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

TECHNICAL NOTE: 2.8

HACCP-ANALYSIS AND TESTING OF MONITORING PROCEDURES

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1 INTRODUCTION

This report gives study on the HACCP (Hazard Analysis Critical Control Point) method for Fibrobacter unit and the degradation of substrate from LabMET.

HACCP method 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.
First, a recall of the method is made. This method consists in four parts; the preliminary analysis (characterization of the product), the hazard analysis (identification of the hazards), the CCPs determination (identification of the Critical control points) and the establishment of a monitoring system. Documentation on HACCP method shows the difficulty of applying HACCP analysis on Fibrobacter unit. However we listed and selected the most interesting techniques to prevent and control biological, chemical and physical hazards in *Fibrobacter succinogenes* unit.

Finally, according to the last meeting, degradation of substrate from partner 1 was performed. In the previous TN 2.7 substrate from metanogenic unit was degraded by *Fibrobacter succinogenes* with a rate of 32% and with VFA production. To obtain this degradation rate, the optimal residence time was determined and the totality of the substrate was added at the beginning of the fermentation. But a stop of production and of the degradation was observed after 150h of culture probably due to an inhibition by the VFA produced. So for this loop we have decided to remove the culture media after 150h of culture.

2 HACCP METHOD

The general objectives of hazard analysis are:
- The assessment of the level of safety of a system in a deterministic way
- The increase of the level of safety of a system through hazard reduction
- The initiation of the use of hazard reduction to drive the definition and implementation of, for example, design and operation requirements, specifications, concepts, procedures
- The provision of a basis for defining adequate safety requirements, determining the applicability of safety requirements, implementing safety requirements
- verifying their implementation, and demonstrating compliance or non-compliance
- The provision of input to safety risk assessment and overall risk management
- The support of safety related project decisions
- The support of safety submissions and reviews through documented evidence
- The support of safety certification of a system through documented evidence

The complete HACCP procedure was provided by Labmet (Annex 1).

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Each step in the iterative four-step hazard analysis process includes a number of tasks (Figure 1):

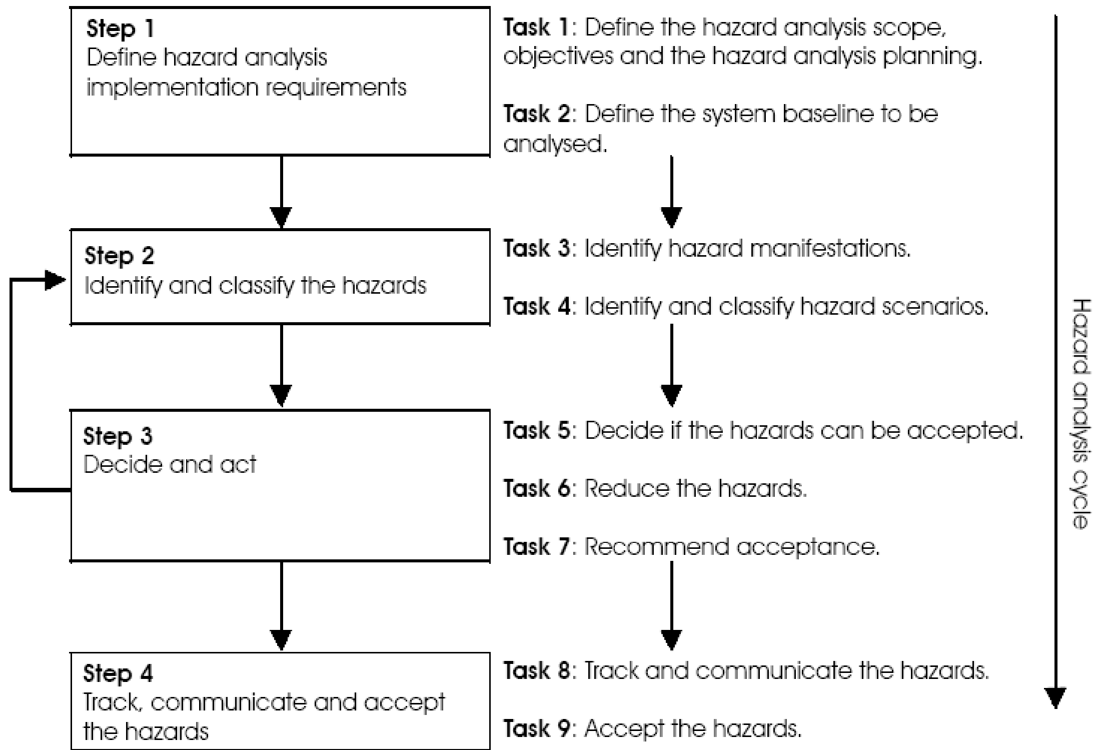

Figure 1: The nine tasks associated with the four steps of the hazard analysis process

3 HACCP IN *FIBROBACTER* UNIT

3.1 Definition of the hazard analysis, implementation requirements

3.1.1 Definition of the hazard analysis

Within the framework of this study we are interested exclusively in *Fibrobacter succinogenes* culture in bioreactor. This bacterium is strictly anaerobic with the ability to degrade vegetable

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wastes. The study thus covers the process of fibres digestion by *Fibrobacter succinogenes* on bioreactor since the reception of the raw materials until vegetable lyses.
The objectives of the HACCP analysis on *Fibrobacter* unit are to define the hazard which can disturb the correct operation of the system. These hazards can be of different severity (Table 1).

Category	Severity	Severity of safety consequence
I	Catastrophic	Loss of the culture
II	Critical	Temporally disabling
III	Marginal	Minor disability
IV	Negligible	Les than minor disability

Table 1: Safety consequence severity categorization

In this study only the hazards of catastrophic severity will be regarded as unacceptable hazards. These hazards will have thus to be controlled and regularly checked in order to avoid them. The others could be corrected by direct corrective actions.
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. A monitoring system and a control system of the process are established in order to ensure a monitoring continuously.

There are procedures to follow when a deviation occurs and actions to confirm that the system functions according to the plan. These actions must be planned for each hazard, in order to allow an immediate action and a fast elimination of the hazard. The information led to appreciate later the appearance of the hazards and the elements necessary to their control.

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...).

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3.1.2 Definition of the system baseline to be analysed

Fibrobacter succinogenes fermentation is performed in order to degrade vegetable wastes and to produce VFA and NH_4. The degradation rate must be maximal in the same way for VFA and NH_4 production.

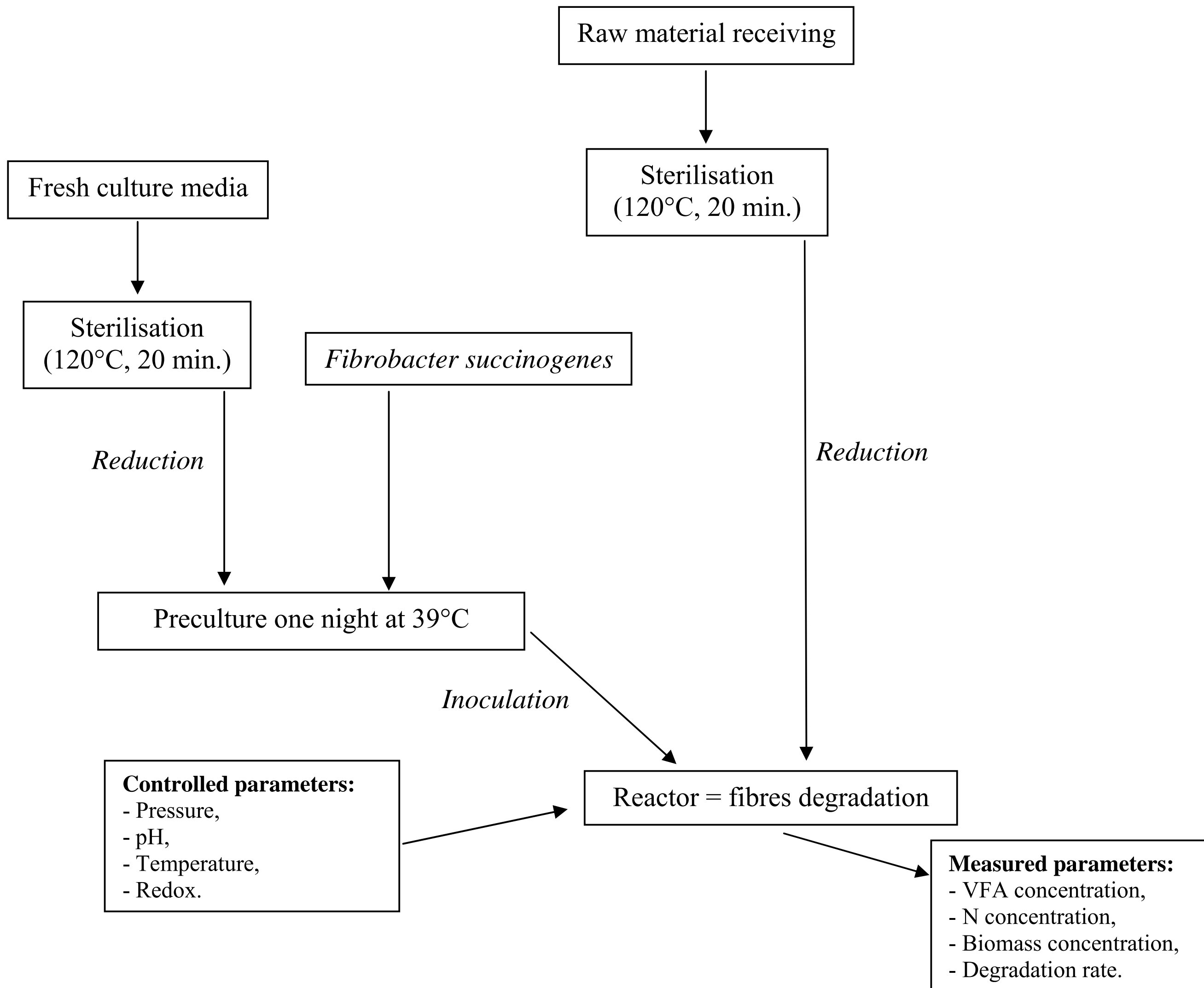

Figure 2: Diagram of the process

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The diagram of the process is described in the figure 2:

1. All the raw material added with the fresh medium is sterilized in order to eliminate microbial contamination risks,
2. A preculture is performed with fresh and sterile culture medium,
3. This one is incubated a night at 39°C and introduced in the bioreactor,
4. *F.s.* in the bioreactor degrade the vegetable wastes,
5. The growth is controlled by several parameters such as pH, temperature, redox…Samples are performed regularly to determinate VFA and N concentrations,
6. At the end of the fermentation degradation rate is determined.

The inputs of the culture are constituted by two parts, a part witch concerns the medium, the raw material and carbonate for the pH regulation and a part witch concerns the bacteria. The outputs of the culture are monomers of glucose which stem from the degradation of cellulose and hemicellulose, lignin, CO_2, NH_4 and VFA produced during the fermentation, no CH_4 has ever been detected.

The inputs and outputs specifications are presented in appendix 1.

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HACCP analysis Laboratoire LGCB	Appendix 1: SPECIFICATION Bioreactor data sheet	Identification: SPE002a
		Date: 30/09/05
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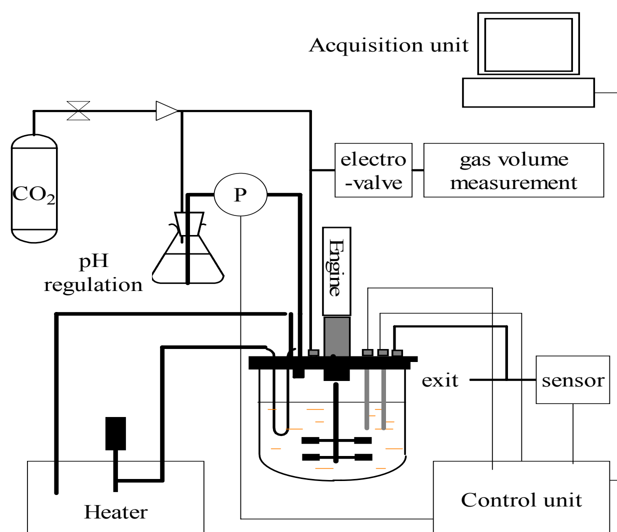
Fibrobacter succinogenes was grown in a 5 L total volume reactor (SETRIC, France) with a working volume of 4 L.



The reactor is equipped with pH, redox potential (Ingold) and temperature probes, all connected to a control unit. An absolute pressure sensor (JPB, France) is connected on the gas loop and related to the control and acquisition units. An electro-valve (2 ways, PVDF, range 2 bars over atmospheric pressure) is also connected on the gas loop in order to respect the specifications of the reactor concerning resistance to pressure. This electro-valve is 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. On-line acquisition of these parameters is realised on a computer with acquisition software. Data acquisition of each of these parameters is performed every 4 minutes. Temperature (regulation at 39°C by an external bath), rotation speed (100 rpm) and pH (6.0) are kept at constant values. The pH regulation is performed with a 100 g/L Na_2CO_3 solution. This solution is connected to a peristaltic pump under the order of the control unit. Each time pH value become fewer than 6.0, the pump starts and introduces the 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

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HACCP analysis Laboratoire LGCB	Appendix 1: SPECIFICATION Culture media composition	Identification: SPE002a Date: 30/09/05 2/5

Culture media composition:

Solution	Preculture (500 mL)	Reactor (3 L)
SI	37.5 mL	225 mL
SII	37.5 mL	225 mL
SIII	100µL	600 mL
VFA	1.5 mL	9 mL
Biotin	100 µL	600 µL
PABA	50 µL	300 µL
Resazurine	1.5 mL	9 mL
Hemin	350 µL	2.1 mL
Substrate	4 g	200 g
H_2O	420 mL	2520 mL
Sterilisation 20 min. 120 °C flush with CO_2 during one night for the reactor and 3 hours for the preculture		
Na_2CO_3	1.6 g	11.2 g
Cystein	0.2 g	1.4 g

Solution composition and conditioning:

Solution	Composition		Conditioning
I	K_2HPO_4	0,6%	7°C
II	KH_2PO_4	0,6%	
	$(NH_4)_2SO_4$	1,2%	
	NaCl	1,2%	
	$MgSO_4$	0,12%	
	$CaCl_2$	0,12%	
III	$MnSO_4$, $6H_2O$	0,015%	
	$CoCl_2$, $6H_2O$	0,0015%	
	$FeSO_4$, $7H_2O$	0,040%	
VFA	Acetate	17mL	
	Propionate	6 mL	
	Butyrate	4 mL	
	Isobutyrate	1 mL	
	n-Valerate	1 mL	
	Isovalerate	1 mL	
	DL 2-methylbutyrate	1 mL	

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HACCP analysis Laboratoire LGCB	Appendix 1: SPECIFICATION Substrate	Identification: SPE002a Date: 30/09/05 3/5

Three types of substrate are used: Glucose, vegetable wastes and LabMET substrate.

1. The glucose must be perfectly sterile before being introduced into the reactor.

2. The vegetable wastes are constituted by sterilized (20’, 121°C) wheat straw, soya bean cake and green cabbage (30.66 g DM, 1/3 of each). The mixture is introduced in the initial culture media and then once a week. Wheat straw and soya bean cake are ground in dry conditions in a blender. Fresh green cabbage is first cut in small parts and then ground in a kitchen mixer. All the substrates are mixed in order to increases the surface area for the cells attachment.

3. The substrate is sent to us by LabMET and must be perfectly sterile before being introduced into the reactor. All the substrate is sterilised but only the solid effluent is introduced into the fermentor.

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HACCP analysis Laboratoire LGCB	Appendix 1: SPECIFICATION Fibrobacter succinogenes	Identification: SPE002a
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Fibrobacter succinogenes:

Fibrobacter succinogenes S85 (ATCC 19169) a bacillus Gram +, 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 litre) : 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 $FeSO_4$ 7 H_2O, 0.25 mg biotin, 0.005 mg para-aminobenzoic acid (PABA), 0.01 mg hemin, a mixture of volatile fatty acids (VFA) (Gaudet et al., 1992) and carbon substrate.

Fibrobacter succinogenes have culture conditions defined by Bryant and Doestch (1954) i.e. a temperature of 39°C and a pH of 6.

Metabolism:

Fibrobacter succinogenes uses cellulose, glucose or cellobiose as carbon and energy source and ammonia as only nitrogen source to produce acetate, succinate and carbon dioxide mainly.

This bacterium (Wells and Russel, 1996) requires for its growth: ammonia, volatile fatty acids: valerate, isobutyrate and DL 2 methylbutyrate, acid para aminobenzoïc, the ions such as Na+, K+, Mg2+, Ca2+, phosphate and carbonate.

The depletion of these factors in the medium causes the loss of the capacity to degrade cellulose by *Fibrobacter succinogenes*.

Fibrobacter succinogenes produces primarily four metabolites: succinate, acetate which is obtained starting from acetyl CoA, during the fermentation of hexoses (Miller, 1978), but also, butyrate and some traces of formate obtained by the reduction of the carbon dioxide or by the oxidation of the pyruvate (Matheron, 1997).

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HACCP analysis Laboratoire LGCB	Appendix 1: SPECIFICATION Fibrobacter succinogenes	Identification: SPE002a
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Fibres degradation:
Fibrobacter succinogenes is one of the major cellulolytic bacteria found in the bovine rumen (Stewart and Flint, 1989). This bacterium digests very efficiently the more crystalline forms of cellulose such as wheat straw. Moreover it becomes predominant among the cellulolytic bacteria when ruminants are fed with poor diet i.e. highly lignified material. 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. Xylanases or disconnecting enzymes (Forsberg et al., 1994) allow the separation of hemicellulose and lignin from the rest of the matrix and lead to improve the degradation of hemicellulose (Mc Dermid et al., 1990). After a narrow adhesion with the cell walls of the vegetables (Fields et al., 2000) the cellulose is depolymerized and releases in cellodextrine which will be hydrolyzed in glucose and cellobiose in the periplasm. Glucose and cellobiose are then fermented in succinate, acetate (Miller, 1978; Stewart and Flint, 1989), CO_2 as main metabolites and a short amount of formate.

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3.2 Identification and classification of the hazard

3.2.1 Identify hazard manifestations

The hazards in this study are microbiological, physical and chemical. These dangers relate to the process just as the users. i.e. the process hazards as an explosion due to pressure increase can cause damage to the users and bad handling of the operator as an introduction of a chemical into the reactor can cause damage to the process (stop of growth for example)

Microbiological hazards: Control of the axenicity of the MELiSSA compartments is needed because contaminants (bacteria, fungi, protests, viruses, bacteriophages) constitute a major concern in the proper functioning and maintenance of a closed artificial ecosystem. Contamination by biomolecules, normally not produced in a given compartment, may result in reactor instability or even collapse of the system, rendering the biomass of Arthrospira sp. Unsuitable for human consumption. In addition, contamination of compartment IV by pathogenic organisms will pose serious risks to human health.

The source of contamination of Fibrobacter unit can be germs with the same cellulolytic properties as *Fibrobacter succinogenes* and able to growth in anaerobic conditions and with very low redox conditions. Among these micro-organisms we can quote:

- Cellulolytic microorganisms of the rumen such as *Ruminoccoccus albus* or *R. flavefacians*. These bacteria degrade vegetables and produce soluble sugars like *F.s.*.
- Anaerobic bacteria of the ground such as *Clostridium naviculum* and *C. Botulicum*.

Physical hazards: The physical hazards are mainly parameters such as temperature, pressure, pH, liquid or gas leak and anaerobic conditions which would be badly regulated and could disturb bacterial growth. Pressure built up due to malfunction of the pressure release valve might lead to explosion and consequent loss of the culture. Additionally, there are risks to the users.

For the same reason as for microbiological hazards, it can also be a dysfunction of the material of measurement or sterilization but also contaminants such as the plastics, metal or glass which could be introduced into the medium following bad handling.

Chemical hazards

In the same way if chemical compounds are introduced into the culture medium they could contaminate the culture and pose serious risks to human health and to culture health. The material can also be a chemical contamination of the medium if is damaged. Although the components of the fermentor are essentially in glass or stainless steel the risk remains present. This is due to the fact that the medium is corrosive and thus could deteriorate stainless steel.

The generic hazards applicable to the system design are presented in table 2

Generic hazards	**Subsystem elements**			
	F. succinogenes	Fermentor	Substrates	Autoclave
Microbiological	X	-	X	-
Physical	X	X	-	X
Chemical	X	-	X	-

Table 2: Hazard matrix on *F. succinogenes* unit

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The description of the hazard manifestations are given in table 3.

Subsystems	Manifestations
F. succinogenes	No growth / no production of metabolites or production of other metabolites / no degradation of wastes
Fermentor controls	Increase of the pressure or of the temperature
Substrates	Presence of microorganisms
Autoclave	Bad sterilisation of the fermentor or of the raw material so contamination of the strain

Table 3 Manifestation of the hazards

3.2.2 Identification and classification of the hazard scenarios

Hazard manifestation	Cause-Events-Consequence	Consequence severity	Observable symptoms
Microbial contamination	Faulty operation of the autoclave-contamination-growth of a non-desired bacterium	Catastrophic	None or production of an other metabolite
	No sterile inoculation-contamination-growth of a non-desired bacterium	Catastrophic	
Chemical contamination	Faulty preparation of the culture media-stop of F.s. growth-no degradation	Catastrophic	None
Variation of the pressure	Malfunction of the control unit-pressure increase-explosion	Critical	Pressure increase
	Electric breakdown-stop of the monitoring control-decrease of the growth	Negligible	Pressure drop
Variation of the temperature		Marginal	Temperature drop
Variation of the pH	Electric breakdown-stop of the monitoring control-decrease of the growth	Marginal	pH drop
	Electric breakdown-stop of the pH regulation-decrease of the growth	Critical	

Table 4: Hazard scenarios

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3.3 Decide and act

3.3.1 Decide if the hazards can be accepted

At this step, the hazards are identified but not all can be accepted. An acceptable hazard is a point at witch control can be applied to prevent or eliminate a safety hazard or reduce it to an acceptable level.
Hazards should not be too important in order to focus on a point in particular. It is necessary for each hazard to apply, in complement of the preventive measures defined previously, a control monitoring. A minimum and/or maximum value (critical limit), which respect is necessary to ensure the effective control of the hazard, are defined for each parameters. This identification of the critical limits is the first corrective action.
In this study, fermentor hazards are acceptable because they are online monitored. Online monitoring allows making direct corrective actions. Similarly, the hazards are acceptable for substrate. Indeed, substrate is sterilised before being introduced in the fermentor.
However, contamination can still occur during inoculation of the reactor, and no direct corrective actions can be applied. This highlights the needs for controls.

3.3.2 Reduction of the hazard

The respect of the critical limits insures the control of the hazards (table 5).

Step	Critical limits
Sterilisation	Sensibility of the luminometer (0.1pg ATP in 100 µg luciferase buffer)
inoculation	Limits of biology molecular tests

Table 5: Critical limits

The first hazard is associated to the sterilization by autoclave of the process container and culture medium. The risks are due to a bad adjustments or a dysfunction of the autoclave. The preventive measures which must be taken are

To respect the autoclaving procedure

To respect the maintenance plan and tests procedures (appendix 2).

The second hazard is associated to bacterial contamination during inoculation. Regular checking of culture parameters (VFA…) associated to molecular biology tests allow to determine whether the culture is contaminated or not.
The preventive measure is to respect thoroughly the inoculation procedure (appendix 2).

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HACCP analysis Laboratoire LGCB	Appendix 2: Verification of the autoclave efficiency	Identification: I.PRO001a
		Date: 30/09/2005
		1/2

Objective
The purpose of this instruction is to give the precautions to check the good effectiveness of the autoclave on the laboratory equipments.

Who?
Only the people competent and authorized to handle the autoclave can use it.

Where?
The autoclave must be located in a particular room.

When?
According to a planning elaborated and recorded by the person in charge for the fermentor.

How?
The efficiency of sterilisation is checked by carrying out sterilisation tests using thermosensitive bacteria. To perform this test thermosensitive bacteria are cultivated in flask and sterilised by autoclave 20 min at 121°C then this culture is inoculated in sterile conditions on solid medium. If bacteria growth, the autoclave must be control.

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HACCP analysis Laboratoire LGCB	Appendix 2: *Fibrobacter succinogenes* inoculation	Identification: I.PRO002a
		Date: 30/09/2005
		2/2

Objective
The purpose of this instruction is to give the precautions for the inoculation of *F.s.*.

Who?
Only the people competent and authorized to inoculate in the fermentor can do it.

When?
The inoculation is performed at the beginning of every culture in bioreactor. Before inoculation the fermentor must be sterilised and the atmosphere must be saturated with CO_2.

How?
Sterile material (grips, funnel….) must be used at all inoculation steps.

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3.4 Track, communicate and accept the hazards

3.4.1 Tack and communicate the hazards (Table 6)

	Corrective actions	
Step	**Means of action**	**Responsible**
Autoclave	Calibration of the autoclave set values (Temperature, Pressure, Time)	Responsible for the fermentor
Inoculation	To throw all the medium and sterilisation of all the material	

Table 6: Corrective actions

3.4.2 Establishment of a documentary system

The monitoring and control system are constituted of the 4 types of documents:

- Specification (appendix 1). This documentation is used as complement to all the other procedure documents,
- Procedures: these documents aim to describe the protocols used to achieve the objectives,
- Instructions: these documents aim to explain the preventive and corrective actions (appendix 2 and 3),
- Recording supports: With these documents it’s possible to keep a copy of work performed and results obtained.

HACCP plan is re-examined every year by the person in charge according to the recordings of the previous year. In the same way it is re-examined in the course of year to update the new internal requirements (modifications of the raw materials...) and external requirements (environment, customers...); a new version of the plan is then worked out by the person in charge of the quality control.

In all the HACCP system the staff training is essential and necessary. This training is intended for all people who work on *F.s.* fermentation. The aim of this training is to inform the personnel about the control of the critical points and to make them sensitive to the various risks existing in the laboratory and their consequences on the productivity of *F.s.*.

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HACCP analysis Laboratoire LGCB	Appendix 3: Hazards analysis	Identification: HACCP tables Date: 30/09/2005 1/1

		Danger	Hazard	Preventive actions	Corrective actions
Fermentor inoculation	Media	Physical Chemical Microbiological	Contaminations	Respect of the cleaning planning	
	Methods	Chemical	Not respect of the protocol	Internal audit and control cards	To throw the solution and new staff training
	Material	Chemical Microbiological Physical	Contamination by an other chemical product Contamination of the containers Leak of the medium False concentrations of a component because of a badly regulated balance	Cleaning of sample materiel and use materiel of single use Microbiological analysis Maintenance Calibration	Cleaning of sample materiel Sterilisation of the containers Regular maintenance
	Raw material	Microbiological Chemical	Contamination of the container out-of-date product	Microbiological analysis Checking of the expiry dates	Removal of the product
	Manuel labour	Physical Microbiological Chemical	Contamination by the hair, the clothes…. Not respect of the protocol	To put on a blouse… Internal audit and control cards	New staff training

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4 LOOP

4.1 Introduction

The first tests of closed loop experiments (substrate exchanges between the different MAP project processes) gave encouraging results. After the loop in TN 2.6, we were confronted to a difficulty. It was to define a feeding procedure in terms of quantity (200g once) and of frequency (33g once a week). So for the loop in TN 2.7, we proposed to add the totality of the substrate at the beginning of the fermentation to try to obtain a better rate of degradation. To know if this type of alimentation is better than those of the previous loop, we compared the different loops (Table 7).

TN and date	Total added (g DM)	Addition	Total time of culture (h)	Regulation of pH	Degradation rate (% DM)
2.3 (07/2002)	44	2	400	N	28
2.4 (12/2002)	44	2	450	N	25
2.6 (10/2004)	200	6	660	Y	28
2.7 (03/2005)	200	1	325	Y	32

Table 7: Comparison between the different loops

Experiments were performed in order to determine the existing standard deviation on the degradation rates, this ones show the same standard deviation between the loops.
Comparison of the second and the third loop shows that the pH regulation increases the degradation rate but the difference is not very important (25 and 28%) however the DM (g) added in culture 3 is 200g what was not possible in the culture 2 or in culture 1 where the degradation rate is the same because of the low pH caused by degradation. Indeed degradation by F.s. produces VFA which decrease the pH and inhibit the growth, so the difference between the culture with and without the pH regulation is on the level of the degraded quantity. Indeed, in loop 2, 11g (25% of 44g) were degraded and in loop 3, 56g (28% of 200g) were degraded.
The only difference between the third and the last loop is the type of alimentation and we show that the degradation rate is better when we performed the fermentation with batch process.
So we have added a pH regulation to the process. This one increases the degradation rate. After, we have determined the optimal residence time, which allowed us to do the fermentation on batch. But we observed a stop of the productions and of the degradation after 150 h of culture probably due to an inhibition by the VFA produced. So we proposed for this loop to add all the substrate at the beginning of the culture but to remove the culture media to reduce VFA concentration and to eliminate the inhibitor. Figure 4 shows the organisation of the substrate exchanges between MAP partners.

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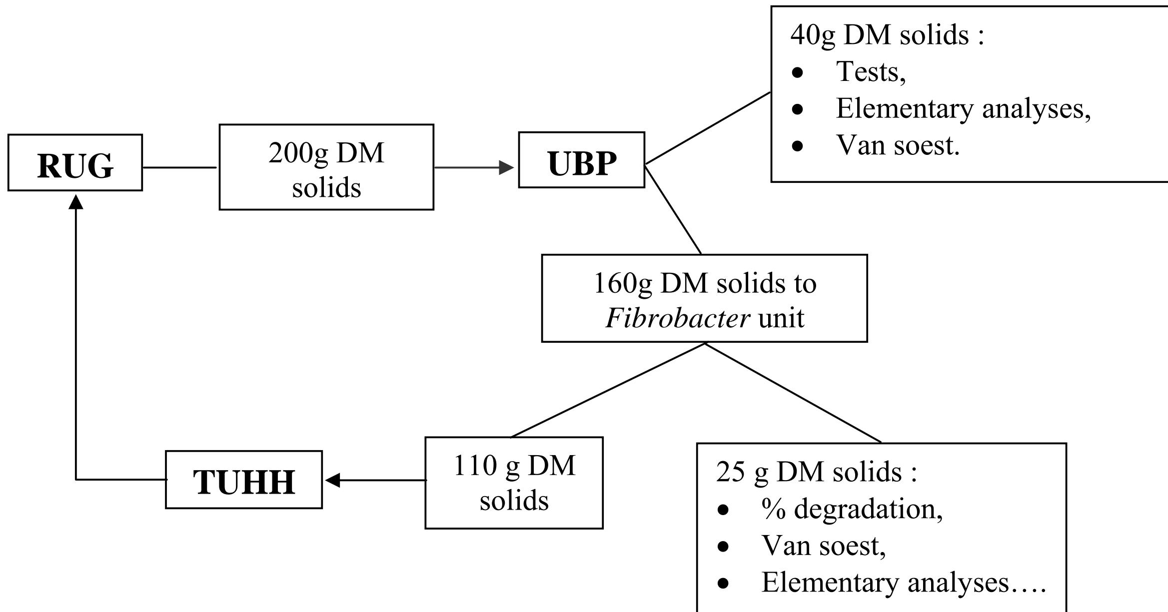

Figure 3: Organisation of the substrate exchanges between MAP partners

4.2 Glucose culture

First the experiments were performed on glucose to test the feasibility of the removal of the culture media. Indeed, the change of the culture media could modify the culture conditions (pH, redox…) and disturb Fibrobacter growth. It is thus necessary to test in first on glucose, which is a simple substrate.

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 described in TN 2.5.

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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).

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-NH_3 concentration was measured in the liquid phase by Patton and Crouch procedure. pH was automatically (peristaltic pump) maintained at 6.0 at least by addition of a concentrated solution of Na_2CO_3 (100 g/l) under CO_2 atmosphere.

The process was maintained during 1250h including 15 additions of glucose (10-12 g/L) in a N-NH_3 solution and 2 changes of the culture media .At 625h the stirring was stop to decant and separate liquid and solid phase during 25 hours. At 650h the liquid phase was replaced by fresh medium.. The same thing was made between the 1020th and 1030th hours.

4.2.2 Liquid phase

4.2.2.1 pH and redox

Figure 4 shows the evolution of pH during the time course of the experiment. The profile of the redox curve is not represented because of a problem with the redox probe. But the redox value is always comprised between –300 and –450 mV (relative value) during all the experiments, which validates the strictly anaerobic conditions during the culture. The pH decreases during the first hours when *Fibrobacter succinogenes* grows and is regulated at 6.01. At 700h and 1030h the pH increases because of the change of the culture media and decreases quickly.

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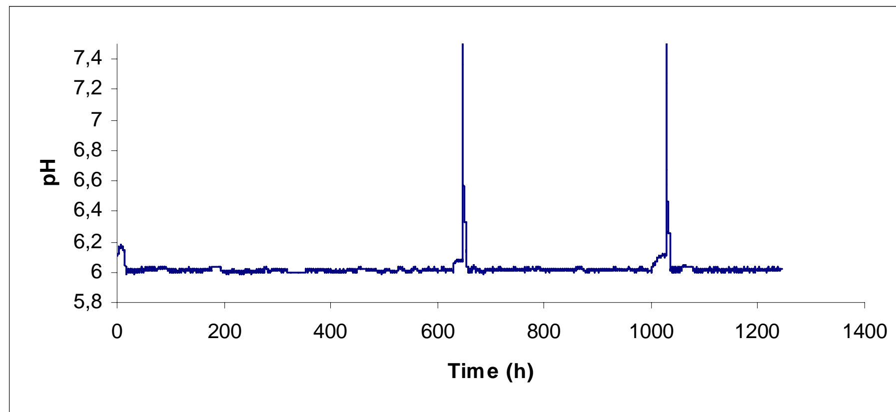

Figure 4: pH evolution

4.2.2.2 VFA and glucose

Figure 5 represents the evolution of metabolites concentrations during the experiment with the first culture media. After 640 hours the concentration of butyrate, acetate, succinate, propionate and formiate was respectively 13.9g/L, 4.41g/L, 2.8g/L, 0g/L and 0.95g/L. The total VFA production during all the process was around 22.06g/L.

Figure 6 represents the evolution of metabolites concentrations during the experiment with the second culture media. After 380 hours the concentration of butyrate, acetate, succinate, propionate and formiate was respectively 2.24g/L, 3.22g/L, 1.5g/L, 6.03g/L and 1.4g/L. The total VFA production during all the process was around 14.4g/L. We can observe the same metabolites with the second culture media except for the propionate. However the concentrations are lower that those obtained with the first media. But this is probably due to the number of glucose addition (8 for the first culture media and 5 for the second) and to the time of culture (650h for the first culture media and 380h for the second)

Figure 7 represents the evolution of metabolites concentrations during the experiment with the third culture media. After 218 hours the concentration of butyrate, acetate, succinate, propionate and formiate was respectively 4.17g/L, 3.23g/L, 1.15g/L, 5.47g/L and 0.78g/L. The total VFA production during all the process was around 14.8g/L. We can observe the same metabolites with the third culture media in the same proportions. However the concentrations are lower that those obtain with the first media. But this is probably for the same reasons as previously (8 for the first culture media in 650h and 2 for the third in 210h).

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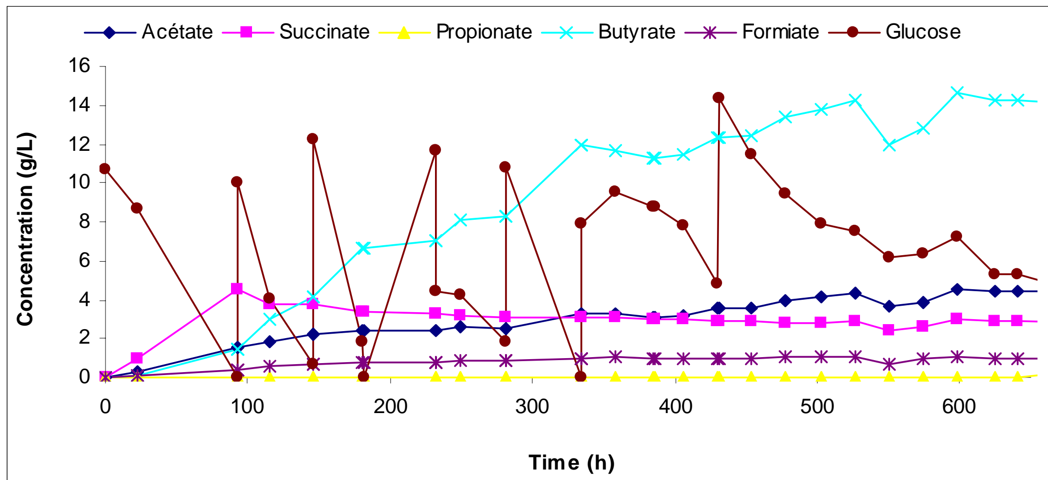

Figure 5: VFA concentrations during the experiment with the first culture media

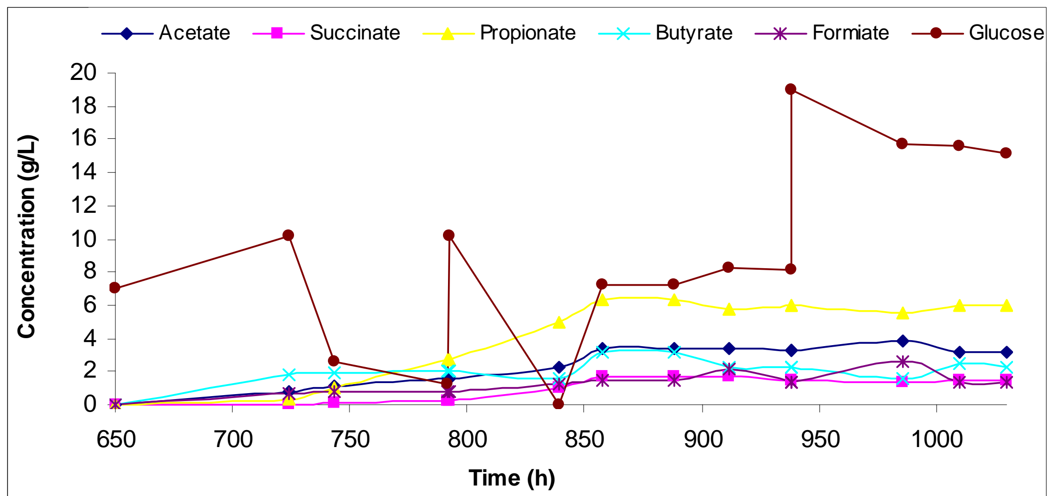

Figure 6: VFA concentrations during the experiment with the second culture media

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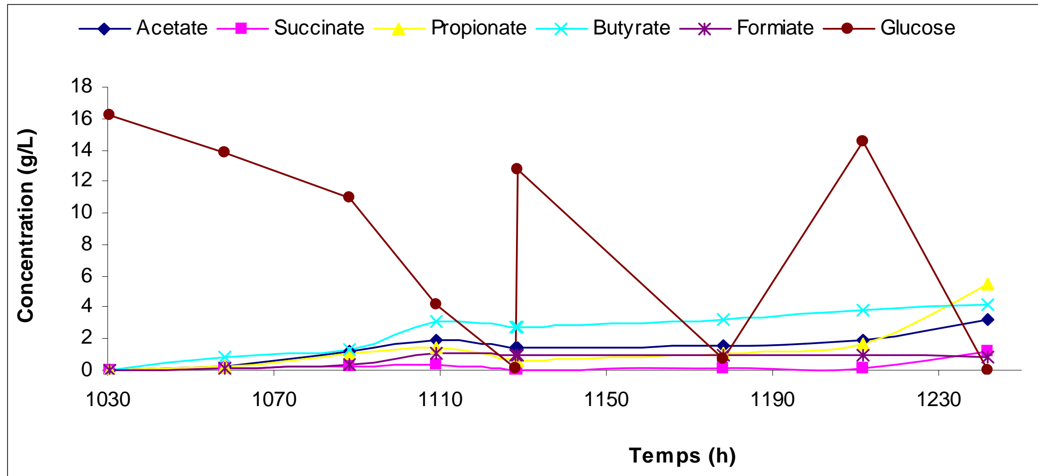

Figure 7: VFA concentrations during the experiment with the third culture media

4.2.2.3 Nitrogen

The additions of glucose were performed in liquid medium containing N-NH_3. This addition of glucose in a N-NH_3 solution explains the increases of nitrogen concentration. We can observe a decrease of N concentration after each addition. This increase shows the growth of *Fibrobacter succinogenes.*

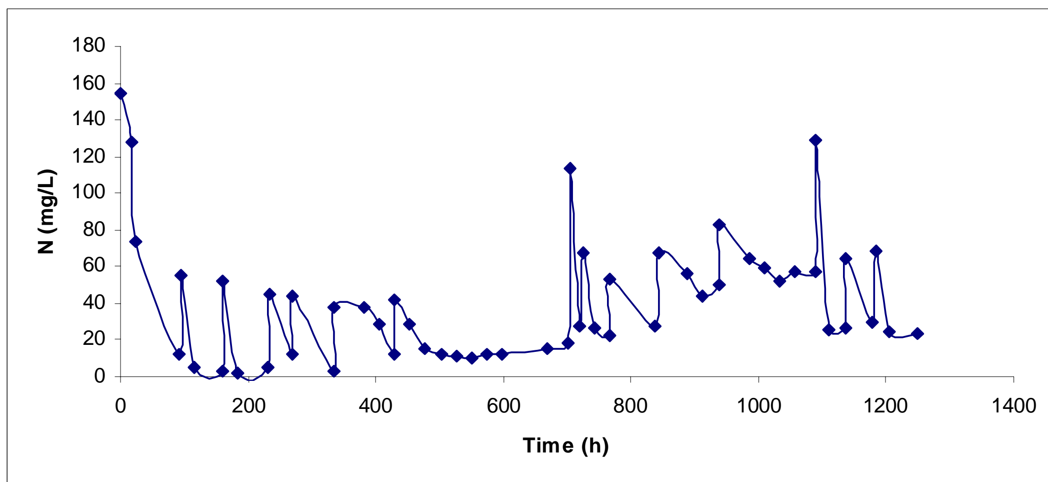

Figure 8: Evolution of N concentration

4.2.3 Gas phase

Figure 9 describes the evolution of gas pressure during the fermentation and figure 10 focuses on one batch. Pressure, which is manually increased to 1.2 bars just after substrate addition of glucose, 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 70 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 glucose. So each time this decrease of production is observed we decided to add glucose, this allows the resumption of the growth.

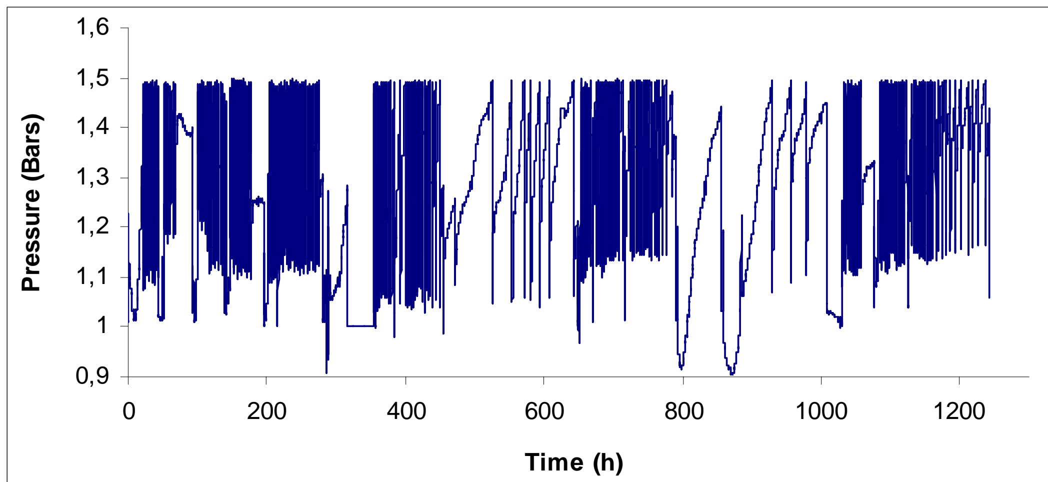

Figure 9: Evolution of gas pressure during the experiment

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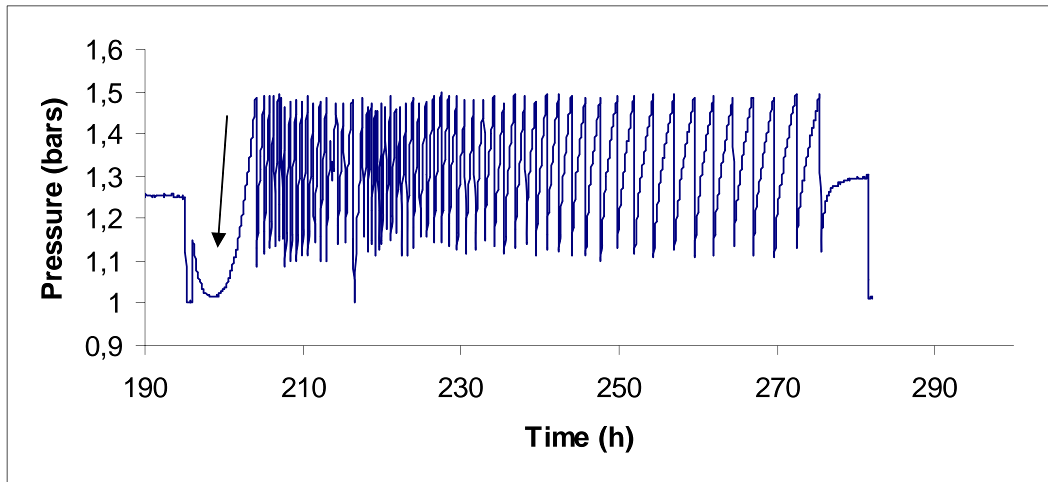

Figure 10: Evolution of gas pressure during the fourth batch (201-281h of culture)
(→ Addition of glucose)

4.2.3.1 Evolution of gas pressure for the three culture media

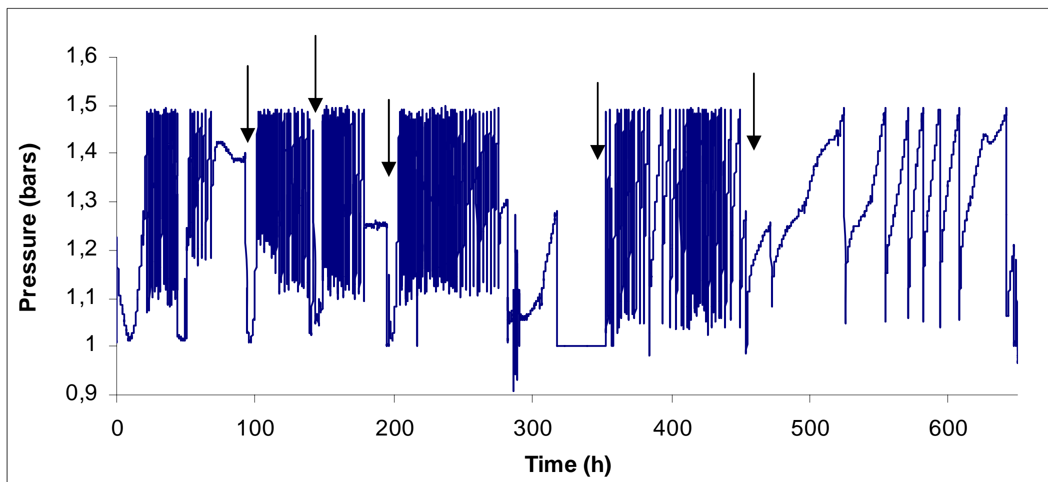

Figure 11: Evolution of gas pressure during the first medium (0-650h of culture)
(→ Addition of glucose)

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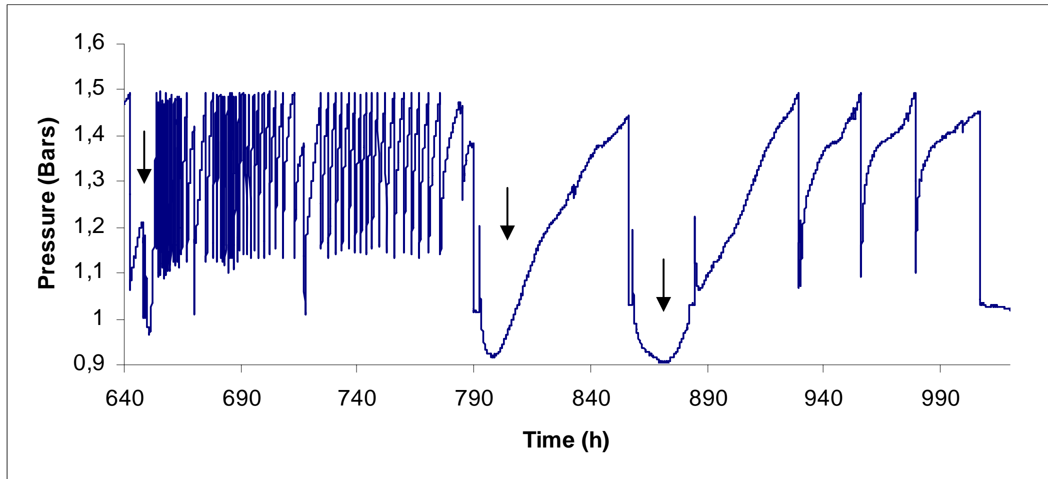

Figure 12: Evolution of gas pressure during the second medium (650-1030h of culture)
(→ Addition of glucose)

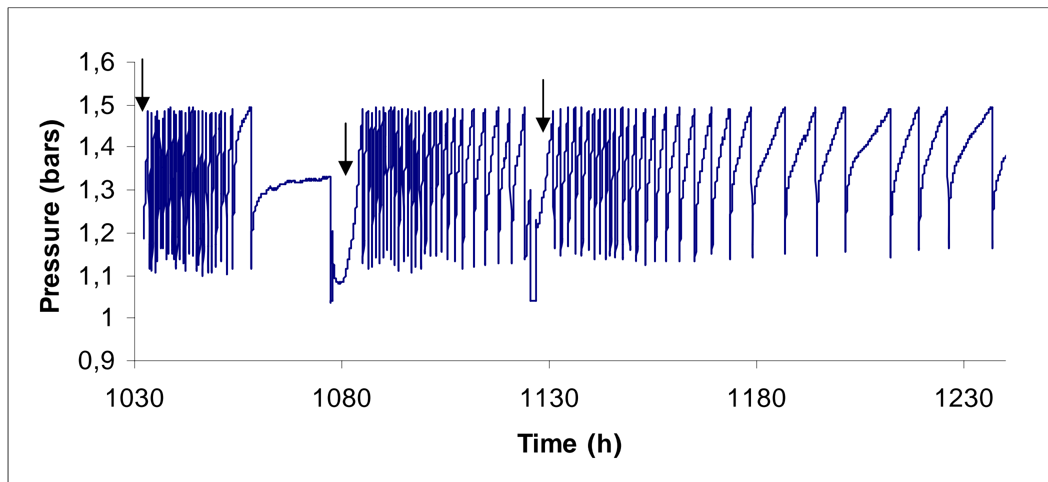

Figure 13: Evolution of gas pressure during the third medium (1030-1248h of culture)
(→ Addition of glucose)

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Figures 11, 12 and 13 focus on the 3 medium. As for a classical culture each time a decrease of CO_2 production is observed we decided to add glucose, this allows the resumption of the growth. However we observe that this culture is getting weaker with time. Consequently, we decided to change the culture medium. Once the medium is refreshed, we observe that the production increases and is important until the next addition of glucose.
Additionally, we observed that the refreshment of culture media must be done more and more frequently after 650h for the first, after 480h for the second.
3 changes of culture media were performed in this experiment but more could have been done. Indeed the change of the culture medium eliminates the inhibitor and allows a recovery for the growth. This can be applied to the degradation of vegetables or of LabMET substrates with which we observe a stop of the growth and production after some time.

4.2.4 Determination of C and N mass balance

	Input		**Output**			
Source	Substrates	Carbonate	Residues	VFA	CO2	Biomass
Total C (g)	199,10	24,68	33,19	110,57	58,88	6,01
Total C (g)	223,78		208,68			
Mass balance	**93 %**					

Table 8: Determination of C mass balance

The results for the determination of C mass balance are summed up in table 8. The C mass balance was 93% for the overall experiment, which confirms the efficiency of the control and regulation of *Fibrobacter succinogenes* process.

	Input		**Output**		
Source	substrates	$N-NH_3$	solid residue	$N-NH_3$	Biomass
Total N (g)	0	1.54	0	0.45	0.93
Total N (g)	1.54		1.38		
Mass balance	**90 %**				

Table 9: Determination of N mass balance

As well as for C mass balance, the results obtained for N mass balance (90 %, see table 9) is very promising and confirms the efficiency of the control of *Fibrobacter* process.

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4.3 LabMET substrate degradation

4.3.1 Preliminary analysis

The analysis classically performed for *Fibrobacter succinogenes* process characterisation were realised on the liquid effluent (table 10). The pH value was found to be 7.94 and the VFA concentrations were determined by HPLC. The liquid effluent contained 0 g/L of acetate, 0 g/L of propionate and 0 g/L of butyrate. These concentrations are lower than those found in the TN 2.4, TN 2.6 and 2.7.

	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
TN 2.7	3.25	1.21	0.86	7.6
TN 2.8	0	0	0	7.94

Table 10: Comparison of VFA concentrations and pH of LabMET substrate between TN 2.4, TN 2.6, TN 2.7 and TN 2.8.

4.3.2 Experiment set-up

Fibrobacter succinogenes was grown under 100% CO_2 on a basal medium. The general set up of the culture was the same as described previously. However we decided to add all the substrate at the beginning of the culture to try to obtain a better rate of degradation, according to the previous loop results (TN 2.7) and we also decided to add with the substrate 10 g/L of glucose to improve the bacterial growth. Moreover as the change of medium does not affect the growth of *Fibrobacter succinogenes* on glucose we decided to do the same thing on LabMET substrates. The process was maintained during 307h with a change of the culture media to eliminate the VFA produced after 150 hours of culture. We choose to change the culture media after 150 hours of culture because it is the optimal residence time determined in the previous TN for degradation of LabMET substrates.
Absolute pressure, redox potential, pH and temperature were on-line monitored with probes connected on the reactor and related to acquisition software. HPLC analyses were performed on liquid samples in order to follow the production of volatile fatty acids. N-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).

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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.

4.3.3 Liquid phase

4.3.3.1 pH and redox

Figure 14 shows the evolution of pH during the time course of the experiment. The profile of the redox curve is not represented because of a problem with the redox probe acquisition. But the redox value is always comprised between –300 and –450 mV (relative value) during all the experiments, which validates the strictly anaerobic conditions during the culture. The profile of the redox curve is due to a problem with the redox probe because of the density of the substrate.

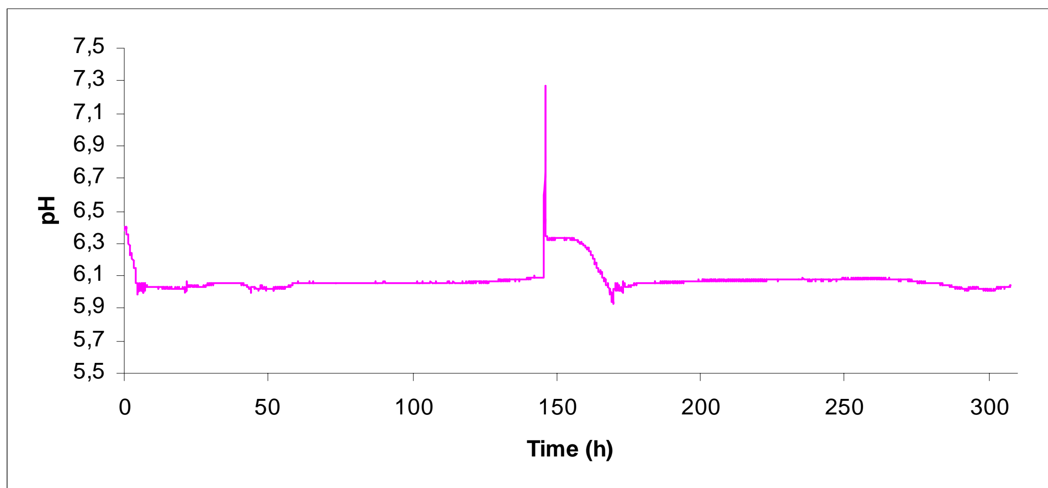

Figure 14: pH and redox evolution

4.3.3.2 VFA

Figure 15 represents the evolution of metabolites concentrations during the experiment. No sample was performed in the first hours of the experiment in order to preserve the pressure into the reactor. After 150 hours (Figure 16) the concentration of butyrate, formiate, acetate and succinate was respectively 1.84g/L, 0.17g/L 0.58g/L and 0.43g/L. At 150 hours the medium was changed and after 150 hours with the new medium the production (Figure 17) of butyrate,

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formiate, propionate, acetate and succinate was respectively 1.75g/L, 0.33g/L, 0.04g/L, 0.79g/L and 0.09g/L. The total VFA production during all the process was around 6.02g/L.

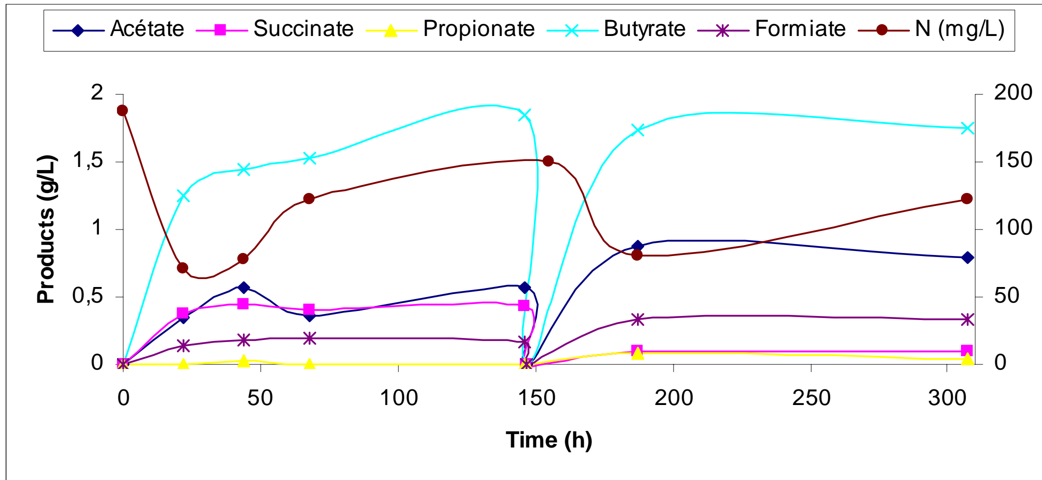

Figure 15: VFA and N concentrations during the experiment

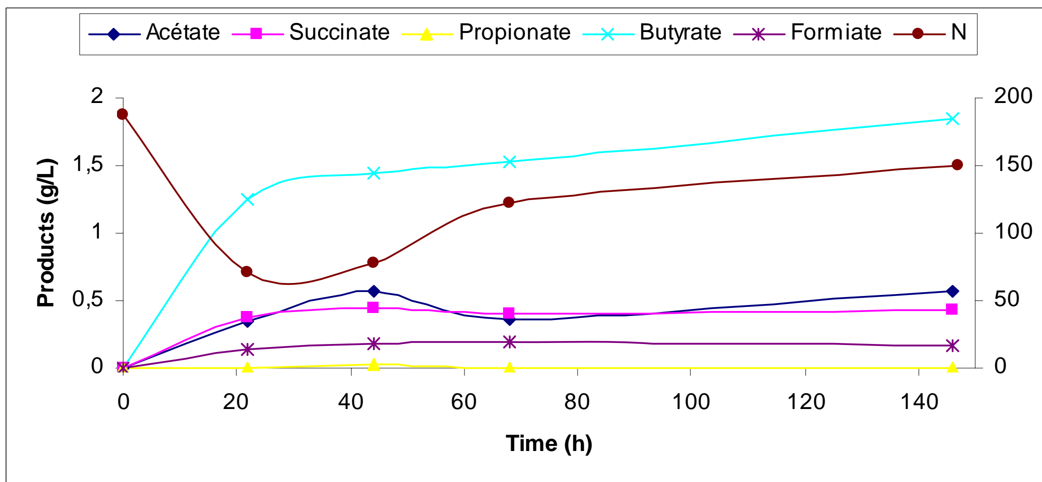

Figure 16: VFA and N concentrations during the experiment on the first medium

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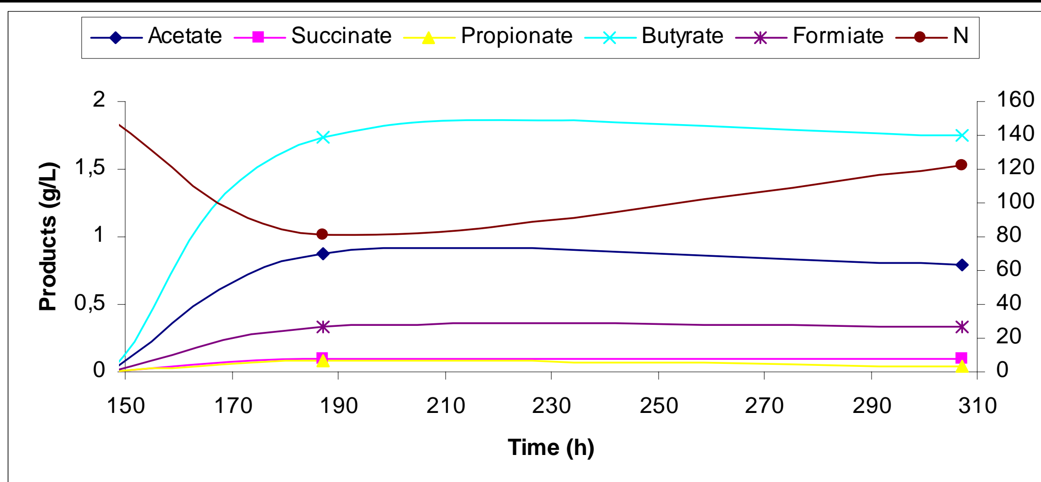

Figure 17: VFA and N concentrations during the experiment on the second medium

The metabolic profile was similar to the previous loop and 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) but about the same as during previous loops (8g/L at the end of the degradation of LabMET substrate). So the change of the culture media does not affect *Fibrobacter succinogenes* growth. Indeed the productions of VFA go on normally.
The final N-NH_3 concentration is about 120 mg/l. N-NH_3 production is different to what was observed during the previous culture in bioreactor. Indeed during the first hours N is consumed when *Fibrobacter succinogenes* grows on glucose. Then, as biodegradation starts, N from vegetable wastes is released in the culture medium in larger quantities than the amount consumed by *F.S.* Consequently, we observe an increase of N content in the bioreactor.

TN and date	Acetate g/L	Butyrate g/L	Propionate g/L	N mg/L
2.3 (07/2002)	4	0.7	1.2	400
2.4 (12/2002)	2.6	1.5	2.2	350
2.6 (10/2004)	4.5	1.2	2.5	800
2.7 (03/2005)	1.87	3.82	2.08	800
2.8 (09/2005)	1.35	3.6	0.04	120

Table 11: Comparison of VFA productions between TN2.3, TN 2.4, TN 2.6, TN 2.7 and TN 2.8.

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4.3.4 Gas phase

Figure 18 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.

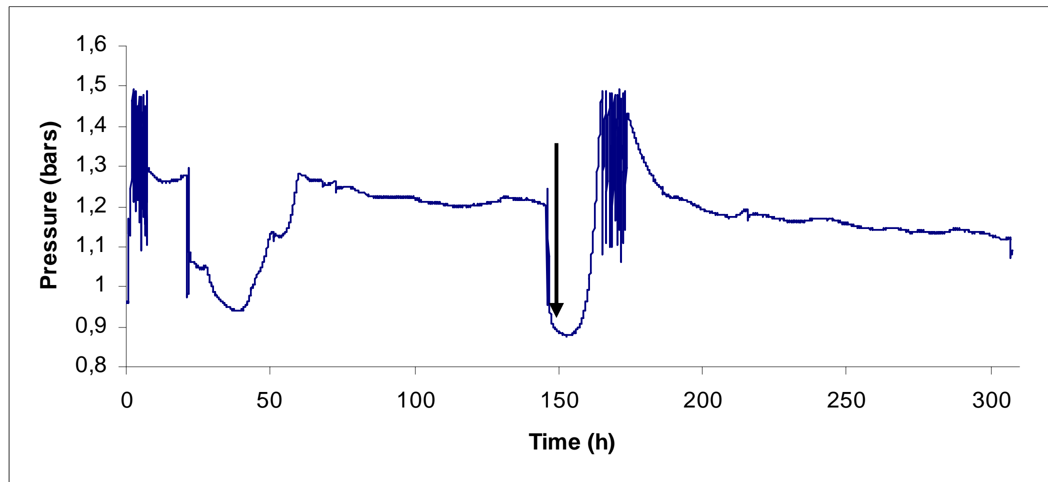

Figure 18: Monitoring of the CO_2 pressure (⟶ : change of the culture media)

Pressure increases (Figure 19) during the 10 first hours of the culture and the curve has a profile similar to degradation of vegetables. After this phase, CO_2 production became linear and has a low rate. So, two phases are observed: a high production and a low production. This may be explained by the degradation of two types of substrates: during a first step, the more easily degradable substrate (single sugars liberated by autoclave, cellulose with simple access and degradation of the glucose added at the beginning of the culture…) is degraded, which allow a very active metabolism of *Fibrobacter succinogenes*; then, the degradation of recalcitrant substrates. The kinetic of degradation of these substrates are clearly lower and results in a deceleration of *Fibrobacter succinogenes* metabolism. After medium refreshment gas production is very important. This production is due to the medium refrehment which allows the elimination of the VFAs which inibited the growth of F.s and due to the introduction of glucose (8g/L) with the new medium to improve the bacterial growth. Without glucose the bacterial growth goes on but with glucose the kinetic of degradation is faster. So the new medium without all these VFAs allow a very active metabolism of *Fibrobacter succinogenes.*

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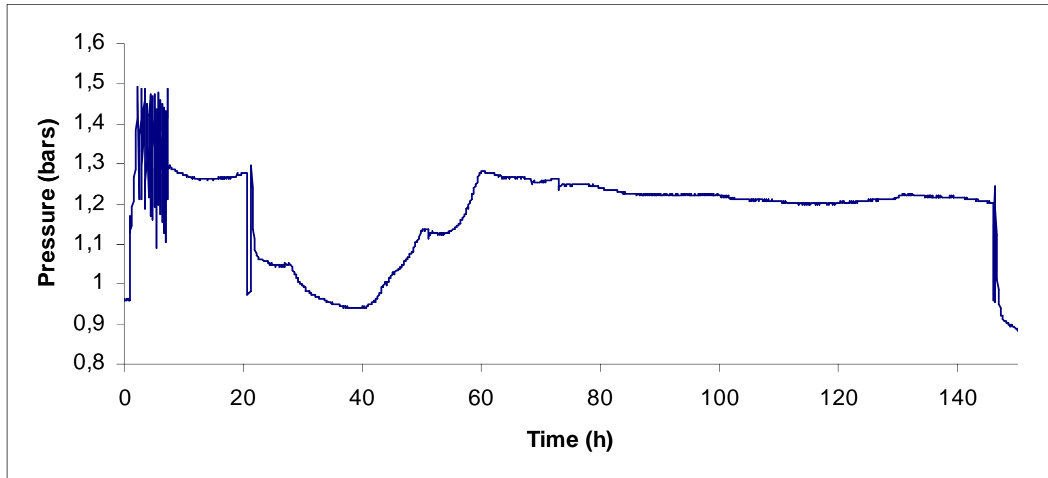

Figure 19: Monitoring of the CO_2 pressure during the first 150 hours

After the change of the culture media (Figure 20) the same thing is observed i.e. a high production at the beginning and a low production. So we can think that the change of the culture media does not affect *Fibrobacter succinogenes* CO_2 production on Lab MET substrates

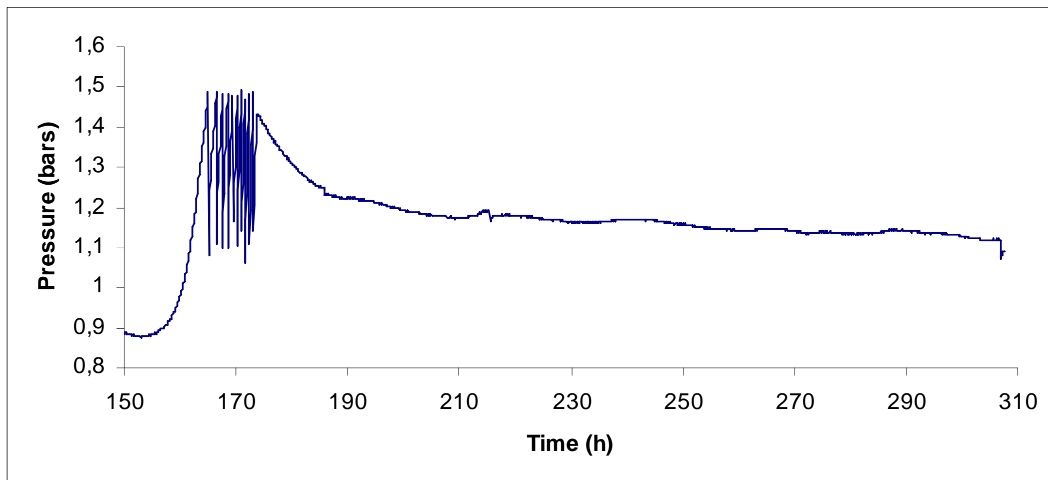

Figure 20: Monitoring of the CO_2 pressure after the change of the culture media

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4.3.5 Solid phase

4.3.5.1 Van soest

	Total fibers (% DM)	Hemicellulose (% DM)	Cellulose (% DM)	Lignin (% DM)
Substrate (160 g)	59.38	31.93	11.81	15.625
Residue (111 g)	40.19	13.86	9.9	21.44
Degradation (%)	53.03	69.88	41.87	4.82

Table 12: Analysis of cell wall degradation by Van Soest method

The percentage of degradation was determined by: $\% = \frac{(\%s \times Ms)-(\%r \times Mr)}{\%s \times Ms} \times 100$ (TN 2.6)

The cell wall components were determined by Van Soest (1967) method described in the TN 2.6. Table 12 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.

4.3.5.2 Degradation

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 2.5 (desiccation and filtration). The percentage of degradation estimated by desiccation was 32.19% and by filtration was 36.4%. This also confirms the efficiency of *Fibrobacter succinogenes* to degrade LabMET substrate.

4.3.6 Determination of C and N mass balance

	Input		**Output**			
Source	substrates	carbonate	solid residue	VFA	CO_2	Biomass
Total C (g)	83.57	1.29	53.50	11.74	5.70	5.07
Total C (g)	84.87		76.01			
Mass balance	**90 %**					

Table 13: Determination of C mass balance

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The results for the determination of C mass balance are summed up in table 13. The C mass balance was 90% for the overall experiment, which confirms the efficiency of the control and regulation of *Fibrobacter succinogenes* process.

	Input		**Output**		
Source	substrates	N-NH_3	solid residue	N-NH_3	Biomass
Total N (g)	6.86	0.48	4.9	0.41	0.78
Total N (g)	7.35		6.10		
Mass balance	**83%**				

Table 14: Determination of N mass balance

As well as for C mass balance, the results obtained for N mass balance (83 %, see table 14) 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.

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5 CONCLUSION / OUTLOOKS

Technical studies on HACCP were carried on in order to apply it into *Fibrobacter succinogenes* process. Documentation on HACCP method shows the difficulty of applying HACCP analysis on this kind process. We have listed and selected the most interesting techniques to prevent and control biological, chemical and physical hazards in *Fibrobacter succinogenes* unit. Two hazards were identified and the control of these hazards should allow the respect of standards HACCP in Fibrobacter unit.

The first experiments on glucose showed that the change of culture medium allowed a better growth of *Fibrobacter succinogenes* indeed it allow eliminating the VFA responsible of the growth inhibition.

Finally, the degradation of recalcitrant solids from the methanogenic reactor for the closed loop experiment gives results better than for previous closed loop experiment. Indeed according to the results on glucose, substrate from metanogenic unit was degraded by *Fibrobacter succinogenes* with a change of the culture media after 150h of culture. The change of the culture media was performed to increase the degradation rate and indeed the rate obtained is 36%. Moreover, 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.

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ANNEX 1

HACCP PROTOCOL

The establishment of the HACCP method described in this chapter was based on the ECSS-Q-40-02A report (2003) on Space Product Assurance - HACCP analysis.

Hazard analysis objectives

The general objectives of hazard analysis are:

- The assessment of the level of safety of a system in a deterministic way
- The increase of the level of safety of a system through hazard reduction
- The initiation of the use of hazard reduction to drive the definition and implementation of, for example, design and operation requirements, specifications, concepts, procedures
- The provision of a basis for defining adequate safety requirements, determining the applicability of safety requirements, implementing safety requirements
- verifying their implementation, and demonstrating compliance or non-compliance
- The provision of input to safety risk assessment and overall risk management
- The support of safety related project decisions
- The support of safety submissions and reviews through documented evidence
- The support of safety certification of a system through documented evidence

Hazard analysis concept

The general concept of hazard analysis is depicted in Figures 1-4.

Hazards which are present through hazard manifestations in the system, are activated if initiating events (i.e. cause) occur. Hazard scenarios reflect the system behaviour to the activated hazards in terms of event propagation from causes to safety consequences, as depicted in Figure 1. The occurrence of events is coupled to observable symptoms in the system. Safety consequences are characterized by their severity.

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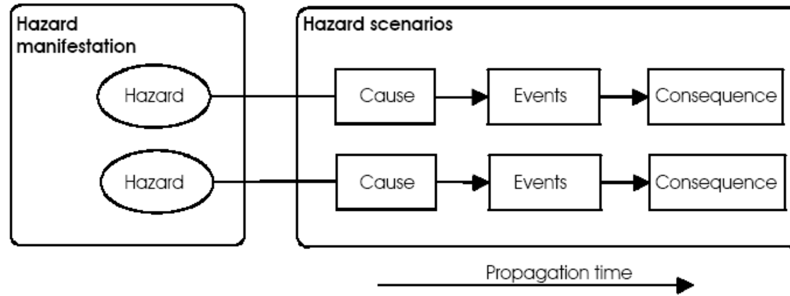

Figure 1 Hazards and hazard scenarios

Different hazard scenarios can originate from the same hazard. Furthermore, different hazard scenarios can lead to the same safety consequence (Fiure 2 & 3).

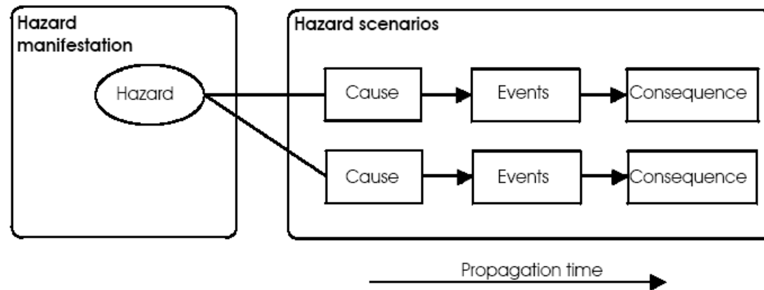

Figure 2 Example of a hazard tree

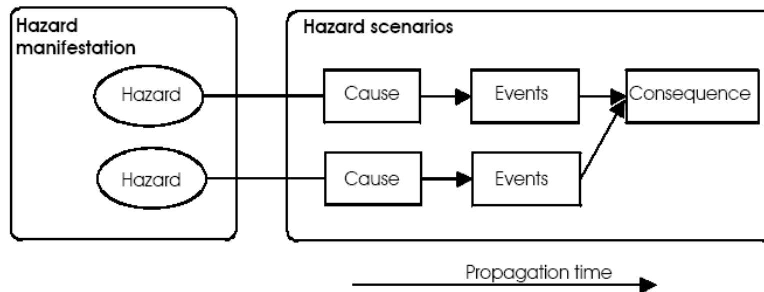

Figure 3 Example of a consequence tree

Hazards are reduced by either (Figure 4)
- elimination - remove specific potentially safety threatening system characteristics

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- minimization - reduce level or amount of specific potentially safety threatening system characteristics
- control - prevent occurrence or reduce likelihood and mitigate effects of events

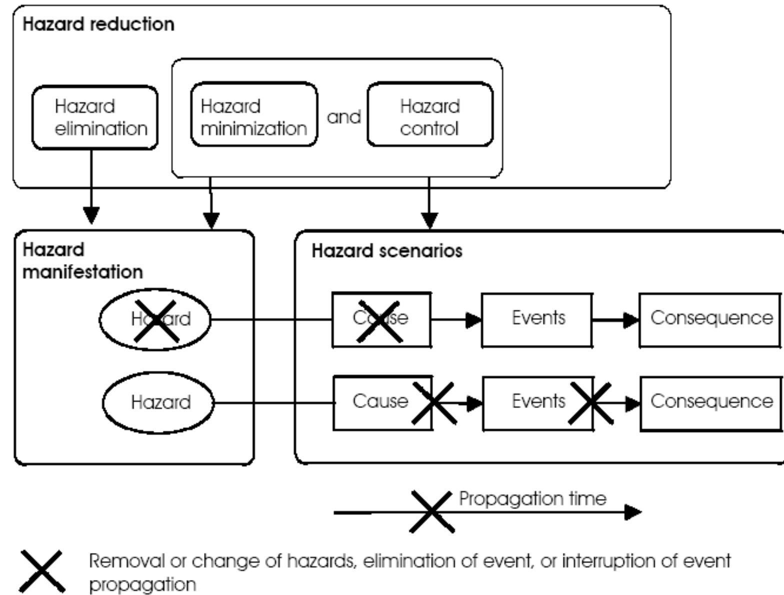

Figure 4 Reduction of hazards

Hazard analysis process

The basic steps comprised in hazard analysis are (Figure 6):
- Step 1: define the hazard analysis implementation requirements;
- Step 2: identify and classify the hazards;
- Step 3: decide and act on the hazards;
- Step 4: track, communicate and accept the hazards.

Each step in the iterative four-step hazard analysis process includes a number of tasks (Figure 5):

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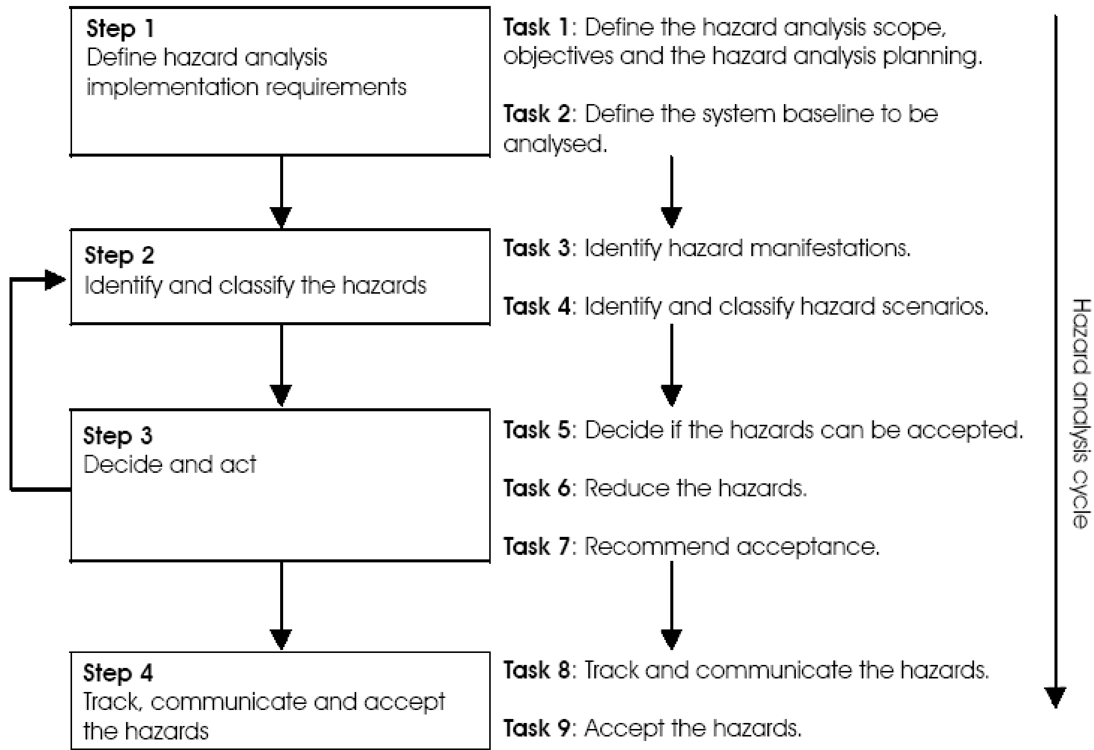

Figure 5 The nine tasks associated with the four steps of the hazard analysis process

Step 1: Define hazard analysis implementation Requirements

Task 1: Define the scope, the objectives of hazard analysis and the hazard analysis planning

Establish the purpose and application boundaries of hazard analysis; define the type of project; identify applicable safety requirements, define the hazard analysis approach; establish scoring schemes for the severity of safety consequences for the classification of hazard scenarios (example Figure 6); establish criteria to determine the actions to be taken on hazards, hazard reduction and the associated decision levels in the project structure; define hazard acceptance criteria for individual hazards and hazard scenarios; define the strategy, and the formats to be used for documenting hazard analysis data and communication of relevant data to the decision-makers, and for monitoring the hazards; describe the review, decision and implementation flow within the project concerning all hazard analysis matters.

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Category	Severity	Severity of safety consequence
I	Catastrophic	Loss of life, life-threatening or permanently disabling injury or occupational illness; Loss of an element of an interfacing manned flight system; Loss of launch site facilities or loss of system; Severe detrimental environmental effects.
II	Critical	Temporarily disabling, but not life-threatening injury or illness; Major damage to flight systems or loss of or major damage to ground facilities; Major damage to public or private property; Major detrimental environmental effects.
III	Marginal	Minor injury, minor disability, minor occupational illness; Minor system or environmental damage.
IV	Negligible	Less than minor injury, disability, occupational illness; Less than minor system or environmental damage.

Figure 6 Example of a safety consequence severity categorization

Task 2: Define the system baseline to be analysed

Define and describe the design and operation subjected to hazard analysis, such as drawings, procedures and test reports; revise the system baseline definition for each hazard analysis cycle with the level of detail available at that time.

Step 2: Identify and assess the hazards

Task 3: Identify hazard manifestations

Identify generic hazards applicable to the system design and operation using a hazard matrix (example in Figure 7); identify and give a detailed definition of system specific hazards and describe them in the form of hazard manifestations (example in Figure 8).

Hazard matrix for ground operation			
Generic hazards	Subsystem elements		
	Propulsion subsystem	Instruments	Communication subsystem
High pressure	X	-	-
High temperature	-	-	-
Toxicity	X	X	-
Flammability	X	-	-
X = applicable	- = not applicable		

Figure 7 Example of a hazard matrix

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Hazard manifestation list		
Mission phase	**Subsystem**	**Hazard manifestation**
Ground operation	Propulsion	Filling of Y litres of toxic propellant into two tanks at a pressure of X1 Pa
	Instruments	Painting and seal material used in instrument cabinet A emitting toxic fumes if exposed to fire
In-orbit operation	Propulsion	Propellant lines under pressure at X2 Pa
	Instruments	Painting and seal material used in instrument cabinet A emitting toxic fumes if exposed to fire

Figure 8 Example of a hazard manifestation list

Task 4: Identify and classify the hazard scenarios

Identify the hazard scenarios associated with the hazard manifestations by identifying the causes, events and safety consequences, according to the hazard analysis planning; identify the propagation time, the observable symptoms and the detection time for each hazard scenario; determine the consequence severity of each hazard scenario according to the severity categorization defined in Task 1; determine the hazard trees by identifying all hazard scenarios originating from one and the same hazard manifestation; determine the consequence trees by identifying all hazard scenarios leading to one and the same safety consequence; use the hazard and consequence trees to screen for additional hazard scenarios.

An example is given below.

Hazard scenario list for in-orbit phase				
Hazard Manifestation	**Cause - Events - Consequence**	**Consequence Severity**	**Observable Symptoms**	**Propagation and reaction time**
In-orbit - pressurized manned module: Meteorite debris environment	Meteorite debris impact - shell rupture - explosion - loss of spacecraft and astronauts	Catastrophic	None	Ptime: 1 s Rtime: N/A
	Meteorite debris impact - shell damage - leakage - loss of spacecraft and astronauts	Catastrophic	Module pressure drop	Ptime: 3 min Rtime: < 3 min

Figure 9 Example of a hazard scenario

Step 3: Decide and act

Task 5: Decide if the hazards can be accepted

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Apply the hazard acceptance criteria to the hazards as defined in Task 1; identify the acceptable hazards and those that are subjected to hazard reduction; for acceptable hazards, proceed directly to Step 4; for unacceptable hazards proceed to Task 6.

Task 6: Reduce the hazards
Determine measures in the form of design and operation features through which the hazards can be eliminated; where hazards cannot be eliminated, determine measures in the form of design and operation features through which hazards can be minimized and controlled; for hazard control, identify the preventive and mitigation measures; further determine hazard reduction success, failure and verification criteria; determine verification means and methods for the implementation of hazard reduction; select and prioritize the hazard reduction measures; verify hazard reduction through application of the verification means and methods; identify the resolved and unresolved hazards.

Task 7: Recommend acceptance
Submit the hazard data; present the unresolved hazards for further action; provide the rationale and supporting data for resolution and acceptance of the hazards.

Step 4: Track, communicate and accept the hazards

Task 8: Track and communicate the hazards
Periodically assess and review all identified hazards and update the results after each iteration of the hazard analysis process; identify changes to existing hazards, and subsequently initiate new hazard analysis; verify the performance and the effect of the hazard reduction activities; identify and communicate the evolution of hazards over the project life cycle.

Task 9: Accept the hazards
Submit the residual hazards to formal hazard acceptance; assess the performance of the hazard analysis processes and implement improvement of the effectiveness based on experience with project progress.

Documentation of hazard analysis

Hazard analyses are documented to ensure that all associated decisions are traceable and defensible. Every task of the hazard analysis process is documented. Example forms for summarizing the results of the tasks are presented below.

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Hazard and safety risk register *(Example, see also ECSS-Q-40-03 and ECSS-M-00-03)*

Project WBS Ref.	Organization	Source Controlled by Supported by	Date and issue Approved by

Hazard description and safety risk magnitude

No.	Hazard scenario title
Hazard manifestation	Cause, events and safety consequence

Safety consequence severity (S)				Likelihood *(L)*					Risk Index	Risk	Red*	Yellow*	Green*
Negligible	Marginal	Critical	Catastrophic	Minimum	Low	Medium	High	Maximum	*(R = S x L)*	Safety			
IV	III	II	I	E	D	C	B	A	Numerical risk and uncertainty contribution:				
				Numerical estimate:									

Hazard and safety risk decision and action

Accept hazard and safety risk ☐		Reduce hazard and safety risk ☐
Hazard reduction measures Hazard elimination: Hazard minimization: Hazard control:	Hazard reduction verification means	Expected safety risk reduction Severity, likelihood, risk index: Numerical estimates: Safety risk rank:
Actions		Status
Agreed by project management		Hazard status

* Enter "R" in the appropriate column: correspondence of the risk index scores for red, yellow and green are defined in the project risk management policy

Ranked hazard and safety risk log *(Example)*

Project			Organization				Date and issue
Rank	**No.**	**Hazard scenario title**	**Risk ***	**Red**	**Yellow**	**Green**	**Actions and status**
			Safety				
			Safety				
			Safety				
			Safety				
			Safety				
			Safety				
			Safety				
			Safety				

* Enter "R" from Hazard and safety risk register

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