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TECHNICAL NOTE: 2.6

OPTIMIZATION OF *FIBROBACTER SUCCINOGENES* UNIT : FERMENTATION PARAMETERS

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1 INTRODUCTION

Fibrobacter succinogenes is one of the major cellulolytic bacteria found in the bovine rumen (Stewart and Flint, 1989). Except for the lignin, all the parietal polymers of the vegetable cells are degraded by *Fibrobacter succinogenes* thanks to a complex cellulolytic system. These enzymes have different modes of action but act as synergy in order to degrade parietal polymers in an optimal way (Forsberg et al., 1984). This complex equipment and the synergistic actions are probably the main explanation of the efficiency of vegetables degradation and the determination and the characterisation of the enzymes responsible of degradation can probably help to increase *Fibrobacter succinogenes* efficiency. In this study, we tested for the presence of endoglucanases and determined the activities of related fibrolytic enzymes produced when *Fibrobacter succinogenes* grown on three different carbon sources.

The rumen is assimilated to a semi-continuous system of fermentation in solid (food) and continuous in liquid (salivate). For several years, artificial fermentation systems have been primarily elaborate to replace the animals. One of these systems is the RUSITEC (rumen simulator technique). This one allows to simulate the degradation of the vegetable wastes and to envisage their digestibility in the bovine rumen. If this system allows *Fibrobacter succinogenes* growth with a production of VFA similar to those in the bioreactor and allows the monitoring of pH and CO_2 production which are important growth parameters, this system can be use as a useful tool to *Fibrobacter succinogenes* culture and to determine the optimal residence time. So after some improvement of the initial system we tested it for *Fibrobacter succinogenes* culture.

Finally, according to the last meeting in Copenhague, degradation of substrate of partner 1 was performed.

2 CELLULASE ACTIVITIES

2.1 Growth conditions (incubation culture)

Fibrobacter succinogenes has been grown anaerobically under 100% CO_2 in a basal medium that contained (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 $MnSO_4$,6 H_2O, 0.3 mg $CoCl_2$,6 H_2O, 8 mg $FeSO_4$,7H_2O, 0.25 mg biotin, 0.005 mg para-aminobenzoic acid (PABA), 0.01 mg hemin, 8 g carbon substrate and a mixture of volatile fatty acids (Gaudet et al., 1992).
100 mL of this media was introduced in serum bottles and sterilized (20', 121°C). Cystein (0.5 g/l) and Na_2CO_3 (4 g/l) were introduced in the hot medium to decrease redox potential (–350 mV) and increase pH (6.0-6.5). Then, as *Fibrobacter succinogenes* is a strictly anaerobic

organism, serum bottles were flushed with CO_2 during 3 hours in order to completely fill the atmosphere with this gas.

Three carbon sources have been used to test cellulase induction. Those were glucose, microcristalline cellulose (Avicel PH 101) and vegetable wastes constituted of green cabbage, soya and wheat straw (400 mg/L of each)

After these preparation steps, the serum bottles were inoculated with an overnight preculture (10 mL) grown on cellobiose (8g/l) (figure 1). The serum bottles were then incubated 48h at 39°C.

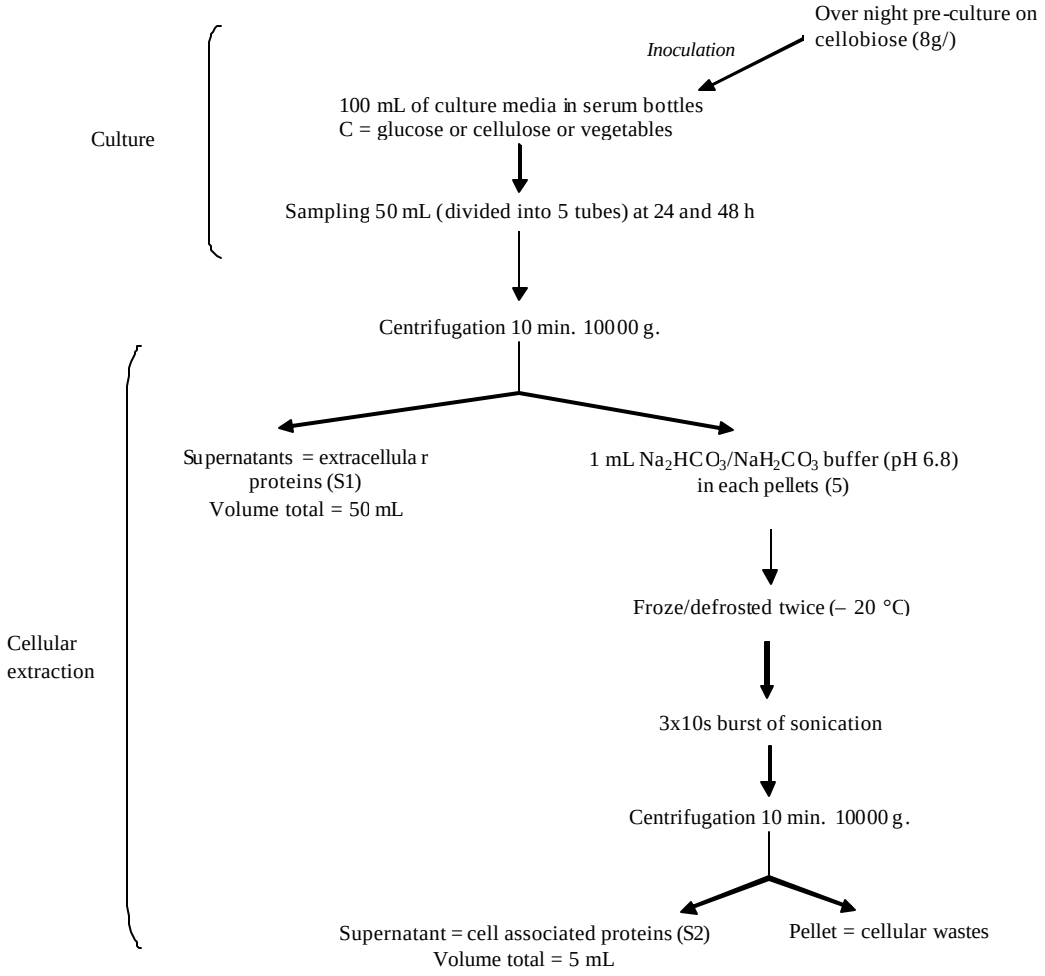


Figure 1 : Cellular extraction

2.2 Cellular extraction

A sample of 50 ml of this culture was performed at 24h or 48h of incubation time (Figure 1). This one was divided into tube (10 mL) and then centrifuged 10 min. 10000 g. The supernatants which represent the extracellular protein (S1) were removed and preserved on ice. The pellets were suspended in 1 ml of 50 mM Na_2HPO_4/NaH_2PO_4 buffer (pH 6.8) and then frozen and defrosted twice, sonicated with three 10s burst with 10s intervals on ice because this process increases the temperature of the medium and thus denatures the enzymes. The sonic extract was centrifuged 10 min.10000 g. and preserved on ice. This fraction represents cell associated proteins (S2)

2.3 Enzyme essays

S1 and S2 (5mL) were incubated with 1% of carboxymethylcellulose (CMC) in a 50 mM Na_2HCO_3/NaH_2CO_3 buffer (pH 6.8) (7.5 mL) at 37°C during 3h. Samples were performed every 30 min. and preserved on ice until the end.
Cellulase activities were measured by quantifying the reducing sugars produced during the experiment thanks to the DNS reagent as described by Miller (1971) with glucose as standard. Indeed the cellulases degrade CMC in reducing sugars which react with the 3,5 dinitrosalisilic acid to form an orange colouring whose intensity is proportional to the reducing sugars concentration at 540 nm. Proteins are determined by the Bradford method (1976) with bovine serum albumin as standard in order to define specific activities (nmol/min/mg of proteins).

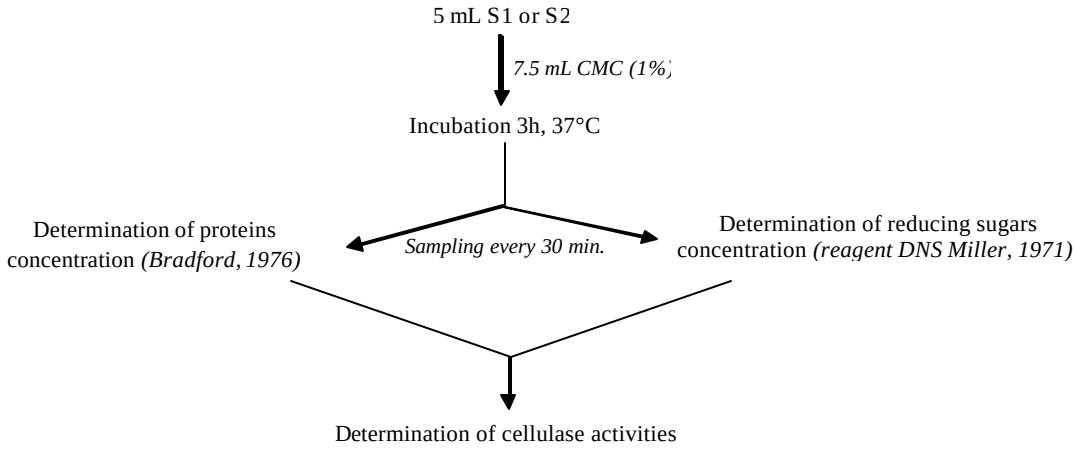


Figure 2 : Determination of cellulase activities

2.4 Results

Figures 3 and 4 represent the concentration of reducing sugars produced by the extracellular proteins during 3h of incubation with CMC.

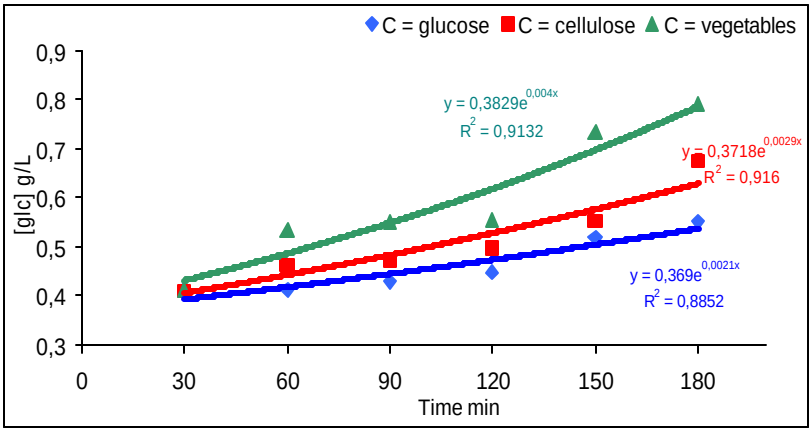


Figure 3 : Reducing sugar produced by S1 of a 24h incubation culture

More reducing ends are produced by extracellular proteins from cultures performed on vegetable wastes after 24h of incubation (Figure 3). On the contrary after 48h of incubation, more reducing ends are produced by extracellular proteins from cultures performed on cellulose (Figure 4). Reducing ends produced by extracellular proteins from cultures performed on glucose are lower and don't increase a lot with the time.

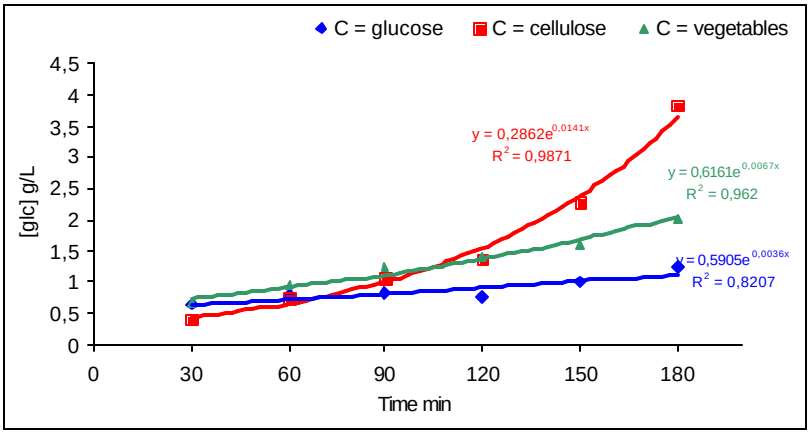


Figure 4 : Reducing sugar produced by S1 of a 48h incubation culture

The present curves had a characteristic profile of the endoglucanase and exoglucanase activities. The presence of these enzymes is confirmed by Field et al. (1997) in a study on *Prevotella ruminicola* B14, an anaerobic bacterium of the rumen, which also had endoglucanases.

Here only sugars with reducing ends were measured out. These ends are produced by the lysis of cellulose by the endoglucanases initially, and then by the endoglucanases and the exoglucanases (Figure 5). In the first step of lysis the endoglucanases generate reducing ends in small quantity; there are thus more and more ends during incubation and thus more possibilities for the exoglucanases. Consequently the number of reducing ends increases in an exponential way. That why we decided to plot the curves with this exponential profile.

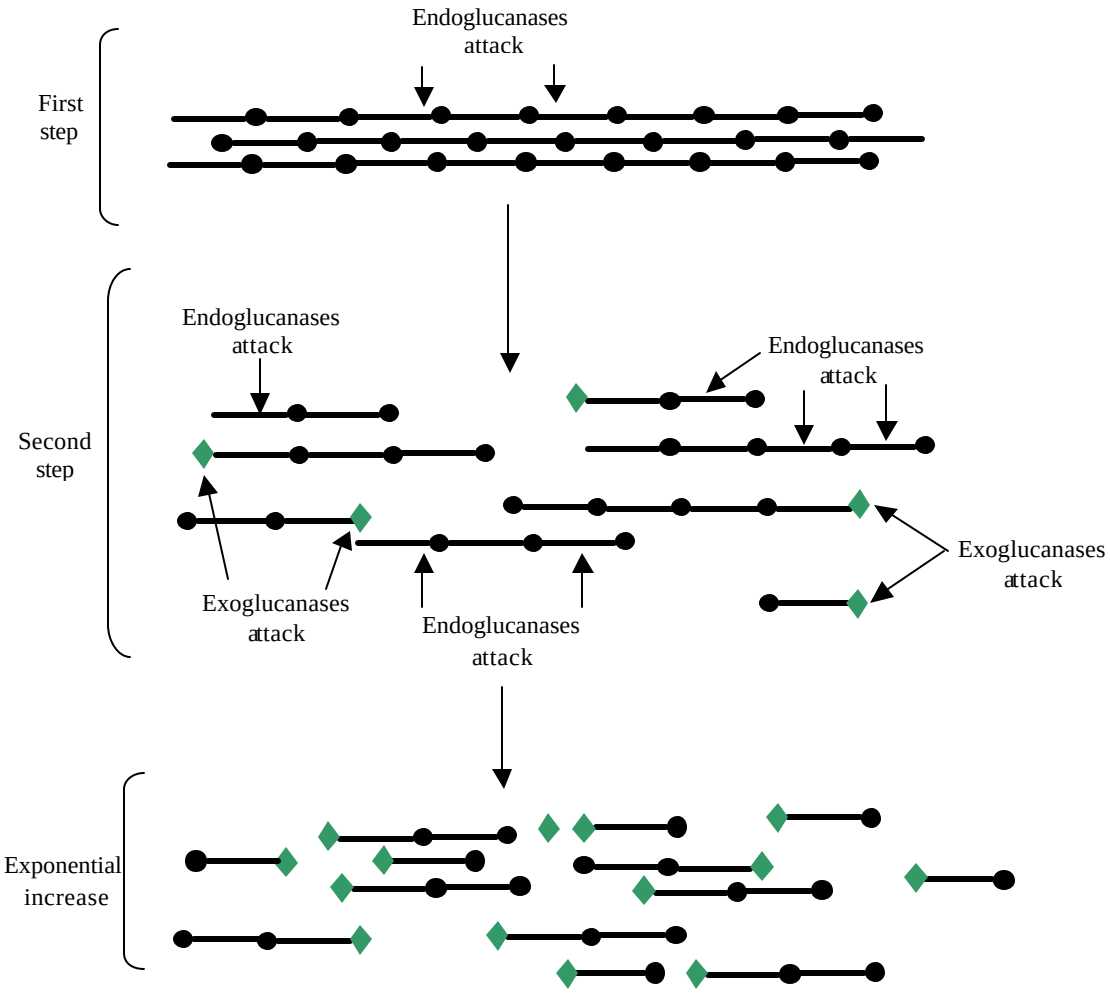


Figure 5 : Steps of the cellulose lysis by endo and exoglucanase s

With the same profile the concentration of reducing sugars produced by the cell associated enzymes are represented by figure 6 and 7.

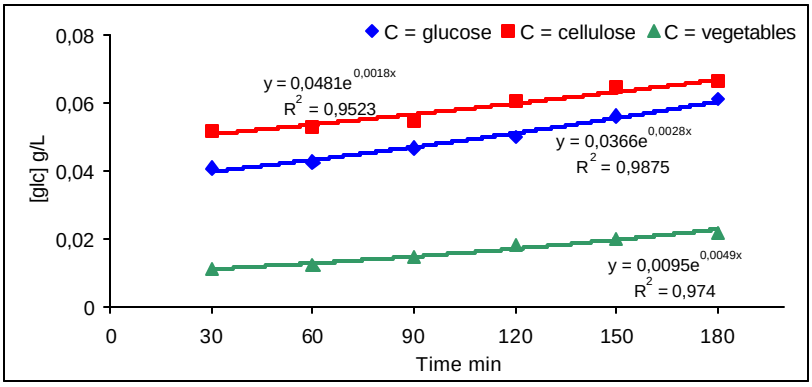


Figure 6 : Reducing sugar produced by S2 of a 24h incubation culture

After 24 (Figure 6) or 48h (Figure 7) of incubation the concentration of reducing sugars detected are very weak. The three main explanations are either the protocol of cellular lysis is not effective enough or all the enzymes are in the extra cellular part or there is a natural lysis before the first separation and the cell associated enzymes are detected in the extra cellular part.

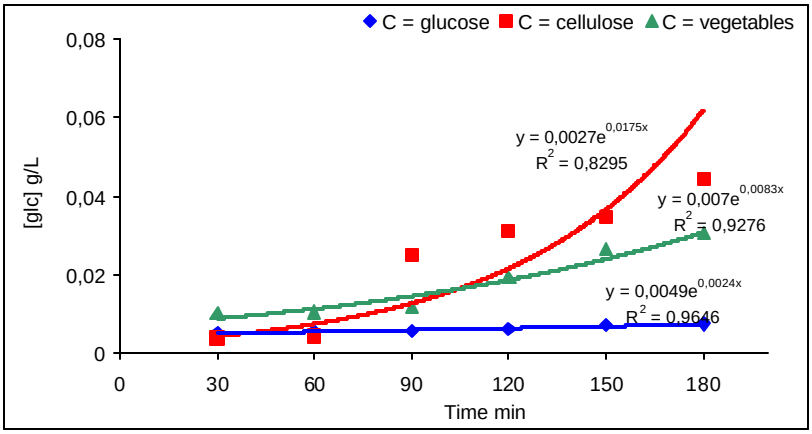


Figure 7 : Reducing sugar produced by S2 of a 48h incubation culture

Thanks to the equation curves, the activities were calculated and presented in table 1. These activities represent the endoglucanase activities which reflect the global activity of the cells (endo/e xoglucanases mainly) (Béra, 1998).

	enzymatic activities (nmol/min/mg of proteins)					
	glucose		cellulose		Vegetable wastes	
Time of culture (h)	24	48	24	48	24	48
S1	408	497	430	713	493	580
S2	41,3	0,4	29,8	41	6,2	8,57

Table 1 : Enzymatic activities of the different cultures

Generally, an enzymatic activity is present in all the extracts and this activity increases with the age of the culture, except for the activity of S2 on glucose. Mc Dermid et al. (1990), report that the presence of a simple sugar in the culture medium decreases the cellulose digestion. Under these conditions, *Fibrobacter succinogenes* uses simple sugars to provide energy necessary to synthesize proteins; the cellulases synthesis in this case is not necessary. This explains why the activities of the extracts from glucose culture are lower than that the activities of the extracts from cellulose or vegetation wastes culture.
In addition, the activities of the cells associated extracts are systematically lower than those of the extracellular extracts. This fact is in agreement with the observations of Béra (1998) which had observed that the majority of *Fibrobacter succinogenes* enzymes are salted out in the media and had studied for this reason only the extracellular or total extracts.
In the same way, the activity of S1 from a vegetable wastes culture is important after 24h of culture but the increase at 48h is lower than the increase of S1 from a cellulose culture. Mc Dermid et al. (1990) notes that all hydrolases, such as endoglucanases, identified in *Fibrobacter succinogenes* cells seem to be synthesized in the presence of cellulose, what suggests a mechanism of regulation. These enzymes have a basal level of expression in the cell, when the substrate is glucose or any other simple sugar and this level increase if the substrate is a more complex sugar such as cellulose. On vegetable wastes *Fibrobacter succinogenes* hydrolysis first of all substrates easily degradable, such as cellulose, then recalcitrant substrates. The degradation require the synthesis of more specific enzymes which do not degrade cellulose but other polymers of the vegetable walls, the activity of cellulases will be thus less important.

The dosage by the DNS Miller reagent is the most usually used in the literature (Reguera et al, 2001; Sigoillot et al, 2002) to quantify the reducing sugars. However some problems were encountered. First of all a step of centrifugation post-incubation was added to the protocol in order to remove cellulose of the culture media before proportioning. Indeed interference between cellulose and the DNS reagent was discovered by internal standards, the DNS reacting with cellulose by a strong coloring which masks the coloring produced by reaction with reducing sugars. Then the results were heterogeneous. Although the cultures were carried out in duplicates, the results were not always comparable. The results presented in this report are thus those most often met. A step of the protocol seems to be very sensitive but we are not able yet to identify it.

3 RUSITEC

3.1 RUSITEC system

3.1.1 History of the system

The first system to simulate the rumen was developed by Hoover et al. in 1976. It was a system to control independently one of the other the dilution rate of the solid and liquid phase and to maintain the turn over necessary to the substrate. Gijzen et al. (1986) improved Hoover's work by adding a continuous system of feeding. In order to ameliorate the nutrition of the ruminants Czerkawski and Breckenridge (1977) developed the RUSITEC (RUmen SImulation TEChnique). This system of fermentation allows the maintain of a normal microbial population of the rumen under controlled condition over long periods, the determination of fermentation characteristics according to animals feeding, and the simulation of vegetables degradation with the type and the quantity of products very similar to those of the rumen of donor animals.

3.1.2 Design

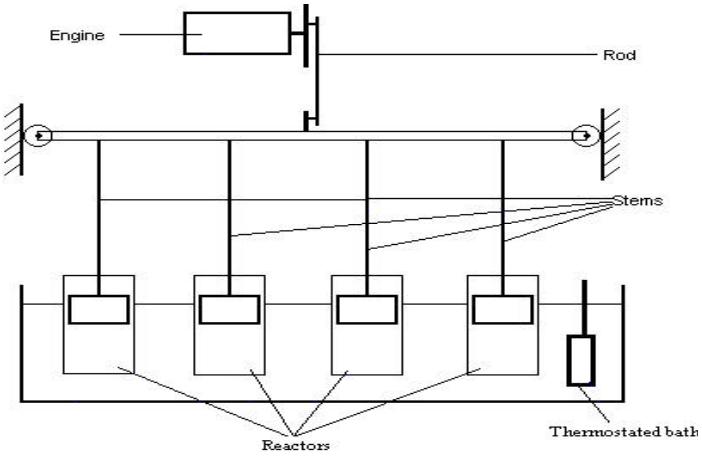


Figure 8 : Design of the RUSITEC

The RUSITEC has four identical units of culture (Figure 8). Each unit or reactor is connected to an evacuation system of the effluents. The four units are in a thermostated bath (39°C). The system is equipped with a mechanic pump in a vertical axis to stirring at 40 rpm.

Each unit is a PVC cylinder (figure 9) (1L) containing a perforated food container, with a vertical movement, which contains the substrate closed in a nylon bag (100µm of porosity). This porosity lets penetrate the bacteria but limits the exit of food particles.
On the lid three connections are possible : the first to connect the reactor to the CO_2 in order to maintain anaerobic conditions, the second for the exit of gas effluents (this exit is connected to a pressure gauge in order to measure the pressure into the reactor) and the third is reserved to sample in anaerobic condition using a plastic syringe (50 mL) connected to the gas.

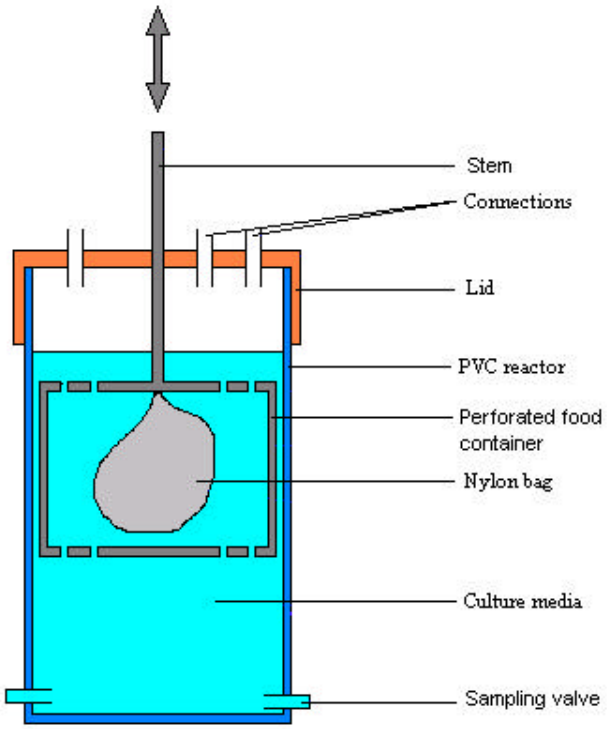


Figure 9 : Design of a reactor

3.1.3 Improvements

We obtained the RUSITEC from INRA (institut national de recherche agronomique). They used it to simulate the rumen and to cultivate a consortium of bacteria. Modifications were carried out to adapt this system of fermentation to *Fibrobacter succinogenes* culture. Indeed, the growth of *Fibrobacter Succinogenes* requires particular conditions of culture, like pH (6),

temperature (39°C) and redox potential (-330 mV). Moreover, as it's a strictly anaerobic bacteria, the atmosphere must be saturated with CO_2 and the pressure in the reactor after the inoculation must be stabilized at around 1.3 bars. With these objectives, several improvements were performed in order to:

- regulate the temperature without evaporation of the external bath water,
- found an overpressure at the beginning of the culture and to use the pressure like a growth parameter,
- sample during the culture without breaking the pressure and the CO_2 atmosphere,
- ensure a perfect sterility.

3.1.3.1 Improvements of the lid of the thermostated bath

The optimal growth temperature for *Fibrobacter succinogenes* is 39°C, at this temperature the water contained into the bath tends to evaporate. So the lid must have a special form to keep water in the bath, it must allow the retirement of the reactor most easily possible and must be transparent to visualize the contents of the reactor during the culture.
For these reasons, it was decided to do the lid into three parts in PVC material, to well adjust the different part and to put it in the bath.
To validate the efficiency of the lid, tests were performed with and without the lid (table 2). The experiments temperature was 39°C and the safety autonomy is the time during which the evaporation increases the water level of 10 cm. In this case heating resistance would not be any more in water and will be likely deteriorate.

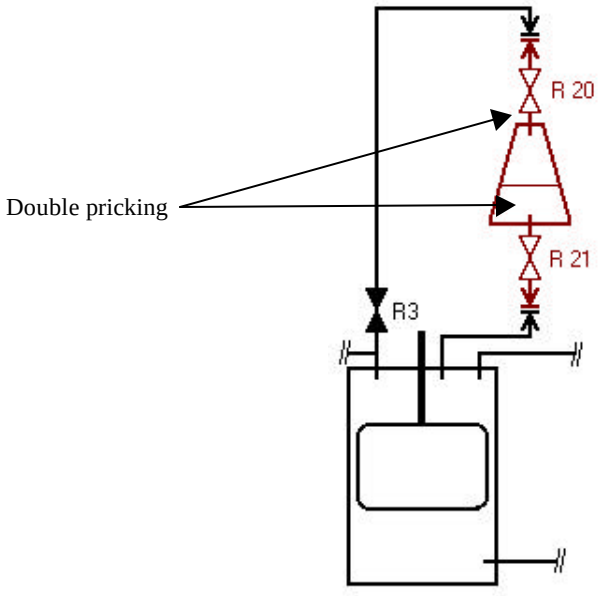
	loss (L/h)	Safety autonomy (h)
Without lid	0.386	60
With lid	0.076	307

Table 2 : Validation of the lid

The addition of the lid limits considerably the evaporation. However to carry out an experiment during a long time (more than 300h) a manual addition of water is necessary. The presence of a sensor of level connected to a peristaltic pump will be able to solve this problem.

3.1.3.2 The system of inoculation (Figure 10)

During inoculation, the reactor could be opened but oxygen is likely to enter so it is necessary to find a solution to inoculate without opening the reactor. A flask with double pricking hermetically closed seems to be the best solution. Higher pricking is connected to the atmosphere of the reactor and lower pricking is connected to the media. After pressure equilibrium, the contents of the flask run out by gravity. The interest of such a method is to ensure a good sterility and to prepare the preculture directly in the flask.



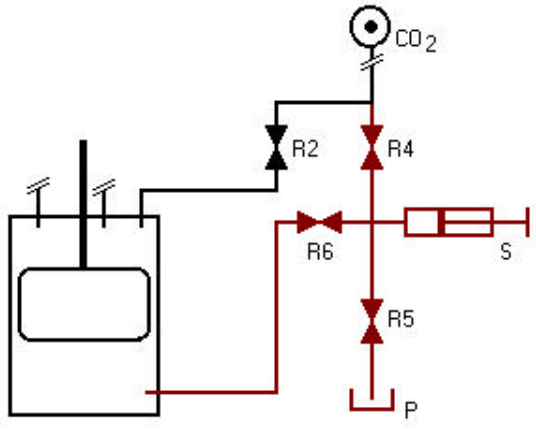
R3, R20, R21 : insulation valves

In red the inoculation circuit which can be sterilized.

Figure 10 : System of inoculation

3.1.3.3 Sampling of liquid phase (Figure 11)

As *Fibrobacter succinogenes* is a strictly anaerobic bacteria, a sample cannot be perform by opening the reactor. Moreover culture volume is weak and the bag containing the substrate must plunge in the media. It is thus not possible to sample more than 5-8 ml. Finally, for an effective agitation, the food container was adjusted according to the reactor, so there is no possibility of sampling by the top of the reactor. So a syringe is used to sample thanks to a flexible tube connected to a valve at the bottom of the reactor. This syringe is also connected to CO_2 flow to ensure the anaerobic conditions.



Gas flow :
R2 = CO_2
Sampling circuit :
R4, R5, R6
P = Sampling
S = Syringe.

Figure 11 : Sampling system of liquid phase

3.1.3.4 Sterilisation

The reactor is in PVC so it is impossible to sterilize it with high temperature. So we choose to sterilise it using a chemical method in order to eliminate the residue and the micro-organisms of previous experiments. It is an acid-base disinfection used in food industries. The reactor is first place under a laminar flow hood, brushed thanks to a flue brush and rinsed with sterile water. Then the fermentor is washed with NaOH solution (1%) and with a HNO_3 solution (0.5%) and finally with sterile water to eliminate all product traces.

3.2 Culture conditions

600 mL of basal medium is prepared in a flask with the nylon bag (porosity 100 µm) containing vegetable wastes (2.8g of green cabba ge, 2.8g soya and 2.8g of wheat straw). After the sterilisation (20', 121°C), Na_2CO_3 (4g/L) and cystein (0.5g/L) are added to induce the reduction of the medium under CO_2 atmosphere. Finally this medium with the nylon bag is introduced in the reactor under a laminar flow hood.
In the same time, 100 mL of medium are prepared in a flask with a double picking with cellobiose (8g/L) as carbon source. This double picking allows the connection to the gas flow to preserve anaerobic conditions and the inoculation. The medium is then inoculated with an overnight culture (5mL) and incubated one night at 39°C.
The reactor inoculation is done with the overnight preculture prepared previously under CO_2 atmosphere and the vertical agitation is launched and the pressure brought to 1.35 bars.

In order to test all the modifications performed in the RUSITEC and validate the system to *Fibrobacter succinogenes* culture, several culture were carried out. During this cultures, OD at 600nm, pH, VFA and CO_2 production were followed thanks to sampling every 3 hours the first 24h and then once per day.

3.3 Validation of the process

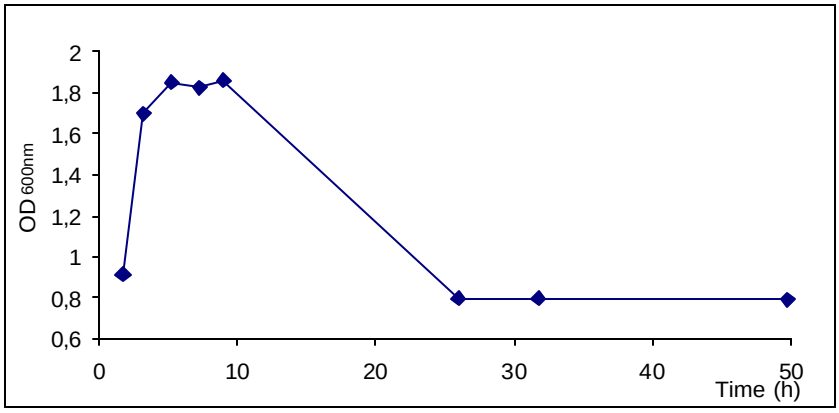


Figure 12 : Biomass estimation

Figure 12 represents the evolution of the OD (600nm) of a *Fibrobacter succinogenes* culture. The measurement of the OD is only associated to the no-adherent bacteria, the main bacteria are in the nylon bag in adhesion with the substrate. This curve shows an exponential phase of growth and a phase of bacterial lysis characteristic of *Fibrobacter succinogenes* growth (no-adherent bacteria).

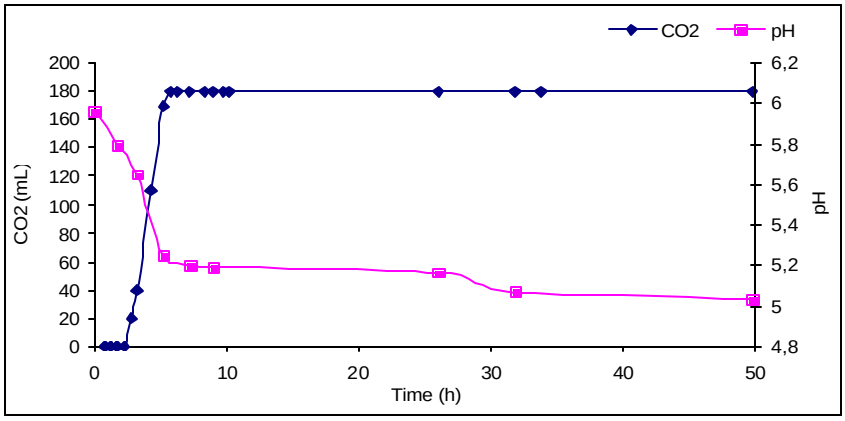


Figure 13 : Monitoring of pH and CO_2

Figure 13 shows the evolution of the pH and the CO_2 production. The pH decreases and CO_2 increases the first hours and then are stable. The profile of the curves is similar to these observe d in bioreactor.

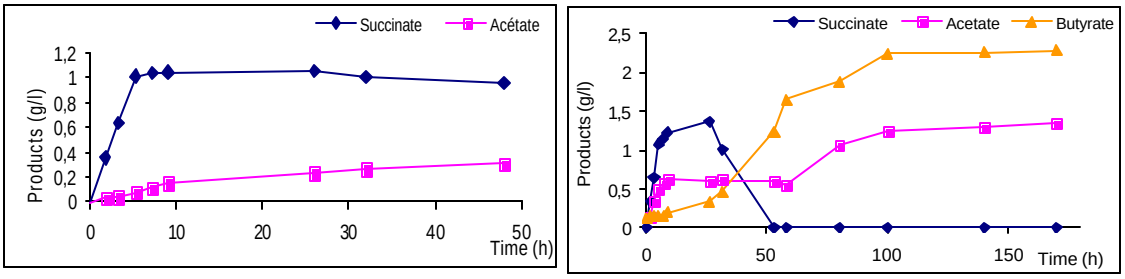


Figure 14 : VFA at 48h and 170h of culture

Succinate and acetate (figure 14) are produced during the first 48h, and then a re-consumption of succinate and a production of acetate and butyrate are observed like in bioreactor.

The modified RUSITEC allows the growth of *Fibrobacter succinogenes*, the monitoring of the pH and CO_2 can be used as growth parameters. Moreover the productions of VFA are similar to those in bioreactor. The RUSITEC is so a useful tool to *Fibrobacter succinogenes* culture and to determine the optimal residence time. Indeed, the nylon bag allows to recover the residue of fermentation more easily and so the dry mass measurement become an easily value obtained with the RUSITEC.

3.4 Determination of the optimum residence time

To determine the optimal residence time several culture were perform with different time of incubation. At the end of the culture the nylon bag was recovered and dried (48-72h, 110°C). The percentage of degradation was determined by : $\% = \frac{(Dmi-DMf)*100}{DMi}$

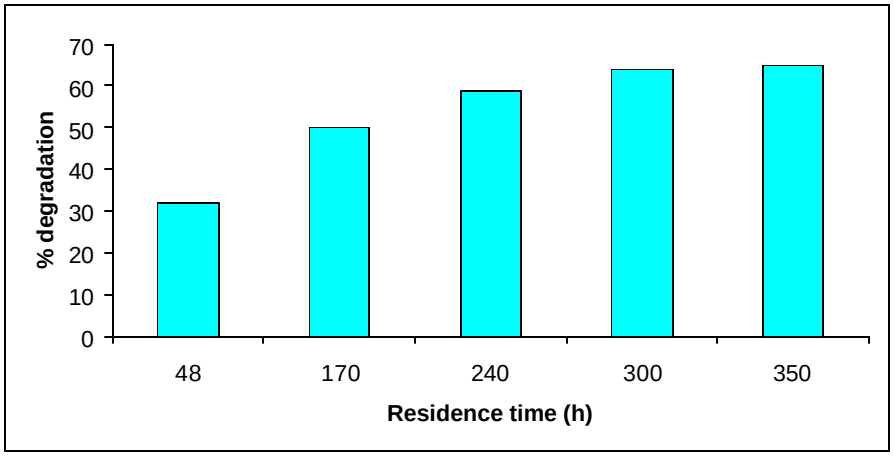


Figure 15 : Percentage of degradation with the residence time

The optimal residence time corresponds to a maximum degradation in a minimum of time. From that time, the profit of degradation is low compared to additional time. According to this definition, the optimal residence time obtained with the RUSITEC for the degradation of vegetables by *Fibrobacter succinogenes* is 300h (12 days) with 64% of degradation (Figure 15), after this time the profit is not enough large to continue the culture and before of this time the degradation is not enough important. Indeed the profit between 240h and 300h is 0.08% of degradation per hour whereas the profit between 300h and 350h is 0.02% of degradation per hours.

4 LOOP

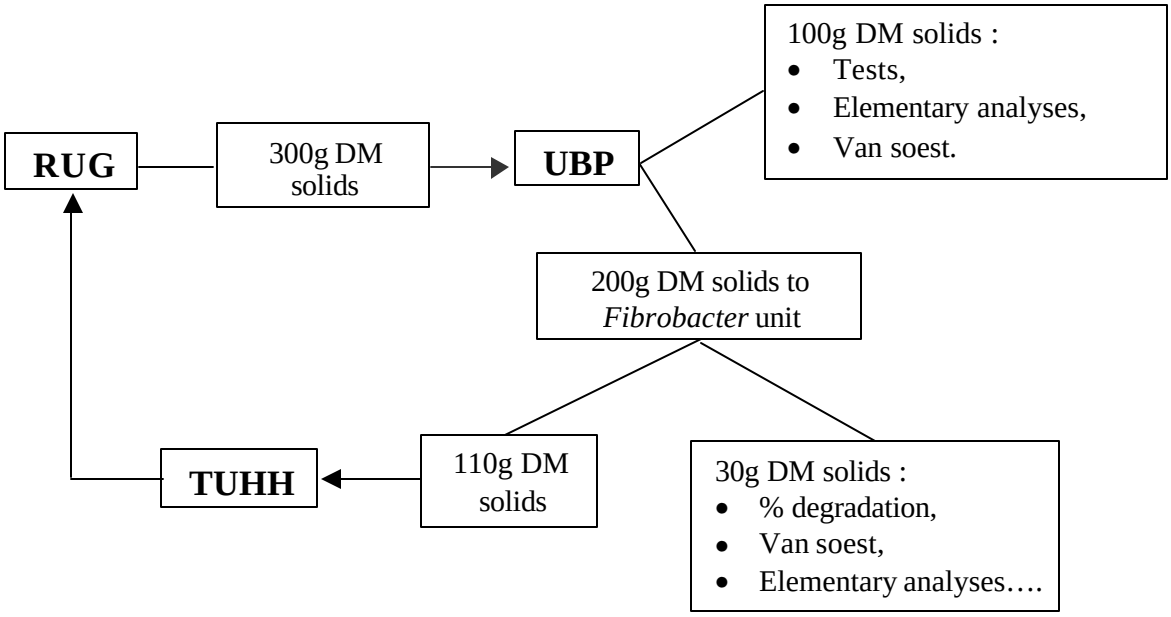


Figure 16 : Organisation of the substrate exchanges between MAP partners

4.1 Preliminary analyses

4.1.1 Analysis of the liquid phase

The analysis classically performed for *Fibrobacter succinogenes* process characterisation were realised on the liquid effluent. The pH value was found to be 7.2 and the VFA concentrations were analysed by HPLC. The liquid effluent contained 1.7 g/L of acetate, 0.9 g/L of propionate, 1.1 g/L of butyrate and 453.95 mg/L of N-NH3. These concentrations are higher than those found in the TN 2.4 (table 3).

	Acetate (g/L)	Propionate (g/L)	Butyrate (g/L)	pH
TN 2.4	0.7	0.87	0.017	10.2
TN 2.6	1.7	0.9	1.1	7.2

Table 3 : Comparison of VFA concentrations and pH of LabMET substrate between TN 2.4 and TN 2.6

4.1.2 Results of small batch experiments

Preliminary batch experiments in serum-bottles were carried out in order to test the capacity of *Fibrobacter succinogenes* to grow on the liquid and solid effluent from partner 1.
100 ml of the culture medium were prepared in serum-bottles. The substrate was glucose or the liquid or solid phase. Mediums were introduced and sterilized (20', 121°C), then cystein (0.5g/L) and Na2CO3 (4g/L) were added in the hot medium under CO_2. After 3 hours at 60°C for the medium reduction, serum bottles were inoculated with an overnight preculture (10 mL). The growth was determined by measuring the VFA production determined by HPLC (table 4).

	Liquid	**Solid**	**Glucose**
Acetate (g/L)	0,31	0,33	1,09
Succinate (g/L)	0,21	0,36	1,89

Table 4 : VFA production during the time of the culture .

After 100h of culture, the VFA production determined was the same on the liquid than those on the solid. These productions are lower than those on glucose however these experiments allow to conclude that *Fibrobacter succinogenes* can growth on LabMET substrates.

4.2 Degradation in bioreactor

4.2.1 Experiment set-up

Fibrobacter succinogenes was grown under 100% CO2 on a basal medium. The general set up of the culture was the same as describe in TN 2.5.
Absolute pressure, redox potential, pH and temperature were on-line monitored with probes connected on the reactor and related to an acquisition software. HPLC analyses were performed on liquid samples in order to follow the production of volatile fatty acids. $N\text{-}NH_3$ concentration was measured in the liquid phase by Patton and Crouch procedure. pH was automatically (peristaltic shear) maintained at 6.0 at least by addition of a concentrated solution of Na_2CO_3 (100 g/l) under CO_2 atmosphere.
Percentage of degradation was estimated by dry mass measurement of substrates and residue of fermentation (105° C, 48 h).
Percentage of carbon in the solid substrates, in the solid residue of fermentation and in biomass was determined by elementary analysis. Biomass concentration in the reactor was estimated by a correlation with succinate and acetate concentration.

4.2.2 First results

The substrate was only constituted with recalcitrant solids from partner 1.
After 96 hours, 70.9 mg/L of acetate and 75 mg/L of succinate was produced whereas usually in the same time more than 1 g/L of each VFA is produced and a production of butyrate and propionate is observed. Moreover we did not observe an increase of the pressure or a decrease of the pH. All this parameters show that the growth did not started, so we decided to stop the experiment.

4.2.3 Results of the second experiment

According to the previous results, it was decided to create a population of bacteria by doing the first batch on glucose (8 g/L). The following batches were on LabMET substrate and the process was maintained during about 660 hours including 6 additions of substrate (33 g each time).

4.2.3.1 Analysis of the liquid phase

- Monitoring of the pH and the redox potential
-

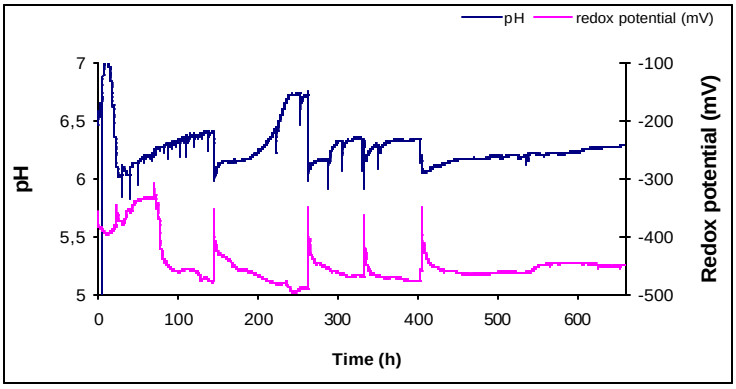


Figure 17 : Monitoring of the pH and the redox potential

Figure 17 shows the evolution of redox potential and pH during the time course of the experiment. The redox value is always comprised between –350 and –500 mV (relative value) during all the experiments, which validates the strictly anaerobic conditions during the culture. Each time the reactor is opened (addition of substrate), an increase of redox value can be observed and the value decreases again as soon as the reactor is completely closed. The pH decrease the first hours when *Fibrobacter succinogenes* growth on glucose then it increases so

we decided to regulate it manually by adding HCL each time substrate was added in order to decrease the pH to a value of 6.

➢ Monitoring of the VFA production

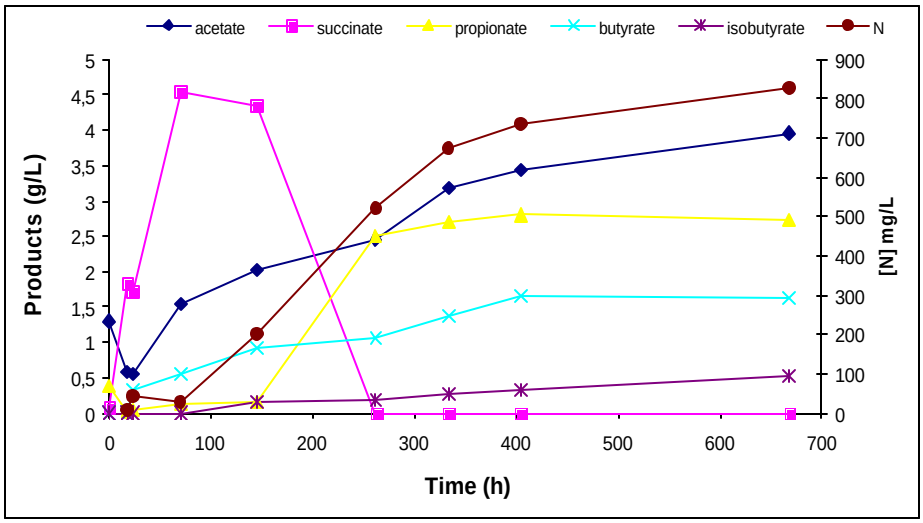


Figure 18 : Evolution of VFA production during the degradation

Figure 18 represents the evolution of metabolites concentrations during the experiment. The first hours of culture are characteristic of *Fibrobacter succinogenes* metabolism with production of mainly succinate and acetate (4.5 and 1.5g/l). As it was observed in the experiment on vegetable wastes, all the succinate was reconsumed after 150 hours of culture and production of other VFA (butyrate and propionate) began. The final concentration of this two metabolites was respectively 1.2g/L and 2.5g/L. The total VFA production during all the process was around 9g/L. The metabolic profile was similar to degradation of vegetable wastes (soya, green cabbage and wheat straw) but with lower VFA production (20g/L at the end of the process on vegetable wastes).

After a consumption of nitrogen source during the first hours of culture, a regular increase of this concentration is observed during all the culture. The final $N\text{-}NH_3$ concentration is about 800 mg/l. $N\text{-}NH_3$ production is similar to what was observed during the previous culture in bioreactor. However, at the end of the culture the ratio [VFA]/[$N\text{-}NH_3$] is lower than these observed previously on vegetable wastes. The difference between the two ratios could explain the profile of the pH curve. Indeed, we observed an increase of the pH (ever observed until there), this is probably due to the more important production of ammonium ions in the media.

4.2.3.2 Analysis of gas phase

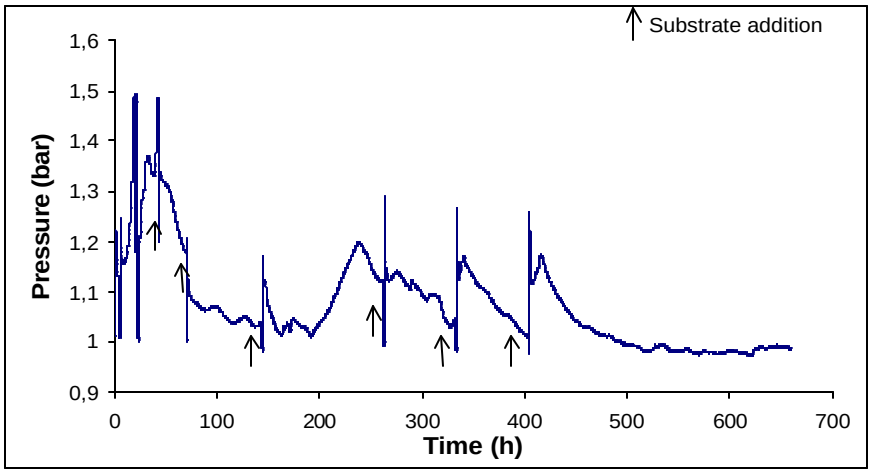


Figure 19 : Monitoring of the CO2 pressure

Figure 19 describes the evolution of gas pressure during the fermentation. Pressure, which is manually increased to 1.2 bars just after substrate addition, decreases during a few minutes because of chemical equilibrium between gaseous and dissolved CO_2. Pressure increases during the first batch and the curve has a profile similar to degradation of vegetables. After this first batch the pressure became linear and has a low rate, except each time the reactor was opened. This profile can be explain, in LabMET substrate there is less easily degradable particles than in vegetable wastes and as it was explained in the TN 2.5, when *Fibrobacter succinogenes* grown on recalcitrant substrates, CO_2 production is lower.

4.2.3.3 Analysis of the solid phase

- Van Soest analysis

	Total fibers (% DM)	**Hemicellulose (% DM)**	**Cellulose (% DM)**	**Lignin (% DM)**
Substrate (200 g)	89.4	30.9	32.9	20.6
Residue (140g)	55.2	11.1	19.1	29
Degradation (%)	56.8	74.9	59.36	1.4

Table 5 : Analysis of cell wall degradation by Van Soest method

The percentage of degradation was determined by : $\% = \frac{[(\%s \times Ms)-(\%r \times Mr)]}{\%s \times Ms} \times 100$

%s = % DM of a component of the substrate as hemicellulose
%r = % DM of a component of the residue
Ms = mass of the substrate (200g)
Mr = mass of the residue (140g)

Example of degradation of total fibers : $\frac{[(89.4x200)-(55.2x140)]x100}{(89.4x200)} = 56.8$

The cell wall components were determined by Van Soest (1967) method described in the TN 2.3. Table 5 shows the composition of the substrate from LabMET and the composition of the residues after *Fibrobacter succinogenes* unit.
The proportions of fibers are different before and after *Fibrobacter* unit. This confirms the efficiency of *Fibrobacter succinogenes* to degrade fibers.

- Dry mass measurements

All the content of the reactor was centrifuged (15', 13 000g) and the liquid and solid phase were separated. Percentages of degradation were determined by the two methods described in previously TN (desiccation and filtration). The percentage of degradation estimated by desiccation was 26% and by filtration was 28%. This also confirms the efficiency of *Fibrobacter succinogenes* to degrade LabMET substrate.

4.2.3.4 Determination of carbon and nitrogen mass balances

	Input		**Output**			
Source	substrates	carbonate	solid residue	VFA	CO_2	Biomass
Total C (g)	92.54	7.93	67.01	15.79	1.76	5.52
Total C (g)	100.47		90.08			
Mass balance	**89.7%**					

Table 6 : Determination of C mass balance

The results for the determination of C mass balance are summed up in table 6. The C mass balance was 89.7% for the overall experiment, which confirms the efficiency of the control and regulation of *Fibrobacter succinogenes* process.

	Input		**Output**		
Source	substrates	$N\text{-}NH_3$	solid residue	$N\text{-}NH_3$	Biomass

Total N (g)	9.19	0.034	4.95	2.65	0.85
Total N (g)	9.22		8.45		
Mass balance	**91.6%**				

Table 7 : Determination of N mass balance

As well as for C mass balance, the results obtained for N mass balance (91.6%, see table 7) also confirms the efficiency of the control of *Fibrobacter* process.
C and N mass balances indicates that the process is controlled for the two principal elements of the culture. These results obtained for carbon and nitrogen mass balances also show the efficiency of the control on *Fibrobacter succinogenes* process and confirm the methods and techniques used to determine the quantities consumed and produced.

5 CONCLUSION / OUTLOOKS

It seems that *Fibrobacter succinogenes* in our culture conditions has endo and exoglucanases. These enzymes have a basal level but can be induce with the substrate. To confirm the presence of endoglucanases and the activities of related fibrolytic enzymes produced by *Fibrobacter succinogenes* other methods can be perform like a viscosimetric method. In the same way we could test for the presence of other enzymes which act in the degradation of the cell wall components, such as xylanases.

After several improvements on the initial system we have used the RUSITEC to cultivate *Fibrobacter succinogenes* like in bioreactor i.e. with using CO_2 production and VFA production like growth parameters. One of the advantages of the RUSITEC is to determine more exactly the percentage of degradation of the vegetable wastes. Moreover, the mode of culture on the RUSITEC allows the determination of the optimum residence time for the degradation of vegetable wastes 64% in 300h) by *Fibrobacter succinogenes* and the system gives also the possibility to work in the same time on two reactors.

Finally, substrate from metanogenic unit was degraded by *Fibrobacter succinogenes* with a rate of 28% and with VFA production. But we were confronted to two difficulties. The first was to define a feeding procedure in terms of quantity (200g once) and of frequency (33g once a week). So for the following loop we propose to add the totality of the substrate at the beginning of the fermentation to try to obtain a better rate of degradation. The second was a difficulty to define the metabolic reactions between the substrates and the products. Enzymatic measurements seem to be the best way to solve this difficulty.

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