



Pretreatment of *Miscanthus* for hydrogen production by *Thermotoga elfi*

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Abstract

Pretreatment methods for the production of fermentable substrates from *Miscanthus*, a lignocellulosic biomass, were investigated. Results demonstrated an inverse relationship between lignin content and the efficiency of enzymatic hydrolysis of polysaccharides. High delignification values were obtained by the combination of mechanical, i.e. extrusion or milling, and chemical pretreatment (sodium hydroxide). An optimized process consisted of a one-step extrusion-NaOH pretreatment at moderate temperature (70°C). A mass balance of this process in combination with enzymatic hydrolysis showed the following: pretreatment resulted in 77% delignification, a cellulose yield of more than 95% and 44% hydrolysis of hemicellulose. After enzymatic hydrolysis 69% and 38% of the initial cellulose and hemicellulose fraction, respectively, was converted into glucose, xylose and arabinose. Of the initial biomass, 33% was converted into monosaccharides. Normal growth of *Thermotoga elfi* on hydrolysate was observed and high amounts of hydrogen were produced.

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1. Introduction

The utilization of hydrogen for the purpose of replacing carbonized fuels in the energy and chemical industry is gaining worldwide interest. However, when fossil fuels are used for the production of hydrogen, there is no advantage with respect to the reduction of CO₂ emission. Therefore, attempts are being made to produce hydrogen in a CO₂ neutral way. The biological production of hydrogen by fermentation using biomass as energy source is one of these new developments [1–3].

A successful biological conversion of biomass to hydrogen depends strongly on the processing of raw materials to produce feedstock which can be fermented by the microorganisms. Lignocellulosics are especially interesting as a source of biomass due to their abundance and low costs.

Various pretreatment methods are described which promote the accessibility of polysaccharides in a lignocellulose complex for enzymatic hydrolysis. Examples are steam explosion and wet oxidation under alkaline conditions, supercritical CO₂ pretreatment, mild and concentrated acid hydrolysis and solvent extractions [4–7]. These methods often involve conditions, e.g. high temperatures, which may lead to the formation of degradation products which act as inhibitors in fermentations [8]. An alternative mechanical pretreatment method is extrusion. In this study, a corotating twin-screw extruder is used. The biomass is transported via transport screws to a reversed screw element (RSE). This results in accumulation and compression of the material in the space between the transport screws and the RSE. High compression and shear forces cause defibration, fibrillation and shortening of the fibers in the biomass [9,10]. The efficiency of this pretreatment method is determined by comparing hydrolysis yields of extruded biomass and milled material of different particle size.

As an example of a lignocellulosic material, *Miscanthus* has been tested as substrate for the bioprocess. It is a woody

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rhizomatous *C₄* perennial grass species which grows rapidly and gives high yields per hectare. Extrusion in combination with sodium hydroxide pretreatment caused a substantial delignification of *Miscanthus* material and significantly improved *C₅* and *C₆* sugar yields by subsequent enzymatic hydrolysis of cellulose and hemicellulose. Many heterotrophic bacteria are known to produce hydrogen from saccharides [11]. Compared to mesophilic and moderate thermophilic bacteria, extreme and hyperthermophiles produce higher hydrogen yields (close to the theoretical maximum of 4 mol of H_2 /mol of hexose) [12–14]. A *Miscanthus* hydrolysate promoted hydrogen production by the extreme thermophilic bacterium *Thermotoga elfii*. There were no indications for the presence of inhibitors in the hydrolysate.

2. Material and methods

2.1. *Miscanthus*

Miscanthus used in this study was collected in the spring of 2000 and 2001 from a location in Groningen, The Netherlands. Stems were harvested and chopped to a length of 0.5–5 cm. The dry weight of the harvested material varied between 86% and 90%. The samples were stored, protected from the weather under dry conditions.

2.2. Pretreatment

Miscanthus was pretreated by a combination of a mechanical and chemical method. Mechanical treatment existed of either milling or extrusion. A Retsch mill equipped with a 1 mm or 0.25 mm sieve, or a ZPS50 sifter mill (Hosokawa–Alpine, Germany) was used for size reduction. Lengths of the two latter samples were determined by particle size analyses which showed a mean length of 0.22 mm and 17 μ m, respectively. A Clextral BC45 corotating twin-screw extruder (Clextral, Firminy, France) with a screw diameter of 55 mm and a total axis length of 1.25 m was used for the extrusion experiments. The following conditions were applied: screw speed, 100 rpm; biomass throughput, 15–30 kg dry matter/h; temperature of extruder, 100°C; reversed screw element RSE-15H6; specific energy consumption, approximately 300 kWh/ton dry matter.

Miscanthus was chemically pretreated with 12% NaOH (w/w dry matter) at variable solid:liquid ratios. Standard condition of the incubation was at 70°C for 4 h. Only sample E1 was treated differently. NaOH-treated samples were washed with excess water to reduce the alkalinity of the material prior to enzymatic hydrolysis. Pretreated samples were stored at 4°C or at room temperature after drying at 45°C.

2.3. Enzymatic hydrolysis

Enzymatic hydrolyses of pretreated *Miscanthus* samples were performed using commercial cellulase and

β -glucosidase preparations (Novozymes, Bagsvaerd, Denmark). The filter paper activity of Celluclast 1.5 LFG (34.5 FPU/ml) was determined according to the International Union of Pure and Applied Chemistry (IUPAC) procedure [15]. β -Glucosidase activity of Celluclast 1.5 LFG (12.5 U/ml) and Novozym 188 (172 U/ml) was determined by measuring the amount of *p*-nitrophenol liberated from *p*-nitrophenyl- β -glucopyranoside after 10 min. Both assays were done in citrate buffer (50 mM), pH 4.8 at 45°C. One unit was defined as the amount of enzyme required to liberate 1 μ mol of glucose or *p*-nitrophenol per minute for filter paper and β -glucosidase activity, respectively.

Standard conditions of enzymatic hydrolysis of *Miscanthus* were: substrate concentration, 5% w/v; enzyme concentrations, 1.6 FPU (Celluclast) and 2.3 β -glucosidase units (Celluclast plus Novozym) per gram dry matter; buffer, 50 mM citrate, pH 4.8; temperature, 45°C; incubation time, 0–72 h. Oxy-tetracycline, gentamycin and cycloheximide (200, 100 and 50 μ g/ml, respectively) were added as preservatives to hydrolysates-containing substrates which were not treated with NaOH. Analytical and larger batch experiments of 20 and 500 ml, respectively, were carried out in duplicate. Samples were collected at 0 and approximately 7, 24, 48 and 72 h. After pelleting of the remaining solids the liberated, soluble sugars in the supernatant were determined by enzymatic method (glucose) or by HPLC (total sugars). The glucose yield was taken as a measure of the hydrolysis efficiency and is expressed as a percentage of the maximum amount of glucose obtained after complete chemical hydrolysis.

2.4. Microorganism and medium

T. elfii DSM 9442 was purchased from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany). A modified DSM664 medium consisted of (per liter): NH_4Cl 1.0 g, K_2HPO_4 0.3 g, KH_2PO_4 0.3 g, $MgCl_2 \cdot 6H_2O$ 0.2 g, $CaCl_2 \cdot 2H_2O$ 0.1 g, KCl 0.1 g, $NaCl$ 10.0 g, Na-acetate 0.5 g, yeast extract 4 g, cysteine-HCl $\cdot H_2O$ 0.5 g, Na_2CO_3 2.0 g, resazurine 0.5 mg, trace elements 10.0 ml. Glucose was used as carbon and energy source (7 or 10 g/l in medium with hydrolysate). The pH was adjusted to 8.0 with HCl. The trace elements were (per liter): nitrilotriacetic acid 1.5 g, $MgSO_4 \cdot 7H_2O$ 3.0 g, $MnSO_4 \cdot 2H_2O$ 0.5 g, $NaCl$ 1.0 g, $FeSO_4 \cdot 7H_2O$ 0.1 g, $CoSO_4 \cdot 7H_2O$ 0.18 g, $CaCl_2 \cdot 2H_2O$ 0.1 g, $ZnSO_4 \cdot 7H_2O$ 0.18 g, $CuSO_4 \cdot 5H_2O$ 0.01 g, $KAl(SO_4)_2 \cdot 12H_2O$ 0.02 g, H_3BO_3 0.01 g, $Na_2MoO_4 \cdot 2H_2O$ 0.01 g, $NiCl_2 \cdot 6H_2O$ 0.025 g, $Na_2SeO_3 \cdot 5H_2O$ 0.3 mg. The medium was made anaerobic by flushing with nitrogen (100%) and sterilized by autoclaving. Separate sterile, anaerobic stock solutions were prepared of Na_2CO_3 , trace elements and glucose. An anaerobic, non-sterile *Miscanthus* hydrolysate was used for the experiments.

2.5. Cultivation

T. elfii was cultivated in anaerobic serum bottles (100 ml) in culture volumes of 30 ml at 65°C. The flasks were inoculated with approximately 1 ml of a culture in the exponential phase in the same medium (4 g/l glucose). Experiments were carried out in duplicate.

2.6. Analytical methods

The chemical composition of *Miscanthus* was determined using methods described by the Technical Association of the Pulp and Paper Industry (Tappi). Milled samples (Retsch mill with 0.5 mm sieve) were successively extracted with ethanol/toluene (2:1, v/v) in a Soxtec system and by hot water (100°C) [16]. Lignin and neutral sugars of dried extracted material were determined after sulfuric acid hydrolysis. Samples were either directly hydrolyzed in 1 M H₂SO₄ (3 h, 100°C, polysaccharides without cellulose) or first dispersed in 72% H₂SO₄ (1 h, 30°C) followed by hydrolysis in 1 M H₂SO₄ (3 h, 100°C, total polysaccharides). Neutral sugars were determined as alditol acetates by GC or directly by HPLC. The gas chromatograph was equipped with a CP-SIL 88 WCOT fused silica column (Chrompack, The Netherlands) and a flame ionization detector. Helium was the carrier gas. Sugars were separated by HPLC on a Shodex ionpak KC811 column (Waters, The Netherlands) at 80°C with differential refractometric detection and 3 mM H₂SO₄ as the mobile phase (flow, 1 ml/min). Acid-insoluble lignin was determined gravimetrically as Klason lignin and acid-soluble lignin by spectrophotometric analysis [17]. Uronic acids were measured spectrophotometrically using galacturonic acid as the standard [18]. The protein content was determined from the total nitrogen content (Kjeldahl method) using a conversion factor of 6.25. Ashes were determined after combustion of the samples at 525°C for 4 h [19].

Untreated *Miscanthus* was analyzed in octuple. The applied hydrolysis procedure allowed to discriminate between non-cellulose and total sugars. Pretreated *Miscanthus* samples were analyzed in duplicate. The glucose content in the total polysaccharide fraction was representative for the cellulose (glucan) content and the sum of xylose, arabinose and galactose for the hemicellulose (xylan) content.

Glucose in hydrolysates was determined enzymatically (modification of the Trinder method [20]; Sigma, The Netherlands). Soluble sugars in hydrolysates and fermentation medium were analyzed by HPLC as described. The same HPLC method was used for determination of the organic acid content in fermentation medium. Reducing sugars in the filter paper assay were determined using the DNS method [21] with glucose as the standard. Dry weight contents were determined after drying at 105°C for 24 h. Hydrogen was measured by GC using a RVS MolSieve 5A, 60/80 mesh, 3 m × 1/8" column. The temperature of the thermal conductivity detector, injector and column was

100°C, 80°C and 50°C, respectively. N₂ was used as the carrier gas.

3. Results

3.1. Chemical composition of *Miscanthus*

Results on the chemical composition of *Miscanthus* showed that polysaccharides represent the largest fraction, i.e. 62.5% of the total dry matter (Table 1). The most abundant residue of the polysaccharides was glucose, representative of cellulose (glucan). The hemicellulose was of a xylan type because of a relatively high amount of xylose residues in the remaining polysaccharide fraction. The total lignin content was 25.0% and the lignin consisted mainly of acid-insoluble Klason lignin.

3.2. Pretreatment

In a first experiment, conditions for the chemical pretreatment of milled *Miscanthus* (0.22 mm) were studied. Based on earlier experiments with *Miscanthus* [22], a solid:liquid ratio of 1:6 and a high NaOH load of 12% (w/w) were applied. Incubation was for 4 h at variable temperatures. NaOH treatment resulted in efficient delignification of *Miscanthus* which increased at higher incubation temperatures (Table 2). At the same time, a relative increase of the glucan content in the dry solid residue was observed. Control (water) treatment at 95°C did not show significant effects on lignin and glucan contents. Enzymatic hydrolysis of the glucan fraction of the residual solids was higher after NaOH treatment and increased with increasing temperatures during NaOH pretreatment. Because no further significant increase of hydrolysis was observed at 95°C a pretreatment temperature of 70°C was applied in further experiments.

The effect of various pretreatment methods (Table 3) on the composition of *Miscanthus* and the yield of the

Table 1
Chemical composition of *Miscanthus* expressed as percentage of dry matter

Component	Amount %	Monomeric residues in polysaccharide fraction	Amount %
Cellulose	38.2 (3.2)	glucose	39.5 (3.2)
Hemicellulose	24.3 (1.4)	xylose	19.0 (1.3)
Lignin		arabinose	1.8 (0.2)
acid insoluble	24.1 (0.9)	galactose	0.4 (0.2)
acid soluble	0.9 (0.1)	uronic acids	1.8 (0.3)
Protein	1.3 (0.1)		
Solvent extract	4.2 (0.2)		
Hot water extract	1.4 (0.2)		
Ash	2.0 (0.0)		

Standard deviations are shown within parenthesis.

Table 2

Effect on lignin and glucan content and on enzymatic hydrolysis of glucan of milled *Miscanthus* (0.22 mm) chemically pretreated with NaOH at different temperatures

Sample	Lignin (%) ^a	Glucan (%) ^a	Glucan hydrolysis (%) ^b
Control, 95°C	25.1 (1.4)	32.0 (1.3)	15.5 (1.6)
NaOH, 50°C	14.2 (0.6)	48.9 (1.6)	38.5 (0.4)
NaOH, 70°C	11.5 (0.1)	49.1 (0.7)	49.9 (0.4)
NaOH, 95°C	9.2 (0.6)	52.9 (2.3)	50.7 (0.5)

^aContents are expressed as percentage of dry solids after pretreatment.

^bHydrolysis to glucose is expressed as percentage of glucan content after pretreatment.

Standard deviations are shown within parenthesis.

residual dry matter has been determined (Table 4). For all samples, except E1, the relative glucan content was increased compared to the initial material (Table 1). Relative xylan contents were not significantly altered or increased. Pretreatment resulted in partial solubilization of the solids. The solubilized products consisted of hydrolyzed hemicellulose and decomposed lignin. Delignification values of more than 70% have been reached for most of the pretreatment methods, only delignification of sample E1 was less. Xylan solubilization increased with decreasing particle size and was high for extruded samples, except E1. In general, the glucan yield was high, but there was a notable loss of glucan for material of 17 µm (sample M3).

3.3. Enzymatic hydrolysis

Miscanthus polysaccharides were enzymatically hydrolyzed by commercially available cellulase and

β -glucosidase preparations. To determine the optimal conditions of hydrolysis, Celluclast 1.5 LFG and Novozym 188 were tested in various concentrations and ratios. Glucose yields were measured for an extruded and NaOH pretreated sample (E3) (Fig. 1). At increasing concentrations of Celluclast (2.5–10 FPU/g glucan) and Novozym in a fixed ratio, a significantly higher hydrolysis rate was observed. In addition, the glucose yield at 72 h incubation was higher, and increased from 50% at a low cellulase concentration to 71% at 10 FPU/g glucan. In all cases, a high initial rate was followed by a decreasing hydrolysis rate. Hydrolysis continued after 72 h (results not shown). Higher β -glucosidase activities did not effect the reaction rate or the glucose yield, suggesting that decreasing hydrolysis rates were not due to product inhibition by cellobiose (Fig. 1). The presence of 1% glucose in the reaction mixture did lower the hydrolysis rate and the glucose yield (Fig. 1). In this case cellobiose accumulated in the hydrolysate suggesting inhibition of β -glucosidase activity by glucose.

Subsequently, all NaOH pretreated milled or extruded *Miscanthus* samples and some controls were tested for glucan hydrolysis applying a low Celluclast concentration of 1.6 FPU/g dry matter. The glucose yield of milled NaOH pretreated samples increased as the particle size of the material decreased (Fig. 2). Maximum glucan hydrolysis of 17 µm material after 72 h incubation amounted to 56%. Milled material, not chemically pretreated showed inefficient hydrolysis and the maximum value was reached within 24 h of incubation. Also in this case glucan hydrolysis was higher for material with a smaller particle size. Extruded samples which were treated with NaOH during or following extrusion (samples E2–E4) showed a comparable hydrolysis efficiency reaching a maximal value of 50% after 72 h incubation (Fig. 3). Glucan hydrolysis was significantly lower for material which had undergone chemical (and steam)

Table 3

Survey of applied pretreatment methods

Sample code	First pretreatment	Dry weight, % (L:S ratio)	Second pretreatment	Dry weight, % (L:S ratio)
M1	Milling, approx. 1 mm	86	NaOH	14 (6:1)
M2	Milling, 0.22 mm	90	NaOH	14 (6:1)
M3	Milling, 17 µm	86	NaOH	14 (6:1)
E1	NaOH → steam ^a	9 (10:1)	extrusion	25
E2	Extrusion	86	NaOH	14 (6:1)
E3	Extrusion + NaOH feed ^b	86 → 30 (2.3:1)		
E4	Extrusion + NaOH feed ^c	52 → 20 (4:1)		

^a*Miscanthus* with a length between 0.5 and 5 cm was impregnated with 8% NaOH (w/w) at room temperature for 24 h. After allowing the liquid to drain through a perforated screen the impregnated material was preheated with saturated steam at atmospheric pressure for 10 min.

^b*Miscanthus* (0.5–5 cm) was manually fed in a dry form to the extruder. An NaOH solution was supplied through an injection port downstream of the RSE.

^c*Miscanthus* (0.5–5 cm) was first impregnated with excess water for 16 h at room temperature. After drainage of water the material was fed to the extruder and treated as in footnote b.

The liquid:solid ratio of the NaOH treatment is shown within parenthesis.

Table 4

Effect of pretreatment of *Miscanthus* on the composition of the solids remaining after treatment, the yield of solids and the removal of various components from the solid fraction

Sample	Composition (%) ^a			Solid yield (%) ^b	Removal (%) ^c		
	Glucan	Xylan	Lignin		Glucan	Xylan	Lignin
M1	56.5	29.1	10.8	64	–2	20	70
M2	54.3	27.7	10.9	n.d.			
M3	48.8	26.5	14.1	57	24	36	67
E1	41.4	25.2	16.4	88	–2	6	37
E2	55.6	26.9	9.8	58	9	33	75
E3	59.9	22.1	9.3	52	1	44	77
E4	57.7	22.0	9.4	57	5	44	77

^aComposition is shown as percentage of the remaining solid fraction after pretreatment.

^bSolid yield is shown as percentage of the initial amount of dry matter.

^cLoss of components is shown as percentage of the amount in the initial material.

n.d. Not determined. The maximum standard deviation was 3.7, 1.7 and 1.2 for glucan, xylan and lignin composition values, respectively.

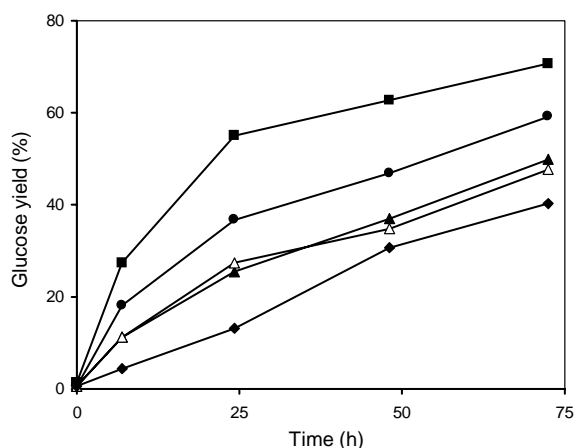


Fig. 1. Enzymatic hydrolysis of extruded and NaOH-pretreated *Miscanthus* (sample E3) at substrate concentrations of 5% (w/v) and various enzyme concentrations. Cellulase: (▲, △) 2.5; (●) 5 and (■) 10 FPU/g glucan and β -glucosidase: (▲) 2.3; (△) 4.2; (●) 2.9; and (■) 3.9 U/g dry matter. (◆) enzyme concentrations as (▲) plus 10 g/l glucose. Glucose yield is expressed as percentage of the maximum amount of glucose in glucan. The maximum standard deviation was 0.8.

pretreatment prior to extrusion (E1) and amounted to 21%. No hydrolysis was observed for steamed extruded material.

The presence of other monomeric sugars in the hydrolysates was determined (Table 5). Besides glucose hydrolysis products of hemicellulose, xylose and arabinose were present. The concentration of these sugars was dependent on the applied pretreatment method and showed a similar pattern as the glucose concentration. Hardly any hydrolysis of xylan occurred for material without NaOH treatment. Enzymatic hydrolysis of xylan could occur because of the presence of xylanase activity in Celluclast and a

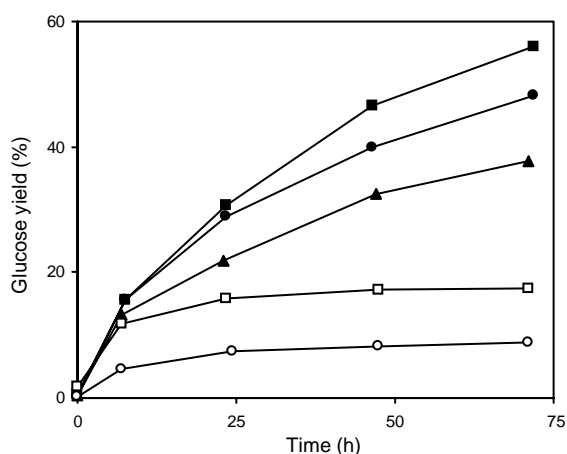


Fig. 2. Enzymatic hydrolysis of milled *Miscanthus*. NaOH treated: (▲) 1 mm; (●) 0.22 mm and (■) 17 μ m particle size. Non-treated: (○) 0.22 mm and (□) 17 μ m particle size. Enzyme activities: cellulase, 1.6 FPU/g dry matter; β -glucosidase, 2.3 U/g dry matter. The maximum standard deviation was 0.4.

low β -xylosidase activity in both Celluclast and Novozym. Starting with an initial concentration of 50 g/l biomass a maximum concentration of 32 g/l monomeric sugars was reached after 72 h incubation. Under standard conditions no cellobiose was present in the hydrolysates, but possibly xylobiose and other oligomeric hydrolysis products of xylan were present. This could be caused by the low β -xylosidase activity (10 times lower than β -glucosidase activity under the conditions applied).

Efficiencies of hydrolysis and conversion of glucan, xylan and total biomass are depicted in Table 6. The conversion efficiency is based on the initial biomass taken into account the loss of dry matter by pretreatment. Because of the

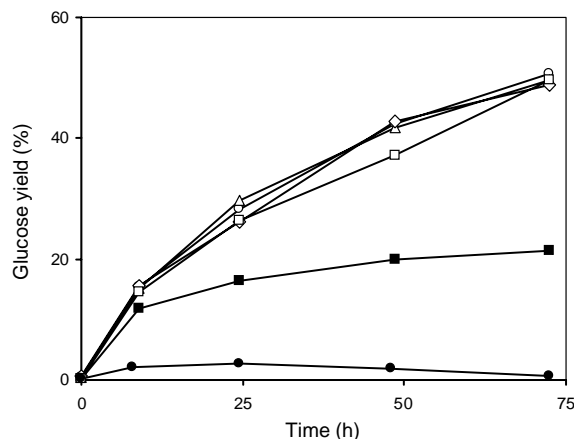


Fig. 3. Enzymatic hydrolysis of extruded and NaOH-pretreated *Miscanthus*. (■) E1; (○), E2; (□) E3 and (◇) E4. (●) extruded and steam pretreated *Miscanthus*. Enzyme activities: cellulase, 1.6 FPU/g dry matter; β -glucosidase, 2.3 U/g dry matter. The maximum standard deviation was 0.7.

Table 5

Enzymatic hydrolysis of NaOH-pretreated *Miscanthus* and control samples

Sample	Glucose (g/l)	Xylose + arabinose (g/l)	Monomeric sugars (g/l)
Control M2	1.7	0.7	2.4
Control M3	3.4	1.1	4.6
M1	11.7	7.2	18.9
M2	13.5	6.9	20.4
M3	14.4	8.3	22.7
Control E1	1.1	0.3	1.4
E1	4.8	4.8	9.6
E2	15.3	8.3	23.6
E3	16.7	6.8	23.4
E4	15.6	6.4	22.0
E3, high FPU	23.5	8.5	32.0

Hydrolysis of biomass (50 g/l) to monomeric sugars under standard amounts of Celluclast (1.6 FPU/g dry matter) and Novozym and using a four times higher concentration (6.3 FPU/g) in case of sample E3. Incubation time was 72 h. Control M2 and M3 were milled *Miscanthus*, control E1 was extruded and steam pretreated *Miscanthus*, but without NaOH treatment. The maximum standard deviation was 0.5 and 0.3 for glucose and xylose + arabinose concentration, respectively.

minimal loss of glucan, the hydrolysis and conversion efficiencies are almost equal except for sample M3. The conversion efficiency of xylan was much lower than the hydrolysis efficiency, mainly because of the significant loss of xylan caused by NaOH pretreatment. In general, biomass conversion of milled or extruded *Miscanthus* in combination with a chemical treatment did not significantly differ (except sample E3). Maximal conversions of 69%

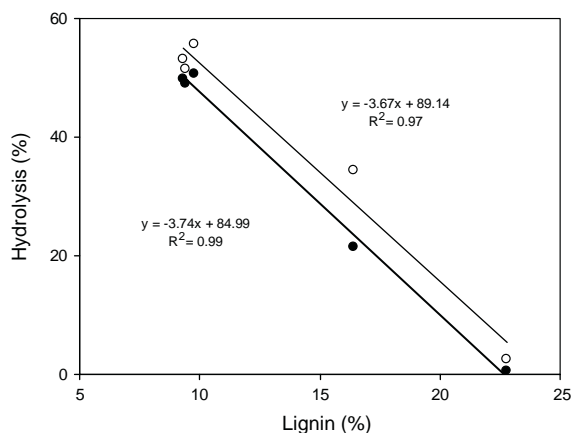


Fig. 4. Relation between lignin content and hydrolysis of glucan (●) and xylan (○) of extruded *Miscanthus* under standard conditions.

and 38% of glucan and xylan, respectively, into monomeric sugars were obtained, which corresponds to 34% of the biomass (sample E3, 10 FPU/g glucan).

A linear relationship was observed between lignin content of extruded *Miscanthus* and the level of enzymatic hydrolysis of glucan and xylan (Fig. 4). At equal lignin contents xylan conversion was on average 4% higher than glucan conversion. Under the standard conditions at low cellulase concentrations, a maximal theoretical conversion can be derived at 0% lignin and amounts to 85% and 89% for glucan and xylan, respectively. For milled samples no relationship could be obtained with lignin content. Here, hydrolysis of polysaccharides seemed to be dependent on the particle size of the material.

3.4. Thermophilic fermentation

Growth of the extreme thermophilic bacterium, *T. elfii*, on hydrolysate was compared with growth on glucose. A batch culture on glucose medium showed a normal growth curve with a short lag phase followed by an exponential phase. After 3 days of growth a maximal optical density was reached (Fig. 5A, Table 7). During fermentation glucose was converted to hydrogen and acetate. At the end of the fermentation glucose was only partially consumed possibly because of growth inhibition due to a low pH and/or the accumulation of metabolites. *T. elfii* was able to grow on a *Miscanthus* hydrolysate containing glucose as the main monosaccharide and reached a similar optical density as on glucose medium (Fig. 5B, Table 7). Hydrogen production by *T. elfii* on hydrolysate was slightly higher and acetate production was significantly higher than on glucose medium. Glucose consumption, which was comparable to that on glucose medium, occurred simultaneously with the consumption of xylose during growth of *T. elfii* on

Table 6
Hydrolysis and conversion efficiencies of glucan, xylan and total *Miscanthus* biomass

Sample	Glucan		Xylan		Biomass Conversion (%)
	Hydrolysis (%)	Conversion (%)	Hydrolysis (%)	Conversion (%)	
M1	37.8	38.4	44.2	35.3	21.9
M2	48.1	n.d.	47.2	n.d.	n.b.
M3	56.0	43.6	57.7	37.1	24.2
E1	21.5	21.9	34.4	32.4	15.4
E2	50.7	46.3	55.6	37.4	25.2
E3	49.7	48.7	53.2	29.9	24.7
E4	48.9	46.1	51.4	28.7	23.5
E3, high FPU	70.7	69.4	67.7	38.0	34.2

Hydrolysis is shown as percentage of the content of pretreated material and conversion as percentage of the content of initial material (glucan and xylan conversion) or of the total biomass (biomass conversion). n.d. Not determined.

Table 7
Growth, consumption of glucose and xylose, and production of hydrogen and acetate by *Thermotoga elfii* on hydrolysate of extruded and NaOH-treated *Miscanthus* and on glucose (control) medium

Sample	OD ₅₈₀	pH	Glucose consumption (mM)	Xylose consumption (mM)	H ₂ production (mM)	Acetate production (mM)
Control	1.22	5.2	21.7	—	71.1	19.2
Hydrolysate	1.21	5.6	18.1	11.8	82.2	42.4

The OD₅₈₀ and pH values were reached at the end of the fermentation after 93 h of incubation at 65°C. Consumption and production values were determined for the whole fermentation period.

hydrolysate. No clear preference for one of the two sugars was observed.

4. Discussion

The present study describes results on pretreatment and enzymatic hydrolysis of a lignocellulosic biomass. The chemical composition of *Miscanthus* is similar to that of, e.g. willow (hard wood), wheat straw, and bagasse [8,23,24] and shows a relatively high amount of lignin. Lignin content and efficiency of enzymatic hydrolysis appeared to be inversely related (Fig. 4) which confirms the importance of biomass delignification [25,26]. Chemical pretreatment is required for delignification and in this study NaOH was used to decompose and remove lignin. Conditions for NaOH pretreatment of *Miscanthus* were mainly based on earlier results [22]. Our results showed that NaOH treatment of chopped material was less effective, while milled material (approximately 1 mm and smaller) was delignified for 70%. More than 75% delignification was obtained when the material was treated with NaOH in combination with extrusion. In this case, the most practical and efficient method is the addition of NaOH to the biomass during extrusion. The pressure drop created in passing the RSE

allowed rapid impregnation of a NaOH solution supplied via an injection port downstream the RSE.

Enzymatic hydrolysis of milled or extruded material was low. Significantly higher yields were obtained for NaOH-treated *Miscanthus* samples with low lignin contents. Only at a very small particle size (approximately 10 µm), the lignin content appeared to be less important (Fig. 2, Table 4) and the substrate surface area seemed to become an influential factor [27]. Additionally, the crystallinity of cellulose is an important feature determining its susceptibility to enzymatic hydrolysis [28,29]. The effects of the applied mechanical and chemical pretreatment methods on the crystallinity of *Miscanthus* cellulose were, however, not studied. At a 5% (w/v) substrate concentration and a cellulase concentration of 10 FPU/g cellulose, which is often used in laboratory experiments and for techno-economical evaluations [30], approximately 70% of the cellulose of NaOH-treated and extruded *Miscanthus* was hydrolyzed in 72 h (Fig. 1, Table 6). Enzyme kinetics showed a typical course with a high initial rate resulting in 55% hydrolysis in the first 24 h. Higher enzyme concentrations and longer incubation times will probably result in complete hydrolysis but these conditions are no options for commercial processes. End product inhibition appears to be a major cause of decreasing hydrolysis rates with

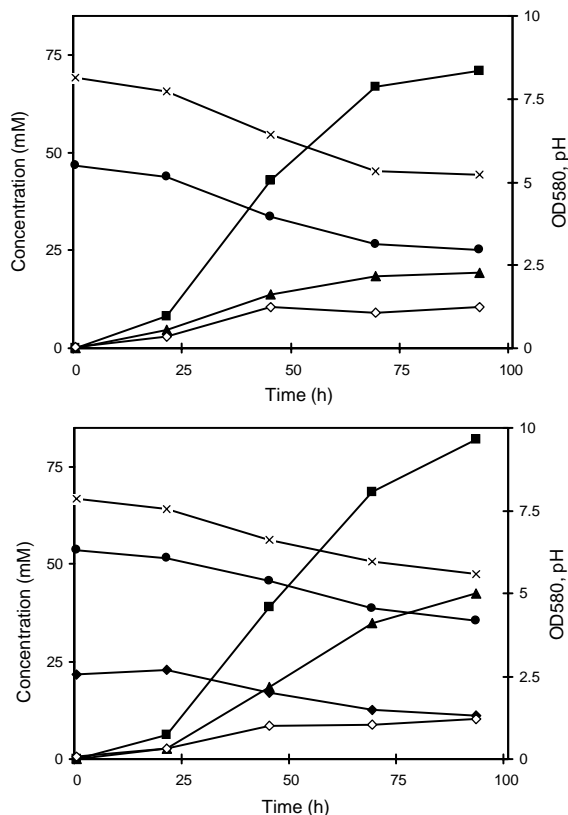


Fig. 5. Growth of *Thermotoga elfii* on glucose medium (A) and *Miscanthus* hydrolysate (B) at 65°C. (●) Glucose consumption; (◆) xylose consumption; (■) hydrogen production; (▲) acetate production; (◇) OD₅₈₀; and (x) pH.

lignocellulosic materials. Suggested approaches to overcome end product inhibition are simultaneous saccharification and fermentation of the substrate [31,32] or removal of the sugars from the hydrolysate by ultrafiltration [33,34]. A promising strategy for low lignin-containing materials is the recycling of enzymes in combination with short residence times in the hydrolysis step (reviewed by Gregg and Saddler [30]).

Although biomass conversion efficiencies for milled *Miscanthus* are not necessarily less than for extruded material (in combination with a chemical treatment) the latter process seems to be more favorable. Extrusion and chemical pretreatment can be combined in one step, gaining potential higher delignification values. The mass balance of such a process is shown in Fig. 6. Pretreatment resulted in 77% delignification and a loss of hemicellulose of 44%. Cellulose yield was more than 95%. Approximately 70% of the polysaccharides of the pretreated biomass was enzymatically hydrolyzed. Of the initial cellulose fraction of *Miscanthus* 69% was converted into glucose. Thirty-three percent of the total initial biomass was converted into

monosaccharides with 62.5% being the theoretical maximum. The concentration of monosaccharides in the final product, the hydrolysate, was 32 g/l. Two side streams were generated in the process. The 'black liquor', after concentration of the solids, can be used for generation of energy and recovery of the chemical. The solid residue obtained after the enzymatic hydrolysis, with a similar composition as the starting material, can be recycled back to the pretreatment stage. With respect to conversion efficiencies, comparison of this process with other pretreatment methods described in literature is difficult. The type of feedstock determines for a considerable part the results, and enzymatic hydrolysis yields greatly depend on assay conditions.

An important advantage of the extrusion technique over other pretreatment methods is the moderate operation temperature, preventing the formation of degradation and oxidation products of lignin and saccharides, respectively, which are potential inhibitors of fermentation. A first experiment on small scale demonstrated that a hydrolysate prepared from extruded *Miscanthus* was able to sustain growth of the extreme thermophilic bacterium, *T. elfii*, at a comparable level as glucose medium. C₅ and C₆ sugars, present in this hydrolysate, were simultaneously consumed and high amounts of hydrogen and acetate were produced. In future experiments performances of hydrolysates in large-scale hydrogen fermentations under controlled conditions will be tested.

The cost for hydrogen produced in a small scale biohydrogen production plant with a capacity of 500 m³ H₂/h has been estimated [35]. In this two-stage bioprocess, H₂ and organic acids are produced from biomass in a first fermentation by thermophilic bacteria. In the second stage, photoheterotrophic bacteria convert the organic acids to hydrogen with the help of light. This bioprocess theoretically yields 12 mol of hydrogen per mole of hexose. In the cost estimation, a biomass conversion efficiency of 40% and energy consumption of the extruder of 150 kWh/ton dry biomass was taken into account. The *Miscanthus* process described in this article yielded a conversion efficiency of 33% and an extruder energy demand of approximately 300 kWh/ton dry *Miscanthus*. This finding will increase the costs by 3 Euro cents to €0.24/m³ H₂, but leave the costs within the range of sustainable hydrogen costs from other small-scale production processes [33].

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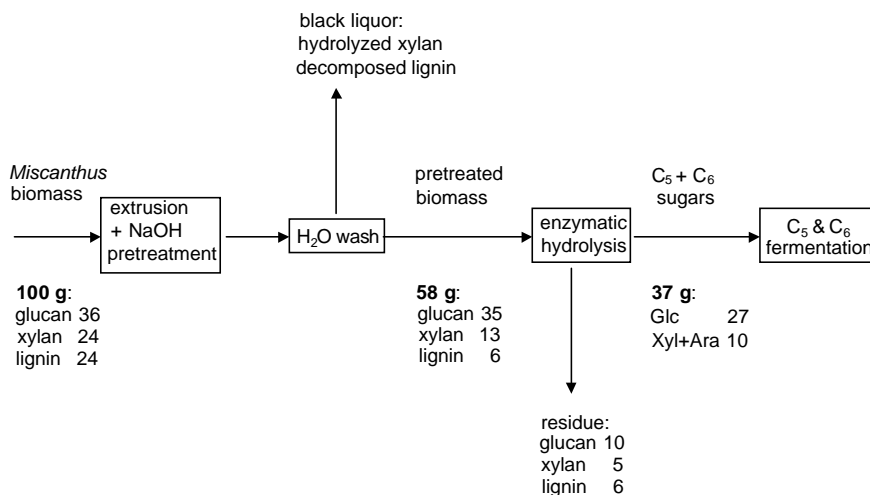


Fig. 6. Flowsheet of pretreatment and enzymatic hydrolysis of *Miscanthus*. Conversion and hydrolysis efficiencies of glucan and xylan and yields of soluble sugars starting with 100 g dry matter. NaOH pretreatment was at 70°C. Enzymatic hydrolysis of pretreated biomass (5% w/v) was carried out at cellulase concentrations of 10 FPU/g glucan.

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