BIOCONVERSION OF A MIXTURE OF PAPER SLUDGE AND EXTRACTION LIQUOR FROM WATER PREHYDROLYSIS OF *EUCALYPTUS* CHIPS TO ETHANOL USING SEPARATE HYDROLYSIS AND FERMENTATION

Zhi-Sheng Zhu,[a] Xu-Hui Li,[b] Qiu-Mei Zheng,[a] Zeng Zhang,[c] Yang Yu,[d] Ju-Fang Wang,[a] Shi-Zhong Liang,[a] and Ming-Jun Zhu [a,]*

Paper sludge and extraction liquor from water prehydrolysis of *Eucalyptus* chips before pulping are potential raw materials for ethanol production in an integrated forest biorefinery concept. Ethanol production from paper sludge, extraction liquor from water prehydrolysis of *Eucalyptus* chips, and a mixture of both using separate hydrolysis and fermentation were investigated. The hydrolysate composed of 51.01±0.72 g/L glucose, 30.11±0.09 g/L xylose, and 13.65±0.94 g/L cellobiose, which was obtained by enzymatic saccharification of the mixture at an initial consistency of 6% (w/v, expressed in terms of total carbohydrate mass), was used for ethanol production by yeast SHY07-1 without prior detoxification and nutrient supplementation. A final ethanol concentration of 36.82±0.35 g/L was achieved, corresponding to an ethanol yield of 0.45±0.04 g/g with a fermentation efficiency of 80.71±0.03% and an ethanol productivity of 0.31±0.01 g/(L h). This confirmed the feasibility of co-fermentation of these two materials for bioconversion to ethanol.

Keywords: Paper sludge; Extraction liquor; Fermentation; Ethanol; Bioconversion; Yeast

*Contact information: a: School of Bioscience and Bioengineering, South China University of Technology, Guangzhou Higher Education Mega Center, Panyu, Guangzhou, People's Republic of China, 510006; b: Institution of ZhiZhongHe Liquor Company, Ltd, Tech-industrial park, east of Jiande city, Zhejiang Province, People's Republic of China, 311607; c: State Key Laboratory of Pulp and Papermaking Engineering, South China University of Technology, Guangzhou, People's Republic of China, 510640; d: Forest Bioproducts Research Initiative, Chemical and Biological Engineering, University of Maine, Orono ME 04469; * Corresponding author: mjzhu@scut.edu.cn*

INTRODUCTION

The increasing demand for energy as well as the unavoidable depletion of world petroleum resources in the near future has aroused worldwide interest in seeking an alternative, nonpetroleum-based source of energy. Bioethanol is increasingly used as an alternative renewable fuel in the transportation sector, being a cleaner fuel with a higher octane rating than gasoline (Li et al. 2009; Lau and Dale 2009). First-generation ethanol is produced mainly from sugar cane, corn, and cassava (Fang et al. 2010; Talebnia et al. 2010). However, a dramatic increase in ethanol production using these crops has caused undue pressure on the global food supply. It is well known that lignocellulosic materials such as agricultural residue and industrial waste are the most abundant and cheap source of renewable energy in the world. Vast environmental and social benefits could result

from the replacement of petroleum-based fuels with second-generation bioethanol converted from lignocellulosic biomass (Katahira et al. 2008). In this context, pulp and paper industry wastes, paper sludge (PS), and the extraction liquor from water prehydrolysis of *Eucalyptus* (EL) were evaluated as feedstocks for bioconversion to ethanol.

Paper sludge is a solid waste material that is composed of pulp residues and ash generated from pulping and papermaking processes (Boni et al. 2004). It is estimated that there are approximately 4000 dry tons of PS produced per day in China as a consequence of paper production. The disposal of these sludges by combustion and land application or landfilling is costly and undesirable; therefore, new methods are sought to unlock their full potential commercial value. Because of its high carbohydrate content and well-dispersed structure, PS could be biologically converted to value-added products such as ethanol without pretreatment (Kang et al. 2010). Compared with traditional pretreated fiber such as corncobs, wheat straw, and rice straw, the polysaccharides in PS are much more susceptible to enzymatic digestion (Fan et al. 2007a,b; Domke et al. 2004; Lark et al. 1997; Wayman et al. 1992; Lee et al. 2004), since the sludge has already been subjected to the extensive mechanical and chemical processing previously imposed on the paper raw material through pulping (Marques et al. 2008). Therefore, PS is believed to be one of the most promising feedstocks for bioconverting to commodity products (Fan et al. 2003).

EL is a kind of wastewater obtained from *Eucalyptus* wood by a hot-water extraction process before pulping. In the pulp and paper industry, some wood components, such as hemicelluloses, representing approximately 25% (w/w) of the raw material, as well as lignin, are dissolved in the black liquor due to the severe conditions in the kraft cooking process. This black liquor is burned to produce electricity and thermal energy. Because hemicelluloses have a lower calorific content than lignin, they could preferably be extracted before cooking to produce value-added products such as bioethanol (Mendes et al. 2009). To maximize hemicellulose use, the authors have adopted a primary auto-hydrolysis treatment process so that the hemicelluloses (mainly xylan) are dissolved in the liquor and partly decomposed to monosaccharides.

So far, little attention has been paid to the joint use of these two waste materials for conversion to ethanol. Therefore the objective of the present work was to evaluate the feasibility of their co-fermentation and potential conversion to ethanol.

EXPERIMENTAL

Materials

The PS used in this study came from paper mills using commercial wood pulp with a kraft pulping process (Zhongshan, Guangdong) and was kept at 4°C for short-term storage and at -20°C for long-term storage. The EL was obtained using a water prehydrolysis process at 150°C for 3 h with a solid:liquid ratio of 1:5 using a computer-controlled digestion unit (M/K 609-2-10, M/K Systems, United States). The *Eucalyptus* used was an improved variety of Myrtaceae, which was kindly provided by Leizhou Forestry Bureau of Guangdong Province, with a pentosan content of 20.64%. The

Eucalyptus was chipped into approximately 2.5 cm×1.5 cm×0.5 cm sections before use. The EL was kept at 4°C for short-term storage and at -20°C for long-term storage.

Microorganisms and Media

SHY07-1 yeast (an intergeneric protoplast fusant between *Saccharomyces cerevisiae* (a gift from Sanhe ethanol factory, Zanjiang, Guangdong province) and *Pichia stipitis* (a gift from Guangzhou Sugarcane Industry Research Institute, Guangzhou)), which possesses the ability to convert not only glucose, but also xylose, into ethanol, was adapted in EL continuously for 155 days and used in this study (Zhu et al. 2011). Adaptation of the yeast was performed by serial transfers in crimp-sealed 25 mL BELLCO tubes. The tubes containing 9 mL EL and 10 g/L corn steep liquor were sealed and sterilized by steam autoclaving at 121^{o}C. Ethanol was added to each tube using a 1 mL syringe for a final concentration of 0 to 60 g/L with an increment of 5 g/L, and each inoculation/transfer was 10% volume (1 mL). Subsequently, cultures were grown in an incubator with temperature controlled at 30^{o}C and rotation speed set at 150 rpm. For the serial transfer to obtain mutants, inoculation alternated between the use of medium with elevated ethanol concentrations and medium with no added ethanol. The parameter R was defined as the ratio of the final OD over the initial OD 24-hour incubation. The criteria for transfer were (a) transfer to a higher ethanol concentration if R >=5, (b) maintain the current ethanol concentration if 2 <= R <5, and (c) transfer to a previous ethanol concentration if R <2. Single colonies of the mutants were isolated from the final cultures and denoted as SHY07-1.

The strain was stored in a glycerol mixture at -80°C and was grown at 30°C for two days on a YPX-agar plate containing 20 g/L yeast extract (Oxoid, Ltd., Basingstoke, Hampshire, England), 10 g/L peptone (AoboXing Universeen BioTech Company, Ltd., Beijing, China), and 20 g/L xylose (QiYun BioTech Company, Ltd., Guangzhou, China). The medium was sterilized by steam autoclaving at 115°C for 20 min, and the strain was periodically subcultured on YPX medium and EL to maintain its activity and purity. Subculturing was performed once a month and the subcultures were stored under sterile conditions at 4°C.

Inoculum Preparation

The seed culture for the fermentation inoculum was prepared from culture by transferring a loopful of active SHY07-1 cells to 10 mL of YPX medium in sterile test tubes and incubating them at 30°C, 200 rpm for 12-18 h in an incubator shaker (C24KC refrigerated incubator shaker, Edison, New Jersey, United States). Then 10 mL of the active cells were aseptically transferred to 100 mL of sterile YPX medium in a 250-mL Erlenmeyer flask. The flask was incubated at 30°C, 200 rpm for 12 h in the same incubator shaker. After cultivation, the cells were harvested by centrifugation in a 50-mL sterilized centrifuge tube for 10 min at 5000 rpm using a TDL-5000B centrifuge (Shanghai Anke Company, Ltd., China). The pellets were resuspended in a sterilized 0.9% NaCl solution to obtain a cell suspension with a cell mass concentration of 200 g wet weight per liter. The time between cell harvesting and initiation of the separate hydrolysis and fermentation (SHF) was no longer than 2 h. Finally, a 6% inoculum of the cell suspension was used for initiating fermentation experiments.

Analytical Method

Composition Analysis of PS and EL

PS was analyzed gravimetrically for water (by oven drying at 105°C to constant weight) and ash content (by igniting at 575°C and burning for 5 h). Protein content was estimated by the Kjeldahl method using a nitrogen-to-protein conversion factor of 6.25. Fat was determined by extraction with petroleum ether using conventional Soxhlet glassware and gravimetric extract analysis. Hemicellulose and cellulose contents analysis was conducted according to the method described by Fan et al. (2003), and reducing sugars were measured by high-performance liquid chromatography (HPLC) using an Aminex HPX-87P column (300 mm × 7.8 mm) and a Carbo-P Micro-Guard column (30 mm × 4.6 mm, Bio-Rad, Hercules CA, United States), operating at 60°C with ultrapure water as the mobile phase at a flow rate of 0.6 mL/min.

Lactose, xylose, cellobiose, galactose, arabinose and furfural in the samples of EL were analyzed by HPLC using an Aminex HPX-87P column as described above. Other components such as xylitol, lactic acid, glycerol, and acetic acid in EL were determined by HPLC using an Aminex HPX-87H column (300 mm × 7.8 mm) and a Cation H Cartridge Micro-Guard column (30 mm × 4.6 mm, Bio-Rad, Hercules CA, United States), operating at 60°C with 2.5 mM H_2SO_4 as the mobile phase at a flow rate of 0.6 mL/min (Lynd et al. 1989).

Assay of enzyme activity

Total cellulase activity was described by filter paper activity (FPU) with the Whatman No. 1 filter paper strip, 1.0 × 6.0 cm (approximately 50 mg) as a substrate. Xylanase activity in the cellulase was assayed using 1% (w/w) oat spelts xylan (Sigma, St. Louis, USA) as substrate. The total reducing sugars were estimated by the 3, 5-dinitrosalicylic acid (DNS) method (Miller 1959). Enzyme activity was expressed in international units (U) as the amount of enzyme required to release 1 μmol of either glucose (FPU/mL) or xylose (U/mL) per minute under the assay conditions (pH 4.8, 50°C).

β-glucosidase (EC 3.2.1.21) enzyme activity was expressed in terms of cellobiohydrase units (CBU) with cellobiose as a substrate. An international unit of β-glucosidase enzyme activity (CBU/mL) was considered to be the amount of enzyme required to produce 2 μmol of glucose per minute under standard conditions (pH 4.8, 50°C).

HPLC analysis for sugars and products

For determination of products such as ethanol, organic acids and xylitol, 1-mL samples were acidified with 10% sulfuric acid, centrifuged in 1-mL Eppendorf tubes at 12000 rpm for 10 min (TGL-16H Centrifuge, HEMA Company, Ltd., Zhuhai, China), and then filtered through a membrane of 0.22 μm pore size. The supernatant (pH 1-3) was analyzed for soluble sugars and ethanol using a Waters 2695 (Millford LA, United States) analyzer equipped with a refractive-index detector (RID) and an Aminex HPX-87H column as described above.

For determination of sugars and furfural, 1-mL samples were adjusted to pH 6-9 with $CaCO_3$, centrifuged in 1-mL Eppendorf tubes at 12000 rpm for 10 min (TGL-16H

Centrifuge, HEMA Company, Ltd., Zhuhai, China), and then filtered through a membrane of 0.22 mm pore size. The supernatant (pH 6-9) was analyzed for sugars and furfural using an Aminex HPX-87P column as described above.

*Hydrolysis and fermen*tation

PS and EL were adjusted to pH 5.0 with either 1 M hydrochloric acid or 10% sodium hydroxide and then were sterilized by steam autoclaving at 121°C for 30 min. Each hydrolysis was conducted at a working volume of 50 mL in a 125-mL serum bottle. The calculated amount of either tap water or EL was added to ensure a total final volume of 50 mL. PS and EL were enzymatically hydrolyzed by the Celluclast 1.5L cellulolytic complex (Novozymes, Denmark, exhibiting a cellulase activity of 12.54 FPU/mL and a xylanase activity of 0.97 U/mL). Cellulase loading was 15 FPU/g substrate (expressed in terms of total carbohydrate mass). Novozyme™188 (Sigma-Aldrich Co., 192 CBU/mL), a fungal β-glucosidase, was used to supplement the β-glucosidase activity of the cellulase at an enzyme loading of 30 CBU/g substrate (expressed in terms of total carbohydrate mass). Sludge was incubated with the filter-sterilized enzyme solution in an orbital shaker (40°C, 250 rpm) for 96 h. After four days, the hydrolysis mixture was centrifuged at 8000 rpm for 10 min to separate the liquid solution from the solids. The supernatant was adjusted to pH 5.5 with 5% NaOH and then used for fermentation. As for EL, a hydrolysis process catalyzed by sulfuric acid (4% v/v) at 121°C for 30 min was used. Enzyme hydrolysis was also carried out with commercial enzymes. The hydrolysates obtained, after residual solids removal by filtration (through a membrane filter of 0.22-μm pore size), were analyzed for sugar profiles using HPLC.

Fermentations were conducted at a working volume of 50 mL in a 125-mL serum bottle. All of the serum bottles were sealed with butyl rubber stoppers and aluminium seals. The fermentation medium was inoculated with 6% (v/v) inoculum. The fermentation temperature was kept constant at 30±0.5°C in an orbital shaker for 120 h, and the broth was kept under agitation at 200 rpm. 2-mL samples were taken aseptically during fermentation. Glucose, xylose, cellobiose, glycerol, organic acid, and ethanol profiles were measured using HPLC, as previously described. For the hydrolysis and fermentation experiments, at least one experimental condition was duplicated for each set of experiments to ensure consistency and accuracy of results.

The fermentation calculations were as follows,

$$\text{Fermentation efficiency} = \frac{\text{Ethanol formed concentration, g/L}}{\text{Theoretical ethanol concentration, g/L}} \times 100\% \quad (1)$$

$$\text{Theoretical ethanol concentration} = (\text{Glucose concentration} + \text{Cellobiose concentration} \times \frac{360}{342}) \times 0.51 + \text{Xylose concentration} \times 0.46, \text{g/L} \quad (2)$$

$$\text{Enzymatic hydrolysis efficiency} = \frac{\text{Glucose concentraion, g/L}}{\text{Cellulose concentration, g/L}} \times \frac{162}{180} \times 100\% \quad (3)$$

$$\text{Glucose/xylose used} = \frac{\text{Glucose/xylose consumption, g/L}}{\text{Initial glucose/xylose concentration, g/L}} \times 100\% \quad (4)$$

$$\text{Glucose/xylose consumption rate} = \frac{\text{Glucose/xylose consumption, g/L}}{\text{Fermentation time, h}} \quad (5)$$

where 0.51 is the coefficient of ethanol from glucose, and 0.46 is the coefficient of ethanol from xylose.

RESULTS AND DISCUSSION

Characterization of PS and EL

The main compositions of PS were determined as follows (on a dry weight basis): 57.6% glucan, 13.41% xylan, 12.6% ash, 1.52% protein, 2.74% fat, 1.07% acid-soluble lignin, and 5.27% acid-insoluble lignin. The PS material consisted of 71.01% carbohydrate on a dry-matter basis (57.6% glucan and 13.41% xylan). This value is slightly higher than the average carbohydrate content (Marques et al. 2008) and similar to the values determined by others (Lark et al. 1997; Fan et al. 2003). It is worth noting that due to the low ash content, this sludge was considered desirable for enzymatic hydrolysis and bioconversion to ethanol.

The components of the EL were as follows: 2.37 g/L lactose, 4.39 g/L xylose, 2.22g/L cellobiose, 2.11 g/L galactose, 0.73 g/L arabinose, 0.13 g/L furfural, 0.97 g/L xylitol, 0.47 g/L lactic acid, 1.31 g/L glycerol, 2.65 g/L acetic acid, and 18 g/L of xylan. It was shown that the hydrolysate contained pentose and hexose and that the xylose content was as high as 4.4 g/L, which was less than the 18 g/L of xylan content, as roughly measured by the Douglas colorimetric method (Cerning et al. 1972), suggesting that part of the pentosan was not completely hydrolyzed. In addition, the pentose sugar content was high, while the hexose sugar content was low in the EL, revealing that the process of hot-water extraction has damaged the hemicellulose structure, but has only a slight effect on the cellulose of *Eucalyptus* wood. However, this process would not endanger pulp quality and yield when used before the papermaking process (Liu et al. 2008; Mendes et al. 2009). Besides, a broad range of substances either derived from the raw material or as reaction products from sugar and lignin degradation also existed in the EL. Some of these substances, such as furfural which presented at 0.13 g/L, may have had an inhibitory effect on the microorganisms in subsequent fermentation steps (Kumar et al. 2009; Walton et al. 2010 and Sreenath et al. 2000). Moreover, the content of the substances could vary considerably depending on the various extraction conditions of EL (data not shown).

Fermentable Sugars Released by Hydrolysis

Before ethanol fermentation by a microorganism, the feedstock needs to be processed by a saccharification technology to release fermentable sugars. For PS and for the PS-EL mixture, only enzymatic hydrolysis was carried out, whereas for EL alone, both the dilute-acid hydrolysis process and the enzymatic hydrolysis process were

attempted. The enzymatic digestibility of PS was studied at an initial substrate loading of 6% (w/v, expressed in terms of total carbohydrate mass) with cellulase with a dosage of 15 FPU/g effective cellulose (glucan and xylan) and with Novozyme™188 on a dosage of 30 CBU/g effective cellulose.

Monosaccharides (glucose and xylose) were the main products obtained, but cellobiose was also present in the six different hydrolysates (Fig. 1). For the PS, the PS-EL mixture, and the PS-acid-prehydrolyzed EL mixture (represented by A, B, and C respectively in Fig. 1), the glucose content in the hydrolysates was as high as 50 g/L after enzymatic hydrolysis for 96 h. The achievement of complete hydrolysis confirmed that the polysaccharides in PS were much more susceptible to enzymatic digestion than those in traditional native lignocellulose feedstocks, thus avoiding the need for sludge pretreatment. These results were similar to those reported by others (Marques et al. 2008; Kang et al. 2010). For EL, the xylose content in the hydrolysates (represented by D, E, and F in Fig. 1) was substantially increased by 63.8% and 75.6% respectively compared with the acid-prehydrolyzed EL and the enzyme-prehydrolyzed EL. It was confirmed, as previously mentioned, that EL needed a secondary hydrolysis to convert oligomers to fermentable sugars. It was also apparent that enzymatic prehydrolysis was superior to acid prehydrolysis.

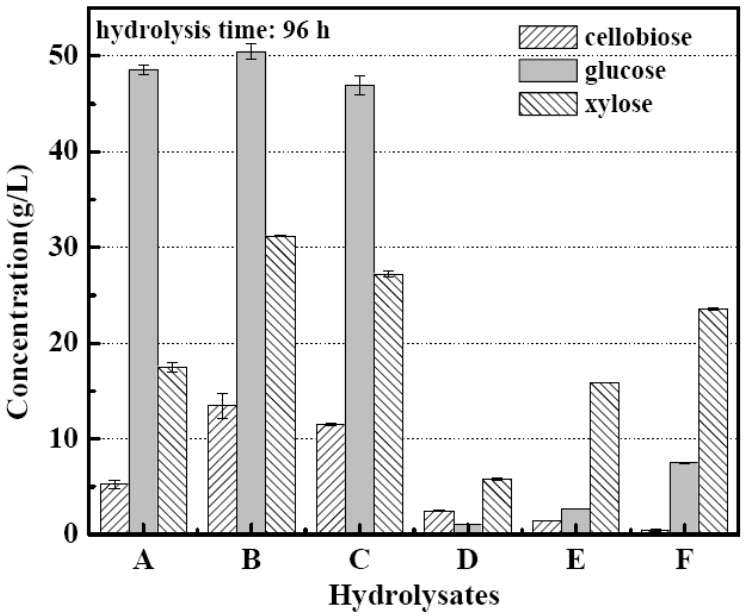

Fig. 1. Sugar content of hydrolysates.
Results are means±standard deviation for duplicate experiments. A: PS + tap water; B: PS + EL; C: PS + EL which had already been hydrolyzed with 4% (v/v) sulfuric acid at 121°C for 30 min; D: EL + tap water (steam autoclaving at 115°C for 20 min); E: EL + 4% (v/v) sulfuric acid (steam autoclaving at 121°C for 30 min); F: EL. A, B, C, and F were performed by 96 h enzymatic hydrolysis process with an initial substrate loading of 6% (w/v, expressed in terms of total carbohydrate mass) at pH 4.8-5.0, 40°C, 250 rpm, and the enzyme loadings were: 15 FPU Celluclast 1.5L + 30 CBU Novozyme™188/g effective cellulose. Some increasing sugars resulted from the addition of enzyme solution.

It is worth noting that the enzymes used in enzymatic pre-hydrolysis of EL were not severely inhibited by the degradation products present in EL (F in Fig. 1). What was of concern was the total sugar content in the individual and mixed raw materials. Obviously, the contents of both glucose and xylose in hydrolysates obtained from mixed materials (B and C in Fig. 1) were less than the sum of the glucose and xylose contents in hydrolysates obtained from the corresponding single material (A + F or A + E in Fig. 1). This suggested that there was an synergistic inhibition by the degradation products or the presence of inhibitory compounds such as furfural and acetic acid in the PS and EL, because the acetic acid and furfural detected in EL were 2.65 g/L and 0.13 g/L, respectively, which were reported to interact negatively on ethanol fermentation (Palmqvist et al. 1999). Furthermore, the concentration of the inhibitory compounds had to be higher in the hydrolysates of PS-EL mixture, and this had a significant impact on the overall ethanol yield and the ethanol productivity of the subsequent fermentation.

A higher sugar concentration, and consequently a higher final ethanol concentration, could be obtained by increasing the raw-material concentration. For the mixture of these two materials, prolonged hydrolysis (for 168 h) with an initial consistency ranging from 6% to 12% (w/v, expressed in terms of total carbohydrate mass) was performed to maximize the sugar concentration in the hydrolysates that were to be used for subsequent fermentation. However, when the hydrolysis occurred at a higher solids concentration, the high initial viscosity made mixing difficult (Marques et al. 2008). The glucose and xylose release displayed a similar trend, with approximately 80% of the total sugar released after 24 h, regardless of the varied initial consistency. As shown in Fig. 2, a low substrate loading resulted in a low sugars concentration but a high hydrolysis efficiency. When the initial substrate loadings increased from 6% to 12% (w/v, expressed in terms of total carbohydrate mass), the enzymatic hydrolysis efficiency decreased from 95.75% at 6% to 80.90% at 12%, although the sugars concentration increased accordingly, which indicated that the enzymatic hydrolysis at a high substrate loading was significantly inhibited by a high solids concentration and releasing sugars, resulting in a low hydrolysis efficiency.

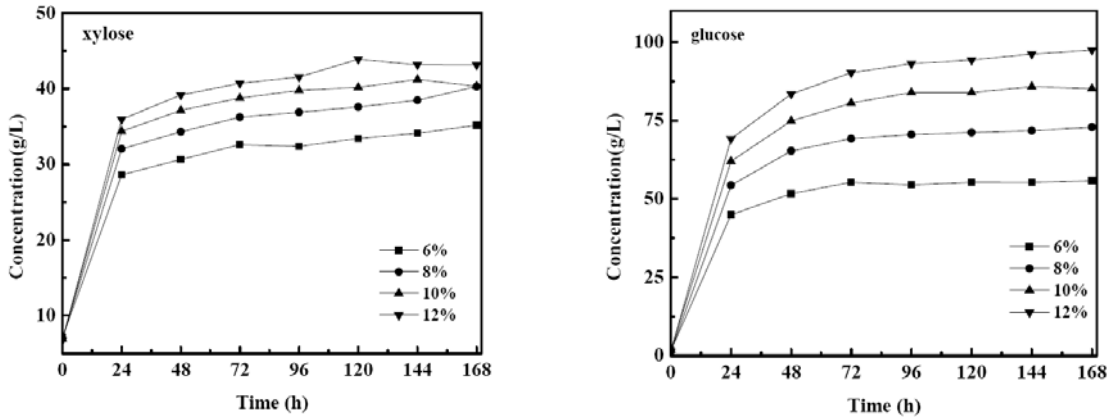

Fig. 2. Trends over time of sugar release from enzymatic saccharification (pH 4.8-5.0, 40°C, 250 rpm) of the PS-EL mixture with various initial consistencies ranging from 6% to 12% (w/v, expressed in terms of total carbohydrate mass). Enzyme loadings were: 15 FPU Celluclast 1.5L + 30 CBU Novozyme™188/g effective cellulose.

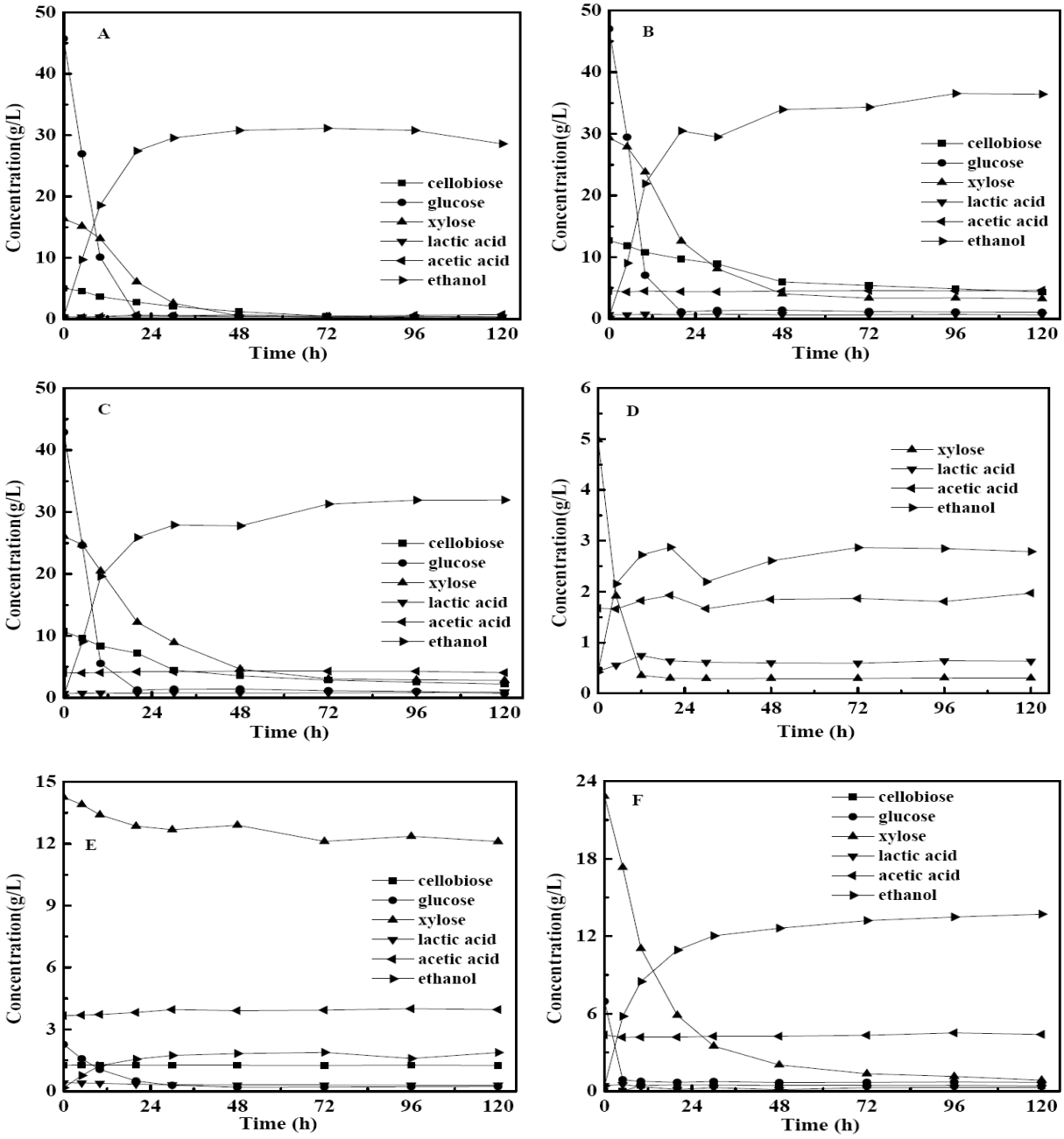

Fig. 3. Trends over time of substrate and product concentrations in the fermentation of hydrolysates. Results are means for duplicate experiments. Fermentations were conducted at 30°C, initial pH 5.5, and 200-rpm agitation under largely anaerobic conditions for 120 h. A: hydrolysate from PS; B: hydrolysate from PS + EL; C: hydrolysate from PS + EL which already had been hydrolyzed with 4% (v/v) sulfuric acid at 121°C for 30 min; D: EL + tap water (steam autoclaving at 115°C for 20 min); E: EL + 4% (v/v) sulfuric acid (steam autoclaving at 121°C for 30 min); F: EL. A, B, C, and F were performed by an enzymatic hydrolysis process with an initial substrate loading of 6% (w/v, expressed in terms of total carbohydrate mass) at pH 4.8-5.0, 40°C, 250 rpm for 96 h, and the enzyme loadings were: 15 FPU Celluclast 1.5L + 30 CBU Novozyme™188/g effective cellulose.

Fermentation of Different Hydrolysates to Ethanol

Hydrolysates obtained from PS at an initial consistency of 6% (w/v, expressed in terms of total carbohydrate mass), EL, and a mixture of both were used as fermentation media without prior detoxification for ethanol production with SHY07-1 yeast. The profiles over time obtained for the fermentation experiments are presented in Fig. 3. In the fermentation of hydrolysates that consisted of glucose/xylose as mixed substrates, glucose was used first (A, B, and C in Fig. 3). Xylose was fermented faster only after a 12-h period, during which the glucose concentration was reduced to a low level. This observation was in agreement with previous studies, which reported that *Pichia stipitis* grown in glucose/xylose mixtures fermented glucose preferentially because xylose uptake was competitively inhibited by high levels of glucose (Agbogbo et al. 2006; Marques et al. 2008; Zhao et al. 2008).

Glucose was depleted completely after 24 h of fermentation, and xylose was almost exhausted after 72 h in the case of hydrolysate from PS (A in Fig. 3). However, xylose could not be consumed completely in the case of hydrolysates from PS-EL mixtures (B and C in Fig. 3). Ethanol concentration increased rapidly at the beginning of fermentation with fast glucose consumption, then it increased slowly after glucose had been used up after 24 h (A, B, and C in Fig. 3). In the case of fermentation of EL alone, which contained mainly xylose, ethanol accumulated as xylose was consumed, indicating that the SHY07-1 fusant performed well in EL even without detoxification after a lengthy adaptation to EL (D and F in Fig. 3).

However, it was observed that xylose assimilation was inhibited in the EL which had already been hydrolyzed with 4% sulfuric acid at 121°C for 30 min (E in Fig. 3), probably due to the toxic compounds derived from the degradation of the lignin under such a severe condition, for example at 4.0 g/L acetic acid. Residual xylose was also found as shown by B and C in Fig. 3. This phenomenon may have resulted from the high acetic acid concentration (approximately 4.0 g/L) and the synergistic inhibition of toxic compounds and was similar to that observed by other research groups (Saha et al. 2010; Kumar et al. 2009; Huang et al. 2009). These results indicated that the SHY07-1 fusant should be improved further for ethanol production in hydrolysates such as acid-hydrolyzed EL in spite of its xylose-fermenting ability and ethanol tolerance in EL. Furthermore, it was shown that enzymatic hydrolysis was superior to acid hydrolysis, as mentioned above, because it generated fewer inhibitors in a relatively mild condition and because xylose could be completely consumed as shown by A, D and F in Fig. 3. Furthermore, cellobiose concentration also decreased gradually throughout the fermentation process (A, B, C, and F in Fig. 3). A possible reason for this was that β-glucosidase, which was added in the previous hydrolysis step, remained active, converted cellobiose to glucose, and then glucose was subsequently fermented by the yeast strain (Marques et al. 2008).

Table 1 provides a summary of the fermentation parameters used (data from Fig. 3). The average glucose consumption rate (2.08-2.29 g/(L h)) was higher than the average xylose consumption rate (0.07-0.52 g/(L h)), and the glucose used was also higher than the xylose used. The results indicated that the strain SHY07-1 prefers glucose to xylose. The highest ethanol concentration (36.42±0.29 g/L) was obtained in the hydrolysate from the PS-EL mixture at the end of the fermentation, corresponding to an ethanol yield of

0.45±0.02 g/g with an ethanol productivity of 0.30±0.01 g/(L h) and a fermentation efficiency of 81.33±0.05% (B in Table 1). However, for the hydrolysate from PS or EL alone catalyzed by commercial enzymes, the ethanol yields were 0.46±0.06 g/g and 0.47±0.02 g/g respectively, corresponding to fermentation efficiencies of 91.64±0.03% and 93.97±0.04% respectively (A and F in Table 1). The results demonstrated that the SHY07-1 fusant performed worse in the hydrolysate of the PS-EL mixture than in the hydrolysate of either PS or EL separately with an enzymatic hydrolysis process. In addition, it was further determined that the enzymatic hydrolysis process was better than the dilute-acid hydrolysis process for fermentation of EL, because the ethanol concentration of 13.70±0.32 g/L and fermentation efficiency of 93.79±0.04% obtained with enzymatic hydrolysis process were much higher than the values of 1.88±0.73 g/L and 20.28±0.09% obtained from the dilute-acid hydrolysis process (E and F in Table 1). Similar results were also observed for the PS-EL mixture, because the ethanol concentration of 36.42±0.29 g/L and the fermentation efficiency of 81.33±0.05% obtained with the enzymatic hydrolysis process were slightly higher than the values of 31.94±0.28 g/L and 79.58±0.01% obtained with the dilute-acid hydrolysis process with EL (B and C in Table 1).

Table 1. Summary of Fermentation Results (120 h) from Different Hydrolysates with SHY07-1 Fusant

Hydrolysates	A	B	C	D	E	F
Ethanol concentration (g/L)	31.14±0.37	36.42±0.29	31.94±0.28	2.78±0.01	1.88±0.73	13.70±0.32
Ethanol yield (g/g)	0.46±0.06	0.45±0.02	0.43±0.05	0.51±0.05	0.42±0.07	0.47±0.02
Fermentation efficiency (%)	91.64±0.03	81.33±0.05	79.58±0.01	103.3±0.01	20.28±0.09	93.79±0.04
Ethanol productivity (g/(L h))	0.26±0.02	0.30±0.01	0.26±0.03	0.02±0.04	0.01±0.01	0.11±0.02
Glucose used (%)	99.34±0.02	97.74±0.01	98.37±0.02	N.A.	90.06±0.03	90.12±0.01
Xylose used (%)	97.74±0.03	88.84±0.02	89.22±0.04	93.94±0.05	15.06±0.17	96.27±0.01
Glucose consumption rate (g/(L h))	2.26±0.02*	2.29±0.03*	2.08±0.03*	N.A.	N.A	N.A
Xylose consumption rate (g/(L h))	0.33±0.03**	0.52±0.01**	0.45±0.01**	0.23±0.02*	0.07±0.04*	0.43±0.02**

Fermentations were conducted at 30°C, initial pH 5.5, and 200 rpm under anaerobic conditions for 120 h.A: hydrolysate from PS; B: hydrolysate from PS + EL; C: hydrolysate from PS + EL which had already been prehydrolyzed with 4% (v/v) sulfuric acid at 121°C for 30 min; F: EL; D: EL + tap water (steam autoclaving at 115°C for 20 min); E: EL + 4% (v/v) sulfuric acid (steam autoclaving at 121°C for 30 min).A, B, C, and F were performed by an enzymatic hydrolysis process with an initial substrate loading of 6% (w/v, expressed in terms of total carbohydrate mass) at pH 4.8-5.0, 40°C, 250 rpm for 96 h, and the enzyme loadings were: 15 FPU Celluclast 1.5L + 30 CBU Novozyme™188/g effective cellulose.
N.A. means not available.
*Based on data at fermentation time of 20 h.**Based on data at fermentation time of 48 h.

Hydrolysate Fermentation with No Nutrient Supplementation

The hydrolysate obtained from enzymatic hydrolysis of the PS-EL mixture, containing cellobiose 13.65±0.94 g/L, glucose 51.01±0.72 g/L, and xylose 30.11±0.09 g/L (certain amounts of increasing sugars resulted from the addition of enzyme solution) at an initial substrate consistency of 6% (w/v, expressed in terms of total carbohydrate mass), was used as a fermentation medium without nutrient supplementation and detoxification. An ethanol concentration of 36.82±0.35 g/L was obtained at 120 h, corresponding to an ethanol yield of 0.45±0.04 g/g with an ethanol productivity of 0.31±0.01 g/(L h) and a fermentation efficiency of 80.71±0.03% (Fig. 4 A). Supplementation of 3 g/L corn steep liquor resulted in a slightly higher ethanol concentration of 38.03±0.21 g/L at 120 h and faster xylose utilization rate and ethanol production rate at 20 h (Fig. 4 B).

The results showed that supplementation with corn-steep liquor had no significant effect on fermentation in the hydrolysate derived from the PS-EL mixture by SHY07-1 yeast, indicating that some of the nutrients released from enzymatic saccharification of PS and EL and those resulting from mill processing were sufficient to support microbial growth and ethanol fermentation. Similar results were observed in a fermentation using corn stover as feedstock (Lau et al. 2009).

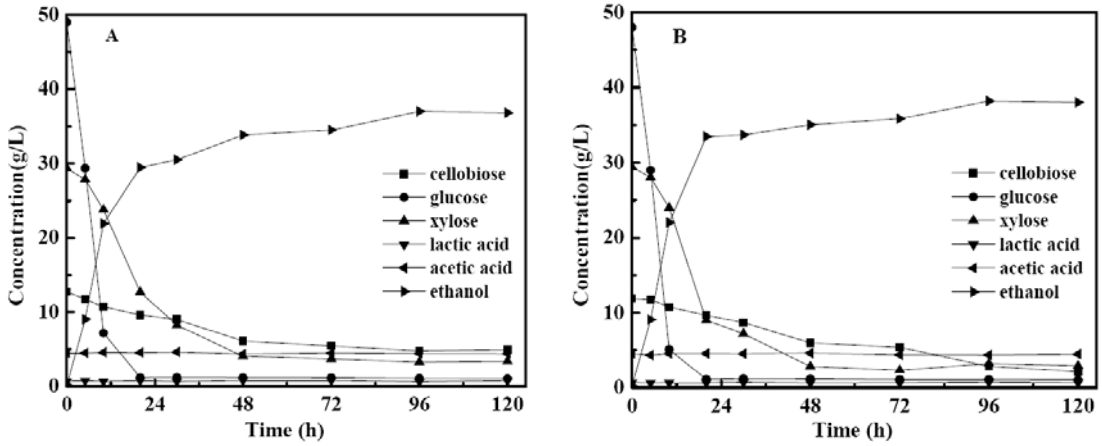

Fig. 4. Trends over time of substrate and product concentrations in the fermentation of hydrolysate from enzymatic hydrolysis of PS + EL.
A: No nutrient supplementation; B: 3g/L corn-steep liquor. Fermentations were conducted at 30°C, initial pH 5.5, and 200-rpm agitation under largely anaerobic conditions for 120 h.

CONCLUSIONS

The present study has validated the feasibility of a PS-EL mixture as a feedstock for bioconversion to ethanol by separate hydrolysis and fermentation (SHF) using yeast as an ethanologenic strain. The yeast SHY07-1 can utilize xylose released from the mixture efficiently. An ethanol concentration of 36.82±0.35 g/L was obtained, corresponding to an ethanol yield of 0.45±0.04 g/g with an ethanol productivity of 0.31±0.01 g/(L h) and a fermentation efficiency of 80.71±0.03%. These promising results

demonstrated that the bioconversion of these two wastes together into ethanol was efficient even without pretreatment, detoxification, and nutrient supplementation. This approach may therefore represent an opportunity for reducing residues generated from the papermaking industry.

ACKNOWLEDGMENTS

The authors are grateful for the support provided by the National Natural Science Foundation of China (No. 51078147) and the Guangdong Provincial Science and Technology Program (No. 2010B031700022 and No. 2010A010500005).

REFERENCES CITED

Agbogbo, F. A., Kelly, G. C., Smith, M. T., and Wenger, K. (2006). "Fermentation of glucose/xylose mixtures using *Pichia stipites*," *Process Biochemistry* 41, 2333-2336.

Boni, M. R., Aprile, L. D., and Casa, G. D. (2004). "Environment quality of primary paper sludge," *Journal of Hazardous Materials* B108, 125-128.

Cerning, J., and Guilbot, A. (1972). "A specific method for determination of pentosans in cereals and cereal products," *Cereal Chemistry* 23, 176-178.

Domke, S. B., Aiello, M. C., and Holtzapple, M. T. (2004). "Mixed acid fermentation of paper fines and industrial biosludge," *Bioresource Technology* 91, 41-51.

Fan, Z., and Lynd, L. R. (2007a). "Conversion of paper sludge to ethanol. I: Impact of feeding frequency and mixing energy characterization," *Bioprocess and Biosystems Engineering* 30, 27-34.

Fan, Z., and Lynd, L. R. (2007b). "Conversion of paper sludge to ethanol. II: Process design and conomic analysis," *Bioprocess and Biosystems Engineering* 30, 35-45.

Fan, Z., South, C., Lyford, K., Munsie, J., Walsum, P. V., and Lynd, L. R. (2003). "Conversion of paper sludge to ethanol in a semicontinuous solids-fed reactor," *Bioprocess and Biosystems Engineering* 26, 93-101.

Fang, X., Shen, Y., Zhao, J., Bao, X. M., and Qu, Y. (2010). "Status and prospect of lignocellulosic bioethanol production in China," *Bioresource Technology* 101, 4814-4819.

Huang, C. F., Lin, T. H., Guo, G. L., and Hwang, W. S. (2009). "Enhanced ethanol production by fermentation of rice straw hydrolysate without detoxification using a newly adapted strain of *Pichia stipites*," *Bioresource Technology* 100, 3914-3920.

Kang, L., Wang, W., and Lee, Y. Y. (2010). "Bioconversion of kraft paper mill sludges to ethanol by SSF and SHF," *Applied Biochemistry and Biotechnology* 161, 53-66.

Katahira, S., Ito, M., Takema, H., Fujita, Y., Tanino, T., Tanaka, T., Fukuda, H., and Kondo, A. (2008). "Improvement of ethanol productivity during xylose and glucose co-fermentation by xylose-assimilating *S.cerevisiae* via expression of glucose transporter Sut1," *Enzyme and Microbial Technology* 43, 115-119.

Kumar, A., Singh, K. A., and Ghosh, S. (2009). "Bioconversion of lignocellulosic fraction of water-hyacinth (*Eichhornia crassipes*) hemicellulose acid hydrolysate to ethanol by *Pichia stipites*," *Bioresource Technology* 100, 3293-3297.

Lark, N., Xia, Y. K., Qin, C. G., Gong, C. S., and Tsao, G. T. (1997). "Production of ethanol from recycled paper sludge using cellulase and yeast," *Biomass and Bioenergy* 12, 135-143.

Lau, M. W., and Dale, B. E. (2009). "Cellulosic ethanol production from AFEX-treated corn stover using *Saccharomyces cerevisiae* 424A (LNH-ST)," *Proceedings of the National Academy of Sciences of the United States of America* 106, 1368-1373.

Lee, S. M., Koo, Y. M., and Lin, J. (2004). "Production of lactic acid from paper sludge by simultaneous saccharification and fermentation," *Advances in Biochemical Engineering/Biotechnology* 87, 173-194.

Li, H., Kim, N. J., Jing, M., Kang, J. W., and Chang, H. N. (2009). "Simultaneous saccharification and fermentation of lignocellulosic residues pretreated with phosphoric acid-acetone for bioethanol production," *Bioresource Technology* 100, 3245-3251.

Liu, X., Zhang, Z., Chi, C.-C., and Ge, W.-W. (2008). "Influence of water pre-hydrolyzing eucalyptus chips on the following alkaline pulping," *Proceedings of International Conference on Pulping, Papermaking and Biotechnology*, Nanjing, China, 120-123.

Lynd, L. R., Grethlein, H. G., and Wolkin, R. H. (1989). "Fermentation of cellulosic substrate in batch and continuous culture by *Clostridium thermocellum*," *Applied Environmental Microbiology* 55, 3131-3139.

Marques, S., Alves, L., Roseiro, J. C., and Girio, F. M. (2008). "Conversion of recycled paper sludge to ethanol by SHF and SSF using *Pichia stipites*," *Biomass and Bioenergy* 32, 400-406.

Mendes, C. V. T., Carvalho, M. G. V. S., Baptista, C. M. S. G., Rocha, J. M. S., and Soares, B. I. G. (2009). "Valorisation of hardwood hemicelluloses in the kraft pulping process by using an integrated biorefinery concept," *Food and Bioproducts Processing* 87, 197-207.

Miller, G. L. (1959). "Use of dinitrosalicylic acid reagent for determination of reducing sugar," *Analytical Chemistry* 31, 426-428.

Palmqvist, E., Grage, H., Meinander, N., and Hahn-Hägerdal, B. (1999). "Main and interaction effects of acetic acid, furfural, and p-hydroxybenzoic acid on growth and ethanol productivity of yeasts," *Biotechnology and Bioengineering* 63, 46-55.

Saha, B. C., and Cotta, M. A. (2010). "Comparison of pretreatment strategies for enzymatic saccharification and fermentation of barley straw to ethanol," *New Biotechnology* 27, 10-16.

Sreenath, H. K., and Jeffries, T. W. (2000). "Production of ethanol from wood hydrolyzate by yeasts," *Bioresource Technology* 72, 253-260.

Talebnia, F., Karakashev, D., and Angelidaki, I. (2010). "Production of bioethanol from wheat straw: An overview of pretreatment, hydrolysis and fermentation," *Bioresource Technology*. 101, 4744-4753.

Walton, S., Heiningen, A. V., and Walsum, P. V. (2010). "Inhibition effects on fermentation of hardwood extracted hemicelluloses by acetic acid and sodium," *Bioresource Technology* 101, 1935-1940.

Wayman, M., Chen, S., and Doan, K. (1992). "Bioconversion of waste paper to ethanol," *Process Biochemistry* 27, 239-245.

Zhao, L., Zhang, X., and Tan, T. W. (2008). "Influence of various glucose/xylose mixtures on ethanol production by *Pachysolen tannophilus*," *Biomass and Bioenergy* 32, 1156-1161.

Zhu, M. J., and Zhu, Z. S. (2011). "Construction of a fusant with improved xylose fermentation by intergeneric protoplast fusion of *Pichia stipitis* and *Saccharomyces cerevisiae* and its application in the fermentation of the hydrolysate from paper sludge," China Patent 201110304975.X.

Article submitted: August 17, 2011; Peer review completed: September 14, 2011; Revised version received: October 13, 2011; Accepted: October 18, 2011; Published: October 21, 2011.