Université Blaise Pascal
CLERMONT-FERRAND

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Substrate exchange and characterization of *Fibrobacter succinogenes* process

Jérôme SEON, Catherine CREULY, Claude-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

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1.0 INTRODUCTION

The efficiency of *Fibrobacter succinogenes* for the degradation of ESA-substrate was shown in TN 2.300. This bacteria is able to grow on a mixture of substrates containing only wheat straw, soja bean cake, cabbage, spirulina and faeces. The degradation of the vegetable part of this mixture can reach 80%.
The first tests of closed loop experiments (substrate exchanges between the different MAP project processes) gave encouraging results. 40% of recalcitrant solid materials from the methanogenic reactor were degraded by *Fibrobacter succinogenes*.
As it was demonstrated in the previous TN, F.s produces CO2 during its growth. Thus, it was decided to try to follow the growth of *F.s.* by the evolution of pressure. Kinetic analyses were also performed on the three vegetable ESA-substrate by sequential addition of substrates.
Finally, according to the last MAP meeting in Hambourg, closed loop experiments were performed with liquid and solid effluent from the methanogenic reactor.

Remark : The evolution of the MAP project strategy has involved a modification of the WP 2.400 title

2.0 DIFFERENTIAL ADDITION OF SUBSTRATE

F.s is very efficient for the degradation of vegetable substrates. Experiments of growth on each of the three vegetable models have been performed in batch tests (serum bottles of 100 mL, 15 days of culture, see TN 2.100) and showed that the degradation was more important for cabbage than for soya than for wheat straw, for the same time of culture.
Following these results, it was decided to test the differential addition of these three substrates in bioreactor culture conditions and to focus on two particular points: firstly, the monitoring of pressure evolution in the reactor as a bacterial growth indicator and secondly, the kinetic of degradation of these three substrates.

2.1 General conditions of culture

F.s was grown under 100% CO2 on a basal media described in TN 2.100.

2.2 Preparation of substrate

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.

All the substrates were sterilized at 121°C during 20'.

2.3 Experimental set-up

The general set up of the culture was the same as described in TN 2.200.
Absolute pressure, redox potential, pH and temperature values were on-line monitored with probes connected on the reactor and related to an acquisition software.
HPLC analysis were performed on liquid samples in order to follow the metabolites production.
GC analysis were performed on gas phase samples in order to determine its composition.

2.4 Substrate addition

As we wanted to test the effect each substrates on the growth in classical conditions, it was decided to create a population of bacteria by the introduction of the mix of the three vegetable substrates (wheat straw, soya and cabbage, in proportion 1/3) at the beginning of the culture. Two first batches were performed with 30g of this mix during 150h.. Then, each time that the pressure increase and the pH drop was finished, a new addition of only one substrate was performed. By this way, 10.2 g of wheat straw were added and the culture was maintained during 30h. The same quantity of cabbage and finally of soya bean cake was added and the culture was maintained during respectively for 70 and 100h (Table 1).

Composition of the substrate added	Quantity (g DM)	Time (h)
Mixture of wheat straw, soja and cabbage	30.6 g (1/3 of each)	0
Mixture of wheat straw, soja and cabbage	30.6 g (1/3 of each)	150
Wheat straw	10.2 g	300
Cabbage	10.2 g	330
Soja	10.2 g	400

Table 1 : Chronology of the substrate additions in the reactor

2.5 Results

2.5.1 Addition of wheat straw

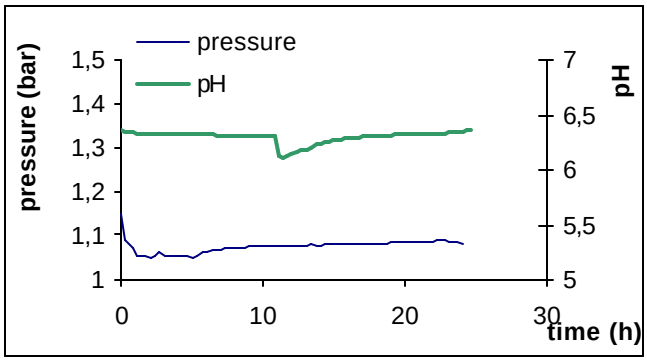

Figure 1 : Evolution of pH and pressure during the degradation of wheat straw

Figure 1 represents the evolution on of gas pressure (absolute pressure in bar) and pH during the degradation of wheat straw. The addition of wheat straw in the bioreactor resulted in a very short increase of the pressure value and drop of pH.
The addition was performed under CO2 flux. Then, pressure in the biorector was manually increased up to 1.15 bar in order to improve the bacterial growth. The first decrease of pressure during the first hour of culture is due to chemical equilibrium of CO2 between the liquid and the gas phase.

After that, pressure become stable and then begin to increase very slowly. The drop of pH value was also very slow. After 30 hours of culture, the increase of pressure was going on with the same low rate. Nevertheless, it was decided to perform a new substrate addition (cabbage).
The kinetic of degradation of wheat straw by *F.s.* is very slow and an efficient degradation take probably a rather long time of culture. The two main reasons to explain these results are probably the size of the particles and the high lignin rate of the fibers (see paragraph 2.7).

2.5.2 Addition of cabbage

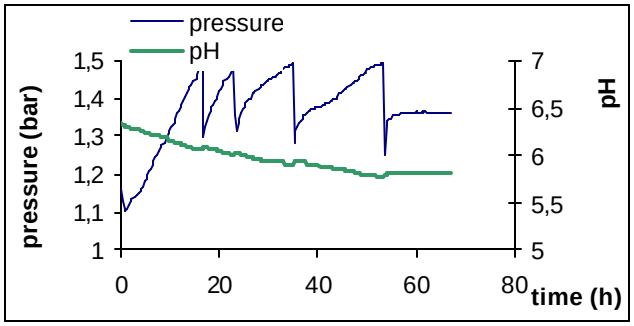

Figure 2 : Evolution of pH and pressure during the degradation of cabbage

Contrary to wheat straw, figure 2 shows that the addition of cabbage is responsible for a very important production of CO2 and drop of pH.

After the first pressure decrease due to chemical equilibrium, the production of CO2 begins and becomes more and more important. When the value reaches 1.5 bar, the control unit induces the opening of the electro-valve, which decreases the value of pressure to 1.15 bar in the reactor. This important CO2 production goes on during about 60 hours and generates four opening of the electro-valve. The drop of pH was regular during all this time and finally reaches 0.8 unit. After 70 hours, the pH and pressure values become stable ; a new substrate addition could be performed.

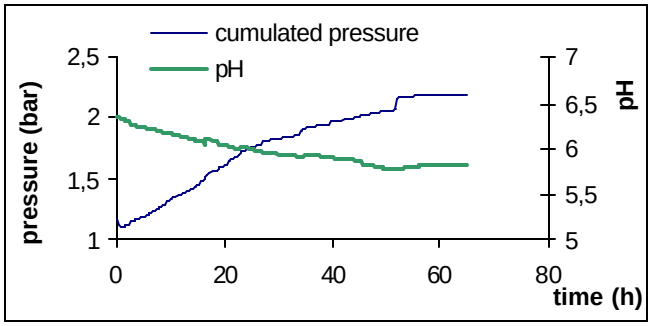

Figure 3 : Evolution of pH and cumulated pressure during the degradation of cabbage

Figure 3 represents the evolution of cumulated pressure values in the reactor. This shows a regular gas production during about 50 hours. The total pressure increase is 1.1 bar.
The kinetic of degradation of cabbage is clearly very high and a degradation of the main part of this substrate could be obtained in only 60 hours.

2.5.3 Addition of soya bean cake

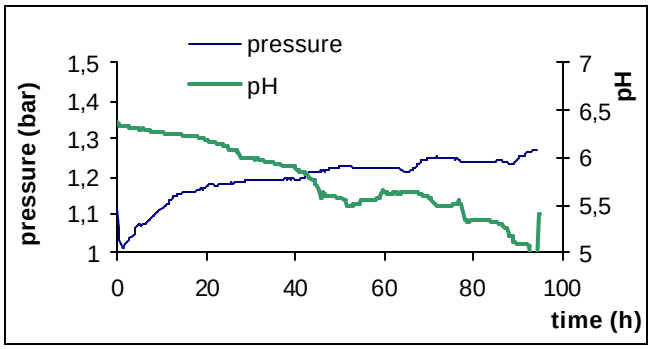

Figure 3 : Evolution of pH and pressure during the degradation of soja

The addition of soya results in an intermediary CO2 production drop of pH. The pressure increased from 1 to 1.3 bar in 90 hours. During the same time the drop of pH was 0.6 unit. The final decrease of pH values (last 40 hours) is probably due to a technical problem on the pH probe.

After 100 hours of culture on this substrate, the pressure value goes on increasing with a slow rate. This clearly shows that *F.s.* is able to degrade efficiently the soya but the kinetic is slower than for cabbage.

2.6 Discussion on the efficiency of gas pressure to follow bacterial growth

Substrate	Quantity (g DM)	pH drop	Pressure increase (bar)	VFA production (g/l)	Degradation (%DM)
cabbage	10,22	0,8	1,02	1,72	78,2
soya	10,22	0,6	0,21	1,28	62,6
wheat straw	10,22	0,05	0,03	0,42	31,7
mix (1/3)	30,66	1,2	1,5	3,46	60

Table 2 : Evolution of the parameters during the degradation of each individual or mix vegetable substrates

Table 2 represents the evolution of the different parameters measured for each substrate addition. The comparison of the evolution of pressure in the reactor with the other parameters classically controlled seems to be interesting. The increase of pressure in the reactor (due to CO2 production) is the most important (mix and cabbage) when the drop of pH (due to VFA production) and the percentage of degradation represent also the highest values.
The metabolism of *F.s.* appears clearly more efficient for the degradation of cabbage than for soja and for wheat straw. This is confirmed by the global efficiency of degradation which is largely higher for cabbage.

These data markedly indicates that the monitoring of gas pressure in the reactor could be a interesting indicator of the bacterial growth and consequently of the efficiency of degradation.

2.7 Discussion on the kinetic of vegetable substrate degradation by *F.s.*

substrates	Total fibers (%DM)	Hemicellulose (%DM)	Cellulose (%DM)	Lignin (%DM)	Degradation by *F.s.* (%DM)
Wheat straw	72	26	39	7	31,7
Soya	12	5	6	1	62,6
Cabbage	16	2	13	1	78,2

Table 3 : Cell wall composition of the vegetable substrate and efficiency of *F.s.* degradation

Table 3 shows the cell wall composition of the three vegetable substrates obtained by Van Soest analyses (see TN 2.300) related to the global degradation (DM) obtained with *F.s.*

In wheat straw, the fiber part of cell wall represents more than 70% and the proportion of lignin is quite important (7%). These data are one of the explanation to the complexity of the degradation of this substrate. The complex and fibrous structure with a high proportion of undegradable lignin and attached hemicellulose make wheat straw very difficult to degrade. The second point is the size of the particles which are much more important for wheat than for the two other substrates. The use of shorter particles should probably improve the efficiency of the degradation.

The fiber and lignin proportions are slightly the same for soja and for cabbage (12 and 16% respectively). Nevertheless, the proportion of hemicellulose is much more important for soja than for wheat straw. This is probably the reason why *F.s.* is more efficient to degrade cabbage than soya. Indeed, hemicellulose is a branched glucose polymer which participates to the complexity of the fibrous matrix. This involves that the enzymatic equipment necessary is more important to degrade hemicellulose than cellulose and therefore a slower kinetic of degradation.

Fibrobacter succinogenes possesses the specific enzymatic equipment necessary for the degradation of vegetable cell walls. The presence of various cellulases allow an efficient degradation of cellulose. Xylanases and debranching enzymes allow the separation of hemicellulose and lignin from the rest of the matrix and which leads to improve the degradation of hemicellulose. Lignin can not be degraded by *F.s.* This equipment explained that a substrate containing lignin and hemicellulose in high proportion will be more complicated to degrade.

Some preliminary tests have been performed to determine the composition and activity of the enzymatic equipment of *Fibrobacter succinogenes* in our culture conditions. It clearly appeared that the enzymes which are mobilized are not the same and have different activities when the culture is performed on vegetable wastes or single substrate like glucose (data not shown). These analyses will have to be carried on.

3.0. THIRD CLOSED LOOP EXPERIMENT

As it was decided between the MAP partners in the last meeting in Hambourg and according to the good results obtained for the second closed loop experiment, a third experiment was tested, as described in the following scheme.

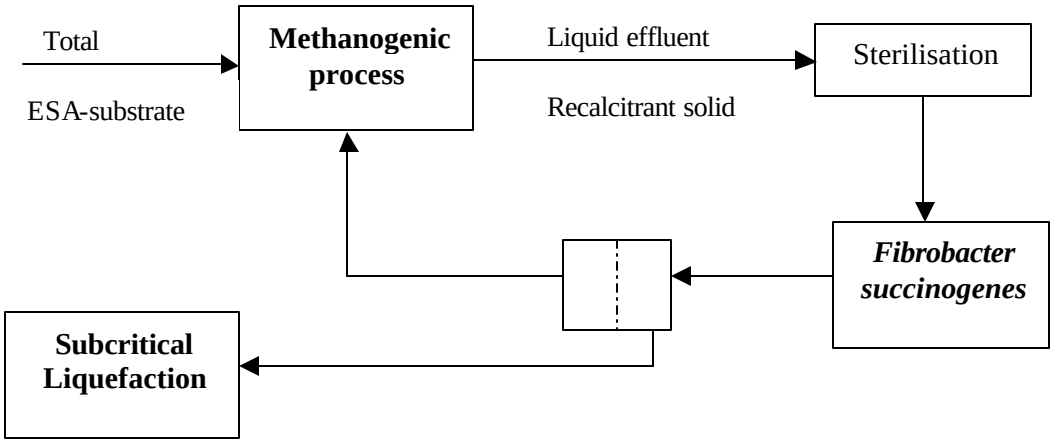

Figure 5 : Organization of the substrate exchanges between MAP partners for the third closed loop experiment

3.1 Experimental set up

The liquid and solid effluents received separately from Ghent University were sterilized by autoclave (20', 121°C). A 2.8L culture media containing for a half the liquid effluent from methanogenic process and for the other half a classical *Fibrobacter* culture media (description in TN 2.100) was prepared. The substrate used was only constituted with recalcitrant solids from methanogenic process.

3.2 Development of a method for pH regulation

The production of VFA by *F.s.* is responsible for an important decrease of pH value during the culture. For the previous experiments, pH was manually regulated by addition of Na_2CO_3 powder in the culture media each time the value reached about 5.7. The possibility to maintain this value at 6.0, which is the optimum for this bacteria, could improve the growth and consequently the degradation. The other interesting point is the suppression of the risks of CO_2 formation from carbonate due to the diminution of pH.

For all these reasons, it was decided to connect a regulation system for pH. The regulation is performed with a 50 g/L carbonate solution. This solution is connected to a peristatic pump under the order of the control unit. Each time pH value become under 6.0, the pump starts and introduces the carbonate solution to the reactor. The gas phases of the bottle and the reactor are also connected in order to ensure that the solution introduced is perfectly anaerobic.

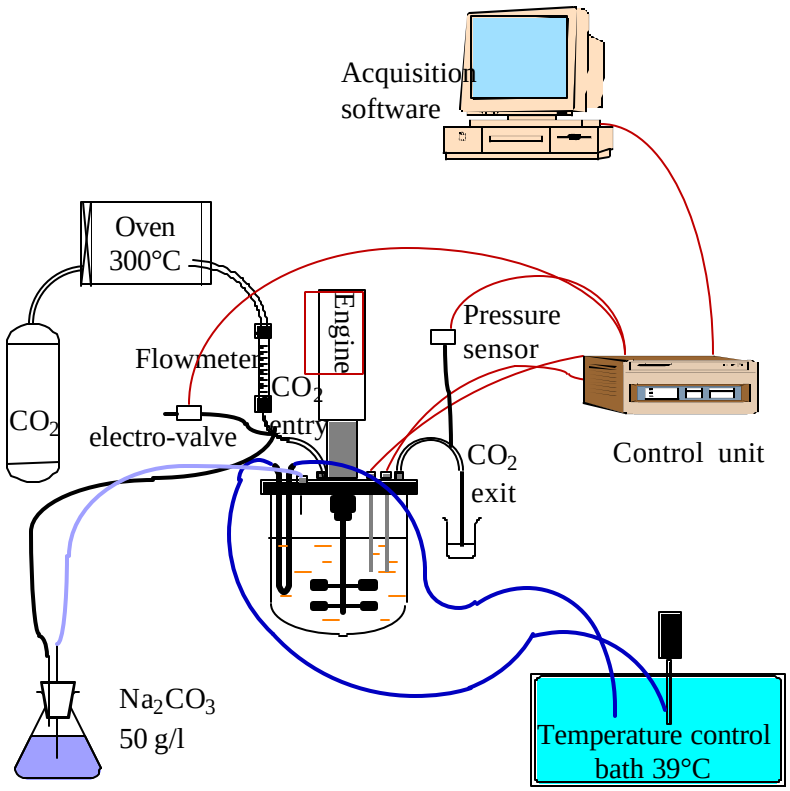

Figure : Scheme of *Fibrobacter* process with pH regulation system

3.3. Analyses on the liquid and solid effluent from the methanogenic reactor

As it was the first time we received liquid effluent from methanogenic compartment, the analyses classically performed for the *Fibrobacter* process characterization were realized on this effluent. pH value of the liquid phase was found to be 10.2. which is higher than the values observed for classical methanogenic process. Consequently, the culture media prepared with this effluent was acidified with HCl
The liquid was also analysed by HPLC in order to determine the VFA concentrations.

VFA	Acetate	Propionate	Isobutyrate	Butyrate	Isovalerate	Total VFA
Concentration (mg/l)	700	870	45	17	100	1700

Table 4 : Concentration of VFA in the liquid effluent from methanogenic compartment.

These concentrations clearly appeared higher than the concentrations found in the TN 1.100.
The COD of this effluent was 4825 mg O_2/l.

3.4 Results of small batch experiments

Two preliminary batch experiments were performed in serum bottles of 100 mL in order to test the capacity of *F.s.* to grow on the liquid and solid effluent from methanogenic compartment.
For the first one, *Fibrobacter* was grown on glucose in a culture media containing only the liquid effluent (+ carbonate and cystein which are indispensable for the reduction of the media). For the second one, *F.s.* was grown on the solids from methanogenic unit in the classical *Fibrobacter* culture media.

The first test did not result in any growth of the bacteria. For the second one, a bacterial growth was observed and 18% of the solids were degraded.

These results indicate that the presence of the liquid media could inhibit *F.s.* growth. It can be assumed that some toxic or inhibitory compounds are present in this effluent. This can maybe also explained by the dilution of some organic compounds added in the classical *Fibrobacter* culture media.

3.5 Results of the culture in bioreactor

3.5.1 First phase of the culture

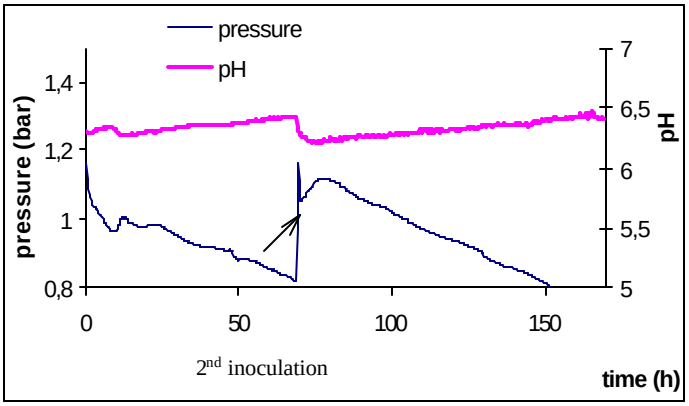

Figure 6 : Evolution of pH and pressure during the first hours of culture

Contrary to what was observed for the first and second closed loops (TN 2.300), this experiment did not result in a classical growth of *F.s.* After the chemical equilibrium between gas and liquid phase, pressure did not increase in the reactor. The culture did not start. Moreover, pressure decreased under the atmospheric pressure value until about 0.8 bar, which has never been observed before.

After 50 hours, it was decided to inoculate the reactor another time with a new fresh preculture. The result was the same with a decrease of the pressure under the atmospheric value. pH was quite stable during all this phase of the culture at an average value around 6.3, which was the initial pH of the culture media.

It was assumed that toxic compounds for *Fibrobacter* could be present in the liquid effluent of the methanogenic reactor. Indeed, the first and second loop were performed with only the solid recalcitrant material of this compartment. The second assumption was the lack of easily degradable material to initiate the *Fibrobacter* culture.
For these reasons, it was decided to prepare a third fresh preculture of *F.s.* and to add glucose (4g/l) at the same time in order to improve the start of the culture.

3.5.2 Second phase : Re-inoculation and addition of glucose

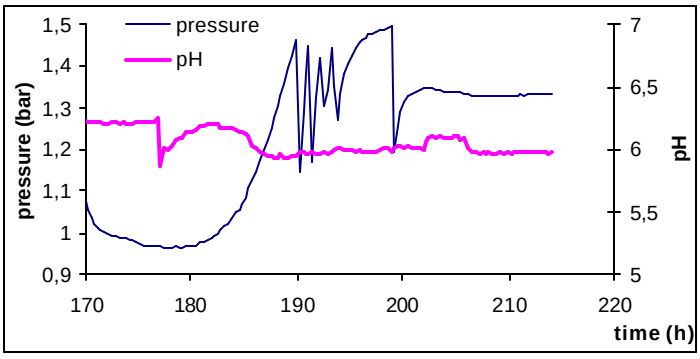

Figure 7 : Evolution of pH and pressure after glucose addition

Figure 7 clearly shows the effect of glucose addition. After a 10 hours lag phase, the culture started and resulted in a very important and quick gas production. The pH curve indicates the efficiency of the regulation system. The VFA production associated to bacterial growth implicated a decrease of pH value from the initial value (6.3) to 6.0. Then, pH was stable at 6.0 during all the growth phase because of the addition of carbonate solution.
This important gas production lets draw two conclusions : first, there is probably no toxic compounds in the liquid effluent of the methanogenic compartment for *F.s.* The difficulty to obtain a bacterial growth is probably due to the lack of easily degradable substrates. Second, an addition of glucose allow the formation of a important bacterial population which will may be able to degrade the recalcitrant solids.

3.5.3 Third phase : addition of solid substrates

At the end of the last step (170-220 hours of culture), it appeared that all the glucose added has been consumed (HPLC analysis). It was decided to add substrates from the methanogenic reactor while the bacterial population was important.

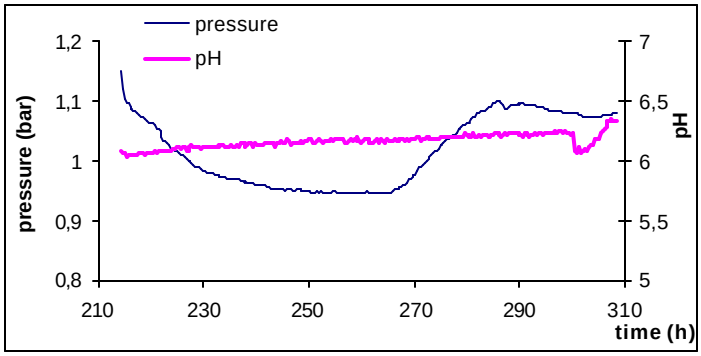

Figure 8 : Evolution of pH and pressure after the second substrate addition

Figure 8 shows the difficulty for *F.s.* to degrade this recalcitrant material. Pressure decreased during 40 hours. Then, a short pressure increase was observed during 20 hours which let suppose a degradation of the solid substrates.

3.5.4 Fourth phase : addition of glucose and substrates.

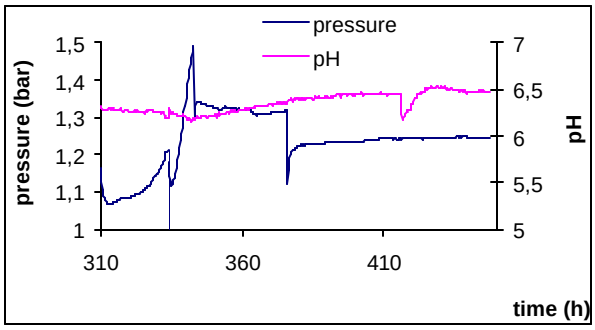

Figure 9 : Evolution of pH and pressure after the third addition of substrate (+ glucose)

The addition of glucose after 310 hours of culture allowed a new CO2 production. The addition of solid substrates after 350 hours of culture did not stop this production and on the contrary, gas pressure went on increasing during a few hours. This probably indicates the degradation of all the glucose and a part of the solids.
After this third addition, the culture was stopped.

3.5.5 Evolution of VFA concentration during all the culture

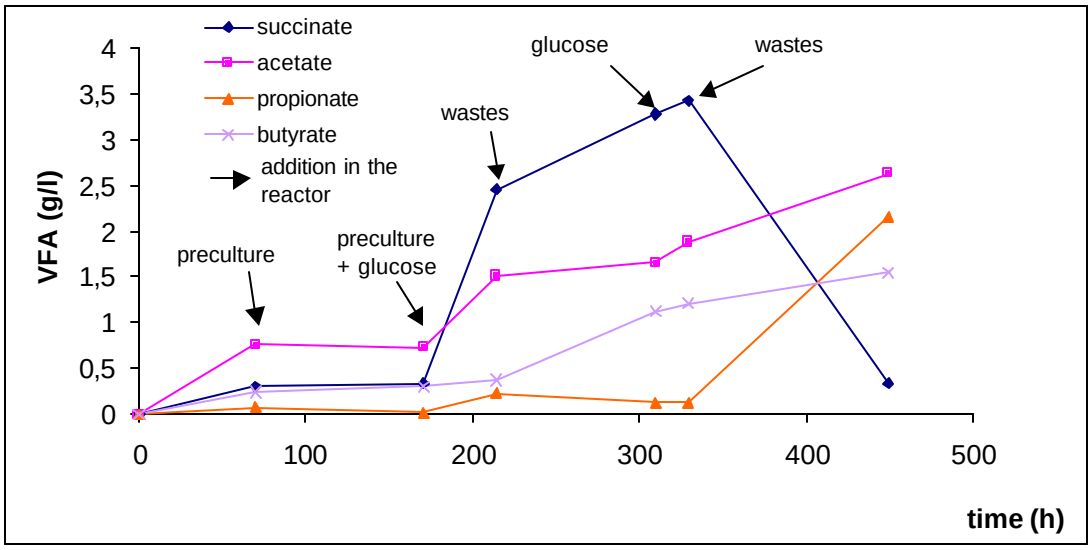

Figure 10 : Evolution of VFA concentration during the culture

Figure 10 shows the evolution of the various VFA produced during the culture and estimated by HPLC analysis. The curves confirm what was observed during the first and second closed loop experiments.

The inoculation with the first and second preculture did not result in any VFA production. The short increase of succinate and acetate value are due to the input of the first preculture.
As it was observed for the gas pressure monitoring, the re-inoculation and addition of glucose after 170 hours of culture induced a very important VFA production. The typical metabolism for a culture of *F.s.* on single substrate as glucose was observed, with an high succinate (2.2 g/l) and acetate (0.8 g/l) production.

The following solid wastes addition allowed a short production of succinate, acetate and butyrate which probably indicates a weak degradation.
The second glucose addition after 310 hours resulted in a new succinate and acetate production until values of 3.5 g/l and 2 g/l respectively.
The last substrate addition resulted in the change of the metabolism of *F.s.* which has already been described in all the previous cultures in bioreactor. Succinate is very quickly re-consumed and acetate and propionate are produced in very large amounts.

The final VFA concentrations are 0.3 g/l succinate, 2.6 g/l acetate, 2.2 g/l propionate, 1.5 g/l butyrate.

3.5.6 Results of degradation

The total content of the bioreactor was centrifuged (16 000g, 15'). The liquid effluent was sent to the first MAP team (Ghent University) for second methanogenesis step.
The solid recalcitrant material was dried (vacuum desiccator, 48h, 65°C) and sent to the fourth MAP team (TUHH) for the subcritical liquefaction.

The global *Fibrobacter* degradation of the solid recalcitrant wastes, calculated by dry mass measurement, was 25%.

3.6 Discussion on the third loop

The results obtained for this third loop, concerning the degradation of recalcitrant solids from methanogenic reactor by *F.s.*, are clearly less interesting than those obtained for second loop experiment. Indeed, the global degradation obtained for the second closed loop experiment reached 40% instead of 25% for the third one.

The introduction of liquid effluent from the methanogenic reactor in the *Fibrobacter* culture media is the only experimental difference between the two loops and could probably be an explanation. This is supported by the results of the batch tests in serum bottles which showed that *Fibrobacter* could not grow in a media containing only the liquid methanogenic effluent.
Further tests will be necessary to determine if the presence of toxic or inhibitory compounds in this effluent are responsible for this worth results.

Nevertheless, it also appeared that it is difficult to start and to maintain the *Fibrobacter* culture in these conditions. Regular glucose additions were necessary to obtain the degradation of the solids. The lack of easily degradable substrates will probably involves an augmentation in the frequency of substrate additions. An analyze of the ideal retention time of the solids in the reactor could probably allows a better efficiency of the degradation.

4.0 DETERMINATION OF THE C AND N MASS BALANCES

The determination of C and N mass balances have been performed on a previous reactor described in TN 2.300. For this experiment, the culture of *F.s.* was made on the classical ESA substrate (23.3 % wheat straw, 23.3 % soya, 23.3 % cabbage, 20 % faeces and 10 % spirulinas) in a bioreactor containing 3.5L of total culture media. The culture was maintained during 1000 h with 6 substrate additions and the total substrate added during this period correspond to 262 g DM.
All the analyses necessary for the characterization of input and output were performed for this experiment.

4.1 Characterization of the input

- *substrate*

A sample of each of the 5 substrates was sent to the CNRS (National Center for Scientific Research, France) for an elementar analysis (C,H,N,O,S,P). The DM of the total substrate added in the bioreactor was determined after 24 h, 105°C).

- *culture media*

The only significant C source in the culture media is the carbonate added for the initial reduction of the media and the regulation of pH during the culture. The total quantity added was calculated.

4.2 Characterization of the output

- *solid residue*

A sample of the recalcitrant solid at the end of the experiment was sent to the CNRS (National Center for Scientific Research, France) for an elementar analysis.
The total quantity of this residue was estimated by dry mass measurement (vacuum desiccation 48h, 65°C)

- *CO_2 production*

The total CO_2 overpressure evacuated after each switch of the electro-valve of the system was collected in a graduated flask. The total volume of CO_2 produced was estimated by this way.

- *biomass*

The biomass concentration in the bioreactor was estimated by a correlation with the succinate and acetate produced during the experiment. These correlations were first established for culture on a single substrate (glucose) and then used for these culture on solid wastes.

- *VFA concentration*

The concentrations of succinate, acetate, propionate, isobutyrate, butyrate, isovalerate and valerate were determined at the end of the experiment by HPLC analysis.

4.3 Determination of C balance

source	*%C*	*Total added in the reactor (g)*	*Total C (g)*
wheat straw	42,55	61,32	26,09
soya	44,92	61,32	27,54
cabbage	39,63	61,32	24,30
spirulina	47,24	26,34	12,44
faeces	52,33	52,68	27,57
carbonate	11,32	20	2,26
Total			117,95

Table 5 : Carbon composition of the input

VFA	*Production (g/l)*	*Total (g)*	*Total C (g)*
acetate	9,83	34,41	13,75
propionate	2,44	8,54	4,15
isobutyrate	1,32	4,63	2,52
butyrate	6,63	23,22	12,65
isovalerate	0,73	2,55	1,50
valerate	0,24	0,84	0,49
Total	21,20	74,18	35,06

Solid residue	*%C*	*Total weighed (gDM)*	*masse C (g)*
	45	120	54

CO_2	*Volume (l)*	*Total CO2 (g)*	*Total C (g)*
	21	38,42	10,48

Biomass	*Concentration (g/l)*	*%C*	*Total C*
	7,5	44	11

Table 6 : Carbon composition of the output

	INPUT			**OUTPUT**		
Source	Solid wastes	Carbonate	Residue	VFA	CO_2	Biomass
Total C (g)	117,95	2,26	54	35,06	10,47	11
Total C (g)	120,21		112,03			
Mass balance(%)	93					

Table 7 : Determination of C mass balance

The C mass balance was 93 % on the overall experiment, which indicated the efficiency of the control on the *Fibrobacter* process. The method used for the determination of biomass concentration (correlation with succinate and acetate production) seems to give interesting results. This determination will have to be validated by further experiments.

The VFA, which are produced in very large amounts for culture on ESA substrate, represent a very important part of the output, as well as recalcitrant residue. The impact of this huge amount of VFA (more than 20 g/l in the liquid effluent) on the bacterial growth will have to be studied. The outlook of maintain a lower value (membrane separation) could probably increase the efficiency of the substrate degradation by *F.s.*

4.4 Determination of N balance

Source	*%N*	*Total added in the reactor (gDM)*	*Total N (g)*
wheat staw	0,34	61,32	0,21
soya	8,14	61,32	4,99
cabbage	2,27	61,32	1,39
spirulina	9,97	26,34	2,63
faeces	4,35	52,68	2,29
sum			11,51

Table 8 : Composition of the input

N-NH3	*Concentration (g/l)*	*Total N-NH3 (g)*	*Total N (g)*
	1	3,5	2,72

Solid residue	*%N*	*Total weighed (g DM)*	*Total N (g)*
	6,1	120	7,32

Biomass	*%N*	*Concentration (g/l)*	*Total N (g)*
	4,2	7,5	1,05

Table 9 : Composition of the output

	INPUT	**OUTPUT**		
	Solid wastes	Residue	N-NH3	Biomass
Total C (g)	11,51	7,32	2,72	1,05
Total C (g)	11,51	11,09		
Mass balance (%)		96		

Table 10 : Determination of N mass balance

As well as for C mass balance, the result obtained for N mass balance is found near 100% for this experiment. This confirms the efficiency of the control on the *Fibrobacter* process and the validity of the technicals and methods used for the determination of each input and output. These interesting results will have to be validated by the determination of mass balances on other experiments in bioreactor.

5.0 CONCLUSION / OUTLOOKS

Fibrobacter succinogenes is a very efficient bacteria for the degradation of vegetable wastes. The sequential addition of the three vegetable substrates during a culture in bioreactor clearly showed that the kinetic of degradation is markedly higher for cabbage than for soya than for wheat straw. The analysis of the cell wall composition of the vegetable wastes showed that total fiber proportion and the percentage of lignin and hemicellulose could give one explanation. This experiment also proved the interest of gas pressure monitoring for the determination of bacterial growth and consequently the efficiency of solid substrates degradation.

The degradation of recalcitrant solids from the methanogenic reactor for the third closed loop experiment did not give results as good as for second closed loop experiment. Only 25% of these solids were degraded by *F.s.* and the start of the culture needed an addition of a single substrate (glucose). The introduction of liquid effluent from the methanogenic reactor in *Fibrobacter* culture media could be one explanation. Further experiments will probably clarify this point.
Finally, the determination of C and N mass balances on *Fibrobacter* process gives very interesting results and highlights the deep knowledge of the control of this process.

Nevertheless, the efficiency of the degradation of vegetable wastes by *Fibrobacter succinogenes* can probably be increased. Some analysis on the optimal retention time of the solids in the reactor will have to be performed as well as the identification and characterisation of the enzymes involved in the cell wall degradation.
Finally, substrate exchange with partner 1 (methanogenic process) and parter 4 (subcritical liquefaction will have to be carried on in order to optimize the global degradation of the solid wastes.

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