Université Blaise Pascal
CLERMONT-FERRAND

Laboratoire de Génie Chimique et Biochimique
LGCB

A TOTAL CONVERTING AND BIOSAFE LIQUEFACTION COMPARTMENT FOR MELISSA

TECHNICAL NOTE: 86.2.2

Optimization of cellulose breakdown

-Gilles DUSSAP

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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

The results of the preliminary biodegradation test of ESA-substrate by pure culture of *Fibrobacter succinogenes* were presented in TN 2.100. A significant hydrolysis was noticed on different substrates as glucose, cellobiose, cellulose, wheat, tomato, soya. Based on the results of these experiments it was decided to test the degradation of complete ESA-substrate in a controlled reactor under CO_2 atmosphere to maintain the anaerobic conditions (table1). One of the conclusions was the unability of *F.succinogenes* to grow when it is placed in the vicinity of faeces.
This report gives tests performed in order to improve the degradation using the vegetable part of ESA-substrate (wheat, soya and cabbage) on fed-batch process.

Table 1 : Schematic overview of the content of the technical note 2.100

CONCEPT
The potentialities of *F.succinogenes*
Production of the ESA-substrate
Degradation of part of ESA-substrate one by one
METHODS
Quantification of extracellular metabolites (glucose, succinate, acetate, VFA) by HPLC
Estimation of degradation by developement of COD method suitable to our process and by dry mass measurement of the substrate residue at the end of experimentation.
EXPERIMENTS
* Tests in bottle which give results of degradation on single part of ESA-substrate

Substrate	Degradation (%)
Wheat	32
Soya	63
Cabbage	78
Faeces	0
Spirulina	0

* Tests in anaerobic reactor with specific substrate: cellobiose, wheat and the previous composition of ESA-substrate (wheat + tomato + faeces + spirulina). The cellobiose experiment serves as a standard degradation. The experiment on wheat has shown that kinetics are lower than the standard growth. The growth on the previous ESA-substrate has confirmed the inhibition due to the presence of faeces.

2.0. PROCESS DESIGN

2.1 General conditions of cultivation

F.succinogenes was grown under 100% CO2 in a basal medium described in TN2.100.

2.2 ESA conditions of cultivation

The basal medium (without carbon source) was added with 2% of dry matter.
All ingredients (table2) were mixed in a kitchen blender.
Fresh green cabbage were first cut in smaller parts and then subsequently mixed in a kitchen mixer. Wheat straw was grind in dry conditions with a kitchen mixer and then a blender until about 5 mm particules were obtained.
Table 2 : Composition of ESA-substrate in the three reactors examined in this technical note

	Reactor 1		Reactor 2 and reactor 3	
Component	DM (g)	proportion (% w/w)	DM (g)	proportion (% w/w)
wheat	28	33,3	33,6	29,2
soya	28	33,3	33,6	29,2
cabbage	28	33,3	33,6	29,2
spirulina	0	0	14,2	12,5

We have chosen to add a fraction of cellobiose at the beginning of the experimentation in reactors 1 and 2 to allow a rapid colonization of fibers by *F.succinogenes*. In these conditions, we could delete the lag phase which is usual on complex substrates like lignocellulosic components. The reactor 3 was started without cellobiose in the initial medium.
During the fed-batch procedure, the dry matter was added once a week and in this case the substrate was made up only with ESA-substrate : wheat straw 5.6 g (%dry matter 94.2), soya waste 5.6 g (%dry matter 90.6), fresh cabbage 76g (%dry matter 7.4), and spirulina 2.4 g. The percentage of DM were determined by weighing after 24 hours at 110°C in oven.

2.3 Experimental set-up

The experiments were performed using the anaerobic reactor described in TN 2.100.
At the beginning of experimentation, the reactor of 2 liters was filled with 1.2 liters of medium (basal medium + vegetable matter) and sterilized at 120°C during 20 min. After sterilization, the reactor was flushed under CO_2 effluent (in the gazeous part in the top of the glass). Cystein and Na_2CO_3 were added in the hot medium to decrease the redox potential. When redox conditions were obtained (-300mV), the temperature was regulated to 40°C and 200 ml of preculture were added. In this process, the reactor was filled up and the gaseous phase was taken up a small part in comparison with the liquid phase. After inoculation, the reactor was sealed gaz tight and the flush of CO_2 was connected only when samples were taken for the analysis of the liquid phase.

2.4 Analysis of the liquid phase

Measurement of pH value : on-line with Ingold probe

Measurement of redox value : on-line with Ingold probe

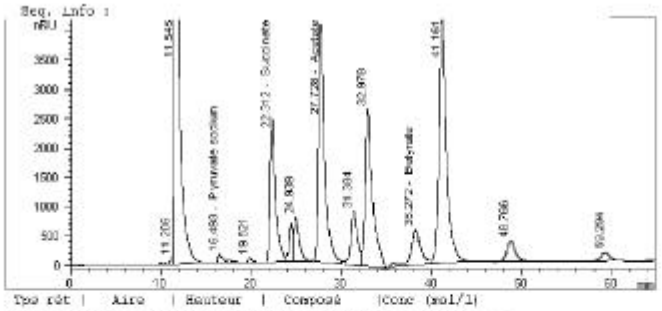
Extracellular metabolites : off-line, quantification of glucose, succinate, acetate, VFA by high pressure -liquid chromatography after calibration of each component. An example of high liquid pressure chromatogram is given in table 3.

2.5 Analysis of the solid phase

Degradation of solid substrate : off-line, at the end of the culture, by COD method described in TN 2.100 and by filtration/weighing of the residue. At the end of the experiment (after 1000 hours of culture), the entire volume of the reactor is filtered. The cake is dried (24 hours at 110°C in a oven) and weighed.

Determination of lignocellulosic residue : off-line, at the end of the culture, by sequential determination of cell-wall components. This method uses a treatment with neutral and acid detergent and sulfuric acid (Van Soest, 1963) . The protocol is described in the french standard (AFNOR, NF V 18-122, 1997)

Table 3 : Analysis of soluble products by HPLC



Tps rét	Aire	Hauteur	Composé	Conc (mol/l)
10.202	1549	42		0.00000
11.206	1196	115		0.00000
11.545	412004	13756		0.00000
0.000	0	0	Cellobiose	0.00000
0.000	0	0	Citrate	0.00000
16.493	4122	142	Pyruvate sod	0.00048
17.523	1290	39	Gluconate	0.00007
0.000	0	0	Glucose	0.00000
18.303	415	15		0.00000
0.000	0	0	Fructose	0.00000
19.821	2638	81		0.00000
22.712	97995	2450	Succinate	0.01029
0.000	0	0	Lactate	0.00000
24.370	18199	648	Glycerol	0.00236
24.939	31165	754		0.00000
27.720	177477	4046	Acetate	0.05809
31.384	45772	917		0.00000
0.000	0	0	Propionate	0.00000
31.978	141605	2697		0.00000
0.000	0	0	Isobutyrate	0.00000
0.000	0	0	Ethanol	0.00000
30.272	45806	590	Butyrate	0.00685
41.161	246001	4226		0.00000
0.000	0	0	Isovalerate	0.00000
48.766	22885	357		0.00000
0.000	0	0	Valerate	0.00000
59.294	10027	141		0.00000
				0.07804

3.0 RESULTS

3.1 Standard culture on cellobiose

In standard conditions, which means on soluble substrate like cellobiose and with only one addition of substrate in the course of process, the extracellular products are basically succinate and acetate The results in figure 1 show a production of succinate 4 g/l and acetate 3 g/l. They are consistent with the results published by Matheron et al, 1997.

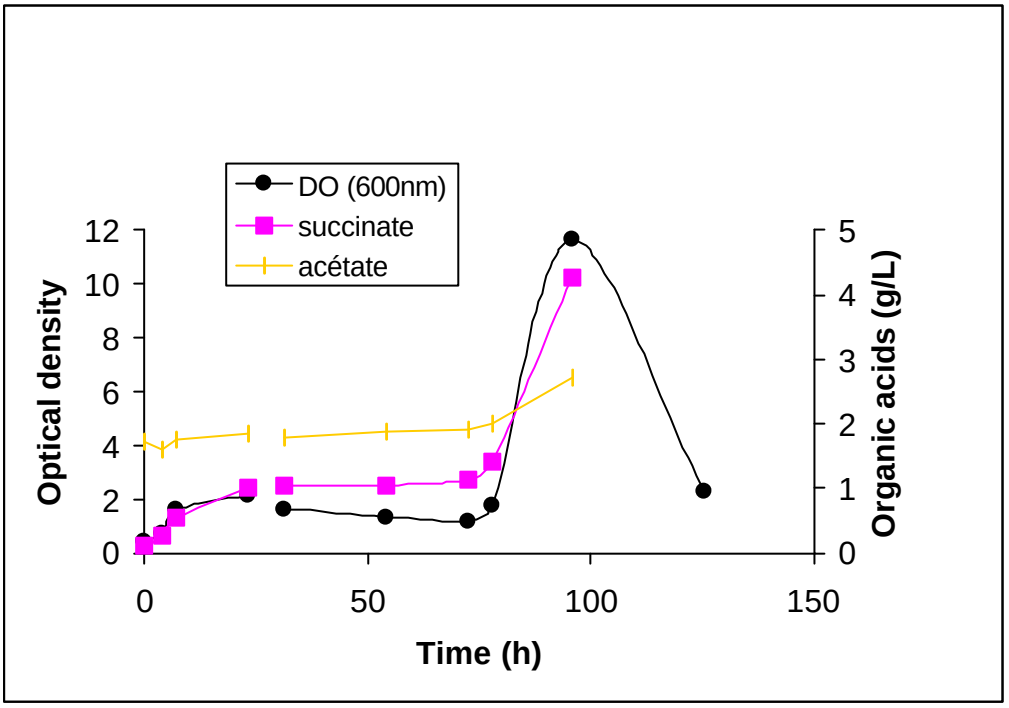


Figure 1 : Growth of *Fibrobacter succinogenes* in reactor on cellobiose.
Report of optical density (●), production of succinate (■) and acetate (◆)

The cellobiose is cleaved in two molecules of glucose by a cellobiase or in one glucose and one glucose-1-phosphate by a cellobiose phosphorylase (Matheron et al, 1996). Actually, we could firstly observed a decrease of cellobiose concentration and an increase of glucose concentration. The increase of cellobiose concentration after 25 hours is due to the fact that we added an other fraction of cellobiose (Figure 2).

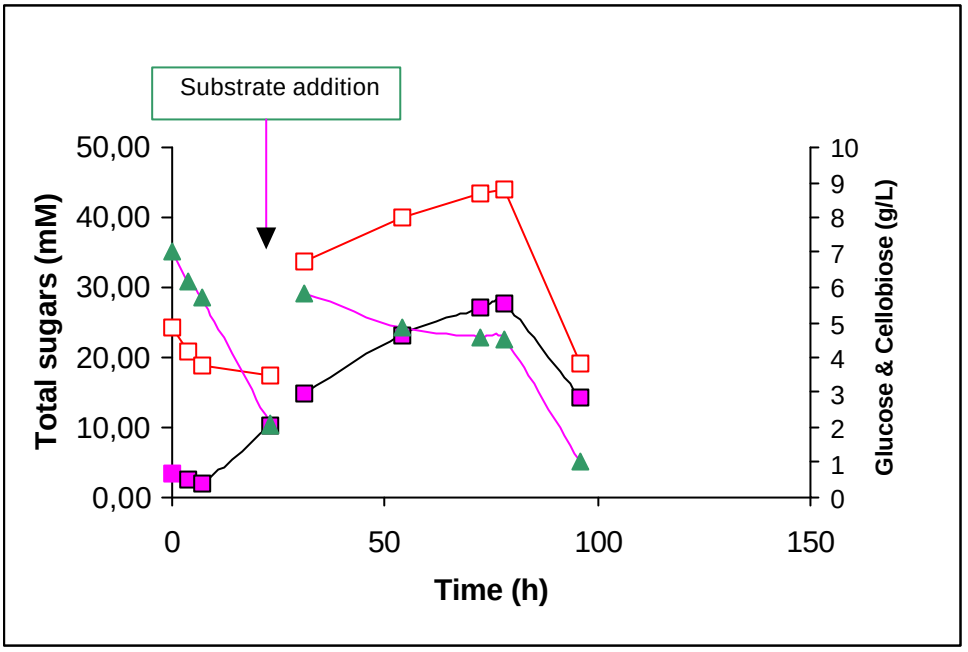


Figure 2 : Growth of *Fibrobacter succinogenes* in reactor on cellobiose.
Report of consumption of substrates: cellobiose (▲), glucose (■) and total sugars (□)

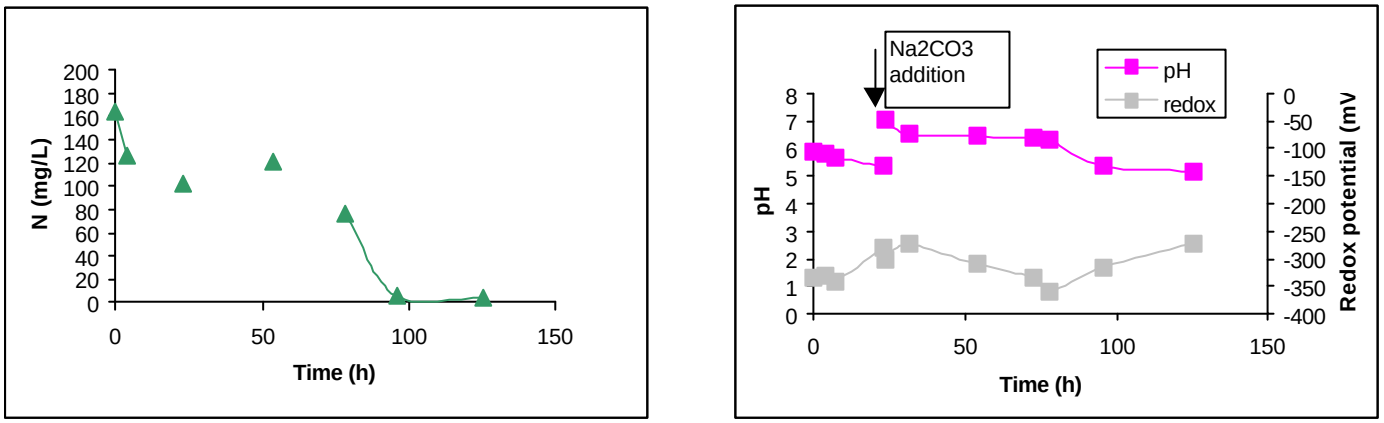


Figure 3 : Growth of *Fibrobacter succinogenes* in reactor on cellobiose.
Report of NH3 consumption (▲), values of pH (■) and redox potential (■)

After 100 hours of culture, there was a limitation by the nitrogen source and the reactor was stopped. This culture would be weighing as a standard behaviour of *F.succinogenes* on soluble substrate and would be just used to compare the metabolism of this bacteria on ESA-substrate.

3.2 Vegetable degradation of ESA-substrate, Reactor 1 : Wheat straw + soya waste + cabbage With cellobiose at t = 0

Within the results of the previous TN , *F.succinogenes* is unable to grow on faeces. In spite of this problem, we have shown that *F.s* remains a good alternative to the first compartment of MELISSA observing that it could degrade lignocellulosic substrates. In the TN 2.100, we have begun to test in reactor the degradation of single substrate one by one: wheat, soya and cabbage. In this report, we have stand up for testing the association of the three vegetables in concentration decided in the meeting of Gent.
The figure 4 gives a general view of all parameters measured during this experiment.

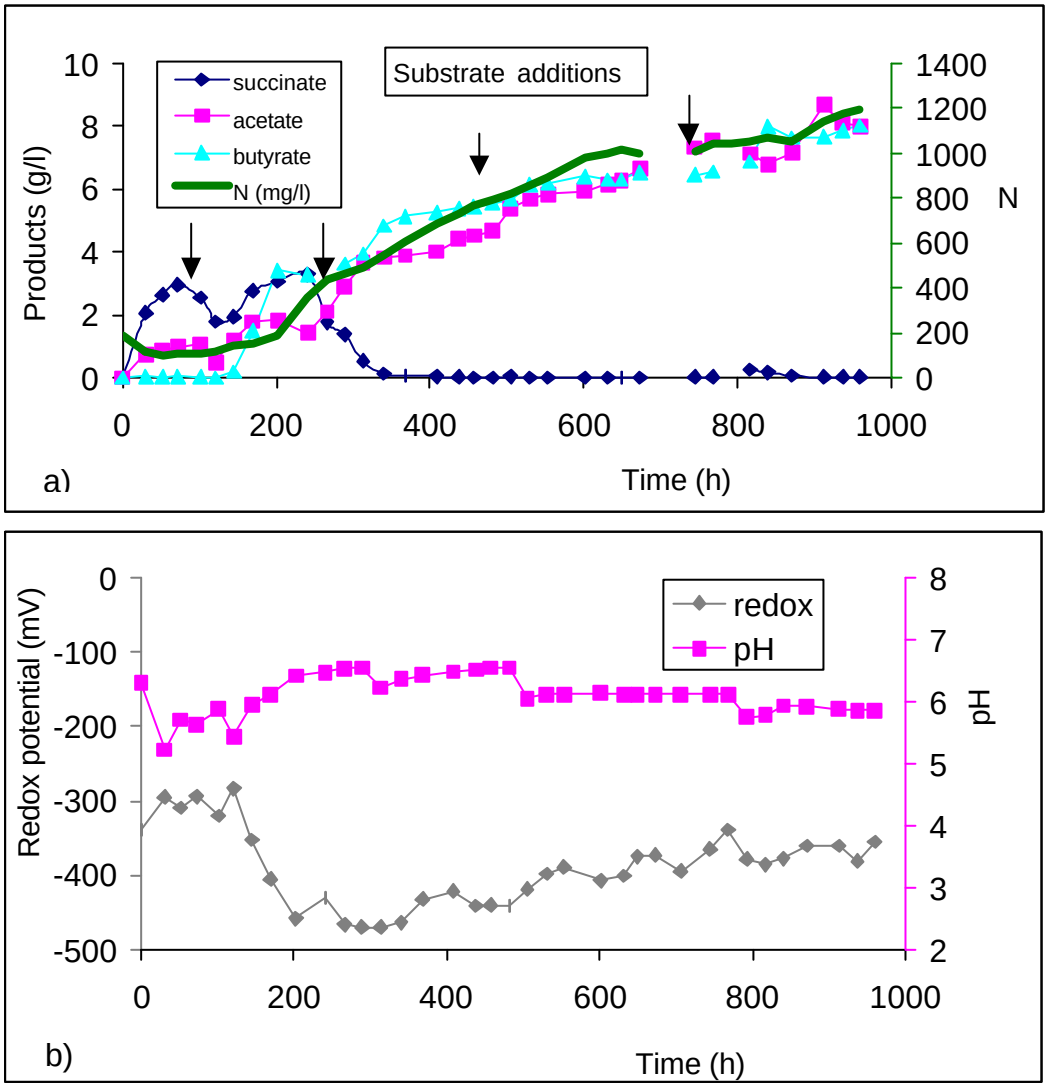


Figure 4 : Growth of *Fibrobacter succinogenes* in reactor 1 (on vegetable part of ESA-substrate).
a) Report of products: succinate (◆), acetate (■), butyrate (▲),NH3 evolution (–)
b) Report of pH values (■) and redox potential (◆)

3.2.1 Analysis of the first step : 0-100h

Figure 5 reports for the first hundred hours.

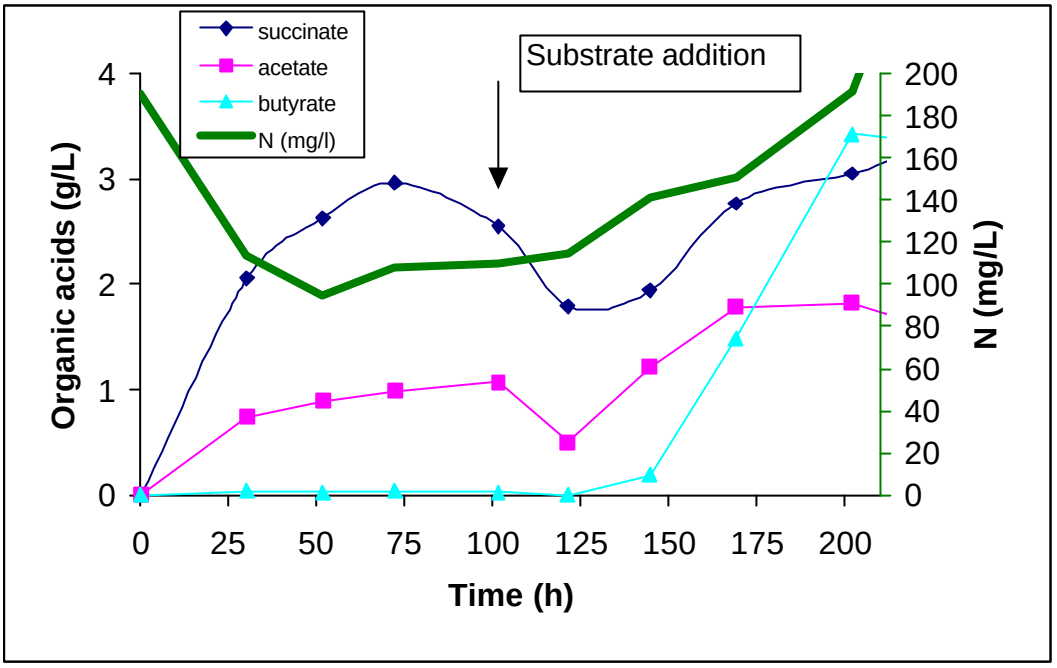


Figure 5 : Growth of *Fibrobacter succinogenes* in reactor on vegetable part of ESA-substrate.
Report of products: succinate (◆), acetate (■), butyrate (▲),NH3 evolution (–)

The comparison between the figure 5 and figures corresponding to a standard culture on cellobiose (figures 1 at 3) gives to believe that in this step of culture, *F.s* degrades at once the cellobiose present in the initial medium. In this case, it developes a classical metabolism, producing succinate (3g/l) and acetate (1g/l). The HPLC analysis shows that after 40 hours of culture, there was no more cellobiose in the medium which could prove that after this time, *F.s* degrades only the non-soluble part of substrate but always produces succinate and acetate. During this step, there was also a consumption of $(NH_4)2SO_4$.

3.2.2 The second step : 100-250 h

At 100 hours, we added a dose of vegetable part: wheat (5.6g), soya (5.6g) and fresh cabbage (76g ≅ 5.6g DM). The same figure (5) shows that the concentrations in succinate and acetate decrease during 24 hours before increasing again.

3.2.3 The third step : 250 –1000 h

After 250 hours, the substrate had been added three times (figure 4). During this stage, succinate was consumed and the products were acetate and butyrate with high level of concentration, near 8 g/l. This particular metabolism with production of butyrate was never described in the literature.

On the other hand, the metabolism of cellulose was investigated using in vivo ^{13}C NMR and ^{1}H NMR (Bibollet et al., 2000).The quantitative determination of metabolic fluxes showed the reversibility of different metabolic pathways in *F.succinogenes*: reversibility of glycolysis and reversibility of succinate synthesis pathway toward the production of acetate. This phenomenon could be an explanation to the change of metabolism observed on our experimentation.

Matheron et al.(1999) showed that the presence of ammonia increased the reversal of succinate synthesis pathway while the other reversed routes remained unchanged. Therefore, it was exactly our case in this reactor. The analysis of N curve reveals a high level of nitrogen excreted in the medium in the form of ammonia. Maybe, it will be correlated to the acetate concentration.

3.2.4 Quantification of degradation

Substrate added (1/3 wheat+1/3 soya+1/3 cabbage) = 84 g DM

Substrate non treated after 1000 hours of culture = 28 g DM

Substrate degraded = 56 g DM , ie **67%** of the initial quantity in 41 days

Degradation yield = 1.36 g DM /day

Total volume of the reactor : 1.6 L

Specific degradation yield : 1.05 g /L.day

3.3 Degradation of ESA –substrate , Reactor 2 : Wheat straw + soya waste + cabbage + spirulina With cellobiose at t = 0

Using the same initial condition (cellobiose 8 g/L) that the previous experimentation, reactor 2 was implemented with spirulina in addition to vegetable part of ESA-substrate.

3.3.1 Analysis of the liquid phase

The figure 6 gives the general profile of the degradation which could be correlated to the profile shown in figure 5. During the first step (0 - 100 h), *Fibrobacter succinogenes* develops an usual metabolism with production of succinate and acetate. Between 100 and 250 h , we could make the assumption that we observed a metabolic balance characterized by a stabilisation of acetate and succinate concentration and production of propionate (butyrate in the reactor 1). During the last step, until 1000 hours, this production increases in large amounts (about 8 g/L) after consumption of succinate.

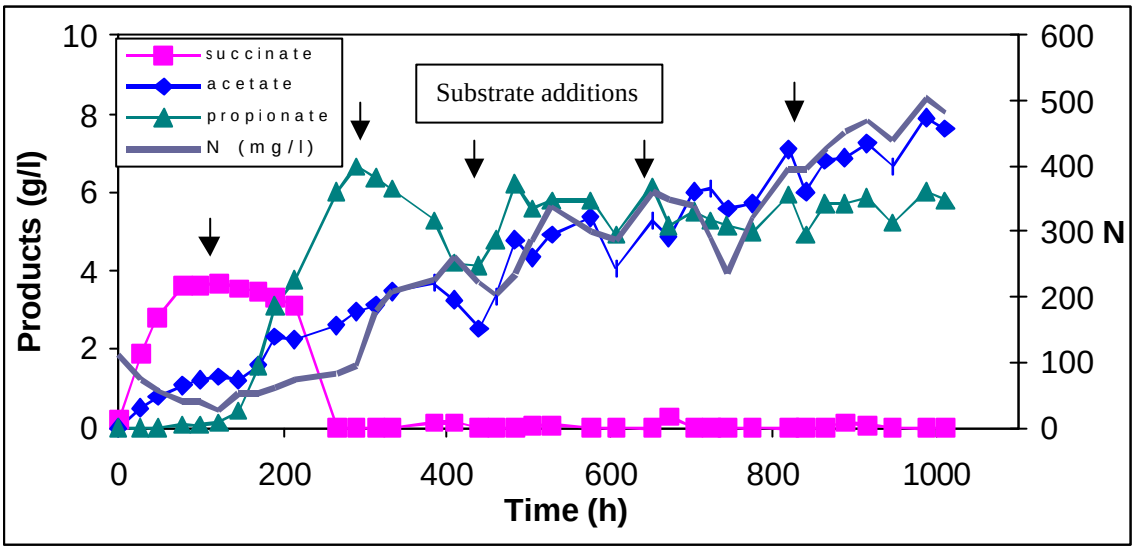


Figure 6 : : Growth of *Fibrobacter succinogenes* in reactor 2 (vegetable part + spirulina).
a) Report of products: succinate (■), acetate (◆), propionate (▲),NH3 evolution (–)
b) Report of pH values (■) and redox potential (◆)

3.3.2 Quantification of degradation by dry mass measurement

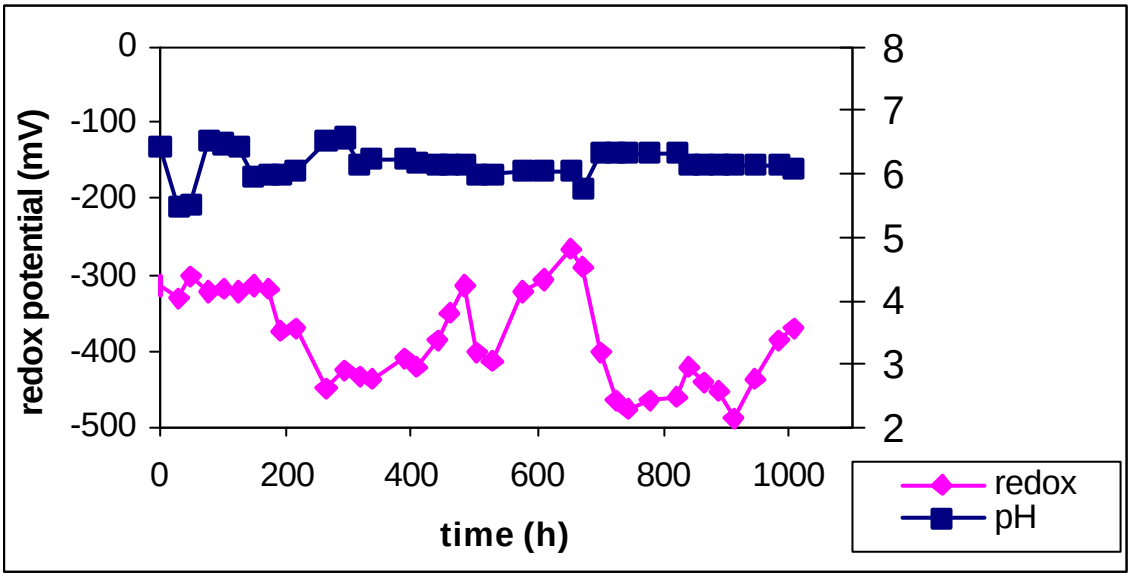
Substrate added (wheat straw+soya+cabbage+spirulina) = 115 g DM

Substrate non treated after 1000 hours of culture = 49 g DM

Substrate degraded = 66 g DM ie 57 % of the initial quantity in 41 days

Degradation yield = 1.61 g DM / day

Specific degradation yield : 1.01 g DM / L.day



The previous tests in bottle have demonstrated that there is no growth of *F.succinogenes* on spirulina, probably because of the proteic composition of spirulina which can't be used as carbon and energy sources by F.s. Therefore, the quantity added during the fed-batch could be recovered at the end of the process. The substrate really involved in the degradation process will be only **100.8 g** DM and from the same point of view, the part of substrate after 1000 h which could be take in account is only **34.5 g DM**. The percentage of vegetable degradation is **66%**, value similar to the one obtained in reactor 1 (67%). That confirms in the opinion that there is no degradation of spirulina.

3.3.3 Quantification of degradation by COD measurement

The $_{substrate}$ COD is defined as the value obtained by the standard curve considering wheat straw + soya waste + cabbage + spirulina :

$_{substrate}$ COD (g O2/L) = 0.725 x dry mass (g substrate added in the medium)

After 1000 h of culture, the $_{total}$ COD and the $_{soluble}$ COD were measured by a representative sample of the heterogeneous medium.

On the reactor 2, these analyses give the following results :

Sum of $_{substrate}$ COD added : $_{substrate}$ COD = 84.3 g O_2/L

Final value obtained after 1000 hours of culture : $_{total}$ COD = 71.4 g O_2/L

Final value in liquid phase : $_{soluble}$COD = 27.9 g O_2/L

Final value of solid COD : 71.4 – 27.9 = 43.5 g O_2/L

According to a previous analysis (TN 2.100), the substrate degradation by *F.s.* does not result in a decrease of total COD in a reactor, the solid substrate being mainly split in small organics (succinate, acetate, propionate and CO_2). Consequently, the total COD is supposed to marginally decrease during the time course of the culture.
The previous results lead to calculate that 84.3 g O_2/L – 71.4 g O_2/L = 12.4 g O_2/L are not recovered in the reactor volume which could indicate that 12.4 / 84.3 = **15 %** of initial COD is flushed out the reactor in the gas phase during the culture. This part can be volatile fatty acids and CO_2.
The percentage of COD solid degradation is calculated by 43.5 / 84.3 = **50 %**. This is significantly lower than the evaluation of degradation by dry mass measurement which is 57 %.

3.3.4 Quantification of degradation by cell-wall measurement

Sequential determination of cell-wall components by Van Soest method was improved on single substrate, on ESA-substrate and on the dry residue obtained after 1000 h of culture. The raw data are shown in table 4.

Table 4: Raw data about cell-wall determination

	Total fibers %DM	Hemicellulose %DM	Cellulose %DM	Lignin %DM
Wheat straw	75.5	23.2	45.2	6.3
Soya waste	9.7	4.7	5	0
Cabbage	15.6	3.4	11.6	0.6

Mix (1/3,1/3,1/3)	33.3	10.3	20.4	2.3
Reactor 2 residue	49.1	18.2	27.2	3.7

Knowing data from dry mass measurement and using hypothesis that spirulina could not be a substrate, degradation of each component could be calculated :

Total substrate added : 115 g DM
Total spirulina added : 14.4 g DM
Total substrate after 1000 hours of culture : 49 g DM

Total fibers : 51 % DM degraded $\dfrac{0.333 \times (115-14.4) - 0.491 \times (49-14.4)}{0.333 \times (115-14.4)}$

Hemicellulose : 40 % DM degraded $\dfrac{0.103 \times (115-14.4) - 0.182 \times (49-14.4)}{0.103 \times (115-14.4)}$

Cellulose : 54 % DM degraded $\dfrac{0.204 \times (115-14.4) - 0.272 \times (49-14.4)}{0.204 \times (115-14.4)}$

Lignin : 45 % DM degraded $\dfrac{0.023 \times (115-14.4) - 0.037 \times (49-14.4)}{0.023 \times (115-14.4)}$

These results point out an homogeneous degradation of cell-wall components, near 50%. Importantly, it has to be emphasized that for soya and cabbage, the fiber fraction to be degraded remains low (10 and 15 % respectively) compared to the total dry matter to remove.

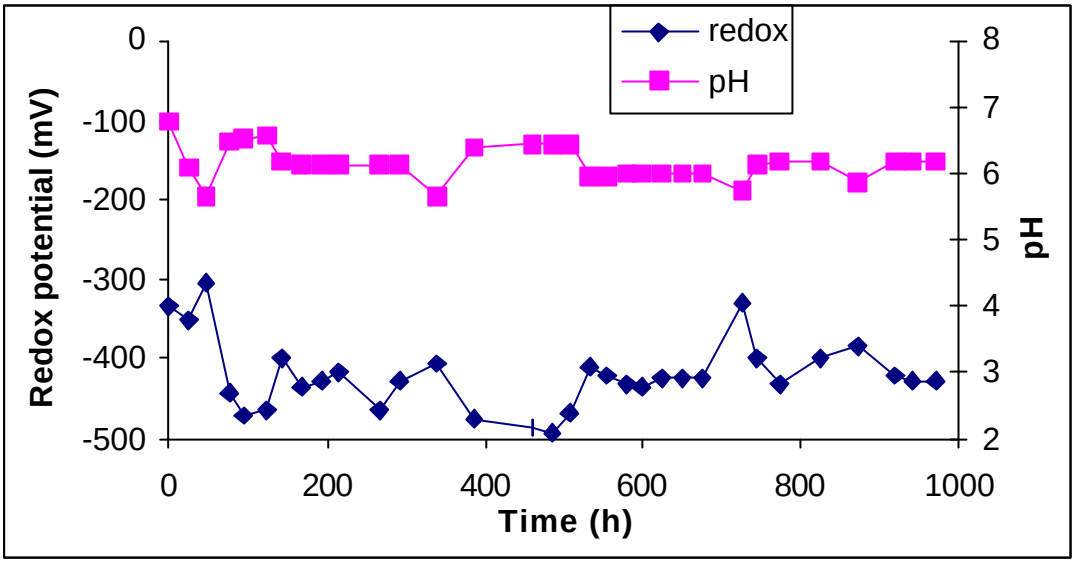
3.4 Degradation of ESA –substrate , Reactor 3 : Wheat straw + soya waste + cabbage + spirulina Without cellobiose at t = 0

The third experiment was improved without addition of cellobiose in the initial medium.

3.4.1 Analysis of the liquid phase

The general profile of the curves (figure 7) is similar to those obtained in reactor 1 or 2 but not in the same scale of products concentrations. Succinate and propionate concentrations are half for the same production of acetate (8 g/L). In the rumen ecosystem, propionate is well-known to be produced from succinate by direct decarboxylation (Gottschalk, 1986).This pathway is employed by *Selenomonas ruminantium* which is the major propionate-producing organism in the rumen and which get rid of succinate produced by cellulolytic bacteria (Scheifinger et al., 1973). None published data indicates that *F .succinogenes* is able to use this pathway on standard

substrate. Two hypothesis could be advanced; the first is that our process involves high concentration of complex lignocellulosic substrates which dictate a metabolic balance to allow a detoxification of the medium. The second hypothesis is that the culture is systematically contaminated by the wheat straw addition even if this substrate is always sterilized. At the present time, several tests are operated to try to find an explanation and will be presented in the next TN.



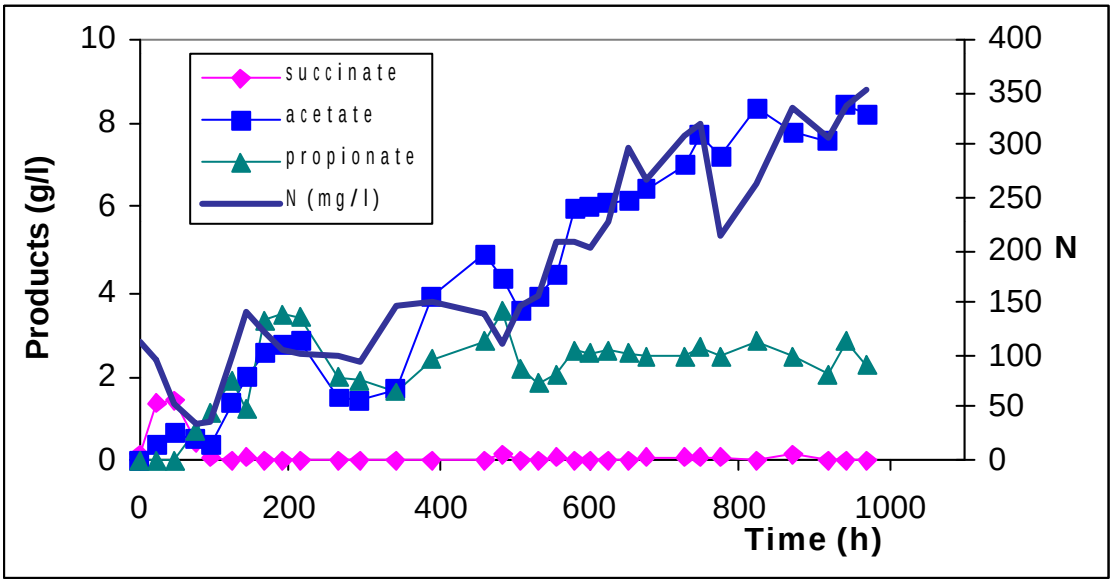


Figure 7 : : Growth of *Fibrobacter succinogenes* in reactor 3 (vegetable part + spirulina).

a) Report of products: succinate (■), acetate (◆), propionate (▲),NH3 evolution (–)
b) Report of pH values (■)and redox potential (◆)

3.4.2 Quantification of degradation by dry mass measurement

Substrate added (wheat straw+soya+cabbage+spirulina) = 115.2 g DM
+ faeces at the mid term
Substrate non treated after 1000 hours of culture = 46.6 g DM

Substrate degraded = 68.6 g DM ie 59 % of the initial quantity in 41 days

Degradation yield = 1.45 g DM / day

Specific degradation yield = 0.91g DM /L.day

According to the hypothesis that spirulina and faeces could not be considered as potential substrates, the dry mass corresponding are removed from the total value. The results become :
Substrate added (wheat straw+soya+cabbage) = 90.7 g DM

Substrate non treated after 1000 hours of culture = 22 g DM

Substrate degraded = 68.7 g DM ie **75.7 %** of the initial quantity in 41 days

3.4.3 Quantification of degradation by COD measurement

On the reactor 3, these analyses gives the next results :

$_{substrate}$ COD = 78.8 g O_2/L (wheat + soya + cabbage + spirulina + faeces)

$_{total}$ COD = 65.5 g O_2/L which could indicates that 16.8 % COD is flushed in the gazeous phase

$_{soluble}$ COD = 38.8 O_2/L

According to these values, the percentage of degradation obtained by COD measurement is **66 %.**

There is still a slight discrepancy between degradation yield estimation by COD (66 %) and dry mass measurements (76 %).

The comparison of the results between reactor 3 (no initial cellobiose) and reactor 2 (initial 8 g/L cellobiose) seems to indicate that the degradation is more efficient in reactor 3. This must be confirmed. An explanation could be that the production of VFA is lower on reactor 3 due to lower total initial sugars concentration which could result in a lower inhibition of *F.s* metabolism. This could lead to interesting strategy of elimination during the culture.

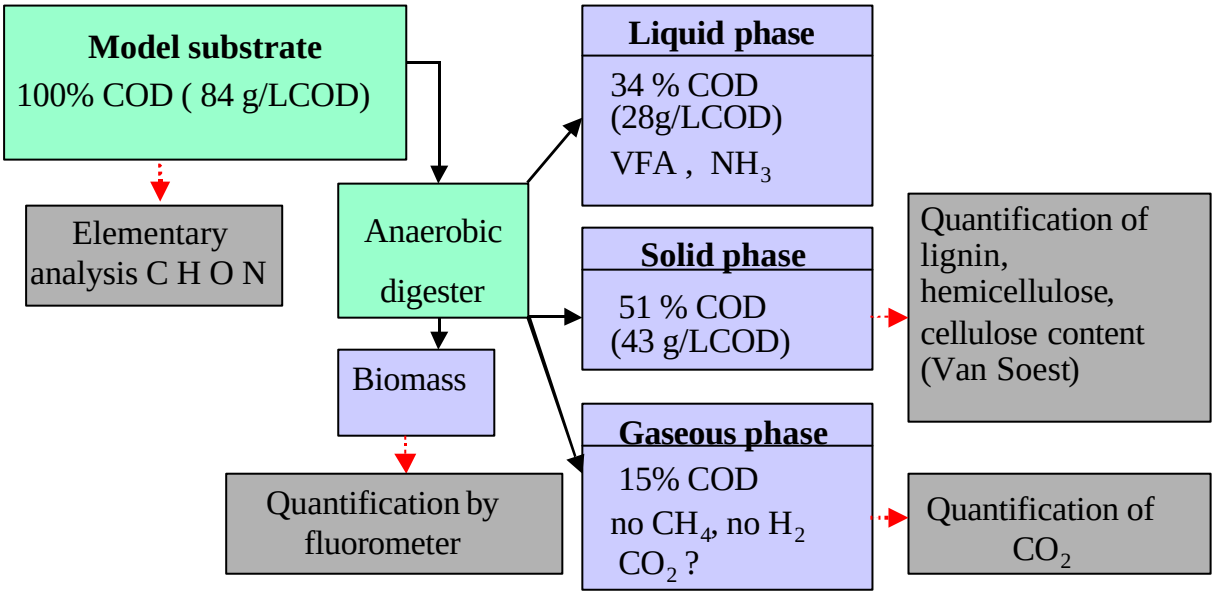
3.4.4 Quantification of degradation by cell-wall measurement

Reactor 3 was stopped at the end of january and these values are not yet available.

4.0 SUMMARY / OUTLOOK

Actually, the process using *Fibrobacter succinogenes* can degrade vegetable part of ESA-substrate with a very good efficiency. Tests on single substrates show that degradation of wheat straw was performed with 32%, soya waste 63%, cabbage 78% (dry mass estimation).
Spirulina which is a proteic substrate, is not degraded by *F.s*.
An ambiguity remains about the influence of faeces because a recent test on a new faeces sample shows that *F.s* can growth when placed in the vicinity of this material in association with a cellulosic substrate but it could not be considered as a substrate source. This is in contradiction with results presented in the previous TN and could be explained by a bad sample of faeces (antibiotic ?). An addition of faeces at the mid term of reactor 3 shows that degradation of vegetable part is not affected by faeces.

In all cases, *F.s* does not seem to be able to degrade faeces and spirulina.
The association of wheat straw, soya waste and cabbage gives between 56 % and 76 % of degradation based on dry mass measurement and between 50 and 66 % of degradation based on COD analysis. The dry mass measurement 67 % (reactor 1), 66% (reactor 2) and 76 % (reactor 3) seems to be the best method because it concerns the whole solid part but there is on the one hand an overestimation because it includes the biomass content and on the other hand an underestimation because there are significant losses during the filtration.
Van Soest method for fibers fraction estimation has been implemented which enables to follow the fibers fraction degradation.
The analytical methods are presently improved according to those presented in Barcelona:



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