAD/NADH dependence and isocitrate dehydrogenase NADP / NADPH specific (Table 1 ; J82). Matheron et al (1999) have isolated a specific enzyme that produce alanine from pyruvate and NH_3 (Table 1 ; J80).

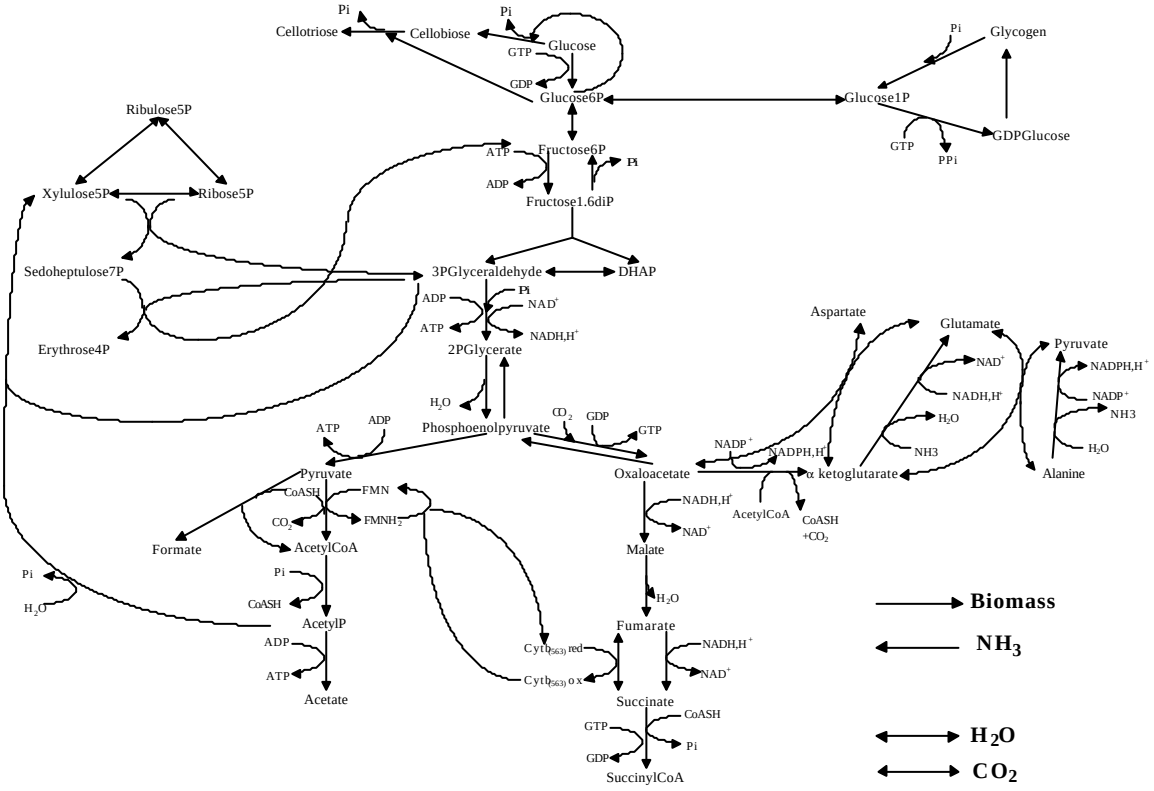

Figure 3: Metabolic network built for *Fibrobacter succinogenes*
Table 1: Catabolic reactions considered in the network

Flux	Stoichiometric reactions
J1	glucose + GTP → glucose-6P + GDP
J2	glucose-6-P → fructose-6-P
J3	fructose-6-P + ATP → fructose-1,6-diP + ADP
J4	fructose-1,6-diP → dihydroxyacetone-P + glyceraldehyde-3-P
J5	glyceraldehyde-3-P → dihydroxyacetone-P
J6	glyceraldehyde-3-P + NAD + ADP + Pi → 3-P-glycerate + ATP + $NADH_2$
J7	3-P-glycerate → P-enolpyruvate + H_2O
J8	P-enolpyruvate + ADP → pyruvate + ATP
J9	pyruvate + CoASH + FMN → acetyl-CoA + $FMNH_2$ + CO_2
J10	pyruvate + CoASH → acetyl-CoA + formiate
J11	acetyl-CoA + Pi → acetyl-1P + CoASH
J12	acetyl-1P + ADP → acetate + ATP
J13	fumarate + $NADH_2$ → succinate + NAD
J14	fumarate + H_2O → malate
J15	malate + NAD → oxaloacetate + $NADH_2$
J16	ribulose-5P → ribose-5P
J17	ribulose-5P → xylulose-5P
J18	xylulose-5P + ribose-5P → glyceraldehyde-3-P + sedoheptulose-7-P
J19	glyceraldehyde-3-P + sedoheptulose-7-P → fructose-6-P + erythrose-4-P
J20	P-enolpyruvate + CO_2 + GDP → oxaloacetate + GTP
J76	xylulose-5P + Pi → glyceraldehyde-3-P + acetyl-1P + H_2O
J77	$Cytb_{red}$ + fumarate → $Cytb_{ox}$ + succinate
J78	xylulose-5P → ribose-5P
J79	$Cytb_{red}$ + $FMNH_2$ → $Cytb_{ox}$ + FMN
J80	pyruvate + $NADPH_2$ + NH_3 → alanine + NADP + H_2O
J81	CoASH + GTP + succinate → succinylCoA + GDP + Pi
J82	oxaloacetate + acetyl-CoA + NADP + H_2o → α-ketoglutarate + CoASH + $NADPH_2$ + CO_2
J83	glucose + glucose-6P → cellobiose + Pi
J84	glucose-6P + cellobiose → cellotriose + Pi
J85	cellobiose + H_2O → 2 glucose
J21-J75	**Anabolic network reactions**

2. 0 PROCESS DESIGN

2.1 Strain and general conditions of cultivation

Fibrobacter succinogenes S85 (ATCC 19169) was originally isolated from the bovine rumen (Bryant and Doetsch, 1954) and has been maintained as pure culture in laboratory ever since. It was grown anaerobically under 100% CO_2 in a medium containing (per liter) : 450 mg KH_2PO_4,

450 mg K_2HPO_4, 900 mg NaCl, 1.8 g $(NH_4)_2SO_4$, 90 mg $MgSO_4$, 90 mg $CaCl_2$, 3 mg $MnSO_4$,$6H_2O$, 0.3 mg $CoCl_2$,$6H_2O$, 8 mg $FeSO_4$,$7H_2O$, 0.25 mg Biotin, 0.005 mg para-aminobenzoic acid, 500 mg Cystein, 4 g Na_2CO_3, 8 g carbon substrate and a volatile fatty acid mixture (Gaudet et al, 1992). The above medium was sterilized (120°C, 20 min) in the reactor (2 litters total volume) or in serum-bottle (100 mL). The entire procedure is described in appendix 1 and 2.

2.2 Experimental design in reactor

After redox potential reduction at -350 mV and temperature equilibration at 37°C, the thermostated, stirred (set at 40 rpm) fermentor (1.5 L) was inoculated with 200 mL of an overnight culture. pH and redox were measured on line. Growth was monitored by the increase of optical density (600 nm). Growth was correlated with the decrease of the pH since the initial value (6.9) until value 5.5. At this value, pH was readjusted at neutral value by Na_2CO_3 addition. In the same order, a substrate feed was performed when substrate concentration became limiting (fed-batch technique).

.

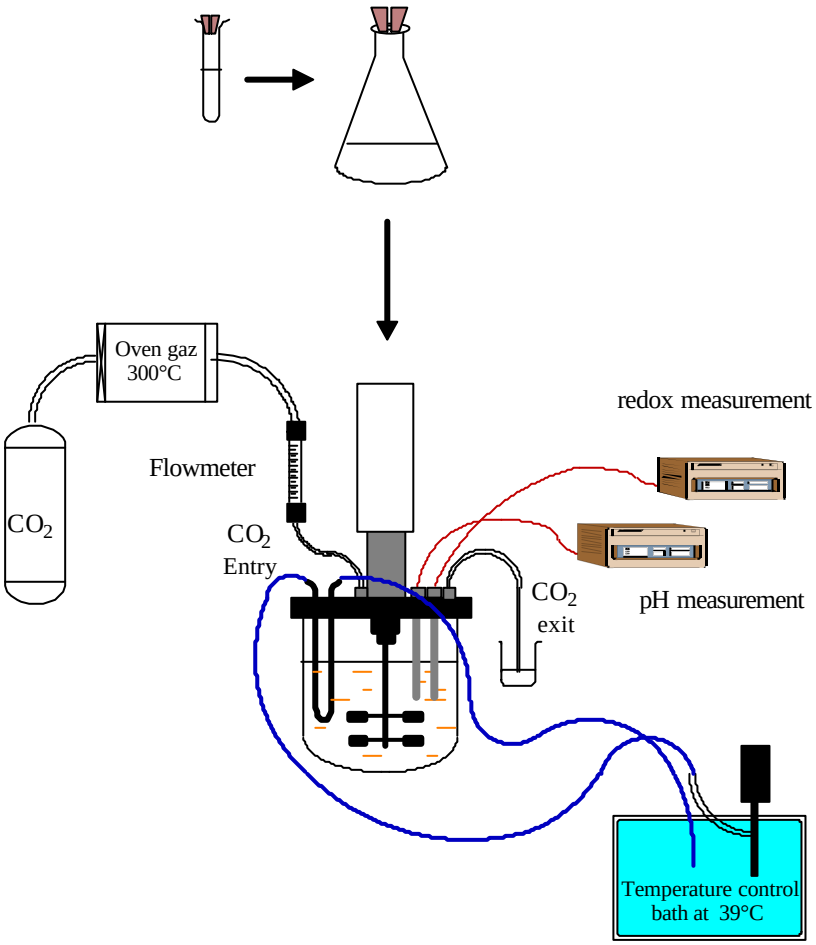

Figure 4: The anaerobic reactor

2.3. Metabolites assays

According to the type of substrate used in the culture, the analyses could be different. We speak about "reference culture" when *F.s* was grown on glucose or cellobiose which are soluble substrates and "fiber culture" when *F.s* was grown on cellobiose or ESA-substrate.

On reference culture, cell mass was determined by dry weight at 100°C after a centrifugation step and by the increase of optical density (600 nm). On fiber culture, these methods are unapplicable because the major part of the bacteria adheres on the substrate.

Extracellular metabolites (glucose, succinate, acetate, volatile fatty acids) were quantified in cell free samples by high pressure-liquid chromatography (1100 series Hewlett Packard, 1047A refractometer analyzer) fitted with two ionic exclusion columns (Phenomenex rezex organic acid – 300*7.8 mm) maintained at 80°C and isocratic eluted with solvent 5 mM H_2SO_4.

The degradation of the fiber was estimated by a COD method described in the next section and by filtration/weighing at the end of the culture.

2.4. COD approach

During the growth of *Fibrobacter succinogenes* , there is no variation of $_{total}$COD because these bacteria do not produce any gazes. The substrate is converted in biomass and in soluble products which the majors are succinate and acetate. In accordance with the tasks defined in the work package, we have developed a method to estimate the part of COD that could be attribute to the substrate degradation.
We implement the method on reference culture where we are able to quantify all the substrate and products (Figure 5). In fact, this method allows to obtain a $_{glucose}$COD value and this value could be validated by an other method : a calibration $_{glucose}$COD = f(glucose concentration).

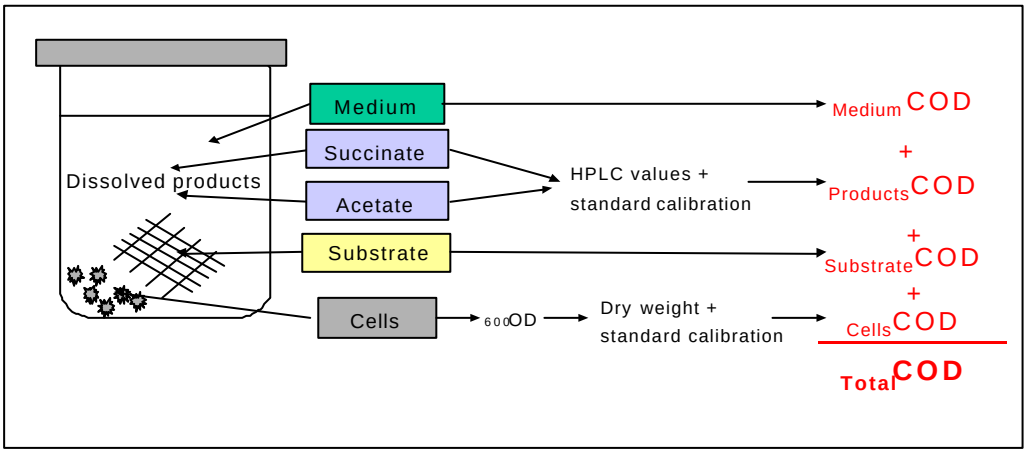

Figure 5: Analysis of chemical oxygen demand during the culture of *Fibrobacter succinogenes*

Firstly, three standard calibration are drawn up to correlate the COD values to the increase of products during the culture (Figure 6).

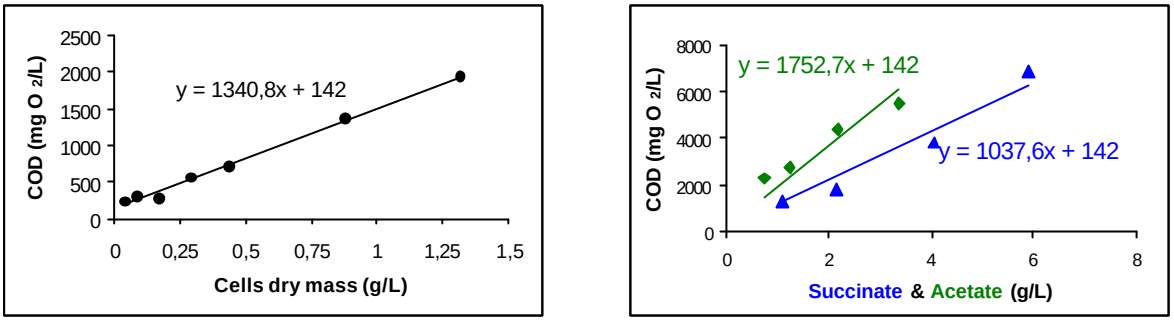

Figure 6: Standard calibration curves of COD for cell dry mass, succinate and acetate

Secondly, the following equation (Figure 7) defines the substrate COD which could be calculated.

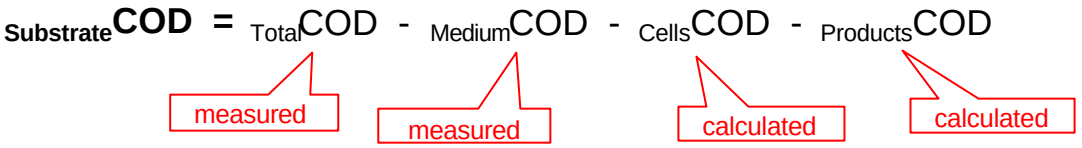

Figure 7: Estimation of $_{Substrate}$COD

During a reference culture, the HPLC analyses allow to follow the consumption of glucose and production of succinate and acetate (Figure 8).

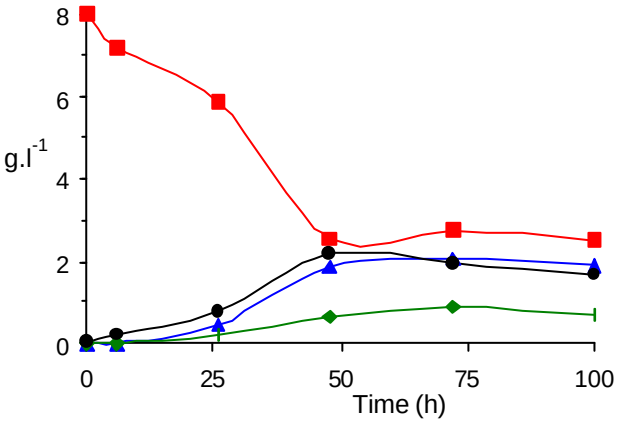

Figure 8: Evolution of substrate (glucose, ☝) and products (succinate, ▲ and acetate, ◆) during the growth of *Fibrobacter succinogenes* (dry weight, ●).

The final step is the determination of $_{glucose}$COD by the equation defined in the figure7 and the comparison with $_{glucose}$COD value obtained by HPLC measurement. The results show that there is a good correlation between the two values (Figure 9). In this way, we validate the method which can be applied to fiber substrate. *Fibrobacter succinogenes* was cultivated in two serum-bottle on cellulose and wheat (Figure10). Testing the previous approach, we have calculated the decrease of COD corresponding to the degradation of fiber and we have compared it to the succinate and acetate production (Table2).

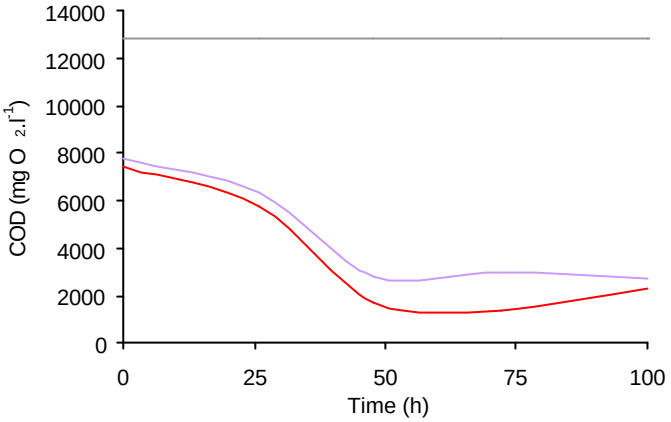

Figure 9: Evolution of chemical oxygen demand for estimation of glucose consumption during the growth of *Fibrobacter succinogenes*. COD was calculated from the previous equation (—) and measured from HPLC data and standard calibration (—).

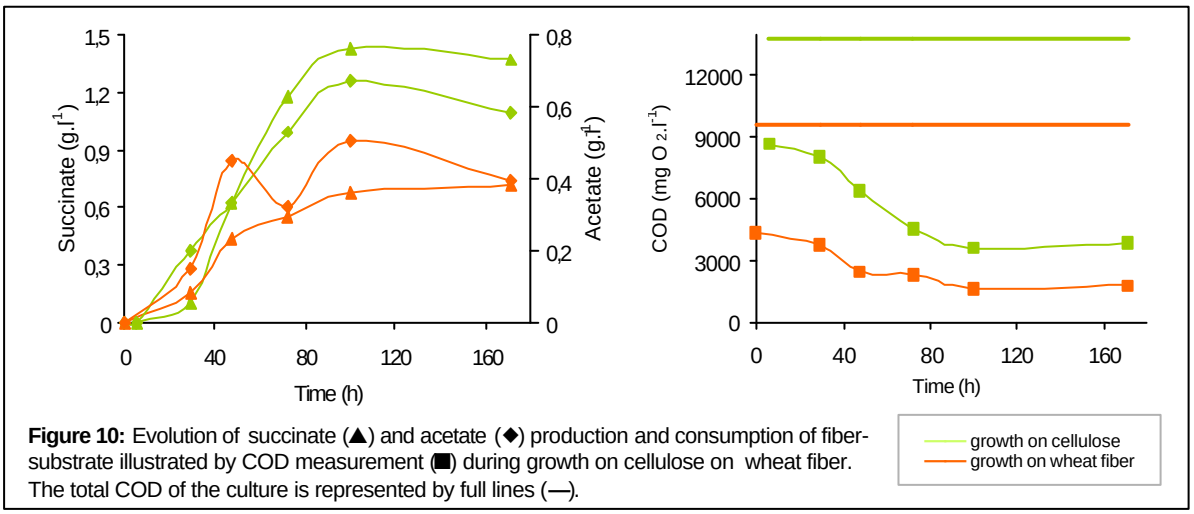

Figure 10: Evolution of succinate (▲) and acetate (◆) production and consumption of fiber-substrate illustrated by COD measurement (■) during growth on cellulose on wheat fiber. The total COD of the culture is represented by full lines (—).

Table 2: Validation of COD analyses on different substrates in serum-bottles.

Substrate	Glucose	Cellulose	Wheat
Culture duration (h)	100	110	110
Final $_{substrate}$COD – Initial $_{ubstrate}$COD (mgO_2/L)	5137	4803	2560
Succinate production (g/L)	2.06	1.43	0.70
Acetate production (g/L)	1.18	0.67	0.50

This method allows to show the influence of the polymerization degree on the degradation of cellulose fibers (Table 2). In fact, the growth of F.s was better on glucose than on cellulose or wheat; this was correlated to the production of metabolites and to the $_{substrate}$COD values.

3.0 DEGRADATION IN BIOREACTOR

3.1 Production of the ESA-substrate

We have prepared the substrate according to the decision took during the meeting in Gent : faeces, spirulina, wheat, cabbage and soya at 2% DM in the reactor (20 g/L).

Table 3: The composition of ESA substrate

			Non-edible plant material		
Substrate	Human Faeces	Spirulina	Wheat straw	Cabbage	Soya waste
Distribution (% DM)	20	10	23.3	23.3	23.3
Suppliers	laboratory	La vie en Bio	farm	shop	France melasses
Concentration (g DM/L)	4	2	4.7	4.7	4.7
% Humidity	<1	<1	5.8	83.3	9.4
% Fibers	-	-	45 * (1)	3 * (2)	22 * (3)

*These values are obtained from Food Composition and nutrition tables (1999/2000) Souci S.W., Fachmann W., Kraut H., CRCpress and will be specified in the next TN. because its only concern a fraction of ours substrates.

The values correspond to the part of all high-molecular substances which cannot be split up by the enzymes of the human digestive system. Basically, they include certain polysaccharides (cellulose, hemicellulose) as well as polymer phenolic compounds (lignin).

(1) wheat bran
(2) white cabbage
(3) Soya bean (seed, dry)

3.2 Cellobiose degradation in anaerobic reactor using *Fibrobacter succinogenes*

In this part, cellobiose substrate was chosen for validating the developed method, since this soluble substrate allows a standard growth . No problem was detected in our process.
In the first stage (0 to 30 h), *Fibrobacter succinogenes* was grown on cellobiose (8 g/L) which decreased in parallel with the pH (Figure 11). These decreases were correlated to the production of succinate and acetate. After 30 h, we added a new fraction of cellobiose and Na_2CO_3 to recover the initial pH. In the second stage (30 to 125 h) , we observed a lowest rate of cellobiose consumption but the production rate of metabolites are more important than in the first stage.
We decided to stop the degradation at 125 h, because the growth was limited by the concentration in $(NH_4)_2SO_4$ (mineral nitrogen source).

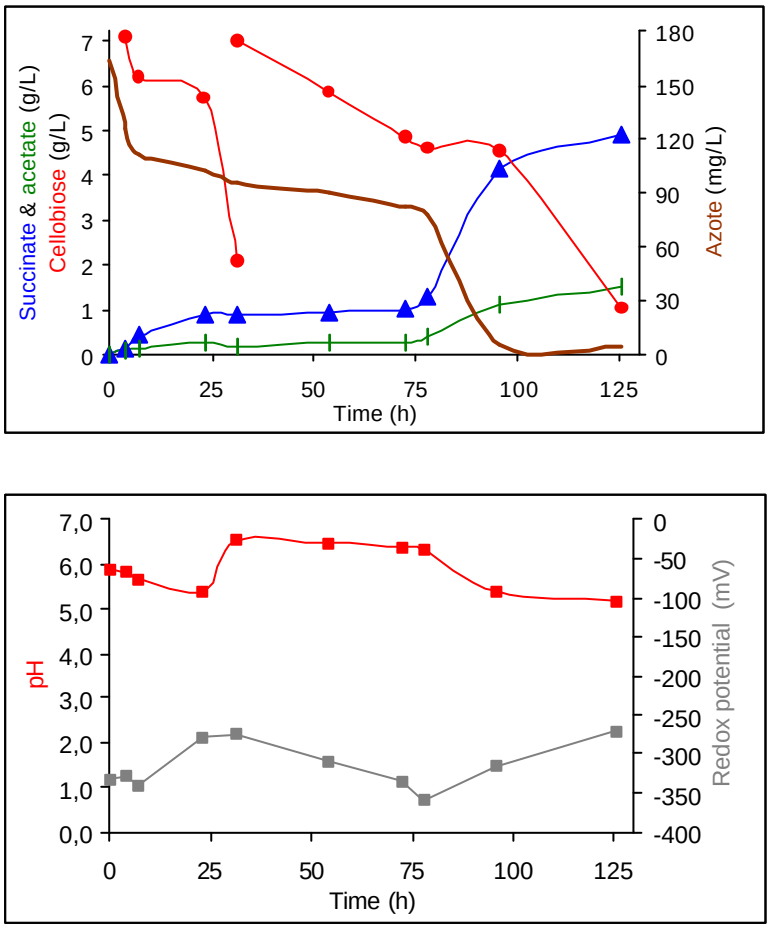

Figure 11: Growth of *Fibrobacter succinogenes* in reactor on cellobiose.
The production of succinate ( ) and acetate (◆) was estimated by HPLC analyses.
Evolution of NH3 consumption (—), cellobiose consumption (●), values of pH (■) and redox potential (■) were also reported.

3.3 Wheat degradation in anaerobic reactor using *Fibrobacter succinogenes*

To determine whether the degradation process occurred with non-soluble substrate, the growth was monitored by the production of succinate (Figure 12). Using a standard correlation curve (Cell dry mass = 1.28 succinate in g/L) we have estimated at 1.5 g/L the biomass production after 300 h of culture.
The results obtained during the course of wheat degradation are in sharp contrast with those observed in the previous experiment on cellobiose. The wheat system exhibited a best stability but the substrate degradation was slower on wheat than on cellobiose. It seems that this feature could be attributed to the increase in the complexity of the substrate which made the microorganism to produce specific enzymatic equipment. An other difference observed was a gradual buffering of the medium which appeared after 50 h.
The substrate feeding (9.6 g at the beginning) was performed by a fed-batch procedure at 115 h and 210 h. When the experiment was stopped at 300 h, 16.6 g of wheat had been feed and the microorganism had degraded 48 %.

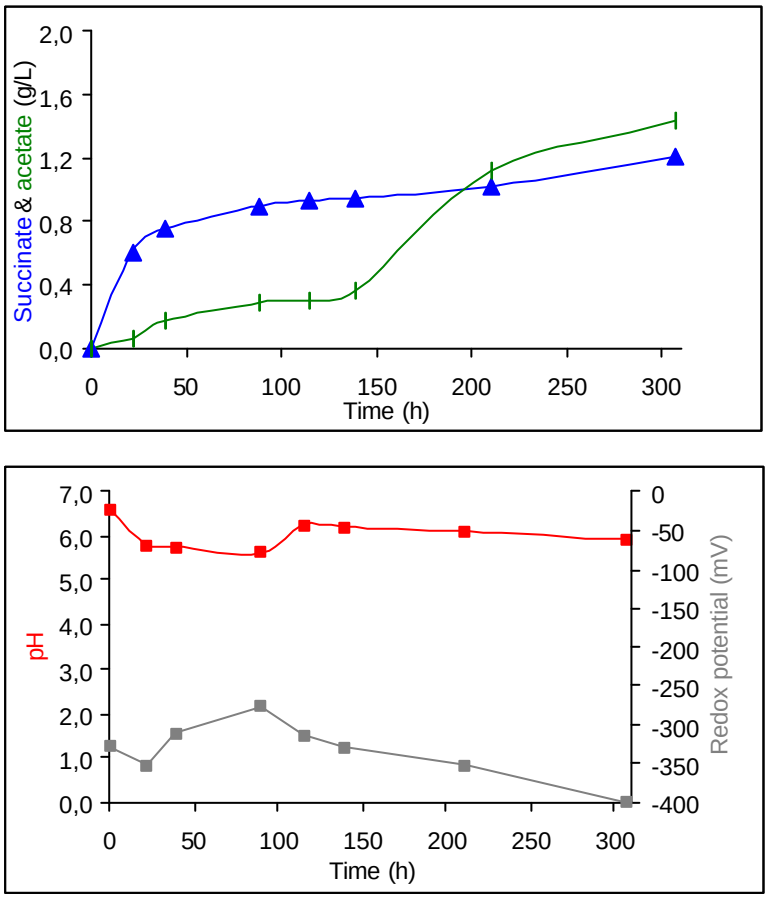

Figure 12: Growth of *Fibrobacter succinogenes* in reactor on wheat substrate.
The production of succinate ( ) and acetate (◆) was estimated by HPLC analyses.
Evolution of pH (■) and redox potential (■) were also reported.

3.4 ESA-substrate degradation in anaerobic reactor using *Fibrobacter succinogenes*

For this experiment, the substrate was prepared before the meeting in Gent. It was made up of wheat (3.84 g), mixed tomato (0.96 g), faeces (0.6 g) and spirulina (2.6 g).
With these conditions, the results showed that the succinate and acetate production were very low in comparison with the previous result on wheat only. In parallel the pH value was very stable (Figure 13). It seems that we could expect a problem of inhibition on this ESA-substrate.

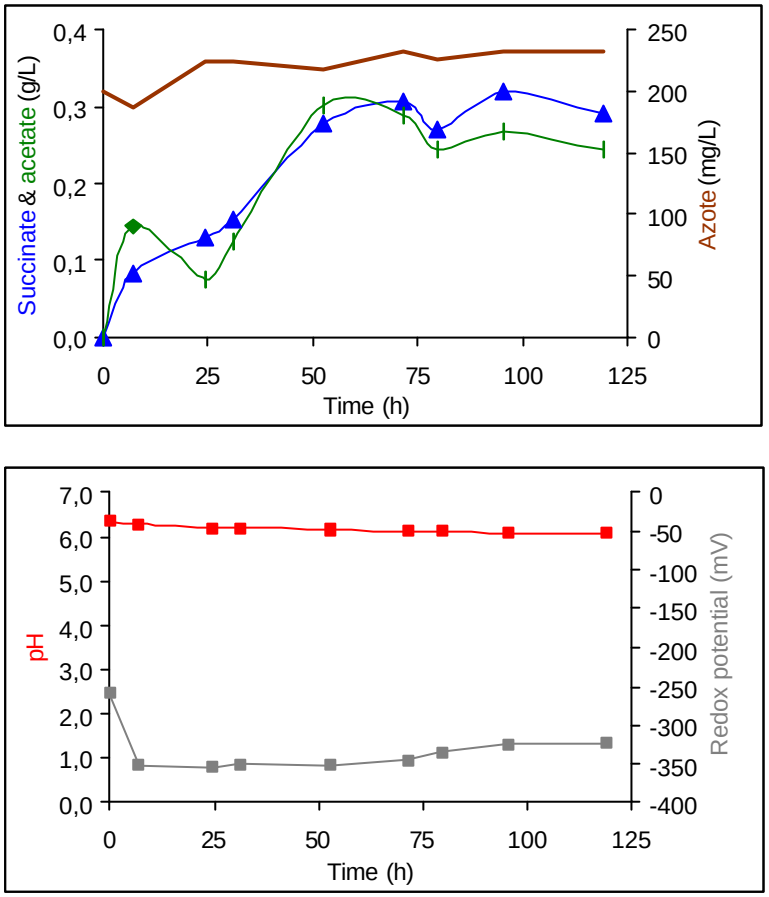

Figure 13: Growth of *Fibrobacter succinogenes* in reactor on ESA - substrate.
The production of succinate ( ) and acetate (◆) was estimated by HPLC analyses.
Evolution of NH3 consumption (—), values of pH (■) and redox potential (■) were also reported.

4.0 GROWTH ON SINGLE SUBSTRATE

Trying to find an explanation to the bad result on ESA-substrate, we had decided to make single substrate culture, one by one and two by two. Firstly we have expected to target the hypothetical inhibition and secondly it was a good procedure to know the potentialities of *Fibrobacter succinogenes* on each substrate. These experiments are provided in serum-bottle during 15 days to be sure that the degradation is all completed.

The cultivation conditions involved production of succinate and acetate but not in same concentrations. The results show clearly that in front of faeces and in spite of the association of another substrate, *F.succinogenes* is unable to grow on this waste (Figure 14). A similar result is obtained with two concentrations in faeces (0.5 and 1 g/L) which are very low in comparison with the composition of the ESA-substrate defined in Gent.

In a second part, the influence of initial concentration was tested on wheat substrate and the results show that there is no significant difference between 30, 20 and 15 g/L.
At 8 g/L the degradation will be less important but we estimate that in our conditions the total fibers concentration is near 15 g/L.

The table 3 gives the percentage of degradation of each ESA-substrate. The best result is obtained with cabbage then soya and then wheat which is a logical advance in order to the percentage of fibers in each substrate.

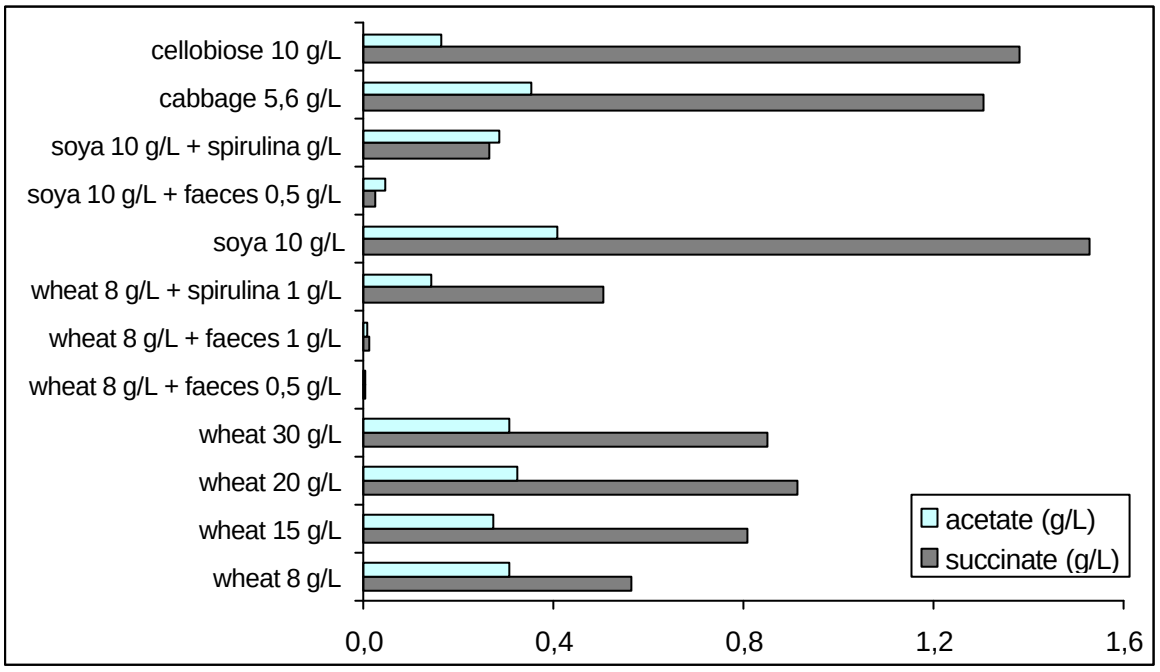

Figure 14: Degradation in serum bottle with separated substrates

Table 4: The percentage of specific degradation by *F. succinogenes*

	degradation (%)
wheat 8 g/L	31.7
wheat 8 g/L + faeces 0.5 g/L	8.3
wheat 8 g/L + faeces 1 g/L	8.3
wheat 8 g/L + spirulina 1 g/L	23.7
soya 10 g/L	62.6
cabbage 5.6 g/L	78.2

5.0 CONCLUSIONS

ESA-substrate was prepared and characterized.

We have designed an operational reactor for the fermentation of *Fibrobacter succinogenes* with the constraints of strictly anaerobic condition. The process was controlled by measurements of pH, redox, temperature and CO_2 pressure.

We have developed protocols to follow bacterial growth in terms of metabolites production (succinate and acetate), solid substrate consumption by dry mass assay and control of non-limitation by the azote source.

As it was decided in the project, we have developed a specific method to analyze the solid-substrate degradation by COD . Firstly we have implemented the theoretical model by the growth on glucose and validated the equation by comparison between these predicted data and the HPLC values. So we have tested this method on consumption of solid-substrates (cellulose and wheat).

This TN includes a report about the first tests of lignocellulosic degradation on cellobiose, wheat and ESA-substrate in reactors.
The cellobiose experiment serves as a standard calibration of the degradation. *Fibrobacter succinogenes* grows on wheat, however the results of this culture have shown that kinetics are lower than standard growth on cellobiose for metabolites production, biomass production and the percentage of wheat's degradation. Growth on ESA-substrate have revealed an inhibition probably due to the presence of faeces.

This direct approach failed, so we have decided to conduct single substrates tests in bottles in order to obtain specific data according to each compounds. These cultures allowed to confirm inhibitions of growth when *Fibrobacter succinogenes* is cultivated on fiber-substrates associated with faeces. Nevertheless, the results of degradation on single wastes prove that *Fibrobacter succinogenes* carried out the alternative of the MELISSA project for degrading wastes issued from higher plants compartments.

APPENDIX 1

Composition of primary solutions used for the preparation of the culture medium

- Mineral solution I

K_2HPO_4	0.6 %

- Mineral solution II

KH_2PO_4	0.6 %
$(NH4)_2SO_4$	1.2 %
NaCl	1.2 %
$MgSO_4$	0.12 %
$CaCl_2$	0.12 %

- Mineral solution III

$MnSO_4$, $6H_2O$	15 mg/L
$CoCl_2$, $6H_2O$	1.5 mg/L
$FeSO_4$, $7H_2O$	40 mg/L

- VFA solution

Acetic acid	17 mL
Propionic acid	6 mL
Butyric acid	4 mL
Isobutyric acid	1 mL
Isovaleric acid	1 mL
n-valeric acid	1 mL
D-L-2methylbutyric acid	1 mL

- Rezasurin solution: 1 tablet in 50 mL (redox indicator)
- biotin: 0.125 mg/mL
- PABA: 0.5 mg/L
- hemin: 0.1 %.

APPENDIX 2

Preparation of culture medium

Products	Bottle 100 mL	Reactor 1.2L
Solution I	7.5 mL	90 mL
Solution II	7.5 mL	90 mL
Solution III	300 µL	3.6 mL
VFA solution	300 µL	3.6 mL
Biotin	20 µL	240 µL
PABA	10 µL	120 µL
Hemin	70 µL	1 ml
Resazurin	300 µL	240 µL
Substrate	0.8 g	9.6 g
distilled H_2O	84 mL	1008 mL
Sterilization 120°C , 20 min. After sterilization, the bottle or the reactor is put under CO_2 flux.		
Na_2CO_3	0.4 g	4.8 g
Cystein	0.05 g	0.6 g
Cystein and Na_2CO_3 are added in the hot medium to induce the redox potential.		

References:

Bryant M.P. and Doetsch R.N. (1954)
A study of actively cellulolytic rod-shaped bacteria of the bovine rumen , *J.Dairy Sci*., 37, 1176-1183.

Chesson A. and Forsberg C.W. (1997)
Polysaccharide degradation by the rumen microorganisms, Hobson, P.N., Stewart, C.S., *the Rumen Microbial Ecosystem*, 2nd ed. Blackie Academic. London, 329-381.

Creuly C., Pons A. and Dussap C.G. (2001)
Metabolic investigation of an anerobic cellulolytic bacterium: *Fibrobacter succinogenes* , in *Focus on biotechnology*, volume 4, Hofman M. and Thonart P.(eds), Kluwer Academic Publishers, Dordrecht, 157-167.

Dussap C.G., Pons A., Pequignot C. and Gros J.B. (1997)
Application d'une méthode de réconciliation de données à des cultures menées en fermenteur discontinu, *Récents Progrès en Génie des Procédés*, 11, 7-12.

Gaudet G., Forano E., Dauphin G. and Delort A.M. (1992)
Futile cycle of glycogen in *Fibrobacter succinogenes* as shown by in situ ^{1}H-NMR and ^{13}C-NMR investigation, *Eur.j.Biochem*., 207, 155-162.

Glass T.L. and Sherwood J.S. (1994)
Phosphorylation of glucose by a guanosine-5'-triphosphate (GTP)-dependent glucokinase in *Fibrobacter succinogenes* S85, *Arch. Microbiol*.,162,180-186.

Gottschalk G. (1986)
Bacterial metabolism. 2nd ed, Springer-Verlag, New-York.

Hungate R.E. (1950)
The anaerobic mesophilic cellulolytic bacteria, *Bacteriol.Rev*., 14, 1-49.

Joyner Jr A.E. and Baldwin R.L. (1966)
Enzymatic studies of pure cultures of rumen microorganisms, *J.Bacteriol*., 92, 1321-1330.

Matte A., Forsberg C.W. and Verrinder A.M. (1992)
Enzymes associated with metabolism of xylose and other pentoses by *Prevotella (Bacteroides) ruminicola* strains, *Selenomenas ruminantium* D and *Fibrobacter succinogenes* S85, *Can.J.Microbiol*., 38, 370-376.

Matheron C., Delort A.M., Gaudet G. and Forano E. (1996)

Simultaneous but differential metabolism of glucose and cellobiose in *Fibrobacter succinogenes* cells, studied by in vivo 13C-NMR, *Can.J.Microbiol*.,42, 1091-1099.

Malburg L.M.Jr, Smith D.C., Schellhorn H.E and Forsberg C.W. (1993)
Fibrobacter succinogenes S85 has multiple xylanase genes. J. Appl. Bacteriol., 75, 564-573.

Miller T.L. (1978)
The pathway of formation of acetate and succinate from pyruvate by *Bacteroides succinogenes*, *Arch.Microbiol*., 117, 145-152.

Selinger L.B., Forsberg C.W., Cheng K.J. (1996)
The rumen : a unique source of enzymes for enhancing livestock production, *Anaerobe*, 2, 263-284.

Stewart C.S. and Flint H.J. (1989)
Bacteroides (Fibrobacter) succinogenes. A cellulolytic anaerobic bacterium from the gastrointestinal tract, *Appl.Microbiol.Biotechnol*., 30, 433-439.

Wells J.E., Russel J.B., Shi Y. and Weimer P. J. (1995)
Cellodextrin efflux by the cellulolytic ruminal bacterium *Fibrobacter succinogenes* and its potential role in the growth of non adherent bacteria, *Appl.Environ.Microbiol*., 61, 1757-1762.