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Enhanced methane productivity from manure fibers by aqueous ammonia soaking pretreatment

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Abstract
The necessity of increasing the methane productivity of manure based biogas plants has triggered the application of anaerobic digestion to the separated solid fraction of manure, with the challenge that its high lignocellulosic fibers content is difficult to digest and thus makes anaerobic digestion process slow and economically unfavourable. In the present study, aqueous ammonia soaking (AAS) was investigated as a pretreatment method to increase methane potential of swine manure fibers. 3 days at 22°C were the optimal conditions among the ones tested (1, 3, and 5 days at 22 and 55°C) for increasing the methane potential of manure fibers. AAS pretreatment exhibited a significant effect on methane production rate and potential. It was found that AAS for 3 days at 22°C resulted at a 30-80 and 178% increase in methane yield from digested and raw manure fibers, respectively. Batch anaerobic digestion of AAS-treated
digested manure fibers could stand loadings as high as 100 g TS/l inoculum with no inhibition problems. Enzymatic hydrolysis tests applied to AAS-pretreated fibers resulted to 80 and 65% hydrolysis efficiency of glucan and xylan compared to insignificant numbers for non-pretreated fibers confirming thus that AAS effect on methane yield and production rate is due to the facilitation of hydrolysis step of anaerobic digestion process. This is attributed to AAS directly affecting the disintegration step and thus releasing carbohydrates, which can be further hydrolysed, from the lignocellulosic matrix.

Keywords: anaerobic digestion; aqueous ammonia soaking; manure fibers; methane potential; pretreatment.
1. Introduction

Biogas production and utilization has become a major part of the rapidly growing renewable energy sector. In recent years, biogas technology has progressed significantly and its application has experienced an explosive growth worldwide in municipalities, industry and agriculture. Biogas is arguably a versatile renewable energy source due to its determinate energy value and ease of storage, hence, potential utilization is significantly independent of factors such as geographical location and season. It can be used directly for heating and electricity generation and as substitute for fossil fuel applications, e.g., transport fuel [1]. Currently, most agricultural biogas plants are used to ferment liquid manure [2] due to several advantages; anaerobic digestion is based on microorganisms already existing in manure and therefore methane production occurs naturally. Additionally, production of biogas from manure results in much higher reduction in greenhouse gas emission than other biofuels producing processes, such as bioethanol, and equals savings in fossil fuels. As cost aspects point to the same direction, manure based biogas should have higher priority than other biofuels [3].

Denmark is one of the largest producers of pig meat and the use of swine manure in biogas production is a common practice. Swine manure, when not properly treated, results in pollution of the environment. Anaerobic digestion is considered as an efficient and cost-effective treatment capable of reducing organic content of swine waste effluents while producing energy from methane valorisation [4]. However, biogas plants digesting liquid manure alone are not economically viable due to the relatively low organic content of the manure, usually 3-5%. Therefore, current biogas production in Denmark is based on the codigestion of at least 75% animal manure and up to 25%
other (additional) biomasses characterized by high methane potential, such as slaughterhouse wastes, glycerine, crops, animal fat, fish oil, etc. The addition of this type of biomasses increases the methane efficiency and thus the process profitability [3]. However, due to the increased demand for biomass feedstock in the bioenergy sector, the prices of these additional biomasses have increased significantly and supply of alternative organic fractions from industrial and agricultural sectors for production of biogas is becoming increasingly limited. This scarcity is causing fluctuation in prices and biomass supply insecurity, which directly affects the income and overall economy of the biogas plants. Moreover, the frequent change of additional biomass supply results in frequent adaptation of the anaerobic digestion processes and thus reduced the overall biogas efficiency. Two possible alternatives have been studied most recently in order to solve this problem: a) the addition of alternative materials to increase dry matter concentration, such as wheat straw [5], which creates a dependency on another extra material and b) the development and application of solid-liquid separation technologies [6]. The solid-liquid separation has the advantage of more efficient and cheaper transportation since only the solid fraction of the manure will be transported to the biogas plant, resulting in a considerable decrease of transportation cost per cubic meter of methane produced. Meanwhile, the liquid fraction will remain in the farm, where it will be used as fertilizer [7]. Separation technologies available include for: mechanical screen separators, filter presses, sedimentation, centrifugation, biological treatment and reverse osmosis [8].

The solid fraction of manure consists mainly of swine faeces, lignocellulosic plant fibers and some additional elements (as hair, skin, and soil from roughage). Biogas
production from manure fibers presents difficulties mainly due to the rigid lignocellulosic structure. Native lignin, which binds and encapsulates carbohydrates, is generally resistant to microbial/enzymatic decomposition [9]. Therefore, pretreatment is a prerequisite process for accessing fermentable carbohydrates. The main purpose of pretreatment of lignocellulosic feedstocks is disrupting the lignocellulosic matrix, facilitating the hydrolysis of cellulose and hemicellulose by cellulases and/or xylanases produced by cellulytic and xylanolytic microorganisms and allowing thus the subsequent anaerobic fermentation and methanogenesis steps [10, 11]. Several pretreatment methods for increasing the biodegradability of methane production from fibers and other lignocellulosic materials have been reported. Mechanical treatment, such us milling, increases the surface available for enzymatic attack and has been proven effective in increasing methane yields of lignocellulosic substrates up to 25%. Physical treatments, e.g microwave treatment, chemical treatments with acids, bases and oxidants and combined physicochemical treatments have also been tested [11-13]. Among them, alkaline hydrolysis with NaOH has been proven a satisfactory treatment when applied on lignocellulosic materials like straw. Biological treatment using white rot fungi with delignification ability has been reported to increase methane yield to some extent. The use of some commercial enzymes increases also methane yield when it is combined with other pre-treatment technologies, such as steam treatment [14].

In the present study, aqueous ammonia soaking (AAS) and subsequent ammonia removal has been used as a method to increase methane potential and biogas productivity of manure fibers. AAS has been so far tested for bioethanol and chemicals production with satisfactory results [15-18]. In these studies, AAS has been proven an
effective treatment of low lignin content feedstocks such as agriculture residues [19]. In addition AAS presents certain advantages as a pretreatment method; Ammonia is relatively safe to handle, non-polluting and non-corrosive and can be easily recovered due to its high volatility [9]. Ammonia is a weak base and has high selectivity toward the lignin reactions, preserving the carbohydrates [19]. It cleaves the ether bonds in lignin and the ether and ester bonds between lignin and hemicellulose. It can also penetrate the crystalline structure in cellulose and cause swelling [9]. In most processes, ammonia pretreatment has been performed at high temperatures, resulting thus in high delignification. However, a number of disadvantages to these processes, such as high energy input, formation of toxic compounds and loss of sugars have been reported. Low reaction temperature has been presented as an alternative approach to alleviate these challenges [9]. Diverse studies have indicated that the use of ammonia at room temperature minimized its interaction with hemicellulose and the formation of toxic compounds while increasing bioconversion and fermentation yields [9]. However, studies on the effect of AAS on methane production from various biomasses are scarce with just that of Himmelsbach et al. [20] applying AAS on switch grass found so far in the international literature.

It has to be emphasized that the ammonia used for the pretreatment can be easily recycled in a full-scale plant resulting in actually no chemicals consumption. Ammonia recovery from digested manure is technically and in many cases economically feasible as well [21]. The ammonia recovery methods, currently used in commercial scale, focus on the whole digestate effluent stream and the production of nitrogen fertilizers. The AAS method tested in the present study requires the ammonia recovery from only the
manure fibers which is a very small fraction (less than 10%) of the total mass of the liquid manure and therefore the energy requirements are expected to be significantly lower. Therefore, application of AAS on manure fibers in biogas plants already equipped with ammonia removal infrastructure (as ammonia removal is in most cases necessary for manure based plants) is expected to constitute a cost-efficient and sustainable pretreatment (or post-treatment) option. A costs and benefits analysis of the proposed AAS method combined with an ammonia recovery system is not part of the present study but it is certainly included in future research plans.

In the present study, AAS was applied to both raw and digested (before and after anaerobic digestion) swine manure fibers. The objectives were to study the effect of AAS on the methane yield and hydrolysis step as well as to investigate if AAS treated fibers exhibited any inhibition on anaerobic digestion with increasing TS loadings.

2. Methods

2.1. Substrate, reagents enzymes and inoculum

Manure fibers were kindly provided by Morsø BioEnergi (a mesophilic biogas plant treating manure and manure fibers) and stored at -20°C until used. Two kinds of manure fibers were used in this study: those collected directly in the farm after separation using a decanter centrifuge - called raw manure fibers - and those which were collected at the Morsø BioEnergi biogas plant after decanting the effluent of the anaerobic digester – called digested manure fibers. Aqueous ammonia solution 32% w/w was used for the pretreatment. Novozymes Biomass Kit was kindly provided by Novozymes A/S (Bagsværd, Denmark). The inoculum for the methane potential tests came from a 3-L
active volume mesophilic digester treating swine manure at an organic loading rate of 2.58 g COD/l d and a methane productivity of 0.55 l/l d. The Volatile Solids (VS) content of the inoculum was 17.6 ± 1.4 g/l. It was estimated that 2.9, 4.3 and 7.8% of the VS of the inoculum corresponded to acidogenic, acetogenic and methanogenic microbial population, respectively. This estimation was based on simulations of the reactor performance based on Anaerobic Digestion Model 1 [22].

2.2. Analytical methods

All characterisations of AAS-pretreated fibers were done after removal of ammonia (as described in section 2.3). The results are given per g TS in order to be comparable with those coming from non-pretreated fibers as the mass of TS before and after AAS pretreatment remained the same. Determination of total solids (TS) and volatile solids (VS) was carried out according to standard methods [23]. In order to measure total Chemical Oxygen Demand (COD), the material was dried at 105°C and milled to powder. After that, the material was diluted in Millipore water and measured with Hach Lange kit LCK_914 (5-60 g/l range). For soluble COD the material was centrifuged at 4000 rpm for 10 min, the supernatant was filtered through high flow filter (pore size 0.2 µm) and analysis was performed with Hach Lange kit LCK_514 (100-2000 mg/l range).

For soluble ammonium nitrogen (NH₄-N) measurement, the material was centrifuged and filtered as previously described and analysis was performed with Hach Lange kit LCK_305 (1-12 mg/l range).

Detection and quantification of sugar monomers (glucose, xylose and arabinose) was made with HPLC-RI equipped with an Aminex HPX-87H column (BioRad) at 60°C. A solution of 4 mmol/l H₂SO₄ was used as eluent at a flow rate of 0.6 mL/min. Samples
for HPLC analysis were acidified with a 10% w/w solution of H$_2$SO$_4$, centrifuged at 10,000 rpm for 10 min and finally filtered through a 0.45μm membrane filter. Two groups of carbohydrates were determined in the samples of raw and pretreated manure fibers: the first group was the total carbohydrates, including those bound in the lignocellulosic biomass and the second group was the simple sugars [24]. Analysis of the two groups of carbohydrates was carried out based on the NREL analytical procedures [25]. Enzyme activity was determined by Filter Paper assay [26, 27]. Biogas composition in methane was measured with a gas chromatograph (SRI GC model 310) equipped with a thermal conductivity detector and a packed column (Porapak-Q, length 6ft and inner diameter 2.1 mm). The temperature for injector, column and detector was set to 80°C. The volume of methane produced in sealed vials during methane potential tests was calculated multiplying the biogas composition with the headspace volume. The increase in methane yield of the AAS-pretreated fibers compared to the non-pretreated fibers was calculated according to equation 1:

$$\text{% increase} = \frac{Y_{\text{CH}_4}^{\text{AAS-fibers}} - Y_{\text{CH}_4}^{\text{control-fibers}}}{Y_{\text{CH}_4}^{\text{control-fibers}}} \times 100$$  \hspace{1cm} (1)

Where $Y_{\text{CH}_4}^{\text{AAS-fibers}}$ and $Y_{\text{CH}_4}^{\text{control-fibers}}$ corresponded to the methane yield in ml CH$_4$/g TS obtained from AAS-pretreated fibers and non-pretreated fibers, respectively.

2.3. Ammonia pretreatment

Samples of manure fibers were soaked in ammonia reagent (32% w/w in ammonia) with a ratio of 10 mL reagent per 1 g TS. The pretreatment was performed in closed glass flasks to avoid ammonia evaporation. After the completion of the pretreatment, water was added at a ratio of 10 mL per g TS to facilitate the subsequent ammonia distillation
Distillation was performed using a rotary evaporator (Buchi RII Rotavapor) with a vertical condenser under 320 psi and gradually increased temperature from 40 to 90°C with a step of 20 degrees from 40 to 80°C. The retention time was 10 and 20 min at the two first and two last temperature levels, respectively.

2.4. Effect of AAS duration and temperature on methane production

Three different AAS pretreatment durations (1, 3 and 5 days) and two moderate temperatures (22 and 55°C) were applied in digested manure fibers. Methane potential tests of pretreated fibers (AAS-fibers) were carried out at triplicates in 300 ml sealed serum vials and anaerobic mixed liquor from a mesophilic digester treating liquid manure was used as inoculum. An amount equivalent to 0.25 g TS of fibers per 10 mL of inoculum was added in the serum vials. A control triplicate was run in parallel by using non-pretreated fibers (control-fibers) while a triplicate containing only inoculum was served as control for background (coming from the inoculum) methane production. The vials were incubated under mesophilic conditions (37°C) for 35-50 days with periodic shaking and methane production was monitored throughout the duration of the experiments. Methane potential was calculated as the volume of methane produced per g of TS of manure fibers added after subtracting the methane produced in the control vials with only inoculum added. Thus, the suitability and the efficiency of the aqueous ammonia soaking as a pretreatment method for enhanced methane production from manure fibers were assessed and the optimal conditions among the ones tested were chosen for subsequent experiments.

2.5. Effect of AAS on the composition of raw and digested manure fibers
Raw and digested manure fibers were subjected to AAS pretreatment for 3 days at 22°C and their composition in terms of lignin, carbohydrate polymers and free sugars, total and soluble COD and NH$_4$-N was determined before and after the pretreatment.

2.6. Effect of organic loading of AAS pre-treated manure fibers on methane production

Methane production rate and yield were evaluated in batch experiments at different organic loadings in order to assess any inhibitory effects due to the pretreatment. Digested and raw manure fibers were pretreated for 3 days at 22°C. Four different TS loadings were tested: 0.16, 0.25, 0.5 and 1 g TS per 10 ml of inoculum.

Methane potential tests of pretreated fibers (AAS-fibers) were carried out as described in section 2.4. Control triplicates by using non-pretreated fibers (control-fibers) at the same TS loadings and a triplicate containing only inoculum were also run in parallel as previously described. Methane potential was again calculated as the volume of methane produced per g of TS of manure fibers added after subtracting the methane produced in the control vials with only inoculum added.

2.7. Effect of AAS on enzymatic hydrolysis step

As it is reported previously, AAS is expected to disrupt the lignocellulosic matrix, facilitating and speeding-up thus the hydrolysis of cellulose and hemicellulose during the hydrolysis step of the anaerobic digestion process. One way to evaluate the effect that AAS has on the hydrolysis’ efficiency is to add commercial cellulases and xylanases to AAS-pretreated and non-pretreated digested fibers and to compare the sugars release in each case. pH of the non-pretreated fibers (control-fibers) and AAS pretreated fibers (AAS-fibers) was adjusted to 5.5 with 30% H$_2$SO$_4$ prior to enzymatic
hydrolysis. Cloranphenicol, tetracycline and ampiciline were added at a final concentration of 50µg/mL, 50µg/mL and 100 µg/mL, respectively, in order to prevent microbial growth and consumption of the released sugars. Novozymes Biomass Kit was used for performing enzymatic hydrolysis. It consisted of five different enzymes (NS50013, NS50010, NS50012, NS50030, NS22002) exhibiting cellulolytic, beta-glucosidic, arabinolytic, hemicellulolytic, pectinolytic and xylanolytic activities. The above enzymes were mixed at a mass ratio of 15:1.5:1:1.25:5 (NS50013: NS50010: NS50012: NS50030: NS22002) and the resulted enzymes solution exhibited an activity of 62 FPU/g as determined by the filter Paper assay. Three different enzymatic loadings were tested: 5, 15 and 25 FPU/g TS, respectively. Enzymatic hydrolysis tests were carried out in a shaking incubator at a temperature of 50°C and 120 rpm continuous mixing for four days. Free sugars in control-fibers and AAS-fibers were analyzed before and after the completion of enzymatic hydrolysis tests and the hydrolytic efficiency regarding glucose and xylose was calculated based on equations 2 and 3, respectively.

\[
\text{Hydrolytic efficiency} = \frac{[\text{glucose}]}{[\text{cellulose}]} \cdot \frac{1}{1.11} \cdot 100
\]  

(2)

\[
\text{Hydrolytic efficiency} = \frac{[\text{xylose}]}{[\text{xylan}]} \cdot \frac{1}{1.14} \cdot 100
\]  

(3)

Where [glucose], [xylose], [cellulose] and [xylan] are the respective concentrations given in g per 100 g TS.

3. Results and discussion

3.1. Effect of AAS duration and temperature on methane production

AAS was investigated as a pretreatment method for enhancement of the methane production rate and potential from digested manure fibers. The efficiency of AAS has
been tested at different durations (1, 3 and 5 days) and temperatures (22 and 55°C) as described previously. The methane yield after 35 days of anaerobic digestion of non-pretreated (control-fibers) and AAS pretreated digested fibers (AAS-fibers) is shown in Fig. 1. Except from the sample treated for 1 day at 22°C, which exhibited a slight decrease in the methane yield compared to the control, an increase between 17-80% in the methane yield of AAS-fibers compared to the control-fibers was observed. AAS for 3 days at 22°C was the most efficient one exhibiting 139 ml CH₄/g TS. The methane production rate was also positively affected by AAS as the maximum yield, 135 mL CH₄/g TS was obtained after 16 days incubation of AAS-fibers (AAS for 3 days at 22°C) while 32 days were needed for control-fibers to achieve the maximum methane yield, approximately 77 mL CH₄/g TS, as shown in Fig. 2. Moreover, the methane yield of AAS-fibers was 128% higher than the one of control-fibers after 16 days incubation. As it was anticipated, the methane production from AAS pretreated fibers was higher and faster probably due to the higher accessibility of the organic compounds. It is well known that the extracellular hydrolysis is slow and incomplete during anaerobic digestion of lignocellulosic material [2], thus being the rate-limiting step in the biogas production. Due to the manure fibers structural changes induced by the AAS pretreatment, faster and more complete hydrolysis occurred resulting to higher methane production rate and potential (Figs. 1-2).

No higher methane yield was obtained when AAS was performed at 55°C compared to that at 22°C for 3 days (139 and 130 ml CH₄/g TS at 22 and 55°C, respectively) and 5 days (122 and 123 ml CH₄/g TS at 22 and 55°C, respectively) pretreatment duration. According to the literature an increase in AAS temperature results in a greater delignification degree [16]; in the present study, nevertheless, a temperature increase
from 22 to 55 °C did not have any positive effect on the methane potential a finding that may be translated in reduced cost for the pretreatment process. It has to be noted, however, that in the study of Kim et al. [16] the pretreated biomass (barley hull) was different than the biomass used in the present study. Whether the optimal conditions for AAS pretreatment depend on the biomass type could be an interesting subject for future investigations. In the present study, 3 days at 22°C were chosen as the optimal conditions among the ones tested for AAS pretreatment of manure fibers since they exhibited the higher methane yield and hence they were applied to all subsequent experiments.

3.2. Effect of AAS on the composition of manure fibers

The composition of raw and digested manure fibers before and after AAS pretreatment for 3 days at 22°C is shown in Table 1. The TS content of raw and digested fibers was 31.98 ± 0.2% and 27.82 ± 0.98%, respectively. The TS/VS ratio in raw and digested fibers accounted for 1.27 and 1.44, respectively and it remains the same for AAS-treated fibers as well.

Soluble COD values imply that solubilisation of the solid matrix took place with AAS pretreatment (7.6% and 14.8% for digested fibers and 5.0% and 7.6% for raw fibers before and after AAS pre-treatment, respectively). Solubilisation was significantly higher in digested than in raw fibers.

As it was expected and according to the literature [9] cellulose (glucan fraction) was not degraded during the pretreatment. Xylan (corresponding to hemicelluloses fraction) did not seem to be degraded either. The non-destruction of sugars was attributed to the mild nature of AAS, not involving high temperature and/or pressure and oxidative
conditions. The decreased concentration of glucan and xylan in digested fibers compared to the raw fibers could be attributed to the fact that digested fibers had already undergone an anaerobic digestion step resulting thus to a small decomposition of the carbohydrate fractions. The same explanation could apply for the higher lignin content in digested fibers: part of TS was removed during anaerobic digestion while lignin remained intact, comprising thus a higher fraction of TS in digested samples. A first-order solubilisation of the lignin during AAS could explain the higher lignin removal for the higher initial concentration samples (digested) in comparison to the raw fibers. This hypothesis needs, however, further investigation. In order to verify that AAS did not result in solubilisation of sugars, free sugars were determined as well before and after AAS treatment and in all cases they remained below detection limit (Table 1).

Ammonia concentration differed slightly among different types of fibers and in all cases the amount of ammonia was low enough to allow anaerobic digestion to proceed without inhibition. Specifically for the AAS-pretreated fibers, the NH$_4$-N concentration reached 0.15 and 0.28 g NH$_4$-N/l for raw and digested fibers, respectively, after ammonia removal. According to the literature, unadapted microorganisms can tolerate ammonia concentration up to 1.5-2.5 g NH$_4$-N/l before inhibition is observed [28]. Moreover, the methanogenic inoculum used for the methane potential tests was adapted to an NH$_4$-N concentration of around 4 g/l.

3.3. Effect of organic loading of AAS pre-treated manure fibers on methane production
Different organic loadings of AAS pretreated raw and digested fibers were tested in order to analyze possible inhibition due to components that may be formed during the pretreatment, as it was described in detail in §2.5.

The final methane yields of non-pretreated (control fibers) and AAS-pretreated (AAS-fibers) digested fibers after 40 days of batch anaerobic digestion at different organic loadings are shown in Fig. 3a. It was noticed that increasing of TS loading up to 1 g TS per 10 ml of inoculum did not affect the final methane yield of either control- or AAS-fibers. Methane yield of control-fibers was $90 \pm 7$ ml CH$_4$/g TS while the final methane yield of AAS-fibers was $117 \pm 4$ ml CH$_4$/g TS taking into account the values obtained from all loadings. Increase of the final methane yield of the AAS-fibers compared to the control fibers was calculated as $30.3 \pm 7.9\%$.

In case of AAS-fibers, even though the final methane yield was the same for all loadings tested, the methane production rate was different (Fig. 4b). Specifically, during the first 10 days, the methane production rate in the vials with 0.5 and 1 g TS per 10 ml of inoculum was lower than the rate observed for the first two loadings (0.16 and 0.25 g TS per 10 ml of inoculum) with the rate decreasing with increasing loading. This implies that an inhibition, most probably due to inhibitors formed during the pretreatment, occurred. The inhibition was overcome after 18 days of digestion where the methane production reached the same level for all loadings. Apparently, the microbial culture could quickly adapt and this is an indication that inhibition will not constitute a problem in a continuous process for anaerobic digestion of AAS pretreated digested fibers, at least up to the loading tested.

For raw fibers the profiles of methane yield at different loadings were different than those of digested fibers. Fig. 3b shows the final methane yields of non-pretreated...
(control fibers) and AAS-pretreated (AAS-fibers) raw fibers after approximately 40 days of batch anaerobic digestion. Increment in TS loading from 0.16 to 1 g TS/10 ml of inoculum did not affect the methane yield of control-fibers. Methane yield was 116 ± 9 ml CH₄/g TS taking into account all loadings. On the other hand, the same increment in TS loading of AAS-fibers resulted in reduced methane yield for loadings higher than 0.16 g TS/10 ml of inoculum. Specifically, the vials loaded with 0.16 gTS/10 ml of inoculum exhibited a methane yield of 320 ml CH₄/g TS (corresponding to a 177.8% increase compared to that of control-fibers) while the methane yield decreased to 180 ml CH₄/g TS for the vials loaded with 0.25 and 0.5 gTS/10 ml inoculum. The methane yield became even lower (138 ml CH₄/g TS corresponding to an increase of just 7.3 % compared to that of control-fibers) in the vials loaded with the higher 1 gTS/10 ml of inoculum. This implies the presence of methanogens’ inhibitors possibly due to the AAS pretreatment of raw fibers. Furthermore, the inhibitory effect could not be overcome with time (at least within 40 days), contrary to what happened with AAS-pretreated digested fibers.

The methane production rate of control-fibers (Fig. 5a) was also the same for all loadings tested, while it was significantly reduced in the vials with a loading of 0.25, 0.5 and 1 g TS AAS-fibers/10 ml of inoculum (Fig. 5b) compared to the vials with the lowest loading of 0.16. An increase of the production rate was observed for the highest loading after 12 days of digestion; however the methane production reached a significantly lower level than in the vials with the lowest loading. The results obtained clearly imply that a strong inhibition of anaerobic digestion process occurred with AAS-pretreated raw fibers at increased loadings. It could be either of the four distinct steps, hydrolysis, acidogenesis, acetogenesis and methanogenesis, or more than one step that
are inhibited. It is also likely that this inhibition will be overcome in a continuous
system where the microbial community will be exposed for long enough time to adapt
to the inhibitors. Moreover, the different inhibition intensity observed between the
AAS-pretreated digested and AAS-pretreated raw fibers could be due to the interaction
of the ammonia and the easily biodegradable organic matter present in the raw fibers.
All the three abovementioned issues regarding inhibited step(s), possibility of
adaptation and inhibition causes exhibit profound scientific interest and deserve further
investigation.

3.4. Effect of AAS on enzymatic hydrolysis step

Enzymatic hydrolysis tests were performed in digested fibers. AAS-fibers (pretreated
for 3 days at 22°C) and control-fibers were hydrolyzed using enzymes from Novozymes
biomass kit (as described in § 2.6) in order to compare the effect of hydrolytic enzymes
on pretreated and non-pretreated digested fibers. Free sugar concentrations at different
enzymatic loadings (5, 15 and 25 FPU/g TS) as well as hydrolytic efficiencies based on
glucose and xylose release are presented in Figs. 6a and b, respectively.

The addition of enzymes to the control-fibers had insignificant effect on the release of
free sugars. This could be attributed to the intact lignocellulosic structure of non-
pretreated fibers, which did not allow the enzymes to reach cellulose and
hemicelluloses. On the other hand, enzymatic hydrolysis had a significant effect on
AAS-fibers. Free sugars reached a maximum total concentration of 19 g per 100 g-TS
corresponding to a hydrolytic efficiency of 80 and 65% for glucose and xylose
respectively. It was concluded that the AAS pretreatment had affected the
lignocellulosic structure allowing the enzymes to hydrolyse the released cellulose and
hemicellulose. The effect of such a pretreatment on anaerobic digestion process would have been a faster hydrolysis step and consequently an increased biogas production rate. An increase of the enzymatic loading from 15 to 25 FPU/g TS had a relatively small effect on the free sugars release.

4. Conclusions

In the present study, aqueous ammonia soaking (AAS) was investigated as a moderate and sustainable method for enhancing the methane potential of swine manure fibers. AAS pretreatment exhibited a significant enhancement of methane potential. AAS for 3 days at 22°C resulted at a 30-80 and 178% increase in methane yield from digested and raw manure fibers, respectively. The positive effect of AAS on the methane yield was due to the facilitation of hydrolysis step of anaerobic digestion process and could be attributed to AAS directly affecting the disintegration step of the lignocellulosic matrix. That was indicated by enzymatic hydrolysis tests of AAS-pretreated fibers, which resulted to 80 and 65% hydrolysis efficiency of glucan and xylan compared to insignificant numbers for non-pretreated fibers. Moreover, batch anaerobic digestion of AAS-pretreated digested manure fibers could stand loadings as high as 100 g TS /l inoculum with no inhibition problems. On the other hand, batch anaerobic digestion of AAS-pretreated raw manure fibers exhibited an inhibition of the methane production rate and potential for loadings higher than 16 g TS /l inoculum.

Acknowledgement

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Figure captions

**Fig. 1** - Methane yield after 35 days of anaerobic digestion of control-fibers and AAS pretreated digested fibers (AAS-fibers) at different pretreatment durations and temperatures.

**Fig. 2** - Methane yield profile during 50 days anaerobic digestion of control fibers and AAS pretreated digested fibers (AAS-fibers). AAS was applied for 3 days at 22°C.

**Fig. 3** - Methane yield after 40 days of anaerobic digestion of control-fibers and AAS-fibers at different g TS per 10 ml of inoculum ratios for (a) digested fibers and (b) raw fibers. AAS was applied for 3 days at 22°C.

**Fig. 4** - Methane yield after 40 days of anaerobic digestion of (a) control-fibers and (b) AAS-fibers at different gTS per 10 ml inoculum ratios for digested fibers. AAS was applied for 3 days at 22°C.

**Fig. 5** - Methane yield after 40 days of anaerobic digestion of (a) control-fibers and (b) AAS-fibers at different g TS per 10 ml inoculum ratios for raw fibers. AAS was applied for 3 days at 22°C.

**Fig. 6** - (a) Glucose and xylose monomers released after AAS pretreatment (3 days, 22°C) and enzymatic hydrolysis (4 days, 50°C, 120 rpm) and (b) enzymatic hydrolysis efficiency.
Table 1 - Composition of control-fibers and AAS-fibers.

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<th>Digested fibers</th>
<th>Raw fibers</th>
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<tr>
<td></td>
<td>Control-fibers</td>
<td>AAS-fibers</td>
</tr>
<tr>
<td>Glucan, g/100g TS</td>
<td>11.8 ± 0.1</td>
<td>10.9 ± 0.2</td>
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<tr>
<td>Xylan, g/100g TS</td>
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<td>11.6 ± 0.0</td>
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<td>Arabinan, g/100g TS</td>
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<td>Klason Lignin, g/100g TS</td>
<td>26.8 ± 4.3</td>
<td>17.5 ± 5.0</td>
</tr>
<tr>
<td>Free glucose, g/100g TS</td>
<td>a</td>
<td>0.1 ± 0.0</td>
</tr>
<tr>
<td>Free xylose, g/100g TS</td>
<td>a</td>
<td>a</td>
</tr>
<tr>
<td>Free arabinose, g/100g TS</td>
<td>a</td>
<td>a</td>
</tr>
<tr>
<td>Soluble COD, g/100g TS</td>
<td>6.6 ± 0.4</td>
<td>14.7 ± 1.4</td>
</tr>
<tr>
<td>Total COD, g/100g TS</td>
<td>97.9 ± 4.7</td>
<td>99 ± 6.1</td>
</tr>
<tr>
<td>NH₄-N, g/100g TS</td>
<td>0.6 ± 0.66</td>
<td>0.6 ± 0.1</td>
</tr>
</tbody>
</table>

a below detectable levels
Fig. 1 - Methane yield after 35 days of anaerobic digestion of control-fibers and AAS pretreated digested fibers (AAS-fibers) at different pretreatment durations and temperatures.
Fig. 2 - Methane yield profile during 50 days anaerobic digestion of control fibers and AAS pretreated digested fibers (AAS-fibers). AAS was applied for 3 days at 22°C.
Fig. 3 - Methane yield after 40 days of anaerobic digestion of control-fibers and AAS-fibers at different g TS per 10 ml of inoculum ratios for (a) digested fibers and (b) raw fibers. AAS was applied for 3 days at 22°C.
Fig. 4 - Methane yield after 40 days of anaerobic digestion of (a) control-fibers and (b) AAS-fibers at different gTS per 10 ml inoculum ratios for digested fibers. AAS was applied for 3 days at 22°C.
Fig. 5 - Methane yield after 40 days of anaerobic digestion of (a) control-fibers and (b) AAS-fibers at different gTS per 10 ml inoculum ratios for raw fibers. AAS was applied for 3 days at 22°C.
Fig. 6 - (a) Glucose and xylose monomers released after AAS pretreatment (3 days, 22°C) and enzymatic hydrolysis (4 days, 50°C, 120 rpm) and (b) enzymatic hydrolysis efficiency.