

**A TOTAL CONVERTING AND BIOSAFE LIQUEFACTION  
COMPARTMENT FOR MELISSA**

**TECHNICAL NOTE: 86.2.3**

**Production and characterisation of substrate coming from  
*Fibrobacter succinogenes***

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# TABLE OF CONTENTS

<b>1.0 INTRODUCTION .....</b>	<b>2</b>
<b>2.0 PROCESS .....</b>	<b>2</b>
2.1 GENERAL CONDITIONS OF CULTIVATION.....	2
2.2 PREPARATION OF ESA SUBSTRATES .....	2
2.3 EXPERIMENTAL SET -UP .....	2
2.4 ANALYSIS OF THE LIQUID PHASE.....	3
2.5 ANALYSIS OF GASEOUS PHASE.....	3
2.5.1 Pressure measurement .....	3
2.5.2 Determination of gas phase composition.....	3
2.6 ANALYSIS OF SOLID PHASE.....	4
2.6.1 Dry mass measurement .....	4
2.6.2 DCO analysis .....	4
2.6.3 Determination of cell wall components degradation by Van Soest method.....	4
<b>3.0 RESULTS AND DISCUSSION .....</b>	<b>7</b>
3.1 CULTURE ON ESA-SUBSTRATES .....	7
3.1.1 Analysis of the liquid phase.....	8
3.1.2 Analysis of the gas phase.....	9
3.1.3 Analysis of the solid phase .....	11
3.2 ASSAYS OF CLOSED LOOP EXPERIMENTS .....	13
3.2.1 First closed loop experiment.....	13
3.2.2 Second closed loop experiment.....	15
<b>4.0 CONCLUSIONS / OUTLOOKS .....</b>	<b>17</b>
<b>REFERENCES.....</b>	<b>18</b>
<b>APPENDIX.....</b>	<b>19</b>

## 1.0 INTRODUCTION

The efficiency of *Fibrobacter succinogenes* for the degradation of the vegetable part of ESA-substrates was shown in TN2.200. Hydrolysis of 55-75% of substrates containing wheat straw, soya, cabbage, spirulina were obtained. The problem of inhibition of *Fibrobacter succinogenes* to grow in presence of faeces was solved by the change of the source of faeces and it was demonstrated that *F.s.* could go on growing when fecal material was added in the time course of the culture.

Following these results, it was decided to test the ability of *F.s.* to grow on ESA-substrates containing faeces since the beginning of the culture. During this culture, improvements of the analysis of liquid, solid and gas phase were performed.

Finally, according to the last meeting in Barcelona, degradation of substrate of partner 1 were performed for 1<sup>st</sup> and 2<sup>nd</sup> loop.

## 2.0 PROCESS

### 2.1 General conditions of cultivation

*F.s.* was grown under 100% CO<sub>2</sub> in a basal medium described in TN2.100.

### 2.2 Preparation of ESA substrates

Wheat straw and soya were ground in dry conditions in a blender.

Fresh cabbage was first cut in small parts and then ground in a kitchen mixer.

Spirulina and fecal material were dried and ground in a blender.

All the substrates were sterilized 20' at 121°C

Component	DM (g)	Proportion (% w/w)
Wheat straw	61.3	23.3
Soya	61.3	23.3
Cabbage	61.3	23.3
Spirulina	26.3	10.0
Faeces	52.7	20.0

**Table 1** : Composition of ESA substrates in the reactor

### 2.3 Experimental set-up

The general set-up of the culture was the same as described in TN2.200.

In order to improve the experimental process, the culture of *F.s.* was performed in an anaerobic 4L reactor. It was equipped with pH, redox (Ingold) and temperature probes and a pressure sensor connected to a control unit. On-line data acquisition of these four variables was realized on a computer with Biowatch software. Data acquisition of each of the parameters was realized every 15 minutes.

## 2.4 Analysis of the liquid phase

Measurement of pH value : on-line with Ingold probe.

Measurement of redox values : on-line with Ingold probe, absolute values.

Extracellular metabolites : off-line, quantification of glucose, succinate, acetate and VFA by HPLC analysis.

Nitrogen analyses : N-NH<sub>4</sub> concentration by Patton and Crouch method.

## 2.5 Analysis of gaseous phase

### 2.5.1 Pressure measurement

As it was supposed that *F.s* could produce CO<sub>2</sub> in our culture conditions, a pressure sensor (range 2 bars) was installed on the anaerobic reactor and connected to the control and acquisition units.

At the beginning of the culture (just after inoculation), pressure in the reactor was manually increased at about 1.1 bar in order to assist the beginning of the growth. This action was repeated after each opening of the reactor (substrate addition, sampling of liquid phase).

An electro-valve (2 ways, PVDF, range 2 bars) was also installed on the reactor in order to respect the specification of reactor concerning resistance for pressure. This electro-valve was first calibrated and programmed to be opened during 2.5 seconds each time that pressure in the reactor reaches 1.5 bar. This opening results in the diminution of pressure at about 1.15 bar.

### 2.5.2 Determination of gas phase composition

GC analysis were performed to determine the composition of gaseous phase.

Technical specification	GC model	Hewlett Packard 5890
	Columns Types	Porapak Q 50/80, 2m – 1/8 Molecular sieve 40/60, 5Å
	Detector Type	Thermo Conductivity Detector
Operating conditions	Carrier gas	CO <sub>2</sub> or H <sub>2</sub>
	Oven temperature	60 → 150°C
	Injector temperature	150°C
	Detector temperature	120°C

**Table 2:** Characteristics of GC system used for determination of gas phase composition

GC analyses were performed with two carrier gas (H<sub>2</sub> or CO<sub>2</sub>) in order to be sure that no peak could be hidden under the major CO<sub>2</sub> peak.

Gas samples were taken by a gas tight syringe and injected in the gas chromatograph.

## 2.6 Analysis of solid phase

### 2.6.1 Dry mass measurement

One of the problems for the determination of percentage of degradation by filtration/drying was the possibility for short particles to cross over the filter. In order to solve this problem and to keep liquid and solid phase safe for the sent to partner 1 (2<sup>nd</sup> loop), a new protocol for dry mass measurement was tested.

All the content of the reactor was centrifuged (15', 16400 g). The supernatants were kept and all the pellets containing the residues of fermentation were weighed. A part of these pellets was desiccated (heated vacuum desiccator, 60°C, 24h) and then weighed. A second part was re-suspended, filtrated, dried (24h, 110°C) and weighed. The third part was re-suspended with the supernatant.

Percentages of degradation were calculated at the same time by filtration and desiccation for the total content of the reactor.

### 2.6.2 DCO analysis

The protocol used is described in TN2.100.

### 2.6.3 Determination of cell wall components degradation by Van Soest method

Degradation of fibrous part of vegetal cell wall is one of the most important obstacle to a efficient degradation of vegetable wastes. The strict connection between chains of cellulose, hemicellulose and lignin form a matrix difficult to reach for a micro-organism. For this reason, the estimation of degradation of these polymers could be a good indicator for the efficiency of the process.

Many methods have been developed to determine cell wall composition in a vegetable substrate. Gravimetric methods allow to calculate an estimation of the proportion of each the components whereas other methods allow a more precise determination of the concentration of only one of the three polymers.

#### - *Gravimetric methods*

They are based on dry mass measurement of the sample analyzed after successive hydrolyses with different solutions (detergent and/or acid solutions). Each hydrolysis eliminates one of the component and dry mass difference between before and after the hydrolysis allow to calculate the proportion of this component. These methods give an *estimation* of cell wall composition in a vegetable sample.

All these gravimetric methods have been initiated by Van Soest (Van Soest, 1963). Many modification and evolution have been performed since the initial protocol, concerning the order of the hydrolyses and the nature of the hydrolysis solutions in order to get more accurate results (Jarrige, 1963 ; Van Soest and Wine, 1967 ; Christian, 1971). They present many advantages. First, they give the proportion of total fibers and of the three different polymers with the same experiment. Second, they do not use a very specific and costly material. Third, they can be used in the same way with all the vegetable substrates (Pochet, 1987).

- *Measurement of specific component*

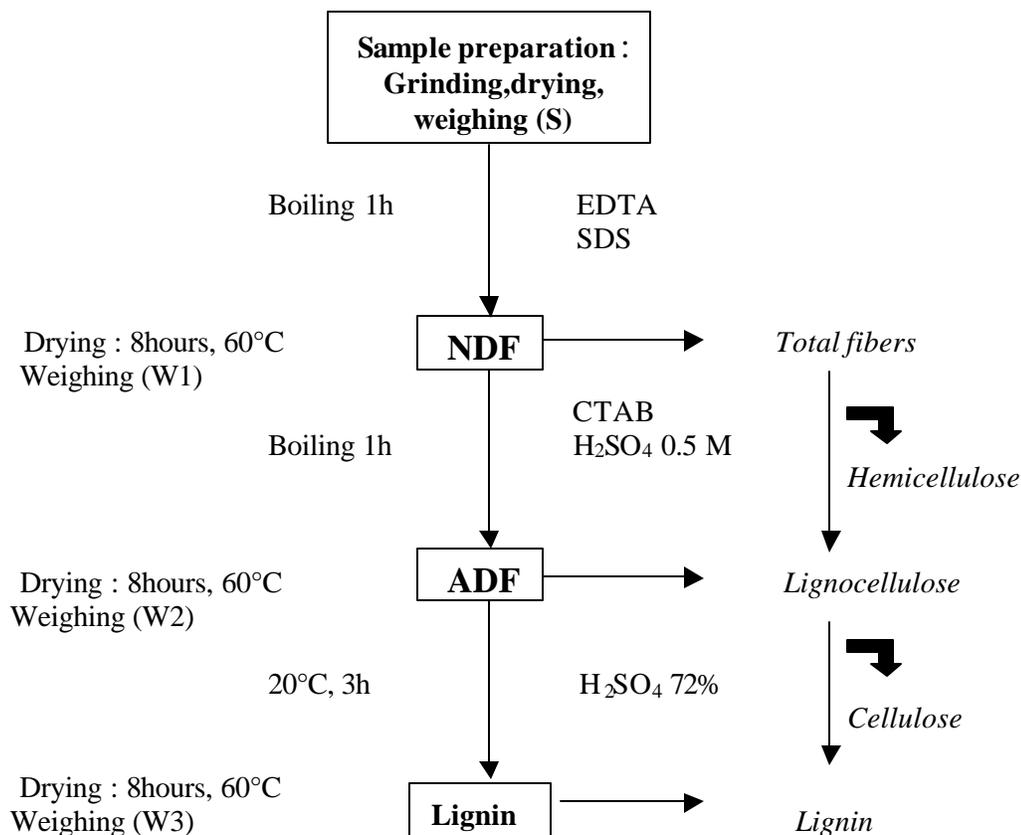
Many other methods have been developed for a more *accurate determination* of the concentration of one of the cell wall component in a sample. These methods require more specific techniques like for example IR-spectroscopy or chromatography (Morrison, 1972).

They are based on measurement of products of degradation of cellulose, hemicellulose or lignin. If the results obtained with these methods are slightly more precise, they need the realization of a standard. This can be done easily for cellulose which is a homopolymer of glucose units. On the contrary, it is more complicated for hemicellulose and especially for lignin that are heteropolymers with a high heterogeneity in the monomers composition in function of the species, the age, the organ and the tissue of the vegetal. As a matter of fact, it becomes difficult to obtain an efficient standardization.

For all these reasons, it appeared that the best choice for the determination of cell wall composition of our samples was one of the version of Van Soest method. We have chosen one of the accurate precise and used gravimetric method which can be performed either with a semi-automatic system or with simple laboratory materials.

A team from INRA (National Institute for Agronomic Research) which routinely performs this kind of analysis with the semi-automatic system was contacted to have more information on the protocol and have the possibility to compare our results obtained with simple laboratory materials and their results with the semi-automatic system.

The protocol used is described in the following figure and in the appendix. It is based on three successive hydrolysis with neutral then acid detergent solution and finally concentrate sulfuric acid. Each of these hydrolyses separates respectively total fibers, hemicellulose, cellulose and lignin. The percentage of each component is calculated from drying / weighing after each step.



$\text{Total fibers} = \frac{S - W1}{S} \times 100$	$\text{Hemicellulose} = \frac{W1 - W2}{S}$
$\text{Cellulose} = \frac{W2 - W3}{S} \times 100$	$\text{Lignin} = \frac{W3}{S} \times 100$

**Figure 1** : Schematic representation of Van Soest method

Table 3 gives the comparative results between experiments performed with laboratory equipment in UBP and semi-automatic system used in INRA. The assays were made on the three vegetable ESA-substrates (wheat straw, soya and cabbage) and the solid culture residue after 1000 hours of fermentation of a previous reactor described in TN 2.200 (containing wheat, soya, cabbage and spirulina as substrate).

All the experiments was repeated twice and the percentages presented in table 3 represent the average of the two values.

Sample	Team	Total Fibers %DM	Hemicellulose %DM	Cellulose %DM	Lignin %DM
Wheat straw	UBP	71.3	25.0	39.1	7.2
	INRA	74.7	23.2	45.2	6.3
Soya bean	UBP	11.4	4.7	5.6	1.1
	INRA	9.7	4.7	5.0	0
Cabbage	UBP	15.9	1.6	13.4	0.9
	INRA	15.6	3.4	11.6	0.6
Residue of reactor	UBP	50.4	10.1	26.6	13.7
	INRA	39.1	8.2	27.2	3.7

**Table 3:** Comparison of the results obtained in UBP and INRA for cell wall composition (%DM) of the three vegetable substrates and the residue of reactor by Van Soest method.

Concerning the vegetable substrates (wheat, soya, and cabbage), there is no significant difference between INRA and UBP results. It clearly appears that cabbage and soja, which contains respectively 15% and 10% of fibers and less than 1% of lignin are much easier to degrade than wheat straw (72% for total fibers, 7% for lignin). The method used in UBP without the semi automatic system can be validated for these single vegetable substrates.

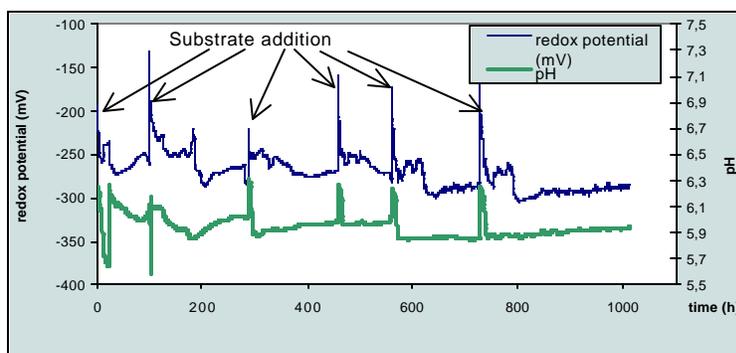
Concerning the analysis of solid residue after 1000 hours of culture, the difference between the two methods is much more important, especially for lignin proportion. Furthermore, it appears a more important heterogeneity in the values between the two tests performed as well in UBP and INRA (data not shown). This problem clearly points out the necessity to obtain samples as homogenate as possible and particularly for the quantity of recalcitrant fibers from wheat straw. The preparation of the sample, and especially the size and the homogeneity of the particles, is a key step for the validity of the results.

### 3.0 RESULTS AND DISCUSSION

#### 3.1 Culture on ESA-substrates

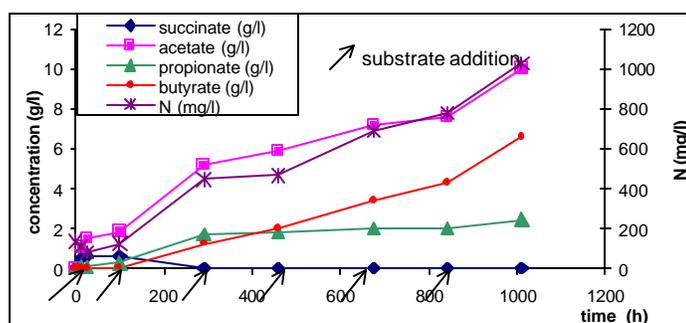
In the previous TN, it was shown that *F.s.* could grow when faeces were added in the time course of the process. It was decided to perform a standard culture of *F.s.* on total ESA-substrates since the beginning of the culture. The process was maintained during about 1000 hours including 6 additions of substrate.

### 3.1.1 Analysis of the liquid phase



**Figure 2 :** Evolution of pH and redox potential during growth of *F.s.* on ESA-substrates.

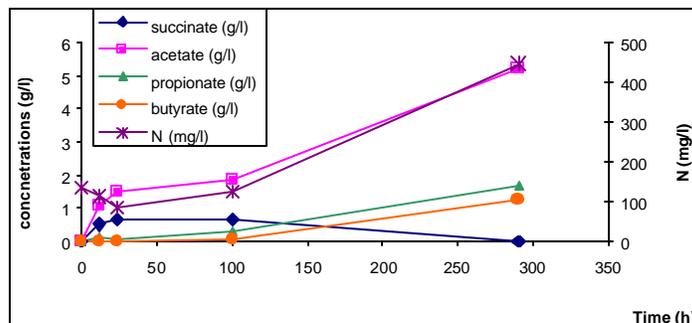
Figure 2 shows the evolution of pH and redox potential during the time course of the fermentation process. During each addition of substrate, the opening of the reactor results in an increase of redox value. This is regulated by addition of  $\text{Na}_2\text{CO}_3$  which has the double effect of decreasing redox potential and increasing pH values. The important production of organic acids during the first hours after a substrate addition results in an important decrease of pH.



**Figure 3 :** Evolution of extracellular metabolites during the growth of *F.s.* on ESA-substrates

Figure 3 shows a general profile similar to profiles observed in previous culture performed without cellobiose and fecal material at the beginning of the culture.

If we focus on the beginning of the culture (figure 4), we can note a metabolic evolution in 3 steps. During the 24 first hours, succinate and acetate are the only metabolites produced. Then, their concentration remain stable until 100 hours. After this time, all the succinate is re-consumed and acetate and others VFA (propionate and butyrate) are produced in large amounts.



**Figure 4** : Evolution of extracellular metabolites concentration during the beginning of the culture (0-280h)

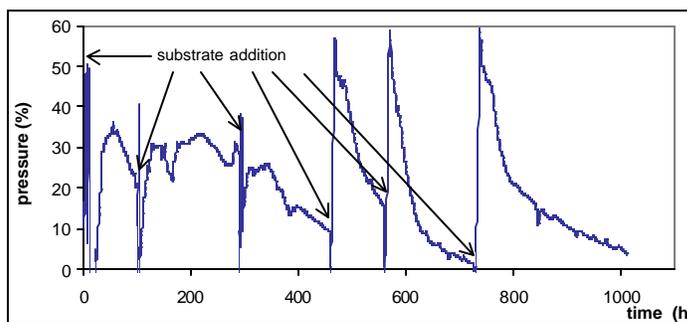
The final concentration of acetate is about 10 g/l whereas final propionate and butyrate concentrations are respectively 2 and 6 g/l after 1000 hours of culture. Contrary to the two previous reactors (TN 2.200), the major VFA produced in this reactor is butyrate.

Figure 3 and 4 finally show a classical evolution of N concentration with a diminution during the first hours of culture. The concentration then increases at the same time as VFA until a final concentration of 1g/l

If the production of acetate in large amounts (concentrations between 8 and 10 g/l) is a constant for all the reactors performed with ESA-substrates, it appears that the final concentration of the other VFA is variable and particularly for propionate and butyrate.

In the previous TN (TN2.200), it was proposed that one explanation for the important production of propionate might be the contamination by an another micro-organism from wheat straw. To check this hypothesis, classical culture media with wheat straw or total ESA-substrate as only carbon and energy sources were prepared in usual conditions but were not inoculated with the pure culture of *F.s*. No growth was observed in the serum bottles during the two weeks after inoculation. This tends to show that the production of propionate or butyrate is due to a change in the metabolism of *Fibrobacter succinogenes* in function of the substrate (presence of spirulina, faeces) and not a bacterial contamination of the media.

### 3.1.2 Analysis of the gas phase



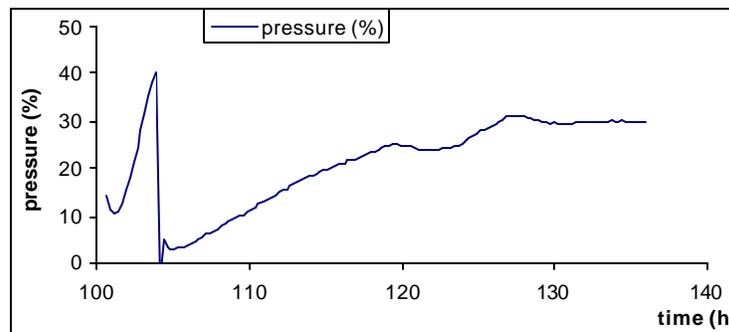
**Figure 5** : Evolution of pressure in the reactor. Pressure is expressed in % (100% represents 1bar in relative pressure)

Figure 4 shows the general evolution of pressure in the reactor during all the process. As explained before, pressure decreased to 1 bar during each opening of the reactor and was manually increased to 1.15 bar after.

It appears that substrates addition results in a large gas production during about 24 hours, which represents the same period as for pH diminution. These two observations give to understand that there are two steps in the degradation for each batch : a first one corresponding to the hydrolysis of substrate easily degradable (cabbage, soya) and a second one corresponding to the degradation of recalcitrant fibers. This hypothesis could be supported by the results of degradation on single substrate, which clearly showed a better efficiency of *F.s.* to degrade cabbage and soya than wheat straw, and by cell wall composition of each substrate determined by Van Soest method (percentage of fibers higher for wheat straw than soya than cabbage).

It also seems on figure 4 that total gas production was more important for the two first batches which could probably be explained by a higher increase of the biomass. The maximum gas flow production was 1.73 NI / l culture / day.

The diminution of pressure in the reactor a few hours after substrate addition is due to leaks. Indeed, it can probably be imagined that after a step of important gas production, the flow became lower and in a first time is compensated by the leaks on the reactor and then leaks became more important than gas flow production. It will be a problem to solve in order to determine the total gas volume produced and correlate gas production to bacterial growth.

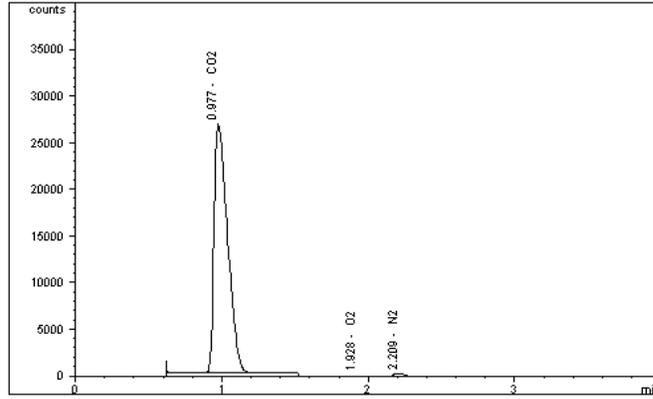


**Figure 6** : Evolution of pressure in the reactor during the first hours after the second substrate addition (100-136 h)

If we focus on the evolution of pressure during the second batch (figure 5), we can note a important gas flow production during the first hours after substrates addition. The opening of the valve after a few hours decreased pressure at about 1.05 bar. Gas production went on during 24 hours and then pressure in the reactor began approximately stable until the next batch. This stability could probably be explained by a reduction of the gas flow production which became equivalent to the gas leak.

Analysis of gas phase composition by GC showed that the only gas present in the reactor is CO<sub>2</sub>. Indeed, N<sub>2</sub> and O<sub>2</sub> short peaks observed in GC graph correspond to contamination during the gas analysis process.

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 Fichier : C:\HPCHEM\1\DATA\JEROME\5F3.D  
 Echantillon: sensitivity low all the time  
 methode Fmolaire ->  
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 Méthode: FMOLAIRE.M



N°	TR	Composé	F. molaire	Aire	Aire %
1	0.977	CO2	0.0187	169859	98.1151
2	1.928	O2	0.0023	742	0.4285
3	2.209	N2	0.0000	2521	1.4563
Total:			0.0210	173122	100.0000

**Figure 7** : Analysis of gas composition by GC

### 3.1.3 Analysis of the solid phase

#### 3.1.3.1 Dry mass measurement

- *measurement by desiccation*

total substrate added (wheat, soya, cabbage, spirulina and faeces) = 263g

substrate not treated after 1000 hours of culture = 130g

substrate degraded = 133g DM ie **51 %** of the initial quantity in 41 days

degradation yield = 3.24 g DM / day

total volume of the reactor = 3.6L

specific degradation yield = **0.9g DM /L.day**

- measurement by filtration / drying

substrate not treated after 1000 hours of culture = 113 g

substrate degraded = 150 g DM ie **57 %** of the initial quantity in 41 days

degradation yield = 3.66g DM / day

total volume of the reactor = 3.6L

specific degradation yield = **1.02g DM /L.day**

If we consider that spirulina and faeces are not potential substrates for *F.s.*, the dry mass corresponding is removed from the total values.

The new percentage of degradation become **72 %** and **81 %** respectively for desiccation and filtration / drying processes.

The percentage of degradation determined by the two different processes of dry mass measurement are roughly the same. The difference could be explained by the crossing of short particles through the filter.

The efficiency of degradation is approximately the same as for last reactor performed with wheat, soya, cabbage, spirulina and faeces at the mid term of culture (57% / 59%). Nevertheless, if it is considered that *F.s.* is able to degrade only vegetable part of ESA-substrates, this percentage of degradation become higher than the previous reactor (81% / 76%). These results confirm the high efficiency of *F.s.* for the degradation of vegetable wastes.

### 3.1.3.2 COD analysis

substrate COD = 71.9 g O<sub>2</sub>/L

total COD = 62.0g O<sub>2</sub>/L which indicates that 14 % COD is flushed in the gas phase.

soluble COD = 39.2 g O<sub>2</sub>/L

According to these values, the percentage of degradation obtained by COD measurement is about 68%.

### 3.1.2.3 Cell wall determination

Sequential determination of cell wall components was performed on the residue obtained after fermentation. These results were compared with the results obtained on the mix of the 3 vegetables substrate (wheat, soya and cabbage) in previous experiments

	Total fibers (%)	Hemicellulose (%)	Cellulose (%)	Lignin (%)
Mix (1/3 wheat, 1/3 soya, 1/3 cabbage)	33.3	10.3	20.4	2.3
Residue of reactor	41.0	16.8	18.3	5.9
Percentage of degradation	66	54	81	55

**Table 4** : Analysis of cell wall degradation by Van Soest method

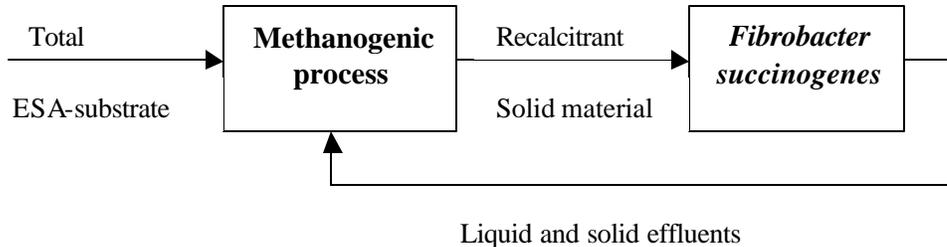
The percentages of degradation were calculated with DM of substrates added in the reactor and recalcitrant material after fermentation. Spirulina and faeces, which do not contain fibers, were not taken account in these calculations.

The percentage of degradation obtained for total fibers, hemicellulose and cellulose fractions seemed to be a good estimation of the potentialities of *F.s.* Indeed, the efficiency of *F.s.* for the degradation of hemicellulose and especially cellulose is described in literature.

Nevertheless, it is also documented that this bacteria does not possess the total enzymatic equipment for an efficient degradation of lignin. The 55% of degradation obtained for lignin is probably due to a experimental problem. Indeed, the presence of a large proportion of faeces in the sample analyzed results in the formation of a powder which is not eliminated during the three hydrolysis and modified the percentage of degradation of lignin.

### 3.2 Tests of closed loop experiments

In order to evaluate the complementarity of the different process of MAP project, it was decided to perform closed loop experiments. Recalcitrant solid material after the full methanisation compartment was dried and then used as only substrate for *Fibrobacter succinogenes*. The solid and liquid effluents after fermentation was then sent back to the methanogenic process (Ghent University).

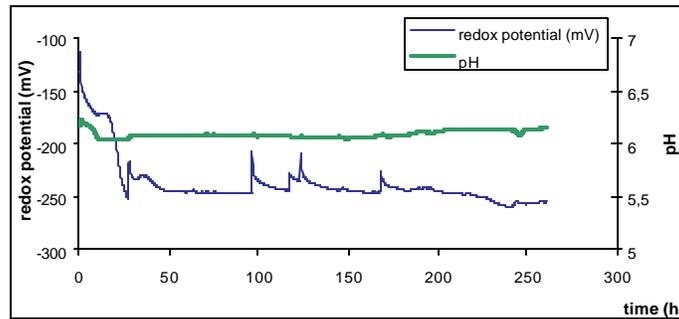


**Figure 8 :** Schematic representation of closed loop experiments

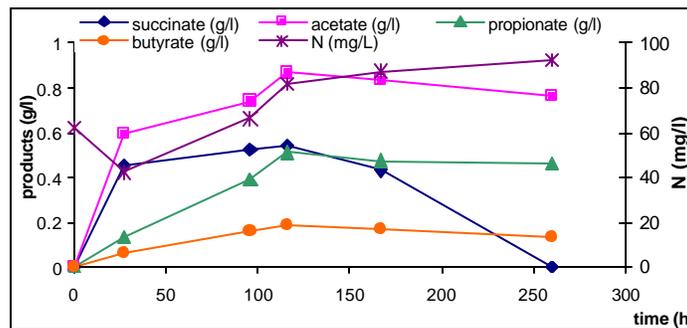
#### 3.2.1 First closed loop experiment

1<sup>st</sup> loop experiment was performed in a reactor of 4 liters in a batch process. The concentration of substrate in the reactor was roughly lower than usually. Indeed, only 28g of recalcitrant material were added in 2.8L of culture media prepared in usual conditions which corresponds to a twice lower concentration than our culture on ESA-substrates. This culture was maintained during about 260 hours.

### 3.2.1.1 Analysis of liquid phase



**Figure 9 :** Evolution of pH and redox potential during 1<sup>st</sup> loop analysis

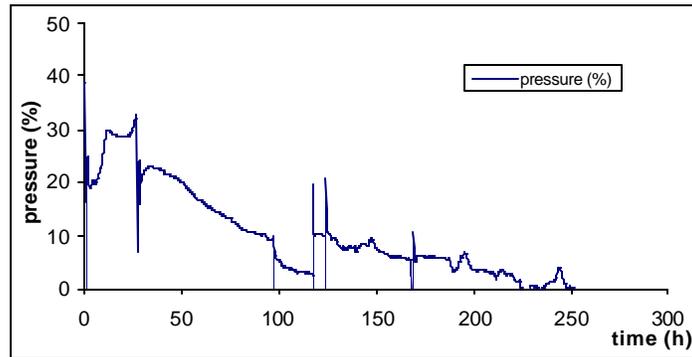


**Figure 10 :** Evolution of extracellular metabolites during 1<sup>st</sup> loop analysis

According to the figures 7 and 8, the general profile of the evolution of the liquid phase is similar to profile of culture on ESA-substrate. During the first hours of culture, there was a production of succinate and acetate as only metabolites and values of pH and redox potential decrease. After 24 hours, concentrations of succinate and acetate became stable and other VFA began to be produced. After 100 hours, the production of metabolites stopped which seemed to indicate that there was no more substrate that *F.s.* could degrade.

Final concentrations of metabolites are lower than those observed during culture on ESA-substrates for the same time. This could probably be explained by the lower concentration of substrate and the lack of easily degradable substrates.

### 3.2.1.2 Analysis of the gas phase



**Figure 11** : Evolution of pressure in the reactor during 1<sup>st</sup> loop analysis

The results concerning gas phase confirm the conclusions drawn for the liquid phase. The pressure in the reactor increased during the 24 first hours of the process and then decreased slowly because of the leak on the reactor. Gas production appears clearly less important than for the first batch of the culture on ESA-substrate

The same process than for the previous reactor was used to check that CO<sub>2</sub> was the only gas present in the reactor.

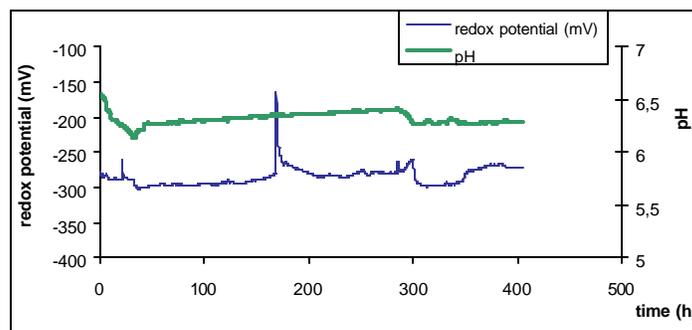
### 3.2.1.3 Analysis of the solid phase

Quantification of degradation by dry mass measurement was performed only by filtration /drying protocol. The non degraded substrate after 250 hours of culture represented 16.6 g which allowed to calculate a percentage of degradation of 28%.

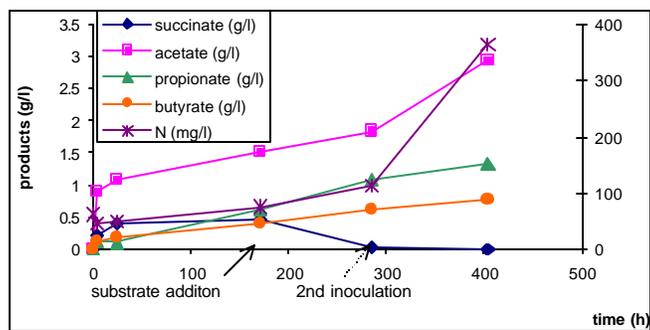
## 3.2.2 Second closed loop experiment

2<sup>nd</sup> loop was performed in fed batch conditions (2 addition of substrates) at a concentration similar to usual culture on ESA-substrates (44g of substrates in 2.8L of culture media). Culture was maintained during about 400 hours of culture.

### 3.2.2.1 Analysis of liquid phase



**Figure 12** : Evolution of pH and redox potential during 2<sup>nd</sup> loop



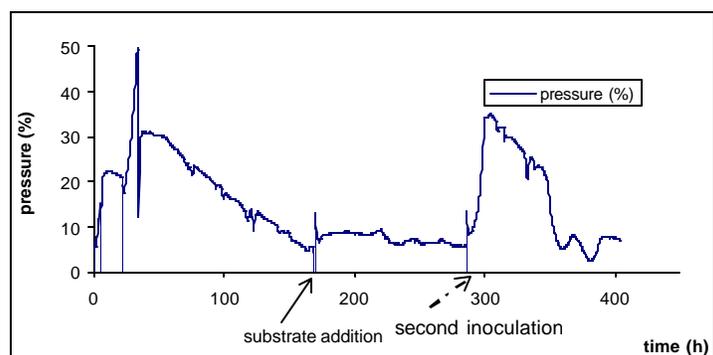
**Figure 13** : Evolution of extracellular metabolite s concentration during 2<sup>nd</sup> loop

The profile for pH, redox potential and extracellular metabolites is similar to 1<sup>st</sup> loop. The production of succinate and acetate during the beginning of the culture was more important which can be explained by a higher concentration of substrate in the reactor. Contrary to what was expected, the addition of substrate after 180 hours of culture did not result in a diminution of pH value. It was decided to re-inoculate the reactor with a second preculture prepared on glucose as substrate (280 hours). The effect was clear with a diminution of pH a few hours after, corresponding to a new VFA production.

As observed in all the culture in fed batch process, all the succinate produced at the beginning of the culture was re-consumed. The final concentrations of acetate, propionate and butyrate are logically higher than for 1<sup>st</sup> loop ( 4, 1.2, 0.7 g/l respectively).

### 3.2.2.2 Analysis of the gas phase

The profile observed for the following of pressure in the reactor can be correlated with the evolution of the liquid phase. An important gas production was noted during the first 24 hours of the culture and after the second inoculation at 280 hours. This result are in accordance with the observations made on liquid phase analysis and confirms the efficiency of the second inoculation performed for this second loop.



**Figure 14** : Evolution of pressure in the reactor during 2<sup>nd</sup> loop process

### 3.2.2.3 Analysis of the solid phase

Percentages of degradation were determined by the two protocols described for culture on ESA-substrate (filtration and desiccation). These values were respectively 41% and 36%. The degradation has consequently been more efficient than for first loop, which was supposed with the observations made on the metabolites concentration and the gas production.

In the same way as for culture on ESA-substrate, the percentage of degradation obtained by filtration is slightly lower than by desiccation. It is possible that the passage of short particles through the filter could explain this small difference.

Analyses for the determination of percentage of degradation by COD and Van Soest method have also been performed. The percentage calculated for COD analysis was 58% which is much higher than those obtained by dry mass measurement. Furthermore, values for cell wall degradation were not in accordance with the values of dry mass measurement. As explained before, it appears that the presence of an important proportion of faeces in the media is responsible for problems in COD and Van Soest analyses.

## 4.0 CONCLUSIONS / OUTLOOKS

*Fibrobacter succinogenes* is able to grow on total ESA-substrates even if faeces are present in the culture media since the beginning of the process. The percentage of degradation obtained on total ESA-substrate are about 55% and about 75% if we take account the inability of *F.s.* to degrade proteic substrates like spirulina and faeces. This value demonstrates its high efficiency for the degradation of vegetable wastes.

The gas phase has been characterized by the connection of a pressure sensor on the bioreactor and the determination of gas composition by GC. An important volume of CO<sub>2</sub> is produced during the first hours of culture and after each addition of substrates in the reactor. This production becomes less important after 24 hours, which probably corresponds to the end of the degradation of easily degradable substrates like cabbage.

As decided in the last meeting in Barcelona, 1<sup>st</sup> and 2<sup>nd</sup> closed loop experiments were also performed. The profiles obtained for liquid and gas phases analyses were similar to classical culture on ESA-substrate. A percentage of degradation of about 40% was obtained for 2<sup>nd</sup> loop experiment. These first results let to imagine a good efficiency for the association between methanogenic and *Fibrobacter succinogenes* processes.

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## APPENDIX : Estimation of cell wall composition by Van Soest method

Van Soest method is a technique which allows an estimation of cell wall composition. The successive treatments with neutral and acid detergent and then with 72 % sulfuric acid lead to obtain 3 residues : neutral detergent fiber (NDF) containing all the fibers, acid detergent fiber (ADF) containing cellulose and lignin and finally sulfuric lignin.

### 1- Materials

- chemical products : Sodium Dodecyl Sulfate (SDS)  
EDTA  
Sodium borate decahydrate  
Disodium hydrogen phosphate anhydrous  
2 ethoxy ethanol  
Cetyltrimethylammonium bromide (CTAB)  
Sulfuric acid  
Acetone
- materials : sintered glass funnel (porosity 40-90  $\mu\text{m}$ )  
round bottom flask and heating system  
condenser  
vacuum filtration system  
oven

### 2- Reagents preparation

- Neutral Detergent Solution (NDS) 1L

SDS	30 g
EDTA	18.61 g
Sodium borate decahydrate	6.81 g
Disodium hydrogen phosphate anhydrous	4.56 g
2 ethoxy ethanol	10 ml
distilled H <sub>2</sub> O	up to 1L

pH must be between 6.9 and 7.1

- Acid detergent solution (ADS) 1L

Cetyltrimethylammonium bromide	20 g
Sulfuric acid 0.5 mol/L	up to 1L

- Sulfuric acid 72 % (d = 1.634)

### **3- Method**

- Sample preparation

The sample to analyze is first dried and ground in very small and homogeneous particles (ultra-turrax). 1 g of this sample is weighed with a precision scale.

The sintered glass funnel used for the experiment is also weighed. It must be the same during all the process (mass of sample + mass of sintered glass funnel = S)

- NDF separation

100 ml of NDS is poured in the round bottom flask containing the sample. After the installation of the condenser, the round bottom flask is heated until the boiling point in 5 or 10 minutes. When the boiling begins, temperature of the heating system is reduced to obtain a weak but constant boiling.

After 1 hour of boiling, the sample is vacuum filtered with a sintered glass funnel (porosity 40-90  $\mu\text{m}$ ).

The sample is washed with boiling water until the elimination of all the detergent and then washed twice with acetone.

The sintered glass funnel is put in a oven at 60°C during at least 8 hours and then weighed with the precision scale (W1).

- ADF separation

The residue obtained after the first hydrolysis is used for the second hydrolysis. The same method is used to obtain the ADF but the NDS is replaced by the ADS.

The new residue obtained after hydrolysis, filtration, washing, drying with acetone and then in a oven (at least 8 hours, 60°C) is weighed with precision scale (W2).

- Lignin separation

The sintered glass funnel containing ADF residue is placed in a container. 72% sulfuric acid is poured until filling the sintered glass funnel at mid-height.

The solution is regularly mixed and the reaction is maintained during 3 hours. After 3 hours, the solution is vacuum filtered and washed with hot distilled water until neutralization of the sample.

Finally, the sintered glass funnel is put in the oven during at least 8 hours at 60°C and then weighed (W3).

#### **4- Estimation of cell wall composition**

$$\text{Total fibers : } \frac{S - W1}{S} \times 100$$

$$\text{Hemicellulose : } \frac{W1 - W2}{S} \times 100$$

$$\text{Cellulose : } \frac{W2 - W3}{S} \times 100$$

$$\text{Lignin : } \frac{W3}{S} \times 100$$