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Optimization of Aqueous Ammonia Soaking of manure fibers by Response Surface Methodology for unlocking the methane potential of swine manure

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ABSTRACT

Swine manure mono-digestion often results to economically non-feasible processes, due to the high dilution and ammonia concentration together with the low degradation rates it presents. The effects of different parameters of Aqueous Ammonia Soaking (AAS) as a pretreatment for improving the digestion of manure fibers when coupled to an ammonia removal step were investigated in this study. Response Surface Methodology was followed and the influence and interactions of the following AAS parameters were studied: NH_3 concentration, duration and solid-to-liquid ratio. The mild conditions found to be optimal (7% w/w NH_3, 96 h, and 0.16 kg/L) in combination to a significant increase of the short term CH_4 yield (244% in 17 days), make this pretreatment a promising solution for improving swine manure mono-digestion. Furthermore, compositional analysis of the manure fibers revealed significant solubilization of hemicellulose, while no lignin removal or loss of cellulose occurred under optimal conditions.

1. Introduction

The interest in developing efficient renewable energy production processes grows continuously as a response to the future limited availability of fossil fuel resources and to the greenhouse effect. Anaerobic digestion constitutes one of the oldest and most established renewable energy production processes, both in developed and developing countries (Bond and Templeton, 2011). The fact that the biogas produced can be used for direct heating, electricity production, to replace natural gas or as a vehicle fuel (when upgraded), ensures that anaerobic digestion processes will represent a major role in the future energy production sector (Holm-Nielsen et al., 2009).

Livestock manure is one of the most extensively used substrates for anaerobic digestion due to the worldwide expansion of the livestock production sector and to its rich content in nutrients and microorganisms, that lead to the spontaneous production of biogas under anaerobic conditions. However, due to the low price of biogas, the low conversion rate of manure to biogas and the high water content attributed to current management practices in farms, the anaerobic digestion process of solely manure often results to be economically non-feasible (Møller et al., 2007). This fact has led to the concept of co-digestion where manure is enriched with diverse organic materials that present a higher biogas production rate, such as whole-grain crops and residues from crops or from the food industry (Asam et al., 2011). Undoubtedly this practice offers some benefits to the operation of a biogas plant, i.e. improves the characteristics of the input material by facilitating the adjustment of the C:N ratio and the dry matter content. However, it renders biogas plants dependent on the availability of these extra materials that might be scarce in comparison to manure. For instance, the amount of livestock manure treated anaerobically in Denmark does not exceed 8% of the total annual production, whereas common substrates for co-digestion are of limited availability (Mikkelsen et al., 2016). This fact in turn, increases the potential pollution of the atmosphere by greenhouse gas and ammonia emissions due to other manure management practices (Chadwick et al., 2011). Therefore, it is important to develop technologies that will improve the methane efficiency when manure is the sole substrate and thus they will lead to an increase of the amount of manure treated and of the energy recovered.

The challenge of improving the conversion of manure to biogas is mainly due to the refractory nature of the lignocellulosic content as well as to the high ammonia content that often characterizes it (Sawatdeenarunat et al., 2015). Usually only 30–50% of the organic matter of manure is degraded during anaerobic digestion in biogas plants (Christensen et al., 2007). Aiming at overcoming these limitations, various researchers have tested different pretreatments, e.g. mechanical, thermal and chemical treatments (Angelidaki and Ahring, 2000; Bonmati et al., 2001; Bonmatí and Flotats, 2003; Carrère et al., 2009; González-Fernández et al., 2008), and in some cases a significant increase of digestibility in terms of biogas production or methane yield has been observed. Nevertheless, only the solid fraction of manure

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(manure fibers) could be targeted as it presents the concentrated recalcitrant fraction with the highest theoretical methane potential (Angelidaki and Ahring, 2000) as well as the majority of organic N. This way, the volume of material to be pretreated is significantly reduced which results to be economically more attractive for large scale applications. A separation of animal slurry to a solid and liquid fraction, which is possible by various available technologies (Hjørth et al., 2011; Møller et al., 2000), reduces transportation costs (Asam et al., 2011) and provides more flexibility on increasing the dry matter content of the influent addressing this way the high dilution of manure (usually less than 8% dry matter content while typical anaerobic digesters can operate with an influent of up to 12% dry matter) (Frandsen et al., 2011).

Similarly to other lignocellulosic biomasses, many pretreatments have been suggested for improving the degradability of manure fibers. Chemical pretreatments, while often highly efficient, are considered more difficult to implement in large scale due to the extra costs of chemical consumption. Among them, Aqueous Ammonia Soaking (AAS) has been identified as a promising pretreatment for lignocellulosic biomasses since ammonia is the only chemical used and is expected to be relatively easy to recover due to its high volatility. Especially in the case of swine manure digestion, an ammonia removal step could also partly alleviate the process from high ammonia loadings. On this line, some biogas plants are already equipped with ammonia stripping installations, facilitating thus an implementation of the pretreatment (Frandsen et al., 2011). Moreover AAS can take place at low temperatures and ambient pressure, reducing thus the energy input requirements. AAS has been tested so far on various biomasses under different configurations mainly for increasing ethanol production and sugar release (Kim et al., 2016, 2008; Ko et al., 2009; Yoo et al., 2013) but also for enhancing methane production (Antonopoulou et al., 2015; Hashimoto, 1986; Jurado et al., 2013a, 2013c; Li et al., 2015; Mirtsou-Xanthopoulou et al., 2014). Generally, AAS is considered to improve the hydrolysis of lignocellulosic biomasses by acting selectively on lignin while preserving carbohydrates (Carrère et al., 2016; Kim et al., 2016), facilitating thus the access of enzymes to carbohydrates. Besides the promising results of AAS applied to different feedstocks for anaerobic digestion, it has been optimized so far only for wheat straw at elevated temperature (Li et al., 2015). Screening experiments on the effects of AAS under different conditions on the methane yield of manure fibers, showed that temperature was the least influencing factor, permitting thus a low energy input of the process (Lymperatou et al., 2015a). A comprehensive study on how the efficiency of the pretreatment is affected by the most influencing parameters (ammonia concentration, duration, and solid-to-liquid ratio) is essential prior to scaling up, as it facilitates the process design and the evaluation of the techno-economic feasibility, elucidating the actual potential of a pretreatment.

In the present study, AAS was applied on swine manure fibers in order to evaluate the efficiency of the pretreatment on increasing the methane yield under different conditions. For this purpose, Response Surface Methodology (RSM) was followed and the optimal conditions for maximizing the methane yield of pretreated manure fibers were determined. Furthermore, the solubilization of the biomass under different conditions was assessed and practical limitations are discussed. Finally, empirical models, able to predict the methane yield of the AAS-treated fibers as a function of the pretreatment conditions were developed.

2. Materials and methods

2.1. Substrate and inoculum

The substrate used for the experiments was collected at the biogas plant Limfjordens Bioenergi (Mors, Denmark) that received manure fibers separated from raw swine manure by means of a mobile decanter centrifuge. Once collected they were sealed in plastic bags and stored at −20 °C until used. The content of the manure fibers in total solids (TS) was 35.13 ± 1.76% of wet mass, and in Volatile Solids (VS) 23.59 ± 0.84% of wet mass. The total Chemical Oxygen Demand (COD) of the manure fibers was 1.20 ± 0.01 g O_2/g VS. The inoculum used for the Biochemical Methane Potential (BMP) tests originated from a centralized full-scale mesophilic biogas plant operating on livestock manure and organic waste (Hashøj Biogas, Denmark). The inoculum was incubated at 37 °C for 9 days prior to use, for minimizing the endogenous biogas production. The main characteristics of the inoculum were 5.3% TS, 3.7% VS, 3.1 g NH_4^+-N/l, 6.73 g soluble COD/l, and pH 8.05.

2.2. Optimization designs – Response Surface Methodology

The performance of the AAS pretreatment of manure fibers was tested under different conditions in order to find the optimal values of the parameters that were found to be the most influencing on the resulting CH_4 yield, based on screening experiments (Lymperatou et al., 2015a). These were the NH_3 concentration in the reagent, the duration of AAS and the solid-to-liquid (S:L) ratio. All AAS pretreatments were conducted at room temperature (20 °C). Initially, a circumscribed Central Composite Design (cCCD) was followed, with the 3 independent variables varying at 5 levels: 0.9, 7, 16, 25, and 31.1% w/w NH_3 concentration, 4.8, 28, 62, 96 and 119.2 h of duration, and 0.12, 0.16, 0.18, 0.22 and 0.32 kg fibers/L reagent for the S:L ratio. In total, nineteen AAS pretreatments of manure fibers were performed, comprising of 8 cube points (2^3), 6 axial points where 1 variable was set to the maximum or minimum value while the rest of them were set at the middle values, and the central point (all variables set at the middle value) was replicated 5 times for allowing of estimation of the experimental error. Additionally, given that the VS determination is an indirect measurement, it was decided to model the CH_4 yield per g TS instead, in order to reduce the associated errors. A similar approach has been followed in more studies where CH_4 yield of lignocellulosic substrates is modelled (Monlau et al., 2012). The responses of the system were the cumulative CH_4 yield after 17 days of digestion (*CH_4 17d*) and the corresponding increase of CH_4 yield as compared to the non-pretreated (NP) fibers (*CH_4 yield increase*), expressed in mL/g TS and % respectively, as resulted from biochemical methane potential (BMP) tests. Values of the volume of CH_4 yield reported are given at 20 °C and 1 atm, unless otherwise stated. Additionally, the soluble COD (*solCOD*) was set as a response in order to evaluate which AAS parameters mostly affected the solubilization of manure fibers. The *CH_4 yield increase* achieved by the AAS-treated Manure fibers was calculated as:

$$CH_4\ yield\ increase = \frac{CH_4\ yield_{AAS} - CH_4\ yield_{NP}}{CH_4\ yield_{NP}} * 100, \tag{1}$$

where $CH_4\ yield_{AAS}$, the average of triplicates of the CH_4 yield of AAS-treated fibers under each set of conditions expressed in ml CH_4/g TS; $CH_4\ yield_{NP}$, the average of triplicates of the CH_4 yield of the NP fibers expressed in mL CH_4/g TS.

The first set of optimization experiments was followed by a second experimental design using a faced Central Composite Design (fCCD), where the independent variables were the NH_3 concentration and the duration of AAS. The ranges of the independent parameters studied in the fCCD were 1, 4, and 7% w/w NH_3 concentration and 96, 120 and 144 h of duration.

The experimental results obtained from both designs (cCCD, fCCD) were analyzed by using Response Surface Methodology (RSM) with the statistical software Design Expert 9 (Stat-Ease, USA). RSM is a statistical tool for studying the effects of independent parameters on one or more responses (dependent parameters), and permits the construction of an empirical model with the form:

$$Y = b + b_1x_1 + b_2x_2 + b_3x_3 + b_{12}x_1x_2 + b_{13}x_1x_3 + b_{23}x_2x_3 + b_{11}x_1^2 + b_{22}x_2^2 + b_{33}x_3^2 \quad (2)$$

where Y is the dependent parameter (response); x_1,x_2,x_3 are the independent parameters NH_3 concentration, duration of AAS and S:L ratio respectively; b is the intercept coefficient; b_1,b_2,b_3 are the regression coefficients expressing the main effect of each parameter on the response; b_{12},b_{13},b_{23} are the regression coefficients for the interaction effect of 2 independent parameters on the response; b_{11},b_{22},b_{33} are the regression coefficients for the quadratic effect of each independent parameter on the response. The regression coefficients are calculated by regression analysis of the experimental data. The results obtained are assessed by ANOVA. All terms expressing main effects, while only interaction and quadratic effects found statistically significant ($p < 0.05$) were included in the models. The quality of the models was assessed by the fit of the experimental data to the model (R^2), the closeness of R^2 to the adjusted R^2 (indicating that the terms included are sufficient for modelling the response), and the lack-of-fit test being insignificant ($p > 0.05$). Furthermore, validation experiments were run for ensuring the CH_4 yield predictions of models were satisfactory. Based on the empirical models obtained, Response Surface graphs are constructed where the predicted response is plotted as a function of two independent interacting parameters in a three-dimension graph.

2.3. Aqueous Ammonia Soaking (AAS) pretreatment

The corresponding amount of swine manure fibers was placed in 2 L screw-capped laboratory bottles with 600 mL of the solution of aqueous NH_3 of the corresponding concentration and sealed for avoiding losses of the NH_3. The mixture was left intact until the end of the pretreatment. Once the pretreatment was finalized, an equal-to-reagent volume of tap water was added to the mixture for facilitating the vacuum evaporation of NH_3. A rotary evaporator (Buchi Rotavapor, Switzerland) was used for this purpose and all batches were evaporated until reaching a concentration of less than 1 g NH_4^+/L. This way it was ensured that no inhibition of the anaerobic microorganisms would occur, since the NH_4^+-N level of the inoculum was higher than the level of the substrate. The evaporation was performed at 130 mbar with initial temperature of the evaporator's water bath set at 20 °C and progressively raised up to 40 °C, 50 °C, 60 °C, 80 °C with a total duration of 80 min. The initial solution of aqueous NH_3 used for the AAS pretreatment was of 32% w/w purity (Merck KGaA, Germany).

2.4. Biochemical Methane Potential (BMP) tests

The BMP tests were set in 320 mL infusion bottles with 1.5 g TS of manure fibers and 60 mL of inoculum. In each series of experiments, two additional BMP tests were set up, one with inoculum and NP manure fibers, used as control, and one only with inoculum used as blank. All BMPs were set in triplicate. Inoculum was added and the bottles were flushed with a mixture of 80% N_2/20% CO_2 for ensuring anaerobic conditions. The bottles were sealed with rubber stoppers, secured with aluminum crimps and placed in an incubator at 37 °C. The CH_4 production was monitored periodically until the end of the experiments. The CH_4 production of the BMP tests of both pretreated and NP fibers were corrected for the residual production of the inoculum by subtracting the CH_4 production of the blank tests. Preliminary BMP experiments of AAS-treated fibers showed similar yields when varying the organic loading from 0.3 to 3.0 g TS/60 mL of inoculum, ensuring thus that no inhibition would occur due to the organic loading. The same behavior was observed from BMP tests of NP manure fibers at different organic loadings (data not shown).

2.5. Analytical methods

Determination of TS, VS and ash was carried out according to Standard Methods (APHA, 2005). NH_4^+-N and soluble COD determination was performed after the NH_3 evaporation step by Hach Lange kit LCK 305 and LCK 514 (Hach Lange ApS, Denmark) respectively; samples of the pretreated fibers were centrifuged at 10,000 rpm for 10 min and filtered through 0.45 µm. For the total COD determination, Manure fibers were dried at 105 °C, milled to powder with a commercial coffee grinder and diluted with Millipore-grade water. Measurement was done by Hach Lange kit LCK914 (Hach Lange ApS, Denmark). Determination of biogas content in CH_4 was carried out by Gas Chromatography (G-C82–22, Mikrolab Aarhus, Denmark). The GC was equipped with a Porapak Q packed column (6 ft. and I.D. 3 mm), coupled with a Thermal Conductivity Detector (TCD) and N_2 was used as a carrier gas.

Compositional analysis of NP and AAS-treated fibers was performed based on NREL's protocols. Specifically, samples followed a two-step extraction based on Sluiter et al. (2008); the first extraction step was performed with Millipore-grade water during 6 h, followed by an ethanol (96% v/v) extraction for 24 h using a Soxhlet apparatus (EV6 ALL/16 No. 10–0012, Gerhardt, Germany). Structural sugars, acid-insoluble lignin and acid-soluble lignin of the extracted samples were determined by following NREL's protocol (Sluiter et al., 2011). In order to determine the soluble components of the biomasses, NP manure fibers were diluted in Millipore-grade water, while for the pretreated fibers the liquid fraction was used. Oligosaccharides were determined after a dilute acid hydrolysis of the samples as described in Bjerre et al. (1996). For determination of free sugars, samples were acidified with H_2SO_4 0.1 M, centrifuged at 10,000 rpm for 10 min and filtered through a 0.22 µm filters. Carbohydrates and acetic acid detection and quantification was performed by HPLC (Shimadzu, USA) equipped with a refractive index and an Aminex HPX-87H column (Bio-Rad) at 63 °C. A solution of 12 mM H_2SO_4 was used as an eluent at a flow rate of 0.6 mL/min. Acid-soluble lignin was also measured from the dilute acid hydrolysates. Elemental Analysis (EA3000, EuroVector Instruments & Software, Italy) of NP fibers and AAS-treated fibers was performed on both as-received and extractives-free basis samples in order to determine the insoluble and soluble N content. Acetanilide was used as a standard. For determination of organic N, the values obtained from Elemental analysis were corrected for the inorganic N content of each biomass; only the NH_4^+-N content of each biomass was taken into consideration for the corrections, and the amount of N in form of nitrates and nitrites was assumed to be negligible. The soluble organic N was calculated as the difference between N content of as-received biomasses and N content of extractives-free biomasses.

3. Results and discussion

3.1. Effect of AAS parameters on methane yield of swine manure fibers – Optimization (cCCD)

The cumulative CH_4 yields as resulted from the BMP tests of the first optimization step (cCCD) are presented in Fig. 1. The control experiments (NP fibers) lasted in total 94 days, and the ultimate CH_4 yield observed was 182.56 mL/g TS, which is similar to the values reported by Møller et al., (2004) for swine manure fibers obtained from centrifugation. AAS affected positively the production rate under all conditions tested, though in a different degree. Here, it has to be mentioned, that in further experiments where NP manure fibers were soaked in water and then subjected to the same evaporation process as AAS-treated fibers, showed no effects on the resulted CH_4 yield due to the evaporation step (data not shown), thus any changes in the subsequent anaerobic digestion could be attributed to the AAS pretreatment. As it can be observed in Fig. 1, the BMP tests of AAS-treated fibers reached the CH_4 yield of 38 days of the NP fibers in only 6–10 days depending on the conditions applied. The ultimate CH_4 yield of the AAS-treated fibers varied significantly based on the different conditions. While generally the same or higher ultimate yield as compared to the NP fibers was observed (Fig. 1), a lower ultimate CH_4 yield was

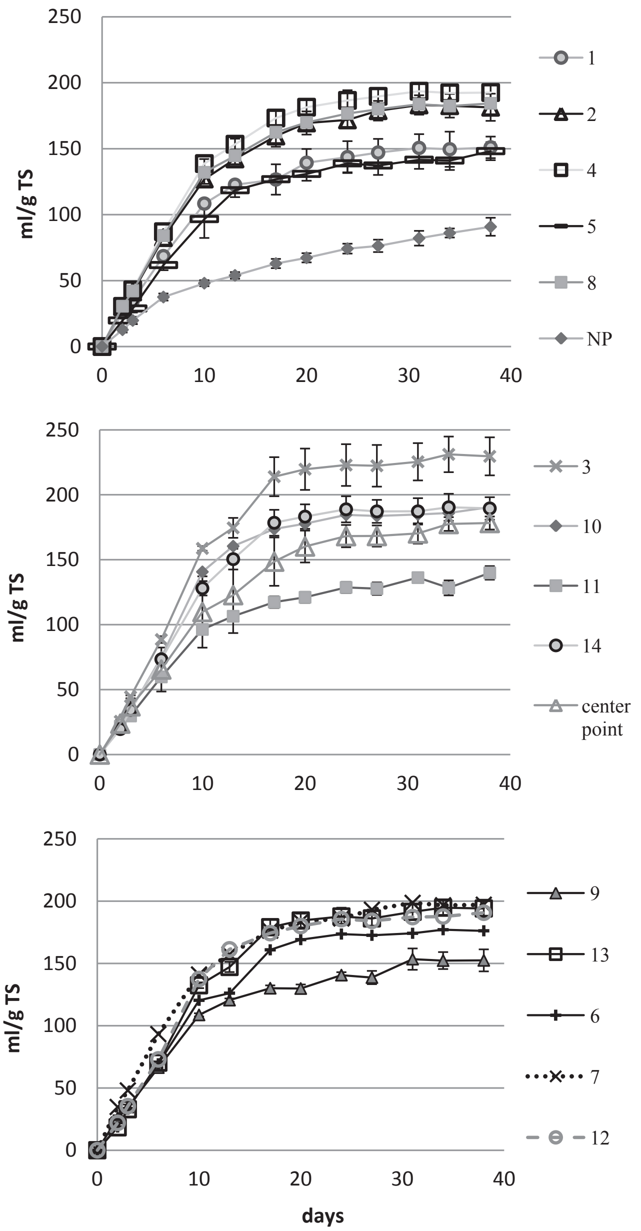

Fig. 1. Cumulative CH_4 yields of BMP tests of Manure fibers treated under different conditions of AAS according to the circumscribed CCD (Table 1). Center point corresponds to the average of experiments 15–19. Vertical bars correspond to standard deviation of triplicates.

found from the batches pretreated with the lowest NH_3 concentration (experiment 9) or the lowest duration of AAS (experiment 11). Nevertheless, after 17 days of digestion the cumulative yields of the AAS-treated fibers were very close (> 75%) to their ultimate CH_4 yields (38 days). The cumulative CH_4 yields after 17 days of digestion were chosen for modelling, as the short-term yield could be a better indicator of a continuous process in comparison to the ultimate CH_4 yields for estimating the effects of the pretreatment within a reasonable duration of digestion. However, the effect of the AAS parameters on the ultimate CH_4 yields was also assessed in order to evaluate how the final biodegradability of the biomass was affected.

Two models were constructed based on the experimental results of the cCCD, namely Eqs. (3) and (4). The models constructed were highly significant ($p = 0.0001$) and the test of lack-of-fit was satisfactory ($p = 0.4177$). The effects that were found to be statistically significant to the responses ($p < 0.0500$) were the duration of AAS (x_2, $p < 0.0001$), the interaction effect of the duration and the NH_3 concentration ($x_1 * x_2$, $p = 0.0020$), and the quadratic effect of the S:L ratio (x_3^2, $p = 0.0086$). The R^2 (0.84) was in good agreement with the adjusted R^2 (0.77), indicating that the effects included in the models are sufficient for modelling the responses. The final models for predicting the CH_4 yield of AAS-treated manure fibers after 17 days of digestion are:

$$CH_4\ 17d = 195.499 + 3.774 * x_1 + 1.328 * x_2 - 1176.160 * x_3 - 0.050 * x_1 * x_2 + 2540.255 * x_3^2 \tag{3}$$

$$CH_4\ yield\ increase = 213.21 + 5.94 * x_1 + 2.10 * x_2 - 1882.07 * x_3 - 0.08 * x_1 * x_2 + 4056.78 * x_3^2 \tag{4}$$

where CH_4 *17d* and CH_4 *yield increase*, are expressed in mL/g TS and % as compared to NP fibers, respectively. All predictive models presented in this study, can be used for estimation of the CH_4 yield within the ranges of the experimental region, i.e. Eqs. (3) and (4) should be used for x_1, x_2 and x_3 values within the ranges [7, 25], [28, 96] and [0.16, 0.28] respectively.

The Response Surface graph of Eq. (3) is presented in Fig. 2*a*, where the predicted CH_4 yield is plotted as a function of the NH_3 concentration of the reagent and of the duration of AAS. The S:L ratio was set to a constant value of 0.16 kg/L as it was found not to interact significantly with the rest of parameters and corresponded to the optimum value. The duration of AAS appears to have had the strongest effect on the cumulative CH_4 yield, which is also depicted in the Response Surface graph, as the CH_4 yield rapidly increases along the axis of duration. On the other hand, the NH_3 concentration appears to have been important when the duration decreased down to 28 h of pretreatment. Lastly, the S:L ratio resulted in high CH_4 yield when set at the maximum or minimum level of the experimental range. This is expressed in the model by the quadratic term of this parameter (x_3^2). According to

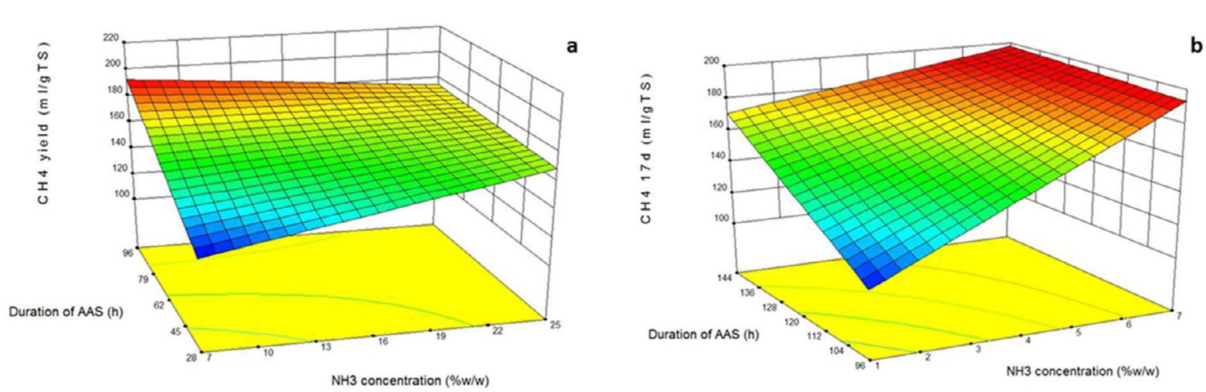

Fig. 2. Response surface graphs of a) 1st (cCCD) and b) 2nd (fCCD) set of optimization experiments. The cumulative CH_4 yield of swine manure fibers is plotted as a function of the ammonia concentration and the duration of AAS.

Fig. 2a, the optimal conditions of the AAS pretreatment of manure fibers for maximum CH_4 yield corresponded to 7% w/w NH_3 (aq.) and 96 h of duration of AAS and at a S:L ratio of 0.16 kg fibers/L reagent. The prediction of Eq. (3) for the CH_4 yield produced under these conditions, corresponds to 192.86 ± 11.14 mL/g TS, and based on Eq. (4) to a 206.60% ± 17.68 increase of CH_4 yield as compared to NP manure fibers. Finally, it was verified by RSM that the ultimate CH_4 yields (after 38 days of digestion) presented the same trends (results not shown).

AAS has been optimized in the past for other lignocellulosic biomasses mainly for sugar release and ethanol production. For instance, AAS of barley straw was optimized for maximizing sugar recovery and the optimal conditions were found to be 15% w/w NH_3, 77.6 °C, 12.1 h and 1:8 S:L ratio (Yoo et al., 2013). In the case of rice straw, the optimal conditions were 21% w/w NH_3, 69 °C and 10 h of AAS for maximizing enzymatic digestibility (Ko et al., 2009). Optimal conditions of AAS reported for oil palm empty fruit brunches (Jung et al., 2011) and for oil palm fronds (Jung et al., 2012) corresponded to 21% w/w NH_3, 60 °C, 12 h and 7% w/w NH_3, 80 °C, 20 h, 1:12 S.L ratio respectively for maximizing ethanol production. Generally, the optimal conditions of the pretreatment appear to depend highly on the biomass studied, though a comparison of optimal conditions for different bioconversion processes is not that straightforward. For instance, in ethanol fermentation, hemicellulose is not consumed by wild-type yeasts, while in anaerobic digestion processes it is converted into CH_4 (Barakat et al., 2012). Consequently, optimal conditions of AAS of the same biomass but for different desired products may differ. As commented in Section 1, ammonia pretreatment has been optimized in the past for biogas production only for wheat straw (Li et al., 2015) and the optimal conditions reported were 14.8% w/w NH_3, 51 °C and 27 h of pretreatment, leading to a 56% increase of biogas yield. While harsher conditions appear to be necessary for pretreating wheat straw in comparison to manure fibers, direct conclusions could be misleading as the parameters chosen to be optimized and their ranges differ among the two studies.

3.2. Effect of AAS parameters on solubilization of COD

The AAS pretreatment resulted in significant solubilization of the swine manure fibers. As shown in Table 1, the NP fibers presented a soluble COD of 0.12% of total COD, while for the pretreated fibers soluble COD varied between 7.25% and 14.62% of total COD depending on the conditions of AAS. The factors that affected the solubilization of COD were mainly the duration of AAS ($p < 0.0001$) and to a less extent the NH_3 concentration ($p = 0.0275$). On the other hand, the S:L ratio appeared not to affect the solubilization of manure fibers ($p > 0.05$). An increase of the two influencing factors produced an increase of the soluble COD measured. These findings are in line with previous studies where harsher conditions of AAS reduced the recovery of solids of pretreated rice straw (Ko et al., 2009). The model constructed was highly significant ($p < 0.0001$), and the test of lack-of-fit was satisfactory ($p = 0.1587$). The fit of the experimental data, while somewhat low ($R^2 = 0.77$), was in good agreement with the adjusted R^2 (*0.74*) indicating that the effects excluded from the model (S:L ratio and interaction and quadratic effects) did not provide significant information. The final model was:

$$solCOD = 6.73 + 0.074 * x_1 + 0.056 * x_2 \tag{5}$$

where *solCOD* is expressed in% of total COD with standard deviation equal to 1.02, and the ranges for x_1 and x_2 are [7, 25] and [28, 96] respectively.

Soluble COD, which serves as an indirect measurement of the solubilization of particulate matter, could be expected to indicate the CH_4 potential of a substrate the hydrolysis of which is the limiting step. Experiment 3 that produced the highest CH_4 yield, showed also the maximum soluble COD though not that different from experiment 12 (Table 1). Additionally, experiments 7, 13 and 14 yielded similar CH_4 as experiment 12, besides having lower soluble COD values. According to Eq. (5), the maximum soluble COD would correspond to harsh AAS conditions while the maximum CH_4 yield corresponded to milder conditions and resulted to be dependent on more factors (see Section 3.1). Therefore, a lack of correlation among soluble COD and CH_4 yield can be hypothesized, a conclusion to which other studies in the field of anaerobic digestion have also reached (Tsapekos et al., 2015). Anaerobic digestion is a relatively slow process with HRTs of usually > 15 days, and partly solubilizing the particulate matter might indicate improvement only of the initial conversion rate. On the other hand, the facilitated access to structural carbohydrates due to a pretreatment is not accounted for when considering only the initially solubilized fraction. Hence, only a slight effect on the structure of the biomass can result in an increased CH_4 yield without observation of significant solubilization. However, soluble COD is an interesting factor to take into account for the configuration of the process. For instance, in order to

Table 1
Experimental conditions and results of soluble COD, cumulative CH_4 yields and CH_4 yield increase of AAS-treated fibers from the cCCD experiments.

No of experiment	NH_3 concentration (% w/w)	Duration of AAS (hours)	S:L ratio (kg fibers/L reagent)	% soluble COD	CH_4 yield 17d (mL CH_4/g TS)[1]	% increase CH_4 yield 17d[1]
1	7	28	0.16	8.31	126.64 ± 11.52	101.31 ± 18.31
2	25	28	0.16	10.75	159.56 ± 8.11	153.65 ± 12.89
3	7	96	0.16	14.62	213.96 ± 15.04	240.12 ± 23.91
4	25	96	0.16	12.53	173.23 ± 5.40	178.78 ± 8.59
5	7	28	0.28	9.47	126.84 ± 3.55	101.63 ± 5.65
6	25	28	0.28	9.54	160.87 ± 4.89	155.73 ± 7.78
7	7	96	0.28	11.89	176.27 ± 3.93	180.21 ± 6.24
8	25	96	0.28	13.39	162.47 ± 3.90	158.27 ± 6.20
9	0.9	62	0.22	8.89	130.06 ± 2.28	106.74 ± 3.62
10	31.1	62	0.22	13.17	173.60 ± 5.47	175.97 ± 8.70
11	16	4,8	0.22	7.25	117.38 ± 4.49	86.59 ± 7.13
12	16	119.2	0.22	14.17	174.29 ± 8.74	177.07 ± 13.90
13	16	62	0.12	12.17	179.05 ± 6.28	184.63 ± 9.98
14	16	62	0.32	12.99	178.50 ± 9.98	183.72 ± 15.86
15	16	62	0.22	12.16	170.74 ± 5.17	171.41 ± 8.22
16	16	62	0.22	11.80	159.66 ± 6.23	153.80 ± 9.90
17	16	62	0.22	10.63	153.11 ± 8.27	143.39 ± 13.15
18	16	62	0.22	10.85	143.15 ± 6.40	127.56 ± 10.18
19	16	62	0.22	11.69	155.48 ± 12.84	147.16 ± 20.41
NP	–	–	–	0.12	62.91 ± 3.49	–

[1] Values correspond to average values from triplicates ± the standard deviation.

reduce the volume of the pretreated fibers that need to pass through an NH_3 removal step, a separation of the solids could be preferable. In such case, the soluble COD that would mainly remain in the liquid fraction may indicate the loss of biomass that will occur from such a separation, affecting the final CH_4 potential of the pretreated biomass. In this line, depending on the configuration of the pretreatment process, one could aim at maximizing the CH_4 yield while keeping the soluble COD at minimum levels. It is important to mention here that the models produced in this study express the specific system and might not be apt for describing a wider application of AAS to Manure fibers of different origin. Nevertheless, these can be used for assessing general trends as well as the existence or not of interaction effects, information that can be valuable for the design of the process configuration.

3.3. Extension of optimization experiments (fCCD)

Given that the optimal conditions for maximizing the CH_4 yield of AAS-treated fibers were found on the edge of the experimental area (minimum NH_3 concentration and maximum duration of AAS tested), a second optimization step took place where the ranges of the interacting parameters of AAS (NH_3 concentration and duration) were further investigated towards the optimum region following a faced CCD (fCCD). The S:L ratio was kept constant to the optimum value of 0.16 kg/L, as it was found not to interact with the rest of parameters.

Based on the cumulative CH_4 yield after 17 days of digestion of the second set of batch tests (Table 2), two new models were constructed for the experimental region covered by the fCCD (Eqs. (6) and (7)). Eq. (6) corresponds to the empirical model constructed for predicting the cumulative CH_4 yield after 17 days of digestion of the AAS-treated fibers and Eq. (7) to the prediction of the *CH_4 yield increase* as compared to the NP fibers. According to the ANOVA results for the constructed models, the most influencing effect in this range of AAS parameters was the NH_3 concentration of the reagent (x_1, $p = 0.0006$), followed by the effect of the duration (x_2, $p = 0.0110$) and lastly by the interaction effect of these parameters (x_1*x_2, $p = 0.0485$). The models were significant ($p = 0.0011$) and no lack-of-fit was detected ($p = 0.1770$). The fit of the experimental data to the models was found to be satisfactory ($R^2 = 0.85$) and in good agreement with the reduced models (*adjusted* $R^2 = 0.80$).

$$CH_4\ 17d = -52.409 + 33.809*x_1 + 1.519*x_2 - 0.203*x_1*x_2 \quad (6)$$

$$CH_4\ yield\ increase = -198.87 + 63.91*x_1 + 2.87*x_2 - 0.38*x_1*x_2 \quad (7)$$

The high influence of the NH_3 concentration on the response can also be observed in Fig. 2*b* where the Response Surface graph of Eq. (6) is depicted; the CH_4 yield increases rapidly when the NH_3 concentration increases from 1 to 7% w/w. On the other hand, the duration of AAS appears to affect the CH_4 yield significantly mostly at low NH_3 concentrations, while at the maximum concentration tested in this design (7% w/w) the duration of AAS does not affect significantly the CH_4 yield when varied from 96 to 144 h. This observation clearly indicates that the influence of the parameters depends greatly on the ranges chosen for optimization. Even though the duration that resulted at the maximum CH_4 yield would correspond to 144 h, the experimental difference found in comparison to 96 h was 3 mL CH_4/g TS (and 5 mL/g TS predicted by Eq. (6), making the difference insignificant. Thus, it appears that the optimal conditions of AAS as resulted from the 2nd set of optimization experiments correspond to the same optimum with the 1st set of experiments (cCCD), that is 7% w/w NH_3 and 96 h. Based on Eq. (6) a cumulative CH_4 yield of 193.43 ± 12.59 mL/g TS would result under these conditions, which is in line with the prediction of the first model Eq. (3) at the same conditions. According to Eq. (7), this corresponds to a 265.92% ± 23.84 increase of CH_4 yield as compared to the yield of NP manure fibers. The prediction of Eq. (7) lies closer to the values obtained experimentally as compared to the prediction of Eq. (4).

In order to validate the models, experiments under optimal conditions were repeated and the average CH_4 yield observed was 190.05 ± 6.70 mL/g TS, which is in line with the predictions of Eqs. (3) and (6). Taking into account all the experiments run under optimal conditions (cCCD, fCCD and Validation experiments) the average CH_4 yield observed was 198.95 ± 9.49 mL/g TS (274.56 mL/g VS), corresponding to a 243.73% increase of the CH_4 yield after 17 days of digestion. Based on total COD measurements and assuming a CH_4 yield of 0.35 m^3/kg O_2, it was calculated that the theoretical CH_4 yield of the manure fibers used in this study corresponded to 283.15 mL/g TS (421.66 mL/ g VS). Thus, considering a yield of 185.37 mL/g TS (255.81 mL/g VS) at STP conditions (0 °C, 1 atm) the AAS-treated manure fibers reached a 65.5% of the theoretical CH_4 yield in only 17 days of digestion under optimal conditions.

Based on the results from both optimization designs, all three AAS parameters appeared to be influencing on the resulted CH_4 yield in some way. Generally the interaction among the duration of the pretreatment and the concentration of NH_3 was pronounced along the entire experimental region. It seems that in order to decrease the NH_3 concentration needed for a successful AAS process, the duration has to be increased and vice versa. In a large scale application though, it is more likely that the reagent concentration will limit the process configuration rather than the duration of AAS. High durations of pretreatment would be translated to an increased volume of pretreatment vessel, or to the need for additional pretreatment vessels running in parallel, affecting thus mainly the initial investment for the implementation of AAS. However, low reagent concentrations lead to easier handling, as well as to an easier target to fulfill in case a surplus of NH_3 is requested (Lymperatou et al., 2015b). On the other hand, the effect of the S:L ratio resulted to be independent of the other parameters, although highly significant. The highest S:L ratio tested in this study corresponded to the minimum volume of reagent in order to ensure the entire biomass was soaked. Thus a further increase would result to a partially pretreated batch, which is undesirable. On the other hand, the low S:L ratio is expected to be more expensive to perform, as the majority of the pretreatment mixture volume corresponds to the reagent. However, the NH_3 removal step of a mixture with low solids would probably be facilitated. All in all, the interaction of the NH_3 concentration with the duration of AAS presents some flexibility on how the pretreatment can be applied.

3.4. Compositional changes of optimally-pretreated Manure fibers

Ammonia, as an alkaline reagent, is known to produce partial delignification and occasionally swelling of the lignocellulosic structure. In the present study, no apparent delignification occurred on manure

Table 2
Cumulative CH_4 yields and CH_4 yield increase of AAS-treated fibers from the fCCD as resulted after 17 days of digestion.

No of experiment	NH_3 concentration (% w/w)	Duration of AAS (hours)	CH_4 yield 17d (mL CH_4/g TS)[1]	% increase CH_4 yield 17d[1]
1	1	96	104.17 ± 7.07	97.06 ± 13.38
2	7	96	192.85 ± 6.73	264.84 ± 12.72
3	1	144	163.23 ± 6.04	208.80 ± 11.43
4	7	144	193.41 ± 10.47	265.90 ± 19.80
5	1	120	135.75 ± 6.94	156.82 ± 13.14
6	7	120	186.19 ± 8.51	252.24 ± 16.11
7	4	96	136.14 ± 8.35	157.55 ± 15.80
8	4	144	178.07 ± 5.66	236.88 ± 10.72
9	4	120	180.85 ± 8.42	242.13 ± 15.93
10	4	120	190.09 ± 4.98	259.61 ± 9.42
11	4	120	178.86 ± 11.99	238.36 ± 22.67
12	4	120	170.45 ± 8.89	222.46 ± 16.82
NP	–	–	52.86 ± 11.47	–

[1] Values correspond to average values from triplicates ± the standard deviation.

Table 3
Composition of raw Manure fibers (NP) and optimally AAS-treated Manure fibers.[1]

Composition	NP manure fibers (% TS)	AAS-treated manure fibers (% $TS_{initial}$)
Glucan	27.20 ± 0.14	27.88 ± 1.03
Xylan	16.04 ± 0.28	10.58 ± 0.45
Arabinan	5.71 ± 0.20	3.10 ± 0.22
Total structural carbohydrates	48.95 ± 0.34	41.57 ± 1.69
Acid-insoluble lignin	16.30 ± 0.68	16.35 ± 0.81
Acid-soluble lignin	0.33 ± 0.00	0.38 ± 0.00
Extractives & volatiles	9.10 ± 0.17	12.88 ± 2.70
Free sugars	0.09 ± 0.00	0.09 ± 0.03
Soluble sugars	0.08 ± 0.00	0.68 ± 0.03
Acetic Acid	0.03 ± 0.00	0.68 ± 0.01
NH_4^+-N content	0.37 ± 0.00	0.98 ± 0.00
Soluble Organic N	0.80 ± 0.14	1.72 ± 0.06
Non-soluble organic N	1.61 ± 0.01	1.45 ± 0.06
C/N ratio	14.37 ± 0.25	11.85 ± 1.01
Total Ash	32.62 ± 1.34	29.81 ± 1.52
Solid Recovery	–	98.65

[1] Values correspond to average values from replicates ± the standard deviation. Solid Recovery was calculated as g TS after pretreatment divided by the g TS before treatment and multiplied with 100. C/N ratio and Solid recovery values are unitless.

fibers, and the lignin content in both the control and the pretreated biomass accounted for ca. 16.3% TS (Table 3). Interestingly, when harsher conditions of AAS (32% w/w NH_3) were applied to swine manure fibers, a similar observation was reported (Jurado et al., 2013b). Delignification is often set to be the principal goal of a pretreatment as the bonding of lignin with hemicellulose and cellulose presents a barrier for enzymatic attack. Nonetheless, it has been reported that accessibility to cellulose is more important than actual removal of lignin for improving digestibility (Rollin et al., 2011). A correlation between lignin removal and temperature increase has been reported by different authors (Li et al., 2015; Yoo et al., 2013). The low temperature applied during AAS could partially explain why no lignin removal was observed in this study. However, AAS might affect differently each type of lignocellulosic biomass. For instance, (Antonopoulou et al., 2015) observed partial lignin removal in sunflower straw after application of AAS at ambient temperature, in contrast to grass and poplar where no apparent delignification occurred under the same conditions.

The cellulose fraction of the biomass also seems not to have been affected by AAS, as the glucan content of both biomasses (Table 3) was similar (assuming that all glucose was derived from cellulose). This is in line with the observations of other studies (Kim et al., 2008; Li et al., 2015). On the contrary, solubilization of hemicellulose was observed during the NH_3 treatment, as the xylan and arabinan contents were significantly reduced in the pretreated biomass (10.58% TS and 3.10% TS as compared to an initial 16.04% TS and 5.71% TS of xylan and arabinan respectively). This is also evident from the increase of soluble sugar concentration detected in the liquid fraction of the pretreated biomass, as well as from a significant increase of the acetic acid content (Table 3). The reduction of the insoluble organic N found in the pretreated biomass indicates that a slight solubilization of proteins might have occurred, whereas the significant increase of soluble organic N found (1.72% TS as compared to 0.80% TS in the NP fibers), could be attributed to the formation of nitrogenous compounds from the reaction of the NH_3-N reagent and the biomass. More studies have shown an increase of organic N in AAS-pretreated biomasses (Mirtsou-Xanthopoulou et al., 2014; Song et al., 2012). A description of possible reactions that can occur during NH_3-based pretreatments of lignocellulosic biomasses is presented in Chundawat et al., (2010). Further investigation of the fate of the reagent-N should be carried out in order to better understand how NH_3 interacts with the organic substances present in the Manure fibers.

In conclusion, the mechanism of AAS appears not to be the same for all lignocellulosic biomasses. In comparison to the delignification effect of AAS on other biomasses, the AAS pretreatment appears to have a mild effect on manure fibers producing though surprisingly high increases of the CH_4 yield. A future systematic optimization of the same AAS parameters and ranges of different lignocellulosic biomasses for anaerobic digestion could further contribute on understanding the mechanism of this pretreatment on different lignocellulosic biomasses. Additionally, the identification of common characteristics of the biomasses that better respond to AAS could be assisted.

3.5. Comparison of pretreatments on increasing the CH_4 yield of manure fibers

Many different approaches have been tested so far for increasing the CH_4 yield of swine manure fibers. The majority of studies have focused on thermal pretreatments as these pose certain advantages such as short duration, inactivation of pathogens, and energy requirements can be reduced if the residual heat from associated Combined Heat and Power (CHP) plants is exploited. Raju et al. (2013) reported a 29% increase of CH_4 yield by thermal pretreatment of manure fibers at the range of 100–225 °C. Menardo et al. (2011) reported a 171% increase by pretreating swine manure fibers at 120 °C for 30 min. Ferreira et al. (2014) investigated the effect of thermal explosion of manure fibers under different combinations of temperature (120–180 °C) and duration (15–60 min), and demonstrated a 107% increase of CH_4 yield for 170 °C and 30 min pretreatment. Other pretreatments tested include mechanical, chemical, and biological processing; Hjørth et al. (2011) tested extrusion as a method for increasing the digestibility of the solid fraction of manure and reported an increase of 27% of cumulative CH_4 yield of pretreated Manure fibers (both swine and cattle manure fibers). A biological pretreatment of fiber-rich swine manure for biogas production was reported to produce a 55% increase of CH_4 yield (Tuesorn et al., 2013). González-Fernández et al. (2008) compared the effectiveness of an acidic and an alkaline pretreatment and reported a negative effect of the acid to the CH_4 yield of pretreated fibers while the increase achieved by NaOH treatment was 13%.

From the work presented here, it appears that AAS has the potential to unlock the CH_4 potential of swine manure fibers at a great degree. Nevertheless, it is important to stress that not all pretreatments have been performed under optimal conditions. While a possible application of AAS of manure fibers in a larger scale would still need further investigation, especially in regards to the NH_3 recovery technology to be applied, a continuous process of manure mono-digestion enriched with AAS-treated fibers can further verify the efficiency of the process. While batch tests can facilitate optimization goals in regards to pretreatment conditions, experiments on continuous mode are more appropriate for evaluating real applications (Carrère et al., 2016). Previous work on a continuous anaerobic digester of manure enriched with manure fibers treated with 32% w/w NH_3 led to a 98% increase of CH_4 yield of manure fibers (Jurado et al., 2016). Based on the present study, AAS of manure fibers could further improve the CH_4 yield by using considerably milder conditions than previously thought.

4. Conclusions

Optimization experiments of AAS through RSM revealed a strong interaction among the NH_3 concentration and the duration of AAS. The optimal conditions of AAS at ambient temperature corresponded to 7% w/w NH_3 (aq.), 96 h of AAS, and 0.16 kg fibers/L reagent, resulting to a 244% increase of the CH_4 yield in only 17 days of batch digestion. The degree of solubilization of the biomass increased with increased severity of AAS and compositional analyses showed that significant solubilization of hemicellulose occurred during optimized AAS, while no delignification or loss of the cellulose fraction was observed.

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