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Original Article

Enhancing Enzymatic Digestibility of *Miscanthus sinensis* using Steam Explosion Coupled with Chemicals[1]

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ABSTRACT

The effect of steam explosion coupled with alkali (1% sodium hydroxide, 1% potassium hydroxide and 15% sodium carbonate) or organosolv solvent (85% methanol, 70% ethanol and dioxane) on the production of sugar, changes in the chemical composition of *M. sinensis* were evaluated. The steam explosion coupled with 1% potassium hydroxide and dioxane were better as compared with other treatments based on the removals of acid insoluble lignin, and about 89.0% and 85.4%. Enzymatic hydrolysis of steam explosion with 1% potassium hydroxide and dioxane treated *M. sinensis*, gave a 98.0% and 96.5% of glucose conversion, respectively. These results suggested that pretreatment of *M. sinensis* with either potassium hydroxide or dioxane could be a promising pretreatment method for glucose production.

Keywords : *Miscanthus sinensis*, steam explosion, pretreatment, enzymatic hydrolysis, potassium hydroxide, dioxane

1. INTRODUCTION

Bioethanol can be produced from renewable resources other than starches or sugars such as lignocellulosic biomass. Lignocellulosic biomass continues to be investigated as a source of fermentable sugars for bioethanol production because of their high availability. The efficient conversion of lignocellulosic biomass to fermentable sugars is essential for the realization of economic bioethanol (Sheehan and Himmel, 1999). The utilization of lignocellulosic residues in a bioconversion process involving enzymatic hydrolysis requires a pretreatment of raw material.

Steam explosion is one of the most intensive investigated pretreatment technology of lignocellulosic material for both ethanol and biogas production (Cara *et al.*, 2006). The steam explosion treatment is applied for a few minutes and then the pressure is abruptly reduced, which make the material suffer an explosive decompression. This produces the hydrolysis of the hemicellulose into water-soluble oligomers

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or to individual sugars, and also generates a good substrate for enzymatic hydrolysis by cellulases (Fernández-Bolaños *et al*., 2001).

Recently, a small number of studies have been published on the steam explosion pretreatment coupled with chemicals and enzymatic hydrolysis. The chemicals applied include sodium hydroxide (Sun *et al*., 2015; Huang *et al*., 2015; Sun *et al*., 2014; Montané *et al*., 1998), hydrogen peroxide/sodium hydroxide (Yang *et al*., 2002), sodium chlorite (Fernández-Bolaños *et al*., 2001; Ballesteros *et al*., 2006), sulfuric acid (Martín *et al*., 2002), ethanol (Hongzhang and Liying, 2007) and methanol (Sasaki *et al*., 2014). Alkali cleaves lignocellulosic biomass structure, such as significantly alters and solubilizes lignin to enhance the fermentable sugar. Mild alkali pretreatment have also recommended mild alkali pretreatment as an effective option for the pretreatment of lignocellulosic biomass. In previous studies, it was reported that the different components are dissolved in accordance with the alkali reagent type (Sun and Hughes, 1998). Organic solvent has some advantages as follows: (1) Organic solvents are always easy to recover by distillation and recycled for treatment; (2) the chemical recovery in organosolv treatment can isolate lignin as a solid material and carbohydrates as syrup, both of which show promise as chemical (Zhao *et al*., 2009). Organosolv treatment is an effective method in the biorefinery concept.

M. sinensis has attracted considerable attention as a possible dedicated energy crop. *M. sinensis* is a perennial grass that requires little nitrogen or herbicide, it can grow over 3 m tall per year to produce from 20 to 25 tons of dry matter per hectare, and it is noninvasive. Moreover, it is a rhizomatous C4 grass species that has a high carbon dioxide fixation rate (Sørensen *et al*., 2008).

In this study, the *M. sinensis* was pretreated with steam explosion followed by various chemicals (1% sodium hydroxide, 1% potassium hydroxide and 15% sodium carbonate, 85% methanol, 70% ethanol and dioxane : water (9 : 1)), and the sequential biomass enzymatic saccharification was conducted. In addition, the glucose conversion after various pretreatments was also evaluated in order to find out an optimal biomass conversion process for *M. sinensis*.

2. MATERIALS and METHODS

2.1. Materials

The *Miscanthus sinensis* used in this study was obtained from the SK Energy Institute of Technology. The *M. sinensis*, cut to an average size 2 - 3 cm for steam explosion pretreatment. The *M. sinensis* and steam exploded *M. sinensis* was milled into 1 - 2 mm and then sieved through a -20 mesh/+80 mesh screen and collected for chemical composition analysis as described below.

2.2. Steam explosion pretreatment coupled with chemicals

2.2.1. Steam explosion condition

The "severity factor : log (*Ro*)" is used to map the destruction, desegregation, and depolymerization of soybean hull *Ro* is calculated using the following relation, Eq. (1) (Fernández-Bolaños *et al.*, 2001):

$$Ro = \{t \times \exp[(T\text{-}100)/14.75]\} \cdots\cdots\cdots \text{Eq. (1)}$$

where T is the temperature (℃) and t the time (min). A steam temperature of 225 - 250℃ and pretreatment time of 5 - 10 min were applied to realize a log (*Ro*) = 4.38, 4.68, 5.12, 5.41 and 5.59. The exploded *M. sinensis* was recovered in a cyclone and after cooling to about 40℃ filtered for liquid and solid fraction.

2.2.2. Steam explosion pretreatment coupled with chemical extraction

In the alkali extraction, the *M. sinensis* was soaked in alkali solutions at 30℃, 100 rpm for 3 h. The solid fraction (cellulignin) from steam explosion pretreatment was extracted using the alkali solution (Fig. 1). The ratio of the liquid and solid was maintained was 25 : 1. The chemicals used were 1% (w/v) sodium hydroxide, 1% (w/v) potassium hydroxide and 15% (w/v) sodium carbonate. The extracted *M. sinensis* was extensively washed with distilled water to remove impurities. It was then neutralized with 1 N HCl, washed with distilled water and dried at 60℃ for 24 h.

In the organosolv extraction, the *M. sinensis* was soxhlet extracted in organic solvent at 80℃ for 3 h. The solid fraction (cellulignin) from steam explosion pretreatment was extracted using the organic solvent. The ratio of the liquid and solid was maintained was 25 : 1. The organic solvent used were 85% (w/v) methanol, 70% (w/v) ethanol and dioxane : water (9 : 1 (v/v)). The extracted *M. sinensis* was extensively washed with distilled water and dried at 60℃ for 24 h.

2.3. Enzymatic hydrolysis

Enzymatic hydrolysis was carried out with commercial enzyme complexes, Celluclast 1.5 ℓ (15 FPU/g biomass; cellulase) and Viscozyme ℓ (24 CBU/g biomass; β-glucosidase) in 50 mM citrate buffer (pH 4.8) with 5% (w/v) of untreated *M. sinensis* and treated *M. sinensis*. The reaction was then incubated at 50℃, 150 rpm for 96 h. Samples were withdrawn after 0, 24, 48, 72 and 96 h to monitor the progress of hydrolysis. Each sample taken from the hydrolysis solution was heated to 100℃ immediately for 10 min to denature the enzymes, cooled to room temperature, and then centrifuged for 15 min at 3,000 rpm (Hanilmicro-12 from Hanil Science Industrial Co., Korea). The supernatant was used for sugar analysis. The percent glucan conversion was calculated as follows (Eq. (2)):

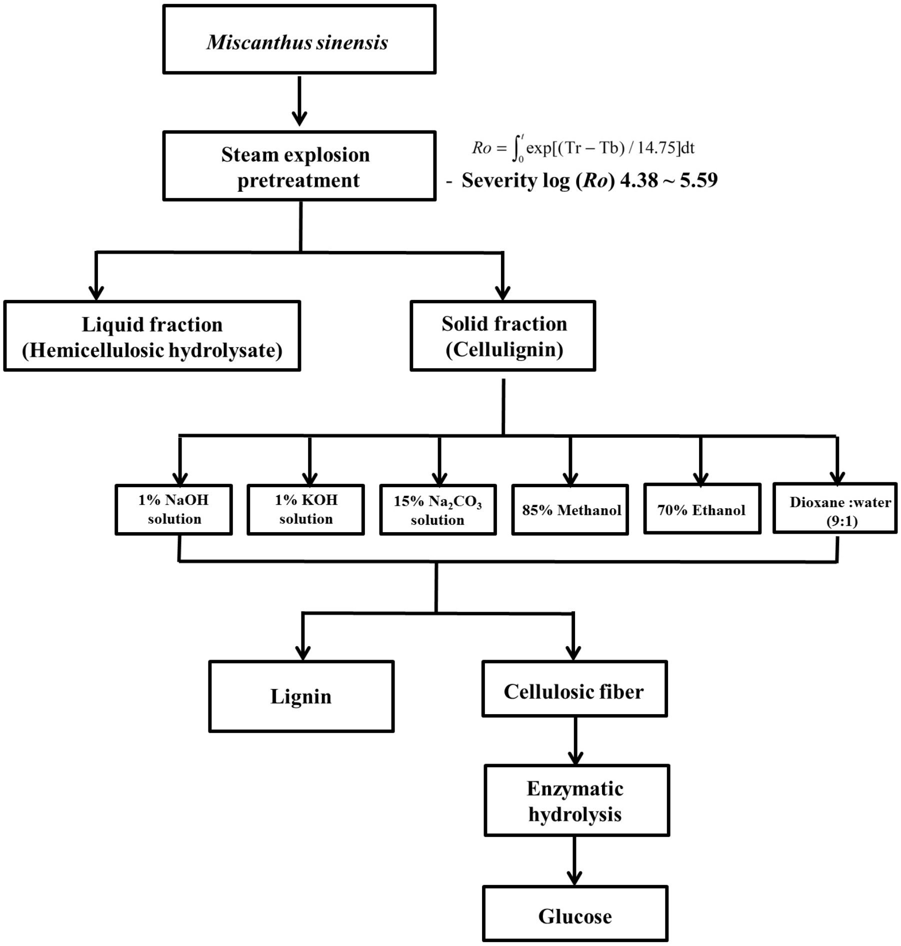

Fig. 1. Flowsheet of procedures for the production of glucose of *M. sinensis*.

Glucose conversion (%) =

$$\frac{\text{GH (\%)}}{\text{GP (\%)}} \times 100 \quad \cdots \text{Eq. (2)}$$

where GH is the dry-weight percentage of glucose in enzyme hydrolysis supernatant (g glucose/g solids hydrolyzed %), GP is the dry-weight percentage glucose in pretreated solids (g glucose/g solids pretreated %).

2.4. Analytical methods

The chemical composition of untreated *M. sinensis* and treated *M. sinensis* were determined by the National Renewable Energy Laboratory (NREL) using Standard Biomass Analytical Procedures (Sluiter *et al*., 2005). The ash content was determined after combustion of the samples at 525℃ for 4 h. Extractives content was determined as the solubilized material after a soxhlet extraction with ethanol for at boiling point for 24 h. Acid insoluble lignin content was assessed gravimetrically as Klason lignin. Acid soluble lignin content was measured spectrophotometrically by reading the UV absorbance at 205 nm in the filtrate. Total lignin content in the sample was the sum of the Klason lignin and the acid soluble lignin. Cellulose and hemicellulose content was determined based on monomer content measured after a two-step acid hydrolysis procedure to fractionate the fiber. A first step with 72% H_2SO_4 at 30℃ for 60 min was used. In a second step, the reaction mixture was diluted to 4% H_2SO_4 and autoclaved at 121℃ for 1 h. This hydrolysis liquid was then analyzed for sugar content by high performance liquid chromatography (HPLC) in a Waters 2695 liquid chromatography with refractive index detector. An Aminex HPX-87P carbohydrate analysis column (Bio-Rad, Hercules, CA) operating at 85℃ with deionized water as mobile-phase (0.6 mℓ/min) was used. Prior to HPLC injection, all samples were neutralized with calcium carbonate, and filtered through 0.2 μm syringe filters. The cellulose and hemicellulose contents were calculated using Eq. (3) and (4), where 0.90 and 0.88 is the correction coefficient for hydration:

$$\text{Cellulose (\%)} = \frac{\text{glucose released (g)} \times 0.90}{\text{sample dry weight (g)}} \times 100 \cdots \text{Eq. (3)}$$

$$\text{Hemicellulose (\%)} = \frac{\begin{array}{c}\text{(xylose + arabinose (g)} \times 0.88) \\ \text{+ (mannose + galactose} \\ \text{released (g)} \times 0.90)\end{array}}{\text{sample dry weight (g)}} \times 100 \cdots \text{Eq. (4)}$$

All analytical determinations were performed in duplicate and average results are shown.

3. RESULTS and DISCUSSION

3.1. Raw material composition

The chemical composition of the untreated *M. sinensis* can be seen in Table 1. The carbohydrate content was the most abundant fraction (cellulose and hemicellulose) followed by lignin (acid insoluble lignin and acid soluble lignin) content and extractive. Glucose (44.2%) represented the largest fraction of carbohydrate, followed by xylose (9.1%). Arabinose represented only a small portion (2.1%) of the *M. sinensis*. However, galactose and mannose were not detected. Classification of hemicelluloses is generally done according to the main sugar residue in the backbone. Because of the large fraction of the pentose xylose, the hemicellulose of *M. sinensis* is of xylan type (Vanderghem *et al*., 2012). The lignin content of the *M. sinensis* was 28.5% (w/w) of which acid insoluble being 28.1% (w/w) and soluble lignin being 0.4% (w/w). The results found for untreated *M. sinensis* are similar to those found in the literature (de Vrije *et al*., 2002; Sørensen *et al*., 2008). The sum of cellulose and hemicellulosic

Table 1. Chemical composition of *M. sinensis*[a]

Chemical composition	Dry matter (%)
Cellulose	44.2 ± 0.0[b]
Hemicellulose[c]	11.2 ± 0.1
Xylose	9.1 ± 0.1
Galactose	-[d]
Arabinose	2.1 ± 0.1
Mannose	-
Acid insoluble lignin	28.1 ± 0.3
Acid soluble lignin	0.4 ± 0.2
Extractives	12.5 ± 0.4
Ash	2.7 ± 0.1

[a] Data in the table are based on oven dry samples.
[b] Mean values of duplicate samples with standard deviations.
[c] Hemicellulose: xylose + galactose + arabinose + mannose
[d] Not detected.

sugar gave a carbohydrate content of 55.4%. This result is comparable to that of other sources of lignocellulosic biomass, such as switchgrass (64.5%) (Li *et al.*, 2010a), corn stover (64.1%) (Li *et al.*, 2010b), poplar (58.6%) (Kumar *et al.*, 2009), rice straw (53.4%) (Chen *et al.*, 2011), sweet sorghum bagasse (41.8%) (Li *et al.*, 2010c), wheat straw (48.9%) (Ballesteros *et al.*, 2006).

3.2. Steam explosion pretreatment

The chemical composition change of *M. sinensis* was important indices for the effectiveness of its pretreatment. The compositions of the steam exploded *M. sinensis* (solid fraction) and liquid fraction (Fig. 1) with different steam explosion conditions are listed in Table 2 and Table 3. The total gravimetric recovery of steam exploded *M. sinensis* and their compositions were obviously different depending on steam explosion conditions. The total gravimetric recovery ranged between 72.0 and 93.0%. A decrease of total gravimetric recovery was detected as the severity factor increased. The glucose decreased from 44.0 to 31.0% and xylose from 9.0 to 1.0%, respectively, with severity factor increased from log (*Ro*) 4.38 to log (*Ro*) 5.59. The arabinose was removed at log (*Ro*) 4.68. The largest decrease is for xylose, which decreases by 97% during steam explosion at log (*Ro*) 5.12. These results are in good agreement with Liu and Wyman, who reported that xylan removal increases with both temperature and residence time, and confirms that xylan is mostly solubilized as oligomers (Liu and Wyman, 2003). Removal of hemicelluloses from the microfibrils is believed to expose the cellulose surface and increase enzyme accessibility to the cellulose microfibrils (Kabel *et al.*, 2007).

The acid insoluble lignin contents of the steam exploded *M. sinensis*, referred to raw material, showed a slight decreased at log (*Ro*) 5.59. The acid insoluble lignin contents after pretreatment is affected by several parameters, such as degradation and solubilization of sugars (and also mineral, protein and wax content) leading to a concentration of lignin in the fiber fraction. However, some of the lignin is also degraded and dissolved in the liquid fraction during pretreatment e.g. as free phenols (Petersen *et al.*, 2009).

The composition of liquid fraction after steam explosion pretreatment is shown in Table 3.

The glucose ranged between 0.7 and 1.9

Table 2. Total gravimetric recovery rate (%) and composition (%) of solid fraction from different steam explosion conditions in *M. sinensis*[a]

Severity value	Total gravimetric recovery	Glucose	Xylose	Arabinose	AIL[b]
4.38	93.0	44.0 (40.9)	9.0 (8.4)	0.8	29.4 (27.3)
4.68	90.0	40.0 (36.0)	4.0 (3.6)	-[c]	30.1 (27.1)
5.12	86.0	38.0 (32.7)	1.0 (0.9)	-	31.4 (27.0)
5.41	89.6	38.0 (34.0)	-	-	30.8 (27.6)
5.59	72.0	31.0 (22.3)	-	-	36.7 (26.4)

[a] Data are expressed in parentheses as a percentage based on dry weight of raw material.
[b] Acid insoluble lignin
[c] Not detected.

Table 3. Sugar weight (g/100 g raw material) of the liquid fraction from different steam explosion conditions in *M. sinensis*[a]

Severity value	Glucose	Xylose	Arabinose	pH
4.38	1.0	0.9	0.3	4.48
4.68	1.9	2.0	- [b]	4.31
5.12	1.1	2.3	-	4.18
5.41	0.9	1.8	-	4.09
5.59	0.7	0.8	-	4.01

[a] Data in the table are based on oven dry samples.
[b] Not detected.

g/100 g raw material, the xylose g/100 g raw material ranged 0.8 and 2.3, respectively. The arabinose was only detected at log (*Ro*) 4.38 (0.3 g/100 g raw material). Sugars were recovered mainly in glucose and xylose in the liquid fraction. Considerable amounts of sugar as a result of steam pretreatment have also been reported by other authors (Shevchenko *et al.*, 2000). The pH values of liquid fraction, ranging from log (*Ro*) 4.01 to log (*Ro*) 4.48, are also shown on Table 3. At higher steam explosion severity factor lower final pH values were obtained. Steam explosion pretreatment often result in the generation of by-products such as acetic, levulinic acids, 5-hydroxymethyl furfural (HMF), furfural and phenolic compounds (Kim *et al.*, 2011). The reduction of pH is mainly attributed to the amount of by-products.

3.3. Steam explosion pretreatment coupled with chemicals

The main component (cellulose and acid in soluble lignin) of untreated *M. sinensis*, steam exploded *M. sinensis* (log (*Ro*) 4.38 and log (*Ro*) 4.68), and steam exploded - chemical treated *M. sinensis* are presented in Table 4. Lignin is removed only to a limited extent during the steam explosion but is redistributed on

Table 4. Cellulose content and lignin content (g/100 g raw material) of resulting from different chemical treatment in steam exploded *M. sinensis*[a]

Sample	Severity log (*Ro*) 4.38		Severity log (*Ro*) 4.68	
	Cellulose, g	Lignin, g	Cellulose, g	Lignin, g
Steam exploded	40.9	27.3	36.0	27.1
Steam exploded - 1% sodium hydroxide	28.0	4.2	27.4	4.5
Steam exploded - 1% potassium hydroxide	28.2	4.2	27.7	3.1
Steam exploded - 15% sodium carbonate	31.9	18.1	30.7	19.0
85% methanol	36.0	13.6	32.8	10.2
70% ethanol	32.3	10.1	30.8	7.2
Dioxane : water (9 : 1)	32.9	6.9	31.3	4.1

[a] Data in the table are based on oven dry samples.

the fiber surfaces as a result of melting and depolymerization/repolymerization reactions (Kabel *et al*., 2007).

Overall, alkali and organic solvents changed the main components in the *M. sinensis*. The lowest lignin content indicated the steam exploded (log (*Ro*) 4.68) - 1% potassium hydroxide treated *M. sinensis.* Also, the highest cellulose content indicated that steam exploded (log (*Ro*) 4.38) - 85 % methanol treated *M. sinensis*. Generally, the steam exploded - alkali treatment results showed a low lignin contents, steam exploded - organic solvent treatment has the high cellulose content.

Alkali solvent can mostly cause the dissociation of entire wall polymers (Li *et al*., 2007). Therefore, alkali gives damage to the cellulose. Sodium hydroxide solvent has been extensively studied to investigate its effect to improve glucose conversion (Chandra *et al*., 2012). High loading of sodium hydroxide may cause serious environmental problems due to the difficulty of recycling. While potassium hydroxide is also a strong base and may be recovered from the black liquor and used as fertilizer (Pang *et al*., 2008).

The steam exploded - dioxane : water (9 : 1) treated *M. sinensis* contained higher amount of cellulose content and lower amounts of lignin compared to chemical treated *M. sinensis*. In a previous study, considering the yield, separation process, and chemical activity, dioxane extraction was considered to be the most favorable method to isolate lignin from bio-ethanol production residue (Guo *et al*., 2013).

3.4. Enzymatic hydrolysis

The cellulosic fiber obtained after steam exploded - chemical extraction was hydrolyzed enzymatically to assess the effects of pretreatment. The glucose conversion during enzymatic hydrolysis is presented in Fig. 2. The result indicated that pretreatment of substrate enhanced the glucose conversion of *M. sinensis*.

The glucose conversion of steam exploded (log (*Ro*) 4.38) - 1% sodium hydroxide, steam exploded (log (*Ro*) 4.38) - 1% potassium hydroxide, steam exploded (log (*Ro*) 4.38) - 15% sodium carbonate, steam exploded (log (*Ro*) 4.38) - 85% methanol, steam exploded (log (*Ro*) 4.38) - 70% ethanol and steam exploded (log (*Ro*) 4.38) - dioxane : water (9 : 1) were founds to be 88.3%, 96.5%, 68.8%, 59.8%, 83.5% and 95.9%, respectively. Also, the glucose conversion of steam exploded *Ro* 4.68 - 1% sodium hydroxide, steam exploded (log (*Ro*) 4.38) - 1% potassium hydroxide, steam exploded (log (*Ro*) 4.38) - 15% sodium carbonate, steam exploded (log (*Ro*) 4.38) - 85% methanol, steam exploded (log (*Ro*) 4.38) - 70% ethanol and steam exploded (log (*Ro*) 4.38) - dioxane : water (9 : 1) were founds to be 95.7%, 98.0%, 73.1%, 63.9%, 88.5% and 96.5%, respectively. Results show that when compared with the untreated *M. sinensis* (20.1%) and steam exploded *M. sinensis* (56.6% and 60.3%), both alkali and organosolv extraction increase glucose conversion. The highest conversion of cellulose into glucose is found when *M. sinensis* is pretreated at steam exploded (log (*Ro*) 4.38) - 1% potassium hydroxide (98.0%).

The increase in the extent of glucose conversion was proportional to the delignification (Table 3), which occurred when the delignification was increased. In previous papers (Timilsena *et al*., 2013), it appears that enzymatic hydrolysis of untreatd *M. sinensis*, autohydrolysed *M. sinensis* and organosolv *M. sinensis* showed low cellulose-to-glucose conversions (11%, 8% and 7% respectively). By using the combinative pretreatment, the cellulose was made more amenable to enzyme, yielding 48% cellulose-to-glucose conversion.

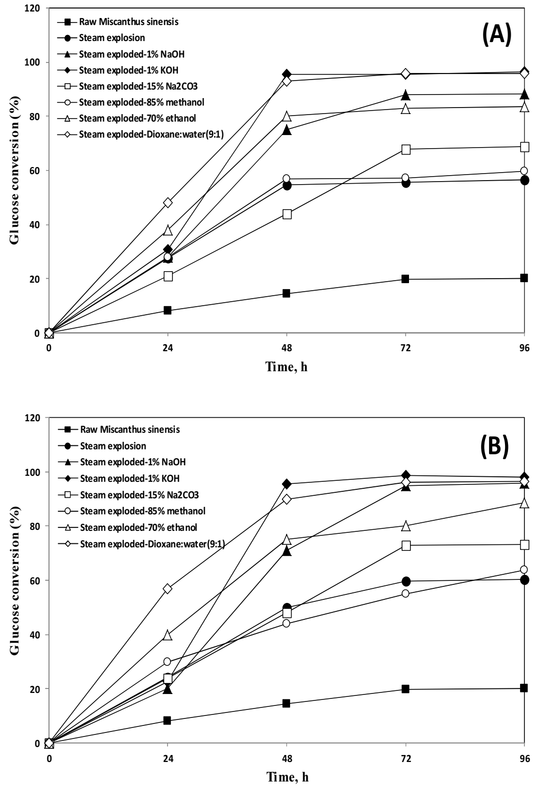

Fig. 2. Glucose conversion of steam exploded *M. sinensis* followed 1% sodium hydroxide, 1% potassium hydroxide, 15% sodium carbonate, 85% methanol, 70% ethanol, dioxane : water (9 : 1), respectively. (A) Severity log (*R*o) 4.38; (B) Severity log (*R*o) 4.68.

A mass balance of steam exploded (log (*Ro*) 4.38) - 1% potassium hydroxide and steam exploded - dioxane : water (9 : 1) treated *M. sinensis* is shown in Fig. 3. This pretreatment resulted in steam exploded (log (*Ro*) 4.38) - 1%

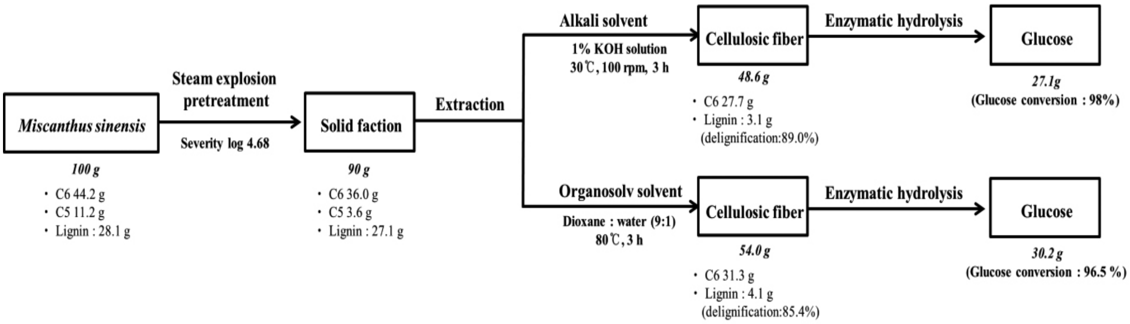

Fig. 3. Material balance of steam exploded-alkali and organosolv treated *M. sinensis*.

potassium hydroxide and steam exploded (log (*Ro*) 4.38) - dioxane : water (9 : 1) treated *M. sinensis* with a delignification of 89.0% and 85.4%. The glucose recovery in steam exploded (log (*Ro*) 4.38) - 1% potassium hydroxide and steam exploded - dioxane : water (9 : 1) treated *M. sinensis* represented 27.1 g and 30.2 g.

4. CONCLUSION

The solid recovery and chemical composition showed that the steam explosion pretreatment resulted in partial removal of hemicelluloses and lignin. The removal of lignin and hemicellulose was essential to increase cellulose digestibility of the *M. sinensis* pretreated by steam exploded (log (*Ro*) 4.38) - 1% potassium hydroxide and steam exploded (log (*Ro*) 4.38) - dioxane : water (9 : 1). Pretreatment of *M. sinensis* with steam exploded (log (*Ro*) 4.38) - 1% potassium hydroxide and steam exploded (log (*Ro*) 4.38) - dioxane : water (9 : 1) were effective method for glucose production by commercial enzyme complexes. This study showed a promising pretreatment for *M. sinensis* using steam explosion coupled with alkali and organic solvent. Nevertheless, research on the improvement of sugar yields must be continued.

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