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Pretreatment of the macroalgae *Chaetomorpha linum* for the production of bioethanol – Comparison of five pretreatment technologies

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HIGHLIGHTS

- Comparison of 5 pretreatment methods for *Chaetomorpha linum* for ethanol production.
- Pretreatment with wet oxidation and ball milling resulted in highest ethanol yield.
- Ball milling resulted in 64% higher ethanol yield compared to unpretreated *C. linum*.
- The ethanol yield was higher than found in literature for other macroalgae.

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ABSTRACT

A qualified estimate for pretreatment of the macroalgae *Chaetomorpha linum* for ethanol production was given, based on the experience of pretreatment of land-based biomass. *C. linum* was subjected to hydrothermal pretreatment (HTT), wet oxidation (WO), steam explosion (STEX), plasma-assisted pretreatment (PAP) and ball milling (BM), to determine effects of the pretreatment methods on the conversion of *C. linum* into ethanol by simultaneous saccharification and fermentation (SSF). WO and BM showed the highest ethanol yield of 44 g ethanol/100 g glucan, which was close to the theoretical ethanol yield of 57 g ethanol/100 g glucan. A 64% higher ethanol yield, based on raw material, was reached after pretreatment with WO and BM compared with unpretreated *C. linum*, however 50% of the biomass was lost during WO. Results indicated that the right combination of pretreatment and marine macroalgae, containing high amounts of glucan and cleaned from salts, enhanced the ethanol yield significantly.

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1. Introduction

The production of 2nd generation bioethanol from land-based biomass such as wheat straw has widely been investigated, and processes have been optimized (Olsson, 2010). Attention is now drawn to the use of marine-based biomass to supplement land-based biomass as a feedstock for biofuel production. In this context, macroalgae are under consideration as a candidate feedstock (Roesijadi et al., 2010).

Macroalgae represent a diverse group of eukaryotic, photosynthetic marine organisms that occur worldwide, commonly referred to as seaweeds. Macroalgae are multicellular and possess plant-like characteristics compared to unicellular microalgae. Macroalgae are abundant in coastal areas, with suitable substrate for attachment. They can also be found floating in the open sea, and are considered as one of the most important natural biomass resources (Roesijadi et al., 2010; Vandendriessche et al., 2006).

All macroalgae assimilate CO_2 in the form of bi-carbonate directly from the slightly alkaline seawater. Therefore the photosynthetic efficiency can be in the order of 7%, which is significantly

Abbreviations: BM, ball milling; DM, dry matter; HTT, hydrothermal treatment; NCWM, none cell wall material; NHOC, non-hydrolysable organic components; PAP, plasma assisted pretreatment; STEX, steam explosion; WO, wet oxidation.

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higher than the typical efficiency of 2% for terrestrial biomass resulting in higher primary production rates (Bruhn et al., 2011; Park et al., 2009; Roesijadi et al., 2010; Ross et al., 2009). Macroalgae could be a realistic contributor to the supply of sustainable feedstock for the production of renewable fuels, since they are not competing with land-based energy crops. However, their full potential can only be exploited if efficient cultivation, harvesting and conversion technologies are available (Roesijadi et al., 2010).

The green macroalgae *Chaetomorpha linum* has rigid cell walls with an outer lamellar part consisting mainly of highly crystalline cellulose and an inner amorphous part consisting of a complex branched polymer of arabinose, xylose and galactose (Bastianoni et al., 2008; Wang et al., 2011). Similar to other cellulosic biomasses, glucan from macroalgae can be converted by enzymes into sugars suitable for ethanol fermentation. The establishment of efficient pretreatment methods in order to facilitate the conversion of sugars in the enzymatic hydrolysis is the key issue for bioethanol production (Lee et al., 2013). However, the conversion technology for the production of liquid biofuels from macroalgae is at an early stage. The polysaccharides in seaweed require new economic pretreatment methods in order to make the monomers available for fermentation (Burton et al., 2009; Roesijadi et al., 2010).

Such a pretreatment method must be simple and the products must be highly fermentable with limited non-digestible residues in order to be efficient. Furthermore, polysaccharides from the biomass should be hydrolyzed directly without sugar degradation which might produce fermentation inhibitors. Energy requirements and costs for pretreatment are additional important aspects (Lee et al., 2013).

Different examples of biofuel production and pretreatment technologies for macroalgae have been described in the literature. *Saccharina japonica* for example was successfully converted to bioethanol using an optimized extremely low acid pretreatment (ELA) followed by simultaneous saccharification and fermentation (SSF) (Lee et al., 2013). This method had the advantage that no further neutralization and/or water treatment was necessary, minimizing the operational costs. Fermentation studies of *Saccharina latissima* demonstrated that no elevated temperature pretreatment was required for the conversion of the brown algae to glucose and fructose (Adams et al., 2009). This indicates that the production of ethanol from macroalgae may be simpler compared to lignin containing terrestrial plants. A third study dealt with the development of a saccharification method for *Gracilaria salicornia.* Enhanced ethanol production was reported after a combination of diluted acid (2.5% sulfuric acid) hydrolysis with enzymatic hydrolysis, compared to a one-stage process (Wang et al., 2011).

C. linum was chosen as target, since it contains more cellulose (35–40 g/100 g DM) than other algae such as *S. japonica* (Lee et al., 2013) and *G. salicornia* (Wang et al., 2011). Furthermore, the cellulose content of *C. linum* is similar to that of land-based biomass. The aim of the investigation was to show that thermal treatment of *C. linum* makes cellulose more accessible for cellulase enzymes. Consequently, this facilitates the enzymatic hydrolysis resulting in enhanced ethanol yield. Furthermore, it was tested if the same range of ethanol yield could be obtained by reducing the particle size of the biomass (BM) and by oxidative removal of lignin-like materials (PAP). Pioneer research was done on the pretreatment of *C. linum* for ethanol production by SSF with yeast. The effect of the five pretreatment technologies, wet oxidation (WO), hydrothermal pretreatment (HTT), steam explosion (STEX), plasma-assisted pretreatment (PAP) and ball milling (BM) on the chemical composition and the conversion of *C. linum* into ethanol by SSF was investigated at pre-selected reaction conditions.

2. Methods

2.1. Harvest and preparation of macroalgae

C. linum was harvested in the Roskilde fjord (N55.69°, E12.08° (WGS84)). The depth of Roskilde fjord is between 2 and 5 m, has a salinity of 8 g salts/L and a temperature between 15 and 22 °C in summer (Flindt et al., 1997). Harvested *C. linum* was sorted, and impurities were removed in order to establish a reproducible biomass balance. A washing protocol was developed to remove salts which can interfere with chemical analysis and enzymatic cellulose hydrolysis. NaCl was removed from the algae by washing with cold tap water, until chloride was no longer detected in the washing water (tested by precipitation with $AgNO_3$). *C. linum* harvested in July 2009 was used for HTT, WO, and PAP experiments and *C. linum* harvested in September 2010 was used for BM and STEX experiments.

2.2. Pretreatment methods

2.2.1. Hydrothermal treatment and wet oxidation

C. linum was dried at 40 °C for 48 h and milled to a particle size of 2 mm (Mill: MF 10 basic, IKA-Werke GmbH & Co. KG, Germany). Former studies on wheat straw revealed that a particle size of 2 mm performed best with this particular method (Bjerre et al., 1996). Hydrothermal treatment and wet oxidation of *C. linum*, were done in a 2 L loop batch autoclave at the Department of Chemical and Biochemical Engineering, Technical University of Denmark. The autoclave was fed with 1 L tap water and 40 g dry matter (DM) of biomass, and operated with continuous stirring (Bjerre et al., 1996). The residence time was 10 min, and the tested temperatures were 180, 190 and 200 °C at an H_2O pressure of 10.0, 12.5 and 15.5 bars, respectively. The pretreatment was done either at atmospheric pressure without additional O_2 (HTT) or with 12 bars of O_2 added (WO) to the 1 L headspace at 20 °C. The liquid and solid phases were separated prior to analysis and the solid phase was washed with tap water in order to remove soluble compounds. HTT and WO were performed in duplicate at 200 °C.

2.2.2. Steam explosion

C. linum was compressed by means of an hydraulic press at 1.9 MPa pressure to increase the DM content to 35% by removal of liquid (Tinkturenpressen HP5M, Fisher Maschinenfabrik GmbH, Germany). Subsequently, STEX was applied at 200 °C and 210 °C for 5 min in a 10 L reactor (Palmqvist et al., 1996; Rudolf et al., 2008). 1.2 kg of *C. linum* (35% DM) was treated resulting in 2.4 kg slurry. 1 kg of the slurry was compressed similar to the algae resulting in 0.5 kg of liquid and 0.5 kg as pressed cake.

2.2.3. Plasma assisted pretreatment

C. linum was dried for 48 h at 40 °C and milled to a particle size of 1 mm and adjusted to a DM content of 50%. Former studies on wheat straw revealed that a particle size of 1 mm performed best with this particular method (Schultz-Jensen et al., 2011). The PAP treatment of *C. linum* was performed in an in-house-built reactor (ø: 7 cm, length: 2 cm) under atmospheric pressure and at room temperature. *C. linum* (2.5 g DM) was placed into the reactor vessel and sealed with a lid before pretreatment. *C. linum* was pretreated for 20, 40 and 60 min with a gas flow rate of 0.01 L/s. The ozone concentration in the gas stream was 1%. Further details of the PAP method (generation of O_3 by means of plasma, diagnostic of O_3 consumption, pretreatment reactor and set up) were described previously (Leipold et al., 2011; Schultz-Jensen et al., 2011). *C. linum* was dried for 48 h in an incubator (Adolf Kühner Lab-Therm LT-V, Switzerland) after pretreatment, with air circulated heating

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at 40 °C. Dried samples were weighed and analyzed for chemical composition. The 60 min pretreatment time experiment was performed in duplicate.

2.2.4. Ball milling experiment

C. linum was dried for 48 h at 40 °C and milled to a particle size of 2 mm. BM was performed with 25 g of dried *C. linum* and 25 balls (15 mm diameter) for a period of 18 h in a cylinder (15 cm diameter) at a rotational speed of 180 rpm. Extra washing of *C. linum* with water was required, in order to decrease the risk of microbial decay. The ball milling process was optimized by varying the DM content of *C. linum* and the number of balls used in order to obtain powdered *C. linum* (data not shown). The BM experiment was performed in duplicate.

2.3. Analysis of sugars and ethanol in solid and liquid samples

2.3.1. HPLC analysis

Glucan, xylan, arabinan and ethanol were determined in subsequent analysis procedures in a HPLC-system (High-Performance Liquid Chromatography; Shimadzu Corp., Kyoto, Japan) with a Rezex ROA column (Phenomenex, Denmark) at 63 °C using 4 mM H_2SO_4 as eluent and a flow rate of 0.01 mL/s. Detection was done by a refractive index detector (Shimadzu Corp., Kyoto, Japan). The conversion factors for dehydration on polymerization were 0.90 for glucose and 0.88 for xylose and arabinose (Kaar et al., 1991). The results were analyzed by the use of glucose, xylose, arabinose and ethanol standards.

2.3.2. Analysis of liquid samples

The determination of the water soluble carbohydrates released into the liquid phase during pretreatment was made by means of diluted acid hydrolysis (Thomsen et al., 2009), in which 10 mL of 80 g/L sulphuric acid was mixed with 10 mL sample and hydrolyzed at 121 °C for 10 min. The hydrolysates were analyzed by means of HPLC (see above). The total content of phenolic compounds in the liquid samples was analyzed based on the color reaction with $FeCl_3$. Phosphoric acid was used to terminate the reaction by precipitating excessive Fe^{3+} resulting in constant color density. Catechol was used as phenol standard and the absorbance was measured at a wavelength of 700 nm (Graham, 1992). Furans were detected by means of HPLC with UV detection at 280 nm with authentic compounds as calibration standards (Thomsen et al., 2009).

2.3.3. Soluble products obtained by BM and PAP

C. linum pretreated by means of BM and PAP were mixed with water to a ratio of 2 g DM per 20 g water and shaken in a lab-shaker at 25 °C and 100 rpm. Samples of liquid were taken after 30 min by filtration. The liquid was analyzed for carbohydrates, volatile fatty acids, furans and total phenol content (Schultz-Jensen et al., 2011).

2.3.4. Analysis of solid samples

The composition of the untreated and the pretreated algae solids were measured by means of concentrated acid hydrolysis of the carbohydrates. Dried and milled samples (160 mg) were treated with 1.5 mL of 72 w/w% H_2SO_4 at 30 °C for 1 h. The solutions were diluted with water to a total volume of 43.5 mL and were autoclaved at 121 °C for 1 h. The hydrolysates were filtered, and the non hydrolysable organic components (NHOC) were determined by subtracting the ash content from the weight of the filter cake. The hydrolysates were analyzed by means of HPLC (see above) (Kaar et al., 1991; Thomsen et al., 2008). In addition, the untreated algae biomass was analyzed for wax and lipids by 5 h soxlet extraction with a mixture of 50 mL ethanol, 50 mL acetone and 200 mL toluene. Pectin was detected as an acid–EDTA complex formed during 4 h extraction at 85 °C with 30 g/L EDTA at pH 4.0 (EthyleneDiamineTetraAcetic acid) (Thygesen et al., 2011). It was found that pectin can occur in the primary cell wall in macroalgae (Eder and Lütz-Meindl, 2008).

2.4. Ethanol fermentation experiments

2.4.1. Yeast strain

Saccharomyces cerevisiae ATCC 96581 was obtained from the American Type Culture Collection (Manassa, VA, USA). The strain was kept at −85 °C in a mixture of 50 vol.% glycerol and 50 vol.% medium containing 20 g of bacto peptone, 10 g of yeast extract, and 20 g of glucose per liter demineralized water.

2.4.2. Yeast cultures

Starter culture of *S. cerevisiae* ATCC 96581 was grown in 250 mL cap flasks containing 100 mL of culture medium. The medium containing 20 g of bacto peptone, 10 g of yeast extract, and 20 g of glucose per liter demineralized water was sterilized at 121 °C for 20 min. The flasks were incubated in an orbital shaker at 130 rpm for 24 h at 30 °C. The cultures were then centrifuged at 3000 rpm for 10 min (Sigma 4-15, SIGMA Laborzentrifugen GmbH, Germany), washed with demineralized water and harvested in demineralized water.

2.4.3. Simultaneous saccharification and fermentation (SSF)

In order to evaluate the convertibility of *C. linum* pretreated with HTT, WO, STEX, PAP and BM into ethanol, SSF was performed under semi-anaerobic conditions in blue capped flasks with 10 g of dry solid pretreated material in a total volume of 100 mL. The flasks were equipped with yeast lockers and shaken at 100 rpm (Lab-Therm LT-X, Adolf Kühner AG, Switzerland). The pH was adjusted to 4.8 with 3 M H_2SO_4.

The commercial cellulase enzymes (Celluclast 1.5 L and Novozyme 188, Novozymes A/S, Denmark) were applied with a concentration of 15 FPU/g dry pretreated substrate. The volume ratio between Novozyme 188 and Celluclast 1.5 L in the mixture was 0.2. The fermentation medium contained 10 g pretreated sample in water. Prehydrolysis (initial liquefaction) was performed before inoculation with yeast for 24 h at 50 °C. The temperature was reduced to 32 °C and 20 FPU/g dry substrate of enzymes and 2 mL urea/L were added after prehydrolysis. The content of the flask (liquefied algae) was inoculated with harvested yeast to initiate the SSF. The initial yeast concentration was 2 g DM/L. The flasks were incubated in a rotary shaker at 32 °C for 200 h.

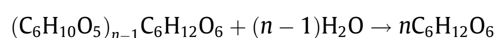
The evolution of carbon dioxide was monitored via measuring the weight loss of the flask content. Samples were taken at the end of the fermentation and centrifuged in a laboratory desktop centrifuge at 3000 rpm for 5 min. The supernatants were analyzed for carbohydrates and ethanol concentration using the same HPLC system as described above.

2.5. Calculations

2.5.1. Theoretical calculation of ethanol production from glucan

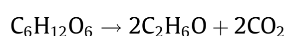
Reactions for ethanol fermentation from glucan:

1. Enzymatic glucan hydrolysis with degree of polymerization DP of n:



$$(C_6H_{10}O_5)_{n-1}C_6H_{12}O_6 + (n-1)H_2O \rightarrow nC_6H_{12}O_6$$

2. Ethanol fermentation from the produced glucose:



$$C_6H_{12}O_6 \rightarrow 2C_2H_6O + 2CO_2$$

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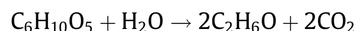
3. Overall reaction for n at infinity for hydrolysis and fermentation of glucan:



$$C_6H_{10}O_5 + H_2O \rightarrow 2C_2H_6O + 2CO_2$$

The theoretical yield of ethanol from glucan was calculated considering 100% conversion/fermentation efficiency:

$$\text{Yield}_{\text{Ethanol,glucan}} = \frac{2M_w(C_2H_6O)}{M_w(C_6H_{10}O_5)} = \frac{2 * 46\ \text{g/mol}}{162\ \text{g/mol}} = 0.57 \quad (1)$$

The theoretical calculation of ethanol production from 100 g DM *C. linum* containing 40 g glucan:

$$\frac{40\ \text{g glucan}}{100\ \text{g DM}} \cdot \frac{0.57\ \text{g ethanol}}{\text{g glucan}} = 23\ \text{g ethanol}/100\ \text{g DM} \quad (2)$$

2.5.2. Equations to Table 3

The relative loss of DM of *C. linum* is the ratio between the difference of the DMs before and after pretreatment and the DM before pretreatment:

$$\text{Relative loss of DM} = \frac{\text{DM}_{\text{before}} - \text{DM}_{\text{after}}}{\text{DM}_{\text{before}}} \quad (3)$$

The relative expected glucan content after pretreatment prorated to 100 g DM due to loss of DM is given by the relative glucan content of 40 g/100 g multiplied by the ratio of DM before and DM after pretreatment:

$$\text{Relative glucan content after pretreatment} = \frac{40\ \text{g}}{100\ \text{g}} \times \frac{\text{DM}_{\text{before}}}{\text{DM}_{\text{after}}} \quad (4)$$

The relative gain in glucan content is given by the difference between the measured relative glucan content after pretreatment and the relative expected glucan content after pretreatment:

$$\text{Relative gain in glucan cont.} = \text{Measured relative glucan cont.} - \text{expected glucan cont.} \quad (5)$$

2.5.3. Equations to Table 4

Yield 1 was calculated as total amount of g ethanol obtained per g raw material of *C. linum* before pretreatment:

$$\text{Yield 1} = \frac{\text{Total amount of ethanol obtained}}{\textbf{Raw material}\ (C.linum, \text{before pretr.})} \quad (6)$$

Yield 2 was calculated as total amount of g ethanol obtained per g raw glucan of *C. linum* before pretreatment:

$$\text{Yield 2} = \frac{\text{Total amount of ethanol obtained}}{\textbf{Raw glucan}\ (C.linum, \text{before pretr.})} \quad (7)$$

3. Results and discussion

3.1. Chemical composition of the macroalgae C. linum

C. linum consisted of 34–38 g glucan, 6 g xylan, 9–10 g arabinan, 7 g non hydrolyzable organic components (NHOC), 21–23 g ash, 14 g pectin and 6 g wax per 100 g DM (Table 1). NHOC could not be hydrolyzed or extracted into solution by the concentrated acid hydrolysis. This included phenolic compounds and polysaccharides. *C. linum* was washed with cold tap water in order to remove salts (mainly NaCl), sand and insects prior to analysis. Salts (mainly NaCl) interfered with the biomass analysis, e.g. concentrated and dilute acid hydrolysis. The washing was done to assure a clear and reproducible analysis of the chemical composition of the macroalgae.

Previous investigations of the chemical composition of *C. linum*, were not as complete as the present study and mainly focused on the concentration of cellulose (Kiran et al., 1980). The reported cellulose content of 37 g/100 g DM in *C. linum* was also found in this study. In many cases, macroalgae appeared to have lower carbohydrate contents than lignocellulosic biomass due to their high ash content (Bird et al., 2011). It could be confirmed that the high amount of salt disturbed the analysis of the biomass by the concentrated acid hydrolysis (72 w/w% H_2SO_4) and lead to an underestimation of the sugar contents (Bird et al., 2011). In accordance with the presented results (Table 1) Bird et al. (2011) reported that the carbonate free ash content of macroalgae varied between 11 and 34 g/100 g DM.

3.2. Effect of pretreatment on chemical composition of C. linum

Three thermochemical pretreatment methods HTT (180, 190 and 200 °C), WO (180, 190 and 200 °C) and STEX (200 and 210 °C) were tested at various temperatures. HTT and WO, performed at 200 °C, resulted in the highest glucan concentration of 64 and 74 g/100 g DM after pretreatment (Table 1). The xylan (1.8 and 0.0 g/100 g DM) and arabinan concentrations (0.6 and 0.3 g/100 g DM) were significantly reduced during HTT and WO, due to hydrolysis and thermal degradation (Table 1). The amount of NHOC was doubled to 14 g/100 g DM by HTT. This was probably caused by the degradation of sugar monomers, rendering the carbohydrates non extractable (Table 1). In contrast, STEX performed at 200 °C resulted in a lower concentration of glucan (46 g/100 g DM) but similar concentrations of xylan and arabinan (0–2 g/100 g DM) after pretreatment (Table 1). Obviously, the process conditions during STEX were less severe compared to WO due to addition of oxygen to the process. NHOC were accumulated and the total amount raised from seven to a three times higher value (Table 1, 21 g NHOC/100 g DM) during the STEX process.

PAP pretreatment of *C. linum* conserved the concentration of glucan (C6) and arabinan (C5) in the pretreated biomass, independent of the treatment time (Table 1). The xylan concentration decreased to 70% compared to the concentration before pretreatment, however, remaining at a much higher concentration than achieved with HTT, WO and STEX (Table 1). BM experiments were done in order to test the effect of mechanical pretreatment on washed and dried *C. linum* (95 g DM/100 g). BM had no effect on the total amount of carbohydrates, therefore the concentration of C6 sugars (glucan) and C5 sugars (xylan, arabinan) remained unchanged after pretreatment (36 g glucan, 6 g xylan and 12 g arabinan per 100 g DM).

3.3. Carbohydrate recovery after pretreatment of C. linum in the solid and liquid fraction

The carbohydrate recovery of *C. linum* in the solid and liquid fractions was investigated after pretreatment with HTT, WO, STEX, PAP and BM (Table 2). Generally, HTT, WO and STEX provided high extractions of xylan (22–26 g/100 g xylan) and arabinan (30–54 g/100 g arabinan) into the hydrolysates (liquid). However, the amount of glucan (8 g/100 g), xylan (26 g/100 g) and arabinan (54 g/100 g) extracted was highest after STEX (Table 2). This was probably due to the lower water to DM ratio in the STEX process, compared to HTT and WO (Table 2). The highest glucan contents of 100 g/100 g DM and 98 g/100 g DM were recovered after HTT and WO (Table 2). This was in congruency with the results of the chemical composition of *C. linum* after WO (Table 1).

No C6 and C5 sugars were detected in the liquid fraction after BM, indicating that most of the sugars remained in the fibers after

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Table 1
Chemical composition of *C. linum* as obtained with the pretreatment methods tested such as hydrothermal pretreatment (HTT), wet oxidation (WO), steam explosion (STEX), plasma-assisted pretreatment (PAP) and ball milling (BM). The NCWM* fraction of 22 g/100 g DM was found to contain 14 g pectin and 6 g wax per 100 g DM.

Condition	Glucan (C6) g/100 g DM	Xylan (C5) g/100 g DM	Arabinan (C5) g/100 g DM	NHOC g/100 g DM	Ash g/100 g DM	NCWM g/100 g DM	∑
Raw 2009	38	5.4	10.1	7	21	19	100
Raw 2010	34	5.7	8.5	7	23	22*	100
STDEV	*1.5*	*1.2*	*0.4*	*0.2*	*0.1*	*1.5*	
HTT 180 °C	55	2.6	2.0	14	17	11	100
HTT 190 °C	60	2.2	0.9	14	16	8	100
HTT 200 °C	64	1.8	0.6	14	17	3	100
STDEV	*1.8*	*0.0*	*0.0*	*0.2*	*0.2*	*1.8*	
WO 180 °C	66	0.0	1.2	6	16	11	100
WO 190 °C	71	0.0	0.8	6	17	6	100
WO 200 °C	74	0.0	0.3	5	19	2	100
STDEV	*1.8*	*0.0*	*0.0*	*0.2*	*0.2*	*1.8*	
STEX 200 °C	46	0.0	0.8	19	23	11	100
STEX 210 °C	50	1.9	1.9	21	26	0	101
STDEV	*3.0*	*1.6*	*0.7*	*0.2*	*3.4*	*3.0*	
PAP 20 min	38	3.6	9.7	7	19	23	100
PAP 40 min	36	3.8	9.6	6	20	25	100
PAP 60 min	38	4.1	9.9	5	20	23	100
STDEV	*1.5*	*0.1*	*0.4*	*0.5*	*0.1*	*1.5*	
BM	36	5.7	12.3	12	10	24	100
STDEV	*1.5*	*0.1*	*0.4*	*0.5*	*0.1*	*1.5*	

Table 2
Carbohydrate recovery after hydrothermal treatment (HTT), wet oxidation (WO), steam explosion (STEX), plasma assisted pretreatment (PAP), and ball milling (BM) of *C. linum* in the resulting solid and liquid fractions.

Pretreatment	Glucan g/100 g glucan		Xylan g/100 g xylan		Arabinan g/100 g arabinan	
	Solid	Liquid	Solid	Liquid	Solid	Liquid
HTT (200 °C)	100 ± 6	2 ± 0	22 ± 5	22 ± 5	4 ± 0	30 ± 1
WO (200 °C)	98 ± 6	2 ± 0	0 ± 0	25 ± 6	2 ± 0	39 ± 2
STEX (200 °C)	92 ± 8	8 ± 0	21 ± 7	26 ± 6	14 ± 1	54 ± 2
PAP (60 min)	100 ± 6		75 ± 17		98 ± 5	
BM (18 h)	91 ± 5		90 ± 20		98 ± 5	

pretreatment (Table 2). The chemical analysis of *C. linum* after BM revealed no significant changes in biomass composition after pretreatment. This supported the observation, that no sugars were detected in the liquid fraction (Tables 1 and 2). The recovery of the carbohydrates was similar after pretreatment with PAP and BM. This was in accordance with the results presented in Table 1. Glucan and arabinan were recovered to 100 and 98 g/100 g DM in the solid fraction of *C. linum* after PAP, while xylan was recovered to 75%.

3.4. Estimation of loss of biomass during pretreatment

The estimation of the loss of DM and the gain of glucan during pretreatment of *C. linum* with HTT, WO, STEX, PAP and BM was investigated. *C. linum* pretreated with HTT showed the highest gain of glucan (+7 g/100 g DM) compared to WO, STEX, PAP and BM (Table 3). This was in congruency with the low extraction of glucan into the hydrolysate after HTT (Table 2), indicating easier accessibility of glucan for the acidic hydrolysis. HTT extracted less glucan, xylan and arabinan into the hydrolysate compared to the two other thermal pretreatment methods (WO, STEX) investigated (Table 2). Only 30% of the DM of *C. linum* was lost after HTT, while 50% was lost after WO (Table 3). This might explain the higher gain of glucan after pretreatment with HTT compared to WO (Table 3).

HTT produced less formic acid and acetic acid than WO of *C. linum* (Fig. 1). It could be speculated that there was little conversion of C6 and C5 sugars into inhibitors (Tables 1–3), supporting the highest gain of glucan after HTT of *C. linum.* STEX showed the highest extraction of glucan into the hydrolysate (Table 2) compared to HTT and WO, matching with the highest loss of glucan (−11 g/100 g DM) estimated in Table 3. PAP and BM showed no loss of DM after pretreatment, indicating that only a little amount of undesirable, non-digestible residues and fermentation inhibitors was produced.

3.5. Fermentation inhibitors measured in the hydrolysates after pretreatment

The inhibitor concentration of *C. linum* in the hydrolysates was measured after pretreatment with HTT, WO, STEX, PAP and BM (Fig. 1). HTT and WO resulted in the highest concentrations of

Table 3
Estimation of the loss of DM (g/100 g DM) and the gain of glucan during pretreatment of the macroalgae *C. linum* with hydrothermal treatment (HTT), wet oxidation (WO), steam explosion (STEX), plasma assisted pretreatment (PAP) and ball milling (BM). The measured glucan values were taken from Table 1.

Pretreatment	Eq. (3)					Eq. (4)		Eq. (5)
	DM before pretreatment g	DM after pretreatment g	Difference g	Loss of DM g/100 g DM	Remain DM (=100 − loss) g/100 g DM	Relative expected glucan content after pretreatment g/100 g DM	Relative measured glucan content after pretreatment g/100 g DM	Difference between measured and expected relative glucan content after treatment g/100 g DM
HTT	40	28	12	30	70	57 ± 2	64 ± 2	+7 ± 3
WO	40	20	20	50	50	80 ± 3	74 ± 2	−6 ± 3
STEX	425	295	130	31	69	57 ± 2	46 ± 3	−11 ± 4
PAP	50	50	0	0	100	40 ± 2	38 ± 2	−2 ± 2
BM	25	25	0	0	100	40 ± 2	36 ± 2	−4 ± 2

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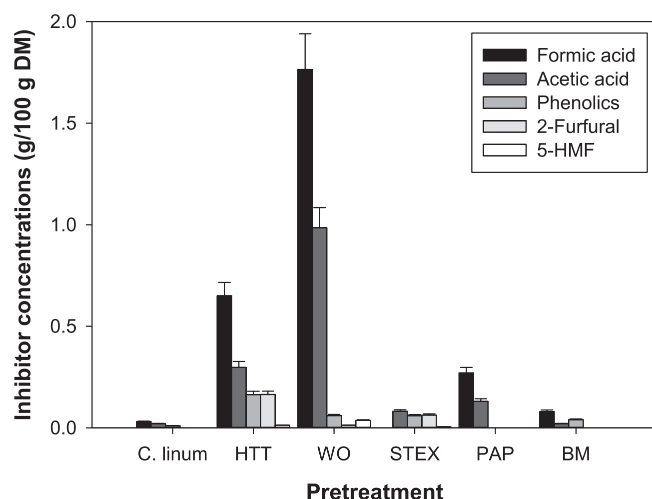

Fig. 1. Concentration of inhibitors (g/100 g DM) measured in the hydrolysates after pretreatment of *C. linum* with hydrothermal treatment (HTT), wet oxidation (WO), steam explosion (STEX), plasma assisted pretreatment (PAP) and ball milling (BM).

formic acid (0.7 and 1.8 g/100 g DM, respectively) and acetic acid (0.2 and 1.0 g/100 g DM, respectively) (Fig. 1). However, no formic acid and less acetic acid (0.1 g/100 g DM) were found after STEX. The highest amount of furfural (0.2 g/100 g DM) was measured after HTT. This is probably due to the chemical and thermal conversion of the C5 sugars to 2-furfural (Fig. 1).

Interestingly, neither 2-furfural nor 5-HMF(5-Hydroxymethylfurfural) was measured after PAP and BM (Fig. 1). This is in agreement with the observation that samples pretreated with PAP and BM resulted in almost 100% recovery of glucan, xylan and arabinan (Tables 1 and 2). It can be speculated, that there was no conversion of C6 and C5 sugars into inhibitors (Tables 1 and 2). Generally, low concentrations of fermentations inhibitors were found for all 5 pretreatment technologies applied and no effect on the conversion of pretreated *C. linum* into ethanol was observed.

3.6. Ethanol yield after pretreatment of C. linum

Pretreated *C. linum* was used for SSF. The yield of ethanol after pretreatment was calculated in g ethanol/100 g DM (yield 1) and in g ethanol/100 g raw glucan (yield 2) as shown in Table 4. Differences in the ethanol yields of pretreated *C. linum* were observed (Table 4). HTT, WO, PAP and BM increased the ethanol yield (yield 1) from 11 to 15–18 g ethanol/100 g DM (Table 4). Furthermore, HTT, WO, PAP and BM increased the ethanol yield (yield 2) from 31 to 38–44 g ethanol/100 g glucan. The maximum was found for ball milled *C. linum* (Table 4). STEX, however, resulted in the lowest ethanol yield 1 and yield 2 of pretreated *C. linum* (13 g ethanol/100 g DM and 38 g ethanol/100 g glucan). The reason might be the high loss of glucan during STEX pretreatment (Tables 2–4). The theoretical ethanol yields (yield 1 and yield 2) at complete DM and glucan conversion were 23 g ethanol/100 g DM and 57 g ethanol/100 g glucan (Eq. (2)).

Among the thermal pretreatment methods, WO resulted in the highest glucan content of 74 g/100 g DM and in the highest ethanol yield (yield 1) of 17 g/100 g DM (Tables 1 and 4). The low extraction of glucan during WO (Table 2), contributed to the high amount of glucan available for ethanol production. The highest ethanol yield (yield 1) of 18 g ethanol/100 g raw glucan was achieved with BM (Table 4). This was a clear improvement of the ethanol yield (yield 1) of 64% compared to the ethanol production form unpretreated *C. linum* (Table 4). The ethanol yield (yield 2) achieved with WO and BM (44 g ethanol/100 g raw glucan) was 77% of the theoretical ethanol yield (yield 2) (57 g/100 g glucan) (Table 4).

A mechanical grinding process, highly different from our grinding process (BM), enhanced the ethanol yield from *S. latissima* (Adams et al., 2009). Therefore it can be speculated, that BM could be sufficient for the pretreatment of *C. linum* for ethanol production. Obviously, the surface which can be active for reaction (with e.g. enzymes) is inversely proportional to the particle size. This might explain the higher ethanol yield when smaller sized particles were used for the SSF. Those results indicate that the production of ethanol from *C. linum* might be rather simple compared to terrestrial lignocellulosic biomass.

In conclusion, WO was the preferred pretreatment method, if high glucan outcome was desired. WO resulted in an 80% higher concentration of glucan in the pretreated biomass compared to unpretreated *C. linum* (Table 1). Consequently, WO resulted in the yield (yield 2) of ethanol of 44 g ethanol/g of raw glucan. However, about 50% loss of DM (especially C5 sugars) was observed during the pretreatment process, decreasing the yield of ethanol per g DM (Tables 3 and 4). Hence, BM should be the pretreatment method of choice, if high conservation of sugars and little loss of DM are in focus.

3.7. Perspectives and discussion of macroalgae utilization in ethanol production

Pioneer research on the comparison of 5 different pretreatment methods for *C. linum* for ethanol production has been performed, based on the experience of the pretreatment of land-based biomass. To the knowledge of the authors, no other study compared the effect of thermochemical, chemical and mechanical pretreatment methods on the biomass composition of macroalgae systematically. *C. linum* was chosen for ethanol production, since it contained a higher amount of cellulose compared to similar algae such as *S. japonica* (Lee et al., 2013) and *G. salicornia* (Wang et al.,

Table 4
Comparison of the ethanol yield produced from *C. linum* pretreated with hydrothermal treatment (HTT), wet oxidation (WO), steam explosion (STEX), plasma assisted pretreatment (PAP) and ball milling (BM) compared to raw material.

Pretreatment	Raw material(*C. linum* before pretr.[a]) g	Glucan before pretreatment g	Total DM after pretreatment g	Total amount of ethanol obtained g	Yield 1 (total ethanol/raw material) g ethanol/100 g DM	Yield 2 (total ethanol/raw glucan) g ethanol/100 g C6
None	100	34 ± 1	100	11 ± 0.6	11 ± 1	31 ± 2
HTT (200 °C)	40	15 ± 1	28	6 ± 0.3	15 ± 1	39 ± 3
WO (200 °C)	40	15 ± 1	20	7 ± 0.4	17 ± 1	44 ± 3
STEX (200 °C)	425	146 ± 6	295	55 ± 3.2	13 ± 1	38 ± 3
PAP (60 min)	50	19 ± 1	50	8 ± 0.5	15 ± 1	41 ± 3
BM (18 h)	25	10 ± 0	25	4.4 ± 0.3	18 ± 1	44 ± 3
Theoretical yield					23 ± 1	57

[a] Referred to g raw material in column 1.

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2011). Other macroalgae used for biofuel production e.g. *Ulva lactuca* and *Gracilaria longissima* contained less glucan (6 g/100 g DM and 20 g/100 g DM) (Coppalo et al., 2009).

Consequently, the maximum ethanol yield (yield 1) of *C. linum* found in our study (18 g ethanol/100 g DM) was up to 125% higher than reported in other studies using different types of macroalgae such as *G. salicornia* and *S. japonica* (Jang et al., 2012; Wang et al., 2011). The ethanol yield for *G. salicornia* for example, pretreated in a 2-stage hydrolysis processs, was 8 g ethanol/100 g DM (Wang et al., 2011). The ethanol yield of *S. japonica* after pretreatment with thermal acid hydrolysis resulted in 7.7 g ethanol/100 g DM (Jang et al., 2012). Apparently, the type of pretreatment and the type of macroalgae are significantly contributing factors for enhancement of the ethanol yield from marine biomass.

4. Conclusions

A comprehensive research on the comparison of five different pretreatment methods for *C. linum* for ethanol production was performed based on the experience of the pretreatment of land-based biomass. Results have shown that the pretreatment with BM resulted in 64% higher raw material based ethanol yield compared to unpretreated *C. linum*. Furthermore, the pretreatment with WO and BM resulted in 42% higher raw glucan based ethanol yield compared to unpretreated *C. linum.* Finally, the pretreatment of *C. linum* with WO and BM resulted in 77% of the theoretical ethanol yield (57 g/100 g glucan).

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