USE OF SURFACTANTS IN ENZYMATIC HYDROLYSIS OF RICE STRAW AND LACTIC ACID PRODUCTION FROM RICE STRAW BY SIMULTANEOUS SACCHARIFICATION AND FERMENTATION

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Rice straw (RS) is one of the most abundant lignocellulosic waste by-products worldwide and provides an alternative substrate to produce useful chemicals such as bioethanol and lactic acid. However, higher enzyme loadings are needed to obtain a higher product yield, which makes the large-scale utilization economically difficult. The presence of non-ionic surfactants and poly(ethylene glycol) (PEG) during the enzymatic hydrolysis of lignocellulosics has been found to increase the conversion of cellulose into fermentable sugars. We have found that adding 0.2g g^{-1} substrate of polyoxyethylene(20) sorbitan monooleate (PSM) or high-mass PEG increased the sugar yield by 22% and 12%, respectively, when enzyme loading was at 10FPU g^{-1} for 24h. PSM behaved better than PEG when different substrate concentrations, temperatures, and enzyme loadings were investigated. PSM provides an opportunity to reduce enzyme dosage while still keeping the same extent of hydrolysis. We also investigated the effect of PSM on the simultaneous saccharification and fermentation of pretreated RS to lactic acid. Results showed that addition of 0.7g L^{-1} PSM improved the lactic acid production by 24% compared to the reference without PSM addition at 72h.

Keywords: Rice straw, Cellulose, Surfactant, Lactic acid, Simultaneous saccharification and fermentation

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INTRODUCTION

Lactic acid and its derivatives have been widely used in the food, pharmaceutical, cosmetic and industrial applications. Recently, lactic acid has gained increasing interest as a raw material for manufacture of poly(lactic acid), a bio-compatible and biodegradable plastic (Hujanen and Linko 1996). However, the economics of ultimate lactic acid fermentation is still a problem, and medium compositions contribute most to the overall cost of lactic acid production. In order to reduce the production cost of lactic acid, many studies have been published using the hydrolysate of cellulosic materials as carbon sources to produce lactic acid (Hofvendahl and Hahn-Hagerdal 2000; Wee et al. 2006). The research along this line has yielded two efficient bioprocess schemes, which are termed "simultaneous

saccharification and fermentation" (SSF) and "separate hydrolysis and fermentation" (SHF), of which the SSF of lignocellulosic materials for production of lactic acid has been extensively investigated (Bustos et al. 2005; John et al. 2006; Tanaka et al. 2006).

In the bioconversion of lignocellulosic biomasses to value-added products, hydrolysis of pretreated lignocellulosic materials to fermentable sugars is of great importance. It is the rate-limiting step in both SSF and SHF (Lyer and Lee 1999; Alkasrawi et al. 2003). From previous studies by different authors (Wyman et al. 1992; Gregg and Saddler 1996), it is apparent that a high degree of bioconversion of lignocellulose to reducing sugars requires low substrate concentration, prolonged hydrolysis time, and high enzyme loading. However, high enzyme concentration contributes significantly to the overall process cost due to the high enzyme cost. For instance, enzyme loading can amount to as much as 60% of the process cost of biomass conversion (Wilke et al. 1981; Nguyen and Saddler 1991; Helle et al. 1993). Therefore, it is urgent to find methods to enhance the saccharification yield of cellulose while reducing cellulase dosage. Towards this aim, it has been reported that applying both non-ionic surfactants and bovine serum albumin (BSA) treatment can effectively increase the hydrolysis rate of lignocellulose (Eriksson et al. 2002; Yang and Wyman 2006).

In China, rice straw (RS) is the most abundant agricultural residue, and the annual production is estimated to be approximately 200 million tons (Zhu et al. 2006). In this study, the effect of poly(ethylene glycol) (PEG), an ethylene oxide (EO) containing polymer, and polyoxyethylene(20) sorbitan monooleate (PSM) (Tween® 80) on the enzymatic cellulose hydrolysis was investigated using pretreated RS as substrate. Further, the effect of PSM on SSF of pretreated RS to lactic acid by *Lactobacillus casei* YQ-06 was also studied. To the best of our knowledge, there have been few studies on lactic acid production by SSF with addition of surfactant.

EXPERIMENTAL

Microorganism

Trichoderma viride YQ-02, isolated by our laboratory, was used for cellulase production. The stock culture was stored on potato-glucose-agar (PDA) and subcultured every two months.

Lactobacillus casei YQ-06 was acclimatized *from Lactobacillus casei* GIM 1.159 (originally from ATCC 334). The strain can grow well at 45 °C and was maintained on deMan, Rogosa and Sharpe (MRS) slant (DeMan et al. 1960).

Lignocellulosic Materials and Pretreatment

Raw RS was obtained from local farmers in Hefei, Anhui province, China. Prior to pretreatment, it was cut to 1-2 cm length and washed thoroughly with tap water until the washings were clean and colorless and then dried in a oven at 65 °C to constant weight. Oven-dried RS was then ground to 80-120 mesh with an electric grinder and was used in the experiments. The smashed RS was stored at room temperature until the time of use. The initial composition of the RS was determined to be 33.3% cellulose, 23.32% hemicellulose, and 17.5% lignin.

A two-step chemical pretreatment: dilute acid pretreatment and alkaline peroxide pretreatment was carried out as described by Curreli et al. (2002). The cellulose, hemicellulose, and lignin contents of pretreated RS were 68.8%, 9.4%, and 8.9%, respectively.

Cellulase Production

Cellulase was produced by solid state fermentation. A two-stage technique was employed. In the first stage, the fungus was grown on PDA slants for 5 days at 30 ℃. In the second, 10 mL of deionized sterile water was added to a PDA slant, and a spore suspension containing 10^7 spores mL^{-1} was used to inoculate Erlenmeyer flasks (250 mL) containing 5 g of RS and wheat bran (dry weight ratio is 3:2) moistened with the following mineral medium (g L^{-1}): $(NH_4)_2SO_4$ 10; KH_2PO_4 4; $MgSO_4 \cdot 7H_2O$ 0.5, and $CaCl_2$ 0.5. (Chen et al. 2004). The initial pH value of the medium was adjusted to 5.0 after sterilization at 121 ℃ for 30 min, and the water content of the substrate was 75%. The flasks were incubated at 30℃ for 96h under static conditions.

Enzyme Extraction and Concentration

The enzyme was extracted by a simple contact method (Krishna and Chandrasekaran 1996). The fermented samples were shaken (150rpm) with 50mL of 0.1M citrate buffer of pH=4.8 for one hour and filtered through filter paper. The filtrates were centrifuged at 10,000 rpm (4℃) for 15 min to remove spores of the organism, and supernatants were further concentrated by ammonium sulfate precipitation. The enzyme salt solutions were dialyzed with 0.1M citrate buffer of pH4.8 for 24h with continuous stirring and the buffer was changed from time to time (3 to 5 times) for removing ammonium salt. The enzyme was concentrated with PEG20,000 to the required concentration. Concentrated cellulase solutions with a filter paper activity (FPU) of 5.028 IU mL^{-1} and a cellobiase activity (CBU) of 3.017 IU mL^{-1} were used in the hydrolysis and SSF experiments.

Enzymatic Hydrolysis

Hydrolysis experiments were carried out in 250 mL flasks with a working volume of 100mL in the presence of different concentrations of polyoxyethylene(20) sorbitan monooleate (PSM) or high molecular mass polymer of ethylene oxide (PEG). The PSM was Tween® 80, provided by Shanghai Shenyu Pharmaceutical & Chemical Co. Ltd., China. The PEG was PEG 6000, provided by Shantou Xilong Chemical Industry Factory Co., Ltd., China.

Pretreated RS was saccharified with different volumes of concentrated cellulose solutions in 0.1M citrate buffer (pH 4.8) at a substrate concentration of 2% (w/v). Sodium azide (30 μg mL^{-1}) was added to the reaction mixture to prevent microbial and fungal contamination. The flasks were incubated at 50℃ on an orbital shaker agitated at 150rpm. Sample aliquots of 2mL were taken at different times, centrifuged, and the supernatants were analyzed for reducing sugars. Sugar yield was expressed as mg reducing sugars produced by enzymatic action per g dry biomass.

Simultaneous Saccharification and Fermentation (SSF)

L. casei inoculum was prepared by transferring a loop of stock culture to

10mL sterile MRS medium in 50mL screw cap tubes and maintained at 45℃ for 24h. This culture (1mL) was then transferred to 10mL MRS medium in a 100mL screw cap conical flask and incubated at 45℃ for 24h.

SSF experiments were carried out in 250mL conical flasks (working volume 100mL) placed in orbital shakers (150rpm) under anaerobic conditions. SSF reaction mixtures contained 6g of pretreated RS (previously autoclaved for 15min at 121℃); 12mL of crude cellulase extract (10FPU g^{-1} substrate); 5mL *L.casei* inoculum; different concentrations of PSM, ranging from 0 to 5.6 g L^{-1}; and basal medium were added to make up the volume to 100mL. The medium pH was adjusted to 5.0 with 0.1M citrate buffer (Lyer and Lee 1999). The basal medium contained (g L^{-1}): peptone 10; yeast extract 5; $NaCH_3CO_2$ 0.5; $MnSO_4 \cdot H_2O$ 0.05; $MgSO_4 \cdot 7H_2O$ 0.2; $FeSO_4 \cdot 7H_2O$ 0.01; NaCl 0.01; and $ZnSO_4 \cdot 7H_2O$ 0.2.

Analytical Methods

Filter paper activity and cellobiase activity were assayed according to a standard cellulase activities analytical procedure recommended by the International Union of Pure and Applied Chemistry (IUPAC) (Ghose 1987). One unit of filter paper activity (FPU) was defined as the amount of enzyme required to liberate 1 μmol of reducing sugars as glucose per minute per milliliter of crude enzyme extract under assay conditions. Cellulose, hemicellulose, and lignin were determined according to the procedures described elsewhere (Goering and VanSoest 1970). The amount of reducing sugars was determined by the DNS method (Miller 1959). L-lactic acid was estimated by a colorimetric method (Kimberley and Taylor 1996).

Three parallel samples were used in all analytic determination, and data are presented as the mean of three replicates. Relative standard deviations in the assays of reducing sugars and lactic acid were below 5%.

RESULTS AND DISCUSSION

Evaluation of Saccharification Conditions with Surfactant Addition

Hydrolysis at lower temperature with addition of surfactants

The temperature 50 ^oC has been used as the optimum for enzymatic hydrolysis of different lignocellulosics (Vlasenko et al. 1997; Krishna et al. 1999; Ortega et al. 2001; Adsul et al. 2005; Chen et al. 2006; Xu et al. 2006). This is also true of for the cellulase from *T. viride.*

Hydrolysis of pretreated RS with addition of 0.2g g^{-1} surfactants was investigated at 40 ^oC and 50 ^oC (Fig. 1). As expected, samples with surfactant additions had higher conversion, with PSM performing better than PEG at both temperatures. At 40 ^oC, sugar yields were 184 and 212 mg g^{-1} dry biomass with PEG and PSM addition, respectively, while for the sample without surfactant addition, the sugar yield was only 148mg g^{-1} dry biomass at 72h. At a temperature of 50 ^oC, the conversion increased by 13% and 8% when adding PSM and PEG in the reaction mixture, compared to the control without surfactant addition, respectively. It has been reported that surfactants could increase the stability of cellulase (Kaar and Holtzapple 1998). Surfactant as an enzyme stabilizer possibly contributes to better performance

of cellulase at lower temperature.

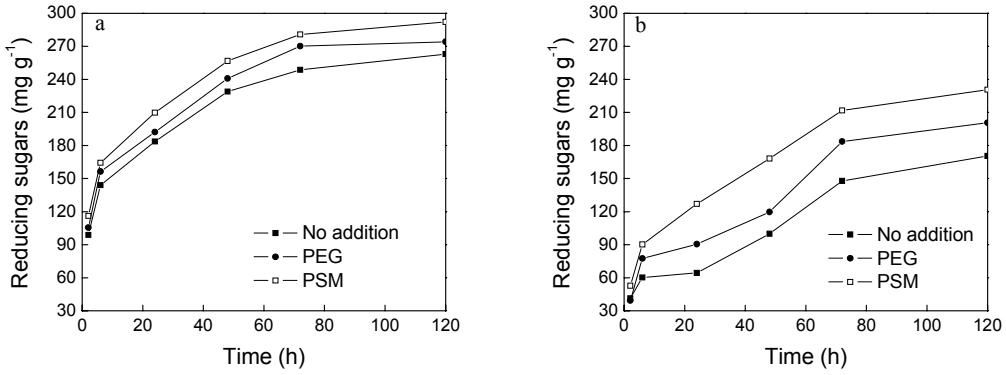

Fig. 1. Time course of RS hydrolysis with surfactant addition at different temperatures. Enzyme loading 10FPU g^{-1} substrate, substrate concentration 2% (w/v), pH 4.8. Surfactant concentration 0.2g g^{-1} substrate.(a) 50°C; (b) 40°C.

Effect of substrate concentration

The effect of substrate concentration on enzymatic hydrolysis was investigated with addition of surfactant at a fixed ratio of cellulase dosage to substrate (10FPU g^{-1} substrate and 6CBU g^{-1} substrate). As shown in Fig. 2, the rate of conversion decreased when higher substrate concentration than 2% (w/v) was used. Further increase in substrate concentration limits the saccharification yield because of stirring difficulties, reduction of the aqueous movable phase, and end-product inhibition (Lee and Fan 1982; Szczodrak 1987). Substrate concentrations of 2% have also been found optimum to release reducing sugars from different lignocellulosic biomass (Krishna. et al. 1998; Krishna et al. 1999). Accumulation of glucose and cellobiose as end-products, especially cellobiose, strongly inhibits the cellulase in the reaction mixture. In order to reduce the inhibition of end-product, several methods have been developed, including supplementation with excess β-glucosidase during hydrolysis, and the use of a high enzyme loading and removal of sugars during hydrolysis by SSF (Stenberg et al. 2000; Chen et al. 2006).

Effect of surfactant concentration

A portion of the data from surfactant loading experiments is plotted in Fig. 3. Addition of surfactants to the reaction mixture enhanced the enzymatic hydrolysis of pretreated RS in comparison to the surfactant-free samples. Addition of 0.2 g g^{-1} of PSM and PEG increased the sugar yield to 210 and 192mg g^{-1} dry biomass from 172mg g^{-1} dry biomass at 24h, respectively. Higher surfactant concentration than 0.2g g^{-1} substrate resulted in decreased conversion. A likely explanation for this may be that the hydrophobic part of the lignin in the lignocellulosic materials needs a certain concentrations of non-surfactants to be occupied through hydrophobic interactions to prevent unproductive binding of cellulases to lignin (Eriksson et al. 2002). Thus, more free cellulose is available for hydrolysis of cellulosic substrate. When higher surfactant concentration is used, micelles of non-surfactant may be formed (Jonstromer and Strey 1992; Schomacker and Strey 1994). Formation of micelles may prevent cellulase from contacting the cellulose substrate, thus reducing the conversion rate.

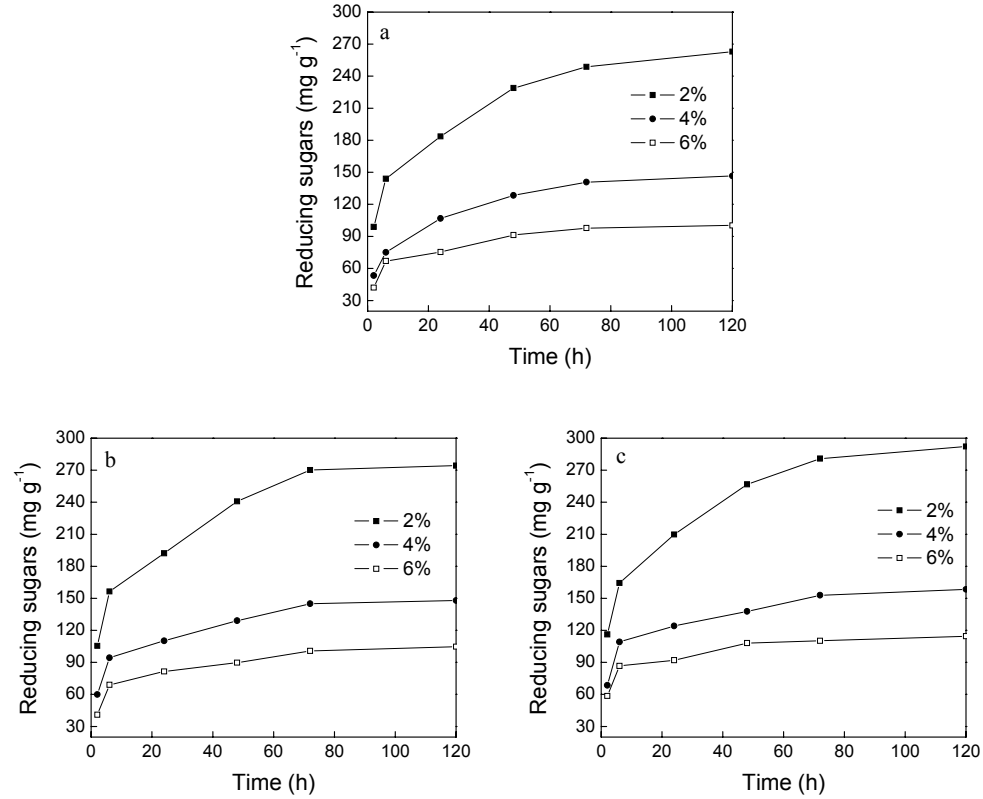

Fig. 2. Effect of substrate concentration (% (w/v)) on enzyme hydrolysis with addition of surfactants. Enzyme loading 10FPU g^{-1} substrate, pH 4.8, surfactant concentration 0.2g g^{-1} substrate, temperature 50 °C. (a) no surfactant addition; (b) PEG addition; (c) PSM addition

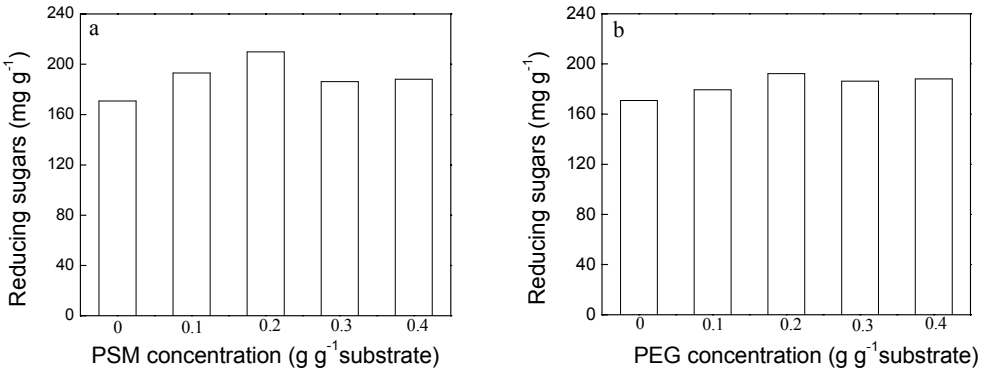

Fig. 3. Effect of surfactant concentration on enzyme hydrolysis. Enzyme loading 10FPU g^{-1} substrate, substrate concentration 2% (w/v), temperature 50 °C, pH 4.8, incubation time 24h. (a) PSM addition; (b) PEG addition.

Effect of enzyme loading

The effect of enzyme loading (5-25FPU g^{-1} substrate) on the enzymatic hydrolysis of pretreated RS has been studied and the results are presented in Fig. 4. Higher enzyme loading resulted in higher sugar yield. The sugar yield increased sharply when increasing enzyme dosage from 5FPU g^{-1} to 10FPU g^{-1} substrate for all of the samples. Thereafter the increase observed was less. Most samples leveled off after 72h, but the sample of enzyme concentration at 5FPU g^{-1} substrate without surfactant addition leveled off after 6h.

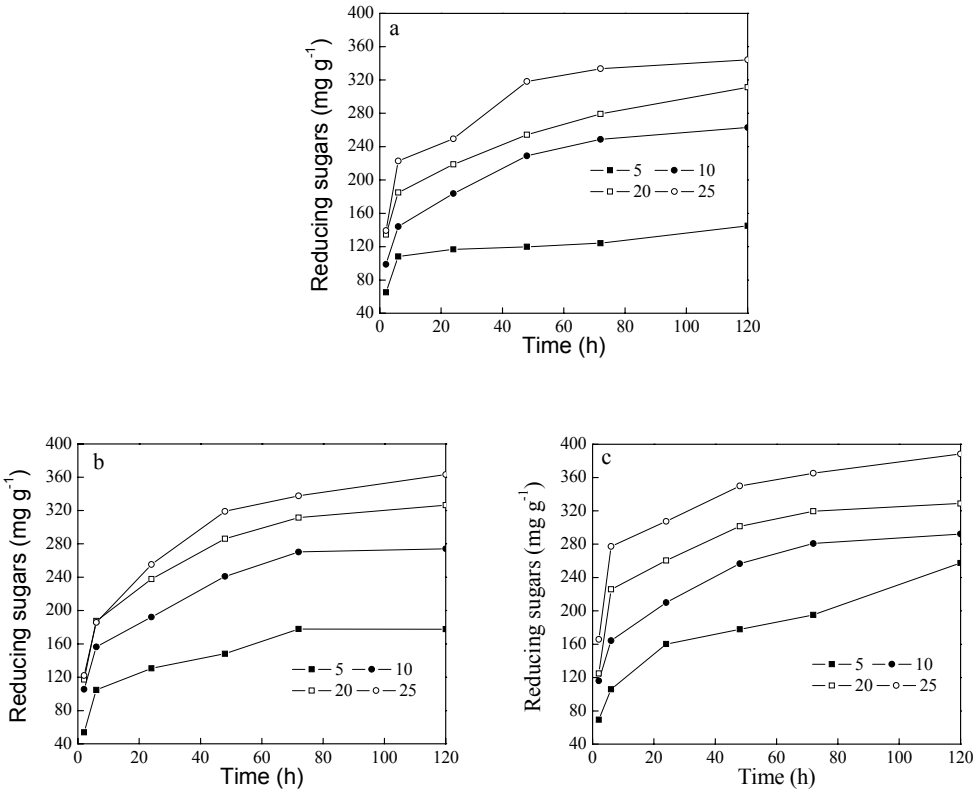

Fig. 4. Effect of enzyme loading (FPU g^{-1} substrate) on enzyme hydrolysis with addition of surfactants. substrate concentration 2% (w/v), pH 4.8, surfactant concentration 0.2g g^{-1} substrate, temperature 50°C. (a) no surfactant addition; (b) PEG addition; (c) PSM addition.

SSF of RS to Lactic Acid with PSM Addition

As mentioned above, PSM performed better in the enhancement of enzymatic hydrolysis of pretreated RS. So PSM was chosen to investigate the effect of surfactant on SSF of pretreated lignocellulosics to lactic acid.

The effect of PSM on the SSF of cellulosic materials to lactic acid is plotted in Fig. 5. Results indicated that a small amount of PSM (0.7g l^{-1}) could increase the production of Lactic acid by *L. casei*, while higher PSM concentration had a negative effect on lactic acid production. The positive effect of PSM in the SSF experiments was likely due to the increase in conversion rate of lignocellulosic materials by

preventing the unproductive adsorption of cellulase to the lignin and allowing more enzyme to be available for hydrolysis of cellulose. But when higher PSM concentration is used, the PSM may dissolve the lipid in the cell membrane and cause the death of the cell. PSM provides an opportunity to reduce the enzyme loading while still maintaining the same extent of lactic acid production.

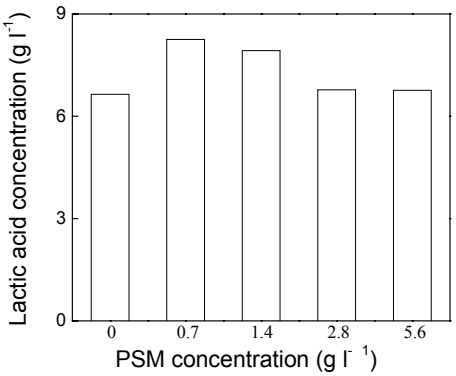

Fig. 5. Effect of PSM concentration on SSF of pretreated RS to Lactic acid. Enzyme loading 10FPU g^{-1} substrate, substrate concentration 6% (w/v), temperature 45 °C, pH 5.0, incubation time 72h.

Further research on the effects of surfactant in SSF of lignocellulosics to lactic acid and economic analysis of the production process of lactic acid from cellulosic substrate with addition of surfactants are being carried out in our laboratory.

CONCLUSIONS

Addition of a non-ionic surfactant polyoxyethylene(20) sorbitan monooleate (PSM, Tween® 80) or a high-mass polymer of ethylene oxide (PEG) can effectively improve the enzymatic hydrolysis of pretreated rice straw (RS). But PSM performed better than PEG when different substrate concentrations, temperatures, and enzyme loading were investigated. The simultaneous saccharification and fermentation (SSF) of pretreated RS to lactic acid was improved by the addition of PSM. Lactic acid production increased by 24% when adding 0.7g L^{-1} PSM, compared to the reference without PSM addition at 72h.

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