Final Thesis

Evaluation of emergent macrophytes as a source for biogas production after mechanical, alkaline and fungal pretreatments.

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The results showed that emergent macrophytes have a biogas yield similar to other plants already tested (grasses) and commonly used (pasture crops) in large scale reactors. However, emergent macrophytes and grasses cause mechanical problems in a reactor due to their structure. Probably some kind of milling must be done to decrease the fiber length of the emergent macrophytes.
The costs for harvest, transport, handling and possible pretreatment of the emergent macrophytes have to be estimated and included in the overall cost calculations. This can tell if emergent macrophytes should be used as a substrate for biogas production.

Nyckelord
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Alkaline, anaerobic digestion, biogas, fungi, plants, pretreatments, milling, wetland.

Content

1 Abstract

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The methane yield for *Typha latifolia* and *Phalaris arundinacea* was determined to 300 and 323 mL methane per g VS, respectively. There was no statistical difference in methane yield between the two species. Milling pretreatment increased the biogas yield with 16 % by average compared to untreated plant. Alkaline pretreatment with lime increased the biogas yield with 27 % at room temp. and 22 % at 55 °C. The fungal pretreatment decreased the biogas production by 20 % and is probably not suitable for this kind of substrate.

The results showed that emergent macrophytes have a biogas yield similar to other plants already tested (grasses) and commonly used (pasture crops) in large scale reactors. However, emergent macrophytes and grasses cause mechanical problems in a reactor due to their structure. Probably some kind of milling must be done to decrease the fiber length of the emergent macrophytes.

The costs for harvest, transport, handling and possible pretreatment of the emergent macrophytes have to be estimated and included in the overall cost calculations. This can tell if emergent macrophytes should be used as a substrate for biogas production.

Keywords: alkaline, anaerobic digestion, biogas, fungi, plants, pretreatments, milling, wetland.

2 List of abbreviations

AD – Anaerobic digestion.
Degradation of organic material under anaerobic conditions by microorganisms.

BD1/BD4 – Batch digestion test 1 and 4.
Four separate batch digestion tests were carried out and the results presented in this report are from BD1 and BD4.

GC-FID - Gas-chromatography with flame ionization detector.
Machine used for analyzing the methane content of a sample.

OLR – Organic loading rate.
The amount of organic material that is added to a biogas process. Here expressed in g VS per liter working volume .

TS – Total solids.
The weight of an material after water removal.

VS – Volatile solids.
The amount of volatile solids in a material after water and ash subtraction.

3 Introduction

3.1 Introduction

The global need for alternative energy sources is rapidly growing today. Biogas is a biofuel that has many advantages compared to other biofuels. It can be produced from a wide range of organic materials, including many waste products. Common substrates for biogas production are household waste, food industry waste, sewage sludge from waste water treatment plants and agricultural substrates like manure or pasture crops (Ward *et al*., 2008). The biogas can be used to produce electricity and heat or upgraded to biofuel. The rest product from the process can be used as a fertilizer in agriculture. Biogas can therefore be an important component of a system for effective disposal of our waste products and simultaneously produce a highly needed biofuel. However the supply of organic material to the biogas industry must increase if biogas is to become a major alternative to fossil fuels. The biogas industry is constantly looking for new substrates. A possible source of additional organic material could be biomass from highly productive wetlands. Natural or constructed wetlands are often used to reduce the amount of nutrients in nutrient rich water (Eno, 2007). The plants absorb nutrients into their biomass as they grow and contribute to decreasing the amount of nutrients that end up in the target waters. These plants could be harvested instead of leaving the biomass in the wetland to decompose. In temperate climate, wetlands can be harvested two or three times per growing season without lowering the biomass production or nutrient uptake (Geber, 2000). This combination of two environmental measures would strengthen both the incentives for constructing and restoring wetlands and also contribute to the biogas production.

Plant biomass can however be quite resistant to degradation. The external and internal structures of the plant, its lignin content and many other factors determine how well the anaerobic bacteria in the biogas process can digest the plant. Lignin content and the available surface area are considered to be the two most important factors (Taherzadeh and Karimi, 2008; Hendriks and Zeeman 2009). A pretreatment can be used to break the structures and increase the surface area. Many pretreatments have previously been evaluated; mechanical, thermal, acid, alkaline, biological and others (Taherzadeh and Karimi, 2008; Hendriks and Zeeman, 2009). Milling increases the surface area, makes the cellulose more amorphous and has been proven to increase the hydrolysis in most cases with 5-25 % (Hendriks and Zeeman, 2009). Alkaline pretreatment has been proven to be effective; it raise the pH which causes the sulubilization of the hemicelluloses and lignin. The use of lime is a feasible solution for economical reasons, it is also safe to handle and the lime can be recycled. It is therefore very attractive for large scale applications (Saha and Cotta, 2007). Paylostathis and Gosset (1985) reported a 100 % increase in methane yield for *Triticum* spp (wheat) straw pretreated with NaOH, compared to untreated straw.

It seems that the biological pretreatments have not been evaluated as much as the other more conventional pretreatments. Biological pretreatments are mostly carried out with microorganisms. Fungal pretreatment is an enzymatic pretreatment where fungi excrete extracellular enzymes that degrade structural carbohydratates and lignin (Taherzadeh and Karimi, 2008; Montañez-Valdez 2008). An effective fungi is the *Pleurotus spp* of the group

basidiomycetes and the most common species to use is *P.ostreatus.* It has proven to be selective and degrade a higher amount of lignin relative to cellulose than other studied microorganisms (Kerem *et al*., 1992). It is however a slow process that requires long incubation times. Taniguchi *et al.* (2005) concluded that the optimum pretreatment time is 60 days when studying the net yield of sugars after hydrolysis of the pretreated substrate. Taherzadeh and Karimi (2008) concluded that pretreatments with steam, liquid hot water, lime and ammonia are the most promising pretreatments considering economical aspects and other factors together.

Not much has been done to evaluate emergent macrophytes as a biogas substrate before. There are hardly any specific methane yields for emergent macrophytes reported in the literature. Seppälä *et al*. (2009) evaluated the digestibility and biogas yield for a number of grasses, including *Phalaris arundinacea* (reed canary grass). They obtained biogas yields from batch studies very similar to the methods used in this master thesis. The total methane yield for *P.arundinacea* was 296 mL per g VS (volatile solids). There was a big difference in the digestibility depending on the harvest date; a 35 % higher biogas yield was obtained from material harvested in the end of June compared to the end of August. The other grasses in that study gave higher methane yields, most likely because they contained less lignin. This study was performed without any kind of pretreatments. Hu and Yu (2006) evaluated the anaerobic digestion of *Typha latifolia* (common cattail) but did not provide any information about its biogas yield. They had a focus on VS-reduction and obtained a maximum of 66 % and concluded that cattail has a good yield for anaerobic digestion. Geber (2000) evaluated the digestibility of *P. arundinacea* and compared different cutting regimes and cutting heights in a production wetland. Again, only the digestibility of the biomass was evaluated and the author concluded that the digestibility of *P.arundinacea* decreased slightly with more than two cuts per year, and that the plants height of cutting had no effect on the digestibility of the crop. Martins (2009) state that pasture crops are the most similar to wetland plants, and that the biogas yield for pasture crops can give an indication how much to expect from the wetland plants. Pasture crops is a mix of grasses (e.g. timothy) and herbs (e.g. clover), when referred to in this thesis. Methane yield for pasture crops was determined to 340 mL CH_4 per g VS from a study by Lehtomäki (2006**)** and 300 mL per g VS in a review article made by Carlsson and Uldal (2009).

3.2 Aim of study

This master thesis determined the specific methane yield for two species of emergent macrophytes, *T.latifolia* and *P.arundinacea*. They were chosen because of their high production rate as fast growing emergent macrophytes. They establish quickly and are therefore suitable for a production wetland (Fraser *et al*., 2004).

The effect of three different pretreatments were chosen to be evaluated; an alkaline pretreatment with lime, milling (mechanical pretreatment) and a biological pretreatment with *Pleurotus ostreatus* (white rot fungi).

4 Background

4.1 Production Wetlands

Natural and constructed wetlands can be used to capture nutrients and pollutants from eutrophicated waters.(Eno, 2007) The plants absorb both nitrogen and phosphorus which are identified as the main cause for eutrophication of our waters. The plants also absorb pollutants like heavy metals in their biomass. Wetland release nitrogen gas into the atmosphere when anaerobic bacteria perform denitrificaton in the sediments. The sediments themselves capture phosphorus, heavy metals and other pollutants through sedimentation, precipitation and sorption reactions. These processes can be optimized in a constructed wetland when having the opportunity to control water depth, water flow and species composition. As a result, the phytodepuration can be more efficient across the entire wetland bed (Barbera *et al*., 2009).

4.1.1 Constructed wetlands for biomass yield

A production wetland is a constructed wetland that is adjusted to enhance a rapid growth, efficient nutrient uptake and high biomass production. In a natural wetland, the plants will decompose and the nutrients captured in the biomass will be released again. The purpose of a production wetland is to harvest the biomass instead and use it for biogas production or other benefits. In temperate climate, wetlands can be harvested two or three times per growing season without lowering the biomass production or nutrient uptake, depending on the species compositional changes (Geber, 2000).

4.1.2 Design and structure

A production wetland should be harvested, therefore a solution where the wetland is temporary flooded with water is optimal. The water can be kept inside the wetland by a river bank and released when needed. The wetland can then be harvested with normal agricultural machines and urban discharges which would keep down the cost of harvesting. A suitable site can be next to a river that is rich in nutrients from waste water and occasionally flooded. The dryer parts along the edge of the wetland can act as a barrier between the wetland and the surrounding cropland. (Eno, 2007)

4.1.3 Species and plantation

The species suitable for a production wetland are mostly large emergent macrophytes. The species must grow and spread rapidly and establish easily after seeding or transplanting. Species like *T.latifolia*, *Phagmites australis*(common reed) and *Glycera maxima* (reed manna grass) are suitable in permanently flooded, nutrient rich wetlands while species like, *P. arundinacea* or *Carex sp.* (large ruches species) dwells in temporary flooded areas. According to Barbera *et al*. (2009) the most commonly used plants in constructed wetlands today are: *P. australis*, *Juncus spp* (rushes), *Scirpus spp* (bulrushes), *Typha angustifolia* (narrow-leaved cattail), *T.latifolia*, *Iris pseudacorus* (yellow flag), *Acorus calamus* (sweet flag), *G. maxima* and *Carex spp*.

4.2 Anaerobic digestion

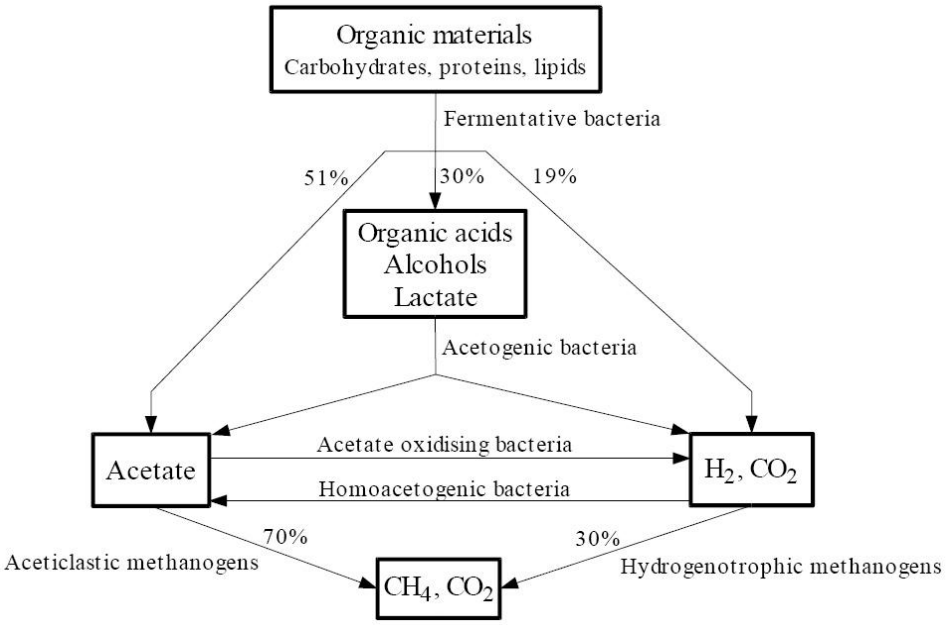

Figure 1. Carbon cycle for the biogas process. Modified after Kanokwan 2006.

In the biogas process, bacteria digest organic matter under anaerobic conditions and produce methane and carbon dioxide as final products. It is a multi-step reaction where at least three groups of different bacteria are involved. The process is often divided into four main steps (Fig. 1).

Hydrolysis. The first step is called the hydrolysis, where hydrolytic bacteria excrete enzymes that break insoluble polymers to soluble oligomers and monomers. In this step carbohydrates are converted into sugars, lipids are broken down to fatty acids and proteins are split into amino acids. The hydrolysis is often known to be the rate limiting step in the anaerobic digestion(AD) process (Kanokwan, 2006).

Acidogenesis. The smaller units from the hydrolysis can be taken up through the bacterial cell wall and are digested in the second step by acidogenic fermentative bacteria. Some of the products from this fermentation (mainly acetate, CO_2 and hydrogen) can be utilized directly in the fourth step; methanogenesis. Products like alcohols and volatile fatty acids on the other hand must be oxidized in the third step by acetogenic bacteria (Yadvika *et al*., 2004; Kanokwan, 2006).

Acetogenesis. The acetogenic step is where alcohols and volatile fatty acids produced during the fermentation are converted to acetic acid, carbon dioxide and hydrogen, which is used in the last step (methanogenesis). These acetogenic bacteria live in symbiosis with the methanogenic organisms (Yadvika *et al*., 2004).

Methanogenesis. This is the final step where methanogens convert acetic acid, hydrogen and CO_2 to methane, CO_2 and some trace gases (Yadvika *et al*., 2004).

4.3 Lignocellulotic structure

The structural units and cell walls of higher plants make them resistant to enzymatic attacks and therefore harder to degrade. This may cause difficulties in the first step of the biogas process (*hydrolysis)*. The possibility to degrade a plant depends much on the cell wall structure and relative content of its different components. Below is a description of the main components of the plant cell wall:

Cellulose. Cellulose is built up by D-glucose molecules that are bound together into linear strains. These strains bundle together and form so called "microfibrills" which is the backbone of the cell wall. These microfibrills have both crystalline and amorphous regions. The proportion of the amorphous regions varies from plant to plant. Studies have shown that more amorphous area increases the available surface area and favors the degradation (Taherzadeh and Karimi, 2008).

Hemicellulose. Hemicellulose is a complex structure built up from different kinds of carbohydrates. The hemicelluloses act as glue between the cellulose and are not supportive in itself. The carbohydrates in hemicelluloses are highly soluble and are therefore the most sensitive part of the lignocellulotic structure. The solubility depends on factors like temperature, moisture content and pH. The key to unlock the lignocellulotic structure is to dissolve the hemicelluloses (Hendriks and Zeeman, 2009).

Lignin. Lignin is a very branched, amorphous and insoluble structure that is cross linking the hemicelluloses, filling the gaps between the cellulose. Lignin has many different functions in the plant; to give it rigidity, support water transportation and a support against microbial attacks. Thus, lignin is what makes lignocellutotic biomass hard to degrade and inhibits the hydrolytic step (Caffall and Mohnen, 2009).

4.4 Lignocellulotic biomass

Plant biomass with relatively high content of lignin is called lignocellulotic biomass. The mix of herbs and grasses in pasture crops are relatively easily hydrolyzed and degraded. Martens *et al.* (2004) determined the lignin content of a pasture to be around 4.4 %. Hardwood species however are much more complicated to degrade. The lignin content in three hardwood species as determined by Bose *et al*. (2009), where *Populus deltoids* (eastern cottonwood), *Betula sp* (birch) and *Acer sp (*maple) had a lignin content of 17.7, 21.5 and 25.0 % of TS respectively. Emergent macrophytes in general have a lower lignin content than hardwood species but higher then pasture crops. Hu and Yu (2005) found that the lignin content of *T. latifolia* was 10.5 % of total solids. Bridgeman *et al.* (2007) found that *P. arundinacea* contained between 7.1 and 9.4 % lignin.

4.5 Limiting factors in lignocellulotic degradation

There no consensus on what is the most limiting factor for degradation of lignocellulotic biomass (Taherzadeh and Karimi, 2008). The available surface area has, however, been defined as important as it determines how well the hydrolytic enzymes can get into the material and the cellulose. One important factor linked to this is the crystallinity of the cellulose; more amorphous cellulose increases the surface area and makes it easier to degrade. The pore size of the substrate in relation to the enzyme size is also important and decides how far the enzymes can penetrate into the material itself. The degree of polymerization in the cellulose structure is another factor which is being discussed; longer polymers are harder to degrade. Also moisture content can have a considerable impact, optimum water content has been determined to be 30 % of maximum fiber saturation (Hendriks and Zeeman, 2009). The % lignin of the total biomass is however the most recognized factor when it comes to limitations. The removal of lignin makes it possible for enzymes to get to the cellulose and to dissolve the hemicelluloses (Hendriks and Zeeman, 2009).

5 Materials and Methods

5.1 Collection of plants

Figure 2. The plants were collected from this small wetland situated in southern Sweden.

The plant material used in this experiment were collected from a small wetland in Roma, Gotland, Southern Sweden (Figure 2). The wetland is part of a wetland system that has previously been used to trap excessive nutrients in water from a sewage treatment plant. The harvest was done the 15th of June 2009. The plants were cut by a machete at around 10 cm stubble heights and dried in the sun for 14 days. The dried plant material was cut by scissors to 1x1 cm pieces.

5.2 Experimental set-up

5.2.1 Basic method description

Evaluations of biogas wields were carried out in batch digestion tests. The material to be evaluated was placed in glass bottles together with water, nutrient solution and inoculum. The bottles were prepared under anaerobic conditions and placed in a 37°C climate room for approximately six weeks. Measurements were performed (2-3 times per week during the first weeks, less frequent later in the experiment) to monitor the biogas and methane production. This method gives the methane yield for the substrates as well as a rough estimation of the methane production rate of the process during the test period.

Four separate batch runs were carried out to evaluate the two untreated plants and the three different pretreatment methods (Table 1).

Table 1. An overview of the four batches and the respective pretreatment evaluations. Every treatment was evaluated in triplicate and for both plant species, except batch four that was carried out with only T.latifolia.

Batch	**Untreated**	**Milling**	**Alkaline. Incubated 24h**		**Fungal. All three incubated 37 days.**			**Bottles**
			Room temp.	55 °C	Untreated	Sterilized	Applied Fungi	
Batch 1	X	X						21
Batch 2					X	X	X	27
Batch 3			X	X			X	31
Batch 4	X	X	X	X	X	X	X	30

Figure 3. Batch digestion bottles after preparation.

5.2.2 Batch digestion bottle preparation

In the experiment 330 mL serum bottles sealed with rubber stopper and Al-screw caps were used (Figure 3). Open bottles and containers were continuously flushed with N2-gas to obtain an oxygen free environment. The following procedure was done for all substrate bottles:

- Substrates were added (see *5.2.3* for detailed description of substrates and respective amounts).
- 2 mL of nutrient solution was added to each bottle, containing: NH_4Cl (0,3g/l), NaCl (0,3g/l), $CaCl_2 * 2H_2O$ (0,1g/l), $MgCl_2 * 6H_2O$ (0,1g/l). This is to provide the microorganisms with the basic nutrients they need.
- 20 mL of inoculums (see *5.2.4* for more information about the specific content) was added.
- Boiled ultra pure water (MilliQ) was added to obtain a working volume of 100 mL in all bottles. The bottles were then sealed.
- The gas phase was changed to a mix of $N_{2(}$ (80%) and CO_2 (20%).
- The last step before incubation was to add 0.3 mL 100mM Na_2S.

Three sets of controls were included in every batch and treated in the same way as the other bottles.

- Three bottles containing no substrate, only the inoculum. The methane production from these bottles were subtracted from the methane production of the substrate bottles so that the biogas yield of the substrates don't include gas production from the inoculum.
- Three bottles containing 0.5 gram VS of pure cellulose (filter paper) cut in pieces of 1x1 cm. These bottles are regarded as positive controls and give an estimation of the inoculums degradation capacity .
- Three bottles containing no substrate and no inoculum but only 15 mL of methane. This serves as a control for a known amount of methane when analyzing methane content on a GC-FID (Gas-chromatography with flame ionization detector).

All bottles were placed in a 37°C climate room.

5.2.3 Substrates and VS contents

The TS content of dried *T.latifolia* and *P.arundinacea* was determined to 88 % and 90 % respectively. This was determined by incubation at 110 °C for 20 h. The VS of TS content was determined to 80 % and 83 % of dried plant material respectively. This was determined by incubation in a muffle furnace at 550°C for 2 h. In batch 1-3 an organic loading rate of 2.5 g VS per liter of working volume in the bottles was used. This showed to be insufficient due to high production from the inoculum control. The OLR was therefore raised to 4g VS per liter in batch 4 (see discussion for method development).

5.2.4 Inoculum

An inoculum was added to supply the process with the necessary microorganisms. The inoculum used was reactor liquid from small scale semi-continuously stirred 5 liter reactors. The inoculum added to the bottles was a mixture of material from reactors running on sewage treatment plant sludge, manure and sludge from paper- and pulp industry. However the mixture was not identical in every batch and the different inoculum properties is a variable that can have affected the results. The TS content of the inoculum varied between 3.3 % and 5.8 % in the different batches. The VS content varied between 2.4 % and 4.0 %.

5.2.5 Measurements

The batch bottles were monitored for biogas production and methane content over the whole test period. In the beginning of the test, production was high and the measurements were taken frequently. About three times per week for the first two weeks, two times per week the two weeks after that and one time per week for the two last weeks.. The same sampling procedure was used at all times:

- The pressure of each bottle was measured using a 10 mL glass syringe. The obtained value was used to calculate how much biogas that had been formed since the last measurement.
- A 1 mL sample was taken out from the 330 mL bottle and injected into a 30.7 mL glass vial.
- The overpressures of the incubation bottles were released.

3 x 0.3 mL of the diluted sample was injected on a GC-FID to determine % methane in the gas phase in the incubation bottle.

5.2.6 Calculations

A standard curve was prepared by injecting samples from three bottles containing 0,07, 0,63 and 1,71 % methane were injected into the GC-FID prior to every measurement of methane content. A standard curve was created so that the values from the GC-FID could be transformed to methane content percentage. This percentage was multiplied with 30.7(the volume of the vial) to obtain the percentage in the actual batch bottle. The methane content of the bottle at the point of measure was compared with the methane content of the bottle after release of overpressure at the last point of measure. In this way the newly formed amount of methane could be calculated and added to the amount methane accumulated over time. The methane production from the inoculums was subtracted from the substrate bottles at every point of measurement before adding it to the accumulated methane production.

5.3 Pretreatment methods

5.3.1 Milling

The dried plant material was milled using a "TECATOR Cyclotec 1093 Sample mill" to 0,5 mm mesh size.

5.3.2 Alkaline pretreatment

A strongly alkaline solution was prepared mixing ultra pure water (MilliQ) and $Ca(OH)_2$ to a concentration of 67,5 mM. This solution was distributed into six 100 mL beakers with 10 mL in each. 0.5 g of dried plant material was added to every beaker and pH was measured using a pH meter (PHM 93 Reference; Radiometer Copenhagen). Values around pH 12.6 were obtained. The beakers were stirred by hand until the plant material had absorbed the water and then incubated for 24 h. Three were incubated in room temperature and three in 55 °C. The pH was adjusted to 7 after the incubation using 0,1 M HCl.

5.3.3 Fungal pretreatment

Prior to the preparation of the incubated bottles a strain of *Pleurotus ostreatus* was cultivated on agar for about two weeks at 30°C.

6 grams of plant material was added to 250 mL E-flasks and filled with ultra pure water. The plant material was saturated and the excessive water was poured out. Four different treatments were carried out in the four E-flasks:

- **Incubation:** The E-flask was plugged with a cotton plug to allow for oxygen diffusion (Figure 4).
- **Incubation + applied fungi:** Two agar plugs of the mycelia from *Pleurotus streatus* were added to the material in the E-flask. The agar plugs were meshed and stirred slightly to get an even distribution of mycelia in the bottle. The agar residues were removed from the bottle after the incubation period (Figure 5).
- **Sterile incubation:** This flask was sterilized in an autoclave for 20 min at 2 atm before incubation (Figure 6).
- **Sterilized incubation + applied fungi.** Sterilization in autoclave as for treatment 3 above followed by applied fungi as for treatment 2 above (Figure 7).

This pretreatment was done in a 30°C climate room over 46 days. Sterile water was added to the bottles to maintain equal moisture content. 2 mL of water was added at tree occasions, spread out over the time period. The autoclaved E-flask lost more water in the beginning and therefore got 5 x 2 mL of water. Figure 4 show the content of the flasks looked after 46 days incubation before they were taken out:

A.

B.

C.

D.

Figure 4. The four different treatments in the fungal experiment. A – incubation. B – incubation + appl. Fungi. C - Sterile incubation. D - Sterile incubation + appl. fungi.

5.4 Statistics

Independent t-test were carried out to determine if the difference between plants and pretreatments where statistically significant. This was done both after an 11 day period in the batch digestion test and for the last point of measurements at either 37 or 44 days. This was done using the statistics program SPSS.

In order to use more data than a single point of measurements on triplicates, a repeated measures analysis is advantageous. However the different dates of measurements are not independent of each other. Therefore Hodges Leehman estimation was carried out as it is more robust to independency problems. (Helsel and Hirsch, 1992). A set of measurements points along the graph was chosen, where the production had leveled out, temporary or terminal. All triplicates for the chosen points of measurements were then compared to all corresponding data points of the other treatment. An estimation whether the difference between the two datasets were significant of not was obtained.

6 Results

In this section only the results from BD1 and BD4 are presented due to methodological problems that arose during BD2 and BD3. Method improvements are discussed in chapter 7.

6.1 Plant methane yeild

The total methane production for *T.latifolia* in BD1 was about 220 mL methane per g VS after 11 days and 300 mL after 37 days.(Fig. 5) The total methane production for *P.arundinacea* in BD1 was about 210 mL after 11 days and 320 mL after 37 days. There was no statistically significant difference between the species at either 11 days (p=0.73) or 37 days (p=0.43). Hodges leehmann estimation confirms that there is no statistical difference between day 18 to 37.Therefore only *T.latifolia* was used in BD4.

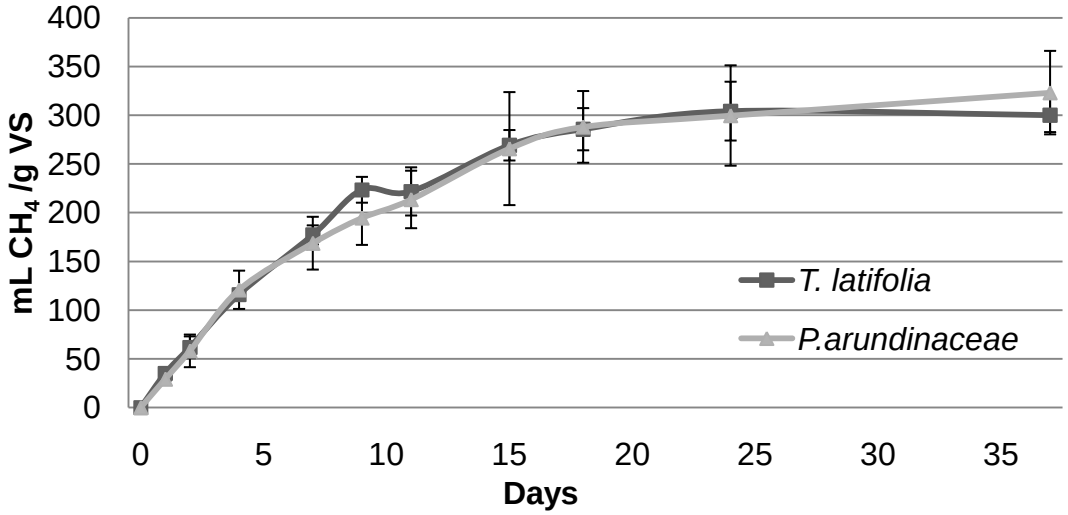

Figure 5. Mean methane production (n=3) for T.latifolia and P.arundinace in BD1 (Batch digestion 1) over 37 days. Error bars show standard deviation.

In the incubation BD4, the total methane production for *T.latifolia* was about 190 mL after 11 days and 270 mL after 37 days.(Fig. 6) The difference from BD1 is likely due to different content and properties of the inocula used. The paper control could not be used to adjust the results to each other, due to different kind of papers used in the different batch digestion tests.

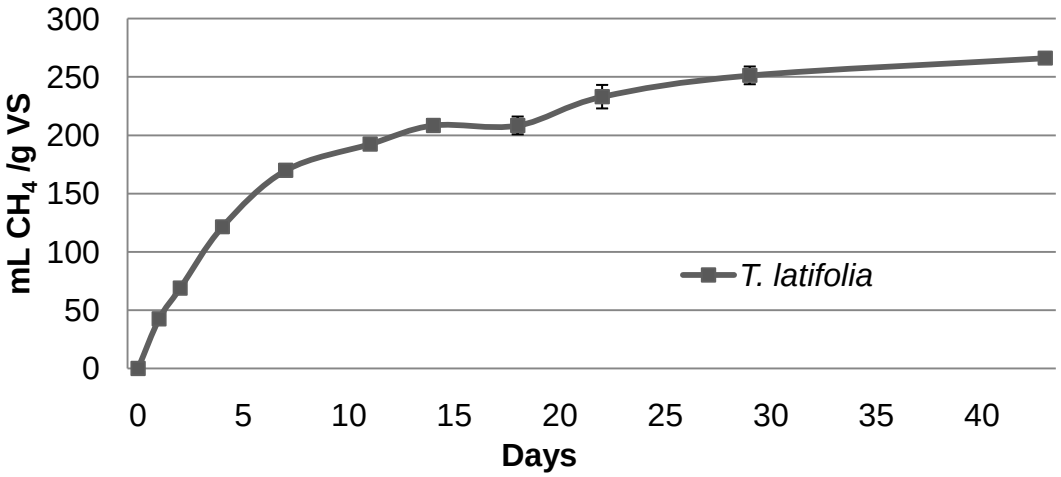

Figure 6. Mean methane production (n=3) for T.latifolia in BD4 over 44 days. Error bars show standard deviation.

6.2 Milling Pretreatment

Milling pretreatment significantly increased the methane production, by 22 % after 11 days (P = 0.047) and 16 % after 38 days (P = 0.013) in BD1(Fig. 7). Hodges Leehman estimation confirms that the difference was significant between days 18 to 37.

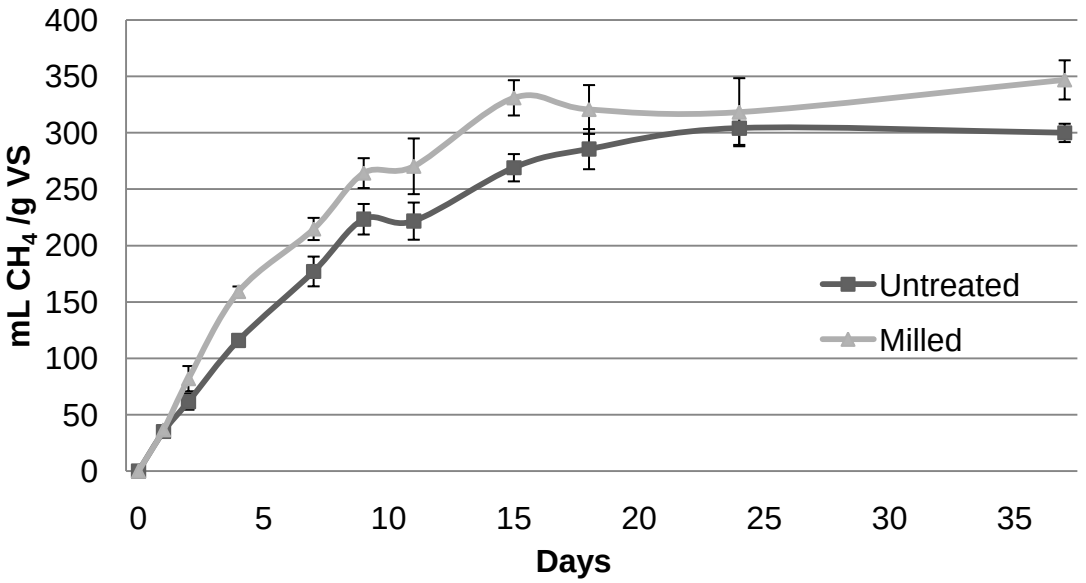

Figure 7. Mean methane production (n=3) for untreated and milled T.latifolia in BD1over 37 days. Error bars show standard deviation.

The effect of milling was also seen in BD4, where the yield was 11 % higher after 11 days (P = 0.047) and 3 % after 44 days (P = 0.016). Hodges Leehman estimation confirmed that the difference was significant between days 11 to 18 (Figure 8).

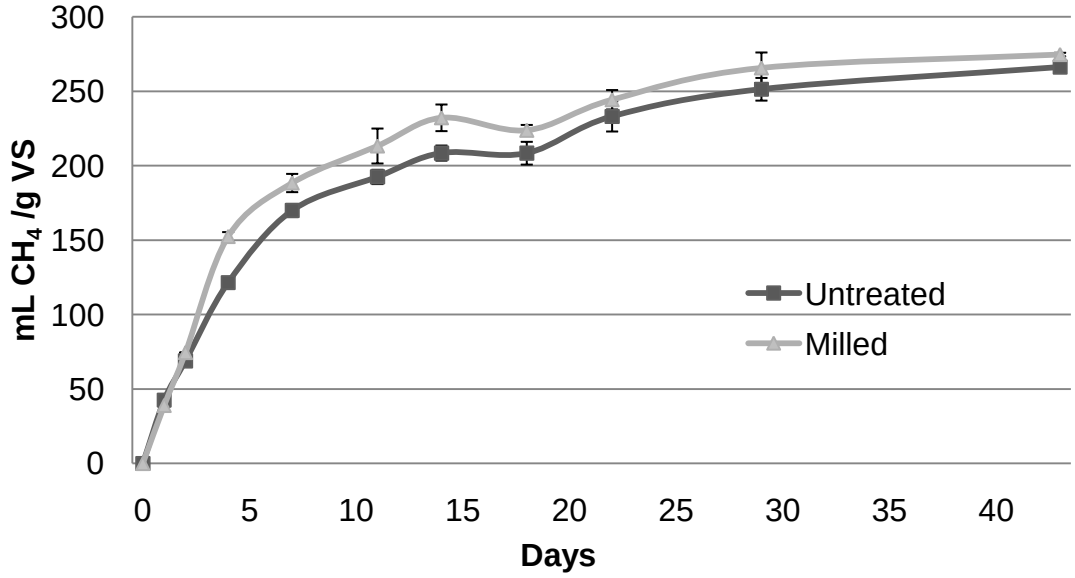

Figure 8. Mean methane production (n=3) for untreated and milled T.latifolia in BD4 over 44 days. Error bars show standard deviation.

6.3 Alkaline pretreatment

Alkaline pretreatment at room temperature increased the methane yield by 27 % after 11 days and 7 % after 44 days (Fig 9). The difference was significant after 11 days (P = 0.009) but not after 44 days (P = 0.154). Hodges Leehman estimation indicated significant differences between both day 11-18 and day 22-44.

Alkaline pretreatment at 55 °C increased the methane yield by 22 % after 11 days and decreases the methane yield with 3 % after 44 days. The difference to was significant after 11 days (P = 0.009) but not after 44 days (P = 0.642). Hodges Leehman estimation confirm that the difference between day 11 and 18 was significant but not day 22 to 44.

No significant difference between the two temperature treatments after either 11 days (P = 0.595) or 44 days (P = 0.253).

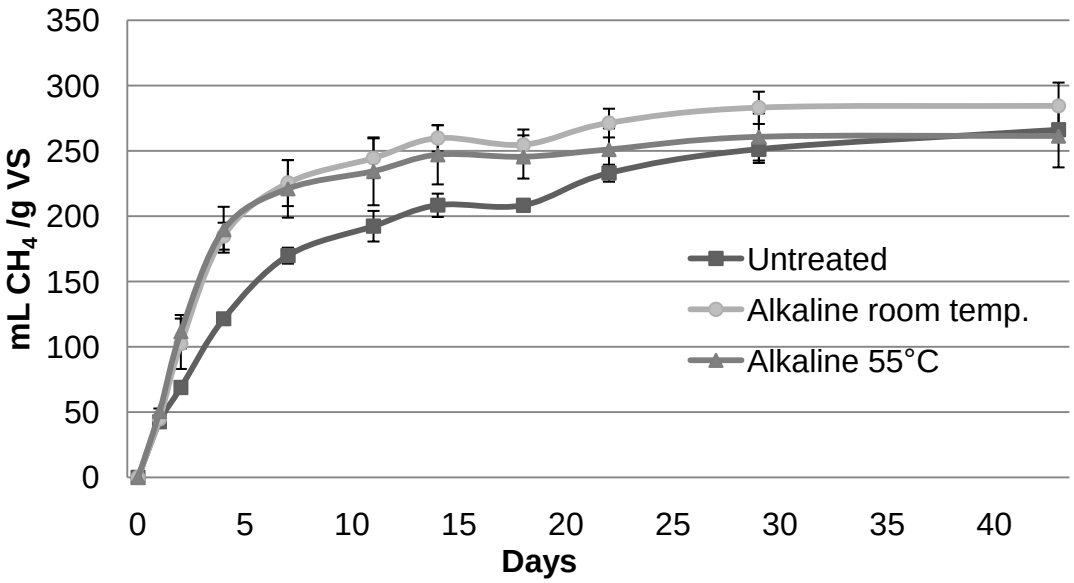

Figure 9. Mean methane production (n=3) for untreated and alkaline pretreatment in BD4.Alkaline pretreatment at room temp. and 55 °C; both incubated with lime for 24 h. Error bars show standard deviation.

6.4 Fungal pretreatment

Plant material that was incubated, sterilized and applied with white rot fungi decreased the methane production by 20 % after 11 days and 27 % after 44 days (Fig 10). The difference to untreated material is significant after both 11 days ($P = 0.005$) and 44 days ($P > 0.001$). Hodges Leehman estimation confirms that the difference between day 14 to 43 is significant.

Plant material that was incubated but not sterilized had a lower methane yield than untreated plant material, 59 % after 11 days and 54 % after 44 days (Fig. 10). The difference is significant both after 11 days ($P > 0.001$) and 44 days ($P > 0.001$). Hodges Leehman estimation confirms that the difference between day 14 to 43 is significant.

Plant material incubated and sterilized increased the methane production by 2 % after 11 days and 19 % after 44 days (Fig 10). The difference to untreated material is not significant after 11 days ($P = 0.684$) but after 44 days ($P = 0.016$). Hodges Leehman estimation confirms that the difference between day 22 to 43 is significant.

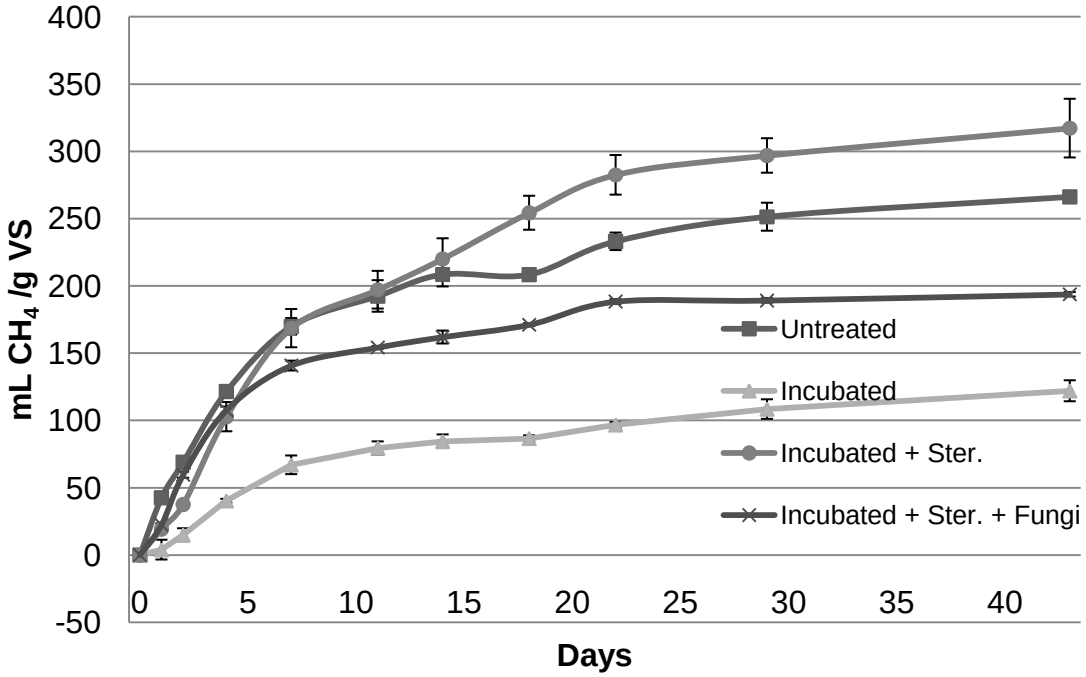

Figure 10. Mean methane production (n=3) for untreated and incubated pretreatments in fungal experiment at BD4 over 44 days. Untreated has not been incubated as the others (30 °C for 46 days). Two of the incubated treatments has been sterilized and one of them also applied with white rot fungi. Error bars show standard deviation.

7 Discussion

7.1 Plant methane yield

The final methane yield for *T.latifolia* was 300 mL (BD1) and 270 mL (BD4) CH_4 per g VS. The final methane yield for *P.arundinacea* was 320 mL. That is slightly higher than Sepälä *et al*. (2009) who obtained 296 mL from *P.aruninaceea.*Those authors also obtained methane yields for *Dactylis glomerata* (cooksfoot), *Festuca arundinaceae* (tall fescue) and *Phleum pratense* (timothy) that were 342, 336 and 335 mL, respectively. Thus, it seems as the emergent macrophytes evaluated in the present study do not differ much from other grasses in terms of biogas yield. Some grasses have already been tested for anaerobic digestion in large scale applications (Prochnow *et al*, 2009). However, pasture crops seems to be preferred before grasses mainly due to the more problematic structure of the grasses. Studies have shown that pasture crops contain less fibers and are softer plants(Carlsson and Uldal, 2009). The methane yield for pasture crops was about the same as for grasses, 340 mL (Lehtomäki, 2006) and 300 mL (Carlsson & Uldal, 2009). The results for emergent macrophytes gives an indication that they can be used in the same way as pasture crops, as long as some kind of milling is carried out, and probably co-digestion with other substrates would be a good way to secure substrate availability, improve the nutrient balance and consistency of the whole process (Martins, 2009). Manure is an abundant substrate at farm scale reactors, and can be more economically feasible to use if co-digested with energy rich crops (Seppälä *et al.*, 2009). Linné et al. (2008) list the methane yield for cattle manure to around 170 mL methane per g VS. They also refer to co-digestion studies where there in many cases has been an increase in methane production compared to individual digestion of the substrates. Sewage sludge and food waste gave a 6 % increase, potatoes and sugar beet bagasse gave an increase of 60 % due to the superior nutrient balance. The present study showed that emergent macrophytes could be one of the candidates for such co-digestion as long as it is economically feasible to harvest them.

7.2 Pretreatments

Milling pretreatment increased the biogas yield with 11% and alkaline with 25 % (average for both temperatures) in BD4. Fungal pretreatment decreases the biogas yield with 20 % and also lost a lot of organic material during the pretreatment (40-50%). It is clear that the alkaline pretreatment is the most effective in this case, but milling is probably the most necessary pretreatment for practical reasons when applied in large scale reactors. The fungal pretreatment should obviously not be used on this kind of species. Economical assessments and further studies considering the costs and use of the alkaline and milling pretreatments can tell which one that is most effective in reality.

7.2.1 Milling pretreatment

Milling increased the biogas yield in both BD1 (22%) and BD 4 (11%). This is in correspondence with previous results for lignocellulotic material. Milling is however very energy demanding, at least with the techniques used today, and it is likely that it is not economically feasible (Taherzadeh and Karimi, 2008). However, if the plants material would be applied untreated to a large scale biogas reactor, it could be troublesome. Grasses and similar substrates often twine around mechanical stirring parts and also float on the surface of the reactor liquid (Prochnow *et al* 2009). The untreated plant in this thesis was cut to pieces of 1x1 cm, and some cutting of the plants is probably necessary to avoid this kind of problems in large scale. Practical issues and economical benefits must be evaluated in order to know if, or to what mesh size, milling should be applied.

7.2.2 Alkaline pretreatment

Alkaline pretreatment with lime incubated for 24 h increased the yield with 27 % and 22 % (11 days; room temp and 55 °C respectively). The difference between the temperature treatments was however not significant. That lime had an effect on the methane production is in agreement with previous studies of alkaline pretreatments (Lehtomäki, 2006). Taherzadeh and Karimi (2008) stated that alkaline pretreatment works well even in low temperatures and the results from this study confirm that statement. Still, an economical assessment of chemical costs must be done. In this study a pH of 12.6 was used. Different pH could be evaluated is future studies to see what pH minimum that would be needed to obtain a clear positive effect.

7.2.4 Fungal pretreatment

In contrast to the milling and alkali treatments the fungal pretreatment did not increase the biogas yield, instead it decreased. 40 to 50 % of the organic material was consumed by fungal respiration during the pretreatment incubation period. The biogas yield from unsterilized incubated plant material was 59 % lower than the untreated plant (11 days). It is clear that if the material was not sterilized; other microorganism dwelled in the material and consumed the most easily available carbohydrates. Montanez *et al*. (2008) described a process where easily digestible carbohydrates are converted to simple sugars. This is called *fungus primary metabolism* and explains the low biogas production after pretreatment in the present study. The added white root fungi could apparently not compete and did not dominate among the other fungi and microorganisms when the material was not sterilized. The biogas yield was 20 % lower after 11 days compared to the untreated plant material even if it was sterilized before the application of white rot fungi, so the pretreatment did not work. However, the plant material that was only sterilized and with no added fungus produced 19 % more biogas than the untreated plant. The reason for this is probably that autoclaving at high temperature and pressure works as a pretreatment in itself. But to apply sterilizing on a large scale would be very energy demanding and obviously not beneficial in this case with emergent macrophytes, unless massive amounts of excess steam is available from some other industrial process. Fungal pretreatment is probably a more beneficial method when applied on species that contains more lignin, for example hard wood species and residues from saw mills.

7.3 Methodological problems and improvement

BD2 was supposed to evaluate the fungal pretreatment. The pretreated plant material was mixed together with water in order to obtain a homogenous substrate. However, the material from the different treatments needed different amount of time in the electrical mixer to obtain a homogeneous texture. Some treatments received up to approximately four times more impact from the mixer. This is a major source of error. It was resolved in BD4 by not using the mixer; the material was instead put directly in the incubation bottle as was the untreated.

In addition to this, the amount of organic material added to the bottles became too low in BD2. The calculated rough weight that was added to the bottles before the exact VS/TS analysis was based on the organic content before the pretreatment. The loss of organic material during respiration was thus not taken into account. As said above, this loss was about 40-50 % resulting in an organic loading per liter working volume of about half of the target load (2.5 g/L). This was resolved in BD4 by doing a VS/TS analysis only three days prior to the experiment. In that way the actual VS/TS content was the same as the calculated one used when adding the substrate.

BD3 was supposed to evaluate the alkaline pretreatment as well as re-examine the fungal pretreatment. However, the inoculum in this batch had a very high methane yield compared to that of the previous experiments. This gave a high "methane background" thus making it hard to distinguish the production from the substrates added. The small difference between the controls and substrate amended bottles resulted in methane production values with very high standard deviations. This was resolved in BD4 by raising the organic load of substrate from 2.5 to 4g VS/ L working volume.

This demonstrated that the inoculum is a factor that makes each batch vary in production slightly, which makes comparisons between batches (and other studies) hard. Therefore the untreated plant material was re-digested in BD4 together with all other treatments for better comparisons.

7.4 Conclusion and future prospects

The two emergent macrophytes seem to have methane yields around 300 mL per g VS in anaerobic digestion. The yield is similar to that of other grasses already tested in large scale biogas reactors. Pasture plants are mostly used in practice because of more suitable fiber lengths and structure. Emergent macrophytes probably need some kind of milling, but this thesis has shown that the biomass can generate the same amount of biogas as, for example, pasture crops. If used, they would probably be co-digested with sludge or manure in farm scale reactors. Economical assessments now need to be made considering the harvest, transports, reactor usage, value of rest products and other factors. This can tell if it is worth using emergent macrophytes for biogas production.

Milling and alkaline pretreatment increased the biogas yield by about 15 and 25 %, respectively, but the pretreatment must be cost effective and energy efficient when applied in a large scale. Common obstacles are high consumption of electricity/heat, high cost for chemicals or a high water demand (Taherzadeh and Karimi, 2008). Economical assessments

need to be done in order to know if it is worth applying any pretreatment. Further studies can show how small milling size that is necessary and at which pH alkaline pretreatments could be conducted. Both the fine milling size and the high pH in this study was shown effective but could be very costly in large scale. When it comes to the fungal pretreatment, is not worth doing for this kind of plants and a waste of energy since it did actually decrease the biogas yield. The effect on hardwood species is still unknown and could be evaluated in future studies.

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