Simultaneous Saccharification and Fermentation of Waste Textiles for Ethanol Production

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Ethanol production in a simultaneous saccharification and fermentation (SSF) process using waste textiles as feedstock was studied. The dissolution pretreatment of waste textiles in ortho-phosphoric acid resulted in at least 2 fold improvement in enzymatic hydrolysis rate and reducing sugar yield. The reducing sugars obtained from dyed or discolored waste textiles by cellulase hydrolysis demonstrated no inhibitory effect on ethanol fermentation activity of *Zymomonas mobilis* employed in SSF. SSF with a high waste textile loading (75 g L^{-1}) could still be operable due to the fast liquefaction of the pretreated substrate via enzymatic hydrolysis. Approximately 50 g L^{-1} ethanol was achieved within 24 h. In addition to 100% cotton textiles, the 40/60 polyester/cotton (T/C) blend waste textile could also be pretreated under the same condition to achieve the comparable ethanol production yield (~0.4 g EtOH g^{-1} glucose) from its cotton fraction in SSF.

Keywords: Simultaneous saccharification and fermentation; Ethanol; Waste textile; Phosphoric acid; Pretreatment

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INTRODUCTION

Cotton comes from the seed hairs of the plant *Gossypium*. China is the largest producer of cotton in the world, with annual production of about 34 million bales (Adams *et al.* 2013). Cotton must be scoured and usually bleached before use, which then leaves about 99% of the cotton cellulose to be consumed by the textile industries. Generally, most cotton is used for the preparation of cellulose-based clothes and textiles. The annual world-wide production of cotton fiber and regenerated cellulosic fibers (such as Rayon and Lyocell) is more than 26 and 3.1 million tons, respectively (JCFA 2008). However, these textiles are eventually disposed as waste to be treated in landfills or by incineration (Bartl *et al.* 2005; Miranda *et al.* 2007; Shen and Patel 2008). These waste textiles are mostly composed of natural cellulosic fibers and can be considered an alternate renewable biomass for the production of higher-value products *via* enzymatic hydrolysis of cellulose to glucose, followed by microbial fermentation.

Glucose can be obtained from enzymatic hydrolysis of cellulosic materials. However, cellulose has a highly crystalline structure and is resistant to enzymatic (Zhang

et al. 2006) and microbial attacks (Castro *et al*. 2012). The crystalline structure of cellulose can be disrupted by dissolving cellulose in a proper solvent, followed by regenerating it in a non-solvent. Recently, ionic liquids (Spiridon *et al.* 2011), concentrated phosphoric acid (81%) (Hong *et al.* 2012; Sathitsuksanoh *et al.* 2012; Zhang *et al.* 2007), N-methylmorpholine-N-oxide (NMMO) (Goshadrou *et al.* 2013; Kuo and Lee 2009a; Shafiei *et al.* 2011), and sodium hydroxide (NaOH)/urea solution at cold temperature (Jeihanipour and Taherzadeh 2009; Li *et al.* 2010; Zhao *et al.* 2008) have been employed to dissolve cellulosic materials, with the regenerated products showing a great improvement on the enzymatic hydrolysis rate and glucose yield. Among these dissolution pretreatments, phosphoric acid pretreated cellulose has been shown to have a higher initial hydrolysis rate and glucose yield (Kuo and Lee 2009b).

Simultaneous saccharification and fermentation (SSF) can improve ethanol production yield by removing end-product inhibition of the enzymatic saccharification process and eliminating the need for separate saccharification and fermentation reactors (Hasunuma and Kondo 2012). To achieve a high ethanol concentration from cellulosic materials in a SSF system, a high substrate loading is required, which results in a high insoluble solids content in the system. In practice, it is difficult to achieve a good ethanol yield with an insoluble solids content higher than 10% in SSF systems (Olofsson *et al.* 2008) because of the poor enzymatic saccharification rate caused by the highly crystalline structure of intact cellulosic materials. In addition, cellulose absorbs most of the water, making it difficult to achieve good mixing at high substrate loadings in stirred tank reactors. A major concern in the SSF process is fast liquefaction of cellulose to overcome the mixing problem at high loadings of cellulosic materials for microbial simultaneous fermentation of releasing glucose. Therefore, phosphoric acid-pretreated cellulose may be a method for increasing the enzymatic saccharification rate in a high-substrate loading SSF to enhance the ethanol production yield.

In this paper, cellulose-based waste textiles, including cotton linters, colored 100% cotton fabrics, and 40/60 polyester/cotton (T/C) blend fabric, were pretreated with concentrated phosphoric acid. The effect of pretreated dyed or decolored waste textiles on the enzymatic hydrolysis was investigated. In addition, SSF of pretreated waste textiles at different substrate loadings to ethanol was also evaluated.

EXPERIMENTAL

Materials

The waste textiles used in this work were 100% cotton linters (C), red T-shirt (RWT), and blue polyester/cotton (40/60) blended shirt (T/C). Ortho-phosphoric acid (85%) was obtained from Acros (New Jersey, USA). Cellulase AP3 (8.5 FPU g^{-1}) from *Aspergillus niger* strain was supplied by Amano Enzyme Inc. (Nagoya, Japan). Yeast extract was purchased from Difco. *Zymomonas mobilis* (ATCC 29191) was purchased from BCRC (Hsinchu, Taiwan).

Methods

Pretreatment and regeneration

Waste textiles were cut by scissors into small pieces (approximately 0.5 cm x 0.5 cm). The decolored waste textile was prepared by soaking the waste textile in 5 g L^{-1} of

sodium hydrosulfite ($Na_2S_2O_4$) and sodium carbonate (Na_2CO_3) solution at 100 °C for 1 h.

Next, 5.2% (%; w solid/w acid loading) cellulose solution was prepared by mixing 5.6 mL (9.5 g) of 85% phosphoric acid dissolved waste textile (0.5 g) in a 50 mL glass vial with magnetic stirring at 50 °C, 100 rpm for 2 h. To regenerate the dissolved fabrics, 60 mL of 50 °C deionized water was rapidly added to the dissolved fabric solution with continuous mixing until room temperature was reached. The regenerated products with solution were then transferred into polypropylene–centrifuge tubes, collected by centrifuging at 7000 rpm for 20 min, and washed with 50 mL of deionized water three times. Water (50 mL) was added to suspend the regenerated fabrics, and the solution was neutralized with 3 N NaOH to pH 7. Finally, the regenerated fabrics were collected on a filter paper (Advantec 5A), washed with 150 mL water to remove the salt, and stored in wet form at 4 °C until enzymatic saccharification or SSF was carried out.

Enzymatic hydrolysis

Wet pretreated waste textiles were employed in the enzymatic saccharification reaction, because dehydration of oven-dried regenerated waste textiles results in an enzymatic saccharification rate that is much lower than in the case of waste textiles that are not oven-dried (Sun and Chen 2008). Regenerated product collected on the filter paper from the regeneration mixture of 0.5 g dry weight waste textiles (based on the initial amount loaded in the pretreatment step) was suspended in 25 mL of phosphate buffer (pH 5, 100 mM). Cellulase AP3 (10 FPU g^{-1}) substrate was added to the mixture for the hydrolysis reaction. The hydrolysis was carried out at 50 °C with magnetic stirring at 250 rpm for 48 h, and the release of soluble reducing sugars was periodically measured. Because the enzyme Cellulase AP3 itself may contain reducing sugars, its content was measured and considered as the background concentration for calculating reducing sugars released during saccharification. A conversion factor of 1.11 was used to calculate the amount of glucose released from the amount of cellulose consumed. The saccharification conversion was defined as the ratio of reducing sugars produced to 1.11 fold of the weight of cellulosic fabric loaded.

Medium and Z. mobilis cultivation

The freshly grown *Z. mobilis* was cultured in phosphate buffer (pH 5, 100 mM) consisting of 50 g L^{-1} glucose, 5 g L^{-1} yeast extract, 2 g L^{-1} KH_2PO_4, and 1.5 g L^{-1} $(NH_4)_2HPO_4$. After incubation at 30 °C for 24 h, the optical density (OD_{660}) was measured and the dry cell weight calculated from the standard curve to convert OD value to g L^{-1}. The cells were concentrated by centrifugation at 3500 rpm for 10 min. The cells were re-suspended in sterile water for SSF use.

SSF of regenerated waste textile

Simultaneous saccharification and fermentation was carried out in 20 mL of fermentation medium at 37 °C in a 50-mL sealed vessel under mild stirring conditions from 60 to 250 rpm (after 2 h) for 48 h and the ethanol and glucose concentration were periodically measured. The fermentation medium containing regenerated waste textile (50 to 75 g L^{-1}), Cellulase AP3 (10 FPU g^{-1} substrate), and freshly grown *Z. mobilis* (3 g L^{-1}; dry weight) with yeast extract (5 g L^{-1}), KH_2PO_4 (2 g L^{-1}), $(NH_4)_2HPO_4$ (1.5 g L^{-1}), and $MgSO_4 \cdot 7H_2O$ (1 g L^{-1}). The conversion yield of glucose to ethanol in SSF was calculated as shown in Eq. 1,

$$Yield = \frac{E}{S \times 1.1 \times C + G - R} \quad (1)$$

where E is the ethanol concentration (g L^{-1}), S is the cellulose substrate concentration (g L^{-1}), C is the enzyme hydrolysis conversion of cellulose to glucose, G is the initial glucose concentration from Cellulase AP3 powder (g L^{-1}), and R is residual glucose in the medium (g L^{-1}).

Analysis

The cellulose content of waste textiles was determined by Taiwan textile research institute using the AATCC test method 20 fiber analysis: qualitative. The reducing sugar was measured by the DNS assay (Miller 1959) using glucose as a standard. The glucose concentration in SSF was monitored using a glucose analyzer (YSI 2700 SELECT). Cellulase activity was determined by the standard filter paper assay and expressed as filter paper units per gram of glucan (FPU) (Ghose 1987). One FPU is defined as the enzyme that releases 1 μmol of glucose equivalent per min from Whatman No.1 filter paper. The ethanol was quantified with a gas chromatograph equipped with a flame ionization detector (Shimadzu 14A, Japan) utilizing a Stabilwax-Da capillary column (Restek, Bellefonte, PA, USA). Injector and detector temperatures were set at 200 °C and 250 °C, respectively. The initial temperature of the column oven was set at 40 °C for 2 min, then elevated to 230 °C at 20 °C min^{-1}. Pure nitrogen was used as a carrier gas at a flow rate of 20 mL min^{-1}.

RESULTS AND DISCUSSION

Enzymatic Hydrolysis of Regenerated Waste Textiles

After the phosphoric acid dissolution pretreatment, the wet precipitates recovered from the regeneration mixture were subjected to enzymatic saccharification. The time course of enzymatic hydrolysis of untreated and regenerated waste textiles is shown in Fig. 1. After 1 h of enzymatic hydrolysis, approximately 7.3, 7.7, 6.6, 4.6, and 5.1 mg mL^{-1} of reducing sugars were released from regenerated cotton linter (C), red T-shirt (RWT), decolored red T-shirt (DRWT), light blue polyester/cotton (40/60) blended shirt (T/C), and decolored light blue polyester/cotton (40/60) (DT/C), respectively. In contrast, only around 2 to 2.5 mg mL^{-1} of reducing sugars were released from the untreated samples. The result indicates that the initial saccharification rates of waste textiles were enhanced at least 2-fold after the phosphoric acid dissolution pretreatment. Similarly high initial saccharification rates have also been observed at cellulase loading of 15 FPU g^{-1} glucan in the saccharification of phosphoric acid pretreated lignocellulose (Zhang *et al*. 2007).

In the case of pretreated waste textiles, the amount of reducing sugar released leveled off after 7 h of digestion. However, the increase of reducing sugar after 3 h of hydrolysis of untreated substrates was limited. The lower reducing sugar yield and a short time to achieve level-off value of released reducing sugar are probably due to the fact that most of crystalline structure of untreated substrates was kept intact and only the area around the substrate surface was accessible to cellulase digestion. The final saccharification conversion in the range of approximately 60% to 91% was achieved after 48 h of hydrolysis, which is comparable to those obtained at 7 h for the pretreated waste

textiles. Without pretreatment, only 25% to 35% saccharification conversions were obtained. This implies that approximately 2.5-fold enhancement of cellulose saccharification conversion can be achieved by employing phosphoric acid pretreatment. The conversions of regenerated T/C and DT/C were only approximately 60% because the constituent of those substrates were 60% cotton and 40% PET. The results indicate that cellulose in the regenerated T/C and DT/C was nearly digested. Since the same amount of enzyme was added in the saccharification reaction, the 60% cellulose content of polyester/cotton blended textiles had a higher enzyme/cellulose ratio than cellulose-based textiles. Besides, the effect of decoloration of waste textile on enhancing saccharification rate and yield was not significant, as indicated by the fact that similar reducing sugars releasing curves were obtained for the dyed and decolored waste textile substrates.

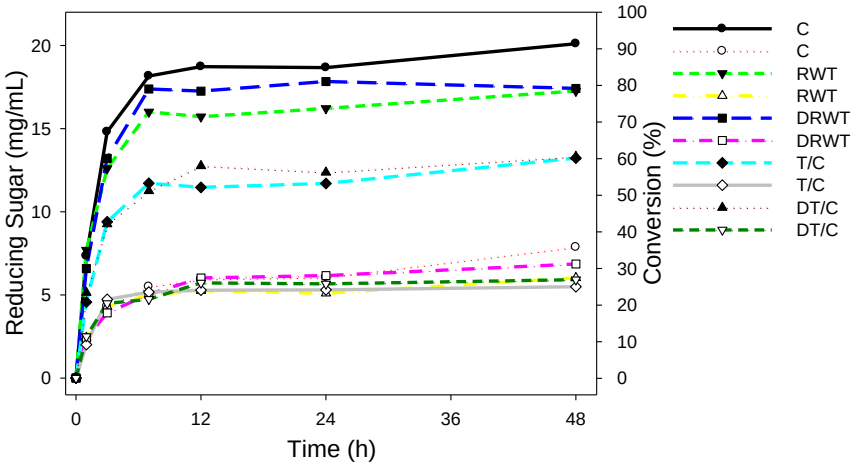

Fig. 1. Time course of reducing sugars released from the hydrolysis of untreated (empty symbol) and pretreated waste textile (filled symbol) with a substrate loading of 20 mg ml^{-1}. C, RWT, DRWT, T/C, and DT/C are cotton linter, red waste T-shirt, decolor of red waste T-shirt, light blue waste shirt (cotton/PET blend), and decolor of light blue waste shirt, respectively. The content of cellulose was 100% for C, RWT, DRWT; 60% for T/C, DT/C.

A successful SSF with high substrate loading is dependent on the saccharification rate. This is because the loaded solid substrate will be liquefied *via* enzymatic saccharification, thus facilitating good mixing and enhancing fermentation efficiency. Increased substrate concentration enables higher potential ethanol concentration, reducing the size equipment, the consumption of energy in heating and distillation, and the downstream processing requirements (Romaní *et al.* 2012). The much faster enzymatic saccharification rate of the phosphoric acid pretreated waste textiles observed in Fig. 1 indicates that high substrate loading of the never-dried regenerated waste textile in SSF is highly possible.

SSF of Regenerated Waste Textile

Production of ethanol from cotton linter and waste textiles *via* SSF was first investigated by loading 50 g L^{-1} of the regenerated substrates into the solution containing Cellulase AP3 and *Z. mobilis*. The ethanol and glucose concentration in the SSF medium are summarized in Table 1. The initial glucose concentration of 29 g L^{-1} measured in the fermentation medium was carried over from the added Cellulase AP3 powder, which contains a large amount of glucose as a bulking agent. The carried-over glucose and the release from enzymatic hydrolysis of waste textiles were rapidly metabolized to generate

ethanol. No appreciable ethanol and glucose concentration increases were observed after 12 h of SSF for all waste textiles. After prolonged SSF operation for 48 h, the ethanol concentration reached 29.2, 30.9, 28.4, 23.3, and 25.4 g L^{-1} for the substrate C, RWT, DRWT, T/C, and DT/C, respectively. In the dyed cellulosic waste textiles, the dye content is about 2 wt% (based on averaged dye molecular weight of 700 Daltons) (Czilik *et al.* 2002). The dye and dye-conjugated reducing sugars released from enzymatic hydrolyzed waste textiles may inhibit the fermentation activity of microorganisms. However, the χ^2 test result (p-value=0.96, degree of freedom=3) showed the ethanol concentration obtained from cotton linter and dyed waste textile were significantly the same. This result indicates that the effect of dye-conjugated reducing sugars on inhibiting the fermentation activity of *Z. mobilis* was insignificant, based on the saccharification conversion of approximate 80% and 100% for cellulose based and PET blended textiles observed in Fig. 1. After 48 h, the conversion yields of glucose to ethanol in SSF were estimated from Eq. 1 to be 0.4, 0.43, 0.40, 0.38, and 0.41 g EtOH g^{-1} glucose for the substrates C, RWT, DRWT, T/C, and DT/C, respectively. In the case of blended T/C and DT/C textiles, which contained about 40% polyester, only cellulose fiber was digested in the SSF process.

Table 1. Ethanol Production from Simultaneous Saccharification and Fermentation using 50 g L^{-1} Pretreated Waste Textile

Sample	Ethanol (g/L)					Glucose (g/L)					Yields (g/g)
	0 h	6 h	12 h	24 h	48 h	0 h	6 h	12 h	24 h	48 h	
C	0.0	22.3±0.5	26.6±0.2	29.8±1.4	29.2±0.5	29.0	0.9±0.0	0.4±0.0	0.2±0.0	0.4±0.0	0.40±0.01
RWT	0.0	23.7±0.0	28.3±1.4	29.7±0.2	30.9±0.6	29.0	0.7±0.0	0.4±0.0	0.3±0.0	1.5±0.1	0.43±0.01
DRWT	0.0	23.8±0.1	28.4±0.5	31.6±0.1	28.4±0.0	29.0	0.8±0.0	0.6±0.0	0.4±0.0	1.7±0.2	0.40±0.00
T/C	0.0	19.6±0.4	22.8±0.8	23.8±0.4	23.3±0.2	29.0	0.7±0.0	0.4±0.0	0.2±0.0	0.4±0.0	0.38±0.00
DT/C	0.0	22.6±0.5	23.2±0.1	24.3±1.1	25.4±0.4	29.0	0.9±0.0	0.5±0.0	0.2±0.0	0.3±0.0	0.41±0.01

C, RWT, DRWT, T/C and DT/C are cotton linter, red waste T-shirt, decolored red waste T-shirt, light blue waste shirt (cotton/PET blend) and decolored light blue waste shirt, respectively.

Time Course of High Substrate Loading SSF

The time course of ethanol production and glucose consumption in SSF with 75 g L^{-1} of cellulosic waste textiles is shown in Fig. 2. The initial high glucose concentration (45 g L^{-1}) in the fermentation medium was carried over from the Cellulase AP3 powder. The glucose concentration decreased to a very low level, and was accompanied by a rapid increase of ethanol concentration within the first 6 h of operation. During the rest of the operation, the glucose and ethanol concentration had no differences. This indicated that *Z. mobilis* has a high glucose uptake rate and rapidly metabolizes glucose into ethanol. Several advantages of employing *Z. mobilis* to produce ethanol have been reported, such as lower biomass production compared to yeast, higher volumetric sugar uptake and ethanol productivity, no requirement for the controlled addition of oxygen to maintain the cell viability, and amenability of this prokaryote to genetic improvement (Panesar *et al.* 2006). The final ethanol concentration of 49.2, 49.5, and 47.3 g L^{-1} and residual glucose concentration of 2.2, 4.1, and 3.0 g L^{-1} were obtained for substrates C, RWT, and DRWT, respectively. Based on the saccharification conversion of approximately 80%, the conversion yields of glucose to ethanol in SSF were estimated from Eq. 1 to be 0.45, 0.46, 0.44 g EtOH g^{-1} glucose for the substrate C, RWT, and DRWT, which corresponds to 90%, 92%, and 88% of the theoretical ethanol yield (0.5 g EtOH g^{-1} glucose). Several

articles have reported that the maximum ethanol concentration produced by *Z. mobilis* using starch-based substrates in SSF was approximately 54.0 g L^{-1} (Bandaru *et al.* 2006; Cazetta *et al.* 2007; Davis *et al.* 2006). Our results show that the high loading of water-insoluble substrate of waste textiles produced a comparable ethanol concentration to that of employing a starch-based substrate. Obviously, the phosphoric acid pretreatment increases the saccharification rate of waste textile and the fast hydrolysis rate of insoluble substrates in SSF can overcome the poor mass transfer usually encountered in a high substrate loading SSF operation.

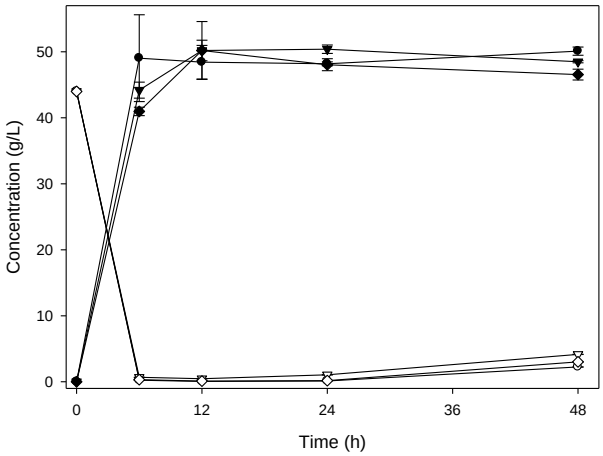

Fig. 2. Ethanol production from simultaneous saccharification and fermentation of 75 g L^{-1} of pretreated C (●), RWT (▼), and DRWT (◆). The empty symbol is glucose concentration and the filled symbol is ethanol concentration. C, RWT, and DRWT are cotton linter, red waste T-shirt, and decolored red waste T-shirt, respectively.

Scale-up of SSF

The scale-up operation of SSF using 75 g L^{-1} of regenerated C and RWT was performed in a 1-L fermentor, and the results are shown in Fig. 3. The ethanol concentration increased rapidly to approximately 38 g L^{-1} in 6 h. The SSF was completed in 24 h, and 45.5 and 48.8 g L^{-1} of ethanol was produced from C and RWT, respectively.

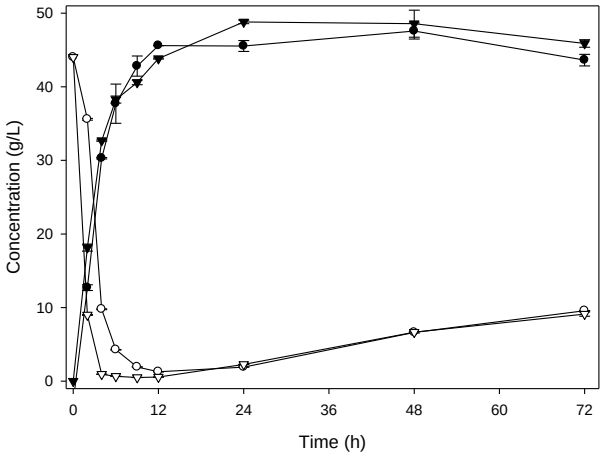

Fig. 3. Scale-up of simultaneous saccharification and fermentation using 75 g L^{-1} of C (●) and RWT (▼) for ethanol production in a 1 L fermentor. The empty symbol is glucose concentration and the filled symbol is ethanol concentration. C and RWT are cotton linter and red waste T-shirt, respectively.

No appreciable increase of ethanol concentration was observed after 24 h. After 24 h, the residual glucose concentration of 1.9 and 2.3 g L^{-1} were obtained for substrate C and RWT, respectively. The ethanol concentration leveled off between 24 to 72 h, but the residual glucose concentration increased slightly from ~2.1 to ~9.2 g L^{-1}. This might arise from the occurrence of some evaporation during the extended times of exposure, accompanied by some ethanol removal and the consequent concentration of non-volatile compounds in the culture broth. Based on the saccharification conversion of approximate 80%, the conversion yields of glucose to ethanol in SSF at 24 h were estimated from Eq. (1) to be 0.42 and 0.45 g EtOH g^{-1} glucose for the substrate C and RWT, which corresponds to 84% and 90% of the theoretical ethanol yield (0.5 g EtOH g^{-1} glucose). This result shows that the scale-up of SSF for the production of high ethanol concentration from waste textiles using *Z. mobilis* can be achieved within 24 h.

CONCLUSIONS

1. The ortho-phosphoric acid dissolution pretreatment of cellulosic waste textile significantly improved its cellulase hydrolysis rate and sugar conversion yield. Higher than 80% of pretreated waste textile was converted into reducing sugars while less than 40% was achieved for the untreated one.
2. Pretreatment made the water-insoluble cellulosic textile much easier to be hydrolyzed by cellulase, which led to fast liquefaction of the SSF mixture that overcomes the usually encountered poor mixing condition in high substrate loading SSF.
3. A high substrate loading of 75 g L^{-1} waste textile was operable in SSF to produce 50 g L^{-1} ethanol at 24 h. The dyed textile did not show significant inhibitory effect on ethanol fermentation. Besides, the polyester/cotton (T/C) blend textile could also be pretreated by ortho-phosphoric acid dissolution and resulted in a comparable ethanol conversion yield based on its cotton fraction (~0.4 g EtOH g^{-1} glucose) in SSF.

ACKNOWLEDGMENTS

The authors are grateful for the kind support from the Committee of the 4th Conference on Biorefinery towards Bioenergy (ICBB2013) in Xiamen, China.

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Article submitted: January 10, 2014; Peer review completed: March 5, 2014; Revised version received and accepted: March 26, 2014; Published: April 3, 2014.