Journal of Basic & Applied Mycology (Egypt) 6 (2015): 17-25

Production of bioethanol from sugarcane bagasse using recombinant *Saccharomyces cerevisiae* strain

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Abstract: An improvement in bioethanol production from glucose-xylose sugars has been achieved by a recombinant xylose fermenting *Saccharomyces cerevisiae* SK-NY. The recombinant strain was used to ferment sugars hydrolyzed from hemicellulose combined with enzymatically saccharified cellulose of sugarcane bagasse. The main components of bagasse were cellulose, hemicellulose, lignin and ash representing 37.3%, 30.5%, 30.2% and 1.99% of total weight respectively. Hemicellulose hydrolysis conditions were optimized by pretreatment of bagasse with 1% sulfuric acid at 135ºC for 25 min. Delignification was accomplished by three oxidation-bleaching cycles of treatment using sodium chlorite and acetic acid at 70 ºC for one hour per cycle. Liberation of integrated lignin takes place in the final oxidation cycle by the swelling action of sodium bicarbonate. Simultaneous saccharification of cellulignin and cellulose with cofermentation (SSCF) of sugars hydrolyzed from hemicellulose were compared using cellulase enzyme and recombinant *S. cerevisiae* SK.NY. Released glucose-xylose sugars were successfully fermented and the productivity of ethanol was raised from 13.7 g/l to 18.9 g/l from cellulignin and cellulose respectively. Enzymatic saccharification of cellulose showed 37% higher production of glucose compared with that produced from cellulignin. Consequently, the bioethanol production from sugar obtained by SSCF of cellulose increased to 28%.

Keywords: sugarcane bagasse, bioethanol, delignification, simultaneous saccharification and cofermentation (SSCF), recombinant *Saccharomyces cerevisiae.*

Introduction

Sugarcane (*Saccharum officinarum*) bagasse is one of the lignocellulosic materials that composed of up to 75% carbohydrates. Small amounts of pectin, extractives and ashes were also included in biomass composition. Bagasse has high tensile strength crystalline cellulose fibers, embedded in an amorphous matrix of cellulose, hemicellulose and lignin. Lignin acts as a physical barrier and lowers the biodegradability of both cellulose and hemicelluloses (Fox *et al.* 1987, Bidlack *et al.* 1992, Pandey *et al.* 2000, Jørgensen *et al.* 2007). High costs of conversion of cellulose and hemicellulose to fermentable sugars is a bottleneck to be applied on an industrial scale (Ragauskas *et al.* 2006). A number of different pretreatment strategies have been envisioned to convert biomass into fermentable sugars. These pretreatment technologies are being under developments using physical, chemical, biological or combinations of those methods. Multiple steps of pretreatment are needed to overcome the hardness of bagasse e.g. hydrolysis of hemicelluloses and/or removing lignin to increase surface area and decrease the crystallinity of cellulose in order to improve enzyme saccharification and prevents the formation of byproducts that can inhibit the fermentation process. Pretreatment also has great potential for improving the efficiency and lowering the costs of fermentation (Lee *et al.* 1994, Lynd 1996). Researchers are going on to reduce the costs of pretreatments via mechanical pulverization, pyrolysis as well as addition of acid, alkali, hydrogen peroxide, ammonia fiber explosion, wet-oxidation, lime, CO2 explosion and organic solvent treatment. Each of these pretreatment methods has its distinct advantages and disadvantages (Martín *et al.* 2002, Ramos and Fontana 1996).

On the other hand, finding the robust microorganism strains that have the ability to ferment hydrolyzed sugars efficiently was the other obstacle for production of bioethanol from biomass. Intensive studies were done to modify the microorganisms genetically in order to efficiently ferment the hydrolyzed sugars of biomass to bioethanol. Many investigations have indicated that native *Saccharomyces cerevisiae* ferment significant amounts of glucose in adverse conditions and tolerate the action of inhibitors present in the medium, but it cannot utilize xylose, which is the second most abundant sugar in biomass after glucose. *S. cerevisiae* acquired the ability of efficiently ferment xylose via some genetic strategies. One of them was recycling cofactors NADPH/NAP^+ between *Pichia stipitis* xylose reductase (PsXR) and xylitol dehydrogenase (PsXDH) genes via genetic and protein engineering (Khattab *et al.* 2011 a & b). Chromosomal integration of overexpressed endogenous *S. cerevisiae*

xylulokinase ScXK with PsXR and PsXDH was also done to boost the production of bioethnol (Khattab *et al.* 2013). Furthermore, the production of bioethanol from glucose and xylose mixture was improved by recombinant *S. cerevisiae* SK-NY in which NADPH-dependent aldose reductase (GRE3) and ScXK of *S. cerevisiae* genes combined with mutated $NADP^+$-dependent PsXDH gene were cloned in overexpression state to chromosomal DNA (Khattab and Kodaki 2014).

In this study, a combination of diluted acid hydrolysis and oxidation bleaching pretreatments prior to simultaneous saccharfication and cofermentation (SSCF) of hydrolzed bagasse by a recombinant *S. cerevisiae* SK-NY was carried out for enhancing production of bioethanol.

Material and Methods

Sugarcane bagasse and analytical methods

Sugarcane bagasse used in this study was obtained from a local squeezer in Damietta Governorate, Egypt, during April 2013. The bagasse was dried under sunlight, pulverized in domestic machine (0.1 to 25 mm long) and kept in a plastic bag in dry place until use. Moisture content, cellulose, hemicellulose, lignin and ash of bagasse were analyzed according to the National Renewable Energy Laboratory (NREL) standard procedure (Ehrman 1994 a & b, Ehrman 1996, Sluiter 2008). Glucose and xylose of liquid hydrolyzates were analyzed and determined using HPLC (JASCO, Tokyo) equipped with an Aminex HPX-87H column (Bio-Rad Laboratories, Hercules, CA, USA).

Hydrolysis of hemicellulose

Different sulfuric acid concentrations 0.5, 1~2.5 % combined with three different temperatures 110º, 121º and 135ºC for 25 minutes were used to determine the best conditions for hydrolysis of hemicellulose. Hydrolysis was achieved using 1/10 W/V (bagasse/sulfuric acid) in TOMY LSX 500 high-pressure steam sterilizer. Optimal conditions of hydrolysis were determined based on monitoring the maximum sugars produced using HPLC as described by Khattab *et al.* (2013). Liquid hydrolyzates of hemicellulose were collected by filtration, using glass microfiber filter GF/A 110 mm diameter. The percent of digestibility was calculated as following:

Digestibility % = [Total sugar(s) concentration g/l × volume] / [biomass weight (g)] × 100

Neutralization process

Neutralizing liquid hydrolyzates of first treatment during acid hydrolysis was achieved by calcium hydroxide (powder- Wako Company) added gradually to the hydrolyzed hemicellulose liquid fraction- A (Fig.1) in order to react with excess H_2SO_4 and produce calcium sulphate (Ranatunga *et al.* 2000; Mohagheghi *et al.* 2006). Calcium sulfate (gypsum) was precipitated by overliming with keeping temperature at 40ºC for 30 min using METTLER TOLEDO pH meter and IKA® C-MAG HS4 magnetic stirrer. After gypsum (calcium sulfate) had been precipitated, it was separated by filtration using vacuum pressure ULVAC MDA-006 and glass microfiber filter GF/A 110mm diameter. Neutralized liquid fraction (Fraction A) was kept at 4ºC for further use during SSCF.

Delignification Process

Oxidation bleaching using sodium chlorite acidified by acetic acid was used in delignification of cellulignin (Residual part; RP after dilute sulfuric acid pretreatment) according to the modified methods of Hubbell *et al.* (2010) and Kahar (2013). Cellulignin was suspended in 1/30 W/V of 0.2 M acetate buffer (pH 3.0) and three oxidation/bleaching cycles using gentle swirling in glacial acetic acid (0.04 ml/g RP) and sodium chlorite (0.2 g/g RP) per cycle/h at 70ºC. Sodium bicarbonate (0.1g/g RP) was added in the final cycle in order to swell cellulose fiber and liberate integrated lignin. Cellulose fiber was self-separated from hydrolyzed lignin fraction by repeated filtration cycles via porcelain Büchner funnel without filter paper, then washed by 10 % acetone solution and dried.

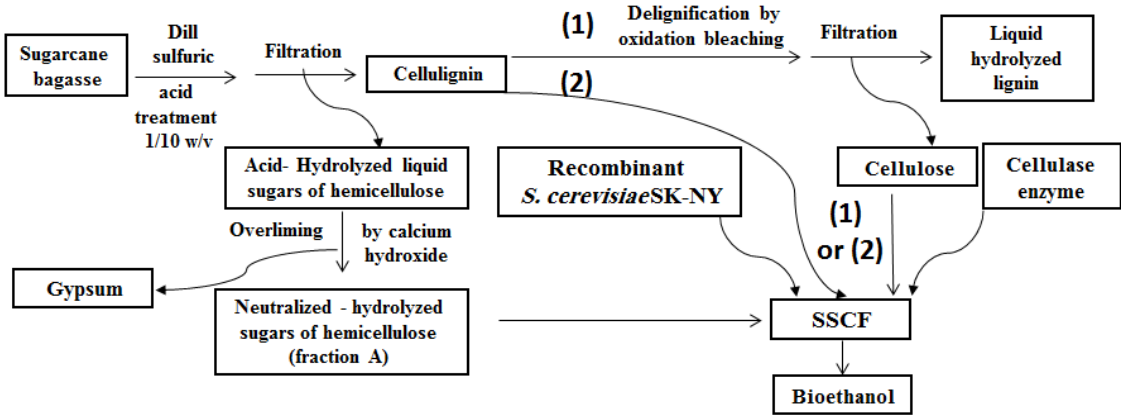

Figure 1: Schematic representation of sugarcane bagasse pretreatments prior to simultaneous saccharfication and cofermentation (SSCF) of cellulignin or cellulose by a recombinant *S. cerevisiae* SK-NY for production of bioethanol.

Simultaneous saccharification and cofermentation of pretreated sugarcane biomass

Cellulignin and cellulose powders (Fig. 1) were individually suspended in fraction A (1/20 W/V) buffered in 50 mM of citrate. Two doses of cellulose enzyme (168 and 350 EGU) obtained from Sigma-Aldrish, novozyme (*Trichoderma reesei* ATCC 26921 which contained ≥700 units/gram) were tested during simultaneous saccharification (SS) and SSCF, where one unit of enzyme liberates one µmole of glucose from carboxymethyl cellulose (CMC) in one hour at pH 5.0 and 37 ºC. Additionally, one mg of Almond β-glucosidase enzyme (11 CBU/mg protein) was added to each 84 U of cellulase enzyme SSCF.

Recombinant *S. cerevisiae* SK-NY (Khattab and Kodaki 2014), which has the ability to ferment glucose/xylose mixtures, was cultivated in liquid yeast peptone dextrose (YPD) medium with 150 rpm of shacking for 24 h at 30 ºC. Cell pellets were collected by centrifugation at 3000g for 4 min then washed twice by YP medium prior to SSCF process (Fig. 1). The reaction of 100 ml was performed in 300 ml Erlenmeyer flasks and incubated at 30 ºC under continuous agitation (150 rpm). The initial cell density in the fermentation culture was approximately adjusted to an optical density of 1.0 before addition to slurry mixture. All growth rates were monitored using spectrophotometer (DEN-1B McFarland Densitometer). For SSCF, concentration of sugars and ethanol were measured at 0, 3, 6, 12, 24, 48, 72, 96, 120, 144, 168, 192h. In case of SS, replicated flasks were prepared without *S. cerevisae* SK-NY in order to determine sugar production rate during saccharification. Samples were centrifuged and then filtered via 0.45 µm millipore filter prior to high performance liquid chromatography (HPLC) analysis. Concentrations of ethanol, glucose and xylose were determined using HPLC as described in a previous work (Khattab *et al.* 2013). To assess the effects of removing lignin, comparative studies of SSCF using cellulose and cellulignin were achieved (Fig. 1).

Results and Discussion

Sugarcane bagasse analysis

The main components of sugarcane bagasse analyzed in this study included cellulose, hemicellulose, lignin and ash. These substances represented respectively 37.3, 30.5, 30.2 and 1.98 % of dry mass (Table 1). Reports from Brazil showed that these components accounted for 38.8 % to 45.5% in case of cellulose; 25.2 % - 27.0 % for hemicelluloses, 19.1 % - 32.4 % for lignin and 1.0 % - 2.8 % for ash (Table 1).

According to Hatfield and Fukushima (2005), variations in the chemical composition of sugarcane bagasse could be attributed to several factors including sugarcane species, the hybrids, environmental conditions, harvesting system, crop age and the analytical methods employed.

Effect of pretreatment of bagasse by dilute sulfuric acid

Pretreatments are the most expensive steps of converting biomass to fermentable sugars and then to ethanol due to complexity of biomass, crystallinity of cellulose and degree of polymerization. Furthermore, lignin that covers and bind cellulose-hemicellulose network lowers the process of the hydrolysis (Binod *et al.* 2012). Hydrolysis of bagasse's hemicellulose was achieved by sulfuric acid. The effects of sulfuric acid concentrations 0.5~ 2.5 % at three different temperatures were investigated (Table 2). Results showed that the total amount of released sugars increased with increasing acid concentrations to 1% and temperatures 135 ºC, then the amount of sugars declined by the action of excess acid. Autoclaving 1/10 w/v bagasse in 1% sulfuric acid at 135 ºC for 25 min was found to be the best conditions to achieve maximum sugars concentrations (Table 2). Data also show that xylose was the main constituent of hemicellulose-hydrolyzed sugars (18.55 g/l) matching 15.16 % of total weight of bagasse. Decrease in glucose and xylose concentrations was accompanied with excess acid concentration and/or rise in temperature during the first pretreatment certainly by decomposition of xylose and glucose to furfural and hydroxy methyl furfural (Rodríguez-Chong *et al.* 2004). A maximum concentration of glucose produced from hemicellulose hydrolysis in the first pretreatment was 8.11 g/l (Table 2).

Table 1: Chemical composition (% w/w, dry basis) of sugarcane bagasse compared with other references

Component (%)	Brienzo *et al.* (2009)	da Silva *et al.* (2010)	Canilha *et al.* (2011)	Rabbelo *et al.* (2011)	Rocha *et al.* (2011)	Present study*
Cellulose	42.4	38.8	45.0	38.4	45.5	37.3
Hemicellulose	25.2	26.0	25.8	23.2	27.0	30.5
Lignin	19.6	32.4	19.1	25.1	21.1	30.2
Ash	1.6	2.8	1.0	1.5	2.20	1.98

* The values are the average of three independent experiments.

Table 2: Effect of pretreatment of bagasse by different concentrations of sulfuric acid at three different temperatures for 25 minutes of treatments on glucose and xylose release.

Sulfuric acid concentration (%)	Temperature (ºC)	Glucose (g/l)	Xylose (g/l)
0.5	110	6.31±0.31	13.55±0.32
	121	7.11±0.12	15.55±0.37
	135	7.71±0.26	15.85±0.11
1	110	7.51±0.08	16.85±0.18
	121	7.81±0.32	17.04±0.20
	135	8.11±0.22	18.55±0.35
1.5	110	7.01±0.14	16.55±0.08
	121	7.45±0.42	17.65±0.16
	135	7.67±0.36	17.11±0.19
2	110	7.78±0.31	17.32±0.24
	121	7.89±0.12	16.75±0.29
	135	7.98±0.20	14.45±0.31
2.5	110	6.23±0.19	16.66±0.33
	121	6.11±0.21	14.36±0.25
	135	5.70±0.22	13.33±0.27

The values of glucose and xylose concentrations are average ± SD, n=3.

Numerous attempts have been done to investigate effective methods to alleviate harmful effect of hydrolyzates (furfural and hydroxymethylfurfural) in order to improve fermentation efficiency (Gong *et al.* 1993, Rodríguez-Chong *et al.* 2004). Direct neutralization was found to be somewhat effective in removing sulfate anions, although sulfate toxicity is considerably less than that of acetic acid (Ranatunga *et al.* 2000). Overliming was reported as an effective method of reducing toxicity of various hydrolyzates (Ranatunga *et al.* 2000; Mohagheghi *et al.* 2006). As a consequent of overliming by calcium hydroxide for 30 minutes at 40ºC and pH 6, calcium sulfate (Gypsum) precipitated and completely

hydrolyzed sugars were successfully fermented to ethanol. Diluted sulfuric acid pretreatments effectively hydrolyzed hemicelluloses, nonetheless only 0.85% (w/w) of lignin was removed from the bagasse. Furthermore, cellulose has been well cited as being more resistant to attack by diluted acids than hemicelluloses and needs more pretreatment processes (Lee *et al.* 1994; Mussatto and Roberto 2006).

Acid oxidation for bleaching lignin

Lignin represents a physical barrier to accessible cellulose chains by cellulases enzymes. It adsorbs cellulose hydrolyzing enzymes. In addition, depolymerized lignin acts as an inhibitor for saccharification process (Converse *et al.* 1990). For this reason, a second pretreatment was proposed to hydrolyze and separate lignin from cellulignin using thermo-chemical acid oxidation-bleaching processes before enzymatic saccharification. Treatment of cellulignin by sodium bicarbonate changes the properties of cellulose surface by swelling cellulose microfibers and cellulose matrix for further removal of the integrated lignin (Kahar 2013). Bleaching by acidified sodium chlorite after hydrolysis of hemicellulose prevents degradation of glucan or xylan where chlorite is a strong oxidant and acts selectively on lignin (Ahlgren and Goring, 1971). These double pretreatments of diluted acid hydrolysis and delignification increased pure cellulose content and decreased lignin content (Yasuda *et al.* 2013).

Simultaneous saccharification and cofermentation

Residual cellulose and cellulignin powder were individually saccharified by cellulase enzyme in liquid hydrolyzates of hemicellulose (fraction A) and simultaneously fermented the released sugars to bioethanol using recombinant *S.cerevisiae* SK-NY (Fig. 1). Seventy-four grams of cellulose were obtained from double pretreated 200g crude bagasse. At saccharification dose of 0.18g cellulase enzyme/ g cellulose, a maximum released glucose was reached 22.05 g/l (data not showed in figures). Simultaneous fermentation of these released sugars produced 15.12 g/l ethanol (Table 3). By increasing saccharification enzyme dose to 0.39 g cellulase / g cellulose, a maximum released glucose was increased to 29.46 g/l (Fig. 3) and simultaneous fermentation the released sugars produced 18.9 g/l ethanol represented 0.151 g ethanol / g of bagasse (Fig. 2 & Table 3). Surprisingly, there were no improvements in SS or SSCF at higher enzymes loading than 0.39g cellulase/g of cellulose. Furthermore, there were no improvements in SS or SSCF by addition of β-glucosidase enzyme with 0.39g cellulase / g cellulose, where the accumulated cellobiose was depleted after 72 h of saccharification (data not shown in figures), where cellulase cocktail hydrolyzed cellulose to cellobiose and then to glucose through the combined action of endo-exoglucanases and β-glucosidase enzymes.

The effects of delignification from cellulignin were also evaluated during SS and SSCF at saccharification dose 0.39 g cellulase enzyme/ g cellulignin and cellulose. A maximum released glucose from cellulignin was reached (18.75 g/l) while that from cellulose was reached (29.46 g/l) (Fig. 3). Simultaneous fermentation of these released sugars produced 13.7 g/l and 18.9 g/l ethanol respectively. The potential effects of delignification process were clarified by 37% improvement in saccharification and 28% higher ethanol production respectively; nonetheless, 41 % of cellulose was rested, for unknown reason, without hydrolysis to fermentable sugars (Figure 3). Tan *et al.* (2013) recovered 92.3% of theoretical glucose by acid–alkali combination (1% H_2SO_4, 150º C, 10 min, followed by 1% NaOH, 80ºC and 60 min) and enzymatic saccharification. Fermentation efficiency for released glucose and xylose from sugarcane bagasse by recombinant *S. cerevisiae* SK-NY reached 77 % of theoretical released sugars. Khattab and Kodaki (2014) reported its fermentation efficiency to mixture of glucose-xylose sugars by 85.7% of the theoretical sugars. This is the first study using recombinant *S. cerevisiae* SK-NY to ferment hydrolyzed sugars from bagasse or any kind of biomass. Furthermore, the positive effects of delignification on both saccharification and fermentation process were an emphasized by this study where the total amount of released glucose increased from 18.75 g/l to 29.46 g/l and bioethanol production increased from 13.7 g/l to 18.9 g/l from cellulignin and cellulose respectively. Effectiveness of removing lignin by alkali-pretreatment prior to simultaneous saccharification of glucan and fermentation by native *S. cerevisiae* was also reported by Yasuda *et al.* (2013); nevertheless there was a lower production of bioethanol than the results reported in this study, probably due to fermentation of xylose by recombinant *S. cerevisiae* SK-NY.

Table 3: Simultaneous saccharification and cofermentation of cellulignin and cellulose suspended in hydrolyzed hemicellulose sugars of sugarcane bagasse using cellulase enzyme and recombinant *Saccharomyces cerevisiae* SK-NY.

Enzyme concentration (g / g Biomass)	Cellulase 0.18 g / cellulose			Cellulase 0.39 g / cellulignin			Cellulase 0.39 g / cellulignin		
Time (h)	Glucose	Xylose	Ethanol	Glucose	Xylose	Ethanol	Glucose	Xylose	Ethanol
0	8.11±0.31	18.55±0.31	0.00±0.00	8.11±0.31	18.55±0.31	0.0±0.0	8.11±0.31	18.55±0.31	0.00±0.00
3	1.21±0.11	17.87±0.21	4.30±0.10	1.22±0.25	17.77±0.23	4.14±0.20	1.40±0.21	17.56±0.21	4.76±0.10
6	0.31±0.06	16.91±0.11	6.97±0.22	0.21±0.31	16.75±0.24	5.98±0.31	0.52±0.11	16.72±0.19	6.89±0.20
12	0.21±0.01	15.21±0.21	7.00±0.24	0.19±0.01	15.62±0.40	6.84±0.24	0.44±0.02	15.35±0.20	8.24±0.21
24	0.21±0.01	12.78±0.31	8.88±0.34	0.17±0.03	12.45±0.34	7.21±0.41	0.32±0.02	12.25±0.27	10.21±0.21
48	0.15±0.01	9.25±0.33	10.01±0.10	0.15±0.02	10.1±0.28	8.05±0.31	0.25±0.01	9.78±0.21	12.01±0.29
72	0.14±0.01	7.82±0.24	11.20±0.21	0.13±0.01	7.78±0.15	8.99±0.39	0.20±0.01	6.89±0.18	14.22±0.30
96	0.12±0.01	4.35±0.28	12.31±0.09	0.11±0.02	4.74±0.25	9.79±0.44	0.16±0.01	4.68±0.15	15.01±0.18
120	0.10±0.01	2.64±0.16	13.46±0.22	0.08±0.0l	2.56±0.30	10.88±0.26	0.10±0.0l	2.79±0.10	16.20±0.22
144	0.09±0.01	1.46±0.17	14.52±0.20	0.06±0.01	1.85±0.26	12.03±0.21	0.08±0.00	1.81±0.05	17.18±0.19
168	0.07±0.01	0.88±0.11	14.75±0.24	0.05±0.01	0.83±0.28	13.57±0.23	0.04±0.00	0.98±0.01	18.09±0.20
196	0.07±0.01	0.42±0.02	15.12±0.31	0.04±0.01	0.30±0.18	13.70±0.33	0.03±0.00	0.32±0.01	18.90±0.23

The values of glucose and xylose and ethanol concentrations are average ± SD, n=3.

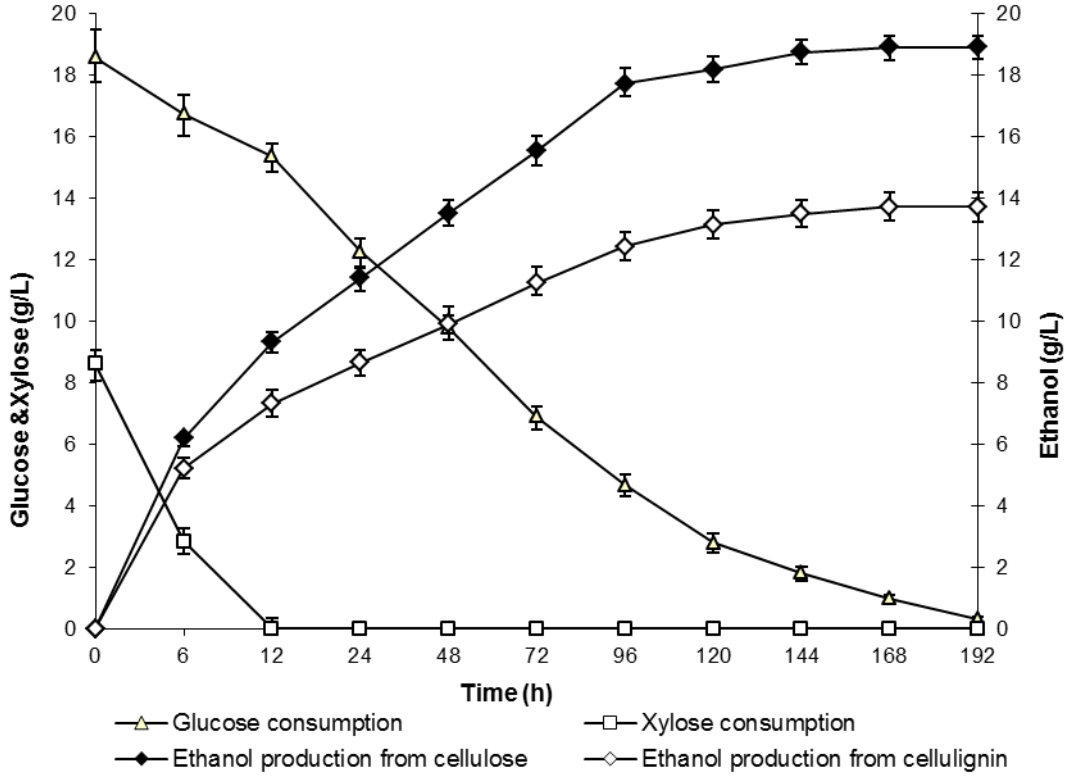

Figure 2: Simultaneous saccharification of cellulignin and cellulose suspended in liquid hydrolyzed hemicellulose (Fraction A) of sugarcane bagasse using 0.39g cellulase/ g biomass and fermentation of released sugars by recombinant *S. cerevisiae* SK-NY.
Values are average ± SD, n=3.
Fermentation efficiency % = [(Ethanol produced g/l/ total consumed sugars g/l) * 0.51] * 100

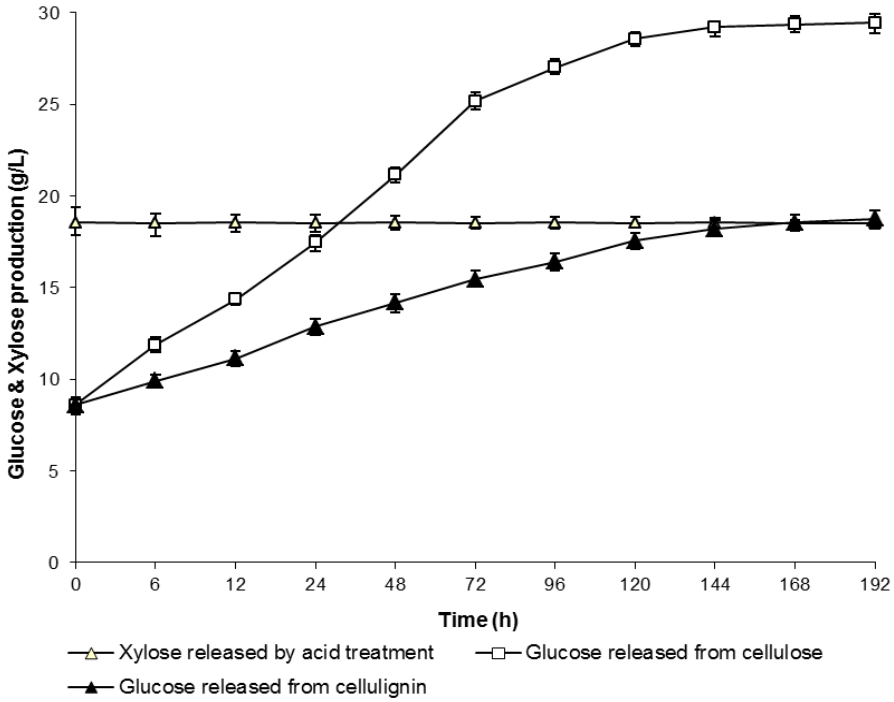

Figure 3: Total released glucose and xylose during saccharification of cellulignin and cellulose suspended in liquid hydrolyzed hemicellulose (Fraction A) of sugarcane bagasse using 0.39g cellulase/ g biomass.
Values are average ± SD, n=3.

Conclusion: The main components of Egyptian sugarcane bagasse were matched with other ratios in the literatures where cellulose, hemicellulose, Klason lignin and ash. Xylose was the dominant sugar in the hemicelluloses fraction and the second most abundant sugar after glucose in the total hydrolyzates, which represented 15.16 % of total weight. Double pretreatments were integrated to overcome the complexity structures of bagasse prior to SSCF. First, hemicellulose was thermo-chemically hydrolyzed using 1% sulfuric acid at 135 °C for 25 min. Second, lignin was bleached by acid oxidation. The potential effect of delignification process was clarified by 37% and 28% improvements in released glucose and produced ethanol respectively compared with those of cellulignin. Bioethanol production during SSCF was increased from 0.110 to 0.151 g ethanol / g of bagasse from cellulignin and cellulose respectively. Fermentation efficiency of saccharified glucose and xylose sugars using recombinant *S. cerevisiae* SK-NY reached 77 % of released sugars during SSCF of acid pretreated and delignified sugarcane bagasse.

Acknowledgements

I greatly thank Dr. Tsutomu Kodaki, Institute of Advanced Energy, Kyoto University, Japan. He kindly provided me with chemicals and laboratory equipments to achieve and complete this work. In addition, I would like to thank the Egyptian Cultural Affairs Sector and Missions for the grant of postdoctoral scholarship. The author wish to thank Dr. Ahmed M. Mahmoud, Botany and Microbiology Department, Faculty of Science, Al-Azhar University, Assiut branch for fruitful discussion of the written manuscript.

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