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Fermentable Sugar Production from Lignocellulosic Waste

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Effective and economically supported conversion of lignocellulosic biomass is not only related to high sugar yields but also to simple, environmentally friendly and sustainable technological solutions. Commercialization of the biofuel and biogas production technologies from lignocellulose is of high interest in many countries, however, costs and various yield affecting factors are still not fully understood. The aim of this research was to evaluate and define a mild-hydrolysis technique, involving the combination of simple physical, thermal and enzymatic methods. Use of medium-fine biomass (< 0.5 cm) wetted in purified (nanofiltrated) water, boiled to remove indigenous biomass microorganisms and enzymatically hydrolysed for 24 h at 30 °C was enough to obtain similar sugar yields than with sulphuric acid hydrolysis.

1. Introduction

The potential of lignocellulosic biomass as a renewable energy resource has been evaluated worldwide over many decades. Effective conversion of biomass to electrical and heat energy has been shown in many countries as a considerable share in the total produced energy (Šturc, 2012). At the same time biomass resources, like grass, shrubs, straw, hay and agricultural waste could effectively replace energy crops as feedstock for biofuel and biogas production which take up considerable areas of agricultural land unavailable for food production. However, due to highly complex lignocellulosic matrix consisting of cellulose, hemicellulose and lignin, direct conversion of these biomass resources is not possible. To release fermentable sugars, various physical, thermal, physicochemical, chemical and biological techniques or their combinations have been offered over the years (Kumar et al., 2009). Sugar yields of up to 99 % have been obtained (Ballesteros et al., 2002), however, generally the yields vary from 12 to 98 % depending on the biomass source and treatment method used (Chaturvedi and Verma, 2013). Therefore there is a great interest in the optimization of laboratory techniques to increase fermentable sugar yields and decrease the production costs to subsequently introduce them into a commercial scale.

On average 40% of the lignocellulosic biomass consists of cellulose which is the main source of fermentable sugars. The amount of cellulose varies from the biomass type assessed (Jorgensen et al., 2007), thus, affecting the product yields when a single pre-treatment/hydrolysis technique is used for various biomass sources. This in turn can strongly affect the availability and cost of the substrate, especially in the areas with a distinct seasonality and limited uniform feed material. At the same time high productivity techniques, e.g., oxidative and physicochemical, producing more than 80 % sugar yields (Chaturvedi and Verma, 2013) and, thus, not so strongly affected by cellulose concentration, are mostly regarded as too energy and labor consuming, environmentally unfriendly (Brodeur et al., 2011) and fermentation inhibitor generating (Larsson et al., 1999), thus, limiting their application in small and medium scale biofuel production units.

The aim of this study was to evaluate and define a mild-hydrolysis technique, involving combination of simple physical, thermal and enzymatic methods, for effective conversion of lignocellulose to fermentable sugars applicable in small-scale biofuel production. During the research various factors, like, biomass type, grinding (particle size), heat pre-treatment, solvent type and hydrolysis conditions were tested on the total outcome of fermentable sugar yields. To enhance the efficiency of enzymatic hydrolysis special laboratory made enzymes from the assessed substrates were used in the study.

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2. Materials and Methods

2.1 Lignocellulosic biomass

Hay mowed in late June from lowland hay meadows located in Latvia was used as a reference material. For biomass comparison studies wheat straw and fresh biomass from lowland hay meadows, dry grasslands (collected in July), cultivated grass (collected in July), *Heracleum sosnowskyi* (collected in early June) and freshwater green algae were used.

2.2 Preparation of cellulolytic enzymes

Laboratory scale preparation of cellulolytic enzymes was performed according to the methodology described by Mezule et al. (2012). In brief, pre-homogenized liquid cultures of *Irpex lacteus* IBB 104 were transferred to liquid media (2.0 g/L NH_4NO_3; 0.8 g/L KH_2PO_4; 0.4 g/L K_2HPO_4; 0.5 g/L $MgSO_4 \cdot 7H_2O$; 2.0 g/L yeast extract; pH = 5.5–5.8) containing hay as a carbohydrate source. After 10 days the biomass was removed and $(NH_4)_2SO_4$ (0.5 kg/L) was added to the supernatant, dissolved and incubated at 4 °C for 24–48 h. After the incubation the enzyme was sedimented by centrifugation (8500 rcf; 10 min) and stored in 0.05 M sodium citrate buffer at 4 °C for further use. Enzyme activity (FPU) was estimated according to IUPAC recommendations (Ghose, 1987).

2.3 Substrate pre-treatment and hydrolysis

The dried biomass was grounded by grinder (Retsch GM200) and screened with a sieve to obtain various biomass fractions (powder, < 0.5 cm, 0.5 – 1 cm, 1 – 2 cm, > 2 cm). Fresh biomass was ground to a size < 0.5 cm. Prior heat treatment the biomass was diluted in 0.05 M sodium citrate buffer (3 % w/v) and either boiled for 5 minutes or heat treated at 121 °C for 15 minutes.
For enzymatic hydrolysis, the prepared enzyme (0.2 FPU/mL, 20 FPU/g) was added to the diluted substrates and incubated on an orbital shaker for 24 – 48 h at 30 – 50 °C depending on the experiment setup. For process optimization sterile distilled, nanofiltered or tap water was used as a solvent.
As a reference acid hydrolysis was used for the same substrates. The grounded biomass samples were diluted in H_2SO_4 (5 % final concentration) and heat treated at 121 °C for 15 min. Prior sampling for the released sugars, adjustment to pH 5 with concentrated NaOH was performed.
All tests were prepared in triplicates and at least 2 samples from each test were collected for produced sugar measurements.

2.4 Measurements of sugar concentration

Reducing sugar concentration was measured by dinitrosalicylic acid (DNS) method (Ghose, 1987). In brief, all samples were centrifuged (6,600 g, 10 min). Then 0.1 mL of the supernatant was mixed with 0.1 mL of 0.05 M sodium citrate buffer and 0.6 mL of DNS. For blank control, distilled water was used instead of the sample. Then all samples were boiled for 5 minutes and transferred to cold water. Then 4 mL of distilled water was added. Absorption was measured with spectrophotometer at 540 nm (Camspec M501, UK). To obtain absolute concentrations, a standard curve against glucose was constructed.

2.5 Statistical analyses

MS Excel 2007 ANOVA single parameter tool (significance level ≤ 0.05) was used for analysis of variance on data from various sample setup`s. To determine if the data sets are significantly different or not, t-test analyses (MS Excel 2007) were performed for two tailed distributions. Probabilities of ≤ 0.05 were considered as significant.

3. Results and discussion

3.1 Pre-treatment and solvent effect

To make the handling of the biomass material easier, reduction of the particle size by mechanical treatment is often used. Increased surface/volume ratio increase the effectivity of the biomass hydrolysis (Kumar et al., 2009). Research related to the effect of particle size has shown high inconsistencies among the reported results (Zhang et al., 2013), indicating on the need for the evaluation of this parameter within each biomass treatment technique. To analyze the effect of hay particle size on enzymatic hydrolysis, reducing sugar concentration in the biomass with the size range of powder, < 0.5 cm, 0.5 – 1 cm, 1 – 2 cm and > 2 cm was measured directly after grinding and after enzymatic hydrolysis. The results showed that the highest variations among the results of the size groups occurred with the size above 0.5 cm. Significantly different (p < 0.05)

sugar yields were observed in-between samples of > 2 cm and those below 0.5 cm. No difference (p > 0.05) between > 2 cm and 1-2 cm was observed in samples both after grinding and after hydrolysis (Figure 1). The same observation was also for the samples of powder and < 0.5 cm with only difference that samples of < 0.5 cm gave the highest standard deviation values. This could be explained by variations among the produced grinded biomass size – from powder to 0.5 cm phase in a single sample; creating a real situation – no detailed size checkup during the production process. Other samples showed variable yields in between the experiments or treatments (wetting and hydrolysis), giving raise to the constant inconsistencies of the reported results. Nevertheless, the analysis of the reducing sugar concentrations after the hydrolysis showed 24-35 % higher sugar yields in samples with lower particle size (< 0.5 cm). Due to the observations particle size of < 0.5 cm was used in all further tests. The use of powder type biomass was omitted due to almost double energy consumption when compared to <0.5 cm and the observed yields were only 13 % higher for powder (p >0.05). Subsequent fractionation in particle size was omitted due to the fact that commercial availability of such grinding technologies is either limited or highly expensive.

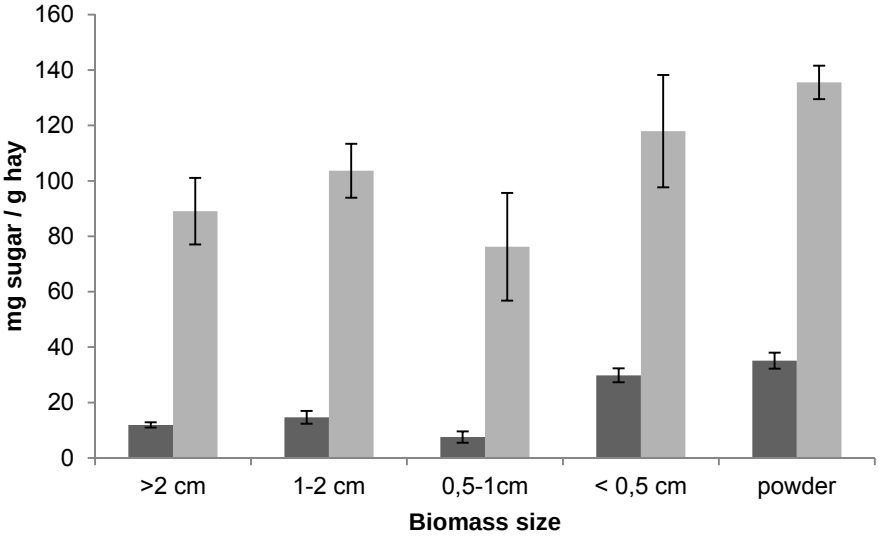

Figure 1: The amount of reducing sugars produced from various biomass sizes directly after wetting (dark) and after enzymatic hydrolysis (light). The bars represent average values from 6 replicates.

Another feature assessed within the research was the type of solvent used for biomass wetting prior mild thermal treatment (5 min boiling as reported by Mezule et al. (2012)) and subsequent enzymatic hydrolysis. Generally this issue is not addressed during the technology evaluation by limiting to laboratory pure (deionised) (Chen et al., 2012) or unidentified water (Diaz et al., 2013). No matter as insignificant it seems the production of distilled or ultra pure water has an effect on the overall production costs. During the study three water types were evaluated: laboratory pure deionised water, tap water complying all regulations (European Comission, 1998) and nanofiltered tap water. The latter was used due to the wide availability of such devices intended for commercial scale, thus, increasing the competitiveness and minimizing production costs. The results of the research showed that the highest sugar yields from hay were obtained when pure deionised water was used for biomass wetting. A 6% decrease was observed for nanofiltered and 14 % for tap water. Thus, giving no significant difference (p > 0.05) in between pure deionised and nanofiltered water, however, for pure deionised and tap water sugar yields differed significantly (p < 0.05). Slightly different results were obtained for fresh biomass obtained from dry grasslands where almost no difference was observed for pure deionised and nanofiltered water and a 3.4 % decrease for tap water (p > 0.05). Despite the relatively low variations it was concluded that tap water due to its changing nature and quality (regionally and in time) can affect the produced sugar yields.

3.2 Hydrolysis conditions

Enzymatic hydrolysis is generally linked to very slow processes with often low yields (Chaturvedi and Verma, 2013), however, the method does not require high temperatures and is regarded as environmentally friendly (Brodeur et al., 2011). Enzyme producing fungi are incubated with the lignocellulosic biomass for 2 to 23 days

(Chaturvedi and Verma, 2013). Shorter incubations are achieved with commercially available or pre-prepared enzymes at temperatures around 48 – 50 °C when fungal cellulases are regarded as the most effective (Brodeur et al., 2011). At the same time white-rot fungus *Irpex lacteus* is cultured at 28 – 30 °C (Novotný et al., 2000). Thus, to estimate the necessity of increased temperature (50 °C) hydrolysis, a comparative study was performed. The results (Table 1) showed that higher reduced sugar yields from hay biomass were obtained at the temperatures closer to the natural growth and enzyme production temperature of *I. lacteus.* 30 % higher reducing sugar yields were obtained in samples incubated at 30 °C than at 50 °C. A reduction in 9 % of sugar yield was observed when the temperature was increased from 30 °C to 37 °C ($p < 0.05$). It is regarded that mesophilic bacteria found in the biomass are not effective at 50 °C temperatures, thus, non-sterile saccharification of lignocellulose has been proposed (Song et al., 2013). The results of this study showed that there is a significant difference ($p < 0.05$) in between the sugar yields of heat pre-treated and untreated samples.

Table 1: The amount of reduced sugars produced from hay at various enzymatic hydrolysis conditions. Standard deviation represents the average values from 6 replicates

Time, h	Temperature, °C	mg/g reducing sugar
24	30	176.1 ± 7.4
	37	160.3 ± 10.4
	50	122.6 ± 7.9
	50 (no heat pre-treatment)	99.7 ± 4.6
48	30	172.6 ± 11.6
	37	172.6 ± 7.7

An additional release of sugars during the heat pre-treatment and reduced effect of sugar consuming microorganisms are the reasons for the choice of the selected thermal treatment. As it was shown previously (Mezule et al., 2012) there is no need for energy intense sterilization – simple 5 min boiling is sufficient. Within the study it was observed that irrespective of the generally low sugar concentration, the released sugars, vary ($p < 0.05$) before and after thermal treatment. Thus, 10 minutes of boiling were compared to the previously used 5 minutes. The results showed that the yields increased ($p < 0.05$) from 13 % (straw) to 18 % (hay), however, due to the double energy consumption; this was not regarded as essential improvement in the overall technology.

To increase the sugar yields longer hydrolysis was introduced. No significant ($p > 0.05$) improvement in the sugar yields was observed when the samples are incubated at 30 °C for 24 or 48 h (Table 1). Additionally there was no difference in sugar yields hydrolysed at 30 °C or 37 °C after 48 h.

3.3 Technology effectivity and substrate evaluation

To assess the efficiency of the technology, sugar production yields were compared with acid hydrolysis (Figure 2). Mechanical grinding released low amount of reducing sugars in wetted biomass of all samples ($p < 0.05$). No significant increase ($p > 0.05$) in reducing sugar concentration after thermal treatment was observed for samples containing only wetting buffer. At the same time reducing sugar concentration in samples undergoing acid hydrolysis increased significantly ($p < 0.05$). The results showed that boiling is not as efficient (24% lower sugar yields, $p < 0.05$) than heating at 121 °C. This corresponds to the data available for efficient acid hydrolysis where the temperatures are closely linked to the acid concentrations (Janga et al., 2012). Subsequent 24 h enzymatic hydrolysis (pH 5 adjusted for all samples) increased reducing sugar concentration in non-acid treated samples to an average level of 216 mg/g substrate which was only slightly lower than 220 mg/g substrate obtained with acid hydrolysis ($p > 0.6$). The results supported the described advantages and disadvantages of both techniques where long treatment time comes up against the use of chemicals (Brodeur et al., 2011). Nevertheless, enzymatic hydrolysis was regarded as superior due to the fact that final liquid was directly available for fermentation and no presence of elevated salt concentration generated during pH adjustment was affecting the growth of fermenting microorganisms.

Assessment of the described technique was evaluated on various biomass resources available in temperate climate zones (Figure 3). Due to limited seasonal availability of various biomass both dried and fresh biomass was tested. The results showed low differences between hay and fresh grass from cultivated and lowland hay meadows (163-194 mg/g dry mass). Higher yields from hay could be affected by the earlier biomass collection period, which also supported the more than 300 mg sugar/g dry mass from *H. sosnowskyi* collected in early June. The lowest reducing sugar yields (below 50 mg/g) were obtained from straw showing the potential drawback of this material.

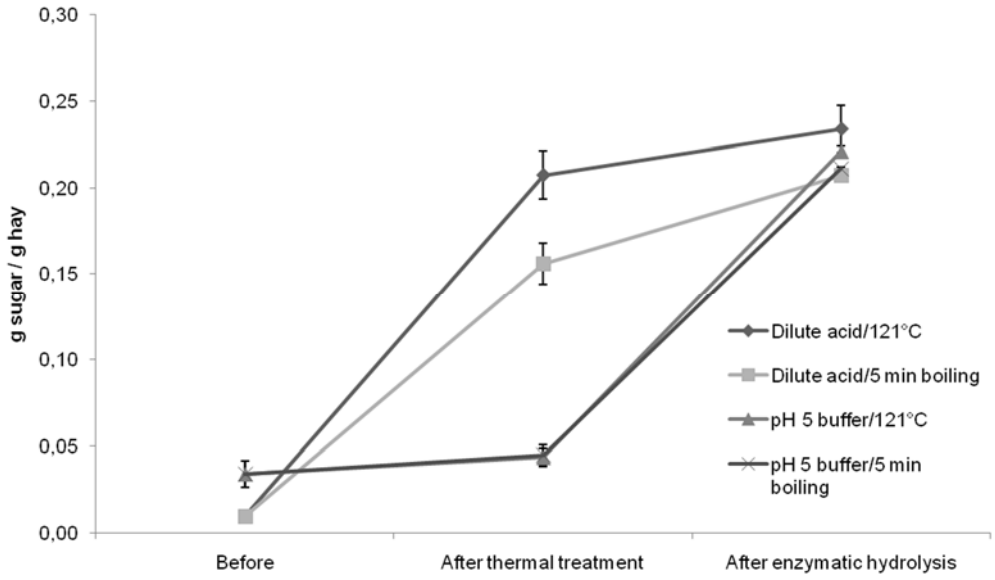

Figure 2: The amount of reducing sugars produced from hay with enzymatic and acid hydrolysis with 5 min of boiling or 30 min at 121 °C thermal treatment. Standard deviation represents the average values from 3 replicates.

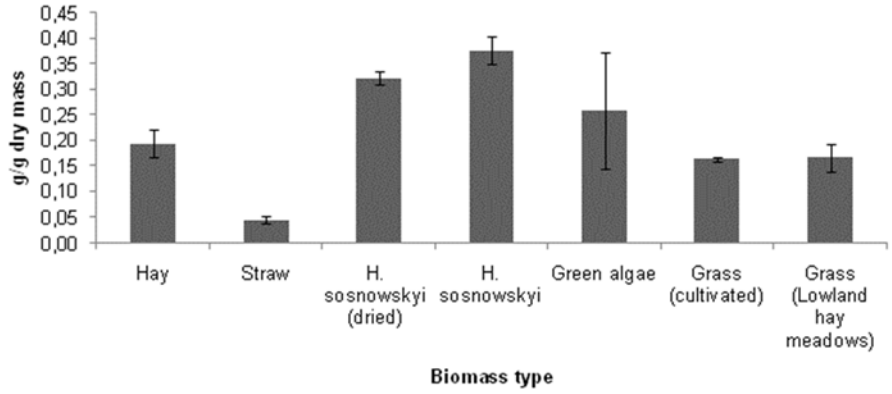

Figure 3: Comparison of sugar yields obtained from various biomass sources with mild-enzymatic hydrolysis technique. Standard deviation represents the average values from 3 replicates.

Despite the observed outcomes, the results showed that in none of the cases increased sugar productivity was observed when compared to the available yields of other techniques (Chaturvedi and Verma, 2013). Variability within the substrates, pre-treatment conditions and enzymes used can affect the outcome. To apply the technology in a commercial scale such issues should be minor. One of the potential solutions could be enzyme recovery and sugar concentration by membrane technologies (Knutsen and Davis, 2004), thus, excluding any potential shifts in productivity of fermentation feed. However, the potential application and introduction of this technology is still not fully described.

4. Conclusions

Effective conversion of lignocellulosic biomass to fermenting sugars is not only limited to selection of most efficient pre-treatment/hydrolysis technique. Factors like, biomass type and collection period, size, preparatory reagents and treatment conditions affect the outcome.
This study showed that enzymatic hydrolysis can be as efficient as acid hydrolysis and the overall enzymatic treatment time can be reduced to 24 h at 30 °C which is more energy efficient than described before.

The study allowed to come up with a simple technique for lignocellulose hydrolysis which can be applied at a small-scale and do not require extensive resource and technological involvement.

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