Enhancing the hydrolysis yield in thermophilic anaerobic digestion of cattle manure

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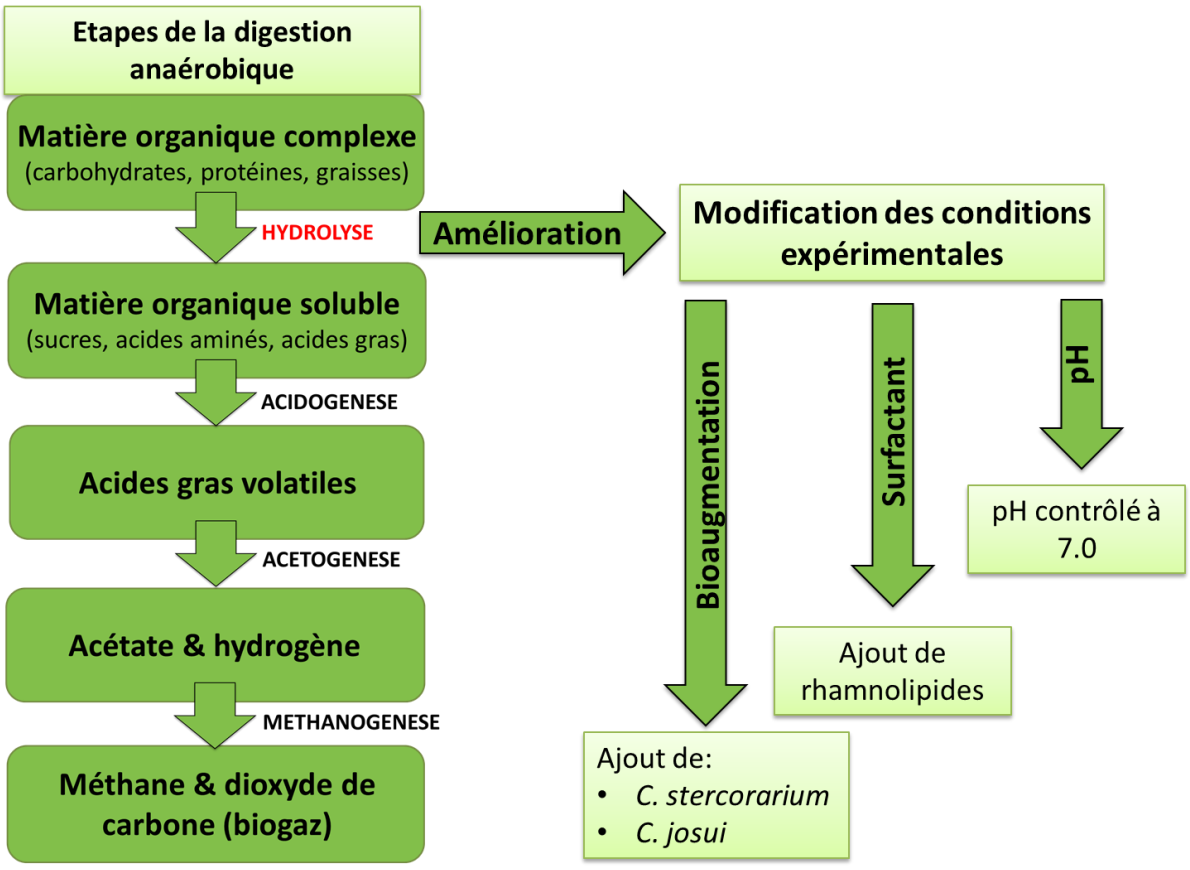
Résumé

La recherche vise à améliorer le taux de production et le rendement de biogaz au cours de la digestion anaérobique du fumier de bovins.

L'hydrolyse étant une étape limitante de la digestion anaérobique, une recherche bibliographique a été réalisée sur les différentes façons de l'améliorer en faisant varier les conditions expérimentales. La bio-augmentation, l'ajout de tensioactifs et la diminution du pH à 7.0 sont choisis afin d'améliorer la production de biogaz. La croissance des organismes sélectionnés a été étudiée pour leur addition dans les réacteurs. Le pH a été contrôlé à 7.0 dans deux réacteurs mais l'expérience a dû être stoppée à cause de l'ajout d'acide qui a été excessif.

Parallèlement, des expériences ont été réalisées afin de construire un protocole efficace pour l'extraction protéique du digestat des réacteurs et ainsi comparer la production d'enzymes hydrolytiques en fonction des différentes conditions. Les résultats ont démontré que la grande majorité des protéines étaient contenues dans la partie liquide du digestat.

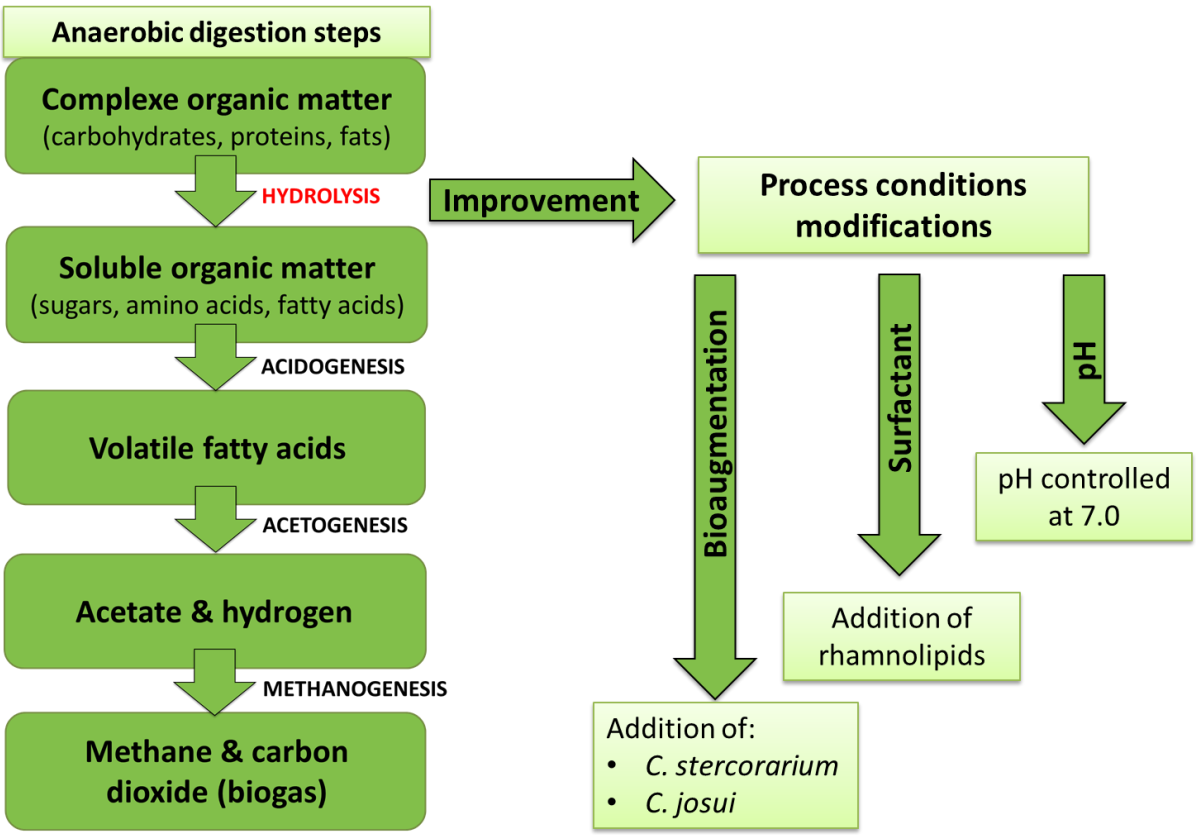


Summary

The purpose of the research is to improve the biogas production rate and yield during anaerobic digestion of cattle manure.

Hydrolysis being an anaerobic digestion-limiting step, a literature study was carried out on the ways to improve it by varying the experimental conditions. Bioaugmentation, addition of surfactant and decreasing the pH to 7.0 were expected to enhance biogas production. Growth of selected organisms was studied for their addition into reactors. Two reactors were operated under pH-control at 7.0 but the experiment had to be stopped because of the acid addition that was excessive.

Meanwhile, experiments were made to design an efficient protocol for extracting proteins from the reactor digestate and comparing produced hydrolytic enzymes depending of the conditions. It was shown that proteins are mostly present in the liquid fraction.



Abbreviation list

AD = anaerobic digestion

C. stercorarium = *Clostridium stercorarium*

C. josui = *Clostridium josui*

VS = Volatile solids

TS = Total solids

VFA = Volatile fatty acids

OD = Optical density

Rpm = Revolution per minute

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I. Introduction

Due to global warming and the depletion of fossil fuels, there is a trend towards renewable energy sources, such as solar power, bio-ethanol and biogas. Digestion of agricultural biowaste, such as animal manure or plant residues, is of interest because it enables the recovery of chemical energy as biogas (Ward et al., 2008) while treating wastes at the same time.

Although anaerobic digestion (AD) is a proven technology, at present, biogas yield for anaerobic digesters of manure is limiting for process profitability and research is required to increase biogas yield and production rate. The production of biogas is limited by the hydrolysis step (Pavlostathis et al., 1991). Even if the hydrolysis step is important, it has been poorly described and not very well understood. Improving the hydrolysis step of the recalcitrant organic matter (biofibers) contained in the manure could significantly increase the biogas yield from manure (Angelidaki et al., 2000) and make the digestion more profitable.

1. Background

In AD, organic material is microbiologically converted to biogas, viz. methane and carbon dioxide, which can be used to produce electricity and heat or as a vehicle fuel while reducing greenhouse emissions. AD is a multistep process, consisting of successively hydrolysis, acidogenesis, acetogenesis and methanogenesis with a number of microbial interrelationships and dependencies. The first step of AD is hydrolysis, which consists of a wide range of depolymerization and solubilization processes breaking down complex polymeric organic compounds into soluble monomers. Acidogenesis subsequently converts monomeric compounds into volatile fatty acids (VFA), alcohols, NH_3, lactic acid, H_2, CO_2 and H_2S. Then, acetogenic bacteria oxidize these products to acetate, formate, H_2, and CO_2. Finally, methanogenic archaea cleave the acetate (acetoclastic methanogens) and others reduce H_2 and CO_2 (hydrogenotrophic methanogens) in order to produce CH_4 and CO_2 (Angelidaki et al., 2011). The process is mediated by organisms present in the digesters and the enzymes they excrete. Hydrolytic enzymes, like cellulases, are essential for AD because they degrade cellulose and hemicellulose in the substrate.

AD is dependent on different environmental conditions like temperature, pH and retention time. These conditions can impact activity of enzymes or their excretion by organisms. In order to select the best conditions to modify, a bibliographic research was made.

2. Literature review

pH

Organisms, enzymes and reactions are highly dependent on pH and have different pH optima. Therefore, selection of one optimal value for the whole sequence of processes involved in AD is difficult. However, for AD the optimum pH mean is 7 (Chen et al., 2008).

Moreover, ammonia concentration depends on pH and ammonia is known as the principal inhibitor of AD (Zeeman et al., 1991), the decrease of its concentration likely results in a higher hydrolysis rate. Furthermore, there is a relief of ammonia-induced inhibition at lower pH. Braun et al. (1981) showed that lowering the pH from 8 to 7.4 during anaerobic digestion of liquid piggery manure resulted in a reduction of the concentration of ammonia from 316 mg l^{-1} to 84 mg l^{-1} and an increased biogas production. Zeeman et al. (1985) observed that decreasing the pH from 7.5 to 7.0 during thermophilic anaerobic digestion of cow manure resulted in four times increased methane production.

At Sävsjö the pH in the digester is 7.8 – 8.9. The above mentioned studies indicate the importance of controlling the pH at a lower value for a better hydrolysis rate and biogas production.

Bioaugmentation

Chemical and physical pretreatments are used to improve the hydrolysis rate of other wastes like wood or straw. However, addition of organisms producing hydrolytic cellulolytic enzymes would be more cost-effective (Angelidaki et al., 2000) because they will produce their own enzymes, add new degradation pathways for manure and improve the final hydrolysis-rate (Schwarz et al., 2001).

For bioaugmentation, organisms that grow under thermophilic and anaerobic conditions, and that produce enzymes that are not already present in the digesters should be selected.

Clostridium josui and *Clostridium stercorarium* were selected, since both of them are known for producing hydrolytic enzymes, being thermophilic and anaerobic.

C. josui produces a complex of cellulases interacting with each other, called cellulosome (Kakiuchi et al., 1998) and has an optimum temperature of 45°C, while *C. stercorarium* does not have a cellulosome but produces cellobiose and cellodextrine phosphorylases instead (Reinchenbecher et al., 1997) and its optimum temperature is at 65°C.

Surfactants

Since hydrolysis is limited by the available surface area of cellulose, increasing surface area should improve the hydrolysis. Helle et al. (1993) showed that surfactants increased hydrolysis rate by 67 %, probably by lowering the nonactive binding sites that decrease the effectiveness of enzymes. Several tests conducted by Eriksson et al. (2002) indicated that a major obstacle in the enzymatic conversion of lignocellulose is the adsorption of significant amounts of enzyme on exposed lignin surfaces without being able to degrade it. Surfactants prevented unproductive binding of cellulases to lignin, by binding lignin in the lignocellulose fibers to the hydrophobic part of the surfactant by hydrophobic interactions. Then, adding surfactants in digesters should increase the available substrate and its hydrolysis rate by hydrolytic enzymes.

Rhamnolipids are surfactants that can be produced either by chemical synthesis or by means of microbial cultivation; it is ecologically well acceptable and biodegradable (Mohan et al., 2006). The use of rhamnolipids for solid substrate fermentation resulted in a better cellulase and xylanase activity, the last one being 119.6% higher than the control (Liu et al., 2006). Zhang et al. (2009) tried to explain mechanisms of the stimulatory effect of rhamnolipids on rice straw hydrolysis. Rhamnolipids increased the activity and stability of hydrolytic enzymes and prevented unproductive binding of enzymes to lignin.

3. Research questions

The aim of the present research is to acquire a deeper understanding of the processes taking place in anaerobic digestion of manure, especially the hydrolysis, while increasing the biogas yield and production rate of a full scale thermophilic digester of Göteborg Energi AB located in Sävsjö.

- Does addition of *Clostridium josui* and *Clostridium stercorarium* to the digester improve hydrolysis yield and rate?

- Does operating anaerobic digesters at a decreased pH of 7.0 result in an increased hydrolysis yield and rate?
- Does addition of rhamnolipids to digesters increase hydrolytic enzyme activity by preventing unproductive binding?

4. Thesis outline

The first part of the project was to revive and growth enough of *C. stercorarium* and *C. josui* to add them to the reactors for the bioaugmentation.

Secondly, the amount of protein and the fraction they were (liquid or solid part) had to be determined to design a protein extraction protocol. It was made thanks to a plate assay with three different substrates and a Bradford test.

Finally, Reactor were operated mimicking the conditions of a full-scale digester fed with cattle. The pH was set at 7.0 and rhamnolipids were added as well as the organisms since they were all known for having an effect on the anaerobic digestion.

II. Material and methods

1. Bioaugmentation

Revival and culturing

Clostridium josui (strain JCM 17888, RIKEN BioResource Center, Japan) and *Clostridium stercorarium* (strain NCIMB 11754, NCIMB, Scotland) were revived (following supplier procedure) and cultured according to Bayer et al., 1983. The organisms were grown in a growth medium for *Clostridium thermocellum*, described by Johnson et al. (1981). In addition to the procedure by Johnson et al. (1981), the solutions were degassed in a Büchner filter. Thereafter, base medium, 10 x concentrated cellobiose and 10 x concentrated salts were added to serum bottles and closed with a crimp camp. Hereafter, the solutions were flushed with sterile nitrogen for 10 minutes and autoclaved. Due to the addition of the redox indicator resazurin to the base medium, it could be visually checked if the medium was anaerobic, viz. colorless.

The inoculation of the serum bottles was performed under anaerobic conditions, in an anaerobic bag, filled with nitrogen. After breaking the ampoule, 0.5 ml of medium was added to suspend the biomass; then the solution was transferred into a serum bottle and pressurized with some nitrogen from the anaerobic bag. Cultures were incubated overnight at their optimal temperature, *C. josui* at 45 °C (Sukhumavas et al., 1988) and *C. stercorarium* at 65 °C (Madden, 1983).

Growth curve

Culture growth was checked with OD measurements at 600 nm in duplicates with a spectrophotometer.

Samples were taken every two hours in order to make a growth curve and determine the exponential growth phase. After 24 hours at their optimal growth temperature, cultures were still sampled for 4 days.

A growth curve was made for *C. stercorarium* at 65°C and 52°C in order to know when they reached their maximal OD_{600} for their enrichment and addition in reactors.

Amplification

600 ml of medium was prepared and inoculated with *C. stercorarium* cultures to have a starting OD_{600} of 0.1. Following the growth curves, *C. stercorarium* cultures were harvested after 33h cultivation and put in the fridge at 4°C.

Because of some encounter problems in *C. josui* cultivation, 600 ml bottles were inoculated with an OD_{600} below 0.1 and they were left in incubation until their OD_{600} was sufficient for the reactors inoculation.

C. stercorarium was then added directly to reactors.

2. Protein extraction

Plate assay

Plate assays were developed to quickly check where cellulolytic and hemicellulolytic activities were localized after different kind of fractionation, e.g. liquid/solid. The goal of this was to design the protein extraction method for the proteomics study because it enabled to know how much proteins needed to be "stripped" from the solids and how much were in solution in the liquid instead.

Three chromogenic substrates (Megazyme) were used:

- Azo-Alpha-Cellulose was used to detect activity of the endo-1,4-β-D-glucanase (endo-cellulase).
- AZCL-Galactomannan was used to detect activity of the endo-1,4-ß-mannanase
- AZCL-Arabinoxylan used to detect activity of the endo-xylanase.

Agarose plates were made, containing 0.2 % w/v of one of the three substrates, 2% agarose, 100 mM HEPES buffer, mQ H_2O. pH was set at 7.8 via addition of 1M hydrochloric acid.
Digestate solid and liquid fractions were separated by centrifugation (5,525 *g* for 10 minutes). Three spots (triplicates) of solid fraction were put on each substrate plate, as well as liquid fraction, and on a control one without substrate. Plates were incubated at 52 °C for 24 hours.

Protein quantification

- Fraction preparation

Sävsjö digestate samples were centrifuged at 7,000 *g* for 10 min then at 15,000 *g* for 30 min in order to separate as much as possible liquid (supernatant) and solid fraction.

The solid fraction was resuspended in two different buffers in order to separate the enzymes bound to the solids:

- Buffer I : [Na_2HPO_4 100 mM, NaCl 0.5 M] + [NaH_2PO_4 100 mM, NaCl 0.5 M]. The second solution was mixed to the first one to pH 7.8.
- Buffer II : [Na_2HPO_4 100 mM, NaCl 0.5 M, TEAB 50 mM, SDS 4%] + [NaH_2PO_4 100 mM, NaCl 0.5 M, TEAB 50 mM, SDS 4%]. The second solution was mixed to the first one to pH 7.8.

The addition of SDS and TEAB was supposed to increase the solubilization of proteins bound to solids.

After being suspended in buffers for 1 hour, solutions were centrifuged at 15,000 *g* for 30 minutes, to separate newly solubilized enzymes from the solids, and the supernatant was kept and used for analyses.

Samples from the same digestate were centrifuged at 7000 *g* for 10 min, their supernatant was weighted and centrifuged again at 15000 *g* for 30 min to determine the solid and liquid percentage in the digestate.

- Gel SDS-PAGE

For each sample of 15 µl, 15 µl loading dye (50 mM Tris-HCl, pH 6.8, 2 % SDS, 10 % glycerol, 1 % β-mercaptoethanol, 12.5 mM EDTA, 0.02 % bromophenol blue) was added and heated up to 95°C for 5 minutes in order to denature all proteins.

Samples were loaded to the gel and the migration started at 200 V for 30 min. After washing 3x10 minutes with demineralized water, the gel was incubated 1 h with PageBlue protein staining solution for coloration. Then it was washed again for the removal of the excess dye (not binded to proteins) and the result was photographed.

- Well-plate assay protocol

The protein concentration in the samples was measured using a Bradford assay (Bradford reagent protocol from Sigma–Aldrich). Two standard curves were made using a BSA standard, from 0.2 to 1.4mg/ml with the buffer I and buffer II. Liquid fraction was diluted 5 times with the buffer I and solid fractions were diluted 2 times with their own buffer.

The analysis was performed in 96 well plates in triplicates. Bradford reagent was added to the wells containing the samples (one from the liquid fraction and two from the solid fraction) and the BSA standard. After 10 minutes the absorbance was measured at 595nm and the protein concentration was calculated by comparing the net A_{595} value against the standard curve.

3. Reactor operation

8 reactors were operated mimicking the conditions of the full scale digester in Sävsjö. Reactors were heated at 52°C and stirred at 100 revolutions per minutes (rpm) during all the experiment. Performance of reactors was evaluated based on analysis of total solids, volatile solids, biogas production rate and composition.
Reactors were fed every day from Monday to Friday, 250 ml of digestate were removed and 250 ml of manure (from Sävsjö) were added.

Once a week, digestate was analyzed following methods of Sluiter et al. (2005 & 2008) for the TS and VS analyzes and reactors stirred up at 200 rpm for 30 min, likewise for every new batch of manure. Gas samples were also taken once a week in every reactor to be analyzed with a biogas analyzer from Agilent Technologies (490 micro GC).

Reactors were operated for 44 days with two pH-controlled at 7.0. All analyses were done during that time. Due to problems with the acid addition, all reactors were stopped and started again with all new conditions at the end.

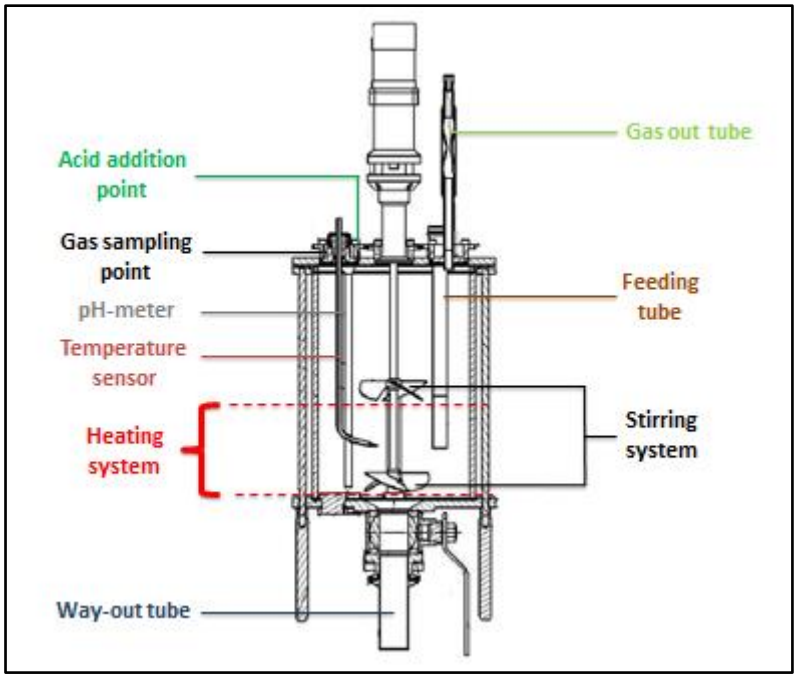


Figure 1: Reactor scheme

pH

In two reactors, the pH was controlled at 7.0 with a pH-meter. Since during anaerobic digestion pH was only expected to increase pH control was only made by addition of 2 M hydrochloric acid as soon as the pH went above the settled range (6.95-7.05).

However, a problem occurred with the pH control, addition of acid was excessive and pH in the reactor was below 7.0. To counterbalance it, sodium hydroxide (NaOH) 3M was added.

Surfactants addition

90% pure Rhamolipid biosurfactant (R90) were bought from AGAE Technologies LLC (Corvallis, Oregon, USA).

It was a glycolipid anionic biosurfactant produced by fermentation/separation/purification process using *Pseudomonas aeruginosa* in Canola Oil substrate. Its full name is: Decanoic acid, 3-((6-deoxy-2-O-(6-deoxy-alpha-L-mannopyranosyl)-alpha-L-mannopyranosyl)alpha-L-mannopyranosyl)oxy)-, 1-(carboxymethyl)octyl ester, mixt. with 1-(carboxymethyl)octyl 3-((6-deoxy-alpha-L-mannopyranosyl)oxy)decanoate [CAS 869062-42-0].

III. Results & discussion

1. Organisms growth

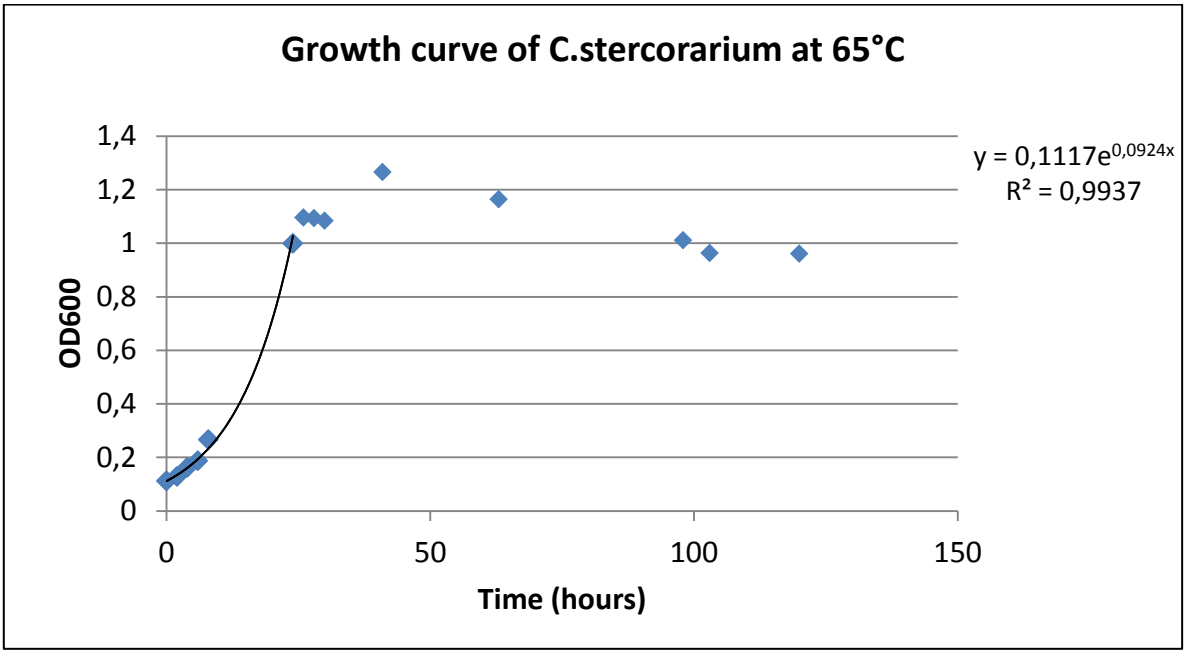


Figure 2: Growth curve of C.stercorarium at 65°C for 5 days

After being inoculated with an OD_{600}=0.1, *C. stercorarium* entered in exponential phase for more than 24h, before reaching a transient stationary phase and started decreasing slowly. The decrease of OD was caused by sporulation of organisms (spores having a lower OD than organisms) because of some limiting substrate in the medium.

The equation of the growth curve during the exponential phase was: $DO_{600}=Initial_{OD}*e^{\mu t}$

μ_{max} being the growth rate, its maximum was: $\mu_{max} = 0.0924$

Following this curve, enrichment bottles were harvested after 33 hours, to have the highest OD_{600} as possible before it started to decrease. They were then put in the fridge waiting for the reactor enrichment.

To avoid sporulation due to non-optimal conditions, organisms should be added to reactors directly during their exponential phase.

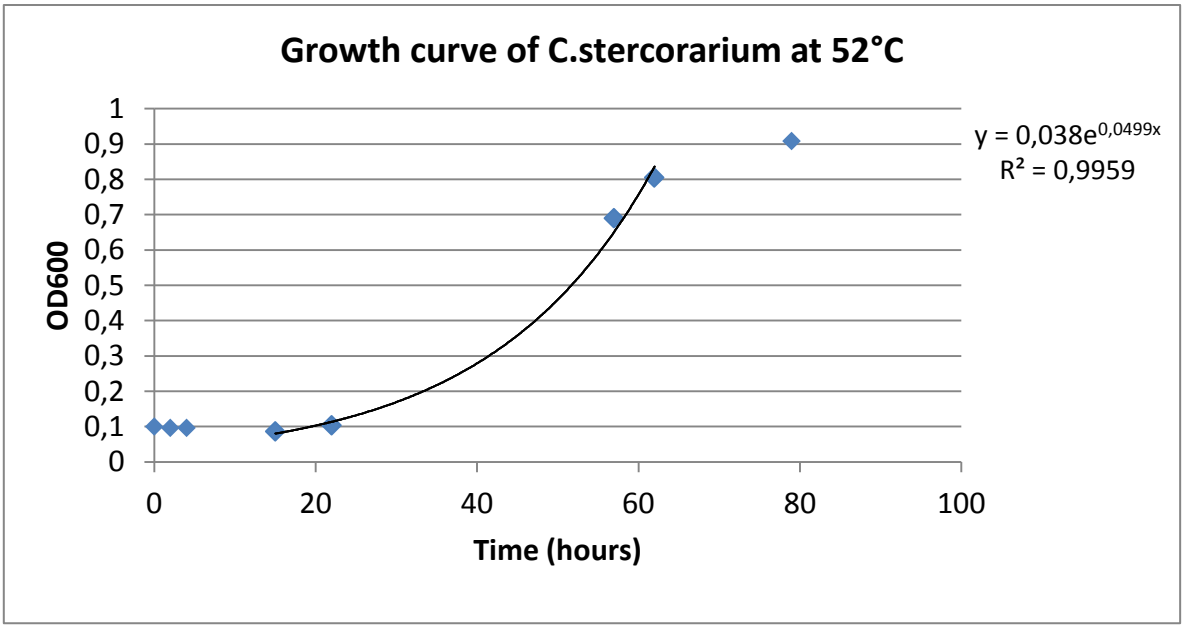


Figure 3: Growth curve of C.stercorarium at 52°C for 3 days

The growth curve of *C. stercorarium* cultivated at 52°C showed that at this temperature, exponential phase started 20 hours after inoculation and needed around 50 hours to reach its maximal DO_{600}.

Here, the growth rate was $\mu_{max} = 0.0499$

Under non-optimal temperature conditions there was still a growth of the organisms, even if it was slower than at 65°C with a maximal growth rate of 0.0336 instead of 0.0916. Reactors temperature was then not expected to be restraining for the growth of *C. stercorarium*.

2. Plate assay

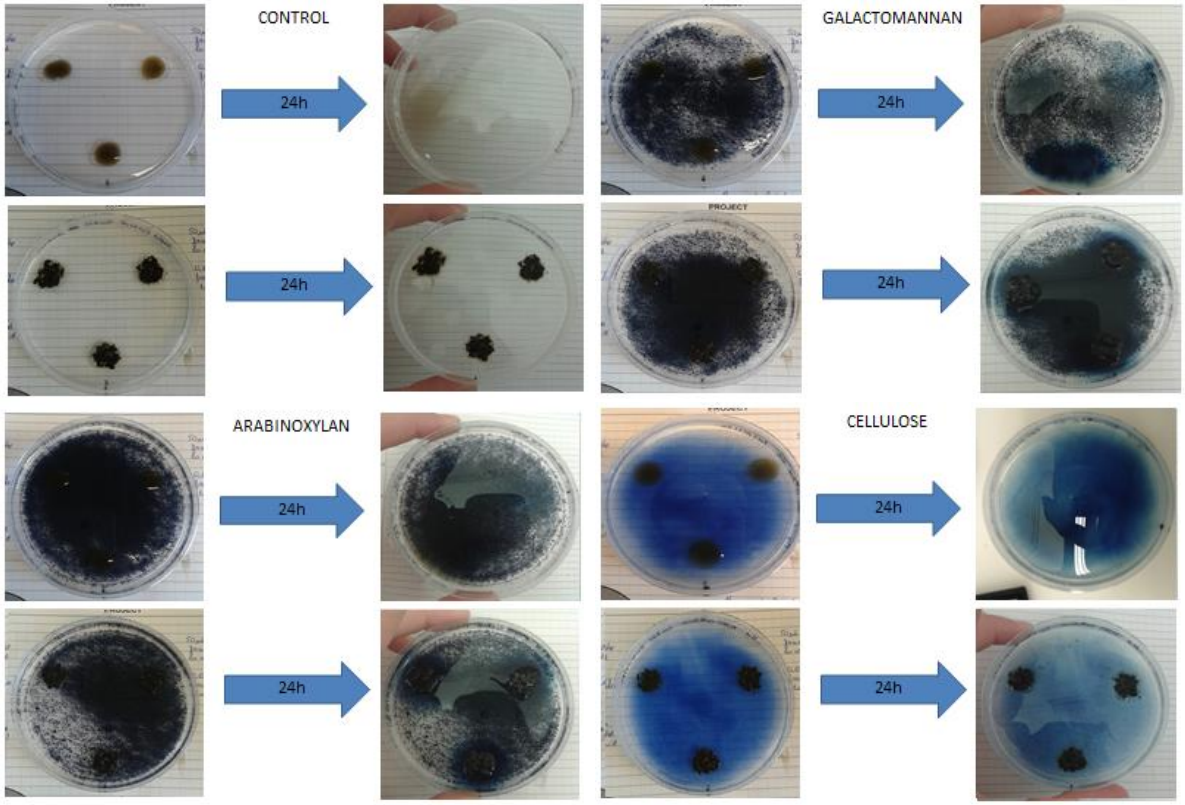


Figure 4: Results of the plate assays after 24h incubation at 52°C

After 24 hours incubation, there was a colored halo around the spots of the AZCL-Arabinoxylan and the AZCL-Galactomannan for both of liquid and solid fraction. Nevertheless there was not any change for the Azo-Alpha-Cellulose.

The halo around the spots of the AZCL-Arabinoxylan and the AZCL-Galactomannan plates showed a release of the dye contained in the substrate.

To be released, the substrates had to be degraded by specific enzymes: some endo-xylanases for the AZCL-Arabinoxylan and some endo-1,4-β-Mannases for the AZCL-Galactomannan.

The absence of results with the Azo-Alpha-Cellulose plate suggested that the specific enzyme, endo-1,4-β-D-glucanase, able to degrade it was not in the liquid nor the solid fraction of the digestate.

The fact that degradations were observed in around the solid and liquid spots proved that the enzymes were present in both of these fractions. Consequently, the protein quantification needed to be done with both solid and liquid fraction.

3. Protein quantification

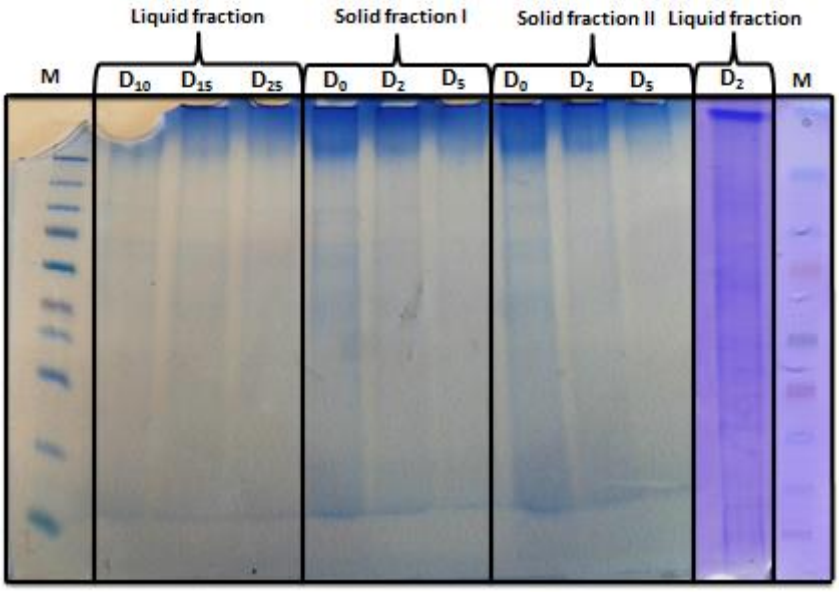


Figure 5: Photo of SDS-Page electrophoresis gel result

On the gel, there were smears for every fraction, more or less pronounced depending of the different dilutions. Smears represented all of the present proteins in fractions.

The smear was more significantly distinct on the liquid fraction with a 2 times dilution than on both solid fractions without any dilutions.

According to this gel results a Bradford assay was made to quantify liquid and solid fraction proteins concentration.

Buffer I		Buffer II	
mg/ml	DO595	mg/ml	DO595
0	0,308	0	0,300
0,2	0,337	0,2	0,330
0,4	0,366	0,4	0,378
0,6	0,388	0,6	0,411
0,8	0,384	0,8	0,474
1	0,426	1	0,507
1,2	0,374	1,2	0,534
1,4	0,511	1,4	0,629

Table 1: Standard OD_{595} values of buffers

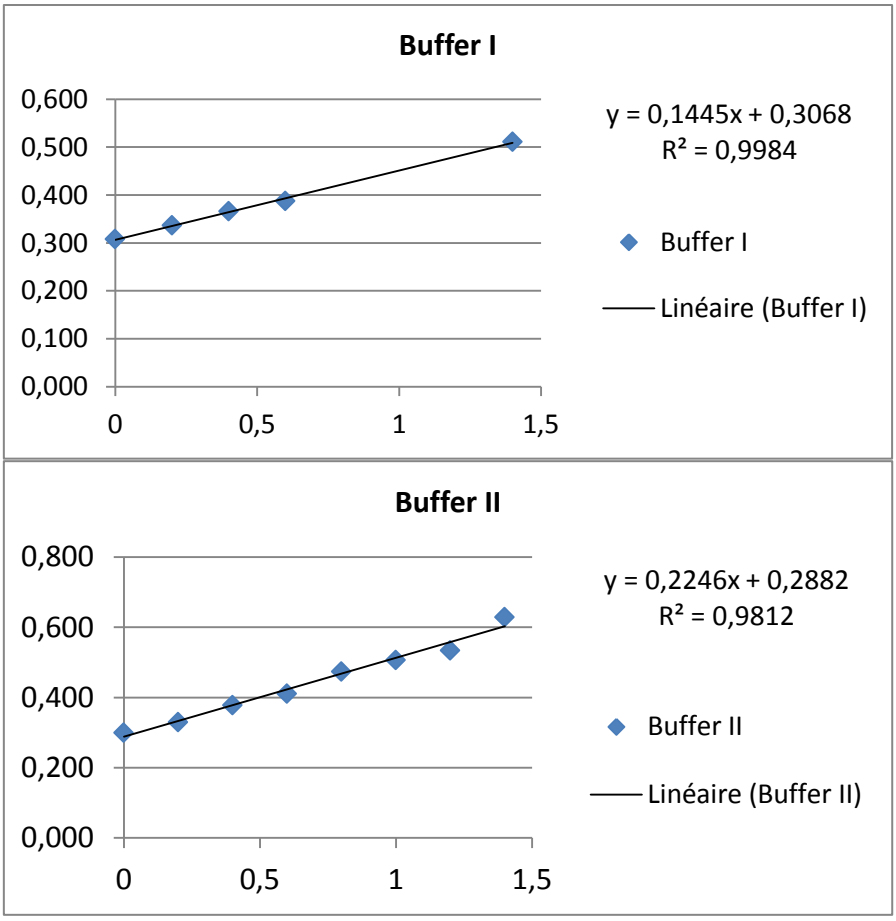


Figure 6: Standard curves of buffers

	Liquid fraction			
	DO595	mg/ml	Before dilution mg/ml	Mean mg/ml
Dilution 5	0.393	0.60	2.98	**2.96**
	0.377	0.49	2.43	
	0.407	0.69	3.47	

	Solid fraction I			
	DO595	mg/ml	Before dilution mg/ml	Mean mg/ml
Dilution 2	0.351	0.31	0.61	**0.65**
	0.351	0.31	0.61	
	0.36	0.37	0.74	

	Solid fraction II			
	DO595	mg/ml	Before dilution mg/ml	Mean mg/ml
Dilution 2	0.374	0.38	0.76	**0.65**
	0.357	0.31	0.61	
	0.352	0.28	0.57	

Table 2: Concentration of solid and liquid fractions depending of their OD_{595} and the standard curve.

The standard curve for buffer I rejected values of 0.8, 1.0 and 1.2 mg/ml because they were too distant from the standard line. Proteins were mostly present in the liquid fraction, with 2.96 mg/ml.

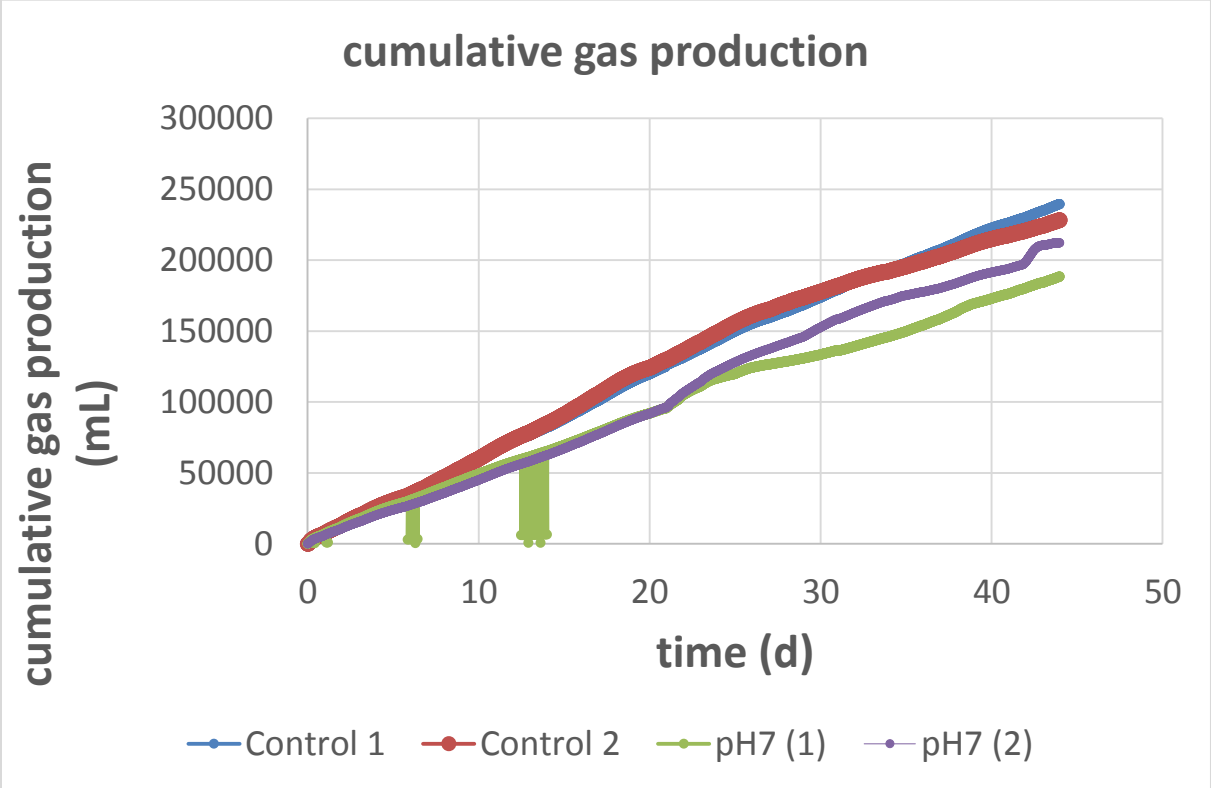
Both of solid fractions had a protein concentration of 0.65mg/ml, 4.5 times lower than in liquids. There was no difference between both of solid fractions, so the addition of SDS and TEAB did not increase the solubilization of proteins.

	Duplicata 1	Duplicata 2	Mean	Protein concentration (mg/ml)
Liquid percentage	82%	79%	80.5%	0.805*2.96 =2.38
Solid percentage	18%	21%	19.5%	0.195*0.65 =0.13

Table 3: Liquid and solid percentage of the digestate.

Following the liquid and solid percentage, the total protein concentration in the digestate is 2.51 mg/ml. It was possible that positive results in the solids for the plate assay and the protein quantification were due to proteins remaining from the liquid. To have more significant results, the same tests should be done on a completely dried solid fraction.

4. Reactors data



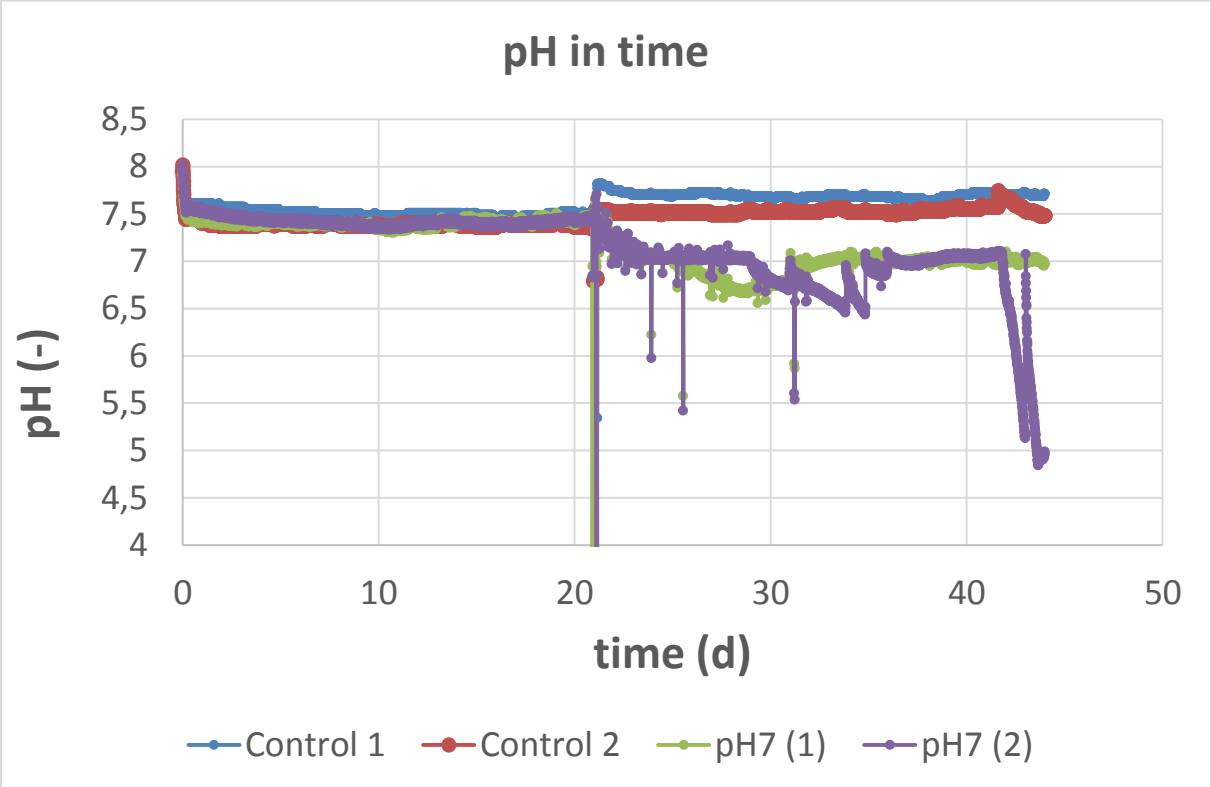


Figure 7: Cumulative gas production and pH of reactors; GB1 & GB2 were pH controlled at 7.0 while GA1 & GA2 were under standard conditions (control)

Reactors with the pH-control had a slightly lower gas production than the control one, 233870 ml of gaz production against 200260 ml for the pH-controlled.

The pH of GB1 & GB2 got lower 7.0 because of an acid addition too important. NaOH 3M was added to reverse the situation, unsuccessfully.

The problem had might be caused by some solid part at the top of the digestate that didn't let the HCl go in the liquid part and be stirred step by step. pH measured by the pH-meter did not change because of this and more acid was added until everything went through in one time, provoking a lower pH than expected. To avoid this problem, pH addition should be done lower, next to the stirring system directly into the liquid part. However conclusions couldn't be made with this experiment, especially because to have significant results, reactors should have been running for 75 days.

Conclusion

Anaerobic digestion is a complex process that needs to be improved in order to be used in biogas production cost-effectively. Hydrolysis being one of the limiting steps, bioaugmentation, pH control and addition of surfactants were selected to improve the hydrolysis yield.

The hydrolytic organisms *C. josui and C. stercorarium* grow under anaerobic and thermophilic conditions, they were selected for their enzymes production and used for the bioaugmentation of digesters. Cultivation showed some sporulation after their exponential phase or under non-optimal conditions, that is why they need to be harvested during their exponential phase to avoid spores formation.

Rhamnolipids are the best candidates for the addition of surfactant since they are from a renewable source, biodegradable and their properties on hydrolytic enzymes are already studied.

Due to operational problems, the pH in the two pH-controlled reactors decreased below 7.0 and the reactors were restarted. Their gas production was lower than in control reactors. The control of pH needs to be improved by a modification of the acid addition point, by being added directly inside the digestate or with a longer time between two acid additions to give enough time to pH to stabilize.

Reactors are currently operated and have to be maintained at steady state for 75 days (3 hydraulic retention times) before being able to interpret results of biogas production and composition, and then know if the overall process of anaerobic digestion was improved.

Hydrolysis rate and yield will also have to be evaluated with total solid and volatile solid analyses, proving if the hydrolysis improvement was achieved or not.

Protein plate assay showed that endo-1,4-ß-mannanases and endo-xylanases are present in both liquid and solid fraction of the digestate. According to these results, an electrophoresis and a Bradford assay were made to quantify protein concentration in digestate. An extraction protocol has to be designed in order to be able to analyze hydrolytic enzymes and supply more information on their secretion and activity during anaerobic digestion.

If the selected conditions are really improving biogas production by an increased hydrolysis rate, they should be tested together and on a full-scale thermophilic digester.

Improvement of anaerobic digestion of the manure would lead to more renewable energy extract from wastes and a better fertilizer for farmers that take back the digestate to fertilize their land.

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