MAP T E C H N I C A L N O T E

Contract 14719/00/NL/SH MAP AO-99-LSS-015

TECHNICAL NOTE: 86.2.5

CHARACTERISATION OF *FIBROBACTER SUCCINOGENES* UNIT MASS BALANCES

prepared by/*préparé par* Gwendoline CHRISTOPHE, Jérome SEON, Catherine CREULY, Claude-Gilles DUSSAP

reference/*réference* (contract number)
issue/*édition* 1
revision/*révision* 0
date of issue/*date d'édition*
status/*état* Draft
Document type/*type dedocument* Technical Note
Distribution/*distribution*

C O N F I D E N T I A L D O C U M E N T

APPROVAL

Title *titre*	issue 1 *issue*	revision 0 *revision*

author *auteur*	date *date*

approved by *approuvé by*	date *date*

CHANGE LOG

reason for change /*raison du changement*	issue/*issue*	revision/*revision*	date/*date*

CHANGE RECORD

Issue: 1 Revision: 0

reason for change/*raison du changement*	page(s)/*page(s)*	paragraph(s)/*paragraph(s)*

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1 INTRODUCTION

Fibrobacter succinogenes is able to grow on a mixture of substrates containing only wheat straw, soya bean and green cabbage (TN2.300). The sequential addition of the three vegetables during the culture in bioreactor showed a different kinetic of degradation. Based on these results, this report gives tests performed in order to overlook the efficiency of the degradation using all vegetables on fed batch process including all the control parameters.

Fibrobacter succinogenes S85 (ATCC 19169) was originally isolated from the bovine rumen (Bryant and Doestch, 1954) and has been maintained as a pure culture in laboratory ever since. It has been grown anaerobically under 100% CO_2 in a basal medium that contained (per liter) : 450 mg KH_2PO_4 (SI), 450 mg K_2HPO_4, 900 mg NaCl, 1.8 g $(NH_4)_2SO_4$, 90 mg Mg SO_4, 90 mg $CaCl_2$, 3 mg Mn SO_4,6 H2O (SII), 0.3 mg $CoCl_2$,6 H_2O, 8 mg $FeSO_4$,7H_2O (SIII), 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 *and al.,* 1992). The complexity of this medium is incompatible with the process, so it was decided to simplify it.

Finally, technical studies on HACCP were carried on in order to apply it into *Fibrobacter succinogenes* process.

2 DEGRADATION IN BIOREACTOR

2.1 General condition of culture

2.8L of this media was introduced in the 5L reactor (total volume) 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 until –350 mV and increase pH to values compatible to *Fibrobacter succinogenes* growth (6.0-6.5). Then, as *Fibrobacter succinogenes* is a strictly anaerobic organism, the reactor was flushed with CO_2 during one night in order to completely fill the atmosphere with this gas.

After these preparation steps, the thermostated (regulation at 39°C by an external bath) and stirred (100 rpm) reactor was inoculated with an overnight preculture (400 mL) grown on cellobiose (8g/l).

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The carbon and energy sources were constituted only of vegetable wastes. A mixture of sterilized (121°C, 20') wheat straw, soya bean cake and green cabbage (44g DM, 1/3 of each in DM) was introduced in the initial culture media and then once a week. The total substrate added in the reactor in the time course of the culture is described in table 1.

Substrate	DM (g)	Proportion (%)
Wheat straw	61.3	33
Soya bean cake	61.3	33
Green cabbage	61.3	33

Table 1 : Composition and total quantity of the substrate

2.1.1 On-line monitoring and control

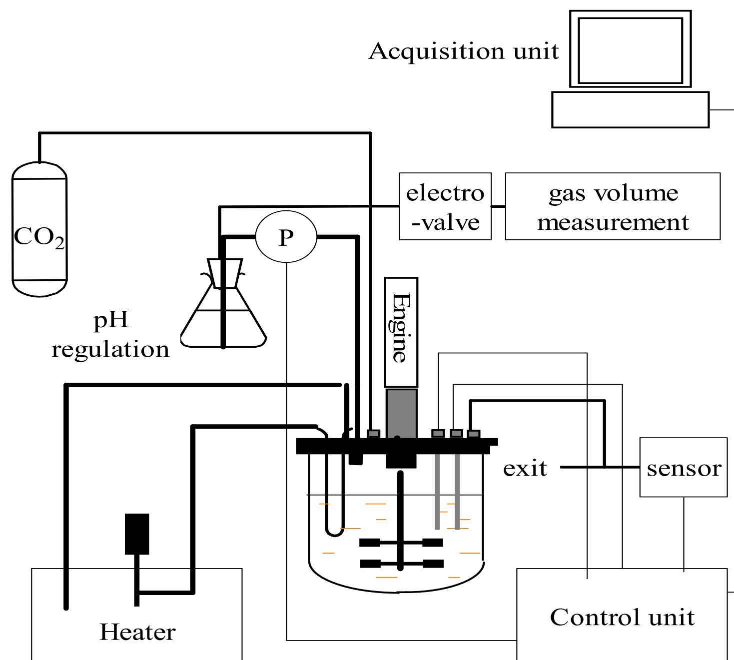

Figure 1 : Design of the strictly anaerobic reactor

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The 5L reactor (Figure 1) was equipped with pH, redox potential (Ingold) and temperature probes, all connected to a control unit. On-line acquisition of these three parameters was realised on a computer with acquisition software. Data acquisition of each of these parameters was performed every 4 minutes.
A gas pressure sensor (range 2 bars over atmospheric pressure) was connected on the gas loop and related to the control and acquisition units. Data acquisition of this parameter was realised every 4 minutes.

An electro-valve (2 ways, PVDF, range 2 bars over atmospheric pressure) was also connected on the gas loop in order to respect the specifications of the reactor concerning resistance to 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 bars. This results in the reduction of pressure down to 1.15 bars.
After inoculation, the reactor was closed with gas tight seals and the flush of CO_2 was then only connected for addition of new substrate and / or sampling of liquid phase.

2.1.2 Analytical procedures

Volatile fatty acids were determined by HPLC analysis (Hewlett Packard). N-NH_3 concentration was measured in the liquid phase by Patton and Crouch procedure. Gas phase composition was determined on gas samples by gas chromatography (Hewlett Packard 5890). 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.

2.1.3 Determination of percentage of degradation

Percentage of degradation was estimated by dry mass measurement of all the content of the reactor (substrates, residue of fermentation and bacteria) (105° C, 48 h).
The composition in cellulose, hemicellulose and lignin of fibrous substrate and residue of fermentation was determined by Van Soest method.

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2.2 *Results*

2.2.1 Monitoring of pH and redox potential

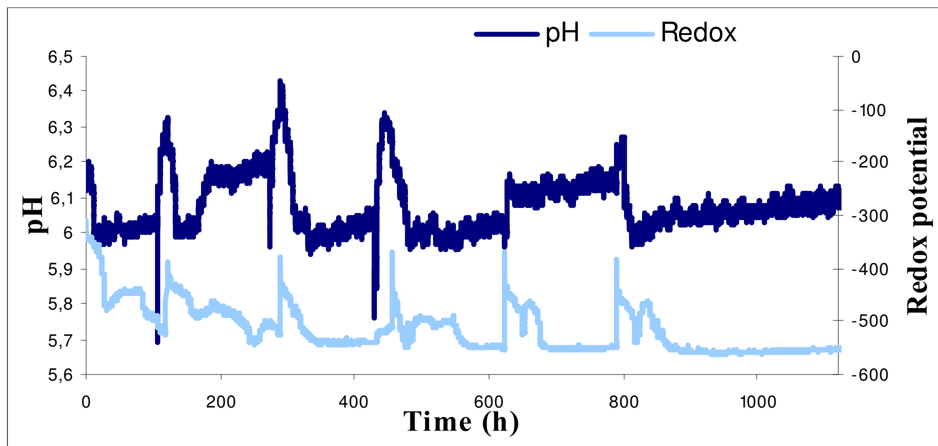

Figure 2 : Evolution of redox potential and pH

Figure 2 shows the evolution of redox potential and pH during the time course of the experiment. The redox value is always comprised between –300 and –550 mV (relative value) during all the experiments, which validates the maintain of strictly anaerobic conditions during the culture. Each time the reactor is opened (addition of substrates), an increase of redox value can be observed, but never over –400 mV, thanks to the flush with CO_2. The value decreases again as soon as the reactor is completely closed.
pH curve shows the efficiency of pH regulation. The high VFA production rate which characterizes *Fibrobacter succinogenes* growth is compensated by the addition of the concentrated carbonate solution (Na_2CO_3 100 g/l). As the same way as for redox potential, the opening of the reactor for substrate addition result in a modification of chemical equilibrium in the liquid phase so that pH is increased over 6.0.
Finally, this graph clearly shows the strong chemical relation between these two parameters.

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2.2.2 Monitoring of gas pressure

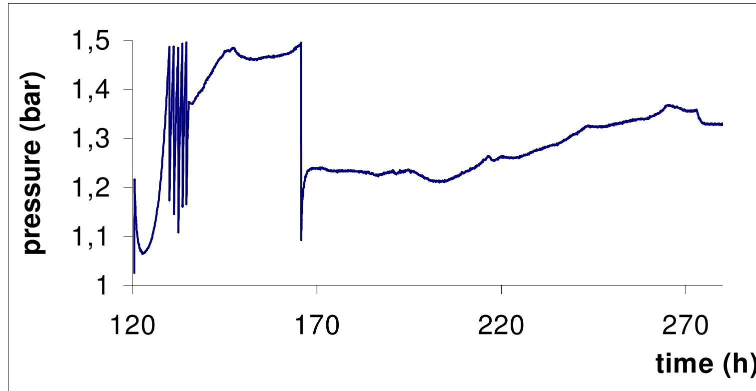

Figure 3: Evolution of gas pressure during the second batch (120-275h of culture)

Figure 3 describes the evolution of gas pressure between the second addition of substrates. 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. Indeed the reactor was flushed with CO2 during the addition of substrate so gaseous phase contain more CO_2 than the liquid phase. Then, CO_2 production begins and is very important during about 10 hours. Each time pressure reaches 1.5 bars, the electro-valve is opened and it results in a drop of pressure value down to 1.15 bars. After this first phase, gas production goes on but appears clearly lower until the next addition of fresh wastes.

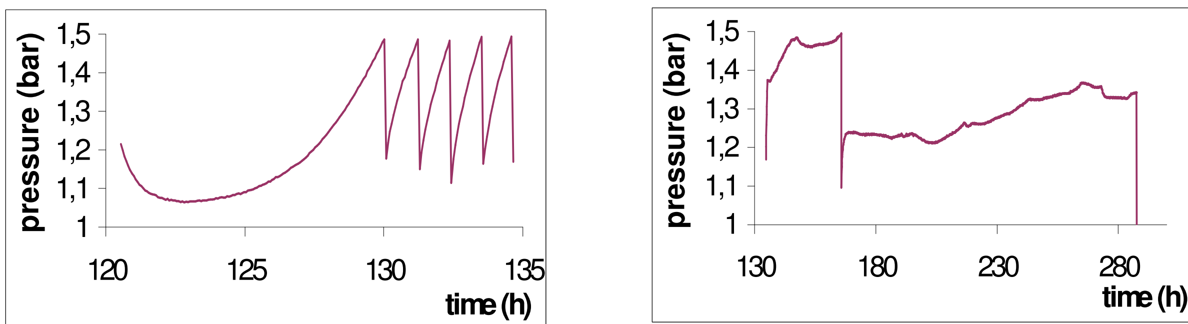

Figure 4 : Evolution of gas pressure during the second batch (120-275h of culture)

This separation in two phases has been observed for each batch. It appears very clearly on figure 4 which focuses on each one of these two steps. This may be explained by the degradation of two types of substrates : during a first step, the more easily degradable substrate

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(single sugars liberated by autoclave, cellulose with simple access…) is degraded, which allow a very quick kinetic of degradation and a very active metabolism of *Fibrobacter succinogenes* ; then, the degradation of fibers of cellulose and hemicellulose, which are strongly cross-linked with lignin. The kinetic of degradation of these substrates are clearly lower and results in a deceleration of *Fibrobacter succinogenes* metabolism.

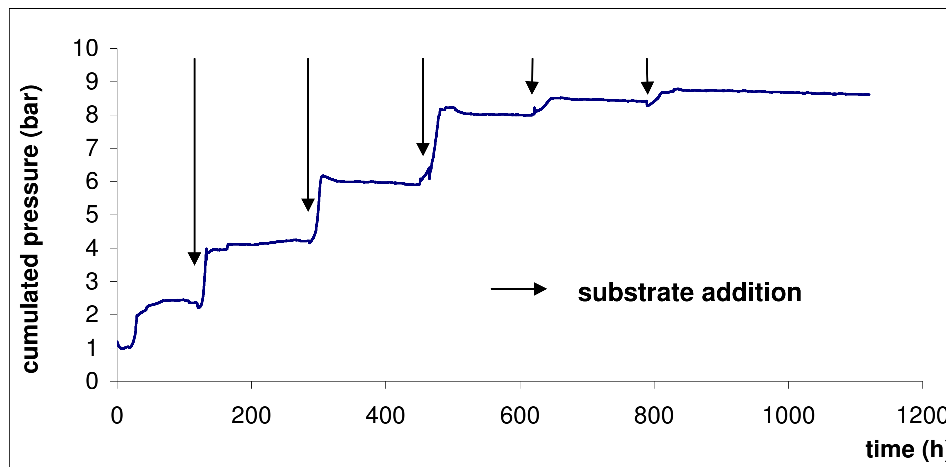

Figure 5 : Evolution of cumulated pressure in the reactor

Figure 5 shows the evolution of cumulated gas pressure in the reactor during the 1100 hours of culture. The results observed on the focus of the second batch are confirmed by this curve. During about 10 to 20 hours after each addition of new vegetables wastes, an important gas production can be observed. Then the production is clearly lower until the next addition of substrates and sometimes compensated by some gas leaks on the reactor. This production is around 1 to 2 bars for the four first batches and clearly lower (0.5 bars) for the two last one. As it was described for figure 4 the decrease of pressure may be explained by the degradation of two types of substrates, so two types of metabolisms.
The total gas production during all the culture represents around 7 bars, which means about 20 liters of CO_2.

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2.2.3 Evolution of metabolite concentrations

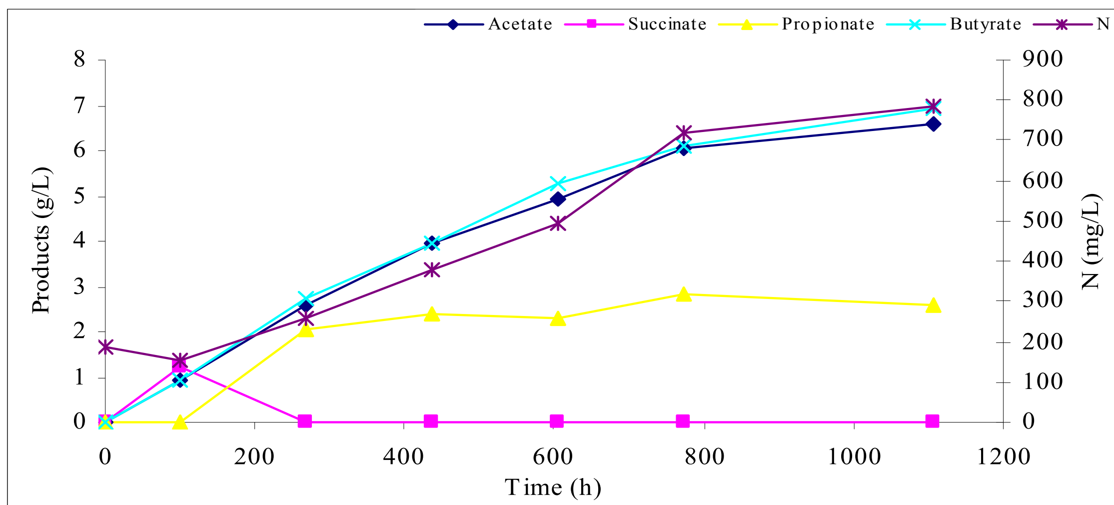

Figure 6 : Metabolic evolution during the culture

Figure 6 represents the evolution of metabolites concentration during the experiment. The first hours of culture are characteristic of *Fibrobacter succinogenes* metabolism with production of mainly succinate and acetate (1.2 g/l). As it was observed in the previous tests, all the succinate was reconsumed after 100 hours of culture and production of other VFA (butyrate and propionate) began. The evolution of acetate and butyrate concentration became linear and quite important. The final production of these two metabolites after 1100 hours of culture was about 8 g/l each. The production of propionate decreases after 300 h of culture and its total production after 1100 h was 2.5 g/l. The total VFA production during all the experiment was around 20 g/l.

N-NH_3 curve is also similar to what was observed during the previous culture in bioreactor. After a consumption of nitrogen source during the first hours of culture, a regular increase of this concentration is observed during all the culture, probably due to the liberation of ammonium ions from vegetables wastes. The final N-NH_3 concentration is about 800 mg/l.

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2.2.4 Percentage of degradation

All the content of the reactor was centrifuged (15', 13 000g) and the liquid and solid phase were separated. The solids were dried (vacuum desiccation, 48 h, 60°C) and weighed. The global percentage of degradation estimated by dry mass measurement was 64%. This result is higher than the degradation obtained when spirulinas and / or faeces (50 / 57%) are added in the reactor. This confirms the better efficiency of *Fibrobacter succinogenes* when the substrates are only vegetables.

2.2.5 Determination of carbon and nitrogen mass balances

	Input		**Output**			
Source	substrates	carbonate	solid residue	VFA	CO2	Biomass
Total C (g)	77,94	2,53	29,14	28,25	9,13	8,54
Total C (g)	80,47		75,06			
Mass balance	**93%**					

Table 2 : Determination of C mass balance

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.

The results for the determination of C mass balance are summed up in table 2. The C mass balance was 93% for the overall experiment, which confirms the efficiency of the control and regulation of *Fibrobacter succinogenes* process.
$_{t}$VFA, which are produced in very large amounts during the culture (20 g/l), represents approximately the same part of carbon as the solid recalcitrant solid (38 % each). Only 12% of total carbon is got in the gas phase (CO_2) and 12 % in the biomass.

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	Input		Output		
Source	substrates	$N-NH_3$	solid residue	$N-NH_3$	Biomass
Total N (g)	6,59	0,64	2,98	2,5	1,32
Total N (g)	7,23		6,79		
Mass balance	**94%**				

Table 3 : Determination of N mass balance

As well as for C mass balance, the results obtained for N mass balance (94 %, see table 3) is very promising and confirms the efficiency of the control of *Fibrobacter* process. 44% of the total N is set in the recalcitrant solids. 37 % is observed in the form of $N-NH_3$ and 20 % in the biomass.

C and N mass balances are higher than 90%, which 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.

3 SIMPLIFICATION OF THE CULTURE MEDIUM

3.1 Experiment set up

The first step was to determine the essential elements of the medium. Cultures were carried on in tubes according to the Hungate technique (1954). For each culture a component of the basal medium was deleted like hemin, SIII, biotine, PABA, all the VFA and the no essential VFA (neVFA) (acetate, propionate, butyrate and isovalerate), except for the control which contained all the elements of the basal medium. Mediums were prepared under CO_2 and sterilized (20', 121°C). Culture were anaerobically transferred with hypodermic syringes (2 mL) to tubes and incubated 60 hours at 39°C. The growth was followed by measuring culture turbidity at 600 nm. All the tests were carried on in triplicate. Lag times, generation times and biomass at the end of the growth were calculated.

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The second step consists in the validation of a minimum medium established from the previous results, this medium containing only the elements considered as essential for *Fibrobacter succinogenes* growth.

The experiments were performed in serum-bottles (100 ml) when the carbon source was glucose and in bioreactor (4L) when it was solid wastes.
For culture in serum bottles, 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 culture turbidity at 600 nm and by VFA production.
The preparation steps for cultures in bioreactor are the same those made previously. The growth on wastes cannot be followed in a simple way such as a measurement of culture turbidity because of wastes. It was consequently followed by the metabolites production, the CO_2 production and by the percentage of degradation of the vegetable wastes.

Metabolites concentrations were determined by HPLC analysis and the degradation by dry mass measurement (105°C, 48h).
The figure 7 illustrates the protocol using to validate the minimum medium.

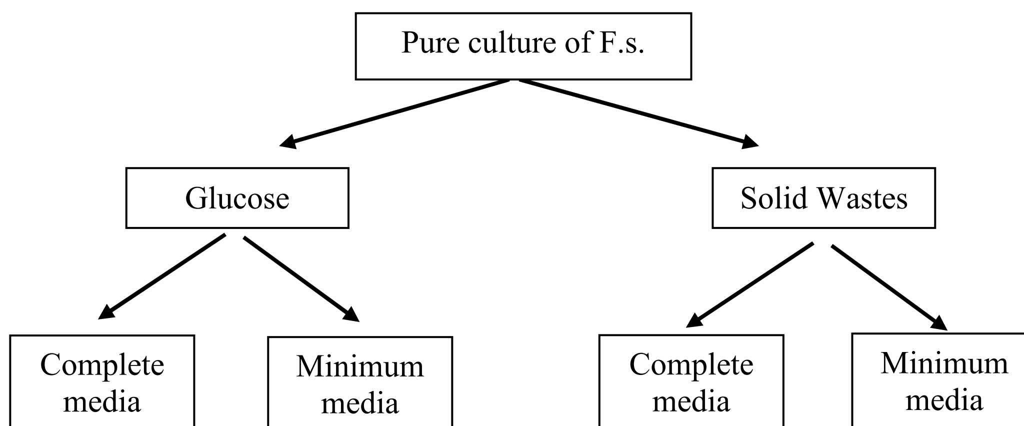

Figure 7 : Protocol of validation of a minimum medium

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3.2 Results

3.2.1 Preliminary analyses

The pH was controlled at the beginning and at the end of the cultures. The pH remained in ranges accepted by *Fibrobacter succinogenes* (5, 5 to 7,7). In all case, a medium acidification was observed, except in the medium without VFA. This acidification, general characteristic of *Fibrobacter succinogenes* (Weimer, 1993), is probably coming from the production of VFA. This was controlled by HPLC : VFA concentrations, in all the mediums where an acidification was observed, are identical to the control.

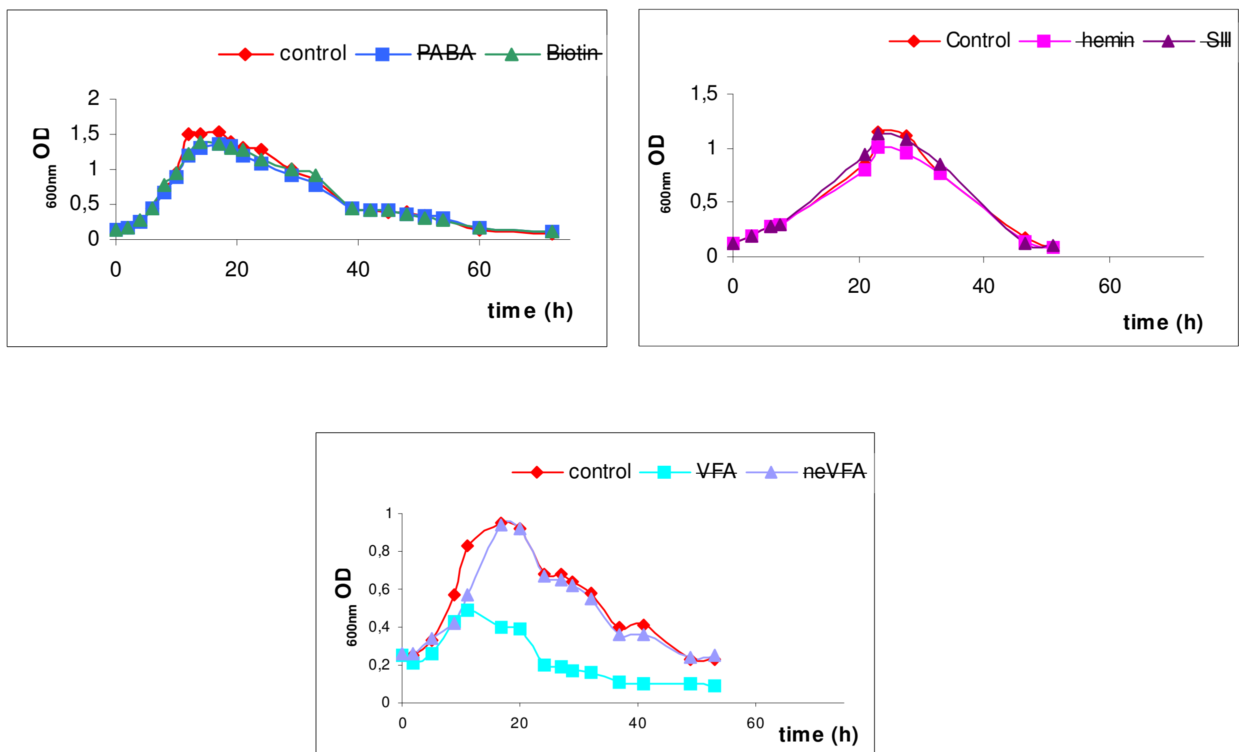

Figure 8 : Growth of Fibrobacter succinogenes in the different culture medium

Figure 8 represents the evolution of the optical density during the time of culture in different conditions. In the medium which does not contain hemin or SIII, the growth *of Fibrobacter succinogenes* is almost identical to the control. In the same way, no significant difference was observed when biotin or PABA was deleted. So, the presence of hemin or SIII or PABA or

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biotin seems to be non essential in the culture medium for the growth. These results are in agreement with Wells and Russel (1996) with Miller (1978) experiments. Moreover a growth comparable to the control was also obtained if the non essential VFA were suppressed and there is an important difference for the medium without VFA. This confirms that valerate, isobutyrate and DL-2-methylbutyrate are essentials for *Fibrobacter succinogenes* growth (Dehority, 1967)

Generation times, lag times and the biomass concentrations at the end of the culture are summed up in table 4. The results observed on growth curve in figure 8 are confirmed by this table. No difference were calculated when the culture medium was prepared without hemin or SIII or biotin or PABA or neVFA. The only significant difference was observed when all VFA were depleted, where the generation time is more important and the quantity of biomass is lower.

Supressed elements	Lag time (h)	Generation time (h)	Biomass (g/l)
Control	3	4,1	3
hemin	3	4,1	2,7
SIII	3	4,4	3
Control	2	1,8	4
biotin	2	2,2	3,6
PABA	2	1,8	3,7
Control	2	3,4	2,5
VFA	2	4,9	1,3
neVFA	2	3,7	2,5

Table 4: *Fibrobacter succinogenes* growth parameters

The tests show that the presence of PABA, biotine, SIII, hemin is non essential for *Fibrobacter succinogenes* growth and only the absence of the VFA essential (valerate, isobutyrate and DL-2-methylbutyrate) strongly slowed down the growth.

Based one this preliminary tests, we can defined a minimum medium containing only SI, SII and eVFA and valid it.
The tests were performed on glucose in serum-bottles (100mL) and on solid wastes in a bioreactor (5L).

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3.2.2 Validation of a minimum media on glucose as carbon source

The growth on glucose was determined by measuring the turbidity of the culture (600 nm) and by VFA production.

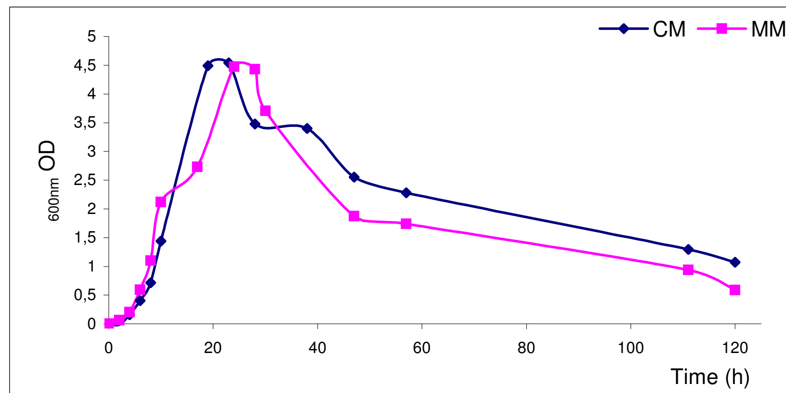

Figure 9 : *Fibrobacter succinogenes* growth on glucose

Figure 9 shows *Fibrobacter succinogenes* growth on glucose in a minimum medium (MM) and in a complete medium (CM). The growth of *Fibrobacter succinogenes* on glucose is the same in the two mediums. The generation times (2.8 h), the lag time (5.71 h) and quantity of biomass (1.8 g/L) at the end of the exponential phase are also similar. These results also show that the minimum medium doesn't affect the growth.

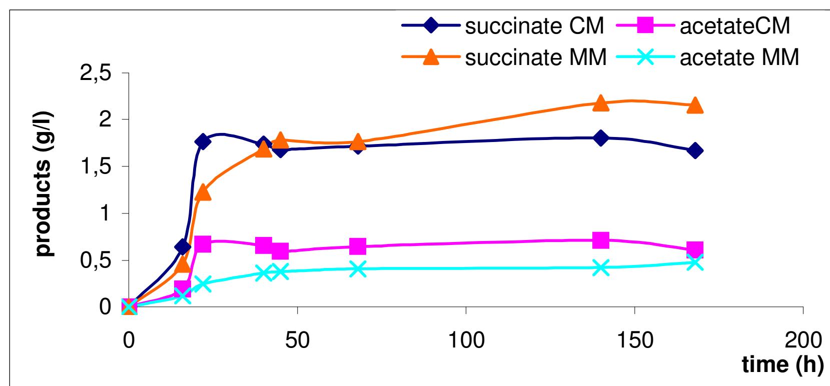

Figure 10 : Evolution of VFA production on glucose

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The general profile of the curves (figure 10) obtained with the minimum medium is the same obtained with the complete medium and the metabolites are produced in the same concentrations. So on glucose, there are few effects on succinate or acetate production.

There is thus no effect of the minimum medium use on *Fibrobacter succinogenes* growth and no effect on the production of the metabolites (acetate and succinate) when the only source of carbon is glucose. So, this medium can be tested on vegetable wastes as carbon source.

3.2.3 Validation of a minimum media on solid wastes as carbon source

The growth on solid wastes (ESA-substrate) was determined by measuring the production of VFA, CO_2 and by the percentage of degradation.

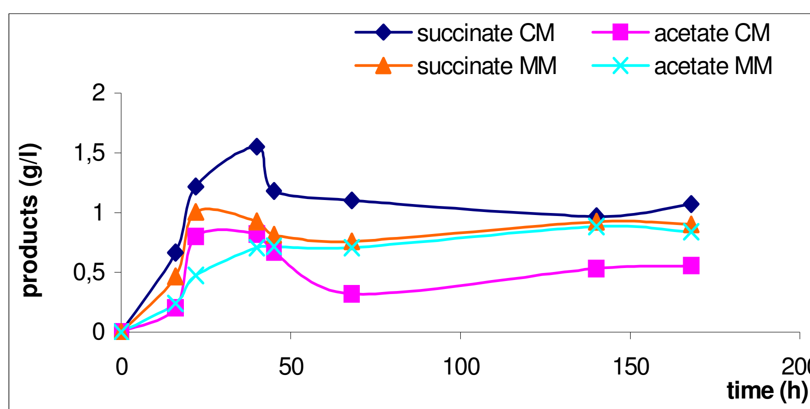

Figure 11 : Evolution of acetate and succinate concentration during 200h.

At the end of the culture, few effects of the minimum medium utilisation were observed on acetate and succinate production (figure 11). Incubation of cells on solid wastes with only SI, SII and eVFA, shows that *Fibrobacter succinogenes* can produce acetate and succinate in the same scale.

	Succinate g/L	Acetate g/L	Propionate g/L	Lactate g/L
waste / CM	1,07	0,56	0,78	0
waste / MM	0,90	0,84	0	1,52

Table 5 : VFA productions on solid wastes

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Table 5 confirms that acetate and succinate are produced in the same scale in the two medium but propionate was not detected when cells were incubated with the minimum medium and in the opposite, lactate was detected. This indicates that *Fibrobacter succinogenes* presents a metabolic reversion but further analyses must been made to explain the production of lactate.

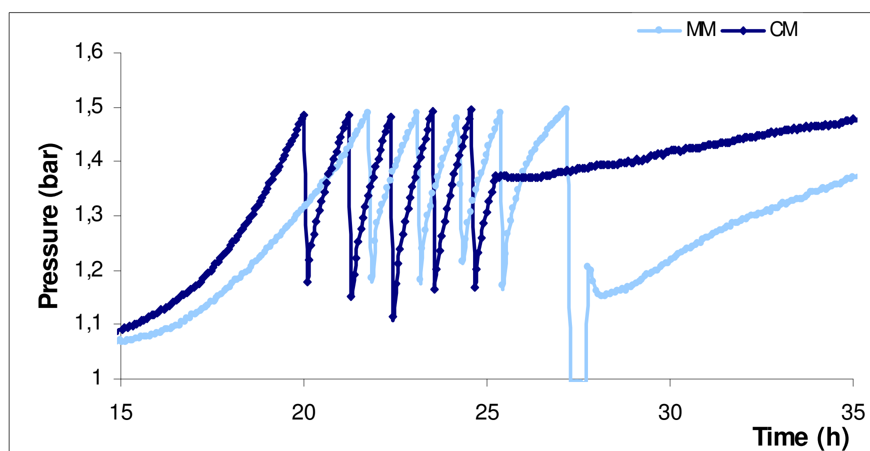

Figure 12 : Evolution of gas pressure

Figure 12 describes the evolution of gas pressure during the first batch. CO_2 production begins after 15 hours and is very important during about 10 hours. After this phase, gas production increase and stop. This figure also shows two steps in the degradation : the more easily substrate is degraded then the recalcitrant substrate. Those two steps in the degradation are observed in the two mediums. The use of the minimum medium thus does not disturb *Fibrobacter succinogenes* CO_2 production.

The degradation rate was performed by dry mass measurement of residues of fermentation. 59% of degradation was obtained with the complete medium and 57% with the minimum medium. The comparison between the two rates of degradation seems to indicate that the minimum medium had no effect on the efficiency of *Fibrobacter succinogenes* degradation.

The defined minimum medium can be used without changing neither the growth of *Fibrobacter succinogenes* neither VFA nor CO_2 production nor the degradation rate.

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4 HACCP

4.1 Definition

It is a preventive method which identifies and evaluates the hazards associated to the various stages of the process, and so defines the means necessary to their control. HACCP is a management tool used to protect the food supply against biological, chemical and physical hazards. First is used for food and then could be developed for space programs.

4.2 Objectives

The objective of this method is to guarantee the safety and to set up the quality control of a product. It led to identify seven principles of action :

- Conduct hazard analysis and identify preventive measures,
- To identify critical control points (CCPs) in the process,
- To establish critical limits,
- Monitor each CCP,
- To establish corrective actions,
- To establish verification procedures,
- To establish record-keeping and documentation procedures.

4.3 The step

4.3.1 Preliminaries phases

The first phase consists in a definition of the study plan. The type of hazards and the extent of the plan are chosen. Then, an HACCP group is constituted. This team must be multi-field because the step utilizes various and varied concepts (physical, chemical, microbiological, human…). An organizer is named in order to direct the team. A secretary is also named, this one is responsible for the HACCP meeting reports.

The product must be described. The objective of this step is to describe precisely the raw materials, ingredients, intermediate products and end products (storage, conditioning, treatments, use…). The information led to appreciate later the appearance of the hazards and the elements necessary to their control. During this phase, a diagram of the process must be

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established. It is a representation of the various operations of the process. The diagram is then checked “in situ”.

4.3.2 Hazard analyses

At each stage, it is necessary to identify, to list and to describe the modes and the causes of appearance of the hazards and their effects in order to apply preventive measures. To carry out correctly this step, it is advisable to utilize the "5M" method (medium, method, material, raw materials and experimenter). For each M, hazards, causes and preventive measures are indexed. These preventive measures are actions, activities, techniques, means, materials or factors necessary to control the hazard causes identified by HACCP team.

4.3.3 Characterisation of the critical points and critical limits

At this step, the CCPs are identified. A CCP is a point at witch control can be applied to prevent or eliminate a safety hazard or reduce it to an acceptable level.
CCPs should not be too important in order to focus on a point in particular. It is necessary for each CCP to apply, in complement of the preventive measures defined previously, a control monitoring. A minimum and/or maximum value (critical limit), whose respect is necessary to ensure the effective control of the CCP, are defined for each parameters. This identification of the critical limits is the first corrective action.

Example :
Hazard = bacterial (pathogens)
CCP = sterilisation
Critical limit = ≥ 121°C 20 min for elimination

4.3.4 Establishment of a monitoring system

A monitoring system and a control system of the process are established in order to ensure a monitoring continuously. However, in practice the monitoring is discontinuous, it is thus necessary to define the frequency and the number of the operations of control.
The monitoring system must contain a corrective action plan, documentation and a validation of the HACCP plan.

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4.3.5 Checking of the performance of system HACCP

There are procedures to follow when a deviation occurs and actions to confirm that the system according to the plan. These actions must be planned for each CCP, in order to allow an immediate action and a fast elimination of the hazard.
The last step is the installation of a documentary system thanks to two types of documents : documents on HACCP system showing all points of the method (procedures...) and registers to prove the application and the control of the system (report...).

5 CONCLUSION / OUTLOOKS

The process using *Fibrobacter succinogenes* can degrade solid wastes vegetable wastes with a good efficiency (64%) and all the parameters of the culture are controlled (temperature, pH, redox, CO_2 pressure…). The results obtained for carbon and nitrogen mass balances confirm the efficiency of the control and validate the techniques used.

Moreover we define a minimum medium which contains SI, SII and eVFA. This minimum medium can be use without disturbing the growth, acetate, succinate and CO_2 production and the rate of degradation. It however remains to explain the production of lactate and the absence of production of propionate.

Documentation on HACCP method shows the difficulty of applying HACCP analysis on this kind process. We must now list and select the most interesting techniques to prevent and control biological, chemical and physical hazards in *Fibrobacter succinogenes* unit. But it's not so easy to adapt HACCP method to a biological process using microorganism. So, as we decided in DTU meeting, we should add in our reactors a persistent chemical product that is biologically not degradable and not toxic for the system.

Nevertheless *Fibrobacter succinogenes* efficiency to degrade can probably be increased in particular by the knowledge of the optimal retention time or by the identification and the characterisation of the enzymes responsible for degradation. Finally substrates exchange with partner 1 will have to be carried on in order to optimize the global degradation of solid wastes.

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