The influence of different pretreatment methods on biogas production from *Jatropha curcas*oil cake.

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Abstract

Drought and pest resistance, together with high oil content in its seeds, make*Jatropha curcas*a good oil source for biodiesel. Oil cake from *J. curcas*is not suitable for animal feeding and thus may be profitably used for additional energy production by conversion into biogas; however, the anaerobic digestion process must be optimized to obtain good efficiency.We subjected oil cake to thermal and acidic pretreatment to deactivate protease inhibitors and partially hydrolyze phytate. We then digestedthe samples in batch conditions to determine the effects of pretreatment on biogas production. Thermal pretreatment changed the kinetics of anaerobic digestionand reduced protease inhibitor activity and the concentration of phytate; however, biogas production efficiency was not affected.To evaluate the possibility of recirculating water for SSF hydrolysis,ammonium nitrogenrecovery from effluent was evaluated by its precipitation in the form of struvite(magnesium ammonium phosphate).We proposea water-saving concept based on percolation of *J. curcas*cake using anaerobic digestion effluent and feeding that percolate into a methanogenic bioreactor.

1. Introduction

Growing world energy demand and the incoming depletion of fossil fuels has increased interest in development of renewable energy sources. Renewable fuels may be obtained from biomass by anaerobic digestion and transesterification of vegetable oils. Due to its advantages (such as drought and pest resistance and high oil content in its seeds),*Jatropha curcas* is considered a suitable candidate for biodiesel production(Achten et al., 2007). The oil cake remaining after the removal of oil, however, cannot be utilized as a fodder due its toxicity(Martínez-Herrera et al., 2006);production of additional energy from this substrate seems a reasonable solution. Several technologies may be used to achieve this: anaerobic digestion, alcohol fermentation, pyrolysis, and combustion(Liang et al., 2010; Şen and Kar, 2011; Staubmann et al., 1997). The two latter methods offer high energy recovery, butanaerobic digestion enables the recovery of nitrogen from this material in the form of ammonium ions, which may be used as fertilizer(Batstone and Virdis, 2014). Nitrogen recovery is valuable, especially in developing countries with limited access to synthetic fertilizers.

Anaerobic digestion of *J. curcas*oil cake was previously attempted(Chandra et al., 2012; Staubmann et al., 1997); surprisingly, the biogas production efficiency obtained in those experiments was low compared to results obtained for oil cakes derived from other plants (Jabłoński et al., 2015).It may be a result of high concentration of lignin and cellulose in *J. curcas*seed's shells (3). Oil cake derived from seeds with shells contain up to 20 % of lignin and 20 % of cellulose. Since this biopolymers are hardly biodegradable in anaerobic

conditions biogas production would require pretreatment, allowing more efficient utilization of this biopolymers.

Plants from the *Jatropha* genus contain many substances regarded as antinutritional and toxic(Martínez-Herrera et al., 2006), including phytate and protease inhibitors that reduce animals' digestion rate of *Jatropha* seeds' biomass (Makkar et al., 1998). The concentration of these two compounds in *J. curcas*seeds is much higher than in oil cakes derived from other oil plants. Since the digestion process in animal intestinal tracts, in many aspects, resembles the anaerobic digestion of organic matter, it is probable that protease inhibitors and phytate may cause reduced biogas production efficiency from *Jatropha* oil cake.

Since many protease inhibitors are proteins, their activity may be reduced by denaturation after thermal treatment, which can also partially degrade phytate. Thus the thermal pretreatment of *J.curcas* oil cake may positivelyaffect the anaerobic digestion of this substrate.

Another problem concerning the anaerobic digestion of *J.curcas* in dry regions of the world is the demand for processed water;traditional wet digestion would consume a significant amount of water. The solution to this problem may be application of a combined digestion system, in which water is used in a closed circuit(Kothari et al., 2014);however, in the case of protein-rich oilcake digestion, the resulting accumulation of ammonia will inhibit digestion (Yenigün and Demirel, 2013). Thus, ammonium nitrogen removal is required. One possible solution is the precipitation of ammonium ions in the form of struvit mineral, which may then be recovered and used as a fertilizer(Uysal et al., 2010). Struvit is a mineral salt ($(NH_4)Mg[PO_4]x\ 6\ H_2O$) formed at aboutpH 9.

Optimal utilization of *Jatropha*cake should exploit all potential biomass components (chemical energy and minerals) with minimal energy input and water usage. This could be achieved by: optimization of pretreatment methods, increasing the efficiency of biogas production; recovery of minerals used as fertilizer from the effluent and waterrecirculation within the system; and the use of the remaining biomass and the recovered minerals as fertilizer.The aim of this work was to evaluate different pretreatment methods on the protease inhibitors and phytate anddetermine the kinetics and efficiency of biogas production from *J.curcas* oil cake. The recovery of ammonium nitrogen fromthe effluent was evaluated by struviteprecipitation. We then proposed the concept of water-saving digestion technology.

2. Materials and methods

2.1.*Jatropha curcas oil cake*

J.curcas oil cake was obtained from the Chemical Department of the WrocławUniwersity of Technology (WUoT). *J.curcas* seeds were purchased from EKOMOTOR Sp. z o.o. in 2011. The oil from *J.curcas*whole seeds(with shells) was removed by cold pressing in the laboratory press in the Chemical department of WUoT. After oil pressing, the oil cake was ground in a MKM 6000 grinder (BOSH). This material was used for further experiments.

2.2. Oil cake pretreatment

16.7 g samples of oil cake were suspended in 33 mL of 0.1 mol·L^{-1}HCl or in 33 mL of 0.1 mol·L^{-1}NaCl. The samples were then incubated for 1 hour at 20°C, 70°C, or 100°C or for 15 minutes at 115°C. After incubation, the samples were cooled to room temperature. NaOH was added to the samples containing HCl to neutralize the solution. The samples were stored at -20°C until further use.

2.3. Anaerobic digestion of pretreated samples

Anaerobic biodegradation tests were prepared in 120 mL serum bottles. FiftymLof the inoculum and 0.5 g of the substrate sample were placed in each serum bottle (in control bottles, the substrate was omitted). The inoculum material was obtained from a laboratory anaerobic reactor fed with cow manure. In the next step, the air was removed from the bottles by flushing them with nitrogen gas. The digestion test took place at 37°C. The samples were stirred, every 24 hours, just before the gas measurements. Produced gas was measured every 24 h across 21 days by water displacement(Kida et al., 2001). All the samples were prepared in quadruplicate. The amount of biogas produced from biomass was calculated as the difference between the production in the sample bottles and the production in the control bottles. Biogas volumes were calculated for standard state (100kPa, 273.15K).

2.4. Precipitation of struvite

For the precipitation of struvite, solid particles were removed from the sludge by centrifugation (10,000 RCF for 10 minutes). Supernatant was transferred to fresh test tubes and stored at 4 °C before further use. For precipitation experiments,1 mol · L^{-1}solutions of $MgCl_2$ and Na_2HPO_4 were prepared. To precipitate struvite, 1 mLof MgCl solution and 1 mLof Na_2HPO_4were added to 10 mLof supernatant and NaOH solution (40% w/w) was added to obtain a pH of9. After the precipitation occurred, the total nitrogen concentration in the solution was determined.

2.5. Oil cake composition analysis

The dry weight, ash, and volatile solids were prepared by standard methods for the examination of water and wastewater(Clesceri et al., 1998). The organic nitrogen was prepared according to the Kieldahl method(Persson, 2008). The total lipid concentration was determined by extraction of dry samples with petroleum ether, 60/80 fraction (from POCH), in a Soxleth apparatus. 0.5 g of sample was dried at 105 °C for 4 hours, placed in a Soxleth apparatus, and extracted with 100 mLof petroleum ether for 3 hours. The solvent was removed from the extract with a vacuum evaporator. The obtained extract was weighted on analytical scales. Dietary fiber analysis was performed with detergent extraction method (van Soest, 1967).

2.6. Determination of trypsin inhibitor activity

Protease inhibitor activity was evaluated bymeasuring trypsin activity in a casein assay in the presence of oil cake extracts. To obtain extracts, 5 mL of 0.9% NaClwere added to 5 mL of oil cake suspension after

pretreatment. The samples were incubated at 20 °C for 30 minutes with shaking and then centrifuged at 20,000 RCF for 10 minutes. The obtained supernatant was filtered through a 0.22 µm filter and transferred to a new test tube. Samples were stored at 4 °C for 3 days before further analysis.

The trypsin activity was determined as follows. 50 µL of trypsin solution (80 $\mu g \cdot mL^{-1}$) in 1 $mmol \cdot L^{-1}$HCL and 80$mmol \cdot L^{-1}$ $CaCl_2$were mixed with 25 µL of 0.1$mol \cdot L^{-1}$ $CaCl_2$, 50 µL of diluted oil cake extract, and 175 µL of 0.2 $mol \cdot L^{-1}$Tris/HCL buffer (pH=7.5). The samples were incubated at 37 °C for 5 minutes, after which 500 µL of 0.5% casein solution were added to the samples. The reaction was stopped after 15 minutes by addition of 500 µL of 10% trichloroacetic acid (TCA); in blank samples, the TCA solution was added before the casein solution. Afterward, the samples were centrifuged for 10 min at 20,000 RCF at 4 °C. After centrifugation, the absorbance of the supernatant was measured at 275nm (Cary Bio-50 Spectrophotometer (Agilent)). The amount of released tyrosine was calculated on the basis of the calibration curve, and the trypsin activity was calculated. 1 U of protease activity was defined as the amount of enzyme releasing 1 µmol of tyrosine per minute.

2.7. Determination of phytate concentration

Determination of the concentration of phytate in oil cake samples was carried out according to the colorimetric method described by Latta and Eskin(Latta and Eskin, 1980). Pre-treated oil cake was diluted with water (1:1); 6 mL of this suspension were added to 15 mL of 3.2% HCl. The samples were incubated for 1 hour at 28°C with shaking (2.5 Hz). After incubation, samples were centrifuged for 15 minutes at 20,000 RCF at 4 °C. The clear supernatant was filtered through a 0.22 µm filter and transferred to a new test tube. In the next step, samples were extracted with petroleum ether (1:4 ether to sample ratio) to remove lipids and precipitated proteins. After extraction, samples were centrifuged for 5 minutes at 20,000 RCF. The clean water phaseswere then transferred to new micro-test tubes. Before the preparation of the colorimetric assay, the samples were diluted in water (1:10 dilution). To determine the concentration of phytate, 250 µL of the diluted extracts were added to 750 µL of modified Wade reagent (0.03% $FeCl_3x6H_20$ and 0.3% sulfosalicylic acid in distilled water). The absorption of the solution was measured at 500 nm (DR/2500 Spectrophotometer (Hach)). The concentration of phytate was calculated on the basis of the calibration curve prepared with phytic acid, sodium salt hydrate (Sigma-Aldrich).

2.8. Determination of total nitrogen concentration

The concentration of total nitrogen in digestion fluid was determined by the photometric method based on the 2,6-dimethylphenol reaction. Reagent set Total nitrogen (TN_b 22) was purchased from Machery-Nagel GmbH. Samples were diluted with distilled water to fit the method's concentration range. The analysis wasperformed according to the manufacturer's instructions.

2.9. Calculation of theoretical biogas production

For the calculation of the maximal theoretical volume of biogas which can be obtained from 1 kg of substrate, the following equation was used:

$$V = V_{mol} \sum C_i C_{Ci}$$

where:

V – volume of biogas,

C_i – concentration of the biopolymer in substrate (1-proteins, 2-carbohydrates , 3-lipids),

C_{Ci}- concentration of carbon in the polymers (C1-proteins, C2-carbohydrates , C3-lipids),

Vmol – volume of 1 mol of gas at standard conditions (22.7 L at 100 kPa, 273.15 K).

3. Results and discussion

3.1. Theoretical biogas production

The composition of oil cake derived from *J.curcas* is similar to materials obtained from other oil crops (Table1). All oil cakes contain low amounts of water and ash and a high amount of volatile matter. *Jatropha* oil cake contains fewerprotein (19.9%) than the other two plantscommonly used to generate oil cakes (rape, 30.1 %, and flux, 36.3%) and fewer lignin and cellulose fibers. In all oil cakes, the concentration of lipids is around 10%. The most important difference is the concentration of lignin. In case of *J. curcas* lignin stands for about 20 % of total weight of oil cake. In two other oil cakes concentration of lignin is much lower.

Table 1. Composition of oil cake derived from different plants

Plant	*Jatropha curcas*	Rape (*Brassica napus*)	Flux (*Linumusitatissimum*)
Dry weight	93.0%	90.5%	91.8%
Ash	5.6%	6.1%	4.9%
Volatile solids	87.4%	84.4%	86.9%
Protein	19.9%	27.3%	33.3%
Lipids	8.0%	10.1%	10.7%
Lignin	19.5%	8.2%	2.5%
Cellulose	27.9%	14.5%	12.5%
Other carbohydrates	12.1%	24.3%	27.9%

Based on the composition presented in Table 1, the theoretical maximum biogas volume obtained from 1 kg was calculated (Table 2); the theoretical biogas production from *J. curcas*oil cake is lower than the volumes obtained for flux and rape due to the lower content of protein in this substrate (carbohydrates contain less carbon than proteins).

Table 2. Biogas production from different oil cakes.

	Theoretical [$m^3 \cdot kg^{-1}$]	Experimental [$m^3 \cdot kg^{-1}$]	Efficiency
Jatropha curcas	0.616	0.281	45.6%
Rape (*Brassica napus*)*	0.711	0.507	71.3%
Flux (*Linumusitatissimum*)*	0.792	0.545	68.8%

* - data from (Jabłoński, 2014)and(Jabłoński et al., 2015)

The biogas produced in the laboratory experiments, for all plants, was lower than the theoretical values calculated in Table 2. Production efficiency from flux and rape was around 70% of the theoretical value, probably caused by partial use of organic matter as a building material for microorganisms' cells. Some carbon dioxide remained dissolved in the liquid or was converted into bicarbonate. The production from *Jatropha* oil

cake was,however, much lower, around 45% of the theoretical value, which cannot be explained by carbon dioxide dissolution or carbon use by microorganisms.

3.2. Protease inhibitor activity

In all samples that underwent pretreatment, the activity of trypsin inhibitors was determined. The extract obtained from the sample incubated in 0.1 mol·L^{-1}NaCl at 20°C showed considerablyless activity (Figure 1). The trypsin activity was reduced by 63% comparedto the reaction conducted without extracts (p value 0.042%). The inhibitory activity of the samples incubated at 20°C with HCl, 70°C with HCl or NaCl, and 100°Cwith HCl or NaClwas statistically identical to that observed in the sample incubated with NaCl at 20 °C. The inhibitory activity in samples incubated at 115 °C with HCl or NaCl was statistically lower than in untreated samples; however, the trypsin activity was still lower than in the control.The trypsin inhibitors present in *J.curcas* seeds are thermostable and are deactivated only by temperatures exceeding 100 °C.

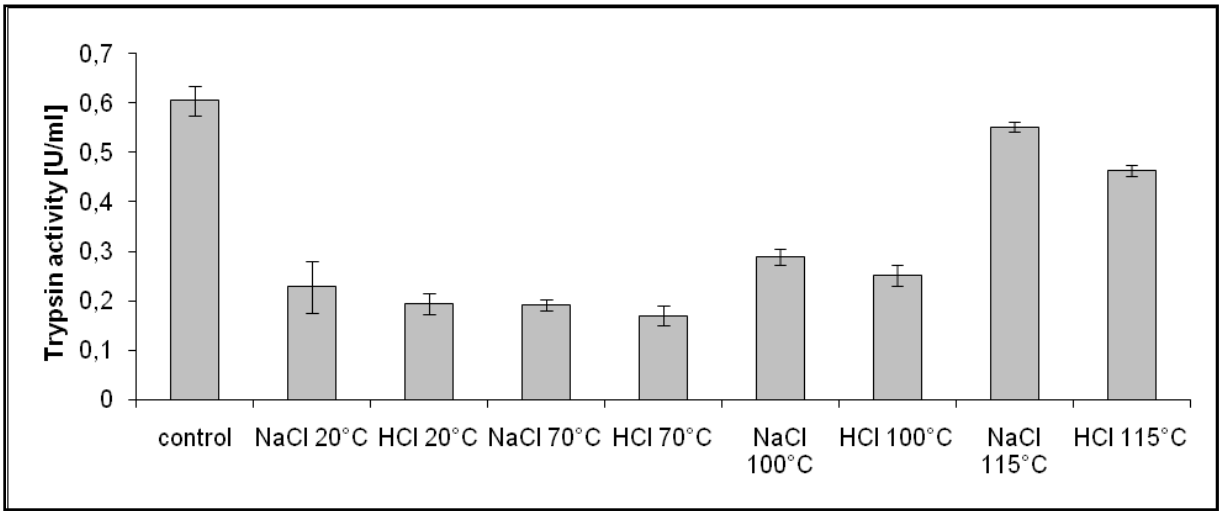

Figure 1. The trypsin inhibitory activity of extracts obtained from samples after different pretreatments.

3.3. Phytate concentration

The concentration of phytate in samples incubated with NaCl at 20°C and with HCl at 20°C was around 10.5% (Figure 2). The concentration of phytate in all other samples was significantly lower, varying between 6.0% and 6.6%. The concentrations of phytate in samples were similar to previously-reported values (Martínez-Herrera et al., 2006). The thermal treatment at temperatures as low as 70 °C effectively reduced phytate in *J.curca*oil cake.Moreover, the application of NaCl or HCl seems to be more efficient than pretreatment with $NaHCO_3$to reduce phytate in *J.curcas* oil cake(Martínez-Herrera et al., 2006).

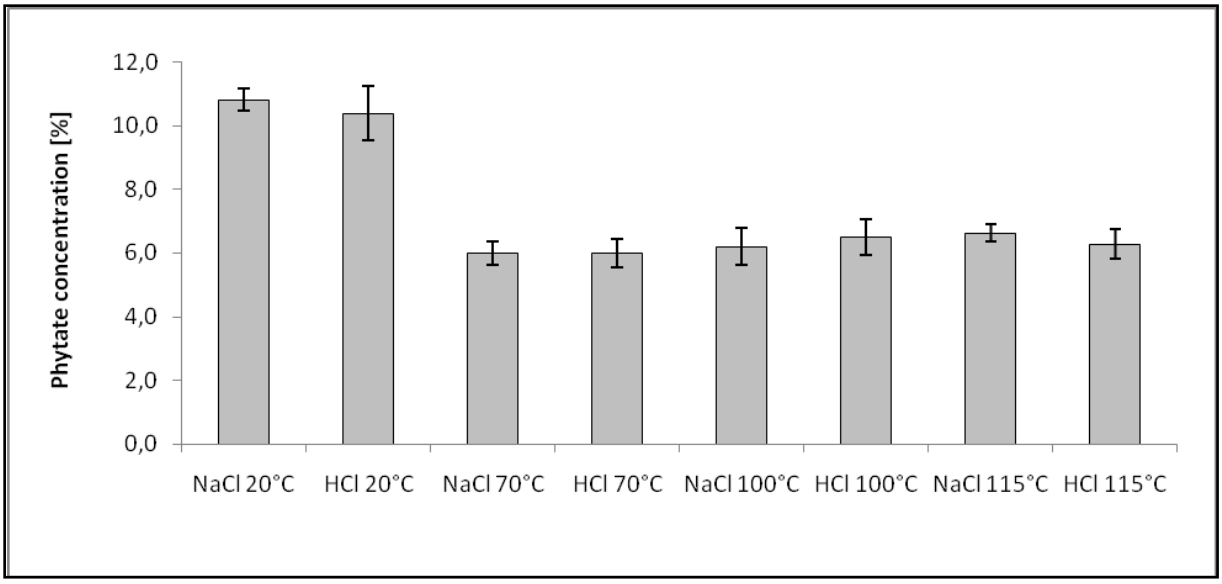

Figure 2. The concentration of phytate in samples after pretreatment (calculated as a percent of dry mass of sample).

3.4. Anaerobic digestion of oil cake

The biogas production from oil cake after pretreatment was based on batch fermentation experiments. Biogas production rates after different pretreatment methods are presented in Figure 3.

The fastest fermentation rate was observed when oil cake was incubated in NaCl at 20 °C; a similar fermentation pattern was observed when samples were incubated at 115 °C with NaCl. In these two cases,the biogas production rate rapidly increased from 5 $cm^3 \cdot day^{-1}$ to 25 $cm^3 \cdot day^{-1}$between the 1st and 5th days of fermentation. After this period, a sharp decrease in biogas production rate was observed. In samples incubated with NaCl at 70 and 100 °C, the initial biogas production rate increase took place between the 1st and 5th days of fermentation (Figure 3A), after which it remained relatively stable at 15 $cm^3 \cdot day^{-1}$ until the 12th day of fermentation;after that, biogas production rapidly decreased.

For all samples incubated with HCl, the biogas fermentation rate rose between the 1st and 5th days of fermentation to reach 17 $cm^3 \cdot day^{-1}$; this remained stable until the 9th day of fermentation (Figure 3B). A rapid decrease in biogas production rate was observed between the 9th and 12th days of fermentation.

Total 21-day biogas production from oil cake after different pretreatmentsis presented in Figure 5. While the biogas production rate showed differences between samples, the total biogas volume was statistically equal for all samples.

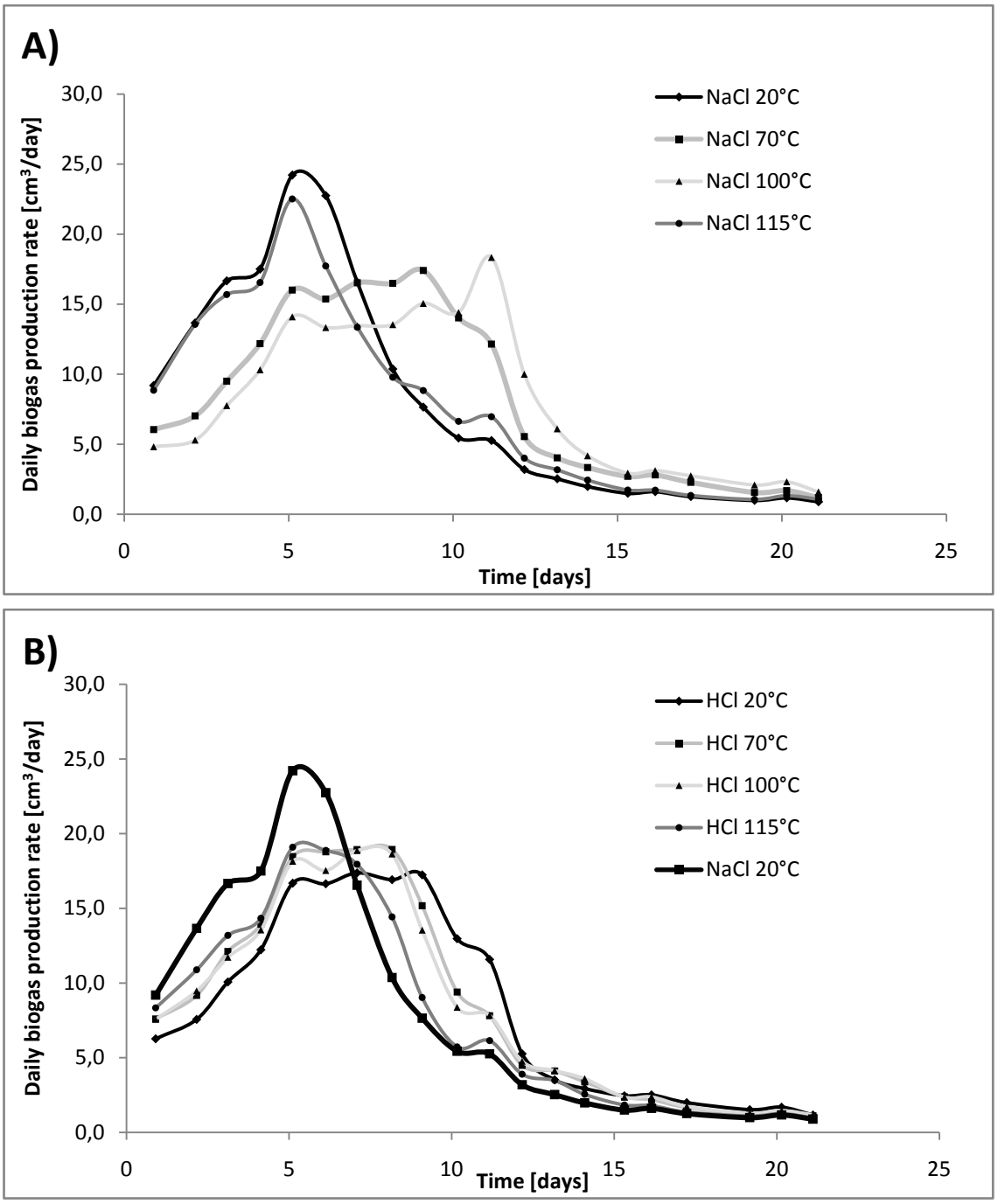

Figure 4. Changes in biogas production rates after different pretreatment methods of *Jatropha curcas* oil cake.

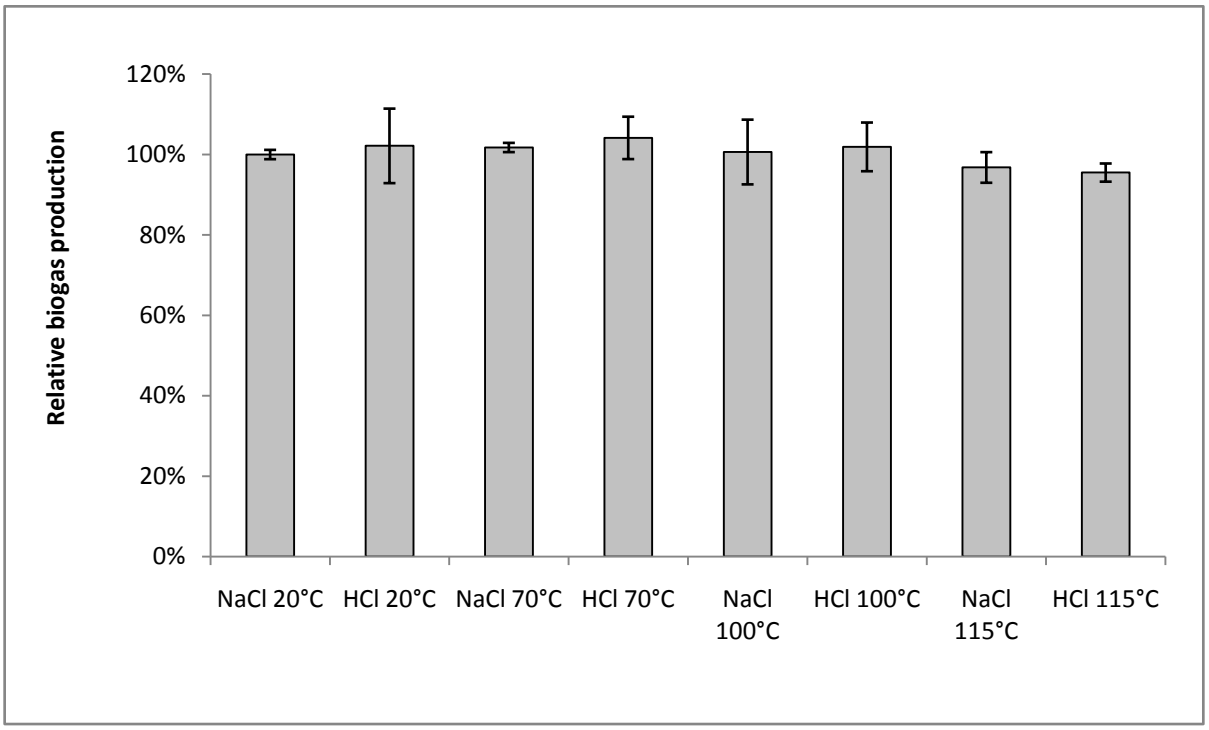

Figure 5. Relative total biogas production from *Jatropha curcas* oil cake after different pretreatments and 21 days of fermentation.

Low biogas production may be the result of reduced protein biodegradation in *J.curcas*oil cake. Thermal treatment at 115 °C successfully reduced the inhibitory activity of the oil cake but did not change the biogas production efficiency nor improved the fermentation rate of the pretreated sample. The biogas production rate of oil cake incubated with NaCl at 20°C was similar to results obtained for incubation with NaCl at 115 °C;this suggests that protease inhibitors are not essential to hydrolysis of biopolymers in *J.curcas*oil cake. This may be result of our experimental design, since in batch experiments,the applied organic load is relatively low;the influence of protease inhibition may be observed in systems working with higher loading rates. Additionally, proteolytic enzymes produced by anaerobic microorganisms may be resistant to the inhibitors present in *J.curcas* seeds, and thus the deactivation of trypsin inhibitors does not influence protein digestion in anaerobic reactors.

Since the *J. curcas* oil cake digestion efficiency is not improved by thermal pretreatment, the application of technologies otherthan wet digestion may by beneficial. For instance,a multi-stage high solid process offers easy recovery of undigested biomass, lower water demand, and potentially higher digestion efficiency. In this process, hydrolysis and acidogenesis take place in micro-aerobic conditions. The substrate is washed with digester effluent and the percolate is returned to the reactor. Since undigested organic matter remains in the percolation tank, it does not require dewatering. However, in systems with closed water circuits, substances like ammonium ions may accumulate, whichmay inhibit methane production.

3.5. Nitrogen in digestion fluid.

The concentration of nitrogen in the digestion fluid increased after fermentation of *J.curcas* oilcake. The concentration of nitrogen in the fluid in control samples was around 1570 $mg \cdot L^{-1}$; in samples after batch

fermentation of *J. curcas* oilcake, the nitrogen concentration was around 2100 $mg \cdot L^{-1}$. Since the nitrogen in anaerobic conditions is reduced to ammonium ions, it may be assumed that total nitrogen represents the concentration of ammonium with good approximation. Ourresults clearly show that ammonium ions accumulate during digestion of protein-rich oilcake. Thus, the ammonium ions should be removed from digestion fluid to prevent ammonia inhibition.

The ammonium ions could be removed from the solution by struvite formation. For*J. curcas* oil cake digestion, due to the low concentrations of phosphate and magnesium ions,struvite formation required the addition of these compounds. The concentration of nitrogen in digestion fluid was reduced by 53% after addition of magnesium and phosphate ions. This reaction was less efficient than the results presented by other authors. Nitrogen removal efficiency in landfill leachate reached about 80 % (Huang et al., 2014) and anaerobically-treated wastewater reached 95 % (Escudero et al., 2015). The low efficiency of nitrogen removal may be the result of calcium ions (around 0.01 $mol \cdot L^{-1}$). Escudero et al. showed that calcium ions also precipitated in the conditions required for struvite formation (Escudero et al., 2015). Thus, the presence of calcium and other cations may result in reducedammonium removal efficiency.

4. Final conclusions

Reduction of trypsin inhibitor activity does not correlate with anaerobic digestion activity or the concentration of phytic acid. Moreover, thermal treatment does not influence biogas production efficiency but may change the digestion rate.

The concentration of ammonium ions during anaerobic digestion of *J. curcas*oil cake significantly increased. The accumulation of ammonium ions may cause inhibition problems if the digestion was performed usingdry digestion technology. The concentration of ammonium ions may be efficiently reduced by precipitation of struvite. Due to low concentrations of phosphate and magnesium,struviteprecipitation requires addition of these compounds.

Our results indicate that anaerobic digestion of protein-rich substrates may be conducted usingdry technology with limited demand for water. Struvite precipitation allows the recovery of macroelements in a form thatcan be applied as mineral fertilizer. Such a technology can recover macronutrients from wastewater streams, resulting in lower energy costs required for fertilizer production (Batstone and Virdis, 2014).

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