MAP **TECHNICAL NOTE**

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

Université Blaise Pascal LGCB
Laboratoire de Génie Chimique et Biochimique

TECHNICAL NOTE: 86.2.9
MODELLING AND DEMONSTRATION

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reference/*réference* 14719/00/NL/SH MAP AO-99-LSS-015
issue/*édition* 1
revision/*révision* 0
date of issue/*date d'édition* 30/06/2006
status/*état* Draft
Document type/*type dedocument* Technical Note
Distribution/*distribution*

CONFIDENTIAL DOCUMENT

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

During the course of the project, promising adaptations of the culture conditions have been achieved with regard to *Fibrobacter succinogenes* performances in terms of vegetal fibres degradation efficiency. Indeed at the beginning of the project we were confronted to three difficulties.
The first was a difficulty to define a feeding procedure in terms of quantity (200g once) and of frequency (33g once a week). After the optimal residence time has been determined using the RUSITEC system, we propose to add the totality of the substrate at the beginning of the fermentation. We have obtained a better degradation rate, 28%.
The second was to eliminate the VFA responsible for growth inhibition. To solve this difficulty we decided to remove the culture media and replace it by a fresh solution after 150h of culture (optimal residence time). This allowed obtaining a better degradation rate, 32%.
The last difficulty was to define the metabolic reactions between substrates and products. Enzymatic measurements seem to be the best way to solve this difficulty. So several experiments were performed in order to identify and characterize the enzymes responsible for degradation. Moreover in all experiments in bioreactor, not only production of the metabolites classically produced by *Fibrobacter succinogenes*, but also butyrate production was observed. This butyrate production either comes from a reversion of *F.s.* metabolism or results from a contaminant. In this report we look for the presence of a contaminant in our culture by molecular techniques.

Finally, during MAP project 3 loops were performed between MAP partners. Each partner has collected his data of the best loop in order to verify the mass balances of the loop. *Fibrobacter succinogenes* will be used as model. Thanks to simulations, we will be able to compare the experimental results obtained on the bioreactor with those obtained during simulations with the AWC-ms-[V2.0.Ob] program and to thus evaluate the differences between the theory (simulation) and the practice (experimental studies)

2 DETECTION OF CONTAMINANTS

2.1 Study goal

Fibrobacter succinogenes S85, uses cellulose and the other vegetable polymers as carbon source to produce acetate and succinate (Bryant and Doestch, 1954). However after a first phase of production of these two metabolites, succinate is consumed and butyrate is produced sometimes in great quantity (up to 13 g/L) and generally proportionally to acetate at the nearly the same concentration. Figure 1 represents the typical profile of the productions obtained

with our process during degradation of vegetables (green cabbage, soy bean, wheat straw, 1/3 each).

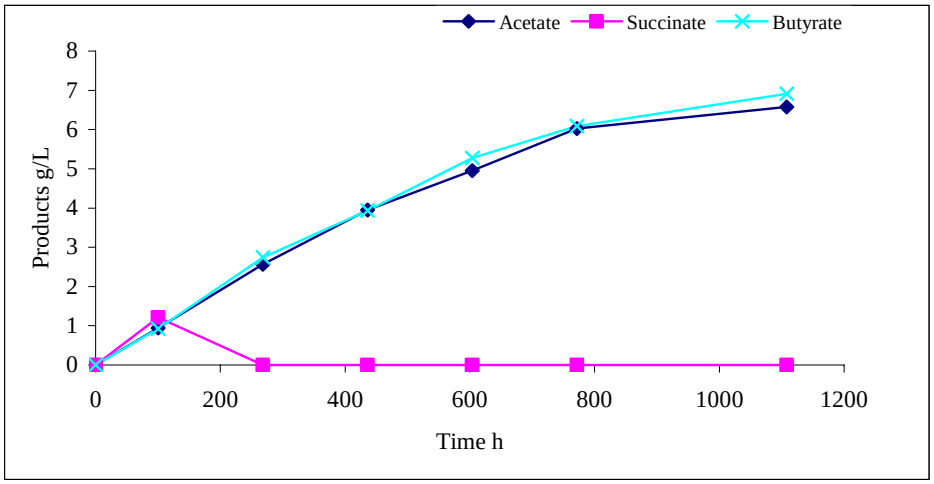


Figure 1: Typical profile of productions during vegetable degradation

Two assumptions could be made to explain this succinate consumption followed by butyrate production. The first could be a change of *Fibrobacter succinogenes* metabolism i.e. a reversion of succinate metabolic partway and the second the presence of bacterial contaminants which enter in symbiosis with F.s. This last assumption was checked by several means.

First, we supposed that the contamination came from our pure culture. The strain was thus re-started from new ATCC inoculum. No change was observed. Indeed during culture with the new strain of *Fibrobacter succinogenes* the production of butyrate is always observed as well as the succinate consumption.

Then we supposed that the contamination came from the vegetables. The vegetables were thus put under the same conditions as the *F.s.* culture conditions but without inoculation of *Fibrobacter succinogenes.* No metabolite was produced under these conditions. As the production of butyrate is also observed during cultures on glucose in the bioreactor, it can be deduced that the contamination doesn't come from the vegetables.

The conclusions we can draw from these results are either there is a reversion of the metabolism or there is one or more bacterial contaminants which need the succinate produced by *Fibrobacter succinogenes* to develop themselves. The only techniques to check this last assumption are traditional techniques of microbiology and molecular microbiology to identify the contaminants.

2.2 *Material and methods*

2.2.1 Introduction

We checked the presence of one or more bacterial contaminants in 3 steps (Figure 2):

- A global identification of the contaminants: to check the presence of the contaminants,
- An analyse of microbial diversity of the samples: to know number of contaminants,
- An identification of cultivable contaminants: to know who are the contaminants.

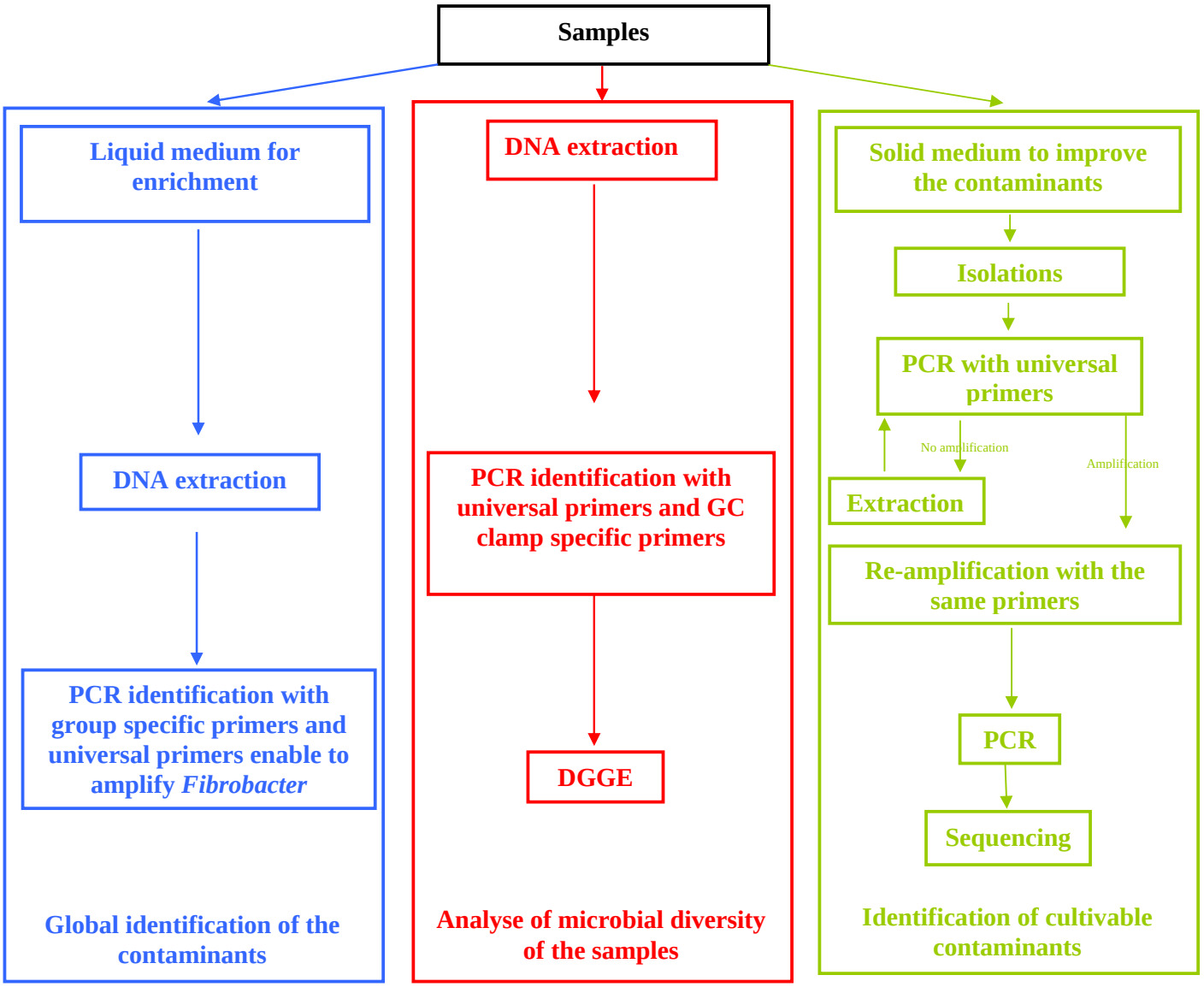


Figure 2: Experimental design

2.2.2 Cultures

Several samples of culture in bioreactor were cultivated on various solid and liquid media. Mediums 869, 284 glucose, 284 succinate, sistrom glucose and sistrom succinate were used at 39°C in aerobic and anaerobic conditions. The use of various media with different carbon sources allows the discrimination of certain bacteria from others. For example the use of a medium with succinate as the only carbon source allows improving the growth of the contaminant which consumes succinate produced by *Fibrobacter succinogenes* to produce butyrate.

2.2.2.1 869

This medium is composed by (per litre): 10g peptone, 5g of yeast extract, 5g NaCl, 1g glucose, 0.345g $CaCl_2$,$6H_2O$ to this medium 1.5% of agar are added in order to obtain solid media.

2.2.2.2 284

This medium is composed by (for 985mL) : 6.06g trisHCl, 4.68g NaCl, 1.49g KCl, 1.07g NH_4Cl, 0.43g Na_2SO_4, 0.20g $MgCl_2$,$6H_2O$, 0.03g $CaCl_2$,$2H_2O$, 0.2% carbon (glucose or succinate), 4mL of a 1% Na_2HOP_4 solution, 10mL of a solution containing Fe(III) NH_4 citrate (48mg/100mL) et 1mL of a SL7 solution(1.3mL HCl 25%, 144mg $ZnSO_4$,$7H_2O$, 100mg $MnCl_2$,$4H_2O$, 62mg H_3BO_3, 190mg $CoCl_2$,$6H_2O$, 17mg $CuCl_2$,$6H_2O$, 24mg $NiCl_2$,$6H_2O$, 36mg Na_2MoO_4,$2H_2O$). pH is adjusted to 7.8 with HCl ou NaOH. For solid media 2% of agar are added.

2.2.2.3 Sistrom

918mL of H_2O with 0.2% of the carbon source (glucose or succinate) are sterilised and 1.5% of agar are added before the sterilisation for the solid media. To this sterilised solution (70°C) are added: 20mL of solution C (10g nitrilotriacetic acid, 29.5g $MgSO_4$,$7H_2O$, 3.835g $CaCl_2$,$2H_2O$, 99 mg $FeSO_4$,$7H_2O$, 9,25g $(NH_4)_6Mo_7O_{24}$,$4H_2O$, 50mg nicotinic acid, 25mg thiamine HCl, 0.5mg biotine), 20mL of calcium phosphate (1M), 5mL a 10% $(NH_4)_2SO_4$ solution, 20mL of a 10% potassium succinate solution, 2mL of a 5% L-glutamic acid solution, 4mL of a 1% L-aspartique acid solution, 10ml of a 5% NaCl solution, 30mg L-cystein and 1mL of traces solution (1.097g $ZnSO_4$,$7H_2O$, 250mg ethylendiamine tetraacetic acid, 500mg $FeSO_4$,$7H_2O$, 154mg $MnSO_4$,H_2O, 89.2mg $CuSO_4$,$5H_2O$, 24.8mg $Co(NO_3)_2$, $6H_2O$, 11.4mg H_3BO_3).

2.2.3 DNA extraction

3mL of the sample (2*1,5mL) were centrifuged 5 min at 10000rpm, the pellet was suspended in 200µL of TE (Trisbase 10 mM, EDTA 1 mM, pH 8) with 50µL of lysozyme (5mg/mL) pH

7 and 30µL of RNase (10mg/mL). The mix was incubated 30 min at 37°C. Then the mix was transferred in a Fastprep tube which contains 0,2g of glass ball (212-300µ, Sigma) to which 30µL of a 10% SDS solution and 200µL of phenol-chloroform is added. After two cycles (40 s power 6) in the fastprep machine and a centrifugation 5 min at 14000rpm, the supernatant was taken delicately. 1/10 volume of sodium acetate and 2.5 volume of ethanol were added to the supernatant and incubated 1h at -20°C. After a centrifugation during 20 min at 12000rpm (4°C) and a washing with ethanol 70% (-20°C), a second centrifugation was carried out 5 min at 14000rpm in order to recover the DNA in the pellet. This one was dried and suspended in TE (100µL) and preserved at -20°C. DNA purity was determined using the ratio of sample absorbance at 260nm and 280nm. A ratio of 1.8 was regarded as pure DNA. The ratio of sample absorbance at 230 and 260nm was used as secondary measure of purity. The 230/260 values between 1.8 and 2.2 was regarded as pure. In all experiments, only pure DNA was used.

2.2.4 PCR (Polymerase chain reaction)

The PCR is a cycling process for DNA amplification. This process allow an exponential increase of the target DNA sequence after n cycles of denaturation, primer annealing and chain elongation. The PCR mix contained (per reaction) 5 µL of 2.5mM of dNTP, 2 µL of 10x TaKaRa *ExTaq*™ buffer, 1 µL of forward primer (3 µM), 1 µL of reverse primer (3 µM), 5 µL DNA sample and 1 µL of high purity water.
The PCR program was 5 min at 94°C, with a hold at 80°C to add the polymerase (5µL of TaKaRa *Ex Taq*™ polymerase (1U/µL)). These two steps are the hotstart. The hotstart is followed by 35 cycles of 1 min at 94°C, 1 min at the primer specific annealing temperature (Table 1) and 1 min at 72°C, followed by a final amplification at 72°C for 10 min (Figure 3).

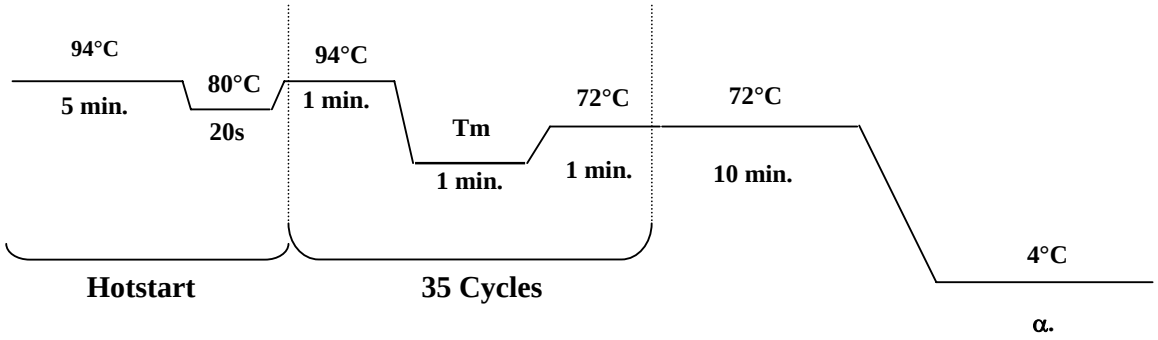


Figure 3: PCR program

Samples were loaded on 1% agarose gels, containing 1/10000 volume ethidium bromide. The electrophoreses were run in a Tris-Borate-EDTA (TBE) buffercontainig 5.5g boric acid, 0.744g EDTA and 10.8g Tris per litre of MilliQwater. 10µL of PCR amplicons were loaded mixed with 2µL of loading dye. All agarose gels were run at constant voltage of 100V for 2h. To visualise the migrated PCR amplicons, the agarose gels were subjected to UV irradiation.

Nom	Temperature °C	Sequence (5'-3')	Position	References
ALF968r	56	AAC GCG CAG AAC CTT ACC	968	a
BET42af	52	GCC TTC CCA CTT CGT TT	42	a
GAM42af	52	GCC TTC CCA CAT CGT TT	42	a
LGC354Ar	54	GCA GTA GGG AAT CTT CCA	354	a
LGC354Br	54	GCA GTA GGG AAT CTT CCG	354	a
LGC354Cr	54	GCA GTA GGG AAT CTT CGG	354	a
CBF286f	56	TCC TCT CAG AAC CCC TAC	286	a
CFB563f	52	GGA CCC TTT AAA CCC AAT	563	a
CFB719f	58	AGC TGC CTT CGC AAT CGG	719	a
CFB972r	56	CGA GGA ACC TTA CCA AGG	972	a
CFB1083f	56	TGG CAC TTA AGC CGA CAC	1083	a
CF319af	56	TGG TCC GTG TCT CAG TAC	319	a
Bac303f	54	CCA ATG TGG GGG ACC TT	303	a
Univ787R	55	TAC CAG GGT ATC TAA T	787	b
Univ926Ar	54	CCG TCA ATT CCT TTA AGT TT	926	c
Univ926r	54	CCG TCA ATT CCT TTG AGT TT	926	b
Univ9-27f	54	GAG TTT GAT CCT GGC TCA G	9	a
Univ1406Ar	56	GAC GGG CGG TGT GTA CA	1406	d
Univ1406Gr	56	GAC GGG CGG TGT GTG CA	1406	b
Univ786f	58	GAT TAG ATA CCC TGG TAG	786	a
Univ529CfGC	55	GCC AGC CGC CGC GGT	529	a
Univ529AfGC	55	GCC AGC AGC CGC GGT	529	a
EUB515r	55	CCG TMT TTA CCG CGG CTG CTG GCA	515	b
EUB338R	55	ACT CCT ACG GGA GGC AGC	338	a
EUB1492r	55	GGT TAC CTT GTT ACG ACT T	1492	a
GC Cclamp : 5'- CCG CCG CGC GGC GGG CGG GGC GGG GGC ACG GGG-3' covalently bound to the 5' end of the primers EUB63f, Univ529CfGC, Univ529AfGC et Univ9-27f GC. Muyzer *et al.*, 1996[c]				

Table 1: The primer specific annealing temperature a: Probase, b: DasSarma and Fleischmann1995, c: Muyzer *et al.*, 1996, d: Baker *et al.*, 2003

2.2.5 DGGE (Denaturant Gradient Gel Electrophoresis)

The DNA migration is done in a polyacrylamide gel, in this gel the DNA meets increasing concentrations of denaturing solutions (urea+formamide), which role is to imitate a linear increase of the temperature from the top to the bottom of the gel. Dissociation transforms the DNA segment into a partially opened structure and thus creates a reduction of its mobility. DNA concentrates in a point of the gel. The molecules migration in the gel is thus very dependent on the DNA sequence (Figure 4). This gradient of denaturing solution allows the separation of bacteria according to the migration speed of their DNA in the gel. The DNA used for DGGE experiments are amplified beforehand by PCR with DGGE primers; CG-clamp primer (Table 1). The DGGE gels were prepared by mixing an 24mL 8% acrylamide solution containing 35% or 65% urea/formamide with 100µL of 10% ammonium persulfate (APS) and 5µL TEMED (N,N,N',N'-tetra methyl ethylen diamine) per gel. The gradients were prepared using a system based on communicating vessels. After the denaturing gel had polymerised, a stacking gel was added on top, prepared by mixing 8% acrylamide, 200µL APS and 10µL TEMED. All denaturing gels were run first at constant voltage of 200 V for 15 min, to the migration in the stacking, and then at constant voltage of 120V for 16h with a constant circulation of the buffer at 60°C.

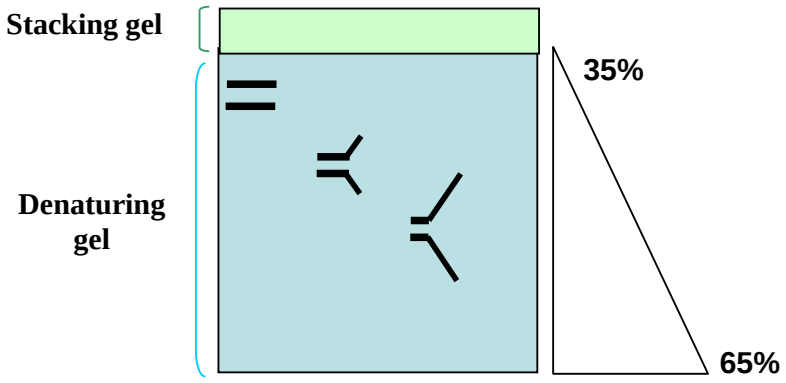


Figure 4: DGGE gel

The migrated DGGE amplicons were fixed by 200mL TAE containing 0.5% acetic acid and were left for 5min. After removing the TAE with acetic acid, 100mL TAE containing 30µL SYBR®Gold (nucleic acid gel strain) was added and left for 30 min in the dark at room temperature. After removing the buffer, the gel was subjected to UV irradiation.

2.2.6 Sequencing

First, PCR amplicons were purified. The purification was carried out with the kit Wizard® SV gel and PCR clean-up system (Progema). The quantities of DNA obtained after purification were checked to be between 1.8 and 2.2 (ratio 230/260). Purified PCR amplicons

were diluted in order to obtain between 3 and 10 ng of DNA in 9µL for amplicons obtained with the primers Univ786f /univ926r and between 5 and 20 ng for amplicons obtained with the primers Univ927f/EUB1492r. These diluted PCR amplicons were re amplified with a mix included in a kit (BigDye®Terminator v1.1 cycle sequencing kit) and the same primers but one by one. For this PCR 9µL of DNA were mixed with 8µL of the kit mix and 3µL of primer (water q.s.p. for 20µL). The PCR program was 5 min at 94°C then 30 cycles of 30 s at 95°C, 30 s at 50°C and 4 min at 60°C. The products obtained were purified on columns centri-sep and 20µL of TSR were added to avoid evaporation. Then the products were denatured by heat 4 min at 70°C followed by 2 min to 96°C. Finally they were introduced in the sequencing machine.

2.3 Results

The results are presented according to the experimental design in § 2.2.1.

2.3.1 Global identification of contaminants

2.3.1.1 Liquid Cultures

The samples were cultivated on various liquid media: 869, 284 glucose, 284 succinate, sistrom glucose and sistrum succinate were used at 39°C in anaerobic and aerobic conditions. The results obtained are presented in table 2. 32F glucose means that the carbon source in the bioreactor was glucose, in the same way for 33F straw the carbon source was the straw, for 34F LabMET the carbon source was the substrates sent by LAbMET (MAP) and 35F the carbon source was a mixture of straw, cabbage and soya (1/3 of each).

	Milieux	**Fs**	**32F glucose**	**33F paille**	**34F Labmet**	**35F mix**
Ae	869	+	+	+	+	+
	284 glc	+	-	-	-	-
	284 succ	-	-	-	-	-
	Sis glc	+	-	-	+	+
	Sis succ	-	-	+	+	-
An	869	++	+	+	+	+
	284 glc	-	+	-	-	+
	284 succ	-	-	-	-	-
	Sis glc	+	+	+	-	+
	Sis succ	-	-	+	+	+

Table 2: Results of cultures on liquid medium. + growth, - no growth,

Several things can be noted in these results: first a growth is observed in aerobic conditions for a great number of medium what should not be observed if *Fibrobacter succinogenes* was the only bacterium present. This is probably due to bacterial contamination of the sample. The second thing interesting to observe is a growth in some medium containing succinate as only carbon source (+). Indeed a growth is observed on the medium sistrum succinate. However no growth is observed on the medium 284 succinate.

2.3.1.2 Extraction

The extractions of DNA were carried out according to the method described in the material and methods part on the liquid media.

2.3.1.3 PCR

Primers	Amplicon size (pb)	Temperature (°C)
EUB515f / ALF968r	453	56
BET42f / EUB515r	473	52
GAM42f / EUB515r	473	52
Univ927f / LGC354r	573	54
Univ927f / EUB515r	412	56
CFB286f / EUB515r	229	56
CFB563f / EUB1492r	929	52
CFB719f / univ1492r	773	58
EUB515f / CFB972r	457	56
CFB1083f / EUB515r	568	56
CF319af / EUB515r	196	56
Bac303f / EUB515r	212	54
Univ927f / univ1492r	565	55
Univ927 / EUB515r	412	55

Table 3: Primers used on extracted DNA from liquid cultures

First, PCR with group specific primers (Table 3) were performed in order to focus on the contaminants rather than on *F.s.*. No amplification was obtained both on controls as well as on the samples. It appears that the use of these primers requires an optimization.
We thus decided to give up this strategy and use universal primers, which should not amplify *Fibrobacter succinogenes*: Univ927f/univ1492r and Univ927/EUB515r.
Amplification was observed for a great number of samples (Figure 5) with the universal primers. However some were not amplified.

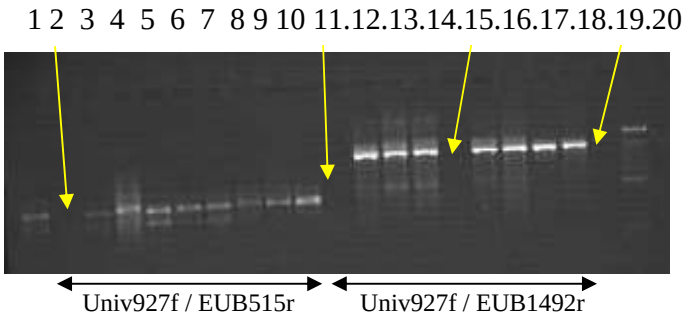


Figure 5: Agarose gel obtained for DNA amplicons from liquid cultures.

The samples which weren't amplified are indicated by an arrow on figure 5 (wells 2, 11, 15 and 19). All these samples are coming from *Fibrobacter succinogenes* in pure culture (ATCC strain) cultivated in tube, with better sterility conditions. These results show the presence of bacterial contamination in all the other samples.

2.3.2 Analyse of microbial diversity of the samples

2.3.2.1 Extraction

DNA extractions were performed according to the method described in the material and methods part directly on the samples from the bioreactor.

2.3.2.2 PCR

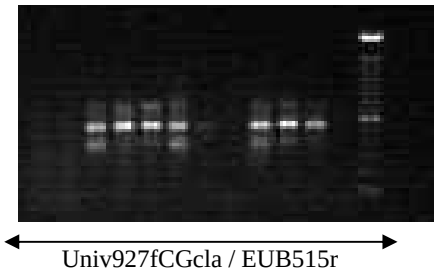
The primers in table 4 have been selected. As some primers (e.g. 63f/EUB338r) don't amplify *Fibrobacter succinogenes* and others (e.g. 9-27fGC/EUB515r) do, it is possible to discriminate the contaminants from *Fibrobacter succinogenes*.

Primers	Amplicon size (pb)	Temperature (°C)
Univ9-27fGC / EUB515r	506	55
Univ9-27fGC / EUB338r	329	55
Univ9-27fGC / EUB518R	509	55
63f / EUB338r	275	55
63f / EUB518r	455	55
Univ529CfGC + univ529AfGC / univ926Ar + univ926r	397	55
Univ529CfGC + univ529AfGC / univ787r	258	55

Table 4: Primers used directly on the sample

Amplification was only observed with the primers univ927fCG/EUB515r (primers are in table 1) (Figure 6) Are these primers are not specific of F.s.. This primer will thus be used in DGGE gel.

1.2.3.4.5.6.7.8.9.10.11.12.13.



Univ927fCGcla / EUB515r

Figure 6: Agarose gel obtained with DNA amplicons

Only this amplification gave satisfactory results (figure 6). All the other amplifications with GC-clamp primers gave the same result: DNA was visualized on the gel but there is not enough DNA amplified on the gel to perform a gel extraction of this DNA.

2.3.2.3 DGGE

Only one DGGE gel was performed because a single amplification with GC clamp primers was obtained.

1.2. 3. 4. 5. 6.7.8.9.10.11.12.

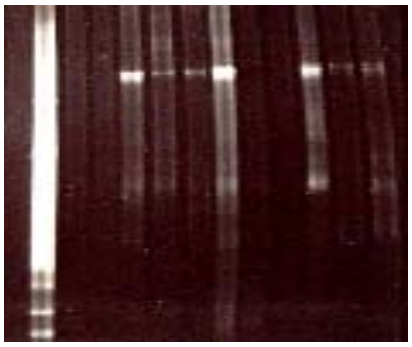


Figure 7: DGGE gel

In the first two wells (Figure 7) are the "amplicons" of *Fibrobacter* in pure culture. As no signal was detected after the PCR (samples were deposited in the same order on both gels), no signal is observed after DGGE.
In wells 3 to 7 and 12 are the amplicons corresponding to cultures with glucose as carbon source.

Wells 6 and 7 correspond to the same culture but two different times of culture respectively 1000h and 1240h.
Two different bands are observed on wells 3 and 6, indicating is the presence of one or two contaminants. Only one band is observed on wells 4 and 5, indicating the presence of a single contaminant. No signal is observed on wells 7 and 12. Even though wells 6 and 7 come from the same culture, they do not exhibit the same signal. Either skew of PCR amplification by universal primers or survival of contaminant for more than 1000h is observed.
No signal is observed on well 8 (culture on straw), which is consistent with the outcome of the PCR (no signal).
On well 9, (culture on waste of LabMET) a quite distinct band is observed indicating the presence of a single contaminant.
DGGE results show the presence of contaminants in a large number of samples. However it should be noticed that the absence of signal does not necessarily mean the absence of contaminants: the primers used may not perfectly match the contaminants present.

2.3.3 Identification of cultivable contaminants

2.3.3.1 Cultures

	Medium	Fs	32F glucose	33F straw	34F Labmet	35F mix
Ae	869	+	+	+	+	+
	284 glc	+	-	+	+	-
	284 succ	-	-	+	+	-
	Sis glc	+	+	+	+	+
	Sis succ	-	+	+	+	-
An	869	+	+	+	+	+
	284 glc	+	-	+	+	-
	284 succ	-	-	-	+	-
	Sis glc	+	-	+	+	+
	Sis succ	-	-	+	+	-

Table 5: Results of cultures on solid medium. + growth, - no growth,

As for the cultures in liquid medium, it is interesting to notice that growth is observed in aerobic conditions (Table 5) for a large number of medium. Additionally, growth was observed on media containing succinate as only carbon source (+). Indeed, growth is observed on medium “284 succinate” in samples 33F and 34F, as well as on medium “Sistrum succinate” (samples 32F, 33F and 34F).

On cultures carried out from *F.s.* in pure culture, the colonies are small and white (for both aerobic and anaerobic conditions).
On cultures from 32F samples, two types of colonies are observed: either some small and white colonies (869 anaerobe, sistrom) or the medium took a brown colour (869 aerobe).
On cultures from 33F samples, two types of colonies are observed: either some small and white (869 anaerobe), sistrom aerobic glucose, 284 aerobic succinate) or a sticky white carpet.
On cultures from 34F samples, four types of colonies are observed: either small, round and yellow (869 aerobe, 284), or red in very small quantity (sistrom aerobic glucose), or white with a mushroom aspect (sistrum glucose) or a sticky white carpet.
Finally, on cultures from 35F samples, the colonies are all the same.

2.3.3.2 Isolation

Isolations of the cultures were performed on the same medium as for the cultures and the morphology of each isolate is summed in table 6.
Other isolations were carried out on the same medium as for the cultures but no growth was observed. It mainly concerns (table 6) the cultures coming from F.s. in pure culture, which isolation was not possible and cultures coming from "sistrum succinate" medium.
It may be thus that the colonies observed for the first road repair used the carbon source brought by the inoculum and not succinate. When we performed the first culture, we put on the solid medium 1mL of sample so we brought with the inoculum some glucose or vegetable.

N°	Origin, medium, condition	Morphology
55	32, 869, Ae	Small, round, white
56	32, 869, Ae	Small, round, white
57	33, 869, Ae	White carpet
58	33, 284 glc, Ae	Little white carpet
59	33, 284 Succ, Ae	Small, round, white
60	33, Sistrum glc, Ae	Small, round, white
61	34, 284 glc, Ae	Small, dry, yellow
62	34, 284 Succ, Ae	Small, dry, yellow
63	34, Sistrum glc, Ae	Small, dry, yellow
65	35, 869, Ae	1 small, round, white
68	32, 869, An	Small, round, white
70	33, 869, An	Small, round, white
71	33, 284 glc, An	Small, round, white in a little quantity
72	33, Sistrum glc, An	White, extensive
75	34, 284 Succ, An	Small, dry, yellow
76	35, 869, An	Small, round, white
77	35, Sistrum glc, An	Small, round, white in a little quantity

Table 6: Source and morphology of the isolated colonies

2.3.3.3 Extraction

Only some extractions were carried out by the Fastprep method. The others were amplified by PCR without preliminary extraction of their DNA. No isolation was possible for colonies 54 and 74 (Table 6) (data not shown). We consequently used the colonies from first culture.

2.3.3.4 PCR

PCR (Table 7) were performed either directly on a colony sample on the solid medium (this colony was integrated to the PCR mix) or after DNA extraction with the Fastprep method.

Primers	Amplicon size (pb)	Temperature (°C)
Univ927f / EUB338r	589	55
Univ927f / P518r	409	55
Univ927f / EUB1492r	565	55
Univ786f / univ926r	140	47
Univ786f / univ1406Gr + univ1406Ar	620	55

Tableau 7: Primers used

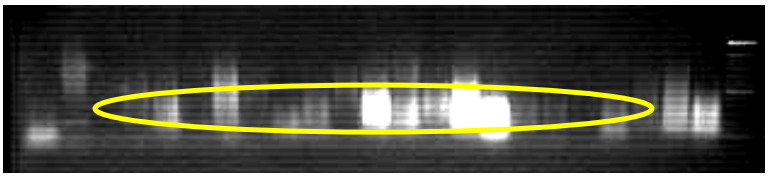
- **First amplification**

Figures 8A and 8B show the results obtained after the first amplification.

A 1.2.3.4.5.6.7.8.9.10.11.12.13.14.15.16.17.18.19.20.21

Univ927f / EUB1492r

B 1.2.3.4.5.6.7.8.9.10.11.12.13.14.15.16.17.18.19.20.21.22.23



Univ786f / univ926r

Figure 8: Agarose gel obtained after amplification, A directly on colonies; B after extraction

Amplifications (figures 8A and 8B) were observed but the bands were not separated enough from each other for sequencing. These amplicons were thus re-amplified.

➢ **Second amplification**

Figure 9 shows the results of the re-amplification of the previous amplicons with the same primers.

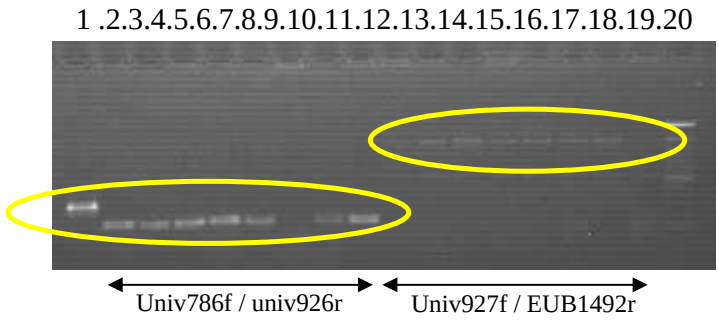


Figure 9: Agarose Gel obtained after re-amplification

For amplification performed with the primers Univ786f / univ926r, amplicons are not separated enough to perform the sequencing. However, for primers Univ927f / EUB1492r a third amplification seems to be necessary before sequencing.

2.3.3.5 Sequencing

Amplicons were purified with Wizard® SV gel kit and PCR clean-up system (Progema). DNA concentrations obtained after purification are reported in table 8.

N°	Source	DNA concentration ng/µL	Primers
1	869 Ae 32F	42	Univ786f / univ926r (140bp)
2	869 Ae 32F	37,9	
3	Sis glc Ae 33F	40,3	
4	284 succ Ae 34F	55,3	
5	Sis glc Ae 34F (colonies jaune)	30,2	
6	869 An Fs	33	
7	869 An 33F	38,5	
8	284 succ An 34F	29,8	
9	869 Ae 35F	23,3	
10	869 Ae 35F	33	
11	869 An 33F	30,4	
12	284 succ An 34F	29,9	
13	869 Ae Fs	36,9	Univ927f / EUB1492r (565bp)
14	869 Ae 32F	36,2	

Tableau 8: DNA concentration

Amplicons were then diluted in order to obtain:

A range of concentration from 3 to 10 ng of DNA in 9µL for the PCR amplicons obtained with the primers Univ786f / univ926r and

A range of concentration from 5 to 20 ng in 9µL for the PCR amplicons obtained with the primers Univ927f / EUB1492r.

These diluted amplicons were re-amplified with a BigDye®Terminator v1.1 cycle sequencing kit using the same primers as before. The amplicons obtained with this kit were further purified on a centri-sep column, denatured during 4 min at 70°C and 2 min at 96°C, and introduced in the sequencing machine.

14 isolates were successfully sequenced and 6 groups of isolates (Table 9) were identified with the online NCBI BLAST program: *Staphylococcus, Bacillus, Enterococcus, Sphingomonas, Mycobacterium* and *Clostridium*, notably *Clostridium butyricum*.

N°	Source	Culture media	Primers	Identified bacteria	Forward primer		Reverse primer	
					% Similarity	% Gap	% Similarity	% Gap
1	32F	869 Ae	Univ786f / univ926r	Staphylococcus	98	0	98	0
14	32F	869 Ae	Univ927f / EUB1492r	Mycobacterium	92	0	-	-
2	33F	869 Ae	Univ786f / univ926r	Bacillus	98	0	98	0
3	33F	Sis glc Ae		Bacillus	96	0	99	0
7	33F	869 An		Enterococcus	97	0	98	1
11	33F	869 An		Bacillus	98	0	96	1
4	34F	284 suc Ae		Shingomonas	96	2	98	0
5	34F	Sis glc Ae		Shingomonas	99	0	98	0
8	34F	284 suc An		Shingomonas	97	0	98	0
12	34F	284 suc An		Shingomonas	95	0	98	1
6	35F	869 Ae		Enterococcus	97	0	98	0
9	35F	869 Ae		Enterococcus	99	0	97	0
10	35F	869 Ae		Clostridium	97	0	98	0
13	35F	869 An	Univ927f / EUB1492r	Enteroccocus	93	0	83	1

Table 9: Isolates identified

2.3.4 Characterization of identified contaminants

2.3.4.1 Enterococcus (*Enterococcaceae*)

Enterococcus is a Gram positive, aerobic bacterium. They are often in long chains.
Enterococci are generally parasitic bacteria of the digestive mucous membranes and commensally of the intestinal flora.

2.3.4.2 Sphyngomonas

They are Gram positive, strictly aerobic bacilli, which produce yellow colonies. They are largely widespread in nature and are frequently isolated from the aquatic environments, ground, sludges.... Sphyngomonas were also isolated from biological wastes treatment processes.

2.3.4.3 Bacillus

They are Gram positive, aerobic and sporulated bacilli. *Bacillus* germs are ubiquitary in the environment, particularly the ground. They are frequently isolated as contaminants.
Spores resistance to heat plays an important role in the frequency of isolation as contaminant. *Bacilli* such as *B subtilis* or *B cereus* are found in small quantity in the rumen (Bryant, 1959).

2.3.4.4 Clostridium

Clostridium are strictly anaerobic and sporulated bacilli. *Clostridium argentinense*, *Clostridium baratii*, *Clostridium botulinum* and *Clostridium butyricum* are largely widespread in environments (ground, fresh water, marine sediments, decomposing plants) where spores are able to survive for long periods.
Clostridium are faeces hosts detecting them in ground samples generally reveals faecal contamination.
These bacteria can be present in the digestive tract of men and of animals and can contaminate food. *C. butyricum*, C. *lochheadii* and *C. longisporum* were isolated from the rumen (Bryant, 1959).

2.3.4.5 Staphylococcus

Staphilococcus are Gram positive cocci, classically found in cluster. They are frequently isolated from men and warm-blooded animals. However, they are eliminated in the external medium with human wastes.
These very resistant bacteria are frequently found in the environment.

2.3.4.6 Mycobacterium

Mycobacteria are pathogenic opportunist bacteria, often associated with deficit of the human immune system (HIV). Some *Mycobacteria* are also found as saprophytes in water, the ground… in particularly *Mycobacterium avium*.

2.3.5 Conclusions

During F.s. cultures in bioreactor we are confronted to a production of butyrate which isn't an F.s. end product. We have looked for the presence of a bacterial contaminant by PCR at SCK premises. The results obtained show 6 species possibly responsible for contamination.
However, among these 6 contaminants, 3 are aerobic and 2 don't produce butyrate. Consequently, the only contaminant which can be present seems to be *Clostridium butyricum*.
Even though complementary PCR were performed with *Clostridium butyricum* specific primers, no amplification was observed.
The discrepancy in results calls for more experiments in order to fully assess the contamination.

3 MAP MASS BALANCES

3.1 MAP mass balances

The data collected from the various partners allow performing the carbon and nitrogen mass balance of each technology as well as the mass balances of the substrate exchange experiments.
Carbone balance of the substrate exchange experiments are represented in figure 10. This figure shows that only solid residues were exchanged between the partners.

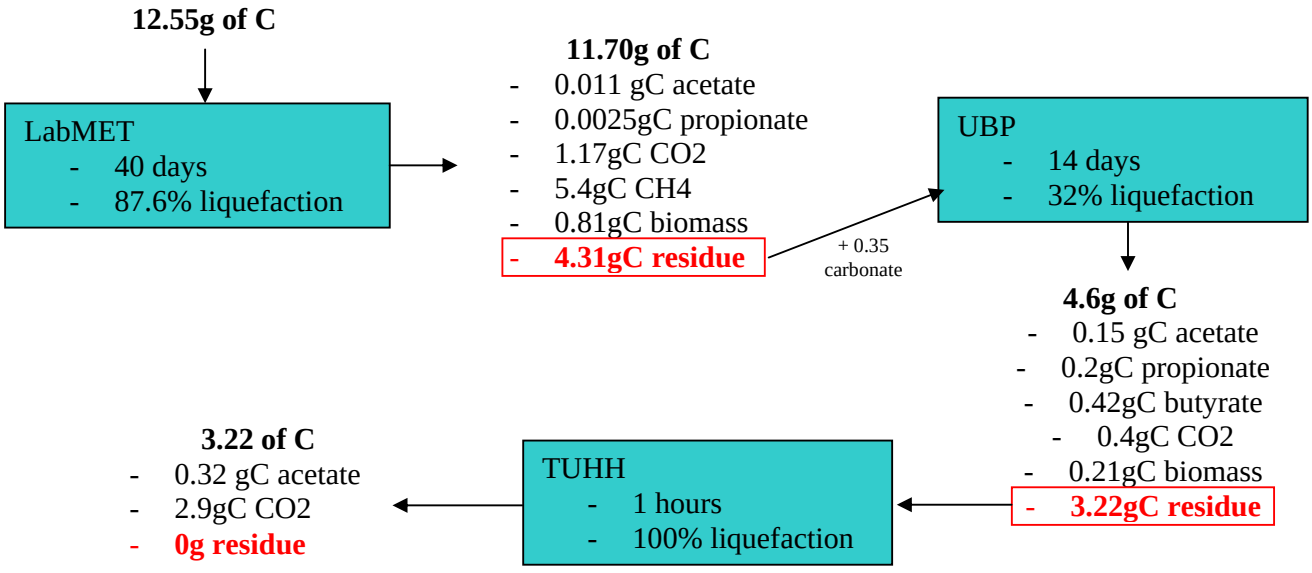


Figure 10: C exchanges

The results for the determination of C and N mass balance are summed up in tables 10, 11 and 12.

C mass balance	**IN**	**OUT**					
Compounds	Substrate	Acetate	Propionate	CO_2	CH_4	Biomass	Residue
g of C	12.55	0.011	0.0025	1.17	5.4	0.81	4.31
Total	12.55	11.7					
Mass balance	**93.25%**						

N mass balance	**IN**		**OUT**		
Compounds	Substrate	NH_3	Biomass	Residue	NH_3
g of N	1.20	0.35	0.13	0.43	0.96
Total	1.55		1.52		
Mass balance	**98.06%**				

Table 10: C and N high load methanogenesis unit mass balances (40 days of culture)

C mass balance	**IN**		**OUT**					
Compounds	Substrate	Carbonate	Acetate	Propionate	Butyrate	CO_2	Biomass	Residue
g of C	4.31	0.35	0.15	0.2	0.42	0.4	0.21	3.22
Total	4.66		4.6					
Mass balance	**98.71%**							

N mass balance	**IN**	**OUT**		
Compounds	Substrate	Biomass	Residue	NH_3
g of N	0.43	0.03	0.24	0.13
Total	0.43	0.40		
Mass balance	**93.02%**			

Table 11: C and N *Fibrobacter* unit mass balances (14 days of culture)

C mass balance	IN	OUT	
Compounds	Substrate	Acetate	CO_2
g of C	3.22	0.32	2.9
Total	3.22	3.22	
Mass balance	**100%**		

N mass balance	IN	OUT
Compounds	Substrate	NH_3
g of N	0.24	0.24
Mass balance	**100%**	

Table 12: C and N hydrothermal liquefaction mass balances (1 hour)

The C mass balances (93.25%, 98.71% and 100%) and the N mass balances (98.06%, 93.02% and 100%) closure for each technology confirms the efficiency of the experimental methods used for the 3 processes.

Global carbon balance of the substrate exchange experiments is represented in figure 11. This figure shows the solids which were exchanged between MAP partners.

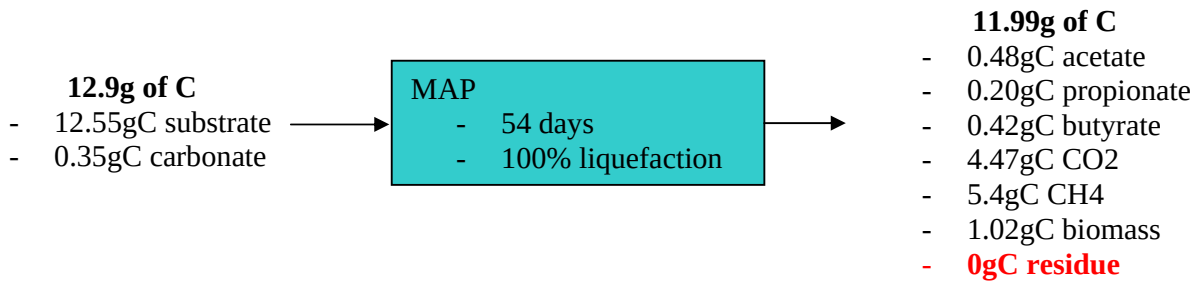


Figure 11: C balance for overall process

C mass balance	IN		OUT					
Compounds	Substrate	Carbonate	Acetate	Propionate	Butyrate	CO_2	CH_4	Biomass
g of C	12.55	0.35	0.48	0.2	0.42	4.47	5.4	1.02
Total	12.9		11.99					
Mass balance	**92.95%**							

N mass balance	IN		OUT	
Compounds	Substrate	NH_3	Biomass	NH_3
g of N	1.2	0.35	0.16	1.33
Total	1.55		1.49	
Mass balance	**96.13%**			

Table 13: C and N global mass balances of MAP exchange

The C mass balance was 92.95% and the N mass balance was 96.13% for the overall experiment (Table 13). The experimental methods used are validated by these results.

3.2 Anaerobic Waste Compartment Modelling and Simulation program

The "Anaerobic Waste Compartment Modelling and Simulation" program was made in order to simulate cultures of microorganisms used in MELiSSA loop. This program allows:

- The study and the development of dynamic models for the first compartment of MELiSSA,
- The determination of the most reliable model,
- The determination of the experimental parameters.

Several steps are necessary before performing the actual simulation. Simulation is the last step of the process (Figure 12).

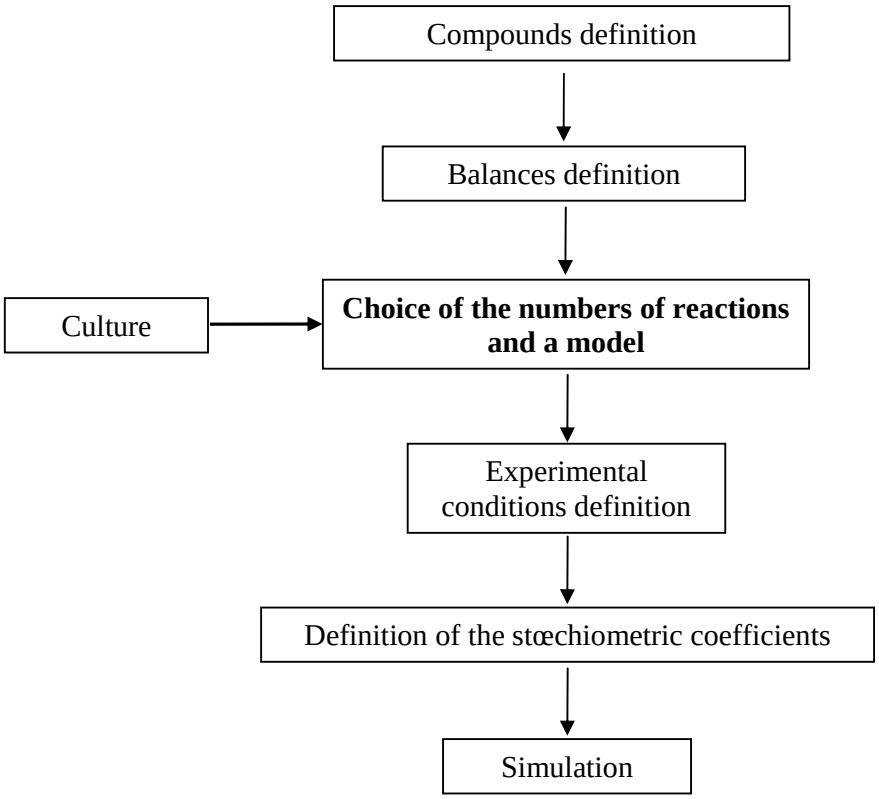


Figure 12: Various steps of the simulation

3.3 Example: Fibrobacter unit

3.3.1 Compounds definition

For each compound (figure 13), there are four fields:
- The name,
- The composition,
- The molar mass,
- The density.

The fields "name of the compound" and "molar mass" are mandatory. The molar masses allow checking if the total mass balance matches the stoechiometric description.

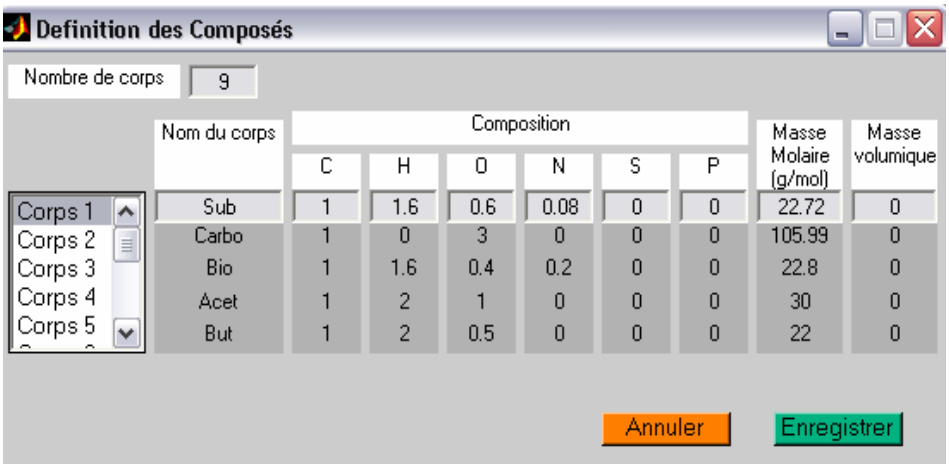


Figure 13: Compounds definition

3.3.2 Balances definition

For each compound in the reaction, it is necessary to define the liquid/gas balance and the acid/base balance (Figure 14 and 15).

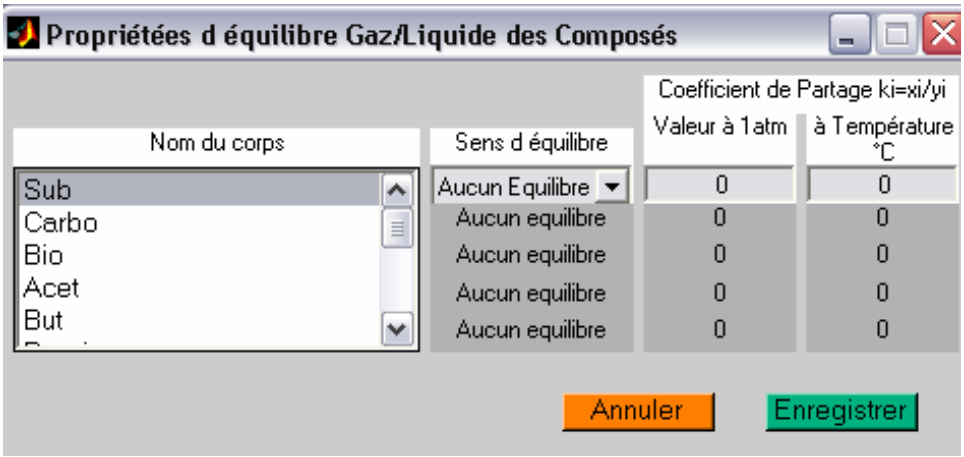


Figure 14: Liquid/gas balance

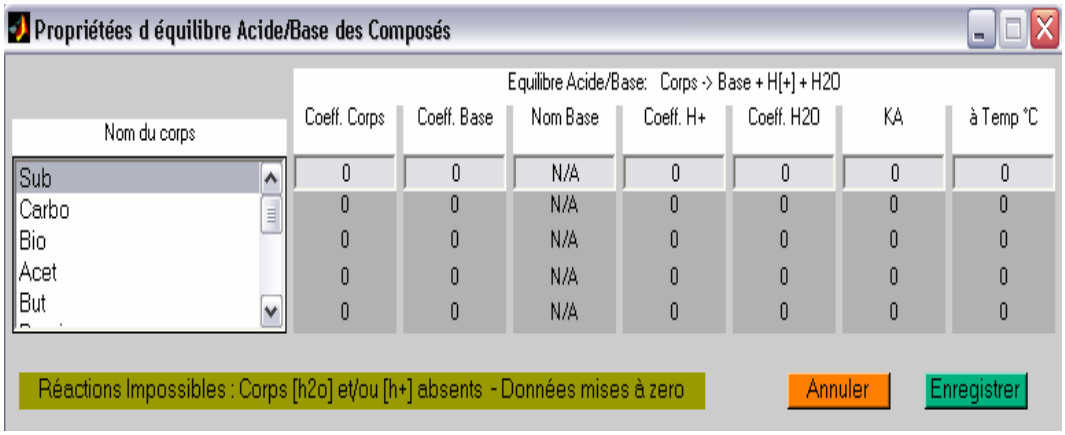


Figure 15: Acid/base balance

For all the compounds defined, liquid/gas balances are negligible and we considered that all compounds remained in liquid form during the reaction except for CO_2 which was supposed to be in gas form during the reaction. Similarly, acid/base balance was not defined.

3.3.3 Choice of the reactions and of a model

The biological behaviour can be represented by one or more reactions, each reaction requiring the definition of a model (Monod/Pirt/Andrews equation, enzymatic reaction…) (Figure 16). The program requires the stoechiometric coefficients in order to carry out the simulation. Some of these coefficients must be determined from experimental yields when the elements conservation equations are not sufficient for calculating all stoechiometric coefficients.

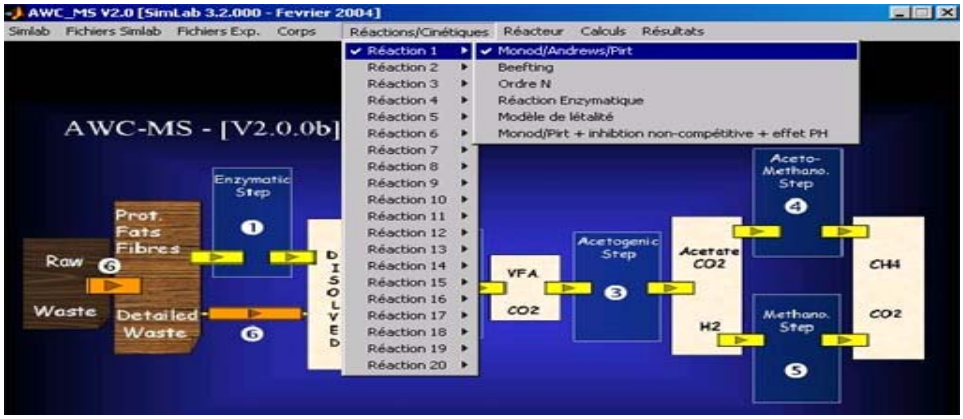


Figure 16: Choose of the reaction and of the model

We have chosen a standard chemostat setup and Monod equation without maintenance. This type of model requires the definition of the maximum growth rate (µmax) and of Ks (corresponding to limiting concentration in substrate).
We made the assumption that there was no substrate limitation during the reaction. We have consequently chosen a very low value of Ks (Ks=1*10-8) (Figure 17).

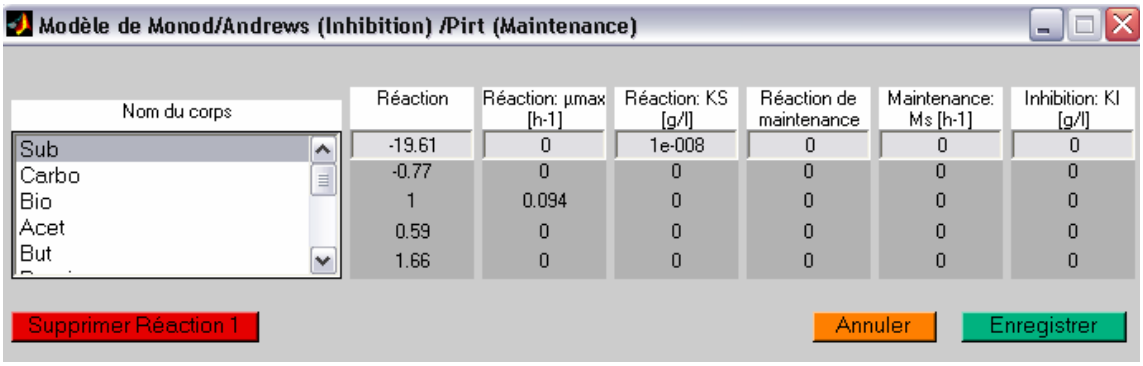


Figure 17: Model of Monod (Molar yields)

3.3.4 Definition of the experimental conditions/data

3.3.4.1 Experimental data: Fibrobacter culture

Before simulation, experimental data, such as substrates and products concentration, biomass concentration… are needed.
µmax, degradation rate, stoechiometric coefficients…can be calculated with these data.

Determination of the µmax value
We performed a culture on glucose in a 10mL tube to calculate µmax. Figure 18 represents the evolution of optical density during the time.
Plotting logarithm OD vs. time during the exponential growth phase allows calculating µmax (slope of the obtained curve). The exponential phase of growth starts at 4h and finishes after 12h.

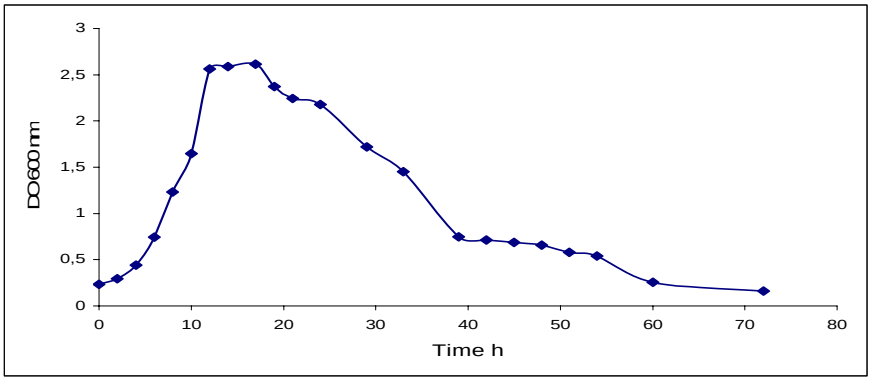


Figure 18: Evolution of the optical density.

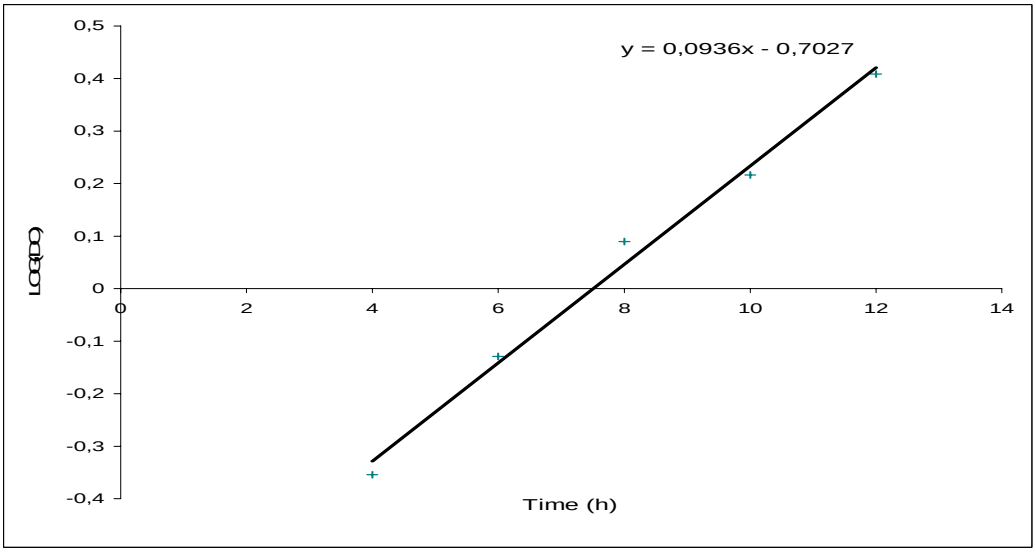


Figure 19: Logarithm of OD

The maximum growth rate corresponds to the slope of the curve so μmax = 0.094 h^{-1}. We found in the literature a range of μ_{max} 0.07 h^{-1} to 0.11 h^{-1} for cultures performed on glucose.

Determination of C-molar composition of the substrate and the residue

C, N, O, H… composition of the substrate and the residue (Table 14) is obtained by elementary analysis (CNRS).

	C	H	O	N	S	P
Substrate (%)	47.32	6.17	32.71	3.51	1.75	1.02
Residue (%)	43.14	5.88	34.54	4.29	0.88	0.80

Table 14: CNRS elementary analysis

With the elementary composition (mass percentage) of the substrate and the residue, we can determine the C-molar composition of each element (Table 15). This composition is related to a specific experiment presented in TN 2.700.
Substrate:
Mass percentage of C = 47.32%
M (C) = 12 g/mol; M(N) = 14 g/mol, M(H) = 1 g/mol; M(O) = 16 g/mol
% mol (C) = 47.32/12 = 3.94%
% mol (N) = 3.51/14 = 0.25%
% mol (H) = 6.17/1 = 6.17%
% mol (O) = 32.71/16 = 2.04%

In order to use the AWC-MS, the molar composition needs to be normalised with respect to C content.
This implies:
mol (C) = 1
mol (N) = 0.25/3.94 = 0.064
mol (H) = 6.17/3.94 = 1.564
mol (O) = 2.04/3.94 = 0.518.

	C	H	O	N
C-molar composition of the residue	1	1.564	0.518	0.064
C-molar composition of the substrate	1	1.64	0.6	0.085

Table 15: C-molar composition

Determination of the products concentrations

Table 16 summarizes information concerning the compounds C-molar composition, the corresponding molar masses, the initial concentrations.

			Experimental values	
Compounds	**Composition**	**Molar mass (g/mol)**	**Initial (g)**	**Final (g)**
Eau	H_2O	18		
Carbon dioxide	CO_2	44		39.85
Acetate	$C_1H_2O_1$	30		7.48
Butyrate	$C_1H_2O_{0.5}$	22		15.28
Propionate	$C_1H_2O_{0.67}$	24.67		8.32
NH3	N_1H_3	17		3.2
Biomass	$C_1H_{1.6}O_{0.4}N_{0.2}$	22.8		9.56
Substrate	$C_1H_{1.64}O_{0.6}N_{0.085}$	24.43	200	
Carbonate	Na_2CO_3	105.99	34.22	
Residue	$C_1H_{1.564}O_{0.518}N_{0.064}$	22.72		135

Table 16: Composition and quantities of the compounds

3.3.4.2 Experimental equation

In the experiment performed, all the substrate wasn't degraded. At the end of the reaction there is a solid residue. In order to take account the partial degradation, two solutions can be envisaged:

- To consider two coupled equations: A first equation splits the total substrate into the degradable substrate and residual substrate fractions and a second equation describes the degradation of the degradable substrate to various products.

This method requires knowledge related to enzymes (Ks) and knowledge of the evolution of the degradable and residual substrate during culture time in order to determine the stoechiometric coefficients of the equation.

Substrate + Carbonate ⟶ Degradable substrate + Residual substrate

Degradable substrate ⟶ Biomass + CO_2 + H_2O + Acetate + Butyrate + Propionate + NH_3

- To consider only one equation which starts with the initial substrate and leads to the residual substrate and the products. This second method does not take into account the intermediate steps of degradation, but only the initial and final states.

This method is less accurate but easier to use because it necessitate less information than the first one.

Substrate + Carbonate $\longrightarrow$ Biomass + CO_2 + Acetate + Butyrate + Propionate + NH_3 + Residual substrate

As we didn't have all the information necessary to follow the first method (unknown evolutions of the substrates degraded and residual during time), we chose the second method, which uses only one equation, similar to the following one:

A Substrate + B Carbonate $\longrightarrow$ C Biomass + D Acetate + E Butyrate + F Propionate + G CO_2+ H NH_3 + I Residue

A, B, C, D, E, F, G, H and I are the stoechiometric coefficients which must be determined using experimental data.

Two types of yields can be calculated: either with respect to biomass concentration or with respect to substrate masses.

Two of three processes are biological. For these we decided to use the yields with respect to the biomass concentration because simulations using the yields with respect to the biomass concentration seem to be closer to reality than those with respect to substrate masses (data not shown). For the third one, we decided to use yields with respect to the substrate because it's a chemical process.

With these yields (related to the biomass) we can calculate the stœchiometric coefficients. There are 9 stoechiometric coefficients, which are determined as follows:

- The stoechiometric coefficients are established for one C-mole of biomass i.e. C=1,
- The ratio A/B depends on substrate compositions i.e. the ratio of solid substrate versus carbonate which experimentally fixed,
- There are 4 element conservation equations for C, H, O, N. Therefore, 6 equations are involved in the calculation of the 9 stoechiometric coefficients. The remaining necessary information results from the experimental knowledge of 9-6=3 measured yields

In practice, considering the C and N mass balances are satisfactory, we have calculated the stoechiometric coefficients for all experimental yields available. The resulting stoechiometric equations will not match perfectly the C, H, O, N mass balances (knowing that all products and substrates are probably not completely known). But in any case, these stoechiometric equations are considered representative for mass balance calculation purposes.

3.3.5 Determination of the equations

3.3.5.1 Determination of the yields

For each metabolite produced, we have measured the concentration evolution during time then the yields (Y) with respect to the biomass concentration (Table 17). e.g. butyrate:
$[butyrate]_{g/L}$ = 15.28 g/L
Ybutyrate/biomass = 15.28/9.56 = 1.598 g(but)/g(biomass)
We obtain the molar yields with molar masses: Mbiomasse = 22.8 and Mbutyrate = 22 so
Ybutyrate/biomass (mole) = 1.598*22.8/22 = 1.66 mole (but)/mole (biomass)

YSubstrate/biomass	19.61
Ycarbonate/biomass	0.77
Ybiomass/biomass	1
Yacetate/biomass	0.59
Ybutyrate/biomass	1.66
YPropionate/biomass	0.8
YCO_2/biomass	2.16
YResidue/biomass	14.17
YNH_3/biomass	0.452

Table 17: Metabolites/biomass molar yields (mole/mole)

3.3.5.2 Determination of the stœchiometric coefficients

A	B	C	D	E	F	G	H	I
Substrate	Carbonate	Biomass	Acetate	Butyrate	Propionate	CO_2	NH_3	Residue
-19.61	-0.77	1	0.59	1.66	0.8	2.16	0.45	14.17

Table 18: The stœchiometric coefficients (mole/mole)

3.3.5.3 Stœchiometric equation

19.61 Substrate + 0.77 Carbonate $\longrightarrow$ Biomass + 0.59 Acetate + 1.66 Butyrate + 0.8 Propionate + 2.16 CO_2 + 0.52 NH_3 + 14.17 Residue

3.3.6 Determination of the degradation rates

3.3.6.1 Experimental degradation rate

The degradation yield reported in TN 2.7 was 32.5%.

3.3.6.2 Degradation rate by the equation

From the stoechiometric coefficients obtained we calculate the theoretical degradation rate, in the absence of any limitation or inhibition.
% of degradation = (I*M(I)-J*M(J))/(I*M(I))
Where I is the stoechiometric coefficient of the substrate, J is the stoechiometric coefficient of residue, M(I) is the molar mass of substrate and M(J) is the molar mass of the residue

% of degradation = ((19.61*24.32)-(14.17*22.72))/(19.61*24.32) = 32.49%

The percentage of degradation calculated is the same (32.5%) as the experimental results, which validates this method of calculation of the stoechiometric coefficients.

3.3.7 Simulation

The simulation with the program AWC-ms-[V2.0.0b] was performed with the stoechiometric equation obtained previously. The results obtained are reported in figures 20 and 21.

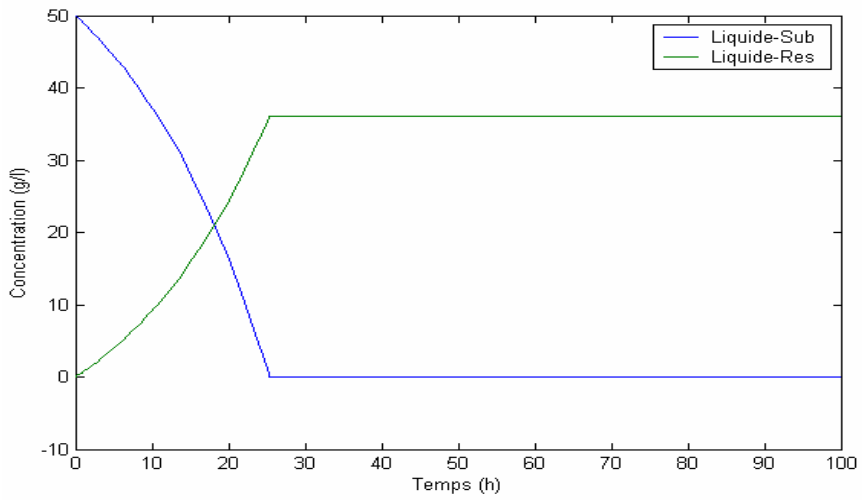


Figure 20: Substrate and residue evolution during the time

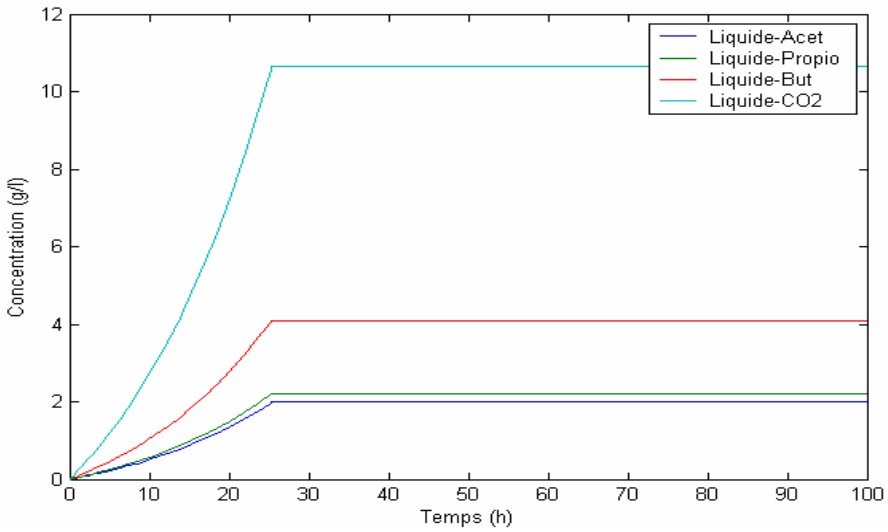


Figure 21: Biomass and metabolites evolution during the time

The first observation that can be made is that the reaction stops after 25 hours of culture, after all substrate have been consummed. This reaction time is much shorter than that observed in experiments. Indeed, the reaction was stopped after 325 hours in the experiment (TN2.7) whereas there still was remaining substrate.
This difference comes from the fact that simulation doesn't take into account inhibition whereas during the culture VFA inhibition may have occurred. Inhibition slowed down the reaction speed. This can explain why the simulated process is faster. Moreover the µmax used is a µmax obtained during a culture on glucose and not during a culture on methanogenesis residues. (µmax can not be calculated on waste).

From the results obtained with the simulation, we can check that the degradation yields are the same with the simulation as with the experiment.

$R = [(substrate)_{initial} – (residue)_{final}] / [(substrate]_{initial}]$
$R = (50 – 35) / 50 = 0.30$

The same theoretical degradation rate was found with the simulation than this measured during the experiment. So the simulation can be validated because the same degradation rate was found with the simulation and with the stoechiometric equation.

This simulation allows the determination of the initial and final concentration (Figure 20) of the metabolites (Table 19) and allows the comparison between experimental and simulation concentration (Table 20).

	Initial concentration (g/L)	Final concentration (g/L)
Biomass	0,1	2.9
Substrate	50	0
Residue	0	33.75
Acetate	0	2
Propionate	0	2.29
Butyrate	0	4.1
CO_2	0	10.9

Table 19: Initial (TN 2.700) and final concentration with the simulator

	Final experimental concentration (g/L)	Final concentration with the simulation (g/L)
Acetate	1.87	2
Propionate	2.08	2.19
Butyrate	3.82	4.1
CO_2	9.96	10.9

Table 20: Final experimental concentration and final concentration with the simulation

A difference is observed between the concentrations obtained from the simulation and the experimental concentration but this difference is very weak. This can be explained by the fact that inhibitions are not taken into account in the simulation. Moreover, the µmax used is a µmax obtained during a culture on glucose and not during a culture on methanogenesis residues. (µmax can not be calculated on waste)
However, if a µmax of $0.02h^{-1}$ is used (literature value for cultures on vegetables); better results are obtained (Figure 22 and 23).

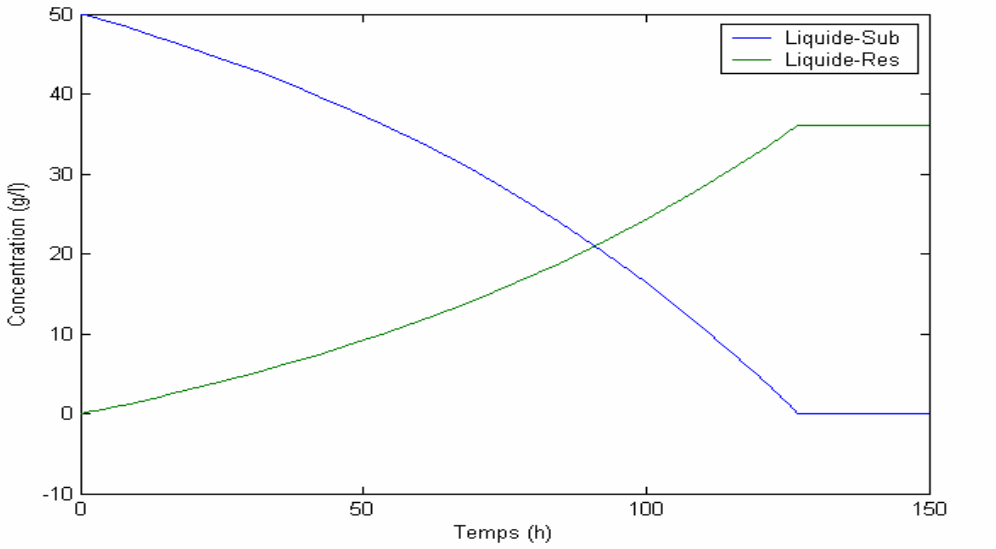


Figure 22: Substrate and residue evolution

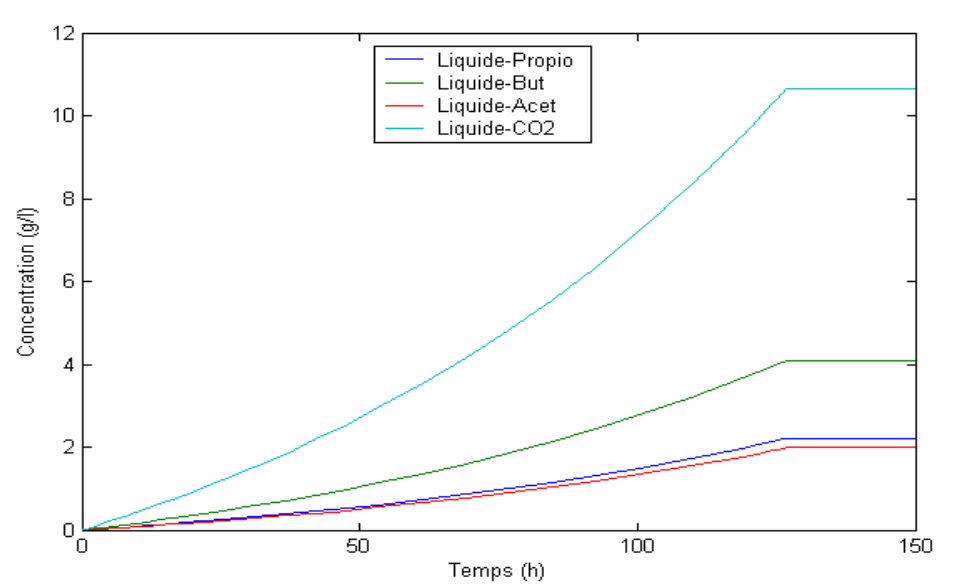


Figure 23: Biomass and metabolites evolution

As previously, the reaction time is shorter than in experiments with this µmax. The explanation seems to be the same one as previously, indeed during the simulation there is no inhibition or vegetable access limitation. However the reaction time obtained is the time

obtained in TN 2.7 as the optimal residence time (150h). The same metabolite concentrations of are found with this μmax.

3.4 MAP simulation

3.4.1 Partner 1 (LabMET)

3.4.1.1 Determination of the conversion yields

Determination of the yields
As for *Fibrobacter*, for each metabolite produced, we have calculated the concentration evolution during time then the yields (Y) compared to the biomass (Table 21),

YSubstrate/biomass	29.95
Ybiomass/biomass	1
Yacetate/biomass	0.001
YPropionate/biomass	0.0003
YCH4/biomass	2.34
YCO_2/biomass	1.38
YResidue/biomass	3.78
YNH_3/biomass	2.5

Table 21: Yields compared to the biomass

Stœchiometric equation

29.95 Substrate $\longrightarrow$ 1 Biomass + 0.001 Acetate +0.0003 Propionate + 2.34 CH_4 + 1.38 CO_2 + 2.5 NH_3 + 3.78 Residue + 13.28 H_2O

Determination of the degradation yield
The degradation yield obtained by the experimentation was 87.6%.

From the stoechiometric coefficients obtained we can calculate the theoretical degradation yields, in the absence of any limitation or inhibition: % of degradation =87.58%
The percentage of degradation calculated is very close (87.6%) to the experimental results, which validates this method of calculation of the stoechiometric coefficients.

3.4.1.2 Simulation

The simulation with the program AWC-ms-[V2.0.0b] was performed with the stoechiometric equation obtained previously the yields are calculated versus biomass and we used the µmax obtained with *Fibrobacter* ($0.02h^{-1}$) because we didn't have the µmax for this process. The results obtained are reported in figures 24, 25 and 26.

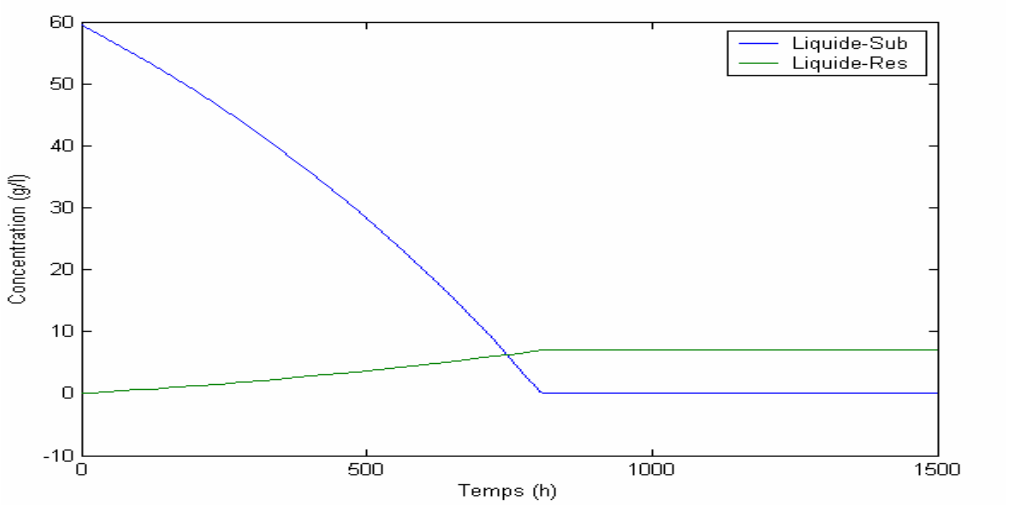


Figure 24: Substrate and residue evolution

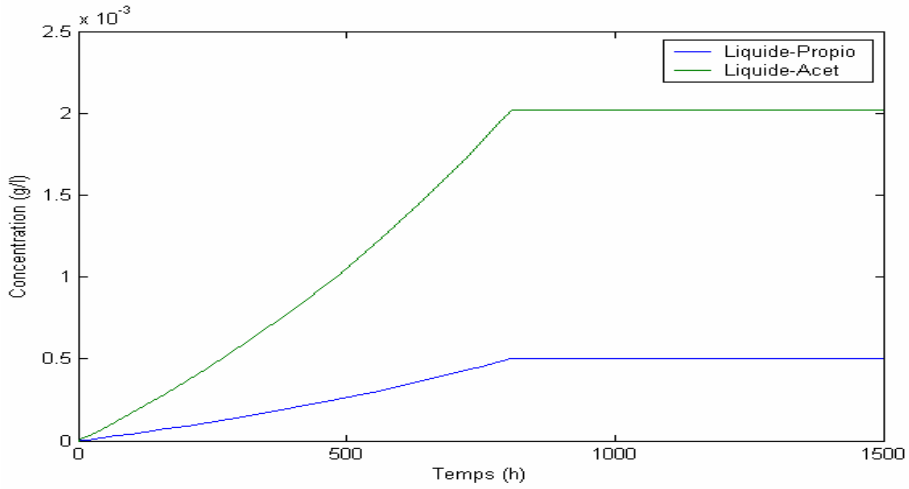


Figure 25: Metabolites evolution

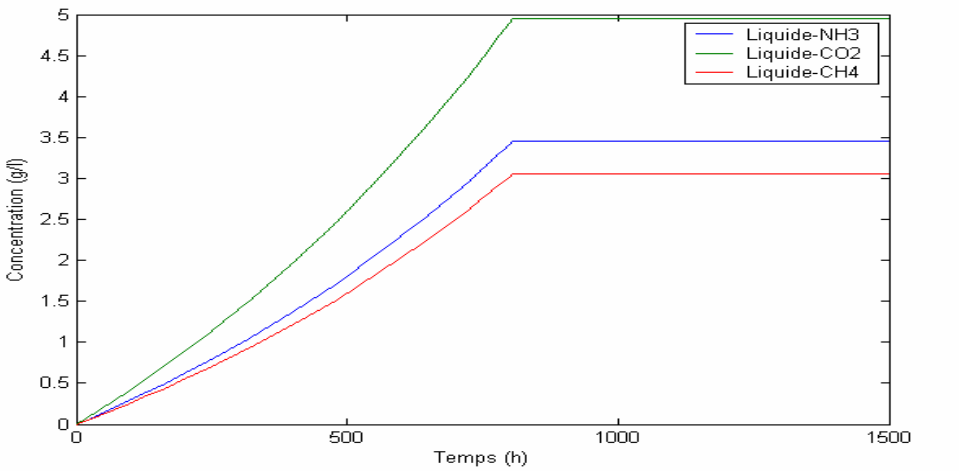


Figure 26: Biomass, NH_3, CH_4 and CO_2 evolution

The reaction stops after 803 hours of culture and all the substrate is consumed. This reaction time is very close to that observed in experiments (960h).

From the results obtained with the simulation, the degradation yield can be checked.
$R = [(substrate)_{initial} – (residue)_{final}] / [(substrate]_{initial}]$
R = 90%
The experimental degradation yield (87.58%) found was very close to the one found with the simulation. So the simulation can be validated because the same degradation rate was found with the simulation and with the stoechiometric equation.

This simulation allows the determination of the initial and final concentration (Figure 25 and 26) of the metabolites and allows the comparison between experimental and simulation concentration (Table 22).

	Final experimental concentration (g/L)	**Final concentration with the simulation (g/L)**
Acetate	0.0027	0.002
Propionate	0.0005	0.0005
CH_4	4.1	3.1
CO_2	6.7	4.97

Table 22: Final experimental concentration and final concentration with the simulation

A difference is observed between the concentrations obtained from the simulation and the experimental concentration but this difference is very weak. The reason is that these concentrations were obtained with the simulation are those which can be obtained without

limitation or inhibition. Moreover, the µmax used is a µmax obtained for *Fibrobacter* because we didn't have the µmax for this process.

3.4.2 Partner 4 (TUHH)

For each metabolite produced, we have calculated the concentration evolution during the time then the yields (Y) (Table 23). Because hydrothermal treatment is a physical-chemical process , yields can only be calculated with respect to substrate.

YAcetate/Substrate	0.1
YCO_2 /Substrate	0.9
Yresidue/Substrate	0
YNH_3/Substrate	0.06

Table 27: Yields compared to the substrate (mole/mole)

Stœchiometric equation

$$\text{Substrate} + 0.675\ O_2 \longrightarrow 0.1\ \text{Acetate} + 0.9\ CO_2 + 0.06\ NH_3 + 0.59\ H_2O$$

Determination of the degradation yield
The degradation yield obtained during the experiment was 100%.
From the stoechiometric coefficients obtained we calculated the theoretical degradation rate, in the absence of any limitation or inhibition.
% of degradation = 100%
The percentage of degradation calculated is the same as the degradation rate during the experiment, which validates this method of calculation of the stoechiometric coefficients.

For the same reason as for the determination of the stoechiometric coefficients (versus substrate) a simulation with the program AWC-ms-[V2.0.0b] is impossible.

3.4.3 MAP

For the simulation of global MAP process, only the input and the output data of the whole MAP process were used. So the equation represents what occurs overall and not individually. Moreover we reduced the biological and physical-chemical processes to one single biological process. We chose to reduce MAP to a biological process because two of three processes are biological and because the biological degradation time (1300h) is largely higher than the chemical degradation time (1h). We also fixed only one volume (10L) and one µmax ($0.02h^{-1}$)….

3.4.3.1 Determination of the equations

Determination of the yields

We added all the metabolites produced by the 3 processes and calculated the concentration evolution during time. Then, yields (Y) are calculated with respect to the biomass (Table 24).

YSubstrate/biomass	25.17
Ybiomass/biomass	1
Yacetate/biomass	0.32
YPropionate/biomass	0.13
YButyrate/biomass	0.27
YCH4/biomass	5.04
YCO_2/biomass	1.96
YResidue/biomass	0
YNH_3/biomass	2.31

Table 24: Yields compared to the biomass (mole/mole)

Stœchiometric equation

25.17Substrate
+ 0.12 carbonate $\longrightarrow$ 1 Biomass + 0.32 Acetate +0.13 Propionate + 0.27 Butyrate + 1.96 CH_4 + 5.04 CO_2 + 2.31 NH_3 + 0 Residue + 9.81 H_2O

3.4.3.2 Determination of the degradation yields

The degradation yield calculated during the experiment is 100% considering the biomass produced can be further used for other applications.

3.4.3.3 Simulation

The simulation with the program AWC-ms-[V2.0.0b] was performed with the stoechiometric equation obtained previously. Moreover, the µmax used is a µmax obtained for *Fibrobacter* because we didn't have the µmax for the overall process.
The results obtained are in figures 27, 28 and 29.

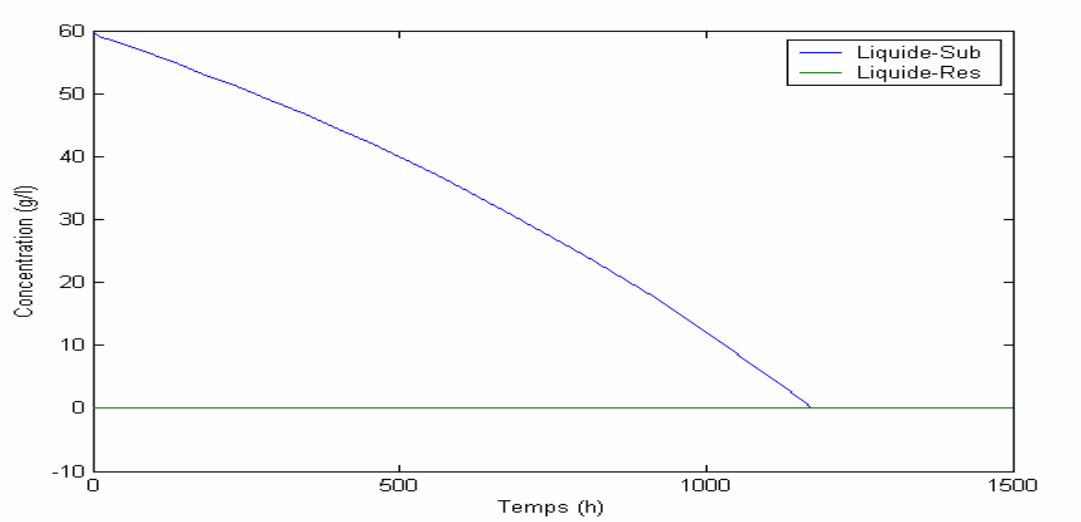


Figure 27: Substrate and residue evolution

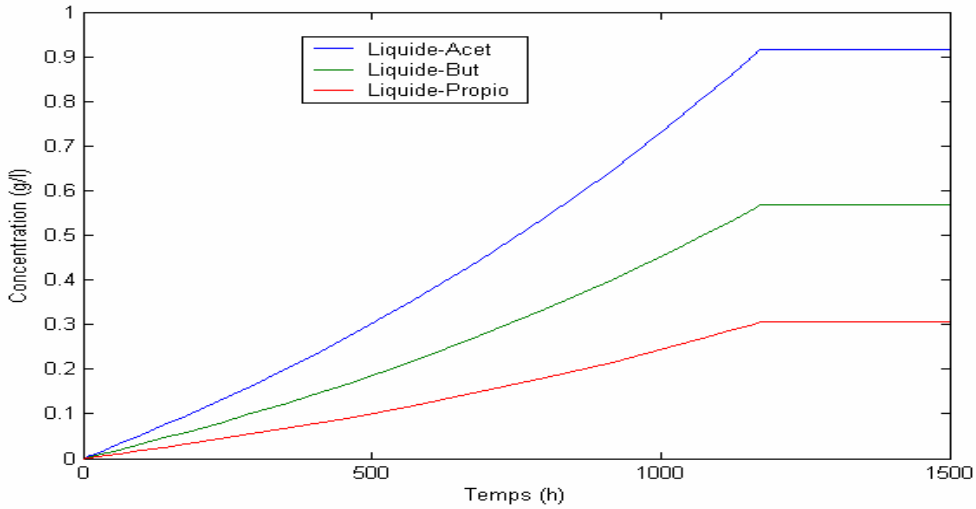


Figure 28: Metabolites evolution

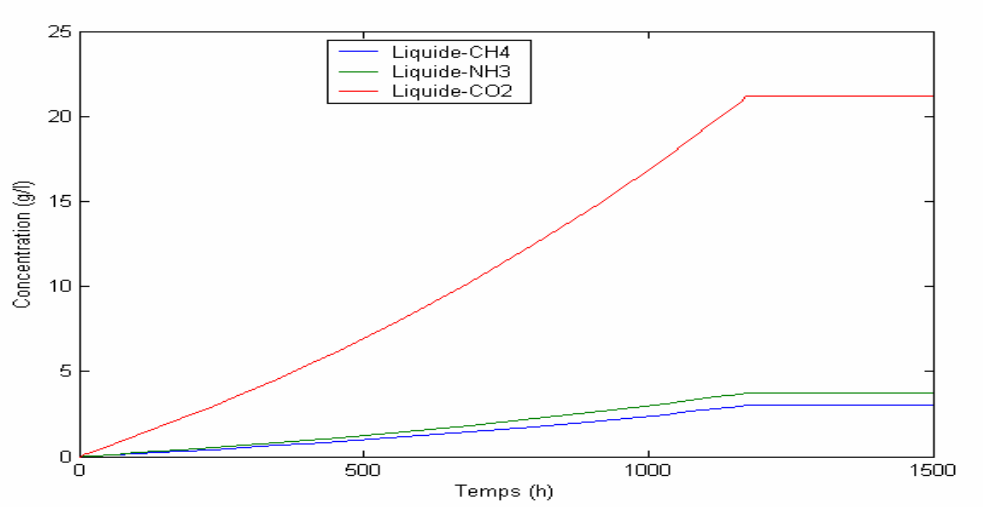


Figure 29: NH_3, CH_4 and CO_2 evolution

The reaction stops after 1170 hours of culture. This reaction time is very close to that observed in experiments (≅1300).

A new global yield of degradation can be calculated thanks to the results obtained with the simulation. This degradation yield allows checking the stoechiometric coefficients of the equation.

$R = [(substrate)_{initial} - (residue)_{final}] / [(substrate]_{initial}]$

$R = 100\%$

The same theoretical degradation rate (100%) was found with the simulation than this calculated with the stoechiometric equation. So the simulation can be validated because the same degradation rate was found with the simulation and with the stoechiometric equation despite the incorporation of physical-chemical process with biological ones.

From the figures 28 and 29 we can calculate the initial and final concentration of the metabolites and we can compare experimental and simulation concentrations (Table 25).

	Final experimental concentration (g/L)	**Final concentration with the simulation (g/L)**
Acetate	3.077	0.9
Propionate	2.08	0.3
Butyrate	2.08	0.58
CH_4	4.1	3.5
CO_2	29.63	21.67

Table 25: Final experimental concentration and final concentration with the simulation

A difference is observed between the concentrations obtained from the simulation and the experimental concentration but this difference is very weak. The difference is certainly due to the fact that for the simulation we have reduced the several biological and physical-chemical processes to one single bioreactor with only one volume, one µmax…

3.5 Partner 3

The data given by partner 3 are summarised in figure 30.

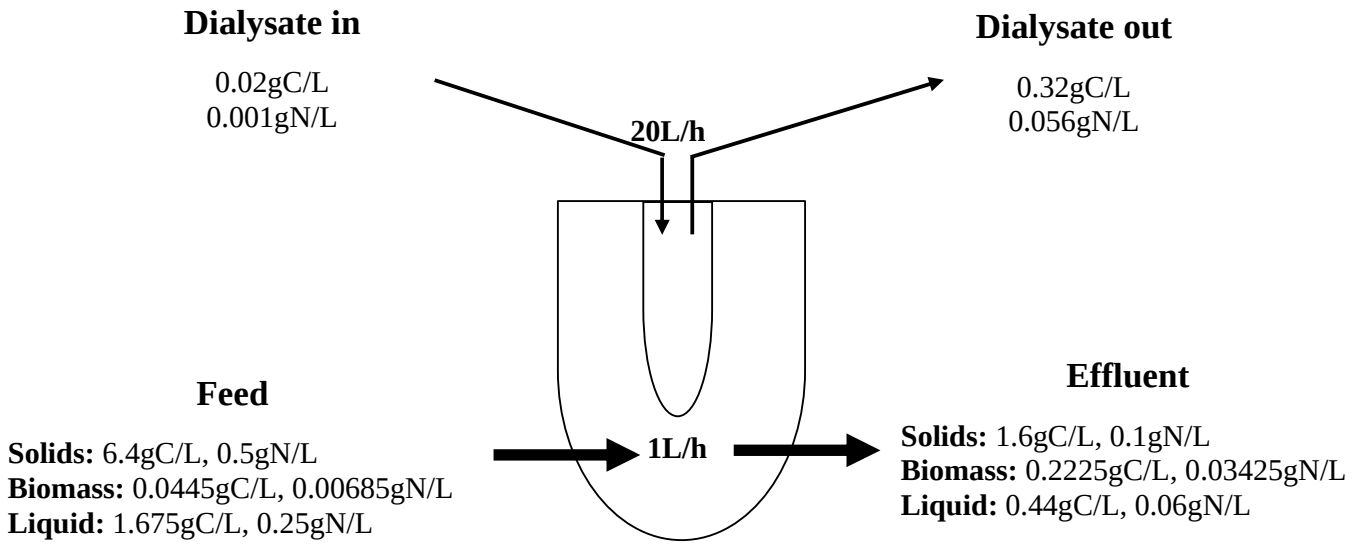


Figure 30: Input and output of partner 3

3.5.1 Mass balances

These data from the partner 3 allow performing the carbon and nitrogen mass balance (tables 26). However, a culture duration is necessary (1 hour was selected as culture duration) to be able to calculate the quantity of C and N in and the quantity of C and N out.

C mass balance	**IN**		**OUT**		
	Dialysate	Feed	Dialysate	Effluent	CO_2
gC	0.4	8.06	6.4	2.26	0.04
Total	8.46		8.50		
Mass balance	**100.5%**				

N mass balance	IN		OUT	
	Dialysate	Feed	Dialysate	Effluent
gN	0.02	0.76	1.12	0.19
Total	0.78		1.31	
Mass balance	**168%**			

Table 26: Mass balance

The nitrogen comparison between input and output seems to indicate that some input have not been taken into account.

3.5.2 Simulation

3.5.2.1 Determination of C-molar composition of the substrate and the residue

First, it is necessary to determine the C, N, O, H… composition of the substrate and the residue (Table 27). With the data collected we know that there is 6.4gC/L in 20g/L of substrate so C represents 32% of the substrate in the same way there is 2.5% of N in the substrate and 32% of C and 2% of N in the residue. For H and O we use known value from LabMET and UBP.

	C	H	O	N
Substrate (%)	32	6.5	33	2.5
Residue (%)	32	6.2	32.7	2

Table 27: Elementary composition of the substrate and of the residue

With the elementary composition (mass percentage) of the substrate and residue for each element, we can determine the C-molar composition of each element (Table 28).

	C	H	O	N
C-molar composition of the substrate	1	2.44	0.77	0.07
C-molar composition of the residue	1	2.33	0.77	0.05

Table 28: C-molar composition

Determination of the products concentrations

Table 29 summarizes information concerning the compounds C-molar composition, the corresponding molar masses, the initial concentrations.

Compounds	Composition	Molar mass (g/mol)	Initial (g/L)	Final (g/L)
Eau	H_2O	18		
Carbon dioxide	CO_2	44		0.15
H_2	H_2	2		0.35
Acetate	$C_1H_2O_1$	30		15.52
NH_4^+	N_1H_4	18		0.68
Biomass	$C_1H_{1.6}O_{0.4}N_{0.2}$	22.8		0.4
Substrate	$C_1H_{2.44}O_{0.77}N_{0.07}$	27.74	20	
Residue	$C1H_{2.33}O_{0.77}N_{0.05}$	27.35		5

Table 29: Composition and quantities of the compounds

3.5.2.2 Determination of the equations

Determination of the yields

For each metabolite produced, we have calculated the concentration evolution during time. The global conversion yields (Y) are then expressed versus biomass synthesis (Table 30).

YSubstrate/biomass	41.1
Ybiomass/biomass	1
Yacetate/biomass	29.49
YH_2/biomass	10.03
YCO_2/biomass	0.19
YResidue/biomass	10.42
YNH_4/biomass	2.16

Table 17: Yields compared to the biomass

Stœchiometric equation

$$41.1\ \text{Substrate} + 6.64\ H_2O \longrightarrow \text{Biomass} + 29.49\ \text{Acetate} + 10.03\ H_2 + 0.19\ CO_2 + 2.16\ NH_4 + 10.42\ \text{Residue}$$

3.5.2.3 Determination of the degradation rates

The degradation yield obtained during the experiment was 75%.

From the stoechiometric coefficients obtained the theoretical degradation rate is 75%, in absence of any limitation or inhibition. The percentage of degradation calculated is the same as the experimental results, which validates this method of calculation of the stoechiometric coefficients.

3.5.2.4 Simulation

The simulation with the program AWC-ms-[V2.0.0b] was performed with the stoechiometric equation obtained previously. Moreover, the µmax used is a µmax obtained for *Fibrobacter* because we didn't have the µmax for this process. Figures 31 and 32 represent the results obtained by the simulation.

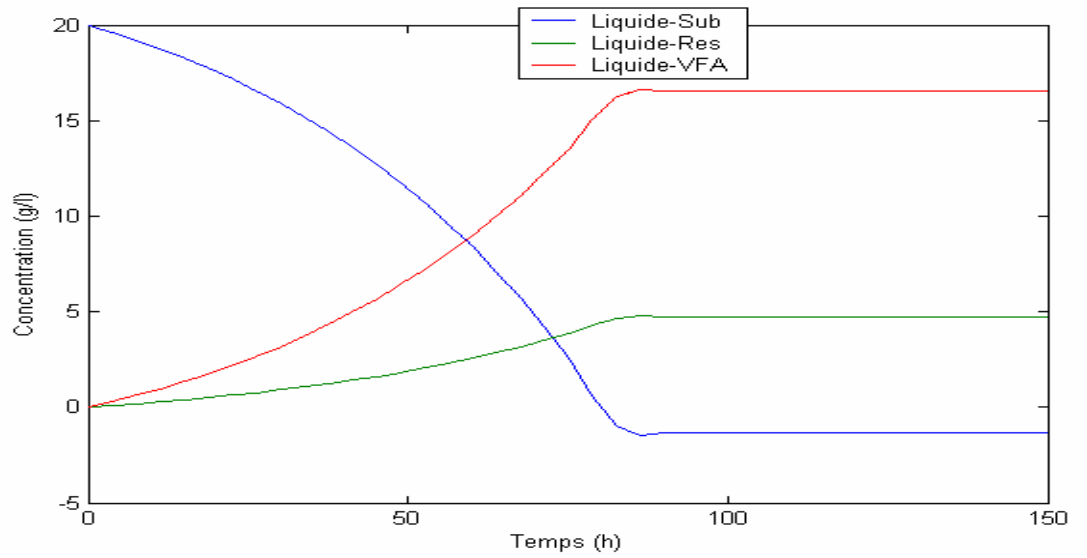


Figure 31: Substrate, residue and VFA evolution during the time

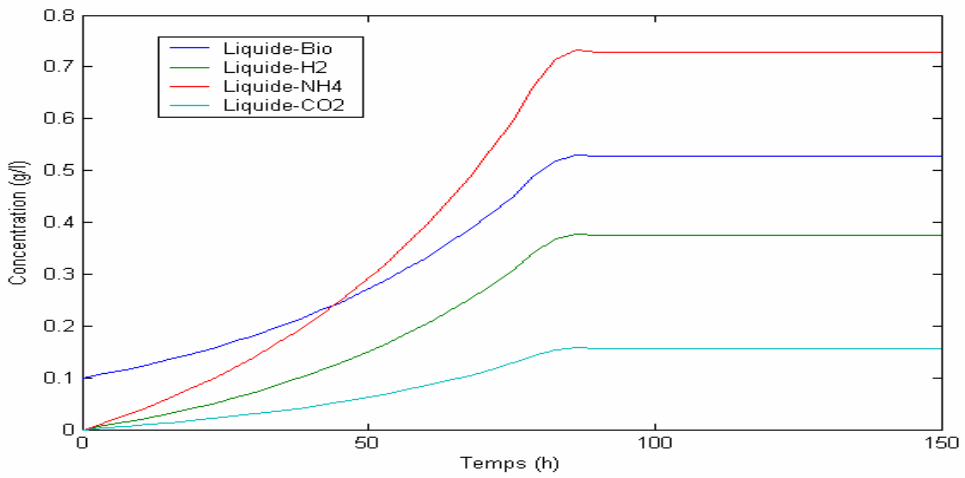


Figure 32: Biomass and metabolites evolution during the time

The reaction time observed with the simulation is 84h of culture. This reaction time is the time we can obtain without inhibition or limitation by a product

These results (figure 31) allow the determination of the degradation rate without inhibition or limitation.

$R = [(substrate)_{initial} – (residue)_{final}] / [(substrate]_{initial}]$
$R = (20 – 4.5) / 20 = 0.77$

This degradation rate (77 %) found with the simulation is very close to the degradation rate obtained during the experiment or calculated with the stoechiometric equation. So the simulation can be validated because the same degradation rate was found with the simulation and with the stoechiometric equation.

This simulation (Figure 32) allows the comparison between experimental and simulation concentration (Table 20).

	Final experimental concentration (g/L)	**Final concentration with the simulation (g/L)**
Acetate	15.52	16.19
Biomass	0.4	0.52
H_2	0.35	0.38
CO_2	0.15	0.16

Table 20: Final experimental concentration and Final concentration with the simulation

A difference is observed between the concentrations obtained from the simulation and the experimental concentration but this difference is very weak for all the metabolites. The reason is that these concentrations were obtained with the simulation are those which can be obtained without limitation or inhibition. Moreover, the µmax used is a µmax obtained during a culture on glucose with *Fibrobacter succinogenes*. Moreover this simulation used a model of reactor which must be adapted to this process. In spite of that, the results obtained with the simulation are rather close to reality.

4 CONCLUSION / OUTLOOKS

Fibrobacter succinogenes S85 is a strict anaerobic bacterium and uses glucose, cellobiose, cellulose and other parietal polymers as carbon source to produce acetate and succinate. However a production of butyrate is observed during culture in the bioreactor. The tests carried out in SCK show that there is a possibility of bacterial contamination. First, when there is growth under aerobic conditions we know it's not F.s.. Moreover the sequencing allowed the isolation of 6 potential bacterial contaminants including:

Sphingomonas, able to use succinate for his growth, this bacterium being strictly aerobic;

Clostridium, in particular *C butyricum*, able to produce butyrate.

The 4 others even if they are present do not produce butyrate and are not known to consume succinate.

These results must be verified because the contamination and/or the contaminant are not definitively proved.

A PCR was performed with *C butyricum* specific primers to confirm the presence of this bacterium but no amplification was observed. An explanation could be that during amplifications with universal primers (before the sequencing), sequences can preferentially be amplified than others resulting in nondetection of target contaminant. This frequently happened when checking contamination (Amann et al., 1995).

We have carried out the C and N mass balances of each process and of MAP loop. The results of the mass balances show the importance to have all the input and output of the various processes.

Finally, the simulation of each partner process and of MAP loop was performed. The program AWC-ms-[V2.0.0b] used for the simulation gives satisfactory results on all processes, but this program must be adapted according to the study process.

Better simulation could be performed provided all the necessary data (µmax, volume, time of culture, inhibition or limitation…) are available. Indeed, the differences between experimental and simulated results obtained for the metabolite concentrations or for the degradation yields are due to the absence of inhibition or limitation in the proposed model.

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